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A Novel Type of Triterpenoid Quinone Methide Pigment from the Toadstool *Russula flavida* (Agaricales)

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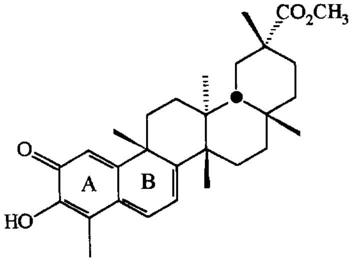
Abstract: The structures of two quinone methide pigments from fruiting bodies of *Russula flavida* have been elucidated. Russulaflavidin and its dihydro derivative contain the same chromophore as pristimerin and represent the first triterpenoids with a shionane (D:A friedo-18,19-secolupane) skeleton isolated from fungi.

Russula flavida (Frost & Peck apud Peck) is a bright orange-yellow agaric which grows in mixed woods with pines or oaks in the eastern USA, Japan and China. Its pigments are confined to the outer layer of the fruiting bodies and are soluble in organic solvents such as *tert*-butyl methyl ether. Gel chromatography of the *n*-hexane extract on Sephadex LH-20 followed by preparative HPLC yielded a mixture of two orange yellow pigments which were finally separated by isocratic HPLC on silica gel under carefully defined conditions.

The main pigment, russulaflavidin (**2**), $C_{28}H_{36}O_3$, is readily decolorized by the addition of sodium borohydride to its ethanolic solution. Its UV/Vis maxima at 240 nm (lg ε = 3.85) and 400 nm (3.54) and IR bands at 3440 and 1600 cm^{-1} are similar to those of pristimerin (**1**)[1] and indicate the presence of an extended quinone methide system.



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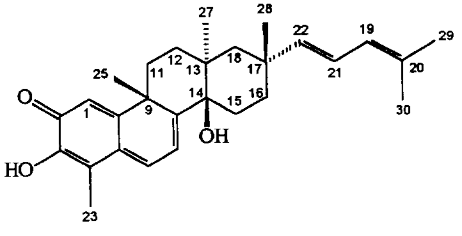

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This is confirmed by the ^1H NMR and ^{13}C NMR signals of russulaflavidin (Table 1) which correspond closely to those of **1**[2] so that **2** must therefore contain the same A and B rings. A further set of olefinic signals at δ 5.51 (d, J = 15.5 Hz), 6.16 (dd, 15.5 and 10.5 Hz), and 5.77 (d, 10.5 Hz) and two methyl signals at δ_H 1.73 can be ascribed to a (*E*)-4-methyl-penta-1,3-dienyl side chain.

Table 1. ^{1}H and ^{13}C NMR spectra of russulaflavidin (**2**) in comparison with selected data of pristimerin (**1**)^2 (400 and 100.6 MHz, respectively, ppm, J in Hz, $CDCl_3$)

Position[a]	δ_C (**2**)		J	δ_H (**2**)		J	^2J,^3J\{^{1}H-^{13}C\}	δ_C (**1**)	δ_H (**1**)		J
1	120.08	D	161	6.53	d	1.5		119,6	6.53	d	1.3
2	178.74	s, br.					3-OH	178.4			
3	146.08	ddq	5, 5, 5				1, 23, 3-OH	146.1			
3-OH				6.97	s				6.99	s	
4	116.62	ddq	6, 6, 6				3-OH, 6, 23	117.0			
5	129.10	m					1, 7, 23	127.5			
6	132.70	D	159	6.98	dd	7, 1.5		133.9	7.02	dd	7, 1.3
7	118.30	D	160	6.38	d	7		118.1	6.35	d	7
8[b]	163.31	dq	4, 4				6, 25	169.9			
9	43.36	m					1, 7, 12α, 25	42.9			
10[b]	160.87	m					6, 11β, 25	164.7			
11	34.29	Tm	130	Hα 1.80	m		12β, 25				
				Hβ 2.08	ddd	14, 5, 2.5					
12	31.57	Tm	128	Hα 1.08	ddd	14, 5, 2.5	27				
				Hβ 2.21	ddd	14, 14, 5					
13	39.68	m					11β, 12α, 15β, 18β, 27				
14	75.28	m					12α, 15β, 16α, 18α, 27				
15	28.91	Tm	123	Hα 1.97	ddd	14, 14, 6	16α				
				Hβ 1.64	ddd	14, 4, 3					
16	30.02	Tm	128	Hα 1.95	dm	14	18α, 28				
				Hβ 1.83	m						
17	35.48	m					18α, 18β, 15β, 28				
18	47.76	Tm	126	Hα 1.25	dd	13.5, 2	16α, 27, 28				
				Hβ 1.77	d	13.5					
19	125.47	Dm	150	5.77	dm	10.5	22, 29, 30				
20	132.70	m					29, 30				
21	121.20	D	148	6.16	dd	10.5, 15.5					
22	142.42	Dm	148	5.51	d	15.5	16β, 18β, 28				
23	10.32	Q	128	2.18	s			10.2	2.21	s	
25	36.11	Qm	131	1.56	s		11α, 11β	38.3	1.45	s	
27	20.73	Qm	125	0.66	s		12β, 18α, 18β				
28	33.81	Q	127	0.97	s		18β				
29	25.95	Qm	126	1.73	s		19, 30				
30	18.37	Qm	126	1.73	s		19, 29				

a) Numbering as in D:A-friedo-18,19-secolupane according to *Chem. Abstr.* **1992**, *117*, 4221 CS.

b) Assignments may be interchanged.

According to the DEPT spectrum, the remaining ten carbon atoms can be assigned to two methyl groups, five methylenes and three quarternary carbons. A DQF {^{1}H-^{1}H} COSY experiment[3] determined the arrangement of the methylene groups in two isolated CH_2CH_2 units and a single methylene group.

The separate units can be connected via their {^{1}H-^{13}C} long range couplings (Fig. 1). Thus, the ethylene carbons at δ_C 34.29 and 31.57 are coupled to the angular methyl groups at C-9 and C-13, respectively, and can therefore be assigned to C-11 and C-12. The isolated methylene group at δ_C 47.76 exhibits long-range couplings to both quaternary methyl groups at C-13 and C-17. Since the methyl group at C-17 couples both to the dienyl side chain and one of the methylene groups of the second ethylene unit, the latter must represent C-15 and C-16. Finally, the free valences at C-8, C-13 and C-15 can be joined to the remaining quarternary C(OH) center at δ_C 75.28 which leads to formula **2** for russulaflavidin.

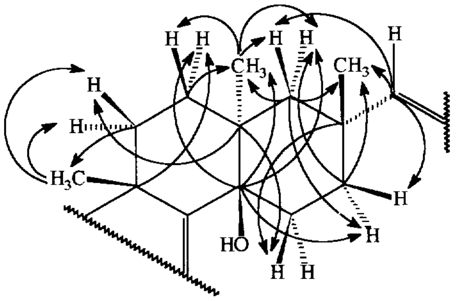
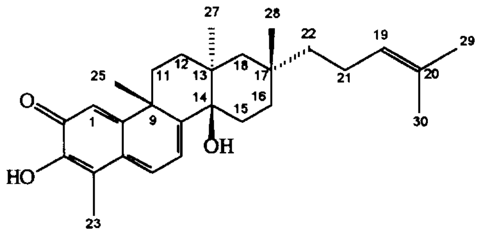


Fig. 1. Selected correlations observed in the long-range {^{1}H-^{13}C} COSY spectrum of **2**

The minor pigment, $C_{28}H_{38}O_3$, differs from russulaflavidin only in that it has two additional hydrogen atoms which are located in a $CH_2CH_2CH{=}C(CH_3)_2$ side chain. formula **3** for dihydro-russulaflavidin is in accordance with its spectroscopic data. Both pigments exhibit nearly identical MS fragmentations at masses below *m/z* = 300.



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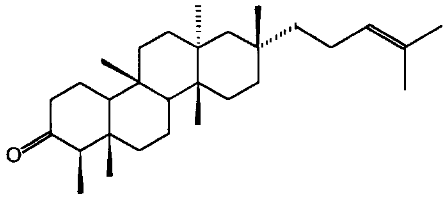

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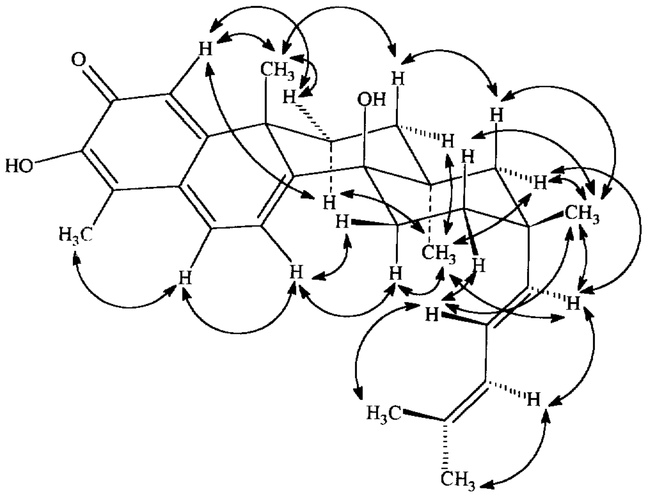

Fig. 2. NOEs observed for russulaflavidin (**2**)

The relative stereochemistry of russulaflavidin follows from its NOE difference spectra and phase sensitive NOESY experiments[4] (Fig. 2). Since strong NOE interactions are observed between the axial hydrogen at C-12 and both the angular methyl group at C-9 and 18-Hβ, the latter substituents must occupy axial positions. The observed NOE correlation between H-18β and the neighbouring methyl group at C-17 proves the axial conformation of the dienyl side chain at the same carbon. The methyl group at C-13 exhibits NOEs to the axial protons at C-11 and C-15 and must therefore be axial. This proves the all-chair conformation of the *trans*-decalin system and explains why the olefinic proton at C-7 exhibits NOEs to both protons at C-15.

The absolute configuration of the *Russula* pigments was established by comparing their CD spectra with that of pristimerin (**1**). The absolute configuration of this compound has been unambiguously determined by X-ray crystallography.[5] As can be seen from Fig. 3, the CD spectra of dihydrorussulaflavidin (**3**) and **1** are nearly identical which confirms the absolute configuration of the pigments as given in the formulas.

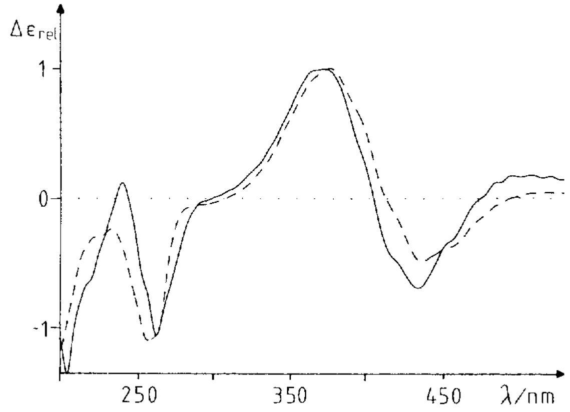

Fig. 3. CD spectra of dihydrorussulaflavidin (**3**) (——) and pristimerin (**1**) (----) in *n*-hexane

Russulaflavidin and its dihydro derivative are the first natural products with a 24,26-bisnorshionane skeleton. Their stereochemistry corresponds to that of shionone (**4**), a constituent of several *Aster* species.[6] Interestingly, the *Russula* compounds contain the same chromophore as members of the nor-friedelane family of quinone methides from Celastraceae and Hippocrateaceae.[7] These compounds exhibit interesting antibacterial and antitumor activities.[8]

Specimens of *Russula flavida* from Japan and the eastern USA can not be differentiated by TLC or HPLC analysis of their extracts which points to a surprisingly high conservation of their secondary metabolites despite the long geographic separation of east Asia and eastern North America.[9]

To our knowledge, *R. flavida* is the only *Russula* which produces lipophilic pigments. All other species investigated so far contain highly polar pigments related to the riboflavin and pteridine metabolism.[10]

EXPERIMENTAL SECTION

General. NMR spectra were obtained on Bruker AM 400 and AMX 600 spectrometers in deuteriochloroform solutions with solvent peak as the internal standard. High resolution MS were obtained on an AEI MS 50 instrument at 35 eV. UV spectra were taken with a Hewlett-Packard 8452 A diode array spectrophotometer and CD spectra with an Instruments S.A. Jobin Yvon CD6 Dichrograph. Optical rotations were measured with a Perkin Elmer 214 polarimeter. IR spectra were recorded on a Perkin Elmer 1420 infrared spectrophotometer. Melting points were determined using a Reichert hot-stage microscope and are uncorrected. Solid phase extraction was performed on Macherey-Nagel Chromabond $(OH)_2$ cartridges. TLC was carried out on Merck silica gel 60 F_{254} aluminium sheets. Column chromatography was performed using Sephadex LH-20 (Pharmacia).

Preparative HPLC was performed at room temperature with two type 590EF pumps, gradient controller 680, injector U6K (Waters/Millipore) and a Knauer Variable Wavelength Monitor with a superpreparative flow cell. System 1: 16 x 250 mm column and 16 x 30 mm guard column with LiChrosorb Diol, 7 μm (Merck), linear gradient, 0 min: *n*-hexane/*tert*-butyl methyl ether = 5:1, 60 min: *tert*-butyl methyl ether/2-propanol = 5:1, flowrate 6.8 ml min^{-1}, detection at 410 nm. System 2: 16 x 250 mm column and 16 x 30 mm guard column with Nucleosil 50, 5 μm (Macherey-Nagel), eluent *n*-hexane/acetone = 7:3, flowrate 4.5 ml min^{-1}, detection at 410 nm.

Analytical HPLC was performed at room temperature with two type 510 pumps, gradient controller 721/710, injector U6K, photodiode array detector 990 (Waters/Millipore). System 3: 4 x 250 mm column and 4 x 30 mm guard column with LiChrosorb Diol, 7 μm (Merck), linear gradient, 0 min: *n*-hexane/*tert*-butyl methyl ether = 5:1, 60 min: *tert*-butyl methyl ether/2-propanol = 5:1, flowrate 1.0 ml min^{-1}, range of detection 230-550 nm. System 4: 4 x 250 mm column with Nucleosil 50, 5 μm (Macherey-Nagel), eluent *n*-hexane/acetone = 7:3, flowrate 0.3 ml min^{-1}, range of detection 330-550 nm.

The fruiting bodies were collected at Coweeta, Macon County, North Carolina, USA and Okuike, Ashiya, Hyogo, Japan.

Isolation of the pigments. Fresh fruiting bodies of *Russula flavida* were dried in a stream of warm air (Sigg Dörrex) and lyophilized (Leybold Lyovac GT2) to remove traces of humidity. 28 g of this material was pulverized and extracted subsequently with *n*-hexane (3 x 600 ml) and *tert*-butyl methyl ether (3 x 600 ml). Evaporation of the combined yellow extracts *in vacuo* at room temperature yielded an orange-colored oily residue (354 mg). This was dissolved in 2-propanol and after filtering off some ergosterol was separated by chromatography on a column with Sephadex LH-20 (eluent: 2-propanol). The yellow fractions afforded an orange, glassy residue (32 mg) which was dissolved in *tert*-butyl methyl ether and filtered through a cartouche filled with diol phase to remove a green impurity. Preparative gradient HPLC (diol phase, system 1) afforded a mixture of the pure pigments (17 mg) which was separated by isocratic preparative HPLC on silica gel (system 2). This procedure caused severe losses of material, however, it was the only way to separate the two components. Dihydrorussulaflavidin (**3**) (1.5 mg) was eluted first and proved to be pure whereas russulaflavidin (**2**) (5.5 mg) still contained traces of **3**. Both compounds were obtained as yellow powders on concentration of their solutions in *tert*-butyl methyl ether.

Russulaflavidin **(2)**. Amorphous powder, m. p. 78-80 °C, soluble in organic solvents such as *n*-hexane, ether, ethanol and chloroform, $[\alpha]^{23}_{D}$ = -130 (*c* =0.10, chloroform), TLC: R_f = 0.33 (silica gel, *n*-hexane/2-propanol = 10:1; yellow spot), HPLC: t_R = 27 min (system 3), 13 min (system 4); UV (*n*-hexane): λ_{max} (lg ε) = 240 nm (3.85), 394 (3.54); CD (*n*-hexane): λ ($\Delta\varepsilon$) = 202 nm (-2.5), 218 sh (-0.5), 230 (0), 241 (+1.9), 252 (0), 263 (-2.2), 289 sh (-0.2), 306 (0), 368 (+1.8), 399 (0), 416 sh (-1.6), 434 (-2.1), 453 sh (-1.2); IR (KBr): ν_{max} = 3440 cm^{-1} (s, br.), 2970 (w), 2940 (m), 2865 (w), 1740 (w), 1635 sh, 1600 (m), 1520 (w), 1450 (w), 1380 (w), 1270 (s), 1215 (w), 1100 (m), 1025 (m), 875 (w), 805 (s); ^{1}H NMR and ^{13}C NMR (see Table 1); MS (EI, DI 210 °C, 35 eV): *m/z* (%) = 420.2676 (14, M^+, calc. for $C_{28}H_{36}O_3$: 420.2664), 214 (100, $C_{14}H_{14}O_2$), 201 (38, $C_{13}H_{13}O_2$), 199 (16, $C_{13}H_{11}O_2$), 196 (23, $C_{14}H_{12}O$).

Dihydrorussulaflavidin **(3)**. Amorphous powder, m. p. 81-83 °C, soluble in organic solvents such as *n*-hexane, ether, ethanol and chloroform, $[\alpha]^{23}_{D}$ = -125 (*c* =0.04, chloroform), TLC: R_f = 0.33 (silica gel, *n*-hexane/2-propanol = 10:1; yellow spot), HPLC: t_R = 27 min (system 3), 12 min (system 4); UV (*n*-hexane): λ_{max} (lg ε) = 234 nm (3.90), 390 (3.90); CD (*n*-hexane): λ ($\Delta\varepsilon$) = 204 nm (-1.3), 216 sh (-0.6), 236 (0), 240 (+0.2), 245 (0), 263 (-1.0), 292 sh (0), 373 (+1.0), 406 (0), 420 sh (-0.4), 434 (-0.6), 453 sh (-0.3); IR (KBr): ν_{max} = 3440 cm^{-1} (m, br.), 2960 (m), 2930 (m), 2860 (w), 1730 (w), 1625 sh, 1600 (m), 1520 (w), 1445 (w), 1380 (w), 1275 (s), 1210 (w), 1105 (s), 1030 (s), 870 (w), 810 (s); MS (EI, DI 220 °C, 35 eV): *m/z* (%) = 422.2831 (18, M^+, calc. for $C_{28}H_{38}O_3$: 422.2821), 214 (100, $C_{14}H_{14}O_2$), 201 (38, $C_{13}H_{13}O_2$), 199 (19, $C_{13}H_{11}O_2$), 196 (36, $C_{14}H_{12}O$).

Table 2. ^1H and ^{13}C NMR spectra of dihydrorussulaflavidin (3) (400 and 100.6 MHz, respectively, ppm, J in Hz, $CDCl_3$)

Pos.	δ_C (3)	δ_H (3)		J	Pos.	δ_C (3)	δ_H (3)		J
1	120.14	6.54	d	1.5	15	28.28	H_α 2.02	ddd	14, 14, 4
2	178.73						H_β 1.62	ddd	14, 4, 3.5
3	146.08				16	34.25	H_α 1.45	dm	14
3-OH		6.97	s				H_β 1.77	ddd	14, 14, 4
4	116.63				17	32.44			
5	129.10				18	42.86	H_α 1.31	dd	14, 2
6	132.73	7.00	dd	7, 1.5			H_β 1.48	d	14
7	118.12	6.40	d	7	19	124.82	5.06	ddm	7, 7
8	163.48				20	131.16			
9	43.39				21	22.92	1.92	m	
10	160.98				22	40.33	1.15	ddd	14, 13, 5
11	34.44	H_α 1.82	ddd	14, 14, 5.5			1.67	dd	14, 6
		H_β 2.12	ddd	14, 5, 2	23	10.33	2.20	s	
12	31.93	H_α 1.11	ddd	14, 5.5, 2	25	36.34	1.56	s	
		H_β 2.22	ddd	14, 14, 5	27	20.57	0.77	s	
13	39.34				28	31.93	0.93	s	
14	75.47				29	25.71	1.65	s	
					30	17.60	1.54	s	

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