Sesquiterpene Lactones from *Artemisia genipi* Weber: Isolation and Determination in Plant Material and in Liqueurs

Giovanni Appendino, Flavio Belliardo, Gian Mario Nano,* and Silvio Stefenelli

The sesquiterpene lactones of *Artemisia genipi* Weber, a rare Alpine plant, were investigated with the purpose of following this plant during the experiments of cultivation and establishing the authenticity of liqueurs call "genepi". Costunolide, dehydroartemorin, santamarine, reynosin, and artemorin were isolated from the flowered aerial parts of the plant, and techniques for their determination in plant material and in liqueurs were developed. Plants of different origin (cultivated; wild) and karyotype ($2n = 18$; $2n = 36$) as well as various brands of liqueurs sold under the denomination of genepi were analyzed. The production of sesquiterpene lactones, responsible for the bitterness of *A. genipi*, did not seem to be affected by factors such as polyploidy or cultivation, while the presence of this plant was not demonstrable in some liqueurs analyzed.

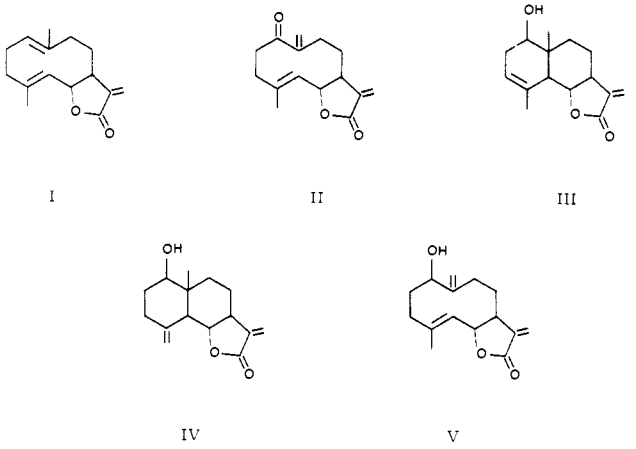
Artemisia genipi Weber is an Alpine plant of the Compositae family used by liqueur-producing industries. The indiscriminate picking of this plant has diminished its already rare presence almost everywhere, and special laws to limit its collection have been promulgated (e.g., Regione Autonoma Valle d'Aosta, Regional Law No. 4, April 26, 1977). The cultivation of *A. genipi* has been suggested both to satisfy market requests and to exploit the high mountain agriculture. This would make it more remunerative on account of the high commercial value of this plant. In this connection experiments of cultivation of this species in its natural habitat have been in progress for several years in the alpine botanical garden Paradisia (Cogne, Italy). Plants with particular racemes and a somatic number ($2n = 36$) not previously found in this species, whose only reported karyotype was $2n = 18$ (Tutin et al., 1976), have been noticed with a not negligibile frequency (18%) during the cultivation (Stefenelli and Busanelli, 1980). Chemical studies on this plant seemed indicated, and research efforts to identify metabolites characteristic of this plant, particularly the ones responsible for its organoleptic properties, were taken up with the purpose of following this species during the cultivation and being able to ascertain its actual presence in liqueurs.

The isolation of five sesquiterpene lactones, responsible for the bitterness of the plant, and the development of techniques for their determination in plant material and in liqueurs are the subject of this paper; results obtained analyzing plants of different origin (wild; cultivated) and karyotype ($2n = 18$; $2n = 36$) as well as various brands of liqueurs sold under the denomination of "genepi" are also reported.

EXPERIMENTAL SECTION

General Instrumentation. Melting points were taken on a Büchi SMP-20 apparatus and are uncorrected; optical rotations were measured on a Perkin-Elmer 141 automatic polarimeter; UV spectra were obtained on a Beckman DB-GT spectrophotometer; IR spectra were taken on a Perkin-Elmer Model 237 spectrophotometer; proton nuclear magnetic resonance (^{1}H NMR) spectra were measured on a Varian EM360L instrument, with Me_4Si as the internal standard; mass spectra (MS) were recorded by direct inlet at 70-eV ionization on a Varian Mat CH7A instrument; silica gel 60 (70–230 mesh) (Merck) was used for column chromatography (CC) separation; HPLC analyses were run on a Perkin-Elmer Series 3B liquid chromatograph equipped with an LC-75 spectrophotometric detector and an LC-75 autocontrol; a Hibar-Lichrosorb RP-8, 10 μm (25 × 0.4 cm i.d.) (Merck) column was used; graphs were generally obtained with an attenuation setting corresponding to 0.64 AUFS on a Perkin-Elmer 561 recorder.

Chart I. Structural Formulas of Sesquiterpene Lactones Isolated from *A. genipi* Weber



I II III IV V

Isolation of Sesquiterpene Lactones. Air-dried powdered aerial parts (800 g) of *A. genipi*, collected during its flowering time in the alpine botanical garden Paradisia (voucher 1AG-80 in its herbarium), were extracted with $CHCl_3$ (5 × 10 L) at room temperature. The residue (63 g) remaining after evaporation of the solvent at reduced pressure was dissolved in hot EtOH (800 mL) and then an aqueous 5% lead(II) acetate solution (800 mL) was added; after removal of the precipitate by filtration, the filtrate was evaporated in vacuo to remove most of the EtOH and then extracted exhaustively with $CHCl_3$; the combined $CHCl_3$ extracts gave 22 g of a dark syrup that was chromatographed on a silica gel (400-g) column, eluted with $CHCl_3$. Eluates (300 mL) were collected, and each fraction was monitored by TLC (eluent: $CHCl_3$–Me_2CO, 6:1), ^{1}H NMR ($CDCl_3$ solution), and IR ($CHCl_3$ solution) spectroscopy.

Costunolide (I). Fractions 4–7 gave a solid residue that crystallized spontaneously, yielding 5 g of costunolide (I)

Laboratorio RMN e Spettroscopie applicate alla Tossicologia, Facoltà di Farmacia, Corso Raffaello 31, 10125 Torino, Italy (G.A., F.B., and G.M.N.), and Giardino Botanico Alpino "Paradisia", Valnontey, 11012 Cogne, Italy (S.S.).

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(see Chart I): mp 104–105 °C [lit. mp 105–106 °C (El-Feraly and Benigni, 1980)]; $[\alpha]^{25}_D$ +105° ($CHCl_3$, *c* 0.47) [lit. $[\alpha]^{25}_D$ +117° (El-Feraly and Benigni, 1980)]; UV λ_{max}^{EtOH} (log ϵ) 210 nm (3.90); IR (KBr disk) ν_{max} no –OH bands, 1765, 1667, 1285, 1136, 995 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.27 (1 H, d, J = 3.7 Hz, H-13$_a$), 5.54 (1 H, d, J = 3.7 Hz, H-13$_b$), 4.95 (1 H, m, H-1), 4.80 (1 H, m, H-5), 4.59 (1 H, t, J = 10 Hz, H-6), 1.70 (3 H, br s, H-15), 1.40 (3 H, br s, H-14); MS m/z (rel intensity) 232 (M^+, 36).

Dehydroartemorin (II). Fractions 14–15 gave a thick oil that by trituration with Et_2O afforded 30 mg of dehydroartemorin (II) as a white powder: mp 121–123 °C [lit. mp 123–124 °C (El-Feraly and Benigni, 1980)]; $[\alpha]^{25}_D$ +142° ($CHCl_3$, *c* 0.52) [lit. $[\alpha]^{22}_D$ +144° (El-Feraly and Benigni, 1980)]; UV λ_{max}^{EtOH} (log ϵ) 230 nm (sh), 210 (4.08) [lit. 233 nm (ϵ 4000), 300 (ϵ 68) (El-Feraly et al., 1979)]; IR (KBr disk) ν_{max} no –OH bands, 1760, 1680, 1260, 1145, 950 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.20 (1 H, d, J = 3.3 Hz, H-13$_a$), 5.86 (1 H, br s, H-14$_a$), 5.66 (1 H, br s, H-14$_b$), 5.48 (1 H, d, J = 3.2 Hz, H-13$_b$), 5.10 (1 H, br d, J = 9.7 Hz, H-5), 4.32 (1 H, t, J = 9.7 Hz, H-6), 1.75 (3 H, s, H-15); MS m/z (rel intensity) 246 (M^+, 12).

Santamarine (III). Fractions 17–19 afforded a solid residue that by crystallization from diisopropyl ether gave 80 mg of santamarine (III): mp 139–140 °C [lit. mp 134–135 °C (El-Feraly and Benigni, 1980)]; $[\alpha]^{25}_D$ +92.6° ($CHCl_3$, *c* 0.51) [lit. $[\alpha]^{25}_D$ +72.8° (EtOH, *c* 0.18) (El-Feraly and Benigni, 1980)]; UV λ_{max}^{EtOH} (log ϵ) 210 nm (4.30); IR (KBr disk) ν_{max} 3350, 1770, 1130, 1005, 975 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.10 (1 H, d, J = 3.2 Hz, H-13$_a$), 5.44 (1 H, d, J = 3.0 Hz, H-13$_b$), 5.35 (1 H, m, H-3), 3.98 (1 H, t, J = 10 Hz, H-6), 3.68 (1 H, dd, J = 6.0 and 9.6 Hz, H-1), 1.87 (3 H, s, H-15), 0.87 (3 H, s, H-14); MS m/z (rel intensity) 248 (M^+, 100).

Reynosin (IV). Fractions 20–23 gave a yellowish solid. Recrystallization from Et_2O–$CHCl_3$ afforded 100 mg of reynosin (IV): mp 144–145 °C [lit. mp 145–146 °C; (El-Feraly and Benigni, 1980)]; $[\alpha]^{25}_D$ +170.8° ($CHCl_3$, *c* 0.45) [lit. $[\alpha]^{25}_D$ +122° (EtOH, *c* 0.10) (El-Feraly and Benigni, 1980)]; UV λ_{max}^{EtOH} (log ϵ) 210 nm (3.90); IR (KBr disk) λ_{max} 3450, 1770, 1645, 1125, 980, 940 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.12 (1 H, d, J = 3.5 Hz, H-13$_a$), 5.46 (1 H, d, J = 3.0 Hz, H-13$_b$), 5.03 (1 H, br s, H-15$_a$), 4.90 (1 H, br s, H-15$_b$), 4.08 (1 H, t, J = 10.8 Hz, H-6), 3.56 (1 H, dd, J = 4.0 and 10.2 Hz, H-1), 0.85 (3 H, s, H-14); MS m/z (rel intensity): 248 (M^+, 69).

Artemorin (V). A total of 920 mg of artemorin (V) was obtained from fractions 24–33 by crystallization from Et_2O–$CHCl_3$: mp 119–121 °C [lit. mp 120–121 °C; (El-Feraly and Benigni, 1980)]; $[\alpha]^{25}_D$ +95° ($CHCl_3$, *c* 0.47) [lit. $[\alpha]^{25}_D$ +89° (El-Feraly and Benigni, 1980)]; UV λ_{max}^{EtOH} (log ϵ) 210 nm (4.03); IR (KBr disk) λ_{max} 3450, 1758, 1665, 1310, 1260, 960, 895 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.18 (1 H, d, J = 3.4 Hz, H-13$_a$), 5.45 (1 H, d, J = 3.1 Hz, H-13$_b$), 5.24 (1 H, br d, J = 10 Hz, H-5), 5.20 (1 H, br s, H-14$_a$), 4.90 (1 H, br s, H-14$_b$), 4.40 (1 H, t, J = 10 Hz, H-6), 4.00 (1 H, t, J = 6.2, H-1), 1.73 (3 H, d, J = 1.2 Hz, H-15); MS m/z (rel intensity) 248 (M^+, 8).

Further elution of the column with $CHCl_3$ and more polar solvents (EtOAc; MeOH) gave in all 5 g of a gum that, after IR and ^1H NMR analysis, was discarded, as it did not contain lactones.

Analysis of Sesquiterpene Lactones in Plant Material. Dried powdered aerial parts (5 g) were turboextracted in a Waring Blendor with EtOH (1 × 200 mL; 4 × 100 mL). The pooled extracts were concentrated in vacuo at 40 °C and made up to 50 mL with EtOH; to this an equal volume of an aqueous 4% lead(II) acetate solution was added. After standing for about 12 h, the solution was filtered, concentrated, and then extracted with $CHCl_3$ (5 × 100 mL). The pooled $CHCl_3$ extracts were dried ($MgSO_4$) and the solvent was removed in vacuo to give a dark syrup (lactone fraction), which was quantitatively transferred to a 3-mL calibrated vial with $CHCl_3$; the volume was adjusted to 3 mL with $CHCl_3$, and this solution was analyzed by IR spectroscopy between 2000 and 1600 cm^{-1}. A sealed cell with sodium chloride windows of standard path length 0.1 mm was used; a salt plate was placed in the reference beam. A strong absorption at 1770 cm^{-1}, the intensity of which was taken to be proportional to the content of sesquiterpene lactones in the plant material, was observed. Absorbance values were obtained by using the base-line method; six standard solutions of costunolide (I), with concentrations ranging from 3 to 0.5%, were used to determine a calibration curve.

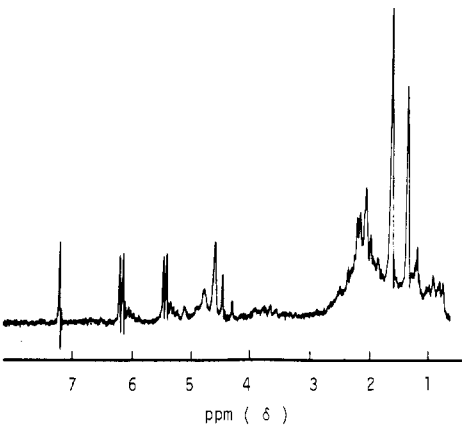

Figure 1. ^1H NMR spectrum of the crude lactone fraction from *A. genipi* Weber.

The composition of every lactone fraction was analyzed by using both chromatographic (TLC; HPLC) and spectral (^1H NMR) techniques.

(*a*) *TLC Analysis.* Silica gel 60 F-254 (0.25 mm) precoated plates (Merck) were used. The solvent system was $CHCl_3$–Me_2CO (6:1). Lactones were detected by spraying the plates with H_2SO_4 or vanillin (Picman et al., 1980) and heating them at 70 °C for 10 min. The R_f values for compounds from I to V were 0.88, 0.74, 0.55, 0.52, and 0.45, and the colors produced were respectively green, brown, brown, brown, and red with H_2SO_4 and brown, brown, dark blue, dark blue, and blue with vanillin.

(*b*) *^1H NMR Analysis.* Spectra of the lactone fractions were run in $CDCl_3$; peaks corresponding to the protons of costunolide (I) are clearly recognizable (Figure 1).

(*c*) *HPLC Analysis.* Analyses of the lactone fractions were achieved under the following conditions: mobile phase, 75% MeOH (Lichrosolv, Merck), 25% water purified by a Milli-Q system (Millipore); flow rate, 1 mL/min; temperature, 20 °C; wavelength, 210 nm; chart speed, 0.5 cm/min. Three peaks corresponding to costunolide (I), artemorin (V), and santamarine (III) plus reynosin (IV), that we found convenient not to separate in order to obtain enhancement of the peak, are clearly recognizable (Figure 2).

Determination of Sesquiterpene Lactones in Liqueurs. A 75-mL sample of the liqueur was diluted with an equal volume of water and extracted in an apparatus for continuous extraction with 100 mL of CH_2Cl_2 for 6 h. The CH_2Cl_2 extract was dried ($MgSO_4$) and then evaporated in vacuo. The residue was dissolved in 1 mL of MeOH (Lichrosolv) and analyzed by HPLC with the same conditions previously described for the analyses of plant extracts. Due to the complexity of the extracts of liqueurs,

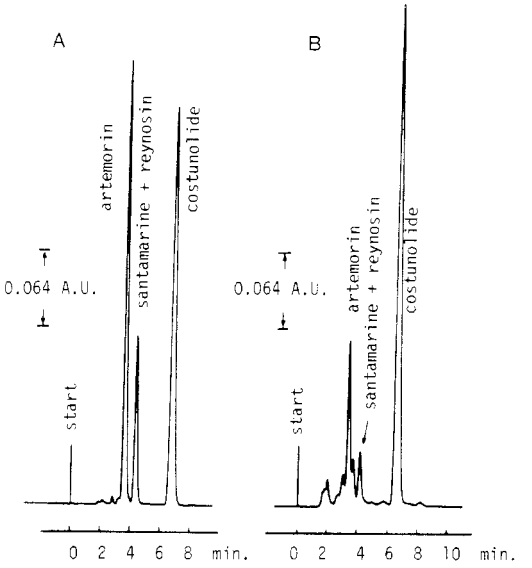

Figure 2. (A) HPLC of the main sesquiterpene lactones isolated from *A. genipi* Weber. (B) HPLC profile of the crude lactone fraction from *A. genipi* Weber (see Experimental Section for the conditions).

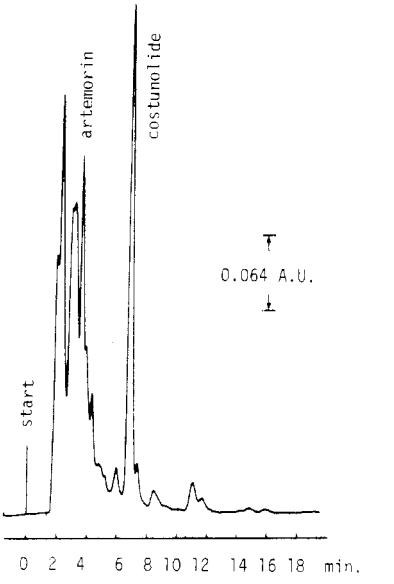

Figure 3. HPLC profile of a CH_2Cl_2 extract of liqueur genepì (sample no. 11) (see Experimental Section for the conditions).

only costunolide (I) and artemorin (V) were unambiguously detectable in them (Figure 3). The identification of the peaks corresponding to these two substances was accomplished by addition of pure compounds and duplication of the enhancement of the peaks by using different conditions of operation. Standard solutions of these compounds were made by weighing pure samples of costunolide (I) and artemorin (V). The validity of this estimation was checked by adding known amounts of these compounds to the liqueurs and comparing the results with the ones obtained without internal standard addition.

Liqueurs of the following brands were analyzed: Ottoz, St. Rock, Herbetet Alpe, La Valdotaine, Savio, Vertosan, Treves, Aval, Aresca, Cortese, and Verginal Crête noire (data in Table II do not reflect this order of listing).

RESULTS AND DISCUSSION

A purified $CHCl_3$ extract of *A. genipi* Weber yielded five sesquiterpene lactones upon chromatography on a silica gel column with $CHCl_3$ as the eluent. They were identified according to their physical and spectral properties as

Table I. Total Sesquiterpene Lactone (SL) Content of Various Samples of *A. genipi* Weber

type of plant	place of collection	somatic no.	total SL content, %
cultivated	Paradisia	$2n = 18$	1.08
cultivated	Paradisia	$2n = 18$	1.14
cultivated	Paradisia	$2n = 18$	1.21
cultivated	Paradisia	$2n = 36$	1.24
cultivated	Paradisia	$2n = 36$	1.10
cultivated	Paradisia	$2n = 36$	1.23
wild	Cime Bianche	$2n = 18$	1.15
wild	Valsavaranche	$2n = 18$	1.29
wild	Elva	$2n = 18$	1.10

costunolide (I), dehydroartemorin (II), santamarine (III), reynosin (IV), and artemorin (V). The amounts of sesquiterpene lactones in the plant material was determined by using, as proposed by Bloszyk et al. (1978), the characteristic absorption of γ-lactones at 1780–1750 cm^{-1} in their IR spectrum. A calibration curve was obtained by using costunolide (I), the most abundant compound we isolated from this plant. Chromatographic (TLC; HPLC) and spectral (^1H NMR) techniques were used for the analysis of plant extracts. Cultivated plants with different somatic number ($2n = 18$; $2n = 36$) and wild plants were analyzed with regard to their contents of sesquiterpene lactones. The total quantity of sesquiterpene lactones showed little variation in the samples analyzed (Table I), ranging from 1.29 to 1.08% on dried plant material. Qualitatively no difference was found among the various extracts. The estimation of the lactone content in separate parts of the plant showed that the highest concentration of sesquiterpene lactones was in seeds (mean value = 1.85%), followed by flowers (mean value = 1.42%) and leaves (mean value = 1.30%). Lesser amounts were detected in stems (mean value = 0.38%).

The large quantities of costunolide (I) and artemorin (V) in the plant under investigation and the intense absorption of these substances at 210 nm allowed us to determine quantitatively their presence in liqueurs called genepì. HPLC was the technique used. Along with *A. genipi*, some other botanically related Alpine plants of the Compositae family of lesser commercial value (*Artemisia mutellina* L., *Artemisia glacialis* L., and *Achillea erba-rotta* All. and its subspecies, *Achillea nana* L.) are sometimes used in the production of this liqueur. They were all investigated with regard to their contents of sesquiterpene lactones: very small amounts of artemorin (V) (0.005% on dried plant material) were isolated from *Achillea erba-rotta* All. subsp. *ambigua* Heimerl (Nano et al., 1981), while all the other plants were found to be lacking in sesquiterpene lactones or to produce sesquiterpene lactones different from the ones isolated from *A. genipi* Weber (Appendino, 1981). The simultaneous presence of costunolide (I) and artemorin (V) in liqueurs was therefore taken to be due to the presence of *A. genipi* Weber.

Liqueurs, chosen to include the best known brands, were analyzed. A wide range in the amounts of the compounds we took as markers was observed (Table II), and they were not detectable in three samples. While the ratio between the amounts of costunolide (I) and artemorin (V) was about 6:1 in plant extracts, ratios of 2:1 or even lesser were noticed in liqueurs, probably on account of the much lower solubility of costunolide (I) in diluted alcoholic solutions. Mixtures of different plants were generally used in the production of the liqueurs, and only in four samples was *A. genipi* Weber the main constituent of the liqueur extract.

While costunolide (I), a cytotoxic (Doskotch and El-Feraly, 1969) and allergenic (Evans and Schmidt, 1980)

Table II. Concentration of Costunolide and Artemorin in Various Liqueurs Sold under the Common Denomination of Genepi

sample	concn, μg/100 mL	
	costunolide	artemorin
1	86.6	57.8
2	33.0	18.2
3	466	230
4		
5	149	83.2
6		
7	338	*a*
8	598	320
9	250	*a*
10		
11	608	326
12	203	99.6

[a] Not detectable as it is covered with other peaks.

sesquiterpene lactone, is only slightly bitter, all the other compounds we isolated are intensely bitter, particularly artemorin (V), which, due also to its concentration, should be considered the main bitter principle of the plant.

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Evidence for the Occurrence and Formation of Diazonium Ions in the *Agaricus bisporus* Mushroom and Its Extracts

Allan E. Ross,* Donald L. Nagel, and Bela Toth

N^{β}-[(+)-γ-Glutamyl]-4-(hydroxymethyl)phenylhydrazine (agaritine), a component of the common cultivated commercial mushroom *Agaricus bisporus*, is hydrolyzed to the 4-(hydroxymethyl)benzenediazonium ion, a carcinogen in mice, by an enzyme system present in the mushroom. This system, together with others, may be responsible for a 0.6-ppm level of the 4-(hydroxymethyl)benzenediazonium ion in the mushroom. A second diazonium ion is generated in acid extracts of *A. bisporus* from a precursor of unknown structure in the mushroom.

The common cultivated mushroom of commerical *Agaricus bisporus* contains relatively large amounts of the hydrazide N^{β}-[(+)-γ-glutamyl]-4-(hydroxymethyl)-phenylhydrazine (agaritine) (Levenberg, 1960, 1961). Kelly et al. (1962) reported a 0.04% yield of agaritine, based on the fresh weight of the mushroom. Using an HPLC assay system, we have found between 0.1 and 0.3% (wet weight) of agaritine in locally purchased mushrooms (unpublished data). Accordingly, an average-sized mushroom (15 g) could contain 45 mg of agaritine.

Levenberg (1961) also described an enzyme that catalyzes the hydrolysis of agaritine to glutamate and 4-(hydroxymethyl)phenylhydrazine (**2**) (Figure 1), and in 1963 Gigliotti isolated and partially characterized an enzyme that converts **2** to the 4-(hydroxymethyl)benzenediazonium ion (**3**). The enzyme is specific for hydrazines and, therefore, inactive on hydrazides. The presence of enzymes capable of generating a diazonium ion from agaritine supports the finding of **3** in the fresh mushroom by Levenberg (1962). In that report, however, **3** was poorly characterized.

To date, tumors have been induced in laboratory animals by 55 hydrazine derivatives (Toth, 1975, 1980), including **3** which, as the tetrafluoroborate salt, caused tumors in the subcutis and skin of mice (Toth et al., 1981). Two other compounds, closely related to **2**, *N'*-acetyl-4-(hydroxymethyl)phenylhydrazine and a reduction product of **2**, 4-methylphenylhydrazine, both significantly increased the incidence of lung and blood vessel tumors in mice (Toth et al., 1977, 1978) (Table I). *N'*-Acetyl-4-(hydroxymethyl)phenylhydrazine, like agaritine, contains the $HOCH_2(C_6H_4)$–NH–NH–CO– substructure. These recent carcinogenicity findings on compounds closely related to agaritine or, in the case of **3**, present in the mushroom motivated an investigation of the fate of agaritine in mushroom extracts and the confirmation and quantitation of **3** in the fresh mushroom.

MATERIALS AND METHODS

Mushrooms. Common commercial mushrooms, *A. bisporus*, were purchased from local food stores and used within 2 days of purchase.

Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska 68105.

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