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First asymmetric epoxidation catalysed by cyclohexanone monooxygenase

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Abstract—The first example of a totally enantioselective epoxidation mediated by cyclohexanone monooxygenase for a model compound related to fosfomycin is reported.

Cyclohexanone monooxygenase (CHMO) (EC 1.14.13.22) from *Acinetobacter calcoaceticus* NCIMB 9871 is a flavoenzyme of about 60,000 Daltons, containing one non-covalently bound FAD unit per enzyme molecule.[1] Its high versatility has been exploited in the manufacture of fine chemicals, based on the Baeyer–Villiger oxidation, which transforms racemic ketones into enantiomerically pure esters. The only reagents consumed are dioxygen, NADPH and the substrate ketone.

CHMO can also oxygenate heteroatoms, due to the high reactivity of the 4a-hydroperoxyflavin intermediate, which can act as an electrophile to trimethyl phosphite and iodide ions, and as a nucleophile to boronic acids and ketones, as shown by Walsh and co-workers.[1] This enzyme also catalyses the asymmetric sulfoxidation of alkyl aryl sulfides,[2] 1,3-dithioacetals[3] and dialkyl sulfides.[4] Organic cyclic sulfites are transformed into sulfates,[5] whereas tertiary amines give the amine *N*-oxides.[6]

We were interested in exploring whether cyclohexanone monooxygenase was also able to epoxidise olefins, a reaction that, to the best of our knowledge, has not been described previously in the literature. Repeated efforts to epoxidise olefine double bonds having very different structural features such as dimethyl styrene, methyl enol ethers or enamines have all failed with this enzyme.[7] We considered this behaviour rather striking, since it is known that for *N*- or *S*-oxygenation the N5-alkylflavin-4a-hydroperoxide is almost 200,000 times more reactive than *tert*-butyl hydroperoxide.[8]

In view of the ambivalent reactivity of the 4a-hydroperoxyflavine intermediate, which behaves as an electrophile or as a nucleophile oxidising agent, we chose olefins containing either electrondonating or electron withdrawing groups.[†] The enzyme was used as a partially purified preparation obtained from an *E. coli* strain in which the gene of CHMO was cloned and overexpressed.[9]

In spite of numerous olefins tested,[†] only dimethyl and diethyl vinyl phosphonate are accepted as substrate by CHMO (Table 1).

We were pleased to find that in these cases the reaction was completely enantioselective, as determined by chiral GC. Fresh aliquots of enzyme had to be added in the course of the reaction in order to have appreciable chemical conversions (10–40%).

The (*R*) absolute configuration of diethyl phosphonate epoxide **1a** (Scheme 1) was attributed by chiral GC comparison with an authentic specimen obtained via kinetic resolution of a racemic mixture with (*R*,*R*)-

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[†] The substrates were: styrene; α-methyl styrene; 2-phenylpropene; 2-methyl-2-propen-1-ol; 3-methyl-3-buten-1-ol; 2,3,3-trimethyl-1-butene; 2,4,4-trimethyl-1-pentene; *N*,*N*-diethylacrylamide; ethyl 3,3-dimethylacrylate; dimethyl vinyl phosphonate; diethyl vinyl phosphonate; diethyl 2-propenyl phosphonate; diethyl 2-methyl vinyl phosphonate; diethyl 1-propenyl phosphonate.

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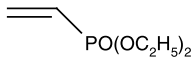
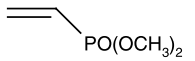
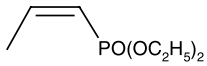
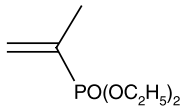
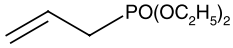
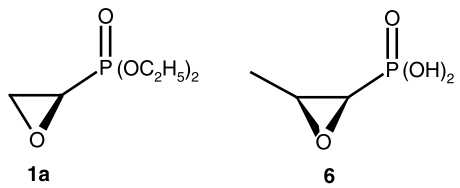
Table 1.

SUBSTRATE		YIELD (%)	Ee (%)
1		40	≥98
2		10	≥98
3		No reaction	
4		No reaction	
5		No reaction	

The olefins (12 mM) were reacted, at 25°C, under stirring, in 10 ml of 0.05 M Tris–HCl buffer, pH 8.6, containing NADPH (0.5 mM), glucose-6-phosphate (50 mM), 100 units of CHMO and 100 units of glucose-6-phosphate dehydrogenase (glucose-6-phosphate and glucose-6-phosphate dehydrogenase served to regenerate NADPH). After 48 h, the reaction mixtures were extracted with ethyl acetate and the organic extracts dried and analysed. The degree of conversion and the enantiomeric excess of the epoxide products were determined by chiral GC with a CP-cyclodextrin column (50 m, 0.25 ID, Chrompack). The separation factor (α) and the resolution factor (R) were 1.10 and 1.58, respectively, for the enantiomers of the epoxide obtained from **1**, and 1.11 and 1.79 for the enantiomers of the epoxide obtained from **2**.



Scheme 1.

N,*N*′-bis-(3,5-di-*tert*-butylsalicydene)-1,2-cyclohexanediamino cobalt(III) acetate as a resolving agent.[10] Its structure was confirmed by CI mass spectrometry, that showed a m/z peak 181 corresponding to the molecular ion.

The data obtained with CHMO expressed in *E. coli* were confirmed by oxidising the same substrate with CHMO from *Acinetobacter calcoaceticus*, thus excluding any interference by other enzymatic systems deriving from *E. coli* (the host cell).

We have also determined the kinetic constants for the oxidation of diethyl vinyl phosphonate.‡ These data showed that the olefin was not a good substrate for the enzyme, since it was transformed at a rate that was about 1 tenth of that of thioanisole, the affinity ($K_m = 3$ mM) being almost 30 times lower than that of the sulfide.

The reason why we chose the commercially available diethyl vinyl phosphonate as a model substrate for this study is due to its structural analogy with (*Z*)-propenyl phosphonic acid, the precursor of (–)-(1*R*,2*S*)-1,2-epoxypropylphosphonic acid **6** (fosfomycin) (Scheme 1), a broad spectrum antibiotic effective against both Gram-positive and Gram-negative infections in mammals, having an effectiveness comparable with that of tetracycline or chloramphenicol.

Another significant result was that obtained in the oxidation of the racemic phenyl vinyl sulfoxide, a substrate having two sites susceptible to attack by the oxidant, the double bond and the sulfur atom. The oxidation occurred preferentially at the sulfur with the formation of phenyl vinyl sulfone, and the concomitant kinetic resolution afforded the (*R*) sulfoxide with e.e. ≥98%.

In conclusion, we here report the first example of a highly enantioselective epoxidation reaction catalysed by cyclohexanone monooxygenase of a model compound related to fosfomycin. The substrate specificity is very high since the only substrates accepted by the enzyme are vinyl phosphonates. Interestingly, it has been recently shown that FMN or FAD greatly enhance the production of fosfomycin from (*S*)-2-hydroxypropylphosphonic acid (HPP) catalysed by HPP epoxidase.[11] This could possibly be connected to the fact that CHMO is only active with substrates

‡ The kinetic constants of CHMO for diethyl vinyl phosphonate were determined in 50 mM Tris–HCl buffer at pH 8.6, 25°C, in 1 ml cuvettes, 1 cm path length. The reaction mixture contained CHMO (40 milliunits), 0.1 mM NADPH and 3-60 μM substrate. The consumption of NADPH was spectrophotometrically monitored at 340 nm.

structurally related to fosfomycin precursors. Although the accepted substrates do not denature CHMO, the reaction rate is rather low. The presence of electron withdrawing groups is essential for performing the asymmetric epoxidation, as shown by the two successful results; this is in line with the nucleophilic attack by the peroxyflavin anion intermediate.[12]

The nature of the mechanism involved in CHMO-catalysed epoxidation is not known. Model data for *N*- and *S*-oxygenation have been reported by Bruice and co-workers.[8] On the basis of structure–activity studies they have found that the oxygen transfer potential of 4a-hydroperoxyflavin value tallies with those of other ROOH species and correlates with the pK_a of the leaving group alcohol.

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References

1. Walsh, C. T.; Chen, Y.-C. J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 333–343.
2. Colonna, S.; Gaggero, N.; Pasta, P.; Bernardi, A. *J. Chem. Soc., Chem. Commun.* **1996**, 2303–2307 and references cited therein.
3. Colonna, S.; Gaggero, N.; Bertinotti, A.; Carrea, G.; Pasta, P.; Bernardi, A. *J. Chem. Soc., Chem. Commun.* **1995**, 1123–1124.
4. Colonna, S.; Gaggero, N.; Carrea, G.; Pasta, P. *J. Chem. Soc., Chem. Commun.* **1997**, 439–440.
5. Colonna, S.; Gaggero, N.; Carrea, G.; Pasta, P. *J. Chem. Soc., Chem. Commun.* **1998**, 415–416.
6. Ottolina, G.; Bianchi, S.; Belloni, B.; Carrea, G.; Danieli, B. *Tetrahedron Lett.* **1999**, *40*, 8483–8486.
7. Latham, J.; Chen, Y.-C. J.; Walsh, C. T., unpublished results.
8. Bruice, T. C.; Noar, J. B.; Ball, S. S.; Venkataram, U. V. *J. Am. Chem. Soc.* **1983**, *105*, 2452.
9. Doig, S. D.; O'Sullivan, L. M.; Patel, S.; Ward, J. M.; Woodley, J. M. *Enzyme Microb. Technol.* **2001**, *28*, 265–274.
10. Wygatt, P. B.; Blakskjaer, P. *Tetrahedron Lett.* **1999**, *40*, 6481–6483.
11. Liu, P.; Murakami, K.; Seki, T.; He, X.; Yeung, S.-M.; Kuzuyama, T.; Seto, H.; Liu, H.-W. *J. Am. Chem. Soc.* **2001**, *123*, 4619–4620.
12. Sheng, D.; Ballou, D. P.; Massey, V. *Biochemistry* **2001**, *40*, 11156–11167.