

# Activity and enantioselectivity of serine proteases in transesterification reactions in organic media

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The activity and enantioselectivity of  $\alpha$ -chymotrypsin and subtilisin Carlsberg in the transesterification of a series of *N*-acetyl-alanine and -phenylalanine esters in cyclohexane were investigated at constant water activity, both in the absence and presence of 18-crown-6. Isosteric variation of the leaving ability of the alcoholate group in the substrates by fluoro substitution provided information about the acylation step of the reaction. For less reactive esters, the rate of acylation is determined by expulsion of the leaving group, whereas for activated esters the rate is dictated by a physical step, most likely a relatively slow conformational change of the enzyme. This makes the enantioselectivity of the enzymes strongly dependent of the substrate activity and the composition of the reaction medium. The catalytic effect of 18-crown-6, observed for all reactions, can be attributed to an enhanced enzyme-substrate binding and an increase of the rate of acylation.

## Introduction

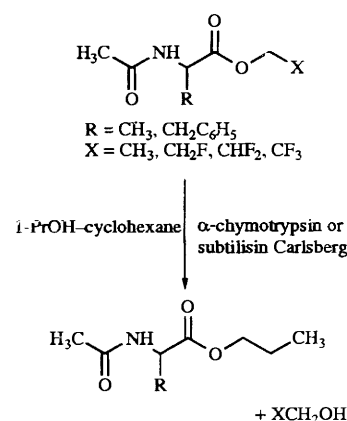
In the last decade, non-aqueous enzymology has received considerably increased attention. It is now generally accepted that enzymes in organic media can enlarge the application of biocatalysts in synthetic chemistry.<sup>1-5</sup> However, an important reason why this approach is still hardly used in commercial processes is the strongly reduced activity of the enzymes under anhydrous conditions. We have reported that one method to enhance the enzyme activity by one to two orders of magnitude is the addition of crown ethers to the enzyme suspension in organic solvent.<sup>6-9</sup> A macrocyclic interaction is responsible for the effect, since an open chain analogue, pentaglyme, does not change the enzyme activity.<sup>7</sup>

This paper describes the effects of substrate activation and addition of 18-crown-6 on the activity and enantioselectivity of  $\alpha$ -chymotrypsin and subtilisin Carlsberg in the transesterification of a series of *N*-acetyl-D,L-alanine and *N*-acetyl-D,L-phenylalanine esters in cyclohexane-propan-1-ol. In this study the reactivity of a series of serine protease substrates is *isosterically* varied (Scheme 1). The similar shape and polarity within a series of substrates of different reactivity eliminates effects due to differences in enzyme-substrate (ES) binding and makes kinetically visible that for activated esters a relatively slow physical process is determining the rate of conversion of the ES complex into the acyl-enzyme intermediate. The kinetic data also reveal that by addition of 18-crown-6 (18-C-6) to the organic medium both the enzyme-substrate binding and acylation rate can be increased.

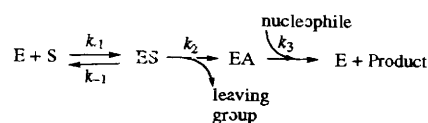
## Results

The mechanism of action of serine proteases can be represented by a minimum of a three-step process, as is given in Scheme 2.<sup>10a</sup>

Binding of the substrate to the active site gives the enzyme-substrate complex (ES) from which an acyl-enzyme intermediate (EA) is formed by nucleophilic attack of the serine hydroxy group to the carbonyl group of the substrate and expulsion of the leaving group. The acylated enzyme is subsequently



Scheme 1 Transesterification of *N*-acetylalanine and *N*-acetylphenylalanine ethyl esters by propan-1-ol (1-PrOH)



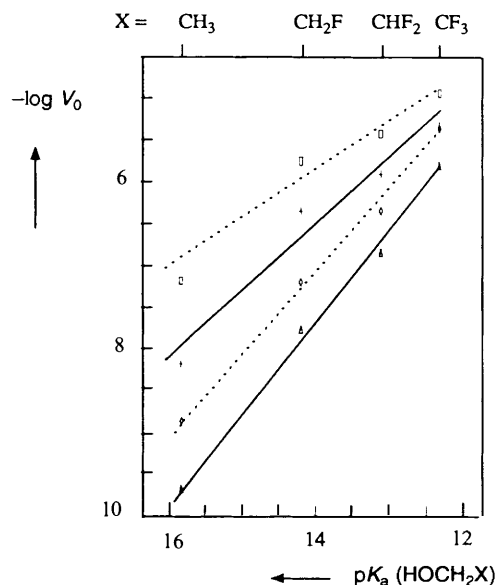
Scheme 2 General acylation-deacylation mechanism of serine proteases

deacylated by attack of a nucleophile from the reaction medium. Under the organic solvent conditions studied all reactions were found to be first order in enzyme and substrate concentration and the initial rate ( $V_0$ ) for substrate conversion can be represented by the second order rate equation  $V_0 = k_2/K_S[E_0][S]$ , in which  $K_S (= k_{-1}/k_1)$  is the dissociation constant of the ES complex and  $k_2$  involves all chemical and physical processes related to the acylation of the enzyme.<sup>‡</sup> The addition of enzyme inhibited with benzylsulfonyl fluoride has no influence on the rate of the transesterification reactions.

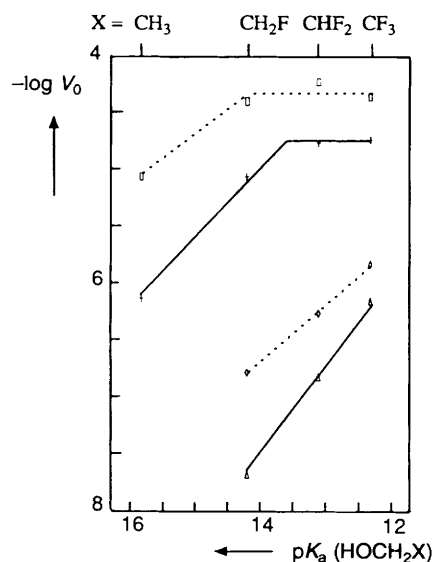
Figs. 1-4 give the activity of  $\alpha$ -chymotrypsin and subtilisin Carlsberg towards a series of *N*-acetyl-D,L-alanine and *N*-

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<sup>‡</sup> In this reaction, where  $k_{-1} \gg k_2$ , the second order rate constant of the process  $k_2/K_S$  is equal to the second order rate constant  $k_{\text{cat}}/K_M$  derived from the well-known Michaelis-Menten equation.<sup>10a</sup>



**Fig. 1** Effect of the base strength of the leaving group of *N*-acetyl-D,L-alanine esters on the activity of  $\alpha$ -chymotrypsin in the transesterification with propan-1-ol in cyclohexane. Standard deviations in  $\log V_0$  are less than 0.06, except for the L-OC<sub>2</sub>H<sub>5</sub> ester (0.13), the L-OCH<sub>2</sub>CHF<sub>2</sub> ester (0.11), the D-OCH<sub>2</sub>CHF<sub>2</sub> ester (0.11), and the D-OCH<sub>2</sub>CH<sub>2</sub>F ester + 18-C-6 (0.07). + =  $V_0(L)$ ;  $\Delta$  =  $V_0(D)$ ;  $\square$  =  $V_0(L)$  + 2 mmol dm<sup>-3</sup> 18-C-6;  $\diamond$  =  $V_0(D)$  + 2 mmol dm<sup>-3</sup> 18-C-6.



**Fig. 2** Effect of the base strength of the leaving group of *N*-acetyl-D,L-phenylalanine esters on the activity of  $\alpha$ -chymotrypsin in the transesterification with propan-1-ol in cyclohexane. Standard deviations in  $\log V_0$  are less than 0.06, except for the L-OCH<sub>2</sub>CH<sub>2</sub>F ester (0.10), the D-OCH<sub>2</sub>CH<sub>2</sub>F ester (0.20), the D-OCH<sub>2</sub>CH<sub>2</sub>F + 18-C-6 (0.25), and the D-OCH<sub>2</sub>CHF<sub>2</sub> ester + 18-C-6 (0.10). + =  $V_0(L)$ ;  $\Delta$  =  $V_0(D)$ ;  $\square$  =  $V_0(L)$  + 2 mmol dm<sup>-3</sup> 18-C-6;  $\diamond$  =  $V_0(D)$  + 2 mmol dm<sup>-3</sup> 18-C-6.

acetyl-D,L-phenylalanine esters with ethoxy, 2-fluoroethoxy, 2,2-difluoroethoxy, and 2,2,2-trifluoroethoxy leaving groups in the transesterification of these substrates by propan-1-ol in cyclohexane at a fixed water activity. The influence of 2 mmol dm<sup>-3</sup> 18-crown-6 on these reactions is also included in these figures. Initial rates have been determined from racemic mixtures under steady state conditions in which both enantiomers compete for the free enzyme. The enzyme specificity thus obtained is the most relevant for biological systems.<sup>10d</sup> The  $V_0^L$  and  $V_0^D$  values were calculated from the measured rate  $V_0^{D,L}$  after the enantioselectivity was established, using the relation given by Chen *et al.*<sup>11</sup> It should be noted that this approach yields the relative rate values for the two enantiomers which can deviate from those obtained from measurement of the pure enantiomers.

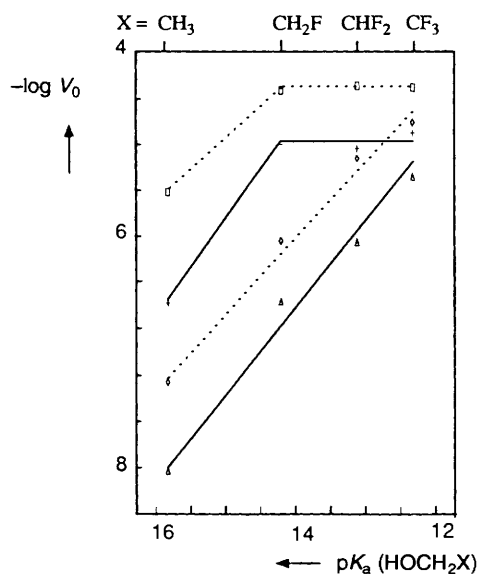
#### Effect of leaving group variation on the enzyme activity

In Figs. 1–4 the initial rate ( $\log V_0$ ) is given as a function of the leaving ability of the alcoholate group [expressed as the  $pK_a$  values for the conjugate alcohols, which are 15.83 (ethanol), 14.20 (2-fluoroethanol), 13.11 (2,2-difluoroethanol), and 12.32 (2,2,2-trifluoroethanol)<sup>12</sup>]. The reactivity of the amino acid esters is systematically varied by isosteric variation of the electronic effect of the leaving group. Due to the comparable size of hydrogen and the fluoro substituents in the leaving group the steric effect of the substrates will not greatly differ. In addition, the leaving groups have similar polarities (the polarity parameter  $\log P$  is 0.702 for the CH<sub>3</sub> group and 0.757 for the CF<sub>3</sub> group<sup>13</sup>). Consequently, within the series of isosteric amino acid esters the binding constant  $K_S$  may be expected to remain nearly constant. The change in initial rate, which is linearly proportional to  $k_2/K_S$ , gives therefore direct information about the sensitivity of the  $k_2$  step for substrate activation. If expulsion of the leaving group is determining the rate of the  $k_2$  step, then the reaction will be strongly enhanced by the inductive effect of the fluoro atoms. On the other hand, if a physical process such as a conformational change of the enzyme is determining the rate of the  $k_2$  step, then the reaction will become independent of substrate activation.

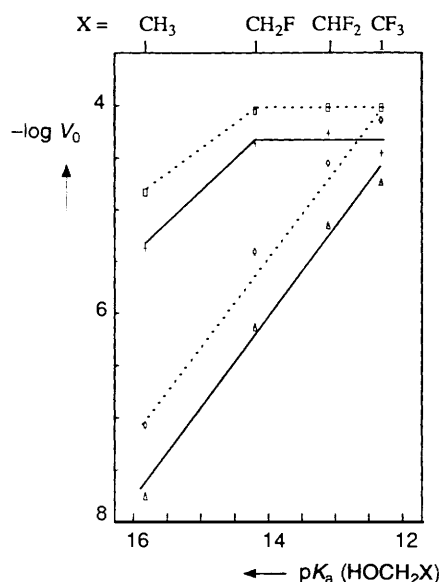
**$\alpha$ -Chymotrypsin and D,L-alanine esters.** Fig. 1 shows that for the  $\alpha$ -chymotrypsin catalysed transesterification of the D,L-alanine esters  $\log V_0$  increases linearly upon increase of the leaving ability of the alcoholate group. The linear behaviour indicates that no change of mechanism is occurring in the rate-limiting step under these conditions. The slope of the free energy relation for the L-enantiomers gives a reaction constant  $\rho$  of  $-0.8 (\pm 0.1)$ , and this value is within the range of  $\rho$  values observed for  $\alpha$ -chymotrypsin catalysed reactions of L-substrates in water in which leaving group expulsion is rate-limiting in the acylation process ( $\rho$   $-0.6$  to  $-1.0$ ).<sup>14</sup> The more negative  $\rho$  value of  $-1.1$  found for the D-enantiomers indicates that for these substrates a larger fraction of negative charge is developed in the transition state, reflecting the less favourable positioning of these unnatural enantiomers in the active site of the enzyme.

**$\alpha$ -Chymotrypsin and D,L-phenylalanine esters.** Fig. 2 gives the activity of  $\alpha$ -chymotrypsin in the transesterification of the *N*-acetyl-D,L-phenylalanine esters. For these more reactive esters a distinctly different response on leaving group variation is found. The change of the leaving group in the L-phenylalanine ester from ethoxy to 2-fluoroethoxy gives a  $\rho$  value of  $-0.4$ , which points to an earlier transition state for these substrates compared with the alanine esters. This is in accordance with the higher specificity of this enzyme towards phenylalanine substrates.<sup>15</sup> Further activation of the leaving group leads to a plateau value in the reaction rate, indicating that for these relatively fast reacting substrates a process other than expulsion of the leaving group becomes rate limiting during the acylation process. Also for these activated substrates the reaction rate is found to be first order in substrate concentration. Most likely, a physical process, like a conformational change of the enzyme determines the rate of acylation ( $k_2$ ) for these activated esters.

In the series of D-enantiomers a linear increase of the reaction rate upon activation of the substrate is found and expulsion of the leaving group remains rate determining in the acylation process. The rate of the D-ethyl ester is not included in the figure



**Fig. 3** Effect of the base strength of the leaving group of *N*-acetyl-D,L-alanine esters on the activity of subtilisin Carlsberg in the transesterification with propan-1-ol in cyclohexane. Standard deviations in  $\log V_0$  are less than 0.06, except for the D-OCH<sub>2</sub>CH<sub>3</sub> ester + 18-C-6 (0.14), and the D-OCH<sub>2</sub>CHF<sub>2</sub> ester + 18-C-6 (0.10). + =  $V_0(L)$ ;  $\Delta$  =  $V_0(D)$ ;  $\square$  =  $V_0(L)$  + 2 mmol dm<sup>-3</sup> 18-C-6;  $\diamond$  =  $V_0(D)$  + 2 mmol dm<sup>-3</sup> 18-C-6.



**Fig. 4** Effect of the base strength of the leaving group of *N*-acetyl-D,L-phenylalanine esters on the activity of subtilisin Carlsberg in the transesterification with propan-1-ol in cyclohexane. Standard deviations in  $\log V_0$  are less than 0.06. + =  $V_0(L)$ ;  $\Delta$  =  $V_0(D)$ ;  $\square$  =  $V_0(L)$  + 2 mmol dm<sup>-3</sup> 18-C-6;  $\diamond$  =  $V_0(D)$  + 2 mmol dm<sup>-3</sup> 18-C-6.

because it could not be determined with sufficient accuracy from the conversion of the racemic mixture due to the relatively high preference of the enzyme for the L-enantiomer.

**Subtilisin Carlsberg and D,L-alanine esters and D,L-phenylalanine esters.** For subtilisin Carlsberg, leaving group activation in the substrates leads to a plateau value in the transesterification rate for both the L-alanine esters and the L-phenylalanine esters. For the D-enantiomers such a plateau value is not observed and leaving group expulsion is rate-limiting in the enzyme acylation by the D-esters (Figs. 3 and 4).

**Table 1** Effect of 2 mmol dm<sup>-3</sup> 18-crown-6 on the initial rate of the transesterification of amino acid esters by  $\alpha$ -chymotrypsin and subtilisin Carlsberg

	$V_0(+2 \text{ mmol dm}^{-3} \text{ 18-C-6})/V_0$	
	$\alpha$ -Chymotrypsin	Subtilisin Carlsberg
<i>N</i> -Ac-L-Ala-OCH <sub>2</sub> CH <sub>3</sub>	10 ± 5	15.8 ± 0.8
<i>N</i> -Ac-L-Ala-OCH <sub>2</sub> CF <sub>3</sub>	2.6 ± 0.4	3.1 ± 0.2
<i>N</i> -Ac-L-Phe-OCH <sub>2</sub> CH <sub>3</sub>	12 ± 1	3.5 ± 0.6
<i>N</i> -Ac-L-Phe-OCH <sub>2</sub> CF <sub>3</sub>	2.4 ± 0.1	2.7 ± 0.1

#### Effect of 18-crown-6 on the transesterification reactions

Addition of 2 mmol dm<sup>-3</sup> 18-crown-6 enhances the rate of  $\alpha$ -chymotrypsin and subtilisin Carlsberg in reaction with all esters (Figs. 1–4). Generally, the transesterification of the unactivated ethyl esters shows more enhancement than that of the activated fluoro esters (Table 1).

The crown ether activation is specific for organic media since we found that addition of 18-crown-6 does not have any effect on the activity of these enzymes in aqueous solution. Active site titrations of suspended  $\alpha$ -chymotrypsin in cyclohexane–1 mol dm<sup>-3</sup> propan-1-ol, both in the presence and in the absence of 2 mmol dm<sup>-3</sup> 18-crown-6, showed comparable percentages (11 ± 3%) of accessible active sites. This excludes the possibility that the 18-crown-6 activation is due to an increase of the effective enzyme concentration. Also interaction of crown ether with buffer cations present in the enzyme preparation is of minor importance, since the crown ether activation shows only slight dependency of the type of buffer cation used in the lyophilization process.<sup>7</sup> We have investigated whether interaction of the crown ether with the enzyme backbone could increase the enzyme flexibility, resulting in an increased catalytic activity of the enzyme. However, time resolved fluorescence depolarization studies on  $\alpha$ -chymotrypsin labelled with an anthraniloyl group in the active site revealed that the rotational mobility of this probe is not increased by addition of crown ether to the organic medium.<sup>8,16a</sup>

These results indicate that 18-crown-6 must be directly involved in the enzymatic transesterification process by influencing enzyme-substrate binding (lowering of  $K_S$ ) and/or increase of processes involved in the  $k_2$  step.

#### Effect of leaving group variation on the enzyme enantioselectivity

Comparison of the  $V_0$  values of the L- and D-enantiomers shows that the enantioselectivity of the enzymes becomes more strongly reduced for the more activated esters. The increased leaving ability of the alcoholate group causes a decrease in the discrimination of the enzyme for the two enantiomers in the acylation process. This is kinetically reflected in the convergent lines of  $V_0^L$  and  $V_0^D$  for the  $\alpha$ -chymotrypsin-catalysed reaction of the alanine ester in Fig. 1. Since  $K_S$  should remain approximately constant upon variation of the leaving group, the decreased enantioselectivity is due to a decrease in the  $k_2^L/k_2^D$  ratio. The plateau value in  $V_0$  observed in Figs. 2–4 for the activated L-esters causes a further reduction of the enzyme enantioselectivity, since the rate of the D-enantiomers continues to increase upon activation of the substrate. The absolute rate of the physical step in the enzyme acylation related to the plateau value is of large influence on the ultimate enantioselectivity of the enzyme.

The rate of this physical process is strongly dependent on the solvent composition. For example, for the reaction of subtilisin

Carlsberg with *N*-acetyl-D,L-phenylalanine 2,2,2-trifluoroethyl ester a decrease of the propan-1-ol concentration in the reaction medium from 1 mol dm<sup>-3</sup> to 5 mmol dm<sup>-3</sup> reduces  $V_0^L$  eight times more than  $V_0^D$ . As a result, the enantioselectivity,  $E = (k_2 K_S)^L / (k_2 K_S)^D$  drops from 2.4 to 0.37, implying that the enantioselectivity of the enzyme under these conditions is inverted.<sup>16b</sup> When hydrogen-bonding cosolvents were added to the cyclohexane medium containing 5 mmol dm<sup>-3</sup> propan-1-ol, the rate of the L-enantiomer increases again, while the rate of the D-enantiomer remains almost constant. The  $V_0^L$  increased upon addition of 0.33 mol dm<sup>-3</sup> cosolvent in the order butyronitrile < propionitrile < propan-1-ol < acetonitrile < ethanol. In the presence of 0.33 mol dm<sup>-3</sup> acetonitrile as the additive, the enantioselectivity of subtilisin Carlsberg is 15.1, which is 41 times higher than in its absence and 6 times higher than in the presence of 1 mol dm<sup>-3</sup> propan-1-ol.

### Discussion

The activity of  $\alpha$ -chymotrypsin and subtilisin Carlsberg in cyclohexane-1 mol dm<sup>-3</sup> propan-1-ol is reduced by several orders of magnitude compared with water as the reaction medium. This is a generally observed phenomenon for enzyme reactions in organic solvents.<sup>2,3</sup> As no saturation kinetics is observed, the initial reaction rate can be described by the second order rate equation  $V_0 = k_2 K_S [E_0][S]$ , according to the kinetic Scheme 2. Based on this kinetic scheme, the lower enzyme activity in organic media can in principle be attributed to a decreased  $k_2$ , an increased  $K_S$ , and/or to a much lower effective concentration of the suspended enzyme ( $E_0$ ) compared with water as the solvent. The active site titrations have shown that the latter effect cannot be responsible for the dramatically lower enzyme activity since in the organic medium still 11 ( $\pm 3$ )% of the active sites of the enzyme are accessible for the substrate.<sup>8</sup> The major contribution must therefore originate from an increased dissociation constant ( $K_S$ ) and/or a reduction of the processes involved in the  $k_2$  step. An increase of  $K_S$  in organic solvent can be expected due to a larger stabilisation of the free substrate in the organic solvent<sup>17</sup> and a higher free energy of transfer of water molecules from the active site to the bulk solution upon binding of the substrate.<sup>18</sup> Indeed, high  $K_S$  values have been recently reported for subtilisin Carlsberg towards amino acid esters in organic media.<sup>19</sup> In addition, the binding of the substrate may be reduced by the restricted flexibility of the enzyme in organic solvent, hampering the formation of energetically favourable enzyme-substrate interactions.<sup>16a</sup> A reduced enzyme flexibility may also lead to a significant decrease of the rate of the processes involved in the  $k_2$  step due to restrictions in adopting the conformational changes necessary before or during the acylation process.

Comparison of the log  $V_0$  values of the  $\alpha$ -chymotrypsin-catalysed reaction of the ethyl esters of L-alanine (Fig. 1) and L-phenylalanine (Fig. 2) shows that the transesterification proceeds 115 times faster for the phenylalanine ester. The  $k_2 \cdot K_S$  values for these two esters are approximately 0.01 and 1 dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup>, respectively. In water, however,  $\alpha$ -chymotrypsin is ca. 25 000 times more active towards *N*-acetyl-L-phenylalanine ethyl ester than towards its alanine analogue ( $k_2 \cdot K_S$  values are 1.7 and 42 000 dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup>, respectively<sup>20</sup>). This large preference for the phenylalanine ester in water has been attributed to hydrophobic interactions of the phenyl group which directs the ester carbonyl in a more productive mode in the oxyanion hole of the active site.<sup>10b</sup> In organic solvent, however, hydrophobic interactions as a driving force for substrate binding are much reduced which explains the much smaller difference in activity between both substrates.<sup>10c</sup>

Isosteric variation of the leaving ability of the alcoholate group has given important mechanistic information about the rate-limiting steps in the enzyme acylation and the enantioselectivity of the reactions. The variation of the leaving ability of the alcoholate group of the esters is reflected in the acylation process. The linear increase of the rate with increasing nucleofugicity of the alcoholate group indicates that expulsion of the leaving group in the acylation process is rate determining for the D-enantiomers and for the less activated L-esters. The  $\rho$ -values found from the slopes of plots of  $-\log V_0$  vs.  $pK_a$  of the conjugate leaving groups are in the range found for  $\alpha$ -chymotrypsin-catalysed reactions in water in which expulsion of the leaving group ( $\rho = -0.6$  to  $-1.0$ )<sup>14</sup> is rate determining. The plateau values observed for the activated L-esters in Figs. 2-4 show that the transesterification reaction becomes independent of the substrate reactivity. Also for the activated substrates no saturation kinetics could be observed upon increase of the substrate concentration. Therefore, the observed rate values in the plateau region are still those of the second order rate process of the reaction, i.e.  $V_0 = k_2 K_S [E][S]$ , and do not reflect the rate of deacylation of the enzyme ( $k_3$ ). However, for the activated esters the chemical steps in the acylation process have become relatively fast and apparently another, physical process becomes rate determining.

In principle, a number of physical processes can be considered as being responsible for the plateau value in catalytic activity: (i) external diffusion limitation of the substrate, (ii) internal diffusion limitation of the substrate, (iii) a slow conformational change of the enzyme in the acylation step, possibly accompanied by a further dehydration of the active site.<sup>21</sup> If slow external diffusion of the substrate is rate limiting, due to the formation of stagnant layers around the enzyme suspension particles, then the reaction rate should be increased by vigorous stirring. This is not observed, which makes slow external diffusion as the rate-limitation process unlikely. Also internal diffusion limitation of the substrate, i.e. slow diffusion of the substrate through the pores of the enzyme suspension, could be ruled out by experiments comparing the activity of several different enzyme preparations.<sup>22</sup> Active  $\alpha$ -chymotrypsin and inactivated  $\alpha$ -chymotrypsin (inhibited with benzylsulfonyl fluoride) were mixed in various ratios before lyophilization, and the activities of the different preparations in the transesterification of *N*-Ac(L)PheOCH<sub>2</sub>CF<sub>3</sub> in cyclohexane-1 mol dm<sup>-3</sup> propan-1-ol were compared with the activities towards the specific substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide in water.<sup>23,24</sup> A linear correlation between the activities of the various mixed preparations in aqueous and organic solvent was found (Fig. 5). Since diffusion limitation does not play a role in the homogeneous aqueous solution, the linear behaviour of these mixed enzyme preparations proves that this process is also not occurring in the organic enzyme suspension. Comparable results were found in the case of subtilisin Carlsberg (data not shown).

Taking into account these experimental results, the most likely explanation for the cause of the plateau values in reaction rates of the activated L-esters is a relatively slow conformational

§ Diffusion-controlled encounter of enzyme and substrate is not very likely to be rate limiting, considering the low turnover rates which are observed. For  $\alpha$ -chymotrypsin in water, diffusion-controlled encounter of enzyme and substrate requires  $k_2 \cdot K_S$  values of approximately 10<sup>7</sup>-10<sup>9</sup> dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup>. In the organic reaction medium, the  $k_2 \cdot K_S$  values corresponding to the  $V_0$  values are ca. 10 dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup> or lower for  $\alpha$ -chymotrypsin, and 10<sup>1</sup>-10<sup>2</sup> dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup> for the subtilisin-catalysed reactions of the activated L-esters. This indicates that diffusion-controlled encounter of enzyme and substrate is clearly not a rate limiting step under these conditions.<sup>21</sup>

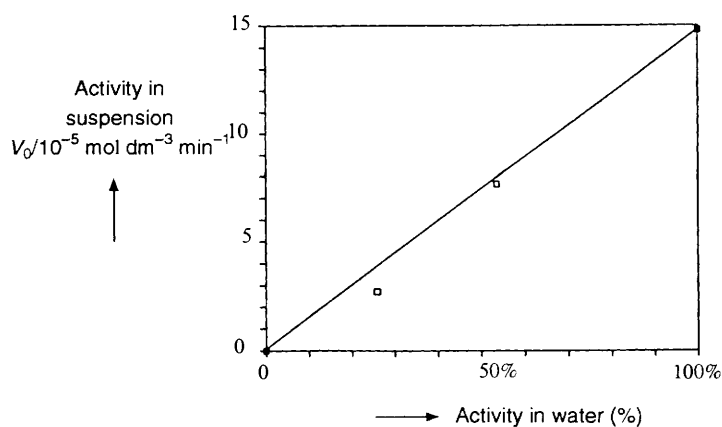


Fig. 5 Relation between the catalytic activity of  $\alpha$ -chymotrypsin in cyclohexane-1 mol dm<sup>-3</sup> 1-PrOH and the activity in aqueous solution (0.1 mol dm<sup>-3</sup> Tris-HCl, pH 8.0, 25 °C)

change of the enzyme during the acylation process (reflected in a relatively low, constant,  $k_2$ ).<sup>¶</sup>

Addition of 18-crown-6 increases the enzyme activity for all substrates. Therefore, 18-crown-6 influences both the reactions in which expulsion of the leaving group is the rate determining step in the enzyme acylation as well as reactions in which this process is controlled by a physical step. It was found earlier that the enzyme activating effect of crown ethers of various structures correlates with their water binding capability. In addition, small crown ethers of intermediate polarity, like 18-crown-6, have a larger activating effect than large lipophilic crown ethers, like decyl 18-crown-6 and dicyclohexyl 18-crown-6.<sup>7</sup> This behaviour thus well corresponds with their ability to enter the active site of the enzyme and to bind water molecules at this site. Formation of the ES complex must proceed with (partial) dehydration of the active site of the enzyme, a process which is more unfavourable in organic solvent than in water. The 18-crown-6 can assist in the dehydration process by acting as a water carrier between the relatively polar active site and the apolar reaction medium. The log  $P$  value of 18-crown-6 is about zero<sup>26</sup> which indicates that there may be a rather balanced distribution of this crown ether between both pseudophases.‡ Therefore an important role of 18-crown-6 is most likely to act as a water complexing agent to promote the transport of water molecules from the active site, causing an increased binding of substrate to the enzyme active site (lowering of  $K_S$ ). The decrease of the dissociation constant  $K_S$  results in higher  $V_0$  values for all esters.\*\*

Martinek and co-workers<sup>27</sup> have concluded from their study of the thermodynamics of  $\alpha$ -chymotrypsin with amino acid esters that during the acylation process a further dehydration of the active site takes place. This dehydration may also be assisted

by 18-crown-6 present in the active site, causing an increase of the rate constant  $k_2$  of the acylation process. This additional catalytic role of 18-crown-6 may be reflected in the relatively larger rate enhancing effect of 18-crown-6 found for the reactions of unactivated ethyl esters (Table 1). The second order rate constant  $k_2/K_S$  increases by both an increase of  $k_2$  and a decrease of  $K_S$  in these cases. The data in Table 1 show that the effect of crown ether is strongly dependent on the leaving group, indicating that at least a part of the crown ether activation is expressed in  $k_2$ .

The enantioselectivity of the enzymes turned out to be strongly dependent on the leaving ability of the alcoholate group in the ester substrates. Only a few papers have addressed the relation between enantioselectivity and leaving group ability.<sup>15b,28</sup> It has been reported that the enantioselectivity of  $\alpha$ -chymotrypsin and subtilisin Carlsberg in aqueous solution is much lower in reactions with *p*-nitrophenyl esters than with the corresponding methyl esters. The decreased necessity of the enzyme to act as a general acid catalyst in the expulsion of the *p*-nitrophenolate leaving group has been proposed as a reason for this phenomenon.<sup>28</sup> However, as pointed out by Polgar and Fejes<sup>28</sup> and Silver and Matta,<sup>15b</sup> the different binding properties of the leaving groups of these substrates (methyl *vs.* 4-nitrophenyl) complicate the assignment of the different enantioselectivities exclusively to an electronic effect.

The isosteric variation of the electronic properties of the leaving group by fluoro substitution gives direct information about the effect of substrate activation on the acylation process. For the reaction of the *N*-acetylalanine ethyl esters catalysed by  $\alpha$ -chymotrypsin (Fig. 1) the convergency of the lines of log  $V_0$  *vs.*  $pK_a$  for both enantiomers shows that the enantioselectivity decreases upon activation of the esters. This may be attributed to the decreased role of His-57 to act as general acid catalyst in the expulsion of the leaving group.<sup>28</sup> A sharp reduction of the enantioselectivity of the enzymes is observed in the region where the rate of the L-enantiomer is determined by a relatively slow physical process which is most likely a conformational change during the acylation process. The rate of this physical process is very sensitive to the concentration and structure of hydrogen bonding species in solution. A change of the reaction medium by decreasing the propan-1-ol concentration from 1 mol dm<sup>-3</sup> to 5 mmol dm<sup>-3</sup> gives a considerably

¶ For subtilisin Carlsberg, insensitivity for leaving group variation in activated substrates has a precedent in aqueous solution. Matta and co-workers found fairly constant  $k_2/K_S$  values for the hydrolysis of a series of differently *para*-substituted phenyl esters of *N*-(methoxycarbonyl)phenylalanine, although the base strength of the leaving groups differed markedly. The observed  $k_2/K_S$  values (*ca.*  $3 \times 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ ) were approximately  $10^3$  times lower than the diffusion-controlled encounter frequency. No significant change in  $k_2/K_S$  was observed when the reaction was performed in D<sub>2</sub>O, which led the authors to the conclusion that the levelling off of the enzyme activity is due to an isotopically 'silent' physical process, like a conformational change.<sup>25</sup>

‡ In order to get more experimental evidence that 18-crown-6 can indeed penetrate into the active site of the enzymes, the substrate *N*-acetylphenylalanine 18-crown-6-methyl ester was synthesized.<sup>8</sup> Both  $\alpha$ -chymotrypsin and subtilisin Carlsberg were able to transesterify this ester in cyclohexane-propan-1-ol, indicating that the 18-crown-6 moiety can enter the active sites of these enzymes.

\*\* Based on this observation, a rate-limiting dehydration of the active site in the substrate binding step can be ruled out as an alternative explanation for the plateau values in reaction rate. If dehydration would be rate limiting for the activated esters, an increase of the enantioselectivity would be expected upon addition of 18-crown-6 to the reaction medium. However, a decrease of the enantioselectivity was observed for the trifluoroethyl esters.

stronger reduction of the rate of the L-enantiomer than of the D-enantiomer, with the result that the enzyme enantioselectivity is inverted under these conditions. Addition of cosolvents with hydrogen-bonding properties tune the enantioselectivity primarily by influencing the rate of the physical process.<sup>††</sup> It is well documented that addition of water increases the flexibility of enzymes equilibrated at a low water activity, resulting in a higher enzyme activity.<sup>10a,29</sup> The high sensitivity of  $V_0^L$  of the suspended enzyme for additives with small molecular volume and hydrogen-bonding properties strengthens the view that these water-like properties induce flexibility and that the physical rate-limiting step in the acylation process, as shown in Figs. 2–4, is representing a conformational change of the enzyme.

### Conclusions

Isosteric variation of the basicity of the leaving alcoholate group in the transesterification of a series of *N*-acetyl-D,L-alanine ethyl esters and *N*-acetyl-D,L-phenylalanine ethyl esters has resulted in useful mechanistic information about the acylation process and the enantioselectivity of the  $\alpha$ -chymotrypsin and subtilisin Carlsberg catalysed reactions. For the slowly reacting D-enantiomers and for the L-ethyl esters having a relatively poor leaving group, expulsion of the leaving group is the rate determining step in the acylation process. For most of the activated L-esters, the enzyme acylation is limited by a relatively slow physical process, most likely representing a slow conformational change of the enzyme. Addition of 18-crown-6 to the organic reaction medium enhances the reaction rate for all esters and this effect can be reconciled with facilitated water transport from the active site by 18-crown-6, which improves substrate binding and facilitates water release in the acylation process. Since the rates for the L- and D-enantiomers have a different sensitivity for the concentration and structure of hydrogen-bonding species in the reaction medium, the enzyme enantioselectivity can be varied significantly by the reaction medium. These results show that it is possible to optimize the enantioselectivity of serine proteases towards particular substrates by selection of the proper leaving groups and reaction medium.

### Experimental

<sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Bruker AC 250 spectrometer with Me<sub>4</sub>Si as an internal standard. *J* Values are given in Hz. *N*-Acetyl-D,L-alanine and *N*-acetyl-D,L-phenylalanine were from Sigma. 2,2,2-Trifluoroethanol, 2-fluoroethanol, and cyclohexane, uvasol grade, were from Merck and 2,2-difluoroethanol was from Fluorochem. 18-Crown-6 (99.5 + %) was from Aldrich.  $\alpha$ -Chymotrypsin (Boehringer) and subtilisin Carlsberg (Sigma) were dissolved in 0.1 mol dm<sup>-3</sup> KH<sub>2</sub>PO<sub>4</sub> and 0.02 mol dm<sup>-3</sup> NaH<sub>2</sub>PO<sub>4</sub> buffer pH 7.8, respectively, frozen with liquid N<sub>2</sub> and lyophilized. Protein contents were 40 and 75%, respectively.

Conversions of the transesterification reactions were determined from the change of peak areas of the substrate and product enantiomers with a Varian 3400 GC equipped with fused capillary column (J & W liquid phase: DB-5<sup>+</sup>, film thickness: 25  $\mu$ m, 15 m  $\times$  0.32 mm), SPI injector and FID detector. The temperature programme used was: 2 min at 45 °C,

then temperature increase of 30 °C min<sup>-1</sup> to 190 °C, at which the temperature was held for 4 min. Enantioselectivities were measured with a Varian 3600 GC equipped with a split/splitless injector (250 °C), a Chirasil-Val column (0.32 mm  $\times$  50 m) and a FID detector (250 °C). Temperature programme for the alanine esters: 100–115 °C (heating 1 °C min<sup>-1</sup>). For the phenylalanine esters: 150–170 °C (heating 1 °C min<sup>-1</sup>). The enantiomers of all the substrates and products could be baseline separated under these conditions.

Transesterification experiments were carried out with 1 mg cm<sup>-3</sup> enzyme and 5 mmol dm<sup>-3</sup> of substrate. Reactions were started by mixing 2–3 mg of enzyme powder with 2–3 cm<sup>3</sup> of solvent containing the ester, propan-1-ol and 0.5 mm<sup>3</sup> cm<sup>-3</sup> tridecane (Janssen) as internal standard followed by sonification (5–10 s). Periodically, aliquots were taken by a syringe, filtered through a 0.5  $\mu$ m Millex FH<sub>4</sub> filter (Milipore) and analysed by GC. All enzyme reactions have been performed at least twice on a 2–5 cm<sup>3</sup> scale in 7 cm<sup>3</sup> vials (Pierce) with magnetic stirring (350 rpm) at 25 °C in cyclohexane–1 mol dm<sup>-3</sup> propan-1-ol.

Since the level of hydration of a suspended enzyme has a considerable effect on its activity in organic solvent, all reagents have been previously equilibrated above a saturated LiCl solution at 5 °C for at least 24 h.<sup>30</sup> This ensured that the reactions proceeded at a constant water activity, which was at the same time sufficiently low to suppress enzymatic hydrolysis of the substrates.

The enantioselectivity in each reaction, calculated by using the formula given by Chen *et al.*<sup>11</sup> was estimated from at least two determinations at low conversions (generally < 10%). In this way at least four initial rate values for each enantiomer were obtained. Standard deviations are given in the legends of the Figs. 1–4. Initial rates were expressed per mg of enzyme preparation. A slow nonenzymatic transesterification reaction was only found in the  $\alpha$ -chymotrypsin-catalysed reactions of the alanine esters in cyclohexane–1 mol dm<sup>-3</sup> propan-1-ol containing 2 mmol dm<sup>-3</sup> 18-crown-6 (Fig. 1). In this case the percentages of the blank reaction of the trifluoroethyl, difluoroethyl, fluoroethyl and ethyl ester were 8.6, 8.4, 5 and 2%, respectively. The initial rates and enantioselectivities have been corrected for this blank reaction. No spontaneous racemization of the esters under the tested conditions occurred, as was proven with optically enriched *N*-AcPheOCH<sub>2</sub>CF<sub>3</sub> ester. Enantiomeric excesses (ee) of the substrate and product at 0 and near 100% conversion were the same in the transesterification catalysed by  $\alpha$ -chymotrypsin in cyclohexane–1 mol dm<sup>-3</sup> propan-1-ol and 2 mmol dm<sup>-3</sup> 18-crown-6.

The *N*-acetylamino acid esters were synthesized by refluxing a solution of the *N*-acetylamino acid in the corresponding alcohol in the presence of a catalytic amount of benzenesulfonic acid or Amberlite IR 120. The reaction was followed by TLC or NMR. After evaporation of the alcohol, the residue was dissolved in dichloromethane and washed with acid and base. After drying with MgSO<sub>4</sub> the organic phase was evaporated. The alanine esters were further purified by vacuum distillation (bulb-to-bulb) 1–5  $\times$  10<sup>-2</sup> Torr/100–140 °C. The phenylalanine esters were crystallized from dichloromethane–cyclohexane. Isolated yields (not optimized) were in most cases about 50%.

#### *N*-Acetyl-D,L-alanine 2-fluoroethyl ester

$\delta_{\text{H}}$ (CDCl<sub>3</sub>) 6.08 (1 H, br s, NH), 4.62 (1 H, q, *J* 7.2, CH), 4.60 (2 H, dt, CH<sub>2</sub>F), 4.5–4.2 (2 H, m, *J*<sub>H,F</sub> 47.2, *J*<sub>H,H</sub> 4.0, OCH<sub>2</sub>), 2.01 (3 H, s, CH<sub>3</sub>) and 1.42 (3 H, d, *J* 7.2, CH<sub>3</sub>);  $\delta_{\text{C}}$ (CDCl<sub>3</sub>) 173.0 (s, CO), 169.6 (s, CO), 81.0 (dt, *J* 171.1, CH<sub>2</sub>F), 64.1 (dt, *J* 20.3, CH<sub>2</sub>), 48.1 (d, CH), 23.1 (q, CH<sub>3</sub>) and 18.7 (q, CH<sub>3</sub>) (Found: C, 47.3; H, 6.8; N, 7.8. C<sub>7</sub>H<sub>12</sub>FNO<sub>3</sub> requires C, 47.45; H, 6.83; N, 7.91%).

<sup>††</sup> Klibanov and co-workers<sup>18</sup> have earlier reported that the enantioselectivity of subtilisin Carlsberg decreases in organic solvents of increasing hydrophobicity (expressed in log *P*). This decrease in enantioselectivity was attributed to an increasingly unfavorable displacement of water molecules from the active site into the reaction medium, which affects the rate of the better fitting L-enantiomer more than the D-enantiomer.

***N*-Acetyl-D,L-alanine 2,2-difluoroethyl ester**

$\delta_{\text{H}}(\text{CDCl}_3)$  6.12 (1 H, br s, NH), 5.93 (1 H, tt,  $J_{\text{H,F}}$  54.9,  $J_{\text{H,H}}$  4.0, CHF<sub>2</sub>), 4.61 (1 H, q,  $J_{\text{H,H}}$  7.2, CH), 4.45–4.2 (2 H, m, CH<sub>2</sub>), 2.00 (3 H, s, CH<sub>3</sub>) and 1.42 (3 H, d,  $J_{\text{H,H}}$  7.2, CH<sub>3</sub>);  $\delta_{\text{C}}$  172.4 (s, CO), 169.7 (s, CO), 112.4 (td,  $J$  241.5, CHF<sub>2</sub>), 63.0 (tt,  $J$  29.5, CH<sub>2</sub>), 48.0 (d, CH), 23.0 (q, CH<sub>3</sub>) and 18.2 (q, CH<sub>3</sub>) (Found: C, 42.8; H, 5.6; N, 7.1. C<sub>7</sub>H<sub>11</sub>F<sub>2</sub>NO<sub>3</sub> requires C, 43.08; H, 5.68; N, 7.18%).

***N*-Acetyl-D,L-alanine 2,2,2-trifluoroethyl ester**

$\delta_{\text{H}}(\text{CDCl}_3)$  6.17 (1 H, br s, NH), 4.67 (1 H, q,  $J_{\text{H,H}}$  7.2, CH), 4.63 (1 H, dq,  $J_{\text{H,AB}}$  12.7,  $J_{\text{H,F}}$  8.3, CH<sub>A</sub>H<sub>B</sub>), 4.44 (1 H, dq,  $J_{\text{H,AB}}$  12.7,  $J_{\text{H,F}}$  8.3, CH<sub>A</sub>H<sub>B</sub>), 2.04 (3 H, s, CH<sub>3</sub>) and 1.46 (3 H, d,  $J$  7.2, CH<sub>3</sub>);  $\delta_{\text{C}}$  171.7 (s, CO), 169.8 (s, CO), 122.7 (q,  $J$  277.4, CF<sub>3</sub>), 60.8 (qt,  $J$  37.0, CH<sub>2</sub>), 47.9 (d, CH), 23.0 (q, CH<sub>3</sub>) and 18.0 (q, CH<sub>3</sub>) (Found: C, 39.1; H, 4.5; N, 6.4. C<sub>7</sub>H<sub>10</sub>F<sub>3</sub>NO<sub>3</sub> requires C, 39.44; H, 4.73; N, 6.57%).

***N*-Acetyl-D,L-phenylalanine 2-fluoroethyl ester**

$\delta_{\text{H}}(\text{CDCl}_3)$  7.33–7.21 and 7.13–7.07 (5 H, m, ArH), 5.85 (1 H, br s, NH), 4.93 (1 H, dt,  $J_{\text{NH,H}}$  7.8,  $J_{\text{H,CH}_2}$  5.8, CH), 4.7–4.4 (2 H, dm,  $J_{\text{H,F}}$  46.5, CH<sub>2</sub>F), 4.50–4.20 (2 H, m, CH<sub>2</sub>), 3.22–3.08 (2 H, m, CH<sub>2</sub>Ar) and 1.99 (3 H, s, CH<sub>3</sub>);  $\delta_{\text{C}}$  171.5 (s, CO), 169.6 (s, CO), 135.6 (s, Ar), 129.3 (d, Ar), 128.5 (d, Ar), 127.2 (d, Ar), 80.9 (dt,  $J$  171.3, CH<sub>2</sub>F), 64.2 (dt,  $J$  20.4, CH<sub>2</sub>), 53.1 (d, CH), 37.8 (t, CH<sub>2</sub>Ar) and 23.1 (q, CH<sub>3</sub>) (Found: C, 61.4; H, 6.35; N, 5.5. C<sub>13</sub>H<sub>16</sub>FNO<sub>3</sub> requires C, 61.65; H, 6.37; N, 5.53%).

***N*-Acetyl-D,L-phenylalanine 2,2-difluoroethyl ester**

$\delta_{\text{H}}(\text{CDCl}_3)$  7.35–7.25 and 7.13–7.08 (5 H, m, ArH), 5.84 (2 H, tt,  $J_{\text{H,F}}$  54.9,  $J_{\text{H,H}}$  4.0, CHF<sub>2</sub>), 5.82 (1 H, br s, NH), 4.93 (1 H, dt,  $J_{\text{NH,H}}$  7.7,  $J_{\text{H,CH}_2}$  6.1, CH<sub>2</sub>F), 4.40–4.15 (2 H, m, CH<sub>2</sub>), 3.17–3.05 (2 H, m, CH<sub>2</sub>Ar) and 1.99 (3 H, s, CH<sub>3</sub>);  $\delta_{\text{C}}$  170.9 (s, CO), 169.7 (s, CO), 135.4 (s, Ar), 129.2 (d, Ar), 128.7 (d, Ar), 127.4 (d, Ar), 112.3 (td,  $J$  241.5, CHF<sub>2</sub>), 63.1 (tt,  $J$  29.7, CH<sub>2</sub>), 53.1 (d, CH) and 37.7 (t, CH<sub>2</sub>) (Found: C, 57.2; H, 5.6; N, 5.05. C<sub>13</sub>H<sub>15</sub>F<sub>2</sub>NO<sub>3</sub> requires C, 57.06; H, 5.57; N, 5.16%).

***N*-Acetyl-D,L-phenylalanine 2,2,2-trifluoroethyl ester**

$\delta_{\text{H}}$  7.35–7.22 and 7.13–7.08 (5 H, m, ArH), 5.78 (1 H, br s, NH), 4.96 (1 H, dd,  $J_{\text{H,NH}}$  7.8,  $J_{\text{H,CH}_2}$  6.1, CH), 4.53 (1 H, dq,  $J_{\text{H,AB}}$  12.6,  $J_{\text{H,F}}$  8.3, CH<sub>A</sub>H<sub>B</sub>F), 4.43 (1 H, dq,  $J_{\text{AB}}$  12.6,  $J_{\text{H,F}}$  8.3, CH<sub>A</sub>H<sub>B</sub>F), 3.17 (1 H, dd,  $J_{\text{AB}}$  14.0,  $J_{\text{H,CH}}$  6.1, CH<sub>A</sub>H<sub>B</sub>Ar), 3.11 (1 H, dd,  $J_{\text{AB}}$  14.0,  $J_{\text{H,CH}}$  6.1, CH<sub>A</sub>H<sub>B</sub>Ar) and 1.98 (3 H, s, CH<sub>3</sub>);  $\delta_{\text{C}}$  170.3 (s, CO), 169.8 (s, CO), 135.2 (s, Ar), 129.1 (d, Ar), 128.8 (d, Ar), 127.4 (d, Ar), 122.6 (q,  $J$  277.2, CF<sub>3</sub>), 60.9 (qt,  $J$  37.0, CH<sub>2</sub>), 53.0 (d, CH), 37.5 (t, CH<sub>2</sub>) and 22.9 (q, CH<sub>3</sub>) (Found: C, 53.6; H, 4.8; N, 4.9. C<sub>13</sub>H<sub>14</sub>F<sub>3</sub>NO<sub>3</sub> requires C, 53.98; H, 4.88; N, 4.84%).

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