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A VERSATILE MECHANISM BASED REACTION PROBE FOR THE DIRECT SELECTION OF BIOCATALYSTS

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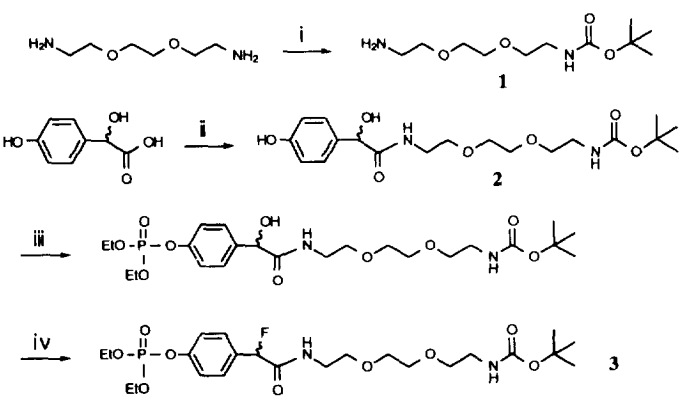
Abstract: A mechanism based reaction probe was synthesized and shown to modify a bacterial phosphotriesterase; this strategy for generating a probe is general and should allow the isolation of a host of unique catalysts. Copyright © 1996 Elsevier Science Ltd

It is of great interest to produce biocatalysts, either enzymes, ribozymes, or catalytic antibodies, with new properties, including catalytic activities, reaction pathways and substrate specificities.[1,2,3] One approach is through phage display of combinatorial libraries of proteins.[4] This tack is attractive because large protein libraries can be rapidly assembled while random mutagenesis or chain shuffling of these libraries can potentially expand or enhance the reaction spectra of these proteins. However, for maximum exploitation of these libraries suitable and effective selection protocols for catalysis must be designed and implemented.[3] As a first step towards such a goal we have demonstrated how semi-synthetic phage displaying antibody libraries may be panned for individual complementary determining regions (CDRs) containing isolated cysteine residues using a disulfide interchange reaction.[5] In this case a covalent reaction allowed selected phage to be trapped onto a matrix displaying the panning reagent, where they could be identified and replicated. Simultaneously another panning approach for a β-lactamase enzyme was disclosed.[6] While both of these methods were successful, neither was designed in a cassette like fashion so as to allow direct selection of catalysis from a variety of different types of reactions. Herein we describe a versatile mechanism based reaction probe that should allow isolation of a host of unique catalysts.

In designing the probe the following criteria were established: (1) Suitability for the attachment of a variety of mechanism based functionalities. (2) An available site for attachment of a reporter group, which will allow recovery of the selected clones. (3) Cassette like qualities that would allow rapid probe synthesis from readily available starting materials. The hydroxymandelic acid derivative **2** was anticipated to meet our needs as it is readily assembled and allows sequential attachment of both the mechanism based functionality and the reporter group (Scheme 1).

To investigate the potential of **2** as a cassette we constructed the phosphotriesterase probe **3** (Scheme 1)[7]. The two components to note on **3** are: (1) The head group, which contains a phosphotriester moiety and a latent quinone methide species. The latter would be generated following the hydrolysis of the triester and a 1,6-elimination of fluoride ion.[8,9] In essence then, this region of the molecule serves as a suicide substrate for both the probing of substrate specificity and ultimately the trapping of any enzyme that can hydrolyze this unit. (2) A spacer unit that contains an innocuous polyethylene glycol-like linker which terminates with a Boc-protected amino moiety. This can be left in its present format to examine enzyme specificity, however, Boc-deprotection and funtionalization with a reporter group (i.e., biotin) allows for discrete selection of catalysis.



Scheme 1 *Reagents and conditions*: i, $(Boc)_2O$, DCM, 0 °C to rt, 14 h 88%; ii, EDC, HOBt, **1**, DMF, 0 °C to rt, 16 h, 85%; iii, $(EtO)_2POCl$, DIEA, DCM, 16 h, 69%; iv, DAST, DCM, 0 °C, 30 min, 59%.

Bacterial phosphotriesterase is a highly efficient zinc-containing enzyme that hydrolyzes paraoxon at a diffusion-controlled rate.[10,11] In addition to paraoxon, it also tolerates a broad range of paraoxon analogues having a variety of substituents on the benzene ring.[10] Compound **3** (13 mM) was incubated with phosphotriesterase (4 μM) in PIPES buffer (100 mM, pH 7.0) and the mixture was monitored by ^{19}F NMR (Figure 1).[12] At time zero (a), the doublet at -22 ppm arises from compound **3** and the singlet at 76 ppm is the TFA internal standard. Compound **3** was quickly consumed by the enzyme; a large portion of the substrate disappeared within five minutes (b), and totally disappeared in ten minutes (c). The new signal at 30 ppm,

identical to that of NaF in the same buffer, corresponds to inorganic fluoride which was eliminated from the substrate when the P-O bond (phosphorus-phenolic) was cleaved.

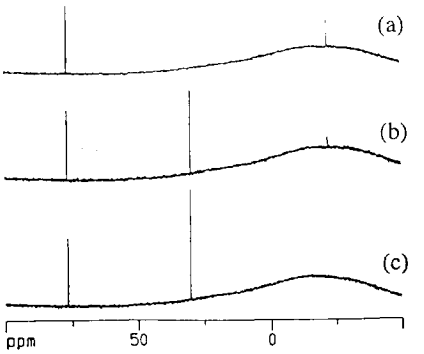


Figure 1 Time course for the bacterial phosphotriesterase hydrolysis of compound **3** as monitored by ^{19}F NMR; (a) 0 min, (b) 5 min, and (c) 10 min.

After three minutes a precipitate started to form in the incubation mixture leading to a loss of hydrolytic activity. Precipitation was prevented by inclusion of NaN_3 (100 mM) in the original incubation mixture, and activity was retained under these conditions. When the protein (MW: 36.3 KD) and a reaction mixture of **3** was followed by LC/MS, multiple modifications were observed. For each modification by the quinone methide intermediate there would be an increase in molecular weight of 381. The number of enzyme site modifications ranged from one to more than four as determined by mass spectral analysis (Figure 2). This increase was time dependent (data not shown) and we believe that it is these multiple modifications that eventually cause precipitation of the protein and hence loss of enzymatic activity. Interestingly this result is very different from that of known suicide substrates of this phosphotriesterase (alkynyl phosphate esters) where a critical histidine residue was acylated by a ketene intermediate, leading to diminished enzymatic activity.[13]

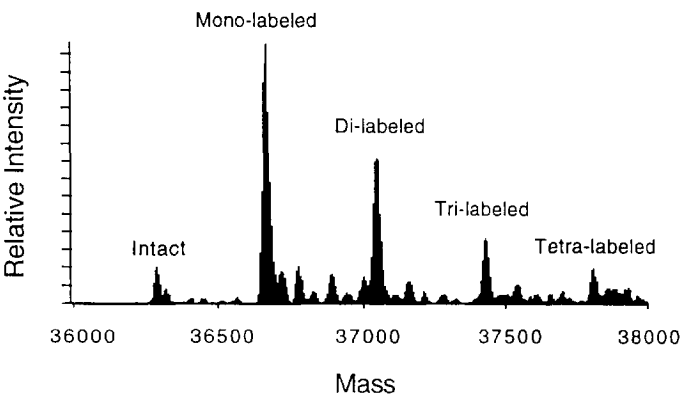


Figure 2 MS profile of the covalently modified phosphotriesterase

This study demonstrates that probe **3** derived from hydroxymandelic acid is suitable for covalently modifying and thus selecting phosphotriesterase activity. It also implies that **2** could serve as the starting point for preparing other hydrolytic probes with a variety of different recognition units and reporter groups. Ongoing studies include the use of this probe for the selection of catalytic antibodies, and to expand this series of activity probes for the selection of phosphodiesterases.

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