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GLC–Mass Spectral Analysis of Fungal Metabolites

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Abstract □ Four metabolites, hispidin, bisnoryangonin, muscimole, and ibotenic acid, from potentially psychoactive mushrooms were analyzed by GLC–mass spectrometry as their trimethylsilyl derivatives. This method was applied to the first two compounds in *Gymnopilus punctifolius* (Peck) Singer and to the last two compounds in *Amanita pantherina* (Fr.) Secr.

Keyphrases □ Hispidin—GLC–mass spectral analysis in mushrooms □ Bisnoryangonin—GLC–mass spectral analysis in mushrooms □ Muscimole—GLC–mass spectral analysis in mushrooms □ Ibotenic acid—GLC–mass spectral analysis in mushrooms □ GLC–mass spectrometry—analyses, hispidin, bisnoryangonin, muscimole, and ibotenic acid in mushrooms □ *Gymnopilus punctifolius*—GLC–mass spectral analysis of various components □ *Amanita pantherina*—GLC–mass spectral analysis of various components

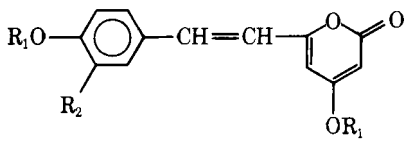
A previous report described the GLC–mass spectral analysis of the trimethylsilyl derivatives of psilocin and psilocybin (1). As part of continuing studies of the chemical constituents of higher fungi, this report describes the GLC analysis of the trimethylsilyl derivatives of four such compounds and their mass spectral features. Bisnoryangonin (Ia), hispidin (IIa), muscimole (IIIa), and ibotenic acid (IVa) occur in various genera of the Hymenomycetes.

BACKGROUND

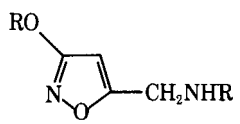
The styrylpyrone derivatives Ia and IIa were analyzed by TLC and IR, UV, and mass spectrometry (2, 3). Compound IIa was first reported (4–6) in certain species of Polyporaceous fungi. One or both of these compounds have been detected in various species of *Gymnopilus* Karsten (Cortinariaceae), *Pholiota* (Fr.) Kummer [including *Flammula* (Fr.) Kummer] (Strophariaceae or Cortinariaceae), and *Naematoloma* Karsten [= *Hypholoma* (Fr.) Kummer] (Strophariaceae) (2, 7–10). These and related compounds may be of considerable use in biochemical systematics (10–13).

The historical literature of Japan suggests the ethnomycological importance of *G. spectabilis* (Fr.) Smith[1] [= *Pholiota spectabilis* (Fr.) Quel.] (15, 16), but only occasional incidents of intoxication due to this species have been reported recently (17, 18). The presence of the related styrylpyrone yangonin (V) in the rhizomes of kava-kava (*Piper methysticum* Forst.) (19), the narcotic pepper used in the South Pacific, lends support to the possibility that this class of compounds might be psychoactive. However, limited pharmacological studies of the pure kava pyrones have not accounted for the activity of the whole plant (20).

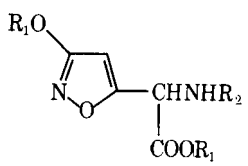
Compounds IIIa and IVa were analyzed previously by paper chromatography (21–23) and paper electrophoresis (21, 24). Instrumental analysis of the pure compounds was performed by IR (21) and mass spectrometry (25). These compounds appear to be confined to a few species of the genus *Amanita* Pers. ex Hook., principally *A. muscaria* (L. ex Fr.) Hooker (21–23, 26), *A. pantherina* (Fr.) Secr. (23, 26), and *A. cothurnata* Atk. (26). The ethnobotanical importance of *A. muscaria* has been established (27). Although these mushrooms are the cause of numerous intoxications, both accidental and intentional (28), pure compounds IIIa and IVa have received limited pharmacological study (29–31).



Ia: $R_1 = R_2 = H$
Ib: $R_1 = (CH_3)_3Si$, $R_2 = H$
IIa: $R_1 = H$, $R_2 = OH$
IIb: $R_1 = (CH_3)_3Si$, $R_2 = (CH_3)_3SiO$
V: $R_1 = CH_3$, $R_2 = H$



IIIa: R = H
IIIb: R = $(CH_3)_3Si$



IVa: $R_1 = R_2 = H$
IVb: $R_1 = R_2$ $(CH_3)_3Si$
IVc: $R_1 = (CH_3)_3Si$, $R_2 = H$

The previously described methods have been adequate for the qualitative analysis of these compounds. However, quantitative analysis by these methods suffers from several disadvantages, especially for the highly polar, zwitterionic IIIa and IVa.

The facile decarboxylation of IVa to IIIa during extraction, analytical, and isolation procedures (23, 26) leads to an inaccurate estimation of individual components present in the mushroom. Occasionally, enrichment of fungal extracts by ion-exchange chromatography is necessary before analysis can proceed (26). Use of colorimetric reagents to visualize spots on a chromatogram can often be misleading, especially in quanti-

Table I—Chromatography Conditions, Retention Times [a], and Percent of Trimethylsilyl Derivatives

	Ib	IIb	IIIb	IVb	IVc
SE-30	250°, R_t 8.5 min	250°, R_t 15 min	150–250°, R_t 2.1 min	150–250°, R_t 5.4 min	150–250°, R_t 3.7 min
OV-101	—	—	100–200°, R_t 6.0 min	100–200°, R_t 13.1 min	100–200°, R_t 10.8 min
Percent [b] present in fungal extracts	0.44 (0.42) [c]	0.25 (0.21) [c]	0.046	0.002	—

[a] Relative to the solvent front. [b] Based on dry weight. [c] By isolation.

[1] This species might be identical with *G. junonious* (Fr.) Orton (14).

tative estimation. Most of these difficulties can be overcome utilizing GLC of the neutral, stable, and easily prepared derivatives I*b*, II*b*, III*b*, and IV*b*.

EXPERIMENTAL[2]

Muscimole monohydrate was prepared according to published procedures (32, 33). The melting point and IR and mass spectra were in agreement with those reported (21, 25).

Pileus tissue of air-dried *G. punctifolius* (Peck) Singer[3] (2) (89 mg) was ground to a powder with sand and transferred to a screw-capped tube. Methanol (5 ml) was added, and the tube was shaken at room temperature for 20 hr. The contents were filtered, and the collected solids were washed with two 5-ml portions of methanol. Then the combined filtrate and washings were concentrated to 5.0 ml, and 0.5 ml of this solution was transferred to a 1.0-ml vial[4].

The solvent was removed by evaporation in a nitrogen stream, and the residue was dried *in vacuo*. Bis(trimethylsilyl)trifluoroacetamide[5] (25 μl) was added, and the vial was closed with a septum-lined seal[6] and heated for 30 min at 140°. Aliquots of 1 μl were used for subsequent analyses.

Pileus tissue of freeze-dried *A. pantherina* (Fr.) Secr.[7] (1.56 g) was ground as already described and extracted with 30 ml of 10% aqueous methanol at 4° for 8 hr. The mixture was filtered, and the volume of the filtrate was adjusted to 30.0 ml. An aliquot of this solution (0.5 ml) was transferred to a reaction vial and treated as already described.

Quantitation was carried out based on the external standard technique used previously (1). Weighed samples of each of the four pure standards were mixed with bis(trimethylsilyl)trifluoroacetamide in reaction vials so that the final concentrations were 0.25% (w/v). The sealed vials were heated at 140°. Aliquots were removed every 15 min and analyzed by GLC for a total of 2 hr.

In each case, the reaction solutions attained homogeneity within 15–30 min; analyses of aliquots removed after 30 min showed no further increase in peak areas. When injection of equal volumes of each sample was repeated, reproducibility of detector response (as measured by peak areas) was within ±2%. Linearity of detector response was verified by injecting successively diluted volumes of each standard solution and comparing the peak areas obtained with the known concentrations of solutes.

Carpophores of *G. punctifolius*[3] (1.911 g) were ground and extracted by stirring with methanol (100 ml) for 20 hr. The solids were collected by filtration, and the filtrate was concentrated *in vacuo* to 10.0 ml. A second 100-ml extract of the filtered solids was shown to be devoid of I*a* and II*a* by TLC. Compounds I*a* and II*a* were isolated from the extract by preparative TLC, using 25% methanol in chloroform as the developing solvent. The separated bands (I*a*, R_f 0.58; and II*a*, R_f 0.44) were removed from the plates, and the compounds were eluted with 40% methanol in ethyl acetate.

The residue of I*a*, obtained by concentration of the eluate, was crystallized from methanol–ether to afford 8.0 mg (0.42%), mp 242–244° dec. [lit. (2) mp 244–246° dec.]. The residue of II*a* was precipitated from ethanol by addition of ethyl acetate, and the precipitate was isolated by centrifugation. After drying *in vacuo*, II*a* weighed 4.0 mg (0.21%), mp 228° [lit. (4) mp 256°]. The compounds were chromatographically homogeneous and indistinguishable from reference standards.

Table II—Mass Spectral Parameters[a] for Trimethylsilyl Derivatives

I*b*	II*b*	III*b*	IV*b*	IV*c*
372 (7%) (M + 2)	464 (12%) (M + 2)	244 (2%)	360 (2%)	272 (1%)
375 (18%) (M + 1)	463 (22%) (M + 1)	243 (8%) (M − 15)	359 (6%) (M − 15)	259 (2%)
374 (51%) (M +)	462 (50%) (M +)	169 (5%)	332 (2%)	258 (4%)
361 (2%)	449 (7%)	156 (2%)	331 (6%) (M − 43)	257 (20%)
360 (6%)	448 (13%)	147 (2%)	259 (7%)	156 (2%)
359 (19%) (M − 15)	447 (24%) (M − 15)	146 (3%)	258 (9%)	147 (3%)
347 (6%)	434 (7%) (M − 28)	144 (2%)	257 (45%) (M − 117)	121 (5%)
346 (19%) (M − 28)	419 (5%)	128 (4%)	218 (3%)	116 (3%)
319 (5%)	407 (3%) (M − 56)	114 (3%)	169 (2%)	100 (4%)
318 (18%) (M − 56)	406 (8%)	102 (10%)	148 (3%)	99 (3%)
317 (2%)	359 (4%)	100 (3%)	147 (15%)	89 (5%)
219 (3%)	346 (7%)	99 (2%)	133 (2%)	75 (15%)
99 (4%)	245 (3%)	89 (1%)	130 (4%)	74 (11%)
75 (13%)	229 (4%)	75 (10%)	103 (2%)	73 (100%)
74 (9%)	219 (4%)	74 (11%)	100 (5%)	45 (17%)
73 (100%)	191 (9%)	73 (100%)	99 (2%)	
69 (4%)	183 (5%)	59 (8%)	75 (11%)	
45 (15%)	147 (10%)	45 (18%)	74 (9%)	
	143 (5%)	44 (3%)	73 (100%)	
	99 (6%)	43 (6%)	59 (3%)	
	75 (33%)		45 (10%)	
	74 (35%)			
	73 (100%)			
	69 (5%)			
	45 (48%)			

[a] *m/e* (relative intensity).

RESULTS AND DISCUSSION

The derivatives I*b* and II*b* were widely separated under the isothermal conditions described. While II*b* was completely resolved from other components in the natural extract, some overlap with an adjacent peak was noted for I*b*. However, a mass spectrum comparable to that of the standard was obtained. The resolution of I*b* was not improved by changes in chromatographic conditions or stationary phases. The isoxazoles III*b* and IV*b* were well resolved under either set of conditions.

The concentrations of I*a* and II*a* in the collection of *G. punctifolius* used in this study were lower than those reported for this species previously (2). The quantities of III*a* and IV*a* in *A. pantherina* also appeared to be low compared to earlier findings (23). Both previous investigations were based on analysis by spot-intensity techniques on paper and thin-layer chromatograms. However, these methods are only semiquantitative (34). The isolated yields of IV*a* recently reported (26) from *A. pantherina* and of I*a* and II*a* from *G. punctifolius* reported here are compatible with data obtained by GLC (Table I).

Several factors could affect the quantities of I*a*, II*a*, III*a*, and IV*a* found in these organisms, including intraspecies variation based on season, year, and environment of collections and differences in handling and storage of specimens. With respect to the former, marked variation in isoxazole content was reported in collections of *A. muscaria* (35). The quantity of hispidin in *Polyporus hispidus* (Bull.) Fr. decreased with the increasing age of the carpophore (5). It was suggested that this decrease was due to the elaboration of hispidin into polymeric lignans; recently, polymeric hispidin derivatives were detected in several species containing I*a* and II*a* (10–13).

The mass spectra of the styrylpyrone trimethylsilyl derivatives (Table II) were characterized by intense molecular ion signals (*m/e* 462 and 374 for II*b* and I*b*, respectively). This result is rather unusual since trimethylsilyl ethers undergo facile loss of a methyl group (36). These M − 15 peaks were also present in the spectra of I*b* and II*b*. The base peak in each spectrum at *m/e* 73 due to the trimethylsilyl ion is a common occurrence in these derivatives (36).

[2] GLC was carried out with a Hewlett-Packard model 402 instrument with flame-ionization detectors. The columns used were: (*a*) a 0.75-m × 2.8-mm i.d. glass U-tube with 3% OV-101 on 100–120-mesh Gas Chrom Q with temperature programmed from 100 to 200° at 7.5°/min, and (*b*) a 1.2-m × 2.8-mm i.d. glass U-tube with 1.5% SE-30 on 100–120-mesh Gas Chrom W with temperature programmed from 150 to 250° at 7.5°/min or isothermally at 250°. The injector block temperature was 250°, the detector was at 280°, and the chart speed was 0.64 cm/min with helium carrier gas at 50 ml/min.

GLC–mass spectrometry was performed with a Finnigan 9500 gas chromatograph coupled to a Finnigan 3100 D quadrupole mass spectrometer through a single-stage glass jet separator. Data were acquired *via* a System/250 digital computer system. The mass spectrometer conditions were: interface temperature, 225°; transfer line, 175°; manifold temperature, 100°; and ion source potential, 70 ev. Chromatography conditions were the same as those used for GLC, except that the helium flow rate was 20 ml/min.

Analytical TLC was carried out on 0.25-mm layers of silica gel GF on glass plates. Preparative TLC was performed with 1-m × 20-cm glass plates coated with 0.75-mm layers of silica gel GF. The solvent system in both cases was 25% methanol in chloroform.

Standards of hispidin and bisnoryangonin were obtained from Dr. Lynn R. Brady, Department of Pharmaceutical Sciences, School of Pharmacy, University of Washington, Seattle, Wash. Natural ibotenic acid was obtained from Dr. William Scott Chilton, Department of Chemistry, University of Washington.

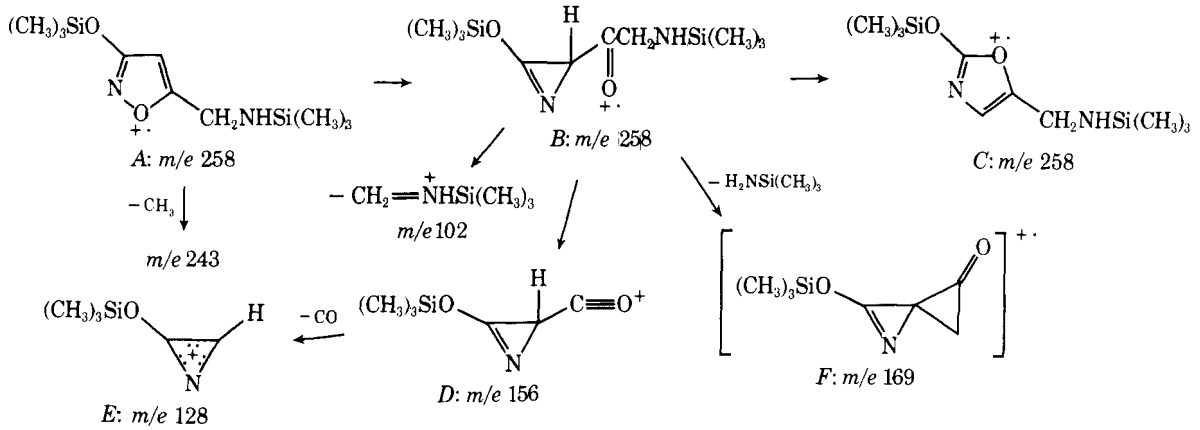
[3] *G. punctifolius* (Peck) Singer was collected in Grays Harbor County, Wash., in October 1975. This material is on deposit at the University of Washington Herbarium, Seattle, Wash., as LESLIE 2663.

[4] Hewlett-Packard 5080-8712.

[5] Regisil, Regis Chemical Co.

[6] Hewlett-Packard 5080-8713.

[7] *A. pantherina* (Fr.) Secr. was collected in Thurston County, Wash., in October 1975 and is on deposit at the University of Washington Herbarium, Seattle, Wash., as LESLIE 2629.



Scheme I

The fragmentation processes and rearrangements in the mass spectra of isoxazoles are complex (37–39). Scheme I shows several pathways proposed for the mass spectrum of III*b*. The rearrangement isoxazole → azirine → oxazole (*A* → *B* → *C*) (37) can lead to parallel fragmentation processes. Loss of methyl from a silyl group from any of the rearranged parent ions accounts for the *m/e* 243 signal. This process occurs so readily that the parent ion (*m/e* 258) is not evident. Production of trimethylsilylmethylimminium ion (*m/e* 102) followed by loss of carbon monoxide can lead to ions *D* (*m/e* 156) and *E* (*m/e* 128). Also, loss of trimethylsilylamine can lead to ions such as *F* (*m/e* 169), which can undergo further rearrangement. Similar fragmentations of ion *C* could also account for the signals at *m/e* 156 and 169.

The mass spectrum of IV*b* exhibited all typical fragmentations of trimethylsilylated α-amino acids (40), including M − 15 (*m/e* 359), M − 43 (331), M − 117 (257) due to loss of carbonyltrimethylsilyloxy, and *m/e* 218 due to loss of the amino acid side chain (36). The parent peak (*m/e* 374) was not present. Shorter reaction times or lower reaction temperatures resulted in the presence of variable amounts of a partially derivatized product, presumably IV*c*. The production of more than one trimethylsilyl derivative when various silylating reagents are used is not an uncommon occurrence with α-amino acids (40). However, quantitative derivatization giving only IV*b* occurred under the described conditions (see *Experimental*). Loss of carbon dioxide and a proton from IV*c* with retention of the trimethylsilyl group would account for the *m/e* 257 signal. This compound also exhibited fragments similar to those discussed for III*b*.

The decarboxylation of IV*a* to III*a* (41) did not occur during silylation of IV*a* since III*b* was not present in gas chromatograms of the reaction solutions. GLC offers accurate quantitative analysis of I*a*, II*a*, III*a*, and IV*a* directly from initial extracts. Sensitivity is in the 10^{-8}–10^{-7}-g range with analyses routinely run on 10–20 mg of dried fungal tissue. The strong signals for the parent ions of I*b* and II*b* and the combination of characteristic fragmentations for III*b* and IV*b* allow positive identification of these substances.

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