VIP Chemical Defense

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Chemical Defense of the Crust Fungus *Aleurodiscus amorphus* by a Tailor-Made Cyanogenic Cyanohydrin Ether**

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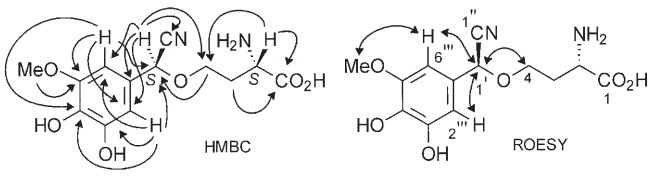
Aleurodiscus amorphus Rabenh. (German name: Orangefarbene Mehlscheibe) is a crust fungus native to Europe, Asia, and North America which occasionally can be found coating the bark of dead or dying silver fir trees (*Abies alba*).[1] It is worthy of note that its pinhead-sized brilliantly pink-orange fruiting bodies are virtually never attacked by predators. Here we report the detection of the tailor-made cyanohydrin aleurodisconitrile (**1**) which, on injury of the fruiting bodies, releases HCN by means of an oxidative mechanism so far unknown in nature, thus deterring predators from feeding.

The characteristic odor of hydrocyanic acid is always perceptible immediately after injury of the fruiting bodies. This observation was confirmed by GC-MS after derivatization of an aqueous crude extract with pentafluorobenzyl bromide.[2] Moreover, the HPLC metabolic profiles of methanolic extracts of intact and injured fruiting bodies differed. Aleurodisconitrile (**1**) was the main component in intact fruiting bodies, while aleurodiscoester (**2**) dominated in injured ones. In contrast, neither **1** nor **2** were detectable in mycelial cultures of *A. amorphus*.

In order to elucidate the structure of the two compounds, 737 mg of the relatively rare fruiting bodies were carefully removed from the bark of the silver firs and immediately extracted with methanol; the crude extract was separated by preparative reverse-phase HPLC, resulting in the isolation of 2.9 mg of aleurodisconitrile and 1.2 mg of aleurodiscoester.

Absorption maxima in the UV spectrum of **1** at $\lambda = 212$ and 246 nm indicate the presence of an aromatic moiety. This is in agreement with the molecular formula $C_{13}H_{16}N_2O_6$ deduced by high-resolution atmospheric pressure chemical ionization mass spectrometry (HR-APCIMS) and the ^{1}H NMR spectrum, which contains signals of two CH protons in the aromatic region at $\delta_H = 6.67$ (H-6‴) and 6.68 ppm (H-2‴) in addition to seven signals in the aliphatic region. The aromatic protons are in a *meta* position to each other as shown by a coupling constant of $J = 1.8$ Hz. The ^{13}C NMR spectrum consists of 13 signals; two of the seven signals in the aromatic region can be attributed based on the HSQC spectrum to the aromatic CH groups mentioned above, the remaining five arise from quaternary carbons. The HMBC spectrum indicates that the resonance at $\delta_C = 118.7$ (C-1″) does not originate from the aromatic ring and that the CH group at $\delta_H = 5.34$ (H-1′) is adjacent to the quaternary carbon atom of the aromatic ring at $\delta_C = 125.5$ (C-1‴), which is flanked by the two aromatic CH groups at $\delta_C = 104.1$ (C-6‴) and 109.5 ppm (C-2‴). A nuclear Overhauser effect (NOE) between the aromatic proton at $\delta_H = 6.67$ (H-6‴) and those at $\delta_H = 3.88$ indicates that a methoxy group is located at C-5‴, while the remaining two downfield-shifted aromatic carbons, C-3‴ and C-4‴, bear OH groups. The CH group at $\delta_H = 5.34$ (H-1′) is in addition linked—according to a $^3J_{CH}$ correlation in the HMBC spectrum—to the OCH_2 group at $\delta_C = 67.7$ (C-4). The COSY, HSQC, and HMBC spectra indicate that the signals of this OCH_2 group ($\delta_H = 3.74$–3.78 and 3.85–3.89 ppm) represent a part of a homoserine residue, to which the resonances of the CH_2 group at $\delta_H = 2.07$–2.13 and 2.24–2.29 (H-3) and the CH group at $\delta_H = 3.66$ ppm (H-2) also belong. According to the molecular formula and a $^2J_{CH}$ correlation of the proton at $\delta_H = 5.34$ (H-1′) to the carbon at $\delta_C = 118.7$ (C-1″), the still undefined substituent at the CH group at $\delta_H = 5.34$ ppm (H-1′) is a CN group. Hence, aleurodisconitrile corresponds to the structure shown in Scheme 1. Comparison of the circular dichroism (CD) spectrum of **1** with that of (*R*)-amygdaline reveals the *S* configuration of the cyanohydrin carbon atom in **1**. In contrast, the *S* configuration of the stereocenter of the homoserine moiety was determined indirectly via the ester **2** (see below). The structure of **1** is in agreement with considerations of its biosynthesis. In analogy to the biosynthesis of cyanogenic glycosides,[3] the cyanohydrin could be



Scheme 1. Aleurodisconitrile (**1**) with selected HMBC and ROESY correlations.

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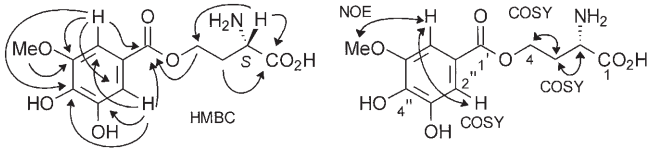
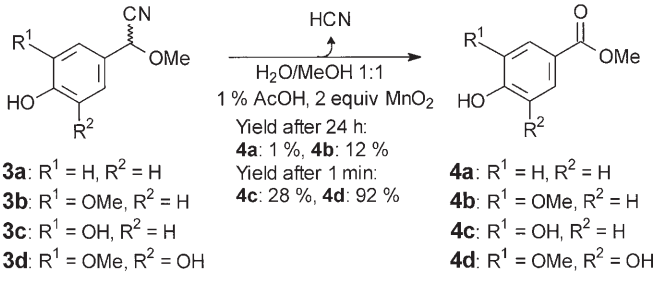
generated from tyrosine while the homoserine residue could originate from (*S*)-adenosylmethionine.

Aleurodiscoester (**2**) is closely related to **1**; the NMR data point to a homoserine residue and an aromatic moiety with the same substituents as those deduced for **1**. However, in the NMR spectra of **2** both the cyanohydrin ^{1}H signal and the ^{13}C resonance of the cyanide are missing. Since a resonance at $\delta_C = 168.2$ (C-1′) occurs instead of that at $\delta_C = 72.0$ ppm, an ester group in **2** must replace the cyanohydrin (Scheme 2). The ester **2** has the *S* configuration as supported by the good agreement of the optical rotation of the natural product with that of a synthetic sample.

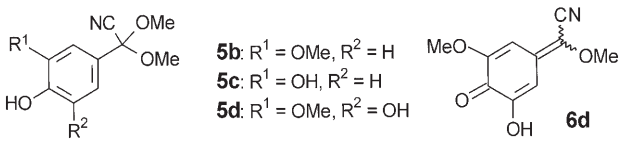


Scheme 2. Aleurodiscoester (**2**) with selected HMBC, COSY, and ROESY correlations.

In contrast to cyanogenic glycosides such as amygdaline in which the cyanohydrin is connected by an acetal to the sugar moiety and undergoes smooth enzymatic hydrolysis,[3] the ether bridge between the cyanohydrin and the homoserine residue in **1** prevents hydrolysis. Therefore, **1** can only be converted oxidatively to **2** and HCN. To study the mechanism of the reaction more precisely we synthesized the compounds **3a–d**[4] in which the homoserine side chain was shortened and the substitution pattern of the aromatic residue was varied. These model compounds were dissolved in aqueous MeOH, MnO_2 was added as oxidant, and the course of the reaction was followed by analyzing aliquots after appropriate periods of time. The samples were trimethylsilylated and investigated by GC-MS. Model compounds with a single hydroxy group in *para* position of the aromatic residue exhibited almost no reaction (**3a**,**b**). In contrast, **3c** degrades quickly to **4c**, because there is a second free OH group directly adjacent to that in *para* position. The reaction is further enhanced by an additional OMe group such as present in **3d** (Scheme 3). Therefore, the aromatic moiety in the natural product **1** is optimized to be easily oxidized. When the oxidation of **3b–d** was performed in methanol, compounds **5b–d** were generated (Scheme 4). When **3d** was oxidized in absolute acetonitrile, the quinonemethide **6d** was identified instead (Scheme 4).

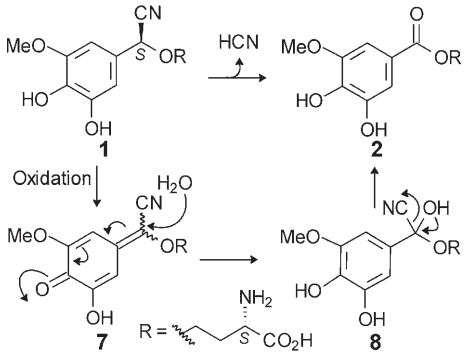


Scheme 3. Oxidative degradation of the model compounds **3a–d** to the corresponding esters **4a–d** by release of HCN.



Scheme 4. Oxidation products of the model compounds **3b–d**.

On the basis of these results we propose the following mechanism for the degradation of aleurodisconitrile (**1**) to the ester **2** and HCN.[5] In the first step **1** is oxidized to the *ortho*-quinone, which rearranges to the corresponding quinine-methide **7**. Then, H_2O is added formally to **7** by generation of the intermediate **8**, from which HCN is eliminated and the ester **2** is formed (Scheme 5).



Scheme 5. Proposal for the oxidative degradation of **1** to **2**.

Mechanical injury often induces oxidative processes in mushrooms.[6] These are often initiated by rather unspecific tyrosinases (or enzymes with similar function), which are widespread in fungi and which become active only on injury.[7] Therefore, it is not surprising that **2** was generated when **1** was incubated with commercially available tyrosinase from mushrooms.[8]

Since we isolated 2.9 mg of **1** from 737 mg of fungus fruiting bodies, it follows that 0.35 mg of HCN can be produced by one gram of fungus, apparently sufficient to protect *A. amorphous* effectively against predators.[9] The production of HCN is not rare in nature and has been observed in plants in particular[10] as well as in algae,[11] bacteria,[12] animals,[13] and fungi.[14] In the plant kingdom cyanogenic compounds occur especially in form of cyanogenic glycosides, which are hydrolyzed enzymatically upon injury of the plants and liberate HCN after decomposition of the cyanohydrin for the protection against predators.[3] In contrast, in bacteria HCN is often generated oxidatively from glycine by the enzyme HCN synthase.[12] In higher fungi the production of HCN is best known in representatives of the families of Tricholomataceae, Pleurotaceae, Polyporaceae, and Stereaceae.[14] *A. amorphous*, which belongs to the latter family, had not been investigated for release of HCN before our experiments. However, until now the mode of hydrocyanic acid generation has been studied only for a mycelial culture of a phytopathogenic, unclassified basidiomycete which probably produces HCN from glycine.[15]

The mechanism of the oxidative degradation of aleurodisconitrile (**1**) to aleurodiscoester (**2**) and HCN differs fundamentally from both that of the oxidation of glycine in bacteria and also from that of the cyanogenic glycosides in plants.[10b] While the ease of hydrolysis of the glycosidic bond by glycosidases is decisive in plants, in the case of *A. amorphus* the oxidizability of the aromatic residue is essential. The degradation of oxidation-prone aromatic cyanohydrins therefore represents a third possibility for the generation of hydrocyanic acid in nature.

Experimental Section

In order to isolate **1** and **2**, fresh or frozen fruiting bodies were extracted with MeOH and separated by preparative HPLC on RP-18.

1: Colorless solid; CD (MeOH): λ ($\Delta\varepsilon$) = 210 nm (+1.8); ^{1}H NMR (600 MHz, CD_3OD, reference: δ = 3.31 ppm, 330 K): δ = 2.07–2.13 (m, 1H; H-3), 2.24–2.29 (m, 1H; H-3), 3.66 (dd, 1H; J = 7.0, 4.9 Hz; H-2), 3.74–3.78 (m, 1H; H-4), 3.85–3.89 (m, 1H; H-4), 3.88 (s, 3H; OCH_3), 5.34 (s, 1H; H-1′), 6.67 (d, 1H, J = 1.8 Hz; H-6‴), 6.68 ppm (d, 1H, J = 1.8 Hz; H-2‴); ^{13}C NMR (151 MHz, CD_3OD, reference: δ = 49.0 ppm, 298 K): δ = 31.9 (C-3), 54.4 (C-2), 56.8 (OCH_3), 67.7 (C-4), 72.0 (C-1′), 104.1 (C-6‴), 109.5 (C-2‴), 118.7 (C-1″), 125.5 (C-1‴), 136.8 (C-4‴), 147.0 (C-3‴), 149.9 (C-5‴), 173.6 ppm (C-1); UV/Vis (MeOH): λ_{max} (lgε) = 212 (4.07), 246 (3.31), 274 nm (2.79); HR-APCIMS: m/z: 297.1079 $[M+H]^+$, calcd for $C_{13}H_{17}N_2O_6$ 297.1087.

2: Colorless solid; $[\alpha]_D^{25} = +70\ cm^3\,g^{-1}\,dm^{-1}$ (c = 0.135 $g^{-1}\,dm^{-1}$, MeOH); ^{1}H NMR (600 MHz, CD_3OD, 300 K): δ = 2.17–2.23 (m, 1H; H-3), 2.34–2.40 (m, 1H; H-3), 3.67 (dd, 1H; J = 7.3, 5.5 Hz, H-2), 3.88 (s, 3H; OCH_3), 4.38–4.46 (m, 2H; H-4), 7.20 (d, 1H, J = 1.9 Hz; H-6″), 7.24 ppm (d, 1H, J = 1.9 Hz; H-2″); ^{13}C NMR (151 MHz, CD_3OD, 300 K): δ = 32.1 (C-3), 53.9 (C-2), 56.7 (OCH_3), 62.3 (C-4), 106.2 (C-6″), 112.1 (C-2″), 121.1 (C-1″), 141.1 (C-4″), 146.4 (C-3″), 149.2 (C-5″), 168.2 (C-1′), 174.8 ppm (C-1); UV/Vis (MeOH): λ_{max} (lgε) = 217 (3.57), 277 nm (3.18); HR-APCIMS: m/z: 286.0916 $[M+H]^+$, calcd for $C_{12}H_{16}NO_7$ 286.0927.

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