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Inhibition of the β-Lactamases of *Escherichia coli* and *Klebsiella aerogenes* by Semi-Synthetic Penicillins

By M. COLE, S. ELSON and P. D. FULLBROOK*
Beecham Research Laboratories, Brockham Park, Betchworth, Surrey, U.K.

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1. A new automated micro-iodometric method is described for screening compounds for inhibitory action against β-lactamase enzymes. 2. Over 1000 semi-synthetic penicillins were tested for inhibitory activity against the β-lactamase of *Escherichia coli* B11 and 18 showed a fractional inhibition similar to or higher than that of methicillin. 3. The best inhibitors were alkoxy- and halogen-substituted phenyl-, naphthyl- or quinolyl-penicillins. 2-Isopropoxy-1-naphthylpenicillin (BRL 1437) was clearly the best and had a K_i value about 1% of that of methicillin. 4. The inhibition of the β-lactamase of *E. coli* B11 by BRL 1437 was shown to be reversible and competitive. The K_i was 0.004 μM and K_i/K_m with ampicillin and *p*-hydroxyampicillin (BRL 2333) was about 0.0001. The K_m and $V_{max.}$ values were determined for the β-lactamases of *E. coli* B11 and *Klebsiella aerogenes* A against a variety of penicillins. Cell-bound and solubilized enzymes gave similar K_i and K_m values. 5. BRL 1437 was superior to cloxacillin and methicillin for inhibition of the β-lactamase of live, fully grown cultures of several strains of *E. coli* and *K. aerogenes*. Of a group of inhibitors BRL 1437 was the most stable to the β-lactamase of *E. coli* B11.

Many Gram-negative bacteria produce the enzyme β-lactamase (penicillin amido-β-lactam-hydrolase, EC 3.5.2.6), and this has been shown to be associated with resistance to penicillins, although it is not always the sole cause of resistance (Ayliffe, 1963; Cole & Sutherland, 1966; Percival *et al.*, 1963; Sutherland, 1964; Sabath & Finland, 1968). This association has created interest in inhibitors of the enzyme as penicillin-protecting agents (synergists) or as tools for studying the mechanism of bacterial resistance.

There have been several reports of the inhibition of Gram-negative β-lactamases by methicillin (BRL 1241), oxacillin (BRL 1400) or cloxacillin (BRL 1621), all compounds selected for their stability to staphylococcal β-lactamase. Initially Jago *et al.* (1963) and Hamilton-Miller (1963) reported inhibition of enzymes from *Pseudomonas pyocyanea* and *Klebsiella aerogenes* respectively, and later Sutherland & Batchelor (1964) and Hamilton-Miller & Smith (1964) demonstrated inhibition of enzymes from strains of *Klebsiella, Aerobacter, Escherichia, Pseudomonas* and *Proteus*. Dicloxacillin has been shown to be a good inhibitor of the β-lactamases of *Shigella flexneri* by Bach *et al.* (1967), and substituted benzamidocephalosporanic acids and the cephalosporin analogue of cloxacillin have been shown to be inhibitors of the β-lactamases of *Aerobacter aerogenes, Proteus morganii* and *Enterobacter cloacae* (O'Callaghan *et al.*, 1967, 1969).

* Present address: Department of Enzyme Chemistry, NOVO Industri A/S, Fuglebakkevej 115, 2200 København N, Denmark.

It has frequently been reported that high concentrations of these inhibitors were required for good antibacterial synergism. Sutherland & Batchelor (1964) reported that the degree of synergism between their β-lactamase inhibitors and penicillins was inadequate for clinical application, although Sabath *et al.* (1967) have suggested that the high concentrations of penicillins obtainable in urine may be sufficient for synergism.

There is thus considerable room for improvement on the existing combinations of β-lactamase inhibitors with penicillins or cephalosporins, and it was the object of the present work to see if superior inhibitors could be found. For this purpose large numbers of semi-synthetic penicillins were examined for ability to inhibit cell-free preparations of the β-lactamases of *Escherichia coli* and *K. aerogenes*. Because previously available methods were not satisfactory for testing and comparing the inhibitory activities of large numbers of compounds at substrate concentrations that are therapeutically attainable, a new automated micro-iodine procedure was devised.

Materials and Methods

Cultures, penicillins and reagents

The *E. coli* strains were: B11 and B41 (infantile gastroenteritis, serotype 055, Public Health Labs., Cork, Ireland), 83 (wound swab, Middlesex Hospital), T506 and T507 (urine, St. Thomas's Hospital) and NCIB9465. The *K. aerogenes* strains were:

A (Beecham typical *Klebsiella* laboratory strain), B (strain from Middlesex Hospital), C (Imperial College), I281 (urine, West Middlesex Hospital), R129 (urine, Redhill General Hospital) and C978 (Middlesex Hospital). The *Klebsiella oxytoca* strains were T200 (St. Thomas's Hospital) and F (Beecham Laboratory strain). *Proteus vulgaris A* and *Pseudomonas aeruginosa A* were Beecham Laboratory strains. *Bacillus cereus* 569/H was NCIB8933. *Staphylococcus pyogenes* (var. aureus) A was a Beecham Laboratory strain and strain H was from Redhill General Hospital.

All novel semi-synthetic penicillins and derivatives were sodium salts synthesized in the Chemistry Department of Beecham Research Laboratories. Sodium salts of benzylpenicillin, ampicillin (BRL 1341), carbenicillin (BRL 2064), methicillin (BRL 1241) and cloxacillin (BRL 1621) were commercial materials available from Beecham Research Laboratories. Sodium nafcillin (BRL 1383) was obtained from Wyeth Laboratories Inc., Philadelphia, Pa., U.S.A. Penicilloic acids were prepared by NaOH hydrolysis of the corresponding penicillins and freeze-drying (Cole & Sutherland, 1966).

Stock phosphate buffer (pH 7.0, 0.5 M) was prepared by dissolving 24.3 g of KH_2PO_4 and 56.0 g of K_2HPO_4 in 1 litre of water containing 1 ml of butyl acetate as preservative.

Laboratory chemicals were purchased from British Drug Houses Ltd., Poole, Dorset, U.K., and bacterial cultures were grown in nutrient broth no. 2, prepared as directed by the manufacturer (Oxoid Ltd., London S.E.1, U.K.).

Preparation of β-lactamases

Small quantities of cultures were grown in 100 ml volumes of nutrient broth in 500 ml conical flasks on a rotary shaker (280 rev./min, 1 in radius) at 37°C for 18 h and cells were collected by centrifugation at 38000*g* for 15 min.

Larger quantities of *E. coli* B11 were prepared as follows. A portion (10 ml) of an overnight nutrient-broth culture was used to inoculate 3 litres of sterile nutrient broth. This was incubated for 22.5 h at 37°C in a 5-litre conical flask. During growth, the culture was aerated at 3 litres/min, 0.2 ml of 10% (v/v) Pluronic L.81 antifoam in soya-bean oil (Jacobson, Van den Burg and Co. Ltd., London W.C.1., U.K.) being used to prevent foaming. Cells were harvested at 21000*g* with an MSE continuous-action rotor at 5°C and a flow rate of 60 ml/min. Cells were removed from the collection basket as a slurry and spun down at 38000*g* for 15 min.

Cells from large- and small-scale preparations were washed by resuspending in 0.02 vol. of 0.05 M-potassium phosphate buffer, pH 7, and re-centrifuging for 15 min at 38000*g*. Finally the cells were resuspended in 0.02 vol. of water, chilled in an ice bath and subjected to ultrasonic treatment for 10–20 min. Alternate 2.5 min periods were used at the two frequencies of maximum amplitude (i.e. 21 and 26 kHz) of an MSE 100 W ultrasonic disintegrator. Cell debris was finally spun down by centrifugation for 30 min at 38000*g* and the supernatant was used as stock enzyme preparation, being deep frozen when not in use.

In a reaction mixture containing a final dilution of 1 in 2500 of the *E. coli* B11 stock enzyme preparation and 40 μg of ampicillin/ml the rate of hydrolysis was 0.55 μg/min per ml at 37°C and pH 7.0 as determined by the starch–iodometric assay. The velocity for the *K. aerogenes* A stock enzyme preparation at a final dilution of 1 in 750 was 0.46 μg/min per ml in a similar reaction mixture. The stock enzyme preparations of *E. coli* B11 and *K. aerogenes* A had activities of 4.5 and 1.14 units/ml respectively; one unit being the amount that will catalyse the transformation of 1 μmol of ampicillin/min under the above conditions.

Hydroxylamine assay

The hydroxylamine assay described by Batchelor *et al.* (1961) was used to determine the purity of test penicillins before evaluation. Purity was expressed in terms of sodium benzylpenicillin, with correction for molecular weight. Some of the inhibitors were very old preparations and gave poor purity on the above basis, but molar extinction does vary with structure. e.g. ampicillin gives a high assay and carbenicillin a low one in terms of benzylpenicillin.

Bioassay and determination of minimum inhibitory concentrations

Bioassay of penicillins was carried out as described by Sutherland (1964). Minimum inhibitory concentration values were determined by Mr. R. Sutherland of these laboratories using nutrient agar.

Starch–iodometric assay

The method, as described by Novick (1962), modified for use with the Unicam SP. 800 double-beam spectrophotometer, was used for measurement of the rate of formation of penicilloic acid. Typical reaction mixtures consisted of 1.0 ml of starch–iodine reagent + 1.0 ml of ampicillin solution (in the range 0.25–25 μg/ml final concentration) + 0.1 ml of enzyme solution + 0.1 ml of inhibitor solution or 0.1 ml of buffer, and the volume was made up to 2.5 ml with buffer. Reaction blanks were set up by replacing the enzyme with buffer, because ampicillin solutions absorb iodine very much more than the enzyme does. Starch–iodine reagent, ampicillin and buffer were left in the constant-temperature cell housing for 5 min to

warm up to a reaction temperature of 37°C before addition of the enzyme. Reactions were followed at 590nm over a 10min period. All solutions were made up in the 0.05M-phosphate buffer.

The starch–iodine solution was prepared as described by Novick (1962), except that BDH AnalaR soluble starch was used. Experiments showed that the starch–iodine complex had an absorption maximum at 590nm and a molar extinction coefficient (ϵ) at this wavelength of 29200. By using this value and also the number of equivalents of iodine reacting with 1mol of α-aminobenzylpenicilloic acid ($n = 6.7$, measured by titration), reaction velocities were transformed from a change in extinction per unit time ($\Delta E/\Delta t$) into μg of ampicillin/ml hydrolysed per unit time (v), by using the expression $v = \frac{M \cdot \Delta E \times 10^3}{n \cdot \epsilon \cdot l \cdot \Delta t}$, where M = molecular weight of ampicillin = 371.4 and l = optical path length = 1cm. Values of n for the penicilloic acids of other penicillins were found to be as follows: benzylpenicillin 6.4, carbenicillin 9.3, *p*-hydroxy-α-aminobenzylpenicillin (BRL 2333) 6.85, α-carboxy-3-thienylmethylpenicillin (BRL 2288) 8.4.

K_m and K_i values

For determinations of K_m and $V_{max.}$ eight substrate concentrations [S] were used, generally in the range 1–100μg/ml. The initial velocity (v) of reaction was determined at pH7 and 37°C by using the above starch–iodometric method and expressed as ΔE_{590}/unit time. The top velocity for ampicillin was about 1 E_{590} unit/200s, or 0.57μg/min per ml. The intercept on the abscissa of a graph of $1/v$ against 1/[S] gave $-1/K_m$ and the intercept on the ordinate gave $1/V_{max.}$.

For K_i determinations two inhibitor concentrations were used each with four substrate concentrations. The substrate concentrations were in the range 8–40μg of ampicillin/ml and the inhibitor concentrations, differing by a factor of two, were chosen to give inhibition in the region of 30–60% as judged by preliminary experiment. The enzyme concentration was kept constant and was such as to give the velocity stated above. Initial velocity, v_i, at pH7 and 37°C was determined by the above starch–iodometric method. For each inhibitor concentration [I] a graph was plotted of $1/v_i$ against 1/[S] and the intercept on the abscissa, $-1/K_m(1+[I]/K_i)$ used to calculate K_i. The lines for the two inhibitor concentrations intersected on the ordinate and gave K_i values usually within 10% of one another.

Automated micro iodine assay

Direct adaption of the above starch–iodometric assay for use with a Technicon AutoAnalyzer proved

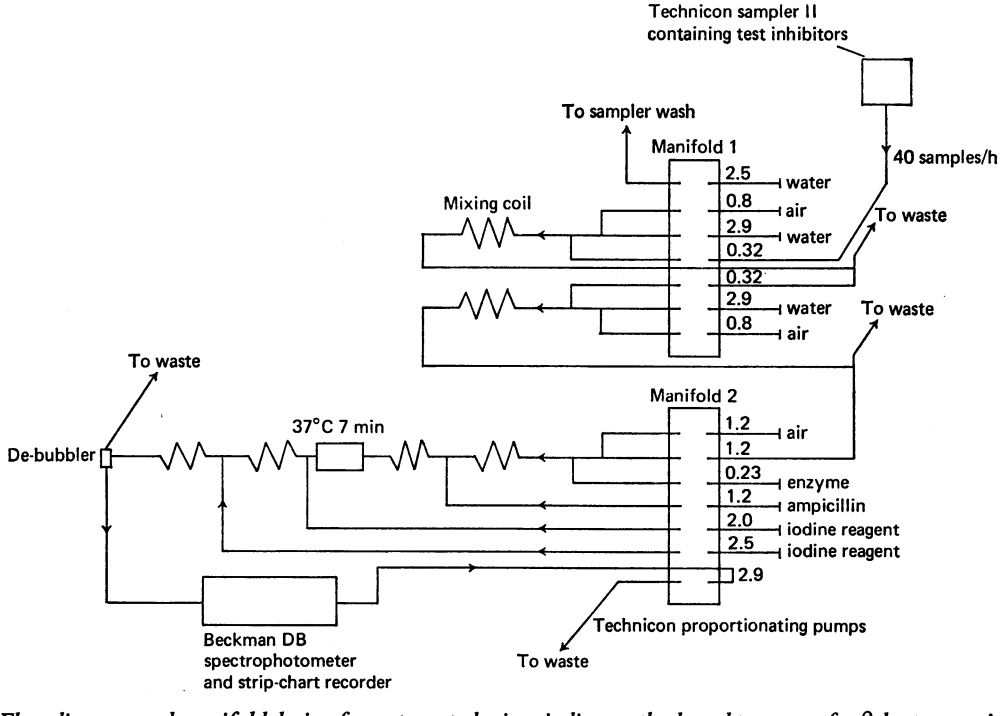


Fig. 1. *Flow diagram and manifold design for automated micro iodine method used to screen for β-lactamase inhibitors and measure fractional inhibition*

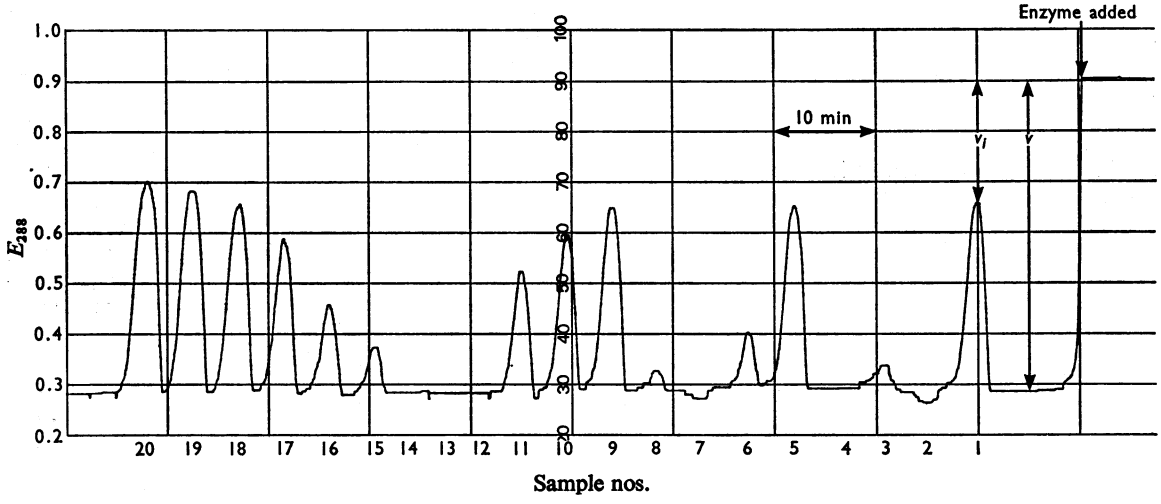


Fig. 2. *Screen trace from automated micro iodine assay*

The trace shows compounds acting as substrates, non-substrates and inhibitors of *E. coli* B11 β-lactamase with ampicillin (10 μg/ml) as substrate. E_{288} was 0.9 without added enzyme and 0.28 after β-lactamase action. In the presence of inhibitor a higher final value of E_{288} was observed, and a peak appears on the trace. Sample numbers 1–11 were 1 μg/ml of the following compounds (structures in Table 1): methicillin (1), benzylpenicillin (2), BRL 1222 (3), BRL 1071 (4), methicillin (5), BRL 1234 (6), BRL 957 (7), BRL 2852 (8), methicillin (9), BRL 1336 (10), BRL 1707 (11). Samples 12, 13 and 14 were water and samples 15–20 were 3-isopropoxy-4-quinolylpenicillin (BRL 3215) at the concentrations 0.05, 0.1, 0.25, 0.50, 0.75 and 1.0 μg/ml respectively. For experimental details see the Materials and Methods section.

unsatisfactory because incomplete mixing between the iodine solution and the colloidal starch solution resulted in non-stoicheiometric reaction. However, an absorption spectrum of iodine solution in potassium iodide (tri-iodide ion) was found to possess two distinct peaks, with $\lambda_{max.}$ at 288 nm and 350 nm. Graphs of extinction against iodine concentration at these wavelengths were straight lines passing through the origin, and gave molar extinction coefficients of 3.33×10^4 at 288 nm and 2.38×10^4 at 350 nm. The height of the 288 nm absorption peak was used to measure the iodine remaining after reaction with penicilloic acid and this was made the basis for an automated screen for *E. coli* B11 β-lactamase inhibitors, the manifold design and flow diagram for which are shown in Fig. 1.

Solutions of compounds under test as inhibitors were automatically sampled and diluted before being mixed with enzyme and substrate. The final concentrations of test compound (purity not corrected) and substrate were respectively 1 and 10 μg/ml. The final enzyme concentration was sufficient to hydrolyse about 1 μg of ampicillin/min. After incubation for 7 min at 37°C iodine was fed into the system to react with the penicilloic acid formed. The iodine reagent consisted of about 0.04 mM-iodine in 4% (w/v) KI and 0.05 M-potassium phosphate buffer.

A test compound acting as an inhibitor decreases the amount of penicilloic acid formed in the reaction between ampicillin and enzyme, causing an increase in residual iodine. The changes in concentration of iodine were recorded continuously at 288 nm by passing the reaction stream through a flow cell in a Beckman DB spectrophotometer connected to a strip-chart recorder. Compounds acting as inhibitors showed up as a series of peaks on the strip chart. We found it convenient to alternate each test compound with a water-wash sample and to use methicillin as a marker after every fourth test sample. This facilitated identification of test compounds and indicated any drift in output signal. Compounds giving higher inhibition peaks than methicillin were selected for further study.

An example of a screen trace is shown in Fig. 2. The trace starts at E_{288} 0.9, which was set by the concentration of the iodine reagent. When enzyme was added to the reaction stream the extinction decreased. This change in extinction is proportional to the amount of α-aminobenzylpenicilloic acid formed during the reaction period of 7 min and is a measure

of the initial velocity of the reaction (v). When an inhibitor was fed into the system the extinction increased as a result of decreased penicilloic acid formation. The difference in extinction between the inhibitor peak and the original base-line gives a measure of the inhibited velocity (v_i).

Results

Screen for β-lactamase inhibitors

Over 1000 semi-synthetic penicillins and their derivatives were screened by using the automated micro iodine assay with ampicillin as substrate and the β-lactamase of *E. coli* B11. Some 64 compounds showed various degrees of inhibitory activity. Fig. 2 is the trace obtained for a small collection of such compounds and illustrates the fractional inhibition (i) given by different substances. Fractional inhibition is defined as

$$i = \frac{v - v_i}{v}$$

where v and v_i are respectively initial reaction rates in the absence and presence of test inhibitors. Thus methicillin causes a change in extinction from 0.29 to 0.66 and hence has a fractional inhibition of 0.61. Benzylpenicillin was next tested and the trace shows a decrease in extinction to 0.26, indicating that this substance is acting as a secondary substrate and not an inhibitor. 1-Ethyl-1-phenylpropylpenicillin (BRL 1222) was the third test compound and shows slight inhibition. This was followed by triphenylmethylpenicillin (BRL 1071) and the trace registers no signal, signifying that this compound is neither an inhibitor nor a substrate for the enzyme. The structures of these and subsequent test inhibitors are given in Tables 1 and 4. The three methicillin reference samples give an indication of the accuracy and reproducibility of the method. Fig. 2 also shows (samples 15–20) the relationship between fractional inhibition and inhibitor concentration for 3-isopropoxy-4-quinolylpenicillin (BRL 3215) from which the concentration giving 50% inhibition (I_{50}) may be determined.

Structure–inhibition relationships

The compounds in Table 1 were selected to illustrate the relationship between degree of inhibition and chemical structure. Analogues of BRL 2852, BRL 1336 and BRL 1707 were examined in more detail for their inhibitory activity against β-lactamase from *E. coli* B11 and *K. aerogenes* A and the K_i results, with ampicillin as substrate, are given in Tables 2, 3 and 4.

Penicilloic acids derived from 6-aminopenicillanic acid, BRL 1437, benzylpenicillin, ampicillin, methicillin or cloxacillin had no inhibitory activity at a concentration of 1.0 μg/ml. Similarly, the following analogues of penicillin had no inhibitory activity: DL-alanyl-DL-alanine, benzoxycarbonyl derivatives of valine, valylvaline, valylvalylvaline and valylvalylvalylvaline, D-biotin, 8-hydroxypenillic acid (*NN'*-dibenzylethylenediamine salt), 2,6-dimethoxy-3,5-di-iodobenzoic acid (side chain of BRL 1540).

Nature of the inhibition

Experiments with the starch–iodometric assay showed that a constant degree of inhibition of the hydrolysis of ampicillin by *E. coli* B11 β-lactamase with BRL 1437 was attained within 100s. Further experiments showed that this inhibition could be overcome by addition of excess of ampicillin. Such results led us to believe that inhibition is reversible and probably competitive in nature. The reversibility of the inhibition was confirmed by a dialysis experiment (Table 5).

The competitive nature of the inhibition was proved by classical kinetic methods. The effect of a range of ampicillin concentrations on the inhibition brought about by 2-isopropoxy-1-naphthylpenicillin (BRL 1437) and methicillin was investigated by the starch–iodometric method with *E. coli* B11 enzyme. Fig. 3 shows that BRL 1437 acted as a competitive inhibitor, since the maximum reaction velocity was unaffected by the presence of BRL 1437. The $V_{max.}$ with methicillin was slightly decreased, but inhibition was mainly competitive.

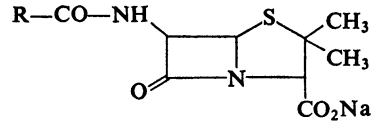
Kinetic constants

The kinetic constants K_m, $V_{max.}$, $V_{max.}/K_m$ and K_i (BRL 1437)/K_m for *E. coli* B11 and *K. aerogenes* A β-lactamases and various substrates are shown in Table 6. BRL 2333 has a similar antibacterial spectrum to ampicillin (Sutherland & Rolinson, 1971) but is much better absorbed in man (Croydon & Sutherland, 1971) and BRL 2288 is more active than carbenicillin against strains of *Pseudomonas* (Sutherland *et al.*, 1971). For each β-lactamase the same enzyme concentration was used for all substrates, thus making the $V_{max.}$ values comparable. The ratio $V_{max.}/K_m$ is a measure of the physiological efficiency of enzymes (Pollock, 1965) and is calculated from the values in Table 6. As the enzyme preparations for the two organisms were made in a similar fashion the physiological efficiencies can be compared.

A comparison of the K_m values for ampicillin and the β-lactamases of the cell-bound and cell-free enzyme from *E. coli* T506 showed that they were similar, being respectively 40.5 μM and 54 μM. For the cell-bound enzyme a washed whole-cell suspension

Table 1. *Structure–inhibition relationships of some penicillins for E. coli B11 β-lactamase*

Fractional inhibition was measured by using the automated micro iodine assay as described in the text. Test compounds were used at a concentration of 2.7 μM (approx. 1 μg/ml) to inhibit the hydrolysis of 27 μM-sodium ampicillin (10 μg/ml) by a cell-free preparation of *E. coli* B11 β-lactamase. The reaction system was incubated at pH 7.0 and 37°C for 7 min.



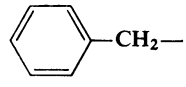
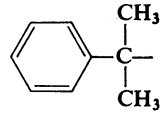
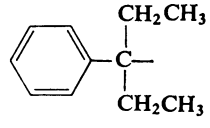
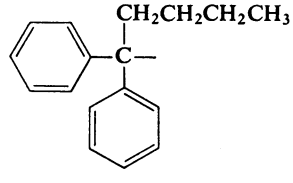
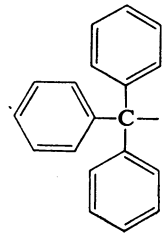
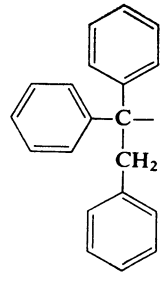
Test compound	Structure R	Fractional inhibition
Benzylpenicillin		A substrate
BRL 1209		0.07
BRL 1222		0.16
BRL 1215		0.07
BRL 1071		0.00
BRL 1307		0.28

Table 1 (*continued*)

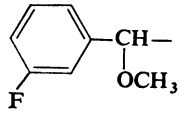
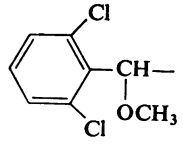
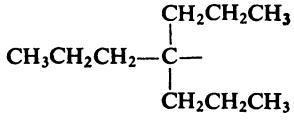
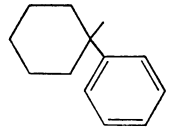
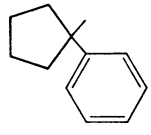
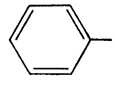
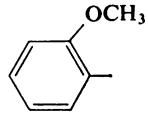
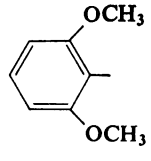
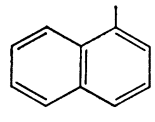
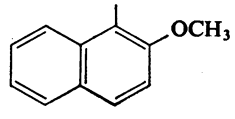
Test compound	Structure R	Fractional inhibition
BRL 2214		A substrate
BRL 2072		0.40
BRL 1201		0.12
BRL 1234		0.27
BRL 1228		0.13
BRL 0957		A substrate
BRL 2852		0.10
BRL 1241, methicillin		0.62
BRL 1371		A substrate
BRL 1336		0.62

Table 1 (*continued*)

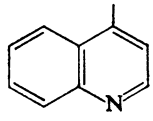
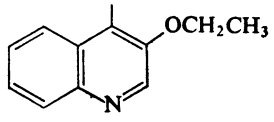
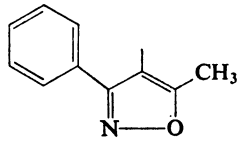
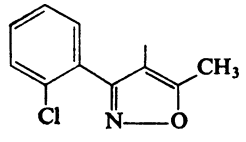
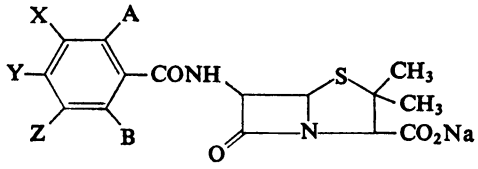
Test compound	Structure R	Fractional inhibition
BRL 1559		A substrate
BRL 1707		0.54
BRL 1400, oxacillin		0.00
BRL 1621, cloxacillin		0.00

Table 2. *Inhibition of β-lactamase by derivatives of phenylpenicillin*

K_i values were measured by using the starch–iodometric assay as described in the Materials and Methods section.



Test compound	Structure A	Structure B	Structure X	Structure Y	Structure Z	K_i (μM) purity corrected: *E. coli* B11 enzyme	K_i (μM) purity corrected: *K. aerogenes* A enzyme
BRL 2852	OCH_3	H	H	H	H	6.75	—
BRL 1241	OCH_3	OCH_3	H	H	H	0.286	0.22
BRL 1593*	$OCH–[CH_3]_2$	$OCH–[CH_3]_2$	H	H	H	0.034	—
BRL 1789	OCH_3	OCH_3	H	Cl	H	0.345	—
BRL 1471	OCH_3	OCH_3	Br	H	H	0.526	—
BRL 1428	OCH_3	OCH_3	Br	H	Br	1.44	—
BRL 1540	OCH_3	OCH_3	I	H	I	0.183	—

* Very poor purity.

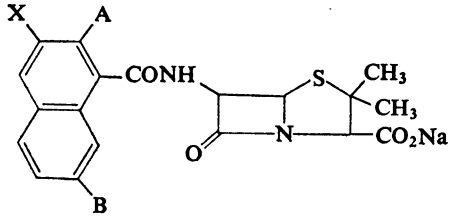
was used and for the cell-free enzyme a sample of the latter suspension was sonicated (see the Materials and Methods section) and the debris removed by centrifugation. The velocities of hydrolysis of ampicillin by these two preparations were within a factor of 2. The addition of 7.5 ng of BRL 1437/ml to the two preparations gave a similar degree of inhibition.

Effect of 2-isopropoxy-1-naphthylpenicillin (BRL 1437) on β-lactamase preparations from various bacteria

BRL 1437 was selected from a large number of compounds as the most effective inhibitor of *E. coli* B11 and *K. aerogenes* A β-lactamases. The effect on β-lactamases of other organisms was tested by

Table 3. *Inhibition of β-lactamase by derivatives of naphthylpenicillin*

K_i values were measured by using the starch–iodometric method.

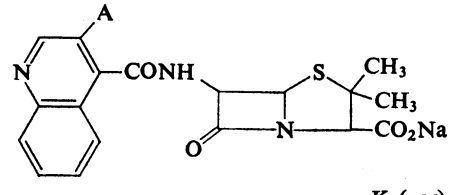


Test compound	Structure A	Structure B	Structure X	K_i (μM) purity corrected: *E. coli* B11 enzyme	K_i (μM) purity corrected: *K. aerogenes* A enzyme
BRL 1336	OCH_3	H	H	0.242	0.048
BRL 1383	OCH_2–CH_3	H	H	0.028	0.059
BRL 1389	OCH_2–CH_2–CH_3	H	H	0.019	0.040
BRL 1396*	OCH_2–CH_2–CH_2–CH_3	H	H	0.016	0.0082
BRL 1437	$OCH(CH_3)_2$	H	H	0.0040†	0.0011
BRL 1403*	OCH_2Ph	H	H	0.022	0.0064
BRL 1652	OCH_3	H	OCH_3	0.316	0.032
BRL 1578	OCH_3	OCH_3	H	0.098	0.029

* Very poor purity.
† = 0.0018 μg/ml.

Table 4. *Inhibition of β-lactamase by derivatives of quinolylpenicillin*

K_i values were measured by using the starch–iodometric method.



Test compound	Structure A	K_i (μM) purity corrected: *E. coli* B11 enzyme	K_i (μM) purity corrected: *K. aerogenes* A enzyme
BRL 1707	OCH_2–CH_3	0.50	0.12
BRL 3214	OCH_2–CH_2–CH_3	0.39	—
BRL 3213	OCH_2–CH_2–CH_2–CH_3	0.46	—
BRL 3215	$OCH(CH_3)_2$	0.037	0.059

measuring the decrease in reaction velocity against benzylpenicillin when the relatively high concentration of 1 μg of BRL 1437/ml was added to the reaction mixtures. The results are shown in Table 7.

Effect of inhibitors on the β-lactamases of viable cultures

All experiments described above have employed β-lactamases released from the cells of *E. coli* or *K. aerogenes*. To demonstrate the effect of a range of good and poor inhibitors on the β-lactamase of live cultures of recently isolated ampicillin-resistant strains the cultures were incubated with 100 μg of ampicillin/ml in the presence or absence of 5 μg of test compound/ml. The residual ampicillin was determined by bioassay and the results are shown in Table 8. The experiment was repeated by using the preferred inhibitor BRL 1437 but with the ampicillin at 5 μg/ml and the inhibitor at 0.1 or 0.5 μg/ml to

Table 5. *Reversible inhibition of E. coli B11 β-lactamase by BRL 1437*

To 5ml portions of B11 enzyme was added either 1ml of water or 1ml of a 2.5μg/ml solution of BRL 1437. Portions (3ml) of these solutions were separately dialysed overnight in 1 litre of water at 4°C in Visking tubing, and the remaining 3ml portions were stored at 4°C overnight. The next morning each sample was diluted to 3.5ml (to allow for any volume change) and the enzymic activity of each fraction assayed by the starch–iodometric assay with 10μg of ampicillin/ml as substrate. The BRL 1437 diffusate was evaporated to dryness on a rotary evaporator, redissolved in 1ml of water and tested for its ability to inhibit the hydrolysis of 10μg of ampicillin/ml by fresh enzyme.

Enzyme fraction	Reaction rate (μg of ampicillin/ml hydrolysed/min)	Reaction rate relative rate (%)
Enzyme not dialysed	0.276	100.0
Enzyme dialysed (retentate)	0.125	45.3
Enzyme + BRL 1437, not dialysed	0.051	18.5
Enzyme + BRL 1437, dialysed (retentate)	0.208	75.5
Fresh enzyme + water	0.513	100.0
Fresh enzyme + diffusate	0.083	16.2

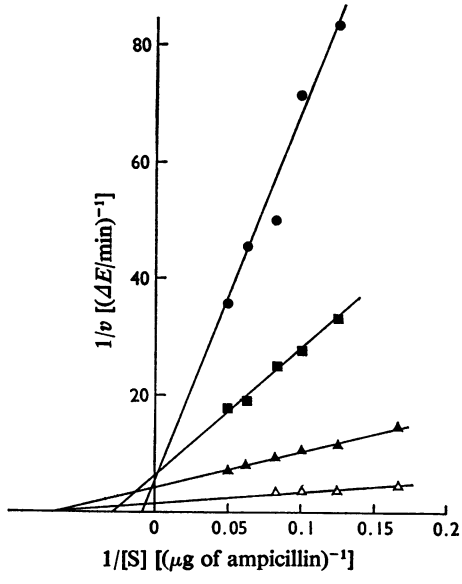


Fig. 3. *Lineweaver–Burk plots showing the determination of K_m value for ampicillin with two concentrations of E. coli B11 β-lactamase, and the competitive inhibition of the hydrolysis by this enzyme with BRL 1437 and methicillin*

Reaction mixtures for K_m determination consisted of 6.0–22.0μg of ampicillin/ml plus high (△) or low (one-third) (▲) concentration of enzyme. Reaction mixtures for inhibition experiments consisted of 6.0–20.0μg of ampicillin/ml plus the low concentration of enzyme plus 0.17μg of methicillin/ml (■) or 0.019μg of BRL 1437/ml (●) (all final concentrations). Reaction velocities were measured by the starch–iodometric assay over the initial 5min period of reaction at pH7.0 and 37°C.

simulate possible clinical conditions. The results are presented in Table 9.

Stability of inhibitors and related penicillins to E. coli B 11 β-lactamase

As all of the inhibitors described above are penicillins, the possibility existed that the poorer ones might be substrates for β-lactamase. A range of compounds each at 100μg/ml was therefore incubated with various concentrations of cell-free *E. coli* B11 β-lactamase. The remaining compound was bioassayed and the results are presented in Table 10. As with the results in Tables 8 and 9 the possibility exists for continued action of β-lactamase during the early part of the incubation of the bioassay plate.

Discussion

Examination of a large number of semi-synthetic penicillins for their ability to inhibit the hydrolysis of ampicillin by *E. coli* B11 β-lactamase has revealed several new potent inhibitors and has provided information on structure–activity relationships. The results in Table 1 illustrate that disubstitution at the α-carbon atom of the substrate molecule benzylpenicillin (or *n*-butylpenicillin as in BRL 1201) confers weak inhibitory activity. However, if these substituents are too bulky, as in triphenylmethylpenicillin (BRL 1071), the compound does not act as an inhibitor and is a poor substrate, suggesting that this compound cannot enter the catalytic site. Oxacillin and cloxacillin behave similarly. Insertion of a β-CH_2 group into the BRL 1071 molecule (BRL 1307) deflects the phenyl group attached to it and allows the molecule access to the catalytic site and to regain inhibitory properties. 2,6-Dichloro-substitution of α-methoxybenzylpenicillin converts the compound

Table 6. *Kinetic results for β-lactamases*

K_m and $V_{max.}$ values (purity corrected) were obtained for cell-free extracts of *E. coli* B11 and *K. aerogenes* A by using the starch–iodometric assay as described in the Materials and Methods section. K_I values were those for the compound BRL 1437 (see Table 3).

Substrate	*E. coli* B11 enzyme				*K. aerogenes* A enzyme			
	K_m (μM)	$V_{max.}$ (nmol/min per ml)	$\frac{V_{max.}}{K_m}$	$10^4 \times \frac{K_I(\text{BRL 1437})}{K_m}$	K_m (μM)	$V_{max.}$ (nmol/min per ml)	$\frac{V_{max.}}{K_m}$	$10^4 \times \frac{K_I(\text{BRL 1437})}{K_m}$
Sodium benzylpenicillin	28.9	2.92	0.10	1.39	9.0	1.18	0.13	1.22
Sodium ampicillin (α-amino-benzylpenicillin)	36.9*	3.63*	0.098	1.09	22.1†	1.53†	0.069	0.5
BRL 2333 (*p*-hydroxy-α-amino-benzylpenicillin free acid)	46.0	3.75	0.082	0.87	17.9	1.09	0.061	0.62
Disodium carbenicillin (α-carboxy-benzylpenicillin)	10.1	0.390	0.039	3.96	2.42	0.102	0.042	4.55
BRL 2288 (α-carboxy-3-thienyl-methylpenicillin, disodium salt)	7.0	0.393	0.056	5.71	3.78	0.111	0.029	2.91

* K_m = 13.7 μg/ml; $V_{max.}$ = 1.35 μg/min per ml.
† K_m = 8.2 μg/ml; $V_{max.}$ = 0.57 μg/min per ml.

into a fairly good inhibitor (BRL 2072) as does incorporation of the α-side-chain carbon atom of benzylpenicillin into a ring system such as cyclohexyl (BRL 1234).

Like benzylpenicillin, phenyl- (BRL 957), naphthyl- (BRL 1371) and quinolyl- (BRL1 559) penicillins are substrates for the *E. coli* B11 enzyme (Table 1), but substitution with an alkoxy group in the 2-position in all three compounds converts them into inhibitors.

Results in Table 2 illustrate further the effect of alkoxy substitution in phenylpenicillins. Insertion of alkoxy groups at both 2- and 6-positions gives better inhibitors, the best substituent group being isopropoxy as in BRL 1593. Insertion of halogen atoms in the 3- and 5-, or 4-positions also had significant effect; 3,5-dichloro substitution also gave a good inhibitor.

Substitution by an alkoxy group in the 2-position of the side-chain of naphthylpenicillin (Table 3) or quinolylpenicillin (Table 4) also confers inhibitory activity, which again increases as this homologous series is ascended. The optimum substituent for maximum inhibitory activity is isopropoxy, as was found in the substituted phenylpenicillin series.

These observations suggest that inhibitory activity conferred on these penicillins is not merely due to bulk of the side chains attached to 6-aminopenicillanic acid but is probably also due to more specific electronic effects. Thus alkoxy substituents in aromatic ring systems will tend to polarize this part of the penicillin molecule, so that there will be a greater electron density in the region of the *meta*-positions of phenylpenicillins and the 3-position of naphthylpenicillins. These electron-dense areas could be envisaged as constituting a secondary binding with adjacent positively charged areas on the enzyme molecule.

The most active inhibitor against the β-lactamase of *E. coli* B11, 2-isopropoxy-1-naphthylpenicillin (BRL 1437) was also the most active against the β-lactamase of *K. aerogenes* A although inhibitor structure–activity profiles were not identical for the two organisms, the *K. aerogenes* enzyme being readily inhibited by a wide range of structures.

Experiments with BRL 1437 revealed that it was a reversible (Table 5) and competitive (Fig. 3) inhibitor. The graphs used to obtain K_I values for other inhibitors suggest that they also act competitively. The stability results in Table 10 show that BRL 3215 and BRL 1437, respectively the *o*-isopropoxy-substituted quinolyl- and naphthyl-penicillins, are highly resistant to the β-lactamase of *E. coli* B11. As cloxacillin and triphenylmethylpenicillin have some stability but are not inhibitors and methicillin is a poor inhibitor but has fair stability, there does not seem to be any correlation between stability and effectiveness as an inhibitor, although clearly an effective inhibitor must be stable.

Table 7. *Effect of BRL* 1437 *on the β-lactamases of various bacteria*

Suitable dilutions of the enzyme preparations were prepared so that they hydrolysed 10 μg of benzylpenicillin/ml at an initial rate of about 0.1–0.3 μg/min per ml. The cell-free extracts of cells were prepared by sonicating cells as described in the Materials and Methods section and centrifuging off cell debris. The enzyme preparations were incubated with 10 μg of benzylpenicillin/ml at pH7 (0.05 M-phosphate buffer) and 37°C in the presence and absence of 1 μg of BRL 1437/ml. The reaction velocities were determined by the starch–iodometric method.

Culture	Type of β-lactamase	Reaction velocity (μg of benzylpenicillin hydrolysed/min per ml)	
		Uninhibited*	BRL 1437 added
E. coli B11	Constitutive; cell free extract of cells	0.26	<0.01
E. coli NCIB 9465		0.27	0.01
K. aerogenes A		0.11	0.00
Pr. vulgaris A	Benzylpenicillin induced; cell-free extract of cells	0.13	<0.01
Ps. aeruginosa A		0.36	0.02
B. cereus 569/H	Constitutive; cell-free supernatant	0.25	0.09
S. pyogenes A	Methicillin induced; whole cells	0.19	0.19
S. pyogenes H	Constitutive; whole cells	0.09	0.09

* These values do not represent the relative activities of different organisms.

Table 8. *Inhibition of ampicillin destruction with live cultures of Escherichia and Klebsiella*

Bacterial cultures were grown for 18 h in test-tubes of sterile Oxoid no. 2 nutrient broth at 37°C. A sterile solution of ampicillin was added to each culture to give a final concentration of 100 μg/ml. Sterile solutions of β-lactamase inhibitors were added where indicated to give final concentrations of 5 μg/ml. The tubes were incubated for a further 4 h at 37°C before bioassay of the residual ampicillin. A certain amount of further destruction of ampicillin by β-lactamase would have occurred during the bioassay. Test inhibitors at 5 μg/ml concentrations have no antibacterial activity against the bioassay organism.

Culture	Ampicillin remaining (%) in absence or presence of β-lactamase inhibitors					Minimum inhibitory concentration of ampicillin for the organism (μg/ml)
	None	BRL 1621	BRL 1241	BRL 1437	BRL 3215	
E. coli B11	0	0	0	60	0	>1000
B41	0	0	0	55	0	>1000
83	45	90	90	90	55	>1000
T506	0	0	0	75	35	>1000
T507	0	0	0	70	20	>1000
K. aerogenes A	0	0	20	70	15	250
I281	0	0	45	70	60	250
R129	0	15	55	75	65	100
C978	15	45	55	90	55	50
B	0	0	60	80	60	250
C	0	0	10	50	15	250
K. oxytoca T200	0	0	0	0	0	>1000
F	0	0	0	0	0	>1000
Control nutrient broth	100	100	100	100	100	—

Compound BRL 1437 also has inhibitory action against the β-lactamases of *Pseudomonas* and *Proteus*, as shown in Table 7. It is without effect on staphylococcal β-lactamase and has weak effect on that of *B. cereus*. Like Nafcillin (2-ethoxy-1-naphthylpenicillin), which is used clinically for penicillin-

resistant staphylococci, BRL 1437 is stable to staphylococcal β-lactamase and is active against staphylococci.

The fact that compounds such as BRL 1437 can have good inhibitory activity against the β-lactamases of live cultures of various ampicillin-resistant strains of *E. coli* and *K. aerogenes* is revealed in Table 8, where the inhibition was good at the low concentration of 5μg of inhibitor/ml with ampicillin concentrations of 100μg/ml. BRL 1437 would appear to be superior to BRL 3215 and methicillin (BRL 1241) whereas cloxacillin (BRL 1621) was ineffective, in agreement with the result in Table 1. Inhibition was also obtained at 0.1 and 0.5μg of BRL 1437/ml with ampicillin at 5μg/ml (Table 9). Inhibition was most marked for *Klebsiella*, but *E. coli* B11, which produced the highest enzyme activity, was not inhibited in this stringent test. These concentrations are in the clinically obtainable range and suggest that this combination could have useful synergistic effects *in vivo* where ampicillin destruction was responsible for bacterial resistance. None of the inhibitors in Table 8 was effective against the β-lactamase of *K. oxytoca*, suggesting that this organism produces a different enzyme.

The *Klebsiella* A enzyme has both K_m and $V_{max.}$ values lower than that of *E. coli* B11 against all substrates, and the $V_{max.}/K_m$ ratios are therefore similar for the two enzymes (Table 6) indicating similar physiological efficiencies (Pollock, 1965). The relatively low $V_{max.}/K_m$ ratio for BRL 2288 and carbenicillin indicates that these compounds have some general stability to the β-lactamases of *E. coli* and *K. aerogenes*, but these compounds do not differ from the others by more than a factor of five. The lower $V_{max.}$ of carbenicillin and BRL 2288 is somewhat compensated by the higher affinity.

From the K_i/K_m ratios for BRL 1437 it is clear that for all compounds in Table 6 the relative affinity of BRL 1437 for both enzymes is over 1000 times the affinity of the benzylpenicillin, carbenicillin and BRL 2288 for the enzyme. With ampicillin and BRL 2333 as substrate the relative affinity for BRL 1437 was 10000 times greater. Thus with ampicillin and BRL 2333 as substrates BRL 1437 would be expected to be very effective at protecting these compounds from destruction by *E. coli* and *K. aerogenes* β-lactamases and therefore to act synergistically. With

Table 9. *Inhibition of β-lactamase by live fully grown bacterial cultures at low concentrations of ampicillin and BRL* 1437

The method was as described in Table 7 except that the final concentration of ampicillin was 5μg/ml. The β-lactamase inhibitor BRL 1437 was added to give final concentrations of 0.5 or 0.1μg/ml and the residual ampicillin determined by bioassay after 4h at 37°C.

Culture	Ampicillin remaining (μg/ml)		
Concn. of BRL 1437 ...	0	0.1μg/ml	0.5μg/ml
E. coli B11	0	0	0
B41	0	0	0.6
83	2.5	2.5	3.3
T506	0	1.3	3.2
T507	0	1.9	3.7
K. aerogenes A	0.5	1.7	2.5
R129	0.5	1.4	2.2
C978	0	2.8	3.7
B	0.2	2.8	3.7
C	0	1.1	1.4

Table 10. *Stability of inhibitors and related penicillins to β-lactamase of E. coli B*11

Test compounds at a final concentration of 100μg/ml (purity corrected) were incubated at pH7 at 37°C for 4h in buffer or in the presence of various dilutions of the stock enzyme preparation of *E. coli* B11. The remaining penicillin was bioassayed against the relevant standard by using *B. subtilis* and expressed as % of compound remaining, after correction for the buffer control.

Test compound	Compound remaining (%)		
Final enzyme concn. (stock = 1.0) ...	1.5×10^{-2}	1.5×10^{-3}	1.5×10^{-4}
Benzylpenicillin	0	0	56
Phenylpenicillin (BRL 957)	0	0	44
1-Naphthylpenicillin (BRL 1371)	0	0	15
Cloxacillin (BRL 1621)	0	85	100
Methicillin (BRL 1241)	37	87	90
Triphenylmethylpenicillin (BRL 1071)	0	46	91
3-Isopropoxy-4-quinolylpenicillin (BRL 3215)	66	75	75
2-Isopropoxy-1-naphthylpenicillin (BRL 1437)	94	94	94

a K_i/K_m ratio of 2×10^{-4} for dicloxacillin and ampicillin, Bach *et al.* (1967) obtained good antibacterial synergism for *Shigella flexneri*. Of course, antibacterial synergism cannot be expected where the bacterium is insensitive to ampicillin (i.e. intrinsically resistant), even though its β-lactamase is effectively inhibited. This appears to be the case with *E. coli* B11 (R. Sutherland, personal communication), which was chosen for our preliminary screen mainly because it produced a high β-lactamase activity.

O'Callaghan *et al.* (1969) have examined many cephalosporins for stability to the β-lactamase of *Ent. cloacae* and found that the most stable were inhibitors of the action of this enzyme on cephaloridine. We initially looked for β-lactamase-stable (*E. coli*) penicillins as a route to inhibitors but abandoned it because of the questionable relationship between stability and ability to inhibit the enzymes. In the present paper this is illustrated by triphenylmethylpenicillin (BRL 1071), which is stable but not an inhibitor.

Hamilton-Miller *et al.* (1965) have reported that cloxacillin is a better inhibitor than methicillin for hydrolysis of benzylpenicillin by *E. coli* β-lactamase (ampicillin was not hydrolysed). This is contrary to the results obtained in the present paper for cultures which hydrolyse ampicillin, and it may be that their *E. coli* strain 419 was atypical and more like our strain 83 (Table 8). Hamilton-Miller *et al.* (1965) reported that methicillin was a better inhibitor than cloxacillin for two strains of *Klebsiella*, which is in agreement with our findings.

There is now no shortage of good inhibitors of the β-lactamases of *E. coli* and *K. aerogenes*, and it is to be hoped that the frequency and degree of synergism between penicillins and the new inhibitors for a collection of isolates might be higher than obtained with previously available inhibitors (Sutherland & Batchelor, 1964).

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