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Paspalum Staggers: Isolation and Identification of Tremorgenic Metabolites from Sclerotia of *Claviceps paspali*

Richard J. Cole,* Joe W. Dorner, John A. Lansden, Richard H. Cox, Countney Pape, Barry Cunfer, Stephen S. Nicholson, and David M. Bedell

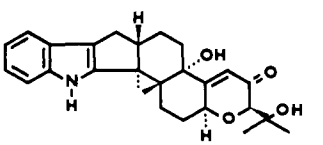
The neurological disorder, Dallisgrass poisoning or paspalum staggers, occurs in cattle that graze *Paspalum dilatatum* infected with the fungus *Claviceps paspali* and occurs sporadically in the southern portions of the U.S. Three tremorgenic metabolites have been isolated from *C. paspali* sclerotia collected from *Paspalum dilatatum*. The identification of these metabolites has been accomplished by spectroscopic methods. One of the metabolites is identical with paspalinine, a previously reported metabolite of *C. paspali*. The remaining two metabolites differ from paspalinine in that they contain an additional isoprene and hydroxyisoprene unit attached to carbon 5 of the six-membered indole ring, 3-methyl-2-butenylpaspalinine and 3-hydroxy-3-methyl-1-butenylpaspalinine, respectively.

A naturally occurring neurological disorder, Dallisgrass poisoning, also called "paspalum staggers", occurs when cattle graze *Paspalum dilatatum* infected with the fungus, *Claviceps paspali* (Brown, 1916). *C. paspali* invades the pistil of the grass flower and destroys the ovary, which is replaced by a mass of fungal tissue. Spores are produced in great abundance along with a sticky, sweetish exudate of the fungal tissue termed "honeydew". Insects attracted to the exudate and cattle or other animals walking through the grass spread the infection to other grass plants. If conditions are favorable, the fungus forms a sclerotium which is toxic to grazing cattle (Grayson, 1941). Other microorganisms, notably *Fusarium heterosporum*, may colonize the honeydew, ultimately inhibiting sclerotium maturation (Cunfer, 1975). Immature sclerotia are smaller in size and appear orange in color.

Clinical signs of "paspalum staggers" are tremors which are exaggerated by enforced movement, hyperexcitability, and ataxia. Mortalities from the disease are generally caused by accident or inability of affected animals to obtain water. Affected animals generally recover from the disease if removed from the toxic pasture.

Paspalum staggers occurs sporadically in the southern portions of the U.S. where the host plant is found. In Louisiana, the disease was more extensive in 1976 than in the three previous years. The increased incidence of the disease was associated with lower than normal rainfall in most parts of the state.

Prompted by the availability of field-produced sclerotia and the observation that paxilline (I), a tremorgenic



I

metabolite of *Penicillium paxilli* (Cole et al., 1974; Springer et al., 1975), was closely related to previously reported metabolites [paspaline (II), paspalicine (III), and

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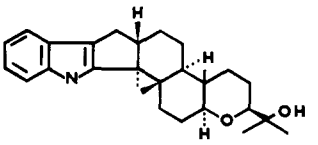
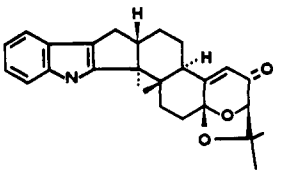
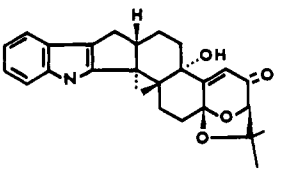


II



III

paspalinine (IV)] of *Claviceps paspali* (Fehr and Acklin,



IV

1966), we analyzed sclerotia of this fungus for chemically related tremorgenic metabolites. We now wish to report the isolation and identification of three tremorgenic metabolites from *C. paspali* sclerotia collected from *Paspalum dilatatum* in toxic pastures.

EXPERIMENTAL SECTION

Extraction and Purification of Tremorgenic Metabolites from *C. paspali* Sclerotia. Sclerotia of *C. paspali* were hand collected from *Paspalum* spp. in Georgia and *Paspalum dilatatum* in Louisiana. Sclerotia were separated from seed with a controlled air blast seed blower (E.L. Erickson Co., S. Dak.), ground in a Wiley Mill, and extracted with chloroform in a blender. The extracted residue was further extracted with chloroform in a Soxhlet apparatus for 6 h. The two extracts were combined, evaporated to dryness, and partitioned between cyclohexane and 80% aqueous methanol. The cyclohexane fraction was discarded after negative results from bioassay. The biologically active aqueous methanol fraction was evaporated to dryness and chromatographed on a silica gel column packed in benzene. The column was eluted with five column volumes of benzene, followed by a linear gradient elution from benzene to ethyl ether. A total of 240 fractions (17 mL each) were collected. Tubes 42–58 (fraction I) and tubes 75–95 (fraction II) were combined to form two distinctly different biologically active fractions.

Fraction I, which contained two major metabolites, was chromatographed on a second silica gel column packed in benzene and eluted with a linear gradient of 0–10% ethyl ether in benzene (240 fractions were collected). Tubes 80–95 contained a nonpolar metabolite (R_f 0.60), tubes 106–120 contained a slightly more polar metabolite (R_f 0.52), and tubes 96–105 contained a mixture of the two metabolites. The metabolites from tubes 80–95 and 106–120 were each isolated by precipitation from benzene solution with petroleum ether.

Fraction II was further purified by preparative thin-layer chromatography (TLC). A band at approximately R_f 0.2 was scraped from the plate and eluted with ethyl ether.

Physical and Chemical Analyses of *C. paspali* Tremorgens. The tremorgens were analyzed by thin-layer chromatography (TLC) on 20 × 20 cm glass plates coated with silica gel GH-R (0.50 mm, analytical and 1.0 mm, preparative). The developing solvent was chloroform–acetone, 93:7 v/v. Toxins were visualized by spraying TLC plates with 50% ethanolic H_2SO_4 and heating for 5 min at 100 °C.

Ultraviolet spectra (UV) of the toxins in methanol solution were taken with a Beckman Model DB-G recording spectrophotometer. Infrared spectra (IR) were taken with a Perkin-Elmer Model 257 IR spectrophotometer equipped with a 4× beam condenser. Samples were analyzed as a thin film coated onto KBr windows.

High-resolution mass spectral analyses (HRP) were made with an A.E.I. MS-9 mass spectrometer. Samples were introduced into the ion source by the direct-probe method and ionization was effected by electron impact at 70 eV. The ion-source temperature was kept at 200 °C, and high-resolution measurements were made by peak matching with perfluorokerosene as the internal standard.

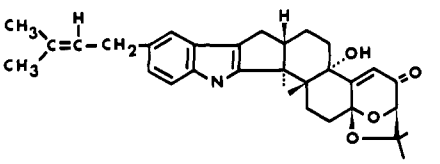
Proton NMR spectra were obtained in $CDCl_3$ solutions on a Varian Associates HA-100 NMR spectrometer. Natural abundance, proton-decoupled ^{13}C spectra were obtained on a JEOL PFT-100 spectrometer equipped with the EC-100 data system. Single-frequency, off-resonance decoupled spectra were obtained by off-setting the decoupling frequency 1000 Hz downfield from the center of proton absorption. Chemical shifts were assigned on the basis of the off-resonance decoupling experiments, known substituent effects, and comparison among the spectra of paspalinine, paxilline, paspalicine, and paspaline.

Biological Tests. Animals used in these studies were 1-day-old Dekalb cockerels (approximately 40 g) and mice (approximately 30–40 g). Cockerels were given a single oral dose of the test material in 1 cm^3 of corn oil carrier (Kirksey and Cole, 1974) while mice were dosed IP (0.1 cm^3 corn oil preparation) or orally (0.2 cm^3 corn oil preparation). Since the tremor produced by these metabolites is more intermittent than sustained (especially at lower levels), animals were forced to move during evaluation for tremor response.

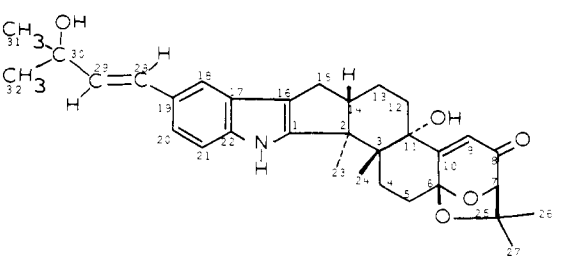
Purification of tremorgenic metabolites from chloroform extracts of *C. paspali* sclerotia was monitored with orally dosed day-old cockerels as bioassay organism. Mice dosed IP were used for preliminary ED_{50} determinations. Mice were dosed within the range of 14–250 mg/kg.

RESULTS AND DISCUSSION

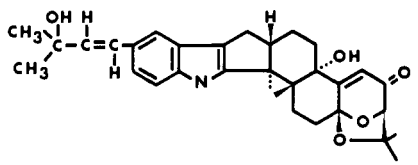
Three tremorgenic metabolites were isolated from sclerotia of *C. paspali*. These appeared on TLC plates at R_f 0.60 (compound A, VI), 0.52 (compound B), and 0.20



VI



(compound C, V). Compound C was visualized on TLC as a green spot immediately after spraying with 50% ethanolic H_2SO_4. Compounds A and B were visualized as



V

grey-blue spots in visible light after the plates were sprayed with 50% ethanolic H_2SO_4 and heated for 5 min at 150 °C. All three were fluorescent under long- and short-wave UV radiation after spraying and heating.

The UV absorption spectrum of compound A was λ_{max}^{MEOH} 234 and 278 nm, compound B was λ_{max}^{MEOH} 232 and 275 nm, and compound C was λ_{max}^{MEOH} 227, 248, 305 and 336 nm. The former two were very similar to the UV absorption of the indole and α-β unsaturated carbonyl chromophores of paxilline (I). Compound C contained additional UV absorption. The IR spectra of all three compounds contained similar features, i.e., 1665–1675 cm^{-1} (α-β unsaturated ketone), 3310–3500 cm^{-1} (OH, indole NH), and doublet 1355–1380 cm^{-1} (*gem*-dimethyl).

The high-resolution mass spectrum showed molecular ion peaks at measured mass m/e 501.2885 ($C_{32}H_{39}O_4N$ requires 501.2878) for compound A, m/e 433.2227 ($C_{27}H_{30}O_4N$ requires 433.2252) for compound B, m/e 517.2857 ($C_{32}H_{39}O_5N$ requires 517.2826) for compound C.

A comparison of UV, IR, mass spectra, ^{13}C NMR, and ^1H NMR of compound B with those of paspalinine (IV) showed the two compounds to be identical. Compound A contained an additional isoprene unit (C_5H_8) while compound C contained an additional hydroxyisoprene unit (C_5H_8O).

The more polar compound C was identified by comparison of its spectral properties with those of paspalinine (IV). The proton NMR spectrum of compound C is similar to that of paspalinine with peaks at δ 1.21 (3 H, s), 1.25 (3 H, s), 1.41 (3 H, s), 1.47 (3 H, s), 1.50 (6 H, s), 1.90 to 2.77 (11 H, multiplet), 4.32 (1 H, s), 5.81 (1 H, s), 6.42 (1 H, d, J = 16.0 Hz), 7.00–7.30 (4 H, multiplet), and 7.76 (1 H, s, N–H). In addition to the peaks of paspalinine, the proton spectrum of compound C (1) shows a peak for two additional methyl groups, (2) peaks for two protons on a trans double bond, and (3) integration and chemical shifts indicate that the indole ring is substituted on the six-membered ring. Compared with the spectrum of paspalinine, the natural abundance proton-decoupled ^{13}C NMR spectrum of toxin C (Table I) shows additional peaks for five carbons. The aliphatic region of the spectrum is identical with that of paspalinine with the addition of a two carbon peak at 29.9 ppm and a single carbon peak at 71.2 ppm (Table I). Single-frequency, off-resonance decoupling experiments show that the two carbons at 29.9 ppm are due to methyl carbons and the peak at 71.2 ppm is due to a quaternary carbon. The peak at 71.2 ppm is characteristic (Stothers, 1972) of a tertiary alcohol. The aromatic and vinyl region of the ^{13}C spectrum of C shows two extra peaks at 124.7 and 137.5 ppm (C-H's) in addition to chemical shift differences expected for a substituted indole ring.

The fact that compound C contains five more carbons than paspalinine suggests that C contains an additional isoprene unit compared with paspalinine. With two methyl carbons, a tertiary alcohol, and a trans double bond, there are several possibilities for the isoprene unit. The UV spectrum (λ_{max} 248 nm) indicates extended conjugation with the indole ring compared with paspalinine. Thus, the carbon–carbon double bond is most likely attached to the indole ring. The remaining question concerning the structure of compound C is the location of the isoprene unit on the indole ring. Comparison of the proton and ^{13}C chemical shifts of C with those of the various methyl indoles (Parker and Roberts, 1970) and calculations using known substituent effects on an aromatic ring (Stothers, 1972) shows that the isoprene unit is located on carbon 5 of the six-membered ring of the indole nucleus. Therefore, all the data are consistent with the proposed structure V for compound C, 3-methyl-3-hydroxy-1-butenylpaspalinine.

During the attempted purification of compound C on a Sephadex LH-20 column (50:50 chloroform–methanol), another compound, D, was eluted from the column. Comparison of the NMR spectral properties of compound D with those of C indicates that the hydroxy group on the isoprene side chain of compound C has been replaced with a methoxy group in compound D (Table I). It is not known at this time whether the column itself, or a trace of acid in the chloroform is responsible for catalyzing this reaction on the column.

The structure of the nonpolar compound A was also deduced by comparison of its spectral properties with those of paspalinine and C. The proton NMR spectrum of compound A showed peaks at δ 1.18 (6 H, s), 1.35 (3 H, s), 1.42 (3 H, s), 1.77 (6 H, s), 1.8–2.9 (11 H, multiplet), 3.62 (2 H, multiplet), 4.29 (1 H, s), 5.38 (1 H, multiplet), 5.73 (1 H, s), and 6.8–8.0 (5 H, multiplet). Compared with paspalinine, the proton spectrum of compound A shows (1) an additional six-proton broad singlet, (2) a two-proton multiplet at 3.62 ppm, (3) a one-proton multiplet at 5.58 ppm, and (4) that the indole ring is substituted on the six-membered ring. The ^{13}C NMR spectrum of A contains 32 carbon peaks (Table I). The aliphatic region of the spectrum is identical with the spectrum of paspalinine with the addition of three peaks at 18.0, 25.8, and 32.0 ppm. A single-frequency, off-resonance proton decoupled spectrum shows these peaks to be due to two methyl carbons and methylene carbon, respectively. In addition to some chemical shift differences, the aromatic and vinyl region of the spectrum contains two more carbons than paspalinine. The off-resonance decoupled spectrum shows that one of these carbons is a doublet and the other a singlet.

Again, the data suggest that compound A is similar to paspalinine with the addition of an isoprene group with two methyl carbons, a methylene carbon, and a double bond. The lack of extended conjugation in the UV spectrum of A and the chemical shift of the methylene carbon suggest that the methylene carbon is attached to the indole ring. The fact that one of the carbons in the double bond contains no attached hydrogens and the ^{13}C chemical shifts of the methyl carbons indicates that the two methyl carbons are attached to the same carbon in the double bond. The proton–proton coupling constants and the fact that the remaining carbon in the double bond has one hydrogen attached suggest that this carbon is attached to the methylene carbon. These data are only consistent with a 3-methyl-2-butenyl group. The similarity of the proton and ^{13}C chemical shifts of compound A and C for the indole ring suggest that the indole ring is substituted similarly in both compounds. All of the data taken together are consistent with the proposed structure VI for compound A, 3-methyl-2-butenylpaspalinine.

The metabolite pattern of *C. paspali* sclerotia collected from *Paspalum* spp. in Georgia was not significantly different than that of sclerotia collected from toxic pastures in Louisiana. Furthermore, there were no significant differences between mature and mixtures of mature and immature sclerotia. Sclerotia contained approximately 0.16% total tremorgens, with a ratio of 0.7, 0.8, 1.0 for

Table I. Carbon-13 Chemical Shifts of Paspalinine and Related Compounds[a,b]

Carbon	Paspalinine	Compound C	Compound C-OCH_3	Compound A	Paspalicine	Paxilline	Paspaline
1	152.1	152.3	152.3	151.2	149.4	152.1	150.7
2	51.5	51.2	51.2	51.2	51.5	50.0	52.9
3	39.9	39.9	39.9	39.9	39.9	39.9	42.2
4	21.1[c]	21.1[c]	21.1[c]	21.1[c]	21.6[c]	20.6[c]	21.9[c]
5	26.3	27.0[c]	27.0[c]	27.0[c]	27.7[c]	25.8[c]	24.6[c]
6	104.5	104.4	104.4	104.4	104.4	72.2	84.7
7	87.9	87.9	87.9	88.0	88.4	83.1	85.7
8	197.2	197.3	197.2	197.3	197.6	196.9	37.7
9	117.5	117.6	117.7	117.6	118.4	117.9	25.2[c]
10	169.9	169.7	169.7	169.8	171.8	169.9	36.4
11	77.4	77.5	77.6	77.6	37.5	75.4	46.4
12	27.4[c]	28.3[c]	28.3[c]	28.3[c]	28.6[c]	26.7[c]	21.9[c]
13	28.2[c]	29.9[c]	29.9[c]	29.4[c]	29.4[c]	28.1[c]	27.5[c]
14	48.6	48.6	48.7	48.7	48.9	49.1	48.7
15	32.8	33.8	33.8	33.8	32.1	32.4	33.8
16	117.1	116.4	116.4	116.7	118.4	114.5	118.3
17	125.2	124.2	124.2	124.6	125.3	124.1	125.1
18	118.4	116.0	116.0	117.6	118.6	117.2	118.3
19	119.5	128.6	128.6	127.9	119.9	118.4	119.4
20	120.3	120.9	121.0	120.9	120.8	118.8	120.4
21	111.5	110.5	110.6	109.4	111.6	111.4	111.3
22	139.9	140.3	140.3	139.8	140.2	139.3	139.9
23	16.2	16.3	16.3	16.3	14.9	16.2	14.5
24	23.0	23.0	23.1	23.1	23.2	18.6	19.9
25	78.6	78.7	78.7	78.7	78.2	70.8	71.9
26	23.5	23.6	23.6	23.6	23.8	25.7[d]	23.7[d]
27	28.8	28.8	28.8	28.9	29.0	25.8[d]	26.1[d]
28		124.7	127.5	32.0			12.7
29		137.5	134.7	123.7			
30		71.2	75.3	133.0			
31		29.9	26.1	18.0			
32		29.9	26.1	25.8			
33			50.5				

[a] In ppm downfield from internal Me_4Si. [b] Most of the saturated carbon assignments are based on those of paxilline by Tanabe (private communication, 1976). [c] Assignments of these carbons uncertain at this time. [d] These assignments may be reversed.

compounds A, B, and C, respectively.

A previous report (Cole, 1976), demonstrated that paspaline (II) and paspalicine (III) were not tremorgenic in mice dosed IP at 500 mg/kg and 250 mg/kg, respectively. However, paspalinine (IV) dosed IP at 80 mg/kg and 160 mg/kg showed gross clinical signs indistinguishable from those for the tremorgen paxilline (I). The most significant chemical feature distinguishing the active metabolites from the inactive metabolites appears to be the tertiary hydroxy group at position C-11. It is interesting to note that all three *C. paspali* tremorgens contain this tertiary OH group.

Initial clinical signs (15–20 min after dosing) in mice dosed IP were lethargy, incoordinated movement, rough hair coat, and diarrhea. Later clinical signs (30–60 min after dosing) were intermittent tremor becoming more sustained at the higher dosage levels and hypersensitivity to sound stimuli. Tremors were most noticeable when the animal tried to move or was forced to move. Perceptible effects by all three toxins were observed down to 14 mg/kg in mice dosed IP (ED_{50} values were <14 mg/kg). Animals continued to feed or attempt to feed. The animals appeared normal after 24 h at all dosage levels.

The production of peripheral gangrene, a typical sign of *C. purpurea* ergotism, has not been observed in paspalum poisoning (Hopkirk, 1936). Peripheral gangrene results from the constriction of arterioles in the extremeties from the action of certain ergoline alkaloids. The absence of peripheral gangrene in *C. paspali* ergotism suggests that the neurological manifestations may be caused entirely by the action of the chemically neutral paspalinine-type metabolites. Conversely, the neurological signs produced by *C. purpurea* ergotism may not be entirely due to ergoline alkaloids. It has been assumed that little difference existed in the chemistry of the two species *C. purpurea* and *C. paspali*. This assumption might have prejudiced investigators to look only for alkaloids as the cause of the neurological manifestations in *C. paspali* ergotism and, therefore, frustrated attempts to isolate the causative toxins.

Tanabe (1976) has studied the biosynthesis of paxilline (I) using ^{13}C-labeled precursors while Acklin et al. (1976) have performed similar studies on paspaline (II), paspalicine (III), and paspalinine (IV). Both laboratories were in precise agreement. They showed that these related metabolites were derived from tryptophan and the diterpene geranylgeraniol. Acklin et al. (1976) reported that the probable biosynthetic sequence was paspaline to paspalicine to paspalinine. It can be further speculated that paspalinine is converted to compound A by the addition of an isoprene unit to the aromatic ring and compound A goes to compound C by the addition of a tertiary OH group and the migration of a double bond.

Further studies are being planned to compare the clinical signs produced by purified *C. paspali* tremorgens in Bovine species with those produced in the natural toxic syndrome.

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Simulation of Smoking Conditions by Pyrolysis

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A rapid, economical method was developed for the evaluation of the potential of different tobacco varieties to produce possibly hazardous smoke compounds. This controlled pyrolysis method produces pyrolyzate fractions very nearly identical with corresponding cigarette smoke fractions, as determined by analyses of their polynuclear aromatic hydrocarbons, neutral constituents, and phenolic contents.

Extensive research efforts are currently underway to develop safer smoking products which are still flavorful and aromatic. Thus, new varieties of tobacco and new methods of harvesting and curing tobacco must be evaluated. Typically, the evaluation involves the preparation of tobacco into cigarettes, the smoking of the cigarettes, and the subsequent testing of the cigarette smoke condensate. Such an evaluation is time consuming and expensive. We have been studying the formation of cigarette smoke components by the controlled pyrolysis (thermal decomposition) of tobacco leaf, tobacco extracts, and individual tobacco leaf compounds. With this improved pyrolysis method, we are able to rapidly evaluate the potential of a tobacco to form deleterious compounds, specifically, the tumor-promoting phenols and the carcinogenic polynuclear aromatic hydrocarbons (PAH) (Schmeltz et al., 1974).

Pyrolysis has been used frequently in studies to establish precursor–product relationships between tobacco components and cigarette smoke constituents (Schlotzhauer and Schmeltz, 1968; Bell et al., 1966; Schlotzhauer et al., 1976). The studies were made so that the smoke-forming process might be understood, and so that leaf constituents responsible for biologically active smoke compounds might be determined. However, the studies have been criticized since the pyrolytic conditions differed from those within a burning cigarette (Jenkins et al., 1970). We have adapted a method of pyrolysis that simulates those conditions closely, and the composition of the pyrolyzate formed resembles closely that of cigarette smoke. We used recent methods of analyzing smoke PAH's (Severson et al., 1976) to determine pyrolytic conditions that produce pyrolyzate with a PAH profile nearly identical with that of smoke PAH's. We now report the developed methodology and results.

EXPERIMENTAL SECTION

Materials. The tobacco used in the pyrolysis experiments was removed from 1R1 University of Kentucky reference cigarettes and was equilibrated at 60% relative humidity for 48 h.

Pyrolytic Apparatus. The pyrolysis apparatus was similar to one previously described (Smith et al., 1975) and is pictured in Figure 1. The Vycor pyrolysis tube was positioned horizontally through a moveable oven containing a 5.08-cm long heating core. The temperature of the oven was monitored and controlled by a temperature controller (±20 °C). Temperature range was ambient to 800 °C. Movement of the oven from 0.16 cm/min to 116.84 cm/min was controlled by a speed control unit. Two timers were used—one controlling operative puffing time and the other, nonoperative time (the intervals between puffs). When the operative timer was in control, puffing conditions were simulated in that nitrogen flowed and the oven moved at a preset rate along the pyrolysis tube. In the nonoperative mode, there was neither flow nor oven movement. Puffing time varied from 5 to 60 s, and the intervals between puffs were the differences between 60 s and puffing time. Nitrogen flow was controlled by a solenoid valve in relay with the timers; and the nitrogen flow rate, controlled by a needle valve, was monitored by an in-line gas flow meter.

Pyrolysis Procedure. A Vycor glass wool plug was placed about 30 cm from the outlet of the pyrolysis tube (1.22-m × 2.54-cm o.d.) and about 30 g of tobacco were loosely packed into the tube to yield a 60.96-cm column of tobacco. A Vycor glass wool plug was then inserted to close the column. Nitrogen flow was adjusted to the desired rate, and the oven temperature was equilibrated over an empty portion of the tube. The oven was then moved to the beginning of the tobacco column to initiate pyrolysis, and then allowed to move over the column at a preset rate, either continuously or in a pulsating motion. The trapping system consisted of a 3-L, uncooled expansion flask, three uncooled traps half-filled with ether, and a gas bubbler containing ether and 0.5% aqueous NaOH.

Pyrolysis Fractionation. The pyrolyzate was quantitatively removed from the traps and fractionated into neutral, basic, acidic, and phenolic fractions by solvent partitioning and pH adjustments (Higman et al., 1970). Residue weights for each fraction, as shown in Table I, were determined by reducing 10% aliquots of the indi-

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