

Spirooxindoles as novel 3D-fragment scaffolds: Synthesis and screening against CYP121 from *M. tuberculosis*

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Abstract:

The search for new scaffolds to complement current HTS and fragment libraries is an active area of research. The development of novel strategies to synthesise compounds with 3D character in order to expand the diversity of a fragment library was explored. A range of substituted bicyclo[2,2,1]spirooxindoles were synthesised using a Diels-Alder [4+2] cycloaddition reaction. Both diastereoisomers were isolated from the reactions and these 3D fragment scaffolds were screened against the cytochrome P450 enzyme CYP121 from *M. tuberculosis*. A number of hits were identified to bind to CYP121 and were shown to exhibit Type I binding interactions with the heme group.

Fragment-based approaches to drug discovery are now firmly established in both academia and industry. This methodology has been applied to a diverse range of target classes, including kinases, protein-protein interactions and GPCRs, and the scope is ever increasing.¹⁻³ At the core of this approach is the fragment library, as it is the composition of these libraries that critically influences the hit rate and nature of inhibitors that are developed. Proteins bind substrates and cofactors that typically have 3D character and it has been argued that fragments or compounds with 3D character would be preferentially favoured for binding.⁴ However, fragment and commercial screening libraries typically have a high proportion of flat heterocycles, and as such there has been a concerted effort in recent years to expand the structural diversity of these libraries. One approach to increasing the structural diversity of libraries has been to introduce sp³ carbon centres onto the fragment scaffold, thereby increasing the 3D character.⁴⁻⁷ However, the routes to these compounds are often challenging and require multiple step syntheses.

A number of research groups have examined various synthetic methodologies to develop fragments containing a high level of 3D character. Young and co-workers used a diversity oriented synthesis (DOS) approach to synthesise novel bicyclic- and spirocyclic-pyrrolidine fragments using the proline scaffold as a startpoint.⁸ Examination of the scaffolds synthesised using cheminformatic tools showed that they had significantly different 3D character to commercial compounds found in the ZINC database and maintained favourable physicochemical properties. Bull and co-workers developed a synthetic route to aryl-sulfonyl-oxetanes, which introduce a high degree of 3D functionality into the fragment scaffold through the non-planar SO₂ group and the vectors provided by the oxetane ring. *Ortho*-metallation and palladium-catalysed reactions were subsequently used to further expand the

structural diversity of these scaffolds.⁹ Neither of these papers reported results the screening of these fragments against a biological target.

Only small proportion of our own in-house fragment library contain 3D character. Analysis of these scaffolds showed that structural diversity was primarily due to functional groups such as sulphonamides and biaryl rings, while spirocyclic containing scaffolds were not represented. In the last few years, interest in spirocyclic scaffolds has increased because they extend beyond the chemical space currently represented by drug molecules.¹⁰ The synthesis of spirocycles is generally difficult, despite a number of single-step routes being available, such as the Diels-Alder [4+2] and the Huisgen [3+2] cycloaddition reactions. These single step syntheses involve the reaction of a diene or 1,3-dipole with an exocyclic double bond and can be used to synthesise spirocycles in high diastereo- and enantioselectivity by using either metal or organocatalytic methods.^{11,12} In a recent report, Carreira and co-workers synthesised a novel thiaazaspiro[3,4]octane ring using a thiocarbonyl ylide, which was generated *in-situ* and subsequently reacted with an α,β -unsaturated ester. Subsequent oxidation of the sulphur to the sulfone using *m*CPBA afforded the spirocycles in good yields.^{12d} A drawback of this method of constructing spirocycles is the limited availability of sufficiently reactive exocyclic dieno/dipolarophile starting materials. Steric crowding around the double bond was found to significantly decrease the reactivity of these 2π -components.

One class of exocyclic dienophiles that has sufficient reactivity and has recently been studied is based on the 2-oxindoles (isatin) scaffold.¹³ These important heterocycles have been incorporated into a range of drug molecules, such as **1** and **2**, and are also found in natural products such as horsfiline and corulescine **3** (Figure 1). A major advantage of the 2-oxindole ring is that it is amenable to modification at various positions, which would allow scope for fragment elaboration and optimisation in a medicinal chemistry program.^{13c-e} We decided to use this scaffold as a start-point to explore the Diels-Alder [4+2] cycloaddition reaction with the highly reactive cyclopentadiene **5**. The resultant products of the cycloaddition reactions contained a novel spirobicyclo[2,2,1]heptene ring system which has not been previously been reported as a fragment scaffold and has only been briefly mentioned in the literature.¹⁴ The aim of this work was to construct a small library of these 3D fragment scaffolds and screen them against the cytochrome P450 enzyme CYP121 from *Mycobacterium tuberculosis* (*Mtb*). CYP121 is an essential enzyme, which has been identified as important drug target for tuberculosis and is a focus of fragment-based drug discovery efforts within our research group. We have previously reported the discovery of a number of fragments and the development of small molecule CYP121 inhibitors with binding affinities in the low micromolar region.¹⁵ The *Mtb* genome encodes 20 cytochrome P450 enzymes (CYP's), and obtaining selectivity for a single CYP isoform is both challenging and desirable.

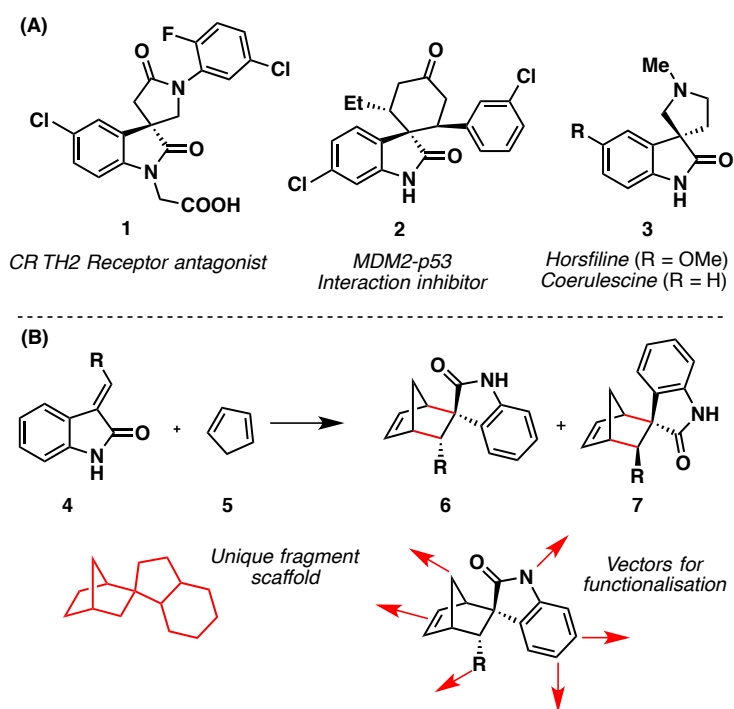
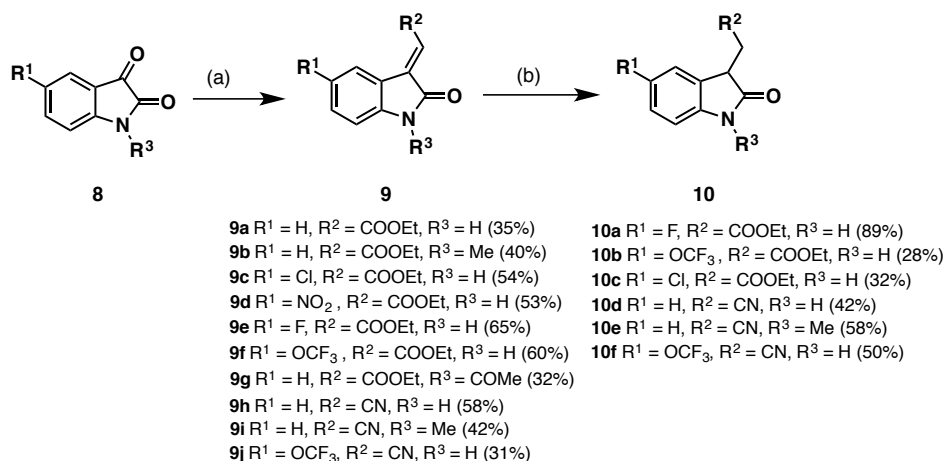


Figure 1. (A) Natural products and drugs containing the spirooxindole scaffold. (B) Synthesis of bicyclo[2,2,1]spirooxindoles using the Diels-Alder [4+2] cycloaddition methodology. Parent scaffold indicating vectors available for functionalisation.

The first step in the preparation of the spirobicyclo[2,2,1]heptene scaffolds was to synthesise the exocyclic dienophile component. A Wittig reaction between (carbethoxymethylene)triphenylphosphorane and a range of commercially available *N*- and 5-substituted indoline-2,3-diones **8** was used to synthesise the substituted 3-alkylidene-2-oxindoles dienophiles **9a-g** as highly coloured crystalline solids in moderate yields (32-65%) (Scheme 1).¹⁶ The structural diversity of the dienophile component was further expanded by employing Horner-Wadsworth-Emmons (HWE) methodology to synthesise a range of cyano-substituted 3-alkylidene-2-oxindoles **9h-j** in moderate yields (31-58%).¹⁷ To explore the scope of the subsequent Diels-Alder reactions, the 3-alkylidene benzofuranone and benzothiophenone (**13a** and **13b**) scaffolds were also prepared according to literature procedures.¹⁸ A number of the substituted 3-alkylidene-2-oxindoles dienophiles were reduced with NaBH₄ to produce the corresponding 2-oxindolines **10a-f** and **13c**, which are themselves interesting fragment scaffolds that were not represented in our fragment library.¹⁹ These reduced intermediates allowed investigation of the inherent binding properties of the oxindoline scaffold. The reaction with NaBH₄ was rapid (1-2 mins) in converting the highly coloured 3-alkylidene-2-oxindoles to a clear solution and resulted in product yields of 28-89%, Scheme 1.



R² = COOEt: (a) Ph₃PCHCOOEt, THF, rt, 2h (b) NaBH₄, MeOH, 30 mins
R² = CN: (a) CNCH₂P(O)(OEt)₂, NaH (60%), THF, 14h (b) NaBH₄, MeOH, 30 mins

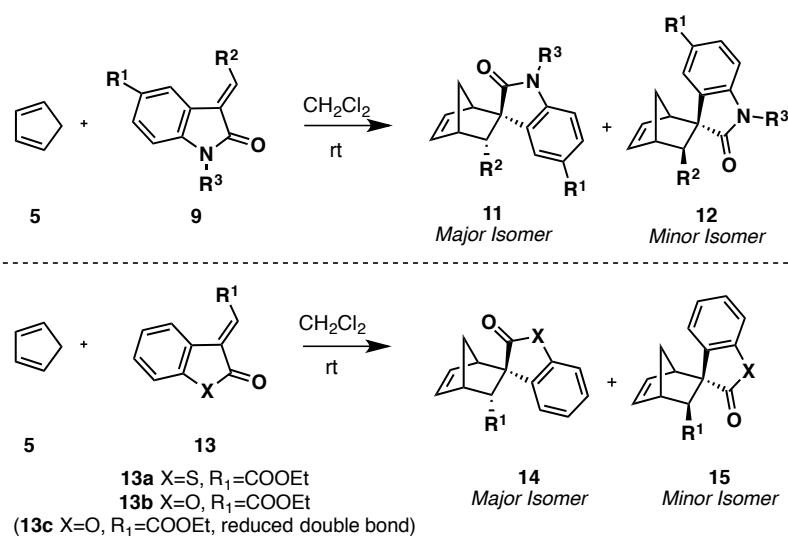
Scheme 1. Synthesis of dienophile components (**9a-j**) for the Diels-Alder cycloaddition reaction and synthesis of reduced 3-alkylidene-2-oxindoles (**10a-f**)

The next step was to react the substituted 3-alkylidene-2-oxindoles **9a-j** in a Diels-Alder [4+2] cycloaddition reaction with cyclopentadiene **5**. The reaction proceeded under mild conditions to give a mixture of two isomers in a ratio of 2-3:1 (Table 1). The isomers were separable in the majority of cases using flash column chromatography. The stereochemistry of the isomers was deduced using 2-D NOESY NMR. An NOE between the C-3 proton of the bicyclo[2,2,1]heptene ring and one of the bridging methylene protons indicated that the major isomer produced from the Diels-Alder cycloaddition had the ester and methylene bridge in the *endo* conformation (Table 1). No NOE was observed for the C3-proton of the minor isomer, however NOEs between the methylene bridgehead and the C5-proton of the original 2-oxindoles ring were identified (Supporting Information Figure S1). The structural assignments of the major and minor isomers are in agreement with those previously reported in the literature, which had been determined by subsequent derivitisation of the products.¹⁴

The low diastereoselectivity of the cycloaddition reaction was considered advantageous as it allowed us to further expand our exploration of chemical space. The isomers were readily separable with the exception of entries 4, 9 and 10, which allowed both the *endo* and *exo* products to be screened independently as fragments. The scope of the Diels-Alder reaction was investigated with a range of 5-substituted and *N*-substituted 3-alkylidene-2-oxindoles (Table 1, entries 2-7). All substrates yielded the desired cycloaddition products in moderate to excellent yields (48-99%), and similar diastereoselectivity (1.8:1 to 3:1). In general, electron-withdrawing groups at the 5-position of the oxindole ring increased the reaction rate, with product formation typically complete within 1-4 hours. In comparison the unsubstituted 3-alkylidene-2-oxindole **9a** and the two *N*-substituted 3-alkylidene-2-oxindoles **9b** and **9g** required reaction times of between 36-48 hours. When the reaction was explored with the cyano-substituted 3-alkylidene-2-oxindoles **9h-9j** (Table 1, entries 8-10) the reaction rate was further reduced compared to the carboxyethyl-3-alkylidene-2-oxindoles and additional equivalents of cyclopentadiene **5** (2 eq.) were also required for the reactions to go to completion. The diastereoselectivity of the reaction was found to be similar to that with the carboxyethyl-3-alkylidene-2-oxindoles, however in the case of the *N*-methyl **9i** (Table 1, entry 9) and 5-trifluoromethoxy substituted derivative **9j** (Table 1, entry 10) the isomers proved difficult to separate using column chromatography. The benzothiopheneone **13a** and

benzofuranone **13b** derivatives were also well tolerated by the Diels-Alder reaction conditions, undergoing reaction with cyclopentadiene **5** (Table 1, entries 11 and 12) to give good yields (up to 76%) and similar diastereoselectivity to that previously observed. The F_{sp^3} (F_{sp^3} = Number of sp^3 hybridised carbons/total carbon count) of each of the compounds isolated was calculated and found to be comparable to that of other 3D like fragments previously reported in the literature.²⁰ However, as both bicyclo[2,2,1]heptane isomers have identical F_{sp^3} values but structurally occupy different 3D space, this comparison likely underrepresents the structural diversity of the fragment scaffolds developed here.

Table 1. Synthesis of bicyclo[2,2,1]heptene scaffolds by the Diels-Alder [4+2] cycloaddition reaction of substituted 3-alkylidene-2-oxindoles with cyclopentadiene



| Entry | R ¹ | R ² | R ³ | Time (h) | Yield (%) | Cpd No. | F_{sp^3} | <i>dr</i> (11:12) |
|-------|------------------|----------------|----------------|----------|-----------|-----------------|------------|--------------------|
| 1 | H | COOEt | H | 48 | 70 | 11a, 12a | 0.41 | 65:35 |
| 2 | H | COOEt | Me | 48 | 73 | 11b, 12b | 0.44 | 73:27 |
| 3 | Cl | COOEt | H | 1 | 99 | 11c, 12c | 0.41 | 65:35 |
| 4 | NO ₂ | COOEt | H | 1 | 48 | 11d, 12d | 0.41 | 75:25 ^a |
| 5 | H | COOEt | Ac | 30 | 56 | 11e, 12e | 0.42 | 72:28 |
| 6 | F | COOEt | H | 4 | 80 | 11f, 12f | 0.41 | 70:30 |
| 7 | OCF ₃ | COOEt | Me | 4 | 87 | 11g, 12g | 0.47 | 72:28 |
| 8 | H | CN | H | 24 | 89 | 11h, 12h | 0.33 | 66:24 |
| 9 | H | CN | Me | 48 | 49 | 11i, 12i | 0.37 | 72:28 ^a |
| 10 | OCF ₃ | CN | H | 48 | 45 | 11j, 12j | 0.37 | 68:32 ^a |
| Entry | R ¹ | R ² | X | Time (h) | Yield (%) | Cpd No. | F_{sp^3} | <i>dr</i> (14:15) |
| 11 | H | COOEt | O | 4 | 74 | 14a, 15a | 0.41 | 62:38 |
| 12 | H | COOEt | S | 4 | 76 | 14b 15b | 0.41 | 59:41 |

^a The two isomers proved inseparable by column chromatography and were isolated as a diastereoisomeric mixture.

With the novel bicyclo[2,2,1]heptane scaffolds isolated, we proceeded to screen this focused library of 3D fragments and reduced 3-alkylidene-2-oxindoles against our chosen target CYP121 from *Mtb*. Fragments were prepared as 100 mM stock solutions in DMSO-d₆ and diluted to a concentration of

1 mM in aqueous buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) to assess their solubility prior to screening. Any compounds which precipitated under these conditions were not screened to avoid false positive/negative results (see Supporting Information, Table S1).

A UV-Vis spectrophotometric assay was used to assess whether fragments bound to CYP121, and specifically if binding interactions were in the vicinity of the catalytic heme group. The UV-Vis assay monitors perturbations in the maximum wavelength (λ_{max}) of the Soret band of the optical spectrum of heme containing proteins. The assay is highly sensitive and allows detection of fragments which bind by either directly ligating the heme iron (Type II), to cause a red-shift in the Soret band, or which displace the axial water ligand but do not directly ligate the heme iron (Type I) which produces a blue-shift in the Soret band. The novel 3D-fragments were screened at 1 mM and the Soret band of CYP121, which occurs at 416.5 nm in the water-ligated ferric resting state, was monitored. The previously identified Type II CYP121 ligand **16** was used as a positive control (screened at 1 mM), and was observed to cause a change in the λ_{max} of the Soret to 421 nm (+3 nm).¹⁵ The X-ray crystal structure of **16** bound to CYP121 has previously revealed the proximity of the aniline NH₂ with the heme iron which would be consistent with the red-shift in the Soret band observed in the UV-Vis spectra.^{15a} None of the 3D fragments or reduced precursors in the present screening library were identified as Type II hits. This was in sharp contrast to a previous fragment screening campaign against CYP121, where the fragment hits produced Type II perturbations of the Soret band.^{15a} However, 7 of the 3D fragments were observed to cause a blue-shift in the λ_{max} of the Soret band consistent with a Type I binding mode. Type I, or substrate-like, hits are relatively uncommon against CYP's in our experience compared to Type II ligands, possibly because binding affinity is more dependent on interactions with diverse active site residues as opposed to conserved metal-binding interactions. Examining the structures of the fragment hits revealed that 4 fragments, **12b**, **12c**, **15a** and **15b**, were the minor product isomers from the cycloaddition reactions. There was one example **11a** of the major isomer, as well as a single example of a reduced 2-oxindoline **10f** and benzofuranone **13b** dienophile. The presence of benzofuranone, benzothiophenone and indolinone containing bicyclo[2,2,1]heptene scaffolds amongst the fragment hits identified, indicated that binding affinity of these compounds was unaffected by the properties of the heterocyclic component itself.

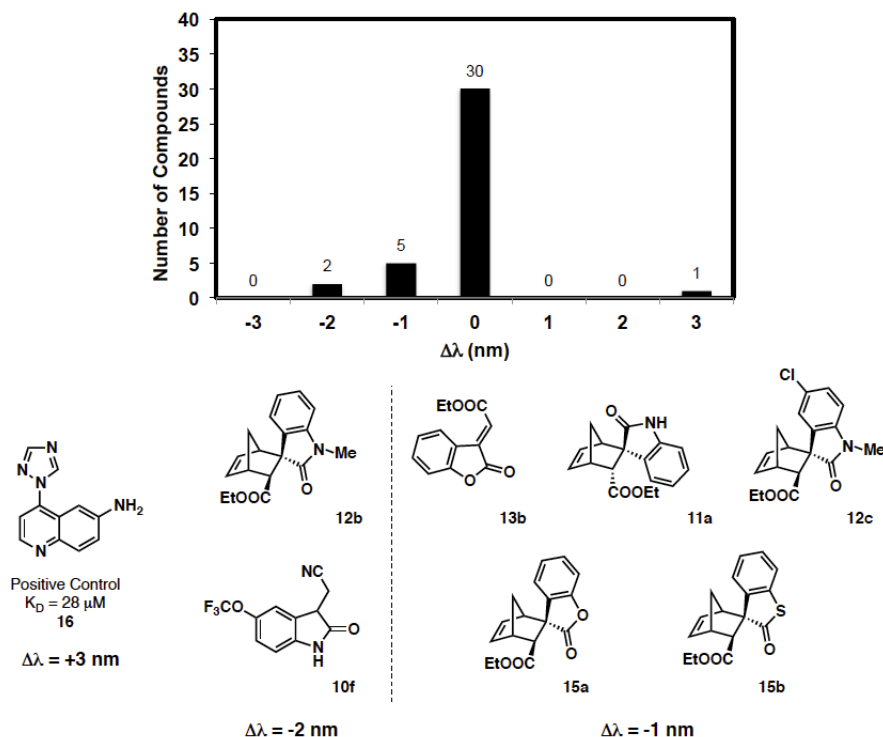


Figure 2. Results from UV-Vis fragment screening assay showing the distribution of compounds plotted against the change in wavelength ($\Delta\lambda_{\text{max}}$) of the Soret band of CYP121.

Attempts were made to determine the binding affinities (K_D values) of the most potent hits, **10f** and **12b**, using both direct isothermal titration calorimetry (ITC) and optical titrations. However, titration curves could not be saturated within the solubility limits of the fragments. As a result, an indirect optical titration was conducted to determine the apparent K_D ($K_{D(\text{app})}$) of the known Type II CYP121 ligand **17** ($K_D = 0.015 \mu\text{M}$) (Figure 3A-C), when it was titrated against CYP121 in the presence of either compound **10f** or **12b** (Figure 3D-F). The change in the $K_{D(\text{app})}$ of **17** from the K_D of **17** determined in the absence of the fragments was then used to calculate the binding affinity of **12b** (Figure 3C-E).²¹ The K_D of compound **12b** was determined to be $360 \mu\text{M}$, which is comparable to the binding affinities of previous fragments which have been identified to bind to CYP121 from our standard fragment library ($K_D = 400\text{--}3000 \mu\text{M}$).^{15a} A K_D value could not be determined for compound **10f**, indicating that it bound too weakly to compete with the type II probe ligand **17**. The ligand efficiency of **12b** ($\text{LE} = 0.21$) is significantly lower than that of previously identified fragments ($\text{LE} = 0.29$ to 0.39) because of the higher molecular weight that is required to functionalise the fragment with the complex spirocycle framework. Murray and Rees from Astex Pharmaceuticals recently highlighted the importance of balancing the advantages of structural and stereochemical diversity with other physicochemical properties.²² They highlighted the need for new synthetic chemistry efforts to develop lower molecular weight 3D fragments, which might enable exploration of uncharted areas of chemical space, while preserving the sampling advantages offered by fragments.

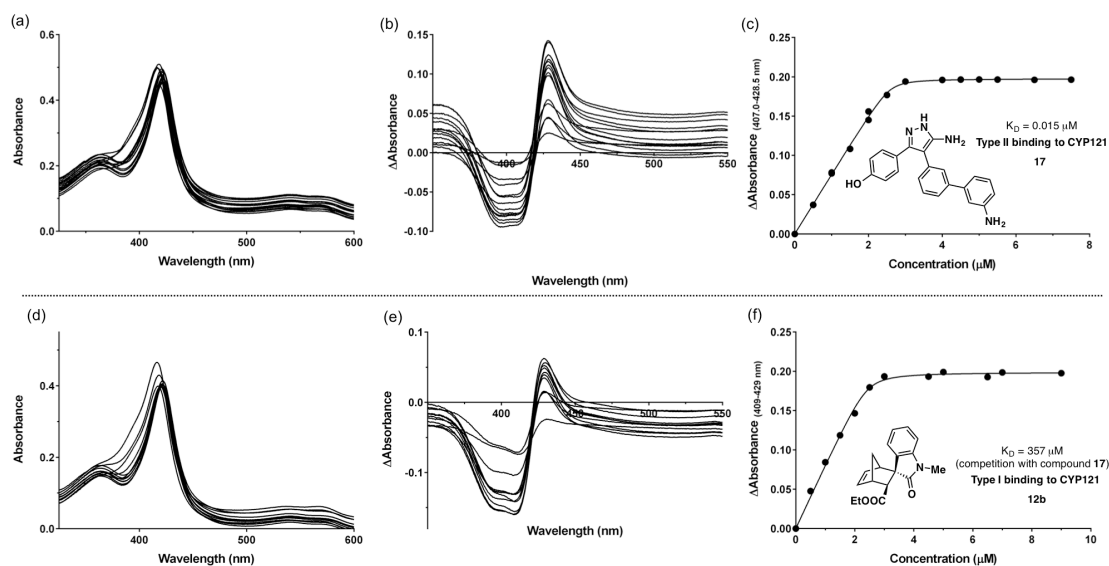


Figure 3. (a) UV-Vis spectra of CYP121 (5 μM) titrated with compound **17**. (b) Difference spectra of generated from the titration of compound **17** with CYP121 (c) Compound **17** induced absorbance change plotted against the concentration of compound **17** (d) UV-Vis spectra of CYP121 (5 μM) titrated with compound in the presence of compound **12b** (500 μM). (e) Difference spectra generated from the competition titration of compound **17** and fragment **12b** with compound **17** with CYP121. (f) Compound **17** induced absorbance change plotted against the concentration of compound **17** when titrated against CYP121 in the presence of fragment **12b**.

A series of docking studies were used to ascertain how the fragment hits bound to CYP121 and to explore the preference of CYP121 for the minor product isomer. A range of different binding scenarios were modelled, employing positional constraints to either restrict binding interactions to the vicinity of the heme cofactor, or to enforce hydrogen-bonding interactions between fragments and the axial heme water ligand. In one scenario this resulted in a conserved binding orientation for the minor product isomers. The fragments docked to form H-bonding interactions between the indolinone carbonyl group and Arg386, which positioned the indole N to point towards the heme iron or axial water ligand. In contrast, the major product isomers docked in a range of conformations. Either displacement of the axial water ligand or weakening the coordination of the axial water ligand to the heme iron through forming H-bonding interactions with fragments would be consistent with the Type I shift in the Soret band that was observed in UV-Vis assays. This binding mode has not been previously observed for fragments identified using our previous fragment-based approaches, in which all the hits coordinated directly to the heme iron using an aniline group, Figure 4.

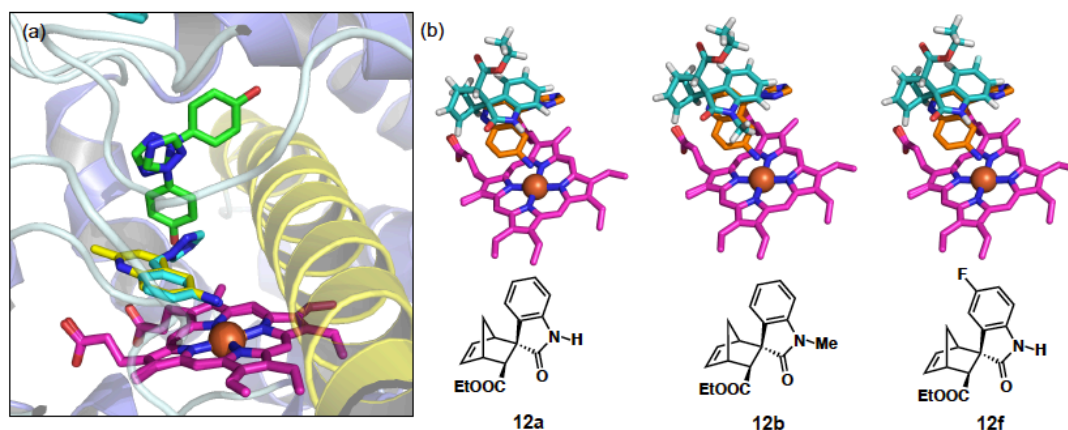


Figure 4. (a) Overlay of X-ray crystal structures of previously identified fragments which bind to CYP121 (pdb codes 4G44 (Blue), 4G47 (Green), 4G45 (Yellow)). (b) Docking of **12a**, **12b** and **12f** (minor isomers) into CYP121 showing conformations relative to the positive control **16** (pdb code 4G1X, the protein was removed to make viewing the binding poses easier to observe).

In conclusion we have explored the Diels-Alder [4+2] cycloaddition reaction of substituted oxindoles with cyclopentadiene to synthesise a series of novel spirocycles. Alternative benzofuranone and benzothiophenone precursors were also explored. The yields obtained for the reactions were good to excellent and the diastereoselectivity was typically in the range of 2:1, which enabled the isolation of both isomers for use in fragment screening. The novel 3D fragments were screened against CYP121 from *Mtb* using a UV-Vis spectrophotometric assay. Seven Type I hits were identified and using indirect titration compound **12b** was identified to have an apparent K_D of 360 μM . Co-crystallisation of a selection of these hits with CYP121 is currently in progress. Owing to the success of this methodology for identifying novel ligand scaffolds, a selection of these 3D fragments will be incorporated into our main fragment library and screened against a range of other protein targets.

Acknowledgements

AGC was supported by the BBSRC for funding (BB/I019669/1). AGC and TB would like to acknowledge BP for funding of a summer studentship through the Department of Chemistry, University of Cambridge. MEK was supported by a Commonwealth (University of Cambridge) Scholarship awarded in conjunction with the Cambridge Commonwealth Trust and Cambridge Overseas Trust.

Supplementary data

Syntheses and characterization of organic molecules, UV-Vis assays and docking conditions are described in the Supporting Information. Additionally, NMR spectra related to this publication are also available at the University of Cambridge data repository (www.repository.cam.ac.uk/handle/1810/254133).

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