Biologically Active Metabolites from the Basidiomycete *Limacella illinita* (Fr.) Murr.

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In the course of our search for new bioactive compounds from basidiomycetes, four new compounds were isolated from fermentations of *Limacella illinita.* Illinitone A (**1**) exhibited weak phytotoxic and moderate nematicidal activities against *Caenorhabditis elegans*, illinitone B (**2**) was moderately cytotoxic, while limacellone (**3**) exhibited weak cytotoxic and phytotoxic activities. The muurolane sesquiterpene **4a** was found to be inactive in the assays performed here. Limacellone (**3**), which appeared to be related with the illinitones **1** and **2**, has a new C_{15} carbon skeleton. It is possible that compounds **1**, **2** and **3** are terpenoids/secoterpenoids, but their biosyntheses were not investigated.

Key words: Limacella illinita, Illinitones, Limacellone, 11-Desoxyeleganthol

Introduction

Within the family Amanitaceae the genus *Limacella* is characterized by morphological characteristics, *e.g.* viscid or glutinous caps and inamyloid spores (Singer, 1986), and by its nrITS and nLSU sequences (Moncalvo *et al*., 2000, 2002; Vellinga, 2004). The genus is of worldwide occurrence but to our knowledge its secondary metabolites have not been explored previously. In the following we wish to describe the production, structure elucidation and biological activities of four new metabolites from mycelial cultures of *L. illinita*.

Materials and Methods

Producing fungus

Mycelial cultures of *L. illinita* strain 99048 were derived from tissue plugs of young fruiting bodies collected in Sardegna. The species was identified by macroscopic and microscopic characteristics. A voucher specimen and mycelial cultures are deposited in the culture collection of the IBWF e. V., Kaiserslautern, Germany. For maintenance on agar slants the fungus was grown on YMG medium (g/l): yeast extract (4), malt extract (10), glucose (4) and agar 2% for solid media. The pH was adjusted to 5. 5.

Fermentation of Limacella illinita

Fermentations were carried out in a Biolafitte C6 fermentor containing 20 l of YMG medium with aeration (3 l air/min) and stirring (120 rpm) at room temperature. A well-grown culture (250 ml YMG medium) in a 500 ml Erlenmeyer flask (grown at 22 °C and 120 rpm) was used as inoculum. When the glucose was used up and the cytotoxic activity in ethyl acetate extracts of the culture fluid had reached the maximal value (after approx. 21 d), the culture fluid was separated from the mycelia by filtration.

*Isolation of compounds **1**, **2**, **3** and **4a***

The culture fluid (14 l) was passed through a column filled with HP21 resin (Mitsubishi Diaion, 25×5.5 cm) using methanol/H_2O 1:1 and methanol (1.5 l each) as eluants. After evaporation of the solvent the crude product of the methanol fraction (1.4 g) was applied onto a silica gel column (Merck 60, 0.063–0.2 mm, 5×10 cm). Elution with 500 ml cyclohexane/ethyl acetate 7:3 (v/v) yielded 19.7 mg of an enriched product containing **3**. Elution with cyclohexane/ethyl acetate 1:1 (v/v) resulted in 105 mg of a product containing **1**, **2** and **4a**. In a last step both enriched products were fractionated by preparative HPLC using a Jasco Model PU-1586 column with a multiwavelength detector

MD-910 (column: Phenomenex, Luna RP-18, 10 μm, 250 × 21 mm; gradient: H_2O/MeCN, 25–75% MeCN in 30 min; flow: 20 ml/min). Yields (retention times): **1**, 9.1 mg (9.7 min); **2**, 13.3 mg (12.8 min); **3**, 8.2 mg (14.5 min); **4a**, 8.9 mg 24.5 min.

Spectroscopy

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The chemical shifts (δ) are given in ppm and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). High resolution mass spectra (HRESIMS) were recorded with a Micromass Q-TOF MICRO instrument. UV and IR spectra were recorded with a Perkin Elmer λ 16 instrument and a Bruker IFS 48 spectrometer. The optical rotations were measured with a Perkin Elmer 141 polarimeter at 22 °C.

*Physicochemical properties of the compounds **1**, **2**, **3** and **4a***

Illinitone A (**1**): Colourless oil, $[\alpha]_D^{22}$ −2° (*c* 0.4 in DMSO). – UV (MeOH): λ_{max} (log ε) = 232 (4.09) and 282 nm (4.05). – IR (KBr): ν = 3430, 2975, 1670, 1610, 1290, 1150, 1080, 1040, 960, 925 and 600 cm^{-1}. – ^{1}H and ^{13}C NMR: see Tables I and II. – HRESIMS: *m/z* = 251.1303 [M+H$^+$] ($C_{14}H_{19}O_4$ requires 251.1283).

Illinitone B (**2**): Colourless oil, $[\alpha]_D^{22}$ +154° (*c* 0.5 in DMSO). – UV (MeOH): λ_{max} (log ε) = 343 nm (3.87). – IR (KBr): ν = 3430, 1725, 1665, 1605, 1300, 1145, 1025, 935 and 675 cm^{-1}. – ^{1}H and ^{13}C NMR: see Tables I and II. – HRESIMS: *m/z* = 249.1122 [M+H$^+$] ($C_{14}H_{17}O_4$ requires 249.1127).

Limacellone (**3**): Colourless oil, $[\alpha]_D^{22}$ +11° (*c* 0.4 in $CHCl_3$). – UV (MeOH): λ_{max} (log ε) 219 nm (4.07). – IR (KBr): ν = 3430, 2985, 1680, 1315, 1130, 980, 910, 840 and 660 cm^{-1}. – ^{1}H and ^{13}C NMR: see Tables I and II. – HRESIMS: *m/z* = 281.1373 [M+H$^+$] ($C_{15}H_{21}O_5$ requires 281.1389).

11-Desoxyeleganthol (**4a**): Colourless oil, $[\alpha]_D^{22}$ +78° (*c* 0.6 in $CHCl_3$). – UV (MeOH): λ_{max} (log ε) 203 nm (3.49). – IR (KBr): ν = 3420, 2950, 1455, 1375, 1125 and 610 cm^{-1}. ^{1}H and ^{13}C NMR: see

Table I. ^{1}H (500 MHz) NMR data (δ; multiplicity; *J* in Hz) of illinitone A (**1**), illinitone B (**2**), limacellone (**3**) and 11-desoxyeleganthol (**4a**). The spectra of **1** and **2** were recorded in CD_3SOCD_3, while those of **3** and **4a** were recorded in $CDCl_3$; the solvent signals (2.50 and 7.26 ppm, respectively) were used as reference.

H	1	2	3	4a
1	–	–	5.46; dd; 2.0, 2.7	–
2α	4.13; d; 3.9	–	2.37; dd; 2.7, 12.3	–
2β	–	–	1.98; dd; 2.0, 12.3	–
3	–	–	–	1.61; m
4α	–	–	–	1.50; m
4β	–	–	–	1.19; m
5	–	–	–	1.26; m
6	7.13; s	7.20; s	–	1.89; m
7	–	–	5.82; s	5.43; d; 4.2
9	7.56; s	7.71; s	2.66; ddq; 5.4, 13.0, 6.4	2.05/2.14; m
10α	–	–	1.95; dd; 13.0, 14.0	1.85; ddd; 7.1, 11.0, 13.7
10β	–	–	2.33; dd; 5.4, 14.0	1.98; m
11	0.65; q	1.13; s	–	1.97; m
12	1.09; q	1.06; s	0.94; s	0.89; d; 7.0
13	1.37; q	1.30; s	1.21; s	0.83; d; 7.0
14	2.12; q	2.16; s	1.30; s	1.34; s
15	–	–	1.15; d; 6.4	1.71; s
	10.32; s (7-OH) 5.08; s (4-OH) 4.86; d; 3.9 (2-OH)	5.79; s (4-OH)	2.79; s (3-OH)	

Table II. ^{13}C (125 MHz) NMR data (δ; multiplicity) of illinitone A (**1**), illinitone B (**2**), limacellone (**3**) and 11-desoxyeleganthol (**4a**). The spectra of **1** and **2** were recorded in CD_3SOCD_3 while those of **3** and **4a** were recorded in $CDCl_3$; the solvent signals (39.51 and 77.00 ppm, respectively) were used as reference. The multiplicities of the carbon signals were determined indirectly from HMQC experiments.

C	**1**	**2**	**3**	**4a**
1	196.4; s	180.0; s	94.2; d	75.1; s
2	77.3; d	200.6; s	35.9; t	75.2; s
3	47.0; s	56.2; s	97.4; s	35.4; t
4	74.1; s	74.4; s	45.8; s	20.7; t
5	151.4; s	152.2; s	79.4; s	48.7; d
6	111.8; s	111.4; d	152.0; s	43.6; d
7	161.3; s	162.8; s	119.8; d	121.8; d
8	123.5; s	125.0; s	200.2; s	134.1; s
9	128.8; d	130.7; d	38.6; d	27.2; t
10	120.5; s	121.8; s	40.2; t	23.2; t
11	16.4; q	17.6; q	95.5; s	26.8; d
12	20.2; q	20.4; q	17.2; q	21.6; q
13	27.3; q	28.6; q	18.6; q	15.2; q
14	15.4; q	15.4; q	16.4; q	23.0; q
15	–	–	14.0; q	23.4; q

Tables I and II. – HRESIMS: m/z = 239.2025 $[M+H^+]$ ($C_{15}H_{27}O_2$ requires 239.2011).

Biological assays

The antimicrobial spectra were determined in an agar plate diffusion assay as described previously (Anke *et al*., 1977). Cytotoxic activities were measured according to Zapf *et al*. (1995) with minor modifications: MDA-MB-231 (ATCC HTB 26, human breast adenocarcinoma), MCF-7 (DSMZ ACC 115, human breast adenocarcinoma) and HEP-G2 (DSMZ ACC 180, human hepatocellular carcinoma) cells were grown in D-MEM medium (Invitrogen, Karlsruhe, Germany). COLO-320 (DSMZ ACC 144, human colon adenocarcinoma), Jurkat (ATCC TIB 152, human acute T cell leukemia) and L1210 (ATCC CCI 219, murine lymphocytic leukemia) cells were cultivated in RPMI medium. Both media contained 10% fetal calf serum (Invitrogen), 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. The cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2. Cytotoxicity was measured in microtiter plates with 5×10^4 cells/ml for monolayer cells and $1 - 2 \times 10^5$ cells/ml medium for suspension cell lines. Cells were incubated with and without test compounds and observed with a microscope after 24, 48, and 72 h. In addition, the effect on the growth of cell lines growing as monolayers was measured by Giemsa staining. The viability of suspension cells was quantified in a test based on XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide].

Phytotoxic activities on *Setaria italica* and *Lepidium sativum* were determined according to Anke *et al*. (1989) and nematicidal effects on *Caenorhabditis elegans* and *Meloidogyne incognita* as described by Stadler *et al*. (1994).

Results and Discussion

Isolation and structure determination

The four new compounds **1**, **2**, **3** and **4a** were isolated as described in the experimental section. Illinitone A (**1**) has, according to high resolution MS experiments, the composition $C_{14}H_{18}O_4$, and the structure has six unsaturations. The aromatic ring could be elucidated by HMBC correlations, from 14-H_3 to C-7, C-8 and C-9, from 6-H to C-7, C-8 and C-10, and from 9-H to C-5 and C-7. In support of this, HMBC correlations from 7-OH to C-6, C-7 and C-8 were observed. The connection between C-5 and C-4 was suggested by the HMBC correlation from 6-H to C-4, and confirmed by the correlations from 13-H_3 as well as 4-OH to C-3, C-4 and C-5. 11-H_3 and 12-H_3 give HMBC correlations to C-2, C-3 and C-4, as well as to each others carbon atom, showing that these two methyl groups are geminal and positioned on C-3. HMBC correlations from 2-OH to C-1, C-2 and C-3, from 2-H to C-1 and C-3, as well as from 9-H to C-1 complete the second ring and thereby the structure of **1**. The relative configuration of **1**

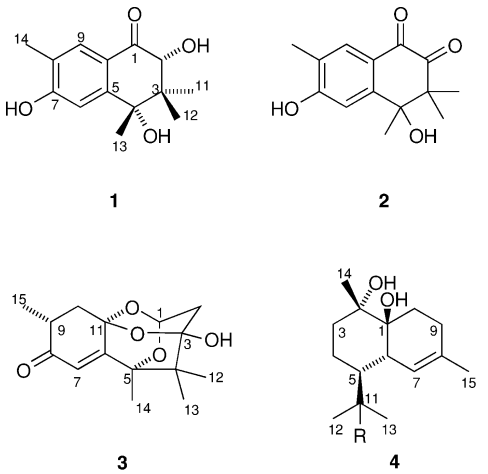

Fig. 1. Structures of illinitone A (**1**), illinitone B (**2**), limacellone (**3**), 11-desoxyeleganthol (**4a**, R = H) and eleganthol (**4b**, R = OH). All configurations are relative.

was shown to be as presented in Fig. 1, by the strong NOESY correlation from 2-H to 13-H_3, suggesting that the two are axial and close in space.

The NMR data of **1** and **2** are similar, except that the secondary alcohol of **1** has been exchanged for a second keto function in **2**. Also the MS data suggest that **2** is oxidized compared to **1**; they establish that the elemental composition of the compound is $C_{14}H_{16}O_4$, and show that the structure has seven unsaturations. The corresponding HMBC correlations as discussed for **1** were observed also for **2**, except that no correlation demonstrating the bond between C-1 and C-2 was observed. However, there is no other possibility to satisfy the elemental composition determined, and the structure of illinitone B (**2**) is consequently as shown in Fig. 1.

For limacellone (**3**), the elemental composition $C_{15}H_{20}O_5$ was suggested by MS experiments, which corresponds to an unsaturation number of six. In the ring containing the unsaturated keto moiety, the COSY correlations between 9-H and 10-H_2 as well as 15-H_3, the HMBC correlations from 15-H_3 to C-8, C-9 and C-10, from 7-H to C-5, C-9 and C-11, from 9-H to C-10, as well as from 10-H_2 to C-6 and C-11, demonstrate the structure of the ring and show that C-5 is attached to C-6. HMBC correlations from 14-H_3 to C-4, C-5 and C-6 as well as from 12-H_3 and 13-H_3 to C-3, C-4 and C-5 indicate that **3** has a similar methylation pattern as the illinitones, however, it is evident that **3** has an extra carbon atom in its structure. 2-H_2 gives HMBC correlations to C-1, C-3 and C-4, while 1-H correlates to C-3, C-5 and C-11. This only comes together as structure **3**, which conforms with the restrictions imposed by the elemental composition and the NMR chemical shifts. C-1 and C-11 are consequently acetals and C-3 is a hemiacetal, and this is confirmed by the appearance of 3-OH at 2.79 ppm in the ^{1}H NMR spectrum. The relative configuration of **3** is effectively demonstrated by the NOESY correlation between 9-H and 13-H_3.

11-Desoxyeleganthol (**4a**) has, according to high resolution MS experiments, the elemental composition $C_{15}H_{26}O_2$, and thereby the unsaturation index 3. With one evident carbon-carbon double bond, **4a** is consequently bicyclic. The isopropyl group is attached to C-5, as evidenced by the HMBC correlations from 12-H_3 and 13-H_3 to C-5 and C-11, and the COSY correlation between 5-H and 11-H. 6-H gives COSY correlations to 5-H and 7-H, and HMBC correlations to C-1, C-5, C-7 and C-8. 4-H_2 gives COSY correlations to 3-H_2 and 5-H, and HMBC correlations to C-2, C-3 and C-5, while 3-H_2 gives HMBC correlations to C-2, C-4 and C-14. With the HMBC correlations from 14-H_3 to C-1, C-2 and C-3 the saturated six-membered ring is established. 10-H_2 gives COSY correlations to 9-H_2, and HMBC correlations to C-1, C-6, C-8 and C-9, while the HMBC correlations from 15-H_3 to C-7, C-8 and C-9 as well as from 7-H to C-1, C-6, C-9 and C-15 close the second ring and establish the structure of **4a**. The relative configuration of **4a** was suggested by the correlations observed in the NOESY spectrum. Correlation between 6-H as well as 4-Hβ and 14-H_3 show that these protons are on the same side of the molecule, and suggest that the 2-methyl group has an axial position. 13-H_3 gives NOESY correlations to 4-Hβ and 6-H, while 12-H_3 correlates with 4-Hα and 5-H. 5-H gives NOESY correlations to 4-Hα, 10-Hα and 11-H, while 7-H correlates with 6-H and 11-H. 10-Hα gives a NOESY correlation to one of the C-3 protons, although they are not resolved, showing that the ring fusion is *cis*. This is consistent with the saturated ring having a chair conformation, and that the 2-methyl group is axial while the isopropyl group is equatorial. **4a** is consequently a muurolane sesquiterpene.

It is reasonable to believe that limacellone (**3**) is biosynthetically related with the illinitones **1** and **2**, as they share large parts of the carbon skeleton.

However, it is not clear whether they are terpenoids (or secoterpenoids) as the carbon skeleton of **3** has not been reported previously. **4a** belongs to a group of sesquiterpenes which are components of essential oils of plants, but are quite rarely isolated from fungal sources. It is similar to eleganthol (**4b**, Fig. 1) reported from *Clitocybe elegans* (Arnone *et al.*, 1993). For eleganthol no biological activities have been described so far, which is in accordance with our findings for **4a**.

Biological properties

In the agar diffusion assay none of the isolated metabolites showed an effect on the growth of *Bacillus brevis, B. subtilis, Micrococcus luteus, Enterobacter dissolvens, Penicillium notatum, Paecilomyces variotii, Mucor miehei* and *Nematospora coryli* at 100 μg/disk.

The cytotoxic activities of illinitone A and B and limacellone against human and murine cell lines were tested as described in the experimental section. Up to a concentration of 100 μg/ml compounds **1** and **4a** did not show inhibitory effects. **2** inhibited COLO-320 cells with an IC_{50} of 45 μg/ml whereas **3** exhibited cytotoxic effects on L1210 cells with an IC_{50} of 90 μg/ml.

It is interesting that **1** exhibits nematicidal activity on *Caenorhabditis elegans* (IC_{50} 25 μg/ml).

At high concentrations (333 μg/ml) **1** inhibits the growth of shoots and roots of *Setaria italica* and *Lepidium sativum* by 60% while **3** only affects shoot growth. **2** and **4a** had no effect on the germination and growth of both plants.

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