OLIVANIC ACIDS, A FAMILY OF β-LACTAM ANTIBIOTICS WITH β-LACTAMASE INHIBITORY PROPERTIES PRODUCED BY *STREPTOMYCES* SPECIES

I. DETECTION, PROPERTIES AND FERMENTATION STUDIES

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The screening of soil actinomycetes for β-lactamase inhibitors is described. Using a plate test a number of strains of *Streptomyces* were found to produce β-lactamase inhibitory activity designated olivanic acid complex. Factors affecting the production of this complex by *Streptomyces olivaceus* ATCC 21379 are reported. The complex showed antibacterial activity and also inhibited a number of different types of β-lactamase in a progressive manner. Certain ampicillin-resistant bacteria are rendered sensitive to ampicillin in the presence of olivanic acid complex at a concentration which alone did not inhibit growth.

The importance of β-lactamase as a resistance determinant for penicillins and cephalosporins and the observation that certain penicillins act as β-lactamase inhibitors[1,2,3,4], prompted us to screen microorganisms for naturally-occurring inhibitors of this enzyme.

Various strains of *Streptomyces* have been found to produce a family of β-lactamase inhibitors which we refer to as olivanic acid complex. This paper describes the production and properties of the olivanic acid complex from *S. olivaceus*. Subsequent work has shown that this comprises at least three components: MM 4550, MM 13902 and MM 17880[5,6,7].

Materials and Methods

Screening of soil actinomycetes

Cultures of aerobic actinomycetes were isolated from soil samples by plating out on selective culture media. The arginine-glycerol-salts medium of EL-NAKEEB and LECHAVALIER[8] was found to be particularly satisfactory for this purpose. Single colonies were picked off and grown on test-tube slants of a medium containing 2% glucose, 1% yeast extract and 1.5% agar (pH 6.8). Submerged culture was carried out at 28°C in 100-ml conical flasks containing 20 ml of liquid medium. Each culture was grown in four different liquid media made up in deionised water and having the following compositions:

Medium A. 2.5% glucose, 4% soybean flour, 0.5% distillers solubles, 0.25% NaCl (pH 7.5).
Medium B. 2.0% glucose, 0.4% yeast extract (paste), 1% malt extract (powder) (pH 7.3).
Medium C. 2% glucose, 0.4% $NaNO_3$, 2% malt extract (pH 7.3).
Medium D. 1% glucose, 0.05% egg albumen, 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $Fe_2(SO_4)_3$ (pH 7.3).

Cultures

The following cultures were used as sources of β-lactamase:—*Klebsiella aerogenes* A (a subculture of *K. aerogenes* NCTC 418, ATCC 15380; now deposited as *K. pneumoniae* ATCC 29665); *Escherichia coli* B11 (clinical isolate, serotype 055); *Escherichia coli* NCIB 9465[9], and *Bacillus cereus* 569/H

(NCTC 9945).

Proteus vulgaris BRL 1054, *Pseudomonas aeruginosa* BRL 1058, *Staphylococcus aureus* BRL 1083 and *Staphylococcus aureus* BRL 1089 were Beecham laboratory strains.

The agar plate test for β-lactamase inhibitors

Fermentation samples were clarified by centrifugation and 80 μl volumes added to 8-mm diameter wells cut in two 25-cm square agar plates; a test plate and a control plate. The test plate contained 150 ml of blood agar base (Oxoid) seeded with 0.4 ml of an overnight broth culture of *K. aerogenes* A. A solution of sodium benzylpenicillin was added to the agar before pouring to give a concentration of 10 μg/ml. The control plate was identical except that the sodium benzylpenicillin was omitted. The plates were incubated at 28°C for 14 hours and the zones of inhibition measured. A difference in zone diameter between the test and control plates was indicative of the presence of a β-lactamase inhibitor. Methicillin which is known to have inhibitory activity against the β-lactamase of *K. aerogenes*[1] was used as a positive control. At a concentration of 200 μg/ml a clear zone of inhibition was obtained on the test plate whereas no zone occurred on the control plate.

Fermentation procedure for production of olivanic acid complex

Streptomyces cultures were grown on the glucose-yeast extract agar medium described above. Slant cultures on this medium produced good sporulation after incubation for 1 week at 28°C.

Shake flask seed and final fermentations were carried out in 500-ml Erlenmeyer flasks containing 100 ml of medium and incubated at 28°C on a rotary shaker at 240 rpm and with a throw of 50 mm. A spore suspension was used to inoculate a seed stage medium consisting of 1% soybean flour and 2% glucose, pH 6.8. In preliminary experiments this medium was also used for the final fermentation. The seed stage was incubated for 48 hours and the final fermentation flasks inoculated with spores or 2.5 ml of vegetative seed growth and incubated for 2~3 days. The fermentation media used are described under Results.

Pilot scale fermentations were carried out in stainless steel fully baffled stirred tanks. A seed stage using the medium described was inoculated with a spore suspension and incubated at 28°C for 48 hours. Final fermentations were carried out in tanks containing 50 liters of medium inoculated with 2.5 liters of vegetative growth and incubated at 30°C. Seed and final fermentations were carried out with aeration at 1.0 vol/vol/min.

β-Lactamase studies

β-Lactamase activity was determined using the starch-iodometric assay described by COLE, ELSON and FULLBROOK[10] except that benzylpenicillin was used in place of ampicillin. All reactions were carried out at pH 7 in 0.05 M phosphate buffer. The method of preparation of β-lactamase from *E. coli* B11 and other bacteria was as already reported[10]. The *E. coli* B11 culture has been shown to produce a plasmid-mediated β-lactamase corresponding to R_{TEM} β-lactamase, as well as a chromosomal β-lactamase[11].

Thin-layer chromatography

The β-lactamase inhibitory substances present in culture filtrate were concentrated by the following procedure before chromatography.

The culture filtrate (25 ml) was extracted with 12.5 ml of 0.2% benzyldimethyl - *n*-hexadecyl-ammonium chloride in dichloromethane. After separation of the phases 2.5 ml of 0.5% sodium iodide in water was added to the organic phase, the mixture shaken and the aqueous phase chromatographed by spotting 5 μl onto Eastman Kodak cellulose "chromogram" sheets. The chromatograms were developed with a solvent consisting of *n*-butanol - isopropanol - water, 7: 7: 6 v/v[5]. The zones of β-lactamase inhibitory activity were detected by contacting the chromatograms with agar seeded with *K. aerogenes* A and containing 6 μg/ml benzylpenicillin.

Results

Screening of Aerobic Actinomycetes

Positive results were obtained in the β-lactamase inhibitor agar-plate test with a number of

cultures isolated from geographically diverse soil samples. Four of these cultures, isolated from soils obtained from Spain, New Zealand, Israel and South Africa, were subsequently identified as strains of *S. olivaceus* by Dr. I. BOUSFIELD, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland.

The four strains have been deposited in the American Type Culture Collection (ATCC 21379, ATCC 21380, ATCC 21381, ATCC 21382) and the olivanic acid complex produced by all four strains was found to display the same characteristics on thin-layer chromatography, namely multiple zones of β-lactamase inhibitory activity with Rf values between 0.4 and 0.8.

A number of cultures obtained from national culture collections were also tested for ability to produce olivanic acid complex employing the fermentation procedure and thin-layer chromatography detection system described in the Methods section. They comprised strains named as *S. olivaceus* as well as species considered by HÜTTER[12] to be synonymous with *S. olivaceus* or which could be classed in the same or closely allied groups[13].

As a result of these tests the following cultures were also found to produce olivanic acid complex: *S. olivaceus* NCIB 8238, *S. olivaceus* NCIB 8509, *S. argenteolus* ATCC 11009, *S. flavovirens* ATCC 3320, *S. flavus* ATCC 3369, *S. fulvoviridis* ATCC 15863 and *S. sioyaensis* ATCC 13989. Of these cultures, *S. flavovirens, S. flavus* and *S. fulvoviridis* are listed by HÜTTER[12] as synonyms of *S. olivaceus*. Three other cultures designated *S. olivaceus* ATCC 21549, ATCC 12019 and ATCC 3335, did not give a positive response for olivanic acid complex when tested by the same methods. ATCC 3335 is the type strain of *S. olivaceus*.

Fermentation Studies with *S. olivaceus* ATCC 21379

In the agar plate test, culture filtrate of *S. olivaceus* ATCC 21379 produced zones of β-lactamase inhibitory activity of 17.0 and 20.0 mm respectively using fermentation media B and D, whereas no zones of antibiotic activity occurred on the control plates. Using media A and C no zones of activity were detected on either the test or control plates. The results in these media indicated that under the conditions of the screening procedure a relatively low nitrogen content and a high C: N ratio was required. In shake flask experiments a range of complex nitrogen sources, including bacteriological peptones, meat extract, yeast extract, cotton seed meal, soybean flour and distillers solubles, were compared at a concentration of 0.5% of the nitrogen-containing material in a basal medium containing 2% glucose. The best results were obtained with soybean flour. Further shake flask experiments showed the optimum level of soybean flour to be in the region of 1% and glucose to be superior to

Table 1. The effect of addition of cobalt chloride on olivanic acid complex production. (Spore inoculated flasks).

$CoCl_2 \cdot 6H_2O$ added to medium (mg/liter)	β-Lactamase-inhibitory activity in plate test (zone diameter—mm)
0	24.1
0.01	30.5
0.05	34.3
1.0	34.4

Table 2. Composition of soybean/glucose fermentation medium for production of olivanic acid complex by *S. olivaceus*.

Ingredient	Concentration (per cent w/v)
Soybean flour	1.0
Glucose	2.0
$CaCO_3$	0.02
Na_2SO_4	0.05
$CoCl_2 \cdot 6H_2O$	0.0001

pH adjusted to 6.0 before sterilisation.

Table 3. Typical stirred tank fermentation for the production of olivanic acid complex by *S. olivaceus* ATCC 21379.

Sample time (hours)	pH	Glucose (mg/ml)	Olivanic acid complex. Agar plate test for β-lactamase inhibitory activity in culture filtrate*. Zone diameter (mm)
0	6.3	20	0
6			0
12	7.0	18	0
18			22.5
24	6.4	12	27.6
30			30.0
36	5.4	7	31.0
42			32.2
48	5.3	3	32.0

* Clarified fermentation samples were diluted 1:10 in phosphate buffer, pH 7. At this dilution no antibacterial activity was detected on the control plate.

Table 4. Antibacterial activity of culture filtrate of *S. olivaceus* ATCC 21379.

Test organism	Zone diameter[d] (mm)
E. coli NCTC 10418	15.3
E. coli B11[a]	11.8
E. coli 83[b]	No zone
Klebsiella aerogenes A	19.0
Enterobacter cloacae	Trace
Shigella sonnei	13.5
Salmonella typhi	17.0
Proteus mirabilis C977	12.3
Proteus mirabilis C889[a]	12.7
Pseudomonas aeruginosa A	No zone
Staphylococcus aureus Oxford	13.8
Staphylococcus aureus Russell[a]	12.0
Staphylococcus aureus BRL 1517[c]	No zone
β-Haemolytic *Streptococcus*	25.5
Streptococcus faecalis	No zone
Bacillus subtilis	17.4

[a] β-Lactamase-producing strains
[b] Intrinsically insensitive to ampicillin and produces some β-lactamase
[c] Intrinsically insensitive to methicillin and other β-lactam antibiotics; produces β-lactamase
[d] Samples placed in 8-mm holes cut in nutrient agar plates seeded with the various organisms; plates incubated overnight at 37°C

other carbon sources including glycerol, lactose, maltose and soluble starch.

As a result of examining the effect of addition of various cations to the fermentation medium it was found that trace amounts of cobalt stimulated the production of olivanic acid complex, as indicated by the agar plate test for β-lactamase inhibitors. The effect of addition of a range of concentrations of cobalt chloride ($CoCl_2 \cdot 6H_2O$) to 1% soybean flour - 2% glucose medium is shown in Table 1. We also observed some stimulation in yield with manganese ion.

Addition of calcium carbonate and sodium sulphate to the medium also resulted in some increase in olivanic acid complex production; the maximum effects were observed at concentrations in the medium of 0.02% w/v for calcium carbonate and 0.05% w/v for sodium sulphate. The composition of the most suitable medium arising from these shake flask experiments is shown in Table 2.

The time-course of a typical 50-liter stirred tank fermentation is shown in Table 3. The medium for this experiment was as shown in Table 2 with addition of 0.2% v/v antifoam agent consisting of 10% Pluronic L81 in soybean oil. Olivanic acid complex reached a maximum at approximately 40 hours, at which stage the glucose in the medium was almost exhausted.

A fermentation broth sample obtained using the medium of Table 2 was tested for antibacterial activity against a range of bacteria. The results are shown in Table 4.

β-Lactamase Inhibitory Properties of *S. olivaceus* Culture Filtrate

Preparations of β-lactamase were made from a variety of bacteria including the *K. aerogenes* A used for the agar-plate test for β-lactamase inhibitors. The enzymes were diluted so that the rates of hydrolysis of 10 μg/ml benzylpenicillin in 0.05 M potassium phosphate buffer at pH 7 anp 37°C

Table 5. Inhibition of β-lactamases by culture filtrate of *S. olivaceus* ATCC 21379.

Source of β-lactamase	Rate of hydrolysis of benzyl-penicillin μg/ml/min 37°C pH 7 over an initial reaction period of 10 minutes		% inhibition by culture filtrate
	In presence of culture filtrate at a dilution of 1/625	In absence of culture filtrate	
Klebsiella aerogenes A	0.02	0.12	83
Escherichia coli B11	0.03	0.19	84
Escherichia coli NCIB 9465	0.04	0.05	20
Proteus vulgaris BRL 1054	0.08	0.17	53
Pseudomonas aeruginosa BRL 1058	0.15	0.17	12
Bacillus cereus B569/H NCTC 9945	0.05	0.16	69
Staphylococcus aureus BRL 1083	0.21	0.21	0
Staphylococcus aureus BRL 1089	0.08	0.10	20

were in the range 0.05~0.20 μg/ml/minute. The reaction rates were determined over a 10-minute reaction time using the starch-iodometric assay procedure. The rate of hydrolysis of the benzylpenicillin was then redetermined in the presence of culture filtrate obtained from *S. olivaceus* ATCC 21379 grown in a 90-liter fermenter at 30°C for 48 hours using a medium consisting of 1% soybean flour, 2% glucose and 0.005% $MnSO_4 \cdot 4H_2O$. The culture filtrate, clarified by centrifugation for 15 minute at 40,000 *g* and filtration through diatomaceous earth, was added to each β-lactamase preparation so that its final dilution was 1/625. The results in Table 5 show that the addition of culture filtrate caused a marked inhibition of the β-lactamase produced by most of the cultures tested.

The rate of hydrolysis of benzylpenicillin (10 μg/ml) by the β-lactamase of *E. coli* B11 in the presence and absence of various dilutions of *S. olivaceus* culture filtrate was followed over a more prolonged period of time using the starch-iodometric procedure mentioned above. From the results shown in Fig. 1 it can be seen that the rate progressively slows down in the presence of the *S. olivaceus* culture filtrate and the effect is more marked in the presence of the higher concentration of culture filtrate.

An examination was made of the effect of incubating the β-lactamase preparations with the culture filtrate for various periods of time before addition of substrate to determine the level of residual enzyme activity. The enzyme preparations were incubated at 37°C in the presence and absence of culture fluid from *S. olivaceus* ATCC 21379 using equal volumes of suitably diluted enzyme and culture filtrate.

Fig. 1. Rate of hydrolysis of benzylpenicillin (10 μg/ml) by the β-lactamase of *E. coli* B11 in presence and absence of diluted culture supernatant of *S. olivaceus* ATCC 21379.
A=Control uninhibited reaction
B=*S. olivaceus* culture added at 1/2,500 dilution
C=*S. olivaceus* culture added at 1/1,000 dilution

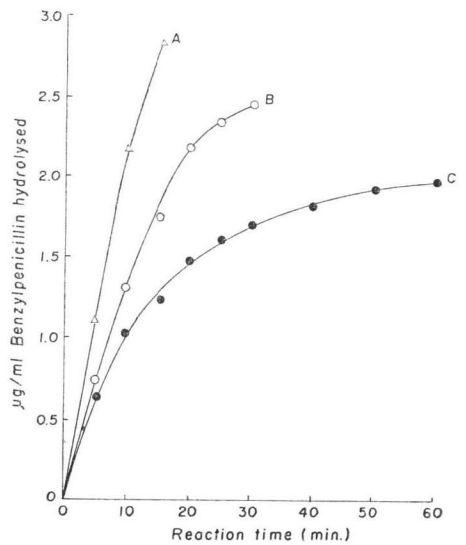

Table 6. Effect of time on the inactivation of β-lactamases by culture filtrate of *S. olivaceus* ATCC 21379.

Source of β-lactamase	Inhibition (%) of β-lactamase after incubation with culture filtrate for various periods of time (hours)			
	0	0.5	1	2
Klebsiella aerogenes A	13	62	79	—
Escherichia coli B11	19	92	92	—
Escherichia coli NCIB 9465	21	43	44	—
Proteus vulgaris BRL 1054	9	36	39	—
Pseudomonas aeruginosa BRL 1058	0	12	12	—
Bacillus cereus B569/H NCTC 9945	0	26	60	70
Staphylococcus aureus BRL 1083	0	30	40	60
Staphylococcus aureus BRL 1089	0	—	20	30

Samples were removed at intervals of time and assayed for initial rate of hydrolysis of 10 μg/ml benzylpenicillin at 37°C using the starch-iodometric assay. The velocities for the reactions with and without culture filtrate were used to calculate the percentage inhibition of the enzymes.

The results in Table 6 show that there was a time-dependent inactivation of β-lactamase which was particularly marked for the enzymes of *E. coli* B11 and *K. aerogenes* A.

Inactivation of the β-lactamase of *E. coli* B11 was demonstrated by showing that enzyme activity was not regenerated by dialysis. Culture filtrate (2.5 ml) from *S. olivaceus* was incubated with 0.5 ml of the B11 β-lactamase preparation for 1 hour at 37°C. The mixture was then dialysed against distilled water for 18 hours using Visking tubing. The diffusate (1 liter) was concentrated to 3 ml on a rotary vacuum evaporator. Using the starch-iodometric procedure it was shown that there was no β-lactamase activity in either the retentate or diffusate. Further tests revealed that the β-lactamase inhibitory activity was no longer detectable in the dialysis sac but was clearly present in the diffusate.

The antibacterial activity of ampicillin against a number of β-lactamase-producing, ampicillin-resistant strains of bacteria, was determined in the presence of a culture filtrate containing olivanic acid complex. The results in Table 7 show a marked reduction in the amount of ampicillin required to inhibit growth.

Table 7. Enhancement of the antibacterial activity of ampicillin against β-lactamase-producing bacteria by culture filtrate of *S. olivaceus* ATCC 21379.

Culture	Minimum inhibitory concentrations (μg/ml)[a]	
	Ampicillin alone	Ampicillin in presence of a 1 in 10 dilution of culture filtrate[b]
Klebsiella aerogenes A	100	<2.5
Klebsiella pneumoniae E70	250	10
Proteus mirabilis C889	>1,000	<2.5
Proteus mirabilis 8	>1,000	5
Staphylococcus aureus Russell	500	<2.5
Staphylococcus aureus BRL 1517 (methicillin-resistant)	1,000	25

[a] Determined by serial dilution of ampicillin in nutrient agar. The inoculum was one drop of overnight culture. The results were read after 18 hours at 37°C.

[b] This sample of culture filtrate showed no antibacterial activity at 1 in 2 dilution against the test bacteria.

Discussion

The production of β-lactamase inhibitors by strains of *S. olivaceus* was first described by us in British Patent 1,363,075 filed on 17th September, 1970 and published on 14th August, 1974.

A preliminary report on these inhibitors and a β-lactamase inhibitor produced by *S. clavuligerus* has been published[14]. Subsequently, it has been shown that the β-lactamase inhibitory complex produced by *S. olivaceus* comprises a number of components including olivanic acids MM 4550, MM 13902 and MM 17880. The structures of these compounds have been reported to be novel bicyclic compounds containing the β-lactam ring[6,7]. Cultures of *Streptomyces* which produce olivanic acids are not uncommon and appear to be widely distributed.

Naturally-occurring β-lactamase inhibitors have also been reported by HATA, *et al.*[15] and by UMEZAWA, *et al.*[16]. HATA, *et al.* reported finding inhibitory activity in culture filtrates of certain *Streptomyces* including *S. olivaceus* and *S. gedanensis*. Highest activity was seen in *S. gedanensis* ATCC 4880 KA-107 from which the inhibitor was isolated and characterized as a protein which unlike the olivanic acid complex did not pass through a dialysis membrane. In the work reported by UMEZAWA, *et al.*[16] two potent β-lactamase inhibitors were found to be produced by *S. fulvoviridis* MC696-SY2 which shows characteristics similar to *S. olivaceus*. One of these inhibitors has now been reported to have a structure closely related to that of MM 4550[17].

The olivanic acid complex in the culture filtrate of *S. olivaceus* ATCC 21379 inhibits the β-lactamase produced by a range of Gram-positive and Gram-negative organisms, the inhibition being progressive in nature. The β-lactamase preparation from the strain of *K. aerogenes* A which was used in the agar plate test for β-lactamase inhibitors, was shown to be very readily inhibited by the olivanic acid complex as was the β-lactamase of *E. coli* B11. The results in Fig. 1 reveal that the rate of inactivation of benzylpenicillin by the latter β-lactamase became progressively slower in the presence of culture filtrate of *S. olivaceus*. After 1 hour, reaction C had almost ceased though apparently 80% of the substrate remained unhydrolysed. When a further quantity of benzylpenicillin (35 μg/ml final concentration) was added to such a reaction there was only a slight temporary increase in rate of benzylpenicillin hydrolysis compared with a control in which buffer replaced the added benzylpenicillin. These results are consistent with a progressive inactivation of the β-lactamase rather than a slowing of the reaction resulting from loss of substrate by β-lactamase action.

The inactivation of the β-lactamase of *E. coli* B11 was confirmed by demonstrating that the activity of the enzyme was not regenerated by removing the β-lactamase inhibitor by dialysis. These results are in marked contrast to those obtained in a very similar experiment with the β-lactamase-inhibiting penicillin BRL 1437 in which the β-lactamase activity of *E. coli* B11 was shown to be regenerated by dialysis[10].

The progressive inactivation of β-lactamase shown by high dilutions of the olivanic acid complex thus distinguishes it from previously reported inhibitors which are penicillins or cephalosporins. The properties of the olivanic acid complex are more like those of clavulanic acid, a metabolite produced by *S. clavuligerus* ATCC 27064, in as much as both are progressive inhibitors and have relatively poor activity against the cephalosporinase of *Pseudomonas* sp.[18].

Of particular interest in the development of an improved fermentation medium was the marked stimulation of yield of olivanic acid complex achieved by addition of trace quantities of cobalt ion. This metal ion has already been reported to have a marked effect in some streptomycete fermentations, for example on the methylation of coumermycin A_2[19] and on stimulation of the yield of phosphonomycin[20].

The improved fermentation media which gave increased yield of β-lactamase inhibitory activity also resulted in the appearance of significant antibiotic activity against a wide range of test bacteria. The spectrum of antibacterial activity shown by the culture filtrate has some of the characteristics of a number of β-lactam antibiotics. Thus no activity was seen against the stains of *E. coli* and *Staph. aureus* which were intrinsically resistant to penicillins. At dilutions of culture filtrate which showed no antibacterial activity it was possible to demonstrate considerable enhancement of the antibacterial activity of ampicillin against β-lactamase-producing bacteria. The mechanism of this synergy is presumed to be the result of the protection of the ampicillin by β-lactamase inhibition.

Enzyme inhibition by olivanic acid complex appears to be highly specific. For example, tests with DOPA decarboxylase, monoamine oxidase, chymotrypsin, trypsin, carbonic anhydrase and urease (Dr. R. A. EDMONDSON and Mr. C. READING, private communication) showed no inhibition by

olivanic acid complex. On the other hand, the carboxypeptidase of *E. coli* B which is known to be inhibited by β-lactam antibiotics[21] was found to be markedly inhibited by the complex (Dr. L. FELLOWS, private communication).

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