

## **Inhibitors of the Plasmodium falciparum M18 Aspartyl Aminopeptidase**

### **Author**

Schoenen, Frank J., Weiner, Warren S., Baillargeon, Pierre, Brown, Chris, Chase, Peter, Ferguson, Jill, Fernandez-Vega, Virneliz, Ghosh, Partha, Hodder, Peter, Krise, Jeffrey P., Matharu, Daljit S., Neuenswander, Benjamin, Porubsky, Patrick, Rogers, Steven, Skinner-Adams, Tina, Sosa, Melinda, Spicer, Timothy, To, Joyce, Tower, Nichole A., Trenholme, Katharine R., Wang, Jenna, Whipple, David, Aubé, Jeffrey, Rosen, Hugh, Lucile White, E., Dalton, John P., Gardiner, Donald L.

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## Probe Report

### Title: Inhibitors of the *Plasmodium falciparum* M18 Aspartyl Aminopeptidase

**Authors:** Frank J. Schoenen<sup>6\*</sup>, Warren S. Weiner<sup>6</sup>, Pierre Baillargeon<sup>1</sup>, Christopher L. Brown<sup>5</sup>, Peter Chase<sup>1</sup>, Jill Ferguson<sup>11</sup>, Virneliz Fernandez-Vega<sup>1</sup>, Partha Ghosh<sup>6</sup>, Peter Hodder<sup>1,7</sup>, Jeffrey P. Krise<sup>6,10</sup>, Daljit S. Matharu<sup>6</sup>, Benjamin Neuenswander<sup>6</sup>, Patrick Porubsky<sup>6</sup>, Steven Rogers<sup>6</sup>, Tina Skinner-Adams<sup>2</sup>, Melinda Sosa<sup>8</sup>, Timothy Spicer<sup>1</sup>, Joyce To<sup>4</sup>, Nichole A. Tower<sup>8</sup>, Katharine R. Trenholme<sup>2</sup>, Jenna Wang<sup>6</sup>, David Whipple<sup>6</sup>, Jeffrey Aubé<sup>6,9</sup>, Hugh Rosen<sup>1,7</sup>, E. Lucile White<sup>8</sup>, John P. Dalton<sup>3,4</sup>, and Donald L. Gardiner<sup>2</sup>

<sup>1</sup>The Scripps Research Institute Molecular Screening Center, Scripps Florida, Jupiter, Florida; <sup>2</sup>Malaria Biology Laboratory, The Queensland Institute of Medical Research, Brisbane, Australia; <sup>3</sup>Institute of Parasitology, McGill University, Quebec, Canada, <sup>4</sup>Institute for Biotechnology of Infectious Diseases (IBID), University of Technology Sydney (UTS), Sydney, Australia, <sup>5</sup>School of Biomolecular and Physical Sciences, Griffith University, Brisbane, Australia <sup>6</sup>The University of Kansas Specialized Chemistry Center (KU SCC), Lawrence, Kansas, <sup>7</sup>Department of Molecular Therapeutics, Scripps Florida, Jupiter, Florida; <sup>8</sup>Southern Research Specialized Biocontainment Screening Center, Birmingham, AL; <sup>9</sup>Department of Medicinal Chemistry, University of Kansas, Lawrence, KS; <sup>10</sup>Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS; <sup>11</sup>The Scripps Research Institute Molecular Screening Center, La Jolla, CA.

\*Corresponding author email: schoenen@ku.edu

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**Screening Center Name(s) & PI(s):** Scripps Research Institute Molecular Screening Center, Hugh Rosen; Southern Research Specialized Biocontainment Screening Center, E. Lucile White

**Chemistry Center Name & PI:** University of Kansas Specialized Chemistry Center, Jeffrey Aubé

**Assay Submitter(s) & Institution(s):** John P. Dalton, Institute of Parasitology, McGill University, Quebec, Canada; Institute for Biotechnology of Infectious Diseases (IBID), University of Technology Sydney (UTS), Sydney, Australia; Donald L. Gardiner, Malaria Biology Laboratory, The Queensland Institute of Medical Research, Brisbane, Australia

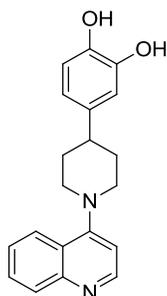
**PubChem Summary Bioassay Identifier (AID):** 1855

### Abstract:

Malaria is one of the most prevalent human parasitic diseases and is a global health issue accounting for >600,000 deaths annually. For survival, the *Plasmodium falciparum* (*Pf*) malaria parasite requires the action of a number of metallo-aminopeptidases that each display restricted amino acid specificities, including *Pf*M1MAA (membrane alanine aminopeptidase), *Pf*M17LAP (leucine aminopeptidase), and *Pf*M18AAP (aspartyl aminopeptidase), which are thought to act in concert to degrade host erythrocyte hemoglobin that the parasite uses as a source of amino

acid building blocks for the synthesis of its own proteins. Since there are several small molecule inhibitors of *PfM1MAA* and *PfM17LAP*, and very few small molecule inhibitors of *PfM18AAP*, we set out to identify small molecule inhibitors of *PfM18AAP*. Biochemical assays employing *rPfM18AAP*, native *PfM18AAP*, recombinant *Fasciola hepatica* cathepsin L1 (*rFhCTSL1*), *rPfM1MAA*, *rPfM17LAP*, and rhM18, and cell-based parasite growth inhibition and cytotoxicity assays were used to identify CID23724194 (from the NIH MLSMR) as a viable starting point for medicinal chemistry optimization. Two rounds of structure-activity relationship studies were performed to generate the probe ML369 (CID56846691). The probe is the best-in-class small molecule inhibitor of *PfM18AAP*; however, certain liabilities discussed in detail in the probe report limit its usefulness. When the probe is used as recommended, the probe is “fit-for-purpose” and should be useful for advancing the field.

### Probe Structure & Characteristics:



**ML369**

CID/ML#	Target Name	IC <sub>50</sub> [SID, AID]	Anti-target Name(s)	IC <sub>50</sub> [SID, AID]	Fold Selective	Secondary Assay(s) Name: EC <sub>50</sub> [SID, AID]
56846691/ ML369	<i>rPfM18</i> AAP	4.6 μM [135378316, 624177]	<i>rPfM1MAA</i>	75 μM [135378316, 624176]	16x	RBC Parasite Growth Inhibition 1.3 μM [135378316, TBD]  Vero Cell Cytotoxicity >50 μM [135378316, 624205]
			<i>rPfM17LAP</i>	38 μM [135378316, 624175]	8.2x	
			rhM18	4.7 μM [135378316, 624174]	1x	

### **Recommendations for scientific use of the probe:**

Using this probe and analogues, researchers may evaluate the *Pf*M18AAP enzyme as a target in culture for developing small-molecule anti-malaria drugs. Moreover, a more detailed understanding of the enzyme's function in the parasite may be elucidated and its putative role alongside other aminopeptidases (e.g. *Pf*M17LAP and *Pf*M1MAA) may be determined. Eventually, this could contribute to the development of multi-drug therapies targeting several enzymes, therapies for which there might be a lower prospect of drug resistance developing.

More specifically, in fluorescence and electron microscopy studies, parasitologists might use the compounds reported here to study when and where the *Pf*M18AAP enzyme acts in cells. Chemical biologists might use the compounds to design biotin, photo-affinity or fluorescent conjugates as an approach to illuminating the distinct roles for *Pf*M18AAP, as has been demonstrated for *Pf*M1MAA and *Pf*M17LAP using bestatin-based conjugates. Crystallographers might use the highly soluble and moderately potent compounds reported here for co-crystallization studies with the recombinant *Pf*M18AAP enzyme, for which the X-ray crystal structure has been reported. Physical chemists might use the compounds reported here to study the relationship between a compound's physical properties, such as, cell permeability and  $pK_a$ , the compound's predicted propensity to accumulate in the parasitophorous vacuole, and the compound's activity in the parasite growth inhibition assay.

The probe is the best-in-class small molecule inhibitor of *Pf*M18AAP; however, certain liabilities discussed in detail in the probe report limit its usefulness. When the probe is used as recommended above, the probe is "fit-for-purpose" [1] and should be useful for advancing the field.

## 1 Introduction

The causative agents of malaria are protozoan parasites of the genus *Plasmodium*. There is an estimated >250 million cases of malaria each year and > 600,000 deaths. *P. falciparum* (*Pf*) causes the most deaths, particularly in Africa, and mainly in children and pregnant women [2]. Due to the spread of drug-resistant parasites, the treatment and prevention of *Pf* malaria is becoming more difficult. Artemisinin and several derivatives are our last line of defense; however, artemisinin-resistant malaria cases have been reported in SE Asia. Accordingly, the need for new antimalarial agents with new modes of action is pressing [3].

The parasite uses an amino acid pool to synthesise their proteins as they grow inside the host erythrocytes. They acquire most of these amino acids by digesting the hemoglobin of the erythrocytes while some are taken from outside [5]. The parasite digests 65-75% of the host cell hemoglobin within a specialized acidic digestive vacuole (DV) which contains a number of aspartic-, cysteine- and metallo-endoproteases, a dipeptidase and some aminopeptidases. The hemoglobin is systematically degraded to small peptide fragments (2-20 residues long, and possibly some free amino acids) which are then transported into the parasite cytosol for further processing to free amino acids by several aminopeptidases [5,6,7].

Eight metallo-aminopeptidases have been identified in the *Pf* genome [8]. Four of these are methionine aminopeptidases and are presumed to play housekeeping roles, including removal of the initiator methionine from newly synthesised polypeptides. The other four may act in concert to degrade the hemoglobin-derived peptides, and consist of a prolyl aminopeptidase, a leucine aminopeptidase (*PfM17LAP*), an alanine aminopeptidase (*PfM1MAA*), and an aspartyl aminopeptidase (*PfM18AAP*). The aminopeptidases *PfM1MAA* and *PfM17LAP* have been shown to be essential for the survival of the parasite; inhibitors of these enzymes kill malaria parasites in culture and in rodent models of malaria (*P. chabaudi*) [9,10].

The *PfM18AAP* gene is present as a single copy on the *Pf* genome. The *PfM18AAP* protein is a ~ 65 kDa metallo-aminopeptidase of the M18 family of metalloproteases [11]. Unlike for the other aminopeptidases, few aspartyl aminopeptidases (M18AAP) have been characterised. Most information has been derived from studies of the aspartyl aminopeptidase of mammals [12], yeast [13] and bacteria [14]. Because of their highly restricted specificities for the N-terminal acidic amino acids, aspartic and glutamic acid, which cannot be cleaved by any other aminopeptidases, aspartyl aminopeptidase are believed to act together with other aminopeptidases to facilitate protein turnover. In humans, a more specific function in the conversion of angiotensin II to the vasoactive angiotensin III within the brain has been implicated [12,15].

Dalton has produced functionally active recombinant forms of the *PfM18AAP*, *PfM17LAP*, and *PfM1MAA* enzymes that exhibit comparable properties to the native forms in malaria cytosolic extracts. Dalton has also produced a functionally active recombinant form of the human M18

enzyme [16, 17]. Studies show that the *PfM18AAP* is expressed in the parasite cytosol and exported to the parasitophorous vacuole of the parasite indicating that while the enzyme may function in the final stages of hemoglobin digestion it may also have an additional function outside the parasite. Antisense-mediated inhibition of the *PfM18AAP* results in a lethal phenotype as a result of significant morphological changes to the parasite, and, therefore, suggests the enzyme as a promising target for new anti-malarial drug development.

Currently, there are very few small molecule inhibitors of *PfM18AAP*. Previous reports have identified phosphinic and phosphonic acid analogs of glutamic and aspartic acid, GluP and AspP, respectively, as modestly active amino-acid-derived inhibitors of *PfM18AAP in vitro* [16]. However, these amino acid derivatives do not reduce malaria growth in culture when tested at concentrations of up to 100  $\mu$ M. Thus, the objective of this effort was to identify novel and selective (against *rPfM17LAP*, *rPfM1MAA*, and recombinant human M18) small molecules that inhibit the activity of recombinant and extracted *PfM18AAP* in biochemical assays that also prevent the growth of the parasite in culture.

## 2 Materials and Methods

See subsections for a detailed description of the materials and methods used for each assay.

### Recombinant enzymes

Recombinant *PfM18AAP*, *PfM17LAP*, *PfM1AAP*, and human M18 were prepared at the University of Technology, Sydney, as described previously [16,17]. Recombinant cathepsin L of the parasitic helminth *Fasciola hepatica* was prepared in the same laboratory as described previously [18].

### Native *PfM18AAP* enzyme

Parasite extracts containing *PfM18AAP* were prepared at the University of Technology, Sydney, as described previously [16].

### 2.1 Assays

A list of the relevant assays and corresponding PubChem assay identifier numbers (AIDs) is provided in the **Appendix A (Table A1)**. Please see the **Appendix B** for the detailed assay protocols.

#### 2.1.1 *rPfM18AAP* QFRET-based Primary Biochemical High Throughput Assay (Primary Assay AID No. 1822)

The purpose of this assay is to identify compounds that inhibit the activity of M18 aminopeptidase of the malaria parasite *Plasmodium falciparum* (*PfM18AAP*). In this biochemical assay, a commercially available fluorogenic peptide substrate (H-Glu-NHMec) is incubated with purified recombinant *PfM18AAP* enzyme (*rPfM18AAP*) in the presence of test compounds. Cleavage of the substrate by *rPfM18AAP* enzyme liberates the NHMec leaving group from the peptide, leading to increased well fluorescence as measured at 340 nm excitation and 450 nm

emission. As designed, compounds that inhibit *PfM18AAP* will block *rPfM18AAP*-mediated cleavage of H-Glu-NHMec and liberation of the NHMec leaving group from the substrate, resulting in decreased well fluorescence. Test compounds were assayed in singlicate at a final nominal concentration of 7.35 micromolar. This assay was performed at the Scripps Research Institute Molecular Screening Center.

### **2.1.2 CTSL1 QFRET-based Counterscreen Biochemical High Throughput Assay (Primary Assay AID No. 1906)**

The purpose of this assay is to identify compounds that inhibit the activity of recombinant *Fasciola hepatica* cathepsin L1 expressed in yeast. Like the HTS for *PfM18AAP* this assay also exploits fluorogenic enzyme substrates. This assay also serves as a counterscreen for a set of previous experiments entitled, "Fluorescence-based primary biochemical high throughput screening assay to identify inhibitors of the *Plasmodium falciparum* M18 Aspartyl Aminopeptidase (*rPfM18AAP*)" (AID 1822). In this biochemical assay, a commercially available fluorogenic peptide substrate (Z-Leu-Arg-MCA) is incubated with purified recombinant cathepsin L1 protein in the presence of test compounds. Cleavage of the substrate by cathepsin L1 releases the fluorogenic MCA leaving group, leading to an increase in well fluorescence. As designed, compounds that inhibit cathepsin L1 will prevent substrate cleavage and liberation of the fluorescent leaving group, resulting in decreased well fluorescence. Test compounds were assayed in singlicate at a final nominal concentration of 5.96 micromolar. This assay was performed at the Scripps Research Institute Molecular Screening Center.

### **2.1.3 rPfM18AAP QFRET-based Confirmatory Biochemical High Throughput Assay (Primary Assay AID No. 2170)**

The purpose of this assay is to confirm activity of compounds identified as active in a set of previous experiments entitled, "QFRET-based primary biochemical high throughput screening assay to identify inhibitors of the *Plasmodium falciparum* M18 Aspartyl Aminopeptidase (*rPfM18AAP*)" (AID 1822). In this biochemical assay, a commercially available fluorogenic peptide substrate (H-Glu-NHMec) is incubated with purified recombinant *PfM18AAP* enzyme (*rPfM18AAP*) in the presence of test compounds. Cleavage of the substrate by *rPfM18AAP* enzyme liberates the NHMec leaving group from the peptide, leading to increased well fluorescence. As designed, compounds that inhibit *PfM18AAP* will block *rPfM18AAP*-mediated cleavage of H-Glu-NHMec and liberation of the NHMec leaving group from the substrate, resulting in decreased well fluorescence as measured at 340 nm excitation and 450 nm emission. Test compounds were assayed in triplicate at a final nominal concentration of 7.35 micromolar. This assay was performed at the Scripps Research Institute Molecular Screening Center.

### **2.1.4 CTSL1 QFRET-based Confirmatory Counterscreen Biochemical High Throughput Assay (Primary Assay AID No. 2178)**

The purpose of this counterscreen assay is to determine whether compounds identified as active in a set of previous experiments entitled, "QFRET-based primary biochemical high throughput screening assay to identify inhibitors of the *Plasmodium falciparum* M18 Aspartyl

Aminopeptidase (r*Pf*M18AAP)" (AID 1822) also inhibit cathepsin L1. This assay also serves to confirm activity of compounds identified as active in a set of previous experiments entitled, "QFRET-based counterscreen for r*Pf*M18AAP inhibitors: biochemical high throughput screening assay to identify inhibitors of the Cathepsin L proteinase (CTSL1)" (AID 1906). In this biochemical assay, a commercially available fluorogenic peptide substrate (Z-Leu-Arg-MCA) is incubated with purified recombinant cathepsin L1 protein in the presence of test compounds. Cleavage of the substrate by cathepsin L1 releases the fluorogenic MCA leaving group, leading to an increase in well fluorescence. As designed, compounds that inhibit cathepsin L1 will prevent substrate cleavage and liberation of the fluorescent leaving group, resulting in decreased well fluorescence. Test compounds were assayed in triplicate at a final nominal concentration of 5.96 micromolar. This assay was performed at the Scripps Research Institute Molecular Screening Center.

#### **2.1.5 r*Pf*M18AAP QFRET-based Confirmatory Concentration-response Biochemical High Throughput Assay (Primary Assay AID No. 2195)**

The purpose of this assay is to determine dose response curves for compounds identified as active in a set of previous experiments entitled, "QFRET-based biochemical high throughput confirmation assay for inhibitors of the *Plasmodium falciparum* M18 Aspartyl Amino peptidase (r*Pf*M18AAP)" (AID 2170). In this biochemical assay, a commercially available fluorogenic peptide substrate (H-Glu-NHMec) is incubated with purified recombinant *Pf*M18AAP enzyme (r*Pf*M18AAP) in the presence of test compounds. Cleavage of the substrate by r*Pf*M18AAP enzyme liberates the NHMec leaving group from the peptide, leading to increased well fluorescence. As designed, compounds that inhibit *Pf*M18AAP will block r*Pf*M18AAP-mediated cleavage of H-Glu-NHMec and liberation of the NHMec leaving group from the substrate, resulting in decreased well fluorescence as measured at 340 nm excitation and 450 nm emission. Test compounds were assayed in triplicate in a 10-point 1:3 dilution series starting at a nominal test concentration of 73.5 micromolar. This assay was performed at the Scripps Research Institute Molecular Screening Center.

#### **2.1.6 CTSL1 QFRET-based Confirmatory Counterscreen Concentration-response Biochemical High Throughput Assay (Primary Assay AID No. 2196)**

The purpose of this counterscreen assay is to determine dose response curves for compounds identified as active in a set of previous experiments entitled, "QFRET-based biochemical high throughput confirmation assay for inhibitors of the *Plasmodium falciparum* M18 Aspartyl Amino peptidase (r*Pf*M18AAP)" (AID 2178). In this biochemical assay, a commercially available fluorogenic peptide substrate (Z-Leu-Arg-MCA) is incubated with purified recombinant cathepsin L1 protein in the presence of test compounds. Cleavage of the substrate by cathepsin L1 releases the fluorogenic MCA leaving group, leading to an increase in well fluorescence. As designed, compounds that inhibit cathepsin L1 will prevent substrate cleavage and liberation of the fluorescent leaving group, resulting in decreased well fluorescence. Test compounds were assayed in triplicate in a 10-point 1:3 dilution series starting at a nominal test concentration of 59.6 micromolar. This assay was performed at the Scripps Research Institute Molecular Screening Center.

### **2.1.7 Fluorescence-based rPfM18AAP Confirmatory Biochemical Assay (Hit Validation Assay AID No. 492974)**

The purpose of this assay is to determine inhibitory activity of powder samples of compounds for recombinant *P. falciparum* M18AAP. In this assay, a fluorogenic peptide substrate (H-Glu-NHMec) that binds to the active site of rPfM18AAP was used to quantify the activity of rPfM18AAP in the presence of inhibitor compounds. The rate of hydrolysis of this substrate in the presence of 5 uM inhibitor compounds was measured by monitoring the release of the -NHMec fluorogenic leaving group at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. As designed, compounds that bind to rPfM18AAP will compete with binding of the fluorogenic peptide substrate, resulting in a decrease in the release of the fluorogenic leaving group and a decrease in well fluorescence. This assay was performed in the labs of the assay provider, John P. Dalton.

### **2.1.8 Fluorescence-based Malarial Cell Lysate PfM18AAP Confirmatory Biochemical Assay (Hit Validation Assay AID No. 492975)**

The purpose of this assay is to determine inhibitory activity of powder samples of compounds for PfM18AAP in a malarial cell lysate. In this assay, a fluorogenic peptide substrate (H-Glu-NHMec) that binds to the active site of PfM18AAP was used to quantify the activity of PfM18AAP in malaria cell lysate in the presence of inhibitor compounds. The rate of hydrolysis of this substrate in the presence of 5 uM inhibitor compounds was measured by monitoring the release of the -NHMec fluorogenic leaving group at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. As designed, compounds that bind to PfM18AAP will compete with binding of the fluorogenic peptide substrate, resulting in a decrease in the release of the fluorogenic leaving group and a decrease in well fluorescence. This assay was performed in the labs of the assay provider, John P. Dalton.

### **2.1.9 Radiolabel-based Pf Parasite Growth Cell-based Assay (Hit Validation and SAR Assays AID No. 489015)**

The purpose of this assay is to determine the ability of powder samples of inhibitor compounds to inhibit the growth of *Plasmodium falciparum* in its asexual erythrocytic stage. In this assay, compounds are incubated with *P. falciparum*-infected red blood cells (RBC) in hypoxanthine-free media. <sup>3</sup>H-hypoxanthine is added, cells incubated 48 hours, and incorporation of <sup>3</sup>H determined. As designed, compounds that inhibit the growth of *P. falciparum* in RBC will decrease the level of <sup>3</sup>H incorporated. Test compounds were assayed in triplicate at 10 micromolar, and each compound was tested in two or three experiments. For the purposes of hit validation and SAR optimization, this assay was performed in the labs of the assay provider, Donald Gardiner.

### **2.1.10 Radiolabel-based Pf Parasite Growth Cell-based Assay (Hit Validation and SAR Assays AID No. 489011)**

The purpose of this assay is to determine the potency of a powder sample of an inhibitor compound identified in a previous assay to inhibit the growth of *Plasmodium falciparum* in its

asexual erythrocytic stage. In this assay, compounds are incubated with *P. falciparum*-infected red blood cells (RBC) in hypoxanthine-free media. <sup>3</sup>H-hypoxanthine is added, cells incubated 48 hours, and incorporation of <sup>3</sup>H determined. As designed, compounds that inhibit the growth of *P. falciparum* in RBC will decrease the level of <sup>3</sup>H incorporated. Compounds were tested in triplicate using a 5-point dilution series starting at a nominal concentration of 25 uM. For the purposes of hit validation and SAR optimization, this assay was performed in the labs of the assay provider, Donald Gardiner.

#### **2.1.11 rPfM18AAP QFRET-based Concentration-response Biochemical High Throughput Assay (SAR Assay AID Nos. 588678, 602222, 624177)**

The purpose of this assay is to determine concentration response curves for compounds to support on-target SAR studies. In this biochemical assay, a commercially available fluorogenic peptide substrate (H-Glu-NHMec) is incubated with purified recombinant PfM18AAP enzyme (rPfM18AAP) in the presence of test compounds. Cleavage of the substrate by rPfM18AAP enzyme liberates the NHMec leaving group from the peptide, leading to increased well fluorescence. As designed, compounds that inhibit PfM18AAP will block rPfM18AAP-mediated cleavage of H-Glu-NHMec and liberation of the NHMec leaving group from the substrate, resulting in decreased well fluorescence as measured at 340 nm excitation and 450 nm emission. Test compounds were assayed in triplicate in a 10-point 1:2 dilution series starting at a nominal test concentration of 100 micromolar. This assay was performed at the Southern Research Specialized Biocontainment Screening Center.

#### **2.1.12 rPfM1MAA QFRET-based Counterscreen Concentration-response Biochemical High Throughput Assay (Counterscreen Assay AID Nos. 588680, 602219, 624176)**

The purpose of this assay is to determine concentration response curves for compounds to support off-target-selectivity SAR studies. In this biochemical assay, a commercially available fluorogenic peptide substrate (H-Leu-NHMec) is incubated with purified recombinant PfM1MAA enzyme (rPfM1MAA) in the presence of test compounds. Cleavage of the substrate by rPfM1MAA enzyme liberates the NHMec leaving group from the peptide, leading to increased well fluorescence. As designed, compounds that inhibit PfM1MAA will block rPfM1MAA-mediated cleavage of H-Leu-NHMec and liberation of the NHMec leaving group from the substrate, resulting in decreased well fluorescence as measured at 340 nm excitation and 450 nm emission. Test compounds were assayed in triplicate in a 10-point 1:2 dilution series starting at a nominal test concentration of 100 micromolar. This assay was performed at the Southern Research Specialized Biocontainment Screening Center.

#### **2.1.13 rPfM17LAP QFRET-based Counterscreen Concentration-response Biochemical High Throughput Assay (Counterscreen Assay AID Nos. 588679, 602220, 624175)**

The purpose of this assay is to determine dose response curves for compounds to support off-target-selectivity SAR studies. In this biochemical assay, a commercially available fluorogenic peptide substrate (H-Leu-NHMec) is incubated with purified recombinant PfM17LAP enzyme (rPfM17LAP) in the presence of test compounds. Cleavage of the substrate by rPfM17LAP enzyme liberates the NHMec leaving group from the peptide, leading to increased well

fluorescence. As designed, compounds that inhibit *Pf*M17LAP will block *rPf*M17LAP-mediated cleavage of H-Leu-NHMec and liberation of the NHMec leaving group from the substrate, resulting in decreased well fluorescence as measured at 340 nm excitation and 450 nm emission. Test compounds were assayed in triplicate in a 10-point 1:2 dilution series starting at a nominal test concentration of 100 micromolar. This assay was performed at the Southern Research Specialized Biocontainment Screening Center.

#### **2.1.14 rhM18 QFRET-based Counterscreen Concentration-response Biochemical High Throughput Assay (Counterscreen Assay AID Nos. 588696, 602221, 624174)**

The purpose of this assay is to determine dose response curves for compounds to support off-target-selectivity SAR studies. In this biochemical assay, a commercially available fluorogenic peptide substrate (H-Glu-NHMec) is incubated with purified recombinant human M18 enzyme (rhM18) in the presence of test compounds. Cleavage of the substrate by rhM18 enzyme liberates the NHMec leaving group from the peptide, leading to increased well fluorescence. As designed, compounds that inhibit hM18 will block rhM18-mediated cleavage of H-Glu-NHMec and liberation of the NHMec leaving group from the substrate, resulting in decreased well fluorescence as measured at 340 nm excitation and 450 nm emission. Test compounds were assayed in triplicate in a 10-point 1:2 dilution series starting at a nominal test concentration of 100 micromolar. This assay was performed at the Southern Research Specialized Biocontainment Screening Center.

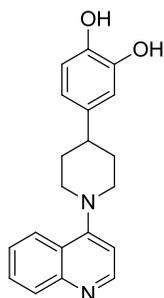
#### **2.1.15 Vero Cell-based Concentration-response Cytotoxicity Assay (Counterscreen Assay AID Nos. 588714, 602225, 624205)**

In this assay, Vero E6 cells were treated with compounds selected as active in the *Pf*M18AAP assay for 72 hours over a 10 point 2-fold dilution series, ranging from 0.19  $\mu$ M to 100  $\mu$ M. Following 72 hours of treatment, relative viable cell number was determined using Cell Titer Glo from Promega. Each plate contained 64 replicates of vehicle treated cells which served as negative controls. This assay was performed at the Southern Research Specialized Biocontainment Screening Center.

## **2.2 Probe Chemical Characterization**

ML369 was synthesized in two steps from commercially available materials and 72% overall yield as described in **Section 2.3**. Analytical characterization data ( $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and HRMS) were all consistent with the structure. The compound is 100% pure as measured on the basis of peak integration (area under the curve) from UV-Vis absorbance at 214 nm as determined using RP HPLC/UV/MS. The procedure for the synthesis of ML369 is found in **Appendix C** and spectral data are provided in **Appendix E**.

## Probe Chemical Structure and Properties:



ML369

PubChem CID: 56846691  
Molecular Weight: 320.385  
Exact Mass: 320.1525  
Molecular Formula: C<sub>20</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub>  
CLogP: 3.56  
H-Bond Donor: 2  
H-Bond Acceptor: 4  
Rotatable Bonds: 2  
Topological Polar Surface Area: 56.06

### Aqueous Solubility:

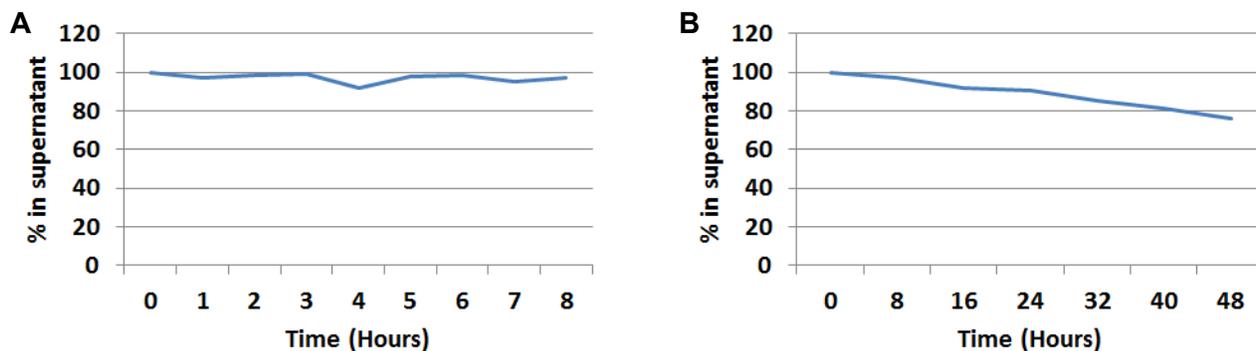
The aqueous solubility for the probe was measured in phosphate-buffered saline (PBS) at room temperature (23 °C). PBS by definition is 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic at a pH 7.4. The probe compound ML369 was found to have a solubility of 2.9 µg/mL, or 9 µM, under these conditions, which is 2X and 7X the IC<sub>50</sub>s in the biochemical and cell-based r*Pf*M18AAP and *Pf*M18AAP inhibition assays, respectively. See the **Appendix D** for the detailed procedure for measuring aqueous solubility.

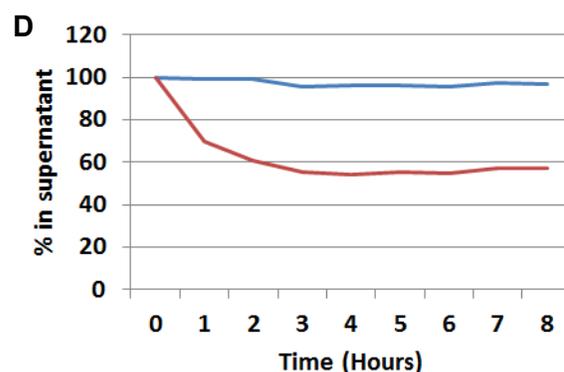
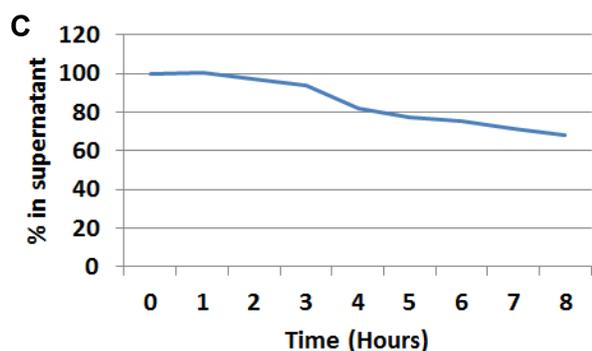
### Aqueous Stability & Thiol Stability:

The aqueous stability for the probe compound was assessed in 1:1 acetonitrile:PBS (no antioxidants or other protectants, DMSO concentration 1%, room temperature) and the results are reported in **Figure 2.2.1**. The probe compound ML369 was found to be moderately stable in 1:1 acetonitrile:PBS, whereby 75% of the compound remained after 48 hours, which is the timeframe for the radiolabel-based *Pf* parasite growth cell-based inhibition assay (**Figure 2.2.1, B**). See the **Appendix D** for the detailed procedure for measuring aqueous stability.

The thiol stability for the probe compound was assessed in 1:1 acetonitrile:PBS and the results are reported in **Figure 2.2.1**. The probe compound ML369 was found to be moderately stable to DTT, whereby 70% of the compound remained after 8 hours (**Figure 2.2.1, C**). See the **Appendix D** for the detailed procedure for measuring thiol stability.

**Figure 2.2.1** Stability of ML369 in 1:1 acetonitrile:PBS & DTT Stability Assay





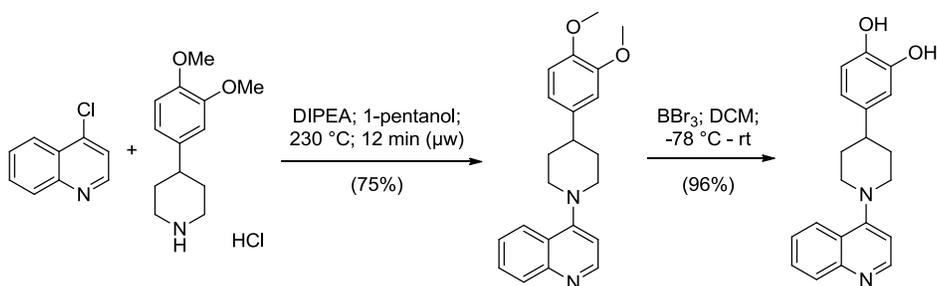
ML369 (CID56846691, SID135378316) was tested over a time course in an aqueous stability assay and thiol stability assays. (A) Stability of the probe over 8 hours in 1:1 acetonitrile:PBS; (B) Stability of the probe over 48 hours in 1:1 acetonitrile:PBS; (C) Stability of the probe to DTT over 8 hours; (D) Stability of Ethacrynic Acid (positive control for the thiol reaction) in the absence (blue) and presence (red) of DTT.

### Submission of the Probe and Analogues to the NIH MLSMR:

Samples of the probe and five analogues were submitted to the NIH MLSMR compound collection on March 21, 2013. Please see the **Table I1, Appendix I**, for the compound ID information.

### 2.3 Probe Preparation

ML369 was synthesized as shown in **Scheme 1**.  $S_NAr$  reaction afforded the O,O-dimethyl catechol-containing compound, which was deprotected to the catechol ML369 using boron tribromide.



**Scheme 1.** Synthesis of Probe ML369

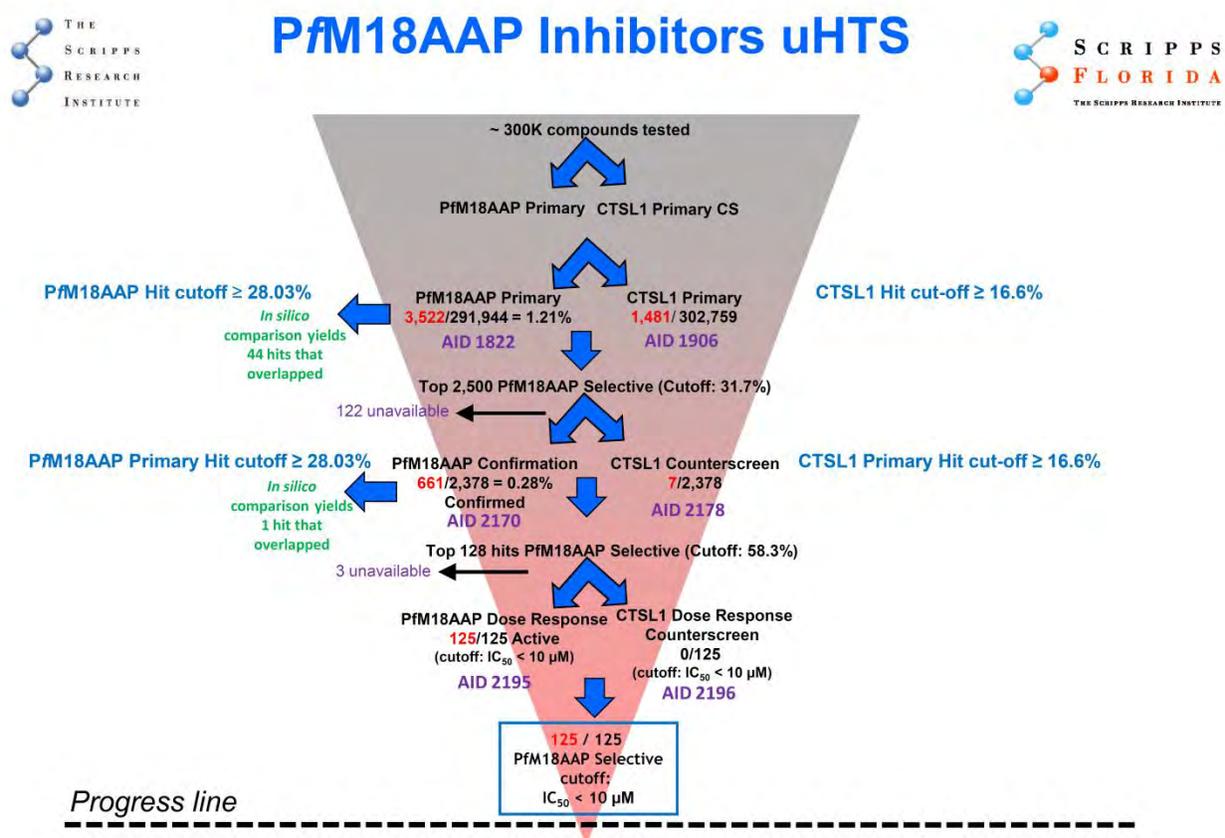
Full experimental details for the preparation of ML369 are provided in **Appendix C**.

### 3 Results

#### 3.1 Summary of Screening Results

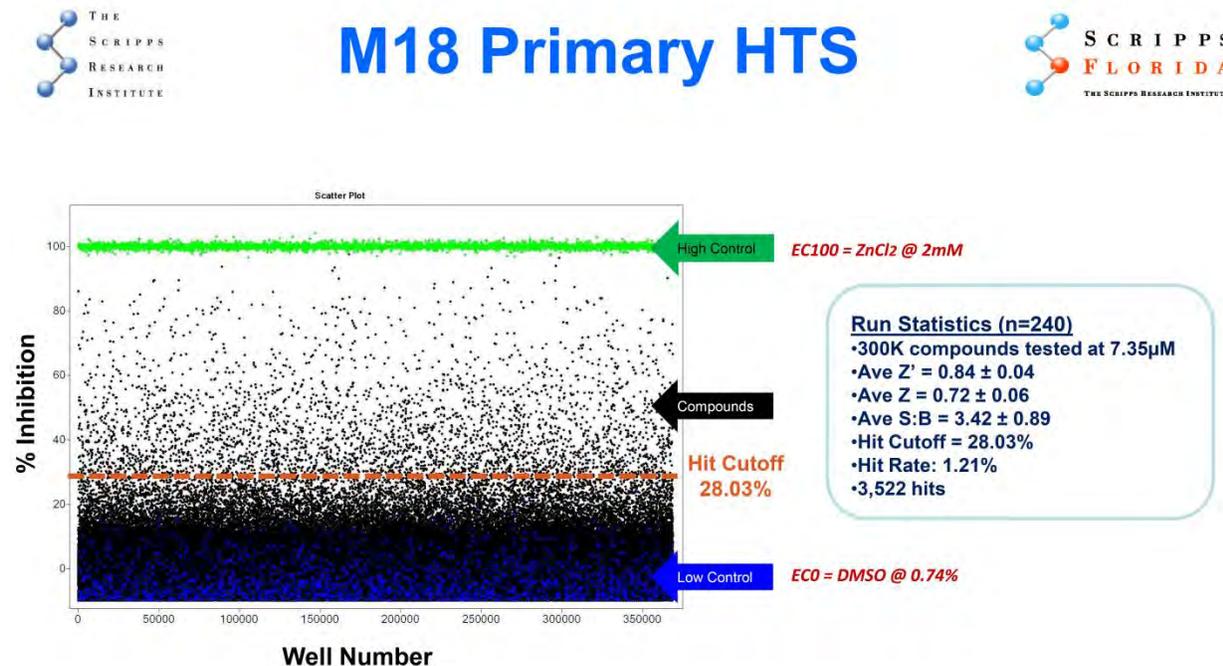
Assay validation and high-throughput screening for the project were carried out as a collaboration between the screening center, the Scripps Research Institute Molecular Screening Center, and the assay providers, John P. Dalton and Donald L. Gardiner. Professor Dalton and his team produced, purified, and functionally characterized the recombinant *Plasmodium falciparum* (rPfM18AAP) and recombinant cathepsin L (rCTSL1) enzymes which were used by the SRIMSC team. The following flowchart summarizes the results for the high-throughput screening campaign (**Figure 3.1.1**).

**Figure 3.1.1.** Critical Path for Primary Screening



The approximately 300k compounds in the NIH Molecular Libraries Small Molecule Repository (MLSMR) were screened in singlicate at 7.35 μM. The results and statistics for the screen are described schematically in the following figure (**Figure 3.1.2**).

Figure 3.1.2. Primary Screening Statistics



Of the approximately 300k compounds screened for rP<sub>M</sub>18AAP inhibition at 7.35 µM, 3,522 compounds showed inhibition >28% and were considered to be active. These screening results are captured in PubChem AID 1822. Of the approximately 300k compounds screened for cathepsin L1 inhibition in singlicate at 5.96 µM, 1,481 compounds showed inhibition >16% and were considered to be active. These screening results are captured in PubChem AID 1906.

The most active 2,500 compounds from the primary screen for rP<sub>M</sub>18AAP inhibition, which were considered to be inactive in the primary counterscreen against capthepsin L1, were ordered from the NIH MLSMR as DMSO stock solutions, and 2,378 of the 2,500 compounds were available and were delivered to the SRIMSC for confirmatory screening.

Of the 2,378 compounds screened for rP<sub>M</sub>18AAP inhibition in triplicate at 7.35 µM, 661 compounds showed inhibition >28% and were considered to be active. These screening results are captured in PubChem AID 2170. Of the 2,378 compounds screened against cathepsin L1 in triplicate at 5.96 µM, 7 compounds showed inhibition >16% and were considered to be active. These screening results are captured in PubChem AID 2178.

The most active 128 compounds from the confirmatory, single-concentration-in-triplicate screen for rP<sub>M</sub>18AAP inhibition, which were inactive in the confirmatory, single-concentration-in-triplicate counterscreen for cathepsin L1 inhibition, were ordered from the NIH MLSMR as DMSO stock solutions, and 125 of the 128 compounds were available, and were delivered to the SRIMSC for concentration-response confirmatory screening.

Of the 125 compounds screened for rP<sub>M</sub>18AAP inhibition in 10-point concentration-response between 0.0037 and 73.5 µM, all 125 showed IC<sub>50</sub>s < 10 µM, and were considered to be active.

These screening results are captured in the PubChem AID 2195. Of the 125 compounds screened against cathepsin L1 in 10-point concentration-response between 0.003 and 59.6  $\mu\text{M}$ , none of the 125 showed  $\text{IC}_{50}\text{s} < 10 \mu\text{M}$ , and all were considered to be inactive. These assay results are captured in the PubChem AID 2196.

Hit validation for the project using compound samples from the solid physical state was carried out as a collaboration between the chemistry center, KU SCC, and the assay providers, John P. Dalton and Donald L. Gardiner. In parallel, the Dalton team performed percent-inhibition biochemical assays using recombinant *PfM18AAP* and *PfM18AAP* in soluble extracts of the malaria parasite, while the Gardiner team performed percent-inhibition and concentration-response parasite growth inhibition cell-based assays.

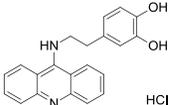
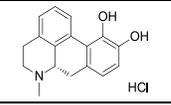
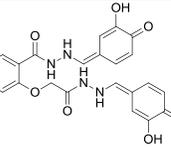
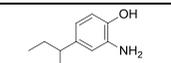
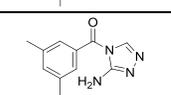
All of the 125 compounds considered active against r*PfM18AAP* and inactive against cathepsin L1 were suggested for follow-up hit validation using compound samples from the solid physical state. While many of the 125 compounds are considered PAINS [19,20], the assay providers suggested that many compounds used to treat malaria might be considered PAINS and that PAINS should not be excluded from hit validation. Of the 125 compounds, 76 compounds were purchased or synthesized, purified, analyzed, and shipped by the University of Kansas Specialized Chemistry Center to the assay providers, John P. Dalton and Donald L. Gardiner.

Of the 76 compounds screened for confirmatory activity against the recombinant *PfM18AAP* in singlicate at 5  $\mu\text{M}$ , 22 compounds showed inhibition  $>50\%$  and were considered to be active. These screening results are captured in the PubChem AID 492974. Of the 60 compounds screened for confirmatory activity against the *PfM18AAP* in soluble extracts of malaria in singlicate at 5  $\mu\text{M}$ , 28 compounds showed inhibition  $>50\%$  and were considered active. These screening results are captured in PubChem AID 492975.

Of the 76 compounds screened for parasite growth inhibition in triplicate at 10  $\mu\text{M}$ , 8 compounds showed inhibition  $>50\%$  and were considered to be active. These screening results are captured in the PubChem AID 489015. Of the 8 compounds screened for parasite growth inhibition in 5-point concentration-response between 1 and 25 micromolar, 5 showed  $\text{IC}_{50}\text{s} < 5 \mu\text{M}$ , and were considered to be active. These screening results are captured in PubChem AID 489011.

The top 5 compounds of interest resulting from the primary screening and hit validation studies are shown in the **Table 3.1.1**. In selecting a compound chemotype for medicinal chemistry optimization, the aqueous solubility and biological promiscuity (i.e., lack of biological promiscuity, as determined from a survey of PubChem) for the top 5 compounds were considered in addition to the requirement for selectivity and reasonable activity in the biochemical and cell-based assays. Ultimately, the compound CID 23724194 (Entry 1) was selected by the greater team for medicinal chemistry optimization. One structural feature worth noting, common to all of the top compounds of interest, is a putative zinc-binding domain (i.e., catechol or other), consistent with the nature of *PfM18AAP* as a metallo-aminopeptidase.

**Table 3.1.1.** Top 5 compounds of interest from the primary screening and hit validation studies.

Entry	CID / SID	Compound Hit Structure	rPM18 %Inh (5 $\mu$ M)	<sup>†</sup> ePM18 %Inh (5 $\mu$ M)	Parasite Growth %Inh (10 $\mu$ M) (n)	Parasite Growth IC <sub>50</sub>	<sup>‡</sup> Solubility at pH 7.4 ( $\mu$ g/mL)	Promiscuity (PubChem)
1	23724194 / 92117383		52	#ND	96 (2)	< 1.6	44.7	13.7
2	6852389 / 87693049		35	84	87 (2)	4	12.5	12.1
3	11958779 / 92117384		76	ND	66 (2)	6	ND	13.0
4	100028 / 87693038		65	ND	95 (3)	6	ND	2.4
5	912604 / 87693047		0	11	45 (3)	7	ND	2.5

#ND = Not Determined. Data are the average of three replicates; n = number of repeat analyses (n = 1 by default).

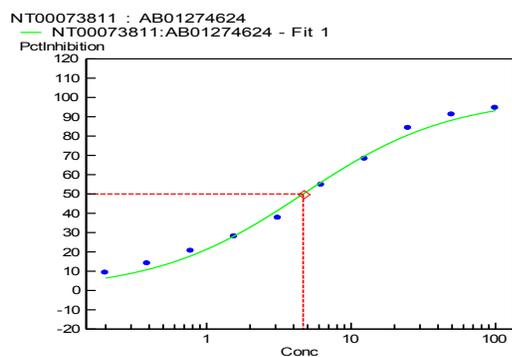
<sup>†</sup>ePM18AAP = PM18AAP in soluble extracts of the *Plasmodium falciparum* parasite.

<sup>‡</sup>As reported in PubChem AID 1996.

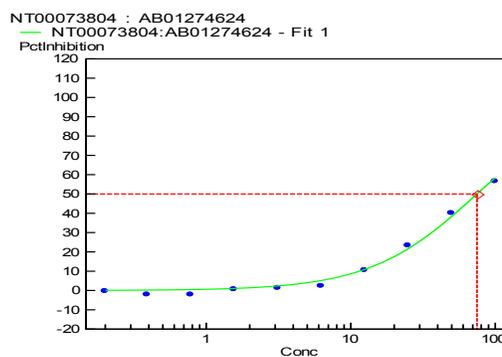
### 3.2 Dose Response Curves for Probe

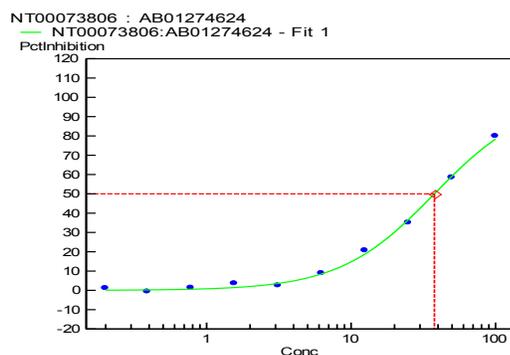
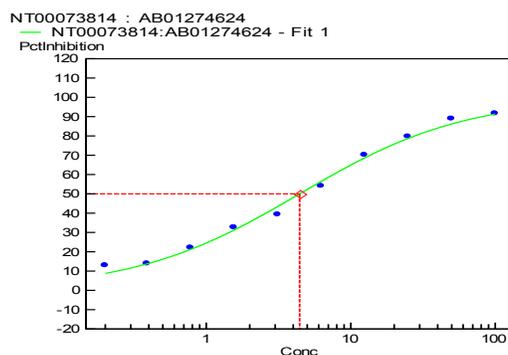
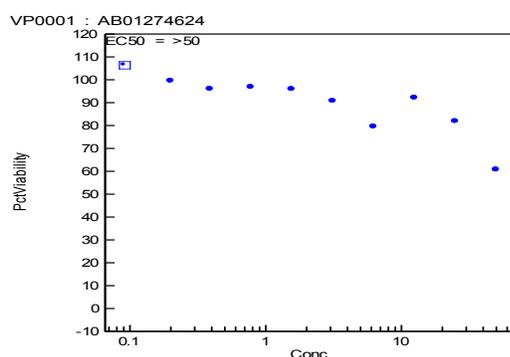
**Figure 3.2.1.** Concentration-response Curves for the Probe (ML369)

**A**



**B**



**C****D****E**

ML369 (CID56846691, SID135378316) was tested across a range of concentrations up to 100  $\mu\text{M}$  in the primary and several secondary assays. Concentration response data was analyzed using a four parameter logistic fit to the data (Excel Fit equation 205) with the maximum and minimum locked at 100 and 0. From these curves  $\text{IC}_{50}$  values were calculated. (A) *rPfM18AAP* biochemical assay (AID 624177),  $\text{IC}_{50} = 4.6 \mu\text{M}$ ; (B) *rPfM1MAA* biochemical assay (AID 624176),  $\text{IC}_{50} = 74.9 \mu\text{M}$ ; (C) *rPfM17LAP* biochemical assay (AID 624175),  $\text{IC}_{50} = 37.7 \mu\text{M}$ ; (D) *rhM18* biochemical assay (AID 624174)  $\text{IC}_{50} = 4.43 \mu\text{M}$ ; (E) Vero cell cytotoxicity assay (AID 624205)  $\text{EC}_{50} > 50 \mu\text{M}$

### 3.3 Scaffold/Moiety Chemical Liabilities

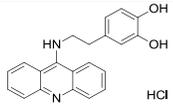
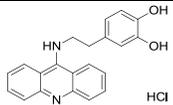
The aqueous solubility for ML369 was determined to be 2.9  $\mu\text{g/mL}$  (9.1  $\mu\text{M}$ ) in phosphate buffered saline (PBS, pH 7.4) containing 1% DMSO, which is 2X and 7X the  $\text{IC}_{50}$ s in the biochemical and cell-based *rPfM18AAP* and *PfM18AAP* inhibition assays, respectively. The probe is stable in 1:1 acetonitrile:PBS, with 100% remaining after 8 hours (see **Figure 2.2.1 A**) and 75% remaining after 48 hours, which is the timeframe for the radiolabel-based *Pf* parasite growth cell-based inhibition assay (see **Figure 2.2.1 B**). The probe was determined to be moderately stable to thiol (DTT), with 70% remaining after 8 hours (see **Figure 2.2.1 C**). See the **Appendix D** for the experimental procedures for aqueous solubility, aqueous stability, and thiol stability measurements. While the catechol functional group may be a liability due to its perceived toxicity, a number of currently prescribed drugs contain the catechol functional group, and methods of circumventing the toxicity of catecholics have been suggested [21].

### 3.4 SAR Tables

SAR optimization for the project was carried out as a collaboration between the chemistry center, the KU SCC, the assay providers, John P. Dalton and Donald L. Gardiner, and the screening center, the Southern Research Specialized Biocontainment Screening Center. In parallel, the Gardiner team performed percent inhibition and concentration-response parasite growth inhibition cell-based assays, while the SRSBSC team performed the biochemical *rPfM18AAP*, *rPfM1MAA*, *rPfM17LAP* and *hM18* assays, and the Vero cell cytotoxicity assays.

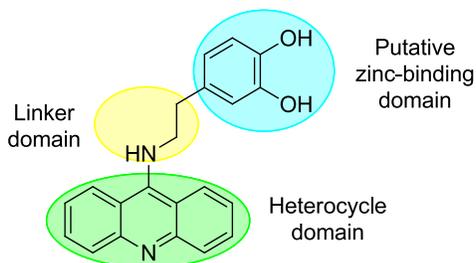
During the primary screening and compound hit validation stages of the project, the main drivers for compound prioritization were activity against the *rPfM18AAP* and *ePfM18AAP* in biochemical assays, selectivity against cathepsin L1 in a biochemical assay, activity against the malaria parasite in a red blood cell cell-based assay, and, finally, acceptable aqueous solubility and biological promiscuity. Based on these considerations, the catechol-containing compound CID 23724194 was chosen for medicinal chemistry optimization. The main project drivers during the SAR optimization stage of the project were activity against the *rPfM18AAP* in a biochemical assay, selectivity against *rPfM1MAA*, *rPfM17LAP* and *rhM18* in biochemical assays, activity against the malaria parasite in a red blood cell cell-based assay, and lack of activity in a Vero cell cytotoxicity assay. While achieving 10-fold selectivity for the *rPfM18AAP* versus the *rPfM1MAA* and *rPfM17LAP* was considered to be quite likely, achieving selectivity against the *rhM18* was considered unlikely. Although there is only a low level of amino acid sequence identity between the *rPfM18AAP* enzyme and the human orthologue (18%), suggesting that selectivity of compound binding for the malaria enzyme might be achieved, significant conservation exists between the active sites of the malaria and human enzymes. For example, three histidine residues (His-94, His-170, and His-440) which are predicted from site-directed mutagenesis studies to be critical for enzymatic activity and another (His-352) essential for stabilization of the quaternary structure of human M18 are conserved in the malaria enzyme. In fact, when the compound CID 23724194/CID 53464134 was tested across the suite of assays that would be used to drive SAR, the above predictions were substantiated, as modest selectivity was observed for the inhibition of *rPfM18AAP* versus *rPfM1MAA* and *rPfM17LAP*, and poor selectivity was observed versus the human M18 orthologue (**Table 3.4.1**).

**Table 3.4.1.** Activity for CID 23724194, the starting point for SAR optimization

CID / SID	Structure	Biochemical Target	Biochemical Anti-target				Red Blood Cell-based Target		Cytotoxicity
		<i>rPfM18AAP</i> IC <sub>50</sub> (μM)	<i>rPfM1MAA</i> IC <sub>50</sub> (μM)	<i>rPfM17LAP</i> IC <sub>50</sub> (μM)	<i>rhM18</i> IC <sub>50</sub> (μM)	<i>Pf</i> Growth %Inh @ 10 μM	<i>Pf</i> Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)	
23724194 / 92117383		ND	ND	ND	ND	96	<1.6	ND	
53464134 / 126490285		4.3	29.8	14.2	1.0	ND	ND	38.13 (n=2)	

ND = Not Determined. Data are the average of three replicates; n = number of repeat analyses (n = 1 by default).

From this starting point, the team's goal was to improve upon CID 23724194 over two rounds of SAR optimization. For the purpose of SAR optimization, the team divided the compound into three domains: (i) a putative zinc-binding domain, (ii) a linker domain, and (iii) a heterocycle domain.



For the first round of SAR the team chose to survey the effect of gross structural changes to these three domains, in isolation, and in combination. As shown in **Table 3.4.2**, while changes to the heterocycle domain were tolerated, none of the changes led to increased activity against *rPfM18AAP*. Although the selectivity against *rPfM1MAA* and *rPfM17LAP* was largely unchanged, the selectivity against *rhM18* was observed to increase quite dramatically for alternative heterocycles (**Table 3.4.2**, entries 1 and 5).

As shown in **Table 3.4.3**, changes to the length of the linker domain had a profound effect on activity across the biochemical assays. Shortening the linker by one carbon atom abolished activity across the biochemical assays (**Table 3.4.3**, entry 1) and lengthening the linker by one carbon atom diminished the activity across the biochemical assays (**Table 3.4.3**, entry 2).

For the compounds listed in **Table 3.4.4**, changes to the putative zinc-binding domain were explored. Exchanging the catechol for a phenol resulted in complete loss of activity across the biochemical assays (**Table 3.4.4**, entry 1). Replacing the catechol moiety using the 3-hydroxy-4-pyridone moiety, a zinc-binding group popularized by Cohen [22], also resulted in complete loss of activity across the biochemical assays (**Table 3.4.4**, entry 2). Replacing the catechol moiety with the carboxyl group resulted in complete loss of activity across the biochemical assays, too (**Table 3.4.4**, entries 3 and 4).

As shown in **Table 3.4.5**, as was the case for changes to the heterocycle domain only (**Table 3.4.2**), while simultaneous changes to the heterocycle and linker domains were tolerated, none of the changes led to increased activity against *rPfM18AAP*. Although the selectivity against *rPfM1MAA* and *rPfM17LAP* was largely unchanged, the selectivity against *hM18* was observed to increase quite dramatically (versus the initial compound hit) in some cases (**Table 3.4.5**, entries 3 and 5).

A curious discrepancy is apparent from inspection of the data in the **Tables 3.4.2, 3.4.3, 3.4.4, and 3.4.5**, which is that there does not appear to be a very good correlation between the activity of the compounds against *rPfM18AAP* and their activity in the parasite growth inhibition assay. One striking example is entry 2 in the **Table 3.4.4**, which shows no activity across the various biochemical assays and no cytotoxicity against Vero cells, yet has an  $EC_{50}$  of 6  $\mu$ M in the parasite growth inhibition assay. In general, across the larger set of compounds in these tables,

this discrepancy might be explained by potential off-target activity. With respect to entry 2, **Table 3.4.4**, specifically, it is possible that the activity observed for this compound might result from the off-target iron-chelating ability of the 3-hydroxy-4-pyridone moiety [23].

The curious discrepancy between the activity of some compounds against *rPfM18AAP* and their activity in the parasite growth inhibition assay was further highlighted by the purposeful circumstance whereby the Gardiner team performed percent inhibition and concentration-response parasite growth inhibition cell-based assays on compounds independent from the SRSBSC team, which performed the biochemical *rPfM18AAP*, *rPfM1MAA*, *rPfM17LAP* and *hM18* assays, and the Vero cell cytotoxicity assays. In other words, all compounds, even compounds that were inactive across the biochemical assays, were tested purposefully for inhibition of parasite growth. **Table 3.4.6** lists the biological activity for target compounds containing the catechol functional group and the respective penultimate target compounds containing the O,O-dimethyl catechol functional group from which the final compounds were prepared. In a number of cases, specifically for entries 5, 8, and 14, **Table 3.4.6**, even though the compounds were inactive across the various biochemical assays and non-toxic to Vero cells, the compounds showed significant activity in the parasite growth inhibition assay. With respect to these entries, it is possible that the activity observed for these compounds might result from off-target hemozoin capping, much as is the case for chloroquine, which shares the 4-aminoquinoline heterocycle with these compounds [24].

On the other hand, most importantly, a few compounds from the first round of SAR, specifically, entries 1 and 5, **Table 3.4.2**, and entry 5, **Table 3.4.5**, showed the expected correlation between activity in the biochemical assay and activity in the parasite growth inhibition assay (see **Table 3.4.6** and compare the activity for entries 2, 6, and 16 to the activities for their respective O,O-dimethyl catechol counterparts, listed in the rows immediately above each entry).

It should be noted, however, that the correlation between activity in the biochemical assays and the activity in the parasite growth inhibition assay may be confounded by the selective accumulation of appropriately basic inhibitor compounds in the low-pH [25] parasitophorous vacuole of the parasite [23], the site in which *PfM18AAP* is believed to function (the pH for the red blood cell cytoplasm and parasite cytoplasm is estimated as 7.0-7.2 and 6.8-7.0, respectively [26]).

With respect to the second round of SAR, the results for only a small subset of the compounds synthesized will be highlighted, where the results augment (as opposed to reproduce) the SAR story from the first round.

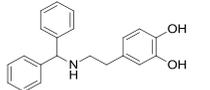
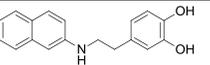
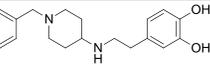
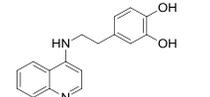
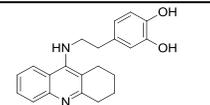
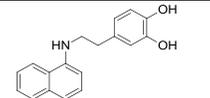
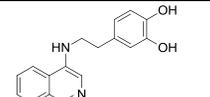
For the second round of SAR the team chose to build on the observation from the first round that selectivity against *rhM18* was observed to increase quite dramatically for alternative heterocycles (**Table 3.4.2**, entries 1 and 5) and for simultaneous changes to the heterocycle and linker domains (**Table 3.4.5**, entries 3 and 5). Armed with this specific information and the general precedent that conformational constraint may influence selectivity, compounds where the N-atom of the linker domain was joined to a carbon atom of the linker domain were targeted for synthesis. Examples of these compounds are shown in the **Table 3.4.7**. In fact, across the

three compounds shown in **Table 3.4.7**, the selectivity across the biochemical assays is improved, relative to the starting compound hit (**Table 3.4.1**).

The discrepancy between the activity of some compounds against *rPfM18AAP* and their activity in the parasite growth inhibition assay continued for this set of compounds, as may be seen in **Table 3.4.8**.

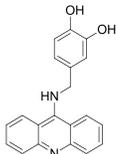
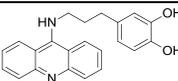
Based on these two rounds of SAR optimization, the compounds in **Table 3.4.9** were considered as probe candidates (catechol-containing compounds, only). While the activity and selectivity for the compounds varies across the biochemical assays, one constant is that none of the catechol-containing compounds is cytotoxic, as judged by the results for the Vero cell assay, and, more importantly, for three of the four compounds, the biochemical activity correlates relatively well with the activity observed in the cell-based parasite growth inhibition assay, suggesting that the activity for these compounds is on-target. Ultimately, the compound highlighted in this table (CID 56846691) was nominated as the probe (and assigned the NIH Molecular Libraries Initiative probe number ML369), based on the good activity against *rPfM18AAP*, good selectivity against the *rPfM1MAA* and *rPfM17LAP* enzymes, and lack of cytotoxicity, even though the compound showed no selectivity against the *rhM18*.

**Table 3.4.2.** Round 1 SAR and Changes to the Heterocycle Domain

Entry	CID / SID	P/S %Purity	Structure	Biochemical Target	Biochemical Anti-target			Red Blood Cell-based Target		Cytotoxicity
				rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)
1	53308670 / 124767902	S 76.6		10.5	55.1	46.8	41.4	52	14	41.64 (n=3)
2	53308684 / 124767875	S 96.8		11.6	34.6	39.7	34.0	20	ND	28.26
3	53308686 / 124767869	S 100		26.6	120.3	129.9	>50	93	6.5	>50
4	13162550 / 124767892	S 95.6		7.3	46.4	30.1	7.3	94	3.9	>50
5	53308667 / 124767868	S 97.3		10.2	35.0	61.8	>50	93	3.6	>50
6	53308675 / 124767874	S 99.2		5.4	29.9	26.9	30.4	19	ND	16.54
7	53308680 / 124767873	S 99.2		11.4	35.7	26.6	50.7	33	ND	37.68

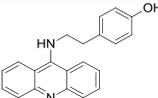
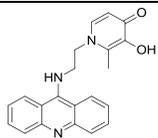
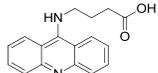
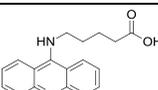
ND = Not Determined. Data are the average of three replicates; n = number of repeat analyses (n = 1 by default).

**Table 3.4.3.** Round 1 SAR and Changes to the Linker Domain

				Biochemical Target	Biochemical Anti-target				Red Blood Cell-based Target		Cytotoxicity
Entry	CID / SID	P/S %Purity	Structure	rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)	
1	53308666 / 124767867	S 98.2		>50	>50	>50	>50	98	2.9	7.78	
2	53308665 / 124767891	S 97.6		27.9	129.5	>50	>50	92	3.7	>50	

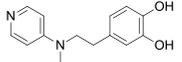
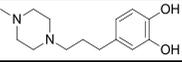
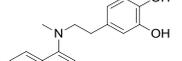
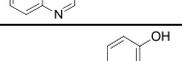
ND = Not Determined. Data are the average of three replicates; n = number of repeat analyses (n = 1 by default).

**Table 3.4.4.** Round 1 SAR and Changes to the Putative Zinc-binding Domain

				Biochemical Target	Biochemical Anti-target				Red Blood Cell-based Target		Cytotoxicity
Entry	CID / SID	P/S %Purity	Structure	rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)	
1	11522530 / 124767886	S 99.7		>50	>50	>50	>50	95	0.7	7.34	
2	53308669 / 124767866	S 100		>50	>50	>50	>50	86	6.1	>50	
3	610254 / 124767900	S 100		>50	>50	>50	>50	8	ND	>50	
4	10565734 / 124767899	S 97		>50	>50	>50	>50	24	ND	>50	

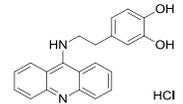
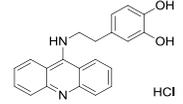
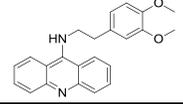
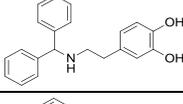
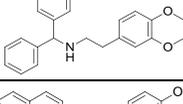
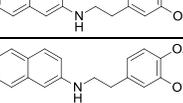
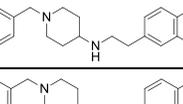
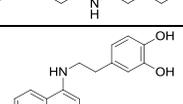
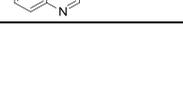
ND = Not Determined. Data are the average of three replicates; n = number of repeat analyses (n = 1 by default).

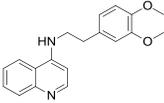
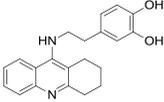
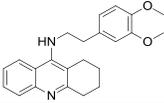
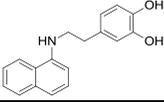
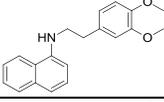
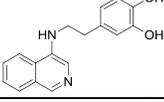
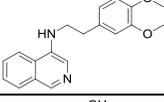
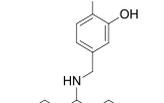
**Table 3.4.5.** Round 1 SAR and Simultaneous Changes to the Heterocycle and Linker Domains

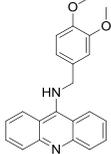
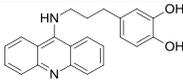
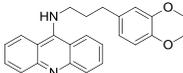
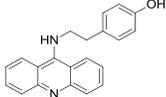
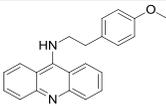
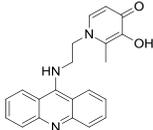
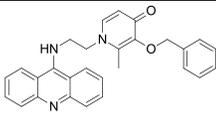
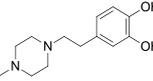
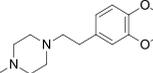
				Biochemical Target	Biochemical Anti-target				Red Blood Cell-based Target		Cytotoxicity
Entry	CID / SID	P/S %Purity	Structure	rPfM18AAP IC <sub>50</sub> (μM)	rPfM1AA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)	
1	53308664 / 124767897	S 97.6		8.4	45.2	116.8	>50	8	ND	>50	
2	53384805 / 125311612	S 92.3		13.3	>50	73.8	6.5	3	ND	>50	
3	53308676 / 124767890	S 99.8		4.9	19.7	18.0	31.1	93	4.7	>50	
4	53384806 / 125311611	S 100		27.1	>50	94.3	29.3	7	ND	>50	
5	53308668 / 124767872	S 98.6		22.3	77.1	58.6	>50	57	7.9	>50	

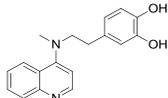
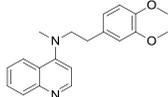
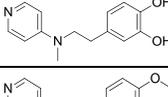
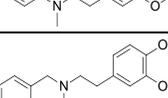
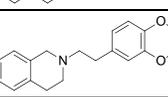
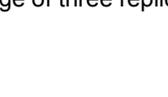
ND = Not Determined. Data are the average of three replicates; n = number of repeat analyses (n = 1 by default).

**Table 3.4.6.** Round 1 SAR and Final Catechol-containing and Penultimate O,O-Dimethyl Catechol-containing Compounds

				Biochemical Target	Biochemical Anti-target			Red Blood Cell-based Target		Cytotoxicity
Entry	CID / SID	P/S %Purity	Structure	rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)
Table 3.4.1	23724194 / 92117383	/ 100		ND	ND	ND	ND	ND	<1.6	ND
Table 3.4.1	53464134 / 126490285	P / 100		4.3	29.8	14.2	1.0	ND	ND	38.13 (n=2)
1	3815572 / 124767904	S 88.5		>50	>50	>50	>50	99	2.3	14.82 (n=3)
Table 3.4.2 Entry 1	53308670 / 124767902	S 76.6		10.5	55.1	46.8	41.4	52	14	41.64 (n=3)
2	318615 / 124767896	S 95.522		>50	>50	>50	>50	0	ND	39.225 (n=2)
Table 3.4.2 Entry 2	53308684 / 124767875	S 96.8		11.6	34.6	39.7	34.0	20	ND	28.26
3	53308685 / 124767885	S 93.1		>50	>50	>50	>50	2	ND	>50
Table 3.4.2 Entry 3	53308686 / 124767869	S 100		26.6	120.3	129.9	>50	93	6.5	>50
4	1131661 / 124767879	S 100		>50	>50	>50	>50	91	9.9	>50
Table 3.4.2 Entry 4	13162550 / 124767892	S 95.6		7.3	46.4	30.1	7.3	94	3.9	>50

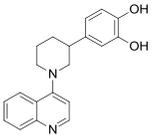
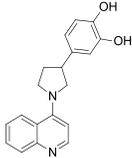
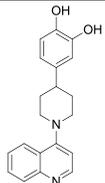
				Biochemical Target	Biochemical Anti-target			Red Blood Cell-based Target		Cytotoxicity
Entry	CID / SID	P/S %Purity	Structure	rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)
5	53308673 / 124767870	S 97.1		>50	>50	>50	>50	92	5.8	>50
Table 3.4.2 Entry 5	53308667 / 124767868	S 97.3		10.2	35.0	61.8	>50	93	3.6	>50
6	22023 / 124767880	S 98.923		>50	>50	>50	>50	47	20	>50
Table 3.4.2 Entry 6	53308675 / 124767874	S 99.2		5.4	29.9	26.9	30.4	19	ND	16.54
7	53308674 / 124767884	S 90.7		>50	>50	>50	>50	15	ND	>50
Table 3.4.2 Entry 7	53308680 / 124767873	S 99.2		11.4	35.7	26.6	50.7	33	ND	37.68
8	53308682 / 124767881	S 94.529		>50	134.8	>50	>50	64	15	>50
Table 3.4.3 Entry 1	53308666 / 124767867	S 98.2		>50	>50	>50	>50	98	2.9	7.78

Entry	CID / SID	P/S %Purity	Structure	Biochemical Target	Biochemical Anti-target			Red Blood Cell-based Target		Cytotoxicity
				rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)
9	53308662 / 124767894	S 97.9		>50	>50	>50	>50	100	1.1	5.37
Table 3.4.3 Entry 2	53308665 / 124767891	S 97.6		27.9	129.5	>50	>50	92	3.7	>50
10	53308661 / 124767876	S 97.99		>50	>50	>50	>50	92	2.2	21.83
Table 3.4.4 Entry 1	11522530 / 124767886	S 99.7		>50	>50	>50	>50	95	0.7	7.34
11	53308681 / 124767888	S 98.8		>50	>50	>50	>50	99	1.2	14.92
Table 3.4.4 Entry 2	53308669 / 124767866	S 100		>50	>50	>50	>50	86	6.1	>50
12	53308683 / 124767882	S 90.398		>50	>50	>50	>50	97	4	>50
Table 3.4.5 Entry 4	53384806 / 125311611	S 100		27.1	>50	94.3	29.3	7	ND	>50
13	20649668 / 124767877	S 97.308		>50	>50	>50	>50	3	ND	>50

				Biochemical Target	Biochemical Anti-target			Red Blood Cell-based Target		Cytotoxicity
Entry	CID / SID	P/S %Purity	Structure	rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)
Table 3.4.5 Entry 3	53308676 / 124767890	S 99.8		4.9	19.7	18.0	31.1	93	4.7	>50
14	53308677 / 124767903	S 87.3		>50	>50	>50	>50	95	11	>50
Table 3.4.5 Entry 1	53308664 / 124767897	S 97.6		8.4	45.2	116.8	>50	8	ND	>50
15	53308663 / 124767893	S 100		>50	>50	>50	>50	6	ND	>50
Table 3.4.5 Entry 5	53308668 / 124767872	S 98.6		22.3	77.1	58.6	>50	57	7.9	>50
16	12553367 / 124767883	S 100		>50	>50	>50	>50	2	ND	>50

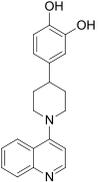
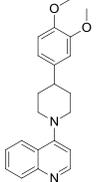
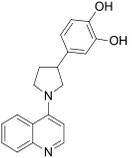
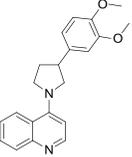
ND = Not Determined. Data are the average of three replicates; n = number of repeat analyses (n = 1 by default).

**Table 3.4.7.** Round 2 SAR and Constrained N-Atom/Linker Analogues

				Biochemical Target	Biochemical Anti-target			Red Blood Cell-based Target		Cytotoxicity
Entry	CID / SID	P/S %Purity	Structure	rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)
1	56840766 / 134970506	S 97.8		7.3	106.7	53.2	5.6	50	4.2	>50
2	56840772 / 134970490	S 100		6.6	47.2	30.3	5.6	95	0.93	>50
3	56846691 / 135378316	S 93.4		4.6	74.6	38.4	4.7	76	1.3	>50

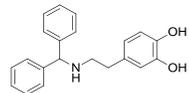
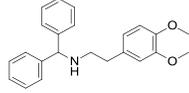
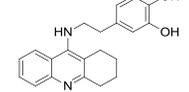
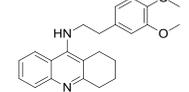
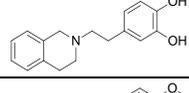
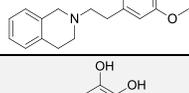
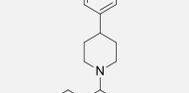
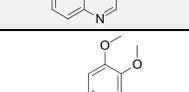
ND = Not Determined. Data are the average of three replicates; n = number of repeat analyses (n = 1 by default).

**Table 3.4.8.** Round 2 SAR and Final Catechol-containing and Penultimate O,O-Dimethyl Catechol-containing Compounds

Entry	CID / SID	P/S %Purity	Structure	Biochemical Target	Biochemical Anti-target			Red Blood Cell-based Target		Cytotoxicity
				rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)
Table 3.4.7 Entry 3	56846691 / 135378316	S 93.4		4.6	74.6	38.4	4.7	76	1.3	>50
1	56846690 / 135378305	S 92.3		>50	>50	>50	>50	1	nd	23.7
Table 3.4.7 Entry 2	56840772 / 134970490	S 100		6.6	47.2	30.3	5.6	95	0.93	>50
2	56840760 / 134970501	S 99.1		>50	>50	>50	>50	94	2	>50

ND = Not Determined. Data are the average of three replicates; n = number of repeat analyses (n = 1 by default).

**Table 3.4.9.** Probe Candidates (Catechol-containing Compounds Only) from Round 1 and 2 SAR

				Biochemical Target	Biochemical Anti-target			Red Blood Cell-based Target		Cytotoxicity
Entry	CID / SID	P/S %Purity	Structure	rPfM18AAP IC <sub>50</sub> (μM)	rPfM1MAA IC <sub>50</sub> (μM)	rPfM17LAP IC <sub>50</sub> (μM)	rhM18 IC <sub>50</sub> (μM)	Pf Growth %Inh @ 10 μM	Pf Growth Inh EC <sub>50</sub> (μM)	Vero EC <sub>50</sub> (μM)
Table 3.4.2 Entry 1	53308670 / 124767902	S 76.6		10.5	55.1	46.8	41.4	52	14	41.64 (n=3)
Table 3.4.6 Entry 2	318615 / 124767896	S 95.522		>50	>50	>50	>50	0	ND	39.225 (n=2)
Table 3.4.2 Entry 5	53308667 / 124767868	S 97.3		10.2	35.0	61.8	>50	93	3.6	>50
Table 3.4.6 Entry 6	22023 / 124767880	S 98.923		>50	>50	>50	>50	47	20	>50
Table 3.4.5 Entry 5	53308668 / 124767872	S 98.6		22.3	77.1	58.6	>50	57	7.9	>50
Table 3.4.6 Entry 16	12553367 / 124767883	S 100		>50	>50	>50	>50	2	ND	>50
Table 3.4.7 Entry 3	56846691 / 135378316	S 93.4		4.6	74.6	38.4	4.7	76	1.3	>50
Table 3.4.8 Entry 1	56846690 / 135378305	S 92.3		>50	>50	>50	>50	1	ND	23.7

### 3.5 Cellular Activity

The malaria parasite growth inhibition assay and the Vero cell cytotoxicity assay are cell-based assays. The majority of compounds screened were active in the malaria parasite killing assay and were inactive in the Vero cell cytotoxicity assay. The cellular permeability (PAMPA) for ML369 and three of the supporting analogues was measured and was determined to be good. See the **Table J1, Appendix J**, for the complete tabulation of the results. The cellular permeability for the probe (PAMPA) was determined to be approximately  $1400 \times 10^{-6}$  cm/s,  $900 \times 10^{-6}$  cm/s, and  $120 \times 10^{-6}$  cm/s at pH 7.4, 6.2, and 5.0, respectively. See the **Appendix D** for the experimental procedure for cellular permeability (PAMPA) measurements.

### 3.6 Profiling Assays

The probe was submitted to Eurofins Panlabs for screening in the LeadProfilingScreen, a screen profiling binding to 68 protein targets of therapeutic or toxicological interest, and the results for this screen are listed in detail in **Appendix H**. In summary, not surprisingly, the probe was observed to be particularly active against a few of the GPCR biological targets, such as the dopamine transporter and norepinephrine transporter, for which it shares structural similarity to the known ligands, dopamine and norepinephrine, respectively. In addition, significant activity was observed against the hERG potassium channel. While the potential for GPCR and ion channel activity would be most important if the probe were recommended for use in in vivo studies, this activity is much less important with respect to the more limited recommended uses for ML369 (i.e., biochemical and cell-based assays; electron microscopy studies; biotin, photo-affinity, or fluorescent conjugates for chemical biology; co-crystallography with *rPfM18AAP*; modeling with *PfM18AAP*; and physical chemistry to study lysosomotropic properties). In any case, as the SAR surrounding ML369 is expanded to include bioisosteric replacements for the putative catechol zinc-binding domain, testing for off-target effects should include GPCR and ion channel targets.

The probe compound and supporting analogues were submitted to the Sanford-Burnham Medical Research Institute for various levels of in vitro pharmacology profiling (i.e., aqueous solubility in Pion's buffer (pH 5.0, 6.2, and 7.4), aqueous solubility in 1x PBS, cell permeability, (PAMPA), plasma stability (human and mouse), plasma protein binding (human and mouse), hepatic microsome stability (human and mouse), and toxicity towards Fa2N-4 immortalized human hepatocytes), and the results are provided in **Table J1, Appendix J**. Overall, the probe showed moderate aqueous solubility that increases as pH decreases, very good permeability that decreases as pH decreases, poor/very poor stability to human/mouse plasma, high binding to human/mouse plasma proteins, very poor/poor stability to human/mouse hepatic microsomes, and no significant toxicity toward immortalized human hepatocytes. See the **Appendix D** for the experimental procedures for the various in vitro PK measurements.

## 4 Discussion

At the outset, the aim of this project was to identify inhibitors of *PfM18AAP* that were selective versus *PfM1MAA* and *PfM17LAP* that also inhibited cell-based parasite growth that were also

non-toxic to Vero cells. Subsequent to the HTS campaign, the previously unreported human recombinant M18 became available (Dalton Lab), and was incorporated into the list of anti-targets. After screening a compound library of almost 300,000 members and two subsequent rounds of SAR optimization, the team identified the compound ML369, and, for the first time, have apparently correlated inactivation of the *Pf*M18MAAP with malaria parasite killing. In general, the non-toxicity of this compound against mammalian cells augurs wells for the development of anti-malaria drugs that do not exhibit toxic off-target effects, although this must be examined in vivo in the future.

While the probe shows no selectivity against the human rM18, it also did not show toxicity against mammalian cells. The lack of toxicity against mammalian cells could suggest that the compound is taken into parasite-infected RBCs via a mechanism that is not active in mammalian cells. It is well known that malaria parasites insert various transport channels into the membrane of the RBC, which facilitates the active up-take of nutrients and other compounds (e.g. amino acids) from the external milieu.

The probe ML369 meets the probe criteria for the project, which are listed in the **Table 4.1**.

**Table 4.1** Comparison of the Observed Probe Properties to the Probe Criteria

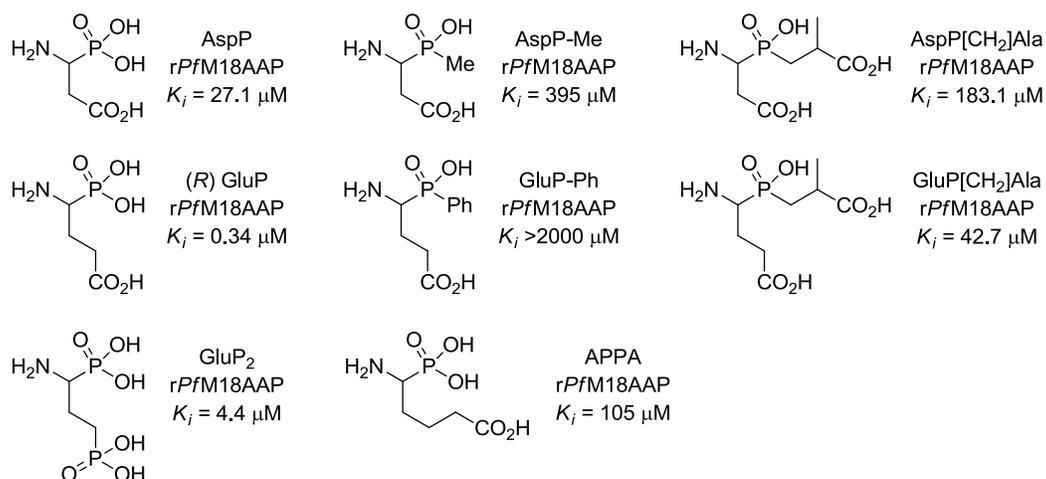
No.	Property	CPDP Probe Criteria	Probe Property
1	r <i>Pf</i> M18AAP Potency	IC <sub>50</sub> < 10 μM	IC <sub>50</sub> = 4.6 μM
2	Extracted <i>Pf</i> M18AAP Potency	IC <sub>50</sub> < 500 nM	To be determined
3	r <i>Pf</i> M1MAA Selectivity	10x	16x
4	r <i>Pf</i> M17LAP Selectivity	10x	8.2x
5	Human rM18 Selectivity	Not Required	1x
6	<i>Pf</i> Parasite Growth Inhibition	Preferably < 1 μM	EC <sub>50</sub> = 1.3 μM
7	Vero Cell Cytotoxicity	> 50 μM	> 50 μM
8	Aqueous Solubility (pH 7.4, 23 °C)	Sufficient	9 μM

#### 4.1 Comparison to existing art and how the new probe is an improvement

The prior art was investigated by searching the Chemical Abstracts database using the SciFinder software. The search terms applied and the statistics for the prior art search are provided in **Table G1, Appendix G**. Abstracts were obtained for all references returned from the search and were analyzed for relevance to the current project. For all references that were deemed relevant, the articles were analyzed and the results are summarized. The search results are current as of April 4, 2013.

The only existing prior art was reported from the labs of the assay providers Dalton and Gardiner [16]. Dalton and Gardiner prepared phosphinic and phosphonic acid derivatives of aspartic and glutamic acid, the N-terminal acidic amino acids for which the specificity of the aspartyl aminopeptidase is restricted, for which the *K<sub>i</sub>*s against r*Pf*M18AAP ranged from a high of >2000 μM to a low of 0.34 μM (**Figure 4.1.1**). While a number of these inhibitors showed moderate inhibition of r*Pf*M18AAP, none of the inhibitors showed any significant inhibition of the growth of *P. falciparum* D10 parasites in culture (even at 100 μM final concentration).

**Figure 4.1.1.** Reported Inhibitors of *rPfM18AAP*



The selectivity for these compounds toward *rPfM1MAA*, *rPfM17LAP*, and *rhM18*, and their cytotoxicity against Vero cells was not reported.

The current probe is an improvement over this prior art, in that, it is the first small-molecule inhibitor for *rPfM18AAP* that is moderately selective against *rPfM1MAA* and *rPfM17LAP* and exhibits good blocking of parasite growth in culture (e.g. lower than the broad-range neutral aminopeptidase inhibitor bestatin,  $\text{IC}_{50}$  10  $\mu\text{M}$ ), and, strikingly, is not toxic to Vero cells. The studies support that *PfM18AAP* is a viable target for anti-malarial drug discovery as the studies suggest the first link between its enzymatic inhibition and parasite killing via small-molecule compounds.

## 4.2 Mechanism of Action Studies

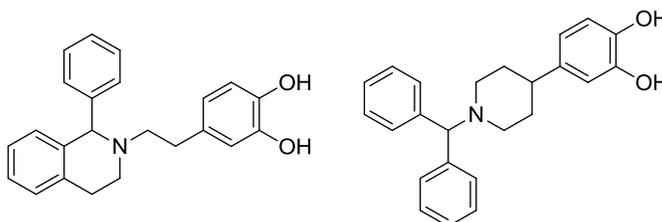
The assays used in this project included a combination of on- and off-target biochemical and cell-based assays. Recombinant *PfM18AAP* was used for the biochemical target assay, while recombinant *FhCTSL1*, *PfM1MAA*, *PfM17LAP*, and human M18 were used for the biochemical anti-target assays. Malaria parasite growth inhibition in red blood cells and cytotoxicity against Vero cells were used as the cell-based target and anti-target assays, respectively. While the probe ML369 and a handful of analogues listed in **Table 3.4.9** show activity across this suite of assays that is consistent with an on-target mechanism of action, some ambiguity does remain, in this regard. Future studies, including additional rounds of SAR, the measurement of  $\text{IC}_{50}$ s against the native *PfM18AAP*, *PfM1MAA*, and *PfM17LAP*, and activity-based protein profiling using derivatives of the current, or improved, probes could address this ambiguity. In addition, one could imagine performing an experiment to help clarify the mechanism of action, whereby, *PfM18AAP* is overexpressed in the cell-based assay, and if the probe and analogues are acting on target their activity might be reduced.

### 4.3 Planned Future Studies

#### SAR Studies:

Additional rounds of SAR could combine the structural features of the compounds listed in **Table 3.4.9**. For example, the first and third compound structures or the first and fourth compound structures could be combined to afford the compounds on the left and right, respectively, in the **Figure 4.3.1**.

**Figure 4.3.1** Example Future SAR Studies



Bioisosteric replacements for the catechol moiety, such as those popularized by Cohen, should also be revisited [20]. While many such catechol replacements were earmarked for synthesis, only a few were prepared due to the challenges associated with their preparation.

#### Mechanism of Action Studies:

The mechanism of action for the probe or analogues could be explored using affinity-based protein profiling. This chemical biology strategy using bestatin-based probes has been used to illuminate the distinct roles for malaria *PfM1MAA* and *PfM17LAP* [27]. While the activity for the current probe is at the upper-end of what might be considered useful for such a probe, ideally, additional SAR studies should lead to significantly more active analogues, with activities more appropriate for such studies. The SAR studies to date suggest that the region of the inhibitor structure that is distal to the catechol moiety is a likely candidate for conjugation to biotin, photo-affinity, or fluorescent labels.

#### Co-Crystallization Studies using *rPfM18AAP* and the Probe or Analogues:

Recently, the 3-D structure of the malaria recombinant *PfM18AAP* was resolved and showed that the enzyme exists as a large complicated multimeric structure [28]. Because of the lack of availability of a potent inhibitor, no structure for an inhibitor-enzyme complex was obtained. However, the activity and solubility of the current probe, and its analogues, should now fill this gap. One of the probe analogues, in particular, CID 53308676 (**Table 3.4.5**, entry 3, and **Table J1, Appendix J**), that shows potency in the low single-digit micromolar range ( $IC_{50}$  against the *rPfM18AAP*) is quite soluble in aqueous solution across the pH range from 5.0 to 7.4 (>0.5 mM). Co-crystallization studies should allow a better understanding of the manner in which these compounds bind and interact with the malaria enzyme.

### **Fluorescent and Electron Microscopy Studies:**

Fluorescent and electron microscopy studies could be used to examine the capacity for the probe and analogues to disrupt the intracellular architecture of the malaria cell.

### **Studies on the Physical Properties of the Probe and Analogues and Their Selective Accumulation in the Parasitophorous Vacuole:**

The uptake and intracellular distribution properties of a compound are fundamentally important variables in achieving a potent and desirable biological response. The ability of compounds to cross cellular lipid bilayers is a necessary step in this process but rarely is it a sufficient one. This is because cells are highly compartmentalized entities with over 50% of their total volume comprised of membrane-bound organelles/compartments that can provide barriers between a probe and its intended target molecule.

Malaria parasite enzymes that interfere with hemoglobin digestion/metabolism are often presumed to be localized, at least partially, within the acidic digestive vacuole. Interestingly, PfM18AAP has been shown to be secreted into the parasitophorous space [16], which is also acidic due to the action of the V-H<sup>+</sup>-ATPase localized at the plasma membrane of the parasite [29]. Being relatively acidic, these compartments have the propensity to significantly concentrate weakly basic compounds according to an ion trapping-type mechanism. Accordingly, we hypothesize that compounds that have ideal structural and physicochemical properties that promote ion trapping (i.e., lysosomotropism) will have enhanced interactions with PfM18AAP relative to non-lysosomotropic counterparts. It is important to note that the presence or absence of lysosomotropic properties should not influence probe interactions with PfM18AAP in such assays. However, the lysosomotropic propensity of probes will be expected to favor interactions with PfM18AAP in the anti-malarial red blood cell cell-based assay since intracellular proton gradients are actively maintained. Interestingly, lysosomotropic compounds show enhanced potency in anti-malarial assays [23].

For next-generation probe analogues we will rationally incorporate functional groups that will impart increasing degrees of lysosomotropic properties and evaluate them using *in vitro* enzyme inhibition and RBC-based anti-malarial assays as described, previously. There are two key physicochemical properties of drugs that influence the degree of ion trapping in acidified intracellular compartments that will be systematically modified. The first parameter is the pK<sub>a</sub> value of the conjugate acid of the weak base [30]. The second is the ratio of the permeabilities of the base in the ionized versus unionized form [31]. We will experimentally estimate the ratio of permeabilities by evaluating octanol/water partition coefficients as a function of pH as previously described [31]. The pK<sub>a</sub> values of probes will be estimated using spectrophotometric approaches. In addition, we will experimentally determine the lysosomotropic characteristics of the probes using a recently developed approach that has been validated using the fluorescent lysosomotropic probe LysoTracker Red (LTR). In this approach we comparatively evaluate the total cell uptake in cells before and after treatment with ionophores nigericin and monensin. The ionophores dissipate the lysosome-to-cytosol pH gradient that provides the driving force for ion trapping, and cells treated with ionophores have markedly reduced lysosomal sequestration of LTR. Using this data we can mathematically arrive at the percentage of total cell uptake that is

driven by an ion trapping-based mechanism. For lysosomotropic molecules such as LTR, greater than 76% of its total cell uptake can be attributed to ion trapping. This measure of lysosomotropic potential will be evaluated for all probe analogues. Using these approaches, together with optimization of  $pK_a$  and permeability ratios of probe analogues, we expect that next-generation probes will have greatly enhanced intracellular distribution properties and therefore fully optimized anti-malarial activity if the lysosomotropic hypothesis is correct.

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## Appendix A: Assay Summary Table

Table A.A. Summary of Assays and Performance Sites

PubChem AID No.	Assay Format	Assay Target	Test Compound Concentration Range ( $\mu\text{M}$ )	Number of Compound Samples Tested	Performance Site	Stage
1855	Summary	rP $\mathcal{M}$ 18 inhibitor project	NA	NA	SRIMSC	NA
1822	Biochemical	rP $\mathcal{M}$ 18 inhibition assay	7.35	291,944	SRIMSC	Primary Screen
1906	Biochemical	rCTSL1 inhibition assay	5.96	302,759	SRIMSC	Primary Screen
2170	Biochemical	rP $\mathcal{M}$ 18 inhibition assay	7.35	2,378	SRIMSC	Primary Screen
2178	Biochemical	rCTSL1 inhibition assay	5.96	2,378	SRIMSC	Primary Screen
2195	Biochemical	rP $\mathcal{M}$ 18 inhibition assay	0.0037-73.5	125	SRIMSC	Primary Screen
2196	Biochemical	rCTSL1 inhibition assay	0.003-59.6	125	SRIMSC	Primary Screen
492974	Biochemical	rP $\mathcal{M}$ 18 inhibition assay	5	76	Dalton Lab	Hit Validation
492975	Biochemical	eP $\mathcal{M}$ 18 inhibition assay	5	60	Dalton Lab	Hit Validation
489015	Cell-based	<i>Pf</i> growth inhibition assay	10	76/TBD	Gardiner Lab	Hit Validation/ SAR
489011	Cell-based	<i>Pf</i> growth inhibition assay	1-25	8/TBD	Gardiner Lab	Hit Validation/ SAR
588678	Biochemical	rP $\mathcal{M}$ 18 inhibition assay	0.19-100	63	SRSBSC	SAR
602222	Biochemical	rP $\mathcal{M}$ 18 inhibition assay	0.19-100	10	SRSBSC	SAR
624177	Biochemical	rP $\mathcal{M}$ 18 inhibition assay	0.19-100	45	SRSBSC	SAR

588680	Biochemical	rPflM1 inhibition assay	0.19-100	63	SRSBSC	SAR
602219	Biochemical	rPflM1 inhibition assay	0.19-100	10	SRSBSC	SAR
624176	Biochemical	rPflM1 inhibition assay	0.19-100	45	SRSBSC	SAR
588679	Biochemical	rPflM17 inhibition assay	0.19-100	63	SRSBSC	SAR
602220	Biochemical	rPflM17 inhibition assay	0.19-100	10	SRSBSC	SAR
624175	Biochemical	rPflM17 inhibition assay	0.19-100	45	SRSBSC	SAR
588696	Biochemical	rhM18 inhibition assay	0.19-100	63	SRSBSC	SAR
602221	Biochemical	rhM18 inhibition assay	0.19-100	10	SRSBSC	SAR
624174	Biochemical	rhM18 inhibition assay	0.19-100	45	SRSBSC	SAR
588714	Cell-based	Vero cytotoxicity assay	0.19-100	63	SRSBSC	SAR
602225	Cell-based	Vero cytotoxicity assay	0.19-100	15	SRSBSC	SAR
624205	Cell-based	Vero cytotoxicity assay	0.19-100	TBD	SRSBSC	SAR

NA = not applicable,

## Appendix B: Detailed Assay protocols

### (2.1.1) rP $\beta$ M18AAP QFRET-based Primary Biochemical High Throughput Assay (Primary Assay AID No. 1822)

#### *List of Reagents:*

rP $\beta$ M18AAP enzyme (supplied by Assay Provider)  
H-Glu-NHMec substrate (Bachem, part I-1180)  
1536-well plates (Greiner, part 789176)  
Tris (Amresco, part 0497)  
CoCl<sub>2</sub> 6H<sub>2</sub>O (Univar, part D3247)  
ZnCl<sub>2</sub> (Sigma, part 208086)

#### *Protocol Summary:*

Prior to the start of the assay, 2.5 microliters of assay buffer (50 mM Tris HCl pH7.5, 4 mM CoCl<sub>2</sub>, 0.1% BSA) containing 5 micrograms/mL rP $\beta$ M18AAP were dispensed into a 1536 microtiter plate. Next, 37 nL of test compound in DMSO, ZnCl<sub>2</sub> (2 mM final concentration), or DMSO alone (0.74% final concentration) were added to the appropriate wells. The plates were then incubated for 30 minutes at 25 degrees Celsius. The assay was started by dispensing 2.5 microliters of 100 micromolar H-Glu-NHMec substrate in buffer (50 mM Tris HCl, pH 8.8) into all wells. Well fluorescence was read immediately (T0) on the Viewlux (Perkin-Elmer) and again after 90 minutes (T90) of incubation at 25 degrees Celsius.

Prior to further calculations, T0 was subtracted from T90 for each individual well. The difference between RFU values read at T0 (RFU T0) and T90 (RFU T90), named delta RFU, was calculated as follows:

$$\text{delta RFU} = \text{RFU T90} - \text{RFU T0}$$

The percent inhibition for each well was then calculated as follows:

$$\text{Percent inhibition} = (\text{test compound delta RFU} - \text{negative control delta RFU}) / (\text{positive control delta RFU} - \text{negative control delta RFU}) * 100$$

Where:

Test Compound is defined as wells containing test compound. Negative Control is defined as the median of the wells containing test compounds. Positive Control is defined as the median of the wells containing ZnCl<sub>2</sub>.

A mathematical algorithm was used to determine nominally inhibiting compounds in the Primary screen. Two values were calculated: (1) the average percent inhibition of all compounds tested, and (2) three times their standard deviation. The sum of these two values was used as a cutoff parameter, i.e. any compound that exhibited greater % inhibition than the cutoff parameter was declared active.

The reported PubChem Activity Score was normalized to 100% observed primary inhibition. Negative % inhibition values were reported as activity score zero.

The inactive compounds of this assay have activity score range of 0 to 28 and the active compounds have activity score range of 28 to 100.

### **(2.1.2) CTSL1 QFRET-based Counterscreen Biochemical High Throughput Assay (Primary Assay AID No. 1906)**

#### *List of Reagents:*

Cathepsin L enzyme (supplied by Assay Provider)  
Z-Leu-Arg-MCA substrate (Peptides International, part MCA-3210-v)  
1536-well plates (Greiner, part 789176)  
Tris (Amresco, part 0497)  
DTT (Invitrogen, part 15508-013)  
Z-Phe-Ala-diazomethylketone (Bachem, part N-1040)  
BSA (Calbiochem, part 126609)

#### *Protocol Summary:*

Prior to the start of the assay, 2.5 microliters of assay buffer (25 mM Tris HCl pH7.5, 1 mM DTT, 0.1% BSA) containing 1.5 micrograms/mL cathepsin L were dispensed into a 1536 microtiter plate. Next, 30 nL of test compound in DMSO, Z-Phe-Ala-diazomethylketone (1micromolar final concentration), or DMSO alone (0.59% final concentration) were added to the appropriate wells. The plates were then incubated for 30 minutes at 25 degrees Celsius. The assay was started by dispensing 2.5 microliters of 100 micromolar Z-Leu-Arg-MCA substrate in buffer (25 mM Tris HCl, pH 7.5, 1mM DTT) into all wells. Well fluorescence was read immediately (T0) on the Viewlux (Perkin-Elmer) and again after 90 minutes (T90) of incubation at 25 degrees Celsius.

Prior to further calculations, T0 was subtracted from T90 for each individual well. The difference between RFU values read at T0 (RFU T0) and T90 (RFU T90), named delta RFU, was calculated as follows:

$$\text{delta RFU} = \text{RFU T90} - \text{RFU T0}$$

The percent inhibition for each well was then calculated as follows:

$$\text{Percent inhibition} = (\text{test compound delta RFU} - \text{negative control delta RFU}) / (\text{positive control delta RFU} - \text{negative control delta RFU}) * 100$$

Where:

Test Compound is defined as wells containing test compound. Negative Control is defined as the median of the wells containing test compounds. Positive Control is defined as the median of the wells containing Z-Phe-Ala-diazomethylketone.

A mathematical algorithm was used to determine nominally inhibiting compounds in the Primary screen. Two values were calculated: (1) the average percent inhibition of all compounds tested, and (2) three times their standard deviation. The sum of these two values was used as a cutoff parameter, i.e. any compound that exhibited greater % inhibition than the cutoff parameter was declared active.

The reported PubChem Activity Score was normalized to 100% observed primary inhibition. Negative % inhibition values were reported as activity score zero.

The activity score range for active compounds was 100-14, for inactive 14-0.

### **(2.1.3) rP<sub>f</sub>M18AAP QFRET-based Confirmatory Biochemical High Throughput Assay (Primary Assay AID No. 2170)**

#### *List of Reagents:*

rP<sub>f</sub>M18AAP enzyme (supplied by Assay Provider)

H-Glu-NHMec substrate (Bachem, part I-1180)

Tris (Amresco, part 0497)

CoCl<sub>2</sub> 6H<sub>2</sub>O (Univar, part D3247)

ZnCl<sub>2</sub> (Sigma, part 208086)

BSA (Calbiochem, part 126609)

1536-well plates (Greiner, part 789176)

#### Protocol Summary:

Prior to the start of the assay, 2.5 microliters of assay buffer (50 mM Tris HCl pH7.5, 4 mM CoCl<sub>2</sub>, 0.1% BSA) containing 5 micrograms/mL rP<sub>f</sub>M18AAP were dispensed into a 1536 microtiter plate. Next, 37 nL of test compound in DMSO, ZnCl<sub>2</sub> (2 mM final concentration), or DMSO alone (0.74% final concentration) were added to the appropriate wells. The plates were then incubated for 30 minutes at 25 degrees Celsius. The assay was started by dispensing 2.5 microliters of 100 micromolar H-Glu-NHMec substrate in buffer (50 mM Tris HCl, pH 8.8) into all wells. Well fluorescence was read immediately (T0) on the Viewlux (Perkin-Elmer) and again after 90 minutes (T90) of incubation at 25 degrees Celsius.

Prior to further calculations, T0 was subtracted from T90 for each individual well. The difference between RFU values read at T0 (RFU T0) and T90 (RFU T90), named delta RFU, was calculated as follows:

$$\text{delta RFU} = \text{RFU T90} - \text{RFU T0}$$

The percent inhibition for each well was then calculated as follows:

$$\text{Percent inhibition} = (\text{test compound delta RFU} - \text{negative control delta RFU}) / (\text{positive control delta RFU} - \text{negative control delta RFU}) * 100$$

Where:

Test Compound is defined as wells containing test compound. Negative Control is defined as the median of the wells containing test compounds. Positive Control is defined as the median of the wells containing ZnCl<sub>2</sub>.

The average percent inhibition and standard deviation of each compound tested were calculated. Any compound that exhibited an average percent inhibition greater than the hit cutoff calculated for the primary screen was declared active.

The reported PubChem Activity Score was normalized to 100% observed primary inhibition. Negative % inhibition values were reported as activity score zero.

The activity score range for active compounds was 100-28, for inactive 28-0.

#### **(2.1.4) CTSL1 QFRET-based Confirmatory Counterscreen Biochemical High Throughput Assay (Primary Assay AID No. 2178)**

##### *List of Reagents:*

Cathepsin L enzyme (supplied by Assay Provider)  
Z-Leu-Arg-MCA substrate (Peptides International, part MCA-3210-v)  
Tris (Amresco, part 0497)  
DTT (Invitrogen, part 15508-013)  
Z-Phe-Ala-diazomethylketone (Bachem, part N-1040)  
BSA (Calbiochem, part 126609)  
1536-well plates (Greiner, part 789176)

##### *Protocol Summary:*

Prior to the start of the assay, 2.5 microliters of assay buffer (25 mM Tris HCl pH7.5, 1 mM DTT, 0.1% BSA) containing 1.5 micrograms/mL cathepsin L were dispensed into a 1536 microtiter plate. Next, 30 nL of test compound in DMSO, Z-Phe-Ala-diazomethylketone (1 micromolar final concentration), or DMSO alone (0.59% final concentration) were added to the appropriate wells. The plates were then incubated for 30 minutes at 25 degrees Celsius. The assay was started by dispensing 2.5 microliters of 100 micromolar Z-Leu-Arg-MCA substrate in buffer (25 mM Tris HCl, pH 7.5, 1mM DTT) into all wells. Well fluorescence was read immediately (T0) on the Viewlux (Perkin-Elmer) and again after 90 minutes (T90) of incubation at 25 degrees Celsius.

Prior to further calculations, T0 was subtracted from T90 for each individual well. The difference between RFU values read at T0 (RFU T0) and T90 (RFU T90), named delta RFU, was calculated as follows:

$$\text{delta RFU} = \text{RFU T90} - \text{RFU T0}$$

The percent inhibition for each well was then calculated as follows:

$$\text{Percent inhibition} = (\text{test compound delta RFU} - \text{negative control delta RFU}) / (\text{positive control delta RFU} - \text{negative control delta RFU}) * 100$$

Where:

Test Compound is defined as wells containing test compound. Negative Control is defined as the median of the wells containing test compounds. Positive Control is defined as the median of the wells containing Z-Phe-Ala-diazomethylketone.

The average percent inhibition and standard deviation of each compound tested were calculated. Any compound that exhibited an average percent inhibition greater than the hit cutoff calculated for the primary screen (AID 1906) was declared active.

The reported PubChem Activity Score was normalized to 100% observed primary inhibition. Negative % inhibition values were reported as activity score zero.

The activity score range for active compounds was 100-18, for inactive 13-0.

### **(2.1.5) rP $\beta$ M18AAP QFRET-based Confirmatory Concentration-response Biochemical High Throughput Assay (Primary Assay AID No. 2195)**

#### *List of Reagents:*

rP $\beta$ M18AAP enzyme (supplied by Assay Provider)  
H-Glu-NHMec substrate (Bachem, part I-1180)  
1536-well plates (Greiner, part 789176)  
Tris (Amresco, part 0497)  
CoCl<sub>2</sub> 6H<sub>2</sub>O (Univar, part D3247)  
ZnCl<sub>2</sub> (Sigma, part 208086)  
BSA (Calbiochem, part 126609)

#### *Protocol Summary:*

Prior to the start of the assay, 2.5 microliters of assay buffer (50 mM Tris HCl pH7.5, 4 mM CoCl<sub>2</sub>, 0.1% BSA) containing 5 micrograms/mL rP $\beta$ M18AAP were dispensed into a 1536 microtiter plate. Next, 37 nL of test compound in DMSO, ZnCl<sub>2</sub> (2 mM final concentration), or DMSO alone (0.74% final concentration) were added to the appropriate wells. The plates were then incubated for 30 minutes at 25 degrees Celsius. The assay was started by dispensing 2.5 microliters of 100 micromolar H-Glu-NHMec substrate in buffer (50 mM Tris HCl, pH 8.8) into all wells. Well fluorescence was read immediately (T0) on the Viewlux (Perkin-Elmer) and again after 90 minutes (T90) of incubation at 25 degrees Celsius.

Prior to further calculations, T0 was subtracted from T90 for each individual well. The difference between RFU values read at T0 (RFU T0) and T90 (RFU T90), named delta RFU, was calculated as follows:

$$\text{delta RFU} = \text{RFU T90} - \text{RFU T0}$$

The percent inhibition for each well was then calculated as follows:

$$\text{Percent inhibition} = (\text{test compound delta RFU} - \text{negative control delta RFU}) / (\text{positive control delta RFU} - \text{negative control delta RFU}) * 100$$

Where:

Test Compound is defined as wells containing test compound. Negative Control is defined as the median of the wells containing rP $\beta$ M18AAP. Positive Control is defined as the median of the wells containing ZnCl<sub>2</sub>.

For each test compound, percent inhibition was plotted against compound concentration. A four parameter equation describing a sigmoidal dose-response curve was then fitted with adjustable baseline using Assay Explorer software (Symyx Technologies Inc). The reported IC<sub>50</sub> values were generated from fitted curves by solving for the X-intercept value at the 50% inhibition level of the Y-intercept value. In cases where the highest concentration tested (i.e. 73.5 micromolar)

did not result in greater than 50% inhibition, the IC<sub>50</sub> was determined manually as greater than 73.5 micromolar. Compounds with an IC<sub>50</sub> greater than 10 micromolar were considered inactive. Compounds with an IC<sub>50</sub> equal to or less than 10 micromolar were considered active.

Any compound with a percent activity value <50% at all test concentrations was assigned an activity score of zero. Any compound with a percent activity value >50% at any test concentration was assigned an activity score greater than zero. Activity score was then ranked by the potency, with the most potent compounds assigned the highest activity scores.

The activity score range for active compounds was 100-1. There were no inactive compounds.

#### **(2.1.6) CTSL1 QFRET-based Confirmatory Counterscreen Concentration-response Biochemical High Throughput Assay (Primary Assay AID No. 2196)**

##### *List of Reagents:*

Cathepsin L enzyme (supplied by Assay Provider)  
Z-Leu-Arg-MCA substrate (Peptides International, part MCA-3210-v)  
1536-well plates (Greiner, part 789176)  
Tris (Amresco, part 0497)  
DTT (Invitrogen, part 15508-013)  
Z-Phe-Ala-diazomethylketone (Bachem, part N-1040)  
BSA (Calbiochem, part 126609)

##### *Protocol Summary:*

Prior to the start of the assay, 2.5 microliters of assay buffer (25 mM Tris HCl pH7.5, 1 mM DTT, 0.1% BSA) containing 1.5micrograms/mL cathepsin L were dispensed into a 1536 microtiter plate. Next, 30 nL of test compound in DMSO, Z-Phe-Ala-diazomethylketone (1 micromolar final concentration), or DMSO alone (0.59% final concentration) were added to the appropriate wells. The plates were then incubated for 30 minutes at 25 degrees Celsius. The assay was started by dispensing 2.5 microliters of 100 micromolar Z-Leu-Arg-MCA substrate in buffer (25 mM Tris HCl, pH 7.5, 1mM DTT) into all wells. Well fluorescence was read immediately (T0) on the Viewlux (Perkin-Elmer) and again after 90 minutes (T90) of incubation at 25 degrees Celsius.

Prior to further calculations, T0 was subtracted from T90 for each individual well. The difference between RFU values read at T0 (RFU T0) and T90 (RFU T90), named delta RFU, was calculated as follows:

$$\text{delta RFU} = \text{RFU T90} - \text{RFU T0}$$

The percent inhibition for each well was then calculated as follows:

$$\text{Percent inhibition} = (\text{test compound delta RFU} - \text{negative control delta RFU}) / (\text{positive control delta RFU} - \text{negative control delta RFU}) * 100$$

Where:

Test Compound is defined as wells containing test compound. Negative Control is defined as the median of the wells containing Cathepsin L. Positive Control is defined as the median of the wells containing Z-Phe-Ala-diazomethylketone.

For each test compound, percent inhibition was plotted against compound concentration. A four parameter equation describing a sigmoidal dose-response curve was then fitted with adjustable baseline using Assay Explorer software (Symyx Technologies Inc). The reported IC<sub>50</sub> values were generated from fitted curves by solving for the X-intercept value at the 50% inhibition level of the Y-intercept value. In cases where the highest concentration tested (i.e. 59.6 micromolar) did not result in greater than 50% inhibition, the IC<sub>50</sub> was determined manually as greater than 59.6 micromolar. Compounds with an IC<sub>50</sub> greater than 10 micromolar were considered inactive. Compounds with an IC<sub>50</sub> equal to or less than 10 micromolar were considered active.

Any compound with a percent activity value <50% at all test concentrations was assigned an activity score of zero. Any compound with a percent activity value >50% at any test concentration was assigned an activity score greater than zero. Activity score was then ranked by the potency, with the most potent compounds assigned the highest activity scores.

There are no active compounds in this assay.

### **(2.1.7) Fluorescence-based rP<sub>1</sub>M18AAP Confirmatory Biochemical Assay (Hit Validation Assay AID No. 492974)**

#### *List of Reagents:*

rP<sub>1</sub>M18AAP (Assay Provider)  
H-Glu-NHMec (Bachem Chemical Co., catalog I-1180)  
Tris buffer (Supplier name, BioShop Canada, catalog TRS001-1)  
96-well plates (Costar Incorporated USA, catalog 3904)

#### *Protocol Summary:*

Prior to the start of the assay, 5 uL of rP<sub>1</sub>M18AAP were dispensed into 96-well black non-binding surface plates. This was followed by 90 uL of assay buffer (25 mM Tris HCl, pH 7.5) and then 5 uL of compound. After 10 minutes, 100 uL of 100 uM fluorogenic peptide substrate (H-Glu-NHMec) in assay buffer was added to each well. The final concentrations in the reaction were 50 uM H-Glu-NHMec, 1 ug/ml rP<sub>1</sub>M18AAP, 25 mM Tris-HCl (pH 7.5), and 5 uM compound. The test plate was immediately transferred to a Synergy HT fluorimeter with microplate reader and fluorescence was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm at 60-second intervals for 30 minutes (30 readings). Each plate had 4 control wells in the eight outside columns with 2 wells containing the complete reaction mixture with carrier control (positive control) and 2 wells in which the rP<sub>1</sub>M18AAP had been left out (background).

The % inhibition for each well was then calculated as follows:

% Inhibition = 100 - % Enzyme Activity

% Enzyme Activity = (Test – Background Control) / (Positive Control – Background Control) \* 100

Where:

Test = RFU in wells with rPfM18AAP treated with test compound. Background Control = mean count of wells containing substrate in assay buffer only. Positive Control = mean count of wells containing rPfM18AAP with substrate but no compound added.

PubChem Activity Outcome and Score:

Compounds resulting in greater than 50% inhibition of rPfM18AAP were considered active.

The reported PubChem Activity Score was normalized to 100% observed inhibition. Negative % inhibition values were reported as activity score zero.

The PubChem Activity Score range for active compounds was 100-55, and for inactive compounds 54-0.

### **(2.1.8) Fluorescence-based Malarial Cell Lysate PfM18AAP Confirmatory Biochemical Assay (Hit Validation Assay AID No. 492975)**

*List of Reagents:*

Soluble malaria cell extract (Assay Provider)  
H-Glu-NHMec (Bachem Chemical Co., catalog I-1180)  
Tris buffer (Supplier name, BioShop Canada, catalog TRS001-1)  
96-well plates (Costar Incorporated USA, catalog 3904)

*Protocol Summary:*

Prior to the start of the assay, 5 uL of malaria cell lysate were dispensed into 96-well black non-binding surface plates. This was followed by 90 uL of assay buffer (25 mM Tris HCl, pH 7.5) and then 5 uL of compound. After 10 minutes, 100 uL of 100 uM fluorogenic peptide substrate (H-Glu-NHMec) in assay buffer was added to each well. The final concentrations in the reaction were 50 uM H-Glu-NHMec, 10 ug/ml malaria cell lysate, 25 mM Tris-HCl (pH 7.5), and 5 uM compound. The test plate was immediately transferred to a Synergy HT fluorimeter with microplate reader and fluorescence was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm at 60-second intervals for 30 minutes (30 readings). Each plate had 4 control wells in the eight outside columns with 2 wells containing the complete reaction mixture with carrier control (positive control) and 2 wells in which the malaria lysate had been left out (background).

The % inhibition for each well was then calculated as follows:

% Inhibition = 100 - % Enzyme Activity

% Enzyme Activity =  $(\text{Test} - \text{Background Control}) / (\text{Positive Control} - \text{Background Control}) * 100$

Where:

Test = RFU in wells with malaria lysate treated with test compound. Background Control = mean count of wells containing substrate in assay buffer only. Positive Control = mean count of wells containing malaria lysate with substrate but no compound added.

PubChem Activity Outcome and Score:

Compounds with greater than 50% inhibition of PfM18AAP in malaria cell lysate were considered active.

The reported PubChem Activity Score was normalized to 100% observed inhibition. Negative % inhibition values were reported as activity score zero.

The PubChem Activity Score range for active compounds was 100-53, and for inactive compounds 49-0.

### **(2.1.9) Radiolabel-based *Pf* Parasite Growth Cell-based Assay (Hit Validation and SAR Assays AID No. 489015)**

#### *List of Reagents:*

*Plasmodium falciparum* clone 3D7

Hypoxanthine Mono-Hydrochloride, [3H(G)]-(Perkin Elmer code Net177)

Plate Microtiter 96 well flat bottom Sterile (Costar, catalog 3595)

Special Gas Mix (5% CO<sub>2</sub> 5% O<sub>2</sub> 90% N<sub>2</sub>) (BOC Gas)

Culture media (85% RPMI, 4% Sodium Bicarbonate, 10% human sera) (GIBCO, catalog 31800-089)

#### *Protocol Summary:*

Prior to the start of the assay, 200 uL of PBS was added to the outside wells of a 96 well flat-bottomed microtiter plate. 100 uL hypoxanthine-free RPMI 1640 media plus 10% serum was added to all other wells except those that contained diluted compound or vehicle control. An appropriate volume of compound (10 uM) or vehicle control (DMSO <= 1%) in hypoxanthine-free RPMI 1640 media plus 10% serum was added to remaining wells. A parasite suspension (1% parasitemia, 2% hematocrit) was prepared in hypoxanthine-free RPMI media containing 10% serum and added to all test wells (except RBC controls) before the addition of 0.5 uCi/well of 3H-hypoxanthine (10 uL). Uninfected RBC controls (in triplicate) were included on each assay plate. Plates were incubated at 37 °C for 48 hours in an atmosphere of 90% N<sub>2</sub>, 5% CO<sub>2</sub>, and 5% O<sub>2</sub>. A cell harvester and Beta counter were used to determine the amount of hypoxanthine incorporated for each well. Three replicates at 10 uM were performed for each compound tested in each experiment, and each compound was tested in two or three experiments.

The % inhibition for each well was then calculated as follows:

$$\% \text{ Inhibition} = 100 - \% \text{ Growth}$$

$$\% \text{ Growth} = (\text{Test} - \text{Background Control}) / (\text{Positive Control} - \text{Background Control}) * 100$$

Where:

Test = count (3H-hypoxanthine incorporation) by parasites treated with test compound.  
Background Control = mean count of uninfected RBC control tests. Positive Control = mean count (3H-hypoxanthine incorporation) by untreated parasites exposed to vehicle only.

PubChem Activity Outcome and Score:

Compounds achieving  $\geq 50\%$  growth inhibition for any replicate were considered active; compounds achieving  $< 50\%$  growth inhibition in all replicates were considered inactive.

#### **(2.1.10) Radiolabel-based *Pf* Parasite Growth Cell-based Assay (Hit Validation and SAR Assays AID No. 489011)**

##### *List of Reagents:*

*Plasmodium falciparum* clone 3D7

Hypoxanthine Mono-Hydrochloride, [3H(G)]-(Perkin Elmer code Net177)

Plate Microtiter 96 well flat bottom Sterile (Costar, catalog 3595)

Special Gas Mix (5% CO<sub>2</sub>; 5% O<sub>2</sub>; 90% N<sub>2</sub>) (BOC Gas)

Culture media (85% RPMI, 4% Sodium Bicarbonate, 10% human sera) (GIBCO, catalog 31800-089)

##### *Protocol Summary:*

Prior to the start of the assay, 200  $\mu$ L of PBS was added to the outside wells of a 96 well flat-bottomed microtiter plate. 100  $\mu$ L hypoxanthine-free RPMI 1640 media plus 10% serum was added to all other wells except those that contained diluted compound or vehicle control. An appropriate volume of compound (10  $\mu$ M) or vehicle control (DMSO  $\leq 1\%$ ) in hypoxanthine-free RPMI 1640 media plus 10% serum was added to remaining wells. A parasite suspension (1% parasitemia, 2% hematocrit) was prepared in hypoxanthine-free RPMI media containing 10% serum and added to all test wells (except RBC controls) before the addition of 0.5  $\mu$ Ci/well of 3H-hypoxanthine (10  $\mu$ L). Uninfected RBC controls (in triplicate) were included on each assay plate. Plates were incubated at 37 C for 48 hours in an atmosphere of 90% N<sub>2</sub>, 5% CO<sub>2</sub>, and 5% O<sub>2</sub>. A cell harvester and Beta counter were used to determine the amount of hypoxanthine incorporated for each well.

The % inhibition for each well was calculated as follows:

$$\% \text{ Inhibition} = 100 - \% \text{ Growth}$$

$$\% \text{ Growth} = (\text{Test} - \text{Background Control}) / (\text{Positive Control} - \text{Background Control}) * 100$$

Where:

Test = count (3H-hypoxanthine incorporation) by parasites treated with test compound.  
Background Control = mean count of uninfected RBC control tests. Positive Control = mean count (3H-hypoxanthine incorporation) by untreated parasites exposed to vehicle only

For each test compound, percent inhibition was plotted against compound concentration. The reported IC<sub>50</sub> value was generated from a fitted curve by solving for the X-intercept value at the 50% inhibition level of the Y-intercept value.

PubChem Activity Outcome and Score:

Compounds with an IC<sub>50</sub> greater than 10  $\mu$ M were considered inactive. Compounds with an IC<sub>50</sub> equal to or less than 10  $\mu$ M were considered active.

Any compound with a percent activity value < 50% at all test concentrations was assigned an activity score of zero. Any compound with a percent activity value >= 50% at any test concentration was assigned an activity score greater than zero.

Activity score was then ranked by the potency of the compounds with fitted curves, with the most potent compounds assigned the highest activity scores.

The PubChem Activity Score range for active compounds was 100-43, and for inactive compounds 1-0.

### **(2.1.11) rP<sub>1</sub>M18AAP QFRET-based Concentration-response Biochemical High Throughput Assay (SAR Assay AID Nos. 588678, 602222, 624177)**

#### *List of Reagents:*

rP<sub>1</sub>M18AAP enzyme (supplied by Assay Provider)  
H-Glu-NHMec substrate (Bachem, part I-1180)  
Tris (Amresco, part 0497)  
CoCl<sub>2</sub> 6H<sub>2</sub>O (Univar, part D3247)  
BSA (Calbiochem, part 126609)

#### *Protocol Summary:*

2.5 uL of rP<sub>1</sub>M18AAP reagent mix, which included the fluorogenic peptide substrate (H-Glu-NHMec) in assay buffer, was added to each well of the previously compound dosed 1536-well plates. The reaction was initiated with the addition of 2.5 uL of the rP<sub>1</sub>M18AAP diluted in assay buffer. The final concentrations in the reaction were 0.1 mM H-Glu-NHMec and 5 ug/ml rP<sub>1</sub>M18AAP diluted in assay buffer (50 mM Tris-HCl (pH 7.5), 2 mM CoCl<sub>2</sub>, 0.1% BSA, 0.01% Triton X-100, and 2% DMSO). The test plate was incubated at room temperature for 90 minutes, then transferred to a Perkin Elmer Envision microplate reader and fluorescence (RFU) was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Each plate had 256 control wells in the eight outside columns with 128 wells containing the complete reaction mixture with carrier control (Full Rxn) and 128 wells in which the rP<sub>1</sub>M18AAP had been left out (Bkg).

128 background control wells containing the peptide substrate only and 128 full reaction control wells containing peptide substrate and 5 ug/ml rP<sub>1</sub>M18AAP were included on each assay plate and used to calculate a Z' value for each plate and to normalize the data on a per plate basis. Data were analyzed using the IDBS Activity Base software. Results for each concentration were expressed as percent inhibition (% Inhibition) and was calculated as:  $100 * ((\text{Med Full Rxn RFU} - \text{Med Bkg RFU}) - (\text{Cmpd RFU} - \text{Med Bkg RFU})) / ((\text{Med Full Rxn RFU} - \text{Med Bkg RFU}))$ . The dose response data was analyzed using a four parameter logistic fit to the data (Excel Fit equation 205) with the maximum and minimum locked at 100 and 0. From these curves IC<sub>50</sub> values were calculated.

Compounds showing 30% or greater inhibition at any concentration were considered "Active". IC<sub>50</sub> values were calculated for these compounds and used to determine the relative score. The following tiered system has been implemented at Southern Research Institute for use with the PubChem Score: Compounds in the primary screen are scored on a scale of 0-40 based on % activity; a score of 40 corresponds to 100% activity. In the confirmatory dose response screen of primary screen hits, active compounds are scored on a scale of 41-80 based on IC<sub>50</sub> result

while compounds where activity was not confirmed are given the score 0. Confirmatory dose response and secondary screens of purified and/or resynthesized compounds, indicating the highest degree of confidence) are scored on a scale of 81-100 based on IC<sub>50</sub> result. Inactive compounds are given the score 0.

**(2.1.12) rP<sub>1</sub>M1MAA QFRET-based Counterscreen Concentration-response Biochemical High Throughput Assay (Counterscreen Assay AID Nos. 588680, 602219, 624176)**

*List of Reagents:*

rP<sub>1</sub>M1MAA enzyme (supplied by Assay Provider)  
H-Glu-NHMec substrate (Bachem, part I-1180)  
Tris (Amresco, part 0497)  
CoCl<sub>2</sub> 6H<sub>2</sub>O (Univar, part D3247)  
BSA (Calbiochem, part 126609)

*Protocol Summary:*

2.5 uL of rP<sub>1</sub>M1MAA reagent mix, which included the fluorogenic peptide substrate (H-Leu-NHMec) in assay buffer, was added to each well of the previously compound dosed 1536-well plates. The reaction was initiated with the addition of 2.5 uL of the rP<sub>1</sub>M1MAA diluted in assay buffer. The final concentrations in the reaction were 0.1 mM H-Leu-NHMec and 5 ug/ml rP<sub>1</sub>M1MAA diluted in assay buffer (50 mM Tris-HCl (pH 7.5), 2 mM CoCl<sub>2</sub>, 0.1% BSA, 0.01% Triton X-100, and 2% DMSO). The test plate was incubated at room temperature for 90 minutes, then transferred to a Perkin Elmer Envision microplate reader and fluorescence (RFU) was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Each plate had 256 control wells in the eight outside columns with 128 wells containing the complete reaction mixture with carrier control (Full Rxn) and 128 wells in which the rP<sub>1</sub>M1MAA had been left out (Bkg).

128 background control wells containing the peptide substrate only and 128 full reaction control wells containing peptide substrate and 5 ug/ml rP<sub>1</sub>M1MAA were included on each assay plate and used to calculate a Z' value for each plate and to normalize the data on a per plate basis. Data were analyzed using the IDBS Activity Base software. Results for each concentration were expressed as percent inhibition (% Inhibition) and was calculated as:  $100 * ((\text{Med Full Rxn RFU} - \text{Med Bkg RFU}) - (\text{Cmpd RFU} - \text{Med Bkg RFU})) / ((\text{Med Full Rxn RFU} - \text{Med Bkg RFU}))$ . The dose response data was analyzed using a four parameter logistic fit to the data (Excel Fit equation 205) with the maximum and minimum locked at 100 and 0. From these curves IC<sub>50</sub> values were calculated.

Compounds showing 30% or greater inhibition at any concentration were considered "Active". IC<sub>50</sub> values were calculated for these compounds and used to determine the relative score. The following tiered system has been implemented at Southern Research Institute for use with the PubChem Score: Compounds in the primary screen are scored on a scale of 0-40 based on % activity; a score of 40 corresponds to 100% activity. In the confirmatory dose response screen of primary screen hits, active compounds are scored on a scale of 41-80 based on IC<sub>50</sub> result while compounds where activity was not confirmed are given the score 0. Confirmatory dose response and secondary screens of purified and/or resynthesized compounds, indicating the highest degree of confidence) are scored on a scale of 81-100 based on IC<sub>50</sub> result. Inactive compounds are given the score 0.

### **(2.1.13) rP<sub>f</sub>M17LAP QFRET-based Counterscreen Concentration-response Biochemical High Throughput Assay (Counterscreen Assay AID Nos. 588679, 602220, 624175)**

#### *List of Reagents:*

rP<sub>f</sub>M17LAP enzyme (supplied by Assay Provider)  
H-Glu-NHMec substrate (Bachem, part I-1180)  
Tris (Amresco, part 0497)  
CoCl<sub>2</sub> 6H<sub>2</sub>O (Univar, part D3247)  
BSA (Calbiochem, part 126609)

#### *Protocol Summary:*

2.5 uL of rP<sub>f</sub>M17LAP reagent mix, which included the fluorogenic peptide substrate (H-Leu-NHMec) in assay buffer, was added to each well of the previously compound dosed 1536-well plates. The reaction was initiated with the addition of 2.5 uL of the rP<sub>f</sub>M17LAP diluted in assay buffer. The final concentrations in the reaction were 0.1 mM H-Leu-NHMec and 5 ug/ml rP<sub>f</sub>M17LAP diluted in assay buffer (50 mM Tris-HCl (pH 7.5), 2 mM CoCl<sub>2</sub>, 0.1% BSA, 0.01% Triton X-100, and 2% DMSO). The test plate was incubated at room temperature for 90 minutes, then transferred to a Perkin Elmer Envision microplate reader and fluorescence (RFU) was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Each plate had 256 control wells in the eight outside columns with 128 wells containing the complete reaction mixture with carrier control (Full Rxn) and 128 wells in which the rP<sub>f</sub>M17LAP had been left out (Bkg).

128 background control wells containing the peptide substrate only and 128 full reaction control wells containing peptide substrate and 5 ug/ml rP<sub>f</sub>M17LAP were included on each assay plate and used to calculate a Z' value for each plate and to normalize the data on a per plate basis. Data were analyzed using the IDBS Activity Base software. Results for each concentration were expressed as percent inhibition (% Inhibition) and was calculated as:  $100 * ((\text{Med Full Rxn RFU} - \text{Med Bkg RFU}) - (\text{Cmpd RFU} - \text{Med Bkg RFU})) / ((\text{Med Full Rxn RFU} - \text{Med Bkg RFU}))$ . The dose response data was analyzed using a four parameter logistic fit to the data (Excel Fit equation 205) with the maximum and minimum locked at 100 and 0. From these curves IC<sub>50</sub> values were calculated.

Compounds showing 30% or greater inhibition at any concentration were considered "Active". IC<sub>50</sub> values were calculated for these compounds and used to determine the relative score. The following tiered system has been implemented at Southern Research Institute for use with the PubChem Score: Compounds in the primary screen are scored on a scale of 0-40 based on % activity; a score of 40 corresponds to 100% activity. In the confirmatory dose response screen of primary screen hits, active compounds are scored on a scale of 41-80 based on IC<sub>50</sub> result while compounds where activity was not confirmed are given the score 0. Confirmatory dose response and secondary screens of purified and/or resynthesized compounds, indicating the highest degree of confidence) are scored on a scale of 81-100 based on IC<sub>50</sub> result. Inactive compounds are given the score 0.

**(2.1.14) rhM18 QFRET-based Counterscreen Concentration-response Biochemical High Throughput Assay (Counterscreen Assay AID Nos. 588696, 602221, 624174)**

*List of Reagents:*

rhM18 enzyme (supplied by Assay Provider)  
H-Glu-NHMec substrate (Bachem, part I-1180)  
Tris (Amresco, part 0497)  
CoCl<sub>2</sub> 6H<sub>2</sub>O (Univar, part D3247)  
BSA (Calbiochem, part 126609)

*Protocol Summary:*

2.5 uL of rhM18 reagent mix, which included the fluorogenic peptide substrate (H-Glu-NHMec) in assay buffer, was added to each well of the previously compound dosed 1536-well plates. The reaction was initiated with the addition of 2.5 uL of the rhM18 diluted in assay buffer. The final concentrations in the reaction were 0.1 mM H-Glu-NHMec and 5 ug/ml rhM18 diluted in assay buffer (50 mM Tris-HCl (pH 7.5), 2 mM CoCl<sub>2</sub>, 0.1% BSA, 0.01% Triton X-100, and 2% DMSO). The test plate was incubated at room temperature for 90 minutes, then transferred to a Perkin Elmer Envision microplate reader and fluorescence (RFU) was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Each plate had 256 control wells in the eight outside columns with 128 wells containing the complete reaction mixture with carrier control (Full Rxn) and 128 wells in which the rhM18 had been left out (Bkg).

128 background control wells containing the peptide substrate only and 128 full reaction control wells containing peptide substrate and 5 ug/ml rhM18AAP were included on each assay plate and used to calculate a Z' value for each plate and to normalize the data on a per plate basis. Data were analyzed using the IDBS Activity Base software. Results for each concentration were expressed as percent inhibition (% Inhibition) and was calculated as:  $100 * ((\text{Med Full Rxn RFU} - \text{Med Bkg RFU}) / (\text{Med Full Rxn RFU} - \text{Med Bkg RFU}))$ . The dose response data was analyzed using a four parameter logistic fit to the data (Excel Fit equation 205) with the maximum and minimum locked at 100 and 0. From these curves IC<sub>50</sub> values were calculated.

Compounds showing 30% or greater inhibition at any concentration were considered "Active". IC<sub>50</sub> values were calculated for these compounds and used to determine the relative score. The following tiered system has been implemented at Southern Research Institute for use with the PubChem Score: Compounds in the primary screen are scored on a scale of 0-40 based on % activity; a score of 40 corresponds to 100% activity. In the confirmatory dose response screen of primary screen hits, active compounds are scored on a scale of 41-80 based on IC<sub>50</sub> result while compounds where activity was not confirmed are given the score 0. Confirmatory dose response and secondary screens of purified and/or resynthesized compounds, indicating the highest degree of confidence) are scored on a scale of 81-100 based on IC<sub>50</sub> result. Inactive compounds are given the score 0.

**(2.1.15) Vero Cell-based Concentration-response Cytotoxicity Assay (Counterscreen Assay AID Nos. 588714, 602225, 624205)**

*Protocol Summary:*

Cell Culture: Vero E6 cells were subcultured every 7 days in E-MEM with 10% fetal bovine serum and 2 mM glutamine (complete growth medium), incubated at 37 degrees C in 5% carbon dioxide, and maintained for no more than 20 passages.

Compound Dosing/Plating: Carrier control / compounds were diluted in complete growth medium to prepare a 6X concentrated dosing solution which was dispensed into 384-well black clear-bottom tissue culture treated plates (5 uL volume).

Cell Plating: Twenty uL of complete growth medium containing 3000 cells were dispensed per well. Plates were incubated at 37 C, 5% CO<sub>2</sub> for 72h prior to endpoint detection.

Endpoint/Detection: At the end of the treatment period, assay plates were removed from the incubator and equilibrated to room temperature for 10 min. Twenty-five uL of Cell Titer Glo reagent was added and plates were incubated for an additional 10 min in the dark. At the end of the incubation, assay plates were analyzed using a PerkinElmer Envision microplate reader in luminescence mode with an integration time of 0.1 s.

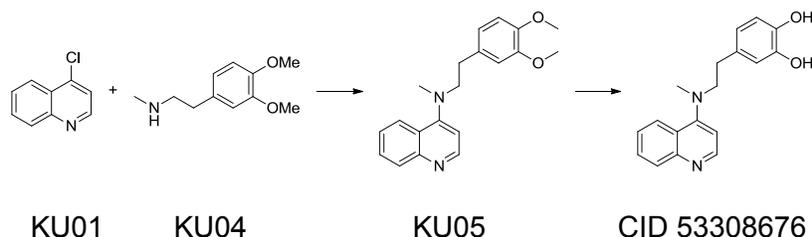
Data Analysis: Sixty-four control wells containing cells treated with DMSO vehicle and were included on each assay plate. Compound data was normalized and reported as % viability which was calculated using the following formula: % viability = 100\*(Cmpd Lum-Med background)/(Med Cell Ctrl - Med background). The normalized % viability was plotted against the tested concentrations. The CC<sub>50</sub> values were calculated using XLfit formula 205, a 4 parameter Levenburg-Marquardt algorithm with maximum and minimum limits set at 100 and 0, respectively and allowing extrapolation to identify weakly active compounds.

Compounds that showed <80% cell viability for at least one concentration were defined as "Active" (toxic). If the % viability at all doses was <80%, the compound was defined as "Inactive" (non-toxic). Instances where replicate data sets conflicted using these criteria are listed as "Inconclusive". The following tiered system has been implemented at Southern Research Institute for use with the PubChem Score: Compounds in the primary screen are scored on a scale of 0-40 based on % activity; a score of 40 corresponds to 100% activity. In the confirmatory dose response screen of primary screen hits, active compounds are scored on a scale of 41-80 based on CC50 result while compounds where activity was not confirmed are given the score 0. Confirmatory dose response and secondary screens of purified and/or resynthesized compounds, indicating the highest degree of confidence) are scored on a scale of 81-100 based on CC50 result. Inactive compounds are given the score 0



reaction was adsorbed onto Celite then purified by column chromatography on silica gel, eluting with a 0 – 20% gradient of 20:1 methanol:conc ammonium hydroxide in dichloromethane. ML369 was afforded as a light tan solid (144 mg; 96%). <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 8.77 (s, 1H), 8.70 (s, 1H), 8.68 (d, *J* = 4.9 Hz, 1H), 8.05 (m, 1H), 7.94 (m, 1H), 7.69 (ddd, *J* = 8.3, 6.9, 1.3 Hz, 1H), 7.55 (ddd, *J* = 8.2, 6.9, 1.2 Hz, 1H), 7.00 (d, *J* = 5.0 Hz, 1H), 6.74 – 6.65 (m, 1H), 6.59 (dd, *J* = 8.1, 2.0 Hz, 1H), 4.11 (m, 1H), 3.70 – 3.57 (m, 2H), 2.99 – 2.81 (m, 2H), 2.59 (m, 1H), 1.99 – 1.82 (m, 4H). <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>) δ 156.8, 150.8, 149.1, 145.1, 143.5, 136.9, 129.5, 128.9, 125.2, 123.9, 123.0, 117.2, 115.5, 114.2, 109.0, 52.8, 41.0, 33.4. HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 321.1598, found: 321.1693. UPLC purity at 214 nm is 100%.

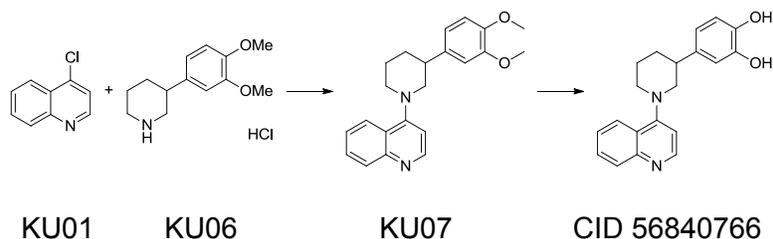
### Synthesis of CID 53308676; SID 162021973:



***N*-(3,4-Dimethoxyphenethyl)-*N*-methylquinolin-4-amine (KU05):** KU05 (tan oil; 368 mg; 76%) was synthesized in a manner analogous to that for KU03 from 4-chloroquinoline (245 mg; 1.50 mmol) and 2-(3,4-dimethoxyphenethyl)-*N*-methylethanamine (360 μL; 1.95 mmol) in 1-pentanol (2.0 mL). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.65 (d, *J* = 5.3 Hz, 1H), 8.10 (d, *J* = 8.0 Hz, 1H), 7.98 (m, 1H), 7.66 (ddd, *J* = 8.4, 6.8, 1.4 Hz, 1H), 7.44 (ddd, *J* = 8.3, 6.8, 1.3 Hz, 1H), 6.82 (d, *J* = 5.3 Hz, 1H), 6.79 (d, *J* = 8.2 Hz, 1H), 6.72 (dd, *J* = 8.1, 2.0 Hz, 1H), 6.65 (d, *J* = 1.9 Hz, 1H), 3.85 (s, 3H), 3.80 (s, 3H), 3.62 – 3.54 (m, 2H), 3.09 (s, 3H), 3.00 – 2.91 (m, 2H).

**4-(2-(Methyl(quinolin-4-yl)amino)ethyl)benzene-1,2-diol (CID 53308676):** CID 53308676 was synthesized in a manner analogous to that for ML369 from *N*-(3,4-dimethoxyphenethyl)-*N*-methylquinolin-4-amine (198 mg; 0.577 mmol) and boron tribromide (1.0 M in DCM; 1.80 mL; 1.80 mmol) in DCM (6.0 mL). After the initial chromatography, the <sup>1</sup>H NMR spectrum showed an extra, broad peak in the downfield region. The reaction product was suspended in methanol (6.0 mL), treated with sodium bicarbonate (273 mg), then adsorbed onto Celite and (re)chromatographed as above yielding CID 53308676 as a pale yellow hard foam (131 mg; 77%). <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 8.75 (s, 1H), 8.69 (s, 1H), 8.60 (d, *J* = 5.1 Hz, 1H), 7.98 – 7.93 (m, 1H), 7.93 – 7.88 (m, 1H), 7.66 (ddd, *J* = 8.3, 6.8, 1.3 Hz, 1H), 7.47 (ddd, *J* = 8.3, 6.8, 1.3 Hz, 1H), 6.93 (d, *J* = 5.1 Hz, 1H), 6.63 (d, *J* = 8.0 Hz, 1H), 6.61 (d, *J* = 2.0 Hz, 1H), 6.48 (dd, *J* = 8.0, 2.1 Hz, 1H), 3.41 – 3.35 (m, 5H), 3.00 (s, 3H), 2.85 – 2.78 (m, 2H). <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>) δ 156.4, 150.3, 149.3, 145.1, 143.6, 129.9, 129.4, 128.8, 124.6, 124.2, 122.6, 119.4, 116.1, 115.5, 108.4, 58.0, 32.5. HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>18</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 295.1441, found: 295.1509. UPLC purity at 214 nm is 99.5%.

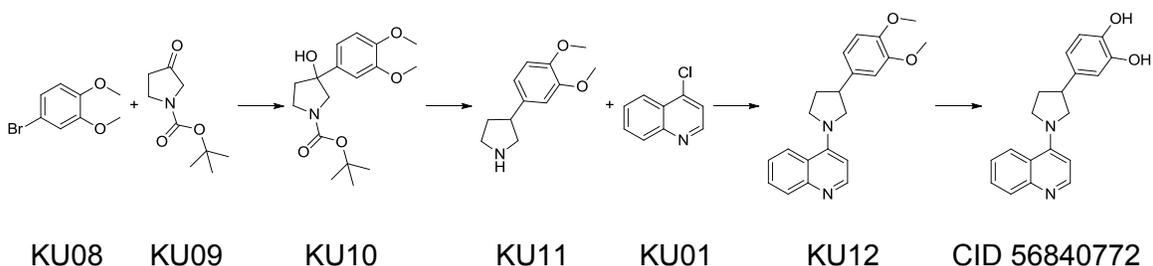
### Synthesis of CID 56840766; SID 162021974:



**(rac)-4-(3-(3,4-Dimethoxyphenyl)piperidin-1-yl)quinolone (KU07):** KU07 (tan oil; 229 mg; 87%) was synthesized in a manner analogous to that for KU03 from 4-chloroquinoline (123 mg; 0.75 mmol), (rac)-3-(3,4-dimethoxyphenyl)piperidine hydrochloride (252 mg; 0.978 mmol) and Hünig's base (300  $\mu$ L; 1.82 mmol) in 1-pentanol (1.5 mL).  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.71 (d,  $J = 5.0$  Hz, 1H), 8.05 (m, 2H), 7.65 (ddd,  $J = 8.4, 6.8, 1.4$  Hz, 1H), 7.49 (ddd,  $J = 8.2, 6.9, 1.2$  Hz, 1H), 6.88 – 6.80 (m, 4H), 3.89 (s, 3H), 3.87 (s, 3H), 3.74 – 3.66 (m, 2H), 3.11 (m, 1H), 2.94 – 2.81 (m, 2H), 2.22 – 1.95 (m, 3H), 1.72 (m, 1H).

**(rac)-4-(1-(Quinolin-4-yl)piperidin-3-yl)benzene-1,2-diol (CID 56840766):** CID 56840766 (tan hard foam; 184 mg; 98%) was synthesized in a manner analogous to that for ML369 from 4-(3-(3,4-dimethoxyphenyl)piperidin-1-yl)quinolone (227 mg; 0.586 mmol) and boron tribromide (1.0 M in DCM; 2.00 mL; 2.00 mmol) in DCM (7.0 mL).  $^1\text{H NMR}$  (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.76 (s, 1H), 8.74 (s, 1H), 8.65 (d,  $J = 5.0$  Hz, 1H), 8.06 – 8.00 (m, 1H), 7.96 – 7.90 (m, 1H), 7.68 (ddd,  $J = 8.3, 6.9, 1.4$  Hz, 1H), 7.55 (ddd,  $J = 8.2, 6.9, 1.2$  Hz, 1H), 6.98 (d,  $J = 5.0$  Hz, 1H), 6.70 (d,  $J = 2.0$  Hz, 1H), 6.67 (d,  $J = 8.1$  Hz, 1H), 6.58 (dd,  $J = 8.1, 2.0$  Hz, 1H), 3.61 – 3.55 (m, 1H), 3.54 – 3.48 (m, 1H), 2.98 – 2.89 (m, 1H), 2.88 – 2.75 (m, 2H), 2.02 – 1.88 (m, 3H), 1.67 – 1.55 (m, 1H).  $^{13}\text{C NMR}$  (126 MHz,  $\text{DMSO}-d_6$ )  $\delta$  156.6, 150.8, 149.1, 145.1, 143.8, 134.6, 129.6, 128.9, 125.2, 123.8, 122.9, 117.6, 115.5, 114.5, 109.1, 59.5, 52.5, 41.5, 31.4, 25.5. HRMS (ESI $^+$ )  $m/z$  calcd for  $[\text{C}_{20}\text{H}_{20}\text{N}_2\text{O}_2 + \text{H}]^+$ : 321.1598, found: 321.1680. UPLC purity at 214 nm is 100%.

### Synthesis of CID 56840772; SID 162021975:



**(rac)-tert-Butyl 3-(3,4-dimethoxyphenyl)-3-hydroxypyrrolidine-1-carboxylate (KU10):** A solution of *n*-butyllithium (1.6 M in hexanes; 10.0 mL; 16 mmol) was added dropwise over 5 min to a degassed solution of 4-bromoveratrole (2.00 mL; 13.90 mmol) in dry THF (70 mL) in a dry ice – acetone bath. After 0.3 h, a solution of *N*-Boc-3-pyrrolidinone (2.854 g; 15.41 mmol) in dry THF (15 mL) was added over 3 min. The reaction was allowed to stir at room temperature after 5 min at  $-78$   $^{\circ}\text{C}$ . After 3.3 h at room temperature the reaction was quenched with 0.5 M citric acid (40 mL), then most of the organic volatiles were removed on a rotary evaporator. The product was extracted into EtOAc (70 mL) and the organic portion was washed with water (2 x 20 mL), satd NaCl (2 x 20 mL), then dried over  $\text{MgSO}_4$  and filtered. KU10 was obtained as a clear, tan, viscous liquid after column chromatography ( $\text{SiO}_2$  eluting with a gradient of 0 – 75%

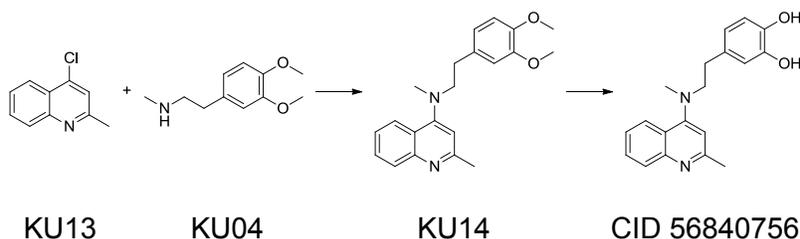
EtOAc in hexanes with ELS detection). The product (2.91 g) contained ca. 1 mole equivalent of EtOAc and was used as is in the next reaction.

**(rac)-3-(3,4-Dimethoxyphenyl)pyrrolidine (KU11):** Neat trifluoroacetic acid (15 mL) was added to a biphasic solution of triethylsilane (5.70 mL; 35.7 mmol) and KU10 (2.91 g as prepared above) in DCM (10 mL). After 1.0 h at 0 °C, the reaction was concentrated on a rotary evaporator then partitioned between aq NaOH (50 mL of 1 M NaOH plus 1 mL of 10 M NaOH) and DCM (10 mL). The aq portion was extracted with additional DCM (3 x 10 mL) and the combined extracts were washed with 1 M NaOH (3 x 10 mL), then dried (Na<sub>2</sub>SO<sub>4</sub>) and filtered. Pure KU11 (749 mg; 26% over two steps) was obtained as a viscous tan liquid after direct injection of the DCM solution onto a silica gel column, eluting with a 0 – 20% gradient of 20:1 methanol:conc ammonium hydroxide in dichloromethane. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 6.85 – 6.75 (m, 3H), 3.88 (s, 3H), 3.86 (s, 3H), 3.37 (dd, *J* = 10.7, 7.6 Hz, 1H), 3.26 – 3.04 (m, 3H), 2.85 (dd, *J* = 10.7, 8.4 Hz, 1H), 2.23 (m, 1H), 1.85 (m, 1H).

**(rac)-4-(3-(3,4-Dimethoxyphenyl)pyrrolidin-1-yl)quinolone (KU12):** KU12 (tan hard foam; 251 mg; 92%) was synthesized in a manner analogous to that for KU03 from 4-chloroquinoline (133 mg; 0.813 mmol) and 3-(3,4-dimethoxyphenyl)pyrrolidine (220 mg; 1.06 mmol) in 1-pentanol (1.5 mL). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.38 (d, *J* = 6.3 Hz, 1H), 8.29 (m, 2H), 7.70 (ddd, *J* = 8.3, 6.9, 1.2 Hz, 1H), 7.44 (ddd, *J* = 8.4, 6.9, 1.3 Hz, 1H), 6.92 – 6.79 (m, 3H), 6.52 (d, *J* = 6.4 Hz, 1H), 4.19 (dd, *J* = 9.9, 7.5 Hz, 1H), 4.02 – 3.84 (m, 3H), 3.90 (s, 3H), 3.89 (s, 3H), 3.57 (m, 1H), 2.51 (m, 1H), 2.30 (m, 1H).

**(rac)-4-(1-(Quinolin-4-yl)pyrrolidin-3-yl)benzene-1,2-diol (CID 56840772):** CID 56840772 (green solid; 105 mg; 66%) was synthesized in a manner analogous to that for CID 53308676 (including a second sodium bicarbonate treatment and chromatography) from 4-(3-(3,4-dimethoxyphenyl)pyrrolidin-1-yl)quinolone (172 mg; 0.514 mmol) and boron tribromide (1.0 M in DCM; 1.60 mL; 1.60 mmol) in DCM (6 mL). <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 8.91 – 8.73 (m, 2H), 8.40 (d, *J* = 13.2 Hz, 1H), 8.28 (m, 1H), 7.82 (m, 1H), 7.59 (ddd, *J* = 8.2, 6.8, 1.1 Hz, 1H), 7.36 (ddd, *J* = 8.3, 6.8, 1.3 Hz, 1H), 6.74 (d, *J* = 2.1 Hz, 1H), 6.70 (d, *J* = 8.0 Hz, 1H), 6.62 (dd, *J* = 8.1, 2.0 Hz, 1H), 6.56 (d, *J* = 5.5 Hz, 1H), 3.91 (dd, *J* = 9.3, 7.5 Hz, 1H), 3.88 – 3.79 (m, 2H), 3.75 – 3.64 (m, 2H), 2.30 (m, 1H), 2.04 (m, 1H). <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>) δ 151.7, 149.8, 149.7, 145.2, 144.0, 131.9, 129.1, 128.4, 125.3, 123.0, 120.6, 117.8, 115.5, 114.6, 102.7, 58.6, 51.7, 42.9, 32.5. HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>19</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 307.1441, found: 307.1424. UPLC purity at 214 nm is 97.2%.

#### Synthesis of CID 56840756; SID 162021976:

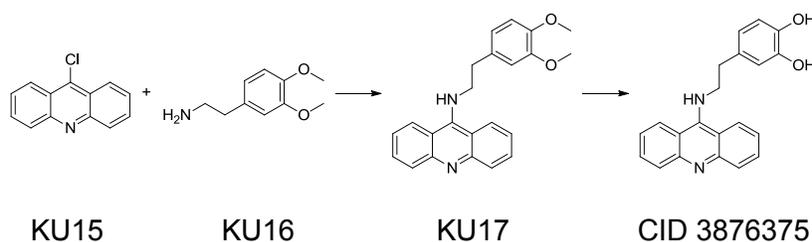


**N-(3,4-Dimethoxyphenethyl)-N,2-dimethylquinolin-4-amine (KU14):** KU14 (light brown waxy solid; 633 mg; 94%) was synthesized in a manner analogous to that for KU03 from 4-chloro-2-methylquinoline (355 mg; 2.00 mmol) and 2-(3,4-dimethoxyphenyl)-N-methylethanamine (480 μL; 2.60 mmol) in 1-pentanol (2.5 mL). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 7.98 (d, *J* = 8.3 Hz, 1H),

7.82 (m, 1H), 7.68 (m, 1H), 7.45 (m, 1H), 6.87 – 6.68 (m, 4H), 3.70 (s, 3H), 3.65 (s, 3H), 3.60 (m, 2H), 3.11 (s, 3H), 2.93 (m, 2H), 2.56 (s, 3H).

**4-(2-(Methyl(2-methylquinolin-4-yl)amino)ethyl)benzene-1,2-diol (CID 56840756):** CID 56840756 (hard tan foam; 233 mg; 86%) was synthesized in a manner analogous to that for CID 53308676 (including a second sodium bicarbonate treatment and chromatography) from *N*-(3,4-dimethoxyphenethyl)-*N*,2-dimethylquinolin-4-amine (297 mg; 0.883 mmol) and boron tribromide (1.0 M in DCM; 3.00 mL; 3.00 mmol) in DCM (10 mL). <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 8.75 (s, 1H), 8.71 (s, 1H), 7.93 (d, *J* = 7.9 Hz, 1H), 7.84 – 7.78 (m, 1H), 7.68 – 7.61 (m, 1H), 7.46 – 7.39 (m, 1H), 6.83 (s, 1H), 6.65 – 6.59 (m, 2H), 6.48 (dd, *J* = 8.0, 2.0 Hz, 1H), 3.46 – 3.40 (m, 3H), 3.04 (s, 3H), 2.86 – 2.77 (m, 2H), 2.56 (s, 3H). <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>) δ 157.5, 157.0, 147.4, 145.1, 143.6, 129.7, 129.3, 127.3, 124.4, 124.0, 120.5, 119.4, 116.1, 115.5, 108.1, 57.9, 40.2, 32.4, 24.2. HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>19</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 309.1598, found: 309.1598. UPLC purity at 214 nm is 98.1%.

#### Synthesis of CID 3876375; SID 162021977:



***N*-(3,4-Dimethoxyphenethyl)acridin-9-amine (KU17):** A mixture of 9-chloroacridine (641 mg; 3.00 mmol), 2-(3,4-dimethoxyphenyl)ethanamine (700 μL; 4.21 mmol) and phenol (1.414 g; 15.0 mmol) was heated at 220 °C under microwave irradiation for 5 min. 1 M NaOH (30 mL) was added and this was extracted with DCM (4 x 10 mL). The combined organic portions were washed with 1 M NaOH (5 x 2 mL) then dried over K<sub>2</sub>CO<sub>3</sub>. The material was purified by direct injection of this solution using silica gel chromatography, eluting with a 0 – 20% gradient of 20:1 methanol:conc ammonium hydroxide in dichloromethane. KU17 was obtained as a red-orange glassy solid (970 mg; 90%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.08 (m, 2H), 7.95 (m, 2H), 7.66 (m, 2H), 7.34 (m, 2H), 6.91 – 6.80 (m, 2H), 6.72 (d, *J* = 1.7 Hz, 1H), 4.09 (t, *J* = 6.7 Hz, 2H), 3.90 (s, 3H), 3.80 (s, 3H), 2.99 (t, *J* = 6.7 Hz, 2H).

**4-(2-(Acridin-9-ylamino)ethyl)benzene-1,2-diol (CID 3876375):** CID 3876375 was synthesized in a manner analogous to that for ML369 from *N*-(3,4-dimethoxyphenethyl)acridin-9-amine (168 mg; 0.469 mmol) and boron tribromide (1.0 M in DCM; 1.50 mL; 1.50 mmol) in DCM (6.0 mL); with an additional purification by pRPLC (C18 Isco Gold column eluting with a gradient of 10-100% acetonitrile in (0.02% formic acid in water)). <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 8.80 (s, 1H), 8.77 (s, 1H), 8.52 (d, *J* = 8.6 Hz, 2H), 7.98 – 7.91 (m, 2H), 7.84 (d, *J* = 8.2 Hz, 2H), 7.56 – 7.49 (m, 2H), 6.68 – 6.59 (m, 2H), 6.52 (dd, *J* = 8.0, 2.0 Hz, 1H), 4.25 – 4.18 (m, 2H), 3.05 – 2.98 (m, 2H). <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>) δ 163.2, 157.0, 145.3, 143.9, 140.3, 134.6, 128.6, 125.8, 123.2, 119.4, 116.1, 115.6, 112.7, 51.0, 34.5. HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>21</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 331.1441, found: 331.1442. UPLC purity at 214 nm is 100%.

## Appendix D: Experimental Procedure for Analytical Assays

**Aqueous Solubility:** Solubility analysis was performed using a direct UV kinetic solubility method in a 96-well format. All liquid dispense and transfer steps were performed with the Freedom Evo automated liquid handler (Tecan US). Solubility measurements were performed in an aqueous buffer solution (System Solution, pION Inc, P/N 110151) at pH 5.0, 6.2 and 7.4, in duplicate. Samples were incubated at room temperature for a minimum of 18 hrs to achieve equilibrium, then filtered (filter plate, pION Inc, P/N 110322) to remove any precipitate formed. The concentration of the compounds was measured by UV absorbance (250-498 nm) using the Infinite M200 (Tecan US) and compared to the spectra of the precipitation-free reference solutions. Spectroscopically pure 1-Propanol (Sigma P/N 256404) was used as a cosolvent to suppress precipitation in the reference solutions. The solubility of each compound was determined using  $\mu$ SOL Evolution Plus software v3.2 (pION Inc) and is expressed as the concentration ( $\mu$ g/mL) of a solute in a saturated solution.

### *Assay details:*

- Diclofenac Na and Dipyridamole were used as standards. Diclofenac Na is highly soluble. Dipyridamole is poorly to moderately soluble.
- Standards and test compound stocks were made in 100% DMSO
- Assay concentration of standards: 500  $\mu$ M and test compound: 300  $\mu$ M
- Cosolvent used in the reference solution to suppress precipitation: 1-Propanol
- Assay DMSO final concentration: 1%

**Aqueous Stability:** Compound was dissolved at 10  $\mu$ M in PBS/acetonitrile (1/1) at pH 7.4 (1% DMSO) and incubated at room temperature. The mixtures were sampled every hour for eight hours or every 8 hours for 48 hours and analyzed by RP HPLC/UV/HRMS. The analytical RP HPLC/UV/HRMS system utilized for the analysis was a Waters Acquity system with UV-detection and mass-detection (Waters LCT Premier). The analytical method conditions included a Waters Acquity HSS T3 C18 column (2.1 x 50mm, 1.8 $\mu$ m) and elution with a linear gradient of 99% water to 100% CH<sub>3</sub>CN at 0.6 mL/min flow rate. Peaks on chromatograms were integrated using the Waters OpenLynx software. Absolute areas under the curve (214 nm) were compared at each time point to determine relative percent compound remaining in supernatant.

**Thiol Stability:** Compound was dissolved at 10  $\mu$ M in PBS/acetonitrile (1/1) at pH 7.4 (1% DMSO) and incubated at room temperature with either no thiol source as a negative control or 50  $\mu$ M dithiothreitol (DTT). The mixtures were sampled every hour for eight hours or every 8 hours for 48 hours and analyzed by RP HPLC/UV/HRMS. The analytical RP HPLC/UV/HRMS system utilized for the analysis was a Waters Acquity system with UV-detection and mass-detection (Waters LCT Premier). The analytical method conditions included a Waters Acquity HSS T3 C18 column (2.1 x 50mm, 1.8 $\mu$ m) and elution with a linear gradient of 99% water to 100% CH<sub>3</sub>CN at 0.6 mL/min flow rate. Peaks on chromatograms were integrated using the Waters OpenLynx software. Absolute areas under the curve (214 nm) were compared at each time point to determine relative percent compound remaining in supernatant. The masses of potential adducts were searched for in the samples to determine if any detectable adduct formed. All samples were prepared in duplicate. Ethacrynic acid, a known Michael acceptor, was used as a positive control and was tested in PBS/acetonitrile (1/1).

**Plasma Stability:** Stability of the compound in human plasma (BioChemMed Services, P/N 752PR-EK3-PMG) was determined. All liquid dispense and transfer steps were performed with the Freedom Evo automated liquid handler (Tecan US). Plasma was allowed to thaw at room

temperature prior to preparing the assay solution of plasma:1X PBS (1:1). The assay solution was warmed up at 37 °C prior of adding the compound. Immediately after compounds were added, time 0 min aliquots were promptly collected and mixed with cold acetonitrile (spiked with an internal standard). The remainder of the reaction volume was incubated at 37 °C with shaking. Additional aliquots were collected 180 min after the start of the reaction and promptly quenched with cold acetonitrile (spiked with an internal standard). Samples were centrifuged at 3000 rpm for 10 min. The amount of compound in the supernatant was determined by LC/MS/MS (Applied Biosystems, Sciex API4000 Q-Trap) and the percent of parent compound remaining after 180 min was calculated by the following formula:

$$\% \text{ parent compound remaining} \left[ \frac{\text{Concentration at 180 min}}{\text{Concentration at 0 min}} \times 100 \right]$$

Results reported are the mean of each reaction duplicate, normalized to the internal standard, and expressed as a percent of compound remaining after the incubation time.

*Assay details:*

- Human Plasma in K3 EDTA
- Procaine and Procainamide were used as standards. Procaine is highly unstable in human plasma, Procainamide is highly stable in human plasma.
- Assay concentrations of standards and test compound: 1 µM
- Incubation Time: 3 hrs
- Reaction pH: 7.4
- Assay DMSO final concentration: 2.5%

**Hepatic Microsome Stability:** Metabolic stability was assessed in the presence of human liver microsomes (XenoTech, P/N H0630) and mouse liver mirosomes (XenoTech, P/N M1000). All liquid dispense and transfer steps were performed with the Freedom Evo automated liquid handler (Tecan US). NADPH, a required cofactor for CYP450 metabolism, was provided by the NADPH Regenerating System, Solutions A (BD Biosciences, P/N 451220) and B (BD Biosciences, P/N 451200). Compound stock solutions were initially prepared in 100% DMSO and subsequently diluted in acetonitrile for the assay. The pH of the reactions was kept at ~ 7.4 with potassium phosphate buffer (BD Biosciences, P/N 451201). The reactions were started after adding NADPH to the reaction plate containing microsomes and compounds and time 0 min aliquots were promptly collected and mixed with ice cold acetonitrile (spiked with internal standards) to quench the reactions. The remainder of the reaction volume was incubated at 37 °C with shaking. Additional aliquots were collected 60 min after the start of the reaction and promptly quenched with ice cold acetonitrile (spiked with an internal standard). Samples were centrifuged at 3000 rpm for 10 min. The amount of compound in the supernatant was determined by LC/MS/MS (Applied Biosystems, Sciex API4000 Q-Trap) and the percent of parent compound remaining after 60 min was calculated by the following formula:

$$\% \text{ parent compound remaining} = \left[ \frac{\text{Concentration at 60 min}}{\text{Concentration at 0 min}} \times 100 \right]$$

All reactions were run in triplicate, except negative controls (no NADPH) which were performed as single reactions. Results reported are the mean of each reaction triplicate, normalized to the internal standard, and expressed as a percent compound remaining after the incubation time.

*Assay details:*

- Human and Mouse Liver Microsomes: 0.5 mg/mL protein concentration
- NADPH Regenerating System: 1.55 mM NADP<sup>+</sup>, 1.33 mM glucose-6-phosphate, 1.33 mM Magnesium chloride, and 0.4 U/mL glucose-6 phosphate dehydrogenase
- Incubation Temperature: 37 °C
- Incubation Time: 60 min
- Standards: Verapamil-HCl and Testosterone, at 20 µM and 50 µM, respectively
- Test compound at 1 µM
- Assay DMSO final concentration: ≤ 0.5%
- Assay ACN final concentration: ≤ 1.2%

**Cellular Permeability:** Permeability was assessed using the Parallel Artificial Membrane Permeability Assay, PAMPA in a 96-well format. All liquid dispense and transfer steps were performed with the Freedom Evo automated liquid handler (Tecan US). Measurements were performed in an aqueous buffer solution (System Solution, pION Inc, P/N 110151) at pH 5.0, 6.2, and 7.4, in duplicate. A “sandwich” plate (pION Inc, P/N 110212) consisting of a donor bottom plate and an acceptor filter plate was used. The donor wells contained the compounds in 180 µl system solution, and magnetic stir bars. The filter on the bottom of each acceptor well was coated with GIT-0 lipid (pION Inc, P/N 110669) and filled with 200 µl of Acceptor Sink Buffer, pH 7.4 (pION Inc, P/N 110139) containing a surfactant to mimic the function of serum proteins. The permeation time was 30 min and moderate stirring (equivalent to 40 µm Aqueous Boundary Layer thickness) was applied using the Gut-Box™ (pION, Inc, P/N 110205). After the permeation time, the sandwich was disassembled and the amount of compound present in both the donor and acceptor wells was measured by UV absorbance (250-498 nm) using the Infinite M200 (Tecan US) and compared to spectra obtained from reference standards. Mass balance was used to determine the amount of material embedded in the membrane filter. The effective permeability,  $P_e$ , was calculated using the software PAMPA Evolution Plus, version 3.2 (pION Inc).

*Assay details:*

- Verapamil HCl, Metoprolol, and Ranitidine were used as reference standards
- Verapamil HCl is considered highly permeable
- Metoprolol is considered moderately permeable
- Ranitidine is considered poorly permeable
- Permeation time: 30 min
- Moderate stirring (equivalent to 40 µm ABL, aqueous boundary layer, also known as the unstirred water layer)
- Donor buffer pH: 5.0, 6.2 and 7.4
- Double-Sink: pH gradients between donor and acceptor compartments; acceptor buffer contains chemical sink
- Assay DMSO final concentration : 0.5%

**Plasma Protein Binding:** Teflon® Base Plate wells were rinsed with 20% ethanol for 10 minutes. Ethanol was then removed and wells were rinsed with ultrapure water and allowed to dry. RED Inserts from Thermo Scientific (Pierce) were placed (open end up) into the wells of the base plate. All liquid dispense and transfer steps were performed with the Freedom Evo automated liquid handler (Tecan US). The sample chambers (red ring) contained 300 µl of a mixture of plasma and compound. And 500µl of dialysis buffer (1X PBS, pH7.4) were added to the buffer chambers of the inserts. Duplicate inserts were made for each concentration tested. The base plate was covered with sealing tape and incubated at 37°C on an orbital shaker at 300 rpm for 4 hours. After the incubation time, equal volume from both chambers were removed and

transferred to a 96 well plate containing either plasma or buffer. To precipitate proteins and release compounds, ice cold acetonitrile (with an internal standard) was added. Samples were centrifuged for 10 minutes at 3000 rpm. The amount of compound in the supernatant was determined by LC/MS/MS (Applied Biosystems, Sciex API4000 Q-Trap). The percent of free and bound compounds were calculated with the following formulas:

$$\% \text{ of free parent compound} = \left[ \frac{\text{amount of compound in receiver chamber}}{\text{amount of compound in donor chamber}} \times 100 \right]$$

$$\% \text{ of bound parent compound} = 100 - \% \text{ of free compound}$$

Results reported are the mean of each reaction duplicate, normalized to the internal standard, and expressed as a percent compound bound after the incubation time.

*Assay details:*

- Human Plasma in K3 EDTA
- Propranolol and Metoprolol were used as standards. Propranolol is highly bound, Metoprolol is poorly bound
- Assay concentrations of standards and test compounds: 1  $\mu\text{M}$  and 10  $\mu\text{M}$
- Incubation Time: 4 hrs
- Reaction pH: 7.4
- Assay DMSO final concentration: 1%

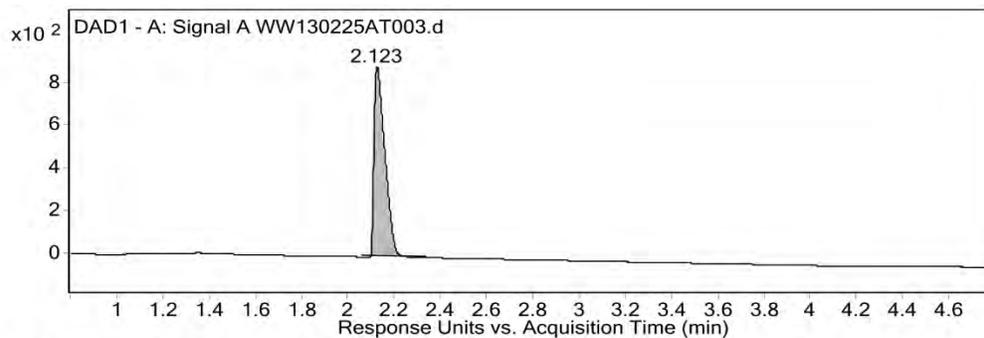
**Cytotoxicity:** Immortalized human hepatocytes, Fa2N-4 cells (XenoTech) were seeded at ~56,000 cells/well, and incubated with a range of concentrations (0.01-50  $\mu\text{M}$ ) of the test compound, in duplicate, for 24 hrs at 37°C, 5% CO<sub>2</sub>. Cell viability was determined by cellular ATP levels using the Luminescence ATP Detection Assay System (ATPlite 1 step, Perkin Elmer, # 6016731) and the Infinite M200 plate reader (Tecan).

*Assay details:*

- Cells used: Fa2N-4, immortalized human hepatocytes
- Media used for Fa2N-4 cells: MFE Plating and MFE Support (with 1% Penicillin, Streptomycin, and Amphotercin mixture)
- Assay DMSO final concentration = 0.5%
- Treatment time: 24 hrs
- Camptothecin and Terfenadine were used as standards. Camptothecin is highly toxic and Terfenadine is highly non toxic.

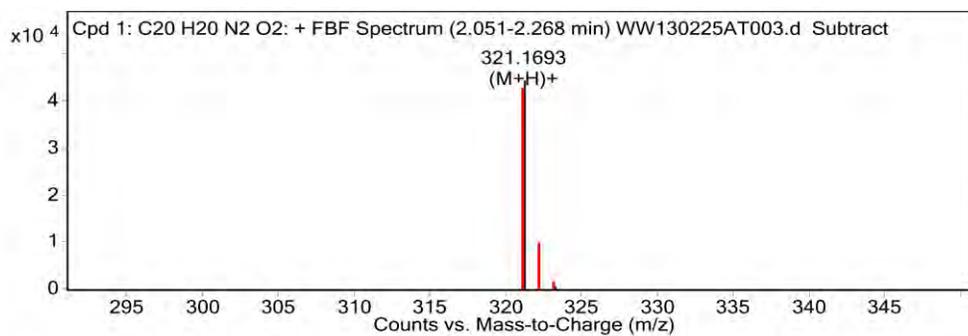


## UPLC-MS Chromatogram



### User Chromatogram Peak List

RT	Height	Height %	Area	Area %	Area Sum %	Symmetry	Width
2.123	883.5	100	2853.03	100	100	0.37	0.08



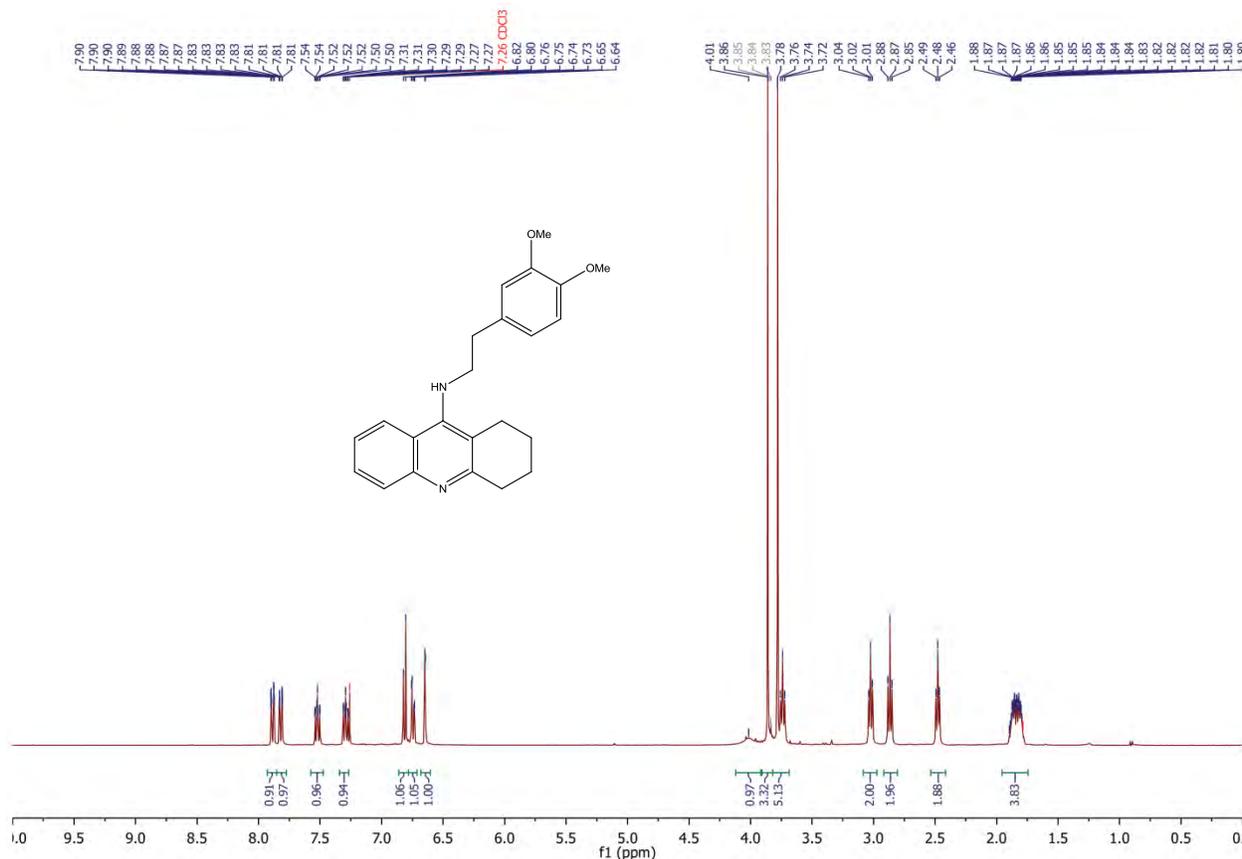
<i>m/z</i>	<i>z</i>	Abund	Formula	Ion
321.1693	1	44455.47	C <sub>20</sub> H <sub>21</sub> N <sub>2</sub> O <sub>2</sub>	(M+H) <sup>+</sup>
322.1717	1	8205.38	C <sub>20</sub> H <sub>21</sub> N <sub>2</sub> O <sub>2</sub>	(M+H) <sup>+</sup>
323.1745	1	903.93	C <sub>20</sub> H <sub>21</sub> N <sub>2</sub> O <sub>2</sub>	(M+H) <sup>+</sup>

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 321.1598, found: 321.1693.

## Appendix F: Chemical Characterization Data for All analogs

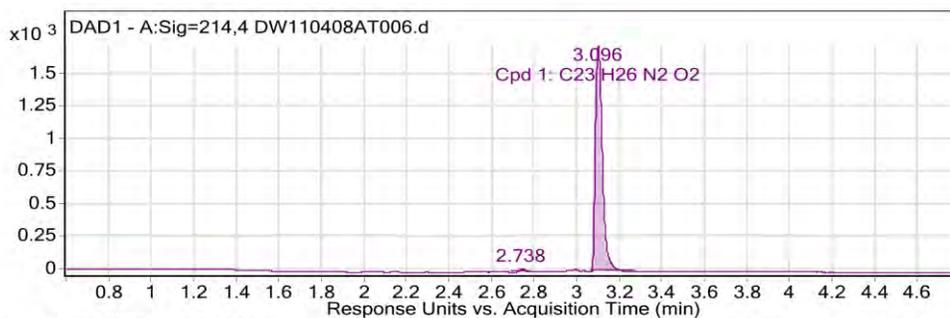
### Analog CID 22023; SID 124767880

#### <sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, CDCl<sub>3</sub>) δ 158.6, 150.4, 149.2, 148.0, 147.5, 131.0, 128.8, 128.3, 123.7, 122.8, 120.9, 120.4, 116.5, 112.0, 111.5, 56.1, 55.9, 50.1, 36.9, 34.1, 24.7, 23.1, 22.8.

#### UPLC-MS Chromatogram



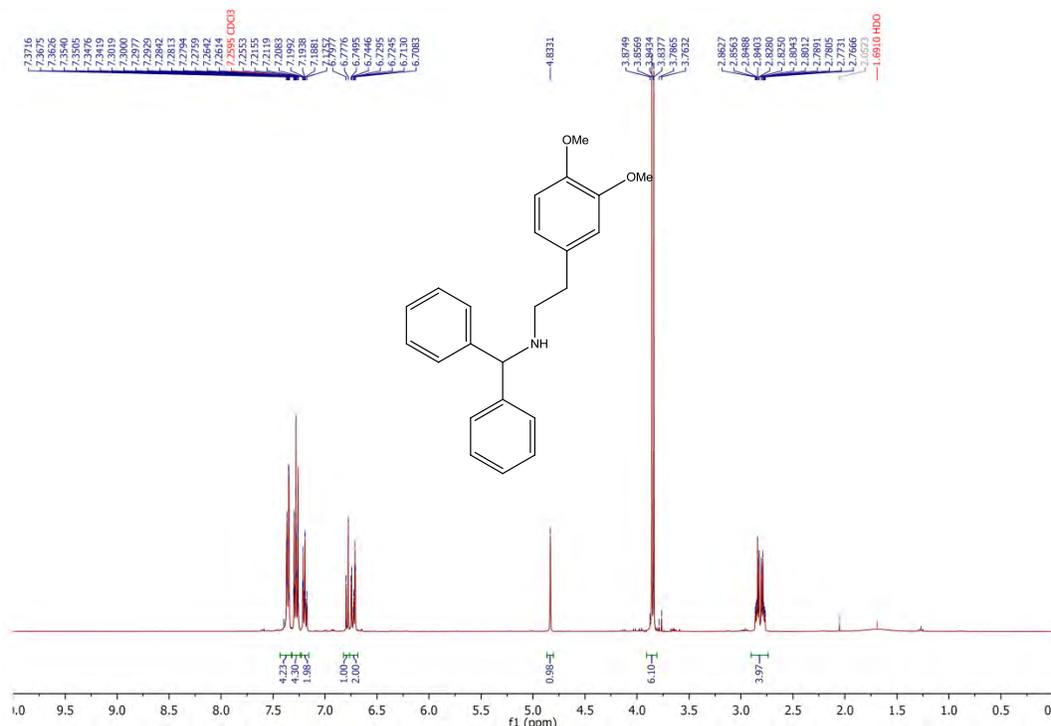
#### User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.738	24.55	1.42	42.83	1.09	1.08	0.027
2	Cpd 1: C <sub>23</sub> H <sub>26</sub> N <sub>2</sub> O <sub>2</sub>	3.096	1722.89	100	3935.4	100	98.92	0.036

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>23</sub>H<sub>26</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 363.2067, found: 363.2095.

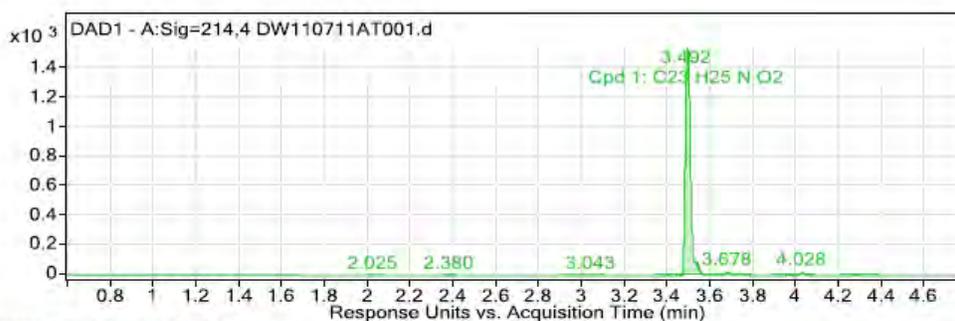
Analog CID 318615; SID 124767896

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, CDCl<sub>3</sub>) δ 149.0, 147.5, 144.0, 132.7, 128.6, 127.4, 127.1, 120.8, 112.1, 111.4, 67.4, 56.0, 55.9, 49.4, 36.0.

UPLC-MS Chromatogram



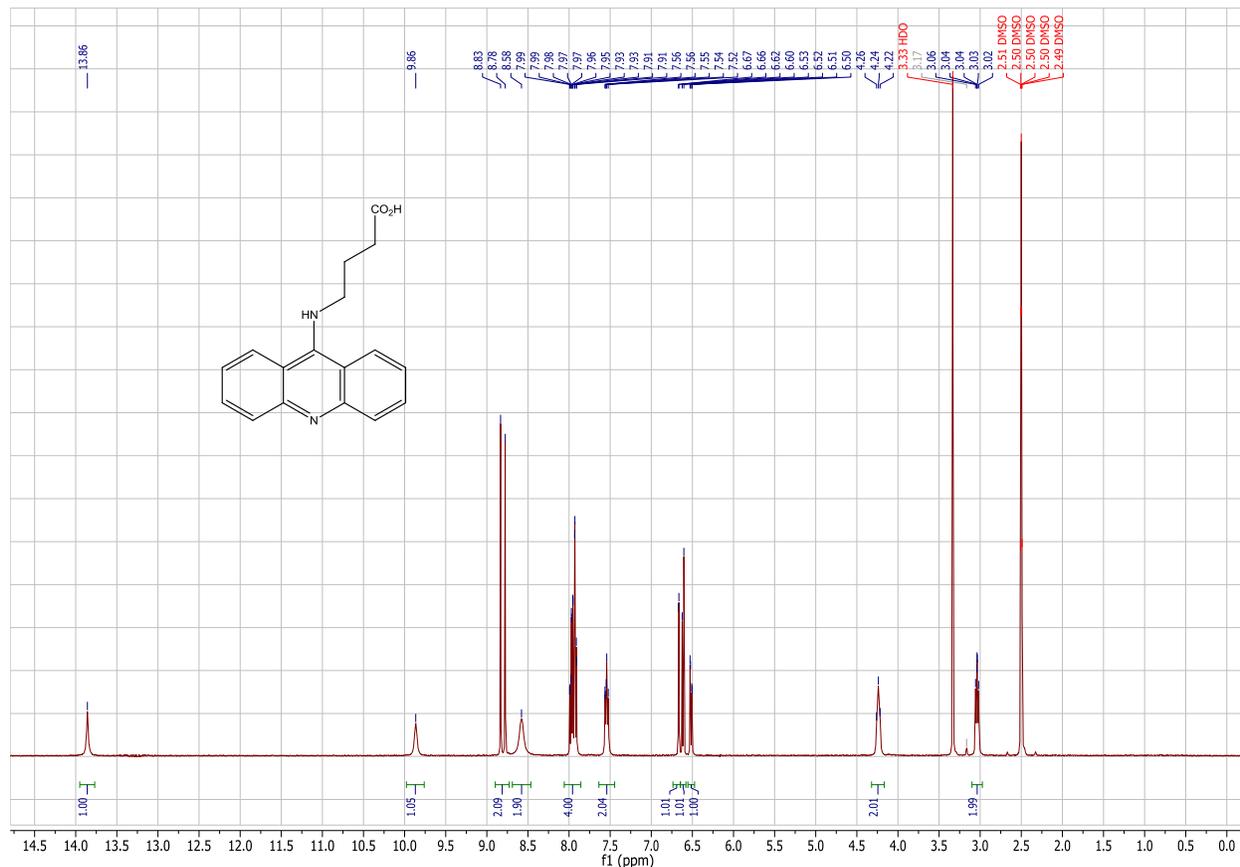
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
2		2.38	2.61	0.17	3.96	0.16	0.16	0.024
3		3.043	1.41	0.09	5.86	0.24	0.23	0.055
4		3.395	1.45	0.09	3.09	0.13	0.12	0.031
5	Cpd 1: C <sub>23</sub> H <sub>25</sub> N O <sub>2</sub>	3.492	1540.61	100	2428.9	100	95.52	0.025
6		3.535	12.55	0.81	10.26	0.42	0.4	0.014
7		3.678	19.05	1.24	26.62	1.1	1.05	0.022
8		3.744	13.6	0.88	18.95	0.78	0.75	0.022
9		3.963	2.31	0.15	4.1	0.17	0.16	0.027
10		4.028	17.57	1.14	27.71	1.14	1.09	0.024
11		4.275	6.67	0.43	10.8	0.44	0.42	0.025

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>23</sub>H<sub>25</sub>NO<sub>2</sub> + H]<sup>+</sup>: 348.1958, found: 348.1955.

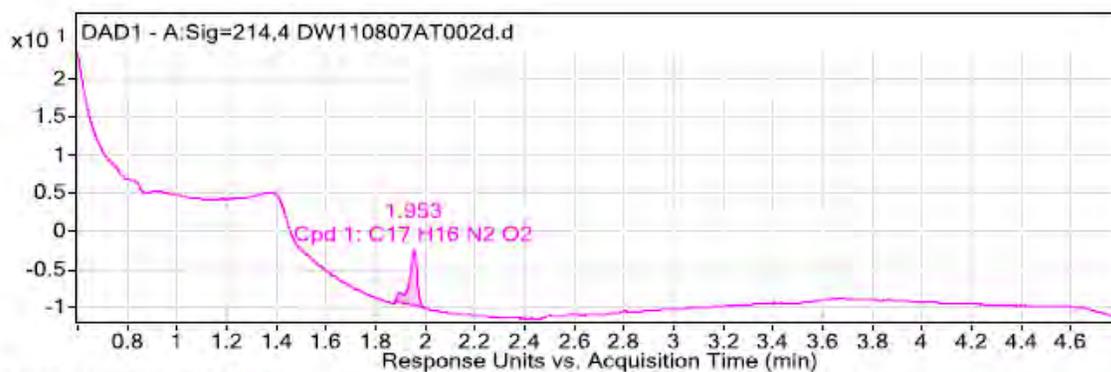
Analog CID 610254; SID 124767900

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 175.204, 174.200, 157.332, 157.328, 134.8, 123.2, 118.6, 118.5, 48.9, 30.9, 24.3.

UPLC-MS Chromatogram



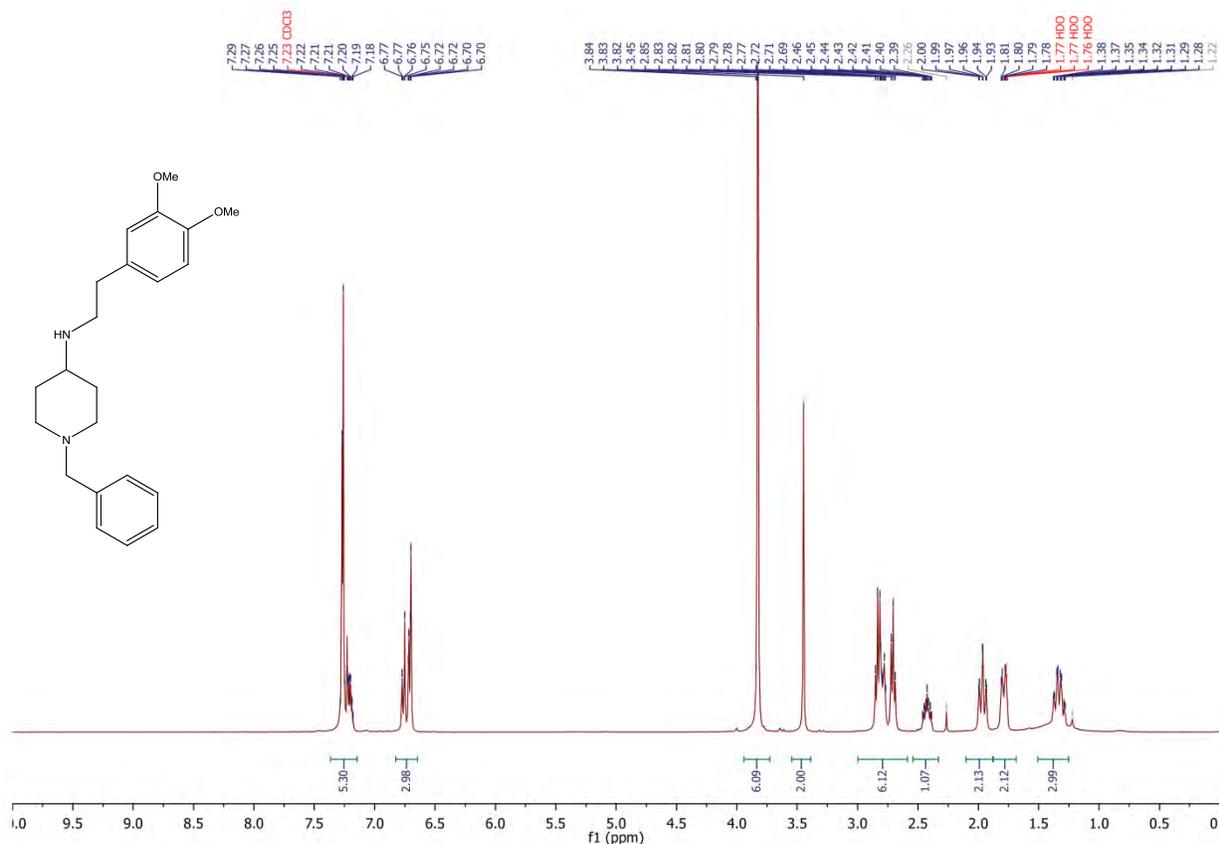
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>17</sub> H <sub>16</sub> N <sub>2</sub> O <sub>2</sub>	1.953	7.46	100	17.88	100	100	0.034

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>17</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 281.1285, found: 281.1284.

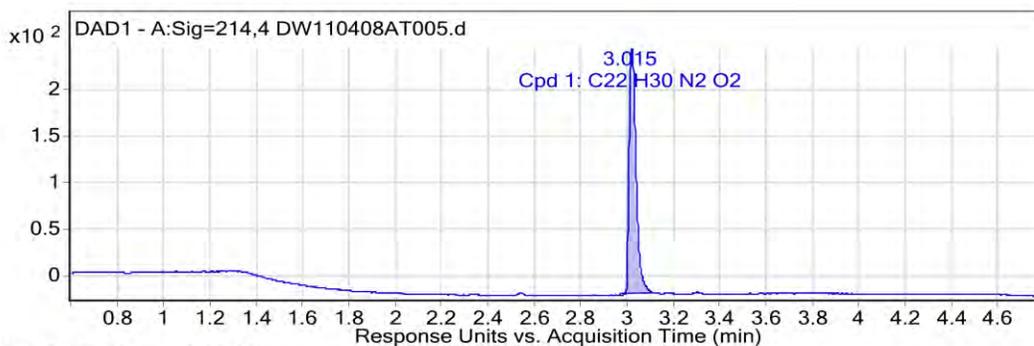
Analog CID 1131661; SID 124767879

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, CDCl<sub>3</sub>) δ 149.0, 147.6, 138.7, 132.8, 129.2, 128.3, 127.0, 120.6, 112.1, 111.5, 63.2, 56.1, 56.0, 55.1, 52.6, 48.3, 36.3, 32.9.

UPLC-MS Chromatogram



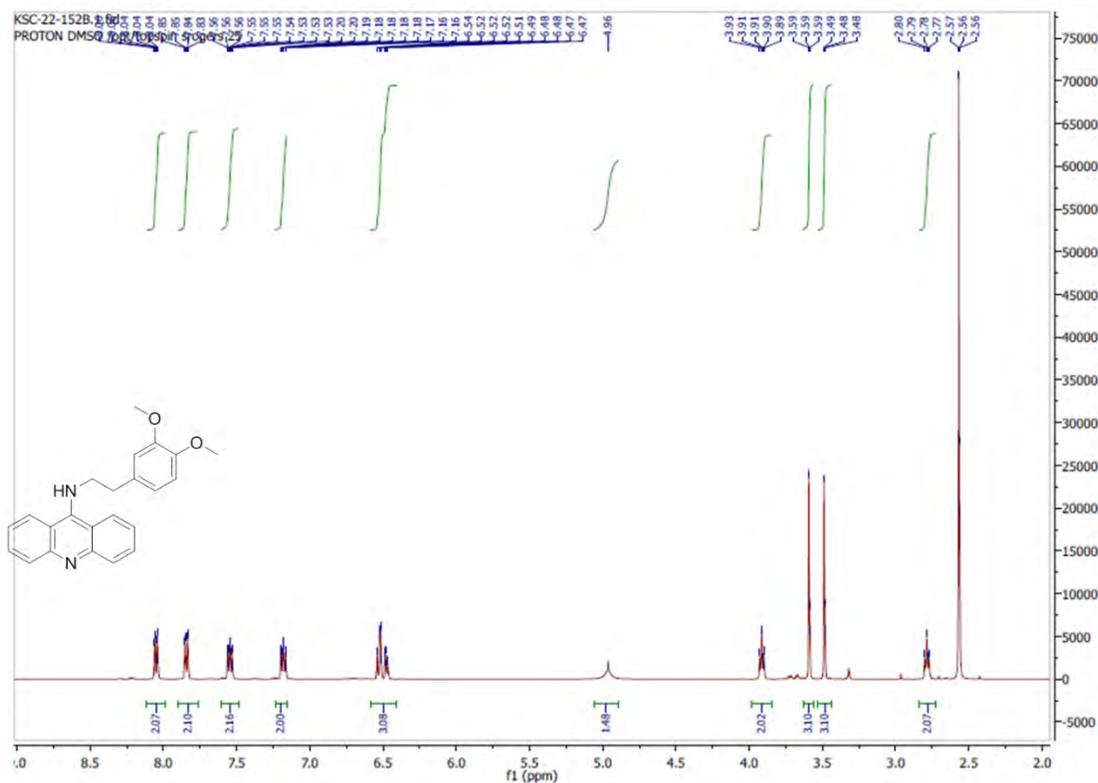
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>22</sub> H <sub>30</sub> N <sub>2</sub> O <sub>2</sub>	3.015	262.68	100	553.77	100	100	0.034

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>22</sub>H<sub>30</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 355.2380, found: 355.2398.

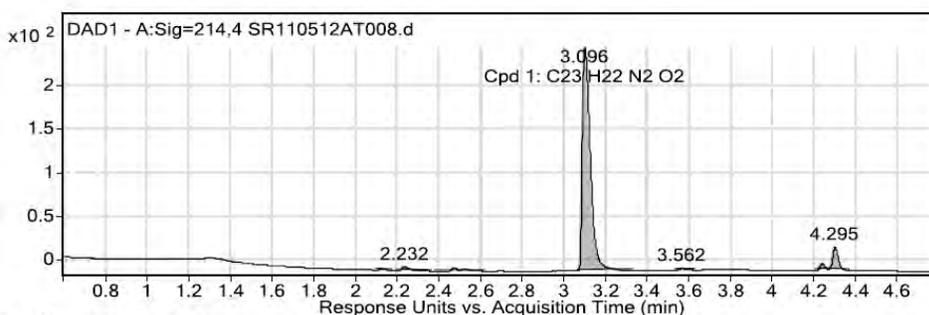
Analog CID 3815572; SID 124767904

<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 153.8, 150.1, 149.7, 148.9, 132.4, 131.4, 128.2, 124.8, 123.3, 122.0, 117.1, 113.5, 112.9, 56.3, 56.1, 52.3, 40.4, 37.5.

UPLC-MS Chromatogram



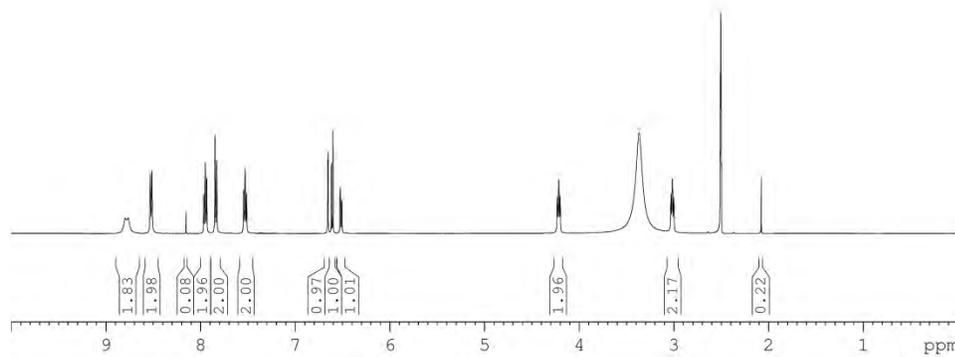
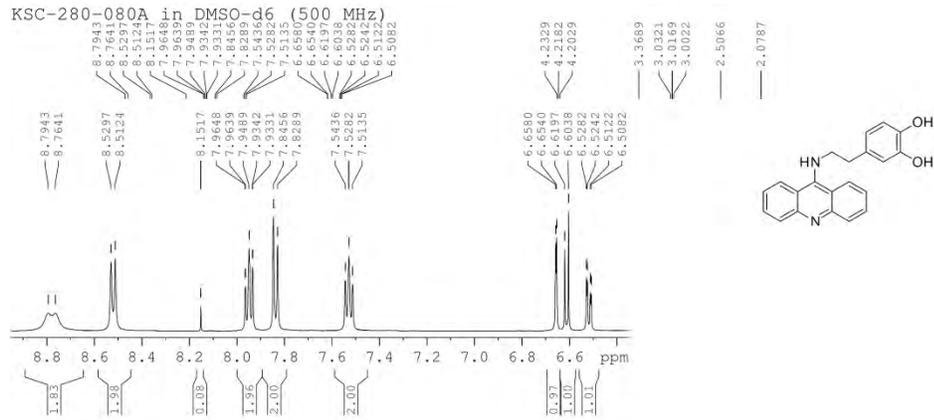
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.124	1.69	0.66	2.98	0.44	0.39	0.028
2		2.232	4.24	1.66	11.11	1.64	1.45	0.039
3		2.47	3.89	1.53	6.81	1.01	0.89	0.027
4		2.515	2.27	0.89	7.19	1.06	0.94	0.045
5	Cpd 1: C <sub>23</sub> H <sub>22</sub> N <sub>2</sub> O <sub>2</sub>	3.096	254.99	100	676.19	100	88.5	0.042
6		3.562	1.71	0.67	3.54	0.52	0.46	0.031
7		4.234	7.28	2.86	11.12	1.65	1.46	0.024
8		4.295	25.6	10.04	45.12	6.67	5.9	0.028

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>23</sub>H<sub>22</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 359.1754, found: 359.1751.

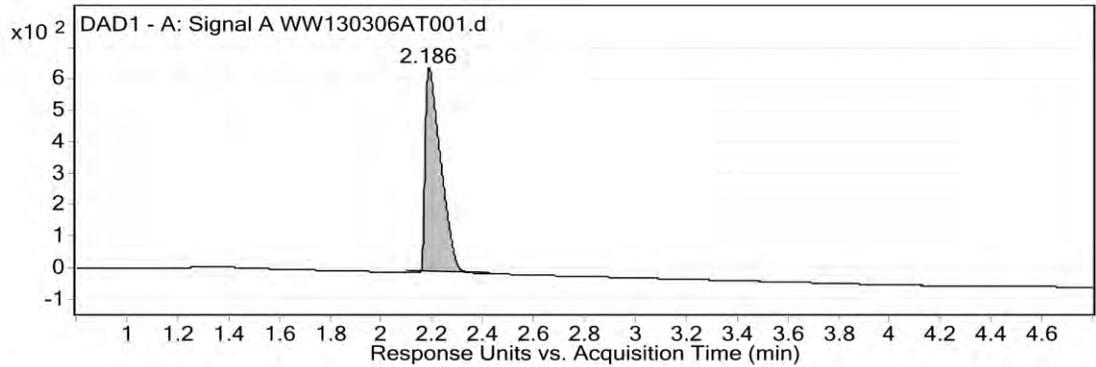
Analog CID 3876375; SID 162021977

<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR (126 MHz, DMSO-d<sub>6</sub>) δ 163.2, 157.0, 145.3, 143.9, 140.3, 134.6, 128.6, 125.8, 123.2, 119.4, 116.1, 115.6, 112.7, 51.0, 34.5.

UPLC-MS Chromatogram



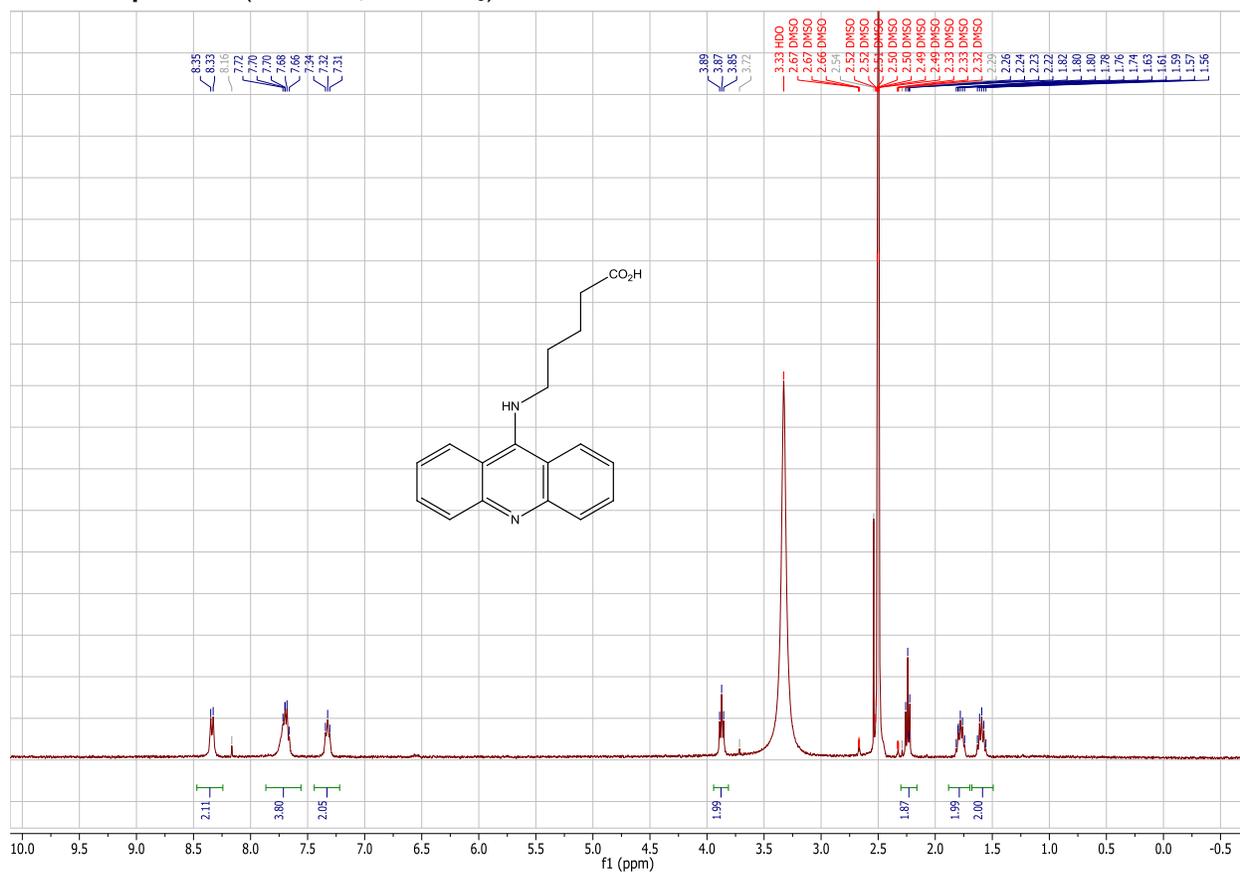
User Chromatogram Peak List

RT	Height	Height %	Area	Area %	Area Sum %	Symmetry	Width
2.186	648.64	100	2753.55	100	100	0.33	0.102

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>21</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 331.1441, found: 331.1442.

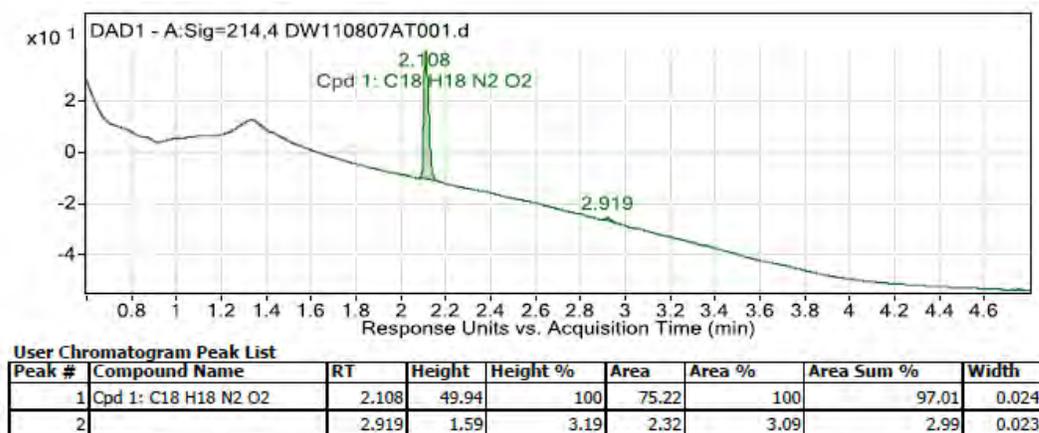
Analog CID 10565734; SID 124767899

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



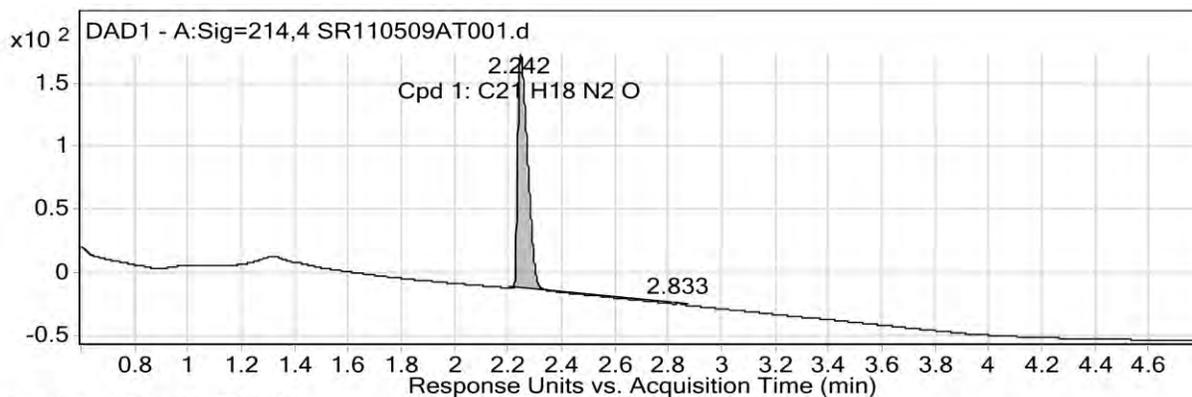
<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 174.221, 174.217, 157.288, 157.285, 134.8, 133.3, 118.44, 118.43, 48.4, 33.1, 28.3, 21.8.

UPLC-MS Chromatogram



HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>18</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 295.1441, found: 295.1449.

UPLC-MS Chromatogram



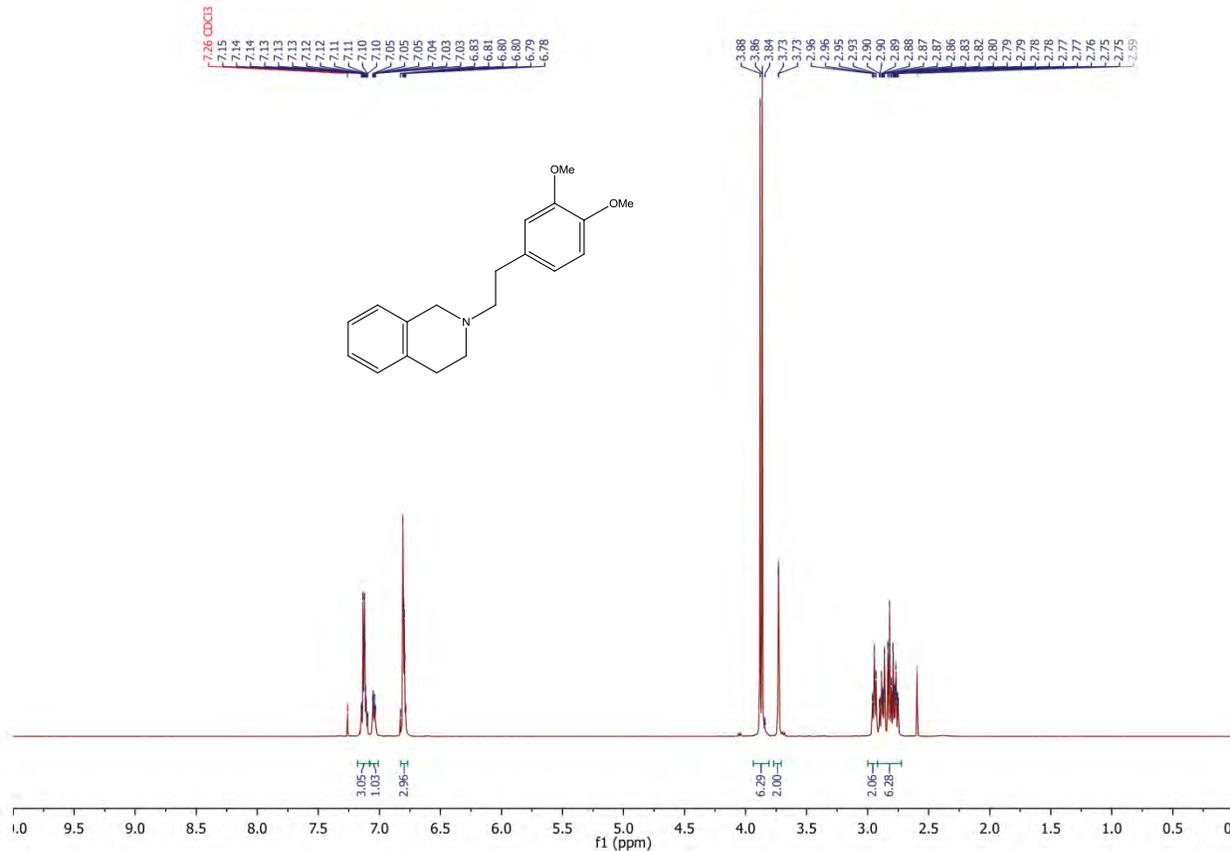
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C21 H18 N2 O	2.242	185.01	100	488.37	100	99.73	0.041
2		2.833	1.1	0.6	1.3	0.27	0.27	0.019

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>21</sub>H<sub>18</sub>N<sub>2</sub>O + H]<sup>+</sup>: 315.1492, found: 315.1496.

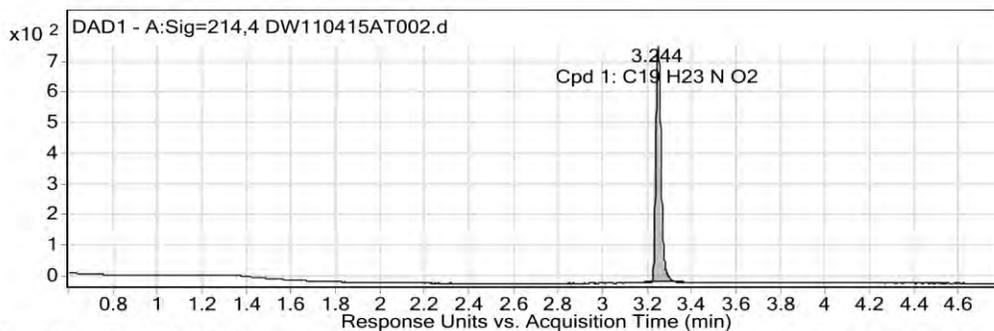
Analog CID 12553367; SID 124767883

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, CDCl<sub>3</sub>) δ 148.9, 147.4, 134.6, 134.2, 133.0, 128.7, 126.6, 126.2, 125.7, 120.6, 112.1, 111.3, 60.5, 56.1, 56.0, 55.9, 51.0, 33.6, 29.1.

UPLC-MS Chromatogram



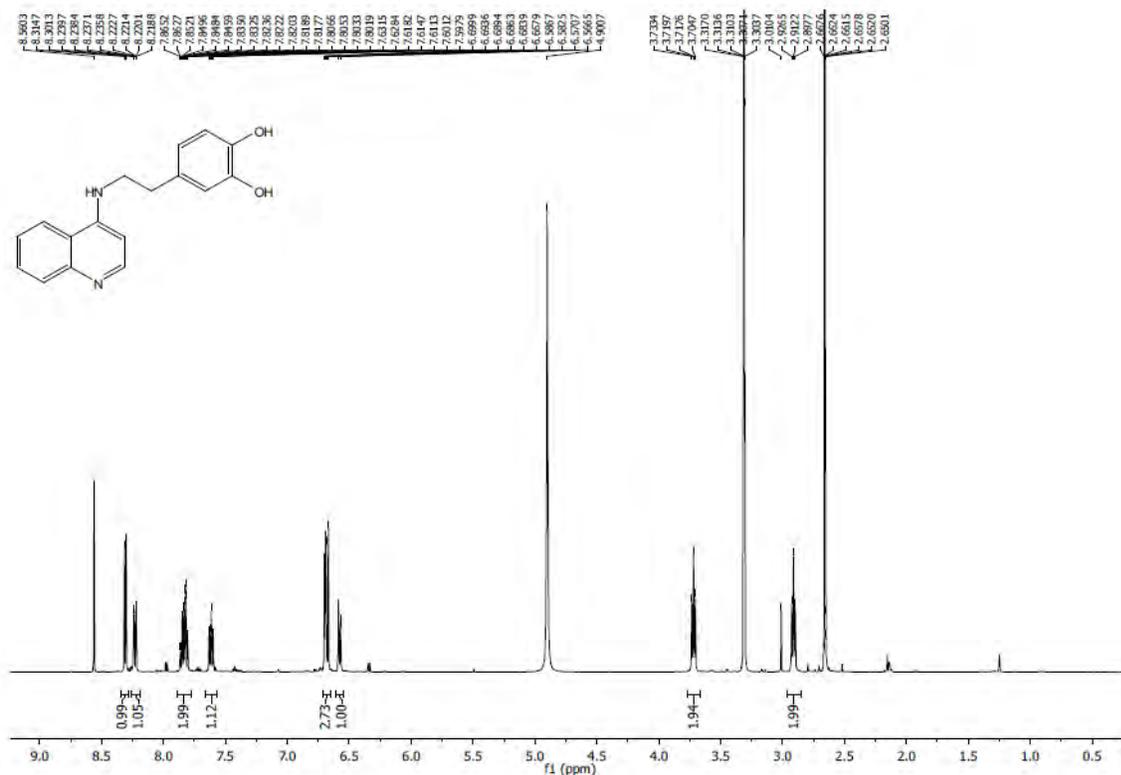
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C19 H23 N O2	3.244	767.66	100	1281.57	100	100	0.026

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>19</sub>H<sub>23</sub>NO<sub>2</sub> + H]<sup>+</sup>: 298.1802, found: 298.1827.

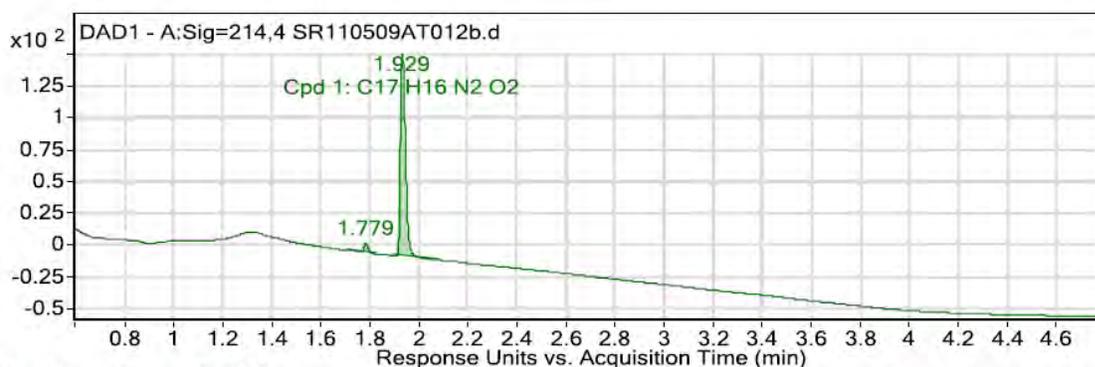
Analog CID 13162550; SID 124767892

<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 170.2, 156.3, 146.5, 145.1, 145.0, 133.7, 131.3, 127.4, 123.3, 123.1, 121.2, 118.8, 117.0, 116.5, 99.2, 46.3, 40.4, 34.9.

UPLC-MS Chromatogram



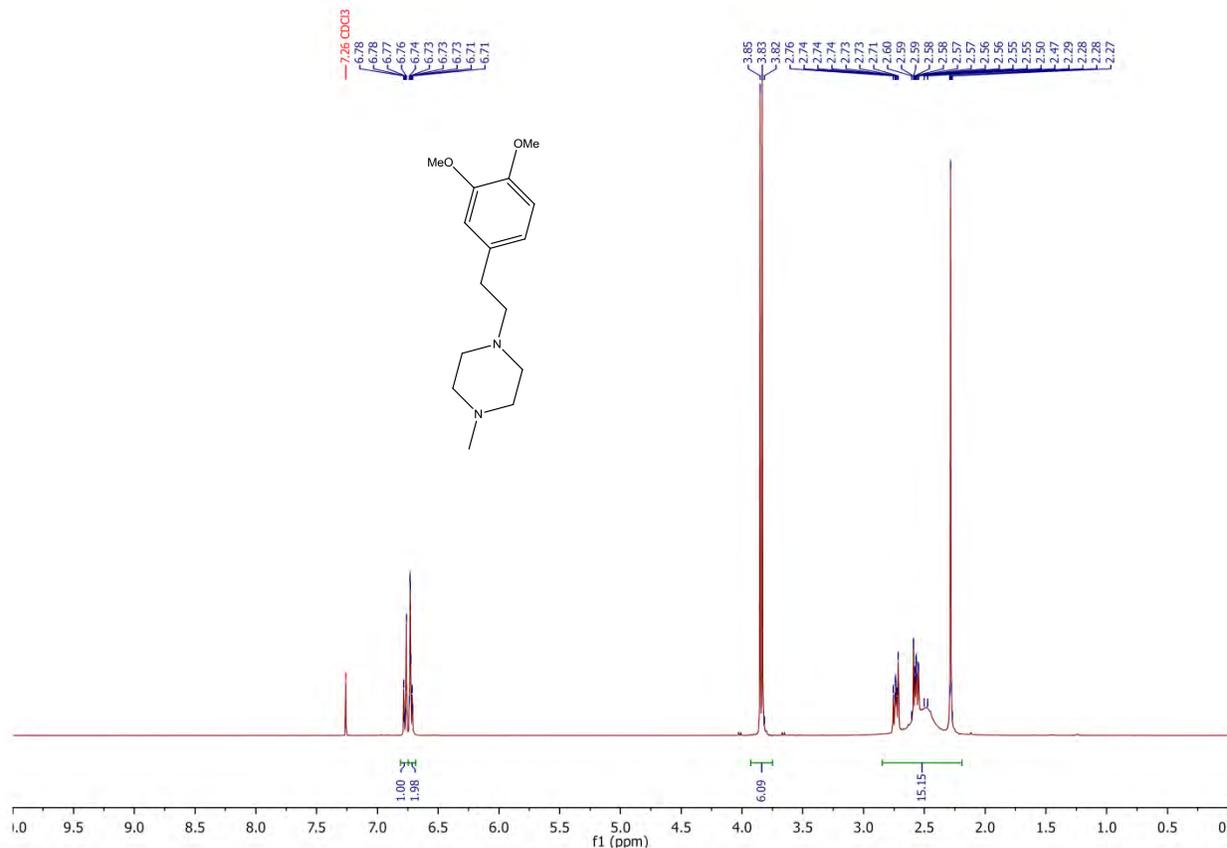
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		1.722	1.22	0.77	1.56	0.68	0.65	0.02
2		1.779	7.44	4.71	8.95	3.93	3.76	0.019
3	Cpd 1: C <sub>17</sub> H <sub>16</sub> N <sub>2</sub> O <sub>2</sub>	1.929	158.15	100	227.71	100	95.59	0.023

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>17</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 281.1285, found: 281.1292.

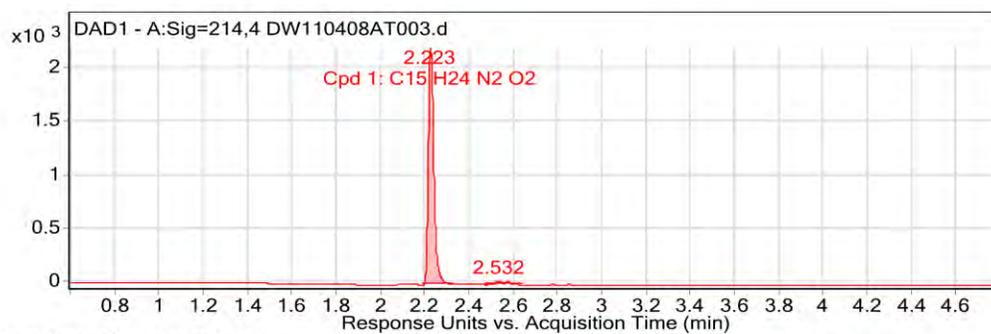
Analog CID 20649668; SID 124767877

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, CDCl<sub>3</sub>) δ 148.8, 147.3, 133.3, 120.5, 112.1, 111.3, 60.6, 55.9, 55.8, 55.2, 53.2, 46.1, 33.2.

UPLC-MS Chromatogram

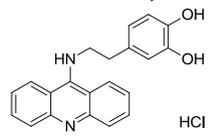


User Chromatogram Peak List

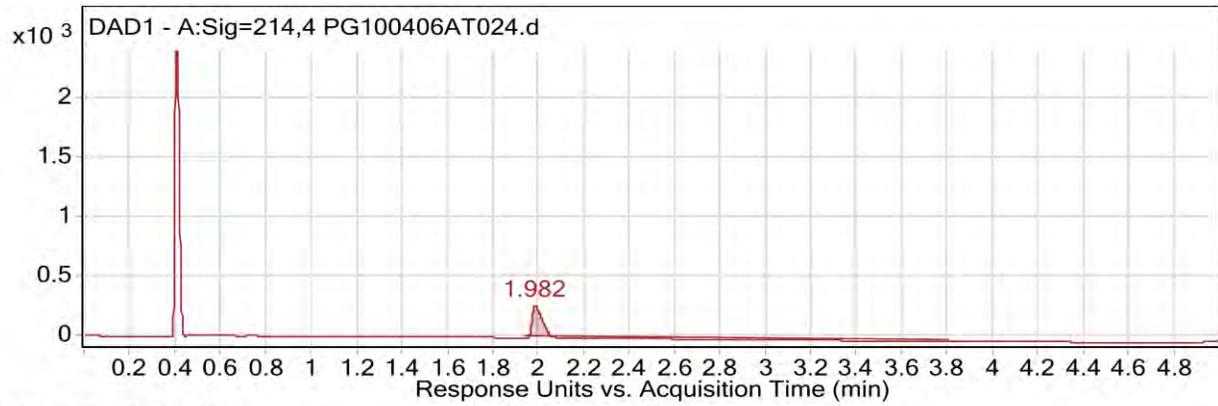
Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C15 H24 N2 O2	2.223	2188.53	100	3838.39	100	97.31	0.027
2		2.532	29.7	1.36	50.95	1.33	1.29	0.027
3		2.57	27.07	1.24	55.25	1.44	1.4	0.03

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>15</sub>H<sub>24</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 265.1911, found: 265.1935.

Analog CID 23724194; SID 92117383



UPLC-MS Chromatogram



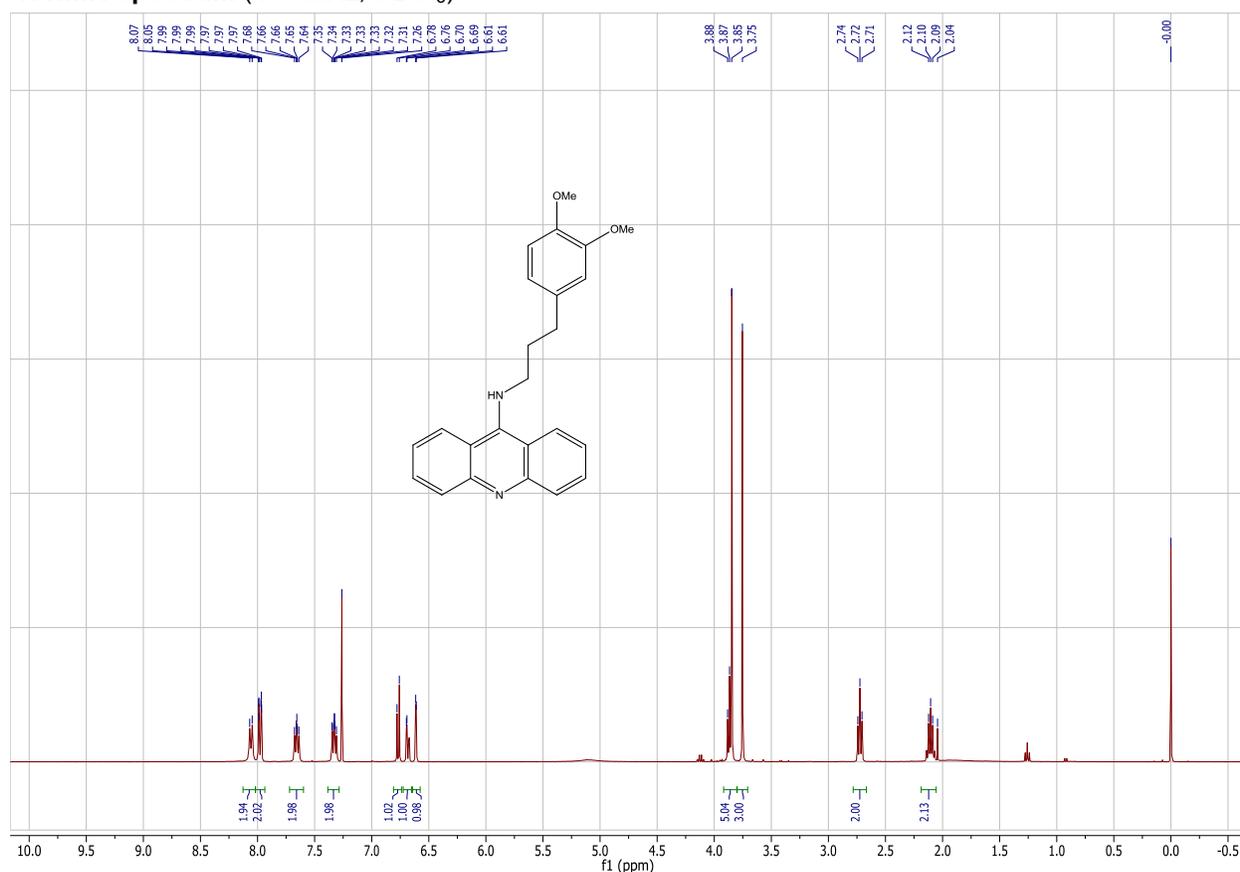
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	1.938	1.982	3.809	269.96	871.8	100

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>21</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 331.1441, found: 331.1441.

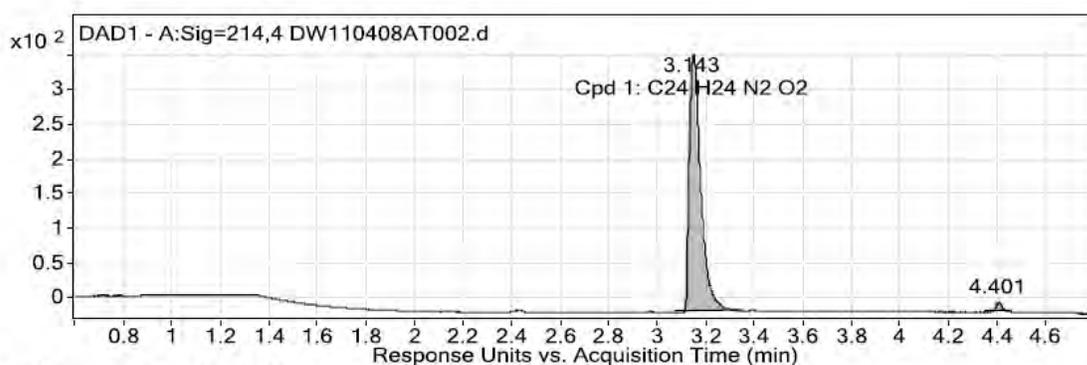
Analog CID 53308661; SID 124767876

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ151.7, 149.137, 149.134, 147.6, 133.5, 130.3, 123.150, 123.146, 122.9, 120.3, 116.4, 111.7, 111.5, 56.1, 55.9, 50.3, 33.4, 33.0.

UPLC-MS Chromatogram



User Chromatogram Peak List

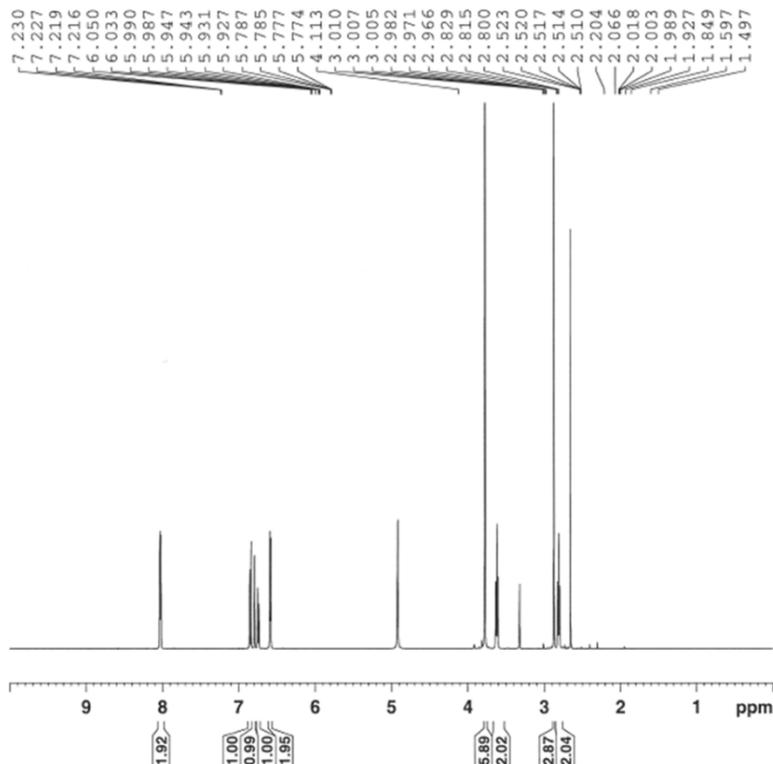
Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>24</sub> H <sub>24</sub> N <sub>2</sub> O <sub>2</sub>	3.143	369.46	100	1228.76	100	97.99	0.051
2		4.401	13.95	3.77	25.2	2.05	2.01	0.027

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>24</sub>H<sub>24</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 373.1911, found: 373.1930.



Analog CID 53308663; SID 124767893

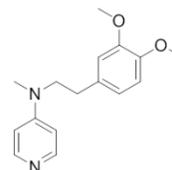
<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



```

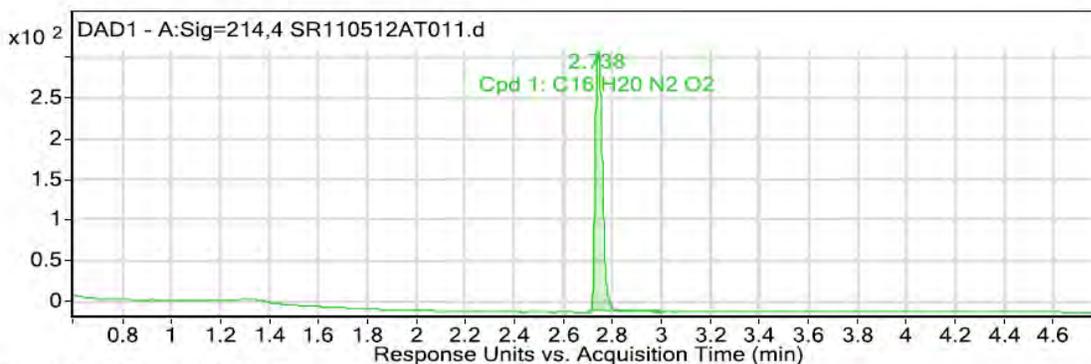
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EXPNO     1
PROCNO    1
Date_     20110630
Time      0.07
INSTRUM   spect
PROBHD    5 mm CPDUL 13C
PULPROG   zg30
TD        65536
SOLVENT   DMSO
NS        16
DS        2
SWH       10330.578 Hz
FIDRES    0.157632 Hz
AQ        3.1719923 sec
RG        36
DW        48.400 usec
DE        6.50 usec
TE        298.2 K
D1        1.00000000 sec
TD0       1

===== CHANNEL f1 =====
NUC1      1H
P1        15.00 usec
PL1       6.00 dB
PL1W      8.64411545 W
SFO1      500.1930889 MHz
SI        32768
SF        500.1895983 MHz
WDW       EM
SSB       0
LB        0.30 Hz
GB        0
PC        1.00
    
```



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 155.3, 150.5, 149.4, 149.2, 133.2, 122.3, 113.9, 113.2, 107.8, 55.5, 56.4, 48.4, 40.4, 38.1, 33.4.

UPLC-MS Chromatogram,



User Chromatogram Peak List

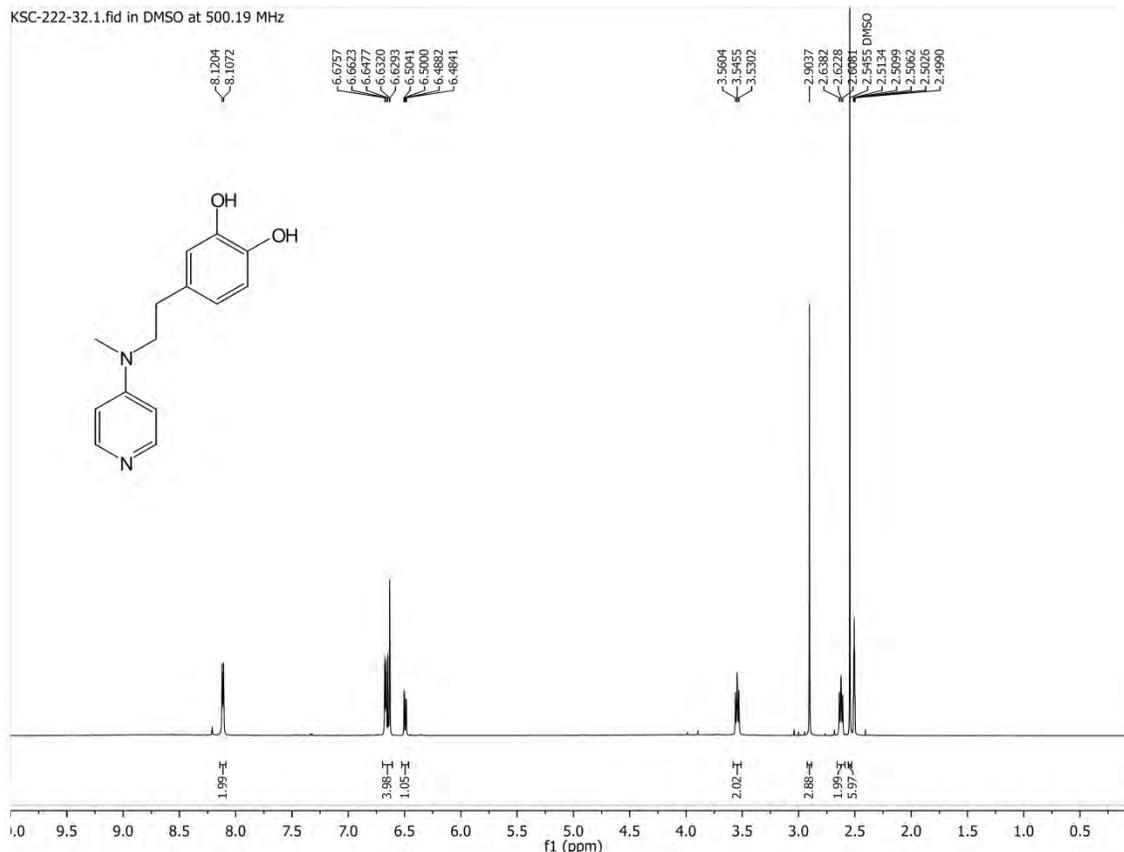
Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C16 H20 N2 O2	2.738	320.02	100	645.28	100	100	0.033

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>16</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 273.1598, found: 273.1594.

**Analog CID 53308664; SID 124767897**

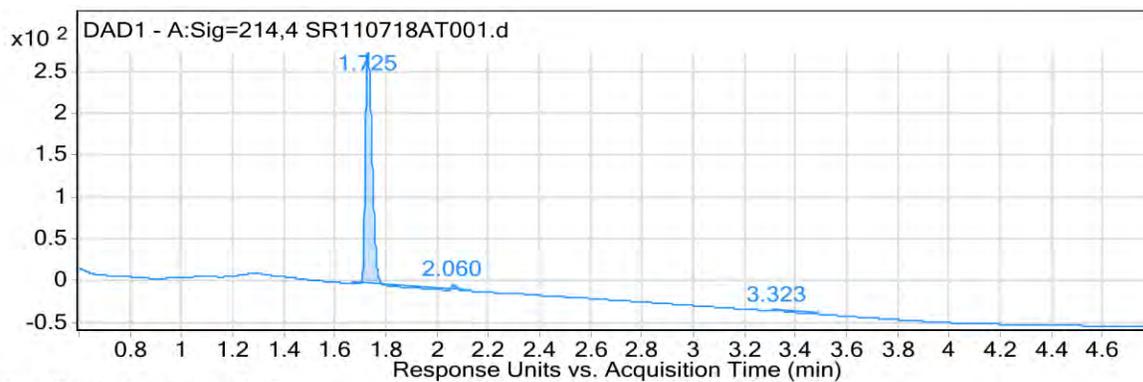
**<sup>1</sup>H NMR Spectrum** (nnn MHz, DMSO-d<sub>6</sub>)

KSC-222-32.1.fid in DMSO at 500.19 MHz



<sup>13</sup>C NMR (126 MHz, DMSO) δ 153.6, 146.8, 145.2, 143.7, 129.5, 119.5, 116.3, 115.5, 106.6, 52.9, 37.5, 31.4.

**UPLC-MS Chromatogram**



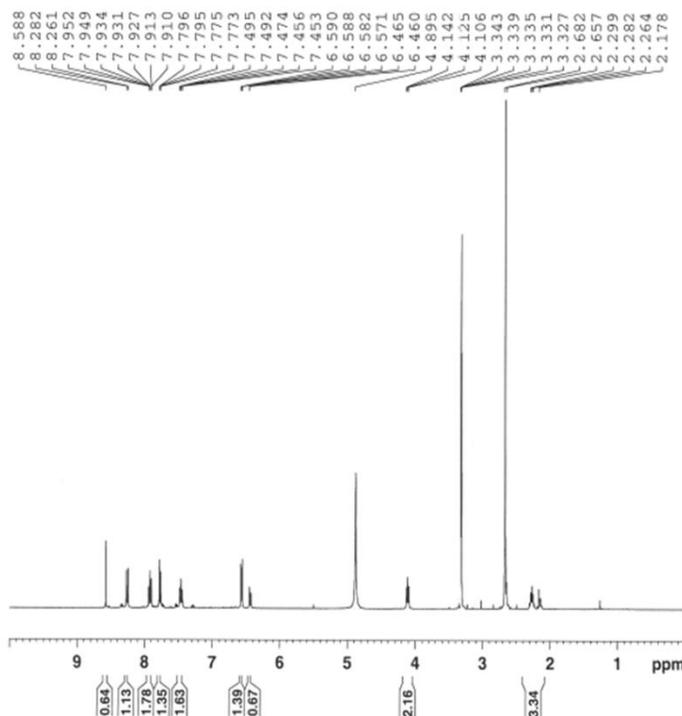
**User Chromatogram Peak List**

Peak #	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	1.679	0.81	0.29	0.87	0.17	0.17	0.016
2	1.725	276.1	100	499.76	100	97.61	0.029
3	2.06	6.99	2.53	9.02	1.81	1.76	0.02
4	3.323	1.57	0.57	2.33	0.47	0.45	0.021

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>14</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 245.1285, found: 245.1290.

Analog CID 53308665; SID 124767891

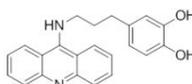
<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



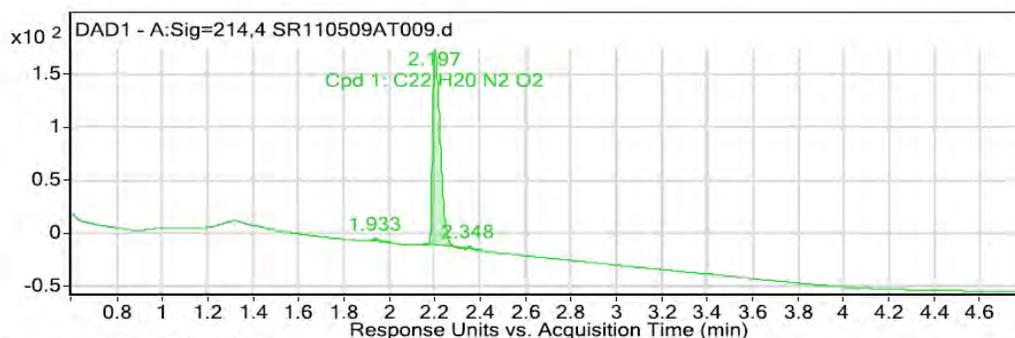
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DS         2
SWH       8278.146 Hz
FIDRES    0.126314 Hz
AQ         3.9584243 sec
RG         256
LW         60.400 usec
DE         6.50 usec
TE         297.6 K
DL         1.00000000 sec
TD0        1

===== CHANNEL f1 =====
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P1         14.80 usec
PL1        1.00 dB
PL1W       14.48560429 W
SFO1       400.2324716 MHz
SI         32768
SF         400.2300059 MHz
WDW        EM
SSB         0
LB         0.30 Hz
GB         0
PC         1.00
    
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UPLC-MS Chromatogram



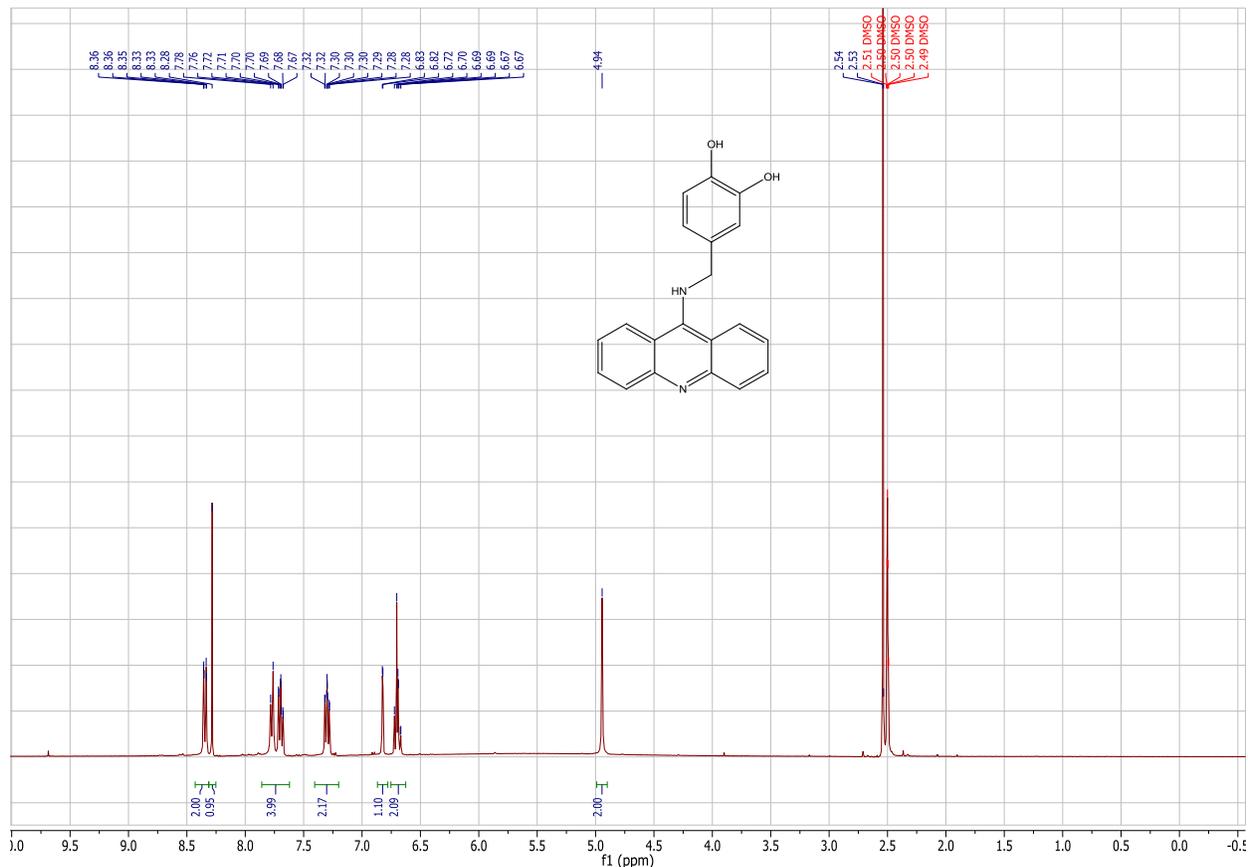
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		1.933	3.66	1.99	6.27	1.47	1.44	0.025
2	Cpd 1: C <sub>22</sub> H <sub>20</sub> N <sub>2</sub> O <sub>2</sub>	2.197	183.64	100	425.6	100	97.59	0.037
3		2.348	3.68	2	4.25	1	0.98	0.018

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>22</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 345.1598, found: 345.1608.

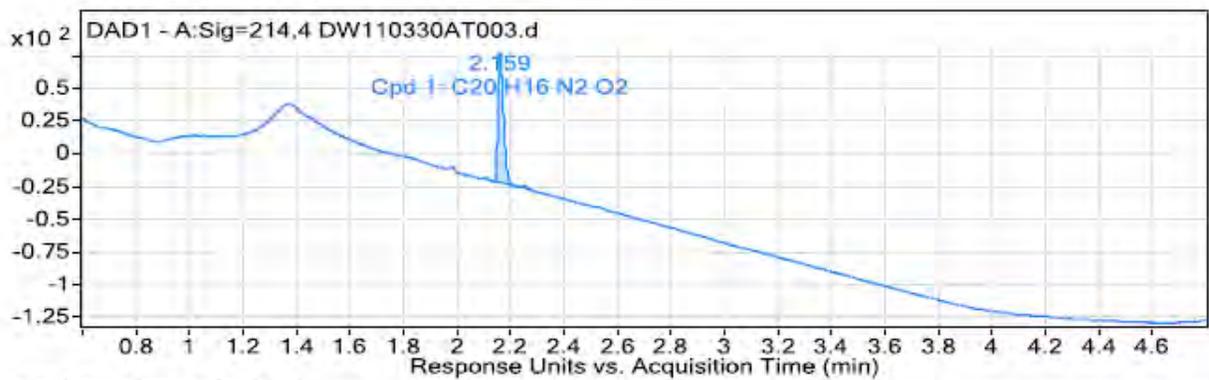
Analog CID 53308666; SID 124767867

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>) δ 153.5, 145.5, 144.7, 144.5, 131.4, 130.1, 125.2, 123.5, 122.0, 117.6, 115.7, 115.3, 114.4, 52.2.

UPLC-MS Chromatogram



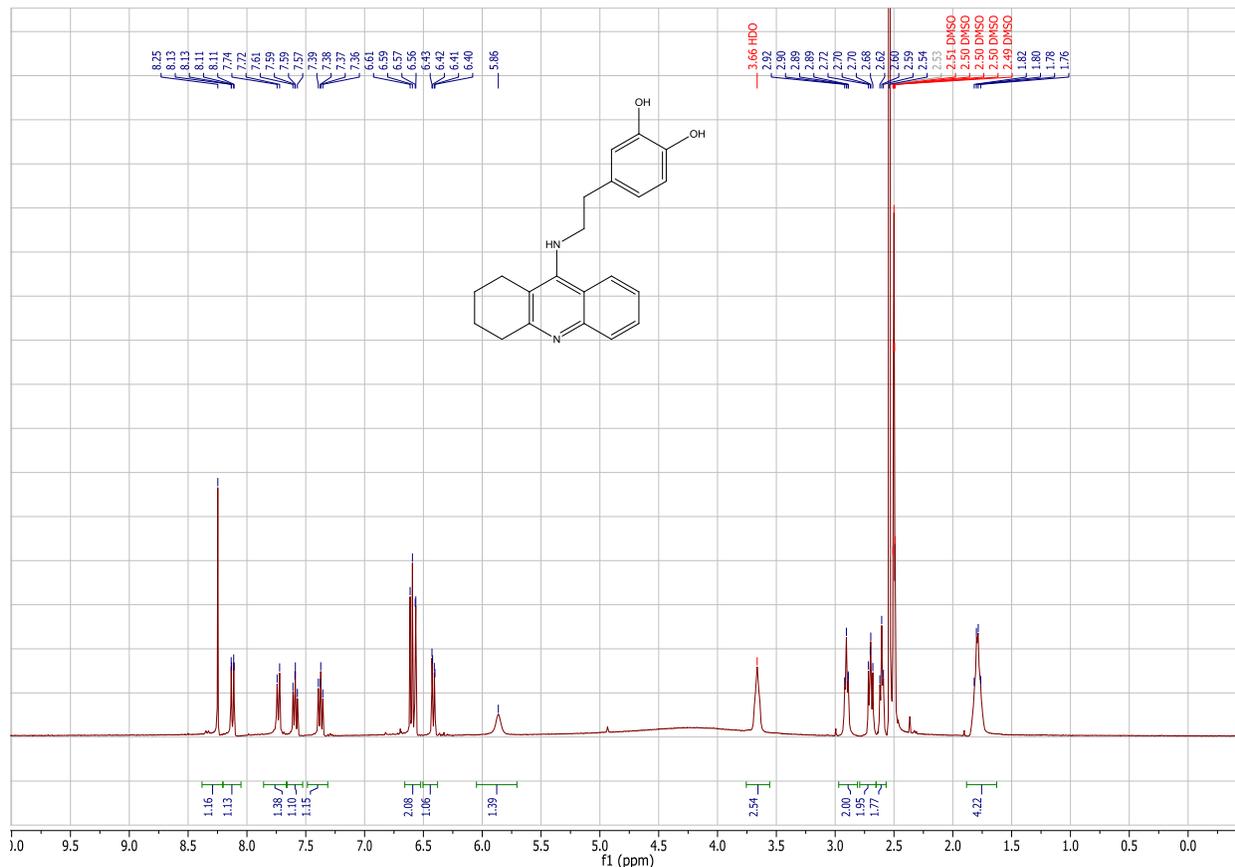
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>20</sub> H <sub>16</sub> N <sub>2</sub> O <sub>2</sub>	2.159	98.18	100	143.16	100	98.15	0.025
2		2.25	2.08	2.12	2.7	1.88	1.85	0.021

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 317.1285, found: 317.1295.

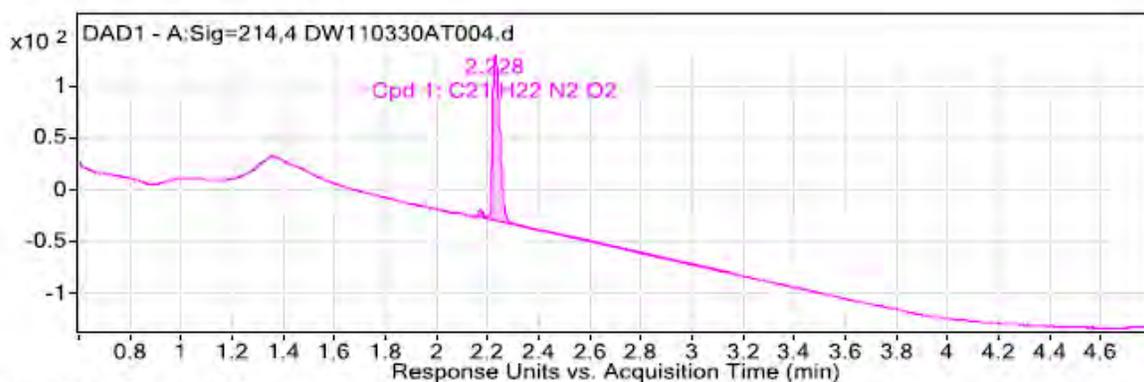
Analog CID 53308667; SID 124767868

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>) δ 156.2, 151.3, 145.2, 144.9, 143.7, 129.5, 128.9, 126.2, 123.6, 123.5, 119.3, 119.1, 116.0, 125.5, 114.8, 49.8, 35.9, 32.2, 24.6, 22.4, 21.9.

UPLC-MS Chromatogram



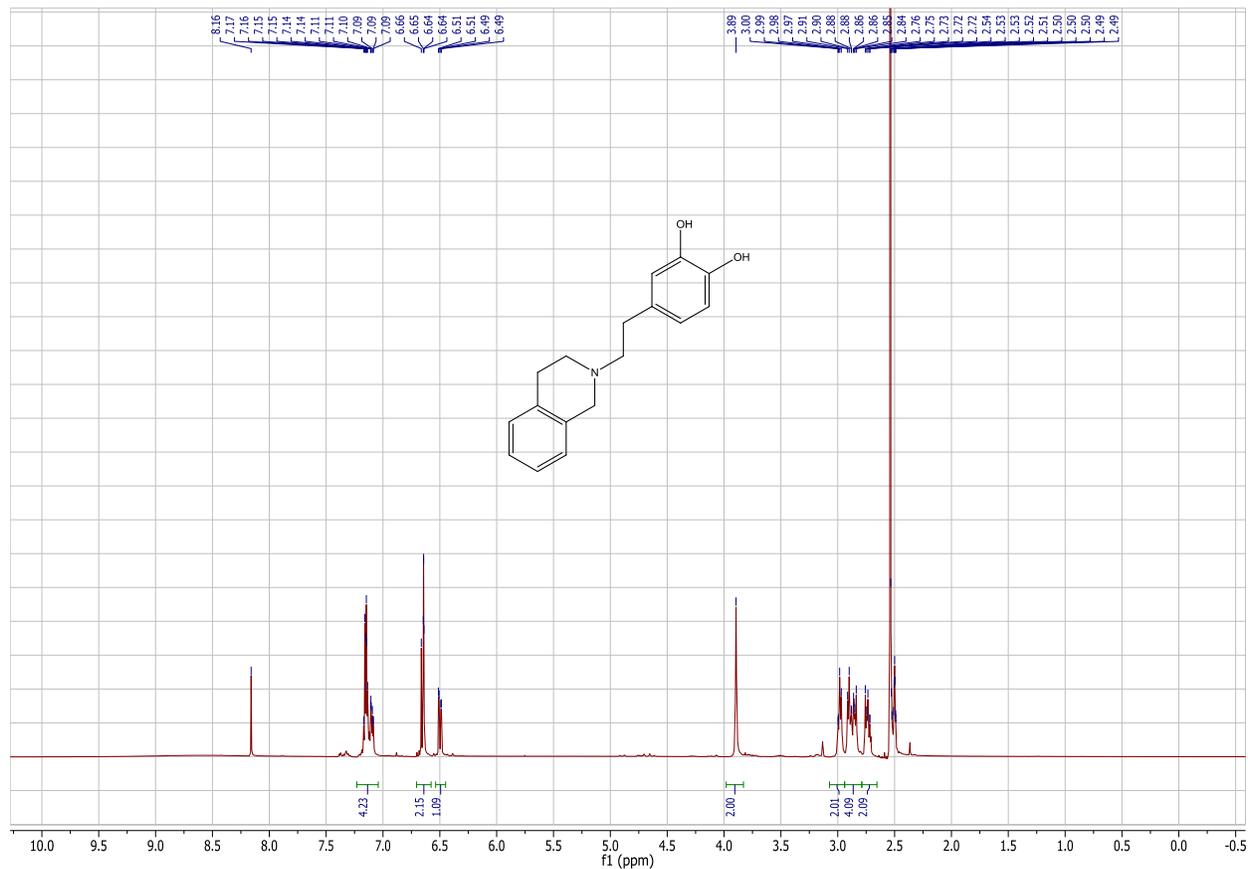
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.169	7.42	4.82	10.01	2.79	2.72	0.022
2	Cpd 1: C <sub>21</sub> H <sub>22</sub> N <sub>2</sub> O <sub>2</sub>	2.228	153.98	100	358.47	100	97.28	0.035

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>21</sub>H<sub>22</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 335.1754, found: 335.1769.

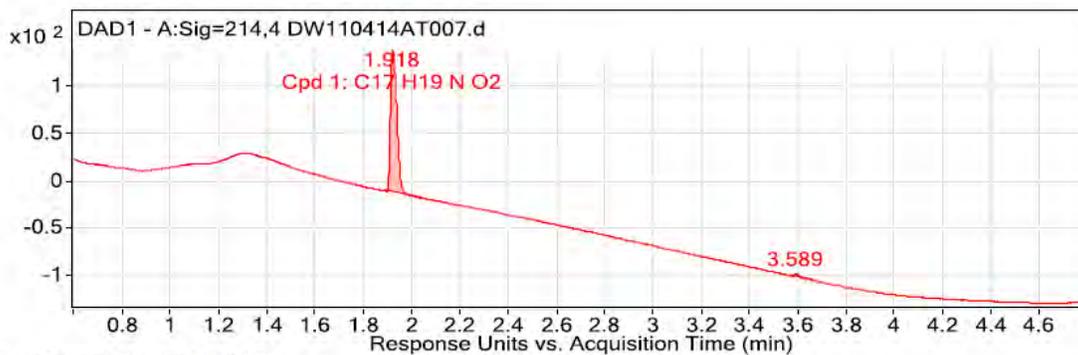
Analog CID 53308668; SID 124767872

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 145.1, 143.6, 133.3, 132.9, 129.9, 128.5, 126.52, 126.49, 125.9, 119.3, 116.1, 115.6, 58.9, 54.3, 50.0, 31.3, 27.5.

UPLC-MS Chromatogram



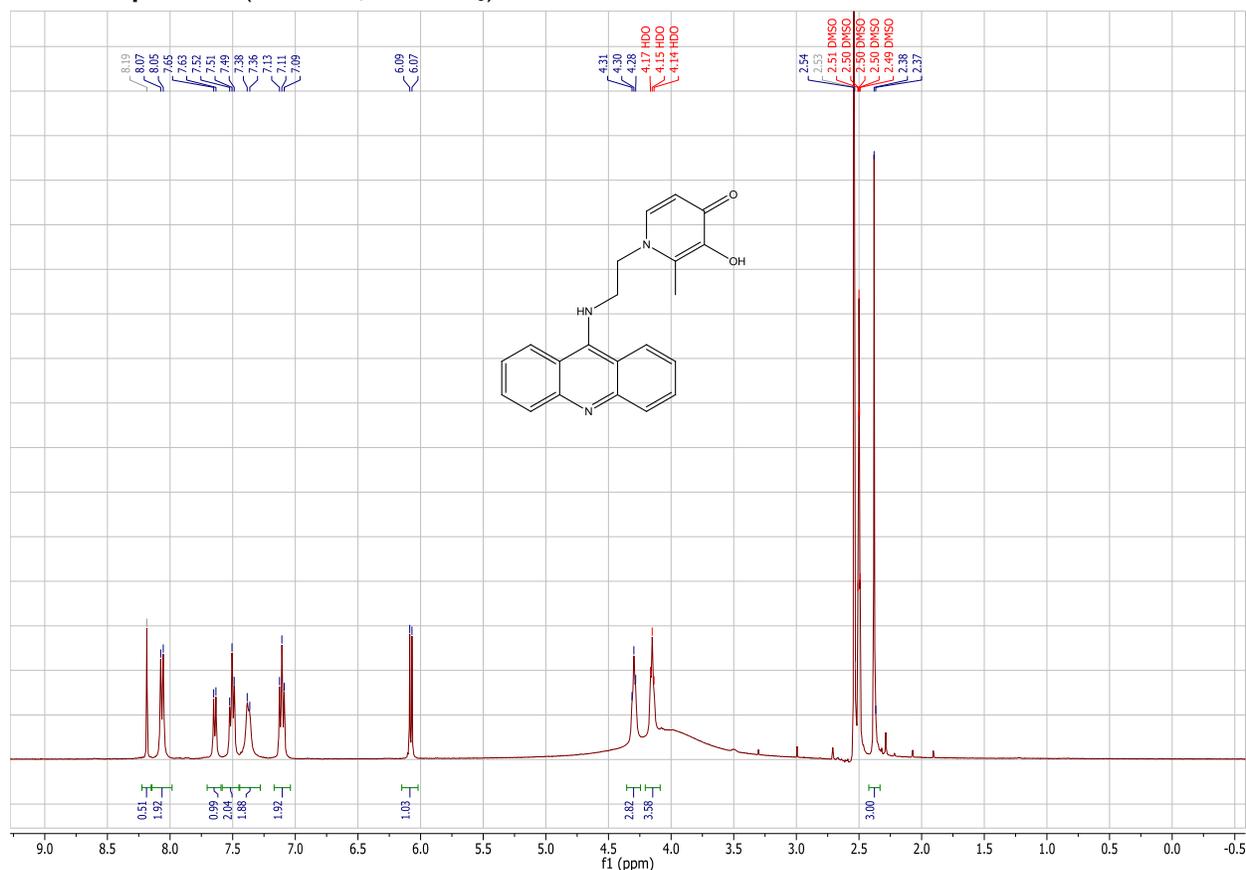
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C17 H19 N O2	1.918	149.36	100	267.25	100	98.63	0.029
2		3.589	3.1	2.08	3.72	1.39	1.37	0.019

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>17</sub>H<sub>19</sub>NO<sub>2</sub> + H]<sup>+</sup>: 270.1489, found: 270.1506.

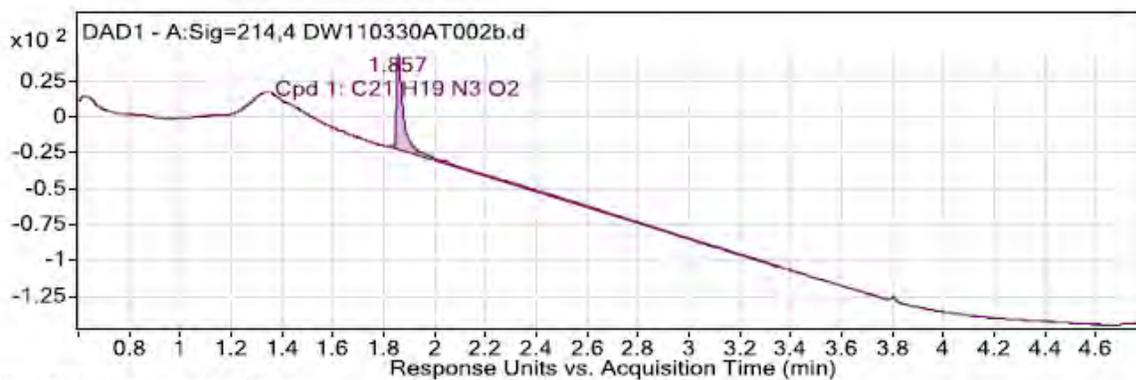
Analog CID 53308669; SID 124767866

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 168.9, 153.0, 145.2, 138.4, 133.6, 130.7, 128.9, 126.4, 121.2, 120.5, 117.7, 110.1, 54.6, 52.2, 11.7.

UPLC-MS Chromatogram



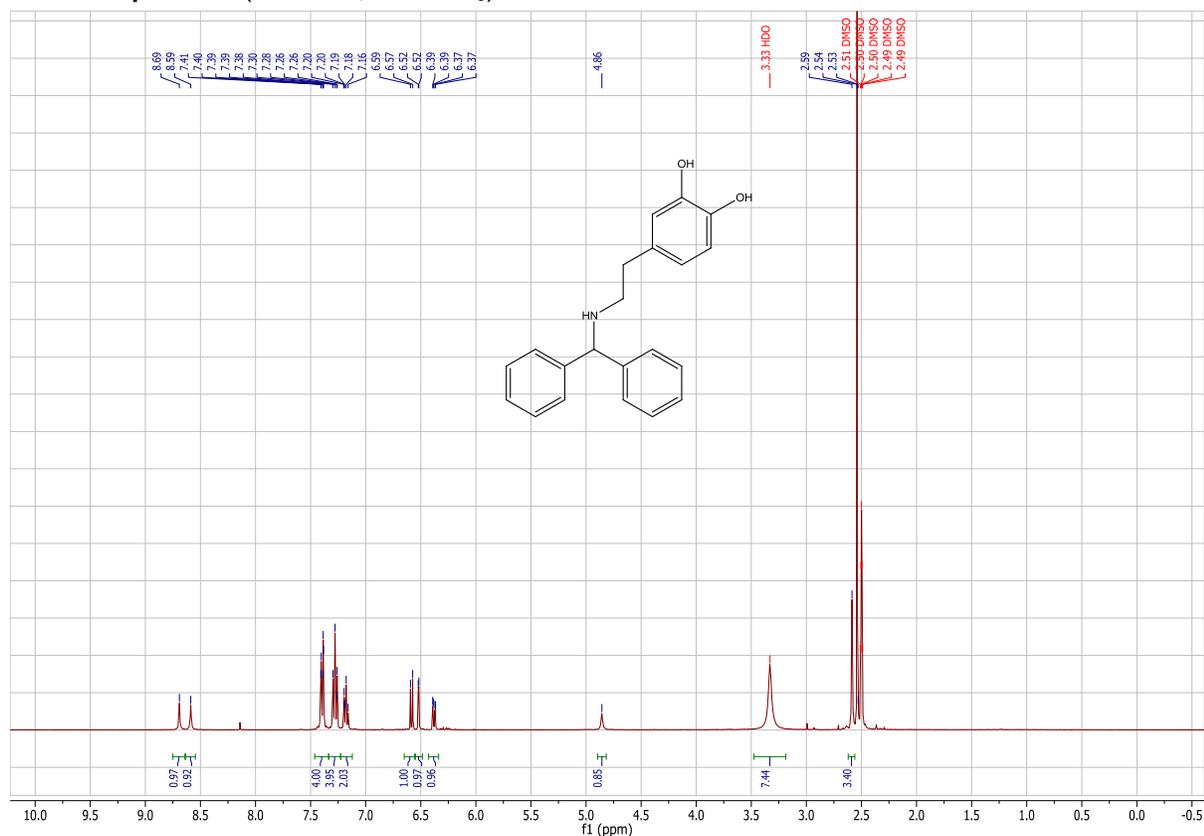
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>21</sub> H <sub>19</sub> N <sub>3</sub> O <sub>2</sub>	1.857	65.55	100	219.24	100	100	0.045

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>21</sub>H<sub>19</sub>N<sub>3</sub>O<sub>2</sub> + H]<sup>+</sup>: 346.1550, found: 346.1555.

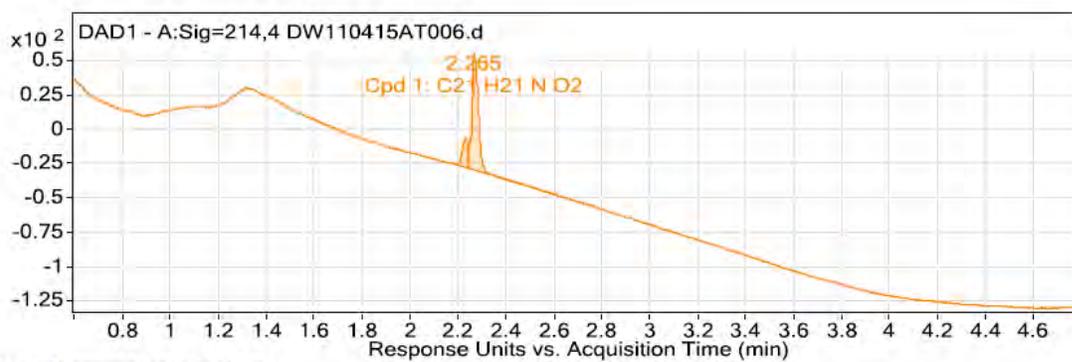
Analog CID 53308670; SID 124767902

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 145.0, 143.3, 130.9, 130.8, 128.3, 127.1, 126.8, 119.1, 115.9, 115.4, 66.2, 49.5, 34.9.

UPLC-MS Chromatogram



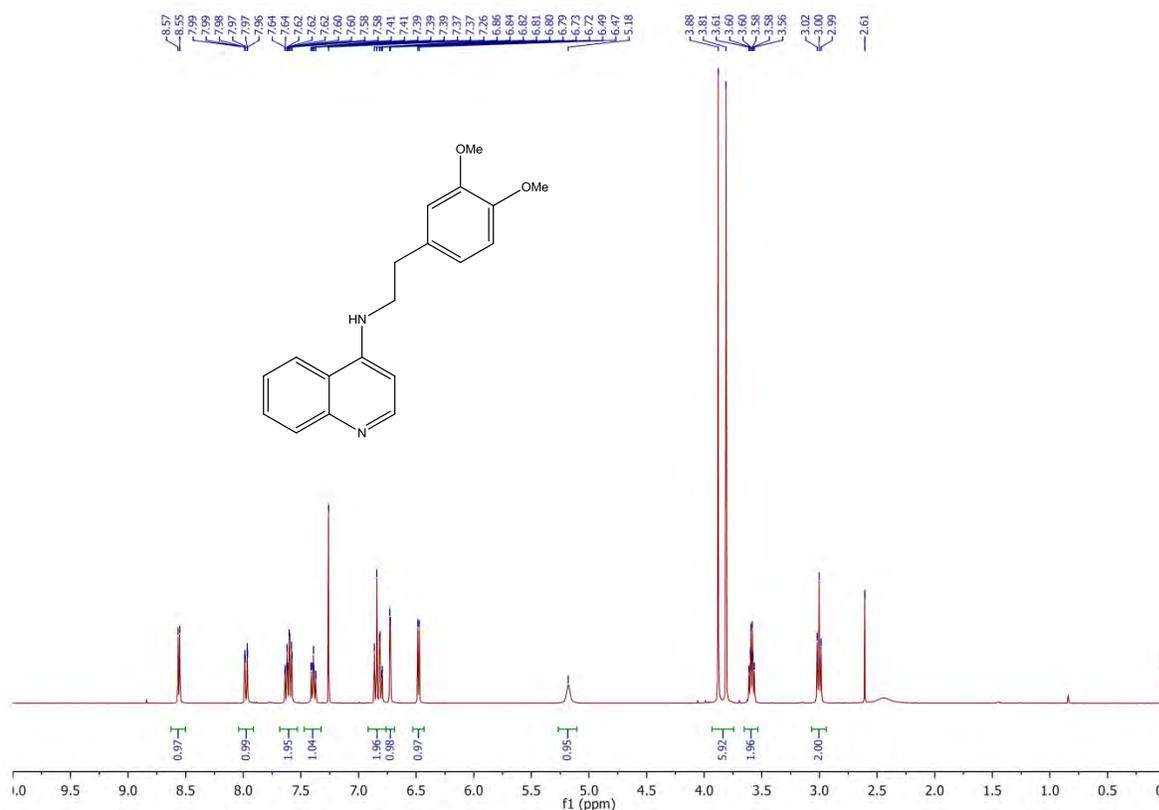
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.23	21.18	24.82	42.1	28.39	21.74	0.032
2	Cpd 1: C <sub>21</sub> H <sub>21</sub> N O <sub>2</sub>	2.265	85.35	100	148.27	100	76.56	0.026
3		2.351	1.03	1.21	3.29	2.22	1.7	0.053

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>21</sub>H<sub>21</sub>NO<sub>2</sub> + H]<sup>+</sup>: 320.1645, found: 320.1670.

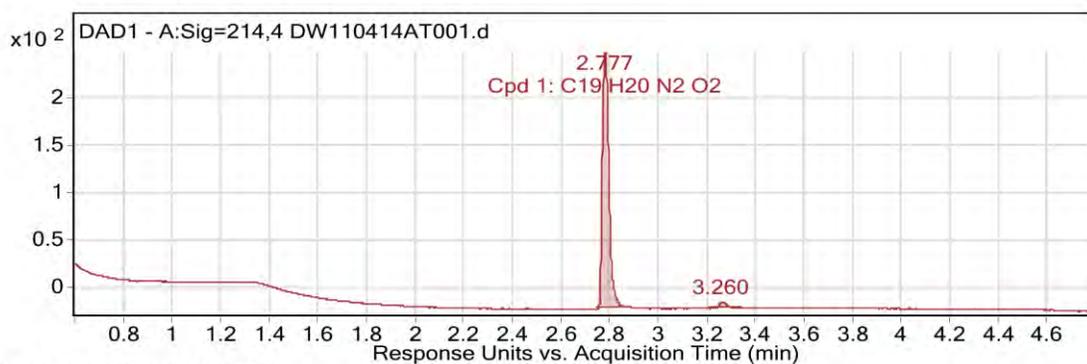
Analog CID 53308673; SID 124767870

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, CDCl<sub>3</sub>) δ 150.2, 150.0, 148.3, 148.1, 147.6, 131.0, 129.6, 129.2, 125.0, 120.8, 119.6, 118.8, 112.0, 111.6, 98.9, 56.1, 56.0, 44.4, 34.4.

UPLC-MS Chromatogram



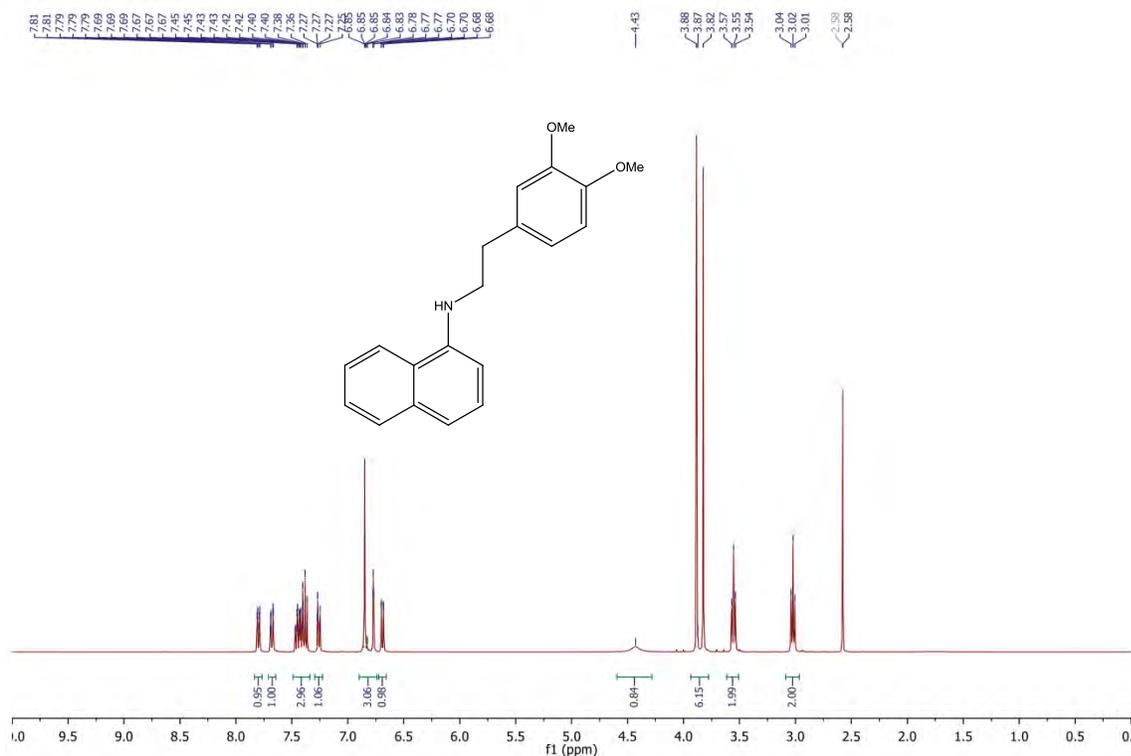
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>19</sub> H <sub>20</sub> N <sub>2</sub> O <sub>2</sub>	2.777	267.24	100	505.85	100	97.06	0.03
2		3.26	6.84	2.56	15.32	3.03	2.94	0.033

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>19</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 309.1598, found: 309.1613.

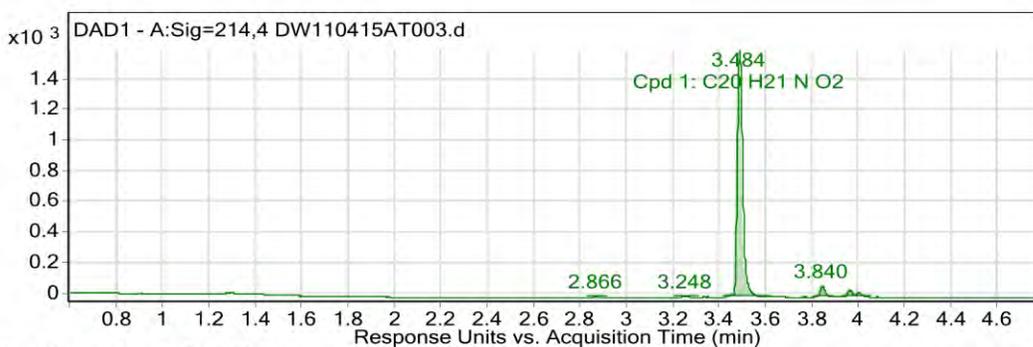
Analog CID 53308674; SID 124767884

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, CDCl<sub>3</sub>) δ 149.2, 147.9, 143.3, 134.5, 132.0, 128.8, 126.7, 125.8, 124.8, 123.6, 120.8, 119.8, 117.5, 112.2, 111.6, 104.6, 56.1, 55.9, 45.3, 34.9.

UPLC-MS Chromatogram



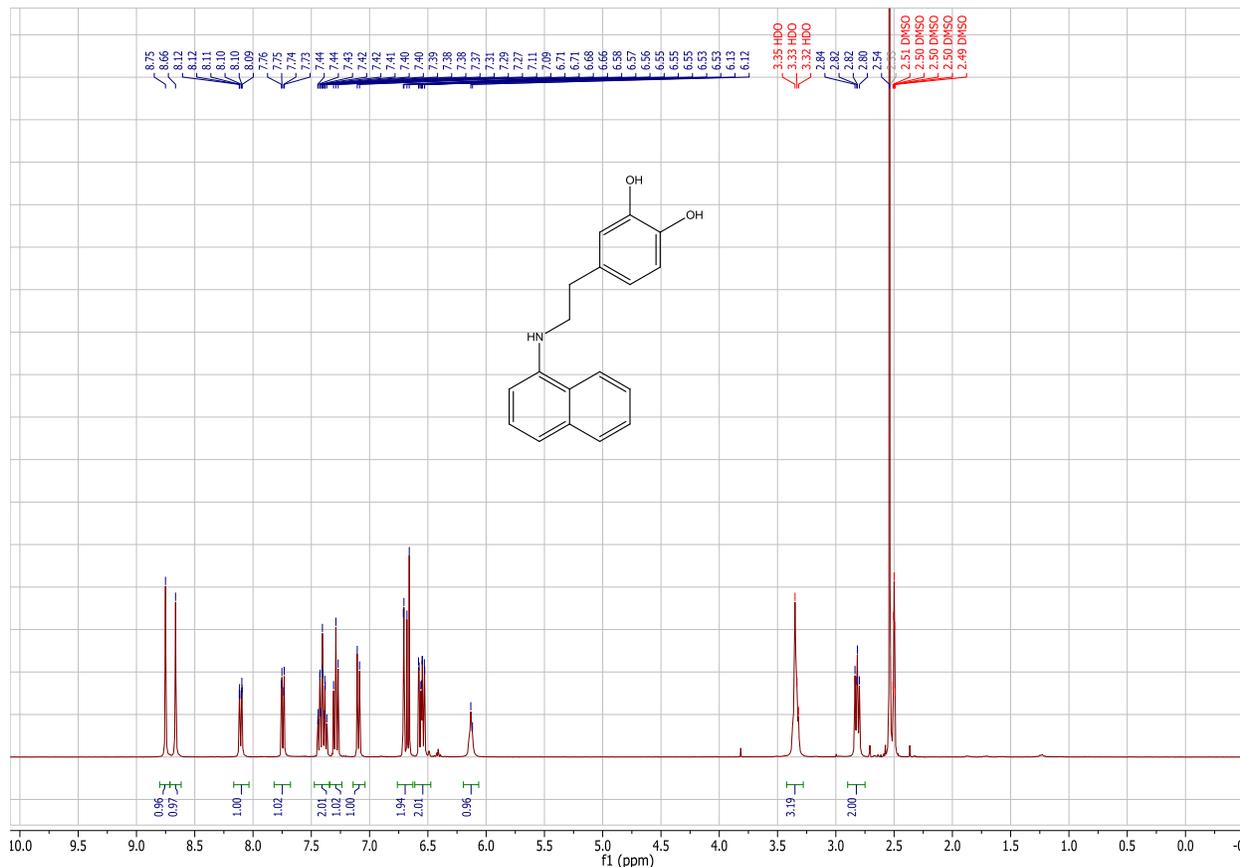
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.866	2.83	0.18	5.39	0.21	0.19	0.029
2		3.248	3.27	0.2	9.54	0.37	0.34	0.043
3		3.445	17.72	1.11	21.88	0.85	0.77	0.019
4	Cpd 1: C <sub>20</sub> H <sub>21</sub> N O <sub>2</sub>	3.484	1601.75	100	2579.69	100	90.73	0.025
5		3.84	71.24	4.45	106.83	4.14	3.76	0.023
6		3.959	47.87	2.99	70.11	2.72	2.47	0.022
7		3.997	29.18	1.82	49.9	1.93	1.76	0.025

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>21</sub>NO<sub>2</sub> + H]<sup>+</sup>: 308.1645, found: 308.1663.

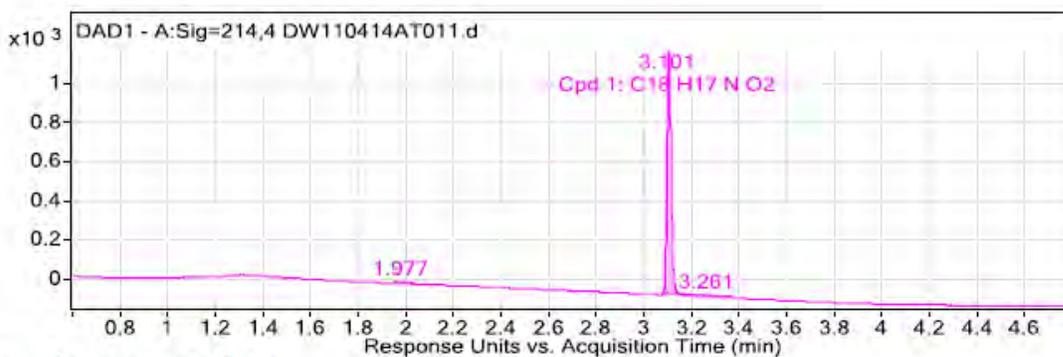
Analog CID 53308675; SID 124767874

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 145.1, 143.9, 143.5, 134.1, 130.7, 128.0, 126.9, 125.6, 123.9, 123.0, 121.6, 119.3, 116.1, 115.5, 115.3, 102.9, 45.4, 33.9.

UPLC-MS Chromatogram



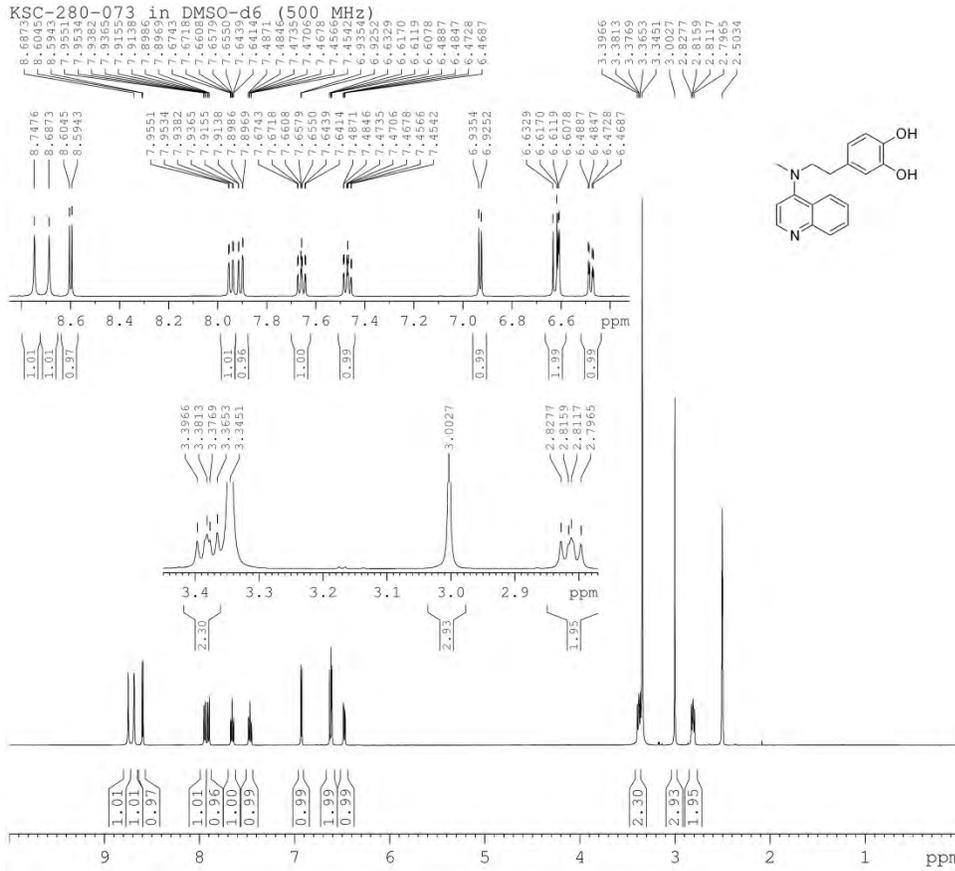
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		1.977	3.74	0.3	5.35	0.37	0.37	0.022
2	Cpd 1: C <sub>18</sub> H <sub>17</sub> N O <sub>2</sub>	3.101	1227.44	100	1445.27	100	99.18	0.019
3		3.261	1.49	0.12	6.64	0.46	0.46	0.054

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>18</sub>H<sub>17</sub>NO<sub>2</sub> + H]<sup>+</sup>: 280.1332, found: 280.1349.

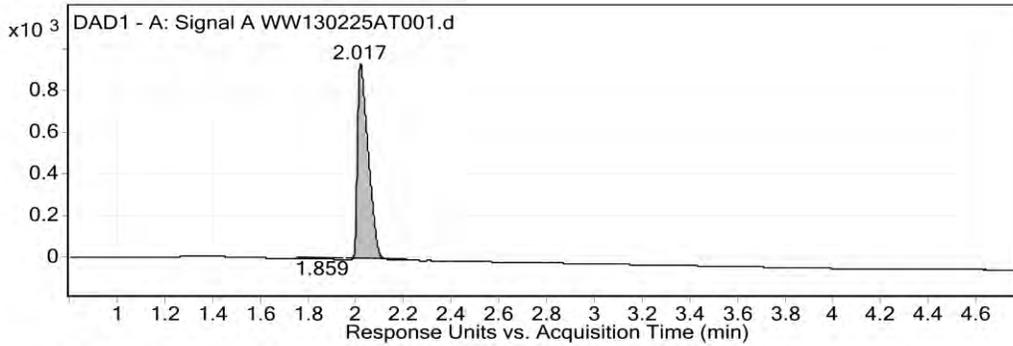
Analog CID 53308676; SID 162021973

<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR (126 MHz, DMSO-d<sub>6</sub>) δ 156.4, 150.3, 149.3, 145.1, 143.6, 129.9, 129.4, 128.8, 124.6, 124.2, 122.6, 119.4, 116.1, 115.5, 108.4, 58.0, 32.5.

UPLC-MS Chromatogram



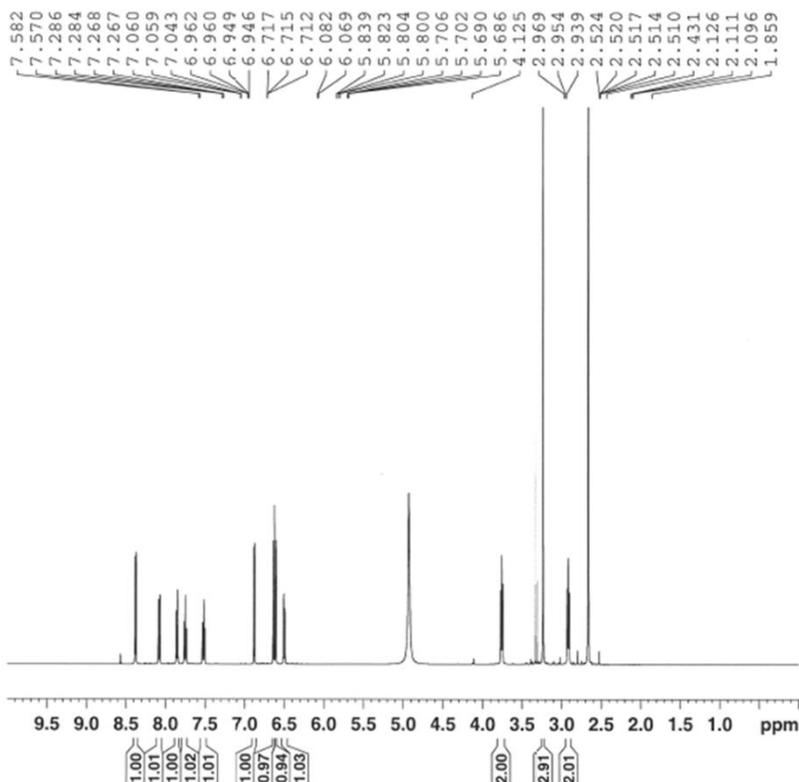
User Chromatogram Peak List

RT	Height	Height %	Area	Area %	Area Sum %	Symmetry	Width
1.859	4.12	0.44	15.81	0.54	0.54	1.56	0.064
2.017	937.1	100	2933.43	100	99.46	0.39	0.047

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>18</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 295.1441, found: 295.1509.

Analog CID 53308676; SID 124767890

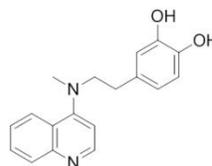
<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



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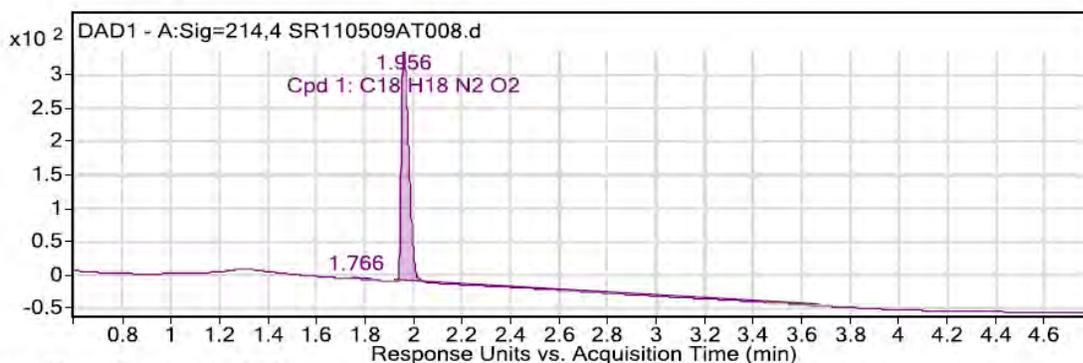
NAME      KSC-22-151B
EXPNO     1
PROCNO    1
Date_     20110630
Time      0.35
INSTRUM   spect
PROBHD    5 mm CPDUL 13C
PULPROG   zg30
TD         65536
SOLVENT   DMSO
NS         16
DS         2
SWH        10330.578 Hz
FIDRES     0.157632 Hz
AQ         3.1719923 sec
RG         114
DN         48.400 usec
DE         6.50 usec
TE         298.2 K
D1         1.00000000 sec
TD0        1

===== CHANNEL f1 =====
NUC1      1H
P1        15.00 usec
PL1       6.00 dB
PL1W      8.64411545 W
SF01      500.1930889 MHz
SI        32768
SF        500.1895984 MHz
WDW       EM
SSB       0
LB        0.30 Hz
GB         0
PC         1.00
    
```



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 157.8, 143.7, 143.3, 142.3, 129.6, 128.5, 124.2, 123.5, 122.8, 119.4, 118.5, 114.2, 113.7, 104.3, 56.4, 36.9, 37.7, 31.2.

UPLC-MS Chromatogram



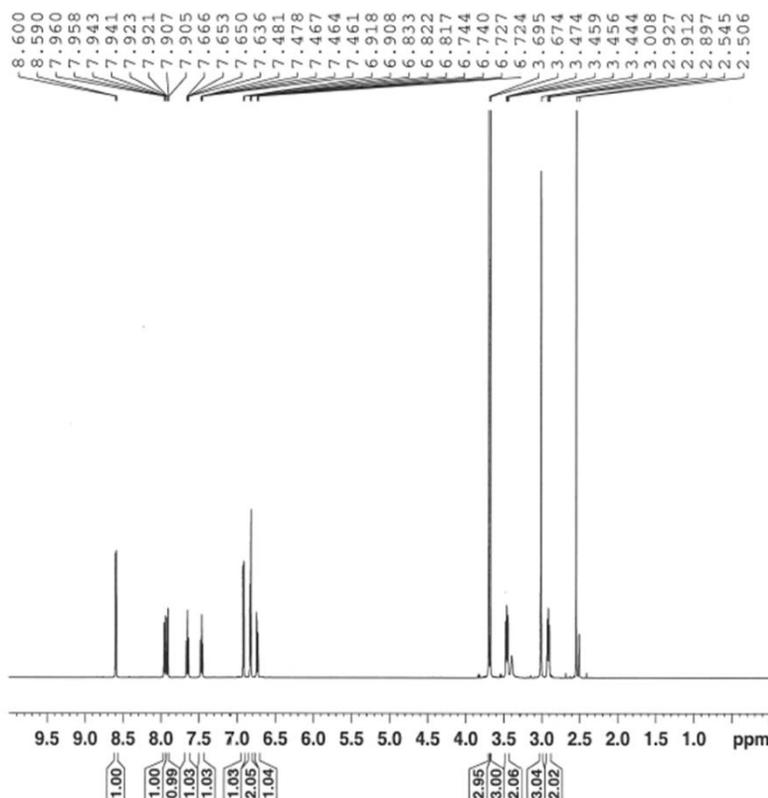
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		1.766	1.22	0.36	1.74	0.22	0.22	0.022
2	Cpd 1: C <sub>18</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	1.956	339.97	100	778.96	100	99.78	0.036

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>18</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 295.1441, found: 295.1446.

Analog CID 53308677; SID 124767903

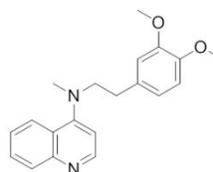
<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



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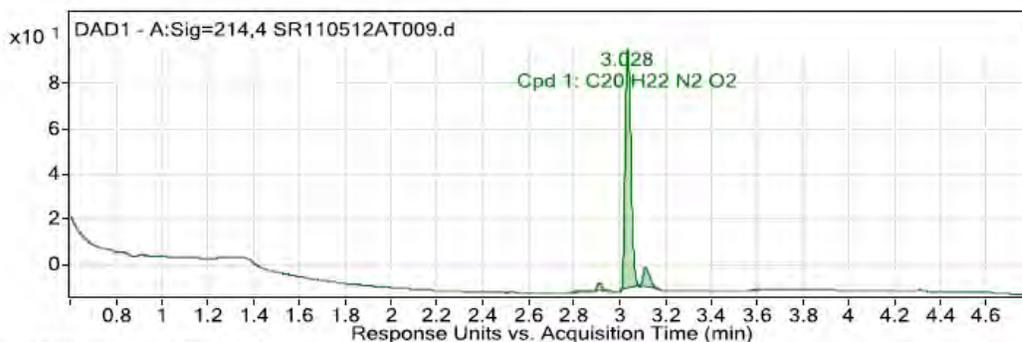
NAME          KSC-222-20
EXPNO         1
PROCNO        1
Date_         20110419
Time          12.27
INSTRUM       spect
PROBHD        5 mm CPDUL 13C
PULPROG       zg30
TD            65536
SOLVENT       DMSO
NS            16
DS            2
SWH           10330.578 Hz
FIDRES        0.157632 Hz
AQ            3.1719923 sec
RG            71.8
DW            48.400 usec
DE            6.50 usec
TE            298.2 K
D1            1.00000000 sec
TD0           1

===== CHANNEL f1 =====
NUC1          1H
P1            15.00 usec
PL1           6.00 dB
PL1W         8.64411545 W
SFO1          500.1930889 MHz
SI            32768
SF            500.1900000 MHz
WDW           EM
SSB           0
LB            0.30 Hz
GB            0
PC            1.00
    
```



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 156.3, 150.3, 149.3, 148.6, 147.2, 131.6, 129.5, 128.7, 124.5, 124.2, 122.6, 120.5, 112.6, 111.8, 108.5, 57.4, 55.5, 55.3, 39.1, 32.6.

UPLC-MS Chromatogram



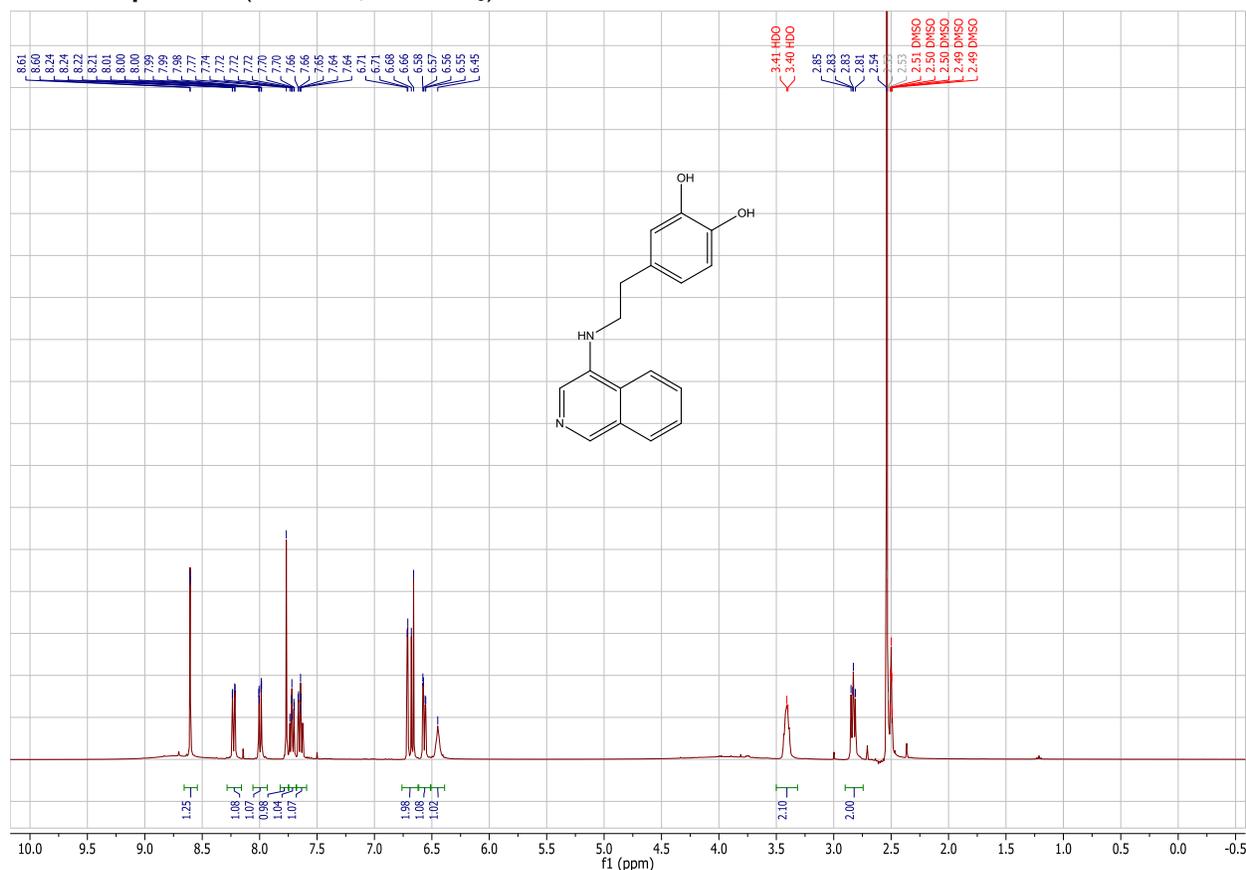
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.905	3.92	3.72	7.11	3.94	3.44	0.028
2	Cpd 1: C <sub>20</sub> H <sub>22</sub> N <sub>2</sub> O <sub>2</sub>	3.028	105.39	100	180.24	100	87.27	0.027
3		3.106	9.11	8.64	19.19	10.64	9.29	0.034

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>22</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 323.1754, found: 323.1751.

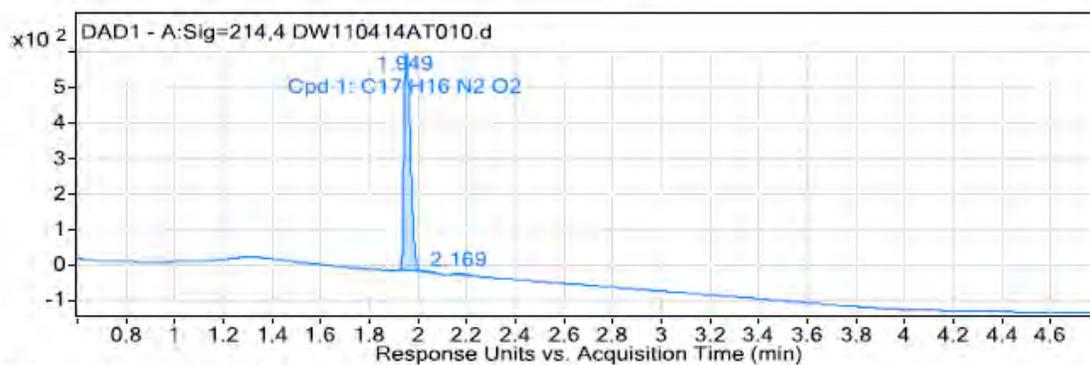
Analog CID 53308680; SID 124767873

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ145.2, 143.6, 138.8, 138.6, 130.5, 129.1, 128.1, 127.7, 127.5, 125.4, 121.1, 120.3, 119.4, 116.2, 115.6, 45.04, 33.8.

UPLC-MS Chromatogram



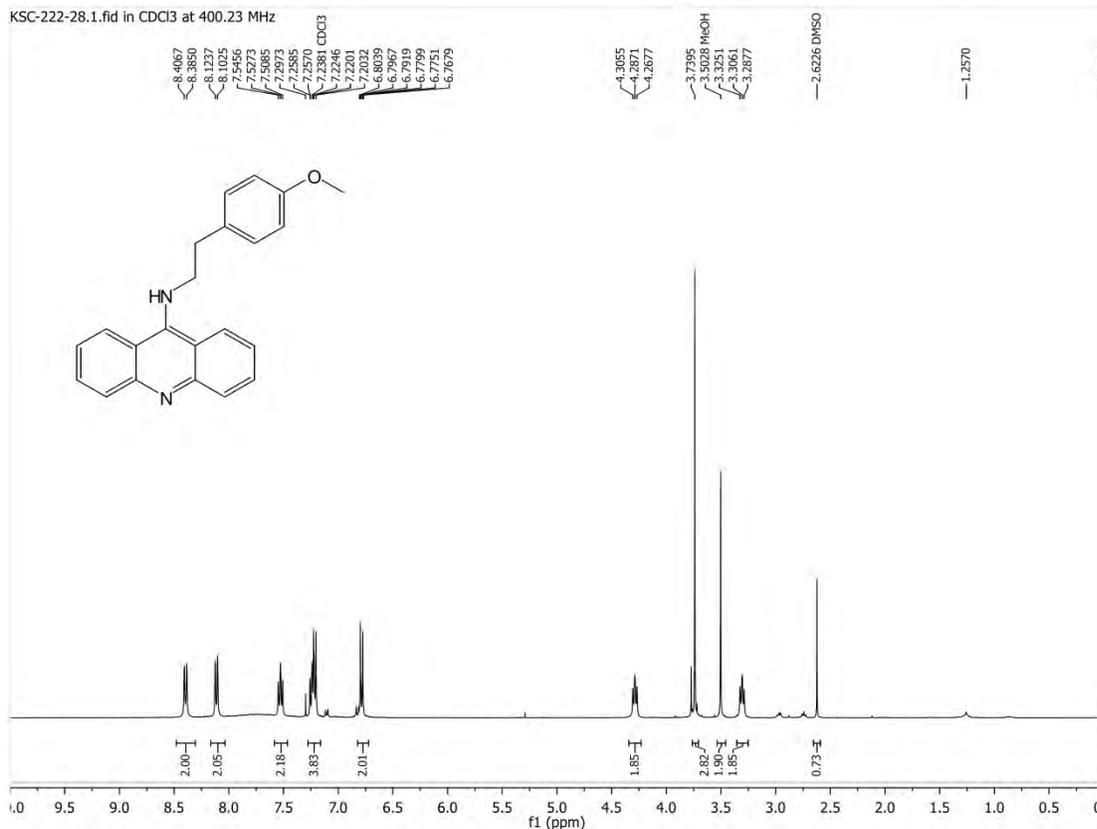
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C17 H16 N2 O2	1.949	616.51	100	1130.55	100	99.16	0.028
2		2.035	2.35	0.38	3.9	0.35	0.34	0.022
3		2.169	3.72	0.6	5.67	0.5	0.5	0.022

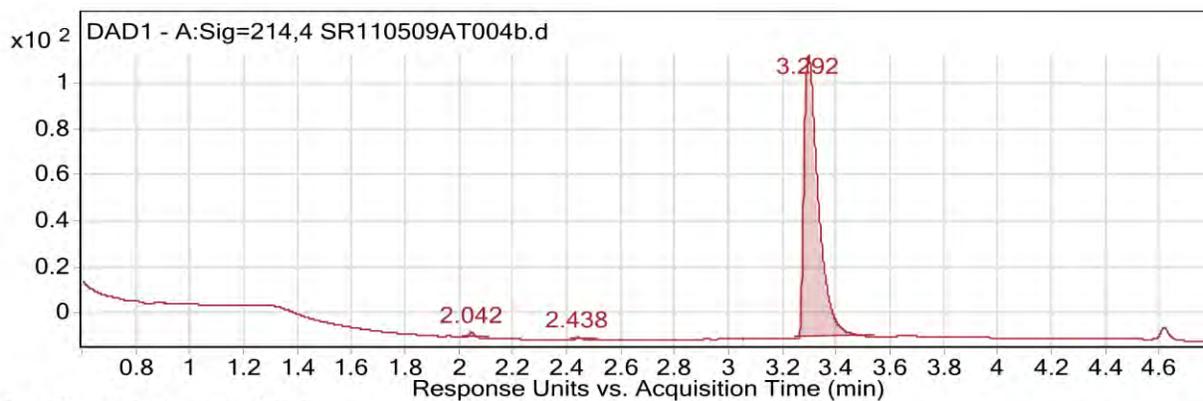
HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>17</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 281.1285, found: 281.1302.

Analog CID 53308681; SID 124767888

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



UPLC-MS Chromatogram



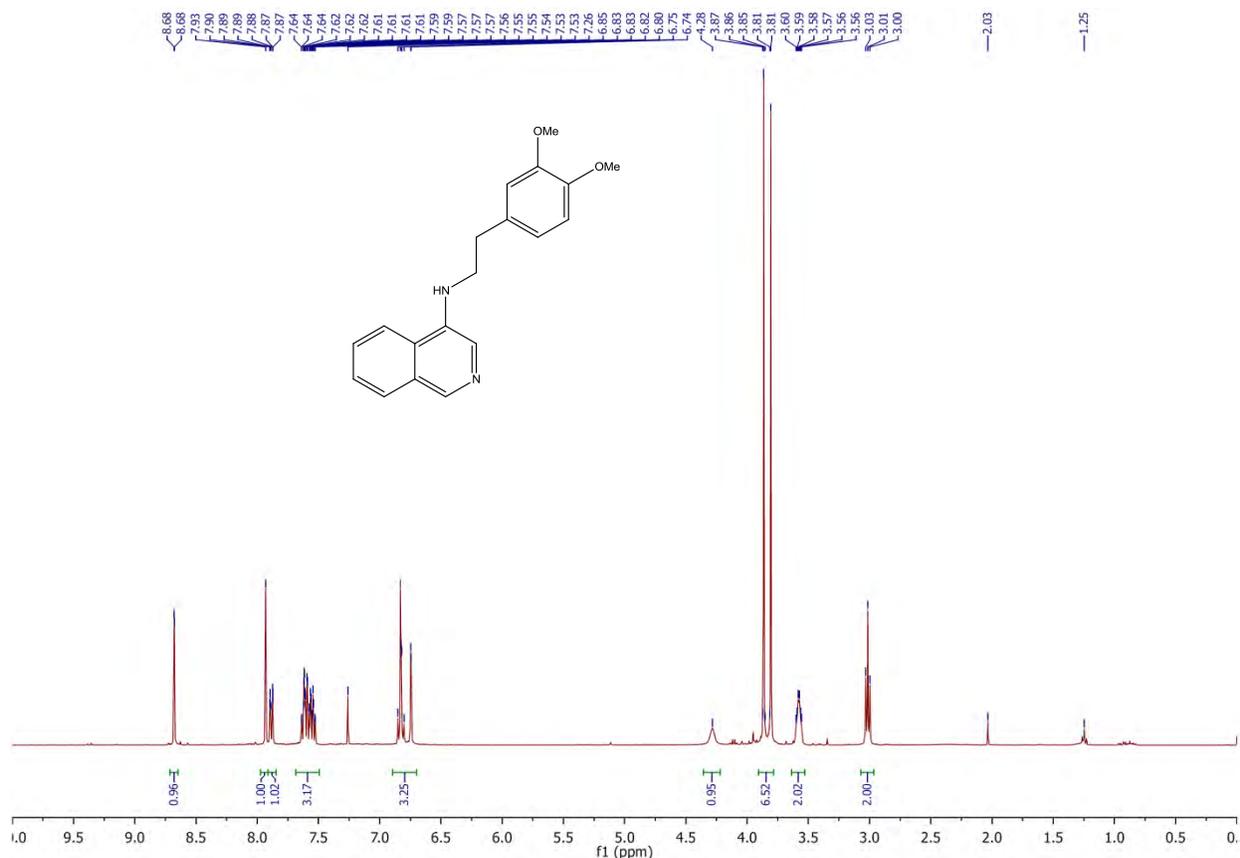
User Chromatogram Peak List

Peak #	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	2.042	2.52	2.07	3.66	0.8	0.79	0.023
2	2.438	1.06	0.87	2.07	0.45	0.45	0.03
3	3.292	122.15	100	455.18	100	98.76	0.055

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>22</sub>H<sub>20</sub>N<sub>2</sub>O + H]<sup>+</sup>: 329.1648, found: 329.1647.

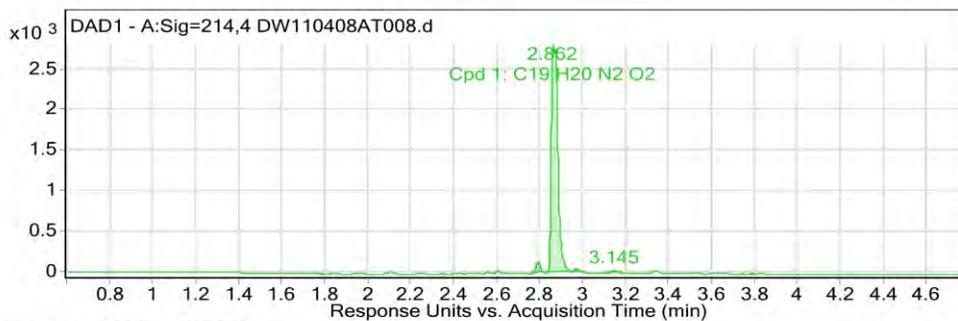
Analog CID 53308682; SID 124767881

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, CDCl<sub>3</sub>) δ 149.2, 148.0, 142.2, 137.6, 131.5, 129.0, 128.6, 128.1, 127.0, 126.0, 123.7, 120.8, 119.1, 112.1, 111.6, 56.0, 55.9, 44.9, 34.8.

UPLC-MS Chromatogram



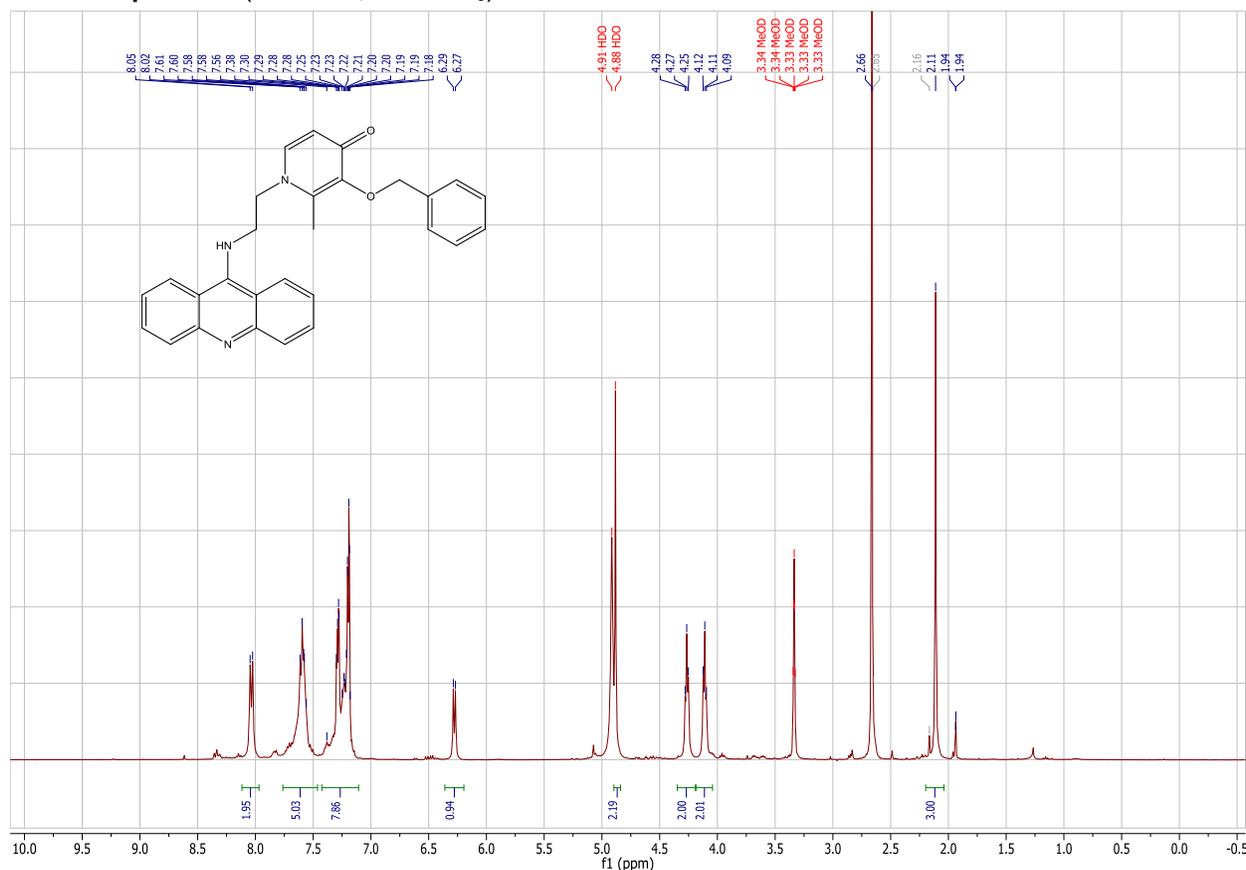
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.788	134.59	4.82	203.14	3.39	3.21	0.025
2	Cpd 1: C <sub>19</sub> H <sub>20</sub> N <sub>2</sub> O <sub>2</sub>	2.862	2791.81	100	5989.19	100	94.53	0.034
3		2.967	46.5	1.67	79.66	1.33	1.26	0.026
4		3.145	37.52	1.34	63.81	1.07	1.01	0.027

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>19</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 309.1598, found: 309.1623.

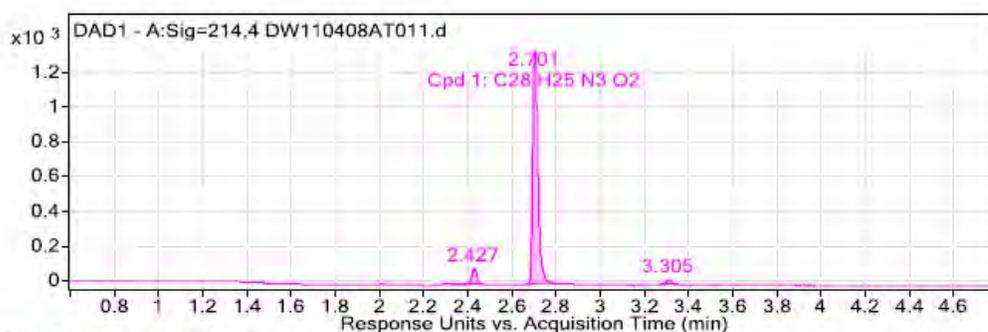
Analog CID 53308683; SID 124767882

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 174.7, 155.1, 146.9, 145.1, 142.5, 141.8, 138.3, 134.9, 132.7, 129.2, 127.2, 122.5, 121.8, 118.3, 116.9, 74.4, 55.9, 42.5, 13.0.

UPLC-MS Chromatogram



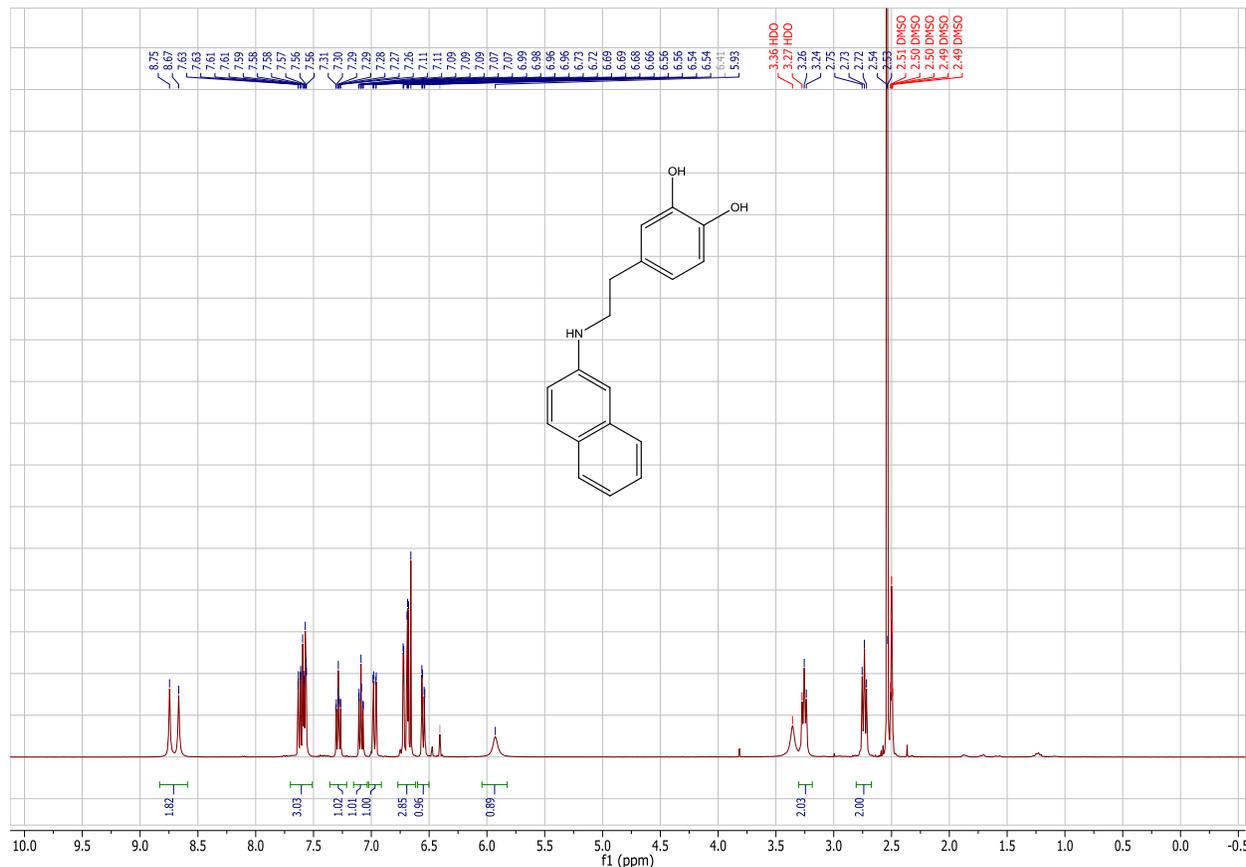
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.309	11.02	0.62	24.2	1.06	0.96	0.033
2		2.427	94.31	7.02	160.24	7.03	6.35	0.026
3	Cpd 1: C <sub>28</sub> H <sub>25</sub> N <sub>3</sub> O <sub>2</sub>	2.701	1344.21	100	2279.78	100	90.4	0.027
4		3.305	33.8	2.51	57.71	2.53	2.29	0.027

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>28</sub>H<sub>25</sub>N<sub>3</sub>O<sub>2</sub> + H]<sup>+</sup>: 436.2020, found: 436.2042.

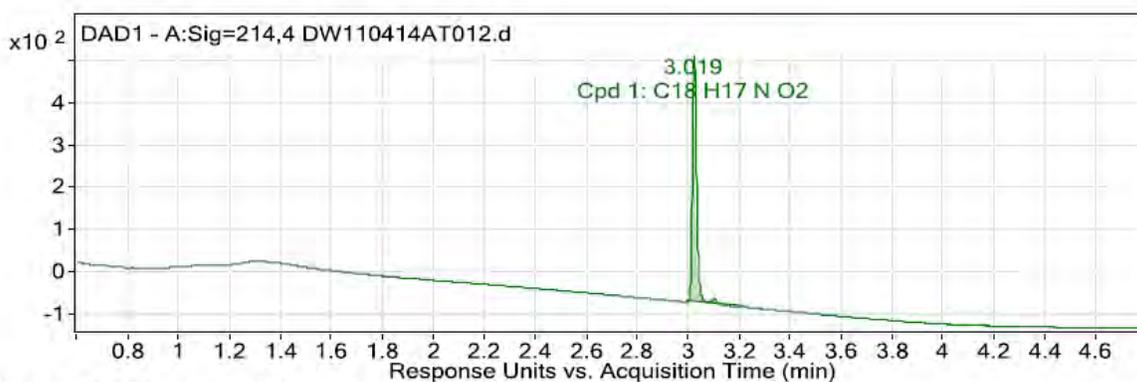
Analog CID 53308684; SID 124767875

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 34.1, 45.1, 102.2, 115.5, 116.1, 118.4, 119.3, 120.9, 125.5, 126.0, 126.4, 127.4, 128.3, 130.6, 135.3, 143.5, 145.1, 146.6.

UPLC-MS Chromatogram



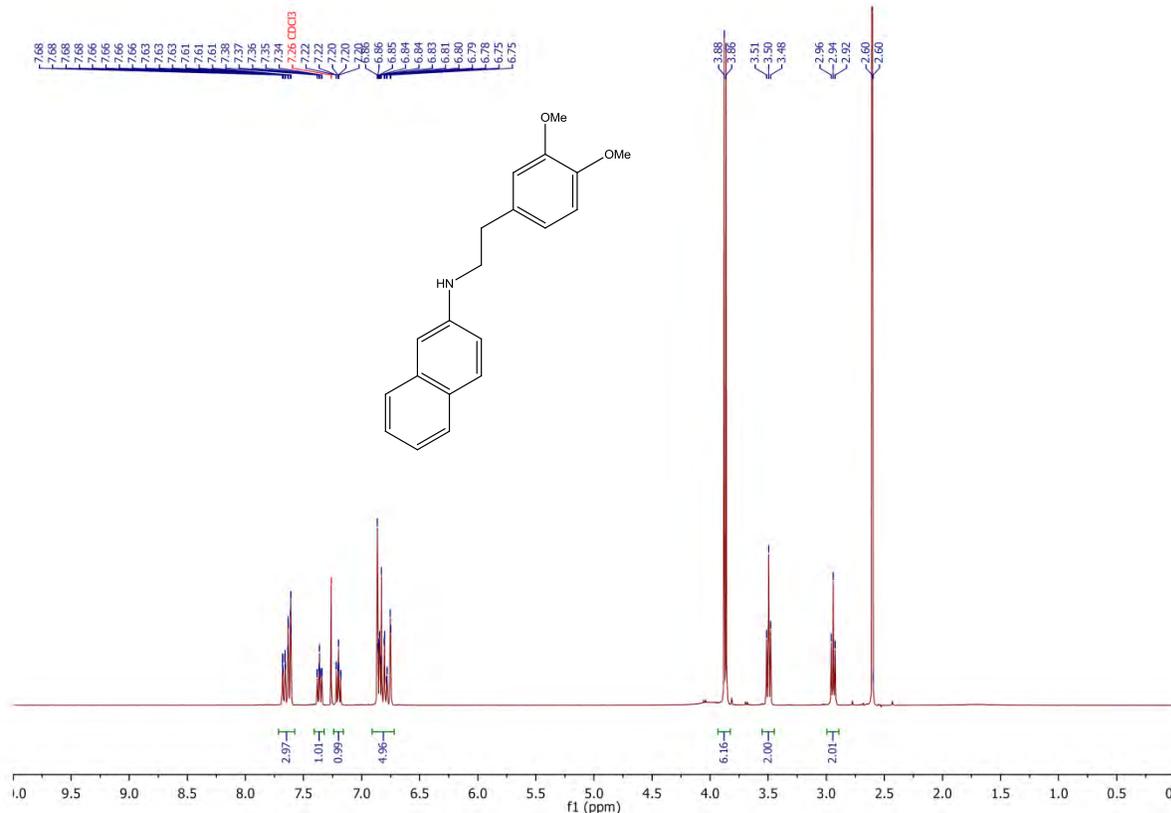
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>18</sub> H <sub>17</sub> N O <sub>2</sub>	3.019	577.06	100	678.61	100	96.85	0.019
2		3.098	14.25	2.47	22.1	3.26	3.15	0.023

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>18</sub>H<sub>17</sub>NO<sub>2</sub> + H]<sup>+</sup>: 280.1332, found: 280.1352.

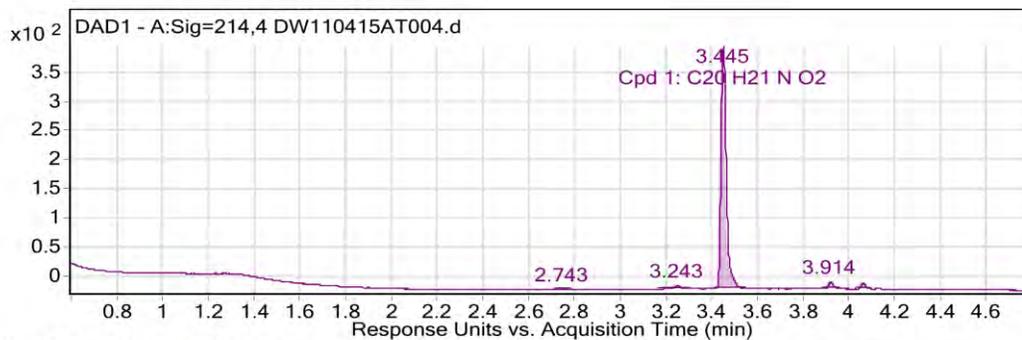
Analog CID 53308685; SID 124767885

<sup>1</sup>H NMR Spectrum (400 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (400 MHz, CDCl<sub>3</sub>) δ 149.1, 147.8, 142.8, 134.8, 131.1, 129.4, 128.9, 127.8, 126.7, 126.5, 123.3, 120.8, 118.7, 112.0, 111.4, 108.9, 56.02, 55.95, 47.3, 34.1.

UPLC-MS Chromatogram



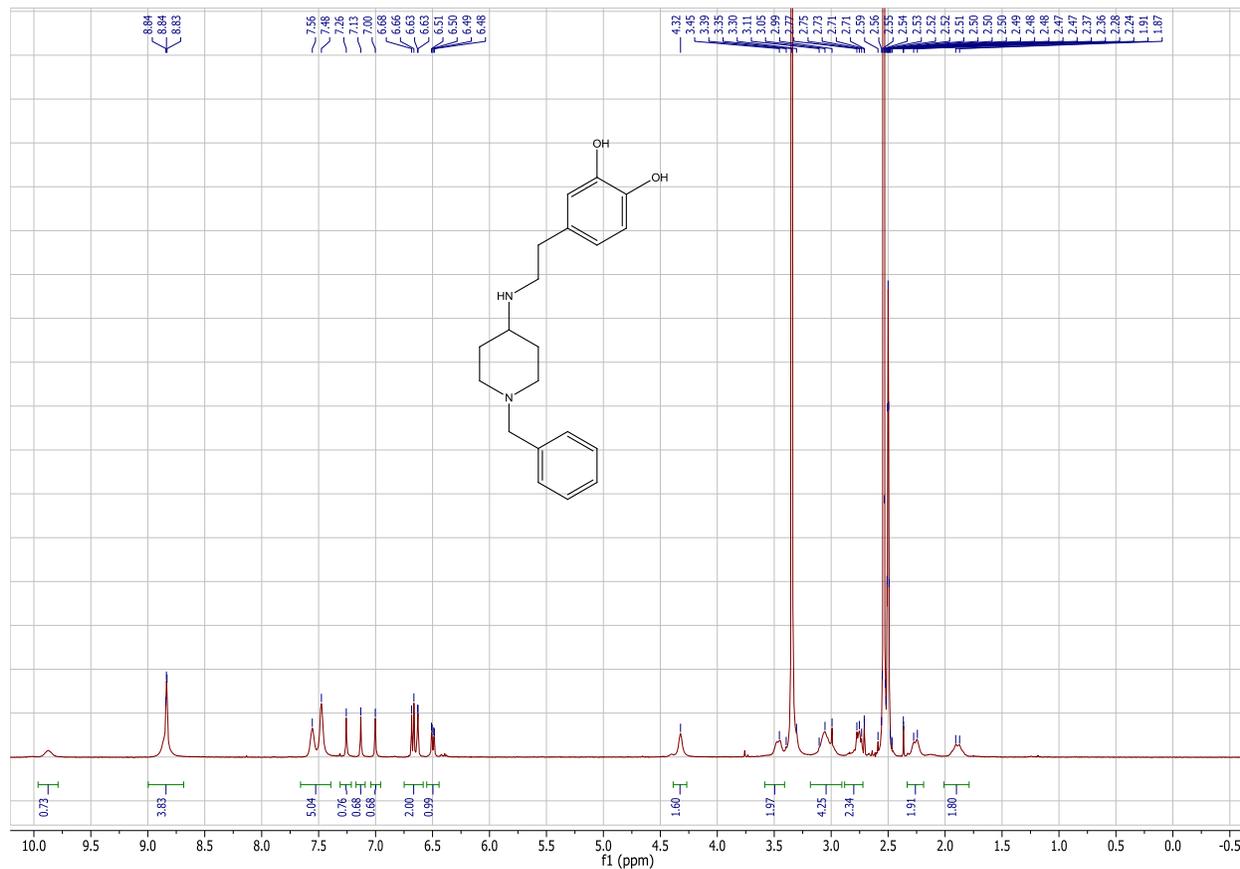
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.743	1.27	0.31	1.88	0.28	0.26	0.023
2		3.195	2.37	0.58	3.25	0.48	0.45	0.02
3		3.243	5.7	1.38	13.05	1.94	1.8	0.033
4	Cpd 1: C <sub>20</sub> H <sub>21</sub> N O <sub>2</sub>	3.445	412.58	100	673.23	100	93.06	0.026
5		3.914	11.48	2.78	17.87	2.65	2.47	0.024
6		4.055	9.3	2.25	14.12	2.1	1.95	0.023

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>21</sub>NO<sub>2</sub> + H]<sup>+</sup>: 308.1645, found: 308.1662.

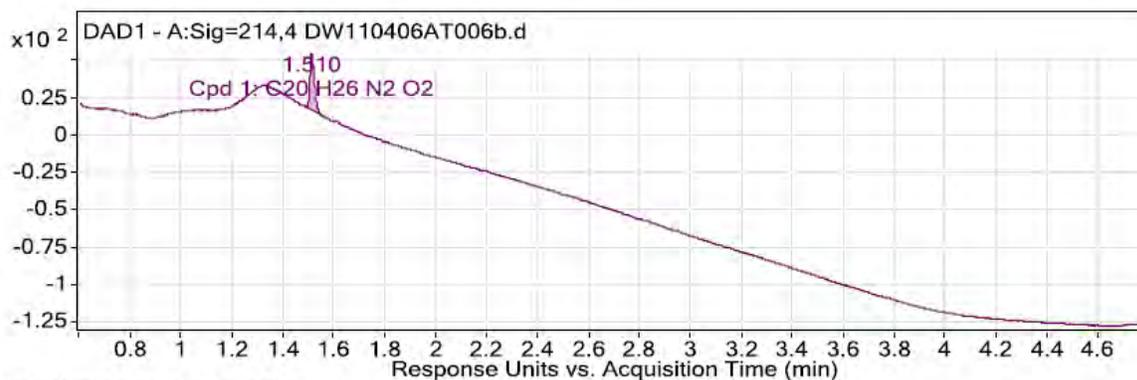
Analog CID 53308686; SID 124767869

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 145.3, 144.1, 131.6, 129.4, 128.8, 127.6, 119.3, 116.0, 115.7, 58.9, 51.7, 49.5, 45.7, 31.0, 25.4.

UPLC-MS Chromatogram



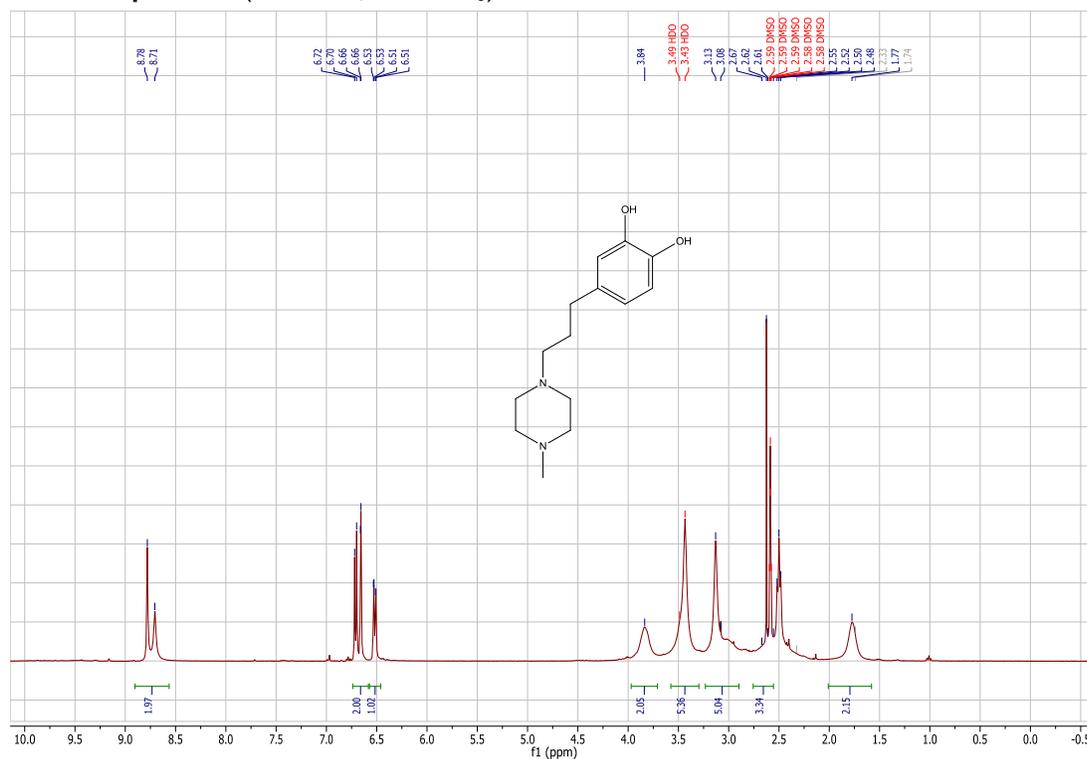
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>20</sub> H <sub>26</sub> N <sub>2</sub> O <sub>2</sub>	1.51	37.17	100	45.45	100	100	0.019

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>26</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 327.2067, found: 327.2063.

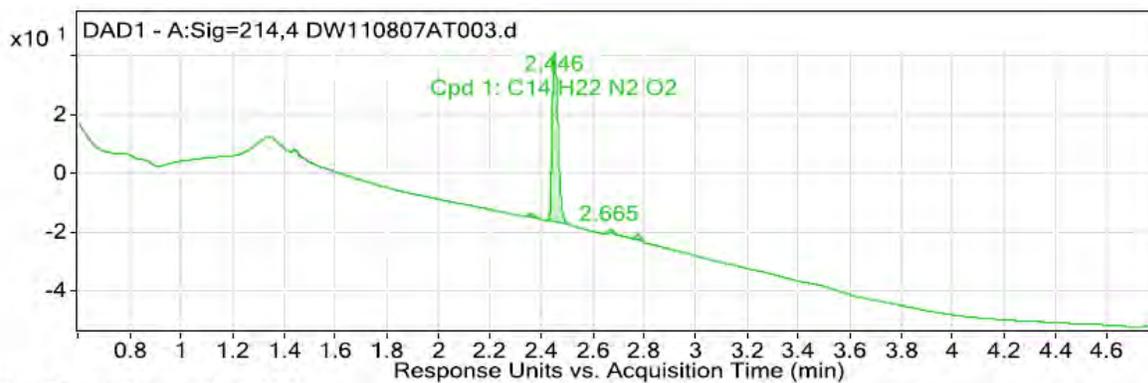
Analog CID 53384805; SID 125311612

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 145.0, 143.2, 132.4, 118.9, 115.7, 115.4, 67.4, 56.0, 45.7, 40.4, 31.9, 18.8.

UPLC-MS Chromatogram



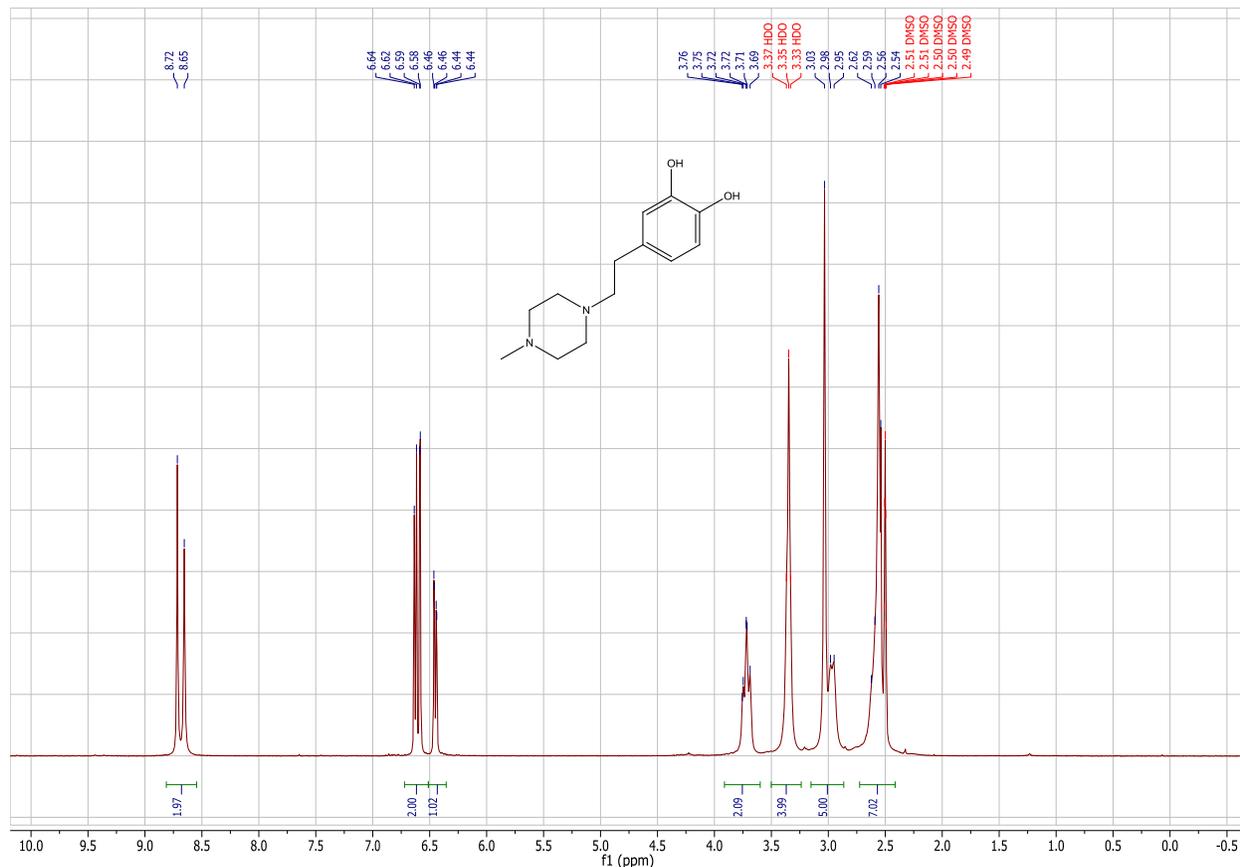
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.354	1.65	2.88	2.77	3.12	2.88	0.024
2	Cpd 1: C <sub>14</sub> H <sub>22</sub> N <sub>2</sub> O <sub>2</sub>	2.446	57.39	100	88.89	100	92.31	0.024
3		2.665	1.73	3.02	1.8	2.03	1.87	0.016
4		2.772	2.43	4.23	2.82	3.18	2.93	0.018

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>14</sub>H<sub>22</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 251.1754, found: 251.1762.

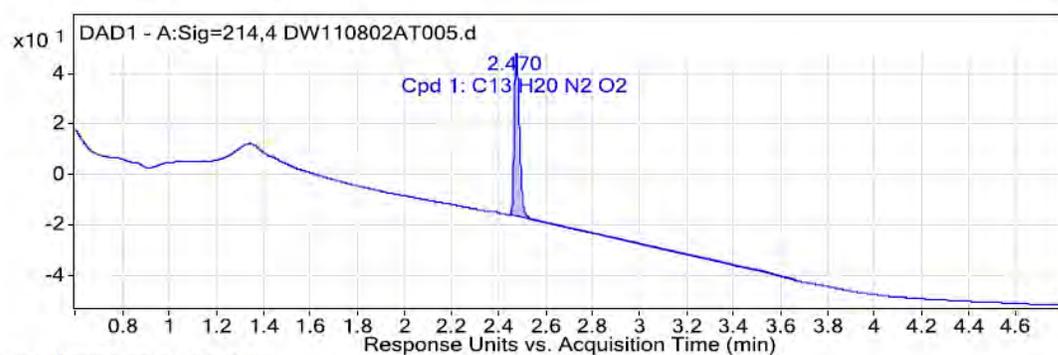
Analog CID 53384806; SID 125311611

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 145.0, 143.4, 130.7, 119.2, 116.0, 115.5, 59.0, 54.4, 45.8, 40.3, 32.0.

UPLC-MS Chromatogram



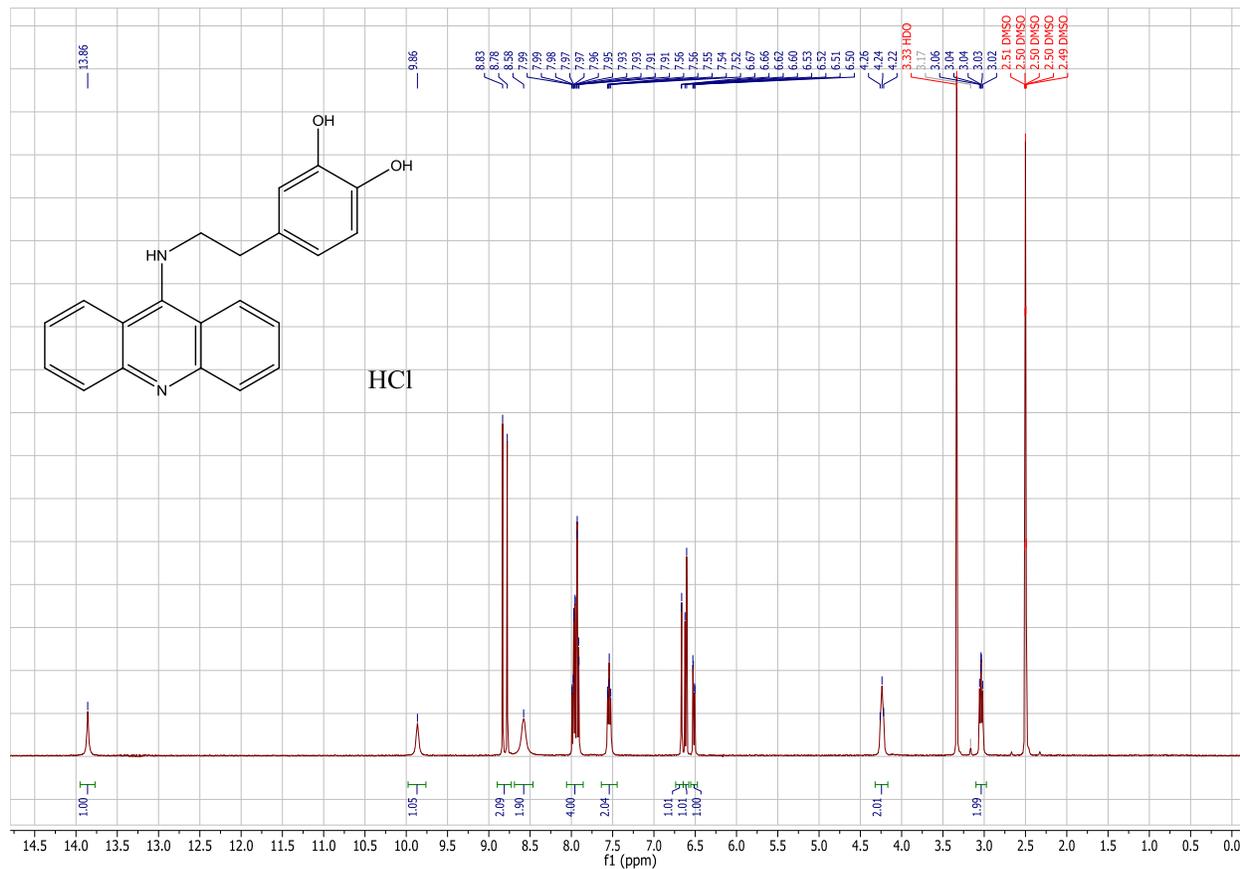
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C13H20N2O2	2.47	64.59	100	135.17	100	100	0.03

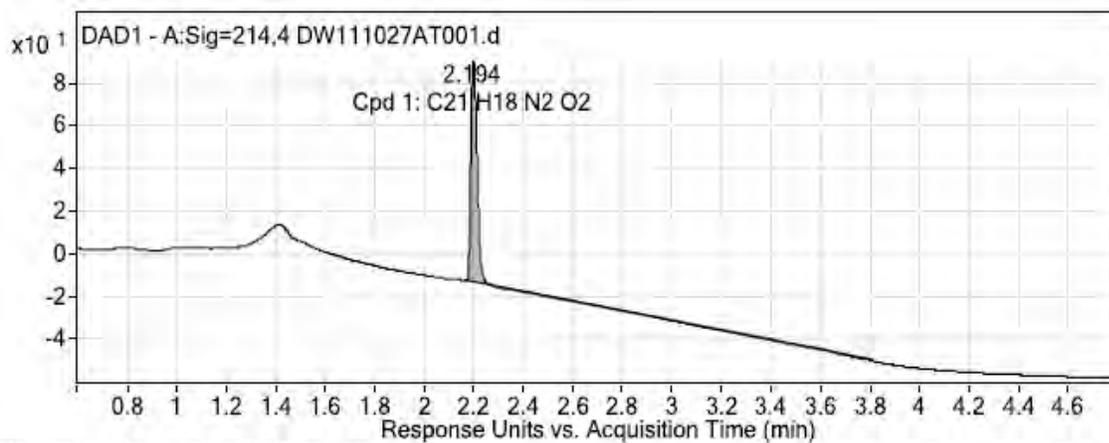
HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 237.1598, found: 237.1602.

Analog CID 53464134; SID 126490285

<sup>1</sup>H NMR Spectrum (400 MHz, DMSO-d<sub>6</sub>)



UPLC-MS Chromatogram



User Chromatogram Peak List

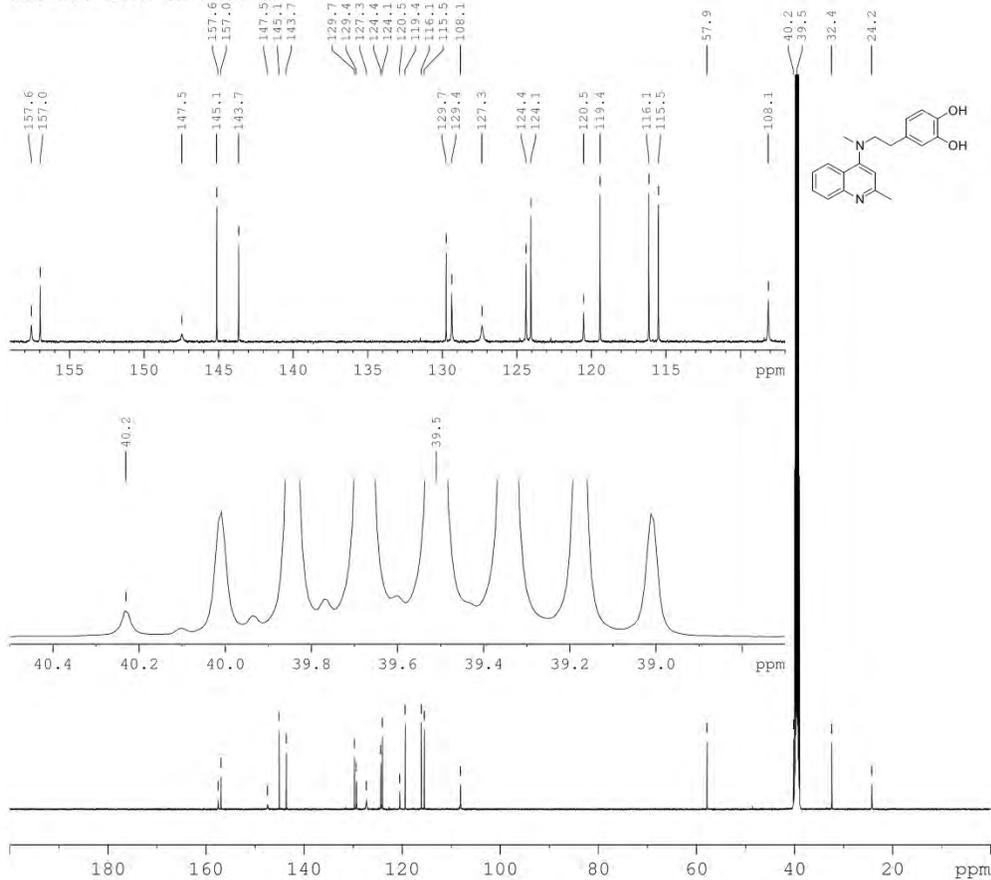
Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>21</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	2.194	102.82	100	235.27	100	100	0.034

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>21</sub>H<sub>19</sub>CIN<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 367.1208, found: 331.1438.

Analog CID 56840756; SID 162021976

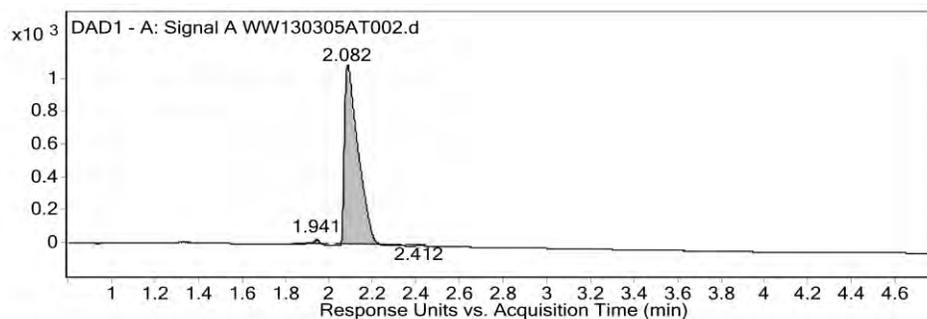
<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)

KSC-280-083A in DMSO-d<sub>6</sub>



<sup>13</sup>C NMR (126 MHz, DMSO-d<sub>6</sub>) δ 157.5, 157.0, 147.4, 145.1, 143.6, 129.7, 129.3, 127.3, 124.4, 124.0, 120.5, 119.4, 116.1, 115.5, 108.1, 57.9, 40.2, 32.4, 24.2.

UPLC-MS Chromatogram



User Chromatogram Peak List

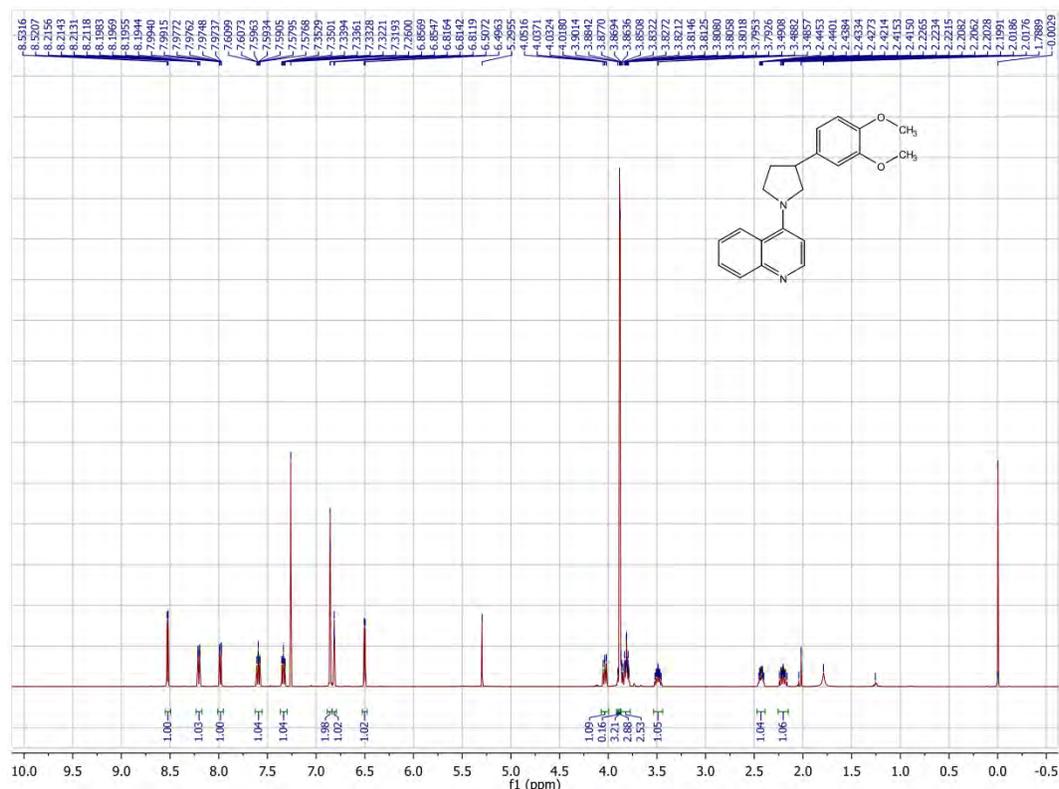
RT	Height	Height %	Area	Area %	Area Sum %	Symmetry	Width
1.941	31.05	2.85	79.38	1.68	1.64	1.85	0.035
2.082	1091.3	100	4736.93	98.11	98.11	0.29	0.062
2.412	8.26	0.76	11.85	0.25	0.25	0.6	0.024

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>19</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 309.1598, found: 309.1598.



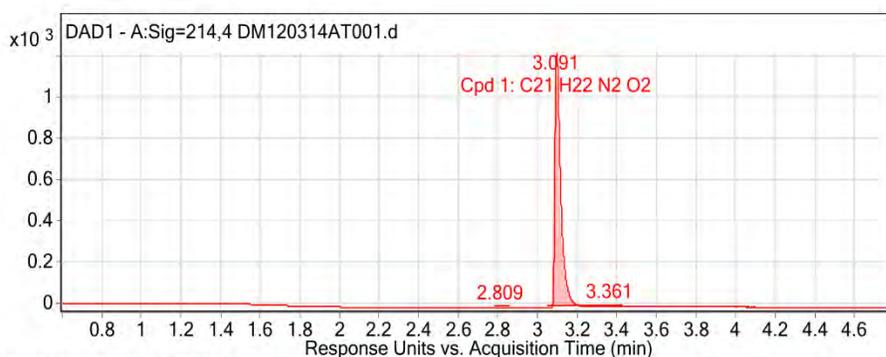
Analog CID 56840760; SID 134970501

<sup>1</sup>H NMR Spectrum (500 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, CDCl<sub>3</sub>) δ 152.5, 150.4, 150.2, 149.3, 148.2, 133.6, 129.9, 128.7, 125.1, 123.4, 121.3, 119.1, 111.5, 110.6, 102.9, 59.3, 56.11, 56.06, 51.9, 44.2, 33.2.

UPLC-MS Chromatogram



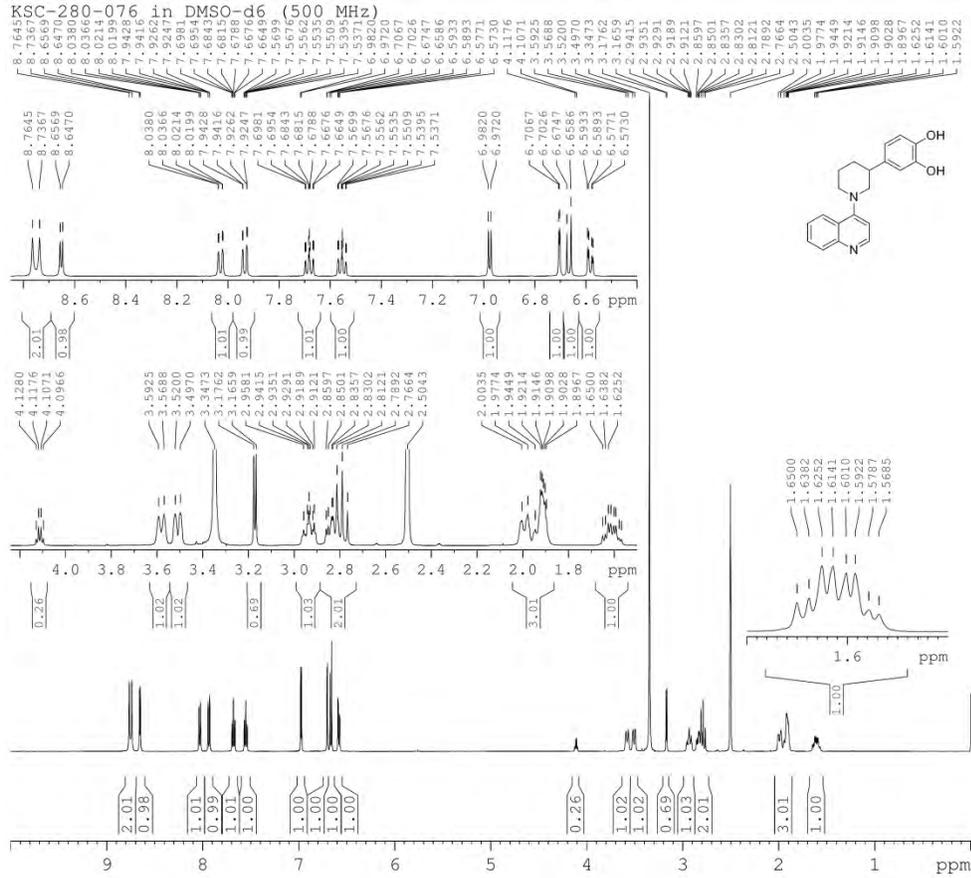
User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		2.809	1.39	0.11	2.84	0.11	0.11	0.031
2	Cpd 1: C <sub>21</sub> H <sub>22</sub> N <sub>2</sub> O <sub>2</sub>	3.091	1229.14	100	2519.31	100	99.11	0.031
3		3.361	3.76	0.31	19.85	0.79	0.78	0.066

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>21</sub>H<sub>22</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 335.1754, found: 335.1770.

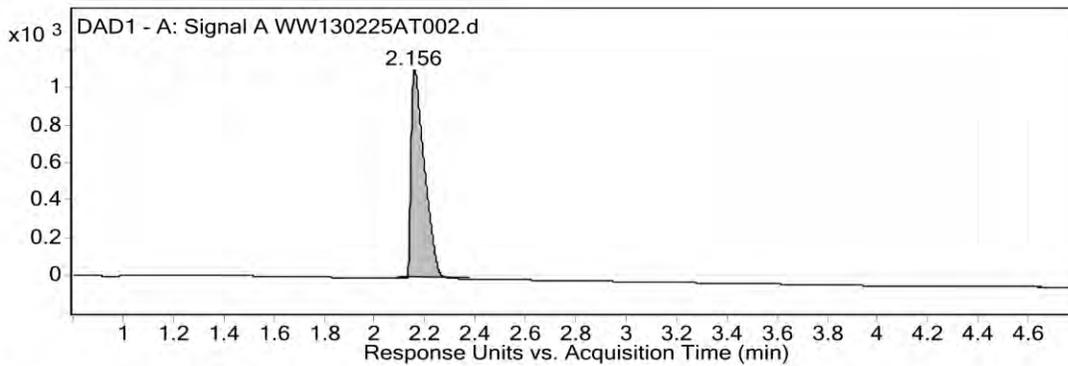
Analog CID 56840766; SID 162021974

<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR (126 MHz, DMSO-d<sub>6</sub>) δ 156.6, 150.8, 149.1, 145.1, 143.8, 134.6, 129.6, 128.9, 125.2, 123.8, 122.9, 117.6, 115.5, 114.5, 109.1, 59.5, 52.5, 41.5, 31.4, 25.5.

UPLC-MS Chromatogram



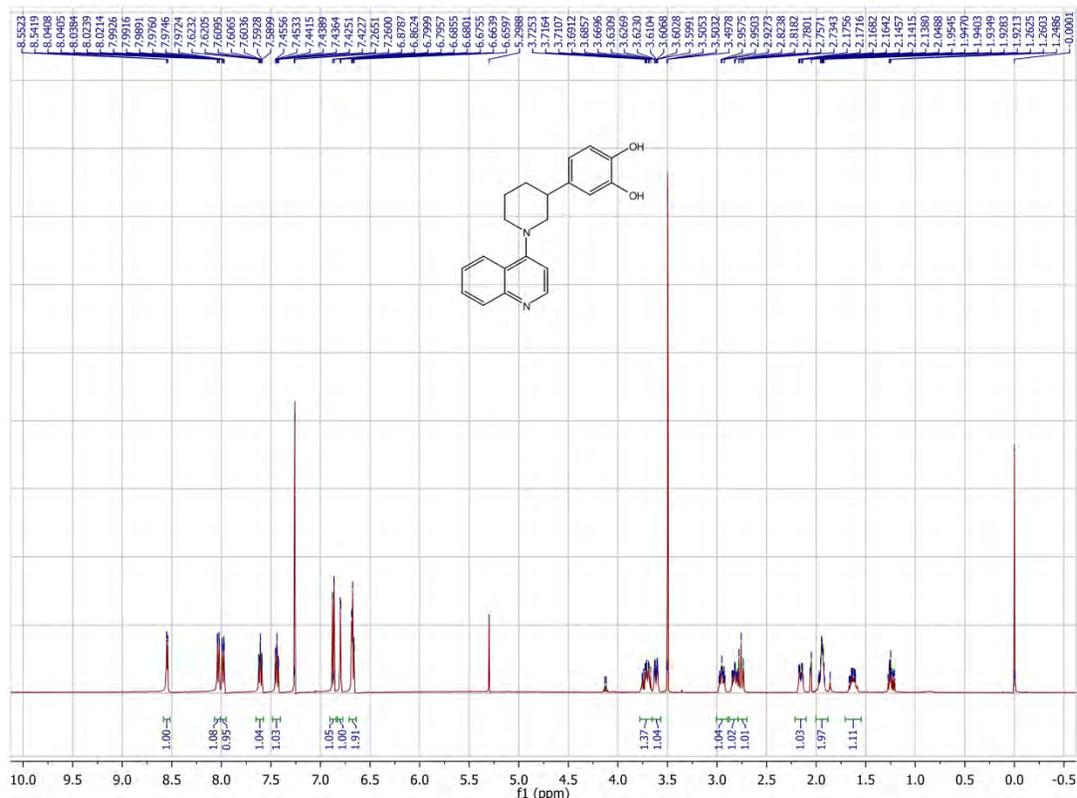
User Chromatogram Peak List

RT	Height	Height %	Area	Area %	Area Sum %	Symmetry	Width
2.156	1107.01	100	4063.71	100	100	0.34	0.087

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 321.1598, found: 321.1680.

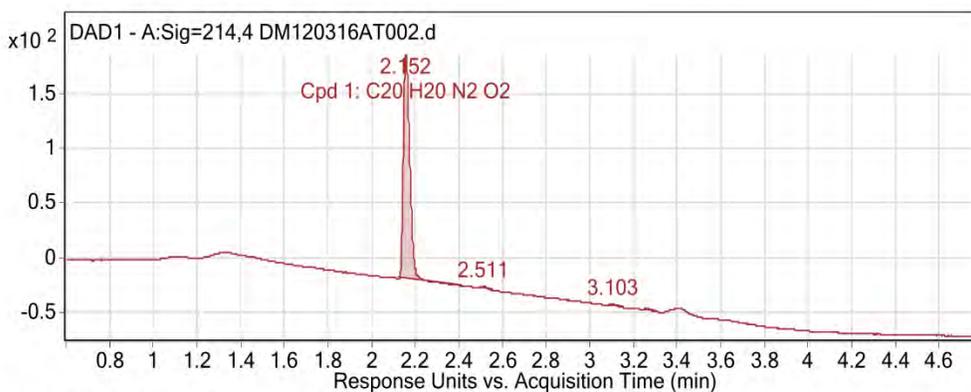
Analog CID 56840766; SID 134970506

<sup>1</sup>H NMR Spectrum (500 MHz, CDCl<sub>3</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, CDCl<sub>3</sub>) δ 158.6, 149.5, 148.2, 145.0, 144.1, 135.2, 129.8, 128.4, 125.6, 124.3, 123.5, 118.2, 114.8, 114.2, 108.5, 59.8, 53.3, 41.7, 31.4, 25.9.

UPLC-MS Chromatogram



User Chromatogram Peak List

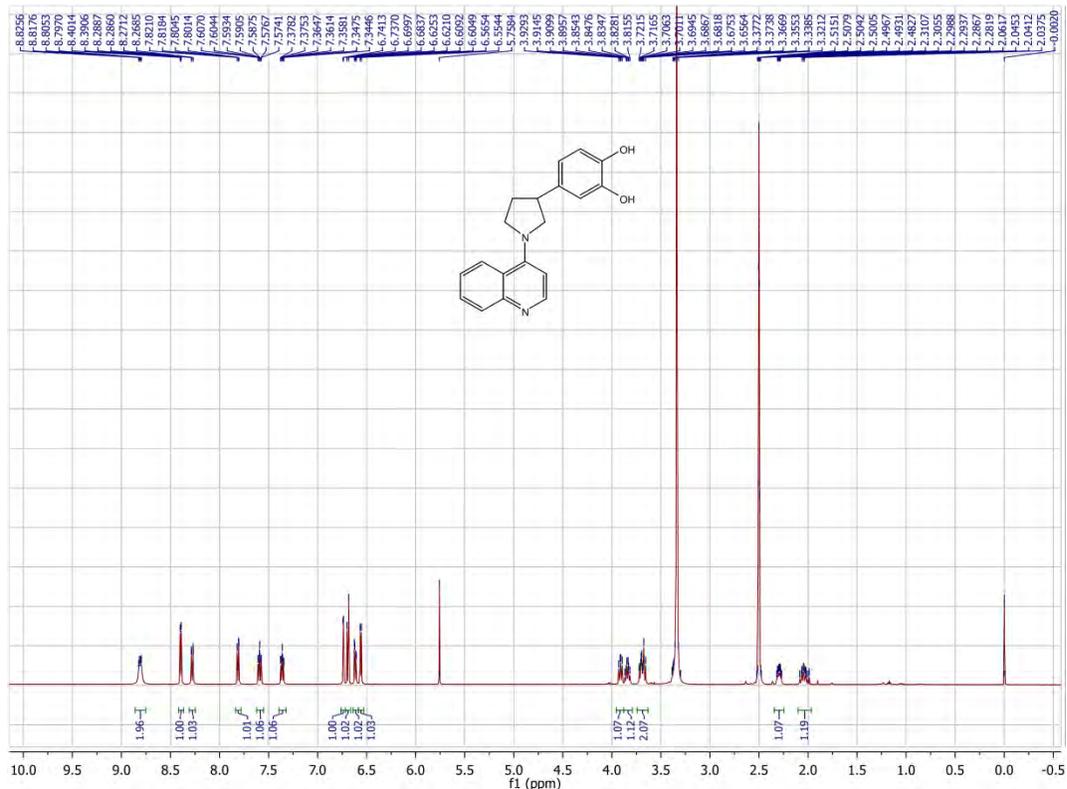
Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>20</sub> H <sub>20</sub> N <sub>2</sub> O <sub>2</sub>	2.152	203.6	100	423.39	100	97.84	0.032
2		2.511	2.34	1.15	4.67	1.1	1.08	0.032
3		3.103	1.5	0.74	2.49	0.59	0.57	0.025
4		3.261	1.52	0.75	2.2	0.52	0.51	0.021

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 321.1598, found: 321.1610.



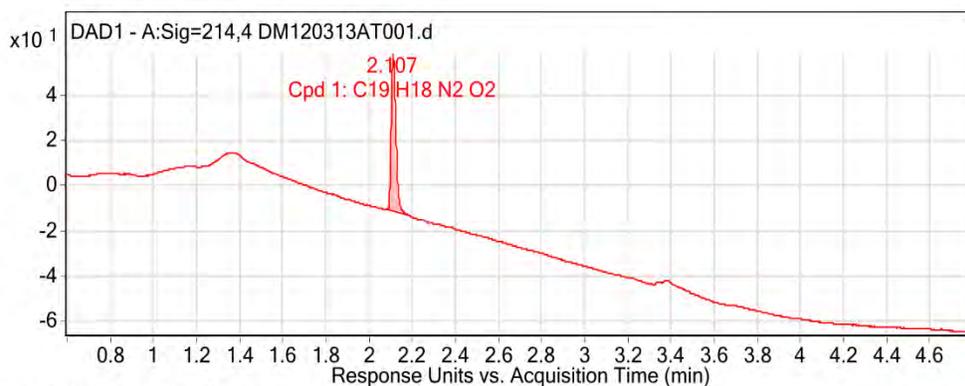
Analog CID 56840772; SID 134970490

<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 151.7, 149.8, 149.7, 145.2, 144.0, 132.0, 129.1, 128.4, 125.3, 123.0, 120.6, 117.8, 115.5, 114.6, 102.7, 58.6, 51.7, 42.9, 32.5.

UPLC-MS Chromatogram



User Chromatogram Peak List

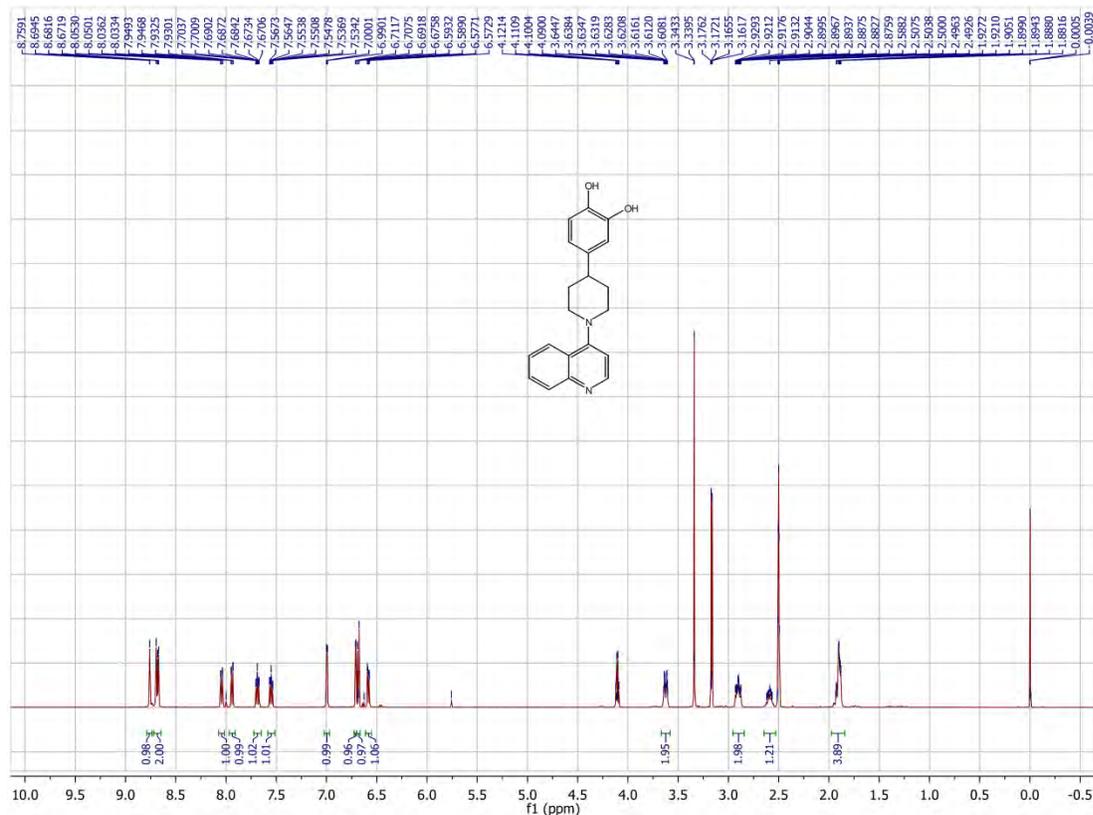
Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1	Cpd 1: C <sub>19</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	2.107	69.44	100	106.27	100	100	0.024

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>19</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 307.1441, found: 307.1440.



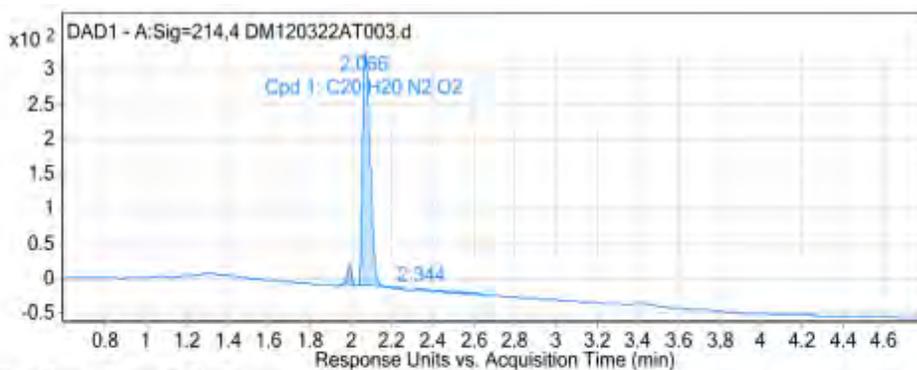
Analog CID 56846691; SID 135378316

<sup>1</sup>H NMR Spectrum (500 MHz, DMSO-d<sub>6</sub>)



<sup>13</sup>C NMR Spectrum (126 MHz, DMSO-d<sub>6</sub>) δ 156.8, 150.8, 149.1, 145.1, 143.5, 136.9, 129.5, 128.9, 125.2, 123.9, 123.0, 117.2, 115.5, 114.2, 109.0, 52.8, 41.0, 33.5.

UPLC-MS Chromatogram



User Chromatogram Peak List

Peak #	Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Width
1		1.989	28.5	8.54	49.4	5.84	5.46	0.026
2	Cpd 1: C20 H20 N2 O2	2.066	333.66	100	845.71	100	93.45	0.041
3		2.195	1.63	0.49	2.83	0.33	0.31	0.023
4		2.344	1.43	0.43	2.58	0.31	0.29	0.026
5		2.425	1.01	0.3	1.6	0.19	0.18	0.021
6		2.522	1.13	0.34	2.91	0.34	0.32	0.034

HRMS (ESI<sup>+</sup>) *m/z* calcd for [C<sub>20</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> + H]<sup>+</sup>: 321.1598, found: 321.1617.

## Appendix G: Prior Art Search

**Table G1.** Search Strings and Databases Investigated in the Prior Art Search

Search Terms	Database	Number of Hits
M18 aminopeptidase inhibitor	Chemical Abstracts Service (SciFinder)	2
<i>Plasmodium falciparum</i> M18 inhibitor	Chemical Abstracts Service (SciFinder)	1
M18 inhibitor	Chemical Abstracts Service (SciFinder)	27
<i>Pf</i> M18 inhibitor	Chemical Abstracts Service (SciFinder)	0
<i>Plasmodium falciparum</i> inhibitor	Chemical Abstracts Service (SciFinder)	11
<i>Pf</i> M18AAP	Chemical Abstracts Service (SciFinder)	2

## **Appendix H: Eurofins Panlabs LeadProfilingScreen Report for ML369**

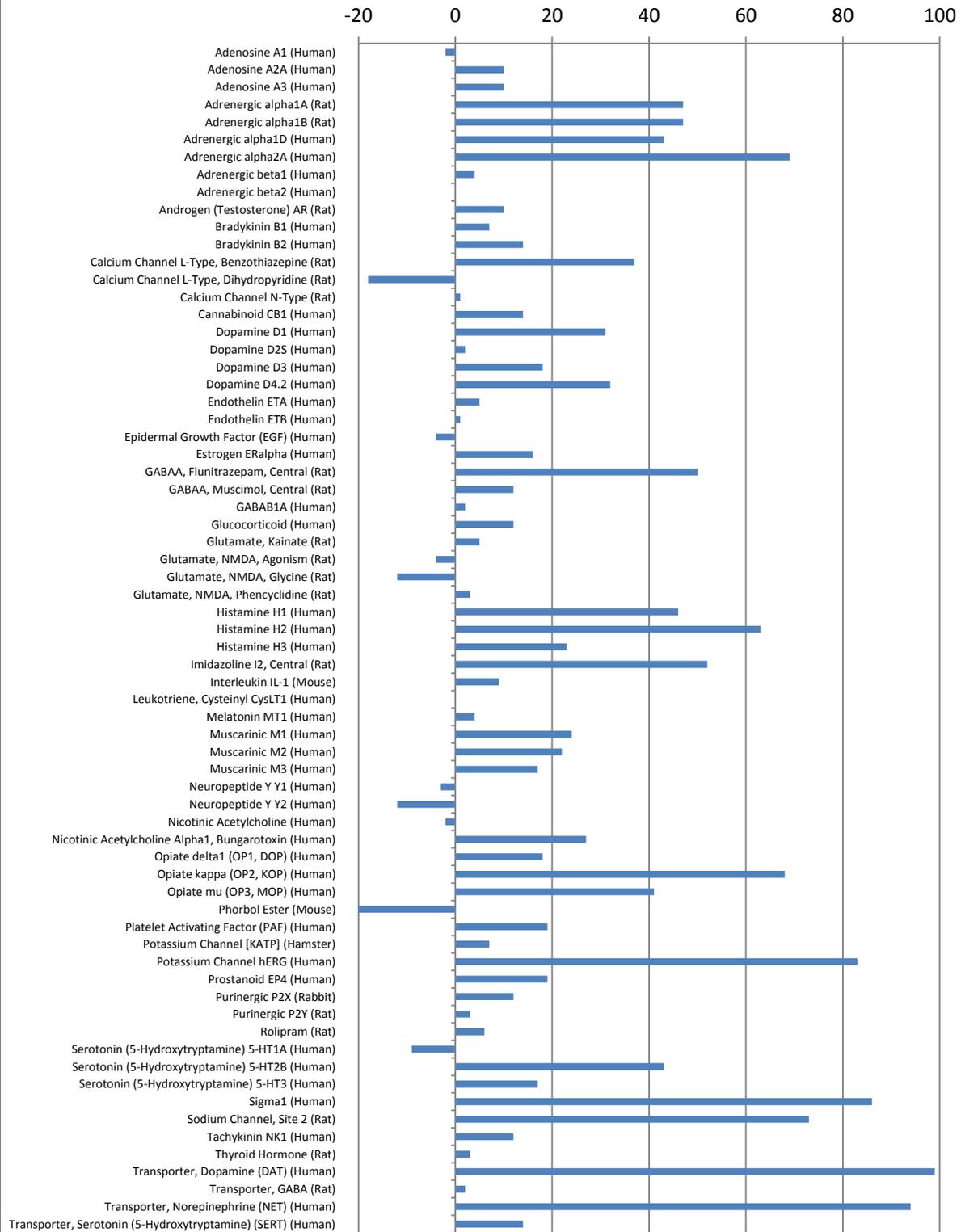
### **Study Objective:**

To evaluate, in radioligand binding assays, the activity of probe compound **ML369** (KUC110948N-02) across a panel of 68 receptors.

### **Methods:**

Methods employed in this study performed at Eurofins Panlabs [32] have been adapted from the scientific literature to maximize reliability and reproducibility. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. Assay results are presented as the percent inhibition of specific binding or activity (for n = 2 replicates) for the probe compound tested at a concentration of 10  $\mu$ M.

## ML369 LeadProfilingScreen Results: % Inhibition at 10 $\mu$ M



## Appendix I: Compounds Provided to Evotec

**Table I1.** Compound Identifier Information for Probe and Supporting Analogues

<b>CID / SID</b>	<b>KU Registry Number / KU Compound Lot Number</b>	<b>P/A</b>	<b>MLS ID</b>	<b>ML ID</b>
56846691 / 162021972	KUC110948N-02 / KSC-280-077	P	MLS004820019	ML369
53308676 / 162021973	KUC109866N-02 / KSC-280-073	A	MLS004820020	NA
56840766 / 162021974	KUC110936N-02 / KSC-280-076	A	MLS004820021	NA
56840772 / 162021975	KUC110920N-02 / KSC-280-078A	A	MLS004820022	NA
56840756 / 162021976	KUC110934N-02 / KSC-280-083A	A	MLS004820023	NA
23724194 / 162021977	KUC105264N / KSC-280-080A	A	MLS004820024	NA

Samples of the probe and five analogues were submitted to the NIH MLSMR compound collection on March 21, 2013.

## Appendix J: Profiling for the Probe ML369 and Supporting Analogues

**Table J1.** In Vitro Pharmacology Profiling

CID SID KU#	Structure	Aqueous Solubility in Pion's buffer pH5.0/6.2/7.4 (µg/mL)	Aqueous Solubility in 1xPBS pH 7.4 (µg/mL) [µM]	PAMPA Permeability, Pe (x10 <sup>-6</sup> cm/s) Donor pH: 5.0/6.2/7.4 Acceptor pH:7.4	Plasma Stability (% Remaining after 3 hrs) Human / Mouse	Plasma Protein Binding (%Bound)		Hepatic Microsome Stability (% Remaining after 1 hr) Human / Mouse	Toxicity towards Fa2N- 4 Immortalized Human Hepatocytes LC <sub>50</sub> (µM)
						Human 1 µM / 10 µM	Mouse 1 µM / 10 µM		
56846691 162021972 KUC110948N-02		>159 / 16.2 / 2	(2.9) [9.1]	116 / 906 / 1376	30.0 / 0.10	99.5 / 99.6	94.1 / 99.2	0.03(0.03)* / 6.26	>50
53308676 162021973 KUC109866N-02		>146 / >146 / >146	(>146) [>496]	7.4 / 87 / 756	ND	ND	ND	ND	>50
56840766 162021974 KUC110936N-02		>159 / 114.6* / 6.6	(5.5) [17]	176 / 1140 / 1516	ND	ND	ND	ND	>50
56840772 162021975 KUC110920N-02		104.6 / 124.6 / 120.6	(101.6) [331.6]	0.61 / 1.3 / 17	ND	ND	ND	ND	>50
56840756 162021976 KUC110934N-02		>92 / >92 / >92	(>92) [>298]	ND	ND	ND	ND	ND	ND
3876375 162021977 KUC105264N		>65 / >65 / 50.8	(50.8) [154]	ND	ND	ND	ND	ND	ND

\*The value in parenthesis corresponds to the reaction without NADPH.

\*\* replicate at >159