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Title Page

Pro-luciferin Acetals as Bioluminogenic Substrates for CYP Activity and Probes for CYP3A Inhibition

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d) ABBREVIATIONS

NCE, new chemical entity; TCEP, tris(2-carboxyethyl)phosphine; LAH, lithium aluminum hydride; NADPH, Nicotinamide adenine dinucleotide phosphate reduced form; KPO_4, potassium phosphate buffered to the stated pH; DMSO, dimethyl sulfoxide; RT, HPLC retention time; PMSF, phenylmethanesulfonylfluoride; LOD, limit of detection; HLM, human liver microsomes, RLU, relative light units.

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ABSTRACT:

Cytochrome P450 assays employ probe substrates to interrogate the influence of NCE's towards CYP enzymes. We report the synthesis and study of a family of bioluminogenic luciferin acetal substrates which are oxidized by CYP enzymes to form luciferase substrates. The luciferin acetals were screened against a panel of purified CYP enzymes. In particular, one pro-luciferin acetal has demonstrated sensitive and selective CYP3A4 catalyzed oxidation to a luciferin ester – K_m and k_{cat} are 2.88 uM and 5.87 pmol metabolite / min / pmol enzyme, respectively. The pro-luciferin acetal was used as a probe substrate to measure IC_{50} values of known inhibitors against recombinant CYP3A4 or human liver microsomes. IC_{50} values for the known inhibitors correlate strongly with IC_{50} values calculated from the traditional HPLC based probe substrate testosterone. Luciferin acetals are rapidly oxidized to unstable hemi-orthoesters by CYP3A resulting in luciferin esters and, therefore, are conducive to simple rapid CYP3A bioluminescent assays.

INTRODUCTION:

Cytochrome P450 enzymes (CYPs) oxidize endogenous compounds, drugs, new chemical entities (NCEs), and other xenobiotics (Guengerich, 2007). CYP enzyme inhibition or *CYP* gene induction by drugs can affect the elimination of concomitantly administered drugs thereby resulting in adverse drug-drug interactions (DDI) (Wienkers and Heath, 2005). Fatalities due to profound DDIs are not uncommon (Lazarou et al., 1998; Kohler et al., 2000), and efforts to identify CYP active NCEs early in the drug discovery process continue (Zientek et al., 2008; Grime et al., 2009).

Commonly, CYP assays employ probe substrates to interrogate the influence of NCE's towards CYP enzymes. Probes based on known drugs are traditionally detected by radiometric assays (Stresser et al., 1996; Moody et al., 1999) and mass spectrometry, yet these assays are disadvantaged by concerns over the safety and disposal of radioactive isotopes or by low throughput, respectively. Chromogenic, fluorogenic (Friden et al., 2006), and bioluminogenic probes (Cali et al., 2006) are rapid and sensitive, yet concerns regarding their analogy to traditional drug probes (Bell et al., 2008) must be addressed by empirical correlation to traditional probes. A complexity in interpreting correlative studies is associated in particular with CYP3A4, which demonstrates substrate-dependent responses to certain analytes (Wang et al., 2000). Even traditional probes such as nifedipine, midazolam, and testosterone interrogate NCEs in a substrate dependent manner.

Bioluminescent luciferase substrates produce photons in a luciferase catalyzed reaction and have been utilized extensively as tools in structural biology and drug development. (Fan and Wood, 2007) Numerous structure/activity papers (White et al., 1965; White and Woerther, 1966; Branchini et al., 1989; Woodroofe et al., 2008) have outlined the structural requirements of bioluminescent luciferase substrates. In contrast to a bioluminescent substrate, a bioluminogenic substrate is a luciferase pro-substrate that does not produce light with luciferase, but can be converted to a light generating bioluminescent substrate by an enzyme or system of interest. The bioluminescent product of the reaction of interest can then be detected as a luminescent signal in a second reaction with firefly

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luciferase. Assays based on bioluminogenic substrates have been reported for proteases (O'Brien et al., 2005), oxidases (Zhou et al., 2006b), phosphatases (Zhou et al., 2008), glutathione S-transferases (Zhou et al., 2006a), and glycosidases (Cali et al., 2008). In the majority of these cases, the bioluminogenic substrates have been modified at the 6'-carbon atom of luciferin with a recognition moiety for the enzyme of interest (see Scheme 1 for luciferin numbering).

The electronic characteristics of substrate molecules play a decisive role in the site of oxidation in CYP catalyzed reactions. A strong correlation has been demonstrated between the oxidation site in a CYP reaction and the most stable radical site in the organic substrates.(Jones et al., 2002) The relative stabilities of the possible radicals formed on an organic molecule relate to the homolytic bond dissociation enthalpy of the carbon-hydrogen bond. Oxygens impart radical stability to neighboring carbons, and CYP catalyzed O-dealkylations result from the oxidation of these carbons. However, the oxygens of an acetal group impart significant radical stability on the carbon atom sandwiched between them. Bond enthalpies for the C-H bond of a carbon between the oxygens in an acetal are some 10 kcal/mol lower than the corresponding C-H bonds of an ether.(Blanksby and Ellison, 2003) Correspondingly, P450 enzymes should oxidize acetals at the carbon between the two oxygens thereby forming an unstable hemi-orthoester, which rapidly decomposes to an ester. This expectation suggested that acetal derivatives of D-Luciferin could serve as bioluminogenic CYP substrates according to the hypothetical scheme shown (scheme 1). The scheme recognizes that luciferin esters do not react with luciferase to produce light, but are rapidly and quantitatively de-esterified to the luminogenic carboxylic acid form in a luciferase formulation supplemented with a carboxyl esterase. (Cali et al., 2006) In such a formulation any luciferin acetal oxidized by a CYP according to the proposed scheme would convert to the luminogenic form and produce light with luciferase.

We report the synthesis and study of a family of bioluminogenic luciferin acetals which are converted to luciferase substrates selectively by the CYP3A enzymes. This study examines the activity of the

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acetals toward a panel of CYP enzymes, and examines a subset of acetals as bioluminescent probes for CYP3A4 inhibition measurements.

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METHODS:

Synthesis. A series of acetals was synthesized to examine the structure/bioluminogenic profile with CYP enzymes. D-cysteine was converted to compound **1** in six steps (Scheme 3). D-cysteine was allowed to react with acetone to form the thiazolidine which was subsequently protected with di-t-butyldicarbonate. The carboxylic acid was converted to the Weinreb amide and subsequently reduced to the aldehyde with LAH to yield **20**. The aldehyde was refluxed in 2-propanol and HCl. The intermediate was allowed to react with the nitrile of **5b** to yield **1**. TCEP was added to prevent any disulfide formation during the reaction. As a control, D,L-cysteine was used in a parallel synthesis to generate the racemized version of **1**. Chiral HPLC was used to show that the reaction starting with D-cysteine results in enantiomerically pure **1** (data not shown).

A series of dimethylacetals based on alternative luciferase substrates were synthesized from the thiazolidine aldehyde **20** (Scheme 4). The dimethyl acetal of thiazolidine aldehyde **22** was condensed with various benzothiazole nitriles (**2b**-**5b**). Under aqueous conditions, compound **22** was proposed to establish equilibrium between the thiazolidine ring and a ring-opened beta-mercaptoamine; the beta-mercaptoamine rapidly reacts with the nitrile **2b**-**5b** to form the thiazoline products (**2**-**5**).

Aryl containing acetals (**8**-**10**), dioxanes and dithianes (**11**-**13**) were constructed in a similar manner (Scheme 5). The acetals containing basic amines (**6**,**7**,**14**) were synthesized from a trans-acetalization of **5** with the appropriate halogenated alcohol followed by substitution with dimethylamine (Scheme 6). Detailed experimental protocols are included in supplemental materials.

Screening of CYPs. Recombinant CYP enzymes in microsomal fractions (Supersomes^{TM}) were purchased from Bectin Dickinson/Discovery Labware. Each CYP enzyme is co-expressed with cytochrome P450 reductase; cytochrome b5 is also co-expressed with some of the CYPs (CYP2A6, -2B6, -2C8, -2C9, -2C19, -2E1, -2J2, -3A4, -3A5, -3A7, -4F2, -4F3A, -4F3B and -4F12). The minus CYP controls are Supersomes^{TM} devoid of CYP enzyme expression. To screen the pro-luciferin acetal compounds as CYP substrates they were first prepared as 50mM stock solutions in DMSO and then

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combined at 50μM with CYP enzymes at 20nM recombinant CYP enzyme and an NADPH generating system (1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$ and 0.4 U/mL glucose-6-phosphate dehydrogenase) in an appropriate buffer as recommended by the CYP enzyme manufacturer (200 mM KPO_4, pH 7.4 for CYP3A4, 100 mM KPO_4, pH 7.4 for CYP1A1, -1A2, -1B1, -2D6, -2E1, -3A5, -3A7, -2J2, -4F12 and -19; 50mM KPO_4, pH 7.4 for CYP2B5, -2C8, -2C19, -4F2, -4F3A and -4F3B; 25mM KPO_4, pH 7.4 for CYP2C9, 100mM Tris-HCl, pH 7.5 for CYP2A6, -2C18 and -4A11). The 50mM luciferin acetal stock solutions contributed 0.1% DMSO to each reaction mixture. Reactions were performed in a volume of 50μl in opaque white 96-well plates that were incubated in a 37 ^oC H_2O bath for 30 minutes. Reactions were initiated by addition of the NADPH generating system. Reactions were stopped and luminescence initiated by adding 50μL of P450-Glo™ Luciferin Detection Reagent (Promega Corporation) supplemented with 20 units/mL of porcine esterase (Sigma). After 20 minutes at room temperature, luminescence was read as relative light units (RLU) on a plate reading luminometer (Polarstar Optima by BMG Labtech or Veritas™ by Turner Biosystems).

HPLC analysis of CYP3A4 catalyzed oxidation of 1. Fluorescence chromatograms (Supplemental Figure 1) were used for the analysis of compound **1** with a control Sf9 membrane preparation, with CYP3A4 membranes, and with CYP3A4 membrane preparation with esterase treatment. Luciferin at 2.2 min was identified with an authentic standard. A peak at 7.5 min disappeared on treatment with esterase and was, therefore, assigned as the putative luciferin ester intermediate. Separation was obtained on an Alltech Adsorbosphere HS C18 column (5u, 150 x 4.6mm) with a 50mM ammonium acetate buffer (pH6) and linearly modified acetonitrile gradient. Component elution was monitored with fluorescence by means of 330nm excitation and 520nm emission.

Measuring CYP Inhibition. Several known CYP3A4 inhibitors were applied in a dose response mode to reactions of recombinant CYP3A4 or human liver microsomes (HLM) with compound **1** or with the CYP3A4 substrate testosterone. The HLMs were a mixed gender pool from 9 male and six female donors (Bectin Dickinson/Discovery Labware). Reaction conditions for interrogation of inhibitors

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were established as described in the results section and GraphPad Prism was used to calculate IC_{50} values. Inhibition reactions were performed in 100mM KPO_4 buffer, pH 7.4 with 20nM recombinant CYP3A4 and 10µg/ml HLM using 50µl and 100µl volumes, respectively, for the compound **1** and testosterone assays. Testosterone reactions were performed as previously described (Kenworthy et al., 1999). Compound **1** concentrations of 3µM for recombinant CYP or 8µM for HLM were used. Testosterone concentrations of 50µM for recombinant CYP or 10µM for HLM were used. Substrates and inhibitors were allowed to incubate for 10 minutes at 37^{o}C with CYP3A4 or HLM before initiating reactions by adding the NADPH generating system. The reactions with compound **1** were stopped and luciferin product detected by luminescence in the same manner as described for CYP screening. The reactions with testosterone were stopped by addition of 50µl of acetonitrile and then centrifuged at 10,000 x g for 3 minutes. The supernatant (50 uL) was injected into a 3 x 100 mm 3u C18 HPLC column and separated with a mobile phase initially of 15% acetonitrile increasing to 100% acetonitrile over 12 min., at a flow rate of 0.75 ml/min. The CYP3A4 reaction product 6β-hydroxytestosterone was detected by its absorbance at 240 nm (RT= 5min) and quantitated by comparison to absorbance of authentic 6β-hydroxyltestosterone standards.

RESULTS

Screening cytochrome P450s for activity with pro-luciferin acetals. A series of pro-luciferin acetals (**Scheme 2**) were screened for bioluminogenic activity against a panel of recombinant human CYP enzymes in microsomal fractions (**Figure 1**). A moderate to high substrate concentration of 50 μM was used with 20nM CYP enzymes and a liberal reaction time of 30 minutes at 37^{o}C. A control microsome fraction with no recombinant CYP expression was used to monitor intrinsic instability or non-CYP catalyzed conversion of the pro-luciferin acetals to luciferin. The bioluminogenic reactions are performed by incubating the CYP enzymes with pro-luciferin CYP substrates and NADPH in a first reaction where an active enzyme/substrate combination generates luciferin ester. In a second reaction, carboxyl esterase and luciferase convert any luciferin ester to a proportional number of photons. CYP activity is detected on a luminometer as relative light units (RLU). Other than **13**, compounds **1** – **18** showed only a minor luminescence signal in the control, indicating that non-CYP enzymes and/or un-catalyzed conversions of the pro-luciferin do not contribute to the signals. The diisopropyl acetal **1** displayed the largest CYP3A4 signal. CYP3A5 activity was highest for **16**, **17**, and **18**. CYP3A7 activity was highest for **8**. Compounds **2**, **7**, and **14**, gave no light in excess of the minus CYP control. The 1,3 dioxane **11** showed some CYP3A activity along with detectable CYP1A and 1B activity. The dithiane **13** displayed CYP3A5 and CYP3A7 activity only with substantial light in the control indicating some level of luciferin contamination in the sample. Fourteen of eighteen pro-luciferin acetals were biolumingenic primarily against CYP3A. Detailed CYP3A net luminescent signals were extracted from Figure 1 and displayed with error bars in 2 dimensions for better visualization (**Figure 2)**.

*Characterization of CYP activity with compound **1***. The diisopropyl acetal **1** was studied in further detail because it displayed the largest signal with CYP3A4, an enzyme of particular interest because of its prominent role in drug metabolism. Preferential activity with CYP3A4, -3A5 and -3A7 suggested this compound could be used as a selective CYP3A probe. The recombinant CYP3A4 reaction with **1**

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was performed using fluorescence HPLC to detect the pro-luciferin acetal and its CYP3A4 reaction products with and without addition of carboxyl esterase. Incubation of **1** with CYP3A4 gave rise to two apparent reaction product peaks (Supplemental info). The smaller of the two peaks co-migrated with an authentic D-luciferin standard. Esterase treatment shifted the second intermediate peak to the position of D-luciferin. This analysis is consistent with the model of Scheme 1 where the CYP enzyme initiates a reaction sequence that yields a luciferin ester that is processed to luciferin by added esterase. The appearance of a small D-luciferin peak in the absence of added esterase is likely due to esterase activity already present in the CYP3A4 microsome preparation and this was confirmed by observing elimination of the peak by pretreatment of the microsomes with the carboxylesterase inhibitor PMSF (data not shown). The absence of luciferin and luciferin ester peaks after incubation with control membranes lacking CYP enzyme confirms that conversion of **1** to these products is CYP-dependent. Non-catalytic or non-CYP enzymatic conversions of **1** do not occur to a significant extent in the Supersome system.

*Kinetic characterization of CYP3A4 catalyzed oxidation of compound **1***. An initial test that varied the concentration of the common CYP reaction buffer KPO_4 (pH 7.4) showed the CYP3A4 reaction with compound **1** was optimal at 100mM KPO_4 (data not shown) and this concentration was used for subsequent characterizations. The reaction was characterized by varying CYP3A4 enzyme concentration, the initial concentration of **1**, and incubation time. Plots of time-dependent (Figure 3A-C) and enzyme concentration-dependent (Figure 3E-H) signal increases showed initial linear phases with decreases in linearity at longer time points, increased enzyme concentration and decreased initial concentrations of **1**, indicating that **1** might become limiting in the non-linear phases. The dose-dependent activity curves of compound **1** were fit to a hyperbola for each of the various reaction times and enzyme concentrations (Figure 3I-K). The average of the K_m values derived from the curve fits for 12 sets of conditions was 2.78 ± 0.80μM. The substrate concentration curve was repeated under linear conditions of reaction time and enzyme concentration (10 minutes, 2nM CYP3A4) (Figure 3L). In

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this case CYP3A4 reaction rates were calculated after converting RLU values to luciferin product concentrations by interpolation from a luciferin standard curve (data not shown). Under these conditions the curve fit found K_m and k_{cat} values of 2.88 μM ± 0.18 and 5.87 ± 0.14 pmol metabolite / min / pmol enzyme, respectively, for CYP3A4 (Table 1). A contribution from substrate depletion to the loss of linearity observed at lower substrate concentration, higher enzyme concentration and greater incubation times is consistent with the reaction rates calculated for Figure 3L. Conversely, steady state reaction conditions can be assumed in 10 minute reactions with 2nM CYP3A4 at K_m substrate concentration where we estimate < 2% of the substrate is consumed, and these conditions are reasonable for interrogation against CYP3A4 inhibitors. Figure 3F includes a CYP3A4 enzyme titration curve performed under nearly identical conditions: 10 minutes with 2.5μM compound **1**. From a linear regression analysis, the limit of detection (LOD) for the CYP3A4 enzyme was estimated at 0.04nM. LOD is defined as the concentration in which the luminescent value falls within three standard deviations of the background.

These steady state CYP3A4 conditions were also used in a repeat screen of compound **1** against the recombinant human CYP enzyme panel where a substantial increase in selectivity for CYP3A4 over CYP3A5 and -3A7 was observed compared to the conditions of the initial screen with 20nM enzyme, 50μM substrate and 30 minute incubations (Figure 1). This preference of compound **1** for CYP3A4 over CYP3A5 and -3A7 is explained in terms of the K_m, k_{cat} and enzymatic efficiencies (k_{cat}/K_m) of the three enzymes (Figure 3D, 3L and Table 1). Ratios of the enzymatic efficiencies, CYP3A4/CYP3A5 or CYP3A4/CYP3A7, indicate a 9-fold and 102-fold selective preference for CYP3A4 over the other two enzymes.

Inhibition assays. Compound **1 (**Luciferin-IPA) was used as a probe substrate to measure IC_{50} values of known inhibitors against recombinant CYP3A4 or HLMs. The linear reaction conditions for recombinant CYP3A4 at substrate K_m were used (3μM compound **1**, 2nM CYP3A4, 10 minute reactions). Recombinant CYP3A4 inhibition was also measured against the commonly used

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testosterone 6-β-hydroxylation reaction (Kenworthy et al., 1999; Wang et al., 2000) and IC_{50} values from this study were compared to those determined with compound **1**. Conditions for the testosterone assays were first established so that reactions were performed at substrate K_m (50 μM) under conditions of linearity with respect to time and CYP3A4 concentration (10 minutes, 20nM). The IC_{50} values for known CYP3A4 inhibitors correlated closely between the compound **1** assay and testosterone assays (Figure 4A). A similar comparison was performed using HLMs in place of recombinant CYP3A4 enzyme. Reaction conditions for interrogation of inhibitors were first established and found to be somewhat different from those observed with recombinant CYP3A4. For compound **1** an apparent K_m concentration of 8μM was observed and used in 10 minute reactions with 20μg/ml human liver microsomes. It was also noted that significant luminescent signals were not observed in HLMs when NADPH was withheld from the reactions (data not shown). This was consistent with a CYP-dependent mechanism and the absence of non-catalytic or non-CYP enzyme dependent substrate conversion. Using testosterone as a substrate an apparent K_m of 50μM was observed and used in 10 minute reactions with microsomes at 20μg/ml. As with recombinant CYP3A4, a close correlation of IC_{50}s between the testosterone and compound **1** assay was observed for the eight inhibitors tested (Figure 4B).

DISCUSSION

The CYP catalyzed oxidation of pro-luciferin acetals results in bioluminescent luciferase substrates. Scheme 1 describes the proposed mechanism by which light is produced from CYP activity. The oxidation of the acetal yields an unstable hemi-orthoester which is proposed to decompose to the luciferin ester. The resulting luciferin ester is converted to D-luciferin by esterase and its concentration is measured using the bioluminescent output from a luciferase reaction. Light output is proportional to CYP activity.

The acetals were designed to explore the structural effects of hydrophobicity (**1, 5, 9, 11, 12, 15**) and charge (**6**, **7**, **14**) on the CYP activity. Mixed acetals (**10**, **16 – 18**) and a dithiane (**13**) were also produced. Furthermore, alkyl aminoluciferin luciferase substrates (Woodroofe et al., 2008) have been reported, and a series of related acetals (**2**, **3**, **4**) were generated. The screen of CYP enzymes was designed to push the system to detect any reasonable activity.

CYP3A converts **1** to a compound that is easily measured in a bioluminescent reaction. A direct relationship between the CYP activity and light output exists because the concentration of luciferin produced by CYP is within the linear range of a standard curve of luciferin bioluminescence. A high degree of selectivity by this compound **1** for the CYP3A subfamily was expected based on screening a panel of recombinant CYP enzymes (Figure 1) and greater selectivity for CYP3A4 could be dialed in with reaction conditions that favor CYP3A4 over CYP3A5 and CYP3A7 (Figure 2 & Table 1). The selectivity of compound **1** was confirmed by inhibition studies in HLMs that contain a mixture of potentially cross-reacting CYP enzymes. Known CYP3A4 inhibitors inhibited the HLM activity at IC_{50} values consistent with selective CYP3A4 inhibition as indicated by close correlation to the IC_{50} values determined by the well established CYP3A4 selective testosterone 6β-hydroxylation reaction (Figure 4A).

The 0.04 nM LOD for CYP3A4 activity with substrate **1** is very low. This allows for an assay of exquisite sensitivity. By reducing the amount of CYP containing microsomes in a reaction the extent

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of non-specific substrate or analyte binding is kept to a minimum or even non-detectable level. A very sensitive assay also enables detection of activity in samples with low CYP expression. The limit of CYP3A4 detection is potentially influenced by three scenarios: contamination of **1** with D-luciferin, non-CYP catalyzed conversion of **1** to D-luciferin or uncatalyzed conversion of **1** to D-luciferin. Due to the drastic difference in functional groups and intrinsic stability of the acetal functionality, contaminating luciferin was typically reduced to less than 0.0001% of pro-luciferin acetals using either silica or RP chromatography. Non-CYP catalyzed and uncatalyzed conversions apparently do not contribute significantly to background signals as there was no evidence of conversion in the minus CYP control of the recombinant CYP system (Figures 1 and S1), and conversion in HLMs was NADPH-dependent, a hallmark of CYP-dependent catalysis.

IC_{50} comparisons for known CYP3A4 inhibitors between the compound **1** assay and the testosterone 6β-hydroxylation assay showed nearly identical rank ordering for both recombinant CYP3A4 and HLM reactions, indicating that **1** can be used as a surrogate for the testosterone CYP3A4 assay (Table 2). The HLM correlation is also consistent with the high degree of selectivity of compound **1** for CYP3A4 enzymes. The IC_{50} values were closely correlated for most of the inhibitors showing fold differences between unity and about 4-fold. A few compounds showed larger fold differences, which is not unexpected given the substrate-dependent nature of CYP3A4 inhibition (Wang et al., 2000). Differences between HLM and recombinant CYP3A4 inhibition are known from the literature though not explained within the scope of this study (Nomeir et al., 2001).

In addition to cell free enzyme inhibition studies, the utility of Compound **1** for non-lytic cell based assays of CYP3A induction in cultured hepatocytes has also been described (Li, 2009; Doshi and Li, 2011). The CYP3A Compound **1** was also applied to hepatocytes in a three part multiplex format that provided luminescent CYP3A, a fluorescent CYP1A and a luminescent viability measurement from a single culture well (Larson et al., 2011).

The simple nature of the luminogenic pro-luciferin acetal assays likely provides an advantage to CYP activity screens of large chemical libraries and other applications that benefit from a high throughput approach. CYP assays based on HPLC or LCMS analysis are widely accepted but often too slow for high throughput applications and limiting when rapid turnaround times are needed. Furthermore, the ease of implementing a bioluminescent CYP assay facilitates expansion from assays of a single concentration of test compound to more informative full dose response screens. Data from such quantitative high throughput CYP screens reveals structure/activity relationships and inhibition trends in large compound sets, and enables the annotation of compound libraries with CYP data.(MacArthur et al., 2009; Veith et al., 2009) The strong correlation between inhibitory data from the CYP3A4 luciferin acetal and the testosterone assays supports the expectation that the bioluminescent assays will provide relevant data while meeting throughput requirements in large CYP screens.

Authorship Contribution

Participated in research design: Meisenheimer, Cali, Ma, Sobol, Klaubert

Conducted experiments: Meisenheimer, Cali, Ma, Sobol, McDougall, Uyeda, Corona, Simpson

Contributed new reagents or analytic tools: Meisenheimer, Simpson

Performed data analysis: Cali, Meisenheimer, Ma, Sobol, Simpson

Wrote or contributed to the writing of the manuscript: Meisenheimer, Cali, Klaubert, Corona, Uyeda, McDougall

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Footnotes

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LEGENDS FOR SCHEMES

Scheme 1. Proposed mechanism for bioluminogenic CYP activity of pro-luciferin acetals

Scheme 2. Series of pro-luciferin acetals tested against CYP activity

Scheme 3. Synthesis of D-luciferin isopropyl acetal (compound 1) from D-cysteine.

Scheme 4. Dimethyl acetal derivatives of N-alkyl aminoluciferins were synthesized by converting **20** (Scheme 3) to the isolated intermediate **22** followed by the condensation with previously reported benzothiazole nitrile **2b** – **5b**.

Scheme 5. Aryl containing acetal derivatives, dioxanes, and dithianes were synthesized from intermediate **20**.

Scheme 6. Mixed acetals and amine containing acetals were synthesized from **5**.

LEGENDS FOR FIGURES.

Figure 1. Recombinant CYP enzyme screen against pro-luciferin acetals. **1**-**18** are the respective acetals illustrated in Scheme 2 exposed at 50μM to 20nM recombinant CYP enzymes for 30 minutes at 37°C. **1a** is compound **1** exposed at 3μM to 2nM recombinant CYP enzymes for 10 minutes at 37°C.

Figure 2. Recombinant CYP3A enzyme screen against pro-luciferin acetals. Net luminescence values are signals from the CYP reactions shown in Figure 1, minus the respective CYP-independent controls (n=3, ±SD).

Figure 3. Characterization of the CYP3A4 reaction with compound **1**. 50 μl reactions in duplicate or triplicate were performed at 37^0C. Recombinant CYP3A4 activity was scored as relative light units (RLU) after adding a luciferase reaction mixture for detection of the compound **1**/CYP3A4 reaction product. For panel D recombinant CYP3A5 or -3A7 were substituted for CYP3A4. For panels D and L rates of D-luciferin production were calculated after conversion of RLU to D-luciferin concentration by comparison to a D-luciferin standard generated with the luciferase reaction mixture.

Figure 4. Correlation of testosterone and compound **1** as probe substrates for CYP inhibition assays. IC_{50} comparisons A: recombinant CYP3A4 assays (r^2=0.96, P<0.0001), B: human liver microsome assays (r^2=0.91, P=0.0003).

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Table 1. Kinetic data for CYP3A catalyzed oxidation of pro-luciferin acetal **1** based on analysis of figures 2D and 2L.

reaction	K_m (μM)	k_{cat}	k_{cat}/K_m
CYP3A4	2.88 ± 0.18	5.87 ± 0.14	2.04
CYP3A5	4.21 ± 0.31	0.94 ± 0.02	0.22
CYP3A7	8.85 ± 0.53	0.17 ± 0.004	0.02

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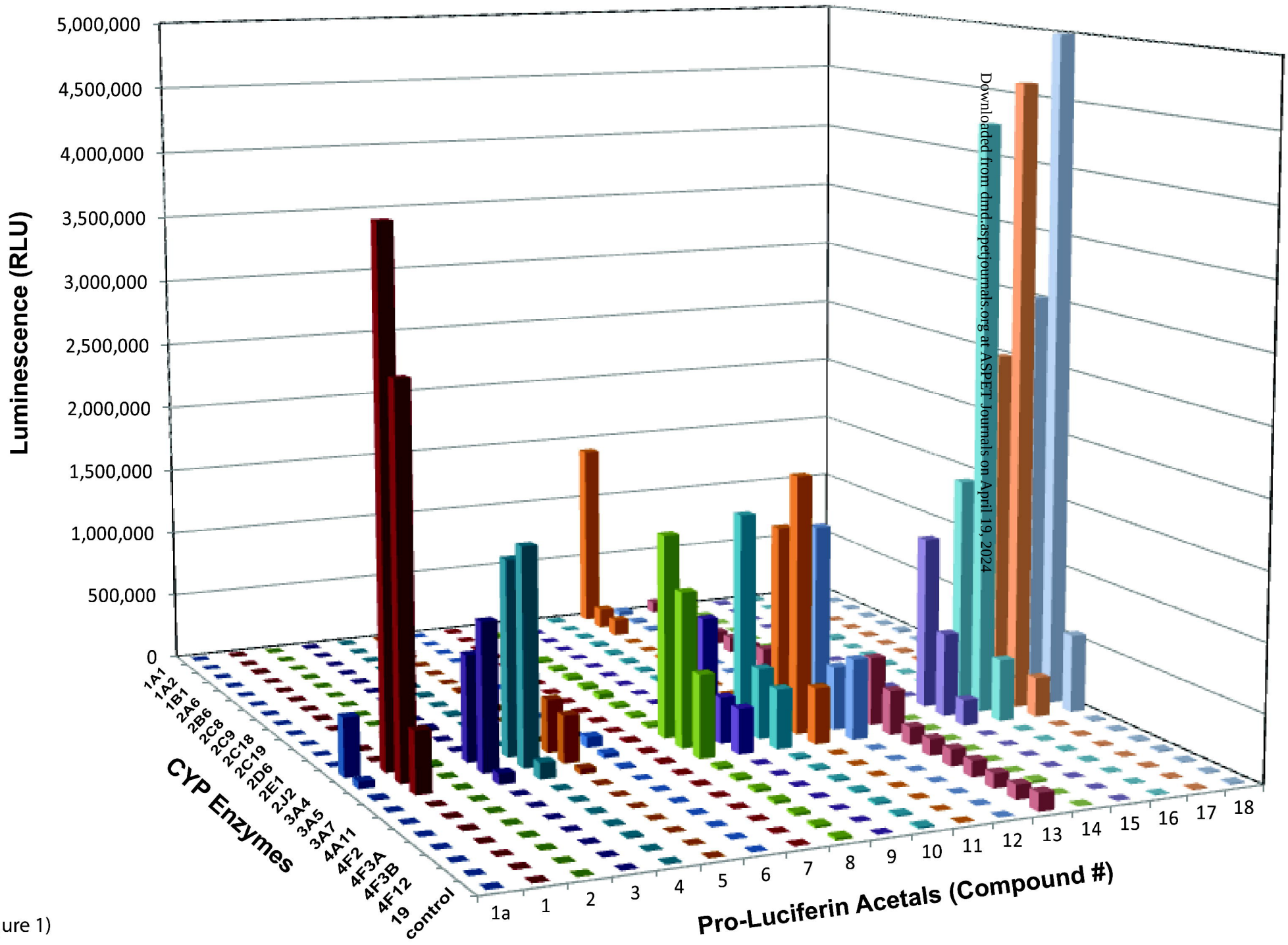
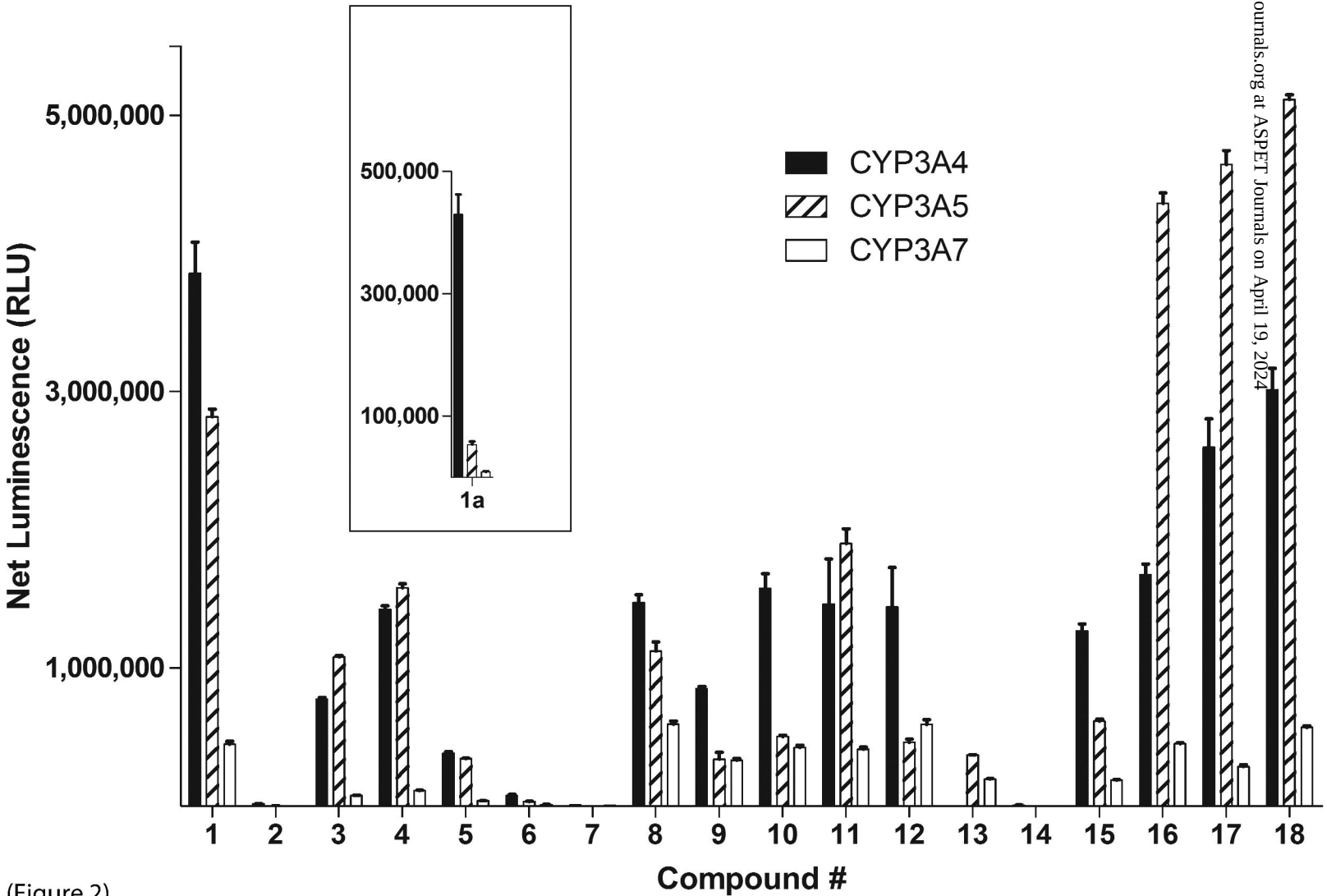
Table 2. IC_{50} comparisons. Data compares inhibition with the compound **1** assay and testosterone 6β hydroxylase assay using recombinant CYP3A4 or human liver microsomes.

	rCYP3A4 *IC_{50} (uM)*			HLM[d] *IC_{50} (uM)*		
CYP3A4 Inhibitors	**1**[a]	TS[b]	Δ[c]	**1**[a]	TS[b]	Δ[c]
Clotrimazole	0.006	0.05	8	0.003	0.052	16.7
Ketoconazole	0.04	0.10	2.5	0.074	0.10	1.3
Troleandomycin	0.52	1.13	2.2	1.96	1.11	1.8
Midazolam	2.06	6.80	3.3	16.82	3.64	4.6
Nifedipine	3.24	13.55	4.2	5.99	6.90	1.2
Verapamil	3.45	12.95	3.8	115	14.86	7.7
Erythromycin	7.33	12.27	1.7	NC[e]	NC[e]	---
Omeprazole	11.21	10.00	1.1	68.27	13.52	5.0
α-naphthoflavone	10.00	36.64	3.7	NC[f]	NC[f]	---
Fluvoxamine	ND[g]	ND[g]	---	46.94	48.31	1.0
Disopyramide	53.29	65.88	1.2	71.09	48.80	1.5

[a] IC_{50}s determined using bioluminescent assay with compound **1**. [b] IC_{50} determined using testosterone 6□ hydroxylase assay [c] Δ (delta) is the fold difference in IC_{50} values between the two assays [d] Human Liver Microsomes [e] IC_{50} was not calculated due to only modest inhibition of both assays [f] IC_{50} was not calculated due to a modest stimulation. [g] Fluvoxamine was not applied to recombinant CYP3A4



(Figure 1)



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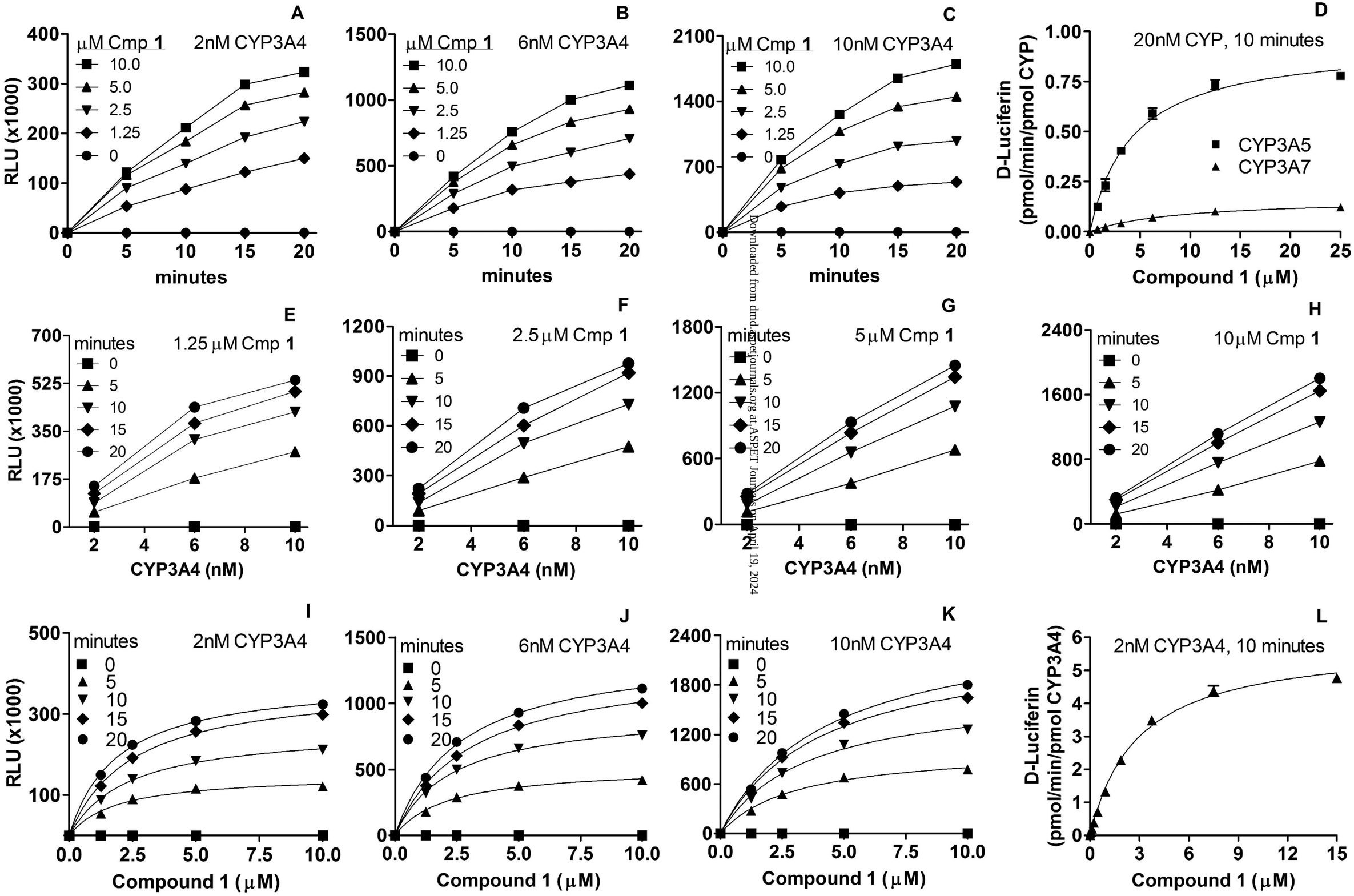

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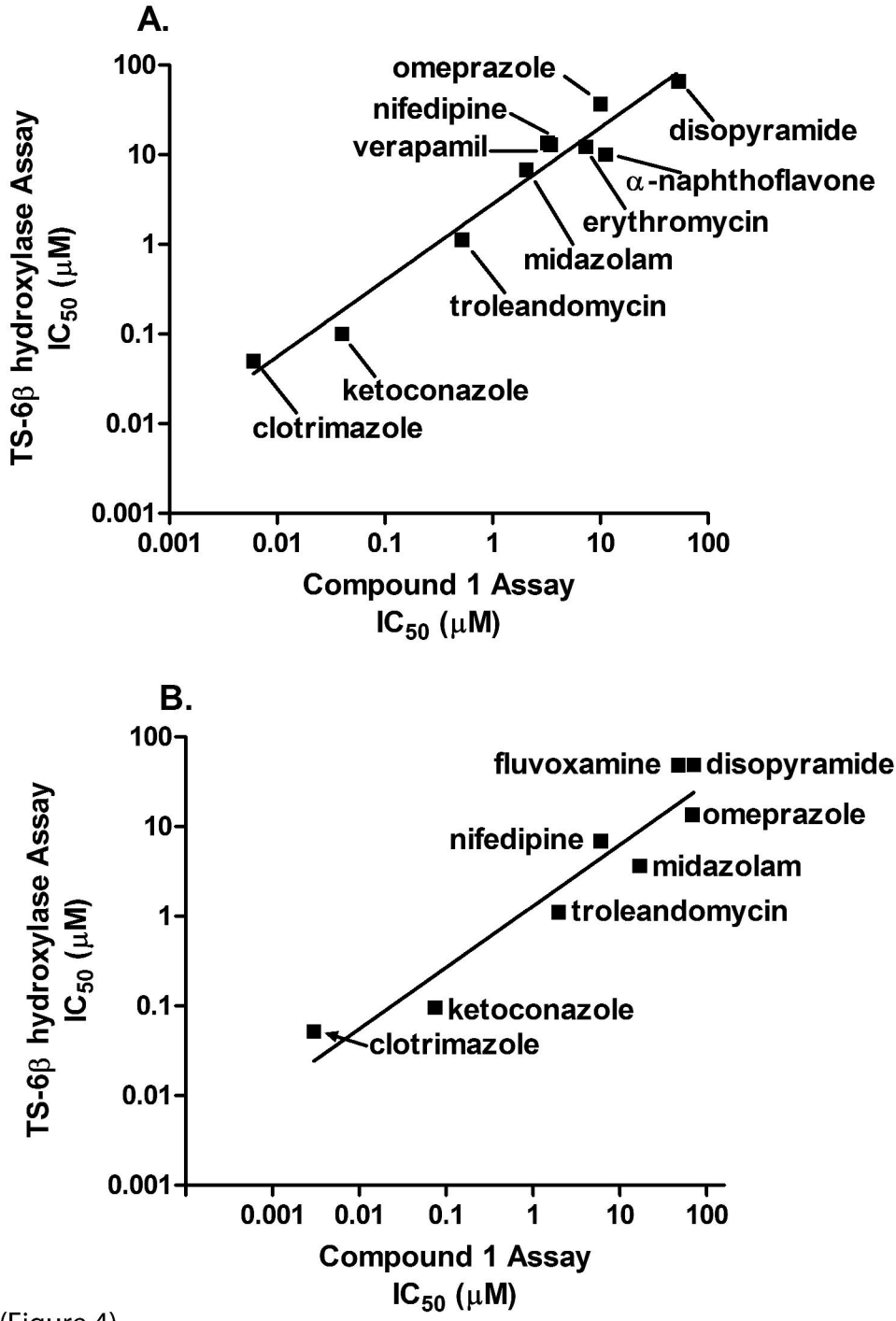
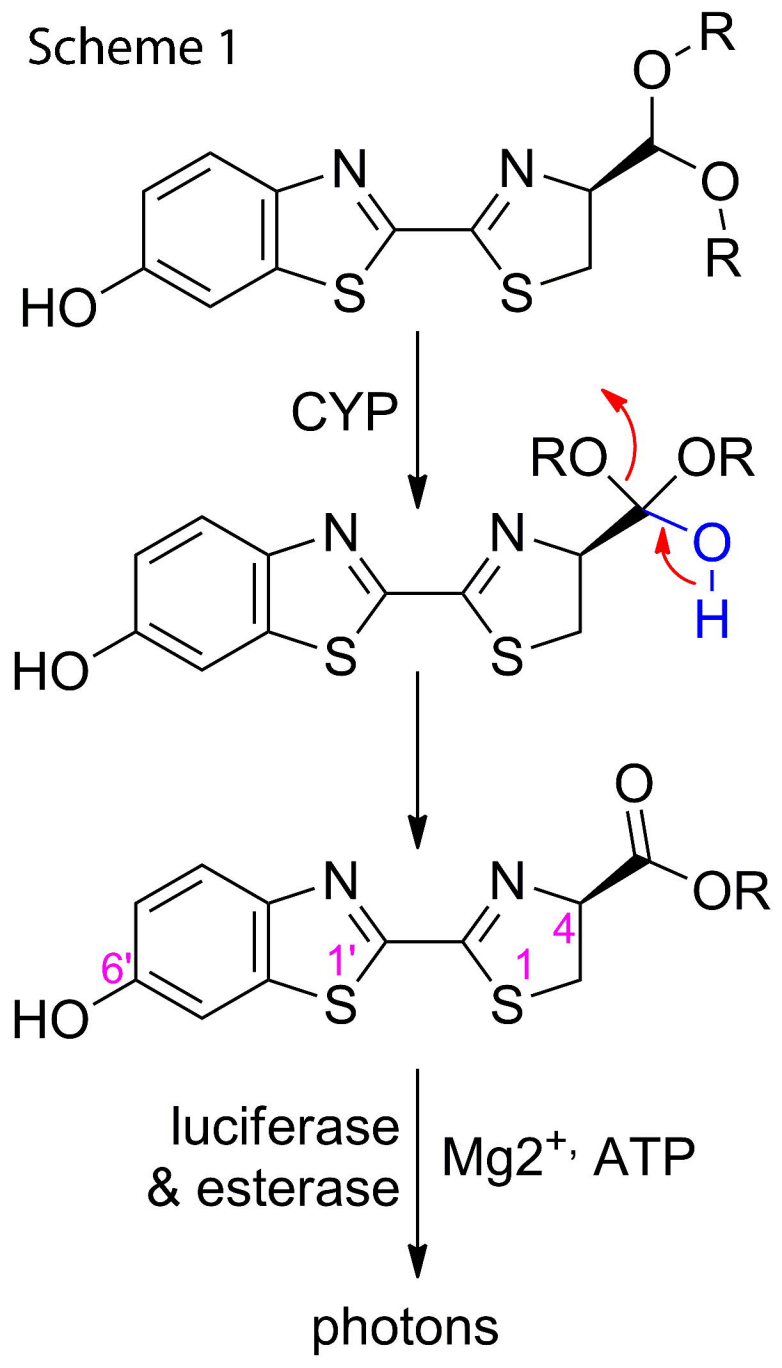
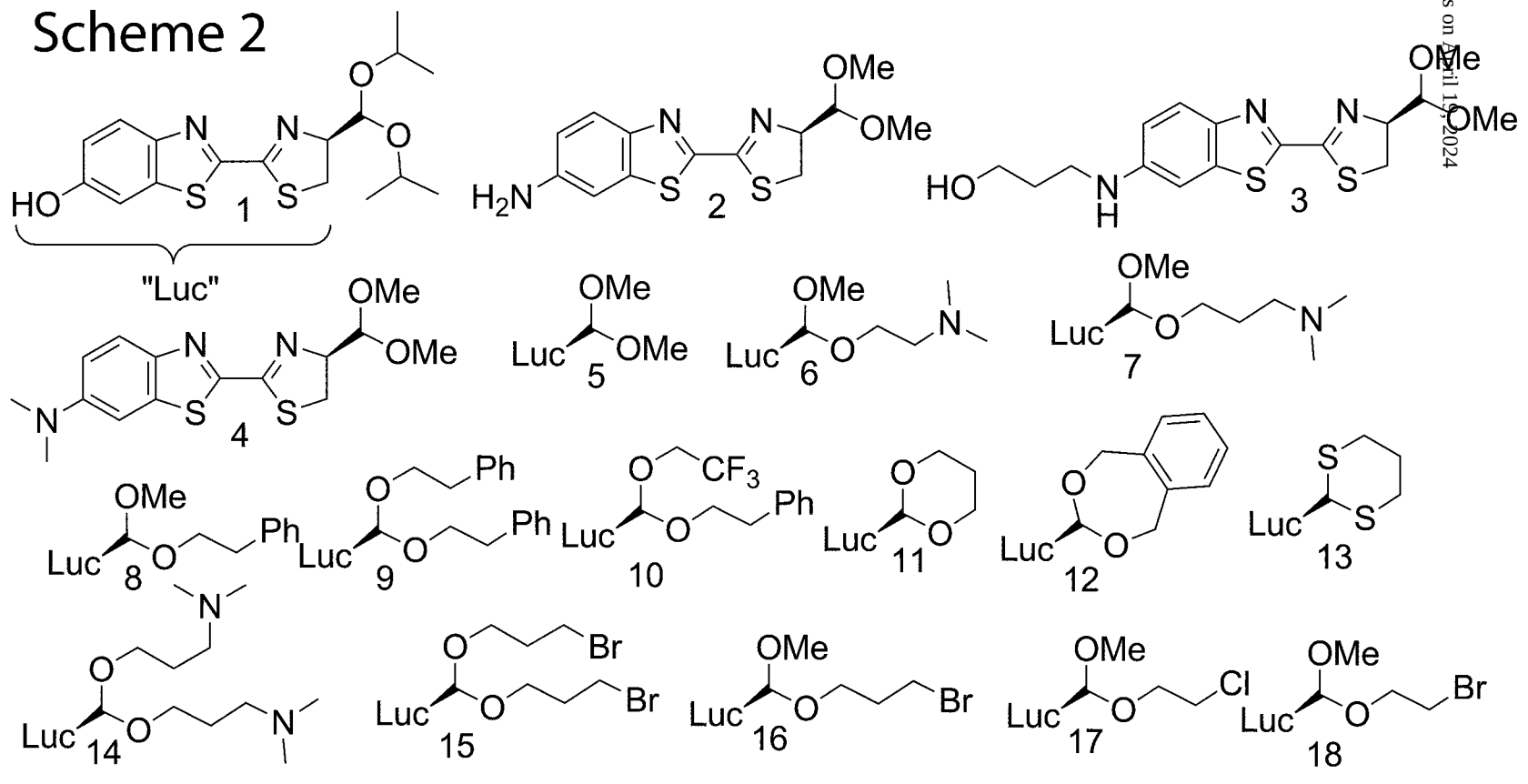

A.
omeprazole
nifedipine
verapamil
disopyramide
α-naphthoflavone
erythromycin
midazolam
troleandomycin
ketoconazole
clotrimazole
TS-6β hydroxylase Assay
IC50 (μM)
Compound 1 Assay
IC50 (μM)
100
10
1
0.1
0.01
0.001
0.001
0.01
0.1
1
10
100
B.
fluvoxamine
disopyramide
omeprazole
nifedipine
midazolam
troleandomycin
ketoconazole
clotrimazole
TS-6β hydroxylase Assay
IC50 (μM)
Compound 1 Assay
IC50 (μM)

(Figure 4)

Scheme 1


R
O
N
N
O
R
HO
S
S
CYP
RO
OR
O
H
6'
1'
1
4
OR
luciferase
& esterase
Mg2+, ATP
photons

Scheme 2

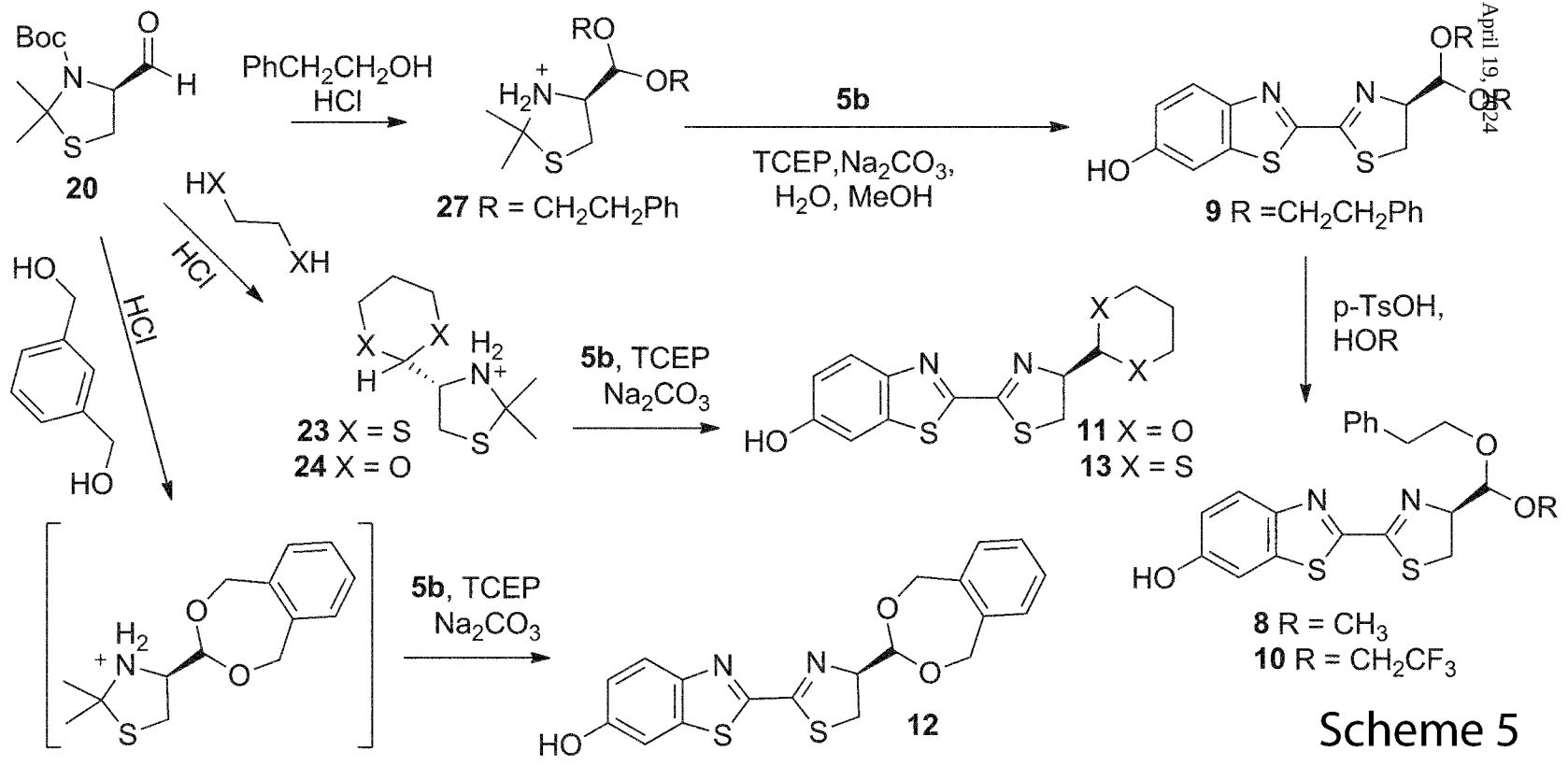


Scheme 3

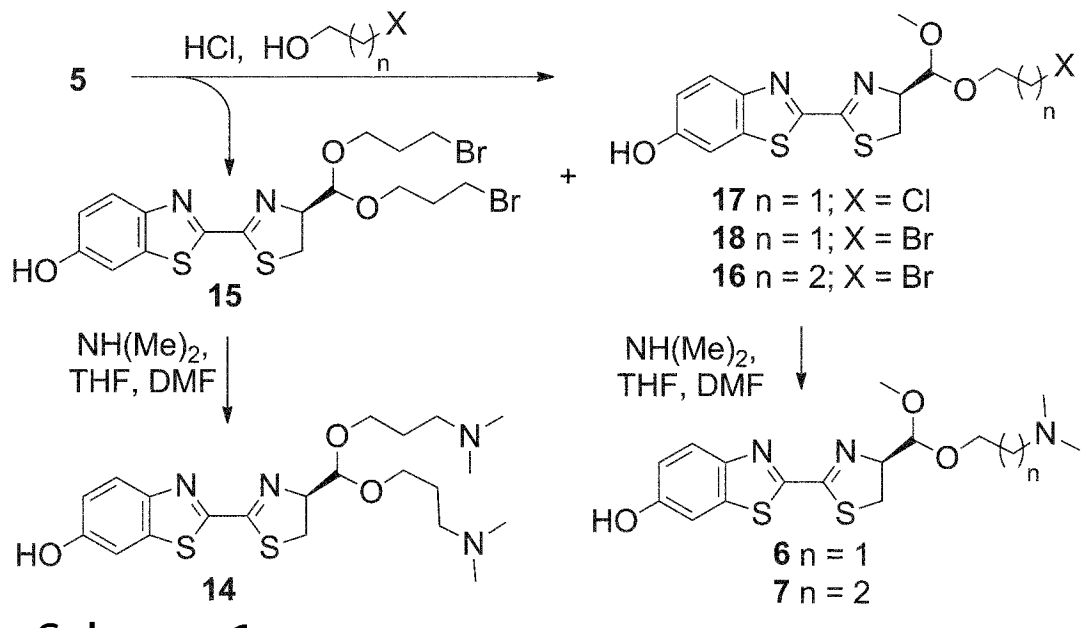
H2N
O
OH
HS
4 steps
Boc
N
O
H
S
20
2-propanol
HCl
5b,
TCEP, Na2CO3
H2O, MeOH
HO
N
S
N
S
O
O
1

MeOH
HCl
20
MeO
OMe
H2N
S
22
N
S
R
≡N
TCEP, Na2CO3,
H2O, MeOH
2b R = NH2
3b R = NH(CH2)3OH
4b R = NMe2
5b R = OH
O
O
N
S
N
S
R
2 R = NH2
3 R = NH(CH2)3OH
4 R = NMe2
5 R = OH

Scheme 4


Boc
N
S
O
H
20
PhCH2CH2OH
HCl
RO
OR
H2N
+
S
27 R = CH2CH2Ph
5b
TCEP,Na2CO3,
H2O, MeOH
OR
N
N
S
S
HO
9 R =CH2CH2Ph
April 19, 2024
HX
XH
HCl
HO
HO
HCl
X
X
H
H2
N+
S
23 X = S
24 X = O
5b, TCEP
Na2CO3
X
X
N
N
S
S
HO
11 X = O
13 X = S
p-TsOH,
HOR
Ph
O
OR
N
N
S
S
HO
8 R = CH3
10 R = CH2CF3
H2
N+
O
O
S
5b, TCEP
Na2CO3
O
O
N
N
S
S
HO
12

Scheme 5


5
HCl, HO
X
n
Br
Br
HO
15
+
17 n = 1; X = Cl
18 n = 1; X = Br
16 n = 2; X = Br
NH(Me)2,
THF, DMF
NH(Me)2,
THF, DMF
14
6 n = 1
7 n = 2

Scheme 6