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STRUCTURE-ACTIVITY RELATIONSHIPS AMONGST β-LACTAMASE INHIBITORS

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Using a variety of β-lactamases including those from *Escherichia coli* (TEM-1), *Enterobacter cloacae* P99 and *Staphylococcus aureus* the inhibition profiles (I_{50} values) were determined for various groups of compounds including penicillins, penicillanic acid derivatives (sulphone and β-halo substitutions), olivanic acids and clavulanic acid derivatives including substituted ethers and amines. Some of the latter compounds had higher activity than clavulanic acid with and without preincubation of enzyme with inhibitor but they still had poor activity against the P99 enzyme. Improvements in activity against Class I cephalosporinases were obtained with some derivatives of clavulanic acid but this was usually achieved at the expense of activity against clavulanate susceptible β-lactamases.The olivanic acids had the highest activity against the widest range of β-lactamases.

KEY WORDS: β-lactamase inhibitors, clavulanic acid derivatives, β-lactamases, structure-activity, penicillanic acids, microbial metabolites.

INTRODUCTION

Many compounds have been shown to have β-lactamase inhibitory activity but by far the most frequently occurring structural feature in these substances is the β-lactam ring. This ring occurs in penicillins and cephalosporins and its hydrolysis by β-lactamase is the chief mechanism of bacterial resistance to these compounds. It would seem that the β-lactam ring of inhibitors mimics the β-lactam ring of substrates and fits closely into the catalytic centre of the enzyme. In this position it can either react with the enzyme to form a catalytically inactive derivative or simply fill the catalytic site and hinder the access of substrate molecules. In the latter situation the compound would have to be stable to β-lactamase action to be an effective inhibitor.

The first compounds to be found with specific β-lactamase inhibitory activity were indeed β-lactamase stable penicillins such as methicillin and cloxacillin, and cephalosporins, for example cephalosporin C and cefoxazole (see review by Cole[1]). Later a variety of microbial metabolites were discovered which were potent inhibitors of a wide range of β-lactamase types. The first of these were the olivanic acids and clavulanic acid.[2] Since these initial discoveries further microbial metabolites, penicillanic acid derivatives and penicillin sulphones have been shown to be potent broad spectrum inhibitors of β-lactamase. These developments have been the subject of recent reviews.[3–7] Some of these compounds have been clinically evaluated and clavulanic acid, potassium salt plus amoxycillin (Augmentin‡) is in clinical use.

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‡Augmentin is a trademark of Beecham Group p.l.c.

The present paper will discuss the structure-activity relationships within various classes of compound including naturally occurring substances and is based on information first presented orally at meetings of the Paul Ehrlich Society held at Bad Honnef in Germany in 1982 and at the European Symposium on Bio-organic Chemistry, Gregynog, Wales, 1983.

METHODS

I_{50} Determinations

The inhibitory activity of the compounds against various β-lactamases was measured by determining an I_{50} value, which is the concentration of inhibitor giving 50% inhibition of substrate hydrolysis under defined test conditions. The values were determined with and without preincubation of the inhibitor with the enzyme. When preincubation was used the inhibitor was allowed to interact with the enzyme for either 5 or 15 minutes at pH 7.3 and 37°C before adding substrate to measure residual enzyme activity. When the preincubation step was omitted, enzyme was usually added to premixed inhibitor and substrate. For inhibitors which progressively inhibit the β-lactamase and form an acyl-enzyme with some degree of stability, the I_{50} values are lower with preincubation in comparison to those obtained without preincubation. Compounds giving similar I_{50} values in the two incubation modes are usually acting in a simple competitive fashion. The activity of progressive inhibitors in the presence of substrate (without preincubation) can also provide a rough guide to their ability to compete with substrate for the enzyme active site. I_{50} values are of course only constant under defined test conditions being influenced by length of preincubation, by the type of substrate and its concentration. In some cases the results have been obtained using benzylpenicillin or ampicillin as the substrate but the majority of I_{50} values were obtained with and without a 5 minute preincubation at pH 7.3 and 37°C before addition of the chromogenic substrate nitrocefin (250 μg/ml). Residual substrate was then measured after a further 5 minute reaction period. These methods have been described and discussed in detail by Reading and Farmer.[8]

Substrate Protection Curves

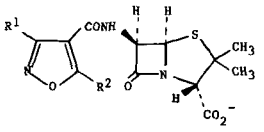
The results in Figure 1 were obtained by following amoxycillin hydrolysis at 37°C by measuring fall in absorbance at 250 nm using a Pye Unicam 8200 recording spectrophotometer as described previously.[8] Reactions of enzyme and amoxycillin with and without 10 μg/ml of the inhibitors were carried out in 1 cm silica cuvettes with 0.05 M sodium phosphate buffer at pH 7.3.

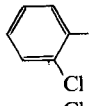
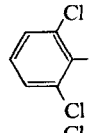
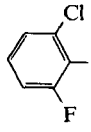
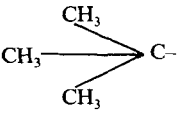
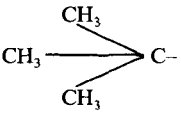
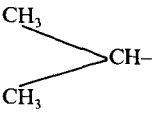
RESULTS AND DISCUSSION

Penicillins, Penicillanic Acid Derivatives and Cephalosporins as β-Lactamase Inhibitors

The structure of the side chain in the 6-position of penicillins appears to have a marked influence on whether the penicillin is a good substrate or an inhibitor of the β-lactamase. Isoxazolyl penicillins such as oxacillin have inhibitory activity against certain β-lactamases, for example the Richmond and Sykes Class I cephalosporinase

TABLE I
Inhibition of the 'Sabath' type β-lactamase of *Pseudomonas aeruginosa* by isoxazolyl penicillins



R^1	R^2		I_{50} value (μg/ml)[a]
	CH_3–	(oxacillin BRL 1400)	0.50
	CH_3–	(cloxacillin BRL 1621)	0.30
	CH_3–	(dicloxacillin BRL 1702)	0.10
	CH_3–	(flucloxacillin BRL 2039)	0.13
	CH_3–	(BRL 1916)	0.04
		(BRL 3249)	0.012

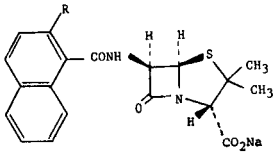
[a]Concentration giving 50% inhibition of hydrolysis of 10 μg/ml benzylpenicillin (no preincubation).

produced by *Pseudomonas aeruginosa*. The results in Table I, which were obtained without preincubation of enzyme and inhibitor, show that introduction of halo substituents in the phenyl ring improved the inhibitory activity, the I_{50} values being lower for dicloxacillin and flucloxacillin. Replacing the phenyl by tertiary butyl (BRL 1916) resulted in a further decrease in I_{50} value and the additional replacement of the methyl by isopropyl (BRL 3249) gave an even lower value.

Unlike the isoxazolylpenicillins, alkoxy substituted penicillins can have good inhibitory activity against the plasmid mediated TEM type of β-lactamase and also the enzyme produced by *Klebsiella pneumoniae*. 1-Naphthyl penicillin was a good substrate for the TEM enzyme produced by *Escherichia coli* B11 but introduction of a methoxy group at position 2 made it an inhibitor as shown in Table II.

Extending the length of the alkoxy side chain improved the inhibitory action, the best compound in the series was BRL 1437 which has a 2-isopropoxy-1-naphthyl side chain and behaves as a competitive inhibitor (Cole *et al.*[9]). BRL 1437 has activity against several β-lactamases but it does not inhibit the staphylococcal enzyme to which it is stable (see Table VII). In this respect it resembles the isoxazolyl penicillins which also do

TABLE II
Inhibition of TEM-1 β-lactamase of *E. coli* B11 by alkoxynapthyl penicillins



R		I_{50} value (μg/ml)[a]
–H	(BRL 1371)	A substrate
$-OCH_3$	(BRL 1336)	0.21
$-OCH_2CH_3$	(BRL 1383)	0.06
$-OCH_2CH_2CH_3$	(BRL 1389)	0.04
$-OCH(CH_3)_2$	(BRL 1437)	0.005
$-OCH_2CH_2CH_2CH_3$	(BRL 1396)	0.06
[Methicillin	(BRL 1241)	0.33]

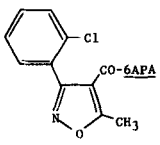
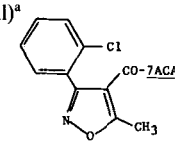
[a]Concentration giving 50% inhibition of hydrolysis of 10 μg/ml ampicillin (no preincubation).

not inhibit staphylococcal β-lactamase at low concentrations although they are stable.

The nucleus to which the side chain is attached also contributes to the level and spectrum of inhibitory activity. This is illustrated in Table III using cloxacillin and the cephalosporin, cephoxazole which has the same side chain. A much higher substrate concentration was used in this experiment and hence the cloxacillin I_{50} value for the β-lactamase of *P. aeruginosa* A is higher than shown in Table I. In recent years most of the new cephalosporins which have improved stability to β-lactamase have been investigated for their β-lactamase inhibitory action. Cephalosporins such as cefuroxime, ceftizoxime, cefonicid, ceftriaxone, and 7-α-methoxy substituted cephalosporins such as cefoxitin, cefotetan and moxalactam (oxacephem) have been shown to have good inhibitory activity against cephalosporin hydrolysing β-lactamases such as that produced by *Enterobacter cloacae* (see review[4]). These compounds have very high affinity for this type of β-lactamase and they are essentially competitive inhibitors but very slow rates of hydrolysis are demonstrable and hence they can also be termed competitive or inhibitory substrates.[7]

Sulphones of certain penicillins for example methicillin, have been reported by Fisher *et al.*[10] to have β-lactamase inactivating activity although they are poor synergists (P. Hunter, Beecham Pharmaceuticals unpublished data) but as can be seen from Table IV methicillin sulphone has poorer inhibitory activity than the sulphone of penicillanic acid, that is, the compound having no acylamino side chain in the 6-position. Penicillanic acid sulphone (Pfizer CP 45899, sulbactam) was first described by English *et al.*[11] to have β-lactamase inhibitory activity. As shown by our data in Table IV, it inhibited the β-lactamases of both Gram-positive and Gram-negative bacteria but the parent penicillanic acid (BRL 4178) had no such inhibitory activity. The 6-α-amino penicillanic acid sulphone BRL 26629 had relatively good activity against the staphylococcal β-lactamase but unlike CP 45899 was very poor against the others. The 2-β-chloromethyl penicillanic acid sulphone (BL-P 2013) has been reported by

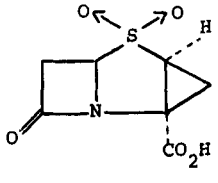
TABLE III
β-Lactamase inhibition spectra of cloxacillin and cephoxazole

Source of β-lactamase	I_{50} (μg/ml)[a]	
		
	Cloxacillin	Cephoxazole
E. coli K12 TEM-1	> 40	> 40
E. coli JT414	0.14	4
P. mirabilis C889	> 40	> 40
K. pneumoniae (*aerogenes*) A (NCTC 418)	> 40	11
Ent. cloacae (NCTC 10005)	2.5	0.9
P. aeniginosa A	2.2	0.4
Staph. aureus MB9	> 40	> 40

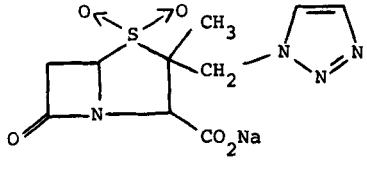
[a]Concentration giving 50% inhibition of hydrolysis of 1 mg/ml benzylpenicillin (no incubation).
6APA = 6-aminopenicillanic acid.
7ACA = 7-aminocephalosporanic acid.

Gottstein *et al.*[12] to have β-lactamase inhibitory activity similar to that of CP 45899, although the data presented by these authors indicated that both compounds were poorer than clavulanic acid at protecting a cephalosporin from the destructive action of several β-lactamases.

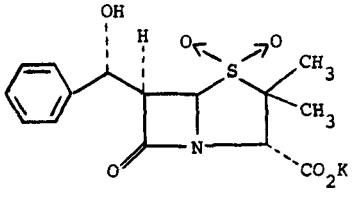
The cyclopropyl analogue of sulbactam (I in Figure 1) ([2,3]-β-methylene penicillanic acid sulphone)[13] has also been shown to inhibit β-lactamases although the reported I_{50} values for staphylococcal and TEM enzymes remained inferior to those obtained



I



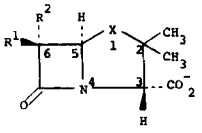
II YTR 830

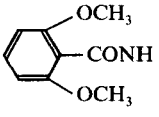


III

FIGURE 1 Structures of penicillanic acid sulphone derivatives I, II and III.

TABLE IV
Substituted penicillanic acid derivatives as β-lactamase inhibitors



	R^1 (β)	R^2 (α)	X	I_{50} value (μg/ml)[a] for β-lactamases from: *Staph. aureus* (Russell)	*E. coli* JT4 (TEM-1)	*Ent. cloacae* (P99)
BRL 4178	H	H	S	> 50	> 50	> 50
Penicillanic acid sulphone (sulbactam)	H	H	SO_2	1.6	1.4	6
BRL 26629	NH_2	H	SO_2	0.4	40	50
Methicillin sulphone		H	SO_2	> 50	16	20
BRL 25959	Cl	H	S	0.3	50	> 50
BRL 25214	Br	H	S	1.5	0.15	1.5
BRL 28701	I	H	S	0.7	0.06	5.5
BRL 25248	H	Cl	SO_2	50	30	30
BRL 26624	Cl	H	SO_2	50	13	> 50
BRL 28712	Br	H	SO_2	4	1.5	> 50

[a]5 minute preincubation of β-lactamase with inhibitor before adding nitrocefin as substrate (250 μg/ml).

for sodium clavulanate. The 2-triazolyl methyl derivative of penicillanic acid sulphone (II in Figure 1), YTR 830[14] has been reported to have β-lactamase inhibitory activity and to be as effective as clavulanate at reducing the minimum inhibitory concentrations of aminopenicillins. Recently (1′*R*,6*R*)-6-(1′-hydroxy)benzylpenicillanic acid SS-dioxide (III in Figure 1) was described[15] but this compound only inhibited chromosomal cephalosporinases such as those produced by *E. coli* and *P. aeruginosa*.

Penicillanic acids with a halogen substituent in the 6-β-position (EP 0 013 617), that is projecting above the plane of the β-lactam ring, have β-lactamase inhibitory activity. The first compound reported to have activity was β-bromopenicillanic acid, the α-bromo epimer being inactive (Pratt and Loosemore;[16] Knott-Hunziker *et al.*[17]). The 6-β-iodo (Pfizer compound UK 38006) and 6-β-chloro penicillanic acids (Kemp *et al.*[18] and Daehne[19]) have also been reported to have β-lactamase inhibitory activity. The relative activity of these compounds as determined by our methods is shown in Table IV. The β-bromo and β-iodo compounds had activity against both staphylococcal and TEM β-lactamases while the β-chloro had significant activity against only the

staphylococcal enzyme. The non-halogenated penicillanic acid (BRL 4178) was inactive against all three β-lactamases tested. The effect of the presence of substrate on the activity of some penicillanic acid derivatives is shown in Table V where I_{50} values both with and without preincubation are listed.

The 6-α-chloro penicillanic acid sulphone was reported by Cartwright and Coulson[20] to inhibit staphylococcal β-lactamase. We found this compound to be less active than penicillanic acid sulphone against several β-lactamases including the staphylococcal enzyme (Table IV). Compared with 6-α-chloro penicillanic acid sulphone, the 6-β-chloro isomer was somewhat more active against the TEM β-lactamase but 6-β-bromo penicillanic acid sulphone was very much more active against both the TEM and staphylococcal β-lactamases (Table IV).

Further 6-substituted penicillanic acid derivatives have followed the 6-β-halo compounds. The 6-acetylmethylene penicillanic acid (RO 151903)[21] has similar activity to sulbactam against the Class I β-lactamases and was reported to be significantly more active than clavulanic acid against plasmid mediated β-lactamases, as well as Class IV and staphylococcal β-lactamases when preincubated with cell-free enzyme preparations. However, this activity is less marked in the presence of substrate (without preincubation) as shown in Table V. The *in vivo* and *in vitro* antibacterial results for this compound, in combination with β-lactam antibiotics,[22] appear disappointing if one compares them with the reported inhibitory activity of the compound against cell-free β-lactamases. The inhibition of TEM β-lactamase by a similar compound, 6-(methoxymethylene)penicillanic acid, has been described recently.[23] The *Z* isomer was active but the *E* isomer did not interact with the enzyme.

Olivanic Acids, Clavulanic Acid and Other Microbial Metabolites as β-Lactamase Inhibitors

Some of the most active β-lactamase inhibitors are members of the olivanic acid family of antibiotics (Table VI) which were originally detected by their β-lactamase inhibitory properties.[2] Like the penicillins and cephalosporins these compounds contain a β-lactam ring system but it is fused to an unsaturated 5-membered ring containing carbon in the place of the sulphur atom of the penicillin molecule. In view of the structure of this nucleus, compounds of this type have been called carbapenems. Included in this group, in addition to the olivanic acids (Beecham), are the thienamycins (Merck), the PS5 group of compounds (Sanraku Ocean), the carpetimycins (Kowa), C19393 (Takeda), the asperenomycins and the pluracidomycins (Shionogi). All of the substances have alkyl or substituted alkyl side chains in the 6-position and a substituted thio side chain at position 2 in various states of oxidation. All of these substances are notable for their antibiotic activity but they also have various degrees of β-lactamase inhibitory activity.[3–7]

Examples of olivanic acids with high β-lactamase inhibitory activity are those with a sulphated 1-hydroxyethyl side chain in the 6-position, namely BRL 17880, BRL 13902 and BRL 4550. These compounds have activity against a wide range of β-lactamases as shown in Table VI, the sulphoxide BRL 4550 being the most active against the TEM and *K. pneumoniae* β-lactamases.

The marked effect of introducing the sulphate ester group can be seen by comparison of the inhibitory activity of BRL 23380 (free hydroxy) with BRL 17880 (sulphated); these compounds have the same side chain in the 2-position and the same stereochemistry, namely 5*R*, 6*R*, 8*S*, the 5,6 protons being in the cis configuration. The effect

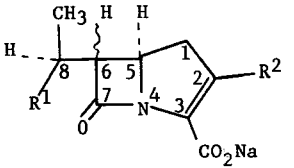
TABLE V

β-Lactamase inhibitory activity of sulbactam, 6-β-halopenicillanic acids and 6-acetylmethylene penicillanic acid compared with clavulanic acid, with and without pre-incubation

	I_{50} (μg/ml)[a] with (+) and without (−) a 5 minute preincubation									
	Sulbactam		6-β-Bromo-penicillanic acid		6-β-Iodo-penicillanic acid		6-Acetyl-methylene penicillanic acid		Clavulanic acid	
β-Lactamase	+	−	+	−	+	−	+	−	+	−
Enterobacter cloacae P99	5.8	> 50	1.5	> 50	5.5	> 50	10	> 50	110.0	> 4000
Proteus mirabilis C889	–	–	–	–	–	–	0.007	15.0	0.015	10.0
K. pneumoniae E70	5.5	20	0.07	12.5	0.014	12.0	0.015	5.2	0.007	0.63
TEM-1	1.4	2.0	0.15	8.0	0.06	5.5	0.005	2.5	0.06	0.7
Staphylococcus aureus (Russell)	1.6	> 50	1.5	50	0.7	> 50	0.15	> 50	0.03	75.0

[a]Concentration required to give 50% protection of 250 μg/ml nitrocefin substrate after a 5 minute reaction time.[8]

TABLE VI
Olivanic acids – β-lactamase inhibitors produced by *Streptomyces olivaceus*



	R¹	5,6 stereochem.	R²	I_{50} values (μg/ml)[a] for β-lactamases from: *Staph aureus* (Russell)	*E. coli* JT4 TEM-1	*K. pneumoniae* E70	*Ent. cloacae* P99
BRL 22380	OH	cis	$SCH_2CH_2NHCOCH_3$	0.1	15	10	0.03
BRL 22381	OH	trans	$SCH_2CH_2NHCOCH_3$	5	0.03	0.01	0.2
BRL 17880	OSO_3Na	cis	$SCH_2CH_2NHCOCH_3$	0.02	0.02	0.006	0.001
BRL 13902	OSO_3Na	cis	$SCH=CHNHCOCH_3$	0.01	0.006	0.003	0.0008
BRL 4550	OSO_3Na	cis	O ↑ $SCH=CHNHCOCH_3$	0.006	0.001	0.0003	0.001

[a]5 minute preincubation of β-lactamase with inhibitor before adding nitrocefin substrate (250 μg/ml).

of having the 5*R*, 6*S*, 8*S* stereo-chemistry, that is the 5,6 protons in the trans configuration, is seen when comparing the results for the non-sulphated compounds BRL 22380 with BRL 22381 (Table VI). There was a marked increase in inhibitory activity for the trans compound BRL 22381 against two of the β-lactamases, activity against TEM being associated with increased β-lactamase stability. However, the activity against the *Enterobacter* and staphylococcal β-lactamases was reduced.

Another group of microbial metabolites containing sulphate ester groups and having potent β-lactamase inhibitory activity is represented by the sulphated macrocyclic lactones izumenolide[24] and dotriacolide.[25] These substances are produced by strains of *Micromonospora*. They have low I_{50} values against the β-lactamases of Gram-negative bacteria but not that of *Staphylococcus aureus*. They are reported to be highly toxic to mice when administered parenterally. Structure-activity studies have shown for izumenolide that the β-lactamase inhibitory action is largely associated with the acidic sulphate ester groups.[26] Two β-lactamase inhibitors belonging to the alkylbenzene disulphate group of substances, namely M-4854 I and II were found by Yaginuma *et al.*[27] These substances were detected in a culture of *Chaetomella raphigera* by their inhibitory activity against the β-lactamase of *Citrobacter freundii*. All of these sulphated compounds seem to act by progressively inactivating the β-lactamase.

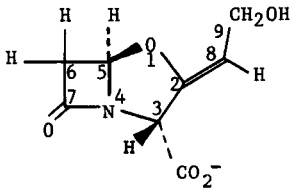
Certain monocyclic β-lactam-containing antibiotics for example the monobactams[28] have been reported as being produced by various bacteria. These compounds have a sulphonic acid group attached to the ring nitrogen. One of these substances, monobactam VIII, produced by *Agrobacterium radiobacter* had pronounced inhibitory activity against the β-lactamase of *Ent. cloacae* P99 but little activity against others. Comparison of various members of the series suggested that the structure of the acylamino side chain affected the β-lactamase inhibitory activity while the 3-α-methoxy substituent was responsible for the high stability to β-lactamase.[28] In the absence of the 3-α-methoxy group monobactams are substrates for β-lactamase but introduction of a 3-β-aminothiazolyl oxime side chain as in aztreonam, restores β-lactamase stability and inhibitory activity against some enzymes.[29] The synthetic monocyclic β-lactam, 3-*p*-nitrophenyl acetamido-4-phenylazetidin-2-one has been shown to have inhibitory activity against staphylococcal β-lactamase.[30]

The β-lactamase inhibitor which has been most extensively studied is clavulanic acid produced by *Streptomyces clavuligerus*.[2] The bicyclic nucleus of this compound resembles that of penicillin but with an oxygen atom in place of the sulphur atom (see Table VII). Unlike penicillins the compound has no acylamino side chain in the 6-position. The β-lactamase inhibitory spectrum of this compound is shown in Table VII alongside that for one of the best β-lactamase-inhibiting penicillins, BRL 1437. As can be seen, clavulanic acid had much improved activity against a number of enzymes including that produced by *Staph. aureus*. For most β-lactamases the I_{50} values obtained for BRL 1437 were similar irrespective of whether or not the compound was preincubated with the enzyme indicating that the compound was acting as a competitive inhibitor. On the other hand the I_{50} values for clavulanic acid were much lower when the compound was preincubated with the enzyme before addition of the substrate (Table V). This illustrates the ability of clavulanic acid to progressively inactivate many β-lactamases.

Structure-Activity Relationships for Derivatives of Clavulanic Acid

Clavulanic acid has a β-lactam ring and is a subtrate analogue. Its mechanism of action relies on the formation of an acyl-enzyme intermediate which is followed by an

TABLE VII
Clavulanic acid – a β-lactamase inhibitor produced by *Steptomyces clavuligerus*



Source of β-Lactamase	β-Lactamase Class	I_{50} values (μg/ml)[a] for Clavulanic Acid (BRL 14151)	2-Isopropoxy-1-naphthyl penicillin (BRL 1437)
E. coli K12 TEM-1	TEM	0.08	0.2
E. coli JT414	I	50	16
P. mirabilis C889	II	0.01	13
K. pneumoniae (*aerogenes*) A	IV	0.01	0.9
Staph. aureus MB9	Gram-positive	0.02	< 50

[a]15 minute preincubation of β-lactamase with inhibitor before adding 1 mg/ml benzylpenicillin as substrate.

elimination reaction with resultant cleavage of the oxazolidine ring. Tautomerisation of the initial imine acyl intermediate to the enamine is believed to produce an acyl-enzyme of moderate stability whilst further covalent interactions at the active site can also result in permanent inactivation as reported for TEM and other β-lactamases.[6,7]

Certain modifications to clavulanic acid (Table VIII) completely removed activity against all of the β-lactamases listed in Table V. Dihydroclavulanate produced by

TABLE VIII
Clavams with no β-lactamase inhibitory activity

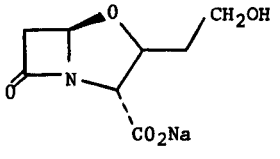
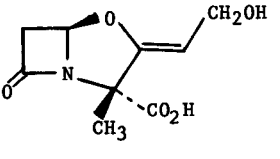
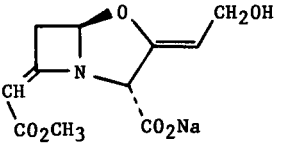
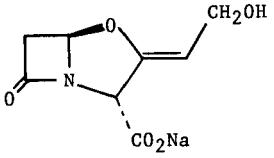
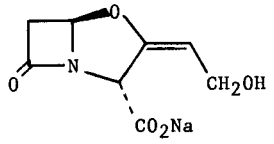
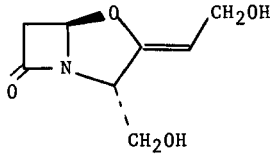
	Dihydroclavulanate (BRL 18905)
	3-β-Methyl clavulanic acid (BRL 21478)
	BRL 25967

TABLE IX
Clavams with reduced β-lactamase inhibitory activity

	I_{50} (μg/ml)[a] β-lactamase from	
	TEM-1	*Staph. aureus* (Russell)
 Sodium clavulanate	0.08	0.07
 Sodium isoclavulanate (BRL 18463)	1.0	0.6
 3-Hydroxymethyl clavulanate (BRL 22113)	0.9	1.2

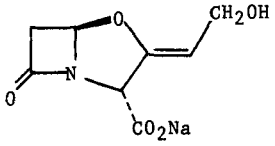
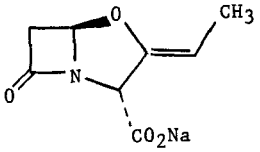
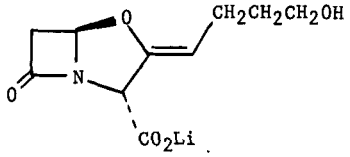
[a] I_{50} value determined with 15 min preincubation of enzyme and inhibitor before adding benzylpenicillin substrate (1 mg/ml).

reduction of the exocyclic double bond is inactive, illustrating the importance of the 2-ethylidene side chain. Substitutents other than a hydrogen atom in the β-position at C_3 also abolishes activity as illustrated in Table VIII by 3-β-methylclavulanate. It seems likely that β-substitution at C_3 prevents interaction at the active site. Replacement of the β-lactam carbonyl by a methoxy carbonyl methylene substituent, not surprisingly also removes inhibitory activity.

In Table IX I_{50} values obtained after preincubation with TEM and staphylococcal β-lactamase illustrate the reduction in activity that usually occurs with iso-clavulanates (*E*-isomer). Replacement of the 3-carboxyl by hydroxymethyl also leads to a general reduction of activity and suggests that the carboxyl function may well be important for binding at the active site of clavulanate-susceptible β-lactamases.

Some modifications have little effect on activity (Table X). The I_{50} values for deoxyclavulanate are virtually identical to those obtained with clavulanic acid. These results enabled us to rule out mechanisms involving the hydroxyl as a leaving group and this was substantiated by more detailed studies on the interaction of deoxy-clavulanate with TEM β-lactamase.[31] Clavulanic acid homologues with extended alkyl side chains (Table X) such as 2-hydroxybutylidine clavam also appear very similar to the parent compound.

TABLE X
Clavams with inhibitory activity similar to clavulanic acid

	I_{50} (μg/ml) with preincubation[a]	
	TEM-1	*Staph. aureus* (Russell)
	0.08	0.1
Sodium clavulanate		
	0.08	0.1
Sodium deoxyclavulanate (BRL 18015)		
	I_{50} (μg/ml) with preincubation[b]	
Sodium clavulanate	0.06	0.03
	0.1	0.02
Lithium 2-hydroxybutylidene clavam (BRL 33437)		

[a]15 minute preincubation before addition of benzylpenicillin substrate (1 mg/ml).
[b]5 minute preincubation before addition of nitrocefin substrate (250 μg/ml).

A characteristic of the spectrum of activity of clavulanic acid is its poor activity against Class I cephalosporinases some of which have also been categorised as Class C β-lactamases[7] on the basis of their structure and mechanism of action. These enzymes however, are slowly inactivated by high concentrations of clavulanic acid. This type of enzyme is exemplified in Table XI by the β-lactamases of *Ent. cloacae* P99 and *P. aeruginosa*. It is interesting that alteration of the 3-carboxyl group of clavulanate by formation of the amide, by esterification or by its complete removal (3-decarboxyclavam) results in dramatic improvement in the I_{50} values obtained for these cephalosporinases (Table XI) but generally causes a decrease in activity against TEM and staphylococcal enzymes (Class A β-lactamases). It would appear that the carboxyl actually hinders binding with these Class C β-lactamases. This is in contrast to penicillins with normal 3-carboxyl functions which generally have high affinity for this type of β-lactamase, although V_{max} values are generally low.

TABLE XI
Clavams with a broader spectrum of β-lactamase inhibitory activity

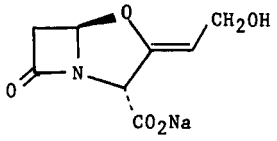
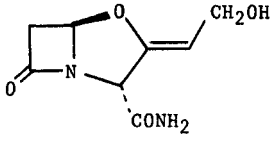
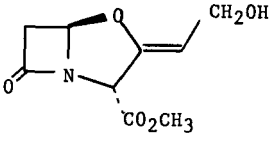
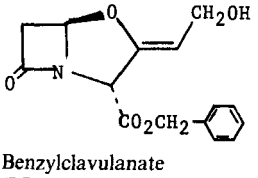
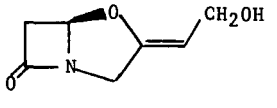
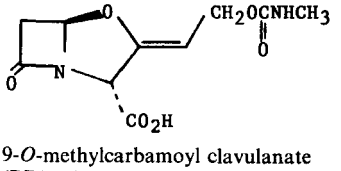
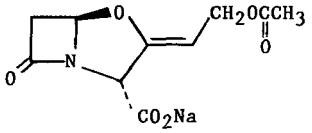
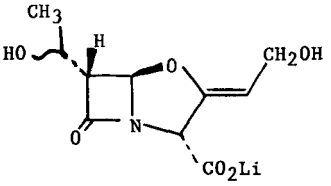
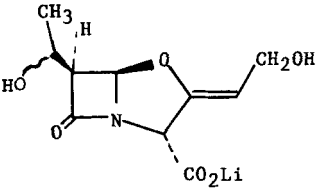
	I_{50} (μg/ml) with a 5 minute preincubation[a]			
	Ent. cloacae P99	*P. aeruginosa*	TEM-1	*Staph. aureus* (Russell)
 Sodium clavulanate	110	> 100	0.06	0.03
 Clavulanamide (BRL 19984)	12	9	40	2.4
 Methyl clavulanate (BRL 15591)	2.2	1.1	3.4	16
 Benzylclavulanate (BRL 16557)	9	–	0.2	3.6
 3-Decarboxyclavam (BRL 25088)	8	0.3	0.1	3
 9-*O*-methylcarbamoyl clavulanate (BRL 18716)	0.45	1.0	2.5	1.5

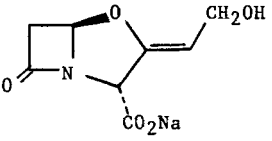
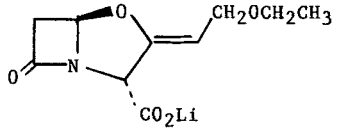
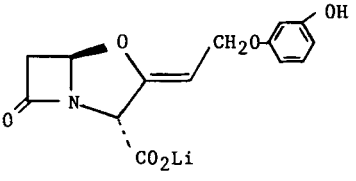
TABLE XI continued

	I_{50} (μg/ml) with a 5 minute preincubation[a]			
	Ent. cloacae P99	*P. aeruginosa*	TEM-1	*Staph. aureus* (Russell)
 9-*O*-acetylclavulanate (BRL 18714)	0.4	1.4	–	0.04
 6-α-(1-Hydroxyethyl)clavulanate (BRL 23546)	2.3	30	> 50	> 50
 6-β-(1-Hydroxyethyl)clavulanate (BRL 23547)	< 0.04	3.7	0.23	5.4

[a] I_{50} values determined using nitrocefin substrate (250 μg/ml).

Penicillins have a 6-acylamino side chain which is absent in clavulanic acid. Substitution of clavulanic acid at position-6 with retention of the 3-carboxyl group can improve activity against the cephalosporinases. This is illustrated in Table XI by 6-β-hydroxyethyl clavulanate, the 6-α-derivative being less active. Alternatively some position-9 substituents can also provide improved inhibition of *Ent. cloacae* and pseudomonal β-lactamases. The 9-*O*-methylcarbamoyl and 9-*O*-acetyl derivatives both show marked improvement over clavulanic acid (Table XI). The 2-ethylclav-2-em carboxylate, i.e. the endocyclic double bond isomer of clavulanic acid, has also been reported[32] to have significant activity against P99 β-lactamase. It is possible therefore to improve the inhibitory activity of clavulanic acid against the cephalosporinases either by removing or blocking the 3-carboxyl group or by retaining the carboxyl group and introducing substituents at either position-9 or -6. These improvements however, are usually at the expense of activity against clavulanic acid susceptible

TABLE XII
Examples of clavams (ethers) having β-lactamase inhibitory activity

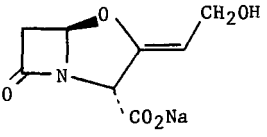
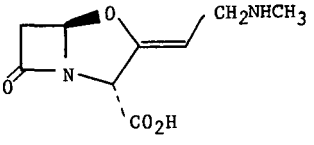
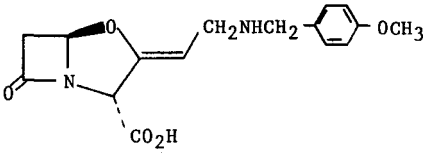
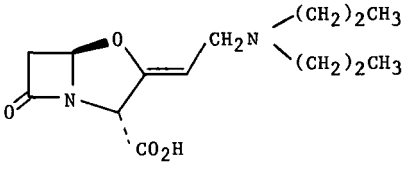
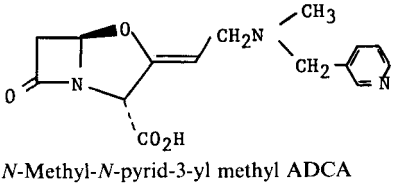
Clavam	I_{50} (μg/ml)[a] with (+) and without (−) 5 min preincubation					
	Ent. cloacae P99		TEM-1		*Staph. aureus* (Russell)	
	+	−	+	−	+	−
 Sodium clavulanate	110	> 4000	0.06	0.6	0.03	80
 Lithium 9-*O*-ethylclavulanate (BRL 22101)	60	> 100	0.05	0.25	0.03	> 50
 Lithium 9-*O*-*m*-hydroxyphenyl clavulanate (BRL 25003)	23	> 100	0.02	0.06	0.01	1.0

[a] I_{50} values determined using nitrocefin substrate (250 μg/ml).

enzymes which is perhaps not surprising in view of the known differences between Class A and Class C β-lactamases.[7]

Some derivatives of clavulanic acid have the same spectrum but have improved activity against clinically important β-lactamases such as TEM and staphylococcal enzymes. In Tables XII and XIII I_{50} values have been determined both with and without preincubation of the enzyme with the inhibitor. The I_{50} values obtained without preincubation when compared to the value with preincubation gives some idea of the ability of the inhibitor to compete with the substrate for the active site of the enzyme. Table XII shows that ether derivatives such as the 9-*O*-ethyl and 9-*O*-hydroxyphenyl compounds are generally very similar to clavulanic acid when comparing I_{50} values obtained with preincubation. However, consistent improvements in the I_{50} values against TEM β-lactamase in the competitive situation (without preincubation) can be seen for these two ethers and suggests an increase in the affinity of these compounds for the TEM β-lactamase. Aromatic ethers also show a marked improvement against staphylococcal β-lactamase when tested in the presence of

TABLE XIII
Examples of clavams (amines) having improved β-lactamase inhibitory activity

Clavam	I_{50} (μg/ml)[a] with (+) and without (−) 5 min preincubation					
	Ent. cloacae P99		TEM-1		*Staph. aureus* (Russell)	
	+	−	+	−	+	−
 Sodium clavulanate	110	> 4000	0.06	0.6	0.03	80
 9-*N*-Methyl ADCA (BRL 25368)	> 50	> 50	0.007	0.09	0.03	25
 9-*N*-*p*-Methoxybenzyl ADCA (BRL 23654)	20	> 50	0.001	0.02	0.002	1.2
 9-*N*-Dipropyl ADCA (BRL 19989)	10	> 50	0.007	0.04	0.02	10.0
 *N*-Methyl-*N*-pyrid-3-yl methyl ADCA (BRL 25197)	3.5	> 50	0.006	0.04	0.007	2.5

[a] I_{50} values determined using nitrocefin substrate (250 μg/ml).
ADCA = amino deoxyclavulanic acid.

substrate (without preincubation). As shown in Table XII sodium clavulanate has an I_{50} of 80 μg/ml in this system compared to 1.0 μg/ml for the hydroxyphenyl ether.

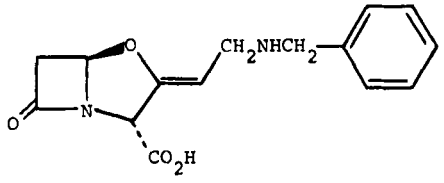
The amine derivatives (9-aminodeoxyclavulanates) show significant improvements in their I_{50} values (Table XIII) both with and without preincubation compared with sodium clavulanate. This applied to both secondary and tertiary amines when tested against clavulanate susceptible β-lactamases. As seen in the ether series, aromatic or heterocyclic rings in the side chain lead to significant improvements against staphylococcal β-lactamase but these substituents have little effect against TEM β-lactamase where I_{50} values are good for both alkyl and aromatic amines.

More detailed studies on the interaction of ether derivatives with TEM and staphylococcal β-lactamase have shown that these compounds appear to behave in a very similar way to clavulanic acid (T. Farmer and C. Reading, unpublished results). With TEM β-lactamase and simple alkyl ethers, improvements in the K_i value can be seen but under saturating conditions the rate of irreversible inactivation, the formation of a transient complex, and the turnover number are very similar to results reported for clavulanic acid.[33] Similarly, with staphylococcal β-lactamase the improvements in the activity of ether and amine derivatives are largely due to improvements in affinity. The acyl-enzyme intermediates have similar half lives to that obtained with clavulanic acid (160 min at pH 7.3, 37° C) and like the clavulanate intermediate are acid labile.[34]

The 9-*N*-alkylated derivatives such as BRL 22127 (Figure 1) whose interaction with TEM β-lactamase has been studied in more detail do behave differently from clavulanic acid (T. Farmer and C. Reading, unpublished results). Although a transiently inhibited intermediate of similar stability to that obtained with clavulanic acid is found (Scheme 1) no permanent inactivation occurs. The results in Figure 1 show the effect of clavulanic acid and BRL 22127 on the rate of degradation of amoxycillin by TEM β-lactamase. With clavulanic acid the rate of amoxycillin loss is dramatically reduced in the inital stages of the reaction compared to a control of amoxycillin and enzyme alone. As more of the enzyme is inactivated the rate slows until no further degradation of substrate occurs (enzyme completely inactivated). The amine derivative BRL 22127 provides better protection of the substrate than clavulanic acid over a period of several hours but loss of amoxycillin is constant and never completely ceases. This constant but markedly reduced rate of substrate hydrolysis probably reflects the slow release of enzyme from the transiently inhibited complex (Scheme 1) which is then free to react with substrate or fresh inhibitor. This experiment illustrates the consequences of the different mechanism of action of these compounds, BRL 22127 giving results essentially similar to a competitive inhibitor or inhibitory substrate. The antibacterial synergy obtained when BRL 22127 is combined with a β-lactamase labile antibiotic and tested against TEM producing bacteria is greater than that obtained for an equal concentration of clavulanic acid (P. Hunter, Beecham Pharmaceuticals, unpublished results) and reflects the better inhibitory activity of the derivative. This also illustrates the fact that permanent enzyme inactivation is not an essential prerequisite for successful antibacterial synergy.

Penetration of β-Lactamase Inhibitors into Gram-Negative Bacteria

The results in this paper have shown that a variety of β-lactams of differing structural type have potent inhibitory activity when tested against cell-free β-lactamase. Despite this intrinsic activity many of these inhibitors have proved disappointing when tested as synergists in combination with a β-lactamase labile antibiotic against β-lactamase producing Gram-negative bacteria.



BRL 22127

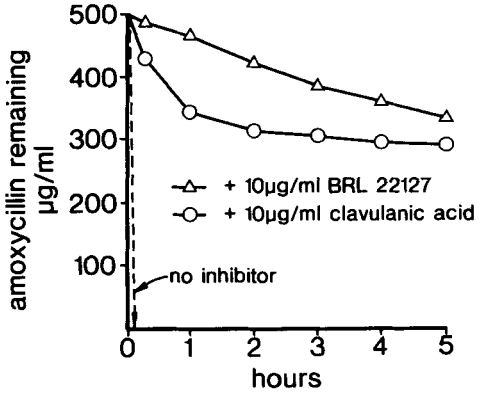
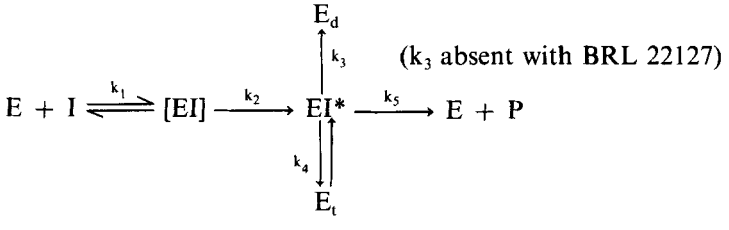


FIGURE 2 A comparison of the effect of BRL 22127 and lithium clavulanate on the hydrolysis of amoxycillin by TEM-1 β-lactamase.



EI* acyl intermediate
E_t transiently stable intermediate
E_d inactivated enzyme

SCHEME 1. Interaction of clavulanic acid with TEM β-lactamase.

Studies[35] on the ability of various inhibitors to inhibit periplasmic β-lactamase in intact cells of a TEM producing strain of *E. coli* go some way to explaining these deficiencies in synergistic activity. The results reproduced in Table XIV illustrate the reduction in activity that can occur when I_{50} values are determined using whole-cell β-lactamase in comparison to cell-free enzyme, having ensured that substrate concentrations in the two systems are similar. These differences reflect the extent to which the outer membrane of *E. coli* prevents the inhibitor from reaching the periplasmic enzyme and a ratio of the I_{50} values can be used as a penetration index. Clavulanic acid, a small hydrophilic molecule, appears to readily inhibit periplasmic

TABLE XIV
The penetration of β-lactamase inhibitors into *E. coli* JT4 (TEM-1)[a]

Inhibitor	I_{50} (μg/ml)		Penetration index (ratio of I_{50} values)
	Cell-free β-lactamase	Whole-cell β-lactamase	
Potassium clavulanate	0.056	0.69	12.3
Sulbactam	0.3	23.0	77.0
β-Bromopenicillanate	0.45	34	76.0
BRL 13902 (an olivanic acid)	0.015	7.0	467.0
BRL 1437 (2-isopropoxy-1-naphthyl penicillin)	0.0032	110.0	34,375.0

[a]Data from Reference 35.

β-lactamase (penetration index = 12). In contrast, the penicillin BRL 1437 which has a hydrophobic side chain and is a potent inhibitor of the cell-free enzyme had a penetration index of 34,000. The sulphated olivanic acids also appear less able to penetrate than clavulanic acid and studies with compounds lacking the sulphate group (T. Farmer and C. Reading, unpublished results) suggest that this strongly acidic function reduced penetration.

CONCLUSIONS

Very many compounds have now been shown to have β-lactamase inhibitory activity. The majority of these are β-lactam-containing bicyclic structures. These inhibitors may be categorised into a number of types. For example those that are purely competitive and reversible, those which have high affinity but are slowly hydrolysed, i.e. competitive or inhibitory substrates, and those that have been termed as "destabilising substrates".[7] The latter describes the interaction of high concentrations of alkoxy and isoxazolylpenicillins with some Gram-positive β-lactamases where conformational changes and acylation of an unidentified site have been implicated. The compounds falling into the above three categories are usually normal penicillins and cephalosporins with acyl amino side chains of varying types and which combine inhibitory activity with a degree of stability to certain β-lactamases. Potent β-lactamase inhibitors such as clavulanic acid, penicillin sulphones and penicillanic acid derivaties are "mechanism based inactivators" where acyl-enzyme formation is followed by an elimination reaction which produces either a relatively stable acyl-enzyme intermediate or which can undergo further covalent interactions at the active site leading to permanently inactivated β-lactamase. The carbapenems such as the olivanic acids are believed to form relatively stable acyl intermediates due to tautomerisation of the Δ^2 pyrroline to the more stable Δ^1 form.[6]

The various types of inhibitors and their mechanisms of action have only been outlined above as they have been reviewed in detail.[5-7] In this paper we have measured the inhibitory activity of a number of inhibitor types by determining I_{50} values under standard conditions, so enabling a direct comparison of their activities. In particular we have considered the activity of various clavams relative to the parent clavulanic acid highlighting the structural changes which lead to loss or reduction of activity, those which have little effect, those which broaden the spectrum to give

improvements over clavulanic acid by inhibiting Class I cephalosporinases and finally those with the same spectrum as clavulanate but with higher activity. Although some improvements in the inhibition of cephalosporinases were noted this usually went hand in hand with a reduction in the activity against enzymes which were well inhibited by clavulanic acid. Clavams with the same spectrum as clavulanic acid but with improved activity were obtained with the ether and amine derivatives. Simple ethers such as 9-*O*-ethylclavulanate gave consistently lower I_{50} values against TEM-1 β-lactamase without preincubation. This suggested an improved affinity for the enzyme and this has been confirmed by K_i determinations. Aromatic ethers and amines showed particularly good activity against staphylococcal β-lactamase in comparison with alkyl ethers and amines, but for TEM these differences were not so marked, all of the compounds showing good activity.

More detailed study of these interactions has shown that simple alkyl ethers apart from having lower K_i values, interact with TEM β-lactamase in an identical fashion to clavulanic acid, the rates of inactivation under saturating conditions being the same and the formation of a transient complex having a similar half life. However, the amines which have been studied do not inactivate TEM-1 β-lactamase but form only a transiently stable complex and rely essentially on their high affinity for the enzyme to ensure protection of a β-lactam substrate. For staphylococcal β-lactamase, with which clavulanate forms only a moderately stable acyl intermediate ($t_{1/2}$ enzyme regeneration = 160 min at pH 7.3 and 37°C), both the amines and ethers give complexes of similar stability to clavulanic acid.

The potent inhibitory activity of the amine derivatives of clavulanic acid is reflected in antibacterial synergy tests. When combined with a β-lactamase labile antibiotic such as amoxycillin, low concentrations of the inhibitor effectively synergise the activity of the penicillin against TEM producing strains of *E. coli*. These results illustrate that permanent inactivation of β-lactamase is not a prerequisite for antibacterial synergy as the amines do not inactivate this enzyme. On the basis of studies[35] on whole cells, an important factor controlling the synergistic activity of a β-lactamase inhibitor appears to be the ability to penetrate the outer membrane of Gram-negative organisms and inhibit periplasmic β-lactamase. Clearly more hydrophobic compounds such as the alkoxy and isoxazolyl penicillins have limited penetrability and strongly acidic olivanic acids are also less able to penetrate than clavulanic acid. However, structural requirements for penetration by various clavams and other classes of inhibitor have yet to be defined.

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