Hydroxylated 2-Amino-3*H*-phenoxazin-3-one Derivatives as Products of 2-Hydroxy-1,4-benzoxazin-3-one (HBOA) Biotransformation by *Chaetosphaeria* sp., an Endophytic Fungus from *Aphelandra tetragona*

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The biotransformation of the phytoanticipin HBOA and its major degradation metabolites 2-hydroxy-*N*-(2-hydroxyphenyl)acetamide (**7**) and *N*-(2-hydroxyphenyl)acetamide (**8**) by *Chaetosphaeria* sp., an endophytic fungus isolated from *Aphelandra tetragona*, was studied. Three new metabolites could be identified as 2-amino-7-hydroxy-3*H*-phenoxazin-3-one (**12**), 2-acetylamino-7-hydroxy-3*H*-phenoxazin-3-one (**13**) and 7-hydroxy-2-(2-hydroxyacetyl)-amino-3*H*-phenoxazin-3-one (**14**). Structure elucidation of **12** and **13** was performed by MS, ^{1}H, ^{13}C NMR and 2D NMR techniques and confirmed by chemical transformation.

Introduction

The benzoxazinones are an important group of secondary metabolites occurring in Gramineae, Acanthaceae, Ranunculaceae and Scrophulariaceae. Their role as defence compounds towards pests like bacteria, fungi and insects is documented for different cereals (Gramineae) including corn, wheat and rye (Niemeyer, 1988).

In *Aphelandra* sp. plants the lactams 2-hydroxy-1,4-benzoxazin-3(2*H*)-one (HBOA, **1**), 2-hydroxy-7-methoxy-1,4-benzoxazin-3(2*H*)-one (HMBOA, **2**) and hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3(4*H*)-one (DIBOA, **3**), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3(4*H*)-one (DIMBOA, **4**) (Fig. 1) and their corresponding C-2 *O*-glucosides are accumulated (Baumeler *et al*., 2000). After tissue damage or pathogen attack the toxic benzoxazolin-2(3*H*)-one, (BOA, **5**) or 7-methoxybenzoxazolin-2(3*H*)-one, (MBOA, **6**) (Fig. 1) are released.

Endophytic fungi, living inside the tissue without causing any disease, were isolated from shoots and roots of *Aphelandra tetragona* (Werner *et al*., 1997). During a recent study on the biotransformation of HBOA and BOA by several endophytic strains two major degradation products of HBOA were identified, namely 2-hydroxy-*N*-(2-hydroxyphenyl)acetamide (**7**) and *N*-(2-hydroxyphenyl)-acetamid (**8**) (Scheme 1) (Zikmundová *et al*., 2002). The degradation of HBOA was accompanied by the production of 2-amino-3*H*-phenoxazin-3-one (**10**) and 2-acetylamino-3*H*-phenoxazin-3-one (**11**), also known as the naturally occurring antibiotics questiomycin and *N*-acetylquestiomycin, respectively. They belong to the important group of actinomycin analogues and were already described as products of BOA transformation by microorganisms (Friebe *et al.*, 1996; 1998; Gagliardo and Chilton, 1992).

1 R^1 = H R^2 = H HBOA
2 R^1 = OMe R^2 = H HMBOA
3 R^1 = H R^2 = OH DIBOA
4 R^1 = OMe R^2 = OH DIMBOA

5 R = H BOA
6 R = OMe MBOA

Fig. 1. Structures of benzoxazinoids occurring in *Aphelandra* sp.

In the case of *Chaetosphaeria* sp. the biotransformation of HBOA and also of its main metabolites **7** and **8** leads additionally to the production of rose-violet pigments. Here we report the isolation and structure characterisation of three hydroxylated aminophenoxazinone derivatives, which are responsible for the brilliant rose-violet

colour of the medium of *Chaetosphaeria* sp. after incubation with HBOA or its two main metabolites **7** and **8**.

Experimental

General

HPLC: HP 1100 HPLC system (Hewlett-Packard, Palo Alto, CA 94304, USA). HPLC conditions: Macherey-Nagel column Nucleosil 100-5 C_{18} (250/3 mm); flow rate 0.4 ml/min; DAD detector setting at 280 and 400 nm; mobile phase: A = H_2O + 0.05% TFA, B = MeOH + 0.05% TFA; gradient: 0–1 min from 3 to 20% B, 1–20 min from 20 to 100% B, 20–25 min 100% B.

HPLC-APCI-MS: The same HPLC system was used as described above. The APCI-MS detector was interfaced directly to the output of the UV-detector. APCI-MS was carried out with a Bruker ESQUIRE-LC quadrupole ion-trap instrument (Bruker Daltonik, Bremen, Germany) connected to an APCI ion source (Hewlett Packard).

ESI-MS: Finnigan TSQ 700 mass spectrometer.

NMR: Bruker AMX-600 spectrometer (^{1}H at 600 MHz, ^{13}C at 150 MHz); chemical shifts in ppm (δ scale). All spectra were run in TFA-*d* with TMS as internal standard.

Commercially available cotton-wool, silica gel Merck 60 (230–400 mesh) for column chromatography, TLC plates Silica-gel 60 F_{254} Merck were used during the isolation of the pigments. *N*-(2-hydroxyphenyl)acetamide (compound **8**) was purchased from Fluka. Synthetic HBOA was kindly provided by Prof. Sicker (Institute of Organic Chemistry, University of Leipzig, Germany). 2-Hydroxy-*N*-(2-hydroxyphenyl)acetamide (compound **7**) was prepared according to Chatterjee *et al.*, 1990.

Fungal material and cultivation

Chaetosphaeria sp., isolated as endophyte from *Aphelandra tetragona* (Vahl) Nees (Acanthaceae) (Werner *et al.*, 1997), was maintained on malt-extract agar. For isolation of the pigments 2.5 l of liquid Czapek-Dox Broth medium (Sigma) (pH 5.7) were supplemented with compound **8** at a final concentration of 1.5 mM (stock solution 0.1 M in EtOH) and inoculated with mycelial plugs (4 × 4 mm square) from the margins of the agar plates. The incubation was carried out in the dark at 25 °C on a rotary shaker (100 rpm) for 14 days.

Biotransformation of HBOA and its metabolites **7** and **8** were also monitored in 50-ml Erlenmeyer flasks and cultivation was carried out under the same condition as described above. In this case the final concentration of the compounds in the medium was 1 mM. Fungi in the medium without any supplements served as control. The stability of the added compounds in the medium was tested in separate uninoculated flasks.

Samples for HPLC analysis were taken at daily intervals from the medium (2 ml). After pH adjusting to 3 with aqueous HCl (0.5 M) the solution was applied to an Extrelut® column (Merck) and eluted with ethyl acetate. The extract was concentrated *in vacuo*, the residue dissolved in 1 ml of MeOH and analysed by HPLC and HPLC-MS.

Extraction and isolation

After 14 days of incubation with compound **8** (1.5 mM) the fungal culture of *Chaetosphaeria* sp. was worked up. Cultivation medium (2.5 l) and mycelial plugs were applied on a column filled with pressed cotton-wool (10 × 15 cm) and eluated with deionized water. A first dark brown fraction containing also the unchanged compound **8** was removed. After that started the elution of the rose-violet fraction, which was evaporated *in vacuo*. The residue was acidified with MeOH saturated with HCl, evaporated again, dissolved in acetone and purified by CC on silica gel. The column was eluted consecutively with $CHCl_3$:acetone (10:2 v/v) and $CHCl_3$:acetone:MeOH (10:2:1 v/v/v) to yield compound **13** (57 mg after recrystallization from MeOH) as brick crystals. Compound **12** (18 mg of dark violet powder) was removed from the column with $CHCl_3$:acetone:MeOH (10:5:1 v/v/v).

2-Amino-7-hydroxy-3H-*phenoxazin-3-one* (**12**)

TLC: R_f: 0.53 ($CHCl_3$:acetone:MeOH, 10:2:1 v/v/v);
R_f: 0.73 ($CHCl_3$:acetone:MeOH:Et_3N, 10:2:4:1 v/v/v); HPLC-UV: R_t: 17.5 min; λ_{max}: 232, 464 nm, λ_{min}: 211, 341 nm; ^{1}H and ^{13}C NMR: see Table I; ESI-MS: 229 ([*M*+H]$^+$).

*2-Acetylamino-7-hydroxy-3*H*-phenoxazin-3-one* (**13**)

TLC: R_f: 0.62 ($CHCl_3$:acetone:MeOH, 10:2:1 v/v/v); R_f: 0.62 ($CHCl_3$:acetone:MeOH:Et_3N, 10:2:4:1 v/v/v/v); HPLC-UV: R_t: 19.8 min; λ_{max}: 243, 464 nm, λ_{min}: 208, 318 nm; ^1H and ^{13}C NMR: see Table I; APCI-MS: 271 ($[M+H]^+$); MS/MS: 229 ($[M-CH_2=C=O+H]^+$).

Chemical transformation of the isolated compound **12** *by acetylation to* **13** *and further methylation to* **15**

Compound **12** (10 mg) was dissolved in a mixture of acetic acid anhydride (10 ml) and pyridine (3 ml). The mixture was heated for 30 min at 80 °C and after stirring for 1 h at r.t. evaporated to dryness *in vacuo*. The residue was dissolved in MeOH saturated with gaseous NH_3, heated for 2 h at 60 °C and evaporated to yield **13** almost quantitatively (HPLC). The residue was dissolved in DMF (5 ml) and K_2CO_3 (200 mg) and dimethylsulfate (150 μl) were added. The mixture was stirred for 1 h at r.t. and directly purified by CC on silica gel (consecutively with CH_2Cl_2 and CH_2Cl_2:MeOH:Et_3N, 10:2:0.5 v/v/v). Compound **15** (7 mg) was obtained as orange crystals after evaporation *in high vacuo* and washing with benzene.

*2-Acetylamino-7-methoxy-3*H*-phenoxazin-3-one* (**15**)

TLC: R_f: 0.82 ($CHCl_3$:MeOH, 9:1 v/v); HPLC-UV: R_t: 22.2 min; λ_{max}: 243, 455 nm; λ_{min}: 211, 315 nm; ^1H and ^{13}C NMR: see Table I; APCI-MS: 285 ($[M+H]^+$); MS/MS: 243 ($[M-CH_2=C=O+H]^+$).

*7-Hydroxy-2-(2-hydroxyacetyl)amino-3*H*-phenoxazin-3-one* (**14**)

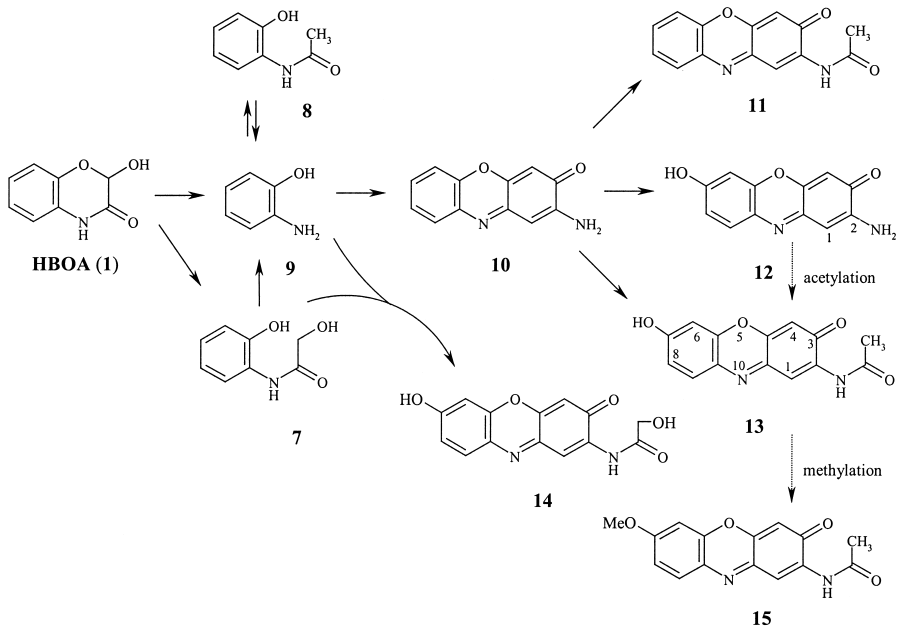
HPLC-UV: R_t: 18.8 min; λ_{max}: 242, 468 nm, λ_{min}: 208, 325 nm; APCI-MS: 287 ($[M+H]^+$); MS/MS: 229 (100, $[M-CH_2=C=O+H]^+$), 269 (10, $[M-H_2O+H]^+$).

Results and Discussion

The incubation of *Chaetosphaeria* sp. with HBOA as well as with its two main metabolites **7** and **8** led to the release of rose-violet pigments into the medium. During the preparation of the samples for the HPLC analysis the pH dependence of this colour was observed. At the range of pH 3–7 the colour was gold-yellow, whereas at higher pH values rose-violet. Also a specific adsorption of these pigments on cotton-wool, cellulose or filter paper was observed. This interesting specificity was used for their isolation from the cultivation medium. To obtain the pigments in preparative amount a large-scale cultivation was undertaken. Because of its commercially availability, *N*-(2-hydroxyphenyl)acetamide (**8**) was chosen as substrate. After two weeks of cultivation two pigments were isolated.

The first pigment (**13**) (Scheme 1), which has a rose-violet colour in basic conditions, exhibits on the HPLC-UV spectrum absorption maxima at 243 and 464 nm. The shape of its absorption curve is similar to that of 2-acetylamino-3*H*-phenoxazin-3-one (**11**) (λ_{max}: 240, 404 nm) but the second absorption maximum is bathochromically shifted by 60 nm. The HPLC-APCI-MS analysis gave a quasimolecular ion ($[M+H]^+$) at *m/z* 271, which is 16 amu more than that of **11** and could correspond to its hydroxylated derivative. The MS/MS fragmentation of the parent ion ($[M+H]^+$ at *m/z* 271) gave a fragment ion at *m/z* 229 ($[M-CH_2=C=O+H]^+$), which is 16 amu more than the quasimolecular ion of 2-amino-3*H*-phenoxazin-3-one (**10**) ($[M+H]^+$, *m/z* 213). This indicates that the additional hydroxy functionality in compound **13** is localized on the aminophenoxazinone moiety. The loss of a ketene fragment ($CH_2=C=O$) from the parent ion ($[M+H]^+$ at *m/z* 271), similar to that of the MS/MS fragmentation of **11**, indicates an *N*-acetyl group in **13**. This is in accordance with the presence of a singlet signal for a $NHCOCH_3$ group in the ^1H NMR spectrum of **13** (Table I).

The second isolated compound (**12**) (Scheme 1) has a dark violet colour under basic conditions. Its HPLC-UV spectrum exhibits strong absorption maxima at 232 and 464 nm and its absorption curve has a very similar shape to that of **13**. The ESI-MS of the compound gave a quasimolecular ion ($[M+H]^+$) at *m/z* 229, which is 42 amu less than that of **13** ($[M+H]^+$, *m/z* 271) and indicates the absence of the acetyl group. Further it is 16 amu more than the quasimolecular ion of compound **10** ($[M+H]^+$, *m/z* 213), which could correspond to a hydroxy derivative of **10**. No additional fragmentation occurred in MS/MS. This pointed to the suggestion of structure **12**. The complete NMR spectra confirmed this proposal (Table I).



Scheme 1. A proposed pathway of HBOA transformation by *Chaetosphaeria* sp. Dotted arrows correspond to the chemical transformation of isolated compound **12** to **13** and **15**. Compound **11** was not detected during the experiments with *Chaetosphaeria* sp.

Table I. ^{1}H and ^{13}C NMR chemical shift assignments of compounds **12**, **13** and **15**.

C-atom	δ_C [ppm]			δ_H [ppm]	J_{HH} [Hz]	
	12	**13**	**15**	**12**[a,b]	**13**[a]	**15**[a]
1	94.8	108.5	108.3	6.73, s	8.99, s	9.02, s
2	144.0	141.9	142.0	–	–	–
3	182.6	176.8	176.7	–	–	–
4	110.5	112.6	113.2	6.87, s	7.21, s	7.27, s
4a	157.4	139.8	139.7	–	–	–
5a	147.8	151.2	151.6	–	–	–
6	107.2	105.6	102.7	7.24, d (2.3)	7.36, d (1.8)	7.38, d (2.3)
7	162.0	169.5	172.2	–	–	–
8	119.9	122.5	122.7	7.20, dd (8.9; 2.3)	7.45, dd (9.1; 1.6)	7.48, dd (9.3; 2.3)
9	123.8	129.7	129.7	7.61, d (8.9)	8.06, d (9.1)	8.1, d (9.3)
9a	119.4	125.6	126.4	–	–	–
10a	154.9	152.8	152.9	–	–	–
Ac–CO		177.9	178.1		–	–
Ac–CH_3		25.0	25.1		2.56, s	2.57, s
OCH_3			58.9			4.19, s

[a] Signals for OH and NH units were not apparent because of a deuterium-proton exchange.
[b] A strong reduction of the singlet of the C-1 proton was observed when the solution of **12** in TFA-*d* was prepared few days before the measurement. This effect did not appear when the ^{1}H NMR spectrum was recorded immediately after dissolution (singlet, δ_H 6.7).

The characteristic signals for an aromatic ABX spin system in the ^{1}H NMR spectra of compounds **12** and **13** are present, however the position of the OH functionality at C-7 or C-8 on the aromatic ring remained uncertain, since both possibilities should have similar chemical shifts and pattern of coupling. Moreover, the heteroatoms O-5 and N-10 separate the molecule into two units and prevent the ^{1}H and ^{13}C long-range correlations between them (Fig. 2). Fortunately, the structure of the *O*-methyl derivative of **13**, 2-acetylamino-7-methoxy-3*H*-phenoxazin-3-one (**15**) (Scheme 1), has been unambiguously determined by X-ray crystallography (Buckley *et al.*, 1982). Therefore

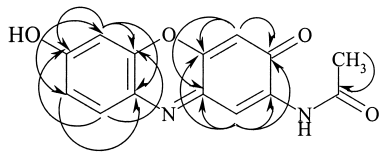


Fig. 2. ^{1}H and ^{13}C long-range correlation signals in the HMBC spectrum of **13**.

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the isolated compound **12** was chemically transformed by *N*-acetylation to **13** and further *O*-methylated to **15**. Since the semisynthetically prepared **15** showed identical spectral properties (^{1}H NMR) as these of **15** prepared by a published method (Buckley and Charalambous, 1982; Charalambous *et al*., 1969), the position of the methoxy respectively the hydroxy functionality at C-7 of **12** and **13** was confirmed.

An additional hydroxylated derivate (**14**) (Scheme 1), which was found in the medium after cultivation of *Chaetophaeria* sp. with HBOA or its metabolite **7** in small flasks (50 ml), could be identified by HPLC-UV and HPLC-APCI-MS spectra. This compound has an identical UV absorption curve as **13**. The quasimolecular ion at *m/z* 287 ($[M+H]^+$) is 16 amu more than that of **13** and obviously indicates again one more hydroxy functionality in the molecule. The MS/MS fragmentation of the parent ion ($[M+H]^+$ at *m/z* 287) gave a main fragment at *m/z* 229 (100, $[M-HOCH=C=O+H]^+$) and a second one at *m/z* 269 (10, $[M-H_2O+H]^+$). The main fragment is identical with the quasimolecular ion of **12** ($[M+H]^+$ at *m/z* 229), which suggests that the additional hydroxy functionality is not attached on the aminophenoxazinone ring, but localised on the N-acyl moiety and corresponds to 7-hydroxy-2-(2-hydroxyacetyl)amino-3*H*-phenoxazin-3-one (**14**). This compound did not occur in the cultivation medium supplemented with metabolite **8**.

The reason, why the aminophenoxazinone derivatives are formed when HBOA or its metabolites **7** and **8** are added to the culture, lies in the fact, that *o*-aminophenol (**9**) is a key intermediate in the metabolic pathway (Scheme 1). This was proved using [$^{13}C_2$]-**7** during the study of biotransformation of HBOA with different endophytic strains (Zikmundová *et al*., 2002). Two molecules of *o*-aminophenol are oxidized to 2-amino-3*H*-phenoxazin-3-one (**10**) (Barry *et al*., 1989) from which by further hydroxylation and acetylation the here described pigments **12** or **13** are formed. Obviously, compound **14** is derived also from *o*-aminophenol, which is coupled by oxidation with **7** and hydroxylated at C-7. This is the reason, why this pigment appears only in case of cultivation with HBOA or metabolite **7**.

Because of known antibiotic effects of **10** and **11** compounds **10**, **11**, **12**, **13** and **15** were preliminary tested for the antibacterial and antifungal activity. The agar overlay technique (Rahalison *et al*., 1991) with *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* was used. This preliminary test reveals that the new two derivatives **12** and **13** possess no antibacterial activity in contrary to the compounds **10**, **11** and **15**.

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