Identification of the Antibiotic Determined by the SCP₁ Plasmid of *Streptomyces coelicolor* A3(2)

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SUMMARY

The antibiotic whose biosynthesis is determined by the SCP₁ plasmid of *Streptomyces coelicolor* A3(2) has been characterized as the recently described methylenomycin A (2-methylene-cyclopentan-3-one-4,5-epoxy-4,5-dimethyl-1-carboxylic acid).

INTRODUCTION

The wild-type *Streptomyces coelicolor* A3(2) harbours a plasmid, SCP₁ (Vivian, 1971). This, and other strains bearing SCP₁ autonomously, have been called SCP₁⁺. The plasmid is readily transmitted to SCP₁[−] derivatives in mixed culture. By interacting with the chromosome in various ways and at several alternative positions, SCP₁ is responsible for high frequencies of chromosomal marker transfer between derivatives of strain A3(2) (Hopwood *et al.*, 1973; Vivian & Hopwood, 1973; Hopwood & Wright, 1976). In NF strains, the plasmid appears to be stably integrated into the chromosome, while in SCP₁' strains, analogous to F' strains of *Escherichia coli* K12, the plasmid acquires chromosomal insertions (Hopwood & Wright, 1973*b*). The plasmid is transferable to certain other *Streptomyces* species (Hopwood & Wright, 1973*a*). These genetic studies provide overwhelming evidence for the existence of SCP₁, even though extrachromosomal DNA corresponding to it has not yet been identified (Schrempf *et al.*, 1975).

In the original description of the phenotypic characteristics attributed to SCP₁, Vivian (1971) found that SCP₁⁺ strains (then called IF) inhibited the growth of SCP₁[−] (UF) strains, through the agency of a diffusible material, when the two cultures were inoculated close together on agar media. The plasmid evidently determined production of and resistance to this inhibitor. It was thought that the action of the inhibitor was specifically against aerial mycelium production by the sensitive SCP₁[−] strain.

Further work, briefly reported by Kirby, Wright & Hopwood (1975), showed that the inhibitor is a low molecular weight material, rather than a bacteriocin or bacteriophage, that it is synthesized by a stepwise process, and that it has a broad antibacterial spectrum; in other words, it is an antibiotic.

This paper describes the identification of this substance as methylenomycin A, a new antibiotic recently isolated from a different streptomycete and characterized by Haneishi *et al.* (1974*a*). We believe that methylenomycin production by *S. coelicolor* A3(2) provides the first adequately documented example of plasmid-determined antibiotic synthesis in an actinomycete.

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Table 1. *Streptomyces* strains

All strains were from the John Innes Institute Culture Collection.

Strain no.	Description	Plasmid status
<i>Streptomyces coelicolor</i> A3(2) derivatives		
A3(2)	wild-type	SCP1 ⁺
12	<i>pheA1</i>	SCP1 ⁺
104	<i>hisA1 uraA1 strA1</i>	SCP1 ⁺
949	<i>cysA15 proA1 argA1 uraA1 nicA1 tps-33</i>	NF
1098	<i>pheA1</i>	SCP1 ⁻
1190	<i>hisA1 uraA1 strA1</i>	SCP1 ⁻
1984	<i>hisD3 cysB6 strA1 SCP1'-cysB⁺</i>	SCP1'-cysB ⁺
2136	<i>adeA13 pheA1 strA5</i>	SCP1 ⁺
A200	<i>adeCv10 pheA1 strA5</i>	SCP1 ⁻
A332	<i>pheA1</i>	NF
A688	<i>uraB2 pheA1 strA1</i>	SCP1 ⁻
A700	<i>proA1 argA1 cysD18</i>	SCP1 ⁻
R70, R72, R74	<i>hisD3 cysB6 strA1 SCP1'-cysB⁺ ant⁻</i>	SCP1'-cysB ⁺ (ant ⁻)
Other strains*		
1152	Sermonti's <i>S. coelicolor</i> 155 wild-type	SCP1 ⁻
1153	Bradley's <i>S. coelicolor</i> 199 wild-type	SCP1 ⁻
1326	Lomovskaya's <i>S. lividans</i> 66 wild-type	SCP1 ⁻
1671	Lomovskaya's <i>S. lividans</i> 66 wild-type	SCP1 ⁺

* For the origin of wild-type strains 155 and 199, see Hopwood & Sermonti (1962); for strain 66, see Lomovskaya *et al.* (1972).

METHODS

General. Complete medium (CM) and standard cultural techniques were those described by Hopwood (1967). Liquid CM was CM with agar omitted. Nutrient agar was Difco Bacto-nutrient agar.

Strains. *Streptomyces* strains, which were derivatives of *Streptomyces coelicolor* A3(2), and other wild-type strains were from the John Innes Institute culture collection (Table 1). The strains of micro-organisms used in testing for sensitivity to SCP1-determined antibiotic are listed in Table 2.

Growth of liquid cultures of Streptomyces coelicolor. Liquid CM (50 ml) in 250 ml Erlenmeyer flasks was inoculated with approximately 10⁷ washed spores from a slant culture of strain 104 (SCP1⁺) or strain 1190 (SCP1⁻). For large quantities, 400 ml medium in 21 Erlenmeyer flasks was inoculated with approximately 10⁸ spores. Cultures were grown at 30 °C in an orbital shaking incubator (Gallenkamp, London) at 230 rev./min for 72 h.

Extraction and detection of SCP1-determined antibiotic. Culture fluid (0.5 ml) was lyophilized in 2 ml vials and dissolved in water or organic solvents to give a final concentration of 10 times the original. Samples were spotted at the origin of silica-gel thin-layer chromatographic plates with fluorescent indicator (no. 13181; Eastman Kodak, Rochester, New York, U.S.A.) and run in two solvents (Snell, Ijichi & Lewis, 1956) in an Eastman Kodak Chromagram Developing Apparatus, no. 6071. Solvent 1 was *t*-butyl alcohol-acetic acid-water (72:3:25, by vol.); solvent 2 was ethyl acetate-acetic acid-water (88:6:6, by vol.). After the chromatograms were dried, spots were detected under u.v. light (90 % emission at 254 nm). Antibiotic activity was tested bioautographically against *Bacillus mycoides* (Kirby *et al.*, 1975).

Adsorption of the SCP1-antibiotic to various resins or other adsorbents was tested as follows. Concentrates of culture-fluids of SCP1⁺ and SCP1⁻ cultures were adjusted to pH 4,

Table 2. *Micro-organisms tested for sensitivity or resistance to the SCP₁-determined antibiotic*

Strain	Source*	Sensitivity (S) or resistance (R)†
<i>Agrobacterium tumefaciens</i> B6	JII	R
<i>Arthrobacter simplex</i> NCIB9770	JII	S
<i>Azotobacter vinelandii</i>	JII	S
<i>Bacillus cereus</i>	UEA	S
<i>B. megatherium</i>	UEA	S
<i>B. mycoides</i>	UEA	S
<i>B. polymyxa</i>	UEA	S
<i>B. subtilis</i>	UEA	S
<i>B. licheniformis</i>	UEA	S
<i>Corynebacterium fascians</i>	FRI	R
<i>C. glutamicum</i> MB1789 (Shapiro)	JII	S
<i>Erwinia carotovora</i> var. <i>atroseptica</i> G120	FRI	S
<i>Escherichia coli</i> K12 J53	JII	R
<i>Klebsiella pneumoniae</i> Kp672	JII	R
<i>Micrococcus lysodeikticus</i> NCTC2655	JII	S
<i>Mycobacterium phlei</i>	UEA	R
<i>M. smegmatis</i>	UEA	R
<i>Nocardia caviae</i>	UEA	S
<i>Penicillium stoloniferum</i> ATCC14586	JII	R
<i>Propionibacterium pentosaceum</i> NCIB8010	JII	R
<i>Proteus mirabilis</i> F67	JII	S
<i>Pseudomonas aeruginosa</i> G23	JII	R
<i>Rhizobium leguminosarum</i> JEB196	JII	R
<i>Saccharomyces cerevisiae</i>	JII	R
<i>Salmonella typhimurium</i> LT2	JII	R
<i>Serratia marcescens</i> H4	JII	R
<i>Staphylococcus aureus</i> NCTC8532	JII	S
<i>Streptococcus faecalis</i> EH69-D5	FRI	S
<i>Streptococcus faecalis</i> N83	FRI	S

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† In certain cases, a micro-organism was inhibited by both SCP₁⁺ and SCP₁⁻ strains of *Streptomyces coelicolor*; sensitivity to the SCP₁-determined antibiotic was indicated when the zone of inhibition caused by the SCP₁⁺ culture was significantly wider than that caused by the SCP₁⁻ culture.

pH 7 and pH 10, and samples (1 ml) were added to polypropylene Microcentrifuge tubes (Quickfit Instrumentation, Stone, Staffs) containing one of the following materials (1 g): Amberlite IRC-50, Amberlite CG-400, Amberlite CG-50, Amberlite XAD-2, Dowex 1-X8, Dowex 50W-X8 (all from BDH), Sephadex-SP (C50), Sephadex QAE (A50) (both from Pharmacia), 'Norite' charcoal and alumina (Analar grade). The tubes were shaken frequently for 30 min at 0 °C before centrifuging on a Microcentrifuge. Samples of the supernatants (20 µl) were analysed for the presence of SCP₁-antibiotic by chromatography and bioautography as before.

Preparation of methylenomycin A. The method was based on that described by Haneishi *et al.* (1974a) for the isolation of methylenomycin A from their *Streptomyces violaceoruber* strain 2416. Culture fluid (2 l), from which the mycelium had been filtered off, was adjusted to pH 2 with HCl and extracted with an equal volume of ethyl acetate. The organic phase was evaporated to dryness and the residue extracted into chloroform (2 ml). The extract was layered on a column (250 × 15 mm) of silica gel equilibrated with chloroform, and fractions were collected while eluting with chloroform. Antibiotic activity was assayed by pipetting a sample (0.1 ml) of each fraction on to a Whatman antibiotic assay disc (13 mm

diam.). The discs were placed on plates of nutrient agar spread with *B. mycoides* and the diameters of inhibition zones were measured after overnight incubation. The active fractions, normally from eluant volume 120 to 160 ml, were pooled and evaporated to 1 ml, carbon tetrachloride (5 ml) was added, and the white precipitate that formed was recovered and dissolved in chloroform; methylenomycin A recrystallized on addition of carbon tetrachloride. A pure sample of methylenomycin A was kindly supplied by Dr T. Haneishi for comparison with extracted material.

Mass spectrometry. This was kindly performed by J. Eagles and R. Self of the Agricultural Research Council Mass Spectrometry Service at the Food Research Institute, Colney Lane, Norwich, using an AEI MS-902 spectrometer at a source temperature of 200 °C and an ionization energy of 70 eV. Accurate masses were obtained at a resolution of 1000 (10 % valley definition), and processed by computer by the method of Johnson, Gordon & Self (1975).

RESULTS

Stages of the SCP1⁻ sensitive response

When SCP1⁺ and SCP1⁻ cultures growing on CM plates were juxtaposed immediately after a period during which the spores were established (approx. 3 to 4 h), the cultures developed normally for some time. Aerial mycelium was observed as fine white branches on the buff-coloured substrate mycelium after about 24 h. After 36 h, the first signs of inhibition began to appear in the SCP1⁻ culture close to the boundary between the strains. In this area, there was no further production of aerial mycelium and the SCP1⁻ culture took on a shiny watery appearance. Between 48 and 72 h, when the characteristic indicator pigment of the organism normally develops, there was no pigment production in the inhibited zone which extended some 8 to 10 mm from the boundary between the two cultures. The remainder of the SCP1⁻ culture outside this zone continued to develop normally, as did the SCP1⁺ culture right up to the boundary. After 96 h incubation, the mycelium in the inhibited zone was observed to be lysing.

Spores of strains 104 (SCP1⁺) and 1190 (SCP1⁻) were plated separately on CM to give confluent growth on the surface of the plates. After 3 days' incubation, cylinders of agar were cut from the plates with a sterile 16 mm cork-borer, and these agar plugs were placed in an empty Petri dish and surrounded by molten CM containing spores of strain 1190. After overnight incubation, the SCP1⁻ indicator culture was found to be inhibited by the SCP1⁺ plug but not by the SCP1⁻ plug. Thus young growth of an SCP1⁻ strain, long before the onset of aerial mycelium production, was sensitive to inhibition by antibiotic produced by an SCP1⁺ culture. By using plugs from SCP1⁺ cultures of various ages, it was found that they caused no inhibition until after approx. 20 h of growth – a time corresponding to the onset of aerial mycelium production. Thus the suggestion of Vivian (1971) that the action of the SCP1-antibiotic was specifically against aerial mycelium production by the sensitive SCP1⁻ culture was not confirmed; rather, synthesis of the antibiotic by the producing culture was correlated with aerial mycelium production.

Spectrum of anti-bacterial activity of the SCP1-determined antibiotic

Plugs from cultures of strains 104 and 1190 were tested against a series of micro-organisms by the method described above. The results are in Table 2.

Four of the sensitive bacterial strains were chosen for further experiments to confirm SCP1 specificity of inhibition: *Bacillus cereus*, *B. mycoides*, *Staphylococcus aureus* and *Proteus mirabilis*. *Streptomyces coelicolor* strains, either lacking SCP1 or having it in

Table 3. *Susceptibility of Bacillus mycoides to various Streptomyces strains grown on CM agar plugs*

Streptomyces strain	Plasmid status	Strain nos	Inhibition of <i>B. mycoides</i> *
<i>S. coelicolor</i> A3(2)	SCP1 ⁺	A3(2), 12, 104, 2136	+
<i>S. coelicolor</i> A3(2)	NF	949, A332	+
<i>S. coelicolor</i> A3(2)	SCP1 ⁻	1098, 1190, A200, A688, A700	-
<i>S. coelicolor</i> A3(2)	SCP1'- <i>cysB</i> ⁺	1984	+
<i>S. coelicolor</i> A3(2)	SCP1'- <i>cysB</i> ⁺ (<i>ant</i> ⁻)	R70, R72, R74	-
<i>S. coelicolor</i> (other wild types)	SCP1 ⁻	1152, 1153	-
<i>S. lividans</i> 66	SCP1 ⁻	1326	-
<i>S. lividans</i> 66	SCP1 ⁺	1671	+

* *Bacillus cereus*, *Proteus mirabilis* and *Staphylococcus aureus* gave identical reactions.

various states [autonomous (SCP1⁺), autonomous but mutant, autonomous and carrying a chromosomal locus (SCP1'), or integrated (NF)] were tested by the agar plug method against these bacteria (Table 3). The mutant strains were isolated by R. Kirby from a strain carrying the SCP1'-*cysB*⁺ plasmid (Hopwood & Wright, 1973*b*) after mutagenesis and selection for lack of inhibition of an SCP1⁻ strain. Hopwood & Wright (1973*a*) described the transfer of SCP1 from *S. coelicolor* A3(2) to *Streptomyces lividans* 66. As seen in Table 3, there was a complete correlation between the presence of SCP1 in non-mutant form and inhibition of the tested bacteria.

On the *Bacillus* spp. and on *Staph. aureus*, SCP1⁻ strains of *S. coelicolor* produced a narrow clear zone of inhibition, much narrower than the zone produced by the SCP1⁺ strain. This inhibition was due to another antibiotic, which is unrelated to that determined by SCP1, and is not plasmid specific (Wright & Hopwood, unpublished results).

Physical properties of the SCP1-determined antibiotic

The antibiotic was shown to diffuse through cellophane. Visking tubing was embedded in CM plates in a U-shape. Spores of strain 104 (SCP1⁺) were sown on the agar surface between the arms of the U, and strain 1190 (SCP1⁻) was inoculated outside. After growth for 72 h, inhibition of the SCP1⁻ culture was observed.

The SCP1-specified antibiotic was mobile in polar solvents on silica-gel thin-layer chromatograms. Crude fluid concentrates from cultures of strains 104 (SCP1⁺) and 1190 (SCP1⁻) gave the following spot patterns in solvent 1: as well as material left at the origin of both chromatograms, there were three u.v.-absorbing spots on the SCP1⁺ trace (A, *R_F* 0.95; B, *R_F* 0.85; and C, *R_F* 0.65 to 0.80) and one (A, *R_F* 0.95) on the SCP1⁻ trace. These patterns were also observed in solvent 2, although the *R_F* values of the two SCP1⁺-specific spots were marginally increased.

When the chromatograms were assayed for antibiotic activity against *B. mycoides* by bioautography (Kirby *et al.*, 1975), spot B was inhibitory and therefore corresponded to the SCP1-specified antibiotic. No biological activity was detected from spots A or C. Two-dimensional chromatography, running first in solvent 1 and then in solvent 2, confirmed the presence of the SCP1-specific spot, inhibitory to *B. mycoides* (Kirby *et al.*, 1975).

Chromatograms of material extracted from lyophilized SCP1⁺ culture fluid by various organic solvents were run in solvent 2 and tested for the presence of spot B (the SCP1-

Fig. 1. Antibacterial activity (width of inhibition zone against *Bacillus mycoides*) and extinction at 248 nm ($\blacktriangle$) of an extract of strain 104 ($SCP1^+$) eluted from a silica-gel column.

specified antibiotic) by u.v. absorbance and bioautography. The antibiotic was soluble in methanol, ethanol, propanol, ethyl acetate, 1,4-dioxan, chloroform and water, but insoluble in butanol, amyl alcohol, acetone, benzene and carbon tetrachloride.

The antibiotic was taken up from aqueous solutions at pH 4, pH 7 and pH 10 by Amberlite CG-400, Dowex 1-X8 and charcoal, and at pH 4 by Amberlite XAD-2. It was not adsorbed by the other resins at any of the three pH values tested. From the solubility and adsorption characteristics we concluded that the antibiotic was polar and probably anionic.

Comparison of SCP1-specified antibiotic with methylenomycin A

After completing the work described, we became aware of a paper on the isolation and purification of two new antibiotics, methylenomycins A and B, from a newly isolated strain of *Streptomyces violaceoruber* (Haneishi *et al.*, 1974a). *Streptomyces coelicolor* A3(2) resembles strains classified as *S. violaceoruber*; moreover, the physical and biological properties of methylenomycin A were similar to those of our SCP1-determined antibiotic. When the extraction procedure for methylenomycin A was applied to liquid culture filtrates of $SCP1^+$ and $SCP1^-$ strains, material inhibiting *B. mycoides* was found in extracts of $SCP1^+$ but not $SCP1^-$ cultures.

The elution profile of the silica-gel column eluate of an extract of strain 104 ($SCP1^+$) is shown in Fig. 1 in terms of the antibiotic activity of each fraction, and of its extinction at 248 nm, a criterion that we found useful in detecting the SCP1-antibiotic. Antibiotic activity was located in fractions collected between eluant volume 120 and 175 ml, with a peak at 130 to 135 ml. This corresponded to a sharp peak of extinction at 248 nm. There was an extinction shoulder at eluant volume approx. 150 to 170 ml, but there was no corresponding shoulder of antibiotic activity. The fractions between 150 and 170 ml absorbed strongly at 266 nm, with a peak at approx. 155 ml. It appeared that another compound had been

Fig. 2. Mass spectra of: (a) an impure sample of SCP1-antibiotic from strain 104; (b) a pure sample of methylenomycin A from Dr T. Haneishi; (c) a pure sample of SCP1-antibiotic from strain 104.

carried through the purification procedure, along with the SCP1-antibiotic, and was responsible for the shoulder in the extinction curve at 248 nm.

Fractions containing antibiotic activity were pooled in different combinations, and the antibiotic was precipitated by adding carbon tetrachloride, and analysed by mass spectrometry. The solid products from pooled fractions collected between eluant volume 120 and 170 ml gave the mass spectrum shown in Fig. 2(a). Two principal ions, with molecular weights of 182 and 166, were present in almost equimolar amounts. The spectrum obtained from a sample of methylenomycin A supplied by Dr T. Haneishi is shown in Fig. 2(b). The simplest interpretation of the spectrum of Fig. 2(a) was that the sample contained approximately equimolar quantities of methylenomycin A (mol. wt 182) and of another compound (mol. wt 166). The latter apparently had a similar breakdown pattern to methylenomycin A, also losing ions of 44 a.m.u. and 29 a.m.u., suggesting a structural relationship between the two substances. The compound of molecular weight 166 was probably that which absorbed strongly at 266 nm in silica-gel column eluate analysis.

Fig. 3. Structure of methylenomycin A (from Haneishi *et al.*, 1974*b*).

The identity of the SCP1-antibiotic was confirmed by mass spectral analysis of material precipitated by carbon tetrachloride from pooled fractions collected between eluant volume 120 and 140 ml. The spectrum obtained from this material, recrystallized from chloroform, is shown in Fig. 2(c) and is identical with that of methylenomycin A (Fig. 2*b*). The structure of methylenomycin A, elucidated by Haneishi *et al.* (1974*b*), is shown in Fig. 3.

Sensitivity of cultures to methylenomycin A

Methylenomycin A incorporated into antibiotic assay discs in amounts ranging from 1 to 200 $\mu\text{g}/\text{disc}$ was tested for inhibitory activity by placing the discs on plates of CM spread with spores of the SCP1⁻ strain 1190. At concentrations of 100 $\mu\text{g}/\text{disc}$ and above, the cultures were inhibited. The zones of inhibition were small and the cultures slowly overcame the inhibitory effect and grew to the edges of the discs. In view of the instability of methylenomycin A (Haneishi *et al.*, 1974*a*), a reasonable explanation for the slight and temporary inhibition of the SCP1⁻ culture by pure methylenomycin A is that rapid diffusion and inactivation of the antibiotic soon reduced its effective concentration to a sub-inhibitory level.

These findings were extended by minimum inhibitory concentration (m.i.c.) experiments against SCP1⁻ spores. Growth of SCP1⁻ strain 1190 on CM was prevented by 50 μg methylenomycin A per ml agar. Growth of SCP1⁺ colonies was suppressed at 100 $\mu\text{g ml}^{-1}$. It is possible, therefore, that both SCP1⁺ and SCP1⁻ strains are sensitive to methylenomycin A during early stages of growth.

The inhibitory response of *B. mycoides* to methylenomycin A was estimated by constructing a dose-response curve. For a 10-fold increase in concentration the inhibitory response increased by 300 %. By extrapolation of the curve to zero zone radius, the m.i.c. was estimated as 10 $\mu\text{g ml}^{-1}$. This agrees with the figure estimated from preliminary experiments (Kirby *et al.*, 1975), by calculating the amount of antibiotic produced per ml of culture broth. This value was derived from the amount of purified material obtained from preparative chromatography, and assuming 20 % recovery. Approximately 3 $\mu\text{g ml}^{-1}$ broth culture was produced. Broth concentrates of threefold and higher were inhibitory to *B. mycoides* and hence the m.i.c. was estimated at 10 $\mu\text{g ml}^{-1}$.

DISCUSSION

The antibiotic determined by the SCP1 plasmid of *S. coelicolor* A3(2) has been identified as methylenomycin A, the compound characterized as 2-methylene-cyclopentan-3-one-4,5-epoxy-4,5-dimethyl-1-carboxylic acid by Haneishi *et al.* (1974*b*). They isolated it from a strain of *S. violaceoruber* but reported no evidence on the genetic control of its biosynthesis.

Methylenomycin A is active against a wide range of eubacteria. Members of all Gram-positive genera tested were inhibited by it, as were a few of the Gram-negative strains tested: *Proteus mirabilis* and *Erwinia carotovora*. These results agreed with those of Haneishi *et al.* (1974a) who found *Proteus vulgaris* and some strains of *Escherichia coli* were sensitive. The minimum inhibitory concentration of methylenomycin A against a *B. mycoides* wild type was 10 $\mu\text{g ml}^{-1}$.

Methylenomycin A contains an epoxide ring between carbon atoms 4 and 5. The occurrence of epoxide rings in antibiotics, though not novel, is rare. In two well-studied examples, phosphonomycin (Kahan *et al.*, 1974) and cerulenin (D'Agnolo *et al.*, 1973), the evidence suggests that the antibiotic in question behaves as an analogue of a biosynthetic intermediate and reacts through the epoxide grouping to form a covalent linkage with the corresponding biosynthetic enzyme. It is therefore a reasonable prediction that methylenomycin A may have a similar mode of action on an unknown target enzyme.

The nature of the molecule of molecular weight 166 present in culture fluids of SCP1⁺ strains of *S. coelicolor* A3(2) is unknown. It was present in equimolar amounts with methylenomycin A, but this may have been fortuitous. The compound probably bears a structural relationship to methylenomycin A, since it showed a similar breakdown pattern in its mass spectrum. Certainly it is not methylenomycin B (mol. wt 138), which has a hydrogen atom replacing the 1-carboxyl group. Moreover, the unknown molecule, like methylenomycin A, appears to lose a 44 a.m.u. fragment readily, presumably CO₂ from the carboxyl group. The most probable cause of the 16 a.m.u. difference between methylenomycin A and the unknown compound is the lack of an oxygen atom. Removal of the epoxide oxygen, with formation of a double bond between carbon atoms 4 and 5, would give a hypothetical compound of molecular weight 166, which would probably be antibiotically inactive, and would be expected to have an extinction maximum at a longer wavelength, such as the observed 266 nm. Only studies of purified preparations of this substance will clarify its nature.

Much interest has resulted from reports suggesting the implication of plasmids in antibiotic synthesis by streptomycetes. These findings, on species other than *S. coelicolor*, were summarized by Kirby *et al.* (1975). In most cases, the evidence for plasmid involvement in antibiotic production is circumstantial, although a recent report on *Streptomyces venezuelae* (Akagawa, Okanishi & Umezawa, 1975) provides good evidence for an extra-chromosomal determinant involved in chloramphenicol production. However, in none of these other systems is there evidence that genes determining steps in the biosynthetic pathway of antibiotic synthesis itself are plasmid-borne. In contrast, it is clear that the SCP1 plasmid carries genes determining several steps in the biosynthetic pathway of methylenomycin A. The evidence for this is the isolation by Kirby (1976) of several distinct classes of SCP1-linked mutants, some of which co-synthesized antibiotic in pairwise combinations. We believe that this is the first case in which there is direct evidence that genes determining steps in the biosynthetic pathway of an antibiotic are located on a genetically well-characterized plasmid.

Plasmid-carrying as well as SCP1⁻ strains are inhibited by methylenomycin A in their early stages of development, although the minimum inhibitory concentration for SCP1⁻ strains appears to be lower. This may be an example of the generalization that resistance to antibiotics in producing strains is not achieved until the time at which synthesis of antibiotic begins (Demain, 1974). However, synthesis itself is not required for the onset of methylenomycin resistance, since the antibiotic non-producing mutants isolated by Kirby (1976) were resistant to the antibiotic. Certainly the SCP1 plasmid confers resistance to

methylenomycin on mature cultures of *S. coelicolor* and it will be interesting to discover the nature of this resistance, particularly whether or not it results from destruction or modification of the antibiotic. It is well established that modifying enzymes for aminoglycoside antibiotics (Benveniste & Davies, 1973) and for chloramphenicol (Shaw & Hopwood, 1976), analogous to those found on R factors in eubacteria, are present in many streptomycetes, but nothing is known about their genetic determination. Thus the present example of plasmid-determined resistance in *S. coelicolor* is interesting in relation to the theory that the actinomycetes may have served as the evolutionary source of eubacterial plasmid-borne resistance determinants (Benveniste & Davies, 1973).

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