Brief Articles

Hydrolytic Stability versus Ring Size in Lactams: Implications for the Development of Lactam Antibiotics and Other Serine Protease Inhibitors†

Peter Imming,* Bernhard Klar, and Dorothea Dix

Institut für Pharmazeutische Chemie, Philipps-Universität, Marbacher Weg 6, 35032 Marburg, Germany

Received June 15, 2000

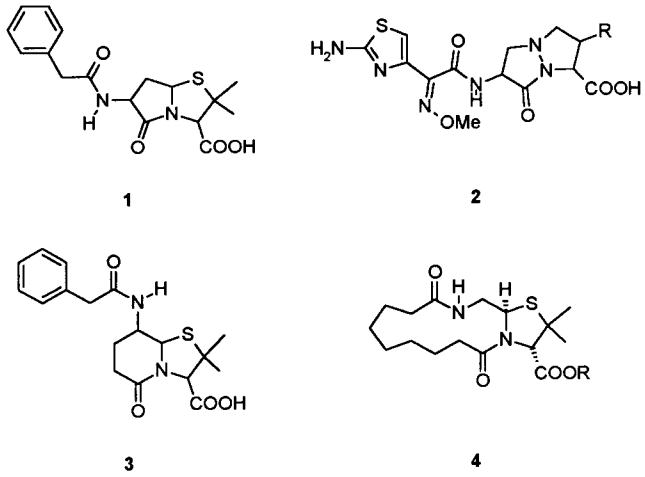
β-Lactam antibiotics act by acylating a serine hydroxyl group in the catalytic center of bacterial proteases. This requires, among other things, suitable reactivity of the lactam moiety. To evaluate the possible suitability of other lactam systems, kinetic studies were performed using the model reaction of lactams with hydroxide. Following the pace of the reaction by NMR, we found γ-butyrolactam to be hydrolyzed considerably slower than β-propiolactam. Surprisingly, δ-valerolactam and β-propiolactam had the same reactivity. β-Lactam antibiotics were more reactive than both by approximately a factor of 10^3. Medium-sized lactams were least susceptible to hydrolysis. The study highlights the as yet overlooked six-membered lactam ring as a promising vantage point for the development of new classes of antiinfectives and other serine protease inhibitors.

Introduction

Inhibitors of serine proteinases are therapeutically indispensable. The most prominent example is the β-lactam antibiotics, suicide inhibitors of bacterial (trans)peptidases. They acylate a serinyl residue in the catalytic center. For this, two prerequisites must be met: one concerning the 3D structure, the other reactivity. Focusing on the latter, they must not be so labile as to become hydrolyzed on their way toward the target, either nonenzymatically in body fluids or enzymatically by peptidases. Lability toward bacterial peptidases (lactamases) cannot be overcome by modification of the acylating power.[1] Lactams stable enough to withstand hydrolysis by a lactamase will also not react with a transpeptidase. So the hydrolytic lability or acylating power of antiinfective β-lactams is confined within narrow boundaries.

Modifications have rarely touched the β-lactam core.[2] Chart 1 depicts examples. The γ-lactam analogue **1** of benzylpenicillin, prepared during the first years of penicillin research, showed no activity and was not hydrolyzed by hydroxide at room temperature.[3] By the end of the 1980s, a lot of research effort went into the preparation of antiinfective γ-lactams.[4] The diazacyclopentanones **2** are the only 'expanded' lactams for which good activity was reported.[5] Larger than five-membered lactams were hardly ever taken into consideration. The only published examples − the six-membered derivative **3**[6] and the medium-sized penicillin analogues we prepared, e.g. **4**[7] − have no antibiotic activity. Their reactivity toward base hydrolysis is not known.

Chart 1



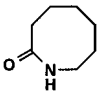
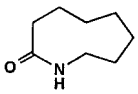
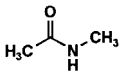
In view of the somewhat unpredictable outcome of the exchange of the β-lactam moiety for a larger ring, we reasoned that a comparative study of the hydrolytic stability of lactams might be able to guide future efforts. Within the various subclasses of β-lactam antiinfectives, a number of studies tried to correlate activity and acylating reactivity. There is no simple correlation between biological activity and chemical reactivity since (a) the target proteins (PBPs) exhibit different sensitivity, (b) lactamases may destroy the antiinfectives to an a priori unknown extent, and (c) diffusion to the target may be the limiting parameter.[8] Nevertheless, sufficient acylating power is necessary. The difference in reactivity between an amide and a β-lactam was estimated to be approximately 100-fold, increased by another 100-fold by fusing the β-lactam to the thiazolidine ring.[9] In recent years, the model reaction with hydroxide has indeed guided the development and selection of, for example,

† The material in this paper was partly presented in a lecture at the Dyson Perrins Laboratory, Department of Organic Chemistry, Oxford University, U.K., August 1999.

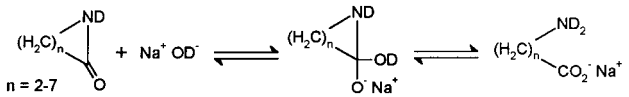
* To whom correspondence should be addressed. Tel: +49-6421-825893. Fax: +49-6421-827052. E-mail: imming@mailer.uni-marburg.de.

10.1021/jm000921k CCC: $19.00
Published on Web 10/13/2000

Table 1. Results of Hydrolysis Kinetics for Various Lactams

formula	no.	name	k_2 (M^{-1} s^{-1})	log k_2	r^2 of regression curve
	**8**	β-propiolactam	2.37×10^{-4}	−3.62	0.998
	**9**	γ-butyrolactam	5.59×10^{-6}	−5.34	0.999
	**10**	δ-valerolactam	1.21×10^{-4}	−3.92	0.999
	**11**	ϵ-caprolactam	3.21×10^{-6}	−5.49	0.983
	**12**	ω-oenantholactam	1.36×10^{-7}	−6.87	0.990
	**13**	ω-caprolactam	2.72×10^{-7}	−6.57	0.999
	**14**	*N*-methylacetamide	3.32×10^{-6}	−5.48	1.000

Scheme 1



5 **6** **7**

pyrazolidinones with excellent antibacterial activity[10] and benzoxazinones as elastase inhibitors.[11]

Results and Discussion

Unsubstituted Lactams. We determined the rate of hydrolysis of amides, beginning with unsubstituted lactams of ring size 4−9 and the open-chain *N*-methylacetamide by treating them with sodium [D]hydroxide in deuterium oxide solution at 30 °C (Scheme 1). In the literature, rate constants were determined by titrimetric methods, UV, or HPLC.[12] Since the hydrolysis of simple lactams may be accompanied by polymerization and the hydrolysis of β-lactam antibiotics triggers fairly complicated degradation sequences,[13] we chose ^{1}H NMR to follow the pace of the reaction. Thus we could be sure we were observing the actual hydrolysis of the amide function. A series of NMR spectra were recorded at constant temperature. The decrease and increase of the integrals of the educt (lactam or amide) and product (ω-aminocarboxylate or amine and carboxylate) yielded the data necessary for calculation of rate constants.

Only in the case of β-propiolactam (**8**) did we observe a set of signals of a further product which turned out to be the dipeptide from the reaction of two propiolactam moieties. The amount of dimer formation was taken into account quantitatively for the calculation of the rate constant as the signals of β-propiolactam, β-alanine, and the dimer were well-separated.

On the basis of second-order kinetics, the rate constants k_2 were calculated from the gradients of a plot of the logarithm of the concentration ratio of lactam and hydroxide against time (Table 1). The plot of log k_2 over ring size (Figure 1) revealed that the amides investigated fall into three groups. Defining the hydrolysis rate of the open-chain amide as 'normal', the five- and seven-membered lactams fall into this category.

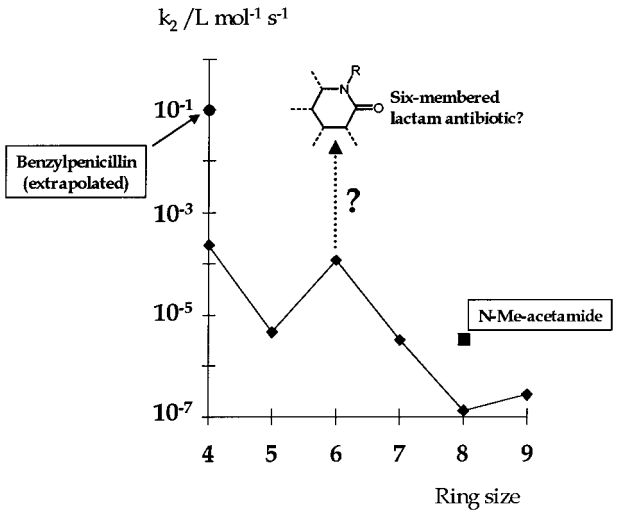


Figure 1. Plot of rate constants versus ring size.

The medium-sized lactams, by comparison, were hydrolyzed very slowly. We explain this by the fact that the addition of another substituent − hydroxide − would increase the amount of transannular strain that the medium-sized rings suffer. We concluded that formation of the tetrahedral intermediate was rate-determining, rather than C−N bond fission. There has been some discussion in the literature concerning the rate-limiting step of amide hydrolysis. An early study[14] was concerned with hydrolysis rates of simple lactones and lactams (e.g. *N*-acetyl-2-pyrrolidone, *N*-acetylcaprolactam, γ-butyrolactone) that are of interest in the production of polymers. Polymerizability was found not to be correlated with hydrolysis rate. Since polymerizability

is correlated with ring strain, it follows from this study that the ring was not broken in the rate-determining step; the latter must consist of hydroxide addition to the ring. Later, for a series of acylic anilides the evidence pointed to C−N fission to be rate-limiting, whereas with N-substituted acyclic amides, penicillins, and cephalosporins, again the formation of the tetrahedral intermediate seemed to be critical.[8b,15] This was inferred from the Brönsted β_{lg} values.

For the design of new non-β-lactam protease inhibitors, the question is important if the ring strain or high carbonyl reactivity is rate-limiting. In this connection it is of interest that Page[8b] has already noted that the rate enhancement of 30−500-fold shown by β-lactams of amines of p$K_a \leq 6$ may be adequately rationalized by the change in coordination number and hybridization of the carbonyl carbon as the tetrahedral intermediate is formed. The magnitude is similar to the 500-fold faster rate of reduction of cyclobutanone by borohydride compared with that of acetone.[16] The results we obtained with medium-sized lactams and with the third category of lactams, the four- and six-membered rings, confirm that the formation of the tetrahedral intermediate was rate-limiting.

Six-Membered Lactam Ring. β-Propiolactam (**8**) and, surprisingly, δ-valerolactam (**10**) were hydrolyzed at about the same rate and much faster than the rest. Why would δ-valerolactam have such a high reactivity? This can be rationalized assuming that the rate-limiting step is the addition of hydroxide. The tetrahedral intermediate **6** ($n = 4$) can adopt a chair conformation which is pronouncedly favored over the semi-chair conformation the six-membered ring is forced into in the native lactam.

Antiinfective β-Lactams. Rates of hydrolysis have been published for a number of antiinfective β-lactams. For antiinfective penicillins and cephalosporins, at 37 °C in buffered (pH 12) solution with an initial lactam concentration of 1 mM, the second-order rate constant for reaction with hydroxide was found to be in the range of 0.1−0.4 M^{-1} s^{-1}.[8a,17] Bearing in mind that reaction conditions are not the same, the following calculation is a fair approximation. With the rate constants we found, β-propiolactam and δ-valerolactam were hydrolyzed slower by a factor of 1000 and 2000, and γ-butyrolactam by a factor of 45000, than β-lactam antiinfectives. This approximation implies four conclusions. (1) The acylating power of lactam antibiotics has to be above a certain threshold, approximately 10^3-fold that of the unsubstituted four-membered lactam. (2) Medium-sized lactams seem to be least suitable for the development of protease inhibitors. (3) The six-membered lactam is a better vantage point than the five-membered one for the development of a new series of lactam antiinfectives. (4) The amount of reactivity enhancement that separates β-propiolactam from penicillins and cephalosporins will have to be built into δ-valerolactam.

To compare directly the reactivity of the unsubstituted lactams and an actual antiinfective within our study, we carried out hydrolyses of benzylpenicillin, β-propiolactam, and δ-valerolactam. Under the conditions we had used for the various lactams, penicillins were hydrolyzed too fast as to allow NMR monitoring.

Table 2. Results of Hydrolysis Kinetics of Four- and Six-Membered Lactams and Benzylpenicillins

name	pH	k (h^{-1})	log k	r^2 of regression curve
benzylpenicillin potassium	7.5	0.00337	−2.47	0.977
benzylpenicillin potassium	12	2.30	0.36	1.000
β-propiolactam, **8**	12	0.00103	−2.99	0.996
δ-valerolactam, **10**	12	0.000648	−3.19	0.998

We therefore switched to buffered solutions. In the literature, pseudo-first-order rate constants for active β-lactams were reported in the range of 0.1−17/h.[10,18] These numbers agree well with the 2.30/h we determined for benzylpenicillin. Table 2 summarizes the results we obtained. Again, the four- and six-membered lactams were hydrolyzed at about the same rate and benzylpenicillin approximately 3000 times faster, which is in accord with the above estimate.

Conclusion

The crucial step in the reaction of hydroxide and amides of therapeutic interest seems to be the formation of the tetrahedral intermediate, rather than the rate of ring fission. Taking this reaction as a model for the reaction of lactams with serinyl residues of serine proteases, it follows that one prerequisite for inhibitory activity is sufficient amide carbonyl reactivity. This study highlights δ-valerolactam as a promising vantage point for a new subclass of lactam antiinfectives. Valerolactam derivatives have to be designed and prepared that are more reactive than the parent ring system.

Besides sufficient acylating activity, the rate of deacylation is decisive for the activity and design of β-lactam and non-β-lactam serine protease inhibitors. This was addressed in an X-ray study of the reaction product of a β- and γ-lactam with elastase.[19] The latter was shown to be inactive, not because of lacking reactivity but because deacylation occurred very rapidly. This was due to the fact that the ring-opened β-lactam underwent a conformational change that extruded the water molecule from the catalytic center, whereas with the γ-lactam it remained in place, probably effecting hydrolytic cleavage of the acyl group from the serine moiety. Future studies will show the behavior of a δ-lactam in the catalytic pocket.

Experimental Section

Materials. The lactams, *N*-methylacetamide, and D_2O (99.9 atom % D) were purchased from Aldrich Chemical Corp. Benzylpenicillin-K was a gift from Grünenthal GmbH, Aachen, Germany. The substances were checked for purity (NMR; combustion analysis) and used without further purification. NaOD, KOD, Na_2DPO_4, KD_2PO_4, and K_2DPO_4 were prepared by repeatedly dissolving and evaporating the respective ^1H salts in D_2O.

Methods. NMR experiments were carried out on a JEOL JNM-6X-400 spectrometer at a frequency of 399.785 MHz, recording 16 scans with a frequency range of 5.9966 kHz and a digital resolution of 0.18 Hz/data point. The temperature within the probe was kept at 30 ± 1 °C. The solutions were prepared at this temperature, and the first spectrum was recorded after approximately 10 min, taking the exact time for each spectrum from the time the solutions were prepared. Spectrum editing was performed using the NMR data processing software Win.Nuts, version 2D 5.083 (Acorn NMR Inc., Fremont, CA). Experiments were done twice by two different

workers. The correlation coefficients (r^2) included in Tables 1 and 2 prove the high degree of linearity in all experiments. Calculations were based on the integral of the NCH_2 group of the lactams and ω-aminocarboxylates at approximately 3.3 and 2.6 ppm. These signals were ideal as they showed no overlap with other signals in all compounds investigated. The hydrolysis products were identified by comparison of their spectra with reference materials.

For the determination of the second-order rate constants, the amides were dissolved in D_2O to give a 0.5 M solution, followed by the addition of excess (4:3) NaOD.

For the determination of the pseudo-first-order rate constants, a phosphate buffer (pH 7.5, 0.33 M) was prepared by mixing 85.00 mL of a D_2O solution of Na_2DPO_4 (119.310 g/L) and 15.00 mL of a KD_2PO_4 solution (45.360 g/L). The pH of the mixture was checked potentiometrically. Benzylpenicillin-K was dissolved to give a 0.13 M solution. The phosphate buffer (pH 12) was prepared by adjusting the pH of a 1 M K_2DPO_4 solution in D_2O with 10 M KOD. Benzylpenicillin-K, β-propiolactam, and δ-valerolactam were dissolved to give 0.10 and 0.50 M solutions.

Rate constants were calculated as described elsewhere.[20] With $[A]_0$ and $[B]_0$ as the starting concentrations of amide and NaOD, $[A]_t$ and $[B]_t$ as the concentrations after the reaction time t, and k_2 as the rate constant, the law for second-order reactions

$$\frac{d[A]}{dt} = -k_2 \cdot [A] \cdot [B]$$

transforms into

$$\ln[B]_0 - \ln[A]_0 + ([B]_0 - [A]_0) \cdot k_2 \cdot t = \ln \frac{[B]_t}{[A]_t}$$

This equation was used, calculating the $[A]_t$ and $[B]_t$ values from the relative NMR integrals and the known starting concentrations.

Acknowledgment. We thank Bayer AG for support of this work and Prof. Chr. Schofield, Dyson Perrins Laboratory, Oxford University, for stimulating discussions.

References

(1) Matagne, A.; Dubus, A.; Galleni, M.; Frère, J.-M. The β-lactamase cycle. *Nat. Prod. Rep.* **1999**, *16*, 1−19.

(2) For reviews, see: (a) Marchand-Brynaert, J.; Ghosez, L. Non β-lactam analogues of penicillins and cephalosporins. In *Recent Progress in the Chemical Synthesis of Antibiotics*; Lukacs, G., Ohno, M., Eds.; Springer-Verlag: Berlin, 1990; pp 727−794. (b) Hamilton-Miller, J. M. T. β-Lactams: variations on a chemical theme. *J. Antimicrob. Chemother.* **1999**, *44*, 729−734.

(3) Du Vigneaud, V.; Carpenter, F. H. The γ-lactam of benzylhomopenicilloic acid and related compounds. In *The Chemistry of Penicillin*; Clarke, H. T., Johnson, J. R., Robinson, R., Eds.; Princeton University Press: Princeton, NJ, 1949; pp 1004−1017.

(4) Baldwin, J. E.; Lynch, G. P.; Pitlik, J. γ-Lactam analogues of β-lactam antibiotics. *J. Antibiot.* **1991**, *44*, 1−24.

(5) (a) Ternansky, R. J.; Draheim, S. E. [3.3.0]Pyrazolidinones: An efficient synthesis of a new class of synthetic antibacterial agents. *Tetrahedron Lett.* **1990**, *31*, 2805−2808. (b) Barnett, C. J.; Holmes, R. E.; Jungheim, L. N.; Sigmund, S. K.; Ternansky, R. J. Eur. Pat. Appl. 310214, Apr 5, 1989.

(6) Todd, D. C.; Cornell, R. J.; Wester, R. T.; Wittstruck, T. The synthesis of analogues of penicillin. *J. Chem. Soc. C* **1969**, 408−410.

(7) Imming, P. Synthesis of the first penicillin derivatives with medium-sized lactam ring and of related thiazolidines. *Arch. Pharm. (Weinheim)* **1995**, *328*, 207−215.

(8) (a) Frère, J.-M.; Joris, B.; Varetto, L.; Crine, M. Structure−activity relationships in the β-lactam family: An impossible dream. *Biochem. Pharmacol.* **1988**, *37*, 125−132. (b) Page, M. I. The mechanisms of reactions of β-lactam antibiotics. *Acc. Chem. Res.* **1984**, *17*, 144−151. (c) Page, M. I. The reactivity of β-lactams, the mechanism of catalysis and the inhibition of β-lactamases. *Curr. Pharm. Des.* **1999**, *5*, 895−913.

(9) Gensmantel, N. P.; McLellan, D.; Morris, J. J.; Page, M. I.; Proctor, P.; Randahawa, G. S. In *Recent Advances in the Chemistry of β-Lactam Antibiotics*; Gregory, G. I., Ed.; Royal Society of Chemistry Special Publication No. 38; Royal Society of Chemistry: London, 1981; pp 227−239.

(10) Indelicato, J. M.; Pasini, C. E. The acylating potential of γ-lactam antibacterials: Base hydrolysis of bicyclic pyrazolidinones. *J. Med. Chem.* **1988**, *31*, 1227−1230.

(11) Krantz, A.; Spencer, R. W.; Tam, T. F.; Liak, T. J.; Copp, L. J.; Thomas, E. M.; Rafferty, S. P. Design and synthesis of 4H-3,1-benzoxazin-4-ones as potent alternate substrate inhibitors of human leukozyte elastase. *J. Med. Chem.* **1990**, *33*, 464−479.

(12) See e.g.: Bowden, K.; Bromley, K. The alkaline hydrolysis of substituted *N*-methylformanilides, *N*-methylacetanilides, 1-phenylazetidin-2-ones, 1-phenyl-2-pyrrolidones, and 1-phenylpiperidones. *J. Chem. Soc., Perkin Trans. 2* **1990**, 2103−2116.

(13) NMR studies of degradation products: (a) Casy, A. F.; Lipczynski, A. 8-Hydroxypenillic acid: NMR characteristics. *J. Pharm. Pharmacol.* **1994**, *46*, 533−534. (b) Vilanova, B.; Muñoz, F.; Donoso, J.; Frau, J.; Blanco, F. G. Alkaline Hydrolysis of Cefotaxim. *J. Pharm. Sci.* **1994**, *83*, 322−327.

(14) Hall, K. H.; Brandt, M. K.; Mason, R. M. Hydrolysis rates and mechanisms of cyclic monomers. *J. Am. Chem. Soc.* **1958**, *80*, 6420−6427.

(15) Proctor, P.; Gensmantel, N. P.; Page, M. I. The chemical reactivity of penicillins and other β-lactam antibiotics. *J. Chem. Soc., Perkin Trans. 2* **1982**, 1185−1192.

(16) Brown, H. C.; Ichikawa, K. The effect of ring size on the rate of reaction of the cyclanones with sodium borohydride. *Tetrahedron* **1957**, *1*, 221−230.

(17) Frère, J.-M.; Kelly, J. A.; Klein, D.; Ghuysen, J.-M. Δ^2- and Δ^3-cephalosporins, penicillinate and 6-unsubstituted penems. *Biochem. J.* **1982**, *203*, 223−234.

(18) Yamana, T.; Tsuji, A. Comparative stability of cephalosporins in aqueous solution. *J. Pharm. Sci.* **1976**, *65*, 1563−1574.

(19) (a) Wilmouth, R. C.; Westwood, N. J.; Anderson, K.; Brownlee, W.; Claridge, T. D. W.; Clifton, I. J.; Pritchard, G. J.; Aplin, R. T.; Schofield, C. J. Inhibition of elastase by N-sulfonylaryl β-lactams: Anatomy of a stable acyl-enzyme complex. *Biochemistry* **1998**, *37*, 17506−17513. (b) Wilmouth, R. C.; Kassamally, S.; Westwood, N. J.; Sheppard, R. J.; Claridge, T. D. W.; Aplin, R. T.; Wright, P. A.; Pritchard, G. J.; Schofield, C. J. Mechanistic insights into the inhibition of serine proteases by monocyclic lactams. *Biochemistry* **1999**, *38*, 7989−7998.

(20) See e.g.: Pilling, M. J.; Seakins, P. W. *Reaction Kinetics*; Oxford University Press: Oxford, 1996.

JM000921K