Synthesis of DL-*threo*-3-(1-fluoro-1-methylethyl)- and DL-*threo*-3-(1,1-difluoroethyl)malic acids. Mechanistic studies of 3-isopropylmalate dehydrogenase

Tetsuya Aoyama,[a] Tadashi Eguchi,[a] Tairo Oshima[b] and Katsumi Kakinuma *,[a]

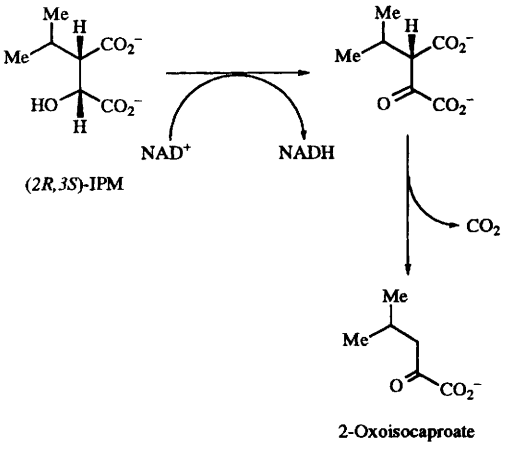
[a] *Department of Chemistry, Tokyo Institute of Technology, Meguro-ku, Tokyo 152, Japan*
[b] *Department of Life Science, Tokyo Institute of Technology, Midori-ku, Yokohama 227, Japan*

For both mechanistic studies and the development of novel inhibitors of 3-isopropylmalate dehydrogenase enzyme (IPMDH), which is involved in the rate-determining step in the biosynthetic pathway of the essential amino acid L-leucine, (2*R**,3*S**)-3-(1-fluoro-1-methylethyl)- and (2*R**,3*S**)-3-(1,1-difluoroethyl)malic acids (F-IPM and F_2-EM) were designed based on the concept of mechanism-based inhibition, and the reaction kinetics with these fluorinated substrates were analysed. The reaction of F-IPM with IPMDH was studied by NMR spectroscopy and product isolation. F-IPM underwent, after the normal enzyme reaction, the expected additional elimination reaction to afford an α,β-unsaturated carbonyl product, which turned out not to participate in any covalent-bond-forming reaction. The conformation of the reaction intermediate during the IPMDH reaction and the functional-group arrangement in the active site of IPMDH are discussed.

Introduction

Mechanistic enzymology is among the major interests in biochemistry. Ultimate goals of this field include precise understanding of the molecular interactions in enzyme reactions and development of potential chemicals for medicinal as well as agricultural use.

We have been interested in the reaction mechanism and substrate recognition of a thermostable 3-isopropylmalate dehydrogenase (IPMDH, E.C. 1.1.1.85) derived from the extremely thermophilic bacteria *Thermus thermophilus* HB8.[1] IPMDH catalyses an oxidation–decarboxylation reaction of (2*R*,3*S*)-3-isopropylmalate (IPM) giving rise to 2-oxoisocaproate in the presence of nicotinamide adenine dinucleotide (NAD^+) in the biosynthetic pathway of the essential amino acid L-leucine, as shown in Scheme 1.

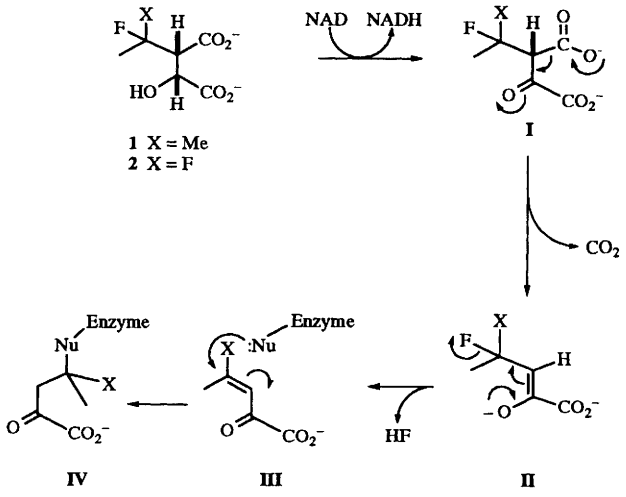


Scheme 1

The cryptic stereochemistry of these transformations has already been elucidated; *i.e.*, (i) the transfer of hydride from the C-2 position of IPM to the pyridine ring of NAD^+ is a so-called A-type specific [transfer of hydride to the *pro-R* position of the dihydropyridine of 1,4-dihydronicotinamide adenine dinucleotide (NADH)], and (ii) the decarboxylation at C-3 occurs with retention of configuration.[2] These stereochemical features clearly suggested a significant similarity of the pertinent regions of IPMDH with those of isocitrate dehydrogenase (ICDH, E.C. 1.1.1.42) functioning in the tricarboxylate cycle.[3] Recent genetic analyses of both IPMDH and ICDH also suggested an evolutional relationship between the two enzymes.[4]

While X-ray crystallographic analyses of IPMDH and IPMDH–NAD^+ complex have recently been carried out[4,5] and a non-competitive inhibitor and competitive inhibitors have been developed recently by us and others,[6,7] precise features of substrate recognition or formation of enzyme–substrate complex have yet to be clarified. One plausible approach to this end is to utilize specific inhibitors which can covalently bind to the target enzyme.[8] Fluorinated analogues of enzyme substrates have been extensively utilized in mechanistic studies of various enzymes,[9] and, in particular, attention has been focused on mechanism-based (suicide) inhibitors in order to analyse the structure of the enzyme active site as well as the features of substrate recognition.[9] The resulting modified enzyme may subsequently be analysed by various ways. With these factors in mind, we designed and synthesized fluorinated analogues of the IPM substrate, as potential suicide inhibitors.

Such an inhibitor was designed with the expectation of the following sequence of reactions with IPMDH, *i.e.*, a well recognized tendency of the fluorine atom to facilitate an elimination of HF from the enzyme reaction product after the first step of oxidation or after the decarboxylation. In either case, an electron-deficient, conjugated olefin functionality would accept an attack of a nearby nucleophilic group of the IPMDH active centre to form a covalent bond between the reaction product and the enzyme as shown in Scheme 2. Racemic *threo*-3-(1-fluoro-1-methylethyl)malic acid and *threo*-3-(1,1-difluoroethyl)malic acids (F-IPM **1** and F_2-EM **2**) were our designed targets. It is well established that, while the true substrate of IPMDH is (2*R*,3*S*)-IPM, its antipode has no effect at all on the IPMDH reaction, and racemic IPM has been utilized as a substrate for the tracing of enzyme during the purification procedures.[4]

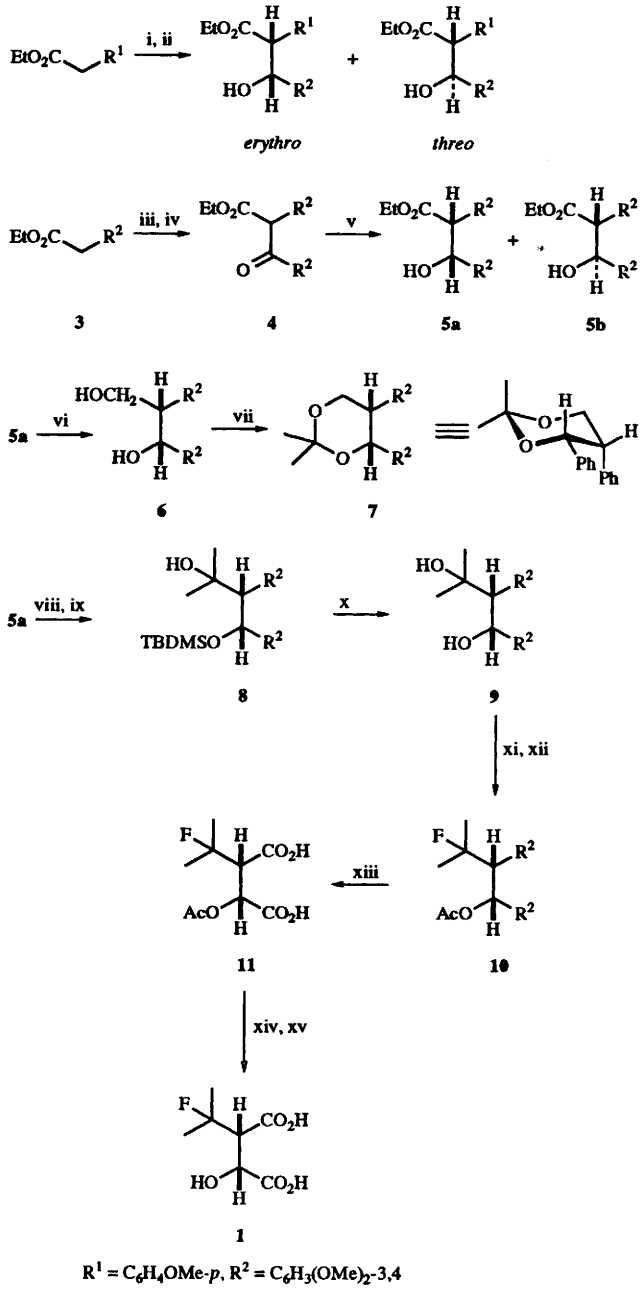


Scheme 2

Results and discussion

A monofluorinated analogue of IPM, F-IPM **1** was first synthesized by modification of our previous preparative method of racemic IPM, as shown in Scheme 3.[2] Attempted aldol condensation of ethyl (4-methoxyphenyl)acetate with 3,4-dimethoxy benzaldehyde (veratraldehyde) was not efficiently selective. The reaction in tetrahydrofuran (THF) solvent gave a 1:1 mixture of *erythro*/*threo* products, and the reaction in THF–hexamethylphosphoric triamide (HMPA) afforded a mixture of 1:10 (*erythro*/*threo*) products (Scheme 3). The desired product should have had an *erythro*-configuration as shown in Scheme 3. As an alternative, a route comprising a Claisen condensation and subsequent reduction was attempted. The enolate of ethyl (3,4-dimethoxyphenyl)acetate **3** was treated with ethyl 3,4-dimethoxybenzoate to afford β-keto ester in 98% yield, which was subjected to hydride reduction. Sodium boranuide reduction of keto ester **4** was less reactive but more promising in terms of stereochemistry to give an *erythro*-predominant 4:1 mixture, whereas $Zn(BH_4)_2$ was extremely selective and more desirable in that it afforded a mixture of compounds **5a** and **5b** in the ratio 23:1 in 80% yield. Presumably, strong co-ordination of the zinc atom by two carbonyl oxygens was effective enough to fix the conformation of the substrate **4** so that the direction of the introduction of hydride was controlled to afford preferentially the *erythro*-product **5a**.[10] The configuration of compound **5a** was confirmed as follows. After purification of compound **5a** by recrystallization, $LiAlH_4$ reduction gave, in 47% yield, 1,3-diol **6**, which was subsequently treated with 2,2-dimethoxypropane in the presence of pyridinium toluene-*p*-sulfonate (PPTS) to afford 1,3-dioxane **7** in 73% yield. The ^1H NMR coupling constant between the benzylic methine protons was 3.4 Hz, thereby suggesting an axial–equatorial relationship between the bulky phenyl groups as shown above. The equatorial orientation of the C-5 proton was further supported by the coupling constants with the adjacent methylene protons.

Further manipulation of the hydroxy ester **5a** was carried out, first by protecting the hydroxy group with a *tert*-butyldimethylsilyl (TBDMS) group. The ester function was then converted into a dimethyl carbinol (isopropyl alcohol moiety) by Grignard reaction with MeMgBr to afford compound **8** in 87% overall yield. Subsequent deprotection with Bu_4NF (TBAF) afforded diol **9** quantitatively. After selective acetylation of the secondary hydroxy group of diol **9**, the key transformation of the alcohol into a fluoride **10** was carried out by treatment of compound **9** with (diethylamino)sulfur



Scheme 3 *Reagents:* i, LDA; ii, 3,4-dimethoxybenzaldehyde; iii, NaH; iv, ethyl 3,4-dimethoxybenzoate; v, $Zn(BH_4)_2$; vi, $LiAlH_4$; vii, $Me_2C(OMe)_2$, PPTS; viii, TBDMSCl, imidazole, DMF; ix, MeMgBr, THF; x, TBAF; xi, Ac_2O, py; xii, DAST; xiii, $RuCl_3$, $NaIO_4$; xiv, LiOH; xv, Dowex 50W-X2 (H^+-form)

trifluoride (DAST)[11] at −78 °C in 73% overall yield. Two aryl rings were exhaustively oxidized with RuO_4 in the presence of $NaIO_4$ in MeCN–CCl_4–H_2O[12] to give, after reversed-phase chromatography, a dicarboxylic acid **11** in 14% yield. Degradation of the substrate and/or the product due to the extremely acidic pH of the system could be a major reason for the low yield of this oxidation. The fluorinated acetate was finally hydrolysed with LiOH and the product was purified by ion-exchange resin chromatography to yield, after recrystallization, the targeted fluorinated hydroxydicarboxylic acid **1** (F-IPM) in 64% yield.

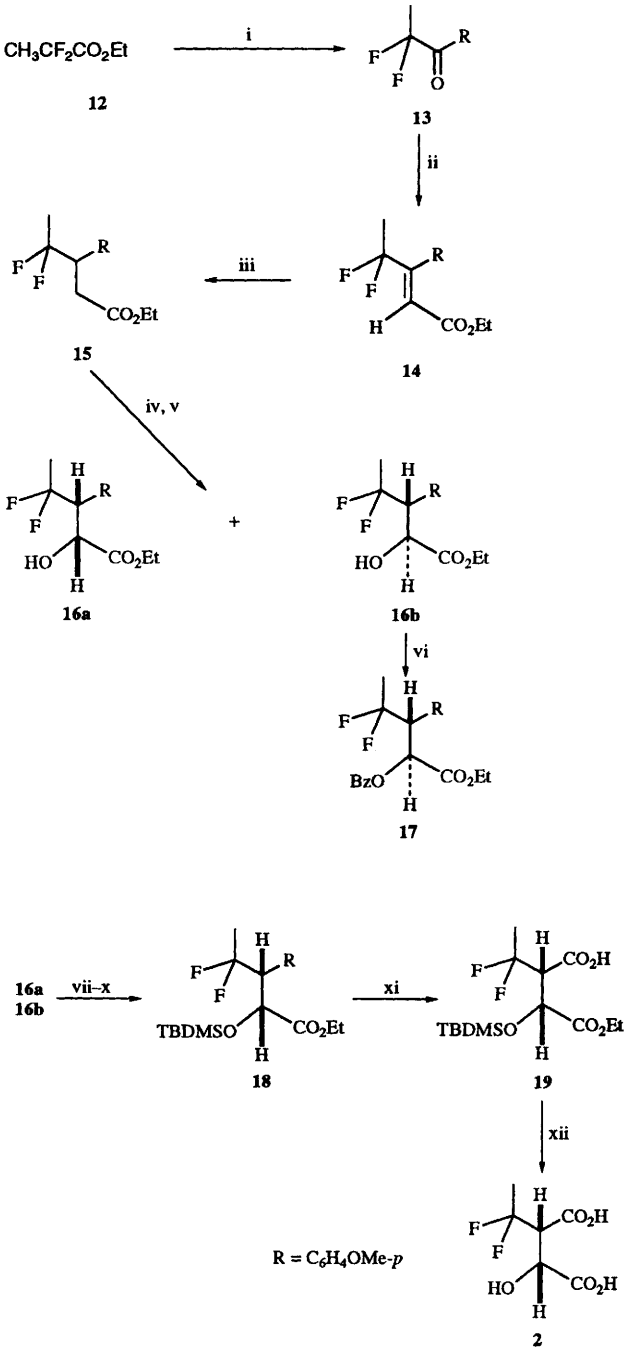
Attention was turned next to another designed target, F_2-EM **2**. The expected enzyme reaction product from F_2-EM, 4-fluoro-2-oxopent-3-enoic acid, would have been a less hindered

and more reactive Michael acceptor than that of F-IPM. Although the difluoromethylene group could be constructed from a ketone by reaction with DAST, it seemed less likely that synthesis of F_2-IPM could be carried out by a modification of the preparation of F-IPM, because this type of reaction was known to cause dehydrofluorination as a side reaction, and the yield of the difluorinated methylene compound was generally low.[11] Therefore, our synthesis of F_2-EM was started with a compound containing a difluoromethylene group as shown in Scheme 4. Thus, ethyl α,α-difluoropropanoate **12** was treated with *p*-methoxyphenyllithium, prepared from *p*-methoxyphenyl bromide and *tert*-butyllithium, to afford α,α-difluorinated ketone **13** in 43% yield. The Wittig reaction of ketone **13** with ethoxycarbonylmethylene(triphenyl)phosphorane proceeded in highly stereoselective manner to give *E*-olefin **14** in high yield as reported previously.[13] After hydrogenation of the resulting double bond of compound **14**, the enolate of saturated ester **15** was treated with molybdenum peroxy complex (MoO_5–pyridine–HMPA, MoOPH)[14] at −78 °C to afford an *erythro*-isomer **16a** and a *threo*-congener **16b** in the ratio 85:15. To elucidate the relative stereochemistry of diastereoisomers **16a** and **16b**, isomer **16b** was isolated and repeatedly crystallized. The benzoyl derivative **17** was subjected to a single-crystal X-ray analysis. As reported previously, benzoate derivative **17** was found to have the 2*R**,3*S** configuration,[15] thus indicating that the major product **16b** has the undesired diastereoisomeric configuration.

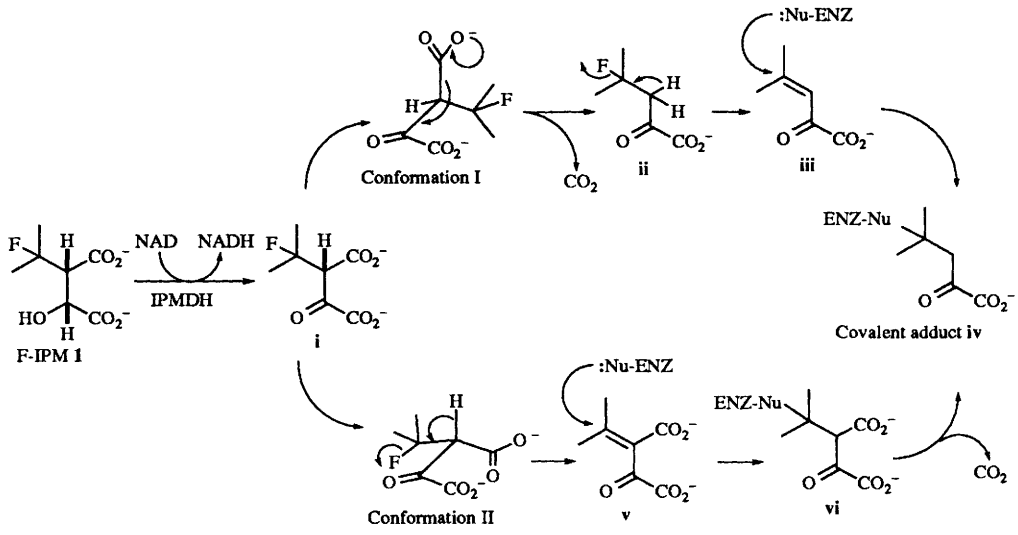
Since several attempts at inversion of the hydroxy group of the major isomer **16b**, such as Mitsunobu reaction and reaction with KO_2 with the corresponding mesyl ester, were unsuccessful, an oxidation–reduction procedure was employed in order to reverse the isomer ratio. Thus, Swern oxidation of a mixture of hydroxy esters **16a** and **16b** gave a relatively unstable α-keto ester derivative, which was directly reduced with K-Selectride™ at −78 °C in THF to afford selectively a mixture of **16a** and **16b** in the ratio 9:1. Since the major isomer **16a** was not separable, the mixture of diastereoisomers **16a** and **16b** was converted into their TBDMS derivatives. At this stage, the desired isomer **18** was easily separable by column chromatography. The aryl ring of compound **18** was oxidatively degraded into a carboxy group with $RuCl_3$–$NaIO_4$ in MeCN–CCl_4–water[12] in the presence of Na_2HPO_4 to give the acid **19** in 56% yield. The fluorinated acid **19** was finally hydrolysed with 4.5 mol dm^{-3} hydrochloric acid to give the targetted difluorinated hydroxy dicarboxylic acid **2** (F_2-EM) in 65% yield.

Racemic specimens of F-IPM **1** and F_2-EM **2** were subjected to reaction with the thermophilic IPMDH derived from *T. thermophilus* HB8 as described previously.[16] The reactions were monitored by measuring the formation of NADH from NAD^+ and the reaction kinetics were analysed by a double-reciprocal plot. In these experiments, F-IPM **1** and F_2-EM **2** were observed to act as substrates (K_m-values: 30 and 28 μmol dm^{-3}; k_{cat} values: 0.02 and 0.02 s^{-1}, respectively). No inhibitory effect was observed in either case against the formation of NADH. These observations suggested that the first oxidation process took place to form an intermediary fluorinated oxalacetate (**I**) as shown in the Scheme 2. However, any direct information as to the second step of the decarboxylation could not be obtained. If the resulting intermediates (**III**) could not be released from the enzyme, the overall reaction should have been inhibitory. This was actually not the case. Therefore, a question remained as to what the reaction products were in the case of F-IPM and F_2-EM.

As can be expected from molecular orbital considerations, a particular conformation should be required for the decarboxylation step in the overall IPMDH reaction, *i.e.*, the σ-bond between the C-4 carboxylate group and C-3 of the intermediate isopropyloxalacetate should be in the same plane as the π*-orbital of the C-2 carbonyl group. In other words, the most preferred conformation for decarboxylation is the one in which the bond between C-3 and the carboxylate is perpendicular to the carbonyl plane as in the conformation **I** illustrated in Scheme 5. It is interesting to speculate as to whether or not any significant bond rotation of the intermediate is required immediately after the oxidation for the decarboxylation step within the active site of the enzyme molecule. The reaction of F-IPM may provide a clue to this intriguing problem. Thus, the expected intermediate would be fluoroisopropyloxalacetate (**i**). If the conformation **I** in Scheme 5 was in the preferred conformation, an overall reaction product would be 4-fluoro-2-oxoisocaproate (**ii**), which would, in turn, be transformed into



Scheme 4 *Reagents and conditions:* i, *p*-lithioanisole; ii, $Ph_3P{=}CH$-CO_2Et; iii, H_2, Pd/C; iv, LDA; v, MoOPH; vi, BzCl, py; vii, Swern oxidation; viii, K-Selectride™; ix, TBDMSCl, imidazole; x, separation of isomers; xi, $RuCl_3$, $NaIO_4$; xii, H_3O^+



Scheme 5

the enoxocarboxylate (**iii**) by elimination of HF. However, the acid–base theory would suggest that the proton at C-3 of the most plausible intermediate (**i**) from F-IPM is more acidic than that of the natural intermediate or that of 4-fluoro-2-oxoisocaproate (**ii**) and is prone to undergo direct elimination to form isopropylideneoxalacetate (**v**). Thus, it could be conceivable that, if the actual conformation of the intermediate oxaloacetate (**i**) were different from the preferred conformation for decarboxylation, and if bond rotation between C-2 and C-3 were required prior to decarboxylation, then preferential elimination of HF from the intermediary oxalacetate structure (**i**) would alternatively give rise to isopropylideneoxalacetate (**v**) instead of 4-fluoro-2-oxoisocaproate (**ii**) as shown in Scheme 5. To get some insight into the preferred conformation of the oxalacetate intermediate in the enzyme active site, the enzymic incubation with F-IPM was subjected to direct NMR analysis, and identification of the product was pursued.

An aliquot of a large-scale incubation of F-IPM with IPMDH was taken when approximately 50% of IPM had been consumed as judged by UV absorbance, and was subjected to ^{1}H NMR spectroscopy. In addition to the typical fluorine-coupled methyl signals at δ_H 1.65 and 1.68 ppm of the substrate F-IPM, two singlet signals emerged at δ_H 2.16 and 2.28 ppm, which were apparently ascribable to allylic methyl groups. Further, a ^{19}F NMR spectrum of the incubation mixture clearly showed the formation of fluoride ions, with signals at −117.6 ppm (Fig. 1). Apparently, elimination of HF took place as originally expected; however, it was necessary to distinguish whether the product was either enoxocarboxylate (**iii**) or isopropylideneoxalacetate (**v**). To do this, the reaction product was isolated from the enzyme reaction mixture as follows; first by solvent extraction, followed by treatment with diazomethane to afford a methyl ester, and final purification by preparative HPLC. The isolated methyl ester showed a molecular-ion peak of *m*/*z* 142 in electron ionization mass spectrometry. The data obtained strongly implied that the product ester was methyl 4-methyl-2-oxopent-3-enoate. Furthermore, the isolated product was directly compared on HPLC with a synthesized authentic sample.[17] Thus, the product from F-IPM by the reaction with IPMDH was 4-methyl-2-oxopent-3-enoate.

This result suggests that a conformation of the product isopropyloxalacetate in the enzyme active site is closely similar to the preferred conformation for the subsequent decarboxylation reaction; that is, the bond between C-3 and C-4 is almost perpendicular to the plane of the carbonyl group. Additional bond rotation may not be required for the decarboxylation.

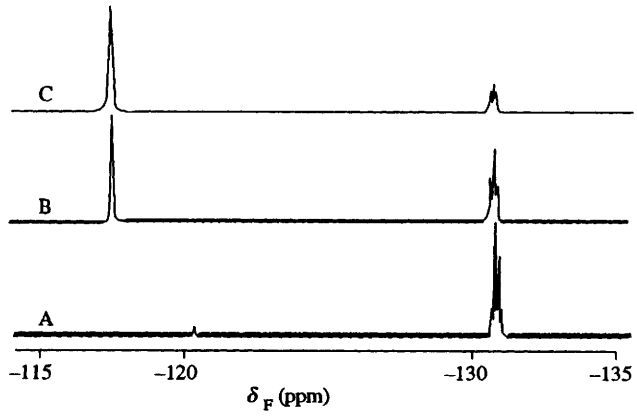


Fig. 1 ^{19}F NMR spectra of (A): F-IPM **1** in D_2O; (B): enzymic reaction mixture of F-IPM with IPMDH; (C): after addition of 1 mol dm^{-3} KF (0.1 cm^3) to solution (B). The enzymic reaction mixture consisted of 15.4 mmol dm^{-3} F-IPM **1**, 10 mmol dm^{-3} $MgCl_2$, 20 mmol dm^{-3} NAD^+, 1 mol dm^{-3} KCl, and 0.1 mg of IPMDH in 50 mmol dm^{-3} HEPES buffer (pH 8.0, pH meter, total volume 6.0 cm^3) and was incubated at 60 °C for 2.5 h. An aliquot of the enzyme reaction mixture (0.5 cm^3) was directly analysed by ^{19}F-NMR spectroscopy after addition of 0.1 cm^3 of D_2O. ^{19}F NMR spectra were recorded on the JEOL GSX-500 spectrometer at 470.4 MHz: 45° fluorine pulse of 6 μs, acquisition time of 0.655 s, 2 s recycling delay, 100 kHz sweep, data points of 128 K, 4 scans, and chemical shifts referenced to external $CFCl_3$ (δ_F 0.0).

This is well in accord with the minimal-motion concept in enzyme reactions.[18] Therefore, IPMDH has been so evolved as to carry out its required reaction such that additional significant motions of either the substrate or the enzyme are not required.

The reaction product from F-IPM with IPMDH turned out to the one which we designed as a substrate for mechanism-based suicidal inhibition. It seems likely that F_2-EM reacts with IPMDH through oxidation–decarboxylation–elimination in analogy with the case of F-IPM, since F_2-EM was shown to be as active as F-IPM toward the thermophilic IPMDH. However, unfortunately, enzyme reaction products were not inhibitory against IPMDH either. This result may be explained in two ways. The enzyme reaction products could be active in accepting a nucleophilic functionality of the enzyme but the addition product might be reversibly eliminated to regenerate the original, unaffected enzyme. This possibility cannot, unfortunately, be tested further by the present approach. The

other explanation would be that no 1,4-addition took place due to the lack of a suitable functional group in the active site. It may be pointed out, as to this possibility, that thermophilic IPMDH has no cysteine residue at all which would have had a suitable nucleophilic SH group;[1] alternatively, although potentially nucleophilic carboxylate functions are available around the active site,[4,5] subsequent elimination reaction, *i.e.*, reversible addition and elimination as described above, from the resulting β-acyloxy ketone function would also be facilitated in this case. It seems possible, therefore, that a suitably oriented reactive nucleophilic functionality is not available in the IPMDH active site. Closer examination of, for example, a ternary complex of enzyme–coenzyme–substrate analogue will provide further insight into these intriguing questions.

Experimental

Mps were measured with a Yanagimoto BY-1 micromelting point apparatus and are uncorrected. IR spectra were taken on a Hitachi 285 infrared spectrometer. ^{1}H, ^{13}C and ^{19}F NMR spectra were recorded on JEOL FX-200, GSX-270, and/or JEOL GSX-500 spectrometers. Deuteriochloroform (99.8% atom enriched, Aldrich) or deuterium oxide (99.9% atom enriched, Aldrich) were used for the NMR solvent throughout. ^{1}H and ^{13}C NMR chemical shifts were reported in δ-values based on internal $SiMe_4$ ($\delta_H = 0$) or dioxane ($\delta_C = 66.5$), or solvent signal ($CDCl_3$: $\delta_C = 77.0$; HOD: $\delta_H = 4.8$) as reference. ^{19}F NMR chemical shifts were expressed in δ_F-values from internal or external $CFCl_3$ ($\delta_F = 0$). *J*-Values are in Hz. High-performance liquid chromatography (HPLC) was carried out on a Hitachi L-6000 chromatograph equipped with an L-4000 UV detector, and using a column of Zorbax Sil (4.6 mm × 250 mm) with hexane–CH_2Cl_2 (5:1) as solvent. Mass spectra were taken on a JEOL AX-505 mass spectrometer. Column chromatography was carried out with Kieselgel 60 (70–230 mesh, Merck) or Lobar Fertigsaule RP-18 (40–63 mm, Merck). All reactions, except for catalytic hydrogenation reactions, were carried out in an inert (Ar or N_2) atmosphere. THF and diethyl ether were distilled from sodium–benzophenone ketyl; pyridine was distilled from potassium hydroxide; and benzene, dimethyl sulfoxide (DMSO), dichloromethane and toluene were distilled from calcium hydride.

Ethyl 2,3-bis-(3,4-dimethoxyphenyl)-3-oxopropanoate 4

Sodium hydride (7.70 g; 60% in mineral oil) was washed with hexane and the residual hexane was removed *in vacuo*. A solution of ethyl 3,4-dimethoxybenzoate (15.4 g, 73.3 mmol) in THF (50 cm^3) was added and the mixture was heated under reflux. After 2 h, a solution of ethyl (3,4-dimethoxyphenyl)-acetate (12.7 g, 56.4 mmol) in THF (10.6 cm^3) was added dropwise over a period of 6 h under reflux and stirring of the mixture was continued for another 4 h. The mixture was cooled to 0 °C, saturated aq. NH_4Cl was added, and the mixture was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (10:1–3:1) to give *title compound* **4** (21.4 g, 98%) as an oil, ν_{max}(thin film)/cm^{-1} 1740 and 1660; $\delta_H(CDCl_3)$ 1.08 (3 H, t, *J* 7), 3.85 (3 H, s), 3.88 (3 H, s), 3.90 (3 H, s), 3.92 (3 H, s), 4.23 (2 H, q, *J* 7), 5.51 (1 H, s), 6.89 (4 H, m) and 7.57 (2 H, m); $\delta_C(CDCl_3)$ 14.1, 55.8, 55.9, 56.1, 59.7, 61.6, 110.0, 111.0, 111.2, 112.4, 121.9, 123.7, 125.9, 128.8, 140.9, 149.1, 153.6, 169.2 and 192.0 (Found: C, 64.7; H, 5.95. $C_{21}H_{24}O_7$ requires C, 64.94; H, 6.23%).

Ethyl (2*R**,3*R**)-2,3-bis-(3,4-dimethoxyphenyl)-3-hydroxy-propanoate 5a

A mixture of zinc chloride (9.28 g, 68.1 mmol) and $NaBH_4$ (5.24 g, 138.5 mmol) in diethyl ether (300 cm^3) was stirred for 1 h at room temp. and cooled to 0 °C. A solution of *keto ester* **4** (21.3 g, 54.8 mmol) in diethyl ether (20 cm^3) was added to the solution and the mixture was stirred for 2 h at 0 °C and then for an additional 2 h at room temp. The solution was cooled to 0 °C and the reaction was quenched by addition of water. The aqueous layer was extracted twice with diethyl ether (300 cm^3). The combined organic layers were washed successively with 2 mol dm^{-3} HCl and brine, dried over Na_2SO_4 and evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (5:1–3:1) and the product was further purified by recrystallization from methanol to give *title compound* **5a** (17.1 g, 80%), mp 115.4–116.2 °C; $\nu_{max}(CHCl_3)$/cm^{-1} 3500 and 1725; $\delta_H(CDCl_3)$ 1.07 (3 H, t, *J* 7), 3.78 (1 H, d, *J* 8), 3.82 (3 H, s), 3.86 (9 H, s), 4.00 (2 H, m), 5.18 (1 H, dd, *J* 2 and 7) and 6.87 (6 H, m); $\delta_C(CDCl_3)$ 14.0, 55.9, 56.0, 59.4, 60.8, 75.1, 110.8, 111.2, 119.1, 121.5, 148.7 and 172.3 (Found: C, 64.8; H, 6.9. $C_{21}H_{26}O_7$ requires C, 64.60; H, 6.71%).

(1*R**,2*S**)-1,2-Bis-(3,4-dimethoxyphenyl)propane-1,3-diol 6

A solution of ester **5a** (336 mg, 0.86 mmol) in THF (2.0 cm^3) was added to a suspension of $LiAlH_4$ (85 mg, 2.24 mmol) in THF (15 cm^3) at 0 °C and the mixture was stirred for 6 h. Water (~0.5 cm^3) was carefully added and the mixture was stirred for 15 min. The resulting precipitate was filtered off by suction and washed with ethyl acetate. The filtrate and washings were combined and evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (3:1) to give *title compound* **6** (142 mg, 47%) as an oil, which was kept in a refrigerator to yield a solid, mp 118–122 °C; $\delta_H(CDCl_3)$ 3.08 (1 H, q, *J* 7), 3.76 (2 H, d, *J* 7), 3.83 (3 H, s), 3.85 (3 H, s), 3.87 (6 H, s), 4.94 (1 H, d, *J* 7) and 6.81 (6 H, m); $\delta_C(CDCl_3)$ 55.4, 55.8, 55.9, 64.2, 75.7, 109.5, 110.7, 111.3, 112.3, 119.0, 120.9, 130.9, 134.5, 148.7, 148.9 and 149.0 (Found: C, 65.5; H, 6.7. $C_{19}H_{24}O_6$ requires C, 65.50; H, 6.94%).

(4*R**,5*S**)-4,5-Bis-(3,4-dimethoxyphenyl)-2,2-dimethyl-1,3-dioxane 7

PPTS (5 mg) was added to the solution of diol **6** (75 mg, 0.22 mmol) and 2,2-dimethoxypropane (162 mm^3, 1.3 mmol) in CH_2Cl_2 (1.5 cm^3), and the solution was stirred for 8 h at room temp. The reaction was quenched by addition of saturated aq. $NaHCO_3$. The mixture was extracted with ethyl acetate (30 cm^3). The organic phase was washed with brine, dried over Na_2SO_4 and evaporated. The residue was chromatographed on silica gel with hexane–ethyl acetate (4:1) to give title compound **7** (62 mg, 73%) as an oil, $\delta_H(CDCl_3)$ 1.64 (3 H, s), 1.66 (3 H, s), 2.67 (1 H, br), 3.60 (3 H, s), 3.78 (3 H, s), 3.80 (6 H, s), 4.12 (1 H, dd, *J* 11.7 and 0.9), 4.55 (1 H, dd, *J* 11.7 and 3.7), 5.32 (1 H, d, *J* 3.4) and 6.6 (6 H, m).

(3*R**,4*R**)-4-(*tert*-Butyldimethylsiloxy)-3,4-bis-(3,4-dimethoxyphenyl)-2-methylbutan-2-ol 8

Solid TBDMSCl (41 g, 0.27 mol) was added to a stirred solution of ester **5a** (21.2 g, 54.2 mmol) and imidazole (36.9 g, 0.540 mol) in dimethylformamide (DMF) (74 cm^3) at 0 °C. The mixture was stirred for 4 h at room temp. Water was then added to the solution, and the mixture was stirred for 15 min before being extracted with diethyl ether (300 cm^3). The organic phase was washed with brine, dried over Na_2SO_4 and evaporated to give a crude TBDMS ether (27.4 g) as an oil, which was used for the next step without further purification.

A solution of methylmagnesium bromide in THF (230 cm^3; 1 mol dm^{-3}) was added to a stirred solution of the above crude TBDMS ether (27.4 g) in THF (50 cm^3). The solution was stirred for 3.5 h at room temp. The reaction was quenched by addition of saturated aq. NH_4Cl and the mixture was extracted with ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4 and evaporated. The residue was

chromatographed over silica gel with hexane–ethyl acetate (5:1) and the product was recrystallized from hexane–ethyl acetate to yield *title compound* **8** (23.1 g, 87%), mp 92.5–94 °C; $\nu_{max}(CHCl_3)/cm^{-1}$ 3550; $\delta_H(CDCl_3)$ −0.37 (3 H, s), 0.14 (3 H, s), 0.94 (9 H, s), 1.08 (3 H, s), 1.52 (3 H, s), 2.46 (1 H, d, *J* 3), 3.48 (3 H, s), 3.80 (6 H, s), 3.82 (3 H, s), 5.50 (1 H, d, *J* 3) and 6.20 and 6.90 (6 H, m); $\delta_C(CDCl_3)$ −4.9, −4.0, 17.9, 25.9, 29.1, 30.4, 55.3, 55.7, 55.9, 62.1, 73.6, 76.3, 110.0, 110.8, 118.6, 130.7, 135.9, 147.8 and 148.0 (Found: C, 66.3; H, 8.3. $C_{27}H_{42}O_6Si$ requires C, 66.09; H, 8.63%).

(1*R,2*R**)-1,2-Bis-(3,4-dimethoxyphenyl)-3-methylbutane-1,3-diol 9**

A solution of TBAF in THF (27.2 cm^3; 93.8 mmol) was added to a stirred solution of silyl ether **8** (23.0 g, 46.9 mmol) in THF (250 cm^3) and the solution was stirred for 4 h at room temp. The reaction was quenched by addition of saturated aq. NH_4Cl and the mixture was extracted with ethyl acetate (400 cm^3 × 2). The combined organic layers were washed successively with saturated aq. NH_4Cl and brine, dried over Na_2SO_4 and evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (3:1) to give *title compound* **9** (19.0 g, quant.) as an oil, ν_{max}(thin film)/cm^{-1} 3400; $\delta_H(CDCl_3)$ 1.11 (3 H, s), 1.62 (3 H, s), 2.57 (1 H, d, *J* 3), 3.62 (3 H, s), 3.77 (3 H, s), 3.79 (3 H, s), 3.80 (3 H, s), 5.53 (1 H, d, *J* 3.0) and 6.7 (6 H, m); $\delta_C(CDCl_3)$ 30.0, 55.7, 55.9, 60.8, 74.2, 74.7, 109.7, 110.3, 114.7, 118.1, 123.6, 130.1, 135.7, 147.7 and 148.3 (Found: C, 66.75; H, 7.6. $C_{21}H_{28}O_6$ requires C, 67.00; H, 7.50%).

(1*R,2*R**)-1,2-Bis-(3,4-dimethoxyphenyl)-3-fluoro-3-methylbutyl acetate 10**

Acetic anhydride (10 cm^3) was added to a solution of diol **9** (17.5 g, 46.5 mmol) and 4-(dimethylamino)pyridine (DMAP) (100 mg) in pyridine (50 cm^3). The solution was stirred for 3 h at room temp. and the reaction was then quenched by addition of water. The mixture was extracted with ethyl acetate (400 cm^3 × 2) and the combined organic layers were washed successively with 2 mol dm^{-3} HCl, saturated aq. $NaHCO_3$, and brine, dried over Na_2SO_4 and evaporated to give the mono-acetate (19.9 g) as an oil, which was used for the next step without further purification.

A solution of this mono-acetate (19.9 g) in CH_2Cl_2 (100 cm^3) was added to a stirred solution of DAST (12.3 cm^3, 93.0 mmol) in CH_2Cl_2 (100 cm^3) over a period of 5 min at −78 °C. The solution was stirred for 4 h at −78 °C and then the mixture was warmed to 0 °C. The reaction was quenched by addition of water and the reaction mixture was extracted with ethyl acetate (500 cm^3 × 2). The combined organic layers were washed successively with saturated aq. $NaHCO_3$ and brine, dried over Na_2SO_4 and evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (5:1) to give *title compound* **10** (14.3 g, 73%) as an oil, ν_{max}(thin film)/cm^{-1} 1740; $\delta_H(CDCl_3)$ 1.33 (3 H, d, *J* 22), 1.39 (3 H, d, *J* 22), 2.00 (3 H, s), 2.94 (1 H, dd, *J* 4 and 22), 3.70 (3 H, s), 3.81 (3 H, s), 3.84 (3 H, s), 3.88 (3 H, s), 6.47 (1 H, d, *J* 4) and 6.7 (6 H, m); $\delta_C(CDCl_3)$ 21.2, 26.1 (d, *J* 23), 27.3 (d, *J* 23), 55.6, 55.7, 55.8, 60.3 (d, *J* 19), 73.7 (d, *J* 3), 96.7 (d, *J* 160), 110.2, 110.3, 110.5, 114.0, 119.2, 123.5, 129.2, 148.1, 148.4 and 169.4 (Found: C, 65.9; H, 7.1. $C_{23}H_{29}FO_6$ requires C, 65.70; H, 6.95%).

(2*R,3*S**)-2-*O*-Acetyl-3-(2-fluoropropan-2-yl)malic acid 11**

Ruthenium trichloride (300 mg) was added to a stirred mixture of compound **10** (11.5 g, 27.3 mmol), $NaIO_4$ (96.0 g, 0.449 mol), CCl_4 (110 cm^3), MeCN (110 cm^3) and water (120 cm^3) at 0 °C. The mixture was stirred for 26 h at room temp. and filtered through sintered glass, and the residue was washed with saturated aq. $NaHCO_3$. The filtrate was acidified with 6 mol dm^{-3} HCl (to pH 1 by pH paper) and was extracted overnight with diethyl ether by continuous extraction. The extract was dried over Na_2SO_4 and evaporated. Chromatography over a Lobar RP-18 column with water–methanol (2:1) and recrystallization from $CHCl_3$–hexane gave *title acid* **11** (924 mg, 14%) as a powder, mp 119.8–121.7 °C; ν_{max}(KBr)/cm^{-1} 1720, 1380 and 1240; $\delta_H(CDCl_3)$ 1.56 (6 H, d, *J* 23), 2.17 (3 H, s), 3.59 (1 H, dd, *J* 3 and 9) and 5.69 (1 H, dd, *J* 4 and 22); $\delta_C(CDCl_3)$ 20.5, 24.0 (d, *J* 22), 27.7 (d, *J* 21), 54.9 (d, *J* 23), 68.4, 93.9 (d, *J* 150), 169.7, 174.0 and 174.5 (Found: C, 45.6; H, 5.3. $C_9H_{13}FO_6$ requires C, 45.77; H, 5.55%).

(2*R,3*S**)-3-(2-Fluoropropan-2-yl)malic acid (F-IPM) 1**

Lithium hydroxide monohydrate (723 mg, 17.2 mmol) was added to a stirred solution of acetate **11** (810 mg, 3.43 mmol) in water (8 cm^3) and the solution was stirred for 20 min at room temp. Then Dowex 50W-X2 (H^+-form) was added (to pH 3 by pH paper) and the mixture was filtered by suction. The filtrate was evaporated and the residue was recrystallized from water–acetone to yield *title acid* **1** (434 mg, 64%) as a powder, mp > 340 °C; ν_{max}(KBr)/cm^{-1} 3420 and 1600; $\delta_H(D_2O)$ 1.48 (3 H, d, *J* 23), 1.50 (3 H, d, *J* 23), 2.93 (1 H, dd, *J* 4 and 16) and 4.22 (1 H, d, *J* 4); $\delta_C(D_2O)$ 24.9 (d, *J* 22), 25.9 (d, *J* 22), 59.4 (d, *J* 19), 71.1 (d, *J* 4), 97.0 (d, *J* 154), 177.7 and 180.4; $\delta_F(D_2O)$ −130.8 (double septet, *J* 16 and 23) (Found: C, 39.2; H, 4.6. $C_7H_{10}FNaO_5$ requires C, 38.90; H, 4.63%).

α,α-Difluoro-*p*-methoxypropiophenone 13

A solution of *tert*-butyllithium in hexane (2.4 cm^3; 2.13 mol dm^{-3}) was added dropwise to a solution of *p*-bromoanisole (477 mg, 2.55 mmol) in diethyl ether (1 cm^3) at 0 °C and the mixture was stirred for 2 h at room temp. This solution (1.4 cm^3) was added to a solution of ethyl α,α-difluoropropionate **12** (100 mg, 0.85 mmol) in diethyl ether (1.5 cm^3) at −78 °C and the mixture was stirred for 15 min at the same temp. The reaction was quenched by addition of water and the mixture was extracted with diethyl ether (50 cm^3 × 2). The combined organic layers were successively washed with saturated aq. NH_4Cl and brine, dried over Na_2SO_4 and evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (20:1) to give *title ketone* **13** (209 mg, 43%) as an oil, ν_{max}(thin film)/cm^{-1} 1695; $\delta_H(CDCl_3)$ 1.88 (3 H, t, *J* 19), 3.90 (3 H, s), 6.97 (2 H, m) and 8.12 (2 H, m); $\delta_C(CDCl_3)$ 20.8 (t, *J* 12), 55.4, 113.9, 119.6 (t, *J* 125), 124.4 (t, *J* 3), 132.7 (t, *J* 4), 164.4 and 187.6 (t, *J* 31); $\delta_F(CDCl_3)$ −92.7 (q, *J* 19) (Found: C, 59.9; H, 5.15. $C_{10}H_{10}F_2O_2$ requires C, 60.00; H, 5.03%).

Ethyl (*E*)-4,4-difluoro-3-(*p*-methoxyphenyl)pent-2-enoate 14

Solid ethoxycarbonylmethylene(triphenyl)phosphorane (39.1 g, 0.11 mol) was added to a stirred solution of ketone **13** (6.13 g, 28.6 mmol) in toluene (75 cm^3) and the mixture was stirred for 22 h at room temp. The reaction mixture was evaporated to dryness and the resulting oily residue was chromatographed over silica gel with hexane–ethyl acetate (20:1) to yield *title ester* **14** (7.92 g, 98%), ν_{max}(neat)/cm^{-1} 1730; $\delta_H(CDCl_3)$ 1.08 (3 H, t, *J* 7), 1.64 (3 H, t, *J* 19), 3.82 (3 H, s), 4.02 (2 H, q, *J* 7), 6.41 (1 H, t, *J* 2), 6.90 (2 H, m) and 7.17 (2 H, m); $\delta_C(CDCl_3)$ 13.9, 23.5 (t, *J* 28), 55.2, 60.5, 113.5, 120.7 (t, *J* 9), 120.8 (t, *J* 241), 126.0, 129.9, 150.0 (t, *J* 20), 159.8 and 165.4 (Found: C, 61.9; H, 6.0. $C_{14}H_{16}F_2O_3$ requires C, 62.22; H, 5.97%)

Ethyl 4,4-difluoro-3-(*p*-methoxyphenyl)pentanoate 15

A solution of enoate **14** (7.25 g, 25.5 mmol) in ethanol (7.2 cm^3) was added to a suspension of 10% Pd–C (360 mg) in ethanol (72 cm^3). The mixture was vigorously stirred for 44 h under hydrogen. The solution was filtered through a Celite pad by suction and washed with ethanol. The filtrate and washings were combined and evaporated to give *title saturated ester* **15** (7.45 g, quant.) as an oil, ν_{max}(thin film)/cm^{-1} 1740; $\delta_H(CDCl_3)$

1.12 (3 H, t, *J* 7), 1.42 (3 H, t, *J* 19), 2.75 (1 H, dd, *J* 10 and 16), 3.03 (1 H, dd, *J* 5 and 16), 3.53 (1 H, m), 3.79 (3 H, s), 4.01 (2 H, m), 6.85 (2 H, m) and 7.20 (2 H, m); $\delta_C(CDCl_3)$ 14.0, 22.3 (t, *J* 27), 34.5 (t, *J* 4), 48.7 (t, *J* 25), 55.2, 60.5, 113.9, 124.3 (t, *J* 245), 129.2 (d, *J* 8), 130.0, 159.1 and 171.5 (Found: C, 62.0; H, 6.6. $C_{14}H_{18}F_2O_3$ requires C, 61.80; H, 6.66%).

Ethyl 4,4-difluoro-2-hydroxy-3-(*p*-methoxyphenyl)pentanoate 16a and 16b

A solution of butyllithium (1.55 mol dm^{-3} in hexane; 27 cm^3, 44.8 mmol) was added to a solution of 2,2′-bipyridyl (10 mg) and diisopropylamine (6.2 cm^3, 44.8 mmol) in THF (100 cm^3) at 0 °C and the mixture was stirred for 30 min. A solution of compound **15** (6.1 g, 22.4 mmol) in THF (10 cm^3) was added to the solution. The mixture was stirred for 30 min at 0 °C and then cooled to −78 °C. Solid MoO_5·pyridine·HMPA complex (11.9 g, 28.1 mmol) was added and the mixture was stirred for 30 min at −78 °C. The reaction was quenched by addition of saturated aq. Na_2SO_3 and the reaction mixture was extracted with ethyl acetate (400 cm^3 × 2). The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated. The resulting residue was chromatographed over silica gel with hexane–ethyl acetate (15:1) to yield a mixture of diastereoisomers **16a** and **16b** (15:85) (4.75 g, 74%). Repeated recrystallization from acetone–hexane gave the threo-*isomer* **16b** (3.2 g); $\nu_{max}(CHCl_3)/cm^{-1}$ 1730; $\delta_H(CDCl_3)$ 1.24 (3 H, t, *J* 7), 1.54 (3 H, t, *J* 19), 3.12 (1 H, br d, *J* 5), 3.59 (1 H, ddd, *J* 4, 12 and 15), 3.80 (3 H, s), 4.20 (2 H, q, *J* 7), 4.51 (1 H, br t, *J* 4), 6.87 (2 H, m) and 7.40 (2 H, m); $\delta_C(CDCl_3)$ 13.9, 23.3 (t, *J* 26), 54.6 (t, *J* 23), 55.2, 62.1, 71.9 (t, *J* 2), 113.9, 121.5, 125.0, 127.9, 128.6, 130.9, 159.3 and 173.4 (Found: C, 58.45; H, 6.2. $C_{14}H_{18}F_2O_4$ requires C, 58.30; H, 6.29%).

Ethyl (2*R,3*S**)-2-benzoyloxy-4,4-difluoro-3-(*p*-methoxyphenyl)pentanoate 17**

Benzoyl chloride (71.0 cm^3, 0.61 mmol) was added to a solution of hydroxy ester **16b** (120 mg, 0.41 mmol) in pyridine (1.2 cm^3). The mixture was stirred for 10 min and 1 mol dm^{-3} HCl (1 cm^3) was added. The solution was extracted with diethyl ether (50 cm^3). The organic phase was washed successively with 1 mol dm^{-3} HCl, saturated aq. $NaHCO_3$, and brine, dried over Na_2SO_4 and evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (10:1) to give title compound **17** (159 mg, quant.). The residue was recrystallized from hot hexane to give prisms, one of which was used for X-ray analysis; $\delta_H(CDCl_3)$ 1.05 (3 H, t, *J* 7), 1.58 (3 H, t, *J* 19), 3.78 (3 H, s), 3.81 (1 H, m), 4.01 (2 H, m), 5.64 (1 H, d, *J* 7), 6.85 (2 H, m), 7.29 (2 H, m), 7.48 (2 H, m), 7.60 (1 H, m) and 8.14 (2 H, m).

Ethyl (2*R,3*R**)-2-(*tert*-butyldimethylsiloxy)-4,4-difluoro-3-(*p*-methoxyphenyl)pentanoate 18**

A solution of DMSO (3.1 cm^3, 44.5 mmol) in CH_2Cl_2 (11 cm^3) was added dropwise to a solution of oxalyl dichloride (12.4 cm^3, 37.1 mmol) in CH_2Cl_2 (120 cm^3) at −78 °C and the mixture was stirred for 20 min at −78 °C. A solution of a 1:6 mixture of **16a** and **16b** (2.14 g, 7.42 mmol) in CH_2Cl_2 (6 cm^3) was added dropwise over a period of 15 min and the mixture was stirred for 15 min at −78 °C, and then at −20 °C for 90 min. Et_3N (9.0 cm^3, 66.8 mmol) was added to the solution. After the addition was complete, the reaction was quenched by addition of water. The organic phase was separated and the aqueous layer was extracted with ethyl acetate (200 cm^3). The combined organic phase was washed successively with saturated aq. NH_4Cl and brine, dried over Na_2SO_4 and evaporated to give crude keto ester (3.38 g).

A solution of K-selectride™ in THF (8.9 cm^3; 1 mol dm^{-3}) was added to a solution of crude keto ester (3.38 g) in THF (80 cm^3) at −78 °C and the mixture was stirred for 45 min. The reaction was quenched by addition of saturated aq. NH_4Cl and the mixture was extracted with ethyl acetate (200 cm^3 × 2). The organic layer was washed successively with saturated aq. NH_4Cl and brine, dried over Na_2SO_4 and evaporated. The oily residue was chromatographed on silica gel [(10:1) hexane–ethyl acetate] to give a 9:1 mixture of diastereoisomers **16a** and **16b** (1.61 g).

Solid TBDMSCl (2.1 g, 14.0 mmol) was added to a stirred solution of the mixture of compounds **16a** and **16b** (1.61 g) and imidazole (1.90 g, 27.9 mmol) in DMF (20 cm^3), and the mixture was stirred for 19 h. The reaction was quenched by addition of saturated aq. NH_4Cl and the mixture was extracted with ethyl acetate (150 cm^3 × 2). The combined organic layers were washed successively with saturated aq. NH_4Cl and brine, dried over Na_2SO_4 and evaporated. The oily residue was chromatographed on silica gel [(15:1) hexane–ethyl acetate] to yield *title siloxy ester* **18** (1.79 g, 60%) as an oil, ν_{max}(thin film)/cm^{-1} 1760; $\delta_H(CDCl_3)$ −0.02 (3 H, s), 0.02 (3 H, s), 0.86 (9 H, s), 1.12 (3 H, t, *J* 7), 1.48 (3 H, t, *J* 19), 3.46 (1 H, ddd, *J* 4, 9 and 21), 3.78 (3 H, s), 4.43 (2 H, m), 4.82 (1 H, d, *J* 4), 6.82 (2 H, m) and 7.31 (2 H, m); $\delta_C(CDCl_3)$ −5.5, −5.2, 14.0, 18.0, 23.2 (t, *J* 25), 25.6, 55.1, 55.3 (t, *J* 20), 60.8, 71.3, 113.4, 124.2 (dd, *J* 222 and 227), 126.4 (d, *J* 6), 131.6, 159.2 and 172.1 (Found: C, 59.6; H, 7.85. $C_{20}H_{32}F_2O_4Si$ requires C, 59.67; H, 8.01%).

1-Ethyl hydrogen (2*R,3*S**)-2-(*tert*-butyldimethylsiloxy)-3-(1,1-difluoroethyl)succinate 19**

Ruthenium trichloride (280 mg) was added to a stirred solution of ester **18** (7.26 g, 18.0 mmol), Na_2HPO_4·$12H_2O$ (97.0 g, 0.27 mol), $NaIO_4$ (57.8 g, 0.27 mol), water (87.0 cm^3), CCl_4 (58.0 cm^3) and MeCN (58.0 cm^3) at 0 °C. The solution was stirred for 20 h at room temp. The reaction mixture was filtered through a glass filter, and the residue was washed successively with CH_2Cl_2 and saturated aq. $NaHCO_3$. The combined filtrate was acidified by addition of 6 mol dm^{-3} HCl (to pH 1 by pH paper), and extracted with CH_2Cl_2 (500 cm^3 × 3). The extract was washed with saturated aq. Na_2SO_3 and brine, dried over Na_2SO_4 and evaporated. The residue was dissolved in diethyl ether and the solution was filtered through a Celite pad. The filtrate was evaporated and the oily residue was chromatographed over silica gel with hexane–ethyl acetate (5:1) to give *title acid ester* **19** (3.41 g, 56%) as syrup, ν_{max}(thin film)/cm^{-1} 3650 and 1740; $\delta_H(CDCl_3)$ 0.09 (3 H, s), 0.10 (3 H, s), 0.89 (9 H, s), 1.30 (3 H, t, *J* 7), 1.80 (3 H, t, *J* 19), 3.51 (1 H, dt, *J* 18 and 6), 4.23 (2 H, q, *J* 7) and 4.69 (1 H, d, *J* 5); $\delta_C(CDCl_3)$ −5.6, −5.0, 18.0, 22.2 (t, *J* 27 Hz), 25.4, 57.2 (t, *J* 26), 61.8, 69.6 (d, *J* 6), 121.2 (dd, *J* 242 and 246), 170.8 and 171.4 (Found: C, 49.6; H, 7.6. $C_{14}H_{26}F_2O_5Si$ requires C, 49.39; H, 7.70%).

(2*R,3*S**)-3-(1,1-Difluoroethyl)malic acid (F_2-EM) 2**

A solution of siloxy compound **19** (3.34 g, 9.80 mmol) in THF (40 cm^3) and 4.5 mol dm^{-3} HCl (25 cm^3) at 50 °C was stirred for 2 days and then evaporated to dryness. The residue was chromatographed over a LiChroprep RP-8 column with methanol–water (1:10) to yield *title acid* **2** (1.05 g, 65%) as syrup, ν_{max}(thin film)/cm^{-1} 3500 and 1720; $\delta_H(D_2O)$ 2.10 (3 H, t, *J* 20), 3.97 (1 H, ddd, *J* 3, 11 and 15) and 5.00 (1 H, d, *J* 3); $\delta_C(D_2O)$ 20.2 (t, *J* 26), 53.2 (t, *J* 25), 65.7, 121.1 (t, *J* 242), 168.9 (d, *J* 5) and 174.2 (Found: C, 36.4; H, 4.4. $C_6H_8F_2O_5$ requires C, 36.38; H, 4.07%).

Enzyme and substrate assay

The thermophilic IPMDH derived from *T. thermophilus* HB8 was prepared and purified as described previously.[16] IPMDH reaction was monitored by measuring the NADH absorption at 340 nm on a Gilford Response spectrometer. Substrate activity

was assayed as follows; F-IPM (0.01–1.0 mmol dm^{-3}) was added to an assay mixture (500 mm^3 total volume) in 50 mmol dm^{-3} HEPES buffer (pH 8.0) containing 50 mmol dm^{-3} $MgCl_2$, 5 mmol dm^{-3} NAD^+ and 1 mol dm^{-3} KCl. The reaction was started by addition of the enzyme (0.4 μg), and the formation of NADH was measured as described above. Large-scale incubation was carried out using a mixture of 15.4 mmol dm^{-3} F-IPM, 10 mmol dm^{-3} $MgCl_2$, 20 mmol dm^{-3} NAD^+, 1 mol dm^{-3} KCl and 0.1 mg of IPMDH in 50 mmol dm^{-3} HEPES buffer (pH 8.0, pH meter, total volume 6.0 cm^3). The reaction was monitored from time to time by ^1H NMR spectroscopy directly. After incubation for 2.5 h at 60 °C, a portion (1 cm^3) of the reaction mixture was acidified to pH 1.0 by addition of 6 mol dm^{-3} HCl and extracted exhaustively with diethyl ether. The extract was concentrated under reduced pressure and the residue was further treated with an excess of ethereal diazomethane. The mixture was evaporated to dryness under reduced pressure to give an oily residue, which was further purified by preparative HPLC as described above to afford a trace amount (unweighed) of a product; λ_{max}(MeOH): 244.8 nm; $\delta_H(CDCl_3)$ 2.02 (s), 2.25 (s), 3.85 (s) and 6.77 (s); EIMS: *m/z* 142 (M^+), 114 ($M^+ - CO$), 109 ($M^+ - CH_3OH - H$), 83 ($M^+ - CO_2CH_3$) and 55 [$(CH_3)_2C{=}CH^+$].

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References

1 T. Oshima and K. Imahori, *J. Syst. Bacteriol.*, 1974, **24**, 102; T. Tanaka, K. Kawano and T. Oshima, *J. Biochem.* (*Tokyo*), 1981, **89**, 677; Y. Kagawa, H. Nojima, N. Nukiwa, M. Ishizuka, T. Nakajima, Y. Yasuhara, T. Tanaka and T. Oshima, *J. Biol. Chem.*, 1984, **259**, 2956; T. Yamada, N. Akutsu, K. Miyazaki, K. Kakinuma, M. Yoshida and T. Oshima, *J. Biochem.* (*Tokyo*), 1990, **108**, 449.
2 T. Yamada, K. Kakinuma and T. Oshima, *Chem. Lett.*, 1987, 1745; T. Yamada, K. Kakinuma, T. Endo and T. Oshima, *Chem. Lett.*, 1987, 1749; K. Kakinuma, K. Ozawa, Y. Fujimoto, N. Akutsu and T. Oshima, *J. Chem. Soc., Chem. Commun.*, 1989, 1190.
3 T. Nakamoto and B. Vennesland, *J. Biol. Chem.*, 1960, **235**, 202; S. England and I. Listowsky, *Biochem. Biophys. Res. Commun.*, 1963, **12**, 356.
4 K. Imada, M. Sato, N. Tanaka, Y. Katsube, Y. Matsuura and T. Oshima, *J. Mol. Biol.*, 1991, **222**, 725.
5 J. H. Hurley and A. M. Dean, *Structure*, 1994, **2**, 1007.
6 H. Terasawa, K. Miyazaki, T. Oshima, T. Eguchi and K. Kakinuma, *Biosci. Biotechnol. Biochem.*, 1994, **58**, 870.
7 M. C. Pirrung, H. Han and R. T. Ludwig, *J. Org. Chem.*, 1994, **59**, 2423; M. C. Pirrung, H. Han and D. S. Nunn, *J. Org. Chem.*, 1994, **59**, 2430.
8 C. T. Walsh, *Annu. Rev. Biochem.*, 1984, **53**, 493.
9 C. T. Walsh, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1983, **55**, 197.
10 T. Nakata and T. Oishi, *Tetrahedron Lett.*, 1980, **21**, 1641.
11 M. Hudlicky, *Org. React.*, 1988, **35**, 513.
12 B. E. Rossiter, T. Katsuki and K. B. Sharpless, *J. Am. Chem. Soc.*, 1981, **103**, 464.
13 T. Eguchi, T. Aoyama and K. Kakinuma, *Tetrahedron Lett.*, 1992, **33**, 5545.
14 E. Vedejs, D. A. Emgler and J. E. Telescow, *J. Org. Chem.*, 1978, **43**, 188.
15 Y. Takenaka, T. Aoyama, T. Eguchi, Y. Ohashi and K. Kakinuma, *Anal. Sci.*, 1994, **10**, 519.
16 K. Miyazaki, K. Kakinuma, H. Terasawa and T. Oshima, *FEBS Lett.*, 1993, **332**, 35.
17 H. W. Schnabel, D. Grimm and H. Jensen, *Justus Liebigs Ann. Chem.*, 1974, 477.
18 K. R. Hanson and I. A. Rose, *Acc. Chem. Res.*, 1975, **8**, 1.

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