BIOSYNTHESIS OF KALAFUNGIN IN *STREPTOMYCES TANASHIENSIS*

SHIZUKO KAKINUMA, HARUO IKEDA and SATOSHI ŌMURA

School of Pharmaceutical Sciences, Kitasato University,
and The Kitasato Institute,
Tokyo 108, Japan

DAVID A. HOPWOOD

John Innes Institute and AFRC Institute of Plant Science Research,
Norwich NR4 7UH, UK

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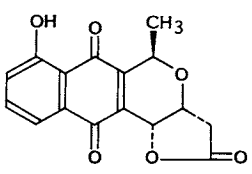
The mutants of *Streptomyces tanashiensis* strain Kala, which were specifically blocked in the synthesis of the benzoisochromanequinone antibiotic kalafungin, were isolated and classified into seven phenotypic classes on the basis of the antibiotic activity and cosynthetic properties. The polarity of cosynthetic reactions and the production of kalafungin by a converter strain showed that the seven mutant classes could be arranged in the most probable linear sequence of biosynthetic blocks. Since kalafungin, which closely resembles the undimerized form of actinorhodin, was accumulated in one of the biosynthetically blocked mutants of the actinorhodin-producing *Streptomyces coelicolor* A3(2), the cosynthesis between kalafungin-nonproducing mutants of *S. tanashiensis* and actinorhodin-nonproducing mutants of *S. coelicolor* was performed. The results of these experiments showed that the early steps in kalafungin biosynthesis in *S. tanashiensis* and actinorhodin biosynthesis in *S. coelicolor* were similar, but the entire biosynthetic pathway of kalafungin in these two streptomycetes was not identical.

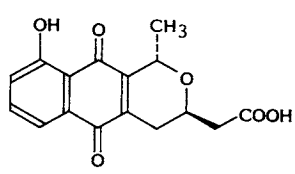
Streptomyces tanashiensis strain Kala produces kalafungin (**1**) (Fig. 1)[1,2], one of the benzoisochromanequinone antibiotics that include actinorhodin (**3**)[3] and the nanaomycins (**2**)[4], which possess antibacterial, antifungal and antimycoplasma activity.

It is known that benzoisochromanequinone antibiotics are synthesized from acetate units *via* a hypothetical intermediate "polyketide"[4,5]. The structure of kalafungin is related to that of actinorhodin, which is a dimer of 6-hydroxydihydrokalafungin. Kalafungin has been suggested to be an intermediate in actinorhodin biosynthesis by *Streptomyces coelicolor* A3(2), since one of the biosynthetically blocked *act* mutants, which was affected at the last step in actinorhodin biosynthesis, accumulated kalafungin and *S. coelicolor* A3(2) was able to convert kalafungin to actinorhodin[6]. Consequently, a similarity between kalafungin and actinorhodin biosynthesis was predicted.

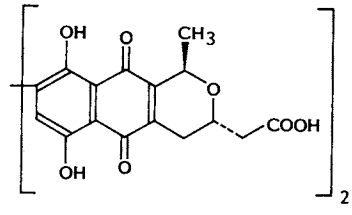
Fig. 1. Structures of benzoisochromanequinone antibiotics.



Kalafungin (**1**)



Nanaomycin A (**2**)



Actinorhodin (**3**)

In this paper, we describe the properties of kalafungin-nonproducing mutants (*kal*) and kalafungin biosynthesis in *S. tanashiensis*, and the relationship between kalafungin and actinorhodin biosyntheses.

Materials and Methods

Bacterial Strains

The bacterial strains used in this study are listed in Table 1.

Growth Media

The sporulation media were M4 (ISP medium 4, Difco) containing 0.25% yeast extract for *S. tanashiensis* and R5[7] for *S. coelicolor*, respectively. The antibiotic production medium used in the cosynthesis experiment was YMS[8] (*S. tanashiensis* cultured alone) or CM[9] (*S. coelicolor* and *S. tanashiensis* cultured together), respectively. Bioassay plates contained a synthetic medium, which consisted of K_2HPO_4 7 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, trisodium citrate 0.5 g, glucose 0.2 g and agar 15 g per liter and were seeded with spores of *Bacillus subtilis* PCI 219.

Isolation of Kalafungin-nonproducing Mutants

Mutagenesis was performed by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as described previously[10]. All mutants evaluated were isolated by the treatment with 1 mg of NTG per ml giving approximately a 97% kill. Each colony surviving mutagenesis was transferred to a 96-hole microtiter plates filled with YMS agar and the plates were incubated at 30°C for 4~5 days. Agar plugs were transferred from the microtiter plates onto bioassay plates and the plates were incubated at 37°C overnight. Kalafungin-nonproducing mutants were identified by the lack of growth inhibition of *B. subtilis*. Nonproducing progenies on the agar plugs were purified on YMS agar by single colony isolation to ensure their genetic homogeneity and were stored at −30°C as mycelial suspension in 50% glycerol or at 4°C as lyophilized cells.

Cosynthesis

Following the method described by DELIĆ *et al.*[11], two kinds of mutants were streaked a few mm apart on a YMS plate and incubated at 30°C for 3 days. The agar block on which mycelium had grown was cut out and transferred onto a bioassay plate, and the plate was incubated at 37°C overnight. The cosynthetic relationship between two mutants, *i.e.*, whether the mutant acted as a converter or a secretor, was revealed by a zone of growth inhibition against *B. subtilis* around one side of the agar block.

Identification of Antimicrobial Products by Cosynthesis

Two kinds of mutants, which cosynthesized antimicrobial products, were spread over opposite halves of 4 ml of YMS medium in a small plate, and the plate was incubated at 30°C for 3 days and then put in the freezer at −30°C until the agar was frozen. After the agar was thawed at room temperature, the products were extracted with 4 ml of methanol. The methanol extract was extracted with 2 ml of chloroform after acidifying with 2 N HCl to pH 2, the phases were separated by centrifugation, and the chloroform layer was evaporated to dryness by a centrifugal concentrator (TAIYO, VC-96). The residue was dissolved in 50 μl of methanol and a portion of solution was applied to a silica gel TLC plate that was developed with chloroform - methanol (10 : 1). The spots corresponding to kalafungin or

Table 1. Bacterial strains.

Strain	Strain	
Streptomyces tanashiensis	*S. coelicolor*	
*kal*1	TK17	*act*I
*kal*2	TK18	*act*III
*kal*3	TK16	*act*IV
*kal*4	JF3	*act*VI
*kal*5	B22	*act*VI
*kal*6	B140	*act*VII
*kal*8		
*kal*9		
*kal*10		
*kal*11		
*kal*12		
*kal*13		
*kal*14		
*kal*15		
*kal*16		

dihydrokalafungin were detected by UV light (365 nm) and compared with authentic samples.

Bioautography

Thin-layer chromatograms were laid on top of the bioassay plate. After 10 minutes, the chromatogram was removed and the bioassay plate was incubated at 37°C overnight. Antimicrobial compounds were identified by the growth inhibition zone that corresponded to their positions on the thin-layer chromatogram.

Results

Characterization of Kalafungin-nonproducing Mutants

Fifteen *S. tanashiensis kal* mutants were isolated among 980 randomly chosen surviving colonies and did not produce any antimicrobial compounds. Almost all of the mutants were completely devoid of aerial mycelium.

The fifteen *kal* mutants were characterized by the method of cosynthesis[11]. Cosynthetic experiments were performed on solid medium, because the results of experiments done in liquid medium were not reproducible and can not define which mutant is a converter or a secretor. These mutants were classified into seven groups by the analysis of their cosynthetic properties. The growth inhibition against *B. subtilis* was detected around the converter strains but not around the secretor strains. The results of cosynthetic experiments are summarized in Table 2. Since strain *kal*16 acted as a converter towards all the other mutants, this mutant must be affected at an early step of kalafungin biosynthesis. Since kalafungin was not cosynthesized by the combination of mutants *kal*6 and 8, the mutation of these two mutants affected identical region, whereas two mutants were classified to class III. The similar results was also observed from the cosynthesis between mutants *kal*11 and 15, and these mutants were classified to class V. Since *kal*2 acted only as a secretor toward all other mutants, this mutant must be affected at one of the last steps of kalafungin biosynthesis. Antimicrobial activity was never detected in the cosynthesis experiments between *kal*4, 9 or 10 and all other *kal* mutants.

Table 2. Cosynthesis pattern between *kal* mutants.

Converter class (*kal*)		Secretor (*kal*)[a]															Production of kalafungins[b]
		16	14	6	8	5	11	15	3	2	12	13	1	4	9	10	
I	16	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+
II	14	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+
III	6	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	+
	8	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	+
IV	5	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−	+
V	11	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	+
	15	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	+
VI	3	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+
VII	2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	12	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−
	13	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−
	1	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] +: Cosynthesis was observed. −: cosynthesis was not observed.

[b] Production of kalafungin and dihydrokalafungin by converter was verified by TLC. +: produced; −: not produced.

Characterization of the Antimicrobial Products Produced in Cosynthesis

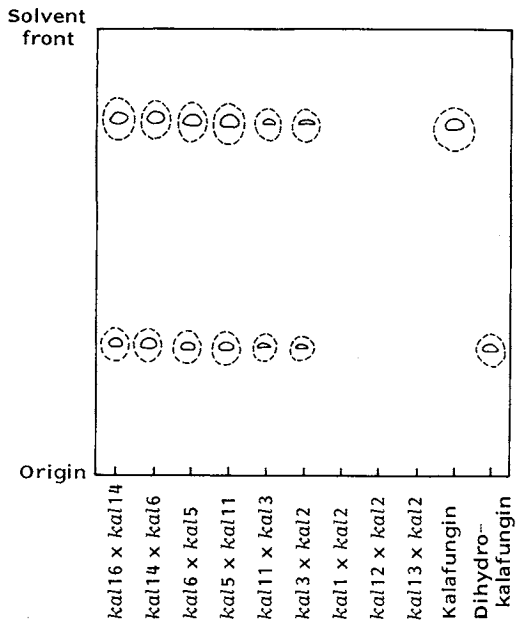
To determine whether the antimicrobial compounds were kalafungin, dihydrokalafungin or both, the compounds extracted from each combination of cosynthetic reaction were analyzed by silica gel TLC and bioautography using authentic samples for comparison. As shown in Fig. 2, the antimicrobial compounds produced by the cosynthesis of the *kal*16, 14, 6, 5, 11, or *kal*3 mutants were identical to kalafungin and dihydrokalafungin. On the other hand, the antimicrobial compounds resulting from the cosynthesis of *kal* 1, 12, or 13 as the converter strain were different from kalafungin or dihydrokalafungin. These compounds were not extracted with chloroform in the isolation procedure described in Materials and Methods.

Cosynthesis between *kal* and *act* Mutants

Since the structure of kalafungin closely resembles the undimerized precursor of actinorhodin and one of the *act* mutants produced kalafungin, the biosyntheses of both antibiotics should be closely related. Thus, cosynthesis in pairwise combination between *kal* and *act* mutants was examined. The conditions for these cosynthetic experiments were critical because the pairwise combinations of *kal* and *act* mutants required a longer cultivation period than that for the cosynthesis between *kal* mutants, and the growth rate of *kal* mutants was not synchronized that of *act* mutants. As shown in Table 3, four kinds of *kal* mutants, which were affected at the early and middle steps in kalafungin biosynthesis, acted as the converter strain which cosynthesized kalafungin by the conversion of substances secreted by *act* mutants. No *act* mutants cosynthesized actinorhodin in these experiments.

Fig. 2. Thin-layer chromatograms and bioautograms of crude extracts from cosynthesis cultures of kalafungin-nonproducing mutants.

Silica gel TLC was developed with $CHCl_3$-MeOH (10:1). Dotted lines indicate the growth inhibition zones against *Bacillus subtilis*.



Discussion

S. tanashiensis kal mutants were isolated at significant frequency only after the induction of the

Table 3. Cosynthesis pattern between *kal* and *act* mutants.

Converter (*kal*)	Secretor (*act*)					Cosynthesized products
	I	III	VII	IV	VI	
I	+	+	+	+	+	Kalafungin
II	+	+	+	+	+	Kalafungin
III	+	+	+	+	+	Kalafungin
IV	+	+	+	+	+	Kalafungin
V	−	−	−	−	−	—
VI	−	−	−	−	−	—
VII	−	−	−	−	−	—

+: Cosynthesis was observed. −: cosynthesis was not observed.

mutation by treatment with NTG. Interestingly, all classes of *kal* mutants lacked the formation of aerial mycelium. The mutation affecting kalafungin biosynthesis apparently also affects the formation of aerial mycelium, or the simultaneous formation of aerial mycelium-defective mutants takes place at high frequency.

The properties of the *kal* mutants indicate that all fifteen mutants evaluated are blocked in the formation of kalafungin. Nine of these mutants cosynthesized kalafungin and its reduced form, dihydrokalafungin. These mutants were classified into seven distinct phenotypic classes in a linear biosynthetic sequence, in which the most probable order of the seven classes of mutations is as follows;

$$kal\text{I}\rightarrow\text{II}\rightarrow\text{III}\rightarrow\text{IV}\rightarrow\text{V}\rightarrow\text{VI}\rightarrow\text{VII}\rightarrow\text{kalafungin}.$$

*kal*4, 9, or 10 mutants, which showed no cosynthesis with any other mutants, can not be included in the biosynthetic sequence. A similar situation exists with the classes of *act* mutants of *S. coelicolor*, in which mutants of one class (*act*II) showed no cosynthetic reactions with mutants of the other classes[12]. The mutation of strains *kal*4, 9, or 10 might be caused by affecting the regulatory region. It was interesting that the *kal*1, 12 or 13 mutants, which acted as converter strains, cosynthesized unidentified antimicrobial compounds that were not detected in the other cosynthetic reactions. These mutants were also able to act as secretors and caused mutants of class I to VI to cosynthesize kalafungin. Therefore, we suggest that these mutants contain at least two mutations in the biosynthetic pathway. One of the *kal* mutations affects the step between class VI and VII *kal* mutants because the *kal*1, 12 or 13 caused mutants class I to VI to cosynthesize kalafungin but not class VII mutant. Another mutation would affect in backward class VII mutation, therefore mutants *kal*1, 12 and 13 did not convert an intermediate derived from class VII mutant to kalafungin. Structural analysis of the unidentified antimicrobial compounds will be necessary before we can understand the above possibility. These mutants thus were not included in the biosynthetic sequence.

Since actinorhodin is the dimer of 6-hydroxydihydrokalafungin and *S. coelicolor act* mutants produced kalafungin, it was expected that the biosynthetic process from acetate to kalafungin in *S. coelicolor* was identical with that in *S. tanashiensis*. If common intermediates existed in both biosynthetic pathway, cosynthetic reaction should occur in combinations of *act* and *kal* mutants. The observation of kalafungin cosynthesis between all classes of the *act* mutants tested and the class I~IV *kal* mutants indicated that the early steps in kalafungin and actinorhodin biosynthesis are similar. However, the lacks of cosynthesis between class V~VI *kal* and the *act* mutants suggests that the process for the formation of kalafungin in *S. coelicolor* is not completely identical to the kalafungin biosynthetic process in *S. tanashiensis*. Although there is a partial similarity between actinorhodin and kalafungin biosynthesis, the following questions still remain: (1) Why did the *act*I and *act*III mutants apparently act as secretors to classes I~IV of the *kal* mutants (since these two classes of *act* mutants have been reported only to be convertor strains[12])? (2) Why did the *act* mutants act only as secretors?

All the genes for actinorhodin biosynthesis by *S. coelicolor* A3 (2) have been cloned[13] and recently we have cloned some (or all) of the genes for kalafungin biosynthesis from *S. tanashiensis* strain Kala. Answers to the above questions may come from introducing the genes for actinorhodin or kalafungin biosynthesis into *kal* or *act* mutants, respectively.

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