FOUR FURTHER ANTIBIOTICS RELATED TO OLIVANIC ACID PRODUCED BY *STREPTOMYCES OLIVACEUS*: FERMENTATION, ISOLATION, CHARACTERISATION AND BIOSYNTHETIC STUDIES

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Four β-lactam antibiotics with β-lactamase inhibitory activity MM 22380, MM 22381, MM 22382 and MM 22383 containing the carbapenem nucleus have been isolated from a culture of *Streptomyces olivaceus* ATCC 31365. Fermentation conditions for their production and methods for their isolation are described. Evidence for a biosynthetic link between these compounds and the previously described olivanic acid derivatives MM 4550, MM 13902 and MM 17880 is presented.

The production, isolation and characterisation of three β-lactam antibiotics with β-lactamase inhibitory properties, MM 4550, MM 13902 and MM 17880 from *Streptomyces olivaceus* has previously been described[1]. Further studies with *S. olivaceus* strains capable of producing these compounds have demonstrated the presence of four further structurally related antibiotics all of which have β-lactamase inhibitory activity. These compounds were designated MM 22380, MM 22381, MM 22382 and MM 22383.

This paper describes fermentation conditions for the production of MM 22380, MM 22381, MM 22382 and MM 22383, a method for their isolation, and preliminary biological and physical properties of the compounds. Studies with a mutant of *S. olivaceus* which provides evidence for a biosynthetic link between these compounds and the previously described olivanic acid derivatives are also described.

Materials and Methods

Fermentation conditions

Streptomyces olivaceus was maintained on agar slopes consisting of V8 vegetable juice (Campbells Soups Ltd., Kings Lynn, Norfolk, U.K.) 20%; Bacto agar (Difco) 2.5%, in deionised water, pH 6.0. After inoculation slopes were incubated at 28°C for 1 week before use. A suspension of spores and mycelium in sterile deionised water (10 ml) was prepared from an agar slope contained in a Universal bottle and a portion (5 ml) used to inoculate the seed stage medium (100 ml) contained in a 500-ml Erlenmeyer flask closed with a foam plastic plug. The seed stage medium consisted of glucose 2%, soybean flour (Arkasoy 50, British Arkady Co., Manchester, U.K.) 1% in deionised water. Inoculated seed stage flasks were incubated at 26°C for 48 hours on a rotary shaker.

Portions of the seed stage culture (1 ml) were used to inoculate the fermentation media (100 ml) contained in 500-ml Erlenmeyer flasks. Fermentations were incubated on a rotary shaker at 26°C, the media had the following compositions:

Medium A: Glucose 2%, Soybean flour (Arkasoy 50) 1.0%, $CaCO_3$ 0.02% $CoCl_2 \cdot 6H_2O$ 0.0001%, Na_2SO_4 0.05% prepared in deionised water.

Medium B: As medium A but without Na_2SO_4.

For the biosynthetic studies the *S. olivaceus* mutant was inoculated directly from agar slopes into fermentation medium C (70 ml) in 250 ml Erlenmeyer flasks.

Medium C: As medium A but with 0.9% soybean flour.
Medium D: As medium C but without Na_2SO_4.

Fermentations with this culture were incubated at 28°C on a rotary shaker.

Where large volumes of culture filtrate were required for isolation of the components the *S. olivaceus* ATCC 31365 was grown in stainless steel fermenters using medium D.

Biosynthetic Studies

A mutant of *S. olivaceus* designated T1 which synthesises predominantly the two *trans* isomers, MM 22381 and MM 22383, was used to investigate the biosynthetic relationship of the five *cis* components. The *cis* olivanic acid derivatives (3.5 mg) were added to separate fermentation flasks after 48 hours and incubation was continued for 17 hours. Culture filtrates were collected and samples were submitted to DEAE cellulose t.l.c. Further samples of culture filtrate (20 ml) were ion pair extracted with 0.125% Aliquat 336 (Tricaprylylmethylammonium chloride, General Mills Chemicals Inc., Minneapolis, Minn, U.S.A.) in dichloromethane (8 ml) and back extracted into 0.4% sodium iodide (1 ml). The extracts were submitted to DEAE cellulose t.l.c. and h.p.l.c. (system B).

Assay Methods

(1) β-Lactamase Inhibition Assay

The levels of MM 4550 were determined using an automated β-lactamase inhibition assay[1].

(2) Thin-Layer Chromatography (t.l.c.)

Samples to be assayed ($1 \sim 5$ μl) were loaded onto diethylaminoethyl cellulose t.l.c. sheets (Polygram CEL 300 DEAE, Macherey-Nagel and Co., 516 Duren, Werkstrasse 6-8, Postfach 307, Germany). The chromatograms were developed with 0.1 M NaCl in 0.05 M potassium phosphate buffer (pH 7.0) for 4 hours at 4°C. The antibiotics were detected by laying the t.l.c. plate for 5 minutes on agar seeded with the test organism.

Two test organisms were used:

1) *Bacillus subtilis* ATCC 6633; after removal of t.l.c. plate the agar was incubated at 28°C for 16 hours.
2) *Escherichia coli* ESS[2] (gift from Prof. A. L. DEMAIN); after removal of the t.l.c. plate the agar was incubated at 37°C for 16 hours.

The t.l.c. method was suitable for detecting MM 4550, MM 13902, MM 17880, MM 22380 and MM 22382, the relatively lower antibacterial activity of MM 22381 and MM 22383 meant that these compounds could not be detected. (Rf's of components are shown in Table 2).

(3) High Performance Liquid Chromatography (h.p.l.c.)

The system comprised a Waters Associates Model 6000A solvent delivery system and a U6K injector connected to a Waters 3.9×300 mm C_{18} μ Bondapak reversed phase column (Waters Associates Ltd., Northwich, U.K.). Monitoring was by a Cecil Model 212 uv spectrophotometer (Cecil Instruments, Cambridge, U.K.) using an 8-μl flow cell with 10-mm path length at 300 nm, the injection volume was 20 μl. Two solvent systems were used for elution depending on the test sample:

Eluant A: 0.05 M ammonium phosphate buffer (pH 4.7) containing 5% acetonitrile, elution at 2 ml/minute. This system was used for fermentation studies although MM 4550 and MM 17880 could not be conveniently assayed in culture filtrate since they were not separated from the large amounts of interfering compounds eluted near the front of the chromatogram.

Eluant B: 0.05 M ammonium phosphate buffer (pH 4.7) containing 3% acetonitrile, elution at 1 ml/minute. This solvent was used for monitoring the ion pair extracts prepared during the biosynthetic studies.

Retention times of the compounds are shown in Table 2.

To aid identification of the olivanic acid derivatives in h.p.l.c. assays, samples were monitored directly and after treatment with a neutral solution of cysteine. The addition of cysteine results in the rapid degradation of the olivanic acid derivatives. The presence of a cysteine degradable peak in the chromatogram with a retention time expected for the derivative confirmed the results.

Assays of the titres of different metabolites in a fermentation sample was achieved by comparing the peak height for the compound to the peak height given by a known concentration of a standard preparation of the compound under the same chromatographic conditions.

Extraction and Isolation

The following column media were used in the isolation procedure outlined in Fig. 3.

Amberlite IRA 458;	acrylic based strongly basic anion-exchange resin.
Amberlite XAD 4;	styrene divinylbenzene cross linked polymeric adsorbent, both from Rohm and Haas co., Philadelphia, Pa, U.S.A.
QAE Sephadex A25;	strongly basic anion-exchange dextran gel from Pharmacia Fine Chemicals, Uppsala, Sweden.
Diaion HP20;	styrene divinylbenzene cross linked polymeric adsorbent from Mitsubishi Chemical Industries, Tokyo, Japan.
Biogel P2;	polyacrylamide gel from Bio Rad Laboratories, Bromley, Kent, U.K.

Results

Fermentation and Isolation Studies

When *Streptomyces olivaceus* ATCC 31365 was grown in fermentation medium A, the presence of antibiotic zones with chromatographic properties consistent with MM 13902 and MM 17880 were detected using the diethylaminoethyl cellulose t.l.c. method with *Bacillus subtilis* as test organism. Assays with the β-lactamase inhibition method showed the level of MM 4550 to be 0.9 μg/ml, which was below the level of detection of

Fig. 1. Thin-layer chromatography of culture filtrate (72 hour) of *S. olivaceus*.

Support: DEAE cellulose
Eluant: 0.1 M NaCl in 0.05 M potassium phosphate buffer (pH 7)
Detection: Bioautography on *B. subtilis*

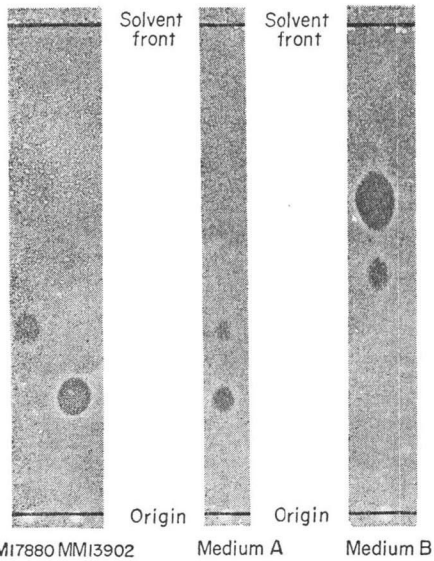
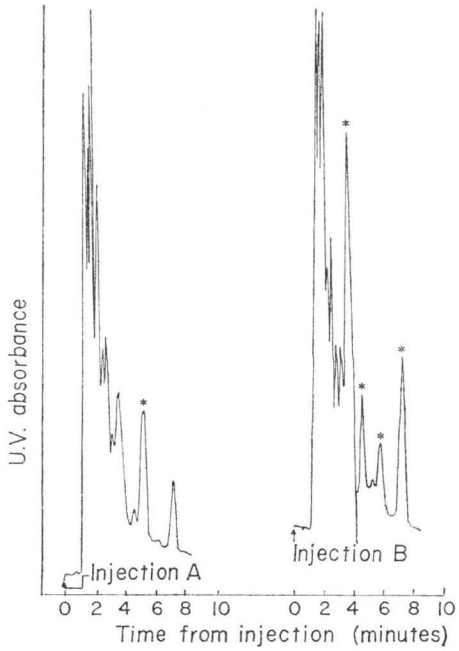


Fig. 2. High performance liquid chromatography of culture filtrate (72 hours) from *S. olivaceus*.

Chromatographic conditions: C_{18} reversed phase column, eluant 0.05 M ammonium phosphate (pH 4.7) containing 5% acetonitrile. (Solvent A) Flow rate 2 ml/min; monitor (uv) at 300 nm.

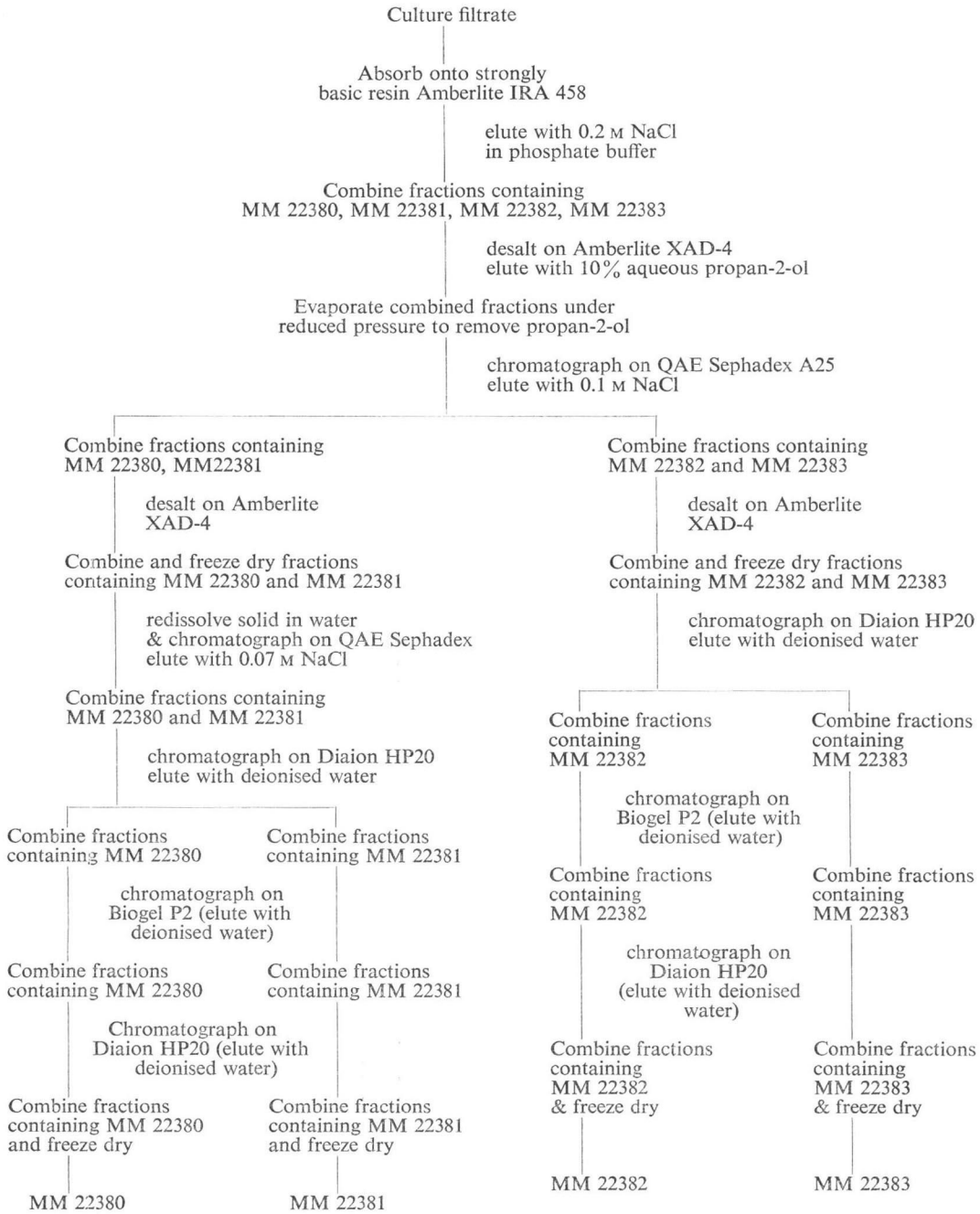
* Peaks degraded on cysteine treatment (see Materials and Methods)

A: Culture grown in medium A.
B: Culture grown in medium B.



the t.l.c. method. When the culture was grown in the medium without sodium sulphate (Medium B) there was a marked change in the antibiotics produced, MM 13902 and MM 17880 could not be detected, and two new antibiotic zones were observed at Rf 0.51 and Rf 0.66. In this medium the level of MM 4550 was assayed at 0.3 μg/ml. The results of t.l.c. analysis of 72 hours fermentation samples from media A and B are shown in Fig. 1. When the same culture filtrate samples were sub-

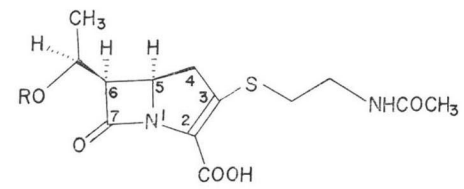
Fig. 3. Isolation procedure for MM 22380, MM 22381, MM 22382 and MM 22383



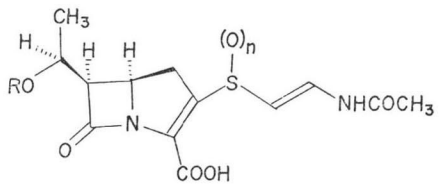
mitted to h.p.l.c. the resulting chromatograms (Fig. 2) showed the presence of four new cysteine degradable components in medium B. The four new components detected in the h.p.l.c. analysis were designated MM 22380, MM 22381, MM 22382 and MM 22383, titres were in the range 1 to 5 μg/ml for each compound.

Isolation of MM 22380, MM 22381, MM 22382 and MM 22383 was achieved by processing culture filtrate yielded by fermentation of *S. olivaceus* ATCC 31365 in a stainless steel fermenter. The culture filtrate was submitted to the extraction procedure outlined in Fig. 3. This process yielded MM 22380, MM 22381, MM 22382 and MM 22383 in substantially pure form as their sodium salts. Their structures have been determined[3] and are shown in Fig. 4. The compounds are two isomeric pairs of antibiotics with structures related to those of the previously described olivanic acid derivatives MM 4550, MM 13902 and MM 17880.

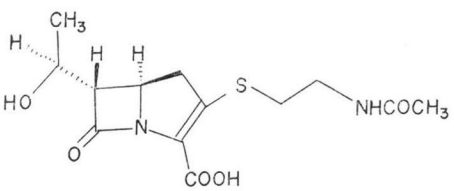
Fig. 4. Structures of antibiotics detected in *Streptomyces olivaceus*.



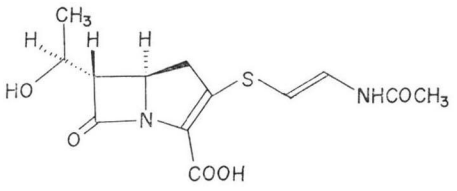
	R
MM 22380	H
MM 17880	SO_3H



	R	n
MM 22382	H	0
MM 13902	SO_3H	0
MM 4550	SO_3H	1



MM 22381



MM 22383

Properties of MM 22380, MM 22381, MM 22382 and MM 22383

Some spectral data of the compounds are shown in Table 1 and chromatographic properties in Table 2.

Each of the four components show a broad spectrum of antibacterial activity, typical minimum

Table 1. UV and IR spectral characteristics of MM 22380, MM 22381, MM 22382 and MM 22383.

	MM 22380	MM 22381	MM 22382	MM 22383
UV (max) nm	298 (ε=8,131)	301 (ε=7,930)	308 (ε=13,627)	309 (ε=13,933)
			228 (ε=13,290)	229 (ε=13,933)
IR (KBr disc)	1750	1750	1750	1750
cm^{-1}	1630(sh)	1735(sh)	1675	1670
	1590	1590	1590~1620	1590~1620

Table 2. Chromatographic properties of olivanic acid derivatives.

	Rf on DEAE cellulose thin-layer chromatography*	Retention time (mins) on reversed phase h.p.l.c.†	
		Solvent A	Solvent B
MM 4550	0.33	2.2	5.7
MM 13902	0.20	5.4	21.2
MM 17880	0.33	3.0	9.6
MM 22380	0.75	3.9	12.4
MM 22381	n.d.	5.2	18.0
MM 22382	0.57	6.6	22.8
MM 22383	n.d.	8.0	29.0

* Eluted with 0.1 M NaCl in 0.05 M potassium phosphate buffer (pH 7.0).

† H.p.l.c. on 3.9×300 mm Waters C_{18} μ Bondapak column.

Solvent A: 0.05 M ammonium phosphate buffer containing 5% acetonitrile (pH 4.7) at 2 ml/minute.

Solvent B: 0.05 M ammonium phosphate buffer containing 3% acetonitrile (pH 4.7) at 1 ml/minute.

n.d. = not detected

Table 3. Antibacterial activity of MM 22380, MM 22381, MM 22382 and MM 22383.

Organism	Minimum inhibitory concentration (μg/ml)			
	MM 22380	MM 22381	MM 22382	MM 22383
Escherichia coli 0111	<0.5	4.0	<0.2	8.0
Escherichia coli JT39	16.0	4.0	4.0	8.0
Enterobacter cloacae N1	4.0	16.0	4.0	16.0
Klebsiella aerogenes A	1.0	4.0	0.5	8.0
Proteus mirabilis 972	0.5	8.0	0.2	31.0
Pseudomonas aeruginosa A	31.0	125	125	125
Staphylococcus aureus Oxford	0.5	4.0	0.5	8.0
Staphylococcus aureus Russell	0.5	4.0	0.5	8.0
Streptococcus faecalis I	1.0	16.0	1.0	31.0
Streptococcus pyogenes CN10	<0.2	0.5	<0.2	2.0

Tests were carried out by serial dilution in nutrient broth by microtitre. Inoculum was prepared by dilution of an overnight broth culture to give the equivalent of approximately 10^6 cells/ml.

inhibitory concentrations are shown in Table 3. The compounds also exhibit potent β-lactamase inhibitory activity, results obtained with a number of cell-free β-lactamase preparations are given in Table 4.

Table 4. β-Lactamase inhibitory activity of MM 22380, MM 22381, MM 22382 and MM 22383.

Compound	I_{50} (μg/ml)				
	Substrate, cephaloridine		Substrate, benzyl penicillin		
	Enterobacter cloacae P99	*Pseudomonas aeruginosa* A	*Proteus mirabilis* C889	*Escherichia coli* JT4	*Staphylococcus aureus* Russell
MM 22380	0.02	4.0	0.1	>2.0	3.25
MM 22381	0.04	4.0	>2.0	0.28	>2.0
MM 22382	0.02	3.0	0.04	>2.0	0.08
MM 22383	0.04	4.0	0.4	0.1	>2.0

I_{50} values were determined by the method described by C. READING and P. HEPBURN[4]

Biosynthetic Studies

Transformation of *cis* olivanic acids MM 22380 and MM 22382 by the *S. olivaceus* blocked mutant T1 was demonstrated by thin-layer chromatography of culture filtrates as shown in Fig. 5. The two *trans* olivanic acids, MM 22381 and MM 22383 produced by the mutant were not detected by the t.l.c./bioautographic method due to their relatively low antibacterial activity. Apart from the zones of inhibition due to the added compound two new zones at Rf 0.20 and 0.33 were detected in both test samples. A zone of Rf 0.20 is characteristic of MM 13902 but two olivanic acids, MM 4550 and MM 17880, have an Rf value of 0.33. Resolution of these compounds was achieved by h.p.l.c. using system B after concentration and partial purification *via* ion pair extraction. The results in Table 5 show the presence of both MM 4550 and MM 17880 in the sample derived from MM 22380, but only

Table 5. High performance liquid chromatography of partially purified extracts derived from the *S. olivaceus* blocked mutant after incubation with olivanic acid derivatives.

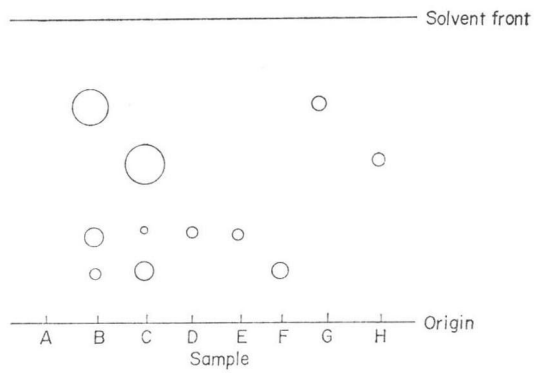
Compound added	Compound detected	
	MM 4550 Rt 5.7 min	MM 17880 Rt 9.6 min
Control	−	−
MM 22380	+	+
MM 22382	+	−
MM 17880	−	+
MM 13902	−	−

\+ Compound detected
− Compound not detected

H.p.l.c. on C_{18} μ Bondapak reversed phase column eluted with 0.05 M ammonium phosphate buffer (pH 4.7) containing 3% acetonitrile.

Fig. 5. Thin-layer chromatography of culture filtrates derived from the *S. olivaceus* blocked mutant incubated with olivanic acid derivatives.

Support:	DEAE cellulose
Solvent:	0.1 M NaCl in 0.05 M potassium phosphate buffer (pH 7)
Assay organism:	*E. coli* ESS
Sample A:	*S. olivaceus* blocked mutant T1 control
Sample B:	*S. olivaceus* blocked mutant T1+MM 22380
Sample C:	*S. olivaceus* blocked mutant T1+MM 22382
Sample D:	MM 4550
Sample E:	MM 17880
Sample F:	MM 13902
Sample G:	MM 22380
Sample H:	MM 22382



MM 4550 in the sample derived from MM 22382. Addition of MM 17880 and MM 13902 to the blocked mutant resulted in the detection of the added compound only. Culture filtrate removed from a control flask at 48 hours was unable to effect the transformations on MM 22380 and MM 22382 observed with a growing culture.

Discussion

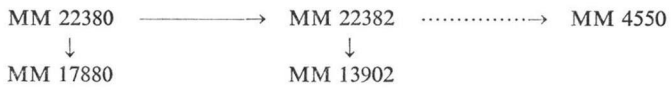
Fermentation studies with *Streptomyces olivaceus* ATCC 31365 demonstrated that substantial changes in the antibiotics produced by the culture could be effected by the presence or absence of sodium sulphate in the medium. Evaluation of these changes using thin-layer chromatography—bioautographic techniques demonstrated the presence of two previously unidentified antibiotic components when sulphate was absent from the medium. The application of h.p.l.c. to the same culture filtrate samples demonstrated the presence of four rather than two new olivanic acid derivatives. These results demonstrated the usefulness of the extremely high resolving power of h.p.l.c. for the separation of series of closely related antibiotics even in fermentation samples. In this case the use of a physical property of the compound, u.v. absorbance, enabled the detection of all four components despite the relatively low antibacterial activity of two members of the series.

Although ion-exchange resins played a most important role in the extraction and isolation of MM 22380, MM 22381, MM 22382 and MM 22383, perhaps the most interesting aspect of the isolation procedure was the resolution of the isomeric pairs of antibiotics. Separation of such closely related components might have been expected to be particularly difficult but was successfully achieved using the polymeric adsorbent Diaion HP20.

Examination of the structures of the olivanic acid derivatives produced by *S. olivaceus* clearly demonstrates the structural relationship between the compounds. MM 17880 and MM 13902 are the sulphate esters of MM 22380 and MM 22382 respectively. MM 22381 and MM 22383 are differentiated from the other olivanic acids in their *trans*-stereochemistry at the β-lactam. The observations that the

addition of sodium sulphate to the fermentation medium stimulated the production of the sulphated antibiotics with concomitant reduction in MM 22380, MM 22381, MM 22382 and MM 22383 suggested all components shared a common biosynthetic pathway. Further evidence for the biosynthetic relationship of the *cis*-compounds was obtained using the feeding experiments with the *S. olivaceus* blocked mutant T1. The addition of MM 22382 resulted in the production of MM 13902 and MM 4550, suggesting MM 22382 is the direct precursor of MM 13902. Since the addition of MM 13902 did not result in the formation of MM 4550, this compound could be formed *via* an alternative pathway from MM 22382. MM 22380 was converted into the three sulphates suggesting that this compound is indeed the direct precursor of MM 17880. Furthermore since there was no evidence for the conversion of MM 17880 to MM 13902, MM 22380 was probably transformed to MM 22382 before the sulphation stage to MM 13902. These results are summarised in Scheme 1.

Scheme 1. Proposed biosynthetic relationship of the *cis*-olivanic acids



Experiments with the *S. olivaceus* blocked mutant T1 have not clarified the relationship between the *trans*-olivanic acid derivatives and the *cis*-compounds.

A number of other antibiotics with the carbapenem nucleus have been described. Thienamycin, N-acetylthienamycin and N-acetyldehydrothienamycin[5] are produced by *Streptomyces cattleya*, all these compounds differ in at least the stereochemistry at position 8 from the olivanic acid derivatives isolated from *S. olivaceus*. A series of compounds produced by strains of *Streptomyces flavogriseus*, epithienamycins A, B, C, and D[6,7], 890 A_9[8] and 890 A_{10}[9], have the same gross structures as the olivanic acid derivatives, excluding MM 4550. *Streptomyces cremeus* var. *auratilis* has been reported to produce a related metabolite PS-5[10,11] having an ethyl group at position 6 with the acetamidoethanethiol side-chain at position 3.

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