Generation of New Protein Kinase Inhibitors Utilizing Cytochrome P450 Mutant Enzymes for Indigoid Synthesis

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Indigoids, a class of bis-indoles, represent a promising protein kinase inhibitor scaffold. Oxidation of indole by cytochrome P450 (P450) has been shown to generate species (indoxyl, isatin) that couple to yield indigo and indirubin. *Escherichia coli*-expressed human P450 2A6 mutants isolated from a randomized library were incubated with 27 substituted indole derivatives. Extracts of the cultures were screened for inhibition of human cyclin-dependent kinases (CDK)-1 and -5 and glycogen synthase kinase-3 (GSK3). The extracts from cultures incubated with 5-methoxyindole were the most inhibitory. High-performance liquid chromatography (HPLC) separation yielded a mixture of seven colored indigoids. These indigoids included indigo, indirubin, the di(5-methoxy) derivatives of indigo and indirubin, and both of the possible mono 5-methoxy derivatives of indirubin, which were all identified by visible, mass, and NMR spectra. Cultures with 5-methylindole added to the media also yielded inhibitory material, and 5- and 5′-methylindirubin were characterized. The most inhibitory of these indigoids were the monosubstituted indirubins and 5,5′-dimethoxyindirubin, which was ≥10× more active than indirubin. Thus, the overall approach involves the use of a library of randomized enzyme mutants to activate component moieties of a desired set of larger molecules, thus yielding a library of drug candidates that can be screened and characterized. The general strategy may have additional applications.

Introduction

Many drugs contain indole moieties, either as a basic template or as an attached group to invoke particular properties. The indole moiety has some similarity to the purine nucleus; thus, these drugs can resemble nucleosides and nucleotides, with the potential to bind and inhibit many enzymes that use adenosine 5′-triphosphate (ATP) or guanosine 5′-triphosphate. Indole-based drugs also interact with other classes of protein targets, including steroid receptors.[1] Indirubin and some of its derivatives are present in some traditional Chinese medicines and inhibit cyclin-dependent kinases (CDKs).[2] These medicines have historically been used to treat chronic diseases including leukemia;[3,4] these traditional medicine practices may have a molecular basis, in that cancer cell lines are sensitive to indirubin.[5] Indirubin inhibits not only CDKs but also glycogen synthase kinase (GSK)-3, another protein kinase involved in abnormal τ-phosphorylation in Alzheimer's disease.[6] Indirubin has also been reported to be a potent agonist of the aryl hydrocarbon receptor,[7] and a variety of other indole derivatives also have agonist activity with this receptor.[8−12]

Protein kinases constitute a very large family of potential targets in a variety of diseases, and indigoids represent one of the promising scaffolds from which pharmacological inhibitors can be optimized for both selectivity and potency. The acquisition of a broad group of indigoids, particularly indirubins, would be of use in the search for new drugs. Indigoids are natural products, and they have a long history but primarily because of the interest in them as dyes.[2,13−15] Ensley et al.[16] reported the bacterial conversion of indole to indigo catalyzed by the dioxygenase naphthalene dioxygenase, and subsequently, a useful process was developed for the bioindustrial production of indigo.[17,18] Work in one of our laboratories demonstrated that human cytochrome P450 (P450) enzymes, expressed in *Escherichia coli*, can oxidize indole to indigo and indirubin[19,20] (Scheme 1). Subsequently, Schmid's group found that a mutated derivative of bacterial P450 102 could also oxidize indole to indigo.[21] In our own work, we used dye formation as a screen in work on random mutagenesis/molecular breeding of human P450 2A6.[22] In that work, we identified mutants that were more active than wild-type P450 2A6 in forming indigo.

In the earlier work with P450 2A6 mutants, substituted indoles were found to be converted to distinctly colored products by P450 mutants.[22] The chemical synthesis of collections of indole derivatives is possible but is not trivial.[23] Few modified indirubins have been isolated or synthesized.[2,6,24−26] One group has reported the concept of feeding substituted indoles to plant tissue cultures to produce substituted indirubins, apparently with the goal of making useful dyestuffs.[26,27]

We coupled the production of indigoids from substituted indoles[22] with screens for kinase inhibition[2,6] and subsequently isolated some indirubins with enhanced

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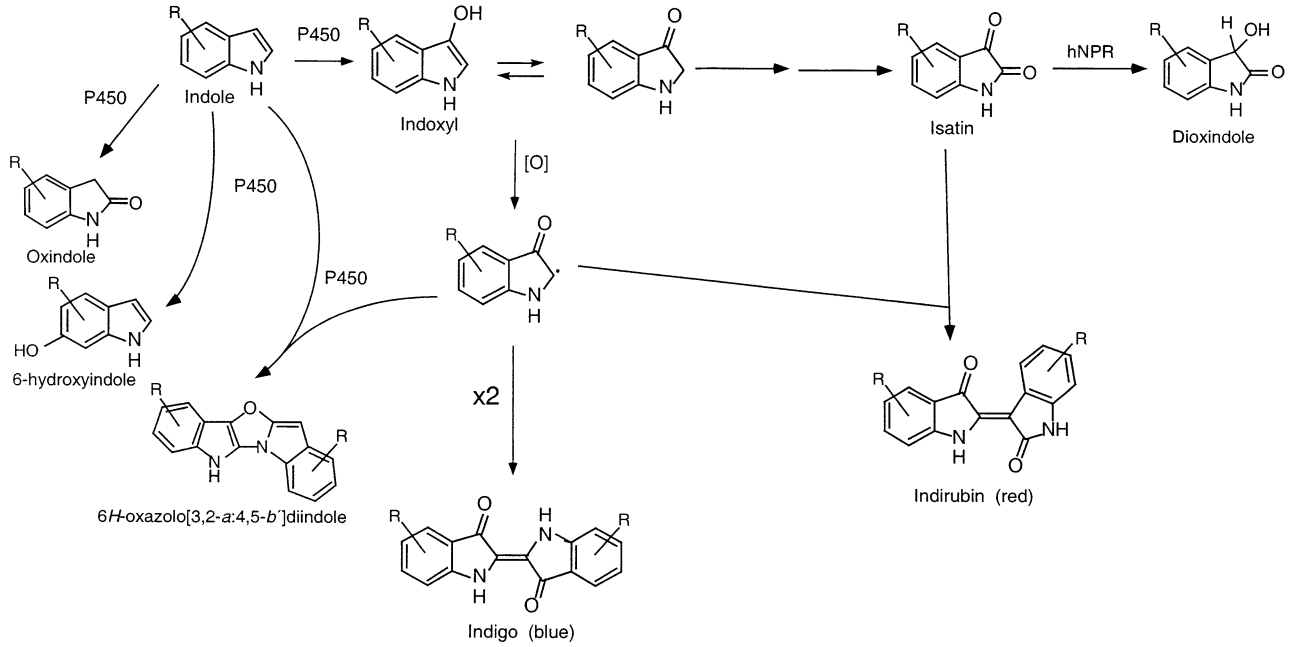
Scheme 1. Pathways of Biosynthesis of Indigoids[a]



[a] See also ref 20.

kinase inhibitory properties attributed to the substitutions. The concept is to utilize this approach as a means of searching for new drug candidates.

Results

Strategy. We have already demonstrated the ability of mammalian P450s to oxidize indole to indigo and indirubin[19,20] and to form colored derivatives from substituted indoles.[22] P450 2A6 mutants that had been selected from randomized libraries were also able to generate variously colored products from substituted indoles.[22] Indirubins have a history as protein kinase inhibitors,[2,6] and we reasoned that the use of a library of P450s with a set of indole precursors might provide a means of generating new and useful kinase inhibitors.

In the initial screening, we selected all P450 mutant/substituted indole combinations that generated colored materials (Table 1).[22] We utilized wild-type P450 2A6 and the L240C and L240C/N297Q mutants. Crude cell extracts (dimethyl sulfoxide, DMSO) were analyzed for inhibition of CDK5/p25 and GSK-3α/β (Table 1), with the results based upon the volume of these unseparated materials and reflecting the total inhibitory "capacity" in each case.

With the compounds tested, the combination of 5-methoxyindole with P450 2A6 L240C/N297Q was the most inhibitory. The incubation of 5-methylindole also yielded inhibitory material, and some work was done with those extracts later.

Large scale incubations were done with P450 2A6 L240C/N297Q and 5-methoxyindole, and the extract was fractionated by solubility differences, high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC). Although HPLC was effective in separating small amounts of the crude mixture into individual components (Figure 1), the resolution decreased considerably when large amounts were applied to the preparative column. The indigoid peaks were repurified using chromatography in the same general system, and each peak was >98% pure. This degree of purity appeared to be substantiated in the subsequent studies using NMR and mass spectrometry.

Table 1. Preliminary Screening for Kinase Inhibitors[a]

P450 2A6	additions to culture (mM)	% control activity CDK5/p25	% control activity GSK-3α/β
wild type	indole (1.0)	78	68
wild type	5-methylindole (1.0)	89	45
L240C	5-bromoindole (1.0)	44	22
L240C	5-methoxyindole (0.2)	87	55
L240C/N297Q	indole (1.0)	81	76
L240C/N297Q	5-aminoindole (1.0)	73	42
L240C/N297Q	7-azaindole (1.0)	103	89
L240C/N297Q	5-bromoindole (0.2)	56	25
L240C/N297Q	indole 5-carboxylic acid, methyl ester (1.0)	99	71
L240C/N297Q	7-chloroindole (0.5)	82	110
L240C/N297Q	5,6-dimethoxyindole (1.0)	80	62
L240C/N297Q	6-fluoroindole (1.0)	97	87
L240C/N297Q	5-methoxyindole (1.0)	3	3
L240C/N297Q	5-methylindole (1.0)	19	15
L240C/N297Q	7-methylindole (1.0)	90	59
L240C/N297Q	6-nitroindole (0.6)	107	63

[a] The table indicates the % residual kinase activity (control = 100%) observed in the presence of 0.3 μL of each extract (in DMSO). Results are presented as means of duplicate experiments.

Chemistry

Spectral Characterization of Indigoids from Incubations with 5-Methoxyindole. The structures were identified as those shown in Chart 1 (spectral properties are indicated in Tables 1S and 2S of the Supporting Information). Indigo (**1**) and indirubin (**4**) were easily identified by comparison with synthetic materials and cochromatography. The colors of the products suggested that they are either indigos (blue) or indirubins (red), and the MS and NMR spectra confirmed this. The presence of one or two methoxy groups was obvious from the mass spectra. The positions of the methoxy groups were predictable, barring unusual rearrangement, and these were confirmed by

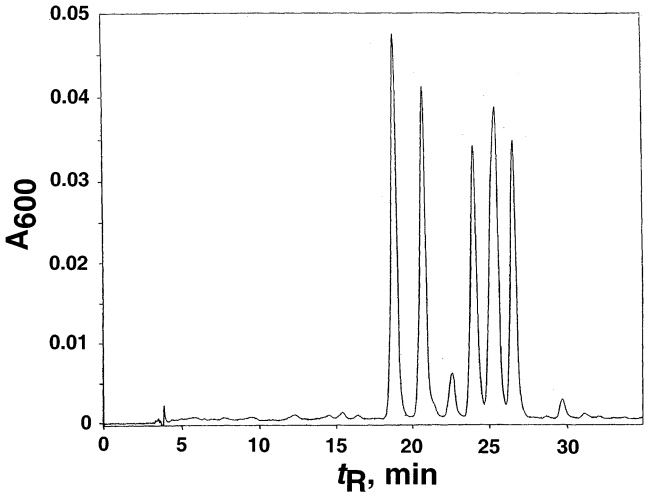
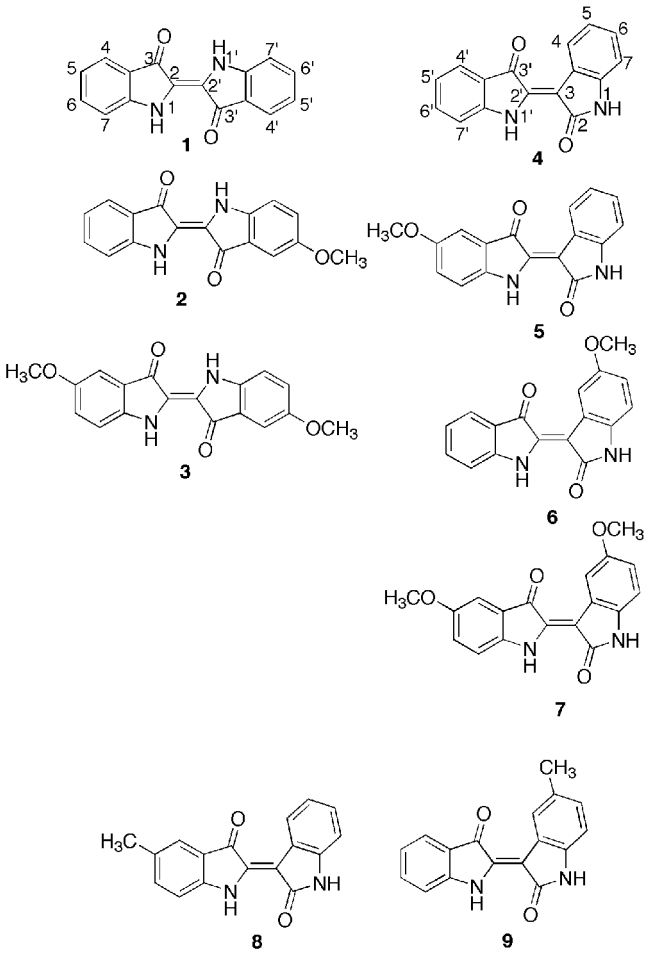


Figure 1. HPLC separation of pigments from a culture of P450 2A6 L240C/N297Q grown with 5-methoxyindole.

Chart 1. Structures of Indigoids Isolated from a Culture of P450 2A6 L240C/N297Q Grown with 5-Methoxyindole (**1**−**7**) Plus Indirubins Isolated from a Culture Grown with 5-Methylindole (**8**, **9**)



NMR spectroscopy. The characterization of 5-methoxyindigo (**2**) was not difficult because of the symmetry of the parent molecule. The methoxyindirubins were less obvious, in terms of which half of the molecule contained the methoxy group. The proton NMR shifts and assignments are shown in Table 1S of the Supporting Information; the assignments were made using a combination of correlation spectroscopy (COSY), nuclear Overhauser correlated spectroscopy (NOESY), heteronuclear multiple quantum correlation spectroscopy (HMQC), and heteronuclear multiple bond correlation spectroscopy (HMBC) experiments (some are presented in the Supporting Information, along with *J* values for proton coupling).

Table 2. Yields of Indigoids from Bacterial Cultures Expressing P450 2A6 L240C/N297Q

compound	nmol (L culture)$^{-1}$
1	600
2	1100
3	190
4	630
5	280
6	440
7	410

The visible spectra showed some differences in λ_{max} among the blue indigos and the red indirubins. The available data suggest that the extinction coefficients are similar, as might be expected.

The indigoids are the products of a mixture of functionalization (3-hydroxylation and subsequent 2-hydroxylation to form isatins, Scheme 1) of endogenous indole and the added indole (Scheme 1). This process gives rise to the "mixed" indigos and indirubins. The presence of unsubstituted indole in the cells is due to the action of tryptophanase on tryptophan. The current procedure is useful in increasing the diversity of reaction products. However, we have developed *trnA*$^-$ (tryptophanase deficient) *E. coli* mutants by recombination (unpublished results) and could use this strain to favor the production of the disubstituted indigoids, e.g., **3** and **7**, if they were found to have a biological activity of interest. The yields of the individual indigoids were estimated from HPLC work using purified indigoids as reference materials (Table 2).

Spectral Characterization of Indigoids from Incubations with 5-Methylindole. Indigoids were isolated from an incubation of the bacteria (with P450 2A6 L240C/N297Q) with 5-methylindole. Preliminary assays showed kinase inhibition by two of the three compounds subsequently identified (Chart 1). The structures of the indigoids paralleled those derived from 5-methoxyindole and were characterized by their MS and NMR spectra as 5′-methylindirubin (**8**) and 5-methylindirubin (**9**). Indirubin was also recovered (5,5′-dimethylindirubin might have been formed in low amounts, but a red peak with the appropriate spectral characteristics was not identified). The structural assignments are based on the more extensive NMR spectral delineation of the methoxyindirubins as reference materials (vide supra). A key to the assignment is the H-4 proton of indirubin, which is the furthest downfield nonexchangeable proton due to its conjugation (Chart 1 and Table 1S of the Supporting Information). The splitting of this signal into a doublet (J = 7.2 Hz) is seen in indirubin and the 5′-substituted indirubins (**5**, **8**) but not the 5-substituted indirubins (**6**, **9**).

Previously, Shim et al.[26] reported the isolation of 5-methylindirubin (from cultures of the plant *Polygonum tinctorium* fortified with 5-methylindole) but apparently only used mass spectrometry for the characterization and did not distinguish between 5- and 5′-methylindirubin by NMR.

Table 3. Inhibition of Kinases by Purified Indigoids[a]

	IC_{50} (μM)		
compound	CDK5/p25	CDK1/cyclin B	GSK-3α/β
1	>100	>100	>100
2	>100	>100	>100
3	26[b]	1.2[b]	6[b]
4	5	6.5	2.5
5	1.2[c]	2.1[c]	0.6[c]
6	1.2[c]	1.5[c]	0.8[c]
7	0.8[c]	0.4[c]	0.2[c]
8	1.9[c]	ND[d]	0.55[c]
9	5	ND[d]	0.8[c]

[a] The kinases were analyzed for inhibition by the indigoids purified from the bacteria. Results are presented based on the use of duplicate assays at each concentration. [b] Significantly less than IC_{50} for **1** ($p < 0.05$, *t*-test). [c] Significantly less than IC_{50} for **4** ($p < 0.05$, *t*-test). [d] ND = not determined.

Biological Activities

The purified indigoids were examined for their abilities to inhibit three kinases: CDK5/p25, CDK1/cyclin B, and GSK-3α/β (Table 3 and Figure 1S of the Supporting Information). Indigo (**1**) and 5-methoxyindigo (**2**) were poor inhibitors; 5,5′-dimethylindigo (**3**) was more active, and its inhibitory potency was roughly similar to that of indirubin (**4**). The substituted indirubins were more potent in inhibiting the kinases, with **5**, **6**, and **8** showing similar inhibitory parameters. The most inhibitory indigoid was 5,5′-dimethoxyindirubin (**7**). As shown previously,[6] indirubins were more potent at inhibiting GSK-3 than CDKs.

Discussion

In the pharmaceutical industry, P450 enzymes are usually considered in the context of drug development as opposed to discovery. However, these catalysts have the potential to be used to prepare novel entities and to be used in the production of fine chemicals including drugs.[28] Thus, P450s can be included in the generally renewed interest in natural products and their improvement through methods of recombinant DNA technology.[29,30]

We report an approach that can probably find other applications in drug discovery. An enzyme library (P450) is generated and utilized with a synthetic library of relatively simple precursor molecules (substituted indoles) to generate a large library of new, complex products. In this particular case, we used the appearance of color as an indicator that a particular system[22] was producing compounds of potential interest. Candidate mixtures were assayed for biological activity, and the most promising preparations were used as sources to purify chemicals. The isolated compunds were reexamined for biological activity, and their structures were identified (Chart 1).

This approach generated novel products with apparent biological activities 10-fold greater than the previously known structures in the area (e.g., indirubin) (Table 3). The increase may not be overly impressive, but we consider this approach to be a model study and the work could be improved in several ways, in future work. The pool of precursor indoles could be expanded, even with commercially available substituted indoles. Another possibility would be the use of the *trnA*$^-$ bacterial strain, free of trypothophanase and low in indole content, thus favoring the synthesis of disubstituted indirubins (Table 3). During the work, we discovered that indirubins display electron capture behavior during mass spectrometry and that the use of negative ion atmospheric chemical ionization technology was advantageous, which would expedite analysis. One of the most laborious aspects of the work was the separation of samples for NMR work, and the availability of modern combined HPLC/NMR would greatly facilitate chemical characterization. Finally, experience with indirubin itself indicates that the oxime derivatives of indirubins have considerably more solubility and should be more useful.[2] Nevertheless, we are of the opinion that the concept of the approach has been validated and may find an application in other systems. We believe that this biochemical approach will allow the generation, from the large number of available indoles, of a large diversity of indirubins from which selective, potent, and pharmacologically optimized kinase inhibitors can be identified and evaluated for their application against various human diseases.[31]

Conclusions

Protein kinases are very numerous and are important targets for many diseases. One group of known inhibitors is the indirubins, which are natural products resulting from indole metabolism. To prepare a library of indirubins, we utilized *E. coli* cells expressing human P450 2A6 mutants that had been selected from random libraries and commercially available substituted indoles to generate mixtures of products, which were screened for the ability to inhibit three kinases—CDK1/cyclin B, CDK5/p25, and GSK-3α/β. From some of the extracts that were mostly inhibitory, we isolated several indirubins that were an order of magnitude more inhibitory than indirubin itself and characterized the structures. The approach can be used as a paradigm for use of enzyme mutagenesis and enzymatic coupling to produce libraries of compounds with pharmacological properties of interest.

Experimental Section

Expression Systems. The system involved expression of human P450 2A6 in *E. coli*.[32] The mutants used here had been selected from libraries generated in selected regions of the P450 2A6 sequence on the basis of their ability to produce colored indigoids.[22] The mutants used in this work were L240C and L240C/N297Q.

Initial Screening. In the initial trials, *E. coli* cells expressing each mutant were grown for 42 h at 30 °C in 50 mL cultures that had been fortified with 0.2−1.0 mM concentrations of several of the 27 indoles used previously[22] (that had yielded colored products in that study). The pelleted cells and the supernatants were separated after centrifugation at $5 \times 10^3 g$ for 15 min. (The supernatants were extracted with 2 volumes of $(C_2H_5)_2O$, and the $(C_2H_5)_2O$ layers were concentrated in vacuo; the residue was dissolved in 10 mL of DMSO.) The cell pellets were extracted with 1.0 mL of DMSO. All samples were diluted in DMSO, and 0.3 mL was added to each CDK5/p25 and GSK-3α/β assay (final volume 30 mL). The activity was expressed as the percentage of the kinase activity measured with 0.3 mL of DMSO. The same final volume of DMSO was used for each bacterial expression of a substituted indole, so that the results then reflect the total inhibitory material per unit volume of bacteria. Results are expressed as means of duplicate assays.

IC_{50} Determinations for CDKs and GSK-3. The following buffers were used—homogenization buffer: 60 mM β-glycerophosphate, 15 mM *p*-nitrophenyl phosphate, 25 mM 3-(*N*-

morpholino)proprane sulfonic acid (MOPS) (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1.0 mM dithiothreitol, 1.0 mM sodium vanadate, 1.0 mM NaF, 1.0 mM phenyl phosphate, 10 μg leupeptin mL^{-1}, 10 μg aprotinin mL^{-1}, 10 μg soybean trypsin inhibitor mL^{-1}, and 100 μM benzamidine; buffer A: 10 mM $MgCl_2$, 1.0 mM EGTA, 1.0 mM dithiothreitol, 25 mM Tris-HCl (pH 7.5), and 50 μg heparin mL^{-1}; and buffer C: homogenization buffer with 5 mM EGTA but no NaF or protease inhibitors.

Kinase activities were assayed in buffer A or C (unless otherwise stated), at 30 °C, at a final ATP concentration of 15 μM. Blank values were subtracted, and activities were calculated as pmol of phosphate incorporated during a 10 min incubation. The activities are expressed as % of the maximal activity, i.e., in the absence of inhibitors. Controls were performed with appropriate dilutions of DMSO.

GSK-3α/β was purified from porcine brain by affinity chromatography on immobilized axin.[33] It was assayed (following a 1/100 dilution in a buffer composed of 1 mg bovine serum albumin mL^{-1} and 10 mM dithiothreitol) with 5 μL of 40 μM GS-1 peptide (YRRAAVPPSPSLSRHSSPHQSpEDEEE, synthesized by the Peptide Synthesis Unit, Institute of Biomolecular Sciences, University of Southampton, Southampton SO16 7PX, U.K.) as a substrate, in buffer A, in the presence of 15 μM [γ-^{33}P]ATP (3000 Ci $mmol^{-1}$; 1 mCi mL^{-1}) in a final volume of 30 μL. After 30 min of incubation at 30 °C, 25 μL aliquots of supernatant were spotted onto 2.5 cm × 3 cm pieces of Whatman P81 phosphocellulose paper, and 20 s later, the filters were washed five times (for at least 5 min each time) in an aqueous solution of 10 mL of 0.15 M H_3PO_4. The wet filters were counted in the presence of 1 mL of ACS scintillation fluid (Amersham, Des Plaines, IL).

CDK1/cyclin B was extracted in homogenization buffer from M-phase starfish (*Marthasterias glacialis*) oocytes and purified by affinity chromatography on $p9^{CKShs1}$-Sepharose beads, from which it was eluted with free $p9^{CKShs1}$ as previously described.[34] The kinase activity was assayed in buffer C, with 1 mg histone H1 mL^{-1}, in the presence of 15 μM [γ-^{33}P]ATP (3000 Ci $mmol^{-1}$; 1 mCi mL^{-1}) in a final volume of 30 μL. After 10 min of incubation at 30 °C, 25 μL aliquots of supernatant were spotted onto P81 phosphocellulose papers and treated as described above.

CDK5/p25 was reconstituted by mixing equal amounts of recombinant mammalian CDK5 and p25 expressed in *E. coli* as glutathione S-transferase fusion proteins and purified by affinity chromatography on glutathione-agarose (vectors kindly provided by Dr. J. H. Wang) (p25 is a truncated version of p35, the 35 kDa CDK5 activator). Its activity was assayed in buffer C as described for CDK1/cyclin B. All assays were performed in duplicate, and both values and their means are presented (e.g., Figure 1S of the Supporting Information).

Large Scale Bacterial Expression for Indigoid Characterization. Six 2.8 L Fernbach flasks were prepared with 1000 mL of media containing 48 g of Terrific Broth (Becton, Dickinson and Co., Sparks, MD), 2.0 g of Bacto-Peptone (Difco, Detroit, MI), and 0.4% (v/v) glycerol and autoclaved for 25 min. Six 10 mL solutions of Luria−Bertani media (Becton, Dickinson, and Co.), each containing 1 μg of ampicillin, were inoculated with a fresh transformation of the pCW′ vector containing P450 2A6 L240C/N297Q and human NADPH-P450 reductase in a bicistronic plasmid.[22,35] These starter cultures were grown for 16 h at 37 °C with gyrorotary shaking at 240 rpm. One 10 mL starter culture was added to each 1000 mL flask of media, along with 1.0 mmol of 5-aminolevulinic acid, 100 μg of ampicillin, 1 mmol of thiamine, and 0.25 mL of a mixture of trace elements.[36] The cultures were incubated for 3 h at 30 °C with gyrorotary shaking at 250 rpm. At that time, 1.0 mL of 1 M D-isopropyl-β-thiogalactoside and 1.0 mL of 1 M 5-methoxyindole (each dissolved in C_2H_5OH) were added, and shaking continued for 45 h at 30 °C.

Extraction and Separation of Pigments from Bulk Cultures. Bacteria recovered from the above procedure (6 L) were pelleted by centrifugation at $5 \times 10^3 g$ for 15 min. Each dark blue pellet (from 1000 mL of bacterial culture) was stirred with 1000 mL of $(CH_3)_2CO$ and filtered through paper. The $(CH_3)_2CO$ extract was concentrated in vacuo, and the resulting solids were stirred overnight (at room temperature) in 65% aqueous CH_3OH, v/v. The solution was filtered to yield a blue solid (on filter, indigo-enriched fraction) and a filtrate (indirubin-enriched fraction).

The blue solid (from a total of 6 L of bacterial culture) was washed with 500 mL of H_2O and dissolved in 500 mL of CH_2Cl_2, which was dried with anhydrous Na_2SO_4. The solvent was removed in vacuo, and the residue was dissolved in a minimum amount of DMSO for HPLC. HPLC was done on a 22 mm × 250 mm Altima octadecylsilane (C_{18}) column (Alltech, Deerfield, IL), with a CH_3OH/H_2O mobile phase (several buffered systems did not yield improved resolution, nor did substitution of CH_3CN or tetrahydrofuran for CH_3OH). A linear gradient of 65−90% CH_3OH (v/v, in H_2O) over 30 min was used with a flow rate of 10 mL min^{-1}. Detection was at 600 nm or, in analytical systems, using an HR-UV3000 rapid-scanning monochromator (ThermoSeparations, Piscataway, NJ). Peaks were collected manually, and most of the CH_3OH was removed with a rotary evaporator. The remaining aqueous phase was extracted three times with equal volumes of CH_2Cl_2, and the CH_2Cl_2 layers were pooled, dried with Na_2SO_4, and concentrated to dryness.

The reddish filtrate resulting from the initial extraction of the cells was extracted three times with 500 mL of CH_2Cl_2, and the CH_2Cl_2 extracts were pooled, dried with Na_2SO_4, and concentrated in vacuo. HPLC was done as in the case of the blue indigo fraction, yielding two large peaks, each of which could be separated into two components using a similar analytical HPLC system or TLC. The two large HPLC peaks (same solvent program as for indigos) were collected and extracted with CH_2Cl_2 as in the case of the indigos. Each of the two HPLC peaks was further separated by preparative TLC (1 mm × 20 cm × 20 cm, silica gel G, CH_2Cl_2/hexane, 80:20, v/v), extracted with $(CH_3)_2CO$, filtered, and evaporated to dryness.

Spectroscopy. Visible spectra were recorded either with the line HPLC/rapid-scanning monochromator system described above or (particularly for quantitative studies) in CH_3OH using a Cary 14/OLIS spectrophotometer (OLIS, Bogart, GA).

Mass spectra of the indigoids were obtained in the Vanderbilt facility, either on-line from HPLC or with direct infusion using a Finnigan TSQ 7000 instrument (Thermo Finnigan, San Jose, CA) triple quadropole mass spectrometer equipped with a standard API-1 atmospheric pressure chemical ionization source running in both positive and negative ion modes. N_2 was used as a sheath gas, which was set to 80 psi for the positive mode and 50 psi for the negative mode. The vaporizer temperature was set to 350 °C, and the corona current was maintained at 5 μA for both the positive and the negative modes. The capillary was set to 200 °C and 34.5 V for the positive mode and 175 °C and −30 V for negative mode. The tube lens voltage was set to 83 V for the positive mode and −80 V for the negative mode. The collision-induced dissociation (CID) offset was to 20 V in the negative mode. In the positive mode, CID spectra were also obtained in some cases with an offset voltage of −50 V. Data acquisition and spectral analysis were conducted with Finnigan ICIS software on a Digital Equipment Corp. Alpha workstation.

High-resolution mass spectra were recorded on compounds **1**−**7** to four decimal places on a magnetic sector instrument at the facility in the Department of Chemistry, University of Notre Dame (South Bend, IN). All NMR experiments were carried out at 298 K on a Bruker Avance DRX 400 MHz instrument (Bruker, Billerica, MA) employing a 5 mm high-resolution broad band probe with ^{1}H decoupling. All samples were dissolved in d_6-DMSO and calibrated to the DMSO proton and carbon signals (δ 2.49 for ^{1}H, 39.50 for ^{13}C). Semiquantitative estimates of the amounts of samples were made by adding known amounts of CH_3CO_2H and referencing to the $-CH_3$ signal. Detailed structural information for the indigoids **5** and **6** was obtained from four separate two-dimensional

NMR experiments (see the Supporting Information section for spectra and assignments). Through-bond H−H connectivity was observed using COSY, and through-space H−H proximity was observed using NOESY with a mixing time of 400 ms. Single C−H bond connectivity was obtained using HMQC, and long-range C−H bond connectivity (2−3 bonds) was obtained using HMBC with a 60 ms delay. All NMR data were processed using XWIN NMR software (Bruker) on a Silicon Graphics O2 visual workstation.

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Supporting Information Available: Tables of low (compounds **1**−**9**) and high-resolution (compounds **1**−**7**) mass spectra and NMR assignments, IC_{50} determinations for compound **6** (CDK1, CDK5, and GSK-3), NMR connectivities, and one- and two-dimensional NMR spectra of compounds **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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