DOI: 10.1002/ejoc.200500869

Nebularic Acids and Nebularilactones, Novel Drimane Sesquiterpenoids from the Fungus *Lepista nebularis*

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Keywords: Antifungal / Drimanes / Fungi / Natural products / Sesquiterpenes

Four (nor-)drimane sesquiterpenoids, nebularic acids A (**1**) and B (**2**) and nebularilactones A (**3**) and B (**4**), were isolated from a cultured *Lepista nebularis* strain by bioassay-guided chromatography, and their structures were elucidated by mass spectrometry and NMR spectroscopy. Nebularic acid A (**1**) and in particular nebularic acid B (**2**), which features an α,β-epoxy carboxylate moiety, show antifungal and antibacterial activities.

Drimanes comprise a class of sesquiterpenoid metabolites[1] that possess a broad spectrum of activities, including antifungal,[2] antibacterial,[3] antiviral,[4,5] cytotoxic,[6] antifeedant,[7] antiallergic, and antiinflammatory properties.[8] Drimane sesquiterpenoids have been isolated from higher plants such as *Canella winterana*,[9,10] *Warburgia salutaris*[11] and *Drymus winteri*,[12] as well as from fungi such as *Aspergillus*,[5] *Panus*,[13] and *Lactarius* species.[14] Here we report the isolation and structural elucidation of two nordrimane sequiterpenoid acids named nebularic acids, and two drimane sesquiterpenoid lactones from a culture of the basidiomycete *Lepista nebularis*.

A strain of *Lepista nebularis* collected in Siberia appeared to produce antimicrobial metabolites, as revealed by an initial screening with crude extracts. The fungus was cultivated under the conditions of surface fermentation at 25 °C in 500 mL Erlenmeyer flasks containing 100 mL medium composed of malt extract (20 g L^{-1}), glucose (10 g L^{-1}), yeast extract (1 g L^{-1}) and $(NH_4)_2SO_4$ (5 g L^{-1}), at pH 6.0. After cultivation for 28 days at 25 °C the mycelium cake from the culture medium (60 L) was harvested and extracted twice with ethyl acetate and methanol (each 10 L). The culture broth was thoroughly extracted with ethyl acetate and the combined extracts were dried and the solvents were evaporated. The residue (3.2 g) was subjected to column chromatography (silica gel 60, Merck, 0.063–0.1 mm, column 4 × 60 cm), with stepwise $CHCl_3$ and $CHCl_3$/MeOH (9:1, 1:1, *v*/*v*) as eluents. Active components were isolated through bioassays (*v.i.*). Final purification was achieved by preparative HPLC with a Spherisorb ODS-2 RP_{18} column (250 × 25 mm, 5 μm), Promochem, and acetonitrile/H_2O (83:17, *v*/*v*) as eluent (flow rate 10 mL min^{-1}, UV detection at 210 nm), yielding **1** (31 mg), **2** (20 mg), **3** (15 mg) and **4** (4 mg).

Compound **1** was obtained as a white powder. Its molecular formula was determined by HR-EIMS (found $[M + H]^+$: 237.1480, calcd. for $C_{14}H_{21}O_3$: 237.1485) and ^{13}C NMR spectroscopy to be $C_{14}H_{20}O_3$. The ^1H NMR spectrum of **1** showed 19 nonexchangeable protons including one olefinic proton at δ = 8.02 ppm and three methyl groups at δ = 0.88, δ = 0.92 and δ = 1.14 ppm. Analyses of ^{13}C, DEPT 135 and HMQC NMR spectra of **1** indicated the presence of two methine C-atoms (one of them sp^2-hybridized) four methylene C-atoms, three methyl C-atoms and three quaternary C-atoms (one of them sp^2-hybridized), a carboxy C atom (δ = 164.0 ppm) and a carbonyl C atom (δ = 204.3 ppm). The occurrence of the carboxy group and the carbonyl group was confirmed by the IR spectrum, which showed strong absorption bands at 1742 cm^{-1} and 1632 cm^{-1}. The ^1H-^1H COSY spectrum revealed the coupling systems H-1/H-2/H-3, and H-5/H-6. By HMBC all connectivities were fully assigned. The geminal arrangement of the methyl groups C-14 (δ = 20.9 ppm) and C-15 (δ = 32.6 ppm) was established by the correlation of H-14 (δ = 0.92 ppm) and H-15 (δ = 0.88 ppm) with C-4 (δ = 33.0 ppm), C-3 (δ = 40.7 ppm) and C-5 (δ = 49.4 ppm). Similarly, the correlation of H-13 (δ = 1.14 ppm) with C-10 (δ = 38.3 ppm), C-1 (δ = 36.8 ppm) and C-9 (δ = 175.4 ppm) showed the connection of the methyl C-13 with C-10. Another important correlation was observed between the olefinic proton H-9 (δ = 8.02 ppm) and C-8 (δ = 123.5 ppm), C-7 (δ = 204.3 ppm) and C-12 (δ = 164.0 ppm), which enabled the unambiguous identification of the positions of the carboxyl and carbonyl groups. The correlation of H-6 (δ = 2.65, 2.50) with C-5, C-7, C-4 and C –8 was also observed, and compound **1** was thus identified as 11-nor-7-oxo-drimen-8-oic acid (nebularic acid A).

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Compound **2** was obtained as a white powder. The ^1H and ^{13}C NMR spectra of **2** were almost identical with those obtained from **1**. However, it appeared from analysis of ^{13}C, DEPT 135 and HMQC NMR spectra of **2** that it contained no olefinic C-atom, the two olefinic C-atoms present in **1** being replaced by a methine and by a quaternary sp^3 C-atom. According to their downfield chemical shifts (δ = 69.5 and δ = 62.2 ppm), both of them are oxygenated. This finding was strongly supported by mass spectrometry, which showed a mass difference of 16 units relative to **1**, the pseudomolecular ion peak obtained by HR-ESIMS corresponding to a molecular formula of $C_{14}H_{20}O_4$. Compound **2** was therefore identified as 8,9-epoxy-11-nor-7-driman-12-oic acid (nebularic acid B). The relative configuration of **2** (Figure 1) was established by NOESY experiments, which showed a correlation between the methine proton H-5 and the oxy methine proton H-9.

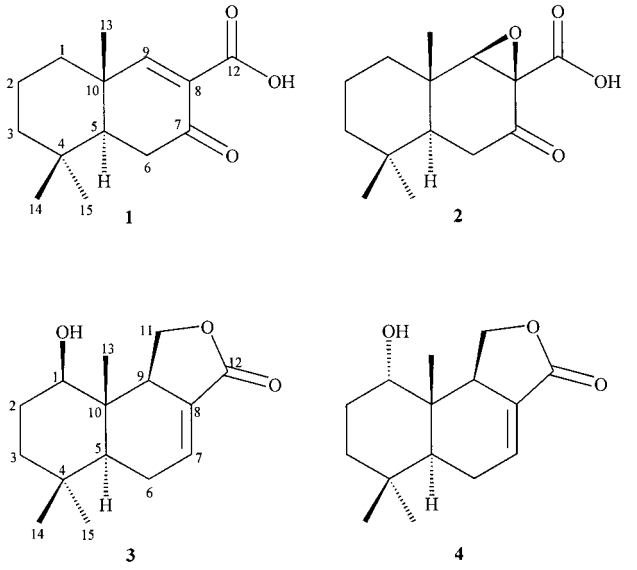

Figure 1. Structures of **1**–**4**.

Compound **3** was obtained as a colourless powder. Its molecular formula was determined by HR-EIMS and ^{13}C NMR spectroscopy to be $C_{15}H_{22}O_3$. The ^1H NMR spectrum of **3** showed 22 non-exchangeable protons including one olefinic proton at 6.85 ppm and three methyl groups at δ = 0.78, 0.94 and 1.34 ppm. Analysis of the ^{13}C, DEPT and HMQC NMR spectra of **3** indicated the presence of four methine C-atoms (two of them sp^2 hybridized and oxygenated according to their chemical downfield shifts of δ = 79.8 and 135.2 ppm, respectively), four methylene C-atoms (one of them oxygenated, δ = 69.2 ppm), three methyl C-atoms, three quaternary C-atoms (one of them sp^2 hybridized), and a lactone C atom (δ = 170.1 ppm). The occurrence of the lactone group was confirmed by the IR spectrum, which showed a strong absorption band at 1729 cm^{-1}. The ^1H-^1H COSY spectrum revealed the coupling systems H-1/H-2/H-3, H-5/H-6/H-7 and H-9/H-11. The other connectivities were fully assigned through the long-range HMBC spectrum (Figure 2). The geminal arrangement of the C-14 (δ = 21.5 ppm) and C-15 (δ = 32.5 ppm) methyl groups, and the connection of the methyl C-13 were established in the same manner as described for compounds **1** and **2**. The connectivity of the lactone ring was established through: the correlation of H-7 (δ = 6.85 ppm) with C-8 (δ = 127.6 ppm), C-9 (δ = 50.1 ppm) and C-12 (δ = 170.1 ppm), the correlation of H-9 with C-8, C-11 (δ = 69.2 ppm) and C-12, and the correlation of the oxymethylene proton H-11 (δ = 4.15 ppm) with C-8, C-9 and C-12. The most important correlations were the correlation of H-9 with C-1 (δ = 79.8 ppm), of H-13 with C-1 and of H-1 (δ = 3.39 ppm) with C-10 (δ = 39.5 ppm) C-13, and C-9. This information enabled the unequivocal identification of the position of the hydroxy group. The relative stereochemistry was achieved by a NOESY experiment, which revealed the correlation between H-1/H-5, H-1/H-9 and H-5/H-9, thus allowing **3** to be identified as 1β-hydroxy-7-drimen-12,11-olide (nebularilactone A).

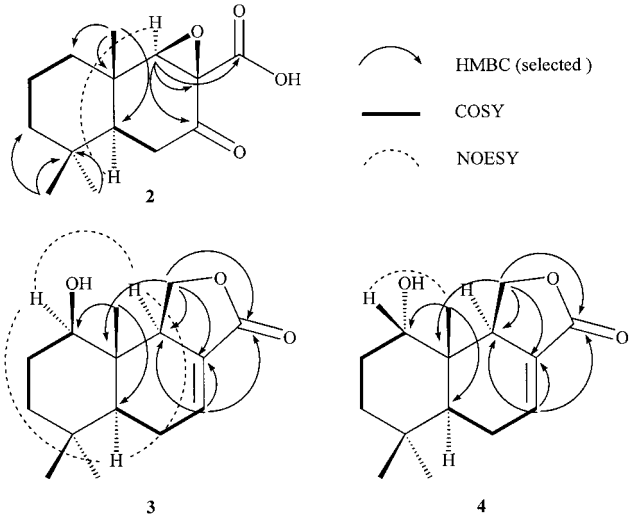

Figure 2. Configurations of **2**–**4** based on 2D NMR spectroscopic data.

Compound **4** was obtained as a colourless oil. Its molecular formula was determined by HR-EIMS (found [M + H]$^+$: 251.1642, calcd. for $C_{14}H_{23}O_3$: 251.1650) and ^{13}C NMR spectroscopy to be $C_{15}H_{22}O_3$. The ^1H, ^{13}C, DEPT 135 HMBC spectra of **4** were almost identical with those obtained from **3**, but it appeared from analysis of the NOESY spectrum of **4** that the proton H-1 (δ = 3.51 ppm) correlated with the methyl group H-13 (0.78). No correlation between the proton H-1 and H-5 (δ = 1.75 ppm) or H-9 (δ = 3.58 ppm) was found, so **4** was identified as 1α-hydroxy-7-drimen-12,11-olide (nebularilactone B).

In the course of this study, four (nor-)drimane sesquiterpenoids were identified and characterized. However, it should be noted that the detection of the methyl ester of **1** from the methylated extract of *Lepista glaucocana* has been reported previously.[15] Compound **1** appears to be the biogenetic progenitor of **2**. This highly substituted metabolite features an α,β-epoxy, α-keto carboxylate moiety, which is unusual for drimanes. Nebularilactones A and B (**3** and **4**) have been tested in various bioassays, but no activities have been identified. Conversely, nebularic acid A (**1**) shows

moderate activity against *Bacillus subtilis* ATCC 6633. Clearly because of its reactive α,β-epoxy moiety, nebularic acid B (**2**) exhibits significant antifungal activities against *Penicillium avellaneum* UC 4376, *Fusarium culmorum* JP15, and the yeast *Kluyveromyces maxianus*, as well as antibacterial activities against, for example, *Staphylococcus aureus* SG511, at 50 μg mL^{-1}.

Experimental Section

General Experimental Procedures: Melting points (uncorrected) were determined on a Wagner & Munz apparatus. Optical rotations were measured with a Propol Digital Automatic Polarimeter. UV was monitored with a Sphericord 200 Carl Zeiss technology spectrometer. IR spectra (film) were recorded on a Satellite FTIR spectrometer fitted with an ATR device. High-resolution electron impact mass spectra (EI-MS) were obtained with a AMD 402 double-focussing mass spectrometer with BE geometry (AMD, Intestra, Harpstedt, Germany). NMR spectra were recorded on a Bruker Avance 500 DRX spectrometer (Bruker, Karlsruhe, Germany) at 300.133 MHz for ^1H and 75.475 MHz for ^{13}C in $CDCl_3$. Chemical shifts are given in ppm relative to internal TMS as standard. HSQC and NOESY (mixing time 0.7 s) data were obtained in the phase-sensitive mode TPPI. Column chromatography was performed on silica gel (Kieselgel 60, Merck; 0.063–0.2 μm) and Sephadex LH-20. For HPLC a Gilson binary gradient HPLC system, fitted with a UV detector (UV/Vis-151, 210 nm) and a preparative column (C18, 7 μm), was used. TLC was carried out with silica gel 60 F_{254} plates. Spots were visualized by spraying with vanillin/H_2SO_4 followed by heating. All solvents used were spectral grade or distilled prior to use.

Fungal Material: *Lepista nebularis* was collected as mycelial culture (derived from tissue plugs of the fruiting body) in Siberia. The taxonomy of the fungus was verified by Dr. Heinrich Dörfelt of the Institute for Nutrition and Environment, Friedrich-Schiller-University, Jena. A specimen (HKI 0411) was deposited in the fungal collection of the Leibniz-Institute for Natural Products Research and Infection Biology, HKI Jena, Germany.

Fermentation: Fifteen-day-old agar plate cultures (25 °C) were prepared as inoculum in a medium composed as follows (g L^{-1}): malt extract (40), yeast extract (4), agar (15), deionized water, pH 6.0. Agar chips (4–5 cm^2) of the plate cultures were used to inoculate a liquid medium composed as follows (g L^{-1}): malt extract (20), glucose (10), yeast extract (1), $(NH_4)_2HPO_4$ (5), pH 6.0. The surface cultivation was carried out under sterile conditions in 500 mL Erlenmeyer flasks containing 100 mL medium.

Extraction and Isolation: The whole culture broth was extracted twice with two volume equivalents of ethyl acetate. The residue from the evaporated ethyl acetate extract from 60 L of culture (3 g) was applied to a silica gel column in chloroform. Elution was performed with 500 mL portions of CH_2Cl_2, CH_2Cl_2/MeOH (9:1), and CH_2Cl_2/MeOH (7:3). Further purification was achieved with a Sephadex LH 20 column and by repeated preparative HPLC on a reversed-phase (RP_8 Spherisorb, 25 mm × 250 mm) column with a binary gradient (water/acetonitrile, 95:5 to 5:95; 30 min) for elution.

Nebularic Acid A (1): White powder, no m.p. (dec.), ($[\alpha]_D^{22}$ = –3, *c* = 1.15, MeOH). ^1H NMR ($CDCl_3$, 300 MHz) data: see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data: see Table 1. IR (film): $\tilde{\nu}_{max}$ = 1742, 1632, 1431, 1410, 864, 165 cm^{-1}. UV (MeOH): λ_{max} = 210 nm. ESIMS: *m*/*z* = 237 [*M* + H]$^+$. HR-EIMS (found [*M* + H]$^+$: 237.1480, calcd. for $C_{14}H_{21}O_3$: 237.1485)

Nebularic Acid B (2): White powder, no m.p. (dec.), ($[\alpha]_D^{22}$ = –83, *c* = 0.25, MeOH). ^1H NMR ($CDCl_3$, 300 MHz) data: see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data: see Table 1. IR (film): $\tilde{\nu}_{max}$ = 1714, 1624, 1424, 748 cm^{-1}. UV (MeOH): λ_{max} = 210 nm. ESIMS: *m*/*z* = 251 [*M* – H]$^-$. HR-EIMS (found [*M* – H]$^-$; 251.1262, calcd. for $C_{14}H_{19}O_4$: 251.1264)

Nebularilactone A (3): Colourless oil, ($[\alpha]_D^{22}$ = –15, *c* = 0.9, MeOH). ^1H NMR ($CDCl_3$, 300 MHz) data: see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data: see Table 1. IR (film): $\tilde{\nu}$ = 1729, 1458, 1364, 1222, 1055, 966 cm^{-1}. UV (MeOH) λ_{max} = 225 nm. ESIMS: *m*/*z* = 251 [*M* + H]$^+$. HR-EIMS (found [*M* + H]$^+$: 251.1642, calcd. for $C_{15}H_{23}O_3$: 251.1650)

Nebularilactone B (4): Colourless oil; ($[\alpha]_D^{22}$ = –21, *c* = 0.2, MeOH). ^1H NMR ($CDCl_3$, 300 MHz) data: see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data: see Table 1. IR (film): $\tilde{\nu}$ = 1730, 1458, 1364, 1222, 1055, 966 cm^{-1}. UV (MeOH): λ_{max} = 225 nm. ESIMS: *m*/*z* = 251 [*M* + H]$^+$. HR-EIMS (found [*M* + H]$^+$: 251.1642, calcd. for $C_{15}H_{23}O_3$: 251.1650)

Table 1. NMR spectroscopic data for **1**–**4**.

No	**1**		**2**		**3**		**4**	
	$\delta = {}^1H^{[a][c]}$	$\delta = {}^{13}C^{[a]}$	$\delta = {}^1H^{[b][c]}$	$\delta = {}^{13}C^{[b]}$	$\delta = {}^1H^{[a][c]}$	$\delta = {}^{13}C^{[a]}$	$\delta = {}^1H^{[a][c]}$	$\delta = {}^{13}C^{[a]}$
1	1.41, 1.80 m	36.8	1.50, 1.60 m	37.3	3.39 dd (4.7, 10.8)	79.8	3.51 br. s	71.4
2	1.61, 1.88 m	18.1	1.58, 1.78 m	19.2	1.63 m	28.3	1.59, 1.98	26.0
3	1.12, 1.51 m	40.7	1.22, 1.48 m	42.4	1.32, 1.52 m	40.0	1.26, 1.76	34.1
4	–	33.0	–	33.5	–	32.5	–	32.7
5	1.78 dd (3.8)	49.4	1.71 dd (4.5)	42.5	1.34 m	48.7	1.75 dd	43.4
6	2.65, 2.50 m	35.2	2.16, 2.35 m	36.5	2.20, 2.40 m	24.7	2.15, 2.43	25.2
7	–	204.3	–	205.1	6.85 dd (3.4, 7.0)	135.2	6.80 dd (3.3, 6.9)	135.2
8	–	123.58	–	62.2	–	127.6	–	127.5
9	8.02 s	175.4	3.16 s	69.5	2.82 m	50.1	3.58 m	42.7
10	–	38.3	–	35.2	–	39.5	–	38.2
11	–	–	–	–	4.15, 4.50 dt (9.6)	69.2	4.00, 4.42 dt (9.3)	67.1
12	–	164.0	–	172.7	–	170.1	–	170.3
13	1.14 s	17.1	1.14 s	17.1	0.78 s	7.28	0.78 s	13.6
14	0.92 s	20.9	0.89 s	21.3	0.94 s	21.5	0.94 s	21.3
15	0.88	32.6	0.85 s	33.0	0.90 s	32.5	0.93 s	32.8

[a] Recorded in $CDCl_3$. [b] Recorded in CD_3OD. [c] Coupling constants (Hz) in parentheses.

Acknowledgments

We thank Mrs. M.-G. Schwinger and Mrs. H. Röhrig for their excellent assistance in fermentation, isolation and assays. We are grateful to the European Community for financial support in the FP5 EUKETIDES Programme.

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Received: November 4, 2005
Published Online: February 9, 2006

