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Biotransformation of Phosphonate Esters

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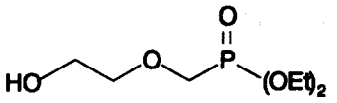
Abstract: Strains of *Bacillus brevis*, *Pseudomonas fluorescens* and *Pseudomonas putida* that display diethyl phosphonate hydrolase activity have been isolated from a soil screen. Also reported is the resolution of a racemic secondary alcohol containing a dibenzyl phosphonate moiety. This was achieved by transesterification under essentially anhydrous conditions using vinyl acetate as the irreversible acyl donor. A second resolution is reported in which hydrolysis of the carboxylate ester of a phosphonyl leucine derivative occurs with resolution of a racemic centre γ to the functional group being hydrolysed.

Several compounds containing the phosphonate functional group are of therapeutic interest. The structural analogy between phosphonate and phosphate groups have resulted in the utilization of phosphonates as inhibitors of enzymes that transform phosphate groups. The ubiquitous nature of the phosphate group in living systems and the intrinsic involvement in cellular reproduction make phosphonate analogues extremely potent inhibitors of many enzyme systems. In the light of their importance it is perhaps surprising that very little material has been published pertaining to the biotransformation of phosphonate containing substrates although their ability to inhibit enzymes may account for this to some extent.

We wish to report here three aspects of the synthetically useful action of enzymes on phosphonate esters; de-esterification of phosphonate esters, resolution of a centre α to the phosphorus by esterification of a secondary alcohol, and resolution of a centre α to the phosphorus by hydrolysis of a remote carboxylate ester in the same molecule.

As part of our investigations, an enzymic system was required which would hydrolyse a diethyl phosphonate ester to the corresponding phosphonic acid. The only literature precedent for such a biotransformation was found in a series of publications by Natchev[1-4]. These papers reported the use of commercially available phosphodiesterases and phosphatases as well as a crude bee venom preparation to achieve hydrolysis and transesterification of a variety of substrates containing the diethyl phosphonate moiety. For our substrate commercially available phosphatases, phosphodiesterases and phospholipases from various sources were screened for the same activity without success. It was subsequently

decided to instigate a general soil sample screen with a view to identifying the required activity.



(1)

Soil samples were screened by incubating a small amount of the soil at 28 °C for 10 days in a minimal medium comprising: ammonium sulphate, di-potassium hydrogen orthophosphate, magnesium sulphate and a trace element mixture in deionised water adjusted to pH 7. The simple diethyl phosphonate (1) was added as the sole carbon source at a concentration of 1mg/ml. After 10 days each sample was examined microscopically for signs of bacterial growth. Three bacterial cultures which were capable of hydrolysing the phosphonate diester were identified (representing a hit rate of 1%). These were found to be strains of *Bacillus brevis, Pseudomonas fluorescens* and *Pseudomonas putida.* All three strains were found to hydrolyse both ester groups to produce the free phosphonic acid. The conversions were poor with all three strains producing the phosphonic acid in <10% yield. Contributing factors to the poor yields could be difficulty in transporting the phosphonic acid out of the cell since at physiological pH the acid will be in an ionised form, or the aforementioned ability of phosphonates to inhibit enzymes.

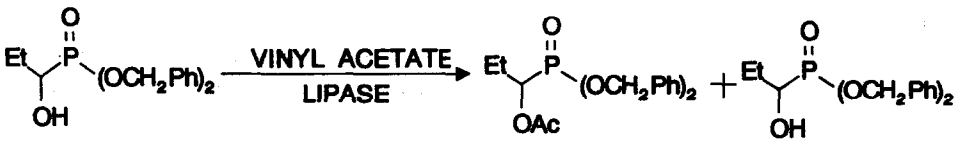
Although these systems require optimisation in order to become of preparative use they represent, to the best of our knowledge, the first reported use of whole cell preparations to hydrolyse phosphonate esters.

A second compound of interest to us is dibenzyl (1-hydroxy)propylphosphonate (2). This is a precursor in the synthesis of a series of compounds currently being evaluated for biological activity. An enzyme based methodology was required for the resolution of this racemic material. This was achieved by utilizing a lipase from *Pseudomonas fluorescens* (ex Biocatalysts Ltd) to catalyse a transesterification from vinyl acetate to the secondary alcohol of our substrate under anhydrous conditions (Figure 1). This reaction proceeded with a high degree of stereoselectivity stopping spontaneously at 50% acylation to yield the dextrorotatory acetate (3) and levorotatory (2) both with enantiomeric excess >99% as determined by NMR with chiral solvating reagent.

In a typical reaction, the phosphonate (2) (700mg) was stirred for 26 hours at ambient temperature in a mixture of vinyl acetate (70ml) and diisopropyl ether (70ml) containing the lipase (1g). The enzyme was removed by filtration and the solution evaporated *in vacuo* to afford a residual oil. This oil was chromatographed on silica gel eluting with diethyl ether to afford the dextrorotatory acetate (3) as an oil and also a single enantiomer of (2) isolated as a

white crystalline solid $[\alpha]^{25}_D$ = -5.1 ($CHCl_3$, c=1.0). The enantiomerically pure alcohol (-) 2 was obtained in 82% yield of available enantiomer.

FIGURE 1

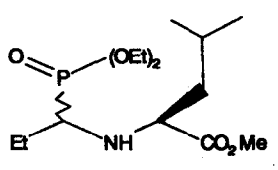


(+/-) (2) (+) 3 (-) 2

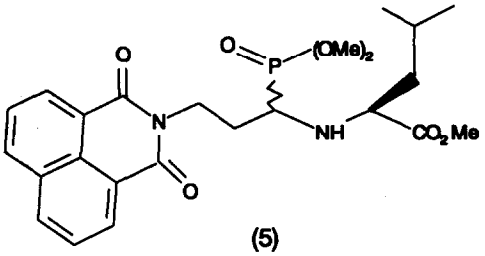
The enzyme was also used to hydrolyse the acetate (+) (3) to produce the dextrorotatory antipode of (2) since the ester is unexpectedly resistant to chemical hydrolysis producing only 13% hydrolysis after 7 hours refluxing in aqueous HCl/THF.

The same degree of stereoselectivity was displayed when the reaction was examined in the hydrolytic direction at pH 7.1 starting with chemically produced racemic acetate. The hydrolysis produced (+) (2) enantiomerically pure.

A third biotransformation on a phosphonate ester containing substrate concerns a remote centre resolution. Resolutions of centres α to the group being transformed are common but resolutions of more remote centres are unusual[5]. In this instance an enzymic method for the diastereomeric resolution of the two phosphonyl leucine derivatives (4) and (5) was required. Each substrate was optically pure at the amino acid centre but racemic at the centre α to the phosphonyl moiety.



(4)



(5)

A screen of commercially available hydrolases was undertaken with a view to hydrolysing the carboxyl ester. In both cases a silica gel tlc system eluting with ethyl acetate separated the pair of diastereomers. Any stereopreference could therefore be seen in terms of a differential rate of hydrolysis of the diastereomeric pair. Two enzyme preparations, namely subtilisin Carlsberg protease and *Aspergillus oryzae* protease (both ex Sigma), displayed stereopreference. In the case of substrate (4) each enzyme preferentially hydrolysed a

different diastereomer in 0.1M phosphate buffer (pH 7) with 5% acetone co-solvent. The reactions were worked-up after the complete hydrolysis of one of the diastereomers as determined by tlc. Unreacted ester was recovered by adjusting the reaction mixture to pH 8.5 and extracting with ethyl acetate. The two esters were confirmed as pure, complementary, diastereomers by 250 MHz ^{1}H-NMR which showed a substantial difference in chemical shift between the proton at the racemic centre in the two different diastereomers. The esters were recovered from subtilisin Carlsberg protease and *Aspergillus oryzae* protease mediated reactions in 24% and 25% yields respectively. The two acids were recovered by acidification of the reaction mixtures to pH 2.5 and extracting with ethyl acetate. The acid from the subtilisin Carlsberg protease catalysed hydrolysis was found to be a 2:1 ratio of diastereomers. The acid from the *Aspergillus oryzae* protease mediated hydrolysis was also found to be a 2:1 ratio but with the complementary diastereomer being the dominant product.

In the case of substrate (5) the same two enzymes showed stereopreference but this time for the same diastereomer. For the hydrolysis catalysed by subtilisin Carlsberg protease the pure diastereomeric ester was recovered in 33% yield and the acid was recovered in 45% yield as a 1.8:1 mixture of diastereomers.

The diastereomeric resolutions discussed here differ from enantiomeric resolutions in that the hydrolytic preference displayed is a function of both chiral centres rather than the conformation at the racemic centre as an isolated moiety. The conformation at the amino acid centre is the most likely recognition site for the enzyme, thus influencing the hydrolytic preference and resolution of the second chiral centre.

The difference in stereopreference displayed by one of the proteases towards the two substrates may be due either to the change from ethyl to methyl phosphonate ester or, more likely, due to conformational considerations at the active site resulting from the necessity to accommodate the bulkier substituent α to the phosphonate group in substrate (5).

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