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Isolation and Biosynthesis of Aurachin P and 5-Nitroresorcinol from *Stigmatella erecta*

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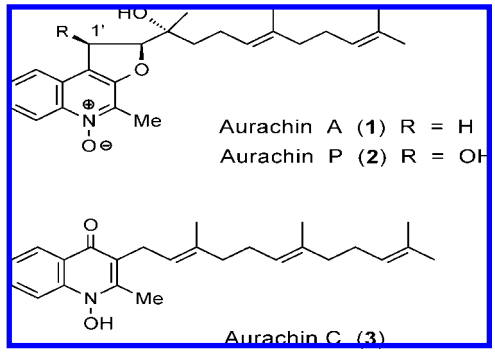
The isolation of aurachin P (**2**) from *Stigmatella erecta* strain Pd e32 is described. Spectroscopic data, in particular NMR data, indicate it is 1′-hydroxyaurachin A with a 1′*R*,2′*S*,3′*R* relative configuration. In addition, a further compound, 5-nitroresorcinol (**4a**), was isolated and identified as a novel natural product. Feeding of ^{13}C- and ^{15}N-labeled precursors indicated this was synthesized solely from glucose and ammonia. To account for the labeling pattern, phloroglucinol (**8**) is postulated as an intermediate branching off from 3-dehydroquinate (**7**) in the shikimate pathway.

The myxobacterial genus *Stigmatella* comprises two species, *S. aurantiaca* and *S. erecta* (*Podangium erectus*).[1] While *S. aurantiaca* has been extensively investigated with regard to its rich secondary metabolism,[2,3] morphogenesis,[4] and underlying genetics,[2,5] very little is known about the biosynthetic potential of *S. erecta*. However, preliminary work in our group identified *S. erecta* strains as producers of myxalamids, myxothiazole, rhizopodin, myxochromid S, and a new natural product, 5-nitroresorcinol.[6] Thus *S. erecta* seems to be as productive as *S. aurantiaca*, but has been unaccountably neglected as a research organism in the past.

Hence, *Stigmatella erecta* strain Pd e32, a producer of antifungal activity, was selected for a detailed investigation. HPLC/MS analysis of the culture extract revealed the presence of myxothiazole A, several myxalamids, several aurachins, and 5-nitroresorcinol, compounds that are also produced by *Stigmatella aurantiaca* strains.[5,7] A study of shake cultures based on a medium of soybean meal and yeast extract showed that the yields of the individual compounds strongly depend on the various additives. Thus, media with increased soybean meal content favor myxalamid production, whereas addition of Probion favors aurachins, myxothiazole, and 5-nitroresorcinol. Thus, an 80 L fermentation batch employing the latter medium in the presence of XAD-16 adsorber resin was run to yield 9.9 g of extract. This extract was separated by consecutive chromatography over Sephadex LH-20, silica gel, and RP-18 silica gel to give, in addition to myxothiazole A (476 mg), a new aurachin variant, P (**2**, 731 mg), and aurachin C (**3**, 132 mg). Fractions containing small amounts of myxalamids were discarded, while 5-nitroresorcinol was inadvertently lost in the first extraction step due to its solubility in water at neutral pH. A second fermentation batch of 12 L was therefore extracted under acidic conditions. The extract gave relatively pure 5-nitroresorcinol (108 mg) as an isolated late-eluting peak using Sephadex LH-20 chromatography.



The NMR and UV data indicate aurachin P (**2**) is closely related to aurachin A (**1**) and possesses one additional oxygen atom according to the HREIMS. This is located as an extra hydroxyl group on C-1′ according to the low-field shifts of the ^{13}C and ^{1}H signals from 29.2 to 71.7 and from 3.55 to 5.69 ppm, respectively. On the basis of the relative configuration of aurachin A,[7a] NOE data indicated aurachin P had the relative 1′*R*,2′*S*,3′*R* configuration (Figure 1). Aurachin P is currently the highest oxidized variant of all the aurachins so far isolated and thus the end point of the biosynthetic sequence.[7a]

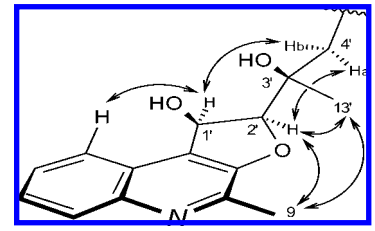
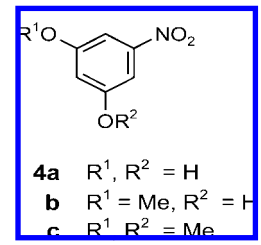


Figure 1. Selected NOEs and preferred conformation of aurachin P (**2**) in $CDCl_3$.

5-Nitroresorcinol (**4a**) was first observed in *S. erecta* strain Pd e21 due to its mild antibacterial activity.[6] Later it was detected by HPLC/MS in culture extracts of strain Pd e32 and *S. aurantiaca*, strain Sg a15. In addition to its elemental composition and melting point, 5-nitroresorcinol was characterized from the presence of four ^{13}C and two ^{1}H NMR signals, the latter showing the expected 2.1 Hz *meta* coupling. In the search for structurally related metabolites, the 5-nitroresorcinol mono- and dimethyl ethers **4b** and **4c** were prepared by methylation with diazomethane. Cochromatography of crude extracts with these reference samples showed that they were absent in strain Pd e32, whereas both were present in comparable amounts in *S. aurantiaca* strain Sg a15.[7a]



5-Nitroresorcinol (**4a**), by analogy with natural 1,3-polyphenols, may be derived from a tetraketide as shown for its close relative phloroglucinol (**8**).[8] On the other hand, it might also be formed from 6-amino-6-desoxyglucose by 1,6-cyclization and elimination of two water molecules, followed by oxidation of the amino group. Indeed, feeding *S. erecta* with 90% [$^{13}C_6$]glucose produced fully labeled 5-nitroresorcinol, albeit with unexpectedly complex coupling patterns in the ^{13}C NMR spectrum. Differences in the estimate of

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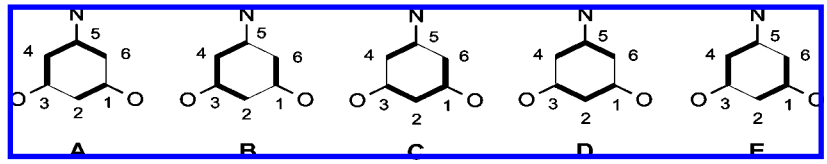
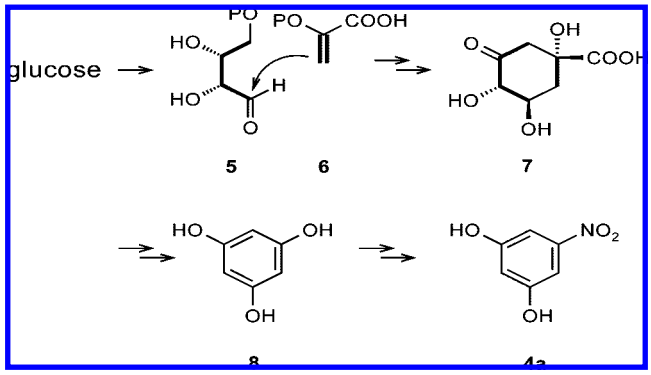
Scheme 1. Selection of Possible Isotopomers **A**−**E** of 5-Nitroresorcinol (**4a**) after Feeding of [$^{13}C_6$]Glucose



the isotope enrichments of ca. 5% from the ^{13}C signal intensities (integrals of the central line and satellites) and of 8−10% from the ^{13}C satellites in the ^{1}H NMR spectrum probably arise from differences in the relaxation behavior of the various species. As a result of the symmetry in the molecule, only four groups of signals are observed, for C-1/C-3, C-4/C-6, C-2, and C-5. All groups contain doublets indicating the presence of two adjacent ^{13}C atoms, as well as double-doublets or triplets indicating three adjacent ^{13}C atoms (Figure 1). This may be rationalized by the presence of five isotopomers arising from the condensation of a C_4 and a C_2 building block (**A**, **B**, **C**) or two C_3 building blocks (**D**, **E**) (Scheme 1). Inspection of the ESIMS (negative ions) indicated a significant incorporation of only two ^{13}C atoms (*m/z* 156, M + 2 − H, 5.1%) and four ^{13}C atoms (*m/z* 158, M + 4 − H, 3.5%). Thus, a [3 + 3] condensation leading to isotopomers **D** and **E** can be excluded. In addition, the observed *gem/vic* C,C couplings established the presence of isotopomers **A** (C-5 ddd), **B** (C-5 dd), and **C** (C-2, ddd), with the consequence that the nitrogen must have been introduced at an intermediate stage that possessed 3-fold symmetry, e.g., phloroglucinol (**8**). It is well known that phloroglucinol reacts readily with aqueous ammonia to give 5-aminoresorcinol.[9] Indeed, ^{15}N is incorporated into 5-nitroresorcinol upon feeding ammonium chloride or [γ-^{15}N]glutamine (18−19% incorporation) or [α-^{15}N]glutamine (10%). The final steps in the proposed biosynthesis would be oxidation of the amino moiety to a nitro group, and, in strain Sg a15, O-methylation.[7a]

Scheme 2. Proposed Biosynthesis of 5-Nitroresorcinol (**4a**) from Erythrose-4-phosphate (**5**) and Phosphoenol-pyruvate (**6**)



Conclusion. The biosynthesis of 5-nitroresorcinol is initiated by condensation of a C_2 and a C_4 building block derived from glucose, indicating the involvement of the shikimate pathway (Scheme 2). Further, the formation of three isotopomers from fully labeled glucose requires an intermediate with 3-fold symmetry, such as phloroglucinol (**8**). Clearly, the crucial step in this sequence is the mechanism of formation of phloroglucinol from glucose, for which we propose a novel branch in the shikimate pathway requiring oxidative decarboxylation of 3-dehydroquinate (**7**) followed by elimination of water. Further feeding studies with differently labeled glucose and advanced intermediates have to be performed to prove the proposed pathway. Moreover, cloning and analysis of the biosynthetic gene cluster from *S. aurantiaca* Sg a15 may provide the molecular details of this pathway.

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 recorded on a Nicolet 20DXB FT-IR spectrometer. NMR spectra were recorded in $CDCl_3$ on Bruker ARX-400 or DMX-600 NMR spectrometers. EI and DCI mass spectra (reactant gas NH_3) were obtained on a Finnigan MAT 95 spectrometer, with high-resolution data acquired using peak matching (M/DM = 10 000). ESI mass spectra were recorded on a Q-Tof II spectrometer from Micromass, and HPLC/DAD/ESI-MS on a PE Sciex Api-2000 LC/MS with a Nucleodur C18 column (5 μm, 2 × 125 mm from Machery-Nagel) using 0.3 mL/min CH_3CN/5 mM NH_4OAc 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 on Nucleosil (column 250 × 21 mm, 7 μm, flow 18 mL/min) with a solvent of CH_3CN/H_2O (gradient) and diode array detection. Analytical TLC used TLC aluminum sheets silica gel Si 60 F_{254} (Merck), with UV detection at 254 nm. Red to violet spots were observed on staining with vanillin/H_2SO_4 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 erecta* (*Podangium erectus*), Strain Pd e32. A 100 L bioreactor with a circulating pump stirrer system (Giovanola Frères, Monthey, Switzerland) was charged with 80 L of culture medium containing 0.8% starch, 0.2% yeast extract, 0.2% soybean meal, 0.5% Probion, 0.1% $CaCl_2 \cdot 2H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, 8 mg/L NaFe-EDTA, and 1 L of Amberlite XAD-16 adsorber resin. After sterilization 0.2% glucose was added and inoculated with 2 L of shake culture grown in the same medium. The aeration rate was adjusted to 150 L/h, and the agitation was 150 rpm. The fermenter was kept at 30 °C, and the initial pH of 6.8 was adjusted to >6.8 by addition of 5% KOH solution. After 4 days HPLC analysis indicated the presence of aurachin P (10.1 mg/L), myxothiazole (8.0 mg/L), myxalamids (<1 mg/L), aurachin C (4.4 mg/L), and 5-nitroresorcinol (8.0 mg/L).

Isolation of Aurachins C (3) and P (2). The XAD adsorber resin was collected with a sieve, washed with H_2O, transferred to an open column, and eluted with MeOH. The eluate was concentrated in the H_2O phase and extracted with EtOAc to give 9.9 g of a dark oil, which was fractionated on Sephadex (column 7 × 64 cm) with MeOH. According to TLC, fraction 2 (5.2 g) contained aurachins and some myxothiazol; fraction 3 (476 mg), myxothiazole and some myxalamids. Fraction 2 was further separated by chromatography on silica gel 100 (column 4.8 × 24 cm) with a *tert*-butyl methyl ether/MeOH gradient (98:2 to 90:10). The aurachin-containing fraction (1.73 g) on RP-18 chromatogagphy (column 4 × 40 cm, MeOH/H_2O, 85:15, 4 batches) gave aurachin P (731 mg) and aurachin C (132 mg).

Isolation of 5-Nitroresorcinol (4a). XAD adsorber resin and culture broth from a 10 L fermentation batch run as described above were collected with a sieve. The culture broth was acidified to pH 4.5 and extracted with 200 mL of adsorber resin with gentle stirring for 4 h. The combined MeOH eluates from the two adsorber resin portions were processed as described above to give 2.2 g of crude extract. This was separated on a Sephadex LH-20 column (7 × 64 cm, detection 313 nm) with MeOH. Relatively pure 5-nitroresorcinol (108 mg) was eluted as an isolated peak at the end of the chromatography and further purified by PDC (53 mg).

Aurachin P (2): colorless solid; R_f 0.70 (CH_2Cl_2/acetone/MeOH, 75:20:5); $[\alpha]_D^{20}$ −70.5 (*c* 3.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (4.28), 236sh (4.30), 248 (4.41), 302 (3.65), 315 (3.72), 350 (3.80), 364 (3.86); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.99 (1H, dbr, J = 8.8 Hz, H-8), 7.66 (1H, dbr, J = 8.0 Hz, H-5), 7.27 (1H, tbr, J = 7.5 Hz, H-6), 7.13 (1H, ddd, J = 8.5, 6.9, 1.1 Hz, H-7), 6.35 (1H, sbr, HO-1′), 5.69 (1H, tbr, J = 5.5 Hz, H-1′), 5.24 (1H, tbr, J = 7.0 Hz, H-6′), 5.11 (1H, tqq, J = 6.8, 1.3, 1.3 Hz, H-10′), 4.33 (1H, d, J = 6.1 Hz, H-2′), 4.21 (1H, sbr, OH-3′), 2.58 (3H, s, H_3-9), 2.36 (3H, m, H-5b′), 2.22 (1H, m, H-5a), 2.09 (3H, m, H-4b, H_2-9′), 2.01 (2H, dd, H_2-8′), 1.90 (1H, ddd, J = 13.2, 11.2, 5.5 Hz, H-4a), 1.70 (3H, sbr, H_3-14′), 1.69 (3H, sbr, H_3-12′), 1.61 (3H, sbr, H_3-15′), 1.56 (3H, s, H_3-13′); strong NOE's H-1′/H-5 and H-4'b, H-2′/H-4'a, H-5′a and H_3-13′; ^{13}C NMR ($DCCl_3$, 75 MHz) δ 152.3 (C-3), 137.1 (C-2), 137.0 (C-8a), 135.8(C-

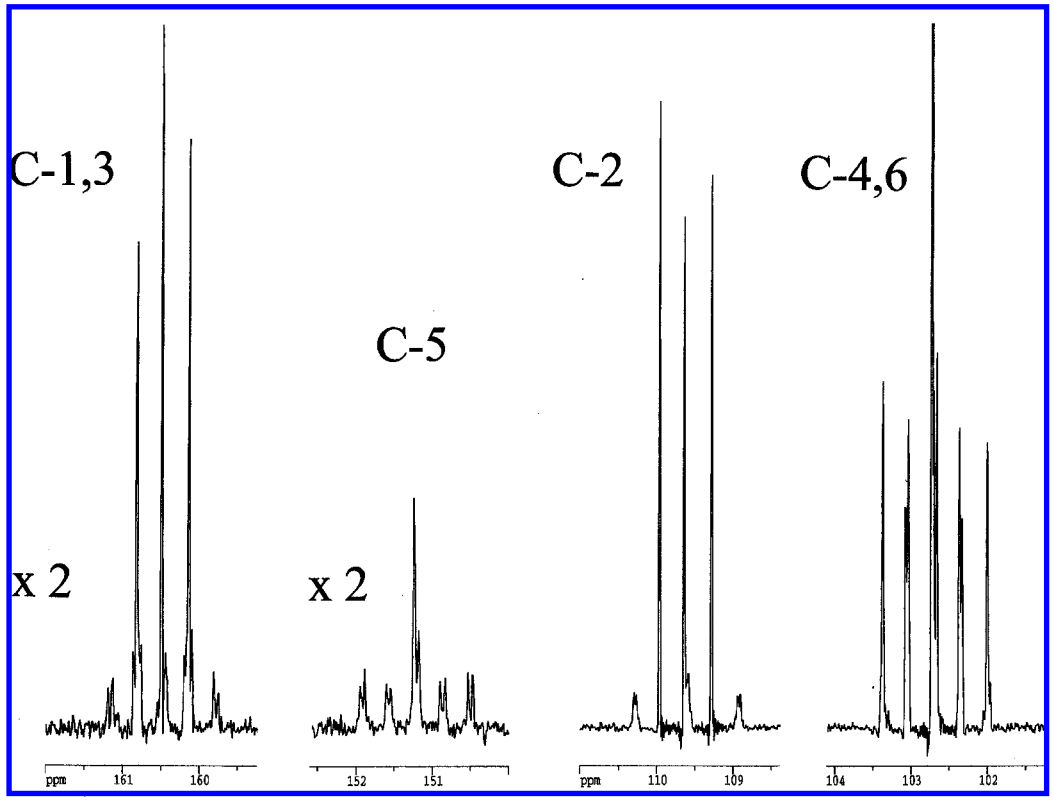


Figure 2. 100 MHz ^{13}C NMR spectrum of 5-nitroresorcinol (**4a**) after feeding of 90% [$^{13}C_6$]glucose.

7′), 131.5 (C-11′), 128.8 (C-6), 127.5 (C-7), 124.3 (C-10′), 124.0 (C-6′), 123.5 (C-4a), 123.0 (C-5), 120.8 (C-4), 119.7 (C-8), 91.9 (C-2′), 74.2 (C-3′), 71.7 (C-1′), 39.8 (C-4′), 39.4 (C-8′), 26.8(C-9′), 25.7 (C-12′), 24.2 (C-13′), 22.1 (C-5′), 17.7 (C-15′), 16.1 (C-14′), 12.1 (C-9); EIMS *m/z* 411 M^+ (6%), 394 (16), 326 (26), 200 (53), 199 (67), 184 (52), 183 (64), 182 (64), 69 (100); HREIMS *m/z* 411.2408 (calcd for $C_{25}H_{33}NO_4$, 411.2410).

5-Nitroresorcinol (4a): light yellow crystals from H_2O, mp 158 °C; t_R 6.5 min; R_f 0.44 (CH_2Cl_2/acetone/MeOH, 90:8:2); UV (MeOH) λ_{max} (log ϵ) 220 (4.08), 291 (3.57), 350sh (3.00); after addition of 1 drop of NH_3 233 (4.07), 314 (3.48), 400sh (2.95); ^1H NMR (MeOH-d_4, 400 MHz) δ 7.12 (2H, d, J = 2.1 Hz, H-4, H-6), 6.62 (1H, t, J = 2.1 Hz, H-2); ^{13}C NMR (MeOH -d_4, 75 MHz) δ 160.5 (C-1, C-3), 151.2 (C-5), 109.8 (C-2), 102.8 (C-4, C-6); ESIMS *m/z* 154 $(M - H)^-$, 309 $(2M - H)^-$.

Labeling Studies. Labeled Precursors. [$^{13}C_6$]Glucose (90 atom %) and $^{15}NH_4Cl$ (99 atom %) were purchased from MSD Isotopes; [2-^{15}N]glutamine and [5-^{15}N]glutamine (99 atom %), from Cambridge Isotope Laboratories.

Feeding of Strain Pd e32 with (a) Glucose and (b) Different Nitrogen Sources. (a) For shake cultures (40 mL), the medium described above, but without glucose, was supplemented with HEPES buffer (1%), pH adjusted to 7.4, sterilized, and inoculated. After 47 h 120 mg of [$^{13}C_6$]glucose and after 67 h 80 mg were added. After 98 h cell mass and adsorber resin were collected by centrifugation and extracted with MeOH, and the supernatant was acidified and extracted with EtOAc. The combined extracts were evaporated, and the residue was distributed between 1 N NaOH and EtOAc. The aqueous phase was acidified and re-extracted with EtOAc to give 44 mg of a refined extract, which on HPLC separation gave 4.5 mg of **4a**: ^1H NMR (MeOH-d_4, 400 MHz) δ 7.12 (2H, d, J = 2.1 Hz, H-4, H-6), ^{13}C-satelites (m, J = 166 Hz, ca. 13%), 6.62 (1H, t, J = 2.1 Hz, H-2), ^{13}C-satelites (m, J = 160 Hz, 10%); ^{13}C NMR (methanol-d_4, 100 MHz) δ 160.50 (s), 160.49 (d, J = 68 Hz, dm, J = 68 Hz), 160.47 (td, J = 68; 6 Hz) (C-1, C-3), 151.24 (s), 151.22 (dd, J = 71; 7 Hz), 151.20 (td, J = 71; 7 Hz) (C-5), 109.65 (s), 109.62 (d, J = 71 Hz, tbr, J = 71 Hz) (C-2), 102.73 (s), 102.71 (d, J = 67 Hz, dd, J = 71; 3.5 Hz, dd, J = 67; 71 Hz) (C-4, C-6), for relative intensities see Figure 2; ESIMS *m/z* 154 $(M - H)^-$ (100%), 155 (10,0%), 156 (5,1%), 157 (2.8%), 158 (3.5%). (b) Three shaken cultures (40 mL) were supplemented in four portions with (I) ammonium chloride (30 mg, 90% ^{15}N), (II) glutamine (30 mg, 90% ^{15}N-γ), and (III) glutamine (30 mg, 90% ^{15}N-α) in four to five portions. After 98 h 5-nitroresorcinol (ca. 0.5 mg) was isolated as described above and analyzed by EIMS (*m/z* 155 (M^+, 100%), 156 (I) 10.5%; (II) 18.6%; (III) 18.9%).

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