

Austrogracilin A and B, two 2-naphthoic acid derivatives from the mushroom *Austroboletus gracilis*

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Dedicated to Professor Rodney W. Rickards on the occasion of his 70th birthday
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Abstract

From air-dried fruit bodies of *Austroboletus gracilis* two unique naphthalene derivatives were isolated and their structures elucidated by spectroscopic and synthetic methods. The chemotaxonomic importance of these compounds is discussed.

Keywords: Austrogracalin, naphthalene derivatives, badione A

Introduction

Austroboletus gracilis (Peck) Wolfe,¹ the Graceful Bolete, is a beautiful mushroom which belongs to a remarkable group of fungi with disjunct occurrence in Eastern North America and East Asia.² It is widely distributed from Canada to Costa Rica and has also been recorded from Papua New Guinea, Japan, Taiwan, and China. In a preliminary investigation of this mushroom we have shown that badione A is responsible for the reddish-brown colour of its cap.³ In this communication we report on the isolation of two novel 2-naphthoic acid derivatives from this species.

Results and Discussion

Investigation of the methanol extract from air-dried fruit bodies of *A. gracilis* by HPLC with diode array-detection revealed the presence of two major constituents with very similar UV spectra. The two compounds, named austrogracilin A (**1**) and B (**3**), could be easily obtained pure by preparative HPLC on reversed phase.

Austrogracilin A is a colourless solid, which becomes yellowish on standing in air. Its UV spectrum (MeOH) exhibits absorption maxima at 229, 266, and 307 nm, and in the IR spectrum intensive bands at 3392 (OH), 1686 (C=O), and 1243 (OH) cm^{-1} are visible. The high resolution mass spectrum of austrogracilin A shows a molecular peak at m/z 220.0369 corresponding to the molecular formula $\text{C}_{11}\text{H}_8\text{O}_5$.

The ^1H NMR spectrum (CD_3OD) of austrogracilin A displays only three singlets at δ_{H} 7.19, 7.52 (br), and 7.88 (br) and a doublet at δ_{H} 7.20 ($J = 1.4$ Hz). According to the ^1H , ^1H -COSY spectrum, the doublet arises by coupling with the proton responsible for the broadened signal at δ_{H} 7.88, thus indicating a *meta*-relationship. In addition, long-range couplings are observed which explain the broadening of the singlets at δ_{H} 7.52 and 7.88.

The ^{13}C NMR spectrum of austrogracilin A exhibits 11 signals in the range between 100 and 175 ppm, in agreement with the molecular formula. In addition to signals for four aromatic CH-groups at δ_{C} 106.2, 106.3, 112.2, and 122.5, three quaternary aromatic C-atoms at δ_{C} 125.0, 127.2, and 131.5 are visible. Further signals at δ_{C} 149.0, 149.5, and 153.6 point to aromatic carbons carrying OH groups, and the signal at δ_{C} 171.4 can be assigned to a carboxyl group. HSQC and HMBC experiments allowed the assignment of all ^1H - and ^{13}C -signals, and from the ^1H , ^{13}C -correlations illustrated in Figure 1 the structure of a 4,6,7-trihydroxy-2-naphthoic acid (**1**) can be proposed for austrogracilin A. It is supported by strong NOE correlations between the two *peri*-protons at δ_{H} 7.19 and 7.88 in the NOESY spectrum.



Figure 1. HMBC correlations of austrogracilin A (**1**).

4-Hydroxy-6,7-dimethoxy-2-naphthoic acid (**2**) is a known compound that can be prepared in three steps from veratraldehyde.⁴ Demethylation of **2** with BBr_3 yielded 4,6,7-trihydroxy-2-naphthoic acid, which was identical with the natural product.

The UV and IR spectra of austrogracilin A and B indicate a close structural similarity of both metabolites. The UV spectrum (MeOH) of austrogracilin B exhibits absorption maxima at 221, 261, and 323 nm, and in the IR spectrum strong bands at 3458 and 3233 (OH), 1687 (CO), and 1254 (OH) cm^{-1} are visible. High resolution mass measurement of the $(\text{M} - \text{H})^-$ peak at m/z 247 in the $(-)$ -ESI MS led to the molecular formula $\text{C}_{12}\text{H}_8\text{O}_6$ for austrogracilin B. The ^1H NMR spectrum (CD_3OD) of austrogracilin B contains signals of four aromatic protons. Two of the signals appear as broadened singlets at δ_{H} 8.43 and 8.52 and one as doublet at δ_{H} 8.60 ($J = 1.3$

Hz). The fourth aromatic proton gives rise to a sharp singlet at δ_{H} 7.33. In DMSO- d_6 additional signals for two phenolic protons at δ_{H} 9.82 and 10.29 are visible as well as a broad signal at δ_{H} 12.92, which corresponds to two carboxyl protons. The ^1H , ^1H -COSY spectrum indicates a strong correlation between the protons at δ_{H} 8.52 and 8.60 as well as long-range coupling between the protons at δ_{H} 8.43 and 8.52. The ^{13}C NMR spectrum of austrogracilin B contains 12 signals. According to the DEPT NMR spectrum, the signals at δ_{C} 109.9, 112.8, 129.0, and 135.0 can be assigned to aromatic CH-groups, whereas the signals at δ_{C} 125.5, 127.1, 131.3, 131.7 belong to quaternary aromatic carbons. Two additional signals at δ_{C} 148.8 and 152.0 can be assigned to aromatic carbon atoms carrying OH groups, and signals at δ_{C} 170.1 and 171.2 point to the presence of two carboxyl groups. HSQC and HMBC experiments (Figure 2) allowed the assignment of all signals and established structure **3** for austrogracilin B. Like austrogracilin A, **3** shows a strong NOE effect between the two *peri*-protons in the NOESY spectrum.

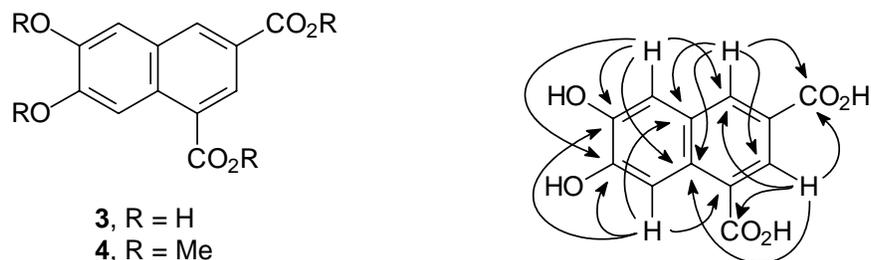


Figure 2. HMBC correlations of austrogracilin B (**3**).

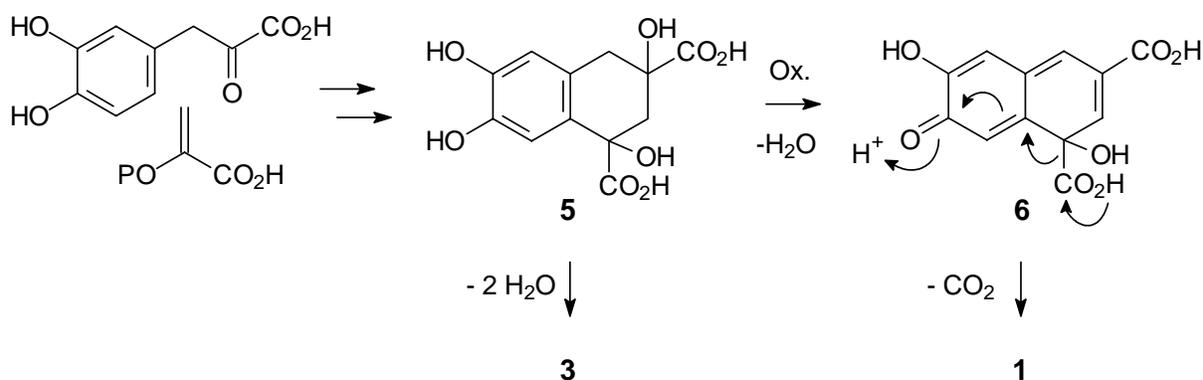
Methylation of austrogracilin B (**3**) with diazomethane yielded the known permethyl derivative **4**. The latter has recently been obtained via an intriguing dimerization followed by oxidative cleavage of methyl 3,4-dimethoxycinnamate.⁵ The spectroscopic data from naturally derived **4** agree very well with those reported for the synthetic material.

Conclusions

The occurrence of the unique naphthalene derivatives **1** and **3** in *Austroboletus gracilis* underlines the special taxonomic position of this genus within the Boletineae. Before considering these compounds as chemotaxonomic markers, further species of *Austroboletus* have to be studied. In a recent phylogenetic investigation based on DNA analysis, Bresinsky and Binder⁶ found that *Austroboletus gracilis*, *A. novae-zelandiae*, and *A. niveus* form a genetically well defined group between *Leccinum* and *Tylopilus*. In contrast, *Austroboletus betula* (Schwein.) E. Horak, a North American species with yellow colours, belongs to a different clade including several *Boletellus* species.⁶ Therefore, the shift of *A. betula* from *Boletellus* to *Austroboletus* because of spore ornamentation^{1b} appears not to be justified and should be reversed. This is also supported by the occurrence of variegatic acid, xerocomic acid, and atromentic acid in *A. betula*,

pigments characteristic for the genus *Boletellus*.^{3,7} According to a preliminary investigation,⁸ most of the variegatic acid is present as a water-soluble sugar conjugate of undetermined constitution which is characterized by a $(M - H)^-$ ion at m/z 1181 in the negative ion FAB MS⁹ suggesting the presence of five hexose residues.

Since most of the Boletales pigments are biosynthetically derived from L-tyrosine,³ it is tempting to assume that this amino acid is also the precursor of the austrogracilins A (**1**) and B (**3**). On condensation of (3,4-dihydroxyphenyl)pyruvic acid with phosphoenol pyruvate, the naphthalenoid dihydroxydicarboxylic acid **5** could be formed, which might either lose two molecules of water to yield austrogracilin B (**3**), or undergo oxidation and dehydration to afford the quinone-methide **6** (Scheme 1). The latter could then deliver austrogracilin A (**1**) on decarboxylation. The possibility, however, that austrogracilin B (**3**) is formed by dimerization of 3,4-dihydroxycinnamic acid to a lignan with subsequent oxidative loss of a 3,4-dihydroxyphenyl residue cannot be excluded. A laboratory analogy for this process is available,⁵ and the common occurrence of thomasic acid and its degradation product 6-hydroxy-5,7-dimethoxy-2-naphthoic acid in *Ulmus thomasi*¹⁰ indicates that such reactions occur in nature.



Scheme 1. Proposal for the biosynthesis of austrogracilin A (**1**) and B (**3**).

Experimental Section

General Procedures. Melting points (uncorrected): Leica Galen hot-plate. UV/Vis: Perkin-Elmer Lambda 16. IR: Perkin-Elmer FT-IR 1000. NMR: Bruker ARX-300 and AMX-600, with TMS or solvent peak as internal standard. MS: Finnigan MAT 90 and MAT 95Q (70 eV, 200°C inlet temperature); MS (ESI): with a Finnigan API-Interface II. Solid phase extraction: Chromabond C18 cartridge, 0.5 g (Macherey and Nagel). TLC: Merck, silica gel 60 F₂₅₄ or RP-18 on aluminum foil, detection at 254 resp. 366 nm. Gel chromatography: Sephadex LH-20 (Pharmacia). Analytical HPLC: Waters 510 pump and System Controller with Photodiode Array Detector 996, Knauer column 4 × 250 mm, Nucleosil 100 C18, 5 μm. System 1: eluent A: H₂O/CH₃CN 9:1 + 0.1% TFA; eluent B: CH₃CN; linear gradient, 0 min: A 100%, B 0%; 25 min:

A 50%, B 50%; flow rate 1^omL/min, detection range 200–400 nm. System 2: same conditions as before, however, 0 min: A 100%, B 0%; 50 min: A 0%, B 100%. Preparative HPLC: Merck Hitachi L-6200 Intelligent Pump, 655A Variable Wavelength UV Monitor, Knauer column 20 × 250 mm, Nucleosil 100 C18, 7 μm; system 3: eluent A: H₂O/CH₃CN 9:1 + 0.1% TFA; eluent B: CH₃CN; linear gradient, 0 min: A 100%, B 0%; 30 min: A 50%, B 50%; 55 min: A 50%, B 50%; flow rate 6.75^omL/min. All solvents for extraction or chromatography were distilled before use. CH₂Cl₂ was dried by distillation from Sicapent[™] (Merck).

Fungal material. *A. gracilis* was collected in autumn 1994, 1996, 1997 in mixed hemlock-rhododendron forests near Highlands, N.C., U.S.A. The fruit bodies were air-dried and stored at room temperature. Voucher samples are kept in the herbarium of N. Arnold, IPB, Halle/Saale.

Extraction and isolation procedure. The air-dried fruit bodies of *A. gracilis* (10 g) were pulverized, defatted with petroleum ether and then exhaustively extracted with degassed MeOH under an argon atmosphere in an ultrasound bath. The combined extracts were concentrated under reduced pressure and the residue distributed between water and EtOAc. The water phase was subsequently extracted with 2-butanol, and both organic phases were dried (Na₂SO₄) and concentrated in vacuo. After solid-phase extraction of the combined residues on a RP-18 cartouche, the compounds were separated by preparative HPLC (system 3). The resulting fractions were lyophilized to yield austrogracilin A (**1**) (14 mg, 0.14% of dry-weight) and austrogracilin B (**3**) (15 mg, 0.15% of dry-weight).

Austrogracilin A (4,6,7-trihydroxy-2-naphthoic acid) (1). Mp >190 °C dec; TLC: *R_f* 0.33 (SiO₂, toluene/HCO₂Et/HCO₂H 10:5:3), 0.70 (RP-18, MeOH + 2 drops of TFA); HPLC: *t_R* 10.0 min (system 1), 13.6 min (system 2); UV-vis (MeOH) λ_{max} (log ε) 205 (sh, 3.74), 229 (4.04), 261 (4.20), 266 (sh, 4.19), 307 (3.53), 316 (sh, 3.51), 349 (sh, 2.87) nm; IR (KBr) ν 3392 (s, br), 2926 (m), 2854 (m), 1686 (s), 1632 (m), 1617 (m), 1594 (m), 1540 (m), 1499 (m), 1410 (s), 1243 (s), 1201 (s), 1159 (m), 1108 (m), 1056 (m), 956 (w), 894 (m), 866 (m), 775 (m), 717 (m), 626 (m) cm⁻¹; ¹H NMR (300 MHz, CD₃OD) δ 7.19 (s, 1 H, H8), 7.20 (d, 1 H, *J* = 1.4 Hz, H3), 7.52 (br s, 1 H, H5), 7.88 (br s, 1 H, H1); ¹³C NMR (151 MHz, CD₃OD) δ 106.2 (C5), 106.3 (C3), 112.2 (C8), 122.5 (C1), 125.0 (C4a), 127.2 (C2), 131.5 (C8a), 149.0 (C7), 149.5 (C6), 153.6 (C4), 171.4 (CO₂H); MS (EI) *m/z* (rel intensity) 220 (M⁺, 100%), 203 (10), 191 (6), 175 (12), 163 (4), 147 (11); HRMS (EI): found M⁺ 220.0369, C₁₁H₈O₅ requires 220.0372.

Austrogracilin B (6,7-dihydroxy-2,4-naphthalene dicarboxylic acid) (3). Mp >250 °C dec; TLC: *R_f* 0.26 (SiO₂, toluene/HCO₂Et/HCO₂H 10:5:3), 0.75 (RP-18, MeOH + 2 drops of TFA); HPLC: *t_R* 11.2 min (system 1); UV-vis (MeOH) λ_{max} (log ε) 205 (sh, 4.27), 208 (sh, 4.30), 214 (sh, 4.32), 221 (4.35), 261 (4.63), 323 (3.91), 348 (sh, 3.81) nm; IR (KBr) ν 3458 (s), 3233 (m), 1687 (s), 1642 (m), 1534 (m), 1477 (m), 1432 (m), 1382 (m), 1254 (s), 1229 (m), 1200 (m), 1144 (m), 1010 (w), 923 (w), 871 (m), 794 (w), 766 (w), 736 (w), 671 (w), 657 (w), 626 (m), 593 (w), 554 (w) cm⁻¹; ¹H NMR (300 MHz, CD₃OD) δ 7.33 (s, 1 H, H8), 8.43 (br s, 1 H, H5), 8.52 (br s, 1 H, H1), 8.60 (d, 1 H, *J* = 1.3 Hz, H3); ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.35 (s, 1 H), 8.33 (s, 1 H), 8.38 (d, *J* = 1.6 Hz, 1 H), 8.44 (br d, *J* = 0.8 Hz, 1 H), 9.82 (br s, 1 H, OH), 10.29 (br s, 1

H, OH), 12.92 (br s, 2 H, 2 × CO₂H); ¹³C NMR (151 MHz, CD₃OD) δ 109.9 (C5), 112.8 (C8), 125.5 (C2), 127.1 (C4), 129.0 (C3), 131.3 (C8a), 131.7 (C4a), 135.0 (C1), 148.8 (C7), 152.0 (C6), 170.1 (2 × CO₂H), 171.2 (4-CO₂H); MS (ESI) *m/z* (rel intensity) 495 (2M – H)[–], 247 (M – H)[–], HRMS [(–)-ESI]: found (M – H)[–] 247.0243, C₁₂H₇O₆[–] requires 247.0243.

Demethylation of synthetic 4-hydroxy-6,7-dimethoxy-2-naphthoic acid (2). To a suspension of **2**^{4a} (497 mg, 2.0 mmol) in dry CH₂Cl₂ (20 mL) was added at –78 °C a solution of BBr₃ (1.50 g, 580 μl, 6.0 mmol) in dry CH₂Cl₂ (10 mL). After stirring at the same temperature for 30 min, the cooling bath was removed and the stirring was continued for 12 h. The mixture was quenched with water and the aqueous phase extracted with 2-butanol. The organic extracts were concentrated in vacuo, and the brown solid residue was purified by preparative HPLC on RP-18 (system 3) to yield 4-hydroxy-6,7-dimethoxy-2-naphthoic acid (**1**) (400 mg, 91%) as a colourless solid, which rapidly turned brown. All physical and spectroscopic data were in agreement with those of austrogracilin A.

Permethylation of Austrogracilin B. A solution of diazomethane in Et₂O was added dropwise at 0 °C to a stirred mixture of **3** (5 mg, 0.02 mmol) and silica gel (50 mg) in MeOH (5 mL) until the gas evolution stopped. The stirring was continued for 30 min, the cold bath removed and the excess of diazomethane destroyed by addition of acetic acid. Then, the solution was evaporated and the residue distributed between water and EtOAc. The dried (Na₂SO₄) organic solution was concentrated in vacuo and the residue purified by TLC on silica gel with hexanes/EtOAc 1:1. Dimethyl 6,7-dimethoxy-2,4-naphthalene dicarboxylate (**4**) (5 mg, 82%) was obtained as a colourless solid. Mp 159–161 °C [lit.⁵ mp 134–136 °C (MeOH)]; TLC: *R_f* 0.76 (SiO₂, hexanes/EtOAc 1:1); UV (MeOH) λ_{max} (log ε) 210 (4.33), 219 (4.34), 262 (4.59), 318 (3.90), 333 (sh, 3.83), 352 nm (sh, 3.68) nm; IR (KBr) ν 3436 (s), 2962 (m), 2924 (m), 2854 (m), 1708 (s), 1624 (m), 1580 (m), 1509 (m), 1484 (m), 1434 (m), 1375 (m), 1332 (m), 1295 (m), 1262 (m), 1195 (m), 1141 (m), 1105 (s), 1029 (s), 1013 (m), 993 (m), 906 (w), 867 (m), 861 (m), 801 (m), 765 (m), 709 (m), 573 (w) cm^{–1}; ¹H NMR (600 MHz, CDCl₃) δ 3.99, 4.01, 4.03, 4.08 (each s, 3 H, CH₃), 7.24 (s, 1 H), 8.55 (s, 1 H), 8.61 (d, *J* = 1.6 Hz, 1 H), 8.71 (d, *J* = 1.6 Hz, 1 H); ¹³C NMR (151 MHz, CDCl₃) δ 52.2, 52.3, 55.9, 56.1 (each CH₃), 104.9 (CH), 107.8 (CH), 124.5 (C_q), 124.8 (C_q), 128.5 (CH), 129.5 (C_q), 130.5 (C_q), 134.0 (CH), 150.0 (C_q), 152.9 (C_q), 166.7 (C_q), 167.7 (C_q); GC/MS (EI) *m/z* (rel intensity) 304 (M⁺, 100%), 273 (50), 230 (12); HRMS (EI) found M⁺ 304.0936, C₁₆H₁₆O₆ requires 304.0947.

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