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STEREOCHEMICAL COURSE OF THE CONVERSION OF α-KETOISOCAPROATE TO β-HYDROXYISOVALERATE BY SOLUBLE, RECOMBINANT MAMMALIAN 4-HYDROXYPHENYLPYRUVATE DIOXYGENASE.[1]

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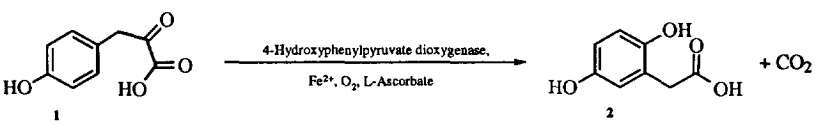
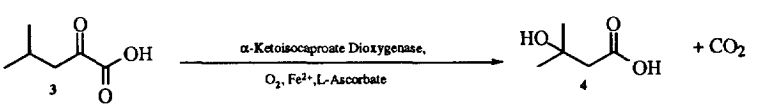
Abstract. *The stereochemical course of the conversion of α-ketoisocaproate to β-hydroxyisovalerate catalysed by the enzyme 4-hydroxyphenylpyruvate dioxygenase is described.*

4-Hydroxyphenylpyruvate dioxygenase (4-HPPD, EC 1.13.11.27) is a key enzyme in the catabolism of the aromatic amino acids phenylalanine and tyrosine. This interesting enzyme catalyses the oxidative decarboxylation of the ketoacid functionality of 4-hydroxyphenylpyruvate **1** and then performs a hydroxylation of the aromatic ring with concomitant migration of the acetic acid group, forming homogentisate **2** (Scheme 1).



Scheme 1

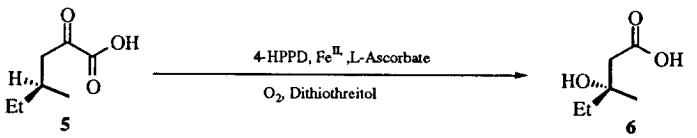
The mechanism of the overall process has been the subject of investigation for some considerable time and despite many proposals, a definitive mechanism still remains obscure. Our recent discovery that 4-HPPD was responsible for the cytosolic catabolism of α-ketoisocaproate (α-KIC) **3** to β-hydroxyisovalerate (β-HIV) **4**,[2,3] an activity previously thought to be due to α-ketoisocaproate dioxygenase (α-KICD) (Scheme 2),[4] prompted us to investigate the mechanistic aspects of this conversion.



Scheme 2

Initially, we were interested in the stereochemistry of hydroxylation at the isopropyl position. Earlier work by Pascal *et al.* with the unnatural substrate, (*S*)-4-methyl-2-oxohexanoate **5** had shown that conversion to 4-hydroxy-4-methylpentanoate **6**, catalysed by what was then believed to be α-KICD, proceeded with complete retention of configuration (Scheme 3).[5] However, nothing was known about the stereochemistry of hydroxylation, for what was considered at the time, the natural substrate of this enzyme, namely α-KIC **3**. It

was envisaged that the results of such an investigation could point to the identity of enzyme-bound intermediates. For example, observation of loss of stereochemical integrity could be indicative of the involvement of a free-radical type intermediate in the reaction mechanism. To examine the stereochemical course of this enzymic conversion, we required a synthesis of the chiral substrates **7a** and **7b** (Scheme 4).

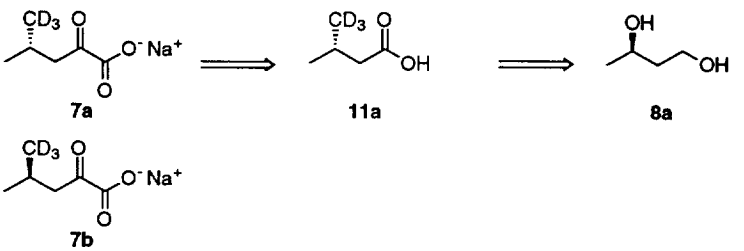


5 **6**

Scheme 3

Results

We reasoned that the chiral isopropyl group could be constructed *via* a bis-trideuteromethyl cuprate displacement of a secondary tosylate.[6] Oxidation and introduction of the ketoacid moiety would then afford the chiral [5-$^2H_3$]-α-KIC **7** (Scheme 4).

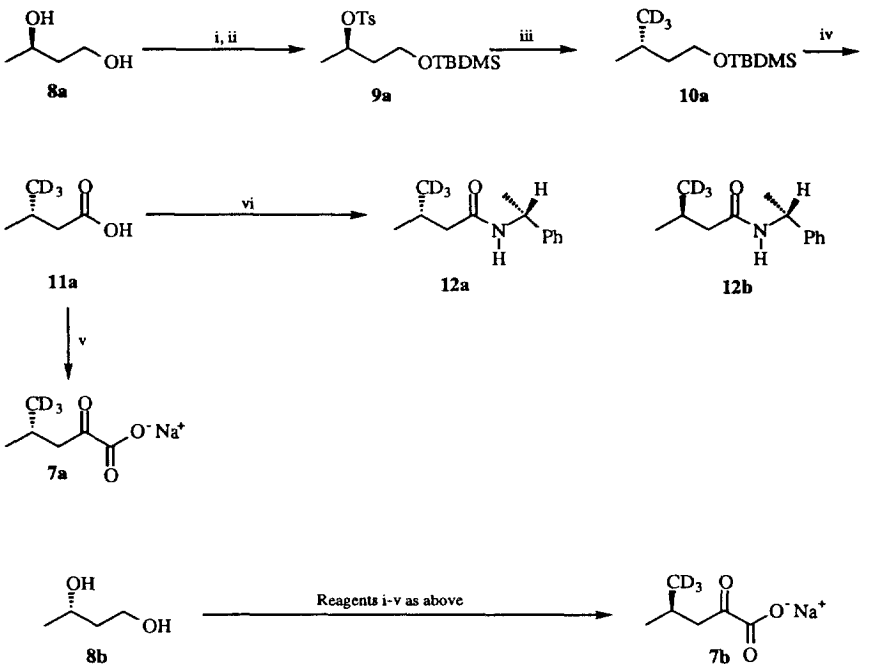


7a **11a** **8a**

7b

Scheme 4

Thus *R*-butane-1,3-diol **8a** (99% e.e., purchased from Aldrich Chemical Company) was selectively silylated and then tosylated to give **9a** (Scheme 5). Commercially available trideuteromethyllithium was reacted with copper (I) iodide to give the bis-trideuteromethylcuprate . Cuprate displacement of the secondary tosylate **9a** proceeded with inversion (*ca.* 80% e.e) to give the simple silyl ether **10a**. Deprotection and oxidation of **10a** with a mixture of KF and Jones' reagent yielded the carboxylic acid **11a**. This was then treated with four equivalents of LDA in THF/TMEDA (1:1 v/v) at 50°C to yield the corresponding dianion which was inverse quenched immediately with diethyl oxalate to give upon base-acid work-up, the ketoacid **7a**. Synthesis of the other enantiomer **7b** (*ca.* 90% e.e) was accomplished via the same route but starting with *S*-butane-1,3-diol **8b**. The optical purity of the product was determined by coupling of the acids **11a** and **11b** to (+)-α-methylbenzylamine to afford the diastereomeric amides **12a** and **12b**. 500 MHz ^1H NMR of these amides allowed discrimination of the diasteromeric isopropyl methyl groups, and integration of the resonances assigned to these established the enantiomeric excesses of **11a** and **11b**. The small loss of enantiopurity observed in both cases was thought to be due to a non-stereospecific $S_{RN}1$ pathway occurring during the displacement reaction.



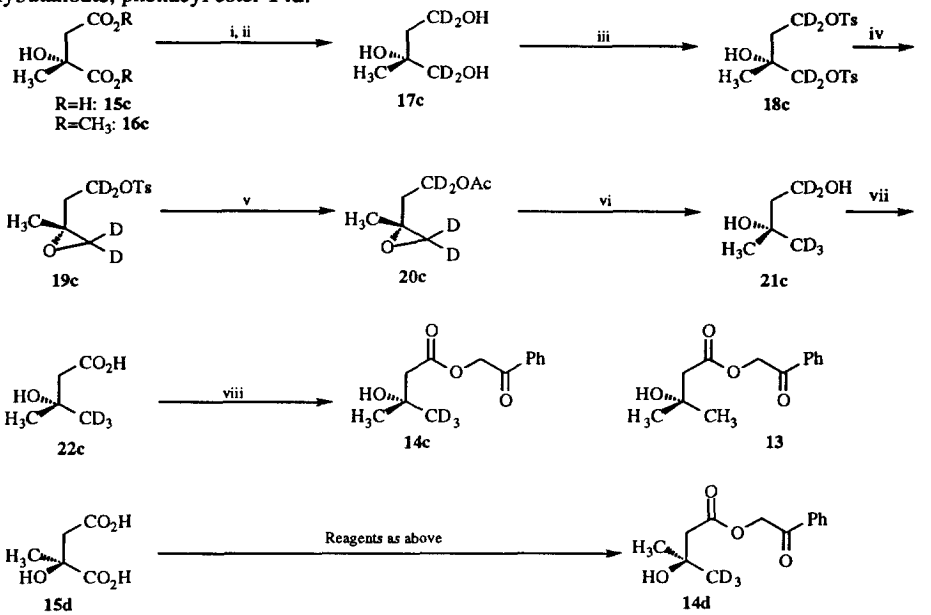
Reagents and Conditions: i) TBDMSCl, Imidazole, DMF, 12h; ii) TsCl, pyridine, 18h, 57% over two steps; iii) $(CD_3)_2CuLi$, diethyl ether; 12h; 45%; iv) KF, Jones, acetone, 12h, 73%; v) a) LDA, TMEDA: THF 1:1, 50°C then diethyl oxalate, 12h; b) HPLC then NaOH, 11%; vi) *R*-α-methylbenzylamine, DCCI, DCM, 18h, 95%.

Scheme 5

Determination of the stereochemical outcome of the hydroxylation reaction required the differentiation of the enantiotopic methyl groups in the product. This was established from the 500 MHz ^{1}H-NMR spectrum of unlabelled β-hydroxyisovalerate, phenacyl ester **13** (Scheme 6) in the presence of varying concentrations of the chiral shift reagent, tris[3-(trifluoromethylhydroxymethylene)-(+)-camphorato], europium (III) derivative ((+)-Eu(tfc)$_3$). At a molar ratio of **13** to Eu(tfc)$_3$ of 1 : 0.6, baseline resolution of the enantiotopic methyl groups was possible. However, it was a requirement for the stereochemical analysis that the pro-*S* and pro-*R* methyl groups of the enzymic products were identified and this called for a synthesis of the chiral 4-(^{2}H$_3$)-β-hydroxyisovalerate, phenacyl esters **14c** and **14d**. With these chiral esters, unambiguous assignment of the methyl groups could be made by performing doping experiments in the presence of the chiral shift reagent. It was decided that the most practicable route to the labelled chiral esters was from the commercially available enantiomers of citramalic acid **15c** and **15d**.[7]

Thus *R*-citramalic acid **15c** was first converted to the corresponding dimethyl ester **16c** by treatment with diazomethane and then reduced with lithium aluminium deuteride to give the deuterated triol **17c** (Scheme 6). This was then converted to the bis-tosylate **18c** with tosyl chloride in pyridine and treated with caesium carbonate to yield the epoxy-tosylate **19c**, the tosylate group of which was then displaced with tetramethylammonium acetate to give the epoxy-acetate **20c**. Treatment of **20c** with lithium aluminium deuteride gave the desired diol **21c**. Oxidation with Jones' reagent was carried out to yield the acid **22c** and esterification with phenacyl bromide gave *S*-(4-^{2}H3)-3-methyl-3-hydroxybutanoate, phenacyl ester **14c**.

Repetition of this route with the other enantiomer of citramalic acid **15d** gave *R*-(4-^{2}H3)-3-methyl-3-hydroxybutanoate, phenacyl ester **14d**.



Reagents and Conditions: i) CH_2N_2, MeOH, quant.; ii) $LiAlD_4$, THF, 18h, 85%, iii) TsCl, pyridine, 18h, 53%; iv) Cs_2CO_3, acetone, 18h, 95%; v) Me_4NOAc; acetone 24h, 96%; vi) $LiAlD_4$, Et_2O, 18h, 28%; vii) Jones, acetone, 15 min, 36%; viii) Phenacyl bromide, Et_3N, acetone, 70°C, 3h, 23%.

Scheme 6

With the chiral esters **14c** and **14d** in hand we performed ^{1}H-NMR doping experiments in the presence of the chiral shift reagent Eu(tfc)$_3$. Thus, the *R* isomer **14d** and the shift reagent (0.6 equiv.) were analysed by ^{1}H-NMR spectroscopy, and the sample doped with **14c**. Another singlet appeared upfield of the main resonance. The converse experiment was carried out (**14c** and shift reagent doped with **14d**). The results obtained enabled us to confirm the enantiopurity of **14c** and **14d**, and also to assign unambiguously the pro-*R* and pro-*S* methyl groups of **13** (Figure 1).

We next incubated the chiral substrates **7a** and **7b** individually with 4-HPPD, dioxygen and cofactors and isolated the products as their phenacyl esters **14a** and **14b**, which were purified by flash silica chromatography. The enzymic ester **14a** was then analysed by 500 MHz ^{1}H NMR spectroscopy in the presence of 0.6 equivalents of (+)-Eu(tfc)$_3$ and there appeared two resonances due to the methyl group in the approximate ratio of 91:9 (high field: low field). When **14b** was analysed in the same way the two signals were in the ratio 4:96 (high field: low field). The results of the two incubations are summarised in Table 1. Thus, the conversion of ketoacid **7a** by 4-HPPD to **22a** had proceeded with at least 95% retention of configuration during the hydroxylation reaction. We confirmed that the resonances observed were indeed due to enzymatically formed β-hydroxyisovalerate by doping the ester **14a** with the synthetic opposite enantiomer **14d** and noting the rise in the relative intensity of the minor (low field) resonance (Figure 2). An analagous result was noted when **14b** was analysed in the same manner. Thus, doping of this sample with authentic **14c** raised the intensity of the minor (high field) resonance.

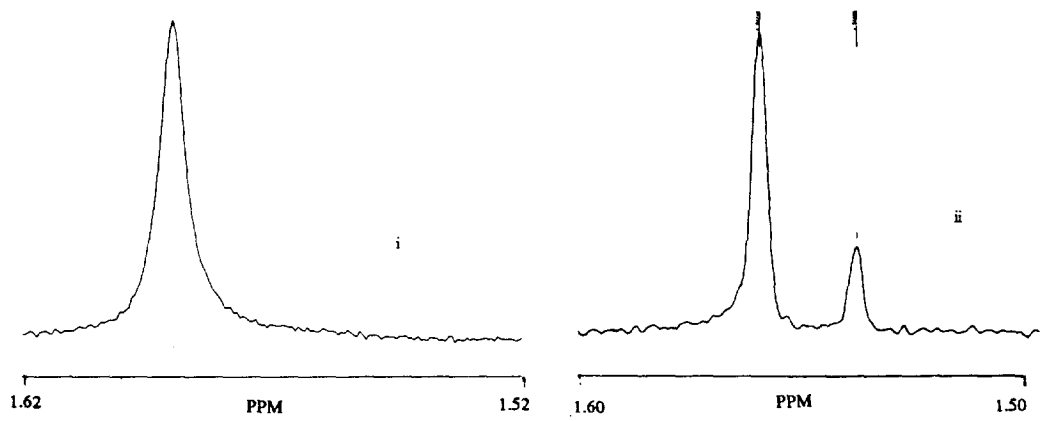


Figure 1: Partial 500 MHz ^{1}H NMR spectra (in the presence of ca. 0.6 equivalents (+)-Eu(tfc)$_3$) of the methyl resonances of i) **14d** (scale δ 1.52-1.62) and **14d** doped with **14c** (scale δ 1.50-1.60). **14c** is also enantiomerically pure.

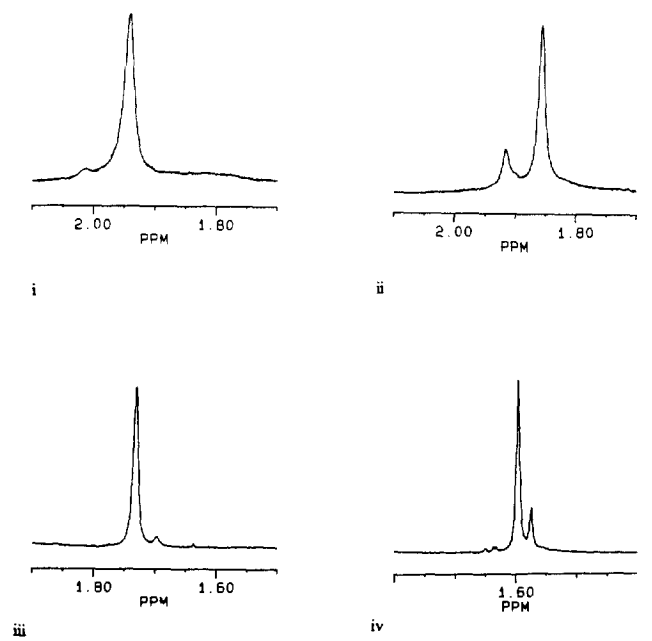


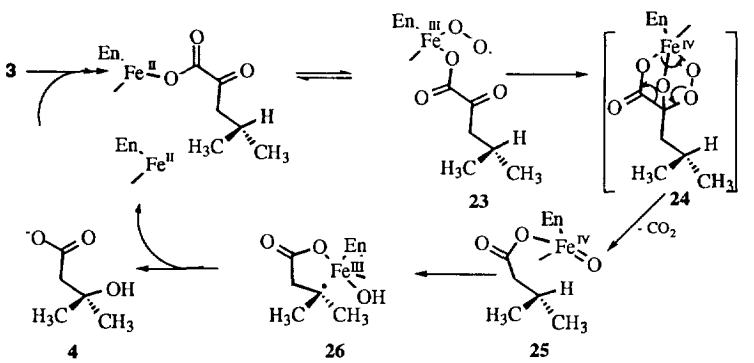
Figure 2: Partial 500 MHz ^{1}H NMR spectrum of the methyl resonances of i) **14a**, ii) **14a** doped with **14d**, iii) **14b**, iv) **14b** and **14c**.

Enzyme Substrate	Substrate enantiopurity (±5%)	Enzyme Product	Product enantiopurity (±5%)	Stereochemical Outcome
7a	90: 10 (*R*:*S*)	**14a**	91: 9 (*S*:*R*)	> 95% retention
7b	4: 96 (*R*:*S*)	**14b**	6: 94 (*S*:*R*)	> 95% retention

Table 1

Discussion

The results reported here and elsewhere[5] prove that the HPPD-catalysed conversion of α-KIC **3** to β-HIV **4** proceeds with retention of stereochemistry (> 95%). A possible reaction mechanism, which is in accord with this result, and the results of ^{18}O labelling experiments[8] is shown in Scheme 7. In this scheme a ferryl (i.e. iron (IV) oxo) species **25** is envisaged as being the active intermediate in the hydroxylation step. This ferryl is produced by a coupled decarboxylation of α-KIC **3** to the splitting of the peroxide **23**, perhaps *via* intermediate **24**. The stereochemical result (>95% retention) is believed to result from a fast radical transfer of hydroxyl to carbon in **26**, which is faster than the rotation of the isopropyl group. It is possible that the topology of the active site may constrain the rotational motion of the isopropyl group. We are currently studying the source of oxygen that is incorporated during several different processes catalysed by 4-HPPD in an attempt to offer a more detailed mechanistic description for this still rather poorly understood enzyme.



Scheme 7

Experimental

General

Protein purification was carried out at 4-8°C using the FPLC™ or Biopilot FPLC™ systems supplied by Pharmacia Biotechnology.

Flash chromatography was performed with Merck Kieselgel 60, 230-400 mesh. Thin layer chromatography was performed with Merck silica gel 60 F254 pre-coated onto aluminium plates. Infra red spectra were recorded on a Perkin Elmer 1750 fourier-transform spectrometer (absorbances recorded as: s strong, m medium, w weak and b broad).

^{1}H-n.m.r. spectra were either recorded at 200 MHz on a Bruker AC 200 or Varian Gemini 200 spectrometer or at 500 MHz on a Bruker AM 500 or spectrometer and are referenced internally to either TMS (samples in $CDCl_3$ $\delta_{ref.}$ = 0.0 ppm) or TSP (samples in D_2O, δ_{ref} = 0.0 ppm) unless otherwise indicated. ^{13}C-n.m.r. spectra were either recorded at 50.31MHz on a Bruker AM 250 spectrometer or at 125.77MHz on a Bruker AM 500MHz spectrometer and are referenced internally to $CDCl_3$ (δ_{ref} = 77.0 ppm).

Mass spectra in the electron-impact (E.I.) mode or chemical ionisation (C.I.) mode were recorded on a VG Micromass ZAB 1F (FAB/CI/DCI), V.G. Masslab 20-250 (CI, DCI/EI) or a V.G. BIO-Q (electrospray) spectrometer. High performance liquid chromatography (h.p.l.c.) of crude incubation mixtures was performed with two Waters Delta Prep 4000 preparative chromatography system.

(*R*)-[(*p*-toluenesulfonyl)oxy]-1-(*tert*-butyldimethylsilyloxy)-butane 10a

The diol **8a** (1.90g, 21.1 mmol, $[\alpha]_D^{22}$=-28.0° (EtOH, c=0.9)) was dissolved in dry DMF (15 ml), imidazole (3.02g, 44.0 mmol) added and the solution cooled to 0°C. *tert*-Butyl dimethylsilyl chloride (3.33g, 22.1 mmol) was added in one portion and the solution stirred overnight. The mixture was poured onto water (100 ml) and extracted with Et_2O (5x 80 ml). The combined organic phases were washed with 1M HCl then saturated brine. Drying ($MgSO_4$), filtration and evaporation of the solvent under reduced pressure gave a mixture containing *(R)-3-hydroxy-1-(tert-butyldimethylsilyloxy)-butane* as a clear oil (4.14g) which was used without further purification; R_f (EtOAc: light petroleum 1:3) 0.50; ν_{max} ($CHCl_3$) 2931s , 2859s , 1472s, 1408m, 1387s, 1363m, 1256s, 939m, 835m; δ_H (200 MHz, $CDCl_3$) 0.09 (6H, s, $(CH_3)_2Si$), 0.92 (9H, s, $(CH_3)_3CSi$), 1.20 (3H, d, *J* 6.5 Hz, $CH_3CH(OH)$), 1.60-1.73 (2H, m, CH_2CH_2OSi), 3.72-4.08 (3H, m, $(CH_3CH(OH)$ and CH_2CH_2OSi); d_C (200 MHz, $CDCl_3$) -5.80 (($CH_3)_2Si$), 17.94 (($CH_3)_3CSi$), 23.21 (($CH_3)_3CSi$), 25.70 ($CH_3CH(OH)CH_2$), 39.81 (CH_2CH_2OSi), 62.80 (CH_2CH_2OSi) and 68.35 ($CH_3CH(OH)CH_2$); m/z (NH_3) 205 (MH^+, 100%), 206 (28) and 207 (9).

This mixture was dissolved in pyridine (50 ml), cooled to 0°C then TsCl (6.30g, 33.1 mmol) and DMAP (400 mg, 3.28 mmol) were added in one portion. The mixture was stirred overnight then more DMAP (400 mg, 3.28 mmol) and TsCl (2.00g, 10.5 mmol) was added. After stirring for a further 12h the pyridine was evaporated under reduced pressure. Water (100 ml) was added and the mixture extracted with Et_2O (4 x 100 ml). The combined organic portions were washed extensively with 1M HCl until the washings were pH=1. 3-(Dimethylamino)-propylamine (2 ml) was shaken with the organic layer and the cloudy solution was washed with further 1M HCl. After drying ($MgSO_4$), filtration and evaporation of the solvent under reduced pressure, the crude product was purified by flash chromatography (SiO_2, DCM: light petroleum (5:4)) to give the *tosylate* **9a** as a colourless oil (4.31g, 57% from diol **8a**); (Found: C, 56.61; H, 8.63, $C_{17}H_{30}O_4SSi$ requires C, 56.95, H, 8.43); R_f (DCM: light petroleum 5:4) 0.28; ν_{max} ($CHCl_3$) 2931s, 2884s, 2858s, 1600m, 1496m, 1463m, 1362s (SO_2), 1308m, 1292m, 1256m, 1214s, 1176s (C-O), 1098s (Si-O), 1021m, 990m cm^{-1}; δ_H (200MHz, $CDCl_3$) -0.02 (6H, s, $(CH_3)_2Si$) , 0.84 (9H, s, $CH_3)_3CSi$, 1.31 (3H, d, *J* 6.3 Hz $CH_3CH(OTs)$), 1.60-1.93 (2H, m, CH_2CH_2OSi), 2.44 (3H, s, CH_3Ar), 3.45-3.58 (2H, m, CH_2CH_2OSi), 4.72-4.85 (1H, m, $CH_3CH(OTs)$, 7.32 (2H, d, J 8.2 Hz, *m*-CH_2, C_6H_4), 7.80 (2H, d, *J* 8.4 Hz *o*-CH_2, C_6H_4); δ_C (200 MHz, $CDCl_3$) -5.74 (($CH_3)_2Si$), 18.00 (($CH_3)_3CSi$), 20.88 (($CH_3)_3CSi$), 21.49 (($CH_3)CH(OTs)$, 25.70 (CH_3-Ar), 39.42 (CH_2CH_2OSi), 58.75 (CH_2CH_2OSi), 78.15 (($CH_3)CH(OTs)CH_2$), 127.87 and 129.9 (*CH*, C_6H_4), 134.13 and 144.66 (quaternary *C*, C_6H_4); m/z (CI^+, NH_3) 376 (MNH_4^+, 5%), 359 (MH^+, 100), 360 (22), 361 (10) and 187 (48).

(*R*)-4-($^2H_3$)-3-Methyl-1-(*tert*-butyldimethylsilyloxy)-butane 10a

Copper (I) iodide (959 mg, 5.02 mmol) was azeotroped three times from toluene and placed under argon. To this was added Et_2O (30 ml) and the suspension cooled to 0°C. Trideuteromethyl lithium (0.5M as lithium iodide complex in Et_2O, 25 ml) was added dropwise until a tan solution persisted. The tosylate **9a** (900 mg, 2.51 mmol) was added as a solution in Et_2O (5 ml) and the solution stirred at room temperature for 5h. Saturated NH_4Cl was added to the black solution and the resulting mixture filtered through Celite. The layers were separated and the aqueous phase extracted with Et_2O (4 x 50 ml). The combined organic phases were washed with saturated brine, dried ($MgSO_4$), filtered and the filtrate evaporated under reduced pressure to give the crude product as a yellow oil (370 mg, 72%). Flash chromatography (SiO_2, light petroleum: EtOAc 50:1) gave the *silyl ether* **10a** as a colourless oil (230 mg, 45%); R_f (EtOAc: light petroleum 1:19) 0.71; ν_{max} ($CHCl_3$) 2957m, 2930m, 2858m, 1473m, 1389m, 1256m (C-O), 1093m, 837m(Si-C); δ_H (200 MHz, $CDCl_3$) 0.06 (6H, s, $(CH_3)_2CSi$), 0.90 (m, 12H, $(CH_3)(CD_3)CH$ and $(CH_3)_3CSi$), 1.42 (2H, m, CH_2CH_2OSi), 1.66 (1H, m, $(CH_3)(CD_3)CH$), 3.64 (2H, t, *J* 6.5 Hz, CH_2CH_2OSi); δ_C (50 MHz, $CDCl_3$) -5.52 ($(CH_3)_2CSi$), 18.19 ($(CH_3)_3CSi$), 22.45 ($(CH_3)_3Si$), 24.28 ($(CH_3)(CD_3)CH$), 25.85 ($(CH_3)(CD_3)CH$), 41.68 (CH_2CH_2OSi), 61.56 (CH_2CH_2OSi); m/z (CI^+, NH_3) 206 (MH+, 100%), 207 (19), 208(7), 165 (46), 132 (15).

(*R*)-4-($^2H_3$)-3-Methyl-butanoic acid 11a

To a solution of the silyl ether **10a** (206 mg, 1.00 mmol) in acetone (10 ml) was added potassium fluoride (116 mg, 2.00 mmol) followed by Jones' reagent (8M) (added dropwise) until an orange colour persisted. There was a time lag between the addition of the first drops of Jones' reagent and the initial exotherm. After stirring overnight, the mixture was diluted with saturated brine, extracted with DCM (5 x 20 ml), and the combined organic layers washed with saturated brine (2x 10 ml). After drying ($MgSO_4$), filtration and evaporation of the solvent under reduced pressure the crude *acid* **11a** was isolated as a yellow oil (74 mg, 73%); ν_{max} (neat) 3600-2600br s, (O-H), 2962s, 2874s, 2688s, 2211s, 2124m, 2065m, 1713s (C=O), 1459m, 1412s, 1380m, 1300s, 1208s, 1095m, 940m; δ_H (200 MHz, $CDCl_3$) 0.98 (3H, d, *J* 6.5 Hz, CH_3), 2.05-2.26 (3H, m, $(CH_3)(CD_3)CHCH_2CO_2H$); δ_C (200 MHz, $CDCl_3$) 22.21 (CH_3CH), 25.19 (CH_3CH), 43.08 (CH_2CO_2H) and 179.98 (CO_2H).

(*R*)-5-($^2H_3$)-4-Methyl-2-oxopentanoic acid 7a

Lithium diisopropylamide (LDA) was prepared by adding n-Butyllithium (2.5M in hexanes, 1 ml, 2.5 mmol) to a solution of diisopropylamine (252 mg, 2.50 mmol) in THF (3 ml) at 0°C under an argon atmosphere. TMEDA (3 ml) was added and the solution cooled to -78°C. A solution of the acid **11a** (70 mg, 0.67 mmol) in anhydrous THF (1 ml) was added and the solution warmed to room temperature then to 50°C. The solution was maintained at this temperature for 2h, cooled to room temperature then injected onto a solution of diethyl oxalate (161 mg, 1.1 mmol) in anhydrous THF (2 ml) at -78°C. The solution was allowed to warm to room temperature then stirred for 16h. Water (5 ml) was added and the THF removed under reduced pressue. The mixture was washed with Et_2O (3 x 10 ml) then the aqueous phase was acidified by careful addition of 6M HCl and extracted with Et_2O (4 x 15 ml). The combined ethereal extracts were dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure to afford a brown oil (70 mg). This oil was treated with 25 mM

ammonium bicarbonate buffer (2 ml) and a drop of 1M NaOH solution to give a clear brown solution, which was filtered and subjected to preparative HPLC (Reversed-phase C18, 90% 25mM ammonium bicarbonate, 10% MeOH, 2.5 ml min^{-1}) in 100 ml batches. The broad peak eluting after 9 min was collected, a few drops of 1M NaOH were added to generate the sodium salt and the water was removed under reduced pressure to afford the ketoacid **67a** (10 mg by ^1H NMR specroscopy, 11%); ν_{max} ($CHCl_3$) 2963w, 2360w, 2341m, 1782m (acid C=O), 1718m (ketone C=O), 1218m, 1218s and 908s; δ_H (500 MHz, D_2O, TSP (δ= 0 ppm) as external reference) 0.94 (3H, d, *J* 6.5 Hz, CH_3), 2.07 (1H, m, $(CD_3)(CH_3)CH$) and 2.61 (2H, d, *J* 6.5 Hz, CH_2); δ_C (125 MHz, $CDCl_3$) 22.31 (CH_3), 24.25 (*C*H), 45.49 (CH_2), 159.12 ($COCO_2H$) and 195.80 ($COCO_2H$); m/z (-ive electrospray) 132 (100%, M^-).

[(*R*)-α-Methylbenzylamido]-3-methylbutyrate 12c

To isovaleric acid (200 mg, 1.96 mmol) in DCM (10 ml) was added α-methylbenzylamine (237 mg, 1.96 mmol,$[\alpha]_D^{22}$ =+27.4° (acetone, lit.(Fluka), +30°±2 (ethanol c=10) and the solution was cooled to 0°C. Dicyclohexylcarbodiimide (444 mg, 2.16 mmol) and a few crystals of DMAP were then added and the mixture stirred at 0°C for 16h. The mixture was filtered through Celite and the filtrate washed with 1M HCl then 1M NaOH, dried ($MgSO_4$), the mixture filtered and the filtrate evaporated under reduced pressure to afford the crude product as a clear oil which was purified by flash chromatography (SiO_2, light petroleum: Et_2O 5:4) to afford the pure *amide* **12c** as a clear oil (422 mg, 95%); (Found: C 75.76, H 9.54 and N 7.02%, $C_{13}H_{19}NO$ requires C 76.06, H 9.33 and N 6.82%); R_f (light petroleum: Et_2O 5:4) 0.09; ν_{max} ($CHCl_3$) 3439w, 3010w, 2962m, 2872w, 1660s, 1494m, 1210s; δ_H (500 MHz, $CDCl_3$) 0.92 (3H, d, *J* 6.5 Hz, $CH(CH_3)(CH_3)$), 0.94 (3H, d, *J* 6.5 Hz, $CH(CH_3)(CH_3)$), 1.50 (3H, d, *J* 6.5 Hz, $C(CH_3)(H)Ph$), 2.03 (2H, d, *J* 6.5 Hz, CH_2), 2.09 (1H, m, $CH(CH_3)_2$), 5.16 (1H, m,$CH(CH_3)Ph$), 5.61 (1H,br s, NH) and 7.24-7.35 (5H, m, ArH); δ_C (50 MHz, $CDCl_3$) 21.65 ($C(CH_3)(H)Ph$), 22.32 ($CH(CH_3)_2$), 26.10 ($CH(CH_3)_2$), 46.04 (CH_2), 48.45 ($CH(CH_3)Ph$), 126.39 (*o*-ArCH), 127.40 (*p*-ArCH), 128.78 (*m*-ArCH) 143.67 (quaternary ArCH) and 171.99 (CONH); m/z (CI^+, NH_3) 207 (12), 206 (100) and 106 (22).

[(*R*)-α-Methylbenzylamido]-3-(R)-4-($^2H_3$)-3-methylbutyrate 12a

The procedure described for the preparation of the undeuterated amide **12c** was followed, with the deuterated acid **11a** (5 mg, 0.05 mmol), as the starting material. The amide **12a** was recovered as a yellow oil; δ_H (500 MHz, $CDCl_3$) 0.92 (3H, d, *J* 6.5 Hz, $CH(CH_3)(CD_3)$), 1.50 (3H, d, *J* 6.5 Hz, $C(CH_3)(H)Ph$), 2.03 (2H, d, *J* 6.5 Hz, CH_2), 2.09 (1H, m, $CH(CH_3)(CD_3)$), 5.16 (1H, m, $CH(CH_3)Ph$), 5.61 (1H, br s, NH) and 7.24-7.35 (5H, m, ArH); δ_C (50 MHz, $CDCl_3$) 21.65 ($C(CH_3)(H)Ph$), 22.32 ($CH(CH_3)(CD_3)$), 26.10 ($CH(CH_3)(CD_3)$, 46.04 (CH_2), 48.45 ($CH(CH_3)Ph$), 126.39 (*o*-ArCH), 127.40 (*p*-ArCH), 128.78 (*m*-ArCH) 143.67 (quaternary ArCH) and 171.99 (CONH); m/z (CI^+, NH_3) 210 (12), 209 (100) and 109 (22).

Synthesis of (*S*)-5-($^2H_3$)-4-Methyl-2-oxopentanoic acid, sodium salt 7b

The acid **7b** was synthesised from (*S*)-butane-1,3-diol **8b** in 2% overall yield, by an identical procedure to the synthesis of **7a** from diol **8a**.

(*R*)-2-Hydroxy-2-methyl-butan-1,4-dioic acid dimethyl ester 16c

A solution of KOH (9.00g, 161 mmol) in water (20 ml) was placed in a 200 ml flask equipped with

magnetic stirrer, dropping funnel and condenser, which was arranged so that the generated diazomethane would condense into a flask containing (*R*)-Citramalic acid **15c** (5.00g, 33.8 mmol) in MeOH (30 ml). Absolute ethanol (50 ml) was added to the KOH solution and stirred at 65°C. DIAZALD™ (38.0g, 178 mmol) was dissolved in Et_2O (120 ml) and added dropwise to the KOH solution until a yellow colour persisted in the flask containing the diacid. The system was flushed with Et_2O then the receiver was carefully detached and acetic acid added dropwise until the yellow colour disappeared. The solvent was evaporated, the residue treated with saturated $NaHCO_3$ solution (30 ml) and the mixture extracted with DCM (2 x 150 ml). The organic layer was dried ($MgSO_4$), filtered, and the solvent evaporated under reduced pressure to afford the *diester* **16c** as a colourless oil (6.01g, 100%); ν_{max} (neat) 3510br s, 3024m, 2957m, 2096w, 1734s, 1638m, 1440m, 1356m, 1214s, 1121s, 1013m, 984m; δ_H (200 MHz, $CDCl_3$) 1.45 (3H, s, C(C*H*$_3$)(OH)(CH$_2$)), 2.68 and 2.95 (2H, ABq, *J* 16 Hz, C*H*$_2$), 3.67 and 3.81 (2x3H, 2s, OC*H*$_3$); δ_C (50 MHz, $CDCl_3$) 26.22 (C(*C*H$_3$)(CD$_3$)OH), 43.90 (*C*H$_2$), 51.84 and 52.91 (2x *C*H$_3$O), 72.49 (C(*C*H$_3$)(CD$_3$)OH) and 171.45 (*C*O$_2$CH$_3$); m/z (CI$^+$ (NH_3) 194 (100, MNH_4^+) and 177 (42, MH^+)

(*R*)-1-($^2H_2$)-4-($^2H_2$)-2-Methyl-1,2,4-trihydroxybutane 17c

Lithium aluminium deuteride (2.20g, 52.3 mmol) was added to a dry flask, flushed with argon, cooled to 0°C and THF (100 ml) added. To this suspension was added, dropwise, a solution of the diester **16c** (6.00g, 34.1 mmol) in THF (30 ml) and the suspension stirred for 18h at 0°C. After this time, the reaction was quenched by the dropwise addition of a mixture of THF and water (5:1). After effervescenece ceased, 1M NaOH (20 ml) was added and the emulsion became a suspension, which was filtered. The filtrate was evaporated to dryness under reduced pressure to afford a yellow oil and the solid residue was refluxed with fresh THF (100 ml) for 18h. The mixture was filtered and the filtrate evaporated to dryness to give a second oil. This was added to the first oil, and the combined products were dissolved in MeOH (40 ml), filtered through Celite and the filtrate evaporated under reduced pressure to afford the *triol* **17c** as a clear oil (3.60g, 85%); R_f (DCM) 0.30; ν_{max} (neat) 3351 br s, 2947s, 2835s, 1656m, 1377m, 1103m, 1029s; δ_H (200 MHz, CD_3OD) 1.15 (3H, s, C*H*$_3$C(OH)) and 1.66-1.80 (2H, ABq, *J* 16 Hz, C*H*$_2$CD$_2$OH); δ_C (50 MHz,CD_3OD) 24.26 (*C*H$_3$C(OH)), 41.05 (*C*H$_2$CD$_2$OH), 58.43 (C(OH)(CH$_3$)*C*D$_2$OH) and 73.44 (*C*(OH)CH$_3$); m/z (NH_3, CI$^+$) 142 (MNH_4^+), 125 (MH^+, 40) and 89 (100).

(*R*)-1-($^2H_2$)-4-($^2H_2$)-Bis-1,4-(*p*-toluenesulfonyloxy)-2-hydroxy-2-methylbutane 18c

The triol **17c** (2.38g, 19.2 mmol), dry pyridine (50 ml) and activated 4Ä molecular sieves (30 ml) were stirred together under argon for 1h, the mixture cooled to 0°C, TsCl (8.10g, 42.5 mmol) added and the resulting solution stirred for 3h at 0°C then a further 18h at room temperature. Further TsCl (1.00g, 5.2 mmol) was added and the mixture stirred for a further 1h, then poured into Et_2O (150 ml) and filtered. The filtrate was washed with 1M HCl (4 x 100 ml). DMAPA (5 ml) was added to the the Et_2O solution and the resultant cloudy solution stirred for 10 min before being washed with further 1M HCl (100 ml). The ethereal extract was dried ($MgSO_4$), the mixture filtered and the filtrate evaporated to dryness under reduced pressure to afford a yellow oil which was purified by flash chromatography (SiO_2, Et_2O: light petroleum 4:1) to afford the *bis-tosylate* **18c** as a clear oil (4.36g, 53%); R_f (Et_2O: light petroleum 4:1) 0.32; Found: C, 52.72, H, 5.32, ($C_{19}H_{20}D_4O_7S_2$ requires C, 52.76, H+D, 5.55%); ν_{max} ($CHCl_3$) 3592w (OH), 3038s, 3011m, 1599m, 1496m, 1367s, 1199s, 1193s, 969s and 813s; δ_H 1.17 (3H, s, C(C*H*$_3$)(CD$_2$)O), 1.76-1.93 (2H, ABq, *J* 15 Hz, C*H*$_2$), 2.47-2.48

(6H, 2xs, Ar-CH_3), 7.36 and 7.78 (8H, 2xm, Ar-*H*); δ_C (50 MHz, $CDCl_3$) 21.53 (C(*C*H_3)(CD_2)O), 23.66 (Ar-*C*H_3), 36.55 (*C*H_2), 70.03 (*C*(CH_3)(CD_2)OH), 128.46 and 130.27 (*o*- and *m*-Ar*C*H) and 132.42, 132.79, 145.31, 145.55 (quaternary Ar*C*); m/z (CI^+ (NH_3)) 450 (100, MNH_4^+), 314 (86) and 278 (34).

(*R*)-1-($^2H_2$)-4-($^2H_2$)-3,4-epoxy-3-methyl-1-*p*-toluenesulfonyloxybutane 19c

A solution of the bis-tosylate **18c** (2.30g, 5.32 mmol) in acetone (40 ml) was stirred for 16h at room temperature with anhydrous caesium carbonate (2.60g, 7.97 mmol). Further caesium carbonate (1.00g, 3.07 mmol) was added and the mixture stirred for a further 2h. The acetone was evaporated, Et_2O (150 ml) added and the solution washed with water (50 ml) and brine (2x 50 ml). The organic layer was dried ($MgSO_4$), the mixture filtered and the filtrate evaporated under reduced pressure to afford the *epoxide* **19c** as a clear oil (1.32g, 95%); R_f (Et_2O: light petroleum 4:1) 0.36; (Found: C 55.70, H+D 6.24%, $C_{12}H_{12}D_4O_4S$ requires C 55.36, H+D 6.15%); ν_{max} ($CHCl_3$) 3066w, 2967s, 2931s, 2305w, 2249w, 2172m, 1924w, 1598s, 1451s, 1364s, 1308s, 1176s, 1152s, 1098s, 1020s and 965s; δ_H (200 MHz, $CDCl_3$) 1.30 (3H, s, C(CH_3)(CD_2)O), 1.86-2.00 (2H, ABq, *J* 15 Hz, CH_2), 2.47 (3H, s, Ar*C*H_3), 7.38 and 7.82 (4H, AA'BB', Ar*H*); δ_C (50 MHz, $CDCl_3$) 21.01 (C(*C*H_3)(CD_2)O), 21.52 (Ar*C*H_3), 35.30 (*C*H_2), 54.16 (*C*(CH_3)(CD_2)O), 128.05 and 130.13 (Ar-*C*H), 132.99 and 145.23 (quaternary Ar*C*); m/z (CI^+ (NH_3)) 278 (MNH_4^+).

(*R*)-1-($^2H_2$)-4-($^2H_2$)-1-Acetoxy-3,4-epoxy-3-methylbutane 20c

To a solution of the epoxide **19c** (1.60g, 6.15 mmol) in acetone (50 ml) was added tetramethylammonium acetate (2.60g, 19.5 mmol, dried at 160°C under vacuum for 16h) in one portion. The mixture was stirred for 8h then the acetone was evaporated, Et_2O (100 ml) added and the solution washed with water (30 ml) and brine (2 x 30 ml). The organic layer was dried ($MgSO_4$), the mixture filtered and the filtrate evaporated under reduced pressure to afford the *acetate* **20c** as a clear oil (870 mg, 96%); R_f (Et_2O: light petroleum 5:3) 0.79; ν_{max} ($CHCl_3$) 3026w, 1732m, 1373w, 1270m, 1228w, 1222s, 1203w, 1159w, 1033w and 910w; δ_H (200 MHz, $CDCl_3$) 1.36 (3H, s, C(CH_3)(CD_2)O), 1.80-1.99 (2H, ABq,*J* 15 Hz, CH_2) and 2.06 (3H, s, CH_3C(O)O); δ_C (50 MHz, $CDCl_3$) 20.97 (C(*C*H_3)(CD_2)O), 24.66 (*C*H_3C(O)O), 35.11 (*C*H_2), 54.67 (*C*(CH_3)(CD_2)O) and 171.21 (CH_3*C*(O)); m/z (CI^+ (NH_3)) 149 (MH^+).

(*S*)-1-($^2H_2$)-4-($^3H_2$)-3-hydroxy-3-methylbutane 21c

To a suspension of lithium aluminium deuteride (100 mg, 2.38 mmol) in THF (10 ml) was added a solution of the epoxyacetate **20c** (300 mg, 2.03 mmol) in THF (5 ml) and the mixture stirred for 1h at 0°C and at room temperature for a further 1h. The mixture was then cooled to 0°C and the reaction was quenched by dropwise addition of a mixture of water and THF (1:4 v/v) until effervesence ceased. A solution of 1M NaOH (2 ml) was added and the emulsion stirred until it became a precipitate. The solid was filtered off and refluxed in fresh THF (15 ml) for 6h. The resultant solution was decanted and combined with the original filtrate then the solvents were evaporated under reduced pressure. The resultant oil was placed under vacuum overnight then dissolved in $CHCl_3$ (20 ml) and the solution filtered. The filtrate was evaporated under reduced pressure to afford an oil containing the *diol* **21c** (62 mg, 28%) which was used without further purification; δ_H (200 MHz, $CDCl_3$) 1.28 (3H, s, CH_3) and 1.70 (2H, s, CH_2); δ_C (50 MHz, $CDCl_3$) 29.45 (q, *C*H_3), 43.87 (t, *C*H_2) and 72.08 (s, C(CH_3)(CD_2)O); m/z (CI^+(NH_3) 127 (31, MNH_4^+), and 110 (63, MH^+).

(*S*)-4-($^3H_2$)-3-hydroxy-3-methylbutanoic acid 22c

To a solution of the diol **21c** (62 mg, 0.57 mmol) in acetone (2 ml) was added, dropwise, 8M Jones' reagent until the orange colour persisted. The acetone was evaporated, water (4 ml) was added and the solution extracted with EtOAc (4 x 10 ml). The combined organic extracts were washed with saturated brine (2 x 5 ml), dried ($MgSO_4$), the mixture filtered and the filtrate evaporated under reduced pressure to afford an orange oil containing the *acid* **22c** (25 mg, 36%); ν_{max} ($CHCl_3$) 3034w, 3008w, 1735w (C=O), 1241m, 1234s, 1193w and 808s; δ_H (200 MHz, $CDCl_3$) 1.37 (3H, s, CH_3) and 2.60 (2H, s, CH_2); δ_C (50 MHz, $CDCl_3$) 25.09 (CH_3), 43.03 (CH_2) 62.90 ($C(CH_3)(CD_3)OH$) and 180.36 (CO_2H); m/z (CI^+ (NH_3)) 139 (100, MNH_4^+) and 122 (30, MH^+).

(*S*)-4-($^2H_3$)-3-hydroxy-3-methylbutanoic acid phenacyl ester 14c

To a solution of the acid **22c** (10 mg, 0.09 mmol) in dry acetone (1 ml) was added Et_3N (12 mg, 0.12 mmol) and phenacyl bromide (30 mg 0.15 mmol) and the solution refluxed for 30 min. The acetone was evaporated, DCM (5 ml) added and the mixture washed with 1M NaOH solution (2 ml). The organic layer was dried ($MgSO_4$), the mixture filtered and the filtrate evaporated under reduced pressure to afford a yellow oil which was purified by flash chromatography (SiO_2, DCM: Et_2O 1:1) to give the *ester* **14c** as a yellow oil (5 mg, 23%) which showed an e.e. of over 98% by ^1H NMR spectroscopy in the presence of a chiral shift reagent; R_f (DCM: Et_2O 3:2) 0.69; ν_{max} ($CHCl_3$) 3041s, 3008s, 1740s (ester C=O), 1708s (ketonic C=O), 1602m, 1241m, 1193m; δ_H (200 MHz, $CDCl_3$) 1.38 (3H, s, CH_3), 2.68 (2H, s,$CH_3C(OH)(CD_3)CH_2$), 3.68 (1H, s, OH), 5.44 (2H, s, $PhC(O)CH_2$), 7.48 (2H, m, *o*-Ar*H*), 7.55 (1H, m, *p*-Ar*H*) and 7.94 (2H, m, *m*-Ar*H*); δ_C ($CDCl_3$, 50 MHz) 29.21 (CH_3), 46.93 ($CH_3C(OH)(CD_3)CH_2$), 66.00 ($PhC(O)CH_2$), 69.07 ($CH_3C(OH)(CD_3)$), 127.85 (*m*-Ar), 128.96 (*o*-Ar), 133.89 (*i*-Ar), 134.18 (*p*-Ar), 171.68 ($C(O)OCH_2$) and 192.33 (Ar $C(O)CH_2$); m/z (CI^+ (NH_3) 257 (18, MNH_4^+), 240 (13, MH^+) and 105 (100).

Synthesis of (*R*)-4-($^2H_3$)-3-hydroxy-3-methylbutanoic acid phenacyl ester 14d

The ester **14d** was synthesised, in an identical manner to the ester **14c**, with (*S*)-citramalic acid **15d** as the starting material. The ester **14d** was obtained in 2% overall yield and over 98% e.e by ^1H NMR spectroscopy in the presence of a chiral shift reagent.

Preparation of Incubation Grade 4-HPPD[9]

The 4-HPPD gene has been cloned into a plasmid as previously described.[3] *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.*

Incubation of (*R*)-5-($^2H_3$)-4-methyl-2-oxopentanoic acid 7a with 4-HPPD

A buffer solution (541 µl, pH 6.5) of Tris (0.74M) and maleic acid (0.74M) was placed in a 5 ml tube. A solution (200 µl) of iron (II) sulfate heptahydrate (2.4 mg), L-ascorbate (0.8 mg) and dithiothreitol (1.4 mg) in water (1 ml) was added to the buffer solution followed by a solution of HPPD from the recombinant DEAE pool (1 ml, 15 mg ml^{-1}). The reaction was started by addition of a solution of the ketoacid **7a** (2.5 mg in 250 µl) which had been adjusted to pH=7 (1M HCl) and the brown solution was incubated at 27°C with shaking for 16h. A fresh solution of the cofactor mixture (100 µl) was added, then further enzyme (0.5 ml) and the solution incubated for a further 12h. A similar incubation mixture was prepared and the two reaction mixtures run concurrently.

The combined solutions were made 70% in acetone and centrifuged (14 000 r.p.m, 20 min). The supernatant was poured off and the acetone evaporated under reduced pressure. Water (5 ml) was added and the mixture acidified to pH 2 then extracted with EtOAc (4 x 20 ml). The combined organic fractions were dried ($MgSO_4$), the mixture filtered and the filtrate evaporated to dryness to afford a clear oil containing the the acid **22a**.

This oil was dissolved in dry acetone (4 ml), Et_3N (0.2 ml) and phenacyl bromide (20 mg) added then the solution was refluxed for 3h.The solvent was evaporated under reduced pressure and the purple oil was chromatographed on silica (DCM:Et_2O 8:1) to afford the pure *ester* **14a** as a clear oil (3 mg) which had the same spectroscopic behaviour as the synthetic ester **14c**.

Incubation of (*S*)-5-($^2H_3$)-4-methyl-2-oxopentanoic acid 7b with 4-HPPD

The enzymic hydroxyacid **22a** was obtained by incubation of the acid **7b** (2.5 mg) with HPPD, in the same manner as described for the hydroxyacid **22a**. The acid **22b** was converted to the ester **14b** and purified in the same manner as described for the ester **14a**.

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References

1. The results described in this publication have previously been communicated; Adlington, R. M.; Baldwin, J. E.; Crouch, N. P.; Lee, M.-H.; MacKinnon, C. H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2721.

2. 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.
3. Baldwin, J. E.; Crouch, N. P.; Lee, M. -H.; MacKinnon, C. H.; Zhang, Z. H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1503.
4. Sabourin, P. J.; Bieber, L. L. *J. Biol. Chem.* **1982**, *257*, 7460.
5. Han, H.; Pascal Jr., R. A. *J. Org. Chem.* **1990**, *55*, 5173.
6. Johnson, C. R.; Dutra, G. A. *J. Am. Chem. Soc.* **1973**, *95*, 7777.
7. Baldwin, J. E; Adlington, R. M; Marquess, D. G.; Pitt, A. R; Porter, M. J.; Russell, A. T. *Tetrahedron* **1996**, *52*, 2515.
8. Sabourin, P. J.; Bieber, L. L. *J. Biol. Chem.* **1982**, *257*, 7468.
9. Crouch, N. P.; Adlington, R. M.; Baldwin, J. E.; Lee, M.-H.; MacKinnon, C. H. *Tetrahedron* **1997**, *53*, 6993.

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