NOTE

Two new aurachins from *Rhodococcus* sp. Acta 2259*

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In the course of our HPLC screening program, we investigated freshly isolated actinomycete strains from selected terrestrial and limnetic habitats with the aim of detecting novel drugs for pharmaceutical applications. The strains were grown in submerged culture in different media, and extracts prepared from mycelia and culture filtrates at various fermentation times. The metabolite patterns of the strains were analyzed by HPLC-diode array monitoring (HP 1090; Agilent, Waldbronn, Germany) in combination with our in-house HPLC-UV-visible database.[2] Strain Acta 2259 was of special interest because of the presence of a prominent peak in the mycelium extract at a retention time of 12.9 min in our standardized gradient elution profile (Figure 1). It also showed an unusual UV-visible spectrum that differed from those of 933 reference compounds stored in our HPLC-UV-visible database. During the isolation and purification procedure, it became obvious that compounds 1–4 co-eluted in the same peak at a retention time of 12.9 min.

Strain Acta 2259 was isolated from activated sludge foam collected at Milcote Pilot Sewage Treatment Plant, Stratford-upon-Avon, UK. The strain was examined for a number of key properties known to be of value in *Rhodococcus* systematics.[3] Thus, it grew well on yeast extract–malt extract agar (ISP medium 2) producing creamy colonies and gave whole-organism hydrolysates that were rich in *meso*-diaminopimelic acid and in mycolic acids. Menaquinones having two of the eight isoprene units hydrogenated, MK-8(H$_2$), were the predominant isoprenologs. Sequencing the complete 16S rRNA gene led to a similarity of 99.8% with *R. baikonurensis* (AB071951), 99.2% with *R. erythropolis* (X81929) and 99.0% with *R. globerulus* (X80619), respectively, which are the nearest phylogenetic neighbors to strain Acta 2259.

Batch fermentations of strain Acta 2259 were carried out in a 20 l fermentor equipped with a turbine impeller system (Biostat b20; B. Braun, Melsungen, Germany) in a medium consisting of soluble starch 10 g, glucose 10 g, glycerol 10 g, corn steep powder 2.5 g

(Marcor, Hackensack, NJ, USA), Bacto peptone 5 g, yeast extract 2 g and NaCl 1 g in 1 l tap water; the pH was adjusted to 7.3 before sterilization. The fermentor was inoculated with 5% by volume of shake flask cultures grown in the same medium at 27 °C in 500 ml Erlenmeyer flasks with a single baffle for 48 h on a rotary shaker at 120 r.p.m. The fermentation was carried out for 48 h with an aeration rate of 0.5 volume air/volume/min and agitation at 1 000 r.p.m. Production of compounds 1–4 started at about 20 h and reached a maximal yield of 394 mg l^{-1} after an incubation period of 48 h. Compounds 1–4 were isolated from the mycelium by extraction with Me$_2$CO. After concentration to an aqueous residue, 1–4 were re-extracted by ethyl acetate and separated by column chromatography on Florisil (E Merck, Darmstadt, Germany) with CH$_2$Cl$_2$–MeOH (50:1) to give 180 mg of 1–4 containing fractions. Separation of 1–4 into single compounds was achieved by semi-preparative reversed-phase HPLC on Grom-Sil 300 ODS-5 (250×20 mm, 10 μm; Alltech-Grom, Rottenburg, Germany) and linear gradient elution H$_2$O–CH$_3$CN (57–61% CH$_3$CN within 39 min) at a flow rate of 15 ml min^{-1}. The fractions yielded 1 (1.68 mg), 2 (3.77 mg), 3 (2.23 mg) and 4 (1.59 mg).

The mass spectra derived from the HPLC-ESI-MS analysis revealed molecular masses for 1 ((M+H)$^+$=362.4), 2 ((M+H)$^+$=364.4), 3 ((M+H)$^+$=378.4) and 4 ((M+H)$^+$=380.4). This led to the assumption that the differences in structure between 1, 2 and 3, 4 were due to the presence or absence of double bounds, and compared to structures 1 and 2, compounds 3 and 4 might have an additional oxygen.

The structures of the compounds were fully elucidated by extensive 1D and 2D NMR analyses. The FT-ICR-MS (*m/z* 362.24777) data of 1 indicated its molecular formula to be C$_{25}$H$_{31}$NO ((M+H)$^+_{theor}$=362.24784; Δm=0.19 p.p.m.). Furthermore, the ^{1}H-NMR and HSQC data of 1 showed a total of 30 carbon-attached protons, of which 5 methyl, 3 methylene and 9 methine carbons could be assigned. Inspection of the 2D-NMR data (COSY, TOCSY, HSQC and HMBC) showed the structure of 1 to be a new metabolite,

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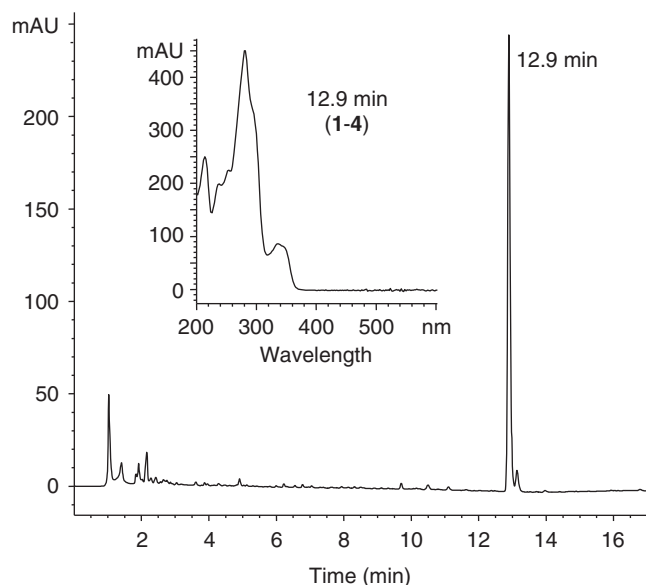Two new aurachins from *Rhodococcus* sp.
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Figure 1 HPLC analysis of a mycelium extract from *Rhodococcus* sp. Acta 2259 at a fermentation time of 48 h, monitored at 260 nm. 12.9 min=aurachins **1–4**; inset: UV-visible spectrum of **1–4**.
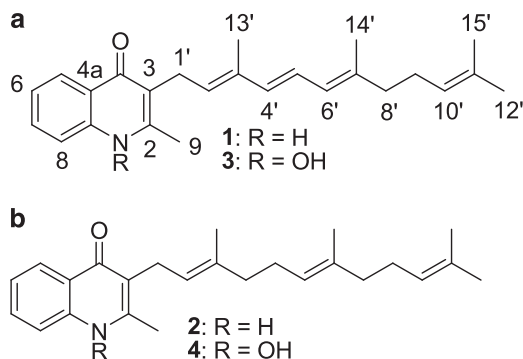

Figure 2 Structures of aurachin Q (**1**) and R (**3**) (**a**), and aurachin D (**2**) and C (**4**) (**b**).

Figure 3 Key correlations in 2D NMR of **1**.

Table 1 ^{1}H and ^{13}C NMR spectral data of 1 in DMSO-d_6

No.	δ (^{1}H) (p.p.m.) J in Hz	δ (^{13}C) (p.p.m.)
1	—	—
2	—	147.5 C
3	—	116.9 C
4	—	173.5 C
4a	—	123.3 C
5	8.12 d (8.03)	125.1 CH
6	7.32 t (7.45)	122.7 CH
7	7.67 t (8.03)	131.4 CH
8	7.80 d (8.60)	114.2 CH
8a	—	139.4 C
9	2.42 s	14.4 CH$_3$
1'	3.46 d (6.95)	24.4 CH$_2$
2'	5.34 t (6.95)	130.3 CH
3'	—	133.3 C
4'	6.11 d (15.4)	135.2 CH
5'	6.31 dd (15.4, 10.7)	122.7 CH
6'	5.83 d (10.7)	125.2 CH
7'	—	137.2 C
8'	2.05 m	39.4 CH$_2$
9'	2.06 m	25.9 CH$_2$
10'	5.05 t (6.95)	123.7 CH
11'	—	130.7 C
12'	1.63 s	25.3 CH$_3$
13'	1.90 s	12.3 CH$_3$
14'	1.76 s	16.3 CH$_3$
15'	1.56 s	17.4 CH$_3$

aurachin Q (Figure 2a). The complete NMR assignments were unambiguously carried out on the basis of COSY, TOCSY and HMBC experiments. The FT-ICR-MS (*m/z* 378.24277) data of **3** indicated its molecular formula to be $C_{25}H_{31}NO_2$ ((M+H)$^+_{theor}$= 378.24276; Δm=0.03 p.p.m.). By comparison with the NMR data of compound **1**, the structure could be assigned as its *N*-hydroxy derivative, aurachin R (**3**; Figure 2a).

The molecular formula of **2** was determined by FT-ICR-MS (*m/z* 364.26351) data to be $C_{25}H_{33}NO$ ((M+H)$^+_{theor}$=364.26349; Δm=0.05 p.p.m.). By comparison with the NMR data of compound **2**, the structure could be assigned to be aurachin D (Figure 2b). The molecular formula of **4** was established as $C_{25}H_{33}NO_2$ ((M+H)$^+_{theor}$=380.25841; Δm=0 p.p.m.) on the basis of FT-ICR-MS (*m/z* 380.25841) data. By comparison of the NMR data of compound **2**, the structure could be considered to be aurachin C (Figure 2b).

The ^{1}H-^{1}H-COSY spectra of **1** revealed protons attached to one phenyl, one ethyl, one propenyl and one propyl moiety as shown by bold lines in Figure 3. The connectivity of these partial structures was elucidated as follows. The ^{1}H-^{13}C-long-range couplings from the
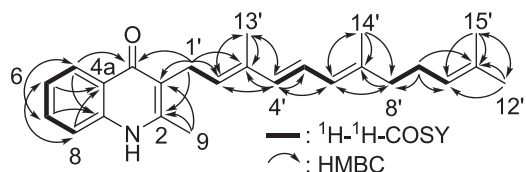
1'-methylene protons (δ$_H$ 3.46) to C-3 (δ$_C$ 116.9), C-4 (δ$_C$ 173.5), C-2' (δ$_C$ 130.3) and C-3' (δ$_C$ 133.3) revealed the connection between the alkyl side chain and the bicyclic core moiety. The 9-methyl protons (δ$_H$ 2.42) coupled with C-2 (δ$_C$ 147.5) and C-3, revealed the vicinal position to the side chain. The 2'-methine proton (δ$_H$ 5.34) showed couplings to C-13' (δ$_C$ 12.3) and to C-4' (δ$_C$ 135.2), and with the couplings of the 13'-methyl group (δ$_H$ 1.90) to C-2', C-3' and C-4' the substructure of the ethyl and the propenyl moiety was determined. The coupling from the 6'-methine proton (δ$_H$ 5.83) to C-14' (δ$_C$ 16.3) and C-8' (δ$_C$ 39.4), the coupling from the 14'-methyl group (δ$_H$ 1.76) to C-6' (δ$_C$ 125.2), C-7' (δ$_C$ 137.2) and C-8' and the coupling from the 8'-methine proton (δ$_H$ 2.05) to C-7' and C-6' underline the connection between the propenyl and the propyl moiety. The isopropenyl end of the alkyl side chain was determined by the coupling from H-9' (δ$_H$ 2.06) to C-11' (δ$_C$ 130.7); the coupling from H-12' (δ$_H$ 1.63) to C-10' (δ$_C$ 123.7), C-11' and C-15' (δ$_C$ 17.4); and the coupling from H-15' (δ$_H$ 1.56) to C-10', C-11' and C-12' (δ$_C$ 25.3), respectively. A summary of the ^{1}H and ^{13}C NMR data of **1** and **3** is shown in Tables 1 and 2.

Comparison of the COSY spectra of **1** and **2** showed that a structural difference could only be seen at the H-4' and H-5' positions;

Table 2 ^{1}H and ^{13}C NMR spectral data of 3 in DMSO-d_6

No.	δ (^{1}H) (p.p.m.) J in Hz	δ (^{13}C) (p.p.m.)
1	11.49 s, br	—
2	—	146.3 C
3	—	117.1 C
4	—	175.1 C
4a	—	123.2 C
5	8.06 d (8.10)	124.9 CH
6	7.26 t (7.70)	122.2 CH
7	7.58 t (8.31)	130.9 CH
8	7.47 d (8.31)	117.3 CH
8a	—	139.1 C
9	2.35 s	17.4 CH$_3$
1′	3.40 m	23.6 CH$_2$
2′	5.37 t (7.52)	130.5 CH
3′	—	133.1 C
4′	6.10 d (15.5)	135.6 CH
5′	6.31 dd (15.5, 10.9)	122.4 CH
6′	5.82 d (10.9)	125.1 CH
7′	—	137.1 C
8′	2.04 t (6.65)	39.5 CH2
9′	2.06 m	25.9 CH2
10′	5.05 t (6.17)	123.8 CH
11′	—	130.8 C
12′	1.62 s	25.5 CH$_3$
13′	1.90 s	12.1 CH$_3$
14′	1.74 s	16.1 CH$_3$
15′	1.55 s	17.5 CH$_3$

these protons are methylene protons in **2** compared to methine protons in **1**. The structural differences of **3** and **4** could be assigned to the N-hydroxy derivatives of **1** and **2** due to the different ^{13}C shifts deduced from the HMBC neighboring the nitrogen atom.

Members of the aurachin family were first isolated from the myxobacterium *Stigmatella aurantiaca*.[4,5] Recently, aurachin RE, which is similar in structure to that of aurachin C with a hydroxy group in position C-9′ of the hydrocarbon chain was isolated from a culture broth of *R. erythropolis* JCM 6824.[6] The aurachin structures are characterized by a bicyclic quinoline core system that is connected to an isoprenoid chain in various positions. In biological assays aurachins show growth inhibitory activity against numerous Gram-positive bacteria and weak antifungal activity. Aurachin C (**4**) and D (**2**) have been extensively studied as they strongly inhibit the quinol oxidation sites of *Escherichia coli*, that is, cytochromes *bo* and *bd*.[7]

Isomers of **2** have been used for electron transfer studies to further characterize the cytochrome oxidase *bd* from *Azotobacter vinelandii*.[8] The different selectivities of **4**—the N-hydroxy derivative of **2**—and **2** toward cytochromes *bd* and *bo* of *E. coli* make them a unique model system to study the selectivity of these oxidases.

Compounds **1**–**4** were tested for antimicrobial activity against several Gram-positive and Gram-negative bacteria using a resazurin-based assay. Compounds **3** and **4** showed moderate antibacterial activity (70–89% growth inhibition at 100 μM) against *Staphylococcus epidermidis* DSM 20044, *Bacillus subtilis* DSM 347 and *Propionibacterium acnes* DSM 1897, whereas compounds **1** and **2** were found to be inactive up to a concentration of 100 μM. Gram-negative bacteria, such as *E. coli* K12 DSM 498, *Pseudomonas fluorescens* NCIMB 10586, *P. aeruginosa* DSM 50071 and *Xanthomonas campestris* DSM 2405 were not susceptible to compounds **1**–**4**. Interestingly, compounds **3** and **4** showed weak inhibitory activity against glycogen synthase kinase 3β at a concentration of 10 μM (19 and 22% inhibition, respectively). Measurement of the glycogen synthase kinase 3β activity was adapted from a luminescent assay described by Baki *et al*.[9]

1 Nachtigall, J., Schulz, D., Beil, W., Süssmuth, R. D. & Fiedler, H.- P. Aranciamycin anhydride a new anthracycline-type antibiotic isolated from *Streptomyces* sp. Tü 6384. *J. Antibiot.* **63**, 397–399 (2010).
2 Fiedler, H.- P. Biosynthetic capacities of actinomycetes. 1. Screening for novel secondary metabolites by HPLC and UV-visible absorbance libraries. *Nat. Prod. Lett.* **2**, 119–128 (1993).
3 Goodfellow, M., Chun, J., Stackebrandt, E. & Kroppenstedt, R. M. Transfer of *Tsukamurella wratislaviensis* Goodfellow *et al*. 1995 to the genus *Rhodococcus* as *Rhodococcus wratislaviensis* comb. nov. *Int. J. Syst. Evol. Microbiol.* **52**, 749–755 (2002).
4 Kunze, B., Höfle, G. & Reichenbach, H. The aurachins, new quinoline antibiotics from myxobacteria: production, physico-chemical and biological properties. *J. Antibiot.* **40**, 258–265 (1987).
5 Augustiniak, H. *et al*. Preparation of aurachins and their use as antibiotics. *Ger. Offen.* 42, DE 3520229 A1 (1986).
6 Kitagawa, W. & Tamura, T. A quinoline antibiotic from *Rhodococcus erythropolis* JCM 6824. *J. Antibiot.* **61**, 680–682 (2008).
7 Meunier, B., Madgwick, S. A., Reil, E., Oettmeier, W. & Rich, P. R. New Inhibitors of the quinol oxidation sites of bacterial cytochromes *bo* and *bd*. *Biochemistry* **34**, 1076–1083 (1995).
8 Jünemann, S., Wrigglesworth, J. M. & Rich, P. R. Effects of decyl-aurachin D and reversed electron transfer in cytochrome *bd*. *Biochemistry* **36**, 9323–9331 (1997).
9 Baki, A., Bielik, A., Molnár, L., Szendrei, G. & Keserü, G. M. A high throughput luminescent assay for glycogen synthase kinase-3β inhibitors. *Assay Drug Develop. Technol.* **5**, 75–83 (2007).