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Des-, syn- and anti-oxyimino-Δ^3-cephalosporins

Intrinsic reactivity and reaction with RTEM-2 serine β-lactamase and D-alanyl-D-alanine-cleaving serine and Zn^{2+}-containing peptidases

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The presence and configuration (syn or anti) of an oxyimino group in the 7(β)-acyl side chain of 3-cephems do not modify the intrinsic reactivity of the β-lactam ring, but have highly enzyme-specific effects. When compared with the corresponding des-oxyimino β-lactam compound: (i) with the plasmid-mediated *Escherichia coli* RTEM-2 serine β-lactamase, the substrate activity of the anti isomer is increased and that of the syn isomer is decreased; (ii) with the *Streptomyces* R61 serine D-alanyl-D-alanine cleaving peptidase (a highly penicillin-sensitive enzyme), the rate of enzyme acylation is not or only little affected when the oxyimino group is in the syn configuration, but is decreased when the oxyimino group is in the anti configuration; (iii) with the *Actinomadura* R39 serine D-alanyl-D-alanine-cleaving peptidase (an exceedingly highly penicillin-sensitive enzyme), the rate of enzyme acylation is unaffected whatever the configuration of the substituent. The oxidation of the sulphur atom of the dihydrothiazine ring on the β-face of the molecule makes it both a poorer inactivator of the DD-peptidases and a poorer substrate of the β-lactamase. The *Streptomyces albus* G Zn^{2+}-containing D-alanyl-D-alanine-cleaving peptidase (a highly penicillin-resistant enzyme) remains highly resistant to all compounds tested.

The oxyimino-cephalosporins (Bucourt, 1981) are characterized by the presence, in the 7(β)-side chain, of an oxime function that may occur in two configurations, syn and anti (Table 1). The syn configuration confers both high antibacterial activity (Bucourt *et al.*, 1978) and high resistance to various β-lactamases (Fu & Neu, 1978; Chabbert & Lutz, 1978; Schrinner *et al.*, 1980). The present paper describes experiments performed in order to elucidate the relationships that might exist between the oxime configuration, the chemical reactivity of the β-lactam ring in the ground-state conformation, its susceptibility to hydrolysis by the plasmid-mediated *Escherichia coli* RTEM-2 serine β-lactamase and its inactivating potency towards various D-alanyl-D-alanine-cleaving peptidases (in short DD-peptidases), namely the Zn^{2+}-containing DD-peptidase of *Streptomyces albus* G and the serine DD-peptidases of *Streptomyces* R61 and *Actinomadura* R39. The RTEM-2 β-lactamase was selected because of its relatively high cephalosporinase activity, when compared with the *Bacillus cereus* I, *B. licheniformis* or *Staphylococcus aureus* β-lactamases (Labia *et al.*, 1979).

Materials and methods

Compounds

Cefuroxime was a gift from Glaxo, Greenford, Middx., U.K. All the other β-lactam compounds listed in Table 1 were gifts from Roussel-Uclaf Laboratories, Romainville, France, or Hoechst A.-G., Frankfurt, Germany. Sodium salts were dissolved in double-distilled water. Acids were dissolved in 0.1 M-sodium phosphate buffer, pH 7.0. The tripeptide $N^{\alpha}N^{\varepsilon}$-diacetyl-L-Lys-D-Ala-D-Ala (the standard substrate of the DD-peptidases) was from UCB-Bioproducts, Brussels, Belgium.

Chemical stability of the β-lactam compounds at 30°C at pH 12.0

The β-lactam compounds (0.6 mM) were incu-

bated at 30°C in 1 M-K_2HPO_4 adjusted to pH 12.0 with KOH. Samples (10 μl) were withdrawn after increasing periods of time, and the extent of hydrolysis was measured by using the starch–iodine test method of Novick as modified by Johnson *et al.* (1975).

Susceptibility to hydrolysis by the RTEM-2 β-lactamase

A freeze-dried sample of the purified β-lactamase (a gift from Dr. J. R. Knox, University of Connecticut, Storrs, CT, U.S.A.) was dissolved in 50 mM-sodium phosphate buffer, pH 7.0 (final concn. 2.93 mg of protein/ml). Enzyme and β-lactam compounds were incubated together in 33 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.0, at 30°C, and the extent of hydrolysis was measured as described above. Benzylpenicillin (from Rhône–Poulenc, Vitry, France) and nitrocefin (from Glaxo) were used in control experiments. Hydrolysis was followed by monitoring the absorbance of the corresponding solutions at 482 nm (O'Callaghan *et al.*, 1972) and 240 nm (Samuni, 1975) respectively.

Reaction with the DD-*peptidases*

Enzyme inactivation. The DD-peptidases, purified as described elsewhere (Frère *et al.*, 1973, 1974; Duez *et al.*, 1981), were used at final concentrations of 15, 30 and 30 μg of protein/ml (for the R39, R61 and G peptidases respectively). Incubation with the β-lactam compounds was carried out at 37°C and in 5 mM-sodium phosphate buffer, pH 7.0, containing 100 μM-EDTA (sodium salt) (R61 peptidase), 100 mM-Hepes/NaOH buffer, pH 8.0, containing 5 mM-$MgCl_2$ (G peptidase) or 100 mM-Hepes/NaOH buffer, pH 8.0, containing 5 mM-$MgCl_2$ and 100 mM-NaCl (R39 peptidase). Residual peptidase activity was assayed by supplementing samples with 2 mM-Ac_2-L-Lys-D-Ala-D-Ala and measuring the amounts of D-alanine released after a 10 min incubation at 37°C. Pseudo-first-order rate constants of inactivation (k_a) were obtained by plotting $\ln(A_t/A_0)$ versus time, where A_0 and A_t are the enzymic activities before and after incubation of the DD-peptidase with the β-lactam compound.

Enzyme recovery. The R61 and R39 serine DD-peptidases (but not the G Zn^{2+}-containing DD-peptidase) could be completely inactivated by an excess of β-lactam compound. In those cases, the unbound β-lactam compound was eliminated by dialysis at 4°C against the relevant buffer, and the inactive enzyme–β-lactam complex was re-incubated at 37°C. Enzyme recovery was measured as a function of time.

Results

The β-lactam compounds listed in Table 1 were studied with respect to their intrinsic chemical stability (as expressed by their susceptibility to hydrolysis by OH^-), their substrate activity towards the RTEM-2 serine β-lactamase and their capacity of inactivating the three selected DD-peptidases. The relevant kinetic data are given in Table 1. The following comments deserve attention.

The opening of the β-lactam amide bond at pH 12.0 followed pseudo-first-order kinetics. The results are expressed in terms of second-order rate constants ($M^{-1}\cdot s^{-1}$) for hydrolysis.

Time courses of RTEM-2 β-lactamase-catalysed hydrolysis of the β-lactam compounds did not suggest any substrate-induced inactivation of the enzyme, such as occurs with some β-lactam compounds and the serine β-lactamase of *Bacillus cereus* (Citri *et al.*, 1976). No biphasic phenomenon was observed, and the hydrolysis obeyed the integrated form of the Henri–Michaelis equation. The K_m and $k_{cat.}$ values were therefore derived from Eisenthal–Cornish-Bowden plots (Eisenthal & Cornish-Bowden, 1974). Note, however, that, with the 3-cephem nitrocefin, time courses revealed a slow and progressive decrease of enzyme efficacy that could not be attributed to substrate depletion or to product inhibition. Rather, the kinetics suggested either a reversible change of the enzyme–substrate complex or an immobilization of part of the protein in the form of an isomerized inactive acyl-enzyme. Furth (1979) reported a similar behaviour during *Pseudomonas aeruginosa* β-lactamase-catalysed hydrolysis of nitrocefin, and more recently Citri & Zyk (1982) described conformational responses of the RTEM β-lactamase to some β-lactam substrates.

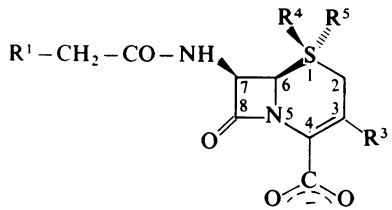
Inactivation of the DD-peptidases by β-lactam compounds usually obeys the following reaction model (Frère *et al.*, 1975*a*,*b*):

$$E + I \overset{K}{\rightleftharpoons} E\cdot I \xrightarrow{k_{+2}} E\text{–}I^* \xrightarrow{k_{+3}} E + \text{degradation products}$$

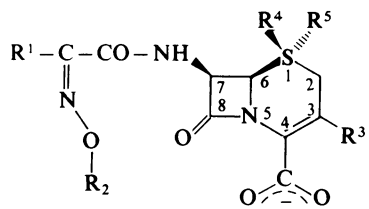
where E represents the DD-peptidase, I the β-lactam compound, E·I the Michaelis complex and E–I* the intermediate (a serine ester-linked acyl-enzyme in the case of the R61 and R39 serine DD-peptidases); K is a dissociation constant, and k_{+3} and k_{+2} are first-order rate constants. The higher the k_{+2}/K value and the lower the k_{+3} value, the better the β-lactam compound as a DD-peptidase inactivator. With the R61 and R39 serine DD-peptidases and at β-lactam compound concentrations of about 1 μM for the R39 DD-peptidase and 100 μM for the R61 DD-peptidase, the velocity of enzyme acylation {i.e. enzyme inactivation; $k_a = (k_{+2}/K)[I]$} remained proportional to [I], thus

Table 1. *Desoxyimino-, syn-oxyimino- and anti-oxyimino-3-cephems*

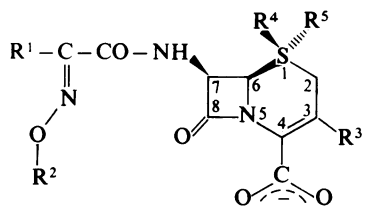
The Table lists second-order rate constants ($M^{-1}\cdot s^{-1}$) for the opening of the β-lactam amide bond by OH^- at 37°C and interaction of the β-lactam compounds with RTEM-2 serine β-lactamase at 37°C and pH 7.0, and with the R61 and R39 D-alanyl-D-alanine-cleaving serine peptidases at 37°C and pH 7.0 (R61 peptidase) or 8.0 (R39 peptidase).



Desoxyimino-3-cephems

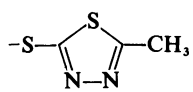
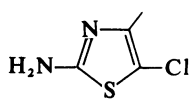


syn-Oxyimino-3-cephems



anti-Oxyimino-3-cephems

Unless otherwise stated: $R^1 = H_2N-$; $R^2 = CH_3$; $R^3 = CH_2O-CO-CH_3$ and there is no substituent at R^4 and R^5.

Compound	R^1	R^2	R^3	R^4	R^5	k_{OH^-} ($M^{-1}\cdot s^{-1}$)	RTEM-2 β-lactamase K_m (mM)	RTEM-2 β-lactamase $k_{cat.}$ (s^{-1})	RTEM-2 β-lactamase $k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	R61* peptidase k_{+2}/K ($M^{-1}\cdot s^{-1}$)	R39* peptidase k_{+2}/K ($M^{-1}\cdot s^{-1}$)
(*a*) Desoxyimino-3-cephem											
(1) RU23345	–	Not applicable	–	–	–	0.06	0.2	8.3	36000	24	2300
(*b*) syn-Oxyimino-3-cephems											
(2) Cefotaxime (HR756)	–	–	–	–	–	0.03	6.2	7.1	1100	16	2600
(3) RU26449	–	–	–	–	O	0.12	1.8	1.3	700	Not determined	
(4) HR109	–	–	–	O	–	0.08	0.5	0.02	40	1.5	200
(5) RU25159	–	–		–	–	0.06	1.0	7.5	7500	23	2000
(6) HR979		–	–	–	–	0.16	0.7	0.4	600	1.5	1200
(7) Cefuroxime		–	CH_2OCONH_2	–	–	0.04	0.9	5.0	5600	350	3900
(*c*) anti-Oxyimino-3-cephems											
(8) RU25238	–	–	–	–	–	0.05	0.32	26	81000	2.0	2300
(9) S810593	–	–	–	–	O	0.12	0.5	41	83000	Not determined	
(10) S810592	–	–	–	O	–	0.34	1.3	6.3	4800	1.5	450
(11) RU25519	–	CH_2-CH_3	–	–	–	0.06	0.21	36	173000	1.5	1300

* Individual k_{+3} values (in $s^{-1} \times 10^6$) for the R61 and R39 peptidases respectively: 5 and <2 [compound (1)]; <4 and <2 [compound (2)]; 4 and <2 [compound (3)]; <2 and <2 [compound (5)]; 1 and <2 [compound (6)]; 4 and <2 [compound (7)]; <2 and <2 [compound (8)]; <5 and <5 [compound (10)]; <2 and <2 [compound (11)].

indicating that $[I] \ll K$. Under these conditions, only the k_{+2}/K ratio, i.e. the second-order rate constant of enzyme acylation (inactivation), could be obtained (Table 1). The k_{+2}/K values thus obtained were all rather low. As discussed by Brocklehurst (1979), such low values indicate that the first step can be considered as a quasi-equilibrium. Moreover, at a given β-lactam concentration, inactivation always appeared to be a pseudo-first-order phenomenon, a behaviour that would not be expected if the first step was not much faster than the second. In turn, the k_{+3} value, which expresses the velocity of the acyl-enzyme E–I* breakdown (or enzyme recovery), was measured as described in the Materials and methods section. The k_{+3} value was at the most equal to $5 \times 10^{-6} s^{-1}$ (see footnote of Table 1), indicating that the acyl-enzyme intermediates had half-lives equal to or longer than 30h. With the G Zn^{2+}-containing DD-peptidase, none of the β-lactams listed in Table 1, at concentrations close to 1 mM, had any inactivating potency, except for compound (10), for which a k_{+2}/K value of $0.5 M^{-1} \cdot s^{-1}$ was obtained (not shown in Table 1).

Discussion

In the ground state conformation, the susceptibility of the β-lactam compounds to nucleophilic attack (as expressed by the second-order rate constant of hydrolysis by OH^-) is the result of a complex interplay between the ring framework and its side chains, in particular the inductive effect of the substituent at C-3 in the 3-cephems (Proctor *et al.*, 1982; Frère *et al.*, 1982; D. B. Boyd, personal communication). Yet, examination of a large series of 2-cephems, 3-cephems, penams, penems and carbapenems showed that these combined effects had relatively little influence on the intrinsic reactivity of the β-lactam compounds, the values of the second-order rate constant (in $M^{-1} \cdot s^{-1}$) for reaction with OH^- ranging at the most from 0.01 for the unsubstituted penam penicillanate to 3.0 for the 3-cephem nitrocefin (Frère *et al.*, 1982). With the 3-cephems listed in Table 1, the observed variations in the values of the second-order rate constant (in $M^{-1} \cdot s^{-1}$) for reaction with OH^- are within one order of magnitude, from 0.03 for compound (2) to 0.34 for compound (10).

Assuming that the RTEM-2 serine β-lactamase and the R61 and R39 serine DD-peptidases operate by the same mechanism, the $k_{cat.}/K_{m(\beta\text{-lactamase})}$, $k_{+2}/K_{(DD\text{-peptidases})}$ and second-order rate constants for hydrolysis by OH^- are comparable data; they all refer to the opening of the β-lactam amide bond. Examination of the data of Table 1 leads to the following observations.

1. The accelerating effect due to enzyme action on a given β-lactam compound and as expressed by the ratio of the values of the second-order rate constant (in $M^{-1} \cdot s^{-1}$) for reaction with the enzyme and OH^- varies from 500 [compound (4)] to about 3×10^6 [compound (11)] with the β-lactamase, from 1300 [compound (10)] to about 1×10^5 [compound (7)] with the R39 DD-peptidase, and from 4 [compound (10)] to about 9000 [compound (7)] with the R61 DD-peptidase. When compared with the R39 DD-peptidase and as already shown by other studies, the R61 DD-peptidase exhibits increased intrinsic resistance to β-lactam action.

2. As shown by the comparable triad compounds (1), (2) and (8), the occurrence of an oxyimino group, irrespective of its configuration, in the 7(β)-side chain of the 3-cephems has very little or no effect on the chemical stability of the β-lactam compound. This substituent, however, is highly enzyme-specific. Depending on whether it occurs in the syn or anti configuration, the oxyimino group causes either a 35-fold decrease (mainly by affecting the K_m value) or a 2-fold increase (mainly by affecting the $V_{max.}$ value) in the substrate activity of the β-lactam compound towards the RTEM-2 β-lactamase; conversely, the syn substituent has no effect on, and the anti substituent causes a 12-fold decrease of, the second-order rate constant of the acylation of the R61 DD-peptidase. Finally, whether the oxyimino group is present or not, and whether it occurs in the syn or anti configuration, is irrelevant to the interaction of the β-lactam compound with the R39 DD-peptidase active site.

3. As shown by the compounds (5), (6), (7) and (11), structural variations at positions R^1, R^2 and R^3 of the (syn or anti) oxyimino 3-cephem derivatives influence both the β-lactamase substrate activity and the DD-peptidase-inactivating potency of the compounds. As shown by the comparable triads compounds (2), (3) and (4) and compounds (8), (9) and (10), oxidation of the sulphur atom of the dihydrothiazine ring either much decreases or has no effect on β-lactamase substrate activity, depending on whether the substitution affects the β- or the α-face of the molecule. β-Sulphoxide derivatives [compounds (4) and (10)] also show low inactivating potency towards the R61 and R39 serine DD-peptidases. It should be noted that, with these two β-sulphoxides, the k_{+2}/K values are similar for the inactivation of the R61 enzyme. Surprisingly, the Zn^{2+}-containing DD-peptidase, an enzyme known to have high intrinsic resistance to β-lactam action, exhibits detectable sensitivity (second-order rate constant of enzyme inactivation $0.5 M^{-1} \cdot s^{-1}$) to the β-sulphoxide compound (10).

In conclusion, it seems that the structural difference between syn and anti isomers has a deep influence on the sensitivity of the molecule to β-

lactamase hydrolysis and relatively little effect on the inactivating potency towards the model enzymes studied in the present paper. The first observation is in good agreement with that reported by Labia *et al.* (1980). The second, however, does not apply to the DD-carboxypeptidase and transpeptidase activities of ether-treated *Pseudomonas aeruginosa* cells, for which Mirelman & Nuchamowitz (1980) reported a much decreased effect on the anti isomer.

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References

Brocklehurst, K. (1979) *Biochem. J.* **181**, 775–778

Bucourt, R. (1981) in *Recent Advances in the Chemistry of β-Lactam Antibiotics* (Gregory, G. I., ed.), pp. 1–25, Royal Society of Chemistry, London

Bucourt, R., Heymès, R., Lutz, A., Pénasse, L. & Perronnet, J. (1978) *Tetrahedron* **34**, 2233–2243

Chabbert, Y. A. & Lutz, A. J. (1978) *Antimicrob. Agents Chemother.* **14**, 749–775

Citri, N. & Zyk, N. (1982) *Biochem. J.* **201**, 425–427

Citri, N., Samuni, A. & Zyk, N. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1048–1052

Duez, C., Frère, J. M., Klein, D., Noël, M., Ghuysen, J. M., Delcambe, L. & Dierickx, L. (1981) *Biochem. J.* **193**, 75–82

Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720

Frère, J. M., Ghuysen, J. M., Perkins, H. R. & Nieto, M. (1973) *Biochem. J.* **135**, 463–468

Frère, J. M., Moreno, R., Ghuysen, J. M., Perkins, H. R., Dierickx, L. & Delcambe, L. (1974) *Biochem. J.* **143**, 233–240

Frère, J. M., Ghuysen, J. M. & Perkins, H. R. (1975*a*) *Eur. J. Biochem.* **57**, 343–351

Frère, J. M., Ghuysen, J. M. & Perkins, H. R. (1975*b*) *Eur. J. Biochem.* **57**, 353–359

Frère, J. M., Kelly, J. A., Klein, D., Ghuysen, J. M., Claes, P. & Vanderhaeghe, H. (1982) *Biochem. J.* **203**, 223–234

Fu, K. P. & Neu, H. C. (1978) *Antimicrob. Agents Chemother.* **14**, 322–326

Furth, A. (1979) in *β-Lactamases* (Hamilton-Miller, J. M. T. & Smith, J. T., eds.), pp. 403–428, Academic Press, London

Johnson, K., Duez, C., Frère, J. M. & Ghuysen, J. M. (1975) *Methods Enzymol.* **43**, 687–698

Labia, R., Barthelemy, M., Fabre, C., Guionie, M. & Peduzzi, J. (1979) in *β-Lactamases* (Hamilton-Miller, J. M. T. & Smith, J. T., eds.), pp. 429–442, Academic Press, London

Labia, R., Kazmierczak, A., Guionie, M. & Masson, J. M. (1980) *J. Antimicrob. Chemother.* **6**, Suppl. A, 19–23

Mirelman, D. & Nuchamowitz, Y. (1980) *Antimicrob. Agents Chemother.* **17**, 115–119

O'Callaghan, C. H., Morris, A., Kirby, S. & Shingler, A. H. (1972) *Antimicrob. Agents Chemother.* **1**, 283–288

Proctor, P., Gensmantel, N. P. & Page, M. I. (1982) *J. Chem. Soc. Perkin Trans. 2* 1185–1192

Samuni, A. (1975) *Anal. Biochem.* **63**, 17–26

Schrinner, E., Limbert, M., Pénasse, L. & Lutz, A. (1980) *J. Antimicrob. Chemother.* **6**, Suppl. 1, 25–30