HOLOMYCIN AND AN ANTIBIOTIC (MM 19290) RELATED TO TUNICAMYCIN, METABOLITES OF *STREPTOMYCES CLAVULIGERUS*

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Streptomyces clavuligerus produces penicillin N, several cephalosporins and the β-lactamase inhibitor clavulanic acid. The detection, isolation and properties of further metabolites of this culture, MM 21801 and MM 19290, are described. MM 21801 was identified as the antibiotic holomycin. MM 19290 was shown to be related to tunicamycin, an antibiotic complex obtained from cultures of *Streptomyces lysosuperificus*.

Streptomyces clavuligerus ATCC 27064 (NRRL 3585) was first described as a producer of penicillin N and of a number of antibiotics structurally related to cephalosporin C, namely the 3-carbamoyloxymethyl analogue and the 7-methoxy derivative of the latter (cephamycin C), and deacetoxycephalosporin C[1,2,3]. More recently, a potent inhibitor of β-lactamase, clavulanic acid, was isolated from *Streptomyces clavuligerus*[4,5], and shown to be a novel fused β-lactam.[6] The culture has since also been found to produce a series of clavams with antifungal activity.[7]

We report here the detection and isolation of further antibiotics from *Streptomyces clavuligerus*, which have been identified as holomycin (MM 21801) and a complex (MM 19290) related to tunicamycin.

Detection Methods

In order to detect the presence of substances with antibacterial activity in cultures of *Streptomyces clavuligerus*, a biochromatographic procedure was used. Samples of the culture filtrate were applied to 1-cm-wide paper strips of Whatman Grade 1, and descending chromatography carried out at 4°C. The dried strips were contacted with agar plates seeded with *Bacillus subtilis* ATCC 6633, *Sarcina lutea* NCTC 8340 or *Klebsiella pneumoniae* ATCC 29665 as test organisms. In addition, a special bioautographic method, in which the agar contained both *Klebsiella pneumoniae* ATCC 29665 and benzylpenicillin, was used to locate the β-lactamase inhibitor clavulanic acid.[5]

MM 21801

Fermentation and Isolation

Mycelium and spores from an agar slope of a mutant strain of *Streptomyces clavuligerus*, designated IT1, produced by far UV irradiation of the parent culture, were used to inoculate 500-ml flasks containing 100 ml of a chemically defined medium at pH 7, consisting of 1.5% glycerol, 2% sucrose, 0.25% proline, 0.15% sodium glutamate, 0.5% NaCl, 0.2% K_2HPO_4, 0.04% $CaCl_2$, 0.01% $MnCl_2 \cdot 4H_2O$, 0.01% $FeCl_3 \cdot 6H_2O$, 0.005% $ZnCl_2$, 0.1% $MgSO_4 \cdot 7H_2O$ in deionised water. Ten ml of the seed culture were transferred after 4 days to each of a series of 2-liter flasks containing 500 ml of the same medium. The flasks were incubated at 26°C on a rotary shaker (240 rpm, 5-cm throw) for 72 hours. Three active metabolites were detected in the culture broth by biochromatography, of

Table 1. Paper chromatography of metabolites of *Streptomyces clavuligerus*.

Metabolite	Rf on Whatman Grade 1 paper strips			Bioautographic detection system
	Butan-1-ol - acetic acid - water (12: 3: 5)	Butan-1-ol - ethanol - water (4: 1: 5, top phase)	Butan-1-ol - pyridine - water (1: 1: 1)	
Cephamycin C	0.15	0.02	0.28	*Klebsiella pneumoniae* ATCC 29665
Penicillin N	0.36	0.03	0.35	*Sarcina lutea* NCTC 8340
Clavulanic acid	0.63	0.17	0.59	*Klebsiella pneumoniae* ATCC 29665 + benzyl penicillin
MM 21801	0.78	0.73	0.78	*Sarcina lutea* NCTC 8340
MM 19290	0.75	0.73	0.85	*Bacillus subtilis* ATCC 6633

Fig. 1. Isolation of MM 21801 from *Streptomyces clavuligerus* culture broth.

Culture broth (10 liters), pH 8.3

↓ extraction with 1/3 vol of butan-1-ol

Butanol extract (3 liters)

↓ washed with equal volume of water. butanol extract vacuum-concentrated to an oil and dissolved in chloroform.

Chloroform extract (10 ml)

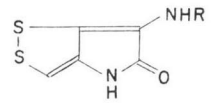
↓ column chromatography on silica gel 60 (Merck) using chloroform-methanol, 9: 1. active fractions combined and dried under vacuum.

MM 21801 (yellow powder, 80 mg)

↓ dissolved in hot ethyl acetate. crystallised at 4°C

Crystalline MM 21801 (orange-yellow prisms, 30 mg)

Fig. 2. Structure of holomycin and N-propionylholothin.



(a) Holomycin R = CH_3CO-
(b) N-Propionylholothin R = CH_3CH_2CO-

which two were identified as cephamycin C and clavulanic acid. The third component had activity against both Gram-positive and Gram-negative bacteria, and was stable to the β-lactamases of *Escherichia coli* JT4 (R factor mediated) and *Enterobacter cloacae* P99. It was chromatographically distinct from other metabolites of *Streptomyces clavuligerus* and was designated MM 21801 (Table 1). The antibiotic MM 21801 was isolated from 10 liters of the culture broth by the procedure outlined in Fig. 1.

MM 21801 was only detected in culture broths of the IT1 mutant of *Streptomyces clavuligerus*, and was not observed to be a metabolite of the parent strain. Three of the β-lactam antibiotics which have been reported to be produced by *S. clavuligerus*, penicillin N, deacetoxycephalosporin C and the 3-carbamoyloxymethyl analogue of cephalosporin C, were not detected in the fermentation broths of either the mutant strain or the parent culture, when grown under the conditions described above.

Physical Properties

The crystalline preparation of MM 21801 was identified as the antibiotic holomycin (Fig. 2a) on the basis of the following spectral data. UV spectrum: λ_{max}^{MeOH} nm (ε); 246 (4665), 301 (2354), 388 (7918). ^1H NMR spectrum (90 MHz, DMSO-d_6) δ: 1.98 (3H, s, CH_3CO–), 6.97 (1H, s, >C=CH–), 9.75 (1H, s, –CONH–), 10.56 (1H, s, –CONH–). Mass spectrum: *m/e* 214 (M^+), 172

($M^+ - CH_2CO$, base peak) and 43 (CH_3CO^+). Precise mass measurement gave *m/e* 213.9874 (M^+, 213.9870 calculated for $C_7H_6N_2O_2S_2$). The IR spectrum was very close to that published for holomycin.

MM 19290

Fermentation and Isolation

An agar slope culture of the *Streptomyces clavuligerus* mutant IT1 was also used to inoculate 500-ml flasks containing 100 ml of a seed medium consisting of 1% malt extract (Oxoid), 1% bacteriological peptone (Oxoid) and 2% glycerol, in tap water (pH adjusted to 7.0 prior to sterilisation). Fifteen ml of the seed culture were transferred after 4 days to each of a series of 2-liter flasks containing 500 ml of production medium consisting of 1.0% triglyceride (Prichem P224, Prices Ltd., Bromborough, Bebington, Cheshire, U.K.), 1.5% soya bean flour (Arkasoy 50, British Arkady Co. Ltd., Old Trafford, Manchester, U.K.) and 0.1% KH_2PO_4, in deionised water (pH adjusted to 7.0 prior to sterilisation). The flasks were incubated at 26°C on a rotary shaker (240 rpm, 5-cm throw) for 92 hours. Four active metabolites were detected in the culture broth by biochromatography. These were clavulanic acid, cephamycin C, penicillin N, and a lipophilic antibiotic, which was separated by paper chromatography from the other metabolites of *Streptomyces clavuligerus* and was designated MM 19290 (Table 1). This substance inhibited the growth of *Bacillus subtilis*, but had no activity against Gram-negative organisms. It was stable to the β-lactamase of *E. coli* JT4. The antibiotic MM 19290 was isolated from 6 liters of the culture broth by the procedure outlined in Fig. 3.

Fig. 3. Isolation of MM 19290 from *Streptomyces clavuligerus* culture broth.

Culture broth (6 liters), pH 7.3
↓ extraction with 1/3 vol of butan-1-ol.
Butanol extract (1,200 ml)
↓ concentrated under vacuum to 150 ml. precipitation with 5 vols of acetone.
Acetone-insoluble precipitate
↓ dissolved in 600 ml of butan-1-ol. butanol extract washed with equal volume of water.
Butanol extract (600 ml)
↓ concentrated under vacuum. column chromatography on silica gel 60 (Merck) using butan-1-ol - ethanol - water, 16: 4: 7; active fractions combined, concentrated, dissolved in ethanol and dried under vacuum.
MM 19290 (white powder, 200 mg)

MM 19290 was produced in significantly smaller amounts by the parent culture of *S. clavuligerus*, compared with the IT1 mutant strain. Deacetoxycephalosporin C and the 3-carbamoyloxymethyl analogue of cephalosporin C were not detected in the fermentation broths of either the mutant strain or the parent culture when grown in the above triglyceride medium.

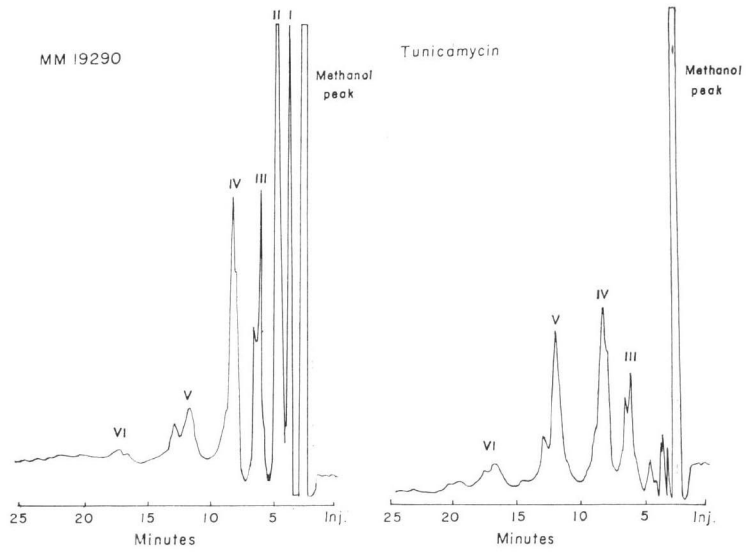
Physical Properties

MM 19290 was obtained as a white powder with UV spectrum: λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$); 214 (162), 259 (74). This data, together with the IR spectrum, indicated that MM 19290 was the same as, or very similar to tunicamycin, a glucosamine-containing antibiotic with activity against Gram-positive bacteria, fungi and viruses, first isolated from *Streptomyces lysosuperificus*[8,9].

The presence of glucosamine in the acid hydrolysate of MM 19290 was shown by both the ELSON-MORGAN reaction and by use of an amino acid analyser. No differences were detected between the UV spectrum of MM 19290 and that of a sample of authentic tunicamycin, and the two

Fig. 4. H.p.l.c. of MM 19290 and tunicamycin.

Column: 5 μm Spherisorb ODS (Phase Separations, Queensferry, Clwyd, U.K.)
Solvent: 55% 0.01 M HCOOH adjusted to pH 4.5 with NaOH, 45% CH_3CN
Flow rate: 1 ml/min
UV Detection: 220 nm, 0.05 Aufs
Loading of compounds: 20 μl of MM 19290 at 200 μg/ml in methanol
20 μl of tunicamycin at 125 μg/ml in methanol



preparations had the same chromatographic mobility on thin-layer plates and paper in several solvent systems. MM 19290 was also compared directly with tunicamycin by h.p.l.c. (Fig. 4). Both samples gave four corresponding groups of peaks with identical retention times (groups III, IV, V and VI, Fig. 4). However, the MM 19290 preparation gave two additional peaks (I and II, Fig. 4) of significant intensity, closer to the solvent front, which were not clearly visible when tunicamycin was chromatographed under the same conditions. In order to determine whether the components responsible for these peaks had antibacterial activity, a separate fraction was collected from the h.p.l.c. column for each of the peaks, or groups of peaks, I ~ VI, produced by the sample of MM 19290 (Fig. 4). When analysed by biochromatography on paper, all six fractions gave zones of inhibition in the position of MM 19290, suggesting that the preparation of MM 19290 was a complex containing at least six related antibiotics.

Discussion

Holomycin is a member of the pyrrothine group of antibiotics and was originally described as a metabolite of *Streptomyces griseus*[10]. Subsequent to our isolation of the antibiotic from *Streptomyces clavuligerus*, OKAMURA *et al.*[11] reported that holomycin and N-propionylholothin (Fig. 2b) were obtained from a mutant of *Streptomyces* sp. P6621, which, in common with *S. clavuligerus*, is a producer of cephamycin C. More recently, KIRBY has discussed the genetic control of the synthesis of holomycin by *S. clavuligerus*.[12]

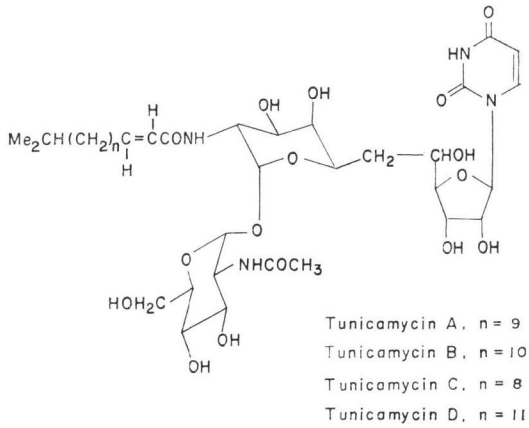
During the isolation of MM 21801 from the mutant culture of *S. clavuligerus* by the column procedure illustrated in Fig. 1, two yellow antibiotics with activity against *Sarcina lutea* were detected

Table 2. T.l.c. of partially-purified preparations of holomycin from *S. clavuligerus*.

Solvent system	Rf Values of components with antibacterial activity	
	Holomycin	Unknown components
Benzene - acetone (1 : 1)	0.30	0.39
Chloroform - methanol (9 :1)	0.50	0.45, 0.58

on t.l.c. plates (Merck silica gel 60) in addition to holomycin (Table 2). This may indicate the production of other members of the pyrrothine group of antibiotics by *Streptomyces clavuligerus*.

Fig. 5. Structure of tunicamycin.



Tunicamycin is a member of the group of streptomycete antibiotics which includes mycospocidin,[13] antibiotic 24010,[14] and the streptovirudins of series II,[15] and is reported to be an inhibitor of peptidoglycan synthesis.[16]

After the present work had been completed, the structure of tunicamycin (Fig. 5) was published by TAKATSUKI *et al.*[17] Tunicamycin was shown to be a mixture of four homologous antibiotics which differ in the length of the carbon chain of the *trans* α,β-unsaturated iso-fatty acid component (where n=8, 9, 10 or 11). The four common groups of peaks (III~VI, Fig. 4) which are observed after h.p.l.c. of both the preparations of tunicamycin and MM 19290, may therefore be due in part to the four components of the tunicamycin complex which have been described by TAKATSUKI *et al.* The chromatogram of tunicamycin shows a number of peaks grouped in pairs, or in the case of group IV (Fig. 4), a main peak with two shoulders, and may indicate that the antibiotic complex consists of more than the four components reported by TAKATSUKI. These extra components may represent isomeric forms. In addition, it would appear that the two peaks with lowest retention time, I and II, observed with MM 19290, which are absent from the h.p.l. chromatogram of the sample of authentic tunicamycin, are due to further, as yet uncharacterised homologous antibiotics with different fatty acid side chains. The relative intensities of the corresponding peaks observed with both the MM 19290 and tunicamycin samples are also different, which suggests that the common components are not present in the same proportions in the two preparations. The h.p.l.c. studies have shown therefore that in these respects, the MM 19290 complex isolated from *S. clavuligerus* differs from the tunicamycin complex (ex. *S. lysosuperificus*).

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