ANTIFUNGAL ANTIBIOTIC FROM THE MUSHROOM *Agrocybe aegerita* (BRIG.) SING.*

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An extract from a submersed culture of the mushroom *Agrocybe aegerita* (BRIG.) SING., containing antifungal antibiotic compounds was chromatographed on a silica gel column. Compounds from fractions which displayed the highest biological activity were concentrated and isolated by means of preparative thin-layer chromatography and preparative high-performance liquid chromatography, and were further characterized by means of gas chromatography, mass spectrometry and nuclear magnetic resonance. They are sesquiterpenic diols predominantly with an illudine skeleton. Structural formulae are proposed for some of them.

Agrocybe aegerita (BRIG.) SING. [synonymum *Agrocybe cylindracea* (DC. ex FR.) MAIRE] is a thermophilic, wood-inhabiting, edible, gilled mushroom[1-3], belonging to the *Basidiomycetes* class, order of *Agaricales*. Submersed cultures of this mushroom produce antibiotic compounds[4-6], soluble in water, passing into the cultivation filtrate. It was found that compounds with bacteriostatic and bactericidal effects on Gram-positive and Gram-negative bacteria are produced first. These compounds have not yet been thoroughly characterized. Later on fungicidal compounds are also formed, active against the yeasts *Candida albicans, Candida kefyr* (synonymum *C. pseudotropicalis*) and *Candida tropicalis*. So far only a biologically inactive compound[7] was isolated from the cultivation medium filtrate, with the composition $C_{30}H_{40}O_6$. Quite recently a polysaccharide[8] with a potent antitumor activity was also isolated from *Agrocybe aegerita*.

The aim of our work was: *a*) to determine the number and the proportion of chromatographed compounds in the extract of the cultivation filtrate; *b*) to find out which of these compounds are responsible for the antifungal activity; *c*) to concentrate or possibly isolate the main components in a pure state; *d*) to determine their

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biological and chromatographic properties; *e*) to acquire structural information on the main compounds.

EXPERIMENTAL

A pure culture was obtained in 1971 by isolation, using the explantate method, from fresh fruit bodies of *Agrocybe aegerita* (BRIG.) SING., collected in south Slovakia. It is deposited in the Culture Collection of Basidiomycetes, Prague (CCBAS 311). This culture was used for submersed cultivation.

Submerse cultivation was carried out in the following way: wort medium (100 ml, 8 °Ball., pH 5·5) in Erlenmayer flask (300 ml) was inoculated with well grown mycelium culture of *Agrocybe aegerita* I (10–20 pieces, 20 days) from slant agar. After shaking (20 days) and filtration the cultivation filtrate (85 ml) was obtained.

The cultivation filtrate (1 l) was extracted with ethyl acetate (three 1 l portions). Combined ethyl acetate extracts were dried over Na_2SO_4 and evaporated to afford 1·34 g of residue. On repetition of this procedure a total of 47 g of extract was obtained.

Antifungal activities of the cultivation filtrate, the extract, chromatographic fractions and concentrated compounds (Table I) were determined by diffusion plate tests using the test culture of *Candida kefyr*. The antifungal antibiotic fungicidine was used as a standard. The weight of the samples tested was 1 mg.

Column chromatography (CC) was carried out in the following way: The extract (44 g, activity 40 I.U./mg) was dissolved in $CHCl_3$ (180 ml) and adsorbed on silica gel according to Pitra[9] (132 g, 60–200 μm). The solvent was evaporated under stirring under an infrared lamp. The adsorbed sample was then chromatographed on a column (6·5 × 47 cm) of silica gel according to Pitra (880 g, 60–200 μm, 12% addition of water for deactivation). The column was prepared in a mixture of light petroleum (b.p. 40–60°C) and diethyl ether (2 : 3) and the same system was used for elution of fractions 1–11 (volume of the fractions 1 l). From fraction 12 (volume 2 l) only diethyl ether was used for elution. Fractions 2, 3 and 4 weighed 305, 241 and 193 mg, respectively.

Preparative high performance liquid chromatography (PHPLC) was carried out on a HP 1090 (Hewlett-Packard) instrument with a diode array detector monitored by a HP-85 B (Software version B 2570/84) computer. The detector was programmed to operate for the 241 nm wave-length. For isocratic separation a 8 × 250 mm column was used, containing silica (particle size 10 μm) (Laboratorní přístroje, Prague, Czechoslovakia). The system hexane–2-propanol (97 : 3 v/v) was used as the mobile phase, column flow rate 2·3 ml/min.

Preparative thin-layer chromatography (PTLC) was carried out on glass plates (20 × 20 cm) coated with silica gel according to Pitra (layer strength 0·2 mm, 10–35 μm particle size, content of gypsum 12%). Fractions 2, 3 or 4 (30 mg in 0·3 ml of $CHCl_3$) were applied with a capillary. Development of the chromatograms was carried out 6 times in a system light petroleum–diethyl ether–methanol (70 : 25 : 1). The same or a similar separation effect was also achieved in the following systems: light petroleum–diethyl ether–2-methyl-2-propanol (85 : 25 : 1) or light petroleum–diethyl ether–2-propanol (85 : 25 : 1). The dry plate was sprayed with a solution of Rhodamine 6G (0·05% in ethanol). Individual zones were marked in UV light (254 nm) and scraped off, transferred into small columns (0·8 cm I.D.) containing silica gel according to Pitra (0·5 g, 25–50 μm) and eluted with diethyl ether (20 ml).

Gas chromatography (GC) was carried out on an HP 5890A instrument (Hewlett–Packard) with flame ionization detector and a split splitless-injection system, used in the split mode. For separation and identification two fused silica capillary columns were used consecutively (J & W

SCIENTIFIC, U.S.A.), length 30 m, I.D. 0·25 mm, film thickness 0·25 μm, carrier gas H_2, 90 kPa. Conditions for chromatography: polar column DB-WAX; detector temperature 250°C, injector temperature 240°C, oven temperature 230°C, average linear velocity of the carrier gas 37·5 cm/s, column flow rate 1·1 ml/min (at 230°C), split ratio 60 : 1. Unpolar column DB-1: detector temperature 250°C, injector temperature 220°C, oven temperature 170°C, average linear velocity of the carrier gas 48·4 cm/s, column flow rate 1·65 ml/min (at 170°C), split ratio 37 : 1. For recording and integration an HP 3393A integrator (Hewlett-Packard) was used. Kováts retention indices of individual compounds (Table II) were calculated by means of a modified Heeg and co-worker method[10]. A homologous series of n-alkanes $C_{24}-C_{34}$ was injected onto the DB-WAX column together with the sample, while with the DB-1 column the $C_{15}-C_{21}$ series was used.

Mass spectrometry (MS) was done on a combined GC/MS instrument HP 5890A/ZAB-EQ (VG Analytical, Ltd., Manchester, U.K.) using a fused silica capillary column with OV-1, at an electron energy of 70 eV. Elemental composition of the isolated compounds was determined

TABLE I

Results of biological titrations

Substance tested	Antifungal activity I.U./mg
The starting extract	40
Fraction 1	50
Fraction 2	225
Fraction 3	2 400
Fraction 4	33 000
Fraction 5	1 250
Fraction 6	1 250
Fraction 7 + 8[a]	810
Fraction 9–11	1 000
Fraction 12	430
Fraction 13	61
Fraction 14	26
Compound **7** (41)[b] + **9** (11)	8
Compound **8** (60) + **29** (21)	1 000
Compound **9** (75)	0
Compound **10A** (37) + **19** (59)	17
Compound **10B** (54) + **7** (35)	480 000
Compound **11** (99)	19
Compound **17** (64)	38
Compound **25** (16) + **29** (38)	6 400

[a] The fractions were combined on the basis of GC analysis. [b] The numbers in brackets mean % of compound in the sample tested (determined by GC). The remainder to 100% is always composed of several further compounds (impurtities), but the percentage of none of them was higher than 6%.

by measurement at a 10 000 resolving power, and the relations between the ions of some compounds were determined by using a B/E linked scan for collision-induced dissociation in the first field region.

Proton and carbon NMR spectra were measured on a FT NMR spectrometer VARIAN XL-200 (^{1}H at 200 MHz and ^{13}C at 50·3 MHz frequency) in deuteriochloroform with tetramethylsilane as internal reference. Mutually interacting hydrogens in the ^{1}H NMR spectra were assigned by selective decoupling experiments. The presence and the character of the hydroxyl groups in compounds *9* and *11* were determined by in situ acylations with trichloroacetyl isocyanate (TAI). TAI-Acylations were carried out in the conventional manner[11] by addition of a slight excess of TAI to a chloroform solution of the sample in an NMR cell. The trichloroacetylcarbamoyl derivatives (TAC) were characterized by ^{1}H NMR spectra without isolation. The ^{13}C NMR spectra of compounds *8*, *11* and *17* were measured with a broad-banded decoupling of hydrogens using pulse sequences APT (ref.[12]), (based on the modulation of the signal amplitudes according to J(C, H)), enabling a resolution of the carbon signals according to the number of directly bound hydrogens. In the case of compound *11* heteronuclear ^{1}H–^{13}C 2D-COSY spectrum was used (measured on a BRUKER AX-400 spectrometer at a 100·6 MHz frequency) for experimental assignment of the signals of carbons and hydrogens of the CH, CH_2 and CH_3 groups.

RESULTS AND DISCUSSION

Chromatography of the extract of the cultivation filtrate on a silica gel column gave 14 fractions. Each of them was tested both for determining antifungal activity (Table I) and analysed by gas chromatography (gas chromatograms of fractions 2, 3 and 4 are in Fig. 1). It was found that at least 70 compounds giving distinctly separated chromatographic peaks were present in all fractions. The more frequent compounds were ordered according to their increasing retention times (RT) on the polar phase DB-WAX and they were numbered accordingly. The compounds are indicated in the text, chromatograms and tables by these numbers. All compounds separated well on the DB-WAX phase with the exception of compounds **10A** and **10B**. On the basis of the analysis of mass spectra it was shown later that compound **10** from the chromatographic fraction 2 is not identical with compound **10** from fraction **4** in spite of the fact that their retention times on the polar phase DB-WAX were almost identical (while on the non-polar phase DB-1 their retention times were distinctly different). In the subsequent calculation of Kováts retention indices (Table II) a difference of only one unit was observed for compounds **10A** and **10B** (on phase DB-WAX). For two compounds to be separated on a medium length capillary column (30 m), it is indispensable that their retention indices should differ at least by 4 units[13,14]. From Table II it is also evident that compounds **11**, **9**, **7**, **10A** and **8** cannot be separated on the non-polar phase DB-1 for the above mentioned reason (they gave practically a single non-separated peak). The same is true of compounds **12** and **13**.

For concentration or isolation of individual compounds (their quantitative representation is given in Table III) fractions 2, 3 and 4 from column chromatography

were used (the last two fractions also displayed the highest antifungal activity – Table I). Using PHPLC compound **11** was isolated in a pure state; mixtures of the pairs of compounds **10A** + **18** and **7** + **10B** were also obtained. In view of considerable losses during PHPLC, further compounds, **7**, **8**, **9** and **17**, were isolated or concentrated by means of PTLC. However, after one development in most various elution systems each of the fractions 2, 3 and 4 gave practically a single unseparated spot. A partial separation was achieved only after application of multiple development in a low-polar system[15].

It follows from the biological tests (Table I) that fractions or enriched mixtures containing compounds **8**, **29** and especially **10B** display the highest activity. Other compounds **7**, **9**, **10A**, **11**, **17** and **19** were practically inactive. However, the most active compound **10B** could not be isolated in an amount and purity necessary for the measurement of its NMR spectrum. Only on the basis of its mass spectrum (Fig. 2) it can be judged that it is a sesquiterpenic compound of elemental composition $C_{15}H_{24}O_2$. The molecular peak in its spectrum is hardly discernible, but from the relations between the peaks of the ions m/z $[(M - 18)^{+\bullet}]$ and m/z 205 $[(M - 31)^+]$ it is evident that $M^{+\bullet}$ is 236. The presence of peaks of masses m/z 234 and 232

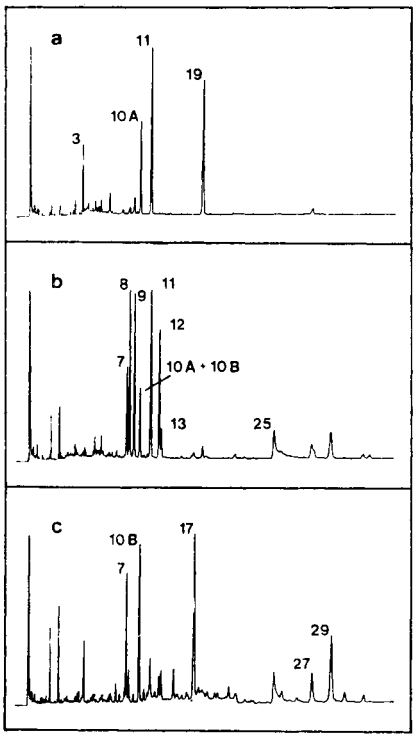

FIG. 1

Gas chromatogram of fractions from column chromatography: a fraction 2, b fraction 3, c fraction 4. The conditions of chromatography are given in Experimental, column DB-WAX

indicates a dehydrogenation of the system and, hence, it may be assumed that the reason is a cycle or two annellated rings with one double bond in which dehydrogenation leads to aromatization. One to two hydroxyl groups are present in the molecule

TABLE II

Kováts Retention Indices. (Individual values represent a mean of at least three measurements, SD ±0·3)

Compound	I_{230}^{DB-WAX}	I_{170}^{DB-1}	$I^{DB-WAX} - I^{DB-1}$
3	2 685·3	1 754·4	930·9
4	2 700[a]	—	
7	2 900[a]	1 788·9	1 111·1
8	2 914·3	1 791·9	1 122·4
9	2 931·0	1 787·5	1 143·5
10A	2 951·2	1 790·6	1 160·6
10B	2 952·2	1 818·2	1 134·0
11	2 984·6	1 785·3	1 199·3
12	3 013·2	1 855·7	1 157·5
13	3 018·3	1 856·1	1 162·2
14	3 051·1	—	
17	3 100[a]	1 876·6	1 223·4
19	3 118·2	1 849·0	1 269·2
25	3 246·0	1 823·4	1 422·6
26	—	1 829·6	
27	3 310·3	1 892·1	1 418·2
29	3 335·0	1 910·3	1 424·7

[a] The peaks of the compounds coincided with the peaks of n-alkanes.

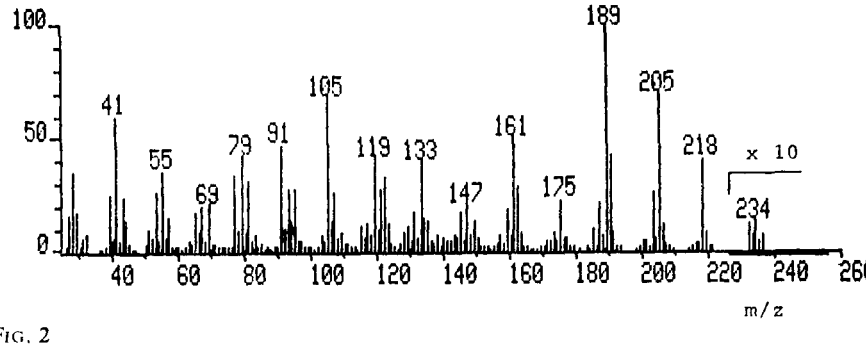

FIG. 2

Mass spectrum of compound **10B**. The conditions are given in Experimental

(m/z 218 = M − 18, m/z 161 = 189 − 18), while one of them is a component of the primary alcohol group (m/z 205 = M − $^{\bullet}CH_2OH$, m/z 31). After comparison with the spectra of identified compounds **11** and **19** it may be assumed that compound **10B** also has an illudine skeleton. In Table IV shortened mass spectra are presented, as well as molecular weights and elemental compositions of all the substances measured.

Compounds **8**, **9**, **11**, **17** and **19** were obtained in amounts higher than 1 mg, which permitted the measurements of at least ^1H NMR spectra. In compounds **8** and **17** their ^{13}C NMR spectra were also measured, but their interpretation was complicated by the insufficient signal-to-noise ratio (due to the very low amount) and purity. Only compound **11** could be studied sufficiently in detail by means of ^1H and ^{13}C NMR spectra. The NMR data obtained are surveyed in Table V.

According to MS all the above mentioned compounds contain 15 carbons, two or three oxygen atoms and three to six unsaturations. The presence of a 1,1-disubstituted cyclopropane ring, two to three tertiary methyl groups completed to the total number of four one-carbon substituents by the secondary methyl group, exomethylene or a CH_2OR group, and the number of unsaturations (indicating the presence of three-carbon cycles) led to the proposal of illudine as the common

TABLE III

Representation of compounds in chromatographic fractions 2, 3 and 4 and in the extract

Compound	Fraction 2[a]	Extract[b]	Fraction 3[a]	Extract[b]	Fraction 4[a]	Extract[b]
3	5·0	0·035	—	—	—	—
7	—	—	6·3	0·035	6·8	0·030
8	—	—	13·8	0·076	1·9	0·0083
9	—	—	12·3	0·067	—	—
10A	12·5	0·087	6·3	0·035	—	—
10B	—	—			9·6	0·042
11	37·9	0·263	16·4	0·090	4·2	0·018
12	—	—	11·1	0·061	1·8	0·0079
13	—	—	2·7	0·015	2·3	0·010
17	—	—	—	—	16·6	0·073
19	26·8	0·186	1·2	0·0066	—	—
25	—	—	5·8	0·032	5·7	0·025
27	—	—	1·8	0·0099	4·3	0·019
29	—	—	5·5	0·030	9·9	0·043

[a] The numbers mean area %, obtained from the integration of the chromatograms of fractions 2, 3 and 4. [b] The numbers mean % obtained by calculation per weight of the starting extract (44 g), chromatographed on a silica gel column.

TABLE IV

Shortened mass spectra and composition formulae of the main compounds

Compound	Mass spectrum[a]									Composition formula
	$M^{+\cdot}$	1	2	3	4	5	6	7	8	
3	236 (3)	124 (100)	109 (61)	41 (42)	137 (36)	95 (32)	55 (28)	150 (23)	203 (21)	$C_{15}H_{24}O_2$
7	250 (9)	43 (100)	189 (69)	232 (39)	91 (26)	138 (26)	105 (24)	55 (23)	203 (23)	$C_{15}H_{22}O_3$
8	236 (67)	203 (100)	41 (83)	91 (82)	105 (78)	236 (67)	119 (63)	205 (60)	189 (57)	$C_{15}H_{24}O_2$
9	248 (64)	43 (100)	248 (64)	91 (40)	201 (40)	187 (36)	160 (34)	147 (31)	77 (29)	$C_{15}H_{20}O_3$
10A	234 (97)	187 (100)	234 (97)	201 (73)	173 (64)	159 (63)	91 (61)	41 (56)	77 (49)	$C_{15}H_{22}O_2$
10B	236 (0)	189 (100)	105 (78)	205 (75)	41 (58)	91 (50)	161 (48)	119 (46)	79 (44)	$C_{15}H_{24}O_2$
11	234 (48)	201 (100)	43 (68)	173 (67)	234 (48)	159 (43)	91 (23)	187 (23)	219 (20)	$C_{15}H_{22}O_2$
12	250 (2)	154 (100)	148 (47)	222 (37)	41 (33)	91 (30)	77 (25)	120 (23)	176 (22)	$C_{15}H_{22}O_3$
13	232 (72)	201 (100)	232 (72)	91 (28)	160 (28)	77 (27)	115 (25)	185 (25)	129 (22)	$C_{15}H_{20}O_2$
17	254 (3)	43 (100)	97 (62)	69 (58)	109 (51)	55 (36)	81 (35)	121 (35)	135 (28)	$C_{15}H_{26}O_3$
19	234 (13)	203 (100)	147 (89)	187 (67)	91 (60)	41 (52)	161 (52)	131 (51)	201 (50)	$C_{15}H_{22}O_2$
27	232 (100)	232 (100)	202 (36)	171 (31)	217 (28)	199 (25)	155 (23)	141 (21)	185 (21)	$C_{15}H_{20}O_2$
29	250 (0)	219 (100)	191 (15)	41 (9)	77 (8)	91 (8)	105 (6)	107 (6)	161 (4)	$C_{15}H_{22}O_3$

[a] The mass of the molecular ion $M^{+\cdot}$ is given in the first place, and it is followed by the eight most intensive peaks. Their relative instensities in % are given in brackets.

TABLE V

Proton and carbon-13 NMR data of compounds 8, 9, 11, 17 and 19

Compound	NMR parameters[a] (chemical shift; multiplicity; structural assignment; coupling constants in Hz)
8[b]	^{1}H NMR ($CDCl_3$)
	0·5–0·7 m, 2 H (cp-H); 1·00 s, 3 H (CH_3—C); 1·09 s, 6 H (2 × CH_3—C); 1·88 dd, 1 H (>CH—, J = 11·9 and 7·2); 2·26 d, 1 H (>CH—, J = 2·0); 3·51 bd 1 H and 3·83 bd, 1 H (CH_2—O, J = 11·5); 3·78 dd, 1 H (CH—O, J = 9·7 and 3·4)
	^{13}C NMR ($CDCl_3$)[c,d]
	9·67 (+); 11·00 (−); 14·53 (+); 29·45 (−); 29·77 (−); 30·57 (−); 37·51 (+); 43·70 (−); 43·83 (−); 43·87 (+); 44·38 (+); 45·04 (+); 45·13 (−); 48·45 (+); 58·07 (+); 66·36 (+); 75·09 (−)
9	^{1}H NMR ($CDCl_3$)
	0·26 m, 1 H and 0·5–1·00 m, 3 H (cp-H); 1·19 s, 3 H (CH_3—C—O); 1·22 s, 3 H (CH_3—C); 1·68 dd, 1 H (CH_aH_b, J = 10·3 and 1·9); 1·77 bd, 1 H (CH_aH_b, J = 10·3); 3·40 bd, 1 H and 3·57 d, 1 H (CH_2—O, J = 7·5); 4·16 bt, 1 H (CH—O, $J \approx$ 3); 4·79 um, 1 H and 4·82 um, 1 H (CH_2=C); 6·33 bs, 1 H (CH=C)
	^{1}H NMR ($CDCl_3$ + TAI)
	0·38 m, 1 H and 0·6–1·2 m, 3 H (cp-H); 1·22 s, 3 H (CH_3—C); 1·72 s, 3 H (CH_3—C—OTAC); 2·02 d, 1 H (CH_aH_b, J = 11·0); 2·41 dd, 1 H (CH_aH_b, J = 11·0 and 2·0); 3·51 d, 1 H and 3·65 d, 1 H (CH_2—O, J = 8·2); 4·94 um, 1 H and 4·97 um, 1 H (CH_2=C); 5·41 b, 1 H (CH—O—TAC); 6·52 s, 1 H (—CH=C); 8·18 s, 1 H (NH); 8·39 s, 1 H (NH)
11	^{1}H NMR ($CDCl_3$)
	0·55 ddd, 1 H (cp-H, J = 9·4, 5·8 and 4·0); 0·87 ddd, 1 H (cp-H, J = 9·7, 6·5 and 4·9); 0·95 ddd, 1 H (cp-H, J = 9·4, 5·8 and 4·9); 1·04 ddd, 1 H (cp-H, J = 9·7, 5·8 and 4·0); 1·06 s, 3 H and 1·16 s, 3 H (CH_3—C—CH_3); 1·07 s, 3 H (CH_3—C—OH); 1·57 dd, 1 H (CH_aH_b, J = 12·7 and 9·8); 1·79 dd, 1 H (CH_aH_b, J = 12·7 and 7·5); 3·08 ddd, 1 H (>CH—, J = 9·8, 7·5 and 2·5); 3·83 d, 1 H and 3·87 d, 1 H (CH_2—OH, J = 13·4); 5·41 d, 1 H (—CH=C, J = 2·5) 6·24 s, 1 H (—CH=C)
	^{1}H NMR ($CDCl_3$ + TAI)
	0·70 ddd, 1 H (cp-H, J = 9·4, 7·1 and 4·6); 0·9–1·2 m, 2 H (cp-H); 1·49 ddd, 1 H (cp-H, J = 9·6, 6·1 and 4·6); 1·06 s, 3 H and 1·17 s, 3 H (CH_3—C—CH_3); 1·57 s, 3 H (CH_3—C—OTAC); 1·82 dd, 1 H (CH_aH_b, J = 12·8 and 7·1); 1·96 dd, 1 H (CH_aH_b, J = 12·8 and 10·1); 3·50 ddd, 1 H (>CH—, J = 10·1, 7·1 and 2·6); 4·43 bd, 1 H and 4·63 bd, 1 H (CH_2—O—TAC, J = 12·7); 5·61 d, 1 H (—CH=C, J = 2·6); 6·41 bs, 1 H (—CH=C); 8·13 s, 1 H (NH); 8·34 s, 1 H (NH)

TABLE V
(*Continued*)

Compound	NMR parameters[a] (chemical shift; multiplicity; structural assignment; coupling constants in Hz)
	^{13}C NMR ($CDCl_3$)
	6·31 and 10·52 (2 × cp-CH_2); 20·13, 27·55 and 29·55 (3 × CH_3); 30·56 (cp-C); 39·80 (CH_2); 44·72 (C); 52·99 (CH); 63·15 (CH_2OH); 71·68 (C—OH); 120·77 (—CH=); 138·56 (>C=); 142·93 (>C=)
17	^{1}H NMR ($CDCl_3$)
	0·4—0·7 m, 2 H (cp-H); 0·79 d, 3 H (CH_3—CH, J = 6·6); 0·99 s, 3 H (CH_3—C); 1·04 s, 3 H (CH_3—C); 1·31 s, 3 H (CH_3—C—O); 2·91 d, 1 H (>CH—, J = 6·0); 3·41 dd, 1 H (CH_aH_b—O, J = 11·7 and 4·4); 3·82 t, 1 H (CH_aH_b—O, J = 11·7); 5·26 bdd, 1 H (CH—O, J = 6·0 and 2·8)
	^{13}C NMR ($CDCl_2$)[c,d]
	14·40 (−); 15·54 (−); 18·71 (+); 19·24 (+); 24·63 (−); 27·51 (−); 28·65 (−); 31·36 (−); 31·55 (−); 35·27 (−); 38·95 (+); 56·63 (−); 61·75 (+); 63·88 (+); 63·91 (−); 73·32 (+); 93·12 (−)
19	^{1}H NMR ($CDCl_3$)
	0·56 m, 1 H (cp-H); 1·03 s, 3 H (CH_3—C); 1·12 s, 3 H (CH_3—C); 1·40 dd, 1 H (CH_aH_b, $J \approx$ 12 and 11); 1·94 dd, 1 H (CH_aH_b, J = 12 and 7·2); 2·30 d, 2 H (CH_2, J = 2·2); 2·67 m, 1 H (CH, J = 11, 10 and 7·2); 3·71 bd, 1 H and 3·80 bd, 1 H (CH_2—O, J = 11·5); 4·11 bd, 1 H (CH—O, J = 10·0); 4·62 d, 1 H and 4·98 d, 1 H (CH_2=C, J = 2·0)

[a] The symbols cp-H and cp-C are used for cyclopropane protons and carbons. [b] The sample used was a mixture with compound 27 — only some of proton signals could be extracted. [c] The amplitudes in APT spectrum are indicated as (+) for CH_2 and/or C and (−) for CH_3 and/or CH carbons. [d] More than 15 signals were detected in ^{13}C NMR spectrum — some of them belong to the admixture.

skeleton for the substances investigated (probably with the exception of compound **17**). Some compounds with the illudine skeleton (or nor-illudine) were isolated and described earlier from mushrooms[3,16–19]. However, according to a comparison with the accessible NMR data the compounds isolated by us are not identical with them. The structural information obtained from the NMR spectra of compounds **8**, **9**, **11**, **17** and **19** will be shortly discussed now. However, structural formulae (Fig. 3) could be proposed only for three of them on the basis of their NMR spectra.

Compound **8** of composition $C_{15}H_{24}O_2$ could not be obtained in pure form and therefore its NMR data are also incomplete. The compound evidently contains a cyclopropane ring, three tertiary methyl groups and hydrogens of the CH_2OR and CHOR type. The ^1H and ^{13}C NMR spectra speak against the presence of a double bond. An attempt at the demonstration of OH groups by means of TAI acylation was unsuccessful, because a mixture of products was formed. The NMR

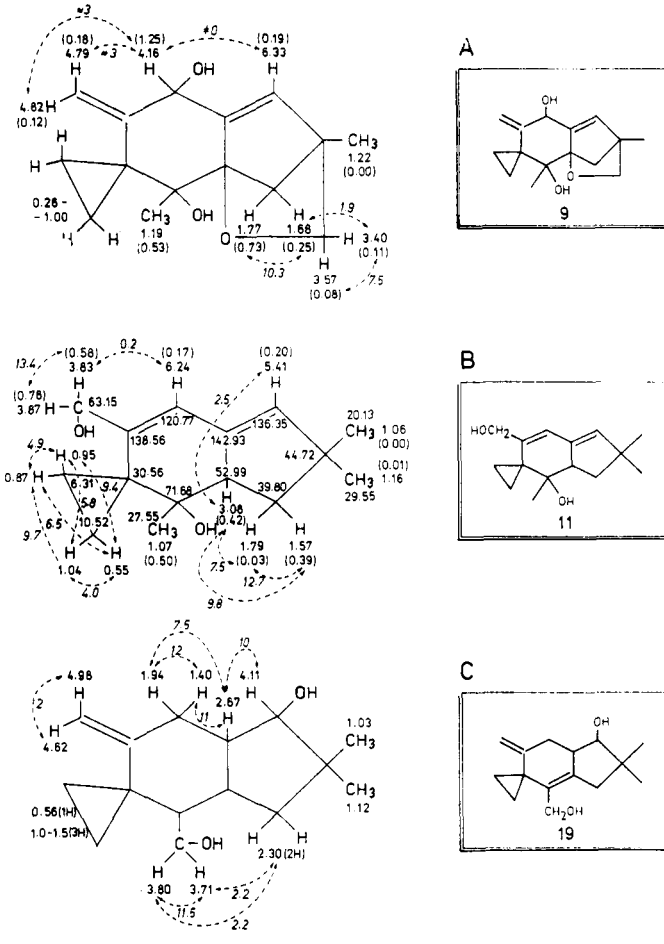

FIG. 3

Structural formulae **A**, **B** and **C** of compounds **9**, **11** and **19** and their NMR parameters. Chemical shifts of hydrogens (or also carbons) are given with the individual atoms. The values of the TAI-acylation shifts are given in brackets. The interacting hydrogens are indicated by an interrupted line with an indication of the value of the coupling constant (italics)

data available and problems with the purity of the sample did not permit us to formulate its probable structure.

Compounds **9** has the composition $C_{15}H_{20}O_3$ and according to its ^{1}H NMR spectra it contains a cyclopropane ring, two tertiary methyls, isolated methylene groups of the C—CH_2—C and C—CH_2—O type, a hydrogen of the >CH—O type, an exomethylene group and a trisubstituted double bond. TAI acylation of compound **9** demonstrated the presence of two OH groups (NH signals at δ 8·18 and 8·39 and characteristic acylation shifts of the >CH—OH hydrogen at δ 4·16 by 1·25 ppm and of the tertiary methyl CH_3—C—OH at δ 1·19 by 0·53 ppm). Very small acylation shifts of the CH_2—O group hydrogens at δ 3·40 and 3·57 (0·11 and 0·08 ppm) and the value of the geminal coupling J = 7·5 Hz, together with the requirement of another unsaturation (following from the elemental composition of compound **9**), show that the CH_2—O group is evidently a component of the five-membered ring, with the oxygen attached to the quaternary carbon of the skeleton. This information, together with the TAI acylation effects on the remaining hydrogens, permitted us to propose the structure **A** for compound **9**, which is also shown by the structural assignment of the hydrogen atoms and the TAI acylation shifts.

In the case of compound **11**, of the coposition $C_{15}H_{22}O_2$, the ^{1}H NMR spectrum indicated a cyclopropane ring, three tertiary methyl groups, a —CH—CH_2— fragment, two trisubstituted double bonds and an isolated CH_2—O group. The TAI acylation proved the presence of two OH groups (singlets of the NH hydrogens at δ 8·13 and 8·34) in the fragments CH_2—OH (acylation shifts of the CH_2—O hydrogens at δ 3·87 and 3·83 by 0·78 and 0·58 ppm) and CH_3—C—OH (acylation shift of the methyl at δ 1·07 by 0·50 ppm). The long-range interaction and the values of the acylation effects on the remaining hydrogens permitted the localization of the structural fragments on the illudine skeleton and the proposal of formula **B** for compound **11**, in which the ^{1}H NMR parameters are also given. The ^{13}C NMR spectrum contains 15 signals the chemical shifts and multiplicity of which are in agreement with the proposed structure. The structural assignment of the CH, CH_2 and CH_3 signals, also given in formula **B**, was carried out by means of heteronuclear ^{1}H–^{13}C 2D-COSY experiment. The signals of five quaternary carbons were assigned on the basis of the chemical shifts values. The relative configurations on the quaternary carbons of the CH_3—C(OH)—CH— fragment were not proved unambiguously, because the information on the conformation of the six-membered ring is not available. The NOE effects observed in the ^{1}H NMR spectrum were generally small and inconclusive. The distinct TAI acylation shift of the angular hydrogen (0·42 ppm) might be evidence for a gauche arrangement of the OH and H in the mentioned fragment and thus of their relative *cis* configuration.

Compound **17** has the composition $C_{15}H_{26}O_3$ and its ^{1}H NMR spectrum showed again the presence of a cyclopropane ring, one secondary and three tertiary methyls

and hydrogens of the CH_2—O and CH—O groups. The third oxygen is probably a component of the CH_3—C—O fragment. Four methyl groups and the CH_2—O group indicate that in this case the illudine skeleton is not present. The TAI acylations afford a mixture of products the spectrum of which cannot be interpreted reliably. The ^1H and ^{13}C spectra exclude the presence of the double bonds. The available information and the small amount of the sample do not permit a proposal of the most probable structure.

Compound **19** of the composition $C_{15}H_{22}O_2$ contains the signals of a cyclopropane ring, two tertiary methyl groups, the fragments HO—CH_2—C=C—CH_2— and —CH_2—CH—CH—OH and the hydrogens of an exomethylene group in its ^1H NMR spectrum. On the basis of these fragments structure **C** may be proposed for compound **19**, which is again based on the illudine skeleton and which also shows the structural assignment of the hydrogen signals.

CONCLUSIONS

As evident from the gas chromatographic records of the fractions 2, 3 and 4 (Fig. 1), they represent very complex mixtures of compounds which are mutually similar, both with respect to their chromatographic behaviour on silica gel (TLC, HPLC) and to that on a capillary chromatographic column. Therefore the majority of compounds could not be obtained in a pure state. It seems that preparative gas chromatography (PGC) which, unfortunately, was not available to us, might be more successful. The isolation was also hampered by the instability of some compounds. This was evident in TLC, PTLC, HPLC and it was also confirmed by GC, but especially by biological tests. Thus, for example, the most active fraction 4 from column chromatography displayed an activity of 33 000 I.U./mg on December 12, 1988, 3 700 I.U./mg on November 30, 1989 and only 300 I.U./mg on April 20, 1990, in spite of the fact that the fraction was kept under argon at −18°C all the time.

From Table I it is evident that the chromatographic fractions 5−11 also display a considerable biological activity. These fractions contained mainly compounds No. **4**, **5**, **6**, **11***, **14**, **15**, **16**, **17**, **18**, **20**, **22**, **23**, **24**, **26**, **32** and **33**. However we have not studied them in greater detail yet.

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* Compound **11**, the main component in fraction 9, is again different from compound **11** from fraction 2, in spite of the fact that their RT on the DB-WAX column are practically identical.

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