Photochromic Agents as Tools for Protein Structure Study: Lapachenole Is a Photoaffinity Ligand of Cytochrome P450 3A4[†]

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ABSTRACT: Cytochrome P450 3A4 is a drug-metabolizing enzyme of extraordinarily broad substrate specificity. This quality imparts upon the enzyme special importance in understanding its determinants of activity and substrate recognition. Limited successes in P450 3A4 active-site structure studies have been achieved by use of mechanism-based inactivators and photoaffinity ligands. We report here the potential of photochromic agents, compounds with the ability to undergo light-induced, reversible reactions, to be used as effective photoaffinity ligands. Four such compounds of the chromene family were shown by ultraviolet and visible spectroscopy to undergo photoinduced rearrangements to highly conjugated and reactive products in buffered aqueous solution. While some of these intermediates were very long-lived (>12 h, photoactivated lapachenole), others existed for milliseconds in their opened forms (precocene I and 2,2-dimethyl-5,6-benzo-2*H*-chromene) and were observed by laser flash photolysis. Each of the tricyclic structures studied rapidly underwent Michael addition reactions with the test nucleophile glutathione upon irradiation to form single conjugated products. The smaller precocene I reacted more extensively to form multiple products. These attributes of the chromenes inspired testing of their potential to label cytochrome P450 3A4 in a light-dependent fashion. Access to the protein active site by lapachenole was demonstrated with the molecule's ability to competitively inhibit P450 3A4-mediated oxidative metabolism of midazolam with an IC_{50} value of 71 μM. This inhibition became irreversible upon irradiation of the enzyme−ligand complex with ultraviolet light. These results clearly demonstrate that chromenes are effective photoaffinity reagents for the cytochrome P450 superfamily of enzymes and probably other proteins as well.

With regard to its broad substrate specificity and complex mechanisms of regulation, cytochrome P450 3A4 (CYP3A4)[1] is one of the most important P450 isoforms studied in the field of drug metabolism (*1*). It is capable of monooxygenating approximately half of all therapeutic agents on the market today and, as it is also abundantly expressed in the intestine, is important in first-pass metabolism of many orally ingested xenobiotics. Its ability to metabolize such a wide range of structurally unrelated compounds ranging from macrolide antibiotics (*2*) to steroids makes it a critical concern in the monitoring of drug−drug interactions. The relatively recent recognition that common human dietary elements such as grapefruit (*3*), garlic (*4*), and red wine (*5*) are also capable of regulating CYP3A4 activity further magnifies this concern. Obviously, future studies in drug structure and therapeutic regimen design will depend critically upon our understanding of CYP3A4 active-site architecture and mechanism of action.

Studies of cytochrome P450 active-site structural components and substrate recognition determinants have commonly included the covalent modification of substrate binding regions with ligands followed by the identification of the adducted peptide. In this fashion, amino acid residues that are in contact with bound ligands can be identified and their roles in normal substrate binding assessed. Data obtained from such studies can serve to validate existing structural models of the P450 or, alternatively, to further refine that model. Mechanism-based inactivators (MBIs, or "suicide substrates") have been used extensively for this purpose in the past. This strategy, which has recently been thoroughly reviewed (*6*), involves the presentation of an inherently inert molecule to the P450 active site that then becomes markedly reactive upon its oxidation through the enzyme's normal

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[1] Abbreviations: CYP 3A4, cytochrome P450 3A4; MBI, mechanism-based inactivator; PAL, photoaffinity ligand; MDZ, midazolam; UV−vis, ultraviolet−visible; 7,8-DMBC, 2,2-dimethyl-7,8-benzo-2*H*-chromene; 5,6-DMBC, 2,2-dimethyl-5,6-benzo-2H-chromene; EDTA, ethylenediaminetetraacetic acid, sodium salt; HPLC, high-pressure liquid chromatography; COSY, correlated spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; TMS, tetramethylsilane; DLPC, L-α-dilaurylphosphatidylcholine; DLPS, L-α-dilaurylphosphatidylserine; DOPC, L-α-dioleoylphosphatidylcholine; GSH, glutathione; QA, quinone allide; TFA, trifluoroacetic acid; APAP, acetaminophen; NAPQI, *N*-acetyl-*p*-benzoquinonimine.

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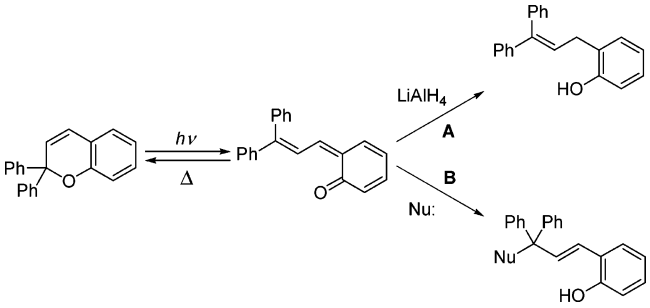
mechanism of action. This reactive intermediate then binds to a closely positioned amino acid residue without first leaving the protein active site, rendering the P450 inactive. Applying this strategy to CYP3A4 structure study, researchers have shown that a furan-containing potential human immunodeficiency virus (HIV) protease inhibitor, the Merck compound L-754,394, is a relatively potent MBI of that enzyme (*7*). Further studies employing [^{14}C]-labeled material resulted in the isolation of a peptide, upon cyanogen bromide digestion, that was proposed to be part of the I-helix lining the substrate recognition region of CYP3A4 (*8*). However, characterization of the modified peptide was prevented by the instability of the adduct itself. On the basis of these results and homology modeling of L-754,394-bound CYP3A4, it was proposed that the adducted amino acid in this case was Glu 307, which served as a nucleophile to attack a furanopyridine epoxide metabolically generated from the parent substrate.

The widespread problem of resultant unstable adducts upon covalent binding of P450s with MBIs is, to some degree, ameliorated with the application of another enzyme active-site covalent modification method, namely, the use of photoaffinity ligands (PALs). In this case, a chemically inert substrate/inhibitor analogue is introduced into the active site of an enzyme after which an extremely reactive species is unmasked upon irradiation of a photosensitive moiety in the molecule with light of the proper wavelength. This strategy has proven very successful in the characterization of several DNA-binding proteins and membrane receptors. However, its utility in the analysis of P450 structure remains underdeveloped relative to the use of MBIs. The application of this technique in P450 studies has recently been reviewed by one of us (*9*), outlining its successes and pitfalls since its first application to studies of P450cam in 1979 (*10*). The potential use of photochromic agents as photoaffinity ligands of cytochrome P450s was suggested at that time.

Photochromism was recently defined as "a reversible transformation in a chemical species between two forms, and in these reversible processes, at least one pathway is induced by photoexcitation (*11*)." This characteristic is shown in Scheme 1 as it applies to members of the chromene family.

Scheme 1: Photochromic Effect[a]



[a] The initial photolysis product can revert to starting material by both thermally and quantum driven processes. Pathway A: reduction of photolyzed products yields stable phenols. Pathway B: Michael addition reaction of photoactivated structure.

In 1966, one of us proposed, on the basis of low-temperature photoactivation and UV−vis spectroscopy, that the irradiation of a chromene nucleus would cause aliphatic ether bond breakage and isomerization to a highly conjugated, nonaromatic colored form (*12*). In the dark, this isomer would rapidly re-form the parent chromene via a thermally driven process. This theory was supported the following year by low-temperature reduction of the proposed ring-opened isomers by lithium aluminum hydride to yield stable phenolic products that could be fully characterized (Scheme 1, pathway A) (*13*).

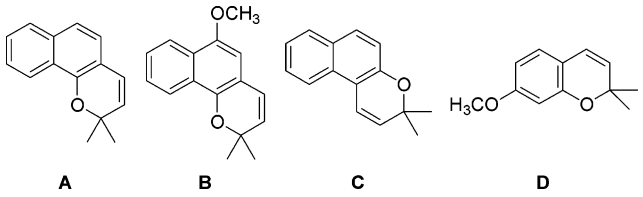


FIGURE 1: Four chromenes under consideration: (A) 2,2-dimethyl-7,8-benzo-2*H*-chromene (7,8-DMBC); (B) lapachenole; (C) 2,2-dimethyl-5,6-benzo-2*H*-chromene (5,6-DMBC); (D) precocene I.

These findings raise the possibility of chromene use as active-site probes of cytochrome P450s. For example, the chromene nucleus described above could potentially participate in Michael addition reactions with a protein nucleophile in a light-dependent manner (Scheme 1, pathway B). The potential to generate a distinct chemical species possessing its own unique reactivity characteristics within the active site of an enzyme on an essentially instantaneous time scale may be considered a hallmark of photoaffinity ligands. The planar and hydrophobic nature of the chromenes also make them likely substrates or inhibitors for several P450 isoforms. Furthermore, light-dependent reaction reversibility dictates that inert and electrophilic forms of the ligands can be readily interconverted within the active site. For these reasons, we present here evidence that, of four chromenes investigtated (Figure 1), all react rapidly with the test nucleophile glutathione in phosphate buffer via a light-dependent manner. One of these agents, the plant-derived benzochromene lapachenole, is shown to bind to the CYP3A4 active site and inhibit its metabolism of acetaminophen (APAP) and midazolam (MDZ). The inhibition of MDZ metabolism is shown to become irreversible upon irradiation of the enzyme−ligand complex, with UV light yielding a fluorescently modified apoprotein. Protection from fluorescent labeling is afforded by the competitive inhibitors ketoconazole and erythromycin. These results, along with UV−vis spectroscopic studies showing profoundly perturbed reduced CO difference spectra of proteins photolyzed in the presence of lapachenole, strongly support the notion that photochromic agents, in particular those of the chromene family, are potentially useful photoaffinity ligands in the study of P450 active-site structure.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were purchased from Sigma−Aldrich (Milwaukee, WI) unless otherwise noted. Precocene I was decolorized by bulb-to-bulb vacuum distillation prior to use. Lapachenole, 2,2-dimethyl-7,8-benzo-2*H*-chromene (7,8-DMBC), and 2,2-dimethyl-5,6-benzo-2*H*-chromene (5,6-DMBC) were synthesized according to published procedures (*14*, *15*). UV−Vis spectra were acquired on a Beckman DU-7400 or a Beckman DU-7 spectrophotometer.

Preparation of Enzymes. Recombinant 6× histidine-tagged CYP3A4 (P450 3A4-HT) was produced in *Escherichia coli* DH5α cells by use of expression vector pCW 3A4-His6 kindly provided by Dr. Ron Estabrook (University of Texas

Southwestern Medical Center, Dallas, TX). Growth and induction of *Escherichia coli* were performed as described by Gillam et al. (*16*). Solubilized membranes were prepared and P450 was purified on a ProBond nickel resin column (Invitrogen, Carlsbad, CA). The column was equilibrated, loaded, and then washed with 20 column volumes of equilibration buffer (100 mM Tris-HCl, pH = 7.4, 20% glycerol, 40 mM imidazole, 0.05% cholate, and 50 μM testosterone). It was then eluted with a minimal volume of elution buffer (100 mM Tris-HCl, pH 7.4, 20% glycerol, 500 mM imidazole, and 0.02% cholate). The eluted protein was dialyzed against 100 mM potassium phosphate, pH 7.4, in 20% glycerol and stored at −80 °C at a concentration of 40 μM. Cytochrome P450 content was determined by reduced carbon monoxide difference spectra after dilution of the enzyme in solubilization buffer (100 mM potassium phosphate, pH = 7.4, 20% glycerol, 0.5% sodium cholate, 1% Emulgen 911, and 1.0 mM EDTA). Rat NADPH−P450 reductase was expressed and purified according to the method of Shen et al. (*17*) with *Escherichia coli* strain C-1A carrying plasmid OR 263 kindly provided by Dr. Charles B. Kasper (University of Wisconsin, Madison, WI).

UV−Vis Spectral Analysis of Chromenes and Their Photoproducts. Stock solutions of each chromene were prepared in methanol at a concentration of 25 mM and diluted to a final concentration of 100 μM in 100 mM potassium phosphate buffer (pH = 7.4) containing 20% glycerol and 0.1% nonionic detergent (Triton X-100). Solutions of 7,8-DMBC or lapachenole were irradiated through Pyrex in a 100 μL quartz cuvette by use of a hand-held UV lamp (Mineralight lamp, model UVG-11, 115 V, 60 Hz, 160 mA; UVP Inc., San Gabriel, CA) at its long-wavelength setting (365 nm maximum).

Flash photolysis experiments were carried out on a Nd:YAG nanosecond laser system with 355 nm excitation. The basic components included a Quantel YG-581-10 Nd:YAG laser controlled through a Kinetic Systems CAMAC interface crate, a LeCroy 6880A 1.35 gigasamples/s waveform digitizer, a LeCroy 6010 Magic controller, a pulsed xenon monitoring lamp, and a Hamamatsu R928 PMT. The data-fitting algorithm for analyzing transient decays employed the Marquart method, an iterative nonlinear least-squares fitting procedure. A solution of 5,6-DMBC in buffer (100 mM KP_i, pH = 7.4, 20% glycerol, and 0.1% Triton X-100) was prepared with OD = 0.39 at 355 nm. Irradiation was carried out at 20 °C by use of a Brinkmann RMS-6 thermal controller. The same procedure was used for precocene I, except that irradiation was performed at 266 nm.

Reactivity of Chromenes during Irradiation. Procedures for analysis of the tricyclic chromenes were identical but differed slightly from that used with precocene I. Solutions containing 50 μM tricyclic chromene and 1 mM GSH were prepared in phosphate buffer (100 mM potassium phosphate, pH = 7.4, 20% glycerol, and 0.1% Triton X-100), and each sample was split in half. A sample of control, 10 μL of unirradiated sample, was immediately analyzed by HPLC. The other half of the sample was transferred to a quartz cuvette and irradiated for 5 min through Pyrex with a hand-held UV lamp operating at its long-wavelength setting. A portion of this irradiated sample, 10 μL, was then anlayzed by HPLC. Reverse-phase chromatographic analysis was performed on an Agilent 1100 HPLC system (San Jose, CA) equipped with a G1312A binary pump and a Thermo Surveyor photodiode array detector (San Jose, CA) controlled by Thermo Xcalibur software. A C18 Vydac column (Hesperia, CA) was used (2.1 × 250 mm, 20 μm beads, 120 Å pore) and operated at a flow rate of 200 μL/min. Solvent A was 5% acetonitrile containing 0.06% TFA. Solvent B was 95% acetonitrile containing 0.06% TFA. In all cases, the column was equilibrated with 5% solvent B, the sample was applied, and then the column was washed for 5 min with 5% solvent B. A 25 min linear gradient to 75% solvent B was then executed. Elution profiles were recorded pertaining to all UV−vis absorbance for wavelengths of 200−500 nm. The chromatograms displayed here for one of these samples, lapachenole, is displayed pertaining only to the 300 nm channel of the diode array detector. Analysis of precocene I was done in a similar fashion. However, no Pyrex was used as a filter during irradiation, and the lamp used for irradiation was operated at its short-wavelength setting (265 nm maximum). The displayed chromatographic data derived from this sample pertains to the total wavelength scan of the diode array detector. This difference in the display of chromatographic data between precocene I and the tricyclic chromenes was a necessity derived from the fact that nonionic detergent eluted in close proximity to regions of interest in the chromatograms of the tricyclic chromene experiments (over 26 min). As the detergent was invisible when monitoring at 300 nm but all detectable products were clearly seen, the data are presented as such. In contrast, precocene I and all its reaction products elute well before the detergent peak. Product peaks, in this case, absorbed very poorly at wavelengths over 280 nm and so chromatograms are displayed pertaining to monitoring of the entire spectral window between 200 and 500 nm. The region showing the elution of detergent in this sample could be safely omitted.

Peaks of interest were manually collected in plastic tubes. A small amount of purified material was diluted 10-fold with a 10% methanol solution containing 6% acetic acid. The sample was then loaded into a metalized glass capillary (Proxeon Biosystems, Odense, Denmark) and subject to nano-electrospray ion trap mass spectrometry on a Thermo Deca XP (San Jose, CA) operating at 900 V ionization potential.

Glutathione conjugates of each chromene were also prepared for NMR characterization by irradiation procedures as described above, except that glutathione was maintained at a 1:1 proportion to the chromene. Multimilligram amounts of conjugates were prepared in this fashion and purified on a semipreparative C18 HPLC column (Phenomenex, Torrance, CA; 10 × 250 mm, 20 μm particle, 100 Å pore size). Samples were then lyophilized to dryness in the dark for NMR analysis.

NMR Analysis of Glutathione Conjugates. All the NMR measurements were performed on a Bruker Avance 750 NMR spectroscopy system (9.4 T) at 298 K. The ^{1}H NMR, COSY, HMQC, and HMBC spectra were collected in methanol-d_4 with TMS as an internal reference. The concentrations of samples were 50 mM. For ^{1}H NMR analysis, 32 transients were acquired with a 1 s relaxation delay and 32K data points. The 90° pulse was 9.7 μs with a spectral width of 4000 Hz. Two-dimensional spectra were acquired with 2048 data points for t_2 and 256 for t_1 increments.

Lapachenole−GSH Conjugate. ^{1}H NMR (methanol-d_4/TMS): δ = 8.17 (d, 1H), 8.12 (d, 1H), 7.50 (m, 1H), 7.44 (m, 1H), 7.05 (d, J = 16.1 Hz, 1H), 6.99 (s, 1H), 6.31 (d, J = 16.1 Hz, 1H), 4.56 (dd, 1H), 4.02 (s, 3H), 3.87 (s, 1H), 3.85 (s, 1H), 3.81 (t, 1H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.51 (m, 2H), 2.11 (m, 2H), 1.60 (s, 3H), and 1.57 (s, 3H). ^{13}C NMR (methanol-d_4/TMS): 174.10, 172.80, 150.95, 150.45, 140.12, 136.86, 128.82, 127.10, 123.90, 123.56, 122.98, 120.10, 101.63, 55.38, 54.98, 54.06, 47.94, 28.10, and 26.40.

5,6-DMBC−GSH Conjugate. ^{1}H NMR (methanol-d_4/TMS): δ = 8.01 (d, 1H), 7.72 (d, 1H), 7.62 (d, 1H), 7.41 (t, 1H), 7.26 (t, 1H), 7.12 (d, 1H), 6.74 (d, J = 16.3 Hz, 1H), 6.27 (d, J = 16.3 Hz, 1H), 4.57 (dd, 1H), 3.91 (m, 2H), 3.86 (t, 1H), 3.07 (dd, 1H), 2.87 (dd, 1H), 2.47 (m, 2H), 2.10 (m, 2H), 1.60 (s, 3H), and 1.57 (s, 3H). ^{13}C NMR (methanol-d_4/TMS): 172.10, 170.26, 151.42, 150.43, 140.12, 134.20, 131.25, 127.10, 123.90, 123.56, 122.98, 115.30, 74.08, 53.10, 51.12, 46.20, 25.98, and 24.36.

7,8-DMBC−GSH Conjugate. ^{1}H NMR (methanol-d_4/TMS): δ = 8.22 (d, 1H), 8.19 (m, 1H), 7.79 (m, 1H), 7.76 (m, 1H), 7.45 (m, 2H), 7.02 (d, J = 16.1 Hz, 1H), 6.31 (d, J = 16.1 Hz, 1H), 4.56 (dd, 1H), 3.92 (m, 2H), 3.89 (t, 1H), 2.97 (dd, 1H), 2.81 (dd, 1H), 2.54 (m, 2H), 2.16 (m, 2H), 1.59 (s, 3H), and 1.40 (s, 3H).^{13}C NMR (methanol-d_4/TMS): 172.12, 170.30, 150.82, 149.85, 136.20, 135.13, 130.25, 126.88, 125.30, 123.96, 119.98, 115.35, 77.12, 60.08, 53.12, 46.70, 27.68, and 25.14.

Precocene I−GSH Conjugate. ^{1}H NMR (methanol-d_4/TMS): δ = 7.33 (d, J = 8.55 Hz, 1H), 6.64 (d, J = 16.3 Hz, 1H), 6.42 (dd, J = 8.53 Hz, 1H), 6.38 (d, 1H), 6.14 (d, J = 16.3 Hz, 1H), 4.54 (dd, 1H), 3.98 (m, 2H), 3.87 (t, 1H), 3.76 (s, 3H), 2.93 (dd, 1H), 2.76 (dd, 1H), 2.53 (m, 2H), 2.18 (m, 2H), 1.51 (s, 3H), and 1.49 (s, 3H). ^{13}C NMR (methanol-d_4/TMS): 174.12, 172.27, 171.60, 161.08, 156.75, 134.60, 127.90, 123.08, 117.98, 106.90, 102.04, 79.42, 55.16, 53.49, 47.80, 32.20, 30.08, 28.36, and 26.95.

IC_{50} Measurement of Lapachenole on CYP3A4. The affinity of P450 3A4 for lapachenole was determined by measuring the molecule's ability to inhibit 1′-hydroxylation of midazolam (MDZ) by the enzyme. Rates of MDZ 1′-hydroxylation measured with various lapachenole concentrations were used to determine an IC_{50} value for the enzyme−lapachenole interaction. Each reaction mixture contained 50 pmol/mL CYP3A4 Supersome with reductase and b_5 (Gentest Corp., Woburn, MA), MDZ (10 μM), and lapachenole (0, 1, 5, 20, 50, 100, or 500 μM) reconstituted in 100 mM potassium phosphate buffer (pH = 7.4). Preincubations were performed in the dark for 5 min at 37 °C before the reactions were initiated by addition of NADPH, 1 mM final concentration. The total reaction volumes were 1 mL. After 5 min, each reaction was stopped by addition of 100 μL of sodium carbonate solution (100 mM) containing internal standards (1 μg/mL [$^{15}N_3$]MDZ and [$^2H_2$]-1′-OH-MDZ). The samples were each extracted once with 5 mL of ethyl acetate and the solvent was evaporated under a dry nitrogen gas stream. The residues were reconstituted with 100 μL of 50% methanol, and 40 μL of each sample was subjected to LC/MS analysis. Metabolites were separated on a Zorbax XDB-C8 column (2.1 × 50 mm, 5 μm particle size) operating at a flow rate of 300 μL/min, and a Shimadzu LC10AD solvent delivery module (Shimadzu Scientific Instruments, Columbia, MD). Conditions used were as follows: solvent A = water and 0.1% acetic acid; solvent B = methanol and 0.1% acetic acid. Column equilibration was performed with 45% solvent B and the column was washed under these conditions for 0.5 min after sample application. A 1.5 min gradient to 60% solvent B was then executed and the solvent components were held at these conditions for a further 0.5 min. A Micromass Quattro II triple-quadrupole mass spectrometer (Manchester, U.K.) equipped with MassLynx NT DS software operating in multiple reaction monitoring mode was used for detection of the metabolites by monitoring the following mass transitions: primary 326 (MDZ); primary 329 ([$^{15}N_3$]MDZ); primary 342 (1′-OH-MDZ); and primary 346 for the ^{37}Cl isotope of [$^2H_2$]-1′-OH-MDZ. Each experiment was performed in duplicate. Ratios of metabolites to internal standards were used for quantification, and data were plotted by using KaleidaGraph 3.5 (Synergy Software, Reading, PA). An IC_{50} value of 70.9 μM was calculated.

P450 3A4-HT Photoinactivation Assay. Assay solutions of reconstituted CYP3A4-HT were prepared by a protocol described elsewhere (*18*). Activity of CYP3A4-HT was determined by measuring midazolam 1′-hydroxylation activity or acetaminophen (APAP) oxidation to *N*-acetyl-*p*-benzoquinoneimine (NAPQI) product after its Michael addition of glutathione to form its GSH-conjugated product. The reconstituted system for the assay contained (midazolam hydroxylation assay) 0.1 μM P450 3A4-HT, 0.2 μM NADPH−P450 reductase, 0.1 μM cytochrome b_5, 50 μM MDZ, 0.1 mg/mL Chaps, 0.02 mg/mL liposomes (DLPC, DOPC, and DLPS), 30 mM $MgCl_2$, 7 mM GSH, and 0.2 mM lapachenole in 50 mM potassium Hepes buffer, pH 7.4. The mixture was preincubated at 37 °C for 5 min. The reaction was then initiated by adding NADPH (1 mM final concentration). The total reaction volume of the assay was 500 μL. After 5 min of incubation at 37 °C, the reactions were stopped by addition of 100 μL of 100 mM sodium carbonate. Controls either contained no lapachenole or were not photolyzed. Control 1 contained no lapachenole and was not photolyzed. Control 2 contained no lapachenole and was treated with UV irradiation. The third control contained lapachenole but was not photolyzed. Each assay was performed in triplicate.

Metabolite extraction was carried out with 1 mL of ethyl acetate. The extracted metabolites were dried under a nitrogen gas stream and the residue was resuspended in 100 μL of 50% methanol. A portion of the mixture (20 μL) was then used for LC/MS quantitative analysis. Samples were injected on a Zorbax Eclipse XDB-C8 column (5 μm, 2.1 × 50 mm, Agilent) operated at a flow rate of 300 μL/min and interfaced with a triple-quadrupole mass spectrometer. Quantification by LC/MS analysis was performed as described above. The amounts of oxidized products were calculated on the basis of the standard curve for metabolites.

Assay solutions for APAP oxidation activity contained 1 mM APAP as substrate and were conducted for 30 min. Relative amounts of GS−APAP conjugate formed from the NAPQI intermediate were measured by HPLC at a monitoring wavelength of 254 nm for detection of products. Controls were performed exactly as described above for the midazolam hydroxylation assay.

CO Reduced Difference Spectra. Spectra were obtained by the method of Omura and Sato (*19*). Photoaffinity labeling

was carried out under the incubation conditions described above. Samples containing photoprobe were dialyzed for 1 h at 4 °C against 1 L of assay buffer (50 mM potassium Hepes, pH 7.4), and this procedure was repeated twice. The absolute spectra and reduced CO difference spectra of CYP3A4-HT (0.5 nmol) were determined by scanning from 400 to 500 nm on a Varian CARY 300 spectrophotometer (Walnut Creek, CA).

Competitive Inhibitor Protection Assays. Labeling conditions were as described above except that ketoconazole and erythromycin were used as competitive inhibitors of protein labeling by lapachenole. Increasing concentrations of these known substrates or inhibitors of CYP3A4-HT (50 and 250 μM ketoconazole, or 60 and 300 μM erythromycin) were included prior to photolysis of lapachenole. Covalent modification by lapachenole was determined by fluorescence of the apoprotein due to the photoactivated lapachenole nucleus, with 320 and 420 nm as excitation and emission wavelengths, respectively. This was done by subjecting the protein preparations to HPLC analysis on a Poros R2 column (2.1 $\times$ 150 mm bed volume) with a Hewlett-Packard 1046A fluorescence detector (Palo Alto, CA). Conditions for chromatographic analysis of P450 proteins have been previously described (*20*).

RESULTS

Generation of Quinone Allides in Buffered Media. The photoinduced rearrangements of chromenes to their quinone allide (QA) counterparts in aqueous buffer were demonstrated by UV−vis spectroscopy. For the cases of lapachenole and 7,8-DMBC, samples irradiated for 5 min turned a deep orange-yellow color. While the colored QA form of lapachenole was found to be very long-lived in aqueous buffer, the QA form of 7,8-DMBC bleached slowly in ambient light. Figure 2 shows the UV−vis spectrum of an aqueous solution of 7,8-DMBC before and after irradiation. The long-wavelength band centered at 458 nm that arose on irradiation is attributed to the highly conjugated, nonaromatic QA formed from the parent chromene. On the benchtop (ambient fluorescent lighting), this band diminished with a half-life of 14.8 min as the chromene was regenerated (inset). Similarly, irradiation of lapachenole in buffer produced a deeply colored QA product. However, this solution did not completely bleach under ambient conditions, even overnight (data not shown).

Irradiation of a 5,6-DMBC sample in buffer produced a QA intermediate that reverted to the parent chromene on a far shorter time scale than did those derived from the 7,8-benzo-substituted chromenes described above. In contrast to the accumulation of colored material upon irradiation in the cases of lapachenole and 7,8-DMBC, no color was evident after the same treatment of a 5,6-DMBC solution in buffer. Evidence for the colored QA form in this case was, however, observed by laser flash photolysis. Irradiation with a pulsed laser (355 nm) revealed a transient with a peak at 420 nm that completely bleached in the dark within 150 ms via multiphasic kinetics (Figure 3).

Laser flash experiments with precocene I could not be effectively conducted in buffer solution due to interference at the shorter irradiation wavelengths (266 nm) required for the molecule's activation. For this reason, the same experiments were performed in ethanol in order to preserve some degree of hydrogen bonding. A transient opened form of photoactivated precocene I was detected at 380 nm under these conditions (Figure 4). In this case, however, reversion to starting material seemed to follow monophasic kinetics and residual absorbance at the wavelength of detection remained well after 150 ms had passed.

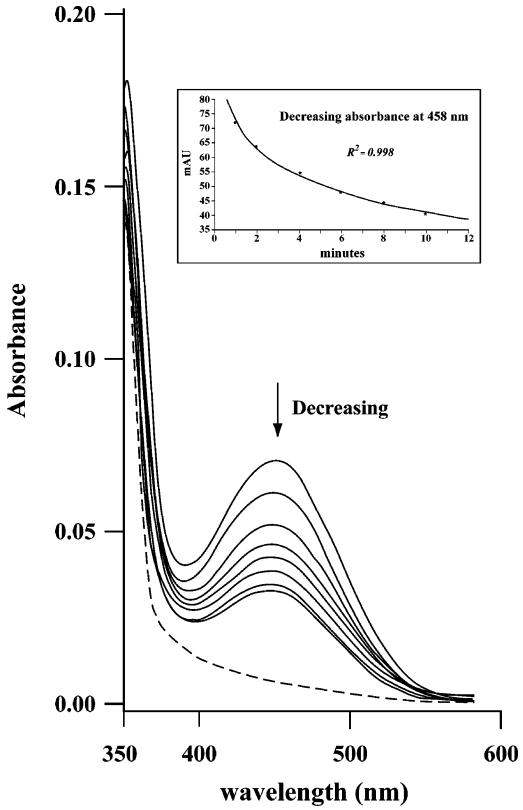


FIGURE 2: Photoactivation of 7,8-DMBC. The ring-opened product absorbs at a maximum wavelength of 458 nm and has a half-life of 14.8 min (inset). The spectrum of the sample before irradiation is also shown (− − −).

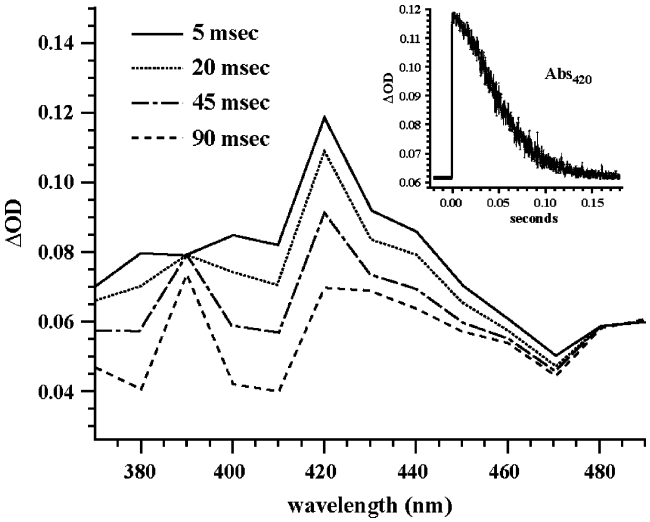


FIGURE 3: Detection of a ring-opened product upon irradiation of 5,6-DMBC in phosphate buffer by laser flash photolysis. The product reverts to starting material within 150 ms following multiphasic kinetics (inset).

Reactivity of QAs with Glutathione. With the confirmation that chromene photoactivation does occur in aqueous media, it was next necessary to establish reactivity profiles for the

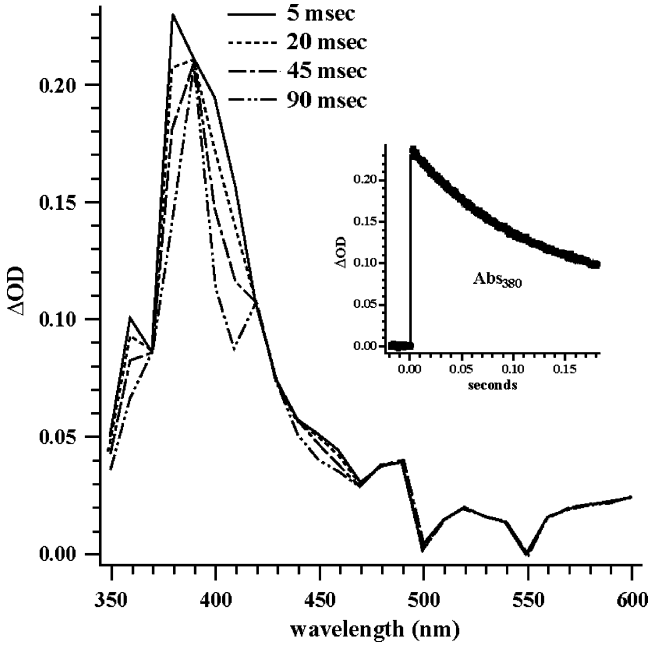


FIGURE 4: Photoactivation of precocene I in ethanol. The ring-opened product absorbs strongly at 380 nm and substantial amounts remain after 150 ms (inset).

chromene nuclei with a test nucleophile, glutathione (GSH), upon irradiation. This was done by preparing samples of each chromene in aqueous buffer containing 1 mM GSH, splitting each into a control and a treated sample, and then exposing the treated samples for 5 min to ultraviolet light passed through a Pyrex filter. Analysis of the resultant solutions by HPLC was then performed by off-line mass spectrometry to confirm the identity of peaks collected. In the cases of all three tricyclic chromenes, only one single product was seen for each of the irradiated samples. When each of these peaks was collected and subjected to mass spectrometric analysis, results were consistent with a Michael addition reaction of GSH to each of the QAs formed by irradiation of the parent chromenes. Glutathione conjugates derived from experiments conducted with 5,6- and 7,8-DMBC showed $[M + H]^{1+}$ peaks at $m/z = 518$. Experiments performed with lapachenole yielded a glutathione conjugate showing a $[M + H]^{1+}$ of $m/z = 548$ (Supporting Information, Figure S1). Chromatographic results from the photoinduced reaction of lapachenole with GSH are shown as an example (Figure 5). Only one major product corresponding to glutathione attack at the terminal carbon of the ring-opened product was visible after 5 min of irradiation (18.09 min). The minor products at approximately 24 min were extremely difficult to observe by off-line mass spectrometry because of poor ionization of the analytes.

The same experiments conducted with precocene I yielded two major GSH-conjugated products eluting at approximately 15.7 min attributed to attack of GSH on the QA terminal and benzylic positions (Figure 6). These assignments were supported by mass spectrometric analysis of the collected products.

It is important to note that although the reactions described did not proceed to any appreciable degree without irradiation, special care was necessary during the handling of samples containing the 5,6-DMBC isoform, as these solutions seemed to be somewhat sensitive to reaction induced by ambient

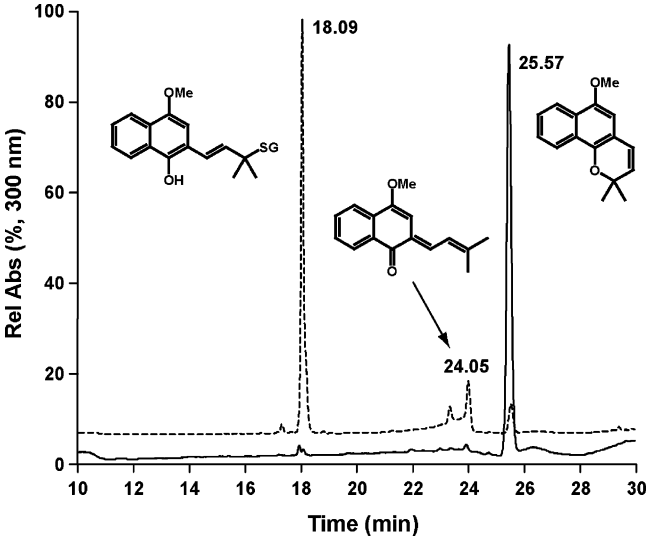


FIGURE 5: Reactivity of lapachenole with glutathione upon irradiation: (—) reaction mixture prior to photolysis; (---) reaction mixture after 5 min of irradiation. Almost all of the starting material is consumed and a single glutathione conjugate is detected.

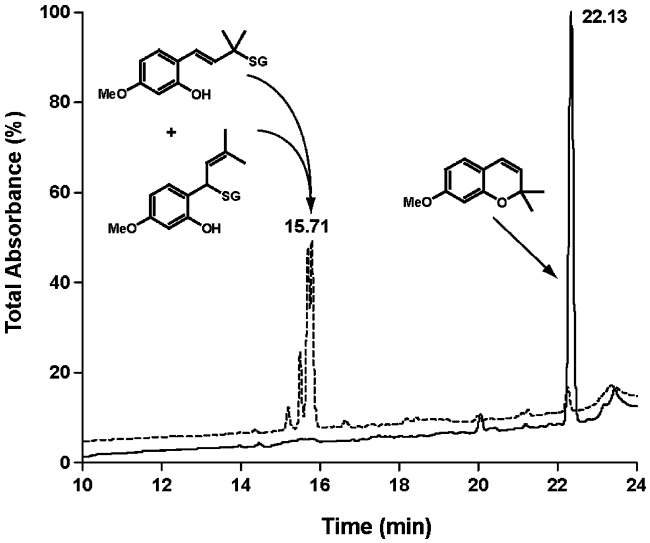
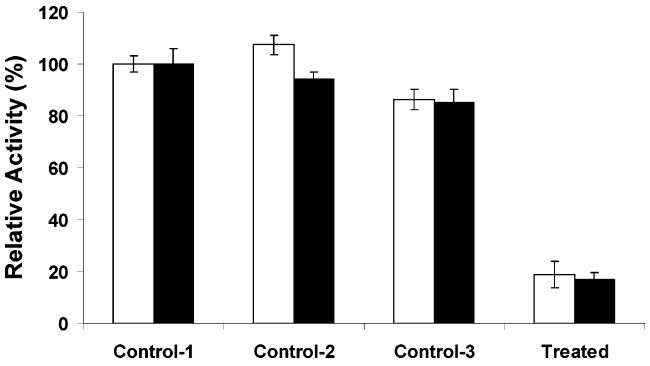


FIGURE 6: Reactivity of precocene I with glutathione upon irradiation: (—) reaction mixture before irradiation; (---) reaction mixture after photolysis. Multiple isomers of glutathione conjugates are formed in this reaction, along with several other earlier eluting unidentified species.

light. These solutions were therefore handled in the dark or in amber glass.

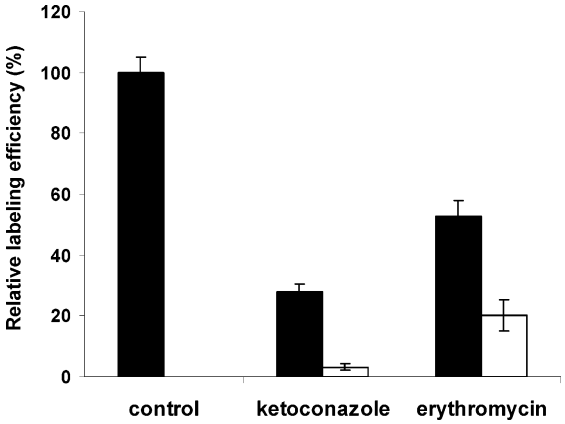
In each of the tricyclic chromene cases, GSH attacks the terminal position of the ring-opened products generated upon irradiation. This was confirmed by larger scale experiments employing semipreparative HPLC purification of GSH conjugates of the photoproducts followed by 1D- and 2D-NMR analysis of the purified material. A one-dimensional ^{1}H NMR spectrum of the lapachenole−glutathione conjugate acquired in methanol-d_4 shows proton signals due to the glutathione moiety, and all signals due to the adducted lapachenole can be assigned as well (Supporting Information, Figure S2). The two aliphatic methyl groups appear as singlets at approximately 1.6 ppm, while the phenolic methyl ether yields a peak at just over 4.0 ppm. All four protons residing on the benzo group are visible as multiplets over

Chart 1: Photoinactivation of P450 3A4 APAP Oxidation and Midazolam 1′-Hydroxylation Activities by Lapachenole[a]



[a] APAP oxidation is shown by open bars; midazolam 1′-hydroxylation is shown by solid bars. Control 1 contained no lapachenole and was not photolyzed. Control 2 was treated with UV irradiation in the absence of lapachenole. Control 3 contained 200 μM lapachenole but was not photolyzed. The treated sample was photolyzed in the presence of 200 μM lapachenole.

Chart 2: Photolabeling Inhibition of CYP3A4-HT by Lapachenole as Judged by Fluorescence of the Treated Protein Preparation[a]



[a] Ketoconazole-treated preparations were exposed to either 50 μM (solid bar) or 250 μM (open bar) ketoconazole prior to irradiation. Erythromycin-treated samples were exposed to either 60 μM (solid bar) or 300 μM (open bar) erythromycin prior to irradiation.

7.4 ppm. Most importantly, vinyl protons corresponding to positions 3 and 4 are seen at 6.31 and 7.05 ppm, respectively, the more downfield signal of position 4 resulting from aromaticity-induced anisotropy. These protons are vicinally coupled, showing a ^3J value of 16.05 Hz, suggesting that these protons are oriented trans across the double bond in the adduct. No signal was detected that corresponded to the cis isoform.

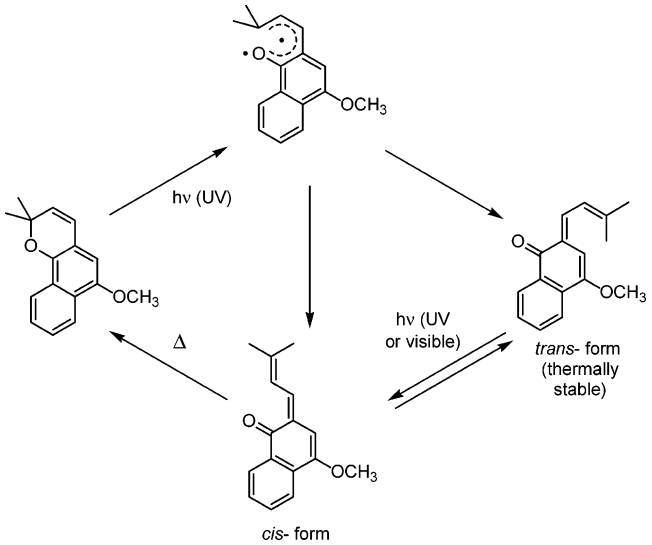
Chromenes Are Capable of Binding to the Active Sites of P450s. When tested for its ability to inhibit hydroxylation of midazolam (MDZ) at the 1′-position by 3A4 in a crude enzyme assay (Supersomes; Gentest, Woburn, MA), lapachenole was shown to block formation of the product with a modest IC_{50} of 71 μM (Supporting Information, Figure S3), although affinity for this ligand was significantly lower in purified 3A4-HT preparations (Chart 1, control 3). This finding strongly supports the assertion that lapachenole binds within the enzyme active site and inhibits metabolism of other molecules in a competitive manner. Photolysis of the chromene while the molecule occupies the enzyme active site may result in covalent binding of the small molecule to a residue critical in normal catalytic mechanisms or for substrate recognition.

Photolysis of the Lapachenole−3A4-HT Complex Results in Enzyme Inactivation through Active-Site Modification. With knowledge pertaining to the photoinduced reactivity of chromenes in hand, as well as confirmation of lapachenole's recognition by 3A4-HT as a substrate/inhibitor, it became possible to test our hypothesis that this molecule is a P450 photoaffinity ligand. Lapachenole displayed the ability to irreversibly inhibit the capacity of purified 3A4-HT to oxidize APAP to NAPQI or to metabolize midazolam to its 1′-hydroxylated metabolitein a light-dependent fashion (Chart 1). Multiple controls served to establish the dependability of these tests. The first controls included enzyme treated with methanol vehicle alone for 5 min without irradiation prior to activity assay, while the second controls consisted of enzyme without lapachenole irradiated for 1 min before the activity assay. The third controls were treated with lapachenole in methanol vehicle but were not irradiated before activity was tested. The treated samples were preincubated for 5 min in the presence of 200 μM lapachenole introduced in a methanol vehicle and then irradiated for 1 min. When the results from the first controls are taken to represent full activity remaining after preincubation, controls 2 and 3 showed retention of their oxidative abilities to an extensive degree. However, the sample treated with lapachenole and irradiated for 1 min showed only between 15% and 20% activity remaining in both activity assays.

To demonstrate that this irreversible inactivation is due to active-site modification by photoactivated lapachenole, we demonstrated protection of the enzyme from lapachenole covalent binding by alternative 3A4-HT competitive inhibitors. This was done by measuring total fluorescence of the protein due to adducted lapachenole after irradiation. Chart 2 shows that ketoconazole blocked approximately 70% of fluorescence labeling when included in the incubation at 50 μM. This labeling is blocked by over 95% when the ketoconazole concentration is raised to 250 μM. Qualitatively similar results were seen when erythromycin was used as a competitive inhibitor, blocking fluorescence labeling to roughly 55% and 17% of that seen with unprotected control incubations when included at 60 and 300 μM concentration, respectively. These results were paralleled by findings from equivalent experiments where activity assays were used rather than measurement of fluorescent modification to evaluate results (Supporting Information, Figure S4). In this case, erythromycin appeared to be superior to ketoconazole in its protective effect on the enzyme.

Further evidence for active-site modification was acquired through observing the effects of irradiation upon reduced CO difference spectra of 3A4-HT (Supporting Information, Figure S5). Control experiments included reduced CO difference spectra from untreated samples (trace 1), one in which the enzyme was irradiated in the absence of lapachenole (trace 2), and one treated with lapachenole without irradiation (trace 3). The third control showed a slightly diminished P450 content and some degree of P420 accumulation. This effect was far more dramatic for the sample treated with lapachnole and irradiated (trace 4). In this case, absorbance at 450 nm was almost completely obliterated while the band at 420 nm became the dominant spectral feature.

Scheme 2: UV Photoactivation of Lapachenole[a]



[a] After carbon−oxygen bond lysis, the ring-opened species can exist in a cis or trans form. Only the cis form can regenerate the starting material.

DISCUSSION

Generation and Reactivity of Quinone Allides in Buffered Media. Chromenes can be isomerized readily and their photoproducts observed in aqueous media. As shown in Figure 2, 7,8-DMBC readily rearranged upon UV irradiation to yield a colored QA isomer with a half-life of 14.8 min. Qualitatively similar results were observed for lapachenole, except that its colored QA form was stable under ambient conditions for a far longer period. It was recently demonstrated that irradiation of this chromene in organic solvent resulted in two main isomers of the QA product, cis and trans forms (*21*) (Scheme 2). In that work, it was shown that one of these products, the trans form, proved to be thermally stable and was therefore purified in the dark and characterized by UV−vis spectroscopy. The cis isomer, however, readily reverted to the parent chromene in a light-independent fashion. This result seems quite reasonable since the trans isomer is prevented from the reverse reaction by simple geometric constraints. Therefore, reversion to the parent compound must occur through the cis isoform. Interconversion between the cis and trans forms of the QA must be mediated exclusively by quantum events, not thermal ones, as is generally required for isomerization of a double bond. It must be emphasized that the rates at which all photochromic reactions occur, however, are critically dependent upon multiple factors including temperature, irradiation wavelength, solvent polarity, and viscosity.

Spectroscopic evidence for the photoinduced generation of QAs from 5,6-DMBC and precocene I was also obtained, but these reactions occurred on far shorter time scales than those observed for the 7,8-substituted benzochromenes. Laser flash photolysis was employed to observe these short-lived species, which both decayed to their respective starting materials in hundreds of milliseconds rather than minutes or hours (Figures 3 and 4). While precocene I followed monophasic kinetics in its reversion to its closed form, the rate at which 5,6-DMBC was regenerated from its QA was multiphasic. The exact nature of these complex kinetics is unclear at this time but certainly reflects intricate processes of isomeric rearrangements and energy transfer. Evaluation of these processes will be the focus of future studies.

The reactivity characteristics of each chromene with the biologically relevant nucleophile GSH upon conditions of irradiation firmly support our proposal that QAs are generated from these compounds in aqueous buffer and that photochromic molecules can serve as effective photoaffinity reagents. All three of the tricyclic chromenes described reacted with GSH upon irradiation to form single Michael addition products by conjugation of the GSH nucleophilic sulfur with the terminal carbon of the QA. The HPLC analysis of this reaction is shown for lapachenole in Figure 5. In addition to the lapachenole−GSH conjugate detected at about 18.1 min, minor products at approximately 24 min were also clearly visible but were difficult to study by off-line mass spectrometric analysis, presumably because of poor ionization of the analytes. However, on the basis of UV−vis spectral similarity of peaks in this region to photolyzed samples of the parent chromene in buffer, it seems safe to assign the structure as ring-opened chromenes that had not yet reacted with glutathione. Note that the reaction does not proceed to any appreciable extent in the absence of irradiation.

In the absence of GSH, none of the tricyclic chromenes reacted with solvent water upon irradiation to any appreciable extent. The QAs generated in these experiments remained stable for extended periods in solution (lapachenole and 7,8-DMBC) or reverted cleanly and rapidly to the parent chromene (5,6-DMBC) as described above. Analysis by mass spectrometry did not reveal any water-conjugated products corresponding to any of these chromenes. Similar negative results were obtained when ϵ-aminocaproic acid was used to test the primary amine moiety as a nucleophile. The fact that hard nucleophiles such as water and amines are not effective in Michael addition to the QAs formed in these cases reflects the soft nature of the conjugated electrophile photoproducts. These data are in accord with the fact that GSH had reacted exclusively as a sulfur nucleophile in our experiments.

Several characteristics of precocene I make it unique from the tricyclic chromenes considered here. For one, this molecule requires shorter wavelengths for photoinduced isomerization to occur, and so irradiation had to be performed without Pyrex glass as a filter. This may cause some difficulties in future studies of precocene I as a photoaffinity ligand, since the researcher would optimally avoid irradiation of a protein's aromatic amino acids (approximately 280 nm) during reagent photolysis so as not to inflict damage to the protein mediated by these residues in photoexcited states. Second, in contrast to the other chromenes each yielding a single adduct upon irradiation in the presence of GSH, two major isomeric products were observed during experiments with precocene I, corresponding to attack by the nucleophile at both electrophilic exocyclic positions (Figure 6). This is in accord with previous findings in which the less substituted 2,2-dimethyl-2*H*-benzopyran formed both of these isomeric solvent adducts when that chromene was irradiated in methanol (*22*). These unique characteristics of precocene I relative to the tricyclic chromenes can reasonably be attributed to its lesser degree of conjugation. Its UV spectrum shows absorbance bands at shorter wavelengths than the other chromenes, as would be expected for a less conjugated

molecule; therefore shorter wavelengths are required to photoactivate the molecule to ring opening. The QA derived from it is also a less conjugated, harder electrophile than those derived from the tricyclic analogues and therefore shows a capacity to react with harder nucleophiles, such as alcohols (*22*).

The reactivity profiles we describe here for the chromenes are generalized for such compounds in aqueous media. However, one must be careful when extrapolating these findings to conclusions from experiments in which these molecules are used as photoaffinity probes for enzyme ligand binding determinants. In the case of the P450s, enzyme active sites are generally considered to be very hydrophobic, in contrast to the aqueous conditions tested here. This fact certainly affects lifetimes and reactivities of QAs formed within this environment so different from bulk buffer. In addition, a very specific network of hydrophobic and hydrophilic interactions must exist in the ligand−enzyme complex that is impossible to take into consideration without a preexisting knowledge of active-site structure. Such interactions will certainly affect reactivity of QAs in Michael addition reactions. We may find that certain active-site nucleophiles react with the QAs of tricyclic chromenes described here only within the unique environment of the ligand binding region, although we see no such reactivity in bulk aqueous media. Alternatively, the position of attack by the nucleophile may not be the terminal carbon of the QA generated, in contrast to results obtained here, but may be on the electrophilic carbon adjacent to the ring system. Any such outcome is likely to arise from specific positioning of a QA within an active site and enhancement of its electrophilicity due to altered hydrogen bonding or other interactions with the protein.

Interactions of Lapachenole with P450 3A4. Certain attributes of the chromene nucleus suggested that this class of molecules would be effective substrates or inhibitors of cytochrome P450s. They are generally planar hydrophobic molecules likely to bind P450s of the 1A family. In fact, precocene I is known to be metabolized to hydroxylated (*23*) and toxic products by P450s in rat liver (*24*). Structurally related cannabinoids are known to bind P450s from the 3A and 2C families as well (*25*). Our inclination to try human P450 3A4 as a target for binding by one of the chromenes being studied here, lapachenole, stems from the great variety of substrates metabolized by that isoform. Inhibition of 3A4-mediated MDZ 1′-hydroxylation activity by lapachenole supported this contention (Supporting Information, Figure S3), and so photolysis of the chromene while the molecule occupies the enzyme active site was thought to potentially result in covalent binding of the small molecule to a residue critical in normal catalytic mechanisms or for substrate recognition. This suspicion was confirmed by experiments showing that inhibition of CYP3A4-HT MDZ hydroxylation activity by lapachenole became irreversible only upon irradiation (Chart 1), an effect that could be qualitatively reproduced with acetaminophen as substrate. We attribute this loss of activity to lapachenole's photoactivation to its QA form within the enzyme's active site. The resultant ring-opened form of the molecule then reacts with a nearby amino acid residue to form a covalent adduct with the apoprotein.

This contention is strongly supported by the finding that competitive inhibitors known to occupy the active site, namely, ketoconazole and erythromycin, protect P450 3A4-HT from fluorescent labeling of the apoprotein by irradiation in the presence of lapachenole (Chart 2). This protective effect is also extended to prevention of enzyme activity loss by the same treatment (Supporting Information, Figure S4). In these experiments, however, erythromycin was found to be far more effective than ketoconazole in its protective effect. This can be rationalized by the fact that ketoconazole is 1000-fold more potent an inhibitor of CYP3A4 ($K_i = 27.8$ nM) than is erythromycin ($K_i = 33.2$ μM) and is certainly more difficult to remove by dialysis (*26*). Ketoconazole remaining bound to the enzyme active site after dialysis is therefore attributed to loss of activity.

The fact that covalent modification strongly perturbs the ability of the enzyme to yield standard P450 spectra in UV−vis difference spectroscopy of reduced CO-bound samples (Supporting Information, Figure S5) also suggests specific labeling of the active site or substrate access channel. In 1996, Martinis and colleagues reported that $P450_{cam}$ converted to its P420 counterpart by exposure of the sample to high pressure was associated with subtle changes in the protein structure that led to a restricted substrate binding site or an altered substrate access channel (*27*). The authors further concluded that P420 corresponded to a cysteine-ligated heme iron protein that was protonated at the sulfur position. A neutral thiol might have then been lost upon reduction during acquisition of a ferrous-CO difference spectrum. These conclusions were recently supported by experiments in which mutants of myoglobin containing neutral thiol-ligated heme iron were generated and studied by UV−vis and magnetic circular dichroism (MCD) spectroscopy (*28*). Taken together, these findings suggest that only modest modifications in P450 protein structure are necessary for conversion to P420 and that these changes can be affiliated with substrate recognition regions, as described here.

Our results strongly support the contention that photochromic agents, particularly the chromenes, can serve as effective photoaffinity ligands for cytochrome P450 enzymes. The claim that the inactivation observed is a result of a very specific covalent modification of the apoprotein by the chromene is also supported by mass spectrometric studies in which 3A4-HT is shown to be adducted by lapachenole at only two residues upon irradiation of the enzyme/inhibitor complex (companion paper in this issue).

The data presented here provide strong evidence that photochromic agents can serve as PALs in the structural analysis of biological macromolecules. We have presented here results from the investigation of one class of such agents, the chromenes, that highlight physical characteristics of these molecules that are typical of PALs. All chromenes studied served as very effective electrophiles in the Michael addition reaction only when irradiated, but not in the dark. One member of the chromene family, lapachenole, was shown to occupy the active site of P450 3A4 and competitively inhibit that enzyme's oxidative metabolism of MDZ. Lapachenole's ability to irreversibly inactivate 3A4-HT only upon irradiation of the enzyme/ligand complex, but not in the dark, confirmed the characterization of chromenes as PALs of cytochrome P450s. Although we only show a limited scope of reactivity for tricyclic chromenes with test nucleophiles in aqueous buffer, it is impossible to predict

the true reactivity of such photoactivated chromenes within the unique environment of an enzyme active site. More extensive reactivity toward Michael addition of QAs formed upon irradiation is likely, and radical reactions induced by triplet states of chromenes and their photoproducts remain to be investigated as well.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Five figures showing a mass spectrum of the lapachenole−glutathione conjugate with a $[M + H]^{+1}$ ion clearly visible at 548 amu [also evident are peaks corresponding to glutathione with loss of the lapachenole nucleus ($m/z = 308$) and the lapachenole nucleus itself with loss of its propyl group ($m/z = 199$)]; ^{1}H NMR spectrum of the lapachenole−glutathione conjugate formed only upon irradiation of a buffered solution of lapachenole and glutathione, in which protons derived from the main structural elements of each of the structural components are labeled and clearly visible; results of affinity measurements pertaining to competitive inhibition of crude CYP3A4 (Supersomes) midazolam metabolism by lapachenole; protective effects of ketoconazole and erythromycin on P4503A4-HT activity from damage due to photolysis of the enzyme−lapachenole complex; and effect of lapachenole on CYP3A4-HT CO reduced difference spectra under differing conditions of lapachenole and/or UV light exposure. This material is available free of charge via the Internet at http://pubs.acs.org.

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