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The role of a β-proton transfer donor in the degradation of benzylpenicillin

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Abstract

The thiolysis mechanism of benzylpenicillin has been determined by ^{1}H NMR and HPLC. The degradation of benzylpenicillin was accelerated when the thiol used presented a β-group capable of acting as a general acid catalyst, such as α-monothioglycerol, 2-mercaptoethanol and mercaptoethylamine. With these thiols, after the formation of the thioester, an intramolecular acyl transfer reaction occurs at a pH far below the pK_a of the group acting as a general acid catalyst, which shows that the proton transfer has already occurred, probably in a concerted mechanism with the nucleophilic attack. Rate constants were calculated. The system can be taken as a simple model of the general acid catalyst in serine and cysteine proteases.

Keywords: Thiolysis; Penicillin; β-Lactam antibiotics; Proton transfer reaction

1. Introduction

Chemical reactivity of β-lactam antibiotics has been extensively studied due to the extraordinary importance of these compounds as antibacterial agents [1,2]. The carbonyl carbon of β-lactam ring shows a high susceptibility towards nucleophilic reagents. The nucleophilic substitution at the carbonyl center is an acyl transfer process involving covalent bond formation between the carbonyl carbon and the nucleophile and C–N bond fission of the β-lactam ring. This reaction goes through the reversible formation of a tetrahedral intermediate [3,4].

Kinetic studies on that kind of reactions show the formation of tetrahedral intermediate as being the rate-limiting step in hydroxylic hydrolysis of β-lactams [5–7]. In alcoholysis [8] and aminolysis [9] the rate-limiting step is the breakdown of such tetrahedral intermediate.

Alkaline hydrolysis of β-lactam antibiotics has been taken as a measure of their reactivity against the penicillin binding proteins (their target enzymes) and β-lactamases (their inhibitory enzymes) [10,11]. For this reason the alkaline hydrolysis has been more thoroughly studied than the other nucleophilic reactions in antibiotics [3–7,12–14].

In a previous work, we studied the thiolysis of benzylpenicillin using thiols of different basicity [15]. Results indicated that, as in alcoholysis and aminolysis, breakdown of tetrahedral intermediate was the rate-limiting step. Results suggest the existence of an intramolecular acid catalysis when thiols used as reagents bear β-groups capable of acting as proton donors.

In this paper, we investigate in detail that aspect of thiolysis. Thiols with different β-groups have been used in order to check the presence of the intramolecular acid catalysis. The mechanisms of formation and breakdown of the thio–acyl intermediate

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are studied and the corresponding associated rate constants related to the influence of that proton donor β-group are determined.

The thiolysis reaction of β-lactams is of interest because of the potential of these compounds to inhibit cysteine proteases. Furthermore the basic aspects of this chemical mechanism could be considered as a simple model of the mechanism of some proteases (serine, cysteine and aspartic proteases) which catalyse fission of C–N bond in protein's amide linkage [16] and β-lactamases which perform the same function in β-lactam structures [17].

2. Experimental section

2.1. Materials

Thiols (α-monothioglycerol, 1-mercapto-2-propanol, 3-mercaptopropanol, 2-mercaptoethanol, mercaptoethylamine) were purchased from Sigma and used without further purification. Benzylpenicillin (potassium salt) was of general reagent grade and other materials of AnalaR grade. Freshly boiled deionised water was used throughout and the ionic strength maintained at 0.5 mol dm^{-3} with potassium chloride. Deuterium oxide (99.9% D) was obtained from Sigma. The thiol buffers were prepared by partial neutralisation just prior to the kinetic run.

2.2. HPLC

Shimadzu-LC-9A chromatograph with a Rheodyne 7125 universal injector and a Shimadzu-SPD-M6A UV–VIS photodiode array detector were used for the HPLC experiments. The column was a Spherisorb ODS-25 × 0.46 cm × 5 μm; eluent 0.1 mol dm^{-3} NH_4OAc/MeCN 88:12; flow rate 1.2 ml min^{-1}. Reaction conditions, such as temperature and pD where chosen in order to obtain adequate reaction times.

Hydrolysis reactions were studied using this technique due to the low rate constants of the hydrolysis reactions compared with the rate constants of the thiolysis of benzylpenicillin. The kinetics of those reactions were studied by following the decrease in HPLC peak areas for benzylpenicillin at 225–228 nm and at the desired temperature using carbonates (I = 0.5 mol dm^{-3}) as buffer at the same pD as the thiolysis reaction. The buffer solutions were preincubated at constant temperature prior to the kinetic run.

2.3. NMR spectroscopy

The NMR spectra were obtained on Bruker AMX-300 spectrometer. Sample tube diameter, 5 mm. 3-(Trimethylsilyl)-1-propane-sulfonic acid (DSS) was used as internal reference. Chemical shift values (δ) are given in ppm. Coupling constants are in Hertz.

Benzylpenicillin (33 mg) was dissolved in 1 ml of thiol buffer (mercaptoethanol, pD = 10.1 or α-monothioglycerol, pD = 9.60) and ionic strength of 0.5 mol dm^{-3}. Progress of the reaction was monitored by ^{1}H NMR at 20°C.

The expected hydrolysis product, 5*R*-, 6*R*-benzylpenicilloic acid was synthesised enzymatically. Benzylpenicillin (110 mg) was dissolved in 5 ml of D_2O to which 200 ml of a solution of *Enterobacter cloacae* P99 β-lactamase was added. The reaction was followed by UV and ^{1}H NMR and after 2 h benzylpenicillin was no longer detected and the solution was lyophilised. The subsequent degradation of 5*R*-, 6*R*-benzylpenicilloic acid (37 mg) dissolved in 1 ml of thiol buffer of mercaptoethanol (pD = 10.1) or α-monothioglycerol (pD = 9.60) was followed by ^{1}H NMR at 20°C.

2.3.1. Benzylpenicillin (**1**)

^{1}H NMR (300 MHz, DSS) δ: 1.48 (s, 3H, 2-α-CH_3), 1.58 (s, 3H, 2-β-CH_3), 3.68 (s, 2H, PhCH_2), 4.24 (s, 1H, 3-H), 5.45 (d, 1H, 6-H, $J_{6.5}$ 3.9), 5.52 (d, 1H, 5H, $J_{5.6}$ 3.9), 7.4 (m, 5H, Ph).

2.3.2. 5R-, 6R-monothioglycerol benzylpenicilloate (**2**)

^{1}H NMR (300 MHz, DSS) δ: 1.22 (s, 3H, 2-α-CH_3), 1.53 (s, 3H, 2-β-CH_3), 3.46 (s, 1H, 3-H), 3.85 (s, 2H, PhCH_2), 4.61 (d, 1H, 5-H, $J_{5.6}$ 6.7), 5.14 (d, 1H, 6-H, $J_{6.5}$ 6.7), 7.4 (m, 5H, Ph). Rest of signals buried under thiol buffer signals.

2.3.3. 5R-, 6R-mercaptoethanol benzylpenicilloate (**3**)

^{1}H NMR (300 MHz, DSS) δ: 1.23 (s, 3H, 2-α-CH_3), 1.56 (s, 3H, 2-β-CH_3), 3.45 (s, 1H, 3-H), 3.71 (s, 2H, PhCH_2), 4.64 (d, 1H, 5-H, $J_{5.6}$ 6.6), 5.15 (d, 1H, 6-H,

$J_{6.5}$ 6.6), 7.4 (m, 5H, Ph). Rest of signals buried under thiol buffer signals.

*2.3.4. 5R-, 6R-benzylpenicilloic acid (**6**)*

^{1}H NMR (300 MHz, DSS) δ: 1.20 (s, 3H, 2-α-CH_3), 1.49 (s, 3H, 2-β-CH_3), 3.40 (s, 1H, 3-H), 3.70 (s, 2H, PhCH_2), 4.21 (d, 1H, 5-H, $J_{5.6}$ 6), 5.02 (d, 1H, 6-H, $J_{6.5}$ 6), 7.4 (m, 5H, Ph).

*2.3.5. 5S-, 6R-benzylpenicilloic acid (**7**)*

^{1}H NMR (300 MHz, DSS) δ: 1.02 (s, 3H, 2-α-CH_3), 1.55 (s, 3H, 2-β-CH_3), 3.39 (s, 1H, 3-H), 3.79 (s, 2H, PhCH_2), 4.77 (d, 1H, 6-H, $J_{6.5}$ 3.3), 5.05 (d, 1H, 5-H, $J_{5.6}$ 3.3), 7.4 (m, 5H, Ph).

2.4. Determination of dissociation constants

The apparent pK_a's values of α-monothioglycerol and mercaptoethylamine were determined potentiometrically in D_2O and H_2O at an ionic strength of 0.5 mol dm^{-3}.

The calculated α-monothioglycerol pK_a value at 20°C in D_2O was 10.1 ± 0.1 and for mercaptoethylamine at 20°C the calculated pK_a's were 8.7±0.1 and 11.6±0.1 in D_2O, and 8.4±0.1 and 11±0.1 in H_2O.

2.5. Determination of free thiol content

Aliquots of the reaction mixture ([thiol] = 0.5 mol dm^{-3}, [PenG] = 1.1 × 10^{-2} mol dm^{-3}) were diluted 500 times and then assayed for free thiol content by using the method of Ellmann [18]. A stock solution of 5,5′-dithiobis(2-nitrobenzoic acid), DTNB, 41.3 mg in 10 ml of 0.1 mol dm^{-3} phosphate buffer pH = 7, was prepared and stored in dark. Samples of the diluted reaction (100 μl) were withdrawn at different times, mixed with 2.5 ml of phosphate buffer pH = 8 and 40 μl of stock DTNB solution added. After 2 min the absorbance at 412 nm was measured against a blank solution lacking thiol. A new blank was prepared for every measurement.

3. Results and discussion

The kinetics of the degradation of benzylpenicillin (in aqueous solutions) with thiols were studied using HPLC and ^{1}H NMR using the thiol both as a reagent and buffer. A major problem of studying the reactions of thiols is their ready oxidation to disulphides, particularly in alkaline solution. Disulphide formation was monitored by measuring the loss of thiol using DTNB under the same conditions used to follow the kinetics of benzylpenicillin with thiol aqueous solution. For α-monothioglycerol, i.e. the loss of thiol after 4 days was 4% at 20°C and 12% at 30°C, while for mercaptoethylamine the loss of thiol was 80% after 6 h. Doing the experiments in flame-sealed ampoules under Argon solved this problem and formation of disulphide bridges was kept to a minimum.

Fig. 1 shows the evolution of the ^{1}H NMR spectra with time for the thiolysis of benzylpenicillin using α-monothioglycerol by ^{1}H NMR at 20°C and pD = 9.6. The first spectrum (4 min after the initiation of the reaction) shows signals of benzylpenicillin (**1**). After 20 min, signals corresponding to the thioester (**2**), benzylpenicillin (**1**) and 5*R*-, 6*R*-benzylpenicilloic acid are present (**6**). After 3 h, all the signals corresponding to the benzylpenicillin and the thioester have disappeared. At this time, the main product is 5*R*-, 6*R*-benzylpenicilloic acid (**6**). After 160 h the main product is 5*S*-, 6*R*-benzylpenicilloic acid (**7**), with the 5*R*-, 6*R*-epimer, in a ratio of ca. 7:1. Signals at 3.10 and 4 ppm correspond to the dimerisation of α-monothioglycerol via disulphide bridges formation, as discussed later on.

The ^{1}H NMR spectrum over the range 4.3–5.5 ppm for the α-monothioglycerol thiolysis of benzylpenicillin at 20°C is shown in Fig. 2. Signals at 5.15 and 4.60 ppm correspond to H5 and H6 of the thioester, with a coupling constant of 6.7 Hz. The H6 signal at 4.6 ppm actually appears as two doublets which are attributed to diastereoisomers of the thioester, arising from the chirality of the α-monothioglycerol and the fact that the racemic thiol was used, rather than coupling to the 6-acylamido NH. This assignment was confirmed by decoupling experiments. Similar behaviour was observed with other racemic thiols such as 1-mercapto-2-propanol (two doublets at 4.6 ppm but only one at 5.1 ppm). With non-chiral thiols such as 2-mercaptoethanol a simple doublet was seen for H6.

As the reaction develops another set of signals appear at 5.1 ppm (a new doublet) and at 4.58 ppm (two new doublets). The intensity of these new signals

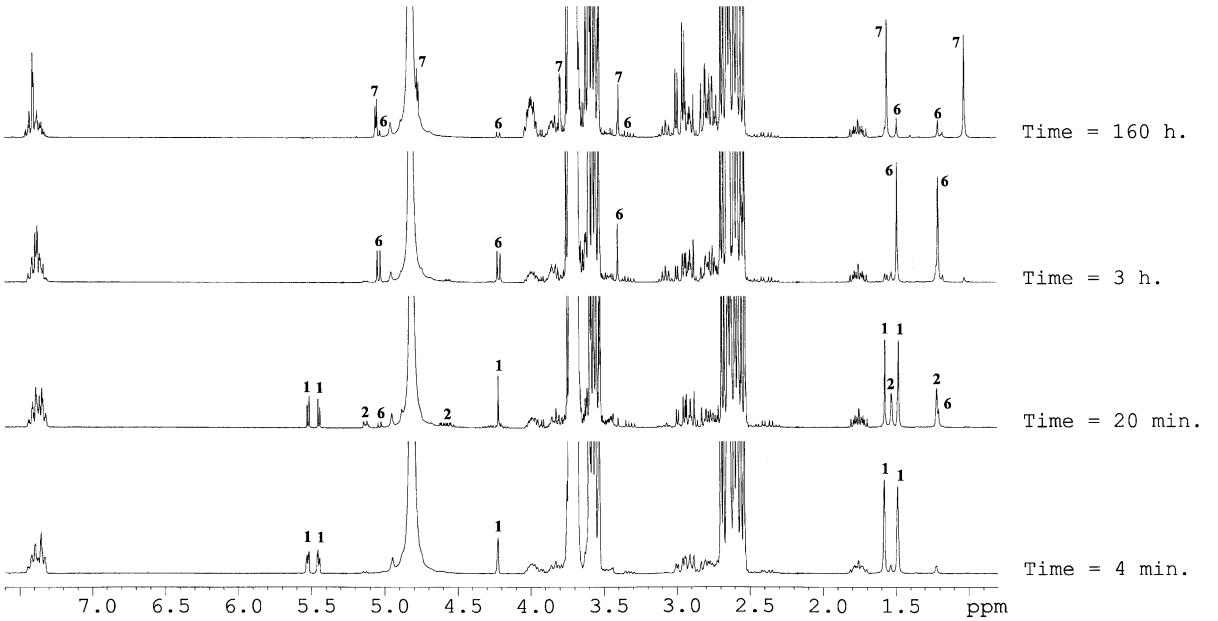


Fig. 1. Evolution of ^{1}H NMR spectra of the thiolysis of benzylpenicillin by α-monothioglycerol with time at pD = 9.6 and $T = 20^\circ$C. Numbers correspond to structures of Scheme 1.

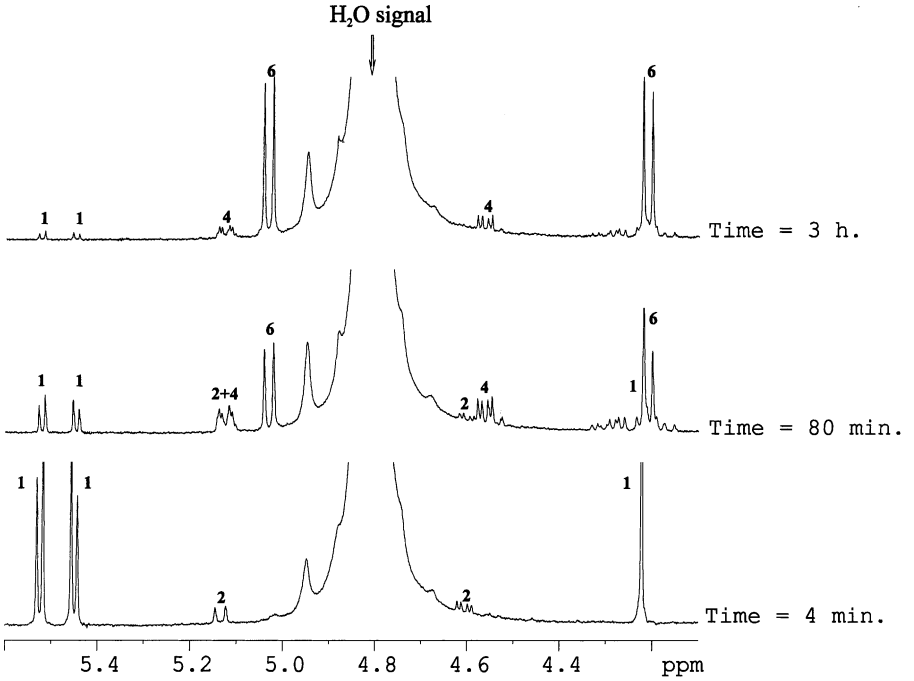
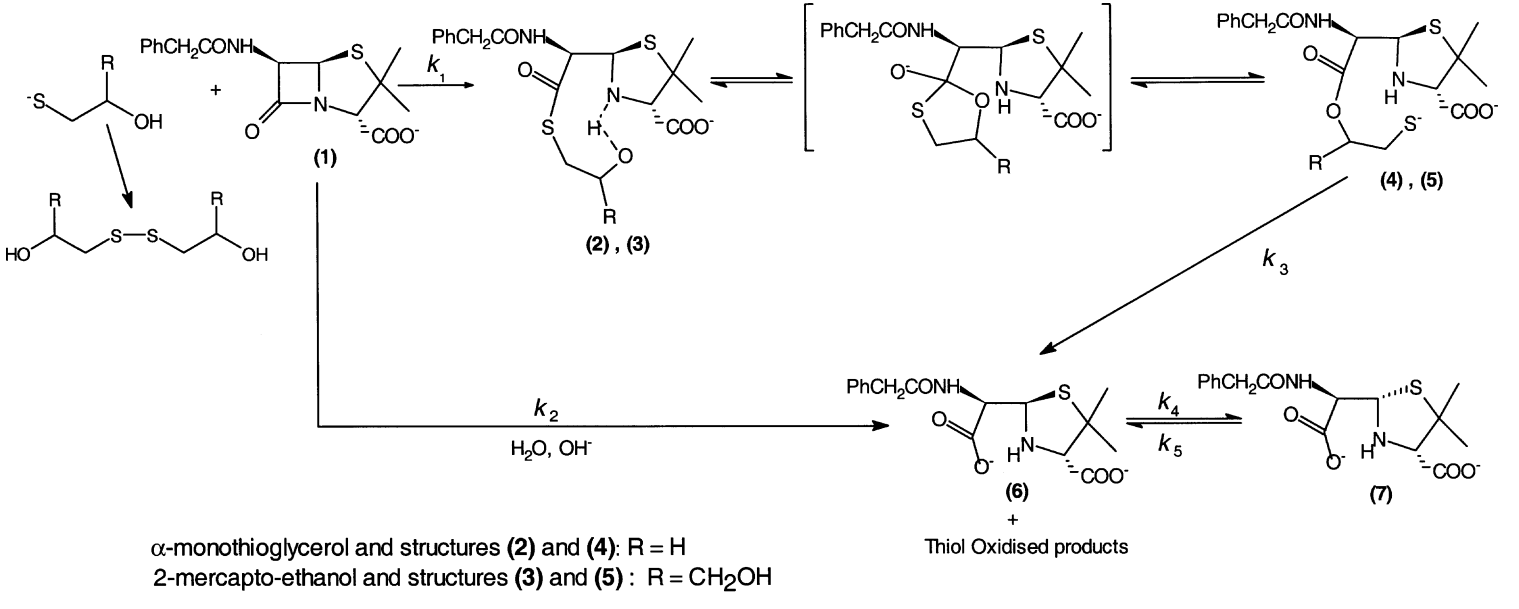


Fig. 2. Evolution of ^{1}H NMR spectra of the degradation of the 5*R*-, 6*R*-monothioglycerol benzylpenicilloate with time at pD = 9.6 and $T = 20^\circ$C. Magnified area between 4.3 and 5.5 ppm. Numbers correspond to structures of Scheme 1.


PhCH2CONH
R
S
OH
+
O
N
COO-
(1)
k1
(2) , (3)
(4) , (5)
k3
HO
S—S
k2
H2O, OH-
(6)
+
Thiol Oxidised products
k4
k5
(7)
α-monothioglycerol and structures (2) and (4): R = H
2-mercapto-ethanol and structures (3) and (5) : R = CH2OH

Scheme 1.

increases as the former ones, corresponding to the thioesters decrease. This can be explained by intramolecular alcoholysis to give *S*- to *O*-acyl transfer [19–24]. At the pD of this reaction is 9.6, direct alcoholysis by the hydroxyl group of α-monothioglycerol is not expected to occur. In the thioester, the β-hydroxyl group is suitably placed to attack the carbonyl of the thioester, through the formation of a five-membered ring intermediate, to give the mercaptoglycerate ester (Scheme 1). The mercaptoglycerate ester can suffer a rearrangement giving 5*R*-, 6*R*-benzylpenicilloic acid and an oxidised form of the thiol, which cannot act as a nucleophile again. 5*R*-, 6*R*-benzylpenicilloic acid slowly epimerises to the more stable form 5*S*-, 6*R*-benzylpenicilloic acid. There is no evidence that epimerisation occurs at C6 at the thioester or the ester state.

The ^{1}H NMR spectrum over the range 1.2–1.7 ppm is shown in Fig. 3. The signal at 1.53 ppm, corresponding to the 2-β-CH_3 of the thioester, becomes broader as the reaction develops and the thioester undergoes intramolecular rearrangement to the ester. This effect is not observed on the signal corresponding to the 2-α-CH_3 (1.2 ppm) and probably results from the closer proximity of the 2-β-CH_3 to the groups attached to C6.

The reaction pathway outlined in Scheme 1 may be used to explain the observations. Thiols facilitate hydrolysis by nucleophilic catalysis involving the formation of a penicilloyl thioester (**2**). If the thiol contains an alcohol group in the β-position (as in the case of the α-monothioglycerol or 2-mercaptoethanol), the thioester undergoes a *S*- to *O*-acyl transfer via an intramolecular alcoholysis to give the ester (**4**) which intramolecularly rearranges to give benzylpenicilloic acid (**6**). The hydrolysis product 5*R*-, 6*R*-benzylpenicilloic acid (**6**) slowly epimerises to the more stable 5*S*-, 6*R*-benzylpenicilloic acid (**7**) as previously shown. These thiols therefore do not follow the general reaction mechanism previously described [15] where the thioesters formed are hydrolysed giving the expected 5*R*-, 6*R*-benzylpenicilloic acid.

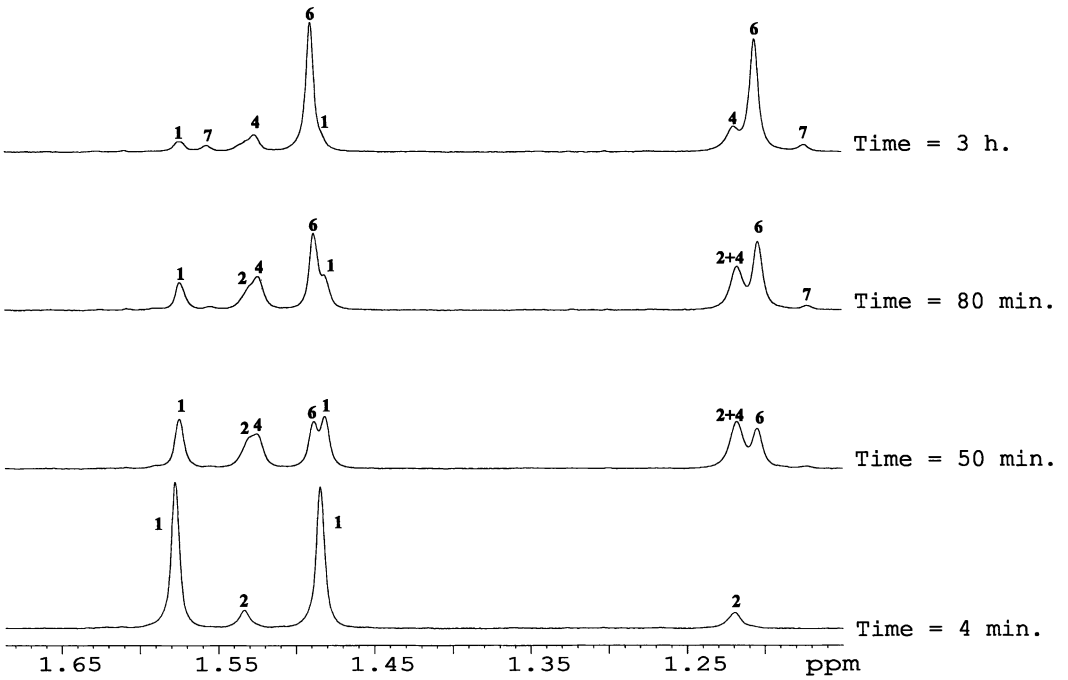


Fig. 3. Evolution of ^{1}H NMR spectra of the degradation of 5*R*-, 6*R*-monothioglycerol benzylpenicilloate with time at pD = 9.6 and $T = 20^\circ$C. Magnified area between 1.2 and 1.7 ppm. Numbers correspond to structures of Scheme 1.

The hydrolysis constant, k_2, of Scheme 1 was determined by a separate experiment following the degradation of benzylpenicillin in carbonates buffer, pD = 9.60 and $T = 20^{\circ}$C by ^{1}H NMR. The isomerisation constants, k_4 and k_5 were determined directly from the isomerisation of the 5*R*-, 6*R*-benzylpenicilloic acid (which was synthesised, isolated and purified, using *Enterobacter clocae* P99 β-lactamase) in α-monothioglycerol and carbonates buffers, pD = 9.60 and $T = 20^{\circ}$C. Kinetic constants obtained when α-monothioglycerol was used as buffer were identical to those obtained when carbonates were used as buffer, proving that the reaction of epimerisation of the 5*R*-, 6*R*-benzylpenicilloic acid is not α-monothioglycerol catalysed. Adjusting the experimental data to the kinetic Scheme 1 all kinetic constants were determined. The value of these rate constants k_2, k_4 and k_5 were identical to those obtained when these constants were introduced as variables into the general scheme. Individual rate constants were calculated using the software program GIT [25,26], which performs numerical integration of the differential equations corresponding to a given kinetic scheme. The peak areas of the NMR signals were transformed into concentration values and introduced into the program GIT which determines the best values of the rate constants to fit the data.

Fig. 4 shows the concentration change of the thiolysis products of benzylpenicillin with α-monothioglycerol as the reaction develops. Dots are experimental data obtained from the ^{1}H NMR experiments. Experimental data fit perfectly to the theoretical behaviour shown by Scheme 1 (lines). The final rate constant values obtained were: $k_1 = 1.7 \times 10^{-3}\,\mathrm{mol^{-1}\,dm^3\,s^{-1}}$, $k_2 = 8.6 \times 10^{-7}\,\mathrm{s^{-1}}$, $k_3 = 2.7 \times 10^{-4}\,\mathrm{s^{-1}}$, $k_4 = 4.3 \times 10^{-6}\,\mathrm{s^{-1}}$ and $k_5 = 5.6 \times 10^{-7}\,\mathrm{s^{-1}}$. The rate constant k_3 is in fact an apparent rate constant.

The possibility of the thiolate anion acting as a general base catalyst over a molecule of water was studied and introduced into the general mechanism. The rate constant for this catalysis was insignificant and taking it into account in the general mechanism did not affect to the rest of rate constants significantly.

The thiolysis of benzylpenicillin was also studied with 2-mercaptoethanol by using ^{1}H NMR technique at 20°C and pD = 10.1, in order to characterise and monitor the degradation products.

Fig. 5 shows the evolution of the ^{1}H NMR spectra of the thiolysis of benzylpenicillin by 2-mercaptoethanol with time. The first spectrum (1 min after the initiation

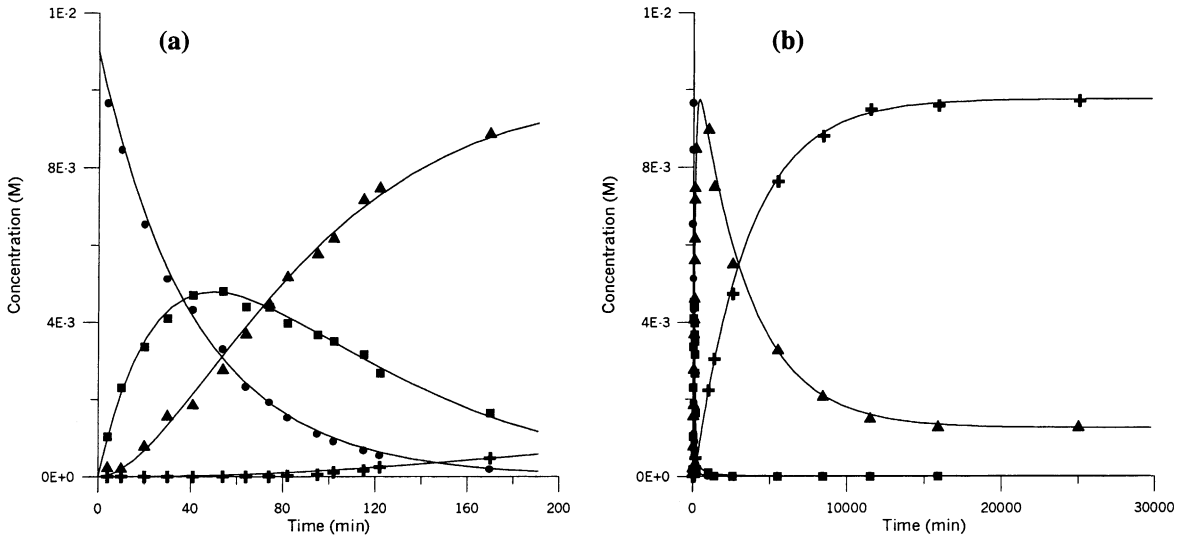


Fig. 4. Degradation of benzylpenicillin with α-monothioglycerol at pD = 9.6 and 20°C at different time scale: (a) 0–200 min; (b) 0–30,000 min. Symbols correspond to: (●) benzylpenicillin; (■) benzylpenicilloyl thioester and ester; (▲) 5*R*-, 6*R*-benzylpenicilloic acid; (✚) 5*S*-, 6*R*-benzylpenicilloic acid.

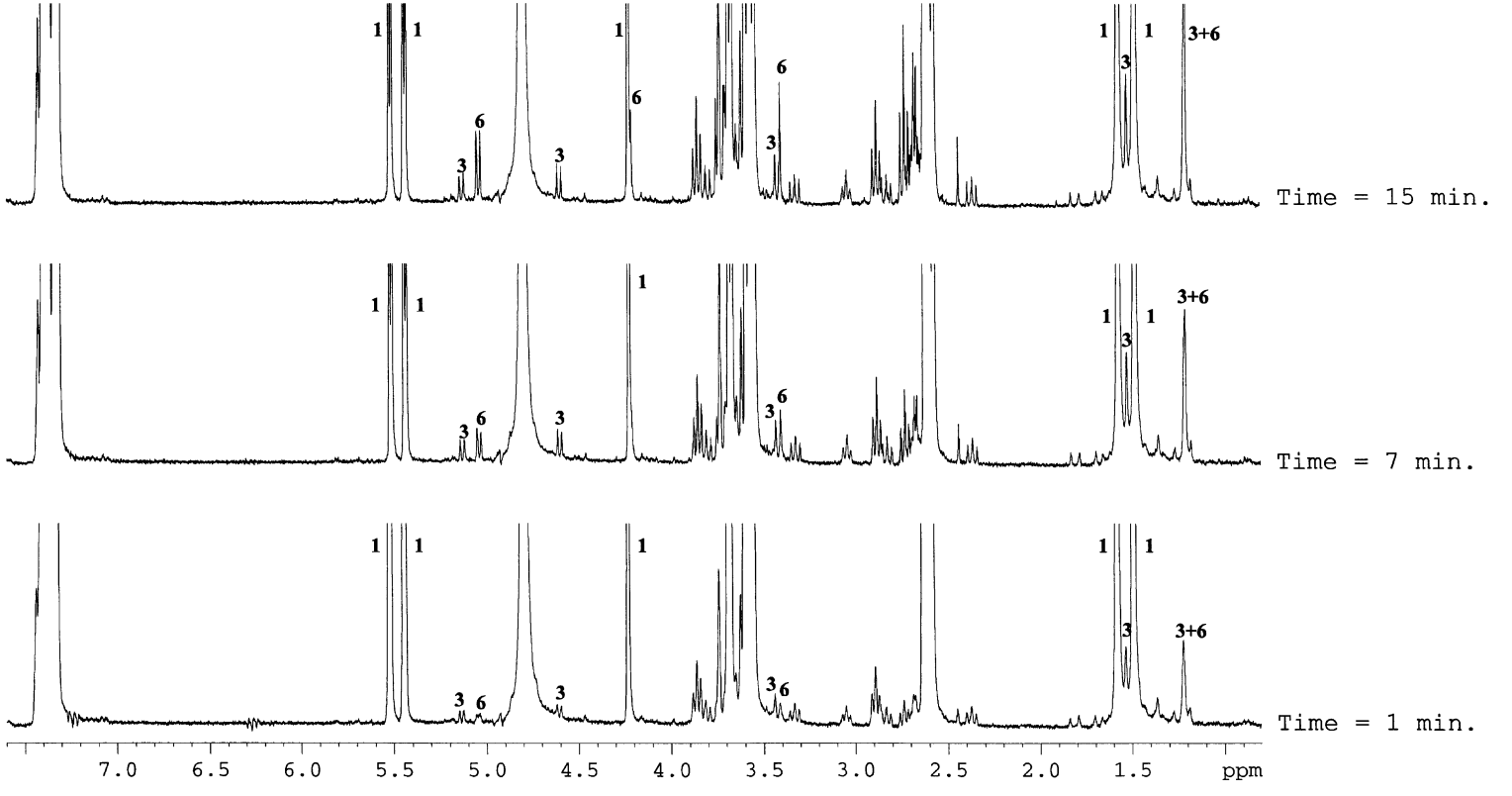


Fig. 5. Evolution of ^{1}H NMR spectra of the thiolysis of benzylpenicillin by 2-mercaptoethanol with time at pD = 10.1 and $T = 20^{\circ}$C. Numbers correspond to structures of Scheme 1.

of the reaction) shows basically signals of benzylpenicillin (**1**) and the buffer (2-mercaptoethanol) together with some new increasing signals compatible with the early formation of the thioester (**3**) (characteristic signals at 4.64 ppm (5-H) and 5.15 ppm (6-H), coupled together with $J = 6.6$ Hz). The signals corresponding to the thioester increase with time. As the reaction develops (7 min) a new set of signals, corresponding to the 5*R*-, 6*R*-benzylpenicilloic acid (**6**), appear. Signals corresponding to the thioester and benzylpenicillin decrease with time, while the signals corresponding to the 5*R*-, 6*R*-benzylpenicilloic acid become more important (15 min). 5*R*-, 6*R*-benzylpenicilloic acid starts epimerising at the fifth position after long reaction times.

In this case we observe the formation and evolution of the same kind of signals upfield, next to the thiol ones (2.65–2.80 and 3.60–3.80 ppm), as in the α-monothioglycerol case (2.70–3 and 3.75–4 ppm). These compounds are assigned to different oxidised forms of the thiol, and they do not present a free thiol. Signals at 2.90 and 3.85 ppm correspond to the dimerisation of 2-mercaptoethanol via disulphide bridges formation. Experiments done using 2-mercaptoethanol (pD = 10.1, 20°C) on its own (without benzylpenicillin) in carbonate buffer show just one oxidation product corresponding to the formation of the disulphide derivative. Several experiments done using 2-mercaptoethanol just as reagent (and not as buffer) show no recovery of the thiol. The Ellmann's test performed on flame-sealed ampoules during the reaction show that the concentration of free thiol (R-SH) decreased at the same rate as the benzylpenicillin one (taking into account the loss of thiol by disulphide formation). Several tests on these isolated products lead us to think that these products are formed by an intramolecular rearrangement of the thioester giving ethylene sulphide which readily can be oxidised to different degrees and hydrolysed. These products were assayed for free thiol content by using the method of Ellmann giving a negative test.

Scheme 1 can also account for the reaction pathway for the thiolysis of benzylpenicillin using 2-mercaptoethanol. Individual rate constants were elucidated by using the software program GIT [25,26]. In order to obtain the best values for these constants, the kinetic constant for the hydrolysis of benzylpenicillin was calculated by HPLC from a separate experiment using carbonates as buffer, in the same conditions that in thiolysis experiments. The hydrolysis constant obtained from this experiment was fixed in the general scheme and the rest of constants were fitted to the experimental data.

Fig. 6 shows the concentration change of the thiolysis of benzylpenicillin with 2-mercaptoethanol as the reaction develops. Experimental ^{1}H NMR data (dots) fits perfectly to the theoretical behaviour. Rate constants obtained were: $k_1 = 1.4 \times 10^{-3}$ mol^{-1} dm^3 s^{-1}, $k_2 = 2 \times 10^{-6}$ s^{-1}, $k_3 = 1.28 \times 10^{-3}$ s^{-1}, $k_4 = 7.8 \times 10^{-6}$ s^{-1} and $k_5 = 9 \times 10^{-7}$ s^{-1}.

The possibility of having the thiolate anion acting as a general base catalyst over a molecule of water was again taken into account and introduced into the general mechanism. Rate constant for this catalysis was insignificant and the effect on the rest of the constants negligible.

The faster degradation of the mercaptoethanol benzylpenicilloate ester compared with the rate of degradation of the monothioglycerol benzylpenicilloate ester could explain why the rearrangement was detected when α-monothioglycerol was used but not when it was 2-mercaptoethanol, even though this reaction could occur through a five-membered ring intermediate. In order to prove that the *S*- to *O*-acyl transfer occurs via the β-hydroxyl group and not the γ one the thiolysis reaction of benzylpenicillin with 3-mercaptopropanol was followed. This thiol would give a thioester which would require the terminal alcohol to form a six-membered ring intermediate for *S*- to *O*-acyl transfer to occur by intramolecular alcoholysis and this is not seen. In addition to the relatively favourable formation of a five-membered ring, intramolecular attack by a β-hydroxy group must also be controlled by other factors. The additional α′-hydroxymethyl substituent at the β-position in the case of the α-monothioglycerol could enhance the intramolecular reaction because of its effect on the pK_a of the β-hydroxyl and/or because of the Thorpe–Ingold effect of alkyl substituents favouring ring closure [27,28].

A final experiment was done in order to show the kinetic effect of a β-proton transfer donor on the degradation rate of penicillin. The mechanism of thiolysis of benzylpenicillin has been investigated using mercaptoethylamine under Argon by ^{1}H NMR

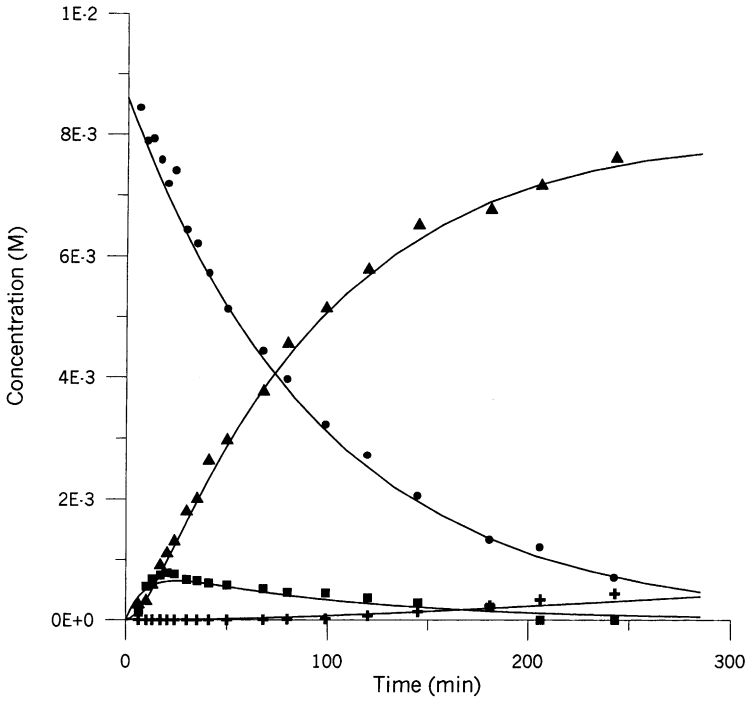


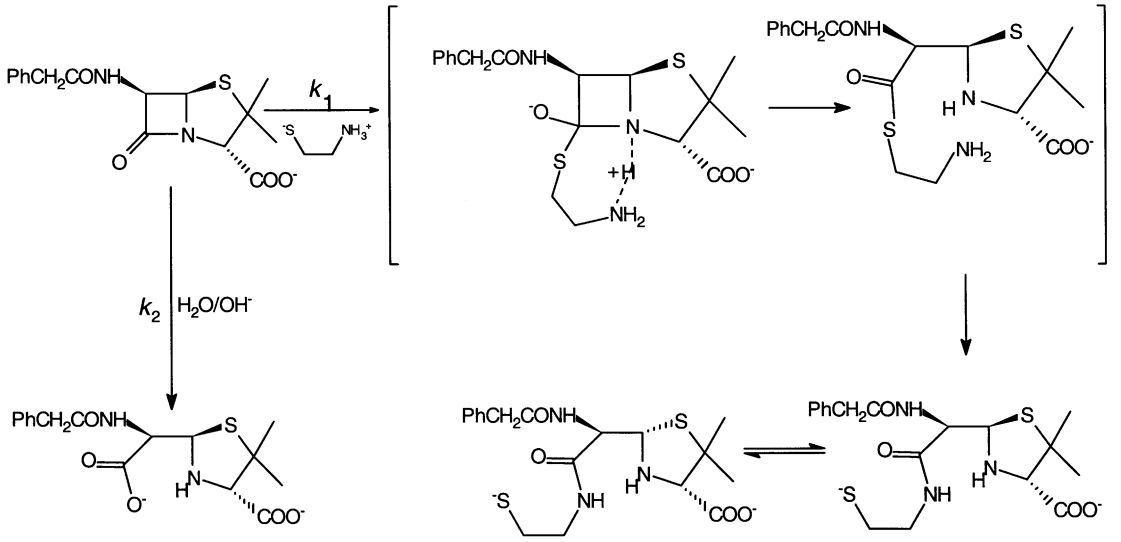
Fig. 6. Degradation of benzylpenicillin with 2-mercaptoethanol at pD = 10.1 and 20°C. Plots represent the reaction evolution with time from $t = 0$ to 300 min. Symbols correspond to: (●) benzylpenicillin; (■) benzylpenicilloyl thioester; (▲) 5*R*-, 6*R*-benzylpenicilloic acid; (✚) 5*S*-, 6*R*-benzylpenicilloic acid. Points represent experimental data. Lines represent the theoretical fit.

and HPLC in order to characterise and monitor the degradation products.

The rate constants were calculated by HPLC at 20°C using carbonates as buffer and at pH = 9.80. Mercaptoethylamine was used as reagent. Several experiments were done following the decrease in the peak areas for benzylpenicillin at 225–228 nm at different mercaptoethylamine concentrations, i.e. when the relation between the mercaptoethylamine and the benzylpenicillin concentrations was 1:1 the degradation of benzylpenicillin was complete, but when the relation was 1:2, degradation of benzylpenicillin only reached the 50% of completion, showing a "normal" hydrolysis rate of degradation once the mercaptoethylamine reacted. During these experiments the total thiol concentration was followed by the Ellmann's method [18] showing a constant value identical to the initial thiol concentration.

^{1}H NMR studies showed that no benzylpenicilloic acids were formed during the course of this reaction. The only final products were the amide and its 5-epimer, which were identified by ^{1}H NMR and ^{13}C experiments but no attempt to isolate these products was done. This data would explain why the total thiol concentration is constant and equal to the initial value. As soon as the thioester is formed (Scheme 2) the terminal amine acts as a general acid catalyst donating a proton to the leaving β-lactam nitrogen which allows the tetrahedral intermediate to breaks down and leaving the terminal amine deprotonated ready to attack intramolecularly as a nucleophile the thioester carbonyl via an acyl transfer S–N reaction. The resulting amide formed will give a positive test in the Ellmann's test. Although no benzylpenicilloic acids were detected during the time that the reaction was followed, the hydrolysis pathway for the degradation of benzylpenicillin was taken into account and introduced in the general scheme.

Individual rate constants were calculated as before, using the GIT program. As in the former experiments with other thiols, the hydrolysis constant was calculated in a separate experiment using the



Scheme 2.

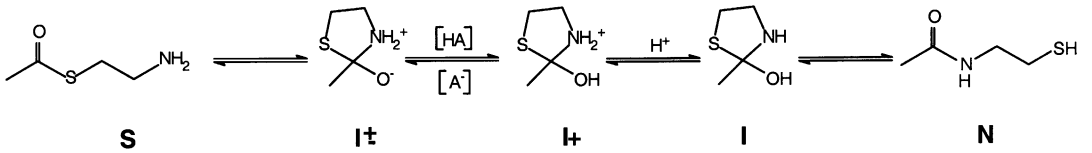
same conditions as used for the thiolysis experiments ([carbonates] = 0.2 mol dm^{-3}, pH = 9.80 and 20°C). This constant was introduced as a fixed value in the kinetic scheme proposed (Scheme 2). The rate constants obtained in all the experiments were almost identical if k_2 (hydrolysis rate constant) was fixed or not. The best rate constant values for the thiolysis of benzylpenicillin by mercaptoethylamine were: $k_1 = 1.3 \times 10^{-1}$ mol^{-1} dm^3 s^{-1} and $k_2 = 1.8 \times 10^{-6}$ s^{-1}.

The exact mechanism for the S–N transfer reaction is not straight forward. It has been suggested that the proton transfer is the rate-determining step in reactions of this kind, as it occurs with simple proton transfer reaction of carbon acids, but a concerted mechanism is nowadays accepted [19,21–24,29].

Jencks and co-workers suggested the mechanism shown in Scheme 3 for the S–N transfer reaction [29]. According to this mechanism at basic pH the protonation of $I\pm$ occurs through a water catalysed reaction. Under conditions in which either of these proton transfer steps are rate-determining the barrier of the thioester formation from *I* is larger than that for amide formation and no thioester is formed as a product of thiazoline hydrolysis, as it occurs when mercaptoethylamine is used.

In a previous paper [15] we reported the Brönsted plot for the thiolysis degradation of benzylpenicillin, together with the activation energy of this process. The α-monothioglycerol presented a positive deviation from the theoretical behaviour of the Brönsted plot, indicating that the thiolysis of benzylpenicillin by this thiol is accelerated.

The activation energy corresponding to the thiolysis of benzylpenicillin by α-monothioglycerol has a very low value (6.9 kcal mol^{-1}) [15], compared with the "normal" values for a nucleophilic attack on penicillins (10–11 kcal mol^{-1} for hydrolysis [30], 15–16 kcal mol^{-1} for aminolysis [31]), indicating



Scheme 3.

that the transition state for this reaction is somehow stabilised.

This rate enhancement of the degradation of penicillins by polyhydric alcohols has been previously reported by Yamada and co-workers [32]. In that paper it was shown that the degradation of ampicillin was accelerated only by polyhydric alcohols which have an adjacent hydroxy group, while the rate constant was not affected by monohydric alcohols such as ethanol, propanol and others. These authors [32] suggested that nucleophilic attack of a hydroxy anion on the β-lactam ring became easier as a result of a simultaneous hydrogen bonding among the two adjacent hydroxy groups of alcohols and the amide carbonyl and the β-lactam carbonyl group of ampicillin. This hypothesis was supported by an inhibition of this rate enhancement when an aldehyde was added together with a polyhydric alcohol. We observed the same rate enhancement effect when a polyhydric alcohol was used, but the formation of a simultaneous hydrogen bond among the two adjacent alcohols would not explain the rate enhancement observed when a protonated amine is placed in a β-position. It has been proved that the catalytically form of an alcohol is the alkoxide anion [8], therefore a rate enhancement should not be observed using weak acidic alcohols such as ethanol or propanol at pH = 9, but a rate enhancement is expected if the pK_a of an alcohol group is sensibly lower, as it occurs when an adjacent alcohol is placed next to another, in this case a little amount of alkoxide is formed and alcoholysis takes place, accelerating the degradation of penicillins when a polyhydric alcohol is used.

The fact that the rate accelerating effect of polyhydric alcohols on the ampicillin degradation is depressed by the addition of aldehydes is not due to the steric hindrance and resonance effect of Schiff's base formation between the α-amino group of ampicillin and the aldehyde, as it was suggested by Yamada and co-workers [32], but to the well known acetal formation between a polyhydric alcohol with an adjacent hydroxy group and an aldehyde [33].

In our case, the accelerating effect is even larger due to the fact that the alcohol with a lower pK_a is placed next to the leaving group (tertiary amine) of the tetrahedral intermediate, which will act as a general base, accepting the proton from the alcohol and stabilising the transition state, probably in a concerted mechanism. A kinetic isotopic effect has been calculated in a previous work [15]. A value of 2.2 in this reaction suggests that a general acid catalysis is taking place in the breakdown of the intermediate by a proton transfer either from a water molecule or from the alcohol to the nitrogen. If the proton transfer comes from the β-alcohol, no accelerating effect should be seen in the thiolysis rate constant when the attacking nucleophile does not present a β-proton transfer donor, and its activation energy should be higher, about the same order as the hydrolysis one. In the case of methyl thioglycolate, which does not present any group capable of donating a proton, no accelerating effect is seen on the rate constant and its activation energy presents an identical value as the hydrolysis one, both values are 10.4 kcal mol^{-1}. By contrast, if the attacking thiolate has a β-proton transfer donor with a pK_a higher than the α-monothioglycerol one its rate enhancement should be less and its activation energy value should be in between the values of the α-monothioglycerol and the methyl thioglycolate ones. The reported activation energy value for the 2-mercaptoethanol is 8.2 kcal mol^{-1} [15].

If this is true an opposite effect is expected if instead of having a β-proton donor, which is able to stabilise the transition state, acting as a general acid catalyst, a general base catalyst is placed in this position. This is the case of 3-mercaptopropionic acid. In this case two negative charges are developed in the transition state. This would increase the activation energy of the process and because the rate-limiting step of thiolysis was proved to be the breakdown of the tetrahedral intermediate, which implies the protonation of the amine as a leaving group. The subsequent rate constant should reflect this fact and therefore 3-mercaptopropionic acid should have a negative deviation of the Brönsted plot, as it is shown in a previous paper [15].

It has been previously shown that the rate constant for the reaction of benzylpenicillin with the monocation of 1,2-diaminoethane is ca. 100-fold greater that that predicted from the Brönsted plot for a monoamine of the same basicity as 1,2-diaminoethane [9]. In the case of aminoethanethiol, the rate enhancement is ca. 1000-fold greater than that predicted from the Brönsted plot for a thiol of the same basicity as aminoethanethiol. This rate enhancement is due to an intramolecular general acid catalysis caused by the protonated amine which gives a proton to the leaving negatively charged nitrogen stabilising in this

way the transition state as described in Scheme 2. The effect is analogous to that already mentioned of α-monothioglycerol and 2-mercaptoethanol.

From these results seems to be clear that proton donor groups suitable placed in a β-position respect to the nucleophile group facilitates breakdown of tetrahedral intermediate by proton transfer to the β-lactam nitrogen and considerably increases the rate constant of lactam ring fission.

The mechanism of most of the proteases is based on what has been called the Catalytic Triad [34–37] in which one residue is acting as a nucleophile (serine, cysteine), another residue is acting as a general base catalyst (proton acceptor) and finally another residue acts as a general acid transferring a proton to the amide nitrogen of the tetrahedral intermediate. β-Lactamases also show the same mechanistic basic trends. For example, the proposed mechanism to class A includes the Ser70 [17] acting as the nucleophile whose nucleophilic character is increased by proton abstraction by the carboxylic group of the Glu166 [38–41] or by the free amine of the Lys74 [42,43]. Finally the breakdown of the tetrahedral intermediate is favoured by the proton donation by Ser130 to β-lactam nitrogen and proton transfer from Lys74 to Ser130.

Although the exact mechanisms involved in catalysis by individual enzymes can only be defined by direct studies of the enzyme–substrate reactions, there is no doubt that the elucidation of the general mechanism of catalysis by enzymes can be enormously aided by studies of the mechanisms of simple reactions in solution. In this way, the system studied here could be considered as a simple model of enzymes catalysing C–N cleavage in amide or lactam linkages such as proteases and β-lactamases.

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