Biosynthesis of anthracyclines: enzymic conversion of aklanonic acid to aklavinone and ϵ -rhodomycinone by anthracycline-producing streptomycetes

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Aklanonic acid, a substituted anthraquinone, is the earliest stable intermediate isolated so far in the biosynthesis of anthracycline antibiotics. Desalted, soluble cell extracts of *Streptomyces* sp. C5 and *Streptomyces peucetius* (ATCC 29050) converted aklanonic acid to ϵ -rhodomycinone in an *S*-adenosyl-L-methionine- and NADPH-dependent sequence of reactions. Aklanonic acid was methylated to form aklanonic acid methyl ester (AAME), which was cyclized to form aklaviketone. Aklaviketone was reduced to aklavinone by an NADPH-linked reductase. When extracts of *Streptomyces* sp. C5 and *S. peucetius* were used, aklavinone was hydroxylated at C-11 by an NADPH- and oxygen-dependent reaction to form ϵ -rhodomycinone. Cell extracts of *Streptomyces galilaeus* strains ATCC 31133 and 31671 converted aklanonic acid to aklavinone in the same manner, but neither of the *S. galilaeus* strains hydroxylated aklavinone to ϵ -rhodomycinone. Mutants of *Streptomyces* sp. C5 blocked at steps in the conversion of aklanonic acid to aklavinone, and which accumulated aklanonic acid (SC5-39; *dauC*), AAME (SC5-138; *dauD*, *dauE*), and aklaviketone (SC5-159; *dauE*, *dauF*), lacked the expected enzymic activities. In a mutant (SC5-24; *dauE*) lacking aklaviketone reductase, but retaining aklavinone 11-hydroxylase activity, maggiemycin was formed as an apparent shunt product. An enzymic pathway for ϵ -rhodomycinone and maggiemycin formation is consistent with the data presented.

Introduction

Aklavinone, one of the important precursors common to the formation of the clinically significant anthracyclines, daunomycin, doxorubicin, carminomycin, aklavin and aclacinomycin A, is produced by the condensation of nine acetyl equivalents with a propionyl-CoA starter molecule (Kitamura *et al.*, 1981). Aklavinone is glycosylated directly to produce aklavin and aclacinomycin A (Oki *et al.*, 1979; Strohl *et al.*, 1989). In the formation of daunomycin, however, aklavinone is hydroxylated to form ϵ -rhodomycinone, a red-pigmented compound (McGuire *et al.*, 1980b), which is then converted to the final product via several reactions (Strohl *et al.*, 1989). Recently, Eckardt and his colleagues isolated aklanonic acid, an anthraquinone metabolite accumulated by

Streptomyces sp. ZIMET 43717 (Eckardt *et al.*, 1985b). They showed that aklanonic acid and its chemically produced methyl ester derivative could be converted *in vivo* by blocked mutants of the anthracycline-producing strain, *S. griseus* IMET JA 5142, to aklavinone (Wagner *et al.*, 1984; Eckardt *et al.*, 1985a), and by *S. peucetius* strain 21/8 and *S. galilaeus* strain S727 to anthracyclines derived from aklavinone (Schumann *et al.*, 1986). Eckardt & Wagner (1988) recently proposed a pathway for the formation of anthracyclines, which included aklanonic acid as the first aromatic intermediate in the pathway.

In an accompanying paper (Bartel *et al.*, 1990a), we describe the isolation and characterization of mutants of *Streptomyces* sp. C5, a baumycin- and daunomycin-producing strain (McGuire *et al.*, 1980a, b), blocked at each of the steps proposed in the formation of aklavinone and ϵ -rhodomycinone. Here we describe the use of cell extracts of those mutants, along with extracts of several wild-type strains, to test each of the enzymic steps proposed for the conversion of aklanonic acid to aklavinone and ϵ -rhodomycinone.

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Abbreviations: AAME, aklanonic acid methyl ester; [methyl-¹⁴C]SAM, *S*-adenosyl[methyl-¹⁴C]-L-methionine.

Table 1. *Strains and mutants used in this study*

Streptomyces strain or mutant	Mutant class*	Relevant characteristics or metabolites accumulated	Source or reference†
C5 (SC5-1)	pt	ϵ -rhodomycinone, daunomycin, baumycins	FCRC; McGuire <i>et al.</i> (1980a, b)
SC5-39	<i>dauC</i>	Aklanonic acid	Bartel <i>et al.</i> (1990a)
SC5-24	<i>dauE</i>	Maggiemycin	
SC5-138	<i>dauD,E</i>	AAME	
SC5-159	<i>dauE,F</i>	Aklaviketone	
<i>S. galilaeus</i> 31133	pt	Aclacinomycin A	ATCC; Oki <i>et al.</i> (1979)
<i>S. galilaeus</i> 31671	<i>dauB</i>	2-Hydroxyaklavinone	ATCC; Matsuzawa <i>et al.</i> (1981)
<i>S. peucetius</i> 29050	pt	Daunomycin	ATCC; Dekleva <i>et al.</i> (1985)

* pt, Parental type (for description of *Streptomyces* C5 lineage, see Bartel *et al.*, 1990). *dauB* indicates a 2-hydroxyanthracycline-producing strain lacking the polyketide reductase function (see Strohl *et al.*, 1989). The other mutant classes are those described by Bartel *et al.* (1990a).

† FCRC, Frederick Cancer Research Center; ATCC, American Type Culture Collection.

Methods

Organisms, culture maintenance and growth conditions. The streptomycetes used in this study are described in Table 1. Spores of the strains were maintained in 20% (v/v) glycerol at -20°C . Cultures used frequently were also maintained at 4°C as mycelia grown on R2YE agar medium (Hopwood *et al.*, 1985). Nitrate-defined medium (NDM; Dekleva & Strohl, 1987), which was used to grow the streptomycetes for the *in vitro* experiments, contained, per litre: glucose, 22.5 g; NaNO_3 , 4.28 g; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.23 g; HEPES, 4.77 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12 g; and $20 \times$ trace elements (Dekleva *et al.*, 1985), 1 ml. The glucose was autoclaved separately and added after the medium was cooled. Maltose, which was substituted for the glucose in NDM when *S. peucetius* ATCC 29050 was grown (Dekleva & Strohl, 1987), was filter-sterilized and added to the medium after cooling. NDM was adjusted initially to pH 7.3 before autoclaving. NDYE consisted of NDM medium plus 0.5% yeast extract (Difco). To limit foaming in NDYE medium, one drop of Mazu DF-60P antifoam (Mazer Chemicals) was added per 50 ml of medium before autoclaving.

Preparation of cell-free extracts for *in vitro* bioconversions. For growth of each streptomycete to make crude mycelial extracts, 100 μl of a concentrated spore suspension, or alternatively mycelia scraped from solid R2YE medium, were used to inoculate a 250 ml flask containing 50 ml of NDYE medium and a coiled spring for dispersion of the mycelia (Dekleva *et al.*, 1985). For growth of *S. peucetius*, NDYE medium containing maltose as the carbon source instead of glucose was used to prevent spontaneous acidogenesis. The flasks were incubated with rotary shaking (approx. 250 r.p.m.) at 30°C for 2 d. Then 5 ml volumes of these 50 ml cultures were added to 500 ml flasks containing 100 ml NDM medium and a coiled spring. These cultures were incubated for 3 d under the same conditions as described above. Mutants of *Streptomyces* sp. C5 were grown by inoculating a 500 ml flask containing 100 ml NDYE and a coiled spring with mycelia scraped from R2YE agar medium, and incubating for 3 d under the same conditions as described above. The mutant strains were grown in NDYE because they grew poorly in NDM.

The mycelia were harvested by vacuum filtration, washed with 10 mM- or 100 mM-phosphate buffer, pH 7.5, as indicated, resuspended in 3–4 ml of buffer, and broken using an automated French pressure cell at 15000 p.s.i. (103.5 MPa). For preparation of desalted extracts, the broken mycelia were pelleted by centrifugation at 20000 g for 30 min at 4°C to sediment cell walls and unbroken cells. The supernatant was passed through a G-25 Sephadex column (Pharmacia LKB), pre-equilibrated with 10 mM or 100 mM-phosphate buffer

(pH 7.5), to remove contaminating low-molecular-mass compounds. The soluble proteins were eluted from the G-25 column using either 10 mM or 100 mM-phosphate buffer (pH 7.5). This desalted, soluble preparation, which contained 1–2 mg soluble protein ml^{-1} , was used as the source of enzymes for the *in vitro* assays.

Isotopic *in vitro* bioconversion analyses. The first method used to analyse the enzymic conversion of the various substrates was based on the ability of cell-free extracts to methylate aklanonic acid using *S*-adenosyl-[methyl- ^{14}C]-L-methionine ([methyl- ^{14}C]SAM). The radioactive products were extracted, concentrated, and detected by autoradiography after separation by thin-layer chromatography (TLC). The complete mixture for this isotopic enzymic assay, contained, in a volume of 250 μl : aklanonic acid, 0.75 nmol; [methyl- ^{14}C]SAM, 2.15 nmol (46 Ci mol^{-1} ; 1.7 TBq mol^{-1}); NADPH, 25 nmol; phosphate ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$) buffer, 25 μmol (100 mM, 'high ionic strength' buffer as denoted later; pH 7.5); and desalted, cell-free extract, 0.20 ml (approx. 0.2 mg soluble protein). The reaction mixtures were incubated at 37°C for times from 5 s to 5 min as denoted in the figure legends, after which the antibiotic intermediates synthesized in the reactions were extracted by the addition of 3 vols chloroform/methanol (9:1, v/v). The chloroform/methanol-soluble material was air-dried and reconstituted in 10 μl chloroform (plus authentic standards for cochromatography) for TLC on 0.25 mm layers of silica gel (Whatman). The TLC plates were then developed using one or more of the following solvent systems: chloroform/heptane/methanol (5:5:1, by vol.; SS1), chloroform/methanol (20:1, v/v; SS2), benzene/methanol (20:1, v/v; SS3), or benzene/ethyl acetate/methanol 0.1 M-HCl (5:5:1:5:0.15, by vol.; SS4). The developed TLC plates containing the radioactive products were then exposed to X-ray film (Kodak X-Omat AR) for 3 to 5 d. The enzymic reaction products were compared to authentic standards by cochromatography and by chromatography in parallel.

Non-isotopic *in vitro* bioconversion analyses. To confirm the results obtained in the autoradiography experiments, unlabelled reaction products were analysed by a combination of HPLC and TLC in which their R_f values, colouration, and fluorescence under ultraviolet (365 nm) light were compared (Bartel *et al.*, 1990a). For analysis of products by HPLC or TLC, the complete assay mixture contained, in 1.0 ml: anthraquinone or anthracyclinone substrate, 40 μg (approx 100 nmol); SAM (added only when aklanonic acid was used as the substrate) 5 μmol ; NAD(P)H, 5 μmol ; phosphate buffer, 100 μmol ; and desalted, cell-free extract, 900 μl (approx 1 mg soluble protein). When 'low ionic strength' buffer was required, 10 mM-phosphate buffer (pH 7.5) was used. In the experiments in which oxygen was

removed from the reaction mixture, the extracts, cooled on ice, were sparged with nitrogen gas for 5 min, the reactants were added under nitrogen sparging, and the tubes were sealed and incubated under anaerobic conditions. In all of the non-radioisotope experiments, the reaction mixtures were incubated at 37 °C for 1 h, and then extracted with chloroform/methanol (9:1). The extract was air-dried. In control experiments to determine background anthracyclines in the cell extract, no substrate was added. The products were reconstituted in 100 µl of methanol and analysed by HPLC using two different solvent systems: HPLC-SS1 consisted of methanol/H₂O/glacial acetic acid (65:30:5; by vol.; final pH 2.5) and HPLC-SS2 contained 65% (v/v) methanol and 35% (v/v) water, adjusted to pH 2.0 with phosphoric acid (Pandey & Toussaint, 1980). An Altex 100A HPLC pump was used at an elution rate of 1 ml min⁻¹, and the compounds were detected by their *A*₂₅₄ values using an Altex 100-40 flow-through cell and a Hitachi spectrophotometer. Alternatively, the reaction products were redissolved in 10 µl chloroform, and examined by TLC in solvent systems SS1–SS4, as described previously. In both methods, reaction products were compared to authentic standards run in parallel.

Chemicals. Authentic standards of aklavinone and ϵ -rhodomycinone were obtained from Adria and the Frederick Cancer Research Center. [*methyl*-¹⁴C]SAM was obtained from Dupont NEN. Authentic samples of aklanonic acid, AAME and aklaviketone were obtained from Klaus Eckardt and his colleagues, Academy of Sciences, Jena, GDR.

Results

Enzymic conversion of aklanonic acid by Streptomyces sp. C5

In the presence of [*methyl*-¹⁴C]SAM, NADPH and 100 mM-phosphate buffer, cell extracts of *Streptomyces* sp. C5 synthesized ϵ -[¹⁴C]rhodomycinone from aklanonic acid (Fig. 1). The ϵ -rhodomycinone formed in this reaction was identified by cochromatography with an authentic standard in TLC solvent systems SS1–SS4. The aklanonic acid and NADPH-dependent incorporation of [*methyl*-¹⁴C]SAM into anthracyclines extractable with chloroform/methanol (9:1) was linear for about 15 min under the reaction conditions described, with approximately 15% of the aklanonic acid converted within the first 15 min. After 1 h, about 50% of the aklanonic acid had been methylated and converted to other anthracyclines (data not shown). If NADPH was omitted from the reaction mixture, [¹⁴C]aklaviketone (identified by cochromatography with authentic aklaviketone in TLC solvent systems SS1–SS4) was produced instead of ϵ -rhodomycinone (Fig. 1). Moreover, [¹⁴C]aklaviketone, generated by incubating *Streptomyces* sp. C5 cell-free extracts with aklanonic acid and [*methyl*-¹⁴C]SAM and extracted with chloroform/methanol (9:1), was converted to [¹⁴C]rhodomycinone by incubation with *Streptomyces* sp. C5 cell extract and NADPH. This confirmed that the *in vitro* product from aklanonic acid and [*methyl*-¹⁴C]SAM in the absence of NADPH was aklaviketone, which subsequently could be reduced and

hydroxylated in the presence of NADPH and the cell extract to form ϵ -rhodomycinone.

One difficulty in detecting the formation of radioactive ϵ -rhodomycinone by TLC was that in all four solvent systems used (as well as with all other TLC solvent systems tested; see accompanying paper: Bartel *et al.*, 1990), ϵ -rhodomycinone and aklavinone were almost inseparable (cf. Fig. 1a). therefore, the enzymic conversion of aklanonic acid to ϵ -rhodomycinone by cell extracts of *Streptomyces* sp. C5 was confirmed by HPLC analysis using two different solvent systems. Incubation of cell extract with aklanonic acid, SAM, NADPH and 100 mM-phosphate buffer (pH 7.5) yielded a compound which eluted in a single peak with retention times of 10.7 and 15.2 min in HPLC-SS1 and HPLC-SS2, respectively. These values were identical to those obtained with authentic ϵ -rhodomycinone. Authentic aklavinone was eluted as a single peak at 8.7 and 11.3 min in HPLC-SS1 and HPLC-SS2, respectively, under these chromatographic conditions. If NADPH was omitted from the reaction mixture, the reaction product eluted in a single peak at 7.3 and 7.8 min in HPLC-SS1 and HPLC-SS2, respectively, as did authentic aklaviketone. If NADH was substituted for NADPH in the reaction, an unidentified compound with a retention time of 12.5 min in HPLC-SS2 was produced.

The ionic strength of the phosphate buffer used in the extractions had a marked effect on the ability of *Streptomyces* sp. C5 cell extracts to convert aklanonic acid to ϵ -rhodomycinone. When cell extracts were prepared using high-ionic-strength phosphate buffer (100 mM, pH 7.5), the product synthesized was always ϵ -rhodomycinone. When low-ionic-strength phosphate buffer was used (10 mM, pH 7.5), very little aklanonic acid was converted all the way to ϵ -rhodomycinone; aklavinone was the major product formed, as determined by HPLC (data not shown). Similarly, the presence or absence of oxygen altered the type of product formed. If cell extracts were incubated with the complete reaction mixture, but in the absence of oxygen, the major product was aklavinone, not ϵ -rhodomycinone (data not shown). On the other hand, sparging with pure oxygen stimulated conversion of aklavinone to ϵ -rhodomycinone.

Conversion of aklanonic acid by other Streptomyces species.

When the enzymic conversion of aklanonic acid was tested using desalted cell extracts of *Streptomyces peucetius* ATCC 29050, a daunomycin-producing strain (Strohl *et al.*, 1989), the results were similar to those described above, i.e. ϵ -rhodomycinone was produced in the complete assay system (using 100 mM-phosphate buffer, pH 7.5), and aklaviketone was produced when

Fig. 1. Autoradiograms of silica gel TLC plates developed with chloroform/heptane/methanol (5:5:1). The products were synthesized during 5 min (unless otherwise noted) in the radioisotopic assay system with cell extracts of the strains indicated. (a) Lanes: A–C, *Streptomyces* C5; D–F, *S. peucetius* 29050; G–I, *S. galilaeus* 31133; J–L, *S. galilaeus* 31671. Lanes: A, D, G, and J, complete reaction; B, E, H and K, without NADPH; C, F, I and L, without aklanonic acid (control). (b) Lanes A–E contain cell extract of strain SC5-138 with the complete assay mixture for 5, 10, 30, 60 and 120 s, respectively, showing accumulation of AAME and, at 120 s, also minor quantities of aklaviketone. (c) Lanes A and B contain cell extract of strains SC5-24 and SC5-159 with the complete assay mixture for 5 min; both show accumulation of aklaviketone.

NADPH was omitted. With *S. peucetius* cell extract, however, aklavinone was the product synthesized when NADH was substituted for NADPH in the complete reaction.

When cell extracts of *Streptomyces galilaeus* ATCC 31133 (aclacinomycin A producer; Oki *et al.*, 1979) or *S. galilaeus* ATCC 31671 (2-hydroxyaklavinone producer; Matsuzawa *et al.*, 1981) were incubated in the complete non-isotopic assay system (with either NADPH or NADH as the electron donor and using 100 mM-phosphate buffer, pH 7.5), aklavinone was synthesized instead of ϵ -rhodomycinone. In both, omitting NAD(P)H from the reaction mixture gave aklaviketone as the only product.

Enzymic conversion of anthracycline precursors by mutants of *Streptomyces* sp. C5

To confirm the steps blocked in various anthracycline biosynthesis mutants, representatives of the mutant

classes blocked at putative steps between aklanonic acid and ϵ -rhodomycinone (Eckardt & Wagner, 1988; Bartel *et al.*, 1990a) were tested in our *in vitro* conversion system using a variety of substrates. Cell extracts of the *dauC* mutant (SC5-39), an aklanonic acid-accumulating strain, failed to incorporate [*methyl*- ^{14}C]SAM into chloroform/methanol (9:1)-soluble reaction products when incubated with the complete radioisotopic assay mixture (data not shown). Cell extracts of this strain also failed to convert aklanonic acid, aklaviketone or aklavinone to ϵ -rhodomycinone in the non-isotopic assay system (Table 2). Cell extracts of mutant SC5-39 converted AAME to aklaviketone, but the conversion was very poor.

Cell extracts of the *dauE* mutant (SC5-24), a maggie-mycin-accumulating strain (Bartel *et al.*, 1990a), converted aklanonic acid and AAME to aklaviketone when incubated with the complete isotopic conversion assay mixture (Fig. 1c). The extract also converted aklavinone to ϵ -rhodomycinone *in vitro* (Table 2). Thus the only reaction that the cell extract of this strain was unable to

Table 2. Products synthesized from various precursors by cell-free extracts of *Streptomyces* C5 blocked mutants

Substrate provided*	Product formed by cell extracts of mutant strains†			
	SC5-24 (<i>dauE</i>)	SC5-39 (<i>dauC</i>)	SC5-138 (<i>dauD,E</i>)	SC5-159 (<i>dauE,F</i>)
Aklanonic acid	Aklaviketone	NC	AAME	Aklaviketone
AAME	Aklaviketone	Aklaviketone	NC	Aklaviketone
Aklaviketone	NC	NC	NC	NC
Aklavinone	ϵ -Rhodomycinone	NC	ϵ -Rhodomycinone	NC

NC, No conversion of the substrate under conditions tested.

* The complete reaction contained (in 1.0 ml): substrate, 40 μ g; SAM, 5 μ mol; NAD(P)H, 5 μ mol; phosphate buffer, 100 μ mol; desalted cell extract, about 1 mg of soluble protein. Cell extracts were prepared in 100 mM-phosphate buffer (pH 7.5).

† Mutants and their metabolites are described in Table 1. Products of reactions were analysed by TLC using SS1 (R_F values and colours of substrates and products: aklanonic acid, 0.28 (yellow); AAME, 0.56 (brown-red); aklaviketone, 0.45 (wine-coloured); aklavinone, 0.48 (yellow); ϵ -rhodomycinone, 0.49 (red)).

Table 3. Presence of enzyme activities in anthracycline-producing strains and blocked mutants

Strain	Enzymic activity observed in cell extracts			
	Aklanonic acid methyltransferase	AAME cyclase	Aklaviketone reductase	Aklavinone 11-hydroxylase
SC5-1	+	+	+	+
SC5-24 (<i>dauE</i>)	+	+	—	+
SC5-39 (<i>dauC</i>)	—	+	—	—
SC5-138 (<i>dauD,E</i>)	+	—	—	+
SC5-159 (<i>dauE,F</i>)	+	+	—	—
<i>S. peucetius</i>	+	+	+	+
<i>S. galilaeus</i>	+	+	+	—

The presence (+) or absence (—) of activities is based on the enzymic conversion of the various anthracycline precursors using the radioisotopic and non-radioisotopic assays described. See Fig. 2 for placement of these enzymes in the pathway.

catalyse was the reduction of aklaviketone to aklavinone (Tables 2 and 3). This is consistent with the hypothesis that strains producing maggiemycin lack aklaviketone reductase activity but retain 11-hydroxylase activity (Table 3).

Using the complete isotopic conversion assay mixture, cell extracts of the *dauD,E* mutant, SC5-138, which accumulates AAME (Bartel *et al.*, 1990), converted aklanonic acid to AAME (Fig. 1*b*). After longer incubation periods, small amounts of aklaviketone were produced (Fig. 1*b*), but aklavinone was never produced from aklanonic acid by this strain. Cell extracts of SC5-138 did, however, convert aklavinone to ϵ -rhodomycinone (Table 2), a result consistent with the fact that SC5-24 (parent of SC5-138) lacks aklaviketone reductase, but retains 11-hydroxylase activity (Table 3).

Cell extracts from the *dauE,F* mutant (SC5-159), which accumulates aklaviketone (Bartel *et al.*, 1990*a*), synthesized aklaviketone from aklanonic acid or AAME (Fig. 1*c*; Table 2), in the presence or absence of NADPH (data not shown). The enzymes of this strain did not convert aklaviketone or aklavinone to any other products, indicating that both aklaviketone reductase and 11-hydroxylase activities were missing (Table 3).

Discussion

We have used cell extracts of several mutants of *Streptomyces* sp. C5 blocked in the early steps of a baumycin and daunomycin biosynthesis (Bartel *et al.*, 1990), along with those of several wild-type strains, to

test the biosynthetic reactions proposed for the conversion of aklanonic acid to ϵ -rhodomycinone. The *dauC* mutant, SC5-39, which accumulates aklanonic acid, appears similar to blocked mutants of *S. peucetius* 601-F1 and *S. galilaeus* F198 which also accumulate aklanonic acid (Shlegel *et al.*, 1987). Thus it appears that aklanonic acid is a primary intermediate in the daunomycin production pathway of several organisms, as previously suggested (Eckardt & Wagner, 1988). Because strain SC5-39 did not convert aklanonic acid, AAME, aklaviketone or aklavinone to ϵ -rhodomycinone under the standard bioconversion assay conditions, it may possess multiple mutations. Alternatively, a single polar mutation or a deletion might have caused these results. From these data, SC5-39 might also be a regulatory mutant; however, this is unlikely because this strain, as well as all other strains in the *dauC* class, cosynthesized daunomycin and baumycins with other blocked mutants (Bartel *et al.*, 1990a).

The *dauD,E* mutant, strain SC5-138, which accumulates AAME (Bartel *et al.*, 1990), was capable of converting aklanonic acid to AAME *in vitro* (Fig. 1). AAME, chemically synthesized from aklanonic acid, was shown to be converted to anthracyclines *in vivo* by blocked mutants (Wagner *et al.*, 1984; Eckardt *et al.*, 1985a), indicating its probable importance as an intermediate in the pathway. The esterification of aklanonic acid helps to render C-10 a good nucleophile, so that nucleophilic attack on the C-9 carbonyl can be catalysed by a cyclization enzyme (Fig. 2). It has been proposed that this cyclization even can occur spontaneously under mild conditions while maintaining stereoselectivity (Eckardt & Wagner, 1988). Accumulation of AAME as a product of SC5-138 and formation in cell extracts of AAME *in vitro* indicate that a cyclization is probably responsible for the majority of aklaviketone formation *in vivo*. Although small quantities of aklaviketone were produced from aklanonic acid by cell extracts of this mutant (Fig. 1b), AAME is by far the predominant metabolite (Bartel *et al.*, 1990a); it was the predominant product of the *in vitro* reactions as well. Moreover, as mentioned in an accompanying paper (Bartel *et al.*, 1990a) we have subcloned a 6 kb *Sst*I fragment from *S. peucetius* anthracycline biosynthesis gene cluster IV (Stutzman-Engwall & Hutchinson, 1989) that complements both aklanonic acid methyltransferase (*dauC*) mutants and AAME cyclase (*dauD*) mutants. Thus, we propose that closure of the A-ring to form the first true anthracyclinone, aklaviketone, is catalysed by AAME cyclase, which is encoded by the *dauD* gene.

McGuire *et al.* (1980a) reported the isolation of a blue-purple 7-oxo anthracyclinone, maggiemycin, produced by a mutant strain, A21, related to *Streptomyces* sp. C5. The complete description of maggiemycin has recently

been published by Pandey *et al.* (1989). Similarly, Eckardt *et al.* (1988) isolated the brown-red 7-oxo anthracyclinone, aklaviketone, from cultures of a mutant of *S. galilaeus* S-383. Aklaviketone, which is 11-deoxymaggiemycin, was proposed to be an intermediate in the formation of aklavinone (Eckardt *et al.*, 1988; Eckardt & Wagner, 1988). Our *dauE* mutant strain, SC5-24, accumulates maggiemycin and is therefore similar to the previously described maggiemycin-accumulating strain, A21 (McGuire *et al.*, 1980a; Pandey *et al.*, 1989). According to the hypothesis proposed by Eckardt & Wagner (1988), maggiemycin-accumulating strains are deficient in their ability to convert aklaviketone to aklavinone, the normal 11-hydroxylase substrate. In the absence of the aklavinone intermediate, aklaviketone is apparently hydroxylated at C-11 to form maggiemycin. This hypothesis is supported by our observation that SC5-24 synthesized only aklaviketone from aklanonic acid *in vitro* (Table 3). Aklaviketone apparently serves as an alternative substrate for the 11-hydroxylase which then generates maggiemycin as a shunt product. Interestingly, SC5-24 did not produce maggiemycin *in vitro* from aklanonic acid, AAME or aklaviketone, suggesting that the enzymic conditions used did not favour aklaviketone as an alternative substrate to aklavinone for the 11-hydroxylase. Under identical conditions, however, the cell extracts did convert aklavinone to ϵ -rhodomycinone.

Aklavinone was generated by the NAD(P)H-dependent reduction of the 7-keto group of aklaviketone to a hydroxyl group. A 7-hydroxyl would be the most stable tautomer because of chelation with the 6-hydroxyl group, and therefore the reduction of the 7-keto group to a hydroxyl group is required for the subsequent attachment of the sugars at C-7 through an ether linkage. In the absence of NADPH, cell extracts of *Streptomyces* sp. C5, *S. peucetius*, and the two *S. galilaeus* strains produced aklaviketone from aklanonic acid. With the exception of *Streptomyces* sp. C5, extracts of these strains also synthesized aklavinone from aklanonic acid when NADH was substituted for NADPH, indicating that the aklaviketone reductase of those strains could utilize either coenzyme. Since aklaviketone was not the product of the NADH-dependent reaction with *Streptomyces* sp. C5 cell extracts, it must be assumed that this enzyme did not use NADH as an alternative coenzyme. When maggiemycin (11-hydroxyaklaviketone) was incubated with NADPH and cell-free extracts of *Streptomyces* sp. C5, no ϵ -rhodomycinone was formed (N. C. Connors, unpublished), indicating that maggiemycin was not an alternative substrate for the aklaviketone reductase under the conditions tested. This result was expected, however, because chloroform/methanol (9:1) extracts of the *dauE* mutants (including SC5-24) could not be converted to daunomycin or baumycins *in vivo* by strains

of other mutant classes (Bartel *et al.*, 1990a). Taken together, these data demonstrate that maggiemycin is a shunt product and not an intermediate in the daunomycin biosynthesis pathway.

Hydroxylation of aklavinone at C-11 produces ϵ -rhodomycinone, a reaction which occurs in *Streptomyces* sp. C5 and *Streptomyces peucetius* ATCC 29050, but not in *Streptomyces galilaeus* or other strains which produce anthracyclines lacking the 11-hydroxyl group (Oki *et al.*, 1979; Cassinelli *et al.*, 1982; Fujiwara & Hoshino, 1983; Wagner *et al.*, 1985). The biosynthesis of ϵ -rhodomycinone from aklanononic acid by cell extracts of the daunomycin-producing strains, *S. peucetius* or *Streptomyces* C5, indicates that all of the essential precursors were available for the aklavinone 11-hydroxylase reaction. When either NADPH or oxygen was omitted from the reactions, or when the molarity of phosphate buffer was 10 mM instead of 100 mM, aklavinone was not converted to ϵ -rhodomycinone, indicating that NADPH, O₂ and high ionic strength were required for this reaction to occur. Other oxygen- and NADPH-dependent polyketide hydroxylase reactions have recently been characterized for the erythromycin (Shafiee & Hutchinson, 1987) and tetracycline (Vančurová *et al.*, 1988) biosynthesis pathways. Furthermore, the 6-deoxy-erythronolide B hydroxylase was found to be a cytochrome P-450 type monooxygenase (Shafiee & Hutchinson, 1987). Our data, indicating that aklavinone 11-hydroxylase required both NADPH and oxygen, suggest that aklavinone 11-hydroxylase may also belong to this class of monooxygenase reaction. The requirement for high molarity buffer conditions is probably related to stabilization of the enzymes or electron transfer components in the cell extract.

Based on our results, we propose a set of enzymic reactions for the conversion of aklanononic acid to aklavinone, ϵ -rhodomycinone and maggiemycin by *Streptomyces* sp. C5 (Fig. 2). Our data indicate that aklanononic acid is methylated by SAM-dependent methylase to form AAME, which is enzymically cyclized to form aklaviketone. Aklaviketone is reduced by an NAD(P)H-dependent reductase to form aklavinone, which is hydroxylated by an NADPH- and oxygen-dependent enzyme to form ϵ -rhodomycinone (Fig. 2). Alternatively, aklaviketone can be hydroxylated to form maggiemycin in strains lacking the aklaviketone reductase (Fig. 2). In strains lacking the aklavinone 11-hydroxylase (e.g. *S. galilaeus* strains), aklavinone is glycosylated directly to form the final products (Fujiwara & Hoshino, 1983). The proposed pathway is entirely consistent with the pathway proposed by Eckardt & Wagner (1988), which was based on mutant analysis and *in vivo* bioconversions. Moreover, our results indicate that two *S. galilaeus* strains and *S. peucetius* ATCC 29050

Fig. 2. Proposed enzymic reactions in the biosynthesis of aklavinone, ϵ -rhodomycinone and maggiemycin from aklanononic acid. The carbons on AAME are numbered according to the standard anthracycline numbering system, and carbon numbers are provided on other structures where significant. Asterisks indicate the incorporation of the [methyl-¹⁴C] group from [methyl-¹⁴C]SAM.

also carry out these conversions to aklavinone and ϵ -rhodomycinone respectively. This indicates that the pathway for the formation of aklavinone and ϵ -rhodomycinone is apparently universal in those organisms in which these compounds are intermediates in the formation of the final anthracycline product.

The conversion of aklanononic acid to aklavinone by *S. galilaeus* ATCC 31671, a mutant that produces 2-hydroxyaklavinone, suggests that the reaction that causes production of the 2-hydroxy derivative must precede formation of aklanononic acid. This is consistent with data from Tobe *et al.* (1982), who discovered a 2-hydroxyanthraquinone methyl ester (compound '58B') very similar in structure to AAME. Since *S. galilaeus* ATCC 31671 was derived from a strain that originally produced aklavinone, it is apparent that loss of a function causes the formation of 2-hydroxy derivatives. The formation of anthracyclines lacking the 2-hydroxyl group is apparently due to reduction of the putative polyketide precursor; if no reduction occurs, the

cyclized compounds contain the 2-hydroxyl group (Eckardt & Wagner, 1988; Strohl *et al.*, 1989). Hallam *et al.* (1989) recently sequenced and characterized a polyketide reductase gene for the actinorhodin biosynthesis pathway, for which a similar mechanism is proposed. We have found that this actinorhodin polyketide reductase gene (*actIII* gene; Hallam *et al.*, 1989), when cloned into *S. galilaeus* ATCC 31671, causes production of aklavinone rather than 2-hydroxyaklavinone (Bartel *et al.*, 1990b), indicating that *S. galilaeus* ATCC 31671 produces 2-hydroxy derivatives because it is mutated in its polyketide reductase gene. This also indicates that the enzymes involved in aklavinone formation can utilize as substrates compounds which contain either a hydrogen or a hydroxyl group at C-2.

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