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`<latex>β</latex>-Lactams' as <latex>β</latex>-Lactamase Inhibitors

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'β-Lactams' as β-lactamase inhibitors

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The application of inhibitors to block the β-lactamase destruction of penicillins and cephalosporins by resistant bacteria is a potentially useful way of improving the efficacy of established compounds. Certain semi-synthetic penicillins and cephalosporins have been found to be competitive inhibitors of selected β-lactamases but an examination of streptomycete culture fluids has revealed two new types of β-lactam compound: clavulanic acid, which is a progressive inactivator of a wide range of β-lactamases, and the olivanic acids, which are both broad-spectrum antibiotics and potent β-lactamase inhibitors. Penicillanic acid sulphone and 6-β-bromopenicillanic acid have been shown to be significant inhibitors of β-lactamase. The chemotherapeutic application of these compounds is discussed.

1. Introduction

The continuing problem of the β-lactamase mediated resistance of clinically important bacteria to β-lactam antibiotics has encouraged the search for more effective inhibitors of this enzyme. This communication deals mainly with developments that have taken place recently particularly in the area of naturally occurring inhibitors and related substances, and their application as potentiators of the antibacterial activity of β-lactam antibiotics.

The first β-lactam containing substance reported to have β-lactamase inhibitory activity was the naturally occurring antibiotic cephalosporin C (Abraham & Newton 1956). Later, methicillin, the semi-synthetic penicillin selected for its stability to staphylococcal β-lactamase, was also found to be an inhibitor (Rolinson *et al.* 1960). These publications only reported the inhibition of *Bacillus cereus* β-lactamase and no synergy data were presented but they heralded the start of an extensive investigation of available β-lactamase-stable penicillins and cephalosporins as β-lactamase inhibitors. Some of these compounds have been shown to be very potent inhibitors in biochemical terms, the type of inhibition being competitive at low concentration. Probably the most extensively investigated inhibitor is the penicillin, cloxacillin. Unfortunately, although it has been possible in selected instances to show marked enhancement of activity of the labile penicillin or cephalosporin in the presence of the inhibitor, the concentration of the latter usually had to be far higher than expected and the number of different types of bacteria or β-lactamases for which an effect was shown was rather low. The result is that such combinations are not in general use to treat resistant strains. The literature on penicillins and cephalosporins as β-lactamase inhibitors and potentiators of the antibacterial activity of β-lactam antibiotics has recently been reviewed (Cole 1979*a*, *b*).

Some years ago in the Beecham laboratories, in addition to mounting a screen to examine for improved β-lactamase inhibitory properties the very large number of penicillins which our chemists had made, we also started to examine a variety of microorganisms for the production of such substances.

As cephalosporin C was produced by a microorganism and had been described as having β-lactamase inhibitory properties, and in view of the variety of structures of penicillins produced by many organisms including a *Streptomyces* sp., it seemed possible that other naturally occurring β-lactamase inhibitors might exist.

2. Screen of semi-synthetic penicillins and cephalosporins for improved β-lactamase inhibitors

The results of the screen of over 1000 semi-synthetic penicillins have been published (Cole *et al.* 1972). We found one compound, BRL 1437 (2-isopropoxy-1-naphthylpenicillin) to be an excellent competitive inhibitor of the β-lactamases produced by *Klebsiella aerogenes* and an R plasmid-carrying strain of *Escherichia coli*.

The affinity of BRL 1437 for these β-lactamases was in the region of 10000 times greater than the affinity of ampicillin for the enzymes. Such a favourable ratio of K_i/K_m suggested that the inhibitor should be a good protecting agent for labile β-lactam antibiotics thereby potentiating their antibacterial activity. Although BRL 1437 did potentiate the antibacterial activity of ampicillin, relatively high concentrations were required. A greater potentiating effect was seen with cephalothin and cephaloridine, illustrating the part played by the 'substrate' in such a situation (Greenwood & O'Grady 1975; Cole 1979*b*). The compound was poorly absorbed in man after oral administration.

A screen of semi-synthetic cephalosporins for β-lactamase inhibitory activity was also carried out in the Glaxo laboratories (O'Callaghan & Morris 1972). The cephalosporin with a naphthyl side chain (443/1) showed good inhibition of the β-lactamase of *Enterobacter cloacae* P99 and *E. coli* R^{+}_{TEM} and enhanced the antibacterial activity of cephaloridine against the *E. coli* but not the *Ent. cloacae*. The cephalosporin cephoxazole, which has the same substituted isoxazolyl side chain as the penicillin cloxacillin, was found to be a potent inhibitor of the *Ent. cloacae* β-lactamase and enhanced the activity of cephaloridine against this organism. As with penicillins that are inhibitors, fairly high concentrations were required to potentiate antibacterial activity and no single cephalosporin was a good inhibitor of a wide range of different β-lactamases. The β-lactamase inhibition profiles for methicillin, cloxacillin, BRL 1437, cephoxazole, cephalothin and the newer cephalosporins, cefoxitin and cefuroxime, have been compared for a range of β-lactamases (Cole 1979*a*). Cephalothin was the only compound at low concentration of this set to show significant inhibition of staphylococcal β-lactamase. More recently, Fu & Neu (1978) have reported that the new cephalosporin HR756 has inhibitory activity against the β-lactamases of *Citrobacter* and *Proteus morganii* but no potentiation of antibacterial activity was seen.

3. β-Lactamase inhibitors from microorganisms: olivanic acids, clavulanic acid and other substances

Using an agar plate procedure to test culture fluids for ability to inhibit the β-lactamase of *K. aerogenes* (*pneumoniae*) (ATCC 29665) we found various strains of *Streptomyces olivaceus* to give positive results (Brown *et al.* 1976). The presence of a β-lactamase inhibitor in the culture fluid of *S. olivaceus* was confirmed by demonstrating that the culture fluids would reduce the rate of hydrolysis of benzylpenicillin by a variety of β-lactamases. Furthermore, prolonging the time

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of contact between the culture fluid and the enzyme appeared to enhance the inhibitory effect. Culture fluid was also shown to enhance the activity of ampicillin against a range of β-lactamase-producing, ampicillin-resistant bacteria (Butterworth *et al.* 1979).

The very unusual properties of this *S. olivaceus* culture fluid encouraged us to embark on the isolation of the active components, a task that turned out to be very protracted primarily because the titre was extremely low. The isolation stages were monitored with a β-lactamase inhibition assay. The first stage involved an ion-pair solvent extraction procedure to effect a big reduction in volume. This was followed by resin adsorption and cellulose chromatography to give three β-lactamase-inhibiting acidic substances which were designated olivanic acids MM 4550, MM 13902 and MM 17880 (Hood *et al.* 1979). Other species of *Streptomyces* have also been found to produce members of this family of substances (Butterworth *et al.* 1979).

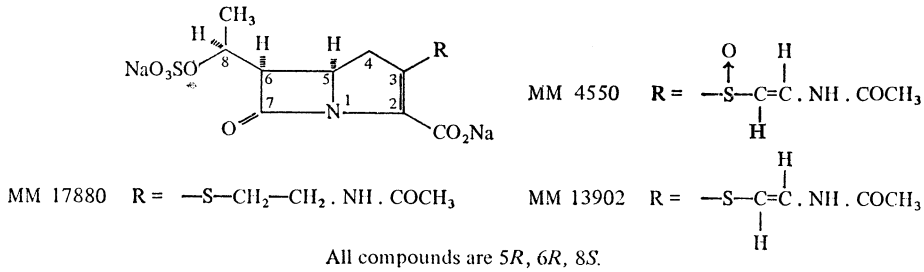

FIGURE 1. Structure of MM 4550, MM 13902 and MM 17880.

Structure elucidation work on MM 4550 and MM 13902 (Brown *et al.* 1977) and MM 17880 (Corbett *et al.* 1977) revealed them to be novel fused bicyclic compounds containing a β-lactam ring system (figure 1). There are two side chains attached to this ring system, one an acetylated aminoethyl thio group in various states of oxidation and the other a sulphated hydroxyethyl group. The unsubstituted nucleus has been named olivanic acid (Brown *et al.* 1977) and clearly resembles the nucleus of penicillins and cephalosporins, including the spatial arrangement at carbons 5 and 6, the protons here being *cis* to one another.

Under fermentation conditions limiting for sulphate, *S. olivaceus* and related cultures accumulate the desulphated forms of MM 13902 and MM 17880 and their 6*S* isomers. These substances are also β-lactamase inhibitors (Beecham German Patent 2 808 563).

During the course of our work, Umezawa *et al.* (1973) described the detection of β-lactamase-inhibiting substances in the culture fluid of *Streptomyces fulvoviridis*, a culture very similar to *S. olivaceus*. Two very active β-lactamase inhibitors were isolated, MC696-SY2A and B. Compound A was a very potent inhibitor of the β-lactamase of *E. coli* K12 R^{+}_{75} and was a competitive inhibitor. Determination of its structure (Maeda *et al.* 1977) revealed it to be related to the Beecham olivanic acid MM 4550 but the stereochemistry was not specified. Compound B was not completely competitive in its inhibition and was said to have distinct antibacterial activity.

Other Japanese workers also described the detection of β-lactamase inhibitors in streptomycete fermentation broths during the course of our work. Thus Hata *et al.* (1972), Iwai *et al.* (1973) and Ohno *et al.* (1973) described the detection and isolation of a protein (KA107) from *Streptomyces gedanensis* ATCC 4880 with inhibitory activity against staphylococcal β-lactamase. Miyamura & Ochiai (1974) also found a macromolecular inhibitor (M540) of the β-lactamases

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of *E. coli* and *K. pneumoniae* to be produced by an unidentified streptomycete. Both of these substances enhanced the activity of penicillins against penicillinase-producing strains.

In our own laboratories we have also observed what appears to be a high molecular mass β-lactamase inhibitor in streptomycete fermentations. For example, such a material was isolated from *Streptomyces fimbriatus* NRRL 3594 and was found to have proteolytic activity against casein and bovine serum albumin although it was not fully purified (S. J. Box & R. Edmondson, unpublished results).

More recently, Okamura *et al.* (1978) have described an antibiotic, PS-5, as having β-lactamase inhibitory activity and being able to potentiate the activity of ampicillin and cephaloridine against *Proteus vulgaris*. This substance was produced by an unidentified species of *Streptomyces* (ATCC 31358) and found to have a structure resembling MM 17880 but without the sulphated hydroxyl group on the ethyl side chain.

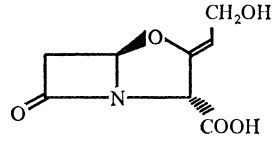

FIGURE 2. Structure of clavulanic acid.

A new β-lactam antibiotic called thienamycin has been reported to be produced by *Streptomyces cattleya* (Kahan *et al.* 1979). The structure of this substance (Albers-Schönberg *et al.* 1978) clearly resembles that of the olivanic acids, but it has a different stereochemical configuration at carbons 6 and 8, has no sulphate and is not acetylated. This compound has not been reported to be a β-lactamase inhibitor.

While we were still working on the isolation and characterization of the olivanic acids, we found that *Streptomyces clavuligerus* ATCC 27064 also produced potent inhibiting activity against the β-lactamases of *K. aerogenes* and an R plasmid-containing strain of *E. coli* (Brown *et al.* 1976; Reading & Cole 1977). *S. clavuligerus* had been previously described as a producer of cephalosporins including the 7-methoxy-substituted cephalosporin, cephamycin C. However, we were able to show that these substances were not responsible for the β-lactamase inhibitory activity that we observed. By using inhibition of β-lactamase to monitor the extraction stages, MM 14151 was isolated and subsequently named clavulanic acid. This substance has also been found to be produced by *Streptomyces jumonjinensis* NRRL 5741 (Beecham U.S.A. Patent no. 4,072,569) and by *Streptomyces katsurahamanus* FERM 3944 (Takeda Kokai no. 52-104796).

Structure determination (Howarth *et al.* 1976) revealed that clavulanic acid was a fused bicyclic compound containing a β-lactam ring (figure 2). The nucleus of this compound resembles the nucleus of penicillin but with oxygen instead of sulphur. As with the olivanic acids, the shape of such a molecule looks as though it could fit into the position normally occupied by a substrate at the catalytic centre of the β-lactamase. As clavulanic acid was produced in much higher concentrations than the olivanic acids, it was possible to make more rapid progress on its isolation and properties. Information on the biosynthesis of clavulanic acid has been obtained by Elson & Oliver (1978). Very little has been published on the formation of β-lactamase inhibitors by organisms other than streptomycetes. In a very early piece of work, Hutter & Welsch (1948) observed an 'anti-penicillinase' in penicillin-producing strains of *Penicillium* and, more recently, Dennen *et al.* (1971) reported that cephalosporin C inhibited a β-lactamase in *Cephalosporium acremonium*.

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4. β-Lactamase inhibitory properties and antibacterial activity of the olivanic acids

The data in table 1 illustrate the potent β-lactamase inhibitory properties of the three olivanic acids against a wide range of β-lactamases. MM 4550 is a more potent inhibitor of the penicillinases of *Proteus mirabilis*, *K. aerogenes*, *E. coli* and *Staphylococcus aureus* than the other compounds while the reverse is true for the cephalosporinase of *Ent. cloacae* P99. MM 4550 and MM 13902 are also potent inhibitors of the β-lactamases produced by *Streptomyces* species (M. Cole & R. Edmondson, unpublished results), leading to the speculation that they could have a regulating role on the level of β-lactamase or related enzymes in these organisms.

Table 1. β-Lactamase inhibitory activity of the olivanic acids MM 4550, MM 13902 and MM 17880 (Hood *et al.* 1979)

	I_{50}/(ng/ml)†				
substrate ...	benzylpenicillin				cephaloridine
inhibitor	*K. aerogenes* E70	*E. coli* JT4‡	*P. mirabilis* C889	*Staph. aureus* Russell	*Ent. cloacae* P99
MM 4550	1.6	0.5	0.4	15	4.0
MM 13902	50	20	1.0	50	0.6
MM 17880	60	10	2.0	75	0.9

† Concentration of inhibitor giving 50% inhibition when incubated with the enzyme at 37 °C for 5 min before the addition of substrate (1 mg/ml final concentration).
‡ Contains TEM 1 β-lactamase.

Table 2. β-Lactamase inhibitory activity of MM 4550 under various conditions

	I_{50}/(μg/ml)	
source of β-lactamase	inhibitor with substrate	inhibitor added 15 min before substrate
E. coli K12 R_{TEM}	0.0014	0.00046
E. coli JT 414	0.035	0.015
P. mirabilis C889	0.00063	0.00035
K. aerogenes A	0.002	0.0008
Ent. cloacae NCTC 10005	0.20	0.15
Ps. aeruginosa	0.81	0.08
Staph. aureus MB9	0.016	0.0037

Substrate: benzylpenicillin (1 mg/ml).

The data in table 2 demonstrate that although the olivanic acids show some evidence of progressive inhibition of the β-lactamase, they appear to be very good competitive inhibitors by virtue of their high initial inhibitory activity in the presence of substrate (no preincubation). These properties led us to speculate, before the structures had been worked out, that MM 4550 and the related metabolites might contain a β-lactam ring system as all of the known potent β-lactamase inhibitors contain this structural feature. All three olivanic acids were able to enhance the antibacterial activity of ampicillin against β-lactamase producing bacteria.

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As work proceeded on the purification of the olivanic acids it became clear that they were also very active broad spectrum antibacterial agents. Minimum inhibitory concentrations (m.i.c.) in broth were 1 μg/ml and lower for MM 13902 and MM 17880 against many Gram-positive and Gram-negative bacteria. The compounds were less active against *Pseudomonas aeruginosa* and *Ent. cloacae* and MM 4550 had generally lower antibacterial activity (Hood *et al.* 1979).

TABLE 3. β-LACTAMASE INHIBITION SPECTRUM FOR CLAVULANIC ACID

source of β-lactamase	I_{50}/(μg/ml)† benzylpenicillin substrate	source of β-lactamase	I_{50}/(μg/ml)† cephaloridine substrate
E. coli JT4‡	0.07	*E. coli* JT4‡	0.04
E. coli JT20‡	0.3	*E. coli* JT410	40
K. aerogenes Ba95‡	0.07	*P. mirabilis* Q2879‡	0.03
K. aerogenes E70	0.015	*P. morganii* G	40
P. mirabilis C889	0.03	*C. freundii* (Mantio)	15
S. marcescens U39‡	0.4	*Ent. cloacae* P99	15
Ps. aeruginosa 1822‡	0.35	*S. marcescens* US 20	> 20
Ps. aeruginosa (Dalgleish)	0.007	*Ps. aeruginosa* A (Sabath type)	80
H. influenza 4482‡	0.07		
Staph. aureus (Russell)	0.06		
B. cereus (mixture of types I and II)	17		

† Concentration giving 50 % inhibition of rate of substrate hydrolysis after preincubating enzyme and inhibitor for 15 min at pH 7.3. Hydroxylamine assay procedure. Substrates 1 mg/ml.
‡ Contains resistance plasmid of TEM type.

5. β-LACTAMASE INHIBITORY PROPERTIES OF CLAVULANIC ACID

Clavulanic acid inhibits a wide range of clinically important β-lactamases as can be seen from table 3 for cell-free enzyme preparations prepared from cells by ultrasonication. Unlike various penicillins and cephalosporins which have been reported as β-lactamase inhibitors clavulanic acid inhibits the enzyme produced by the Gram-positive organism, *Staph. aureus* as well as that produced by Gram-negative bacteria containing R factors of the TEM type. Clavulanic acid is a poorer inhibitor of the β-lactamases which have good activity against cephalosporins and for which cephaloridine was used as a substrate in table 3. The I_{50} value for the extracellular β-lactamase of *Staph. aureus* was about half that for the enzyme liberated from cells by ultrasonics (Reading & Hepburn 1979).

Clavulanic acid also inhibits the oxacillin hydrolysing β-lactamases classified by Matthew & Hedges (1976) as OXA 1, 2 and 3. The I_{50} value for OXA 1 was similar to that for R_{TEM} while those for OXA 2 and OXA 3 were lower. The I_{50} values for *Branhamella catarrhalis* and *Neisseria pharyngis* were similar to that for R_{TEM} while tests on a *Nocardia* β-lactamase preparation received from Dr Wallace of the Texas Medical Center showed an I_{50} value less than one-tenth of that for TEM. (C. Reading, unpublished results).

The β-lactamase activity of *Bacteroides fragilis* is also inhibited (M. H. Richmond, personal communication) as is that of *Shigella sonnei* (Neu & Fu 1978). Durkin & Viswanatha (1978) have described the kinetics of the inhibition of *B. cereus* β-lactamase I by clavulanic acid. The inhibition of the R_{TEM} type of β-lactamase in strains of *Neisseria gonorrhoeae* is implied from the antibacterial studies reported by Miller *et al.* (1978). Neu & Fu (1978) have demonstrated inhibition of β-lactamases of Richmond & Sykes types II, III, IV & V while the inhibition of

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type I β-lactamase was very poor. Streptomycete β-lactamase has been found to be inhibited by clavulanic acid (M. Cole & R. Edmondson, unpublished results; Ogawara *et al.* 1978).

The progressive nature of the inhibition of β-lactamase by clavulanic acid is illustrated in table 4 for a variety of enzymes. The initial level of inhibition is very probably achieved by competitive means and is then followed by rapid inactivation of the enzyme. This competitive phase is illustrated in table 5, from which it can be seen that for the β-lactamase of *Staph. aureus* the extent of initial inhibition of the enzyme is greater where the affinity between enzyme and substrate is lower (i.e. K_m is higher). Thus better initial inhibition is seen with amoxycillin and ampicillin than for benzylpenicillin. However, by 5 min the inactivation of the enzyme has progressed so far that the initial effect of substrate is no longer seen. Neu & Fu (1978) have also demonstrated the initial competitive nature of the inhibition for type III β-lactamase by using ampicillin and cephaloridine as substrates.

TABLE 4. PROGRESSIVE NATURE OF THE INHIBITION OF β-LACTAMASE BY CLAVULANIC ACID

	I_{50}/(μg/ml)	
source of β-lactamase	substrate† added immediately after inhibitor	substrate† added 15 min after inhibitor
E. coli K12 R_{TEM}	0.56	0.08
P. mirabilis C889	0.69	0.01
K. aerogenes A	0.11	0.01
Staph. aureus MB9	0.84	0.02

† Substrate was amoxycillin at 1 mg/ml.

TABLE 5. EFFECT OF TYPE OF SUBSTRATE AND PREINCUBATION TIME ON INHIBITORY ACTIVITY OF CLAVULANIC ACID AGAINST β-LACTAMASES OF *STAPHYLOCOCCUS AUREUS* (RUSSELL) (READING & HEPBURN 1979)

	I_{50}/(μg/ml)	
substrate (1 mg/ml) and K_m value	no preincubation†	5 min preincubation‡
benzylpenicillin; 14.5 μM	43	0.12
ampicillin; 40.4 μM	6	0.12
amoxycillin; 40.5 μM	7	0.11

† Substrate and inhibitor were mixed and added to the enzyme.
‡ Inhibitor was incubated with the enzyme for 5 min before the substrate was added.

The mechanism by which clavulanic acid inhibits the TEM β-lactamase has been investigated (Fisher *et al.* 1978; Charnas *et al.* 1978; Reading & Farmer 1980) and found to involve the formation of transiently inactivated and irreversibly inactivated enzyme, the latter predominating when the clavulanic acid was in more than 300-fold molar excess over the enzyme. Fisher *et al.* (1978) showed that on average 115 clavulanate molecules were destroyed by each TEM β-lactamase molecule before the latter was inactivated. In contrast, Reading & Hepburn (1979) found that the staphylococcal β-lactamase is only transiently inactivated, activity slowly returning when the inhibitor is removed. Furthermore, no large excess of clavulanate was required to form this transiently inactivated enzyme. Labia & Peduzzi (1978) have studied

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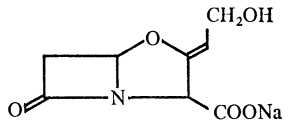
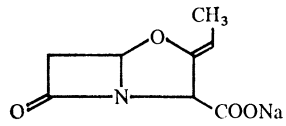
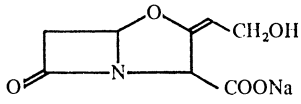
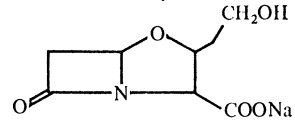
the kinetics of inhibition of the β-lactamases TEM-1, TEM-2 and Pitton's type 2 by clavulanic acid and conclude that it acts as a competitive and irreversible inhibitor of these enzymes i.e. a k_{cat} inhibitor (Rando 1974).

6. Derivatives and analogues of clavulanic acid as β-lactamase inhibitors

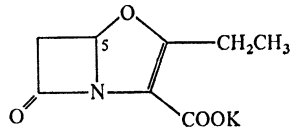
From the small series of derivatives of clavulanic acid shown in table 6 it can be seen that loss of the hydroxyl group in the side chain has not reduced the inhibitory activity against staphylococcal β-lactamase, whereas isomerization of the side chain decreases activity and reduction abolishes it. Cherry *et al.* (1978*a*) have described the conversion of clavulanic acid into an

Table 6. Inhibitory activity of clavulanic acid derivatives against the β-lactamase of *Staphylococcus aureus* (Russell)

compound	I_{50}/(μg/ml) †	compound	I_{50}/(μg/ml) †
			
sodium clavulanate	0.05	sodium deoxy-clavulanate	0.05
			
sodium iso-clavulanate	0.6	sodium dihydro-clavulanate	> 40

† Concentration giving 50% inhibition of rate of hydrolysis of benzylpenicillin (1 mg/ml) after preincubating enzyme and inhibitor for 15 min.

Table 7. β-Lactamase inhibitory activity of an endocyclic double bond isomer of clavulanic acid (Cherry *et al.* 1978*a*)



compound 10; 5*R*, *S*

source of β-lactamase	substrate	I_{50}/(μg/ml)† compound 10	I_{50}/(μg/ml)† lithium clavulanate
Staph. aureus PC1	ampicillin	0.02	8.2
Ent. cloacae P99 (R and S type I)‡	cephaloridine	0.01	> 25
RP1 R factor (R and S type III)	cephaloridine	0.88	0.18
K. aerogenes K1 (R and S type IV)	cephaloridine	0.44	0.15

† Method not described. ‡ Richmond & Sykes (1973).

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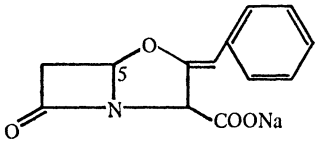
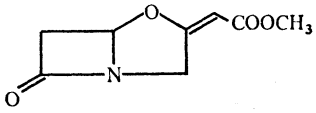
endocyclic double bond isomer (table 7) which, unlike clavulanic acid, has high activity against the cephalosporin hydrolysing β-lactamase produced by *Ent. cloacae* P99.

Various synthetic analogues of clavulanic acid have been described (Bentley *et al.* 1977*b*; Hunt *et al.* 1977). The β-lactamase inhibitory activity of two such compounds is shown in table 8 (C. Reading, unpublished results). The synthesis of racemic clavulanic acid has been described (Bentley *et al.* 1977*a*). Clavulanic acid analogues with substituted thioether side chains have been claimed to be more active inhibitors than clavulanic acid against TEM, staphylococcal and *Klebsiella* β-lactamases (Cherry *et al.* 1978*b*).

TABLE 8. SYNTHETIC ANALOGUES OF CLAVULANIC ACID WITH β-LACTAMASE INHIBITORY ACTIVITY

	I_{50}/(μg/ml)†					
β-lactamase from ...	*E. coli* (JT4)	*K. aerogenes* (E70)	*P. mirabilis* (C889)	*Ent. cloacae* (P99)	*Ps. aeruginosa* (A)	*Staph. aureus* (Russell)
Na clavulanate	0.07	0.015	0.03	15	> 40	0.06
BRL 20780 (5RS) (Bentley *et al.* 1977*b*)	0.2	3.6	< 0.08	> 40	> 40	< 0.08
						
BRL 19378 (5RS) (Hunt *et al.* 1977)	0.18	6.5	4.0	0.16	0.18	0.11
						

† Inhibitor was incubated with enzyme for 15 min before addition of substrate (benzylpenicillin 1 mg/ml).

7. POTENTIATION OF THE ANTIBACTERIAL ACTIVITY OF PENICILLINS AND CEPHALOSPORINS BY CLAVULANIC ACID

It has been shown in our laboratories (Reading & Cole 1977; Hunter *et al.* 1978) that clavulanic acid at low concentrations can potentiate the antibacterial activity of penicillins and cephalosporins against a wide range of bacteria that owe their resistance to β-lactamase production. This effect was seen at concentrations below the level of antibacterial activity shown by clavulanic acid itself, and was only observed to occur where the β-lactamase was readily inhibited by clavulanic acid. Thus the mechanism of the potentiation appears to be dependent on the inhibition of β-lactamase. Because clavulanic acid inhibits the β-lactamases of staphylococci and Gram-negative bacteria it has a considerable broadening effect on the antibacterial spectrum of penicillins such as ampicillin and amoxycillin. However, unlike cloxacillin, clavulanic acid is not a good inhibitor of certain chromosomally controlled, cephalosporin hydrolysing β-lactamases of *Enterobacter*, *Ps. aeruginosa* and certain strains of *E. coli*, and is therefore not a good potentiator for these organisms.

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The β-lactamase-inhibiting properties of clavulanic acid in bacteriological situations has been studied by using BRL 25000, a 2 : 1 ratio of amoxycillin and clavulanic acid (R. Sutherland, K. Comber, A. White & S. Layte, unpublished results). Table 9 illustrates the antibacterial activity of BRL 25000 compared with amoxycillin against a range of amoxycillin resistant bacteria. The broad-spectrum but generally weak antibacterial activity of clavulanic acid by itself is also shown.

TABLE 9. ANTIBACTERIAL ACTIVITY OF BRL 25000 (AMOXYCILLIN:CLAVULANIC ACID, 2:1) COMPARED WITH AMOXYCILLIN AND CLAVULANIC ACID AGAINST β-LACTAMASE-PRODUCING BACTERIA

	m.i.c.†/(μg/ml)		
organism	clavulanic acid	amoxycillin	BRL 25000
E. coli	50	> 500	12.5
K. aerogenes	50	250	2.5
Ent. cloacae	50	> 500	125
P. mirabilis	125	> 500	12.5
P. vulgaris	125	250	5.0
P. rettgeri	125	500	250
Ps. aeruginosa	125	> 500	250
S. marcescens	125	250	125
Bact. fragilis	50	25	1.25
H. influenzae	50	50	2.0
Br. catarrhalis	5	50	0.05
N. gonorrhoeae	5	> 10	1.0
Staph. aureus	25	250	1.25

† Minimum inhibitory concentrations determined in agar by using about 5×10^5 organisms per inoculation site.

Tests with a larger number of bacterial strains of hospital origin were carried out to gain information about the frequency with which improved activity was seen with BRL 25000 compared with amoxycillin or clavulanic acid alone. The percentage of β-lactamase-producing strains for which there was a fourfold or greater potentiation of activity were: *E. coli* (48 %), *K. aerogenes* (58 %), *P. mirabilis* (88 %), *P. vulgaris* (100 %), *Salmonella typhimurium* (100 %), *Br. catarrhalis* (100 %), *Bact. fragilis* (100 %), *N. gonorrhoeae* (85 %), *Haemophilus influenzae* (100 %) and *Staph. aureus* (87 %). No synergy was seen for strains that were already amoxycillin sensitive.

In experiments with the amoxycillin resistant culture *Staph. aureus* (Russell), the bactericidal action of BRL 25000, when measured by counts of viable cells, correlated with the prevention of destruction of the amoxycillin. The small initial fall in concentration of amoxycillin seen in the presence of clavulanic acid probably represents the initial destruction of amoxycillin that occurred during the time that it took for the enzyme to be inactivated.

The β-lactamase-inhibiting effect of clavulanic acid in an *in vivo* situation has been demonstrated for various infections in the mouse. BRL 25000, amoxycillin and clavulanic acid were separately administered by the oral route to mice infected intramuscularly with a β-lactamase-producing strain of *Staph. aureus*. Parallel control tests were set up with a β-lactamase-negative mutant of this strain having the same virulence towards mice. The results in table 10 show the improved efficacy of BRL 25000 at reducing the swelling in the thigh muscle compared with amoxycillin when the animals were infected with a β-lactamase-producing strain.

BRL 25000 was also administered by the oral route to mice infected by the intraperitoneal

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route with β-lactamase-producing strains of *E. coli*, *K. pneumoniae* and *K. aerogenes*. The results in table 11 reveal that a much lower dose of BRL 25000 was required to give protection of 50 % of the animals compared with amoxycillin or the clavulanic acid alone.

TABLE 10. ORAL ACTIVITY OF BRL 25000 COMPARED WITH AMOXYCILLIN AND CLAVULANIC ACID ALONE IN A MOUSE THIGH MUSCLE INFECTION WITH β-LACTAMASE NEGATIVE AND POSITIVE STRAINS OF *STAPH. AUREUS* MB9 (SAME MOUSE VIRULENCE)

		percentage protection by		
total dose/(mg/kg)	strain of *Staph. aureus*†	BRL 25000	amoxycillin	clavulanic acid
100	−ve	97	97	15
100	+ve	64	1	10

Dosage was oral at 1 and 5 h after infection.
† β-lactamase +ve or −ve.

TABLE 11. ORAL ACTIVITY OF BRL 25000 COMPARED WITH AMOXYCILLIN AND CLAVULANIC ACID ALONE AGAINST MOUSE INTRAPERITONEAL INFECTIONS

	c.d.$_{50}$/(mg/kg)†		
organism	BRL 25000	amoxycillin	clavulanic acid
E. coli T778‡	126	> 1000	460
E. coli JT39‡	114	> 1000	340
K. aerogenes I 112§	60	> 2000	> 2000
K. pneumoniae 62 (highly virulent)§	460	> 3200	> 3200

† Total oral dose.
‡ 1 and 5 h oral dosing of drug.
§ 1, 3, 5 and 7 h oral dosing of drug.

Publications by other groups of workers around the world have confirmed and extended our findings on the enzyme inhibitory and antibacterial potentiating properties of clavulanic acid for β-lactamase-labile penicillins and cephalosporins. Thus, enhancement of antibacterial activity by clavulanic acid has been reported for benzylpenicillin, amoxycillin or cephalothin against *Bact. fragilis* and other species (Wise 1977; Wüst & Wilkins 1978), for cephalothin against *Klebsiella* strains (Jackson *et al.* 1978), for benzylpenicillin or amoxycillin against various bacteria including *Staph. aureus*, *H. influenzae*, *E. coli*, *Klebsiella* and *Bact. fragilis* (Wise *et al.* 1978) and for ticarcillin (Paisley 1978). Reeves *et al.* (1978) have reported the potentiating activity of clavulanic acid on amoxycillin and cephaloridine against a variety of Gram-negative bacteria. Using a mouse infection with *K. pneumoniae*, they demonstrated 100 % protection by 10 mg/kg amoxycillin plus 10 mg/kg sodium clavulanate, while when each was given alone at these doses the protection was no greater than 10 %. Sjöberg *et al.* (1978), using *H. influenzae*, have demonstrated a much greater antibacterial potentiating effect between clavulanic acid and ampicillin or carbenicillin than between mecillinam and these penicillins. In a recent report by Wise (1979), clavulanic acid was shown to enhance the activity of the new cephalosporin HR756 (cefotaxime) against *Bact. fragilis*. The 'legionnaires bacterium' was found to produce a β-lactamase that is particularly active against cephalosporins. However, little or no enhancement of the antibacterial activity of cephalothin or benzylpenicillin was achieved by adding clavulanic acid at a concentration below the 0.2 μg/ml level at which it was active by itself (Thornsberry & Kirven 1978).

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The effect of sodium clavulanate at 0.5 μg/ml on the antibacterial activity of amoxycillin against *N. gonorrhoeae* was demonstrated by Miller *et al.* (1978). The m.i.c. values for amoxycillin against resistant strains were brought into the same range as found for the sensitive cultures. Similar results were obtained by using ampicillin or benzylpenicillin in place of amoxycillin, but the m.i.c. values for the β-lactamase-stable cefoxitin were not significantly reduced by clavulanic acid. Elwell *et al.* (1977) have reported that the β-lactamase produced by gonococci is R-plasmid specified.

In a recent paper, Neu & Fu (1978) reported that clavulanic acid enhanced the activity of ampicillin against bacteria that produced β-lactamase types III, IV and V but not II (classification of Richmond & Sykes 1973). For a strain of *E. coli* containing the plasmid mediated type III β-lactamase, a rapid bactericidal effect was shown with 6.3 μg/ml ampicillin plus 1.6 μg/ml clavulanic acid. The minimum inhibitory concentration for ampicillin alone was more than 1000 μg/ml. Neu & Fu (1978) also reported synergy for certain strains of *Staph. aureus*, *K. pneumoniae* and *Bact. fragilis* that appeared not to make β-lactamase. However, the tests that they used for β-lactamase production were of 5 min duration (personal communication) and it seems likely that they did not detect in these bacteria small amounts of β-lactamase strategically placed in the cell wall to protect the penicillin binding proteins.

Using a large number of penicillin resistant clinical isolates, Dumon *et al.* (1979) report the frequency with which clavulanic acid potentiates the activity of benzylpenicillin against *Staph. aureus* and ampicillin, carbenicillin or cephalothin against Gram-negative bacteria. Potentiation was often seen for *Staph. aureus*, *E. coli*, *K. aerogenes* but infrequently for *Enterobacter*, *Serratia* and *Pseudomonas*.

8. Preliminary clinical results with clavulanic acid

Studies in human volunteers have revealed that clavulanic acid is well absorbed by the oral route. After a 125 mg dose, peak concentrations in serum at 1 h were in the region 2.5–3.0 μg/ml and urine concentrations were over 100 μg/ml for 4 h. Similar values for clavulanic acid were obtained when amoxycillin was simultaneously administered. These results coupled with the bacteriological data encouraged tests in patients.

Ninane *et al.* (1978) have described the treatment of three cases of chronic bronchitis in coal miners. Previous treatment had involved ampicillin or amoxycillin, but culture of transtracheal aspirates revealed the presence of *Br. catarrhalis* in all three cases, *H. influenzae* type b in two and *Streptococcus pneumoniae* in one. The cultures of *Br. catarrhalis* were shown to be β-lactamase positive while the others were negative. The patients were treated for 3 days with two tablets of BRL 25000 three times a day. Each tablet contained the equivalent of 250 mg amoxycillin and 125 mg clavulanic acid. Clinical improvement was seen in all three patients. Transtracheal aspirates taken 48 h after cessation of treatment revealed that the *Br. catarrhalis* and *Strep. pneumoniae* had been eliminated, but in two patients the β-lactamase-negative *H. influenzae* was still present. The m.i.c. values for amoxycillin, clavulanic acid and BRL 25000 against the cultures of *Br. catarrhalis* isolated from these patients were respectively 25–50; 2.5–12.5 and 0.05–< 0.02 μg/ml. As can be seen, the cultures were highly sensitive to BRL 25000 and tests carried out in our laboratories revealed that the β-lactamase was readily inhibited, giving I_{50} values somewhat lower than obtained for the TEM β-lactamase.

Kosmidis *et al.* (1978) treated 20 patients with infections due to ampicillin-resistant bacteria.

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Amoxycillin (≡ 250 mg) plus sodium clavulanate (≡ 125 mg clavulanic acid) administered three times a day was used to treat infections in skin, soft tissues and the lower urinary tract, while double this dose was used to treat pyelonephritis or respiratory infections. Treatment lasted 5–8 days. Fifteen pathogens were eradicated. Four persisted and one relapsed after treatment was stopped. There were 15 cures, 2 improvements, 2 failures and 1 unassessable. No toxicity was detected, but two patients had soft stools and one developed a maculopapular ampicillin-like rash on the ninth day from the beginning of the treatment.

Goldstein *et al.* (1978) carried out a study to test the effectiveness of amoxycillin plus clavulanic acid at eradicating β-lactamase-producing Gram-negative bacteria from urinary tract infections in six patients. Administration of a single dose of 500 mg amoxycillin alone did not significantly reduce the bacteriuria and amoxycillin was very low or absent in the urine. When, 48 h later, a single dose of 500 mg amoxycillin + 100 mg clavulanic acid was given, the infecting organisms were eradicated within 8 h and the amoxycillin in the urine was not inactivated. No undesirable side effects were encountered.

Evidence is thus accumulating to show that clavulanic acid is capable of blocking β-lactamase destruction of penicillins in human infections.

9. Penicillanic acid sulphone (CP 45899) as a β-lactamase inhibitor

English *et al.* (1978) have reported penicillanic acid sulphone (CP 45899, figure 3) to be a β-lactamase inhibitor and a potentiator of the activity of ampicillin against resistant bacteria. The compound at 3.8 μg/ml gave 92–100% inhibition of the rate of hydrolysis of 13 μg/ml ampicillin by the β-lactamases of *Staph. aureus*, *E. coli* and *K. pneumoniae*. The β-lactamase of *Bact. fragilis* was also inhibited but the compound was a poor inhibitor of the β-lactamases of *Ps. aeruginosa* and *Ent. cloacae*. The inhibitory activity was suppressed by the presence of substrate. For *Staph. aureus* and *E. coli*, inhibition increased on incubating the inhibitor with the enzyme before adding substrate. Addition of a large excess of substrate did not reverse the inhibition of the *E. coli* β-lactamase. Thus in several respects these properties are similar to those of clavulanic acid.

CP 45899 alone has very little antibacterial activity except against *N. gonorrhoeae*. Addition of the compound to ampicillin resulted in a significant reduction in m.i.c. value for various ampicillin-resistant bacteria for which the β-lactamase was inhibited, although the results with *E. coli* were far poorer than would be suggested by the enzyme inhibition data. As the compound showed little inhibitory activity against the β-lactamases of *Ent. cloacae* and *Ps. aeruginosa*, it was perhaps surprising that some synergy was seen for these cultures. CP 45899 was also shown to potentiate the activity of penicillin G, carbenicillin and cefazolin against certain resistant bacteria.

In a comparison of clavulanic acid with CP 45899, English *et al.* showed that clavulanic acid was a more active potentiator of the activity of ampicillin against ampicillin resistant strains, particularly for *E. coli*. Oral or parenteral administration of CP 45899 plus ampicillin (1:1 ratio) was shown to act synergistically in mice against various ampicillin resistant infections.

Aswarpokee & Neu (1978) have confirmed the antibacterial potentiating effects of CP 45899 for a wide range of β-lactamase-producing organisms, and also reported antibacterial synergy

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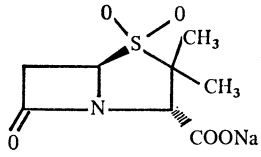
for strains of *Klebsiella* and *Bacteroides* that were recorded as β-lactamase-negative. However, as mentioned earlier in relation to results on clavulanic acid reported by Neu & Fu (1978), their test for β-lactamase may not have detected low levels of β-lactamase activity, particularly if the enzyme was within the cell wall. English *et al.* (1978) showed results that suggested that synergy between ampicillin and CP 45899 was not obtained with ampicillin-sensitive strains of bacteria.

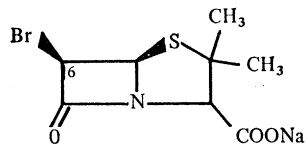
Fu & Neu (1979) have compared the competitive inhibition characteristics of CP 45899, clavulanic acid and dicloxacillin for various β-lactamases. The time-dependent inhibition of β-lactamase by CP 45899 was also reported.

Cartwright & Coulson (1979) have observed β-lactamase-inactivating activity in an α-chloropenicillanic acid sulphone. Staphylococcal β-lactamase appeared to be irreversibly inactivated.



CP 45899

Penicillanic acid sulphone sodium salt



β-bromopenicillanic acid sodium salt

FIGURE 3. Structures of β-lactamase-inhibiting penicillanic acid derivatives.

10. β-BROMOPENICILLANIC ACID AS A β-LACTAMASE INHIBITOR

Pratt & Loosemore (1978) and Loosemore & Pratt (1978) observed that a 30 mM solution of α-bromopenicillanic acid when stored in a 20 mM sodium pyrophosphate buffer at pH 9.1 and 30 °C gradually increased its inhibitory activity against *B. cereus* β-lactamase I over a period of 100 h. This increase in inhibitory activity was found to correlate with the epimerization of the compound to give a mixture containing about 12 % of the β-bromo isomer at equilibrium (figure 3). The best preparation made by hydrogenation of 6,6-dibromopenicillanic acid in dioxan contained 28 % β-bromo epimer as judged by nuclear magnetic resonance.

The inhibition of *B. cereus* β-lactamase I by β-bromopenicillanic acid was progressive. With equimolar amounts of enzyme and compound (0.2 μM), inhibition was about 75 % after 5 min, 90 % after 20 min and 100 % at 1 h. The presence of a very high concentration of benzylpenicillin (0.1 M) markedly slowed the rate of inactivation, only about 50 % inhibition being observed in 15–20 min. The inhibition was not reversed by incubation with 0.1 M hydroxylamine or 0.1 M imidazole or by extensive dialysis. The inhibitor thus seems to be an irreversible inhibitor directed to active sites which reacts with the enzyme in a 1:1 ratio, there being no destruction of the compound.

B. cereus β-lactamase II, an enzyme with activity against cephalosporins, was not readily inhibited by β-bromopenicillanic acid and it was stated that both α and β epimers were in fact substrates for this enzyme. The β-lactamases of *Staph. aureus* and *E. coli* were inhibited, although significantly higher concentrations were required to give high levels of inhibition.

No antibacterial data have been reported for the β-bromo compound, but it seems reasonable

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to suppose that enhancement of the activity of a labile penicillin would be expected in those cases where the β-lactamase was readily inhibited.

Independently, Knott-Hunziker *et al.* (1979*a*) have also shown that *B. cereus* β-lactamase I is inhibited by β-bromopenicillanic acid. Using a preparation containing 5% of the β-epimer, they observed 50% inactivation of 0.1 μM enzyme by 1.0 μM β-epimer in 1 min at pH 7 and 30 °C and determined a rate constant of 10^6 M^{-1} s^{-1}. In a more recent paper, Knott-Hunziker *et al.* (1979*b*) have shown that the compound reacts with serine 44 in the enzyme and is possibly converted to a 2,3-dihydro-1,4-thiazine.

11. Conclusion

In recent years there has been a considerable expansion of effort devoted to the search for new β-lactamase inhibitors, stimulated by the continuing problems of β-lactamase-mediated resistance. This work has led to the discovery of compounds such as clavulanic acid which, unlike the earlier competitive inhibitors, progressively inactivate the enzyme. These substances are undergoing evaluation as potentiators of the antibacterial activity of established β-lactam antibiotics against resistant bacteria. The search for β-lactamase inhibitors in microbial culture fluids has proved to be a useful indirect way of detecting new antibacterial substances such as the olivanic acids, which initially were at concentrations too low to be detected by their antibacterial properties. The availability of inhibitors that react at the catalytic centre of the enzyme has considerably stimulated research on the structure of β-lactamase.

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