Structure–Activity Relationships in the Esterase-catalysed Hydrolysis and Transesterification of Esters and Lactones

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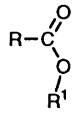
The Brønsted exponents for the alkaline hydrolysis of alkyl esters are 1.3 and 0.4 for substitution in the acyl and alcohol portions, respectively, which is indicative of a transition state which resembles the anionic tetrahedral intermediate with a localised negative charge. By contrast, the rate of the pig liver esterase (PLE)-catalysed hydrolysis shows little dependence upon the electron-withdrawing power of substituents. The values of k_{cat} are independent of the pK_a of the leaving group alcohol suggesting rate-limiting deacylation. There is a small steric effect of α-substitution in both the alcohol and carboxylic acid residues for the enzyme-catalysed reactions but the enzyme rate enhancement factor remains high for most esters. There is no substantial ee observed for the hydrolysis of racemic esters although the kinetic data can be used for determining the regioselective hydrolysis of diesters. Unsubstituted lactones are poor substrates for PLE but derivatives with hydrophobic substituents show k_{cat}/K_m values similar to those for acyclic esters. Dihydrocoumarin undergoes transesterification catalysed by PLE, k_{cat} increases with increasing alcohol concentration indicative of rate-limiting deacylation. There is enantioselectivity in the PLE-catalysed hydrolysis of some racemic lactones but little or none in the transesterification of racemic alcohols with dihydrocoumarin.

Lactones with relatively small rings are forced to adopt a *cis*/*E* ester conformation,[1] **1**, whilst in esters this conformation is higher in energy than the more stable *trans*/*Z* form, **2**. For example, in methyl acetate this energy difference is 8.5 kcal mol^{-1},[2] although it is solvent-dependent.[3] The barrier to rotation about the C–O bond for methyl acetate is 10–15 kcal mol^{-1}.[2] The *E* form has unfavourable alkyl–alkyl and lone pair–



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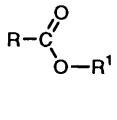

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lone pair steric repulsion and also lacks the favourable dipole–dipole stabilisation which is present in the *Z*-form.[4] The strain energies of 5–9 membered ring lactones are about 8–12 kcal mol^{-1}.[5] Small ring lactones undergo alkaline hydrolysis at an enhanced rate compared with acyclic esters,[1,6,7] but a variety of factors probably contribute to this difference.

The rate enhancement shown by enzymes in catalysing reactions is largely due to the favourable binding energies between the substrate and the protein.[8] In view of the energy differences between the *E* and *Z* forms of esters and the barrier to rotation, it could be anticipated that esterase enzymes would show a strong preference for *Z*-esters and that *E*-lactones would either not be substrates or be only poor ones. Similarly, any selectivity displayed by esterases towards the carboxylic acid and alcohol residues of esters would not be expected to be reflected in any such activity shown towards lactones.

We have already shown that pig liver esterase can catalyse the hydrolysis of β-lactams in preference to esters[9] and can be used enantioselectively to catalyse the hydrolysis of cyclic carbonates[10] and lactones.[11] Herein, we report quantitative data on the enzyme-catalysed hydrolysis and transesterification of some esters and lactones.

Results and Discussion

The effect of changes in substrate structure on enzyme activity is often used to identify specific binding sites between parts of the enzyme and substrate. These changes can affect the ease of bond-making and -breaking by classical 'chemical' electronic factors such as inductive, resonance and steric effects. However, the free energy of activation of an enzyme-catalysed reaction is also affected by the favourable binding energies between the protein and substrate substituents not directly involved with the reaction site.[8] It is therefore important to separate these two effects before conclusions about specific binding sites can be made. We recently suggested[12] that an 'enzyme rate enhancement factor' (EREF) for hydrolytic enzymes could be evaluated by dividing the second-order rate constant for the enzyme-catalysed reaction, k_{cat}/K_m, by that for the hydrolysis of the same substrate catalysed by hydroxide ion, k_{OH}. We therefore report kinetic data for both the alkaline and the esterase-catalysed hydrolysis of esters and lactones.

Pig liver esterase (E.C. 3.1.1.1) is a highly active serine hydrolase which catalyses the hydrolysis of esters. It exists as a trimeric molecule composed of three similar or identical subunits.[13] There is no available crystal structure, although several topographical models of the active site have been suggested.[14] The enzyme acts through the intermediate formation of an acyl enzyme which may be trapped with various nucleophiles.[14–16] The pH dependence of the hydrolysis reaction indicates a catalytic group of pK_a 4.3–5.1, which could represent the perturbed ionisation of an imidazolium group of a histidine residue or the dissociation of a carboxylic acid.[17]

1. *Alkaline Hydrolysis of Esters.*—(*i*) *Variation of the alcohol residue.* Electron-withdrawing substituents in the alcohol residue of esters increase the rate of alkaline hydrolysis.[17] The second-order rate constants for the alkaline hydrolysis of a series of alkyl phenylacetate esters are given in Table 1. This limited set of data covering 6 pK_a units in the leaving-group alcohol generate a Brønsted β_{lg} of −0.4. The second-order rate constants for the esters of secondary alcohols are about ten times less than those of primary alcohols of similar pK_a. This

Table 1 Second-order rate constants, k_{OH}, for the alkaline hydrolysis of esters and the kinetic parameters for the pig liver esterase catalysed hydrolysis of the same esters in water at pH 7.4, 25 °C, I = 0.1 mol dm^{-3} (KCl)

Ester	pK_a of leaving group[a]	k_{OH}/dm^3 mol^{-1} s^{-1} [b]	$(k_{cat}/K_m)/10^5$ dm^3 mol^{-1} s^{-1}	k_{cat}/s^{-1}	$K_m/10^{-4}$ mol dm^{-3}	EREF[c]
$PhCH_2CO_2CH_3$	15.5	0.15 ± 0.01	49.8 ± 2.5	159 ± 11	0.319 ± 0.035	3.3 × 10^7
$PhCH_2CO_2CH_2CH_3$	15.9	0.10 ± 0.01	3.06 ± 0.21	168 ± 12	5.49 ± 0.41	3.1 × 10^6
$PhCH_2CO_2CH(CH_3)_2$	15.6	0.012 ± 0.001	4.52 ± 0.29	143 ± 11	3.22 ± 0.35	3.8 × 10^7
$PhCH_2CO_2CH_2CF_3$	12.4	4.07 ± 0.10	18.0 ± 1.2	562 ± 45	3.18 ± 0.40	4.4 × 10^5
$PhCH_2CO_2CH(CF_3)_2$	9.3	85.5 ± 0.21	2.48 ± 0.15	114 ± 10	4.59 ± 0.48	2.9 × 10^3
$PhCH_2CO_2Ph$	10.0	1.10 ± 0.03	825 ± 66	4144 ± 373	0.505 ± 0.062	7.5 × 10^7
$PhCH_2CH_2CO_2Ph$	10.0	1.46 ± 0.04	1240 ± 100	3964 ± 436	0.320 ± 0.043	8.5 × 10^7
(±)-$PhCH_2CO_2CH(Ph)CH_3$	15.7	0.020 ± 0.001	0.822 ± 0.057	—	—	4.1 × 10^6
(±)-$PhCH_2CO_2CH(CH_3)CH_2CH_3$	15.8	0.024 ± 0.001	0.400 ± 0.004	—	—	1.7 × 10^6
(±)-$PhCH_2CO_2CH_2CH(Ph)CH_3$	15.7	0.27 ± 0.01	5.54 ± 0.33	—	—	2.1 × 10^6
(±)-$PhCH_2CO_2(CH_2)CH(Ph)CH_3$[d]	—	0.045 ± 0.001	3.27 ± 0.72 × 10^{-3}	—	—	7.3 × 10^3
$PhCH_2CO_2(CH_2)CH(OMe)CH_3$	—	—	0.511 ± 0.041	—	—	—

[a] From M. G. Hutchings and J. Gasteiger, *J. Chem. Soc., Perkin Trans. 2*, 1986, 455. [b] 1% v/v dioxane–water, I = 0.2 mol dm^{-3}. [c] EREF, enzyme rate enhancement factor = $(k_{cat}/K_m)/k_{OH}$. [d] 50% v/v DMSO.

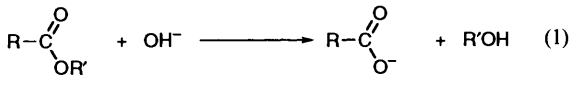
Table 2 The second-order rate constants for the PLE-catalysed and base-catalysed hydrolysis of a series of methyl esters at 25 °C, I = 0.2 mol dm^{-3} (KCl)

Ester	$(k_{cat}/K_m)/10^5$ dm^3 mol^{-1} s^{-1}	k_{OH}/ dm^3 mol^{-1} s^{-1}	EREF[a]
HCO_2Me	8.04 ± 0.46	50.6 ± 1.5	1.6 × 10^4
CH_3CO_2Me	4.04 ± 0.21	0.19 ± 0.01	2.1 × 10^6
$ClCH_2CO_2Me$	4.3 ± 0.3	60.7 ± 2.9	7.1 × 10^3
Cl_2CHCO_2Me	91 ± 11	1240 ± 65	7.3 × 10^3
$PhCH_2CO_2Me$	49.8 ± 4.5	0.15 ± 0.01	3.3 × 10^7
$PhCH_2CH_2CO_2Me$	15.7 ± 0.9	0.15 ± 0.01	1.0 × 10^7
o-$HOC_6H_4CH_2CH_2CO_2Me$	4.86 ± 0.30	0.23 ± 0.01	2.1 × 10^6
(±)-$CH_3CH(Cl)CO_2Me$	1.96 ± 0.12	2.57 ± 0.08	7.6 × 10^4
(±)-$PhCH_2CONHCH(Me)CO_2Me$	0.021 ± 0.002	0.93 ± 0.03	2.3 × 10^3
(±)-$PhCH_2CONHCH(CH_2Ph)CO_2Me$	0.124 ± 0.011	0.55 ± 0.02	2.3 × 10^4
(±)-$PhCH_2CONHCH(CHMeEt)CO_2Me$	*b*	—	
(±)-$CH_3CH(Ph)CO_2Me$	1.96 ± 0.17	0.038 ± 0.002	5.2 × 10^6
(±)-$CH_3CH(Ph)CH_2CO_2Me$	2.60 ± 0.15	0.25 ± 0.01	1.0 × 10^6
(±)-$PhCH(OH)CH_2CH_2CO_2Me$	0.354 ± 0.032	0.08 ± 0.01	4.4 × 10^5

[a] EREF = enzyme rate enhancement factor = $(k_{cat}/K_m)/k_{OH}$. [b] No enzyme-catalysed hydrolysis observed.

presumably reflects an unfavourable steric effect on formation of the tetrahedral intermediate. It is somewhat surprising therefore that the constant for the ester of hexafluoropropan-2-ol appears to 'fit' the Brønsted plot. The β_{1g} of −0.4 is consistent with rate-limiting attack of hydroxide ion upon the carbonyl carbon to generate the anionic tetrahedral intermediate, **3**.

(*ii*) *Variation of the carboxylic acid residue.* Electron-withdrawing substituents in the acyl group increase the rate of alkaline hydrolysis of esters.[17,18] There is an extremely large dependence of the second-order rate constant for the alkaline hydrolysis (log k_{OH}) of various acyl-substituted methyl esters on the pK_a of the carboxylic acid which generates a Brønsted α-value of 1.3 (Table 2). This is an intriguing situation as the product of the ester hydrolysis [eqn. (1)] and that of ionisation of the carboxylic acid [eqn. (2)] are the *same*.



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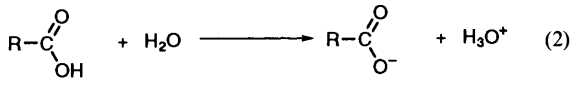

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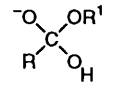
The Brønsted value of 1.3 therefore indicates that the transition state for ester hydrolysis is effectively 'more negative' compared with the neutral ester than is the carboxylate anion compared with the undissociated acid. It seems unlikely that the effective charge distribution is significantly different in the acid and ester. This indicates that there is a greater negative charge density in the transition state for hydrolysis compared with that in the carboxylate anion. The presumed tetrahedral intermediate **3** has a more localised negative charge, the stabilisation



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of which has a greater dependence upon substituents compared with the delocalised carboxylate anion. This is in agreement with other observations of acyl transfer to hydroxide ion progressing through formation of a tetrahedral intermediate.[19]

2. *Esterase-catalysed Hydrolysis of Esters.*—(*i*) *Variation of the alcohol residue.* The pig liver esterase catalysed hydrolysis of the esters follows Michaelis–Menten kinetics, eqn. (3), and the relevant kinetic parameters at pH 7.4 are given in Table 1. The second-order rate constant k_{cat}/K_m shows little dependence on the pK_a of the leaving-group alcohol. The trifluoroethyl ester is only six times more reactive than the ethyl ester for a 3000-fold change in the acidity of the leaving-group alcohol.

$$E + S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} \text{products} \quad (3)$$

Unlike the alkaline hydrolysis, where there is a difference of 7×10^3 in the second-order rate constant for the ester of hexafluoropropan-2-ol compared with that of isopropyl alcohol, there is little difference in the second-order rate constant k_{cat}/K_m for the enzyme-catalysed hydrolysis. If there is any trend in the enzyme-catalysed rate constants, k_{cat}/K_m, it is for them to decrease with increasing steric bulk in the alcohol residue. This may mask a small increase of activity with a better leaving group. The first-order rate constants, k_{cat}, show little dependence on the pK_a of the leaving-group alcohol.

The phenyl ester, however, does show an enhanced rate constant, both in k_{cat}/K_m and k_{cat}. The phenyl ester undergoes alkaline hydrolysis nearly 80 times more slowly than the hexafluoropropan-2-yl ester, whereas it is hydrolysed 300 times faster (k_{cat}/K_m) by the enzyme PLE. This is clearly indicative of a favourable binding site for the phenyl residue, the binding energy of which contributes to the enzyme rate enhancement. It may also be indicative of a separate binding site for phenyl esters compared with alkyl ones.

The pig liver esterase catalysed hydrolysis of esters is thought to proceed through the intermediate formation of an acyl enzyme, eqn. (4).[14] If the acylation step, k_2, is *irreversible* then

$$R'CO_2R + EnzOH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R'CO_2R{\cdot}EnzOH \xrightarrow{k_2} \begin{matrix} R'CO_2Enz + ROH \\ \Big\downarrow k_3\,(H_2O) \\ R'CO_2H + EnzOH \end{matrix} \quad (4)$$

the second-order rate constant k_{cat}/K_m and the first-order rate constant k_{cat} are given, respectively, by eqns. (5) and (6).

$$\frac{k_{cat}}{K_M} = \frac{k_1k_2}{k_1 + k_2} \quad (5)$$

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3} \quad (6)$$

The second-order rate constant, k_{cat}/K_m, is therefore always that for acylation irrespective of the relative values of k_2 and k_3. Conversely, k_{cat}, reflects the microscopic rate constant for either k_2 or k_3 depending on whether acylation or deacylation is rate-limiting. The relatively constant value of k_{cat} (Table 1), is apparently independent of the pK_a of the alcohol leaving group, which is therefore indicative of rate-limiting deacylation, k_3, as the alcohol residue has formally already departed—although it could remain bound to the enzyme. This is in agreement with the work of Jencks and Greenzaid[14] using phenyl acetate as a substrate. However, this constancy of k_{cat} is not absolute—the trifluoroethyl ester is four times more reactive and the phenyl ester shows a 30-fold higher value of k_{cat} compared with the alkyl esters. It appears that the rates of acylation and deacylation are similar and that k_{cat} is a composite rate constant. Alternatively, the alcohol/phenol leaving group could remain bound to the acyl enzyme during deacylation and thus cause moderate changes in the value of k_3.

The enzyme rate enhancement factors (EREF) are large (Table 1) and typical of hydrolytic enzymes.[12] The values decrease with decreasing pK_a of the alcohol because of the different dependence of k_{OH} and k_{cat}/K_m upon the acidity of the leaving group. The large value of 7.5×10^7 for the phenyl ester is again indicative of a specific binding pocket for the aromatic residue.

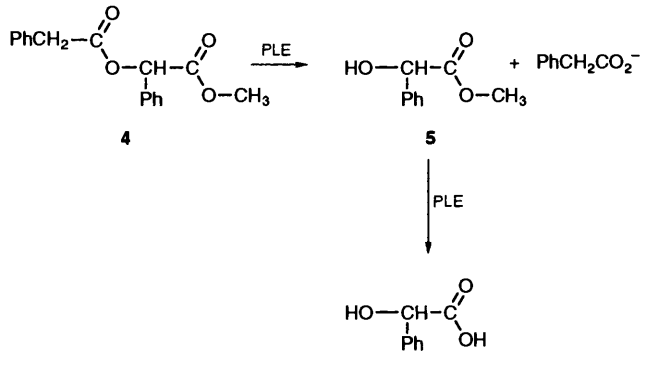
Esters of secondary alcohols, *i.e.*, with substituents at the α-carbon of the alcohol, remain good substrates for pig liver esterase. Substitution of α-methyl, α-ethyl or α-phenyl only reduce k_{cat}/K_m by less than an order of magnitude. Similarly, moving substituents in the alcohol residue so that the chiral centre is at the β- or γ-position has little effect on the rate constant and these esters remain reasonably good substrates with k_{cat}/K_m in the range 10^4–10^5 $dm^3\ mol^{-1}\ s^{-1}$.

(*ii*) *Variation of the carboxylic acid residue.* The second-order rate constants for the alkaline hydrolysis, k_{OH}, and the esterase-catalysed hydrolysis, k_{cat}/K_m, of a series of methyl esters are given in Table 2. Although electron-withdrawing substituents in the α-position of the carboxylic acid residue markedly *increase* the alkaline hydrolysis rate constants, they have much less effect on the enzyme-catalysed rate constant. For example, an α-chloro substituent increases k_{OH} by a factor of 300 but has no effect on k_{cat}/K_m. However, an α-dichloro substituent does increase k_{cat}/K_m. An *N*-acylated amino group in the α-position decreases k_{cat}/K_m by a factor of 200. The rate of acylation of the enzyme by esters is therefore very susceptible to steric bulk of substituents in the α-position. In an extreme case, the last entry in Table 2 shows no significant enzyme-catalysed reaction. Based on the lack of electronic effects upon the rate, the transition state for the enzyme-catalysed acylation cannot resemble the anionic tetrahedral intermediate formed during alkaline hydrolysis of esters. These effects are consistent with a transition state structure which has a semblance to a neutral tetrahedral intermediate, the reactant or product esters.

The substitution of an α-phenyl substituent in the carboxylic acid residue increases k_{cat}/K_m by a factor of ten, indicative of a favourable hydrophobic pocket. The favourable binding of aromatic residues reaches a peak for phenyl 3-phenylpropionate (Table 1) for which the k_{cat}/K_m of 1.2×10^8 $dm^3\ mol^{-1}\ s^{-1}$ approaches the diffusion-controlled limit.

Moving the position of substitution from α to β to γ appears not to have any significant effect. Although the values of k_{cat}/K_m remain smaller than those for the non-substituted straight-chain alkanoates, the substituted esters remain reasonably good substrates with k_{cat}/K_m in the region of 10^4–10^5 $dm^3\ mol^{-1}\ s^{-1}$.

The observation that α-substitution in the carboxylic acid residue has a greater effect in reducing k_{cat}/K_m than does α-substitution in the alcohol residue was used regioselectively to hydrolyse the diester **4** with PLE. As predicted, the unsubstituted carboxylic acid was liberated first to give the methyl ester of mandelic acid, **5**, which was then more slowly hydrolysed (Scheme 1).



Scheme 1

However, there was no substantial enantioselectivity shown in the PLE-catalysed hydrolysis of any of the racemic esters, with the chiral centre in either the alcohol residue (Table 1) or the carboxylic acid residue (Table 2). For example, the hydrolysis of racemic methyl 2-phenylpropionate gives an ee of less than 20% at 50% conversion. The independently

Table 3 Second-order rate constants, k_{OH}, for the alkaline hydrolysis of lactones and the kinetic parameters for the pig liver esterase catalysed hydrolysis of the same lactones in water at pH 7.4, 25 °C, I = 0.2 mol dm^{-3} (KCl)

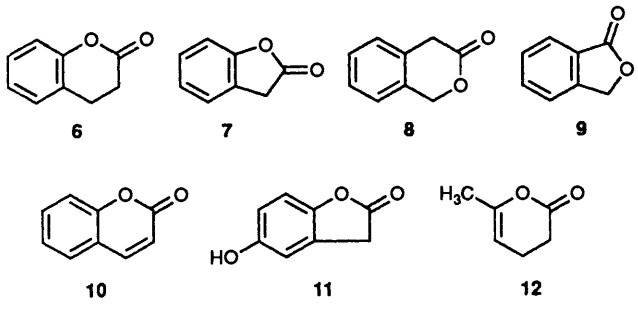
Lactone	(k_{cat}/K_m)/dm^3 mol^{-1} s^{-1}	k_{OH}/dm^3 mol^{-1} s^{-1}	EREF
Methyl acetate	4.04 ± 0.36 × 10^5	0.19 ± 0.01	2.1 × 10^6
β-Propiolactone	<2	2.25 ± 0.03	<1
γ-Butyrolactone	<2	1.43 ± 0.04	<2
δ-Valerolactone	<3	21.1 ± 0.63	<0.2
ε-Caprolactone	<2	1.27 ± 0.04	<2
3-Phenyl-γ-butyrolactone	1.94 ± 0.17 × 10^3	—	—
Dihydrocoumarin, **6**	1.22 ± 0.10 × 10^6	85.5 ± 2.6	1.4 × 10^4
Coumaran-2-one, **7**	1.10 ± 0.10 × 10^5	44.4 ± 1.4	2.5 × 10^3
Isochroman-3-one, **8**	<2	5.50 ± 0.10	<0.4
Phthalide, **9**	<2	1.30 ± 0.04	<2
Coumarin, **10**	<3	1.30 ± 0.04	<2
6-Hydroxycoumaran-2-one, **11**	7.53 ± 0.68 × 10^3	—	—
6-Methyl-3,4-dihydropyran-2-one, **12**	1.14 ± 0.09 × 10^6	79.3 ± 2.5	1.4 × 10^4

determined values of k_{cat}/K_m for the *R* and the *S* enantiomers are almost identical (± 15%). The hydrolysis of (±)-methyl 4-hydroxy-4-phenylbutanoate catalysed by PLE shows no enantioselectivity even though the reaction presumably proceeds through the same acyl enzyme intermediate as that formed from the highly enantioselectively catalysed hydrolysis of 4-phenyl-γ-butyrolactone.[11] The enantioselectivity is expressed in the acylation step, as expressed by the second-order rate constant k_{cat}/K_m.

3. *Hydrolysis of Lactones.*—A significant difference between esters and lactones is that the acyclic derivatives usually adopt the *Z* (*trans*) conformation, **2**, whereas the small ring compounds are normally restricted to the *E* (*cis*) conformation, **1**. This difference contributes to the faster rate of alkaline hydrolysis of lactones compared with esters, although the exact interpretation of the difference remains controversial.[1,6,7]

If acyclic esters bind to esterase enzymes in the *trans*-conformation then it may be anticipated that either *cis*-lactones may not bind or that there will be interesting stereochemical differences between them.

Kinetic data for the alkaline and enzyme-catalysed hydrolysis of some lactones are presented in Table 3. Simple unsubstituted lactones, from four- to seven-membered rings, are not substrates

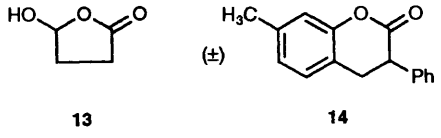


for pig liver esterase. Despite these lactones being chemically more reactive than analogous alkyl alkanoates, the values of k_{cat}/K_m are more than 10^5 times less for the cyclic compared with the acyclic structures. It has been suggested that there is a hydrophobic binding pocket in PLE into which a suitable residue on the ester can fit.[14] The constrained *cis* conformation of the lactone probably inhibits this favourable interaction. However, suitably placed large hydrophobic residues are not a prerequisite for good hydrolytic activity as methyl formate and methyl acetate are both good substrates (Table 2).

Several substituted lactones are, however, good or even excellent substrates for PLE (Table 3). The substitution of a single phenyl residue at the alcohol α-carbon in γ-butyrolactone makes 3-phenyl γ-butyrolactone a reasonable substrate with a k_{cat}/K_m of 1.94 × 10^3 dm^3 mol^{-1} s^{-1}. Hydrolytic activity is increased by a factor of > 10^3 by this additional single phenyl group compared with a fourfold decrease in the analogous acyclic ester (Table 1). Furthermore, unlike with the acyclic ester, PLE discriminates enantioselectively between the two enantiomers of 3-phenyl-γ-butyrolactone giving an ee of > 90% after 50% reaction.[11]

Quite remarkably, dihydrocoumarin **6** is an excellent substrate for PLE-catalysed hydrolysis, showing a k_{cat}/K_m of 1.2 × 10^6 dm^3 mol^{-1} s^{-1}. Clearly a *trans* ester conformation is not a prerequisite for good hydrolytic activity. Both dihydrocoumarin **6** and coumaran-2-one, **7**, are as good substrates as any of the acyclic esters studied. Although increased 'chemical' reactivity may make a contribution to the increased enzyme activity—the k_{OH} values for the two lactones being 40–80 times greater than that for phenyl phenylacetate—this effect alone cannot account for the large values of k_{cat}/K_m. Furthermore, there is little strain energy associated with ring opening of dihydrocoumarin, **6**, and coumaran-2-one, **7**. An α-phenyl substituent in the lactone is not an absolute requirement for good activity, as, for example, the methyl substituted lactone, 6-methyl-3,4-dihydropyran-2-one, **12**, is also an excellent substrate (Table 3).

The position of ring substitution/fusion between the phenyl ring and the lactone is crucial. Neither isochroman-3-one, **8**, or phthalide, **9**, are substrates for PLE. Dihydrocoumarin, **6**, fits into the Jones model of PLE[14] with the phenyl ring binding into the large hydrophobic pocket when the carbonyl carbon is in the vicinity of the active site serine residue. Conversely, with **8**, the leaving group is unfavourably forced into the small hydrophobic pocket. Neither enantiomer of 3-hydroxy-γ-butyrolactone **13** was a hydrolytic substrate for PLE. It appears



that a hydrophobic substituent is necessary for activity. The racemic lactone, 7-methyl-3-phenyldihydrocoumarin, **14**, was a good substrate for PLE and the unchanged lactone could be isolated with an ee of > 90%.

4. *Transesterification Reactions with Dihydrocoumarin as the Substrate.*—Transesterification reactions of dihydrocoumarin, **6**, with alcohols can be catalysed by pig liver esterase in water. At pH 7.4 in the presence of 0.5 mol dm^{-3} methanol, a maximum of 45% of the product is the methyl ester, methyl 3-(2-

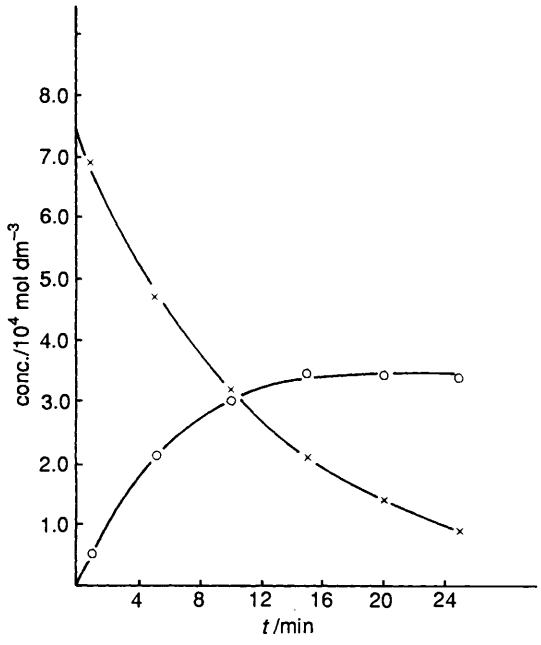
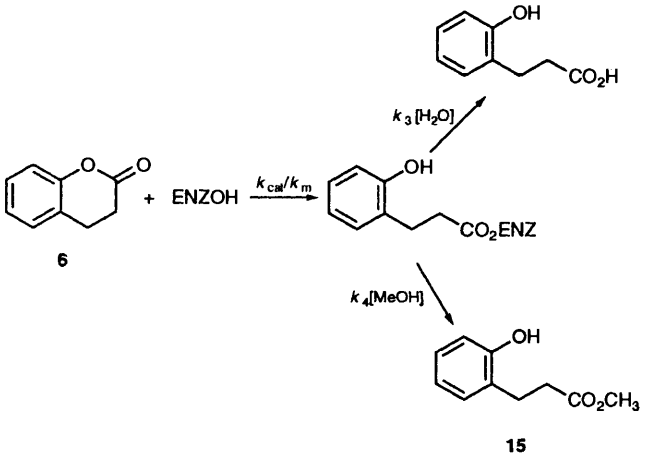


Fig. 1 A plot of the concentration of dihydrocoumarin **6** (×) and methyl 3-(2-hydroxyphenyl)propanoate **15** (○) against time in phosphate buffer, pH 7.4 at 25 °C in the presence of methanol (0.5 mol dm^{-3}) and pig liver esterase (4.4 × 10^{-10} mol dm^{-3})

hydroxyphenyl)propanoate **15**. That the ester accumulates is a reflection of the fact that it is a slightly poorer substrate, k_{cat}/K_m = 4.4 × 10^5 dm^3 mol^{-1} s^{-1}, than dihydrocoumarin, k_{cat}/K_m = 1.2 × 10^6 dm^3 mol^{-1} s^{-1}. In 0.5 mol dm^{-3} methanol, the methyl ester reaches its maximum concentration after about 50% of the dihydrocoumarin has reacted and maintains this concentration until all of the lactone has reacted. The methyl ester then is, relatively, slowly hydrolysed (Fig. 1).



Scheme 2

The PLE-catalysed hydrolysis of dihydrocoumarin, **6**, is thought to proceed through the intermediate formation of an acyl enzyme, which can be trapped with methanol (Scheme 2).

The proportion of the methyl ester formed increases with increasing methanol concentration (Fig. 2). The partitioning of the acyl-enzyme intermediate between hydrolysis and transesterification depends on the ratio of the two rate constants k_3 and k_4 and the concentration of methanol (Scheme 2). The fraction of the ester product formed, assuming no saturation of the enzyme by the alcohol, is given by eqn. (7).

$$\text{Fraction of ester} = \frac{k_4(\text{MeOH})}{k_3(\text{H}_2\text{O}) + k_4(\text{MeOH})} \quad (7)$$

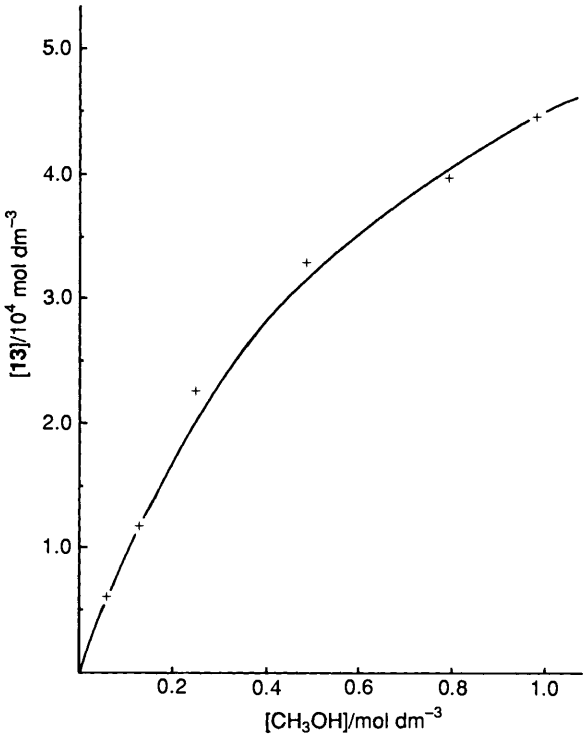
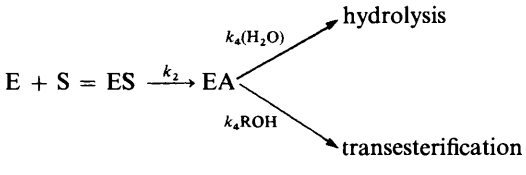


Fig. 2 The concentration of methyl ester formed as a function of methanol concentration from the reaction of dihydrocoumarin (7.5 × 10^{-4} mol dm^{-3}) in the presence of pig liver esterase (1.1 × 10^{-9} mol dm^{-3}) at pH 7.4 phosphate buffer at 25 °C

The line in Fig. 2 is generated from a ratio of k_4/k_3 = 82.5. This is in agreement with the better nucleophilicity of methanol compared with water. This is not the true ratio of rate constants as the product ester is undergoing hydrolysis during the reaction.

Addition of methanol has little or no effect upon the value of k_{cat}/K_m, consistent with the latter constant reflecting simply the rate of acylation of dihydrocoumarin, eqn. (5). However, k_{cat} increases in the presence of methanol but shows a non-linear dependence on increasing alcohol concentration (Fig. 3). This could be indicative of a change in rate-limiting step from deacylation to acylation. The rate of deacylation of the acyl enzyme (EA) is the sum of the two steps for hydrolysis and transesterification (Scheme 3), for which the value of k_{cat} is given by eqn. (8). If the sum of k_3 + k_4 (ROH) becomes greater than k_2, the rate of acylation would show a non-linear dependence on the concentration of alcohol and, when k_4 (ROH) > k_3, k_{cat} would eventually become independent of alcohol concentration as it becomes limited by k_2, the rate of acylation. The values of k_{cat} for esterification in Fig. 3 are calculated from k_2 = 2.23 × 10^3 s^{-1}, k_3 = 2.57 × 10^3 s^{-1} and k_4 = 1.80 × 10^4 dm^3 mol^{-1} s^{-1}. Other, more complicated, events are possible. For example, the acylation step with lactones may be reversible because of intramolecular ring closure back to lactone being competitive with deacylation. There may be a specific binding site for methanol so that the acyl enzyme may become saturated at high alcohol concentrations.



Scheme 3

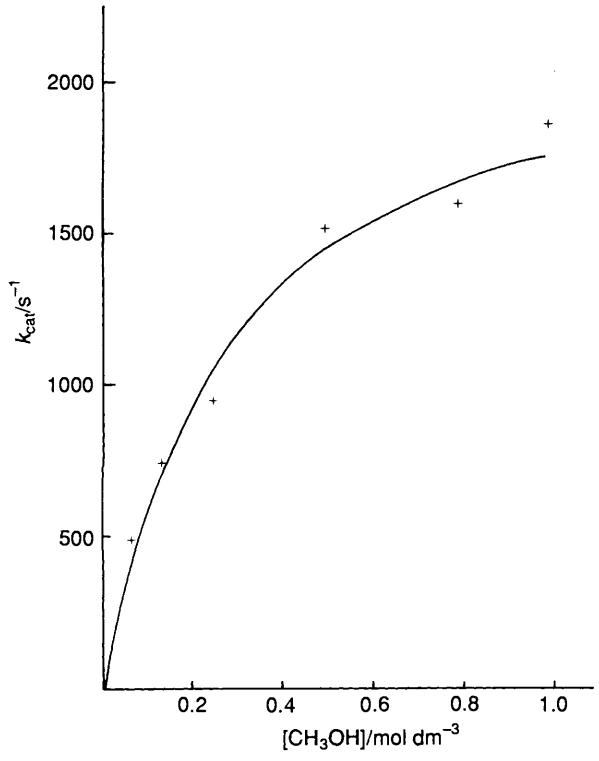


Fig. 3 The dependence of k_{cat} for the appearance of ester formed from the PLE-catalysed (4.4×10^{-10} mol dm^{-3}) reaction of dihydrocoumarin (7.5×10^{-4} mol dm^{-3}) in aqueous buffer at pH 7.4 on the concentration of added methanol

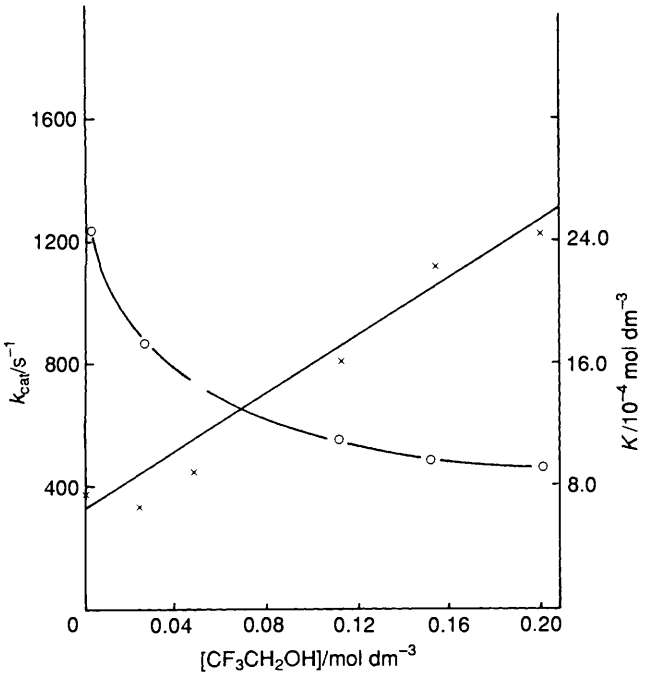


Fig. 4 The effect of trifluoroethanol (TFE) concentration on the observed values of k_{cat} (left-hand scale, ○) and K_m (right-hand scale, ×) for the reaction of dihydrocoumarin in aqueous buffer, pH 7.4 at 25 °C, catalysed by pig liver esterase (2.2×10^{-8} mol dm^{-3})

$$k_{cat} = \frac{k_2(k_3 + k_4[ROH])}{k_2 + (k_3 + k_4[ROH])} \quad (8)$$

In contrast with the effect of methanol or ethanol, the addition of 2,2,2-trifluoroethanol to the PLE-catalysed reaction of dihydrocoumarin caused a *decrease* in both the values of k_{cat} and k_{cat}/K_m (Fig. 4). The value of k_{cat}/K_m decreases by almost a

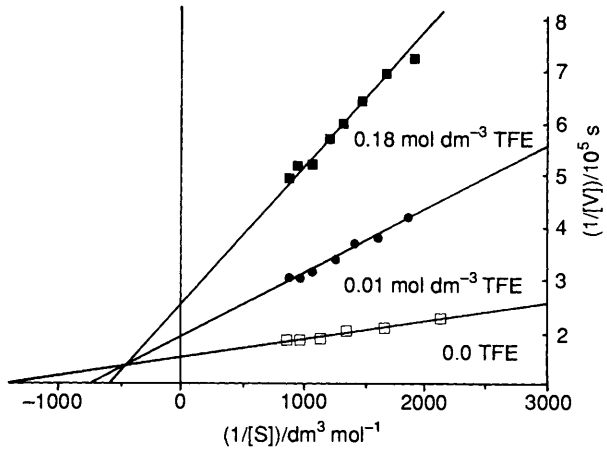
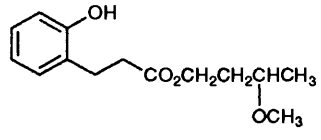


Fig. 5 Lineweaver–Burke plot for the effect of trifluoroethanol (TFE) at the concentrations indicated on the reaction of dihydrocoumarin in aqueous phosphate buffer pH 7.4 at 25 °C, catalysed by pig liver esterase (2.2×10^{-8} mol dm^{-3})

factor of ten in 0.3 mol dm^{-3} trifluoroethanol. Furthermore, no trifluoroethyl ester product was formed—only the hydrolytic product was observed (Scheme 2). This cannot be due to a greater lability of the ester as trifluoroethyl esters are poorer substrates than methyl esters for PLE (Table 1). The decrease in k_{cat} with increasing concentration of trifluoroethanol is indicative of non-competitive inhibition and the kinetic data conform to this behaviour in the Lineweaver–Burke plot (Fig. 5). The weak inhibitory binding of trifluoroethanol to PLE compared with methanol and ethanol could result from the increased acidity of the alcohol making it a better hydrogen-bond donor to a receptor site on the protein. It seems unlikely that it is the trifluoroethoxide anion that binds.

The formation of the methyl ester **15** in water from the reaction of dihydrocoumarin in the presence of methanol and PLE (Scheme 2) demonstrates the feasibility of carrying out enzyme-catalysed transesterification in water and the possibility of aqueous-based resolution of racemic alcohols.

Simple straight-chain primary alcohols, e.g. 3-phenylpropanol, reacted with dihydrocoumarin at pH 7.4 in the presence of PLE to give the corresponding ester. However, a variety of secondary alcohols did not trap the acyl enzyme intermediate and only hydrolysis was observed. Furthermore, a primary alcohol with a chiral centre at the β-position, racemic 2-phenylpropanol, did not give any ester product. However, racemic 3-methoxybutanol, a primary alcohol with an asymmetric centre at the γ-position, did react with dihydrocoumarin in the presence of PLE to give the ester product **16**. The amount of ester product increases with increasing



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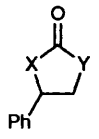
concentration of alcohol, 30% with 0.6 mol dm^{-3} alcohol, but the recovered ester **16** showed only a small specific rotation $[\alpha]_D^{20} = +1.9$ ($c = 1$, $CHCl_3$). Alkaline hydrolysis of **16** regenerated the alcohol with only 10% ee. There appears to be little selectivity in the binding of the incoming nucleophile alcohol to react with the acyl-enzyme intermediate.

For comparison, studies of enzyme-catalysed transesterification using PLE in heptane were examined. The

enzymes were prepared by freeze-drying an aqueous solution of PLE at pH 7.4. There was no enzyme-catalysed transesterification of secondary alcohols in heptane using either ethyl propanoate or dihydrocoumarin as the acylating agent. The same result was obtained with chiral primary alcohols with asymmetry in the β-position. However, transesterification does occur with γ-substituted primary alcohols using ethyl propanoate, but not using dihydrocoumarin. The reaction of 3-methoxybutanol with ethyl propanoate in heptane is catalysed



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by PLE to give the transesterified product from which the optically active alcohol can be recovered in >95% ee.

It is interesting that there is no substantial enantioselectivity in the PLE-catalysed hydrolysis of racemic esters but there is in the hydrolysis of lactones—particularly with hydrophobic substituents α to the alcohol residue. For the general structure, **17**, ee of >90% are observed for X = Y = O,[10] for X = O, Y = CH_2 but not for X = O, Y = NH.

Experimental

Materials.—The enzyme porcine-liver esterase (E.C. 3.1.1.1) was obtained from Sigma and was used without further purification. All compounds that are not given in the Experimental section were purchased from Aldrich or Lancaster Synthesis. Glass-distilled water was used throughout. All other reagents were of AnalaR grade.

Equipment and Analysis.—The instrument used for all experiments involving UV–VIS absorption determinations was a Gilford 2600 single-beam UV spectrophotometer. The cell compartment was thermostatted by a circulating water system which was maintained by a Haake water bath. The spectrophotometer was interfaced to an external microcomputer which allowed the storage, manipulation and analysis of the data collected. In all the experiments carried out, matched quartz glass cuvettes were used each having a path length of 1.0 cm.

All HPLC studies were carried out using an LKB 2150 HPLC pump connected to a Beckman 163 variable wavelength UV absorption detector. The column used was a LiChrosorb C18 RP. The chromatogram was collected on a flat bed chart recorder to give a permanent record. The data was also transferred and analysed on a Trivector 'Trio' integrator.

A method was first developed to quantify the substrate and the hydrolysis products. Initially the column was run at high acetonitrile concentration, typically 80% acetonitrile–water to make sure that all the components of the reaction mixture were eluted. The concentration of the organic phase was then gradually reduced until baseline resolution of the compound under study was achieved. With polar compounds the appropriate ion pairing reagent was added or, more commonly, 0.1% trifluoroacetic acid was added.

pH-stat studies of enzyme-catalysed reactions were carried out using a Radiometer ABU 2 autoburette controlled with a PHM 12 pH-meter. The volume of standard sodium hydroxide against time was plotted on a Radiometer flat bed chart recorder.

Kinetics.—In all the enzyme-catalysed reactions analysed by UV absorption a typical reaction volume would be 2.5 cm^3. The temperature was maintained at 303 K and the ionic strength adjusted to 0.2 mol dm^{-3} with potassium chloride. With all the reactions carried out the pH was measured before and after the enzymic reaction.

A typical assay would consist of the following. A known weight, *ca.* 5.0 mg of substrate was dissolved in 5.0 cm^3 1,4-dioxane to give a concentration approximately 100 times that required for the assay. 2.5 cm^3 of pH 7.4 phosphate buffer would be pipetted into a quartz cuvette and placed in the thermostatted compartment to equilibrate. 25.0 μl of the stock substrate was added to this solution, giving a 100-fold dilution of the substrate and a final solution containing 1% v/v 1,4-dioxane. The reaction would then be initiated by addition of the required amount of enzyme solution.

In all the enzyme-catalysed reactions studied by HPLC the reaction volume was typically 10.0 cm^3, the temperature was maintained by placing the vial containing the reaction mixture in a Haake circulating water bath. The ionic strength was adjusted to 0.2 mol dm^{-3} with KCl and the pH was monitored before and after the reaction.

The substrate, *ca.* 5 mg, was dissolved in 1,4-dioxane at approximately 100 times the required assay concentration. 10.0 cm^3 of pH 7.4 phosphate buffer were pipetted into a clean vial and thermostatted in the water bath at 303 K. After equilibration, 100 μl of the stock substrate were added to give the final reaction mixture of 10.1 cm^3, 1% v/v 1,4-dioxane. An initial injection of the substrate was made before the enzyme was added to give the initial peak area and thus the initial substrate concentration of the compound under study, and to eliminate the possibility of one enantiomer being hydrolysed very rapidly leaving the second isomer to be hydrolysed at a slower rate, giving the false impression of no selectivity. The reaction was then initiated by the addition of the enzyme to the solution containing the substrate.

In all enzyme-catalysed reactions a 'blank' run was carried out to account for any non-enzyme-catalysed reactions, such as buffer catalysis, taking place.

In all the enzyme-catalysed reactions monitored by HPLC an external calibration graph was used. This was usually a plot of concentration of substrate against peak area, as determined from the 'trio' integrator. Serial dilution of the substrate was favoured over an internal standard since it was considered that any internal standard may interfere with the enzymic reaction and lead to spurious results. The substrate was dissolved in 1,4-dioxane at 150 times the required assay concentration. 100, 75, 50, 25, 12 and 5 μl of the stock solution were then added to 10.0 cm^3 of pH 7.4 phosphate buffer and the resulting solutions adjusted to 1% 1,4-dioxane. Duplicate injections of each standard were then made and the average peak area plotted against the concentration of substrate. In most cases the calibration graphs were linear but, when very low wavelengths were used, some deviation from linearity was observed.

All enzyme-catalysed reactions monitored by the pH-stat involved the continuous titration of the acid liberated at constant pH, using standard sodium hydroxide solution.

The substrate was dissolved in dioxane at a concentration 100 times that required for the reaction. 50 μl of the stock substrate was added to 5.0 cm^3 of distilled water and the pH of the resulting solution adjusted to 7.4. The reaction was initiated by the addition of the enzyme and the liberated acid titrated with standard (0.1 mol dm^{-3}) hydroxide solution made up in 1% v/v dioxane. A blank reaction was carried out which involved monitoring the reaction in the absence of enzyme.

Data Analysis.—The Michaelis–Menten kinetic parameters were determined as described previously using both initial rates and the total integrated progress curves.[20]

Enantiomeric Excess.—The enantiomeric excess was deter-

mined from the NMR (270 MHz) using tris[3-(heptafluoropropylhydroxymethylene)(+)-camphorato] europium(III).

Preparation of Simple Esters.—The general method used was simply the acid-catalysed reaction of the carboxylic acid with an alcohol.

Ethyl 2-phenylethanoate. Phenylacetic acid (2.0 g, 1.5 mmol) was dissolved in 20.0 cm^3 of ethanol. 0.1 cm^3 of concentrated sulfuric acid was added and the solution refluxed for 15 h. The solution was cooled to room temperature and neutralised with sodium hydroxide. The remaining alcohol was, in this case, removed by rotary evaporation to yield a viscous yellow oil. With higher boiling alcohols vacuum distillation was required to remove the remaining alcohol. The ester was purified by column chromatography, using Silica gel 60 with hexane–ether (3:1 v/v) as the eluent. TLC (silica gel) R_f = 0.72 (3:1 v/v, hexane–ether), yield = 85% (2.10 g); ν_{max}(thin film)/cm^{-1} 1735, 1600 and 1500; δ_H($CDCl_3$) 1.30 (3 H, t, CH_3), 3.00 (2 H, s, α-CH_2), 4.20 (2 H, q, CH_2) and 7.30 (5 H, m, Ph); δ_C($CDCl_3$) 3.912 (CH_3), 14.151 (CH_2), 60.571 (CH_2), 128.987 (Ph), 133.947 (Ph) and 170.339 (CO); GC–MS (chloroform) *m/z* 164 (M^+).

2,2,2-Trifluoroethyl 2-phenylethanoate. TLC (silica gel) R_f = 0.62 (3:1 v/v, hexane–ether), yield = 62%; ν_{max}/cm^{-1} 1760, 1500 and 1600; δ_H($CDCl_3$) 3.80 (2 H, s, α-CH_2), 4.60 (2 H, q, CH_2) and 7.40 (5 H, m, Ph).

Isopropyl 2-phenylethanoate. TLC (silica gel) R_f = 0.7 (3:1 v/v, hexane–ether), yield = 78%; ν_{max}/cm^{-1} 1735, 1500 and 1600; δ_H($CDCl_3$) 1.10 (3 H, s, CH_3), 1.30 (3 H, s, CH_3), 3.60 (2 H, s, α-CH_2), 5.10 (1 H, m, CH) and 7.30 (5 H, m, Ph); GC–MS (EI, chloroform) *m/z* 178 (M^+).

Methyl 3-phenylpropanoate. TLC (silica gel) R_f = 0.55 (3:1 v/v, hexane–ether), yield = 70%; ν_{max}/cm^{-1} 1736, 1500 and 1600; δ_H($CDCl_3$) 2.90 (2 H, t, β-CH_2), 3.65 (2 H, t, α-CH_2), 4.65 (3 H, s, CH_3) and 7.25 (5 H, m, Ph); δ_C($CDCl_3$) 30.616 (CH_2), 35.348 (CH_2), 51.212 (CH_3), 127.975 (Ph), 28.204 (Ph), 140.227 (Ph) and 172.954 (CO); GC–MS (EI, chloroform) *m/z* 164 (M^+).

1-Phenylethyl 2-phenylethanoate. TLC (silica gel) R_f = 0.65 (3:1 v/v, hexane–ether), yield = 60%; ν_{max}/cm^{-1} 1740, 1500 and 1600; δ_H($CDCl_3$) 130 (3 H, d, CH_3), 3.50 (2 H, s, α-CH_2), 4.60 (1 H, q, CH) and 7.40 (10 H, m, 2 Ph); GC–MS (EI, chloroform) *m/z* 240 (M^+).

3-Phenylbutyl 2-phenylethanoate. TLC (silica gel) R_f = 0.55 (3:1 v/v, hexane–ether), yield = 63%; ν_{max}(thin film)/cm^{-1} 1735, 1500 and 1600; δ_H($CDCl_3$) 1.25 (3 H, d, CH_3), 1.90 (2 H, m, CH_2), 2.65 (1 H, m, CH), 3.60 (3 H, s, α-CH_2), 4.05 (2 H, m, CH_2) and 7.20 (10 H, m, 2 Ph); GC–MS (EI, chloroform) *m/z* 260 (M^+).

Ethyl 2-chloropropanoate. Yield = 85%; ν_{max}/cm^{-1} 1744; δ_H($CDCl_3$) 1.30 (3 H, t, CH_3), 2.10 (3 H, t, β-CH_3), 4.0 (1 H, q, α-CH) and 4.30 (2 H, q, CH_2); GC–MS (EI, chloroform) *m/z* 136 (M^+).

Methyl 3-phenylbutanoate. ν_{max}/cm^{-1} 1737; δ_H($CDCl_3$) 1.45 (3 H, d, γ-CH_3), 2.65 (2 H, octet, α-CH_2), 3.30 (1 H, m, β-CH), 3.65 (3 H, s, CH_3) and 7.25 (5 H, m, Ph); GC–MS (EI, chloroform) *m/z* 178 (M^+).

2-Phenylpropyl 2-phenylethanoate. ν_{max}/cm^{-1} 1732; δ_H($CDCl_3$) 1.35 (3 H, d, CH_3), 2.75 (1 H, m, CH), 3.50 (2 H, s, α-CH_2), 4.05 (2 H, m, CH_2) and 7.25 (10 H, m, 2 Ph); GC–MS (EI, chloroform) *m/z* 254 (M^+).

1-Phenylethyl formate. ν_{max}/cm^{-1} 1756; δ_H($CDCl_3$) 1.55 (3 H, d, CH_3), 6.00 (1 H, d, C*H*CH_3), 7.30 (5 H, m, Ph) and 8.05 (1 H, s, CHO); δ_C($CDCl_3$) 11 031 (CH_3), 72.109 (CH), 126–140 (Ph) and 160.273 (CO); GC–MS (EI, chloroform) *m/z* 150 (M^+).

Synthesis of Esters Containing Alcohol Groups of Low pK_a.—The method used was the condensation of the acid and the alcohol with dicyclohexylcarbodiimide.

1,1,1,3,3,3-Hexafluoropropan-2-yl 2-phenylethanoate. Phenylacetic acid (2.0 g, 14.7 mmol) was dissolved in 50 cm^3 of dry ether and cooled to 0 °C in ice. Dicyclohexylcarbodiimide, DCCI (1.9 g, 14.7 mmol) was added and the resulting solution allowed to stir for 30 min during which time a white precipitate formed. The resulting suspension was then allowed to warm to room temperature. 1,1,1,3,3,3-hexafluoropropan-2-ol (16.0 mmol) was added and the suspension allowed to stir for 48 h. The precipitate was filtered off and the remaining ether removed by rotary evaporation. The product was purified by column chromatography on Silica gel 60 using 3:2 hexane–ether as the eluent.

TLC (silica gel) R_f = 0.77 (3:1 v/v, hexane–ether); ν_{max}/cm^{-1} 1800, 1500 and 1600; δ_H($CDCl_3$) 3.70 (2 H, s, CH_2), 5.80 (1 H, m, CH) and 7.30 (5 H, m, Ph).

Phenyl 3-phenylpropanoate. TLC (silica gel) R_f = 0.77 (3:1 v/v, hexane–ether), yield = 56%; ν_{max}/cm^{-1} 1760, 1500 and 1600; δ_H($CDCl_3$) 2.90 (2 H, t, α-CH_2), 3.10 (2 H, t, β-CH_2) and 7.25 (10 H, m, 2 Ph); δ_C($CDCl_3$) 30.859 (CH_2), 35.888 (CH_2), 121.452 (Ph), 150.546 (Ph) and 171.309 (CO); GC–MS (EI, chloroform) *m/z* 225 (M^+).

Methyl 3-(2-hydroxyphenyl)propanoate. 3-(2-Hydroxyphenyl)propanoic acid (0.5 g, 3.0 mmol) was dissolved in sodium-dried ether (20 cm^3). An ethereal solution of diazomethane was then added dropwise by means of a Pasteur pipette until a permanent yellow colour was observed. The resulting solution was then allowed to stand overnight. The remaining ether was then removed to give a viscous oil. Yield = 98%; ν_{max}/cm^{-1} 3400, 1735, 1500 and 1600; δ_H($CDCl_3$) 3.05 (2 H, t, β-CH_2), 3.80 (2 H, t, α-CH_2), 4.75 (3 H, t, CH_3), 6.90 (2 H, t, Ph), 7.15 (2 H, t, Ph) and 7.48 (1 H, s, OH); δ_C($CDCl_3$) 24.789 (CH_2), 34.732 (CH_2), 52.129 (CH_3), 116.731 (Ph), 120.607 (Ph), 127.103 (Ph), 130.390 (Ph), 154.142 (Ph) and 175.840 (CO); GC–MS (EI, chloroform) *m/z* 180 (M^+).

Methyl 4-hydroxy-4-phenylbutanoate. 3-Phenyl-γ-butyrolactone (1.0 g, 6.10 mmol) was dissolved in methanol (5.0 cm^3). Concentrated sulfuric acid (0.1 cm^3) was added and the resulting solution refluxed for 14 h. The solution was cooled and the excess of methanol removed by rotary evaporation. The product was purified by column chromatography on Silica gel 60 using 3:1 v/v hexane–ether as the eluent. However, TLC analysis showed there to be two products present these being the 4-hydroxy and 4-methoxy derivatives. These two compounds were then separated on a second column with an eluent system of 1:1 hexane–ethylacetate (v/v). Yield = 23%; ν_{max}/cm^{-1} 3488, 1735, 1600 and 1500; δ_H($CDCl_3$) 2.10 (2 H, m, β-CH_2), 2.45 (2 H, t, α-CH_2), 3.25 (1 H, s, OH), 3.65 (3 H, s, CO_2CH_3), 4.15 (1 H, t, CH) and 7.40 (5 H, m, Ph).

N-Phenylacetylalanine methyl ester. (±)-Alanine (5.0 g, 66.0 mmol) was dissolved in 50 cm^3 of 2 mol dm^{-3} sodium hydroxide solution. Phenylacetyl chloride (9.3 g, 66.0 mmol) was added slowly and the resultant solution shaken vigorously for 2 h. The *N*-phenylacetylalanine product was precipitated by the addition of 2 mol dm^{-3} hydrochloric acid and recrystallised from distilled water. Yield = 89% (10.5 g), δ_H($CDCl_3$) 1.40 (3 H, d, CH_3), 4.60 (2 H, s, CH_2), 4.90 (1 H, q, CH), 7.35 (5 H, m, Ph). *N*-Phenylacetylalanine (2.0 g, 10.0 mmol) was then esterified with diazomethane. Yield = 98%, m.p. = 87 °C; ν_{max}/cm^{-1} 1735, 1500 and 1600; δ_H($CDCl_3$) 1.70 (3 H, d, CH_3), 3.60 (2 H, s, CH_2), 3.75 (3 H, s, CH_3), 3.60 (1 H, q, CH) and 7.40 (5 H, m, Ph); δ_C($CDCl_3$) 18.251 (CH_3), 43.475 (CH_2), 48.007 (CH), 52.389 (CH_3), 127.316 (Ph), 128.343 (Ph), 129.315 (Ph), 170.442 (CONH), 173.312 (CO_2Me); GC–MS (EI, chloroform) *m/z* 224 (M^+).

N-Phenylacetylphenylalanine methyl ester. Yield = 98%, m.p. = 77 °C, ν_{max}(Nujol)/cm^{-1} 1737, 1500 and 1600; δ_H($CDCl_3$) 3.10 (2 H, m, CH_2), 3.55 (2 H, s, CH_2), 3.75 (3 H, s, CH_3), 4.75 (1 H, q, CH), 5.80 (1 H, d, NH) and 7.30 (5 H, m, Ph); δ_C($CDCl_3$) 37.540 (CH_2), 43.954 (CH_2), 52.290 (CH), 52.900 (CH_3), 126.933 (Ph), 135.475 (Ph), 170.380 (CONH) and 171.730 (CO_2Me).

O-*Acetylmandelic acid.* 2.0 g (13.1 mmol) of mandelic acid were slowly added to 10 cm^3 of acetyl chloride cooled to 0 °C in an ice bath. When the addition was complete the resulting solution was refluxed for 1 h. The solution was cooled and poured onto 250 cm^3 of ice. The product precipitated as a white solid which was recrystallised from water. ν_{max}(Nujol)/cm^{-1} 3100, 1760 and 1620; δ($CDCl_3$) 2.25 (3 H, s, CH_3), 3.35 (1 H, s, CO_2H), 6.75 (1 H, s, CH) and 7.50 (5 H, m, Ph); *m/z* (EI, chloroform) 150 (M^+).

α-*Methoxycarbonylbenzyl 2-phenylethanoate.* Mandelic acid methyl ester was synthesized using diazomethane and was then condensed with phenylacetic acid using DCCI. ν_{max}/cm^{-1} 1738; δ_H($CDCl_3$) 3.65 (3 H, s, CH_3), 3.80 (2 H, s, CH_2), 6.00 (1 H, s, CH), 7.35 (10 H, m, Ph); GC–MS (EI, chloroform) 284 (M^+).

The Transferification Reaction between Ethyl Propanoate and 3-Methoxybutanol Catalysed by Porcine Liver Esterase.—5.0 cm^3 of stock PLE solution (100 mg/9.1 cm^3) were added to 50 cm^3 of pH 7.4 phosphate buffer solution and the solution freeze-dried to give a white solid (264 mg). 1 g (9.8 mmol) of ethyl propanoate was added to 30 cm^3 of heptane, 3.0 g (19 mmol) of 3-methoxybutanol were added followed by 100 mg of the enzyme preparation. The following reaction was then monitored by HPLC. The reaction was terminated when all of the ethyl propanoate had been consumed. To the solution were added 100 cm^3 of ether which was then washed three times with water, followed by drying and removal of the ether by rotary evaporation. The unchanged ethyl propanoate was then removed by short-path distillation to leave the product as a viscous oil. This oil was then dissolved in 5.0 cm^3 of acetonitrile and the solution was treated with 5.0 cm^3 of 1.0 mol dm^{-3} sodium hydroxide solution with the alcohol being recovered by short path distillation. All the NMR data were found to be consistent with the proposed structure and agreed with the literature values. The optical rotations of the products were as follows: $[\alpha]_D^{20} = -15.9$ (c = 3.1, $CDCl_3$) ester; $[\alpha]_D^{20} = -16.3$ (c = 1.0, $CDCl_3$) alcohol.

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