Cyclodextrins in Conjunction with and as Alternatives to Enzymes

A thesis presented for the degree of Doctor of Philosophy

in the

Research School of Chemistry

by

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The Australian National University

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"Generally, these phenomena are observed,
rather than designed."

Julius Rebek, Jr.

"Enzymes are things invented by biologists to explain things
that otherwise require harder thinking."

Jerome Lettoin

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Contents

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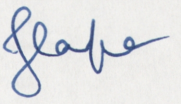
Then, of course, there's Jo. Not much more to say here except this. Thank you.

Jason

Author's Statement

The work described in this thesis is original and has not previously been submitted for a degree or diploma in this or any other University or College, with the exception that the material discussed on pages 47-53 was contained in an undergraduate honours thesis submitted in the Department of Chemistry, Australian National University, 1995. It is resubmitted here for completeness. To the best of my knowledge, this thesis does not contain material previously published or presented by another person, except where due reference is made in the text. Further, I give consent for a copy of this thesis to be deposited in the University Library and be available for loan or photocopying.



Jason Harper
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Publications

Some of the work described in this thesis has been reported in the following publications:

"Use of Cyclodextrins to Limit Product Inhibition of (*S*)-Phenylalanine Ammonia Lyase" C. J. Easton, J. B. Harper and S. F. Lincoln, *J. Chem. Soc., Perkin Trans. 1*, 1995, 2525.

"Acylase I Catalysed Hydrolysis of *para*-Substituted (*S*)-Phenylalanine Derivatives from Mixtures of the Racemic *ortho*- and *para*-Substituted Isomers" C. J. Easton and J. B. Harper, *Tetrahedron Lett.*, 1998, **39**, 3269.

"*N*,*N*'-Bis(6^{A}-deoxy-β-cyclodextrin-6^{A}-yl)urea as a Molecular Template in the Formation of Indigoid Dyes" C. J. Easton, J. B. Harper and S. F. Lincoln, *New J. Chem.*, 1998, **22**, 1163.

"Cyclodextrins as Molecular Templates" C. J. Easton, J. B. Harper and S. F. Lincoln, in *Proceedings of the 9th International Symposium on Cyclodextrins*, ed. J. J. Torres-Labandeira and J. L. Vila-Jota, Santiago de Compostela, Spain, 1999, p. 541.

Abstract

The use of cyclodextrins in conjunction with enzymes, to increase their utility, and as models of enzyme behaviour has been investigated.

Metallocyclodextrins formed from 6^A-(3-aminopropyl)amino-6^A-deoxy-β-cyclodextrin were investigated as possible esterase mimics to catalyse the enantioselective hydrolysis of *O*-acetyltyrosine. Under conditions where enantioselective complexation of *O*-acetyltyrosine was anticipated, the metal(II) ions were found to be insoluble. Altering the reaction conditions revealed that enantioselective hydrolysis of *O*-acetyltyrosine does not occur in reaction mixtures containing a metal(II) ion and 6^A-(3-aminopropyl)amino-6^A-deoxy-β-cyclodextrin.

N,*N*'-Bis(6^A-deoxy-β-cyclodextrin-6^A-yl)urea was shown to act as a molecular template in the competing reactions of indoxyl anion in the presence of either isatin or isatin-5-sulfonate. The preferred alignment of the cyclodextrin annuli in this dimer results in the reagents being complexed in an orientation that disfavours the formation of the linear dye indigo, but allows formation of the non-linear dyes indirubin and indirubin-5'-sulfonate. In the case where indirubin-5'-sulfonate is produced, the proportion of the non-linear dye formed increases by four thousand times on addition of *N*,*N*'-bis(6^A-deoxy-β-cyclodextrin-6^A-yl)urea.

α-Cyclodextrin and β-cyclodextrin were demonstrated to increase the efficiency of the elimination of ammonia and a proton from (*S*)-phenylalanine to afford *trans*-cinnamate, catalysed by (*S*)-phenylalanine ammonia lyase (PAL). These cyclodextrins remove *trans*-cinnamate from solution preferentially, reducing the amount free in solution to act as a product inhibitor of the enzyme and hence the activity of the enzyme increases. PAL was shown to catalyse the deamination of (*S*)-4'-chlorophenylalanine and (*S*)-2'-chlorophenylalanine to the corresponding *trans*-chlorocinnamates. The effect of α-cyclodextrin and β-cyclodextrin on enzyme activity in these cases was shown to be dependent on the extent of product inhibition, which differed from that observed on digestion of (*S*)-phenylalanine.

The effect of cyclodextrins on the relative amounts of (*S*)-phenylalanine and *trans*-cinnamate present at equilibrium in solutions containing PAL was examined. The addition of either α-cyclodextrin or β-cyclodextrin to a reaction mixture increased the

amount of *trans*-cinnamate present in the mixture at equilibrium. This is in agreement with the known association constants of the complexes of (*S*)-phenylalanine and *trans*-cinnamate with these hosts. 6^A-Amino-6^A-deoxy-β-cyclodextrin was found to increase the amount of (*S*)-phenylalanine present in solution at equilibrium. Calculation of the association constants for the complexes of this host with (*S*)-phenylalanine and *trans*-cinnamate gave values inconsistent with this result, suggesting that the association of (*S*)-phenylalanine with 6^A-amino-6^A-deoxy-β-cyclodextrin may be non-inclusive in nature.

The digestion of a range of *ortho*- and *para*-substituted *N*-acetylphenylalanines by acylase I was investigated. The enzyme was found to exhibit selectivity for the digestion of the *para*-isomers of (*S*)-*N*-acetylphenylalanines for a range of substituents. Kinetic experiments established that this selectivity was controlled by steric effects. This selectivity was shown to be synthetically useful through the isolation of *para*-substituted (*S*)-phenylalanines from racemic mixtures of the corresponding *ortho*- and *para*-substituted *N*-acetylphenylalanines.

The effects of cyclodextrins on the hydrolysis of *meta*- and *para*-substituted phenylalanine derivatives by protease VIII were studied. 6^A-Amino-6^A-deoxy-β-cyclodextrin and hydroxypropyl-β-cyclodextrin affected the rates of digestion of the *para*-substituted isomers by protease VIII to a greater extent than for the corresponding *meta*-substituted isomers. This was shown to be due to the cyclodextrins complexing the substrates and decreasing their concentrations free in solution. This affected the hydrolysis of the *meta*- and *para*-isomers to different extents, due to the different stabilities of the enzyme-substrate complexes.

Cyclodextrins were shown to limit substrate inhibition in the hydrolysis of (*S*)-carbobenzoxyglycylphenylalanine and (*S*)-*N*-benzoylglycyl-β-phenyllactate by carboxypeptidase A through sequestering the substrates from solution. The effects were greater for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate, where the enzyme is inhibited at low concentrations of the substrate. The concentration of this substrate at which the maximum rate of hydrolysis occurs increased by a factor of ten on addition of hydroxypropyl-β-cyclodextrin. Calculation of stability constants for the complexes of (*S*)-*N*-benzoylglycyl-β-phenyllactate with β-cyclodextrin and hydroxypropyl-β-cyclodextrin facilitated prediction of their effects on the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate by carboxypeptidase A, which were found to be in good agreement with those observed.

Introduction

Enzymes are highly efficient biological catalysts, with the rate of an enzyme mediated reaction being greatly enhanced relative to that of the corresponding uncatalysed reaction.[1] With the exception of a small group of catalytic RNA molecules, enzymes are proteins, being made up of a series of amino acids joined by amide bonds. The catalytic activity of an enzyme is based on its ability to effectively complex the molecule undergoing reaction, the substrate, in its 'active site'. The active site of an enzyme is complementary to the conformation of the substrate and binding of the substrate to this site brings catalytically significant residues of the peptide adjacent to the site of reaction of the substrate. As such, the primary, secondary, tertiary and quaternary structures of the protein are crucial in the action of an enzyme.

While many different enzymes have been identified, which catalyse a wide range of reactions, each enzyme tends to be quite specific in terms of the substrate digested and the resultant product.[1] This substrate specificity arises from the ability of the enzyme to discriminate between molecules on binding to form the Michaelis complex.[2] An enzyme only catalyses the reaction of a substrate bound in such a manner, to form an enzyme-product complex that dissociates to give the enzyme and the product. This is illustrated in Scheme 1 where E represents an enzyme, S the substrate of that enzyme and P the product of reaction.

$$E + S \rightleftharpoons \underset{\text{(Michaelis complex)}}{E.S} \rightleftharpoons E.P \rightleftharpoons E + P$$

Scheme 1. The formation of a Michaelis complex precedes the enzyme catalysed reaction.

The basis of the selectivity of complexation by the enzyme is the nature of the enzyme active site. To illustrate this, consider the proteolytic enzyme chymotrypsin which is responsible for the hydrolysis of peptides in the small intestine.[3] It is selective for the hydrolysis of peptide bonds on the carboxyl side of (*S*)-amino acids with a large hydrophobic side chains.[4] The nature of the active site (Figure 1) determines how this selectivity is achieved.[4]

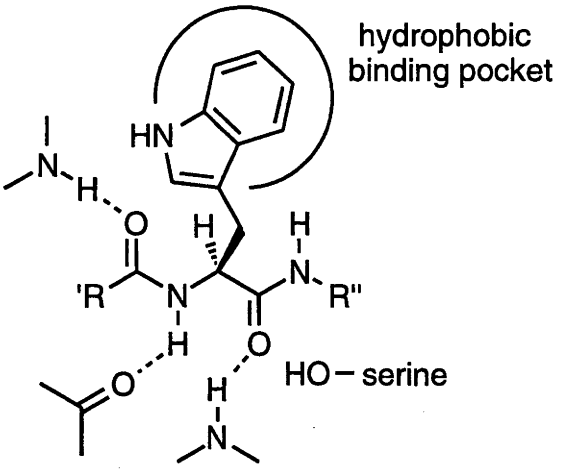


Figure 1. A peptide containing a tryptophan residue complexed in the active site of chymotrypsin.

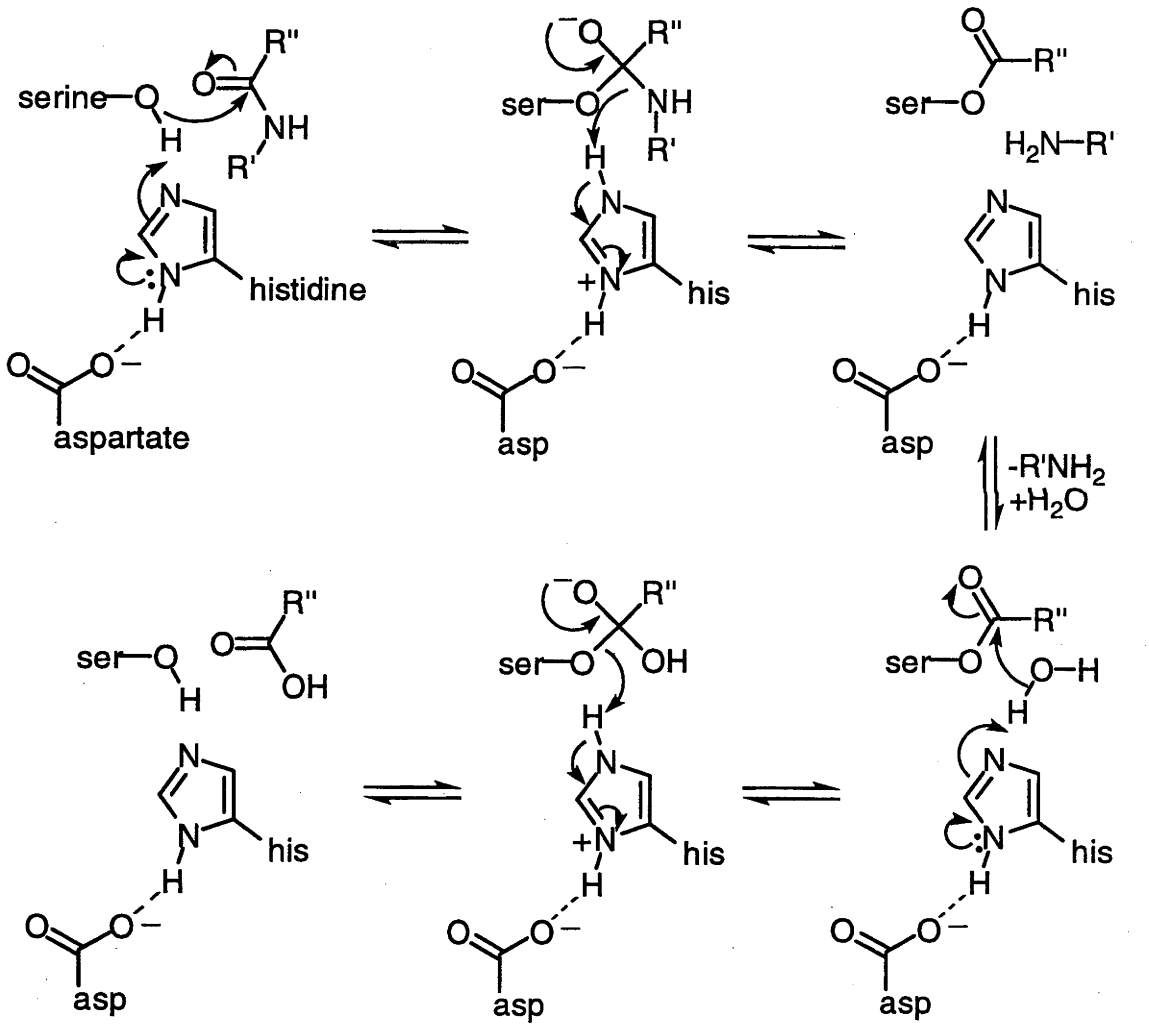
The active site of chymotrypsin is lined with the amide hydrogens and carboxyl oxygens of the enzyme's peptide backbone.[4] These are positioned such that they can form hydrogen bonds with molecules bound in the active site. As a result, molecules with suitable hydrogen bond donors and acceptors, such as peptides, bind efficiently to the active site.

An important structural feature of the active site of chymotrypsin is a large hydrophobic pocket, positioned such that the side chains of amino acids of (*S*)-configuration are accommodated but the side chains of the corresponding (*R*)-enantiomers are not.[4] That is, the position of this binding pocket determines the enantioselectivity of the enzyme as only peptides containing amino acids with the (*S*)-configuration are able to bind to the active site. Further, the hydrophobic interactions of large aromatic side chains such as those of phenylalanine, tryptophan and tyrosine, and to a lesser extent bulky aliphatic side chains such as that of leucine, with the pocket stabilise the complexes of substrates containing these residues.[4] The result is that these substrates are bound more efficiently and selectivity is observed.

Finally, the substrate is complexed in such a way that the residues responsible for catalysis have the necessary orientation. In this case a serine residue which plays a key role in the catalysis is proximal to the carboxyl group of the amino acid residue.[4] As a result, hydrolysis of the corresponding amide linkage is carried out in preference to reaction of any other amide bonds in the substrate.

From this information it is clear that the precise structure of the active site of an enzyme is responsible for the significant enantio-, regio- and substrate-selectivity observed in enzyme catalysis. The structure of the active site is also of particular

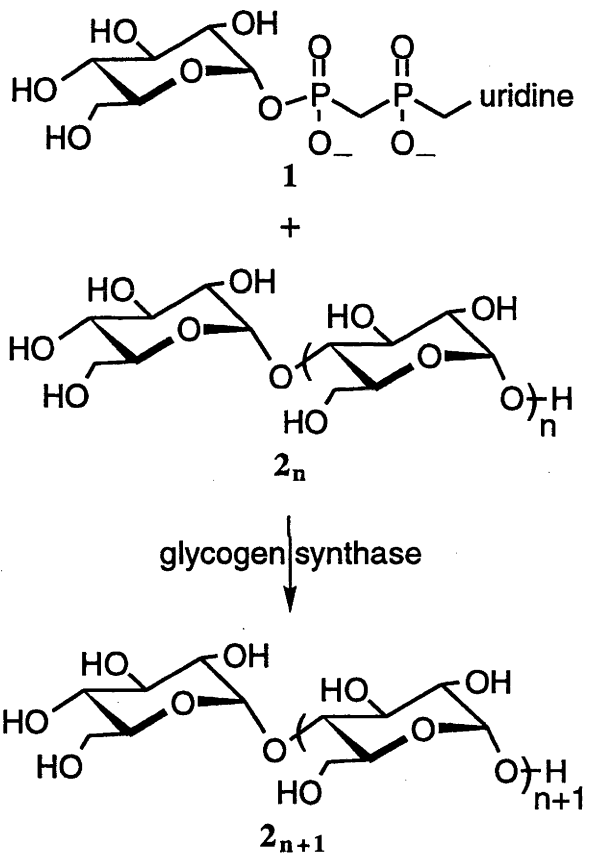
importance to the catalytic activity of the enzyme as demonstrated by the residues responsible for the catalytic activity of chymotrypsin. As mentioned above, there is a serine residue of the enzyme adjacent to the amide bond of the substrate being cleaved. The nucleophilicity of this serine is greatly enhanced by the presence of a histidine residue next to it in the active site. This, in turn, is stabilised in the protonated form by an adjacent aspartate residue. This 'catalytic triad' is an excellent example of how the precise arrangement of the residues of an enzyme results in effective catalysis. The mechanism of hydrolysis by chymotrypsin is illustrated as Scheme 2.[4] This particular catalytic motif is present in a range of enzymes, collectively termed serine proteases.[5]



Scheme 2. The mechanism of hydrolysis of a peptide by chymotrypsin showing the effect of the catalytic triad of serine, histidine and aspartate.

Having discussed the effect of enzyme structure on the selectivity of substrate binding and the nature of the catalysis, the effect on regioselectivity needs to be addressed. Many enzymes, particularly those that involve the condensation of two substrates, catalyse reactions regioselectively, with reaction occurring at only one of

many potential reaction centres. This regioselectivity is observed because the substrates are bound with exact orientations in the active site, resulting in reaction occurring at only one point. An example of this is the formation of glycogen (**2**) in the liver and skeletal muscle by glycogen synthase.[6] This enzyme catalyses the addition of UDP-glucose (**1**), an activated form of glucose, to glycogen (**2**) (Scheme 3). This addition occurs regioselectively, at the 4-position of the terminal glucose unit, and stereoselectively, to form an α-1,4-glycosidic linkage. This regio- and stereo-selectivity is controlled by the orientation of the substrates in the enzyme active site.

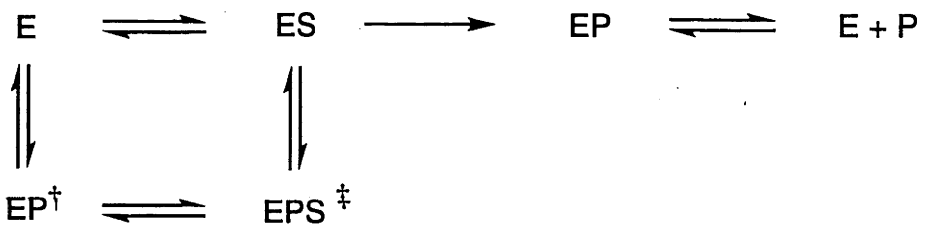


Scheme 3. The stereo- and regio-selective addition of a glucose unit to glycogen (**2**) catalysed by glycogen synthase.

Since enzymes are highly evolved biological molecules, they exhibit specific regulatory mechanisms based on the organisms from which they are derived.[1,7] Typically these involve molecules binding to an enzyme, which either compete for the active site or bind to alternative sites on the enzyme.[1,7] The former tend to have an inhibitory effect on enzyme activity as they compete with the substrate for a single binding site. The latter form of binding, often referred to as allosteric,[1,7] usually results in a conformation change in the enzyme which may increase or decrease the catalytic activity, thus having either an activating or inhibitory effect. The biological imperative on

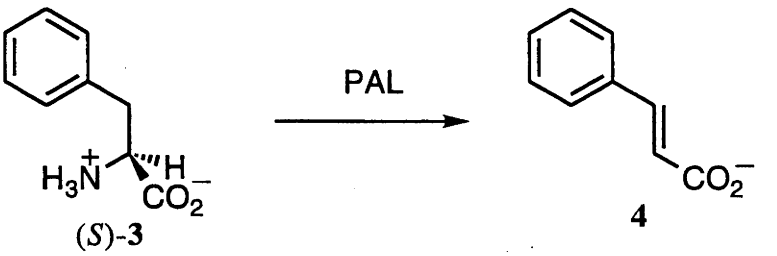
which this control is based is regulation of digestion of the substrate.[1,7] For example, this regulation may prevent unwanted accumulation of an intermediate in a synthetic pathway or prevent over digestion of an abundant substrate.

An excellent example of this type of regulation is product inhibition of enzyme catalysis. Also known as feedback inhibition, this is a key form of metabolic control in biological systems, where the product of the enzyme catalysed reaction reduces enzyme activity, preventing wasteful metabolism of the substrate.[1,7] The product can bind at the active site of the enzyme (competitive inhibition) or at another site on the enzyme (non-competitive or uncompetitive inhibition). The former is more common as the product of an enzyme catalysed reaction often structurally resembles the substrate and hence will be recognised by the active site of the enzyme.[1,7] Each of these forms of inhibition has the effect of reducing the amount of the enzyme-substrate complex in solution (Scheme 4). Since the amount of the enzyme-substrate complex decreases, the rate of reaction and hence the extent of reaction in a given time also decrease.



Scheme 4. The equilibria associated with a system which demonstrates product inhibition.
†This complex forms when the product binds to the enzyme at an allosteric site in the absence of the substrate (non-competitive inhibition).
‡This complex forms when the product binds to the enzyme at an allosteric site in the presence of the substrate (non-competitive or uncompetitive inhibition).

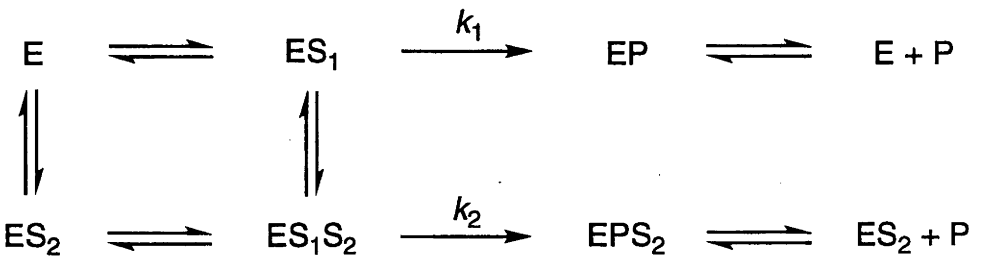
An enzyme which is affected by product inhibition is (*S*)-phenylalanine ammonia lyase (PAL). PAL catalyses the elimination of ammonia and a proton from (*S*)-phenylalanine ((*S*)-**3**) to give *trans*-cinnamate (**4**) (Scheme 5).[8] This reaction represents the principle branching point between the primary and secondary metabolic pathways in higher plants and extensive study has elucidated the mechanism of reaction.[8-15] The cinnamate **4** produced is a potent competitive inhibitor of PAL activity, so this system is an example of product inhibition.[9] The biological compulsion behind this effect is the prevention of wasteful digestion of the substrate (*S*)-**3**, controlling the degree to which the phenylalanine (*S*)-**3** is directed into the primary and secondary metabolic pathways.



(*S*)-**3** **4**

Scheme 5. The removal of ammonia and a proton from (*S*)-phenylalanine ((*S*)-**3**) as catalysed by PAL.

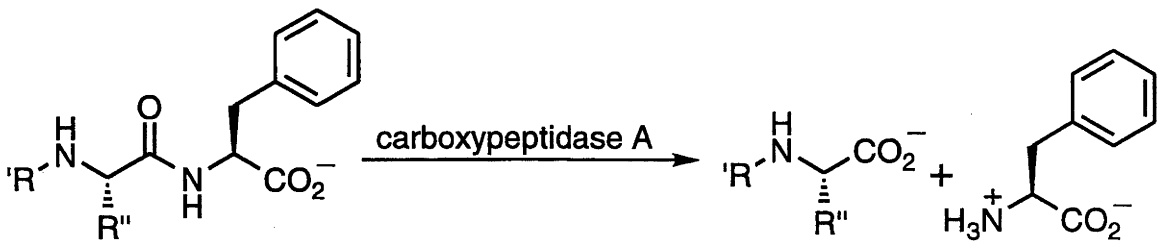
Another form of regulation of enzyme catalysis is substrate inhibition, where the activity of the enzyme decreases at high concentrations of the substrate.[1,7] Physiologically, this prevents wasteful digestion of an abundant substrate and the accumulation of the corresponding product. Typically, substrate inhibition is due to the binding of a second substrate molecule to the enzyme, which can take place either at the active site or elsewhere, and which alters the conformation of the enzyme.[1,7] This allosteric change decreases the activity of the ternary enzyme-substrate complex, hence reducing the rate of digestion of the substrate. Substrate inhibition can be described kinetically as in Scheme 6, where S_1 and S_2 represent substrate molecules bound at two binding sites, with S_1 being bound at the active site and able to react.[1,7] The rate constants for reaction of the bound species (k) will differ depending on whether a second substrate molecule is bound to the enzyme and, in the case of substrate inhibition, $k_1 > k_2$.[1,7] At low concentrations of the substrate, the ES_1 complex dominates so the rate of reaction increases with increasing substrate concentration. As the substrate concentration increases further, more of the ternary complex forms and the rate of reaction decreases.



Scheme 6. The equilibria associated with an enzyme that is affected by substrate inhibition ($k_1 > k_2$).

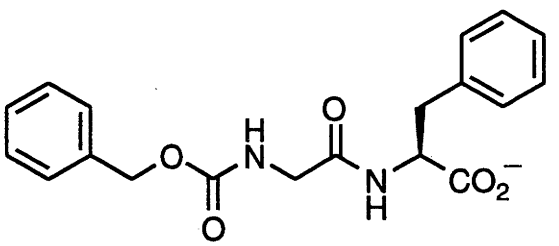
An example of an enzyme that is affected by substrate inhibition is carboxypeptidase A. A zinc containing proteolytic enzyme, its natural function is to hydrolyse the carboxyl-terminal residue from a peptide, the reaction occurring most

readily if the carboxyl-terminal residue has an aromatic or bulky aliphatic side chain (Scheme 7).[3] *In vitro*, it has been shown to also hydrolyse esters with similar configurations to the natural peptide substrates.[16] In both cases, the enzyme is enantiospecific, hydrolysing only the (*S*)-enantiomers of the substrates.



Scheme 7. The hydrolysis of a peptide with a carboxyl-terminal phenylalanine residue, catalysed by carboxypeptidase A.

This enzyme has been shown to exhibit substrate inhibition on catalysis of reaction of a range of substrates.[17-26] Of note is the inhibition observed in the hydrolysis of (*S*)-carbobenzoxyglycylphenylalanine (**5**) and the structurally related lactate esters, including (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**). These two substrates offer a comparison of the effects of binding a second substrate molecule. In the case of the peptide derivative **5**, while they still differ, the rate constants k_1 and k_2 are of comparable magnitude and, as a result, the rate of reaction observed at high concentrations of substrate is approximately half of the maximum rate observed. Thus, the inhibitory effect of the second substrate molecule binding to the enzyme is small. In the case of the lactate **6**, $k_1 >> k_2$, so at high concentrations of the substrate, enzyme activity is reduced to negligible levels. The inhibitory effect of the second substrate molecule binding to the enzyme is clearly very large in this case.



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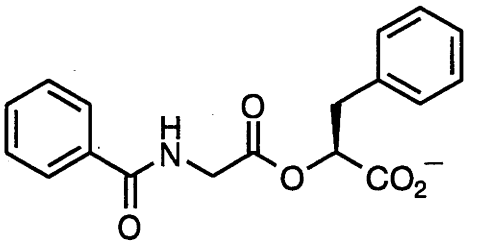

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It should be noted that, despite their biological complexity, enzymes are simply catalysts.[1] They offer an alternative pathway to reaction with a lower energy of activation than is observed in their absence. The free energies of the substrates and products remain unchanged. Thus, the position of equilibrium, which is determined by the relative free energies of the substrates and products, remains unchanged while the rate at which the equilibrium is attained, which is dependent on the energy of activation of the reaction, is increased. While some enzyme catalysed reactions can be considered essentially irreversible under the conditions where they operate, such as the hydrolysis of peptides described above, this is simply because the difference in free energies of the substrates and products is so great that the equilibrium constant is very large.

Enzymes are efficient catalysts and this makes them attractive for use in organic synthesis. Further, the growing availability of enzyme preparations, their safety, ease of handling and the mild conditions (typically room temperature and around neutral pH) under which they operate means that they are being considered more frequently for use in organic synthesis.[16,27-30] However, almost invariably, the enzymes are required to operate under conditions that are inconsistent with their biological origin. These biological origins place notable restrictions on the use of enzymes in organic synthesis.

Modification of enzymes has been used to overcome these limitations in some cases. Total synthesis of enzymes, while academically feasible, is impractical.[31] Of more utility is the overproduction of enzymes in bacterial cells using recombinant DNA technology.[32] In such cases, modifying an enzyme involves mutating the gene responsible for the it, so that there is a change in the peptide chain of the protein produced.[33,34] The residues that make up the active site are often targeted, in an effort to change the nature of the active site and the selectivity of the enzyme. This method is quite time consuming, since the effect of such a point mutation is difficult to predict, with the potential to disrupt the entire tertiary and quaternary structure of the enzyme. Further, the effect of multiple point mutations is not necessarily additive.

Given the difficulties associated with the modification of enzymes, much work has gone into the development of synthetic enzymes not based on proteins. These enzyme models or enzyme mimetics must exhibit the same properties described above as being characteristic of enzyme behaviour. That is, they must exhibit selectivity on binding of the desired substrate, they must have appropriate functional groups for catalysis, the bound species must undergo reaction stereoselectively and the enzyme mimetics must be regenerated unchanged. A class of compounds which have been investigated as models of enzyme behaviour are the cyclodextrins.

Naturally occurring cyclodextrins are homochiral 1-4 linked cyclic oligomers of α-D-glucopyranose.[35] They are formed through the degradation of starch by the enzyme cyclodextrin glucosyl transferase.[36] Cyclodextrins were originally isolated by Villiers from the digest of potato starch by *Bacillus amylobacter*[37] and later characterised by Schardinger.[38,39] The most common of the series are the six, seven and eight membered homologues, being referred to as α-cyclodextrin (**7**), β-cyclodextrin (**8**) and γ-cyclodextrin (**9**), respectively.[40] Conformational restrictions cause these cyclodextrins to be toroidal in shape (Figure 2),† with a hydrophilic exterior and a hydrophobic cavity.[41] These properties of cyclodextrins make them well suited for the formation of water soluble inclusion complexes of hydrophobic molecules.[42-46]

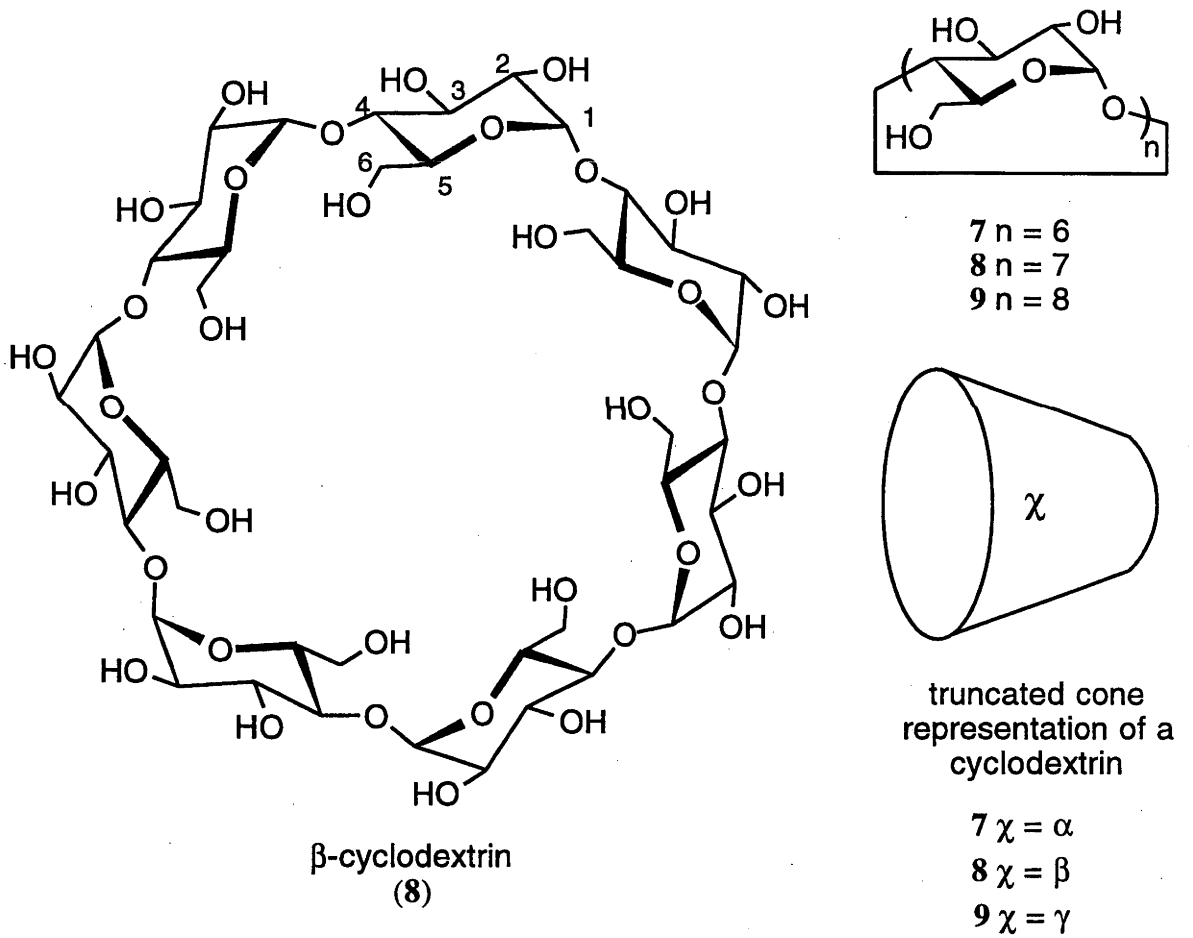
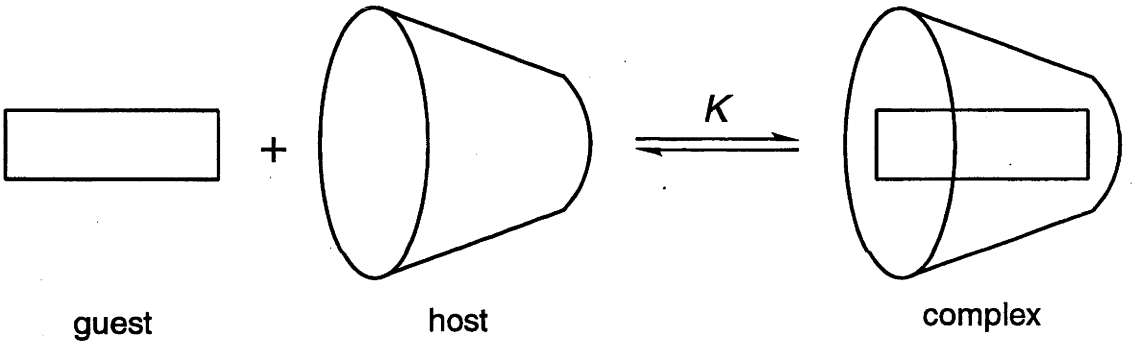


Figure 2. Structure and conformation of the three most common naturally occurring cyclodextrins.

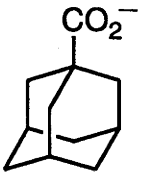
† Cyclodextrins are commonly represented by truncated cones. When a substituent is shown on the narrow end, it represents a moiety that has replaced one of the C(6) primary hydroxyl groups, while a substituent shown on the wider end represents a moiety that has replaced either a C(2) or C(3) secondary hydroxyl group.

An inclusion complex is said to form when a molecule with a cavity, or capable of forming one (the host), encapsulates a smaller molecule (the guest) by purely non-covalent interactions.[43,45,47] These complexes can be thermodynamically quite stable and each is characterised by a thermodynamic stability constant or association constant (K) defined by the position of equilibrium between the host, the guest and the complex (Equation 1).[43,45,47] This is illustrated in Scheme 8 for the case where the host is a cyclodextrin. It should be noted that, as expressed below, K is a concentration stability constant rather than a true stability constant where activities of the equilibrium participants are employed instead of concentrations. However, concentration stability constants are usually determined in solutions where a constant ionic strength is maintained by a supporting electrolyte.[46]

$$K = \frac{[\text{complex}]}{[\text{guest}].[\text{host}]} \qquad (1)$$



Scheme 8. The inclusion of a guest within a host to form a complex.



10

The size, shape and nature of the guest involved will affect the stability of the complex and hence the value of the association constant.[45] The former two constraints are easily understood, as for the guest to be included within the cyclodextrin cavity, it must be able to be accommodated. On the first point, the more closely matched the size of the guest to the size of the cavity, the less distortion of the host occurs on inclusion and the more thermodynamically stable the resulting inclusion complex. This is illustrated by the differing association constants of adamantane-1-carboxylate (**10**), which has a molecular diameter of approximately six angstroms,[48] with the cyclodextrins **7-9**, whose

annuli vary in size (Table 1). For the formation of the inclusion complex with the smallest host **7**, the guest **10** cannot include completely into the host cavity. The cavity size of the seven membered homologue **8** is larger and the guest **10** fits well within it. The result is a more stable complex and a markedly higher value for the association constant of the complex. A further increase in size of the host, however, increases the degrees of freedom of the guest **10** complexed in the host **9**, decreasing the stability of the complex and hence decreasing the value of the association constant of the complex. This relationship between the size of the guest and the size of the host cavity has been referred to as the "Goldilocks' Effect".[45]

Cyclodextrin	**7**	**8**	**9**
Annular diameter measured from the C(5) hydrogens (Å)[49]	4.7	6.0	7.5
Annular diameter measured from the C(3) hydrogens (Å)[49]	5.3	6.5	8.3
Annular depth from the primary to the secondary hydroxyl groups (Å)[49]	7.9 ± 1	7.9 ± 1	7.9 ± 1
Annular volume ($Å^3$)[49]	174	262	427
Association constant of the complex formed with the guest **10** (mol^{-1} dm^3)[a]	150 ± 20	20000 ± 2000	3300 ± 400

Table 1. Geometric dimensions of the cyclodextrins **7-9**, and the association constants of the complexes formed with adamantane-1-carboxylate (**10**).
[a]Calculated from data presented by Cromwell and co-workers.[50]

The influence of the shape of a guest on the association constant of the complex it forms with a given cyclodextrin is probably best demonstrated by considering the two steroids, 5α-cholanic acid 3-β-ol (**11**) and lithocholic acid (**12**). The association constants of the complexes formed with β-cyclodextrin (**8**) differ by a factor of twenty (Table 2) with the complex of the *cis*-fused steroid **12** being more stable. Molecular modelling indicates that the *cis* ring fusion of the AB ring system causes the A ring of the steroid **12** to better fit the wider end of the cyclodextrin cavity than is the case when the AB ring fusion is *trans*.[51] In both cases, the remaining rings thread through the cavity. Hence, the stability of the association complex formed is dependent on the shape of the guest that is included.

The nature of the guest is important in determining the association constant of any complex that it may form, as it determines whether or not a range of stabilising factors affect the complex. These factors include relief of conformational strain in the cyclodextrin host, ion-dipole, dipole-dipole and induced dipole-dipole interactions, and London dispersion forces.[45] As well as these, an important consideration is the change

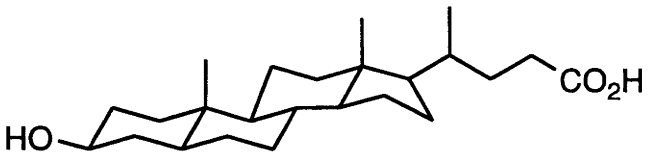
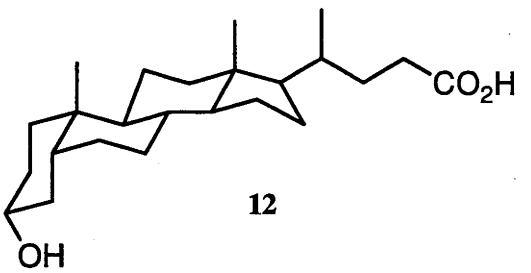
Steroid	K (mol^{-1} dm^3)
 **11**	1.07 x 10^4
 **12**	2.01 x 10^5

Table 2. Association constants for the complexes of β-cyclodextrin (**8**) with the steroids **11** and **12**.[51]

in hydration of the host and the guest in the complexation process, since almost all association constants are measured in aqueous solution.[45,46] The number of waters that hydrate the guest will depend on its nature. Upon formation of the complex, the guest displaces water molecules from the cyclodextrin cavity, while also losing some of the water molecules associated with it. The result is that several molecules of water enter the bulk phase. Since water within the cavity of a cyclodextrin cannot form hydrogen bonds as in bulk water and is often termed "high energy", its expulsion from the cavity is a driving force for guest complexation. Clearly, the guest which can shed its hydration shell to the greatest extent and expel the most water molecules from the cavity of the cyclodextrin will form the more stable complex, all other factors being equal. The latter effect is again related to the size of the guest, while the former is more dependent on the nature of the guest. The more hydrophobic a molecule, the lesser the degree to which it is hydrated and hence the greater the stability constants for the complexes that it forms. For example, the stability constant for the complex of adamantane-1-carboxylate (**10**) and β-cyclodextrin (**8**) is fifteen times smaller than the association constant of the corresponding complex with the parent acid **13** (Table 3).[52]

While guest discrimination by a host can be qualitatively assessed by considering the factors discussed, it is necessary to quantify the association constants to accurately gauge the relative stability of inclusion complexes. The spectroscopic properties of guest molecules often change upon complex formation and, as a result, various spectroscopic

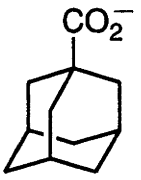
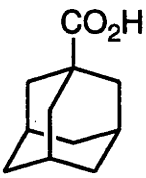
Guest	K (mol^{-1} dm^3)
 **10**	2.0×10^4
 **13**	3.0×10^5

Table 3. Association constants for the complexes of adamantane-1-carboxylate (**10**) and adamantane-1-carboxylic acid (**13**) with the cyclodextrin **8**.[52]

methods have been used to calculate association constants for inclusion complexes.[45,53-67] The use of nuclear magnetic resonance (NMR) spectroscopy for this purpose is well documented.[60-67] When no cyclodextrin is present, the observed chemical shift of a given nucleus is that of the free guest, while varying the concentration of the host changes the chemical shift, which is generally observed as a weighted average of that for the free and included species (Equation 2). Extrapolation to infinite cyclodextrin concentration gives the chemical shift of the included guest. The stability constant of the complex can then be quantified by applying non-linear regression analysis to Equations 1 and 2 (See Appendix 1).

$$\delta_{observed} = \frac{([guest].\delta_{free} + [complex].\delta_{complexed})}{([guest] + [complex])} \qquad (2)$$

For a given cyclodextrin, if the value of the association constant for one complex differs from that of another, this difference is an indication of guest discrimination by the host. This discrimination is the basis for selective binding and hence an element necessary in the formation of enzyme mimics.

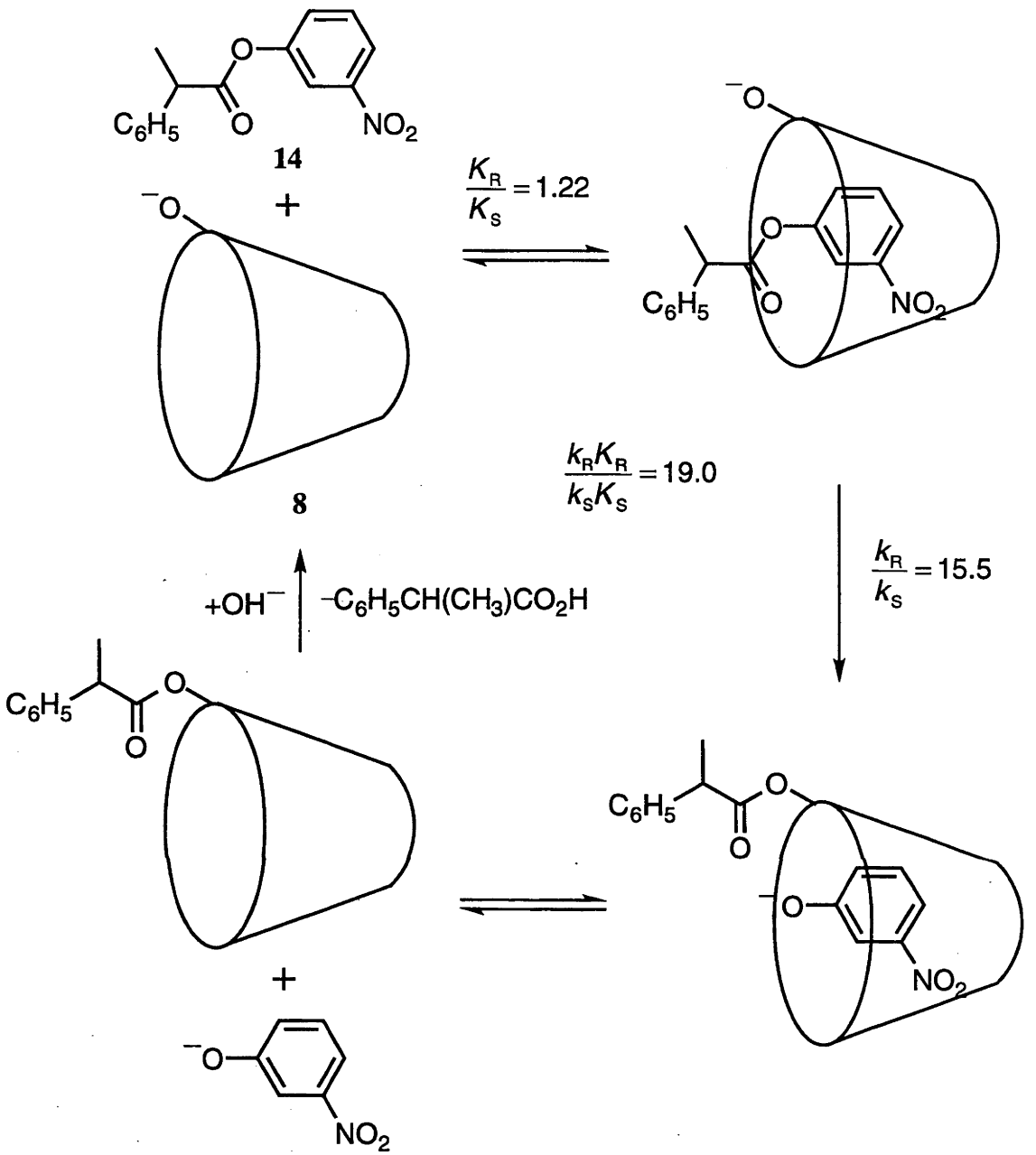
In terms of functional groups with the potential for catalytic activity, the naturally occurring cyclodextrins are limited in utility, with only hydroxyl groups being present. This restricts the natural cyclodextrins to being considered as mimics of behaviour where a nucleophilic reaction centre is required. The hydroxyl groups delineating each end of the cyclodextrin annulus do have different properties, with the secondary hydroxyl groups having a lower pK_a, allowing for regioselectivity of reaction.[68,69]

A good example of a naturally occurring cyclodextrin acting as an enzyme mimic is the hydrolysis of 3'-nitrophenyl 2-phenylpropanoate (**14**) by β-cyclodextrin (**8**) (Scheme 9).[70] The ester **14** includes within the cavity of the cyclodextrin **8**. Hydrolysis occurs through nucleophilic attack of a secondary hydroxyl group of the host **8**, which is deprotonated under the reaction conditions, on the carboxyl group of the guest **14**. The product cyclodextrin ester is subsequently hydrolysed to regenerate β-cyclodextrin (**8**). The cyclodextrin discriminates between the enantiomers of the guest **14** on formation of the inclusion complex, with the ester (*R*)-**14** being complexed in preference to its enantiomer (*S*)-**14**, though the magnitude of this discrimination is small. A greater discrimination is observed on reaction of the complexed ester **14**, with the (*R*)-enantiomer (*R*)-**14** being hydrolysed *ca.* fifteen times faster than the (*S*)-enantiomer (*S*)-**14**. The overall enantioselectivity in the hydrolysis of 3'-nitrophenyl 2-phenylpropanoate (**14**) by β-cyclodextrin (**8**) is a factor of nineteen, with the (*R*)-enantiomer (*R*)-**14** being hydrolysed preferentially.

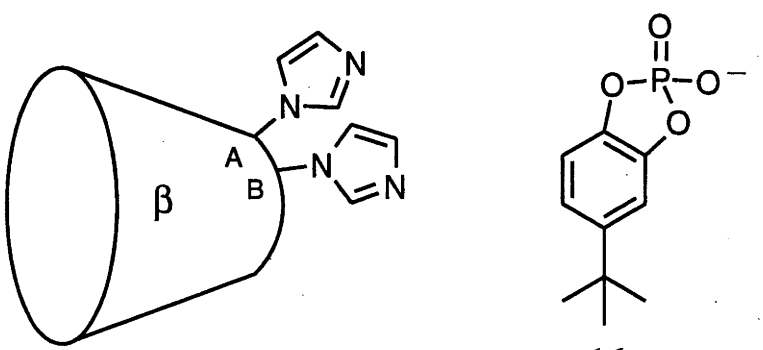
Modification of the hydroxyl functionalities of the naturally occurring cyclodextrins provides a way to increase the utility of cyclodextrins as enzyme mimics. Modifications have been shown to affect the solubility of the cyclodextrins, but the major advantages are for introducing specific catalytic functionality and to modify the selectivity of the complexation.[71,72]

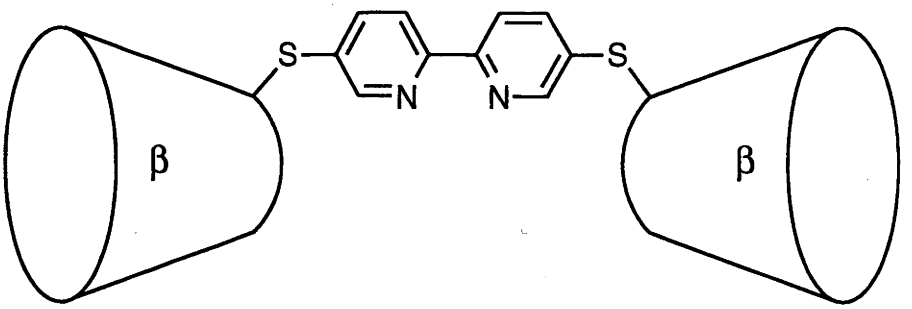
Of particular note has been the work carried out by the research group of Breslow.[73,74] As an example, the modified cyclodextrin **15**, with two imidazole units attached to adjacent glucose residues (designated A and B), was synthesised to introduce the potential for concerted acid-base catalysis. Many enzymes, notably the ribonucleases, use the cooperative functioning of an imidazole base and an imidazolium ion acid, present in the protein as the side chains of histidine residues, for this form of catalysis.[3] The hydrolysis of the cyclic phosphate ester **16** as catalysed by the modified cyclodextrin **15** demonstrates this effect.[75]

The modification of a cyclodextrin also allows the introduction of functionality that may complex a metal, to form a metallocyclodextrin. In enzymes, metals are used to complex reagents at the active site, such as in peroxidases, or act as Lewis acids and increase the reactivity of the bound substrate, such as in the hydrolysis of peptides by carboxypeptidase A.[3] Both of these effects have been mimicked by metallocyclodextrins based on the modified cyclodextrin **17**. The lanthanum(III) complex of the modified cyclodextrin **17** was shown to dramatically increase the rate of hydrolysis of the phosphate ester **18** in the presence of hydrogen peroxide, with rate accelerations greater than 10^7.[76]



Scheme 9. Enantioselective hydrolysis of 3'-nitrophenyl 2-phenylpropanoate (**14**) by β-cyclodextrin (**8**) at pH 10.5.[70]





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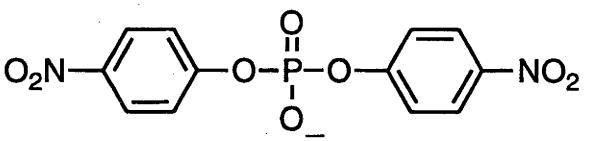

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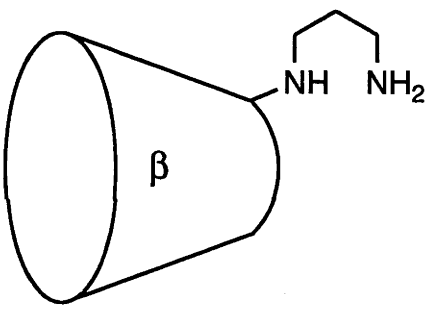

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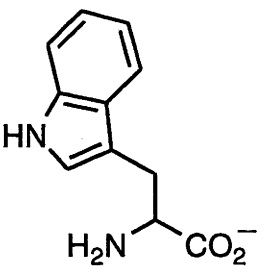

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Metallocyclodextrins based on the modified cyclodextrin **19** have been widely studied and shown to exhibit enantioselectivity on complexation of the anions of the naturally occurring aromatic amino acids.[77-79] The orientation of each of these complexes is controlled by interaction of the amino and carboxylate moieties of the amino acid with the metal centre and inclusion of the aromatic moiety within the annulus of the cyclodextrin. This is illustrated in Figure 3 for the complex of tryptophan anion (**20**) with the nickel(II) metallocyclodextrin based on the modified cyclodextrin **19**. The enantioselectivity on formation of this complex is the greatest reported, with the association constant of the complex of the guest (*S*)-**20** being ten times larger than that of the complex of its enantiomer (*R*)-**20**.[78,79] It was envisaged that the chiral discrimination exhibited by metallocyclodextrins based on the modified cyclodextrin **19** might be exploited in the development of enantioselective enzyme mimics. Studies towards this goal are described in Chapter I of the Results and Discussion.

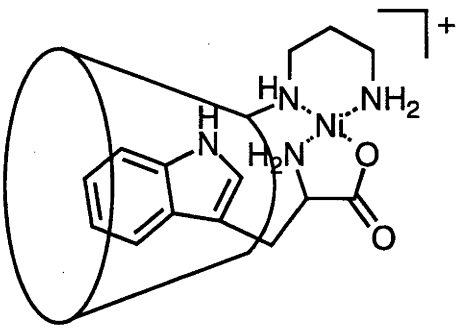
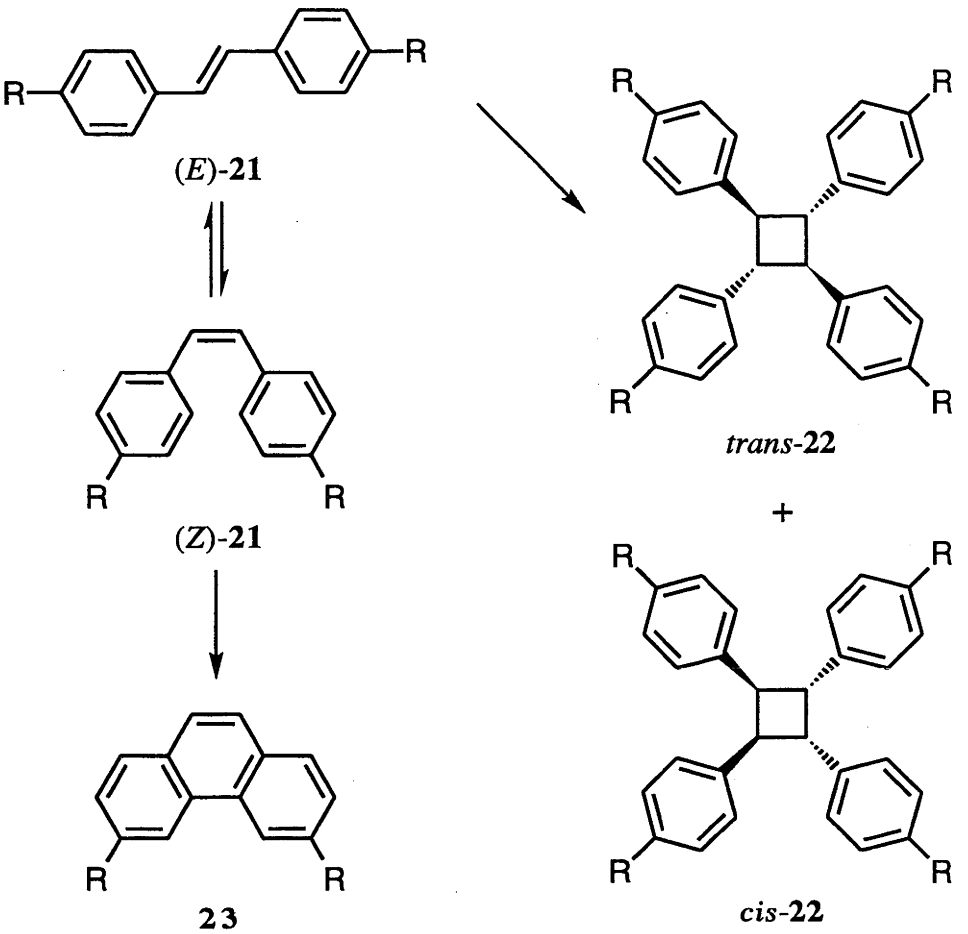


Figure 3. Orientation of the guest **20** in the association complex formed with the nickel(II) metallocyclodextrin based on the modified cyclodextrin **19**.

Native cyclodextrins have been reported to alter the ratio of products formed on photoirradiation of (*E*)-4,4'-bis(dimethylammoniomethyl)stilbene ((*E*)-**21**).[80] On photoirradiation, the (*E*)-isomer (*E*)-**21** can dimerise to give the cyclobutanes *trans*-**22** and *cis*-**22** or undergo reversible isomerisation to give the (*Z*)-isomer (*Z*)-**21**, which can cyclise to give the phenanthrene **23** (Scheme 10). The ratios of the products of the reaction in the absence of a cyclodextrin and in the presence of the cyclodextrins **7**-**9** are summarised in Table 4. In the presence of the cyclodextrins **7** and **8**, none of the cyclobutanes *trans*-**22** and *cis*-**22** is observed. This suggests that the cyclodextrins **7** and **8** complex the stilbene (*E*)-**21** and prevent it from dimerising. The effect of adding γ-cyclodextrin (**9**) to the reaction mixture is a dramatic increase in the amount of the dimers *trans*-**22** and *cis*-**22** produced. Two molecules of the stilbene (*E*)-**21** were shown to be accommodated in the cavity of γ-cyclodextrin (**9**) by microcalorimetric titration.[80] The results suggest formation of this ternary complex favours dimerisation of the stilbene (*E*)-**21**, presumably due to the proximity of the reagents. Similar results have subsequently been reported for the photochemical reactivity of a *trans*-2-styrylpyridine. [81]

Cyclodextrin	(*E*)-**21**	(*Z*)-**21**	*trans*-**22**	*cis*-**22**	**23**
-	10	62	7	2	19
7	20	60	0	0	20
8	16	83	0	0	1
9	0	0	79	19	2

Table 4. The ratio of products of reactions involving the irradiation of the stilbene (*E*)-**21** with ultraviolet (UV) light (312 nm) in aqueous solution in the absence of a cyclodextrin and in the presence of either α-cyclodextrin (**7**), β-cyclodextrin (**8**) or γ-cyclodextrin (**9**).[80]

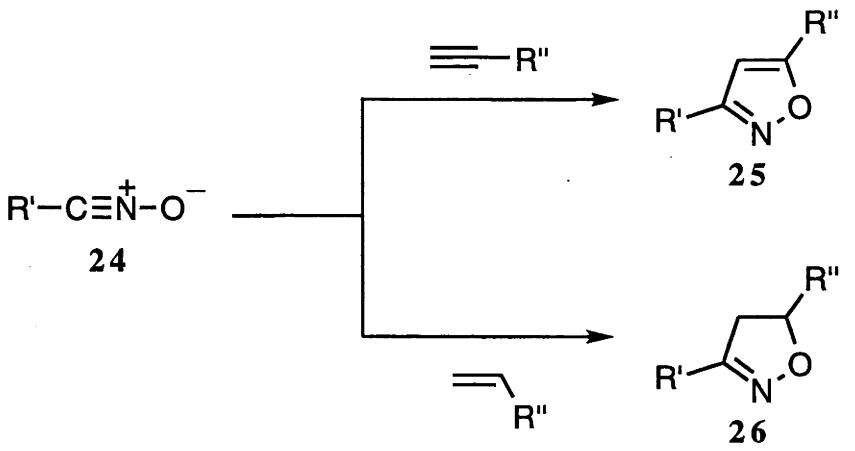


Scheme 10. Competing reactions to form the phenanthrene **23** and the dimers *trans*-**22** and *cis*-**22** on irradiation of the stilbene (*E*)-**21** ($R=CH_2NH(CH_3)_2^+$).

While in these cases the natural cyclodextrin **9** is shown to act as a molecular template, the effects observed must be considered serendipitous as the host **9** was not designed for such a purpose. In contrast, recent work using cyclodextrins as molecularscaffolds for nitrile oxide cycloadditions involved a cyclodextrin host being specifically designed and synthesised to template a reaction.[82-84]

Nitrile oxides **24** undergo [3+2] cycloadditions with alkynes and alkenes to give isoxazoles and 4,5-dihydroisoxazoles, respectively.[85] The use of a mono-substituted and therefore asymmetric dipolarophile gives the possibility of a mixture of products, however steric effects are found to predominate producing mainly 5-substituted isoxazoles **25** and dihydroisoxazoles **26** (Scheme 11).

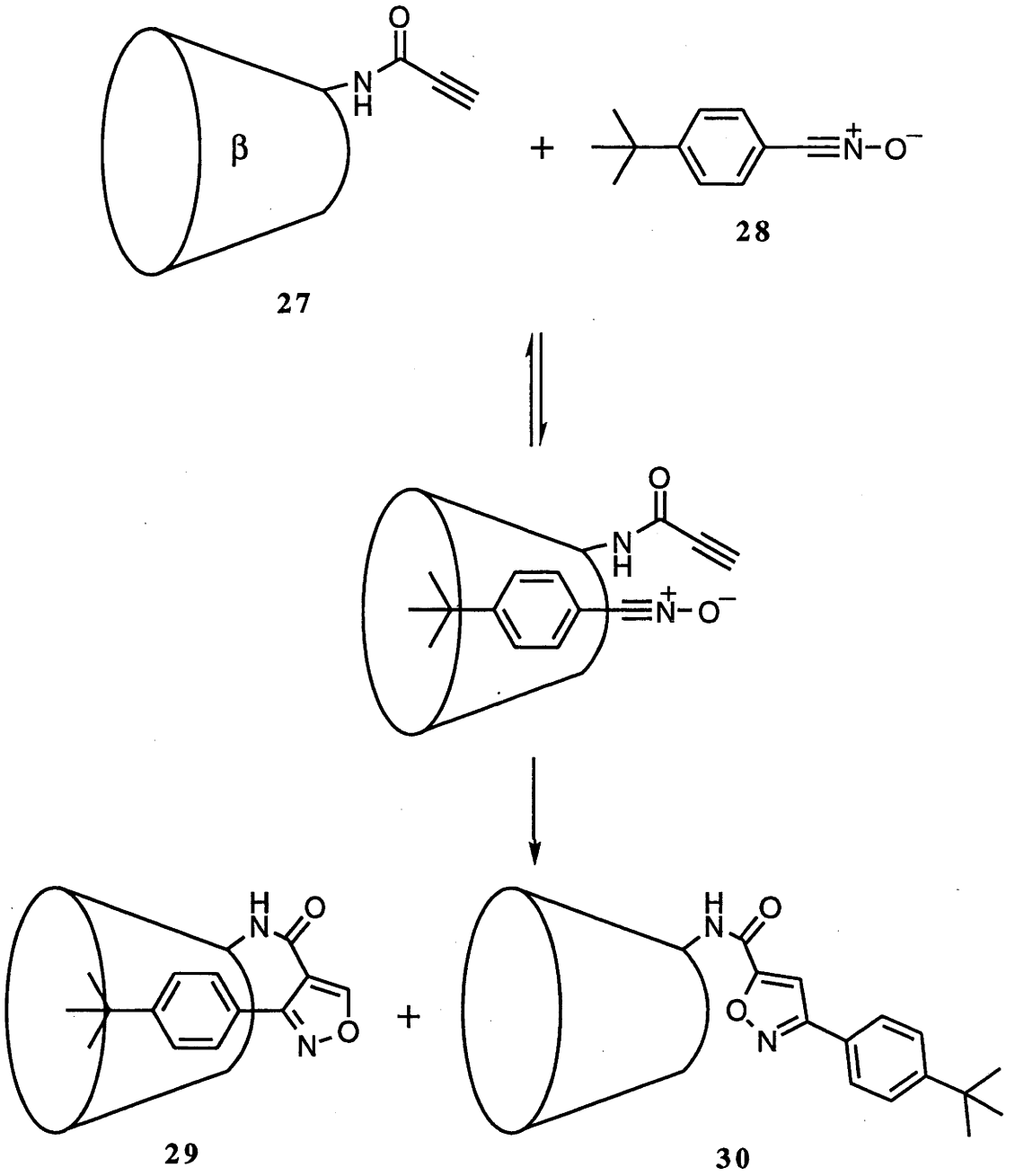
The attachment of a dipolarophile to β-cyclodextrin (**8**) gives the modified cyclodextrin **27**. This cyclodextrin has been used to predetermine the regioselectivity of nitrile oxide cycloadditions by controlling the relative orientation of the dipolarophile and



Scheme 11. Usual regioselectivity of the addition of mono-substituted alkenes and alkynes to nitrile oxides **24**.

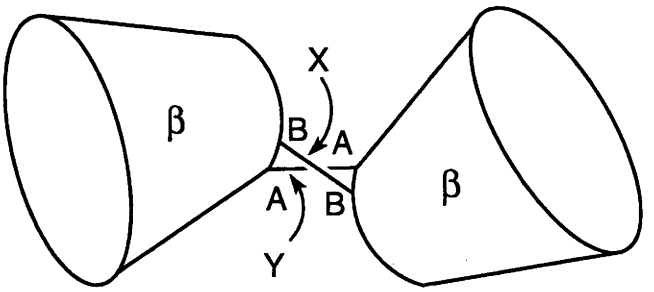
nitrile oxides through the formation of inclusion complexes. This is illustrated by the reaction of the modified cyclodextrin **27** with 4-*tert*-butylbenzonitrile oxide (**28**) (Scheme 12). The orientation of the complex of the guest **28** with the host **27** favours formation of the 4-substituted product **29**. It is produced in 67% yield, compared to 4% for the corresponding 5-substituted product **30**. Thus, attachment of the dipolarophile to a cyclodextrin reverses the normal regioselectivity of nitrile oxide cycloaddition through preassembly of the reagents.

It was envisaged that an alternative method for controlling the regioselectivity of reactions using cyclodextrins might be through the use of linked cyclodextrins, which have been studied widely due to the cooperative guest binding displayed by cyclodextrin annuli in such species.[86-94] It has been shown that the extent of the cooperativity is dependent on the match of the shape of the guest to the alignment of the host cavities. This is illustrated by the work of Breslow and Chung,[90] in which the complex formation properties of the cyclodextrin dimers **31** and **32** were studied. In these doubly linked cyclodextrins, the relative positions of the cyclodextrin annuli are well defined. The dimer **31** is referred to as the aversive or 'loveseat' isomer, while the dimer **32** is referred to as the occlusive or 'clamshell' isomer. In the latter case, the annuli are positioned such that they may close on a guest in much the same way as a clamshell, while the annuli act independently in the former case. Hence, the relative orientation of the annuli determines whether cooperative binding can take place. The complexes formed between the dimer **32** and each of the guests **33** and **34** demonstrate the effect of matching the shape of the guest to the alignment of the annuli in the host. The guest **33** is 'bent' and has a shape corresponding to the relative orientation of the cyclodextrin annuli in the dimer **32**, meaning little distortion of the host or guest is necessary on formation of the



Scheme 12. Cyclodextrins can be used to reverse the normal regioselectivity of nitrile oxide cycloaddition.

corresponding inclusion complex. The resulting complex is thus particularly stable, with an association constant of *ca.* 10^{10} mol^{-1} dm^3.[90] For the linear guest **34** to be included in both annuli of the dimer **32**, significant distortion of either the host **32** or the guest **34** or both is necessary. The complex formed is thus less stable than that formed between the guest **33** and the host **32**, with an association constant of *ca.* 10^5 mol^{-1} dm^3.[90]



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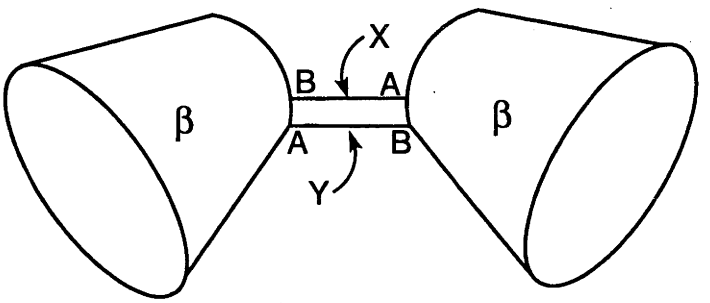

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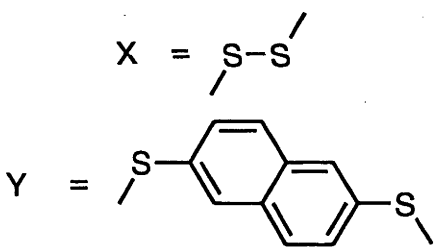
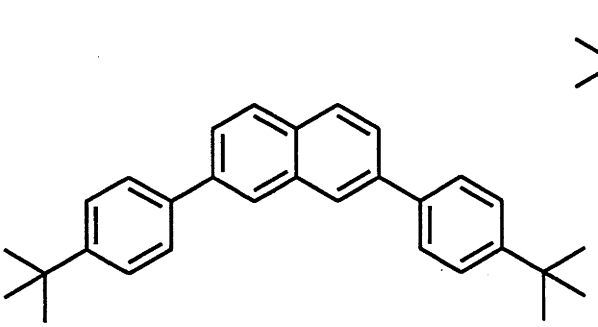
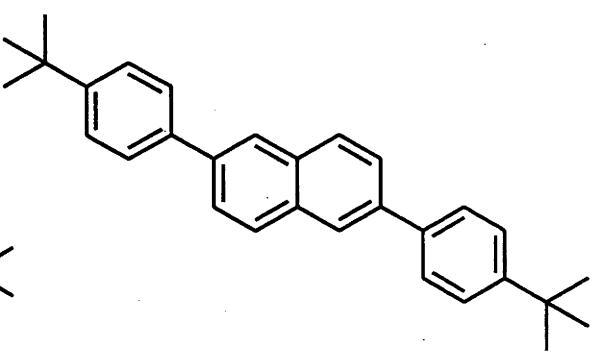


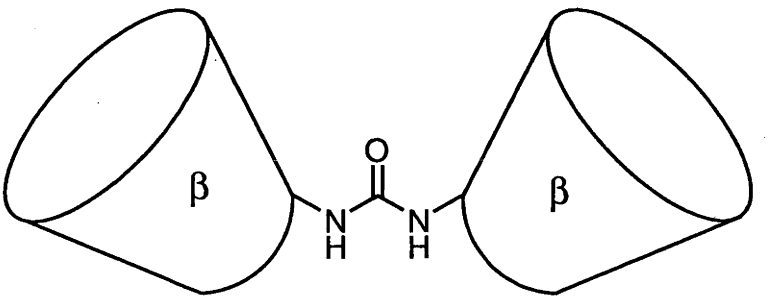


33



34

The dependence of the extent of cooperativity on the match of the shape of the guest to the relative orientation of the host cavities has been used to determine the preferred alignment of the annuli of the cyclodextrin dimers **35**-**37**.[91,92] The complexation of the anions of methyl orange (**38**) and Tropaeolin 000 No. 2 (**39**) by the cyclodextrins **35**-**37** shows maximum binding of the linear dye **38** in the oxalamide **36** and of the non-linear guest **39** in the host **35**. This implies a preferred non-linear orientation of the cyclodextrin annuli in the urea **35** while the annuli of the host **36** are arranged more linearly. The succinamide **37** is observed to complex both of the guests **38** and **39** less strongly than the dimers **35** and **36** though still to a greater degree than the parent cyclodextrin **8**.



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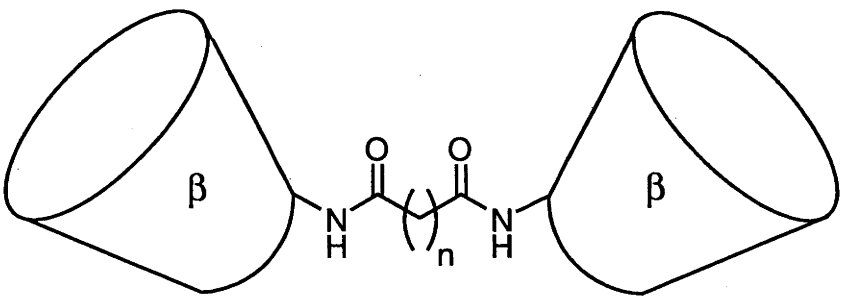
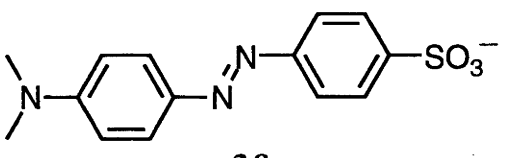


36 n = 0
37 n = 2



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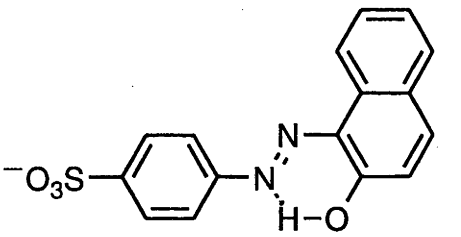


39

It was envisaged that the cyclodextrins **35**-**37** might be used to bias the outcome of condensation reactions through preassembly of the reagents within their annuli. Particularly, it was anticipated that the preferred non-linear alignment of the cyclodextrin

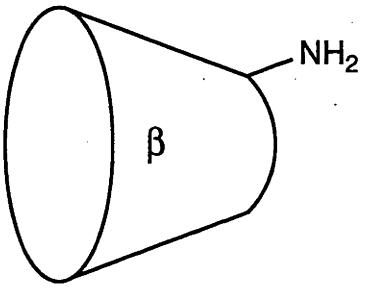
annuli of the urea **35** might be used to complex reagents in a non-linear arrangement, resulting in preferential formation of a non-linear product. Studies examining the use of the cyclodextrins **35-37** as molecular templates for condensation reactions are described in Chapter II of the Results and Discussion

As an alternative to the development of enzyme mimetics, improving the use of enzymes which are normally of limited utility, due to restrictions associated with their biological origins, was also considered. An enzyme that is affected by product inhibition is inappropriate for use in synthetic chemistry as the extent of reaction in a given time is limited by the inhibitory effect of the product. It was anticipated that if the product of reaction was removed from solution, in preference to the substrate, the amount free in solution would decrease. This would result in less product being able to bind to and inhibit the enzyme, without substantially affecting the concentration of the substrate, hence resulting in an increase in the activity of the enzyme and in the extent of reaction in a given time. It was envisaged that a method to remove the product of an enzyme catalysed reaction from solution preferentially might be through inclusion within a molecular host, such as a cyclodextrin.

Consider the elimination of ammonia and a proton from (*S*)-phenylalanine ((*S*)-**3**) to give the cinnamate **4** catalysed by PAL, which is affected by product inhibition. An examination of the substrate (*S*)-**3** and the product **4** suggests that both may be included within a cyclodextrin as the aromatic moieties provide hydrophobic sites of similar size to the cavity of the cyclodextrins **7** and **8**. However, it was expected that the cinnamate **4** would include in the cyclodextrins **7** and **8** in preference to the substrate (*S*)-**3**, due to its reduced ionic character and hence reduced degree of hydration. The reported association constants for the complexes of the substrate (*S*)-**3** with the native cyclodextrins **7** and **8** are 8 and 3 mol^{-1} dm^3, respectively,[95] while the equivalent complexes with the cinnamate **4** as the guest have association constants of 109 and 313 mol^{-1} dm^3, respectively.[96] It was therefore envisaged that the cyclodextrins **7** and **8** could be used to sequester the cinnamate **4** preferentially, reducing the amount free in solution to inhibit PAL. Accordingly, the work described in Chapter III of the Results and Discussion investigates the use of cyclodextrins to reduce product inhibition of enzyme activity.

In the previous discussion, only naturally occurring cyclodextrins were considered. While the cyclodextrins **7**, **8** and **9** are readily available, each has low aqueous solubility, being limited to 14.5 g / 100 cm^3, 1.85 g / 100 cm^3 and 23.2 g / 100 cm^3, respectively.[97,98] This places a constraint on the amount of host that can be present in solution, restricting the degree to which the host can sequester a guest. The limited solubility of the cyclodextrins **7-9** has provided some of the impetus for the investigation of chemically modified cyclodextrins, as they have been shown to have

increased aqueous solubility.[71,72] These modifications may also alter the thermodynamic stability of any complexes formed, relative to the case where the host is the parent cyclodextrin. It was envisaged that modification of a cyclodextrin might be used to increase or decrease the degree to which a particular guest is sequestered and hence alter the effect of the cyclodextrin on an enzyme catalysed reaction. The work described in Chapter IV of the Results and Discussion compares the effects of the naturally occurring cyclodextrins **7** and **8** and the modified cyclodextrin **40** on the reaction catalysed by PAL.



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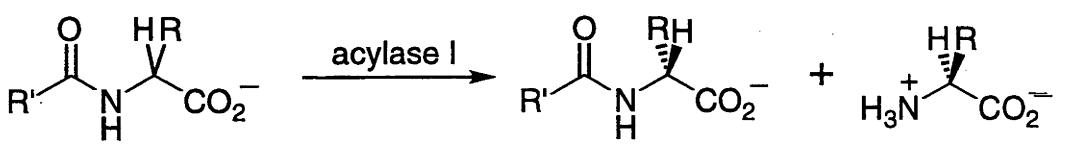
Many enzymes exhibit high specificity for a group of substrates, yet there is limited selectivity within this group. Enzymes which catalyse the reactions of amino acids and peptides are examples of this, as are those involved with the phosphorylation of aldohexaoses.[3,16] As a result, in these cases digestion of a mixture of substrates tends to lead to a mixture of products which is unsuitable as a synthetic procedure, as the products then have to be separated.

Two examples of enzymes which digest a class of molecules are acylase I (also known as aminoacylase or hog renal acylase) and protease VIII (also known as Subtilisin Carlsberg). Acylase I has long been used as a tool for resolution of amino acids, as it selectively deacylates the (*S*)-enantiomers of acylated amino acids, the products being easily separable from the acylated (*R*)-forms (Scheme 13).[99] Further, it has been shown to have a broad specificity, digesting a wide range of substrates.[100,101] Protease VIII has also been shown to be useful for resolution of amino acids, selectively hydrolysing the (*S*)-enantiomers of a range of amino acid esters (Scheme 14).[102-104]



Scheme 13. Deacylation of the (*S*)-enantiomers of acylated amino acids by acylase I.

Scheme 14. Hydrolysis of the (*S*)-enantiomers of amino acid esters by protease VIII.

It was anticipated that if all but one of the components of a mixture being digested by one of these enzymes were removed from solution then only one substrate would remain in solution to be digested, resulting in only one product. Thus, it was envisaged that cyclodextrins might be used to affect the digestion of a mixture of substrates by an enzyme. Chapters V and VI of the Results and Discussion of this thesis examine the digestion of mixtures of substrates by enzymes and investigate the effect of cyclodextrins on these systems.

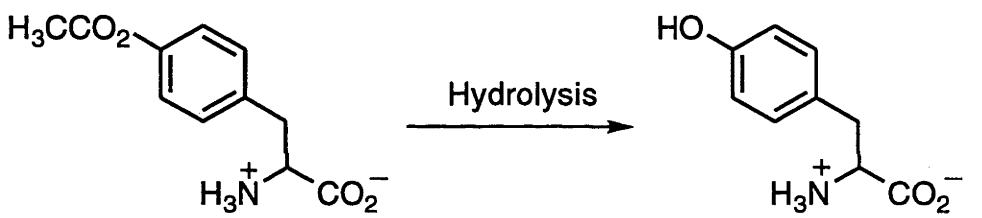
Finally, consider an enzyme that is affected by substrate inhibition. While this form of inhibition prevents excessive digestion of an abundant substrate in biological systems, it limits the utility of the enzyme for synthesis as, at the low concentrations of substrate necessary to maintain enzyme activity, the scales required for reactions are impractical. It was envisaged that if the substrate being digested was removed from solution, then the amount free in solution would decrease. As a result, the proportion of the enzyme present in the less productive ternary complex would decrease, the rate of reaction would increase and the substrate concentration at which enzyme activity was maintained would increase, increasing the practical utility of the process. Further, it was expected that if the substrate was removed from solution by reversible encapsulation in a molecular host, then this host would act as a 'substrate reservoir'. It was anticipated that a cyclodextrin might be used to reduce substrate inhibition and hence increase the utility of enzymes that are adversely affected in this manner. Studies examining this are described in Chapter VII of the Results and Discussion.

Results and Discussion

Chapter I

A Metallocyclodextrin as a Possible Esterase Mimic

As described in the Introduction, metallocyclodextrins based on the modified cyclodextrin **19** exhibit enantioselectivity on complexation of naturally occurring aromatic amino acids[77-79] and it was envisaged that this selectivity might be exploited in the development of an enantioselective enzyme mimic. The reaction chosen to examine this was the hydrolysis of *O*-acetyltyrosine (**41**) to afford tyrosine (**42**) (Scheme 15).



41 **42**

Scheme 15. The hydrolysis of *O*-acetyltyrosine (**41**).

Since the acetate **41** is a derivative of phenylalanine (**3**), it was anticipated that its complexation with a metallocyclodextrin would occur with significant enantioselectivity. The orientation of this complex would be controlled by the interaction of the amino and carboxylate moieties of the amino acid derivative **41** with the metal centre and inclusion of the aromatic ring within the annulus of the cyclodextrin (Figure 4).[77-79] In this orientation, the electrophilic site of the acetate **41** is adjacent to the secondary hydroxyl groups of the metallocyclodextrin. Under basic conditions, the secondary hydroxyl groups of cyclodextrins are deprotonated and have been shown to act as nucleophiles in the cleavage of phenyl esters.[105,106] Thus, under basic conditions it was envisaged that the complexed tyrosine derivative **41** would be hydrolysed by the deprotonated secondary hydroxyl groups of the metallocyclodextrin. Since the acetate **41** might be expected to complex to a metallocyclodextrin enantioselectively and to be hydrolysed once

complexed, it was thought suitable to examine the possible application of metallocyclodextrins as enantioselective esterase mimics.

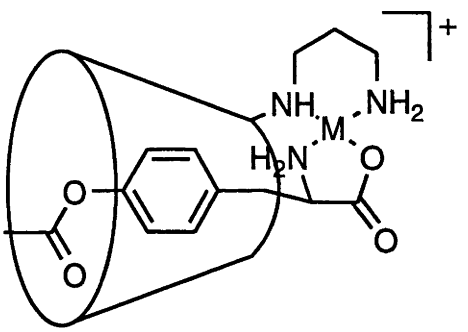
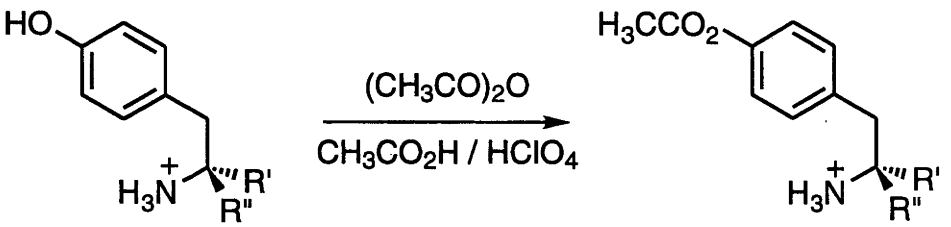


Figure 4. Predicted orientation of *O*-acetyltyrosine (**41**) in the association complex formed with a metallocyclodextrin of the modified cyclodextrin **19**.

The reactions were carried out in buffered solutions to ensure that the pH did not change as they proceeded. A pH value of 10.0 was used as speciation plots of solutions containing a naturally occurring aromatic amino acid, a metal(II) ion and the modified cyclodextrin **19** indicate that the major species present in solution at this pH is the monocationic metallocyclodextrin complex of the amino acid.[77-79] It is on formation of this complex that enantioselectivity is observed.[77-79] Thus, at pH 10.0 in the equivalent system containing the tyrosine derivative **41** it was envisaged that the major species present in solution would be that shown in Figure 4 and that enantioselectivity would be observed on its formation. The secondary hydroxyl groups of the naturally occurring cyclodextrins **7** and **8** have pK_a values of 12.2.[68,69] Thus, at pH 10.0 approximately 1% of the secondary hydroxyl groups of the metallocyclodextrin complex would be expected to be deprotonated and able to facilitate hydrolysis of the complexed acetate **41**. Sodium tetraborate has a pK_{a1} value of 9.14,[107] so a borate buffer was used in the reactions as it has a large buffering capacity at pH 10.0.

Each of the tyrosine derivatives (*R*)-**41** and (*S*)-**41** was prepared using a variation of the method of Sakami and Toennies,[108] through treatment of the corresponding tyrosines (*R*)-**42** and (*S*)-**42** with acetic anhydride in a solution of perchloric acid / acetic acid (Scheme 16).[109] Under these strongly acidic conditions, the amino group of each of the tyrosines (*R*)-**42** and (*S*)-**42** is completely protonated and not nucleophilic. As a result, the phenolic oxygen was the only nucleophilic site in the substrate able to be acetylated. Each of the esters (*R*)-**41** and (*S*)-**41** was isolated in zwitterionic form through precipitation from diethyl ether / acetone, using butylamine to neutralise the acid present. The physical data for each of the esters (*R*)-**41** and (*S*)-**41** are consistent with those reported.[108] The ^{1}H NMR spectrum of each of the products (*R*)-**41** and (*S*)-**41** includes a signal at *ca.* δ2.3 corresponding to the hydrogen nuclei of a phenyl acetate.

No signals were observed at *ca.* δ1.8, at which frequency the protons of an acetamide might be expected to resonate,[110] confirming that only the phenol of the starting materials (*R*)-**42** and (*S*)-**42** had been acetylated. Enantiomeric purity of each of the esters (*R*)-**41** and (*S*)-**41** was established by optical rotation measurements.[108]



(*R*)-**42** R' = CO_2^-, R" = H
(*S*)-**42** R' = H, R" = CO_2^-

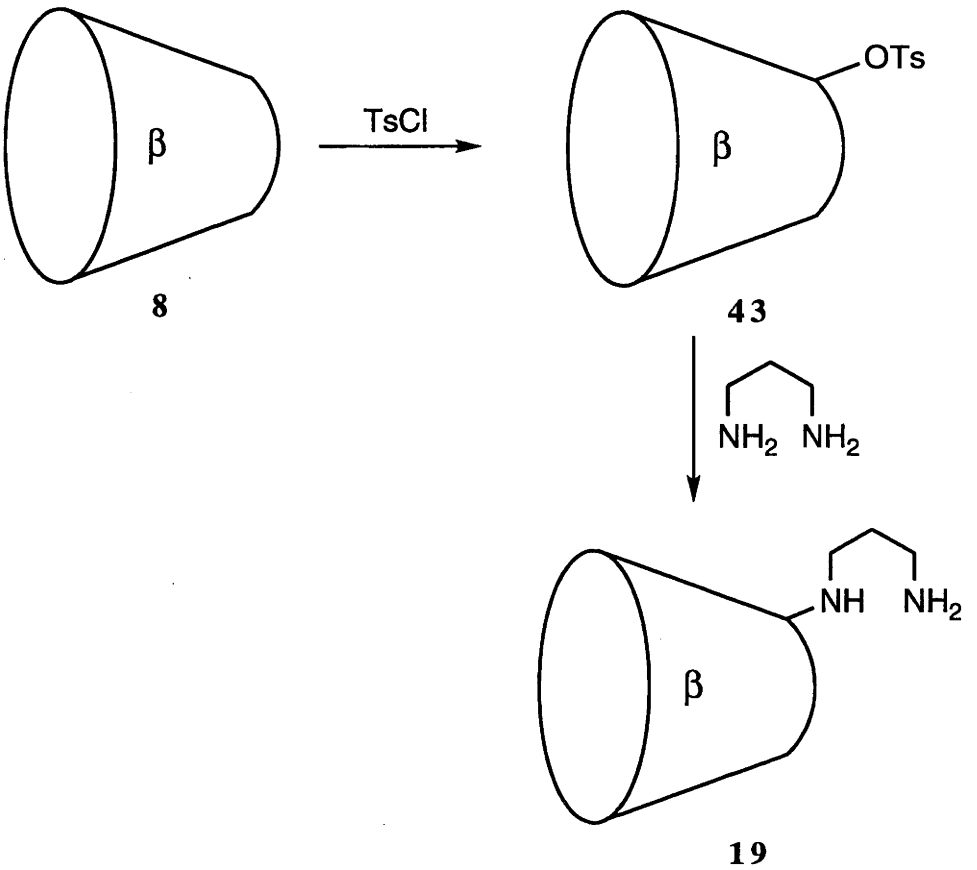
(*R*)-**41** R' = CO_2^-, R" = H
(*S*)-**41** R' = H, R" = CO_2^-

Scheme 16. Synthesis of the tyrosine derivatives (*R*)-**41** and (*S*)-**41** from the corresponding tyrosines (*R*)-**42** and (*S*)-**42**.

6^A-(3-Aminopropyl)amino-6^A-deoxy-β-cyclodextrin (**19**) was prepared from β-cyclodextrin (**8**) *via* the tosylate **43** (Scheme 17), as described in the literature.[111,112] Treatment of the parent cyclodextrin **8** with 4-toluenesulfonyl chloride in pyridine afforded the tosylate **43**. This was purified to remove residual starting material **8** through multiple recrystallisations of the crude product from water. The tosylate **43** was identified through comparison of the observed spectroscopic information with that reported in the literature.[111] Reaction of the tosylate **43** with 1,3-diaminopropane in *N*-methylpyrrolidinone gave the product **19**. The crude product **19** was contaminated with β-cyclodextrin (**8**), a byproduct resulting from hydrolysis of the tosylate **43**, so it was purified using ion exchange chromatography. The modified cyclodextrin **19** was identified through comparison of the observed and reported spectroscopic data.[112] The ^{1}H NMR spectrum of the amine **19** included characteristic resonances corresponding to the hydrogen nuclei of the pendant arm methylene groups at δ1.7, 2.6 and 2.8.

Reactions involving hydrolysis of the esters (*R*)-**41** and (*S*)-**41** were monitored by observing the change in absorbance at 283 nm. Comparison of the absorption spectrum of the starting material **41** and that of the product **42** indicated that this wavelength corresponds to the maximum difference in absorbance between these two species.

Each of the esters (*R*)-**41** and (*S*)-**41** underwent hydrolysis under the reaction conditions in the absence of a cyclodextrin. This is presumably due to nucleophilic attack of the hydroxide ions present in solution on the acetate moiety and was taken into account when evaluating the effect of cyclodextrins.



Scheme 17. Synthesis of the pendant arm cyclodextrin **19** from β-cyclodextrin (**8**) *via* the tosylate **43**.

The effect of the cyclodextrin **19** (1.4 x 10^{-3} mol dm^{-3}) on the hydrolysis of the acetyltyrosines (*R*)-**41** and (*S*)-**41** was examined as a control. The concentration of the esters (*R*)-**41** and (*S*)-**41** was varied over the range 0.2-1.0 x 10^{-3} mol dm^{-3}. The initial rates of reaction (v_0) were quantified at each concentration of the esters (*R*)-**41** and (*S*)-**41**. The rates of reaction in the absence of cyclodextrin (v_{un}) were subtracted from the initial rates of reaction to quantify the rate increase due to the cyclodextrin **19**. This information was interpreted using a modified Lineweaver-Burk analysis.[113] The reciprocal of the observed initial rate after subtraction of the rate in the absence of cyclodextrin ($1/(v_0-v_{un})$) was plotted against the reciprocal of the concentration of the tyrosine derivative **41** for each of the esters (*R*)-**41** and (*S*)-**41** (Figure 5). These data describe a straight line in each case. This indicates that the hydrolysis of each enantiomer of the tyrosine derivative **41** is preceded by the formation of an association complex with the host **19**. The information for each of the acetates (*R*)-**41** and (*S*)-**41** was fitted to Equation 3, and values for the rate constant of the reaction of the bound guest (k_{inc}) and

the association constant of the host-guest complex (K) were calculated for each case using the values of the coordinate axis intercept and the slope (Table 5).

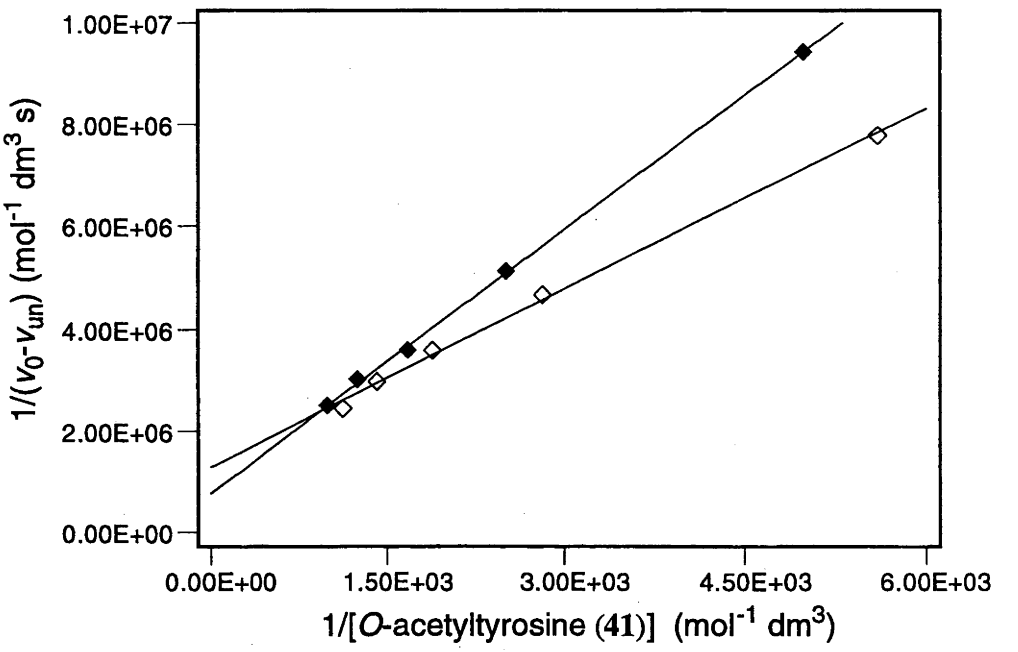


Figure 5. Lineweaver-Burk plot for the hydrolysis of each of the acetyltyrosines (*R*)-**41** (♦) and (*S*)-**41** (◊) in the presence of the modified cyclodextrin **19** (1.4 x 10^{-3} mol dm^{-3}) in 0.01 mol dm^{-3} pH 10.0 borate buffer at 298 K.

$$\frac{1}{v_0 - v_{un}} = \frac{1}{k_{inc}[\text{cyclodextrin}]} + \frac{1}{k_{inc}K[\text{cyclodextrin}][\text{substrate}]} \qquad (3)$$

Substrate	k_{inc} (x 10^3 s^{-1})	K (mol^{-1} dm^3)	$k_{inc}K$ (mol^{-1} dm^3 s^{-1})
(*R*)-**41**	0.84 ± 0.06	480 ± 40	0.40 ± 0.06
(*S*)-**41**	0.56 ± 0.04	1100 ± 100	0.6 ± 0.1

Table 5. Kinetic parameters for interaction between each of the tyrosine derivatives (*R*)-**41** and (*S*)-**41** and the modified cyclodextrin **19** in 0.01 mol dm^{-3} pH 10.0 borate buffer at 298 K.

The association constants for the complexes formed between the tyrosine derivatives (*R*)-**41** and (*S*)-**41** and the modified cyclodextrin **19** were determined to be 480 and 1100 mol^{-1} dm^3, respectively, indicating an enantiodiscrimination on binding of *ca.* 2.3. The rate constants for the reactions of the bound species (0.84 x 10^{-3} and 0.56 x 10^{-3} s^{-1} for the acetates (*R*)-**41** and (*S*)-**41**, respectively) indicate an enantiodiscrimination of *ca.* 1.5. That is, the complexation of the acetate **41** and the

reaction of the bound acetate **41** each proceed enantioselectively, however, the former process is selective for the (*S*)-enantiomer (*S*)-**41**, while the latter is selective for the (*R*)-enantiomer (*R*)-**41**. The product of these two kinetic parameters is a measure of the catalytic efficiency of the host **19** and comparison of this value for each of the esters (*R*)-**41** and (*S*)-**41** gives an indication of the overall enantioselectivity of the process. The product of k_{inc} and K in the case of the (*S*)-isomer (*S*)-**41** is 0.6 mol^{-1} dm^3 s^{-1}, one and a half times that observed for the (*R*)-isomer (*R*)-**41** (0.4 mol^{-1} dm^3 s^{-1}). That is, the overall enantioselectivity of the hydrolysis of the acetate **41** in the presence of the host **19** is 1.5, in favour of the (*S*)-enantiomer (*S*)-**41**.

The perchlorate salts of nickel(II), copper(II) and cobalt(II) (*ca.* 0.9 mole equivalents) were added to solutions containing the modified cyclodextrin **19** in an effort to form a metallocyclodextrin. However, each of these metal ions was found to be insoluble in borate buffer at pH 10.0. Addition of a solution of sodium hydroxide to neutral solutions of these metal ions caused them to precipitate, presumably as the hydroxides, indicating that they are not soluble at high pH.

The reported speciation plots for solutions containing an aromatic amino acid, a divalent metal ion and the cyclodextrin **19** are based on pK values calculated from titration data.[77-79] The titrations were limited by precipitation of the metal ions used, so the pK values were calculated at values of pH below 10.0 and the speciation plots represent an extrapolation of the data. Thus, the speciation plots do not take into account precipitation of the metal ion as the hydroxide. When this is considered it becomes clear that it is impractical to form metallocyclodextrins based on the diamine **19** at pH 10.0. Therefore, in order to form a metallocyclodextrin, lower values of pH, at which the metal ions are soluble, were required.

Copper(II) perchlorate and nickel(II) perchlorate were found to dissolve in water adjusted to pH 8.0 using a solution of sodium hydroxide. Consequently, reactions monitoring the hydrolysis of each of the esters (*R*)-**41** and (*S*)-**41** in the presence of an excess of the cyclodextrin **19** and either copper(II) or nickel(II) (*ca.* 0.9 molar equivalents, based on the cyclodextrin **19**) were carried out in water adjusted to pH 8.0 in this manner. A five fold increase in the initial rate of hydrolysis of each of the acetates (*R*)-**41** and (*S*)-**41** was observed on addition of either of the metal(II) ions and the amine **19**. No enantioselectivity was observed, and this is consistent with the speciation plots for the analogous system containing phenylalanine (**3**) which suggest little of the enantioselective complex illustrated in Figure 4 is formed at pH 8.0.[77] Rather, the hydrolysis may be due to the formation of the protonated form of the complex shown in Figure 4, which might be expected to be the major species in solution based on the

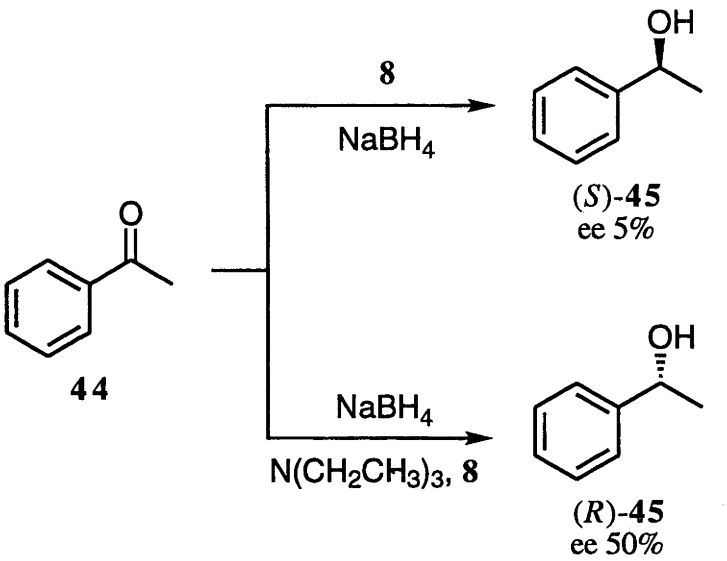
speciation plots of the analogous systems containing phenylalanine (**3**), where the corresponding complex forms without enantioselectivity.

The reactions monitoring the effect of the cyclodextrin **19** and either copper(II) or nickel(II) on the hydrolysis of each of the esters (*R*)-**41** and (*S*)-**41** at pH 8.0 were repeated in water adjusted to pH 7.0. Under these conditions the hydrolysis of the ester **41** proceeded at a rate that was indistinguishable from that in the absence of a cyclodextrin, irrespective of the enantiomer being hydrolysed. By analogy with the equivalent system containing phenylalanine (**3**), the speciation plots predict that the majority of the acetate **41** will not be included in a cyclodextrin at pH 7.0,[77] as is consistent with this result.

From these last two series of experiments, it is evident that enantioselective hydrolysis of the ester **41** does not occur in reaction mixtures containing the cyclodextrin **19** and a metal(II) ion, presumably because the enantioselective complex illustrated in Figure 4 does not form to a significant extent. Thus, it was necessary to investigate an alternative method to induce enantioselective hydrolysis of the ester **41**.

It was envisaged that an external nucleophile added to a solution containing the cyclodextrin **19** and a metal(II) ion might facilitate enantioselective catalysis of the acetate **41** hydrolysis through the formation of a quaternary complex with the ester **41**, the cyclodextrin **19** and the metal(II) ion. Complexes involving the interaction of more than one species within a cyclodextrin have been shown to be responsible for chiral induction in some reactions, notably the reduction of acetophenone (**44**) using sodium borohydride (Scheme 18).[114,115] In the presence of β-cyclodextrin (**8**) alone, the (*S*)-enantiomer of the alcohol **45** is produced in 5% enantiomeric excess (ee). The addition of a variety of alcohols and amines alters this enantioselectivity, the most marked effect occurring on addition of triethylamine. In this case, the enantioselectivity of the reduction is reversed with the (*R*)-enantiomer of the alcohol **45** being produced in 50% ee. This was shown by NMR spectroscopy to occur through the formation of a complex between the host **8**, acetophenone (**44**) and triethylamine.[114]

The reactions monitoring the hydrolysis of each of the esters (*R*)-**41** and (*S*)-**41** in the presence of an excess of the cyclodextrin **19** and either copper(II) or nickel(II) (*ca.* 0.9 molar equivalents, based on the cyclodextrin **19**) at pH 8.0 were repeated in the presence of external nucleophiles (*ca.* 5 molar equivalents, based on the cyclodextrin **19**). On addition of either methanol, ethanol or ethylene glycol to a reaction mixture, no change in the initial rate of reaction of either of the esters (*R*)-**41** or (*S*)-**41** was observed. Addition of either mercaptoethanol or benzyl thiol to a reaction mixture resulted in a dramatic increase in the initial rate of change in absorbance at 283 nm. Further



Scheme 18. The reduction of acetophenone (**44**) in the presence of β-cyclodextrin (**8**). On addition of triethylamine, the enantioselectivity of the reduction is reversed

experiments in the absence of the acetate **41** and the cyclodextrin **19** showed that these changes were due to interaction of the metal(II) ion with the nucleophile, presumably resulting in oxidation of the sulfur. This made monitoring any hydrolysis of the acetate **41** impractical in these cases. Given the lack of success with the nucleophiles used, it was deemed impractical to continue this study.

Since the effect of solutions containing the cyclodextrin **19** and a metal(II) ion on the hydrolysis of the acetate **41** was small in magnitude and not enantioselective, studies examining the effects of cyclodextrins on other reactions were pursued instead.

Results and Discussion

Chapter II

Rational Design of a Molecular Template for Condensation Reactions

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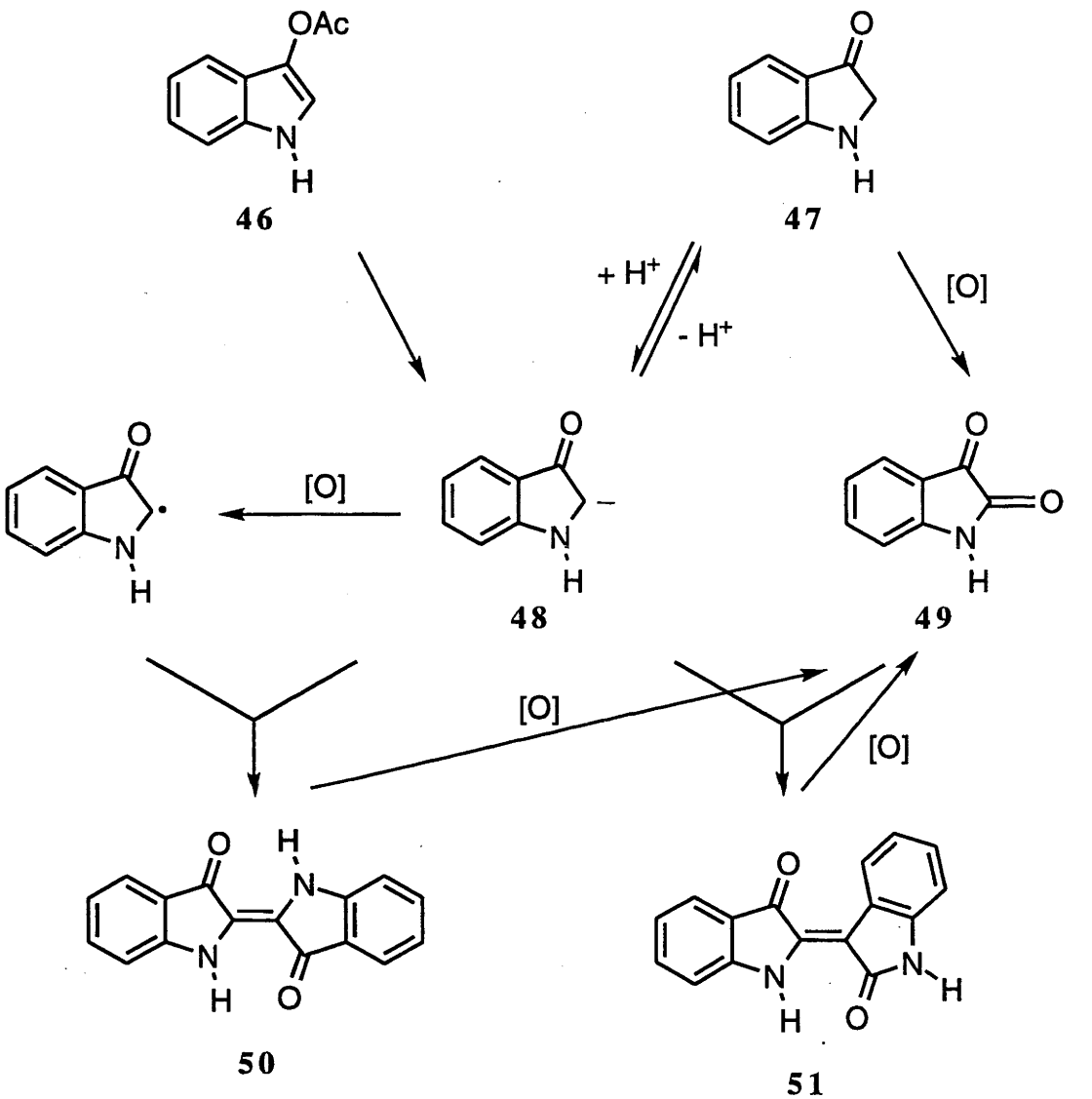
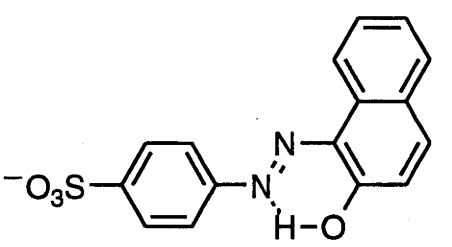
As discussed in the Introduction, the cyclodextrin dimers **35-37** have been shown to exhibit a preferred geometry of their annuli. It was envisaged that this geometry might be exploited to control the alignment of reagents in a condensation reaction, and hence control the regioselectivity of condensation. Thus, the cyclodextrin dimers **35-37** might be used as molecular templates. The system chosen for study was the competitive formation of $\Delta^{2,2'}$-biindoline-3,3'-dione (indigo) (**50**) and $\Delta^{2,3'}$-biindoline-2',3-dione (indirubin) (**51**).

Indigo (**50**) has been used as a dye since ancient times, having a distinctive blue colour, and is still in widespread use today.[116] It is formed through the oxidative dimerisation of 1*H*-indol-3-ol anion (indoxyl anion) (**48**). The non-linear isomer **51** is a pink dye and is the condensation product of the anion **48** and 1*H*-indoline-2,3-dione (isatin) (**49**).[117,118] Isatin (**49**) is formed in the reaction mixture as an oxidation product of indoxyl (**47**), indigo (**50**) and indirubin (**51**). The indoxyl anion (**48**) may be formed through the deprotonation of indoxyl (**47**) or through base catalysed hydrolysis of indoxyl acetate (**46**) (Scheme 19). Industrial syntheses of indigo (**50**) require carefully controlled conditions to ensure no isatin (**49**) is present and hence prevent contamination of indigo (**50**) with indirubin (**51**).[119]

Since indigo (**50**) is linear, it is reasonable to assume that its formation requires a linear alignment of the reagents. Similarly, it follows that a non-linear arrangement of the precursors is required for the formation of indirubin (**51**). It was envisaged that the cyclodextrin dimers **35-37** might be used to bias the ratio of dye formation by preassembly of the reagents **48** and **49**. Particularly, the preferred non-linear alignment of the cyclodextrin annuli of the urea **35** was anticipated to complex the reagents **48** and **49** in a non-linear arrangement, resulting in a preference for the formation of the non-linear dye **51**. This expectation was based on the structural similarity of indirubin



Scheme 19. The formation of indigo (**50**) and indirubin (**51**).



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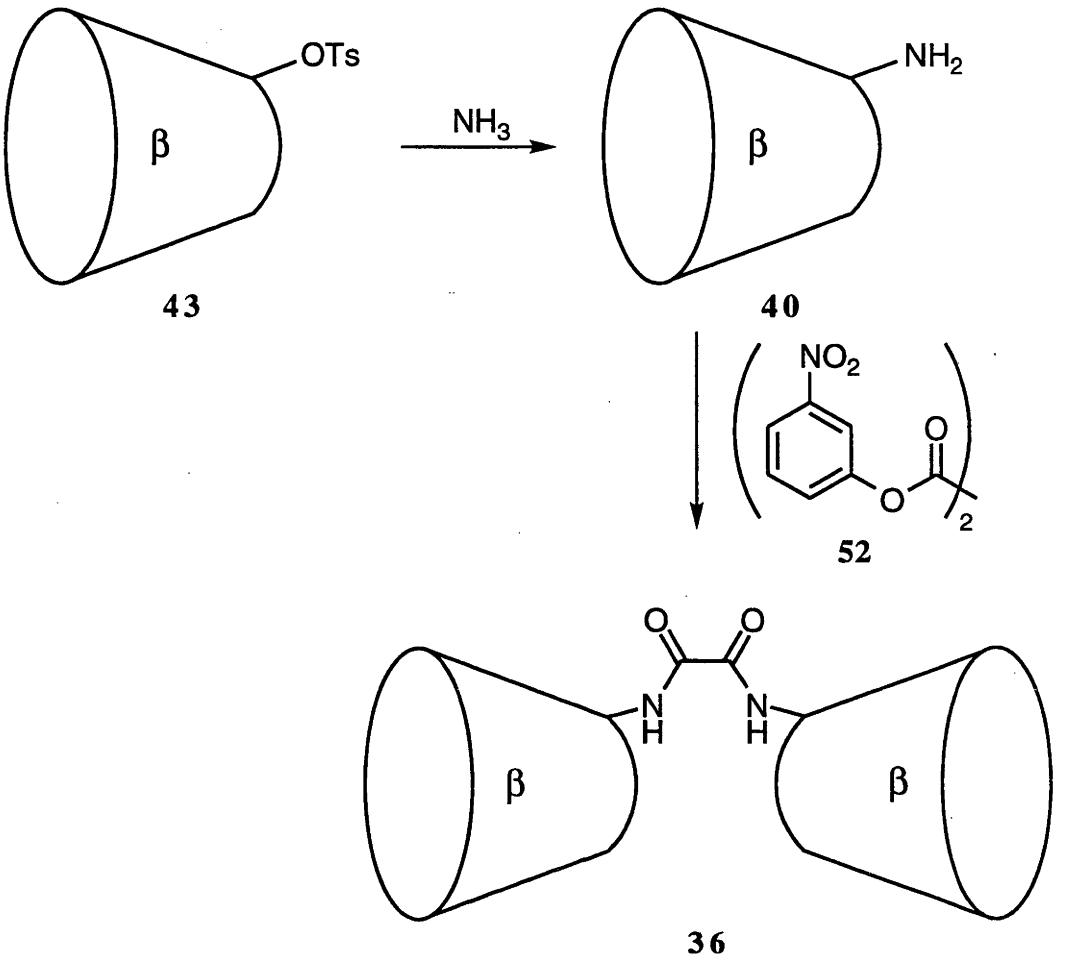
and the anion of Tropaeolin 000 No. 2 (**39**), which is known to be complexed efficiently in the dimer **35**.[92]

It should be noted that where the preferred alignment of the annuli of a cyclodextrin dimer was used to restrict the stereochemistry of a condensation reaction, the product of that reaction would be expected to be complexed efficiently by the dimer. Thus, if the association constants of the complexes formed between each of the dyes **50** and **51** and each of the cyclodextrins **35-37** were known, the effect of the cyclodextrins **35-37** on competing reactions to form indigo (**50**) and indirubin (**51**) might be more reasonably predicted. However, both indigo (**50**) and indirubin (**51**) are insoluble in water, so measurement of the corresponding association constants was impractical.

Authentic samples of the dyes **50** and **51** were required to calibrate the analytical methods used in these studies. Indigo (**50**) was commercially available while indirubin (**51**) was synthesised from isatin (**49**) and indoxyl acetate (**46**) under basic conditions in the absence of oxygen. The product **51** was purified by chromatography and identified by comparison of its spectroscopic information with that reported in the literature.[118]

The cyclodextrin dimers **35** and **37** were available in the laboratory,[120] while the oxalamide **36** was prepared as illustrated in Scheme 20. The tosylate **43**, prepared as described in Chapter I of the Results and Discussion, was treated with ammonia in a high pressure reaction vessel to give the amine **40**. The crude product **40** was contaminated with the parent cyclodextrin **8**, a byproduct resulting from hydrolysis of the tosylate **43**, so it was purified using ion exchange chromatography. The identity of the amine **40** was confirmed through comparison of observed spectroscopic information with that reported.[111,121] The ^{13}C NMR spectrum of the product **40** contained a characteristic resonance at δ43.5, corresponding to the carbon at the 6-position of the substituted glucose unit.[111,121] The dimer **36** was produced through reaction of the amine **40** with the ester **52**. The amine **40** was added to the reaction mixture in excess, to facilitate complete reaction of the ester **52**, so it contaminated the product **36**. The dimer **36** was readily separated from the residual starting material **40** using ion exchange chromatography and identified by comparison of observed with reported spectroscopic data.[94]

As a source of the indoxyl anion (**48**) the parent indoxyl (**47**) could be used, but it is particularly air sensitive and difficult to handle, and therefore impractical. Indoxyl acetate (**46**) is a stable solid and can be hydrolysed to give the anion **48** under basic conditions. Thus, it is suitable for the *in situ* preparation of the indoxyl anion (**48**) and was used in the present work.



Scheme 20. Synthesis of the dimer **36** from the tosylate **43** *via* the amine **40**.

Basic conditions are required to saponify the acetate **46** and deprotonate the indoxyl (**47**) formed. However, isatin (**49**) is known to undergo ring opening at high pH.[122-125] Since both indoxyl anion (**48**) and isatin (**49**) must be present in solution for the competing formation of the dyes **50** and **51** to be monitored, it was necessary to determine a value of pH at which indoxyl anion (**48**) was formed and the isatin (**49**) was not hydrolysed.

The competing reactions of indoxyl anion (**48**) were therefore examined in the absence of cyclodextrins over the pH range 8-13 in either borate or phosphate buffer at 298 K. A buffer was used in each case to ensure that the pH of the mixture did not change as the reaction proceeded. Sodium tetraborate has a pK_{a1} of 9.14,[107] so borate buffer was used for the reactions at pH 8, 9 and 10 as it has significant buffering capacity at these values. Similarly, sodium phosphate has a pK_{a3} of 12.67,[107] so phosphate buffer was used for the reactions at pH 11, 12 and 13 as it has significant buffering capacity at these values. Isatin (**49**) was added in *ca.* five molar excess with respect to

the acetate **46** and the total concentration of indole moieties used was *ca.* 0.7 x 10^{-3} mol dm^{-3}. After 16 h, the products **50** and **51** were extracted into chloroform. Since the two dyes **50** and **51** have very large absorbances in the visible region, these extracts were analysed using high-performance liquid chromatography (HPLC) monitoring at 550 nm with a visible detector. Authentic samples of the dyes **50** and **51** were used to calibrate the detector, which was found to have a linear response of absorbance to the quantity of dye injected. The results of these experiments are shown in Table 6.

pH	Percentage yields of indigo (**50**) and indirubin (**51**) as the ratio
8.0	0 : 0
9.0	2.2 : 2.6
10.0	16 : 13
11.0	64 : 11
12.0	18 : 0.2
13.0	8 : 0.05

Table 6. Yields of indigo (**50**) and indirubin (**51**) from reaction mixtures containing indoxyl anion (**48**) and isatin (**49**) at pH values in the range 8-13.

At pH values below 10, the overall yields of the dyes **50** and **51** were low, being 4.8% at pH 9.0 and below the limits of detection at pH 8.0. This indicated that either indoxyl acetate (**46**) is not saponified or indoxyl (**47**) is not sufficiently deprotonated. The latter explanation is supported by literature.[126]

The yield of each of the dyes **50** and **51** was observed to increase, from 2.2% to 16% and 2.6% to 13%, respectively, on increasing the pH from 9.0 to 10.0. This is consistent with more indoxyl anion (**48**) being formed at pH 10.0, reducing side reactions of indoxyl (**47**). The ratio of the yields of indigo (**50**) and indirubin (**51**) was *ca.* 1 : 1 at both pH 9.0 and 10.0.

At pH 11.0, the total yield of the dyes **50** and **51** is 75%, compared to 29% at pH 10.0. As above, this is consistent with more indoxyl anion (**48**) being formed at the higher pH, reducing the effect of side reactions of indoxyl (**47**). However, at this pH the ratio of indigo (**50**) to indirubin (**51**) is considerably different to that observed at pH 10.0. Rather than the dyes **50** and **51** being produced in approximately equal amounts, the yield of indigo (**50**) is *ca.* six times that of indirubin (**51**). This is consistent with the ring opening of isatin (**49**), which is known to be catalysed by phosphate buffer at high pH.[124,125] As the concentration of isatin (**49**) decreases, so does the rate of its reaction to form indirubin (**51**).

Increasing the pH above 11 was seen to further affect the ratio of the yields of the dyes **50** and **51**. The preference for the formation of indigo (**50**) increased from six times at pH 11.0 to ninety times at pH 12.0 and one hundred and sixty times at pH 13.0. This is consistent with the isatin (**49**) being hydrolysed more efficiently at higher pH,[124,125] resulting in a more rapid decrease in its concentration and hence a greater reduction in the yield of indirubin (**51**). Further, the total yield of the dyes **50** and **51** was seen to drop dramatically on increasing the pH, from 75% at pH 10.0 to 18% at pH 12.0 and 8% at pH 13.0. This suggests that side reactions of indoxyl (**47**) or the corresponding anion **48** are important at the higher pH values.

These results were used to identify the pH range 10-11 as the most suitable for monitoring the effect of cyclodextrins on the competing formation of indigo (**50**) and indirubin (**51**). While the previous experiments were analysed after 16 h, it was envisaged that the ratio of the dyes **50** and **51** would change with time as the isatin (**49**) was hydrolysed. To determine a suitable reaction time, the experiments described above were repeated at pH 11.0 and 298 K in phosphate buffer and the products **50** and **51** were analysed after 2 min, 15 min and 16 h (Table 7).

Reaction Time	Percentage yields of indigo (**50**) and indirubin (**51**) as the ratio
2 min	15 : 5.2
15 min	47 : 12
16 h	64 : 11

Table 7. Yields of indigo (**50**) and indirubin (**51**) from reaction mixtures containing indoxyl anion (**48**) and isatin (**49**) in 0.05 mol dm^{-3} phosphate buffer at pH 11.0.

These results indicate that no indirubin (**51**) is formed after 15 min. This implies that no isatin (**49**) remains in solution to react after this time, presumably having been completely hydrolysed. Thus, the competition being monitored has ceased, so subsequent reactions carried out at pH 11.0 were analysed after 15 min. A longer time (16 h) was allowed for the reactions at lower values of pH as the hydrolysis of the isatin (**49**) was expected to be slower, as was the production of indoxyl anion (**48**) from the acetate **46**.

To examine the effects of cyclodextrins on the competing formation of the dyes **50** and **51**, reactions were carried out in the presence of either no cyclodextrin, β-cyclodextrin (**8**) or one of the linked species **35-37**, at pH 10.0, 10.5 and 11.0. The reaction mixtures were prepared using either borate buffer (pH 10.0 and 10.5) or phosphate buffer (pH 11.0) and they were analysed after 16 h (pH 10.0 and 10.5) or 15 min (pH 11.0). In comparison with the total concentration of the indole moieties

(*ca.* 0.7 x 10^{-3} mol dm^{-3}), each of the cyclodextrin dimers **35-37** was added in *ca.* ten fold molar excess and β-cyclodextrin (**8**) was added in *ca.* twenty fold molar excess. That is, in the cases in which a cyclodextrin was present, the total concentration of the annuli was approximately twenty times that of the indole groups. Given that the association constants of complexes of indoles and cyclodextrins are of the order of 10^3 mol^{-1} dm^3,[46] based on Equation 1 *ca.* 90% of the indole moieties will be complexed at the concentrations of cyclodextrins used. Thus, these cyclodextrin concentrations were chosen to ensure that the majority of the reagents **48** and **49** was complexed. The yields of the dyes **50** and **51** produced were calculated using HPLC analyses of the chloroform extracts of the reaction mixtures and are shown as Table 8.

Cyclodextrin	Percentage yields of indigo (**50**) and indirubin (**51**) as the ratio		
	pH 10.0	pH 10.5	pH 11.0
-	16 : 13	41 : 38	47 : 12
8	2.5 : 2.5	8.5 : 9.0	9.5 : 12
35	0.03 : 1.0	0.4 : 7.5	2.5 : 17
36	0.2 : 0.6	9.0 : 6.5	15 : 14
37	0.5 : 0.7	5.5 : 8.5	6.0 : 6.5

Table 8. Yields of indigo (**50**) and indirubin (**51**) from reaction mixtures containing indoxyl anion (**48**) and isatin (**49**).

Each of the cyclodextrins **8** and **35-37** was observed to decrease the total yield of the dyes **50** and **51**. This is consistent with the complexation of the reagents **48** and **49** within the cyclodextrin annuli, reducing the opportunity for their productive collisions, hence favouring their monomeric reactions at the expense of their second order reactions to form the dyes **50** and **51**.

It is noteworthy that the ratio of the yields of indigo (**50**) and indirubin (**51**) produced at pH 11.0 was *ca.* 1 : 1 in the presence of the monomer **8** compared to *ca.* 4 : 1 in the absence of a cyclodextrin. At the other pH values, this ratio was unchanged by addition of the cyclodextrin **8**, remaining at *ca.* 1 : 1. The effect at pH 11.0 is consistent with the cyclodextrin **8** complexing the isatin (**49**) and protecting it from hydrolysis. In separate experiments, the orange colour of isatin (**49**) was seen to persist in the presence of β-cyclodextrin (**8**) at pH 11.0, while it disappeared when no cyclodextrin was present.

In comparison to the other cyclodextrins **8**, **36** and **37**, the dimer **35** has a marked effect on the ratio of formation of indigo (**50**) and indirubin (**51**). In each case that the host **35** was present, the yield of indirubin (**51**) remained similar to that observed at the same pH value in the presence of the cyclodextrins **8**, **36** and **37** while the yield of indigo (**50**) decreased. Consequently, the ratio of the yields of indirubin (**51**) and

indigo (**50**) increased from *ca.* 1 : 1 in the presence of the other hosts **8**, **36** and **37**, with a maximum preference for the formation of indirubin (**51**) of 3 : 1, to 33 : 1 at pH 10.0, 18 : 1 at pH 10.5 and 6 : 1 at pH 11.0 in the presence of the urea **35**. Any interpretation of these results must account for the particular effect of the cyclodextrin **35**, which is not to produce a higher yield of indirubin (**51**), but to reduce sharply the amount of indigo (**50**) that forms.

The reaction carried out at pH 10.0 in the presence of the urea linked dimer **35** afforded indigo (**50**) in only 0.03% yield, compared to 2.5% in the presence of the monomer **8** and 16% in the absence of a cyclodextrin at the same pH. This indicates that in the presence of the dimer **35** at this pH, indoxyl anion (**48**) is almost completely complexed and in an orientation that prevents oxidative dimerisation so that almost no indigo (**50**) is formed. At higher pH values, where larger amounts of indoxyl anion (**48**) would be present, more would be expected to remain free in solution. This is consistent with the results observed in the presence of the dimer **35** at higher pH and the increases in the yield of indigo (**50**) to 0.4% at pH 10.5 and 2.5% at pH 11.0.

While not being able to dimerise, the anion **48** complexed in the urea **35** must still be able to react with isatin (**49**), to form indirubin (**51**), as it does in solution. Since the anion **48** is complexed under these conditions, it is reasonable to assume that the isatin (**49**), being more hydrophobic, would also be complexed. This is consistent with the effect of the cyclodextrins **8**, **36** and **37** on the competing formation of the dyes **50** and **51** at pH 11.0, which is to increase the proportion of indirubin (**51**) formed through complexation of the isatin (**49**), protecting it from hydrolysis. Therefore, indirubin (**51**) formed in reactions containing the cyclodextrin **35** is the product of the two reagents **48** and **49** complexed in the cyclodextrin annuli.

The most probable orientation of the anion **48** in a cyclodextrin annulus is with the enolate portion protruding from the end delineated by the primary hydroxyl groups (Figure 6). This is consistent with the antiparallel alignment of the dipole moments of the hosts and guests in cyclodextrin host-guest complexes.[127-129] Further, it is consistent with the reduced formation of indigo (**50**) in the presence of the dimer **35** as in the opposite orientation each of the cyclodextrins **8** and **35-37** would be expected to have a similar effect on the yield of indigo (**50**).

Oxidative dimerisation of the anion **48** complexed in the dimer **35** to give indigo (**50**) is disfavoured. This is presumably a result of the unsuitable alignment of the cyclodextrin annuli (Figure 6). At the same time, the geometry of the cyclodextrin dimer **35** allows reaction of the anion **48** with complexed isatin (**49**) to give indirubin (**51**) (Figure 6). When the anion **48** is complexed in one of the other dimers **36** and **37**

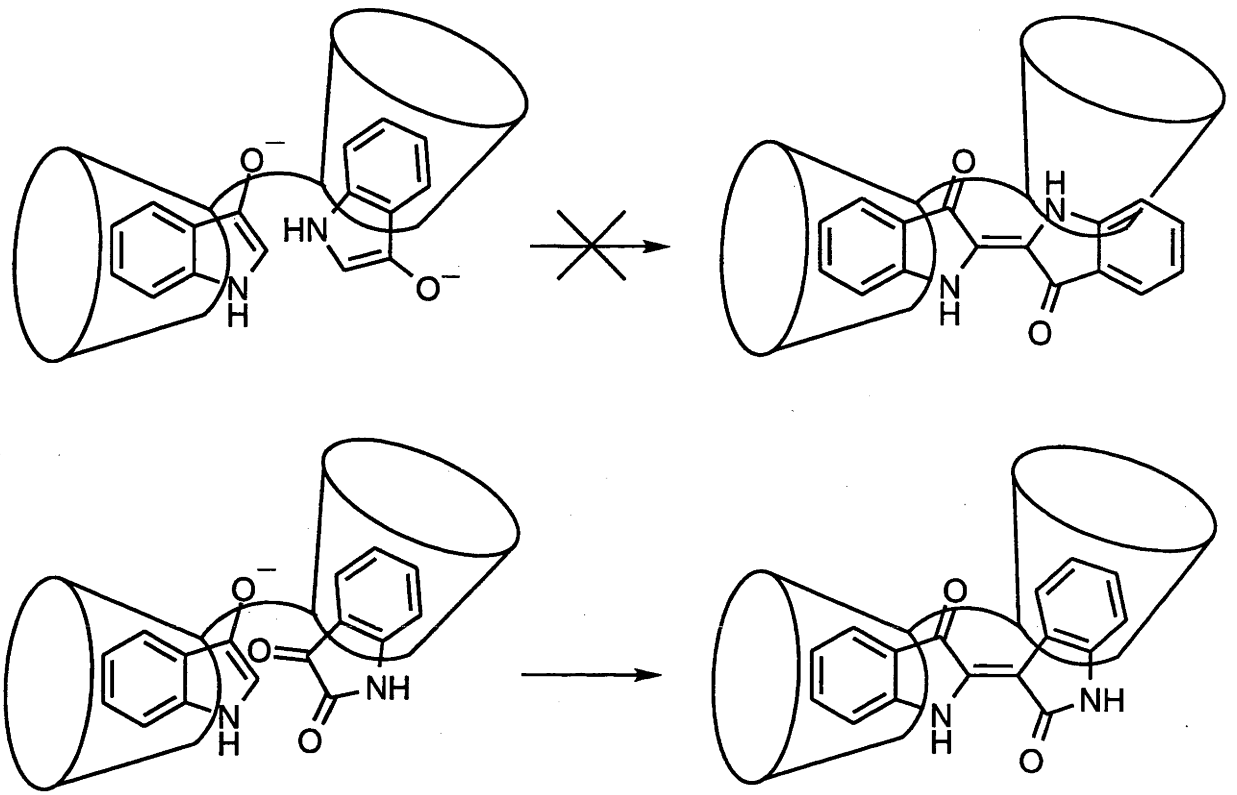


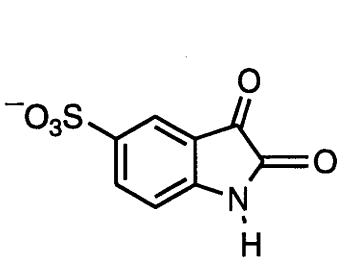
Figure 6. Representation of the effect of the cyclodextrin **35** as a template for condensation of indoxyl anion (**48**) with isatin (**49**) while disfavouring oxidative dimerisation of the anion **48**.

oxidative dimerisation occurs, presumably because in these cases the necessary alignment of the cyclodextrin annuli is more favourable.

The dimer **35** represents the first rationally designed template for the control of geometry in a condensation reaction. The template effect is a result of the preferred alignment of the cyclodextrin annuli of the dimer **35**. The cyclodextrin **35** acts to limit the formation of indigo (**50**) while allowing the production of indirubin (**51**), through the preassembly of the reagents **48** and **49**.

In the experiments described above, it is not clear to what extent the isatin (**49**) which reacts to form indirubin (**51**) is that added initially or that formed by oxidation of one of the other components of the reaction mixture (Scheme 19). To examine the origin of the indoline moieties of indirubin (**51**) in these reactions, a derivative of isatin (**49**), 1*H*-indoline-2,3-dione-5-sulfonate (isatin-5-sulfonate) (**53**) was used. The condensation product of the anion **48** and the isatin derivative **53** is the sulfonated analogue of indirubin (**51**), $\Delta^{2,3'}$-biindoline-2',3-dione-5'-sulfonate (**54**). It was envisaged that through monitoring reaction mixtures containing indoxyl anion (**48**) and the isatin

derivative **53**, the products observed would indicate whether any isatin (**49**) is formed during the reaction. Any indirubin (**51**) would be the product of indoxyl anion (**48**) reacting with isatin (**49**) formed during the reaction.



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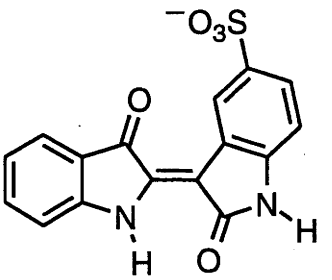

54

Further, the competing reactions of indoxyl anion (**48**) in mixtures containing the isatin derivative **53** offered the opportunity to examine the effect of cyclodextrins on a second pair of competing reactions. It was envisaged that in the presence of the dimer **35**, the indirubin derivative **54** might be formed in preference to indigo (**50**) through preassembly of the reagents **48** and **53** in the cyclodextrin annuli, in a manner analogous to the affect of the dimer **35** on competing reactions to form the dyes **50** and **51**.

To provide an authentic sample for the calibration of the analytical methods used in these studies, the sulfonated indirubin analogue **54** was synthesised from indoxyl acetate (**46**) and the isatin derivative **53** under basic conditions in the absence of oxygen. The product **54** was purified using affinity chromatography and fully characterised. The ^{1}H NMR spectrum of the sulfonate **54** consists of seven signals in the aromatic region. These include one resonance significantly downfield from the remaining signals, at δ9.62, corresponding to the 4'-hydrogen. The downfield shift of this signal is due to the proximal sulfonate group and the ketone moiety of the adjacent indoline.

During trial experiments observing the competing reactions of indoxyl anion (**48**) in the presence of the sulfonate **53**, it was noted that on extraction of the reaction mixtures with chloroform, a pink colour, consistent with each of the indirubins **51** and **54**, remained in the aqueous phase. HPLC analysis of the two phases showed that each contained only one component with an absorption at 550 nm. These compounds were identified as indigo (**50**) in the organic phase and the indirubin derivative **54** in the aqueous phase, through comparison of the HPLC analyses with those of authentic samples. Since no indirubin (**51**) was detected, there is no evidence of isatin (**49**) being produced in the reaction mixture and reacting with the anion **48**. By analogy, it is reasonable to assume that no isatin (**49**) is produced during the competitive formation of

the dyes **50** and **51**. As a result, indirubin (**51**) formed in that case is the product of indoxyl anion (**48**) and isatin (**49**) added initially.

Partitioning between aqueous and organic phases also allowed the analysis of reaction mixtures containing indigo (**50**) and the indirubin derivative **54** to be simplified. Since HPLC analyses established that the dyes **50** and **54** were the only components of the organic and aqueous phases, respectively, with an absorbance at 550 nm, the amount of each could be determined through measuring the absorbance spectra of the phases and calculating product concentrations using molar extinction coefficients determined with authentic samples. It is notable that a cyclodextrin present in the reaction mixture would be expected to remain in the aqueous phase and to complex the indirubin derivative **54** to some extent. While complexation is known to alter the spectroscopic properties of a guest, reported data for other similar dyes suggests that the change in the molar extinction coefficient on complexation is likely to be small,[45,92] allowing the yield of the sulfonate **54** to be estimated using the molar extinction coefficient of the species free in solution.

The assay examining the competing reactions of indoxyl anion (**48**) in a solution containing isatin (**49**) but no cyclodextrin at pH 10.0, was repeated using the sulfonate **53** in place of isatin (**49**) but under otherwise identical conditions, except that the reaction was analysed through extracting the reaction mixture with chloroform and measuring the absorbance spectrum of each phase. This experiment was used to determine if these conditions were suitable for monitoring the competing formation of indigo (**50**) and the sulfonate **54**. The dyes **50** and **54** were afforded in 25% and 1.4% yield, respectively. In the equivalent reaction with the isatin (**49**) rather than the sulfonate **53** added, the dyes **50** and **54** were produced in 16% and 13% yield, respectively. Thus, while the total yield of dyes produced in each case is similar, the ratio is not. This suggests that, relative to the isatin (**49**), the sulfonate **53** is being hydrolysed more quickly under the reaction conditions.

The assays examining the effects of cyclodextrins on competing formation of the dyes **50** and **51** at pH 10.0 were repeated using the sulfonate **53** in place of isatin (**49**) but under otherwise identical conditions, except that reactions were analysed through extracting the mixtures with chloroform and measuring the absorbance spectrum of each phase. The reactions were carried out at pH 10.0 as at lower pH it was expected that the acetate **46** would not be saponified to afford indoxyl anion (**48**), the precursor necessary for the formation of the dyes **50** and **51**. At higher pH it was expected that hydrolysis of the isatin derivative **53** would be faster, removing it from solution and terminating one of the competing reactions being monitored. The results are summarised in Table 9.

Cyclodextrin	Percentage yields of indigo (**50**) and the sulfonated indirubin **54** as the ratio at pH 10.0
-	25 : 1.4
8	1.6 : 11
35	<0.1 : 22
36	1.8 : 36
37	6.0 : 16

Table 9. Yields of indigo (**50**) and the indirubin derivative **54** from reaction mixtures containing indoxyl anion (**48**) and the isatin derivative **53** in 0.01 mol dm^{-3} pH 10.0 borate buffer at 298 K after 16 h.

The ratios of the dyes **50** and **54** produced in the reactions containing no cyclodextrin and the dimer **35** were *ca.* 16 : 1 and <1 : 220, respectively, while the total yields were similar. Thus, the addition of the cyclodextrin **35** changes the ratio of the dyes **50** and **54** by a factor of *ca.* four thousand in favour of the non-linear species **54**, without affecting the overall yield.

A major effect of addition of the urea dimer **35** to a reaction mixture containing the reagents **48** and **53** is to markedly reduce the amount of indigo (**50**) produced. Based on the detection limit of the method, the yield of indigo (**50**) was <0.1%. This indicates that the indoxyl anion (**48**) is almost completely complexed by the urea **35** and in an orientation which prevents oxidative dimerisation. While not being able to dimerise, the complexed anion **48** must still be able to react with the isatin derivative **53**, as the indirubin derivative **54** was produced in 22% yield. This yield is *ca.* sixteen times that observed in the absence of a cyclodextrin (1.4%), suggesting that either the cyclodextrin **35** favours formation of the indirubin derivative **54** or that it protects the isatin derivative **53** from hydrolysis, presumably through complexation. The latter argument is supported by the results of the assays with the other cyclodextrins **8**, **36** and **37** present, in which the yields of the sulfonate **54** were increased relative to the case in which no cyclodextrin was present.

The results of these assays indicate that the cyclodextrin **35** is a very effective template for the formation of the sulfonate **54**. As for the effect of the dimer **35** on competing reactions to form the dyes **50** and **51**, the orientation of the reagents **48** and **53** complexed in the annuli disfavours formation of the linear isomer **50** and allows the formation of the non-linear isomer **54**. However, in this case the change in the ratio of the dyes **50** and **54** on addition of the urea **35** is not solely due to a templating effect. The dimer **35** also acts to protect the isatin derivative **53** from hydrolysis, presumably through complexation, hence increasing the yield of the sulfonate **54**. Nevertheless, this system represents a second example of controlled geometry of reagents by design and

further illustrates that cyclodextrins can be used to control the regioselectivity of a condensation reaction.

Results and Discussion

Chapter III

Effect of Cyclodextrins on Product Inhibition of (*S*)-Phenylalanine Ammonia Lyase

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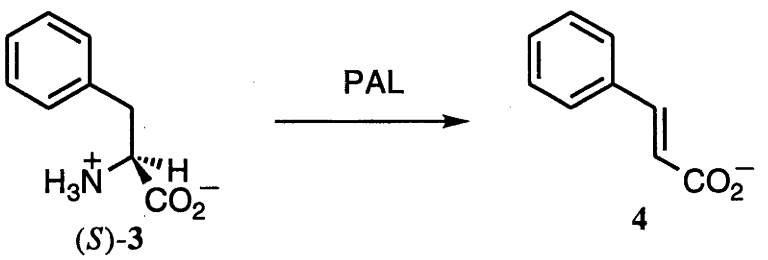
The work described in Chapter II of the Results and Discussion established that cyclodextrins could be used as templates for condensation reactions. As outlined in the Introduction, it was anticipated that cyclodextrins could also be used to manipulate enzyme catalysed reactions. Accordingly, the prospect of reducing product inhibition of an enzyme with cyclodextrins was examined using (*S*)-phenylalanine ammonia lyase (PAL). This enzyme catalyses the deamination of (*S*)-phenylalanine ((*S*)-**3**) to give *trans*-cinnamate (**4**) (Scheme 5, reproduced below).[8] The cinnamate **4** is a potent competitive inhibitor of PAL so the enzyme displays product inhibition.[9] As discussed in the Introduction, the decreased charge and hydration of the product **4** relative to the substrate (*S*)-**3** results in the former being preferentially included in each of the cyclodextrins **7** and **8**. It was anticipated that this preferential binding could be used to selectively complex, and remove from solution, the cinnamate **4**, thus reducing the concentration free in solution to inhibit the enzyme. In this manner it was expected that the cyclodextrins **7** and **8** could be used to limit product inhibition of PAL.



(*S*)-**3** **4**

Scheme 5. The removal of ammonia and a proton from (*S*)-phenylalanine ((*S*)-**3**) as catalysed by PAL.

Since complexation of a guest by a host is a bimolecular reaction, a higher concentration of the host results in a greater proportion of the guest being complexed. Where the guest is an inhibitor of an enzyme, it was anticipated that a larger amount of the host would lead to less inhibitor remaining free in solution and hence a greater effect of

the host to limit enzyme inhibition. Thus, to examine any effect each of the cyclodextrins **7** and **8** would have on limiting product inhibition of PAL they were each used at *ca.* 50% of saturation. The aqueous solubility of α-cyclodextrin (**7**) (14.5 g / 100 cm^3)[97] is greater than that of β-cyclodextrin (**8**) (1.85 g / 100 cm^3)[97] so the former **7** was used at a higher concentration.

The reactions were carried out in 0.05 mol dm^{-3} phosphate buffer at pH 7.0 and 303 K. A buffer was used to ensure that the pH did not change as the reactions proceeded. The pH value was chosen as that where the enzyme shows maximum activity.[9] Further, at this pH the ionic state of the substrate (*S*)-**3** and the product **4** are known and the association constants of these species with the cyclodextrins **7** and **8** have been reported.[95,96] *trans*-Cinnamic acid has a pK_a of 4.44[107] and therefore, at pH 7.0, it is present as the cinnamate **4**. Protonated phenylalanine has pK_a values of 2.6 and 9.2.[107] Consequently the zwitterionic form of phenylalanine (**3**) predominates at pH 7.0. The temperature used is that often reported in the literature for studies involving PAL.[8,9] The reactions were followed by observing the increase in absorbance at 268 nm, which is the wavelength corresponding to the maximum absorption of the cinnamate **4**.[107] This absorption band arises from conjugation in the cinnamate **4** which is absent in phenylalanine (**3**), so the molar absorption of phenylalanine (**3**) at this wavelength is negligible.[107] Hence the change in absorbance can be used to determine the amount of the cinnamate **4** produced, using its molar absorption of 20.0 x 10^3 mol^{-1} dm^3 cm^{-1}.[107]

Each reaction mixture contained the same quantity of enzyme (*ca.* 70 units dm^{-3}) and the substrate (*S*)-**3** (0.25 x 10^{-3} mol dm^{-3}). This concentration of the substrate (*S*)-**3** was chosen to give a convenient change in the spectrum of 0.50 absorbance units for complete reaction in the absence of a cyclodextrin when observed through a quartz cell with an optical pathlength of 10^{-3} m.

A series of experiments was carried out observing the digestion of (*S*)-phenylalanine ((*S*)-**3**) in the presence of either no cyclodextrin, α-cyclodextrin (**7**) or β-cyclodextrin **8** (Figure 7, assays a-c). A second series of experiments was carried out to mimic the latter stages of reactions catalysed by PAL, where a substantial quantity of the cinnamate **4** has been produced and product inhibition more substantially influences the activity of the enzyme. In this latter set of experiments, the cinnamate **4** was present initially at a concentration similar to that of the phenylalanine (*S*)-**3** and, again, the mixtures contained either no cyclodextrin, α-cyclodextrin (**7**) or β-cyclodextrin (**8**) (Figure 7, assays d-f).

The result of the experiment with no cyclodextrin and no added *trans*-cinnamate (**4**) (assay c, Figure 7) illustrates the effect of product inhibition of the enzyme. Initially

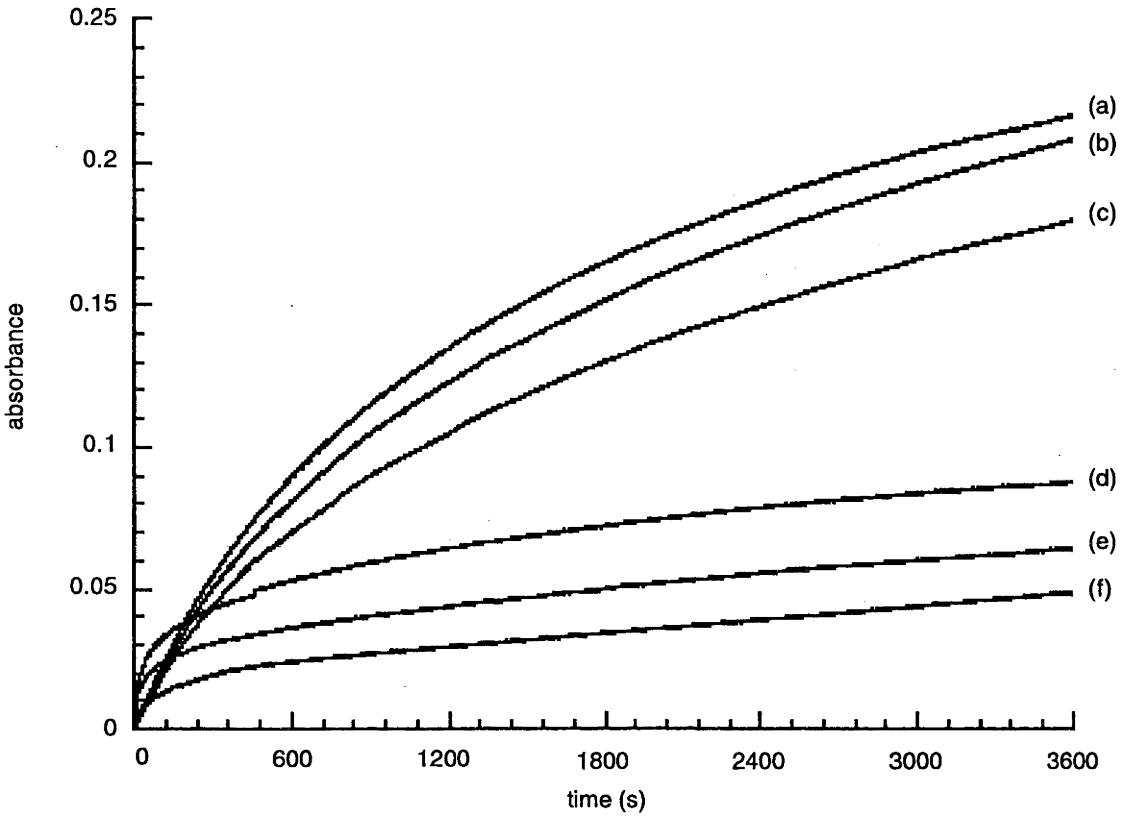


Figure 7. Change in ultraviolet absorbance at 268 nm of solutions containing (*S*)-phenylalanine ((*S*)-**3**) (0.25 x 10^{-3} mol dm^{-3}), PAL (*ca.* 70 units dm^{-3}) and either a) α-cyclodextrin (**7**) (80 x 10^{-3} mol dm^{-3}), b) β-cyclodextrin (**8**) (6.9 x 10^{-3} mol dm^{-3}), c) no cyclodextrin, d) α-cyclodextrin (**7**) (75 x 10^{-3} mol dm^{-3}) and the cinnamate **4** (0.26 x 10^{-3} mol dm^{-3}), e) β-cyclodextrin (**8**) (6.5 x 10^{-3} mol dm^{-3}) and the cinnamate **4** (0.26 x 10^{-3} mol dm^{-3}), or f) the cinnamate **4** (0.26 x 10^{-3} mol dm^{-3}) but no cyclodextrin, in 0.05 mol dm^{-3} phosphate buffer at pH 7.0 and 303 K.

the rate of production of the cinnamate **4** was high, as evident from the rapid increase in absorbance. The rate of change in absorbance, and hence production of the cinnamate **4**, was then observed to decrease with time. This reduction as the concentration of the cinnamate **4** increases is consistent with product inhibition of the enzyme by the cinnamate **4**. Based on the change in absorbance, the overall extent of reaction in 1 h in this case was calculated to be 36%.

The effect of product inhibition is more marked in the experiment where the cinnamate **4** was added initially with no cyclodextrin present (assay f, Figure 7). The

overall change in absorbance decreased by 70% relative to the case in which no cinnamate **4** was present initially (assay c, Figure 7). The extent of reaction after 1 h was calculated to be 10%.

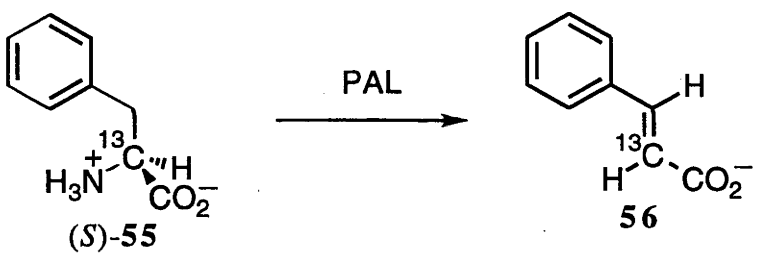
On addition of β-cyclodextrin (**8**) to a reaction mixture with no *trans*-cinnamate (**4**) present initially (assay b, Figure 7), the change in absorbance was observed to increase relative to the case in which no cyclodextrin was present (assay c, Figure 7), while the shapes of the absorbance versus time curves are similar. This assay indicates that the enzyme retains its activity in the presence of β-cyclodextrin (**8**). While complexation of a guest may change its spectroscopic properties, the molar absorption of the cinnamate **4** at 268 nm decreases only slightly, by *ca.* 0.5%, on complexation within β-cyclodextrin (**8**).[96] Hence, the overall change in absorbance in this case corresponds to an extent of reaction of *ca.* 41%. That is, the extent of reaction increased by *ca.* 5%, corresponding to a 15% increase in the overall activity of the enzyme, in the presence of β-cyclodextrin (**8**).

Addition of α-cyclodextrin (**7**) to the reaction mixture in which none of the cinnamate **4** was present initially resulted in an increase in absorbance of *ca.* 20% (assay a, Figure 7). This experiment shows that the enzyme also retains its activity in the presence of α-cyclodextrin (**7**). As is the case when the host is β-cyclodextrin (**8**), the molar absorption of the cinnamate **4** decreases on complexation within α-cyclodextrin (**7**), to 18.6 x 10^3 mol^{-1} dm^3 cm^{-1}.[96] Therefore, the change in absorbance represented by assay a, Figure 7, corresponds to an overall extent of reaction of *ca.* 44% in this case. That is, the extent of reaction increased by *ca.* 9%, corresponding to a 25% increase in the overall activity of the enzyme in the presence of α-cyclodextrin (**7**).

The experiments involving the cyclodextrins **7** and **8** with the cinnamate **4** present initially (assays d and e, respectively, Figure 7) show total changes in absorbance larger than that of the assay in which no cyclodextrin was present (assay f, Figure 7), while the shapes of the absorbance versus time curves are similar. Relative to the cases in which each of the cyclodextrins **7** and **8** but no *trans*-cinnamate (**4**) was present initially (assays a and b, Figure 7) the enzyme activity was greater over the first few minutes. The phenylalanine (*S*)-**3** is known to act as an allosteric inhibitor of PAL,[15] one that binds and conformationally alters the enzyme, decreasing its activity. The initial burst in the reactions with added cinnamate **4** is consistent with competitive binding of the cinnamate **4** over the phenylalanine (*S*)-**3** at an allosteric site on the enzyme. Presumably this competition reduces the negative allosteric effect of the phenylalanine (*S*)-**3** and hence increases the enzyme activity in the early stages of reaction.

In the assay with added cinnamate **4**, when β-cyclodextrin **8** was also added (assay e, Figure 7), the extent of reaction after 1 h increased to *ca.* 13%, corresponding to an increase of *ca.* 30% in the overall activity of the enzyme. The effect of the addition of α-cyclodextrin (**7**) was more marked (assay d, Figure 7), increasing the extent of reaction after 1 h to *ca.* 18%, corresponding to an increase of *ca.* 80% in the activity of the enzyme. The effect of the cyclodextrins **7** and **8** was greater with the cinnamate **4** added than without. Reduction of product inhibition is more effective under these circumstances where more of the product **4** is present.

The summary interpretation of these results is that the cyclodextrins **7** and **8** sequester the cinnamate **4** in preference to the phenylalanine (*S*)-**3**, whether it is added to the solution initially or produced during the reaction. This reduces the amount of *trans*-cinnamate (**4**) free in solution and hence the amount that can inhibit the enzyme. The outcome of the experiments described above is consistent with the reported stability constants of the complexes of (*S*)-phenylalanine ((*S*)-**3**) in the cyclodextrins **7** and **8** of 8 and 3 mol^{-1} dm^3, respectively,[95] and of the analogous complexes with *trans*-cinnamate (**4**) as the guest, of 109 and 313 mol^{-1} dm^3, respectively.[96]



(*S*)-**55** **56**

Scheme 21. The removal of ammonia and a proton from >99% 2-^{13}C-labelled (*S*)-phenylalanine ((*S*)-**55**) as catalysed by PAL.

As independent confirmation of the effect of cyclodextrins to increase the extent of reaction catalysed by PAL, a product study was carried out using the cinnamate **4** added initially as an internal standard. The (*S*)-phenylalanine ((*S*)-**3**) was replaced with >99% 2-^{13}C-labelled (*S*)-phenylalanine ((*S*)-**55**) (Scheme 21) and digested under the same conditions as used in the ultraviolet assays, in the absence of a cyclodextrin, and in the presence of either α-cyclodextrin (**7**) or β-cyclodextrin (**8**). In these experiments, comparison of the amount of the labelled cinnamate **56** to the amount of the unlabelled cinnamate **4** gives the extent of reaction. Since they have the same physical and chemical properties, it may be assumed that the labelled cinnamate **56** and the unlabelled cinnamate **4** are isolated in the same ratio as they are present in the reaction mixture, and the unlabelled cinnamate **4** is therefore an ideal internal standard for the reaction. The relative amounts of each of the cinnamates **4** and **56** present in a mixture can be easily quantified

using spectroscopic techniques. In ^{1}H NMR spectroscopy, the resonance due to the α-proton of the cinnamate **4** appears as a doublet, while the labelled compound **56** gives rise to ^{13}C satellites of these peaks. The ratio of the integration of these satellites to the integration of the central peaks gives the ratio of the labelled cinnamate **56** to the unlabelled cinnamate **4**. By mass spectrometry, the extent of ^{13}C incorporation may be calculated by relating the observed spectra to one of unlabelled *trans*-cinnamate **4**, and comparing the ratios of two consecutive peaks in each spectrum.

The concentrations of the components of these reaction mixtures were the same as those used in the ultraviolet assays. After incubation at 303 K for 1 h, the solutions were acidified to *ca.* pH 1 with concentrated hydrochloric acid. This denatured the enzyme, preventing further reaction, and protonated the cinnamates **4** and **56** in each reaction mixture. This allowed the cinnamates **4** and **56** to be extracted into chloroform. The residue from concentration of the organic extract of each reaction mixture was analysed by ^{1}H NMR spectroscopy (Figure 8).

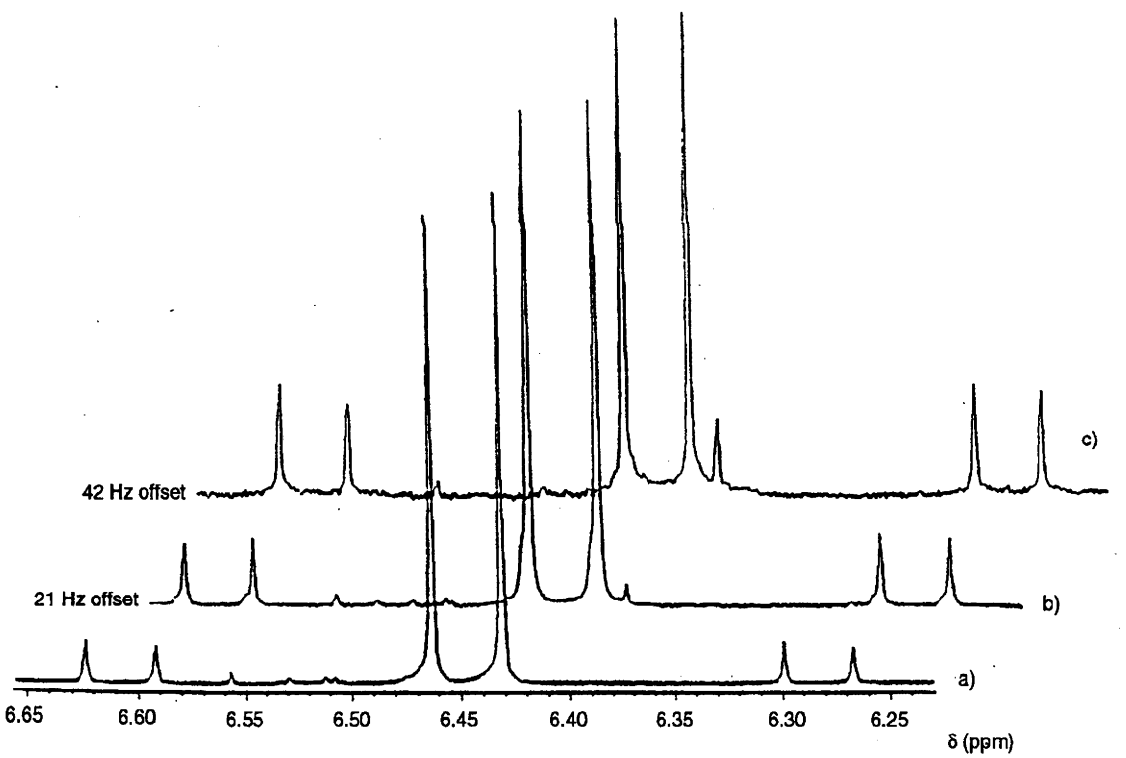


Figure 8. ^{1}H NMR spectra (500 MHz, $CDCl_3$) of the material obtained by the treatment of >99% 2-^{13}C-labelled (*S*)-phenylalanine (*S*)-**55** (0.25 x 10^{-3} mol dm^{-3}) and the cinnamate **4** (0.26 x 10^{-3} mol dm^{-3}), containing either a) no cyclodextrin, b) β-cyclodextrin (**8**) (6.5 x 10^{-3} mol dm^{-3}), or c) α-cyclodextrin (**7**) (75 x 10^{-3} mol dm^{-3}), in 0.05 mol dm^{-3} phosphate buffer at pH 7.0, with PAL (*ca.* 70 units dm^{-3}) at 303 K for 1 h.

The spectra show signals due to the protonated form of the labelled cinnamate **56**, produced in the reaction, at δ6.45 (dd, J_H = 16 Hz, J_C = 164 Hz), and due to the protonated form of the unlabelled cinnamate **4** added initially, at δ6.45 (d, J_H = 16 Hz). Integration of these signals shows that the labelled phenylalanine **55** was converted to the cinnamate **56** to an extent of 16% in the absence of a cyclodextrin. The analogous reactions, carried out with either β-cyclodextrin (**8**) or α-cyclodextrin (**7**) present, proceeded to extents of 29% and 41%, respectively.

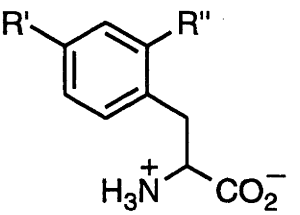
These results were confirmed using gas chromatography / mass spectrometry. The chromatography separates the protonated cinnamates **4** and **56** from impurities present in the extract. A sample of the protonated form of the unlabelled cinnamate **4** was used to identify the retention time on the chromatography column used and to calculate the ratio of the peak at *m/z* 148 due to the molecular ion (M^+) to the peak corresponding to one mass unit less than the molecular ion ($[M-1]^+$). This was repeated for the extract of each of the reaction mixtures, and the increase in the relative height of the peak at *m/z* 148 was noted. This gave the extents of reaction as 16%, 26% and 42%, for the assays with no cyclodextrin, β-cyclodextrin (**8**) and α-cyclodextrin (**7**) present, respectively.

The values determined using the ^{1}H NMR spectroscopy and mass spectrometry methods are in good agreement but higher than those determined using the ultraviolet assays (Table 10). This suggests that while the same concentration of enzyme was used in each experiment, the activity of the enzyme was lower in the ultraviolet assays, and hence the final extents of reaction were less. Such variations in enzyme activity are expected with commercial preparations. Nevertheless, the trends observed in each study are the same with the addition of either α-cyclodextrin (**7**) or β-cyclodextrin (**8**) to the reaction mixtures reducing product inhibition and hence increasing the extents of reaction.

	Extent of Reaction		
Cyclodextrin	Ultraviolet Assays	^{1}H NMR Spectroscopy	Mass Spectrometry
-	10%	16%	16%
8	13%	29%	26%
7	18%	41%	42%

Table 10. Extents of reaction in the conversion of the phenylalanines (*S*)-**3** and (*S*)-**55** to the cinnamates **4** and **56**, respectively, in solutions containing (*S*)-phenylalanine ((*S*)-**3**) or >99% 2-^{13}C-labelled-(*S*)-phenylalanine (*S*)-**55** (0.25 x 10^{-3} mol dm^{-3}), PAL (*ca.* 70 units dm^{-3}), the cinnamate **4** (0.26 x 10^{-3} mol dm^{-3}) and either no cyclodextrin, β-cyclodextrin (**8**) (6.5 x 10^{-3} mol dm^{-3}) or α-cyclodextrin (**7**) (75 x 10^{-3} mol dm^{-3}), in 0.05 mol dm^{-3} phosphate buffer at pH 7.0 and 303 K after 1 h.

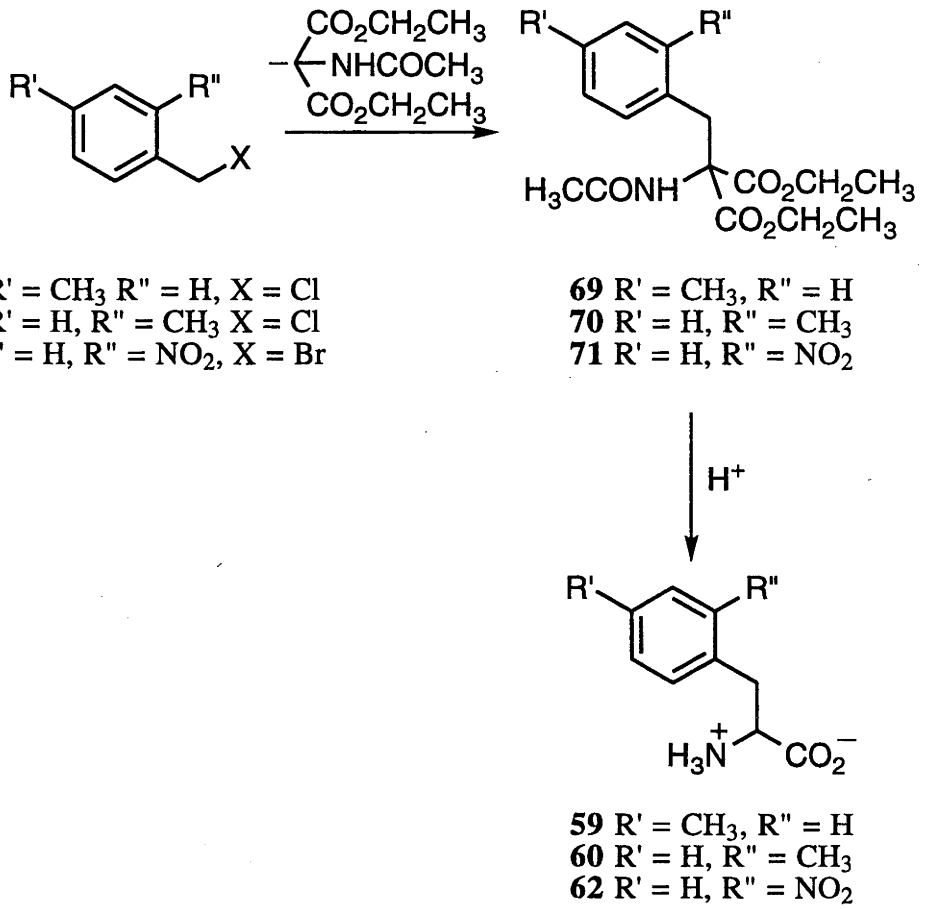
The results discussed above establish that the cyclodextrins **7** and **8** limit product inhibition of PAL, by preferentially complexing, and hence removing from solution, the cinnamate **4**. PAL has been shown previously to digest ring substituted phenylalanines,[130,131] though the scope of this tolerance is not well defined. The association constants of the complexes of a range of ring substituted cinnamates with each of the cyclodextrins **7** and **8** have been reported,[96] and differ from the corresponding association constants where the guest is the parent cinnamate **4**. Thus, the digestion of ring substituted phenylalanines by PAL was investigated to examine the generality of the effect of cyclodextrins on product inhibition and to determine if changing the extent of complexation of the cinnamate altered this effect. The compounds initially examined were the phenylalanines **42** and **57-65**, which each have a substituent at either the *ortho-* or *para*-position of the aromatic ring. The substituents represent a range of steric and electronic properties.



57 R' = F, R" = H; **58** R' = H, R" = F
59 R' = CH_3, R" = H; **60** R' = H, R" = CH_3
61 R' = NO_2, R" = H; **62** R' = H, R" = NO_2
63 R' = Cl, R" = H; **64** R' = H, R" = Cl
42 R' = OH, R" = H; **65** R' = H, R" = OH

The substituted phenylalanines **59**, **60** and **62** were not commercially available so they were prepared from the benzyl halides **66-68** through condensation with diethyl acetamidomalonate to give the adducts **69-71** followed by acid catalysed hydrolysis and decarboxylation (Scheme 22).[132,133] Each of the adducts **69-71** and each of the phenylalanines **59**, **60** and **62** was identified by comparison of observed physical and spectral data with those reported.[132,133] The remainder of the phenylalanines used were commercially available.

Initially, a series of experiments was carried out to determine if the enzyme was able to digest the phenylalanines **42** and **57-65**. All reactions were carried out using the racemates **42** and **57-65**. It is reasonable to assume that the (*R*)-enantiomers (*R*)-**42** and (*R*)-**57-65** do not compete with the (*S*)-isomers (*S*)-**42** and (*S*)-**57-65** to interact with PAL but this was confirmed at least for phenylalanine (**3**). (*R*)-Phenylalanine ((*R*)-**3**) has been shown previously to be a poor substrate and a poor competitive inhibitor of PAL, binding to the enzyme *ca.* 10% as well as the natural substrate (*S*)-**3**.[9] The extents of reaction in trial experiments involving digestion of either the racemate **3** or the



66 R' = CH_3 R" = H, X = Cl
67 R' = H, R" = CH_3 X = Cl
68 R' = H, R" = NO_2, X = Br

69 R' = CH_3, R" = H
70 R' = H, R" = CH_3
71 R' = H, R" = NO_2

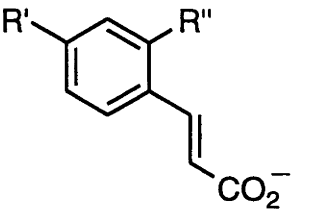
59 R' = CH_3, R" = H
60 R' = H, R" = CH_3
62 R' = H, R" = NO_2

Scheme 22. Preparation of the phenylalanine derivatives **59**, **60** and **62**.

enantiomer (*S*)-**3** by PAL were the same, indicating that the presence of the non-natural isomer (*R*)-**3** had a negligible effect on PAL activity. Consequently the racemates **42** and **57-65** were used but any activity is assumed to reflect the digestion of the (*S*)-isomers (*S*)-**42** and (*S*)-**57-65**.

The addition of a substituent to the aromatic ring of the cinnamate **4** changes the absorption spectrum. Both the wavelength of maximum absorption (λ_{max}) and the molar extinction coefficient at that wavelength change. While the λ_{max} values for the cinnamates **72-81** are not all reported, those that are range from 265 to 285 nm.[134] In comparison, the corresponding phenylalanines **42** and **57-65** have negligible absorbance in this range, due to reduced conjugation. As a result, the experiments to investigate digestion of the phenylalanines **42** and **57-65** were monitored by observing the change in absorbance at 275 nm, corresponding to a wavelength intermediate to the reported λ_{max} values.

Each of the phenylalanines **42** and **57-65** was treated with PAL in 0.05 mol dm^{-3} phosphate buffer at pH 7.0 and 303 K. The concentration of the phenylalanines **42** and



72 R' = F, R" = H; **73** R' = H, R" = F
74 R' = CH_3, R" = H; **75** R' = H, R" = CH_3
76 R' = NO_2, R" = H; **77** R' = H, R" = NO_2
78 R' = Cl, R" = H; **79** R' = H, R" = Cl
80 R' = OH, R" = H; **81** R' = H, R" = OH

57-65 used was such that the (*S*)-enantiomers (*S*)-**42** and (*S*)-**57-65** were present at the same concentration as that of (*S*)-phenylalanine ((*S*)-**3**) in the experiments described previously. The concentration of enzyme used in each case (*ca.* 300 units dm^{-3}) was greater than the concentration used in the experiments described previously with the parent phenylalanine (*S*)-**3**.

Reaction mixtures containing the phenylalanines **42**, **57**, **58**, **60**, **62-64** showed an increase in absorbance at 275 nm over 1 h, while those containing the phenylalanines **59**, **61** and **65** did not. This indicates that the former are digested by PAL, while the latter are not.

The chlorinated phenylalanine derivatives **63** and **64** were subsequently used to further examine the effect of cyclodextrins on PAL activity. These two were chosen because, along with them being substrates of PAL, the cyclodextrin complexation properties and absorption spectrum of each of the corresponding products **78** and **79** have been reported. The cinnamate **78** has a λ_{max} of 275 nm and a molar extinction coefficient at this wavelength of 22.8 x 10^3 mol^{-1} dm^3 cm^{-1}.[134] The association constants of the complexes formed between the cinnamate **78** and the cyclodextrins **7** and **8** are 286 and 689 mol^{-1} dm^3, respectively.[96] Complexation within the cyclodextrins **7** and **8** increases the molar extinction coefficient of the cinnamate **78** to 27.9 x 10^3 mol^{-1} dm^3 cm^{-1} and 28.5 x 10^3 mol^{-1} dm^3 cm^{-1}, respectively.[96] The cinnamate **79** has a λ_{max} of 265 nm and a molar extinction coefficient at this wavelength of 14.9 x 10^3 mol^{-1} dm^3 cm^{-1}.[134] The association constants of the complexes formed between the cinnamate **79** and each of the cyclodextrins **7** and **8** are 283 and 501 mol^{-1} dm^3, respectively.[96] Complexation within the cyclodextrins **7** and **8** increases the molar extinction coefficient of the cinnamate **79** to 18.7 x 10^3 mol^{-1} dm^3 cm^{-1} and 17.4 x 10^3 mol^{-1} dm^3 cm^{-1}, respectively.[96]

A series of experiments was carried out observing the digestion of the chlorinated phenylalanine **63** in the presence of either no cyclodextrin, α-cyclodextrin (**7**) or

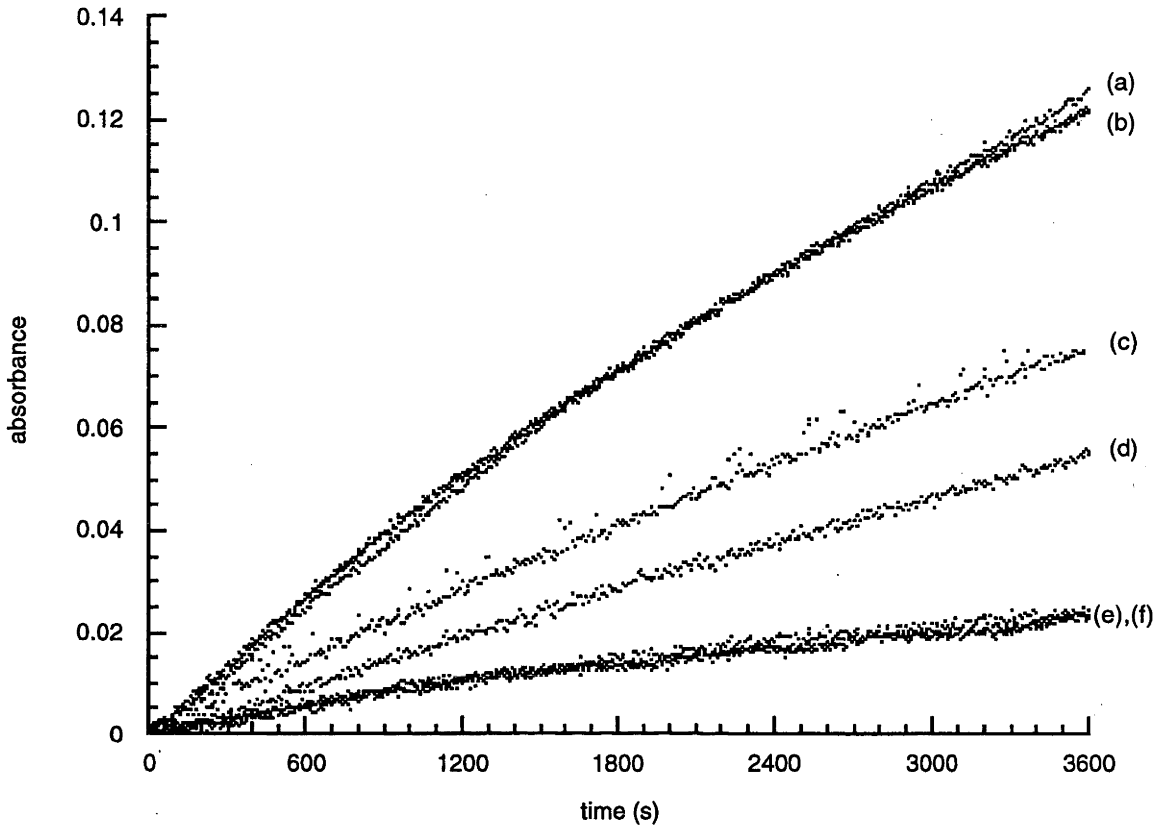


Figure 9. Change in ultraviolet absorbance at 275 nm of solutions containing 4'-chlorophenylalanine (**63**) (0.53 x 10^{-3} mol dm^{-3}), PAL (*ca.* 325 units dm^{-3}) and either a) β-cyclodextrin (**8**) (7.7 x 10^{-3} mol dm^{-3}), b) no cyclodextrin, c) β-cyclodextrin (**8**) (7.7 x 10^{-3} mol dm^{-3}) and the cinnamate **78** (0.27 x 10^{-3} mol dm^{-3}), d) the cinnamate **78** (0.27 x 10^{-3} mol dm^{-3}) but no cyclodextrin, e) α-cyclodextrin (**7**) (77 x 10^{-3} mol dm^{-3}), or f) α-cyclodextrin (**7**) (77 x 10^{-3} mol dm^{-3}) and the cinnamate **78** (0.27 x 10^{-3} mol dm^{-3}) in 0.05 mol dm^{-3} phosphate buffer at pH 7.0 and 303 K.

β-cyclodextrin (**8**) (Figure 9, assays a, b and e). A second series of experiments was carried out to mimic the latter stages of reactions catalysed by PAL where a substantial quantity of the cinnamate **78** has been produced and product inhibition would more substantially influence the activity of the enzyme. In this latter set of experiments, the cinnamate **78** was present initially at a concentration approximately half that of the phenylalanine **63**. As such, the concentration of the cinnamate **78** was similar to that of each enantiomer of the phenylalanine **63** allowing comparison with the assays illustrated in Figure 8. Again, the reaction mixtures contained either no cyclodextrin, α-cyclodextrin (**7**) or β-cyclodextrin (**8**) (Figure 9, assays c, d and f, respectively). All

reactions were carried out in 0.05 mol dm^{-3} phosphate buffer at pH 7.0 and 303 K and monitored for 1 h at 275 nm. At this wavelength, an absorbance change of 0.60 in the absence of a cyclodextrin would correspond to the reaction proceeding to completion.

It is notable that the assays shown in Figure 9 have decreased curvature relative to the assays shown in Figure 8. That is, the rate of change in absorbance, and hence production of the cinnamate **78**, is less affected by the increasing concentration of the product **78**. This suggests that product inhibition of PAL is less significant in these assays. In the assay in which only the phenylalanine **63** was present initially (assay b, Figure 9) the change in absorbance corresponds to an extent of reaction of 20% in 1 h. On addition of β-cyclodextrin (**8**) to the mixture with none of the cinnamate **78** present initially (assay a, Figure 9), the change in absorbance during reaction was similar to the case in which no cyclodextrin was present (assay b, Figure 9). However, since the molar extinction coefficient of the cinnamate **78** increases on complexation with the cyclodextrin **8**, from 22.8 x 10^3 mol^{-1} dm^3 cm^{-1} [134] to 28.5 x 10^3 mol^{-1} dm^3 cm^{-1},[96] the change in absorbance observed with the cyclodextrin **8** present corresponds to a decrease in the extent of reaction. The ratio of the cinnamate **78** free in solution to the cinnamate **78** complexed in the cyclodextrin **8** is *ca.* 1 : 5, based on the known association constant of the complex (689 mol^{-1} dm^3),[96] the concentration of the cyclodextrin **8** used in this assay (7.7 x 10^{-3} mol dm^{-3}) and Equation 1. Thus, the extent of reaction in this case in 1 h is *ca.* 17%, corresponding to a 15% decrease in the overall activity of the enzyme in the presence of β-cyclodextrin (**8**). While the cyclodextrin complexes the majority of the cinnamate **78**, it is clear that this does not increase the activity of the enzyme. This implies that any reduction in product inhibition of PAL through complexation of the cinnamate **78** is more than offset by the effect of the cyclodextrin **8** to reduce the activity of the enzyme through complexation of the phenylalanine **63**, reducing the amount free in solution to be digested. Again, this indicates that product inhibition of PAL by the cinnamate **78** is limited in extent.

Nevertheless, it is apparent. The experiment in which the cinnamate **78** was added initially in the absence of any cyclodextrin (assay d, Figure 9) proceeded to an extent of 9% after 1 h. This indicates that the overall activity of the enzyme is only 45% of that observed in the assay without the cinnamate **78** added initially. On addition of β-cyclodextrin (**8**) to a reaction mixture with the cinnamate **78** present initially (assay c, Figure 9), the change in absorbance after 1 h increased by *ca.* 30%. The majority of this increase is due to inclusion of the cinnamate **78** in the cyclodextrin **8** because the complex has a greater molar extinction coefficient than the free cinnamate **78**.[96,134] Accounting for this, the extent of reaction in 1 h in this case is *ca.* 10%, corresponding to a *ca.* 10% increase in the activity of the enzyme caused by the cyclodextrin **8**. The observation that

the cyclodextrin **8** complexes the majority of the cinnamate **78** but has only this slight effect on enzyme activity supports the previous observation that there is limited inhibition of PAL by the cinnamate **78**.

On addition of α-cyclodextrin (**7**) to the reaction mixture with none of the cinnamate **78** added initially (assay e, Figure 9), the change in absorbance during reaction was observed to decrease relative to the case in which no cyclodextrin was present (assay b, Figure 9). Based on the known association constant of the cinnamate **78** with the host **7** (286 mol^{-1} dm^3),[96] the concentration of the cyclodextrin **7** used (77 x 10^{-3} mol dm^{-3}) and Equation 1, *ca.* 96% of the cinnamate **78** will be complexed in the cyclodextrin **7** in this assay. Taking into account that the molar extinction coefficient of the cinnamate **78** increases on inclusion in the cyclodextrin **7** from 22.8 x 10^3 mol^{-1} dm^3 cm^{-1} [134] to 27.9 x 10^3 mol^{-1} dm^3 cm^{-1},[96] the change in absorbance in this assay corresponds to an extent of reaction of *ca.* 3%. That is, the extent of reaction decreased by *ca.* 17% compared to the assay with no cyclodextrin present (assay b, Figure 9), corresponding to a *ca.* 85% decrease in the overall activity of the enzyme in the presence of α-cyclodextrin (**7**). Thus, despite complexing the majority of the cinnamate **78**, the cyclodextrin **7** does not increase the activity of the enzyme. The complexation of the phenylalanine **63** by the host **7**, reducing the amount free in solution to be digested, significantly outweighs any reduction in product inhibition of PAL through complexation of the cinnamate **78**. Once more, this suggests that product inhibition of PAL is limited in this case.

The assay involving the cyclodextrin **7** with the cinnamate **78** added initially (assay f, Figure 9) showed a total change in absorbance less than that of the assay in which the cinnamate **78** but no cyclodextrin was present (assay d, Figure 9) and the same as the assay with the cyclodextrin **7** but none of the cinnamate **78** added (assay e, Figure 9). Since the changes in absorbance during reaction and the concentration of the cyclodextrin **7** in the reaction mixtures in assays e and f are the same, the extent of reaction is also the same (*ca.* 3%). Since the extent of reaction in the case in which the cinnamate **78** was added initially but no cyclodextrin was present (assay d, Figure 9) was 9%, the effect of adding the cyclodextrin **7** was to decrease the extent of reaction after 1 h by *ca.* 6%. This corresponds to a 66% reduction in the overall activity of the enzyme in the presence of the cyclodextrin **7**. Once again, the activity of the enzyme is reduced even though the majority of the product **78** is removed from solution by the host **7**. Any effect the reduction in the concentration of the cinnamate **78** free in solution has to limit product inhibition is exceeded by the effect of the host **7** to remove the phenylalanine **63** from solution, hence reducing the amount free in solution to be digested.

Overall the results illustrated in Figure 9 indicate that the chlorocinnamate **78** is less potent than the cinnamate **4** as an inhibitor of PAL. Consequently, the

cyclodextrins **7** and **8** do not substantially increase the efficiency of the enzyme because any increase resulting from removal of the product **78** from solution is counterbalanced by the cyclodextrins **7** and **8** also complexing the phenylalanine **63**.

The reactions illustrated in Figure 9 were repeated using the isomeric chlorinated phenylalanine **64** as the substrate but under otherwise very similar conditions, except that they were monitored at 265 nm, corresponding to the λ_{max} of the cinnamate **79**.[134] At this wavelength, an absorbance change of 0.39 in the absence of a cyclodextrin would correspond to the reaction proceeding to completion. The results are shown in Figure 10.

The assays shown in Figure 10 have notably increased curvature relative to the assays shown in Figure 9. That is, the rate of change in absorbance, and hence production of the cinnamate **79**, is more affected by the increasing concentration of the product **79**. This suggests that product inhibition of PAL is more significant in these assays. The experiment with only the phenylalanine **64** present initially (assay c, Figure 10) shows a change in absorbance corresponding to an extent of reaction in 1 h of 16%. In the assay in which the cinnamate **79** was added initially with no cyclodextrin present (assay d, Figure 10) only a very small change in absorbance was noted after 1 h. The extent of reaction in this case was calculated to be 1%, corresponding to a *ca.* 95% decrease in the overall activity of the enzyme on addition of the cinnamate **79**. This indicates that the cinnamate **79** is a potent inhibitor of PAL.

On addition of β-cyclodextrin (**8**) to a reaction mixture with none of the cinnamate **79** added initially (assay b, Figure 10), the change in absorbance during reaction was observed to increase by *ca.* 30% relative to the case in which no cyclodextrin was present (assay c, Figure 10). The ratio of free to complexed product **79** in this assay was calculated using Equation 1, the concentration of the cyclodextrin **8** (7.7×10^{-3} mol dm^{-3}) and the association constant for the complex of the cinnamate **79** with the host **8** (501 mol^{-1} dm^3)[96], to be *ca.* 1 : 4. Since the molar extinction coefficient of the cinnamate **79** increases on complexation in the cyclodextrin **8** from 14.9×10^3 mol^{-1} dm^3 cm^{-1} to 17.4×10^3 mol^{-1} dm^3 cm^{-1},[96] the change in absorbance in this assay corresponds to an extent of reaction of *ca.* 18%. Thus, the overall activity of the enzyme increases by *ca.* 13% on addition of the host **8**. This indicates that product inhibition is limited through complexation of the cinnamate **79** by the host **8**. No effect of the cyclodextrin **8** to remove the phenylalanine **64**, and hence reduce the activity of the enzyme, is observed. Given that the concentration of the phenylalanine **64** is greater than that of the cinnamate **79**, this suggests that the cyclodextrin **8** is selectively sequestering the cinnamate **79**. This is consistent with the decreased hydration and charge of the cinnamate **79** relative to phenylalanine **64** resulting in the former being preferentially included in the cyclodextrin **8**.

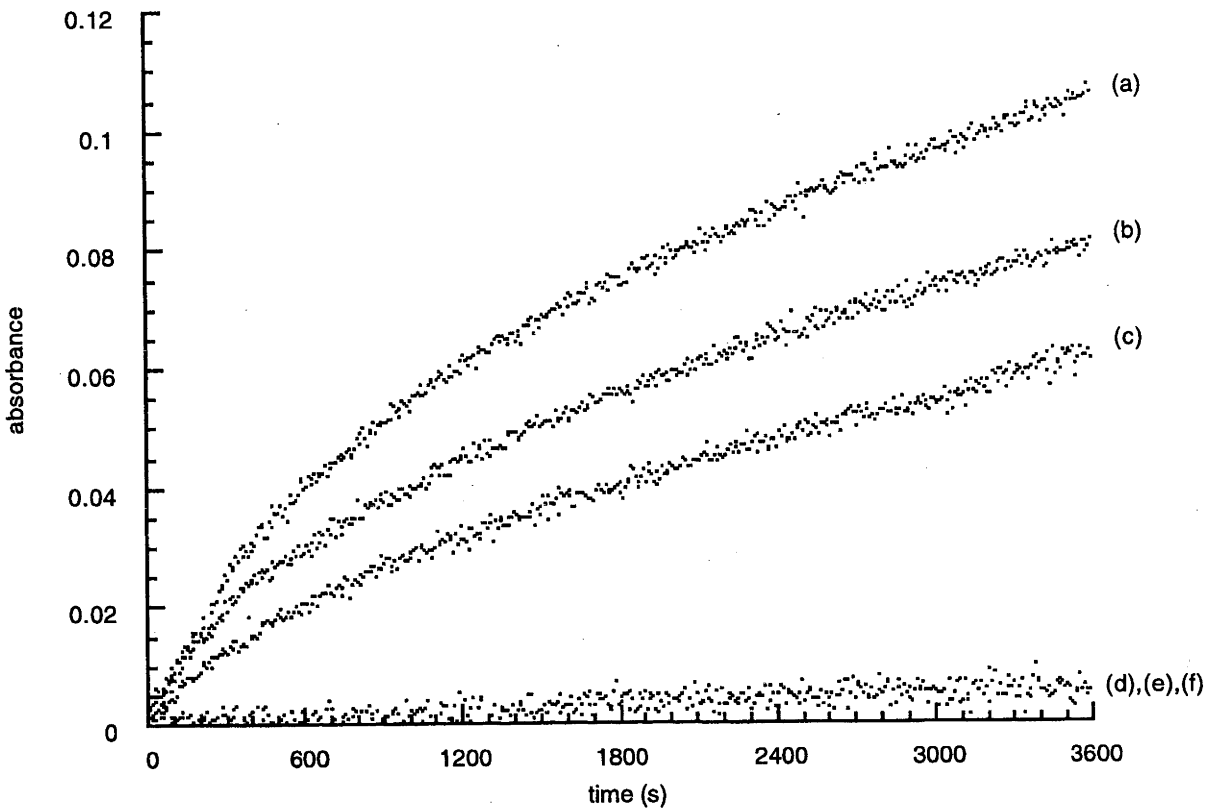


Figure 10. Change in ultraviolet absorbance at 265 nm of solutions containing 2'-chlorophenylalanine (**64**) (0.52 x 10^{-3} mol dm^{-3}), PAL (*ca.* 325 units dm^{-3}) and either a) α-cyclodextrin (**7**) (72 x 10^{-3} mol dm^{-3}), b) β-cyclodextrin (**8**) (7.7 x 10^{-3} mol dm^{-3}), c) no cyclodextrin, d) the cinnamate **79** (0.26 x 10^{-3} mol dm^{-3}) but no cyclodextrin, e) β-cyclodextrin (**8**) (7.7 x 10^{-3} mol dm^{-3}) and the cinnamate **79** (0.26 x 10^{-3} mol dm^{-3}), or f) α-cyclodextrin (**7**) (77 x 10^{-3} mol dm^{-3}) and the cinnamate **79** (0.26 x 10^{-3} mol dm^{-3}), in 0.05 mol dm^{-3} phosphate buffer at pH 7.0 and 303 K.

The reaction mixture containing α-cyclodextrin (**7**) with none of the cinnamate **79** added initially (assay a, Figure 10) gave a change in absorbance during reaction *ca.* 75% greater than that observed in the case in which no cyclodextrin was present initially (assay c, Figure 10). The host **7** removes *ca.* 96% of the cinnamate **79** from solution, based on Equation 1, the concentration of the host **7** in this assay (77 x 10^{-3} mol dm^{-3}) and the association constant for the complex of the cinnamate **79** with the host **7** (283 mol^{-1} dm^3).[96] This allows the extent of reaction in this assay to be calculated, using the molar extinction coefficient for the free cinnamate **79** (14.9 x 10^3 mol^{-1} dm^3 cm^{-1})[134]

and the cinnamate **79** complexed in the host **7** (18.7×10^3 mol^{-1} dm^3 cm^{-1}),[96] as *ca.* 23%. This corresponds to an increase in the overall activity of the enzyme of *ca.* 44% in the presence of α-cyclodextrin (**7**). As for the equivalent case containing the host **8** (assay b, Figure 10), this indicates that the reduction of product inhibition through complexation of the cinnamate **79** has a greater effect than removal of the phenylalanine **64** from solution and suggests that the host **7** preferentially sequesters the product **79** from solution.

The experiments with the cinnamate **79** added initially and either α-cyclodextrin (**7**) or β-cyclodextrin (**8**) present (assays e and f, respectively, Figure 10) each gave a change in absorbance indistinguishable from that of the assay in which the cinnamate **79** was added but no cyclodextrin was present (assay d, Figure 10). Irrespective of any changes in the molar extinction coefficient of the cinnamate **79** on inclusion in each of the hosts **7** and **8**, the extents of reaction in assays d-f are indistinguishable. Thus, at latter stages of reaction, the cyclodextrins **7** and **8** each have a negligible effect on the overall activity of the enzyme, despite complexing *ca.* 80% and 96% of the cinnamate **79**, respectively. This further indicates that the cinnamate **79** is a potent inhibitor of PAL.

The experiments involving the removal of ammonia and a proton from each of the phenylalanines **3**, **63** and **64** catalysed by PAL illustrate the relative proficiency of the cinnamates **4, 78** and **79** as inhibitors of the enzyme. The chlorocinnamate **79** is a more potent inhibitor than the parent cinnamate **4**, which in turn is a more potent inhibitor than the isomeric chlorocinnamate **78**. Further, these experiments indicate that the effectiveness of the cyclodextrins **7** and **8** to limit product inhibition of PAL by sequestering the product, and hence removing it from solution, is dependent on the potency of the product as an inhibitor of PAL. Where the product is a poor inhibitor of PAL, as is the case for the chlorocinnamate **78**, removing it from solution has little effect on PAL activity. Where the product is a potent inhibitor of PAL, as is the case for the chlorocinnamate **79**, removing it from solution noticeably increases PAL activity in the early stages of reaction. However, this effect is reduced as the extent of reaction increases as insufficient product is removed to limit product inhibition. Where the potency of the product as an inhibitor is intermediate, as is the case for the parent cinnamate **4**, then its removal from solution increases PAL activity irrespective of the extent of conversion of the substrate to the product. Thus, the cyclodextrins **7** and **8** have been shown to reduce product inhibition of PAL, by selectively sequestering the product, and there is the potential to exploit this principal to increase the activity of other enzymes which are affected by product inhibition.

Results and Discussion

Chapter IV

Effect of Cyclodextrins on Equilibrations Catalysed by (*S*)-Phenylalanine Ammonia Lyase

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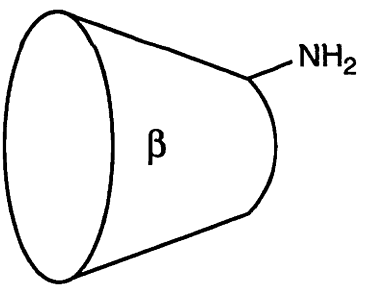
The work described in Chapter III of the Results and Discussion illustrated that the naturally occurring cyclodextrins **7** and **8** can be used to reduce product inhibition of (*S*)-phenylalanine ammonia lyase (PAL), through complexation of the product. It was anticipated that modified cyclodextrins might be used to limit product inhibition of PAL in the same manner. The modified cyclodextrin investigated was 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**), prepared as discussed in Chapter II of the Results and Discussion.



40

The protonated form of the amine **40** has a pK_a of 8.7,[111] so the amine **40** is present in solution predominantly in the protonated form under the conditions used to monitor the effect of cyclodextrins on product inhibition of PAL described in the previous Chapter. This form of the amine **40** has an increased aqueous solubility when compared to the naturally occurring cyclodextrins **7** and **8**,[111] so it can be present in solution at greater concentration. Since complexation of a guest by a host is a bimolecular reaction, a higher concentration of the host results in a greater proportion of the guest being complexed. Where the guest is an inhibitor of an enzyme, it was expected that a larger amount of the host would lead to less of the inhibitor free in solution and hence a greater effect of the host to limit inhibition. Thus, it was anticipated that the amine **40** might be more effective than the naturally occurring cyclodextrins **7** and **8** to limit product inhibition of PAL through removing a greater proportion of the product from solution.

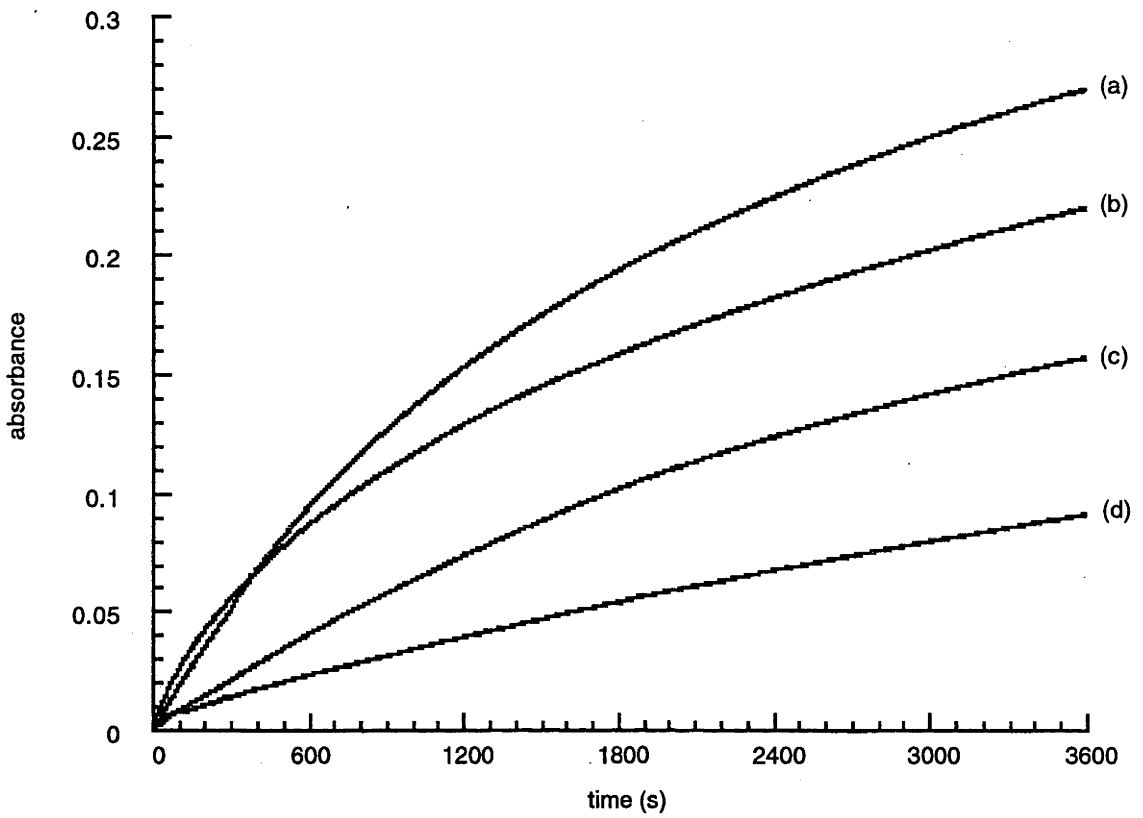


Figure 11. Change in ultraviolet absorbance at 268 nm of solutions containing (*S*)-phenylalanine ((*S*)-**3**) (0.25 x 10^{-3} mol dm^{-3}), PAL (*ca.* 70 units dm^{-3}) and either a) 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**) (27.6 x 10^{-3} mol dm^{-3}), b) no cyclodextrin, c) 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**) (55.2 x 10^{-3} mol dm^{-3}), or d) 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**) (0.104 mol dm^{-3}) in 0.05 mol dm^{-3} phosphate buffer at pH 7.0 and 303 K.

A series of experiments was carried out monitoring the digestion of (*S*)-phenylalanine ((*S*)-**3**) (0.25 x 10^{-3} mol dm^{-3}) by PAL in the presence of either no cyclodextrin or the amine **40** (27.6 x 10^{-3} mol dm^{-3}, 55.2 x 10^{-3} mol dm^{-3} or 0.104 mol dm^{-3}) in 0.05 mol dm^{-3} phosphate buffer at pH 7.0 and 303 K for 1 h at 268 nm (Figure 11). At this wavelength, an absorbance change of 0.50 in the absence of a cyclodextrin would correspond to all of the substrate (*S*)-**3** having been converted to *trans*-cinnamate (**4**).

The experiment with the amine **40** present at a concentration of 27.6 x 10^{-3} mol dm^{-3} (assay a, Figure 11) showed an increase in the change in absorbance after 1 h when

compared to the change in absorbance for the assay in the absence of a cyclodextrin (assay b, Figure 11). This result indicates that the enzyme retains activity in the presence of the amine **40**. Separate experiments indicated that the molar absorption of the cinnamate **4** is reduced by *ca.* 5% on complexation with the host **40**. Thus, the extent of reaction after 1 h in the presence of the cyclodextrin **40** at this concentration is *ca.* 54%, compared to 44% in the absence of a cyclodextrin. This corresponds to an overall increase in the activity of the enzyme of *ca.* 23%.

On addition of the amine **40** at a concentration of 55.2 x 10^{-3} mol dm^{-3} to a reaction mixture (assay c, Figure 11), the change in absorbance during reaction was observed to decrease relative to the case in which no cyclodextrin was present (assay b, Figure 11). The change in absorbance corresponds to an extent of reaction of *ca.* 32%. That is, the extent of reaction decreases by *ca.* 12% on addition of the amine **40** at this concentration, corresponding to a *ca.* 27% decrease in the overall activity of the enzyme.

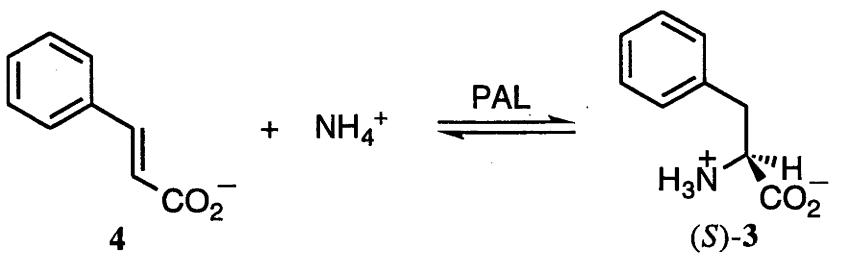
The assay with the amine **40** present at the highest concentration used (0.104 mol dm^{-3}) (assay d, Figure 11) showed a decrease in absorbance after 1 h relative to the case in which no cyclodextrin was present initially (assay b, Figure 11). The change in absorbance equates to an extent of reaction of *ca.* 19%, compared to 44% in the absence of a cyclodextrin. This corresponds to a *ca.* 55% decrease in overall activity of the enzyme on addition of the amine **40** at this concentration.

The interpretation of these results is that at the lowest concentration of the amine **40** used, the cinnamate **4** produced is removed from solution, resulting in less product inhibition. This more than offsets any effect of the host **40** to reduce enzyme activity through complexation of the substrate (*S*)-**3**. As the concentration of the amine **40** is increased, the substrate (*S*)-**3** is removed from solution to a greater extent, resulting in a larger decrease in the amount free in solution to be digested. This effect outweighs any reduction in product inhibition through complexation of the cinnamate **4** at concentrations of the amine **40** of 55.2 x 10^{-3} mol dm^{-3} and 0.104 mol dm^{-3}, and results in a decrease in overall enzyme activity. The larger decrease is seen at the highest concentration of the amine **40** used, at which a greater amount of the substrate (*S*)-**3** is removed from solution.

This series of experiments suggests that the complexation properties of the amine **40** differ from those of the naturally occurring cyclodextrins **7** and **8**. The assays monitoring the digestion of (*S*)-phenylalanine ((*S*)-**3**) by PAL in the presence of the cyclodextrins **7** and **8** (Figure 7) indicate that any effect of the hosts **7** and **8** to remove the substrate (*S*)-**3** from solution is exceeded by their effect to reduce product inhibition through complexation of the cinnamate **4**. Since the activity of PAL decreases on

addition of the amine **40** at the higher concentrations used, this suggests that it removes more of the substrate (*S*)-**3** from solution than do the native cyclodextrins **7** and **8**. It was anticipated that this difference in complexation properties might be exploited to increase the degree to which phenylalanine **3** is removed from solution in related systems, so it was investigated further.

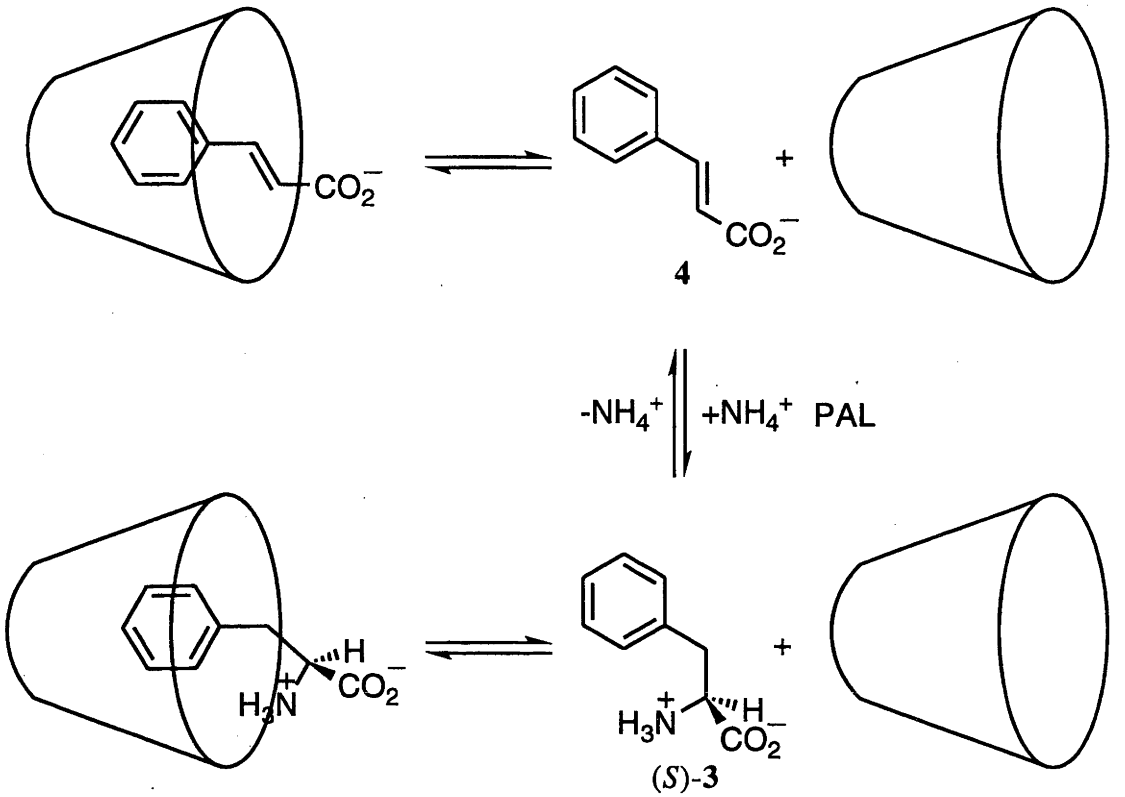
Under the conditions used for the assays summarised in Figure 11, the digestion of the substrate (*S*)-**3** by PAL can be considered irreversible. However, in solutions containing ammonium ions PAL also catalyses the conversion of the cinnamate **4** to (*S*)-phenylalanine ((*S*)-**3**) (Scheme 23).[9] The equilibrium constant for the reaction shown in Scheme 23 is 0.19 mol^{-1} dm^3 at pH 6.8 and 303 K.[9] The utility of this reaction to synthesise (*S*)-phenylalanine ((*S*)-**3**) from *trans*-cinnamate (**4**) has been investigated, but is limited as the large concentrations of ammonium ions necessary to produce the phenylalanine (*S*)-**3** in reasonable yield are deleterious to the activity of the enzyme.[135-137] The same investigations also found that the use of a high pH is advantageous for the production of the phenylalanine (*S*)-**3**, but that this also adversely affects enzyme activity. It was envisaged that the ratio of the cinnamate **4** to the phenylalanine (*S*)-**3** at equilibrium in a mixture containing ammonium ions and PAL might change through complex formation on addition of the cyclodextrins **7**, **8** and **40**. The ratio in free solution would not change, but complexation to different extents would lead to different ratios of the total free and bound species. Any effects observed would allow comparison of the complexation properties of the cyclodextrins **7**, **8** and **40**.



4 (*S*)-**3**

Scheme 23. The equilibrium between *trans*-cinnamate (**4**), ammonium ions and (*S*)-phenylalanine ((*S*)-**3**).

The equilibria of note in a solution containing (*S*)-phenylalanine ((*S*)-**3**), the cinnamate **4**, ammonium ions, PAL and a cyclodextrin are illustrated in Scheme 24. It was anticipated that addition of a cyclodextrin which complexes the cinnamate **4** in preference to the phenylalanine (*S*)-**3** would result in more (*S*)-phenylalanine ((*S*)-**3**) being converted to the cinnamate **4** to maintain the ratio of these species free in solution, hence increasing the ratio of the total amount of cinnamate **4** to the total amount of (*S*)-phenylalanine ((*S*)-**3**) present at equilibrium. By analogy, it was expected that the



Scheme 24. Equilibria in a solution containing (*S*)-phenylalanine ((*S*)-**3**), the cinnamate **4**, ammonium ions, PAL and a cyclodextrin

ratio of the total amount of the cinnamate **4** to the total amount of (*S*)-phenylalanine ((*S*)-**3**) present at equilibrium would decrease on addition of a cyclodextrin which complexes (*S*)-phenylalanine ((*S*)-**3**) in preference to the cinnamate **4**.

It was noted that the association constants of complexes of divalent metal ions and amino acids are very large ($>10^4$ mol^{-1} dm^3).[138] It was anticipated that the addition of metal(II) ions might greatly increase the total amount of (*S*)-phenylalanine ((*S*)-**3**) relative to the total amount of the cinnamate **4** present at equilibrium in a solution containing ammonium ions and PAL, in a manner analogous to that described for cyclodextrins. Thus the effect of metal(II) ions was investigated also.

The assays used to examine the effect of cyclodextrins and metal(II) ions on the ratio of the total amount of the cinnamate **4** to the total amount of (*S*)-phenylalanine ((*S*)-**3**) present at equilibrium in solutions containing ammonium ions (0.5 mol dm^{-3}) and PAL (*ca.* 300 units dm^{-3}), all initially contained the cinnamate **4** (0.26×10^{-3} mol dm^{-3}) and benzoic acid (0.39×10^{-3} mol dm^{-3}) in 0.05 mol dm^{-3} tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.0. Trial experiments indicated that a buffer was necessary to maintain the pH as the reactions proceeded. The phosphate

buffer used in previous experiments was replaced with Tris buffer as, unlike phosphate buffer, divalent metal ions are soluble in it. The concentration of the substrate **4** and the pH are consistent with those used in the experiments examining the effect of cyclodextrins on product inhibition of PAL. The ammonia concentration was chosen as it is known not to denature the enzyme.[9] Under these conditions, the equilibrium mixture in the absence of a cyclodextrin would be expected to contain the cinnamate **4** and (*S*)-phenylalanine ((*S*)-**3**) in a ratio of *ca.* 10 : 1.[9] The reaction mixtures were thermostatted at 303 K and monitored using HPLC. The total amount of the cinnamate **4** present in solution was determined, using the benzoate added initially as an internal standard, and hence the total amount of the phenylalanine (*S*)-**3** formed could be calculated. This information gave the ratio of the total amount of the cinnamate **4** to the total amount of the phenylalanine (*S*)-**3** present in solution. Reactions were monitored until no further change in this ratio was observed. The addition of more of the starting material **4** resulted in further reaction as necessary to restore this ratio, indicating that an equilibrium position had been reached rather than that the enzyme activity had been lost.

Initial studies involving the addition of the metal ions, copper (II), zinc(II) and nickel(II), to the reaction mixture showed they have deleterious effects on the activity of the enzyme. Enzyme activity was reduced to negligible levels in the presence of either 0.25 x 10^{-3} mol dm^{-3} copper(II), 2.5 x 10^{-3} mol dm^{-3} zinc(II) or 25 x 10^{-3} mol dm^{-3} nickel(II). At concentrations below these noted, the ratio of the total amount of the cinnamate **4** to the total amount of the phenylalanine (*S*)-**3** present at equilibrium was unchanged from the case in which no metal was present. Thus, metal(II) ions had no effect on the equilibration catalysed by PAL, other than to denature the enzyme.

The results of experiments to determine the ratio of the total amount of the cinnamate **4** to the total amount of the phenylalanine (*S*)-**3** present at equilibrium in reactions containing ammonium ions, PAL and either no cyclodextrin, α-cyclodextrin (**7**) β-cyclodextrin (**8**) or the amine **40** are shown in Table 11.

Cyclodextrin	Ratio of the cinnamate **4** to the phenylalanine (*S*)-**3** at equilibrium
-	8.5 : 1
8	23 : 1
7	>100 : 1
40	3.5 : 1

Table 11. The ratio of the cinnamate **4** to the phenylalanine (*S*)-**3** (total concentration 0.26 x 10^{-3} mol dm^{-3}) present at equilibrium in mixtures containing PAL (*ca.* 300 units dm^{-3}), ammonium ions (0.5 mol dm^{-3}) and either no cyclodextrin, α-cyclodextrin (**7**) (67 x 10^{-3} mol dm^{-3}), β-cyclodextrin (**8**) (8.2 x 10^{-3} mol dm^{-3}) or 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**) (81 x 10^{-3} mol dm^{-3}) in 0.05 mol dm^{-3} pH 7.0 Tris buffer at 303 K, as observed by HPLC.

In the assay in which no cyclodextrin was present, the ratio of the total amount of the cinnamate **4** to the total amount of the phenylalanine (*S*)-**3** at equilibrium was 8.5 : 1. Addition of the cyclodextrin **8** to the reaction mixture resulted in this ratio increasing to 23 : 1. That is, more of the cinnamate **4** is present at equilibrium in this case than in the absence of a cyclodextrin. In the assay in which α-cyclodextrin (**7**) was present, the total amount of the cinnamate **4** in solution did not change with time which, based on the accuracy of the method, gives the ratio of the total amount of the cinnamate **4** to the total amount of the phenylalanine (*S*)-**3** at equilibrium as >100 : 1. When the reaction mixture contained the amine **40**, this ratio was 3.5 : 1. This corresponds to more (*S*)-phenylalanine ((*S*)-**3**) being present at equilibrium in this case than in the absence of a cyclodextrin.

The results of the experiments involving the addition of the cyclodextrins **7** and **8** to the reaction mixtures are consistent with the reported stability constants of the complexes of (*S*)-phenylalanine ((*S*)-**3**) in α-cyclodextrin (**7**) and β-cyclodextrin (**8**) of 8 and 3 mol^{-1} dm^3, respectively,[95] and of the analogous complexes with *trans*-cinnamate (**4**) as the guest, of 109 and 313 mol^{-1} dm^3, respectively.[96] When added to a reaction mixture, β-cyclodextrin (**8**) preferentially complexes the cinnamate **4**, removing it from solution and hence resulting in more of the phenylalanine (*S*)-**3** being converted to the cinnamate **4** to maintain the ratio of free *trans*-cinnamate (**4**) to free (*S*)-phenylalanine ((*S*)-**3**) in solution. Thus, the ratio of the total amount of the cinnamate **4** to the total amount of the phenylalanine (*S*)-**3** present at equilibrium increases, relative to the case in the absence of a cyclodextrin. By the analogous argument, α-cyclodextrin **7**, which preferentially complexes the cinnamate **4**, also causes an increase in the ratio of the total amount of the cinnamate **4** to the total amount of the phenylalanine (*S*)-**3** present at equilibrium. Using the concentrations of the cyclodextrins **7** and **8** present in the assays and their reported association constants with the cinnamate **4**, the proportion of the cinnamate **4** removed from solution is calculated to be greater in the case of the cyclodextrin **7**. Correspondingly, so is the ratio of the total amount of the cinnamate **4** to the total amount of (*S*)-phenylalanine ((*S*)-**3**) present at equilibrium.

In the experiment in which the amine **40** was added to the reaction mixture, more of the phenylalanine (*S*)-**3** was present at equilibrium. This is consistent with the amine **40** complexing the phenylalanine (*S*)-**3** in preference to the cinnamate **4**. That is, the effect of the host **40** appears to be to remove the phenylalanine (*S*)-**3** from solution to a greater extent than the cinnamate **4**. As a result, more of the cinnamate **4** is converted to (*S*)-phenylalanine ((*S*)-**3**), to maintain the ratio of these species free in solution. Hence, the total amount of the phenylalanine (*S*)-**3** present at equilibrium increases, so the ratio of the total amount of the cinnamate **4** to that of the phenylalanine (*S*)-**3** decreases. To

quantify this effect, studies to calculate the association constants of the complexes formed between the host **40** and each of the guests (*S*)-**3** and **4** were undertaken.

As described in the Introduction, the spectroscopic properties of a guest may change on complexation within a cyclodextrin and the observed spectrum is generally the weighted average of those of the free and complexed species. The extent of this change as a function of the concentration of the host can be used to quantify the association constant of the complex formed. Initially, the effects of differing concentrations of the amine **40** on the chemical shifts of resonances in the ^{1}H NMR spectra of the cinnamate **4** and the phenylalanine (*S*)-**3** were investigated. It should be noted that these measurements were carried out in deuterium oxide containing phosphate buffer, rather than the Tris buffer used in the assays measuring the effect of the cyclodextrins **7**, **8** and **40** on the ratio of the total amounts of the two species **4** and (*S*)-**3** present at equilibrium. This was necessary as resonances due to the hydrogen nuclei of Tris buffer overlap and obscure the resonances of the protons of the guests **4** and (*S*)-**3**, making analysis impossible. Further, the temperature used was 298 K, rather than 303 K as used in the equilibria studies, as this is more convenient for NMR. Nevertheless the results should be comparable.

The difference in chemical shift between the signal due to the α-proton and the signal due to the 4'-proton in the ^{1}H NMR spectrum of the cinnamate **4** was observed to change as a function of the concentration of the host **40** (Figure 12). The association constant for the complex formed between the amine **40** and the cinnamate **4** was calculated, using Equations 1 and 2, to be 270 ± 20 mol^{-1} dm^3. In trial experiments using the phenylalanine (*S*)-**3**, no changes in the signals corresponding to it in the ^{1}H NMR spectrum were observed on addition of the host **40**. This indicates that either there is very little spectroscopic change on complexation of the guest (*S*)-**3** with the host **40**, or that the association constant of this complex is small. Either way, this method is inappropriate for the calculation of the association constant of the complex formed between the host **40** and the guest (*S*)-**3**.

Alternative methods were investigated in order to calculate the association constant for the complex of the guest (*S*)-**3** with the host **40**. The ultraviolet (UV) spectrum of the phenylalanine (*S*)-**3** did not change as a function of the host **40** concentration, so a method analogous to that described above using the change in the UV absorbance of the guest (*S*)-**3** was impractical. The Hummel-Dreyer method uses the effect of a host on the passage of a guest through a chromatography column to determine the association constant of the host with the guest.[57] This method has been applied to HPLC and used to calculate the association constants of complexes in which the host is a

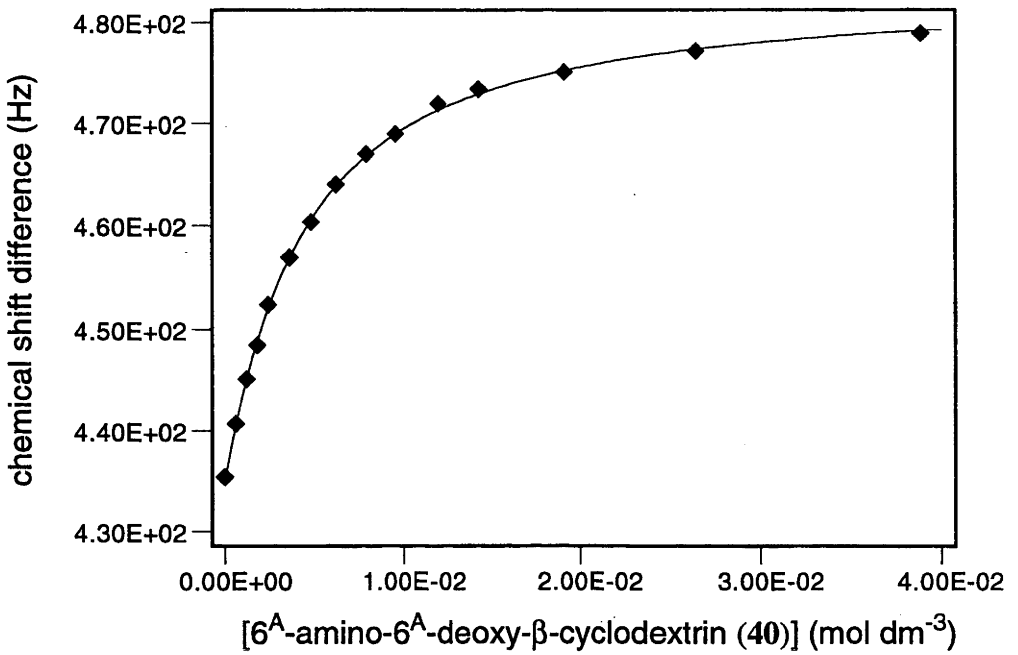
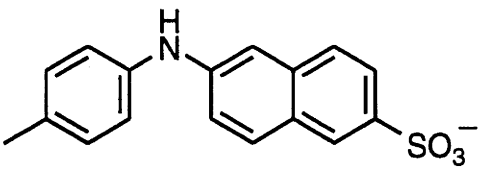


Figure 12. ^{1}H NMR chemical shift difference plotted against the concentration of 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) for the difference in chemical shifts between the resonances corresponding to the α-proton and the 4'-proton of *trans*-cinnamate (**4**) in 0.05 mol dm^{-3} phosphate buffered deuterium oxide at pH 7.0 and 298 K.

cyclodextrin.[139-141] However, the conditions used for the chromatography are limited to those for the system in which the association constant is required, in this case, 0.05 mol dm^{-3} phosphate buffer at pH 7.0. Under these conditions, the signals corresponding to the host **40** and the guest (*S*)-**3** could not be resolved, due to the similar chromatographic properties of these species, so this method could not be used.

Since direct methods of measurement proved unsuitable, the use of an indirect method of association constant calculation was considered. The displacement of a fluorophore from a cyclodextrin cavity by another guest has been used previously to calculate the association constant of the complex formed.[142-147] Initial studies using 2-(4-toluidino)naphthalene-6-sulfonate (**82**) as the fluorophore, indicated that complexes consisting of one guest **82** and two host **40** molecules are formed, as well as 1 : 1 complexes. This complicates any analysis of changes in fluorescence, so this method was not pursued.



82

It was anticipated that the displacement of the cinnamate **4** from the cavity of the cyclodextrin **40** by (*S*)-phenylalanine ((*S*)-**3**) could be used to calculate the association constant of the complex of the guest (*S*)-**3** with the host **40**. Any displacement of the cinnamate **4** by (*S*)-phenylalanine ((*S*)-**3**) would result in an increase in the proportion of the cinnamate **4** free in solution and hence a change in the chemical shifts of the resonances due to it in the ^{1}H NMR spectrum of the solution. It was envisaged that any change in chemical shift of the signals due to the cinnamate **4** on addition of the phenylalanine (*S*)-**3** could be used to calculate the association constant of the complex of the guest (*S*)-**3** with the host **40**. A complete summary of the calculations involved is described in Appendix 2.

To evaluate this method, a trial experiment involving the addition of the adamantane derivative **10** to a solution of the cinnamate **4** and the host **40** was carried out. The adamantane derivative **10** is reported to include in β-cyclodextrins with association constants of $>10^4$ mol^{-1} dm^3,[46] so it was expected to displace the guest **4** from the cavity of the cyclodextrin **40**. On addition of the carboxylate **10**, the chemical shifts of the signals corresponding to the cinnamate **4** protons in the ^{1}H NMR spectrum of the solution were observed to change, indicating that the method was appropriate to monitor displacement of the cinnamate **4** from the cavity of the cyclodextrin **40**.

The association constant for the complex of the phenylalanine (*S*)-**3** with the amine **40** was calculated by observing the change in chemical shift difference between the signals due to the α-proton and the 4'-proton of the cinnamate **4** in the ^{1}H NMR spectrum of the solution at various concentrations of the phenylalanine (*S*)-**3** and fixed concentrations of the host **40** and the cinnamate **4**. Only a very small change in the chemical shift of the signals corresponding to the cinnamate **4** protons was observed on addition of the phenylalanine (*S*)-**3**, with the association constant of the complex between (*S*)-phenylalanine ((*S*)-**3**) and the host **40** thus being calculated as 9.6 ± 0.3 mol^{-1} dm^3.

This value is much lower than had been expected. The effect of the amine **40** on the ratio of the total amount of the cinnamate **4** to the total amount of the phenylalanine (*S*)-**3** present at equilibrium in a solution containing ammonium ions and PAL suggests that the amine **40** complexes the phenylalanine (*S*)-**3** in preference to the cinnamate **4**. That is, it was expected that the association constant for the complex of the phenylalanine (*S*)-**3** with the amine **40** would be greater than that of the cinnamate **4** with the amine **40**. However, this is not the case, as the association constants were calculated as 9.6 ± 0.3 mol^{-1} dm^3 and 270 ± 20 mol^{-1} dm^3, respectively. Further, given that the cyclodextrins **7** and **40** were used at similar concentrations and have similar association constants for their complexes with the guest (*S*)-**3**, their effects to remove the phenylalanine (*S*)-**3** from solution and hence limit its digestion by PAL would be

expected to be similar. However, while no effect of the cyclodextrin **7** to decrease PAL activity by removing the substrate (*S*)-**3** from solution was observed (Figure 7), the amine **40** significantly decreased PAL activity (Figure 11) suggesting that more of the substrate (*S*)-**3** was removed from solution in that case.

The effect of the adamantane derivative **10** to disrupt the complex of the cinnamate **4** and the amine **40** indicates that the cinnamate **4** is complexed in the cavity of the cyclodextrin **40**. The experiments which showed that the phenylalanine (*S*)-**3** does not effectively displace the cinnamate **4** from the cyclodextrin annulus, and the low value of the association constant for the complex of the host **40** and the guest (*S*)-**3** reflecting this do not take into account binding to the exterior surface of the host **40**. The inconsistency of the calculated association constant with the observed effects of the host **40** on systems containing (*S*)-phenylalanine ((*S*)-**3**) and the cinnamate **4** suggests that the interaction of (*S*)-phenylalanine ((*S*)-**3**) with the host **40** may be non-inclusive in nature. The interaction of molecules with the surface of cyclodextrins has been reported and is particularly significant when cyclodextrins are used as chiral stationary phases in separation technology.[148]

In any event, the work described in this Chapter has illustrated that in a solution containing two compounds which are interconverted by an enzyme which is also present, the ratio of the amounts of these compounds present at equilibrium may be altered on addition of a cyclodextrin. Further, different cyclodextrins were shown to be capable of altering the ratio in different directions and to different extents. A limitation of the use of enzymes in synthesis is that the thermodynamics of an enzyme catalysed reaction might be such that, at equilibrium, little of the desired product is present. It now appears that there is the potential to overcome this limitation through the addition of a cyclodextrin which preferentially complexes the desired product, resulting in an increase in the total amount of the desired product present at equilibrium.

Results and Discussion

Chapter V

Acylase I Catalysed Hydrolysis of *para*-Substituted (*S*)-Phenylalanine Derivatives From Mixtures of the Racemic *ortho*- and *para*-Substituted Isomers

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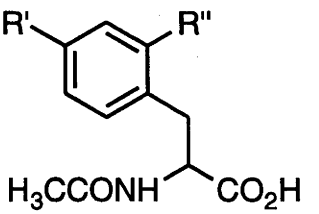
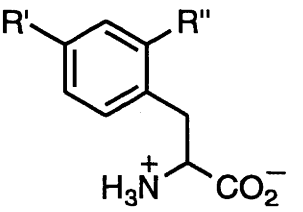
In the previous two Chapters, it has been demonstrated that cyclodextrins can affect the outcomes of enzyme catalysed reactions through interaction with components of the reaction mixtures. It was anticipated that cyclodextrins might also be used to affect the digestion of a mixture of substrates by an enzyme. As discussed in the Introduction, treatment of a mixture of substrates with an enzyme which exhibits broad substrate selectivity results in a mixture of products. This limits the utility of the process, as the products have to be separated. It was envisaged that the addition of a cyclodextrin might be used to complex, and hence remove from solution, all but one of the substrates, resulting in the mixture of substrates affording only one product, thereby increasing the utility of the enzyme.



83 R' = H, R" = H
84 R' = F, R" = H; **85** R' = H, R" = F
86 R' = CH_3, R" = H; **87** R' = H, R" = CH_3
88 R' = NO_2, R" = H; **89** R' = H, R" = NO_2
90 R' = Cl, R" = H; **91** R' = H, R" = Cl
92 R' = OH, R" = H; **93** R' = H, R" = OH

The reactions chosen to examine the effects of cyclodextrins on digestion of mixtures of substrates by an enzyme were the hydrolyses of the *ortho*- and *para*-substituted *N*-acetylphenylalanines **84-93** on treatment with acylase I. As described in the Introduction, acylase I is a practical tool for resolution of a range of *N*-acetylamino acids.[100,101] The synthesis of ring-substituted phenylalanines is of interest as they are important constituents of natural products such as vancomycin.[149,150] Mixtures of *ortho*-

and *para*-substituted phenylalanines may be synthesised, often as the racemates, through aromatic substitution of phenylalanine derivatives [151-153] and other aromatic starting materials.[132,133,154] Cyclodextrins are known to discriminate between the *ortho*- and *para*-regioisomers of a range of disubstituted benzenes.[46,106,155,156] Thus, it was anticipated that a cyclodextrin might discriminate between *ortho*- and *para*-substituted *N*-acetylphenylalanines. As a result, while the treatment of a racemic mixture of the *ortho*-and *para*-isomers of a substituted *N*-acetylphenylalanine by acylase I was expected to afford a mixture of the *ortho*- and *para*-isomers of the corresponding (*S*)-phenylalanines, it was envisaged that a cyclodextrin added to the reaction mixture might selectively complex one of the substituted *N*-acetylphenylalanines. Thus, the enzyme would digest only the phenylalanine derivative remaining in solution, to give only that regioisomer of the substituted (*S*)-phenylalanine. That is, it was anticipated that the addition of a cyclodextrin to these reactions might be used to facilitate the synthesis of a single regioisomer of a ring-substituted (*S*)-phenylalanine from a racemic mixture of the corresponding *ortho*- and *para*-substituted *N*-acetylphenylalanines.



3 R' = H, R" = H
57 R' = F, R" = H; **58** R' = H, R" = F
59 R' = CH_3, R" = H; **60** R' = H, R" = CH_3
61 R' = NO_2, R" = H; **62** R' = H, R" = NO_2
63 R' = Cl, R" = H; **64** R' = H, R" = Cl
42 R' = OH, R" = H; **65** R' = H, R" = OH

Samples of the phenylalanine derivatives **84-91** were prepared from the corresponding phenylalanines **57-64**, which were available either commercially (**57**, **58**, **61**, **63** and **64**) or from the work discussed in Chapter III of the Results and Discussion (**59**, **60** and **62**). The transformations were accomplished using acetic anhydride under basic aqueous conditions.[157] The ^{1}H NMR spectrum of each of the phenylalanine derivatives **84-91** includes a characteristic resonance in the range δ1.7-1.9 corresponding to the hydrogen nuclei of a methyl group adjacent to an amide carbonyl.[110] Further, each of the phenylalanine derivatives **84-91** has physical data consistent with those reported.[132,158-162]

The situation was more complicated for the preparation of the tyrosine derivatives **92** and **93**, since the phenol group in each of the tyrosines **42** and **65** represents a

second nucleophilic site. This problem was overcome by acetylating at both sites with acetic anhydride under basic conditions, then allowing the more labile phenolic ester to hydrolyse under acidic conditions.[163,164] The ^{1}H NMR spectrum of the product of each of these reactions includes only one signal corresponding to the hydrogens of a methyl group. This indicates that the method affords a monoacetylated product in each case. Further, the methyl signal is at *ca.* δ1.8, as is consistent with the hydrogens of a methyl group adjacent to an amide carbonyl.[110] The methyl group of a phenyl acetate would be expected to give rise to a signal further downfield, as is observed in the ^{1}H NMR spectrum of *O*-acetyltyrosine **41** prepared as discussed in Chapter I of the Results and Discussion in which the resonance corresponding to the methyl group is at δ2.3. Thus, these spectral data indicate that the *N*-acetyltyrosines **92** and **93** were produced using this method. Further, the phenylalanine derivative **92** has physical characteristics consistent with those reported,[164] while its regioisomer **93** was fully characterised.

In order for any effects of a cyclodextrin to be quantified, control experiments were carried out examining the treatment of the phenylalanine derivatives **84-93** with acylase I in the absence of a cyclodextrin. These reactions were carried out in phosphate buffer at pH 7.0. A buffer was used to ensure that the pH did not change as the reactions proceeded and the pH value was chosen as that where the enzyme shows maximum activity.[100] Racemic mixtures of regioisomeric pairs of the phenylalanine derivatives **84-93** (*ca.* 10 x 10^{-3} mol dm^{-3}) were treated with acylase I (*ca.* 7 x 10^{-9} mol dm^{-3}) at 298 K for 2 h. Dilute hydrochloric acid was then added to denature the enzyme and protonate the carboxylate form of the residual starting materials and the zwitterionic form of the products. The acetylphenylalanines **84-93** are soluble in organic solvents, while the cationic form of each of the phenylalanines **42** and **57-65** is not. Thus, the acidified reaction mixtures were partitioned between ethyl acetate and water to give the residual starting materials and products, respectively.

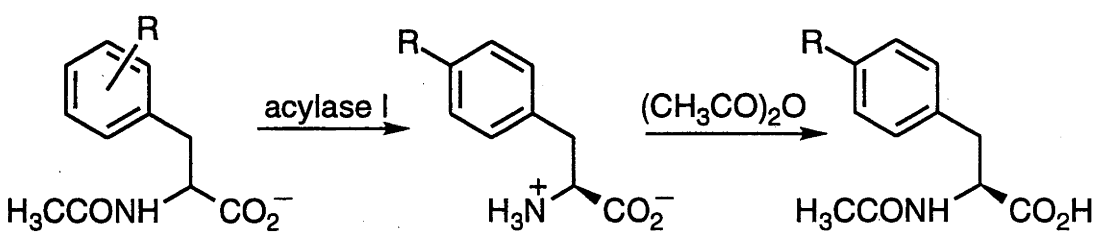
Initial experiments involving the treatment of regioisomeric pairs of the *N*-acetylphenylalanines **84-93** with acylase I in the absence of a cyclodextrin were analysed through treatment of the residual starting materials with thionyl chloride pretreated methanol to afford the corresponding methyl esters. These were analysed through gas chromatography (GC) using a chiral stationary phase on an instrument calibrated with authentic racemic samples. Since the phenylalanine derivatives (*R*)-**84-93** were not expected to be digested by acylase I, the extent of reaction in each case was calculated using the ratio of the integration of the signals due to the (*S*)- and (*R*)-enantiomers, the latter serving as an internal standard.

The results of these experiments indicated that only in the case of the fluorides **84** and **85** does acylase I digest both the *ortho*- and *para*-isomers of ring-substituted

(*S*)-*N*-acetylphenylalanines. In the remaining cases, the *para*-substituted phenylalanines (*S*)-**86**, (*S*)-**88**, (*S*)-**90** and (*S*)-**92** were digested by acylase I, while the corresponding *ortho*-substituted phenylalanines (*S*)-**87**, (*S*)-**89**, (*S*)-**91** and **93** were not. That is, acylase I selectively hydrolyses the *para*-substituted phenylalanine derivatives (*S*)-**86**, (*S*)-**88**, (*S*)-**90** and (*S*)-**92** in solutions containing racemic mixtures of the corresponding *ortho*- and *para*-substituted phenylalanine derivatives **86-93**.

It is clear from these results that the treatment of racemic mixtures of regioisomeric pairs of the phenylalanine derivatives **84-93** with acylase I is unsuitable to examine the effect of cyclodextrins on the digestion of a mixture of substrates by an enzyme, as acylase I does not digest the *ortho*-substituted phenylalanine derivatives (*S*)-**87**, (*S*)-**89**, (*S*)-**91** and (*S*)-**93**. As a result, studies on the effects of cyclodextrins on these systems were not pursued. However, from these results it was expected that the *para*-substituted phenylalanines (*S*)-**42**, (*S*)-**59**, (*S*)-**61** and (*S*)-**63** might be isolated from racemic mixtures of the corresponding *ortho*- and *para*-substituted phenylalanines **86-93** using acylase I. Thus, experiments were carried out to determine the utility of this process.

The experiments involving the treatment of regioisomeric pairs of the *N*-acetylphenylalanines **84-93** with acylase I were repeated on a larger scale under otherwise identical conditions. In these cases, after partitioning the acidified reaction mixtures between ethyl acetate and water, the aqueous layers were concentrated and the residues were treated with acetic anhydride under basic conditions. After acidification, the acetamides thus formed from the initial product phenylalanines were extracted into organic solvent and analysed. Separate experiments established that the conditions used for acetylation did not affect the stereochemistry of the products at the α-centre. The acetamides produced were identified through comparison of their physical and spectroscopic data with those reported in the literature.[161,164-166] Their enantiopurity was determined through conversion to the corresponding methyl esters with thionyl chloride pretreated methanol and analysis by GC using a chiral stationary phase. The results of these experiments are illustrated in Table 12 and a summary of the overall process is shown as Scheme 25 as it applies to compounds **86-93**.



Scheme 25. Isolation of *para*-substituted (*S*)-*N*-acetylphenylalanines from racemic regioisomeric mixtures of the corresponding *ortho*- and *para*-substituted *N*-acetylphenylalanines through treatment with acylase I, then treatment of the product (*S*)-phenylalanines with acetic anhydride under basic aqueous conditions.

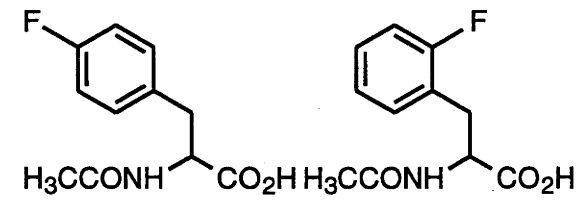
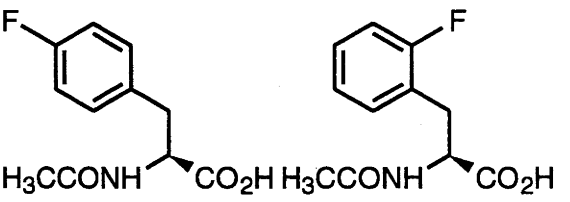
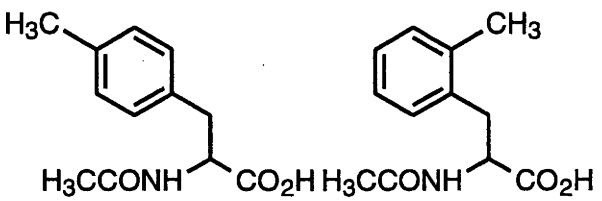
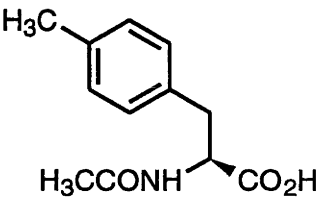
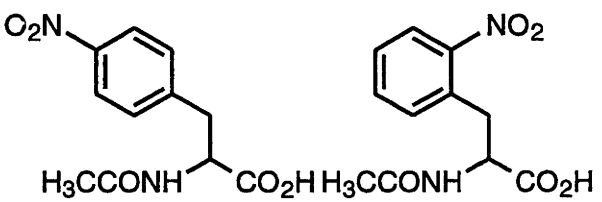
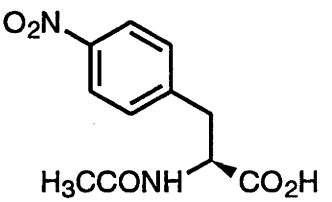
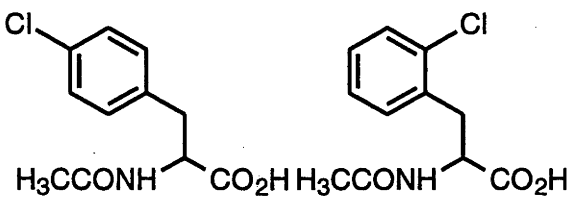
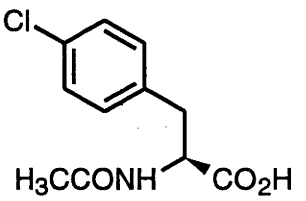
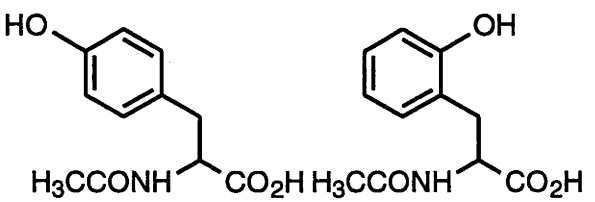
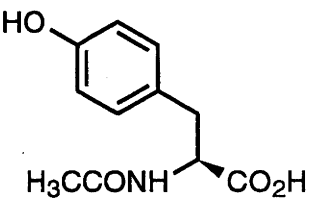
Starting Materials (1 : 1)	Product(s) (Yield[a])
 **84** **85**	 (*S*)-**84** (58%) (*S*)-**85** (29%)
 **86** **87**	 (*S*)-**86** (72%)
 **88** **89**	 (*S*)-**88** (83%)
 **90** **91**	 (*S*)-**90** (71%)
 **92** **93**	 (*S*)-**92** (73%)

Table 12. Results of treatment of regioisomeric pairs of the *N*-acetylphenylalanines **84-93** (*ca.* 10 x 10^{-3} mol dm^{-3}) with acylase I (*ca.* 7 x 10^{-9} mol dm^{-3}) in 0.05 mol dm^{-3} pH 7.0 phosphate buffer at 298 K for 2 h, followed by treatment with acetic anhydride under basic aqueous conditions at room temperature for 3 h.
[a]Yield is calculated for two steps, based on reaction of the (*S*)-enantiomer only.

Based on the signal to noise ratio of the instrument used for the analysis of the substituted *N*-acetylphenylalanine methyl esters by GC and the resolution of the enantiomers of authentic racemic samples, each of the acetamides (*S*)-**84-86**, (*S*)-**88**, (*S*)-**90** and (*S*)-**92** was isolated with an ee >95%. These results show that in four of the five cases investigated the enzyme selectively digests the *para*-substituted (*S*)-*N*-acetylphenylalanine, affording the *para*-substituted (*S*)-phenylalanine which can be isolated as the corresponding acetamide in good yield. The systems studied included ring-substituted phenylalanines with hydrophobic, hydrophilic, electron-donating and electron-withdrawing groups. This indicates the broad tolerance of the enzyme for the nature of the ring substituent at the *para*-position. It was only in the case of the fluoride (*S*)-**85** that any hydrolysis of an *ortho*-substituted *N*-acetylphenylalanine was observed. This is presumably due to fluorine having similar steric bulk to hydrogen[167] and, therefore, little effect on the interaction with the enzyme. Nevertheless, these experiments indicate the synthetic utility of acylase I to hydrolyse a *para*-substituted (*S*)-*N*-acetylphenylalanine from a racemic mixture of the corresponding *ortho*- and *para*-substituted *N*-acetylphenylalanines.

It was anticipated that the basis of the selectivity exhibited by acylase I might be determined through kinetic experiments observing the treatment of the *N*-acetylphenylalanines (*S*)-**84-89** with acylase I. This subset of the acetylphenylalanines **84-93** was chosen as the substituents provide representative examples of both steric and electronic factors. The fluoro substituent is sterically similar to hydrogen and is a powerful electron withdrawing group.[167] The methyl substituent has a small electron donating effect, but is sterically large.[168] The nitro group is a powerful electron withdrawing group and is sterically bulky.[168] Thus, these substituents are of varying size and will affect the electron distribution in the aromatic ring in different ways. Through determining the kinetic parameters of any interactions of the acetylphenylalanines (*S*)-**84-89** with acylase I, it was envisaged that the effects of varying the size of the substituent and the electronic distribution in the aromatic ring on interaction of the substrate with the enzyme might be probed. Further, it was expected that the kinetic parameters for the hydrolysis of (*S*)-*N*-acetylphenylalanine ((*S*)-**83**) by acylase I would provide a basis for comparison.

Each of the *N*-acetylphenylalanines **86**, **87** and **89** was only available in racemic form, having been prepared from racemic starting materials. Consequently, it was necessary to determine if the kinetic parameters of the treatment of the phenylalanines (*S*)-**83-89** with acylase I were affected by the presence of the corresponding (*R*)-enantiomers (*R*)-**83-89**. To examine this, the hydrolysis of the phenylalanine

derivatives (*S*)-**83-85** by acylase I was observed in the presence and absence of the corresponding enantiomers (*R*)-**83-85**.

The phenylalanine derivatives **83**, (*S*)-**83** and (*S*)-**84** were prepared through treatment of the corresponding phenylalanines **3**, (*S*)-**3** and (*S*)-**57**, which are commercially available, with acetic anhydride under basic aqueous conditions. The fluorinated phenylalanine (*S*)-**58** is not commercially available, though the corresponding racemic fluoride **58** is. The phenylalanine derivative **85** was prepared from the corresponding phenylalanine **58**, as discussed earlier in this Chapter, and subsequently treated with acylase I in 0.05 mol dm^{-3} pH 7.0 phosphate buffer at 298 K. This afforded the fluorinated phenylalanine (*S*)-**58** which, after separation from residual starting material **85**, was treated with acetic anhydride under basic aqueous conditions to afford the fluoride (*S*)-**85**. The acetylated phenylalanines **83** and (*S*)-**83-85** were identified by comparison of their physical and spectroscopic properties with those reported.[158,163,165] The ^1H NMR spectrum of each of the phenylalanine derivatives **83** and (*S*)-**83-85** includes a characteristic resonance in the range δ1.7-1.9, corresponding to the hydrogen nuclei of a methyl group adjacent to an amide carbonyl.[110] Each of the acetamides (*S*)-**83-85** was determined to have an ee >95%, through conversion to the corresponding methyl ester with thionyl chloride pretreated methanol and analysis by GC, using a chiral stationary phase.

The hydrolysis of each of the acetamides (*S*)-**83-85** by acylase I was monitored by observing the change in absorbance at 228 nm at 298 K in 0.05 mol dm^{-3} pH 7.0 phosphate buffer. The wavelength used corresponds to the absorbance of the amide being hydrolysed. The initial rates of reaction (v_0) were quantified over a range of substrate concentrations (S). The rate constants of the enzyme catalysed reactions (k_{cat}) and the dissociation constants of the enzyme-substrate complexes (K_M) were calculated by means of Lineweaver-Burk analysis.[113] This involves plotting the reciprocal of the initial rate of reaction against the reciprocal of the substrate concentration. The information in each case was fitted to Equation 4 and values for k_{cat} and K_M were calculated using the values for the slope and coordinate axis intercept. A typical plot is illustrated as Figure 13. From this information, values of k_{cat}/K_M, a measure of the catalytic efficiency of the enzyme, were calculated.

$$\frac{1}{v_0} = \frac{1}{k_{cat}[\text{enzyme}]} + \frac{K_M}{k_{cat}[\text{enzyme}]} \cdot \frac{1}{S} \qquad (4)$$

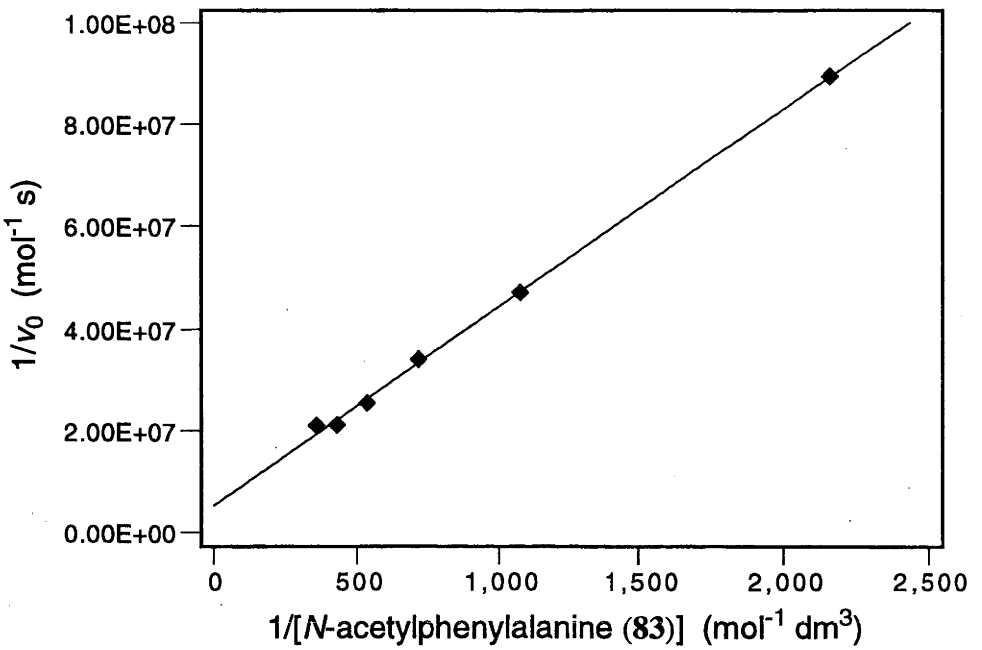


Figure 13. Lineweaver-Burk plot for the treatment of *N*-acetylphenylalanine (**83**) with acylase I.

The kinetic parameters for the treatment of the acetylphenylalanines **83-85** with acylase I in the presence and absence of the corresponding (*R*)-enantiomers are summarised as Table 13. The values of k_{cat} and K_M calculated are within experimental error whether the (*S*)-enantiomer or the racemate was used in each case. These results are consistent with those published previously,[169] where the (*R*)-enantiomers of other acylated amino acids do not affect digestion of their (*S*)-antipodes by acylase I. By analogy, it was expected that the kinetic parameters of any interactions of the acetylphenylalanines (*S*)-**86-89** with acylase I would not be affected by the presence of the corresponding (*R*)-enantiomers (*R*)-**86-89**.

Substrate	k_{cat} (s^{-1})	K_M (x 10^3 mol dm^{-3})	k_{cat} / K_M (x 10^{-3} mol^{-1} dm^3 s^{-1})
(*S*)-**83**†	20 ± 3	3.5 ± 0.5	6 ± 2
(*S*)-**83**	24 ± 4	3.9 ± 0.8	6 ± 2
(*S*)-**84**†	58 ± 7	4.1 ± 0.5	14 ± 4
(*S*)-**84**	66 ± 1	3.3 ± 0.1	20 ± 1
(*S*)-**85**†	7.7 ± 0.2	1.00 ± 0.07	7.7 ± 0.7
(*S*)-**85**	7.3 ± 0.4	1.0 ± 0.1	7 ± 1

Table 13. Rate constants for the reactions of bound substrates and dissociation constants for the Michaelis complexes of the *N*-acetylphenylalanines (*S*)-**83-85** with acylase I in 0.05 mol dm^{-3} pH 7.0 phosphate buffer at 298 K.
†Reaction mixture contains one equivalent of the corresponding (*R*)-enantiomer.

Experiments were carried out to determine the kinetic parameters for any interactions of the phenylalanine derivatives (*S*)-**86-89** with acylase I under the same conditions as used for the series of experiments in which the values of k_{cat} and K_M for the phenylalanine derivatives (*S*)-**83-85** with acylase I were determined. The reaction mixtures were prepared using the racemic forms of the phenylalanine derivatives **86-89**. Combining the results of these experiments with those in Table 13 gives Table 14.

Substrate	k_{cat} (s^{-1})	K_M (x 10^3 mol dm^{-3})	k_{cat} / K_M (x 10^{-3} mol^{-1} dm^3 s^{-1})
(*S*)-**83**	20 ± 3	3.5 ± 0.5	6 ± 2
(*S*)-**84**	58 ± 7	4.1 ± 0.5	14 ± 4
(*S*)-**85**	7.7 ± 0.2	1.00 ± 0.07	7.7 ± 0.7
(*S*)-**86**	2.7 ± 0.2	0.32 ± 0.04	8 ± 2
(*S*)-**87**		no hydrolysis detected	
(*S*)-**88**	15 ± 1	0.50 ± 0.07	30 ± 6
(*S*)-**89**		no hydrolysis detected	

Table 14. Rate constants for the reactions of bound substrates and dissociation constants for the Michaelis complexes of the (*S*)-*N*-acetylphenylalanines (*S*)-**83-89** with acylase I in 0.05 mol dm^{-3} pH 7.0 phosphate buffer at 298 K. Reaction mixtures contain one equivalent of the corresponding (*R*)-enantiomer in each case.

These results indicate that the fluoride (*S*)-**84** is bound to the enzyme as tightly as the unsubstituted phenylalanine (*S*)-**83** but less tightly than its regioisomer (*S*)-**85**. The rate of reaction of the phenylalanine derivative (*S*)-**84** bound to the enzyme is greater than that of the unsubstituted phenylalanine (*S*)-**83** which is, in turn, greater than that of the fluoride (*S*)-**85**. In terms of the catalytic efficiency of the enzyme, the fluoride (*S*)-**84** is digested approximately twice as fast as its regioisomer (*S*)-**85** and the acetylphenylalanine (*S*)-**83**. This is consistent with the observed digestion of a regioisomeric mixture of the fluorides **84** and **85** by acylase I. After acetylation of the phenylalanines (*S*)-**57** and (*S*)-**58** produced, the ratio of the acetamides (*S*)-**84** and (*S*)-**85** was 2 : 1.

The *para*-substituted phenylalanine derivative (*S*)-**88** binds to the enzyme more strongly than each of the acetylphenylalanines (*S*)-**83-85** and reacts at a rate similar to that of the phenylalanine derivative (*S*)-**83** once bound. The interaction of the methylphenylalanine derivative (*S*)-**86** with the enzyme is the strongest observed, while its rate of reaction when bound to the enzyme is slower than that of each of the acetylphenylalanines (*S*)-**83-85** and (*S*)-**88**. Further, in terms of their k_{cat}/K_M values, each of the *para*-substituted phenylalanine derivatives (*S*)-**86** is digested as fast as or faster than the acetylphenylalanine (*S*)-**83** by acylase I.

As is consistent with the results from the treatment of regioisomeric mixtures of the phenylalanine derivatives **86-89** with acylase I, no hydrolysis of the *ortho*-substituted

phenylalanines (*S*)-**87** and (*S*)-**89** was detected. Since the phenylalanine derivatives (*S*)-**83**-**86** and (*S*)-**88** are digested by acylase I with similar catalytic efficiency and the phenylalanine derivatives **87** and **89** are not hydrolysed by acylase I, this suggests that it is the steric bulk of a substituent, rather than its electronic effects, which is the basis for the selectivity exhibited by acylase I.

It was envisaged that there might be two possible explanations for the lack of hydrolysis of the phenylalanine derivatives (*S*)-**87** and (*S*)-**89**. They might not bind to the enzyme, so the Michaelis complexes necessary for catalysis by the enzyme might not form. Alternatively, the phenylalanines (*S*)-**87** and (*S*)-**89** might bind to the enzyme, but not react once bound. It was anticipated that these two explanations could be differentiated through examining the effect on digestion of the phenylalanine derivative (*S*)-**83** by acylase I of the addition of each of the phenylalanine derivatives **87** and **89**. Any decrease in the enzyme activity observed would be consistent with the phenylalanines **87** and **89** binding to the enzyme and reducing the amount free to digest the substrate (*S*)-**83**.

Experiments were carried out observing the treatment of *N*-acetylphenylalanine **83** (*ca.* 10 x 10^{-3} mol dm^{-3}) with acylase I (*ca.* 12 x 10^{-3} mol dm^{-3}) in 0.05 mol dm^{-3} pH 7.0 phosphate buffer at 298 K in the presence of either no *ortho*-substituted phenylalanine or *ca.* three equivalents of one of the phenylalanines **87** and **89**. An excess of each of the *ortho*-substituted phenylalanines **87** and **89** was used to ensure that any effect of these compounds on digestion of the phenylalanine derivative (*S*)-**83** by acylase I would be readily observed. The reaction mixtures were acidified to *ca.* pH 1 after 1 h, and partitioned between ethyl acetate and water. The organic layer was concentrated and the residual starting material **83** present was converted to the corresponding methyl ester with thionyl chloride pretreated methanol. In each case the methyl ester produced was analysed through GC using a chiral stationary phase and the extent of reaction was calculated through comparison of the integration of the signals due to the (*S*)- and (*R*)-enantiomers, the latter serving as an internal standard.

In these experiments, the addition of each of the phenylalanines **87** and **89** to the reaction mixture reduced the extent of reaction of the phenylalanine derivative (*S*)-**83** from 50% to <5%. That is, the phenylalanines **87** and **89** bind to acylase I, reducing the observed enzyme activity. It is reasonable to assume that they bind to the enzyme at the active site, hence acting as competitive inhibitors of acylase I. For an inhibitor present at three times the substrate concentration to decrease the enzyme activity by greater than a factor of ten, the dissociation constant of the enzyme-inhibitor complex (K_i) must be at least three times smaller than the K_M of the substrate. Thus, these results show that the phenylalanines **87** and **89** bind more tightly to the enzyme than does the

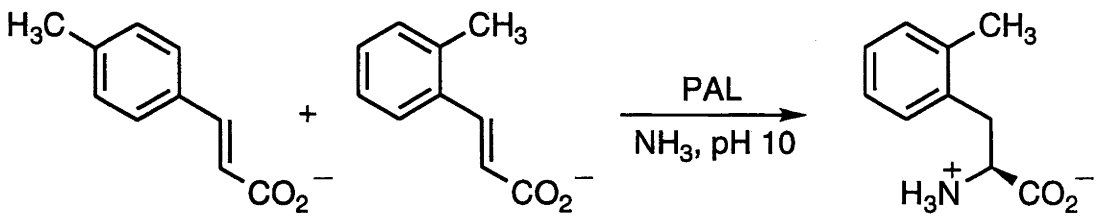
substrate (*S*)-**83**, indicating that a bulky substituent at the *ortho*-position does not adversely affect binding. This suggests that the basis for the selectivity observed on treatment of regioisomeric mixtures of the *ortho*- and *para*-substituted *N*-acetylphenylalanines **84-93** with acylase I is that a bulky substituent at the 2'-position of a substituted *N*-acetylphenylalanine prevents reaction of that species bound to the enzyme.

It is of interest to consider the origins of this selectivity. Acylase I has evolved to digest the derivatives of a wide range of naturally occurring (*S*)-amino acids. This suggests that the principal recognition sites on a substrate of acylase I are near the chiral centre of the amino acid. The natural substrates include derivatives of (*S*)-phenylalanine ((*S*)-**3**) and (*S*)-tyrosine ((*S*)-**42**), suggesting that the enzyme has developed to tolerate *para*-substituted phenylalanine derivatives. It is likely that the corresponding *ortho*-isomers are not processed because natural substrates of this type are not prevalent. As it is envisaged that the major recognition sites on the substrates of acylase I are near the chiral centre of the amino acid, rather than the side chain, this tolerance for substituents at the *para*-position might be explained by the aromatic ring protruding from the active site into free solution. However, the crystal structure of this enzyme has not been solved[170] so this remains conjecture. In any event, acylase I effectively discriminates between *ortho*- and *para*-substituted (*S*)-phenylalanine derivatives.

A comparison of the results presented in this Chapter and those discussed in Chapter III of the Results and Discussion indicates that PAL demonstrates a substrate selectivity which is complementary to that of acylase I. PAL digests the *ortho*-substituted phenylalanines (*S*)-**60** and (*S*)-**62** but not the corresponding *para*-isomers (*S*)-**59** and (*S*)-**61**. As discussed in Chapter IV of the Results and Discussion, under suitable conditions PAL catalyses the conversion of the cinnamate **4** to (*S*)-phenylalanine ((*S*)-**3**). Thus, it was envisaged that PAL might be used to synthesise, enantio- and regio-selectively, the *ortho*-substituted phenylalanines (*S*)-**60** and (*S*)-**62** from mixtures of the corresponding *ortho*- and *para*-substituted cinnamates **74-77**.

To examine this hypothesis, an equimolar mixture of *trans*-4'-methylcinnamate (**74**) and *trans*-2'-methylcinnamate (**75**) (0.150 mol dm^{-3}) was treated with PAL (*ca.* 10^3 units dm^{-3}) at pH 10 in a solution containing ammonia (7.5 mol dm^{-3}) (Scheme 26). These conditions have been identified previously as being ideal for phenylalanine synthesis, although they are detrimental to the activity of the enzyme.[136] They differ from those used in Chapter IV of the Results and Discussion, as monitoring the equilibration catalysed by PAL required enzyme activity to be retained. The reaction mixture was thermostatted at 303 K and monitored for 7 days. Aliquots of the reaction mixture were acidified to *ca.* pH 1 with concentrated hydrochloric acid, which denatured

the enzyme and protonated any residual starting materials **74** and **75**, causing them to precipitate. The suspensions were filtered, and the filtrates were evaporated to dryness *in vacuo* before *tert*-butanol was added. The ^{1}H NMR spectra of these residues were used to determine if any phenylalanine had formed, using the *tert*-butanol added as a standard to determine the yield.



74 **75** (*S*)-**60**

Scheme 26. Synthesis of the substituted phenylalanine (*S*)-**60** from a mixture of the substituted cinnamates **74** and **75** using PAL.

The ^{1}H NMR spectrum of an aliquot of the reaction mixture after 1 day treated as described above includes signals corresponding to aromatic hydrogens and hydrogens of methyl groups attached to an aromatic ring, but no vinylic hydrogens. This, and thin layer chromatography of the reaction mixture, indicated that the phenylalanines (*S*)-**59** and (*S*)-**60** had been formed. Through comparison of the integration of the signals due to the methyl groups of the phenylalanines (*S*)-**59** and (*S*)-**60**, and *tert*-butanol, the reaction was found to proceed to an extent of 27% with the ratio of the phenylalanines (*S*)-**59** and (*S*)-**60** produced being <1 : 20. No change in either the yield or the ratio of the phenylalanines (*S*)-**59** and (*S*)-**60** was observed at longer reaction times, suggesting that the enzyme was denatured under the reaction conditions after 1 day.

This result indicates that (*S*)-2'-methylphenylalanine ((*S*)-**60**) can be produced in >95% regioisomeric purity by treatment of the cinnamates **74** and **75** with PAL, in a solution containing ammonia at pH 10. However, the yield of the process is low and a significant quantity of the enzyme is required, as the reaction conditions are deleterious to enzyme activity. This is consistent with previous reports examining the utility of PAL to synthesise phenylalanines.[131,135-137] Nevertheless, selectivity on digestion of a regioisomeric mixture of cinnamates by PAL is observed.

Thus, acylase I and PAL both exhibit significant substrate selectivity on treatment with mixtures of regioisomers of ring-substituted phenylalanine derivatives. While these systems are inappropriate for use in examining the effect of cyclodextrins on digestion of mixtures of substrates by enzymes, the substrate selectivity of acylase I was shown to be synthetically useful to obtain *para*-substituted (*S*)-phenylalanines from racemic mixtures of the corresponding *ortho*- and *para*-substituted *N*-acetylphenylalanines.

Results and Discussion

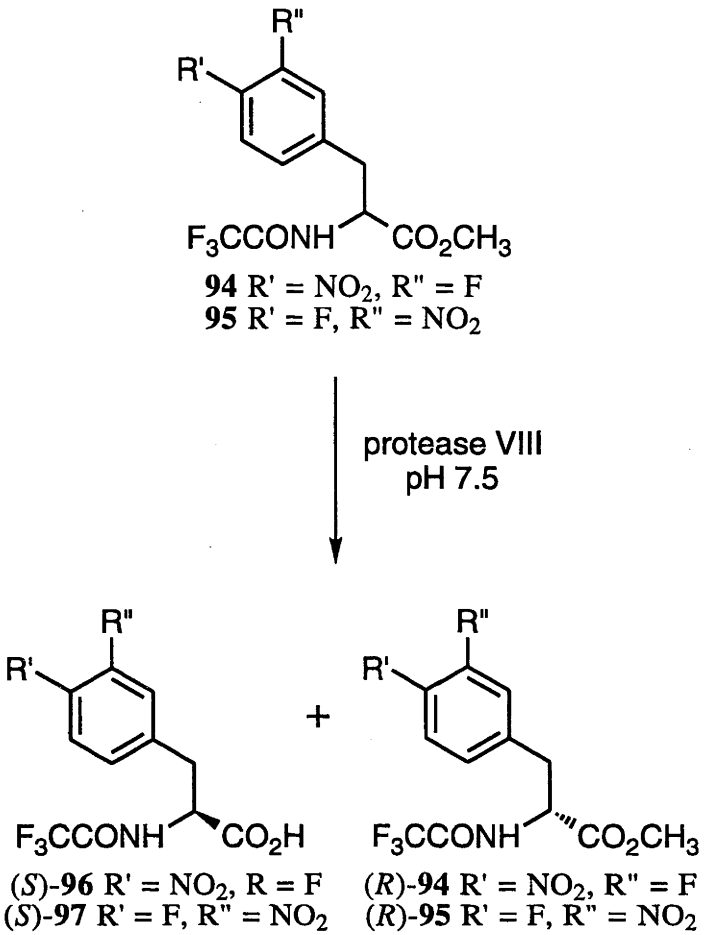
Chapter VI

Effect of Cyclodextrins on Digestion of Ring-Substituted Phenylalanine Derivatives by Protease VIII

As outlined in the previous Chapter, it was envisaged that cyclodextrins might be used to affect the digestion of a mixture of substrates by an enzyme. It was anticipated that they might be used to complex, and hence remove from solution, all but one of the components of a substrate mixture. This might result in the digestion of a mixture of substrates by an enzyme affording only one product and hence increase the utility of the process. The work described in the previous Chapter illustrated that the substrate selectivity exhibited by acylase I on treatment with the phenylalanine derivatives **84-93** made the system unsuitable to study the effects of cyclodextrins on digestion of a mixture of substrates by an enzyme. To carry out such studies, an enzyme which exhibits less selectivity was required.

As discussed in the Introduction, protease VIII has been shown to exhibit broad substrate selectivity, hydrolysing the esters of a range of (*S*)-amino acid derivatives.[102-104] As part of an ongoing project directed towards the synthesis of vancomycin and related phenyl ether structures Zhu and coworkers[104] have reported the enzymatic resolution of ring-substituted nitrophenylalanine derivatives. The enantiospecific hydrolysis of the *N*-trifluoroacetylphenylalanine methyl esters **94** and **95** to the corresponding acids (*S*)-**96** and (*S*)-**97** is catalysed by protease VIII (Scheme 27). That is, phenylalanine derivatives with a nitro group at either the 3'- or 4'-position are digested by the protease VIII. The nitrophenylalanine (*S*)-**94** is hydrolysed approximately four times as fast as its regioisomer (*S*)-**95**.

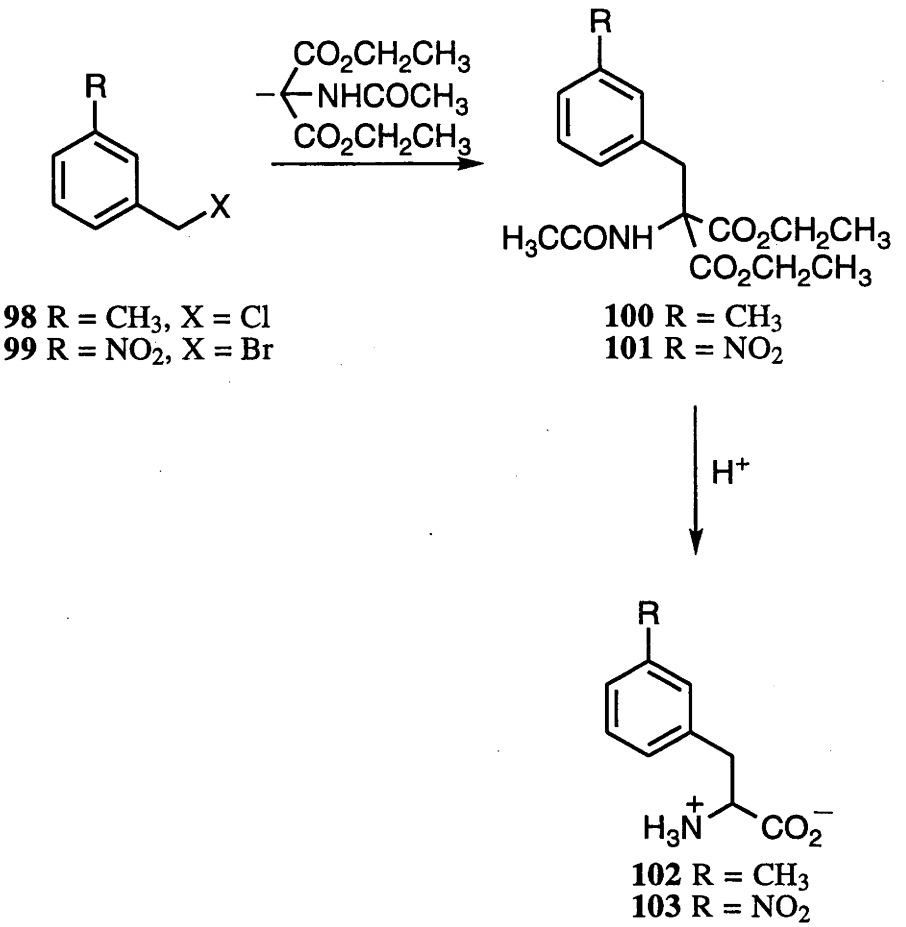
Given that the phenylalanine derivatives **94** and **95** are hydrolysed by protease VIII, it was anticipated that the more easily accessible nitrophenylalanine derivatives **106** and **107** would be also, as replacing a fluorine with a hydrogen, which is of similar size,[167] would be expected not to significantly affect the interaction with the enzyme.



Scheme 27. Enantioselective hydrolysis of the phenylalanine derivatives **94** and **95** by protease VIII.

Further, it was anticipated that the methylphenylalanine derivatives **104** and **105** might also be hydrolysed by protease VIII. Cyclodextrins have been shown to discriminate between the *meta*- and *para*-regioisomers of a range of disubstituted benzenes.[46] Thus, it was anticipated that a cyclodextrin might complex the phenylalanine derivatives **104-107** to differing extents. As a result, it was expected that cyclodextrins might be used to alter the relative rates of hydrolysis of the phenylalanine derivatives **104-107** by protease VIII, through reducing their concentrations free in solution to different degrees.

The phenylalanine derivatives **104-107** were prepared from the phenylalanines **59, 61, 102** and **103**. The nitrophenylalanine **61** is commercially available while the phenylalanines **59, 102** and **103** are not. The methylphenylalanine **59** was prepared as discussed in Chapter III of the Results and Discussion while the phenylalanines **102** and **103** were prepared as illustrated in Scheme 28, through condensation of the benzyl halides **98** and **99** with diethyl acetamidomalonate to give the adducts **100** and **101**

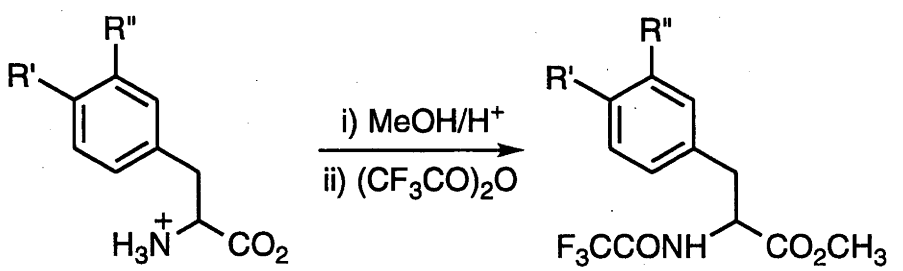


98 R = CH_3, X = Cl
99 R = NO_2, X = Br

100 R = CH_3
101 R = NO_2

102 R = CH_3
103 R = NO_2

Scheme 28. Preparation of the substituted phenylalanines **102** and **103**.



59 R' = CH_3, R" = H
102 R' = H, R" = CH_3
61 R' = NO_2, R" = H
103 R' = H, R" = NO_2

104 R' = CH_3, R" = H
105 R' = H, R" = CH_3
106 R' = NO_2, R" = H
107 R' = H, R" = NO_2

Scheme 29. Preparation of the phenylalanine derivatives **104-107** from the phenylalanines **59**, **61**, **102** and **103**.

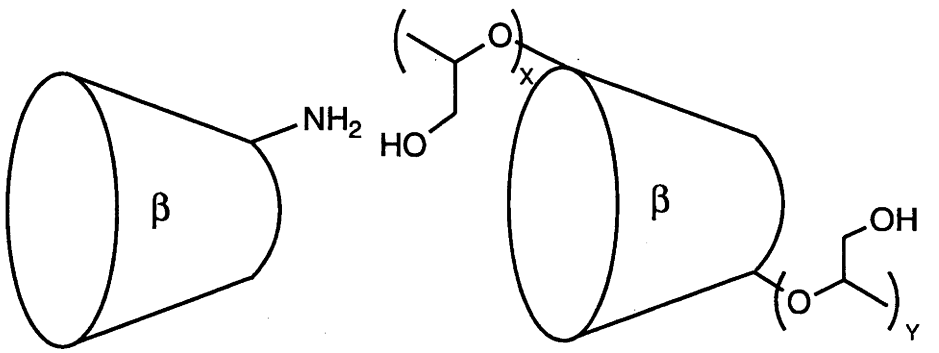
followed by acid catalysed hydrolysis and decarboxylation.[133,171] Each of the adducts **100** and **101** and each of the phenylalanines **102** and **103** was identified by comparison of its observed physical and spectroscopic data with those reported.[133,171]

Treatment of the phenylalanines **59**, **61**, **102** and **103** with thionyl chloride pretreated methanol and then with trifluoroacetic anhydride afforded the phenylalanine derivatives **104-107** (Scheme 29), which were fully characterised. The ^{1}H NMR spectrum of each of the products **104-107** includes a resonance corresponding to the hydrogen nuclei of a methyl ester at *ca.* δ3.8. The presence of a trifluoroacetyl group in the products **104-107** is confirmed by the presence of characteristic peaks in their ^{13}C NMR spectra. Signals corresponding to the quaternary carbon of a trifluoroacetyl group (q, J_F = 288 Hz) at *ca.* δ115 and to the carbonyl carbon of a trifluoroacetyl group (q, J_F = 38 Hz) at *ca.* δ155 are observed in each case.

The phenylalanine derivatives **104-107** are insoluble in water. For the hydrolysis of each of the nitrophenylalanines **94** and **95** by protease VIII reported in the literature,[104] the limited aqueous solubility of the substrates **94** and **95** was overcome through carrying out the reactions in a biphasic system consisting of dichloromethane and pH 7.5 phosphate buffer. Such a system is incompatible with the use of cyclodextrins, as cyclodextrins have been shown to form insoluble complexes with halogenated hydrocarbons.[172] The phenylalanine derivatives **104-107** are soluble in 10% acetonitrile in water. Trial experiments established that protease VIII retains activity in this solvent mixture. Thus, subsequent reactions monitoring the hydrolysis of the phenylalanine derivatives **104-107** by protease VIII were carried out in 10% acetonitrile in pH 7.5 phosphate buffer at 291 K. The buffer was used to ensure the pH of the mixtures did not change as the reactions proceeded. The pH value was chosen as that where the enzyme shows maximum activity.[103,104] The temperature used corresponded to room temperature in a controlled laboratory.

The cyclodextrins used in this study were β-cyclodextrin (**8**), 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**), prepared as discussed in Chapter II of the Results and Discussion, and hydroxypropyl-β-cyclodextrin (**108**). The modified cyclodextrin **108** is commercially available and is the reaction product of β-cyclodextrin (**8**) and propylene oxide. Reaction occurs on both the primary and secondary hydroxyl groups of the cyclodextrin **8** and the average number of hydroxypropyl groups added per cyclodextrin moiety is characteristic of the modified cyclodextrin **108** batch. This degree of substitution has previously been calculated using soft-ionisation mass and NMR spectroscopic techniques.[109,173] In this case, ^{1}H NMR spectroscopy was used to determine the degree of substitution of the batch of hydroxypropyl-β-cyclodextrin (**108**)

used. This involved comparison of the ratio of the integration of the resonance due to the methyl protons (δ1.12, d, J = 8 Hz) to those of the signals corresponding to the acetal protons (δ5-5.3, m) and the remaining protons (δ3.4-4.1, m). This comparison gave an average degree of substitution of 3.65 hydroxypropyl side chains per cyclodextrin moiety for the modified cyclodextrin **108** batch used.



40 **108**

The modified cyclodextrin **108** has an increased aqueous solubility when compared to β-cyclodextrin (**8**).[174,175] The protonated form of the amine **40** has a pK_a of 8.7,[111] so the amine **40** is present in solution predominantly in the protonated form at pH 7.5. This form of the amine **40** has an increased aqueous solubility when compared to the parent cyclodextrin **8**.[111] Thus, the modified cyclodextrins **40** and **108** can be present in solution at greater concentrations than β-cyclodextrin (**8**). Since complexation of a guest by a host is a bimolecular reaction, a higher concentration of the host results in a greater proportion of the guest being complexed. That is, it was envisaged that the cyclodextrins **40** and **108** might have greater effects than the naturally occurring cyclodextrin **8** on the rates of hydrolysis of the phenylalanine derivatives **104-107** by protease VIII, through removing a greater proportion of the phenylalanines **104-107** from solution.

A series of experiments was carried out observing the hydrolysis of each of the phenylalanines **104-107** by protease VIII in the presence of either no cyclodextrin, β-cyclodextrin (**8**) (9.42 x 10^{-3} mol dm^{-3}), 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) (93.5 x 10^{-3} mol dm^{-3}) or hydroxypropyl-β-cyclodextrin (**108**) (86.0 x 10^{-3} mol dm^{-3}). The extent of reaction in each case was monitored through analysis of aliquots of the reaction mixture using HPLC. This involved comparison of the integrations of the signals due to the acid produced and the residual starting material. Hydrolysis of each of the phenylalanines **104-107** by protease VIII ceased at 50% reaction. This is consistent with only the (*S*)-isomer of each of the phenylalanine derivatives **104-107** being hydrolysed, in agreement with literature reports,[102-104] though this was not further

verified. Through trial experiments, the amount of enzyme required in each assay for the extent of reaction to reach *ca.* 40% in a convenient time (4-15 h) was determined and used in the subsequent experiments. The amount of enzyme used in each case was different, so any comparison of the results had to allow for this. As there is an inverse relationship between enzyme concentration and reaction time, comparison of the experiments was made using a plot of the extent of reaction versus the product of enzyme concentration and reaction time. Such a plot is shown as Figure 14, allowing comparison of the rates of hydrolysis of the phenylalanine derivative **104** in the presence of either no cyclodextrin or one of the cyclodextrins **8, 40** and **108**. The equivalent plots for the hydrolysis of the phenylalanine derivatives **105-107** are shown as Figures 15-17.

The information shown in Figures 14-17 indicates that, in the absence of a cyclodextrin, the methylphenylalanine **104** is hydrolysed by protease VIII approximately four times as fast as its regioisomer **105**, while the *para*-substituted phenylalanine derivative **106** is hydrolysed approximately seven times faster than the *meta*-substituted phenylalanine derivative **107**. These results are consistent with those reported,[104] in which the 4'-nitrophenylalanine derivative **94** was digested faster than the 3'-nitrophenylalanine derivative **95**. The addition of β-cyclodextrin (**8**) to the reaction mixtures results in a reduction in the rate of reaction of the phenylalanines **104-107**. This effect is slight for the methylphenylalanines **104** and **105**, while the rates of reaction of the nitrophenylalanines **106** and **107** are reduced to approximately half of those observed in the absence of a cyclodextrin. The addition of either of the modified cyclodextrins **40** or **108** to the reaction mixtures results in a significant decrease in the rate of hydrolysis of each of the phenylalanines **104-107**. The magnitude of this effect ranges from a decrease in the rate of reaction of the nitrophenylalanine **107** of *ca.* 80% on addition of the amine **40** to a reduction of *ca.* 98% in the rate of hydrolysis of the methylphenylalanine **104** on addition of the modified cyclodextrin **108**. For regioisomeric pairs of the phenylalanines **104-107**, the extent of the reduction in the rate of reaction on addition of either of modified cyclodextrins **40** or **108** is greater for the *para*-substituted phenylalanines **104** and **106** than for the corresponding *meta*-substituted phenylalanines **105** and **107**. The largest difference is seen on addition of the modified cyclodextrin **108** to the reaction mixtures containing the methylphenylalanines **104** and **105**, where the *para*-regioisomer **104** is hydrolysed at *ca.* 2% of the rate in the absence of a cyclodextrin while the rate of reaction of the corresponding *meta*-regioisomer **105** is *ca.* 13% of that in the absence of a cyclodextrin. The ratio of the rates of hydrolysis of the phenylalanine derivatives **104** and **105** is *ca.* 1 : 1.5 in reaction mixtures containing the host **108**, compared to *ca.* 4 : 1 in the absence of a cyclodextrin. These results indicate that the modified cyclodextrins **40** and **108** can be used to alter the relative rates of digestion of the phenylalanines **104-107**.

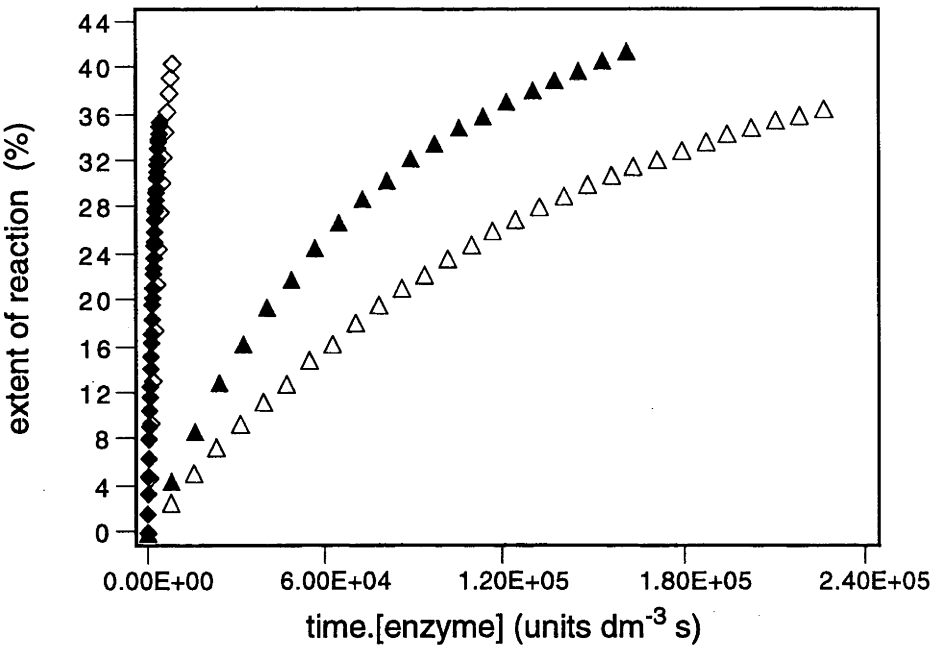


Figure 14. Extent of reaction plotted against the product of reaction time and enzyme concentration for the hydrolysis of *N*-trifluoroacetyl-4'-methylphenylalanine methyl ester (**104**) (1.4 x 10^{-3} mol dm^{-3}) treated with protease VIII in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of either no cyclodextrin (♦), β-cyclodextrin (**8**) (9.42 x 10^{-3} mol dm^{-3}) (◊), 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**) (93.5 x 10^{-3} mol dm^{-3}) (▲) or the modified cyclodextrin **108** (86.0 x 10^{-3} mol dm^{-3}) (△).

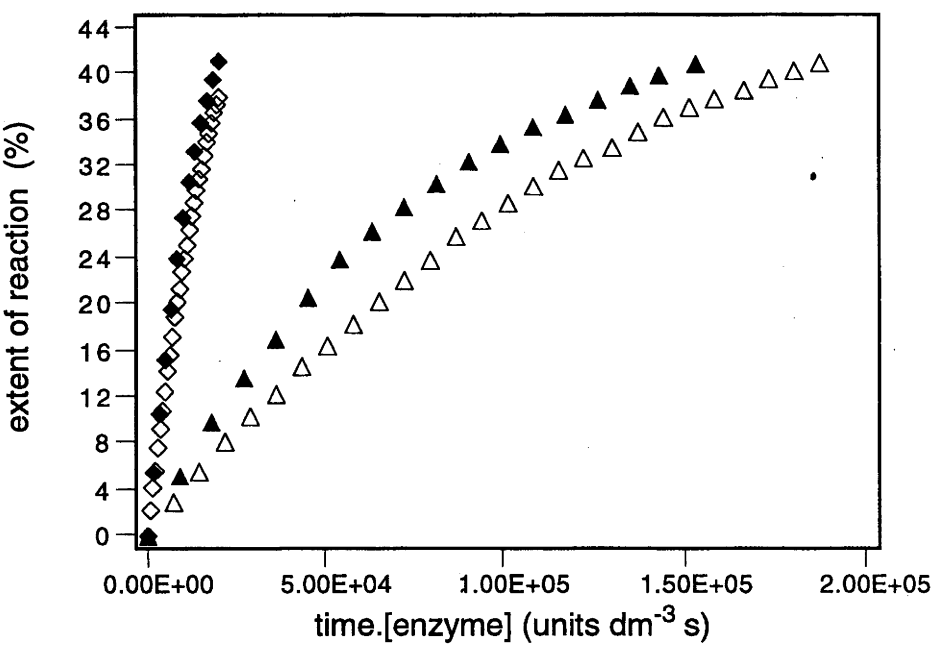


Figure 15. Extent of reaction plotted against the product of reaction time and enzyme concentration for the hydrolysis of *N*-trifluoroacetyl-3'-methylphenylalanine methyl ester (**105**) (1.4 x 10^{-3} mol dm^{-3}) treated with protease VIII in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of either no cyclodextrin (♦), β-cyclodextrin (**8**) (9.42 x 10^{-3} mol dm^{-3}) (◊), 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**) (93.5 x 10^{-3} mol dm^{-3}) (▲) or the modified cyclodextrin **108** (86.0 x 10^{-3} mol dm^{-3}) (△).

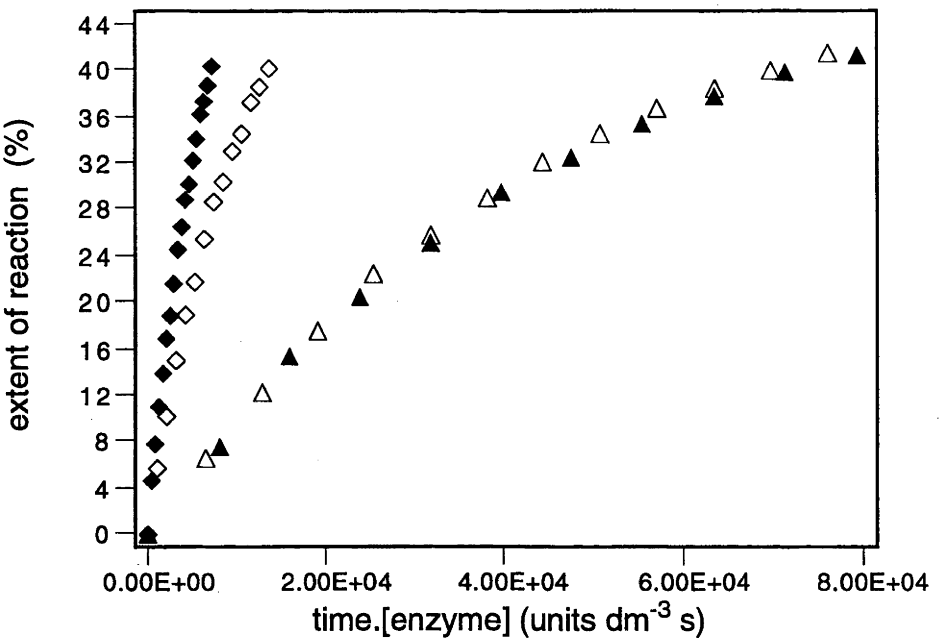


Figure 16. Extent of reaction plotted against the product of reaction time and enzyme concentration for the hydrolysis of *N*-trifluoroacetyl-4'-nitrophenylalanine methyl ester (**106**) (1.4 x 10^{-3} mol dm^{-3}) treated with protease VIII in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of either no cyclodextrin (♦), β-cyclodextrin (**8**) (9.42 x 10^{-3} mol dm^{-3}) (◊), 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**) (93.5 x 10^{-3} mol dm^{-3}) (▲) or the modified cyclodextrin **108** (86.0 x 10^{-3} mol dm^{-3}) (Δ).

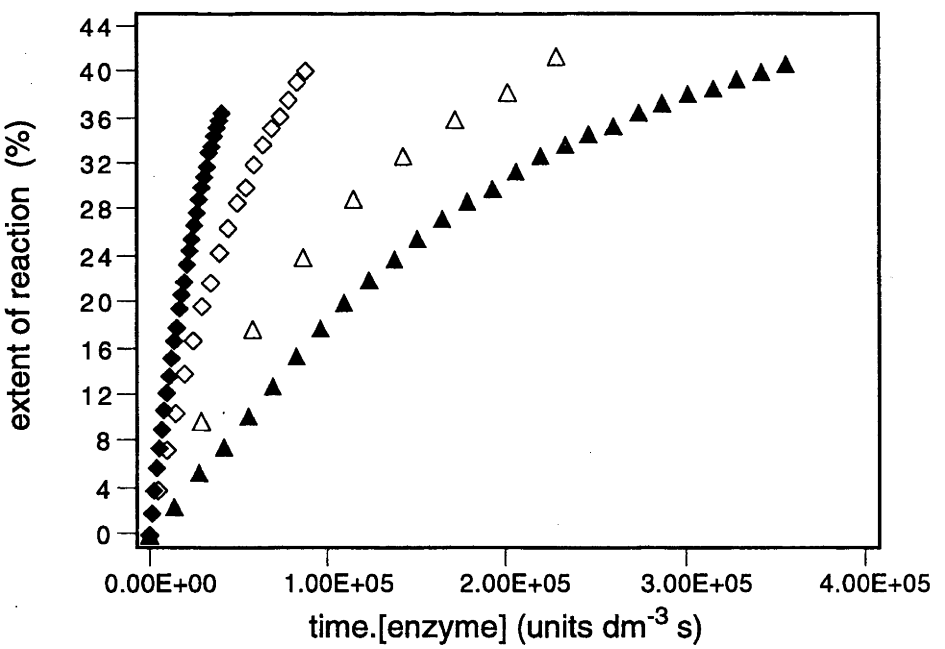


Figure 17. Extent of reaction plotted against the product of reaction time and enzyme concentration for the hydrolysis of *N*-trifluoroacetyl-3'-nitro-phenylalanine methyl ester (**107**) (1.4 x 10^{-3} mol dm^{-3}) treated with protease VIII in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of either no cyclodextrin (♦), β-cyclodextrin (**8**) (9.42 x 10^{-3} mol dm^{-3}) (◊), 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**) (93.5 x 10^{-3} mol dm^{-3}) (▲) or the modified cyclodextrn **108** (86.0 x 10^{-3} mol dm^{-3}) (Δ).

The decrease in the rate of hydrolysis of each of the phenylalanine derivatives **104-107** on addition of β-cyclodextrin (**8**) is consistent with the cyclodextrin **8** complexing the phenylalanines **104-107**, reducing the amount free in solution to be hydrolysed. Similarly, the reduction in the rates of reaction of the phenylalanines **104-107** on addition of the modified cyclodextrins **40** and **108** is consistent with the hosts **40** and **108** complexing the guests **104-107**. Further, the greater effect of the cyclodextrins **40** and **108** suggests that a greater proportion of the phenylalanines **104-107** is complexed in these cases, as is consistent with a larger concentration of a host complexing a greater proportion of a guest.

The effect of the cyclodextrins **40** and **108** to reduce the rates of reaction of the *para*-substituted phenylalanines **104** and **106** to a greater extent than those of the corresponding *meta*-substituted phenylalanines **105** and **107** would be explained by the hosts **40** and **108** complexing, and hence removing from solution, the *para*-substituted phenylalanines **104** and **106** in preference to their *meta*-regioisomers **105** and **107**. To examine this hypothesis, the association constants of the complexes of each of the guests **104** and **105** with the host **108** were quantified. These complexes were chosen as the largest difference in the effect of addition of a host on the rates of reaction of a regioisomeric pair of the phenylalanine derivatives **104-107** is seen on addition of the modified cyclodextrin **108** to the reaction mixtures containing the methylphenylalanines **104** and **105**. Thus, it was envisaged that the difference in the association constants of the complexes of the guests **104** and **105** with the host **108** would be large.

As described in Chapter IV of the Results and Discussion, the change in spectroscopic properties of a guest as a function of host concentration can be used to quantify the association constant of the complex formed. The effects of different concentrations of the modified cyclodextrin **108** on the chemical shifts of resonances in the ^{1}H NMR spectra of the phenylalanine derivatives **104** and **105** were investigated. The temperature used was 298 K, rather than 291 K as used in the studies monitoring the hydrolysis of the methylphenylalanines **104** and **105** by protease VIII, as this is more convenient for NMR. Further, these measurements were carried out in deuterium oxide containing phosphate buffer, rather than the phosphate buffer used in the hydrolysis studies as the resonance due to the hydrogen nuclei of the water overlaps and obscures the resonances of the protons of the guests **104** and **105**, making analysis impractical. Nevertheless the results should be comparable. The difference in chemical shift between the signal due to the aromatic protons and that due to the protons of the methyl group attached to the aromatic ring in the ^{1}H NMR spectrum of the phenylalanine derivative **104** was observed to change as a function of the host **108** concentration (Figure 18). The association constant for the complex formed between the modified cyclodextrin **108**

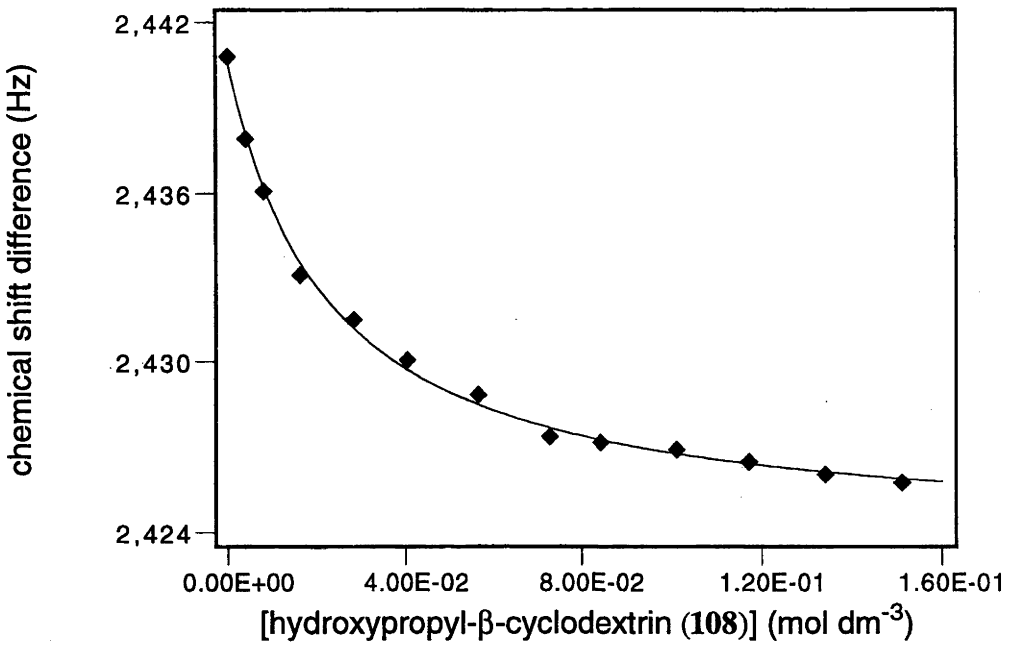


Figure 18. ^{1}H NMR chemical shift difference plotted against the concentration of hydroxypropyl-β-cyclodextrin (**108**) for the difference in chemical shifts between resonances corresponding to the protons of the methyl group attached to the aromatic ring and the aromatic protons of *N*-trifluoroacetyl-4'-methylphenylalanine methyl ester (**104**) in 10% acetonitrile in 0.05 mol dm^{-3} phosphate buffered deuterium oxide at pH 7.5 and 298 K.

and the methylphenylalanine **104** was calculated from this change, using Equations 1 and 2, to be 50 ± 10 mol^{-1} dm^3. Similarly, the difference in chemical shift between the signal due to the 5'-proton and that due to the protons of the methyl group attached to the aromatic ring in the ^{1}H NMR spectrum of the methylphenylalanine **105** was observed to change as a function of the concentration of the host **108** (Figure 19). From this change, the association constant for the complex formed between the host **108** and the guest **105** was calculated to be 90 ± 10 mol^{-1} dm^3, using Equations 1 and 2.

These results indicate that at a given concentration of the cyclodextrin **108**, the proportion of the *meta*-regioisomer **105** complexed would be larger that of the corresponding *para*-substituted phenylalanine **104**. This is the opposite of that expected based on the results illustrated in Figures 14-17. Therefore, the effect of the host **108** on the rates of digestion of the phenylalanines **104** and **105** is not due to the host complexing the *para*-regioisomer **104** in preference to the *meta*-regioisomer **105**. Consequently an alternative possible explanation was considered.

It was envisaged that the effect of the cyclodextrins **40** and **108** on the hydrolysis of the phenylalanine derivatives **104-107** by protease VIII might be due to each of the

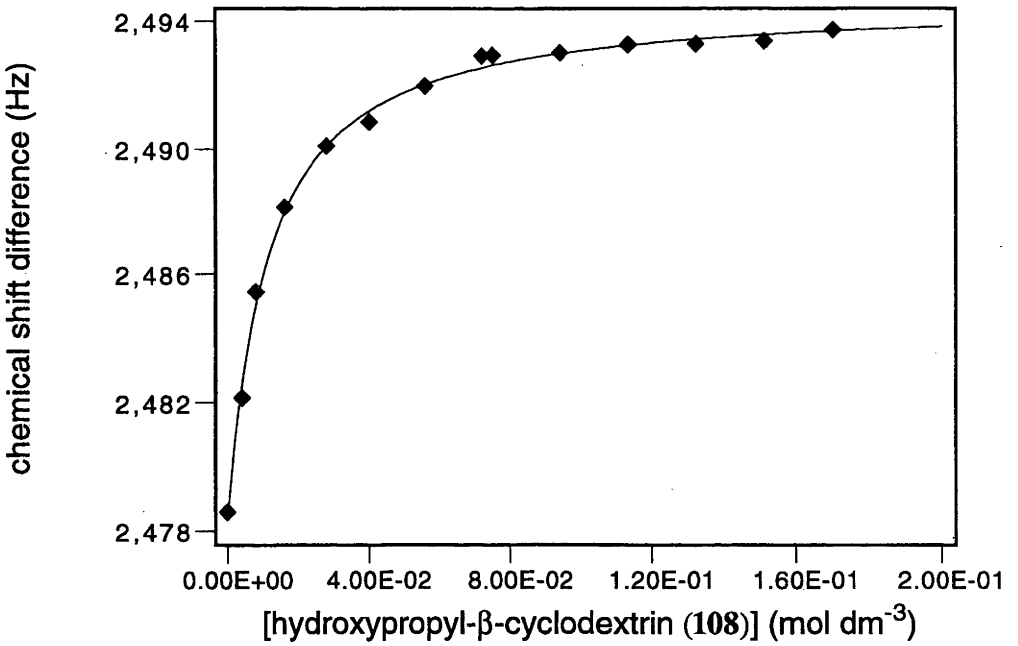


Figure 19. ^{1}H NMR chemical shift difference plotted against the concentration of hydroxypropyl-β-cyclodextrin (**108**) for the difference in chemical shifts between the protons of the methyl group attached to the aromatic ring and the 5'-proton of *N*-trifluoroacetyl-3'-methylphenylalanine methyl ester (**105**) in 10% acetonitrile in 0.05 mol dm^{-3} phosphate buffered deuterium oxide at pH 7.5 and 298 K.

$$v = v_{max} \frac{S}{S + K_M} \tag{5}$$

phenylalanine derivatives **104-107** interacting differently with the enzyme. The rate of an enzyme catalysed reaction (v) is usually related to the rate of reaction of the bound species (v_{max}), the substrate concentration (S) and the dissociation constant of the enzyme-substrate complex (K_M) as shown in Equation 5. Such a system is said to exhibit Michaelis-Menten kinetics.[2] Clearly, if two substrates have different values of K_M, then decreasing their concentrations by the same extent would result in the rate of digestion of the substrate with the larger K_M being reduced to a greater degree. Further, the magnitude of the effect of reducing the substrate concentration on the rate of reaction in such a system depends on the relative values of S and K_M. Thus, it was envisaged that the kinetics of the hydrolysis of the phenylalanines **104-107** by protease VIII might be approximated to Michaelis-Menten kinetics and that the values of K_M for the *para*-isomers **104** and **106** might be greater than for the corresponding *meta*-isomers **105** and **107**. That is, the effect of the cyclodextrins **40** and **108** on hydrolysis of the phenylalanines **104-107** might be to reduce the concentrations of the phenylalanines **104-107** free in solution, with any changes in the relative rates of reaction being due to the phenylalanines **104-107** having different K_M values. To examine this hypothesis, the experiments

monitoring the hydrolysis of the phenylalanines **104-107** by protease VIII in the absence of a cyclodextrin were repeated at concentrations of the substrates **104-107** one-tenth and one-hundredth of those used initially. The results for the hydrolysis of the methylphenylalanines **104** and **105** are summarised in Figure 20 and the results for the reactions of the nitrophenylalanines **106** and **107** are summarised in Figure 21.

In the initial experiments, the ratio of the rates of hydrolysis of the phenylalanine derivatives **104** and **105** by protease VIII was *ca.* 4 : 1. The results illustrated in Figure 20 indicate that this ratio changes to *ca.* 3.5 : 1 on reducing the concentrations of the methylphenylalanines **104** and **105** by a factor of ten and to *ca.* 1.7 : 1 on reducing their concentrations by a factor of one hundred. Similarly, the ratio of the rates of hydrolysis of the nitrophenylalanines **106** and **107** by protease VIII in the initial experiments was *ca.* 7 : 1, which changes to *ca.* 6 : 1 and *ca.* 1.1 : 1 on decreasing their concentrations by factors of ten and one hundred, respectively.

These results indicate that decreasing the concentrations of the phenylalanines **104-107** reduces the rates of hydrolysis of the *para*-isomers **104** and **106** by protease VIII to a greater extent than those of the corresponding *meta*-isomers **105** and **107**. This is consistent with the values of K_M for the *para*-isomers **104** and **106** with protease VIII being greater than the those for the corresponding *meta*-isomers **105** and **107**. Further, since complexation of the phenylalanines **104-107** by a cyclodextrin decreases their concentrations free in solution, it also alters their relative rates of hydrolysis by protease VIII. That is, the change in the relative rates of hydrolysis of the phenylalanines **104-107** by protease VIII on addition of the modified cyclodextrins **40** and **108** is the result of the cyclodextrins **40** and **108** reducing the concentrations of the phenylalanines **104-107** free in solution rather than complexing them to different extents.

It is apparent that cyclodextrins can be used to alter the relative rates of digestion of substrates by an enzyme. However, this effect is due to differences in the interactions of the substrates with the enzyme rather than to discrimination on formation of the complexes of the substrates with cyclodextrins.

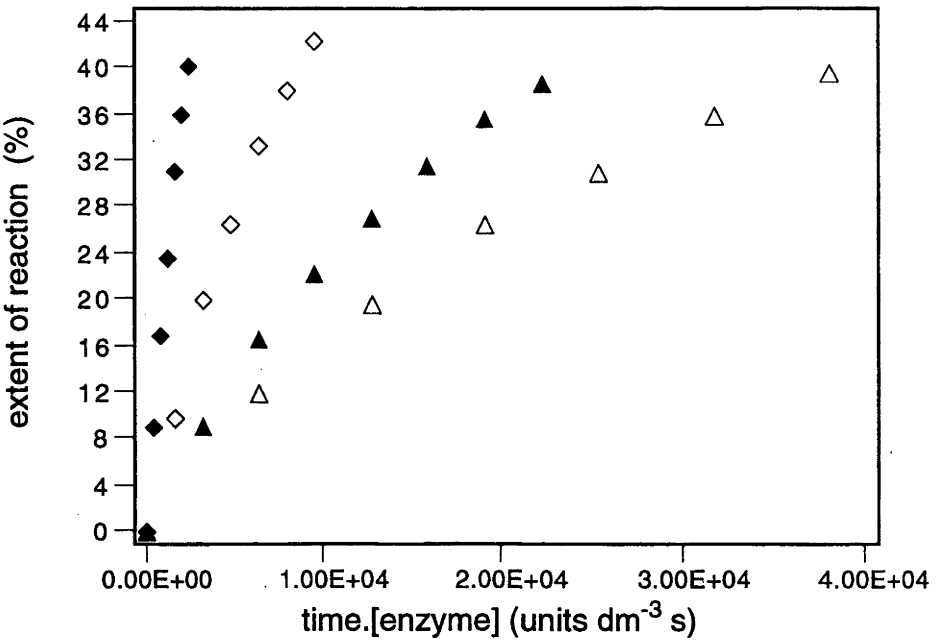


Figure 20. Extent of reaction plotted against the product of reaction time and enzyme concentration for the hydrolysis of each of the phenylalanine derivatives **104** (♦ 0.14 x 10^{-3} mol dm^{-3}, ▲ 14 x 10^{-6} mol dm^{-3}) and **105** (◊ 0.14 x 10^{-3} mol dm^{-3}, Δ 14 x 10^{-6} mol dm^{-3}) by protease VIII in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K.

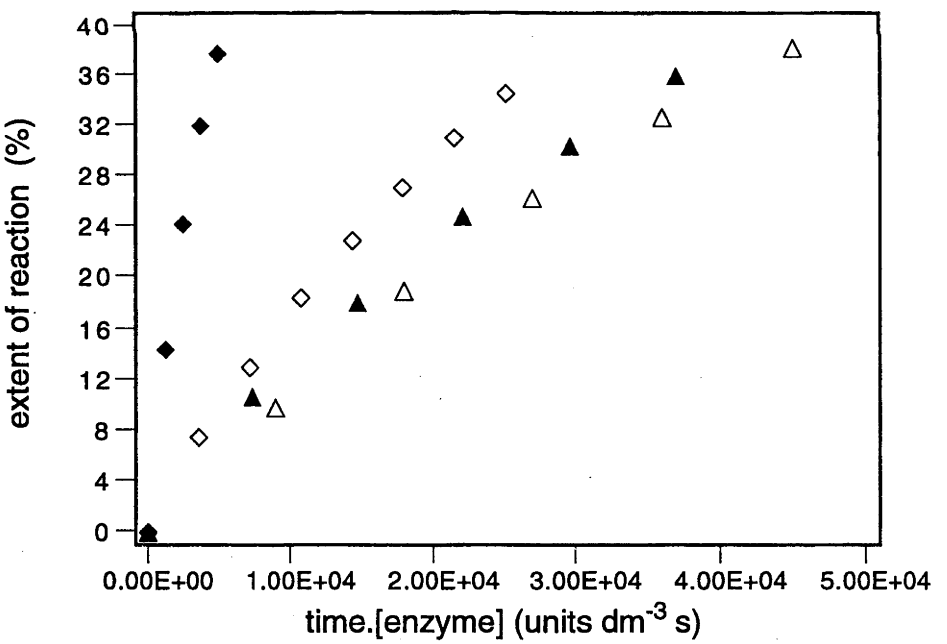


Figure 21. Extent of reaction plotted against the product of reaction time and enzyme concentration for the hydrolysis of each of the phenylalanine derivatives **106** (♦ 0.14 x 10^{-3} mol dm^{-3}, ▲ 14 x 10^{-6} mol dm^{-3}) and **107** (◊ 0.14 x 10^{-3} mol dm^{-3}, Δ 14 x 10^{-6} mol dm^{-3}) by protease VIII in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K.

Results and Discussion

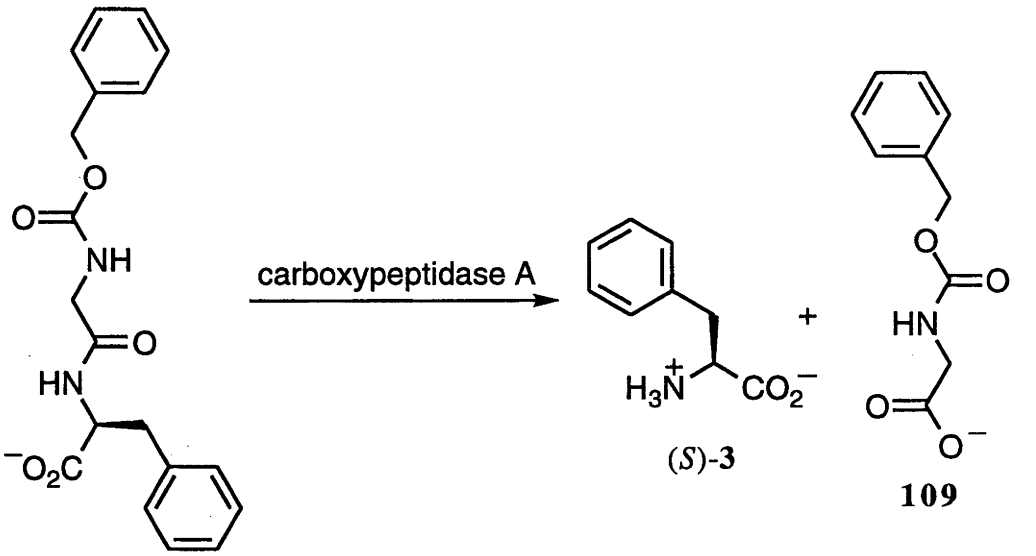
Chapter VII

Effect of Cyclodextrins on Substrate Inhibition of Carboxypeptidase A

The results described in the previous Chapter illustrate that cyclodextrins can act as 'substrate reservoirs', complexing a substrate and hence reducing its concentration free in solution. Further, the work described in Chapter III of the Results and Discussion established that product inhibition of an enzyme could be limited by complexing the inhibitor within a cyclodextrin, reducing the amount free in solution to inhibit the enzyme. It was envisaged that addition of a cyclodextrin might also be used to reduce substrate inhibition of an enzyme by sequestering the substrate from solution, decreasing the proportion of the enzyme present in the less productive ternary complex and hence increasing the rate of reaction. Enzymes that are affected by substrate inhibition are often not practical for use in organic synthesis due to the low concentrations of substrate necessary to retain their activity. The scales required for reactions at these concentrations are frequently too large to be feasible. Thus, it was anticipated that a cyclodextrin might be used to limit product inhibition, retaining enzyme activity at higher concentrations of substrate and increasing the practical utility of the enzyme.

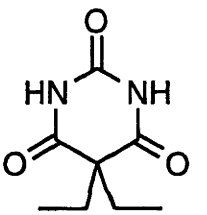
Carboxypeptidase A is affected by substrate inhibition on catalysis of reactions of a range of substrates, including that of the peptide derivative **5** (Scheme 30).[17-19] It was anticipated that a cyclodextrin might complex the peptide derivative **5**, through inclusion of an aromatic moiety of the guest **5** within its annulus, reducing its concentration free in solution and hence limiting substrate inhibition of carboxypeptidase A. This hypothesis was examined in the present work.

Literature reports[17-19] of substrate inhibition of carboxypeptidase A catalysed hydrolysis of the peptide derivative **5** involved experiments carried out in veronal buffer, a solution of the sodium salt of 5,5-diethyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione (**110**). Based on the size and shape of the pyrimidine **110** it was envisaged that it might be included in the annulus of a cyclodextrin. Consequently, it was anticipated that veronal



5 (*S*)-**3** **109**

Scheme 30. Hydrolysis of the dipeptide derivative **5** catalysed by carboxypeptidase A.



110

buffer might interfere with the inclusion of the substrate **5** within a cyclodextrin, so it was not used in these studies. A phosphate buffer was chosen instead as, despite having a minor inhibitory effect on carboxypeptidase A,[17] it was expected that it would not interfere with complexation of the substrate **5** with a cyclodextrin. A pH of 7.5 was used, chosen as that where the enzyme shows maximum activity.[17-19] A previous report[17] indicates that carboxypeptidase A activity increases as the ionic strength of the reaction mixture increases, up to a value of 0.25 mol dm^{-3}. Above this, the activity of the enzyme is independent of ionic strength. In these studies, potassium chloride was added to each of the reaction mixtures to give an ionic strength of 0.5 mol dm^{-3}, ensuring that the change in the ionic strength as the substrate is hydrolysed does not affect enzyme activity.

Reactions involving the hydrolysis of the dipeptide derivative **5** by carboxypeptidase A were carried out at 298 K. They were monitored by acidifying aliquots with dilute hydrochloric acid, which denatured the enzyme and caused the substrate **5** and the *N*-protected glycine **109** to precipitate. The protonated forms of the

substrate **5** and the product **109** are soluble in organic solvents, so they were separated from the other components of the reaction mixtures through extraction of the suspensions with ethyl acetate. Through analysis of these organic extracts using HPLC, the relative amounts of the substrate **5** and the product **109**, and hence the extent of reaction at a given time, were determined. From this information, the rate of reaction (v) of the substrate **5** was calculated.

Initially, the hydrolysis of the dipeptide derivative **5** by carboxypeptidase A (*ca.* 7.5 units dm^{-3}) was examined in the absence of a cyclodextrin at concentrations of the substrate **5** over the range 0.001 - 0.30 mol dm^{-3} to study the effect on enzyme activity. As shown in Figure 22, the rate of hydrolysis of the peptide derivative **5** increases with increasing substrate **5** concentration up to a value of *ca.* 0.06 mol dm^{-3}. Above this, the rate of reaction decreases with increasing substrate **5** concentration to approximately two-thirds of the maximum observed. These results show that the rate of hydrolysis decreases at high concentrations of the substrate **5**, as is consistent with substrate inhibition of carboxypeptidase A. This substrate inhibition is further illustrated in Figure 23, which is a plot of the form derived by Lineweaver and Burk,[113] with the reciprocal of the substrate **5** concentration used as the ordinate axis and the reciprocal of the rate of reaction used as the coordinate axis. The notable deviation from linearity of the plot near the coordinate axis is consistent with substrate inhibition.[176]

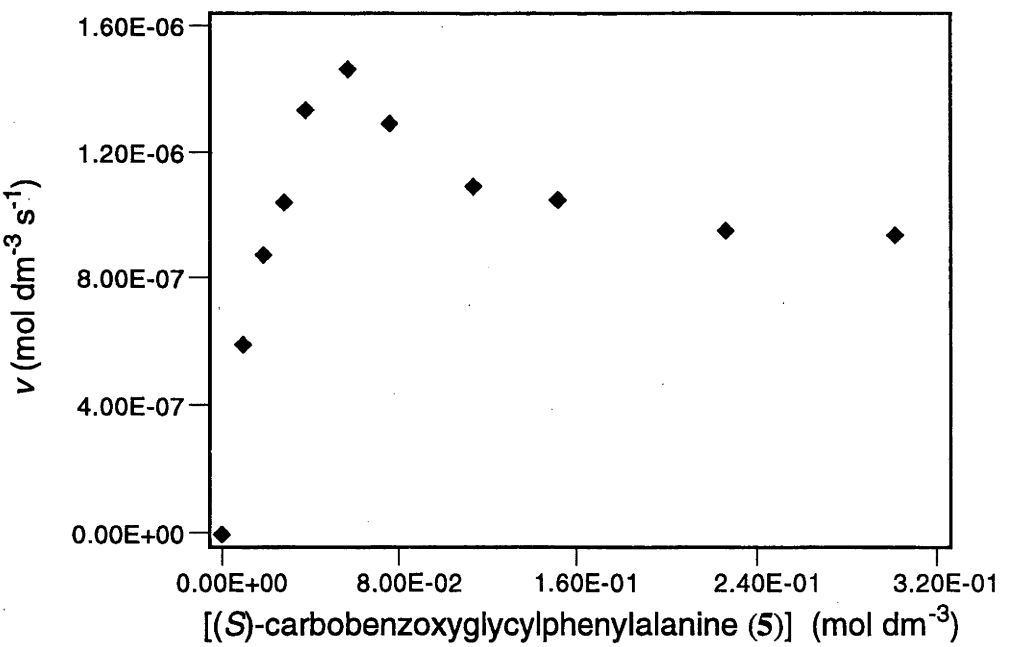


Figure 22. Rate of reaction plotted against substrate concentration for the hydrolysis of carbobenzoxyglycylphenylalanine (**5**) by carboxypeptidase A (*ca.* 7.5 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 phosphate buffer (I = 0.5 mol dm^{-3}) at 298 K.

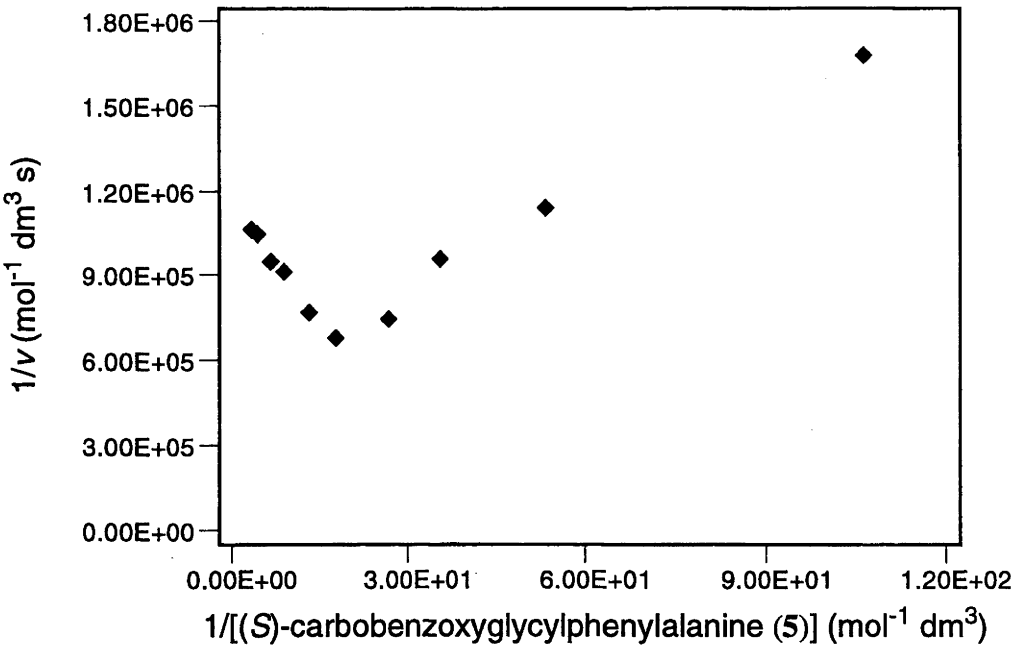
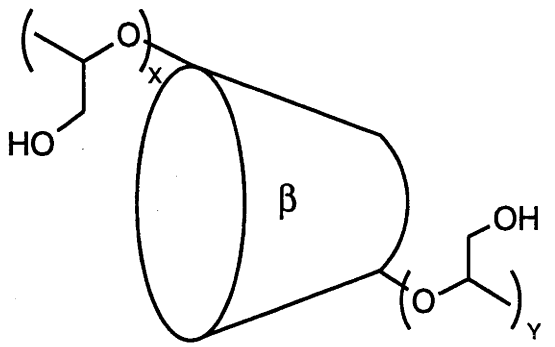


Figure 23. Lineweaver-Burk plot for the hydrolysis of the peptide derivative **5** by carboxypeptidase A (*ca.* 7.5 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 phosphate buffer (I = 0.5 mol dm^{-3}) at 298 K.

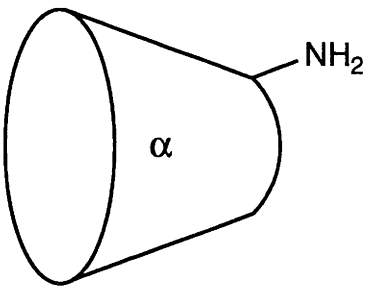
At concentrations of the substrate **5** greater than *ca.* 0.06 mol dm^{-3} substrate inhibition of carboxypeptidase A is observed. The use of β-cyclodextrin (**8**) to limit substrate inhibition is impractical in this case as, due to limited solubility, it can be present in aqueous solution at a maximum concentration of *ca.* 0.016 mol dm^{-3}.[97] Therefore, the maximum reduction in substrate **5** concentration on addition of the cyclodextrin **8** is 0.016 mol dm^{-3}. This would clearly have a negligible effect on substrate inhibition of carboxypeptidase A in the hydrolysis of the peptide **5**.

Hydroxypropyl-β-cyclodextrin (**108**), as used in the work discussed in Chapter VI of the Results and Discussion, has an increased aqueous solubility when compared to β-cyclodextrin (**8**).[174,175] The protonated form of 6^A-amino-6^A-deoxy-α-cyclodextrin (**111**) has a pK_a of 8.7,[111] so the amine **111** would be present in solution predominantly in the protonated form at pH 7.5. This form of the amine **111** also has an increased aqueous solubility when compared to β-cyclodextrin (**8**).[111] Thus, both the amine **111**, available in the laboratory,[120] and the modified cyclodextrin **108** can be present in solution at a greater concentration than β-cyclodextrin (**8**), so the maximum amount of a guest they can remove from solution is larger. Since complexation of a guest by a host is a bimolecular reaction, a higher concentration of the host results in a greater proportion of the guest being complexed. Where the guest is an inhibitor of an enzyme, it was expected that a larger amount of the host would lead to less of the inhibitor free in solution and

hence a greater effect of the host to limit inhibition. Thus, it was anticipated that the modified cyclodextrins **108** and **111** would be more effective than β-cyclodextrin (**8**) to limit substrate inhibition of carboxypeptidase A through removing a greater proportion of the substrate **5** from solution.



108



111

The effect of each of the cyclodextrins **108** and **111** on substrate inhibition of carboxypeptidase A in the hydrolysis of the dipeptide derivative **5** was examined by repeating the previous experiments in the presence of the modified cyclodextrin **108** (0.186 mol dm^{-3}) and the amine **111** (0.221 mol dm^{-3}). Since the amine **111** is present mainly in the protonated form at pH 7.5, the ionic strength of the reaction mixtures containing the amine **111** was *ca.* 0.9 mol dm^{-3}, compared to 0.5 mol dm^{-3} in the other cases. Based on previous studies,[17] it is reasonable to assume that this change will have a negligible effect on carboxypeptidase A activity.

The results of the experiments monitoring the hydrolysis of the dipeptide derivative **5** by carboxypeptidase A in the presence of the cyclodextrins **108** and **111** are illustrated in Figure 24 with comparison to the case with no cyclodextrin present. They indicate that carboxypeptidase A retains activity in the presence of the modified cyclodextrins **108** and **111**. At the lower concentrations of the substrate **5** used, addition of each of the modified cyclodextrins **108** and **111** reduces the rate of reaction, markedly in the case of the amine **111**. At the higher concentrations of the peptide derivative **5** used, substrate inhibition of carboxypeptidase A is observed in each case though the rate of reaction is greater in the presence of the cyclodextrins **108** and **111** than in the absence of a cyclodextrin. Further, the concentration of the substrate **5** at which the maximum rate of reaction occurs increases on the addition of each of the modified cyclodextrins **108** and **111**.

These results are consistent with the cyclodextrins **108** and **111** complexing the substrate **5** and reducing its concentration free in solution. At the lower concentrations of the substrate **5** used, this results in less of the Michaelis complex being formed and hence a decrease in the observed rate of hydrolysis. At the higher concentrations of the

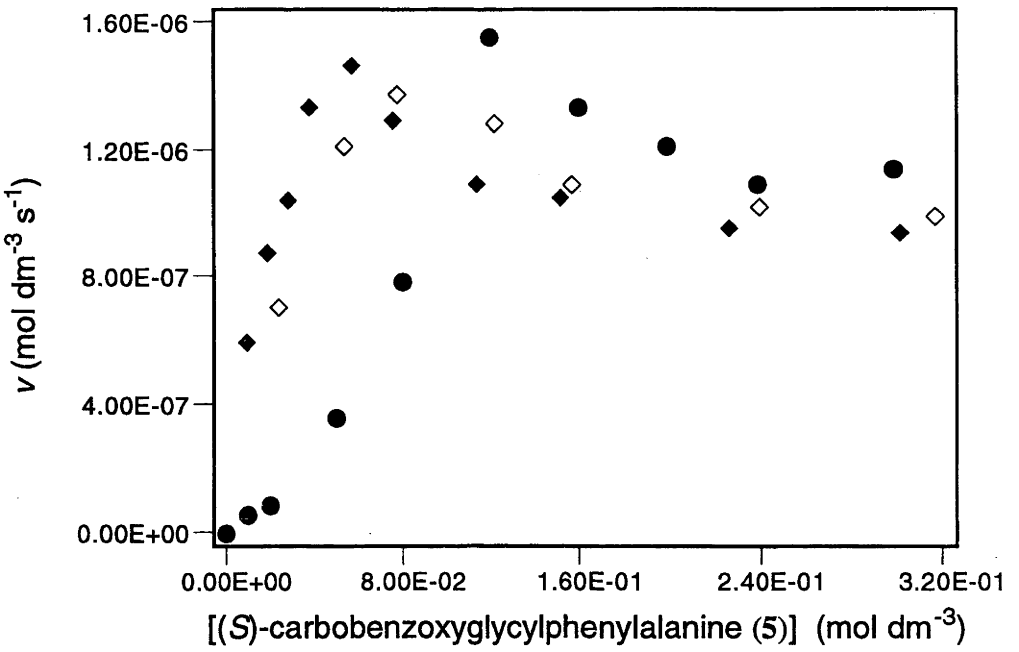


Figure 24. Rate of reaction plotted against substrate concentration for the hydrolysis of (*S*)-carbobenzoxyglycylphenylalanine (**5**) by carboxypeptidase A (*ca.* 7.5 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 298 K in the presence of either no cyclodextrin (I = 0.5 mol dm^{-3}) (♦), hydroxypropyl-β-cyclodextrin (**108**) (0.186 mol dm^{-3}, I = 0.5 mol dm^{-3}) (◊) or the amine (**111**) (0.221 mol dm^{-3}, I = 0.9 mol dm^{-3}) (●).

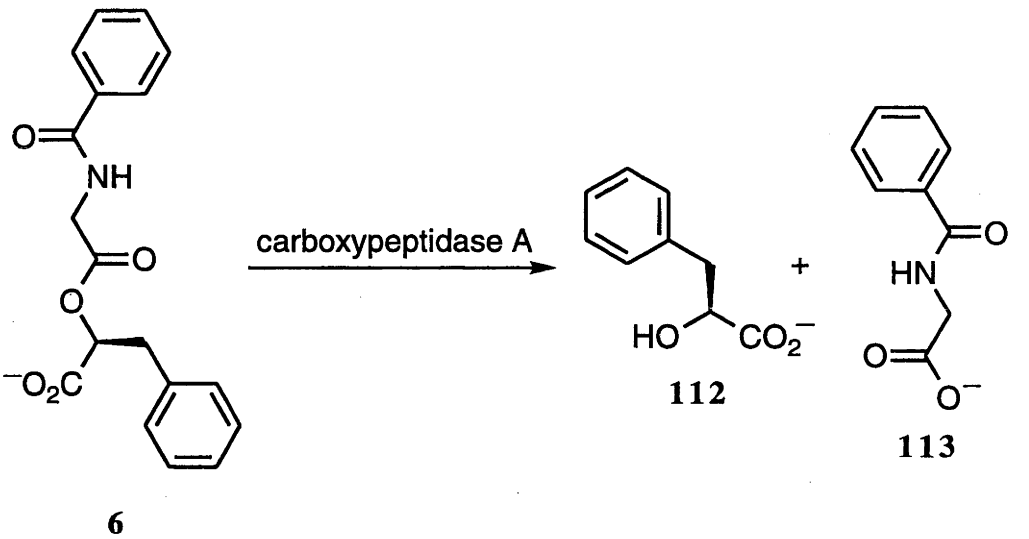
substrate **5** used, decreasing its concentration free in solution results in an increase in the proportion of the Michaelis complex present in solution and hence an increase in the rate of reaction of the substrate **5**. That is, the cyclodextrins **108** and **111** limit substrate inhibition and increase the concentration at which the maximum rate of reaction occurs in the carboxypeptidase A catalysed hydrolysis of the peptide derivative **5**, through sequestering the substrate **5** and reducing its concentration free in solution.

It was anticipated that cyclodextrins would have a greater effect on substrate inhibition of an enzyme which occurs at lower concentrations of substrate than is the case for the hydrolysis of the peptide derivative **5** by carboxypeptidase A. In such a system, it was expected that less substrate would have to be removed from solution by a cyclodextrin for it to have a significant effect on enzyme activity.

Experiments reported by Bunting[22-26,177,178] and others[18-21] on the hydrolysis of (*S*)-lactate derivatives of *N*-benzoylglycine by carboxypeptidase A have shown that substrate inhibition of the enzyme occurs at much lower substrate concentrations than observed for the hydrolysis of the peptide derivative **5**. While dependent on the side chain of the lactate portion of the molecule, the maximum rates of reaction were observed at substrate concentrations of *ca.* 10^{-3} mol dm^{-3}, with only negligible activity being

observed above 10 x 10^{-3} mol dm^{-3}. Thus, it was anticipated that the hydrolysis of an *N*-benzoylglycyllactate by carboxypeptidase A would be more suitable to examine the effect of cyclodextrins on substrate inhibition.

The lactate chosen for study was (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**), which is hydrolysed by carboxypeptidase A as shown in Scheme 31. It was anticipated that a cyclodextrin would complex the lactate **6** reducing its concentration free in solution, hence limiting substrate inhibition of carboxypeptidase A.



6 **112** **113**

Scheme 31. Hydrolysis of the lactate derivative **6** catalysed by carboxypeptidase A.

It should be noted that the racemate of compound **6**, which is commercially available, was used in the preparation of all solutions in the experiments monitoring the hydrolysis of the substrate **6** by carboxypeptidase A. Previous studies have shown that (*R*)-*N*-benzoylglycyl-β-phenyllactate is not hydrolysed by the enzyme and does not affect digestion of the substrate **6**.[18-22,24,177] Thus, it is assumed that any hydrolysis of the racemate of compound **6** observed is due to digestion of the (*S*)-lactate **6**, though this was not independently verified.

The hydrolysis of the lactate **6** by carboxypeptidase A (*ca.* 20 units dm^{-3}) was examined in the absence of a cyclodextrin at concentrations of the substrate **6** over the range (4 x 10^{-6}) - (2.5 x 10^{-3}) mol dm^{-3} to examine the effect of the concentration of the lactate **6** on enzyme activity. Reaction conditions were otherwise identical to the those used for the assays monitoring the hydrolysis of the dipeptide derivative **5** by carboxypeptidase A and reaction mixtures were analysed in a similar fashion. The results of these experiments are illustrated in Figures 25 and 26.

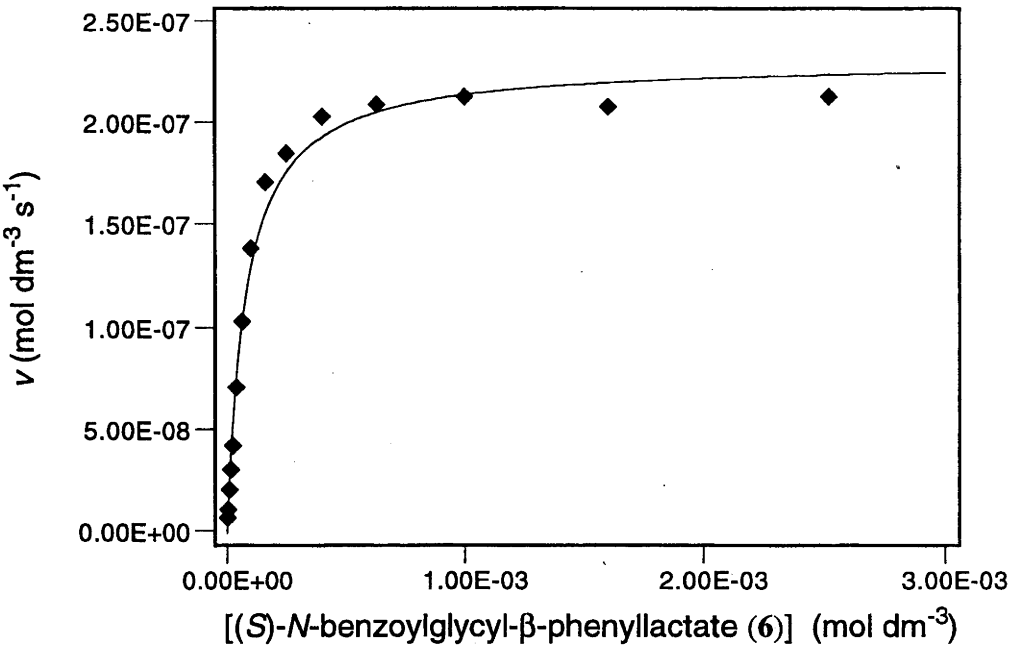


Figure 25. Rate of reaction plotted against substrate concentration for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 phosphate buffer (I = 0.5 mol dm^{-3}) at 298 K.

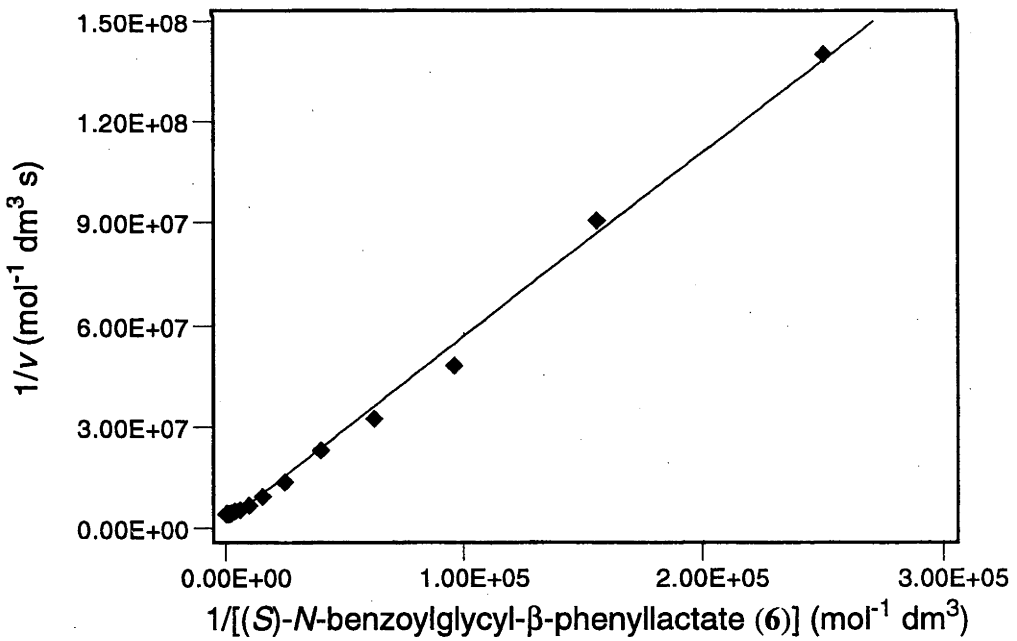


Figure 26. Lineweaver-Burk plot for the hydrolysis of the phenyllactate **6** by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 phosphate buffer (I = 0.5 mol dm^{-3}) at 298 K.

It is clear from Figure 25 that there is no substrate inhibition in the hydrolysis of the lactate **6** by carboxypeptidase A, as enzyme activity does not decrease at high concentrations of the substrate **6**. Further, the plot of the reciprocal of the rate of reaction against the reciprocal of substrate concentration (Figure 26) does not show the deviation from linearity near the coordinate axis indicative of substrate inhibition.[176] The reaction can be described by Michaelis-Menten kinetics[2] under these conditions and the data can be fitted to the Michaelis-Menten equation (Equation 5). This equation relates the observed rate of reaction, v, to the rate of the reaction of the bound species, v_{max}, the dissociation constant of the Michaelis complex, K_M, and the substrate concentration, S. Using it, values of v_{max} and K_M for the complex of carboxypeptidase A with the lactate **6** were calculated as (0.230 ± 0.005) x 10^{-6} mol dm^{-3} s^{-1} and (0.77 ± 0.07) x 10^{-6} mol dm^{-3}, respectively.

$$v = v_{max} \frac{S}{S + K_M} \qquad (5)$$

The lactate **6** does not inhibit carboxypeptidase A under the conditions used in these experiments. The only difference between the experimental conditions reported in the literature and those used for these experiments is the use of phosphate salts in the buffer. Experiments described in the literature used either no buffer, maintaining the pH and analysing the rate of reaction by titration of the acid produced,[22,24,177] or Tris buffer.[18-21] In order to identify conditions under which substrate inhibition of carboxypeptidase A occurs, so the effect of cyclodextrins could be examined, assays monitoring hydrolysis of the substrate **6** by carboxypeptidase A at varying concentrations of the substrate **6** ((4 x 10^{-6}) - (2.5 x 10^{-3}) mol dm^{-3}) were carried out in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K. The reaction mixtures were monitored by observing the decrease in absorbance at a wavelength in the range 260-285 nm. This method has been used previously[18-21] and allows continual monitoring of the reaction progress. Hydrolysis of the ester **6** results in a decrease in absorbance at 260-285 nm, the absorbance change being greater at 260 nm than at 285 nm. At the higher concentrations of the lactate **6** used, the absorbance of the reaction mixtures at 260 nm is large, with less than 0.01% of the light transmitted, so any changes in absorbance at this wavelength cannot be measured accurately. As a result, the reaction mixtures containing the substrate **6** at the higher concentrations used were monitored at the longer wavelengths. Each reaction was monitored until no further change in absorbance was observed and the initial rate of reaction was calculated using the total absorbance change and the initial rate of change in absorbance.

The results of the experiments monitoring the hydrolysis of the lactate **6** by carboxypeptidase A in Tris buffer with no cyclodextrin present are illustrated in Figure 27, indicating that under these conditions the enzyme is affected by substrate inhibition. The rate of reaction increases as the concentration of the substrate **6** increases, up to a value of *ca.* 0.4 x 10^{-3} mol dm^{-3}. Above this, the rate of reaction decreases as the substrate **6** concentration increases. The rate of hydrolysis of the lactate **6** is negligible at concentrations higher than 10 x 10^{-3} mol dm^{-3}. These effects can also be observed in Figure 28, where the ordinate axis is the logarithm of the concentration of the substrate **6**. The curves illustrated in Figures 27 and 28 are based on an expression derived by Murphy and Bunting[24] for the rate of digestion of a substrate by an enzyme which is affected by substrate inhibition. This expression can be written in the form shown as Equation 6, where v is the rate of reaction, S is the substrate concentration and a-d are constants related to the kinetic parameters of the reaction.

$$v = \frac{aS + \frac{bS^2}{c}}{d + S + \frac{S^2}{c}} \qquad (6)$$

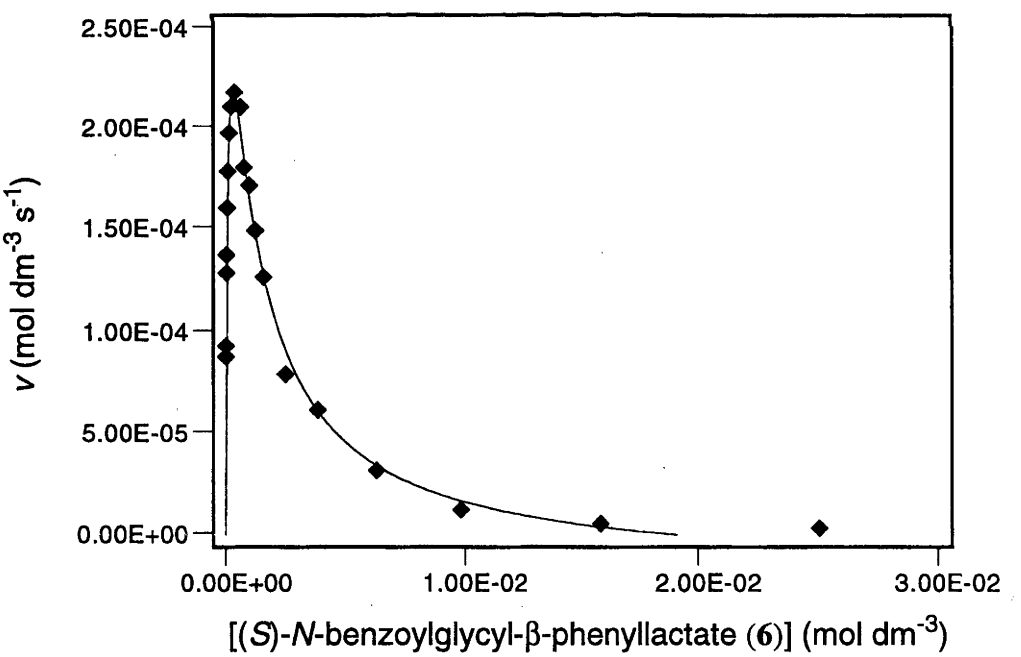


Figure 27. Rate of reaction plotted against substrate concentration for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K.

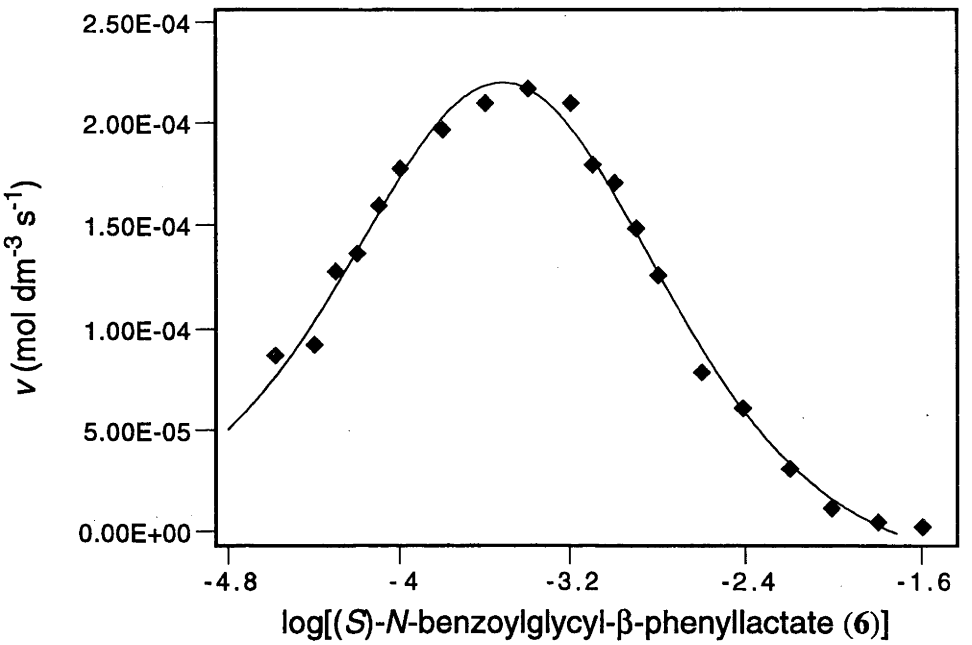


Figure 28. Rate of reaction plotted against the logarithm of the substrate concentration for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K.

While carboxypeptidase A is affected by substrate inhibition on hydrolysis of the lactate **6** under these conditions, approximate values of v_{max} and K_M for the Michaelis complex can still be calculated, allowing comparison with the results of the experiments carried out in phosphate buffer. At low concentrations of the substrate **6**, where a negligible amount of the enzyme is present as the ternary complex, the system can be described by Michaelis-Menten kinetics. Thus, a Lineweaver-Burk[113] plot (Figure 29) can be used to calculate approximate values of v_{max} and K_M for the Michaelis complex by fitting the data that corresponds to low concentrations of the substrate **6** to Equation 4. Through this method, approximate values of v_{max} and K_M for the Michaelis complex were calculated to be 0.27 x 10^{-3} mol dm^{-3} s^{-1} and 60 x 10^{-6} mol dm^{-3}, respectively. The latter is intermediate to the reported values of 52.8 x 10^{-6} mol dm^{-3} [20] and 88 x 10^{-6} mol dm^{-3}.[19,21]

$$\frac{1}{v} = \frac{1}{v_{max}} + \frac{K_M}{v_{max}} \cdot \frac{1}{S} \qquad (4)$$

The values of v_{max} and K_M for the Michaelis complex formed between the lactate **6** and carboxypeptidase A in Tris buffer are *ca.* 0.27 x 10^{-3} mol dm^{-3} s^{-1} and *ca.* 60 x 10^{-6} mol dm^{-3}, respectively, compared to *ca.* 0.230 x 10^{-6} mol dm^{-3} s^{-1} and *ca.* 0.77 x 10^{-6} mol dm^{-3}, respectively, for the equivalent complex in phosphate buffer under otherwise identical conditions. That is, the Michaelis complex formed between the lactate **6** and carboxypeptidase A is more thermodynamically stable in phosphate buffer,

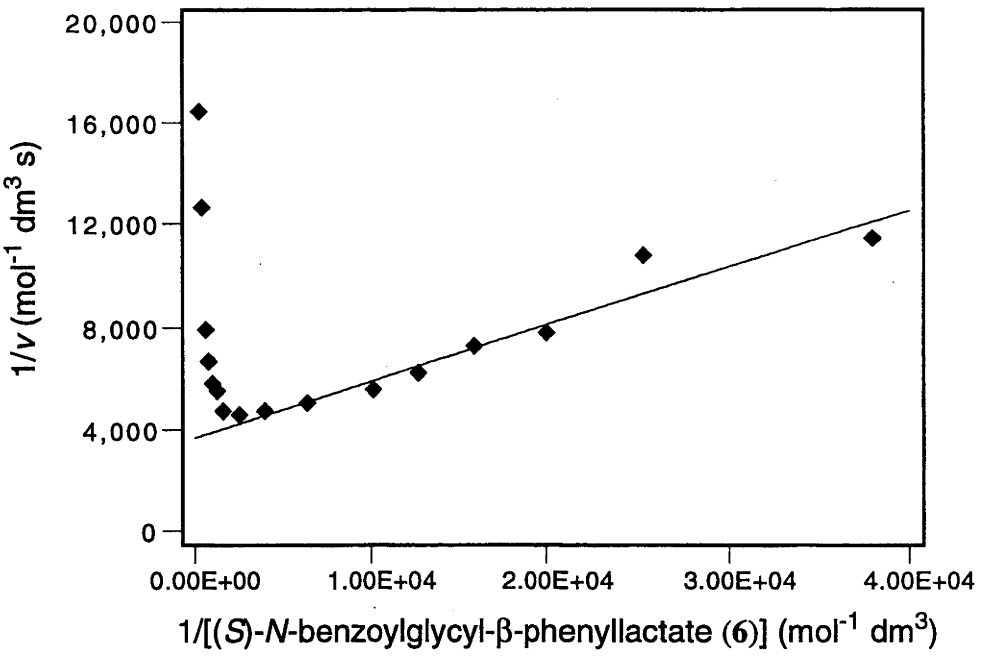


Figure 29. Lineweaver-Burk plot for the hydrolysis of the lactate **6** by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K.

but the rate of reaction of the complexed lactate **6** is three orders of magnitude greater in Tris buffer. The value of v_{max}/K_M, a measure of the catalytic efficiency of the enzyme, is *ca.* 0.30 mol^{-1} dm^3 s^{-1} in phosphate buffer and 4.5 mol^{-1} dm^3 s^{-1} in Tris buffer. That is, the efficiency of carboxypeptidase A catalysed hydrolysis of the lactate **6** is greater in Tris buffer than in phosphate buffer under conditions where substrate inhibition does not affect enzyme activity.

Since conditions had been established under which substrate inhibition of carboxypeptidase A was observed at low concentrations of the substrate **6**, the system was considered suitable to examine the effects of cyclodextrins. It was anticipated that β-cyclodextrin (**8**), despite its limited solubility, would be able to remove sufficient substrate **6** to affect the activity of the enzyme. Consequently, the experiments monitoring the hydrolysis of the hippuric acid derivative **6** catalysed by carboxypeptidase A in Tris buffer over a range of concentrations of the substrate **6** were repeated in the presence of β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}) under otherwise identical conditions.

The effect of β-cyclodextrin (**8**) on the hydrolysis of the substrate **6** by carboxypeptidase A in Tris buffer is shown in Figures 30 and 31. Carboxypeptidase A is seen to retain activity in the presence of β-cyclodextrin (**8**). At the lower concentrations

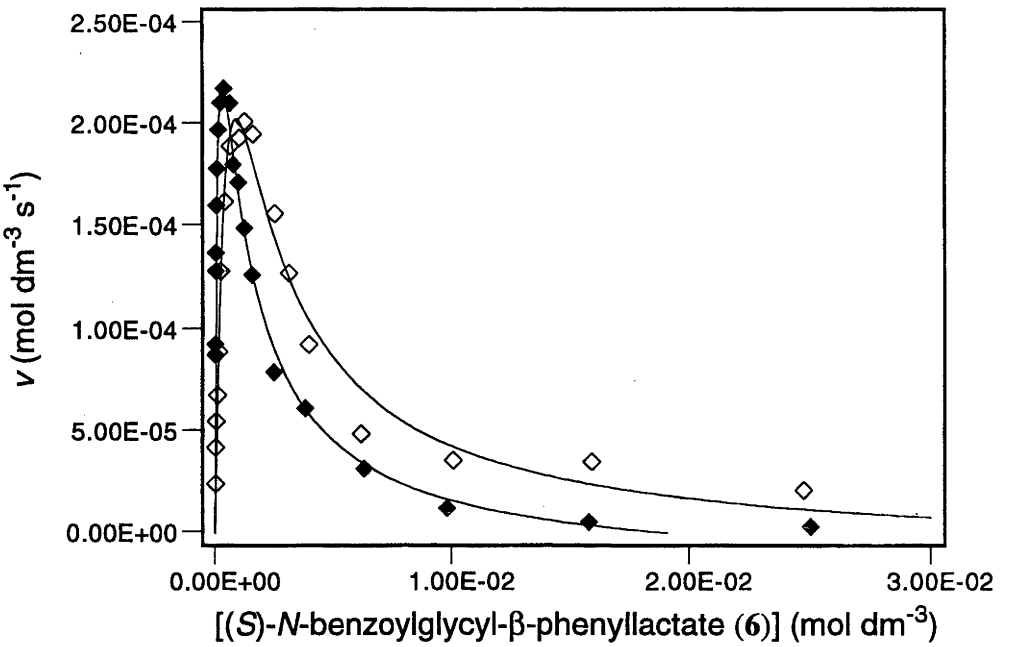


Figure 30. Rate of reaction plotted against substrate concentration for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of either no cyclodextrin (♦) or β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}) (◊).

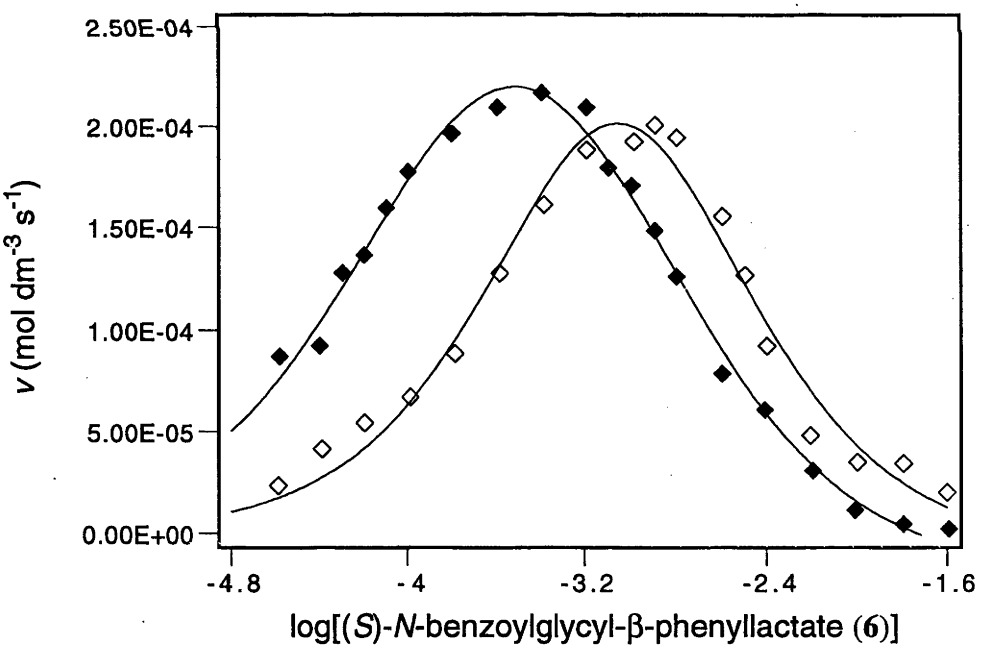


Figure 31. Rate of reaction plotted against the logarithm of the substrate concentration for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of either no cyclodextrin (♦) or β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}) (◊).

of the substrate **6**, the rate of reaction in the presence of β-cyclodextrin (**8**) increases with increasing concentration of the substrate **6** and is slower than in the absence of a cyclodextrin. At the higher concentrations of the substrate **6**, the rate of reaction in the presence of β-cyclodextrin (**8**) decreases with increasing concentration of the substrate **6** and is faster than in the absence of a cyclodextrin.

These results are consistent with the host **8** complexing the lactate **6** and reducing its concentration free in solution. At the lower concentrations of the substrate **6**, where the principal enzyme-substrate complex is the Michaelis complex, reducing the concentration of the free lactate **6** causes a decrease in the concentration of the Michaelis complex and a decrease in the rate of reaction. At the higher concentrations of the substrate **6**, reducing the free substrate **6** concentration results in the proportion of the enzyme present as the less productive ternary complex being reduced. The result is that more of the Michaelis complex is formed and, consequently, the rate of reaction increases on addition of the cyclodextrin **8**.

The maximum rate of hydrolysis of the lactate **6** by carboxypeptidase A in the presence of the cyclodextrin **8** is slightly less than that in the absence of a cyclodextrin. While this might be the result of a slight decrease in enzyme activity on addition of the cyclodextrin **8**, it is more likely that the enzyme solution used to prepare the reaction mixtures in this case had slightly less activity. Given that carboxypeptidase A is commercially available as a suspension, some error is to be expected in the preparation of the reaction mixtures. Irrespective of this, the effect of the cyclodextrin **8** is marked. On addition of the cyclodextrin **8** to the reaction mixtures, the concentration of the substrate **6** at which the maximum rate of reaction occurs increases from *ca.* 0.40×10^{-3} mol dm^{-3} to *ca.* 1.4×10^{-3} mol dm^{-3}.

Given the effect of β-cyclodextrin (**8**) on substrate inhibition of the hydrolysis of the lactate **6** by carboxypeptidase A, it was envisaged that with more of a cyclodextrin in solution, the effect on substrate inhibition would be magnified. The modified cyclodextrin **108**, as used in the work discussed in Chapter VI of the Results and Discussion and earlier in this Chapter, was chosen for this purpose as it has an enhanced solubility in water relative to the parent cyclodextrin **8**.[174,175] The experiments monitoring the hydrolysis of the hippuric acid derivative **6** by carboxypeptidase A in Tris buffer over a range of concentrations of the substrate **6** were repeated in the presence of the modified cyclodextrin **108** (0.105 mol dm^{-3}) under otherwise identical conditions. The results of these experiments are illustrated, with comparison to the case in which no cyclodextrin was present, in Figures 32 and 33.

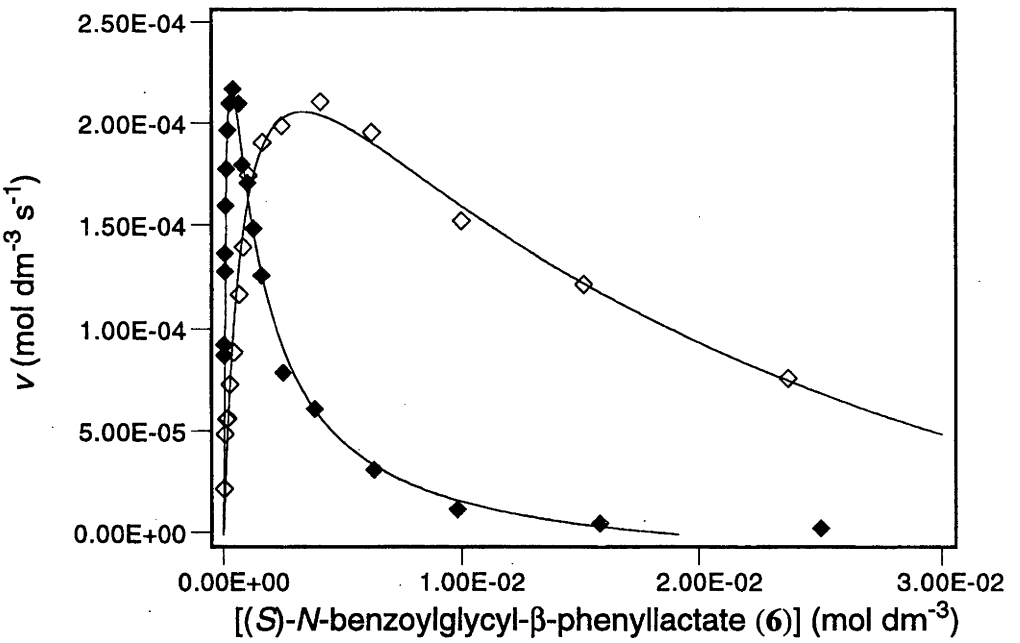


Figure 32. Rate of reaction plotted against substrate concentration for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of either no cyclodextrin (♦) or hydroxypropyl-β-cyclodextrin (**108**) (0.105 mol dm^{-3}) (◊).

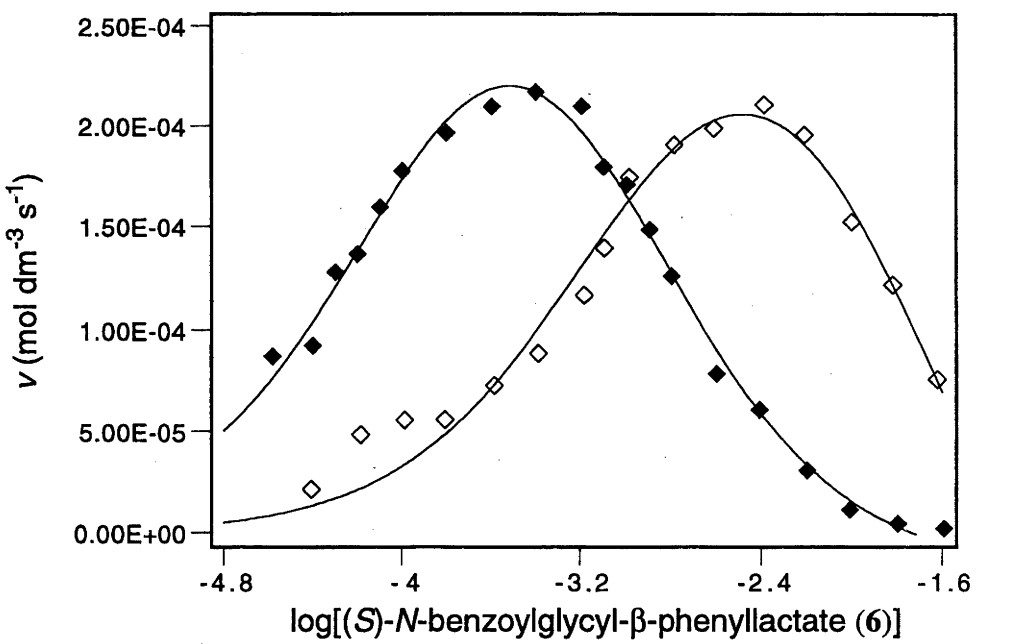


Figure 33. Rate of reaction plotted against the logarithm of the substrate concentration for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A (*ca.* 20 mol dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of either no cyclodextrin (♦) or hydroxypropyl-β-cyclodextrin (**108**) (0.105 mol dm^{-3}) (◊).

As with the naturally occurring cyclodextrin **8**, carboxypeptidase A was found to retain activity in the presence of the modified cyclodextrin **108**. The effect of addition of the modified cyclodextrin **108** to the reaction mixtures is similar to that of addition of β-cyclodextrin (**8**), though greater in magnitude. At the lower concentrations of the substrate **6**, addition of the modified cyclodextrin **108** results in a decrease in the rate of reaction. At the higher concentrations of the substrate **6**, addition of the modified cyclodextrin **108** results in an increase in the rate of reaction.

These results are consistent the modified cyclodextrin **108** complexing the substrate **6**, hence removing it from solution. At the lower concentrations of the substrate **6**, where the principal enzyme-substrate complex is the Michaelis complex, removing the substrate from solution results in a decrease in the concentration of the Michaelis complex and a decrease in the rate of reaction. At the higher concentrations of the substrate **6**, where most of the enzyme is present as the ternary complex, reducing the substrate **6** concentration decreases the proportion of the enzyme present as this complex. The result is that more of the Michaelis complex is formed and the rate of reaction increases relative to the case with no cyclodextrin present. Once again, the maximum rate of hydrolysis of the lactate **6** is slightly decreased relative to the case in the absence of a cyclodextrin though, as described previously, this is within the error expected for the experiment.

As seen with the assays in which the parent cyclodextrin **8** was present, the rate of reaction of the substrate **6** is near the maximum value for a greater range of substrate **6** concentrations in the presence of the modified cyclodextrin **108** than is the case in the absence of a cyclodextrin. This can be seen as an increase in breadth of the rate of reaction versus substrate concentration curve on addition of the modified cyclodextrin **108** (Figure 32), which is more significant than on addition of β-cyclodextrin (**8**). Further, the addition of the modified cyclodextrin **108** to the reaction mixtures results in the concentration of the substrate **6** at which the maximum rate of hydrolysis occurs increasing from *ca.* 0.40×10^{-3} mol dm^{-3} to *ca.* 4.0×10^{-3} mol dm^{-3}. Thus, the addition of the cyclodextrin **108** significantly increases the practical utility of this process, by increasing the concentration at which the enzyme operates efficiently by a factor of ten and hence reducing the scale of a reaction necessary to afford a given amount of product by a factor of ten.

On addition of the cyclodextrins **8** and **108** to reaction mixtures containing the lactate **6** at 15×10^{-3} mol dm^{-3}, a concentration at which substrate inhibition substantially decreases carboxypeptidase A activity in the absence of a cyclodextrin, the rate of hydrolysis of the substrate **6** is observed to increase by factors of three and twelve, respectively. Thus, it was expected that the extent of reaction in a given time would

increase also, the greatest effect being on addition of the host **108**. In order to quantify this and further gauge the effect of cyclodextrins on the reaction, Equation 7, which enables the extent of hydrolysis of the lactate **6** at a given time ($x(t)$) to be related to the time (t) and the initial substrate concentration (S) was derived from Equation 6 (Appendix 3). Calculations were carried out using an initial substrate concentration of 15 x 10^{-3} mol dm^{-3} and the sets of parameters *a-d* determined from the experiments containing either no cyclodextrin, β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}) or the modified cyclodextrin **108** (0.105 mol dm^{-3}) to determine the time taken to reach given extents of reaction under these conditions (Appendix 4). The results are illustrated as Figure 34. As was expected, the addition of each of the cyclodextrins **8** and **108** results in an increase in the extent of reaction in a given time, with the largest effect being observed on addition of the modified cyclodextrin **108**. When the cyclodextrin **108** is present the reaction proceeds to >99.99% before the corresponding reaction in the absence of a cyclodextrin has reached 5%. These findings further illustrate the effect of cyclodextrins to limit substrate inhibition of carboxypeptidase A catalysed hydrolysis of the lactate **6** and increase the utility of the process.

$$t = \frac{x(t)}{b} - \frac{d}{a}\ln(S-(x(t)) + \frac{a^2c+b^2d-abc}{ab^2}\ln(ac+bS-bx(t)) + \frac{d}{a}\ln(S) - \frac{a^2c+b^2d-abc}{ab^2}\ln(ac+bS) \quad (7)$$

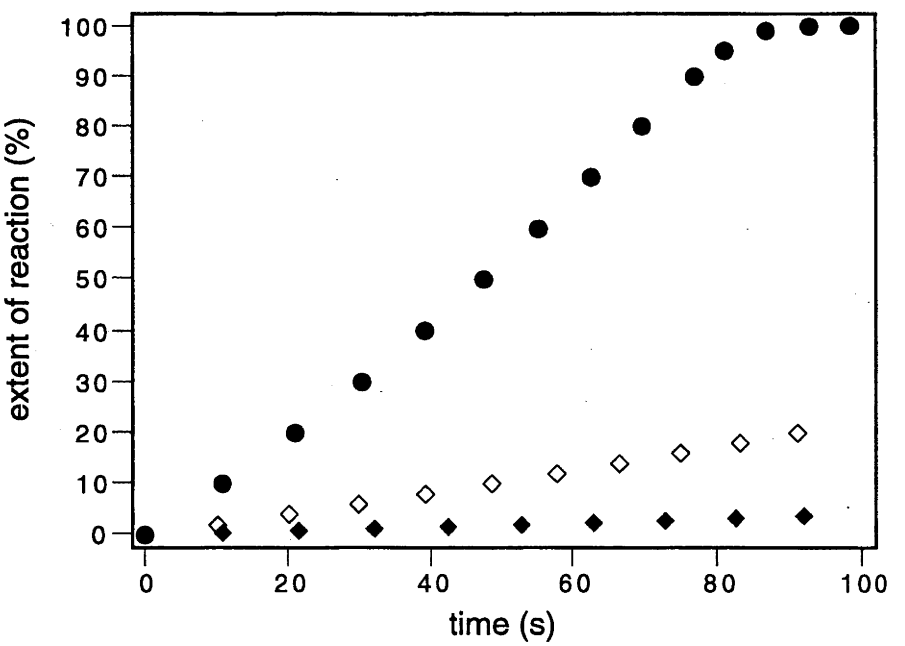


Figure 34. Calculated extent of reaction in a given time for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) (15.0 x 10^{-3} mol dm^{-3}) by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of either no cyclodextrin (♦), β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}) (◊) or hydroxypropyl-β-cyclodextrin (**108**) (0.105 mol dm^{-3}) (•).

It was of interest to determine whether the effects of cyclodextrins on substrate inhibition of an enzyme could be predicted. In order to predict the effects of the cyclodextrins **8** and **108** on hydrolysis of the lactate **6** by carboxypeptidase A, so that a comparison with the observed effects could be made, the association constants of the complexes of the lactate **6** with each of the cyclodextrins **8** and **108** were required, so studies to calculate them were undertaken. The stability constant determinations were carried out using the commercially available racemate of compound **6**. However, it is reasonable to assume that there is little or no enantioselectivity on complexation of the racemate of the lactate **6** with each of the hosts **8** and **108**, as enantioselective complexation by a cyclodextrin is the exception,[70,179-184] rather than the rule.[46] Thus, the association constants for the complexes of the (*S*)-lactate **6** with each of the cyclodextrins **8** and **108** would be expected to be the same as those for the corresponding complexes in which the guest is the racemate of compound **6**, and this assumption is used in the remainder of the discussion.

As described in Chapters IV and VI of the Results and Discussion, the change in spectroscopic properties of a guest as a function of host concentration can be used to quantify the association constant of the complex formed. The effects of different concentrations of the cyclodextrins **8** and **108** on the chemical shifts of resonances in the ^{1}H NMR spectrum of the racemate of compound **6** were investigated. The difference in chemical shift between the signals due to the *ortho*- and *meta*-protons of the benzoyl group in the ^{1}H NMR spectrum of the racemate of the ester **6** was observed to change as a function of the host **108** concentration (Figure 35). The association constant for the complex formed between the modified cyclodextrin **108** and the racemate of compound **6** was calculated, using Equations 1 and 2, to be 120 ± 20 mol^{-1} dm^3. The association constant of the complex of β-cyclodextrin (**8**) and the racemate of the lactate **6** was calculated as 250 ± 60 mol dm^{-3} using the same method. It should be noted that since there are two aromatic moieties in the racemate of the guest **6**, there is the potential for two cyclodextrins to complex one guest molecule. However, the effect of varying the concentration of the hosts **8** and **108** on the ^{1}H NMR spectrum is consistent with the formation of 1 : 1 complexes as can be seen by the good correlation observed between the observed data and the curve based on 1 : 1 binding in Figure 35. This suggests that any formation of 2 : 1 complexes is negligible.

Comparison of the association constants of the complexes of the guest **6** with each of the cyclodextrins **8** and **108**, 250 ± 60 mol dm^{-3} and 120 ± 20 mol^{-1} dm^3, respectively, indicates that the modified cyclodextrin **108** does not form a more stable complex with the guest **6** than the host **8** does. Thus, the greater effect of the modified cyclodextrin **108** when compared to that of β-cyclodextrin (**8**) on the hydrolysis of the

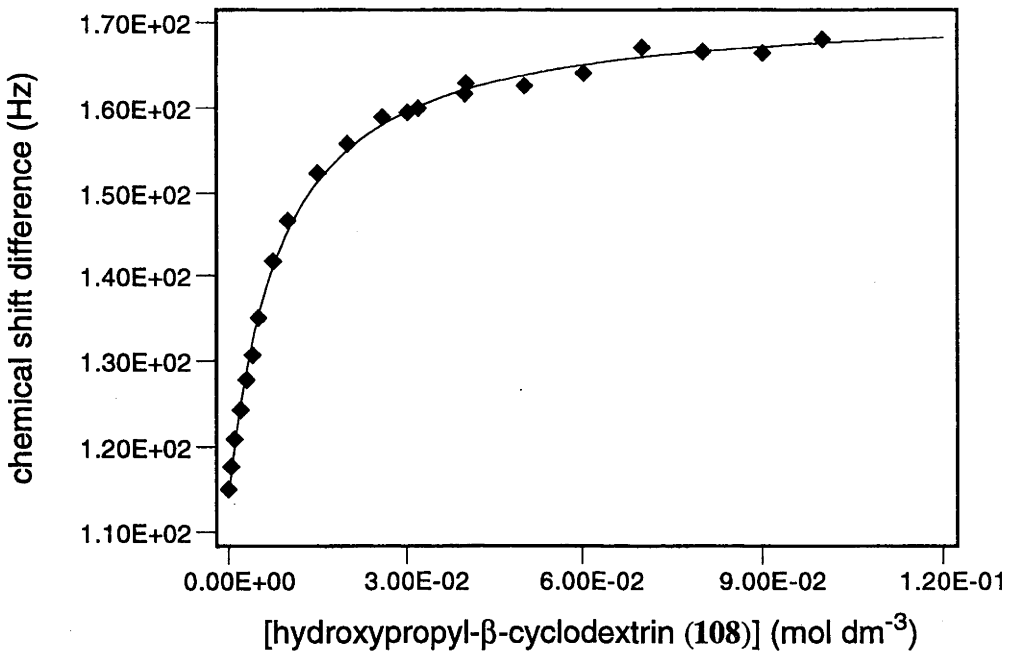


Figure 35. ^{1}H NMR chemical shift difference plotted against the concentration of hydroxypropyl-β-cyclodextrin (**108**) for the difference in chemical shifts between the resonances corresponding to the *ortho*- and *meta*-protons of the benzoyl group of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) in 0.05 mol dm^{-3} Tris buffered deuterium oxide at pH 7.5 and 298 K (I = 0.5 mol dm^{-3}).

lactate **6** by carboxypeptidase A is based entirely on the increased concentration of host present in solution.

The values of the association constants calculated for the complexes of the guest **6** with each of the hosts **8** and **108** were used to predict the effect of each of the cyclodextrins **8** and **108** on hydrolysis of the lactate **6** by carboxypeptidase A. Using them and Equation 1, the predicted concentration of the substrate **6** free in solution was calculated for a range of its total concentrations in the presence of either β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}) or the modified cyclodextrin **108** (0.105 mol dm^{-3}). From these values of the substrate **6** concentration free in solution, the predicted rates of hydrolysis of the lactate **6** were calculated, using Equation 6 and the values of *a-d* calculated in the experiment in the absence of a cyclodextrin. A summary of these calculations is given as Appendix 5. The calculations take into account that the assays monitoring the reaction of the ester **6** were prepared using the racemate of compound **6**, so the concentration of guest is twice that of the substrate **6**. The predicted rates of hydrolysis of the lactate **6** by carboxypeptidase A over a range of concentrations of the substrate **6** in the presence of either β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}) or the modified cyclodextrin **108** (0.105 mol dm^{-3}) are shown as Figures 36 and 37, respectively, with comparison to experimental results.

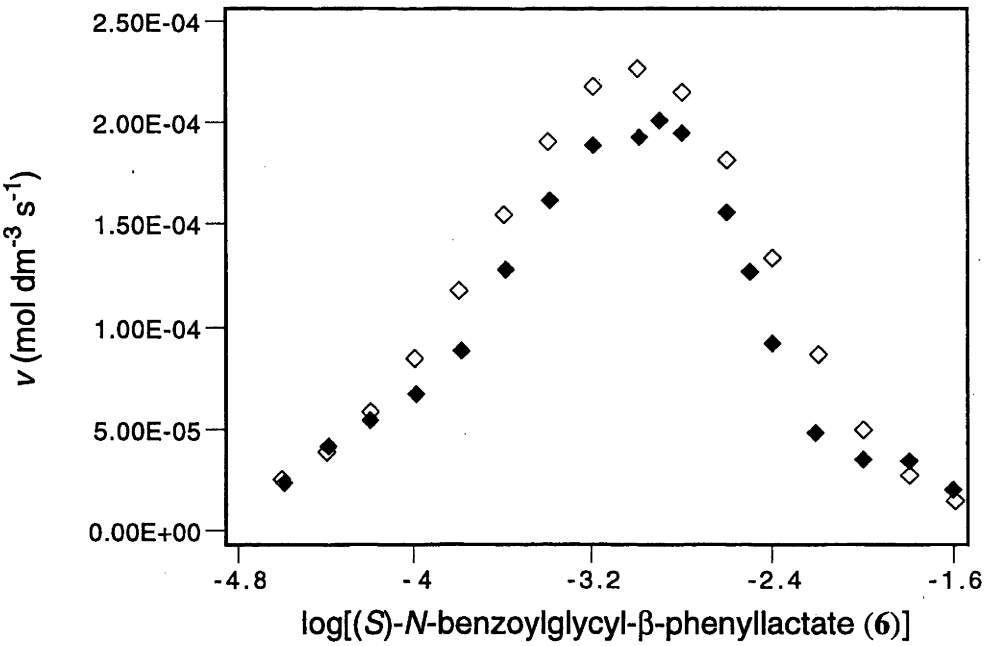


Figure 36. Rate of reaction plotted against the logarithm of the substrate concentration for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}) showing the experimental data (♦) and the data predicted based on the calculated stability constant of the complex between the host **8** and the guest **6** (◊).

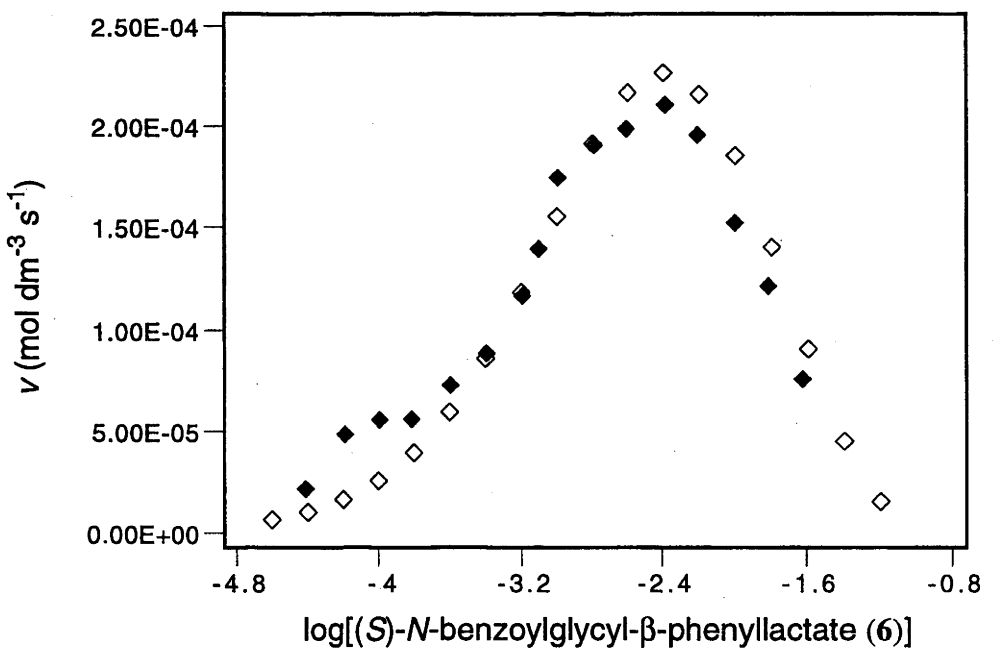


Figure 37. Rate of reaction plotted against the logarithm of the substrate concentration for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of hydroxypropyl-β-cyclodextrin (**108**) (0.105 mol dm^{-3}) showing the experimental data (♦) and the data predicted based on the calculated stability constant of the complex between the host **108** and the guest **6** (◊).

The correlation between the observed and predicted rates of hydrolysis of the lactate **6** by carboxypeptidase A in the presence of β-cyclodextrin (**8**) is excellent (Figure 36). The observed rates of reaction are slightly smaller than predicted, though this is probably due to differences in the amount of enzyme present between assays with no cyclodextrin present, on which the predicted rates are based, and the assays containing β-cyclodextrin (**8**). A similar correlation between the observed and predicted rates of hydrolysis of the lactate **6** by carboxypeptidase A is seen when the modified cyclodextrin **108** (Figure 37) is present. The information in Figures 36 and 37 illustrates that the effect of a cyclodextrin on substrate inhibition of an enzyme can be reasonably predicted if the association constant of the complex of the substrate and the cyclodextrin is known.

Thus, it has been shown that cyclodextrins can be used to reduce substrate inhibition of reactions catalysed by carboxypeptidase A through complexation of the substrate and that this effect can be predicted. There is the potential to exploit this principle to increase the utility of other enzymes which are affected by substrate inhibition, by increasing the concentration of the substrate at which the maximum rate of reaction occurs and hence decreasing the scale of reaction required to afford a given amount of product. Moreover, the effect of cyclodextrins can be predicted, using the association constant of their complexes with the substrate.

Conclusion

Cyclodextrins have been shown to limit product inhibition of (*S*)-phenylalanine ammonia lyase (PAL) by selectively complexing the cinnamate produced, thus reducing the amount free in solution to inhibit the enzyme. Similarly, cyclodextrins were shown to reduce substrate inhibition of carboxypeptidase A by removing the substrate from solution, increasing the total concentration of substrate at which the enzyme operates efficiently. These results indicate that there is the potential to increase the utility of enzymes which are affected by product or substrate inhibition, by reducing the concentration of the inhibitor present in solution through inclusion in a cyclodextrin.

The ratio of (*S*)-phenylalanine to *trans*-cinnamate at equilibrium in a solution containing ammonium ions and PAL has been shown to change on addition of a cyclodextrin that complexes (*S*)-phenylalanine and *trans*-cinnamate to different extents. It is envisaged that this principle might be used to increase the amount of the desired product of an enzyme catalysed reaction present at equilibrium, hence increasing the utility of the process.

The addition of cyclodextrins was shown to affect the hydrolysis of regioisomers of phenylalanine derivatives by protease VIII to different extents. This effect is due to differences in the interactions of the substrates with the enzyme, rather than to discrimination on formation of the complexes of the phenylalanine derivatives with cyclodextrins. Nevertheless, cyclodextrins can be used to alter the relative rates of digestion of substrates by an enzyme.

It has been demonstrated that cyclodextrins do not denature PAL, carboxypeptidase A or protease VIII. Therefore, it is reasonable to assume that other enzymes will retain their catalytic activity in the presence of cyclodextrins. As a result, it is expected that the principles outlined above might be applied to other systems to overcome the limitations of the use of enzymes in organic synthesis. In these cases, there may be specific requirements of the system and it may be necessary to tailor the cyclodextrin to suit the needs of the system in question.

N,*N*'-Bis(6^{A}-deoxy-β-cyclodextrin-6^{A}-yl)urea has been shown to act as a molecular template in the competing formation of indigoid dyes. This effect is based on preassembly of the reagents within the annuli of the dimer, which are known to have a

preferred alignment. Using this principle, there is the potential to extend the application of this, and other, linked cyclodextrins to control the regiochemistry of condensation reactions.

It was found that acylase I and PAL exhibit substrate selectivity on treatment with mixtures of regioisomers of ring-substituted phenylalanine derivatives. The substrate selectivity of acylase I was demonstrated to be synthetically useful to obtain *para*-substituted (*S*)-phenylalanines from racemic mixtures of the corresponding *ortho*- and *para*-substituted *N*-acetylphenylalanines. It is anticipated that the substrate selectivity of other enzymes could be exploited to separate the regioisomers of other compounds in an analogous manner.

Experimental

General

NMR spectroscopy was carried out on either a Varian Inova 500S or a Varian Gemini 300 spectrometer using the solvent system specified. Spectra were referenced against residual protonated solvent. The following abbreviations have been used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad.

Melting points were recorded on a Kofler hot-stage apparatus, equipped with a Reichert microscope, and are uncorrected.

Electron impact mass spectra were obtained on a VG Autospec mass spectrometer.

Ultraviolet (UV) spectra were obtained on a temperature controlled Shimadzu UV-2101 PC spectrophotometer coupled to a Shimadzu CPS temperature controller.

Infrared (IR) spectra were performed on either a Perkin Elmer 1800 FT IR spectrophotometer as nujol mulls between sodium chloride plates or on a Perkin Elmer Spectrum One FT IR spectrophotometer as solutions in chloroform.

Microanalyses were performed by the Microanalytical Services Unit of the Research School of Chemistry, Australian National University, Canberra, Australia.

Optical rotations were carried out in a 10 cm pathlength cell on a Perkin-Elmer 241 polarimeter equipped with sodium and mercury lamps.

Gas chromatography / mass spectrometry was carried out on a Hewlett Packard GC / MS / DS system composed of a Model 5890 GC (with a 12 m x (0.20×10^{-3}) m HP-1.HP-MSD column) coupled to a 5970B series MSD with a Model 59970C Chemstation.

Gas chromatography was carried out on a Varian 3400 Gas Chromatograph using the columns and temperatures specified and a flow rate of rate of 20 cm^3 min^{-1}.

TLC analyses of reaction mixtures were performed on aluminium backed plates of Kieselgel 60_{F254} silica. Preparative TLC was carried out on plates pre-coated with 2×10^{-3} m Kieselgel 60_{F254} silica. Both were visualised using either a 254 nm lamp or

an ethanol solution of 5% phosphomolybdic acid. Column chromatography was carried out on Merck silica gel 60_{PF254}.

High-performance liquid chromatography (HPLC) was carried out using Waters™ 510 HPLC pumps connected to a Waters™ 717plus Autosampler and monitoring elution using a Waters™ 486 Tunable Absorbance Detector and a Waters™ 410 Differential Refractometer using the columns and solvent systems specified.

Fluorescent spectra were obtained on a SLM/AMINCO Limited 8100C Spectrofluorometer using a 450 W ozone-free Xenon arc lamp as the excitation source.

Water, where mentioned, was deionised and then purified using the Waters Milli-Q™ Reagent system to ensure a resistivity of > 15 MΩ cm.

For all pH measurements, BDH Standard Buffer reference solutions at pH 4.00, 7.00 and 10.00 were used with an Orion 520A pH meter and a Ross 81-56 pH electrode.

α-Cyclodextrin (**7**) and β-cyclodextrin (**8**) were the generous gifts of Nihon Shokuhin Kako Co. Hydroxypropyl-β-cyclodextrin (**108**) was purchased from Cyclolab Cyclodextrin Research and Development Laboratories, Limited. The modified cyclodextrins **35**, **37** and **111** were the generous gifts of Mr. B. L. May. All cyclodextrins were dried to constant weight *in vacuo* over phosphorous pentoxide prior to use.

All other compounds used were purchased from Sigma-Aldrich Chemical Company, except isatin (**49**), which was purchased from BDH Ltd, *trans*-cinnamic acid, which was purchased from Ajax Chemicals Incorporated in the protonated form, and >99% 2-^{13}C-labelled (*S*)-phenylalanine ((*S*)-**55**), which was purchased from Cambridge Isotope Laboratories. Compounds were used as received.

All enzymes used were purchased from Sigma-Aldrich Chemical Company. (*S*)-Phenylalanine ammonia lyase (grade I from *Rhodotorula glutinis*) was obtained as a solution in 60% Glycerol, 3 x 10^{-3} mol dm^{-3} tris-hydrochloric acid, pH 7.5 with an activity of *ca.* 3 x 10^3 units dm^{-3}. Acylase I (grade I from porcine kidney) was obtained as a lyophilised powder with an activity of *ca.* 2 x 10^6 units g^{-1}. Protease VIII (from *Bacillus licheniformis*) was obtained as a lyophilised powder with an activity of *ca.* 12 x 10^3 units g^{-1}. Carboxypeptidase A (type I from bovine pancreas) was obtained as an aqueous suspension with toluene added with an activity of *ca.* 1 x 10^6 units dm^{-3}.

Preparation of Buffer Solutions[107,185]

Preparation of 0.05 mol dm^{-3} pH 7.0 phosphate buffer

Potassium dihydrogen phosphate (1.71 g, 12.6 x 10^{-3} mol) and disodium hydrogen phosphate (1.77 g, 12.5 x 10^{-3} mol) were dissolved in water. The pH was adjusted to 7.0 with 1 mol dm^{-3} sodium hydroxide solution and the volume was made up to 500 cm^3. The pH was checked and found to be as required.

Preparation of 0.05 mol dm^{-3} pH 7.0 Tris buffer (with $[NH_4^+]$ = 0.5 mol dm^{-3})

Tris(hydroxymethyl)aminomethane (1.49 g, 12.3 x 10^{-3} mol) and ammonium sulfate (8.16 g, 61.8 x 10^{-3} mol) were dissolved in water and the pH was adjusted to 7.0 with 2 mol dm^{-3} hydrochloric acid. The volume was made up to 250 cm^3, with checking and adjusting of the pH.

Preparation of 0.05 mol dm^{-3} pH 7.5 phosphate buffer

Potassium dihydrogen phosphate (3.42 g, 25.1 x 10^{-3} mol) was dissolved in water. The pH was adjusted to 7.5 with 1 mol dm^{-3} sodium hydroxide solution and the volume was made up to 500 cm^3 with checking and adjusting of the pH.

Preparation of 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3})

Tris(hydroxymethyl)aminomethane (1.49 g, 12.3 x 10^{-3} mol) and potassium chloride (7.46 g, 0.100 mol) were dissolved in water and the pH was adjusted to 7.5 with 2 mol dm^{-3} hydrochloric acid. The volume was made up to 250 cm^3, with checking and adjusting of the pH.

Preparation of 0.01 mol dm^{-3} pH 10.0 borate buffer

Sodium tetraborate (0.967 g, 2.53 x 10^{-3} mol) was dissolved in water. The pH was adjusted to 10.0 with 1 mol dm^{-3} sodium hydroxide solution and the volume was made up to 250 cm^3 with checking and adjusting of the pH.

Preparation of 0.01 mol dm^{-3} pH 10.5 borate buffer

Sodium tetraborate (9.63 g, 25.2 x 10^{-3} mol) was dissolved in water. The pH was adjusted to 10.5 with 1 mol dm^{-3} sodium hydroxide solution and the volume was made up to 250 cm^3 with checking and adjusting of the pH.

Preparation of 0.05 mol dm^{-3} pH 11.0 phosphate buffer

Sodium phosphate (4.07 g, 24.8 x 10^{-3} mol) was dissolved in water. The pH was adjusted to 11.5 with 1 mol dm^{-3} hydrochloric acid and the volume was made up to 500 cm^3 with checking and adjusting of the pH.

Experimental I: Investigation of the Use of a Metallocyclodextrin as a Possible Esterase Mimic

(*R*)-*O*-Acetyltyrosine ((*R*)-41)

(*R*)-Tyrosine ((*R*)-**42**) (0.507 g, 2.80 x 10^{-3} mol) was dissolved in a 0.60 mol dm^{-3} solution of perchloric acid in acetic acid, prepared by diluting 70% perchloric acid (0.28 cm^3) to 5.6 cm^3 with glacial acetic acid. Acetic anhydride (1.24 cm^3, 13.1 x 10^{-3} mol) was added and the solution was stirred for 1 h at room temperature. Water (0.11 cm^3, 6.11 x 10^{-3} mol) was added and the solution was stirred for a further 1 h at room temperature. *n*-Butylamine (0.54 cm^3) was added and the resulting solution was poured into a 1:3 acetone / diethyl ether mixture (60 cm^3) and the mixture was allowed to stand at 277 K overnight. The resulting precipitate was collected, washed with diethyl ether (40 cm^3) and recrystallised from 50% aqueous ethanol to give the title compound as a colourless powder (0.412 g, 67%). m.p. 213-215 °C (dec.) (lit.,[108] 213-214 °C); α_D^{20} = -1.16° (lit.,[108] α_D^{29} = -1.22°), α_{Hg}^{20} = -1.31° (lit.,[108] α_D^{29} = -1.38°) in 1.10 mol dm^{-3} sodium hydroxide solution; ^{1}H NMR (300 MHz, d_6-DMSO) δ2.27 (s, 3H, $-OCH_3$), 2.86 (dd, *J* = 8, 14 Hz, 1H, β-H), 3.14 (dd, *J* = 4, 14 Hz, 1H, β-H), 3.38 (dd, *J* = 4, 8 Hz, 1H, α-H), 3.50 (br s, 2H, $-NH_2$), 7.04 (d, *J* = 8 Hz, 2H, Ar-H), 7.30 (d, *J* = 8 Hz, 2H, Ar-H).

(*S*)-*O*-Acetyltyrosine ((*S*)-41)

(*S*)-Tyrosine ((*S*)-**42**) (0.472 g, 2.60 x 10^{-3} mol) was dissolved in a 0.60 mol dm^{-3} solution of perchloric acid in acetic acid, prepared by diluting 70% perchloric acid (0.28 cm^3) to 5.6 cm^3 with glacial acetic acid. Acetic anhydride (1.15 cm^3, 12.2 x 10^{-3} mol) was added and the solution was stirred for 1 h at room temperature. Water (0.1 cm^3, 5.55 x 10^{-3} mol) was added and the solution was stirred for a further 1 h at room temperature. *n*-Butylamine (0.5 cm^3) was added and the resulting solution was poured into a 1:3 acetone / diethyl ether mixture (60 cm^3) and the mixture was allowed to stand at 277 K overnight. The resulting precipitate was collected, washed with diethyl ether (40 cm^3) and recrystallised from 50% aqueous ethanol to give the title compound as a colourless powder (0.317 g, 51%). m.p. 211-212.5 °C (dec.) (lit.,[108] 213-214 °C); α_D^{20} = 1.14° (lit.,[108] α_D^{29} = 1.22°), α_{Hg}^{20} = -1.28° (lit.,[108] α_D^{29} = 1.38°) in 1.10 mol

dm^{-3} sodium hydroxide solution; ^1H NMR (300 MHz, d_6-DMSO) δ2.30 (s, 3H, $-OCH_3$), 2.87 (dd, J = 8, 14 Hz, 1H, β-H), 3.15 (dd, J = 4, 14 Hz, 1H, β-H), 3.40 (dd, J = 4, 8 Hz, 1H, α-H), 3.51 (br s, 2H, $-NH_2$), 7.05 (d, J = 8 Hz, 2H, Ar-H), 7.31 (d, J = 8 Hz, 2H, Ar-H).

6^A-*O*-4-Toluenesulfonyl-β-cyclodextrin (43)

To a stirred solution of β-cyclodextrin (**8**) (50.1 g, 44.1 x 10^{-3} mol) in pyridine (800 cm^3) was added 4-toluenesulfonyl chloride (9.52 g, 50.0 x 10^{-3} mol) in four portions over 10 min. The resultant solution was stirred for 16 h at room temperature and the solvent was removed *in vacuo*. The residue was triturated with acetone (2 x 400 cm^3), then water (2 x 400 cm^3), recrystallised twice from hot water (363 K) and dried *in vacuo* over phosphorous pentoxide to give the title compound as a colourless powder (8.82 g, 16%) m.p. >250 °C; ^1H NMR (300 MHz, D_2O) δ2.57 (s, 3H, Ar-CH_3), 3.5-4 (m, 42H, remaining cyclodextrin H), 5.05 (m, 7H, H_1), 7.53 (d, J = 9 Hz, 2H, Ar-H), 7.73 (d, J = 9 Hz, 2H, Ar-H). Spectroscopic data is consistent with those reported.[111]

6^A-(3-Aminopropyl)amino-6^A-deoxy-β-cyclodextrin (19)

To a solution of the tosylate **43** (2.04 g, 1.58 x 10^{-3} mol) in *N*-methylpyrrolidinone (5 cm^3) was added 1,3-diaminopropane (5 cm^3, 59.9 x 10^{-3} mol) and the reaction mixture was stirred for 4 h at 343 K. The solution was then cooled to room temperature and added to ethanol (100 cm^3). The resultant precipitate was filtered, then washed with ethanol (100 cm^3) and diethyl ether (50 cm^3). The residue was dissolved in water (100 cm^3) and the resultant solution was loaded onto a Biorex cation exchange column in the protonated form. The eluant was recycled 10 times and the column was washed with water (1 dm^3). The column was washed with concentrated ammonia solution until no more cyclodextrin was present in the eluant. The basic eluants were combined and the solvent was removed *in vacuo* to give the title compound as a colourless powder (1.33 g, 70%). m.p. >250 °C; ^1H NMR (300 MHz, D_2O, pH~9) δ1.70 (m, 2H, $-CH_2-\underline{CH_2}-CH_2-$), 2.55 (t, J = 7 Hz, 2H, $-\underline{CH_2}-NH_2$), 2.65 (d, J = 11 Hz, 1H, H_{6A}), 2.81 (t, J = 8 Hz, 2H, $-NH-\underline{CH_2}-$), 2.95 (d, J = 11 Hz, 1H, H_{6A}), 3.32 (t, J = 9 Hz, 1H, H_{4A}), 3.4-3.6 (m, 13H, H_2, H_{4B-G}), 3.65-3.9 (m, 26H, H_3, H_5, H_{6B-G}), 5.00 (br s, 7H, H_1). Spectroscopic data is consistent with those reported.[112]

Monitoring the rate of hydrolysis of (*R*)-*O*-acetyltyrosine ((*R*)-41) in the presence of 6^{A}-(3-aminopropyl)amino-6^{A}-deoxy-β-cyclodextrin (19)

6^{A}-(3-Aminopropyl)amino-6^{A}-deoxy-β-cyclodextrin (**19**) (0.110 g, 91.2 x 10^{-6} mol) was dissolved in 0.01 mol dm^{-3} pH 10.0 borate buffer and the volume was made up to 50.0 cm^{3} with the buffer. (*R*)-*O*-Acetyltyrosine ((*R*)-**41**) (3.6 x 10^{-3} g, 16.1 x 10^{-6} mol) was dissolved in 0.01 mol dm^{-3} pH 10.0 borate buffer and the volume was made up to 2.00 cm^{3} with the buffer. Reaction mixtures were immediately prepared (Table 15), the volumes were made up to 4.00 cm^{3} with 0.01 mol dm^{-3} pH 10.0 borate buffer and the change in absorbance at 283 nm was monitored by UV spectroscopy at 298 K. The initial rate of reaction, v_0, was calculated (Table 15), using Kaleidagraph® to determine the initial slope and the data was analysed by the method of Lineweaver and Burk[113] (Figure 5). This gave values for the rate of reaction of the included species, k_{inc}, and the association constant of the complex formed between the tyrosine derivative (*R*)-**41** and the amine **19**, *K*, as (0.84 ± 0.06) x 10^{-3} s^{-1} and 480 ± 40 mol^{-1} dm^{3}, respectively.

Host **19** solution (cm^{3})	Ester (*R*)-**41** solution (cm^{3})	[Ester (*R*)-**41**] (mol dm^{-3})	[Ester (*R*)-**41**]$^{-1}$ (mol^{-1} dm^{3})	v_0 (mol dm^{-3} s^{-1})	v_0-v_{un} (mol dm^{-3} s^{-1})	$(v_0$-$v_{un})^{-1}$ (mol^{-1} dm^{3} s)
-	0.100	0.201 x 10^{-3}	4980	27.4 x 10^{-9}	n/a	n/a
3.00	0.100	0.201 x 10^{-3}	4980	0.130 x 10^{-6}	0.103 x 10^{-6}	9.71 x 10^{6}
3.00	0.200	0.402 x 10^{-3}	2490	0.243 x 10^{-6}	0.188 x 10^{-6}	5.32 x 10^{6}
3.00	0.300	0.604 x 10^{-3}	1660	0.351 x 10^{-6}	0.269 x 10^{-6}	3.72 x 10^{6}
3.00	0.400	0.805 x 10^{-3}	1240	0.428 x 10^{-6}	0.318 x 10^{-6}	3.14 x 10^{6}
3.00	0.500	1.01 x 10^{-3}	990	0.521 x 10^{-6}	0.384 x 10^{-6}	2.60 x 10^{6}

Table 15. Sample preparation and observed rates of reaction for the hydrolysis of varying concentrations of (*R*)-*O*-acetyltyrosine ((*R*)-**41**) in the presence of the modified cyclodextrin **19** in 0.01 mol dm^{-3} pH 10.0 borate buffer at 298 K.

Monitoring the rate of hydrolysis of (*S*)-*O*-acetyltyrosine ((*S*)-41) in the presence of 6^{A}-(3-aminopropyl)amino-6^{A}-deoxy-β-cyclodextrin (19)

6^{A}-(3-Aminopropyl)amino-6^{A}-deoxy-β-cyclodextrin (**19**) (0.110 g, 91.2 x 10^{-6} mol) was dissolved in 0.01 mol dm^{-3} pH 10.0 borate buffer and the volume was made up to 50.0 cm^{3} with the buffer. (*S*)-*O*-Acetyltyrosine ((*S*)-**41**) (3.2 x 10^{-3} g, 14.3 x 10^{-6} mol) was dissolved in 0.01 mol dm^{-3} pH 10.0 borate buffer and the volume was made up to 2.00 cm^{3} with the buffer. Reactions were immediately prepared (Table 16), the volumes were made up to 4.00 cm^{3} with 0.01 mol dm^{-3} pH 10.0 borate buffer and the change in absorbance at 283 nm was monitored by UV spectroscopy at 298 K. The initial rate of reaction, v_0, was calculated (Table 16), using Kaleidagraph® to determine the initial slope and the data was analysed by the method of Lineweaver and Burk[113] (Figure 5). This

gave values for the rate of reaction of the included species, k_{inc}, and the association constant of the complex formed between the tyrosine derivative (*S*)-**41** and the amine **19**, *K*, as (0.56 ± 0.04) x 10^{-3} s^{-1} and 1100 ± 100 mol^{-1} dm^3, respectively.

Host **19** solution (cm^3)	Ester (*S*)-**41** solution (cm^3)	[Ester (*S*)-**41**] (mol dm^{-3})	[Ester (*S*)-**41**]$^{-1}$ (mol^{-1} dm^3)	v_0 (mol dm^{-3} s^{-1})	v_0-v_{un} (mol dm^{-3} s^{-1})	$(v_0-v_{un})^{-1}$ (mol^{-1} dm^3 s)
-	0.100	0.179 x 10^{-3}	n/a	24.0 x 10^{-9}	n/a	n/a
3.00	0.100	0.179 x 10^{-3}	5590	0.152 x 10^{-6}	0.128 x 10^{-6}	7.81 x 10^6
3.00	0.200	0.358 x 10^{-3}	2790	0.261 x 10^{-6}	0.213 x 10^{-6}	4.69 x 10^6
3.00	0.300	0.536 x 10^{-3}	1870	0.350 x 10^{-6}	0.278 x 10^{-6}	3.60 x 10^6
3.00	0.400	0.715 x 10^{-3}	1400	0.431 x 10^{-6}	0.335 x 10^{-6}	2.99 x 10^6
3.00	0.500	0.894 x 10^{-3}	1120	0.525 x 10^{-6}	0.405 x 10^{-6}	2.47 x 10^6

Table 16. Sample preparation and observed rates of reaction for the hydrolysis of varying concentrations of (*S*)-*O*-acetyltyrosine ((*S*)-**41**) in the presence of the modified cyclodextrin **19** in 0.01 mol dm^{-3} pH 10.0 borate buffer at 298 K.

Experimental II: Investigations Toward the Rational Design of a Molecular Template for Condensation Reactions

$\Delta^{2,3'}$-Biindoline-2',3-dione (indirubin) (51)

Isatin (**49**) (76.7 x 10^{-3} g, 0.589 x 10^{-3} mol), indoxyl acetate (**46**) (0.102 g, 0.582 x 10^{-3} mol) and potassium carbonate (0.135 g, 0.976 x 10^{-3} mol) were stirred in degassed methanol (2 cm^3) under nitrogen for 24 h. The solvent was removed *in vacuo* and the residue was purified by flash column chromatography (1 : 19, acetone : chloroform) to give the title compound as a dark red solid (0.135 g, 89%) m.p. >300 °C (lit.,[118] >300 °C); $\nu_{max}(cm^{-1})$ 1662, 1609, 1403, 1211, 1003, 962; *m/z* (EI) 223 (M^+, 100%), 234 (62), 205 (34). Spectroscopic data is consistent with those reported.[118]

6^A-Amino-6^A-deoxy-β-cyclodextrin (40)

To liquid ammonia (30 cm^3) condensed in a high pressure reaction vessel was added a solution of the tosylate **43** (6.66 g, 5.16 x 10^{-3} mol) in *N*,*N*-dimethylformamide (50 cm^3) and the vessel was sealed. The reaction vessel was allowed to warm to room temperature, then it was heated at 323 K for 18 h which gave an internal pressure of *ca.* 15 atm. The reaction vessel was allowed to cool to room temperature and the pressure was released slowly. The remaining solution was transferred to a round-bottomed flask and the solvent was removed *in vacuo*. The residue was taken up in water (100 cm^3) and the resultant solution was loaded onto a Biorex cation exchange column in the protonated form. The eluant was recycled 10 times and the column was washed with water (1 dm^3). The column was washed with concentrated ammonia solution until no more cyclodextrin was present in the eluant. The basic eluants were combined and the solvent was removed *in vacuo* to give the title compound as a colourless powder (3.31 g, 50%). m.p. >250 °C (lit.,[111] >250°C); ^1H NMR (300 MHz, D_2O) δ3.4-3.7 (m, 14H), 3.8-4.0 (m, 28H), 5.00 (br s, 7H); ^{13}C NMR (75.5 MHz, D_2O) δ42.1 (C_{6A}), 61.2, 72.7, 72.9, 73.0, 74.0, 81.9, 82.1, 83.2, 102.8. Spectroscopic data is consistent with those reported.[111]

Bis-3-nitrophenyl oxalate (52)

To a solution of oxalyl chloride (0.149 g, 1.17 x 10^{-3} mol) in dichloromethane (5 cm^3) was added 3-nitrophenol (0.373 g, 2.68 x 10^{-3} mol). Triethylamine (0.4 cm^3, 2.86 x 10^{-3} mol) was added dropwise over 15 min with stirring. The resultant suspension was stirred at room temperature for 45 min, and the precipitate was collected and washed with ethyl acetate. Recrystallisation from nitrobenzene gave the title compound as a colourless powder (0.193g, 48%). m.p. 224-227°C (lit.,[186] 226-228 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ7.81 (m, 2H, Ar-H), 8.22 (m, 2H, Ar-H).

***N,N*-Bis-(6^A-deoxy-6^A-β-cyclodextrinyl)oxalamide (36)**

To a stirred solution of the amine **40** (0.870 g, 0.767 x 10^{-3} mol) in pyridine (18 cm^3) was added bis-3-nitrophenol oxalate (**52**) (83.0 x 10^{-3} g, 0.250 x 10^{-3} mol) over 1 h in four portions and the solution was stirred for 48 h. The solution was then added dropwise to a vigorously stirred ether/acetone (1 : 1) mixture (200 cm^3). The resultant suspension was centrifuged and the supernatant was discarded. The pellet was resuspended in acetone, the suspension was centrifuged and the supernatant was discarded. This process was repeated with ether. The pellet was dissolved in water (10 cm^3) and the resultant solution dropped into stirred acetone (100 cm^3). The suspension was centrifuged and the supernatant was discarded. The pellet was suspended in acetone, the suspension was centrifuged and the supernatant was discarded. The pellet was redissolved in water (50 cm^3) and the resultant solution was loaded onto a Biorex Ion Exchange column in protonated form. The column was washed with water (150 cm^3) and the eluant was recycled six times. The column was then washed with water until the eluant contained no cyclodextrin by TLC (250 cm^3). The eluants were combined and the solvent was removed *in vacuo* to give the title compound as a colourless powder (0.378 g, 65%). m.p. >250°C (lit.,[94] >250°C); ^{1}H NMR (300 MHz, D_2O) δ3.4-3.9 (m, 72H), 4.95 (br s, 14H); ^{13}C NMR (75.5 MHz, D_2O) δ55.0, 62.8, 74.3, 74.5, 75.5, 83.5, 104.3, 162.5. The spectroscopic data is consistent with that previously reported.[94]

Calibration of detectors for monitoring the formation of indigo (50) and indirubin (51) by HPLC

Indigo (**50**) (2.3 x 10^{-3} g, 8.77 x 10^{-6} mol) was dissolved in acetone (5 cm^3) and the volume was made up to 50 cm^3 with chloroform. Indirubin (**51**) (3.1 x 10^{-3} g, 11.8 x 10^{-6} mol) was dissolved in acetone (5 cm^3) and the volume was made up to 50 cm^3 with chloroform. An aliquot of each of these solutions (1.00 cm^3) was diluted to 10 cm^3 with acetone / chloroform (1 : 19) to give stock solutions of the dyes **50** and **51** Samples of these stock solutions were analysed by HPLC on an Alltech Econosil 5 μ silica column (4.6 x 10^{-3} m by 0.250 m) (acetone : chloroform, 1 : 19) monitoring at 550 nm (Table 17). The detector response was plotted against the quantity of dye injected and the detector response per mole of dye was calculated in each case (Figure 38). This gave a response of 0.453 x 10^{15} units mol^{-1} for indigo (**50**) and 0.602 x 10^{15} units mol^{-1} for indirubin (**51**).

Dye	Injection volume (cm^3)	Quantity of dye (mol)	Detector response ($units^2$)
50	10.0 x 10^{-3}	0.175 x 10^{-9}	78906
50	15.0 x 10^{-3}	0.263 x 10^{-9}	117755
50	20.0 x 10^{-3}	0.351 x 10^{-9}	155373
50	25.0 x 10^{-3}	0.439 x 10^{-9}	207296
50	30.0 x 10^{-3}	0.526 x 10^{-9}	244961
50	35.0 x 10^{-3}	0.614 x 10^{-9}	268683
50	40.0 x 10^{-3}	0.702 x 10^{-9}	317073
50	45.0 x 10^{-3}	0.789 x 10^{-9}	361324
50	50.0 x 10^{-3}	0.877 x 10^{-9}	395329
51	10.0 x 10^{-3}	0.236 x 10^{-9}	144681
51	15.0 x 10^{-3}	0.354 x 10^{-9}	218593
51	20.0 x 10^{-3}	0.472 x 10^{-9}	296814
51	25.0 x 10^{-3}	0.590 x 10^{-9}	359729
51	30.0 x 10^{-3}	0.708 x 10^{-9}	427923
51	35.0 x 10^{-3}	0.826 x 10^{-9}	502226
51	40.0 x 10^{-3}	0.944 x 10^{-9}	573015
51	45.0 x 10^{-3}	1.06 x 10^{-9}	635523
51	50.0 x 10^{-3}	1.18 x 10^{-9}	715645

Table 17. Detector response at 550 nm on analysis of either indigo (**50**) or indirubin (**51**) by HPLC on an Alltech Econosil 5 μ silica column (4.6 x 10^{-3} m by 0.250 m) (acetone : chloroform, 1 : 19).

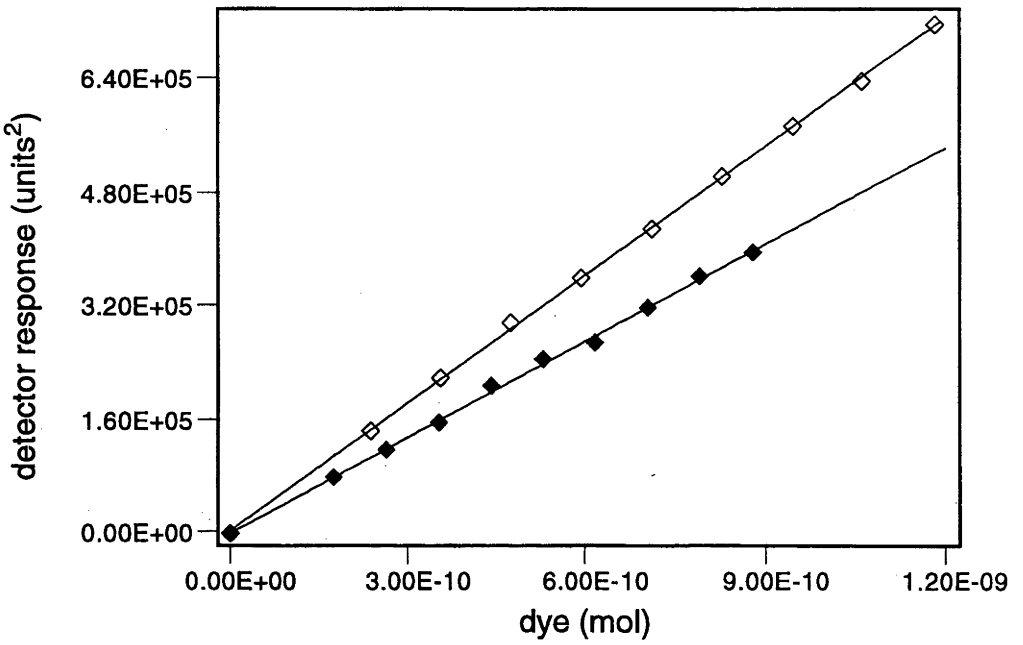


Figure 38. Detector response plotted against concentration of dye analysed by HPLC on an Alltech Econosil 5 μ silica column (4.6 x 10^{-3} m by 0.250 m) (acetone : chloroform, 1 : 19) for indigo (**50**) (♦) and indirubin (**51**) (◊).

Preparation of solutions and analysis of reaction mixtures containing indoxyl acetate (46) and isatin (49) over the pH range 8-13

Borate buffers at pH 8.0 and pH 9.0 were prepared by adjusting 0.01 mol dm^{-3} pH 10.0 borate buffer to the appropriate pH with 2 mol dm^{-3} hydrochloric acid. Phosphate buffers at pH 12.0 and pH 13.0 were prepared by adjusting 0.05 mol dm^{-3} pH 11.0 phosphate buffer. Indoxyl acetate (**46**) (1.7 x 10^{-3} g, 9.7 x 10^{-6} mol) and isatin (**49**) (8.8 x 10^{-3} g, 60 x 10^{-6} mol) were dissolved in 10.0 cm^3 of the appropriate buffer. Aliquots of these solutions (0.100 cm^3) were diluted to 1.00 cm^3 with the appropriate buffer. The reactions were stirred at 298 K for 16 h, then brought to *ca.* pH 7 with 2 mol dm^{-3} hydrochloric acid. The product mixtures were extracted with chloroform (2 x 1 cm^3), the extracts were analysed by HPLC on an Alltech Econosil 5 μ silica column (4.6 x 10^{-3} m by 0.250 m) (acetone : chloroform, 1 : 19) and the amount of indigo (**50**) and indirubin (**51**) present in each sample was quantified (Table 18). The yield quoted in each case is based on the quantity of indoxyl acetate used.

pH	Detector response of the dyes **50** : **51** (units^2)$^{\neq}$	Quantity of the dyes **50** : **51** (mol)	Yield of the dyes **50** : **51** (%)
8.0	¢	0 : 0	0 : 0
9.0	48.5 x 10^6 : 0.152 x 10^9	0.107 x 10^{-6} : 0.252 x 10^{-6}	2.2 : 2.6
10.0	0.355 x 10^9 : 0.781 x 10^9	0.784 x 10^{-6} : 1.30 x 10^{-6}	16 : 13
11.0	1.40 x 10^9 : 0.644 x 10^9	3.10 x 10^{-6} : 1.07 x 10^{-6}	64 : 11
12.0	0.396 x 10^9 : 11.7 x 10^6	0.874 x 10^{-6} : 19.4 x 10^{-9}	18 : 0.2
13.0	0.176 x 10^9 : 2.92 x 10^6	0.389 x 10^{-6} : 4.85 x 10^{-9}	8.0 : 0.05

Table 18. Analysis of reaction mixtures containing indoxyl acetate (**46**) and isatin (**49**) at pH values in the range 8-13 by HPLC.
$^{\neq}$Results are the average of replicate injections from two separate reactions.
¢No reaction products observed

Preparation and analysis of reaction mixtures containing indoxyl acetate (46) and isatin (49) in 0.05 mol dm^{-3} pH 11.0 phosphate buffer

Indoxyl acetate (**46**) (1.7 x 10^{-3} g, 9.7 x 10^{-6} mol) and isatin (**49**) (8.7 x 10^{-3} g, 59 x 10^{-6} mol) were dissolved in 10.0 cm^3 of 0.05 mol dm^{-3} pH 11.0 phosphate buffer. Aliquots of this solutions (0.100 cm^3) were diluted to 1.00 cm^3 with the 0.05 mol dm^{-3} pH 11.0 phosphate buffer. The reactions were stirred at 298 K for a given time (Table 19), then brought to *ca.* pH 7 with 2 mol dm^{-3} hydrochloric acid. The product mixtures were extracted with chloroform (2 x 1 cm^3), the extracts were analysed by HPLC on an Alltech Econosil 5 μ silica column (4.6 x 10^{-3} m by 0.250 m) (acetone : chloroform, 1 : 19) and the amount of indigo (**50**) and indirubin (**51**) present in each sample was quantified (Table 19). The yield quoted in each case is based on the quantity of indoxyl acetate used.

Reaction time	Detector response of the dyes **50** : **51** (units^2)$^{\neq}$	Quantity of the dyes **50** : **51** (mol)	Yield of the dyes **50** : **51** (%)
2 min	0.330 x 10^9 : 0.303 x 10^9	0.728 x 10^{-6} : 0.504 x 10^{-6}	15 : 5.2
15 min	1.09 x 10^9 : 0.691 x 10^9	2.41 x 10^{-6} : 1.15 x 10^{-6}	47 : 12
16 h	1.40 x 10^9 : 0.644 x 10^9	3.10 x 10^{-6} : 1.07 x 10^{-6}	64 : 11

Table 19. Analysis of reaction mixtures containing indoxyl acetate (**46**) and isatin (**49**) in 0.05 mol dm^{-3} phosphate buffer at pH 11.0 in given times by HPLC.
$^{\neq}$Results are the average of replicate injections from two separate reactions.

General procedure for the preparation and analysis of reaction mixtures containing indoxyl acetate (46) and isatin (49) to examine the effect of addition of cyclodextrins

Indoxyl acetate (**46**) and isatin (**49**) (in a mole ratio of *ca* 1 : 6) were dissolved in 10.0 cm^3 of the appropriate buffer. Aliquots of these solutions (0.100 cm^3) were added to solutions containing either no cyclodextrin or one of the species **8** and **35-37** in the appropriate buffer (0.900 cm^3). The reactions were stirred at 298 K, then brought to *ca.* pH 7 with 2 mol dm^{-3} hydrochloric acid. The product mixtures were extracted with chloroform (2 x 1 cm^3), the extracts were analysed by HPLC on an Alltech Econosil 5 μ silica column (4.6 x 10^{-3} m by 0.250 m) (acetone : chloroform, 1 : 19) and the amount of indigo (**50**) and indirubin (**51**) present in each sample was quantified. The yield quoted in each case is based on the quantity of indoxyl acetate used.

0.01 mol dm^{-3} pH 10.0 Borate buffer

Indoxyl Acetate (**46**): 1.7 x 10^{-3} g, 9.7 x 10^{-6} mol; Isatin (**49**): 8.6 x 10^{-3} g 58 x 10^{-6} mol; Reaction time: 16 h.

Cyclodextrin	Cyclodextrin concentration (mol dm^{-3})	Detector response of the dyes **50** : **51** (units^2)≠	Quantity of the dyes **50** : **51** (mol)	Yield of the dyes **50** : **51** (%)
-	n/a	0.355 x 10^9 : 0.781 x 10^9	0.784 x 10^{-6} : 1.30 x 10^{-6}	16 : 13
8	14.1 x 10^{-3}	57.0 x 10^6 : 0.144 x 10^9	0.126 x 10^{-6} : 0.245 x 10^{-6}	2.5 : 2.5
35	6.8 x 10^{-3}	0.656 x 10^6 : 60.0 x 10^6	1.45 x 10^{-9}: 99.7 x 10^{-9}	0.03 : 1.0
36	6.6 x 10^{-3}	4.50 x 10^6 : 34.7 x 10^6	9.93 x 10^{-9}: 57.6 x 10^{-9}	0.2 : 0.6
37	6.7 x 10^{-3}	10.6 x 10^6 : 41.0 x 10^6	23.5 x 10^{-9}: 68.1 x 10^{-9}	0.5 : 0.7

Table 20. Analysis of reaction mixtures containing indoxyl acetate (**46**) and isatin (**49**) in 0.01 mol dm^{-3} pH 10.0 borate buffer by HPLC.
≠Results are the average of replicate injections from two separate reactions.

0.01 mol dm^{-3} pH 10.5 Borate buffer

Indoxyl Acetate (**46**): 1.7 x 10^{-3} g, 9.7 x 10^{-6} mol; Isatin (**49**): 8.5 x 10^{-3} g, 58 x 10^{-6} mol; Reaction time: 16 h.

Cyclodextrin	Cyclodextrin concentration (mol dm^{-3})	Detector response of the dyes **50** : **51** (units^2)≠	Quantity of the dyes **50** : **51** (mol)	Yield of the dye **50** : **51** (%)
-	n/a	0.903 x 10^9 : 2.20 x 10^9	1.99 x 10^{-6} : 3.65 x 10^{-6}	41 : 38
8	13.7 x 10^{-3}	0.187 x 10^9 : 0.527 x 10^9	0.413 x 10^{-6} : 0.875 x 10^{-6}	8.5 : 9.0
35	6.8 x 10^{-3}	9.41 x 10^6 : 0.439 x 10^9	20.8 x 10^{-9}: 0.729 x 10^{-6}	0.4 : 7.5
36	6.8 x 10^{-3}	0.197 x 10^9 : 0.385 x 10^9	0.435 x 10^{-6}: 0.640 x 10^{-6}	9.0 : 6.5
37	6.9 x 10^{-3}	0.132 x 10^9 : 0.497 x 10^9	0.291 x 10^{-6}: 0.825 x 10^{-6}	6.0 : 8.5

Table 21. Analysis of reaction mixtures containing indoxyl acetate (**46**) and isatin (**49**) in 0.01 mol dm^{-3} pH 10.5 borate buffer by HPLC.
≠Results are the average of replicate injections from two separate reactions.

0.05 mol dm^{-3} pH 11.0 Phosphate buffer

Indoxyl Acetate (**46**): 1.7 x 10^{-3} g, 9.7 x 10^{-6} mol; Isatin (**49**): 8.6 x 10^{-3} g, 58 x 10^{-6} mol; Reaction time: 15 min.

Cyclodextrin	Cyclodextrin concentration (mol dm^{-3})	Detector response of the dyes **50** : **51** (units^2)≠	Quantity of the dyes **50** : **51** (mol)	Yield of the dyes **50** : **51** (%)
-	n/a	1.09 x 10^9 : 0.691 x 10^9	2.41 x 10^{-6} : 1.15 x 10^{-6}	47 : 12
8	14 x 10^{-3}	0.206 x 10^9 : 0.702 x 10^9	0.455 x 10^{-6} : 1.17 x 10^{-6}	9.5 : 12
35	6.8 x 10^{-3}	55.0 x 10^6 : 1.01 x 10^9	0.125 x 10^{-6} : 1.68 x 10^{-6}	2.5 : 17
36	6.6 x 10^{-3}	0.326 x 10^9 : 0.797 x 10^9	7.20 x 10^{-6} : 1.32 x 10^{-6}	15 : 14
37	6.7 x 10^{-3}	0.132 x 10^9 : 0.380 x 10^9	2.91 x 10^{-6} : 6.31 x 10^{-6}	6.0 : 6.5

Table 22. Analysis of reaction mixtures containing indoxyl acetate (**46**) and isatin (**49**) in 0.05 mol dm^{-3} pH 11.0 phosphate buffer by HPLC.
≠Results are the average of replicate injections from two separate reactions.

$\Delta^{2,3'}$-Biindoline-2',3-dione-5'-sulfonic acid H-(54)

The sodium salt of isatin-5-sulfonate (**53**) (0.244 g, 1.05 x 10^{-3} mol), indoxyl acetate (**46**) (0.185 g, 1.07 x 10^{-3} mol) and ammonium carbonate (0.121 g, 3.17 x 10^{-3} mol) were stirred in degassed methanol (2 cm^3) under nitrogen for 24 h. The solvent was removed *in vacuo*, the residue was taken up in water (100 cm^3) and the resultant suspension was filtered. The filtrate was adjusted to *ca.* pH 1 with concentrated hydrochloric acid and the solvent was removed *in vacuo*. The residue was extracted using a Soxhlet apparatus (ethyl acetate) for 72 h. The solvent was removed *in vacuo* and the residue was purified using Supelco Diaion® HP-20 resin, washing with water (2 dm^3) and eluting with methanol (200 cm^3). The solvent was removed *in vacuo* to give the title compound as a dark purple solid (0.243 g, 62%) m.p. >300 °C; ^{1}H NMR (300 MHz, CD_3OD) δ7.00 (d, J = 8 Hz, 1H, H_7†‡), 7.08 (dd, J = 7, 8 Hz, 1H, H_5≠), 7.24 (d, J = 8 Hz, 1H, $H_{7'}$‡), 7.59 (dd, J = 7, 8 Hz, 1H, H_6≠), 7.75 (d, J = 8 Hz, 1H, H_4†), 7.80 (dd, J = 2, 8 Hz, 1H, $H_{6'}$), 9.62 (d, J = 2 Hz, 1H, $H_{4'}$); ^{13}C NMR (75.5 MHz, CD_3OD) δ109.9, 113.6, 122.9, 124.5, 125.9, 128.2, 138.2; λ_{max}(nm) 221, 291, 358, 545; ν_{max}(cm^{-1}) 3365, 1665, 1630, 1605, 1318, 1118, 850, 780; *m/z* (EI) 364 ([M+Na]$^+$, 27%), 341 ([M-H]$^+$, 57), 280 (45), 199 (72), 122 (100); Found: C, 53.7; H, 4.5; N, 7.4. Calc. for $C_{16}H_{10}N_2O_5S.1.5CH_3OH$: C, 53.8; H, 4.1; N, 7.2%.

†Assignment may be reversed.
‡Assignment may be reversed.
≠Assignment may be reversed.

Preparation and analysis of reaction mixtures containing indoxyl acetate (46) and the isatin derivative 53 in 0.01 mol dm^{-3} pH 10.0 borate buffer

Indoxyl acetate (**46**) (1.6 x 10^{-3} g, 9.13 x 10^{-6} mol) and the sodium salt of isatin-5-sulfonate (**53**) (8.4 x 10^{-3} g, 33.7 x 10^{-6} mol) were dissolved in 10.0 cm^3 of 0.01 mol dm^{-3} pH 10.0 borate buffer. Aliquots of this solution (0.100 cm^3) were added to solutions containing either no cyclodextrin or one of the species **8** and **35-37** in 0.01 mol dm^{-3} pH 10.0 borate buffer (0.900 cm^3) . The reaction mixtures were stirred for 16 h, then brought to *ca.* pH 7 with 2 mol dm^{-3} hydrochloric acid. The product mixtures were extracted with chloroform (2 x 1 cm^3) and the solvent was removed from both the organic and aqueous layers *in vacuo*. The residues were taken up in known volumes of chloroform (1 cm^3) and water (3 cm^3), respectively, and the absorbances were noted at 600 nm and 545 nm, respectively. The molar extinction coefficients under these conditions were found using authentic samples to be 14.0 x 10^3 mol^{-1} dm^3 cm^{-1} for indigo (**50**) and 7.89 x 10^{-3} mol^{-1} dm^3 cm^{-1} for the indirubin derivative **54**. This allowed calculation of the yield of each of the dyes **50** and **54** based on the quantity of indoxyl acetate (**46**) used (Table 23).

Cyclodextrin	Cyclodextrin concentration (mol dm^{-3})	Absorbance of organic layer$^{\neq}$	Absorbance of aqueous layer$^{\neq}$	Yield of the dyes **50** : **54** (%)
-	n/a	0.325	0.010	25 : 1.4
8	12.8 x 10^{-6}	0.021	0.078	1.6 : 11
35	7.0 x 10^{-6}	¥	0.162	<0.1 : 22
36	7.3 x 10^{-6}	0.023	0.257	1.8 : 36
37	7.0 x 10^{-6}	0.077	0.117	6.0 : 16

Table 23. Analysis of reaction mixtures on reaction of indoxyl acetate (**46**) and the isatin derivative **53** in 0.01 mol dm^{-3} pH 10.0 borate buffer by UV spectroscopy.
$^{\neq}$Results are the average of replicate readings from two separate reactions.
¥Below detection limit of the method.

Experimental III: Investigation of the Effect of Cyclodextrins on Product Inhibition of (*S*)-Phenylalanine Ammonia Lyase

Monitoring the digestion of (*S*)-phenylalanine ((*S*)-3) by PAL in the presence of α-cyclodextrin (7) or β-cyclodextrin (8) by ultraviolet spectroscopy

(*S*)-Phenylalanine ((*S*)-**3**) (81.5 x 10^{-3} g, 0.493 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 100 cm^3 with the buffer. Protonated *trans*-cinnamate **4** (37.8 x 10^{-3} g, 0.255 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 50 cm^3 with the buffer. β-Cyclodextrin (**8**) (0.525 g, 0.463 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 50 cm^3 with the buffer. α-Cyclodextrin (**7**) (5.60 g, 5.34 x 10^{-2} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 50 cm^3 with the buffer. All solutions were sonicated to homogeneity. Commercially available PAL solution (0.20 cm^3, *ca.* 0.280 units) was diluted with 0.05 mol dm^{-3} pH 7.0 phosphate buffer (0.20 cm^3) prior to use.

In a quartz cell, with a pathlength of 10^{-3} m, aliquots of the stock solutions of (*S*)-phenylalanine ((*S*)-**3**) (10 x 10^{-3} cm^3) and *trans*-cinnamate (**4**) (10 x 10^{-3} cm^3), as appropriate, were diluted to 0.160 cm^3 with either the buffer solution, the α-cyclodextrin (**7**) stock solution or the β-cyclodextrin (**8**) stock solution. The resulting solutions were equilibrated for *ca.* 10 min at 303 K, then an aliquot of the PAL stock solution (40 x 10^{-3} cm^3, *ca.* 28 x 10^{-3} units) was added. The ultraviolet absorbance was then monitored at 268 nm over a period of 1 h, with the cell-holder thermostatted at 303 K (Figure 7).

Product studies to determine the extent of digestion of (*S*)-phenylalanine ((*S*)-3) by PAL in the presence of α-cyclodextrin (7) or β-cyclodextrin (8)

The appropriate solutions described above for the ultraviolet assays were used in these product studies. 2-^{13}C-Labelled (*S*)-phenylalanine ((*S*)-**55**) (4.1 x 10^{-3} g, 25 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5 cm^3 with the buffer. An aliquot of the *trans*-cinnamate (**4**) stock solution (0.50 cm^3) and an aliquot of the 2-^{13}C-labelled (*S*)-phenylalanine ((*S*)-**55**) stock solution (0.50 cm^3) were mixed in a flask. This mixture was diluted to 8.00 cm^3 with 0.05 mol dm^{-3} pH 7.0 phosphate buffer solution. The resulting solution was equilibrated for
ca. 10 mins at 303 K in a Lauda RC6 constant temperature bath, then PAL solution (2.00 cm^3, *ca.* 0.7 units) was added. The mixture was allowed to stand in the constant temperature bath for 1 h at 303 K, after which it was acidified to pH 1 with concentrated hydrochloric acid and was extracted with chloroform (8 x 40 cm^3). The combined extracts were dried over magnesium sulfate and then concentrated *in vacuo*. The residue was analysed using ^{1}H NMR spectroscopy and gas chromatography / mass spectrometry. ^{1}H NMR (500 MHz, $CDCl_3$) δ6.45 (dd, J_H = 16 Hz, J_C = 164 Hz, 0.16H), 6.45 (d, J_H = 16 Hz, 1H); m/z (EI) 148 (91), 147 (100).

From the NMR data, the reaction proceeded to 16% of completion. By comparison of the mass spectrum with that of a sample of protonated *trans*-cinnamate (**4**) (m/z (EI) 148 (75), 147 (100)), the extent of reaction by mass spectrometry was 16% of completion.

The process was repeated as above, except that the dilution to 8.00 cm^3 was with the β-cyclodextrin (**8**) stock solution. ^{1}H NMR (500 MHz, $CDCl_3$) δ6.45 (dd, J_H = 16 Hz, J_C = 164 Hz, 0.29H), 6.45 (d, J_H = 16 Hz, 1H); m/z (EI) 148 (100), 147 (99).
The extents of reaction calculated in this case from the NMR data and the mass spectrum were 29% and 26% of completion, respectively.

The process was repeated a further time, except that the dilution to 8.00 cm^3 was with the α-cyclodextrin (**7**) stock solution. ^{1}H NMR (500 MHz, $CDCl_3$) δ6.45 (dd, J_H = 16 Hz, J_C = 164 Hz, 0.41H), 6.45 (d, J_H = 16 Hz, 1H); m/z (EI) 148 (100), 147 (88).
The extents of reaction calculated in this case from the NMR data and the mass spectrum were 41% and 42% of completion, respectively.

Diethyl 2-(acetamido)-2-[(4-methylphenyl)methyl)]malonate (69)

To a stirred solution of 4-methylbenzyl chloride (**66**) (2.35 cm^3, 17.8 x 10^{-3} mol) in benzene (2.5 cm^3) was added diethyl acetamidomalonate (3.61 g, 16.6 x 10^{-3} mol) and sodium hydride, as a 60% suspension in oil (0.76 g, 18.8 x 10^{-3} mol NaH). The resultant mixture was heated at reflux under nitrogen for 16 h. The mixture was allowed to cool to room temperature and 1 : 1 benzene / diethyl ether (30 cm^3) and ethanol (10 cm^3) were added. This was extracted with water, which was washed with diethyl ether (30 cm^3). The combined organic layers were washed with water (2 x 30 cm^3), dried over magnesium sulfate and the solvent was removed *in vacuo*, to give the title compound as a colourless powder (4.54 g, 88%). m.p. 113-114.5 °C (lit.,[133] 111-113 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ1.30 (t, *J* = 6 Hz, 6H, $-CH_2C\underline{H}_3$), 2.03 (s, 3H, $-COCH_3$), 2.30 (s, 3H, Ar-CH_3), 3.60 (s, 2H, β-H), 4.26 (q, *J* = 6 Hz, 4H, $-C\underline{H}_2CH_3$), 6.52 (s, 1H, NH), 6.88 (d, *J* = 7 Hz, 2H, Ar-H), 7.06 (d, *J* = 7 Hz, 2H, Ar-H).

4'-Methylphenylalanine (59)

Diethyl 2-(acetamido)-2-[(4-methylphenyl)methyl)]malonate (**69**) (2.70 g, 8.73 x 10^{-3} mol) was heated at reflux in 25% hydrochloric acid (35 cm^3) for 16 h. After being allowed to cool to room temperature, the resulting mixture was filtered to give a pale yellow powder. The residue was redissolved in hot water, and concentrated ammonia solution added to adjust solution to *ca.* pH 7. The precipitate that formed was collected by filtration and was recrystallised from water to give the title compound as a colourless solid (1.23 g, 79%). m.p. 250-253 °C (dec.) (lit.,[133] 276-279 °C (dec.)); ^{1}H NMR (300 MHz, NaOD/D_2O) δ2.32 (s, 3H, Ar-CH_3), 2.80 (dd, *J* = 7, 14 Hz, 1H, β-H), 2.95 (dd, *J* = 5, 14 Hz, 1H, β-H), 3.95 (dd, *J* = 5, 7 Hz, 1H, α-H), 7.1-7.3 (m, 4H, Ar-H).

Diethyl 2-(acetamido)-2-[(2-methylphenyl)methyl)]malonate (70)

To a stirred solution of 2-methylbenzyl chloride (**67**) (2.35 cm^3, 17.8 x 10^{-3} mol) in benzene (2.5 cm^3) was added diethyl acetamidomalonate (3.60 g, 16.6 x 10^{-3} mol) and sodium hydride, as a 60% suspension in oil (0.79 g, 19.7 x 10^{-3} mol NaH). The resultant mixture was heated at reflux under nitrogen for 16 h. The mixture was allowed to cool to room temperature, and 1 : 1 benzene / diethyl ether (30 cm^3) and ethanol

(10 cm^3) were added. This was extracted with water, which was washed with diethyl ether (30 cm^3). The combined organic layers were washed with water (2 x 30 cm^3), dried over magnesium sulfate and the solvent was removed *in vacuo* to give the title compound as a colourless powder (4.32 g, 84%). m.p. 85-88 °C (lit.,[133] 88-89 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ1.29 (t, *J* = 7 Hz, 7H, -$CH_2C\underline{H}_3$), 2.03 (s, 3H, -$COCH_3$), 2.24 (s, 3H, Ar-CH_3), 3.70 (s, 2H, β-H), 4.30 (q, *J* = 7 Hz, 4H, -$C\underline{H}_2CH_3$), 6.57 (s, 1H, NH), 6.9-7.1 (m, 4H, Ar-H).

2'-Methylphenylalanine (60)

Diethyl 2-(acetamido)-2-[(2-methylphenyl)methyl)]malonate (**70**) (2.94 g, 9.50 x 10^{-3} mol) was heated at reflux in 25% hydrochloric acid (35 cm^3) for 24 h. The reaction mixture was allowed to cool to room temperature and the solvent was removed *in vacuo* until crystals formed. The mixture was cooled on an ice bath and the crystals were collected. These were recrystallised from water (adjusted to *ca.* pH 7 with concentrated ammonia solution) to give the title compound as a colourless solid (0.932 g, 55%). m.p. 234-235 °C (dec.) (lit.,[133] 260-262 °C); ^{1}H NMR (300 MHz, $NaOD/D_2O$) δ2.28 (s, 3H, Ar-CH_3), 2.73 (dd, *J* = 7, 14 Hz, 1H, β-H), 2.95 (dd, *J* = 7, 14 Hz, 1H, β-H), 3.40 (t, *J* = 7 Hz, 1H, α-H), 7.1-7.3 (m, 4H, Ar-H).

Diethyl 2-(acetamido)-2-[(2-nitrophenyl)methyl)]malonate (71)

Sodium metal (0.36 g) was dissolved with stirring in ethanol (22 cm^3) and diethyl acetamidomalonate (3.32 g, 15.3 x 10^{-3} mol) was added to the still warm solution. This was allowed to stir for 10 min, then 2-nitrobenzyl bromide (**68**) (3.25 g, 15.0 x 10^{-3} mol) dissolved in benzene (9 cm^3) was added dropwise over 10 min. The resulting mixture was stirred for 4 h at room temperature before being cooled to 273 K. The mixture was filtered and the filtrate was collected. The solvent was removed *in vacuo* to give a pale yellow residue. This was recrystallised from ethanol to give the title compound as colourless crystals (4.22 g, 80%). m.p. 104-105.5 °C (lit.,[132] 103-105 °C); ^{1}H NMR (300 MHz, d_6-DMSO/$CDCl_3$) δ1.16 (t, *J* = 7 Hz, 6H, -$CH_2C\underline{H}_3$), 1.87 (s, 3H, -$COCH_3$), 2.85 (s, 2H, β-H), 4.12 (q, *J* = 7 Hz, 4H, -$C\underline{H}_2CH_3$), 7.25 (d, *J* = 8 Hz, 1H, Ar-H), 7.54 (t, *J* = 8 Hz, 1H, Ar-H), 7.66 (t, *J* = 8 Hz, 1H, Ar-H), 7.90 (d, *J* = 8 Hz, 1H, Ar-H).

2'-Nitrophenylalanine (62)

Diethyl 2-(acetamido)-2-[(2-nitrophenyl)methyl)]malonate (**71**) (1.35 g, 3.83 x 10^{-3} mol) was heated at reflux in concentrated hydrochloric acid (15 cm^3) for 6 h. After being allowed to cool to room temperature, the resulting mixture was filtered. The residue was redissolved in water and neutralised to *ca.* pH 7 with triethylamine. The solvent was removed *in vacuo* and the residue was recrystallised from ethanol / water to give the title compound as an off-white solid (0.646 g, 80%). m.p. 210-211 °C (dec.) (lit.,[132] 206-208 °C); ^{1}H NMR (300 MHz, D_2O) δ3.43 (dd, J = 7, 14 Hz, 1H, β-H), 3.60 (dd, J = 7, 14 Hz, 1H, β-H), 4.28 (t, J = 7 Hz, 1H, α-H), 7.5-7.6 (m, 2H, Ar-H), 7.73 (t, J = 8 Hz, 1H, Ar-H), 8.14 (d, J = 8 Hz, 1H, Ar-H).

Monitoring the digestion of the phenylalanines 42 and 57-65 by PAL using ultraviolet spectroscopy

4'-Fluorophenylalanine (**57**) (9.5 x 10^{-3} g, 52 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. 2'-Fluorophenylalanine (**58**) (1.9 x 10^{-3} g, 10 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 1.00 cm^3 with the buffer. 4'-Methylphenylalanine (**59**) (10.0 x 10^{-3} g, 52.2 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. 2'-Methylphenylalanine (**60**) (9.3 x 10^{-3} g, 48 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. 4'-Nitrophenylalanine (**61**) (10.4 x 10^{-3} g, 49.5 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. 2'-Nitrophenylalanine (**62**) (10.5 x 10^{-3} g, 50.0 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. 4'-Chlorophenylalanine (**63**) (10.5 x 10^{-3} g, 52.6 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. 2'-Chlorophenylalanine (**64**) (2.0 x 10^{-3} g, 10 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 1.00 cm^3 with the buffer. Tyrosine (**42**) (9.1 x 10^{-3} g, 50 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. 2'-Hydroxylphenylalanine (**65**) (9.1 x 10^{-3} g, 50 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. Aliquots of each of these solutions (20 x 10^{-3} cm^3) were taken, diluted to 0.350 cm^3 with 0.05 mol dm^{-3} pH 7.0 phosphate buffer and commercial PAL solution (50 x 10^{-3} cm^3,

ca. 0.125 units) was added. These reaction mixtures were placed in a quartz cell, with a pathlength of 10^{-3} m, thermostatted at 303 K and the change in absorbance at 275 nm was monitored for 1 h.

Monitoring the digestion of 2'-chlorophenylalanine (64) and 4'-chlorophenylalanine (63) in the presence of either α-cyclodextrin (7) or β-cyclodextrin (8) by ultraviolet spectroscopy

4'-Chlorophenylalanine (**63**) (10.5 x 10^{-3} g, 52.6 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. 2'-Chlorophenylalanine (**64**) (10.4 x 10^{-3} g, 52.1 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. Protonated *trans*-2'-chlorocinnamate (**79**) (4.7 x 10^{-3} g, 25.7 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. Protonated *trans*-4'-chlorocinnamate (**78**) (5.0 x 10^{-3} g, 27.4 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. α-Cyclodextrin (**7**) (0.467 g, 0.480 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. β-Cyclodextrin (**8**) (58.0 x 10^{-3} g, 51.1 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. All solutions were sonicated to homogeneity.

In a quartz cell, with a pathlength of 10^{-3} m, aliquots of the stock solutions of the phenylalanine, and cinnamate where appropriate, were diluted with the appropriate cyclodextrin solution and the volume was made up to 0.350 cm^3 with either the buffer solution, the α-cyclodextrin (**7**) stock solution or the β-cyclodextrin (**8**) stock solution (Table 24). The resulting solutions were equilibrated for *ca.* 10 min at 303 K, then an aliquot of PAL solution (50.0 x 10^{-3} cm^3, *ca.* 0.130 units) was added. The ultraviolet absorbance of the resulting solutions was monitored at either 265 nm (2'-chlorophenylalanine (**64**) case) or 275 nm (4'-chlorophenylalanine (**63**) case) for a period of 1 h with the cell-holder thermostatted at 303 K. The results are shown as Figures 9 and 10.

Phenylalanine	Volume of phenylalanine solution (cm^3)	Cinnamate	Volume of cinnamate solution (cm^3)	Cyclodextrin	Volume of cyclodextrin solution (cm^3)
63	20.0×10^{-3}	-	-	-	-
63	20.0×10^{-3}	**78**	20.0×10^{-3}	-	-
63	20.0×10^{-3}	-	-	**7**	0.300
63	20.0×10^{-3}	**78**	20.0×10^{-3}	**7**	0.300
63	20.0×10^{-3}	-	-	**8**	0.300
63	20.0×10^{-3}	**78**	20.0×10^{-3}	**8**	0.300
64	20.0×10^{-3}	-	-	-	-
64	20.0×10^{-3}	**79**	20.0×10^{-3}	-	-
64	20.0×10^{-3}	-	-	**7**	0.300
64	20.0×10^{-3}	**79**	20.0×10^{-3}	**7**	0.300
64	20.0×10^{-3}	-	-	**8**	0.300
64	20.0×10^{-3}	**79**	20.0×10^{-3}	**8**	0.300

Table 24. Preparation of solutions for monitoring the digestion of the chlorinated phenylalanines **63** and **64** by PAL.

Experimental IV: Investigation of the Effect of Cyclodextrins on Equilibrations Catalysed by (*S*)-Phenylalanine Ammonia Lyase

Monitoring the digestion of (*S*)-phenylalanine ((*S*)-3) by PAL in the presence of the 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (40) by ultraviolet spectroscopy

(*S*)-Phenylalanine ((*S*)-**3**) (82.4 x 10^{-3} g, 0.499 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 100 cm^3 with the buffer. 6^{A}-Amino-6^{A}-deoxy-β-cyclodextrin (**40**) (1.57 g, 1.38 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer, the pH was adjusted to 7.0 with 2 mol dm^{-3} hydrochloric acid and the volume was made up to 10 cm^3 with the buffer. The pH was checked and found to be as required. All solutions were sonicated to homogeneity. Commercially available PAL solution (0.20 cm^3, *ca.* 0.280 units) was diluted with 0.05 mol dm^{-3} pH 7.0 phosphate buffer (0.20 cm^3) prior to use.

In a quartz cell, with a pathlength of 10^{-3} m, an aliquot of the stock solution of (*S*)-phenylalanine ((*S*)-**3**) (10 x 10^{-3} cm^3) was taken and a quantity of the amine **40** stock solution was added (Table 25). These reaction mixtures were diluted to 0.160 cm^3 with 0.05 mol dm^{-3} pH 7.0 phosphate buffer. The resulting solutions were equilibrated for *ca.* 10 min at 303 K, then an aliquot of the PAL stock solution (40 x 10^{-3} cm^3, *ca.* 28 x 10^{-3} units) was added. The ultraviolet absorbance was then monitored at 268 nm over a period of 1 h, with the cell-holder thermostatted at 303 K (Figure 11).

Vessel	Amine **40** stock solution added (cm^3)	Concentration of amine **40** in final solution (mol dm^{-3})
(b)	0	0
(a)	40.0 x 10^{-3}	27.6 x 10^{-3}
(c)	80.0 x 10^{-3}	55.2 x 10^{-3}
(d)	150 x 10^{-3}	0.104 x 10^{-3}

Table 25. Preparation of 6^{A}-amino-6^{A}-deoxy-β-cyclodextrin (**40**) solutions for ultraviolet assays.

Monitoring the ratio of *trans*-cinnamate 4 to (*S*)-phenylalanine ((*S*)-3) in solutions containing ammonium ions, PAL and either α-cyclodextrin (7), β-cyclodextrin (8) or 6^A-amino-6^A-deoxy-β-cyclodextrin (40)

The protonated form of the cinnamate **4** (19.0×10^{-3} g, 0.128×10^{-3} mol) and benzoic acid (24.0×10^{-3} g, 0.197×10^{-3} mol) were dissolved in 0.05 mol dm^{-3} pH 7.0 Tris buffer ($[NH_4^+]$ = 0.5 mol dm^{-3}) and the volume was made up to 5.00 cm^3 with the buffer. β-Cyclodextrin (**8**) (59.1×10^{-3} g, 52.1×10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 Tris buffer ($[NH_4^+]$ = 0.5 mol dm^{-3}) and the volume was made up to 5.0 cm^3 with the buffer. α-Cyclodextrin (**7**) (0.414 g, 0.426×10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 Tris buffer ($[NH_4^+]$ = 0.5 mol dm^{-3}) and the volume was made up to 5.0 cm^3 with the buffer. 6^A-Amino-6^A-deoxy-β-cyclodextrin (**40**) (0.234 g, 0.206×10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 Tris buffer ($[NH_4^+]$ = 0.5 mol dm^{-3}), the pH was adjusted to 7.0 with 2 mol dm^{-3} hydrochloric acid and the volume was made up to 2.0 cm^3 with the buffer, with monitoring and adjusting of the pH. An aliquot of the cinnamate **4** solution (10.0×10^{-6} cm^3), was diluted with cyclodextrin solution (0.800 cm^3), where appropriate, and the volume was made up to 0.900 cm^3 with 0.05 mol dm^{-3} pH 7.0 Tris buffer ($[NH_4^+]$ = 0.5 mol dm^{-3}). Commercial PAL solution (0.100 cm^3, *ca.* 0.30 units) was added and the reaction analysed by HPLC on a YMC ODS-AQ column (4.6×10^{-3} m by 0.250 m) (acetonitrile : water : trifluoroacetic acid, 50 : 49.9 : 0.1) monitoring at 260 nm. Analysis continued until no further change in the amount of the cinnamate **4** present was observed. From the ratio of benzoate to the cinnamate **4**, the amount of (*S*)-phenylalanine ((*S*)-**3**) present in solution was determined, and hence the ratio of the cinnamate **4** to the phenylalanine (*S*)-**3** was calculated (Table 11, reproduced below).

Cyclodextrin	Ratio of the cinnamate **4** to the phenylalanine (*S*)-**3** at equilibrium§
-	8.5 : 1
8	23 : 1
7	>100 : 1
40	3.5 : 1

Table 11. The ratio of the cinnamate **4** to the phenylalanine (*S*)-**3** (total concentration 0.26×10^{-3} mol dm^{-3}) present at equilibrium in reactions containing PAL (*ca.* 300 units dm^{-3}), ammonium ions (0.5 mol dm^{-3}) and either no cyclodextrin, α-cyclodextrin (**7**) (67×10^{-3} mol dm^{-3}), β-cyclodextrin (**8**) (8.2×10^{-3} mol dm^{-3}) or 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) (81×10^{-3} mol dm^{-3}) in 0.05 mol dm^{-3} pH 7.0 Tris buffer at 303 K, as observed by HPLC.
§Average of values, which differ by less than 20%, from duplicate reactions .

Calculation of the association constant of the complex of *trans*-cinnamate (4) with 6^A-amino-6^A-deoxy-β-cyclodextrin (40)

The protonated form of *trans*-cinnamate (**4**) (14.8 x 10^{-3} g, 99.9 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer, and the volume was made up to 50 cm^3 with the buffer. Stock solutions were prepared by dissolving the amine **40** (I: 0.269 g, 0.237 x 10^{-3} mol, II: 0.176 g, 0.155 x 10^{-3} mol) in 0.05 mol dm^{-3} pH 7.0 phosphate buffer, the pH was adjusted to 7.0 with 2 mol dm^{-3} hydrochloric acid and the volumes were made up with the buffer (I: 5.00 cm^3, II: 1.00 cm^3), with checking and adjusting of the pH. All solutions were sonicated to homogeneity. The amine **40** stock solution was taken (Table 26), cinnamate **4** stock solution (0.500 cm^3) and deuterium oxide (0.100 cm^3) were added, and the solution was made up to 1.00 cm^3 with the buffer. The amount of cyclodextrin **40** stock solution added was varied to give from 0 to 40 equivalents of the host **40** with respect to the guest **4**. An aliquot of each sample was analysed by ^1H NMR spectroscopy at 298 K. The chemical shift of the α-proton was observed to change with the concentration of the host **40**. Sample preparation and observed chemical shift changes between the signals corresponding to the α-proton and 4'-proton of the cinnamate **4** are summarised below (Table 26).

Chemical shift information was plotted against concentration of the amine **40** and a non-linear regression analysis (using Equations 1 and 2) gave the association constant for the complex formed as 270 ± 20 mol^{-1} dm^3 (Figure 12).

Stock solution	Cyclodextrin **40** stock solution (cm^3)	[Amine **40**] (mol dm^{-3})	Observed chemical shift difference (Hz)
I	-	-	435.57
I	12.5 x 10^{-3}	0.593 x 10^{-3}	440.73
I	25.0 x 10^{-3}	1.19 x 10^{-3}	445.10
I	37.5 x 10^{-3}	1.78 x 10^{-3}	448.43
I	50.0 x 10^{-3}	2.37 x 10^{-3}	452.42
I	75.0 x 10^{-3}	3.56 x 10^{-3}	457.06
I	0.100	4.74 x 10^{-3}	460.48
I	0.130	6.16 x 10^{-3}	464.14
I	0.165	7.82 x 10^{-3}	467.07
I	0.200	9.48 x 10^{-3}	469.03
I	0.250	11.9 x 10^{-3}	471.96
I	0.300	14.2 x 10^{-3}	473.42
I	0.400	19.0 x 10^{-3}	475.13
II	0.170	26.4 x 10^{-3}	477.10
II	0.250	38.8 x 10^{-3}	478.92

Table 26. Sample preparation and observed chemical shift difference between the resonances corresponding to the α-proton and the 4'-proton of *trans*-cinnamate (**4**) used for the calculation of the association constant of the complex of *trans*-cinnamate (**4**) with 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) in 0.05 mol dm^{-3} phosphate buffered deuterium oxide at pH 7.0 and 298 K.

Effect of 6^A-amino-6^A-deoxy-β-cyclodextrin (40) on the fluorescence spectrum on 2-(4-toluidino)naphthalene-6-sulfonate (82)

The sodium salt of 2-(4-toluidino)naphthalene-6-sulfonate (**82**) (3.4 x 10^{-3} g, 10.1 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer and the volume was made up to 50.0 cm^3 with the buffer. 6^A-Amino-6^A-deoxy-β-cyclodextrin (**40**) (I: 9.4 x 10^{-3} g, 8.29 x 10^{-6} mol, II: 0.116 g, 0.102 x 10^{-3}) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer, the pH was adjusted to 7.0 with 2 mol dm^{-3} hydrochloric acid and the volume was made up to 10.0 cm^3 with the buffer, with checking and adjusting of the pH. All solutions were sonicated to homogeneity. The amine **40** stock solution was taken (Table 27), fluorophore **82** stock solution (7.5 x 10^{-3} cm^3) was added and the solution was made up to 1.50 cm^3 with the buffer. The amount of cyclodextrin **40** stock solution added was varied to give from 0 to 10000 equivalents of the host **40** with respect to the guest **82**. Each sample was excited at 324 nm (excitation slit widths 8 nm) and the fluorescence spectrum recorded (emission slit widths 8 nm). The fluorescent intensity at 440 nm was noted (Table 27) and plotted against cyclodextrin concentration (Figure 39).

Stock solution	Cyclodextrin **40** stock solution (cm^3)	[Amine **40**] (mol dm^{-3})	Observed fluorescent intensity at 440 nm
-	-	-	4760
I	75.0 x 10^{-3}	41.4 x 10^{-6}	8000
I	0.150	82.9 x 10^{-6}	11500
I	0.225	0.124 x 10^{-3}	15000
I	0.300	0.166 x 10^{-3}	18000
I	0.375	0.207 x 10^{-3}	20800
I	0.450	0.249 x 10^{-3}	21300
I	0.600	0.332 x 10^{-3}	24500
I	0.750	0.414 x 10^{-3}	27800
I	1.13	0.624 x 10^{-3}	36000
I	1.43	0.790 x 10^{-3}	39000
II	0.100	0.680 x 10^{-3}	34200
II	0.200	1.36 x 10^{-3}	48800
II	0.300	2.04 x 10^{-3}	63100
II	0.400	2.72 x 10^{-3}	71000
II	0.500	3.40 x 10^{-3}	79600
II	0.600	4.08 x 10^{-3}	94700
II	0.750	5.10 x 10^{-3}	106000
II	0.900	6.12 x 10^{-3}	123000
II	1.05	7.14 x 10^{-3}	134000
II	1.20	8.16 x 10^{-3}	149000
II	1.25	8.50 x 10^{-3}	152000
II	1.43	9.73 x 10^{-3}	170000

Table 27. Sample preparation and observed fluorescent intensity at 440 nm (after excitation at 324 nm) for 2-(4-toluidino)naphthalene-6-sulfonate (**82**) in the presence of varying concentrations of 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) in 0.05 mol dm^{-3} pH 7.0 phosphate buffer at 298 K.

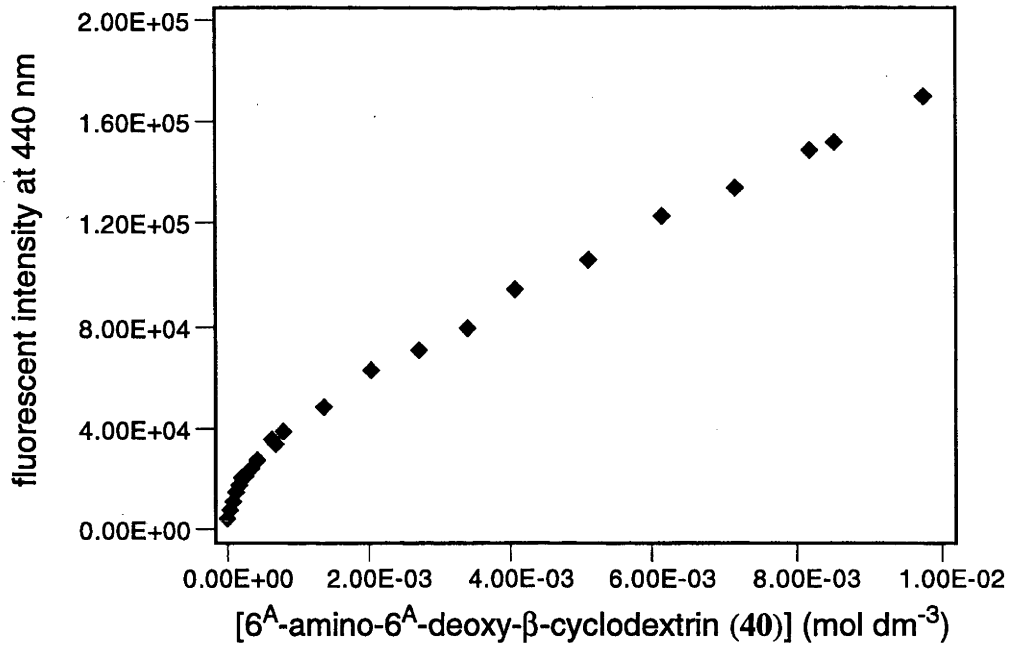


Figure 39. Fluorescence intensity at 440 nm plotted against the concentration of 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) for 2-(4-toluidino)naphthalene-6-sulfonate (**82**) in 0.05 mol dm^{-3} pH 7.0 phosphate buffer at 298 K excited at 324 nm.

Calculation of the association constant of the complex of (*S*)-phenylalanine ((*S*)-3) in 6^A-amino-6^A-deoxy-β-cyclodextrin (40)

The protonated form of *trans*-cinnamate **4** (72.3 x 10^{-3} g, 0.488 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer, and the volume was made up to 50.0 cm^3 with the buffer. An aliquot of this solution (1.00 cm^3) and deuterium oxide (1.00 cm^3) were used to dissolve 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) (0.115 g, 0.101 x 10^{-3} mol), the pH was adjusted to 7.0 with 2 mol dm^{-3} hydrochloric acid and the volume was made up to 5.00 cm^3 with 0.05 mol dm^{-3} pH 7.0 phosphate buffer, with checking and adjusting of the pH. (*S*)-Phenylalanine ((*S*)-**3**) (1.65 g, 9.99 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer, and the volume was made up to 100 cm^3 with the buffer. All solutions were sonicated to homogeneity. An aliquot of the amine **40** / cinnamate **4** solution was taken (0.500 cm^3), (*S*)-phenylalanine ((*S*)-**3**) stock solution was added (Table 28) and the solution was made up to 1.00 cm^3 with the buffer. An aliquot of each sample was analysed by ^{1}H NMR spectroscopy at 298 K. The chemical shift changes observed between the signals corresponding to the α-proton and the 4'-proton of the cinnamate **4** were observed and the association constant of the complex formed between the amine **40** and (*S*)-phenylalanine ((*S*)-**3**) was calculated as per Appendix 2 (Table 28). This gave a value for the association complex as 9.6 ± 0.3 mol^{-1} dm^3.

Phenylalanine (*S*)-**3** stock solution (cm^3)	[Phenylalanine (*S*)-**3** ($mol\ dm^{-3}$)	Observed chemical shift difference (Hz)	Association constant ($mol^{-1}\ dm^3$)
0.100	9.99×10^{-3}	469.10	9.52
0.200	20.0×10^{-3}	468.23	10.0
0.300	30.0×10^{-3}	467.53	9.60
0.400	40.0×10^{-3}	466.85	9.41
0.500	50.0×10^{-3}	466.17	9.37

Table 28. Sample preparation, observed chemical shift difference between the signals corresponding to the α-proton and the 4'-proton of the cinnamate **4** and calculated association constant for the complex of (*S*)-phenylalanine ((*S*)-**3**) with the amine **40** in 0.05 mol dm^{-3} phosphate buffered deuterium oxide at pH 7.0 and 298 K.

Experimental V: Investigation of Acylase I Catalysed Hydrolysis of *para*-Substituted (*S*)-Phenylalanine Derivatives from Mixtures of the Racemic *ortho*- and *para*-Substituted Isomers

General preparation of the *N*-acetylphenylalanines 83-91

The appropriate phenylalanine and triethylamine (1.2 cm^3 per g of starting material) were added to water (90 cm^3 per g of starting material) and the mixture was stirred until the solid dissolved. Acetic anhydride (0.6 cm^3 per g of starting material) was added and the mixture was stirred for 3 h at room temperature. The mixture was acidified to *ca.* pH 1 with 2 mol dm^{-3} hydrochloric acid and was extracted with ethyl acetate (4 x 100 cm^3 per g of starting material). The combined organic extracts were dried over magnesium sulfate and the solvent was removed *in vacuo*. The residue was recrystallised from ethyl acetate / hexane to give the desired *N*-acetylphenylalanine. A small sample of the product was added to thionyl chloride (5 cm^3 per g) pretreated methanol (500 cm^3 per g) and the solution was stirred for 2 h at room temperature. The solvent was removed *in vacuo* and the residue was analysed by GC on a Chirasil-Val capillary column (0.3 x 10^{-3} m x 25 m).

N-*Acetylphenylalanine (83)*
Starting Material: 1.00 g Yield: 1.19 g, 95% Appearance: Colourless powder m.p. 146-147 °C (lit.,[163] 150 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.77 (s, 3H, -$COCH_3$), 2.81 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.03 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.39 (m, 1H, α-H), 7.1-7.3 (m, 5H, Ar-H), 8.19 (d, *J* = 8 Hz, 1H, NH).
Methyl Ester: GC (180 °C): t_R = 4.4, 4.6 min

(S)-N-*Acetylphenylalanine ((S)-83)*
Starting Material: 0.900 g Yield: 1.04 g, 93% Appearance: Colourless powder m.p. 172-174 °C (lit.,[165] 170-171 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.79 (s, 3H, -$COCH_3$), 2.84 (dd, *J* = 10, 13 Hz, 1H, β-H), 3.04 (dd, *J* = 5, 13 Hz, 1H, β-H), 4.40 (m, 1H, α-H), 7.1-7.3 (m, 5H, Ar-H), 8.20 (d, *J* = 8 Hz, 1H, NH).
Methyl Ester: GC (180 °C): t_R = 4.6 min

(R)-N-Acetylphenylalanine ((R)-83)

Starting Material: 0.106 g Yield: 0.127 g, 98% Appearance: Colourless powder m.p. 173-175 °C (lit.,[163] 172 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.79 (s, 3H, -$COCH_3$), 2.84 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.05 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.40 (m, 1H, α-H), 7.1-7.3 (m, 5H, Ar-H), 8.20 (d, *J* = 8 Hz, 1H, NH).

Methyl Ester: GC (180 °C): t_R = 4.4 min

N-*Acetyl-4'-fluorophenylalanine (84)*

Starting material: 0.487 g Yield: 0.488 g, 89% Appearance: Colourless powder m.p. 152-152.5 °C (lit.,[158] 150.5-152 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.77 (s, 3H, -$COCH_3$), 2.78 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.02 (dd, *J* = 4, 14 Hz, 1H, β-H), 4.40 (m, 1H, α-H), 7.1 (m, 2H, Ar-H), 7.3 (m, 2H, Ar-H), 8.35 (d, *J* = 8 Hz, 1H, NH).

Methyl Ester: GC (190 °C): t_R = 4.25, 4.40 min

(S)-N-Acetyl-4'-fluorophenylalanine ((S)-84)

Starting material: 0.297 g Yield: 0.299 g, 90% Appearance: Colourless powder m.p. 141-142 °C (lit.,[158] 142-143 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.79 (s, 3H, -$COCH_3$), 2.82 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.04 (dd, *J* = 4, 14 Hz, 1H, β-H), 4.37 (m, 1H, α-H), 7.1 (m, 2H, Ar-H), 7.3 (m, 2H, Ar-H), 8.22 (d, *J* = 8 Hz, 1H, NH).

Methyl Ester: GC (190 °C): t_R = 4.40 min

N-*Acetyl-2'-fluorophenylalanine (85)*

Starting material: 0.495 g Yield: 0.581g, 95% Appearance: Colourless powder m.p. 154-154.5 °C (lit.,[158] 147-149 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.78 (s, 3H, -$COCH_3$), 2.84 (dd, *J* = 5, 10 Hz, 1H, β-H), 3.15 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.46 (m, 1H, α-H), 7.1-7.3 (m, 4H, Ar-H), 8.26 (d, *J* = 8 Hz, 1H, NH), 12.70 (s, 1H, CO_2H).

Methyl Ester: GC (180 °C): t_R = 5.25, 5.5 min

N-*Acetyl-4'-methylphenylalanine (86)*

Starting material: 0.245 g Yield: 0.299 g, 98% Appearance: Colourless powder m.p. 166-167 °C (lit.,[159] 164 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.89 (s, 3H, -$COCH_3$), 2.37 (s, 3H, Ar-CH_3), 2.89 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.10 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.45 (m, 1H, α-H), 7.15-7.25 (m, 4H, Ar-H), 8.26 (d, *J* = 8 Hz, 1H, NH).

Methyl Ester: GC (140 °C): t_R = 18.1, 20.0 min

N-*Acetyl-2'-methylphenylalanine (87)*

Starting material: 0.171 g Yield: 0.199 g, 95% Appearance: Colourless solid m.p. 162-162.5 °C (lit.,[160] 148 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.89 (s, 3H, -$COCH_3$), 2.40 (s, 3H, Ar-CH_3), 2.91 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.18 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.51 (m, 1H, α-H), 7.1-7.2 (m, 4H, Ar-H), 8.37 (d, *J* = 8 Hz, 1H, NH).

Methyl Ester: GC (140 °C): t_R = 18.9, 20.1 min

N-*Acetyl-4'-nitrophenylalanine (88)*

Starting material: 0.197 g Yield: 0.225 g, 95% Appearance: Colourless powder m.p. 206-207 °C (lit.,[161] 207-209 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.72 (s, 3H, -$COCH_3$), 2.99 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.21 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.5 (m, 1H, α-H), 7.53 (d, *J* = 7 Hz, 2H, Ar-H), 8.17 (d, *J* = 7 Hz, 2H, Ar-H), 8.28 (d, *J* = 8 Hz, 1H, NH).

Methyl Ester: GC (180 °C): t_R = 23.0, 25.0 min

(S)-N-*Acetyl-4'-nitrophenylalanine ((S)-88)*

Starting material: 0.598 g Yield: 0.681 g, 95% Appearance: Pale yellow powder m.p. 167-168 °C (lit.,[161] 170-172 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.79 (s, 3H, -$COCH_3$), 3.00 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.22 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.5 (m, 1H, α-H), 7.54 (d, *J* = 9 Hz, 2H, Ar-H), 8.19 (d, *J* = 9 Hz, 2H, Ar-H), 8.30 (d, *J* = 8 Hz, 1H, NH).

Methyl Ester: GC (180 °C): t_R = 23.0 min

N-*Acetyl-2'-nitrophenylalanine (89)*

Starting material: 0.335 g Yield: 0.345 g, 86% Appearance: Off-white powder m.p. 201-203 °C (lit.,[132] 200-202 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.77 (s, 3H, -$COCH_3$), 3.02 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.22 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.6 (m, 1H, α-H), 7.4-7.7 (m, 3H, Ar-H), 7.98 (d, *J* = 9 Hz, 1H, Ar-H), 8.30 (d, *J* = 10 Hz, 1H, NH).

Methyl Ester: GC (180 °C): t_R = 12.5, 13.0 min

N-*Acetyl-4'-chlorophenylalanine (90)*

Starting material: 0.248 g Yield: 0.285 g, 95% Appearance: Colourless powder m.p. 183-184 °C (lit.,[162] 184 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.70 (s, 3H, -$COCH_3$), 2.67 (dd, *J* = 9, 14 Hz, 1H, β-H), 2.95 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.19 (dd, *J* = 5, 9 Hz, 1H, α-H), 7.02 (d, *J* = 8 Hz, 2H, Ar-H), 7.14 (d, *J* = 8 Hz, 2H, Ar-H).

Methyl Ester: GC (160 °C): t_R = 14.1, 14.7 min

*(S)-N-Acetyl-4'-chlorophenylalanine ((S)-**90**)*

Starting material: 90 x 10^{-3} g Yield: 85 x 10^{-3} g, 86% Appearance: Colourless solid m.p. 150-151 °C (lit.,[166] 149-153 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.79 (s, 3H, -$COCH_3$), 2.84 (dd, *J* = 9, 14 Hz, 1H, β-H), 3.05 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.40 (dd, *J* = 5, 9 Hz, 1H, α-H), 7.26 (d, *J* = 8 Hz, 2H, Ar-H), 7.35 (d, *J* = 8 Hz, 2H, Ar-H).

Methyl Ester: GC (160 °C): t_R = 14.7 min

N-*Acetyl-2'-chlorophenylalanine (**91**)*

Starting material: 51.1 x 10^{-3} g Yield: 58.5 x 10^{-3} g, 95% Appearance: Colourless solid m.p. 164 °C (lit.,[162] 166 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.74 (s, 3H, -$COCH_3$), 2.83 (dd, *J* = 9, 14 Hz, 1H, β-H), 3.22 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.44 (m, 1H, α-H), 7.2-7.4 (m, 4H, Ar-H), 8.25 (d, *J* = 7 Hz, NH).

Methyl Ester: GC (160 °C): t_R = 27.0, 27.6 min

*(S)-N-Acetyl-2'-chlorophenylalanine ((S)-**91**)*

Starting material: 43 x 10^{-3} g Yield: 50 x 10^{-3} g, 96% Appearance: Colourless powder m.p. 158-160 °C; ^{1}H NMR (300 MHz, d_6-DMSO) δ1.64 (s, 3H, -$COCH_3$), 2.73 (dd, *J* = 9, 14 Hz, 1H, β-H), 3.16 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.33 (dd, *J* = 5, 9 Hz, 1H, α-H), 7.2-7.4 (m, 4H, Ar-H).

Methyl Ester: GC (160 °C): t_R = 27.6 min

(*S*)-*N*-Acetyl-2'-fluorophenylalanine ((*S*)-85)

N-Acetyl-2'-fluorophenylalanine (**85**) (0.110 g, 0.488 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer (10 cm^3), and the pH was adjusted to 7.0 with ammonia solution. Acylase I (10 x 10^{-3} g, 0.12 x 10^{-6} mol) was added and the mixture was stirred at 298 K for 6 days. The reaction mixture was treated with charcoal at 333 K for 10 min, filtered and the filtrate was acidified to *ca.* pH 1 with 2 mol dm^{-3} hydrochloric acid and was extracted with ethyl acetate (4 x 10 cm^3). The solvent was removed *in vacuo* and the residue was taken up in water (2.5 cm^3). Triethylamine (0.3 cm^3) and acetic anhydride (0.3 cm^3) were added and the mixture was stirred at room temperature for 3 h. The mixture was acidified to *ca.* pH 1 with 2 mol dm^{-3} hydrochloric acid and was extracted with ethyl acetate (4 x 10 cm^3). The combined organic extracts were dried over magnesium sulfate and the solvent was removed *in vacuo*. The residue was purified by preparative thin layer chromatography (ethanol : methanol : water : acetic acid, 19 : 3 : 2 : 1) and recrystallised from ethyl acetate / hexane to give the title compound as a colourless solid (28.1 x 10^{-3} g, 25%). m.p. 164-166 °C (lit.,[158] 168-170 °C); ^{1}H NMR

(300 MHz, d_6-DMSO) δ1.71 (s, 3H, -$COCH_3$), 2.69 (dd, J = 9, 14 Hz, 1H, β-H), 3.17 (dd, J = 6, 14 Hz, 1H, β-H), 4.1 (m, 1H, α-H), 7-7.30 (m, 4H, Ar-H), 8.24 (d, J = 8 Hz, 1H, NH), 12.75 (br s, 1H, CO_2H).

A sample (*ca.* 2 x 10^{-3} g) of the product was added to thionyl chloride (0.01 cm^3) pretreated methanol (1 cm^3) and the solution was stirred for 2 h at room temperature. The solvent was removed *in vacuo* and the residue was analysed by GC on a Chirasil-Val capillary column (0.3 x 10^{-3} m x 25 m). GC (180 °C): t_R = 5.50 min

_N_-Acetyltyrosine (92)

Tyrosine (**42**) (1.45 g, 8.00 x 10^{-3} mol) was dissolved in water (2.5 cm^3) and 2 mol dm^{-3} sodium hydroxide solution (0.65 cm^3) and the solution cooled in ice. Acetic anhydride (2.0 cm^3) and 2 mol dm^{-3} sodium hydroxide solution (20 cm^3) were each added in 8 equal portions with cooling and shaking over 20 min and the resultant mixture was allowed to sit at room temperature for 40 min. To this was added 6 mol dm^{-3} sulfuric acid (8.4 cm^3) and the resultant solution was cooled at 277 K for 16 h. The solvent was removed *in vacuo* and the residue triturated with acetone. The residue was taken up in ethyl acetate (50 cm^3) and washed with water (50 cm^3), which was subsequently washed with ethyl acetate (2 x 50 cm^3). The combined organic layers were dried over magnesium sulfate and the solvent was removed *in vacuo*. The residue was recrystallised from ethyl acetate / hexane to give the title compound as colourless crystals (1.14 g, 64%). m.p. 121-122 °C (lit.,[187] 94-95 °C for the hydrate); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.78 (s, 3H, -$COCH_3$), 2.73 (dd, J = 9, 14 Hz, 1H, β-H), 2.85 (dd, J = 6, 14 Hz, 1H, β-H), 4.3 (m, 1H, α-H), 6.64 (d, J = 8 Hz, 2H, Ar-H), 6.97 (d, J = 8 Hz, 2H, Ar-H), 8.27 (d, J = 8 Hz, 1H, NH).

A sample (*ca.* 10 x 10^{-3} g) of the product was added to thionyl chloride (0.05 cm^3) pretreated methanol (5 cm^3) and the solution was stirred for 2 h at room temperature. The solvent was removed *in vacuo* and the residue was analysed by GC on a Chirasil-Val capillary column (0.3 x 10^{-3} m x 25 m). GC (140 °C): t_R = 13.0, 13.6 min

(*S*)-*N*-Acetyltyrosine ((*S*)-92)

A suspension of triethylamine (14 cm^3) in water (50 cm^3) was prepared. (*S*)-Tyrosine ((*S*)-**42**) (1.51 g, 8.33 x 10^{-3} mol) was taken up in water (2.5 cm^3) and triethylamine suspension (4.25 cm^3). Acetic anhydride (2.0 cm^3) and triethylamine suspension (20 cm^3) were each added in 8 equal portions with cooling and shaking over 20 min and

the reaction mixture was allowed to sit at room temperature for 40 min. To this was added 6 mol dm^{-3} sulfuric acid (8.4 cm^3) and the resultant solution was cooled at 277 K for 16 h. The solvent was removed *in vacuo* to give an orange oil, which was partitioned between water and ethyl acetate (3 x 50 cm^3). The combined organic layers were dried over magnesium sulfate and the solvent was removed *in vacuo* to give a yellow solid. This was recrystallised from ethyl acetate / hexane to give the title compound as a colourless powder (0.989 g, 54%). m.p. 151-152 °C (lit.,[164] 153-154 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.77 (s, 3H, -$COCH_3$), 2.70 (dd, J = 9, 14 Hz, 1H, β-H), 2.90 (dd, J = 5, 14 Hz, 1H, β-H), 4.3 (m, 1H, α-H), 6.64 (d, J = 9 Hz, 2H, Ar-H), 7.00 (d, J = 9 Hz, 2H, Ar-H), 8.10 (d, J = 8 Hz, 1H, NH), 9.19 (s, 1H, Ar-OH)

A sample (*ca.* 20 x 10^{-3} g) of the product was added to thionyl chloride (0.1 cm^3) pretreated methanol (10 cm^3) and the solution was stirred for 2 h at room temperature. The solvent was removed *in vacuo* and the residue was analysed by GC on a Chirasil-Val capillary column (0.3 x 10^{-3} m x 25 m). GC (140 °C): t_R = 13.6 min

_N_-Acetyl-2'-hydroxyphenylalanine (93)

2'-Hydroxyphenylalanine (**65**) (0.228 g, 1.26 x 10^{-3} mol) was dissolved in water (0.38 cm^3) and 2 mol dm^{-3} sodium hydroxide solution (0.65 cm^3) and this solution cooled in ice. Acetic anhydride (0.30 cm^3) and 2 mol dm^{-3} sodium hydroxide solution (3.0 cm^3) were each added in 6 equal portions with cooling and shaking over 20 min and the resultant mixture was allowed to sit at room temperature for 40 min. To this was added 6 mol dm^{-3} sulfuric acid (1.3 cm^3) and the resultant solution was cooled at 277 K for 16 h. The solvent was removed *in vacuo* and the residue was triturated with acetone. The residue was taken up in ethyl acetate (10 cm^3) and washed with water (10 cm^3), which was subsequently washed with ethyl acetate (2 x 10 cm^3). The combined organic layers were dried over magnesium sulfate and the solvent was removed *in vacuo*. The residue was recrystallised from ethyl acetate / hexane to give the title compound as a hygroscopic colourless powder (0.201 g, 72%). m.p. 142-144 °C; ^{1}H NMR (300 MHz, d_6-DMSO) δ1.92 (s, 3H, -$COCH_3$), 3.09 (m, 2H, β-H), 4.76 (m, 1H, α-H), 7.1-7.2 (m, 4H, Ar-H), 8.54 (d, J = 8 Hz, 1H, NH); ^{13}C NMR (75.5 MHz, d_6-DMSO) δ22.5 (β-C), 32.1 (-CO<u>C</u>H_3), 52.0 (α-C), 114.9, 118.8, 123.9, 127.8, 130.9 (aromatic C), 155.5 (aromatic C-OH), 169.4 (-<u>C</u>OCH_3), 173.8 (-<u>C</u>O_2H); ν_{max}(Nujol, cm^{-1}) 3000-3500, 1705, 1638, 1506, 1458, 1376, 1250, 758; *m/z* (EI) 223 (M^+, 18%), 205 (19), 164 (19), 146 (100), 130 (49), 118 (83), 112 (60), 91 (26), 77 (39); Found: C, 56.9; H, 6.1; N, 5.7. Calc. for $C_{11}H_{13}NO_5.0.5H_20$: C, 56.9; H, 6.1; N, 6.0%.

General procedure for the treatment of regioisomeric pairs of substituted *N*-acetylphenylalanines with acylase I, followed by isolation of the phenylalanines produced

Samples of racemic *ortho*- and *para*-substituted *N*-acetylphenylalanines (*ca.* 20 x 10^{-3} g of each) were dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer (10 cm^3) and the pH checked. Acylase I (*ca.* 6 x 10^{-3} g, 70 x 10^{-9} mol) was added and the mixtures were stirred at 298 K for 2 h, acidified to *ca.* pH 6 with 2 mol dm^{-3} hydrochloric acid and heated with charcoal at 333 K for 10 min. After cooling to room temperature, the reaction mixtures were filtered, the filtrates were acidified to *ca.* pH 6 with 2 mol dm^{-3} hydrochloric acid and were extracted with ethyl acetate (4 x 20 cm^3). The aqueous layers were taken and the solvents were removed *in vacuo*. The residues were taken up in water (5 cm^3), triethylamine (0.2 cm^3) and acetic anhydride (0.2 cm^3) were added and the solutions were stirred at room temperature for 3 h. The solutions were acidified to *ca.* pH 1 with 2 mol dm^{-3} hydrochloric acid and were extracted with ethyl acetate (4 x 10 cm^3). In each case, the combined organic layers were dried over magnesium sulfate and the solvent was removed *in vacuo*. Where analysis of the ^{1}H NMR spectrum of this residue indicated a single regioisomeric product, the residue was recrystallised from ethyl acetate / hexane. In all cases, a small sample (*ca.* 10^{-3} g) of the residue or product was dissolved in thionyl chloride (0.01 cm^3) pretreated methanol (1.0 cm^3) and the reaction mixture was stirred for 2 h at room temperature. The solvent was removed *in vacuo* and the residue was analysed by GC on a Chirasil-Val capillary column (0.3 x 10^{-3} m x 25 m).

N-*Acetyl-4'-fluorophenylalanine (84) and* N-*acetyl-2'-fluorophenylalanine (85)*
Starting Materials: *N*-Acetyl-4'-fluorophenylalanine (**84**) 18.8 x 10^{-3} g;
N-Acetyl-2'-fluorophenylalanine (**85**) 19.2 x 10^{-3} g
Products: (*S*)-*N*-Acetyl-4'-fluorophenylalanine ((*S*)-**84**) and
(*S*)-*N*-Acetyl-2'-fluorophenylalanine ((*S*)-**85**)
Ratio: 2 : 1 (based on ^{1}H NMR spectrum and GC.) Yield: 8.4 x 10^{-3} g (58%, 29%); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.76 (s, 3H, *o*-$COCH_3$), 1.79 (s, 6H, *p*-$COCH_3$), 2.8 (m, 3H, *p*-β-H and *o*-β-H), 3.04 (dd. *J* = 5, 14 Hz, 2H, *p*-β-H), 3.14 (dd, *J* = 5, 14 Hz, 1H, *o*-β-H), 4.5 (m, 3H, *p*-α-H and *o*-α-H), 7-7.40 (m, 12H, *p*-Ar-H and *o*-Ar-H), 8.18 (d, *J* = 8 Hz, 2H, *p*-NH), 8.22 (d, *J* = 8 Hz, 1H, *o*-NH).
Methyl Ester: GC (160 °C): t_R = 9.20, 9.60 min with integrations of 1 and 2 respectively. (These correspond to the methyl esters of the substituted phenylalanines (*S*)-**84** and (*S*)-**85** as determined by injection of standards.)

N-*Acetyl-4'-methylphenylalanine (86) and* N-*acetyl-2'-methylphenylalanine (87)*
Starting Materials: *N*-Acetyl-4'-methylphenylalanine (**86**) 19.0 x 10^{-3} g;
N-Acetyl-2'-methylphenylalanine (**87**) 19.2 x 10^{-3} g
Product: (*S*)-*N*-Acetyl-4'-methylphenylalanine ((*S*)-**86**)
Yield: 7.0 x 10^{-3} g (72%) Appearance: Colourless solid. m.p. 170-171 °C (lit.,[165] 170-171 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.99 (s, 3H, -$COCH_3$), 2.24 (s, 3H, Ar-CH_3), 2.77 (dd, *J* = 10, 14 Hz, 1H, β-H), 2.97 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.4 (m, 1H, α-H), 7.1-7.2 (m, 4H, Ar-H), 8.15 (d, *J* = 8 Hz, 1H, NH), 12.60 (br s, 1H, -CO_2H).
Methyl Ester: GC (140 °C): t_R = 20.0 min

N-*Acetyl-4'-nitrophenylalanine (88) and* N-*acetyl-2'-nitrophenylalanine (89)*
Starting Materials: *N*-Acetyl-4'-nitrophenylalanine (**88**) 19.6 x 10^{-3} g;
N-Acetyl-2'-nitrophenylalanine (**89**) 22.8 x 10^{-3} g
Product: (*S*)-*N*-Acetyl-4'-nitrophenylalanine ((*S*)-**88**)
Yield: 9.5 x 10^{-3} g (83%) Appearance: Colourless solid. m.p. 168-170 °C (lit.,[161] 170-172 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.79 (s, 3H, -$COCH_3$), 2.99 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.25 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.5 (m, 1H, α-H), 7.51 (d, *J* = 9 Hz, 2H, Ar-H), 8.15 (d, *J* = 9 Hz, 2H, Ar-H), 8.26 (d, *J* = 8 Hz, 1H, NH).
Methyl Ester: GC (180 °C): t_R = 23.0 min

N-*Acetyl-4'-chlorophenylalanine (90) and* N-*acetyl-2'-chlorophenylalanine (91)*
Starting Materials: *N*-Acetyl-4'-chlorophenylalanine (**90**) 20.7 x 10^{-3} g;
N-Acetyl-2'-chlorophenylalanine (**91**) 20.2 x 10^{-3} g
Product: (*S*)-*N*-Acetyl-4'-chlorophenylalanine ((*S*)-**90**)
Yield: 7.4 x 10^{-3} g (71%) Appearance: Colourless solid. m.p. 148-150 °C (lit.,[166] 149-153 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.77 (s, 3H, -$COCH_3$), 2.81 (dd, *J* = 10, 14 Hz, 1H, β-H), 3.02 (dd, *J* = 5, 14 Hz, 1H, β-H), 4.4 (m, 1H, α-H), 7.24 (d, *J* = 8 Hz, 2H, Ar-H), 7.31 (d, *J* = 8 Hz, 2H, Ar-H), 8.19 (d, *J* = 8 Hz, 1H, NH).
Methyl Ester: GC (160 °C): t_R = 14.7 min

N-*Acetyltyrosine (92) and* N-*acetyl-2'-hydroxyphenylalanine (93)*
Starting Materials: *N*-Acetyltyrosine (**92**) 20.1 x 10^{-3} g;
N-acetyl-2'-hydroxyphenylalanine (**93**) 19.1 x 10^{-3} g
Product: (*S*)-*N*-Acetyltyrosine ((*S*)-**92**)
Yield: 7.3 x 10^{-3} g (73%) Appearance: Colourless solid. m.p. 150-152 °C (lit.,[164] 153-154 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.79 (s, 3H, -$COCH_3$), 2.70 (dd, J = 9, 14 Hz, 1H, β-H), 2.95 (dd, J = 5, 14 Hz, 1H, β-H), 4.4 (m, 1H, α-H), 6.70 (d, J = 8 Hz, 2H, Ar-H), 7.04 (d, J = 8 Hz, 2H, Ar-H), 8.17 (d, J = 8 Hz, 1H, NH).
Methyl Ester: GC (140 °C): t_R = 13.6 min

General procedure for the calculation of the kinetic parameters for the treatment of the (*S*)-*N*-acetylphenylalanines (*S*)-83-89 with acylase I

Samples of a (*S*)-*N*-acetylphenylalanine, either as the (*S*)-enantiomer or the racemate, were dissolved in 0.05 mol dm^{-3} pH 7.0 phosphate buffer. The absorbance at 228 nm at 298 K was observed in each case for a range of dilutions, made up by diluting aliquots of stock solution to 4.0 cm^3 with 0.05 mol dm^{-3} pH 7.0 phosphate buffer. The molar extinction coefficient at 228 nm ($\varepsilon_{228\ nm}$) for each species was calculated by plotting the absorbance observed against the concentration.

Stock solutions of the *N*-acetylphenylalanines and acylase I (*ca.* 5 x 10^{-3} g, *ca.* 60 x 10^{-9} mol) were prepared in 0.05 mol dm^{-3} pH 7.0 phosphate buffer. Reaction mixtures were made up by taking varying amounts of the *N*-acetylphenylalanine solution, adding an aliquot of the enzyme stock (0.3 cm^3, *ca.* 9 x 10^{-9} mol) and making the total volume to 4.0 cm^3 with 0.05 mol dm^{-3} pH 7.0 phosphate buffer. The reactions were followed by observing the change in absorbance at 228 nm at 298 K for 16 h and the initial rate (v_0) calculated using Kaleidagraph® to calculate the initial slope. The data was analysed by the method of Lineweaver and Burk[113] to give values for the rate constant of the enzyme-catalysed reaction (k_{cat}) and the dissociation constant of the enzyme-substrate complex (K_M).

Treatment of N-*acetylphenylalanine (83) with acylase I*

N-Acetylphenylalanine (**83**) stock solution: 23.6 x 10^{-3} g (0.114 x 10^{-3} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.4	1.14 x 10^{-3}	0.342
0.8	2.29 x 10^{-3}	0.695
1.2	3.43 x 10^{-3}	1.016
1.6	4.58 x 10^{-3}	1.354
2.0	5.72 x 10^{-3}	1.670
2.4	6.87 x 10^{-3}	2.004

Table 29. Observed absorbances of solutions of *N*-acetylphenylalanine (**83**) at 228 nm.

Phenylalanine (**3**) stock solution: 81.1 x 10^{-3} g (0.491 x 10^{-3} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.4	4.91 x 10^{-3}	0.165
0.8	9.82 x 10^{-3}	0.337
1.2	14.7 x 10^{-3}	0.553
1.6	19.6 x 10^{-3}	0.731
2.0	24.5 x 10^{-3}	0.878
2.4	29.5 x 10^{-3}	1.096

Table 30. Observed absorbances of solutions of phenylalanine (**3**) at 228 nm.

This data gives $\varepsilon_{228\ nm}$(*N*-acetylphenylalanine (**83**)) = 289 ± 1 mol^{-1} dm^3 cm^{-1} and $\varepsilon_{228\ nm}$(phenylalanine **3**) = 38 ± 1 mol^{-1} dm^3 cm^{-1}.

N-Acetylphenylalanine (**83**) stock solution: 31.9 x 10^{-3} g (0.155 x 10^{-3} mol) in 50 cm^3.
Acylase I stock solution: 4.9 x 10^{-3} g (57 x 10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.6	0.464 x 10^{-3}	1.12 x 10^{-8}
1.2	0.928 x 10^{-3}	2.12 x 10^{-8}
1.8	1.39 x 10^{-3}	2.92 x 10^{-8}
2.4	1.86 x 10^{-3}	3.91 x 10^{-8}
3.0	2.32 x 10^{-3}	4.71 x 10^{-8}
3.6	2.78 x 10^{-3}	4.74 x 10^{-8}

Table 31. Initial rates of reaction of *N*-acetylphenylalanine (**83**), catalysed by acylase I, observed at 228 nm.

Assuming that any hydrolysis observed is of (*S*)-*N*-acetylphenylalanine *((S)-(83))*, this gives the kinetic data as k_{cat} = 20 ± 3 s^{-1} and K_M = 3.5 ± 0.5 x 10^{-3} mol dm^{-3}.

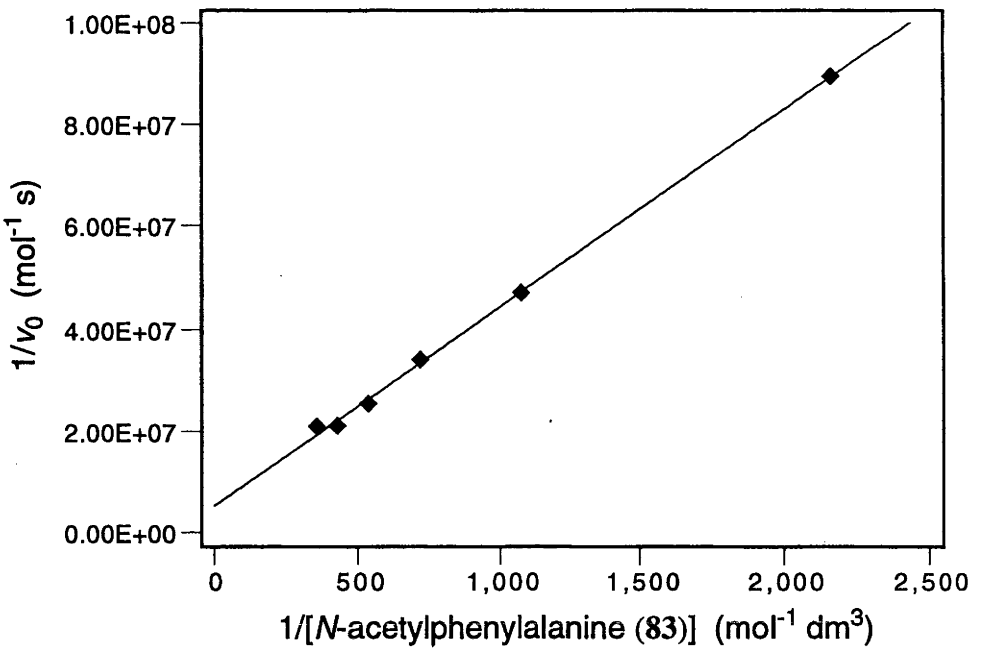


Figure 13. Lineweaver-Burk plot for the treatment of *N*-acetylphenylalanine (**83**) with acylase I. (Reproduced from Results and Discussion, Chapter III)

Treatment of (S)-N-*acetylphenylalanine* (*(S)-(83)) with acylase I*

Used molar absorbtivity data as per the racemate **83**.

(*S*)-*N*-Acetylphenylalanine ((*S*)-**83**) stock solution: 30.6 x 10^{-3} g (0.148 x 10^{-3} mol) in 50 cm^3.

Acylase I stock solution: 4.9 x 10^{-3} g (57 x 10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.6	0.445 x 10^{-3}	2.21 x 10^{-8}
1.0	0.742 x 10^{-3}	3.50 x 10^{-8}
1.2	0.890 x 10^{-3}	3.72 x 10^{-8}
2.4	1.78 x 10^{-3}	6.27 x 10^{-8}
3.0	2.23 x 10^{-3}	8.23 x 10^{-8}
3.6	2.67 x 10^{-3}	9.53 x 10^{-8}

Table 32. Initial rates of reaction of (*S*)-*N*-acetylphenylalanine ((*S*)-**83**) catalysed by acylase I observed at 228 nm.

This gives the kinetic data for the digestion of (*S*)-*N*-acetylphenylalanine ((*S*)-**83**) by acylase I as $k_{cat} = 24 \pm 4\ s^{-1}$ and $K_M = (3.9 \pm 0.8) \times 10^{-3}$ mol dm^{-3}.

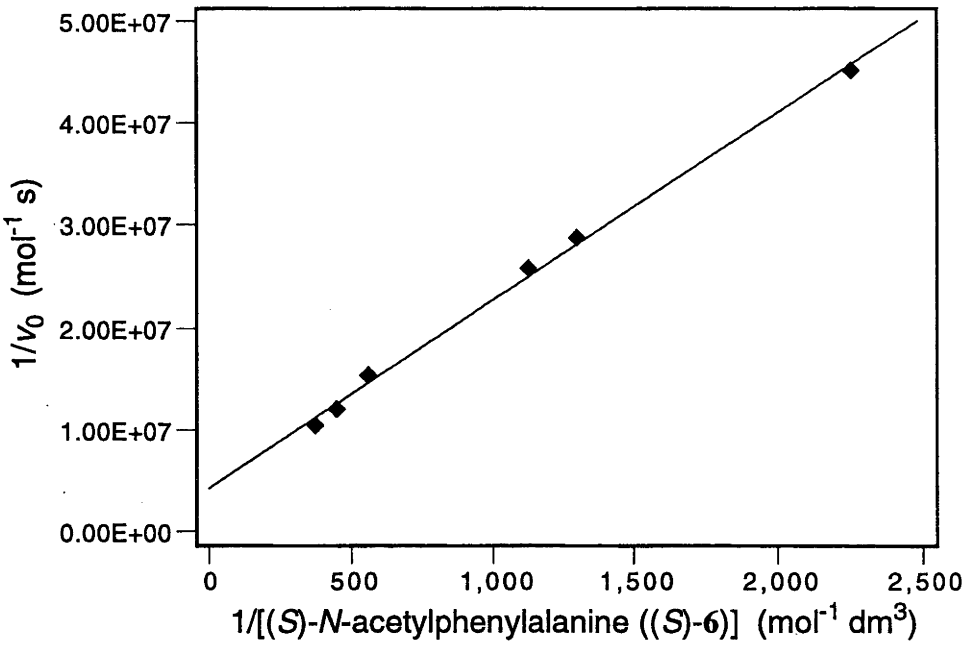


Figure 40. Lineweaver-Burk plot for the digestion of (*S*)-*N*-acetylphenylalanine ((*S*)-**83**) by acylase I.

Treatment of (R)-N-*acetylphenylalanine* ((R)-*83*) *with acylase I*

Used molar absorbtivity data as per the racemate **83**. No hydrolysis was observed by the above procedure. Acylase I (0.5 x 10^{-3} g, 6 x 10^{-9} mol) and (*R*)-*N*-acetylphenylalanine ((*R*)-**83**) (3.2 x 10^{-3} g, 15.5 x 10^{-6} mol) were taken up in 0.05 mol dm^{-3} pH 7.0 phosphate buffer (4.0 cm^3). The reaction was followed by observing the change in absorbance at 228 nm at 298K for 16 h and the initial rate of reaction (v_0) calculated using Kaleidagraph® to determine the initial slope. The observed initial rate was 8.88 x 10^{-10} mol s^{-1}.

Treatment of (S)-N-*acetyl-4'-fluorophenylalanine* (S)-(*84*) *with acylase I*

(*S*)-*N*-Acetyl-4'-fluorophenylalanine ((*S*)-**84**) stock solution: 20.2 x 10^{-3} g (91.3 x 10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.4	9.13 x 10^{-3}	0.146
0.8	18.3 x 10^{-3}	0.373
1.2	27.4 x 10^{-3}	0.578
1.6	36.5 x 10^{-3}	0.810
2.0	45.7 x 10^{-3}	0.980
2.4	54.6 x 10^{-3}	1.208

Table 33. Observed absorbances of solutions of (*S*)-*N*-acetyl-4'-fluorophenylalanine ((*S*)-**84**) at 228 nm.

(*S*)-4'-Fluorophenylalanine ((*S*)-**57**) stock solution: 67.4 x 10^{-3} g (0.368 x 10^{-3} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.4	3.68 x 10^{-3}	0.084
0.8	7.36 x 10^{-3}	0.201
1.2	11.0 x 10^{-3}	0.324
1.6	14.7 x 10^{-3}	0.430
2.0	18.4 x 10^{-3}	0.546
2.4	22.1 x 10^{-3}	0.674

Table 34. Observed absorbances of solutions of (*S*)-4'-fluorophenylalanine ((*S*)-**57**) at 228 nm.

This data gives $\varepsilon_{228\ nm}$((*S*)-*N*-acetyl-4'-fluorophenylalanine ((*S*)-**84**)) = 230 ± 4 mol^{-1} dm^3 cm^{-1} and $\varepsilon_{228\ nm}$((*S*)-4'-fluorophenylalanine ((*S*)-**57**)) = 31.7 ± 0.4 mol^{-1} dm^3 cm^{-1}.

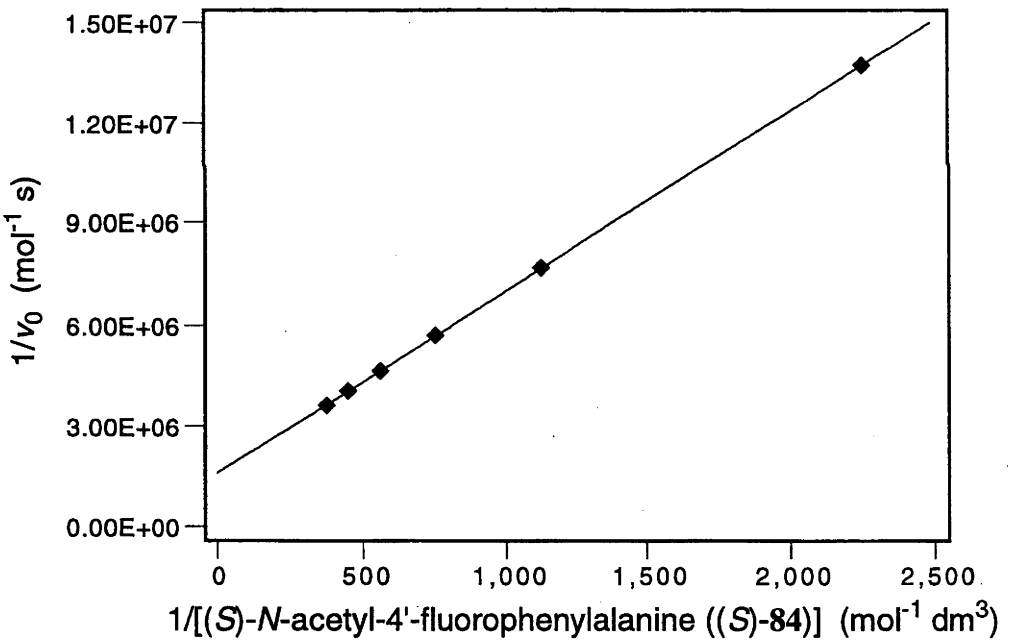


Figure 41. Lineweaver-Burk plot for the reaction of the fluoride (*S*)-**84** by acylase I.

(*S*)-*N*-Acetyl-4'-fluorophenylalanine ((*S*)-**84**) stock solution: 12.3 x 10^{-3} g (55.6 x 10^{-6} mol) in 10 cm^3.

Acylase I stock solution: 5.1 x 10^{-3} g (59 x 10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.4	0.445×10^{-3}	7.28×10^{-8}
0.8	0.890×10^{-3}	1.30×10^{-7}
1.2	1.33×10^{-3}	1.75×10^{-7}
1.6	1.78×10^{-3}	2.14×10^{-7}
2.0	2.22×10^{-3}	2.46×10^{-7}
2.4	2.67×10^{-3}	2.75×10^{-7}

Table 35. Initial rates of reaction of (*S*)-*N*-acetyl-4'-fluorophenylalanine ((*S*)-**84**) catalysed by acylase I observed at 228 nm.

This gives the kinetic data for the digestion of (*S*)-*N*-acetyl-4'-fluorophenylalanine ((*S*)-**84**) as $k_{cat} = 66 \pm 1\ s^{-1}$ and $K_M = (3.3 \pm 0.1) \times 10^{-3}$ mol dm^{-3}.

Treatment of N-*acetyl-4'-fluorophenylalanine (84) with acylase I*

Used molar absorbtivity data as per the (*S*)-enantiomer (*S*)-**84**.

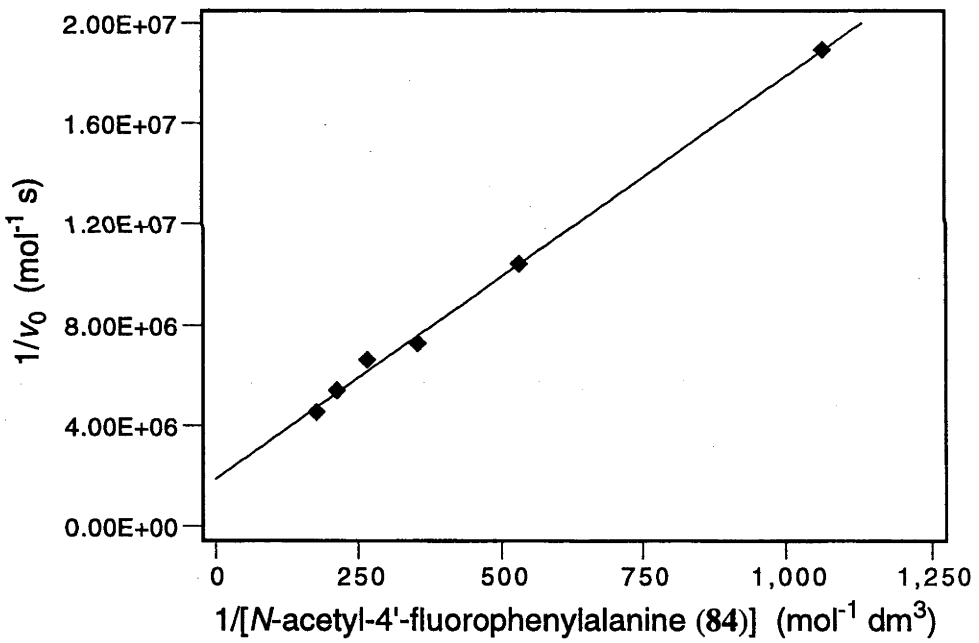


Figure 42. Lineweaver-Burk plot for the hydrolysis of the fluoride **84** by acylase I.

Assuming that any hydrolysis observed is of (*S*)-*N*-acetyl-4'-fluorophenylalanine ((*S*)-**84**), this gives the kinetic data as $k_{cat} = 58 \pm 7\ s^{-1}$ and $K_M = (4.1 \pm 0.5) \times 10^{-3}$ mol dm^{-3}.

N-Acetyl-4'-fluorophenylalanine (**84**) stock solution: 19.4×10^{-3} g (94.1×10^{-6} mol) in 10 cm^3.

Acylase I stock solution: 5.0×10^{-3} g (58×10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.4	0.941×10^{-3}	5.28×10^{-8}
0.8	1.88×10^{-3}	9.59×10^{-8}
1.2	2.82×10^{-3}	1.38×10^{-7}
1.6	3.76×10^{-3}	1.51×10^{-7}
2.0	4.70×10^{-3}	1.84×10^{-7}
2.4	5.64×10^{-3}	2.19×10^{-7}

Table 36. Initial rates of reaction of *N*-acetyl-4'-fluorophenylalanine (**84**) catalysed by acylase I observed at 228 nm.

Treatment of (S)-N-acetyl-2'-fluorophenylalanine ((S)-85) with acylase I

N-Acetyl-2'-fluorophenylalanine (**85**) stock solution: 21.3×10^{-3} g (96.3×10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.4	0.963×10^{-3}	0.240
0.8	1.93×10^{-3}	0.490
1.2	2.89×10^{-3}	0.734
1.6	3.85×10^{-3}	0.983
2.0	4.81×10^{-3}	1.205
2.4	5.78×10^{-3}	1.486

Table 37. Observed absorbances of solutions of *N*-acetyl-2'-fluorophenylalanine (**85**) at 228 nm.

2'-Fluorophenylalanine (**58**) stock solution: 60.4×10^{-3} g (0.330×10^{-3} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.4	3.30×10^{-3}	0.077
0.8	6.59×10^{-3}	0.165
1.2	9.89×10^{-3}	0.342
1.6	13.2×10^{-3}	0.446
2.0	16.5×10^{-3}	0.605
2.4	19.8×10^{-3}	0.700

Table 38. Observed absorbances of solutions of the fluoride **58** at 228 nm.

This data gives $\varepsilon_{228\ nm}$(*N*-acetyl-2'-fluorophenylalanine (**85**)) = 256 ± 3 mol^{-1} dm^3 cm^{-1} and $\varepsilon_{228\ nm}$(2'-fluorophenylalanine (**58**)) = 39 ± 2 mol^{-1} dm^3 cm^{-1}.

N-Acetyl-2'-fluorophenylalanine (**85**) stock solution: 31.7×10^{-3} g (0.143×10^{-3} mol) in 10 cm^3.

Acylase I stock solution: 5.1×10^{-3} g (59×10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.4	1.43×10^{-3}	2.98×10^{-8}
0.8	2.87×10^{-3}	4.05×10^{-8}
1.2	4.30×10^{-3}	4.86×10^{-8}
1.6	5.73×10^{-3}	5.44×10^{-8}
2.0	7.17×10^{-3}	5.43×10^{-8}
2.4	8.60×10^{-3}	5.86×10^{-8}

Table 39. Initial rates of reaction of *N*-acetyl-2'-fluorophenylalanine (**85**) catalysed by acylase I observed at 228 nm.

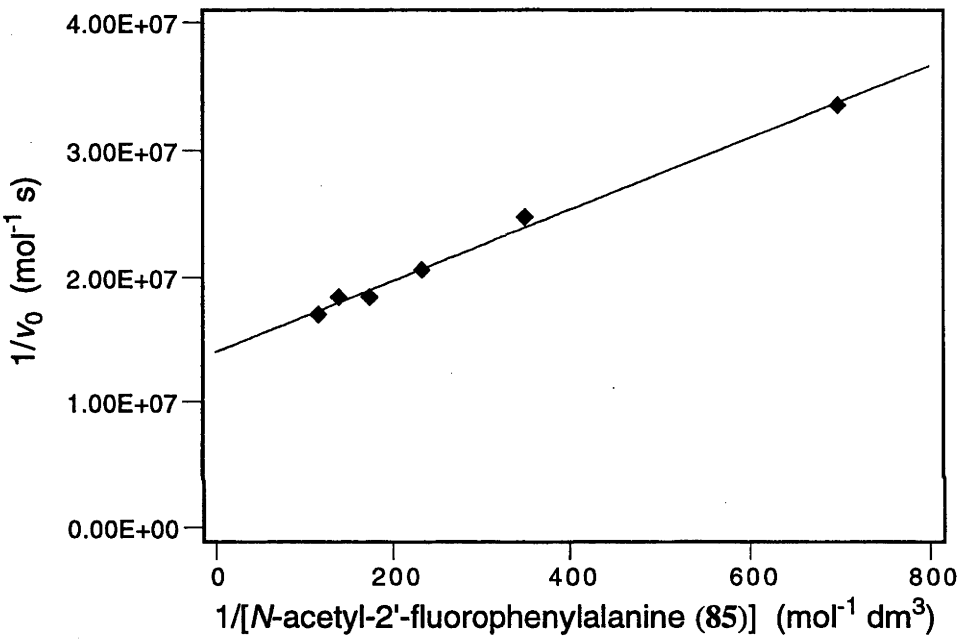


Figure 43. Lineweaver-Burk plot for the hydrolysis of the fluoride **85** by acylase I.

Assuming that any hydrolysis observed is of (*S*)-*N*-acetyl-2'-fluorophenylalanine ((*S*)-**85**), this gives the kinetic data as $k_{cat} = 7.7 \pm 0.2$ s^{-1} and $K_M = (1.00 \pm 0.07) \times 10^{-3}$ mol dm^{-3}.

Treatment of (S)-N-*acetyl-2'-fluorophenylalanine* ((S)-*85*) *with acylase I*

Used molar absorbtivity data as per the racemate **85**.

(*S*)-*N*-Acetyl-2'-fluorophenylalanine ((*S*)-**85**) stock solution: 7.6×10^{-3} g (34.4×10^{-6} mol) in 10 cm^3.

Acylase I stock solution: 5.4×10^{-3} g (63×10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.4	0.344 x 10^{-3}	1.79 x 10^{-8}
0.8	0.687 x 10^{-3}	2.97 x 10^{-8}
1.2	1.03 x 10^{-3}	3.55 x 10^{-8}
1.6	1.37 x 10^{-3}	3.81 x 10^{-8}
2.0	1.72 x 10^{-3}	4.69 x 10^{-8}
2.4	2.06 x 10^{-3}	4.72 x 10^{-8}

Table 40. Initial rates of reaction of (*S*)-*N*-acetyl-2'-fluorophenylalanine ((*S*)-**85**) catalysed by acylase I observed at 228 nm.

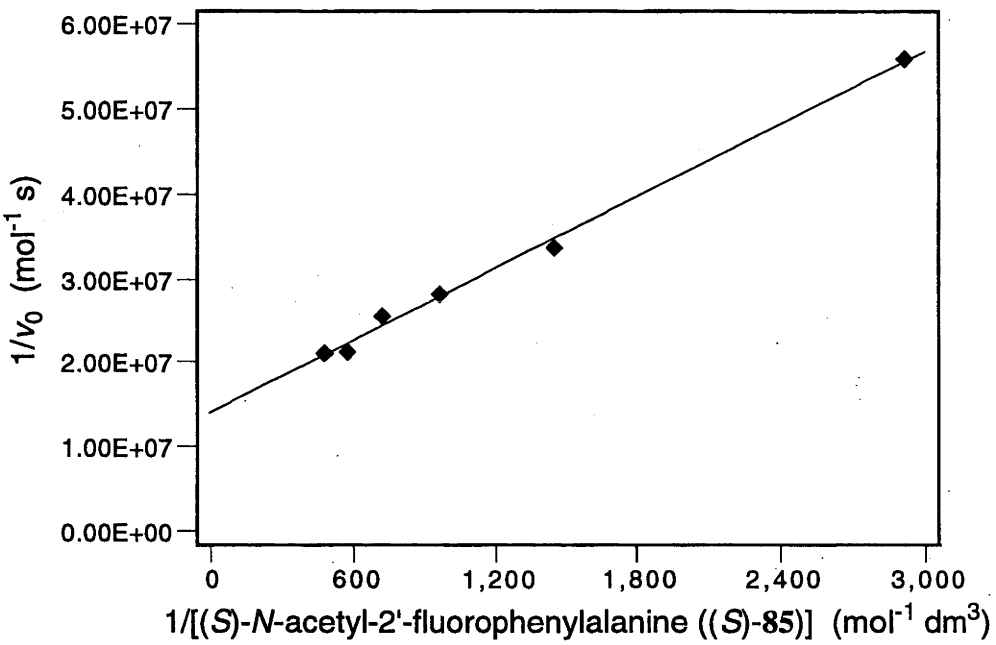


Figure 44. Lineweaver-Burk plot for the digestion of the fluoride (*S*)-**85** by acylase I.

This gives the kinetic data for the digestion of (*S*)-*N*-acetyl-2'-fluorophenylalanine ((*S*)-**85**) as k_{cat} = 7.3 ± 0.4 s^{-1} and K_M = (1.0 ± 0.1) x 10^{-3} mol dm^{-3}.

Treatment of N-*acetyl-4'-methylphenylalanine (86) with acylase I*

N-Acetyl-4'-methylphenylalanine (**86**) stock solution: 14.0 x 10^{-3} g (63.3 x 10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.133	0.211 x 10^{-3}	0.397
0.267	0.422 x 10^{-3}	0.850
0.400	0.633 x 10^{-3}	1.204
0.533	0.844 x 10^{-3}	1.585
0.667	1.06 x 10^{-3}	1.906
0.800	1.27 x 10^{-3}	2.169

Table 41. Observed absorbances of solutions of the phenylalanine **86** at 228 nm.

4'-Methylphenylalanine (**59**) stock solution: 17.3 x 10^{-3} g (96.6 x 10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.2	0.483 x 10^{-3}	0.280
0.4	0.966 x 10^{-3}	0.654
0.6	1.45 x 10^{-3}	0.953
0.8	1.93 x 10^{-3}	1.280
1.0	2.41 x 10^{-3}	1.517
1.2	2.90 x 10^{-3}	1.826

Table 42. Observed absorbances of solutions of the phenylalanine **59** at 228 nm.

This data gives $\varepsilon_{228\ nm}$(*N*-acetyl-4'-methylphenylalanine (**86**)) = 1680 ± 30 mol^{-1} dm^3 cm^{-1} and $\varepsilon_{228\ nm}$(4'-methylphenylalanine (**59**)) = 630 ± 20 mol^{-1} dm^3 cm^{-1}.

N-Acetyl-4'-methylphenylalanine (**86**) stock solution: 3.1 x 10^{-3} g (14.0 x 10^{-6} mol) in 10 cm^3.

Acylase I stock solution: 4.9 x 10^{-3} g (57 x 10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.4	0.140 x 10^{-3}	4.30 x 10^{-9}
0.8	0.280 x 10^{-3}	6.81 x 10^{-9}
1.2	0.421 x 10^{-3}	9.56 x 10^{-9}
1.6	0.561 x 10^{-3}	1.12 x 10^{-8}
2.0	0.701 x 10^{-3}	1.29 x 10^{-8}
2.4	0.841 x 10^{-3}	1.35 x 10^{-8}

Table 43. Initial rates of reaction of *N*-acetyl-4'-methylphenylalanine (**86**) catalysed by acylase I observed at 228 nm.

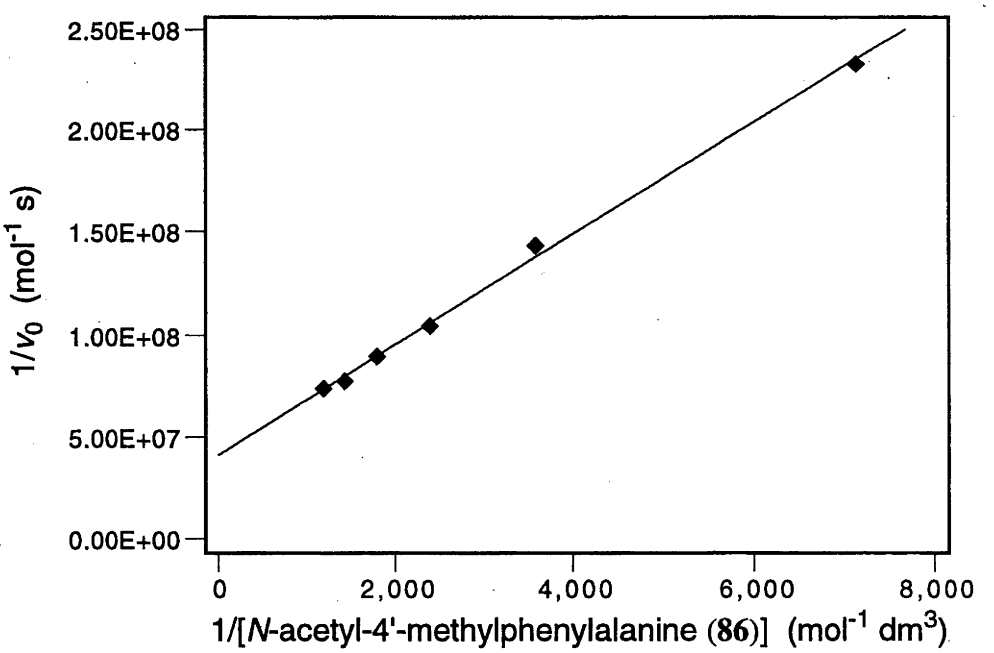


Figure 45. Lineweaver-Burk plot for the hydrolysis of the methylphenylalanine **86** by acylase I.

Assuming that any hydrolysis observed is of (*S*)-*N*-acetyl-4'-methylphenylalanine ((*S*)-**86**), this gives the kinetic data as k_{cat} = 2.7 ± 0.2 s^{-1} and K_M = (0.32 ± 0.04) x 10^{-3} mol dm^{-3}.

Treatment of N-*acetyl-2'-methylphenylalanine (87) with acylase I*

N-Acetyl-2'-methylphenylalanine (**87**) stock solution: 4.6 x 10^{-3} g (20.8 x 10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.4	0.208 x 10^{-3}	0.125
0.8	0.416 x 10^{-3}	0.306
1.2	0.624 x 10^{-3}	0.475
1.6	0.832 x 10^{-3}	0.678
2.0	1.04 x 10^{-3}	0.900
2.4	1.25 x 10^{-3}	1.049

Table 44. Observed absorbances of solutions of *N*-acetyl-2'-methylphenylalanine (**87**) at 228 nm.

2'-Methylphenylalanine (**60**) stock solution: 7.7 x 10^{-3} g (43.0 x 10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.4	0.430 x 10^{-3}	0.095
0.8	0.860 x 10^{-3}	0.181
1.2	1.29 x 10^{-3}	0.250
1.6	1.72 x 10^{-3}	0.342
2.0	2.15 x 10^{-3}	0.468
2.4	2.58 x 10^{-3}	0.526

Table 45. Observed absorbances of solutions of 2'-methylphenylalanine (**60**) at 228 nm.

This data gives $\varepsilon_{228\ nm}$(*N*-acetyl-2'-methylphenylalanine (**87**)) = 900 ± 20 mol^{-1} dm^3 cm^{-1} and $\varepsilon_{228\ nm}$(2'-methylphenylalanine (**60**)) = 207 ± 7 mol^{-1} dm^3 cm^{-1}.

N-Acetyl-2'-methylphenylalanine (**87**) stock solution: 3.5 x 10^{-3} g (15.8 x 10^{-6} mol) in 10 cm^3.

Acylase I stock solution: 5.1 x 10^{-3} g (59 x 10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.4	0.158 x 10^{-3}	*a*
0.8	0.317 x 10^{-3}	*a*
1.2	0.475 x 10^{-3}	*a*
1.6	0.633 x 10^{-3}	*a*
2.0	0.791 x 10^{-3}	*a*
2.4	0.950 x 10^{-3}	*a*

Table 46. Initial rates of reaction of *N*-acetyl-2'-methylphenylalanine (**87**) catalysed by acylase I observed at 228 nm.
[a]No hydrolysis detected within the limitation of the method.

Since no hydrolysis was observed, no kinetic data was calculated.

Treatment of (S)-N-acetyl-4'-nitrophenylalanine ((S)-88) with acylase I

(*S*)-*N*-Acetyl-4'-nitrophenylalanine ((*S*)-**88**) stock solution: 16.7 x 10^{-3} g (66.2 x 10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.2	0.331 x 10^{-3}	0.955
0.3	0.497 x 10^{-3}	1.398
0.4	0.662 x 10^{-3}	1.940
0.5	0.828 x 10^{-3}	2.331
0.6	0.993 x 10^{-3}	2.875
0.8	1.32 x 10^{-3}	3.874

Table 47. Observed absorbances of solutions of (*S*)-*N*-acetyl-4'-nitrophenylalanine ((*S*)-**88**) at 228 nm.

(*S*)-4'-Nitrophenylalanine ((*S*)-**61**) stock solution: 22.3 x 10^{-3} g (9.77 x 10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.1	0.244 x 10^{-3}	0.628
0.2	0.489 x 10^{-3}	1.199
0.3	0.733 x 10^{-3}	1.821
0.4	0.977 x 10^{-3}	2.405
0.5	1.22 x 10^{-3}	2.968
0.6	1.47 x 10^{-3}	3.460

Table 48. Observed absorbances of solutions of (*S*)-4'-nitrophenylalanine ((*S*)-**61**) at 228 nm.

This data gives $\varepsilon_{228\ nm}$((*S*)-*N*-acetyl-4'-nitrophenylalanine ((*S*)-**88**)) = 2950 ± 50 mol^{-1} dm^3 cm^{-1} and $\varepsilon_{228\ nm}$((*S*)-4'-nitrophenylalanine ((*S*)-**61**)) = 2340 ± 40 mol^{-1} dm^3 cm^{-1}.

(*S*)-*N*-Acetyl-4'-nitrophenylalanine ((*S*)-**88**) stock solution: 3.3 x 10^{-3} g (13.1 x 10^{-6} mol) in 10 cm^3.

Acylase I stock solution: 4.4 x 10^{-3} g (51 x 10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.6	0.196 x 10^{-3}	3.30 x 10^{-8}
0.8	0.262 x 10^{-3}	3.87 x 10^{-8}
1.2	0.392 x 10^{-3}	5.04 x 10^{-8}
1.6	0.523 x 10^{-3}	6.23 x 10^{-8}
2.0	0.654 x 10^{-3}	6.81 x 10^{-8}
2.4	0.785 x 10^{-3}	6.84 x 10^{-8}

Table 49. Initial rates of reaction of (*S*)-*N*-acetyl-4'-nitrophenylalanine ((*S*)-**88**) catalysed by acylase I observed at 228 nm.

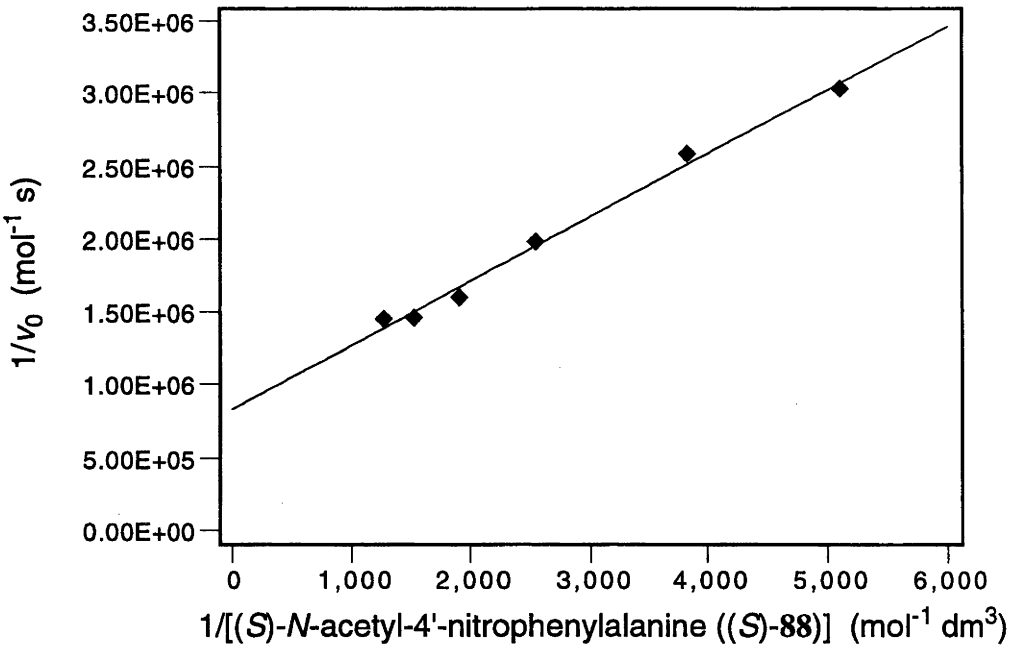


Figure 46. Lineweaver-Burk plot for the digestion of the phenylalanine derivative (*S*)-**88** by acylase I.

This gives the kinetic data for the digestion of (*S*)-*N*-acetyl-4'-nitrophenylalanine ((*S*)-**88**) as $k_{cat} = 15 \pm 1$ s^{-1} and $K_M = (0.50 \pm 0.07) \times 10^{-3}$ mol dm^{-3}.

Treatment of N-*acetyl-2'-nitrophenylalanine (89) with acylase I*

N-Acetyl-2'-nitrophenylalanine (**89**) stock solution: 7.0×10^{-3} g (27.8×10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.2	55.5×10^{-6}	0.139
0.6	0.167×10^{-3}	0.615
1.0	0.278×10^{-3}	1.006
1.4	0.389×10^{-3}	1.422
1.8	0.500×10^{-3}	1.854
2.2	0.611×10^{-3}	2.340

Table 50. Observed absorbances of solutions of *N*-acetyl-2'-nitrophenylalanine (**89**) at 228 nm.

2'-Nitrophenylalanine (**62**) stock solution: 7.7×10^{-3} g (3.37×10^{-6} mol) in 10 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	$A_{228\ nm}$
0.2	0.0674×10^{-3}	0.173
0.6	0.202×10^{-3}	0.655
1.0	0.337×10^{-3}	1.125
1.4	0.472×10^{-3}	1.632
1.8	0.607×10^{-3}	2.095
2.2	0.742×10^{-3}	2.547

Table 51. Observed absorbances of solutions of 2'-nitrophenylalanine (**62**) at 228 nm.

This data gives $\varepsilon_{228\ nm}$(*N*-acetyl-2'-nitrophenylalanine (**89**)) = 3900 ± 60 mol^{-1} dm^3 cm^{-1} and $\varepsilon_{228\ nm}$(2'-nitrophenylalanine (**62**)) = 3530 ± 30 mol^{-1} dm^3 cm^{-1}.

N-Acetyl-2'-nitrophenylalanine ((*S*)-**89**) stock solution: 2.9 x 10^{-3} g (11.5 x 10^{-6} mol) in 10 cm^3.
Acylase I stock solution: 5.1 x 10^{-3} g (59 x 10^{-9} mol) in 2 cm^3.

Stock solution (cm^3)	Concentration (mol dm^{-3})	v_0 (mol s^{-1})
0.4	0.115 x 10^{-3}	*a*
0.8	0.230 x 10^{-3}	*a*
1.2	0.345 x 10^{-3}	*a*
1.6	0.460 x 10^{-3}	*a*
2.0	0.575 x 10^{-3}	*a*
2.4	0.690 x 10^{-3}	*a*

Table 52. Initial rates of reaction of *N*-acetyl-2'-nitrophenylalanine (**89**) catalysed by acylase I observed at 228 nm.
[a]No hydrolysis detected within the limitation of the method.

Since no hydrolysis was observed, no kinetic data was calculated.

Effect of *N*-acetyl-2'-methylphenylalanine (87) and *N*-acetyl-2'-nitrophenylalanine (89) on the digestion of (*S*)-*N*-acetylphenylalanine ((*S*)-83) with acylase I

Reaction mixtures were prepared by dissolving *N*-acetylphenylalanine (**83**) (4.0 x 10^{-3} g, 19.3 x 10^{-6} mol), acylase I (1.0 x 10^{-3} g, 12 x 10^{-9} mol), ammonium carbonate (either 0.9 x 10^{-3} g, 9.3 x 10^{-6} mol or 3.3 x 10^{-6} g, 34.4 x 10^{-6} mol) and either no substituted *N*-acetylphenylalanine, *N*-acetyl-2'-methylphenylalanine (**87**) (12.8 x 10^{-3} g, 57.9 x 10^{-6} mol) or *N*-acetyl-2'-nitrophenylalanine (**89**) (13.4 x 10^{-3} g, 53.2 x 10^{-6} mol) in 0.05 mol dm^{-3} pH 7.0 phosphate buffer (1.0 cm^3). The pH of the reaction mixtures was checked and found to be as required. The reaction mixtures were stirred at 298 K for 1 h, before being acidified to *ca.* pH 1 with 2 mol dm^{-3} hydrochloric acid and extracted with ethyl acetate (5 x 5 cm^3). The combined organic layers were dried over magnesium sulfate and the solvent was removed *in vacuo*. The residue was dissolved in thionyl chloride (0.05 cm^3) pretreated methanol (5 cm^3) and the reaction mixture was stirred for 2 h at room temperature. The solvent was then removed *in vacuo* and the mixture was analysed by GC on a Chirasil-Val capillary column (0.3 x 10^{-3} m x 25 m). Extent of reaction was calculated by comparing the integration of the signal due to the (*S*)-isomer to that of the undigested (*R*)-isomer (Table 53).

Solution contains	Extent of reaction (%)
-	50
87	<5
89	<5

Table 53. Extent of reaction of (*S*)-*N*-acetylphenylalanine ((*S*)-**83**) in the presence of either no other acetylphenylalanine, *N*-acetyl-2'-methylphenylalanine (**87**) or *N*-acetyl-2'-nitrophenylalanine (**89**).

Treatment of a mixture *trans*-4'-methylcinnamate (74) and *trans*-2'-methylcinnamate (75) with PAL in ammonia buffer at pH 10

The protonated forms of *trans*-4'-methylcinnamate (**74**) (0.204 g, 1.26 x 10^{-3} mol) and *trans*-2'-methylcinnamate (**75**) (0.208 g, 1.28 x 10^{-3} mol) were dissolved in concentrated ammonia solution (8.45 cm^3), the pH was adjusted to 10.25 with concentrated hydrochloric acid and the volume was made up to 10.0 cm^3, with checking and adjusting of the pH. Reaction mixtures were prepared by combining aliquots of this solution (0.60 cm^3) with commercial PAL solution (0.40 cm^3, *ca.* 1 unit). The reaction mixtures were thermostatted at 303 K for 1, 3 or 7 days, before being acidified to *ca.* pH 1 with concentrated hydrochloric acid. The residual started material was removed by vacuum filtration, and the filtrates were evaporated to dryness *in vacuo*. To each residue was added *tert*-butanol (4.0 x 10^{-6} cm^3, 41.9 x 10^{-6} mol) and the mixtures were analysed by ^{1}H NMR spectroscopy. The ratio of the integration of the signals corresponding to the aromatic methyl groups of the substituted phenylalanines **59** and **60** was calculated, as was the ratio of the integration of these signals to the signal corresponding to the *tert*-butanol in each case. Hence, the extent of reaction was calculated (Table 54) in each case.

Reaction time (days)	Phenylalanines **59** : **60**	Ratio of the integrations of Ar-CH_3 : *tert*-butanol	Extent of reaction (%)
1	< 1 : 20	1 : 6.1	27
3	< 1 : 20	1 : 6.3	26
7	< 1 : 20	1 : 5.8	28

Table 54. Ratio of products and extent of reaction with time in the treatment of a mixture *trans*-4'-methylcinnamate (**74**) and *trans*-2'-methylcinnamate (**75**) with PAL in ammonia buffer at 303 K as measured by ^{1}H NMR spectroscopy.

Experimental VI: Investigation of the Effect of Cyclodextrins on the Digestion of Substituted Phenylalanine Derivatives by Protease VIII

Diethyl 2-(acetamido)-2-[(3-methylphenyl)methyl)]malonate (100)

To a stirred solution of 4-methylbenzyl chloride (**98**) (9.60 cm^3, 72.6 x 10^{-3} mol) in toluene (10 cm^3) was added diethyl acetamidomalonate (13.3 g, 61.3 x 10^{-3} mol) and sodium hydride, as a 60% suspension in oil (3.31 g, 82.7 x 10^{-3} mol NaH). The resultant mixture was heated at reflux under nitrogen for 8 h. The mixture was allowed to cool to room temperature and the solvent was removed *in vacuo*. The residue was taken up in hot 95% aqueous ethanol and filtered, the filtrate being evaporated to dryness *in vacuo*. The residue was recrystallised from 95% aqueous ethanol to give the title compound as a colourless solid (19.1 g, 97%). m.p. 112 °C (lit.,[133] 108-109 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ1.29 (t, *J* = 7 Hz, 6H, $-CH_2C\underline{H}_3$), 2.02 (s, 3H, $-COCH_3$), 2.28 (s, 3H, Ar-CH_3), 3.59 (s, 2H, β-H), 4.26 (q, *J* = 7 Hz, 4H, $-C\underline{H}_2CH_3$), 6.56 (br s, 1H, NH), 6.7-6.8 (m, 2H, Ar-H), 7-7.2 (m, 2H, Ar-H).

3'-Methylphenylalanine (102)

Diethyl 2-(acetamido)-2-[(3-methylphenyl)methyl)]malonate (**100**) (2.70 g, 18.0 x 10^{-3} mol) was heated at reflux in 25% hydrochloric acid (70 cm^3) for 18 h. The reaction mixture was cooled to room temperature and the solvent was removed *in vacuo*. The residue was redissolved in hot water, concentrated ammonia solution was added to adjust solution to *ca.* pH 7 and the solvent was removed *in vacuo*. The residue was recrystallised from water to give the title compound as a colourless solid (2.59 g, 84%). m.p. 232-233 °C (dec.) (lit.,[133] 244-246 °C (dec.)); ^{1}H NMR (300 MHz, NaOD/D_2O) δ2.28 (s, 3H, Ar-CH_3), 2.74 (dd, *J* = 7, 13 Hz, 1H, β-H), 2.90 (dd, *J* = 5, 13 Hz, 1H, β-H), 3.42 (m, 1H, α-H), 7.0-7.1 (m, 3H, Ar-H), 7.21 (t, *J* = 7 Hz, 1H, Ar-H).

Diethyl 2-(acetamido)-2-[(3-nitrophenyl)methyl)]malonate (101)

Sodium metal (0.45 g) was dissolved with stirring in ethanol (50 cm^3) and diethyl acetamidomalonate (4.06 g, 18.7 x 10^{-3} mol) was added to the solution while still warm. This was allowed to stir for 10 min, then 3-nitrobenzyl bromide (**99**) (4.01 g, 18.6 x 10^{-3} mol) dissolved in benzene (11 cm^3) was added dropwise over 10 min. The resulting mixture was stirred for 3 h at room temperature and the solvent was removed *in vacuo*. The residue was taken up in warm methanol (100 cm^3), the resultant suspension was filtered and the filtrate was evaporated to dryness *in vacuo*. The pale yellow residue was recrystallised from ethanol to give the title compound as colourless needles (4.92 g, 75%). m.p. 161-162 °C (lit.,[171] 159-160 °C); ^{1}H NMR (300 MHz, d_6-DMSO) δ1.20 (t, *J* = 7 Hz, 6H, -$CH_2C\underline{H}_3$), 1.96 (s, 3H, -$COCH_3$), 3.59 (s, 2H, β-H), 4.18 (q, *J* = 7 Hz, 4H, -$C\underline{H}_2CH_3$), 7.47 (d, *J* = 8 Hz, 1H, Ar-H), 7.62 (t, *J* = 8 Hz, 1H, Ar-H), 7.81 (s, 1H, Ar-H), 8.16 (d, *J* = 8 Hz, 1H, Ar-H), 8.23 (s, 1H, NH).

3'-Nitrophenylalanine (103)

Diethyl 2-(acetamido)-2-[(3-nitrophenyl)methyl)]malonate (**101**) (3.89 g, 11.0 x 10^{-3} mol) was heated at reflux in concentrated hydrochloric acid (60 cm^3) for 18 h. The reaction mixture was allowed to cool to room temperature and the solvent was removed *in vacuo*. The residue was dissolved in ethanol, the solvent was neutralised to *ca.* pH 7 with triethylamine and the resultant precipitate was collected by vacuum filtration. The residue was recrystallised from ethanol / water to give the title compound as colourless needles (1.52 g, 66%). m.p. 218-220 °C (dec.) (lit.,[171] 209-212 °C); ^{1}H NMR (300 MHz, D_2O) δ3.21 (dd, *J* = 7, 15 Hz, 1H, β-H), 3.42 (dd, *J* = 6, 15 Hz, 1H, β-H), 3.98 (dd *J* = 6, 7 Hz, 1H, α-H), 7.58 (t, *J* = 7 Hz, 1H, Ar-H), 7.67 (d, *J* = 7 Hz, 1H, Ar-H), 8.15 (s, 1H, Ar-H), 8.18 (d, *J* = 7 Hz, 1H, Ar-H).

General preparation of the *N*-trifluoroacetylphenylalanine methyl esters 104-107

The appropriate substituted phenylalanine was dissolved in thionyl chloride (1 cm^3 per g of starting material) pretreated methanol (100 cm^3 per g of starting material) and the solution was stirred at room temperature for 48 h. The solvent was removed *in vacuo* and the residue was partitioned between ethyl acetate (30 cm^3) and saturated sodium carbonate solution (30 cm^3). The aqueous layer was washed with ethyl acetate

(3 x 30 cm^3), the organic layers were combined, dried over magnesium sulfate and the solvent was removed *in vacuo*. The resulting oil was analysed (^1H NMR spectroscopy) and used without further purification.

The appropriate phenylalanine methyl ester was dissolved in dichloromethane (15 cm^3 per g of starting material) to which triethylamine (1.1 mol equivalent) had been added. Redistilled trifluoroacetic anhydride (1.1 mol equivalent) was added and the solution was stirred at room temperature for 3 h. The volatiles were removed *in vacuo* and the residue was purified by flash column chromatography (dichloromethane) to give the desired *N*-trifluoroacetylphenylalanine methyl ester.

N-*Trifluoroacetyl-4'-methylphenylalanine methyl ester* ***(104)***

Intermediate ester: Starting Material: 0.406 g Yield: 0.440 g, 95% Appearance: yellow oil; ^1H NMR (300 MHz, $CDCl_3$) δ2.25 (s, 3H, Ar-CH_3), 2.75 (dd, J = 7, 12 Hz, 1H, β-H), 2.84 (dd, J = 6, 12 Hz, 1H, β-H), 3.55 (m, 4H, α-H and -OCH_3), 7-7.1 (m, 4H, Ar-H).

Product: Intermediate ester: 0.400 g Yield: 0.430 g, 73% Appearance: colourless rosettes m.p. 78-79 °C; ^1H NMR (300 MHz, $CDCl_3$) δ2.32 (s, 3H, Ar-CH_3), 3.12 (dd, J = 6, 14 Hz, 1H, β-H), 3.21 (dd, J = 6, 14 Hz, 1H, β-H), 3.78 (s, 3H, -OCH_3), 4.86 (q, J = 6 Hz, 1H, α-H), 6.98 (d, J = 8 Hz, 2H, Ar-H), 7.06 (br s, 1H, NH), 7.12 (d, J = 8 Hz, 2H, Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ20.8 (Ar-$\underline{C}H_3$), 36.6 (Ar-$\underline{C}H_2$-), 52.6 (α-C†), 53.4 (O$\underline{C}H_3$†), 115.4 (q, J_F = 288 Hz, -CF_3), 128.8 (Ar-C), 129.3 (Ar-C), 131.3 (Ar-C), 137.1 (Ar-C), 156.4 (q, J_F = 38 Hz, -$\underline{C}OCF_3$), 170.3 (-$\underline{C}O_2CH_3$); ν_{max}($CDCl_3$, cm^{-1}) 3683, 3620, 3019, 2976, 2400, 1725, 1530, 1438, 1290, 1190; *m/z* (EI) 289 (M^+, 3%), 230 (10), 176 (78), 145 (28), 105 (100); Found: C, 54.3; H, 4.8; N, 5.0. Calc. for $C_{13}H_{14}F_3NO_3$: C, 54.0; H, 4.9; N, 4.8%.

†Assignment may be reversed.

N-*Trifluoroacetyl-3'-methylphenylalanine methyl ester* ***(105)***

Intermediate ester: Starting Material: 0.911 g Yield: 0.730 g, 70% Appearance: orange oil; ^1H NMR (300 MHz, $CDCl_3$) δ2.24 (s, 3H, Ar-CH_3), 2.73 (dd, J = 8, 13 Hz, 1H, β-H), 2.97 (dd, J = 5, 13 Hz, 1H, β-H), 3.6-3.65 (m, 4H, α-H and -OCH_3), 6.9-7.0 (m, 3H, Ar-H), 7.11 (t, J = 7 Hz, 1H, Ar-H).

Product: Intermediate ester: 0.700 g Yield: 0.696 g, 67% Appearance: colourless rosettes m.p. 62-63 °C ; ^1H NMR (300 MHz, $CDCl_3$) δ2.32 (s, 3H, Ar-CH_3), 3.11 (dd, J = 6, 14 Hz, 1H, β-H), 3.22 (dd, J = 6, 14 Hz, 1H, β-H), 3.78 (s, 3H, -OCH_3), 4.87 (q, J = 6 Hz, 1H, α-H), 6.9-7.2 (m, 5H, NH and Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ20.8 (Ar-$\underline{C}H_3$), 36.7 (Ar-$\underline{C}H_2$-), 52.4 (α-C†), 53.4 (O$\underline{C}H_3$†), 115.4 (q, J_F = 288 Hz, -CF_3), 125.8 (Ar-C), 127.9 (Ar-C), 128.3 (Ar-C), 129.7 (Ar-C), 134.5 (Ar-C), 138.1 (Ar-C), 156.4 (q, J_F = 38 Hz, -$\underline{C}OCF_3$), 170.3 (-$\underline{C}O_2CH_3$); ν_{max}($CDCl_3$,

cm^{-1}) 3407, 3337, 2926, 1752, 1609, 1534, 1438; *m/z* (EI) 289 (M^+, 3%), 230 (10), 176 (83), 145 (33), 105 (100), 85 (56), 83 (80); Found: C, 53.8; H, 5.1; N, 5.2. Calc. for $C_{13}H_{14}F_3NO_3$: C, 54.0; H, 4.9; N, 4.8%.

N-*Trifluoroacetyl-4'-nitrophenylalanine methyl ester (**106**)*

Intermediate ester: Starting Material: 0.400 g Yield: 0.355 g, 83% Appearance: yellow oil ; ^1H NMR (300 MHz, $CDCl_3$) δ2.90 (dd, *J* = 8, 14 Hz, 1H, β-H), 3.10 (dd, *J* = 6, 14 Hz, 1H, β-H), 3.64 (s, 3H, -OCH_3), 3.70 (dd, *J* = 6, 8 Hz, 1H, α-H), 7.32 (d, *J* = 9 Hz, 2H, Ar-H), 8.07 (d, *J* = 9 Hz, 2H, Ar-H).

Product: Intermediate ester: 0.295 g Yield: 0.340 g, 83% Appearance: pale yellow solid m.p. 101-102 °C; ^1H NMR (300 MHz, $CDCl_3$) δ3.17 (dd, *J* = 6, 14 Hz, 1H, β-H), 3.32 (dd, *J* = 6, 14 Hz, 1H, β-H), 3.73 (s, 3H, -OCH_3), 4.82 (q, *J* = 6 Hz, 1H, α-H), 6.75 (br s, 1H, NH), 7.10 (d, *J* = 9 Hz, 2H, Ar-H), 8.10 (d, *J* = 9 Hz, 2H, Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ37.0 (Ar-$\underline{C}H_2$-), 53.0 (α-C†), 53.1 (O$\underline{C}H_3$†), 115.3 (q, J_F = 288 Hz, -CF_3), 123.8 (Ar-C), 130.0 (Ar-C), 142.2 (Ar-C), 147.3 (Ar-C), 156.6 (q, J_F = 38 Hz, -$\underline{C}OCF_3$), 169.8 (-$\underline{C}O_2CH_3$); ν_{max}($CDCl_3$, cm^{-1}) 3401, 1726, 1525, 1439, 1348, 1217, 1171; *m/z* (EI) 320 (M^+, 2%), 261 (24), 244 (15), 207 (100), 176 (50), 137 (13), 105 (24), 90 (22); Found: C, 45.2; H, 3.8; N, 8.9. Calc. for $C_{12}H_{11}F_3N_2O_5$: C, 45.0; H, 3.5; N, 8.8%.

†Assignment may be reversed.

N-*Trifluoroacetyl-3'-nitrophenylalanine methyl ester (**107**)*

Intermediate ester: Starting Material: 1.03 g Yield: 0.990 g, 83% Appearance: colourless oil; ^1H NMR (300 MHz, $CDCl_3$) δ2.83 (dd, *J* = 8, 14 Hz, 1H, β-H), 3.03 (dd, *J* = 5, 14 Hz, 1H, β-H), 3.57 (s, 3H, -OCH_3), 3.64 (dd, *J* = 5, 8 Hz, 1H, α-H), 7.34 (d, *J* = 8 Hz, 1H, Ar-H), 7.45 (t, *J* = 8 Hz, 1H, Ar-H), 7.92 (d, *J* = 8 Hz, 1H, Ar-H), 7.94 (s, 1H, Ar-H).

Product: Intermediate ester: 0.790 g Yield: 0.808 g, 72% Appearance: colourless powder m.p. 96-97 °C; ^1H NMR (300 MHz, $CDCl_3$) δ3.27 (dd, *J* = 5, 14 Hz, 1H, β-H), 3.41 (dd, *J* = 5, 14 Hz, 1H, β-H), 3.84 (s, 3H, -OCH_3), 4.91 (q, *J* = 5 Hz, 1H, α-H), 6.90 (br s, 1H, NH), 7.44 (d, *J* = 8 Hz, 1H, Ar-H), 7.52 (t, *J* = 8 Hz, 1H, Ar-H), 7.98 (s, 1H, Ar-H), 8.16 (d, *J* = 8 Hz, 1H, Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ36.7 (Ar-$\underline{C}H_2$-), 53.0 (α-C†), 53.2 (O$\underline{C}H_3$†), 115.2 (q, J_F = 288 Hz, -CF_3), 122.4 (Ar-C), 123.9 (Ar-C), 129.6 (Ar-C), 135.3 (Ar-C), 136.9 (Ar-C), 148.1 (Ar-C), 156.5 (q, J_F = 38 Hz, -$\underline{C}OCF_3$), 169.9 (-$\underline{C}O_2CH_3$); ν_{max}($CDCl_3$, cm^{-1}) 3400, 1724, 1533, 1439, 1353, 1292, 1221, 1175; *m/z* (EI) 320 (M^+, 11%), 303 (19), 261 (37), 207 (100), 190 (27), 184 (35), 176 (56), 136 (30), 129 (21), 120 (25), 90 (39); Found: C, 45.2; H, 3.7; N, 8.7. Calc. for $C_{12}H_{11}F_3N_2O_5$: C, 45.0; H, 3.5; N, 8.8%.

†Assignment may be reversed.

Calculation of the degree of substitution of the hydroxypropyl-β-cyclodextrin (108) batch used

The ^{1}H NMR spectrum of a sample of the modified cyclodextrin **108** used was taken and the integration of the resonances due to the methyl protons on the hydroxypropyl substituents was compared to the integrations of the signals due to the acetal protons and the remaining protons. This gives each cyclodextrin moiety an average of 3.65 hydroxypropyl side chains, hence the effective molecular weight is 1347 g mol^{-1}.

^{1}H NMR (300 MHz, D_2O) δ1.12 (d, J = 8 Hz, 0.208H, side chain -CH_3), 3.4-4.1 (m, 1H, remaining H), 5-5.3 (m, 0.132H, H_1)

Monitoring the effect of cyclodextrins on the hydrolysis of the phenylalanine derivatives 104-107 by protease VIII

N-Trifluoroacetyl-4'-methylphenylalanine (**104**) (4.0 x 10^{-3} g, 14 x 10^{-6} mol) was dissolved in acetonitrile and the volume was made up to 1.00 cm^3. *N*-Trifluoroacetyl-3'-methylphenylalanine (**105**) (4.0 x 10^{-3} g, 14 x 10^{-6} mol) was dissolved in acetonitrile and the volume was made up to 1.00 cm^3. *N*-Trifluoroacetyl-4'-nitrophenylalanine (**106**) (4.4 x 10^{-3} g, 14 x 10^{-6} mol) was dissolved in acetonitrile and the volume was made up to 1.00 cm^3. *N*-Trifluoroacetyl-3'-nitrophenylalanine (**107**) (4.4 x 10^{-3} g, 14 x 10^{-6} mol) was dissolved in acetonitrile and the volume was made up to 1.00 cm^3. β-Cyclodextrin (**8**) (60.0 x 10^{-3} g, 52.9 x 10^{-6} mol) was dissolved in 0.05 mol dm^{-3} pH 7.5 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer. 6^A-Amino-6^A-deoxy-β-cyclodextrin (**40**) (0.596 g, 0.525 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.5 phosphate buffer, the pH was adjusted to 7.5 with 2 mol dm^{-3} hydrochloric acid and the volume was made up to 5.00 cm^3 with the buffer, with checking and adjusting of the pH. Hydroxypropyl-β-cyclodextrin (**108**) (0.650 g, 0.483 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.5 phosphate buffer and the volume was made up to 5.00 cm^3 with the buffer.

Samples of the appropriate derived phenylalanine solution (0.100 cm^3) were taken and either 0.05 mol dm^{-3} pH 7.5 phosphate buffer or one of the cyclodextrin stock solutions was added (0.890 cm^3). Protease VIII solution (10.0 x 10^{-3} cm^3) was added, prepared by serial dilution and containing sufficient enzyme that *ca.* 40% of the phenylalanine derivative was digested in 4-15 h. The reaction mixtures were thermostatted at 291 K and aliquots (10.0 x 10^{-3} cm^3) analysed by HPLC every 20 min until the reaction reached

between 35-40% reaction. The extent of reaction was calculated in each case by comparing the integrations of the peaks corresponding to the starting material and the product acid. Samples in which large amounts of enzyme were present gave detector response information. The product of the reaction time and the enzyme activity in each case allowed comparison of the rates of digestion in separate reaction mixtures. The results are summarised in Tables 55-70 and Figures 14-17.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	107	1.58
2.4×10^3	213	3.30
3.6×10^3	320	4.80
4.8×10^3	426	6.38
6.0×10^3	533	8.03
7.2×10^3	639	9.14
8.4×10^3	746	10.51
9.6×10^3	852	11.66
10.8×10^3	959	12.57
12.0×10^3	1.07×10^3	14.11
13.2×10^3	1.17×10^3	15.14
14.4×10^3	1.28×10^3	16.37
15.6×10^3	1.39×10^3	17.10
16.8×10^3	1.49×10^3	18.35
18.0×10^3	1.60×10^3	19.60
19.2×10^3	1.70×10^3	20.21
20.4×10^3	1.81×10^3	21.04
21.6×10^3	1.92×10^3	22.26
22.8×10^3	2.02×10^3	22.79
24.0×10^3	2.13×10^3	23.64
25.2×10^3	2.24×10^3	24.74
26.4×10^3	2.34×10^3	25.04
27.6×10^3	2.45×10^3	25.90
28.8×10^3	2.56×10^3	26.92
30.0×10^3	2.66×10^3	27.63
31.2×10^3	2.77×10^3	27.87
32.4×10^3	2.88×10^3	28.61
33.6×10^3	2.98×10^3	29.28
34.8×10^3	3.09×10^3	29.56
36.0×10^3	3.20×10^3	30.51
37.2×10^3	3.30×10^3	31.06
38.4×10^3	3.41×10^3	31.59
39.6×10^3	3.52×10^3	32.12
40.8×10^3	3.62×10^3	33.04
42.0×10^3	3.73×10^3	33.59
43.2×10^3	3.84×10^3	33.85
44.4×10^3	3.94×10^3	34.28
45.6×10^3	4.04×10^3	34.96
46.8×10^3	4.16×10^3	35.30

Table 55. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-methyphenylalanine methyl ester (**104**) by protease VIII (88.8×10^{-3} units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	605	4.70
2.4×10^3	1.21×10^3	9.43
3.6×10^3	1.81×10^3	13.08
4.8×10^3	2.42×10^3	17.45
6.0×10^3	3.02×10^3	21.39
7.2×10^3	3.62×10^3	24.45
8.4×10^3	4.23×10^3	27.57
9.6×10^3	4.84×10^3	30.09
10.8×10^3	5.44×10^3	32.29
12.0×10^3	6.05×10^3	34.50
13.2×10^3	6.65×10^3	36.18
14.4×10^3	7.26×10^3	37.83
15.6×10^3	7.86×10^3	39.10
16.8×10^3	8.47×10^3	40.31

Table 56. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-methylphenylalanine methyl ester (**104**) by protease VIII (0.504 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of β-cyclodextrin (**8**) (9.42×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	8.06×10^3	4.41
2.4×10^3	16.1×10^3	8.69
3.6×10^3	24.2×10^3	12.93
4.8×10^3	32.3×10^3	16.25
6.0×10^3	40.3×10^3	19.39
7.2×10^3	48.4×10^3	21.81
8.4×10^3	56.5×10^3	24.49
9.6×10^3	64.5×10^3	26.65
10.8×10^3	72.6×10^3	28.67
12.0×10^3	80.7×10^3	30.30
13.2×10^3	88.7×10^3	32.17
14.4×10^3	96.8×10^3	33.42
15.6×10^3	0.105×10^6	34.82
16.8×10^3	0.113×10^6	35.82
18.0×10^3	0.121×10^6	37.07
19.2×10^3	0.130×10^6	38.05
20.4×10^3	0.137×10^6	38.92
21.6×10^3	0.145×10^6	39.70
22.8×10^3	0.153×10^6	40.55
24.0×10^3	0.161×10^6	41.36

Table 57. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-methyphenylalanine methyl ester (**104**) by protease VIII (6.72 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) (93.5×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	7.78×10^3	2.60
2.4×10^3	15.6×10^3	5.09
3.6×10^3	23.2×10^3	7.34
4.8×10^3	31.1×10^3	9.34
6.0×10^3	38.9×10^3	11.27
7.2×10^3	46.7×10^3	12.80
8.4×10^3	54.4×10^3	14.86
9.6×10^3	62.2×10^3	16.26
10.8×10^3	70.0×10^3	18.06
12.0×10^3	77.8×10^3	19.60
13.2×10^3	85.6×10^3	21.05
14.4×10^3	93.3×10^3	22.21
15.6×10^3	0.101×10^6	23.56
16.8×10^3	0.109×10^6	24.80
18.0×10^3	0.116×10^6	26.01
19.2×10^3	0.124×10^6	26.97
20.4×10^3	0.132×10^6	28.07
21.6×10^3	0.140×10^6	28.96
22.8×10^3	0.148×10^6	29.93
24.0×10^3	0.156×10^6	30.75
25.2×10^3	0.163×10^6	31.48
26.4×10^3	0.171×10^6	32.06
27.6×10^3	0.179×10^6	32.85
28.8×10^3	0.187×10^6	33.60
30.0×10^3	0.194×10^6	34.32
31.2×10^3	0.202×10^6	34.85
32.4×10^3	0.210×10^6	35.44
33.6×10^3	0.218×10^6	35.85
34.8×10^3	0.226×10^6	36.43

Table 58. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-methylphenylalanine methyl ester (**104**) by protease VIII (6.48 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of hydroxypropyl-β-cyclodextrin (**108**) (86.0×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	691	2.22
2.4×10^3	1.38×10^3	4.17
3.6×10^3	2.07×10^3	5.62
4.8×10^3	2.76×10^3	7.64
6.0×10^3	3.46×10^3	9.25
7.2×10^3	4.15×10^3	10.78
8.4×10^3	4.84×10^3	12.46
9.6×10^3	5.53×10^3	14.20
10.8×10^3	6.22×10^3	15.59
12.0×10^3	6.91×10^3	17.16
13.2×10^3	7.60×10^3	18.89
14.4×10^3	8.29×10^3	20.17
15.6×10^3	8.99×10^3	21.28
16.8×10^3	9.68×10^3	22.78
18.0×10^3	10.4×10^3	23.94
19.2×10^3	11.1×10^3	25.09
20.4×10^3	11.8×10^3	26.37
21.6×10^3	12.4×10^3	27.62
22.8×10^3	13.1×10^3	28.76
24.0×10^3	13.8×10^3	29.83
25.2×10^3	14.5×10^3	30.79
26.4×10^3	15.2×10^3	31.69
27.6×10^3	15.9×10^3	32.79
28.8×10^3	16.6×10^3	34.00
30.0×10^3	17.3×10^3	34.78
31.2×10^3	18.0×10^3	35.69
32.4×10^3	18.7×10^3	36.60
33.6×10^3	19.4×10^3	37.31
34.8×10^3	20.0×10^3	37.91

Table 59. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-methylphenylalanine methyl ester (**105**) by protease VIII (0.576 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	1.68×10^3	5.47
2.4×10^3	3.36×10^3	10.50
3.6×10^3	5.04×10^3	15.12
4.8×10^3	6.72×10^3	19.52
6.0×10^3	8.40×10^3	23.85
7.2×10^3	10.1×10^3	27.43
8.4×10^3	11.8×10^3	30.54
9.6×10^3	13.4×10^3	33.16
10.8×10^3	15.1×10^3	35.67
12.0×10^3	16.8×10^3	37.60
13.2×10^3	18.5×10^3	39.41
14.4×10^3	20.2×10^3	41.03

Table 60. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-methylphenylalanine methyl ester (**105**) by protease VIII (1.40 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of β-cyclodextrin (**8**) (9.42×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	9.00×10^3	5.18
2.4×10^3	18.0×10^3	9.83
3.6×10^3	27.0×10^3	13.60
4.8×10^3	36.0×10^3	16.92
6.0×10^3	45.0×10^3	20.52
7.2×10^3	54.0×10^3	23.82
8.4×10^3	63.0×10^3	26.22
9.6×10^3	72.0×10^3	28.33
10.8×10^3	81.0×10^3	30.37
12.0×10^3	90.0×10^3	32.29
13.2×10^3	99.0×10^3	33.84
14.4×10^3	0.108×10^6	35.27
15.6×10^3	0.117×10^6	36.39
16.8×10^3	0.126×10^6	37.62
18.0×10^3	0.135×10^6	38.81
19.2×10^3	0.144×10^6	39.73
20.4×10^3	0.153×10^6	40.75

Table 61. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-methylphenylalanine methyl ester (**105**) by protease VIII (7.50 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) (93.5×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	7.2×10^3	2.94
2.4×10^3	14.4×10^3	5.54
3.6×10^3	21.6×10^3	8.14
4.8×10^3	28.8×10^3	10.30
6.0×10^3	36.0×10^3	12.20
7.2×10^3	43.2×10^3	14.62
8.4×10^3	50.4×10^3	16.38
9.6×10^3	57.6×10^3	18.22
10.8×10^3	64.8×10^3	20.17
12.0×10^3	72.0×10^3	22.03
13.2×10^3	79.2×10^3	23.73
14.4×10^3	86.4×10^3	25.82
15.6×10^3	93.6×10^3	27.20
16.8×10^3	0.101×10^6	28.68
18.0×10^3	0.108×10^6	30.15
19.2×10^3	0.115×10^6	31.57
20.4×10^3	0.122×10^6	32.61
21.6×10^3	0.130×10^6	33.51
22.8×10^3	0.137×10^6	34.86
24.0×10^3	0.144×10^6	36.14
25.2×10^3	0.151×10^6	36.98
26.4×10^3	0.158×10^6	37.74
27.6×10^3	0.166×10^6	38.52
28.8×10^3	0.173×10^6	39.49
30.0×10^3	0.180×10^6	40.17
31.2×10^3	0.187×10^6	40.82

Table 62. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-methyphenylalanine methyl ester (**105**) by protease VIII (6.00 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of hydroxypropyl-β-cyclodextrin (**108**) (86.0×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	418	4.67
2.4×10^3	835	7.83
3.6×10^3	1.25	11.00
4.8×10^3	1.67×10^3	13.90
6.0×10^3	2.09×10^3	16.88
7.2×10^3	2.51×10^3	18.82
8.4×10^3	2.92×10^3	21.58
9.6×10^3	3.34×10^3	24.49
10.8×10^3	3.76×10^3	26.44
12.0×10^3	4.18×10^3	28.80
13.2×10^3	4.59×10^3	30.10
14.4×10^3	5.01×10^3	32.16
15.6×10^3	5.43×10^3	34.03
16.8×10^3	5.85×10^3	36.13
18.0×10^3	6.26×10^3	37.24
19.2×10^3	6.68×10^3	38.61
20.4×10^3	7.10×10^3	40.28

Table 63. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-nitrophenylalanine methyl ester (**106**) by protease VIII (0.348 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	1.04×10^3	5.75
2.4×10^3	2.07×10^3	10.23
3.6×10^3	3.11×10^3	15.01
4.8×10^3	4.15×10^3	18.93
6.0×10^3	5.18×10^3	21.75
7.2×10^3	6.22×10^3	25.43
8.4×10^3	7.26×10^3	28.60
9.6×10^3	8.29×10^3	30.33
10.8×10^3	9.33×10^3	32.98
12.0×10^3	10.4×10^3	34.47
13.2×10^3	11.4×10^3	37.15
14.4×10^3	12.4×10^3	38.52
15.6×10^3	13.5×10^3	40.08

Table 64. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-nitrophenylalanine methyl ester (**106**) by protease VIII (0.864 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of β-cyclodextrin (**8**) (9.42×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	6.34×10^3	6.60
2.4×10^3	12.7×10^3	12.30
3.6×10^3	19.0×10^3	17.55
4.8×10^3	25.3×10^3	22.41
6.0×10^3	31.7×10^3	25.73
7.2×10^3	38.0×10^3	28.96
8.4×10^3	44.3×10^3	32.06
9.6×10^3	50.7×10^3	34.50
10.8×10^3	57.0×10^3	36.64
12.0×10^3	63.4×10^3	38.36
13.2×10^3	69.7×10^3	39.92
14.4×10^3	76.0×10^3	41.41

Table 65. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-nitrophenylalanine methyl ester (**106**) by protease VIII (5.28 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) (93.5×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	7.92×10^3	7.56
2.4×10^3	15.8×10^3	15.36
3.6×10^3	23.8×10^3	20.44
4.8×10^3	31.7×10^3	25.08
6.0×10^3	39.6×10^3	29.45
7.2×10^3	47.5×10^3	32.40
8.4×10^3	55.4×10^3	35.29
9.6×10^3	63.4×10^3	37.67
10.8×10^3	71.3×10^3	39.76
12.0×10^3	79.2×10^3	41.19

Table 66. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-nitrophenylalanine methyl ester (**106**) by protease VIII (6.60 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of hydroxypropyl-β-cyclodextrin (**108**) (86.0×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2 x 10^3	1.38 x 10^3	1.86
2.4 x 10^3	2.76 x 10^3	3.77
3.6 x 10^3	4.14 x 10^3	5.72
4.8 x 10^3	5.52 x 10^3	7.42
6.0 x 10^3	6.90 x 10^3	9.02
7.2 x 10^3	8.28 x 10^3	10.67
8.4 x 10^3	9.66 x 10^3	12.16
9.6 x 10^3	11.0 x 10^3	13.58
10.8 x 10^3	12.4 x 10^3	15.16
12.0 x 10^3	13.8 x 10^3	16.66
13.2 x 10^3	15.2 x 10^3	17.76
14.4 x 10^3	16.6 x 10^3	19.46
15.6 x 10^3	17.9 x 10^3	20.63
16.8 x 10^3	19.3 x 10^3	21.76
18.0 x 10^3	20.7 x 10^3	23.22
19.2 x 10^3	22.1 x 10^3	24.45
20.4 x 10^3	23.5 x 10^3	25.42
21.6 x 10^3	24.8 x 10^3	26.62
22.8 x 10^3	26.2 x 10^3	27.70
24.0 x 10^3	27.6 x 10^3	28.84
25.2 x 10^3	29.0 x 10^3	29.86
26.4 x 10^3	30.4 x 10^3	30.81
27.6 x 10^3	31.7 x 10^3	31.69
28.8 x 10^3	33.1 x 10^3	32.90
30.0 x 10^3	34.5 x 10^3	33.47
31.2 x 10^3	35.9 x 10^3	34.35
32.4 x 10^3	37.3 x 10^3	35.08
33.6 x 10^3	38.6 x 10^3	35.73
34.8 x 10^3	40.0 x 10^3	36.36

Table 67. Observed extents of reaction for the hydrolysis of the phenylalanine derivative **107** by protease VIII (1.15 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2 x 10^3	4.84 x 10^3	3.86
2.4 x 10^3	9.67 x 10^3	7.30
3.6 x 10^3	14.5 x 10^3	10.45
4.8 x 10^3	19.3 x 10^3	13.83
6.0 x 10^3	24.2 x 10^3	16.67
7.2 x 10^3	29.0 x 10^3	19.68
8.4 x 10^3	33.9 x 10^3	21.68
9.6 x 10^3	38.7 x 10^3	24.26
10.8 x 10^3	43.5 x 10^3	26.38
12.0 x 10^3	48.4 x 10^3	28.56
13.2 x 10^3	53.2 x 10^3	29.90
14.4 x 10^3	58.0 x 10^3	31.90
15.6 x 10^3	62.9 x 10^3	33.61
16.8 x 10^3	67.7 x 10^3	35.04
18.0 x 10^3	72.5 x 10^3	36.09
19.2 x 10^3	77.4 x 10^3	37.53

Table 68. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-nitrophenylalanine methyl ester (**107**) by protease VIII (4.03 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of β-cyclodextrin (**8**) (9.42 x 10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	13.7×10^3	2.41
2.4×10^3	27.4×10^3	5.31
3.6×10^3	41.0×10^3	7.50
4.8×10^3	54.7×10^3	10.15
6.0×10^3	68.4×10^3	12.72
7.2×10^3	82.1×10^3	15.33
8.4×10^3	95.8×10^3	17.71
9.6×10^3	0.109×10^6	19.99
10.8×10^3	0.123×10^6	21.90
12.0×10^3	0.137×10^6	23.67
13.2×10^3	0.150×10^6	25.49
14.4×10^3	0.164×10^6	27.18
15.6×10^3	0.178×10^6	28.71
16.8×10^3	0.192×10^6	29.80
18.0×10^3	0.205×10^6	31.31
19.2×10^3	0.219×10^6	32.63
20.4×10^3	0.233×10^6	33.64
21.6×10^3	0.246×10^6	34.55
22.8×10^3	0.260×10^6	35.23
24.0×10^3	0.274×10^6	36.43
25.2×10^3	0.287×10^6	37.25
26.4×10^3	0.301×10^6	38.04
27.6×10^3	0.315×10^6	38.50
28.8×10^3	0.328×10^6	39.30
30.0×10^3	0.342×10^6	39.92
31.2×10^3	0.356×10^6	40.59

Table 69. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-nitrophenylalanine methyl ester (**107**) by protease VIII (11.4 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of 6^A-amino-6^A-deoxy-β-cyclodextrin (**40**) (93.5×10^{-3} mol dm^{-3}).

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	28.6×10^3	9.73
2.4×10^3	57.2×10^3	17.69
3.6×10^3	85.6×10^3	23.85
4.8×10^3	0.114×10^{36}	28.89
6.0×10^3	0.142×10^6	32.67
7.2×10^3	0.171×10^6	35.86
8.4×10^3	0.200×10^6	38.18
9.6×10^3	0.228×10^6	41.29

Table 70. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-nitrophenylalanine methyl ester (**107**) by protease VIII (23.8 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K in the presence of hydroxypropyl-β-cyclodextrin (**108**) (86.0×10^{-3} mol dm^{-3}).

Calculation of the association constant of the complex of *N*-trifluoroacetyl-4'-methylphenylalanine methyl ester (104) in hydroxypropyl-β-cyclodextrin (108)

N-Trifluoroacetyl-4'-methylphenylalanine methyl ester (**104**) (5.9 x 10^{-3} g, 20.4 x 10^{-6} mol) was dissolved in d_3-acetonitrile, and the volume was made up to 2.00 cm^3. Stock solutions were prepared by dissolving hydroxypropyl-β-cyclodextrin (**108**) (I: 0.541 g, 0.402 x 10^{-3} mol, II: 1.27 g, 0.943 x 10^{-3} mol) in 0.05 mol dm^{-3} pH 7.5 phosphate buffer and the volumes were made up to 5 cm^3 with the buffer. All solutions were sonicated to homogeneity. Hydroxypropyl-β-cyclodextrin (**108**) stock solution was taken (Table 71), phenylalanine derivative **104** stock solution (0.100 cm^3) was added, and the total volume was made up to 1.00 cm^3 with the buffer. The amount of cyclodextrin **108** stock solution added was varied to give from 0 to 150 equivalents of the host **108** with respect to the guest **104**. An aliquot of each sample was analysed by ^1H NMR spectroscopy at 298 K. The signals due to the aromatic protons and the protons of the methyl group attached to the aromatic ring were observed to vary in chemical shift with the concentration of the host **108**. Sample preparation and observed chemical shift changes between signals corresponding to the methyl group and the aromatic protons are summarised below (Table 71).

Chemical shift information was plotted against concentration of the modified cyclodextrin **108** and a non-linear regression analysis (using Equations 1 and 2) gave the association constant for the complex formed as 50 ± 10 mol^{-1} dm^3 (Figure 18).

Stock solution	Cyclodextrin **108** stock solution (cm^3)	[Cyclodextrin **108**] (mol dm^{-3})	Observed chemical shift difference (Hz)
-	-	-	2440.77
I	50.00 x 10^{-3}	4.02 x 10^{-3}	2437.89
I	0.100	8.03 x 10^{-3}	2436.06
I	0.200	16.1 x 10^{-3}	2433.13
I	0.350	28.1 x 10^{-3}	2431.56
I	0.500	40.2 x 10^{-3}	2430.13
I	0.700	56.2 x 10^{-3}	2428.90
I	0.900	72.3 x 10^{-3}	2427.42
II	0.500	83.9 x 10^{-3}	2427.21
II	0.600	0.101	2426.95
II	0.700	0.117	2426.53
II	0.800	0.134	2426.08
II	0.900	0.151	2425.80

Table 71. Sample preparation and observed chemical shift difference for the calculation of the association constant of the complex of *N*-trifluoroacetyl-4'-methylphenylalanine methyl ester (**104**) with hydroxypropyl-β-cyclodextrin (**108**) in 10% d_3-acetonitrile in 0.05 mol dm^{-3} phosphate buffer at pH 7.5 298 K.

Calculation of the association constant of the complex of *N*-trifluoroacetyl-3'-methylphenylalanine methyl ester (105) in hydroxypropyl-β-cyclodextrin (108)

N-Trifluoroacetyl-3'-methylphenylalanine methyl ester (**105**) (5.7 x 10^{-3} g, 19.7 x 10^{-6} mol) was dissolved in d_3-acetonitrile, and the volume was made up to 2.00 cm^3. Stock solutions were prepared by dissolving the modified cyclodextrin **108** (I: 0.541 g, 0.402 x 10^{-3} mol, II: 1.13 g, 0.839 x 10^{-3} mol) in 0.05 mol dm^{-3} pH 7.5 phosphate buffer and the volumes were made up to 5 cm^3 with the buffer. All solutions were sonicated to homogeneity. Hydroxypropyl-β-cyclodextrin (**108**) stock solution was taken (Table 72), phenylalanine derivative **105** stock solution (0.100 cm^3) was added, and the total volume was made up to 1.00 cm^3 with the buffer. The amount of cyclodextrin **108** stock solution added was varied to give from 0 to 170 equivalents of the host **108** with respect to the guest **105**. An aliquot of each sample was analysed by ^{1}H NMR spectroscopy at 298 K. The signals due to the aromatic protons and the protons of the methyl group attached to the aromatic ring were observed to vary in chemical shift with the concentration of the host **108**. Sample preparation and observed chemical shift changes between signals corresponding to the methyl group and the 5'-hydrogen are summarised below (Table 72).

Chemical shift information was plotted against concentration of the modified cyclodextrin **108** and a non-linear regression analysis (using Equations 1 and 2) gave the association constant for the complex formed as 90 ± 10 mol^{-1} dm^3 (Figure 19).

Stock solution	Cyclodextrin **108** stock solution (cm^3)	[Cyclodextrin **108**] (mol dm^{-3})	Observed chemical shift difference (Hz)
-	-	-	2478.65
I	50.00 x 10^{-3}	4.02 x 10^{-3}	2482.21
I	0.100	8.03 x 10^{-3}	2485.50
I	0.200	16.1 x 10^{-3}	2488.15
I	0.350	28.1 x 10^{-3}	2490.08
I	0.500	40.2 x 10^{-3}	2490.83
I	0.700	56.2 x 10^{-3}	2491.96
I	0.900	72.3 x 10^{-3}	2492.90
II	0.400	75.4 x 10^{-3}	2492.91
II	0.500	95.3 x 10^{-3}	2492.99
II	0.600	0.113	2493.24
II	0.700	0.132	2493.26
II	0.800	0.151	2493.37
II	0.900	0.170	2493.70

Table 72. Sample preparation and observed chemical shift difference for the calculation of the association constant of the complex of *N*-trifluoroacetyl-3'-methyphenylalanine methyl ester (**105**) with hydroxypropyl-β-cyclodextrin (**108**) in 10% d_3-acetonitrile in 0.05 mol dm^{-3} phosphate buffer at pH 7.5 298 K

Monitoring the effect of substrate concentration on the digestion of the phenylalanine derivatives 104-107 by protease VIII

N-Trifluoroacetyl-4'-methylphenylalanine (**104**) (8.0×10^{-3} g, 28×10^{-6} mol) was dissolved in acetonitrile and the volume was made up to 2.00 cm^3. *N*-Trifluoroacetyl-3'-methylphenylalanine (**105**) (8.0×10^{-3} g, 28×10^{-6} mol) was dissolved in acetonitrile and the volume was made up to 2.00 cm^3. *N*-Trifluoroacetyl-4'-nitrophenylalanine (**106**) (8.8×10^{-3} g, 27×10^{-6} mol) was dissolved in acetonitrile and the volume was made up to 2.00 cm^3. *N*-Trifluoroacetyl-3'-nitrophenylalanine (**107**) (8.8×10^{-3} g, 27×10^{-6} mol) was dissolved in acetonitrile and the volume was made up to 2.00 cm^3.

Samples of the appropriate derived phenylalanine solution (10.0×10^{-3} cm^3) were taken and diluted to either 0.100 cm^3 or 1.00 cm^3 with acetonitrile. These were subsequently diluted to either 0.990 cm^3 or 9.99 cm^3 with 0.05 mol dm^{-3} pH 7.5 phosphate buffer. Enzyme solution (10.0×10^{-3} cm^3) was added, prepared by serial dilution and containing enough enzyme that *ca.* 40% of the phenylalanine derivative was digested in 1.5-3 h. The reaction mixtures were thermostatted at 291 K and aliquots (25.0×10^{-3} or 50.0×10^{-3} cm^3) analysed by HPLC every 20 min until the reaction reached between 35-40% reaction. The extent of reaction was calculated in each case by comparing the integrations of the peaks corresponding to the starting material and the product acid. Samples where large amounts of enzyme were present gave detector response information. The product of the reaction time and the enzyme activity in each case allowed comparison of the rates of digestion in separate reaction mixtures. The results are summarised in Tables 73-80 and Figures 20 and 21.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	396	8.93
2.4×10^3	792	16.82
3.6×10^3	1.19×10^3	23.45
4.8×10^3	1.58×10^3	30.96
6.0×10^3	1.98×10^3	35.85
7.2×10^3	2.38×10^3	40.04

Table 73. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-methylphenylalanine methyl ester (**104**) by protease VIII (0.330 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K at a starting concentration of 0.14×10^{-3} mol dm^{-3}.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	3.17×10^3	9.06
2.4×10^3	6.34×10^3	16.52
3.6×10^3	9.50×10^3	22.13
4.8×10^3	12.7×10^3	26.92
6.0×10^3	15.8×10^3	31.43
7.2×10^3	19.0×10^3	35.53
8.4×10^3	22.2×10^3	38.54

Table 74. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-methylphenylalanine methyl ester (**104**) by protease VIII (2.64 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K at a starting concentration of 14×10^{-6} mol dm^{-3}.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	1.58×10^3	9.76
2.4×10^3	3.17×10^3	19.90
3.6×10^3	4.75×10^3	26.40
4.8×10^3	6.34×10^3	33.22
6.0×10^3	7.92×10^3	37.95
7.2×10^3	9.50×10^3	42.23

Table 75. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-methylphenylalanine methyl ester (**105**) by protease VIII (1.32 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K at a starting concentration of 0.14×10^{-3} mol dm^{-3}.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	6.34×10^3	11.89
2.4×10^3	12.7×10^3	19.50
3.6×10^3	19.0×10^3	26.36
4.8×10^3	25.3×10^3	30.86
6.0×10^3	31.7×10^3	35.79
7.2×10^3	38.0×10^3	39.49

Table 76. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-methylphenylalanine methyl ester (**105**) by protease VIII (5.28 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K at a starting concentration of 14×10^{-6} mol dm^{-3}.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	1.24×10^3	14.30
2.4×10^3	2.47×10^3	24.14
3.6×10^3	3.71×10^3	31.90
4.8×10^3	4.94×10^3	37.58

Table 77. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-nitrophenylalanine methyl ester (**106**) by protease VIII (1.03 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K at a starting concentration of 0.14×10^{-3} mol dm^{-3}.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	7.38×10^3	10.57
2.4×10^3	14.8×10^3	18.01
3.6×10^3	22.1×10^3	24.76
4.8×10^3	29.5×10^3	30.31
6.0×10^3	36.9×10^3	35.85

Table 78. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-4'-nitrophenylalanine methyl ester (**106**) by protease VIII (6.15 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K at a starting concentration of 14×10^{-6} mol dm^{-3}.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	3.59×10^3	7.44
2.4×10^3	7.18×10^3	12.91
3.6×10^3	10.8×10^3	18.41
4.8×10^3	14.4×10^3	22.92
6.0×10^3	17.9×10^3	27.08
7.2×10^3	21.5×10^3	31.05
8.4×10^3	25.1×10^3	34.55

Table 79. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-nitrophenylalanine methyl ester (**107**) by protease VIII (2.99 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K at a starting concentration of 0.14×10^{-3} mol dm^{-3}.

Time (s)	Time.[enzyme] (s units dm^{-3})	Extent of reaction (%)
1.2×10^3	8.98×10^3	9.77
2.4×10^3	18.0×10^3	18.94
3.6×10^3	26.9×10^3	26.25
4.8×10^3	35.9×10^3	32.59
6.0×10^3	44.9×10^3	38.07

Table 80. Observed extents of reaction for the hydrolysis of *N*-trifluoroacetyl-3'-nitrophenylalanine methyl ester (**107**) by protease VIII (7.48 units dm^{-3}) in 10% acetonitrile in 0.05 mol dm^{-3} pH 7.5 phosphate buffer at 291 K at a starting concentration of 14×10^{-6} mol dm^{-3}.

Experimental VII: Effect of Cyclodextrins on Substrate Inhibition of Carboxypeptidase A

Treatment of the peptide derivative 5 with carboxypeptidase A

The protonated form of (*S*)-carbobenzoxyglycylphenylalanine (**5**) (0.807 g, 2.26 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.5 phosphate buffer and the volume was made up to 5 cm^3 with the buffer, adjusting the pH to 7.5 with 2 mol dm^{-3} sodium hydroxide solution. Carboxypeptidase A solution (25.0 x 10^{-3} cm^3, *ca.* 30 units) was diluted to 50 cm^3 with 0.05 mol dm^{-3} pH 7.5 phosphate buffer. Reaction mixtures were prepared as described below (Table 81), carboxypeptidase A stock solution (25 x 10^{-3} cm^{-3}, *ca.* 15 x 10^{-3} units) was added, the total volume was made up to the 2.0 cm^3 with the buffer and the solutions were thermostatted at 298 K for 2 h. Aliquots were taken at intervals during this time, adjusted to *ca.* pH 1 with 2 mol dm^{-3} hydrochloric acid and the suspensions were extracted with ethyl acetate (2 x 1 cm^{-3}). The organic layers were dried over magnesium sulfate, the solvent was removed *in vacuo* and the residues were analysed by HPLC on an Alltima 5 μ C18 column (4.6 x 10^{-3} m by 0.250 m) (acetonitrile : water : trifluoroacetic acid, 50 : 49.9 : 0.1) monitoring at 260 nm. The data from the case where a large excess of enzyme was used gave detector response information. From this information, the initial rate of reaction was determined for each concentration of the substrate **5** used (Table 81). This data was used to plot the rate of reaction against the concentration of the substrate **5** (Figure 22) and a Lineweaver-Burk[113] plot (Figure 23).

Substrate **5** stock solution (cm^3)	Potassium chloride (g)	[Substrate **5**] (mol dm^{-3})	Rate of reaction (mol dm^{-3} s^{-1})
41.7 x 10^{-3}	58.1 x 10^{-3}	9.42 x 10^{-3}	0.597 x 10^{-6}
83.3 x 10^{-3}	56.6 x 10^{-3}	18.8 x 10^{-3}	0.876 x 10^{-6}
0.125	55.2 x 10^{-3}	28.3 x 10^{-3}	1.04 x 10^{-6}
0.167	53.6 x 10^{-3}	37.7 x 10^{-3}	1.33 x 10^{-6}
0.250	50.7 x 10^{-3}	56.6 x 10^{-3}	1.46 x 10^{-6}
0.333	47.7 x 10^{-3}	75.3 x 10^{-3}	1.29 x 10^{-6}
0.500	41.8 x 10^{-3}	0.113	1.09 x 10^{-6}
0.667	35.8 x 10^{-3}	0.151	1.05 x 10^{-6}
1.00	23.9 x 10^{-3}	0.226	0.954 x 10^{-6}
1.33	11.9 x 10^{-3}	0.301	0.939 x 10^{-6}

Table 81. Sample preparation and rate of reaction for the hydrolysis of (*S*)-carbobenzoxyglycylphenylalanine (**5**) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 phosphate buffer (I = 0.5 mol dm^{-3}) at 298 K.

Treatment of (*S*)-carbobenzoxyglycylphenylalanine (5) with carboxypeptidase A in the presence of hydroxypropyl-β-cyclodextrin (108)

Carboxypeptidase A solution (25.0×10^{-3} cm^3, *ca.* 30 units) was diluted to 50 cm^3 with 0.05 mol dm^{-3} pH 7.5 phosphate buffer. Reaction mixtures were prepared as described below (Table 82), hydroxypropyl-β-cyclodextrin (**108**) (0.500 g, 0.371×10^{-3} mol dm^{-3}) was added and the solids were dissolved in 0.05 mol dm^{-3} pH 7.5 phosphate buffer, adjusting the pH to 7.5 with 2 mol dm^{-3} sodium hydroxide solution. Carboxypeptidase A stock solution (25×10^{-3} cm^{-3}, *ca.* 15×10^{-3} units) was added, the total volume was made up to the 2.0 cm^3 with the buffer and the solutions were thermostatted at 298 K for 2 h. Samples were taken at intervals during this time, adjusted to *ca.* pH 1 with 2 mol dm^{-3} hydrochloric acid and the suspensions extracted with ethyl acetate (2×1 cm^{-3}). The organic layers were dried over magnesium sulfate, the solvent was removed *in vacuo* and the residues were analysed by HPLC on an Alltima 5 μ C18 column (4.6×10^{-3} m by 0.250 m) (acetonitrile : water : trifluoroacetic acid, 50 : 49.9 : 0.1) monitoring at 260 nm. The data from the case where a large excess of enzyme was used gave detector response information. From this information, the initial rate of reaction was determined for each concentration of the substrate **5** used (Table 82). This data was used to plot the rate of reaction against the concentration of the substrate **5** (Figure 24).

Protonated peptide **5** (g)	Potassium chloride (g)	[Peptide **5**] (mol dm^{-3})	Rate of reaction (mol dm^{-3} s^{-1})
17.0×10^{-3}	56.6×10^{-3}	23.8×10^{-3}	0.708×10^{-6}
37.9×10^{-3}	55.2×10^{-3}	53.2×10^{-3}	1.21×10^{-6}
54.9×10^{-3}	47.7×10^{-3}	77.0×10^{-3}	1.37×10^{-6}
86.4×10^{-3}	41.8×10^{-3}	0.121	1.28×10^{-6}
0.111	35.5×10^{-3}	0.156	1.09×10^{-6}
0.170	23.9×10^{-3}	0.239	1.02×10^{-6}
0.225	11.9×10^{-3}	0.316	0.991×10^{-6}

Table 82. Sample preparation and rate of reaction for the hydrolysis of (*S*)-carbobenzoxyglycylphenylalanine (**5**) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 phosphate buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of the modified cyclodextrin **108** (0.186 mol dm^{-3}).

Treatment of (*S*)-carbobenzoxyglycylphenylalanine (5) with carboxypeptidase A in presence of 6^A-amino-6^A-deoxy-α-cyclodextrin (111)

The protonated form of (*S*)-carbobenzoxyglycylphenylalanine (**5**) (0.884 g, 2.48 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.5 phosphate buffer and the volume was made up to 5 cm^3 with the buffer, adjusting the pH to 7.5 with 2 mol dm^{-3} sodium hydroxide solution. Carboxypeptidase A solution (25.0 x 10^{-3} cm^3, *ca.* 30 units) was diluted to 50 cm^3 with 0.05 mol dm^{-3} pH 7.5 phosphate buffer. Reaction mixtures were prepared as described below (Table 83), amino-6^A-deoxy-α-cyclodextrin (**111**) (0.500 g, 0.441 x 10^{-3} mol) was added and the solids were dissolved in 0.05 mol dm^{-3} pH 7.5 phosphate, adjusting the pH to 7.5 with 2 mol dm^{-3} hydrochloric acid. Carboxypeptidase A solution (25 x 10^{-3} cm^{-3}, *ca.* 15 x 10^{-3} units) was added, the volume was made up to 2.00 cm^{-3} with the buffer and the solutions were thermostatted at 298 K for 2 h. Aliquots were taken at intervals during this time, adjusted to *ca.* pH 1 with 2 mol dm^{-3} hydrochloric acid and the suspensions extracted with ethyl acetate (2 x 1 cm^{-3}). The organic layers were dried over magnesium sulfate, the solvent was removed *in vacuo* and the residues were analysed by HPLC on an Alltima 5 μ C18 column (4.6 x 10^{-3} m by 0.250 m) (acetonitrile : water : trifluoroacetic acid, 50 : 49.9 : 0.1) monitoring at 260 nm. The data from a case where a large excess of enzyme was used gave detector response information. From this information, the extent of reaction was calculated and the rate of reaction determined (Table 83). This data was used to plot the rate of reaction against the concentration of the substrate **5** (Figure 24).

Peptide **5** stock solution (cm^3)	Potassium chloride (g)	[Peptide derivative **5**] (mol dm^{-3})	Rate of reaction (mol dm^{-3} s^{-1})
40.0 x 10^{-3}	52.2 x 10^{-3}	9.92 x 10^{-3}	58.7 x 10^{-9}
80.0 x 10^{-3}	51.1 x 10^{-3}	19.8 x 10^{-3}	88.6 x 10^{-9}
0.200	47.1 x 10^{-3}	49.6 x 10^{-3}	0.361 x 10^{-6}
0.320	41.8 x 10^{-3}	79.4 x 10^{-3}	0.786 x 10^{-6}
0.480	36.1 x 10^{-3}	0.119	1.55 x 10^{-6}
0.640	29.4 x 10^{-3}	0.159	1.33 x 10^{-6}
0.800	23.5 x 10^{-3}	0.198	1.21 x 10^{-6}
0.960	28.0 x 10^{-3}	0.238	1.09 x 10^{-6}
1.20	6.3 x 10^{-3}	0.298	1.14 x 10^{-6}

Table 83. Sample preparation and rate of reaction for the hydrolysis of (*S*)-carbobenzoxyglycylphenylalanine (**5**) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 phosphate buffer (I = 0.9 mol dm^{-3}) at 298 K in the presence the amine **111** (0.221 mol dm^{-3}).

Treatment of (*S*)-*N*-benzoylglycyl-β-phenyllactate (6) with carboxypeptidase A in phosphate buffer

Sodium *N*-benzoylglycyl-β-phenyllactate (0.140 g, 0.401 x 10^{-3} mol) and potassium chloride (82 x 10^{-3} g, 1.1 x 10^{-3} mol) were dissolved in 0.05 mol dm^{-3} pH 7.5 phosphate buffer and the volume was made up to 5 cm^3 with the buffer. Potassium chloride (2.98 g, 40.0 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.5 phosphate buffer and the volume was made up to 100 cm^3 with the buffer. Carboxypeptidase A solution (25.0 x 10^{-3} cm^3, *ca.* 30 units) was diluted to 50 cm^3 with 0.05 mol dm^{-3} pH 7.5 phosphate buffer. Reaction mixtures were prepared as described below (Table 84), made up to the total volume with potassium chloride solution and thermostatted at 298 K for 15 min (over which time the reaction profile had been shown to be linear.). Reactions were adjusted to *ca.* pH 1 with 2 mol dm^{-3} hydrochloric acid and the suspensions were extracted with ethyl acetate (2 x 1 cm^{-3}). The organic layers were dried over magnesium sulfate, the solvent was removed *in vacuo* and the residues were analysed by HPLC on an Alltima 5 μ C18 column (4.6 x 10^{-3} m by 0.250 m) (acetonitrile : water : trifluoroacetic acid, 50 : 49.9 : 0.1) monitoring at 260 nm. The data from a case where a large excess of enzyme was used gave detector response information. From this information, the extent of reaction was calculated and the rate of reaction determined (Table 84), and normalised for the amount of enzyme present. An arbitrary value of 20 units dm^{-3} was chosen, corresponding approximately to the concentration of enzyme in the first case. This data was used to plot the rate of reaction against the concentration of the substrate **6** (Figure 25) fitting the data to Equation 5 to give the kinetic parameters of the system as v_{max} = (0.230 ± 0.005) x 10^{-6} mol dm^{-3} s^{-1} and K_M = (0.770 ± 0.070) x 10^{-6} mol dm^{-3}.

Racemate of compound **6** stock solution (cm^3)	Enzyme stock solution (cm^3)	Total volume (cm^3)	[Lactate **6**] (mol dm^{-3})	Extent of reaction of the lactate **6** after 15 min (%)	Rate of reaction (mol dm^{-3} s^{-1}) normalised to 20 units dm^{-3}
5.00×10^{-3}	40×10^{-3}	5.00	4.01×10^{-6}	19.2	7.13×10^{-9}
8.00×10^{-3}	40×10^{-3}	5.00	6.42×10^{-6}	18.5	11.0×10^{-9}
13.0×10^{-3}	40×10^{-3}	5.00	10.4×10^{-6}	21.5	20.7×10^{-9}
20.0×10^{-3}	40×10^{-3}	5.00	16.0×10^{-6}	20.7	30.6×10^{-9}
31.0×10^{-3}	40×10^{-3}	5.00	24.9×10^{-6}	18.5	42.5×10^{-9}
50.0×10^{-3}	40×10^{-3}	5.00	40.1×10^{-6}	19.1	70.8×10^{-9}
32.0×10^{-3}	16×10^{-3}	2.00	64.1×10^{-6}	17.3	0.103×10^{-6}
50.0×10^{-3}	16×10^{-3}	2.00	0.100×10^{-3}	15.0	0.139×10^{-6}
80.0×10^{-3}	16×10^{-3}	2.00	0.161×10^{-3}	11.5	0.171×10^{-6}
0.125	16×10^{-3}	2.00	0.251×10^{-3}	7.98	0.185×10^{-6}
0.200	32×10^{-3}	2.00	0.401×10^{-3}	10.9	0.203×10^{-6}
0.315	32×10^{-3}	2.00	0.632×10^{-3}	7.14	0.209×10^{-6}
0.250	24×10^{-3}	1.00	1.00×10^{-3}	6.90	0.213×10^{-6}
0.400	24×10^{-3}	1.00	1.60×10^{-3}	4.22	0.208×10^{-6}
0.625	32×10^{-3}	1.00	2.51×10^{-3}	3.67	0.213×10^{-6}

Table 84. Sample preparation, extent of reaction and rate of reaction for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate **6** by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 phosphate buffer (I = 0.5 mol dm^{-3}) at 298 K.

Treatment of (*S*)-*N*-benzoylglycyl-β-phenyllactate (6) with carboxypeptidase A in Tris buffer

Stock solutions were prepared by dissolving sodium *N*-benzoylglycyl-β-phenyllactate (I: 69.1 x 10^{-3} g, 0.198 x 10^{-3} mol, II: 0.689 g, 1.97 x 10^{-3} mol) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) and the volumes were made up to 25 cm^3 with the buffer. Carboxypeptidase A solution (25.0 x 10^{-3} cm^3, *ca.* 30 units) was diluted to 50 cm^3 with 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}). Reaction mixtures were prepared as described below (Table 85) and made up to 3.00 cm^3 with the buffer. Reactions were thermostatted at 298 K and monitored at the wavelength noted until no further absorbance change was observed. The initial rates of reaction were calculated using the UV 2101 PC Kinetics software package and normalised for the amount of enzyme present. An arbitrary value of 20 units dm^{-3} was chosen, consistent with that chosen previously. This data was used to plot the rate of reaction against the concentration of the substrate **6** (Figure 27) and against the logarithm of the concentration of the substrate **6** (Figure 28). From these plots, the data was fitted to Equation 6 and gave values for the constants *a-d* as 0.364 x 10^{-3} s^{-1}, -19.4 x 10^{-6} s^{-1}, 1.04 x 10^{-3} mol dm^{-3} and 93.8 x 10^{-6} mol dm^{-3}, respectively. Further, Lineweaver-Burk analysis[113] (Figure 29) gave approximate values of v_{max} and K_M as 0.27 x 10^{-3} mol dm^{-3} s^{-1} and 60 x 10^{-6} mol dm^{-3}, respectively, for the Michaelis complex.

Stock solution	Racemate of compound **6** solution (cm^3)	Enzyme stock solution (cm^3)	[Lactate **6**] (mol dm^{-3})	log [Lactate **6**]	Wavelength reaction monitored at (nm)	Rate of reaction (mol dm^{-3} s^{-1}) normalised to 20 units dm^{-3}
I	20.0×10^{-3}	20.0×10^{-3}	26.4×10^{-6}	-4.58	250	86.9×10^{-6}
I	30.0×10^{-3}	20.0×10^{-3}	39.6×10^{-6}	-4.40	250	92.3×10^{-3}
I	38.0×10^{-3}	20.0×10^{-3}	50.2×10^{-6}	-4.30	250	0.128×10^{-3}
I	48.0×10^{-3}	20.0×10^{-3}	63.3×10^{-6}	-4.20	250	0.137×10^{-3}
I	60.0×10^{-3}	20.0×10^{-3}	79.2×10^{-6}	-4.10	250	0.160×10^{-3}
I	75.0×10^{-3}	20.0×10^{-3}	99.0×10^{-6}	-4.00	250	0.178×10^{-3}
I	0.120	20.0×10^{-3}	0.158×10^{-3}	-3.80	250	0.197×10^{-3}
I	0.190	40.0×10^{-3}	0.251×10^{-3}	-3.60	260	0.210×10^{-3}
I	0.300	40.0×10^{-3}	0.396×10^{-3}	-3.40	260	0.217×10^{-3}
I	0.480	40.0×10^{-3}	0.634×10^{-3}	-3.20	260	0.210×10^{-3}
I	0.600	80.0×10^{-3}	0.792×10^{-3}	-3.10	260	0.180×10^{-3}
I	0.750	80.0×10^{-3}	0.990×10^{-3}	-3.00	260	0.171×10^{-3}
I	0.900	80.0×10^{-3}	1.26×10^{-3}	-2.90	285	0.149×10^{-3}
I	1.20	80.0×10^{-3}	1.58×10^{-3}	-2.80	285	0.126×10^{-3}
I	1.90	80.0×10^{-3}	2.51×10^{-3}	-2.60	285	78.7×10^{-6}
I	2.92	80.0×10^{-3}	3.85×10^{-3}	-2.41	285	60.8×10^{-6}
II	0.480	40.0×10^{-3}	6.30×10^{-3}	-2.20	290	31.4×10^{-6}
II	0.750	40.0×10^{-3}	9.85×10^{-3}	-2.01	290	12.2×10^{-6}
II	1.20	40.0×10^{-3}	$15.8x\ 10^{-3}$	-1.80	290	5.07×10^{-6}
II	1.90	40.0×10^{-3}	25.0×10^{-3}	-1.60	290	2.89×10^{-6}

Table 85. Sample preparation, observation wavelength and rate of reaction for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K.

Treatment of (*S*)-*N*-benzoylglycyl-β-phenyllactate (6) with carboxypeptidase A in Tris buffer in the presence of β-cyclodextrin (8)

Stock solutions were prepared by dissolving sodium *N*-benzoylglycyl-β-phenyllactate (I: 8.90×10^{-3} g, 25.5×10^{-6} mol, II: 22.0×10^{-3} g, 63.0×10^{-6} mol, III: 87.9×10^{-3} g, 0.252×10^{-3} mol) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) and the volumes were made up to 5 cm^3 with the buffer. Carboxypeptidase A solution (20.0×10^{-3} cm^3, *ca.* 24 units) was diluted to 50 cm^3 with 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}). β-Cyclodextrin (**8**) (11.3×10^{-3} g, 9.96×10^{-6} mol) was placed in volumetric flasks, reaction mixtures were prepared as described below (Table 86) and made up to 1.00 cm^3 with 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}). Reactions were thermostatted at 298 K and monitored at the wavelength noted until no further absorbance change was observed. The initial rates of reaction were calculated using the UV 2101 PC Kinetics software package and normalised for the amount of enzyme present. An arbitrary value of 20 units dm^{-3} was chosen, consistent

with that chosen previously. This data was used to plot the rate of reaction against the concentration of the substrate **6** (Figure 30) and against the logarithm of the concentration of the substrate **6** (Figure 31). From these plots, the data was fitted to Equation 6 and gave values for the constants *a-d* as 0.584 x 10^{-3} s^{-1}, -10.5 x 10^{-6} s^{-1}, 1.07 x 10^{-3} mol dm^{-3} and 0.755 x 10^{-3} mol dm^{-3}, respectively.

Stock solution	Racemate of compound **6** solution (cm^3)	Enzyme stock solution (cm^3)	[Lactate **6**] (mol dm^{-3})	log [Lactate **6**]	Wavelength reaction monitored at (nm)	Rate of reaction (mol dm^{-3} s^{-1}) normalised to 20 units dm^{-3}
I	10.0 x 10^{-3}	10.0 x 10^{-3}	25.5 x 10^{-6}	-4.59	250	24.1 x 10^{-6}
I	16.0 x 10^{-3}	10.0 x 10^{-3}	40.8 x 10^{-6}	-4.39	250	42.7 x 10^{-6}
I	25.0 x 10^{-3}	10.0 x 10^{-3}	63.8 x 10^{-6}	-4.20	250	54.6 x 10^{-6}
I	40.0 x 10^{-3}	20.0 x 10^{-3}	0.102 x 10^{-3}	-3.99	250	67.4 x 10^{-6}
I	63.0 x 10^{-3}	20.0 x 10^{-3}	0.161 x 10^{-3}	-3.79	250	88.8 x 10^{-6}
I	0.100	20.0 x 10^{-3}	0.255 x 10^{-3}	-3.59	250	0.128 x 10^{-3}
I	0.160	40.0 x 10^{-3}	0.408 x 10^{-3}	-3.39	260	0.162 x 10^{-3}
I	0.250	40.0 x 10^{-3}	0.638 x 10^{-3}	-3.20	260	0.189 x 10^{-3}
I	0.400	40.0 x 10^{-3}	1.02 x 10^{-3}	-2.99	280	0.193 x 10^{-3}
II	0.200	40.0 x 10^{-3}	1.26 x 10^{-3}	-2.90	280	0.195 x 10^{-3}
I	0.630	80.0 x 10^{-3}	1.61 x 10^{-3}	-2.80	280	0.201 x 10^{-3}
II	0.400	80.0 x 10^{-3}	2.52 x 10^{-3}	-2.60	285	0.156 x 10^{-3}
III	0.125	80.0 x 10^{-3}	3.15 x 10^{-3}	-2.50	285	0.127 x 10^{-3}
II	0.630	80.0 x 10^{-3}	3.97 x 10^{-3}	-2.40	285	92.1 x 10^{-6}
II	0.980	80.0 x 10^{-3}	6.17 x 10^{-3}	-2.21	285	48.7 x 10^{-6}
III	0.400	80.0 x 10^{-3}	10.1 x 10^{-3}	-2.00	285	35.5 x 10^{-6}
III	0.630	80.0 x 10^{-3}	15.9 x 10^{-3}	-1.80	290	34.9 x 10^{-6}
III	0.980	20.0 x 10^{-3}	24.7 x 10^{-3}	-1.61	290	20.9 x 10^{-6}

Table 86. Sample preparation, observation wavelength and rate of reaction for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}).

Treatment of (*S*)-*N*-benzoylglycyl-β-phenyllactate (6) with carboxypeptidase A in Tris buffer in the presence of hydroxypropyl-β-cyclodextrin (108)

Stock solutions were prepared by dissolving sodium *N*-benzoylglycyl-β-phenyllactate (I: 9.0 x 10^{-3} g, 25.8 x 10^{-6} mol, II: 37.8 x 10^{-3} g, 0.108 x 10^{-3} mol, III: 66.0 x 10^{-3} g, 0.189 x 10^{-3} mol) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) and the volumes were made up to a given volume with the buffer (I: 5 cm^3, II: 2 cm^3, III: 1 cm^3). Carboxypeptidase A solution (20.0 x 10^{-3} cm^3, *ca.* 24 units) was diluted to 50 cm^3 with 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}). Hydroxypropyl-β-cyclodextrin (**108**) (2.82 g, 2.09 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.5 Tris buffer

($I = 0.5$ mol dm^{-3}) and the volume was made up to 10.0 cm^3 with the buffer. Reaction mixtures were prepared as described below (Table 87), hydroxypropyl-β-cyclodextrin (**108**) stock solution was added (0.500 cm^3) and the volume was made up to 1.00 cm^3 with the buffer. Reactions were thermostatted at 298 K and monitored at the wavelength noted until no further absorbance change was observed. The initial rates of reaction were calculated using the UV 2101 PC Kinetics software package and normalised for the amount of enzyme present. An arbitrary value of 20 units dm^{-3} was chosen, consistent with that chosen previously. This data was used to plot the rate of reaction against the concentration of the substrate **6** (Figure 32) and against the logarithm of the concentration of the substrate **6** (Figure 33). From these plots, the data was fitted to Equation 6 and gave values for the constants *a-d* as 0.314×10^{-3} s^{-1}, -0.129×10^{-3} s^{-1}, 22.5×10^{-3} mol dm^{-3} and 0.751×10^{-3} mol dm^{-3}, respectively.

Stock solution	Racemate of compound **6** solution (cm^3)	Enzyme stock solution (cm^3)	[Lactate **6**] (mol dm^{-3})	log [Lactate **6**]	Wavelength reaction monitored at (nm)	Rate of reaction (mol dm^{-3} s^{-1}) normalised to 20 units dm^{-3}
I	15.0×10^{-3}	10.0×10^{-3}	38.7×10^{-6}	-4.41	250	22.1×10^{-6}
I	25.0×10^{-3}	10.0×10^{-3}	64.5×10^{-6}	-4.19	250	48.8×10^{-6}
I	40.0×10^{-3}	10.0×10^{-3}	0.103×10^{-3}	-3.99	260	56.0×10^{-6}
I	60.0×10^{-3}	20.0×10^{-3}	0.155×10^{-3}	-3.81	260	56.4×10^{-6}
I	0.100	20.0×10^{-3}	0.258×10^{-3}	-3.59	260	73.1×10^{-6}
I	0.160	20.0×10^{-3}	0.412×10^{-3}	-3.39	260	88.5×10^{-6}
I	0.250	40.0×10^{-3}	0.644×10^{-3}	-3.19	280	0.117×10^{-3}
I	0.310	40.0×10^{-3}	0.800×10^{-3}	-3.10	280	0.140×10^{-3}
I	0.400	40.0×10^{-3}	1.03×10^{-3}	-2.99	280	0.175×10^{-3}
II	60.0×10^{-3}	40.0×10^{-3}	1.62×10^{-3}	-2.79	280	0.191×10^{-3}
II	90.0×10^{-3}	40.0×10^{-3}	2.43×10^{-3}	-2.61	285	0.199×10^{-3}
II	0.150	40.0×10^{-3}	4.05×10^{-3}	-2.39	285	0.211×10^{-3}
II	0.230	80.0×10^{-3}	6.21×10^{-3}	-2.21	285	0.196×10^{-3}
II	0.370	80.0×10^{-3}	9.99×10^{-3}	-2.00	290	0.153×10^{-3}
III	0.160	80.0×10^{-3}	15.1×10^{-3}	-1.82	290	0.122×10^{-3}
III	0.250	80.0×10^{-3}	23.6×10^{-3}	-1.63	290	76.0×10^{-6}

Table 87. Sample preparation, observation wavelength and rate of reaction for the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenyllactate (**6**) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 Tris buffer ($I = 0.5$ mol dm^{-3}) at 298 K in the presence of hydroxypropyl-β-cyclodextrin (**108**) (0.105×10^{-3} mol dm^{-3}).

Calculation of the association constant of the complex of the racemate of compound 6 in hydroxypropyl-β-cyclodextrin (108)

The sodium salt of *N*-benzoylglycyl-β-phenyllactate (35.0 x 10^{-3} g, 0.102 x 10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}), and the volume was made up to 50 cm^3 with the buffer. Stock solutions were prepared by dissolving hydroxypropyl-β-cyclodextrin (**108**) (I: 0.670 g, 0.497 x 10^{-3} mol, II: 1.685 g, 1.25 x 10^{-3} mol) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) and the volumes were made up to 5 cm^3 with the buffer. All solutions were sonicated to homogeneity. Hydroxypropyl-β-cyclodextrin (**108**) stock solution was taken (Table 88), *N*-benzoylglycyl-β-phenyllactate stock solution (0.500 cm^3) and deuterium oxide (0.100 cm^3) were added, and the solution was made up to 1.00 cm^3 with the buffer. The amount of cyclodextrin **108** stock solution added was varied to give from 0 to 100 equivalents of the host **108** with respect to the racemate of compound **6**. An aliquot of each sample was analysed by ^1H NMR spectroscopy at 298 K. The chemical shifts of the aromatic protons of the racemate of compound **6** were observed to change with the concentration of the host **108**. Sample preparation and observed chemical shift changes between the signals due to the *ortho*-protons and the *meta*-protons of the benzoyl group of the racemate of compound **6** are summarised below (Table 88). Chemical shift information was plotted against concentration of the hydroxypropyl-β-cyclodextrin (**108**) and a non-linear regression analysis (using Equations 1 and 2) gave the association constant for the complex formed as 120 ± 20 mol^{-1} dm^3 (Figure 35).

Stock solution	Cyclodextrin **108** stock solution (cm^3)	[Cyclodextrin **108**] (mol dm^{-3})	Observed chemical shift difference (Hz)
-	-	-	115.23
I	5.00×10^{-3}	0.497×10^{-3}	117.92
I	10.0×10^{-3}	0.994×10^{-3}	121.09
I	20.0×10^{-3}	1.99×10^{-3}	124.51
I	30.0×10^{-3}	2.98×10^{-3}	127.97
I	40.0×10^{-3}	3.98×10^{-3}	130.85
I	50.0×10^{-3}	4.97×10^{-3}	135.25
I	75.0×10^{-3}	7.45×10^{-3}	141.88
I	0.100	9.94×10^{-3}	146.72
I	0.150	14.9×10^{-3}	152.34
I	0.200	19.9×10^{-3}	155.76
I	0.260	25.8×10^{-3}	158.93
II	0.120	30.0×10^{-3}	159.42
I	0.320	31.8×10^{-3}	159.92
I	0.400	39.8×10^{-3}	161.60
II	0.160	40.0×10^{-3}	162.89
II	0.200	50.0×10^{-3}	162.59
II	0.240	60.0×10^{-3}	164.06
II	0.280	70.1×10^{-3}	166.99
II	0.320	80.1×10^{-3}	166.55
II	0.360	90.1×10^{-3}	166.42
II	0.400	0.100	167.96

Table 88. Sample preparation and observed chemical shift difference for the calculation of the association constant of the complex of *N*-benzoyl-β-phenyllactate with hydroxypropyl-β-cyclodextrin (**108**) in 0.05 mol dm^{-3} Tris buffered deuterium oxide at pH 7.5 and 298 K (I = 0.5 mol dm^{-3}).

Calculation of the association constant of the complex of the racemate of compound 6 in β-cyclodextrin (8)

The sodium salt of *N*-benzoylglycyl-β-phenyllactate (35.0×10^{-3} g, 0.102×10^{-3} mol) was dissolved in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}), and the volume was made up to 50 cm^3 with the buffer. β-Cyclodextrin (**8**) was taken (Table 89), *N*-benzoylglycyl-β-phenyllactate stock solution (0.500 cm^3) and deuterium oxide (0.100 cm^3) was added, and the solution was made up to 1.00 cm^3 with the buffer. The amount of cyclodextrin **8** stock solution added was varied to give from 0 to 10 equivalents of the host **8** with respect to the racemate of compound **6** An aliquot of each sample was analysed by ^1H NMR spectroscopy at 298 K. The chemical shift of the aromatic protons of the racemate of compound **6** were observed to change with the concentration of the host **8**. Sample preparation and observed chemical shift changes between the signals due to the *ortho*-protons and the *meta*-protons of the benzoyl group of the racemate of compound **6** are summarised below (Table 89). Chemical shift information was plotted against concentration of the β-cyclodextrin (**8**) and a non-linear

regression analysis (using Equations 1 and 2) gave the association constant for the complex formed as 250 ± 60 mol^{-1} dm^3 (Figure 47).

Cyclodextrin **8** (g)	[Cyclodextrin **8**] (mol dm^{-3})	Observed chemical shift difference (Hz)
-	-	60.4
2.7×10^{-3}	2.38×10^{-3}	69.6
5.7×10^{-3}	5.02×10^{-3}	74.6
8.5×10^{-3}	7.49×10^{-3}	77.4
11.8×10^{-3}	10.4×10^{-3}	79.5

Table 89. Sample preparation and observed chemical shift difference for the calculation of the association constant of the complex of *N*-benzoyl-β-phenyllactate with β-cyclodextrin (**8**) in 0.05 mol dm^{-3} Tris buffered deuterium oxide at pH 7.5 and 298 K (I = 0.5 mol dm^{-3}).

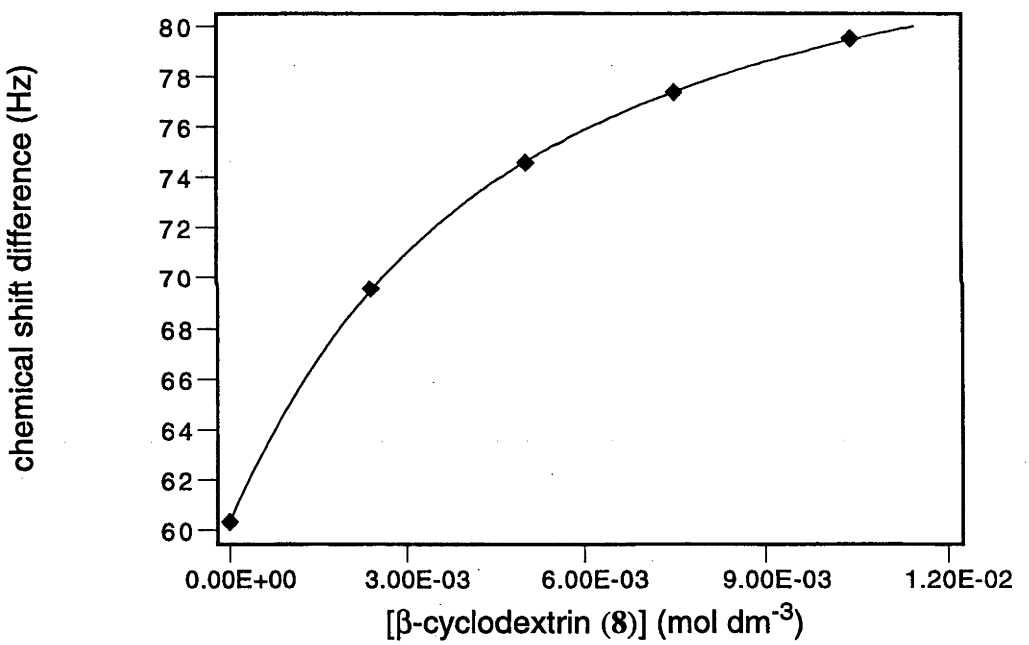


Figure 47. ^1H NMR chemical shift difference plotted against the concentration of β-cyclodextrin (**8**) for the difference in chemical shifts between to the *ortho*-protons and the *meta*-protons of the benzoyl group of *N*-benzoyl-β-phenyllactate in 0.05 mol dm^{-3} Tris buffered deuterium oxide at pH 7.5 and 298 K (I = 0.5 mol dm^{-3}).

References

1. *The Enzymes*, ed. P. D. Boyer, Academic Press: New York and London, 1970.

2. L. Michaelis and M. L. Menten, *Biochem. Z.*, 1913, **49**, 333.

3. L. Stryer, *Biochemistry*, W. H. Freeman and Co.: New York, 1988.

4. D. M. Blow, *Acc. Chem. Res.*, 1976, **9**, 145.

5. J. Kraut, *Ann. Rev. Biochem.*, 1977, **46**, 331.

6. H.-G. Hers, *Ann. Rev. Biochem.*, 1976, **45**, 167.

7. I. H. Siegel, *Enzyme Kinetics: Behaviour and Analysis of Rapid Equilbrium and Steady-State Enzyme Systems*, Wiley-Interscience: New York, 1975.

8. E. A. Havir and K. R. Hanson, *Biochem.*, 1968, **7**, 1896.

9. E. A. Havir and K. R. Hanson, *Biochem.*, 1968, **7**, 1904.

10. K. R. Hanson, R. H. Wightman, J. Staunton and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1971, 185.

11. K. R. Hanson and E. A. Havir, *Arch. Biochem. Biophys.*, 1970, **141**, 1.

12. E. A. Havir and K. R. Hanson, *Biochem.*, 1975, **14**, 1620.

13. D. S. Hodgins, *J. Biol. Chem.*, 1971, **246**, 2977.

14. J. R. Parkhurst and D. S. Hodgins, *Arch. Biochem. Biophys.*, 1972, **152**, 597.

15. S. E. Iredale and H. Smith, *Phytochem.*, 1974, **13**, 575.

16. C.-H. Wong and G. M. Whitesides, *Enzymes in Synthetic Organic Chemistry*, Pergamon: Oxford, 1994.

17. R. Lumry, E. L. Smith and R. R. Glantz, *J. Am. Chem. Soc.*, 1951, **73**, 4330.

18. R. C. Davies, J. F. Riordan, D. S. Auld and B. L. Vallee, *Biochem.*, 1968, **7**, 1090.

19. J. R. Whitaker, F. Menger and M. L. Bender, *Biochem.*, 1966, **5**, 386.

20. W. O. McClure, H. Neurath and K. A. Walsh, *Biochem.*, 1964, **3**, 1897.

21. M. L. Bender, J. R. Whitaker and F. Menger, *Proc. Nat. Acad. Sci., U.S.A.*, 1965, **53**, 711.

22. J. W. Bunting, *Bioch. Biophys. Res. Commun.*, 1972, **48**, 1316.

23. J. W. Bunting and J. Murphy, *Can. J. Chem.*, 1974, **52**, 2640.

24. J. Murphy and J. W. Bunting, *Can. J. Chem.*, 1975, **53**, 283.

25. J. W. Bunting and S. S.-T. Chu, *Biochim. Biophys. Acta*, 1978, **524**, 142.

26. J. W. Bunting and S. S.-T. Chu, *Biochim. Biophys. Acta*, 1978, **524**, 393.

27. *Preparative Biotransformations*, ed. S. M. E. Roberts, Wiley: New York, 1993.

28. S. M. Roberts, *J. Chem. Soc., Perkin Trans. 1*, 1998, 157.

29. A. J. Humphrey and N. J. Turner, in *Enzyme Chemistry. Impact and Applications*, ed. C. J. Suckling, C. L. Gibson and A. R. Pitt, Blackie Academic and Professional Publishers: London, 1998, p. 105.

30. S. M. Roberts, *J. Chem. Soc., Perkin Trans. 1*, 1999, 1.

31. B. Gutte and R. B. Merrifield, *J. Am. Chem. Soc.*, 1969, **91**, 501.

32. J. D. Watson, J. Tooze and D. T. Kurtz, *Recombinant DNA. A Short Course*, Scientific American Books: New York, 1983.

33. M. M. Ling and B. H. Robinson, *Anal. Biochem.*, 1997, **254**, 157.

34. F. H. Arnold, *Acc. Chem. Res.*, 1998, **31**, 125.

35. M. L. Bender and M. Komiyama, *Cyclodextrin Chemistry*, Springer-Verlag: New York, 1977.

36. J. Szejtli, *Chem. Rev.*, 1998, **98**, 1743.

37. A. Villiers, *Compt. Rend. Acad. Sci.*, 1891, **112**, 536.

38. F. Schardinger, *Zeitschrift fur Untersuchung der Nahrungs - und Genubmittel*, 1903, **6**, 865.

39. F. Schardinger, *Zentralbl. Bakteriol. Abt. II*, 1909, **22**, 98.

40. K. Freudenberg and F. Cramer, *Z. Naturforsch. B. Anorg. Chem. Org. Chem.*, 1909, **3**, 464.

41. W. J. Schlenk, *Fortschr. Chem. Forsch.*, 1951, **2**, 92.

42. W. Saenger, *Angew. Chem., Int. Ed. Engl.*, 1980, **19**, 344.

43. R. J. Clarke, J. H. Coates and S. F. Lincoln, *Adv. Carb. Chem. Biochem.*, 1988, **46**, 205.

44. J. Szejtli, in *Comprehensive Supramolecular Chemistry*, ed. J. Szejtli and T. Osa, Pergamon: Exeter, 1996, p. 189.

45. K. A. Connors, *Chem. Rev.*, 1997, **97**, 1325.

46. M. V. Rekharsky and Y. Inoue, *Chem. Rev.*, 1998, **98**, 1875.

47. A. E. Martell and R. J. Motekaitis, *Determination and Use of Stability Constants*, VCH Publishers: New York, 1988.

48. J. Donohue and S. H. Goodman, *Acta Cryst.*, 1967, **22**, 352.

49. J. Szejtli, *Cyclodextrin Technology*, Kluwer Academic Publishers: Dordrecht, 1988.

50. W. C. Cromwell, K. Byström and M. R. Eftink, *J. Phys. Chem.*, 1985, **89**, 326.

51. Z. Yang and R. Breslow, *Tetrahedron Lett.*, 1997, **38**, 6171.

52. M. R. Eftink, M. L. Andy, K. Bystrom, H. D. Perlmutter and D. S. Kristol, *J. Am. Chem. Soc.*, 1989, **111**, 6765.

53. C. J. Seliskar and L. Brand, *Science*, 1971, **171**, 799.

54. K. A. Connors and J. M. Lipari, *J. Pharm. Sci.*, 1976, **65**, 379.

55. K. Flohr, R. M. Paton and E. T. Kaiser, *J. Am. Chem. Soc.*, 1975, **97**, 1209.

56. B. Siegel and R. Breslow, *J. Am. Chem. Soc.*, 1975, **97**, 6869.

57. J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, 1962, **63**, 530.

58. K. A. Connors, in *Comprehensive Supramolecular Chemistry*, ed. J. Szejtli and T. Osa, Pergamon: Exeter, 1996, p. 205.

59. Á. Buvári-Barcza, J. Katjár and L. Barcza, *J. Inc. Phen.*, 1996, **24**, 211.

60. P. V. Demarco and A. L. Thakkar, *J. Chem. Soc., Chem. Commun.*, 1970, 2.

61. D. J. Wood, F. E. Hruska and W. Saenger, *J. Am. Chem. Soc.*, 1977, **99**, 1735.

62. R. J. Bergeron, M. A. Channing, G. J. Gibeily and D. M. Pillor, *J. Am. Chem. Soc.*, 1977, **99**, 5146.

63. S. E. Brown, J. H. Coates, S. F. Lincoln, D. R. Coghlan and C. J. Easton, *J. Chem. Soc., Faraday Trans.*, 1991, **87**, 2699.

64. I. M. Brereton, T. M. Spotswood, S. F. Lincoln and E. H. Williams, *J. Chem. Soc., Faraday Trans. 1*, 1984, **80**, 3147.

65. D. L. Pisaniello, S. F. Lincoln and J. H. Coates, *J. Chem. Soc., Faraday Trans. 1*, 1985, **81**, 1247.

66. N. J. Smith, T. M. Spotswood and S. F. Lincoln, *Carbohydr. Res.*, 1989, **192**, 9.

67. A. Ashnagar, P. T. Culnane, C. J. Easton, J. B. Harper and S. F. Lincoln, *Aust. J. Chem.*, 1997, **50**, 447.

68. R. I. Gelb, L. M. Schwartz and D. A. Laufer, *Bioorg. Chem.*, 1982, **11**, 274.

69. R. I. Gelb, L. M. Schwartz, J. J. Bradshaw and D. A. Laufer, *Bioorg. Chem.*, 1980, **9**, 299.

70. R. Fornasier, F. Reniero, P. Scrimin and U. Tonellato, *J. Chem. Soc., Perkin Trans. 2*, 1987, 193.

71. S. F. Lincoln and C. J. Easton, in *Structural Diversity and Functional Versatility of Polysaccharides*, ed. S. Dumitriu, Marcel Dekker, Inc.: New York, 1998.

72. C. J. Easton and S. F. Lincoln, *Chem. Soc. Rev.*, 1996, **29**, 163.

73. R. Breslow and S. D. Dong, *Chem. Rev.*, 1998, **98**, 1997.

74. R. Breslow, *J. Chem. Ed.*, 1998, **75**, 705.

75. R. Breslow and C. Schmuck, *J. Am. Chem. Soc.*, 1996, **118**, 6601.

76. R. Breslow and B. Zhang, *J. Am. Chem. Soc.*, 1994, **116**, 7893.

77. S. E. Brown, C. A. Haskard, C. J. Easton and S. F. Lincoln, *J. Chem. Soc., Faraday Trans.*, 1995, **91**, 1013.

78. S. E. Brown, J. H. Coates, C. J. Easton, S. J. van Eyk, S. F. Lincoln, B. L. May, M. A. Stile, C. B. Whalland and M. L. Williams, *J. Chem. Soc., Chem. Commun.*, 1994, 47.

79. S. E. Brown, J. H. Coates, C. J. Easton and S. F. Lincoln, *J. Chem. Soc., Faraday Trans.*, 1994, **90**, 739.

80. Q. Herrman, S. Wehrle and G. Wenz, *J. Chem. Soc., Chem. Commun.*, 1997, 1709.

81. H. S. Banu, A. Lalitha, K. Pitchumani and C. Srinivasan, *J. Chem. Soc., Chem. Commun.*, 1999, 607.

82. A. G. Meyer, C. J. Easton, S. F. Lincoln and G. W. Simpson, *J. Chem. Soc., Chem. Commun.*, 1997, 1517.

83. A. G. Meyer, C. J. Easton, S. F. Lincoln and G. W. Simpson, *J. Org. Chem.*, 1998, **63**, 9069.

84. A. G. Meyer, C. J. Easton, S. F. Lincoln and G. W. Simpson, in *Proceedings of the 9th International Symposium on Cyclodextrins*, ed. J. J. Torres-Labandeira and J. L. Vila-Jota, Santiago de Compostela, Spain, 1999, p. 609.

85. C. J. Easton, C. M. M. Hughes, G. P. Savage and G. W. Simpson, *Adv. Het. Chem.*, 1994, **60**, 261.

86. K. Fujita, S. Ejima and T. Imoto, *J. Chem. Soc., Chem. Commun.*, 1984, 1277.

87. R. Breslow, N. Greenspoon, T. Guo and R. Zarzycki, *J. Am. Chem. Soc.*, 1989, **111**, 8296.

88. R. C. Petter, C. T. Sikorski and D. H. Waldeck, *J. Am. Chem. Soc.*, 1991, **113**, 2325.

89. F. Venema, C. M. Baselier, E. van Dienst, B. H. M. Ruël, M. C. Feiters, J. F. J. Engbersen, D. N. Reinhoudt and R. J. M. Nolte, *Tetrahedron Lett.*, 1994, **35**, 1773.

90. R. Breslow and S. Chung, *J. Am. Chem. Soc.*, 1990, **112**, 9659.

91. C. A. Haskard, C. J. Easton, B. L. May and S. F. Lincoln, *J. Phys. Chem.*, 1996, **100**, 14457.

92. C. A. Haskard, B. L. May, T. Kurucsev, S. F. Lincoln and C. J. Easton, *J. Chem. Soc., Faraday Trans.*, 1997, **93**, 279.

93. J. H. Coates, C. J. Easton, S. J. van Eyk, S. F. Lincoln, B. L. May, C. B. Whalland and M. L. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2619.

94. C. J. Easton, S. J. van Eyk, S. F. Lincoln, B. L. May, J. Papageorgiou and M. L. Williams, *Aust. J. Chem.*, 1997, **50**, 9.

95. M. V. Rekharsky, F. P. Schwarz, Y. B. Tewari and R. N. Goldberg, *J. Phys. Chem.*, 1994, **98**, 10282.

96. K. Uekama, M. Otagiri, Y. Kanie, S. Tanaka and K. Ikeda, *Chem. Pharm. Bull.*, 1975, **23**, 1421.

97. W. Saenger, J. Jacob, K. Gessler, T. Steiner, D. Hoffmann, H. Sanbe, K. Koizumi, S. M. Smith and T. Takaha, *Chem. Rev.*, 1998, **98**, 1787.

98. M. J. Jozwiakowski and K. A. Connors, *Carbohydr. Res.*, 1985, **143**, 51.

99. V. E. Price, J. B. Gilbert and J. P. Greenstein, *J. Biol. Chem.*, 1949, **179**, 1169.

100. H. K. Chenault, J. Dahmer and G. M. Whitesides, *J. Am. Chem. Soc.*, 1989, **111**, 6354.

101. A. S. Bommarius, K. Drauz, K. Günther, K. Gnaup and M. Schwarm, *Tetrahedron: Asymm.*, 1997, **8**, 3197.

102. A. V. Guntelberg and M. Ottesen, *Compt. Rend. Trav. Lab. Carlsberg*, 1954, **29**, 36.

103. A. Berger, M. Smolarsky, N. Kurn and H. R. Bosshard, *J. Org. Chem.*, 1973, **38**, 457.

104. C. Vergne, M. Bois-Choussy, J. Ouazzani, R. Beugelmans and J. Zhu, *Tetrahedron: Asymm.*, 1997, **8**, 391.

105. R. L. Van Etten, G. A. Clowes, J. F. Sebastian and M. L. Bender, *J. Am. Chem. Soc.*, 1967, **89**, 3253.

106. R. L. Van Etten, J. F. Sebastian, G. A. Clowes and M. L. Bender, *J. Am. Chem. Soc.*, 1967, **89**, 3242.

107. *Handbook of Chemistry and Physics*, ed. R. C. Weast, CRC Press, Inc.: Cleveland, 1977.

108. W. Sakami and G. Toennies, *J. Biol. Chem.*, 1942, **144**, 203.

109. J. B. Harper, Honours Thesis, Australian National University, Canberra, 1995.

110. R. M. Silverstein, G. C. Bassler and T. C. Morrill, *Spectrometric Identification of Organic Compounds*, John Wiley & Sons, Inc.: Singaporte, 1991.

111. S. E. Brown, J. H. Coates, D. R. Coghlan, C. J. Easton, S. J. van Eyk, W. Janowski, A. Lepore, S. F. Lincoln, Y. Luo, B. L. May, D. S. Schiesser, P. Wang and M. L. Williams, *Aust. J. Chem.*, 1993, **46**, 953.

112. B. L. May, S. D. Kean, C. J. Easton and S. F. Lincoln, *J. Chem. Soc., Perkin Trans. 1*, 1997, 3157.

113. H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 1934, **56**, 658.

114. A. Deratani, E. Renard, F. Djedaïni-Pilard and B. Perly, *J. Chem. Soc., Perkin Trans. 2*, 1997, 1517.

115. A. Deratani, T. Maraldo and E. Renard, *J. Inc. Phen.*, 1995, **23**, 137.

116. R. H. J. Clark, C. J. Cooksey, M. A. M. Daniels and R. Withnail, *Endeavour*, 1993, **17**, 191.

117. A. Wahl and P. Bagard, *Bull. Chem. Soc. Fr.*, 1910, **7**, 1090.

118. G. A. Russell and G. Kaupp, *J. Am. Chem. Soc.*, 1969, **91**, 3851.

119. G. Whited, Genencor International, personal communication.

120. These compounds were the generous gifts of Mr. B. L. May.

121. R. C. Petter, J. S. Salek, C. T. Sikorski, G. Kumaravel and F.-T. Lin, *J. Am. Chem. Soc.*, 1990, **112**, 3860.

122. R. G. Ault, E. L. Hirst and R. A. Morton, *J. Chem. Soc.*, 1935, 1653.

123. A. F. Isbell and H. R. Henze, *J. Am. Chem. Soc.*, 1944, **66**, 2096.

124. W. C. Sumpter, J. L. Williams, P. H. Wilken and B. L. Willoughby, *J. Org. Chem.*, 1949, **14**, 713.

125. W. C. Sumpter, P. H. Wilken, J. L. Williams, R. Wedenmeyer, F. L. Boyer and W. W. Hunt, *J. Org. Chem.*, 1950, **15**, 1777.

126. R. J. Sundberg, *The Chemistry of Indoles*, ed. A. T. Blomquist, Academic Press: New York, 1970.

127. K. Harata, *Bull. Chem. Soc. Jpn.*, 1977, **50**, 1416.

128. M. Kitagawa, H. Hoshi, M. Sakurai, Y. Inoue and R. Chujo, *Carbohydr. Res.*, 1987, **163**, c1.

129. M. Sakurai, M. Kitagawa, H. Hoshi, Y. Inoue and R. Chujo, *Chem. Lett.*, 1988, 895.

130. E. A. Havir, P. D. Reid and H. V. Marsh Jr., *Plant Physiol.*, 1971, **48**, 130.

131. J.-S. Zhao and S.-K. Yang, *Acta Chim. Sin.*, 1997, **55**, 196.

132. M. N. Vasil'eva, E. N. Shkodinskaya and A. Y. Berlin, *Zh. Obsch. Khim.*, 1961, **31**, 1027.

133. R. R. Herr, T. Enkoji and J. P. Dailey, *J. Am. Chem. Soc.*, 1957, **79**, 4229.

134. O. H. Wheeler and C. B. Covarrubias, *J. Org. Chem.*, 1963, **28**, 2015.

135. A. Hädener and C. Tamm, *J. Label. Comp.*, 1987, **24**, 1291.

136. S. Yamada, K. Nabe, N. Izuo, K. Nakamichi and I. Chibata, *Appl. Environ. Microbiol.*, 1981, **42**, 773.

137. S. Takac, B. Akay and T. Ozdamar, *Enzyme Microb. Technol.*, 1995, **17**, 445.

138. *Critical Stability Constants*, ed. R. M. Smith and A. E. Martell, Plenum Press: New York, 1975.

139. B. Sébille, N. Thuaud, J. Piquion and N. Behar, *J. Chrom.*, 1987, **409**, 61.

140. N. Thuaud, B. Sébille, A. Deratani and G. Lelievre, *J. Chrom.*, 1990, **503**, 453.

141. A. K. Croft, C. J. Easton, S. F. Lincoln, B. L. May and J. Papageorgiou, *Aust. J. Chem.*, 1997, **50**, 857.

142. Y. Liu, B.-H. Han, S.-X. Sun, T. Wada and Y. Inoue, *J. Org. Chem.*, 1999, **64**, 1487.

143. A. Ueno, A. Ikeda, H. Ikeda, T. Ikeda and F. Toda, *J. Org. Chem.*, 1999, **64**, 382.

144. M. Eddaoudi, H. Parrot-Lopez, S. F. de Lamotte, D. Ficheux, P. Prognon and A. W. Coleman, *J. Chem. Soc., Perkin Trans. 2*, 1996, 1711.

145. R. Corradim, A. Dossena, G. Galaverna, R. Marchelli, A. Panagia and G. Sartor, *J. Org. Chem.*, 1997, **62**, 6283.

146. H. Ikeda, M. Nakamura, N. Ise, N. Oguma, T. Ikeda, F. Toda and A. Ueno, *J. Am. Chem. Soc.*, 1996, **118**, 10980.

147. H. Ikeda, Nakamura, M., Ise, N., Toda, F., and Ueno, A., *J. Org. Chem.*, 1997, **62**, 1411.

148. A. Berthod, W. Li and D. W. Armstrong, *Anal. Chem.*, 1992, **64**, 873.

149. D. H. Williams, *Acc. Chem. Res.*, 1984, **17**, 364.

150. A. V. Rama Rao, M. K. Gurjar, K. L. Reddy and A. S. Rao, *Chem. Rev.*, 1995, **95**, 2135.

151. F. Bergel and J. A. Stock, *J. Chem. Soc.*, 1954, 2409.

152. T. Shimidzu, T. Iyoda and N. J. Kanda, *J. Chem. Soc., Chem. Commun.*, 1981, 1206.

153. H. H. Coenen, W. Bodsch, K. Takahashi and A. Hossman, *Stoecklin. Nuklearmedizin, Suppl.*, 1986, **22**, 600.

154. G. H. Cleland, *J. Org. Chem.*, 1969, **34**, 744.

155. C. J. Easton, S. F. Lincoln, J. Papageorgiou and D. M. Schliebs, *J. Chem. Res. (S)*, 1995, 381.

156. N. Bodor, M.-J. Huang and J. D. Watts, *J. Inc. Phen.*, 1996, **25**, 97.

157. K. Kociuba, Ph.D. Thesis, University of Adelaide, Adelaide, 1995.

158. E. L. Bennett and C. Niemann, *J. Am. Chem. Soc.*, 1950, **72**, 1800.

159. T. Okuda and Y. Fuji, *Bull. Chem. Soc. Jpn.*, 1957, **30**, 598.

160. J. Porter, J. Dykert and J. Rivier, *Int. J. Pept. Prot. Res.*, 1987, **30**, 13.

161. F. Bergel, V. C. E. Burnop and J. A. Stock, *J. Chem. Soc.*, 1955, 1223.

162. T. N. Ghosh and S. Dutta, *J. Ind. Chem. Soc.*, 1955, **32**, 755.

163. V. Du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, 1933, **98**, 295.

164. R. R. Sealock, *J. Biol. Chem.*, 1947, **170**, 1.

165. W. H. Schuller and C. Niemann, *J. Am. Chem. Soc.*, 1951, **73**, 1644.

166. J. Kovács, I. Teplán and I. Mezô, *Acta Chim. Acad. Scient. Hung.*, 1975, **84**, 109.

167. J. H. Clark, D. Wails and T. W. Bastock, *Aromatic Fluorination*, CRC Press Inc.: Boca Raton, 1996.

168. J. March, *Advanced Organic Chemistry*, Wiley-Interscience: New York, 1985.

169. S. N. Birnbaum, L. Levinton, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, 1952, **194**, 455.

170. Brookhaven National Laboratory, Protein Data Bank, http://pdb.pdb.bnl.gov/.

171. H. F. Gram, C. W. Mosher and B. R. Baker, *J. Am. Chem. Soc.*, 1959, **81**, 3103.

172. D. French, M. L. Levine, J. H. Pazur and E. Norberg, *J. Am. Chem. Soc.*, 1949, **71**, 353.

173. T. Irie, K. Fukunaga, A. Yoshia, K. Uekama, H. Fales and J. Pitha, *Pharm. Res.*, 1988, **5**, 713.

174. K. Uekama and M. Otagiri, *Crit. Rev. Ther. Drug Carrier Syst.*, 1987, **3**, 1.

175. A. Yoshida, H. Arima, K. Uekama and J. Pitha, *Int. J. Pharm.*, 1988, **46**, 1125.

176. A. R. Schulz, *Enzyme Kinetics. From Diastase to Multi-Enzyme Systems*, Cambridge University Press: New York, 1994.

177. J. W. Bunting and S. S.-T. Chu, *Biochem.*, 1976, **15**, 3237.

178. J. W. Bunting and C. D. Myers, *Can. J. Chem.*, 1973, **51**, 2639.

179. F. Cramer and W. Dietsche, *Chem. Ber.*, 1959, **92**, 378.

180. M. Mikolajczyk, J. Drabowicz and F. Cramer, *J. Chem. Soc., Chem. Commun.*, 1971, 317.

181. M. Mikolajczyk and J. Drabowicz, *J. Am. Chem. Soc.*, 1978, **100**, 2510.

182. D. Greatbanks and R. Pickford, *Mag. Res. Chem.*, 1987, **25**, 208.

183. K. B. Lipkowitz, S. Raghothama and J. Yang, *J. Am. Chem. Soc.*, 1992, **114**, 1554.

184. S. E. Brown, J. H. Coates, P. A. Duckworth, S. F. Lincoln, C. J. Easton and B. L. May, *J. Chem. Soc., Faraday Trans.*, 1993, **89**, 1035.

185. *Biochemist's Handbook*, ed. C. Long, Spon: London, 1961.

186. K. Honda, K. Miyaguchi and K. Imai, *Anal. Chim. Acta*, 1985, **177**, 103.

187. J. P. Greenstein and M. Winistch, *Chemistry of the Amino Acids*, Wiley: New York, 1961.

Appendix 1

Derivation of Curve Fitting Equation

Given the equation for the stability constant of an association complex, Equation 1, the concentration of free substrate can be calculated.#

$$K = \frac{[\text{complex}]}{[\text{guest}]_{\text{free}}[\text{host}]_{\text{free}}}$$

$$K = \frac{[\text{complex}]}{([\text{guest}]-[\text{complex}])([\text{host}]-[\text{complex}])}$$

$$K = \frac{[\text{complex}]}{[\text{complex}]^2 - ([\text{guest}]+[\text{host}])[\text{complex}]+[\text{guest}].[\text{host}]}$$

$$K[\text{complex}]^2 - \left(K([\text{guest}]+[\text{host}])+1\right)[\text{complex}]+K[\text{guest}].[\text{host}] = 0$$

$$[\text{complex}] = \frac{\left(K([\text{guest}]+[\text{host}])+1\right) - \sqrt{\left(K([\text{guest}]+[\text{host}])+1\right)^2 - 4K^2[\text{guest}].[\text{host}]}}{2K}$$

The observed chemical shift of a given signal by NMR can be expressed as the weighted average of the signals due to the free and complexed species (Equation 2).

$$\delta_{\text{observed}} = \frac{([\text{guest}].\delta_{\text{free}} + [\text{complex}].\delta_{\text{complexed}})}{([\text{guest}]+[\text{complex}])} \qquad (2)$$

This can be rewritten in terms of the total concentration of host and guest.

$$\delta_{\text{observed}} = \frac{(([\text{guest}]_{\text{total}} - [\text{complex}]).\delta_{\text{free}} + [\text{complex}].\delta_{\text{complexed}})}{[\text{guest}]_{\text{total}}}$$

Substituting the expression for the concentration of the complex gives an equation which relates the observed chemical shift of a given signal to the total amount of host present in solution. Since the total amount of guest remains constant, this allows calculation of the association of the complex formed, as well as the chemical shifts of the observed signal of the guest in free solution and when complexed.

It should be noted that there are two mathematical solutions to the quadratic equation, with only the physically realistic one being shown here.

$$\delta_{observed} = \frac{\left(\left([guest]_{total} - \frac{\left(K([guest]_{total} + [host]_{total}) + 1 - \sqrt{(K([guest]_{total} + [host]_{total}) + 1)^2 - 4K^2[guest]_{total}[host]_{total}} \right)}{2K} \right) . \delta_{free} + \left(\frac{\left(K([guest]_{total} + [host]_{total}) + 1 - \sqrt{(K([guest]_{total} + [host]_{total}) + 1)^2 - 4K^2[guest]_{total}[host]_{total}} \right)}{2K} \right) . \delta_{complexed} \right)}{[guest]_{total}}$$

Appendix 2

Calculation of Association Constants by Displacement

Consider a guest, A. The association constant, K_A, of the complex formed with the cyclodextrin, CD, is known. A is displaced by a second guest, B. The association constant, K_B, of the complex formed with the cyclodextrin is unknown. Starting from Equation 2 gives

$$\delta_{observed} = \frac{([A].\delta_{free} + [CD.A].\delta_{complexed})}{([A] + [CD.A])}$$

$$\delta_{observed}[A]_{Total} = \delta_{free}[A]_{Total} - \delta_{free}[CD.A] + \delta_{complexed}[CD.A]$$

$$[CD.A] = \frac{(\delta_{observed} - \delta_{free})[A]_{Total}}{(\delta_{complexed} - \delta_{free})}$$

From Equation 1

$$K_A = \frac{[CD.A]}{[A][CD]}$$

$$[CD] = \frac{[CD.A]}{K_A([A]_{Total} - [CD.A])}$$

$$[CD] = \frac{\left(\frac{(\delta_{observed} - \delta_{free})[A]_{Total}}{(\delta_{complexed} - \delta_{free})}\right)}{K_A\left([A]_{Total} - \left(\frac{(\delta_{observed} - \delta_{free})[A]_{Total}}{(\delta_{complexed} - \delta_{free})}\right)\right)}$$

$$[CD] = \frac{(\delta_{observed} - \delta_{free})[A]_{Total}}{K_A\left((\delta_{complexed} - \delta_{free})[A_{Total}] - (\delta_{observed} - \delta_{free})[A]_{Total}\right)}$$

$$[CD] = \frac{(\delta_{observed} - \delta_{free})}{K_A(\delta_{complexed} - \delta_{observed})}$$

It follows that

$$K_B = \frac{[CD.B]}{[B][CD]}$$

$$K_B = \frac{([CD]_{Total} - [CD] - [CD.A])}{[CD]([B]_{Total} - ([CD]_{Total} - [CD] - [CD.A]))}$$

$$K_B = \frac{\left([CD]_{Total} - \left(\frac{(\delta_{observed} - \delta_{free})}{K_A(\delta_{complexed} - \delta_{observed})}\right) - \left(\frac{(\delta_{observed} - \delta_{free})[A]_{Total}}{(\delta_{complexed} - \delta_{free})}\right)\right)}{\left(\frac{(\delta_{observed} - \delta_{free})}{K_A(\delta_{complexed} - \delta_{observed})}\right)\left([B]_{Total} - [CD]_{Total} + \left(\frac{(\delta_{observed} - \delta_{free})}{K_A(\delta_{complexed} - \delta_{observed})}\right) + \left(\frac{(\delta_{observed} - \delta_{free})[A]_{Total}}{(\delta_{complexed} - \delta_{free})}\right)\right)}$$

Further simplification of this equation is not possible.

Appendix 3

Derivation of Equation 7

The extent of reaction after time t, $x(t)$, can be expressed in terms of the rate of reaction, v.

$$x(t) = \int_0^t v.dt$$

The rate of reaction is a function, f, of the substrate concentration at time t, S_t.

$$x(t) = \int_0^t f(S_t).dt$$

The substrate concentration at time t is further related to the starting substrate concentration, S.

$$S_t = S - x(t)$$

$$x(t) = \int_0^t f(S - x(t)).dt$$

$$dx(t) = f(S - x(t).dt$$

$$\frac{d(x(t))}{f(S - x(t))} = dt$$

$$\int \frac{d(x(t))}{f(S - x(t))} = \int dt$$

$$RHS = \int dt$$

$$= t + z \qquad (z = \text{constant})$$

$$LHS = \int \frac{d(x(t))}{f(S - x(t))}$$

$$= \int (f(S - x(t))^{-1} d(x(t))$$

Given that the function, f, can be approximated to the formula below, as derived by Murphy and Bunting,[24] the left hand side can be further expanded.

$$f(y) = \frac{ay + \frac{by^2}{c}}{d + y + \frac{y^2}{c}}$$

$$LHS = \int (f(S - x(t))^{-1} d(x(t))$$

$$= \int \frac{d + (S - x(t)) + \frac{(S - x(t))^2}{c}}{a(S - x(t)) + \frac{b(S - x(t))^2}{c}} . dx(t)$$

$$= \int \frac{cd + c(S - x(t)) + (S - x(t))^2}{ac(S - x(t)) + b(S - x(t))^2} . dx(t) \qquad (c \neq 0)$$

$$= \int \frac{cd + cS - cx(t)) + S^2 - 2Sx(t) + (x(t))^2}{acS - acx(t) + bS^2 - 2bSx(t) + bx((t))^2} . dx(t)$$

$$= \int \frac{abcd + abcS - abcx(t) + abS^2 - 2abSx(t) + ab(x(t))^2}{a^2bcS - a^2bcx(t) + ab^2S^2 - 2ab^2Sx(t) + ab^2(x(t))^2} . dx(t) \qquad (ab \neq 0)$$

$$= \int \frac{1}{b} . dx(t) - \int \frac{d}{a} . \frac{-1}{(S - x(t))} . dx(t)$$

$$+ \int \left(\frac{-1}{b} - \frac{d}{a} . \frac{1}{(S - x(t))} + \frac{abcd + abcS - abcx(t) + abS^2 - 2abSx(t) + abx((t))^2}{a^2bcS - a^2bcx(t) + ab^2S^2 - 2ab^2Sx(t) + ab^2(x(t))^2} \right) . dx(t)$$

$$= j - k + l$$

Evaluating each of these separately gives

$$j = \int \frac{1}{b}.dx(t)$$
$$= \frac{x(t)}{b}$$

$$k = \int \frac{d}{a}.\frac{-1}{(S-x(t))}.dx(t)$$
$$= \frac{d}{a}\ln(S-(x(t))$$

$$l = \int \left(\frac{-1}{b} - \frac{d}{a}.\frac{1}{(S-x(t))} + \frac{abcd+abcS-abcx(t)+abS^2-2abSx(t)+ab(x(t))^2}{a^2bcS-a^2bcx(t)+ab^2S^2-2ab^2Sx(t)+ab^2(x(t))^2}\right).dx(t)$$

$$= \int \left(\frac{-1}{b} - \frac{d}{a}.\frac{1}{(S-x(t))} + \frac{abcd+abcS-abcx(t)+abS^2-2abSx(t)+ab(x(t))^2}{ab(S-x(t))(ac+bS-bx(t))}\right).dx(t)$$

$$= \int \frac{-a(S-x(t))(ac+bS-bx(t))-bd(ac+bS-bx(t)+abcd+abcS-abcx(t)+abS^2-2abSx(t)+ab(x(t))2}{ab(S-x(t))(ac+bS-bx(t))}.dx(t)$$

$$= \int \frac{-a^2cS+a^2cx(t)-b^2dS+b^2dx(t)+abcS-abcx(t)}{ab(S-x(t))(ac+bS-bx(t))}.dx(t)$$

$$= \int \frac{-(S-x(t))(a^2c+b^2d-abc)}{ab(S-x(t))(ac+bS-bx(t))}.dx(t)$$

$$= \int \frac{-(a^2c+b^2d-abc)}{ab(ac+bS-bx(t))}.dx(t) \qquad (s - x(t) \neq 0)$$

$$= \int \frac{a^2c+b^2d-abc}{ab^2}.\frac{-b}{(ac+bS-bx(t))}.dx(t)$$

$$= \frac{a^2c+b^2d-abc}{ab^2}\ln(ac+bS-bx(t))$$

It follows that

$$t+z = \frac{x(t)}{b} - \frac{d}{a}\ln(S-(x(t)) + \frac{a^2c+b^2d-abc}{ab^2}\ln(ac+bS-bx(t))$$

$$t = \frac{x(t)}{b} - \frac{d}{a}\ln(S-(x(t)) + \frac{a^2c+b^2d-abc}{ab^2}\ln(ac+bS-bx(t)) - z$$

Since the reaction has not proceeded to any extent at zero time

$$t = 0 \Rightarrow x(t) = 0$$

$$0 = -\frac{d}{a}\ln(S) + \frac{a^2c + b^2d - abc}{ab^2}\ln(ac + bS) - z$$

$$z = -\frac{d}{a}\ln(S) + \frac{a^2c + b^2d - abc}{ab^2}\ln(ac + bS)$$

This gives the following expression for the time taken to get a specific extent of reaction.

$$t = \frac{x(t)}{b} - \frac{d}{a}\ln(S - (x(t)) + \frac{a^2c + b^2d - abc}{ab^2}\ln(ac + bS - bx(t))$$
$$+\frac{d}{a}\ln(S) - \frac{a^2c + b^2d - abc}{ab^2}\ln(ac + bS)$$

Appendix 4

Calculation of Extents of Reaction in the Presence and Absence of Cyclodextrins

All calculations use the values for the constants in Equation 6 as given in the Experimental section for the appropriate system. As such, they assume enzyme and cyclodextrin concentrations as present in that experiment. The results are shown as Tables 90-92.

Extent of reaction in absence of a cyclodextrin (%)	Time taken as calculated by Equation 7 (s)
0.4	10.90
0.8	21.62
1.2	32.17
1.6	42.52
2.0	52.74
2.4	62.77
2.8	72.64
3.2	82.37
3.6	91.93

Table 90. Calculation of time taken to reach a given extent of reaction in the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenylalanine (**6**) (15.0 x 10^{-3} mol dm^{-3}) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) in the absence of a cyclodextrin at 298 K.

Extent of reaction in presence of the cyclodextrin **8** (%)	Time taken as calculated by Equation 7 (s)
2	10.22
4	20.18
6	29.89
8	39.34
10	48.54
12	57.51
14	66.23
16	74.72
18	82.97
20	90.98

Table 91. Calculation of time taken to reach a given extent of reaction in the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenylalanine (**6**) (15.0 x 10^{-3} mol dm^{-3}) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) in the presence of β-cyclodextrin (**8**) (9.96 x 10^{-3} mol dm^{-3}) at 298 K.

Extent of reaction in presence of the cyclodextrin **108** (%)	Time taken as calculated by Equation 7 (s)
10	10.87
20	21.01
30	30.43
40	39.21
50	47.41
60	55.09
70	62.37
80	69.40
90	76.60
95	80.86
99	86.70
99.9	92.64
99.99	98.19

Table 92. Calculation of time taken to reach a given extent of reaction in the hydrolysis of (*S*)-*N*-benzoylglycyl-β-phenylalanine (**6**) (15.0×10^{-3} mol dm^{-3}) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) in the presence of hydroxypropyl-β-cyclodextrin (**108**) (0.105 mol dm^{-3}) at 298 K.

Appendix 5

Calculation of Predicted Rates of Reaction

Given the equation for the stability constant of an association complex, Equation 1, the concentration of free substrate can be calculated as per Appendix 1.

$$[\text{complex}] = \frac{\left(K([\text{guest}]+[\text{host}])+1\right) - \sqrt{\left(K([\text{guest}]+[\text{host}])+1\right)^2 - 4K^2[\text{guest}].[\text{host}]}}{2K}$$

This allows the concentration of free guest in solution to be calculated, by subtracting the concentration of the complex from the total concentration of guest. Using the constants calculated from the case in which no cyclodextrin was present, this value can then be substituted into Equation 6, to calculate the rate of reaction. It should be noted that the amount of guest in solution is twice that of the substrate, since the (*R*)-isomer is also present.

This was carried out for the cases where either β-cyclodextrin (**8**) (9.96×10^{-3} mol dm^{-3}, K = 250 mol^{-1} dm^3) or hydroxypropyl-β-cyclodextrin (**108**) (0.105 mol dm^{-3}, K = 120 mol^{-1} dm^3) were present. This data is summarised in Tables 93 and 94 below and presented in Figures 36 and 37 presented in Chapter VII of the Results and Discussion.

Log [Guest]	$[Guest]_{free}$ (mol dm^{-3})	Rate of reaction (mol dm^{-3} s^{-1})
-4.6	7.22×10^{-6}	26.0×10^{-6}
-4.4	11.5×10^{-6}	39.6×10^{-6}
-4.2	18.2×10^{-6}	59.0×10^{-6}
-4.0	28.9×10^{-6}	85.3×10^{-6}
-3.8	46.2×10^{-6}	0.118×10^{-3}
-3.6	73.9×10^{-6}	0.155×10^{-3}
-3.4	0.119×10^{-3}	0.191×10^{-3}
-3.2	0.193×10^{-3}	0.218×10^{-3}
-3.0	0.318×10^{-3}	0.227×10^{-3}
-2.8	0.535×10^{-3}	0.215×10^{-3}
-2.6	0.931×10^{-3}	0.182×10^{-3}
-2.4	1.70×10^{-3}	0.134×10^{-3}
-2.2	3.23×10^{-3}	87.0×10^{-6}
-2.0	6.23×10^{-3}	50.3×10^{-6}
-1.8	11.6×10^{-3}	28.1×10^{-6}
-1.6	20.6×10^{-3}	15.7×10^{-6}

Table 93. Calculation of predicted rates of reaction for the digestion of (*S*)-*N*-benzoyl-β-phenyllactate (**6**) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of β-cyclodextrin (**8**) (9.96×10^{-3} mol dm^{-3}) .

Log [Guest]	$[Guest]_{free}$ (mol dm^{-3})	Rate of Reaction (mol dm^{-3} s^{-1})
-4.6	1.84×10^{-6}	7.03×10^{-6}
-4.4	2.93×10^{-6}	11.0×10^{-6}
-4.2	4.65×10^{-6}	17.2×10^{-6}
-4.0	7.36×10^{-6}	26.5×10^{-6}
-3.8	11.7×10^{-6}	40.2×10^{-6}
-3.6	18.5×10^{-6}	59.9×10^{-6}
-3.4	29.4×10^{-6}	86.4×10^{-6}
-3.2	46.9×10^{-6}	0.119×10^{-3}
-3.0	74.7×10^{-6}	0.156×10^{-3}
-2.8	0.120×10^{-3}	$0.192x\ 10^{-3}$
-2.6	0.193×10^{-3}	0.217×10^{-3}
-2.4	0.313×10^{-3}	0.227×10^{-3}
-2.2	0.517×10^{-3}	0.216×10^{-3}
-2.0	0.876×10^{-3}	0.186×10^{-3}
-1.8	1.56×10^{-3}	0.141×10^{-3}
-1.6	3.03×10^{-3}	91.0×10^{-6}
-1.4	6.96×10^{-3}	45.5×10^{-6}
-1.2	19.7×10^{-3}	16.4×10^{-6}

Table 94. Calculation of predicted rates of reaction for the digestion of (*S*)-*N*-benzoyl-β-phenyllactate (**6**) by carboxypeptidase A in 0.05 mol dm^{-3} pH 7.5 Tris buffer (I = 0.5 mol dm^{-3}) at 298 K in the presence of hydroxypropyl-β-cyclodextrin (**108**) (0.116 mol dm^{-3}).

Appendix 6

Copies of All Published Work

"Directing Bromination of Piperazine-2,5-diones" T. W. Badran, C. L. L. Chai, C. J. Easton, J. B. Harper and D. M. Page, *Aust. J. Chem.*, 1995, **48**, 1379.

"Use of Cyclodextrins to Limit Product Inhibition of (*S*)-Phenylalanine Ammonia Lyase" C. J. Easton, J. B. Harper and S. F. Lincoln, *J. Chem. Soc., Perkin Trans. 1*, 1995, 2525.

"Complexes of Naproxen and Ibuprofen with 6^A-Amino-6^A-deoxy-β-cyclodextrin" A. Ashnagar, P. T. Culnane, C. J. Easton, J. B. Harper and S. F. Lincoln, *Aust. J. Chem.*, 1997, **50**, 447.

"Acylase I Catalysed Hydrolysis of *para*-Substituted (*S*)-Phenylalanine Derivatives from Mixtures of the Racemic *ortho*- and *para*-Substituted Isomers" C. J. Easton and J. B. Harper, *Tetrahedron Lett.*, 1998, **39**, 3269.

"*N*,*N*'-Bis(6^A-deoxy-β-cyclodextrin-6^A-yl)urea as a Molecular Template in the Formation of Indigoid Dyes" C. J. Easton, J. B. Harper and S. F. Lincoln, *New J. Chem.*, 1998, **22**, 1163.

"Cyclodextrins as Molecular Templates" C. J. Easton, J. B. Harper and S. F. Lincoln, in *Proceedings of the 9th International Symposium on Cyclodextrins*, ed. J. J. Torres-Labandeira and J. L. Vila-Jota, Santiago de Compostela, Spain, 1999, p. 541.

Aust. J. Chem., 1995, 48. 1379–1384

Directing Bromination of Piperazine-2,5-diones

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Abstract

From intermolecular and intramolecular competition experiments, it has been established that, by comparison with an *N*-methyl substituent, an *N*-acetyl group deactivates glycine residues in piperazine-2,5-diones towards free-radical bromination. Combined with the ease of introduction and removal of *N*-acetyl substituents, the deactivating effect provides a method for regiocontrolled functionalization of these compounds.

Introduction

Interest in the synthesis of piperazine-2,5-diones stems from the wide ranging natural occurrence and biological activity of this class of compounds. For example, albonoursin (1) has been isolated from *Streptomyces albus* var. *fungatus*, *Streptomyces noursei* and *Actinomyces tumemacerance*, and has been found to exhibit antibacterial and antitumour activity,[1] bicyclomycin (2) has been obtained from *Streptomyces sapporonensis* and *Streptomyces aizunensis*, and has been shown to be a broad spectrum antibiotic,[2] while gliotoxin (3) has been isolated from a variety of sources including *Aspergillus fumigatus*, *Gliocladium fimbriatum* and *Penicillium obsurum*, and is known to have antibacterial, antifungal, antiviral and immunosuppressive properties.[3]

[1] Brown, R., and Kelley, C., *Annu. Rep. N.Y. State Dep. Health*, 1957, 10; 1958, 47; 1960, 50; 1960, 52; 1961, 40; Rao, K. V., and Cullen, W. P., *J. Am. Chem. Soc.*, 1960, **82**, 1127; Fukushima, K., Yazawa, K., and Arai. T., *J. Antibiot.*, 1973, **26**, 175.
[2] Miyoshi, T., Miyairi, N., Aoki, H., Kohsaka, M., Sakai, H., and Imanaka, H., *J. Antibiot.*, 1972, **25**, 569; Kamiya, T., Maeno, S., Hashimoto, M., and Mine, Y., *J. Antibiot.*, 1972, **25**, 576; Nishida, M., Mine, Y., and Matsubara, T., *J. Antibiot.*, 1972, **25**, 582; Nishida, M., Mine, Y., Matsubara, T., Goto, S., and Kuwahara, S., *J. Antibiot.*, 1972, **25**, 594; Miyamura, S., Ogasawara, N., Otsuka, H., Niwayama, S., Tanaka, H., Take, T., Uchiyama, T., Ochiai, H., Abe, K., Koizumi, K., Asao, K., Matsuki, K., and Hoshino, T., *J. Antibiot.*, 1972, **25**, 610; Miyamura, S., Ogasawara, N., Otsuka, H., Niwayama, S., Tanaka, H., Take, T., Uchiyama, T., and Ochiai, H., *J. Antibiot.*, 1973, **26**, 479.
[3] Waring, R., and Mullbacher, A., *Med. Res. Rev.*, 1988, **8**, 76; Taylor, A., in 'Microbial Toxins' (Eds S. Kadis, A. Ciegler and S. J. Ajl) Vol. 7, p. 337 (Academic: New York 1971).

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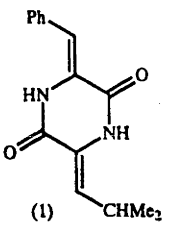
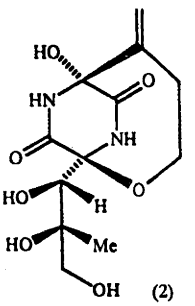
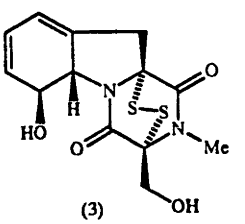
A common approach to the synthesis of the more complex piperazine-2,5-diones is through elaboration of simple precursors derived from proteinogenic amino acids.[4] In this regard, procedures for the regiocontrolled functionalization of piperazine-2,5-diones have considerable potential as many of the target molecules are asymmetrically substituted. The radical bromination of certain symmetric glycine anhydride derivatives with *N*-bromosuccinimide is known,[5–7] but no attempts to direct bromination using different *N*-substituents have been reported. Accordingly, we have now examined the effect of *N*-methyl and *N*-acetyl substituents on the halogenation.



(1)



(2)



(3)

Results and Discussion

Initially, to gauge the effect of the substituents on reactivity, we examined reactions of sarcosine anhydride (4) and 1,4-diacetylpiperazine-2,5-dione (7). Bromination of the sarcosine derivative (4) to give the corresponding bromides (5a) and (6) has been reported.[5] In a similar fashion, the reaction of 1,4-diacetylpiperazine-2,5-dione (7) with *N*-bromosuccinimide in carbon tetrachloride, initiated with azobisisobutyronitrile, gave the bromides (8a) and (9a). Due to their instability, the bromides (8a) and (9a) were characterized by conversion into the corresponding thioethers (8b) and (9b), through treatment with 4-chlorothiophenol and pyridine. The di(thioether) (9b) was only obtained in 15% yield, presumably as a result of the particular instability of the dibromide (9a). The ^{1}H n.m.r. spectrum of the dibromide (9a) showed only one signal for the methyl group hydrogens, at δ 2·65, and one for the hydrogens attached to C3 and C6, at δ 6·93. Likewise, the spectrum of the di(thioether) (9b) showed only one resonance for each type of hydrogen. On this basis, it appears that the dibromide (9a) and the di(thioether) (9b) were each formed as a single diastereomer. Presumably this

[4] Ganem, B., *Tetrahedron*, 1978, **34**, 3353; Trown, P. W., *Biochem. Biophys. Res. Commun.*, 1968, **33**, 402; Fukuyama, T., Nakatsuka, S., and Kishi, Y., *Tetrahedron*, 1981, **37**, 2045; Williams, R. M., *Tetrahedron Lett.*, 1981, **22**, 2341; Williams, R. M., and Rastetter, W. H., *J. Org. Chem.*, 1980, **45**, 2625.

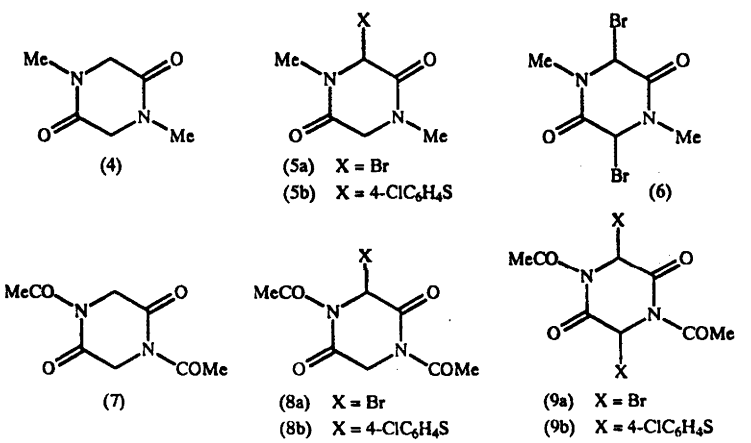
[5] Badran, T. W., and Easton, C. J., *Aust. J. Chem.*, 1990, **43**, 1455.

[6] Chai, C. L. L., and Page, D. M., *Tetrahedron Lett.*, 1993, **34**, 4373.

[7] Williams, R. M., Armstrong, R. W., Maruyama, L. K., Dung, J., and Anderson, O. P., *J. Am. Chem. Soc.*, 1985, **107**, 3246; Williams, R. M., and Kwast, A., *J. Org. Chem.*, 1988, **53**, 5785.

reflects the greater thermodynamic stability of the *cis* isomers of 3,6-disubstituted piperazine-2,5-diones.[8]

The relative reactivity of the piperazinediones (4) and (7) was determined by reaction of an equimolar mixture of each substrate and *N*-bromosuccinimide, in the presence of *N*-t-butylbenzamide (0·1 mole equiv.) as an internal standard. The crude reaction mixture was cooled and concentrated, and the residue was analysed by means of ^{1}H n.m.r. spectroscopy. Integration of signals for the internal standard (δ 1·44, s, 9H, Me_3, 100%), the piperazinediones (4) (δ 3·96, s, 2×CH_2, 4H, 20%) and (7) (δ 4·66, s, 2×CH_2, 4H, 420%), and the bromides (5a) (δ 6·02, s, H3, 1H, 85%) and (6) (δ 6·13, s, H3,6, 2H, 22%) showed that 5% of the sarcosine anhydride (4) remained and the bromides (5a) and (6) were produced in yields of approximately 75 and 10%, respectively, while 95% of the diacetylpiperazinedione (7) remained unreacted. There was no indication of formation of either of the bromides (8a) or (9a), as indicated by the absence of resonances at δ 6·87 and 6·93, respectively.



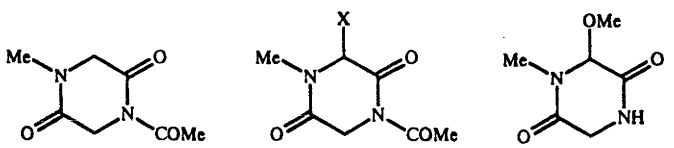
The deactivating effect of the *N*-acetyl substituent was further examined by studying reactions of 1-acetyl-4-methylpiperazine-2,5-dione (10), obtained by acetylation of glycylsarcosine anhydride[9] with acetic anhydride. Reaction of the piperazinedione (10) with *N*-bromosuccinimide under conditions analogous to those described above gave only the unstable bromide (11a), which was characterized by conversion into the thioether (11b) on treatment with 4-chlorothiophenol, and the ether (12) on treatment with methanol. Presumably the reaction of the bromide (11a) with methanol afforded the ether (11c) but the *N*-acetyl substituent of that compound hydrolysed during workup of the reaction mixture and chromatography of the crude product.

The regioselectivity of the halogenation of the piperazinedione (10) was assigned by comparison of the ^{1}H n.m.r. spectrum of the bromide (11a) with those of

[8] Williams, R. M., Anderson, O. P., Armstrong, R. W., Josey, J., Meyers, H., and Eriksson, C., *J. Am. Chem. Soc.*, 1982, **104**, 6092; Williams, R. M., Armstrong, R. W., Maruyama, L. K., Dung, J., and Anderson, O. P., *J. Am. Chem. Soc.*, 1985, **107**, 3246; Benedetti, E., Marsh, R. E., and Goodman, M., *J. Am. Chem. Soc.*, 1976, **98**, 6676.

[9] Levene, P. A., Bass, L. W., Rothen, A., and Steiger, R. E., *J. Biol. Chem.*, 1954, **81**, 697.

the bromides (5a) and (8a). The C3 proton of the bromide (11a) gave rise to a singlet resonance at δ 5·98. This chemical shift is similar to that of the signal for the C3 proton of the dimethylpiperazinedione (5a), at δ 5·79,[5] but different from that of the corresponding diacetyl derivative (8a), at δ 6·87. The ^{1}H n.m.r. spectra of the thioethers (11b), (5b) and (8b) support the assignment of regioselectivity of functionalization of the piperazinedione (10). The resonance for the C3 proton of the thioether (11b) appeared as a singlet at δ 4·99, with a similar chemical shift to that for the dimethyl derivative (5b) at δ 4·94, but 1·23 ppm upfield from that of the corresponding diacetyl derivative (8b). The thioether (5b) was obtained by treatment of the piperazinedione (4) with *N*-bromosuccinimide, followed by reaction of the crude product bromide (5a) with 4-chlorothiophenol.



(10) (11a) X = Br (11b) X = 4-ClC$_6$H$_4$S (11c) X = OMe (12)

Confirmation of the regioselectivity of bromination of the piperazinedione (10) was obtained by heating the ether (12) in refluxing 6 N hydrochloric acid, in the presence of alanine as an internal standard. Analysis of the concentrated product mixture by means of ^{1}H n.m.r. spectroscopy showed that glycine was produced in 60% yield, but there was no evidence of the presence of sarcosine.

From the reactions of the piperazinediones (4), (7) and (10), it is clear that, by comparison with an *N*-methyl substituent, an *N*-acetyl group deactivates glycine residues in piperazine-2,5-diones towards free-radical bromination. This effect is analogous to that observed with amino acid derivatives where the amino group is protected as a benzamide or a phthalimide.[10] Relative to the amido substituent, the greater steric bulk and reduced electron-donating capability of the imido group disfavour radical formation at the adjacent position.

An *N*-acetyl substituent is easily introduced on to a piperazinedione and readily removed,[11] as indicated in the synthesis of the piperazinediones (7) and (10) and the interconversion of the bromide (11a) into the ether (12), outlined above. On this basis, there is considerable scope to exploit the effect of the *N*-acetyl substituent, on reactions of piperazinediones with *N*-bromosuccinimide, in the regiocontrolled halogenation and elaboration of these compounds.

Experimental

Melting points are uncorrected. Light petroleum refers to the fraction with b.p. 66–68°. Radial chromatography was carried out on a Chromatotron 7924T (Harrison Research, Palo

[10] Easton, C. J., Tan, E. W., and Hay, M. P., *J. Chem. Soc., Chem. Commun.*, 1989, 385; Easton, C. J., Hutton, C. A., Rositano, G., and Tan, E. W., *J. Org. Chem.*, 1991, **56**, 5614.
[11] Gallina, C., and Liberatori, A., *Tetrahedron*, 1974, **30**, 667; Badran, T. W., Easton, C. J., Horn, E., Kociuba, K., May, B. L., Schliebs, D. M., and Tiekink, E. R. T., *Tetrahedron: Asymmetry*, 1993, **4**, 197.

Alto/TC Research, Norwich) by using Merck silica gel 60 PF_{254}, eluting with a gradient of light petroleum/ethyl acetate. N.m.r. spectra were recorded on either a Bruker CXP-300 or a Varian FT80A spectrometer, as dilute solutions in (D)chloroform, with tetramethylsilane as the internal standard. Electron impact mass spectra were recorded on either an AEI MS-902 or a Hewlett Packard HP-5995C spectrometer. Microanalyses were performed by the Microanalytical Facility, Otago University, New Zealand.

Glycine anhydride and sarcosine anhydride (4) were purchased from Sigma Chemical Co. 1,4-Diacetylpiperazine-2,5-dione (7) was prepared by treatment of glycine anhydride with acetic anhydride.[9]

1,4-Diacetyl-3-(4-chlorophenylthio)piperazine-2,5-dione (8b)

A mixture of the piperazinedione (7) (0·2 g, 1 mmol), *N*-bromosuccinimide (0·18 g, 1 mmol) and azobisisobutyronitrile (17 mg, 0·1 mmol) in dry carbon tetrachloride (10 ml) was heated at reflux under nitrogen for 2 h, then it was cooled and filtered. The filtrate was concentrated under reduced pressure to give a pale yellow oil, the ^{1}H n.m.r. spectrum of which showed the presence of the bromides (8a) and (9a) in the ratio 13 : 1. Signals for 1,4-diacetyl-3-bromopiperazine-2,5-dione (8a) were observed at δ 2·61, s, 3H; 2·62, s, 3H; 4·30, d, J 19 Hz, 1H; 5·24, d, J 19 Hz, 1H; 6·87, s, 1H.

The crude product of bromination of the piperazinedione (7) was dissolved in dry dichloromethane at 0°, then 4-chlorothiophenol (0·22 g, 1·5 mmol) and pyridine (0·15 g, 1·5 mmol) were added. The mixture was stirred at room temperature for 16 h, before it was washed with dilute hydrochloric acid and brine, then dried and concentrated under reduced pressure. Chromatography of the residual oil afforded a colourless solid which was recrystallized from light petroleum/ethyl acetate to give *1,4-diacetyl-3-(4-chlorophenylthio)piperazine-2,5-dione* (8b) (235 mg, 69%), m.p. 85–87° (Found: C, 49·3; H, 3·8; N, 8·1; S, 9·5. $C_{14}H_{13}ClN_2O_4S$ requires C, 49·3; H, 3·9; N, 8·2; S, 9·4%). ^{1}H n.m.r. δ 2·55, s, 3H; 2·56, s, 3H; 4·09, d, J 18 Hz, 1H; 5·13, d, J 18 Hz, 1H; 6·22, s, 1H; 7·4–7·6, m, 4H.

3-(4-Chlorophenylthio)-1,4-dimethylpiperazine-2,5-dione (5b)

The piperazinedione (4) (0·4 g, 2·81 mmol) was treated with *N*-bromosuccinimide (0·5 g, 2·81 mmol), and that crude product mixture was treated with 4-chlorothiophenol (0·61 g, 4·21 mmol), as described above for the synthesis of the thioether (8b). Chromatography of the crude product afforded a colourless solid which was recrystallized from ethyl acetate/methanol to give *3-(4-chlorophenylthio)-1,4-dimethylpiperazine-2,5-dione* (5b) (54%), m.p. 160–161° [Found: m/z 283·0309. $C_{12}H_{12}{}^{35}ClN_2O_2S$ ($M^{+\bullet} - H$) requires m/z 283·0308]. ^{1}H n.m.r. δ 2·52, d, J 18 Hz, 1H; 2·78, s, 3H; 3·15, s, 3H; 3·46, d, J 18 Hz, 1H; 4·94, s, 1H; 7·3–7·5, m, 4H.

1,4-Diacetyl-3,6-di(4-chlorophenylthio)piperazine-2,5-dione (9b)

The piperazinedione (7) was treated with *N*-bromosuccinimide (2 mole equiv.), and that crude product mixture was treated with 4-chlorothiophenol, as described above for the synthesis of the thioether (8b). The ^{1}H n.m.r. spectrum of the product of bromination showed signals for one diastereomer of 1,4-diacetyl-3,6-dibromopiperazine-2,5-dione (9a) at δ 2·65, s, 6H; 6·93, s, 2H.

Chromatography of the product of the reaction with 4-chlorothiophenol gave one diastereomer of *1,4-diacetyl-3,6-di(4-chlorophenylthio)piperazine-2,5-dione* (9b) (15%) as a white solid after recrystallization from light petroleum/ethyl acetate, m.p. 167–169° (Found: C, 50·0; H, 3·4; N, 5·8; S, 13·3. $C_{20}H_{16}Cl_2N_2O_4S_2$ requires C, 49·7; H, 3·3; N, 5·8; S, 13·3%). ^{1}H n.m.r. δ 2·60, s, 6H; 6·11, s, 2H; 7·3–7·6, m, 8H.

Competitive Reaction of 1,4-Dimethylpiperazine-2,5-dione (4) and 1,4-Diacetylpiperazine-2,5-dione (7) with N-Bromosuccinimide

Treatment of a mixture of the piperazinediones (4) (0·38 g, 2·65 mmol) and (7) (0·52 g, 2·64 mmol) and *N*-t-butylbenzamide (0·047 g, 0·265 mmol) with *N*-bromosuccinimide (0·47 g,

2·64 mmol), as described above for the reactions of the diacetylpiperazinedione (7), afforded a crude product mixture. The ^{1}H n.m.r. spectrum of the mixture indicated the presence of the starting materials (4), (7) and *N*-t-butylbenzamide, and the bromides (5a) and (6), in the ratio 0·05 : 0·95 : 1·0 : 0·75 : 0·10.

1-Acetyl-4-methylpiperazine-2,5-dione (10)

Glycylsarcosine anhydride[9] (200 mg, 1·56 mmol) was dissolved in acetic anhydride (2 ml), and the mixture was heated at reflux for 4 h, then it was cooled and concentrated under reduced pressure. Chromatography of the residual oil afforded a colourless solid which was recrystallized from light petroleum/ethyl acetate to give *1-acetyl-4-methylpiperazine-2,5-dione* (10) (212 mg, 81%), m.p. 60–61° (Found: C, 49·3; H, 5·6; N, 16·4. $C_7H_{10}N_2O_3$ requires C, 49·4; H, 5·9; N, 16·5%). ^{1}H n.m.r. δ 2·56, s, 3H; 3·01, s, 3H; 4·14, s, 2H; 4·37, s, 2H.

1-Acetyl-3-(4-chlorophenylthio)-4-methylpiperazine-2,5-dione (11b)

The piperazinedione (10) was treated with *N*-bromosuccinimide (1 mole equiv.), and that crude product mixture was treated with 4-chlorothiophenol, as described above for the synthesis of the thioether (8b). The ^{1}H n.m.r. spectrum of the product of bromination showed signals for 1-acetyl-3-bromo-4-methylpiperazine-2,5-dione (11a) at δ 2·62, s, 3H; 3·01, s, 3H; 3·82, d, *J* 18 Hz, 1H; 4·99, d, *J* 18 Hz, 1H; 5·98, s, 1H.

Chromatography of the product of the reaction with 4-chlorothiophenol gave *1-acetyl-3-(4-chlorophenylthio)-4-methylpiperazine-2,5-dione* (11b) (74%) as a colourless solid after recrystallization from light petroleum/ethyl acetate, m.p. 115–117° (Found: C, 49·8; H, 4·2; N, 9·1; S, 10·5. $C_{13}H_{13}ClN_2O_3S$ requires C, 49·9; H, 4·2; N, 9·0; S, 10·3%). ^{1}H n.m.r. δ 2·57, s, 3H; 3·13, s, 3H; 3·08, d, *J* 18 Hz, 1H; 4·49, d, *J* 18 Hz, 1H; 4·99, s, 1H; 7·3–7·5, m, 4H.

6-Methoxy-1-methylpiperazine-2,5-dione (12)

A mixture of the piperazinedione (10) (0·57 g, 3·3 mmol), *N*-bromosuccinimide (0·59 g, 3·3 mmol) and azobisisobutyronitrile (5 mg) in carbon tetrachloride (30 ml) was heated at reflux under nitrogen for 0·5 h, then it was cooled. Methanol (1·0 ml) was added and the resultant mixture was stirred at room temperature for 16 h, before it was concentrated under reduced pressure. Chromatography of the residual oil gave *6-methoxy-1-methylpiperazine-2,5-dione* (12) (36%) as an oil, which crystallized from ethyl acetate/light petroleum, in 21% yield, as a colourless solid, m.p. 116–117° (Found: C, 45·7; H, 6·2; N, 17·6. $C_6H_{10}N_2O_3$ requires C, 45·6; H, 6·4; N, 17·7%). ^{1}H n.m.r. δ 3·10, s, 3H; 3·52, s, 3H; 3·96, dd, *J* 4, 17 Hz, 1H; 4·16, d, *J* 17 Hz, 1H; 4·70, s, 1H; 6·3, br, 1H. ^{13}C n.m.r. δ 34·9, 46·8, 58·4, 90·1, 166·5, 167·7.

Hydrolysis of 6-Methoxy-1-methylpiperazine-2,5-dione (12)

A mixture of the piperazinedione (12) (21 mg, 0·13 mmol), alanine (12 mg, 0·13 mmol) and hydrochloric acid (6 N, 10 ml) was heated at reflux for 12 h, then it was cooled and concentrated under reduced pressure. The residue was dissolved in deuterium oxide (3 ml), and the solution was concentrated under reduced pressure. The ^{1}H n.m.r. spectrum (CD_3OD) of that residue showed the presence of alanine (δ 1·56, d, *J* 7 Hz, 3H) and glycine (δ 3·77, s, 2H) in the mole ratio 3 : 2. The presence of glycine and the absence of sarcosine were confirmed by the addition of authentic samples.

Acknowledgments

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Use of cyclodextrins to limit product inhibition of (*S*)-phenylalanine ammonia lyase

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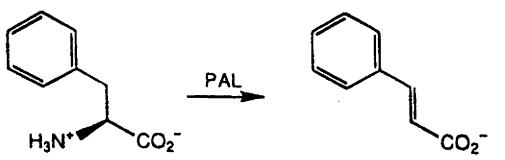
The extent of product inhibition of (*S*)-phenylalanine ammonia lyase, in catalysing the conversion of (*S*)-phenylalanine into *trans*-cinnamate, is reduced, and the efficiency of the reaction increased, through the addition of a cyclodextrin to sequester the cinnamate.

(*S*)-Phenylalanine ammonia lyase (PAL) catalyses the elimination of ammonia and a proton from (*S*)-phenylalanine **1**, to give *trans*-cinnamate **2** (Scheme 1)[1,2] which is a competitive



1 **2**

Scheme 1

inhibitor of the enzyme.[2] While product inhibition of this type is an important form of control of enzyme activity *in vivo*, it limits the utility of enzymes in organic synthesis. In this manuscript we report the use of α- and β-cyclodextrin to limit the effect of the cinnamate **2** on the catalytic activity of PAL, as an illustration of an approach to reduce product inhibition of enzymes.

Reactions of (*S*)-phenylalanine **1** catalysed by PAL (Grade 1 from *Rhodotorula glutinis*, purchased from Sigma Chemical Co.) were followed by monitoring changes in the UV absorbance at 268 nm accompanying formation of the cinnamate **2** (Fig. 1). Comparative experiments using the same quantity of enzyme were carried out with no cyclodextrin and with either α-cyclodextrin or β-cyclodextrin, in the presence and absence of the cinnamate **2**. Owing to its increased solubility in aqueous solutions compared to β-cyclodextrin,[3] it was possible to use α-cyclodextrin at higher concentration.

The results of the experiments show that the addition of the cinnamate **2** increases the extent of reaction over the first 1–3 min, but reduces the extent of reaction in the longer term. The initial increase can be attributed to the effect of the cinnamate **2** to bind competitively to the enzyme and thus slow the negative allosteric effect of the phenylalanine **1**.[4] The later reduction in the extent of each reaction with added cinnamate **2** is a clear illustration of the effect of the cinnamate **2** to inhibit the enzyme, an effect which is also apparent in the reduction in the rate of the reaction as each experiment proceeds and the cinnamate **2** is produced.

At the concentrations used, α- and β-cyclodextrin each marginally reduce the molar UV absorption of the cinnamate **2**.[5] Consequently, the effect of the cyclodextrins to increase the absorption of reaction mixtures clearly demonstrates that both α- and β-cyclodextrin increase the extent of reaction. The obvious interpretation of this effect is that the cyclodextrins complex the cinnamate **2**, irrespective of whether it is only

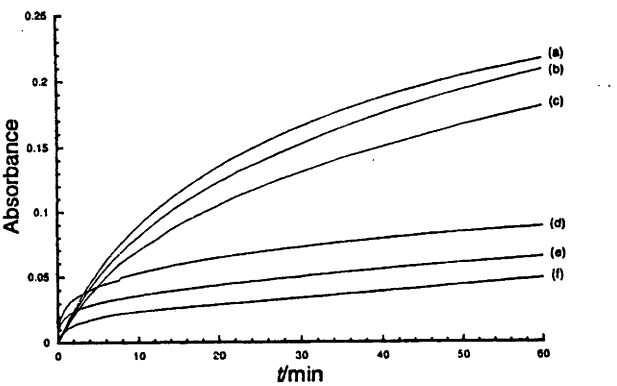


Fig. 1 Change in UV absorbance at 268 nm of solutions containing (*S*)-phenylalanine **1** (0.25×10^{-3} mol dm^{-3}), PAL (*ca.* 70 units dm^{-3}) and either (a) α-cyclodextrin (0.080 mol dm^{-3}), (b) β-cyclodextrin (6.9×10^{-3} mol dm^{-3}), (c) no cyclodextrin, (d) α-cyclodextrin (0.075 mol dm^{-3}) and the cinnamate **2** (0.26×10^{-3} mol dm^{-3}), (e) β-cyclodextrin (6.5×10^{-3} mol dm^{-3}) and the cinnamate **2** (0.26×10^{-3} mol dm^{-3}), or (f) the cinnamate **2** (0.26×10^{-3} mol dm^{-3}) but no cyclodextrin, in 0.05 mol dm^{-3} phosphate buffer at pH 6.9 and 303 K.

produced during the reaction or also added initially. This reduces the concentration of the cinnamate **2** free in solution, thus limiting the inhibitory effect on the enzyme. The results indicate that each cyclodextrin binds the cinnamate **2** in preference to (*S*)-phenylalanine **1**. This is consistent with the reported stability constants of the complexes of α- and β-cyclodextrin with (*S*)-phenylalanine **1**, of 8 and 3 dm^3 mol^{-1}, respectively,[6] and with the cinnamate **2**, of 109 and 313 dm^3 mol^{-1}, respectively.[5] From these stability constants it can be calculated that a solution containing β-cyclodextrin (6.5×10^{-3} mol dm^{-3}) and either the cinnamate **2** (0.26×10^{-3} mol dm^{-3}) or (*S*)-phenylalanine **1** (0.25×10^{-3} mol dm^{-3}) would contain only 34% of the cinnamate **2** or 98% of the phenylalanine **1** free in solution, while in analogous solutions of α-cyclodextrin (0.075 mol dm^{-3}) the amount of the cinnamate **2** and (*S*)-phenylalanine **1** unbound would be 11 and 63%, respectively.

To confirm the above interpretation of the experiments illustrated in Fig. 1, and the effect of the cyclodextrins, the experiments beginning with (*S*)-phenylalanine **1** and the cinnamate **2**, with no cyclodextrin and with either α- or β-cyclodextrin, were repeated using >99% 2-^{13}C-labelled (*S*)-phenylalanine **1** and approximately double the concentration of PAL. After 1 h, each reaction mixture was acidified to pH 1 and extracted with chloroform, and the residue obtained from concentration of the organic extract was analysed by ^{1}H NMR spectroscopy (Fig. 2). In these experiments the unlabelled

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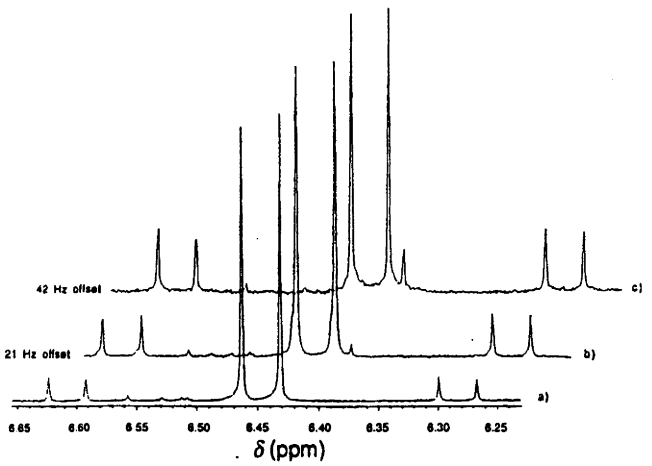

Fig. 2 ^{1}H NMR spectra (500 MHz, $CDCl_3$) of the material obtained by treatment of a solution of 2-^{13}C-labelled (*S*)-phenylalanine **1** (0.25×10^{-3} mol dm^{-3}) and the cinnamate **2** (0.26×10^{-3} mol dm^{-3}), containing either (a) no cyclodextrin, (b) β-cyclodextrin (6.5×10^{-3} mol dm^{-3}), or (c) α-cyclodextrin (0.075 mol dm^{-3}) in 0.05 mol dm^{-3} phosphate buffer at pH 6.9, with PAL at 303 K for 1 h.

cinnamate **2** is an internal standard and the different ratios of unlabelled to labelled cinnamate **2** isolated from the reaction mixtures are a measure of the relative extents of reactions. The ^{1}H NMR spectra show signals due to the ^{13}C-labelled cinnamate **2** produced during reaction, at δ 6.45 (dd, J_H 16 Hz, J_C 164 Hz), and due to the cinnamate **2** added initially to each reaction mixture, at δ 6.45 (d, J_H 16 Hz). Integration of these signals shows that whereas the reaction in the absence of a cyclodextrin proceeded to an extent of 16%, the reaction carried out under otherwise identical conditions, but in the presence of β-cyclodextrin had proceeded to an extent of 29%, while the analogous reaction in the presence of α-cyclodextrin had proceeded even further, to an extent of 41%. These results were confirmed by using gas chromatography–mass spectrometry to determine the ^{13}C-isotope content of the cinnamate **2** recovered from each of the reaction mixtures.

Reducing product inhibition of an enzyme in this manner may be achieved if the cyclodextrins complex a reaction product in preference to a substrate. In a similar manner it may be possible to manipulate enzyme-catalysed equilibrations, or the substrate selectivity in enzyme-catalysed reactions, by selectively complexing components from mixtures. Studies to this effect are underway in our laboratories.

Experimental

Procedures for assaying the effect of cyclodextrins on the catalytic activity of PAL

For UV spectrophotometric studies, aliquots of stock solutions of (*S*)-phenylalanine **1** (5.0×10^{-3} mol dm^{-3} solution in 0.05 mol dm^{-3} pH 6.9 sodium phosphate buffer; 1.0×10^{-5} dm^3) and the cinnamate **2** (5.2×10^{-3} mol dm^{-3} solution in 0.05 mol dm^{-3} pH 6.9 sodium phosphate buffer; 1.0×10^{-5} dm^3), as appropriate, were diluted to 1.6×10^{-4} dm^3 with 0.05 mol dm^{-3} pH 6.9 sodium phosphate buffer containing either no cyclodextrin, α-cyclodextrin (0.107 mol dm^{-3}) or β-cyclodextrin (9.26×10^{-3} mol dm^{-3}). The resulting solutions were equilibrated at 303 K for 10 min and then a thermally pre-equilibrated solution of PAL (4.0×10^{-5} dm^3 of a 30% glycerol, 0.025 mol dm^{-3} pH 6.9 sodium phosphate buffer solution) was added to each one. These mixtures were prepared in a 1 mm path-length cell, and monitored for change in UV absorbance at 268 nm using a Cary 1E spectrophotometer, with the cell-holder thermostatted at 303 K.

For product studies, solutions were prepared as described above, except that >99% 2-^{13}C-labelled (*S*)-phenylalanine **1** was used and the scale of the reactions was increased 50-fold. After incubation at 303 K for 1 h, the solutions were each acidified to pH 1 with concentrated HCl and extracted with $CHCl_3$ (8×0.040 dm^3). For each reaction mixture, the combined extracts were dried ($MgSO_4$) and concentrated under reduced pressure, and the residue was analysed using ^{1}H NMR spectroscopy and mass spectrometry.

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References

1 E. A. Havir and K. R. Hanson, *Biochemistry*, 1968, **7**, 1896; K. R. Hanson and E. A. Havir, *Arch. Biochem. Biophys.*, 1970, **141**, 1; E. A. Havir, P. D. Reid and H. V. Marsh, *Plant Physiol.*, 1971, **48**, 130; E. A. Havir and K. R. Hanson, *Biochemistry*, 1975, **14**, 1620; D. S. Hodgins, *J. Biol. Chem.*, 1971, **266**, 2977; J. R. Parkhurst and D. S. Hodgins, *Arch. Biochem. Biophys.*, 1972, **152**, 597; C. Walsh, *Enzymatic Reaction Mechanisms*, W. H. Freeman and Co., San Francisco, 1979, pp. 563–567; K. R. Hanson and E. A. Havir, in *The Enzymes*, 3rd edn., P. D. Boyer, ed., Academic Press, New York, 1972, vol. 7, pp. 75–166; K. R. Hanson and E. A. Havir, in *The Biochemistry of Plants*, P. K. Stumpf and E. E. Conn, eds., Academic Press, New York, 1981, vol. 7, pp. 577–625.
2 E. A. Havir and K. R. Hanson, *Biochemistry*, 1968, **7**, 1904.
3 M. L. Bender and M. Komiyama, *Cyclodextrin Chemistry*, Springer, New York, 1977.
4 S. E. Iredale and H. Smith, *Phytochemistry*, 1974, **13**, 575.
5 K. Uekama, M. Otagiri, Y. Kanie, S. Tanaka and K. Ikeda, *Chem. Pharm. Bull.*, 1975, **23**, 1421.
6 M. V. Rekharsky, F. P. Schwarz, Y. B. Tewari and R. N. Goldberg, *J. Phys. Chem.*, 1994, **98**, 10282.

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Complexes of Naproxen and Ibuprofen with 6^A-Amino-6^A-deoxy-β-cyclodextrin

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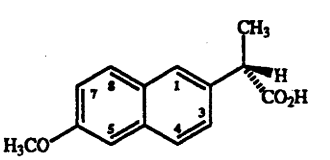
At pD 6·80 in D_2O containing 0·10 mol dm^{-3} phosphate buffer, the association constants of the complexes of Naproxen and Ibuprofen with 6^A-amino-6^A-deoxy-β-cyclodextrin are 810±200 and 8900±2100 mol^{-1} dm^3, respectively, while those of the corresponding complexes with β-cyclodextrin are 940±170 and 8800±1800 mol^{-1} dm^3, respectively. A 2D-ROESY experiment shows that Naproxen includes lengthwise in the substituted cyclodextrin, with the reverse orientation to that of the complex with β-cyclodextrin. The orientation in the host–guest complex of the substituted cyclodextrin results in the alignment of the host amino substituent and the guest carboxy group, which at this pD are predominantly protonated and deprotonated, respectively. The similarity in the association constants of the complexes of Naproxen indicates that any stabilization provided by interactions between the ionic groups in the complex of the substituted cyclodextrin is offset by other factors, such as the extent of desolvation of the host and guest.

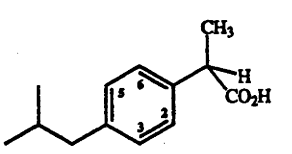
Introduction

Naproxen* (1) and Ibuprofen† (2) are systematic non-steroidal antiinflammatory and analgesic agents which are used widely in the relief of the symptoms of various forms of arthritis.[1–4] Such drugs have deleterious effects on the epithelium of the gastrointestinal tract and it has been proposed that their administration as inclusion complexes within cyclodextrins will result in reduced concentrations of the free drugs available to cause such damage.[5,6] Consequently the formation of host–guest complexes of Naproxen (1) and Ibuprofen (2) with the naturally occurring cyclodextrins has been studied.[7,8] The most stable complexes are formed with β-cyclodextrin, such results indicating that it has the optimal cavity size for binding these guests.

One limitation to using β-cyclodextrin in the preparation of aqueous drug formulations is its low solubility in water, restricted to 18·5 g dm^{-3} at 298·2 K.[9] Modified cyclodextrins which are more soluble provide the opportunity to prepare more concentrated solutions of host–guest complexes, and the modifications also provide additional sites for host–guest interactions, which may affect the thermodynamic stability of the complexes. The solubility of the hydrochloride salt of 6^A-amino-6^A-deoxy-β-cyclodextrin (3)‡ in water at 298·2 K is 705 g dm^{-3}.[10] In solutions near neutral pH the amine is predominantly in the protonated form,



(1)



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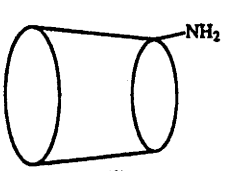

(3)

* (*S*)-2-(6-Methoxy-2-naphthyl)propanoic acid.
† 2-[4-(2-Methylpropyl)phenyl]propanoic acid.
‡ Cyclodextrins are commonly represented as truncated cones with the narrow and wide ends representing the circles delineated by the primary and secondary hydroxy groups respectively. The protons located within the cyclodextrin annulus, ordered from the primary end, are those attached to C6, C5 and C3.

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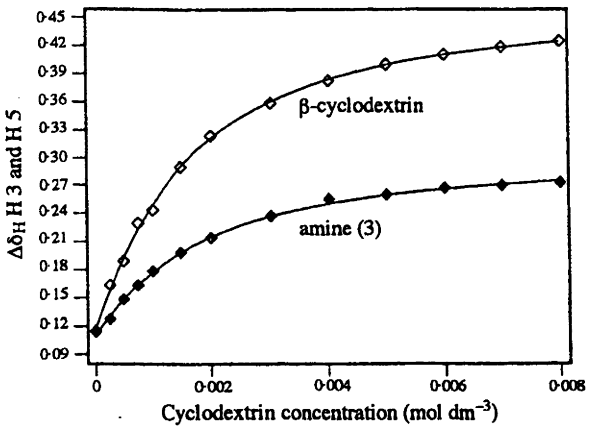

Fig. 1. Differences between chemical shifts of the resonances of H3 and H5 in the ^{1}H n.m.r. spectrum of Naproxen (1) in the presence of varying concentrations of β-cyclodextrin or 6^A-amino-6^A-deoxy-β-cyclodextrin (3) at pD 6·80 in D_2O containing 0·10 mol dm^{-3} phosphate at 298·2 K.

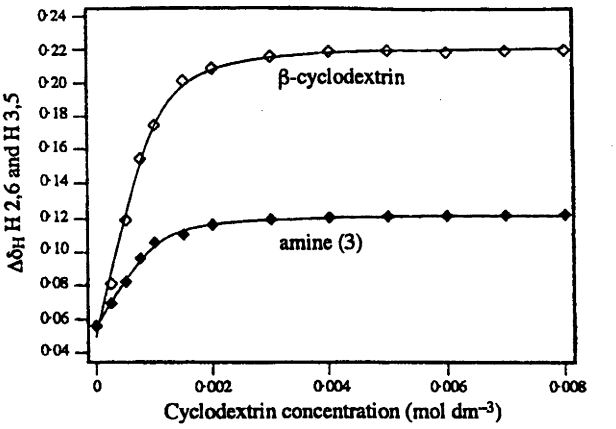

Fig. 2. Differences between chemical shifts of the resonances of H2,6 and H3,5 in the ^{1}H n.m.r. spectrum of Ibuprofen (2) in the presence of varying concentrations of β-cyclodextrin or 6^A-amino-6^A-deoxy-β-cyclodextrin (3) at pD 6·80 in D_2O containing 0·10 mol dm^{-3} phosphate at 298·2 K.

since its pK_b is 8·7,[10] and Naproxen (1) and Ibuprofen (2) are each predominantly in the deprotonated form, with pK_a values of 4·2[11] and 4·4,[11] respectively. We have now studied the complexation of the drugs (1) and (2) by the amine (3), to examine the ability of the modified cyclodextrin (3) to include the guests (1) and (2), and to investigate the possible effects of ionic host–guest interactions on the inclusion complexes.

Results and Discussion

Inclusion of a guest within the cavity of a cyclodextrin changes the physical and spectroscopic properties of the host and guest, and the complexation can be characterized by monitoring the change in one of these properties as a function of increasing cyclodextrin concentration.[12–17] In the present work, the ^{1}H n.m.r. spectra were recorded of solutions of the drugs (1) and (2) ($1·00\times10^{-3}$ mol dm^{-3}) and the cyclodextrin (3) (0–8 mol. equiv.) in D_2O containing 0·10 mol dm^{-3} phosphate buffer, at pD 6·80. For comparison, analogous experiments were performed with α- and β-cyclodextrin, and 6^A-amino-6^A-deoxy-α-cyclodextrin. The chemical shifts of the resonances of the aromatic protons of Naproxen (1) and Ibuprofen (2) varied with changing cyclodextrin concentration. This indicates that the free and complexed guests are in fast exchange on the n.m.r. time scale, and the signals represent environmental averages for the free and complexed species. The effects of 0–8 mol. equiv. of α-cyclodextrin and 6^A-amino-6^A-deoxy-α-cyclodextrin on the ^{1}H n.m.r. spectra of the drugs (1) and (2) were much less than those caused by the same quantities of β-cyclodextrin and the amine (3). Substantial changes were observed when larger excesses of the former cyclodextrins were employed, however, indicating that the association constants of the complexes of these hosts are much lower.

The observed changes in the differences between the chemical shifts of the resonances of H3 and H5 of Naproxen (1), and H2,6 and H3,5 of Ibuprofen (2), induced by β-cyclodextrin and the amine (3), are shown in Figs 1 and 2. The H3 and H5 signals of Naproxen were used because they are shifted the most downfield and upfield, respectively, by the cyclodextrins. By fitting these data according to equations *(1)* and *(2)*, which apply when the free and complexed species are in fast exchange on the n.m.r. time scale and environmentally averaged signals are observed,[17] the association constants (K) of the complexes of Naproxen (1) and Ibuprofen (2) with the amine (3) were calculated to be 810±200 and 8900±2100 mol^{-1} dm^3, respectively, while those of the corresponding complexes with β-cyclodextrin were found to be 940±170 and 8800±1800 mol^{-1} dm^3, respectively. The similarity between the association constants of the complexes of the amine (3) and β-cyclodextrin shows that the introduction of the amino group, protonated at pD 6·80, has little effect on the thermodynamic stability of the complexes of deprotonated Naproxen (1) and Ibuprofen (2).

$$K = \frac{[\text{complex}]}{[\text{host}][\text{guest}]} \qquad (1)$$

$$\delta_{\text{observed}} = \frac{\delta_{\text{free}}[\text{guest}] + \delta_{\text{complexed}}[\text{complex}]}{[\text{guest}] + [\text{complex}]} \qquad (2)$$

From molecular modelling studies,[8] the orientation of Naproxen (1) included within β-cyclodextrin has been proposed. The guest was found to include lengthwise in the cavity, as is typical of 2-substituted naphthalene complexes,[18] with the carboxylate group adjacent to the secondary hydroxy groups of the host (Fig. 3*a*). To determine the alignment of Naproxen (1) in the annulus of the amine (3), a two-dimensional rotating frame ^{1}H–^{1}H nuclear Overhauser effect (2D-ROESY) experiment was conducted on a 1:8 mixture of Naproxen (1) and the amine (3). The resulting spectrum is

shown in Fig. 4. Resonances at *c.* δ 3·7, 3·8 and 3·9 can be assigned to the $C6^{B-G}$, $C5^{A-G}$ and $C3^{A-G}$ protons of the host (3), respectively.[19] The 2D-ROESY experiment shows through-space interactions between H5 of Naproxen (1) (*c.* δ 7·2) and H3 of the host (3), and between H5 and H6 of the host (3) and H1 of the guest (1) (*c.* δ 7·65). These interactions show that the orientation of the guest (1) is such that its carboxylate group is adjacent to the primary hydroxy groups and the protonated amino substituent of the cyclodextrin

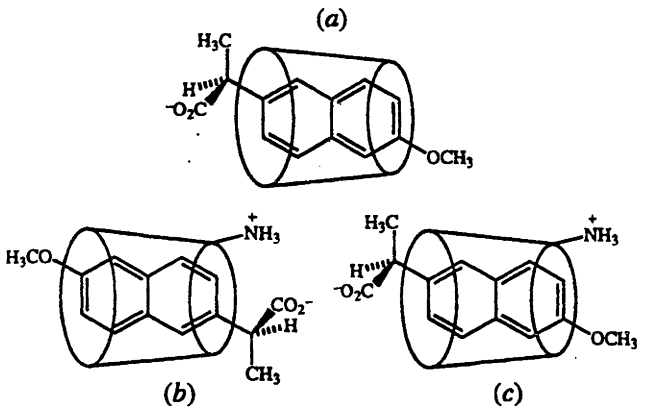

Fig. 3. Complexes of Naproxen (1) in β-cyclodextrin and 6^A-amino-6^A-deoxy-β-cyclodextrin (3).

(3) (Fig. 3*b*), an orientation which is the reverse of that indicated by the molecular modelling studies[8] for the same guest complexed in β-cyclodextrin (Fig. 3*a*).

It appears that the protonated amino substituent alters the orientation of Naproxen (1) in the annuli of β-cyclodextrin and the amine (3), as a result of the ionic interaction between the guest and the modified host (3). This does not lead to an increase in the thermodynamic stability of the inclusion complex, however, indicating that other factors offset the ionic interaction. The extent of desolvation of the host and guest on inclusion complex formation is an important component of the free energy change accompanying complexation.[20,21] It seems likely that this is greater for complexation by β-cyclodextrin than by the amine (3), with the result that the complex of Naproxen (1) with the amine (3) having the orientation shown in Fig. 3*c* is less stable than the analogous complex with β-cyclodextrin (Fig. 3*a*). The reverse orientation of Naproxen in the amine (3) (Fig. 3*b*) is therefore preferred, where ionic host–guest interactions stabilize the complex.

Experimental

α-Cyclodextrin and β-cyclodextrin were the generous gifts of Nihon Shokuhin Kako Co. 6^A-Amino-6^A-deoxy-β-cyclodextrin

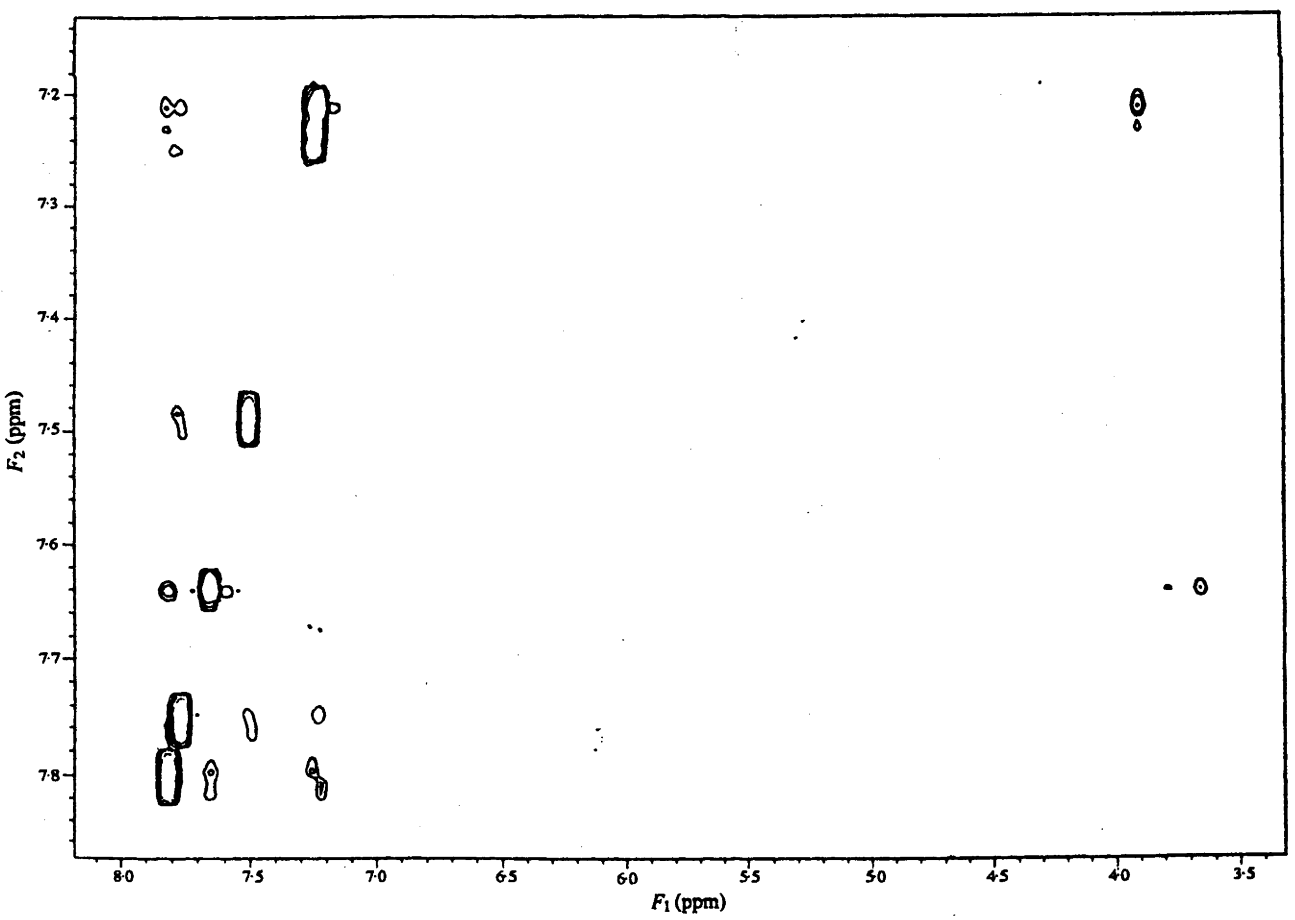

Fig. 4. 2D-ROESY experiment on a solution of Naproxen (1) ($1·00\times10^{-3}$ mol dm^{-3}) and 6^A-amino-6^A-deoxy-β-cyclodextrin (3) ($8·00\times10^{-3}$ mol dm^{-3}) at pD 6·80 in D_2O containing 0·10 mol dm^{-3} phosphate at 298·2 K.

(3) and 6^A-amino-6^A-deoxy-α-cyclodextrin were obtained as reported previously.[10] All cyclodextrins were dried to constant weight under vacuum over phosphorus pentoxide prior to use. Naproxen (1) and Ibuprofen (2) were purchased from Aldrich Chemical Company Inc.

For all pD measurements, BDH Standard Buffer reference solutions at pH 4·00, 7·00 and 10·00 were used with an Orion 520A pH meter and a Ross 81-56 pH electrode.

^{1}H n.m.r. spectroscopy was carried out on a Gemini BB spectrometer for the one-dimensional experiments and on a 500 Unity INOVA spectrometer for the two-dimensional experiment.

Preparation of 0·10 mol dm^{-3} pD 6·80 Phosphate Buffer

Sodium hydroxide (0·100 g, $2{\cdot}50\times10^{-3}$ mol) was dissolved in D_2O, and the volume was made up to 25·0 cm^3 with D_2O. To 11·2 cm^3 of this solution was added potassium dihydrogen phosphate (0·340 g, $2{\cdot}50\times10^{-3}$ mol), and the volume was made up to 50·0 cm^3 with D_2O. The pD was checked and found to be as required.

Preparation of Naproxen (1) and Ibuprofen (2) Stock Solutions

Naproxen (1) ($11{\cdot}5\times10^{-3}$ g, $5{\cdot}00\times10^{-5}$ mol) was dissolved in 0·10 mol dm^{-3} pD 6·80 phosphate buffer, and the volume was made up to 25·0 cm^3 with the buffer. Ibuprofen (2) ($10{\cdot}3\times10^{-3}$ g, $5{\cdot}00\times10^{-5}$ mol) was dissolved in 0·10 mol dm^{-3} pD 6·80 phosphate buffer, and the volume was made up to 25·0 cm^3 with the buffer.

Preparation of Solutions for N.M.R. Experiments

Aliquots of the Naproxen (1) solution (1·00 cm^3) were added to series of 2·00 cm^3 volumetric flasks containing weighed amounts of either α- or β-cyclodextrin, or 6^A-amino-6^A-deoxy-α-cyclodextrin or 6^A-amino-6^A-deoxy-β-cyclodextrin (3). The amount of the cyclodextrin was varied to give from 0 to 8 mol. equiv. of the host relative to the guest. The volume of each solution was made up to 2·00 cm^3 with 0·10 mol dm^{-3} pD 6·80 phosphate buffer. The ^{1}H n.m.r. spectrum of each solution was recorded. In the absence of any cyclodextrin host Naproxen (1) showed: δ 7·252, dd, *J* 2·5, 8·5 Hz, 1H, H7; 7·401, d, *J* 2·5 Hz, 1H, H5; 7·512, dd, *J* 1·5, 8·5 Hz, 1H, H3; 7·791, d, *J* 1·5 Hz, 1H, H1; 7·860, d, *J* 8·5 Hz, 1H, H4; 7·884, d, *J* 8·5 Hz, 1H, H8. The assignments are based on literature values.[22]

The experiments were repeated with Ibuprofen (2). In the absence of any cyclodextrin host Ibuprofen (2) showed: δ 7·143, d, *J* 8 Hz, 2H, H2,6; 7·200, d, *J* 8 Hz, 2H, H3,5.

In addition, the sample containing 8 mol. equiv. of the cyclodextrin (3) relative to the guest (1) was deoxygenated by repeatedly purging with nitrogen, and a 2D-ROESY experiment was performed.

Calculation of Complex Association Constants

The difference between the observed chemical shifts of the resonances of H3 and H5 of Naproxen (1) was plotted against the concentration of the host (Fig. 1) and curves were fitted to the data according to equations *(1)* and *(2)* by using MacCurvefit v1.2. This gave values for the association constants of the complexes of Naproxen (1) with β-cyclodextrin and the modified cyclodextrin (3) of 940±170 and 810±200 mol^{-1} dm^3, respectively. This was repeated for the difference between the observed chemical shifts of the resonances of H2,6 and H3,5 of Ibuprofen (2) (Fig. 2) to give association constants for the complexes with β-cyclodextrin and the modified cyclodextrin (3) of 8800±1800 and 8900±2100 mol^{-1} dm^3, respectively.

Acknowledgments

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References

[1] Roszkowski, A. P., Rooks, W. H., Tomolonis, A. J., and Miller, L. M., *J. Pharmacol. Exp. Ther.*, 1971, **179**, 114.
[2] Harrison, I. T., Lewis, B., Nelson, P., Rooks, W., Roszkowski, A., Tomolonis, A., and Fired, J. H., *J. Med. Chem.*, 1970, **13**, 203.
[3] Adams, S. S., McCullough, K. F., and Nicholson, J. S., *Arch. Pharmacodyn. Ther.*, 1969, **178**, 115.
[4] Chalmers, T. M., *Schweiz. Med. Wochenschr.*, 1971, **101**, 280.
[5] Saenger, W., *Angew. Chem., Int. Ed. Engl.*, 1980, **19**, 344.
[6] Szejtli, J., 'Cyclodextrins and Their Inclusion Complexes' (Akademio Kaido: Budapest 1982).
[7] Brown, S. E., Coates, J. H., Easton, C. J., Lincoln, S. F., Luo, Y., and Stephens, A. K. W., *Aust. J. Chem.*, 1991, **44**, 855.
[8] Bettinetti, G., Melani, F., Mura, P., Monnanni, R., and Giordano, F., *J. Pharm. Sci.*, 1991, **80**, 1162.
[9] Bender, M. L., and Komiyama, M., 'Cyclodextrin Chemistry' (Springer: Berlin 1978).
[10] Brown, S. E., Coates, J. H., Coghlan, D. R., Easton, C. J., van Eyk, S. J., Janowski, W., Lepore, A., Lincoln, S. F., Luo, Y., May, B. L., Schiesser, D. S., Wang, P., and Williams, M. L., *Aust. J. Chem.*, 1993, **46**, 953.
[11] Craig, P. N., in 'Comprehensive Medicinal Chemistry' Vol. 6 (Pergamon: Oxford 1990).
[12] Seliskar, C. J., and Brand, L., *Science*, 1971, **171**, 799.
[13] Connors, K. A., and Lipari, J. M., *J. Pharm. Sci.*, 1976, **65**, 379.
[14] Flohr, K., Paton, R. M., and Kaiser, E. T., *J. Am. Chem. Soc.*, 1975, **97**, 1209.
[15] Siegel, B., and Breslow, R., *J. Am. Chem. Soc.*, 1975, **97**, 6869.
[16] Hummel, J. P., and Dreyer, W. J., *Biochim. Biophys. Acta*, 1962, **63**, 530.
[17] Brown, S. E., Coghlan, D. R., Easton, C. J., and Lincoln, S. F., *J. Chem. Soc., Faraday Trans.*, 1991, **87**, 2699.
[18] Harata, K., and Uedaira, H., *Bull. Chem. Soc. Jpn*, 1975, **48**, 375.
[19] Rekharvsky, M. V., Goldberg, R. N., Schwarz, F. P., Tewari, Y. B., Ross, P. D., Yamashoji. Y., and Inoue, Y., *J. Am. Chem. Soc.*, 1995, **117**, 8830.
[20] Clarke, R. J., Coates, J. H., and Lincoln, S. F., *Adv. Carbohydr. Chem. Biochem.*, 1989, **46**, 205.
[21] Inoue, Y., Haksuhi, T., Liu, Y., Tong, L.-H., Shen, B.-J., and Jin, D.-S., *J. Am. Chem. Soc.*, 1993, **115**, 475.
[22] Dharanipragada, R., and Diederich, F., *Tetrahedron Lett.*, 1987, **28**, 2443.

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TETRAHEDRON LETTERS

Acylase I catalysed hydrolysis of *para*-substituted (*S*)-phenylalanine derivatives from mixtures of the racemic *ortho*- and *para*-substituted isomers

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Abstract

para-Substituted (*S*)-phenylalanines may be obtained by treatment of the corresponding mixtures of *ortho*- and *para*-substituted *N*-acetyl-(*RS*)-phenylalanines with Acylase I from porcine kidney. The selectivity of the enzyme may be attributed to its evolution to digest peptide derivatives of (*S*)-phenylalanine and (*S*)-tyrosine.

Keywords: Amino acids and derivatives; Enzymes and enzyme reactions; Regioselection; Stereoselection

Ring-substituted (*S*)-phenylalanines are important constituents of natural products, such as vancomycin [1,2]. Mixtures of *ortho*- and *para*-substituted phenylalanines may be synthesised, often as racemates, through electrophilic aromatic substitution of phenylalanine derivatives [3-5] and other aromatic starting materials [6,7]. Acylase I is a practical tool for resolution of a range of *N*-acetylamino acids [8,9]. We now report that it selectively digests *para*-substituted *N*-acetylphenylalanines over the corresponding *ortho*-substituted isomers, providing a convenient route for the preparation of *para*-substituted (*S*)-phenylalanines from the corresponding mixtures of *ortho*- and *para*-substituted *N*-acetyl-(*RS*)-phenylalanines.

For the present study, racemic samples of the *ortho*- and *para*-substituted *N*-acetylphenylalanines **1a,b-5a,b** were either prepared as illustrated in Scheme 1 [6] or by acetylation of commercially available substituted phenylalanines. Acylase I was purchased from Sigma Chemical Co. as a lyophilized powder with an activity of approximately 2×10^6 units g^{-1} (one unit will hydrolyse 1.0×10^{-6} moles of *N*-acetyl-(*S*)-methionine at pH 7.0 and 298 K in 1 h). Aqueous solutions containing racemic mixtures of regioisomeric pairs of the phenylalanine derivatives **1a,b-5a,b** (*ca.* 0.01 mol dm^{-3}) were treated with Acylase I (2.2×10^{-6} mol dm^{-3}), in 0.05 mol dm^{-3} pH 7.0 phosphate buffer, at 298 K for 2 h. The mixtures were then acidified, washed repeatedly with ethyl acetate and concentrated. The free amino acids present in the residues were treated with acetic anhydride in water and the

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0040-4039/98/$19.00
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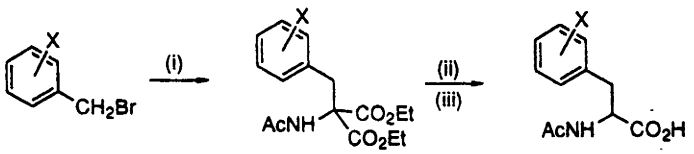


(i) $AcNHCH(CO_2Et)_2$, NaOEt/EtOH, 12 h; (ii) 6N HCl, Δ, 2 h; (iii) NEt_3, Ac_2O, H_2O, r.t., 3 h.

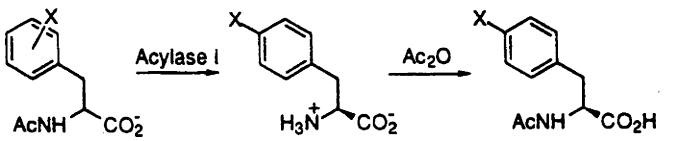
Scheme 1

product acetamides were isolated (Scheme 2, Table 1). They were shown to be enantiomerically pure, within the limits of detection, by conversion to the corresponding methyl esters using thionyl chloride pretreated methanol, followed by gas chromatographic analysis (GC) carried out on a Chirasil-Val® capillary column. The regioisomeric pairs **1a,b-5a,b** were distinguished using GC and ^{1}H NMR spectroscopy.

In four of the five cases investigated, Acylase I was specific for hydrolysis of the *para*-substituted *N*-acetyl-(*S*)-phenylalanine derivative. Control experiments established that separation of the regioisomers **1a,b-5a,b** is not an artefact of the isolation procedure. The systems studied included ring-substituted phenylalanines with hydrophobic, hydrophilic, electron-donating and electron-withdrawing groups, and indicate the broad tolerance of the enzyme for a ring substituent at the *para*-position. It was only in the case of the fluorophenylalanine derivatives **5a** and **5b** that any hydrolysis of the *ortho*-isomer was observed. This is presumably due to the fluoro substituent having comparable steric bulk to hydrogen [10] and, therefore, little effect on the interaction with the enzyme.

To determine the basis of the selectivity exhibited by the enzyme, kinetic experiments observing digestion by Acylase I were carried out on *N*-acetyl-(*S*)-phenylalanine and the derivatives (*S*)-**3a,b-5a,b**. Reactions were monitored by UV spectroscopy at 228 nm, the absorbance of the amide being hydrolysed. The rate constants of the enzyme catalysed reactions (k_{cat}) and the dissociation constants of the enzyme-substrate complexes (K_M) were calculated using Lineweaver-Burk analysis [11] (Table 2). Quite similar results were obtained using the curve-fitting programs KaleidaGraph® and MacCurvefit®. In the case of the methyl derivative **3b**, these values were determined using the racemate. Separate experiments gave values of k_{cat} and K_M for (*S*)-**5a,b** and *N*-acetyl-(*S*)-phenylalanine which were within experimental error whether the enantiomer or the racemate was used. This is consistent with published results [12] in which the (*R*)-enantiomers of other amino acids do not affect digestion of their (*S*)-antipodes.

In terms of their k_{cat}/K_M values, each of the *para*-substituted phenylalanine derivatives **3b-5b** is as good as or better than *N*-acetyl-(*S*)-phenylalanine as a substrate for Acylase I, and binds as or more readily to the enzyme (K_M), although the rate constant for reaction of the bound species (k_{cat}) is not necessarily higher. Although the *ortho*-methyl- and *ortho*-nitro-(*S*)-phenylalanine derivatives **3a** and **4a** were not digested by the enzyme, they each inhibit catalysis of the reaction of *N*-acetyl-(*S*)-phenylalanine. With the methylphenylalanine derivative **3a**, the dissociation constant of the enzyme-inhibitor complex, K_i, was measured as $(1.5 \pm 0.3) \times 10^{-3}$ mol dm^{-3}. Thus it seems likely that *ortho*-substituted phenylalanine derivatives bind to the enzyme, but not with the correct orientation for reaction.



Scheme 2

Table 1
Results of digestion of racemic mixtures of the *N*-acetylphenylalanines **1a,b-5a,b** by Acylase I

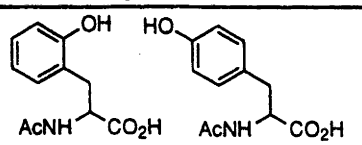
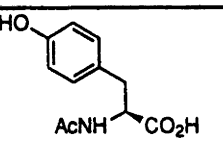
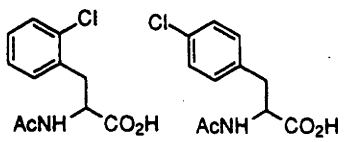
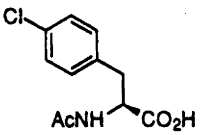
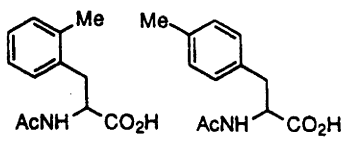
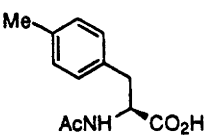
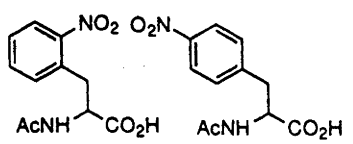
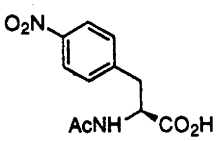
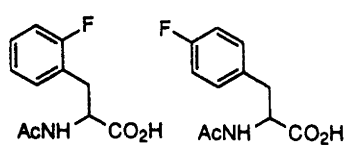
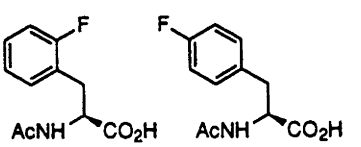
Starting Materials (ratio 1:1)	Products	Yield
 **1a** **1b**	 **(*S*)-1b**	73%
 **2a** **2b**	 **(*S*)-2b**	71%
 **3a** **3b**	 **(*S*)-3b**	72%
 **4a** **4b**	 **(*S*)-4b**	83%
 **5a** **5b**	 **(*S*)-5a** **(*S*)-5b**	29%, 58%

Table 2
Rate constants for the reaction of bound substrates and dissociation constants for the Michaelis complexes of *N*-acetylphenylalanines with Acylase I

N-Acetylphenylalanine	k_{cat} (s^{-1})	K_M (x 10^3 mol dm^{-3})	k_{cat}/K_M (x 10^{-3} mol^{-1} dm^3 s^{-1})
(*S*)-**3a**		no digestion detected	
(*S*)-**3b**	2.7 ± 0.2	0.32 ± 0.04	8 ± 2
(*S*)-**4a**		no digestion detected	
(*S*)-**4b**	15 ± 1	0.50 ± 0.07	30 ± 6
(*S*)-**5a**	7.3 ± 0.4	1.0 ± 0.1	7 ± 1
(*S*)-**5b**	66 ± 1	3.3 ± 0.1	20 ± 1
N-acetyl-(*S*)-phenylalanine	24 ± 4	3.9 ± 0.8	6 ± 2

The natural substrates of Acylase I are phenylalanine and tyrosine derivatives. On this basis it seems likely that the enzyme has evolved to tolerate *para*-substituted phenylalanine derivatives, whereas the *ortho*-isomers are not processed because natural substrates of this type are not prevalent. In any event, Acylase I effectively discriminates between *ortho*- and *para*-substituted (*S*)-phenylalanine derivatives.

Acknowledgment

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References

[1] Williams DH. Acc. Chem. Res. 1984;17:364-369.
[2] Rama Rao AV, Gurjar MK, Reddy KL, Rao AS. Chem. Rev. 1995;95:2135-2167.
[3] Bergel F, Stock JA. J. Chem. Soc. 1954:2409-2417.
[4] Shimidzu T, Iyoda T, Kanda N. J. Chem. Soc., Chem. Commun. 1981:1206-1207.
[5] Coenen HH, Bodsch W, Takahashi K, Hossman A, Stoecklin G. Nuklearmedizin. Suppl. 1986;22:600-602.
[6] Vasil'eva MN, Shkodinskaya EN, Berlin AY. Zh. Obsch. Khim., 1961;31:1027-1033.
[7] Cleland GH. J. Org. Chem. 1969;34:744-747.
[8] Chenault HK, Dahmer J, Whitesides GM. J. Am. Chem. Soc. 1989;111:6354-6364.
[9] Bommarius AS, Drauz K, Günther K, Gnaup K, Schwarm M. Tetrahedron Asymm. 1997;8:3197-3200.
[10] Clark JH, Wails D, Bastock TW. Aromatic fluorination. Boca Raton: CRC Press, 1996.
[11] Lineweaver H, Burk D. J. Am. Chem. Soc. 1934;56:658-666.
[12] Birnbaum SN, Levinton L, Kingsley RB, Greenstein JP. J. Biol. Chem. 1952;194;455-470.

N,N'-Bis(6^A-deoxy-β-cyclodextrin-6^A-yl)urea as a molecular template in the formation of indigoid dyes

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[b] *Department of Chemistry, University of Adelaide, Adelaide SA 5005, Australia*

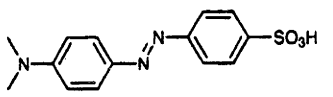
Letter

The title cyclodextrin dimer biases competing reactions of indoxyl anion to give indirubin and indigo against the latter, due to the preferred geometry of alignment of the cyclodextrin annuli.

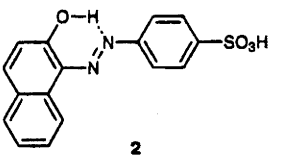
Linked cyclodextrins have been studied widely due to the cooperative guest binding displayed by the cyclodextrin annuli in such species.[1–5] Breslow and Chung[3] demonstrated that the extent of this cooperativity is dependent on the match of the shape of the guest to the relative orientations of the host cavities. Our studies[4] of the complexation of the anions of Methyl Orange (**1**) and Tropaeolin 000 No. 2 (**2**) by the cyclodextrins **4**–**6**[5] showed maximum binding of the linear dye **1** in the oxalamide **5** and of the non-linear guest **2** in the urea **4**, implying a preferred non-linear orientation of the cyclodextrin annuli in the latter host. The present work involved the exploitation of this observation, using the urea **4** as a molecular template, in the formation of a non-linear product from reagents complexed in the cyclodextrin annuli. This is the first example of the controlled orientation of a coupling reaction by a cyclodextrin dimer. $\Delta^{2,2'}$-Biindoline-3,3'-dione (indigo) (**7**) and $\Delta^{2,3'}$-biindoline-2',3-dione (indirubin) (**8**) form competitively from the oxidative dimerisation of the 1*H*-indol-3-ol anion (indoxyl anion) (**9**) and its condensation with 1*H*-indoline-2,3-dione (isatin) (**11**), respectively.[6] Isatin (**11**) is formed in the reaction as an oxidation product of indoxyl, indigo (**7**) and indirubin (**8**). Given the shape of the dyes **7** and **8** and the geometry of the cyclodextrin **4**, it was considered that complexation of the reactants **9** and **11** by the cyclodextrin **4** could bias the ratio of dye formation in favour of indirubin (**8**).

To examine this hypothesis, indoxyl anion (**9**) was generated *in situ*, through hydrolysis of the corresponding acetate **10**, in buffered aqueous solutions containing a *ca.* 6 molar excess of isatin (**11**) and either no cyclodextrin, β-cyclodextrin (**3**) or one of the linked species **4**–**6**. By comparison with the concentration of the linked species **4**–**6**, approximately double the concentration of β-cyclodextrin (**3**) was used, in order to achieve a cyclodextrin annuli concentration *ca.* 130 times that of the acetate **10**, in each case. The reactions were studied at pH 10.0, 10.5 and 11.0. Basic conditions are required for hydrolysis of indoxyl acetate (**10**) to indoxyl and deprotonation of indoxyl to give the anion **9**, but side reactions of indoxyl are prevalent below pH 10[6,7] and hydrolysis of isatin (**11**) is the dominant reaction of that species in phosphate buffer at or above pH 11.[8] Reactions were stopped by acidification of the mixtures, then the products **7** and **8** were extracted into chloroform and the extracts were analysed using HPLC.

The results of these experiments are shown in Table 1. They show that an effect of each of the cyclodextrins **3**–**6** is to decrease the yields of both indigo (**7**) and indirubin (**8**). This is as expected, since complexation of the reactants **9** and **11** by the large excess of cyclodextrin annuli reduces their opportunity for productive collisions and favours their mono-



1



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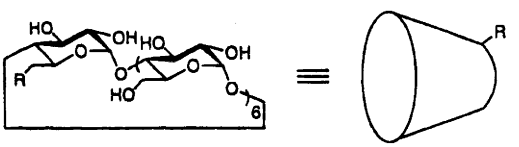
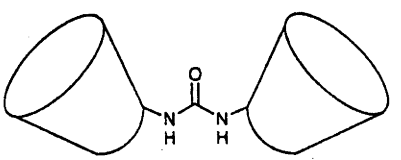


3 R = OH



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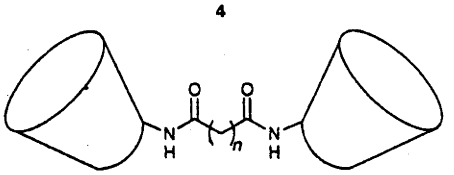
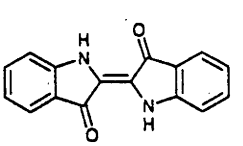


5 *n* = 0
6 *n* = 2



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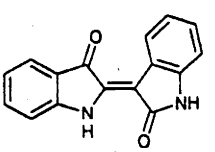

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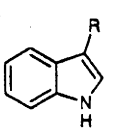

9 R = O⁻
10 R = OAc

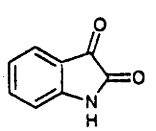

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Table 1 Yields of indigo (7) and indirubin (8) from reactions of indoxyl anion (9) and isatin (11)

Cyclodextrin	Percentage yields of indigo (7) and indirubin (8) as the ratio pH 10.0	pH 10.5	pH 11.0
—	16 : 13	41 : 38	47 : 12‡
3	2.5 : 2.5	8.5 : 9.0	9.5 : 12
4	0.03 : 1.0	0.4 : 7.5	2.5 : 17
5	0.2 : 0.6	9.0 : 6.5	15 : 14
6	0.5 : 0.7	5.5 : 8.5	6.0 : 6.5

‡ The high ratio in this case is consistent with rapid hydrolysis of isatin (11) at the high pH. This is slowed through complexation by the cyclodextrins 3–6, so the product ratio is not as high from reactions in the presence of those species.

meric reactions, at the expense of their second-order reactions to give the dyes 7 and 8. By comparison with the other cyclodextrins 3, 5 and 6, the cyclodextrin 4 has a marked effect on the ratio of formation of indigo (7) and indirubin (8). For example, at pH 10, use of the cyclodextrin 4 affords the dyes 7 and 8 in a 1 : 33 ratio. By contrast, the reactions carried out in the absence of that host give ratios of the products 7 and 8 ranging from 4 : 1 to 1 : 3, with most being approximately 1 : 1.

Any interpretation of the results shown in Table 1 must account for the particular effect of the cyclodextrin 4, which is not to produce a higher yield of indirubin (8), but to reduce sharply the amount of indigo (7) that forms. This decrease shows that most of the indoxyl anion (9) must be complexed by the cyclodextrin 4, and in an orientation that does not allow the oxidative dimerisation, which occurs in free solution to give indigo (7). However, the complexed anion 9 must still be able to react with isatin (11) to form indirubin (8), as it does in solution. It is reasonable to assume this involves isatin (11) that is complexed, since the more hydrophilic anion 9 is complexed under these conditions.[1] This assumption is supported by separate observations that the cyclodextrins 3–6 reduce the rate of degradation of isatin (11) at high pH, presumably by limiting hydrolysis through complexation.

The most probable orientation of the anion 9 in a cyclodextrin annulus is with the enolate portion protruding from the narrow end, delineated by the primary hydroxyl groups (Fig. 1). This is consistent with the antiparallel alignment of the dipole moments of the hosts and guests in cyclodextrin host–guest complexes.[9] It also accounts for the greatly reduced yields of indigo (7) from the reactions carried out in the presence of the dimer 4, compared to those involving any of the cyclodextrins 3, 5 or 6. With the orientation of the anion 9 shown in Fig. 1, the enolate is most shielded when complexed by the dimer 4, but in the opposite orientation; each of the cyclodextrins 3–6 would be expected to have a similar effect on the yield of indigo (7). Oxidative dimerisation of the complexed anion 9, to give indigo (7), is disfavoured by the dimer 4, presumably as a result of the unsuitable geometry of alignment of the cyclodextrin annuli. At the same time, the geometry of the cyclodextrin dimer 4 allows reaction of the anion 9 with complexed isatin (11) to give indirubin (8) (Fig. 1). Therefore, while the cyclodextrin 4 acts to limit the formation of indigo (7) rather than promote the production of indirubin (8), it is clearly acting as a template for the preassembly of reagents, to bias competing reactions of indoxyl anion (9).

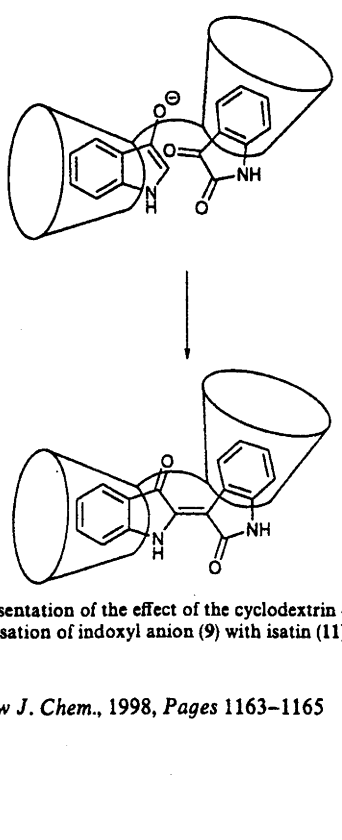

Fig. 1 Representation of the effect of the cyclodextrin 4 as a template for the condensation of indoxyl anion (9) with isatin (11)

Experimental

β-Cyclodextrin (3) was the generous gift of Nihon Shokuhin Kako Co., while the cyclodextrin dimers 4–6 were synthesized as previously reported.[5] All cyclodextrins were dried to constant weight *in vacuo* over phosphorus pentoxide prior to use. Indigo (7) and indoxyl acetate (10) were purchased from Sigma–Aldrich Chemical Company Inc. and isatin (11) was purchased from BDH Ltd. Indirubin (8) was prepared as described previously.[6] Borate buffers (pH 10.0 and pH 10.5, 0.1 mol dm^{-3}) and phosphate buffer (pH 11.0, 5.0×10^{-2} mol dm^{-3}) were prepared using standard procedures.[10]

In a typical procedure, indoxyl acetate (10) (1.7 mg, 9.7 μmol) and isatin (11) (8.5 mg, 59 μmol) were dissolved in 10.0 cm^3 of the appropriate buffer. Samples of this solution (0.10 cm^3) were immediately added to solutions containing either no cyclodextrin, β-cyclodextrin (3) (*ca.* 14 μmol) or one of species 4–6 (*ca.* 7.0 μmol), in 0.90 cm^3 of the appropriate buffer. The reactions at pH 10.0 and 10.5 were stirred for 16 h, then quenched with acid. The reactions at pH 11.0 were quenched after 15 min. The product mixtures were extracted with chloroform and the extracts were analysed using HPLC on an Alltech Econosil column (5 μm silica, 4.6 × 250 mm), eluting with acetone–chloroform (5 : 95) and monitoring at 550 nm using a detector calibrated with authentic samples. The results are shown in Table 1 and represent the average of replicate injections of products from at least two reactions. The product yields from replicate experiments differ by less than 15%.

Acknowledgements

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References

1 For a review see: S. F. Lincoln and C. J. Easton, in *Structural Diversity and Functional Versatility of Polysaccharides*, ed. S. Dumitriu, Marcel Dekker, New York, 1998, pp. 473–511.
2 For examples see: K. Fujita, S. Ejima and T. Imoto, *J. Chem. Soc., Chem. Commun.*, 1984, 1277; R. Breslow, N. Greenspoon, T. Guo and R. Zaryzycki, *J. Am. Chem. Soc.*, 1989, 111, 8296; R. C. Petter, C. T. Sikorski and D. Waldeck, *J. Am. Chem. Soc.*, 1991, 113, 2325; F. Venema, C. M. Baselier, E. van Dienst, B. H. M. Ruël, M. C. Feiters, J. F. J. Engenbersen, D. H. Reinhoudt and R. J. M. Nolte, *Tetrahedron Lett.*, 1994, 35, 1773.
3 R. Breslow and S. Chung, *J. Am. Chem. Soc.*, 1990, 112, 9659.
4 C. A. Haskard, C. J. Easton, B. L. May and S. F. Lincoln, *J. Phys. Chem.*, 1996, 100, 14457; C. A. Haskard, B. L. May, T. Kurucsev,

S. F. Lincoln and C. J. Easton, *J. Chem. Soc., Faraday Trans.*, 1997, **93**, 279.
5 J. H. Coates, C. J. Easton, S. J. van Eyk, S. F. Lincoln, B. L. May, C. B. Whalland and M. L. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2619; C. J. Easton, S. J. van Eyk, S. F. Lincoln, B. L. May, J. Papageorgiou and M. L. Williams, *Aust. J. Chem.*, 1997, **50**, 9.
6 G. A. Russel and G. Kaupp, *J. Am. Chem. Soc.*, 1969, **91**, 3851; A. Wahl and P. Bagard, *Bull. Soc. Chim. Fr.*, 1910, **7**, 1090.
7 R. J. Sundberg, *The Chemistry of Indoles*, Academic Press, New York, 1970.
8 W. C. Sumpter, J. L. Williams, P. H. Wilken and B. L. Willoughby, *J. Org. Chem.*, 1949, **14**, 713.
9 K. Harata, *Bull. Chem. Soc. Jpn.*, 1977, **50**, 1416.
10 *Handbook of Chemistry and Physics*, ed., R. C. Weast, CRC Press, Cleveland, 1977; *Biochemist's Handbook*, ed. C. Long, Spon, London, 1961.

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CYCLODEXTRINS AS MOLECULAR TEMPLATES

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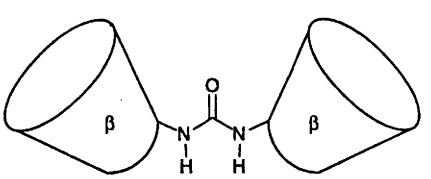
Abstract

The potential of cyclodextrins as molecular templates is demonstrated by the use of *N,N'*-bis(6^A-deoxy-β-cyclodextrin-6^A-yl)urea to bias competing reactions of indoxyl anion to give indigo and indirubin.

Breslow and Chung [1] demonstrated that the extent of cooperative guest binding by linked cyclodextrins [1-4] is dependent on the match of the shape of the guest to the relative orientations of the host cavities. Our studies [4] of the complexation of dyes by cyclodextrin dimers showed a preferred non-linear orientation of the cyclodextrin annuli in the urea **1**. This observation has now been exploited, using the urea **1** as a molecular template, in the formation of a non-linear product from reagents complexed in the cyclodextrin annuli.



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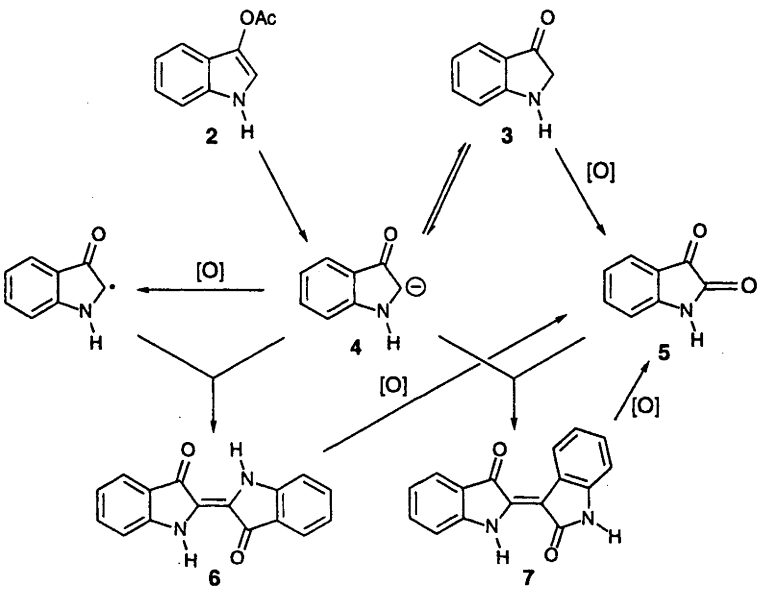

SCHEME 1.

Indigo **6** and indirubin **7** form competitively from the oxidative dimerisation of the indoxyl anion **4** and its condensation with isatin **5**, respectively (Scheme 1) [5]. Isatin **5** is formed in the reaction as an oxidation product of indoxyl **3**, indigo **6** and indirubin **7**. The effect of the cyclodextrin **1** (6.6×10^{-3} mol dm^{-3}) on these reactions was established when indoxyl anion **4** was generated *in situ*, through hydrolysis of the corresponding acetate **2** (9.7×10^{-5} mol dm^{-3}), at pH 10.0 and 298 K, in buffered aqueous solution containing isatin **5** (5.9×10^{-4} mol dm^{-3}). The cyclodextrin **1** sharply reduced the ratio of formation of indigo **6** and indirubin **7**, from 1:1 in the absence of a cyclodextrin, to 1:30 when the cyclodextrin **1** was used. The fact that very little indigo **6** forms shows that most of the indoxyl anion **4** must be complexed by the cyclodextrin **1**, in an orientation that does not allow oxidative dimerisation. However, the complexed anion **4** must still be able to react with isatin **5**, to form indirubin **7**. It is reasonable to assume this involves isatin **5** which is complexed, since the more hydrophilic anion **4** is complexed under these conditions [2]. The most probable orientation of the anion **4** in a cyclodextrin annulus is with

the enolate portion protruding from the narrow end (Figure 1). In this orientation, the enolate is most shielded when complexed by the dimer **1**. Oxidative dimerisation of the complexed anion **5**, to give indigo **6**, is therefore disfavoured by the dimer **1**, as a result of the unsuitable geometry of alignment of the cyclodextrin annuli. At the same time, the geometry of the cyclodextrin dimer **1** allows reaction of the anion **4** with complexed isatin **5**, to give indirubin **7** (Figure 1). Therefore, the cyclodextrin **1** serves to preassemble the reagents and act as a molecular template for the formation of indirubin **7**.

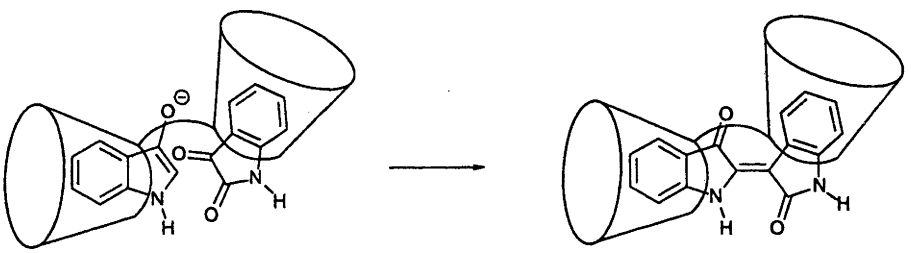

FIGURE 1. Effect of the cyclodextrin **1** as a molecular template in the formation of indirubin **7**.

References

1 For a review see: S. F. Lincoln and C. J. Easton, *Structural Diversity and Functional Versatility of Polysaccharides*, ed. S. Dumitriu, Marcel Dekker, Inc., New York, 1998, pp. 473-511.

2 For examples see: K. Fujita, S. Ejima and T. Imoto, *J. Chem. Soc., Chem. Commun.*, 1984, 1277; R. Breslow, N. Greenspoon, T. Guo and R. Zaryzycki, *J. Am. Chem. Soc.*, 1989, **111**, 8296; R. C. Petter, C. T. Sikorski and D. Waldeck, *J. Am. Chem. Soc.*, 1991, **113**, 2325; F. Venema, C. M. Baselier, E. van Dienst, B. H. M. Ruël, M. C. Feiters, J. F. J. Engenbersen, D. H. Reinhoudt and R. J. M. Nolte, *Tetrahedron Lett.*, 1994, **35**, 1773.

3 R. Breslow and S. Chung, *J. Am. Chem. Soc.*, 1990, **112**, 9659.

4 J. H. Coates, C. J. Easton, S. J. van Eyk, S. F. Lincoln, B. L. May, C. B. Whalland and M. L. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2619; C. A. Haskard, C. J. Easton, B. L. May and S. F. Lincoln, *J. Phys. Chem.*, 1996, **100**, 14457; C. A. Haskard, B. L. May, T. Kurucsev, S. F. Lincoln and C. J. Easton, *J. Chem. Soc., Faraday Trans.*, 1997, **93**, 279; C. J. Easton, S. J. van Eyk, S. F. Lincoln, B. L. May, J. Papageorgiou and M. L. Williams, *Aust. J. Chem.*, 1997, **50**, 9.

5 G. A. Russel and G. Kaupp, *J. Am. Chem. Soc.*, 1969, **91**, 3851; A. Wahl and P. Bagard, *Bull. Soc. Chim. Fr.*, 1910, **7**, 1090.