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ALKALOIDAL PIGMENTS FROM *LACTARIUS NECATOR* AND *L. ATROVIRIDIS*

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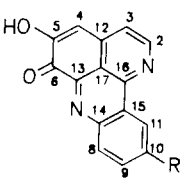
Key Word Index—*Lactarius necator*; *L. atroviridis*; Agaricales; Russulaceae; gilled toadstools; alkaloids; pigments; necatorone; dehydrodimers of necatorone and 10-deoxynecatorone.

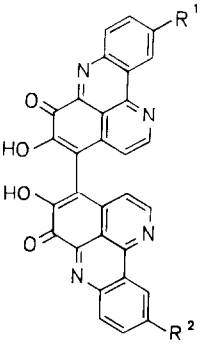
Abstract—Two new alkaloids, 4,4′-binecatorone and 10-deoxy-4,4′-binecatorone, have been isolated from fruiting bodies of *Lactarius necator*. The American species *L. atroviridis* contains 10,10′-dideoxy-4,4′-binecatorone as the main alkaloid which is responsible for the green appearance of this toadstool.

INTRODUCTION

Recently we reported on the isolation and structural elucidation of necatorone (**1**), an alkaloidal pigment from *Lactarius necator* (Bull. em Pers. ex Fr.) Karst. [syn. *L. turpis* (Weinm.) Fr.] [1]. Its 5,10-dihydroxy-6-oxo-dibenzo [*de*, *h*] [1, 6] naphthyridine structure has been confirmed by a total synthesis [2]. Compound **1** is of considerable interest because of its pronounced mutagenicity in the Ames test [3–5]. This paper deals with an improved isolation procedure and the structural elucidation of further pigments from *L. necator* and *L. atroviridis* (Peck).



	R
1	OH
2	H



	R^1	R^2
3	OH	OH
4	OH	H
5	H	H

RESULTS AND DISCUSSION

The peeled skins from caps and stalks of the toadstools were thoroughly extracted with methanol. In order to eliminate large amounts of mannitol, the solvent was evaporated and the aqueous solution of the residue brought to pH 6 with boric acid. After exhaustive extraction with ethyl acetate and repeated chromatography on Sephadex LH-20, necatorone (**1**), 4,4′-binecatorone (**3**) and 10-deoxy-4,4′-binecatorone (**4**) could be isolated from *L. necator*. From the North American species *L. atroviridis* in addition to **1**, **3** and **4**, 10,10′-dideoxy-4,4′-binecatorone (**5**) was obtained as main alkaloid by the same procedure.

4,4′-Binecatorone (**3**) forms dark brown crystals which dissolve readily in DMSO to give a greenish brown colour. Like necatorone, **3** exhibits a purple colour reaction on addition of alkali. The UV and IR spectra of this pigment are very similar to those of necatorone and in the ^{1}H NMR spectrum (Table 1) only the singlet of 4-H at δ6.96 is missing. The lack of a hydrogen atom at C-4 is confirmed by the ^{1}H-coupled ^{13}C NMR spectrum (Table 2) in which this carbon gives rise to a singlet at δ115.0. The upfield shift of 3.9 ppm compared to necatorone excludes hydroxyl substitution at C-4. Because the other δ-values remain more or less unchanged, the assumption was made that the new pigment is the symmetrical 4,4′-dehydrodimer of necatorone. Unfortunately, no mass spectrum of the pigment could be obtained, neither by EI nor FAB. However, its *tetrakis*-trimethylsilyl derivative shows the expected molecular ion at *m/z* 814 and intense fragment ions at *m/z* 799 $[M-Me]^+$ and 786 $[M-CO]^+$ which are in accord with the proposed structure. Despite hindered rotation at the biaryl linkage, **3** exhibits no optical activity.

10,10′-Dideoxy-4,4′-binecatorone (**5**) is responsible for the dark green habit of *L. atroviridis*. It forms blackish green crystals which dissolve readily in methanol and DMSO to give a green-yellow or dark green colour, respectively. In contrast to the other *Lactarius* pigments, **5** gives a dove-grey colour with alkali. Its UV maxima show a hypsochromic shift in comparison to those of **1**. The ^{1}H NMR spectrum of 5 (Table 1) exhibits the typical signal pattern for an *ortho*-substituted aromatic ring. The proton at C-4 of necatorone is missing and the signals of the two isoquinoline protons are found at δ7.33 and 8.85. This leads to structure **5** for this pigment which is

Table 1. ^{1}H NMR spectral data of compounds **1**–**5** (400 MHz, δ-values)

H	**1** (DMSO-d_6)	**3** (DMSO-d_6)	**4** (CD_3OD)	**5** (DMSO-d_6)
2/2′	9.03 *d*	8.94/8.94 *d*	9.02 *d* 8.97 *d*	8.85/8.85 *d*
3/3′	7.66 *d*	7.51/7.51 *d*	7.60 *d* 7.54 *d*	7.33/7.33 *d*
4	6.96 *s*	—	—	—
8/8′	8.23 *d*	8.33/8.33 *d*	8.39 *d* 8.56 *dd*	8.42/8.42 *dd*
9/9′	7.50 *dd*	7.56/7.56 *dd*	7.61 *dd* 8.03* *ddd*	7.97/7.97* *ddd*
10/10′	—	—	— 8.09* *ddd*	8.03/8.03* *ddd*
11/11′	8.33 *d*	8.38/8.38 *d*	8.47 *d* 9.22 *dd*	9.10/9.10 *dd*

J (Hz): Compound **1**: 2, 3 = 4.5; 8, 9 = 9.0; 9,11 = 2.7; compound **3**: 2, 3 = 4.75; 8, 9 = 9.0; 9, 11 = 2.6; compound **5**: 2, 3 = 4.8; 8, 9 = 9, 10 = 10, 11 = 8.2; 8, 10 = 9, 11 = 1.5

*Values may be interchanged.

Table 2. ^{13}C NMR spectral data of compounds **1**, **3** and **5** (100.2 MHz, DMSO-d_6, δ-values, *J* values in Hz in parenthesis)

C	**1**	**3**	**5**
2/2′	154.16 *Dd* (180.4 + 3.2)	154.09 *Dd* (180.5 + 3)	154.50 *Dd* (176 + 2.4)
3/3′	120.93 *Ddd* (170 + 8 + 3.6)	120.06 *Ddd* (166 + 8.2 + 2)	119.83 *Ddd* (166 + 9 + 1)
4/4′	111.08 *Dd* (162.8 + 5.5)	114.96 *d* (5.2)	115.58 *d* (5.0)
5/5′	154.19 *d* (4.4)	152.85 *s*	157.11 *s*
6/6′	178.81 *d* (7.0)	178.82 *s*	180.24*s*
8/8′	133.75 *D* (163.6)	133.83 *D* (163.2)	131.18 *Dd* (163 + 7)
9/9′	122.53 *Dd* (162 + 5)	122.69 *Dd* (161 + 5.8)	130.84* *Ddd* (161 + 8.8 + 1)
10/10′	160.05 *ddd* (9.2 + 2 + 2)	160.29 *ddd* (10 + 2 + 1.5)	130.10* *Ddd* (163 + 8.2 + 1.5)
11/11′	105.79 *Dd* (163 + 4.8)	106.04 *Dd* (163 + 4.2)	123.58 *Dd* (165 + 7)
12/12′	137.65 *d* (6.2)	137.62 *d* (6.1)	139.89 *d* (5.0)
13/13′	143.41 *s*	143.43 *s*	147.71 *s*
14/14′	141.54 *dd* (8.4 + 6.0)	141.57 *dd* (8.8 + 6.0)	146.33 *dd* (8.2 + 6.4)
15/15′	128.40 *d* (5.6)	128.64 *d* (5.2)	126.14 *dd* (8.0 + 4.6)
16/16′	145.34 *ddd* (14 + 12 + 3)	145.55 *ddd* (12 + 3.5 + 2)	146.76 *dd* (12.4 + 3.6)
17/17′	113.86 *ddd* (8 + 7 + 2)	114.28 *dd* (7.1 + 2)	114.14 *d* (6.4)

*These assignments may be interchanged.

confirmed by the ^{13}C NMR spectrum (Table 2). The expected shift from δ 160.3 (in **3**) to 130.1 ppm is observed for C-10 whereas the other δ −values show only small changes. The mass spectrum of the bis-trimethylsilyl derivative shows the expected $[M]^+$ at *m/z* 638 which suffers subsequent losses of Me, CO and $(Me)_3Si$. Again no optical activity could be observed.

10-Deoxy-4,4′-binecatorone (**4**) forms dark brown crystals which dissolve in methanol and DMSO to give a yellow or green colour, respectively. On addition of alkali, the colour of the solutions change to dark purple. The ^{1}H and ^{13}C NMR spectra of the pigment contain two sets of signals corresponding to those of **3** and **5** which leads to the unsymmetrical formula **4**. It is supported by the lack of a 4-H signal in the ^{1}H NMR spectrum and the mass spectrum of its tris-trimethylsilyl derivative which shows the $[M]^+$ at *m/z* 726. The structures of **4** and the other new *Lactarius* alkaloids have been confirmed by total syntheses [6].

By means of HPLC, no further pigments could be detected in crude extracts of *L. necator* and *L. atroviridis*. The pigments were identified by their retention times and characteristic UV spectra. HPLC analyses indicate that only **1**, **3** and **4** are present in *L. necator*, whereas in *L. atroviridis* **5** occurs as an additional pigment. The monomeric 10-deoxynecatorone (**2**), known as a synthetic compound [6], could not be detected in the extracts. For *L. necator* significant differences were observed in the pigment composition of young and aged fruiting bodies. In young, light brown specimens **1** and **3** are about equally represented whereas older, dark brown fruiting bodies contain 95% of **3** and only 5% of **1**.

The occurrence of necatorone type alkaloids in *Lactarius necator* and *L. atroviridis* supports the close taxonomic relationship of both species [7]. Compound **1** and its dehydrodimer **3** are responsible for the characteristic purple colour reaction of the toadstools with alkali.

EXPERIMENTAL

General. UV-Vis spectra were recorded in MeOH, IR spectra in KBr disks. ^1H and ^{13}C NMR spectra were recorded at 400 and 100 MHz respectively; chemical shifts are reported in δ – values with DMSO-d_6 as int. ref. MS were obtained by direct inlet at 70 eV. Pertrimethylsilylation of samples (0.5 mg) was achieved by treatment with *N,O*-(bistrimethylsilyl) acetamide–THF (1:1, 2 ml) at room temp for 24 hr. TLC was performed on silica gel 60 aluminum sheets (Merck No. 5554). Solvent systems: A, $CHCl_3$–MeOH (5:1); B, (2:1); C, (1:1).

Plant material. Lactarius necator was collected in the Taunus region and near Bonn, F.R.G., *L. atroviridis* in the Ball creek area, Macon, County, N.C., U.S.A. Voucher samples are kept by Prof. Dr A. Bresinsky (Regensburg).

Isolation of pigments. Frozen, peeled skins from 7 kg of *L. necator* were extracted with MeOH (8 l). Solvent was evapd under red. pres. and H_2O (1.5 l) was added to the residue. The soln was adjusted to pH 6 by addition of boric acid and the aq. phase exhaustively extracted with EtOAc (250 ml portions). The first extracts were orange-yellow (**1**), those following pale yellow (**3**). During this procedure, a brown ppt. was formed which yielded only traces of **3** on further extraction. Pigments were sepd by repeated CC on Sephadex LH-20 (100 × 4 cm) with MeOH as eluant. Due to their low solubility, only small amounts of pure compounds could be obtained with each run. They were eluted in the order **1** (77 mg), **4** (0.5 mg) and **3** (38 mg). By the same procedure, freshly peeled skins from 0.75 kg of *L. atroviridis* yielded **1** (trace amounts), **5** (13 mg), **4** (4.5 mg), and **2** (4 mg).

Necatorone (**1**), [5,10-dihydroxy-6-oxo-dibenzo[de, h] [1, 6]*naphthyridine*]. Red crystals (MeOH), mp >360°; R_f 0.62 (B), olive-green spot, $+NH_3$ violet. UV/Vis λ_{max} nm: 431 (log ε 4.13), 310 (sh, 3.85), 293 (3.88), 265 (sh, 4.13), 233 (4.60), 212 (sh, 4.38), IR ν_{max} cm^{-1}: 3510 (m), 3200 (br s), 1640 (s), 1610 (ss), 1520 (m), 1460 (s), 1430 (s), 1400 (s), 1360 (m), 1340 (m), 1290 (m), 1260 (m), 1220 (s), 1200 (ss), 1140 (m), 1110 (m), 1085 (w), 1060 (m), 990 (w), 970 (m), 890 (w), 860 (w), 830 (w), 810 (w), 780 (w), 640 (w). ^1H and ^{13}C NMR: see Tables 1 and 2, respectively. HRMS *m/z* (70 eV 240°) (rel. int.): 266 $[M+2]^+$ (15), 265 $[M+1]^+$ (11), 264.0539 $[M]^+$ (calc. for $C_{15}H_8N_2O_3$: 264.0535) (26), 237 (24), 236 $[C_{14}H_8N_2O_2]^+$ (100), 220 (6), 208 (29), 181 (9), 180 (11), 179 (14).

4,4′-*Binecatorone* (**3**), [4,4′-*bi*-(5,10-*dihydroxy*-6-*oxo-dibenzo* [de, h] [1, 6]*naphthyridyl*)]. Dark brown or red crystals (MeOH), mp >360°; R_f 0.5 (C), yellow-brown spot, $+NH_3$ purple. UV/Vis λ_{max} nm: 438 (ε_{rel} 0.31), 315 (sh, 0.21), 268 (sh, 0.5), 250 (sh, 0.73), 236 (1.0), 215 (sh, 0.76). IR ν_{max} cm^{-1}: 3400 (br s), 1650 (s), 1610 (ss), 1510 (s), 1440 (ss), 1350 (s), 1290 (w), 1220 (ss), 1150 (w), 1120 (m), 1090 (m), 1070 (m), 1040 (w), 980 (w), 910 (w), 840 (m), 820 (w), 770 (w), 750 (w), 690 (w), 670 (w), 650 (w), 600 (w). ^1H and ^{13}C NMR: see Tables 1 and 2, respectively. MS of *tetrakis*-TMSi derivative *m/z* (rel. int.): 816 $[M+2]^+$ (4), 815 $[M+1]^+$ (6), 814 $[M]^+$ (8), 799 $[M-Me]^+$ (42), 786 $[M-CO]^+$ (75), 784 (21), 771 (20), 757 (11), 711 (17), 698 (26), 683 (18), 640 (10), 609 (9), 594 (18), 522 (5), 506 (4), 392 (10), 304 (3), 216 (5), 147 (33), 116 (63), 73 (100).

10-*Deoxy*-4,4′-*binecatorone* (**4**), [4-(5′-*hydroxy*-6′-*oxo-dibenzo* [d′e′, h′] [1′, 6′]*naphthyrid*-4′-*yl*)-5,10-*dihydroxy*-6-*oxo-dibenzo* [de, h] [1, 6]*naphthyridine*]. Dark brown crystals (MeOH), mp >360°; R_f 0.33 (A), brown spot, $+NH_3$ purple. UV/Vis λ_{max} nm: 420 (ε_{rel} 0.22), 285 (sh, 0.49), 264 (0.75), 226 (1.0). IR ν_{max} cm^{-1}: 3400 (br s), 3080 (s), 1650 (ss), 1610 (ss), 1580 (s), 1540 (m), 1510 (ss), 1460 (m), 1440 (ss), 1400 (m), 1350 (ss), 1310 (m), 1290 (m), 1230 (s), 1220 (ss), 1210 (s), 1150 (m), 1110 (s), 1070 (s), 1030 (w), 990 (w), 960 (w), 920 (w), 880 (w), 840 (w), 770 (w), 740 (w), 690 (w), 670 (w), 640 (w), 610 (w). ^1H NMR: see Table 1. ^1H-decoupled ^{13}C NMR (d_6-DMSO): δ 105.9 (C-11), 114.1 (C-17′)[a], 114.2 (C-17)[a], 114.7 (C-4), 115.7 (C-4′), 119.3 (C-3)[a], 120.0 (C-3′)[a], 122.2 (C-9), 123.6 (C-11′), 126.1 (C-15′), 128.5 (C-15), 130.1 (C-10′)[a], 130.8 (C-9′)[a], 131.2 (C-8′), 133.4 (C-8), 138.7 (C-12′)[a], 139.8 (C-12)[a], 141.3 (C-14), 143.9 (C-13), 145.5 (C-16), 146.3 (C-14′), 146.7 (C-16′), 147.7 (C-13′), 153.5 (C-2), 154.6 (C-2′), 155.7 (C-5′)[a], 157.2 (C-5)[a], 159.8 (C-10), 179.5 (C-6′)[a], 180.4 (C-6)[a], 157.2 (C-5)[a], 159.8 (C-10), 179.5 (C-6′)[a], 179.5 (C-6′)[a], 180.4 (C-6)[a] ([a]:corresponding δ-values may be interchanged). MS of *tris*-TMSi derivative *m/z* (rel. int.): 727 $[M+1]^+$ (6), 726 $[M]^+$ (6), 712 (13), 711 $[M-Me]^+$ (22), 700 (16), 698 $[M-CO]^+$ (33), 696 (10), 687 (10), 688 (18), 653 (4), 640 (9), 627 (4), 611 (10), 592 (7), 583 (12), 579 (13), 557 (6), 548 (8), 526 (6), 510 (4), 507 (5), 483 (4), 443 (8), 428 (6), 379 (3), 371 (5), 341 (3), 247 (3), 221 (4), 205 (5), 191 (3), 167 (6), 148 (23), 147 (95) 131 (15), 117 (19), 116 (66), 99 (6), 92 (24), 75 (13), 74 (100).

10,10′-*Dideoxy*-4,4′-*binecatorone* (**5**), [4,4′-*bi*-(5-*hydroxy*-6-*oxo-dibenzo* [de, h] [1, 6]*naphthyridyl*)]. Blackish green or orange crystals (MeOH), mp >360°; R_f 0.5 (A), olive-green spot, $+NH_3$ dove-grey. UV/Vis λ_{max} nm: 428 (ε_{rel} sh, 0.12,), 358 (0.22), 285 (sh, 0.39), 264 (0.6), 223 (1.0), 199 (0.69). IR ν_{max} cm^{-1}: 3400 (br s), 1660 (ss), 1640 (m), 1610 (m), 1580 (s), 1550 (m), 1510 (ss), 1470 (m), 1445 (ss), 1400 (m), 1360 (m), 1340 (m), 1290 (s), 1240 (m), 1220 (m), 1185 (w), 1160 (w), 1125 (w), 1110 (m), 1070 (m), 1040 (w), 990 (w), 960 (w), 920 (w), 895 (w), 875 (w), 850 (w), 830 (w), 810 (w), 770 (m), 740 (w), 720 (w), 695 (w), 670 (w), 640 (w), 610 (w). ^1H and ^{13}C NMR: see Tables 1 and 2, respectively. MS of *bis*-TMSi derivative *m/z* (rel. int.): 640 $[M+2]^+$ (6%), 638 $[M]^+$ (11), 624 (33), 623 $[M-Me]^+$ (67), 611 (42), 610 $[M-CO]^+$ (82). 608 (30), 599 (15), 596 (24), 582 (32), 551 (3), 534 (14), 523 (9), 494 (3), 491 (7), 478 (5), 467 (15), 304 (7), 250 (3), 148 (61), 147 (9), 134 (9), 116 (39), 95 (9), 93 (27), 91 (8), 75 (100), 73 (90).

HPLC analyses. Peeled skins (0.5 g) were exhaustively extracted with MeOH (2 l), solvent evapd and the residue (4 mg) dissolved in the HPLC eluant (10 ml; ultrasonic bath for dispersion). After filtration over a Millipore Type HV filter (pore size 0.45 μm), samples of 5–20 μl were inj.

For qualitative analyses of CGC glass cartridge 150-3 filled with LiChrosorb RP-18, 5 μm (Merck), guarded by GuardPak C-18 (Millipore-Waters) was used. Eluant: H_2O–MeOH (1:2) (pH 5 with 1% HOAc); flow rate: 1 ml/min; detection at 254 nm. The pigments showed the following R_t (min): **1**, 2.07; **3**, 2.47; **2**, 2.90; **4**, 4.00; **5**, 5.59.

Quantitative analyses were carried out with a μC 18-Bondapak column (10 μm, 300 × 3.9 mm, Waters) guarded by GuardPak C-18. Eluent: H_2O–MeOH (21:29) (pH 3 with 0.05 mol TFA + NH_4OH); flow rate 1 ml/min; detection at 265 nm. R_t (min): **1**, 7.25; **3**, 12.16. After calibration with ref compounds, aged, dark brown specimens of *L. necator* were found to contain 6.7 mg of **1** (5%) and 134 mg of **3** (95%) in 100 g of fr fruit bodies. In younger, light brown specimens *ca* 40% of **1** and 60% of **3** were found.

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