Biosynthesis of Aurachins A–L in Stigmatella aurantiaca: A Feeding Study

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Biosynthesis of Aurachins A−L in *Stigmatella aurantiaca*: A Feeding Study

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The isolation of aurachins A−L (**1**−**11**) from *Stigmatella aurantiaca* strain Sg a15 is described. Their structures and relative configurations were deduced from spectroscopic data, in particular NMR. Three structural types were identified: A-type aurachins (**1**, **2**, **6**) are C-3 oxygen-substituted quinolines carrying a farnesyl residue on C-4, C-type aurachins (**3**, **4**, **7**−**11**) are C-4 oxygen-substituted quinolines carrying a farnesyl residue on C-3, and C-type aurachin E (**5**) has a [1,1a,8,d]imidazoloquinoline structure. Feeding of ^{13}C-labeled precursors showed that the quinoline ring is constructed from anthranilic acid and acetate, and the farnesyl residue from acetate by both the mevalonate and nonmevalonate pathways. Further, feeding of labeled aurachin C (**3**) indicated the A-type aurachins are derived by a novel intramolecular 3,4-migration of the farnesyl residue that is induced by a 2,3-epoxidation and terminated by a reduction step. ^{18}O-Labeling experiments indicated the new oxygen substituents originate from atomospheric oxygen. On the basis of these results a biosynthetic scheme covering all aurachins is proposed. It is further proposed that quinolones with an unorthodox substitution pattern, such as the 2-geranylquinolones from *Pseudonocardia* sp. and the 3-heptylquinolones from *Pseudomonas* sp., are formed by related rearrangement mechanisms.

During our screening of myxobacteria for antifungal activity *Stigmatella aurantiaca* strain Sg a15 was identified as a particularly prolific producer of structurally unrelated metabolites. In addition to the myxalamids,[1] known from *Myxococcus xanthus*,[2] and the iron chelator myxochelin,[3] known from *Angiococcus disciformis,*[4] the novel chromones stigmatellins A and B[1] and the quinoline alkaloids aurachins A−L (**1**−**11**)[5,6] were isolated. Remarkably three of these groups of compounds turned out to be inhibitors of mitochondrial respiration and photosynthesis: the myxalamids inhibit complex I,[2b] the stigmatellins inhibit complex III,[7] photosystem II, and the cytochrome b_6f-complex,[8] and the various aurachins inhibit complexes I[9,10] and III,[9] as well as the photosystem II and the cytochrome b_6f-complex.[11] Even though the structures of these inhibitors are not related to one another, there is an overall structural resemblance in that a relatively polar headgroup carries a lipophilic side chain about 15 carbon atoms in length, thus mimicking the electron carrier ubiquinone/ubiquinol in the respiratory chain. It is intriguing that *Stigmatella* apparently has invented three different types of inhibitors of the respiratory chain that confer antifungal activity. It may be speculated that their co-occurrence in Sg a15 is a natural archetype of today's antibiotic combination therapy to counteract development of resistance in the target organism.

Whereas the biosynthesis of myxochelin, myxalamid, and stigmatellin has been investigated in great detail by feeding studies[12] and cloning of the biosynthesis genes,[12,13] there is only limited information on the biosynthesis of aurachins. On the basis of an early feeding study a biosynthetic sequence starting with anthranilic acid via aurachins D, C, and B to A has been proposed.[14] Recently, parts of the biosynthetic gene cluster were cloned that are responsible for the synthesis of anthranilic acid and its conversion to aurachin D in the early steps of aurachin biosynthesis.[15] However, those for the following oxidative and rearrangement steps to aurachins A, B, C, and F−L could not be identified.

Herein we report the isolation and structure elucidation of aurachins A−L (**1**−**11**) isolated from *S. aurantiaca* strain Sg a15. Further, results of the incorporation of basic and advanced precursors in aurachins A, B, and C are presented, and a biosynthetic scheme covering all aurachins including aurachin P (**12**)[16] is proposed.

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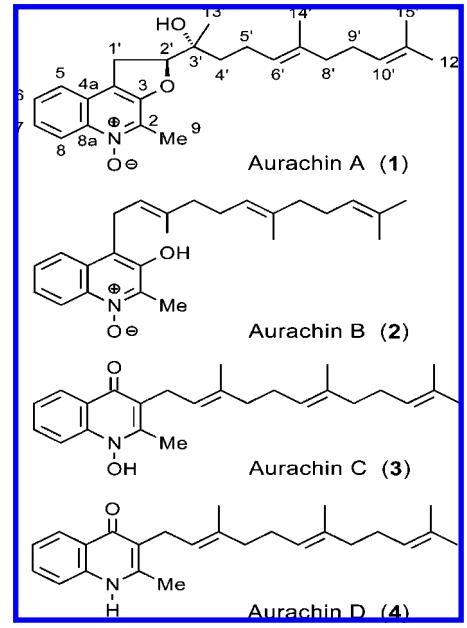
Results and Discussion

The aurachins are a group of unique isoprenoid quinoline alkaloids characterized by a farnesyl residue in either the 3- or 4-position of the quinoline nucleus. Conversely, the 4- or 3-position is occupied by an oxygen substituent. In addition, the aurachins may occur as quinolines, 4-quinolones, or quinoline *N*-oxides. The basic aurachins A−D (**1**−**4**) are formed by *S. aurantiaca* strain Sg a15 at various rates. On large-scale fermentation aurachin C (**3**) predominates during the growth phase, but is consumed during the stationary and decline phases to yield aurachin A (**1**), which accumulates up to 8.8 mg/L, whereas aurachins B and D (**2** and **4**) always remain as minor components[5] (see Supporting Information, Figure S1a). Batchwise feeding of anthranilic acid, a presumed precursor of the aurachins, increased the yields of aurachin A (**1**) to 12 mg/L and aurachin C (**3**) to 14 mg/L.



In the presence of XAD adsorber resin, and continuous feeding of anthranilic acid, the yield of aurachin C (**3**) increased dramatically up to 130 mg/L, while that of aurachin A remained unchanged at

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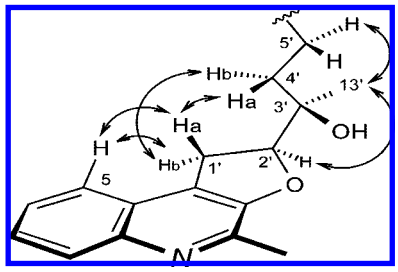


Figure 1. Selected NOEs and preferred conformation of aurachin A (**1**) in $CDCl_3$.

12 mg/L (see Supporting Information, Figure S1b). Aurachins E−L (**5**−**11**) are always very minor components. It is noteworthy that the related species *Stigmatella eracta* strain Pd e32 produces predominantly the new aurachin P (**12**) and small amounts of aurachin C (**3**).[16]

The structural assignment of the aurachins was accomplished straightforwardly from elemental composition, 1D and 2D NMR, and UV data (see Supporting Information, Tables S1 and S2). Thus, a nonmodified farnesyl residue was identified in aurachins B−E (**2**−**5**) by comparison of the chemical shifts with literature values. Similarly, the quinoline nucleus of aurachins A and B (**1** and **2**) was derived from a comparison of ^{13}C NMR data with literature values, whereas the presence of four adjacent protons in the benzene ring was determined from the ^{1}H NMR spectra. From the HMBC spectra the farnesyl residue in **1** and **2** had to be placed at C-4 of the pyridine ring, a methyl group at C-2, and the two oxygen atoms at the remaining C-3 and on the nitrogen. In aurachin A (**1**) the 2′,3′ double bond is oxidized and a 2′,3 ether ring is formed. On the basis of the assumption that this transformation is brought about by epoxidation of the 2′*E* double bond followed by cyclization with the 3-OH, aurachin A (**1**) was assigned the relative *2′S,3′R* configuration. This was readily confirmed by the observation of the NOEs shown in Figure 1. In addition, a strong NOE between the 1′-protons and 5-H in the benzene ring confirmed the attachment of the farnesyl residue at C-4 in aurachin A (**1**), as well as in B (**2**) and F (**6**).

All other aurachins, C−E (**3**−**5**) and G−L (**7**−**11**), showed a strong NOE between the 1′-proton(s) and the methyl group at C-2, indicating the attachment of the farnesyl residue at C-3. Conversely, in these compounds the oxygen is found at C-4, forming a quinolone system in aurachins C−E (**3**−**5**) (C-4 around δ 170 ppm) or an ether in aurachins G−L (**7**−**11**) (C-4 around δ 150 ppm). Aurachins F (**6**) and L (**11**) are cyclo-didehydro derivatives of aurachins B (**2**) and C (**3**) with an extended chromophore shifting the UV bands by 10−20 nm to longer wavelengths. Aurachin K (**10**) is an isomer of L (**11**) with the 1′,2′ double bond moved to the 4′,5′-position, giving rise to an additional strong UV band at 238 nm for the conjugate diene system. Its 4′*E*,6′*E* configuration followed from NOE data. Aurachin H (**8**) is an isomer of A (**1**) with the farnesyl residue at C-3 and oxygen at C-4. It is further modified by a 4′,5′ double bond to give aurachin G (**7**) or by a methoxyl group in the 1′-position to give aurachin I (**9**). On the basis of the assumption that aurachins B (2) and C (3) are epoxidized during biosynthesis by one and the same monooxygenase, the *2′S,3′R* configuration was assigned to aurachins G−I (**7**−**9**). The relative 1′*S* configuration of aurachin I (**9**) followed from the NOE between the 13′-Me and 1′-H and the 3 Hz coupling between 1′-H and 2′-H. Aurachin E (**5**) is related to aurachin D (**4**), but differs from all other aurachins by an extra nitrogen atom and a carbonyl group. From detailed 2D NMR measurements the nitrogen is located at C-8 of the quinoline ring, forming an imidazolone ring with N-1.

The biosynthesis of the basic aurachins A−D (**1**−**4**) was investigated by feeding of ^{13}C- and ^{18}O-labeled anthranilic acid, C-1 and C-2 ^{13}C-enriched acetate, and oxygen $^{18}O_2$, as well as labeled intermediates isolated from previous feedings (Tables 1 and 2). The significant increase of aurachin C production up to 130 mg/L on feeding of anthranilic acid already indicated that it is a biosynthetic precursor and early bottleneck in the multistep biosynthesis. Indeed, on feeding [1′-^{13}C]anthranilic acid, C-4-labeled aurachins A and C were formed (Table 1, expt 1). The remaining carbon atoms C-2, C-3, and C-9 of the quinoline moiety as well as all carbon atoms of the side chain of aurachins A and C were labeled by [1,2-$^{13}C_2$]acetate (Tables 1 and 2, expt 2). The incorporation pattern of intact acetate units and isolated carbons in the 4′-, 8′-, and 12′-position of the farnesyl side chain was deduced from the magnitude of the C,C coupling constants (Table 2, expt 2). This indicates the farnesyl residue is constructed by the classical terpenoid pathway via mevalonate, as demonstrated previously for the myxobacterial menaquinone MK-8.[17] However, on closer inspection of the signal intensities, conspicuous discrepancies were observed. In general, the ^{13}C isotope abundance in the terpenoid side chain was only 50% and less than that of the acetate-derived carbon atoms of the quinoline ring (Tables 1 and 2, expt 2). This can be explained, at least in part, by a shunt mechanism channeling a C_5 unit from leucin to IPP and DMAPP. According to a feeding experiment with Sg a15, about 10% of the farnesyl side chain of the aurachins was labeled by [D_{10}]leucine.[18] To account for the remaining major portion of label, a third pathway has to be considered. The almost equal distribution of label from [2-^{13}C]acetate (Table 2, expt 3) clearly indicated involvement of the

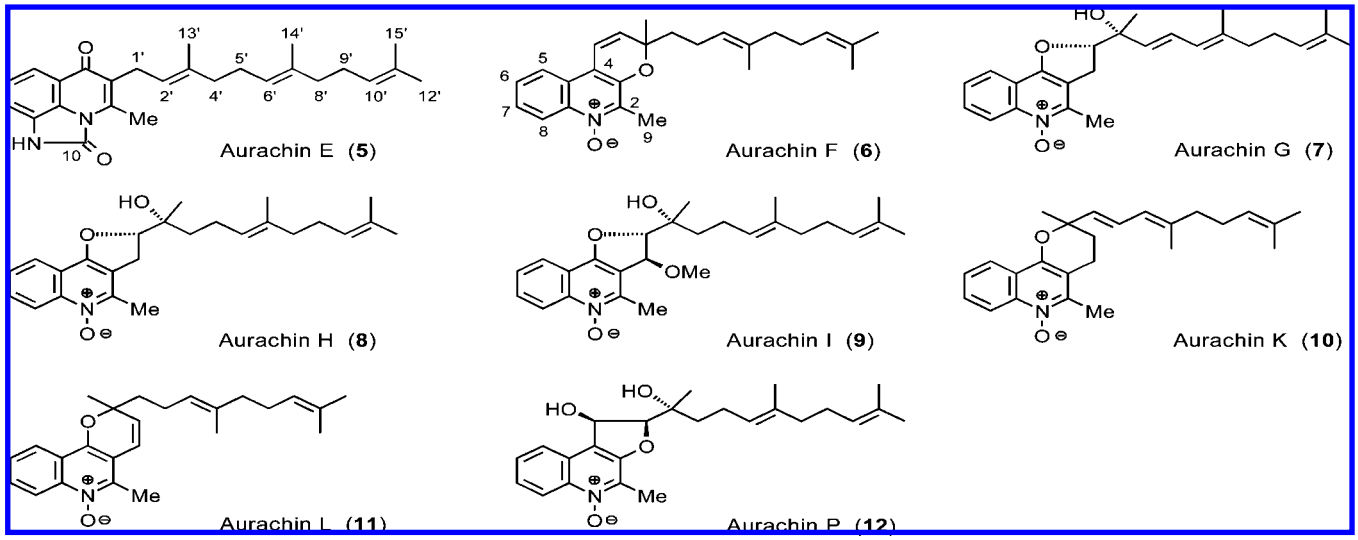
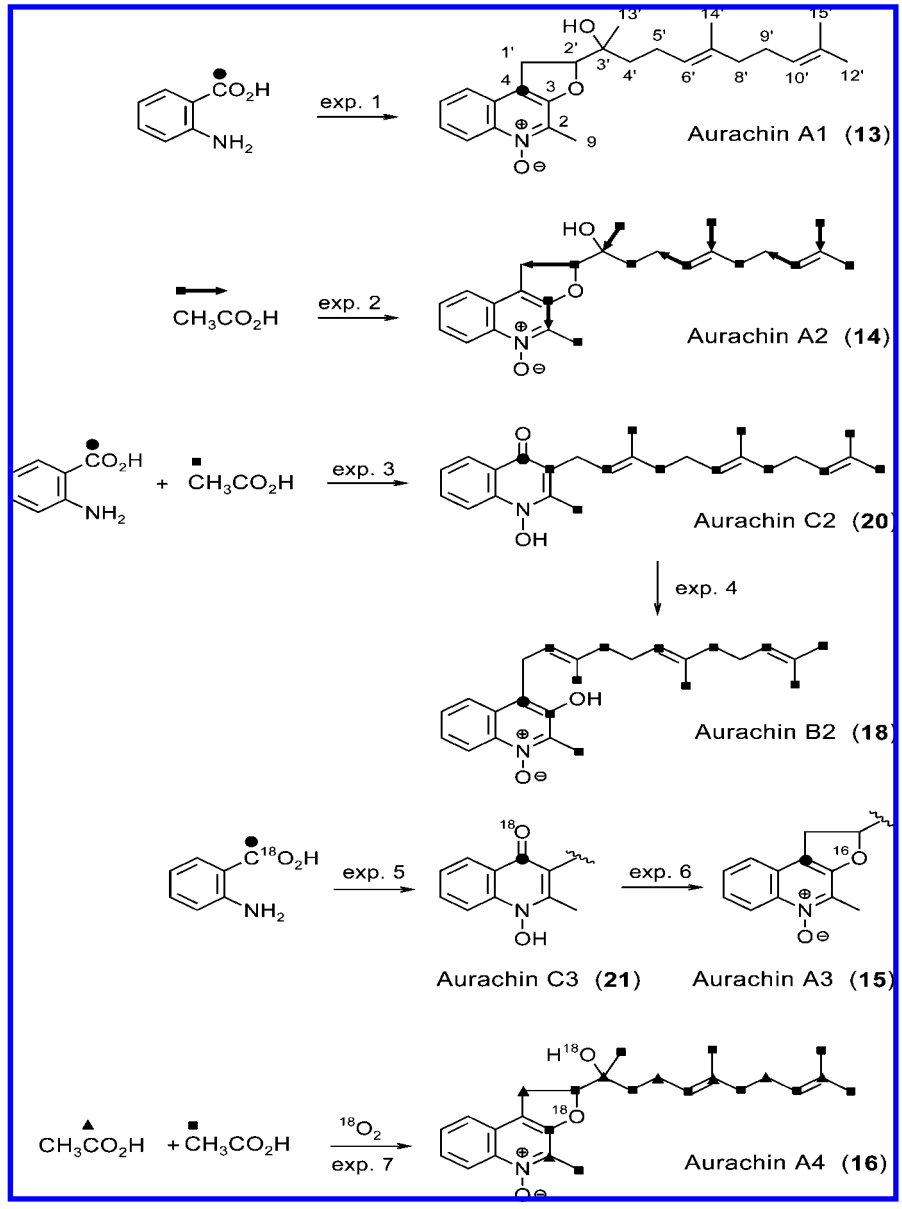


Table 1. ^{13}C NMR Data of the Quinoline Moiety of Labeled Aurachins A1−A4 (**13**−**16**), B1, B2 (**17**, **18**), and C1−C4 (**19**−**22**) in CD_3OD

				^{13}C isotope abundance (%)				
expt no.	precursor fed	isotope abundance	aurachins isolated	C-2	C-3	C-4	C-9	C-1′
1	[1-^{13}C]anthranilate	90%	A1 (**13**)	1.1	1.1	80	1.1	1.1
			C1 (**19**)	1.1	1.1	66	1.1	1.1
2	[1,2-$^{13}C_2$]acetate	90%	A2 (**14**)[a]	14	14	1.1	15	5
3	[1-^{13}C]anthranilate	90%	B1 (**17**)	1.5	14	78	13	n.d.
	[2-^{13}C]acetate	90%	C2 (**20**)	1.5	19	78	16	1.5
4	aurachin C2 (**20**)	C-3 19% C-4 78%	B2 (**18**)	n.d.	18	74	14	2
5	[1-$^{13}C^{18}O_2$]anthranilate	7.5% each	C3 (**21**)	1.1	1.1	7[b]	1.1	1.1
6	[4-$^{13}C^{18}O$]aurachin C3 (**21**)	7% each	A3 (**15**)			5[c]		
			C4 (**22**)			3.5[d]		
7	[1-^{13}C]acetate	90%	A4 (**16**)[e]	15	16	n.d.	16	4
	[2-^{13}C]acetate	90%						
	$^{18}O_2$ on the second day	53%						

[a] Coupling constants for C-2 J_{CC} = 76.17 Hz; C-3 J_{CC} = 76.25 Hz; C-9 singlet. [b] ^{18}O isotope abundance 7%. [c] Isotope abundances determined by EIMS; <0.3% ^{18}O. [d] Isotope abundances determined by EIMS; ^{18}O 3.5%. [e] ^{18}O isotope abundance at C-3 14%, ^{18}O isotope shift $\Delta\delta$ = 0.020 ppm; at C-2′ 16%, $\Delta\delta$ = 0.035 ppm; at C-3′ 20%, $\Delta\delta$ = 0.030 ppm.

Scheme 1. Incorporation of ^{13}C- and ^{18}O-Labeled Precursors and ^{18}O-Labeled Molecular Oxygen in Aurachins A−C (**1**−**3**) (expts 1−7)[a]



[a] For the terpenoid moiety only labeling by the mevalonate pathway is shown. The figures in aurachins A1, A2, A3, etc., designate isotopomers.

Table 2. ^{13}C NMR Data of the Terpene Moiety of Labeled Aurachins A2 (**14**), C2 (**20**), and A4 (**16**) in $CDCl_3$

precursor	expt 2 [1,2-^{13}C]acetate		expt 3 [2-^{13}C]acetate [1-^{13}C]anthranilate	expt 7 [1-^{13}C]acetate [2-^{13}C]acetate
	aurachin ^{13}C isotope abundance (%) $J_{C,C}$ (Hz)			
C atom	A2 (**14**)		C2 (**20**)	A4 (**16**)
C-1′	5	35.05	2	4
C-2′	4	35.01	4	3
C-3′	5	39.67	n.d	3
C-4′	8	singlet	6	5
C-5′	5	43.65	2	5
C-6′	4	43.92	3	3
C-7′	3	42.49	n.d.	3
C-8′	8	singlet	6	6
C-9′	4	44.11	2	4
C-10′	4	44.12	4	3
C-11′		n.d.	n.d	3
C-12′	6	singlet	5	5
C-13′	5	39.89	2	6
C-14′	4	42.29	2	6
C-15′	5	37.09	2	6

glyceraldehydes 3-phosphate/pyruvate pathway to IPP and DMAPP as described by Rohmer et al.[17,19] This is corroborated by the low isotope abundance at C atoms 2′, 3′, 6′, 7′, 10′, and 11′ on feeding a mixture of [1-^{13}C]- and [2-^{13}C]acetate (Table 2, expt 7), as these C atoms receive their label only through the mevalonate pathway. The simultaneous operation of the mevalonate and non-mevalonate pathways in bacteria is well documented for instance in *Streptomycetes*[20] and *Listeria*.[21]

Labeling of C-2 and C-3 by an intact acetate unit and of C-9 by a cleaved acetate unit suggested a 4-quinolone-2-acetic acid intermediate **I** (Scheme 2). As feeding of synthetic **I** or its ethyl ester had no influence on the production of aurachins, it is not the presumed substrate for the farnesyl transferase. This is rather its decarboxylation product 2-methylquinolone **II**, which after feeding to the knockout mutant Sga-D_AS restored aurachin synthesis.[15b] However, its presence in cultures of strain Sg a15 after feeding of anthranilic acid could not be demonstrated by HPLC/MS analysis. Also, in contrast to feeding of anthranilic acid, feeding of synthetic 2-methylquinolone **II** had no influence on the production of aurachins.

The most unusual step in aurachin biosynthesis is the isomerization of aurachin C (**3**) to aurachin B (**2**), which, in a formal sense, interchanges the 3-farnesyl and 4-oxygen substituents. Its mechanism was first investigated by combined feeding of [1′-^{13}C]anthranilic acid and [2-^{13}C]acetate (Table 1, expt 3). The outcome was aurachin B1 (**17**) (the figure in B1 identifies the isotopomer present) and aurachin C2 (**20**) with the label in the expected positions. Feeding of aurachin C2 (**20**) with a ^{13}C isotope abundance of 19% at C-3 and 78% at C-4 to a fresh culture resulted in aurachin B2 (**18**) with an unchanged labeling pattern for the quinoline ring and side chain (Table 1, expt 4). This indicates that an *intramolecular* migration of the farnesyl residue occurs. The fate of the carbonyl oxygen in the rearrangement was investigated by first feeding of [1′-^{13}C^{18}O$_2$]anthranilic acid to produce [4-^{13}C^{18}O]aurachin C3 (**21**) with isotope abundances of 7% (Table 1, expt 5). On feeding of this material to a fresh culture, aurachin A3 (**15**) and in addition some aurachin C4 (**22**) were recovered (Table 1, expt 6). Whereas the isotope ratio in the recovered material was unchanged, the oxygen label in aurachin A3 was lost during the rearrangement. In order to identify the origin of the newly introduced oxygen atom including that on C-3′ of aurachin A, a mixture of [1-^{13}C] and [2-^{13}C]acetate was fed. After 2 days, when the titer of aurachin C was expected to be high, the atmosphere in the culture bottle was replaced by a 4:1 mixture of nitrogen and $^{18}O_2$ oxygen and cultivation was continued for 2 days (Table 1, expt 7). Aurachin A4 (**16**) was isolated and analyzed by NMR spectroscopy. Incorporation of ^{18}O at C-3/C-2′, as well as at C-3′, was clearly seen by upfield isotope shifts of the expected order of magnitude for C-3 (0.020 ppm), C-2′ (0.035 ppm), and C-3′ (0.030 ppm).[22] In summary, the biosynthetic sequence depicted in Scheme 2 is proposed.

Starting from anthranilic acid, the first defined intermediate is 2-methyl-(1,4-dihydroquinoline-4-one) (**II**) according to feeding of a blocked mutant. It is the substrate for a farnesyl transferase leading to aurachin D (**4**),[15b] which is not accumulated but rapidly oxidized to the N-oxide aurachin C (**3**). Epoxidation of its C-2, C-3 double bond to **III** sets the stage for migration of the farnesyl residue to C-4, which is brought about by a push−pull effect between the nitrogen and the carbonyl group. On reduction of the 3-carbonyl group in **IV**, the system aromatizes to aurachin B (**2**). Its intermediate concentration is apparently kept low by rapid epoxidation of the 2′,3′ double bond followed by spontaneous interception of the epoxide by the 3-hydroxy group to give aurachin A (**1**), the final product in *S. aurantiaca*.

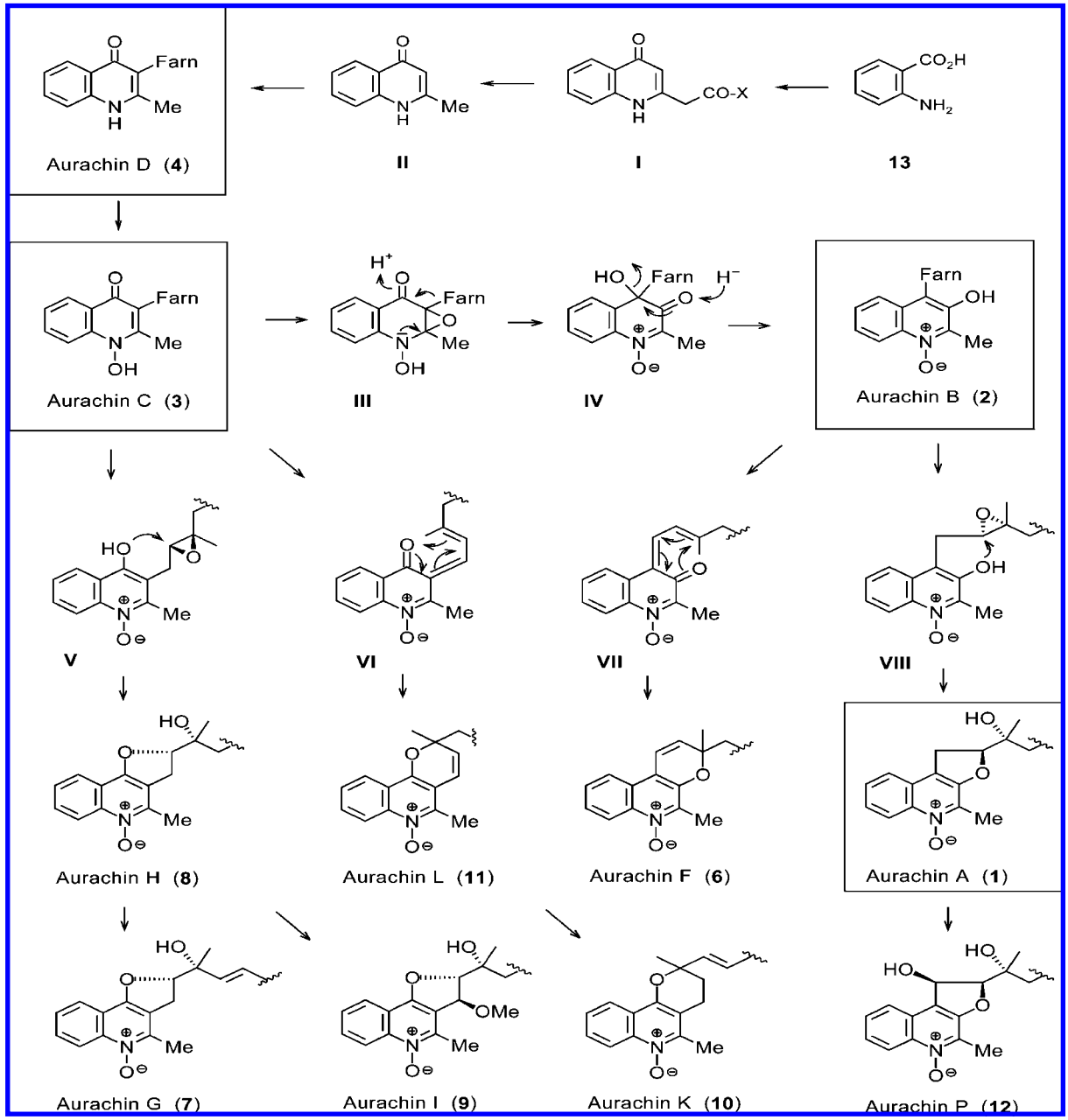
Final and major product in *Stigmatella erecta*, aurachin P (**12**), is formed by a subsequent hydroxylation of **1** at C-1′.[16] Similarly to aurachin B (**2**), side-chain epoxidation of aurachin C (**3**) leads to aurachin H (**8**), which is hydroxylated and O-methylated to aurachin I (**9**). Further, both aurachins B and C may be deydrogenated to **VII** and **VI**, which by 3,3-electrocyclization give aurachins F (**6**) and L (**11**). Finally, dehydrogenation of **8** gives aurachin G (**7**), and double-bond transposition in **11** leads to aurachin K (**10**).

The structurally aberrant aurachin E (**5**) has no reasonable place in the biosynthetic scheme. As it was isolated only in minute amounts, it was suspected that it may be derived from 2,3-diaminobenzoic acid, which was introduced as an impurity with the anthranilic acid fed during cultivation. Thus, 2,3-diaminobenzoic acid was synthesized from 2-amino-3-nitrotoluene[23] and fed as free acid and ethyl ester to *S. aurantiaca*. As this had no influence on the formation of aurachin E, its biosynthesis presumably branches off at a later stage, e.g., from aurachin C or D. Alternatively aurachin E might be an artifact formed from aurachin C or D, ammonia, and a reactive C_1 or a "CN" species during cultivation or isolation. The latter possibility could be demonstrated by the semisynthesis of aurachin E from aurachin C.[24]

Conclusion

The C-type aurachins (**3**−**5**, **7**−**11**) are characterized by an oxygen substituent in the 4-position of the quinoline ring and a farnesyl derived residue at C-3. From their structures they are related to 2,3-substituted 4-quinolones isolated from plants[25] and bacteria,[26,27] although their biosynthesis is different. Most remarkably, the farnesyl residue is constructed in parallel via the mavalonate, the nonmevalonate pathway, and leucine degradation. The relative contribution of these pathways may vary with the physiological state of the organism and has to be investigated in detail by further feeding studies with appropriate precursors. The A-type aurachins (**1**, **2**, **6**, **12**) are derived from aurachin C (**3**) by a unique transposition of the farnesyl residue from C-3 to C-4 by consecutive epoxidation/reduction steps. With this mechanism in mind, the biosynthesis of 2-geranyl-substituted 4-quinolones in *Pseudonocardia* sp.[27] appears in a new light. As direct prenylation of a 4-quinolone at C-2 is not favorable for electronic reasons, we propose a 3-geranyl intermediate related to aurachin D (**4**). This, instead of prior N-oxidation, is directly rearranged by similar epoxidation/reduction steps to the 2-geranyl isomer (see Supporting Information, Scheme S1).[28] By contrast, the 4-quinolones produced by *Pseudomonas aeruginosa*[26] receive their 2-heptyl (and higher alkyl) residues in the course of quinoline ring formation.[29] Like aurachin D (**4**) they are transformed to either the N-oxide or the 2,3-epoxide, which isomerizes to 2-heptyl-3-hydroxyquinolinone. The latter represents an important quorum sensing signal (PQS) in

Scheme 2. Proposed Biosynthetic Relationships of Aurachins A−D (**1**−**4**) and F−P (**6**−**12**)



P. aeruginosa.[26] Interestingly, in addition to the various 2-alkylquinolones a rearranged 3-heptyl-3-hydroxyquinolone has also been isolated from *P. aeruginosa*[30] and *P. methanica*.[31] We suggest that this completely neglected compound, in recent work,[26] is formed from PQS by another 2,3-epoxidation followed by rearrangement (see Supporting Information, Scheme S2) and, moreover, that this is the mechanism by which the quorum sensing signal is turned off.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Perkin-Elmer 241 instrument. UV spectra were recorded on a Shimadzu UV-2102 PC scanning spectrometer. IR spectra were measured with a Nicolet 20DXB FT-IR spectrometer. NMR spectra were recorded in $CDCl_3$ on Bruker ARX-400 and DMX-600 spectrometers. For structural analogues only a selection of significant NMR data is given. EI and DCI mass spectra (reactant gas ammonia) were obtained on a Finnigan MAT 95 spectrometer, with high-resolution data acquired using peak matching (M/DM = 10 000). ESI mass spectra were obtained with a Q-Tof II spectrometer from Micromass. HPLC/DAD/ESI-MS were performed on a PE Sciex Api-2000 LC/MS equipped with a Nucleodur C18 column, 5 μm, 2 × 125 mm (Macherey-Nagel) (0.3 mL/min acetonitrile/5 mM ammonium acetate buffer pH 5.5, gradient 5:95 to 95:5 in 30 min). Large-scale separations were performed on a Prep LC-500 from Merck. Preparative HPLC was performed using a Nucleosil column (250 × 21 mm, 7 μm, flow 18 mL/min) with a solvent of acetonitrile/water (gradients), and diode array detection. Analytical TLC used TLC aluminum sheets of silica gel Si 60 F_{254} (Merck), detection UV absorption at 254 nm. Red to violet spots were formed on staining with vanillin/sulfuric acid followed by heating to 120 °C. Precoated silica gel Si 60 F_{254} plates of 0.25 and 0.5 mm layer thickness were used for preparative TLC.

Cultivation of *Stigmatella aurantiaca* Strain Sg a15. (a) A 100 L bioreactor with a circulating pump stirrer system (Giovanola Frères, Monthey, Switzerland) was charged with 60 L of modified Zein liquid medium and inoculated with 5 L of well-grown shake cultures as described previously.[5] The aeration rate was adjusted to 300 NL/h, and the agitation was 500 rpm. During the cultivation 14 g of anthranilic acid dissolved in 1000 mL of water and adjusted with $KHCO_3$ to pH 7.0 was pumped in at different intervals; in particular, during the logarithmic growth phase 220 mg was added every hour. After 45 h the aeration rate was reduced to 250 NL/h and the agitation to 450 rpm. During the following 60 h the oxygen saturation dropped to 20%

Table 3. Feeding of Labeled Precursors of Aurachins A−C (**1**−**3**) to *Stigmatella aurantiaca*

expt	precursor fed	isotope abundance	amount fed mg/L culture	metabolites isolated
1	[1-^{13}C]anthranilate	90%	75 mg/1 L	4.5 mg **13**
				10 mg **19**
2	[1,2-$^{13}C_2$]acetate	90%	0.42 g/1 L	3 mg **14**
3	[1-^{13}C]anthranilate	90%	107 mg/1.6 L	6 mg **17**
	[2-^{13}C]acetate		0.6 g/1.6 L	10 mg **20**
4	aurachin C2 (**20**)	C-3 19%	6 mg/0.1 L	2 mg **18**
		C-4 78%		
5	[1-$^{13}C^{18}O_2$]anthranilate	7.5% each	88 mg/1.2 L	13 mg **21**
6	[4-$^{13}C^{18}$O]aurachin C3 (**21**)	7% each	12 mg/0.35 L	3 mg **15**
				2 mg **22**
7	[1-^{13}C]acetate	90%	0.2 g/0.2 L	3 mg **16**
	[2-^{13}C]acetate	90%	0.2 g/0.2 L	
	$^{18}O_2$ on the second day	53%	0.22 L	

in spite of increasing the aeration rate to 300 NL/h and agitation to 520 rpm. After 170 h, toward the end of cultivation, the oxygen saturation increased to 80% and pH to 8.0. HPLC analysis indicated the presence of aurachin A (12 mg/L), aurachin B (2 mg/L), and aurachin C (14 mg/L). Three further peaks with identical characteristic UV spectra were identified as 5-nitroresorcinol and its mono- and dimethyl ether.[16] However, these compounds were lost during the standard workup and isolation procedure.

(b) A 100 L bioreactor was charged with 60 L of modified Zein liquid medium as described above, and 2% XAD adsorber resin was added before sterilization. Starting immediately after inoculation the solution of anthranilic acid was continuously pumped in at a rate of 60 mg/h. After 70 h at the end of the cultivation, the pH was regulated with acetic acid at pH 7.6. Samples of culture including the XAD were collected in 5 to 15 h intervals and analyzed by HPLC as described.[5] After 160 h, cell mass and XAD were collected by centrifugation and extracted with acetone as described below for procedure a.

Isolation of Aurachins A−L (1−11). Two 65 L fermentation batches from procedure a were harvested by centrifugation to give 1.6 kg of wet cell mass. This was extracted with 2 L of acetone and then twice with 1 L each of acetone. The combined extracts were concentrated in vacuo to an aqueous slurry (1.5 L). Sodium chloride (150 g) was added followed by extraction with ethyl acetate (4 × 0.4 L). The organic extract was dried with $MgSO_4$ and evaporated to give a dark brown oil (37 g). For gross separation this was dissolved in 100 mL of dichloromethane and applied to an open column (4.5 × 16 cm) packed with Florisil in dichloromethane. After elution with dichloromethane/petroleum ether (8:2, 0.5 L), fraction 1 was obtained by elution with 1.5 L of dichloromethane and dichloromethane/ethyl acetate (8:2), fraction 2 with 0.5 L of ethyl acetate, and fraction 3 with 1 L of ethyl acetate/methanol (8:2). Fraction 1 was further separated by MPLC on Si 60 (Merck) with a dichloromethane/methanol gradient (10:0 to 8:2) to give consecutively aurachins E (**5**, 10 mg), F (**6**, 22 mg), D (**4**, 40 mg), K (**10**, 13 mg), L (**11**, 12 mg), and B (**2**, 10 mg). Fraction 2 was separated by MPLC on silica gel RP-18 with methanol/water/triethyl amine (80:19:1) to give consecutively aurachins C (**3**, 830 mg), B (**2**, 25 mg), and A (**1**, 560 mg). Fraction 3 was further separated by MPLC on Si 60 (Merck) with a dichloromethane/methanol gradient (10:0 to 7:3) to give consecutively aurachins G (**7**, 50 mg), H (**8**, 5 mg), and I (**9**, 7 mg). Physical data of the aurachins A−D (**1**−**4**) are given in ref 5.

Aurachin E (5): colorless needles (diethyl ether); mp 168−169 °C; R_f 0.79 (dichloromethane/acetone/methanol, 80:18:2); UV (MeOH) λ_{max} (log ε) 241 (4.43), 249 (4.49), 300sh (3.68), 339 (4.12) nm; IR ($CHCl_3$) ν_{max} 3445, 1752, 1735, 1654 cm^{-1}; EIMS *m*/*z* 404 [M]$^+$ (2), 5 (32), 267 (100); HREIMS *m*/*z* 404.2445 (calcd for $C_{26}H_{32}N_2O_2$, 404.2464), 335.1757 (calcd for $C_{21}H_{23}N_2O_2$, 335.1760), 267.1140 (calcd for $C_{16}H_{15}N_2O_2$, 267.1134).

Aurachin F (6): colorless solid; R_f 0.59 (dichloromethane/acetone/methanol, 80:18:2); UV (MeOH) λ_{max} (log ε) 241sh (4.27), 253 (4.43), 267sh (4.10), 279sh (3.89), 305 (3.49), 320 (3.62), 336 (3.75), 373 (3.64), 390 (3.69) nm; EIMS *m*/*z* 377 [M]$^+$ (4), 226 (20), 210 (100); HREIMS *m*/*z* 377.2363 (calcd for $C_{25}H_{31}NO_2$, 377.2355).

Aurachin G (7): colorless solid; R_f 0.18 (dichloromethane/acetone/methanol, 75:20:5); UV (MeOH) λ_{max} (log ε) 210 (4.47), 238 (4.52), 247 (4.55), 350sh (4.02) nm; EIMS *m*/*z* 393 [M]$^+$ (4), 377 (8), 172 (100); HREIMS *m*/*z* 393.2299 (calcd for $C_{25}H_{31}NO_3$, 393.2304).

Aurachin H (8): colorless solid; R_f 0.12 (dichloromethane/acetone/methanol, 75:20:5); UV (MeOH) λ_{max} (log ε) 216 (4.62), 249 (4.57), 339 (4.07) nm; IR ($CHCl_3$) ν_{max} 3445, 1752, 1735, 1613 cm^{-1}; EIMS *m*/*z* 395 [M]$^+$ (22), 379 (22), 184 (100); HREIMS *m*/*z* 395.2463 (calcd for $C_{25}H_{33}NO3$, 395.2460).

Aurachin I (9): colorless solid; R_f 0.18 (dichloromethane/acetone/methanol, 75:20:5); UV (MeOH) λ_{max} (log ε) 216 (4.62), 249 (4.56), 350 (3.95) nm; ^{1}H NMR, NOEs H_3-13/H-1′, H_3-9/H-1′, OMe-1′/H-1′, H-2′; EIMS *m*/*z* 425 [M]$^+$ (46), 409 (15), 214 (100); HREIMS *m*/*z* 425.2559 (calcd for $C_{26}H_{35}NO_4$, 425.2566).

Aurachin K (10): colorless solid; R_f 0.50 (dichloromethane/acetone/methanol, 75:20:5); $[\alpha]_D^{20}$ ca. −1.0 (*c* 2.3, MeOH); UV (MeOH) λ_{max} (log ε) 221 (4.58), 242 (4.60), 338 (3.93) nm; EIMS *m*/*z* 377 [M]$^+$ (40), 361 (100), 293 (48), 292 (47).

Aurachin L (11): colorless solid; R_f 0.40 (dichloromethane/acetone/methanol, 75:20:5); $[\alpha]_D^{20}$ ca. −1.0 (*c* 1.3, MeOH); UV (MeOH) λ_{max} (log ε) 225 (4.60), 273 (4.49), 280 (4.45), 352 (3.91) nm; EIMS *m*/*z* 377 [M]$^+$ (37), 361 (100), 293 (37), 292 (45).

Labeling Studies with *Stigmatella aurantiaca* Strain Sg a15. Labeled Precursors. Sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$]acetate (97 at %), [$^{13}C_6$]glucose (90 at %), $^{15}NH_4Cl$ (99 at %), and $^{18}O_2$ (53 at %) were purchased from MSD Isotopes, $Ba^{13}CO_3$ (99 at %) from Promochem, and $^{13}C^{18}O_2$ (99/95 at %), [2-^{15}N]glutamine and [5-^{15}N]glutamine (99 at %) from Cambridge Isotope Laboratories.

[$^{13}CO_2$H]Anthranilic Acid. *o*-Bromoaniline (1.75 g, 1.1 mmol) in diethyl ether was lithiated with *n*-butyllithium in hexane (11.5 mL, 2.6 M).[32] After 15 min stirring at rt the flask was cooled with liquid nitrogen and connected by a vacuum line to a second flask containing $Ba^{13}CO_3$ (2.0 g). By addition of concentrated sulfuric acid $^{13}CO_2$ was liberated and condensed on the lithio compound. The reaction mixture was slowly warmed to rt, and water was added and extracted with diethyl ether. The anthranilic acid was isolated from the aqueous phase and purified via salt formation with sodium bicarbonate. The *p*-isomer byproduct (ca. 10%) was eliminated by repeated recrystallization from methanol. Yield: 220 mg (15%); ^{1}H NMR (CD_3OD) δ 7.81 (1H, ddd, J = 8.0, 1.8, and $^3J_{C,H}$ = 4.2 Hz, H-5).[33]

[$^{13}C^{18}O_2$H]Anthranilic Acid. *o*-Bromoaniline (0.85 g, 0.54 mmol) was lithiated as described above, and the lithio compound quenched with $^{13}C^{18}O_2$ (100 mL, ca. 4 mmol). Due to the excessive formation of (labeled) pentanoic acid, the yield of anthranilic acid was very low and isolation by crystallization was complicated. Two batches were obtained, a fully labeled anthranilic acid (22 mg) and, after addition of nonlabeled anthranilic acid and crystallization, a diluted labeled material (88 mg). EIMS *m*/*z* 142 [M]$^+$ ($^{13}C^{18}O_2$, 5.6%), 137 M$^+$ (65.4), 122 [M − $H_2{}^{18}O$]$^+$ (8.4), 119 (M − H_2O)$^+$ (100).

General Feeding Procedure for Strain Sg a15. Shake cultures with 100 to 400 mL of culture medium containing Zein (1%), peptone (0.1%), and $Mg_2SO_4 \cdot 7H_2O$ (0.1%) at pH 7.3 were inoculated with 5% of a well-grown culture of Sg a15. During 3 to 4 days the labeled precursors were added in 4 to 6 portions. The cell mass was harvested by centrifugation and extracted with acetone. The extract was concentrated in vacuo and partitioned between water and ethyl acetate, and the organic phase was evaporated to give a dark brown oil (1 g/L culture). The aurachins were isolated by chromatography on Florisil, silica gel, and RP-18 silica gel, followed, if necessary, by preparative

TLC. Due to separation problems, the isolated yields were lower than expected from HPLC analysis of the crude extracts.

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Supporting Information Available: Time course of aurachin production, ^{13}C and ^{1}H NMR data of aurachins A−L (**1**−**11**), and biosynthesis schemes for *Pseudonocardia* and *Pseudomonas* quinolones are available free of charge via the Internet at http://pubs.acs.org.

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