STUDIES ON THE BIOSYNTHESIS OF THE ANTIBIOTIC CRISAMICIN A AND CARBON-13 MAGNETIC RESONANCE ASSIGNMENTS

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The biosynthesis of crisamicin A, a novel dimeric isochromanequinone antibiotic from *Micromonospora purpureochromogenes* subsp. *halotolerans* has been investigated by [1-^{13}C] and [2-^{13}C] labeled acetate precursor feeding experiments. Analysis of the proton noise decoupled and off resonance ^{13}C NMR spectra of ^{13}C enriched and unenriched crisamicin A and their acetate derivatives indicated the biosynthesis *via* the polyketide pathway, as expected. Further analysis of the enriched spectra allowed the complete assignment of the carbon signals. Of particular interest was the establishment of the linkage between the two monomeric halves of the molecule and determination of the location of the phenolic hydroxyls.

Crisamicin A (CRS-A) a novel isochromanequinone antibiotic produced by *Micromonospora purpureochromogenes* subsp. *halotolerans*,[1,2] is a symmetrical dimeric molecule, like actinorhodin,[3] and bears structural similarities to the antibiotics nanaomycin D,[4] kalafungin,[5] medermycin[6] and mederrhodin A[6] (Fig. 1). Preliminary investigations indicated that the CRS-A molecule possessed one of two possible structures, (Fig. 2), with **1** being favored based on chemical data.[2] The two alternative structures differ in the location of the phenolic hydroxyl groups and in the position of the linkage bond between the two monomers.

An examination of these possible structures suggests that CRS-A is formed from acetate *via* the polyketide pathway as are other members of this class of antibiotics. In order to distinguish between structures **1** and **2** and to allow for the unambiguous assignment of the ^{13}C NMR spectrum, the biosynthesis of CRS-A was examined by ^{13}C-labeled C-1 and C-2 acetate studies. The present paper describes the results of such experiments leading to **1** as the correct structure for CRS-A.

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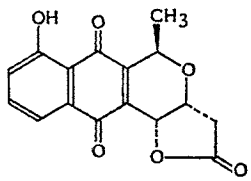
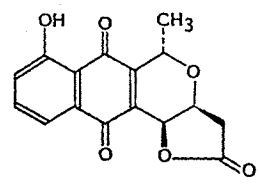
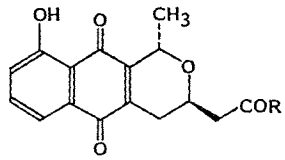
Fig. 1. Related isochromanequinone antibiotics.



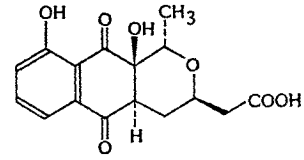
Kalafungin



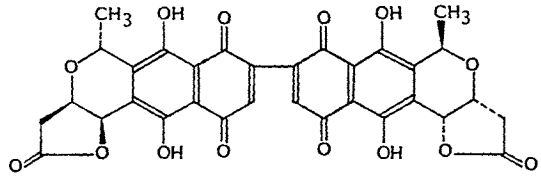
Nanaomycin D



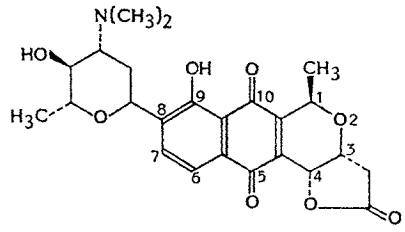
Nanaomycin A R = OH
Nanaomycin C R = NH_2



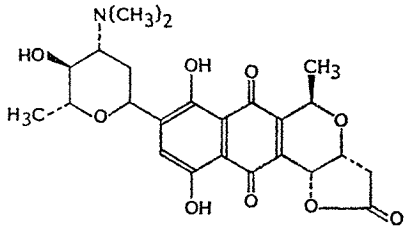
Nanaomycin B



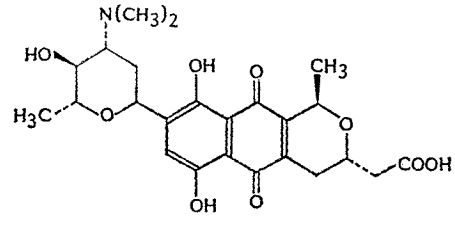
γ-Actinorhodin



Medermycin



Mederrhodin A



Mederrhodin B

Materials and Methods

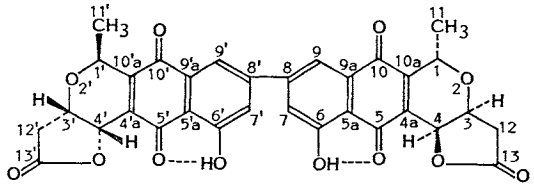
Culture

Micromonospora purpureochromogenes subsp. *halotolerans* (RU-79-9-101) was obtained from the Red V Coconut Products Company Inc., Lucena City, Luzon, Republic of the Philippines. The culture was maintained as frozen vegetative cells stored in the gas phase of a liquid nitrogen freezer.

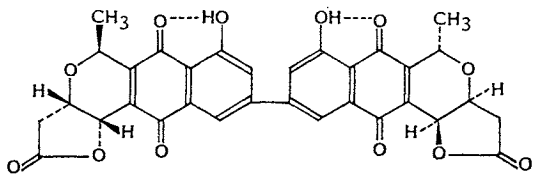
Reagents and Chemicals

Sodium acetate labeled at the C-1 position (93.2% atom enriched) or at the C-2 position (92.8% atom enriched) were obtained from Merck Sharp and Dohme, Stable Isotopes Division, Montreal, Canada. Deuterated solvents were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. All other chemicals were reagent grade and purchased from J. T. Baker Chemical Co. (Phillipsburg, New Jersey) or Fisher Scientific Co, (Springfield, New Jersey).

Fig. 2. Proposed structures for CRS-A.



1



2

NMR Instrumentation

^{13}C NMR spectra of CRS-A were obtained in DMF-d_7 on a Bruker WP 200 SY NMR spectrometer equipped with an IBM Aspect 2000 computer at 50.31 MHz with a 35° pulse width of 10 seconds, acquisition time of 0.5407 seconds and relaxation delay (2 μseconds). Spectra of CRS-A and CRS-A acetate in $CDCl_3$ were recorded on a Varian XL-400 NMR spectrometer at 100.57 MHz with a pulse width of 25° (7 μseconds), acquisition time of 0.40 second, and relaxation delay of 0.50 second.

TLC and Column Chromatography

Samples were analyzed by TLC on Silica Gel-60 F-254 (EM Reagents, Darmstadt, FRG) with an adsorbent thickness of 250 μm. TLC plates were developed in $CHCl_3$ - MeOH - acetic acid (95 : 5 : 1) and compounds of interest were visualized under UV light.

Column chromatography was conducted on Kieselgel 60, 70~230 mesh (EM Reagents, Darmstadt, FRG) slurried in $CHCl_3$. Samples were loaded in $CHCl_3$ and eluted with $CHCl_3$ - MeOH (99 : 1).

HPLC

HPLC was performed on a Waters LC system, equipped with a Model 680 Automated Gradient Controller, two Model 510 pumps and a Model 490 Programmable Multiwavelength Detector. Separations were carried out as described earlier.[1]

Preparation of Labeled CRS-A

Fermentations of labeled CRS-A were carried out as described previously,[1] except that 600 ml of the production medium was used in 2,000-ml baffled flasks (Catalog No. 2542-02000, Bellco Glass Inc., Vineland, New Jersey). Production flasks were inoculated with 30 ml (5%) of the inoculum culture and incubated at 28°C on a Model G-53 Rotary Shaker (New Brunswick Scientific Co., Edison, New Jersey) operated at 200 rpm.

CRS-A titers were monitored in the fermentation broths daily by HPLC assay by extraction of an aliquot of the broth as described previously.[1]

Labeled acetate was added when assays indicated the onset of antibiotic production (approximately 60 hours). Sodium acetate labeled with ^{13}C at the C-1 or the C-2 position was dissolved in distilled water (900 mg/50 ml) and 25 ml of this solution was added to each of two production flasks to give a final concentration of 750 mg/liter of labeled sodium acetate. The fermentations were harvested when CRS-A titers began to decline, at approximately 170 hours.

Whole broths (*ca.* 1.0 liter) were extracted twice with EtOAc at neutral pH. The combined extracts were taken to dryness, and triturated with hexane to remove the oily material and to give *ca* 700 mg of crude product which contains *ca.* 300 mg of CRS-A. The ^{13}C enriched CRS-A was purified by column chromatography on silica gel and analyzed by TLC (Rf 0.48) and by HPLC comparing with standard CRS-A described previously.[1]

Preparation of CRS-A Diacetate

The ^{13}C enriched CRS-A (50 mg) was added with stirring to acetic anhydride (50 ml) at room temperature. A drop of concentrated sulfuric acid was added as a catalyst. Several small pieces of ice were added and the reaction mixture was stirred for 15 minutes. After this time more ice was added to decompose the excess acetic anhydride. Two volumes of water were added, and the reaction mixture was extracted three times with an equal volume of $CHCl_3$. The pooled $CHCl_3$ extract was washed three times with four volumes of water. The organic layer was recovered, dried over Na_2SO_4, and taken to dryness *in vacuo* to give 45 mg of crude product, 40 mg of which on purification on silica gel chromatography gave CRS-A diacetate (28 mg). The eluting solvent was $CHCl_3$ - MeOH (99 : 1). Formation of the diacetate derivative was confirmed by NMR (loss of signal at 11.88 ppm from phenolic hydroxyl, appearance of signal at 2.50 ppm due to a methyl group), IR (KBr pellet; CRS-A, 1759, 1650, 1624 cm^{-1}; CRS-A diacetate, 1783, 1668, 1605 cm^{-1}) and mass spectrometry (fast atom bombardment (FAB) negative ion mode, *m/z* 683 $(M-H)^-$ for hydroquinone form; FAB positive ion mode, *m/z* 685 $(M+H)^+$ for hydroquinone form), as well as by TLC (Rf; CRS-A 0.48; CRS-A diacetate 0.54).

Results and Discussions

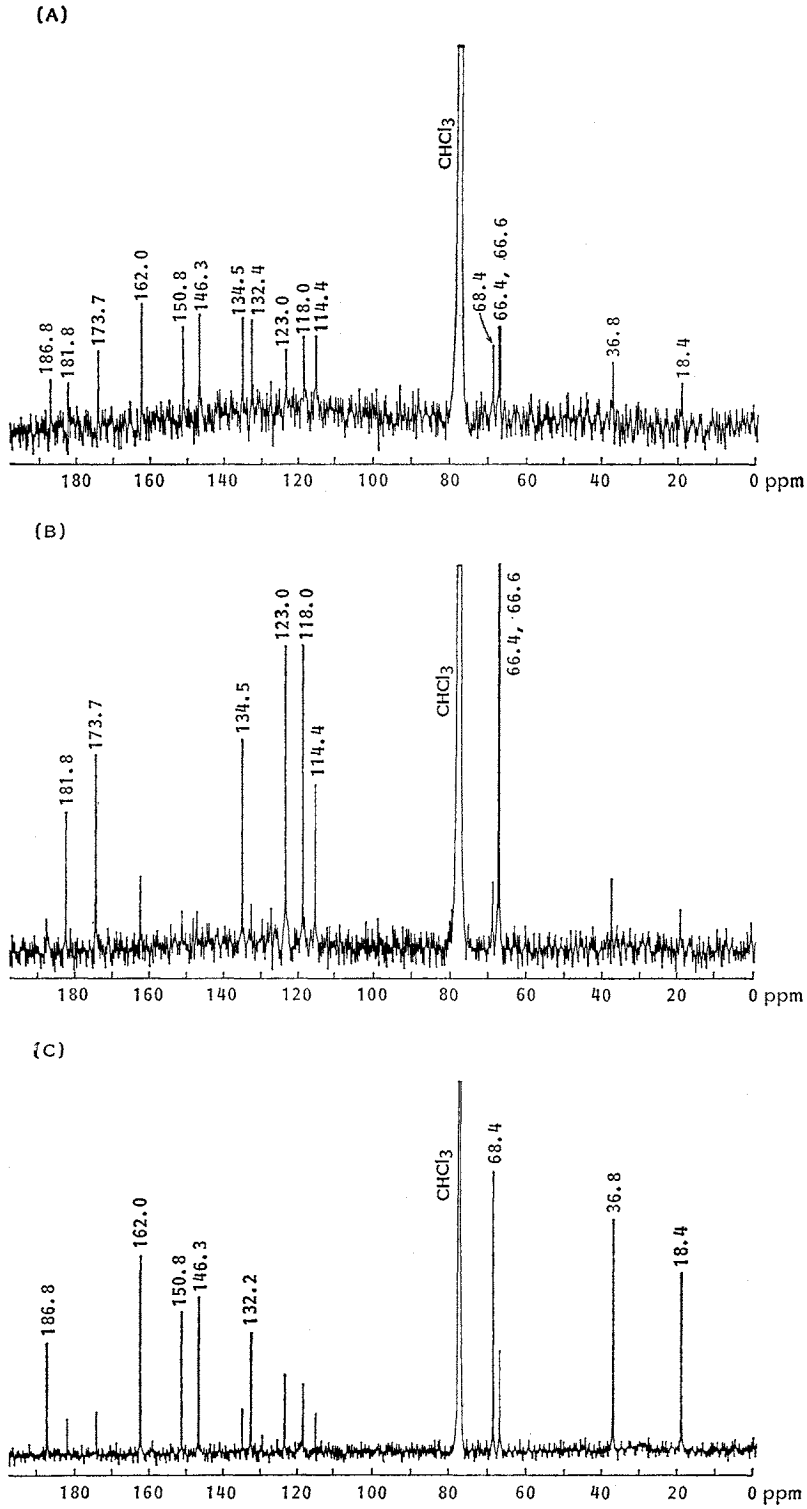
The natural abundance ^{13}C NMR spectrum for CRS-A (Fig. 3A) shows the presence of 16 signals as expected from a symmetrical dimeric molecule with the molecular formula $C_{32}H_{22}O_{12}$.[2] Spectra of [1-^{13}C] and [2-^{13}C]acetate enriched CRS-A are presented in Figs. 3B and 3C, respectively, each of which show the enrichment of eight carbons with no carbon being enriched by the same acetate precursor. The presence of eight enhanced signals in each spectrum thus suggests the alternate labeling pattern anticipated from a polyketide derived compound.

Some peak assignments could be made based on chemical shift data, peak multiplicities and off-resonance decoupling and single bond ^1H-^{13}C coupling.[7,8] Confirmation and differentiation of peak assignments was from labeling patterns, peak intensities and chemical shifts observed from the CRS-A diacetate derivatives (Fig. 4). Reference was also made to the published spectra for juglone[9] and for other isochromanequinone antibiotics.[10~14] Enrichment factors for the C-1 and C-2 acetate labeled compounds were calculated for CRS-A acetate, using the acetate methyl carbon to normalize peak heights. Previously published enrichment factors for CRS-A[15] were in error due to improper normalization of peak height data. The chemical shifts, peak multiplicities, and enrichment factors for CRS-A and CRS-A acetate are shown in Table 1.

In addition to demonstrating the biosynthetic origin of the CRS-A molecule, these data confirm **1** as the correct structure (Fig. 2). In the CRS-A spectrum (Fig. 3) the labeling of the peaks at 118.0 and 123.0 ppm by C-1 of acetate and assignment to aromatic carbons bearing free protons, places these carbons at positions C-9 and C-7 respectively.[9] The phenolic carbon is observed to be enriched by C-2 of acetate in the CRS-A acetate spectrum (peak at 148.7 ppm in Fig. 4) thus placing it at C-6 of the CRS-A molecule.

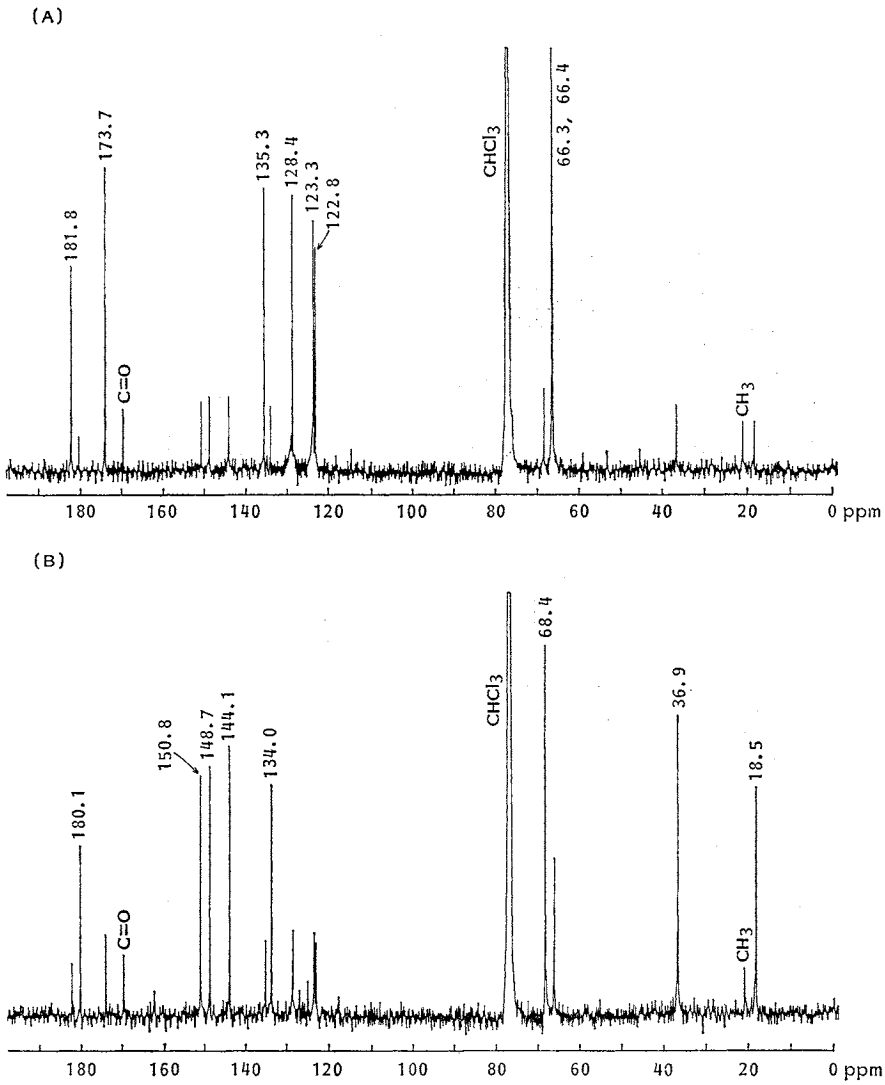
Also, the downfield shift observed for the C-5 quinone carbonyl (chemical shift 186.8 ppm) relative to the C-10 carbonyl (181.8 ppm) is indicative of hydrogen bonding of C-5 quinone carbonyl to the C-6 phenolic hydroxyl. The linkage point between the two monomers can thus be located at

Fig. 3. Proton noise-decoupled ^{13}C NMR spectra of CRS-A at 100.57 MHz in $CDCl_3$.



(A) Natural abundance spectrum, (B) biosynthetically enriched with [1-^{13}C]acetate, (C) biosynthetically enriched with [2-^{13}C]acetate. Chemical shift values relative to TMS.

Fig. 4. Proton noise-decoupled ^{13}C NMR spectra of CRS-A acetate at 100.57 MHz in $CDCl_3$.



(A) Biosynthetically enriched with [1-^{13}C]acetate, (B) biosynthetically enriched [2-^{13}C]acetate. Chemical shift values are relative to TMS.

C-8, *meta* to the phenolic hydroxyl by the observation (under off-resonance decoupling) of a singlet for C-8 and doublets for C-7 and C-9, showing C-8 to be the carbon atom substituted by the adjoining monomer. Taken together, these data confirm **1** as the correct structure for CRS-A.

Biosynthetic studies on the isochromanequinone antibiotics have been reviewed by FLOSS.[16] CRS-A was labeled by C-1 and C-2 of acetate in the manner expected for biosynthesis *via* the polyketide pathway, and is in agreement with the labeling patterns seen for the other isochromanequinone antibiotics (Fig. 5).

A proposed biosynthetic pathway of CRS-A would include the following steps (not necessarily in the order listed):

1) Synthesis of the polyketide backbone of the monomer by head-to-tail condensation of acetate

Table 1. Relative enrichment of carbons in CRS-A labeled with [1-^{13}C] or [2-^{13}C]acetate.

	Carbon[a]	Chemical shifts[b]			Multiplicity[c]	Acetate precursor	Enrichment factor[d]
		CRS-A		CRS-A acetate			
		Solvent A	Solvent B	Solvent B			
CH_3	11,11′	18.3	18.4	18.5	q	C-2	5.12
	Acetyl			21.1	q		
CH_2	12,12′	37.2	36.8	36.9	t	C-2	4.92
CH	1,1′	67.4	66.4	66.3	d	C-1	2.80
	3,3′	67.7	66.6	66.4	d	C-1	2.52
	4,4′	69.5	68.4	68.4	d	C-2	4.83
=CH	9,9′	118.1	118.0	123.3	d	C-1	2.76
	7,7′	122.8	123.0	128.4	d	C-1	2.95
=COH	6,6′	162.6*	162.0	148.7	s	C-2	3.44
=C	4a,4′a	135.7	134.5	135.3	s	C-1	3.38
	5a,5′a	115.4	114.4	122.8	s	C-1	2.69
	8,8′	146.5	146.3	144.1	s	C-2	3.79
	9a,9′a	133.3	132.2	134.0	s	C-2	3.79
	10a,10′a	151.1	150.8	150.8	s	C-2	3.66
C=O	5,5′	188.1	186.8	180.1	s	C-2	4.93
	10,10′	182.7	181.8	181.8	s	C-1	3.40
	13,13′	175.6	173.7	173.7	s	C-1	3.39
	Acetyl			169.4			

[a] Numbering for CRS-A is shown in Fig. 2.
[b] Spectra were recorded at 50.3 MHz in DMF-d_7 (solvent A) and at 100.57 MHz in $CDCl_3$ (solvent B). Chemical shifts (in ppm) are relative to TMS.
[c] The multiplicity arises from one-bond ^{13}C-proton coupling.
[d] Calculated by using the acetate methyl carbon to normalize peak heights. The average enrichment for [1-^{13}C]acetate was 2.99+0.35, and for [2-^{13}C]acetate 4.31+0.70.
* Signal obscured by solvent peak.

or malonate units on an acetate starter;

2) Oxygenation (at C-5 and C-6) and deoxygenation (at C-7 and C-9) reactions;

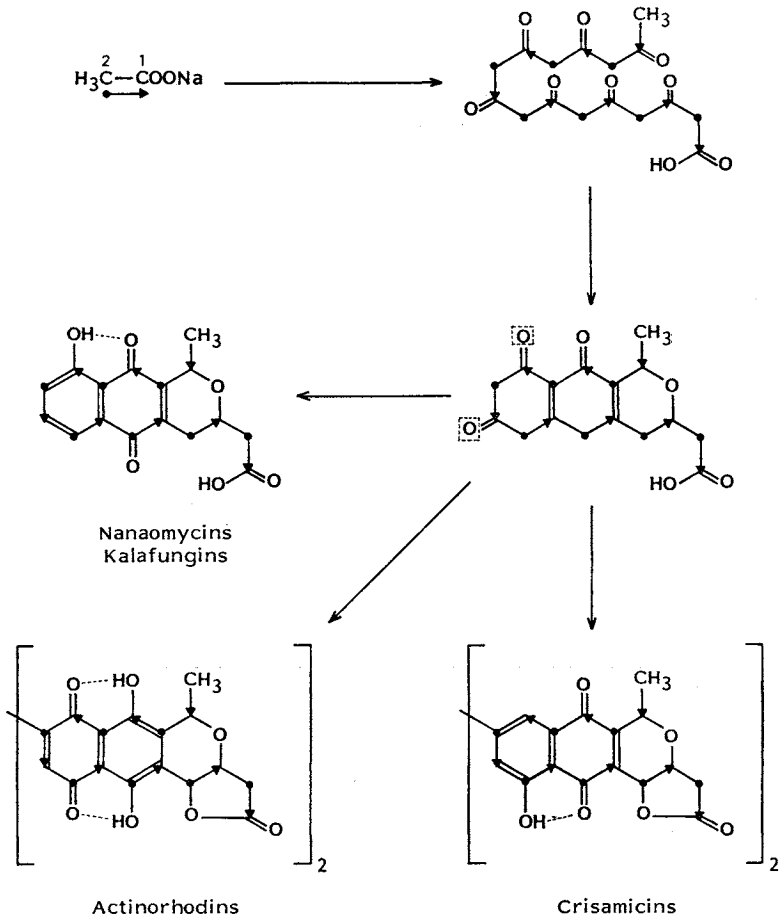
3) Cyclization of the backbone to form the ring system; and,

4) Dimerization to form the completed molecule.

The labeling of equivalent positions in each monomer argues for dimerization, as opposed to cyclization of a single 32 carbon monomer, to form two ring systems.

The location of the phenolic hydroxyl group in CRS-A is of particular interest. In all the previously described isochromanequinone antibiotics, the C-9 position is substituted with oxygen, either as phenolic hydroxyl, or as carbonyl oxygen. CRS-A therefore represents the first compound of this group in which the C-9 has no substitution. The polyketide backbone from which the molecule is synthesized would carry oxygen at this position from the carboxyl group of acetate. Therefore, some mechanism for the removal of this oxygen must exist in the crisamicin producing strain. Also, the existence of an oxygen atom at position C-6 is of interest, as this is the position similarly modified in the formation of the hybrid antibiotics, mederrhodins A and B, from its parent compound, medermycin[6,17] (Fig. 1). In these studies, cloned *act* genes, which led to mederrhodin synthesis when transformed into the medermycin synthesizing organism, were able to complement class *U act* mutants of *Streptomyces coelicolor*. These mutants are blocked in the reaction leading to the hydroxylation

Fig. 5. Biosynthetic pathways for CRS-A and other isochromanequinone antibiotics.



of actinorhodin at the carbon atom equivalent to C-6 of CRS-A. In addition, studies on the genetics of actinorhodin biosynthesis,[18] and the cloning of genes for the complete pathway for biosynthesis of this compound[19] have stimulated interest in this group of compounds.

Acknowledgments

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