5-Hydroxyaloesaponarin II, a minor blue pigment in an actinorhodin-negative mutant of Streptomyces coelicolor A3(2)

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Blue pigmentation in Streptomyces coelicolor A3(2) is attributed to synthesis of the polyketide actinorhodin and its lactone derivative γ-actinorhodin. Therefore, actinorhodin-negative mutants show pigmentation other than blue. When the B22 mutant of S.coelicolor A3(2) [defective in the actVI-ORF1 gene coding for a putative keto(=oxo)reductase] was examined for its secondary metabolite content, the presence of aloesaponarin II (3,8-dihydroxy-1-methyl-9,10-anthraquinone) as the major pigment was confirmed. However, a substantial fraction of a red/blue (acid/alkaline) pigment was detected after separation on HPLC. MS and NMR analysis revealed its structure as 3,5,8-trihydroxy-1-methyl-9,10-anthraquinone. To our knowledge, this anthraquinone has not previously been reported in biological material. A possible route for biosynthesis of this compound is discussed.

Introduction

The phenomenon of blue pigmentation in Streptomyces coelicolor A3(2) was originally ascribed to synthesis of actinorhodin [1]. Recently, another blue pigment, the lactone derivative of actinorhodin, γ-actinorhodin [2], was rediscovered and found to be produced by S.coelicolor A3(2) [3]. It is most probably synthesized as an end product of the actinorhodin pathway during its export from the cell. So far the lack of blue pigmentation has been taken as a reliable indicator of a block in actinorhodin biosynthesis and has been applied for the isolation of various clones of act mutants of S.coelicolor A3(2) [4-6]. All reported act mutants showed no obvious blue colour, but accumulated other pigments as intermediates and shunt products of the pathway (Scheme 1). Among them, structures other than isochromanequinones were revealed [7]. In the case of the actVI-ORF1 mutants defective in a putative keto reductase [8], two anthraquinones were reported, namely aloesaponarin II (3,8-dihydroxy-1-methyl-9,10-anthraquinone) [9] and its carboxylated precursor (3,8-dihydroxy-1-methyl-9,10-anthraquinone-2-carboxylic acid: DMAC2) [10].

Anthraquinones show a high potency as natural food colourants [11,12], so a further search for natural anthraquinones of various colours and structures is important. Here we show that in addition to the major pigment, aloesaponarin II, several minor pigments are produced by an actVI mutant of S.coelicolor A3(2). Among them one blue pigment was found and structurally characterized as 3,5,8-trihydroxy-1-methyl-9,10-anthraquinone, for which the trivial name could be 5-hydroxyaloesaponarin II.

Methods

Strain and growth conditions

Streptomyces coelicolor A3(2) wild-type and its mutants (Table 1) were maintained on agar medium and cultivated in batch flasks (1 litre total volume), 150 ml of liquid, using RG-2 mineral medium supplemented with glucose [3] or soya-mannitol liquid/agar medium [14] as described previously. After 2 days of cultivation in liquid medium the mycelium began to produce pigments. Cultures were incubated for four more days, then harvested and the pigments were extracted and analysed.

Primary extraction and analysis

Liquid cultures were acidified to pH 1-2 with 1 M HCl, cooled on ice and the mycelium was collected by centrifugation (25000 g, 10 min at 4 °C). The cell pellet and culture supernatant were further treated separately. Both samples were extracted twice with chloroform (5:1 vol/vol). The chloroform phase was collected and absorption spectra were recorded using an Aminco DW 2000 recording spectrophotometer. For analytical purposes the sample...
Scheme 1

Schematic representation of the actinorhodin pathway. Note that carbon numbering in anthraquinones is different from that in isochromanequinones. Abbreviations used: PKS, polyketide synthase; KR, ketoreductase; ARO, aromatase; CYC, cyclase.
Table 1 Derivatives of Streptomyces coelicolor A3(2)

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Origin</th>
<th>Phenotype</th>
<th>Major (already known) pigments produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>B385</td>
<td>Parent</td>
<td>redE60 argA1 proA1 SCP1 SCP2*</td>
<td>Actinorhodin*</td>
</tr>
<tr>
<td>1190</td>
<td>Parent</td>
<td>hisA1 uraA1 strA1 SCP1 SCP2*</td>
<td>RED*</td>
</tr>
<tr>
<td>B22</td>
<td>1190</td>
<td>actVI-ORF1</td>
<td>Aloesaponarin II, RED</td>
</tr>
<tr>
<td>B22-14</td>
<td>B22 x B385</td>
<td>actV-O RF1, redE60</td>
<td>Yellow pigment</td>
</tr>
<tr>
<td>B1</td>
<td>1190</td>
<td>actVA-O RF5</td>
<td>Kalafungin, RED</td>
</tr>
<tr>
<td>B1-4</td>
<td>B1 x B385</td>
<td>actVA-O RF5, redE60</td>
<td>Yellow pigment</td>
</tr>
<tr>
<td>B135</td>
<td>1190</td>
<td>actV8</td>
<td>Kalafungin</td>
</tr>
<tr>
<td>B135-33</td>
<td>B135 x B385</td>
<td>actV8, redE</td>
<td></td>
</tr>
</tbody>
</table>

* Actinorhodin is a mixture of mostly actinorhodin and γ-actinorhodin [3].
* RED is a mixture of at least two prodigiosins, undecylprodigiosin and butylcycloheptylprodiginin [13].
Other references given in [3].

was diluted 3-fold with methanol and assayed on HPLC. Pigments were purified and characterized as follows.

**Sephadex LH-20 chromatography**

Samples of mutants B22 and B135 were treated identically. Chloroform extracts, about 50 ml per 250 ml of liquid culture, were dried on a rotary evaporator at 35 °C, redissolved in 2-3 ml of methanol, centrifuged and the pellet was dissolved in 1-2 ml chloroform; any remaining colourless precipitate was removed by centrifugation and discarded. Soluble fractions were combined and applied to a Sephadex LH-20 (2.6 cm x 30 cm) column equilibrated with 75% methanol in 0.5% acetic acid in water. The pigments were eluted in the same solvent, concentrated by extraction with chloroform and drying on a rotary evaporator as above, redissolved in a methanol/chloroform mixture [1:1, 1-2 ml], and applied on a HPLC column.

**HPLC**

A reverse-phase HPLC column, Supelcosil SPLC-18-DB (25 cm x 10 mm internal diameter), was used.

For isolation of pigments from strain B22 the column was equilibrated with solvent A: 60% methanol in 0.5% acetic acid in water. Elution was performed with a flow rate of 1 ml/min in the following gradient: 0-2 min, 100% solvent A; 2-10 min, A-70% solvent B (methanol/acetonitrile, 9:1); 10-40 min, 70-100% solvent B. Sample peaks were recorded on a Shimadzu RF-551S spectrophotometric detector, excitation 430 nm; emission, 530 nm.

Isolation of pigments from strain B135 was performed using the same column as above, equilibrated with solvent A (methanol/acetonitrile/H₂O, 6:2:3). Pigments were eluted with a flow rate of 1 ml/min in the following gradient: 0-10 min, solvent A; 10-30 min, 0-40% solvent B (methanol/acetonitrile/chloroform, 3:2:2).

**NMR**

Nuclear magnetic resonance data were recorded using a Varian VXR 300 apparatus. Both ¹H- and ¹³C-spectra of aloesaponarin II and its 5-hydroxy derivative were recorded in [²H₆]acetone at room temperature.

**MS**

Mass spectra were obtained with an AEI MS9 mass spectrometer (Jeol, Japan) using the electron-impact-ionization method (acceleration voltage, 70 V; temperature, 140 °C).

**Standards**

Mutactin was a gift from Dr Chaitan Kgosla (Stanford, CA, U.S.A.). B1 yellow pigment was isolated as described previously [6]. Isolation of the prodigiosins (‘RED’ pigment) was based on the data of Tsao et al. [13]. Actinorhodin and γ-actinorhodin were isolated as described previously [3].

**Results**

**Primary screening of pigments**

Pigmented metabolites from three previously studied act mutants [4] were characterized: B22 represents the actVI class and is blocked in actVI-ORF1 [8]; B1 represents actVA, blocked in actVA-O RF5 [15]; and B135 represents actV8 [16].

Primary spectral analysis of chloroform extracts confirmed the presence of aloesaponarin II in the B22 mutant (Figure 1, Table 2) as described by Bartel et al. [9]. Similarly, the method of extraction appeared to be working for the ‘B1 Yellow pigment’ from B1 mutant [6]. On the other hand, strain B135 showed a different absorption maximum from that described for kalafungin by Cole et al. [6]. RED pigment (a mixture of prodigiosins [13]) was also detected in these mutants. Its absorption maximum in both methanol and chloroform overlapped with that of γ-actinorhodin. The latter pigment also showed a shoulder at 575 nm, which allowed these two pigments to be distinguished, and also by using TLC as described [3]. To avoid this complica-
tion, red mutant strains were isolated by crossing with the B385 strain (Table 1).

The producer of aloesaponarin II, mutant B22, was further analysed. As well as aloesaponarin II, as previously described, three major bands were usually observed during elution on Sephadex LH-20. The front band was light yellow; it contained multiple products according to HPLC and MS, and was discarded. The second, more intense, yellow-orange band represented mainly aloesaponarin II. The third orange-red fraction represented one major pigment of unknown structure. Pigments of the two last bands were collected and further purified by HPLC (Figure 2). Based on known spectral data (Figure 3) the elution profiles of 3,8-DMAC and aloesaponarin II were assigned to the pigments in the second band. O range-red pigment, which eluted last on both Sephadex LH-20 and HPLC, however, was novel and therefore subjected to structural analysis.

**Identification of aloesaponarin II and 5-hydroxyaloesaponarin II**

The structure of purified aloesaponarin II was confirmed by MS and $^1$H-NMR. MS data showed a compound with a molecular mass of 254 Da. $^1$H-NMR revealed the following peaks (MHz): 2.80 (CH$_3$), 7.13 (dd, $^3$J = 1.9 Hz and $^4$J = 0.8 Hz, 2-H), 7.32 (dd, $^3$J = 6.5 Hz and $^4$J = 3.5 Hz, 7-H), 7.62 (d, $^4$J = 1.9 Hz, 4-H), 7.71 (bs, 5-H), 7.73 (m, 6-H), 13.04 (s, O H). $^{13}$C-NMR showed the following spectrum: 113.0 (C-4), 119.1 (C-5), 124.9 (C-7), 125.4 (C-2), 136.7 (C-6). Absorption ($\lambda_{\text{max}}$, 410 nm) and fluorescent (orange, excitation and emission maxima at 410 and 465 nm respectively) spectra also agreed with previous data for aloesaponarin II [9,18].

The orange-red pigment isolated by chromatography on Sephadex LH-20 and HPLC showed blue colour in alkaline solvents, was poorly soluble in methanol and soluble in chloroform and acetone. The latter pigment showed bathochromic shift both in absorption ($\lambda_{\text{max}}$, 465 nm in acid methanol, 555 nm in 2 M KOH) and fluorescent spectra (red, excitation and emission maxima at 465 and 525 nm respectively) compared with aloesaponarin II. MS analysis revealed a molecular mass of 270 Da. The $^1$H-NMR spectrum showed the following peaks: 2.80 (CH$_3$), 7.17 (dq, $^3$J = 2.8 Hz and $^4$J = 0.7 Hz, 2-H), 7.31 (d, $^4$J = 9.2 Hz, 7-H), 7.37 (d, $^4$J = 9.2 Hz, 6-H), 7.71 (d, $^4$J = 2.8 Hz, 4-H), 12.8 (bs, O H) ,13.04 (s, O H). These results strongly indicated the presence of an extra hydroxy group (mass difference 16 Da and extra OH peak according to NMR) in an otherwise rather similar structure to aloesaponarin II. The position of the extra hydroxy group was confirmed by $^1$H-NMR analysis and further identified by calculation of $^{13}$C-NMR spectra based on known data [21]. The best fit was found for 5-hydroxyaloesaponarin (Table 3). From the UV-visible absorption spectra, it is clear that hydroxylation at carbon 1, 4, 5 or 8 provided the

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**Table 2** Absorption maxima of known polyketide pigments from *S.coelicolor* A3(2) in various organic solvents

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Mutant</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Other</th>
<th>KOH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutactin</td>
<td>actVII</td>
<td>355</td>
<td></td>
<td></td>
<td></td>
<td>Present study</td>
</tr>
<tr>
<td>3,8-DMAC</td>
<td>actVI</td>
<td>407 (3.4)</td>
<td></td>
<td></td>
<td>475</td>
<td>[17]</td>
</tr>
<tr>
<td>Aloesaponarin II</td>
<td>actVI</td>
<td>410 (3.1)</td>
<td></td>
<td></td>
<td>435</td>
<td>[18]</td>
</tr>
<tr>
<td>B1-Yellow</td>
<td>actVA</td>
<td>435</td>
<td>435</td>
<td></td>
<td></td>
<td>Present study</td>
</tr>
<tr>
<td>Kalafungin</td>
<td>actVB</td>
<td>425 (4.46)</td>
<td></td>
<td></td>
<td>525</td>
<td>[19]</td>
</tr>
<tr>
<td>Actinorhodin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>523</td>
<td>[20]</td>
</tr>
<tr>
<td>γ-Actinorhodin</td>
<td></td>
<td>530</td>
<td></td>
<td></td>
<td>542</td>
<td>Present study</td>
</tr>
</tbody>
</table>

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![Figure 1](Image)

Absorption spectra of crude chloroform extracts (recorded in methanol) of various strains of *Streptomyces coelicolor* A3(2). Absorption maxima of major known pigments are indicated.
highest bathochromic shift in the visible region [22]. Some examples of natural 1-methyl-anthraquinones are listed in Table 4. Data available for non-methylated anthraquinones show a similar pattern; a bathochromic shift of more than 50 nm occurs only upon hydroxylation in the para position relative to the hydroxy group already present. The hydroxylation occurs at carbons adjacent to keto (oxo) groups of anthraquinone (in positions 1 and 4 or 5 and 8) [24].

Since aloesaponarin II is methylated at C-1 and already hydroxylated at C-8, and taking into account the NMR results, the most feasible position appeared to be at C-5. Therefore, the structure of the new pigment was concluded to be 3,5,8-trihydroxy-1-methyl-9,10-anthraquinone (suggested trivial name 5-hydroxyaloesaponarin II).

Quantification and optimization of 5-hydroxyaloesaponarin II production

Using HPLC, 5-hydroxyaloesaponarin II was revealed in mutants B22 and B22-14, but not in any other strains listed in Table 1. Quantification via an NMR assay indicated that it was present in about 25% of the quantity compared with aloesaponarin II. Strain B22 was improved by crossing it with red, act S.coelicolor A3(2) strain 385, and picking red, act segregants with the same auxotrophic markers as B22 (selected frequency 10⁻³). This selection improved the productivity of both aloesaponarin II and 5-hydroxyaloesaponarin II about 5-fold. In the absence of the RED pigment, further steps of purification and spectral analysis were undertaken. Both aloesaponarin II and its 5-hydroxy derivative were associated with mycelium (most probably accumulated intracellularly). Use of soya manitol agar medium, compared with liquid RG-2 or soya-mannitol media, resulted in a further 3-fold increase in production of anthraquinones.

Analysis of pigments from strain B135

Strain B135 is a known producer of kalafungin, and therefore was originally thought to be impaired in the formation of octaketide dimer [6]. It was later suggested that it is involved in C-8 hydroxylation. Surprisingly, the absorption spectrum of a chloroform extract showed maxima other than that at 425 nm. Further chromatography on Sephadex LH-20 and HPLC (Figure 4) revealed a major peak of kalafungin, together with other pigments, which were red in acid and blue or green in alkaline solvents. These pig-
Table 4 Visible absorption maxima among various natural 1-methyl-anthraquinones

<table>
<thead>
<tr>
<th>Compound</th>
<th>Modification with respect to aloesaponarin II</th>
<th>( \lambda_{\text{max}} ) (nm) in MeOH</th>
<th>( \Delta \lambda_{\text{abs}} ) upon single hydroxylation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloesaponarin II</td>
<td>-</td>
<td>410</td>
<td>5-OH, 55 nm</td>
<td>[18]</td>
</tr>
<tr>
<td>5-OH-Aloesaponarin II</td>
<td>5-OH</td>
<td>465</td>
<td>5-OH, 20 nm</td>
<td>Present study</td>
</tr>
<tr>
<td>Aloesaponarin II</td>
<td>-</td>
<td>410</td>
<td>5-OH, 55 nm</td>
<td>[18]</td>
</tr>
<tr>
<td>Deoxyerythrolaccin</td>
<td>6-OH</td>
<td>430</td>
<td>6-OH, 28 nm</td>
<td>[18]</td>
</tr>
<tr>
<td>Aloesaponarin I</td>
<td>2-CO(_2)CH(_3)</td>
<td>407</td>
<td>5-OH, 20 nm</td>
<td>[18]</td>
</tr>
<tr>
<td>3,8-DMAC</td>
<td>2-CO(_2)OH</td>
<td>407</td>
<td>5-OH, 20 nm</td>
<td>[17]</td>
</tr>
<tr>
<td>Laccaric acid D</td>
<td>2-CO(_2)CH(_3),6-OH</td>
<td>435</td>
<td>5-OH, 64 nm</td>
<td>[23]</td>
</tr>
<tr>
<td>Kermesic acid</td>
<td>2-CO(_2)OH, 5,6-OH</td>
<td>498*</td>
<td>5-OH, 64 nm</td>
<td>[23]</td>
</tr>
<tr>
<td>Laccaric acid D</td>
<td>2-CO(_2)CH(_3),6-OH</td>
<td>434*</td>
<td>5-OH, 64 nm</td>
<td>[23]</td>
</tr>
<tr>
<td>Erythrolaccin</td>
<td>4,6-OH</td>
<td>466*</td>
<td>4-OH, 32 nm</td>
<td>[24]</td>
</tr>
</tbody>
</table>

* Spectra recorded in EtOH.

ments were found to be very unstable and structural analysis was not possible.

Discussion

HPLC assays were developed to improve assays for pigments in S.coelicolor A3(2). This allowed us to confirm the presence of aloesaponarin II as a major pigment and to find a novel 5-hydroxy derivative in B22. Because of the inability to form the pyran ring in this mutant, formation of the isochromanequinone ring structure failed and an anthraquinone is assumed to be formed as a product of spontaneous cyclization of an unstable carboxylated pre-

cursor [9]. Multiple yellow products could be by-products of this process, or various breakdown products of the uncyclized precursor. The finding of 5-hydroxyaloesaponarin II is, however, rather unexpected. Its spontaneous formation from DMAC or aloesaponarin II is very unlikely. Hydroxylation at C-8 of actinorhodin (the equivalent of C-5 in aloesaponarin II, Scheme 1) is known to be one of the last biochemical events in the actinorhodin pathway. The blue colour of the discussed products is an indication of the presence of a p-hydroxy, p-quinone structure in isochromanequinone as well as anthraquinone, an example being 1,4-dihydroxy-9,10-anthraquinone. The results indicate that 5-hydroxylation may occur as soon as the three-ring structure is formed, although the efficiency is limited. The finding of blue pigmentation in the B135 mutant also fits this idea, because blue pigmentation implies hydroxylation at C-8 of the isochromanequinone structure (theoretically monomeric actinorhodin). Mutant B1 did not reveal any extra blue pigment. However, it does not possess the p-quinone structure, and therefore agrees with the hypothesis that the p-quinone structure can be recognized for 5-hydroxylation.

Although the actinorhodin gene cluster has been completely sequenced and many gene functions have been ascribed, the gene product responsible for C-8 hydroxylation (as carbon numbering used for actinorhodin) is not yet known, although it was successfully introduced into the medermycin-producing strain Streptomyces sp. AM 7161 [25,26] by cloning into it a part of the act gene cluster including the actVA region. Evidently, the C-8 hydroxylation could recognize medermycin or its precursor efficiently. The present results suggest that it may be able to recognize the corresponding carbon in a rather different ring system, albeit with a low efficiency.

1-Methylanthraquinones are rather scarce secondary metabolites in micro-organisms as well as in plants and
animals. However, better knowledge of their synthesis opens a possibility for genetic engineering of anthraquinone pathways by the use of genes from pathways such as those of isochromanequinones. For this objective, S. coelicolor A3(2) is a very promising organism since it is the best genetically studied streptomycete and its genetic manipulation has already yielded a multitude of ‘unusual natural products’ [9,27]. Further manipulations should be possible by identifying and cloning specific enzymes responsible for the diversity of naturally occurring anthraquinones.

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References


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