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Optimization of the Interligand Overhauser Effect for Fragment Linking: Application to Inhibitor Discovery against *Mycobacterium tuberculosis* Pantothenate Synthetase

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Tuberculosis (TB) is a recognized health problem leading to over 2 million deaths each year.[1] Despite the high mortality, no new front-line drugs against TB have been introduced to the market for 40 years.[2] There is evidence that pantothenate synthetase (PtS) may be a potential drug target in *Mycobacterium tuberculosis*.[3] This enzyme catalyzes the magnesium-ATP-dependent condensation of pantoate and β-alanine to form pantothenate (vitamin B_5). A range of approaches have been adopted in the search for inhibitors, including high-throughput screening[4] and mimicking of the reaction intermediate pantoyl adenylate,[5] and most recently, we have reported the use of dynamic combinatorial chemistry[6] and fragment-based methods.[7]

We are interested in the development of techniques for fragment-based discovery of enzyme inhibitors.[8] The interligand Overhauser effect (ILOE) has been used to screen for pairs of ligands that can then be linked to generate inhibitors.[9] It does not require the availability of a 3D protein structure and can be applied to targets that cannot be observed directly by NMR spectroscopy because of their size. However, its application to screening using high concentrations of relatively hydrophobic fragments is problematic because of their aggregation and nonspecific binding effects. Here we report how these problems can be overcome and the ILOE used to guide the iterative assembly of a potent inhibitor of *M. tuberculosis* pantothenate synthetase.

5-Methoxyindole (**1**) and 2-carboxybenzofuranoic acid (**2**) had previously been identified from a fragment screen against pantothenate synthetase. They have K_d values of 1.1 and 1.0 mM, respectively, as measured by isothermal titration calorimetry (ITC).[7] ^{1}H NMR WaterLOGSY[10] and STD[11] competition experiments with known ligands revealed that binding of both is competitive with ATP and that binding of **2** is also competitive with pantoate. Competition between **1** and **2** was not observed in the NMR spectroscopic binding experiments. Docking studies suggested that **1** might bind in the adenine pocket with the pyrrole ring facing the pantoate pocket. No clear binding site for **2** was suggested.

An attempt to use NMR spectroscopy to elucidate the relative binding modes of **1** and **2** was made. Pantothenate synthetase is too large for direct protein observation by NMR spectroscopy (74 kDa for the dimer of His_6-PtS), so observation of ligands through transferred nuclear Overhauser effects (NOEs) in a 2D ^{1}H−^{1}H NOE spectroscopy (NOESY) experiment was attempted. An ILOE (a negative transferred NOE) between **1** and **2** could indicate the formation of a ternary complex involving the protein and both ligands and provide information on their spatial proximity and relative orientation.[12] Interligand NOEs between **1** and **2** were observed. They were protein-dependent and not mediated through the protein (this was shown by repeating the experiment with perdeuterated protein dPtS). However, the peaks were uniform and negative throughout the spectrum (Figure S1 in the Supporting Information), indicative of nonspecific binding to the protein. To elucidate the binding mode of **1**, an independent NOESY experiment with a different probe was needed.

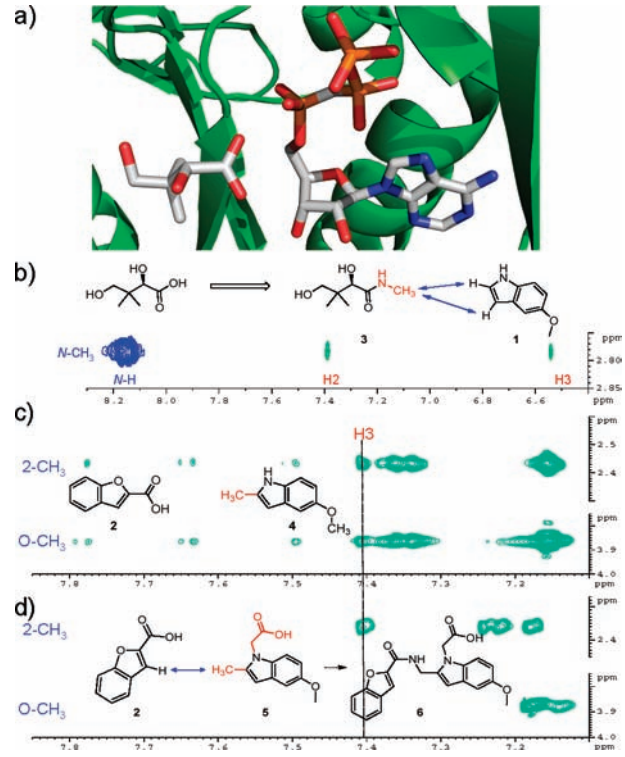

Figure 1. (a) Crystal structure of AMPCPP (an ATP analogue) and pantoate bound to PtS in a ternary complex (PDB entry 1N2E). (b) Fragment of the 2D NOESY spectrum of compounds **1** and **3** in the presence of dPtS. Two negative ILOE peaks between the probe **3** methyl group and H2 and H3 protons of **1** and their interpretation are shown. The large and positive NCH_3/NH peak arises from the high concentration and low affinity of **3**. (c) Fragment of the spectrum with ILOE peaks for **2** and **4**. (d) Fragment of the spectrum with ILOE peaks for **2** and **5** and their proposed relative binding mode to form the linked compound **6**.

The substrate pantoate was modified as the *N*-methyl amide[13] **3** to provide a more sensitive probe having the amide methyl pointing toward the ATP site (Figure 1a).[14] The amide **3** bound specifically in the pantoate pocket, as indicated by a competition STD experiment with pantoate, but its binding affinity was weak (K_d >

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10 mM). A 2D $^1H-^1H$ NOESY experiment with **1** and **3** in the presence of perdeuterated pantothenate synthetase showed two ILOE peaks (Figure 1b), indicating the spatial proximity of the *N*-CH_3 of **3** and H2 and H3 of **1**, suggesting the binding mode of **1**.

The successful use of **3** to obtain ILOE information prompted us to investigate the modified indole 2-methyl-5-methoxyindole (**4**) as a probe to elucidate the binding mode of **2**. The predicted binding mode of **4**, with the 2-CH_3 group facing the pantoate pocket, was confirmed by observation of an ILOE between the *N*-CH_3 of **3** and 2-CH_3 and H3 of **4** (Figure S2). However, NOESY experiments using **4** and **2** with dPtS resulted in ILOEs from both the 2-CH_3 and OCH_3 of **4** to all of the aromatic protons of **2** (Figure 1c). Partial aggregation of **2** and **1** as well as **2** and **4** due to their hydrophobicity may account for the observed spin-diffusion effects. Evidence for the formation of high-molecular-weight nonspecific aggregates by fragments in solution was seen in the NOESY spectra (Figure S3). This suggested that increasing the aqueous solubility of the ligands should be beneficial. Moreover, competition experiments with the substrates ATP and pantoate indicated that the binding of **4** included a significant nonspecific contribution and that the observed ILOEs between **2** and **4** were mostly derived from this (Figure S4).

We envisaged that introduction of an *N*-CH_2COOH group onto **4** not only would increase its water solubility but also might pick up additional interactions, as previously observed for **1**.[7] Hence, compound **4** was modified to give **5** ($K_d = 800\ \mu M$). Its predicted binding mode was confirmed by observation of an ILOE with compound **3** (Figure S5). When a NOESY experiment was performed with **2**, **5**, and perdeuterated protein, an ILOE peak between H2 of **2** and 2-CH_3 of **5** was seen. Lack of an ILOE to the OCH_3 of **5** (Figure 1d) suggested that the problems due to aggregation had been resolved by the introduction of the carboxylate. Also, the ILOE peak disappeared when the fragments were displaced by ATP and pantoate (Figure S6). Achieving this specific binding is the key to elucidating the binding modes using the ILOE and was not possible with the initial fragment hits.

On the basis of the structural information from these experiments, fragment linking was attempted. Observation of ILOE peaks coming only from the H3 proton of **2** suggested that the benzene ring must be further away from the methyl probe in **5** and thus deeper in the pantoate pocket, leaving the carboxylate facing the adenine pocket. This information inspired the attempt to link the fragments **2** and **5** directly by an amide bond to give compound **6** (Figure 1d). This linked compound was found to bind tightly to *M. tuberculosis* pantothenate synthetase ($K_d = 860$ nM) and showed competitive inhibition of the enzyme [$K_i = 5.4\ \mu M$, as measured in a coupled enzyme inhibition assay (see Figure S7)].[15]

The successful linking of these fragments confirmed the binding modes derived from the ILOE-driven step-by-step reasoning. This was further confirmed by X-ray crystal structures of pantothenate synthetase in a ternary complex with **1** and **2**[7] and subsequently an X-ray crystal structure of **6** bound to pantothenate synthetase that was obtained by soaking it into crystals of the protein (Figure 2).

In conclusion, this study, which provides the first direct comparison of ILOE and X-ray approaches, has demonstrated that the ILOE may be used even when the initial screen involves high concentrations of hydrophobic and relatively insoluble fragments that give rise to nonspecific binding effects. By the development of more soluble and more specific probes, extraneous peaks in the ILOE spectra that have been seen in some previous studies can be removed.[9,12] This should enhance the application of this technique for screening libraries of fragments.

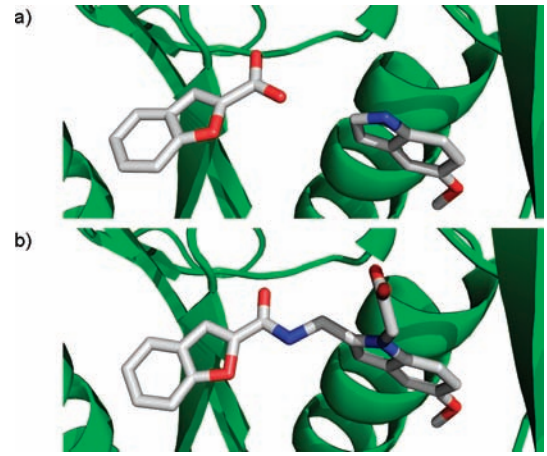

Figure 2. X-ray crystal structures of *M. tuberculosis* pantothenate synthetase with (a) **1** and **2** bound in a ternary complex (PDB entry 3IMG)[7] (an alternative orientation of **2** rotated by 180° around the C2−carboxylate bond is also possible) and (b) the amide **6** bound (PDB entry 3LE8).

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Supporting Information Available: Detailed experimental procedures and additional NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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