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ELECTRONIC SPECTRA OF THE ISOMERIC 4-BENZYLIDENE-2-PHENYL-Δ^2-OXAZOLIN-5-ONES AND THE PRODUCTS OF THEIR REACTION WITH NUCLEOPHILES INCLUDING α-CHYMOTRYPSIN

KINETICS OF THE HYDROLYSIS OF THE ISOMERIC ENZYME DERIVATIVES

K. BROCKLEHURST
Department of Biochemistry and Chemistry, St. Bartholomew's Hospital Medical College, University of London, Charterhouse Square, London, EC1M 6BQ

and

K. WILLIAMSON
Department of Chemistry and Biology, Manchester Polytechnic, All Saints, Manchester, M15 6BX

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Abstract—The 4-*cis*- and *trans*-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones have been prepared and evaluated as chromophoric reagents for the investigation of the active site of α-chymotrypsin. The UV spectra of the isomeric oxazolinones are discussed and compared with those of similar compounds, notably the 1,4-diphenyl-1,3-butadienes. A novel spectroscopic consequence of geometrical isomerism in conjugated chromophores is reported. The facile isomerization of the *cis*-oxazolinone has been investigated by spectrophotometry and product isolation. Both oxazolinones react rapidly with the enzyme to provide products whose UV spectra are consistent with their assignment as α-benzamido-cinnamoyl-enzymes. The uncertainties in these assignments resulting from the presence in the oxazolinones of multiple electrophilic centres are discussed. The pseudo first-order rate constants for the hydrolysis of the products of interaction of α-chymotrypsin with the isomeric oxazolinones were determined at 25·0°, I = 00·1 in the pH range 7–10·5. The pH-rate profile for the hydrolysis of the product formed by reaction of the *trans*-oxazolinone is consistent with this reaction being deacylation of α-benzamido-*trans*-cinnamoyl-α-chymotrypsin catalysed by the enzyme's electron relay system (p*Ka* 7·8, $\bar{k}_3 = 0{\cdot}159\ s^{-1}$). The pH rate profile for the hydrolysis of the product formed by reaction of the *cis*-oxazolinone is more complex. The profile could include a component catalysed by the relay system (p*Ka* approx 8, $\bar{k}_3 = 10^{-3}\ s^{-1}$) but the predominating reaction appears to be an unusually rapid reaction of the derivatized enzyme with hydroxide ion ($k = 233 \pm 10\ M^{-1}s^{-1}$). Possible interpretations of these pH-rate profiles are discussed.

The extensive work of Bender *et al* [1-8] on the α-chymotryptic hydrolysis of *trans*-cinnamoyl derivatives has contributed greatly to the elucidation of the molecular mechanism of action of this enzyme. The utility of cinnamoyl substrates and acylating agents lies in their possession of (a) structures closely related to that of β-phenyl-propionic acid, an important part of the specificity requirements for α-chymotrypsin[9-12] and (b) a conjugated system sensitive electronically to changes in substitution at the carbonyl C atom. The latter allows spectrophotometric observation of the acylation and deacylation steps of the catalysis and of the acyl-enzyme intermediate in which the cinnamoyl moiety is attached by an ester linkage to the oxygen atom of the active centre serine residue.[8]

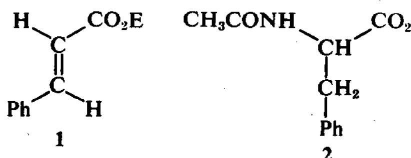
By allowing α-chymotrypsin to react with a cinnamoyl compound that possesses a good leaving group (usually N-*trans*-cinnamoylimidazole or a nitrophenyl-*trans*-cinnamate) Bender *et al* [1-8] prepared solutions of the acyl-enzyme, *trans*-cinnamoyl-α-chymotrypsin which is stable at low pH and whose subsequent deacylation at higher pH values may be followed spectrophotometrically.

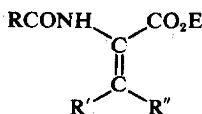
Whilst *trans*-cinnamoyl-α-chymotrypsin (1) is somewhat similar to the acyl-enzyme that would be formed from a "specific" (kinetically good) substrate, e.g. N-acetyl-L-phenylalanyl-α-chromotrypsin (2) these enzyme derivatives differ in a number of important respects. Firstly, N-acetyl-L-phenylalanyl-α-chymotrypsin is a trifunctional acyl-enzyme whose rate of deacylation is controlled by the binding of the N-acetylamino group to the ρ_1 site and of the β-phenyl group to the ρ_2 site of the enzyme which provides the correct orientation of the acyl group in the catalytic, ρ_3,

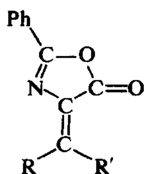
site. By contrast *trans*-cinnamoyl-α-chymotrypsin is at best only a bifunctional acyl-enzyme in which the β-phenyl group may bind at or near either the ρ_1 or the ρ_2 site but which lacks the N-acylamino substituent.

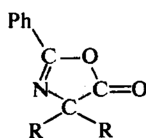
Secondly, the potential free rotation around the single bonds that link the β-phenyl group of **2** to the α-C atom is lacking in **1** which, on account of the rigid geometry associated with the olefinic double bond, will have its β-phenyl group constrained so that it may not be able to bind in the hydrophobic pocket[13] of the protein. Thirdly, the orientation of the acyl group in **1** may be affected by the different geometry (sp^2) of the α-carbon atom in **1** compared with that (sp^3) in **2**. One or more of these differences in the structures of **1** and **2** must be responsible for the fact that **2** undergoes deacylation about 10^4 times faster than **1**.[6]

To investigate the importance of two of the structural differences between **1** and **2**, namely the presence or absence of an α-N-acylamino group and the orientation of the β-phenyl group, it was decided to study the deacylation of α-N-acylamino-*cis*- and *trans*-cinnamoyl-α-chymotrypsins (**3** and **4**). This type of study complements those in which the sp^3 character of the α-C atom of the substrate is retained as in the 2,2′-bridged biphenyl analogue of benzoylphenylalanine methyl ester.[14] The synthesis of these acyl-α-chymotrypsins required the appropriate acylating agents of known configurations. Reactive esters and imidazolyl derivatives of α-N-acylamino-*cis* and *trans*-cinnamic acids have not been reported but one class of acylating agent




3: R′ = H, R″ = Ph, R = alkyl or aryl
4: R′ = Ph, R″ = H, R = alkyl or aryl


5: R = H, R′ = Ph
6: R = Ph, R′ = H


7: R = H
8: R = Me

capable of yielding α-N-acylamino cinnamoyl derivatives, the 4-benzylidene-Δ^2-oxazolin-5-ones, have been known for a long time. It was decided to use the 4-*cis*- and *trans*-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones **5** and **6** because there existed at least a tentative assignment of configuration[15] to these isomers. The stable isomer of this pair reported by Plöchl[16] in 1883 is the first oxazolinone to have been prepared. In spite of the fact that such compounds have been known for so long, they appear to have been neglected as chromophoric acylating agents for the investigation of enzyme mechanism. This is to the authors' knowledge, the first study of the use of unsaturated oxazolinones as enzyme acylating agents. Whilst this work was in progress, de Jersey *et al.*[17] reported a study of the hydrolysis of two saturated oxazolinones **7** and **8** by a number of hydrolytic enzymes.

This paper reports a kinetic and spectrophotometric study of the reactions of α-chymotrypsin with the two isomeric unsaturated oxazolinones **5** and **6**. Part of this work has been the subject of preliminary communications[18–20] and a study of the α-chymotryptic hydrolysis of the stable isomer of the 4-benzylidene-2-phenyl-Δ^2-oxazolin-5-one was reported subsequently by de Jersey and Zerner.[21,22]

RESULTS AND DISCUSSION

The configuration of the isomeric 4-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones and their derived solvolysis products

Assuming that configurational integrity is maintained in the hydrolysis of the oxazolinones (see below) it is now established that the stable oxazolinone, m.p. 165–166° has the configuration **6** in which the potential carboxyl function is *trans* to the phenyl group of the benzylidene moiety and the labile oxazolinone, m.p. 149·5–151·5° has the *cis*-configuration **5**.

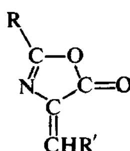
The first tentative assignment of configuration to these compounds was made by Buckles *et al.*[15] Arguing from very indirect evidence (m.p. comparisons) these authors assigned the *cis*-configuration to the stable oxazolinone and the *trans*-configuration to its labile isomer. These assignments have been the subject of some discussion[23–27] but most authors have assumed the assignment of Buckles *et al* to be correct. These assignments, however, were challenged by Morgenstern *et al*[27] who, on the basis of an NMR study of some related α-benzamidocinnamates and their corresponding oxazolinones, suggested that the original assignments of Buckles *et al* were incorrect.

The crystal structure[28] of the stable isomer of α-benzamidocinnamic acid shows that the cinnamic acid moiety of this compound has the *trans*-configuration and this confirms the assignments of Morgenstern *et al.* It is important to point out that the X-ray crystallographic study was carried out on the stable isomer of α-benzamidocinnamic acid,

m.p. 229°. In the report of the X-ray analysis[28] the m.p. of the stable isomer was incorrectly given as 199°. Since it is probable[25,29] that configurational integrity is maintained in the solvolysis of oxazolinones such as **5** and **6** the configuration of the stable isomer of α-benzamidocinnamic acid (*trans*) should be that also of the stable isomers of the oxazolinone and compounds derived from it by nucleophilic attack at C_2 or C_5 of the oxazolinone ring.

Electron absorption spectra of unsaturated oxazolinones

One property that makes unsaturated oxazolinones, particularly those containing aromatic substituents (see **9**), attractive as enzyme acylating agents, is their possession of an intense absorption



9: R and R′ = aromatic substituents

bend in the near UV or visible region of the spectrum. Since the first reports on absorption spectra of oxazolinones by Asahina[30,31] the spectra of a considerable number of unsaturated oxazolinones have been presented. The largest collection of such spectra is that of Bassi *et al*[32] who reported the spectra (above 255 nm) of 71 oxazolinones of type **9** in acetic acid. The spectra of all these oxazolinones were found to contain an intense absorption band with λ_{max} 361–430 nm and in some cases a second, less intense band with λ_{max} 256–310 nm. Although oxazolinones of type **9** exhibit geometrical isomerism, this phenomenon seems to have been neglected by most workers. For example, although the 71 oxazolinones studied by Bassi *et al* presumably exist in both *cis*- and *trans*-forms, spectra are reported only for one of each pair of isomers. These are presumably the spectra of the stable isomers whatever their configurations may be.

The only spectrophotometric study of a pair of isomeric oxazolinones **5** and **6** is that of Buckles *et al.*[15] These authors presented the spectra only graphically. Nevertheless, approximate absorption parameters may be obtained from their graphs and compared with other literature values for the stable isomer and with the values here reported for both isomers.

Electronic absorption spectrum of the stable oxazolinone, 4 - trans - *benzylidene - 2 - phenyl - Δ^2 - oxazolin- 5-one* (**6**)

The main features of the spectrum of the stable oxazolinone in 95% ethanol reported by Buckles *et al*[15] (λ_{max} 360 nm, ϵ_{max} $5{\cdot}5\times10^4\,M^{-1}cm^{-1}$; λ_{max} 260 nm, ϵ_{max} $2{\cdot}1\times10^4\,M^{-1}cm^{-1}$) are qualitatively similar to those of the spectra of this compound reported by other workers: Bassi *et al*[32] give $1{\cdot}56\times10^4\,M^{-1}cm^{-1}$; λ_{max} 259 nm, ϵ_{max} $1{\cdot}56\times10^4\,M^{-1}cm^{-1}$ (solvent acetic acid); λ_{max} 360 nm, ϵ_{max} $3{\cdot}87\times10^4\,M^{-1}cm^{-1}$; λ_{max} 259 nm, ϵ_{max} $1{\cdot}50\times10^4\,M^{-1}cm^{-1}$ (solvent absolute EtOH); Bennett and Hoerger[33] give λ_{max} 366 nm, ϵ_{max} $3{\cdot}9\times10^4\,M^{-1}cm^{-1}$ (solvent chloroform) λ_{max} 360 nm, ϵ_{max} $4{\cdot}2\times10^4\,M^{-1}cm^{-1}$ (solvent ether); this paper gives λ_{max} 360 nm, ϵ_{max} $3{\cdot}90\times10^4\,M^{-1}cm^{-1}$; λ_{max} 259 nm, ϵ_{max} $1{\cdot}49\times10^4\,M^{-1}cm^{-1}$ (solvent acetonitrile; see Table 1).

The spectrum of the stable oxazolinone reported by Buckles *et al*[15] requires further comment because it appears to lack the definition of fine structure which in our hands and in contrast to the findings of Buckles *et al* is one of the main features that distinguishes the spectrum of **6** from that of its *cis*-isomer (**5**), the labile oxazolinone. The spectrum of the stable oxazolinone reported by Buckles *et al* shows some fine structure in the most intense band centre at 360 nm. The fine structure consists of an ill-defined shoulder at approx 380 nm and a somewhat better defined shoulder at approx 340 nm but no defined maxima. The band with λ_{max} 260 nm shows a second maximum at approx 240 nm. By contrast, both Bassi *et al*[32] and Bennett and Hoerger[33] report a clearly defined additional maximum at approx 378–379 nm in the most intense band and a clearly defined shoulder at approx 345–346 nm. These authors report also two extra maxima at 246 nm and 240 nm in the short wavelength band. In our hands the spectrum of **6** in acetonitrile (Fig 1) consists of the bands given in Table 1.

These data are more extensive than those reported previously but the parameters that characterise the main features of the bands are in good agreement with most of the reported values and the shape of the spectrum closely resembles that presented by Bennett and Hoerger.[33]

Electronic absorption spectrum of the labile oxazolinone, 4-cis-benzylidene-2-phenyl-Δ^2-oxazolin-5 one (**5**)

Whereas the spectrum of **6** shown in Fig 1 resembles at least superficially that reported by Buckles *et al*[15] the spectrum of the labile oxazolinone here reported in Fig 1 differs considerably from that reported by these authors. They show the most intense band of the spectrum of the labile oxazolinone to have in 95% ethanol λ_{max} 360 nm and ϵ_{max} $1{\cdot}8\times10^4\,M^{-1}cm^{-1}$, i.e. this band is approx only one-third as intense as the 360 nm band of the stable oxazolinone. No fine structure is shown in this long wavelength band. The lower wavelength band is characterized by λ_{max} 260 nm, ϵ_{max} $9\times10^3\,M^{-1}cm^{-1}$.

In marked contrast to these data, we find that the spectrum of **5** obtained using a soln in acetonitrile freshly prepared from a sample of **5** having m.p.

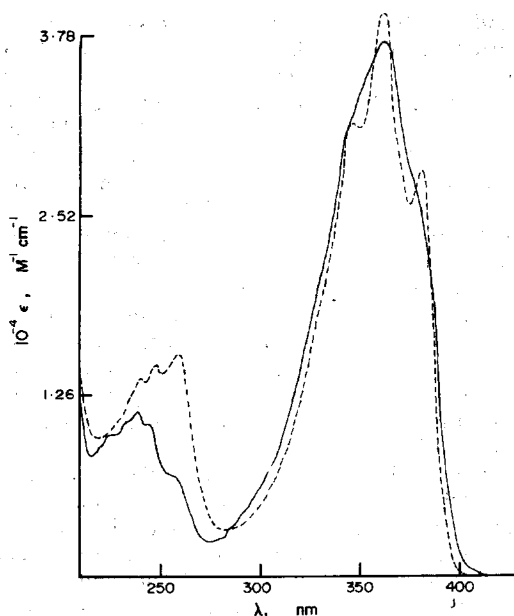


Fig 1. Electronic absorption spectra of the 4-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones in acetonitrile. --------, *trans* (stable)-isomer; ———, *cis* (labile) isomer.

Table 1. Electronic absorption parameters of 4-*cis* and *trans*-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones in acetonitrile

The oxazolinone concentrations were 24·6 μM and the spectra were scanned on the 0–1·0 slidewire of a Cary 15 spectrophotometer; the wavelengths and extinction coefficients refer to maxima except where shoulders are indicated (sh). These spectra are presented graphically in Fig 1.

trans		*cis*	
λ, nm	$10^{-4}\epsilon_{max}M^{-1}cm^{-1}$	λ,nm	$10^{-4}\epsilon_{max}M^{-1}cm^{-1}$
380	2·74	380 sh	2·46
361	3·90	361	3·62
347	3·06	350 sh	3·28
328 sh	1·76	328 sh	1·87
259	1·49	255 sh	0·69
247	1·41	245	1·02
239	1·35	238	1·11
234 sh	1·14	234 sh	1·02
226	0·98	226	0·96

149·5–151·5° is as shown in Fig 1. The absorption parameters are given in Table 1. Whereas according to Buckles *et al*[15] the main difference in the spectra of **5** and **6** is in the intensities of both the 360 nm and 260 nm bands, we find that the main differences are in the intensities of the fine structure maxima in the short wavelength band and in the fine structure content of the long wavelength band. The extinction coefficient of the central maximum of the long wavelength (361 nm) band of **5** is 93% of that of **6**.

Experiments on the isomerization of **5** and **6** (*vide infra*) show that the much greater intensity of the 361 nm band of **5** here reported compared with that reported by Buckles *et al*[15] is not an artifact arising from contamination of our sample of **5** with **6**. We cannot account for the low intensity of the 360 nm band of **5** reported by Buckles *et al.*[15] Bennett and Hoerger[33] pointed out that spectra of oxazolinones in ethanol tend to be unreliable because ethanolysis occurs but this would not account for the shape and intensity of the spectrum reported by Buckles *et al.*[15]

*The electronic spectra of the isomeric 4-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones **5** and **6***

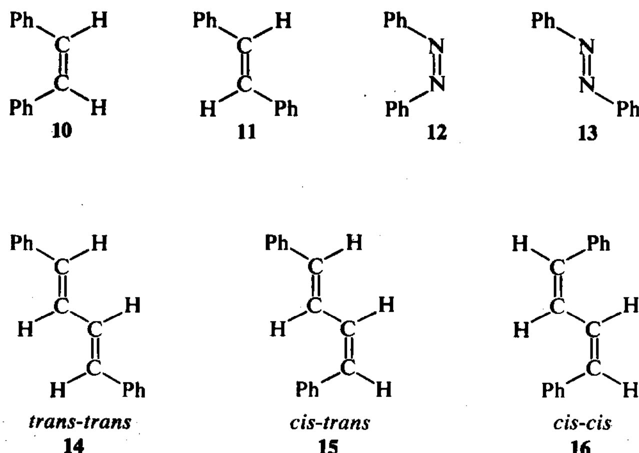
Contrary to the earlier report by Buckles *et al*,[15] the main difference in the long wavelength bands of the spectra of **5** and **6** lies in the fine structure contents of the bands and not in their intensities.

Geometrical isomers that possess conjugated systems may frequently be distinguished by large differences in their electronic spectra. These differences usually arise from steric effects and are generally evaluated in terms of the intensity of the band and its wavelength of maximum absorption.[34]

In the case of the oxazolinones **5** and **6** the long wavelength absorption bands centred at 361 nm probably arise from the lengthwise polarization of the conjugated system linking the two phenyl groups.[33] Both the λ_{max} values and the intensities of these bands are insensitive to the geometrical isomerism of the benzylidene moiety (Fig 1). There is, however, another spectroscopic consequence that can result from geometrical changes in molecules which sometimes accompanies the other sterically induced changes in the absorption parameters, namely a change in the vibrational fine structure content of the band. This is apparent in the spectra of **10** and **11**[35,36] and of **12** and **13**.[37]

The compounds most closely related to the oxazolinones **5** and **6** whose spectra have been studied in any sort of detail are the isomeric 1,4-diphenyl-1,3-butadienes **14–16**. There is considerable theoretical and biological interest in structure-spectra relationships in aromatic polyenes. The isomeric diphenylbutadienes may be considered as model compounds for the photochemical processes occurring in the carotenes, retinenes and vitamin A. The *trans-trans* isomer (**14**) is structurally somewhat similar to the *cis*-oxazolinone (**5**) and the *cis-trans* isomer (**15**) is similar to the *trans*-oxazolinone (**6**).

The UV spectrum of 1,4-diphenyl-1,3-butadiene has been reported by a large number of workers, notably by Pinckard *et al*,[38] Dale[39] and Wettermark and Schor.[40] The main features of the fundamental bands of the isomeric diphenyl butadienes in hexane are: λ_{max}: *trans-trans*, 328 nm; *cis-trans*,



313 nm; *cis-cis*, 299 nm; relative intensities *trans-trans*: *cis*: *trans*: *cis-cis* = 1·0:0·7:0·7; while the *trans-trans* isomer exhibits well defined vibrational structure in the fundamental band, the other two isomers are characterized by essentially continuous spectra.

It is of interest that the shape of the spectrum of 14 is strikingly similar to that of the *trans*-oxazolinone (6), both showing similar types of fine structure in their fundamental bands. However, whereas the isomerization of 14 results not only in loss of the fine structure content of the band but also in a large fall in intensity and shift to shorter wavelengths, isomerization of 6 results only in the loss of the vibrational fine structure.

Wettermark and Schor[40] have carried out LCAO-SCF-CI semiempirical computations using the results of energy calculations for the three isomers of the diphenylbutadienes. On the basis of these they obtained a good overall description of the absorption spectra but were not in a position to do more than speculate about the presence or lack of vibrational fine structure in the bands.

Isomerization of 4 - cis - *benzylidene - 2 - phenyl - oxazolin-5-one to the* trans *isomer*

Although the spectrum of the *cis*-oxazolinone (5) was that given in Fig 1 when the soln of 5 in acetonitrile was prepared immediately before the spectrum is recorded, a spectrum recorded using a soln that was several days old exhibited the fine structure in the 361 nm band characteristic of the *trans*-oxazolinone (6). Accordingly, we undertook a more careful study of the ageing of 5 in acetonitrile soln to ascertain whether isomerization to the *trans*-isomer was occurring in soln and if so how rapidly. The spectrum of an acetonitrile soln of the *cis*-oxazolinone that had been allowed to stand for 4–5 days in a glass flask in a dark cupboard was virtually identical to that of a soln of the *trans*-oxazolinone at equivalent concentration. That these spectral changes result from isomerization of 5 to 6 is further suggested by the melting range of the product obtained by allowing the acetonitrile to evaporate from an "aged" soln of 5 (Table 2). Confirmation of the isomerization is obtained from (i) the IR spectra of 5 and (ii) the rates of hydrolysis of the products of the reaction of α-chymotrypsin with fresh and aged solns of 5 (*vide-infra*). The IR spectra of 5 and 6 differ markedly in several regions (Table 3). The product obtained by rapid evaporation of the solvent from a freshly prepared soln of 5 in acetonitrile had an IR spectrum closely similar to a sample of 5 freshly mulled in Nujol. The product obtained by evaporation of the solvent from a soln of 5 in acetonitrile that had been allowed to stand at room temp (approx 22°) for 5 days in the dark, however, had an IR spectrum closely similar to that of 6.

The progress curves for the *cis* → *trans* isomerization in the dark and in the light as measured by the accompanying increase in extinction at 260 nm (Experimental) are shown in Fig 2. In the light the isomerization appears to reach an equilibrium position (60% of *trans* and 40% of *cis*). The adherance of this process to first order kinetics is demonstrated in Fig 3. In the dark the isomerization is very much slower (Fig 2) but the product consists almost entirely of the *trans*-isomer. When the 60%/40% mixture of isomers is kept in the dark, isomerization continues to yield again a product consisting essentially of only the *trans*-isomer. When the soln of the *trans*-isomer obtained in the dark isomerization is kept in the light the extinction at 260 nm falls rapidly to that of the 60%/40% isomer mixture obtained in the light isomerization experiment. These constant extinction values reached in the dark and light experiments presumably represent the establishment of different thermal and photochemical equilibrium positions.

That the extinction changes shown in Fig 2 are in accord with those predicted from the spectra of the *cis*- and *trans*- oxazolinones shown in Fig 1 supports the claim made above that the spectrum of the

Table 2. Melting ranges of 4-*cis* and *trans*-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones

	Product	Melting Range °C
(i)	Stable (*trans*) isomer	165–166
(ii)	Labile (*cis*) isomer*	149·5–151·5
(iii)	Synthetic mixture of equal weights of (i) and (ii)	125–135
(iv)	Solid obtained by evaporation of the solvent from a solution of (i) in acetonitrile after it had been allowed to stand at room temp for 5 days	163–166
(v)	Solid obtained by evaporation of the solvent from a solution of (ii) in acetonitrile after it had been allowed to stand at room temp for 5 days*	127–129

*The m.p. of the labile isomer appears to be very sensitive to contamination of the compound; its admixture with only small quantities of impurities causes the m.p. to fall by *ca* 20° although it remains reasonably sharp.

Table 3. IR spectra of 4-*cis* and *trans*-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones
Intensity: w, weak; m, medium; s, strong

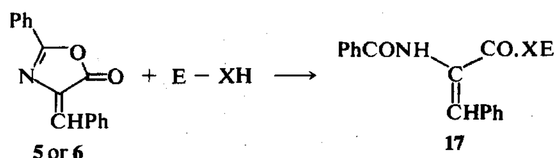
Frequency, cm^{-1} Stable isomer (*trans*)	Labile isomer (*cis*)	Remarks
3000 w	3000 w	
1780 s	1780 m	major difference
1760 m	1760 s	
1640 s	1640 s	
1590 w	1590 w	
	1568 w	
1550 w	1550 w	
1490 m	1490 m	
1450 m	1450 m	
1365 m	1365 m	
1320 m	1320 m	
—	1300 w	
1290 m	1290 m	
1220 m	1220 m	
1160 m	1150 m	
—	1115 m	
1100 w	1100 w	
1090 w	—	
1070 w	1070 w	
1030 w	1040 w	
980 m	1010 s	major difference
—	945 m	
890 w	—	
870 m	900 s	major difference
—	795 m	doublet; major difference
780 w	780 m	
765 m	760 m	
750 w	—	
700 s	700 s	
680 s	680 m	
670 w	670 w	

Spectra were determined as freshly prepared nujol mulls.

cis-oxazolinone here reported is a much close approximation to a correct spectrum than that reported by Buckles *et al.*[15]

Reaction of α-chymotrypsin with the isomeric 4-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones

It was pointed out in the Introduction that a necessary consequence of providing both a cinnamoyl chromophore and an α-N-acylamino substituent in an acyl-enzyme is that the acylating agent be a benzylidene-Δ^2-oxazolin-5-one. One practical advantage of this is that, particularly if the substituent on C_2 of the oxazolinone is aromatic, the oxazolinone absorbs strongly in the near UV, well separated from the region in which either the protein or N-acylamino-cinnamoyl-enzymes would be expected to absorb. The 361 nm absorption bands of **5** and **6** probably arise from lengthwise polarization of the conjugated system linking the two phenyl groups in **5** and **6**. Ring opening of **5** and **6** by the reaction of a nucleophilic centre, e.g. the OH group of serine-195 in α-chymotrypsin, at C_5 of the oxazolinone would destroy this conjugated system. The product of this reaction, the acyl-enzyme **17** (Scheme 1) would possess the substituted cinnamoyl chromophore which would be expected to absorb maximally around 300 nm.



X = a nucleophilic atom, e.g. the oxygen atom of serine-195.

SCHEME 1

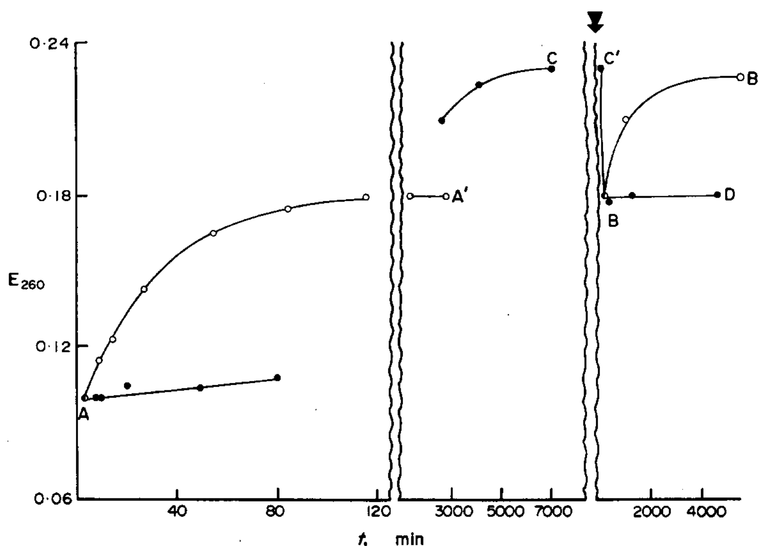


Fig 2. Extinction changes at 260 nm consequent upon the interconversion of the *cis*- and *trans*-isomers of 4-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones in acetonitrile. Temperature, approx. 26°. A—A' (open circles), partial conversion of *cis*- to *trans*-isomer in the light; B—B', isomerization of residual *cis*-isomer at A' to *trans*-isomer in the dark; A—C (closed circles), conversion of *cis*- to *trans*-isomer in the dark; C'—D (closed circles) extinction change observed on illumination of solution at C; the arrow indicates change of illumination: solution from the dark experiment transferred to the light and *vice versa*; for the experimental details, see text.

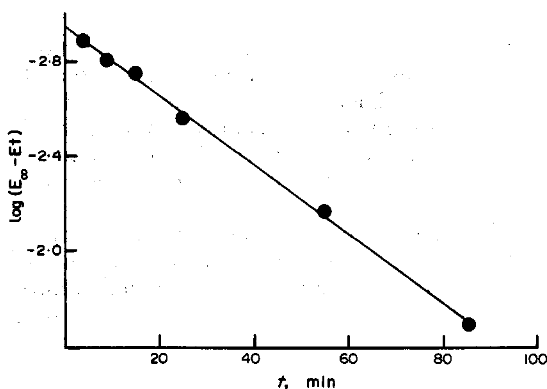


Fig 3. Demonstration of the adherence to first order kinetics by the isomerization of the 4-*cis*-benzylidene-2-phenyl-Δ^2-oxazolin-5-one to its *trans*-isomer in acetonitrile in the light. Temperature, approx. 26°, extinction—time data at 260 nm taken from Fig 2 (A—A'); first order rate constant $5{\cdot}6\times10^{-4}\,s^{-1}$. For other experimental details, see text.

When solns of the oxazolinones **5** and **6** in acetonitrile were added to separate samples of a 5–10 fold molar excess of α-chymotrypsin in sodium acetate buffer pH 5·5 containing approx 10% acetonitrile and absorption spectra were recorded against blanks containing equivalent concentrations of α-chymotrypsin, the 361 nm bands of the oxazolinones had disappeared within the 15 s required for mixing the solns and had been replaced by new absorption bands at shorter wavelengths. The product formed from the *cis*-oxazolinone (**5**) is characterised by λ_{max} 308 nm, ϵ_{max} $2{\cdot}07\times10^4\,M^{-1}cm^{-1}$ and that formed from the *trans*-oxazolinone (**6**) by λ_{max} 302 nm, ϵ_{max} $1{\cdot}75\times10^4\,M^{-1}cm^{-1}$ (Fig 4 and Table 4). These absorption bands did not change either in shape or intensity during at least 10 min at this pH. This stability was expected from the known pH-rate profiles of the deacylation of other acyl-α-chymotrypsins.[8]

Both the positions and intensities of the absorption bands of the products of interaction of α-chymotrypsin with the two oxazolinones are in accord with these products being α-benzamido-*cis*- and *trans*-cinnamoyl-α-chymotrypsins (Scheme 1; for a comparison of these parameters with those of reference compounds, see Table 4). Unfortunately, the values of the absorption parameters of the products of the reactions of α-chymotrypsin with the oxazolinones cannot be regarded as definitive evidence that the products are in fact α-benzamido-cinnamoyl-α-chymotrypsins. The uncertainty arises because of the presence in the benzylidene-Δ^2-oxazolin-5-ones of two other electrophilic centres, in addition to the one at which reaction would give rise to acyl enzymes, i.e. C_2 of the oxazolinone ring and the β-carbon atom of the benzylidene moiety. Reaction of nucleophiles at C_2 of an oxazolinone ring is not unprecedented. This is the electrophilic centre in 4,4-dimethyl oxazolin-5-one that is attacked by the hindered nucleophile,

Table 4. Electronic absorption parameters of α-benzamido-*cis*- and *trans*-cinnamoyl-α-chymotrypsins and reference compounds

Compound	Solvent	λ_{max}, nm	$10^{-4}\epsilon_{max}$, $M^{-1}cm^{-1}$	Reference
α-Benzamido-*trans*-cinnamoyl-α chymotrypsin*	sodium acetate buffer pH 5·50 10% CH_3CN	302	1·75	(i)
α-Benzamido-*cis*-cinnamoyl-α-chymotrypsin*†	sodium acetate buffer pH 5·50 10% CH_3CN	308	2·07	(i)
trans-Cinnamoyl-α-chymotrypsin*	sodium acetate buffer pH 4·28 1·6% CH_3CN	292	1·77	(ii)
O-*trans*-Cinnamoyl-N-acetyl-serinamide	10% CH_3CN	281·5	2·43	(ii)
Methyl-*trans*-cinnamate	3% CH_3CN	279·5	2·21	(ii)
Ethyl-*trans*-cinnamate	95% ethanol	277	241	(iii)
Ethyl-*cis*-cinnamate	95% ethanol	270	1·17	(iii)
Methyl-α-benzamido *cis*-cinnamate	CH_3CN	290	2·2	(i)
Methyl-α-benzamido *trans*-cinnamate	CH_3CN	280	2·2	(i)
α-Benzamido-*cis*-cinnamic acid	ethanol	approx. 300	approx. 1·7	(iv)
	CH_3CN	302	1·78	(i)
α-Benzamido-*trans*-cinnamic acid	ethanol	approx. 280	approx. 1·4	(iv)
	CH_3CN	278	1·44	(i)

*Difference spectra versus α-chymotrypsin.
†The product of the reaction of α-chymotrypsin with the *cis*-oxazolinone, see text.
References: (i) this work; (ii) Refs 1 and 2; (iii) Ref 48; (iv) Ref 15.

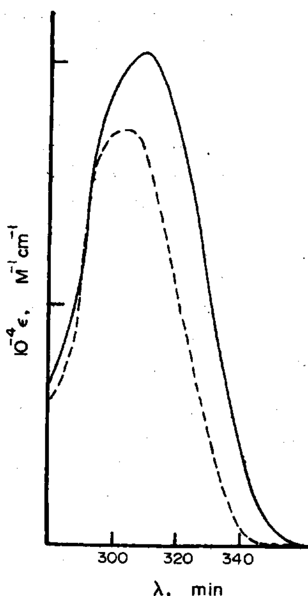
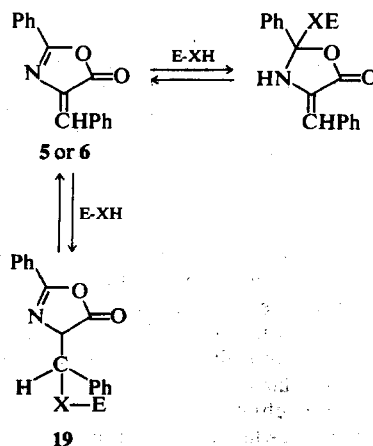


Fig 4. Electronic absorption spectra of the products of the reaction of α-chymotrypsin with the isomeric 4-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones at pH 5·5 which are probably α-benzamido-*cis*- and *trans*- cinnamoyl α-chymotrypsins (see text). Difference spectra *vs* α-chymotrypsin; sodium acetate buffer I = 0·1 containing 10% CH_3CN; ----- *trans*-isomer; ——— *cis*-isomer.

methyl α-amino isobutyrate.[41] Although reactions of nucleophiles at the β-carbon atom of the alkylidene or arylidene moiety of unsaturated oxazolinones have not been reported, such an attack resulting in the well-known 1:4-addition reaction to α,β-unsaturated carbonyl compounds can be envisaged.

The simplest products that could result from reaction of a nucleophilic centre, X, in α-chymotrypsin with these other electrophilic centre in the oxazolinones are shown in Scheme 2. It is



SCHEME 2

necessary to consider the spectra of the products of the reactions in Scheme 2 in addition to that of the reaction in Scheme 1, because of an unusual feature of the pH-rate profile for the hydrolysis of the product of the reaction of α-chymotrypsin with the *cis*-oxazolinone **5** (*vide infra*). Although hydrolysis and methanolysis of **5** and **6** probably proceed by nucleophilic attack at C_5 because they provide the corresponding α-benzamido cinnamic acids and methyl esters, reaction with the enzyme may be directed to one of the other electrophilic centres as a result of the nature of the binding in the adsorptive complex.

The data in Table 4 show that if the products of the reactions of α-chymotrypsin with **5** and **6** are the acyl-enzymes (**17**), then both acyl-enzyme spectra exhibit the red shift relative to the spectra of model esters (in this case approx 20 nm) reported previously[42] for other chromophoric acyl-α-chymotrypsins. A consequence of generating the chromophoric enzyme derivatives *in situ* using an excess of the enzyme is that the calculations of the extinction coefficients of the enzyme derivatives use as their concentrations the initial concentrations of the oxazolinones. This would be invalid if the oxazolinones were undergoing a competing reaction such as aqueous hydrolysis. It was ascertained that under the conditions in which these enzyme derivatives were generated, the concentration of the enzyme was sufficient to ensure that the rate of aqueous hydrolysis of the oxazolinones was negligible compared to the rate of the enzyme reactions as assessed in each case by the fall in extinction at 360 nm.

The products of reaction of the enzyme at C_2 of the oxazolinones (compounds **18** in Scheme 2) are cinnamoyl esters that are constrained to s-*cis* configurations by the cyclic structures. The spectra of such s-*cis* cinnamoyl esters would be expected to exhibit the large red shifts relative to the spectra of simple cinnamoyl esters which exist mainly in the s-*trans* form.[42] As a result the compounds **11** might be expected to absorb maximally at about the wavelengths found for the products of the reactions of α-chymotrypsin with the oxazolinones (Fig 4 and Table 4), although it is difficult to predict the effect on λ_{max} of the secondary amino substituent which may be protonated at pH values approx 5·5. Consideration of the extinction coefficients of these products, however, does not lend strong support to their assignment as the adducts (**18**). The most characteristic spectral property of s-*cis* conformers is that their extinction coefficients (ϵ_{max}) are generally much smaller than (often only approx 50%) those of the corresponding s-*trans* conformers.[42,43] This generalization is supported by quantum mechanical calculations.[44] The values of ϵ_{max} of the products of the reaction of α-chymotrypsin with **5** and **6** are only about 15% lower than that of *trans*-cinnamoyl-α-chymotrypsin and about 20% lower than those of the isomeric methyl-α-benzamido cinnamates (Table 4). Assignment of the adduct structure **18** to the product of the reaction of α-chymotrypsin with the *cis*-oxazolinone (which is the product whose hydrolysis is characterized by the unusual pH-rate profile *vide infra*) is made even less likely because its ϵ_{max} value is 18% higher than that of the product derived from the *trans*-oxazolinone (Table 4). The last three compounds probably exist mainly in s-*trans* conformations.

The products (**19** in Scheme 2) of 1:4-addition of a group in the enzyme to the cinnamoyl moieties of **5** and **6** would be 2-phenyl-5-hydroxy-oxazoles. The UV spectra of these compounds have not been reported and it is not possible therefore to definitely eliminate the compounds **19** as products of the reaction of α-chymotrypsin with **5** and **6** on the basis of the spectra. The longest wavelength absorption band of 5-phenyloxazole occurs at 267 nm (ϵ_{max} 19370 in MeOH) and extension of the conjugated system by another phenyl group (at C_5) i.e. 2,5-diphenyl-oxazole, shifts the absorption band to 314 nm (ϵ_{max} 22080 in MeOH).[45] Extension of a 2-phenyloxazole by the presence of the lone pair of electrons of a hydroxyl substituent at C_5 (as in **19**) would be unlikely to shift the absorption band of 2-phenyloxazole up to 302–308 nm, although the effect on the spectrum of the solvent environment and microenvironment of **19** in the enzyme are unknown quantities.

In summary then, the UV spectra of the products of the reaction of α-chymotrypsin with **5** and **6** suggest that these compounds are probably α-benzamido-cinnamoyl-α-chymotrypsins (**17**). On the other hand, the probable spectra of **18** and possibly also those of **19** may be sufficiently similar to those recorded in Fig 4 that there must still be residual doubts about the assignment of the acyl-enzyme structure to the products.

Kinetics of the hydrolysis of the products of the reaction of α-chymotrypsin with the oxazolinones **5** *and* **6**

When α-chymotrypsin was allowed to react with **5** and with **6** at pH values $> 5{\cdot}5$, where it was predicted that deacylation of acyl-α-chymotrypsins would be relatively rapid, it was possible to follow the progress of the subsequent hydrolysis spectrophotometrically at 310 nm. Using [E] > [oxazolinone] the rapid fall in extinction at 361 nm which is accompanied by an increase in extinction at 310 nm is followed by a relatively slow fall in extinction at 310 nm. This fall in extinction results from a shift to shorter wavelengths of the 302 or 308 nm absorption band of the enzyme derivative as it undergoes hydrolysis to the corresponding α-benzamido-cinnamate (Table 4). Typical progress curves for these hydrolyses are shown in Fig 5 and their adherence to first order kinetics is demonstrated in Fig 6.

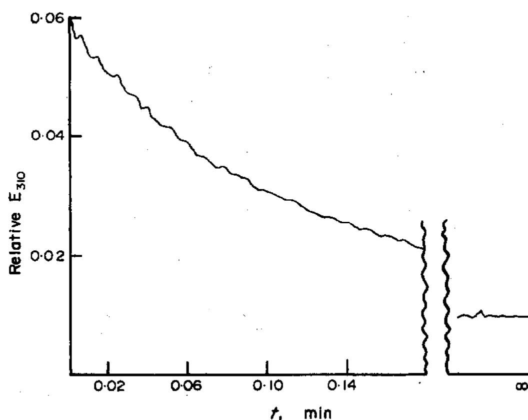


Fig 5a.

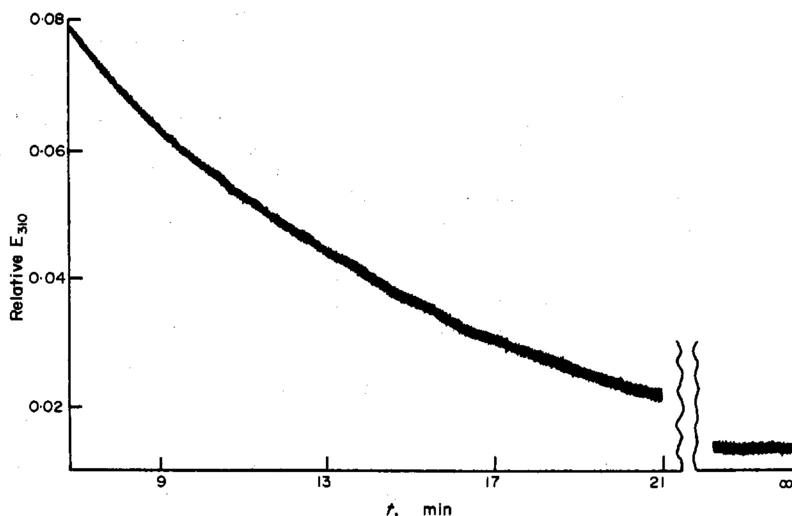


Fig 5b.

Fig 5. Progress curves for the hydrolysis (deacylation) of the isomeric "α-benzamido-cinnamoyl-α-chymotrypsins". (a), *trans*-isomer; (b), *cis*-isomer (for a discussion of the possible doubts about the assignment of these compounds as acyl enzymes, see text); sodium borate buffer, pH 8·50, I = 0·1 containing 10% CH_3CN; 25·0°.

When an acetonitrile soln of the *cis*-oxazolinone (**5**) which had been allowed to stand for 4–5 days was used to generate the enzyme derivative, the first order rate constant for its hydrolysis was found to be very different from that obtained using a freshly prepared soln of **5** and identical with that obtained using a soln of the *trans*-oxazolinone (**6**). This finding adds further support to the spectroscopic and melting point evidence that isomerization of **5** and **6** occurs in acetonitrile solns (*vide ante*).

The pH-rate profiles for the hydrolysis of the enzyme derivatives formed from α-chymotrypsin and the oxazolinones **5** and **6** are presented in Fig 7.

The profile for the hydrolysis of the product formed from the *trans*-oxazolinone (**6**) is of the form expected for the deacylation of an acyl-α-chymotrypsin catalysed by the enzyme's "electron relay system".[46] If this hydrolysis does represent deacylation of α-benzamido *trans*-cinnamoyl α-chymotrypsin, the pH-independent deacylation rate constant (159×10^{-3} s^{-1}) is about an order of magnitude greater than the corresponding rate constant for the deacylation of *trans*-cinnamoyl-α-chymotrypsin ($12 \cdot 5 \times 10^{-3}$ s^{-1}).[1] The presence of the N-acylamino group also appears to result in an increase in the apparent p*Ka* of the relay system from 7·15 (in *trans*-cinnamoyl-α-chymotrypsin) to 7·8. The pH rate profie for what is probably the deacylation of α-benzamido-

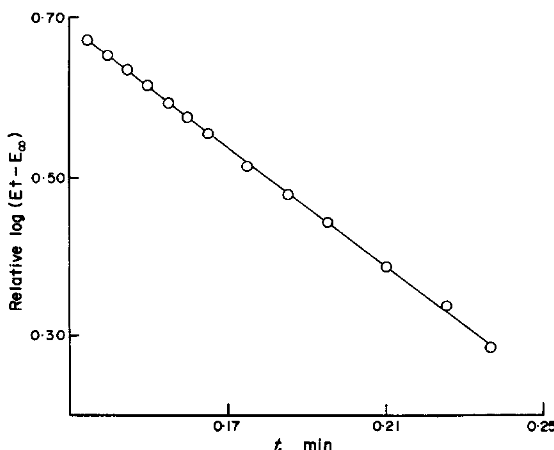


Fig 6a.

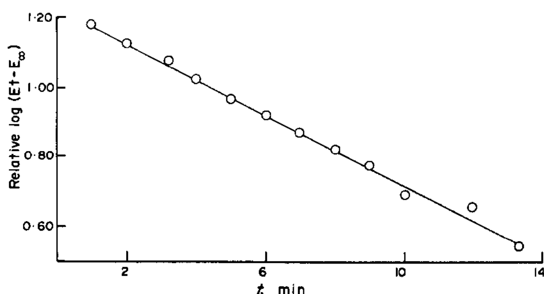


Fig 6b.

Fig 6. Demonstration of the adherance to first order kinetics by the deacylation of the isomeric "α-benzamido-cinnamoyl-α-chymotrypsins" (see also Fig 5 legend). (a), *trans*-isomer; (b), *cis*-isomer; the points were taken from continuous traces of E_{310} *vs* time recorded on the 0–0·1 extinction slidewire of a Cary 15 Spectrophotometer (Fig 5); sodium borate buffer pH 8·50, I = 0·1, containing 10% CH_3CN; 25·0°. Rate constants: (a) $k = 0{\cdot}14\ s^{-1}$; (b) $k = 0{\cdot}002\ s^{-1}$.

trans-cinnamoyl-α-chymotrypsin is very different from that for the hydrolysis of the product of the reaction of α-chymotrypsin with the *cis*-oxazolinone **5** ("α-benzamido-*cis*-cinnamoyl-α-chymotrypsin"). The latter reaction is very much slower in the pH range 7–10 (Fig 7).

A plot of the observed first order rate constant for this reaction against hydroxide ion concentration for the data in the pH range 8–10 (Fig 8) is linear. This suggests that either the hydrolysis of the derivatized enzyme is effected by hydroxide ion with second order rate constant = $233 \pm 10\ M^{-1}s^{-1}$ or that the hydrolysis depends formally on the concentration of a basic centre whose conjugate acid has p*Ka* > 10. A double-log plot of the data in the pH range 7–10 (Fig 9) suggests that the hydrolysis of the "α-benzamido-*cis*-cinnamoyl-α-chymotrypsin" may be complex and may involve not only a hydroxide ion reaction but also a contribution from a reaction dependent upon the base form of a group with p*Ka* about 8 and possibly also another dependent upon an ionization at higher pH values.

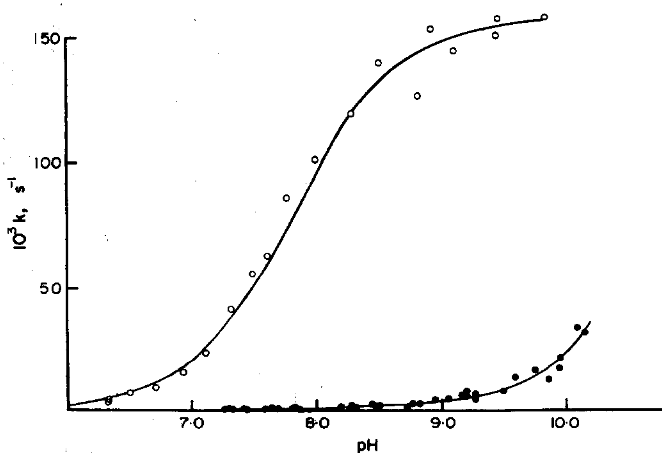


Fig 7. pH-Rate profiles for the hydrolysis of the isomeric "α-benzamido-cinnamoyl-α-chymotrypsins". First order rate constants were determined as described in the text. Buffers: potassium phosphate, sodium borate, sodium bicarbonate, each containing 10% CH_3CN; 25·0°, I = 0·1. Points are experimental for ○, the *trans*-isomer and ●, the *cis*-isomer and the lines theoretical for *trans*: $k = \bar{k}/(1 + [H^+]/Ka)$ in which $\bar{k} = 0{\cdot}159\ s^{-1}$ and pKa = 7·8 and for *cis*; $k = \{\bar{k}/(1 + [H^+]/Ka)\} + k'[HO^-]$ in which $\bar{k} = 0{\cdot}001\ s^{-1}$, $k' = 223\ M^{-1}\ s^{-1}$ and pKa = 8.

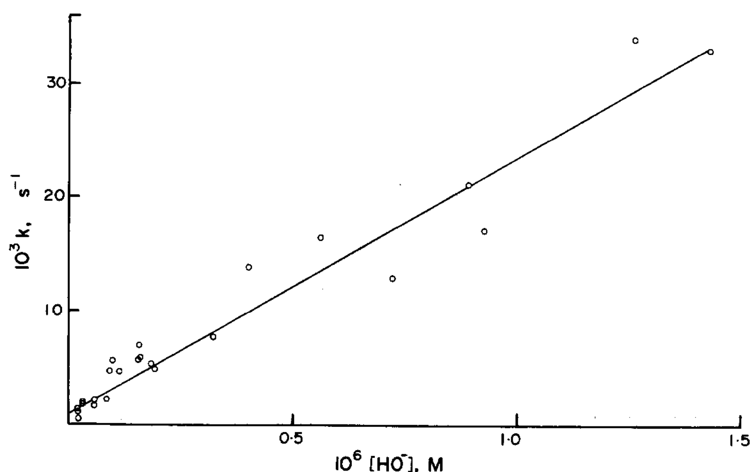


Fig 8. Log plot of the data in Fig 7 for the hydrolysis of the *cis*-isomer in the pH range 8–10. Experimental details are as given in Fig 7 legend; the line is the linear regression line and corresponds to a second order rate constant for the presumed hydroxide ion reactions (k') of 223 $M^{-1}s^{-1}$.

The reaction dependent upon the base form of a group of p*Ka* about 8 suggested by Fig 9 could correspond to hydrolysis by the enzyme's "electron relay system". If this is so, the data in Figs 7 and 9

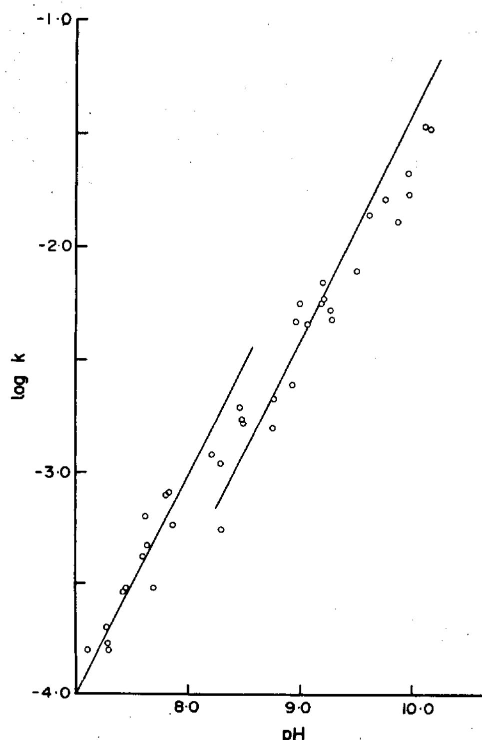


Fig 9. Double log plot of hydrolysis data in Fig 7 for the hydrolysis of the *cis*-isomer in the pH range 7–10. Experimental details are as given in Fig 7 legend; the lines are of slope + 1.

show that deacylation of the *trans*-acyl enzyme occurs about 150 times faster than that of the *cis*-acyl enzyme. This ratio of rate constants is somewhat lower than that found[47] for the deacylation of unsubstituted *trans*- and *cis*-cinnamoyl-α-chymotrypsins where k_{trans}/k_{cis} at pH 7·3 is more than 10^3.

It is unlikely that the high rate of deacylation of α - benzamido - *trans*-cinnamoyl-α-chymotrypsin compared with that of the *cis*-isomer is the result of differential electronic transmission in the isomeric cinnamoyl moieties because of the similarity in the rates of alkaline hydrolysis of the isomeric ethyl cinnamates ($k^{cis}_{HO^-}/k^{trans}_{HO^-}$ = approx 0·6).[48] The forms, both qualitative and quantitative, of the pH-rate profiles for the deacylation of α-benzamido-*trans*- and *cis*-cinnamoyl-α-chymotrypsins lend support to the concept that specific substrates bind productively to α-chymotrypsin such that the β-aryl group (R_2) and the group containing the susceptible linkage (R_3) are transoid with respect to each other.[49–51] That the rate constant for the deacylation of α-benzamido-*trans*-cinnamoyl-α-chymotrypsin is more than an order of magnitude greater than that for the deacylation of unsubstituted *trans*-cinnamoyl-α-chymotrypsin (Table 5) might be taken as an indication that binding of the α-benzamido group to the enzyme increases the effectiveness of the R_3–ρ_3 interaction compared with that in the unsubstituted cinnamoyl-enzyme. Detailed interpretation of this effect, however, is complicated by the increase in the apparent p*Ka* of the electron relay system in the acyl-enzyme by approx. 0·65 unit as a result of introducing the α-benzamido-substituent.

The apparent susceptibility of α-benzamido-*cis*-cinnamoyl-α-chymotrypsin to hydrolysis by hydroxide ion is in marked contrast to the lack of

such reaction in the *trans*-isomer at least up to pH 10. The deacylation of unsubstituted *trans*-cinnamoyl-α-chymotrypsin is essentially invariant from pH 9 to 0·1 M NaOH and possibly up to 1 M NaOH.[2] The enzymic deacylation of *trans*-cinnamoyl-α-chymotrypsin is transformed into a hydroxide ion reaction in 7·74 M urea.[2] It is of some interest that binding of the acyl moiety in α-benzamido-*cis*-cinnamoyl-α-chymotrypsin appears to effect a similar transformation. In this case, however, the effect occurs when the concentration of the perturbing agent (the acyl moiety) and the enzyme are (of necessity) equimolar (approx 10^{-4} M). The second order rate constant for the reaction of hydroxide ion with denatured *trans*-cinnamoyl-α-chymotrypsin in 7·74 M urea is closely similar to that for the reaction of hydroxide ion with model *trans*-cinnamoyl esters in 7·74 M urea[2] (approx 0·05 $M^{-1}s^{-1}$, Table 5). The second order rate constant for the reaction of hydroxide ion with denatured unsubstituted *cis*-cinnamoyl α-chymotrypsin in 8 M urea also is similar to these (0·035 $M^{-1}s^{-1}$)[47]. In marked contrast to these rate constants the second order rate constant for what appears to be a reaction of a hydroxide ion with α-benzamido-*cis*-cinnamoyl-α-chymotrypsin (Fig 8) is 223 ± 10 $M^{-1}s^{-1}$, i.e. approx 4500 times greater. Possible interpretations for this high alkaline rate include (i) a strained acyl-serine (ii) an acyl-histidine and (iii) reaction of a nucleophilic centre in the enzyme at C_2 of the oxazolinone followed by hydrolysis of this adduct (*vide ante*).

EXPERIMENTAL

Enzyme. α-Chymotrypsin was the 3 × crystallised product of Seravac Laboratories (Pty) Ltd. (now Miles Laboratories, Ltd., P.O. Box 37, Stoke Court, Stoke Poges, Slough, U.K. SL2 4L7) and was used without further purification. Stock solutions of the protein approx. 3·5 mM were made up in the appropriate buffer immediately before use. The solutions were centrifuged at 14000 g and 4° for 30 min and the active site concentration then determined by titration with N-trans-cinnamoyl-imidazole.[52]

Oxazolinones, α-benzamidocinnamic acids and α-benzimido methyl cinnamates. The isomeric 4-benzylidene-2-phenyl-Δ^2-oxazolin-5-ones and their derived carboxylic acids and methyl esters were prepared by literature methods.[15,29,53–55]

Stock solns of the oxazolinones in acetonitrile were prepared immediately before use. Storage of the stable (*trans*- see later) isomer in acetonitrile in the dark is not detrimental but the labile (*cis*) isomer undergoes isomerization to the stable isomer (Results and Discussion).

Buffers and solvents. All buffers were made up using AnalaR grade reagents where available, deionized water, saturated KOH and constant boiling HCl. Acetonitrile was the spectrograde product of Eastman Kodak Co., Rochester, N.Y., U.S.A. In experiments in which buffers were to be mixed with acetonitrile the buffers were degassed before use.

pH Measurement. The pH of solns from spectroscopic cells was determined immediately after a spectrum had been recorded or immediately after a kinetic run using the pH meter of a TTT1C automatic titrator (Radiometer, Copenhagen, Denmark).

Melting points. All m.ps are uncorrected.

Spectrophotometric measurements. Electronic spectra and kinetic measurements were recorded at 25·0° in 1 cm cells using a Cary 15 spectrophotometer using the 0·01 and 0–1·0 extinction slidewires.

"Deacylation" kinetics. These were studied at 25·0° by monitoring the change in extinction at 310 nm which followed admixture of enzyme and oxazolinone. Following an initial, virtually instantaneous increase in extinction, there was a relatively slow fall which followed first order kinetics.

Kinetics of the isomerization of the cis-*oxazolinone.* A small crystal of the *cis*-oxazolinone was dropped into 3 ml of acetonitrile in a 1 cm quartz cell. The cell contents were mixed rapidly and the crystal dissolved almost instantaneously. The extinction at 260 nm was measured at intervals of time against an acetonitrile blank after first rapidly scanning a spectrum from 430–220 nm. This type of measurement was made under two sets of conditions. In one case the cell was left in the cell compartment of the spectrophotometer out of the light path between measurements, i.e. relatively "in the dark". In the other cell was exposed to daylight and the artificial lights of the laboratory. In each case when the extinction at 260 nm had reached a constant value the spectrum was again recorded and the state of illumination of the cells was then reversed, i.e. the cell that had been standing in the dark was allowed to stand in the light and *vice versa.* The extinction at 260 nm was again recorded until it had reached a constant value and the spectra of the solutions were again recorded.

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