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Phytochemistry 67 (2006) 1206–1213
PHYTOCHEMISTRY
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Biosynthesis of photodynamically active rubellins and structure elucidation of new anthraquinone derivatives produced by *Ramularia collo-cygni*

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Received 10 February 2006; received in revised form 1 May 2006
Available online 15 June 2006

Abstract

Here we present the first isolation of the anthrachinone derivative rubellin A out of mycelium and culture filtrate of *Ramularia collo-cygni*. Furthermore, two compounds, rubellin E and 14-dehydro rubellin D were isolated and their structures elucidated. In comparison to the other rubellins, rubellin A shows increased photodynamic oxygen activation. By incorporating both [1-^{13}C]-acetate and [2-^{13}C]-acetate into the rubellins, we showed that such anthraquinone derivatives were biosynthesised via the polyketide pathway. The labelling pattern after being fed [U-$^{13}C_6$]-glucose proved the existence of fungal folding mode of the poly-β-keto chain. The ability to produce rubellins is not limited to *R. collo-cygni* in the anamorph genus *Ramularia*.

Keywords: *Ramularia collo-cygni*; *Ramularia uredinicola*; Fungi imperfecti; Rubellins; Anthraquinones; Polyketide biosynthesis; Photodynamic activity; Phytotoxin

1. Introduction

At the end of the 1980s a novel leaf spot disease appeared on barley in Central Europe (Huss et al., 1987). The phytopathogenic fungus *Ramularia collo-cygni* (Sutton and Waller) initiates this disease, known as "Sprenkelkrankheit" in German (Huss and Sachs, 1998; Sachs, 2000). In the early summer *R. collo-cygni*, which belongs to the fungi imperfecti (Hyphomycetes), induces small dark brown spots with a pale halo on the upper leaves of winter barley; these increase in size until the plant ripens. Conidiophores breaking through stomata on the lower side of leaves are visible as rows of small whitish clusters. Early ripening of infected plants causes yields to drop by up to 20% (Huss et al., 1992; Huss, 2000). Since then this plant disease has become known in many countries in Europe (Austria, Germany, Switzerland, Czechia, Ireland, Scotland, Norway) but also in New Zealand, Argentina, and Uruguay (Huss and Formayer, 2003; Sherdian, 2000).

Recently we provided evidence that *R. collo-cygni* produces the anthraquinone derivatives rubellin B (**2**), C (**3**), and D (**4**) as non-host-specific phytotoxins with photodynamic activities (Heiser et al., 2003, 2004; Miethbauer et al., 2003). Until now rubellins has been only described for *Mycosphaerella rubella*, a causal agent of a necrotic spot disease of the medicinal plant *Angelica sylvestris* (Arnone et al., 1986, 1989).

In this paper we report the discovery of rubellin A (**1**) in *R. collo-cygni* and elucidate the structure of two novel anthraquinone derivatives. Furthermore, we present the

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doi:10.1016/j.phytochem.2006.05.003

biosynthesis pathway of the rubellins based on incorporation experiments.

2. Results and discussion

In some hyphae of *R. collo-cygni* grown in a liquid culture, red-coloured compounds accumulate within. Additionally, a compartmentalisation can be observed (Fig. 1). Two red and one yellow compounds were recently isolated from fermentation batches and identified as rubellin B (**2**), D (**4**), and C (**3**) (see Section 1). In addition to these compounds, we isolated another yellow substance with an absorption maximum at 443 nm. The substance was identified by spectroscopic methods as rubellin A (**1**). In particular the ^{1}H and ^{13}C NMR data (shown in Supplementary

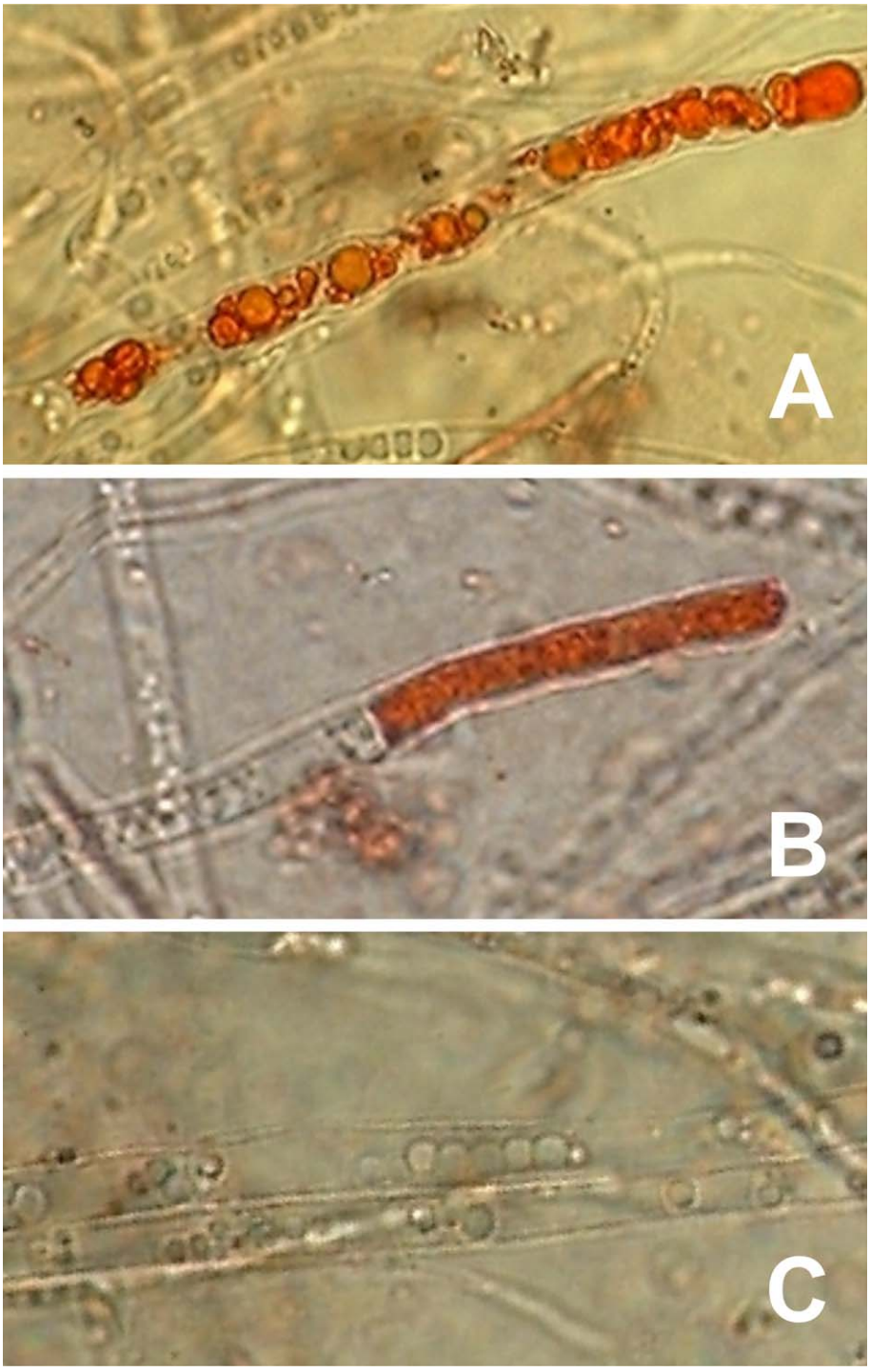
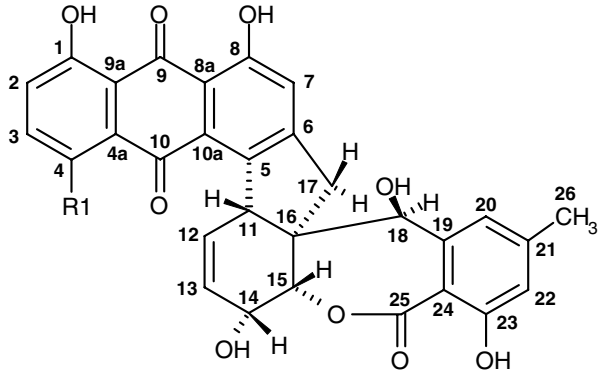


Fig. 1. Hyphae from a submers cultivation: (A) accumulated red compounds within mycelium; (B) terminal hypha containing red ingredients; (C) hyaline hyphae.

data) are in agree with those of Arnone et al. (1986). The minor differences that appear in the spectra are due to a change of solvent. Additionally DEPT, ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-TOCSY and ^{13}C,^{1}H-correlation (HSQC, HMBC) were recorded, the results of which are confirm the structure demonstrated in Fig. 2. Because these data agree, we accept the stereo specificities given by Arnone et al. (1986). The additional discovery of rubellin A (**1**) proves that all rubellins known from *M. rubella* are also biosynthesised by *R. collo-cygni*.

Another minor component was isolated from both mycelium and culture filtrate. The mass difference of 2 Da in comparison to rubellin D (**4**) and B (**2**), respectively, indicates a corresponding dehydro compound. The invariability of the chromophore confirmed by the identical Vis spectrum limits the site of this dehydrogenation. NMR analysis provided an ultimate structure elucidation. The results of DEPT and HSQC spectra are shown in Table 1. All of the phenolic hydroxyl groups appeared at δ_C 12.8; the secondary alcoholic hydroxyl group was not detectable. Irradiation of the C-17 proton at δ_H 3.12 (selective ^{1}H,^{1}H-TOCSY) resulted in distinct couplings to the other proton at δ_H 3.42, the proton at C-7 (δ_H 7.29), and to the protons at C-15 and C-18 (δ_H 4.85 and 5.71, respectively). In comparison to rubellin D (**4**) (see NMR shifts in the Supplementary data), the assignments in the range from C-12 to C-15 are modified; the novel carbonyl group at C-14 is especially conspicuous. Coupling data from ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-NOESY, and ^{13}C,^{1}H-correlation HMBC (shown in Supplementary data) unambiguously confirm the structure as 14-dehydro rubellin D (**5**) presented in Fig. 2. In this respect, a stereo-specific analogy with rubellin D (**4**) is obvious. The dehydro compound (**5**) also produces a high $[\alpha]_D^{20}$ value of +1600°, which can be attributed to the loss of the asymmetry centre at C-14.



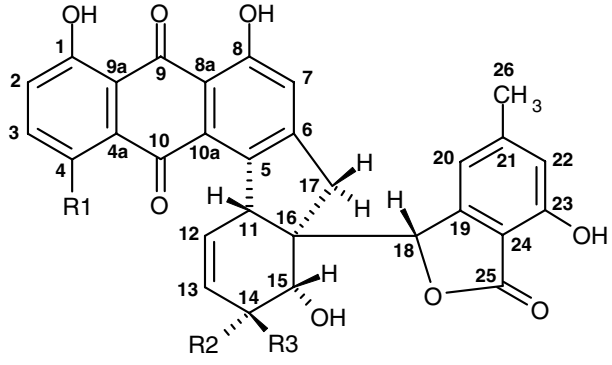


Fig. 2. Structures of rubellin A (**1**), B (**2**), C (**3**), D (**4**), and 14-dehydro rubellin D (**5**).

Table 1
^{1}H and ^{13}C NMR data of 14-dehydro rubellin D (**5**) recorded in THF-d_8

C-atom	14-Dehydro rubellin D (**5**)		
	^{13}C (ppm)	^{1}H (ppm)	DEPT
1	158.3		C
2	130.3	7.33	CH
3	130.4	7.33	CH
4	159.1		C
4a	114.0		C
5	139.2		C
6	157.9		C
7	121.6	7.29	CH
8	165.0		C
8a	115.6		C
9	191.7		C
9a	113.5		C
10	188.9		C
10a	127.9		C
11	48.1	4.57	CH
12	146.4	6.71	CH
13	125.6	5.92	CH
14	198.5		C
15	74.0	4.85	CH
16	56.1		C
17	38.8	3.42/3.12	CH_2
18	85.9	5.71	CH
19	148.7		C
20	116.8	7.00	CH
21	148.4		C
22	118.3	6.74	CH
23	155.9		C
24	111.0		C
25	169.2		C
26	22.2	2.41	CH_3

A conversion from rubellin A (**1**) to C (**3**) and B (**2**) to D (**4**), respectively, is possible in vitro by overnight treatment with 3 N ammonium hydroxide followed by neutralisation. But the reaction is not reversible; obviously the five-membered lactone is more stable than the seven-membered ring. As an intermediate, a related compound with an open lactone ring should exist. We isolated such a rubellin by using MeOH to extract a lyophilised culture filtrate of *R. collo-cygni*; the compound was not detectable in the mycelium. The red substance, whose UV–vis spectrum is identical to that of rubellin D (**4**), shows a more polar behaviour than the known rubellins (**1**–**4**) in all chromatographic procedures. The molecular mass of 560 Da differs from that of rubellin D (**4**) by 18 Da, indicating an opened lactone ring. The typical lactone absorption band can not be observed at

1735 cm^{-1} visible in the IR spectrum of rubellin D (**4**) in the spectrum of the intermediate. Furthermore, rubellin E (**6**) produces a very high $[\alpha]_D^{20}$ value (+2800°).

Significant NMR analysis of the native intermediate is impossible since the compound tends to form the lactone ring. Therefore, the definite structure elucidation was successfully performed after acidification: ^1H and ^{13}C NMR data were identical with those for rubellin D (**4**). This conversion was also detectable by HPLC and TLC analyses. All results militate in favour of the expected structure. The novel compound with an opened lactone ring was called rubellin E (**6**) (Fig. 3).

In addition a yellow substance was detected as a slight impurity of rubellin E (**6**). The molecular mass (544 Da) was in line with a related non-lactone, which the IR data supported (see above). Conversion to rubellin C (**3**) after acidification was also detectable by HPLC and TLC analyses. Because of the small amount, no NMR data were available. We assume that rubellin E (**6**) has a related structure, just without a hydroxyl group at C-4. Therefore, we only propose a structure for the compound called as rubellin F (**7**) (Fig. 3).

It must be noted that both compounds are not artefacts, since pure samples of rubellins A–D (**1**–**4**) were shown to be stable under isolation conditions. Obviously, these substances (**6** and **7**) appear only in the culture filtrate (see above) as intermediates during the extra cellular conversion from the seven-membered to the five-membered lactone ring. Native existence of E and F in the culture filtrate can be directly demonstrated by a TLC analysis (without any sample preparation).

As demonstrated in Fig. 4, rubellins provoke different amounts of ethane formation from α-linolenic acid, indicating different production of singlet oxygen in the photodynamic reaction. Rubellin A (**1**) has the highest activity but rubellin E (**6**) with the opened lactone ring did not show any effect. The emergence of singlet oxygen depends on the duration of the triplet status of the photodynamically active compounds. Since the molecular geometry also affects the stability of the triplet state of the photosensitizer (Schweitzer and Schmidt, 2003), we assume that rubellin E (**6**) exists in the triplet status only for a short time. On the other hand, rubellin E (**6**) is quite able to generate oxygen radicals in a type I photodynamic reaction, which can be confirmed by the release of ethene from KMB (Fig. 5). Both ROS (singlet oxygen and oxygen radicals) cause damage to plants by inducing lipid peroxidation and pigment bleaching, which may contribute to the symptoms seen after infection with the rubellin producer *R. collo-cygni* (for details, see Heiser et al., 2003, 2004).

In preparation for feeding experiments with ^{13}C-acetate, we investigated whether *R. collo-cygni* accepts and utilises this compound during fermentation. The fungus showed normal growth and rubellin production when fed up to 2 g sodium acetate per litre culture medium. By feeding fungus radioactively labelled acetate at the beginning of

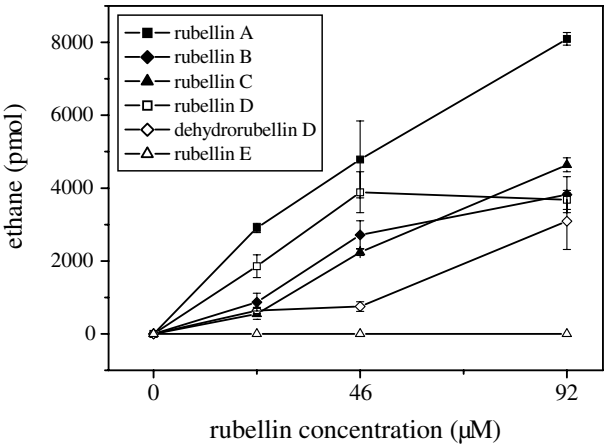
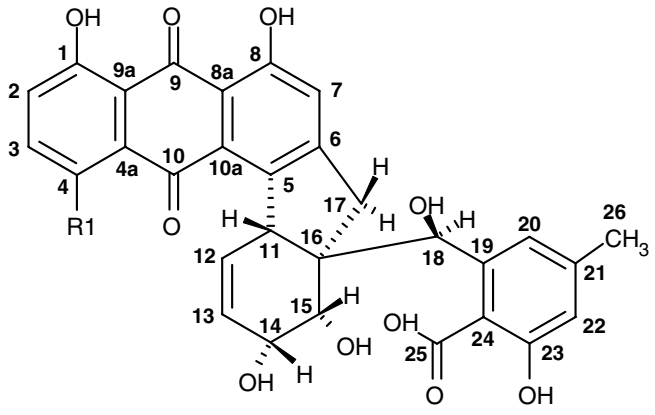


Fig. 4. Ethane formation after 60 min from α-linolenic acid induced by different concentrations of the rubellin A (**1**), B (**2**), C (**3**), D (**4**), E (**6**), and 14-dehydro rubellin D (**5**).



R1 = OH rubellin E (**6**)
R1 = H rubellin F (**7**)

Fig. 3. Structure of rubellin E (**6**), and a proposed structure for rubellin F (**7**).

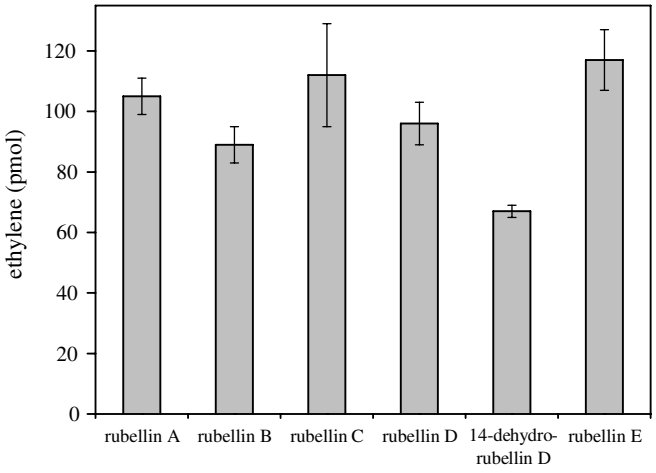
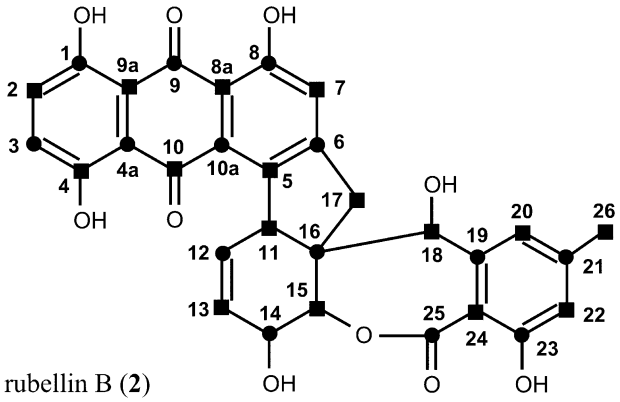


Fig. 5. Release of ethylene from α-keto-4-thiomethyl butyric acid (KMB) by 46.5 μM compound [rubellin A (**1**), B (**2**), C (**3**), D (**4**), E (**6**), and 14-dehydro rubellin D (**5**)].

the fermentation, we got absolute incorporation rates of 2.48% (rubellin D) and 0.68% (rubellin B), respectively. The diminished value of the latter can be explained by the low mass produced. Recently we demonstrated that rubellin B (**2**) was primarily biosynthesised during fermentation and then converted continuously to rubellin D (**4**) (Heiser et al., 2004). This fact makes sense under the presumption of a possible pathway from a dimeric anthraquinone precursor to the rubellins (see below). This conversion is intensified at the end of the fermentation process when broth reacts alkaline, diminishing the yield of rubellin B (**2**). Hence, in the feeding experiments with sodium [^{13}C]-acetate, we used a culture medium with a pH of 5 stabilised by a phosphate buffer, which yielded more rubellin B (**2**).

After feeding of sodium [1-^{13}C]-acetate and sodium [2-^{13}C]-acetate, respectively, and isolating rubellin B (**2**) and D (**4**), incorporation patterns prove unambiguously that the rubellins are biosynthesised via the polyketide pathway (Fig. 6, ^{13}C NMR raw data in Supplementary data). An additional result is remarkable. By feeding of sodium [1-^{13}C]-acetate, the untagged C-atoms reach a normal ^{13}C level explainable by a quick loss of the carboxyl via TCA cycle. But feeding of sodium [2-^{13}C]-acetate yielded a level of 4–5% in the corresponding untagged positions. This means that the methyl group of acetate must be converted in part to a carboxyl one. This is possible via protein turnover, or more specifically, by biosynthesis and the subsequent degradation of lysine in fungi (Michal, 1999).



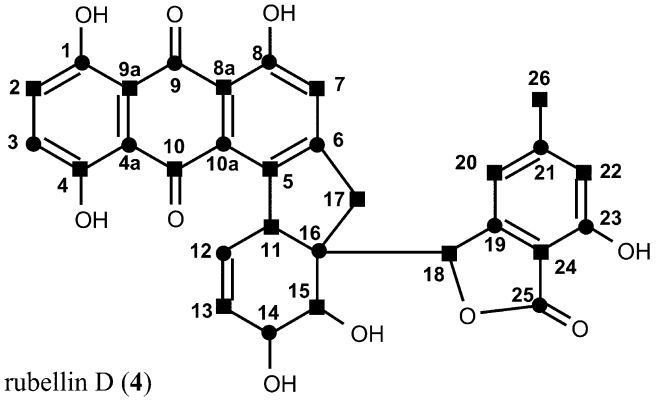
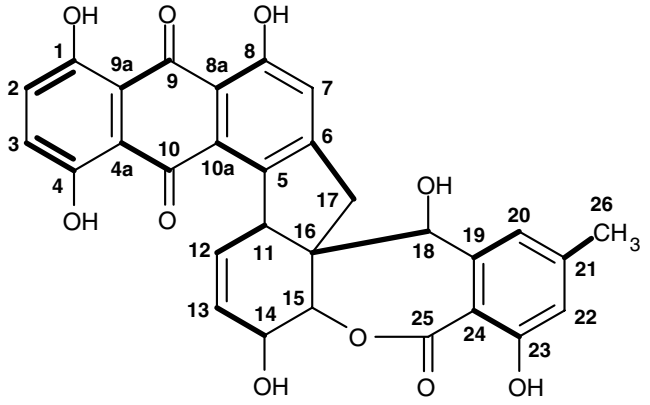


Fig. 6. ^{13}C-labelling patterns after incorporation of sodium [1-^{13}C]-acetate and sodium [2-^{13}C]-acetate by rubellin B (**2**) and rubellin D (**4**). Increased ^{13}C-level caused by sodium [1-^{13}C]-acetate (●) and sodium [2-^{13}C]-acetate (■).

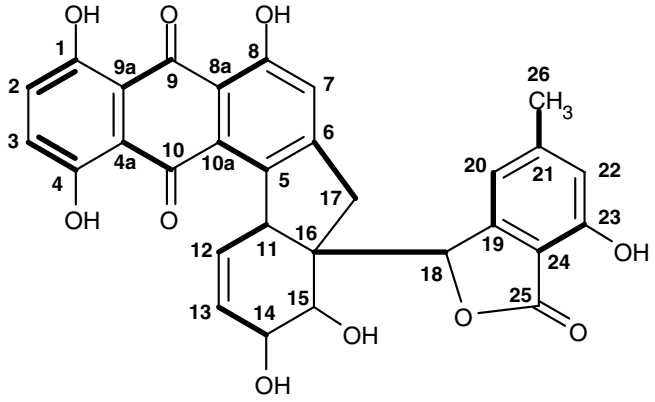
The structures of rubellin A and B (**1** and **2**) suggest that these compounds are generated from a dimer anthraquinone. The incorporation patterns in rubellin B (**2**) clearly prove that two octaketid units are involved in its biosynthesis (Fig. 6). Helminthosporin (3-methyl-1,5,8-trihydroxyanthraquinone), which is known to be a secondary metabolite from other phytopathogenic fungi, for example *Drechslera holmii* and *D. ravenelii* (Van Eijk and Roeymans, 1981), can be considered primordial monomers. The conversion of the keto group into a lactone in the lower part of the rubellins is probably carried out by a biochemical Baeyer-Villiger oxidation catalysed by a monooxygenase. Such biotransformations of aliphatic or cyclic ketones in bacterial and fungal sources have already been described elsewhere (Andrade et al., 2004; Mihovilovic et al., 2005). In the biosynthesis of aflatoxins a Baeyer-Villiger cleavage of anthraquinones is also involved (Henry and Townsend, 2005).

It is well known that fungal anthraquinones are biosynthesised via the polyketide pathway (Gill, 2001). Accordingly, our results support these observations for the rubellins. In order to investigate which of the cyclisation patterns occurs in the biosynthesis of the rubellins we fed a mixture of [U-$^{13}C_6$]-glucose and unlabelled glucose to a fermentation batch of *R. collo-cygni*. After incorporating of sodium [1, 2-$^{13}C_2$]-acetate metabolised from this glucose mixture, the ^{13}C–^{13}C coupling pattern in the rubellins provided information on the mode of cyclisation. Fungi and streptomycetes utilise different polyketide-folding strategies detectable by the number of intact C-2 units incorporated into the initial cyclohexanoid ring (western side) of cyclic polyketides. Streptomycetes make use of three units, fungi of two (Thomas, 2001). Fig. 7 (raw data in Supplementary data) shows the incorporation of only two intact C-2 units into the corresponding cycles of both anthraquinone parts (C-3/C-4, C-1/C-2 and C-11/C-12, C-13/C-14). In conclusion, the fungal folding mode of the poly-β-keto chain is proved.

Sixteen strains of *R. collo-cygni* from different regions (Austria, Germany, Switzerland, Czechia, Scotland, New Zealand, Argentina, Uruguay) were investigated for their ability to produce rubellins. All strains more or less intensively biosynthesised the complete spectrum of the rubellins, whereas two other species of the genus *Ramularia* [*R. pratensis* Sacc. and *R. inaequalis* (Preuss) U. Braun] were unable to do so. But another species recently described by Khodaparast and Braun (2005) as *R. uredinicola* Khodap. & U. Braun produces rubellins as well. This hyperparasitic fungus collected in Iran was associated with the rust fungus *Melampsora* sp. which had infected leaves of *Salix babylonica* L. Colonies of *R. uredinicola* grown on potato dextrose agar show a reddish colour when they age, rubellins can already be detected at this state. A further candidate for rubellin biosynthesis could be the related



rubellin B (**2**)



rubellin D (**4**)

Fig. 7. Incorporation of [U-$^{13}C_6$]-glucose into rubellin D (**4**) and B (**2**), 4% (w/w) diluted with unlabelled glucose; bold bars: carbon atoms involved in ^{13}C–^{13}C coupling.

species *R. rosea* (Fuckel) Sacc. These results demonstrate that although a few *Ramularia* species use the photodynamically active rubellins as non-host-specific phytotoxins, another part of this genus is completely unable to produce these compounds.

3. Experimental

3.1. General

The strain (43/3) used for the investigation originated from Switzerland (Goumoens-la-Ville) and was obtained from Dr. Sachs (BBA Kleinmachnow, Germany). Further fifteen strains of *R. collo-cygni* and two other species (*R. pratensis*, *R. inaequalis*) were received from the same source, and *R. uredinicola* from Prof. Braun (University Halle-Wittenberg).

The complete fermentation procedures of *R. collo-cygni* were performed at 20 °C. Cultures were illuminated in a light-darkness rhythm (12 h artificial light at 1.2 W m^{-2}). Preliminary cultures were grown for 2–3 weeks on V8-medium (vegetable juice 100 ml, $CaCO_3$ 3 g, agar 16 g, 1000 ml of distilled water). Small mycelial plugs were used to inoculate 100 ml (in 500 ml flasks) of V8-medium (vegetable juice 200 ml, $CaCO_3$ 3 g, 1000 ml of distilled water, pH 6.2). The cultures were shaken at 135 rpm. After 7 days the mycelium from 10 flasks was separated, washed and resuspended in 300 ml of distilled water. Five millilitres of this suspension (about 0.1 g dry wt.) were used to inoculate 100 ml of main culture (in 500 ml flasks) with Czapek-Thom-Medium (sucrose 10 g, KNO_3 2 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, distilled water ad 1000 ml; pH 5.8).

The cultures were shaken at 135 rpm and after 21 days separated into culture filtrate and mycelium. Then the former was acidified to pH 4 and exhaustively extracted in a continuous procedure by EtOAc, the latter was extracted twice by the same organic solvent. If we wanted to obtain rubellins E and F (**6** and **7**) a lyophilised culture filtrate was extracted repeatedly with MeOH. A preliminary purification of the respective extracts was carried out by flash liquid chromatography on silica gel [EtOAc–MeOH–H_2O (100:17:13)] in order to remove hydrophilic compounds.

The respective residues were redissolved in the appropriate mobile solvent. The isolation of rubellin A (**1**), B (**2**), C (**3**), D (**4**), and 14-dehydro rubellin D (**5**) was carried out by isocratic preparative HPLC (Shimadzu equipment VP class with photodiode array SPD-M10A) with MeOH–MeCN–H_2O (16:4:5), conditions: Phenomenex Luna C18(2), 250 × 21.2 mm, 10 μm, 100 Å; Security Guard Phenomenex C18(2), 10 × 10 mm, 10 μm, 300 Å; flow rate 8 ml/min. If necessary rechromatographic runs were performed. Finally, isolated compounds were purified by a run on a Sephadex LH-20 column (2.6 × 90 cm) with MeOH–*n*-PrOH (1:1), 0.5% HCOOH.

The isolation of rubellins E and F (**6** and **7**) by preparative HPLC was performed in the same manner, but using a linear gradient: 0–3.4 min 65% organic solvent [MeOH–MeCN (4:1)], 3.4–18 min 65–95% organic solvent and a flow rate of 5 ml/min. Further purification on Sephadex LH-20 is not possible.

Analytical HPLC of rubellin A (**1**), B (**2**), C (**3**), D (**4**), and 14-dehydro rubellin D (**5**) (equipment, see above) was carried out at 25 °C isocratically with MeOH–MeCN–H_2O (16:4:5), under following conditions: Phenomenex Luna C18(2), 250 × 4 mm, 5 μm, 100 Å; Security Guard Phenomenex C18(2), 3 × 4 mm; flow rate 0.6 ml/min. Analytical HPLC of rubellins E and F (**6** and **7**) was performed in the same manner, but using a linear gradient: 0–2 min 65% organic solvent [MeOH–MeCN (4:1)], 2–10 min 65–95% organic solvent and a flow rate of 0.6 ml/min. Quantification of the rubellins was carried out with calibration curves at corresponding λ_{max}.

NMR measurements were performed on a BRUKER AVANCE 400 spectrometer using a 5 mm probe head with a z-gradient (^1H 400 MHz, ^{13}C 100 MHz). Data were acquired with BRUKER standard pulse programmes and processed using the BRUKER TOPSPIN software. HRESI mass spectra (with negative ionisation) were recorded on TSQ Quantum AM (ThermoElectron): temperature of

the ion source 65 °C, sample in MeOH–H_2O (99:1). TLC was done on Merck RP-18 F_{254s}, solvent: MeOH–H_2O (3:1).

The formation of reactive oxygen species (ROS), namely singlet oxygen and oxygen radicals, was determined by fragmentation of α-linolenic acid and α-keto-4-thiomethyl butyric acid, respectively (Heiser et al., 2003). The only modification was that both KMB fragmentation and α-linolenic acid peroxidation were conducted at pH 5.0.

3.2. Physico-chemical properties

3.2.1. 14-Dehydro rubellin D (5)

Analytical HPLC (ret. time): 9.3 min. Preparative HPLC (ret. time): 21.9 min. TLC (R_f): 0.16. UV λ_{max}^{MeOH} nm (log ε): 214 (4.75), 233 (4.65), 500 (4.11), 519 (4.01), 534 (3.92). $[\alpha]_D^{20} + 1600°$ (MeOH; *c* 0.01). FTIR ν_{max}^{KBr} cm^{-1}: 3434, 3047, 2924, 1742, 1691, 1602, 1566, 1452, 1406. HRESI-MS: *m/z* 539.1002 ($M–H^+$), calc. for $C_{30}H_{19}O_{10}$ 539.0978. ^1H NMR and ^{13}C NMR spectra (see Table 1).

3.2.2. Rubellin E (6)

Analytical HPLC (ret. time): 5.5 min. Preparative HPLC (ret. time): 9.5 min. TLC (R_f): 0.48. UV λ_{max}^{MeOH} nm (log ε): 215 (4.63), 490 (3.65), 501 (4.17), 522 (4.07). $[\alpha]_D^{20} + 2800°$ (MeOH; *c* 0.01). FTIR ν_{max}^{KBr} cm^{-1}: 3309, 3038, 2926, 1598, 1580(sh), 1456, 1411. HRESI-MS: *m/z* 559.1213 ($M–H^+$), calc. for $C_{30}H_{23}O_{11}$ 559.1240. After acidification (0.03 N HCl/MeOH, 12 h) ^1H and ^{13}C NMR spectra are identical with those of rubellin D (**4**) (see Supplementary data). This result was supported by TLC, HPLC and ESI-MS spectrum.

3.2.3. Rubellin F (7)

Analytical HPLC (ret. time): 4.5 min. Preparative HPLC (ret. time): 7.4 min. TLC (R_f): 0.52. UV λ_{max}^{MeOH} nm: 215, 251, 449. FTIR ν_{max}^{KBr} cm^{-1}: 3405, 3037, 2959, 1623, 1575, 1460, 1413. HRESI-MS: *m/z* 543.1271 ($M–H^+$), calc. for $C_{30}H_{23}O_{10}$ 543.1291. After acidification (0.03 N HCl/MeOH, 12 h) TLC, HPLC and ESI-MS spectrum prove the presence of rubellin C (**3**).

3.3. Biosynthesis

Altogether 12.4 mg of sodium [1-^{14}C]-acetate (sp. act 4.0×10^6 Bq/mmol) were added to 3×100 ml of culture medium. In the feeding experiments with [^{13}C]-precursors we used a culture medium stabilised by a Soerensen phosphate buffer to pH 5.4. Therewith a phosphate content of 66 mM in the medium was obtained. Two grams of sodium [1-^{13}C]-acetate and 2 g of sodium [2-^{13}C]-acetate (both 99% ^{13}C), respectively, were added to 10×100 ml of culture medium. Feeding with glucose (without sucrose) was performed with 4% (w/w) [U-$^{13}C_6$]-D-glucose (99% ^{13}C) diluted with unlabeled glucose (altogether 25 g in 25×100 ml of culture medium).

Acknowledgements

We are grateful to E. Hänert and A. Muhr for expert technical assistance, A. Perner (HKI Jena) for performing MS and J. Wange (Institute of Pharmacy, Jena) for recording optical rotation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.05.003.

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