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Δ^2- and Δ^3-cephalosporins, penicillinate and 6-unsubstituted penems

Intrinsic reactivity and interaction with β-lactamases and D-alanyl-D-alanine-cleaving serine peptidases

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The intrinsic reactivity of Δ^2- and Δ^3-deacetoxy-7-phenylacetamidocephalosporanates, penicillanate, unsubstituted, 2-methyl- and 2-phenyl-penems and other β-lactam antibiotics has been expressed in terms of the second-order rate constant ($M^{-1} \cdot s^{-1}_{(OH^-)}$) for the hydrolysis of the β-lactam amide bond by OH^- at 37°C. The values thus obtained have been compared with the second-order rate constants ($M^{-1} \cdot s^{-1}_{(enzyme)}$) for the opening of the same β-lactam amide bond during interaction with the β-lactamases of *Streptomyces albus* G and *Actinomadura* R39 and the D-alanyl-D-alanine-cleaving serine peptidases of *Streptomyces* R61 and *Actinomadura* R39. Depending on the cases, the accelerating effect due to enzyme action and expressed by the ratio $M^{-1} \cdot s^{-1}_{(enzyme)}/M^{-1} \cdot s^{-1}_{(OH^-)}$ varies from less than 2 to more than 1×10^6. The primary parameter that governs enzyme action is the goodness of fit of the β-lactam molecule to the enzyme cavity rather than its intrinsic reactivity. With the D-alanyl-D-alanine-cleaving serine peptidases, the three penems studied form intermediate complexes characterized by very short half lives of 14–100s, values significantly lower than those exhibited by most β-lactam compounds.

β-Lactam compounds with widely varying bicyclic fused-ring systems and substituents have been synthesized or isolated from natural sources (Hamilton-Miller & Smith, 1979; Gregory, 1981; Salton & Shockman, 1981). Modifications of the pyramidal character of the β-lactam nitrogen (Woodward, 1980) and the presence of side-chains facilitating electron delocalization outside the β-lactam ring (Boyd & Lunn, 1979) are structural variations that affect the intrinsic reactivity. The question therefore arises to what extent this intrinsic reactivity (in the ground-state conformation) influences the reactions of the β-lactam compounds with β-lactamases and D-alanyl-D-alanine-cleaving serine peptidases. 'Classical' penicillins (cephalosporins) are substrates of the β-lactamases; they are hydrolysed into biologically inactive penicilloate (cephalosporoate) derivatives. 'Classical' penicillins (cephalosporins) are mechanism-based inactivators of the serine D-alanyl-D-alanine-cleaving serine peptidases; they immobilize the enzymes, at least for some time, in the form of serine ester-linked penicilloyl (cephalosporoyl)–enzyme complexes. To answer the question, two β-lactamases and two D-alanyl-D-alanine-cleaving serine peptidases were selected. The β-lactamases were those secreted by *Streptomyces albus* G (the 'G β-lactamase'; Deuz *et al.*, 1981*a*) and *Actinomadura* R39 (the 'R39 β-lactamase'; Deuz *et al.*, 1982). The D-alanyl-D-alanine-cleaving serine peptidases were those secreted by *Streptomyces* R61 (the 'R61 peptidase'; Frère *et al.*, 1973, 1976) and the same *Actinomadura* R39 as above (the 'R39 peptidase'; Frère *et al.*, 1974; Duez *et al.*, 1981*b*). In these and other (Kelly *et al.*, 1981*a*,*b*) studies, the interactions had been described between the four selected enzymes and a large number of compounds belonging to various β-lactam families. To cover a wider range of intrinsic reactivity, the 2-cephem Δ^2-deacetoxy-7-phenylacetamidocephalosporanate, its 3-cephem analogue, the 6-unsubstituted penam penicillanate and three 6-unsubstituted penems have been investigated. The results thus obtained are described in the present report.

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Materials and methods

Enzymes and buffers

The G and R39 β-lactamases and the R61 and R39 peptidases were purified as previously described (Duez *et al.*, 1981*a*, 1982; Frère *et al.*, 1973, 1974). Estimation of enzyme activity and, in the case of the peptidases, enzyme inactivation, were carried out at 30°C for the β-lactamases and at 37°C for the peptidases in the following buffers (unless otherwise stated): 5 mM-sodium phosphate, pH 7.0 (R61 peptidase); 0.1 M-Tris/HCl, pH 8.0, containing 0.1 M-NaCl and 50 mM-$MgCl_2$ (R39 peptidase); and 50 mM-sodium phosphate, pH 7.0 (the G and R39 β-lactamases). The stock solution of the G β-lactamase (and the dilutions made from it) was in 50 mM-sodium phosphate buffer, pH 7.0, containing 10% (v/v) glycerol and 10% (v/v) ethylene glycol. These polyols were not included in the reaction mixtures for the determination of enzyme activity.

β-Lactam compounds

Δ^3-7β-Phenylacetamidodeacetoxycephalosporanic acid was prepared from benzylpenicillin *S*-sulphoxide (de Koning *et al.*, 1975). After esterification with diazomethane, this compound was transformed into Δ^2-7β-phenylacetamidodeacetoxycephalosporanic acid, using the method described for the phenoxyacetamido derivative (Van Heyningen & Ahern, 1968): δ (p.p.m.) (60 MHz, in [^{2}H]chloroform/[^{2}H]dimethyl sulphoxide, with tetramethylsilane as internal standard) 1.9 (broad s, CH_3), 3.6 (s, C_6H_5–CH_2), 4.68 (broad s, H-4), 5.25 (d, $J = 4$ Hz, H-6), 5.55 (double d, $J = 4$ and 8 Hz, H-7), 5.90 (broad s, H-2), 7.30 (s, C_6H_5), 7.85 (d, $J = 8$ Hz, NH). T.l.c. on pre-coated silica gel F254 plates with benzene/acetone/acetic acid (60:39:1, by vol.) gave: Δ^2-cephem, R_F 0.38; Δ^3-cephem, R_F 0.46. Penicillanic acid was a gift from Pfizer Central Research, Sandwich, Kent, U.K. The unsubstituted penem, 2-methylpenem and 2-phenylpenem [racemic mixtures of 5*R*(+)- and 5*S*(−)-isomers] were gifts from Dr. J. Gosteli, formerly of the Woodward Institute, Basel, Switzerland, whose present address is Cerecon, Badendorf B.L., Switzerland. The structure of these six compounds is shown in Table 2. For other β-lactam compounds, see Kelly *et al.* (1981*a*,*b*).

Interaction between β-lactamases and β-lactam compounds

The six β-lactam compounds under study were substrates of the β-lactamases and the kinetic parameters K_m and $V_{max.}$ were calculated from Lineweaver–Burk plots. With the penicillanate and Δ^2- and Δ^3-deacetoxy-7-phenylacetamidocephalosporanates, the determinations were made on 30 μl samples. The reaction was stopped by adding 0.2 ml of 1 M-sodium acetate buffer, pH 4.0, and the amounts of hydrolysed products were estimated with the starch/I_2 procedure as described by Johnson *et al.* (1975). With the three penems, the determinations were made on 300 μl samples and the hydrolysis was followed by spectrophotometric measurements with a Beckman recording DU-8 spectrophotometer at the appropriate wavelengths (Table 1). In all cases, the β-lactam compound concentrations were about the same as the K_m values.

Interaction between D-alanyl-D-alanine-cleaving serine peptidases and β-lactam compounds

'Classical' penicillins and Δ^3-cephalosporins inactivate the D-alanyl-D-alanine-cleaving serine peptidases according to the model:

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} EI^* \xrightarrow{k_{+3}} E + P(s)$$

where E = enzyme, I = β-lactam compound, EI = Michaelis complex, EI* = acyl–enzyme intermediate, P(s) = degradation product(s), K = dissociation constant and k_{+2} and k_{+3} = first-order rate constants (Frère *et al.*, 1975*a*,*b*). The higher the k_{+2}/K value and the lower the k_{+3} value, the better the β-lactam antibiotic as enzyme inactivator. The first step of the interaction is assumed to be a quasi-equilibrium process. No evidence to the contrary has been found by Frère *et al.* (1975*a*) and Fuad *et al.* (1976). Moreover, the value of the second-order rate constant (k_{+2}/K) is always substantially smaller than $10^7\,M^{-1}\cdot s^{-1}$, which, as discussed by Brocklehurst (1979), is the probable lower limit of k_{+1}.

In the cases of penicillanate and both R61 and R39 peptidases, of Δ^3-deacetoxy-7-phenylacetamidocephalosporanate and both R61 and R39 peptidases, and of Δ^2-deacetoxy-7-phenylacetamidocephalosporanate and the R39 peptidase, the k_{+2}/K value (for enzyme inactivation) and the k_{+3} value (for enzyme reactivation) were estimated as described by Kelly *et al.* (1981*b*). To determine the k_{+3} value, the excess of the β-lactam compound used to inactivate the peptidases had to be destroyed with a sufficient amount of β-lactamase; the R39 β-lactamase was used for this purpose. The rate of inactivation of the R39 peptidase ($k_a = k_{+2}/\{1 + (K/[\beta\text{-lactam}])\}$, according to the model) was proportional to [β-lactam] up to 4 μM (3-cephem), 13 μM (2-cephem) and 70 μM (penicillanate). At higher concentrations, the inactivation was too rapid to be followed accurately. The rate of inactivation of the R61 peptidase by the 3-cephem was proportional to [β-lactam] up to 10 μM. With penicillanate, the plot of k_a versus [β-lactam] showed deviation from linearity permitting evaluation of the K and k_{+2} constants.

The interaction between Δ^2-deacetoxy-7-phenylacetamidocephalosporanate and the R61 peptidase proceeded through an intermediate EI*, which was too short-lived to permit evaluation of the k_{+3} and k_{+2}/K values by the reference procedures. (i) To determine the k_{+3} value, the technique used was that previously described (Kelly *et al.*, 1981*b*) for measuring the rate of degradation of the complex formed between the R39 peptidase and 6-aminopenicillanate (and using the R39 β-lactamase to rapidly destroy the excess of the 2-cephem). (ii) To determine the k_{+2}/K value, the procedure made use of measurements of the residual peptidase activity at the steady state (A_{ss}) in the presence of 1.5mM-diacetyl-L-Lys-D-Ala-D-Ala [this concentration was well below the K_m value (12mM), so that the addition of the tripeptide had little effect on the A_{ss} value].

Under these conditions,

$$A_{ss} = \frac{A_0 k_{+3}}{k_a + k_{+3}}$$

where A_0 = enzyme activity in the absence of the 2-cephem. The rate of enzyme inactivation, k_a, was proportional to [2-cephem] for three [2-cephem] values ranging between 20 and 60μM and yielded a k_{+2}/K value of $75\,M^{-1}\cdot s^{-1}$. At the same 2-cephem concentrations, a $60\,M^{-1}\cdot s^{-1}$ value for the k_{+2}/K ratio was calculated from the decrease of the enzyme activity during the first 15min of contact, before the steady state was established. An average value of $68\,M^{-1}\cdot s^{-1}$ was therefore adopted.

With the three penems and both R61 and R39 peptidases, the steady state was so rapidly established that it was impossible to determine the rates of enzyme inactivation. These compounds were then regarded as substrates. With the R61 peptidase, the kinetic parameters K_m and $V_{max.}$ (at 37°C) were estimated on the basis of Lineweaver–Burk plots by spectrometric measurements at the appropriate wavelengths (Table 1). With the R39 peptidase, the K_m values were too small to be determined accurately by this direct procedure; consequently, they were measured by substrate competition experiments under the following conditions. (i) With the 2-methylpenem, samples containing the R39 peptidase (0.2μg), four concentrations of diacetyl-L-Lys-D-Ala-D-Ala (from 0.8 to 3.2mM) and various concentrations of 2-methylpenem (from 0 to 22μM) were incubated for 10min at 37°C in 40μl (total volume) of 100mM-Hepes/HCl buffer, pH8.0, containing 0.1M-NaCl at 50mM-$MgCl_2$ and the amounts of released D-alanine were estimated under initial-rate conditions. The inhibition pattern was clearly competitive and yielded, for this particular penem, a K_i value (which, in fact, is its K_m value) of 1.5μM. (ii) With the unsubstituted penem and the 2-phenylpenem, a fixed diacetyl-L-Lys-D-Ala-D-Ala concentration (2mM) was used and the K_i (i.e. K_m) values were estimated (assuming a competitive model) from the slope of the line v/v_i versus [β-lactam] where v and v_i are the rates of D-alanine release in the absence and in the presence of various penem concentrations. This latter procedure, when applied to the interaction between the unsubstituted penem and the R61 peptidase, yielded a K_i (or K_m) value of 70μM; this value was in excellent agreement with the 80μM value obtained by the direct procedure. (iii) Finally, in another series of experiments, the procedure described above to determine the k_{+3} value for the interaction between the R61 peptidase and Δ^2-deacetoxy-7-phenylacetamidocephalosporanate was also applied to the interaction between the R39 peptidase and the three penems. Samples of the R39 peptidase (1μg) were incubated with saturating concentrations (0.1mM) of the penems at 37°C in a total volume of 200μl of the Hepes/NaCl/$MgCl_2$ buffer (see above). The reaction mixtures were then supplemented with 400nmol of diacetyl-L-Lys-D-Ala-D-Ala and 10μg of G β-lactamase (a quantity sufficient to destroy the excess of the penems in less than 1s) and the amounts of released D-alanine were monitored as a function of time as indicated above. The k_{+3} values thus obtained were close to the $k_{cat.}$ values.

Susceptibility of the β-lactam amide bond to hydrolysis by OH^- at 37°C and pH12.0

The susceptibility to attack by OH^- of the β-lactam amide bond of the six β-lactam compounds under study was investigated in 1M-K_2HPO_4 adjusted to pH12.0 with 10M-KOH. The results were expressed in terms of second-order rate constants ($M^{-1}\cdot s^{-1}$) for hydrolysis. The reaction was shown to be first order in OH^- between pH11 and 12 with cephalosporin C and cephalothin (and was assumed to be so in the other cases). A slight decrease in the reaction rate was observed by decreasing the concentration of K_2HPO_4 from 1.0 to 0.1M, but the effect was negligible when compared with the first-order dependency on OH^-. The same study was extended to 19 other β-lactam compounds (belonging mainly to the 3-cephem and penam families) whose interactions with the G and R39 β-lactamases and the R61 and R39 peptidases had been previously investigated (see the Introduction). Two procedures were used. With the iodometric procedure (which was applied to all the compounds except cephaloridine, nitrocefin and thienamycin), the initial concentration of the β-lactam compounds was 1mM. Samples (5–10μl) were removed after increasing times of incubation at 37°C, supplemented with 0.2ml of 1M-sodium acetate buffer, pH4.0, (to stop the reaction) and the amounts of hydrolysed product were estimated by the starch/I_2 procedure (Johnson *et al.*, 1975). With

Table 1. *Variations of the molar absorption coefficients of β-lactam compounds caused by the hydrolysis of the β-lactam amide bond by either β-lactamase action or treatment with OH^-*

β-Lactam compound	$\Delta\varepsilon$ ($M^{-1} \cdot cm^{-1}$)	Wavelength (nm)
Benzylpenicillin	−570	240
Δ^3-Deacetoxy-7-phenylacetamidocephalosporanate	−2460	258
Cephalothin	−7200	260
Cephaloglycine	−5700	260
Cephaloridine	−10000	260
Δ^2-Deacetoxy-7-phenylacetamidocephalosporanate	+5200	280
N-Formimidoylthienamycin	−7400	298
2-Methylpenem	−3900	298
Unsubstituted penem	−5000	305
2-Phenylpenem	−4000	318
Nitrocefin	−10000	386

the spectrophotometric procedure (which was applied to the compounds listed in Table 1), the initial concentrations of the β-lactam compounds ranged from 0.1–0.5 mM and the total volume of the solutions was 300 μl. The absorbance variations of the solutions were recorded as a function of time at the appropriate wavelengths (Table 1).

Non-planarity of the β-lactam nitrogen of the β-lactam compounds

Non-planarity of the β-lactam nitrogen was expressed by the distance (*h*) between the apex and the base of a trigonal pyramid, where the nitrogen atom is at the apex and its three substituents are at the corners of the base (Sweet & Dahl, 1970; Woodward, 1980). The *h* values for the unsubstituted, 2-methyl- and 2-phenyl-penems were those given by Woodward (1980). The other *h* values were taken from the literature or calculated from the published atomic co-ordinates (see Table 2).

Results

Non-planarity of the β-lactam nitrogen and susceptibility of the β-lactam amide bond to nucleophilic attack by OH^- at pH 12.0 and 37°C

Table 2 shows that depending on the families to which they belong, the β-lactam compounds have varying *h* values (*h* = distance between the β-lactam nitrogen and the plane formed by its three substituents). Table 2 also gives quantitative estimates of the susceptibility of the β-lactam carbonyl carbon to nucleophilic attack by OH^- at pH 12.0 and 37°C. When the two procedures used yielded $M^{-1} \cdot S^{-1}{}_{(OH^-)}$ values differing from each other by a factor larger than 1.5, the individual values are reported.

Interaction of the 2-cephem (compound 1), 3-cephem (compound 2), penicillanate (compound 11) and the penems (compounds 22–24) with the R39 and G β-lactamases and the R61 and R39 D-alanyl-D-alanine-cleaving serine peptidases

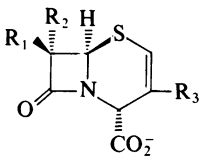
The kinetic parameters for the interaction between the various β-lactam compounds under consideration and the R39 and G β-lactamases are given in Table 3, and for the R39 and R61 peptidases are given in Tables 4 and 5. Note that each of the three penems studied was a racemic mixture of 5*R*(+)- and 5*S*(−)-isomers. As shown in Fig. 1, only one isomer [most likely the 5*R*(+)-isomer] was sensitive to the β-lactamases. Indeed, a prolonged incubation of the penems with the β-lactamases resulted only in a partial disappearance of the absorption band in the near-u.v. (contrary to what was observed by treatment at pH 12.0; results not shown). Consequently, the concentrations used in the calculation of the kinetic parameters for the interactions between penems and both β-lactamases and D-alanyl-D-alanine-cleaving serine peptidases were 50% of the total concentrations.

For comparison purposes (see the Discussion section), benzylpenicillin and 6-aminopenicillanate were included in Tables 3 and 4. All the β-lactam compounds considered were inactivators of the R61 and R39 peptidases except the three penems which were regarded as substrates of the same peptidases (Table 5). Nevertheless, the data of Tables 3, 4 and 5 can be compared with each other on the basis that: (i) for the peptidases, $k_{cat.}$ is equivalent to k_{+3} (see the Materials and methods section and footnote to Table 5); and (ii) if the formation of complex EI is a rapid-equilibrium process, then K, k_{+2} and k_{+3} are related to K_m and $k_{cat.}$ by $K_m = Kk_{+3}/(k_{+2}+k_{+3})$ and $k_{cat.} = k_{+2}k_{+3}/(k_{+2}+k_{+3})$ so that $k_{cat.}/K_m$ and

Table 2. *Structure of β-lactam compounds: pyramidal character of the β-lactam nitrogen and propensity of the β-lactam amide bond to hydrolysis by OH^- at pH 12.0*

IP = iodometric procedure; A = spectrophotometric procedure. See the Materials and methods section for further details. References: (*1*), Simon *et al.* (1972); (*2*), Indelicato *et al.* (1974); (*3*), Domiano *et al.* (1978); (*4*), Sweet & Dahl, 1970; (*5*), Hodgkin & Maslen (1971), (*6*), Van Meerssche *et al.* (1979); (*7*), Albers-Schönberg *et al.* (1978); (*8*), Yamano & Tsupi (1976); (*9*), Indelicato & Wilham (1974); (*10*), Kinget & Schwartz (1968); (*11*), Galdecki & Weifel (1978); (*12*), Sweet (1972); (*13*), Dexter & Van der Veen (1978); (*14*), Domiano *et al.* (1979); (*15*), Blanpain *et al.* (1977*b*); (*16*), Blanpain *et al.* (1977*a*); (*17*), Woodward (1980).

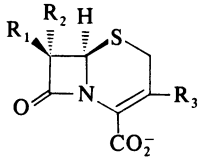
(*a*)



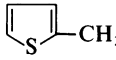
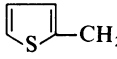
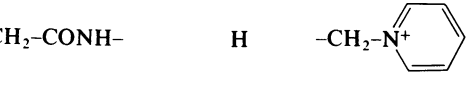
(2-Cephem)

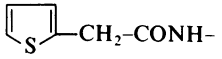
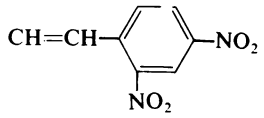
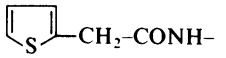
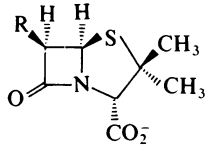
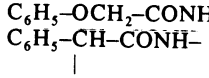
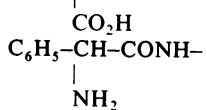
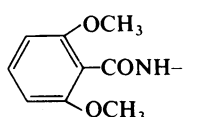
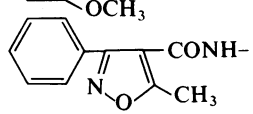
	R_1	R_2	R_3	Non-planarity of the β-lactam nitrogen, *h* (nm)	Second-order rate constant for hydrolysis by OH^- ($M^{-1} \cdot s^{-1}$)	
					At 37°C	At 35°C
					IP	A	Ref. *2*
(1) Δ^2-Deacetoxy-7-phenylacetamido-cephalosporanate	$C_6H_5-CH_2-CONH-$	H	CH_3	0.006[(a)(1)]	0.13	0.06	0.07[(a)]

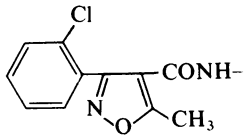
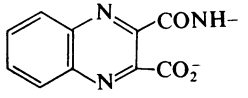
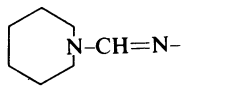
(*b*)



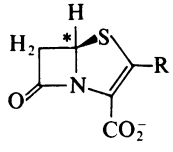
(3-Cephems)

	R_1	R_2	R_3	Non-planarity of the β-lactam nitrogen, *h* (nm)	Second-order rate constant for hydrolysis by OH^- ($M^{-1} \cdot s^{-1}$)				
					At 37°C		At 35°C		At 31.5°C
					IP	A	Ref. *8*	Ref. *2*; *9*	Ref. *10*
(2) Δ^3-Deacetoxy-7-phenylacetylamido-cephalosporanate	$C_6H_5-CH_2CONH-$	H	$-CH_3$	0.020[(a)(3)]	0.18	0.09	—	0.09	—
(3) Cephalexin	$C_6H_5-CH(NH_2)-CONH-$	H	$-CH_3$	—	0.07	—	0.07	0.1–0.4	—
(4) 7-Aminocephalosporanate	NH_2-	H	$-CH_2-O-CO-CH_3$	—	0.24	—	0.06	—	—
(5) Cephaloglycine	$C_6H_5-CH(NH_2)-CONH-$	H	$-CH_2-O-CO-CH_3$	0.022[(4)]	0.70	1.1	0.40	1.7–3	—
(6) Cephalosporin C	^-O_2C, ^+H_3N, C(D)$-[CH_2]_3-CONH-$	H	$-CH_2-O-CO-CH_3$	0.014–0.028[(b)(5)]	0.17	—	—	1.0	—
(7) Cephalothin		H	$-CH_2-O-CO-CH_3$	0.009–0.015[(b)(6)]	0.42 ± 0.12		0.30	0.97	0.14
(8) Cephaloridine		H		0.024[(4)]	—	0.90	1.0	1.9	—

				Non-planarity of the β-lactam nitrogen, *h* (nm)	Second-order rate constant for hydrolysis by OH^- ($M^{-1}.s^{-1}$)				
					At 37°C		At 35°C		At 31.5°C
					IP	A	Ref. *8*	Ref. *2; 9*	Ref. *10*
(9) Nitrocefin		H		—	—	3.0[d]	—	—	—
(10) Cefoxitin		$-OCH_3$	$CH_2-O-CONH_2$	0.018[c][7]	0.25	—	—	—	—
(*c*)  (Penams)									
(11) Penicillanate				—	0.01	—	—		—
(12) 6-Aminopenicillanate		NH_2-		0[e][11]–0.032[12]	0.15	—	0.09	0.15	—
(13) Benzylpenicillin		$C_6H_5-CH_2-CONH-$		0.038–0.042[13]	0.37 ± 0.1		0.33	0.51	0.20
(14) Phenoxymethylpenicillin		$C_6H_5-OCH_2-CONH-$		0.040[14]	0.63	—	—	0.69	0.27
(15) Carbenicillin				—	0.37	—	0.34	—	0.19
(16) Ampicillin				0.038[1]	0.40	—	0.71	0.53–0.56	0.21
(17) Methicillin				0.044[15]	0.33	—	—	—	0.10
(18) Oxacillin				0.038[16]	0.37	—	—	—	0.22

		Non-planarity of the β-lactam nitrogen, *h* (nm)	Second-order rate constant for hydrolysis by OH^- ($M^{-1} \cdot s^{-1}$)				
			At 37°C		At 35°C		At 31.5°C
			IP	A	Ref. *8*	Refs. *2*; *9*	Ref. *10*
(19) Cloxacillin		0.039[f][14]	0.37	—	0.37	—	0.33
(20) Quinacillin		—	0.43	—	—	—	—
(21) Mecillinam		0.042[f][14]	0.03	—	—	—	—

(*d*)

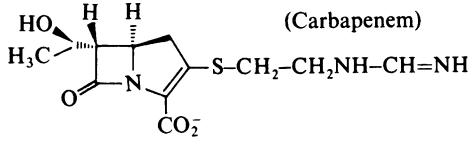


(Penems)

	R		At 37°C	
			IP	A
(22) Unsubstituted	H	0.043–0.044[17]	1.0	—
(23) 2-Methylpenem	$-CH_3$		0.5	1.25
(24) 2-Phenylpenem	$-C_6H_5$		0.8	0.40

* Racemic mixtures of 5*R*(+)- and 5*S*(—)-isomers

(*e*)



(Carbapenem)

(25) Thienamycin (*N*-formimidoyl) — 0.049[7] — 1.60

[a] The indicated values are those obtained for the phenoxymethyl analogues.
[b] Two molecules with different conformations occur in the unit cell.
[c] The indicated value is that of an ester of a cephamycin analogue with different R_1 and R_3 side-chains.
[d] The hydrolysed product rapidly undergoes other transformations as shown by the disappearance of the characteristic absorbance at 482nm. The estimated $M^{-1} \cdot s^{-1}$ value is only approximate.
[e] This value is probably erroneous.
[f] Crystallized as esters.

Table 3. *Interaction of β-lactam compounds with the R39 and G β-lactamases (at 30°C and pH 7.0)*

β-Lactam compounds	R39 β-lactamase K_m (mM)	R39 β-lactamase $V_{max.}$ (μmol·min^{-1}· mg of protein^{-1})	R39 β-lactamase $k_{cat.}$ (s^{-1})	R39 β-lactamase $k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	G β-lactamase K_m (mM)	G β-lactamase $V_{max.}$ (μmol·min^{-1}· mg of protein^{-1})	G β-lactamase $k_{cat.}$ (s^{-1})	G β-lactamase $k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
2-Cephem (1)	0.3	30	7.5	25000	2.0	40	20	10000
3-Cephem (2)	0.4	12	3	7500	1.3	17	8.5	6500
Benzylpenicillin (13)[a]	0.065	256	64	980000	0.74	920	460	620000
6-Aminopenicillanate (12)[a]	0.07	200	50	700000	0.6	700	350	600000
Penicillanate (11)	0.11	56	14	127000	2.5	125	62	25000
Unsubstituted penem (22)	0.3	26	6.5	20000	0.6	670	335	1000000
2-Methylpenem (23)	0.25	66	16	64000	0.25	660	330	2500000
2-Phenylpenem (24)	0.05	30	7.5	150000	0.36	910	455	2500000

[a] From Kelly *et al.* (1981*a*).

Table 4. *Interaction of β-lactam compounds with the R61 and R39* D-*alanyl*-D-*alanine-cleaving serine peptidases [at 37°C unless otherwise indicated and at pH 7 (R61 peptidase) or 8 (R39 peptidase)]*

β-Lactam compounds	R61 peptidase k_{+2}/K ($M^{-1}\cdot s^{-1}$)	R61 peptidase k_{+3} (s^{-1})	R61 peptidase Half-life of complex EI* (min)	R39 peptidase k_{+2}/K ($M^{-1}\cdot s^{-1}$)	R39 peptidase k_{+3} (s^{-1})	R39 peptidase Half-life of complex EI* (min)
2-Cephem (1)	68	3×10^{-3}	4	52	$<2\times10^{-5}$	>575
3-Cephem (2)	77	5×10^{-6}	2270	4850	$<1\times10^{-7}$	>100000
Benzylpenicillin (13)[a]	14000 (25°C)	1.4×10^{-4}	80	300000	2.8×10^{-6}	4100
6-Aminopenicillanate (12)[b]	0.25	$<6\times10^{-5}$	>2000	1200	5.6×10^{-3}	2
Penicillanate (11)	0.06[c]	6×10^{-4}	20	14	6×10^{-4}	20

[a] From Frère *et al.* (1973, 1974).
[b] From Kelly *et al.* (1981*b*).
[c] $K = 36$ mM; $k_{+2} = 2.2\times10^{-3}\,s^{-1}$ (see the Materials and methods section).

Table 5. *Interaction of β-lactam compounds with the R61 and R39* D-*alanyl*-D-*alanine-cleaving serine peptidases [at 37°C and pH 7 (R61 peptidase) or 8 (R39 peptidase)]*

β-Lactam compounds	R61 peptidase K_m (mM)	R61 peptidase $V_{max.}$ (μmol·min^{-1}· mg of protein^{-1})	R61 peptidase $k_{cat.}$[c] (s^{-1})	R61 peptidase $k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	R39 peptidase K_m (mM)	R39 peptidase $V_{max.}$ (μmol·min^{-1}· mg of protein^{-1})	R39 peptidase $k_{cat.}$[c] (s^{-1})	R39 peptidase k_{+3}[d] (s^{-1})	R39 peptidase $k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
Unsubstituted penem (22)	0.08[a] 0.07[b]	0.072	0.05	670	<0.05[a] 0.004[b]	0.008	0.007	0.008	1750
2-Methylpenem (23)	0.125	0.015	0.01	800	<0.05[a] 0.0015[b]	0.009	0.008	0.008	5400
2-Phenylpenem (24)	0.14	0.031	0.02	1400	<0.03[a] 0.001[b]	0.011	0.010	0.010	10000

[a] As obtained from Lineweaver–Burk plot (direct procedure).
[b] As obtained from substrate-competition experiments with diacetyl-L-Lys-D-Ala-D-Ala.
[c] As obtained from direct measurements.
[d] As obtained from measurements of enzyme reactivation (indirect procedure). These values indicated that the EI* complexes have half-lives ranging between 70 and 100 s. On the basis that $k_{cat.} = k_{+3}$ also applies to the interactions with the R61 peptidase, the EI* complexes formed with this latter enzyme have half-lives ranging between 14 and 70 s.

k_{+2}/K are also equivalent. Also, on the basis that $k_{cat.} = k_{+3}$ and $K_m = k_{cat.}/(k_{+2}/K)$, the K_m values (mM) of penicillinate were 10 (R61 peptidase) and 0.04 (R39 peptidase) and those of 6-aminopenicillanate were <0.24 (R61 peptidase) and 0.05 (R39 peptidase). Finally, the $V_{max.}$ values (μmol · min^{-1} · mg of protein^{-1}) of penicillanate were 1×10^{-3} (R61 peptidase) and 7×10^{-4} (R39 peptidase), and those of 6-aminopenicillanate were $<1 \times 10^{-4}$ (R61 peptidase) and 9×10^{-3} (R39 peptidase).

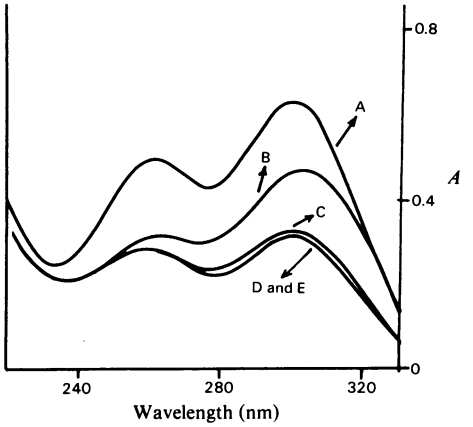


Fig. 1. *Action of the R39 β-lactamase on the racemic 2-methylpenem*

Spectra were recorded at 30°C using a scanning rate of 20nm/min (6min for the whole spectrum) on a Beckman D-U 8 recording spectrophotometer. A, Spectrum of the intact 2-methylpenem (0.2mM, in a total volume of 300μl of 50mM-sodium phosphate, pH7.0). The enzyme (1μg in 5μl) was then added, and spectra B, C, D and E were recorded successively without interruption. No modification of spectrum E was observed after several hours at 30°C.

Comparisons between the second-order rate constants for (i) the hydrolysis of the β-lactam amide bond by OH⁻ and (ii) the opening of the same β-lactam amide bond by the β-lactamases and D-alanyl-D-alanine cleaving serine peptidases

With the D-alanyl-D-alanine-cleaving serine peptidases, formation of the intermediate EI* involves the opening of the β-lactam bond (and enzyme acylation) (Frère *et al.*, 1976; Duez *et al.*, 1981*b*). Assuming that the β-lactamases under study operate by the same or a similar mechanism, the second-order rate constants for hydrolysis of the β-lactam amide bond by OH^- and the second-order rate constants (k_{+2}/K or $k_{cat.}/K_m$) for the opening of the same β-lactam amide bond by both peptidases and β-lactamases are comparable data (Table 6). The selected β-lactam compounds (which include the 2- and 3-cephems, penicillanate and three penems under study) form five overlapping groups. Group (*a*) reveals the effects of a shift 2-cephem → 3-cephem → penam (the three compounds having the same aminoacyl substituent); group (*b*), the shift aminoacyl substitution → amino substitution → no substitution on $C_{(6)}$ of the penam nucleus; and group (*c*), the shift 6-unsubstituted penam → various 6-unsubstituted penems. Groups (*d*) and (*e*) show the

Table 6. *Second-order rate constants for the opening of the β-lactam amide bond by OH⁻, β-lactamases and D-alanyl-D-alanine peptidases*

For explanation of the underlined values, see the Discussion section.

	β-Lactam compounds	OH^- at 37°C ($M^{-1} \cdot s^{-1}$)	R39 β-lactamase at 30°C ($M^{-1} \cdot s^{-1}$)	G β-lactamase at 30°C ($M^{-1} \cdot s^{-1}$)	R39 peptidase at 37°C ($M^{-1} \cdot s^{-1}$) (unless otherwise stated)	R61 peptidase at 37°C ($M^{-1} \cdot s^{-1}$) (unless otherwise stated)
(*a*)	2-Cephem (1)	0.06–0.13	25000	10000	52	68
(*a*)	3-Cephem (2)	0.09–0.18	7500	6500	4850	77
	Benzylpenicillin (13)	0.37 ± 0.10	980000	620000	300000	14000
(*b*)	6-Aminopenicillanate (12)	0.15	700000	600000	1200	0.25
(*b*)	Penicillanate (11)	0.01	127000	25000	14	0.06
(*c*)	Unsubstituted penem (22)	1.0	20000	1000000	1750	670
(*c*)	2-Methylpenem (23)	0.5–1.25	64000	2500000	5400	800
(*c*)	2-Phenylpenem (24)	0.8–0.4	150000	2500000	10000	1400
(*d*)*	Cephalexin (3)	0.07	12300	1200	3000 (10°C)	4
(*d*)*	Cephaloglycine (5)	0.70–1.1	50000	9200	74000 (20°C)	22
(*e*)*	Cephalothin (7)	0.42 ± 0.12	300000	8800	>70000	3000
(*e*)*	Nitrocefin (9)	3.0	2800000	930000	3600000 (10°C)	460 (10°C)

* References for results for β-lactam compounds of groups (*d*) and (*e*): Frère *et al.* (1980); Duez *et al.* (1981*a*, 1982).

effects of various electron-withdrawing substituents on $C_{(3)}$ of the dihydrothiazine molecule in comparable Δ^3-cephalosporins.

Discussion

The data of Table 2 strongly suggest that the degree of non-planarity of the β-lactam nitrogen is mainly imposed by the nature of the ring that is fused to the β-lactam ring (and is relatively little influenced by the substituents that occur on both positions of the ring system). Pairs or groups of analogous or nearly analogous compounds can be selected, for which an increased pyramidal character of the β-lactam nitrogen (as expressed by the *h* value) is accompanied by an increased lability of the β-lactam amide bond (as expressed by the $M^{-1} \cdot s^{-1}_{(OH^-)}$ value). Penicillanate (compound 11), for example, is 50–100 times less labile than the corresponding unsubstituted penem (compound 23). It is dangerous, however, to generalize the rule. The 2-cephem (compound 1) is only slightly more stable than its 3-cephem analogue (compound 2). Mecillinam (compound 21) is 10 times more stable than all the 'classical' penams (which, however, exhibit a very similar *h* value). Doubt has already been cast on the widely held view of the over-riding importance of the non-planarity of the β-lactam nitrogen (Gensmantel *et al.*, 1981). Moreover, one should note (Pfaendler *et al.*, 1981) that a carba-1-penem ($h = 0.54$) is more stable than the analogous carba-2-penem ($h = 0.50$) and penem ($h = 0.42$–0.44). In the 3-cephem series, the electron-withdrawing capacity of the substituent on $C_{(3)}$ seems to supersede any other structural feature [cf. cephalexin and cephaloglycine (compounds 3 and 5), on the one hand, and cephalothin, cephaloridine and nitrocefin (compounds 7–9), on the other]. Remarkably, also, cephaloridine is as reactive as the penems (compounds 22–24), and nitrocefin, whose relevant geometry is very probably closely similar to that of the other 3-cephems, appears to be even more reactive than the carbapenem thienamycin. The chemical reactivity, in the ground-state conformation, of the β-lactam compounds thus appears to be the result of a complex interplay between the fused-ring system and its side chains. Within the limits of the present investigation, these combined effects result, at the most, in a 300-fold variation in the second-order rate constants for hydrolysis of the β-lactam amide bond by OH^- at 37°C, from 0.01 $M^{-1} \cdot s^{-1}$ (penicillanate) to 3.0 $M^{-1} \cdot s^{-1}$ (nitrocefin).

It is reasonable to propose that the higher the intrinsic reactivity of the β-lactam compounds, the lower the barrier energy to formation of the intermediate EI* during interaction with the β-lactamases and the D-alanyl-D-alanine-cleaving peptidases (and therefore the more readily this energy barrier is overcome). In this respect, examination of the data of Table 6 leads to the following observations. (i) The accelerating effect due to enzyme action on a given β-lactam compound can be expressed by the ratio between the second-order rate constant for the opening of the β-lactam amide bond by the enzyme under consideration and the second-order rate constant for hydrolysis by OH^-, i.e. the ratio $M^{-1} \cdot s^{-1}_{(enzyme)} / M^{-1} \cdot s^{-1}_{(OH^-)}$. Depending on both the β-lactam compound and the enzyme, this ratio varies from less than 2 (6-aminopenicillanate and the R61 peptidase) to 1×10^6 or more. (ii) That a higher intrinsic reactivity of the β-lactam compound results in a faster formation of the intermediate EI* with a given β-lactamase or D-alanyl-D-alanine-cleaving serine peptidase depends on which β-lactam compounds are used for the comparison. Thus, with several groups of β-lactam compounds and enzymes, the observed variations in the $M^{-1} \cdot s^{-1}_{(OH^-)}$ values, on the one hand, and the observed variations in the $M^{-1} \cdot s^{-1}_{(enzyme)}$ values, on the other, are roughly commensurate (at least within one order of magnitude). This applies to the β-lactam compounds of groups (*b*), (*d*) and (*e*) and the R39 β-lactamase, those of groups (*b*), (*c*) and (*d*) and the G β-lactamase, those of groups (*c*), (*d*) and (*e*) and the R39 peptidase and those of group (*d*) and the R61 peptidase (the relevant $M^{-1} \cdot s^{-1}$ values are underlined in Table 6). In some cases, however, the observed variations in the $M^{-1} \cdot s^{-1}_{(OH^-)}$ and the corresponding $M^{-1} \cdot s^{-1}_{(enzyme)}$ values are largely disproportionate (compare the 2- and 3-cephems with benzylpenicillin, whatever enzyme is considered). Cases also exist where, contrary to the prediction, increased $M^{-1} \cdot s^{-1}_{(OH^-)}$ values are paralleled by decreased $M^{-1} \cdot s^{-1}_{(enzyme)}$ values (compare the unsubstituted and 2-methylpenems with penicillanic acid in their reaction with the R39 β-lactamase, or nitrocefin with cephalothin in their interaction with the R61 peptidase). This strongly suggests that the goodness of fit of the β-lactam molecule to the enzyme cavity, rather than its intrinsic reactivity, is the primary parameter that governs the enzyme catalytic efficiency. Other observations (Frère *et al.*, 1980; Ghuysen, 1980; Kelly *et al.*, 1981*a*,*b*; Duez *et al.*, 1981*a*,*b*) lead to the same conclusions. Although the penams 13–19 (Table 2) have very similar $M^{-1} \cdot s^{-1}_{(OH^-)}$ values, their $M^{-1} \cdot s^{-1}_{(enzyme)}$ values widely vary: from 15 (methicillin) to 14000 (benzylpenicillin) with the R61 peptidase; from 1100 (methicillin) to 300000 (benzylpenicillin) with the R39 peptidase; from 9200 (methicillin) to 635000 (benzylpenicillin) with the G β-lactamase; and from 46000 (cloxacillin) to 980000 (benzylpenicillin) with the R39 β-lactamase. Conversely, *N*-formimidoylthienamycin, which has a considerably higher intrinsic reactivity than benzylpenicillin but has a

very distinct geometry, is characterized by relatively low $M^{-1} \cdot s^{-1}_{(enzyme)}$ values: 1000 for the R61 peptidase, 10000 for the R39 peptidase, 3000 for the G β-lactamase and 2000 for the R39 β-lactamase. From all these observations, it seems very likely that the structures of the side-chains on the bicyclic fused-ring systems are responsible for the relative positioning of the β-lactam carbonyl group and the active site serine hydroxy group in the case of the D-alanyl-D-alanine-cleaving serine peptidases. Some side-chains can induce a catalytically active geometry, others cannot. The same mechanism probably occurs with the β-lactamases studied (preliminary work strongly suggests that these β-lactamases are also serine enzymes).

A last comment deserves attention. Recent developments have shown that the concept that β-lactam compounds are substrates of the β-lactamases and inactivators of the D-alanyl-D-alanine-cleaving peptidases is an oversimplification. Structural features in the β-lactam molecules can deeply affect the half-lives of the intermediates EI* formed with both types of enzymes. *N*-Formimidoylthienamycin is an inactivator of the R39 β-lactamase (Kelly *et al.*, 1981*a*), whereas, as shown in the present paper, the three penems studied formed very short-lived intermediates (half-life from 14 to 100s) with both R61 and R39 peptidases. Also, with the β-lactamases, some β-lactam compounds are known that behave both a substrates and as inactivators; the reaction pathway appears to be branched and the acyl–enzyme intermediate can undergo either simple hydrolysis or a rearrangement that produces an inactivated enzyme (Fisher *et al.*, 1978; Kelly *et al.*, 1981*a*).

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