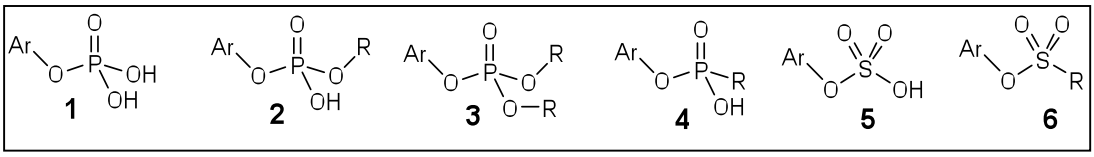
**Final Publishable Summary Report – Mark Mohamed (APcatProm)**

The phenomenon of catalytic promiscuity, the ability of an enzyme to catalyze reactions other than its native one, has gained significant attention in recent years as more and more promiscuous enzymes are identified. The fact that some enzymes can catalyze multiple, chemically distinct, reactions exposes the weakness in our current understanding of enzyme catalysis which typically assumes that catalytic efficiency is derived from perfect optimization of an active site for a single substrate. The overall goal of this project was to use the tools of physical organic chemistry to probe the mechanistic features of promiscuous enzymes in the alkaline phosphatase superfamily, a structurally related family of enzymes that appears to be particularly prone to promiscuous behaviour. Our aim was to gain a better understanding of the origins of promiscuity and, ultimately, to use this information as a guide for the rational design of new enzymes with industrially useful functions. As a model enzyme, we chose to study phosphonate monoester hydrolase (PMH) which was previously shown to be highly promiscuous and which catalyzes a wide range of chemical reactions.

The first phase of the project involved linear free energy relationship (LFER) analysis of the reactions that are catalyzed by the enzyme in order to gain a better understanding of the nature of the transition states for the native and promiscuous reactions. The known activities of PMH include the hydrolysis of phosphate mono-, di-, and triesters (**1**,**2**,**3** respectively), sulfate monoesters (**5**), and sulfonate monoesters (**6**) in addition to its native activity towards the hydrolysis of phosphonate monoesters (**4**).




For each class of substrate (with the exception of substrate **6**), I synthesized a series of compounds with varying leaving groups to construct Brønsted plots (plots of $\log(k_{cat}/K_M)$ vs $pK_a$) where the slopes ($\beta_{LG}$) give information about the nature of the transition state. The $\beta_{LG}$ values for the PMH-catalyzed hydrolysis of substrate classes **1**-**5** can be compared to the known $\beta_{LG}$ values for the uncatalyzed background reactions. This analysis revealed that, compared to the uncatalyzed reactions, the enzyme significantly offsets the build-up of negative charge on the leaving group which is likely one of the major reasons for its catalytic efficiency. Analysis of the crystal structure of the enzyme allowed us to identify several key catalytic residues in the active site. Brønsted analysis of seven active site mutants showed that the mutations cause only modest changes in $\beta_{LG}$ for all of the promiscuous activities. This means that mutation of these catalytically important residues apparently does not disrupt the enzyme's ability to stabilize charge accumulation on the leaving group. These results suggest that there is a system of backups in the active site where several residues can perform the same task, albeit with varying efficiencies. We believe that this is ultimately an important aspect of the promiscuous behaviour.

To further probe the mechanism of the enzyme-catalyzed reactions, we also studied the effects of substrate structure and polarity on the enzyme catalyzed reactions. I synthesized a collection of phosphonate monoesters (**4**) and phosphate diesters (**2**) with varying side chains. Plotting the kinetic

constants ($k_{cat}$, $K_M$, and $k_{cat}/K_M$) against the LogP (a measure of hydrophobicity) of the side chain shows a clear correlation where more hydrophobic substrates show higher $k_{cat}/K_M$ values. These results indicate that hydrophobic binding plays an important role in substrate recognition and catalytic efficiency. Our analysis also reveals that, surprisingly, the rates of enzymatic hydrolysis are determined more by substrate hydrophobicity than by leaving group ability or the functional group undergoing reaction.

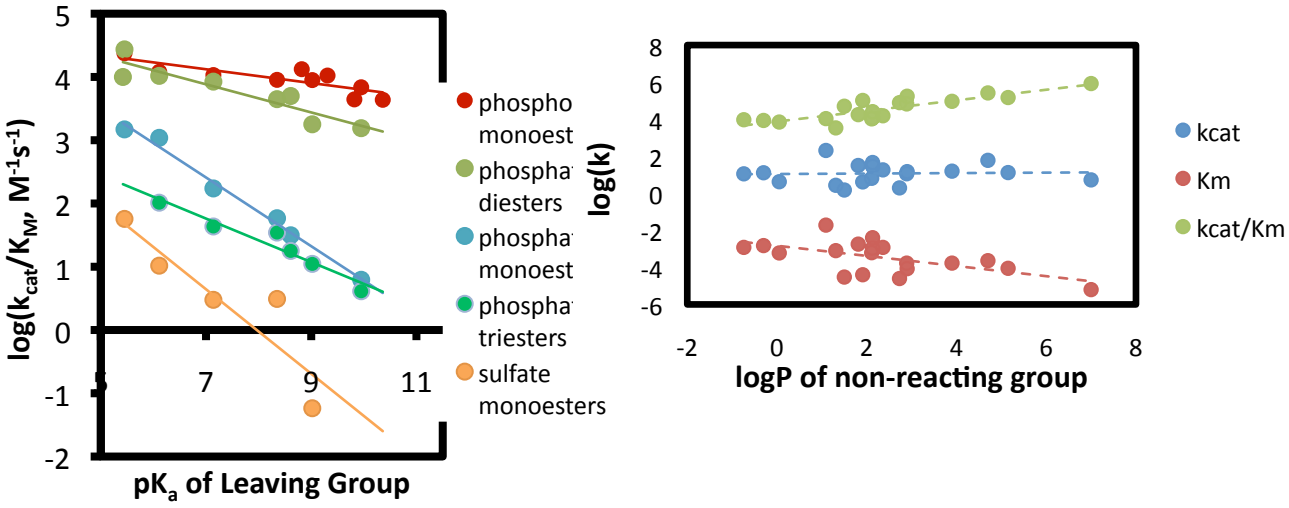



**Figure 1**. The left panel shows the linear free energy relationships for the hydrolysis of substrate classes **1**- **5** catalyzed by the PMH wild-type enzyme. The $\beta_{LG}$ values are given by the slopes of the lines. The right panel illustrates the relationship between the kinetic parameters for the PMH-catalyzed hydrolysis of phosphate diesters (**2**) and phosphonate monoesters (**4**) as a function of the logP of the substrate's side chain. The graph shows a clear positive correlation between catalytic efficiency ($k_{cat}/K_M$) and the hydrophobicity of the substrate.

In addition to the mechanistic studies outlined above, I have also been involved in projects to develop ultra-high throughput techniques to screen large libraries of enzyme mutants in directed evolution experiments. I synthesized a series of substrates with fluorescent leaving groups that could be used to distinguish between active and inactive mutants in a microfluidic sorting device or flow cytometer. The directed evolution experiments were focused on improving the promiscuous activities of various hydrolases, including members of the alkaline phosphatase superfamily, and so the substrates that we employed were fluorescent versions of a phosphonate monoester (**7**), a sulfate monoester (**8**), and a phosphate triester (**9**).