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Rational Development of Novel Activity Probes for the Analysis of Human Cytochromes P450

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The identification and quantification of functional cytochromes P450 (CYPs) in biological samples is proving important for robust analyses of drug efficacy and metabolic disposition. In this study, a novel CYP activity-based probe was rationally designed and synthesised, demonstrating selective binding of CYP isoforms. The dependence of probe binding upon the presence of NADPH permits the selective detection of functionally active CYP. This allows the detection and analysis of these enzymes using biochemical and proteomic methodologies and approaches.

Cytochromes P450 (CYPs) constitute a large family of haem-centred enzymes, with fundamental roles in the biotransformation of endogenous (steroid hormones, fatty acids, prostaglandins) and exogenous molecules (drugs, environmental chemicals, agrochemicals). Given their importance, particularly in xenobiotic and drug metabolism,[1] a great deal of research has been conducted into their roles, the identification of their sequences, and their catalytic mechanisms.[2–4] Whilst a number of CYPs, particularly human liver CYPs and extrahepatic CYPs (i.e., CYP1A1, CYP1B1, and CYP2W1) have been the subject of intense investigation,[5–7] there is still much to be learnt from these mixed-function oxidases. This reflects the difficulties associated with studying these enzymes, because: 1) they are encoded by large gene families, and their functions cannot be predicted from their gene sequence; 2) they are difficult to assay, isolate and purify, so classical biochemical methods are often ineffective in identifying enzymes of interest; 3) these proteins are membrane bound and often depend on co-enzymes and cofactors, making them difficult to express as functional enzymes in cellular systems; and 4) polymorphisms and epigenetic regulation alter their expression levels and functional activity. Therefore, new approaches to identifying, evaluating, and quantifying functionally active CYPs are of utmost importance. One such approach is activity-based protein profiling, which involves 'tagging' the functional protein with a selective small-molecule affinity probe by covalent attachment (Figure 1, pathway B).[8–13]

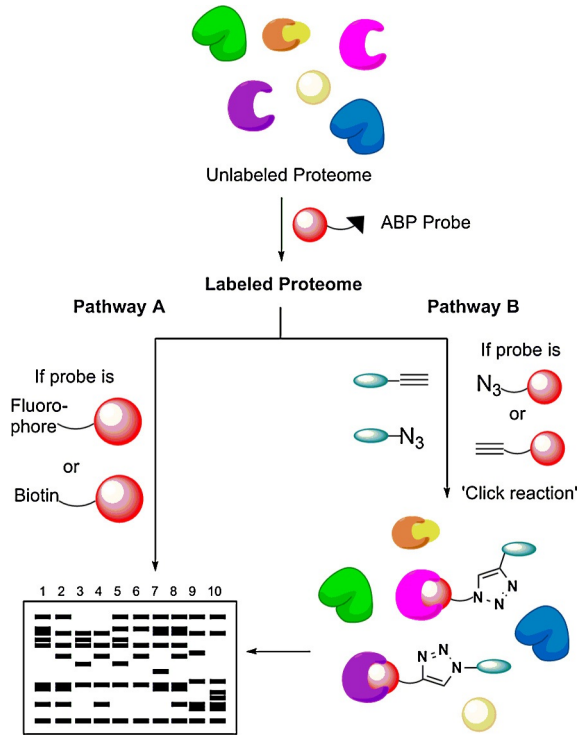

Figure 1. SDS-PAGE activity-based protein profiling, directly with appropriately functionalised probes (pathway A) or orthogonally by using 'click chemistry' (pathway B).

Whilst CYP probes developed to date exhibit the ability to bind these enzymes and thus indicate potential utility, several have failed to demonstrate reactivity as a consequence of steric demands of the probe, and the majority lack differential or selective affinity for functional CYP.[14–18] As a result, there is a requirement for new activity-based probes to study and identify this enzyme class. Herein we report the synthesis of a small group of rationally designed activity-based probes for this purpose, identifying benzofurans as suitable pharmacophores. In addition, evaluation of the probes' efficiency and capacity to selectively label CYPs was achieved using well-established biochemical techniques such as enzyme kinetics, immunoblotting, and proteomic mass spectrometry.

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The probe designs were inspired from the natural product furanocoumarin family, which are known irreversible inhibitors of CYPs.[14–17] Our rationale focused on the structural elements required for a probe to function efficiently and—most importantly, selectively—against CYPs. Furanocoumarins inhibit CYPs following oxidation of the furan moiety to yield a reactive furan epoxide, which undergoes nucleophilic attack from a protein amino acid side chain to covalently bind the apoprotein. Therefore, a rational disconnection approach identified two structural analogues, **7** and **8**, with both retaining the furan moiety and a position for functionalisation with a reporter group; compound **8** possesses an additional aromatic ring to aid for greater selectivity in the active site (Figure 2). A third probe **9** was designed from coumarin, a known substrate of CYPs,[19,20] to understand the requirement of the lactone fragment present in furanocoumarins. Coumarin is hydroxylated at the 7-position, and so it was envisioned that by installing a chloromethyl group at the 6-position, the hydroxylated product would undergo rearrangement in the active site to produce an *ortho*-quinone methide **5**, which would undergo attack from a protein amino acid side chain, covalently linking the probe and enzyme (Figure 2).

Synthesis of the furan and benzofuran probes **11** and **14** was carried out in a linear sequence beginning with the addition of monoprotected cadaverine[21,22] to the activated carboxylic acids **7** and **12**. Use of the reactive acid chloride of **7** led to satisfactory but low-yielding amide **10**, presumably due to the instability of the acid chloride. Notwithstanding this, expedient synthesis of probes **11** and **14** was achieved by trifluoroacetic acid (TFA) deprotection of amides **10** and **13** followed by acylation with biotin–NHS. Synthesis of probe **20** was undertaken with a divergent approach starting from 2-hydroxybenzaldehyde. Following a published procedure,[23] a hydroxymethyl group was introduced with formaldehyde and concentrated hydrochloric acid to produce diol **15**, with subsequent cyclisation of the aldehyde and phenolic hydroxy group with 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid) to provide the coumarin architecture **16**. Following a published procedure[24] biotin was activated as the mixed anhydride, instead of the HOBt ester, and extended with monoprotected cadaverine[21,22] to provide **18** in good yield. Finally, **16** and **18** were joined through activation of the carboxylic acid moiety, and concomitant conversion of the hydroxymethyl group into the chloride with thionyl chloride and deprotection of **18** with TFA to produce **20** in good yield over three steps (Scheme 1).

The ability and potential of probes **11**, **14**, and **20** to bind and inactivate CYP was evaluated with the Vivid assay[25] for two recombinant enzymes: CYP3A4, a central component of many biotransformation processes known to be inhibited by furanocoumarins, and CYP1A2, which has an unknown endogenous substrate, but broad exogenous substrate scope.[14,26] Initially, varying concentrations of methanol (**11** and **14**) and DMSO (**20**) solvent controls were assessed in the assay to determine if they had any deleterious effects. Unsurprisingly, DMSO was observed to be very toxic to the CYP3A4 assay, resulting in 50% inhibition at 0.5% DMSO. This precluded the measurement of **20**, which was soluble only in DMSO.[27,28] The CYP3A4 assay was also sensitive to methanol, but to a lesser extent; methanol could be used up to concentrations of 10% in combination with water to dilute **11** and **14**.[29] The CYP1A2 assay, by contrast, was tolerant of DMSO to 2% as well as methanol to 10%. Consequently, dilutions of **11** and **14** in 4–10% methanol/water for both CYPs, and dilutions of **20** in 4% methanol/2% DMSO for CYP1A2 enabled the determination of IC_{50} values for these enzymes.[29] Probes **11**, **14**, and **20** exhibited no significant inhibition of CYP1A2 activity at concentrations up to 2 mM, whereas **11** and **14** inhibited CYP3A4 at 230 and 90 μM, respectively (Table 1). In addition, the K_{inact} and K_i values were determined for **14** to be 0.042 min^{-1} and 0.5 mM, respectively (Figure 3).

Having established that **11** and **14** are capable of inhibiting CYP3A4, their ability to act as a probe and identify the enzyme–probe complex was assessed. As **11** and **14** possess a biotin moiety, well-established streptavidin-mediated identification was used. Firstly, incubation of **11** and **14** with bactosomes followed by streptavidin blotting revealed that both **11** and **14** are activated by CYP3A4 and appear to covalently label the proteome; however, multiple bands were detected with both probes.[29] These extra bands may be the result of several factors, such as CYP3A4 aggregates and degradation products resulting from overexpression of the gene in *Escherichia coli* or binding to *E. coli* proteins. The greater degree to which **11**

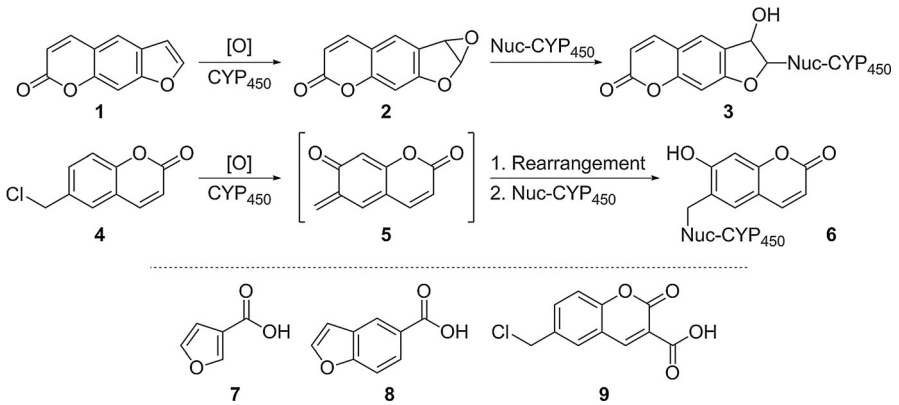
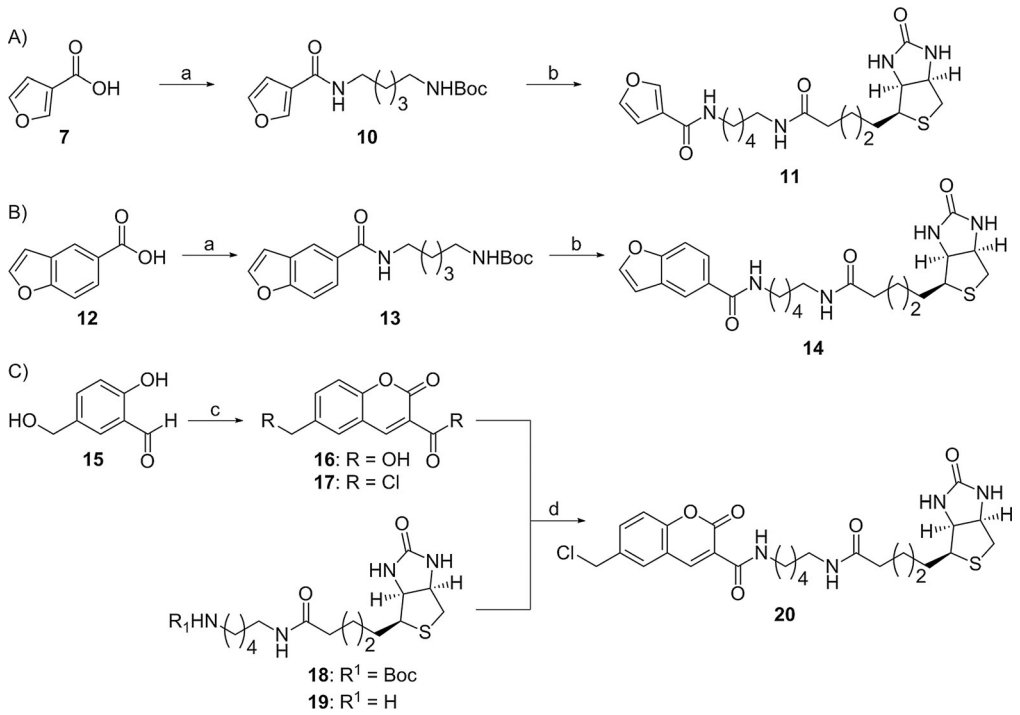


Figure 2. Mechanism of furanocoumarin inhibition; structural analogues for probe synthesis.



Scheme 1. *Reagents and conditions:* a) 1. $SOCl_2$, DMF (cat.), 2. *N*-Boc-1,5-diaminopentane, DIPEA in CH_2Cl_2; b) 1. **10** or **13**, TFA, CH_2Cl_2, 2. biotin–NHS, Et_3N, DMF; c) 2,2-dimethyl-1,3-dioxane-4,6-dione, pyridine, CH_2Cl_2, Δ; d) 1. **16**, $SOCl_2$, Δ, 2. **18**, TFA, CH_2Cl_2, 3. **17** + **19**, excess Et_3N.

Table 1. IC_{50} values for cytochrome P450 3A4 and 1A2.

Probe	IC_{50} [μM]	
	CYP3A4[a]	CYP1A2[b]
11	230 ± 141	> 2000
14	90 ± 7	> 2000
20	ND	> 2000

Values are the mean ± SD of [a] $n = 2$ or [b] $n = 3$ measurements; ND: not determined.

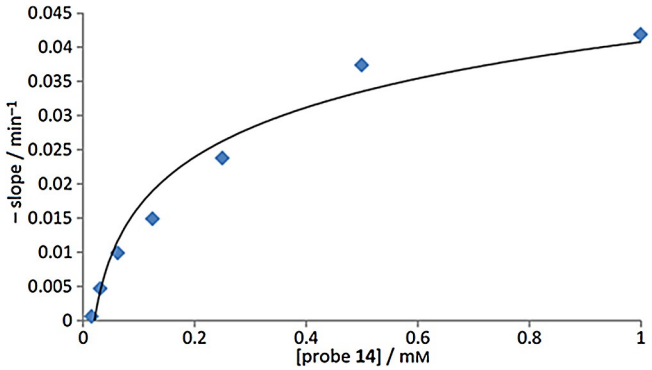


Figure 3. K_{inact} measurement of **14** for CYP3A4.

labels the proteome suggests it may be released from the active site as the long-lived ene–dial intermediate, formed from rearrangement of the furan epoxide (cf. **2**), whereas **14** is a benzofuran which possesses an aromatic ring, increasing its binding affinity to the active site whilst also producing a more reactive benzofuran dioxetane, making labelling of the CYP more competitive.[30–33,34]

As a result, subsequent studies were conducted with **14**, because fewer off-target binding events were observed. Firstly, incubation of **14** with baculosomes followed by desalting of the sample to remove free probe before binding to streptavidin magnetic beads and SDS-PAGE analysis of the bound proteins was performed (Figure 4A). Pleasingly, **14** revealed a distinguishable strong band at ~55 kDa on staining with Coomassie Blue; however, additional bands were still detected. Subsequently, **14** was treated under the same conditions as before, except visualisation was done by western blotting with horseradish peroxidase-conjugated streptavidin and ECL plus detection (Figure 4B). Satisfyingly, **14** produced a band at 55 kDa, indicative of binding and enrichment of CYP3A4.

Having demonstrated that **14** is capable of identifying a band at ~55 kDa and with low off-target binding, our attention turned to identifying the attached protein. Excision, digestion, and mass spectrometric analysis of the 55 kDa band from the Coomassie Blue stained gel identified the protein to be CYP3A4 by peptide mass fingerprinting (Mascot score = 110, with 26 matched peptides; MS threshold > 56 was indicative of extensive homology) and MS–MS analysis of $[M+H]^+ =$ 1812.039 (score = 89, MS–MS threshold > 30 was indicative of extensive homology), 1457.944 (score = 47), and 1960.278 (score = 69).

Satisfied with these results, our attention turned to the selectivity of **14** against an array of bactosome-expressing CYPs using streptavidin blotting. Probe **14** formed clear NADPH-dependent complexes with CYP1B1, CYP2B6, and CYP3A4, and to a lesser extent, CYP2C9. The promiscuity of CYPs is not unex-

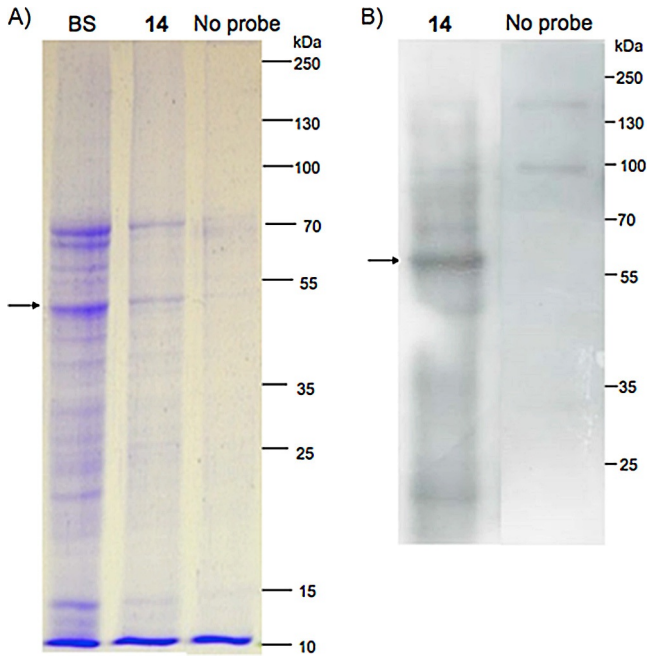


Figure 4. A) Coomassie Blue-stained SDS polyacrylamide gel of CYP3A4 complexed with **14** and recovered from streptavidin magnetic beads; BS: baculosome CYP3A4 starting material. B) Streptavidin blot of **14**, with no-probe control incubated with CYP3A4 baculosomes.

pected, as they have been implicated in the metabolism of a variety of xenobiotic compounds in human liver.[35–37] The level of selectivity displayed by **14** in binding to CYPs in an NADPH-dependent manner with little to no binding observed in its absence is exemplary (Figure 5 A). Using CYP3A4 as an example, the time- and NADPH-dependent binding of **14** was shown to occur within 1 min (Figure 5 B).

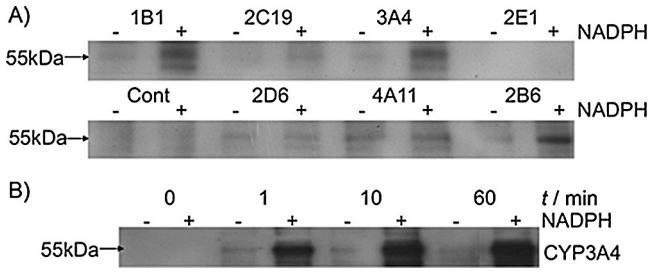


Figure 5. A) Streptavidin blot of probe **14** incubated with bactosomes expressing a specific CYP isoform in the presence or absence of NADPH. B) Time course of **14** reaction with CYP3A4.

In conclusion, a small group of rationally designed probes have been synthesised, with benzofuran probe **14** demonstrating respectable inhibitory activity against CYP3A4 (IC_{50} = 90 μM). In addition, **14** was able to identify CYP3A4 using well-established biochemical techniques, immunoblotting, and mass spectrometry. There have been a number of studies to explore the binding of xenobiotics or activity-based probes to CYPs, founded on coumarin and furan scaffolds. Furan is an established toxicant in liver, and a screen of recombinant CYPs identified that CYP2E1 and CYP2D6 are responsible for its metabolism, with limited activity from CYP3A4.[38] There was inhibition of CYP3A4 activity in the Vivid assay by probe **11** (furan derivative), which may be due to bioactivation of the furan moiety resulting in the production of *cis*-2-butene-1,4-dial, which in turn reacts with lysine side chains and N-termini of proteins.[30] Coumarin and benzofuran derivatives (related to naturally occurring compounds in grapefruit juice) were synthesised and analysed for CYP3A4 inhibition, with the latter having been found to be the most potent.[39] Our benzofuran **14** exhibited the most effective NADPH-dependent binding to CYP3A4 relative to other CYPs tested. It therefore shows great promise for further investigation. Given the limited functionalisation of **14**, the level of selectivity demonstrated in binding CYPs in an NADPH-dependent manner was exemplary. In this respect, it is notable that a number of early CYP probes demonstrate similar selectivity for a range of CYPs.[18] Work is currently in progress to further optimise the selectivity of **14** and extend the library of probes to new CYPs.

Experimental Section

All air- and/or moisture-sensitive reactions were carried out under an argon atmosphere. Solvents were purified and dried following established protocols. All commercially available reagents were used as received unless otherwise stated. Flash column chromatography was performed according to the method of Still et al. using 200–400 mesh silica gel.[40] Yields refer to isolated yields of products of >95% purity as determined by ^{1}H and ^{13}C NMR spectroscopy. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. Infrared spectra were recorded using a Diamond ATR (attenuated total reflection) accessory (Golden Gate) on a PerkinElmer FT-IR 1600 spectrometer. Unless otherwise stated, ^{1}H NMR spectra were recorded in $CDCl_3$ on Varian Mercury 200, Varian VXR 400, Bruker Avance 400, Varian Inova 500, and Varian VNMRS 700 instruments, and are reported as follows: chemical shift δ [ppm] (number of protons, multiplicity, coupling constant J (Hz), assignment). Residual protic solvent $CHCl_3$ (δ_H = 7.26) was used as the internal reference. ^{13}C NMR spectra were recorded at 63 or 126 MHz, using the central resonance of $CDCl_3$ (δ_C = 77.0 ppm) as the internal reference. All ^{13}C NMR spectra were proton decoupled. Assignment of spectra was carried out using DEPT, COSY, HSQC, HMBC, and NOESY experiments. High-resolution mass spectrometry was performed on an LTQ FT mass spectrometer (Thermo Finnigan Corp.) using flow-injection electrospray ionisation.

Standard procedure for the formation of 10 and 13: The carboxylic acid **7** or **12** (1 equiv) was treated with excess thionyl chloride and a drop of DMF. After stirring for the reported time and temperature, the solvent was removed in vacuo, and the resultant material was dissolved and evaporated with CH_2Cl_2 (3×10 mL) to ensure removal of excess thionyl chloride. The resultant material was dissolved in CH_2Cl_2, treated with *N*-Boc-cadaverine (1.2 equiv) and DIPEA (4 equiv) and stirred for the reported time and temperature. The reaction was then poured into saturated aqueous NH_4Cl (10 mL), dried with $MgSO_4$, filtered, and evaporated in vacuo.

***N*-Boc-(5-aminopentyl)-3-furancarboxamide 10**: Following the standard procedure, 3-furoic acid **7** (100 mg, 0.9 mmol) was transformed into a brown solid **10** (153 mg, 58%); R_f = 0.6 (*n*-hexane/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 7.96 (1H, dd, J = 1 Hz,

ArH), 7.44 (1H, dd, $J=2$ Hz, ArH), 6.67–6.62 (1H, m, ArH), 5.99 (1H, bs, NH), 4.62 (1H, bs, NHBoc), 3.44–3.39 (2H, q, $J=8$ Hz, CH_2), 3.15 (2H, m, CH_2), 1.67–1.46 (6H, m, CH_2), 1.45 ppm (9H, s, NHBoc); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta_C=162.7$ (C=O), 156.2 (C=O), 144.7 (ArC), 143.7 (ArC), 122.6 (*ipso*-ArC), 108.3 (ArC), 39.3 (CH_2), 29.8 (CH_2), 29.2 (CH_2), 28.4 (Boc), 23.9 ppm (CH_2); MS *m/z* (+ES): 615.2 $[2M+Na]^+$, 319.6 $[M+Na]^+$, 297.2 $[M+H]^+$.

***tert*-Butyl 5-(benzofuran-5-carboxamido)pentylcarbamate 13**: Following the standard procedure, benzofuran-5-carboxylic acid **12** (100 mg, 0.62 mmol) was transformed into a cream-coloured solid **13** (73 mg, 34%); $R_f=0.8$ (*n*-hexane/EtOAc 1:1); IR (ATR): $\tilde{\nu}=3372$ (NH), 3328 (NH), 2932, 2870, 1685 (C=O), 1628 (C=O), 1522, 1473, 1365, 1164, 1136, 1115, 1044, 1023, 1012, 949, 908, 883, 847, 821 cm^{-1}; ^{1}H NMR (700 MHz, $CDCl_3$): $\delta_H=8.06$ (1H, d, $J=2$ Hz, ArH), 7.72 (1H, dd, $J=9$, 2 Hz, ArH), 7.68 (1H, d, $J=2$ Hz, ArH), 7.52 (1H, d, $J=9$ Hz, ArH), 6.82 (1H, dd, $J=2$ Hz, 1, ArH), 6.24 (1H, bs, NH), 4.57 (1H, bs, NH), 3.49 (2H, q, $J=6$ Hz, CH_2), 3.15–3.12 (2H, m, CH_2), 1.69–1.64 (2H, m, CH_2), 1.58–1.51 (4H, m, CH_2), 1.45–1.41 ppm (9H, s, NHBoc); ^{13}C NMR (700 MHz, $CDCl_3$): $\delta_C=167.0$ (C=O), 156.5 (*ipso*-ArC), 156.1 (*ipso*-ArC), 146.2 (ArC), 130.0 (*ipso*-ArC), 127.5 (*ipso*-ArC), 123.3 (ArC), 120.6 (ArC), 111.4 (ArC), 107.0 (ArC), 40.2 (CH_2), 40.0 (CH_2), 29.8 (CH_2), 29.3(CH_2), 28.4 (Boc), 24.0 ppm (CH_2); MS *m/z* (+ES): 369 $[M+Na]^+$, 244 $[M-Boc]^+$; HRMS (+ES) found: $[M+Na]^+$, 369.1803 ($C_{19}H_{26}N_2O_4Na$ requires 369.1790).

Standard procedure the formation of 11 and 14: The *N*-Boc-protected furancarboxamide **10** or **13** (0.2–0.4 mmol) was dissolved in CH_2Cl_2 (5 mL) and treated with excess TFA (~1 mL). After stirring for the reported time and temperature, the solvent was removed in vacuo, and the resultant material was dissolved and evaporated with CH_2Cl_2 (3×10 mL) to ensure removal of excess TFA. The resultant material was then dissolved in DMF, treated with biotin–NHS (1.1 equiv) and Et_3N (2.1 equiv) and stirred for 4 h. The solvent was then removed in vacuo, and the resultant material was subjected to flash chromatography (CH_2Cl_2/MeOH). Subsequent trituration with CH_2Cl_2 (5 mL) produced the desired product.

***N*-[5-(Furan-3-ylformamido)pentyl]-5-{2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazolidin-4-yl}pentanamide 11**: Following the standard procedure, *N*-Boc-protected furancarboxamide **10** (100 mg, 0.4 mmol) was transformed into a yellow solid **11** (113 mg, 79%); $R_f=0.3$ (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, CD_3OD): $\delta_H=8.06$ (1H, dd, $J=1$ Hz, ArH), 7.58 (1H, dd, $J=2$ Hz, ArH), 6.81 (1H, dd, $J=2$, 1 Hz, ArH), 4.53 (1H, ddd, $J=8$, 5, 1 Hz, CHH), 4.32 (1H, dd, $J=8$, 4 Hz, (CH)H), 3.26–3.18 (4H, m, CH_2), 2.72 (2H, m, CH_2), 2.21 (2H, t, $J=7$ Hz, CH_2), 1.82–1.39 ppm (12H, m, CH_2); MS *m/z* (+ES): 445 $[M+Na]^+$, 424 $[M+H]^+$.

***N*-(5-(5-((3a*S*,4*S*,6a*R*)-2-Oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)pentyl)benzofuran-5-carboxamide 14**: Following the standard procedure, *tert*-butyl 5-(benzofuran-5-carboxamido)pentylcarbamate **13** (100 mg, 0.29 mmol) was transformed into a white gummy solid **14** (100 mg, 73%); $R_f=0.3$ (CH_2Cl_2/MeOH 9:1); IR (ATR): $\tilde{\nu}=3584$–3072, 2940, 2868, 1697 (C=O), 1676 (C=O), 1635 (C=O), 1524, 1461, 1316, 1262, 1179, 1104, 1025, 826 cm^{-1}; ^{1}H NMR (700 MHz, CD_3OD): $\delta_H=8.11$ (1H, dd, $J=2$ Hz, 1, ArH), 7.84 (1H, d, $J=2$ Hz, ArH), 7.78 (1H, dd, $J=9$, 2 Hz, ArH), 7.56 (1H, dt, $J=9$, 1 Hz, ArH), 6.93 (1H, dd, $J=2$, 1 Hz, ArH), 4.47 (1H, ddd, $J=8$, 5, 1 Hz, CH), 4.27 (1H, dd, $J=8$, 5 Hz, CH), 3.42–3.39 (3H, t, $J=8$ Hz, CH_2), 3.21–3.15 (4H, m, CH_2), 2.90 (1H, dd, $J=13$, 5 Hz, CH_2), 2.68 (1H, d, $J=13$ Hz, CH_2), 2.17 (2H, m, CH_2), 1.70–1.55 (4H, m, CH_2), 1.45–1.38 ppm (4H, m, CH_2); ^{13}C NMR (700 MHz, CD_3OD): $\delta_C=176.1$ (C=O), 170.5 (C=O), 166.1 (C=O), 158.0 (*ipso*-ArC), 147.9 (ArC), 130.9 (ArC), 128.9 (*ipso*-ArC), 124.8 (ArC), 121.9 (*ipso*-ArC), 112.1 (ArC), 108.1 (ArC), 63.4 (CH), 61.6 (CH), 56.9 (CH_2), 41.05, 41.0, 40.9, 40.2, 36.8, 30.11, 30.07, 29.7, 29.4, 26.9, 25.3 ppm; MS *m/z* (+ES): 967 $[2M+Na^+]$, 495 $[M+Na]^+$, 473 $[M+H]^+$; HRMS (+ES) found: $[M+Na]^+$, 495.2025 ($C_{24}H_{32}N_4O_4SNa$ requires 495.2042).

6-(Hydroxymethyl)-2-oxo-2*H*-chromene-3-carboxylic acid 16: 2-Hydroxy-5-(hydroxymethyl)benzaldehyde (1.2 g, 7.9 mmol) was dissolved in CH_2Cl_2 and treated with 2,2-dimethyl-1,3-dioxane-4,6-dione (1.4 g, 9.5 mmol) and pyridine (1.3 mL, 15.8 mmol). The solution was held at reflux for 2 h; then, after cooling, the precipitate was collected by filtration and washed with CH_2Cl_2 to afford the title compound as a pale-yellow solid (1.2 g, 70%); ^{1}H NMR (400 MHz, DMSO): $\delta_H=8.43$ (1H, s, ArH), 7.73 (1H, s, ArH), 7.58 (1H, dd, $J=9$, 2 Hz, ArH), 7.34 (1H, s, ArH), 4.55 ppm (2H, s, ArCH_2); MS *m/z* (+ES): 243 $[M+Na]^+$, 221 $[M+H]^+$; all data agree with those reported previously.[23]

6-(Chloromethyl)-2-oxo-*N*-(5-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)pentyl)-2*H*-chromene-3-carboxamide 20: 6-(Hydroxymethyl)-2-oxo-2*H*-chromene-3-carboxylic acid **16** (50 mg, 023 mmol) was dissolved in thionyl chloride (4 mL) and held at reflux for 2 h. At the same time, *tert*-butyl 5-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)pentylcarbamate[24] **18** (97 mg, 0.23 mmol) was dissolved in CH_2Cl_2 (2 mL) and treated with TFA (2 mL). Evaporation of the two reactions to remove the thionyl chloride and TFA was followed by dissolution of the two reactions in CH_2Cl_2 (2 mL). The crude 6-(chloromethyl)-2-oxo-2*H*-chromene-3-carbonyl chloride **17** was then added via cannula to a mixture of crude *N*-(5-aminopentyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-pentanamide **19** and Et_3N (95 μL, 0.7 mmol) at room temperature. The reaction was stirred for 2 h and then evaporated. Flash chromatography (CH_2Cl_2, CH_2Cl_2/MeOH [99:1], [98:2], [95:5], [9:1]) followed by trituration with MeOH afforded the title compound as a white gummy solid (55 mg, 44%); $R_f=0.3$ (CH_2Cl_2/MeOH 9:1); IR (ATR): $\tilde{\nu}=3512$–3150, 2928, 2854, 1697 (C=O), 1653 (C=O), 1614 (C=O), 1573, 1536, 1419, 1246, 1168, 1021, 827 cm^{-1}; ^{1}H NMR (500 MHz, DMSO): $\delta_H=8.80$ (1H, s, ArH), 8.67 (1H, t, $J=6$ Hz, NH), 8.02 (1H, d, $J=2$ Hz, ArH), 7.80 (1H, dd, $J=9$, 2 Hz, ArH), 7.52 (1H, d, $J=9$ Hz, ArH), 6.42 (1H, s, NH), 6.35 (1H, s, NH), 4.84 (2H, s, ArCH_2), 4.29 (1H, m, CH), 4.15 (1H, m, CH), 3.09–3.00 (4H, m, CH_2), 2.80 (2H, dd, $J=12$ Hz, 5, CH_2), 2.64 (2H, m, CH_2), 2.02 (2H, t, $J=6$ Hz, CH_2), 1.62–1.23 (6H, m, CH_2), 1.15 ppm (2H, t, $J=6$ Hz, CH_2); ^{13}C NMR (500 MHz, DMSO): $\delta_C=172.6$ (C=O), 163.4 (*ipso*-ArC), 161.6 (C=O), 161.0 (C=O), 154.2 (C=O), 147.5 (ArC), 135.39 (*ipso*-ArC), 135.20 (ArC), 130.9 (ArC), 120.4 (*ipso*-ArC), 119.2 (*ipso*-ArC), 117.4 (ArC), 61.7, 59.9, 56.1, 46.4, 45.7, 38.9, 35.9, 29.5, 29.4, 28.9, 28.7, 26.0, 24.5, 9.3 ppm; MS *m/z* (+ES): 549 $[(^{35}Cl)M+H]^+$; HRMS (+ES) found: $[(^{35}Cl)M+H]^+$, 549.1928 ($C_{26}H_{34}{}^{35}ClN_4O_5S$ requires 549.1938).

Baculosome CYPs: Baculosome CYP1A2 and CYP3A4 were purchased from Thermo Fisher Scientific and were used for the Vivid assays and streptavidin affinity enrichment.

Cytochrome P450 fluorescence assays: The Vivid® CYP3A4 assay (Life Technologies, Carlsbad, CA, USA) was run according to the manufacturer's instructions. A master pre-mix was prepared, comprising 100 mM potassium phosphate buffer (4500 μL; pH 8.0), regeneration system (100 μL; 333 mM glucose-6-phosphate (G6P) and 30 U mL^{-1} glucose-6-phosphate dehydrogenase (G6PD) in 100 mM potassium phosphate buffer, pH 8.0), and CYP3A4 BACULOSOMES® (400 μL; microsomes from baculovirus-infected cells co-expressing human CYP3A4, NADPH-cytochrome P450 reductase, and human cytochrome b5). An aliquot of master pre-mix (50 μL) was pre-incubated with 40 μL of either 10% MeOH (control) or

serial dilutions of 1 mM solutions of probes **11** or **14** in 10% MeOH, in a 96-well plate (Nunc™, Thermo Fisher Scientific) for 10 min at room temperature. The reaction was initiated with the addition of 10 μL of the substrate solution (920 μL 100 mM PBS, pH 8.0, 30 μL $NADP^+$, and 50 μL 3A4 substrate: 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC)). Final concentrations were as follows: CYP3A4, 40 nM; BOMCC, 10 μM; $NADP^+$, 30 μM; G6P, 3.33 mM; and G6PD, 0.3 U mL^{-1}. The relative fluorescence units (RFU) readings were then taken continuously every minute for 30 min using a Fluoroskan Ascent FL Microplate Fluorimeter and Luminometer (Thermo Fisher Scientific, UK) and Ascent software version 2.6 (Thermo Lab Systems, UK), with respective excitation and emission wavelengths of 390 and 460 nm.

The assay for CYP1A2 was analogous to that described for CYP3A4. A master pre-mix was prepared comprising 10 mM PBS (2800 μL; pH 7.4) regeneration system (100 μL), and CYP1A2 BACULOSOMES® (100 μL; microsomes from baculovirus-infected cells co-expressing human CYP1A2, NADPH-cytochrome P450 reductase, and human cytochrome b5). An aliquot of master pre-mix (30 μL) was pre-incubated with 40 μL of each dilution of a probe for 10 min at 37 °C within the Fluoroskan Ascent FL Microplate Fluorimeter and Luminometer. The reaction was initiated by the addition of 30 μL of the CYP1A2 substrate solution (2940 μL of 10 mM PBS pH 7.4, 30 μL $NADP^+$, and 30 μL 7-ethoxymethoxy-3-cyanocoumarin (EOMCC)), which was also pre-incubated at 37 °C. Final concentrations were as follows: CYP1A2, 10 nM; EOMCC, 2 μM; $NADP^+$, 10 μM; G6P, 3.33 mM; and G6PD, 0.3 U mL^{-1}. The fluorescence readings were taken in the similar way as described above. The effect of each probe was calculated by comparing RFU value with the that of the control assay containing 40 μL PBS instead of the probe. To determine IC_{50} values for both assays, nonlinear regression analysis was carried out with GraphPad Prism 6 software.

Probe–bactosome-expressed CYP reactions: Human CYPs 1B1, 2B6, 2C19, 2D6, 2E1, 3A4, and 4A11, along with control bactosomes expressed in *E. coli*, were purchased from Cypex (Dundee, UK) and used to screen streptavidin blots for specificity, reaction time, and concentration (Figure 5). Each was co-expressed with human cytochrome P450 reductase, and CYPs 3A4 and 4A11 also contained cytochrome b5. The expression levels (nmol or μg CYP per mg of total protein) for each CYP were different for each product. Therefore, each CYP (1 μg, equivalent to 16–18 pmol CYP, Figure 5) was diluted with PBS to a concentration of 0.05 μg μL^{-1}. Control bactosomes were diluted in PBS to give a 4.25 μg μL^{-1} protein solution, which was equivalent to the average protein concentration for the CYP bactosomes used. Each experiment was performed in duplicate to confirm reproducibility. For CYP profiling, each reaction, NADPH (1 mM, Sigma, Poole, UK) or PBS for NADPH-negative controls, was added to a PBS solution of one of the CYPs or control bactosomes (0.05 μg μL^{-1}). CYPs 1B1, 2B6, 2C9, 2D6, 2E1, 3A4, and 4A11 were obtained from Cypex as membrane suspensions containing CYP (1 nmol mL^{-1}) at 580, 88, 306, 282, 370, 327, and 147 pmol (mg protein)$^{-1}$.

Probe **14** (5 mM in PBS, 20% MeOH) was added to each reaction mixture (equivalent to final concentrations of 0.5 mM), and incubated at room temperature for 60 min before the reactions were stopped by transfer to reducing SDS loading buffer. For a time-course experiment, NADPH (1 mM, Sigma, Poole, UK) or PBS for a NADPH-negative control was added to a PBS solution of CYP3A4 (0.05 μg μL^{-1}). An aliquot was removed from each reaction ($t = 0$) and transferred to 4 μL of reducing SDS loading buffer. Probe **14** (5 mM in PBS, 20% MeOH) was added to each reaction, and aliquots were removed at 1, 10, and 60 min. Each aliquot was transferred to reducing SDS loading buffer to stop the reaction.

SDS PAGE/streptavidin–HRP blotting: Samples (16–18 pmol CYP) were applied to 10% SDS polyacrylamide gels at 80 V (Bio-Rad Laboratories, Hemel Hempstead, UK) for 15 min, then at 150 V for 60 min. Following this, gels were transferred to a nitrocellulose membrane (GE Healthcare, Amersham, UK) at 58 mA (Bio-Rad Laboratories, UK) for 2 h. After transferring, membranes were blocked by 5% milk powder at 4 °C overnight followed by a further 30 min at room temperature. After three washes with TBS–Tween 20 (0.05%) for 15 min, the membranes were incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Abcam, Cambridge, UK) at a dilution of 1:500 in 5% blocking buffer for 1 h at room temperature. Membranes were washed a further three times, each time incubated for 15 min at room temperature. ECL plus (GE Healthcare) was used for streptavidin detection followed by exposure using imaging film (GE Healthcare).

Affinity purification using immobilised probes and MS analysis: CYP3A4 baculosomes, equivalent to 5 μg recombinant CYP3A4, were incubated with probe **14** or PBS for 15 min at 37 °C. Each reaction mixture was desalted in NAP-5 size-exclusion columns (GE Healthcare, UK), equilibrated with 10 mM ammonium bicarbonate to remove free probe. The protein–probe complex was lyophilised, resuspended in PBS, and incubated with bovine serum albumin pre-coated streptavidin Dynabeads (Life Technologies, Paisley, UK). After three washes each with PBS, 1 M NaCl/phosphate buffer, and 10 mM ammonium bicarbonate with 10% acetonitrile, the beads were resuspended in SDS-PAGE Laemmli buffer and incubated at 90 °C for 15 min. The proteins released from the beads by the buffer were separated by SDS-PAGE and stained with Coomassie Blue R-250 (Thermo Fisher Scientific). Bands of interest were excised from the gel and subject to in-gel digestion.[41] The resulting peptides were desalted in C_{18} ZipTips (Millipore) and analysed by MALDI MS in reflectron, positive ion mode (Ultraflex II, Bruker Daltonik, Bremen, Germany) for peptide mass fingerprinting and MS–MS for selected peptide fragmentation. The resulting spectra were searched using Mascot version 2.4 (Matrix Science, UK) against SwissProt version 2015_06 (Homo sapiens, 20207 sequences) to identify the proteins.

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