THE INHIBITION OF BACTERIAL β-LACTAMASES BY SOME MONOCYCLIC β-LACTAMS

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(Received for publication February 22, 1982)

The inhibition characteristics of 25 monocyclic β-lactams mainly with an amido function at C3 have been studied against the β-lactamases produced by 4 bacterial types. Significant levels of inhibition were found for only a few of the compounds tested and primarily against *Staphylococcus* β-lactamase. The most active inhibitor tested, 3-*p*-nitrophenylacetamido-4-phenylazetidin-2-one, was found to require a *cis* geometry, the *trans* isomer being almost inactive.

The production of β-lactamase by pathogenic bacteria results in the hydrolysis and inactivation of penicillins and cephalosporins and presents a serious problem by causing resistance to these β-lactam antibiotics. There is considerable interest in the discovery of specific inhibitors which could reduce the activity of the β-lactamases and thereby potentiate antibiotic activity. It has been previously reported[1] that monocyclic azetidinones were antibacterial synergists when formulated with benzylpenicillin and tested against *Staphylococcus aureus*. It was not stated whether these compounds were inhibitors of staphylococcal β-lactamase. We report here the synthesis of 25 monocyclic β-lactams of general formulae I and II, Table 1. These compounds were tested for β-lactamase inhibitory activity against enzymes from four micro-organisms, Table 2, and the structural and stereochemical requirements for inhibitory activity are discussed.

Materials and Methods

Assay of Inhibitory Activity

Enzyme preparations were cell-free extracts of *E. cloacae* P99, *Klebsiella* E70 and *S. aureus* Russell, which were prepared as described previously.[2] TEM-2 β-lactamase was a partially purified preparation from *E. coli* W3110. I_{50} Values were determined by automated analysis using a Pye-Unicam AC-30 discrete analyser. Enzyme (200 μl) was incubated with 10 μl of inhibitor at various concentrations for 5 minutes at 37°C and pH 7.3. Residual enzyme activity was then measured by adding 200 μl of the chromogenic cephalosporin, nitrocefin (500 μg/ml). After a further 5-minute incubation period the reaction was diluted with 1.6 ml water and the A_{482} then measured. Nitrocefin hydrolysis generates a chromophore at this wavelength and increase in absorbance therefore serves as a measure of enzyme activity. Inhibited reactions were measured relative to an uninhibited control and the enzyme activity was adjusted to give 75% hydrolysis of nitrocefin in the 5-minute reaction period.

I_{50} Values were also measured without the preincubation stage by altering the position of the enzyme addition point so that enzyme was added to inhibitor and substrate which had previously been mixed together. After a 5-minute incubation the reaction was diluted and A_{482} measured as described above. All reactions were in 0.05 M sodium phosphate buffer pH 7.3 at 37°C. The I_{50} values, Table 2, quoted are the inhibitor concentrations present with the enzyme after substrate addition.

Synthetic Procedures

Key compounds in this work were 1,4-diaryl-3-aminoazetidin-2-ones and 3-amino-4-arylazetidin-2-ones for which improved syntheses have been achieved and are recorded.

a. 1,4-Diphenyl-3-aminoazetidin-2-one[3]: Phthalimidoacetic acid was prepared by fusion of equimolar quantities of phthalic anhydride and glycine, heating at 130~140°C until solidification occurred. Recrystallization from water gave colorless needles, mp 191~193°C. Phthalimidoacetic acid (30 g) and thionyl chloride (60 ml) were heated under reflux until solution occurred and hexane was then added continuing heating until a homogeneous solution was formed. On cooling phthalimidoacetyl chloride appeared as yellow needles, mp 86~88°C,[4] which were filtered and dried. Equimolar amounts of benzaldehyde and aniline were mixed and, after the reaction had subsided, ether was added and the solution dried and evaporated. Recrystallization from pentane gave benzalaniline as yellow plates, mp 52°C.

A mixture of benzalaniline (36.2 g, 0.2 mole) and triethylamine (14 ml, 0.1 mole) in benzene (350 ml) was cooled to 0~5°C with stirring. A solution of phthalimidoacetyl chloride (22.4 g, 0.1 mole) was dissolved in benzene (100 ml) and added dropwise over 40 minutes maintaining anhydrous conditions. The resulting deep yellow solution was filtered and the precipitated triethylammonium chloride washed with benzene. The combined filtrates were evaporated to dryness at reduced pressure to yield a semisolid mass which was digested with hexane (3×150 ml) and then with ethanol (50 ml) to leave a cream powder mp 227~232°C whose analysis and spectral properties, agreed with those expected for 1,4-diphenyl-3-phthalimidoazetidin-2-one, yield 17 g, 46%. The latter (5 g, 13 mmole) was pulverized and suspended in ethanol (100 ml) and a solution of hydrazine hydrate (14 ml, 1 M) added. After refluxing 2 hours and allowing to stand overnight, the solvent was removed at reduced pressure and the solid product stirred for 2 hours with 5 N hydrochloric acid (100 ml). It was filtered and the residue boiled with water (2× 100 ml) and the filtrates combined. The residue was phthalhydrazide. The combined aqueous extracts were further acidified with 10 N HCl (20 ml) and cooled overnight to give colorless needles of 3-amino-1, 4-diphenylazetidin-2-one, mp 229~236°C as the hydrochloride with expected analyses and spectral properties. The coupling constant for the protons at C-3 and C-4, $J_{3,4}$=2.2 Hz both for the amine and for the phthalimido precursor showing these compounds to have *cis* stereochemistry.

b. 3-Amino-4-phenylazetidin-2-one, 7[5]: Phthalimidoacetyl chloride (4.7 g, 20 mmole) in anhydrous dichloromethane (75 ml) was added to a mixture of hydrobenzamide (6 g, 20 mmole), and triethylamine (2 g, 20 mmole) in dichloromethane (75 ml), maintaining the temperature at 0~5°C with stirring over a 40-minute period. The mixture was allowed to stand overnight when it became dark. Hydrochloric acid (100 ml, 10%) was added and the precipitate of 3-phthalimido-4-phenylazetidin-2-one was filtered off, mp 229~231°C; yield, 1.0 g (50%). The synthesis was repeated to obtain 5.8 g of this product which was suspended in ethanol (100 ml) and hydrazine hydrate added (1.5 ml). The mixture was refluxed for two hours and left to stand overnight: after filtration and evaporation of the solvent, a colorless oil was obtained. This was stirred with HCl (25 ml, 1 M) for 15 minutes, the solution adjusted to pH 8, saturated with sodium chloride and extracted with chloroform (10×10 ml). After washing with cold brine, drying and evaporation, a brown powder was obtained which after digestion with hot benzene (30 ml) gave a fine, white powder identified as *cis*-3-amino-4-phenylazetidinone, mp 107~115°C; yield, 300 mg (10%).

A better yield of this compound was obtained by the following route. Azidoacetyl chloride[6] (14 g, 0.12 mole) in dichloromethane (200 ml) was added dropwise over 40 minutes to a stirred solution of hydrobenzamide (26 g, 0.12 mole) and triethylamine (16.6 ml, 0.12 mole) maintaining the temperature between 0 and 5°C. The dark solution was allowed to stand at room temperature overnight and HCl (100 ml, 10%) was added. A brown precipitate was obtained which was decolorized in methanolic solution by activated charcoal and evaporated to yield 3-azido-3-phenylazetidin-2-one as pale yellow crystals, mp 87~89°C. The coupling constant $J_{3,4}$ indicated that this is the *cis* compound, $J_{3,4}$=5.5 Hz. Yield, 5.0 g, 25%.

This was reduced by catalytic hydrogenation over ADAMS platinum in ethanol solution at 4.0 bar, 200 for 72 hours. After evaporation, 3-amino-4-phenylazetidin-2-one was obtained by recrystallization from dichloromethane - hexane (3: 4) as white flakes (yield 2.0 g, 46%). In a similar manner, 3-amino-

4-*p*-anisylazetidinone, mp 109°C, and 3-amino-4-*p*-chlorophenylazetidin-2-one, mp 130~140°C, were prepared. The attachment of the 3-acyl group was accomplished by dehydration with dicyclohexyldiimine. The following procedure is typical.

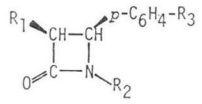
c. *cis*-3-(*p*-Nitrophenylacetamido)-4-phenylazetidin-2-one: To a solution of dicyclohexylcarbodiimide (1.44 g, 7 mmole) in anhydrous tetrahydrofuran (50 ml) was added dropwise a stirring, a solution of *p*-nitrophenylacetic acid (1.26 g, 7 mmole) and *cis*-3-amino-4-phenylazetidin-2-one (1.2 g, 7 mmole) also in anhydrous tetrahydrofuran. The mixture was stirred for 15 minutes and allowed to stand overnight. Dicyclohexylurea was filtered off and the filtrate evaporated. The solid residue was digested with benzene (75 ml) when the amide was obtained as a white crystalline solid, yield 2.0 g (87%). The imide group of **6** was attached using *p*-nitrobenzaldehyde and the sulfonamido group of **11**, by means of *p*-bromobenzenesulfonyl chloride.

Results and Discussion

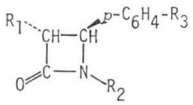
Significant inhibition of β-lactamase activity was detected in only six compounds and only against the staphylococcal enzyme. Several compounds produced weak inhibition of some of the other β-lactamases tested. Compounds **5** and **9** gave weak inhibition of the cephalosporinase from *E. cloacae* P99 with I_{50} values of 12 and 44 μg/ml respectively after preincubation with the enzyme. These two compounds, however, had lost the ability to inhibit staphylococcal β-lactamase. The free acid (Compound **10**) was less active than the ester **9** against *E. cloacae* β-lactamase. Very little inhibition of the *Klebsiella* and TEM-2 β-lactamases was detected in any of the compounds examined.

The synthesis of monocyclic azetidinones was motivated by the philosophy that these simple β-

Table 1. List of azetidinones tested for inhibitory activity.



I (1~23)



II (24, 25)

	R_1	R_2	R_3
1	*p*-O_2N-$C_6H_4CH_2CONH$–	H	H
2	*o*-O_2N-$C_6H_4CH_2CONH$–	H	H
3	*m*-O_2N-$C_6H_4CH_2CONH$–	H	H
4	*p*-O_2N-$C_6H_4CH_2CONH$–	H	OCH_3
5	*p*-O_2N-$C_6H_4CH_2CONH$–	H	Cl
6	*p*-O_2N-C_6H_4CH=N–	H	H
7	$Cl^-H_3N^+$–	Ph	H
8	H_2N–	H	OCH_3
9	*p*-O_2N-$C_6H_4CH_2CONH$–	CH_2COOEt	H
10	*p*-O_2N-$C_6H_4CH_2CONH$–	CH_2COOH	H
11	*p*-Br-C_6H_4-SO_2NH–	H	H
12	O_2N-CH_2-NH–	H	H
13	H_2N–	SO_2Cl	H
14	H_2N–	H	H
15	3-Nitrophthalimido	H	H
16	*p*-O_2N-$C_6H_4CH_2CONH$–	$PhCH_2$–	H
17	Phthalimido-	$PhCH_2$–	H
18	〃	Ph–	H
19	〃	H	H
20	〃	Ph_2CH–	H
21	〃	PhCH=N–	H
22	N_3–	H	H
23	H_2N–	H	H
24	Cl–	H	H
25	*p*-O_2N-$C_6H_4CH_2CONH$–	H	H

Table 2. The inhibition of *Staphylococcus aureus* Russell β-lactamase.

Inhibitor	I_{50} (μg/ml)	
	With preincubation	Without preincubation
1	0.04	23.0
2	0.45	50
3	2.5	>50
4	0.02	—
12	20.0	—
25	10.0	—

I_{50} Values were measured with a 5-minute preincubation of enzyme and inhibitor (37°C and pH 7.3) prior to substrate addition and also without preincubation by adding enzyme to pre-mixed substrate and inhibitor as described in the text.

lactams could be substrate analogues. Hence they would serve as simple competitive inhibitors or react with the enzyme active site to produce inactivated β-lactamase. It would appear that these compounds are highly specific for staphylococcal β-lactamase and it did not prove possible to extend the spectrum of significant activity to other β-lactamase types. There was, however, an indication that a different spectrum of activity was achievable as seen with compounds **5** and **9** and further work is required in order to find derivatives with acceptable levels of activity against β-lactamases other than those from *S. aureus*. Lack of activity against these other enzymes was not due to β-lactamase instability[7] and it seems likely that these monocyclic β-lactams had poor affinity for the β-lactamases of Gram-negative bacteria.

Inhibition of staphylococcal β-lactamase required a penicillin-like side chain, the free amino compound (**8**) being inactive whilst the benzyl acetamido derivative (**4**) had good activity. Similarly compound **14** was inactive whilst compound **1** was highly active. The position of the nitro group in the phenyl acetamido side chain had some effect on activity as shown in compounds **1**~**3**, the *p*-nitro substituted derivative being the most active. A non-aromatic amido side chain, *e.g.* compound **12** gave poor inhibitory activity. It was interesting that substitution of the 4-aryl group enhanced activity in the case of the *p*-methoxy phenyl derivative (compound **4**) whilst the *p*-chloro substituent (compound **5**) was inactive. The introduction of an acetic acid or ester side chain at the β-lactam nitrogen (**9** and **10**) similar to that in the penicillin moiety, removed staphylococcal inhibitory activity but one of these compounds (**9**) did have some affinity for the *E. cloacae* β-lactamase. The *cis* relationship between the amino substituent at C-3 and the C-4 group appears essential for activity which is not surprising in view of this being the natural configuration in the penicillins. Compound **25** which was the *trans* isomer of compound **1** had little activity and even this may have been due to traces of the highly active *cis* isomer in the preparation.

It will be noted in the Table 2 that activity was significantly higher when the staphylococcal β-lactamase was incubated with the inhibitor before addition of the substrate in comparison to the no-preincubation assay results. These results are similar to those reported for clavulanic acid[2] and suggest a time dependent reaction between the inhibitor and the β-lactamase. The kinetic aspects of the interaction of **1** with staphylococcal β-lactamase have been studied and will be discussed at a later date but it appears that the azetidinone is active site directed and forms a relatively stable covalent intermediate on reaction with the enzyme. There are distinct similarities between the reactions of this compound and clavulanic acid with the staphylococcal enzyme.[8]

The structural differences between **1** and other progressive inhibitors of β-lactamase such as clavulanic acid and β-bromopenicillanic acid[9] (PRATT and LOOSEMORE) may prove interesting when considering the chemical mechanisms[10] which lead to covalent enzyme-inhibitor complexes with this clinically important group of enzymes.

Acknowledgement

We are grateful to Messrs. Beecham Ltd. and to the S.R.C. for a C.A.S.E. Studentship to G.S.

References

1) GLADYCH, J.M.Z. & C.W.T. HUSSEY (Allen and Hanbury Ltd.): Azetidine derivatives and their use as enzyme inhibitors. British Patent 1,301,720, 1973
2) READING, C. & M. COLE: Clavulanic acid: a β-lactamase-inhibiting β-lactam from *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. 11: 852~857, 1977

3) SHEEHAN, J. C. & J. J. RYAN: Synthesis of substituted penicillins and simpler structural analogs. I. Alpha amino monocyclic β-lactams. J. Am. Chem. Soc. 73: 1204~1206, 1951
4) KING, F. E.; J. W. CLARK-LEWIS, R. WADE & W. A. SWINDIN: Synthesis from phthalamino-acids. VII. Oxazolones and other intermediates in the synthesis of phthalylpeptides, and an investigation of maleic acid derivatives of amino-acids. J. Chem. Soc. 1957: 873~875, 1957
5) BERTHO, A. & J. MAIER: Sugars containing nitrogen. IV. Catalytic reduction of azides. Liebigs Ann. 498: 51~60, 1932
6) WELLS, J. N. & R. E. LEE: The synthesis of 2-azetidinones. J. Org. Chem. 34: 1477, 1969
7) READING, C.: unpublished results.
8) READING, C. & P. HEPBURN: The inhibition of staphylococcal β-lactamase by clavulanic acid. Biochem. J. 179: 67~76, 1979
9) PRATT, R. F. & M. J. LOOSEMORE: 6β-Bromopenicillanic acid, a potent β-lactamase inhibitor. Proc. Natl. Acad. Sci., U.S.A. 75: 4145~4149, 1978
10) CHARNAS, R. L.; J. FISHER & J. R. KNOWLES: Clavulanic acid: A suicide inactivator for the β-lactamase from *E. coli*. *In* Enzyme-Activated Irreversible Inhibitors (N. SEILER, M. J. JUNG & J. KOCH-WESER, *eds.*), pp. 315~322, Eleseiner-North Holland Biomedical Press, Amsterdam, 1978