Pergamon

Tetrahedron, Vol. 53, No. 20, pp. 6993-7010, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00398-0

A Mechanistic Rationalisation for the Substrate Specificity of Recombinant Mammalian 4-Hydroxyphenylpyruvate Dioxygenase (4-HPPD).

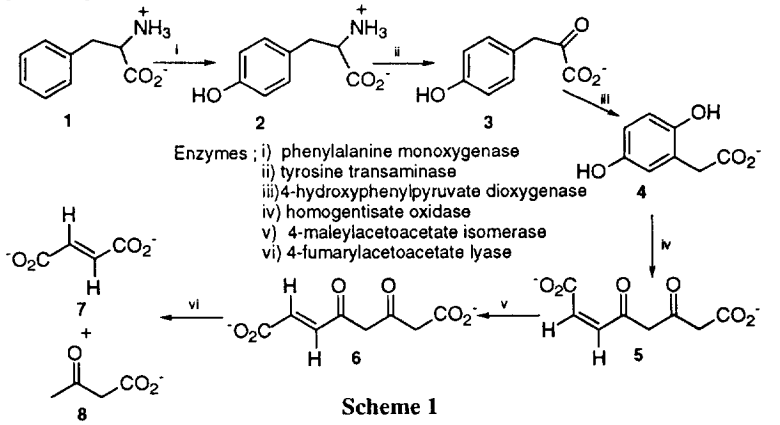
Nicholas P. Crouch*, Robert M. Adlington, Jack E. Baldwin, Meng -Huee. Lee, and Colin H. MacKinnon

The Dyson Perrins Laboratory and The Oxford Centre for Molecular Sciences, South Parks Road,

Oxford OX1 3QY, U.K.

Abstract: The isolation and purification of α-ketoisocaproate dioxygenase [α-KICD] from rat liver is described. Sequence determination of the purified protein revealed it to have complete homology to rat liver 4-hydroxyphenylpyruvate dioxygenase [4-HPPD] which was confirmed by the cloning and expression of the gene encoding 4-HPPD in *E. coli*. Examination of the substrate specificity of the resulting soluble recombinant protein revealed it to be capable of the oxidative decarboxylation of a range of ketoacids derived from proteinogenic amino acids. The significance of the turnover of these different ketoacids is discussed in relation to the mechanism of this fascinating enzyme.

4-Hydroxyphenylpyruvate dioxygenase [4-HPPD][¶] is a key enzyme in the metabolism of the aromatic amino acids, phenylalanine **1** and tyrosine **2** and has recently become the focus of considerable research interest. The degradative pathway for these amino acids share a common sequence of reactions since **1** is initially hydroxylated to **2**, a process catalysed by a tetrahydrobiopterin dependent monooxygenase, phenylalanine monoxygenase. Tyrosine is then transaminated to give 4-hydroxyphenylpyruvate **3** [4-HPP], which undergoes oxidative decarboxylation and hydroxylation to yield homogentisate **4** [HGA]. Cleavage of the aromatic ring by homogentisate oxygenase then yields 4-maleylacetoacetate **5** which is isomerised to 4-fumarylacetoacetate **6** before undergoing cleavage to fumarate **7** and acetoacetate **8** (Scheme 1).

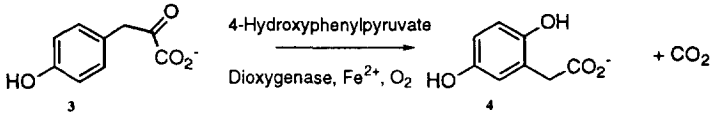


Scheme 1

* Author to whom correspondence should be addressed; FAX :01865 275674,
E-mail : ncrouch@ermine.ox.ac.uk.

¶ Abbreviations: 4-HPPD, 4-hydroxyphenylpyruvate dioxygenase; 4-HPP, 4-hydroxyphenylpyruvate; HGA, homogentisic acid; α-KICD, α-ketoisocaproate dioxygenase; α-KIC, α-ketoisocaproate; β-HIVA, β-hydroxyisovaleric acid; MTG, α-monothioglycerol; PP, phenylpyruvate; MTKB, β-methylthio-α-ketobutyrate; DAOC/DACS, deacetoxycephalo-sporin C / deacetylcephalosporin C synthase.

4-HPPD is responsible for the oxidative decarboxylation, hydroxylation and rearrangement of 4-HPP **3** to HGA **4** (Scheme 2) and achieves this conversion by coupling the oxidative decarboxylation of the ketoacid with molecular oxygen activation.[1]

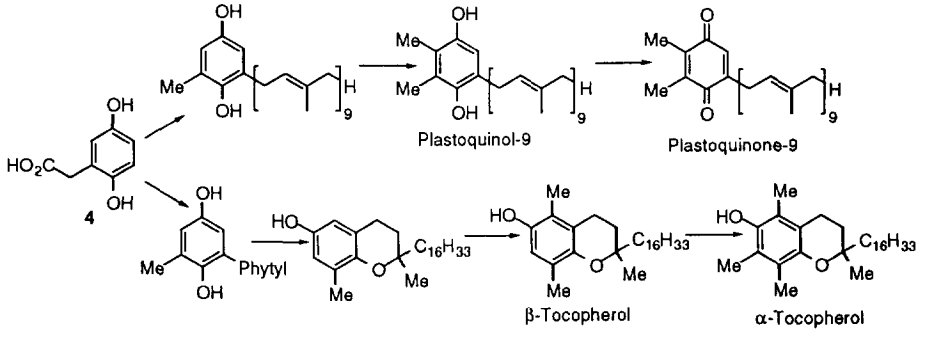


Scheme 2

The powerful oxidant so generated is then used to further modify the original ketoacid substrate. Unlike the α-ketoglutarate dependent dioxygenases, the substrate acts as both the ketoacid and recipient of the generated oxidising power and consequently, 4-HPPD has been referred to as an internal ketoacid dependent dioxygenase, as distinct from the enzymes prolyl hydroxylase[2], deacetoxycephalosporin C / deacetylcephalosporin C synthase[3] or clavaminate synthase[4], and others, which all require α-ketoglutarate as an essential co-substrate and which ultimately perform oxidative-type chemistry on separate substrates.

Several disease states exist due to inborn errors in branched chain and aromatic amino acid metabolism[5], for example maple syrup disease, phenylketonuria, alcaptonuria and tyrosinaemia types I, II and III. Since 4-HPPD occupies a central position in aromatic amino acid metabolism , it is either directly or perhaps indirectly involved in some of these diseases, hence considerable efforts have been made to study this enzyme. As a result, the purification of 4-HPPD from pig[6], beef[7], dog[8], rat[9,10], human[1,11], and frog[12] liver has been reported. The cofactor and co-substrate requirements of 4-HPPD are typical of α-ketoacid dependent dioxygenases i.e. molecular oxygen as co-substrate, ferrous iron as co-factor and ascorbate and dithiothreitol for maximal activity[11]. 4-HPPD from human liver[13], and from *Pseudomonas* sp. strain P.J.874[14] have been sequenced, cloned, and the molecular weight determined. The results indicate that the protein from human liver is a homodimer with sub-units of *ca* 46 KDa, whereas the *pseudomonas* enzyme is a tetramer.

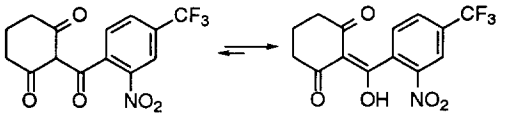
The importance of 4-HPPD extends beyond mammalian metabolism, for example in plants chorismic acid is converted to 4-HPP **3** which is then transformed to HGA by the action of 4-HPPD (Scheme 3). HGA **4** then undergoes polyprenylation and decarboxylation to generate, depending upon the polyprenylating agent, tocopherols and plastoquinones both of which are essential electron carriers involved in photosynthesis[15].



Scheme 3

In addition, 4-HPPD has been shown to be involved in melanin (pyomelanin) biosynthesis in *Streptomyces avermitilis* [16] and likewise in the marine prokaryote, *Shewanella colwelliana D* [17].

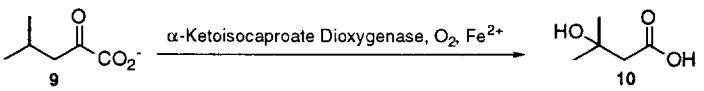
The recent discovery that a class of compounds which act as strong bleaching herbicides are in fact inhibitors of 4-HPPD[18] has led to the use of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexadione (Scheme 4) as an alternative treatment[19] for tyrosinaemia type I . Previous treatment of this life-threatening condition was possible only by liver transplantation.



Scheme 4

The mechanism for this inhibition is currently unknown but since these compounds are strong competitive inhibitors of 4-HPPD with $t_{1/2}$ for dissociation in the order of 10-20h, it is reasonable to assume that the favoured keto-enol form mimics the ketoacid functionality present in the substrate and is capable of binding strongly to the ferrous ion in the active site. Indeed, it has been known for some time that the enol form of 4-HPP acts as an inhibitor of 4-HPPD and gives rise to a 2:1 complex with enzyme-bound ferric iron[20]. Evidence exists which suggests that inactivation of the enzyme in this way may be partly reversed by ascorbate.

The only other example of a so-called internal ketoacid dependent dioxygenase was previously thought to be α-ketoisocaproate dioxygenase (α-KICD), the enzyme believed to be responsible for the cytosolic activity which converts α-ketoisocaproate **9** [α-KIC] to β-hydroxyisovaleric acid **10** [β-HIVA] (Scheme 5).



9 **10**

Scheme 5

α-KICD was reportedly purified from the cytosol of kidney and liver cells of rat[21-24], human, mouse, rabbit, guinea pig, beef and chicken liver[25], but no data relating to the amino acid sequence was ever published. Mechanistic studies[26] with purified enzyme preparations investigated the origin of the hydroxyl oxygen in the hydroxylated product **10**. Incubations performed under an atmosphere of $^{18}O_2$ resulted in the incorporation of label into the hydroxyl group and into the carboxylate group, indicating that both are derived from molecular oxygen. Overall it was shown that the stoichiometry of the reaction involved the consumption of one molecule of dioxygen for each molecule of CO_2 released.

To summarise, it appeared that 4-HPPD and α-KICD were two enzymes which formed a sub-group of the growing family of α-ketoglutarate dependent dioxygenases. We thus decided to examine further the enzymology of one of these so called internal ketoacid dependent dioxygenases and turned our attention to α-KICD.

Results & Discussion.

Initially a crude rat liver extract was prepared *via* a previously published protocol.[22] Further purification was achieved by a G75 gel filtration desalting step, DEAE ion exchange chromatography, phenyl-Sepharose hydrophobic interaction chromatography, Superdex S75 gel filtration and Mono-Q ion exchange chromatography (Table 1). α-KICD activity of column fractions was assayed by monitoring the release of $^{14}CO_2$ from [1-^{14}C]-α-ketoisocaproate[24], prepared by incubating L-[1-^{14}C]-leucine with L-amino acid oxidase.[27]

Mono-Q ion exchange chromatography resolved the α-KICD activity into three major bands. A sample of the protein eluting in the third peak of activity was subjected to N-terminal sequencing but yielded no data and was assumed to be blocked. A tryptic digest was therefore performed on further material and the products purified by reversed-phase HPLC. Selected peptides were sequenced and their sequences compared against the Protein Identification Resource (PIR) package - version 4.0. All the peptides showed total identity with liver specific rat F-antigen[29], which has recently been recognised as a species variant of 4-hydroxyphenyl-pyruvate dioxygenase[13,14] (Figure 1).

```
1    Y W D K G P K P E R G R F L H F H S V T F W V G N A K Q A A
31   S F Y C N K M G F E P L A Y K G L E T G S R E V V S H V I K
61   Q G K I V F V L C S A L N P W N K E M G D H L V K H G D G V
91   K D I A F E V E D C E H I V Q K A R E R G A K I V R E P W V
121  E E D K F G K V K F A V L Q T Y G D T T H T L V E K I N Y T
                      F A V L Q T Y G D T T H T L V E K
151  G R F L P G F E A P T Y K D T L L P K L P S C N L E I I D H
181  I V G N Q P D Q E M E S A S E W Y L K N L Q F H R F W S V D
211  D T Q V H T E Y S S L R S I V V A N Y E E S I K M P I N E P
241  A P G R K K S Q I Q E Y V D Y N G G A G V Q H I A L R T E D
                  S Q I Q E Y V D Y N G G A G V Q H I A L
271  I I T T I R H L R E R G M E F L A V P S S Y Y R L L R E N L
                            G M E F L A V P S S Y Y R
301  K T S K I Q V K E N M D V L E E L K I L V D Y D E K G Y L L
                      E N M D V L E E L K
331  Q I F T K P M Q D R P T L F L E V I Q R H N H Q G F G A G N
361  F N S L F K A F E E E Q A L R G
```

Figure 1. Data Base Sequence of Rat Specific Antigen F and Sequence of Fragments Obtained by Tryptic Digest of a Protein Possessing α-Ketoisocaproate Dioxygenase Activity.

To confirm that the α-KICD activity was due to 4-HPPD, samples of the column fractions which had been retained from all of the purification steps were incubated with [1-^{14}C]-4-HPP and the release of ^{14}C labelled CO_2 monitored. It was found that 4-HPPD activity coincided exactly with the α-KICD activity. It thus appeared that 4-HPPD was capable of catalysing the oxidative decarboxylation and hydroxylation of α-KIC to β-HIVA (Table 1).

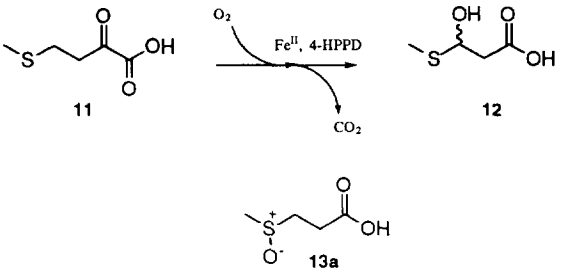
Fraction	Volume (ml)	Protein ($mg.ml^{-1}$)	Activity ($nmol.min^{-1}$)	Recovery %	Specific Activity ($nmol.min^{-1}.mg^{-1}$)	Purification (fold)
Crude	200	36.3	2550	100	0.35	1
G75	672	4.15	1530	60	0.55	1.6
DEAE	450	1.01	900	35	1.99	5.7
Phenyl Sepharose	200	0.26	290	11	6.60	19
Superdex 75	20	0.39	280	11	35.0	100
Mono-Q						
First Peak	5	0.48	19	0.7	8.0	23
Second Peak	3	0.60	34	1.3	18.7	53
Third Peak	7	0.31	68	2.7	30.9	88

Table 1. Purification of α-Ketoisocaproate Dioxygenase from Rat Liver

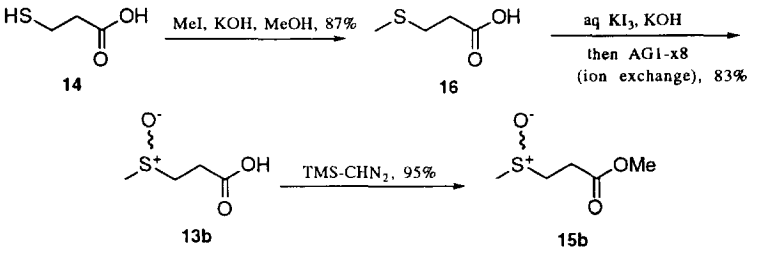
This result was definitively confirmed by the cloning and over expression of the gene encoding 4-HPPD from a rat c-DNA library into *E.coli*. The over expression of soluble protein in this bacterium has permitted the development of a simple one step purification procedure which affords protein of approximately 80% purity. Initial substrate specificity studies of this recombinant 4-HPPD using both conversion of radiolabelled [1-^{14}C]-α-KIC and [1-^{14}C]-4-HPP in addition to NMR scale incubations (2 mg), confirmed that the enzyme did indeed possess both 4-HPPD and α-KICD activity[30,31].

The ketoacids derived from valine (α-ketoisovalerate) and isoleucine (α-keto-β-methyl-*n*-valerate) were also incubated with both the native enzyme and the recombinant protein. N.m.r. scale incubations (*ca.* 2 mg of substrate) suggested that no conversion of these substrates by 4-HPPD had occurred and this was confirmed with the more sensitive radioassay. Thus no $^{14}CO_2$ was detected when the corresponding [1-^{14}C]-ketoacids, prepared from the respective [1-^{14}C]-labelled L-amino acids by the action of L-amino acid oxidase, were incubated with 4-HPPD.

Also of interest to us was the report[23] that α-KICD was capable of converting the ketoacid derived from methionine, α-keto-γ-methiobutyric acid **11** (KMBA), to a product the identity of which had not been established. The authors reasoned on the basis of the shorter retention time of the new product on reversed-phase HPLC, that its identity was likely to be the hemithioacetal **12** (Scheme 6). We considered that this was not the most likely product and reasoned that a more probable product was the corresponding sulfoxide **13a**. To test our hypothesis, we synthesised a racemic sample of the sulfoxide **13b** *via* modification of a literature procedure[32]. Thus commercially available 3-thiopropionic acid **14** was subjected to S-methylation and oxidation followed by purification *via* anion exchange chromatography to give the racemic sulfoxide **13b**. The sulfoxide was then converted to the methyl ester **15b** by treatment with trimethylsilyldiazomethane and the resulting product chromatographed on silica to afford the analytically pure sulfoxide **15b** (Scheme 7).



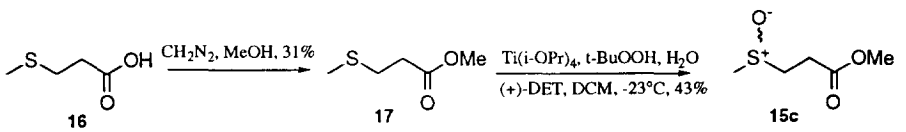
Scheme 6



Scheme 7

We next incubated approximately 20 mg of the ketoacid **11** with recombinant 4-HPPD in the presence of the usual co-substrates and co-factors. After protein precipitation and centrifugation the supernatant was subjected to the same purification and derivatisation protocol as the synthetic sample to afford the pure sulfoxide **15a**. This material was identical to the authentic standard **15b** by ^{1}H-nmr spectroscopy (500 MHz, $CDCl_3$) and mass spectrometry [151 (MH^+) NH_3 chemical ionization], but unlike the racemic synthetic sample, the enzymic product was shown to be optically active.[33] No other products were observed in the conversion of **11** to **13a** and in addition, no conversion occurred when the incubation was performed in the absence of 4-HPPD but in the presence of the other co-factors and oxygen.

In order to determine the stereochemistry of the enzymic product **13a**, we applied the asymmetric oxidation of Kagan[34] to the thioether **17**, itself made by treatment of **16** with diazomethane (Scheme 8), using the (+)-antipode of diethyl tartrate as the chiral inducer. In our hands, we obtained the sulfoxide **15c** but with lower enantiomeric excess (*R*:*S*, 57:43) than reported in the original paper. Both the enzymic and synthetic sulfoxides were analysed by proton ^{1}H-nmr spectroscopy in the presence of two equivalents of *R*-(-)-(dinitrobenzoyl)-α-methylbenzylamine[35] and the sulfinyl-methyl groups showed two resonances of unequal intensity. The bias was similar in magnitude when **15a** and **15c** were compared, but opposite in sense[33]. The same result was obtained when their optical rotations and CD spectra were compared (Fig. 2). From Kagan's assignment of the absolute configuration[34], we conclude that the enzyme converts the ketoacid **11** to the sulfoxide **13a** with a slight bias (15% enantiomeric excess) in favour of the *S* isomer.



Scheme 8

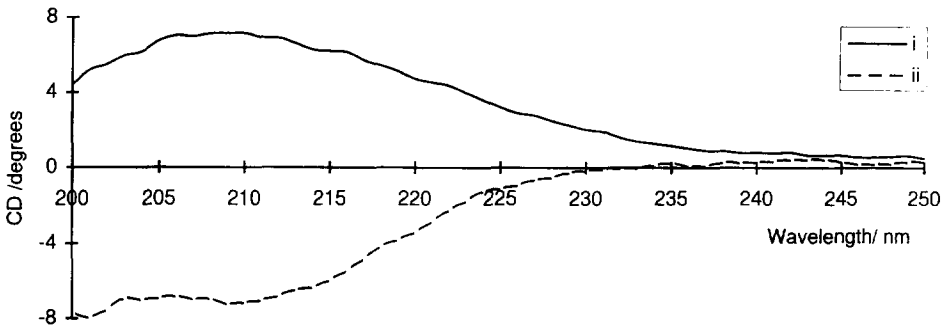
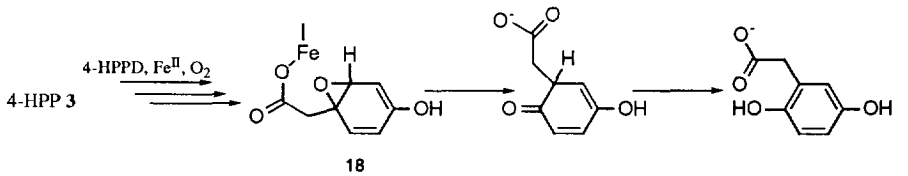


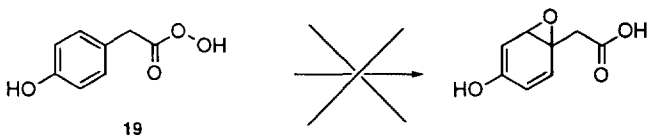
Figure 2. CD spectra of (i) the enzymic sulfoxide **15a** and (ii) the synthetic sulfoxide **15c**

Many mechanisms have been proposed to account for the conversion of 4-HPP to homogentisate by 4-HPPD. These range from the formation of the peracid of 4-HPP by the action of 4-HPPD and molecular oxygen[36] to hydroperoxidation of the aromatic ring followed by rearrangement[37] and more recently to a mechanism involving a metallated ketyl intermediate[38]. A key feature to most of these mechanisms (except Goodwin and Witkop's)[37] has been the proposed intermediacy of an arene oxide **18** which proceeds to product *via* rearrangement to a semi-quinone type intermediate (Scheme 9).



Scheme 9

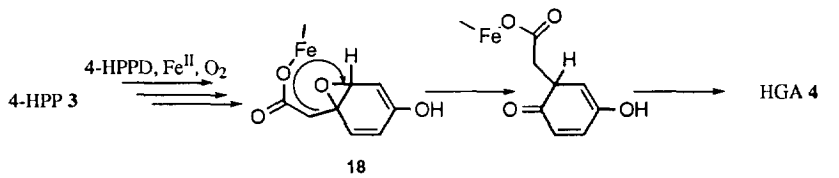
Several key points concerning the proposed mechanism are worthy of comment here. For example, the generation and involvement of a peracid intermediate has been discredited on the basis that 4-hydroxyphenylperacetic acid **19** did not undergo self-epoxidation[39] (Scheme 10).



Scheme 10

The experimental observation that phenylpyruvate and 4-fluorophenylpyruvate[40] are substrates for 4-HPPD, has been presented as evidence against the involvement of the 4-hydroxy group in the conversion of 4-HPP **3** to

HGA **4**. For example, Jefford[38] has proposed a mechanism involving the rearrangement of the proposed arene oxide **18** as below (Scheme 11) and states " A significant aspect of this mechanism is that the 4-hydroxy substituent is not mechanistically implicated, which further explains why the enzymic reaction works equally well when the 4-hydroxy substituent is missing or replaced by a fluorine substituent".

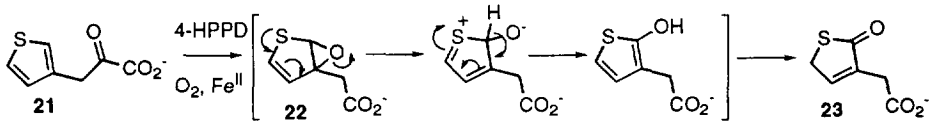


18

Scheme 11

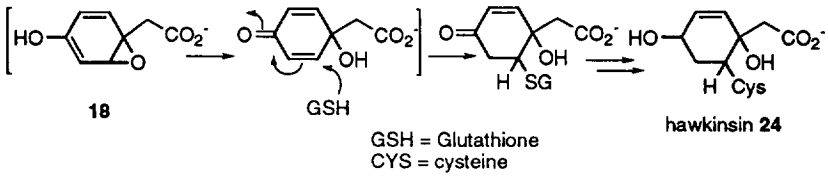
The logic of such argument is obvious but has weak foundation. For example, the rearrangement of the generally proposed arene oxide intermediate **18** would be expected to be greatly facilitated by the presence of the 4-hydroxy group as exemplified in the well known dienone-phenol rearrangement[41]. In the absence of the hydroxy substituent, the rate of rearrangement would be expected to be slower, since the stabilising effect of the hydroxy group is absent. Thus one would conclude phenylpyruvate to be a poorer substrate for the enzyme than 4-HPP, as indeed is found to be the case[1] with values for K_m and V_{max} of 0.03mM and 4 nmol.min.$^{-1}$ g $^{-1}$ for 4-HPP and 0.05mM and 0.02 nmol.min.$^{-1}$ g $^{-1}$ for PP. In addition, the fact that 4-fluorophenylpyruvate is a substrate, despite the strongly electron withdrawing fluoro substituent, seems somewhat surprising. However this observation is readily accounted for, since it is known that the energy levels of the fluorine 2p and carbon 2p orbitals are relatively similar and hence can interact. Such interaction leads to mesomeric stabilisation somewhat similar to, but weaker than, that exhibited by a hydroxy substituent and is generally accepted as the explanation for the experimental observation that fluorobenzene is attacked at very nearly the same rate in electrophilic substitution reactions as benzene itself[42,43]. Thus it is fundamentally wrong to rule out the involvement of the 4-hydroxy substituent in any proposed mechanism.

Further support for the involvement of an arene oxide intermediate, such as **18**, has recently been presented[44]. Studies involving the incubation of the unnatural substrate 3-thienylpyruvate **21** with 4-HPPD resulted in formation of a product best accounted for by the proposal of an epoxide intermediate, i.e. 3-thienylpyruvate was converted to 3-carboxymethyl-3-thiolene-2-one **23** *via* the proposed intermediate **22** (Scheme 12).



Scheme 12

In addition, the observation that some patients suffering from the genetic disease, Hawkinsinuria, attributed to production of defective 4-HPPD, excrete the unusual amino acid hawkinsin **24**[45,46] has been cited as further evidence for the arene oxide intermediate[44] (Scheme 13).



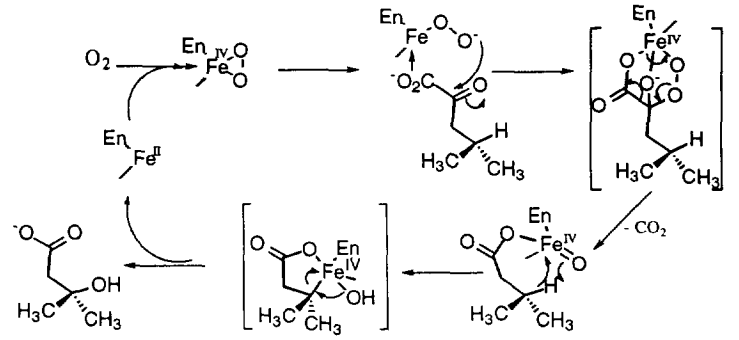
Scheme 13

Overall, it appears that the case for the intermediacy of an arene oxide is strong, but the key point yet to be resolved is the mechanism by which such an arene oxide arises. Whatever mechanism is proposed, it must take into account the wide range of substrates accepted by this enzyme and be capable of rationalising the products generated. With the discovery that α-KICD is actually 4-HPPD, the number of naturally occurring ketoacid substrates converted to products by 4-HPPD has almost doubled, and now includes 4-HPP, PP, α-KIC and KMBA as well as other ketoacid substrates such as 4-fluorophenylpyruvate[40] etc. Far from complicating the problem, it is our belief that the transformation of these other substrates by 4-HPPD enables a more detailed understanding of the overall mechanism to be achieved. Consequently we are of the opinion that the correct manner in which to attempt to resolve mechanistic issues concerning 4-HPPD, is to consider the types of chemistry that this interesting enzyme displays and to correlate them with the known chemistry of other ketoacid dependent dioxygenases.

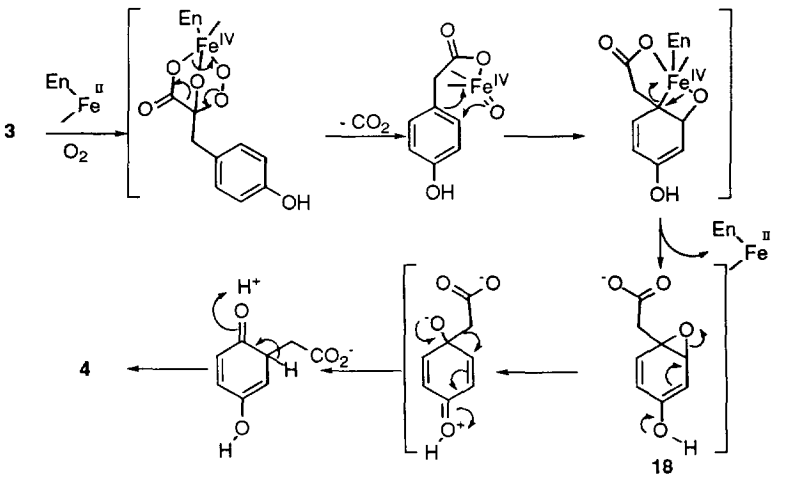
If we disregard the first enzymic event - decarboxylation coupled to reduction of molecular oxygen, a process which is common to all substrates - it seems strange that a single enzyme is capable of catalysing such seemingly different chemical modifications, for example, the 1,2-shift of an acetic acid group on an aromatic ring combined with hydroxylation at the original carbon bearing the acetic acid group, as in the case of 4-HPP **3** to HGA **4** (Scheme 2) and secondly the hydroxylation of an unactivated tertiary centre as in the case of α-KIC to β-HIVA (Scheme 5). However, further consideration of the known chemistry of ketoacid dependent dioxygenases such as deacetoxycephalosporin C/ deacetylcephalosporin C synthase (DAOC/DACS)[3], coupled with the observation that there is a similar internal distance between the α-ketocarboxy group and the site of reaction in both ketoacid substrates 4-HPP and α-KIC, makes the observed promiscuity with respect to substrate specificity more comprehensible. For example, the type of activity displayed by DAOC/DACS appears to be dependent upon the type of functionality present at the site of reaction. Unsaturation at the site of reaction can result in epoxidation[47], as observed with the unnatural substrate, [4-^{2}H]-exomethylene cephalosporin C. Alternatively, with one of the natural substrates of this bifunctional enzyme, deacetoxycephalosporin C, hydroxylation of the vinylic methyl group occurs to give deacetylcephalosporin C.[48]

If we now consider the two ketoacid substrates, α-KIC **9** and 4-HPP **3**, bearing the above mentioned observations in mind, we note that α-KIC presents an unactivated C-H bond to the reactive species at the active site, which considering the mode of enzyme activation, is probably an iron^{IV}oxene[49]. In this case the most likely course of reaction is hydroxylation *via* insertion of the iron^{IV}oxene into the C-H bond followed by reductive elimination / rearrangement (Scheme 14).

Alternatively, unsaturation present in the substrate at the site of reaction, such as the aromatic ring of 4-HPP, can lead to epoxidase activity[47] *via* direct 2 + 2 cycloaddition followed by reductive elimination (Scheme 15). Rearrangement of the proposed epoxide intermediate **18** in a manner analogous to the dienone/phenol rearrangement[41] then yields homogentisate **4**.



Scheme 14



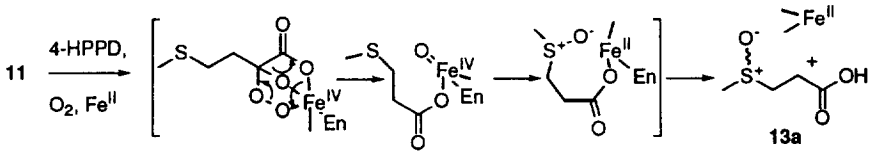
Scheme 15

In both proposed mechanisms above (Schemes 14 and 15) we favour co-ordination of the so-formed iron^{IV}oxene and carboxylate group as this would help to account for the observed regiospecific nature of the oxene's reactivity.

Finally, we have identified[33] the product from the 4-HPPD catalysed conversion of KMBA **11** as being 3-methylsulfinylpropionic acid **13a** thus correcting an erroneous suggestion in the literature[23]. That 4-HPPD should possess the ability to catalyse heteroatom oxidation is not without precedent since Pascal[50] reported the conversion of [(4-hydroxyphenyl)-thio]pyruvate **25** to [(4-hydroxyphenyl)-sulfinyl]acetate **26** (Scheme 16).

Scheme 16

The mechanism for oxidation of **11** probably involves attack by a lone pair of electrons on the sulfur atom onto the electrophilic oxygen of the iron oxene (Scheme 17).



Scheme 17

In the case of KMBA **11** we observed only a slight bias in the stereoselectivity of this process which could be accounted for in terms of increased flexibility of the alkyl chain of the substrate compared to α-KIC. Thus either lone pair of the sulfur atom could be presented to the iron oxene. In the case of the α-KIC **9**, the greater steric bulk could lead to restricted motion of the substrate in the active site and thus possibly explain the observed retention of configuration during the hydroxylation of 9 to β-HIVA **10**[51] and indeed during the conversion of the unnatural substrate, *S*-4-methyl-2-oxohexanoic acid to *R*-3-hydroxy-3-methylpentanoic acid which also proceeds with retention[52].

Perhaps of more interest than the mechanistic aspects of the conversion of KMBA **11** by 4-HPPD, is the catabolic significance of this reaction. It has been thought for some time that alternate pathways probably exist for the catabolism of methionine, other than its conversion *via* homocysteine and cystathionine to cysteine[53]. The basis for this hypothesis arises from the observation that the transamination of methionine is readily catalysed by asparagine and glutamine transaminases. Although the oxidative conversion of KMBA by 4-HPPD *in vitro* has now been established, it is still speculative as to whether this process occurs *in vivo*, and if so, what significance it may have in relation to methionine catabolism. Further work is clearly required to determine what role, if any, 4-HPPD may play in the *in vivo* catabolism of methionine. It is, however, of interest to draw a parallel with cysteine catabolism in which it has been shown that another ketoacid dependent dioxygenase, cysteine dioxygenase[54], is responsible for the oxidation of the thio group of cysteine to cysteine sulfinate.

The discovery that 4-HPPD catalyses the oxidative decarboxylation and oxidation of a variety of ketoacids derived from a range of amino acids may have significant implications for our understanding of mammalian amino acid metabolism. We are continuing our efforts to study this fascinating enzyme and will report our results in due course.

Experimental

1-[^{14}C]-4-methyl-2-oxo-pentanoic acid. The previously described method for the oxidation of radioactive amino acids was followed[27]. Thus [1-^{14}C]-L-leucine (2.5 ml, Amersham, 125 μCi) was converted to [1-^{14}C]-α-KIC by the action of L-amino acid oxidase. The product was purified by Dowex-50WX8 (200 mesh) cation exchange chromatography eluting with water and then diluted with sufficient solid α-KIC to give a stock solution with a specific activity of 100 dpm nmol^{-1}. This solution was divided into 1 ml aliquots and stored at -80°C. For enzyme radioassays, 1 ml of the stock solution was made up to 10 ml with water to give the "working [1-^{14}C]-α-KIC solution" (total α-KIC concentration of 24mM).

Radioassay of α-ketoisocaproate dioxygenase activity. A modified procedure to that previously described in the literature[23] was used. Thus, assay buffer (0.25M Tris, 0.25M maleic acid adjusted to pH 6.5 with 1M NaOH solution, 318 µl) was placed in a 10 ml tube. Freshly prepared cofactor solution ([iron (II) sulfate heptahydrate (22.0 mg), L-ascorbic acid (7.0 mg) and dithiothreitol (12.5 mg) in water (5 ml)], 25µl) was added to the enzyme solution (40 µl). This solution was pre-incubated at 27°C with shaking for 30 min and then [1-^{14}C]-KIC solution (16.5 µl, 40Kdpm, final concentration 1mM) was added. A 1 ml eppendorf vial containing hyamine hydroxide (200 µl) was immediately placed above the solution and the tube sealed with a vaccine cap. After incubation at 27°C for 1h the reaction was quenched by the addition of 20% trifluoroacetic acid (200 µl) administered by syringe through the vaccine cap. Incubation at 27°C was continued for a further 1 h to ensure that all the liberated $^{14}CO_2$ had been released and absorbed, then the vial was removed, placed in a scintillation vial, Optiphase 'Safe' scintillant added and the mixture counted for ^{14}C activity.

1-[^{14}C]-4-Hydroxyphenylpyruvate. 1-[^{14}C]-L-tyrosine (Amersham, 50 µl, 2.5 µCi) was converted to [1-^{14}C]-4-HPP in a similar manner to that described for the preparation of [1-^{14}C]-α-KIC. After purification by cation exchange chromatography, the [1-^{14}C]-4-HPP solution was made up to 7.5 ml with Tris-HCl buffer (0.2M, pH 7.5) and a total 4-HPP concentration of 1 mM. It was found that this solution was generally stable only for 2-3 days and so was normally used immediately after preparation.

Radioassay for 4-Hydroxyphenylpyruvate Dioxygenase activity. The procedure described for α-KICD assays was then followed except that [1-^{14}C]-4-HPP (40Kdpm per assay) was used in place of [1-^{14}C]-α-KIC.

Purification of wild-type α-KICD

General. All purification steps were carried out at 4°C. Buffer A consisted of 0.2M Tris-HCl, pH 7.8, 1% isopropanol. All chromatography was controlled by the BioRad FPLC system and fractions were assayed for α-KICD activity as described above. Protein concentration was measured by the method of Bradford[28] and purity of fractions was determined by SDS-Polyacrylamide gel electrophoresis. All active fractions were made 5% in α-monothioglycerol (MTG).

Preparation of the crude rat liver extract. Rat livers (175g, male, Sprague-Dawley, 6 months old) were thawed in 0.25M sucrose containing 1% isopropanol (350 ml) and the mixture homogenized at 0°C. The resultant suspension was centrifuged (10 Krpm, 40 min, JA-10 rotor) and ammonium sulfate added to the supernatant to 45% saturation. The precipitate was stirred for 40 min, centrifuged (10Krpm, 40 min) and the supernatant decanted. Ammonium sulfate was added to the supernatant to 75% saturation and the mixture again centrifuged (10 Krpm, 40 min, JA-10 rotor). The pellet was resuspended in Buffer A containing 5% MTG, the solution was centrifuged once more (20 Krpm, 20 min, JA-20 rotor) and the supernatant decanted to give a final volume of 210 ml.

Sephadex G-75 gel filtration chromatography. A Sephadex G-75 superfine column (90 x 5 cm) was equilibrated with Buffer A (2 L) and the above crude extract (50 ml, 55 mg ml^{-1}) loaded onto it. Buffer A (600 ml) was pumped through the column at 2 ml min^{-1} and discarded. Fractions (40 x 25 ml) were then collected, assayed and the most active 4 fractions were combined and made 5% in MTG. The procedure was carried out a further 3 times to yield 400 ml (920 mg) of active *G-75 pool.*

DEAE-Sepharose anion exchange chromatography. A DEAE-Sepharose fast-flow anion-exchange column (12 x 5 cm) was equilibrated with 1M NaCl in buffer A (600 ml) followed by buffer A (2 L). The G-75 pool was loaded and the column was washed with Buffer A (500 ml) before running a linear gradient of 0-200 mM NaCl over 3 L (4 ml min^{-1}). Fractions (20 x 150 ml) were collected and the most active 3 fractions were combined, made 5% in MTG, and concentrated by ultrafiltration to 120 ml. This solution constituted the *DEAE pool* (455 mg).

Phenyl-Sepharose hydrophobic interaction chromatography. A phenyl-Sepharose fast-flow column (20 ml) was washed with buffer A (60 ml) and then equilibrated with 2.5M NaCl in buffer A. The DEAE pool was made 2.5M in NaCl and 60 ml applied to the column (2 ml min^{-1}). The column was washed with 2.5M NaCl in buffer A and a linear gradient of 2.5-0M NaCl run over 320 ml with fractions (20 x 20 ml) being collected. The entire process was repeated until all the DEAE pool had been purified. The 5 most active fractions from each run were combined and concentrated by ultrafiltration to 5 ml. This solution constituted the *phenyl-Sepharose pool* (53 mg).

Superdex-75 gel filtration chromatography. A Superdex 75 column (60 x 2.6 cm) was equilibrated with buffer A (350 ml) and the phenyl-Sepharose pool applied. Buffer A was pumped through the column (0.5 ml min^{-1}) and the first 50 ml run to waste. Fractions (30 x 10 ml) were collected and the two most active fractions combined to constitute the *Superdex-75 pool* (8 mg).

Mono-Q 5/5 anion exchange chromatography. A Mono-Q 5/5 column (1 ml) was washed with 1M NaCl in Buffer A and then equilibrated with Buffer A. The Superdex-75 pool was applied and the column washed with buffer A (10 ml). A linear gradient of 0-200 mM NaCl in buffer A was run over 35 ml and 1 ml fractions taken. The α-KICD activity was split into 3 incompletely resolved bands. The three most active fractions of the first peak (2.4 mg), the four most active of the second peak (1.8 mg) and the three most active of the third peak (2.2 mg) were combined and made 5% in MTG. These constituted the *Mono-Q pools 1, 2 and 3* respectively.

Purification of recombinant 4-HPPD

Preparation of Incubation Grade 4-HPPD. *E. coli* cells containing the 4-HPPD-encoding plasmid were grown on 2TY media in a 30L fermentor at 27°C. A portion of the resultant cells (32g) were suspended in buffer A (100 ml, 50 mM Tris.HCl, pH 7.8), lysozyme added (40 mg) and the suspension stirred for 1h at 4°C. DNase type I and magnesium chloride (a few crystals each) were added and the viscous mixture stirred for a further 30 min. The mixture was sonicated (5 x 15 second pulses, 30s cooling between pulses), centrifuged (10Krpm, JA-10 rotor for 30 min) and the supernatant made 50% saturated in ammonium sulfate and the resulting suspension stirred for 30 min. After further centrifugation (14Krpm, JA-20 rotor, 30 min) the supernatant was discarded and the pellet resuspended in buffer A (250 ml). After filtering, the solution was loaded at 20 ml min^{-1} onto a DEAE-Sepharose column (250 ml) which had been pre-equilibrated in 2M NaCl in buffer A (1 L) followed by buffer A (3 L). The column was washed with buffer A until all unbound protein had been eluted and then a NaCl gradient in buffer A (0-350 mM) was run at 20 ml min^{-1} over 1 L, whilst collecting 50 ml fractions. The fractions were assayed for α-KICD activity using the radioassay described above and also examined by SDS-gel

electrophoresis. The purest active fractions were concentrated to 8 ml (15 mg ml^{-1}) and labelled the *recombinant DEAE pool.*

Preparation of Homogenous 4-HPPD

Q-Sepharose Chromatography. *E. coli* cells containing the 4-HPPD-encoding plasmid (160g) were suspended in buffer B (50 mM Tris.HCl, pH 7.5, 1 mM EDTA, final volume 460 ml) at 4°C. The cells were disrupted by sonication (flow-through sonicator) and the DNA precipitated by the addition of PEI (5% w/v solution in water, pH 7.8) to a final concentration of 0.15% w/v. The resulting mixture was stirred for 15 min and centrifuged (10Krpm, 30 min). The supernatant was filtered through a 0.2 μm syringe filter and loaded with a flow rate of 20 ml min^{-1}, onto a column of Q-Sepharose (300 ml bed volume) which had previously been equilibrated in buffer B (2.5 l). Buffer B was then pumped through the column until all unbound protein had been eluted and then a NaCl gradient (0-200 mM) in buffer B was run over 1.8 L, with 20 ml fractions being collected. 4-HPPD eluted at approximately 120 mM NaCl, as judged by radioassay and SDS-PAGE, and the purest fractions were combined. These constitute the *Q-Sepharose pool A*. The later active side-fractions were pooled, constituting the *Q-Sepharose pool B.*

Phenyl-Source Chromatography. The Q-Sepharose pool A was made 1M in NH_4SO_4, filtered and applied at 5 ml min^{-1} to a 50 ml phenyl-Source column which had been pre-equilibrated in buffer B (100 ml) then 1M ammonium sulfate in buffer B (100 ml). After all the protein solution had been applied, the column was washed with 1M NH_4SO_4 in buffer B until no further protein eluted. A gradient of 1-0M NH_4SO_4 in buffer B was run over 450 ml, with 10 ml fractions collected. HPPD eluted at approximately 220 mM NH_4SO_4. The purest fractions were combined to constitute the *phenyl-Source pool A*. The Q-Sepharose pool B was purified in a similar fashion to yield the *phenyl-Source pool B.*

Superdex-75 Chromatography. The phenyl-Source pool A was concentrated by ultrafiltration to 4 ml then applied at 2 ml min^{-1} to a Superdex-75 column (85 x 3.2 cm) which had been pre-equilibrated in buffer A (800 ml, 50 mM Tris.HCl, pH 7.5). Buffer A was pumped onto the column, the first 240 ml was discarded then 10 ml fractions were collected. The protein began to elute after 290 ml had been passed down the column. The purest fractions were combined and concentrated to 850 μl (50 mg ml^{-1}). The phenyl-Source pool B was purified in a similar manner to yield the *Superdex-75 pool B.*

[U-^{14}C]-3-Methyl-2-oxobutanoic acid ([U-^{14}C)]-α-ketoisovalerate). [U-^{14}C]-L-valine (Amersham, 40 μl, 265 mCi $mmol^{-1}$, 50 μCi ml^{-1}) was converted to [U-^{14}C]-3-methyl-2-oxobutanoic acid in a similar manner to that described for the preparation of [1-^{14}C]-α-ketoisocaproate (see above). The product was purified by Dowex 50WX8 (200 mesh) cation exchange chromatography and the ketoacid eluted with water. Fractions of 2 ml were collected and counted for ^{14}C activity. The most active fractions were combined and then made 5 mM in 3-methyl-2-oxobutanoic acid by addition of unlabelled 3-methyl-2-oxobutanoic acid, sodium salt.

Radioassay of α-ketoisovalerate decarboxylation activity. As for α-KICD radioassay except that [1-^{14}C]-α-ketoisovalerate solution (200 μl, 52Kdpm) was used.

Sulfoxide synthesis

3-Methylmercaptopropanoic acid[32] **16**. 3-Mercaptopropanoic acid **14** (7.40 g, 69.8 mmol) was dissolved in methanol (30 ml), a solution of potassium hydroxide (10.10 g, 180.4 mmol) in methanol (10 ml) added and the solution cooled to 0°C. Iodomethane (11.8g, 83 mmol) was added over 10 min and the solution stirred at room temperature for 12h. The solvent was evaporated *in vacuo*, water (50 ml) added and the mixture extracted with ether (3 x 100 ml). The combined organic extracts were washed with saturated brine (30 ml), dried ($MgSO_4$), filtered and evaporated *in vacuo* to give the thioether **16** (7.33g, 87.5%); δ_H ($CDCl_3$, 200 MHz) 2.14 (3H, s, CH_3S) and 2.66-2.81 (4H, m, $CH_2CH_2CO_2H$); δ_C DEPT ($CDCl_3$, 50 MHz) 15.33 (CH_3S), 28.50 ($CH_2CH_2CO_2H$), 34.15 ($CH_2CH_2CO_2H$) and 178.86 (CO_2H); m/z (EI^+) 121 (31%, MH^+), 120 (100%, M^+), and 103 (96%, $[M\text{-}OH]^+$).

(R/S)-3-Methylsulfinylpropanoic acid[32] **13b**. The thioether **16** (100 mg, 0.83 mmol) was dissolved in water (4 ml) and a solution of potassium iodide (1.00 g, 6.02 mmol) and iodine (500 mg, 1.96 mmol) in water (5 ml) was added dropwise. The pH was maintained at pH 7 by the simultaneous addition of aqueous potassium hydroxide (1M). After the red colour persisted a saturated aqueous solution of sodium sulfite was added until the colour disappeared. The solution was applied to the top of a column of AG1-X8 (BioRad) (20 ml) which had been extensively washed with aqueous sodium hydroxide (1M), water, acetic acid (2M) and water. Once the sample had been loaded, the column was washed with water (50 ml) then acetic acid (25 ml of 0.5M followed by 25 ml of 0.75M). The latter fraction was evaporated and the sulfoxide **13b** (94 mg, 83.3%) was used without further purification; δ_H (200 MHz, D_2O, TSP δ= 0.00 ppm) 2.72 (3H, s, $CH_3S(O)$); 2.87 (2H, t *J* 7.5 Hz, CH_2CO_2H) and 3.00-3.31 (2H, m, $CH_2CH_2CO_2H$); δ_C DEPT (50 MHz, D_2O, dioxan as internal reference δ_C = 66.37) 26.69 (CH_2CO_2H), 36.38 ($CH_3S(O)$), 47.23 ($CH_2CH_2CO_2H$) and 175.39 (s, CO_2H) m/z (NH_3,CI^+) 154 (90%, MNH_4^+), 137 (16%, MH^+) and 120 (100%, M^+-O).

(R/S)-Methylsulfinylpropanoic acid, methyl ester 15b. The sulfoxide **13b** (21 mg, 0.15 mmol) was dissolved in methanol (5 ml), cooled in ice then trimethylsilyldiazomethane (2M in hexanes) was added dropwise until the yellow colour persisted. One drop of glacial acetic acid was added to decolourise the solution, the solvent evaporated *in vacuo* and the mixture chromatographed on silica (ethyl acetate / methanol, 5:2 v/v) to afford the methyl ester as a clear oil (22 mg, 95%); R_f (ethyl acetate / methanol, 5:2 v/v) 0.6; Found: C 39.76%, H 7.07%, $C_5H_{10}O_3S$ requires C 39.99%, H 6.71%; υ_{max} (neat) 3002w, 2957w, 2924w, 1734s, 1659w, 1439m, 1366m, 1248m, 1201m, 1028m; δ_H (200 MHz, $CDCl_3$) 2.60 (3H, s, $CH_3S(O)$), 2.82-3.05 (4H, m, $CH_2CH_2CO_2CH_3$) and 3.73 (3H, s, CO_2CH_3); δ_C DEPT ($CDCl_3$, 50 MHz) 26.61 ($CH_2CO_2CH_3$), 38.67 ($CH_3S(O)$), 47.81 ($CH_2CH_2CO_2CH_3$), 52.23 (CO_2CH_3) and 175.08 (CO_2CH_3); m/z (NH_3, CI^+) 168 (8%, $(MNH_4)^+$) and 151 (100%, MH^+).

Incubation of 4-methylmercapto-2-oxo-butanoic acid 11 with 4-HPPD. A solution of iron (II) sulfate heptahydrate (2.4 mg), L-ascorbate (0.8 mg) and dithiothreitol (1.4 mg) in water (1 ml) was prepared and an aliquot (200 µl) added to buffer solution (Tris.HCl / maleic acid 0.75M in each, pH 6.5, 540 µl) in a 5 ml vial. The enzyme from the recombinant DEAE pool (1 ml, 2 mg ml^{-1}) was added and the reaction was started by the addition of 4-methylmercapto-2-oxobutyrate **11** (10 mg). The resulting mixture was incubated at 27°C for 18 hours. A similar incubation mixture was prepared and the two reaction mixtures run concurrently. The incubation mixtures were then combined and the protein precipitated by addition of acetone to 70% (v/v) before

being centrifuged (14Krpm, 20 min). The supernatant was partially evaporated to remove the acetone and the aqueous solution applied to the top of a column of AG1-X8 (BioRad) (20 ml) which had been extensively washed with aqueous sodium hydroxide (1M), water, acetic acid (2M) and water. The column was then washed with water (50 ml) and acetic acid (25 ml of 0.5M then 25 ml of 0.75M). The latter fraction was evaporated to afford the pure sulfoxide **13a** (11 mg) which had the same spectroscopic properties (^{1}H NMR, ^{13}C NMR and mass spectrum) as the racemic synthetic material. The enzymic sulfoxide was esterified in the same manner as described for the racemic sulfoxide to give, after chromatography, the pure methyl ester **15a** (9 mg) which also exhibited the same spectroscopic properties as the racemic synthetic ester **15b**; $[\alpha]_D^{22}$=+34.4° (acetone, c=0.4)

3-Methylmercaptopropanoic acid, methyl ester[34] **17**. The acid **16** (1.60g, 13.3 mmol) was dissolved in methanol (50 ml) and cooled to 4°C. A solution of diazomethane in ether was generated by the dropwise addition of an ethereal solution of DIAZALD® (4.00g, 18.7 mmol) to a stirred solution of potassium hydroxide in ethanol / water (70 ml, 5:2 v/v) at 65°C. The resulting diazomethane was distilled into a flask containing the stirred solution of **16** until the yellow colour persisted. Excess diazomethane was quenched with one drop of glacial acetic acid, the solvent evaporated and the crude product chromatographed on silica (petroleum ether: diethyl ether, 9:1, v/v) to afford the pure methyl ester **17** as a colourless oil (555 mg, 31%); R_f (petroleum ether: diethyl ether, 9:1, v/v); υ_{max} ($CHCl_3$) 3023w, 2955w, 1735m, 1439w, 1362w, 1222s, 1216s, 1210s; δ_H ($CDCl_3$, 200 MHz) 2.11 (3H, s, CH_3S), 2.57-2.82(4H, m, $CH_2CH_2CO_2CH_3$) and 3.71 (3H, s, CO_2CH_3); δ_C DEPT ($CDCl_3$, 50 MHz) 15.43 (CH_3S), 29.04 ($CH_2CO_2CH_3$), 34.20 ($CH_2CH_2CO_2CH_3$), 51.70 (CO_2CH_3) and 172.35 (CO_2CH_3); m/z (NH_3, CI^+) 152 (100%, MNH_4^+), 135 (8%, MH^+) and 134 (84%, M^+).

Asymmetric oxidation of 3-Methylmercaptopropanoic acid, methyl ester[34] **17**. Diethyl tartrate (309 mg, 1.50 mmol), dichloromethane (5 ml), titanium (IV) isopropoxide (225 µl, 0.76 mmol) and water (14 µl, 0.78 mmol) were sequentially placed in a dry flask under argon. The solution was stirred for 30 min, the thioether **17** (100 mg, 0.75 mmol) added and the solution cooled to -30°C. *tert*-Butyl hydroperoxide (108 µl, 7.2M in isooctane, 0.78 mmol) was then added such that the internal temperature did not rise, then the solution was stirred overnight at -23°C. Water (1 ml) was added and the resulting gel stirred vigorously for 1h before being filtered through Celite. The plug was washed extensively with dichloromethane and the combined organic fractions washed with saturated brine (10 ml). The organic layer was dried ($MgSO_4$), filtered and the filtrate evaporated to dryness to afford the crude product as an oil which was purified by chromatography on silica (ethyl acetate: methanol, 9:1, v/v). The pure sulfoxide **15c** (48 mg, 43%) exhibited the same spectroscopic properties (mass, IR, ^{1}H and ^{13}C NMR) as the racemic synthetic compound; $[\alpha]_D^{22}$=-38.0° (acetone, c=1.2); [Lit.[34] -50.2° (acetone)].

Acknowledgements.

We thank the EPSRC for a grant (to CHM) and the British Council for an Overseas Research studentship (to MHL). We thank also Prof. R. A. Pascal, Princeton University, USA, for help in the early stages of our work, Dr Y. Fujishima and Mr J.P.N. Pitt for assistance with protein purification, Dr A. C. Willis for peptide sequencing, Dr. G. J. Pritchard for help with the synthetic chemistry and Dr Z. H. Zhang for assistance in the cloning of mammalian 4-HPPD.

References

1. Lindblad, B.; Lindstedt, G.; Lindstedt, S.; Rundgren, M. *J. Biol. Chem.* **1977**, *252*, 5073.
2. Nietfield, J. J.; Kemp, J. A. *Biochem. Biophys. Acta* **1980**, *613*, 349.
3. Baldwin, J. E.; Adlington, R. M.; Coates, J. B.; Crabbe, M. J. C.; Crouch, N. P.; Keeping, J. W.; Knight, G. C.; Schofield, C. J.; Ting, H. -H.; Vallejo, C. A.; Thorniley, M. *Biochem. J.* **1987**, *245*, 831.
4. Baldwin, J. E.; Adlington, R. M.; Bryans, J. S.; Bringhen, A. O.; Coates, J. B.; Crouch, N. P.; Lloyd, M. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H.; Cassells, R.; Nicholson, N. *J. Chem. Soc. Chem. Comm.* **1990**, 617.
5. Watts, R.W.E., (1988), in *Oxford Textbook of Medicine*, Vol.1, section 9.11, 2nd Ed., Oxford University Press.
6. Buckthal, D. J.; Roche, P. A.; Moorhead, T. J.; Forbes, B. J. R.; Hamilton, G. A. *Meth.Enz.* **1987**, *142*, 132.
7. Hager, S. E.; Gregerman, R. I.; Knox, W. E. *J. Biol. Chem.* **1957**, *225*, 935.
8. La Du, B. N.; Zannoni, V. G. *J. Biol. Chem.* **1956**, *219*, 273.
9. Goswami, M. N. D. *Biochim. Biophys. Acta* **1969**, *85*, 390.
10. Lin, K. T.; Crawhall, J. C. *Can. J. Biochem.* **1976**, *54*, 423.
11. Lindstedt, S.; Odelhog, B. *Meth. Enz.* **1987**, *142*, 143.
12. Laskowska-Klita, T. *Acta Biochem. Pol.*, **1969**, *16*, 35.
13. Rüetschi, U.; Dellsen, A.; Sahlin, P.; Stenman, G.; Rymo, L.; Lindstedt, S. *Eur. J. Biochem.* **1993**, *213*, 1081 and Endo, F., Awata, H., Ishiguro, M., Eda, Y., Titani, K., and Matsuda, I. *J. Biol. Chem.* **1992**, *267*, 24235.
14. Rüetschi, U.; Odelhog, B.; Lindstedt, S.; Barros-Sördeling, J.; Persson, B.; Jornvall, H. *Eur. J. Biochem.* **1992**, *205*, 459.
15. Goodwin, T. W.; Mercer, E. I. (1983), *Introduction to Plant Biochemistry*, p458, 2nd Ed., Pergamon Press.
16. Denoya, D. C.; Skinner, D. S.; Morgenstern, M. R. *J. Bacteriology* **1994**, *176*, 5312.
17. Coon, S. L.; Kotob, S.; Jarvis, B. B.; Wang, S.; Fuqua, W. C.; Weiner, R. M. *Appl. Env. Microb.* **1994**, *60*, 3006.
18. Schultz, A.; Ort, O.; Beyer, P.; Kleinig, H. *FEBS Lett.* **1993**, *318*, 162.
19. Lindstedt, S.; Holme, E.; Lock, E.A.; Hjalmarson, O.; Strandvik, B. *Lancet* **1992**, *340*, 813.
20. Lindstedt, S.; Rundgren, M. *J. Biol. Chem.* **1981**, *257*, 11922.
21. Wohlhueter, R. M.; Harper, A. E. *J. Biol. Chem.* **1970**, *245*, 2391.
22. Sabourin, P. J.; Bieber, L. L. *Arch. Biochem. Biophys.* **1981**, *206*, 132.
23. Sabourin, P. J.; Bieber, L. L. *J. Biol. Chem.* **1982**, *257*, 7460.
24. Sabourin, P. J.; Bieber, L. L. *Meth.Enz.* **1988**, *166*, 288.
25. Grant, W. D.; Connelly, J. L. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1974**, *33*, 1570 (Abstract).
26. Sabourin, P. J.; Bieber, L. L. *J. Biol. Chem.* **1982**, *257*, 7468.
27. Rudiger, H. W.; Langenbeck, U.; Goedde, H. W. *Biochem. J.*, **1972**, *126*, 445.
28. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
29. Gershwin, M. E.; Coppel, R. L.; Bearer, E.; Peterson, M. G.; Sturgess, A.; Mackay, I. R. *J. Immunol.* **1987**, *139*, 3828.
30. Crouch, N. P.; Baldwin, J. E.; Lee, M. -H.; MacKinnon, C. H.; Zhang, Z. H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1503.
31. Baldwin, J. E.; Crouch, N. P.; Fujishima, Y.; Lee, M. -H.; MacKinnon, C. H.; Pitt, J. P. N.; Willis, A. C. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1255.
32. Doi, J. T.; Goodrow, M. H.; Musker, W. K. *J. Org. Chem.* **1986**, *51*, 1026.
33. Adlington, R. M.; Baldwin, J. E.; Crouch, N. P.; Lee, M. -H.; MacKinnon, C. H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2003.
34. Duñach, E.; Kagan, H. B. *Nouv. J. Chim.* **1985**, *9*, 1.
35. Desmuckh, E.; Duñach, E.; Juge, S.; Kagan, H. B. *Tet. Lett.* **1984**, *25*, 3467.
36. Hamilton, G. A. *Prog. Bioorg. Chem.* **1971**, *1*, 83-152.
37. Goodwin, S.; Witkop, B. *J. Am. Chem. Soc.* **1957**, *79*, 179.
38. Jefford, C. W. *Adv. Det. Reac. Mech.* **1992**, *2*, 149.
39. Jefford, C. W.; Cadby, P. A., *Experientia*, **1981**, *37*, 1134.
40. Taniguchi, K.; Kappe, T.; Armstrong, M. D. *J. Biol. Chem.* **1964**, *239*, 3389.
41. Miller, B. *Acc. Chem. Res.*, **1975**, *8*, 245.
42. Sykes, P. (1981), *A Guide Book to Mechanism in Organic Chemistry*, p 155, 5th Edition, Longman, London.
43. Isaacs, N. (1995), *Physical Organic Chemistry*, p489, 2nd Ed., Longman, London.
44. Forbes, B. J. R.; Hamilton, G. A. *Bioorg. Chem.* **1994**, *22*, 343.

45. Niederwieser, A.; Wadman, S. K.; Danks, D. M. *Clin. Chim. Acta* **1978**, *90*, 195.
46. Hocart, C. H.; Halpern, B.; Hick, L. A.; Wong, C. O. *J. Chromatography* **1983**, *275*, 237.
47. Baldwin, J. E.; Adlington, R. M.; Crouch, N. P; Pereira, I. A. C.*Tetrahedron* **1993**, *49*, 7499.
48. Baldwin, J. E.; Adlington, R. M.; Crouch, N. P; Aplin, R. T.; Wilkinson, R.*Tetrahedron* **1992**, *48*, 6853.
49. Siegel, B. *Bioorg. Chem.* **1979**, *8*, 219.
50. Pascal, R. A.; Oliver, M. A.; Chen, Y.-C. *J .Biochemistry* **1985**, *24*, 3158.
51. Adlington, R. M.; Baldwin, J. E.; Crouch, N. P.; Lee, M. -H.; MacKinnon, C. H.; Paul, D. R. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2721.
52. Han, H.; Pascal, R. A. *J. Org. Chem.* **1990**, *55*, 5173.
53. Steele, R. D.; Benevenga, N. J. *J. Biol. Chem.* **1978**, *253*, 7844.
54. McCann, K. P.; Mohammed, T. A.; Williams, A. C.; Ramsden, D. B. *Biochim. Biophys. Acta* **1994**, *1209*, 107 and references therein.

(*Received in UK* 3 *March* 1997; *revised* 26 *March* 1997; *accepted* 7 *April* 1997)