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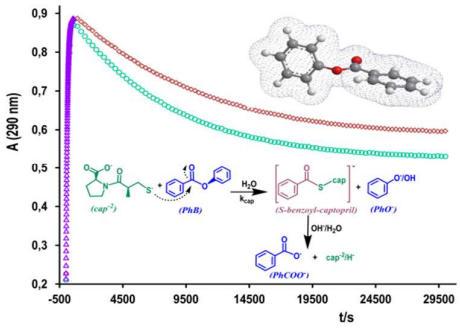

Captopril as a nucleophile for ester cleavage. Formation of the thiolester S-benzoylcaptopril

Emilia Iglesias*, and Isabel Brandariz

The reaction between captopril and phenylbenzoate yields S-benzoylcaptopril that is very stable in carbonate buffer or in aqueous micellar medium.

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The rates of both acyl transfer from phenylacetate (PhA) or acetylsalicylic acid (ASA), and benzyl transfer from phenylbenzoate (PhB) to hydroxide ion and to the thiol anion of captopril (cap) or cysteine (cys) have been determined in aqueous basic solution. The rates of ester cleavage by OH^- are faster than that promoted by the thiol anion of either cap cys. In every case, the pseudo-first order rate constant shows linear dependence on either [OH^-] or [thiol]. Nevertheless, for the reaction between PhB and cap, the absorbance-time profiles obey to consecutive reactions, where the two reaction paths were attributed to the rapid formation of S-benzoylcaptopril that subsequently decomposes in a slower reaction step to form phenolate and benzoate, as stable products. The S-benzoylcaptopril decomposition is accelerated in alkaline medium and is practically suppressed in carbonate buffer of pH 10. In addition, the presence of cationic micelles at concentration values close the cmc, not only accelerates the formation of the thiolester, but also decreases its decomposition. The second-order rate constant for the reaction between the three nucleophiles and PhA or ASA correlates quite well with the basicity (pK_a) of the nucleophile, a fact that suggest the same reaction mechanism. By contrast, in the correlation for PhB, the datum corresponding to the reaction with cap deviates significantly from the linear plot, which evidences a different reaction scheme.

1. Introduction

The cysteine proteases comprise a large group of enzymes that contain the –SH group of cysteine residues in the active site.[1-3] These enzymes can be obtained from plant –such as papain,[4] ficin,[5] or actinidin[6]–, bacterial, and animal sources. The catalytic cycle of cysteine proteases is known to proceed through an intermediate thiolester which is subsequently hydrolysed to regenerate the native enzyme. The intermediate thiolester is formed in the nucleophilic attack of –SH group to the electrophilic centre, the carbonyl group of the substrate. Only a limited number of nucleophiles have been demonstrated to participate in covalent catalysis by enzymes.[7] The wide reactivity of –SH groups in enzymes can be attributed to their different microenvironment and/or to the molecular weight of the thiol compound.

Carbonyl displacement reactions have been extensively investigated. Many biochemical reactions involve nucleophilic attack in the acyl transfer process. The effect of acceptor and leaving group basicities on the reaction rate of the deacylation step is a valuable mechanistic probe. As a general rule, acyl transfer reactions in which the nucleophile is more basic than the leaving group involve rate-determining expulsion of the leaving group from the tetrahedral intermediate, which is consistent with the high sensitivity of the rate constants to the basicity of the acyl acceptor.[8-10] The acyl transfer reaction of p-nitrophenyl acetate has been investigated with an extensive series of nucleophiles.[8-12], including N-acetyl-L-cysteine.[13] The study concludes that the thiolate anion, and not the protonate thiol, is the only species which reacts at a significant rate wi the p-nitrophenyl acetate. Other amides or ester reactions with different thiols or amine thiols have been studied as simple model systems for the acylation step of the active site –SH in cysteine proteases.[14,15]

The present study was undertaken to examine the –SH nucleophilic attack to the carbonyl group of aromatic esters. Among them, it was chose phenylacetate (PhA), phenylbenzoate (PhB), and acetyl salicylic acid (ASA), in order to analyze the effect of the leaving group (phenolate, PhO^-, or salicylate, SA^-), as well as the acyl- or benzyl-transfer. As thiol models, it was studied the behavior of captopril (cap) and cysteine (cys), and the results were compared with the classical OH^- hydrolysis.

Phenylacetate is a common metabolite of phenylalanine, and then a naturally occurring plasma component, which is used in the treatment of hyperammonemia associated with inbo errors of urea synthesis or liver failure. Due to its effectiveness in reducing plasma glutathione levels, PhA is implicated in growth control and differentiation of tumor cells throu nontoxic mechanisms.[16,17] On the other hand, captopril (cap) a mercapto-proline derivative highly effective as angiotens converting enzyme (ACE) inhibitor.[18] The captopril molecu contains two acid ionisable groups, the carboxylic group of

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Electronic Supplementary Information (ESI) available: Figures S1 to S7 and Table S1 are included. See DOI: 10.1039/x0xx00000x

proline and the thiol group of the propionyl moiety. In aqueous medium several forms of cap are then possible depending on the pH; in strong acid medium (pH<2.5) the neutral cap molecule predominates, whereas in strong alkaline medium (pH>11.5) the doubly charged anion (cap^{-2}) is the majority species.[19,20] Reactiviy between both family compounds, thiols and aromatic esters, are of interest from the biochemical perspective.

2. Results and Discussion

2.1. Reaction in alkaline medium

The reaction spectra of the alkaline hydrolysis of either PhA, PhB or ASA show an increasing absorption band between 270-320 nm, approximately. As an example, for the hydrolysis of PhB in water at [OH^-]=0.013 M the band appears centered at 287 nm, while the much stronger absorption at 235 nm shifts to lower wavelengths. Two well-defined isosbestic points are drawn at 270 and 230 nm, see Figure S1 (Electronic Supplementary Information). These are typical characteristics of electronic spectra of the products of the reaction, i.e., phenolate (PhO^-), benzoate ($PhCOO^-$), or salicylate (SA^-) anions that show two main absorption bands due to $\pi \rightarrow \pi^*$ namely B-band (λ_{max}~287nm, ε~2600 $M^{-1}cm^{-1}$) and E_2-band (λ_{max}~235nm, ε~9400 $M^{-1}cm^{-1}$). The reaction spectra of PhA resembles that of PhB except by the lower absorption intensity, as expected, due to in the latter case two absorbing products, PhO^- and $PhCOO^-$, are generated at equal concentration. In the case of ASA, the absorption maximum was stated at 296 nm due to the salicylate ion.[21-25]

The kinetics of alkaline hydrolysis of the aforementioned aryl esters were examined by following the reaction as a function of [OH^-] (I=0.10 M and 25 °C) at the wavelength indicated in Table 1. Under these conditions the hydrolysis is first-order in [OH^-], and the rate constant, k_o, increases with the hydroxide ion concentration according eq. (1), indicating no significant uncatalysed reaction.

$$k_o = k_{OH}[OH^-] \qquad (1)$$

Figure 1 shows comparative data for the three aryl esters. Values of k_{OH} are listed in Table 1, together with the reaction conditions and the net absorbance increase. It is necessary to remark that the net absorbance change is independent of [OH^-]. The estimated value of molar absorptivity of the reaction product for the hydrolysis of PhB is nearly double of the corresponding to PhA, because PhB gives equimolar concentration of PhO^- and $PhCOO^-$, which absorb in the same spectral region and all are in good agreement with published values. Found literature k_{OH} values are also given, including that of *p*-nitrophenylacetate (PNPhA),[26] for comparative purposes. The rate of PhA hydrolysis is more than 3-fold that of PhB and near 10-times that measured for ASA. The leaving group in PhA or PhB is the same; therefore, the reason of different reactivity must be due to the nature of the electrophilic site that in PhB is a poorer electrophile because of the resonance effect of the phenyl ring. The optimization structures and the formal charges on the atoms of interest shown in Scheme 1, confirm this hypothesis. The 3D structure of PhB showed that both phenyl rings are in perpendicular planes. On the other hand, the low reactivity of acetyl salicylate anion can be justified by the electrostatic repulsion of OH^-. The Eyring's plot, $\ln(k_{OH}/T)$ *versus* 1/T, was constructed with the results of ASA (Figure S3 of ESI). Good correlation (cc>0.98) was obtained if data of Tee *et al.*[21] measured at 25 °C and that of Fersth *et al.*[24] at 39 °C were not included. Both data were measured at I=1.0 M, an important factor taking into account the ionic nature of both reagents. Then the temperature effect gives $\Delta H^{\#}$=43 kJ·mol^{-1} and $\Delta S^{\#}$=–119 J·mol^{-1}K^{-1}. The high negative entropy of activation accounts for a highly ordered transition state, which involves the approaching ions of same charge.

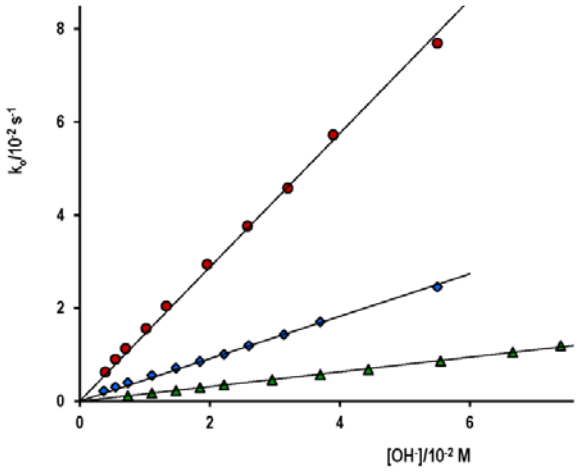
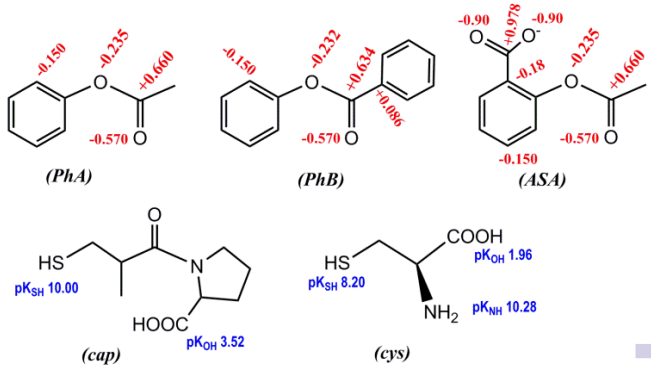


Figure 1. Plot of the observed rate constant, k_o, for the alkaline hydrolysis of (●)PhA, (◆)PhB and (▲)ASA as a function of the OH^- concentration, ionic strength 0.10 M and 25 °C.



Scheme 1. Chemical structures of the studied compounds; formal charges on selected atoms, and pK_a of ionizable groups (including $-NH_3^+$ of cys).

2.2. Ester thiolysis by captopril (cap)

Reaction of PhB in excess of [OH^-]. In alkaline medium the cap molecule is a divalent anion, the pK_{OH} (carboxylic acid)-3.52 while the pK_{SH} (sulfydryl group)=10.0, and aqueous solutions of cap show negligible absorption a λ>270 nm either in acid or basic medium.[19,20]

Table 1. Experimental conditions for the alkaline hydrolysis of PhA, PhB, and ASA and values of the second order rate constant, k_{OH}, obtained in this word and literature values.

ester (c/mM)	λ_{max}/ nm	$A_\infty - A_o$	$\Delta\varepsilon$/ $M^{-1}cm^{-1}$	k_{OH}/ $M^{-1}s^{-1}$ [a]	k_{OH}/ $M^{-1}s^{-1}$ [b]	Ref.
PhA (0.25)	286	0.520	2100	1.44±0.01	1.15 (25 °C) 5.6 (37.5 °C)	Ref. 8(a) Ref. 26
PNPhA	400	---	---	---	14.8 (25 °C) 23.9 (37.5 °C)	Ref. 9 Ref. 26
PhB (0.14)	290	0.590	4250	0.455±0.008	Not found	- - -
ASA (0.29)	296	0.890	3100	0.160±0.020	0.32 (25 °C) 0.16 (30 °C) 0.25 (37 °C) 0.56 (39 °C) 0.81 (56.4 °C)	Ref. 21 Ref. 22 Ref. 23 Ref. 24 Ref. 25

[a]This work; [b] literature values.

In this study, the behavior of cap as a nucleophile towards the hydrolysis of aromatic ester was analyzed. The cap concentration used was lower than 0.020 M and under acidity conditions of total ionization of cap, i.e. $[OH^-]_o = 2\cdot[cap] + [OH^-]_{free}$. Figure 2 shows representative data of A-t profiles for PhB hydrolysis catalyzed by captopril. In the absence of cap, the absorbance reading at 290 nm at the end of the reaction, A_∞, is due to the reaction products, PhO^- and $PhCOO^-$. In the presence of cap, a fast absorbance increase was observed in the first 5-10 min of the reaction; but enlarging the time scale more than 50-fold a second reaction appears, and as a consequence the absorbance decreases to reach the A_∞ readings observed in the absence of cap, compare Figs 2a and 2b. This finding says us that the initial product of the reaction is the thiolester of captopril (S-benzoylcaptopril, CAS: 75107-57-2P) that hydrolyses at much slower rates than its formation. In every case, the A-t profiles fit first-order integrated rate equation. Table 2 contains results of some typical experiments. It can be appreciate the relative rates of its formation(k_o)-to-hydrolysis(k_o'), as well as, the relative catalytic effect of [cap] over $[OH^-]$ for the reaction in water and in aqueous micellar medium of the surfactant tetradecyltrimethylammonium bromide (TTABr).

Table 2. Experimental conditions and parameters observed for the hydrolysis of PhB (0.17 mM) in alkaline medium both in the absence and presence of captopril (cap) and in water or in aqueous micellar medium of the cationic surfactant tetradecyl-trimethylammonium bromide (TTABr)

[cap]/ mM	$[OH^-]_{free}$/ mM	[TTABr]/ M	$A_\infty - A_o$ [a]	k_o/s^{-1}	k_o'/s^{-1}	effect (290 nm)
- - -	12.5	- - -	+0.326	6.05×10^{-3} [b]	N.D.[d]	A-increase
- - -	12.5	0.013	+0.359	8.13×10^{-3} [b]	N.D.[d]	A-increase
3.44	0.95	- - -	+0.717	1.83×10^{-3} [c]	- - -	A-increase
4.60	6.0	0.0027	+0.680	7.89×10^{-3} [c]	- - -	A-increase
			-0.386	- - -	$1.28\cdot10^{-4}$ [e]	A-decrease
4.60	6.0	0.013	+0.685	9.09×10^{-3} [c]		A-increase
			-0.345	- - -	$1.01\cdot10^{-4}$ [e]	A-decrease

[a]Net absorbance change (+, increase; - decrease); [b]rate constant of PhB hydrolysis by OH^-; [c]observed rate constant of thiolester formation; [d]not detected; [e] observed rate constant for thiolester hydrolysis.

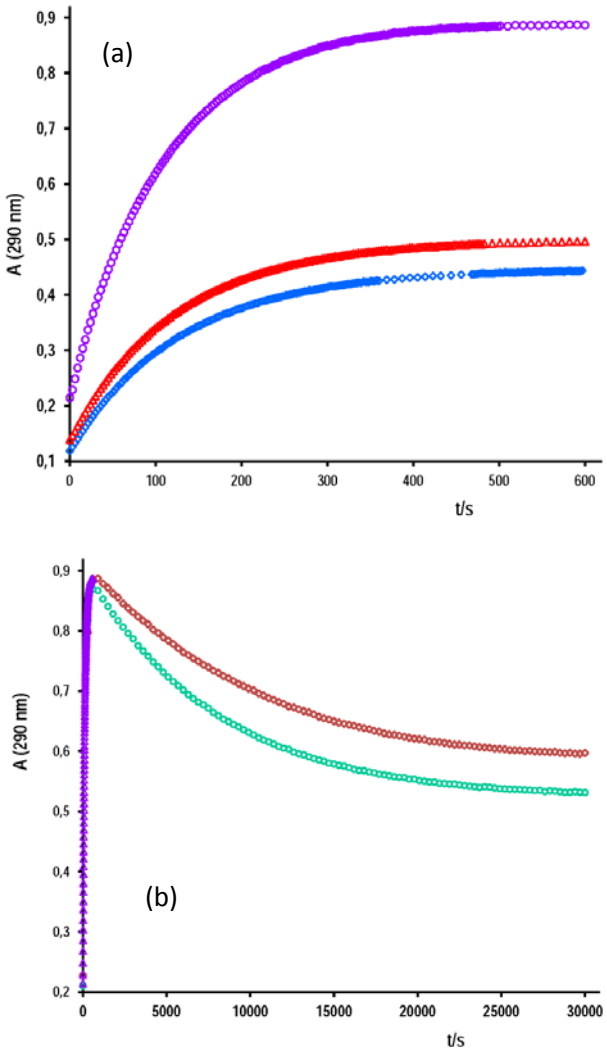


Figure 2. Absorbance-time profiles for (a)the S-benzoylcaptopril formation at (o)[cap]= 4.6 mM, $[OH^-]_f$=6.0 mM and [TTABr]=0.013 M; (△)no cap, $[OH^-]_f$=0.0125 M and [TTABr]=0.013 M, and (◇)no cap, no TTABr, $[OH^-]_f$=0.0125 M; and (b)(△)the same as for the set points (o) in (a) with the time scale enlarged 50-times; and S-benzoyl-captopril hydrolysis at (◇)$[OH^-]_f$=6.0 mM and [TTABr]=0.013 M, and (o)$[OH^-]_f$=6.0 mM and [TTABr]=2.67 mM.

The values of k_o' afford the following second-order rate constant for thiolester hydrolysis k'_{OH}=0.021 $M^{-1}s^{-1}$ or 0.017 $M^{-1}s^{-1}$ at [TTABr]=2.67 and 13 mM, respectively. Similar reaction found in the literature reports for the hydrolysis of the activated *p*-nitrobenzoyl ester of 2-(N,N-dimethylamino) ethanethiol the value of k'_{OH}=5.3 $M^{-1}s^{-1}$ at 50 °C, along with that of other thiolesters.[27]

By increasing [cap], the rate of the reaction path with cap also increases and, consequently, the concentration of S-benzoylcaptopril is higher, which means high values of A_∞ corresponding to the first reaction of thiolester formation. The rate of hydrolysis of S-benzoylcaptopril increases with $[OH^-]_{free}$, but in aqueous micellar medium of TTABr at concentrations higher than the critical micelle concentration, negligible hydrolysis was detected after 24 h.

The pseudo-first order rate constant, k_o, for the thiolester formation in the nucleophilic attack of cap to the carbonyl of

PhB, increases with the concentration of cap according to eq. (2), which suggest the mechanism of competitive reactions stated in Scheme 2.

$$k_o = k_o^w + k_{cap}[cap] \qquad (2)$$

Figure 3a shows three set of experiments performed in water at $[OH^-]_{free}$=1.0 mM and 6.0 mM and at pH 9.90 in a buffer of carbonate-bicarbonate at total buffer concentration 0.20 M. In aqueous alkaline medium, the slope of the straight line is, within the experimental error, independent of $[OH^-]_{free}$, *i.e.* k_{cap}=0.46±0.01 $M^{-1}s^{-1}$ at $[OH^-]_{free}$=6.0 mM and k_{cap}=0.42±0.01 $M^{-1}s^{-1}$ at $[OH^-]_{free}$=1.0 mM, which, as required, evidences the total ionization of cap. Conversely, the uncatalyzed reaction is significantly affected by $[OH^-]_{free}$, e.g. k_o^w=(2.74±0.08)×$10^{-3}s^{-1}$ or (0.45±0.03)×$10^{-3}s^{-1}$ at $[OH^-]$=6.0 or 1.0 mM, respectively, because $k_o^w=k_{OH}[OH^-]$. These extrapolated k_o^w values are in reasonable agreement with that determined from k_{OH} (Table 1) and the $[OH^-]_f$ used.

On the other hand, under excess of $[OH^-]$, the variation of the net absorbance change, A_∞-A_o, with [cap] describes saturation curves. According to Scheme 2, the reaction between PhB and hydroxide yields PhO^- and $PhCOO^-$ as reaction products, which contribute to the absorbance increase; the reaction path with cap yields also PhO^- and the thiolester (ThE), whose molar absortivity must be higher than that of benzoate. Therefore, high [cap] means high [ThE] and, consequently high absorbance change. Taking into account the contributions of the reaction products to A_∞ stated in eq. (3) for 1 cm optical path length and the products concentration at the end of the reaction, eq. (4), it is easy to arrive to eq. (5) that expresses the absorbance variation as a function of captopril concentration.

$$A_\infty - A_o = \varepsilon_{PhO}[PhO^-]_\infty + \varepsilon_{PhCOO}[PhCOO^-]_\infty + \varepsilon_{ThE}[ThE]_\infty \qquad (3)$$

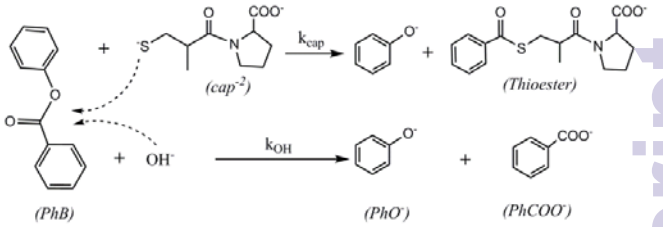
$$[PhO^-]_\infty = [PhB]_o$$

$$[PhCOO^-]_\infty = \frac{k_{OH}[OH^-][PhB]_o}{k_{OH}[OH^-] + k_{cap}[cap]} \qquad (4)$$

$$[ThE]_\infty = \frac{k_{cap}[cap][PhB]_o}{k_{OH}[OH^-] + k_{cap}[cap]}$$

$$A_\infty - A_o = \frac{A_\infty^o + A_\infty^{cap} f_k[cap]}{1 + f_k[cap]} \qquad (5)$$

In eq (5) A_∞^o (=$\{\varepsilon_{PhO}+\varepsilon_{PhCOO}\}$ $[PhB]_o$) is the absorbance at the end of the reaction in the absence of cap; A_∞^{cap} (=$\{\varepsilon_{PhO}+\varepsilon_{ThE}\}$ $[PhB]_o$) is the absorbance at high [cap], i.e. when [thiolester] equals that of PhB, and $f_k=k_{cap}/(k_{OH}[OH^-])$, which is a known parameter. Figure 3b shows the experimental results.



Scheme 2. Reaction paths of PhB cleavage in aqueous basic medium in the presence of captopril.

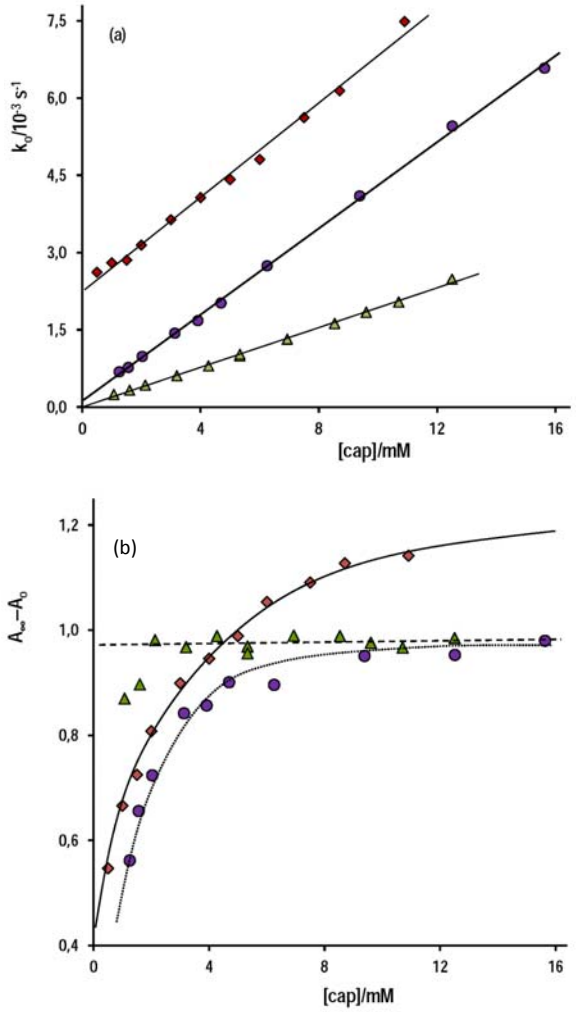


Figure 3. (a)Plot of k_o against [cap] for the reaction of cap and PhB due to S-benzoylcaptopril formation at (◆)$[OH^-]_f$=6.0 mM; (●)$[OH^-]_f$=1.0 mM , and (▲)buffer of carbonate-bicarbonate 0.20 M of pH 9.90; (b)variation of the net absorbance increase as a function of [cap] under the experimental conditions of (a).

The non linear least squares analysis of the experimental values of A_∞-A_o against [cap] gives the optimized parameters of A_∞^{cap} and f_k reported in Table 3 together with the calculated f_k values. There is a good concordance, mainly with the results at high $[OH^-]$, i.e. when the intercept of the straight line (reaction step by OH^-) is more significant. In addition, the molar absorptivity of S-benzoylcaptopril was estimated as (5150±150) $M^{-1}cm^{-1}$).

Table 3. Parameter obtained by fitting the net absorbance change, A_∞-A_o, against [cap] according to eq. (5)

[PhB]/ mM	$[OH^-]_f$/ mM	A_∞^o	A_∞^{cap}	$f_k^{(exp)}$	$f_k^{(cal)}$ (=$k_{cap}/(k_{OH}[OH^-])$)
0.21	6.0	0.487	1.60±0.06	176±20	168
0.145	1.0	0.340	1.075±0.025	620±60	840

Reaction of PhB in carbonate-bicarbonate buffer. When the reaction was performed in aqueous buffered solutions, the A-t profiles fit quite good to first-order integrated rate equation (cc>0.9999) and the absorbance readings at the end of the reaction, A_∞, are independent of [cap], but their values are much higher than those read in the absence of cap, e.g. compare A_∞=1.20 in buffer, and A_∞=0.45 in the absence of [cap] at the same [PhB]=0.145 mM, Figure 3b shows the net absorbance change as a function of [cap].

The lowest data set in Figure 3a corresponds to the variation of k_o as a function of [cap] in carbonate-bicarbonate buffer 0.20 M of pH 9.90. Again, a straight line was observed, but now the uncatalyzed reaction is negligible, i.e., k_o^w~0 and the slope is nearly half the value observed in excess of $[OH^-]$, that is k_o=(0.193±0.001)[cap].

The effect of [buffer] was studied at ionic strength 1.25 M ($NaClO_4$), [cap]=7.13 mM and pH 9.50. It was found a small catalysis: k_o/s^{-1}=(7.0±0.6)×10^{-4}+(7.5±0.3)×10^{-4}[buffer], which means a 20% increase at [buffer]=0.20 M, see Figure S4 of ESI.

Finally, the effect of pH was analyzed at [cap]=4.6 mM and total buffer concentration 0.20M. The observed rate constant increases strongly with the pH. Figure 4 displays the obtained results that describe a sigmoide curve, typical of a titration process, in which the inflection point can be noted at pH~10.2, that is the data k_o *versus* pH fit a 3rd-order polynomic curve, for which the second derivative equals zero at pH~10.2. These results confirm that the reaction occurs by the nucleophilic attack of the thiolate group of captopril on the carbonyl of phenylbenzoate to yield the S-benzoylcaptopril, which is quite stable at these pH values, Scheme 3.

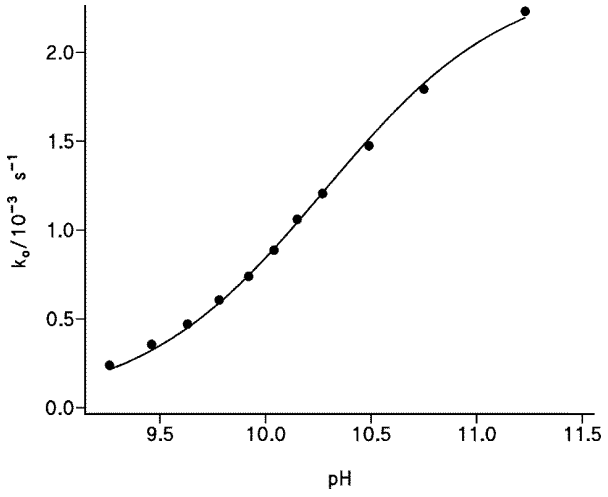
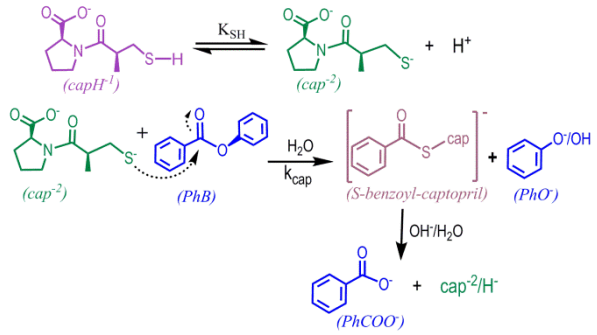


Figure 4. Plot of the pseudo-first order rate constant for the benzyl transfer reaction from PhB to cap ([cap]=4.6 mM) as a function of pH of carbonate-bicarbonate buffered solutions, [buffer]=0.20 M. Solid line fits eq. (6)



Scheme 3. Proposed reaction steps in aqueous buffered solution of CO_3^{-2}/CO_3H^-.

Taking into account that $[cap]_o=[capH^-]+[cap^{-2}]$ and the expression of K_{SH} given in Scheme 3, the observed rate constant is defined by eq. (6)

$$k_o = \frac{k_{cap}[cap]_o}{1 + 10^{(pK_{SH} - pH)}} \qquad (6)$$

The nonlinear correlation of k_o vs pH according to eq (6) yields the optimized values of pK_{SH}=10.27±0.02 and $k_{cap}[cap]_o$=(2.40±0.05)×10^{-3} s^{-1}. Solid line in Fig. 4 shows the calculated points from eq (6) using the optimized parameters. The kinetic pK_{SH} compares quite well with the value obtained from direct potentiometric titration; however, one might take into account the different experimental conditions, especially the ionic strength in 0.20 M of carbonate-bicarbonate buffer and the slight catalytic effect of buffer in the reaction rate.

With this in mind, the slope of the plot k_o *vs* [cap] for experiments at pH 9.90 and shown in Fig. 3a (triangles) is *slope*=$k_{cap}/(1+10^{(pK_{SH}-pH)})$; and then, k_{cap}=0.436 M^{-1}s^{-1}, whose value agrees perfectly with that obtained in excess of OH^-.

Reaction of PhB in cationic micelles. Considering that the thiolysis of PhB by cap in alkaline conditions involves the reaction between a hydrophobic substrate, PhB, and a negatively charged anion, cap^{-2}, the presence of cationic micelles of, for instance, TTABr should affect the reaction. The study of micellar effects on –SH nucleophilic reactivity has relevance to the question of the reactivity differences found in biological systems. For that, it was analyzed the effect of surfactant concentration on the thiolysis of PhB by cap in excess of OH^-. The addition of TTABr causes *ca.* a 4-fold increase in the reaction rate. Typical data of the variation of k_o against [TTABr] are showed in Figure 5. Close the cmc (~2.0 mM), the rate of the reaction increases with the [surfactant], goes through maxima, and decreases on further increment the [surfactant].

We have previously demonstrated that the cap anion binds effectively to cationic micelles of TTABr. The estimated value of the equilibrium binding constant was K_c=70 M^{-1}, but values up to 90 M^{-1} are also possible, since the binding process is mainly governed by the surfactant counterion, instead by the micelle hydrophobicity.[20] Since the ion exchange equilibrium constant between the Br^- surfactant counterions and OH^- *ca.* is K_{OH}^{Br}=55 at 25 °C,[28] at the hydroxide concentration used in these experiments one can neglected the amount of OH^- in

the micellar surface. Consequently, the ester cleavage in the aqueous pseudophase is due to both OH^- and cap^{-2}, whilst that in the micellar pseudophase is only due to cap^{-2}, being the corresponding bimolecular rate constants denote by k_{OH}, k_{cap}, and k_{cap}^m. By considering that the products of the reaction are the PhO^- and the S-benzoylcaptopril, which are stable under the reaction conditions, the k_o *vs* [TTABr] profiles can be quantitatively analysed using a phase separation model[29] that leads to eq. (7), where K_s and K_c are the distribution constants of PhB and the thiolate anion of cap, respectively, defined in terms of concentrations based on the total solution volume; k_{cap} and k_{cap}^m are the second-order rate constants in the aqueous and micellar pseudo-phases, respectively, and k_{OH} refers to the hydrolysis in water of PhB by OH^-; V_m is the molar volume of the micellar reaction region, a necessary datum for a direct comparison between reactivities in water and micellar phase; the assumed value of V_m is 0.37 L/mol (estimated V_m range from 0.14 to 0.37 L/mol),[30] and Dn represents the micellized surfactant, i.e. [Dn]=[surfactant]-cmc.

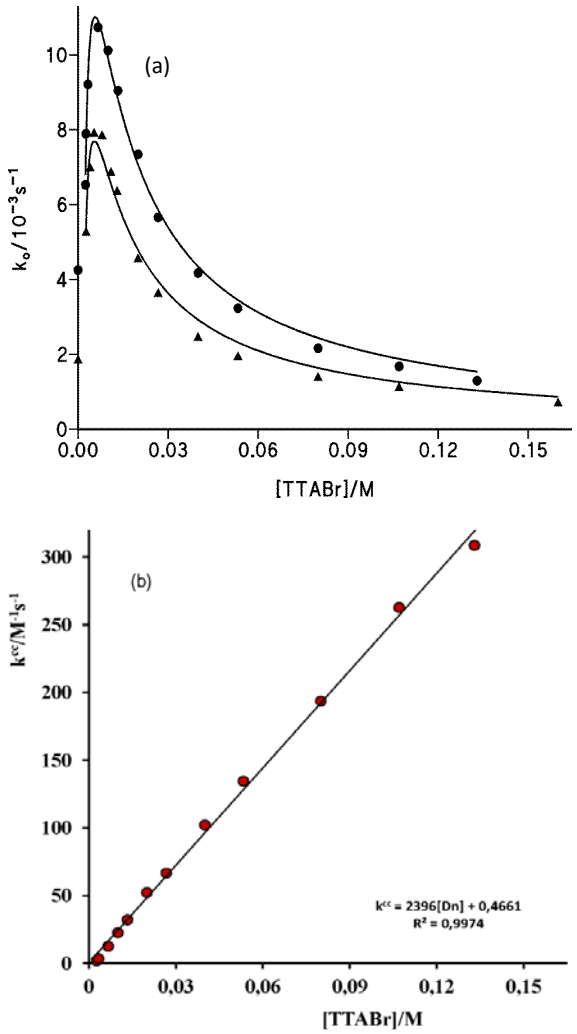


Figure 5. (a)Influence of [TTABr] on the observed rate constant, k_o. for the reaction of S-benzoylcaptopril formation at (•)[cap]=4.6 mM; $[OH^-]_f$=6.0 mM, and (▲)[cap]=3.4 mM, $[OH^-]_f$=1.0 mM. Solid line fits eq. (7). (b)Plot of k^{cc} versus [TTABr], see text, for k_o-values obtained at (•)[cap]=4.6 mM; $[OH^-]_f$=6.0 mM.

$$k_o = \frac{k_{OH}[OH^-]}{(1+K_s[Dn])} + \frac{\left(k_{cap} + \frac{k_{cap}^m}{V_m}K_sK_c[Dn]\right)[cap^{-2}]_o}{(1+K_s[Dn])(1+K_c[Dn])} \quad (7)$$

Equation (7) was fitted to the experimental data of Fig. 5 in the following manner. Values of K_c were determined in previous study and good plots were obtained if K_c=92 M^{-1} was assumed;[20] the cmc was fixed as the minimum required surfactant concentration to observe an increase in k_o (cmc=2 mM) and using the experimental values of k_{OH} (Table 1) and k_{cap}, *vide supra*. The best estimates of k_{cap}^m and K_s were obtained by successive iterations. Solid lines shown in Fig. 5a correspond to the calculated points from eq. (7) with the optimized K_s=650 M^{-1} and k_{cap}^m=0.015 $M^{-1}s^{-1}$.

From these estimated data, the plot of k^c (=$k_o(1+K_s[Dn])$ against [Dn] should be a saturation curve as it can be observed in Figures S5 and S6(a) of ESI. In addition, the plot of k^{cc} (=$\{k_o - k_{OH}[OH^-]\}\cdot\{1+K_c[Dn]\}/[cap^{-2}]_o$) *versus* [Dn] must be a straight line of *intercept*=k_{cap} and *slope*=$K_sK_c(k_{cap}^m/V)$. The resulting graphs shown good linear relationship and the values of k_{cap} are in good agreement with that determined in the absence of surfactant, see Figure 5b and Figure S6(b) of ESI. The rate constant of PhB thiolysis by cap in the micellar interfase is 30-fold lower than that in water, because of the lower polarity of the micellar interfase, which makes the reaction transition state less stable. Charged transition states are stabilized in polar media. Then, the catalysis observed at low surfactant concentrations is due to the concentration effect of reagents, PhB and cap, in the small volume of the micelle.

Reaction of PhA and ASA with cap. The influence of cap on the thiolysis of PhA was followed at 286 nm by registering the increasing absorbance due to PhO^-. Under the experimental conditions of [PhA]=0.23 mM and $[OH^-]_f$=1.0 mM, the observed rate constant increases more than 8-fold with [cap] up to 0.015M, that is $k_o/s^{-1}=(1.3\pm0.2)\times10^{-3}+(0.62\pm0.03)[cap]$. The total absorbance increase at the end of the reaction reaches an average value of $A_\infty-A_o$=0.500±0.020. Assuming that the only absorbing species is the phenolate anion, then the molar absortivity was estimated as ε=2170 $M^{-1}cm^{-1}$, which is in good agreement with data in Table 1 corresponding to alkaline hydrolysis.

The hydrolysis of acetylsalicylic acid (ASA) is near 10-fold slower than that of PhA. The pK_a of ASA is 3.50; then, in alkaline medium it is a negatively charged substrate. Therefore, the nucleophilic cleavage of ASA implies the approach of two anions: acetylsalicylate and cap^{-2} or OH^-. The approach is difficult by electrostatic repulsion (high activation entropy), in fact, under conditions of fully ionized cap, the increase of [cap] from 1.78 to 17.9 mM at $[OH^-]_f$=0.010 M and ionic strength 0.10 M ($NaClO_4$) does not affect at all the observed rate constant, $k_o=(1.51\pm0.05)\times10^{-3}$ s^{-1}, see the upper most line on Figure 6. By supposing the cleavage goes completely through the attack by OH^-, one gets k_{OH}=0.11 $M^{-1}s^{-1}$, that is, the value obtained in the study of the influence of $[OH^-]$ in the absence of cap, see Table 1

Table 4. Experimental conditions and rate constants obtained in the study of the effect of cap on the ester hydrolysis of ASA in aqueous carbonate-bicarbonate buffer.

[buffer]/ M	pH	[cap]/ mM	k_o^w/ $10^{-4}s^{-1}$	*slope*/10^{-3} $M^{-1}s^{-1}$ (eq.2)	k_{cap}/ $M^{-1}s^{-1}$	A_∞-A_o (290 nm)
0.17	10.20	1.8 to 23	0.460±0.005	(7.850±0.005)	0.0128	0.98±0.05
0.15	10.55	2.2 to 21	0.74±0.01	(8.95±0.15)	0.0115	0.97±0.04

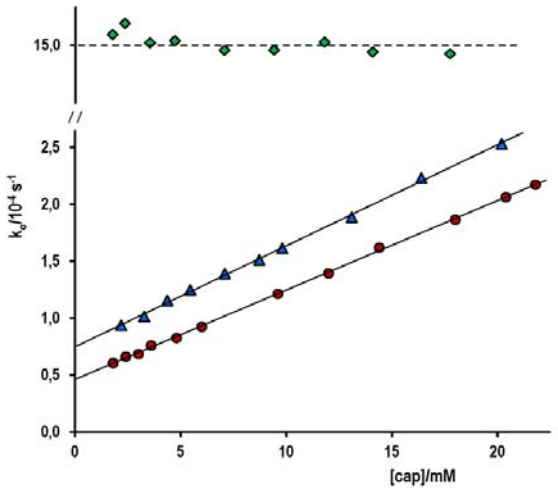


Figure 6. Variation of k_o for the acyl transfer reaction from ASA to cap as a function of [cap] at (◆)$[OH^-]_f$=0.010 M; (▲)carbonate-bicarbonate buffer 0.15 M of pH 10.50, and (●)at [buffer]=0.17 M of pH 10.20.

In order to be able to measure the second-order rate constant due to the attack by cap, the reaction was studied in carbonate-bicarbonate buffer under the experimental conditions listed in Table 4. In both cases, k_o increases nearly 4-fold at the highest [cap], see Figure 6. Again, good straight lines were obtained, but now the corresponding slope is: $slope=k_{cap}/(1+10^{(pK_{SH}-pH)})$, from which one determines the k_{cap} listed in Table 4 for the second-order rate constant for the ASA cleavage by captopril. On the other hand, either in alkaline medium or in buffer solutions of carbonate-bicarbonate, the average absorbance increase at the end of the reaction was observed as A_∞-A_o=0.977±0.050 for [ASA]=0.29 mM, and consequently $\varepsilon_{(290nm)}$=3380 $M^{-1}cm^{-1}$ in excellent agreement with data in Table 1.

2.3. Hydrolysis by cysteine (cys)

For comparison purposes, the acyl transfer reaction of both PhA and ASA, as well as, the benzyl transfer reaction of PhB, to cysteine were analysed in aqueous alkaline medium at constant $[OH^-]$ and [ester].

Cysteine contains three ionizable groups of pK_a 1.96 (-COOH), 8.20 (-SH) and 10.28 ($-NH_3^+$). The total $[OH^-]$ was fixed equal to $[OH^-]=[OH^-]_f+2\cdot[cys]$, in order to keep constant the dianion concentration (^-S-CH_2-$C(COO^-)$-NH_2). The [cys] was varied between 1.6-to-26.7 mM and, in order to reduce the alkaline hydrolysis, the $[OH^-]_f$=1.0 mM. Under these conditions, good first-order kinetics was obtained in every case. The k_o increases with [cys] following linear relations (Figure S7 of ESI) as for cap, eq. (2). The corresponding results are reported in Table 5.

Table 5. Rate constants obtained in the ester cleavage in the presence of cysteine at $[OH^-]_f$=1.0 mM and 25 °C.

rate constants	PhA (0.23 mM)	PhB (0.14 mM)	ASA (0.23 mM)
$k_o^w/10^{-3}s^{-1}$	1.00±0.15	0.515±0.040	0.145±0.003
$k_{cys}/M^{-1}s^{-1}$	0.460±0.010	0.213±0.003	0.0065±0.0003

Under conditions of completely ionization of cys, there are *a priory* three nucleophilic centers. However, the carboxylate group is the poorest nucleophile, so this reaction path can be neglected.[31] On the other hand, the bimolecular rate constant for the attack of amines of similar pK_a than that of the amine group of cys, such as dimethylamine (pK_a 10.64) or n-butylamine (pK_a 10.59), to phenylacetate (PhA) was determined as k_{ami}=0.075 $M^{-1}s^{-1}$, *i.e.* more than 6-fold slower than that determined here, which we assumed to be due to the thiolate attack.[8b]

Finally, and putting all the results together, the Brönsted type correlation plot of *log*(k_{Nu}) *vs.* pK_a yields quite good straight lines for the three nucleophiles studied in this work and whose results a summarized in Table 6 (see Figure 7), with the excepcion of the datum of cap+PhB that, as we have demonstrated, correspond to a different reaction mechanism.

Table 6. Bimolecular rate constants for the nucleophilic attack of hydroxide ion or of the thiolate ion of cap or cys to the carbonyl of aryl esters.

nucleophile	pKa	$k_{Nu}/M^{-1}s^{-1}$	PhA	PhB	ASA
OH^-	15.74	k_{OH}	1.44	0.438	0.157
cap^{-2}	10.00	k_{cap}	0.620	0.488	0.0128
cys^{-2}	8.20	k_{cys}	0.460	0.213	0.0065

3. Conclusions

The best fit rate constants give in Table 6 invite some comparisons. The second-order rate constant for hydroxide attack on the aryl esters span a range near 10-fold with the order being PhA>PhB>ASA. The ordering might be explicable on electrostatic grounds reflecting the stabilization for the transition state for nucleophilic attack on neutral PhA relative to attack on anionic ASA; the relative rate decrease of PhB to PhA is due to the resonance effect in PhB, Scheme 1. The same ordering was observed with cap or cys as nucleophiles, nevertheless, the spanned range enlarges up to 50-fold or 70-fold, respectively to cap or cys, in agreement with reactivity-selectivity principle, in which as the nucleophile reactivity increases, the selectivity decreases. The results indicate also that PhA, PhB or ASA react with the thiolate anion of cap or cys in mild basic medium by the nucleophilic route. An observation which is reconciled by the well-established principle of acyl-, or benzyl-, transfer reactions in which when the nucleophile is less basic than the leaving group the reaction involves rate-determining attack of the nucleophile. This is consistent with the low sensitivity (β_{Nu}~0.1) of the rate constants to the basicity of the thiol. As a particular case, the reaction of cap and PhB leads to the rapid formation of the thiolester:

benzoylcaptopril. This compound shows to be quite stable in buffer solutions of pH~10, as well as, in aqueous micellar solutions. The presence of cationic micelles enhances the rate of S-benzoyl-captopril formation, due to reagents concentration effect, and, practical suppresses its decomposition.

By considering that thiol esters are common intermediates in the catalytic cycle of cysteine proteases, the systems studied here can be seen as biomimetic models of cysteine proteases that cleave ester substrates with the intermediacy of an S-acyl-enzyme, such as it occurs in the phenylbenzoate cleavage by captopril.

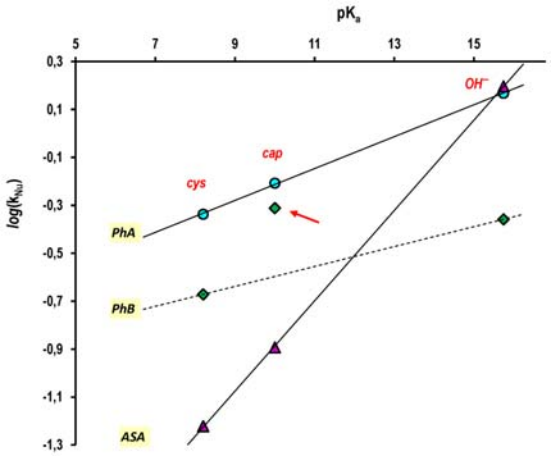


Figure 7. Bronstedt plot for the nucleophilic reaction of (•)PhA; (◆)PhB, and (▲)ASA with cysteine (cys), captopril (cap) or OH^-. The correlations are: (PhA) $\log(k_{Nu})= 0.066 \cdot pK_a-0.88$; (ASA) $\log(k_{Nu})=0.19 \cdot pK_a-2.77$, and (PhB) $\log(k_{Nu})=0.041 \cdot pK_a-1.01$ estimated; the point indicated with a red arrow was not included.

4. Experimental

Materials. All reagents were of the maximum available purity and were used without further purification. Esters (phenylacetate, phenylbenzoate, and acetylsalicylic acid), thiols (cysteine and captotpril or N-(3-mercapto-2-methylpropionyl)-L-proline), and the surfactant tetradecyl-trimethylammonium bromide (TTABr) were obtained from Sigma-Aldrich.

Stock solutions of esters were prepared in dried dioxane, spectrophotometric grade. A small volume of this solution (40 or 50 µL) was added to the reaction sample (V=3 mL) to start the reaction. The percentage of dioxane in the final reaction mixture never exceeded 2% v/v. The rest of the solutions were prepared in water that was firstly deionized and subsequently twice distilled (the first distillation over potassium permanganate). Cysteine solutions were prepared just before using them to avoid oxidation to cystine.

Techniques. The UV-vis spectra and kinetics of slow reactions ($t_{1/2}$>60 s) were recorded with a Kontron-Uvikon double beam spectrophotometer fitted with thermostated multicell holders. Kinetics of fast reactions was studied on a Bio-Logic SFM-20 stopped-flow system interfaced with a computer and operated by Bio-Kine32 software (V4.51, 2009). The pH was controlled using buffer solutions of carbonate-hydrogen carbonate and was measured with a Crison 2001 pH-meter equipped with a GK2401B combined glass electrode and calibrated using commercial buffers of pH 4.01, 7.02, and 9.26 (Crison). The reported [buffer] refers to the total buffer concentration. In alkaline (NaOH) medium the acidity was reported as [OH^-].

Methods. The rate of ester hydrolysis was followed by noting the optical density increase due to the formation of products. Experiments were performed under pseudo-first order conditions with the ester as the limiting reagent concentration. Then, the integrated method was used to fit the experimental data of absorbance (A) *versus* time (t) to the first-order integrated rate equation, $A=A_\infty+(A_o-A_\infty)\cdot exp(-k_o t)$. The non-linear regression analysis gives k_o, A_o, and A_∞ as optimizable parameters, with k_o being the pseudo-first order rate constant and A, A_o, and A_∞, the absorbance readings at times t, zero, and at the end of the reaction. All experiments were carried out at 25 °C.

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