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

II. ISOLATION AND CHARACTERISATION OF THE OLIVANIC ACIDS MM 4550, MM 13902 AND MM 17880 FROM *STREPTOMYCES OLIVACEUS*

J. D. HOOD,* S. J. BOX and M. S. VERRALL

Beecham Pharmaceuticals Research Division,
Brockham Park, Betchworth, Surrey RH3 7AJ, England

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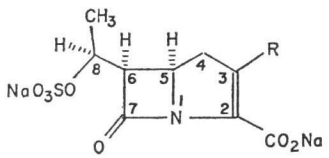
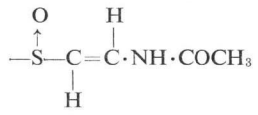
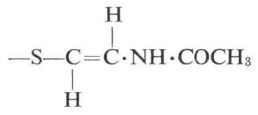
The olivanic acids MM 4550, MM 13902 and MM 17880 are members of a new family of β-lactam antibiotics. An isolation and purification process utilising ion-pair extraction and ion-exchange chromatography is described and the metabolites are characterised by physico-chemical and biological properties.

MM 4550, MM 13902 and MM 17880 are members of a family of β-lactam antibiotics, named the olivanic acids, co-produced by *Streptomyces olivaceus*. The chemical structures of the compounds have been determined[1,2] and are as shown in Fig. 1. The detection of a complex of olivanic acids has been described[3,4]. This paper describes the isolation and purification of MM 4550, MM 13902 and MM 17880 from culture filtrate of *Streptomyces olivaceus* and characterises them by their physico-chemical and biological properties. In recent years other reports of naturally occurring novel bicyclic β-lactams have appeared. A metabolite of *Streptomyces fulvoviridis* was assigned the structure of MM 4550 but the stereochemistry was not specified[5]; clavulanic acid was isolated from *Streptomyces clavuligerus*[6]; the antibiotics thienamycin and N-acetylthienamycin are produced by *Streptomyces cattleya*[7,8,9,10] and a group of substances called the epithienamycins is produced by strains of *Streptomyces flavogriseus*[11,12]. Most recently the antibiotic PS-5 was reported from an unspecified *Streptomycete*[13].

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



	R
MM 4550	
MM 13902	
MM 17880	—S—CH_2—CH_2·NH·$COCH_3$

All compounds are 5R, 6R, 8S.
C8 stereochemistry—D. F. CORBETT, unpublished results.

Materials and Methods

Fermentation

A spore suspension of *Streptomyces olivaceus* ATCC 31126 was used to inoculate seed stage

medium (75 litres) contained in a 100-litre fermenter. For production of MM 4550, MM 13902 and MM 17880, production medium (1,500 litres) was contained in a 2,000-litre fermenter. The composition of each medium is shown in Table 1. Seed stage and production fermentations were continued for 48 hours at 28°C and 30°C respectively under suitable conditions of aeration and agitation.

Table 1. Fermentation media for production of MM 4550, MM 13902 and MM 17880

Constituent	Medium composition (g/litre)	
	Seed	Production
Soybean flour*	10	10
Glucose monohydrate	20	20
$CaCO_3$		0.2
$CoCl_2 \cdot 6H_2O$		0.001
Na_2SO_4		1.0

* Arkasoy 50: British Arkady Co. Ltd., Old Trafford, Manchester.
The production medium was adjusted to pH 6.0 before sterilisation.

Antibacterial assay

Klebsiella pneumoniae ATCC 29665 was the test organism for agar plate diffusion assays using MM 13902 as standard. The relative activities of MM 4550, MM 13902 and MM 17880 in this assay are approximately 1 : 20 : 4 respectively.

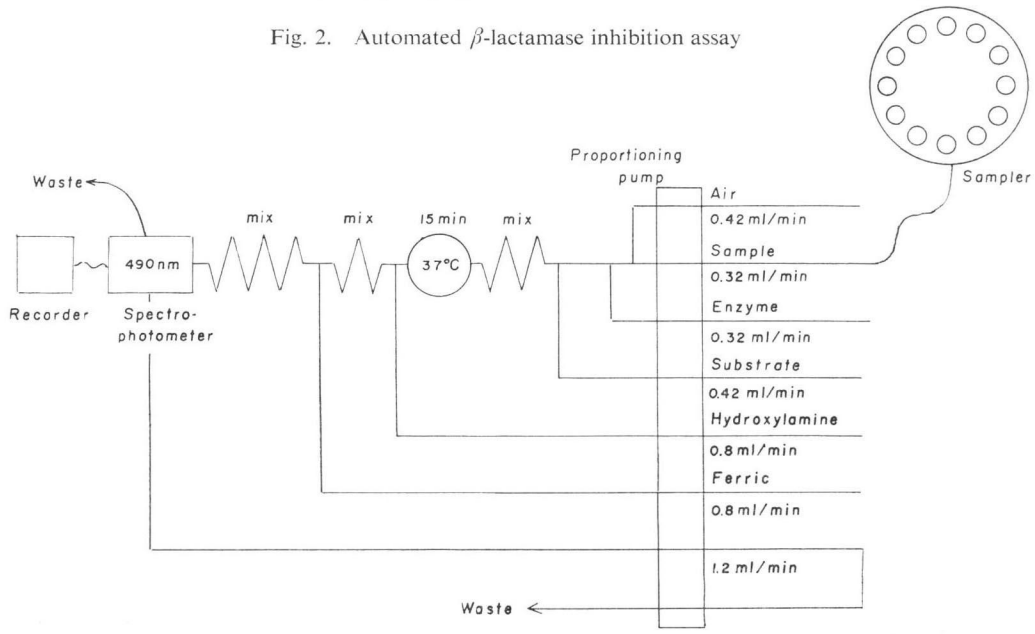
Paper chromatographic assay

Estimation of concentrations of MM 13902 and MM 17880 in culture filtrate samples were made by a bioautographic method. Culture filtrate (40 μl) was applied to anion-exchange paper strips (DEAE paper, DE81, Whatman Ltd., Maidstone, Kent, England) and developed with 0.2 M NaCl in 0.05 M phosphate buffer pH 7 at 4°C. The developed tapes were laid on an agar plate seeded with *Neisseria catarrhalis* NCTC 3622 and incubated at 37°C for 24 hours. *Neisseria catarrhalis* was maintained on slopes of Dorset egg medium (Mast Laboratories Ltd., Liverpool, England). A seed culture was prepared by inoculation into tryptone soya broth (Oxoid Ltd., London, England), 50 ml, contained in a 250-ml flask shaken at 37°C for 6 hours. Molten blood agar base (Oxoid), 300 ml, was cooled to 45~48°C and inoculated (15 ml) from the seed flask for preparation of the bioassay plates. Zones of inhibition due to MM 13902 and MM 17880 (Rf values are shown in Table 3) were traced, cut out and weighed. Comparison with standards of MM 13902 and MM 17880 allowed estimates of titres to be made.

β-Lactamase inhibition assay

An automated method for the assay of penicillins[14] was modified for measurement of β-lactamase

Fig. 2. Automated β-lactamase inhibition assay



inhibition, using standard Technicon autoanalyser equipment. A flow diagram is shown in Fig. 2. The reagents have the following composition:

Substrate: Benzyl penicillin, 4 mg/ml in 0.005 M phosphate buffer, pH 7.

Enzyme: R-TEM mediated β-lactamase, type IIIa[15], prepared by ultrasonication of *E. coli* JT4 cells. The supernatant of the disrupted cells was diluted with 0.005 M potassium phosphate buffer pH 7 to give just less than total degradation of the substrate under the assay conditions.

Hydroxylamine reagent: Mix fresh each day one volume stock hydroxylamine hydrochloride (350 g/litre), one volume stock alkaline buffer (NaOH 173 g/litre, anhydrous sodium acetate 20.6 g/litre), 4 volumes ethanol and 16 volumes distilled water.

Ferric reagent: Ammonium ferric sulphate, $NH_4Fe(SO_4)_2 \cdot 12H_2O$ (150 g/litre) in 0.5 M sulphuric acid.

The samples were monitored at 490 nm in a 10-mm path length flow cell. A standard line of MM 4550 concentration against peak height on the recorder trace (which is proportional to percentage inhibition) was constructed. Known concentrations of MM 13902 or MM 17880 giving peak height values falling on the standard line were then related to MM 4550 concentrations. The relative inhibitory activities of MM 4550, MM 13902 and MM 17880 are dependent on the preincubation time, *i.e.* the time of contact of enzyme with inhibitor before addition of the substrate. With a preincubation time of five seconds MM 4550 is approximately 250 times more active than MM 13902 or MM 17880. Using this system MM 4550 is determined within the range 5~50 μg/ml. By extending the preincubation time to 15 minutes at room temperature, the activities of MM 13902 and MM 17880 are increased to about one third of the MM 4550 activity.

Results and Discussion

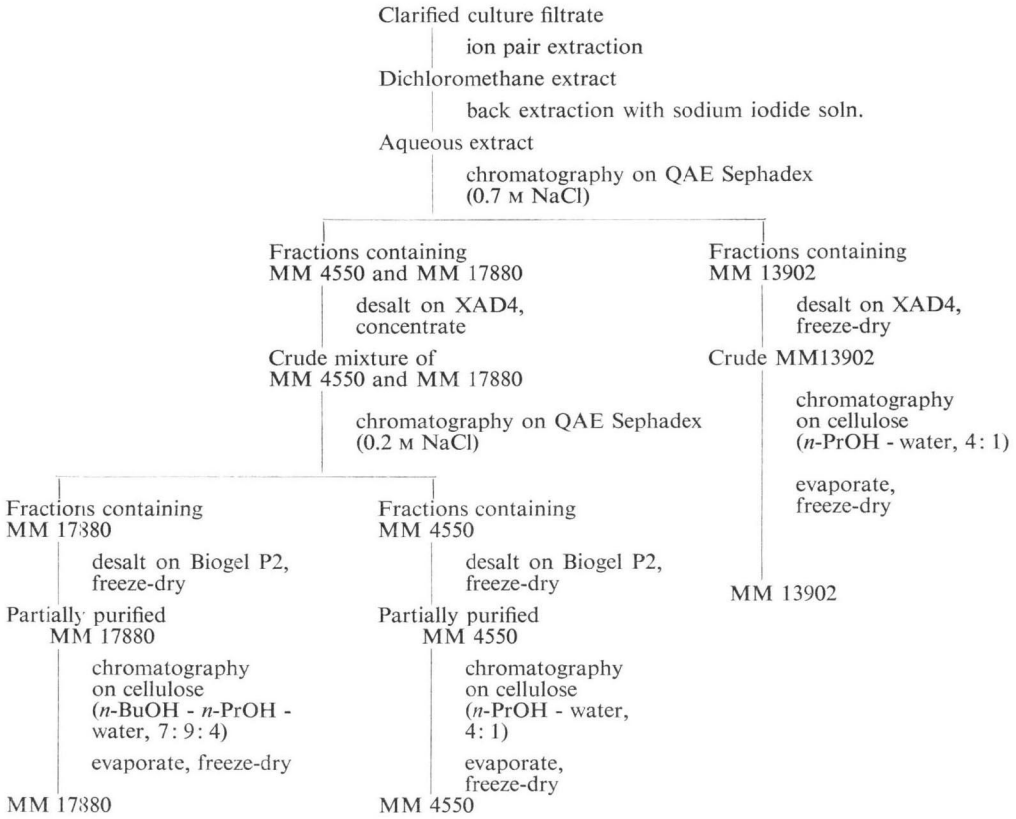
Isolation of MM 4550, MM 13902 and MM 17880

The isolation process is summarised in Fig. 3. Fermentation broth was clarified by filtration and the solids were discarded. Titres in the clarified broth were typically in the range 2~6 μg/ml for each antibiotic. All operations were carried out at 5°C unless otherwise stated. Culture filtrate (4,400 litres) at pH 7 was extracted with dichloromethane (900 litres) containing benzyldimethyl *n*-hexadecyl ammonium chloride (4 g/litre). The solvent phase was back extracted with aqueous sodium iodide (18 litres, 30 g/litre).

The sodium iodide extract was percolated at 60 ml/min through a column (150 mm × 230 mm) of QAE Sephadex A25 (Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated in 0.3 M NaCl in pH 7 phosphate buffer (KH_2PO_4 2.43 g/litre, K_2HPO_4 5.6 g/litre). The adsorbed antibiotics were eluted with 0.7 M NaCl in phosphate buffer, pH 7, at a flow rate of 35 ml/min. A mixture of MM 4550 and MM 17880 was eluted first followed by MM 13902. The metabolites were detected in the collected fractions by their u.v. absorption spectra and antibacterial activity.

Selected fractions were pooled and desalted as follows using Amberlite XAD4 (Rohm and Haas Co., Philadelphia). Sodium chloride (50 g/litre) was added to the pooled fractions, which were percolated through the column of Amberlite XAD4 resin (75 mm × 230 mm) at 20 ml/min. Under these conditions the metabolites were adsorbed to the resin, whereas inorganic salts and some organic impurities were not. The antibiotics were eluted with distilled water at room temperature. The MM 13902 eluate was freeze-dried to yield a partially purified solid. XAD4 eluate containing MM 4550 and MM 17880 was concentrated by evaporation *in vacuo* and submitted to further chromatography on QAE Sephadex. The concentrate was loaded on a column of QAE Sephadex A25 (54 mm × 200 mm) and eluted with 0.2 M NaCl at 3 ml/min. MM 17880 was eluted first, followed by MM 4550. The compounds were detected in the collected fractions by their u.v. absorption spectra and selected

Fig. 3. Isolation procedure for MM 4550, MM 13902 and MM 17880



fractions were pooled, concentrated by evaporation *in vacuo* and desalted by gel filtration on Bio-Gel P2 (Bio-Rad Laboratories, St. Albans, Herts, England). The partially purified MM 4550 and MM17880 were recovered from the eluates by freeze-drying.

Portions of the freeze-dried products (0.5 ~ 1.0 g) were purified by partition chromatography on columns (38 mm × 300 mm) of microgranular cellulose, grade CC31 (Whatman Ltd., Maidstone, Kent, England). For larger quantities of product the column bed volume was increased *pro rata* by using a column of greater diameter.

The columns used for MM 4550 and MM 13902 were eluted with *n*-propanol - water (4: 1) and the MM17880 column was eluted with *n*-butanol - *n*-propanol - water (7: 9: 4). The antibiotics were detected by their u.v. absorption spectra, selected fractions were pooled, evaporated *in vacuo* and freeze-dried. Typical yields of purified products from 4,400-litre culture filtrate were: MM 4550, 0.8 g, MM 13902, 2.0 g and MM 17880, 0.15 g.

The ion pair extraction technique was of considerable importance for the successful isolation of the antibiotics. With fermentation titres in the range 2 ~ 6 μg/ml for each antibiotic an efficient first step giving a good degree of purification and concentration was essential. Solvent extraction is frequently employed in antibiotic isolation for this purpose; however MM 4550, MM 13902 and MM 17880, being salts of very strong acids, are not extractable under neutral conditions and acidification to a pH where the acids are protonated results is very rapid degradation. Extraction in the

presence of a carrier was therefore considered and it was found that several commercially available quaternary ammonium salts formed solvent-extractable ion pairs with the antibiotics under neutral conditions. Some quaternary ammonium salts are compared in Table 2. In general the most lipophilic quaternary ammonium salts gave the highest extraction efficiencies. However the final choice depended not only on the efficiency but also the selectivity, since it was found that those salts giving the highest efficiency also extracted the most impurities. The sodium salts of the antibiotics were readily regenerated by back extraction with sodium iodide solution, leaving the quaternary ammonium iodide in the solvent phase. Using benzyldimethyl *n*-hexadecylammonium chloride as the quaternary salt followed by back extraction, a volume reduction from culture filtrate of greater than two hundred-fold was coupled with a purification of about forty-fold.

Table 2. Comparison of quaternary ammonium salts as ion pair extractants

Quaternary ammonium salt	Extraction efficiency (%)
Benzyldimethyl-*n*-hexadecylammonium chloride[a]	65
Tricaprylylmethylammonium chloride (Aliquat 336)	73
Tetra-*n*-butylammonium chloride[b]	trace
Dimethyldioctylammonium chloride (Bardac LF)	41
Dimethyldidecylammonium chloride (Bardac 22)	73

Culture filtrate (50 ml) was extracted with 5 mM quaternary ammonium salt in dichloromethane (25 ml). Assays were by β-lactamase inhibition with 15 minute preincubation. Dichloromethane extracts were diluted into dimethylformamide and phosphate buffer pH 7 for assay.

[a] BDH Chemicals Ltd., Poole, Dorset, England.

[b] Fluka Chemische Fabrik, CH 9470, Buchs, Switzerland. Aliquat 336 (General Mills Chemicals Inc., Minneapolis, Minn., U.S.A.) Bardac (Lonza Ltd., CH 4002, Basel, Switzerland)

Physico-chemical Properties

The three antibiotics were isolated as disodium salts in substantially pure form.

MM 4550, MM 13902 and MM 17880 are soluble in water, methanol, dimethylformamide and dimethylsulphoxide, but are insoluble in other common organic solvents. Infrared absorption spectra are shown in Fig. 4 and proton magnetic resonance spectra in Fig. 5. Ultraviolet absorption characteristics are as follows: λ_{max} (H_2O): MM 4550, 240 nm and 287 nm ($E^{1\%}_{1cm}$ 268); MM 13902, 227 nm and 307 nm ($E^{1\%}_{1cm}$ 356); MM 17880, 298 nm ($E^{1\%}_{1cm}$ 192). The spectra are illustrated in Fig. 6. Bathochromic shifts of the high wavelength maxima occur in acid, λ_{max} (0.01 M HCl): MM 4550, 293 nm; MM 13902, 319 nm; MM 17880, 310 nm. In acid solution the compounds are unstable and degradation leads to further changes in the spectra.

Chromatographic properties are presented in Table 3. The antibiotics were detected on chromatograms by bioautography on agar seeded with *Klebsiella pneumoniae*. MM 4550 may be visualised by spraying with EHRLICH's reagent—a blue colour develops on warming. MM 13902 and MM 17880 do not give this colour reaction. During high voltage paper electrophoresis the antibiotics migrate towards the anode. At pH 6.5 (pyridine-acetic acid buffer) and 5,000 V the Rm values of MM 4550, MM 13902 and MM 17880 were 2.0, 1.9 and 2.0 respectively compared to benzylpenicillin.

The antibiotics are unstable in aqueous solution outside a narrow pH range. MM 4550 is more acid stable than MM 13902 and MM 17880 but less alkaline stable. The half-lives for a range of pH values are shown in Table 4. The addition of hydroxylamine or cysteine to neutral solutions results in rapid degradation—similar reactions are well known for penicillins[16].

Fig. 4-a. I.r. spectrum of MM 4550 (KBr)

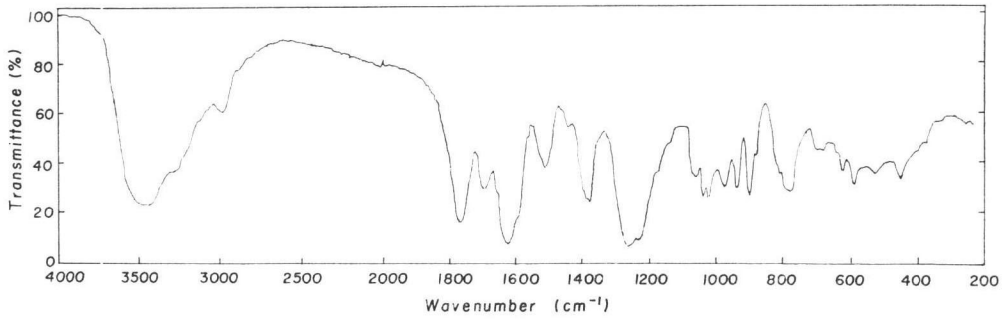


Fig. 4-b. I.r. spectrum of MM 13902 (KBr)

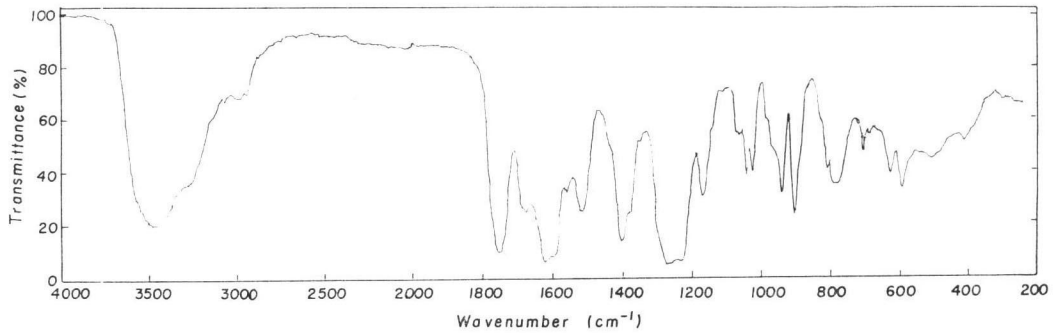


Fig. 4-c. I.r. spectrum of MM 17880 (KBr)

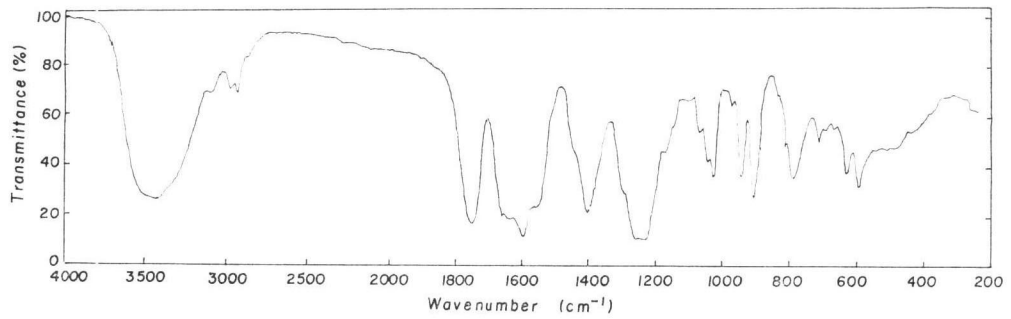


Fig. 5-a. Proton n.m.r. spectrum (90 MHz) of MM 4550 (D_2O, internal standard DSS)

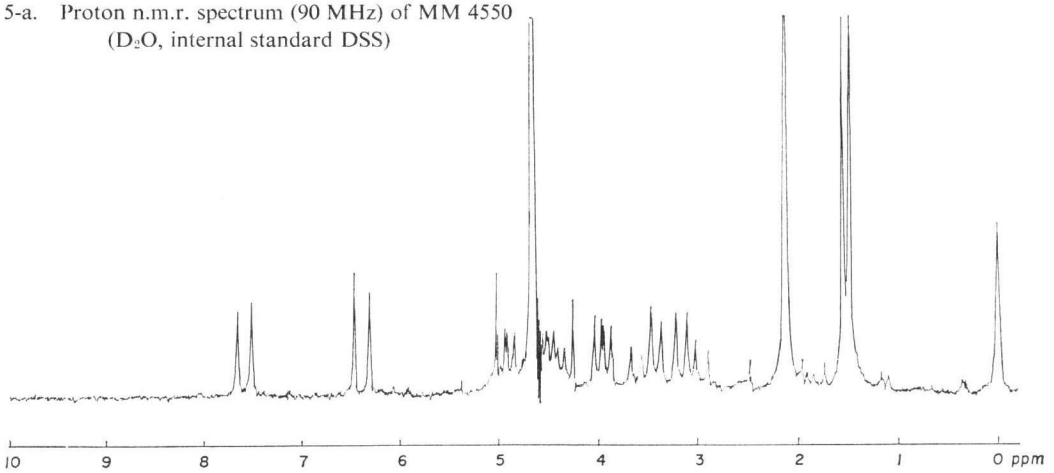


Fig. 5-b. Proton n.m.r. spectrum (90 MHz) of MM 13902
(D_2O, internal standard DSS)

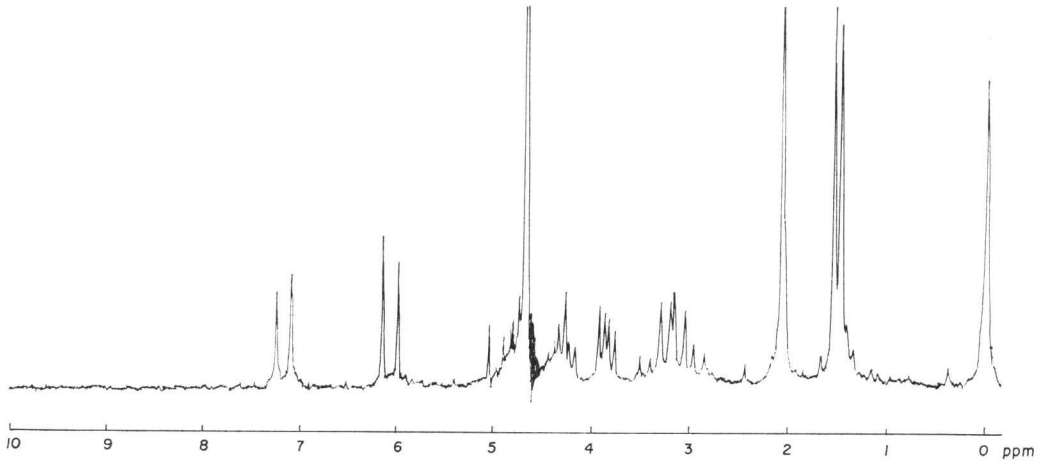


Fig. 5-c. Proton n.m.r. spectrum (90 MHz) of MM 17880
(D_2O, internal standard DSS)

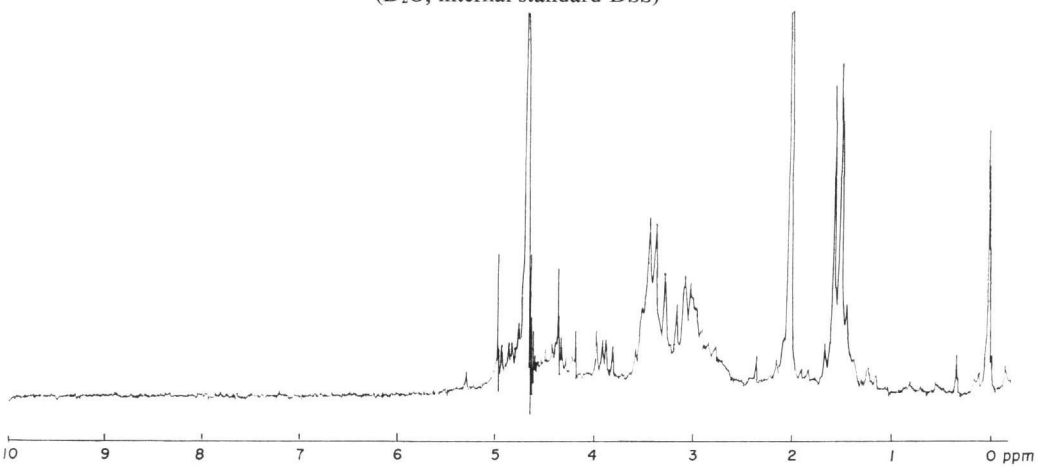
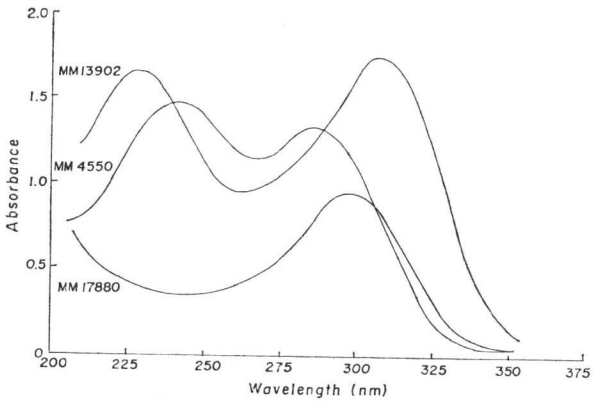


Fig. 6. U.v. spectra of MM 4550, MM 13902 and MM 17880
(50 μg/ml in 0.05 M potassium phosphate buffer pH 7.0)



Biological Properties

MM 4550, MM 13902 and MM 17880 are broad spectrum antibiotics. Some typical minimum inhibitory concentration (M.I.C.) values against a range of bacteria are shown in Table 5. In general good activity is seen against β-lactamase producing strains indicating stability of the antibiotics to these enzymes.

Inhibition of a range of β-lactamases by each of these metabolites is

shown in Table 6. The I_{50} values were obtained against cell-free preparations of the enzymes. Synergism with penicillins and cephalosporins against β-lactamase producing bacteria can be demonstrated.

Table 3. Chromatographic properties

System	Rf		
	MM 4550	MM 13902	MM 17880
T.l.c. cellulose (Eastman-Kodak) *n*-propanol - water (4: 1)	0.45	0.75	0.70
n-Butanol-isopropanol - water (7: 7: 6)	0.55	0.70	0.70
Isopropanol - water (7: 3)	0.70	0.85	0.80
Ion-exchange paper DE81 (Whatman) 0.2 M NaCl in 0.05 M phosphate buffer pH 7	*	0.40	0.60

* MM 4550 is inactivated in this system.

Table 4. Stability in aqueous solution at 25°C

pH	$t_{\frac{1}{2}}$		
	MM 4550	MM 13902	MM 17880
2.2	13.5 minutes	2.5 minutes	5 minutes
4.0	5.5 hours	7.5 minutes	15 minutes
6.0	18 hours	4 hours	5.5 hours
7.0	10.5 hours	12 hours	15 hours
7.8	8.5 hours	27 hours	27.5 hours
10.2	19 minutes	4 hours	2.5 hours

pH values 2.2~7.8: McIlvaines sodium phosphate-citric acid buffer. pH 10.2: sodium carbonate-bicarbonate buffer, 0.1 M
MM 4550 and MM 13902 at 60 μg/ml
MM 17880 at 100 μg/ml
Concentration was measured for each antibiotic by direct u.v. spectrophotometry at the high wavelength maximum.
Hydrolytic degradation results in products of lower extinction value at these wavelengths.

Table 5. Antibacterial spectrum of MM 4550, MM 13902 and MM 17880

Organism	MIC (μg/ml)		
	MM 4550	MM 13902	MM 17880
Bacillus subtilis	3.1	0.1	0.2
Citrobacter freundii Mantio*	12	0.8	0.8
Enterobacter cloacae P99*	200	25	25
Escherichia coli NCTC 10418	6.2	0.4	0.4
Escherichia coli JT39*	15	1.6	0.8
Klebsiella aerogenes A*	12	0.2	0.4
Proteus mirabilis C977*	3.1	0.2	0.4
Proteus vulgaris W096*	3.1	0.2	0.8
Proteus mirabilis C889*	3.1	0.4	0.8
Pseudomonas aeruginosa A*	500	62	125
Salmonella typhimurium CT10	15	0.2	0.4
Serratia marcescens US1*	6	0.4	0.8
Staphylococcus aureus Oxford	25	0.4~0.8	0.8
Staphylococcus aureus Russell*	50	1.6	1.6
Streptococcus pyogenes CN10	3.1	0.05	0.05

* β-lactamase producing strain

The antibiotics were diluted serially in tryptone soya broth (TSB) using microtitre trays and diluters. The inoculum was a 1/500 dilution of an overnight culture in TSB. *Streptococcus pyogenes* CN10 was grown in nutrient broth +10% serum and diluted 1/200.

Acknowledgements

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Table 6. β-Lactamase inhibitory activity of MM 4550, MM 13902 and MM 17880

Inhibitor	I_{50} (ng/ml)				
	Substrate: cephaloridine	Substrate: benzylpenicillin			
	Enterobacter cloacae P99	*Proteus mirabilis* C889	*Klebsiella aerogenes* E70	*Escherichia coli* JT4	*Staphylococcus aureus* Russell
MM 4550	4.0	0.4	1.6	0.5	15
MM 13902	0.6	1.0	50	20	50
MM 17880	0.9	2.0	60	10	75

I_{50} values were determined by the method described by C. READING and M. COLE[6]. Inhibitor was incubated with enzyme at 37°C for 5 minutes prior to addition of substrate (1 mg/ml final concentration).

spectra, C. READING for β-lactamase inhibition data and Mrs. P. HUNTER for antibacterial data.

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