Chromapedic Acid, Pulvinic Acids and Acetophenone Derivatives from the Mushroom *Leccinum chromapes* (Boletales)

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Dedicated to the memory of the late Dr. Ryuji Marumoto

The pigment responsible for the bright-yellow color of the stalk bases of *Leccinum chromapes* is methyl isoxerocomate, which is accompanied by lesser amounts of isoxerocomic acid and atromentic acid. In addition, 4′-hydroxy- and 3′,4′-dihydroxyacetophenone, previously unreported as metabolites of Basidiomycetes, were isolated. The pink color of the cap skin is due to variegatorubin. This pigment is accompanied by chromapedic acid, a γ-butenolide of biosynthetic interest representing a new dimerization mode of 4-hydroxyphenylpyruvic acid in Boletales.

Key words: Boletales, Pulvinic Acids, Mushroom Pigments, Arylpyruvic Acid Dimers

Introduction

Leccinum chromapes (Frost) Singer (= *Tylopilus chromapes* (Frost) A. H. Sm. & Thiers), the ‘chrome-footed mushroom’, is a conspicuous bolete distributed in broad-leaved and coniferous woods in eastern North America, Japan, China, and East Siberia [1]. It can be easily recognized by its pink cap, the pink scabrous dots on the stalk, and its bright-yellow stalk base and mycelium. Despite this attractive appearance, the pigments of this mushroom are hitherto unknown. We now describe an investigation of the pigments and other constituents of *L. chromapes*.

Results and Discussion

Isolation of pulvinic acids and acetophenone derivatives from the stalk

For the isolation of the pigments, air-dried stalks from a North American collection of *L. chromapes* were extracted with MeOH. The combined orange-brown extracts were then purified by preparative HPLC to afford five UV-active compounds: 3′,4′-dihydroxyacetophenone (pungenol, **1a**), 4′-hydroxyacetophenone (piceol, **1b**), isoxerocomic acid (**2a**) [2 – 4], atromentic acid (**3**) [5], and methyl isoxerocomate (**2b**) [2]. The use of EtOH instead of MeOH in the extraction procedure yielded the same set of compounds; thus, methyl ester **2b** must be a genuine constituent of the mushroom.

Compounds **1a**, **1b**, **2a**, and **3** were identified by their spectroscopic data (UV, IR, NMR, MS) and HPLC comparison with authentic samples. The main orange-yellow pigment **2b** exhibited the characteristic ^{1}H NMR pattern of isoxerocomic acid [4] and, in addition, a signal for a methoxy group at $\delta_H = 3.89$. In an HMBC experiment, this methoxy signal correlated to the carboxyl group at $\delta_C = 172.9$, which defines the compound as methyl isoxerocomate (**2b**) [2]. This is supported by the mass spectrum, in which the base peak at $m/z = 338$ is formed by methanol elimination from the molecular ion at $m/z = 370$.

Trimethylsilylation of ester **2b** yielded a tetrakis(trimethylsilyl ether) ($m/z = 658$), and acetylation gave the corresponding tetraacetate ($m/z = 538$). In accordance with their hydroxylation pattern, pulvinic acid **2a** and its methyl ester **2b** showed no blueing with $K_3[Fe(CN)_6]$ [6]. An HPLC comparison of extracts of *L. chromapes* collections from North America and Japan displayed no differences in the pigment pattern.

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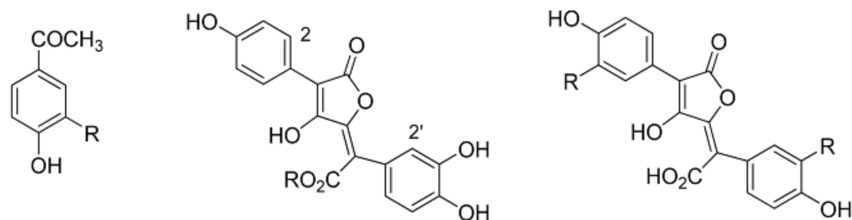


1a, R = OH **2a**, R = H, isoxerocomic acid **3**, R = H, atromentic acid

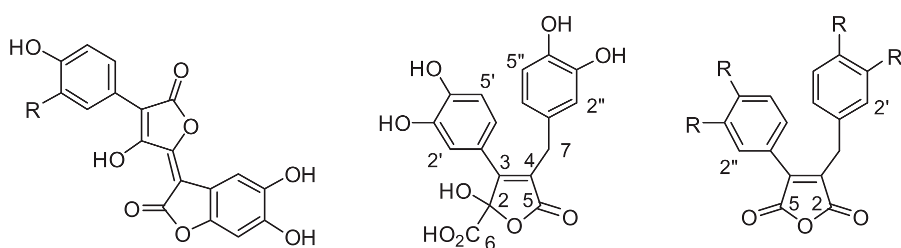
1b, R = H **2b**, R = CH_3, methyl isoxerocomate **4**, R = OH, variegatic acid

While atromentic acid (**3**) is a common pigment of boletes [5], isoxerocomic acid (**2a**) is only known from cultures of *Serpula lacrymans* [7]. Its methyl ester **2b** has been isolated from the fruit bodies of *Rhizopogon roseolus* [8]. The occurrence of the acetophenone derivatives **1a** and **1b** in fruit bodies of mushrooms is most unusual. These compounds and their respective glucosides, which normally occur in the needles of conifers [9 – 11] but have also been reported from several other plants, exhibit interesting biological activities including induction of resistance to spruce budworm [12], fungitoxicity [13], and phytotoxicity [10, 14, 15]. Since the free acetophenones **1a** and **1b** were isolated from *L. chromapes* in considerable quantities, their origin from plant material attached to the fruit bodies and mycelium is highly improbable.

*Isolation of variegatorubin (**5**) and chromapedic acid (**7**) from the cap skin*

The pigment responsible for the pink cap color of *L. chromapes* was identified as variegatorubin (**5**) [16]. This finding is surprising since not even a trace of its precursor, variegatic acid (**4**), could be detected by TLC or HPLC. Rather, it would seem logical to find xerocomorubin (**6**), which is easily formed by oxidation of isoxerocomic acid (**2a**) [2, 3]. Clearly, in the cap skin, isoxerocomic acid is hydroxylated to variegatic acid, which then undergoes oxidative cyclization to variegatorubin.

In the course of these investigations, a pale-yellow compound, chromapedic acid (**7**), was isolated from the acetone extract of air-dried cap skins. The EI, ESI, and FAB mass spectra of this acid did not yield a molecular ion peak nor meaningful fragment ions; unsatisfactory results were also obtained with the corresponding pertrimethylsilyl, permethyl, and peracetyl derivatives. Compound **7** exhibited IR absorptions at 3435, 1757, 1629, and 1521 cm^{-1}, indicating the presence of hydroxy groups, a lactone ring, and conjugated double bonds, respectively. In the ^{1}H NMR spectrum (CD_3OD), signals for diastereotopic methylene protons, at $\delta = 3.71$ and 3.78 ($J = 15.6$ Hz), and for two 3,4-dihydroxyphenyl rings were visible. The ^{13}C NMR spectrum displayed signals for



5, R = OH, variegatorubin **7**, chromapedic acid **8**, R = OH

6, R = H, xerocomorubin **9**, R = H

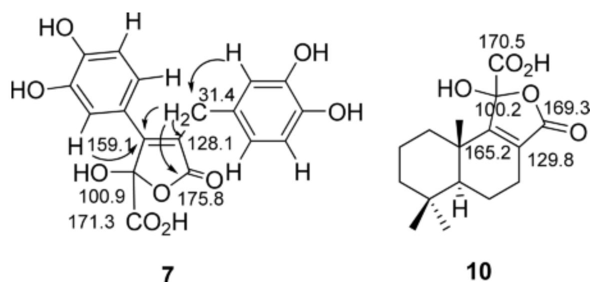


Fig. 1. Selected HMBC correlations and ^{13}C chemical shifts of chromapedic acid (**7**), and comparative ^{13}C NMR data for lactone **10**.

18 carbon atoms, including those for the methylene group at $\delta_C = 31.4$ and the two 3,4-dihydroxyphenyl rings. The remaining five quaternary carbon signals at $\delta_C = 100.9$, 128.1, 159.1, 171.3, and 175.8 could be assigned to a γ-butenolide system with an attached carboxylic group by the HMBC correlations depicted in Fig. 1. The chemical shifts are in reasonable agreement with those reported for the butenolide system in compound **10** [17]. From this evidence, formula **7** could be proposed for chromapedic acid. Compound **7** exhibits a specific rotation $[\alpha]_D^{20}$ of +4; however, the absolute configuration of this metabolite remains to be established.

Structure **7** was supported by the slow formation of a stable, yellow autoxidation product upon leaving chromapedic acid exposed to air. This product was isolated in low yield and was characterized as 3-(3,4-dihydroxybenzyl)-4-(3,4-dihydroxyphenyl)furan-2,5-dione (**8**) from spectroscopic data, including the high-resolution EI mass spectrum. The base peak at $m/z = 255$ is formed from the molecular ion at $m/z = 328$ by the loss of C_2O_3H, a fragmentation characteristic for anhydrides of this type [18, 19]. A metabolite **9** with the same basic structure has been isolated previously from cultures of *Aspergillus nidulans* [20, 21].

Conclusion

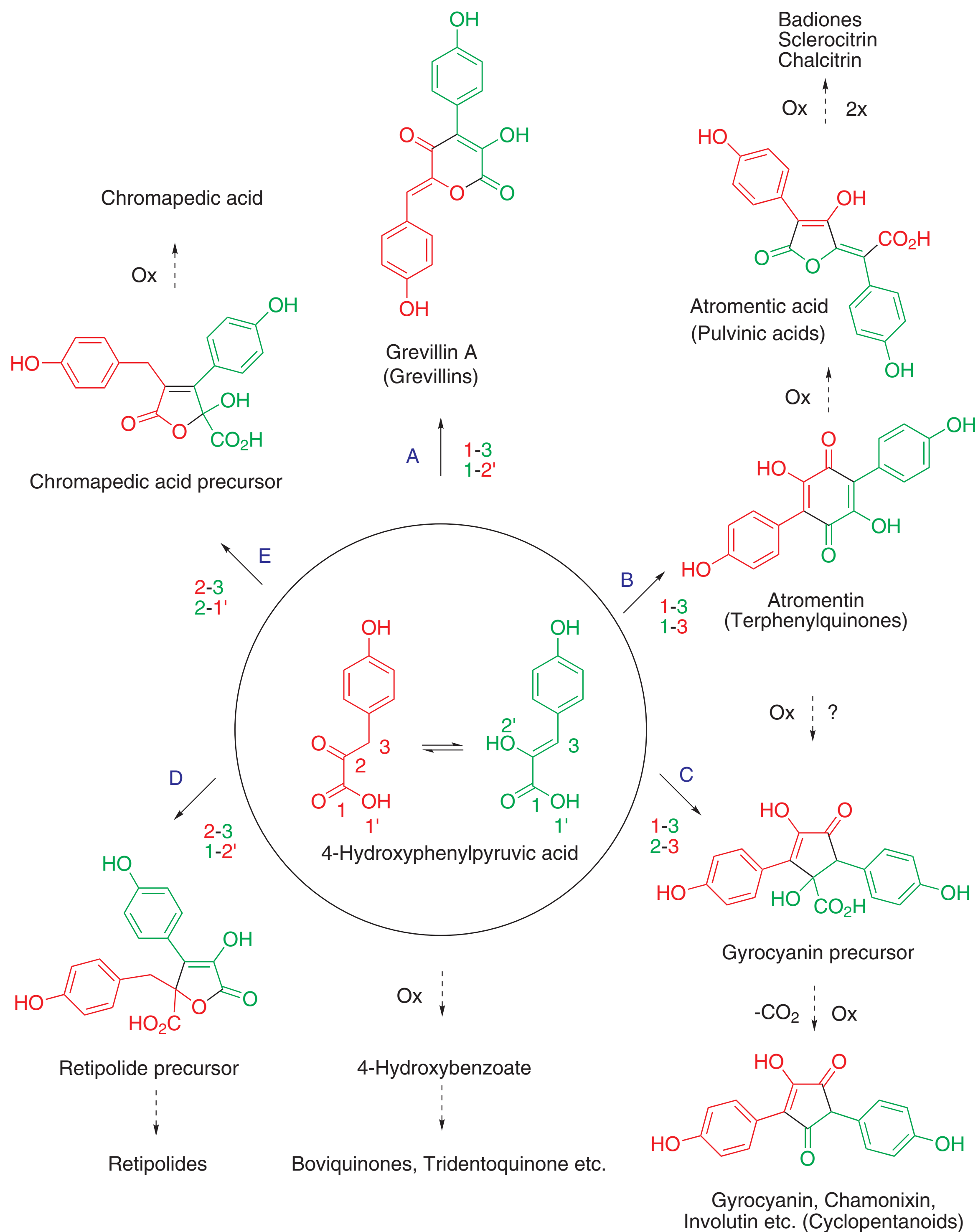
Leccinum chromapes shows a highly characteristic pigment pattern: the rare methyl isoxerocomate (**2b**) is responsible for the bright-yellow color of the stipe base and variegatorubin (**5**) for the pink color of the cap. Since some species of the newly established Boletaceae genus *Zangia* from East Asia exhibit very similar morphological characters [22], it would be prudent to establish if they contain the same pigments.

Chromapedic acid (**7**) represents a new type of dimer formed from 4-hydroxyphenylpyruvic acid [23]. It complements the four possible cyclization modes of this acid in boletes, depicted in Scheme 1: (A) grevillin cyclization (→ grevillin A) [24], (B) terphenylquinone cyclization (→ atromentin) [24], (C) cyclopentanone cyclization (→ gyrocyanin precursor) [25, 26], and (D) retipolide cyclization (→ retipolide precursor) [27].

Recent additions to the cyclopentanone cyclization C are the tylopilusins, which may be formed from the methyl ester of the gyrocyanin precursor *via* water elimination and epoxidation of the resulting double bond. Opening of the oxirane ring with water or methanol, respectively, may then yield the tylopilusins A and B [28], whereas proton-catalyzed ring enlargement of the oxirane intermediate may afford the unusual 4*H*-pyran-4-one derivative tylopilusin C [29].

Interestingly, cyclization mode D is also used for the biosynthesis of a metabolite from *Aspergillus terreus* var. *africanus* [30] and the xenofuranones from cultures of the bacterium *Xenorhabdus szentirmaii* [31]. Cyclizations A to C use an ester condensation (1 – 3 bond formation) as the first step, whereas the chromapedic acid and retipolide cyclizations apparently commence with an aldol addition (2 – 3 bond formation). The different dimerization products are then modified by the introduction of additional hydroxy groups, oxidative ring cleavage (atromentin → atromentic acid), or the incorporation of tyrosol as a building block (retipolides) [27]. The biosynthetically most advanced pigments in this scheme are the badiones, sclerocitrin and chalcitrin, which are formed by three different modes of oxidative dimerization from 3,4,4′-trihydroxypulvinic acid (xerocomic acid), followed by molecular rearrangements [32 – 34].

Thus, mushrooms of the order Boletales are able to produce an impressive variety of chromophores from a single building block, 4-hydroxyphenylpyruvic acid. The scheme may even be expanded by including the boviquinones, bovilactones and tridentoquinone, which are formed from 4-hydroxybenzoate *via* L-tyrosine [35, 36]. With the advent of molecular biology, there is now scope for a detailed analysis of the genes and enzymes responsible for all of these specific dimerizations [37, 38].



Scheme 1. Biogenetic relationships of Boletales pigments derived from 4-hydroxyphenylpyruvate (the positions of the newly formed bonds are indicated by the colored number pairs).

Experimental Section

General

Melting points (uncorrected): Reichert Thermovar hot-stage apparatus. Optical rotations: Perkin-Elmer 241 polarimeter. IR: Bruker IFS 45 FTIR spectrophotometer. Intensity of the bands: ss (very strong), s (strong), m (medium), and w (weak). UV/Vis spectra: Hewlett Packard 8452A Diode Array spectrophotometer. NMR: Bruker AMX 600 instrument; samples in CD_3CN, CD_3OD, or [D$_6$]acetone with the solvent peak as internal standard. MS: Finnigan MAT 90 and 95 Q spectrometers (direct inlet, 70 eV, 200 °C). All solvents were distilled before use. Evaporation of the solvents was performed under reduced pressure using a rotary evaporator. Analytical TLC: silica gel 60 F_{254} aluminum foils (Merck); solvent system A (v/v): toluene-HCO_2Et-HCO_2H (10 : 5 : 3), solvent system B: hexanes-EtOAc (1 : 2). Column chromatography: Sephadex LH-20 (Pharmacia). Analytical HPLC: Waters 600 E pump and system controller with 990+ photodiode array detector [RP-8; solvent A: MeCN-water (1 : 9) + 0.1 % TFA, solvent B: MeCN + 0.1 % TFA; gradient: start 100 % A, 60 min: 50 % A and 50 % B, 65 min: 100 % B; flow rate: 1 mL · min^{-1}]. Preparative HPLC: Waters Millipore system with an M 680 gradient controller, two M 590 EF pumps, and a U 6 K injector equipped with a Knauer variable wavelength monitor with a super-preparative flow cell. Pre-filtration of the solutions over Sep-Pak RP-18 cartridges (Waters), Nucleosil 100 C8 and C18 pre-packed HPLC columns (Macherey-Nagel), and gradient systems with MeCN-H_2O mixtures were used.

Fungal material

The American samples of*Leccinum chromapes* were collected in the Acadia National Park, Maine (*leg. et det.* N. Arnold, W. Helfer, W. Steglich 1996, 1998) and in the White Mountain National Forest, Burnt Mill Brook, New Hampshire (USA) (*leg. et det*. N. Arnold 1999). The Japanese collection was obtained near Okuike, Ashiya, Hyogo (*leg. et det*. R. Marumoto 1997).

Isolation of metabolites from the stalks

The air-dried stalks (44.4 g) of the American collection were pulverized, defatted with hexanes (2 × 1 L), and shaken for 2 h with MeOH (2 × 1 L). The mixture was filtered, and the solvent was removed under reduced pressure. The resulting residue was distributed between water (0.5 L) and EtOAc (1 L), and the aqueous phase was extracted with EtOAc (2 × 0.5 L). Then, the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure to afford an orange-brown residue (9.2 g), which was dissolved in a small volume of MeCN-water (1 : 1), filtered over RP-8 cartridges, and purified by preparative HPLC [Nucleosil 100 C8, 7 μm, 250 × 16 mm; solvent A: MeCN-water (1 : 9) + 0.1 % TFA, solvent B: MeCN + 0.1 % TFA; gradient: start 100 % A, 60 min: 100 % A, 65 min: 50 % A and 50 % B, 70 min: 100 % B; flow rate: 7 mL · min^{-1}] to yield the colorless acetophenones **1a** (t_R = 23.7 min) and **1b** (t_R = 31.6 min), and the orange-yellow pulvinic acids **2a** (t_R = 42.1 min), **3** (t_R = 46.1 min) and **2b** (t_R = 54.9 min). Workup of the air-dried stalks (4.1 g) with EtOH instead of MeOH afforded the same set of compounds.

*3′,4′-Dihydroxyacetophenone (**1a**)*

Yield 71 mg (0.16 % of dried stalks), colorless crystals, m. p. 116 – 119 °C (lit.: 116 °C [39]). – R_f (TLC) = 0.37 (solvent system A), greenish-black spot with $FeCl_3$. – t_R (analytical HPLC) = 11.9 min. – ^{1}H NMR (600 MHz, CD_3OD): δ = 2.54 (s, 3 H, Me), 6.86 (d, J = 8.0 Hz, 1 H, 5′-H), 7.45 (d, J = 1.5 Hz, 1 H, 2′-H), 7.47 (dd, J = 8.0, 1.5 Hz, 1 H, 6′-H). – ^{13}C NMR (150 MHz, CD_3OD): δ = 26.5 (Me), 116.1 (CH-5′), 116.4 (CH-2′), 123.8 (CH-6′), 131.0 (C-1′), 146.7 (C-3′), 152.6 (C-4′), 200.0 (C=O). – MS (EI): *m/z* (%) = 152 (66) [M]$^+$, 138 (39), 137 (100), 123 (5), 121 (31), 109 (25), 81 (7), 69 (4), 55 (6), 43 (16). – HRMS (EI): *m/z* = 152.0471 (calcd. 152.0474 for $C_8H_8O_3$, [M]$^+$).

*4′-Hydroxyacetophenone (**1b**)*

Yield 42 mg (0.1 % of dried stalks), colorless crystals, m. p. 106–109 °C (lit.: 109 °C [39]). – R_f (TLC) = 0.47 (solvent system A), no reaction with $FeCl_3$. – t_R (analytical HPLC) = 16.9 min. – ^{1}H NMR (600 MHz, CD_3OD): δ = 2.57 (s, 3 H, Me), 6.88 (br d, J = 8.8 Hz, 2 H, 3′-H, 5′-H), 7.93 (br d, J = 8.8 Hz, 2 H, 2′-H, 6′-H). – ^{13}C NMR (150 MHz, CD_3OD): δ = 26.6 (Me), 116.5 (CH-3′, CH-5′), 130.5 (CH-2′, CH-6′), 132.4 (C-1′), 164.2 (C-4′), 199.8 (C=O). – MS (EI): *m/z* (%) = 136 (36) [M]$^+$, 122 (8), 121 (100), 93 (23), 65 (12), 43 (5). – HRMS (EI): *m/z* = 136.0523 (calcd. 136.0524 for $C_8H_8O_2$, [M]$^+$).

*Isoxerocomic acid (**2a**)*

Yield 36 mg (0.08 % of dried stalks), orange-yellow solid. – R_f (TLC) = 0.33 (solvent system A). – t_R (analytical HPLC) = 28.0 min. – UV/Vis$_{qual}$ (MeOH): λ_{max} = 203, 260, 375 nm. – MS (EI): *m/z* (%) = 338 (8) [M–H_2O]$^+$, 282 (4), 207 (11), 149 (5), 137 (12), 133 (3), 123 (19), 121 (32), 107 (32), 105 (7), 77 (7), 44 (100). – HRMS (EI): *m/z* = 338.0456 (calcd. 338.0427 for $C_{18}H_{10}O_7$, [M–H_2O]$^+$). Identical in all aspects (UV, NMR, MS, TLC, co-HPLC) with an authentic sample.

*Methyl isoxerocomate (**2b**)*

Yield 129 mg (0.29 % of dried stalks), orange-yellow solid, m. p. 215–220 °C. – R_f (TLC) = 0.47 (solvent sys-

tem B). – t_R (analytical HPLC) = 43.0 min. – UV/Vis (MeCN): $\lambda_{max}(\lg\varepsilon_{max})$ = 248 (4.043), 272 (4.083), 402 nm (3.975). – IR (KBr): ν = 3435 (br, ss), 1746 (w), 1677 (m), 1607 (s), 1516 (m), 1436 (m), 1369 (w), 1279 (m), 1181 (m), 1151 (w), 1118 (w), 1067 (m), 1002 (w), 956 (w), 840 (w), 769 (w) cm^{-1}. – ^1H NMR (600 MHz, $[D_6]$acetone): δ = 3.89 (s, 3 H, OMe), 6.73 (dd, J = 8.1, 2.0 Hz, 1 H, 6′-H), 6.86 (d, J = 8.1 Hz, 1 H, 5′-H), 6.89 (d, J = 2.0 Hz, 1 H, 2′-H), 6.91 (d, J = 8.9 Hz, 2 H, 3-H, 5-H), 7.98 (d, J = 8.9 Hz, 2 H, 2-H, 6-H). – ^{13}C NMR (150 MHz, $[D_6]$acetone): δ = 54.7 (OMe), 105.3, 115.6 (CH-5′), 116.1 (CH-3, CH-5), 116.6, 118.4 (CH-2′), 121.7, 123.1 (CH-6′), 125.1, 130.1 (CH-2, CH-6), 145.2, 146.3, 154.3, 158.4, 159.5, 166.9, 172.9. – MS (EI): m/z (%) = 370 (38) $[M]^+$, 338 (100) $[M–MeOH]^+$, 310 (7), 282 (46), 226 (28), 179 (54), 177 (10), 161 (12), 149 (21), 135 (43), 133 (21), 121 (18), 105 (19), 77 (11), 44 (63). – HRMS (EI): m/z = 370.0677 (calcd. 370.0689 for $C_{19}H_{14}O_8$, $[M]^+$), 338.0420 (calcd. 338.0427 for $C_{18}H_{10}O_7$, $[M–MeOH]^+$).

*Atromentic acid (**3**)*

Yield 40 mg (0.09 % of dried stalks), orange-yellow solid. – R_f (TLC) = 0.43 (solvent system A). – t_R (analytical HPLC) = 33.2 min. Identical in all aspects (UV, NMR, MS, TLC, co-HPLC) with an authentic sample.

Isolation of metabolites from the cap skin

The cap skins (24.7 g) of air-dried fruit bodies of the American collection were separated and treated with a small volume of water (*ca.* 10 – 20 mL). After 10 min, the material was extracted with several 900-mL portions of acetone, acidified with aqueous HCl to pH ∼5, until the extracts remained colorless. The combined orange acetone solutions were concentrated at 40 °C, and the remaining aqueous residue was extracted with EtOAc (4 × 150 mL). The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure to yield a brown-red residue (1.85 g), which was dissolved in acetone-MeOH (15 mL, 4 : 1, v/v) and chromatographed with the same solvent mixture on a Sephadex LH-20 column. The fractions containing the red pigment were purified by preparative HPLC [Nucleosil 100 C18, 7 μm, 250 × 16 mm; solvent A: MeCN-water (1 : 9) + 0.1 % TFA, solvent B: MeCN; gradient: start 100 % A, 10 min: 100 % A, 50 min: 100 % B; flow rate: 6.8 mL · min^{-1}] to yield the yellowish chromapedic acid (**7**) (t_R = 16.3 min) and the red variegatorubin (**5**) (t_R = 35.1 min), along with minute amounts of the orange-yellow pulvinic acids **2a** and **3**.

*Variegatorubin (**5**)*

Yield 9.6 mg (0.039 % of dried cap skins), dark-red solid. – R_f (TLC) = 0.46 (solvent system A). Identical in all aspects (UV, NMR, MS, TLC, co-HPLC) with an authentic sample.

*Chromapedic acid, 4-(3,4-dihydroxybenzyl)-3-(3,4-dihydroxyphenyl)-2-hydroxy-5-oxo-2,5-dihydrofuran-2-carboxylic acid (**7**)*

Yield 8.7 mg (0.034 % of dried cap skins), yellowish oil. – R_f (TLC) = 0.07 (solvent system A), blue-green spot with $FeCl_3$. – $[\alpha]_D^{20}$ = +4 (c = 1.6, MeCN). – UV/Vis (MeOH): $\lambda_{max}(\log\varepsilon_{max})$ = 211 (3.49), 249 (3.22), 296 (3.18), 304 (3.19), 323 nm (3.19). – IR (KBr): ν = 3435 (ss), 2924 (w), 1757 (w), 1629 (m), 1521 (w), 1287 (w), 1024 (w), 997 (w) cm^{-1}. – ^1H NMR (600 MHz, CD_3OD): δ = 3.71 (d, J = 15.6 Hz, 1 H, 7-H_a), 3.78 (d, J = 15.6 Hz, 1 H, 7-H_b), 6.56 (dd, J = 8.4, 2.4 Hz, 1 H, 6″-H), 6.67 (d, J = 8.4 Hz, 1 H, 5″-H), 6.70 (d, J = 2.4 Hz, 1 H, 2″-H), 6.76 (d, J = 8.4 Hz, 1 H, 5′-H), 7.03 (dd, J = 8.4, 2.4 Hz, 1 H, 6′-H), 7.19 (d, J = 2.4 Hz, 1 H, 2′-H). – ^{13}C NMR (150 MHz, CD_3OD): δ = 31.4 (C-7), 100.9 (C-2), 117.3 (CH-5″), 117.5 (CH-5′, CH-2″), 117.9 (CH-2′), 121.5 (CH-6″), 123.9 (CH-6′), 124.0 (C-1′), 128.1 (C-4), 131.3 (C-1″), 145.9 (C-4″), 147.4 (C-3′, C-3″), 150.1 (C-4′), 159.1 (C-3), 171.3 (C-6), 175.8 (C-5). – MS: A meaningful mass spectrum could not be obtained in either the EI or FAB mode.

*3-(3,4-Dihydroxybenzyl)-4-(3,4-dihydroxyphenyl)furan-2,5-dione (**8**)*

Dione **8** was slowly formed from **7** when exposed to air, and was separated and purified by HPLC as described for the cap skin solution, t_R = 15.4 min. Yield 0.3 mg, yellow solid. – R_f (TLC) = 0.47 (solvent system A). – UV/Vis_{qual} (MeOH): λ_{max} = 207, 272, 384 nm. – ^1H NMR (600 MHz, CD_3CN): δ = 3.85 (s, 2 H, CH_2), 6.60 (dd, J = 7.8, 1.7 Hz, 1 H, 6′-H), 6.73 (d, J = 1.7 Hz, 1 H, 2′-H), 6.74 (d, J = 7.8 Hz, 1 H, 5′-H), 6.93 (d, J = 8.3 Hz, 1 H, 5″-H), 7.12 (dd, J = 8.3, 2.2 Hz, 1 H, 6″-H), 7.17 (d, J = 2.2 Hz, 1 H, 2″-H). – MS (EI): m/z (%) = 328 (82) $[M]^+$, 310 (40), 282 (18), 255 (100) $[M–CO–CO_2H]^+$, 239 (15), 237 (14), 210 (25), 181 (11), 163 (18), 152 (54), 133 (7). – HRMS (EI): m/z = 328.0474 (calcd. 328.0583 for $C_{17}H_{12}O_7$, $[M]^+$).

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