MM 14201, A NEW EPOXYQUINONE DERIVATIVE WITH ANTIBACTERIAL ACTIVITY PRODUCED BY A SPECIES OF *STREPTOMYCES*

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A new antibiotic designated MM 14201 has been detected in a culture of *Streptomyces* sp. NCIB 11813. Methods for the production and purification of MM 14201 are described. Biological evaluation has shown it has broad spectrum antibacterial activity being most effective against *Serratia* and *Pseudomonas* sp. Structural studies are reported which have demonstrated MM 14201 is a new epoxyquinone derivative.

During screening of soil isolates a new antibiotic designated MM 14201 was found to be produced by *Streptomyces* sp. NCIB 11813. This compound was of particular interest because of its activity against *Pseudomonas* strains. Despite the unstable nature of MM 14201 its structure has been determined (Fig. 1) and it has been shown to be related to a group of previously described epoxyquinones[1,2].

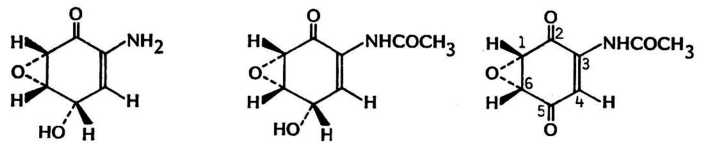
This paper describes the production, purification and properties of MM 14201, and studies undertaken to determine its structure. Some properties of derivatives of MM 14201 prepared during these studies are also reported.

Materials and Methods

Fermentation Conditions

Streptomyces sp. NCIB 11813 was maintained on agar slopes consisting of glucose monohydrate 1.0%, yeast extract 1.0%, Agar No. 3 (Oxoid Ltd) 1.5% prepared in tap water, pH 6.8. Slopes were incubated at 28°C for approximately 7 days. To an agar slope contained in a McCartney bottle was added sterile deionised water (10 ml), and a suspension of spores and mycelium prepared. Portions (2.5 ml) of this suspension were used to inoculate the seed stage medium (50 ml) contained in 250 ml. Erlenmeyer flasks closed with foam plastic plugs. The seed stage had the following composition: glucose monohydrate 3.0%, Bacteriological peptone (Oxoid Ltd) 1.0%, K_2HPO_4 0.2%, NaCl 0.1% prepared in deionised water, pH 7.0. The seed stage was incubated at 28°C on a gyrotary shaker for between 48 and 62 hours. Portions (2.5 ml) of the seed medium were used to inoculate the fermentation medium (50 ml) contained in 250 ml. Erlenmeyer flasks closed with foam plastic plugs. Fermentations

Fig. 1. Structures of MM 14201, MT 35214 and MT 36531.



MM 14201 MT 35214 MT 36531

were incubated on a gyrotary shaker at 28°C. The following basal media were used for the fermentation experiments and supplemented as described in the Results section:

(a) Carbon source basal medium: $NaNO_3$ 1%, Pharmamedia 0.1%, pH 7.0 in deionised water
(b) Complex nitrogen source basal medium: Glucose 1%, $NaNO_3$ 1.0% pH 7.0 in deionised water

The composition of the medium used for production and purification of MM 14201 was glycerol 1.0%, $NaNO_3$ 1.0%, Pharmamedia (cotton seed flour) 0.1%, $CaCO_3$ 0.5% prepared in deionised water, pH 7.0.

All media were sterilised at 121°C for 15 minutes before use.

Assay Methods

Antibacterial Activity: Samples were assayed using an agar plate diffusion assay using one or more of the following test organisms *Pseudomonas aeruginosa* G, *Bacillus subtilis* ATCC 6633, *Acinetobacter lwoffii* LSS7.

High Performance Liquid Chromatography (HPLC): The system comprised a Waters Associates Model 6000A solvent delivery system and U6K injector connected to a Waters RCM 100 radial compression unit containing a C_{18} Radi-pak cartridge (Waters Associates, Northwich, Cheshire, U.K.). Monitoring was with 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. The eluant was 0.05 M potassium phosphate buffer (pH 7.0) at 2 ml/minute giving a retention time of approximately 5.4 minutes for MM 14201.

Purification

The following column media were used in the purification procedure for MM 14201: Amberlite IRA 458; acrylic based strongly basic anion exchange resin from Rohm and Haas Co., Philadelphia, U.S.A., Diaion HP-20; styrene divinylbenzene cross linked polymeric adsorbent from Mitsubishi Chemical Industries, Tokyo, Japan.

Antibacterial and Antifungal Evaluation

Minimum Inhibitory Concentrations: The following media were used for growth of the test organisms; Todd-Hewitt Broth (Oxoid Ltd) for *Streptococcus pyogenes*; Brain Heart Infusion (Oxoid Ltd) plus Levintal Broth (2: 1) for *Haemophilus influenzae*; Schaedler broth (Oxoid Ltd) for *Bacteroides fragilis*; a broth composed of Mycological peptone (1%) and maltose (4%) was used for fungi; Nutrient Broth (Oxoid Ltd) for all remaining cultures.

The test compounds were serially diluted in 0.05 ml volumes of growth medium using microtitre equipment (Dynatech). All microtitre trays were inoculated with a multipoint inoculator (Denley) which delivered 0.001 ml of a 1/10 dilution of an overnight broth culture in the case of bacteria, or a spore/cell suspension in the case of fungi, an inoculum equivalent to 10^6 cfu/ml. Incubation was at 37°C for 18 hours for aerobic bacteria and for 24 hours at 37°C in 95% H_2/5% CO_2 in a Gaspak Jar (BBL) for *B. fragilis*. The yeasts and *Aspergillus niger* were incubated at 30°C for 18 hours and *Trichophyton mentagrophytes* for 72 hours at 30°C. The MIC was determined as the lowest concentration of antibiotic preventing visible growth.

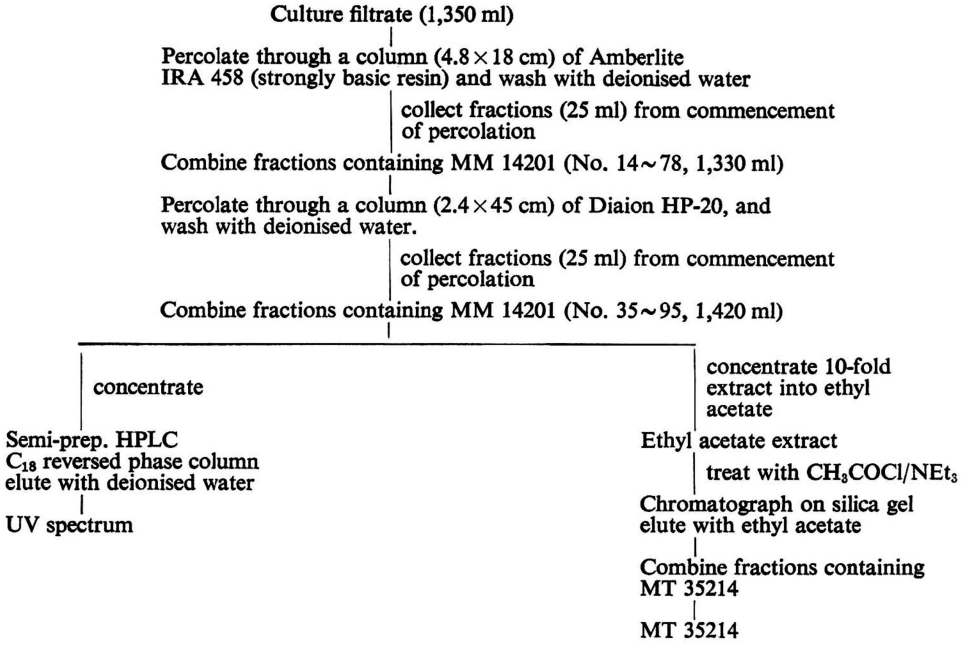
Viable Count Determinations: Solutions of MM 14201 (0.5 ml) were added to 4.5 ml volumes of Nutrient broth (Oxoid) to give a range of dilutions of the antibiotic. The broths were inoculated with 0.1 ml of a 1/10 dilution of an overnight culture of *P. aeruginosa* NCTC 10662, and incubated at 37°C. Samples were removed at intervals for viable count determinations on agar.

Structural Studies

Preparation of the *N*-Acetyl Derivative MT 35214: A purified aqueous solution containing MM 14201 (1,420 ml) prepared essentially as shown in Fig. 2 was evaporated under reduced pressure to give a more concentrated solution (85 ml). After saturation with salt the solution was extracted with ethyl acetate (3 × 80 ml) and the combined extracts dried ($MgSO_4$) and filtered.

The dried extract was stirred at 20°C and treated with triethylamine (150 mg, 1.5 mmol) followed by acetyl chloride (0.105 ml, 1.5 mmol). After stirring for 1 hour the solution was washed with water

Fig. 2. Purification procedure for MM 14201 and preparation of MT 35214.



(20 ml) and dried. Evaporation of the solvent afforded a gum which was purified by preparative TLC (silica gel, ethyl acetate eluant) to give MT 35214 (13 mg). Recrystallisation from ether - acetone gave colorless prisms, mp 149~151°C (dec.), $[\alpha]_D^{20}$ +104° (*c* 1, MeOH). Found: C 52.27, H 5.05, N 7.62. Calcd for $C_8H_9NO_4$: C 52.46, H 4.95, N 7.65. Calcd for $C_8H_9NO_4$: M^+ 183.0532, Found: *m/z* 183.0540.

Preparation of Dione MT 36531: To a stirred solution of the alcohol MT 35214 (21 mg, 0.115 mmol) in methylene dichloride (5 ml) was added sodium acetate (10 mg) followed by pyridinium chlorochromate (38 mg, 0.175 mmol) and the resulting suspension was stirred at 20°C for 1.5 hours. The solvent was evaporated and the residue chromatographed on silica gel. Elution with ethyl acetate - cyclohexane (1: 1) gave MT 36531 as a solid (7 mg, 33%). Recrystallisation from ethyl acetate - ether gave needles, mp 144~145°C, $[\alpha]_D^{20}$ −99° (*c* 0.5, MeOH). Calcd for $C_8H_7NO_4$: M^+ 181.0375, Found: *m/z* 181.0365.

The IR spectra were recorded on a Perkin Elmer 197 and the UV spectra on a Pye Unicam SP7-500 spectrometer. The ^1H and ^{13}C NMR spectra were both recorded at 250 MHz on a Bruker WM250 instrument, with tetramethylsilane as an internal standard. The accurate mass measurement was determined on a double focusing VG 70-70F mass spectrometer coupled to a Multispec 8 data system.

Results

Fermentation Studies

The results of the shake flasks medium development studies are outlined in Figs. 3 and 4. *Streptomyces* sp. NCIB 11813 was not fastidious in its requirements for either nitrogen or carbon sources. MM 14201 was produced at highest levels in complex nitrogen sources originating from plant materials. A range of carbon sources gave adequate MM 14201 levels, however glycerol was of particular interest because antibiotic levels were maintained over a longer time period in the fermentation. A characteristic aspect of these fermentations was the early production of MM 14201, peak titres being achieved at approximately 24 hours.

Fig. 3. Effect of carbon source on the production of MM 14201 by *Streptomyces* sp. NCIB 11813.
MM 14201 was assayed by HPLC (see methods).

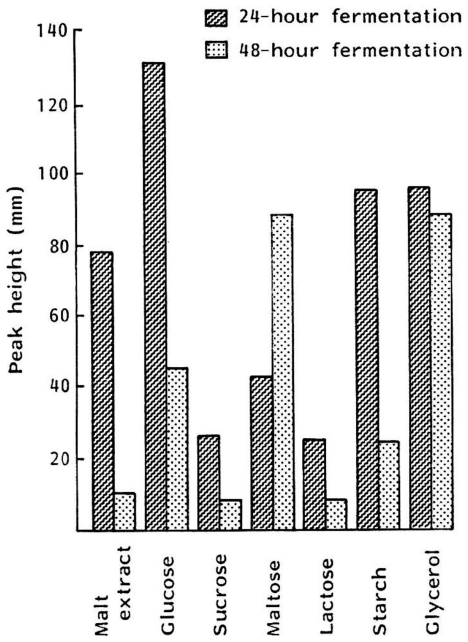
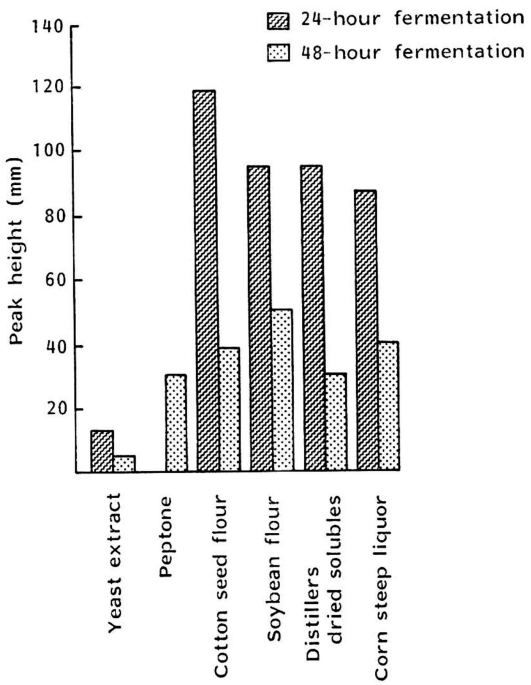


Fig. 4. Effect of complex nitrogen source on the production of MM 14201 by *Streptomyces* sp. NCIB 11813.
MM 14201 was assayed by HPLC (see methods).



Purification Studies

Preliminary attempts at the purification of MM 14201 were hampered by the unstable nature of the antibiotic. These studies showed that in solution, instability of MM 14201 increased as its concentration increased. Therefore the purification procedures developed were directed at the removal of impurities from dilute solutions of MM 14201. During these studies correlation between antibacterial activity and a particular component in reversed phase HPLC studies was observed. The use of this rapid HPLC assay system facilitated the development of the purification procedure outlined in Fig. 2. Attempts to prepare MM 14201 as a solid by freeze drying, precipitation or evaporation led to degradation of the antibiotic. Thus further biological properties were determined using purified solutions of MM 14201 and structural studies involved derivatisation. Characteristic physical properties determined for MM 14201 were its UV spectrum which showed a single maxima at approximately 304 nm, and its electrophoretic mobility. MM 14201 moved towards the cathode when submitted to high voltage paper electrophoresis in 0.05 M sodium phosphate buffer (pH 7).

Biological Properties

Since MM 14201 could not be prepared in solid form MIC determinations were carried out using a standardised solution of the antibiotic. A purified solution of MM 14201, prepared essentially as described in Fig. 2, was concentrated *in vacuo* to give an optical density of 0.6 units at 304 nm using a 1 mm path. The MIC values expressed as dilutions of this standard solution are shown in Table 1. MM 14201 showed broad spectrum antibacterial activity and weak activity against *Candida albicans*. Interestingly it had greatest activity against strains of *P. aeruginosa* and *Serratia marcescens*.

Table 1. Antibacterial activity of MM 14201, MT 35214 and MT 36531.

Organism	MIC (μg/ml)		
	MM 14201*	MT 35214	MT 36531
Escherichia coli ESS	1/4	125	62.5
E. coli NCTC 10418	1/4	125	125
Pseudomonas aeruginosa NCTC 10662	1/16	250	125
P. aeruginosa 1771	1/32	125	—
P. aeruginosa G	1/32	250	—
P. aeruginosa W14	1/16	250	—
Serratia marcescens US 32	1/16	62.5	125
Klebsiella aerogenes A	1/4	250	250
Enterobacter cloacae N1	1/2	62.5	62.5
Proteus mirabilis C977	1/2	125	125
P. morganii	1/4	62.5	31
P. rettgeri	1/2	62.5	31
Haemophilus influenzae Q1	1/2	15.5	—
Bacteroides fragilis 146	1/4	62.5	—
Staphylococcus aureus 'Oxford'	1/4	125	125
Bacillus subtilis	1/2	250	125
Streptococcus pyogenes CN10	1/2	125	—
Candida albicans	1/4	125	—
Saccharomyces cerevisiae	0	>250	—
Trichophyton mentagrophytes	0	>250	—
Aspergillus niger	0	>250	—

MIC's were determined by the microtitre method (see Methods).

* Results for MM 14201 are expressed as dilutions of a standardised solution (see Results).

Fig. 5. Bacteriocidal action of MM 14201 against *P. aeruginosa* 10662.

Levels of MM 14201 expressed as dilutions of standardised solution.

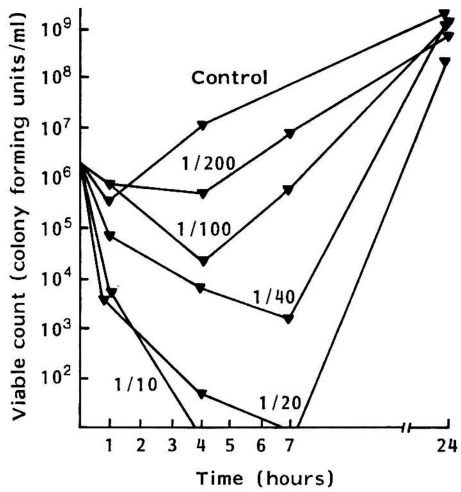


Table 2. IR spectra of MT 35214 and MT 36531.

Assignment	ν_{max} ($CHCl_3$) cm^{-1}	
	MT 35214	MT 36531
NH	3390	3370
OH	3400 (broad)	—
Ketone C=O	1680	1710 1695
Amide C=O	1680 1510	1680 1500
C=C	1630	1610

MT 35214 had weak broad spectrum antibacterial activity but did not display the specific activity against *Pseudomonas* strains which was so characteristic of MM 14201. It retained some activity against *C. albicans*. MT 36531 although tested against a more limited range of organisms had similar activity to MT 35214.

In view of the instability of MM 14201 a viable count determination of its activity against *P. aeruginosa* 10662 was carried out. The results of these determinations at a number of dilutions are shown in Fig. 5. This demonstrated that MM 14201 was bacteriocidal at concentrations several times lower than its MIC. The rate of breakdown of MM 14201 in nutrient broth at 37°C was also deter-

Table 3. ^{1}H NMR spectra of MT 35214 and MT 36531.

Assignment	δ ppm ($CDCl_3$)	
	MT 35214	MT 36531
$COCH_3$	2.14 (s)	2.24 (s)
OH	2.69 (d), J=11 Hz, D_2O exch.,	—
C(1)H	3.60 (d), J=4.0 Hz	3.93 (d), J=3.5 Hz
C(6)H	3.89 (ddd), J=4.0, 3.2, 2.2 Hz	3.85 (dd), J=3.5, 2.0 Hz
C(5)H	4.87 (ddd), J=11, 3.2, 3.0 Hz	—
C(4)H	7.45 (dd), J=3.0, 2.2 Hz	7.54 (d), J=2.0 Hz
NH	7.57 (broad s), D_2O exch.,	7.87 (broad s)

Table 4. ^{13}C NMR spectrum of MT 35214.

δ ppm ($CDCl_3$)	Assignment
24.67 (q)	$COCH_3$
52.75 (d)	C (1) or C (6)
54.29 (d)	C (6) or C (1)
64.68 (d)	C (5)
125.41 (d)	C (4)
128.51 (s)	C (3)
169.64 (s)	$COCH_3$
188.80 (s)	C (2)

mined, the antibiotic lost 43% of its activity in a 3-hour period, which suggested that the regrowth which occurred subsequently to the bacteriocidal action was due to instability of the compound.

Structural Studies

Preparation of the acetyl derivative of MM 14201 was of particular importance in the structural studies on this antibiotic. The UV spectrum of MT 35214 (in EtOH) exhibited absorption maxima at 213 (ε 10,200) and 278 nm (3,360). When this spectrum was compared with that of the parent amino compound it became apparent that the amino function must be closely associated with the chromophore. The mass spectrum of MT 35214 indicated a molecular formula of $C_8H_9NO_4$. The most striking feature of the IR spectrum (Table 2) was the single strong carbonyl absorption at 1680 cm^{-1} characteristic of α,β-unsaturated ketones and amides. The presence of a broad hydroxyl absorption at 3400 cm^{-1} was clearly noticeable under the sharp amide NH absorption. The ^{1}H NMR spectrum of MT 35214 (Table 3) was consistent with that of an α,β-unsaturated ketone and featured the characteristic low-field β-proton at δ 7.45. Two exchangeable protons at δ 7.57 and δ 2.69 supported the presence of a secondary amide NH and a hydroxyl group respectively. Furthermore, it was clear from the coupling of the hydroxyl proton with its α-proton that the compound was a secondary alcohol. From this data it became obvious that the amido group must be located at the α-position of an α,β-unsaturated ketone. Confirmation of this part structure was obtained from the ^{13}C NMR spectrum (Table 4) which indicated a total of 8 carbon atoms including two carbonyl carbons at δ 188.80 and δ 169.64 and two olefinic carbons at δ 128.51 and δ 125.41. The off-resonance decoupled spectrum indicated that only one of the olefinic carbons was attached to a proton, the other presumably bearing the acetamido functionality. Consideration of the above data led directly to the proposed structure (Fig. 1). However, the relative stereochemistry of the hydroxyl and epoxide groups could not be determined by ^{1}H NMR and it was found necessary to resort to X-ray crystallographic analysis to demonstrate their *cis* relationship (T. J. KING, unpublished results). Although the absolute stereochemistry of MT 35214 has not been determined it seems likely that it is as indicated by analogy with the structurally-related natural products, the enaminomycins[3,4].

The UV spectrum of MT 36531 (in EtOH) exhibited λ_{max} 312 nm (ε 6,800). The other spectroscopic data (Tables 2 and 3) was consistent with the epoxy-quinone structure (Fig. 1).

Discussion

The antibacterial activity produced by *Streptomyces* sp. NCIB 11813 was of particular interest because of its unusual spectrum with greatest activity against *Pseudomonas* sp.

MM 14201 was originally detected using a chemically defined medium which produced only low levels of activity and this rapidly degraded. The fermentation experiments were of value not only by increasing the titres of MM 14201 but in yielding a more stable and reproducible fermentation. The unstable nature of MM 14201 presented particular problems during purification. These difficulties were exacerbated by the autodegradative properties of MM 14201 which precluded preparation of concentrated solutions of the antibiotic. The development of an HPLC assay was of particular importance with such a labile compound since it allowed rapid processing during purification studies. Electrophoretic studies showed MM 14201 was basic in character and acylation of the amino function to yield MT 35214 overcame the stability problems with this molecule. Structural studies with MT 35214 led to the elucidation of the structure of MM 14201.

The antibacterial spectrum of MM 14201 was unusual and of interest because of its activity against *Pseudomonas* strains. Although it was not possible to accurately quantify the activity of MM 14201, estimates of concentration related to UV extinction and absorbance values of test solutions suggest the MIC values to be significantly below 10 μg/ml for *Pseudomonas* strains. When considered in relation to the viable count results and instability of MM 14201 the intrinsic activity of the compound is probably much greater than implied by the MIC results.

Similarities between the biological properties of MM 14201, MT 35214 and MT 36531 with those of the enaminomycins[3] are clear. However it is interesting that the presence of the free amino function in MM 14201 was responsible for its relatively greater activity against *Pseudomonas* strains, and greater intrinsic activity in general. Despite its interesting antibacterial spectrum MM 14201 is not of commercial interest due to its poor stability.

Recently the discovery in a *Streptomyces* sp. of a structural isomer of MT 35214 has been reported[2]. The compound LL-C10037 has weak antibacterial and antifungal activity as well as antitumor activity.

Acknowledgements

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