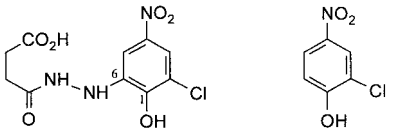
Stephanosporin, a "Traceless" Precursor of 2-Chloro-4-nitrophenol in the Gasteromycete *Stephanospora caroticolor***

Martin Lang, Peter Spiteller, Veronika Hellwig, and Wolfgang Steglich*

Dedicated to Professor Siegfried Hünig on the occasion of his 80th birthday

The carrot truffle, *Stephanospora caroticolor* (BERK.) PAT., is a rare Gasteromycete. It occurs in deciduous forests and mixed wooded areas in loamy, calcareous soil. The fruit bodies are tubers of 1 to 3 cm in diameter that mature underground. They may be recognized by their bright orange appearance on places where the soil is partially removed. The interior of the mushroom (gleba) is also intensively orange colored.

Extraction of the fruit bodies of *St. caroticolor* with methanol gives a yellow solution in which two pigments **1** and **2** (Scheme 1) can be detected by HPLC.[1] Compound **2**



1 **2**

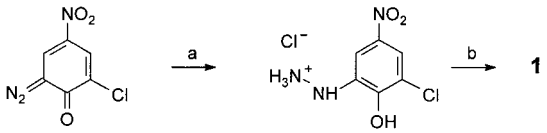
Scheme 1. Chloronitroarenes from *St. caroticolor*.

was identified as 2-chloro-4-nitrophenol from the spectroscopic data and by direct GC/MS comparison.[2] Analysis of the extract by atomic absorption spectroscopy (AAS) and UV/Vis spectrometry indicate that **2** occurs mainly as its potassium salt, which explains the yellow color of the solution. 2-Chloro-4-nitrophenol (**2**) is a fungicide[3a] which was used previously for seed protection[3b] and the conservation of leather.[3c] The compound has not yet been described as a natural product.

2-Chloro-4-nitrophenol (**2**) was always present in the raw extracts of fruit bodies that were either stored in the refrigerator, dried, or crushed before extraction. The amount of **2** can exceed 2% of the dry weight. In contrast, the extraction of young uninjured fruit bodies with methanol immediately after harvesting yields only pigment **1**, which must be isolated instantly by preparative HPLC in order to avoid decomposition to **2**.

The ^{1}H NMR spectrum of **1** shows only two doublets (^4J(H,H) = 3 Hz) in the aromatic region. Thus, in comparison to **2**, the pigment must bear an additional substituent at C6. In addition, a CH_2CH_2 group can be recognized which, according to the HMBC spectrum, is flanked by two COX groups. The molecular mass of **1** was determined as $M_r = 303$ (ref. to ^{35}Cl) by LC/(−)-ESI-MS.[4] The key ion at *m*/*z* 116 indicates that one of the carbonyl groups of the succinic acid unit is present as an amide. These facts allow the structure **1** to be deduced for the pigment, for which the name stephanosporin is proposed (Scheme 1). Like the phenol **2**, stephanosporin is present in the mushroom mainly in the form of its potassium salt, which explains the orange color of the fruit bodies.

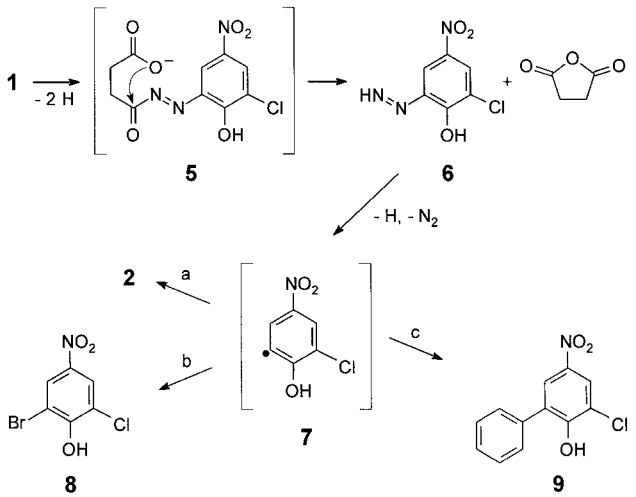
Structure **1** was confirmed by synthesis (Scheme 2). It started from 6-chloro-4-nitro-1,2-quinone-2-diazide[5a] (**3**), which had already been prepared by Griess.[5b] Reduction of **3** with tin(II) chloride afforded the hydrochloride salt of 2-chloro-6-hydrazino-4-nitrophenol (**4**) which underwent reaction with succinic anhydride and triethylamine to furnish stephanosporin (**1**).



3 **4**

Scheme 2. Synthesis of stephanosporin (**1**). a) $SnCl_2$, HCl, HOAc, 0 °C, 77%; b) succinic anhydride, NEt_3, MeOH, 25 °C, 70%.

The oxidative transformation of stephanosporin (**1**) into 2-chloro-4-nitrophenol (**2**) is an enzymatic process induced by injury of the fruit body. It may be explained by a reaction cascade which starts by oxidation of **1** to the acyldiazene **5**. Intramolecular attack of the carboxylate ion at the activated carbonyl group[6, 7a] then leads to the formation of succinic anhydride and 2-chloro-6-diazenyl-4-nitrophenol (**6**). Compounds of this type are known to decompose into nitrogen and an aryl radical by a one-electron transfer mechanism.[8] In the last step, the radical **7** is converted into 2-chloro-4-nitrophenol (**2**) by abstraction of a hydrogen atom (Scheme 3, route a).



5 **6** **7** **8** **9**

Scheme 3. Generation of 2-chloro-4-nitrophenol (**2**), 2-bromo-6-chloro-4-nitrophenol (**8**), and 3-chloro-5-nitro-2-biphenylol (**9**) from stephanosporin (**1**): a) enzymatic in the mushroom, or CAN (3 equiv), MeOH, 5 min, RT, 77%; b) CAN (3 equiv), $CBrCl_3$/MeOH 2/1, 5 min, RT, 59%; c) CAN (3 equiv), benzene/MeOH 2/1, 5 min, RT, 20%. Concentration of **1** 25 mM each. CAN = cerium(IV) ammonium nitrate.

[*] Prof. Dr. W. Steglich, Dipl.-Chem. M. Lang, Dipl.-Chem. P. Spiteller, Dr. V. Hellwig
Department Chemie der Universität
Butenandtstrasse 5–13, 81377 München (Germany)
Fax: (+49) 89-2180-7756
E-mail: wos@cup.uni-muenchen.de

[**] We thank the Deutsche Forschungsgemeinschaft for financial support (SFB 369).

  1433-7851/01/4009-1704 $ 17.50+.50/0

The same degradation can be achieved in vitro by oxidation of **1** with salts such as cerium(IV) ammonium nitrate, iron(III) nitrate, or manganese(III) acetate in methanol or tetrahydrofuran.[9] In these cases, succinic anhydride was detected by GC/MS. When the oxidation was carried out in the presence of bromotrichloromethane (route b), 2-bromo-6-chloro-4-nitrophenol (**8**) was obtained, while benzene (route c) furnished 3-chloro-5-nitro-2-biphenylol (**9**). These results are in accordance with the intermediate formation of an aryl radical (Scheme 3). The loss of the side chain in stephanosporin is comparable with the use of "traceless" hydrazide anchors in solid-phase synthesis.[10]

Since 2-chloro-4-nitrophenol (**2**) has antibiotic properties, its enzymatic generation from precursor **1** by injury of the fruit bodies may serve the mushroom as a defense mechanism. It is interesting that *N*-aryl-*N*′-succinylhydrazines of the stephanosporin type have been patented as fungicides.[11, 12] Moreover, an *N*-succinylphenylhydrazine group plays a crucial role in the action of the antibiotic spinamycin.[7]

In addition to **1** and **2**, *St. caroticolor* contains 4-hydroxyacetanilide (paracetamol), 4-amino-2-chlorophenol, and 4-nitrophenol. The compounds were identified by GC/MS analysis and by comparison with authentic samples. Further investigations are required to clarify their possible role in the biosynthesis of **1**.

Interestingly, we have also found 4-nitrophenol and traces of 2-chloro-4-nitrophenol in the orange-yellow resupinate fruit bodies of *Lindtneria trachyspora* (Bourd. & Galz.) Pil.[13] This observation supports Oberwinkler and Horak's[14] inclusion of *Stephanospora* and *Lindtneria* into their own family Stephanosporaceae. It is unknown, however, whether the nitrophenols in *Lindtneria* are generated from "traceless" precursors.

Experimental Section

Isolation of **1** (dipotassium salt): Two fruit bodies of *St. caroticolor* were detected on September 10th 1999 in a mixed forest near the Pupplinger Au, Wolfratshausen (Oberbayern). The fruit bodies (fresh weight approximately 3 g) were extracted instantly with MeOH (20 mL). The extract was pre-purified by solid-phase extraction (RP18) and the solvent removed in vacuo. The residue was dissolved in MeOH (0.5 mL) and purified by preparative HPLC (column: 25 × 2 cm, 7 μm, RP18) using MeOH/H_2O as the solvent system (linear gradient: from 100% water to 100% MeOH in 40 min); R_t = 15.9 min; yield: about 3 mg. LC/(−)-ESI-MS: *m*/*z* (%): 304 (28), 302 (100) $[M-H]^-$; LC/(−)-ESI-MSMS (parent ion *m*/*z*: 302, 20 eV): *m*/*z* (%): 302 (0.2) $[M-H]^-$, 266 (9) $[M-H-HCl]^-$, 202 (28) [2-chloro-6-hydrazino-4-nitrophenol − H]$^-$, 186 (10), 156 (9) [2-chloro-6-hydrazinophenol − H]$^-$, 154 (9), 116 (100) [succinic acid monoamide − H]$^-$, 98 (16) [succinimide − H]$^-$; ^{1}H NMR (600 MHz, CD_3OD, reference: δ = 3.31, 25 °C): δ = 2.59 (m, 2H, $CH_2CO_2^-$), 2.60 (m, 2H, CH_2CONH), 7.42 (d, ^4J(H,H) = 3.0 Hz, 1H, H5), 7.79 (d, ^4J(H,H) = 3.0 Hz, 1H, H3); ^{13}C NMR (151 MHz, CD_3OD, reference: δ = 49.0, 25 °C): δ = 30.8 ($CH_2CO_2^-$), 32.0 (CH_2CONH), 106.0 (C5), 119.7 (C3), 121.2 (C2), 135.7 (C4), 140.8 (C6), 158.9 (C1), 174.8 (CONH), 178.1 (CO_2^-). **2** (potassium salt): R_t = 30.5 min.

4: A solution of $SnCl_2 \cdot 2H_2O$ (6.77 g, 30.0 mmol) in concentrated hydrochloric acid (7 mL) was added dropwise at 0 °C to a solution of **3**[5a] (2.99 g, 15.0 mmol) in acetic acid (15 mL) and concentrated hydrochloric acid (15 mL). The precipitated product was filtered off, washed several times with half concentrated hydrochloric acid, and then with diethyl ether. Drying the product in a vacuum yielded 2.77 g of **4** (11.5 mmol, 77%) as a greyish brown solid. Decomposition on heating; ^{1}H NMR (300 MHz, CD_3OD, 25 °C): δ = 7.88 (d, ^4J(H,H) = 2.6 Hz, 1H), 8.00 (d, ^4J(H,H) = 2.6 Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD, 25 °C): δ = 109.3, 120.3, 121.5, 136.3, 141.8, 149.2.

1: Triethylamine (0.46 mL, 3.3 mmol) was added dropwise to a stirred solution of succinic anhydride (0.330 g, 3.30 mmol) and 2-chloro-6-hydrazino-4-nitrophenol hydrochloride (**4**; 0.720 g, 3.00 mmol) in MeOH (7 mL). The mixture was then acidified with 2N HCl and diluted with water (15 mL). After several hours, the yellow precipitate was filtered off, washed with water, and dried in vacuum. Yield: 0.638 g (2.10 mmol, 70%). M.p. 191–194 °C (decomp); UV/Vis (MeOH) λ_{max} (ε): 226 (11925), 258 (12579), 312 (4738), 351 nm (3751, sh); ^{1}H NMR (300 MHz, CD_3OD, 25 °C): δ = 2.63 (m, 4H, CH_2CH_2), 7.55 (d, 1H, ^4J(H,H) = 2.5 Hz, H5), 7.71 (d, 1H, ^4J(H,H) = 2.5 Hz, H3); ^{13}C NMR (75 MHz, CD_3OD, 25 °C): δ = 29.5 (CH_2CO_2H), 29.7 (CH_2CONH), 106.5 (C5), 116.8 (C3), 120.5 (C2), 140.3 (C6), 142.3 (C4), 147.2 (C1), 174.6 (CONH), 176.0 (CO_2H); elemental analysis calcd for $C_{10}H_{10}ClN_3O_6$: C 39.55, H 3.32, Cl 11.68, N 13.84; found: C 39.55, H 3.31, Cl 11.47, N 13.70.

1 (dipotassium salt): A 1.0M solution of KO*t*Bu in THF (0.55 mL, 0.55 mmol) was added dropwise to **1** (76 mg, 0.25 mmol) in dry THF (1 mL). The red precipitate was filtered off, washed several times with THF and once with EtOH, and dried in vacuum. Yield: 86 mg (0.23 mmol, 90%). The spectroscopic data were in accordance with those of the natural compound.

Received: November 20, 2000 [Z16132]

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