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# A highly sensitive fluorogenic probe for cytochrome P450 activity in live cells

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ABSTRACT

A derivative of rhodamine 110 has been designed and assessed as a probe for cytochrome P450 activity. This probe is the first to utilize a 'trimethyl lock' that is triggered by cleavage of an ether bond. In vitro, fluorescence was manifested by the CYP1A1 isozyme with $k_{cat}/K_M = 8.8 \times 10^3\ M^{-1}\ s^{-1}$ and $K_M = 0.09\ \mu M$. In cellulo, the probe revealed the induction of cytochrome P450 activity by the carcinogen 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and its repression by the chemoprotectant resveratrol.



The cytochrome P450 (P450) family of enzymes is responsible for the oxidative metabolism of a wide variety of compounds, including chemotherapeutic agents and environmental toxins.[1,2] The catalytic activity of P450 enzymes controls the rate of xenobiotic metabolism, and can produce undesirable byproducts.[3] Originally, this activity had been assessed by using HPLC or other methods to separate and quantify metabolites. In the 1970s, 7-ethoxycoumarin and 7-ethoxyresorufin were introduced as the first fluorogenic substrates for assays of P450 activity.[4] Although these and other fluorogenic substrates have been used to assay P450 activity in vitro and enable assays in cellulo,[5,6] they suffer from background fluorescence.[7] For example, alkoxycoumarins exhibit moderate fluorescence and are used frequently as fluorophores in peptidase substrates based on Förster resonance energy transfer (FRET).[8] In addition, both 7-ethoxyresorufin and resorufin fluoresce brightly.[6] This problem arises because O-alkylation of the hydroxyl group of fluorophores such as coumarin and resorufin does little to deter the oxygen electrons from participating in the resonance that gives rise to fluorescence.

Here, we report on a superior small-molecule probe for assessing P450 activity. Our probe employs the trimethyl lock.[9–11] The trimethyl lock is an *o*-hydroxycinnamic acid derivative in which severe crowding of three methyl groups induces rapid lactonization to form a hydrocoumarin.[12] In this strategy, the phenolic oxygen of the *o*-hydroxycinnamic acid is modified to create a functional group that is a substrate for a designated enzyme, and the carboxyl group is condensed with the amino group of a dye. Unmasking of the phenolic oxygen leads to rapid lactonization with concomitant release of the dye. An important attribute of this strategy is that the fluorescence/absorbance of the dye is masked completely by amidic resonance and the resulting lactonization within the rhodamine moiety.[9,10]

The human genome contains 27 genes encoding P450 isozymes, along with many pseudogenes.[13] Of these isozymes, cytochrome P450 1A1 (CYP1A1) is known to be especially important in the metabolism of xenobiotics.[14] Unlike most P450 isoforms, which are found primarily in the liver, CYP1A1 is present mainly in the lungs, where it plays an important role in the metabolic activation of chemical carcinogens.[1,15] The lung is a primary site of exposure for inhaled toxins along with carcinogens that can ultimately yield lung carcinomas.[16] CYP1A1 is strongly induced by cigarette smoking.[15] Many compounds, including some found in cigarette smoke, are not hazardous until metabolized by CYP1A1.[16,17] Accordingly, CYP1A1 levels could be correlated with human disease.

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We suspected that the trimethyl lock could provide the basis for a useful probe for CYP1A1 activity. As a dye, we chose a morpholino–urea derivative of rhodamine 110 ($Rh_{110}$) that is bright ($\varepsilon \times \Phi = 2.38 \times 10^4\ M^{-1}\ cm^{-1}$) but has no measurable fluorescence after N-acylation.[10] We installed an ethyl group on the phenolic oxygen of the trimethyl lock because ethyl ethers are especially effective substrates for CYP1A1.[15] The synthetic route to fluorogenic probe **1** is shown in Figure 1. Briefly, known intermediate **2**[18] was alkylated with diethyl sulfate to give ethyl ether **3**. Removal of the silyl group followed by Jones oxidation afforded carboxylic acid **5**. Condensation with urea–rhodamine **6** gave fluorogenic probe **1** in 5% overall yield.

Fluorogenic probe **1** was first assayed as a substrate for human CYP1A1 in vitro. Fluorogenesis was rapid, with $k_{cat}/K_M = 8.8 \times 10^3\ M^{-1}\ s^{-1}$ and $K_M = 0.09\ \mu M$ (Fig. 2A). These values are comparable to the highest values obtained with other fluorogenic substrates.[1,2] These data are the first to demonstrate that the trimethyl lock can be activated by the cleavage of an ether bond.

Next, fluorogenic probe **1** was assayed as a substrate for CYP1A1 in live human cells. These experiments employed human lung adenocarcinoma cell line A549, which is especially well suited for studying the expression of the pulmonary CYP system.[16,19] A low but observable level of CYP1A1 was apparent after a 1-h incubation with fluorogenic probe **1** (Fig. 2B).

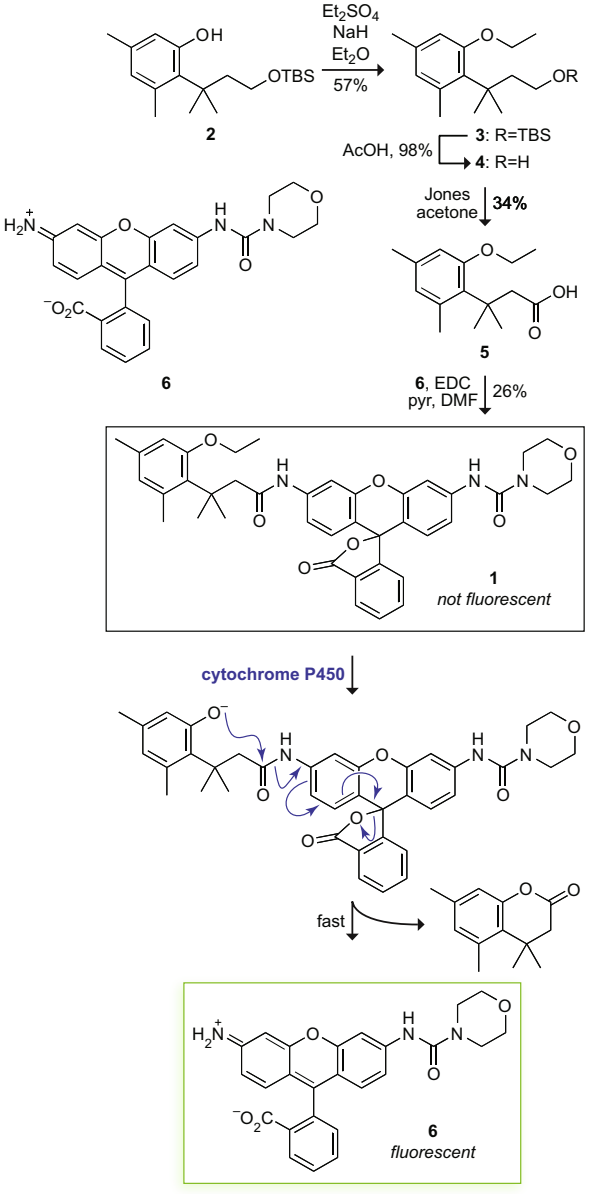
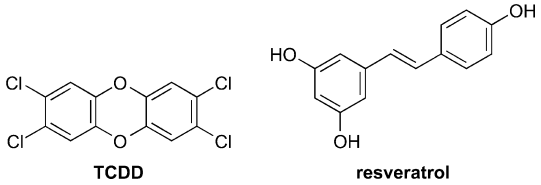



**Figure 1.** Scheme for the synthesis and use of fluorogenic probe **1**.




Then, fluorogenic probe **1** was evaluated as a means to detect an increase in CYP1A1 levels. To do so, A549 cells were incubated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 10 nM), which is the notorious contaminant in the herbicide Agent Orange and the most potent known inducer of CYP1A1.[20] The effect of TCDD on fluorogenesis within A549 cells was dramatic (Fig. 2C).

Finally, fluorogenic probe **1** was used to reveal a more complex modulation of P450 activity. Levels of P450 are highly variable in individuals, and there are many known P450 polymorphisms.[21] Inhibitors of P450 activity have potential as chemotherapeutic agents.[22] For example, resveratrol (3,5,4′-trihydroxystilbene), which is a natural phytoalexin present in grapes and other foods, has been proposed to have a chemoprotective effect against lung cancer by virtue of its ability to decrease CYP1A1 activity.[23] To test this hypothesis with fluorogenic probe **1**, live A549 cells were treated with both TCDD and resveratrol, along the probe. After a 1-h incubation, cells exhibited a dramatic decrease in fluorescence compared with cells treated with TCDD (Fig. 2D). The levels appeared to be even lower than those in untreated cells. These and other data[23] pro-

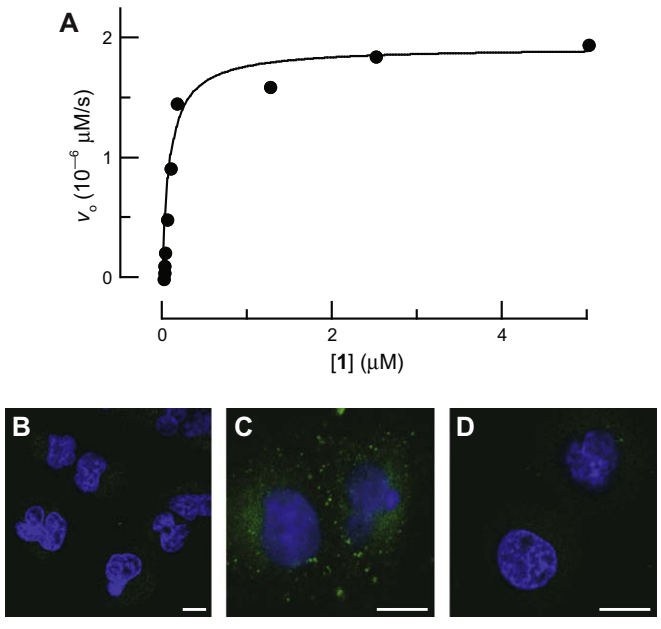



**Figure 2.** Fluorogenesis from fluorogenic probe **1** in vitro and in cellulo. (A) Data for the in vitro cleavage of fluorogenic probe **1** by human CYP1A1 (5.0 pM) in PBS containing NADPH (8 mM) and $MgCl_2$ (8 mM). (B–D) Images of the in cellulo cleavage of fluorogenic probe **1**. A549 cells were incubated with fluorogenic probe **1** (10 μM) and an additive for 1 h and counterstained with Hoechst 33342. (B) No additive. (C) TCDD (10 nM). (D) TCDD (10 nM) and resveratrol (50 μM). Scale bars: 10 μm.

vide direct and conclusive evidence that resveratrol decreases CYP1A1 activity in cellulo.

In conclusion, fluorogenic probe **1** is the first to utilize a 'trimethyl lock' that is triggered by cleavage of an ether bond. This probe has numerous desirable attributes. Its chemical and photophysical properties allow for real-time imaging of P450 levels in cellulo. The modularity of this probe enables its extension to enzymes throughout the P450 family, and its success indicates that the trimethyl lock strategy can be applied to P450-activated prodrugs. Finally, appending the urea group with a trichloromethyl ketone or other weak electrophile would allow the probe to react with an intracellular thiol and enable its retention within a cell, providing additional utility.[24]

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## Supplementary data

Detailed procedures for the synthesis, analysis, and use of fluorogenic probe **1** can be found, in the online version, at doi:10.1016/j.bmcl.2008.06.015.

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