Blennione, a Green Aminobenzoquinone Derivative from *Lactarius blennius*

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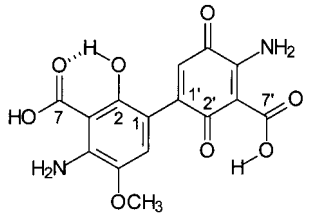
A green pigment, blennione (**1**), was isolated from fruit bodies of the toadstool *Lactarius blennius*. Its structure was established by 2D NMR methods and confirmed by a biomimetic synthesis. Compound **1** represents a diphenylquinone derivative, which may be formed biosynthetically from two 3,6-dihydroxyanthranilic acid units.

Several sesquiterpene lactones have been isolated from fruit bodies of *Lactarius blennius* Fr. (Agaricales) (German: *Graugrüner Milchling*), a toadstool common to European beech woods.[1] During our work on the pigments of *Lactarius* species[2,3] we became attracted to the pale green complexion of this toadstool. In this publication we describe the isolation of blennione, the relevant green pigment.

Blennione (**1**) was extracted from the peeled skins of freeze-dried toadstools using methanol and then isolated by repeated chromatography on Sephadex LH-20. Skins from 1 kg of freeze-dried mushrooms contain only 2−3 mg of the pigment, which crystallizes in beautiful green needles.



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Blennione exhibits absorption maxima at 222, 254, 342, 435, and 684 nm in the UV/vis spectrum. The LC/APCIMS of **1** shows a $[M + H]^+$ ion at *m/z* 349, which corresponds to the molecular formula $C_{15}H_{12}N_2O_8$ according to the HR/APCIMS. The ^{13}C NMR spectrum of **1** displayed a methoxy signal at δ_C 56.0 and 14 carbon signals between δ_C 96.2 and 188.4, which were assigned to two methine (δ_C 116.2 and 122.9) and 12 quaternary carbon atoms.

In the ^1H NMR spectrum (CD_3OD) of blennione only three singlets were detected. The ^1H NMR spectrum in DMSO-d_6 revealed three singlets at δ_H 3.68 (OCH_3), 7.09 (CH), and 7.49 (CH) and six additional signals between δ_H 6.27 and 18.86. This indicated at least six exchangeable NH, NH_2, or OH protons. Therefore, all protons except one were recognized in the ^1H NMR spectrum recorded in DMSO-d_6.

In the HMBC spectrum (Figure 1), the methoxy protons coupled to the carbon at δ_C 138.0, which is apparently located in an aromatic ring. The NOESY correlation between the methoxy protons and the aromatic proton at δ_H 7.09 places the two substituents *ortho* to one another. The OH proton (δ_H 18.86) was evident at an unusually low field and coupled to four carbon signals (δ_C 100.6, 106.1, 167.2, and 173.5). This suggested attachment of this OH group to the aromatic carbon at δ_C 167.2 and the presence of a CO_2H group (δ_C 173.5) in *ortho*-position. In addition, the LC/APCIMS/MS exhibited strong peaks indicating sequential loss of one and two molecules of H_2O followed by loss of CO, respectively. Such prominent loss of H_2O is typical for aromatic acids carrying either an OH or NH_2 group in an *ortho*-position (*ortho*-effect).[4] The observed double loss of H_2O points to the presence of two such structural elements.

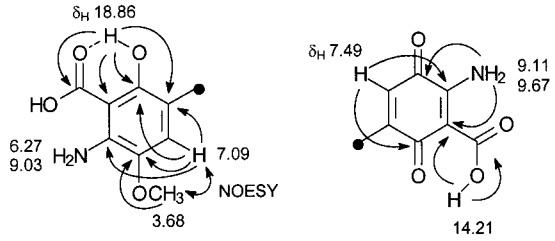

Figure 1. HMBC correlations determining the substitution pattern of the benzene and the quinone ring of blennione.

Another exchangeable proton at δ_H 14.21 was coupled to carbon signals δ_C 96.2 and 169.1. The latter was attributed to a second CO_2H group. The remaining carbonyl signals at δ_C 177.7 and 188.4 were assigned to the carbonyl groups of a 1,4-benzoquinone system. Two exchangeable protons at δ_H 9.11 and 9.67 in the ^1H NMR spectrum coupled to carbon atoms at δ 96.2 and 177.7, indicating the presence of a NH_2 group. A second NH_2 group explains the two signals at δ_H 6.27 and 9.03, which are devoid of any cross-peaks in the HMBC spectrum due to extensive broadening.

The substitution pattern of the quinone ring can be derived from the HMBC spectrum. Correlations of the amino protons with the carbonyl group at δ_C 177.7 and the *ortho*-carbon at δ_C 96.2 carrying the carboxyl group define the $-CO-C(NH_2)=C(CO_2H)-$ moiety. The 1,3-coupling of the ring proton at δ_H 7.49 to the second quinone carbonyl at δ 188.4 and additional correlations to carbons at δ_C 106.1 and 154.7 allowed us to complete the structure of the quinone ring. The substitution of the aromatic ring followed unambiguously from the HMBC correlations given in Figure 1 and the NOE effect between the methoxy protons with the ring proton at δ_H 7.09. Combining both partial structures led to structure **1** for blennione, which was supported by HMBC correlations between 6-H and C-1′ as well as between 6′-H and C-1.

The quinone part of blennione resembles that of paulomycinone A, a degradation product of the antibiotic paulomycin.[5] The ^{13}C NMR data reported for the quinone rings of both compounds are in good agreement.

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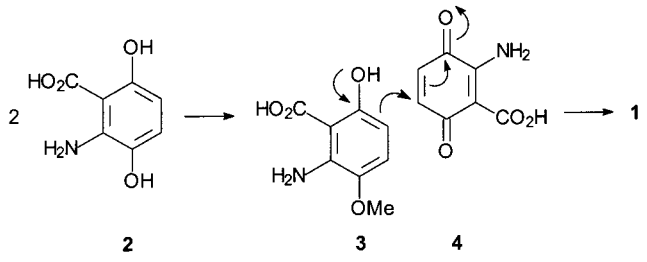

Figure 2. Proposed biosynthesis of blennione.

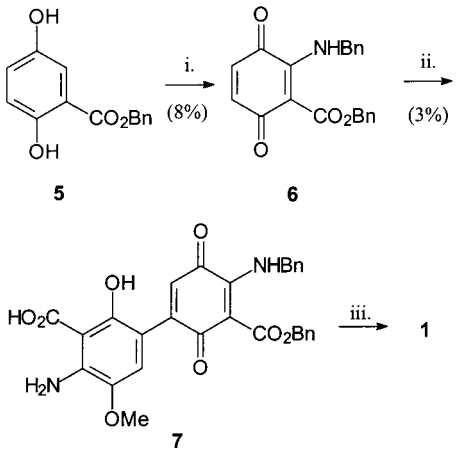

Figure 3. Biomimetic synthesis of blennione. Reagents and conditions: (i) MnO_2, CH_2Cl_2, rt, 24 h; then $BnNH_2$, EtOAc, MnO_2, rt, 5 h; (ii) **3**, silica gel, MeOH, EtOAc, rt, 1 h; HPLC separation; (iii) H_2, PtO_2, MeOH, rt, 10 atm, 12 h.

The identical sequence of substituents in both rings suggests that blennione is formed from 3,6-dihydroxyanthranilic acid (**2**) via the two building blocks **3** and **4** (Figure 2). Compound **2** is the hydroxy derivative of 3-hydroxyanthranilic acid, a well-known intermediate in primary metabolism.[6]

To test this proposal, we prepared the methyl ether **3** in five steps from methyl gentisate.[7] Instead of the highly unstable aminoquinone carboxylic acid **4**, the *N*,*O*-protected derivative **6** was generated in situ by controlled addition of benzylamine to 2-benzyloxycarbonyl-1,4-benzoquinone, prepared by MnO_2 oxidation of the corresponding hydroquinone **5** (Figure 3). Due to the high tendency to form the bis adduct, the yield of **6** did not exceed 20% even at high dilution.

The addition of electron-rich aromatics to electrophilic benzoquinones has been studied by Eugster and co-workers.[8] In the case of **3** and **6**, the reaction presented considerable difficulties. A complex mixture of colored products was formed, from which the desired green dimer **7** could be isolated by HPLC in 3% yield. Catalytic hydrogenation of **7** yielded blennione (**1**), which agreed in its UV/vis and ^{1}H NMR data with the natural product.

Blennione (**1**) constitutes a novel type of fungal pigment. Interestingly, anthranilic acid is also the precursor of the chromoalkaloids necatorone[2] and 10-deoxynecatorone in *Lactarius necator*,[9] a species related to *L. blennius*. Dicinnaquinone, a 4,4′-dimer of 3,5,6-trihydroxyanthranilic acid, is produced by cultures of *Streptomyces griseoflavus* ssp. *thermodiastaticus* TÜ 2486,[10] and lepiotaquinone from the agaric *Lepiota americana* owes its red color to a 2,5-diamino-1,4-benzoquinone chromophore.[11] Green pigments are rare in fungi and are, in most cases, due to extended quinonoid systems, e.g., xylindein[12] and hypoxyxylerone.[13] Interestingly, the dark green color of the North American *Lactarius atroviridis* Peck is caused by the 4,4′-dimer of 10-deoxynecatorone.[3]

Experimental Section

General Experimental Procedures. Reactions requiring anhydrous conditions were conducted under an atmosphere of argon. Evaporation of the solvents was performed under reduced pressure using a rotary evaporator. Column chromatography: Sephadex LH-20 (Pharmacia). Preparative HPLC: Nucleosil RP-18 column (Macherey-Nagel, 250 × 20 mm, 7 μm, flow 6 mL/min, 100% H_2O → 100% MeOH in 40 min). UV: Perkin-Elmer Lambda spectrophotometer. NMR: Bruker AMX-600 spectrometer (^{1}H at 600.1, ^{13}C at 150.9 MHz), chemical shifts in δ relative to $CDCl_3$ (δ_H 7.26, δ_C 77.7), CD_3OD (δ_H 3.31), DMSO-d_6 (δ_H 2.49, δ_C 39.5) as internal standard; temperature of the probe 300 K (unless specified otherwise). EIMS and HREIMS: Finnigan MAT 90 instrument using EI at 70 eV. LC-APCIMS: Gynkotek-HPLC equipped with a Nucleosil RP-18 column (Macherey-Nagel, 250 × 2 mm, 5 μm, operation temperature 40 °C, flow 300 μL/min [solvent A, 99.9% H_2O/0.1% AcOH; solvent B, CH_3CN; gradient 100% A → 100% B in 30 min]) coupled with a Finnigan TSQ 7000, Finnigan API ion source interface, positive APCI mode, ionization 4.5 kV, capillary temperature 200 °C, mass range 50−800 mu, multiplier 1000 V (scan modus). MS/MS: argon collision gas 2.0 mbar, sheath gas (N_2) 2.9 bar, multiplier 1400 V, collision energy automatically rotated at −20, −30, −40 eV.

Organism. Fruit bodies of *L. blennius* were collected in 1998, 1999, and 2000 in beech forests near München (Mühlthal), Ingolstadt (Reisberg), and Regensburg (Girnitztal) (leg. et det. N. Arnold). The toadstools were freeze-dried after collecting. A voucher specimen is kept in the herbarium of the Ludwig-Maximilians-Universität München, Department Chemie.

Isolation Procedure. The grayish skin of the freeze-dried fruit bodies was peeled with a razor blade. The skins were extracted several times with MeOH at 50 °C. The green pigment was obtained as green needles after repeated chromatography on Sephadex LH-20 (eluent MeOH). Approximately 1 kg of freeze-dried fruit bodies was required to obtain 2−3 mg of the pigment.

Blennione (1): emerald green needles; mp >300 °C (dec); UV/vis (MeOH) λ_{max} (log ϵ) 222 (3.64), 254 (3.65), 342 (3.38), 435 (3.07), 684 (3.07) nm; ^{1}H NMR (CD_3OD) δ 3.82 (3H, s, OCH_3), 7.04 (1H, s, 4-H), 7.24 (1H, s, 2′-H); ^{1}H NMR (DMSO-d_6, 292 K) δ 3.68 (3H, s, OCH_3), 6.27 (1H, br s, 4-NH_2), 7.09 (1H, s, 6-H), 7.49 (1H, s, 6′-H), 9.03 (1H, br s, 4-NH_2), 9.11 (1H, s, 4′-NH_2), 9.67 (1H, s, 4′-NH_2), 14.21 (1H, s, 7′-CO$_2H$), 18.86 (1H, s, 2-OH), 7-CO$_2H$ not visible; ^{13}C NMR (DMSO-d_6, 292 K) δ 56.0 (*C*H$_3$O), 96.2 (C-3′), 100.6 (C-3), 106.1 (C-1), 116.2 (C-6), 122.9 (C-6′), 138.0 (C-5), 146.5 (C-1′), 147.1 (C-4), 154.7 (C-4′), 167.2 (C-2), 169.1 (C-7′), 173.5 (C-7), 177.7 (C-5′), 188.4 (C-2′); LC/APCIMS t_R = 16.6 min (detection, UV at λ = 250 nm and APCIMS), *m*/*z* 349 [M + H]$^+$; LC/APCIMS/MS (parent ion *m*/*z* 349, 40 eV) *m*/*z* (%) 331 (4), 313 (62), 285 (100), 229 (23); HR/APCIMS *m*/*z* 349.0685 (M$^+$, calcd 349.0672 for $C_{15}H_{12}N_2O_8$).

2-Amino-6-hydroxy-3-methoxybenzoic Acid (3). To a solution of methyl 6-acetoxy-3-methoxy-2-nitrobenzoate[7] (2.90 g, 10.8 mmol) in THF (30 mL) and H_2O (10 mL) was added LiOH × H_2O (2.00 g). After stirring at 40 °C for 30 min, H_2O (20 mL) was added. The mixture was extracted with EtOAc (2 × 50 mL) and the aqueous layer acidified to pH 1 with 2 N HCl. Extraction with EtOAc (3 × 50 mL) and concentration of the dried (Na_2SO_4) organic layers yielded 6-hydroxy-3-methoxy-2-nitrobenzoic acid (1.96 g, 85%) as pale yellow sheets, mp 221 °C. To a solution of this acid (1.90 g, 8.91 mmol) in MeOH (50 mL) was added PtO_2 (1 mg), and the mixture was stirred in an autoclave under 5 atm of hydrogen at 25 °C for 12 h. HPLC purification of the air-sensitive product (UV detection at 250 nm, t_R = 20.0 min) yielded **3** (1.55 g, 95%) as a colorless solid, mp 62 °C: ^{1}H NMR (600 MHz, CD_3OD) δ 3.81 (3H, s, OCH_3), 6.29 (1H, d, $^3J_{HH}$ = 8.7 Hz, 5-H), 6.95 (1H, d, $^3J_{HH}$ = 8.7 Hz, 4-H); ^{13}C NMR (151 MHz, CD_3OD) δ 57.0

(O*C*H$_3$), 102.8 (C-1), 106.2 (C-5), 117.6 (C-4), 137.4 (C-2), 142.7 (C-3), 156.8 (C-6), 173.9 (*C*O$_2$H); EIMS *m*/*z* (%) 183 (7), 165 (9), 150 (16), 139 (63), 124 (100), 122 (3), 110 (3), 109 (7), 96 (24), 95 (4), 80 (3), 68 (5), 44 (28); HREIMS *m*/*z* 183.0534 (M$^+$, calcd 183.0532 for $C_8H_9NO_4$).

Benzyl 2,5-Dihydroxybenzoate (5). To 2,5-dihydroxybenzoic acid (5.00 g, 32.4 mmol) in acetone (200 mL) was slowly added K_2CO_3 (4.93 g, 35.7 mmol) and benzyl chloride (4.32 g, 35.7 mmol) in acetone (20 mL).[14] After 12 h of stirring at 70 °C, the suspension was cooled to 20 °C and filtered. The solvent was evaporated in vacuo at 40 °C, and the residue was dissolved in EtOAc (10 mL) and filtered through a 5 cm layer of silica gel to give pure benzyl 2,5-dihydroxybenzoate (7.77 g, 98%) as colorless crystals, mp 91 °C: ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.36 (2H, s, PhC*H*$_2$O), 6.88 (1H, d, $^3J_{HH}$ = 8.9 Hz, 3-H), 6.99 (1H, dd, $^3J_{HH}$ = 8.9 Hz, $^4J_{HH}$ = 2.8 Hz, 4-H), 7.32 (1H, d, $^4J_{HH}$ = 2.8 Hz, 6-H), 7.36−7.44 (5H, m, Ph), 10.34 (1H, s, 2-OH); ^{13}C NMR (151 MHz, $CDCl_3$) δ 67.8 (Ph*C*H$_2$O), 112.9 (C-1), 115.5 (C-6), 119.2 (C-3), 124.9 (C-4), 129.0 (C-2′, C-6′), 129.3 (C-4′), 129.4 (C-3′, C-5′), 135.9 (C-1′), 148.4 (C-5), 156.7 (C-2), 170.2 (PhCH$_2$O*C*O); EIMS *m*/*z* (%) 244 (38), 153 (1), 137 (3), 136 (3), 109 (1), 108 (2), 91 (100), 65 (7), 43 (3); HREIMS *m*/*z* 244.0738 (M$^+$, calcd 244.0736 for $C_{14}H_{12}O_4$).

3-Benzylamino-2-benzyloxycarbonyl-1,4-benzoquinone (6). To a solution of benzyl 2,5-dihydroxybenzoate (7.70 g, 31.5 mmol) in CH_2Cl_2 (100 mL) was added activated MnO_2 (5.00 g). After stirring for 24 h at 20 °C, the solid was filtered off and the solvent removed in vacuo. The residue was dissolved in 1 mL of EtOAc/hexanes (1:4) and chromatographed on a silica gel column (20 × 3 cm) with the same solvent mixture. 2-Benzyloxycarbonyl-1,4-benzoquinone (2.90 g, 38%) was obtained as an air-sensitive orange solid: ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.36 (2H, s, PhC*H*$_2$O), 6.71 (2H, m, 3-H, 4-H), 7.08 (1H, s, 6-H), 7.36−7.49 (5H, m, Ph); EIMS *m*/*z* 244 (6) [M + 2 H]$^+$, 242 (0.3), 91 (100), 65 (9).

To a solution of this quinone (100 mg, 0.42 mmol) in EtOAc (500 mL) was added activated MnO_2 (100 mg). The mixture was vigorously stirred under argon and treated dropwise with a solution of benzylamine (45 μL, 0.41 mmol) in 25 mL of EtOAc (6 drops/min). This operation required 3 h. After 2 h of further stirring, the mixture was passed through a fiberglass filter and concentrated in vacuo at 20 °C to 10 mL. Since **6** could not be purified or isolated without decomposition, the crude solution was used for the next reaction step.

2-Amino-5-(4-*N*-benzylamino-5-carboxybenzyl-3,6-dioxocyclohexa-1,4-dienyl)-6-hydroxy-3-methoxybenzoic Acid (7). To a solution of **3** (13 mg, 70 μmol) in MeOH (50 mL) was added silica gel (100 mg), and the mixture was placed in a two-necked 250 mL round-bottom flask and kept under argon. To the vigorously stirred mixture was added dropwise crude **6** in EtOAc (10 mL, see above) at 25 °C. The color of the solution turned greenish brown immediately. After 1 h of stirring, the silica gel was filtered off and the solvent removed in vacuo at 30 °C. The brown residue was dissolved in MeOH (0.5 mL) and chromatographed on a RP-18 HPLC column with UV detection at λ = 280 nm (t_R ~30 min) to yield **7** (1.1 mg, 3%): UV/vis (MeOH) λ_{max} (log ϵ) 214 (3.49), 240 (sh, 3.38), 344 (3.20), 424 (sh, 2.57). 604 (2.25) nm; ^{1}H NMR (CD_3OD, 600 MHz) δ 3.76 (3H, s, OC*H*$_3$), 4.09 (2H, s, PhC*H*$_2$N), 5.12 (2H, s, PhC*H*$_2$O), 6.91 (1H, s, 6-H), 7.20−7.49 (10H, m, Ph), 7.27 (1H, s, 6′-H); ^{13}C NMR (151 MHz, CD_3OD) δ 44.4 (Ph*C*H$_2$N), 56.6 (O*C*H$_3$), 68.0 (Ph*C*H$_2$O), 94.7 (C-3′), 103.0 (C-3), 106.4 (C-1), 117.4 (C-6), 126.9 (C-6′), 128.5, 128.8, 129.1, 129.4, 129.8, 130.2, 132.3, 134.7 (each Ph), 140.4 (C-5), 146.9 (C-1′), 148.3 (C-4), 156.6 (C-4′), 161.7 (C-2), 168.9 (C-7 or C-7′), 175.1 (C-7 or C-7′), 177.2 (C-5′), 186.0 (C-2′).

Catalytic Hydrogenation of 7. Blennione (**1**) was obtained from 1.1 mg of **7** in 5 mL of MeOH in a small autoclave containing 0.5 mg of $PtO_2 \times H_2O$ and a Teflon stirring bar. After 12 h stirring at 20 °C under 10 atm pressure of H_2, the solution was passed through a fiberglass filter. During this procedure the solution turned green again. The solvent was removed in vacuo and the residue purified on a Sephadex LH-20 column (25 × 1 cm, eluent MeOH) to yield **1** (0.44 mg, 60%): UV/vis (MeOH) λ_{max} 222, 255, 342, 434, 678 nm; ^{1}H NMR (600 MHz, CD_3OD) δ 3.81 (3H, s, OC*H*$_3$), 7.07 (1H, s), 7.25 (1H, s), identical to natural **1**.

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