Biosynthesis of anthracyclines: analysis of mutants of *Streptomyces* sp. strain C5 blocked in daunomycin biosynthesis

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Streptomyces sp. strain C5, an organism that normally produces baumycins, daunomycin and ϵ -rhodomycinone, was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Mutants blocked at various points in daunomycin and baumycin production were isolated by screening for altered pigmentation and absence of bioactivity against *Staphylococcus aureus*. Examination of the mutants by thin-layer chromatography of their accumulated anthracycline metabolites, by cosynthesis assays, and by extract feeding experiments allowed a classification into six groups. These were: *dauA*, strains that accumulated no anthracyclines but with other blocked mutants cosynthesized anthracyclines (polyketide-synthase-minus mutants); *dauG*, regulatory mutants that, either alone or mixed with other blocked mutants, accumulated no anthracyclines; *dauC*, mutants that accumulated aklanonic acid; *dauE*, mutants that accumulated maggiemycin; *dauF*, mutants that accumulated aklavinone; and *dauH*, mutants that accumulated only ϵ -rhodomycinone. Mutant SC5-24 (*dauE*), which accumulated the shunt product maggiemycin, was re-mutagenized with NTG to obtain blocked mutants in preceding biosynthetic steps; the three groups of double mutants obtained accumulated aklanonic acid (*dauC,E*), aklanonic acid methyl ester (*dauD,E*) and aklaviketone (*dauE,F*).

Introduction

Several streptomycetes produce 7,8,9,10-tetrahydro-5,12-naphthacene quinone glycosides, commonly known as anthracyclines (Strohl *et al.*, 1989). Three such anthracyclines, adriamycin (also known as doxorubicin), daunomycin (also known as daunorubicin) and aclacinomycin A, are used extensively as chemotherapeutic agents but are cumulatively cardiotoxic and have destructive side-effects when used for long periods of time (Arcamone, 1984). Therefore, new anthracyclines with enhanced clinical properties have been sought intensively (reviewed by Fujiwara & Hoshino, 1983; Strohl *et al.*, 1989). One of the major difficulties has been the absence of a thorough knowledge of the enzymic reactions and intermediates in the formation of anthracyclines. This knowledge would assist efforts to determine how anthracycline biosynthesis is regulated, to analyse recombinant DNA clones containing anthracycline biosynthesis genes, to construct over-producing

strains and to design rational interspecies cloning experiments leading to new 'hybrid' anthracyclines with potentially enhanced clinical properties (Strohl *et al.*, 1989). This paper describes the mutation of the anthracycline-producing strain, *Streptomyces* sp. C5 (McGuire *et al.*, 1980*a, b, c*; Strohl *et al.*, 1989), the isolation and characterization of blocked mutants, and the location of the blocked steps in the daunomycin biosynthesis pathway.

Methods

Organisms, culture maintenance and growth conditions. *Streptomyces* sp. strain C5 was used as the parental strain for mutagenesis. Its isolation as a spontaneous and UV-induced, cerulenin-selected, anthracycline-overproducing mutant of an unidentified streptomycete (Fig. 1) has been described (McGuire *et al.*, 1980*a, b, c*). Nitrate-defined medium (NDM; see accompanying paper: Connors *et al.*, 1990*a*), yeast/malt agar medium (YMA; Dekleva *et al.*, 1985) and R2YE medium (Hopwood *et al.*, 1985) have also been described.

Spore suspensions for culture storage or mutagenic treatment were prepared from sporulated cultures grown on R2YE agar. Agar plates containing R2YE medium were inoculated with spores of *Streptomyces* strains and incubated at 30 °C until sporulation, which was usually in 4–8 d. Sterile, acid-washed 3 mm glass beads were rolled over the surface of the culture to collect spores for mutagenic treatment. The beads were transferred to a sterile beaker and the spores were washed

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Abbreviations: AAME, aklanonic acid methyl ester; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Fig. 1. Derivation of strains C5 and A21 by researchers at the Frederick Cancer Research Center (McGuire *et al.*, 1980*a, b, c*) and the subsequent derivation of SC5-24 and other blocked mutants as described herein.

from them with water, pelleted by centrifugation (6000 *g*, 10 min) and resuspended in 10% (v/v) glycerol. Spore suspensions were stored at -20°C until used. For long-term storage, spores were scraped from the surface of sporulated cultures, suspended in Difco nutrient broth (8 g l⁻¹) containing 10% glycerol, and frozen at -70°C . Cultures used frequently were stored at room temperature after sporulation. For bioassays *Staphylococcus aureus* was grown overnight at 37°C on TSA plates (BBL trypticase soy broth, 30 g; agar, 15 g; distilled water to 1 l) and was stored at 4°C .

Mutagenesis. Spores were mutagenized by exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Sigma) by a slight modification of the method described by Delić *et al.* (1970). Spore preparations of *Streptomyces* sp. C5 or SC5-24 (a maggiemycin-accumulating mutant of *Streptomyces* sp. C5) were pelleted by centrifugation and were resuspended in 50 mM-Tris/maleate buffer, pH 9.0. The spore suspensions were added to pre-weighed crystals of NTG in 1.5 ml microcentrifuge tubes to yield a final NTG concentration of 3 mg ml⁻¹. The suspensions were vortexed vigorously to dissolve the NTG and incubated at room temperature for 90 min. After the spores had been washed four times with water to remove NTG, the titre of survivors was determined by plating on YMA, and the treated spores were resuspended in 10% glycerol for storage at -20°C .

Isolation of mutants with altered pigmentation. Spores treated with NTG were plated on NDM or R2YE agar at a concentration of 100–200 viable spores per plate and incubated at 30°C for 4–7 d; colonies were examined for altered pigmentation. *Streptomyces* sp. C5 sporulates well on both R2YE and NDM media and gives dark-red coloured colonies due to production of various anthracyclines, including ϵ -rhodomycinone and baumycins (McGuire *et al.*, 1980*c*; Strohl *et al.*, 1989). Colonies with altered pigmentation but otherwise normal colonial morphology were transferred to fresh R2YE plates. Candidate mutants were examined during at least five transfers to ensure that they had stable phenotypes.

Mutants blocked in two steps of daunomycin biosynthesis were generated by treating strain SC5-24 with NTG. Colonies of SC5-24 are

blue due to production of maggiemycin (McGuire *et al.*, 1980*b*), a putative pathway shunt product (Eckardt & Wagner, 1988). Mutants which produced yellow or red pigments were chosen from survivors grown on R2YE or NDM agar medium.

Isolation of mutants lacking bioactivity. Mutants inactive against *S. aureus* were detected by a slight modification of the methods used by Motamedi *et al.* (1986). NTG-treated spores of *Streptomyces* sp. C5 were plated on YMA to give about 300 colonies per plate. After 3–4 d incubation at 30°C , mycelia from individual colonies were transferred to an R2YE agar plug in a 96-well immunoassay plate and to fresh YMA (50 colonies per plate on a grid). The immunoassay plates contained approximately 300 μl R2YE agar medium, prepared as described by Hunter (1985), per well. The YMA plates were incubated at 30°C for 2 d and were then stored at 4°C until needed; the 96-well plates were incubated at 30°C for 6–8 d. To avoid choosing pleiotropic mutants, bald and dwarf colonies were marked. Bioassay agar was prepared by adding 5 ml of an overnight nutrient broth culture of *S. aureus* to 125 ml of warm (approx. 45°C) nutrient agar and dispensed into Petri dishes (about 20 ml each). Twenty plugs from the 96-well plates were transferred to each dish and incubated at 37°C overnight. Plugs which produced no zone of inhibition were noted and the corresponding colonies from the previously incubated YMA plates were transferred to fresh YMA. The bioassay of these colonies was repeated three more times to ensure that the strains were stable and had no antibiotic activity against *S. aureus*.

Extraction and analysis of anthracycline metabolites. Spores of mutant strains were spread on R2YE agar and incubated at 30°C for 4–7 d. A 4 × 4 cm piece of agar supporting an area of confluent growth was removed, cut up and placed in 3 ml of methanol. The extraction mixture was incubated at room temperature for 1 h, after which the methanolic extract was decanted, 3 ml each of water and chloroform were added, and the pigments were extracted into the chloroform phase. Alternatively, when the anthracyclines in the extract were hydrolysed to aglycones, 3 ml 0.4 M-HCl was added. The mixture was incubated at 90°C for 45 min before extraction with 3 ml of chloroform. The chloroform extracts were evaporated *in vacuo* at room temperature. The dried extracts were resuspended in 20 μl chloroform for analysis by thin-layer chromatography (TLC) on silica gel (Whatman HP-KP, 4802-400). Chromatograms were developed with solvent system SS1, chloroform/heptane/methanol (10:10:3, by vol.); SS2, benzene/acetone/methanol (100:10:1, by vol.) for separation of aglycones (Table 1); or SS3, chloroform/methanol/water/glacial acetic acid (80:20:2:0.2, by vol.), for separation and analysis of glycones. Compounds resolved from extracts by TLC were compared to authentic anthracyclines and anthracyclinone intermediates (Table 1). The R_F values, colouration in acid and alkali, and fluorescence of the zones irradiated at 365 nm were also compared with standards (Table 1).

Analysis of cocultured mutant strains. Four patches of approximately 4 × 4 cm, each containing spores from two mutants on R2YE agar were incubated at 30°C for 5–7 d. Pieces of agar containing the patches were then removed and extracted as described. The extracts were analysed by TLC for the production of daunomycinone (after hydrolysis with HCl as described above) or baumycins (non-hydrolysed samples).

Analysis of mutants by extract feeding. Spores of a mutant strain were swabbed over the entire surface of an R2YE agar plate and incubated for 7 d, after which the agar was extracted at room temperature for 1 h with 25 ml methanol. The methanolic extract was concentrated *in vacuo* and the amount produced by a culture on one Petri dish was incorporated into a dish of fresh R2YE medium. Four mutant strains were patched individually on each extract-containing plate and incubated at 30°C for 5 d, after which the patches were cut out, extracted and examined for baumycins by the methods described for

Table 1. Analysis of anthracyclines and anthracyclonones by TLC

Compound	R_F^*			Colour of zone†			Fluorescence‡
	SS1	SS2	SS3	Neutral	Acid	Base	
Aglycones							
Aklanonic acid	0.21	0.07	0.55	Y	Y	B	O
AAME	0.69	0.79	0.88	B	Y	B	O
Aklaviketone	0.29	0.30	0.82	B-R	Y	V	V
Aklavinone	0.41	0.42	0.83	Y	Y	O	Y
Daunomycinone	0.25	0.12	0.80	O	O	V	O
ϵ -Rhodomycinone	0.45	0.45	0.83	O	O	V	R
Maggiemycin	0.29	0.30	0.82	V	R	V	V
Glycosides							
Adriamycin	0.00	0.00	0.07	R	O	R	O
Baumycin A1/A2	0.00	0.00	0.27	R	O	R	O
Daunomycin	0.00	0.00	0.14	R	O	O	O
13-Dihydrodaunomycin	0.00	0.00	0.09	R	O	O	O

* Solvent systems used: SS1, chloroform/heptane/methanol (10:10:3, by vol.); SS2, benzene/acetone/methanol (100:10:1, by vol.); SS3, methanol/chloroform/acetic acid/water (80:20:2:0.2, by vol.).

† Colour of zone observed at pH 7.0 (neutral), when 1 M-NaOH was added (base) or when 1 M-HCl was added (acid). B, brown; O, orange; R, red; V, violet; Y, yellow.

‡ Fluorescence under UV (365 nm) light (neutral conditions). For abbreviations see footnote †.

the cosynthesis assays. Representative strains from each class of mutants were used to prepare extracts and to convert extracts from other strains.

Standards. Authentic standards for anthracyclines and anthracyclonones were obtained from Adria, the Frederick Cancer Research Center and Rhône-Poulenc. Authentic samples of aklanonic acid, aklanonic acid methyl ester (AAME) and aklaviketone were obtained from Klaus Eckardt and his colleagues of the Academy of Sciences, Jena, GDR.

Results

Streptomyces sp. C5 mutants blocked in anthracycline biosynthesis

Streptomyces sp. strain C5 grown in nutrient-rich media for 5–7 d normally accumulates about 20 mg l⁻¹ of aglycones, the major species of which is ϵ -rhodomycinone, and about 2 mg l⁻¹ of glycosides, of which there are seven separable forms (P. L. Bartel, J. Duddy & W. R. Strohl, unpublished). Since the final glycosidic products of *Streptomyces* sp. C5, e.g. daunomycin and baumycins A and B, are orange-to-red pigments active against *Staphylococcus aureus*, both the absence of normal pigmentation and lack of bioactivity were used as indicators in screening for blocked mutants. Treatment of *Streptomyces* sp. C5 spores with NTG left 0.93% survivors, of which a total of approximately 80 000 were examined for pigmentation and absence of activity against *S. aureus*. Over 100 blocked mutants were obtained and analysed in greater detail. All of them displayed normal developmental phenotypes, i.e. they

formed aerial mycelia and spores and grew to the normal size.

Of 62 000 colonies from NTG-treated spores plated on either NDM or R2YE and closely examined for altered pigmentation, several hundred with altered pigmentation were isolated. After at least five transfers to examine the stability of the mutations, 101 mutants stably exhibited altered pigmentation but still grew and sporulated normally. In a separate mutant isolation experiment, 12 000 colonies derived from NTG-treated spores were screened by bioassay against *S. aureus*. Using this method, 12 mutant colonies with no bioactivity were isolated (irrespective of colony pigmentation). In a third experiment, four red-pigmented mutants which did not possess bioactivity against *S. aureus* were detected out of 5000 red colonies screened. In all, 117 stable, blocked mutants were obtained out of 79 000 colonies screened, yielding a frequency of 1.28×10^{-3} .

Analysis of anthracycline metabolites accumulated by blocked mutants

The anthracycline metabolites accumulated by each of the 117 mutant strains were extracted with organic solvents and analysed by TLC. The strains were classified into groups based on the type of anthracycline metabolites they accumulated (Table 2). The largest group (30 cream-coloured mutants) sporulated normally but failed to accumulate any anthracycline-like metabolites. Three reddish-yellow mutants accumulated

Table 2. Classification of blocked mutants of *Streptomyces* based on their accumulated anthracycline metabolites and cosynthesis behaviour

Mutant class	Type strain	No. of strains	Colony colour	Anthracycline intermediate accumulated	10 ⁵ × Frequency	Percentage cosynthesis with class*
<i>dauA</i>	SC5-68	25	Cream	None	36	A, 0; C, 99; E, 89; F, 83; G, 1; H, 71
<i>dauG</i>	SC5-38	5	Cream	None	7	A, 1; C, 7; E, 35; F, 3; G, 0; H, 0
<i>dauC</i>	SC5-69	5	Reddish-yellow	Aklanonic acid	4	A, 99; C, 0; E, 100; F, 100; G, 7; H, 75
<i>dauE</i>	SC5-24	4	Blue	Maggiemycin	8	A, 89; C, 100; E, 0; F, 100; G, 35; H, 100
<i>dauC,E</i>	SC5-136	2	Reddish-yellow	Aklanonic acid	ND	ND
<i>dauD,E</i>	SC5-138	3	Red	AAME	6	ND
<i>dauE,F</i>	SC5-159	13	Orange	Aklaviketone	ND	ND
<i>dauF</i>	SC5-31	7	Yellow	Aklavinone	13	A, 83; C, 100; E, 100; F, 0; G, 0; H, 96
<i>dauH</i>	SC5-58	9	Red	ϵ -Rhodomycinone	53†	A, 71; C, 75; E, 100; F, 96; H, 33; G, 0

ND, Not determined.

* Percentage of positive cosynthesis reactions with members of class listed.

† Red-pigmented mutants detected by bioassay; 9 *dauH* mutants were isolated from 17000 colonies examined.

aklanonic acid, four blue-pigmented mutants accumulated maggiemycin, and seven yellow mutants accumulated aklavinone. Nine red mutants accumulated ϵ -rhodomycinone, but a few of these also accumulated small amounts of other biologically inactive, pigmented metabolites. These other metabolites did not comigrate with any standards that were available (data not shown). A large group of 35 mutants accumulated normal amounts of glycosylated end products, i.e. baumycins, but failed to accumulate any ϵ -rhodomycinone (data not shown). Finally, a group of leaky mutants accumulated small quantities of several compounds, at least one of which was bioactive. The leaky strains and those that accumulated only baumycins were not included in any further analyses.

Mutagenesis of SC5-24 and isolation of additional blocked mutants

Eckardt & Wagner (1988) postulated that AAME and aklaviketone were intermediates in the formation of ϵ -rhodomycinone. We did not find, however, any NTG-induced mutants of *Streptomyces* sp. C5 that accumulated these postulated aglycone intermediates. We hypothesized that this might have been due to our inability to distinguish the slight changes in pigmentation exhibited by colonies excreting these compounds, which are reddish to red-brown in colour. Therefore, mutant strain SC5-24, a blue-pigmented, maggiemycin-accumulating mutant (Figs 1 and 2; Table 2), was re-mutagenized with NTG and survivors, plated on R2YE medium, were screened for altered pigmentation. The frequency of such colonies was 6×10^{-4} , and no revertants to the *Streptomyces* sp. C5 phenotype were observed among the 50000 colonies screened. From the

total of 30 NTG-induced mutants of SC5-24, two reddish colonies accumulated aklanonic acid, three brown-pigmented mutants accumulated AAME, 13 reddish blocked mutants accumulated aklaviketone, and the remainder did not accumulate any pigmented metabolites (Table 2). All three of the AAME-accumulating strains also produced small quantities of maggiemycin in addition to AAME. Several of the aklaviketone accumulators also accumulated small quantities of maggiemycin. Since maggiemycin and aklaviketone were not separable on TLC plates (although their pigmentation and fluorescence differ considerably), the accumulation of aklaviketone by these strains was confirmed by separation with HPLC as described in the accompanying paper (Connors *et al.*, 1990a).

Analysis of the cosynthetic behaviour of blocked mutants

Attempts to cosynthesize glycone end-products (e.g. daunomycin and baumycins) by growing mutant strains together in liquid medium (Blumauerová *et al.*, 1979), or by growing mutant strains adjacently on agar medium (Delić *et al.*, 1969; Rudd & Hopwood, 1979), were unsuccessful. However, when spores of two mutant strains were mixed and then grown together on agar medium as a coculture, they cosynthesized baumycins that were detectable by TLC. The cosynthesis assays, in conjunction with analysis of intermediates accumulated by the mutants in each group, placed the mutants in six distinct cosynthetic classes (Table 2). Mutants of classes A, C, E, F and H cosynthesized baumycins when mixed with mutants of all classes except G and those of the same class. Mutants of classes A and G, which were cream-pigmented and did not accumulate any anthra-

Table 3. *Biotransformation of intermediates from extracts*

Converter strain	Source of extract and its mutant class*						
	SC5-68(A)	SC5-69(C)	SC5-138(D,E)	SC5-24(E)	SC5-31(F)	SC5-55(H)	SC5-58(H)
SC5-68 (<i>dauA</i>)	—	+	—	—	+	+	+
SC5-69 (<i>dauC</i>)	—	—	+	—	—	+	—
SC5-138 (<i>dauD,E</i>)	—	—	—	—	+	?	ND
SC5-24 (<i>dauE</i>)	—	—	—	—	+	+	+
SC5-160 (<i>dauE,F</i>)	—	—	—	—	—	+	ND
SC5-31 (<i>dauF</i>)	—	—	—	—	—	ND	+
SC5-38 (<i>dauG</i>)	—	—	—	—	—	—	ND
SC5-55 (<i>dauH</i>)	—	—	ND	—	—	—	—
SC5-58 (<i>dauH</i>)	—	—	ND	—	—	—	—

ND, Not done.

* Symbols: +, extracts transformed to baumycins and/or daunomycin by converter strain; —, extracts not transformed to daunomycin or baumycins by converter strain; ?, data not consistent.

cycline intermediates, could be distinguished by their cosynthesis behaviour. Class A mutants cosynthesized baumycins with mutants from classes C, E, F and H, whereas class G mutants did not cosynthesize baumycins with classes A, C, F and H. Occasionally, class G mutants cosynthesized baumycins with class E mutants but those results were not reproducible (data not shown). Several class H mutants cosynthesized baumycins with other class H mutants, so this appears to be heterogeneous and is perhaps blocked at various biosynthetic steps.

Extract feeding experiments to establish a biosynthetic sequence

The cosynthesis assays did not allow determination of the polarity of the reactions (i.e. which strain was secreting and which was converting). To determine the biosynthetic sequence represented by the mutants, a series of extract feeding experiments was conducted using representative (or 'type') strains from each mutant class (Table 3). The *dauA* strain could convert extract from most of the other strains tested (except *dauE*) to glycosides; the *dauF* strain, SC5-31, and the *dauH* strains, SC55 and SC5-58, produced compounds that could be converted to glycosides by strains from classes *dauA*, *dauC* and *dauE* (Table 3). The *dauE* mutant, SC5-24, which accumulated maggiemycin, converted extracts from classes *dauF* and *dauH*. Extracts from the *dauE* mutant, however, were not converted to daunomycin or baumycins by any of the mutants tested (Table 3), indicating that maggiemycin is not an intermediate in daunomycin biosynthesis. The *dauF* mutant converted extracts from a *dauH* mutant, and extracts from the *dauF* mutant were converted by the earlier mutants from classes A, C, D/E and E. Class H mutants were unable to

convert extracts from other class H mutants. The *dauG* strain, SC5-38, did not convert extracts from any strain to glycosides (Table 3).

Discussion

We chose *Streptomyces* sp. strain C5 for this study after several attempts at obtaining stable blocked mutants of *Streptomyces peucetius* ATCC 29050 (G. D. Gibb & W. R. Strohl, unpublished data). Mutants of *S. peucetius* obtained by UV irradiation, or by NTG or EMS chemical treatments reverted to a wild-type phenotype at a very high frequency. In contrast, this study has demonstrated that NTG-induced mutants of *Streptomyces* sp. strain C5 are stable with a very low frequency of reversion. Earlier attempts to use UV irradiation to generate mutants of *Streptomyces* sp. strain C5 were less successful.

Among the mutants of *Streptomyces* sp. C5 was a large group of strains that did not accumulate any detectable anthracycline-like compounds (based on pigments, fluorescence and R_F after separation by TLC), but which still cosynthesized baumycins with a variety of strains from the other blocked mutant classes. These were assigned to the earliest step in the biosynthetic sequence by extract feeding experiments. This class of mutants, designated *dauA*, appears to represent strains that are mutated within the postulated 'polyketide synthase complex', a group of enzymes thought to be responsible for the successive condensation of malonyl-CoA units to a propionyl-CoA starter unit to produce aklanonic acid (Eckardt & Wagner, 1988; Strohl *et al.*, 1989). Although very little is currently known about the anthracycline polyketide synthase system, it is believed to be similar to

those of other streptomycete polyketide synthase systems, which are composed of a β -ketoacyl synthase, a poly- β -ketone cyclase and an ACP-like protein (Bibb *et al.*, 1989; Sherman *et al.*, 1989). The production of a stable polyketide compound may also require the action of a β -keto reductase (Hallam *et al.*, 1989). Our *dauA* class of mutants probably contains strains which are mutated in different proteins of the polyketide synthase complex, but the determination of this will have to await genetic analysis. From daunomycin-producing strains of *S. griseus*, IMET JA 3933, IMET JA 5142 and IMET JA 5570, thirty-two mutants have previously been isolated, which differ from one another in the amounts of daunomycin produced (Strauss & Fleck, 1975; Wagner *et al.*, 1981, 1985). The largest class (14 members) was made up of mutants that accumulated no anthracycline metabolites. These mutants, designated class I or *lkml*, were probably blocked in the polyketide synthase complex (Wagner *et al.*, 1981), like our class of *Streptomyces* sp. C5 *dauA* mutants.

Eckardt *et al.* (1985) isolated aklanonic acid, an anthraquinone metabolite accumulated by *Streptomyces* sp. ZIMET 43717, and showed that this compound and its chemically produced methyl ester could be converted *in vivo* by blocked mutants of the daunomycin-producing strain, *S. griseus* IMET JA 5142, to aklavinone, an intermediate in daunomycin biosynthesis (Wagner *et al.*, 1984). They also showed that *S. peucetius* strain 21/8 and *S. galilaeus* strain S727 could convert aklanonic acid to anthracyclines similar to aklavinone (Schumann *et al.*, 1986). Eckardt & Wagner (1988) recently proposed a pathway for anthracycline biosynthesis in which aklanonic acid was the first stable intermediate after cyclization and aromatization of the polyketide precursor, and was converted to aklavinone and ϵ -rhodomycinone. Strains of the *Streptomyces* sp. C5 mutant class *dauC*, which accumulate aklanonic acid, appear to be similar to blocked mutants of *S. peucetius* 601-FI and *S. galilaeus* F198 which also accumulate aklanonic acid (Schlegel *et al.*, 1987). Thus it appears that aklanonic acid is a primary intermediate in the daunomycin production pathway of several organisms (Eckardt & Wagner, 1988). An anthraquinone intermediate, similar to aklanonic acid, also has been implicated in oxytetracycline biosynthesis (Thomas & Williams, 1983). This suggests that aklanonic acid or analogous anthraquinones may be universal intermediates in the formation of various naphthacenequinone polyketides.

AAME and aklaviketone have previously been converted to anthracyclines *in vivo* (Wagner *et al.*, 1984; Eckardt *et al.*, 1985; Schumann *et al.*, 1986). When spores of the maggiemycin-accumulating *dauE* mutant, SC5-24, were treated with NTG, doubly-mutated strains that accumulated aklanonic acid (*dauC,E*), AAME

(*dauD,E*) or aklaviketone (*dauE,F*) were isolated. Eckardt & Wagner (1988) proposed that aklaviketone is an intermediate to maggiemycin formation. If this is true, then aklaviketone appears to be a substrate for the formation of both aklavinone by a reduction at C-7 and for maggiemycin by hydroxylation at C-11 (Fig. 2; Connors *et al.*, 1990a). Thus, the only way in which an aklaviketone accumulating mutant could be obtained would be to have a double mutant (aklaviketone 7-reductase- and C-11 hydroxylase-deficient), so that aklavinone would not be produced as an intermediate, and aklaviketone accumulated would not be utilized as a C-11 hydroxylase substrate (producing maggiemycin). Our data, in this and an accompanying paper (Connors *et al.*, 1990a), indicate that these hypotheses are correct.

There are two possible reasons why we were unable to find a strain that accumulated AAME by screening mutants of *Streptomyces* sp. C5 for altered pigmentation or by bioassay. The first is that AAME-accumulating strains are similar in colour to the wild-type strain. Mutants accumulating AAME have not been isolated previously, suggesting that, *in vivo*, AAME might be converted to aklaviketone by a chemical rather than by an enzymic reaction (Eckardt & Wagner, 1988). The fact that we isolated double mutants (*dauD,E*) that accumulated AAME indicates that the cyclization is enzyme-catalysed. Moreover, AAME was the predominant product of the *in vitro* conversion of aklanonic acid by strain SC5-138, a *dauD,E* double mutant (Connors *et al.*, 1990a). The reason these mutants are slightly leaky (i.e. produce small quantities of maggiemycin) is probably that AAME can be chemically cyclized, yielding four possible stereoisomers. Although three of the chemically-formed isomers would probably not be biologically active, the fourth would be aklaviketone. Without a second mutation, these leaky mutants probably would have formed enough aklaviketone to synthesize glycosides that would have inhibited *S. aureus*. This provides the second reason why it is unlikely that we would have found AAME-accumulating mutants by direct mutation of *Streptomyces* sp. C5. Although no mutant accumulating AAME had previously been isolated, anthraquinone methyl esters structurally similar to AAME have been isolated from culture supernatants of *S. galilaeus* ANR-58 and ANR-665 mutant strains (Tobe *et al.*, 1982), *S. galilaeus* J-14 (Královková *et al.*, 1980) and *S. coeruleorubidus* 24-27 (type E) (Jizba *et al.*, 1980).

McGuire *et al.* (1980b) isolated the 7-oxo anthracycline, maggiemycin (described in detail by Pandey *et al.*, 1989), from a mutant strain, A21, which is related to *Streptomyces* sp. C5 (Fig. 1). Eckardt *et al.* (1988) recently isolated the brown-red 7-oxo anthracycline, aklaviketone, from cultures of a mutant of *S. galilaeus* S-383. Aklaviketone, which is 11-deoxymaggiemycin, was

shown to be an intermediate in the formation of aklavinone (Eckardt *et al.*, 1988; Eckardt & Wagner, 1988). The NAD(P)H-dependent reduction of aklaviketone at C-7 results in the formation of aklavinone (Connors *et al.*, 1990a). Our strain, SC5-24, and other *dauE* mutants, accumulated maggiemycin and are therefore similar to the maggiemycin-accumulating strain A21 previously described (Fig. 1). Similarly, among the blocked mutants of *S. griseus* IMET JA 3933 were four 'class VI' mutants that were coloured a deep blue-violet and were shown to accumulate anhydromaggiemycin (Ihn *et al.*, 1984), indicating that they were probably also aklaviketone reductase mutants. According to the hypothesis proposed by Eckardt & Wagner (1988), maggiemycin-accumulating strains are deficient in their ability to convert aklaviketone to aklavinone, which is the normal C-11 hydroxylase substrate. In the absence of the aklavinone reductase, aklaviketone accumulates and is apparently hydroxylated at C-11 to produce maggiemycin (Connors *et al.*, 1990a). The extract feeding experiments indicated that maggiemycin produced by the *dauE* mutants is not converted to glycosides, even though the *dauE* mutant could convert intermediates excreted by other, later mutants, to glycosides. These data, along with experiments showing that maggiemycin cannot be converted to ϵ -rhodomycinone *in vitro* (Connors *et al.*, 1990a), demonstrate that maggiemycin is a shunt product of *dauE* mutants blocked in daunomycin production, rather than an intermediate in the biosynthesis pathway (Fig. 2).

The anthracycline intermediate ϵ -rhodomycinone is found in most daunomycin fermentations (McGuire *et al.*, 1980c; Strohl *et al.*, 1989). ϵ -Rhodomycinone is formed by hydroxylation of aklavinone at C-11 (Connors *et al.*, 1990a). Whereas most early intermediates in the anthracycline biosynthesis pathway are yellow, ϵ -rhodomycinone and other compounds containing the C-11 hydroxyl group are red or orange (reviewed by Strohl *et al.*, 1989). Aclacinomycin-type antibiotics which incorporate aklavinone as their chromophore are produced by strains (e.g. *S. galilaeus*) which do not hydroxylate aklavinone at the C-11 position (Oki *et al.*, 1979). Mutants such as *S. peucetius* var. *aureus* (ATCC 31428), which are presumably deficient in the C-11 hydroxylation step, accumulate aklavinone and 11-deoxy derivatives of daunomycin (Cassinelli *et al.*, 1982). The seven *dauF* mutants of *Streptomyces* sp. C5 accumulated aklavinone and lacked aklavinone 11-hydroxylase activity as shown by *in vitro* reactions reported in the accompanying paper (Connors *et al.*, 1990a). Similarly, Wagner *et al.* (1981) isolated four 'class II' mutants of *S. griseus* that produced several yellow compounds which were later identified as 11-deoxydaunomycin analogues (Wagner *et al.*, 1985). These strains also probably lacked the C-11 hydroxylase

function which normally converts aklavinone to ϵ -rhodomycinone.

A group of nine mutants, comprising the *dauH* class, accumulated ϵ -rhodomycinone but no glycosides. Whereas most blocked mutants were isolated at a frequency of about $6-8 \times 10^{-5}$, *dauH* mutants were isolated at a frequency of 5.3×10^{-4} (Table 2), suggesting that any of several different genes could be mutated to obtain the same phenotype. Similar results were found with *S. griseus*, where mutants in class V were heterogeneous and consisted of eight strains that accumulated ϵ -rhodomycinone and some other minor red compounds (Wagner *et al.*, 1981). Based on cross-feeding between certain members of this class, Wagner *et al.* (1981) subdivided them into class V1, accumulating only ϵ -rhodomycinone, and class V2, accumulating ϵ -rhodomycinone, an ϵ -rhodomycinone-glycoside, 11-deoxydaunomycin and 11-deoxyaureomycin (C. Wagner, personal communication).

The biosynthetic sequence deduced from our accumulated data is shown in Fig. 2. Feeding experiments suggested that *dauA* mutants carry the earliest block in the order of reactions. This is consistent with their failure to accumulate any detectable anthracycline intermediates – they do not feed other blocked mutants – or to convert extracts from *dauC*, *dauF* and *dauH* mutants to glycosides. The next step in the biosynthetic sequence is represented by the *dauC* mutants; these accumulate aklanonic acid, can feed *dauA* mutants, and can be fed by *dauD,E* and *dauH* mutants. The *dauD,E* double mutants, which accumulate AAME and feed *dauC* mutants, are positioned next, followed by the *dauE,F* double mutants, strains that accumulate aklaviketone. The next group consists of the *dauF* mutants which accumulated aklavinone, were able to feed the *dauA*, *dauD,E* and *dauE* mutants, and were fed by some *dauH* mutants. The *dauH* mutants are placed at the final step of the reaction sequence, because they accumulated ϵ -rhodomycinone and fed *dauA*, *dauC*, *dauE*, *dauE,F* and *dauF* mutants.

Compounds in extracts made from cultures of the *dauG* strain, SC5-38, could not be converted to glycosides, nor did this strain convert any compounds produced by other strains (Table 2). Similarly, the *dauG* strains did not cosynthesize glycosides with any strains, except inconsistently with *dauE* strains (data not shown). Furthermore, *dauG* mutants were unable to support the expression of the actinorhodin biosynthesis genes, *actI*, *actIII* and *actVII* (P. L. Bartel and others, unpublished data), whereas the actinorhodin genes were expressed when transformed into blocked mutants from other classes (reviewed by Strohl *et al.*, 1989; P. L. Bartel and others, unpublished data). All five strains of the *dauG* class sporulated, and no noteworthy phenotypic changes

Fig. 2. Biosynthetic scheme for daunomycin showing the order of mutants, and the respective reactions, characterized in this work. Mutant classes A, C, D-E, E-F, F and H are ordered based on their accumulated metabolites and the results of cosynthesis assays and extract feeding experiments. Class E mutants accumulated maggiemycin, which could not be converted *in vivo* (this paper) or *in vitro* (Connors *et al.*, 1990a) to other anthracyclines, indicating that it is a shunt product. The second half of the pathway, from ϵ -rhodomycinone to carminomycin and daunomycin is described by Connors *et al.* (1990b).

were found other than the absence of anthracycline formation. Thus, it appears that the *dauG* strains are *daun*-minus regulatory mutants which may be analogous to *actII* mutants of *S. coelicolor* (Rudd & Hopwood, 1979; Hallam *et al.*, 1989).

Although about 80000 mutants were screened, none appeared to be affected in the reaction between ϵ -rhodomycinone and daunomycin. Both *Streptomyces* sp. C5 (McGuire *et al.*, 1980c) and *S. coeruleorubidus* ME130-A4 (Yoshimoto *et al.*, 1980) convert ϵ -rhodomycinone to glycosides of daunomycinone, demonstrating that ϵ -rhodomycinone is an intermediate of daunomycin biosynthesis. The sequence of enzymic reactions from ϵ -rhodomycinone to daunomycin is considerably less clear than the sequences preceding ϵ -rhodomycinone (see accompanying paper: Connors *et al.*, 1990b), but the following must take place: (i) removal of the methyl group at C-15; (ii) decarboxylation at C-10; (iii) two-step oxidation from a methylene to a keto group at C-13; (iv) *O*-methylation of the C-4 hydroxyl group; and (v) glycosylation with daunosamine of the C-7 hydroxyl group (see Fig. 2; Strohl *et al.*, 1989). We have recently proposed, from data in the literature and some of our own results, a biochemical sequence for these reactions (Strohl *et al.*, 1989). Our hypothetical sequence includes the glycosylation of ϵ -rhodomycinone to rhodomycin, which then would be demethylated and decarboxylated at C-10, followed by oxidation at C-13, and finally *O*-methylation at C-4 (Connors *et al.*, 1990b). If this sequence of reactions is correct, then all intermediates in the pathway after ϵ -rhodomycinone are red- to orange-pigmented glycosides that would inhibit our test organism. Therefore, our primary screening procedures would not have detected mutants blocked in the pathway after ϵ -rhodomycinone formation.

Stutzman-Engwall & Hutchinson (1989) recently cloned five distinct, non-overlapping regions of DNA from *S. peucetius* ATCC 29050, four of which may be involved in anthracycline production. Group IV DNA directed the biosynthesis of ϵ -rhodomycinone in *S. lividans* and caused the production of 100-fold more ϵ -rhodomycinone in *S. peucetius* strain H6125 (Stutzman-Engwall & Hutchinson, 1989). Southern hybridization indicated that DNA isolated from *Streptomyces* sp. C5 had homologous regions in common with DNA within gene clusters III and IV from *S. peucetius* ATCC 29050. Recent data indicate that the group IV cluster, along with DNA contiguous to it, probably encodes daunomycin formation in *S. peucetius* ATCC 29050 (Stutzman-Engwall & Hutchinson, 1989). We have recently subcloned a 2.0 kb *Sst*I fragment from the group IV gene cluster from *S. peucetius* ATCC 29050 that complemented the *dauE* (aklaviketone reductase) mutation in *Streptomyces* sp. C5 mutant strain SC5-138. Similarly, we

have subcloned from the group IV cluster a non-overlapping 6 kb *Sst*I DNA fragment that complemented both *dauC* and *dauD* mutants (T. Miller, P. L. Bartel & W. R. Strohl, unpublished). These experiments indicate that the mutants of *Streptomyces* sp. C5 we have obtained will be useful for analysing the anthracycline biosynthesis gene clusters recently isolated from *S. peucetius* ATCC 29050 (Stutzman-Engwall & Hutchinson, 1989).

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