3. Acute toxicity

The compounds **4, 14, 17** were tested for LD_{50} in male and female mice with body weight of 17–25 g after i.p. injection by Prozorovsky [8].

4. Statistical analysis

The statistical significance of the difference between values of compound-treated versus control animals was evaluated using the Student's *t* test.

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New illudane sesquiterpenes from the basidiomycete *Clitocybe rivulosa HKI* 0273

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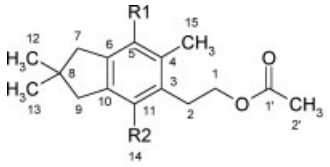
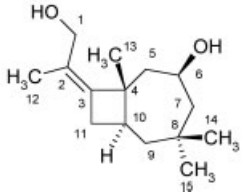
Bicyclic illudanes are frequently occurring fungal sesquiterpenes displaying various biological activities [1–6]. Representatives of this family such as fomajorins [1], radulactone [2], illudone [3], phaliotic acid [4], tsugicoline [5] and 5(2′-hydroyethyl)-2,2,4,6-tetramethyl-1,2-dihydroindane [6] are generated by different genera of basidiomycetes such as *Russula*, *Pholiota*, *Fomitopsis*, *Laurilia*, *Fomes* and *Radulomyces*. Here we report on isolation, structures and biological activities of new metabolites **1, 2** and **3** (Fig. 1) as new illudane sesquiterpenes from cultures of *Clitocybe rivulosa* HKI 0273. Another unusual bicyclic sesquiterpenoid compound (**4**) was co-isolated as a possible shunt metabolite of illudane biosynthesis. The producing strain HKI 0273 from the collection of the Hans-Knöll-Institute was conserved as mycelial culture of *Clitocybe rivulosa* HKI 0273. This was derived from tissue plugs of a fruiting body of *Clitocybe rivulosa* collected from a meadow near Oberbodnitz (Saale-Holzland district, Thuringia, Germany). At the end of the fermentation the whole culture broth (20 l) was extracted three-times under stirring by ethyl acetate (1 : 1). The combined and dried extracts were evaporated to yield 1.5 g oily residue. The material was chromatographed on silica gel 60 (0.063–0.1 mm, elution by $CHCl_3$/MeOH 9 : 1). The obtained fractions were evaporated and rechromatographed on TLC (Merck silica gel aluminium sheets, $CHCl_3$/MeOH; 9 : 1) whereby zones were eluted staining reddish-blueish with 1% vanillin/conc. H_2SO_4. Final purification was achieved by preparative TLC using silica gel aluminium sheets RP_{18} and MeCN/H_2O 83 : 17 with 0.5% TFA as eluent. **1** (15 mg), **2** (12 mg), **3** (5 mg) and **4** (10 mg) were thus obtained as colorless wax in addition to sulcatins A and B [7, 8]. The physico-chemical properties of **1**–**4** are shown in the Experimental part.

Structure elucidation of compounds **1**–**4** was carried out by MS, one- and two-dimensional NMR spectroscopy (^1H, ^{13}C, DEPT 13T, ^1H, ^1H-COSY, HSQC, HMBC, NOESY).





1 (R_1=H, R_2=CH_2OH), **2** (R_1=OH, R_2=CH_2OH), **3** (R_1=H, R_2=OH) and bicyclic metabolite **4** from *Clitocybe rivulosa* HKI 0273

The molecular weight and the chemical formulas of **1**–**3** were readily suggested by HREI-MS (see Experimental). The ESI-MS of **4** displayed m/z 261.2 ($[M + Na]^+$) but in the HREI-MS only m/z 137.1336 (100%) was visible as a prominent fragment ion.

The number of carbon atoms in compounds **1**–**4** and their bonding type was suggested by the ^{13}C and DEPT-135 NMR spectra. The ^{13}C NMR spectra of illudanes **1**–**3** unraveled the presence of carbonyl groups, of an aromatic rings, methylene and methyl groups. Moreover, the occurrence of aromatic and aliphatic quaternary carbon atoms in **1**–**3** was attested by the DEPT-125 and HSQC spectra. In the ^{13}C NMR spectra of **4** signals of two doubly bound carbons were visible at 127.2 ppm and 145.3 ppm. Low intensity of these and singlet multiplicity suggested their quaternary nature. The substitutions of the benzoid rings in **1**–**3** were furnished by the chemical shift and coupling pattern in the ^1H and ^1H, ^1H-COSY spectra. For structural assignment of compounds **1**–**3** the C, H long-range correlations in the HMBC spectra were of pivotal importance.

The structure of **4** was unveiled doubtlessly as an unusual bicyclic metabolite due to MS and NMR spectra (see Experimental). Thus, the observable $^3J_{H,H}$ COSY couplings of H-6/H-5_A, H-6/H-5_B, H-6/H$_{7A}$, H-6/H$_{7B}$, H-10/H-9_A, H-10/H-9_B, H-10/H-11_A and H-10/H-11_B supported the structural assignment as shown in Fig. 1. The structure of **4** was further confirmed by the $^2J_{C,H}$ and $^3J_{C,H}$ long-range coupling pattern in the HMBC spectrum.

The arrangement of substituents at the double bond in **4** was settled on the basis of the strong NOE's between H-1/H13 and H-11/H-12. The relative stereochemical arrangement of substituents at C-4, C-6, C-8 and C-10 was suggested further by the missing NOE between H-13/H-4 and H-13/H-6, and the observable NOE between H-13/H-14. Compound **4** thus appears as an unusual bicyclic structure composed of four- and seven-membered rings which is similar to koraiol [8]. The occurrence of sulcatins A and B amongst the products of the fungal strain *Clitocybe rivulosa* HKI 0273 suggested that metabolites **1**, **2**, **3** and **4** are intermediates and/or end products of a branched sesquiterpenoid pathway.

A moderate antimicrobial activity of compounds **1** and **4** was found in the common agar well diffusion assay against *Bacillus subtilis* ATCC 6633, *Mycobacterium vaccae* IMET 10670 and the yeast *Kluyveromyces marxianus* IMET 25148 concentrations $>50\,\mu g$ per agar well suggesting that these metabolites are capable of interacting with biological structures.

Experimental

1. Cultivation conditions

A small piece of a malt agar culture of *Clitocybe rivulosa* HKI 0273 (malt extract 4%, yeast extract 0.4%, agar 1.5%, pH 6) was used to inoculate 1 l Erlenmeyer bottles containing 250 ml of the nutrient medium composed as follows (g/l): D-glucose 10, malt extract 20, soytone 2, yeast extract 1, KH_2PO_4 1, $MgSO_4 \cdot 7\,H_2O$ 0.5, pH 5.5. The cultivation was carried out for 12 days at 23 °C and 110 rpm on a rotary shaker.

2. Instruments

ESI-MS was recorded on a triple quadrupole instrument Quattro (VG Biotech, Altrincham, England), HREI-MS on a double focussing sector-field instrument AMD 402 equipped with direct inlet system (Harpstedt, Germany), NMR spectra on a Bruker Avance 500 DRX spectrometer and IR spectra (film) on a sattellite FTIR spectrometer equipped with ATR device.

3. Compounds

1: Colorless wax; M.W. (HREI-MS): m/z 276.1732 (M^+; calcd. 276.1738 for $C_{17}H_{24}O_3$); IR (cm^{-1}): 770, 1006, 1024, 1044, 1092, 1237, 1242, 1363, 1383, 1450, 1462, 1580, 1621, 1733, 2867, 2926, 2953, 3328: ^1H NMR (δ in ppm; J in Hz): 0.99 (H-15, s, 3 H), 1.01 (H-12, s, 3 H), 1.61 s (H-2′, s, 3 H), 1.82 (H-13, s, 3 H), 2.2 (14-OH, br), 2.56 (H-7, d, −9.8 Hz, 2 H), 2.58 (H-9, d, −9.3 Hz, 2 H), 2.85 (H-2, dd, 7.0, 3.5, 2 H), 3.62 (H-14, s, br, 2 H), 4.65 (H-1, dd, 7.0, 13.2, 2 H), 6.28 (H-5, s, 1 H). ^{13}C NMR (δ in ppm; multiplicity): 12.2 (C-15, q), 20.7 (C-2′, q), 29.17 (C-12, q), 28.8 (C-2, t), 29.18 (C-13, q), 29.2 (C-8, s), 44.2 (C-7, t), 47.3 (C-9, t), 62.7 (C-14, t), 62.9 (C-1, t), 117.9 (C-5, d), 118.0 (C-3, s), 121.2 (C-4, s), 125.9 (C-6, s), 135.3 (C-11, s), 142.2 (C-10, s), 170.9 (C-1′, s). R_f TLC (silica aluminium sheets, Merck, $CHCl_3$/MeOH 9 : 1, v/v): 0.65.

2: Colorless wax; M.W. (HREI-MS): m/z 292.1709 (M^+; calcd. 292.1742 for $C_{17}H_{24}O_4$); IR (cm^{-1}): 754, 1001, 1028, 1105, 1177, 1240, 1363, 1382, 1437, 1462, 1585, 1645, 1722, 2866, 2926, 2951, 3385. ^1H NMR (δ in ppm, J in Hz): 1.19 (H-13, s, 3 H), 1.19 (H-12, s, 3 H), 2.05 (H-2′, s, 3 H), 2.23 (H-15, s, 3 H), 2.30 (14-O, br), 2.60 (H-7, d, −10.0), 2.78 (H-9, d, −10.0), 3.25 (H-2, dd, 7.0, 3.1, 2 H), 4.25 (H-1, dd, 7.0, 13.0), 4.75 (H-14, d, br, 2 H), 8.01 (5-HO, br). ^{13}C NMR (δ in ppm; multiplicity): 12.0 (C-15, q), 21.0 (C-2, q), 29.3 (C-12, q), 29.3 (C-13, q), 29.7 (C-8, s), 43.7 (C-7, t), 46.8 (C-9, t), 60.3 (C-14, t), 64,3 (C-1, t), 121.1 (C-4, s), 126.5 (C-6, s), 128.0 (C-3, s), 134.4 (C-11, s), 142.7 (C-10, s), 150.3 (C-5, s), 171.2 (C-1, s). R_f on TLC (silica gel 60 aluminium sheets, Merck, $CHCl_3$/MeOH 9 : 1, v/v): 0.55.

3: Colorless wax.; M.W. (HREI-MS): m/z 262.1551 (M^+; calcd. 262.1569 for $C_{16}H_{22}O_3$); ^1H NMR (δ in ppm, J in Hz): 0.99 s (H-15, s, 3 H), 1.00 (H-13, s, 3 H), 2.05 (H-2′, s, 3 H), 2.58 (H-9, d, −10.0, 2 H), 2.60 (H-7, d, −10.0, 2 H), 2.75 (H-2, dd, 7.0, 3.1, 2 H), 4.65 (H-1, dd, 7.0, 13.0, 2 H), 6.8 (H-5, s, 1 H), 8.2 (OH-11). ^{13}C NMR (δ in ppm, multiplicity): 15.3 (C-15, q), 25.6 (C-2, t), 29.2 (C-12, q), 29.2 (C-13, q), 29.3 (C-8, s), 44.9 (C-7, t), 47.3 (C-9, t), 63.1 (C-1, t), 120.3 (C-3, s), 123.3 (C-4, s), 126.3 (C-6, s), 126.7 (C-5, d), 140.7 (C-10, s), 151.5 (C-11, s), 170.9 (C-1, s). R_f on TLC (silica gel aluminium sheets, Merck, $CHCl_3$/MeOH, 9 : 1, v/v): 0.85.

4: colorless wax.; M.W. (ESI-MS): m/z 261.2 ($[M + Na]^+$); (HREI-MS): m/z 137.1336; calcd. 137.1330 for $C_{10}H_{17}$ (M- $C_5H_{10}O_2$); IR (cm^{-1}): 997, 1030, 1059, 1215, 1365, 1452, 1669, 1724, 2864, 2927, 3360. ^1H NMR (δ in ppm, J in Hz): 1.01 (H-12, s, 3 H), 1.15 (H-13, s, 3 H), 1.26 (H-15, s, 3H), 1.29 (H_A-7, dd, 7.0, 7.1, 1 H), 1.51 (H-9_A, dd, 7.5, 1.2, 1 H), 1.59 (H-14, s, 3 H), 1.60 (OH-6), 1.60 (OH-1), 1.61 (H_B-7, dd, 7.0, 1.5, 1 H), 1.62 (H_A-5, dd, 8.9, 1.8, 1 H), 1.76 (H_B-5, dd, 8.9, 7.0, 1 H), 2.31 (H-10, m, 1 H), 2.47 (H-$_A$-11, dd, 8.2, 1.2, 1 H), 2.48 (H_B-11, dd, 8.2, 1.5, 1 H), 3.96 (H_A-1, d, 11.5, 1 H), 4.10 (H-6, m, 1 H), 4.15 (H_B-1, d, 11.5, 1 H). ^{13}C NMR (δ in ppm, multiplicity): 15.1 (C-14, q), 24.4 (C-15, q), 24.5 (C-11, t), 27.8 (C-5, t), 29.8 (C-13, q), 30.4 (C-12, q), 36.6 (C-8, s), 42.8 (C-7, t), 48.2 (C-4, s), 51.4 (C-5, t), 55.8 (C-10, d), 63.1 (C-1, t), 75.3 (C-6, d), 127.2 (C-2, s), 145.3 (C-3, s). R_f on TLC (silica gel aluminium sheets, Merck, $CHCl_3$/MeOH, 9 : 1, v/v): 0.4.

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