ARTICLE

OBC
www.rsc.org/obc

Enantioselective synthesis of non-natural amino acids using phenylalanine dehydrogenases modified by site-directed mutagenesis

Patricia Busca,[a] Francesca Paradisi,[b] Eamonn Moynihan,[a] Anita R. Maguire*[a] and Paul C. Engel*[b]

[a] *Department of Chemistry, Analytical and Biological Chemistry Research Facility, University College Cork, Cork, Ireland. E-mail: a.maguire@ucc.ie*

[b] *Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland. E-mail: paul.engel@ucd.ie*

Received 28th April 2004, Accepted 23rd July 2004
First published as an Advance Article on the web 25th August 2004

The substrate scope of three mutants of phenylalanine dehydrogenase as biocatalysts for the transformation of a series of 2-oxo acids, structurally related to phenylpyruvic acid, to the analogous α-amino acids, non-natural analogues of phenylalanine, has been investigated. The mutant enzymes are more tolerant than the wild type enzyme of the non-natural substrates, especially those with substituents at the 4-position on the phenyl ring. Excellent enantiocontrol resulted in all cases.

Introduction

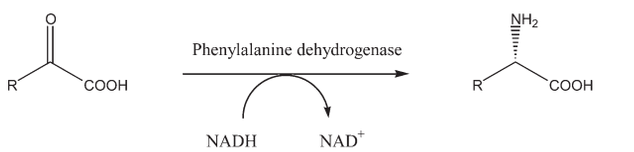
Enantiopure drugs constitute an increasing proportion (35% of drugs on the market in 2000) of pharmaceuticals, and thus there is substantial chemical and economic interest in methods for asymmetric synthesis.[1] Non-natural amino acids, in enantiopure form, are of considerable interest in the synthesis of alkaloids, peptides and other compounds with therapeutic applications *e.g.* in HIV-protease inhibitors.[2] Incorporation of non-natural amino acids into biologically active peptides and proteins can greatly improve their activity, stability, bioavailability and binding specificity.[3] In this context access to both enantiopure series, D- and L-, is important. Non-natural amino acids are increasingly in demand by the pharmaceutical industry for peptidomimetic and other single-enantiomer drugs.[4] They are also in demand as precursors to ligands for asymmetric synthesis.[2] While some enantiopure non-natural amino acids are commercially available they are very expensive.

One of the most important strategies for asymmetric synthesis involves biocatalysts—the application of biological species such as microbial cells or enzymes derived therefrom to catalyse organic reactions.[5] Many biocatalysts exhibit high regio-, chemo- and stereo-selectivity making them superior to chemical catalysts for asymmetric synthesis.[6] Furthermore, biocatalysts are ideal energy-efficient, environmentally acceptable reagents, as virtually all reactions proceed under mild conditions and avoid the use of toxic reagents and disposal of byproducts. Thus biocatalysts offer a good opportunity to prepare industrially useful chiral compounds.[7]

While naturally occurring enzymes have been widely employed in asymmetric synthesis,[8] there are instances in which they cannot be readily employed owing to limited substrate scope. Designer biocatalysts, where the naturally occurring protein is modified structurally to provide the desired reaction site selectivity, offer many advantages.[9]

Cofactor dependent amino acid dehydrogenases catalyse the interconversion of 2-oxo acids and α-amino acids, illustrated in Scheme 1 for phenylalanine dehydrogenase (PheDH).[10] Indeed phenylalanine dehydrogenase has been employed for the enantioselective synthesis of L-2-amino-4-phenylbutanoic acid with excellent enantiopurity by supplying the homologous oxo acid as illustrated in Scheme 1.[6b,11,12]

Seah *et al.*[13] undertook site-directed mutagenesis on PheDH and reported in 1995 that the resulting enzymes displayed reduced activity for L-phenylalanine compared to the wild type enzyme and enhanced activity towards aliphatic amino acid substrates indicating that the substrate profile of the enzyme was varied significantly by the mutations. Enhanced discrimination between phenylalanine and tyrosine in the engineered enzymes is of particular significance.[13b] On this basis we envisaged that engineered PheDH mutants might prove useful as biocatalysts for the asymmetric synthesis of non-natural amino acids, especially phenylalanine analogues. In this paper we report our preliminary results in this area.



Scheme 1

The basis of active site design in this project was initially a recognition of the common chemistry and shared structural features of the amino acid dehydrogenase family,[14] with glutamate dehydrogenase as the archetype for which a high-resolution structure had been solved by X-ray crystallography.[15] More recently this strategy has been further strengthened by the direct solution of the structure of a PheDH.[16] Site directed mutagenesis allows alteration of the amino acid residues surrounding the substrate-binding pocket of the enzyme to alter the size, shape and polarity of the pocket. The active site of phenylalanine dehydrogenase (PheDH) from *Bacillus sphaericus* has been mutated on the basis of homology modeling[13a] and several mutants affecting the contact with the aromatic ring of the amino acid substrate have been studied.

We focused in particular on mutations of the Asn in position 145 (N145) which showed an increased discrimination between phenylalanine and tyrosine,[13b] making the enzyme more selective towards non-polar substrates.

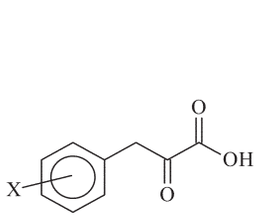
In this paper we report the activities of those mutants in which the asparagine has been substituted by an alanine (N145A), a leucine (N145L) or a valine (N145V).

Results and discussion

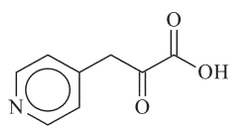
Substrate synthesis

With the objective of synthesizing a series of phenylalanine analogues from the appropriate 2-oxo acids through use of the engineered PheDH mutants, a series of substrates structurally related to phenyl pyruvate was selected. The compounds (**1**–**13**)

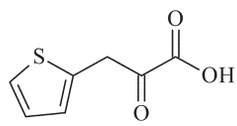
DOI: 10.1039/b406364c



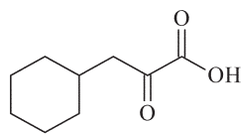
X = 2-Cl (**1**)
3-Cl (**2**)
4-Cl (**3**)
2-F (**4**)
3-F (**5**)
4-F (**6**)
4-Me (**7**)
4-OMe (**8**)
4-CF_3 (**9**)



(**10**)



(**11**)



(**12**)

$CH_3(CH_2)_3COCO_2H$ (**13**)

were chosen for this study, including aromatic, aliphatic and heteroaromatic 2-oxo acids as precursors for the non-natural amino acids.

These compounds were chosen to allow exploration of substrate scope in the modified active site of the enzyme. Both steric and electronic factors have been explored through this series. Evidently we required ready access to this series of 2-oxo acids. 2-Oxo acids, especially analogues of the naturally occurring amino acids are of major importance in intermediary metabolism. They have been used in the therapy of certain conditions such as uraemia and nitrogen accumulation disorders.[17] They are also of interest as intermediates in chemical synthesis, in the development of enzyme inhibitors and drugs, as model substrates of enzymes and in other ways. A general review was published in 1983.[17]

In this work three general synthetic routes to the 2-oxo acids were employed. The first route involved preparation through the addition of Grignard reagents to diethyl oxalate followed by hydrolysis of the resulting 2-oxo ester as illustrated in Scheme 2.[18] This method was employed for the synthesis of the chloro and fluoro substituted phenylpyruvate derivatives (**2**)–(**6**) and for the saturated analogue (**12**), see Table 1.

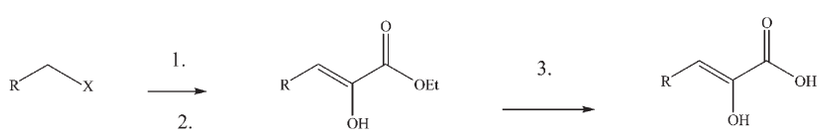
Using a series of benzylic halide derivatives the 2-oxo esters (**20**)–(**25**) were obtained in excellent yields. The Grignard reagents were prepared in Et_2O instead of THF to avoid the Wurtz coupling side reaction.[19] While this approach gave the oxo esters in high yields, the recovery of the acids after hydrolysis was poor, which can be attributed to a number of side reactions. Decarboxylation can occur during hydrolysis at high temperature or in basic media during the extraction process. Rapid decomposition can occur in polar solvents during concentration of the organic layer at reduced pressure giving an unidentifiable mixture by NMR. However, the acids can be isolated in high purity by precipitation from an ethyl acetate/dichloromethane mixture. ^{1}H and ^{13}C NMR spectra in CD_3OD show that only the enol form is present for (**2**)–(**6**).[20] This route also led successfully to the cyclohexyl oxo acid (**12**), the saturated analogue of phenylalanine. In this case only the keto form is observed in d_6-DMSO.

Table 1 Synthesis of 2-oxo acids—Grignard route

Benzyl halide R	Yield of 2-oxo ester (%)	Yield of 2-oxo acid (%)
3-ClC_6H_4 (**14**)	90 (**20**)[a]	45 (**2**)[c]
4-ClC_6H_4 (**15**)	87 (**21**)[a]	33 (**3**)[c]
2-FC_6H_4 (**16**)	95 (**22**)[a]	42 (**4**)[c]
3-FC_6H_4 (**17**)	92 (**23**)[a]	27 (**5**)[c]
4-FC_6H_4 (**18**)	87 (**24**)[a]	39 (**6**)[c]
C_6H_{11} (**19**)	81 (**25**)[b]	33 (**12**)[c]

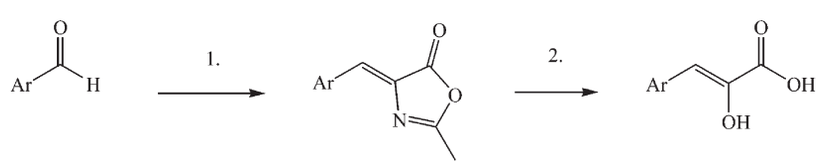
[a] Yields following chromatography. [b] Yield following distillation. [c] Yield following precipitation from EtOAc/DCM.

This method was less successful for the preparation of compounds containing electron-donating aryl substituents such as methyl and methoxy, as the benzyl Grignard reagents bearing these substituents were seen to undergo extensive Wurtz coupling. For these derivatives (**7**)–(**11**) a second method was employed *via* the azlactone (Scheme 3 and Table 2), a route



(**14-19**) (**20-25**) (**2-6**) (**12**)

Scheme 2 1. Mg, Et_2O, reflux, 10 min; 2. $(EtOCO)_2$, Et_2O, 0 °C, 2 h; 3. AcOH, H_2SO_4 10%, Δ, 1 h.



(**26-30**) (**31-35**) (**7-11**)

Scheme 3 1. *N*-Acetylglycine, AcONa, Ac_2O, Δ, 1 h; 2. 3 M HCl, Δ, 3 h.

Table 2 Synthesis of 2-oxo acids—azlactone route

Ar	Yield of azlactone[a] (%)	Yield of 2-oxo acid[b] (%)
4-MeC_6H_4 (**26**)	72 (**31**)	95 (**7**)
4-$MeOC_6H_4$ (**27**)	78 (**32**)	98 (**8**)
4-$CF_3C_6H_4$ (**28**)	81 (**33**)	93 (**9**)
4-pyridyl (**29**)	68 (**34**)	94 (**10**)
2-thienyl (**30**)	79 (**35**)	92 (**11**)

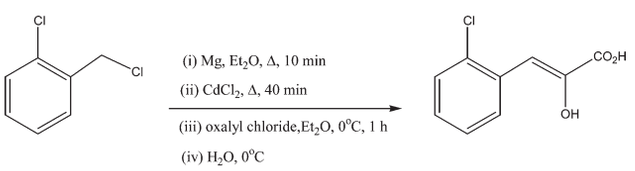
[a] Yield following precipitation from reaction mixture. [b] Yield following crystallisation from reaction mixture.

reported to provide an efficient synthesis of arylpyruvic acids containing electron-donating groups.[21] In this synthesis the starting aldehyde is condensed with *N*-acetylglycine in the presence of sodium acetate in acetic anhydride. The resulting azlactone is then hydrolysed with 3 M hydrochloric acid to yield the 2-oxo acid.

While the literature reports the condensation under reflux overnight, in this work the reaction was found to reach completion within 1 hour at reflux, monitoring by TLC. The reaction was then quenched with ice and filtration of the resulting precipitate afforded the desired azlactones as yellow solids in good yields and purity.[22]

The reaction time for the hydrolysis step was also reduced from a reported 24–48 hours to typically 3 hours again monitoring by TLC. Filtration of the resultant precipitate gave the 2-oxo acids in high yields and purity. Again ^1H and ^{13}C NMR showed that only the enol form of each compound (**7**)–(**11**)was present in CD_3OD or d_6-DMSO.

The 2-chlorophenylpyruvate derivative (**1**) was synthesized as illustrated in Scheme 4. Based on earlier work describing acylation of organocadmium reagents with acid chlorides,[23] reaction of an organocadmium reagent with oxalyl chloride followed by hydrolysis was envisaged as a short synthetic route to the 2-oxo acid. However, while this route did produce the 2-oxo acid (**1**) this synthetic method is less satisfactory than the routes described above so it was not investigated further.



Scheme 4

2-Oxocaproic acid (**13**) is commercially available and was included in this study as an acyclic derivative.

Biocatalysis

The non-natural 2-oxo acids were initially screened for activity in the reductive amination of the oxo acid with the wild type PheDH and three different mutants (N145A, N145L and N145V). The results are reported in Table 3 and Fig. 1. The activity is measured under standard conditions at 25 °C[24] by following the decrease in absorbance of NADH at 340 nm (UV spectrophotometer, Cary 50) according to the reaction in Scheme 5. For convenience each of the results in Table 3 is expressed both as a specific activity (a unit, U, of enzyme is defined as the amount of enzyme which converts one μmole of

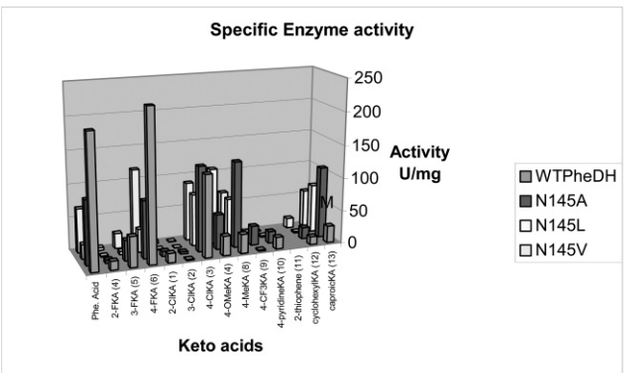


Fig. 1

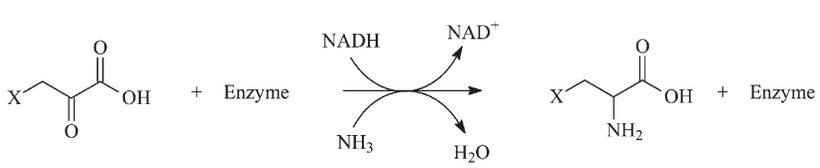
substrate per minute under standard conditions) and also normalized relative to activity with phenylpyruvate for each of the enzymes. Fig. 1 displays the specific activities only.

To test the enantioselectivity of some of these reductive aminations, a new experiment was set up for each oxo acid substituted in position 4 of the aryl ring and also for the pyridine substrate (**10**): working with an excess of NADH (1 mM) and with a suitable amount of enzyme a reaction mixture containing 0.5 mM oxo acid was taken to completion and the crude mixture was then loaded onto a chiral HPLC column. Each enzyme was tested with all the substrates. The D-amino acid was never detected, while the L-amino acid was clearly identified in all cases. Fig. 2 shows an example of the HPLC outcome, in which all the peaks are clearly separated and the L enantiomer is the only one detectable.

Discussion

The wild type enzyme is able to catalyse the reductive amination of oxo acids bearing 4-substituted aromatic groups, although it is necessary to distinguish between halogens and bulky non-polar groups: while the activity with 4-fluoro (**6**) and 4-chloro (**3**) substitution is certainly satisfactory (114 and 62.3%), it drops substantially with 4-methyl (**7**) or 4-methoxy (**8**) (14.6 and 14.9%) and it is relatively poor with 4-trifluoromethyl (**9**) (0.9%). In comparison with these results, the activities of the mutants are remarkable: while the mutants show decreased activity for phenyl pyruvate compared to the WT enzyme, the amino acid substitution in the binding pocket of these enzymes results in generally increased tolerance of substitution at the 4-position of the aromatic ring. In the case of the 4-chloro substitution one of the most striking results is that for N145L (359% as compared with 62% for the wild-type enzyme). However, in assessing this result it is important to note that this mutant shows much the poorest reference activity with phenyl pyruvate (only 22 U mg^{-1}). Thus 359% is only 79 U mg^{-1} which is in fact the lowest figure for the 4-chloro oxo acid (**3**) with the four enzymes tested. With 4-methyl (**7**) or 4-methoxy (**8**) on the other hand, the improvement in catalytic activity is absolute, in that activities are not only higher in most cases than with phenyl pyruvate, but also uniformly much higher than the activity of the wild type enzyme with the same substrates (3.9-fold for N145L with 4-methoxy and 4.6-fold for N145A with 4-methyl). In the case of the 4-trifluoromethyl (**9**) substitution the N145A mutant shows a remarkably high activity of 29.4 U mg^{-1}.

Despite the fact that these mutants were designed to discriminate between Phe and Tyr (different group in the 4-position), the effect of substitution in the 2- and 3-positions has



Scheme 5

Table 3 Activity in reductive amination of 2-oxo acids with WT PheDH and engineered mutants

Oxo acids	WTPheDH	N145A	N145L	N145V
phenylpyruvic acid	100 (200.0 U mg^{-1})	100 (97.0 U mg^{-1})	100 (22.0 U mg^{-1})	100 (67.0 U mg^{-1})
2-F-phenyl pyruvate (**4**)	6.6 (13.2 U mg^{-1})	5.6 (5.4 U mg^{-1})	7.0 (1.5 U mg^{-1})	5.9 (4 U mg^{-1})
3-F-phenyl pyruvate (**5**)	23.0 (46 U mg^{-1})	32.1 (31.1 U mg^{-1})	22.2 (4.9 U mg^{-1})	34.4 (23.0 U mg^{-1})
4-F-phenyl pyruvate (**6**)	114 (228.0 U mg^{-1})	89.3 (86.6 U mg^{-1})	160 (35.2 U mg^{-1})	175 (117.3 U mg^{-1})
2-Cl-phenyl pyruvate (**1**)	7.0 (14.0 U mg^{-1})	7.7 (7.5 U mg^{-1})	7.0 (1.5 U mg^{-1})	8.0 (5.4 U mg^{-1})
3-Cl-phenyl pyruvate (**2**)	0.8 (1.6 U mg^{-1})	3.4 (3.3 U mg^{-1})	1.2 (0.3 U mg^{-1})	1.0 (0.7 U mg^{-1})
4-Cl-phenyl pyruvate (**3**)	62.3 (124.5 U mg^{-1})	133 (129.0 U mg^{-1})	359 (79.0 U mg^{-1})	133 (88.8 U mg^{-1})
4-OMe-phenyl pyruvate (**8**)	14.9 (29.8 U mg^{-1})	54.9 (53.3 U mg^{-1})	522 (114.8 U mg^{-1})	132 (88.4 U mg^{-1})
4-Me-phenyl pyruvate (**7**)	14.6 (29.2 U mg^{-1})	135 (130.5 U mg^{-1})	300 (66.0 U mg^{-1})	102 (68.3 U mg^{-1})
4-CF_3-phenyl pyruvate (**9**)	0.9 (1.8 U mg^{-1})	30.3 (29.4 U mg^{-1})	51.5 (11.3 U mg^{-1})	4.5 (3.0 U mg^{-1})
4-pyridyl pyruvate (**10**)	9.0 (18.0 U mg^{-1})	18.2 (17.7 U mg^{-1})	10.2 (2.2 U mg^{-1})	1.0 (0.7 U mg^{-1})
2-thienyl pruvate (**11**)	Not detectable	Not detectable	Not detectable	Not detectable
cyclohexyl KA (**12**)	6.1 (12.2 U mg^{-1})	17.9 (17.4 U mg^{-1})	10.3 (2.3 U mg^{-1})	22.6 (15.1 U mg^{-1})
α-ketocaproic acid (**13**)	12.8 (25.6 U mg^{-1})	112 (108.4 U mg^{-1})	334 (73.6 U mg^{-1})	85.0 (57.0 U mg^{-1})

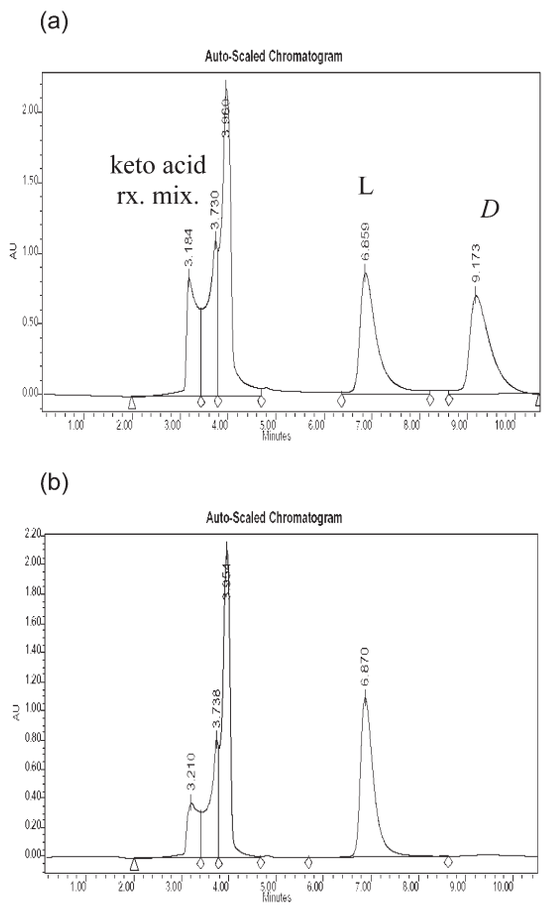


Fig. 2 HPLC separation of 4-Cl-phenyl pyruvate (**3**) + DL-4-Cl-Phe (A) and HPLC chromatogram of the reductive amination of 4-Cl-phenyl pyruvate (**3**) with the mutant N145A (B).

also been investigated for the first time: all the enzymes show the same trend when the substrates are 2-substituted, namely a decrease of about 15-fold in specific activity, regardless of the size of the halogen (F or Cl). On the other hand, 3-fluoro substitution is much better tolerated whereas 3-chloro is a considerably less acceptable substitution, suggesting a more precise spatial fit at this position and reflecting the decreased steric demand of the fluoro substituent.

Finally, a series of substrates which differ more substantially from the natural substrate, phenyl pyruvate, has been tested: heteroaromatic 2-oxo acids (pyridine (**10**) and thiophene (**11**) in place of the phenyl ring), and cyclic (cyclohexyl (**12**) in place of the phenyl ring) and linear aliphatic derivatives (C-6) (**13**).

Pyridine oxo acid (**10**) is well tolerated by N145A and the specific activity is very close to the activity shown by the wild type, while the mutant N145V shows very little activity. Quite surprisingly, each of the enzymes show activity with a cyclic aliphatic substrate indicating a good degree of freedom in the shape of the binding pocket, especially in N145A and N145V. In this work we report the activity towards one linear aliphatic oxo acid (**13**), but several others have been tested in a previous study[13b] and, although the wild type is rather tolerant, all the mutants provide increased activity, offering more versatile catalysis.

In conclusion, in this work we explored the possible application in catalysis of engineered enzymes which in several cases have markedly improved activities with novel non-natural 2-oxo acid substrates. In contrast the activity of the engineered enzymes with the natural substrate phenyl pyruvate decreases significantly. Clearly, site substitution of the asparagine residue at 145 for the less polar alanine, leucine or valine residues results in a binding site which can better accommodate substituted aromatic derivatives of phenyl pyruvate than the WT enzyme. As a general rule 5% of the wild-type activity with phenylpyruvate might be regarded as a borderline for discriminating between a good or a poor catalyst. In cases where the activity achieved at present falls well below this figure, such as 3-chlorophenylpyruvate (**2**), it seems likely that further site-directed mutagenesis may produce better biocatalysts. However, it should be borne in mind that even activities of 1–3 U mg^{-1} represent perfectly usable biocatalysts, especially in view of the possibility of 'over-production' of the biocatalyst in the bacterial host.

The potential for use of the engineered enzymes as biocatalysts for the production of non-natural amino acids is clear from this work. Significantly, we have demonstrated that there is no decrease in enantioselectivity associated with the increased activity.

Experimental

General procedures

All solvents were distilled prior to use as follows. Dichloromethane and ethyl acetate were distilled from phosphorous pentoxide. Diethyl ether was freshly distilled from sodium in the presence of benzophenone. Melting points were measured on a Thomas Hoover Capillary melting point apparatus and are uncorrected. Elemental analyses were performed by the Microanalysis Laboratory, University College Cork, using a Perkin Elmer 240 and an Exeter Analytical CE440 elemental analyser. Infrared (IR) spectra were recorded on a Perkin-Elmer FT-IR Paragon 1000. Liquid samples were examined as thin films interspersed between sodium chloride plates. Solid samples were dispersed in potassium bromide and recorded as pressed discs.

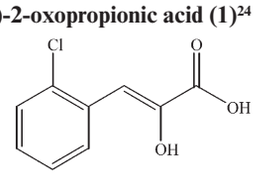
^{1}H NMR spectra were recorded at 300 MHz on a Bruker AVANCE 300 spectrometer. ^{13}C NMR spectra were recorded on a Bruker AVANCE 300 instrument at 75 MHz. Spectra were run in deuteriochloroform ($CDCl_3$) with tetramethylsilane (TMS) as internal standard unless otherwise specified. Chemical shifts (δ_H and δ_C) are expressed as parts per million (ppm), positive shifts

being downfield from TMS. Splitting patterns in ^{1}H spectra are designated as s (singlet), bs (broad singlet), bd (broad doublet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), ABq (AB quartet) and (m) multiplet. Coupling constants are quoted in Hz. For the ^{13}C NMR spectra, assignments are made from ^{13}C DEPT spectra run in the DEPT-90 and DEPT-135 modes and with the aid of COSY and HETCOR experiments in some cases. Mass spectra were measured on a Kratos Profile spectrometer in electron impact (E.I.) mode with an ionisation voltage of 70 eV.

All organic solutions were dried using magnesium sulfate unless otherwise specified. Thin layer chromatography was performed on precoated silica gel (Merck HF_{254}) plates and compounds were visualised under ultraviolet light. Column chromatography was performed using Merck silica gel 60 (typical ratio of silica : crude reaction mixture ~ 30 : 1). Bulb to bulb distillations were carried out on a Buchi GKR-50 Kugelrohr.

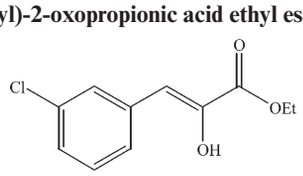
For the Grignard reactions, Et_2O was freshly distilled from $LiAlH_4$ and the molarity of the reagent was determined by titration of aliquots with 1 M HCl. For the preparation of organocadmium reagents $CdCl_2$ was stored in a high temperature oven to keep it dry.

3-(2-Chlorophenyl)-2-oxopropionic acid (1)[24]



To a suspension of Mg turnings (0.51 g, 20.9 mmol, 1.1 eq) in Et_2O (3 mL) was added 1 mL of a solution of 2-chlorobenzyl chloride (2.50 mL, 19.0 mmol, 1 eq) in Et_2O (17 mL). The reaction was initiated by heating and the rest of the halide solution was added dropwise at a rate that maintains reflux. The mixture was stirred for 10 min, cooled to RT and $CdCl_2$ (1.83 g, 10.0 mmol, 0.5 eq) added portion wise. The suspension was stirred at reflux for 40 min, cooled to RT and transferred to a dropping funnel. This was added dropwise to a solution of oxalyl chloride (4.30 mL, 38.5 mmol, 2 eq) in Et_2O (18 mL) previously cooled to 0 °C. After stirring for 1 h at 0 °C, the reaction was quenched with ice (approx. 50 mL). The aqueous layer was extracted twice with Et_2O (2 × 50 mL) and the combined organic layers were washed with brine (50 mL), dried with $MgSO_4$ and finally concentrated under reduced pressure. Precipitation of the residue (EtOAc : DCM ≈ 1 : 19) afforded the title compound as a pale yellow solid (1.20 g, 32%). ^{1}H NMR δ (CD_3OD, ppm) 6.90 (1H, s, H-3), 7.17–7.29 (2H, m, Ar–H), 7.38–7.41 (1H, dd, J = 1.4 Hz, 7.8 Hz, Ar–H), 8.33–8.37 (1H, dd, J = 1.4 Hz, 7.8 Hz, Ar–H); ^{13}C NMR δ (CD_3OD, ppm) 106.55 (CH-3), 128.52, 130.18, 131.09, 132.96 (4 × CH), 134.80, 134.98 (2 × C), 144.63 (C-2), 168.78 (C-1); ν_{max} /cm^{-1} (KBr) 3416 and 3200–2500 (OH), 1682 (C=O).

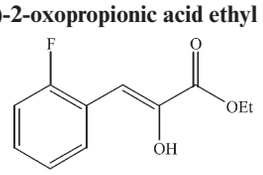
3-(3-Chlorophenyl)-2-oxopropionic acid ethyl ester (20)[25]



To a suspension of Mg turnings (1.02 g, 42.0 mmol, 1.1 eq) in Et_2O (5 mL) was added 2 mL of a solution of 3-chlorobenzyl chloride (5.00 mL, 38.0 mmol, 1 eq) in Et_2O (35 mL). The reaction was initiated by heating and the rest of the halide solution was added dropwise at a rate that maintains reflux. The mixture was stirred for 10 min, cooled to RT and then transferred into a dropping funnel. This was added dropwise to a solution of diethyl oxalate (10.00 mL, 76.0 mmol, 2 eq) in Et_2O (75 mL) previously cooled to 0 °C. After stirring for 2 h at RT, the reaction was quenched with aq. NH_4Cl (1 M, 100 mL). The aqueous layer was extracted twice with Et_2O (2 × 100 mL) and the combined organic layers were washed with brine (100 mL), dried with $MgSO_4$ and finally concentrated under reduced pressure, maintaining the temperature below 25 °C. The excess diethyl oxalate was removed with bulb to bulb distillation and the residue was purified by flash chromatography (hexane : EtOAc = 8 : 2) to yield the title compound as a colourless oil (7.75 g, 90%). ^{1}H NMR δ ($CDCl_3$, ppm) 1.35 (3H, t, J = 7.1 Hz, CH_3), 4.33 (2H, q, J = 7.1 Hz, CH_2), 6.43 (1H, s, H-3), 6.82 (1H, bs, OH), 7.12–7.98 (4H, m, Ar–H).

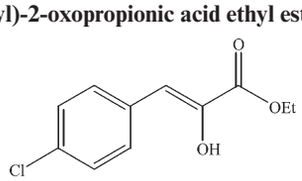
The procedure described above for (**20**) was employed for the synthesis of each of the esters (**21**)–(**25**) using the appropriate benzyl or alkyl halide in each case. Purification by flash chromatography using hexane : EtOAc (8 : 2) gave the pure esters.

3-(4-Chlorophenyl)-2-oxopropionic acid ethyl ester (21)[25]



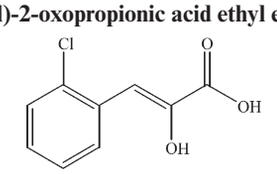
This was prepared as described for (**20**) using 4-chlorobenzyl chloride to give the title compound as a colourless oil (87%). ^{1}H NMR δ ($CDCl_3$, ppm) 1.35 (3H, t, J = 7.1 Hz, CH_3), 4.33 (2H, q, J = 7.1 Hz, CH_2), 6.43 (1H, s, H-3), 6.82 (1H, bs, OH), 7.29 (2H, J = 8.8 Hz, H_A part of ABq, Ar–H), 7.64 (2H, J = 8.8 Hz, H_B part of ABq, Ar–H).

3-(2-Fluorophenyl)-2-oxopropionic acid ethyl ester (22)



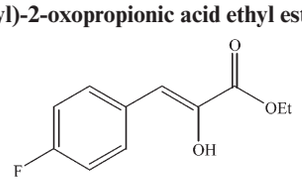
This was prepared as described for (**20**) using 2-fluorobenzyl chloride to give the title compound as a colourless oil (95%). ^{1}H NMR δ ($CDCl_3$, ppm) 1.35 (3H, t, J = 7.1 Hz, CH_3), 4.33 (2H, q, J = 7.1 Hz, CH_2), 6.66 (1H, s, H-3), 6.79 (1H, bs, OH), 6.95–7.37 (3H, m, Ar–H), 8.22–8.24 (1H, m, Ar–H).

3-(3-Fluorophenyl)-2-oxopropionic acid ethyl ester (23)[26]



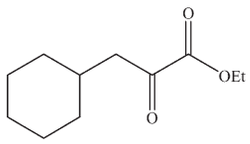
This was prepared as described for (**20**) using 3-fluorobenzyl chloride to give the title compound as a colourless oil (92%). ^{1}H NMR δ ($CDCl_3$, ppm) 1.36 (3H, t, J = 7.1 Hz, CH_3), 4.34 (2H, q, J = 7.1 Hz, CH_2), 6.48 (1H, s, H-3), 6.77 (1H, bs, OH), 6.95–6.99 (1H, m, Ar–H), 7.29–7.33 (1H, m, Ar–H), 7.42–7.44 (1H, m, Ar–H), 7.61–7.80 (1H, m, Ar–H).

3-(4-Fluorophenyl)-2-oxopropionic acid ethyl ester (24)[27]



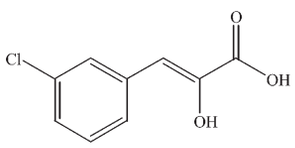
This was prepared as described for (**20**) using 4-fluorobenzyl chloride to give the title compound as a colourless oil (87%). ^{1}H NMR δ ($CDCl_3$, ppm) 1.37 (3H, t, J = 7.1 Hz, CH_3), 4.35 (2H, q, J = 7.1 Hz, CH_2), 6.48 (1H, s, H-3), 6.64 (1H, bs, OH), 7.01–7.07 (2H, m, Ar–H), 7.71–7.78 (2H, m, Ar–H).

3-Cyclohexyl-2-oxopropionic acid ethyl ester (25)[28]



This was prepared as described for (**20**) using bromomethyl cyclohexane to give the title compound as a colourless oil (81%). ^{1}H NMR δ ($CDCl_3$, ppm) 0.64–1.34 (5H, m, ring CH_2), 1.39 (3H, t, J = 7.1 Hz, CH_3), 1.66–1.72 (5H, m, ring CH_2), 1.90–1.98 (1H, m, CH), 2.70 (2H, d, J = 7.1 Hz, CH_2), 4.27 (2H, q, J = 7.1 Hz, CH_2).

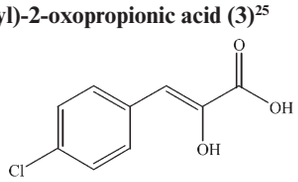
3-(3-Chlorophenyl)-2-oxopropionic acid (2)[25]



A solution of (**20**) (7.00 g, 30.1 mmol) in AcOH (20 mL) and 10% aq. H_2SO_4 (20 mL) was stirred at reflux for 1 hour, under a Dean–Stark apparatus. The mixture was cooled to RT, diluted with water (20 mL) and extracted with Et_2O (3 × 50 mL). The combined organic layers were extracted twice with aq. $NaHCO_3$ (2 × 100 mL). The combined aqueous layers were acidified to pH 1 and extracted three times with Et_2O (3 × 100 mL). The combined organic layers were washed with brine, dried with $MgSO_4$ and finally concentrated under reduced pressure, keeping carefully the temperature below 25 °C. Precipitation of the residue (EtOAc : DCM ≈ 1 : 19) afforded the title compound as a white solid (2.67 g, 45%). ^{1}H NMR δ (CD_3OD, ppm) 6.43 (1H, s, H-3), 7.19–7.32 (2H, m, Ar–H), 7.57–7.59 (1H, dd, J = 7.5 Hz, 1.2 Hz, Ar–H), 7.89 (1H, t, J = 1.8 Hz, Ar–H); ^{13}C NMR δ (CD_3OD, ppm) 110.02 (CH-3), 128.46, 129.37, 130.51, 131.07 (4 × CH), 136.28, 139.12 (2 × C), 144.78 (C-2), 162.03 (C-1); ν_{max} /cm^{-1} (KBr) 3459 and 3200–2500 (OH), 1676 (C=O).

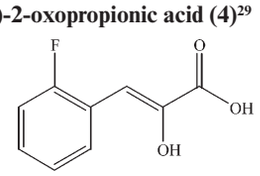
2-Oxo acids (**3**)–(**6**), (**12**) were prepared following the procedure described for (**2**). Precipitation using EtOAc : DCM 1 : 19 was used to purify the acids in each case.

3-(4-Chlorophenyl)-2-oxopropionic acid (3)[25]



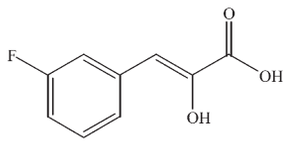
Ester (**21**) was hydrolysed to give acid (**3**) as a white solid (33%). ^{1}H NMR δ (CD_3OD, ppm) 6.44 (1H, s, H-3), 7.31 (2H, J = 8.8 Hz, H_A part of ABq, Ar–H), 7.75 (2H, J = 8.8 Hz, H_B part of ABq, Ar–H); ^{13}C NMR δ (CD_3OD, ppm) 110.68 (CH-3), 130.15, 132.85 (2 × CH), 133.17, 135.30 (2 × C), 143.98 (C-2), 168.83 (C-1); ν_{max} /cm^{-1} (KBr) 3466 and 3200–2500 (OH), 1667 (C=O).

3-(2-Fluorophenyl)-2-oxopropionic acid (4)[29]



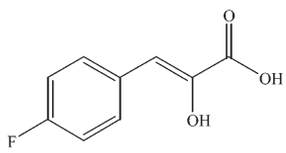
Ester (**22**) was hydrolysed to give acid (**4**) as a white solid (42%). ^{1}H NMR δ (CD_3OD, ppm) 6.68 (1H, s, H-3), 7.02–7.35 (3H, m, Ar–H), 8.28–8.32 (1H, m, Ar–H); ^{13}C NMR δ (CD_3OD, ppm) 102.20 (d, $^3J_{C,F}$ = 8.0 Hz, C-3), 116.49 (d, $^2J_{C,F}$ = 22.4 Hz, C-3′), 125.87 (d, $^3J_{C,F}$ = 3.5 Hz, C-6′), 130.65 (d, $^3J_{C,F}$ = 8.0 Hz, C-4′), 132.75 (C-5′), 146.59 (C-2), 166.17 (d, $^1J_{C,F}$ = 283.0 Hz, C-2′), 168.64 (C-1); ν_{max} /cm^{-1} (KBr) 3478 and 3200–2500 (OH), 1694 (C=O).

3-(3-Fluorophenyl)-2-oxopropionic acid (5)[29]



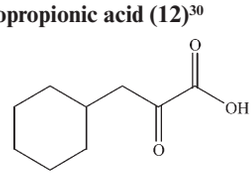
Ester (**23**) was hydrolysed to give acid (**5**) as a white solid (27%). ^{1}H NMR δ (CD_3OD, ppm) 6.46 (1H, s, H-3), 6.94–7.04 (1H, m, Ar–H), 7.27–7.35 (2H, m, Ar–H), 7.43 (1H, d, J = 7.8 Hz, Ar–H); ^{13}C NMR δ (CD_3OD, ppm) 110.36 (CH-3), 115.26 (d, $^2J_{C,F}$ = 21.8 Hz, C-4′), 117.09 (d, $^2J_{C,F}$ = 23.0 Hz, C-2′), 127.02 (C-6′), 131.15 (d, $^3J_{C,F}$ = 8.4 Hz, C-5′), 139.43 (d, $^3J_{C,F}$ = 8.4 Hz, C-1′), 144.78 (C-2), 167.56 (C-1), C–F not detected; ν_{max} /cm^{-1} (KBr) 3477 and 3200–2500 (OH), 1698 (C=O).

3-(4-Fluorophenyl)-2-oxopropionic acid (6)[29]



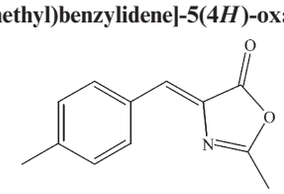
Ester (**24**) was hydrolysed to give acid (**6**) as a white solid (39%). ^{1}H NMR δ (CD_3OD, ppm) 6.47 (1H, s, H-3), 6.98–7.08 (2H, m, Ar–H), 7.76–7.82 (2H, m, Ar–H); ^{13}C NMR δ (CD_3OD, ppm) 111.07 (CH-3), 116.78 (d, $^2J_{C,F}$ = 21.8 Hz, 2 × C-3′), 133.39 (d, $^3J_{C,F}$ = 8.0 Hz, 2 × C-2′), 132.90 (C-1′), 142.88 (C-2), 162.52 (d, $^1J_{C,F}$ = 245.5 Hz, C-4′), 169.02 (C-1); ν_{max} /cm^{-1} (KBr) 3476 and 3200–2500 (OH), 1699 (C=O).

3-Cyclohexyl-2-oxopropionic acid (12)[30]



Ester (**25**) was hydrolysed to give acid (**12**) as a white solid (33%). ^{1}H NMR δ ($CDCl_3$, ppm) 0.88–1.31 (5H, m, ring CH_2), 1.66–1.72 (5H, m, ring CH_2), 1.90–1.99 (1H, m, CH), 2.79 (2H, d, J = 7.1 Hz, CH_2), 8.69 (1H, bs, OH); ^{13}C NMR δ (CD_3OD, ppm) 26.30, 26.36, 33.82 (3 × CH_2), 33.96 (CH), 45.49 (CH_2), 161.40 (C-1), 195.80 (C-2); ν_{max} /cm^{-1} (KBr) 3428 and 3200–2500 (OH), 1694 (C=O).

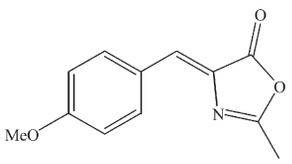
2-Methyl-4-[4-(methyl)benzylidene]-5(4*H*)-oxazolone (31)[31]



A mixture of *p*-tolualdehyde (5.00 mL, 42.4 mmol, 1 eq), *N*-acetyl-glycine (6.45 g, 55.1 mmol, 1.3 eq) and sodium acetate (4.50 g, 55.1 mmol, 1.3 eq) in acetic anhydride (19.00 mL, 0.2 mol, 5 eq) was stirred at reflux for 1 h. The reaction was quenched with ice (approx. 50 mL) and vigorously stirred for 1 h in an ice bath to allow precipitation. Filtration afforded the title compound as a yellow solid (6.14 g, 72%). ^{1}H NMR δ (CD_3OD, ppm) 1.90 (3H, s, CH_3), 2.23 (3H, s, CH_3–Ph), 7.23 (2H, H_A part of ABq, J = 8.8 Hz, Ar–H), 7.48 (1H, s, H-3), 7.49 (2H, H_B part of ABq, J = 8.8 Hz, Ar–H); ^{13}C NMR δ (CD_3OD, ppm) 21.84 (CH_3), 22.97 (CH_3–Ph), 126.51 (C), 130.77, 131.41 (2 × CH), 132.57 (C), 136.25 (CH-3), 141.65 (C), 168.81 (C=N), 173.60 (C=O); ν_{max} /cm^{-1} (KBr) 1794, 1773, 1667.

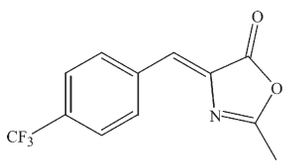
The procedure described above for (**31**) was employed for the synthesis of each of the azlactones (**32**)–(**35**) using the appropriate benzaldehyde in each case. Filtration afforded the pure azlactones in each case.

2-Methyl-4-[4-(methoxy)benzylidene]-5(4*H*)-oxazolone (32)[31]



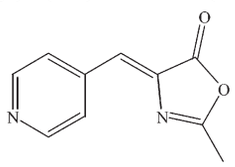
This was prepared as described for (**31**) using 4-methoxybenzaldehyde to give the title compound as a yellow solid (78%). ^{1}H NMR δ ($CDCl_3$, ppm) 2.39 (3H, s, CH_3), 3.87 (3H, s, OCH_3), 6.96 (2H, H_A part of ABq, J = 7.1 Hz, Ar–H), 7.02 (1H, s, H-3), 7.49 (2H, H_B part of ABq, J = 7.1 Hz, Ar–H); ^{13}C NMR δ ($CDCl_3$, ppm) 16.03 (CH_3), 55.84 (OCH_3), 114.84 (2 × CH), 126.53 (C), 130.78 (2 × CH), 131.91 (CH-3), 134.65 (C), 162.45 (C), 165.28 (C=N), 168.59 (C=O); ν_{max} /cm^{-1} (KBr) 1798, 1775, 1665.

2-Methyl-4-[4-(trifluoromethyl)benzylidene]-5(4*H*)-oxazolone (33)[31]



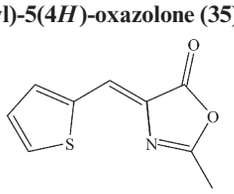
This was prepared as described for (**31**) using 4-trifluoromethyl-benzaldehyde to give the title compound as a yellow solid (81%). ^{1}H NMR δ ($CDCl_3$, ppm) 2.43 (3H, s, CH_3), 7.13 (1H, s, H-3), 7.81 (2H, H_A part of ABq, J = 7.1 Hz, Ar–H), 8.06 (2H, H_B part of ABq, J = 7.1 Hz, Ar–H); ν_{max} /cm^{-1} (KBr) 1797, 1775, 1663.

2-Methyl-4-(4-pyridyl)-5(4*H*)-oxazolone (34)[32]



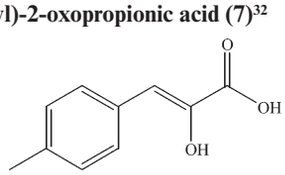
This was prepared as described for (**31**) using 4-pyridinecarboxaldehyde to give the title compound as a grey solid (68%). ^{1}H NMR δ ($CDCl_3$, ppm) 2.45 (3H, s, CH_3), 7.03 (1H, s, H-3), 7.98 (2H, H_A part of ABq, J = 6.2 Hz, ArH), 8.80 (2H, H_B part of ABq, J = 6.2 Hz, ArH); ν_{max} /cm^{-1} (KBr) 1793, 1771, 1662.

2-Methyl-4-(2-thienyl)-5(4*H*)-oxazolone (35)[33]



This was prepared as described for (**31**) using 2-thiophenecarboxaldehyde to give the title compound as a yellow solid (79%). ^{1}H NMR δ ($CDCl_3$, ppm) 2.41 (3H, s, CH_3), 7.12–7.15 (1H, m, Ar–H), 7.38 (1H, s, H-3), 7.56 (1H, d, J = 3.6 Hz, Ar–H), 7.68 (1H, d, J = 5.1 Hz, Ar–H); ν_{max} /cm^{-1} (KBr) 1793, 1779, 1665.

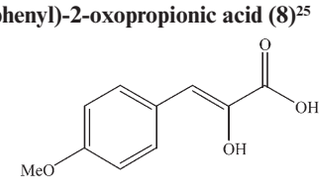
3-(4-Methylphenyl)-2-oxopropionic acid (7)[32]



A suspension of (**31**) (2.60 g, 12.9 mmol) in aq. HCl (3 M, 10 mL) was stirred at reflux for 3 h. The mixture was cooled to RT to allow crystallisation. Filtration afforded the title compound as an orange solid (2.19 g, 95%). ^{1}H NMR δ (CD_3OD, ppm) 2.34 (3H, s, CH_3), 6.47 (1H, s, H-3), 7.14 (2H, H_A part of ABq, J = 8.1 Hz, ArH), 7.65 (2H, H_B part of ABq, J = 8.1 Hz, ArH); ^{13}C NMR δ (CD_3OD, ppm) 21.76 (CH_3), 112.16 (CH-3), 130.32, 131.17 (2 × CH), 133.96 (C), 138.86 (C), 141.98 (C-2), 168.90 (C-1); ν_{max} /cm^{-1} (KBr) 3478 and 3200–2500 (OH), 1669 (C=O).

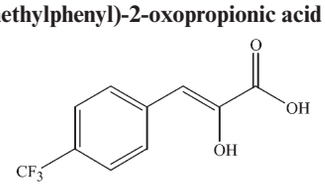
2-Oxo acids (**8**)–(**11**) were prepared following the procedure described for (**7**). Filtration gave the acids in each case.

3-(4-Methoxyphenyl)-2-oxopropionic acid (8)[25]



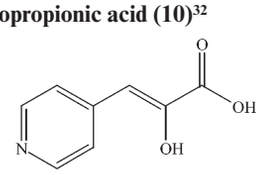
Azlactone (**32**) was hydrolysed to give acid (**8**) as an orange solid (98%). ^{1}H NMR δ (CD_3OD, ppm) 3.81 (3H, s, OCH_3), 6.48 (1H, s, H-3), 6.96 (2H, H_A part of ABq, J = 7.1 Hz, ArH), 7.49 (2H, H_B part of ABq, J = 7.1 Hz, ArH); ^{13}C NMR δ (CD_3OD, ppm) 56.05 (CH_3), 112.15 (CH-3), 115.10 (2 × CH), 129.47 (C), 132.65 (2 × CH), 140.98 (C-2), 160.91 (C), 169.00 (C-1); ν_{max} /cm^{-1} (KBr) 3458 and 3200–2500 (OH), 1662 (C=O).

3-(4-Trifluoromethylphenyl)-2-oxopropionic acid (9)[34]



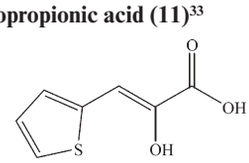
Azlactone (**33**) was hydrolysed to give acid (**9**) as an orange solid (93%). ^{1}H NMR δ (d_6-DMSO, ppm) 6.74 (1H, s, H-3), 7.69 (2H, H_A part of ABq, J = 8.1 Hz, ArH), 7.95 (2H, H_B part of ABq, J = 8.1 Hz, ArH), 9.80 (1H, bs, OH); ν_{max} /cm^{-1} (KBr) 3461 and 3200–2500 (OH), 1667 (C=O).

3-(4-Pyridyl)-2-oxopropionic acid (10)[32]



Azlactone (**34**) was hydrolysed to give acid (**10**) as a red solid (94%). ^{1}H NMR δ (d_6-DMSO, ppm) 6.57 (1H, s, H-3), 8.24 (2H, H_A part of ABq, J = 6.7 Hz, ArH), 8.75 (2H, H_B part of ABq, J = 6.7 Hz, ArH); ^{13}C NMR δ (d_6-DMSO, ppm) 103.71, 125.33, 141.03 (3 × CH), 152.08 (C), 152.36(C-2) 164.93 (C-1); ν_{max} /cm^{-1} (KBr) 3449 and 3200–2500 (OH), 1660 (C=O).

3-(2-Thienyl)-2-oxopropionic acid (11)[33]



Azlactone (**35**) was hydrolysed to give acid (**11**) as a grey-green solid (92%). ^{1}H NMR δ (d_6-DMSO, ppm) 6.74 (1H, s, H-3), 6.99–7.02 (1H, m, ArH), 7.22 (1H, d, J = 3.5 Hz, ArH). 7.50 (1H, d, J = 5.7 Hz, ArH), 9.49 (1H, bs, OH); ^{13}C NMR δ (d_6-DMSO, ppm) 105.93, 127.10, 128.18, 128.27 (4 × CH), 137.80 (C), 140.00 (C-2) 166.03 (C-1); ν_{max} /cm^{-1} (KBr) 3458 and 3200–2500 (OH), 1664 (C=O).

Biochemistry

General details. All the reagents were reagent-grade and used without further purification. HPLC grade solvents were used

Table 4 Retention times (min) of the amino acids on a chirobiotic T ($MeOH/H_2O$ 70/30, flow rate of 1.0 mL min^{-1})

	4-F-phe (**6**)	4-Cl-phe (**3**)	4-Me-phe (**7**)	4-MeO-phe (**8**)	4-CF_3-phe (**9**)	Pyridine (**10**)
L-enantiomer	6.2	6.9	6.9	7.2	4.8	11.3
D-enantiomer	7.9	9.2	9.0	10.0	n.a.	n.a

for HPLC chromatographic separations. The NADH grade II was purchased from Roche. The wild type PheDH and the mutants were over-expressed in *E. coli* TG1 cells and purified as described elsewhere.[13a] The enzymes were stored as precipitates in 60% ammonium sulfate at 4 °C and desalted before use through extensive dialysis against Tris buffer pH 8.0, 50 mM.

Evaluation of enzyme activity—UV assays

Each oxo acid (4 mM) was dissolved to form a component of a reaction mixture containing NH_4Cl (400 mM), KCl (100 mM), 0.1 mM NADH and Tris (50 mM). The pH was adjusted to 8.0 by adding a suitable amount of HCl. 1 mL of reaction mixture was incubated at 25 °C. The reaction was followed at 340 nm over 1 minute after adding an appropriate amount of enzyme to achieve an optimally measurable reaction rate (between 0.01–0.03 min^{-1}). Each of the reactions was carried out in duplicate and the average value is reported.

Determination of enantioselectivity—chiral HPLC conditions

Each oxo acid (~0.5 mM) was dissolved in a solution containing NH_4Cl (400 mM), KCl (100 mM), 1 mM NADH and Tris (50 mM). The pH was adjusted to 8.0 by adding a suitable amount of HCl. The solution was filtered through a sterile filter Acrodisc® 0.45 μm. 1 mL of reaction mixture was incubated at 25 °C and an appropriate amount of enzyme was added to allow approximate completion of the reaction within about 40 min. The formation of the corresponding amino acid was followed by loading 20 μL of the mixture onto a CHIROBIOTIC T, chiral HPLC column (Table 4). The elution mixture was $MeOH/H_2O$ 70/30 at a flow rate of 1.0 mL min^{-1}.

Acknowledgements

Patricia Busca and Francesca Paradisi were supported by Conway Post-doctoral Fellowships under the (Irish) Higher Education Authority's Programme for Research in Third-level Institutions. Eamonn Moynihan and Francesca Paradisi are employed under Enterprise Ireland's Advanced Technology Research Programme. Support from both these funding sources is gratefully acknowledged.

References

1 (*a*) R. M. Williams, J. E. Baldwin and P. D. Magnus, *Synthesis of Optically Active Amino Acids, Vol 7 of Organic Chemistry Series*; Pergamon Press, Oxford, 1989; (*b*) R. O. Duthaler, *Tetrahedron*, 1994, **50**, 1539–1650.
2 A. S. Bommarius, M. Schwarm, K. Stingl, M. Kottenhahn, K. Huthmacher and K. Drauz, *Tetrahedron Asymmetry*, 1995, **6**, 2851–2888.
3 A. Boto, R. Hernandez, A. Montoya and E. Suarez, *Tetrahedron Lett.*, 2002, **43**, 8269–8272.
4 P. P. Taylor, D. P. Pantaleone, R. F. Senkpeil and I. G. Fotheringham, *Trends Biotechnol.*, 1998, **16**, 412–418.
5 (*a*) K. Faber, *Biotransformations in Organic Chemistry—A text book*, 4th edn., Springer-Verlag, Berlin, 2000; (*b*) R. N. Patel, *Stereoselective Biocatalysis*, Marcel Dekker, New York, 2000.
6 (*a*) H. G. Davies, R. H. Green, D. R. Kelly and S. M. Roberts, *Crit. Rev. Biotechnol.*, 1990, **10**, 129–153; (*b*) E. Santaniello, P. Ferraboschi, P. Grisenti and A. Manzocchi, *Chem. Rev.*, 1992, **92**, 1071–1140.
7 (*a*) A. L. Margolin, *Enzyme Microbial Technol.*, 1993, **15**, 266–280; (*b*) K. Mori, *Synlett*, 1995, **11**, 1097–1109; (*c*) R. N. Patel, *Adv. Appl. Microbiol.*, 1997, **43**, 91–140; (*d*) R. N. Patel, *Adv. Appl. Microbiol.*, 2000, **47**, 33–78.
8 O. P. Ward and A. Singh, *Curr. Opin. Biotechnol.*, 2000, **11**(6), 520–526.
9 H. M. Wilks, K. W. Hart, R. Feeney, C. R. Dunn, H. Muirhead, W. N. Chia, D. A. Barstow, T. Atkinson, A. R. Clarke and J. J. Holbrook, *Science*, 1988, **242**, 1541–1544.
10 W. Hummel and M. R. Kula, *Eur. J. Biochem*, 1989, **184**, 1.
11 Y. Asano, A. Yamada, Y. Kato, K. Yamaguchi, Y. Hibino, K. Hirai and K. Kondo, *J. Org. Chem.*, 1990, **55**, 5567–5571.
12 C. W. Bradshaw, C.-H. Wong, W. Hummel and M.-R. Kula, *Bioorg. Chem.*, 1991, **19**, 29.
13 (*a*) S. Y. Seah, K. L. Britton, P. J. Baker, D. W. Rice, Y. Asano and P. C. Engel, *FEBS Lett.*, 1995, **370**, 93–96; (*b*) S. Y. Seah, K. L. Britton, D. W. Rice, Y. Asano and P. C. Engel, *Biochemistry*, 2002, **41**, 11390–11397.
14 K. L. Britton, P. J. Baker, P. C. Engel, D. W. Rice and T. J. Stillman, *J. Mol. Biol.*, 1993, **234**, 938–945.
15 (*a*) P. J. Baker, K. L. Britton, P. C. Engel, G. W. Farrants, K. S. Lilley, D. W. Rice and T. J. Stillman, *Proteins*, 1992, **12**, 75–86; (*b*) T. J. Stillman, P. J. Baker, K. L. Britton, D. W. Rice and H. F. Rodgers, *J. Mol. Biol.*, 1992, **224**, 1181–1184.
16 J. L. Vanhooke, J. B. Thoden, N. M. Brunhuber, J. S. Blanchard and H. M. Holden, *Biochemistry*, 1999, **38**, 2326–2339.
17 A. J. L. Cooper, L. Z. Ginos and A. Meister, *Chem. Rev.*, 1983, **83**, 321–358.
18 (*a*) L. M. Weinstock, R. B. Currie and A. Lovell, *Synth. Commun.*, 1981, **11**(12), 943–946; (*b*) W. J. Middleton and E. M. Bingham, *J. Org. Chem.*, 1980, **45**, 2883–2887.
19 K. V. Baker, J. M. Brown, N. Hughes, A. J. Skarnulis and A. Sexton, *J. Org. Chem.*, 1991, **45**, 698–703.
20 T. Takai, H. Senda and H.-H. Lee, *Spectrosc. Lett.*, 1998, **31**(2), 379–395.
21 H. N. C. Wong, Z. L. Xu, H. M. Chang and C. M. Lee, *Synthesis*, 1992, 793–797.
22 V. Dala, P. Cotelle and J.-P. Catteau, *Tetrahedron Lett.*, 1997, **38**(9), 1577–1580.
23 (*a*) J. Cason, *Chem. Rev.*, 1947, **40**, 15–32; (*b*) D. A. Shirley, *Org. React.*, 1954, **8**, 28–58.
24 H. Hongwen and F. Xianqi, *Gaodeng Xuexiao Huaxue Xuebao*, 1988, **9**, 966–968.
25 H. Yinglin and H. Hongwen, *Gaodeng Xuexiao Huaxue Xuebao*, 1991, **12**(5), 617–620.
26 K. Hirai, E. Ejiri and S. Ooishi, *Jpn. Patent* no. JP 06157488, 1994.
27 H. F. Schuster and G. M. Coppola, *J. Heterocycl. Chem.*, 1994, **31**(6), 1381–1384.
28 S. Brandange, J. Staffan and S. Vallen, *Acta Chem. Scand.*, 1973, **27**(10), 3668–3672.
29 M. Tanaka and K. Otsuka, *Jpn. Patent* no. JP63048244, 1988.
30 D. H. R. Barton, C.-Y. Chern and J. Cs. Jaszberenyi, *Tetrahedron*, 1995, **51**(7), 1867–1886.
31 H. Hoshina, T. Hitoshi, K. Kubo, T. Igarashi and T. Sakurai, *Heterocycles*, 2000, **53**(10), 2661–2274.
32 K. Yamabe, A. Sawada and K. Miyafuchi, *Sasebo Kogyo Koto Senmon gakko Kenkyu Hokoku*, 1988, **25**, 119–122.
33 J. Meiwes, M. Schudok and G. Kretzschmar, *Tetrahedron Asymmetry*, 1997, **8**(4), 527–536.
34 Y. Ueno, K. Morishita, M. Muraoka and N. Ohashi, *Jpn. Patent* no. JP10265464 1998.