Antimicrobial Agents and Chemotherapy

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Antimicrob. Agents Chemother. **1977, 11(5):852. DOI: 10.1128/AAC.11.5.852.**

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1977, p. 852-857
Vol. 11, No. 5
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Clavulanic Acid: a Beta-Lactamase-Inhibiting Beta-Lactam from *Streptomyces clavuligerus*

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Received for publication 29 December 1976

A novel β-lactamase inhibitor has been isolated from *Streptomyces clavuligerus* ATCC 27064 and given the name clavulanic acid. Conditions for the cultivation of the organism and detection and isolation of clavulanic acid are described. This compound resembles the nucleus of a penicillin but differs in having no acylamino side chain, having oxygen instead of sulfur, and containing a β-hydroxyethylidine substituent in the oxazolidine ring. Clavulanic acid is a potent inhibitor of many β-lactamases, including those found in *Escherichia coli* (plasmid mediated), *Klebsiella aerogenes*, *Proteus mirabilis*, and *Staphylococcus aureus*, the inhibition being of a progressive type. The cephalosporinase type of β-lactamase found in *Pseudomonas aeruginosa* and *Enterobacter cloacae* P99 and the chromosomally mediated β-lactamase of *E. coli* are less well inhibited. The minimum inhibitory concentrations of ampicillin and cephaloridine against β-lactamase-producing, penicillin-resistant strains of *S. aureus*, *K. aerogenes*, *P. mirabilis*, and *E. coli* have been shown to be considerably reduced by the addition of low concentrations of clavulanic acid.

Streptomyces clavuligerus ATCC 27064 (NRRL 3585) has been described as producing several antibiotics structurally related to cephalosporin C, namely, the 3-carbamoyloxymethyl analogue, the 7-methoxy derivative of the latter compound (cephamycin C), and deacetoxy cephalosporin C, as well as penicillin N (8, 9, 12, 16; M. Gorman, M. M. Hoehn, R. Nagarajan, L. D. Boeck, E. A. Presti, J. G. Whitney, and R. L. Hamill, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 11th, Atlantic City, N.J., abstr. 14, p. 7, 1971). During an investigation of the metabolites produced by this culture, a pronounced β-lactamase inhibitory activity was detected in the culture filtrate using a special bioassay procedure based on β-lactamase inhibition (4). The substance responsible for the β-lactamase inhibitory activity was named clavulanic acid (4) and has been shown to have the structure given in Fig. 1 (10). We describe below the detection, isolation, and preliminary information on the β-lactamase-inhibitory properties of clavulanic acid.

MATERIALS AND METHODS

Cultural conditions for *S. clavuligerus*. *S. clavuligerus* ATCC 27064 (NRRL 3585) was grown at 26°C on agar slopes containing 1% Yeatex yeast extract, 1% glucose, and 2% Oxoid agar no. 3, pH 6.8. Mycelium and spores from the slope were used to inoculate flasks containing a seed stage medium consisting of (wt/vol) 1% malt extract (Oxoid), 1% bacteriological peptone (Oxoid), and 2% glycerol. The medium was made up using tap water and adjusted to pH 7.0 with sodium hydroxide solution. Inoculated flasks were shaken for 3 days at 26°C.

Production stage flasks containing the DAS medium were inoculated with 5% vegetative inoculum from the seed flasks. The DAS medium consisted of 2% dextrin, 1% Arkasoy 50 soyabean flour (British Arkady Co., Manchester, U.K.), 0.1% Scotasol dried distillers solubles (Thomas Borthwick Ltd, Glasgow, U.K.), and 0.01% $FeSO_4 \cdot 7H_2O$ in deionized water at a final pH of 7.0.

Media for both seed and production stages were dispensed in 100-ml volumes in 500-ml conical flasks which were closed with foam plugs and sterilized by autoclaving at 121°C for 15 min. Seed and production flasks were shaken at 26°C on a rotary shaker with 2-inch (5.08-cm) throw and a speed of 240 rpm.

The cultivation in stainless-steel fermentors was carried out using the DAS medium as described above, except that antifoam was added before steam sterilization. The antifoam was 10% Pluronic L81 (Ugine Kuhlmann Chemicals Ltd, London, U.K.) in soyabean oil and was added to give a final concentration of 0.05% (wt/vol). The seed fermentor containing 50 liters of medium was inoculated with 100 ml of a spore/mycelial suspension prepared by adding a sterile solution of 0.05% Triton X-100 in water to a Yeatex/glucose agar (see above) slope in a roux bottle grown for 10 days at 26°C. The 90-liter baffled seed fermentor was agitated by a 13-cm disk turbine impeller at 240 rpm. Sterile air was supplied at 50 liters/min, and the temperature was 26°C. After 72 h the seed fermentor was used to provide a 5% vegetative inoculum into 150 liters of DAS fermentation medium contained in a 300-liter baffled fermentor. This fermentor was stirred at 210 rpm using a 21-cm

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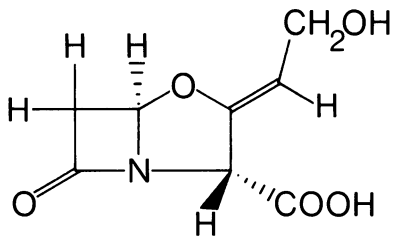


FIG. 1. *Structure of clavulanic acid, Z-(2R,5R)-3-(β-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo-[3,2,0]heptane-2-carboxylic acid.*

disk turbine impeller. The temperature was maintained at 26°C and the air flow at 150 liters/min. The above-mentioned antifoam was added in 10-ml amounts when required.

Preparation of β-lactamases. The β-lactamase preparations consisted of ultrasonic extracts of bacterial cells and were prepared as follows.

Nutrient agar cultures of the organisms listed in Table 2 were used to inoculate flasks containing the tryptone medium. These flasks were shaken overnight and used to provide a 1% inoculum into further flasks of the same medium. The tryptone medium consisted of 3.2% tryptone (Oxoid), 2% yeast extract (Oxoid), and 0.5% NaCl. The final pH was adjusted to 7.4 with dilute NaOH, and the medium was dispensed in 100-ml volumes in 500-ml conical flasks. The flasks were closed with foam plugs and sterilized at 121°C for 15 min.

All flasks were incubated on a rotary shaker running at 240 rpm and having a 2-inch throw. The temperature was 37°C for all cultures except *Escherichia coli* JT410, JT4, and JT39 and *Proteus mirabilis* C889, which were grown at 30°C. After shaking for 2 h, 6-aminopenicillanic acid was added to cultures of *Pseudomonas aeruginosa* A to give a final concentration of 500 μg/ml. Methicillin was added at 2 h to the *Staphylococcus aureus* cultures to give a final concentration of 1 μg/ml. The total incubation time was about 16 h for all cultures except for the two strains of *P. aeruginosa*, which were grown for 7 h.

The bacterial cells were collected by centrifugation and resuspended in deionized water to give a cell concentration 100 times that in the growth medium. This cell suspension was then disrupted in an MSE ultrasonic disintegrator for 5 min at 6.3-μm amplitude with ice cooling. The cell debris was removed by centrifugation, and samples of the supernatant were deep frozen until required.

The β-lactamase of *Bacillus cereus* was an extracellular enzyme preparation obtained from Whatman Biochemicals Ltd, Maidstone, U.K.

β-Lactamase inhibition assay and determination of I_{50} value. Before use in the assay procedures the β-lactamase preparation was diluted in 0.005 M phosphate buffer (pH 7) until it gave about 75% hydrolysis of a 1-mg/ml solution of substrate (see Table 2) in 30 min at 37°C. Suitable dilutions of the clavulanic acid preparation were mixed with the β-lactamase solution and incubated at 37°C for 15 min because of the progressive nature of the inhibition. A control with buffer in place of clavulanic acid preparation was also incubated. Substrate in pH 7.0 buffer was then added to both inhibitor and control reaction mixtures to give a final concentration of 1 mg/ml, and incubation at 37°C was continued for a further 30 min at 37°C. The residual substrate in each mixture was then estimated using the hydroxylamine assay (3), and the results were used to calculate the percentage of inhibition.

Sodium benzylpenicillin (Beecham) was used as a substrate except for the cephalosporinase-producing cultures *P. aeruginosa* A, *E. coli* JT410, and *Enterobacter cloacae* P99, where cephaloridine (Glaxo) was used. The concentration and assay methods were the same for the two substrates except that the reaction time with the hydroxylamine reagent was reduced from 10 to 3 min for cephaloridine to optimize color formation with the ferric reagent.

The I_{50} for clavulanic acid is defined as the concentration giving 50% inhibition of the β-lactamase as measured by the above procedure, I_{50} values being obtained from a plot of percentage of inhibition against inhibitor concentration.

Chromatography of clavulanic acid. Paper chromatography was carried out on 1-cm-wide paper strips of Whatman grade 1. The solvent systems described in the text were used in the descending fashion at 4°C. The position of clavulanic acid was located using the bioautographic method described below.

Thin-layer chromatography was carried out at 20°C using precoated, glass-backed, thin-layer plates (Merck silica gel 60). Clavulanic acid was detected by spraying with the TTC reagent, which consisted of equal volumes of 4% methanolic solution of 2,3,5-triphenyltetrazolium chloride and aqueous 1 N NaOH.

Special bioautographic detection method for clavulanic acid. Clavulanic acid was located on paper strip chromatograms by contacting the dried strips with agar containing benzylpenicillin and seeded with *Klebsiella aerogenes* A, a variant of *K. aerogenes* NCTC 418. Inoculum was prepared by growing this culture overnight in 2.5% Oxoid nutrient broth CM67 in a test tube at 28°C. A 300-ml amount of 4% blood agar base (Oxoid CM55) was cooled to 50°C after sterilization before addition of 0.8 ml of inoculum and 1 ml of 1.8 mg of sodium benzylpenicillin per ml (final concentration, 6 μg/ml). For ordinary bioautography the benzylpenicillin was omitted and the inoculum level was halved. The agar was poured onto glass plates (13 by 16 inches [ca. 33 by 40 cm], the chromatogram strips were laid on the agar, and the plates were incubated for 16 h at 28°C.

Antibacterial synergy tests. The enhancement of the antibacterial activity of β-lactam antibiotics by clavulanic acid was demonstrated using the microtiter method. All organisms were grown in tryptone soy broth (Lab M. Ltd, Salford, Lancs., England) at 37°C overnight to provide inoculum for tests. Serial dilutions of antibiotics were prepared in tryptone soy broth, with or without the addition of sodium clavulanate, and inoculum was added to give a final dilution of 1/500. The trays were incubated overnight at 37°C, and minimum inhibitory concentra-

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tions values were taken as the lowest concentrations producing inhibition of growth.

RESULTS

Detection and isolation of clavulanic acid. *S. clavuligerus* ATCC 27064 was grown for 4 days in shaken conical flasks using the DAS medium. Samples of culture filtrate were tested for β-lactamase inhibitory activity and were found to give a mean inhibition of 56% at a final dilution of 1/2,500 against the R factor-mediated β-lactamase of *E. coli* JT4.

The culture fluid was examined by paper chromatography, the location of β-lactamase-inhibitory material being determined by a special bioautographic procedure based on the fact that the β-lactamase of *K. aerogenes* is readily inhibited by clavulanic acid (Table 2).

The results in Fig. 2 clearly show the presence of a zone of β-lactamase-inhibitory activity. The substance responsible for this zone of activity had an R_f of 0.6 to 0.65 and has been named clavulanic acid. It is quite distinct from cephamycin C and penicillin N, neither of which shows β-lactamase-inhibitory activity in the special bioautographic system used (Table 1, Fig. 2). Furthermore, clavulanic acid has a much higher R_f value than the various naturally occurring, amino adipyl-containing β-lactam antibiotics. It will be noticed in Fig. 2 that chromatograms of fermentation samples showed no antibiotic zones when contacted with *K. aerogenes* seeded agar, the cephamycin C level presumably being just too low to be detected except when added as a marker.

In addition to the above procedure, clavulanic acid can also be detected on thin-layer chromatography plates by spraying with TTC reagent. It appears as a dark red spot after heating the sprayed chromatogram and has an R_f value of 0.44 when using a solvent system consisting of *n*-butanol–ethanol–water (4:1:5, vol/vol/vol, top phase).

To provide culture fluid for the isolation of clavulanic acid, *S. clavuligerus* was grown in a 300-liter fermentor containing 150 liters of the DAS medium for 4 days at 26°C, by which time the clavulanic acid titer was in the region of 150 to 200 μg/ml. Clavulanic acid was isolated as its sodium salt according to the procedure outlined in Fig. 3. During the isolation the metabolite was followed using the enzyme inhibition assay with the β-lactamase from *E. coli* JT4.

β-Lactamase-inhibitory properties of clavulanic acid. The enzyme-inhibitory activity of sodium clavulanate against a range of β-lactamases was determined by measuring the amount required to give 50% inhibition (I_{50}) under the conditions of the test (see Materials and Methods). For all of the β-lactamase preparations it was found that the degree of inhibition of the β-lactamase increased with time. An interaction time of 15 min was arbitrarily chosen for the determination of I_{50} values. The results given in Table 2 show that β-lactamases from both gram-negative and gram-positive organisms are inhibited by very low concentrations of clavulanic acid. The enzymes not so readily inhibited under the test conditions were those with a predominantly cephalosporinase activity and the enzyme preparation from *B. cereus*, which contained both enzymes I and II. Enzymes that are well inhibited include those from clinically important organisms such as *E. coli* harboring the R_{TEM} plasmid. This is exemplified in Table 2 by *E. coli* JT4, which was found to produce a β-lactamase indistinguishable by isoelectric focusing and substrate profile from that found in *E. coli* K-12 pR_{TEM}.

β-Lactamase stability of clavulanic acid. Using paper chromatography and the special bioautographic detection method, no gross destruction of clavulanic acid was observed on incubation with those β-lactamases that were less readily inhibited by clavulanic acid, such as those produced by *P. aeruginosa* A, *E. coli* JT410, and *E. cloacae* P99. The poor inhibitory activity against these β-lactamases thus does not seem to be due to instability of the compound.

The activity of sodium clavulanate was the same against strains of bacteria that produced β-lactamase as against those that did not, reflecting the high stability of the compound to β-lactamase. Thus, the minimum inhibitory concentration for the β-lactamase-producing Russell strain of *S. aureus* was the same as that against the Oxford strain, and the minimum inhibitory concentration for *E. coli* JT39, a potent β-lactamase producer, was the same as that against *E. coli* NCTC 10418, an ampicillin-susceptible strain.

Enhancement of antibacterial activity of β-lactamase-labile antibiotics by clavulanic acid. When low levels of sodium clavulanate were added to β-lactamase-labile compounds, the antibacterial spectrum of the labile compound could be widened to include many previously resistant β-lactamase-producing organisms. Good correlations could be seen between enzyme inhibition data and antibacterial synergy tests. Data in Tables 2 and 3 show that where good inhibition was seen against isolated enzymes the organisms producing those enzymes were rendered susceptible to β-lactam antibiotics in the presence of sodium clavulanate. However, where inhibition was poor against isolated enzymes, such as the chromo-

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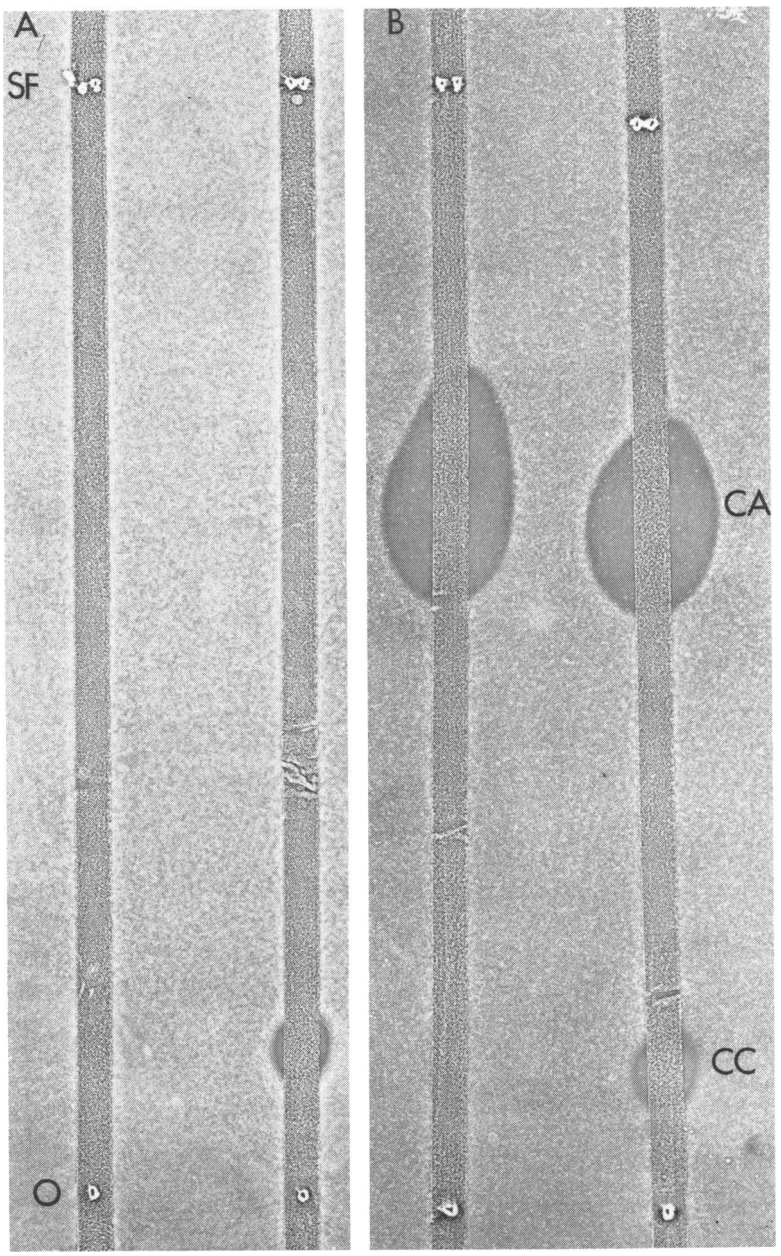


FIG. 2. *Paper chromatographic detection of clavulanic acid in a 4-day fermentation sample of S. clavuligerus grown as described in the text. The chromatograms were contacted with agar seeded with a β-lactamase-producing strain of K. aerogenes without (A) or with (B) the addition of a subinhibitory concentration of benzylpenicillin (6 μg/ml) to the agar. The second chromatogram of each pair was of a fermentation sample supplemented with cephamycin C (10 μl of 1 mg/ml). The solvent system was n-butanol–acetic acid–water (12:3:5, vol/vol/vol), the paper was Whatman grade 1, and the loading was 15 μl of sample. SF, Solvent front; O, origin; CA, clavulanic acid; CC, cephamycin C.*

somal β-lactamase of *E. coli* JT410, no significant change in minimum inhibitory concentration against this organism could be seen when sodium clavulanate was added to cephaloridine or ampicillin.

DISCUSSION

The property of β-lactamase inhibition is well known for certain penicillins and cephalosporins (1). The inhibition exhibited by these compounds is usually restricted to a small num-

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TABLE 1. *Paper chromatography of sodium clavulanate*

Solvent system	R_f on Whatman no. 1 paper strips		
	Sodium clavulanate	Penicillin N	Cephamycin C
n-Butanol–acetic acid–water, 12:3:5 (vol/vol/vol)	0.63	0.3	0.18
n-Butanol–ethanol–water, 4:1:5 (vol/vol/vol, top phase)	0.14	0	0
n-Butanol–pyridine–water, 1:1:1 (vol/vol/vol)	0.56	0.3	0.23

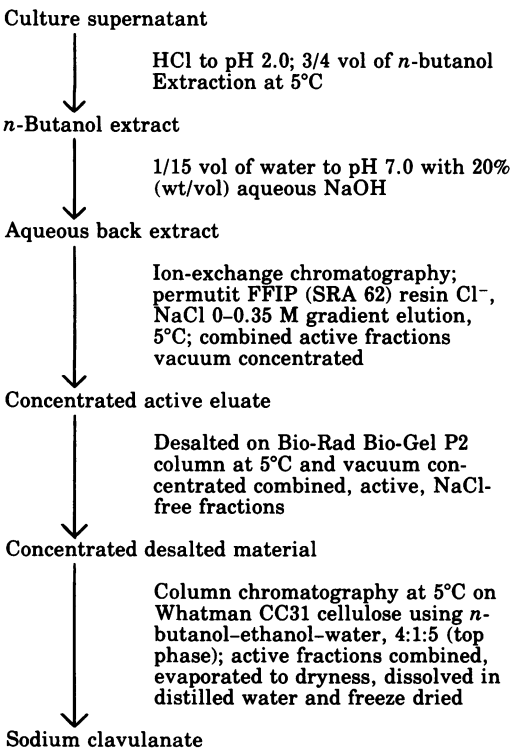


FIG. 3. *Isolation of clavulanic acid as its sodium salt.*

ber of β-lactamases and is of the competitive and mainly reversible type. Furthermore, the inhibitory properties of these compounds are clearly associated with the side chain (5, 13). Some semisynthetic penicillins have been reported to inactivate *B. cereus* and staphylococcal enzymes, but only at very high concentrations (1, 2, 6). In contrast, clavulanic acid, which has no acylamino side chain, inhibits a wide range of β-lactamases and does so in a progressive fashion at low concentrations. The similarity between the stereochemistry of clavulanic acid and the penicillin nucleus could explain the affinity between clavulanic acid and β-lactamases, whereas the reactivity may in some ways be associated with the hydroxyethylidene in position 3.

The only naturally occurring, low-molecular-weight β-lactamase inhibitors apart from clavulanic acid that have been reported are those from our laboratory, namely, MM 4550, MM 13902 and MM 17880 produced by *Streptomyces olivaceus* (4; Belgian Patent 1,467,413, 16 March 1977, D. Butterworth, M. Cole, and J. D. Hood) and MC696-SY2 A and B described by Umezawa et al. from *Streptomyces fulvoviridis* (15, U.S. Patent 3,928,569, 23 December 1975, H. Umezawa, S. Mitsuhashi, R. Utahara, and T. Ishikawa). Chromatographic and spectral data indicate that clavulanic acid is clearly different from these metabolites. Inhibitors of β-lactamase have been reported by Hata et al. from *Streptomyces gedanensis* (7) and by Miyamura and Ochiai, also from a streptomycete (11), but these were macromolecular substances.

Although clavulanic acid has weak broad-spectrum antibacterial activity, its potential application is as an inhibitor of many clinically important β-lactamases. It is thus possible, by adding clavulanic acid at low concentrations, to demonstrate marked improvement in the activity of β-lactamase-labile penicillins and cephalosporins. This improvement of activity is very marked for strains of bacteria that owe their resistance to the production of β-lactamase but only occurs where this enzyme is readily inhibited by clavulanic acid.

TABLE 2. *β-Lactamase inhibitory activity of sodium clavulanate*

Source of β-lactamase	Substrate[a]	I_{50} (μg/ml)
Pseudomonas aeruginosa A (Sabath type [14])	C	160
Escherichia coli JT410 (chromosomally mediated)	C	56
Enterobacter cloacae P99	C	10.0
Klebsiella aerogenes NCTC 418	P	0.03
Escherichia coli JT4 (R factor mediated)	P	0.08
Escherichia coli JT39 (R factor mediated)	P	0.08
Proteus mirabilis C889	P	0.03
Pseudomonas aeruginosa Dalgleish (preparation contains some Sabath-type enzyme)	P	0.1
Staphylococcus aureus Russell	P	0.06
Bacillus cereus (Whatman Biochemicals Ltd.)	P	17.0

[a] C, Cephaloridine; P, benzylpenicillin.

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TABLE 3. *Activity of β-lactamase-labile antibiotics in the presence of sodium clavulanate*

β-Lactam antibiotic plus sodium clavulanate	Minimum inhibitory concn (μg/ml)[a]					
	Staphylococcus aureus Russell	*Klebsiella aerogenes* NCTC 418	*Proteus mirabilis* C889	*Escherichia coli* JT39	*Escherichia coli* JT410	*Pseudomonas aeruginosa* Dalgleish
Sodium clavulanate alone	15	31	62–125	31	31	250
Ampicillin alone	500	250	>2,000	>2,000	250	>2,000[b]
Ampicillin + 1 μg/ml	0.8	0.4	62	31	250	2,000[b]
Ampicillin + 5 μg/ml	0.02	0.1	8	4	250	500[b]
Ampicillin + 20 μg/ml					125	125[b]
Cephaloridine alone	0.6		62	62	62	
Cephaloridine + 1 μg/ml	0.15		8	4	62	
Cephaloridine + 5 μg/ml	0.06		4	2	62	

[a] Microtiter technique using serial dilutions in tryptone soy broth with a 1/500 dilution of overnight broth culture as inoculum. End points read after 18 h at 37°C.

[b] Carbenicillin in place of ampicillin.

It is hoped that clavulanic acid will provide a new aid to the treatment of infections caused by penicillin- and cephalosporin-resistant bacteria.

ACKNOWLEDGMENTS

We thank the following colleagues for their assistance: S. J. Box, A. G. Brown, D. Byrom, S. E. M. Cox, C. J. Davis, G. Hanscomb, P. J. Hepburn, T. T. Howarth, P. A. Hunter, L. R. Jeffries, M. Kenig, A. Lawrence, M. S. Verrall.

We thank D. Hendlin of the Merck Institute of Therapeutic Research for a sample of cephamycin C.

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