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The enzymatic resolution of an α-fluoroamide by an acylase

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Dedicated to Professor Paul Tarrant on the ocassion of his 85th birthday

Abstract

The acylase from *Aspergillus melleus* was able to hydrolyse the amide bond of (*S*)-phenylalanine-*N*-2-(*R*,*S*)-fluoropropionamide and discriminate the diastereoisomers such that the (*S*,*S*)-diastereoisomer was hydrolysed by an order of magnitude faster than the (*S*,*R*)-diasteroisomer. The origin of the kinetic discrimination is attributed to both binding and kinetic effects.

Keywords: ^{19}F-NMR; Acylase; Aspergillus melleus, α-fluoroamide; Enzyme resolution

It is part of the culture of bio-organic chemistry that the fluorine atom is considered to have a steric influence just slightly greater than that of a hydrogen atom [1]. However, electronically fluorine and hydrogen are clearly very different. It is also part of the culture of bio-organic chemistry that lipase and acylase enzymes distinguish substrates through binding interactions which are largely steric in nature. Steric models for the common hydrolytic enzymes have been developed where 'small', 'medium' and 'large' pockets have been identified which define the chirality of the preferred enantiomer of a given substrate enantiomer [2,3]. In general, these models have served well as predictive tools. On the other hand, if an α-fluoroester or α-fluoroamide is presented as a substrate to a hydrolytic enzyme, then the compound is chiral by virtue of the substitution of a hydrogen by a fluorine. It is now not so obvious, on the basis of established steric models, to predict the outcome of such a kinetic resolution, as the fluorine and hydrogen are both 'small' and a distinction based on steric considerations cannot be made, which predicts how these groups will best fit the enzyme surface.

Examples of successful kinetic resolutions with α-fluoroesters are rare [4,5], however, the following one [4] serves to illustrate that certain enzymes can distinguish fluorine and hydrogen at the stereogenic centre.

Lipase P30 was able to selectively hydrolyse the (*S*)-enantiomer of ethyl 2-fluorohexanoate over the (*R*)-enantiomer and on work up the (*R*)-ester was isolated in 99% ee after 60% conversion, as illustrated in Fig. 1. Such a discrimination cannot easily be accounted for on the basis of a steric difference between hydrogen and fluorine, and we have previously suggested that a stereo-electronic effect is responsible for this discrimination [1,6]. The enzyme attacks the ester, via a serine alkoxide nucleophile and becomes acylated. The preferred transition state has the serine alkoxide attacking the carbonyl *anti* to the vicinal fluorine atom. The overlap of the C–F σ* orbital with the carbonyl LUMO stabilises this molecular orbital in transition state A relative to transition state B, and the electron density of the serine nucleophile can donate into the C–F σ* orbital only in transition state A, lowering the energy of *anti* approach to the fluorine atom. Also, the electrostatic repulsion between F and O^- is minimised in transition state A. Calculations suggest up to a 2.5 kcal mol^{-1} difference in the energy of these transition states, and there is a sufficient energy difference to account for the observed kinetic resolution.

In this paper, we report the first study on the enzymatic resolution of an α-fluoroamide with an acylase. We were led to this investigation after a recent evaluation of the preferred conformation of *N*-alkyl α-fluoropropionamides [7]. A number of years ago, both electron [8] and neutron diffraction [9] analyses have demonstrated that the major conformer of monofluoroacetamide ($FCH_2C(O)NH_2$) has the C–F bond *anti* to the C=O bond and *syn* to the N–H bond of the amide in the solid state as illustrated in Fig. 2. The energy difference between this and the next favoured *syn* conformation was calculated to be 7.5 kcal mol^{-1}. In our recent

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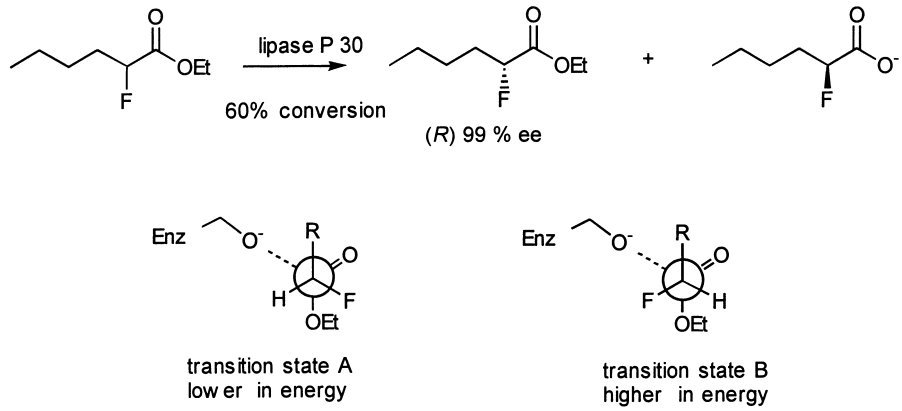


Fig. 1. Rationale for the stereoselectivity observed during the lipase mediated hydrolysis of a racemic α-fluoroester.

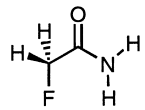


Fig. 2. Conformation of fluoroacetamide.

study [7], we have investigated the preferred conformation of *N*-alkyl α-fluoropropionamides by both X-ray crystallography and *ab initio* calculations. It is found that the *trans* conformation is again favoured in the solid state for these amides where the C–F bond lies *anti* to the C=O bond as shown in Fig. 3. Calculations revealed that this was the only minimum on the rotational energy profile (around the FC–C(O) bond) and that the barrier to rotation was large at 8.0 kcal mol^{-1}. The fluorine atom and not the methyl group dictates the conformation of these amides.

It follows that with such a conformational preference, where the C–F and N–H bonds are *syn* and co-planar, enantiomers of α-fluoropropionamides will necessarily have to locate their methyl groups in opposite spacial locations around the stereogenic centre (Fig. 3). This is anticipated to impose significantly different diastereomeric interactions during the binding of such enantiomers to an enzyme. With this in mind, we have synthesised (*S*)-phenylalanine *N*-(*R*,*S*)-2-fluoropropionamide as a substrate for acylase hydrolysis as shown in Scheme 1.

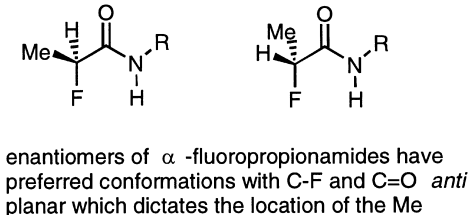
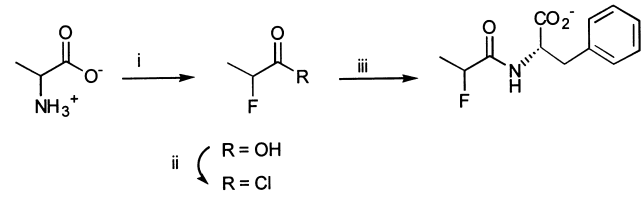


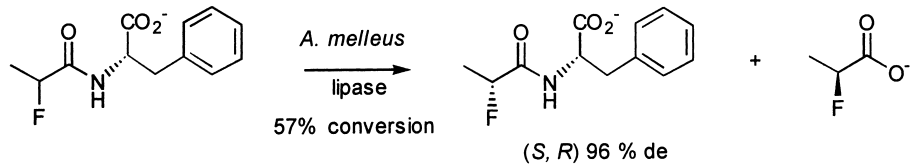
Fig. 3. Conformation of α-fluoropropionamides.

This substrate was chosen as the diastereoisomers are distinguised only by the fluorine atom at the stereogenic centre. Incubation of this substrate with the acylase from *Aspergillus melleus* resulted in a slow hydrolysis. The relative rates of hydrolysis of the two diastereoisomers could be followed analytically, in a very straightforward manner by ^{19}F-NMR, as each diastereoisomer had a unique and resolvable ^{19}F-NMR resonance ((*S*,*S*) −182.1 ppm and (*S*,*R*) −181.67 ppm). The enzymatic reaction was monitored through time in an NMR tube, and the rate slowed down considerably as the reaction approached 50% conversion. The reaction was stopped at 57% conversion where the ratio of isomers was 98 : 2, representing a diastereomeric excess of the residual substrate of 96% de. The major diastereoisomer was (*S*,*R*), the diastereoisomer which is hydrolysed more slowly by the enzyme (Scheme 2).

In order to gain some insight into the origin of the observed selectively, Km and V_{max} data for each diastereoisomer were obtained in separate experiments. This required the preparation of each diastereomer in an enantiomerically enriched form. This was achieved by preparing (*S*)- and (*R*)-2-fluoropropionic acids directly from (*S*)- and (*R*)-alanines by diazotisation and *in situ* treatment with HF-pyridine [10]. The diazotisation reaction is reported to proceed with predominant retention of configuration [11,12]. In the event, it emerged that the product 2-fluoropropionic acids were generated in an enantiomeric excess of 80% ee from the enantiomerically pure alanines. This enantioselectivity was established by recording ^{19}F-NMR of (*S*)-2-(diphenylmethyl)pyrrolidine salts of the resultant



Scheme 1. (i) $NaNO_2$, HF pyridine, 14%; (ii) $SOCl_2$, 83%; (iii) Aq. NaOH, 16%.



Scheme 2

2-fluoropropionic acids in $CDCl_3$ [13]. The enantiomerically enriched 2-fluoropropionic acids were converted to their acid chlorides after treatment with thionyl chloride and were then coupled directly to (*S*)-phenylalanine. This generated diastereomerically enriched compounds of 80% de, a value which was readily determined by integration of the two fluorine signals in the ^{19}F-NMR spectrum, associated with each diastereoisomer. In the case of the (*S*,*R*) stereoisomer, the material was stereochemically purified by an initial short bio-transformation with the *A. melleus* acylase, to hydrolyse the 10% (*S*,*S*) isomer and the diastereomerically pure (*S*,*R*) substrate was recovered as its sodium salt. The (*S*,*R*) stereoisomer was enantiomerically pure within the limits of ^{19}F-NMR detection. For the (*S*,*R*) stereoisomer, improvement of the 80% de was not so readily achievable and this material was used directly for the kinetic study (Table 1).

Initial reaction rates (v) at different substrate concentrations [S] were determined by ^{19}F-NMR analysis, and then plotted as Lineweaver–Burke plots ($1/v$ versus 1/[S]) to determine V_{max} and Km for each substrate [14]. It emerged that the (*S*,*S*) substrate has a Km of 16.26 mM and a V_{max} of 0.92 mM min^{-1}, whereas the (*S*,*R*) substrate had a Km of 40.34 mM and a V_{max} of 0.20 mM min^{-1}. Km is a measure of substrate binding and V_{max} a measure of the rate of product formation (enzyme-substrate complex breakdown). The overall efficiency of the enzyme for each substrate can be estimated by comparing V/K values for each substrate. These values emerge as $V/K = 0.0566$ for the (*S*,*S*) substrate, and $V/K = 0.005$ for the (*S*,*R*) substrate. The study predicts one order of magnitude difference in the rates of hydrolysis in favour of the (*S*,*S*) substrate and the experimental value of 96% de for the residual (*S*,*R*) diastereoisomer in the previous study is consistent with this analysis. The Km values reveal that there is a differential binding affinity of the substrates by the *A. melleus* lipase. Also the V_{max} values indicate a differential rate of product formation, which is perhaps related to the relative rates of enzyme acylation/deacylation.

Table 1
Kinetic parameters for the hydrolysis of (*S*)-phenylalanine-*N*-2-fluoropropionamides with *Aspergillus melleus*. The data are an average of two separate studies in each case

Substrate diastereomer	Km (mM)	V_{max} (mM min^{-1})	V/K
(*S*,*S*)[a]	16.26 ± 1.84	0.92 ± 0.07	0.0566
(*S*,*R*)	40.34 ± 3.21	0.20 ± 0.02	0.0050

[a] 80% de.

In summary, the *A. melleus* lipase can mediate an efficient kinetic resolution between diastereoisomers of (*S*)-phenylalanine-*N*-(*R*,*S*)-2-fluoropropionamide. Although, acylases have hardly been explored in such reactions, this initial study suggests that they offer an excellent method for preparing enantiomerically enriched 2-fluorocarboxylic acids and amides. The preferred conformation of such amides, where the C–F and C=O bonds orientate *anti* to each other, predicts a discernable binding difference between the enzyme and each diastereomeric substrate. Although, the data reveal that both substrates have Km values in the high mM range, and are poor binders, a two fold difference in binding affinity emerges. Perhaps less obviously, the V_{max} data revealed a four fold difference between the diastereoisomers, and reflects the difference in the rate of product formation (or enzyme-substrate breakdown). Perhaps this latter difference lies in off-loading the 2-fluoroacyl ester-enzyme complex by nucleophilic attack by water, a process which should be susceptible to the stereoelectronic effect discussed above for the lipase hydrolysis of α-fluoroesters [1,6].

The overall kinetic profile of such enzyme resolutions are complex and difficult to deconstruct as there are various factors operating at different stages along the enzyme reaction course, however, it emerges from this study that conformational and stereoelectronic effects can combine to result in highly stereoselective enzymatic resolutions with mono-fluorinated ester and amide substrates.

1. Experimental

FTIR spectra were recorded on a Nicolet Magna IR 550 Spectrometer. NMR spectra were obtained on a Jeol EX 270 MHz spectrometer in $CDCl_3$ or D_2O. Chemical shifts are quoted relative to TMS for ^{1}H- and ^{13}C-NMR spectra and ^{19}F-NMR chemical shifts are quoted as negative relative to fluorotrichloromethane. Solvents were dried and distilled prior to use. Reactions requiring anhydrous conditions were carried out under an atmosphere of nitrogen.

1.1. Preparation of (R,S)-2-fluoropropanoic acid

(*R*,*S*)-Alanine, (8.6 g, 96 mmol) was added to a stirred solution of pyridinium hydrogen fluoride (100 ml, 70% w/w) in a Teflon bottle. The solution was cooled to 0°C and

sodium nitrite (22.6 g, 232 mmol) was added in five equal portions over 1 h. The reaction mixture was stirred at 0°C for 1 h and was then warmed to room temperature and then stirred overnight. The reaction was quenched by addition to ice water (100 ml). The aqueous solution was extracted into diethylether (3 × 100 ml), washed with sodium chloride solution (3 × 100 ml, 5% w/v) and dried over anhydrous sodium sulphate. The organics were filtered and the solvent was evaporated. The residue was distilled (50°C, 23 mbar) to afford the product (1.4 g, 14%) as a colourless oil. δ_H(270 MHz, $CDCl_3$) 5.12 (1H, dq, *J* 48.5, 6.9, CFH), 1.61 (3H, dd, *J* 23.5, 6.9, Me); δ_C(67.9 MHz, $CDCl_3$) 85.1 (d, *J* 182.7), 18.2 (d, *J* 22.3); δ_F(254.2 MHz, $CDCl_3$) −184.76 (dq, *J* 48.3, 23.5, CFH).

1.2. Preparation of (R,S)-2-fluoropropanoyl chloride

Thionyl chloride, (2.8 g, 23.5 mmol) was added dropwise to heat (*R*,*S*)-2-fluoropropanoic acid, (1.00 g, 10.9 mmol) at room temperature. The reaction was heated to reflux for 60 min and a sample removed for ^{1}H- and ^{19}F-NMR analysis to confirm the absence of starting material. The product was distilled (bp. 56°C) directly from the reaction mixture at atmospheric pressure to give the product as a colourless oil (1.0 g, 83%). δ_H(270 MHz, $CDCl_3$) 5.14 (1H, dq, *J* 48.6, 7.0, CFH), 1.66 (3H, dd, *J* 22.8, 6.8, Me); δ_C(67.9 MHz, $CDCl_3$) 172.5 (d, *J* 27.9), 90.3 (d, *J* 194.5), 17.6 (d, *J* 21.9); δ_F(254.2 MHz, $CDCl_3$) −171.4 (dq, *J* 49.1, 23.0, CFH).

1.3. Preparation of (S)-phenylalanine-N-2-(R,S)-fluoropropionamide

(*S*)-Phenylalanine (2.63 g, 16 mmol) was dissolved in 4 M NaOH (12 ml, 48 mmol). The reaction was cooled to 0°C and (*R*,*S*)-2-fluoropropanoyl chloride (1.6 g, 14 mmol) was added dropwise. The reaction was warmed to room temperature and stirred for 30 min, acidified to pH = 3 with a HCl solution and the product was extracted into ethyl acetate (100 ml), washed with brine (3 × 100 ml, 5% w/v) and dried over anhydrous sodium sulphate, filtered and evaporated in vacuo to afford the product as an amorphous white solid (0.6 g, 2.5 mmol, 16%). M.p. 87.0–87.5°C; FTIR 3388 (s), 3335 (s), 2979 (s), 2800 (br), 1729 (s), 1624 (s), 1545 (s), 1227 (s); EI (m/z) 239 (5), 194 (10), 148 (100), 131 (13), 120 (30), 103 (22), 91 (95), 77 (27), 65 (27), 47 (33); δ_H(270 MHz, $CDCl_3$), 7.23 (5H, m, ArH), 6.92 (1H, m, NH), 5.03 (1H, dq, *J* 39.1, 6.8, CFH), 4.90 (1H, overlapping dd, *J* 6.8, 6.8, CH), 3.19 (2H, m, CH_2), 1.48 (3H, 2 overlapping dd, *J* 24.5, 6.8, CH_3); δ_C(67.9 MHz, $CDCl_3$) 174.2, 174.1, 171.2 (d, *J* 19.0), 171.1 (d, *J* 20.0), 135.3, 135.3, 129.3, 129.2, 128.6, 128.5, 127.2, 127.2, 88.3 (d, *J* 183.6, CFH), 88.3 (d, *J* 183.5, CFH), 52.6, 52.4, 37.4, 37.24, 18.3 (d, *J* 21.9), 18.1 (d, *J* 20.9); δ_F(254.2 MHz, $CDCl_3$) −182.79 (ddq, *J* 49.2, 24.7, 4.2, CFH), −182.92 (ddq, *J* 49.8, 25.2, 4.3, CFH).

1.4. Acylase enzyme reaction

(*S*)-Phenylalanine-*N*-2-(*R*,*S*)-fluoropropionamide was dissolved in 4 M NaOH (three equivalents) and the solution adjusted to pH = 7 with aq. HCl and then diluted to a known volume of phosphate buffer (pH = 7), to give a 50 mM concentration of substrate in solution. Each diastereoisomer was readily observable by ^{19}F-NMR. δ_F(254.2 MHz, $CDCl_3$) −181.67 (dq, *J* 48.7, 25.2, CFH) *(S,S)*-diastereoisomer, −182.10 (dq, *J* 48.5, 25.2, CFH), (*S*,*R*)-diastereoisomer. The above solution of sodium (*S*)-phenylalanine-*N*-2-(*R*,*S*)-fluoropropionamide (0.2 ml) (for a 10 mM reaction) and phosphate buffer, (pH = 7, 0.6 ml) was placed in a 5 mm NMR tube. A solution (0.2 ml of a 25 mg/ml, 0.5 units/mg) of the *A. melleus* acylase (Sigma Chem.) in phosphate buffer (pH = 7) was added to initiate the reaction and the reaction maintained at 25°C and monitored by ^{19}F-NMR.

For the kinetic analysis of the individual diastereoisomers, the same conditions were used. For the faster reacting (*S*,*S*)-diastereoisomer the initial reaction rate could be calculated after 5–10 min by monitoring the integration of the fluorine signals by ^{19}F-NMR. For the slower reacting (*S*,*R*)-diastereoisomer this required several hours.

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