Reactions of *N*-(*o*-carboxybenzoyl)-L-leucine: intramolecular catalysis of amide hydrolysis and imide formation by two carboxy groups

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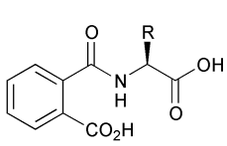
The intramolecular reactions of *N*-(*o*-carboxybenzoyl)-L-leucine (**1**) were studied in aqueous solution, as a function of the hydrogen ion concentration. Two competing reactions were observed: i) cyclization to form the imide *N*-phthaloylleucine, and ii) hydrolysis of (**1**) to phthalic acid and leucine. Individual rate constants for cyclization and hydrolysis were obtained from the overall rate constants and product distributions. Imide formation predominates under highly acidic conditions ($H_0 < -1$) and hydrolysis in the $H_0 > -1$ to pH 5 range. In the hydrolysis reaction, the neighbouring carboxy group participates nucleophilically and in the pH 3–5 range there is a requirement for participation of the second carboxy group as a general acid. Imide formation also requires participation of two carboxy groups in the pH 2–5 range, and shows a bell-shaped pH–rate constant profile. Possible mechanisms for these reactions are discussed.

Introduction

The understanding of enzymatic catalysis on a molecular level has led chemists on a continuing search for simple models where the rates and selectivity of biological catalysts are mimicked.[1–3] Recently, there has been considerable interest in the study of systems involving the intramolecular participation of carboxy groups, in ester and amide hydrolysis, as models for hydrolytic enzymes.[4–15] Because of the predominance of amide groups in proteins, the possibility arises that these groups may also, in some cases, be involved in the mode of action of hydrolytic enzymes.[16]

The intramolecular "models" and the enzymatic reactions share in common the bringing together of reactant species within a single molecule or complex, respectively. We are interested in the study of models of aspartic proteinases which form an interesting and mechanistically well-defined class of hydrolytic enzymes. In general, the optimum pH for catalytic action of aspartic proteinases is in the pH range of 1.9–4.0. In all cases, the hydrolysis of a peptide bond is mediated by two carboxy groups from aspartic acid residues whose p*K* values are 1.4 and 4.5.[17] The observation of essential carboxy groups in the catalytic centre of aspartic enzymes has generated a great interest in the possible role of these groups in catalyzing hydrolytic reactions.[17] In order to ascertain the importance of these groups, we have initiated studies of intramolecular effects in the hydrolysis of *N*-(*o*-carboxybenzoyl)-L-leucine, NCBL, compound **1** with R = $-CH_2CH(CH_3)_2$ and related compounds.[18]

This system could represent a simple model for hydrolysis of a peptide bond catalyzed by enzymes with two carboxy groups at the active site. In this paper, we present the results obtained in a kinetic study of the acid-catalyzed hydrolysis of **1**.



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Experimental section

All chemicals were of reagent-grade and were purchased from Aginomoto, Fluka and Merck. Methanol and chloroform were purchased from Merck and purified by standard methods prior to use. Aqueous solutions were prepared with distilled water. Melting points were recorded on Kofler hot-stage apparatus (Microquímica APF-301) and were not corrected. IR spectra were obtained with a Perkin-Elmer Model 16 PC-FTIR spectrophotometer. Proton NMR spectra were determined on a Bruker AW-200 (200 MHz) instrument with Me_4Si as an internal standard, and ^{13}C NMR spectra were determined on a Bruker (50.3 MHz) spectrometer. Mass spectra were measured on a Shimadzu-CGMS-QP-2000-A. All optical rotation measurements were carried out by use of the Schmidt + Haensch Polartronic E and are given as 10^{-1} deg cm^2 g^{-1}. Elemental analyses were performed on a Perkin-Elmer 2400 instrument. TLC was routinely performed on silica gel F_{254}.

L- Leucine methyl ester hydrochloride[18]

Thionyl chloride (44 mmol) was added slowly, over a period of 20 min, to a stirred and cooled (0 °C) suspension of L-amino acid (40 mmol) in dry methanol (50 mL). Stirring was continued and the mixture was allowed to warm up to room temperature overnight and then refluxed for 2 h, cooled and evaporated. The resulting solid was suspended in diethyl ether (30 mL) and evaporated. This procedure was repeated four times and the white amorphous solid was again suspended in diethyl ether (30 mL), filtered and dried under a N_2 atmosphere. The title compound was obtained (6.75 g, 93%). The melting points and optical rotations are in agreement with those reported in the literature.[19]

N-(*o*-Carboxybenzoyl)-L-leucinate methyl ester (NCBL-ME)[18]

Triethylamine (40 mmol) was added dropwise over *ca.* 10 min to a stirred and cooled (0 °C) suspension of the above compound (33 mmol) in dry chloroform (150 mL) under a dry N_2 atmosphere. After 10 min under the same conditions, phthalic anhydride (30 mmol) was added in one portion. The cooling

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bath was removed and stirring was continued for 18 h at room temperature. The resulting solution was washed with 1 M HCl (2 × 100 mL), dried over Na_2SO_4 and then evaporated. The oily yellow residue was crystallized from diethyl ether–petroleum ether to produce the ester as a white solid (7.4 g, 85%). Mp 124–125 °C (Found: *C*, 61.49; *H*, 6.82; *N*, 4.79%. $C_{15}H_{19}NO_5$ requires *C*, 61.40; *H*, 6.53; *N*, 4.78%); $[\alpha]_D^{20} = -41$ (*c* 1 in $CHCl_3$); ν_{max}(KBr)/cm^{-1} 3300 (br NH), 3200–2500 (OH), 1738 (CO_2Me), 1700 (CO_2H), 1636 (CONH); δ_H(200 MHz, $CDCl_3$) 8.70 (1H, br s, CO_2H, exchange with D_2O), 7.99–7.49 (4H, m, Ar*H*), 6.73 (1H, d,*J* 8.4 Hz, N*H*, exchange with D_2O), 4.87 (1H, m, NC*H*), 3.76 (3H, s, CO_2CH_3), 1.77–1.68 (3H, m, $CHCH_2$), 1.00 (3H, d, *J* 6.46 Hz, CH_3), 0.97 (3H, d, *J* 6.55 Hz, CH_3); δ_C(50.3 MHz, $CDCl_3$) 173.50, 169.91(*C*=O), 169.81, 137.19, 132.46, 131.23, 130.05, 128.87 and 127.91 (Ar), 52.47 (*C*HNH), 51.28 (O*C*H$_3$), 41.38 (CH_2), 24.84 (*C*H), 22.73 and 21.92 ((CH_3)$_2$); *m*/*z* calculated 293.17, found 294.15 $(M + 1)^{+\bullet}$.

N-(*o*-Carboxybenzoyl)-L-leucine (NCBL)[18]

A solution of NCBL-ME (3.48 mmol) in 1 M aqueous NaOH (11 mL) was stirred at room temperature for 2.5 h. The reaction mixture was acidified with 1 M hydrochloric acid up to pH 1 and extracted with ethyl acetate (3 × 50 mL). The organic layers were combined, washed with water (2 × 50 mL) and brine (2 × 50 mL), dried over Na_2SO_4 and evaporated. The resulting solid residue was suspended in $CHCl_3$ (50 mL), stirred for 1 h, and the diacid **4** as a white amorphous solid was collected by filtration and dried under vacuum to give the title compound (0.66 g, 68%). Mp 127–128 °C (Found: *C*, 60.34; *H*, 6.00; *N*, 5.09%. $C_{14}H_{17}NO_5$ requires *C*, 60.21; *H*, 6.13; *N* 5.02%); $[\alpha]_D^{20} = -42$ (*c* 1 in EtOH); ν_{max}(KBr)/cm^{-1} 3300 (br NH), 3200–2500 (OH), 1750 and 1690 (CO_2H), 1616 (CONH); δ_H(200 MHz, DMSO-d_6) 12.80 (1H, br s, CO_2H, exchange with D_2O), 8.63 (1H, d, *J* 8.00 Hz, N*H*, exchange with D_2O), 7.78–7.39 (4H, m, Ar*H*), 4.40–4.30 (1H, m, NC*H*), 1.70 (3H, m, $CHCH_2$), 0.98 (6H, d, *J* 6.37 Hz, (CH_3)$_2$); δ_C(50.3 MHz, DMSO-d_6) 174.07, 168.31, 167.89 (*C*=O), 138.05, 131.04, 130.80, 129.18, 129.06 and 127.76 (Ar), 50.51 (*C*HNH), 39.79 (CH_2), 24.16 (*C*H), 23.01 and 21.24 ((CH_3)$_2$); *m*/*z* calculated 279.28, found 261.15 $(M - 18)^{+\bullet}$.

L-Phthaloylleucine (NPL)

This was prepared by a direct reaction between L-leucine and phthalic anhydride in toluene, according to a method given in the literature[20] (85%, mp 115–116 °C, lit.[21] 116 °C). ν_{max} and δ_H data were consistent with the structure.

Kinetic measurements

All kinetic work was carried out using a Hewlett Packard diode-array spectrophotometer fitted with a thermostated water-jacketed cell holder (Microquímica MQBTZ99-20). All solutions were prepared using distilled water which was boiled and cooled under nitrogen to remove dissolved CO_2. The kinetic solutions were prepared from reagent-grade HCl, chloroacetic acid and acetic acid (Merck). Measurements of pH were made using a Beckman Model Φ 71 pH meter and were checked before use. In the pH region 1 to 5, the stock buffers were made to a total ionic strength of 0.1 M by addition of KCl. A typical kinetic run, 2.7 mL of the appropriate solution, was kept in equilibrium at 50 °C for ten minutes. The reaction was then initiated by adding 0.3 mL of a stock solution of NCBL (1.0×10^{-2} M) prepared in acetonitrile, and placed in the thermostated cell compartment (50 ± 0.1 °C). This procedure thus added 10% v/v acetonitrile into the reaction mixture. The reaction was monitored following changes in absorbance at 310 nm as a function of time. The absorbance values were stored directly on a microcomputer and analyzed using the HP 8452 kinetics software, which showed, for at least 4–5 half-lives, an excellent first-order kinetics behavior.

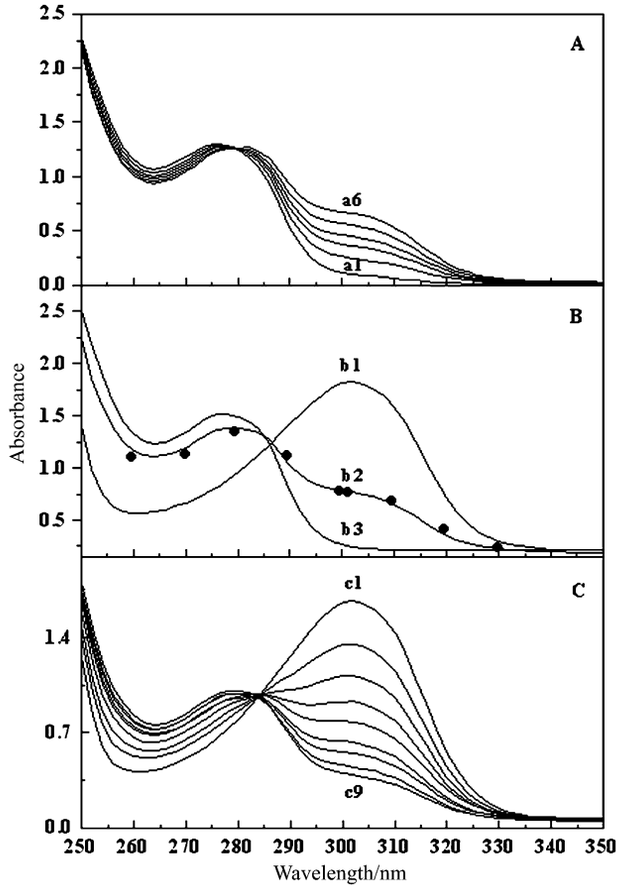


Fig. 1 Ultraviolet spectra in 5 M HCl, at 50 °C: Fig. 1A. Spectral variation obtained during the reaction of NCBL (1.0×10^{-3} M). The time interval between each scan (**a1–a6**) is 3 min. Fig. 1B shows the spectra of (**b1**) *N*-phthaloylleucine (1.0×10^{-3} M); (**b2**), solid line, refers to the observed spectra of the products of the NCBL reaction; the symbol (●) corresponds to the absorbance calculated for a solution composed of 6.67×10^{-4} M phthalic acid and 3.33×10^{-4} M *N*-phthaloylleucine; and (**b3**) corresponds to the spectra of phthalic acid (1.0×10^{-3} M). Fig. 1C shows repetitive scans obtained during the reaction of *N*-phthaloylleucine, 1.0×10^{-3} M. The time interval between each scan (**c1–c9**) is 24 hours.

Results and discussion

The kinetic behavior of *N*-(*o*-carboxybenzoyl)-L-leucine, NCBL, in aqueous solution was studied as a function of hydrogen ion concentration from $H_0 = -3.59$ to pH 5. Fig. 1A shows the spectral variation observed in the 250–350 nm region, upon addition of NCBL to a 5 M HCl solution. Spectrum (**a1**) corresponds to NCBL at the starting time: the intermediate spectra were collected at 3 minute intervals and the final spectrum (**a6**) upon completion of the reaction. As can be seen, there is a smooth absorbance variation from NCBL to products, with an isosbestic point at 278 nm, permitting the use of changes in absorbance at 310 nm to follow the kinetics of the reaction.

Fig. 1B shows the spectrum obtained after 10 half lives for the reaction of NCBL in the presence of 5.0 M HCl. The final spectrum **b2** seems to correspond to a mixture between that of the imide *N*-phthaloylleucine (NPL), spectrum **b1**, and that of phthalic acid, spectrum **b3**, which are included in Fig. 1B for comparison purposes. The dots marked in spectrum **b2** correspond to the calculated absorbances, assuming that the final mixture corresponds to 3.33×10^{-4} M *N*-phthaloylleucine (NPL) and 6.67×10^{-4} M phthalic acid, and that they agree (within 3%) with the final kinetic spectrum. The intensity of the

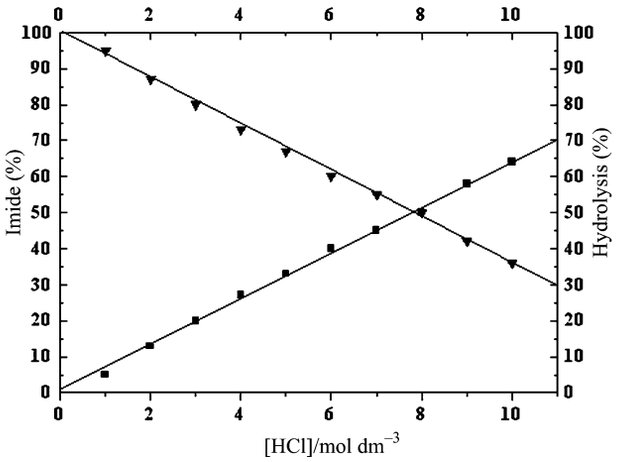
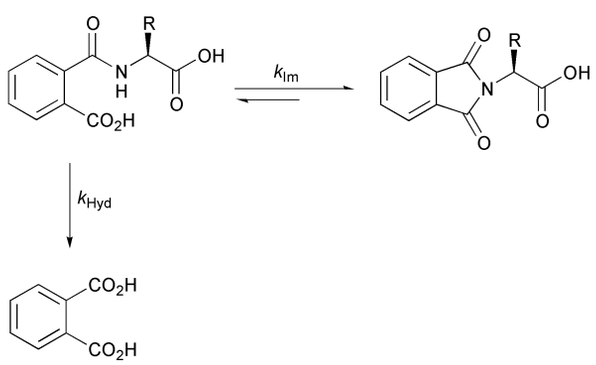


Fig. 2 Plot of the percentage contents of imide (■) and hydrolysis products (▲) obtained in the reaction of NCBL (1.0×10^{-3} M) as a function of the hydrochloric acid concentration at 50 °C.

absorbance in the 290–320 nm region depends on the acidity of the kinetic solution and increases with the rising concentration of HCl. In order to verify the formation of the imide in the kinetic analysis of the reaction, we investigated the hydrolysis of *N*-phthaloylleucine (NPL) in 5 M HCl. As can be seen in Fig. 1C, the final spectrum is identical to that obtained in the reaction of NCBL under similar conditions (compare spectra **a6, b2** and **c9**). The kinetic analysis reveals that the acid hydrolysis of the imide is approximately 120 times slower than the reaction of NCBL, and therefore does not interfere in the kinetic analysis of the reaction of *N*-(*o*-carboxybenzoyl)-L-leucine. Thus, the reaction of NCBL results in the formation of both NPL and phthalic acid under the experimental conditions. Direct evidence for the existence of phthalic anhydride as an intermediate was not obtainable, since the subsequent hydrolysis to phthalic acid or its ionized forms was usually faster than its formation. Scheme 1 adequately describes our experimental results.



Scheme 1

A detailed inspection of data similar to that shown in spectrum **b2** (Fig. 1B), permits the calculation of the percentages of phthalic acid and of *N*-phthaloylleucine formed in the reaction of NCBL at different acidities. This information was obtained from the absorbance spectrum for each individual mixture, since the absorbance measurements at 302 nm are directly proportional to the amount of imide formed in the reaction. The results obtained, in terms of product distribution in the reaction of NCBL, which represent the average of at least three independent determinations, are shown in Fig. 2.

Fig. 3A contains the data obtained for the hydrolysis of *N*-(*o*-carboxybenzoyl)-L-leucine as a function of the acidity of the solution in the range of $H_0 = -3.6$ up to pH 5.

The dependence of the global rate constant k_{obs} on pH shown in Fig. 3A is somewhat different from that reported by Bender[4]

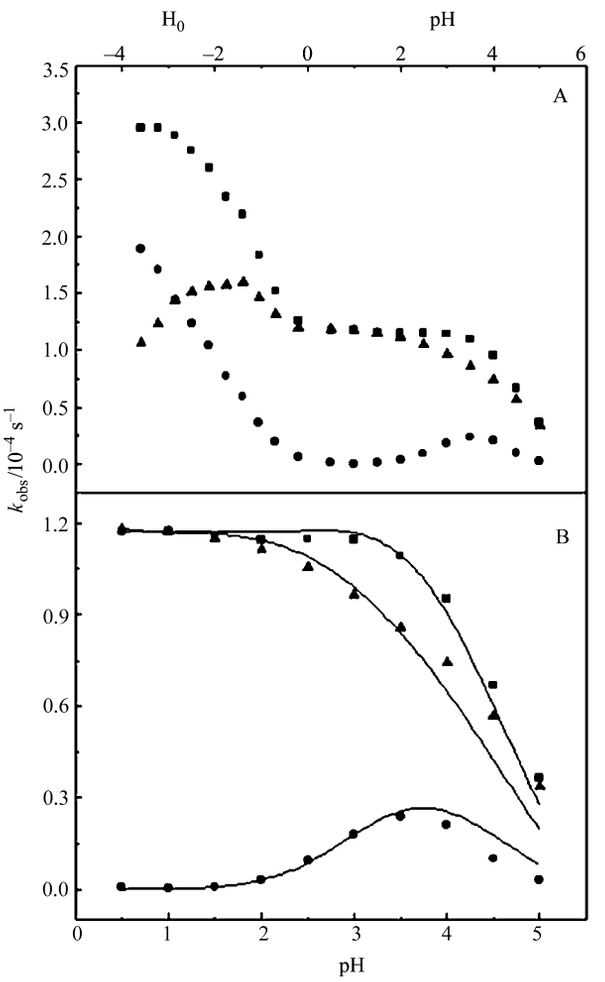


Fig. 3 (**A**) Plot of k_{obs} (■), k_{Im} (●) and k_{Hyd} (▲) against pH or H_0 for the reactions of *N*-(*o*-carboxybenzoyl)-L-leucine at 50 °C. (**B**) Solid lines correspond to the calculated pH–rate constants profiles using eqn. (2) for k_{Im} (●) and eqn. (3) for k_{Hyd} (▲). The line for k_{obs} (■) corresponds to the sum of the individual contributions.

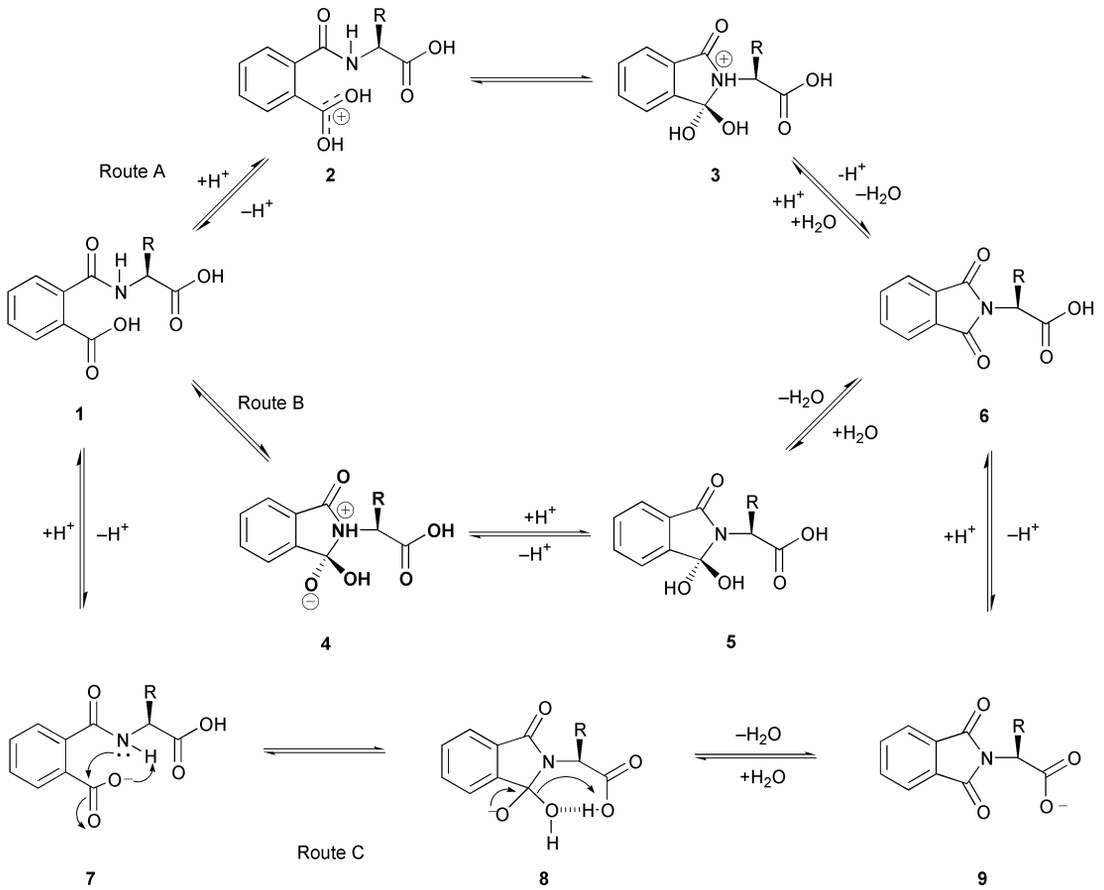
for the hydrolysis of phthalamic acid and derivatives. The differences in experimental behavior between phthalamic acid and NCBL may well be related to the contribution of imide formation in the NCBL reaction given in Table 1, since Bender did not detect contribution of imide formation in the phthalamic acid reaction. Furthermore, in the pH region 1 to 5, the experimental data suggest a pK_a which differs from the expected value by approximately 2 units, and because of this it is not possible to draw direct comparisons with similar reactions reported in the literature.[4,6,7,8,10]

Thus, in order to adequately analyze the experimental data and compare our data with the literature, the contributions of the individual rate constants for cyclization (k_{Im}) and hydrolysis (k_{Hyd}) of *N*-(*o*-carboxybenzoyl)-L-leucine at several hydrogen ion concentrations, were obtained by combining the overall rate constants (eqn. (1)) and the experimental product distribution, which are included in Fig. 3A.

$$k_{obs} = k_{Im} + k_{Hyd} \quad (1)$$

Cyclization of *N*-(*o*-carboxybenzoyl)-L-leucine

As can be seen in Fig. 3A, cyclization depends strongly on the acidity of the solution, becoming linearly dependent on the acidity when $H_0 < -1$ and becomes the dominant reaction, in terms of product distribution, in the high acidity region ($H_0 < -3$). This behaviour is different from that found by Bender and co-workers[4] for the reaction of phthalamic acid in aqueous solutions, where the only reaction was hydrolysis, and



Scheme 2

Table 1 Rate constants for the overall reaction, the cyclization and the hydrolysis of *N*-(*o*-carboxybenzoyl)-L-leucine[a]

pH	H_0	$10^4\ k_{obs}/s^{-1}$	$10^4\ k_{Im}/s^{-1}$	$10^4\ k_{Hyd}/s^{-1}$
	−3.59	2.954	1.890	1.064
	−3.22	2.950	1.711	1.239
	−2.86	2.884	1.442	1.442
	−2.50	2.753	1.238	1.515
	−2.12	2.600	1.040	1.560
	−1.76	2.344	0.774	1.570
	−1.40	2.187	0.591	1.596
	−1.05	1.833	0.367	1.466
	−0.69	1.516	0.197	1.318
	−0.20	1.260	0.063	1.197
0.50		1.172	0.009	1.181
1.00		1.175	0.004	1.171
1.50		1.156	0.008	1.148
2.00		1.146	0.032	1.114
2.50		1.147	0.092	1.055
3.00		1.145	0.178	0.967
3.50		1.093	0.236	0.857
4.00		0.952	0.210	0.742
4.50		0.668	0.100	0.568
5.00		0.363	0.028	0.335

[a] [NCBL] = 1.0×10^{-3} M, at 50 °C.

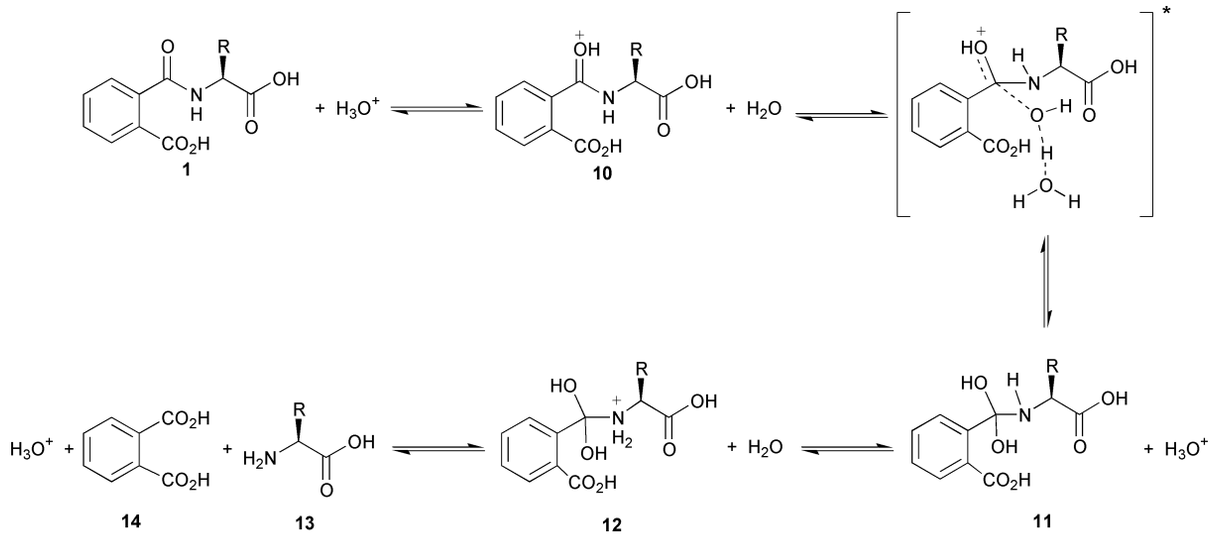
cyclization was not observed. Results reported by Schafer[22] for the hydrolysis of *N*-methylphthalamic acid and Perry[14] for the reaction of *N*-(2-aminophenyl)phthalimide indicate that cyclization is important, whether occurring at high[22] or low[14] pH values. Route A, in Scheme 2, is consistent with our experimental evidence for the cyclization reaction in the H_0 region, and includes protonation equilibria of the neighbouring *o*-carboxylic acid group, which should increase the electrophilicity of this group, facilitating attack of the amidic nitrogen and therefore promoting cyclization and formation of the corresponding tetrahedral intermediates.

In the pH region 0–5, the cyclization is not the dominant reaction in the solution, and the dependence of k_{obs} on pH can be described by a pH-independent component (pH 0 to *ca.* 2), followed by a bell-shaped curve (pH 2 to 5) and eqn. (2) is consistent with the experimental observations:

$$k_{Im} = k_{Im\text{-}DA}/\{1 + (K_{a1}/[H^+]) + (K_{a1}K_{a2}/[H^+]^2)\} + k_{Im\text{-}MA}/\{1 + ([H^+]/K_{a1}) + (K_{a2}/[H^+])\} \quad (2)$$

where $k_{Im\text{-}DA}$ corresponds to the rate constant of cyclization by the diacidic form of NCBL (route B in Scheme 2), and $k_{Im\text{-}MA}$ corresponds to that of the monoanionic species (route C in Scheme 2).

Thus, the first term on the right hand side of the equation corresponds to the pH-independent hydrolysis of NCBL, in the diacidic form, while the second term corresponds to the region where the monoanionic form of NCBL is the predominant species in solution. The dependence of the rate constant on pH in this region was quantitatively fitted with eqn. (2) (Fig. 3B), using values of $pK_{a1} = 3.0$ and $pK_{a2} = 4.5$, $k_{Im\text{-}DA} = 0.01\ s^{-1}$ and $k_{Im\text{-}MA} = 0.37 \times 10^{-4}\ s^{-1}$. The kinetic pK_a values are consistent with expectations, since a value of $pK_{a1} = 3.67$ has been reported for *N*-methylphthalamic acid, and calculations based on the ΔpK method with statistical corrections[23] yielded values of $pK_{a1} = 3.37$ and $pK_{a2} = 4.2$. Clearly, the dicarboxylic acid form is considerably less reactive, probably because (as can be seen in Scheme 2, route B) the driving force provided by the protonation equilibria, in the high acidity region, is absent in this particular route. Also, the initial zwitterionic intermediate most probably favours decomposition to reagents, while the neutral intermediate, resulting from proton transfer, which may include a water molecule, will favour product formation. The increased reactivity observed for the monoanionic form, route C in Scheme 2, may well be related to the contribution of the carboxylate anion, *via* hydrogen bonding, in the attack of the amidic nitrogen on the carboxylate. Thus, formation of the



Scheme 3

C–N bond and proton transfer may occur simultaneously, forming directly a monoanionic intermediate with the negative charge on one of the oxygen atoms (Scheme 2, route C). It is important to note that if proton transfer does not occur simultaneously, the formed intermediate would have a positively charged, protonated, N atom, and two negatively charged oxygen atoms, an intermediate which besides being of high energy would strongly favour regeneration of the reagents.

Hydrolysis of *N*-(*o*-carboxybenzoyl)-L-leucine

As can be seen in Fig. 3A, in the H_0 region ([HCl] > 1.0 M) the reaction of hydrolysis of **1** is subject to hydrogen ion catalysis. Similar to normal amides[24] and phthalamic acid[4], the rate constant increases up to the rate maximum (at approximately $H_0 = -2.0$) and then decreases. The similarities in the pH rate constant profiles, between NCBL and phthalamic acid, indicate that in our case NCBL hydrolyzes, *via* *O*-protonation of the amide group followed by water assisted attack of H_2O, to form a neutral tetrahedral intermediate and hydronium ion (Scheme 3). Not surprisingly, the pK_a for protonation of the amide group is similar to that reported for phthalamic acid and benzamide (p$K_a = -2.5$).[4]

Hydrolysis of NCBL in aqueous solutions over the pH range 0 to 5 shows a rate constant *vs.* pH profile characteristic of intramolecular participation of the neighbouring undissociated carboxylic group. There is a constancy of the rate constant from pH 0.5 to 2.5, followed by a decrease in rate constant from pH 3 to 5. The pH–rate constant profile for the hydrolysis of **1**, in this region, is strongly indicative of the participation of both the diacidic and the monoanionic forms of **1**, and the dependence of the rate constants on pH can be quantitatively fitted using eqn. (3), (Fig. 3B):

$$k_{Hyd} = k_{Hyd\text{-}DA}/\{1 + (K_{a1}/[H^+]) + (K_{a1}K_{a2}/[H^+]^2)\} + k_{Hyd\text{-}MA}/\{1 + ([H^+]/K_{a1}) + (K_{a2}/[H^+])\} \quad (3)$$

where $k_{Hyd\text{-}DA}$ and $k_{Hyd\text{-}MA}$ correspond to the rate constant for hydrolysis of the diacidic and monoanionic forms of NCBL, respectively. The line in Fig. 3B was calculated using values of $k_{Hyd\text{-}DA} = 1.18 \times 10^{-4}$ s^{-1}, $k_{Hyd\text{-}MA} = 0.81 \times 10^{-4}$ s^{-1}, p$k_{a1} = 3.0$ and p$k_{a2} = 4.5$. It is important to note that the kinetic data could not be fitted with a single pK value, and that the reactivity of the diacidic form of NCBL is slightly higher than that of the monoanionic form.

In order to understand the contribution of the –COOH group in the amino acid to the overall rate constant, the hydrolysis of the *N*-(*o*-carboxybenzoyl)-L-leucinate methyl ester (NCBL-ME) was studied in the pH region 0.5 to 5. The

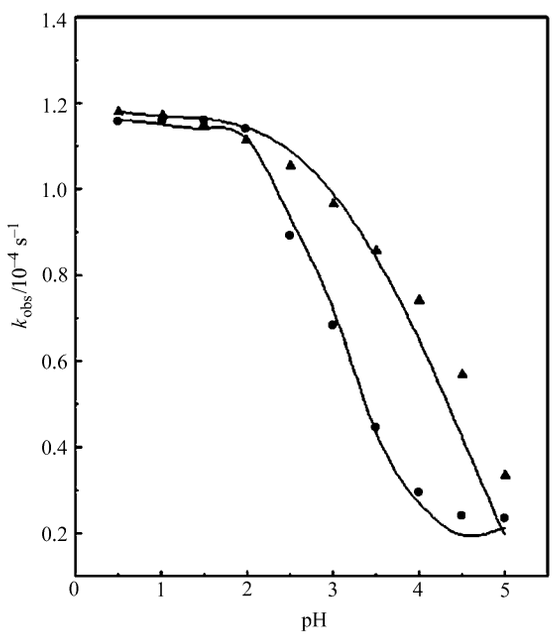
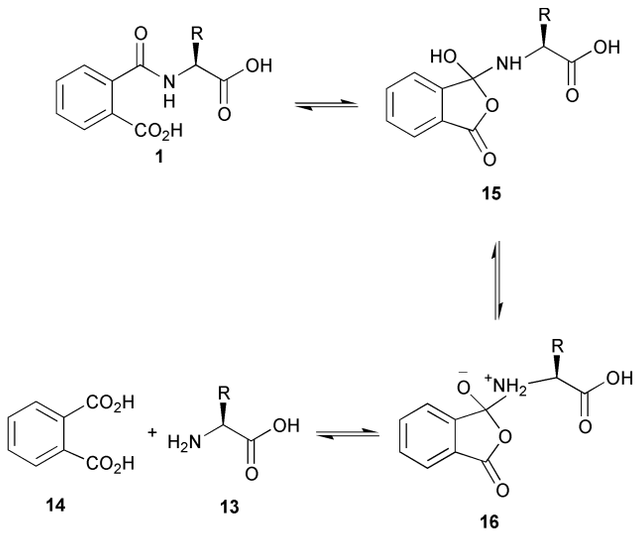


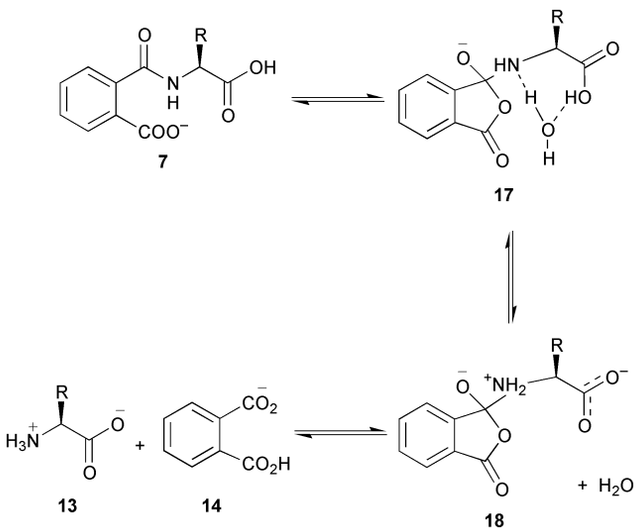
Fig. 4 Plot of k_{obs} against pH for the acid hydrolysis of *N*-(*o*-carboxybenzoyl)-L-leucinate methyl ester (●) at 50 °C. The solid line for NCBL-ME was calculated from $k_{obs} = 1.18 \times 10^{-4}/(1 + k_{a1}/[H^+])$. Values and theoretical fitting of k_{Hyd} for the reaction of NCBL (▲) are included for comparison purposes.

observed pH–rate profile is shown in Fig. 4, where the pH–rate constant profile of NCBL is included for comparison purposes. As can be seen, both NCBL and NCBL-ME show almost identical reactivities in the plateau region, pH range 0.5 and 2. Above pH 2, the reactivity of NCBL-ME decreases faster than that of NCBL, indicating that the monoanion is considerably less reactive. Indeed, a kinetic pk_a value of 3.2 was obtained from the fitting of the data, and the difference between the kinetic curves represents the contribution of the –COOH group in the amino-acid residue to the overall catalytic process in the hydrolysis reaction of NCBL.

Scheme 4 shows a mechanistic route consistent with the $k_{Hyd\text{-}DA}$ term in eqn. (3), which represents the hydrolysis of the diacidic form of NCBL. Since, the reactivity of the undissociated diacid is similar to that of the methyl ester, we can conclude that the terminal –COOH group is not playing an important role in proton transfer. Comparing the reaction of NCBL with that reported by Schafer[22] for the hydrolysis of *N*-methylphthalamic acid, we observe that NCBL hydrolyzes about 4–5 times faster. A similar effect was observed by Kirby and coworkers[9] in the



Scheme 4



Scheme 5

intramolecular catalysis of maleamic acids, and a possible explanation for this result is the facility of proton transfer between intermediates (**15**) and (**16**) in Scheme 4 due to the introduction of a more polar group in the solvation shell surrounding the tetrahedral intermediate. This proton transfer, which is most probably solvent mediated, is particularly important, since it is well known that a tetrahedral intermediate such as (**15**) would be expected to preferentially expel the carboxylate group rather than the amino acid ion, and thus react *via* the zwitterionic form (**16**).

As given in eqn. (3), $k_{\text{Hyd-MA}}$ represents the hydrolysis of the monoanionic form of NCBL, which shows considerable reactivity at pH 3–5 where the monoanionic form is found predominately in solution. The reactivity of the acid anion form of NCBL is approximately 3 times greater than the NCBL methyl ester. Scheme 5 shows a mechanism consistent with the experimental data, and the reaction proceeds *via* initial formation of a negatively-charged tetrahedral intermediate with a negatively-charged oxygen atom (**17**). Since this intermediate is not reactive, and should decompose to reagents, it is not unreasonable to suppose that the proton transfer step for formation of the reactive zwitterionic intermediate (**18**) proceeds *via* general acid catalysis by the COOH group. The relatively small effect is to be expected, since the five-membered ring limits the efficiency of the catalytic process, and it is likely that a water molecule is involved in the process. The zwitterionic intermediate (**18**) decomposes to products, most probably *via* phthalic anhydride, which could not be detected under our experimental conditions, since its hydrolysis to phthalic acid proceeded faster than the observed rate constants.[4,15,22]

Conclusions

The rate constant for the hydrolysis of *N*-benzoyl-L-leucine (8×10^{-6} s^{-1}, at 100 °C, calculated from the data of Capindale and Fan[25]) is approximately 4600-fold smaller than the rate constant for the carboxy-catalyzed hydrolysis of *N*-(*o*-carboxybenzoyl)-L-leucine (3.7×10^{-2} s^{-1}, at 100 °C, according to our kinetic data), a result which substantiates the nucleophilic participation of the neighbouring carboxy group. The requirements for participation of a second carboxy group in both the hydrolysis and cyclization reactions are similar to those observed in the mechanisms of aspartic proteases, where a water molecule is activated by two carboxy groups, one ionized and one in the acid form. The kinetic study of a variety of substrates with two carboxy groups is currently being carried out and should allow a more detailed discussion of the possible analogies of the model systems with closely related enzymatic reactions, such as aspartic proteases.

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