BIOSYNTHESIS OF THE ANTIBIOTIC ACTINORHODIN

ANALYSIS OF BLOCKED MUTANTS OF *STREPTOMYCES COELICOLOR*

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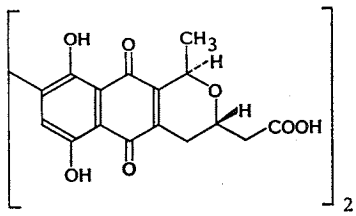
From two types of class V *act* mutants of *Streptomyces coelicolor* two monomeric precursors of actinorhodin have been isolated and their structures determined. One is the known antibiotic kalafungin (**2**) and the other a new compound (**6**). Their relationship to actinorhodin biosynthesis is discussed.

Streptomyces coelicolor A3(2) is the genetically most characterized actinomycete, with a well developed circular chromosome linkage map[1]. A fragment of this chromosome between the markers *gua A* and *his D* contains a cluster of structural genes for the biosynthesis of the isochromanequinone antibiotic, actinorhodin (**1**) (Scheme 1)[2]. RUDD and HOPWOOD isolated 75 mutants which are blocked in the biosynthesis of this antibiotic, and were able to group them into seven classes. The classification was based on the mutants' ability to secrete intermediates which other mutants, blocked earlier in the biosynthetic pathway, could convert into actinorhodin. Production of this antibiotic could easily be recognized by fuming the agar plates over ammonia, since actinorhodin is a red-blue acid-base indicating compound. If a donor strain secreted a precursor which a converter strain could use to produce actinorhodin, then a blue zone would appear on the edge of the converting strain colonies closest to the donor. The classes could thus be ordered as to their place in the actinorhodin biosynthetic pathway.

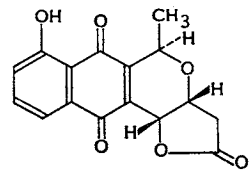
Class V mutants secreted (a) diffusible intermediate(s) into the medium which all other classes (except a putative regulatory class, II) could convert into actinorhodin. Members of this class could not convert compounds secreted by any other mutants into actinorhodin, and therefore must be blocked at the latest stage in the biosynthesis. The steps of isochromanequinone antibiotic biosynthesis following polyketide chain formation have recently been of interest since the *act* genes from class V have been cloned and inserted into another species, *Streptomyces* sp. AM-7161[3]. This latter species normally produces the yellow-brown acid-base indicating antibiotic, medermycin but after transfer of the class V *act* genes, a new, hybrid orange-purple acid-base indicating pigment named mederrhodin A was produced. Another new hybrid structure, dihydrogranatirhodin was produced by a transformant of the dihydrogranticin-producing *S. coelicolor* A3(2). This compound has the granaticin stereochemistry at one chiral center and the actinorhodin stereochemistry at the other.

With this ability to clone antibiotic genes and transfer them between species, the design and syn-

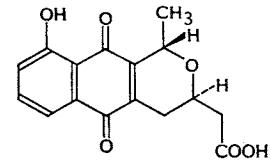
Scheme 1.



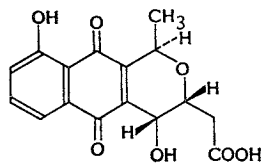
Actinorhodin (**1**)



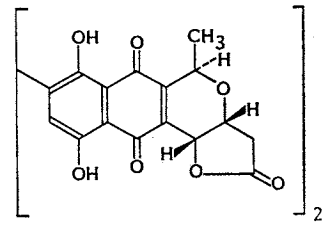
Kalafungin (**2**)



Nanaomycin A (**3**)



Kalafunginic acid (**4**)



γ-Actinorhodin (**5**)

thesis of new antibiotics by genetic manipulation has been shown to be possible. However, the rational use of this technique depends upon the elucidation of the biosynthetic pathway and characterization of the intermediates involved. It is known from [1,2-^{13}C]acetate feeding experiments that actinorhodin is a polyketide-derived antibiotic, originating from 16 acetate units[4]. However, the steps following polyketide chain formation leading to the assembly and modification of the final product have not been studied. The mutants from the last classes blocked in actinorhodin biosynthesis all accumulate intermediates which potentially can be isolated and characterized. In this paper we describe the structure elucidation of two intermediates secreted by class V mutants, from type strains B1 and B135.

Materials and Methods

The UV spectrum of the intermediate from mutant B1 was recorded on a Cary 17 spectrophotometer in acetonitrile. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and circular dichroism spectra were obtained on a Jasco J-500 spectropolarimeter. IR spectra were determined as KBr pellets on a Perkin-Elmer 1420 ratio recording spectrometer with data station. Molecular formulas were established by high resolution mass spectrometry on a Kratos MS 50 spectrometer. ^{1}H NMR spectra of the compound produced by mutant B135 were obtained on a Varian XL-200 spectrometer. Single frequency decoupling experiments on the intermediate from mutant B1 were run on a Nicolet NC-470 spectrometer. Additional spectra, including nuclear Overhauser effect (NOE) difference spectra, were obtained on a GE NC-500 NMR spectrometer. Spin simulations were run on a Varian XL-200 NMR computer using the H2 software.

The selection of *act* mutants derived from *Streptomyces coelicolor* A3(2) has been described by RUDD and HOPWOOD[2]. Seed cultures were inoculated from slants into five 500-ml Erlenmeyer flasks each containing 200 ml of starch-yeast medium[5] for B1 and CM medium[6] for B135. The cultures were grown at 30°C with shaking at 250 rpm in a New Brunswick bench-top rotary shaker. At 1.5 days for B1 and 3 days for B135, the seed cultures were used to inoculate 8 liters of the same medium containing 5 ml of polypropylene glycol 200 (Fluka). The fermentations were carried out in a New

Brunswick Microferm fermenter at 30°C with aeration at 1 ml/minute for 4 days for B1 and 5 days for B135, after which the cultures were harvested by filtration with Celite 545 (Fisher).

The isolation of intermediates was guided by a bioassay in which fractions were tested against an earlier blocked mutant. Strain B18 from class I was grown at 30°C for 4 days as a confluent lawn in Petri plates about 1 cm from the edge on 1.5% agar plus starch-yeast medium for B1 fractions or agar plus CM medium for B135 fractions. Samples to be tested were impregnated into 0.5 cm filter paper discs and dried completely before positioning on the edge of the B18 colonies. After 24~48 hours of further incubation at 30°C, the plates were inverted and exposed to ammonia vapor for 5 minutes. The presence of actinorhodin was noted as a blue zone on the edge of the B18 colonies around the discs.

Fermentation broths of strain B1 were extracted under argon five times with 2 liters of $CHCl_3$. The crude extract was purified by Sephadex LH-20 column chromatography under argon, eluting with CH_3CN - $CHCl_3$ (9 : 1). A pure yellow band having an Rf value of 0.83 on Whatman $KC_{18}F$ TLC plates in H_2O - acetone (1 : 1) gave a positive bioassay. This compound turned brown after a few hours on the TLC plates, and was noted to decompose, particularly when concentrated. Due to the instability of the intermediate, eluates were concentrated to about 25 ml on a rotary evaporator, then freeze-dried.

For the isolation of the product from mutant B135, the broth was acidified to pH 3.0 and extracted under argon twice with 2 liters of $CHCl_3$. The extract was chromatographed on a Sephadex LH-20 column, using the same mobile phase as above. An impure yellow band with Rf 0.82 on Whatman $KC_{18}F$ analytical TLC plates in H_2O - acetone (1 : 1) was rechromatographed on preparative TLC plates using the same conditions. The yellow band was eluted with acetone and chromatographed on analytical TLC plates under the same conditions as above. This analysis showed in addition to the Rf 0.82 spot a new yellow spot at Rf 0.22. Upon further purification of the Rf 0.82 compound by preparative TLC under the same conditions, the upper yellow band became less intense and the lower yellow band became more prominent. Eventually, with an additional chromatographic purification by TLC, the upper band disappeared, leaving only the lower band. This lower band was scraped off, eluted with acetone, evaporated to dryness on a rotary evaporator and then dried *in vacuo* for 36 hours. This yellow compound gave a positive bioassay and its structure was determined by ^1H NMR and co-chromatography to be identical to that of kalafungin (**2**)[7]. On Whatman $KC_{18}F$ TLC plates in H_2O - acetone (1 : 1), the Rf value of both authentic kalafungin (from The Upjohn Company, Kalamazoo) and the compound from mutant B135 was 0.22. Two solvent systems were tried on silica gel TLC plates. In $CHCl_3$ - CH_3CN (4 : 1), the Rf value was 0.53 for both materials, and in toluene - acetone (6 : 1), the two spots co-migrated at Rf 0.42.

The nature of the higher Rf band originally present in the extract was probed by co-chromatography with nanaomycin A (**3**)[8] (from Prof. S. ŌMURA, Tokyo), kalafungin and kalafunginic acid (**4**) (prepared from kalafungin by treatment with 1 N NaOH as described by HOEKSEMA and KRUEGER[7]). In $CHCl_3$ - CH_3OH (8 : 2) on Silica gel 60 F_{254} TLC plates (EM Reagents), the yellow pigment from a fresh ethyl acetate extract of B135 fermentation broth had an Rf value of 0.49, while the Rf values of kalafungin, nanaomycin A and kalafunginic acid were 0.70, 0.69 and 0.32, respectively. This was re-checked in H_2O - acetone (1 : 1) on Whatman $KC_{18}F$ TLC plates, and the Rf values of the B135 yellow pigment, kalafungin, nanaomycin A and kalafunginic acid were, in that order, 0.82, 0.23, 0.90 and 0.92.

Radioactive tracer experiments were carried out to determine the incorporation of [^{14}C]acetate into the intermediates, and of the labeled intermediates into actinorhodin. For the intermediate from mutant B1 a total of 105 μCi of [1-^{14}C]acetate (Amersham) plus 500 μmol of non-labeled sodium acetate was fed to two 200-ml cultures in CM medium. The precursor was added in portions at 12, 24 and 48 hours. Cultures were harvested at day 5, and worked up by extraction, column chromatography and preparative TLC as described above. The yellow pigment was analyzed for radioactivity in a Beckman LS 7000 scintillation counter, and the percent incorporation was determined as described[9]. Since the total radioactivity was only 0.045 μCi, giving an incorporation rate of only 0.042%, the broth and harvested mycelial mass were also counted. The broth contained 12.46 μCi

and the cells only 2.11 μCi, suggesting that the majority of the radioactivity of the precursor fed had been metabolized to $^{14}CO_2$. A solution containing 0.03 μCi of the labeled intermediate isolated from mutant B1 was fed in equal portions at 36 and 48 hours to two 200-ml cultures of *S. coelicolor* 1190 (a derivative of A3(2) carrying mutations for histidine and uracil auxotrophy and streptomycin resistance). Fermentations were conducted in starch-yeast medium, and the cultures were grown for a total of 4 days. Crude actinorhodin was isolated from the mycelium as described by GORST-ALLMAN *et al.*[4]. Since actinorhodin is also present in the broth in starch-yeast medium, the crude actinorhodin precipitate was also collected from the acidified medium. Actinorhodin was extracted from the crude precipitates from both the broth and the mycelium with dioxane, giving a total radioactivity of 0.001 μCi and an incorporation rate of 3.95%. Due to the small amount of radioactivity present in the sample, further proof of radiochemical purity was not possible.

To cultures of mutant B135 growing in two 500-ml Erlenmeyer flasks each containing 200 ml of CM medium were added at 48 and 72 hours equal portions of a solution containing of 179 μCi of [1-^{14}C]acetate plus 120 μmol of unlabeled sodium acetate. The cultures were harvested at day 5 by filtration, extracted and chromatographed three times in H_2O - acetone (1 : 1) on Whatman $KC_{18}F$ TLC plates. A total of 0.0047 μCi of product were isolated, giving an incorporation rate of 0.0026%. Only 13.7 μCi were found in the broth, suggesting again that a large percentage of the administered [^{14}C]acetate had been metabolized to $^{14}CO_2$.

A total of 0.23 μCi of [^{14}C]kalafungin (prepared by Dr. HEIDE HERRMANN) was fed to a 52-hour old culture of *S. coelicolor* 1190. The cultures were harvested and actinorhodin was isolated as described above. After extraction of actinorhodin from the acid precipitate with dioxane, the total radioactivity was determined to be 0.04 μCi, representing an incorporation rate of 17.7%. The amount of actinorhodin isolated was 18.2 mg, indicating a specific activity of 3,064 dpm/μmol. To establish radiochemical purity, the actinorhodin was esterified, acetylated and purified using the procedure described by GORST-ALLMAN *et al.*[4]. The specific activity of the purified actinorhodin derivative was 3,458 dpm/μmol, or 113% of the value before derivatization.

Unlabeled kalafungin (4.98 mg) was also fed to a 58-hour old culture of mutant B18 growing in 200 ml of CM medium, and the culture was harvested at 5 days of growth. The actinorhodin was isolated from the acidified mycelium by extraction with dioxane. The actinorhodin produced in this feeding experiment was compared with that produced by strain 1190, and also with γ-actinorhodin (**5**)[10] (from Prof. A. ZEECK, Göttingen Univ.) by TLC in $CHCl_3$ - acetone (9 : 1), on EM Reagents silica gel 60 F_{254} TLC plates, saturated with 0.5 N malonic acid and oven-dried overnight.

Results

Class V represents the second largest class of *act* mutants, comprising 21 members[2]. It was considered that this class might be genetically inhomogeneous and hence, extracts of all its members were subjected to thin-layer chromatography. This analysis showed the presence of two types of pigment patterns, and two mutants, B1 and B135, representative of the two patterns were chosen for further analysis. The two types of class V mutants did not engage in co-synthesis when grown in proximity to each other, but they mapped in opposite sides of the gene cluster (HOPWOOD and MALPARTIDA, unpublished results), indicating that they are genetically different.

An unstable yellow pigment was isolated from fermentation extracts of mutant B1. This compound gave a positive bioassay, and therefore is an intermediate in actinorhodin biosynthesis. This was confirmed by radioactive feeding experiments. The incorporation of [^{14}C]acetate into the intermediate produced by mutant B1 was only 0.042%, but most of the precursor fed had apparently been metabolized to $^{14}CO_2$, since it was not detected in either the broth or the mycelium at the time of harvest. However, the incorporation of the [^{14}C]-labeled intermediate into actinorhodin when fed to the wild type (strain 1190) was 3.9%, indicating that the compound indeed acts as a precursor.

The yellow pigment from mutant B1 was unstable, especially when concentrated, so that care had to be taken to freeze-dry the sample, rather than dry it on a rotary evaporator. In view of the ready degradation of the compound, fresh samples were prepared from fermentation broths prior to every spectroscopic analysis. The compound melted at 195~197°C, and had a specific rotation of $[\alpha]_D^{25}$ +51.6° (*c* 0.8, CH_3CN). Its exact mass of 286.0844 indicated a molecular formula of $C_{16}H_{14}O_5$ (calcd 286.0841). UV-visible absorbances (in CH_3CN) at 198 nm (ε 9,000), 233 (4,100), 249 (sh, 3,700), 269 (10,100), 276 (sh, 9,100) and 429 (3,800) pointed to the presence of an oxidized naphthalene skeleton, by comparison with other related oxidized antibiotics[8]. The IR spectrum indicated hydroxyl (3433 cm^{-1}), carboxylic acid (1716 cm^{-1}) and extensively conjugated carbonyl (1643 cm^{-1}) groups.

Table 1 lists the ^1H chemical shift values for the intermediate from mutant B1. A triplet at 7.40 ppm was coupled to two doublets at 6.75 and 6.79 ppm. Single frequency decoupling of either of these two doublets caused the collapse of the triplet at 7.40 ppm into a doublet. Similarly, irradiation of the triplet at 7.40 ppm caused the collapse of each aromatic doublet into a singlet. A long range coupling of 0.9 Hz established a three bond link between the protons at 6.75 and 6.79 ppm. In order to establish a connectivity between one of these doublets and the singlet at 6.27 ppm, the latter proton was irradiated, and the NOE difference spectrum was recorded. This revealed an NOE of 13.3% for the proton at 6.75 ppm. In the more downfield part of the spectrum, an exchangeable sharp singlet was observed at 13.92 ppm, suggesting a hydrogen-bonded phenolic proton. A partial structure which follows from the above data is shown below.

In the aliphatic region, a multiplet at 4.76 ppm, denoting a single proton with a complex coupling pattern, could be assigned to a CH-O function. Single frequency decoupling of this proton at 470 MHz in CD_3CN significantly simplified the upfield four-proton signals at 2.74 to 2.97 ppm to eight lines, so that the two pairs of methylene protons could be assigned as one AB-spin system at 2.93 and 2.78 ppm, and another A'B'-spin system at 2.94 and 2.78 ppm. In $CDCl_3$ at 500 MHz, all of the multiplets were well resolved and the coupling patterns could be assigned, especially after decoupling the singlet at 6.27 ppm, which removed all long range coupling. In order to confirm the assignments of the splitting patterns, spin simulations were done for these two ABX-spin systems using a spin simulation program and the coupling constants shown in Table 1. There was good agreement between the observed and simulated spectra. A fragment consistent with these results is illustrated below.

Two protons in one of the methylene groups (2.78 and 2.93 ppm) showed long range couplings of J=1.8 and 1.0 Hz to the proton resonating at 6.27 ppm. A connectivity could therefore be established between this methylene group and the naphthalene skeleton. The structure **6** shown in Table 1 is therefore proposed for the intermediate from mutant B1. The same stereochemistry at C-3 as in actinorhodin is assumed in view of the biosynthetic relationship. The specific rotation of this intermediate is lower than that reported for actinorhodin dimethyl ester ($[\alpha]_D^{20}$ +116.9°, *c* 1.4 in

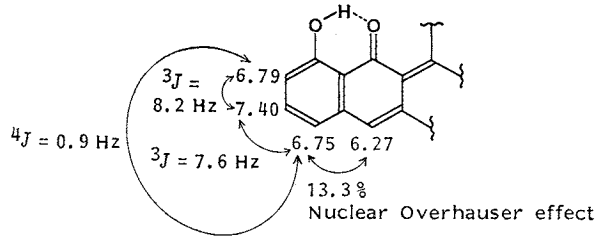
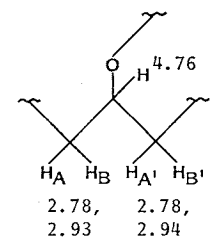


Table 1. ^1H NMR chemical shifts and assignments for the intermediate from mutant B1 in $CDCl_3$.

Chemical shift	Multiplicity (J; Hz)	Assignment
2.94	dd (J=16.4, 7.3)	2
2.78	dd (J=16.4, 5.9)	2
4.76	m (J=10.3, 7.3, 5.9, 3.4)	3
2.93	ddd (J=15.8, 3.4, 1.0)	4
2.78	ddd (J=15.8, 10.3, 1.8)	4
6.27	br s	6
6.75	dd (J=7.61, 0.9)	8
7.40	t (J=7.6, 8.2)	9
6.79	dd (J=8.2, 0.9)	10
2.65	s	16
13.93	s	11-OH



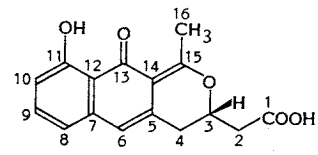


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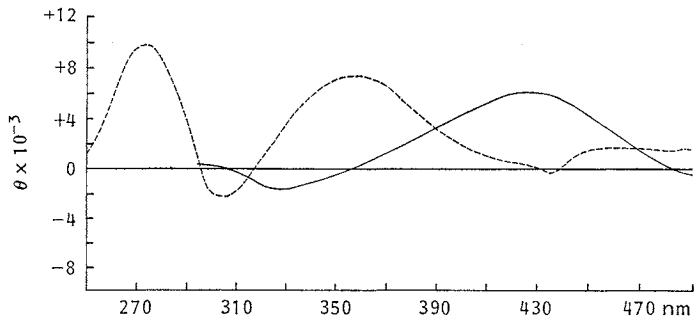
Fig. 1. Circular dichroism spectra of the compound isolated from mutant B1 (—) and actinorhodin dimethyl ester (----).



2 N NaOH with 10% sodium dithionite). The CD spectrum is compared to that of actinorhodin dimethyl ester in Fig. 1.

Another yellow intermediate was produced in small quantities by mutant B135 and this compound was different from the compound isolated from B1. The intermediate from B135 had the same Rf value as that from B1, but it could be distinguished by the fact that it did not decompose (turn brown) on the TLC plate. During purification two yellow bands (Rf 0.82 and 0.22) were noted on the TLC plate, both appearing orange under long wave UV light. The Rf 0.82 band showed decomposition into the lower Rf 0.22 band during TLC on reverse-phase plates. After several elutions on TLC plates, all of the upper band had decomposed into the lower band. The lower band did, however, give a positive bioassay, and therefore was purified and analyzed spectroscopically.

The lower band material was found to be kalafungin (**2**), an isochromanequinone lactone produced by *Streptomyces tanashiensis* Kala[7,11]. The two compounds had identical ^1H NMR spectra, and co-migrated on TLC plates in three different solvent systems. The exact mass observed for the lower band was 300.0671 (calcd for $C_{16}H_{12}O_6$ 300.0633). The specific rotation of the isolated B135 product was $[\alpha]_D^{25}$ +153° (*c* 0.008, $CHCl_3$), which corresponds to the stereochemistry of kalafungin ($[\alpha]_D^{25}$ +159°, *c* 1.0, $CHCl_3$).

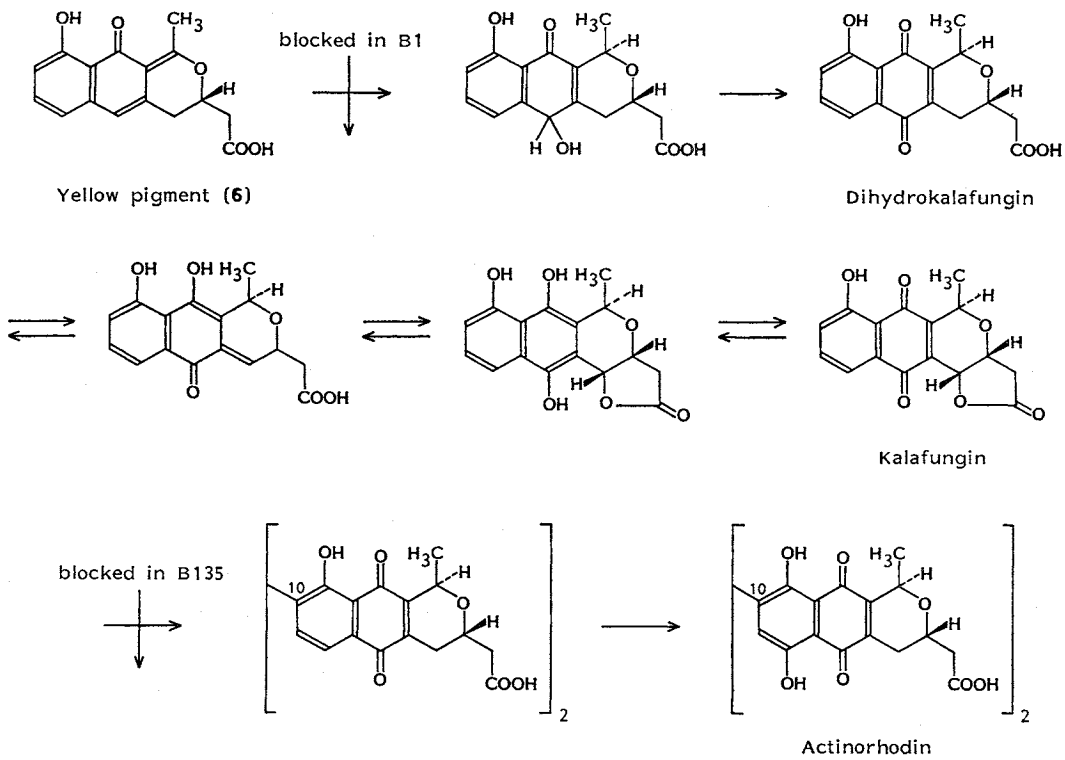
The low Rf yellow compound from B135, kalafungin, gave a positive bioassay on a lawn of mutant

B18. When kalafungin was fed to a liquid culture of mutant B18, the compound produced was actinorhodin, not the lactone γ-actinorhodin (**5**), as confirmed by co-chromatography of the $CHCl_3$ extract with the authentic compounds. The precursor role of kalafungin was again confirmed by radioactive feeding experiments. The incorporation rate from [^{14}C]acetate into the kalafungin produced by B135 was low (0.0026%), but a high incorporation rate (17.7%) was observed for actinorhodin formation from [^{14}C]kalafungin. The higher Rf spot which was present initially in the crude extract of mutant B135 was shown by TLC to be distinct from nanaomycin A (**3**)[8] and therefore also dihydrokalafungin, and from kalafunginic acid (**4**).

Discussion

The two types of class V mutants represent two distinct blocks late in the actinorhodin biosynthetic pathway. The absence of cross-feeding between these two types is peculiar, but may simply reflect lack of diffusibility of the intermediate. Based on the structures of the isolated compounds, mutant B1 must be blocked earlier in the pathway than B135. Scheme 2 presents a suggested interpretation of the relationship between the two compounds and actinorhodin. The B1 intermediate may either add water or undergo 1,4-reduction followed by hydroxylation to give a precursor which would be readily oxidized to dihydrokalafungin, possibly even without the intervention of another enzyme. The conversion into this dihydrokalafungin precursor seems to be blocked in the B1 mutant. Whether kalafungin, isolated from the B135 mutant, is itself a biosynthetic intermediate or is only in equilibrium with a true intermediate, *e.g.*, dihydrokalafungin, cannot be decided from the data. Based on chemical evidence[12] it seems possible that the sequence from dihydrokalafungin to kalafungin is reversible. In any event, the successful incorporation of kalafungin into actinorhodin shows

Scheme 2.



that the lactone ring of **2** can be reductively opened at some point. The analogous process has been demonstrated in the biosynthesis of the nanaomycins[13,14] and naphthocyclinones[15].

The structures of the two isolated compounds, both monomers, indicate that the dimerization is a very late step in the biosynthetic sequence. Since only the symmetrical dimer with the connection through carbons 10 and 10′ is found in nature, it seems plausible that dimerization occurs before the final hydroxylation at C-8, *i.e.*, when only C-10, but not C-9 is activated. If this is true, the block in the B135 mutant quite likely may be in the dimerization step.

Acknowledgments

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