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STEREOCHEMISTRY OF HYDROXYLATION DURING THE CONVERSION OF α-KETOISOCAPROATE TO β–HYDROXYISOVALERATE BY 4-HYDROXYPHENYLPYRUVATE DIOXYGENASE

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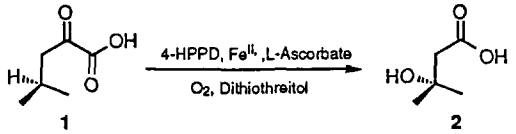
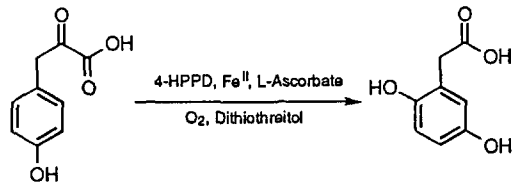
Abstract: 4-Hydroxyphenylpyruvate dioxygenase catalyzes the oxidative decarboxylation and hydroxylation of α-ketoisocaproate **1** to β-hydroxyisovalerate **2** with retention of configuration during the hydroxylation step (Scheme 1).



1 2

Scheme 1

In 1982 Sabourin and Bieber reported an enzyme in rat liver, which they named α-ketoisocaproate dioxygenase (α–KICD), that in the presence of oxygen and the cofactors iron(II), L-ascorbate and dithiothreitol, oxidatively decarboxylated α–ketoisocaproate (α-KIC) **1** to β-hydroxyisovalerate **2**[1]. We have shown[2,3] that this enzyme is identical to the previously reported 4-hydroxyphenylpyruvate dioxygenase (4-HPPD)[4] which catalyzes the conversion of 4-hydroxyphenylpyruvate **3** to homogentisate **4** (Scheme 2).



3 4

Scheme 2

In 1990 Han and Pascal demonstrated that α–KICD could also convert the unnatural substrate *S*-4-methyl-2-oxohexanoic acid **5** to *R*-3-hydroxy-3-methylpentanoic acid **6**, corresponding to complete retention of configuration during the hydroxylation reaction (Scheme 3)[5]. In order to establish the stereochemical outcome associated with the natural substrate **1** we have synthesized both enantiomers of the chiral α-KIC **7** (Scheme 4, only the *R* isomer **7a** shown) and of the chiral β-hydroxyisovalerate **8** (Scheme 5, only the *S* isomer **8c** shown) and thence proved that the hydroxylation of the natural substrate proceeds with retention of configuration.

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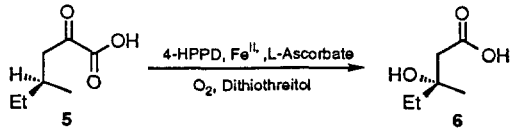
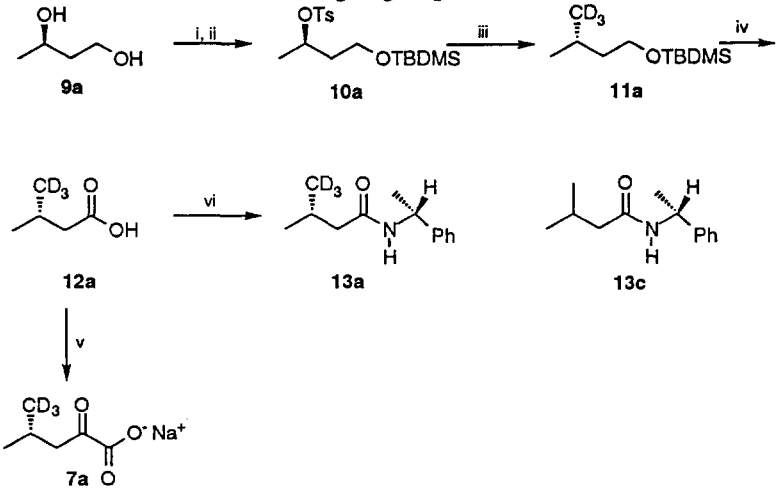


Scheme 3

The *R*-α-KIC **7a** was synthesized as follows. *R*-1,3-butanediol **9a** (Aldrich, 99% ee) was silylated then activated by treatment with *para*-toluenesulfonyl chloride (TsCl) to give, after silica gel chromatography, the tosylate **10a**. The trideuteromethyl group was added, largely with inversion, by the addition of lithium d_6-dimethyl cuprate to an ethereal solution of **10a**, affording the silyl ether **11a**. This was desilylated using potassium fluoride, then oxidized with Jones' reagent giving the labelled isovalerate **12a** (ca. 80% ee)[6].

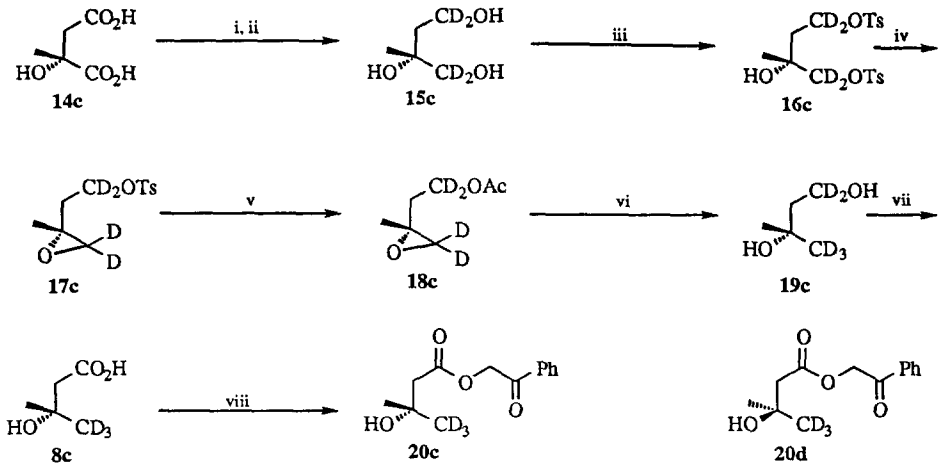


Reagents and Conditions: i) TBDMSCl, Imidazole, DMF, 12h; ii) TsCl, pyridine, 18h, 54% over two steps; iii) $(CD_3)_2CuLi$, diethyl ether; 12h; 45%; iv) KF, Jones, acetone, 12h, 71%; v) a) LDA, TMEDA: THF 1:1, 50°C then diethyl oxalate, 12h; b) HPLC then NaOH, 11%; vi) *R*-α-methylbenzylamine, DCCI, DCM, 18h, 95%.

Scheme 4

The acid **12a** was added to LDA (4 equivalents) in a mixture of THF and TMEDA and the resulting solution transferred to a solution of diethyl oxalate in dichloromethane. After work-up, the free acid was subjected to reverse phase HPLC to afford the ketoacid **7a** which was then basified to form the sodium salt. The antipode **7b** (ca. 92% e.e.) was made in similar fashion starting with *S*-1,3-butanediol **9b** (Aldrich, 96% ee)

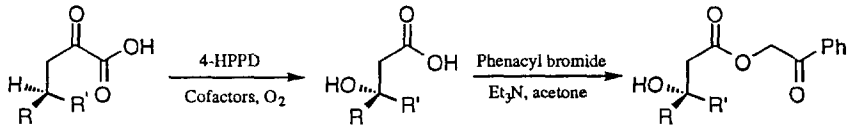
In order to determine the stereochemistry of hydroxylation when **7a** was incubated with 4-HPPD, the chiral hydroxyacids **8c** and **8d** were synthesised from *R* and *S* citramalic acids **14c** and **14d** respectively (Scheme 5). Each enantiomer of the ester **20c, d** was analyzed by 500 MHz ^{1}H NMR spectroscopy in the presence of 0.6 equivalents of the chiral shift reagent tris[3-(trifluoromethylhydroxymethylene)-(+)-camphorato],europium (III) derivative. It was not possible to assign a fully reproducible chemical shift to the methyl group of each isomer under these conditions, due to the sensitivity of the molecule to small changes in chiral shift reagent concentration. However, it was straightforward to demonstrate by doping experiments that the methyl group singlet of the *S* isomer **20c** lay distinctly upfield of that of the *R* isomer **20d.**



Reagents and Conditions: i) CH_2N_2, MeOH, 100%; ii) $LiAlD_4$, Et_2O, 18h, 85%, iii) TsCl, pyridine, 18h, 50%; iv) Cs_2CO_3, acetone, 18h, 95%; v) Me_4NOAc; acetone 24h, 90%; vi) $LiAlD_4$, Et_2O, 18h, 29%; vii) Jones, acetone, 15 min, 35%; viii) Phenacyl bromide, Et_3N, acetone, 70°C, 3h, 85%.

Scheme 5

The ketoacid **7a** was then incubated with 4-HPPD (Scheme 6, 0.2M Tris, 0.2M maleate, iron(II) sulphate, L-ascorbate, dithiothreitol, 25°C, 18h). The mixture was made 70% in acetone, centrifuged and the supernatant was evaporated to remove the acetone, acidified to pH 2 and the aqueous solution extracted four times with ethyl acetate. The organic extracts were dried and esterified with phenacyl bromide and triethylamine in acetone. Purification by silica chromatography gave a NMR pure sample of the enzymic hydroxyacid **8a** as the phenacyl ester **20a**. Incubation of the antipode **7b** and subsequent esterification provided the ester **20b**.



7a: R=CH_3, R'=CD_3
7b: R=CD_3, R'=CH_3

8a: R=CH_3, R'=CD_3
8b: R=CD_3, R'=CH_3

20a: R=CH_3, R'=CD_3
20b: R=CD_3, R'=CH_3

Scheme 6

20a was then analyzed by proton NMR spectroscopy in the presence of 0.6 equivalents of the chiral shift reagent and there appeared two resonances due to the methyl group in the approximate ratio of 91:9 (high field: low field). The results of this incubation and that of **7b** are summarised in Table 1. Thus, the conversion of ketoacid **7a** by 4-HPPD to **8a** had proceeded with apparently full retention of configuration during the C-H to C-OH hydroxylation reaction. We confirmed that the resonances observed were indeed due to enzymically formed β-hydroxyiosvalerate by doping the ester **20a** with authentic **20d** and noting the rise in the intensity of the minor (low field) resonance (Fig 1). An analagous result was noted when **20b** was analyzed. Thus, doping of this sample with authentic **20c** raised the intensity of the minor (high field) resonance.

In conclusion, we have demonstrated that the conversion of α–KIC **1** to β–hydroxyisovalerate **2** proceeds with retention of configuration during the hydroxylation step.

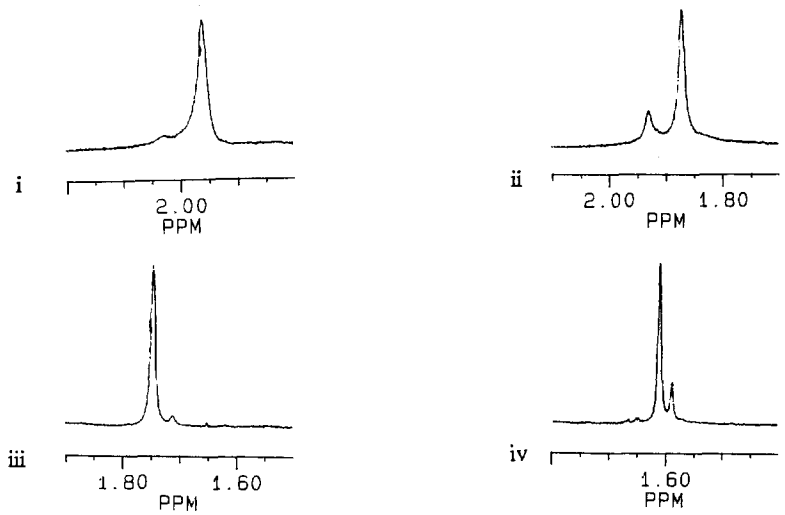


Figure 1: Proton NMR spectrum of the methyl resonances of i) **20a**, ii) **20a** doped with **20d**, iii) **20b** and iv) **20b** doped with **20c** in the presence of chiral shift reagent

Enzyme Substrate	Substrate Enantiopurity (±5%)	Enzyme Product	Product Enantiopurity (±5%)	Stereochemical Outcome
7a	90:10 (*R*:*S*)	**8a**	91:9 (*S*:*R*)	>95% retention
7b	4:96 (*R*;*S*)	**8b**	6:94 (*S*:*R*)	>95% retention

Table 1

Acknowledgements

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4) Lindblad, B; Linstedt, S.; Olander, B. and Omfelt, M., *Acta. Chem. Scand.* **1971**, *25*, 329 and references cited therein.
5) Han, H. and Pascal Jr, R. A. *J. Org. Chem.* **1990**, *55*, 5173.
6) The acid **12a** was coupled to (+)-α-methylbenzylamine with dicyclohexyldiimide (DCCI) and the resultant amide **13a** checked for diastereomeric purity by 500 MHz ^{1}H NMR spectroscopy. The methyl group showed a major resonance (ca. 90%) at δ 0.92 (d, *J* 6.5 Hz) and a minor one (10%) at δ 0.94 (d, *J* 6.5 Hz) in contrast to the two equal intensity doublets observed from the amide **13c**. Likewise, ca 92% e.e. for **12b** was established.

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