7 it is seen that also the aromatic ring of methabenzthiazuron (1) can be attacked oxidatively. This occurrence is generally regarded as the first step leading to ring opening and demonstrates the feasibility of the further degradation of this substance class.

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Hydroxyterphenyllin: A Novel Fungal Metabolite with Plant Growth Inhibiting Properties

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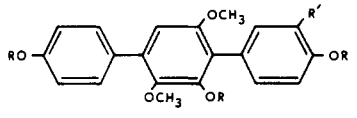
Two metabolites, demonstrating plant growth regulating activity, were isolated from *Aspergillus candidus* found growing on unbleached flour. One of the metabolites was identified as terphenyllin (2′,5′-dimethoxy-4,3′,4″-trihydroxy-*p*-terphenyl); the other was a new metabolite, hydroxyterphenyllin (2′,5′-dimethoxy-3,4,3′,4″-tetrahydroxy-*p*-terphenyl). The former significantly inhibited wheat coleoptile growth at 10^{-3} M; the latter inhibited growth at 10^{-3}, 10^{-4}, and 10^{-5} M. Hydroxyterphenyllin tetraacetate was ineffective against wheat coleoptiles.

During the screening of fungi for metabolites exhibiting plant growth regulating activity, we isolated two biologically active compounds from *Aspergillus candidus* isolated from molded unbleached flour. Both inhibited the growth of wheat coleoptiles. One of these compounds was subsequently identified as terphenyllin (2′,5′-dimethoxy-4,3′,4″-trihydroxy-*p*-terphenyl) (I), a metabolite cytotoxic to HeLa cells (Takahashi et al., 1976) and originally isolated by Marchelli and Vining (1975). The other metabolite, which was more inhibitory to wheat coleoptiles, is a new metabolite, hydroxyterphenyllin (2′,3′-dimethoxy-3,4,3′,4″-tetrahydroxy-*p*-terphenyl) (II). We now wish to report the isolation and identification of hydroxyterphenyllin and the plant growth regulating properties of terphenyllin, hydroxyterphenyllin, and hydroxyterphenyllin tetraacetate (III).



I . R, R′ = H
II . R = H, R′ = OH
III . R = $CH_3C(=O)$, R′ = $CH_3C(=O)O$–

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MATERIALS AND METHODS

Production and Isolation of Active Metabolites. *Aspergillus candidus* (ATCC accession no. 36008) was isolated from a small sample of moldy unbleached flour by culturing on potato dextrose agar (PDA) slants at 26 °C, for 7 days. Cultures were then maintained at 5 °C until transferred to Fernbach flasks (2.8 L), each containing 100

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g of shredded wheat, 200 mL of Difco mycological broth (pH 4.8), 2% yeast extract, and 20% sucrose (Kirksey and Cole, 1974) for production of the metabolites. After 16 days at 27–28 °C, the mycelia and substrate were extracted with approximately 300 mL/flask acetone in a Waring Blendor. The suspension was filtered through cheesecloth and the resulting filtrate was filtered through Whatman No. 1 filter paper on a Buchner funnel. The acetone was removed from the crude extract under vacuum at 50 °C and the remaining aqueous phase partitioned twice against ethyl acetate: the total volume of ethyl acetate was three times that of the aqueous phase. The ethyl acetate fraction was dried over anhydrous sodium sulfate and reduced, under vacuum, to a small volume, then placed on a silica gel (70–230 mesh) chromatography column (9.0 × 10 cm) that had been slurry packed in benzene. Sequential elution of the crude fraction was made by adding 1.0 L of benzene, ethyl ether, chloroform, ethyl acetate, acetone, and methanol. Each solvent drained to the top of the silica gel before the next solvent was added. Each fraction was reduced in volume, under vacuum. On standing at 5 °C, the ethyl ether fraction yielded crystals that inhibited the growth of wheat coleoptiles. Thin-layer chromatography indicated that two compounds were present in the crystalline mass. Additional separation and purification of the two metabolites was achieved by further silica gel column chromatography (4.0 × 50 cm) packed as a slurry in benzene and using a linear gradient of benzene to acetone (1.5 L of benzene and 1.5 L of acetone); 20-mL fractions were collected. The crystalline biologically active mixture was added to the top of the column as a fine suspension in a minimal amount of ethyl acetate. Each 20-mL fraction was evaporated to a small volume and was assayed using wheat coleoptiles.

Plant Bioassay. Wheat seedlings (*Triticum aestivum* L. cv. Wakeland) were grown on moist sand for 4 days at 22 ± 1 °C in the dark (Hancock et al., 1964), then coleoptiles were cut 4 mm long from the apex of the etiolated seedlings, after the first 2 mm had been discarded, in a Van der Weij guillotine under a green safelight (Nitsch and Nitsch, 1956). Crude fractions to be assayed for biological activity were added to test tubes (approximately 15 μL per tube) and then evaporated to dryness under nitrogen. Two milliliters of phosphate–citrate buffer solution containing 2% sucrose at pH 5.6 (Nitsch and Nitsch, 1956) were added to each test tube, followed by the addition of ten coleoptiles to each tube. Purified metabolites were assayed at 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M. A stock solution of each metabolite was made at 10^{-3} M by dissolving each in 50 μL of acetone (Cutler, 1968) and adding phosphate–citrate buffer to a volume of 10 mL. Coleoptiles were incubated and rotated 0.25 rpm in a roller-tube apparatus, in the dark, for 24 h at 22 °C and then measured by projecting their images (×3) from a photographic enlarger (Cutler and Vlitos, 1962). All data were statistically analyzed (Kurtz et al., 1965).

Physical and Chemical Analyses. Ultraviolet (UV) spectra of the metabolites were taken in 95% ethanol solution with a Bausch & Lomb Spectronic 505 recording spectrophotometer. Infrared (IR) spectra were made from samples that had been prepared as thin films on KBr windows with a Beckman IR4210 spectrophotometer equipped with a 4× beam condenser. Both ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Varian Associates XL-100-12 spectrometer equipped with the 620-L data system. ^{1}H spectra were obtained in the continuous wave mode and the ^{13}C spectra were obtained using the Fourier Transform (FT) mode. Operating

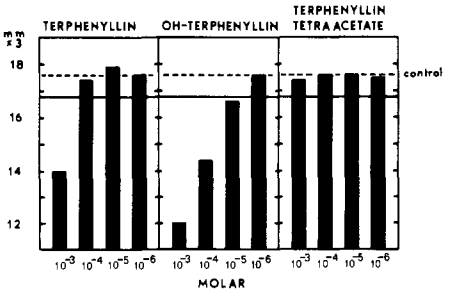

Figure 1. Growth regulating activity of terphenyllin (I), hydroxyterphenyllin (II), and hydroxyterphenyllin tetraacetate (III) in wheat coleoptile bioassays (*Triticum aestivum* L. cv. Wakeland). Control: dotted line. Significant inhibition: below solid line ($P < 0.01$).

parameters in the FT mode were: sweep width 5 KHz; data points 8K; pulse angle 30°; repetition time 1.8 s; and square-wave, broadband decoupling power 11 W. Single-frequency, off-resonance proton decoupled (sford) ^{13}C spectra were obtained by offsetting the decoupler frequency 1000 Hz downfield from the center of proton adsorption. Low-resolution (LRP) and high-resolution (HRP) mass spectra analyses were gathered with an AEI MS-9 mass spectrometer, and ionization was by electron impact at 70 eV, or via chemical ionization with isobutane as the ionizing reagent. Perfluorokerosene was used as the internal standard for HRP. Melting points were determined with a Hoover capillary melting point apparatus and were uncorrected.

To synthesize the tetraacetate of compound II, 50 mg of the parent material were stirred with acetic anhydride (1 mL) and pyridine (1 mL) at room temperature for 24 h. Removal of the acetic anhydride–pyridine mixture by high-vacuum evaporation yielded an off-white solid which was further recrystallized from hexane.

To obtain R_f values, compounds I, II, and III were chromatographed on silica gel-60, F-254, (E. M. Laboratories, Inc.) thin-layer plates with a toluene–ethyl acetate–formic acid (5:4:1, v:v:v) solvent system. The metabolites were visualized under short wave UV.

RESULTS AND DISCUSSION

Effects on Plant Growth. Terphenyllin (I) significantly ($P < 0.01$) inhibited wheat coleoptile growth 35% at 10^{-3} M relative to controls, but did not inhibit growth at 10^{-4}, 10^{-5}, or 10^{-6} M (Figure 1). However, compound II significantly inhibited growth at 10^{-3}, 10^{-4}, and 10^{-5} M; (100, 42, and 8% relative to controls) and was considerably more active biologically than compound I. By contrast, the acetate III was inactive. In comparison, abscisic acid inhibited coleoptiles 100, 90, and 69% at 10^{-3}, 10^{-4}, and 10^{-5} M. It seems probable, then, that the biological activity is a function of the number and placement of the hydroxyl groups, but this remains to be proved.

Physical and Chemical Characteristics. After growing 16 days, 50 Fernbach flasks of *Aspergillus candidus* produced 1.5 g of a mixture of compounds I and II upon sequential elution column chromatography with silica gel. The metabolites eluted in the ethyl ether fraction. Further passage through silica gel using a linear gradient from benzene to acetone yielded compounds I and II in ratios of 1.2:1 with a 20% loss of both compounds. Compound I eluted out in tubes 14–20 (280–400 mL) and compound II in tubes 23–30. Thin-layer chromatography R_f values were 0.57–0.63 for I; 0.49–0.54 for II, and 0.64 to 0.69 for III. Spots were visualized as deep purple areas under short-wave UV. Melting points were 235–236 °C for I, 233–234 °C for II, and 146–148 °C for III. UV

Table I. ^{1}H NMR Chemical Shifts for Some Terphenyl Derivatives[a]

Proton	1[b]	2[b]	3[c]
2	7.22	6.72 (J = 2.0)	[d]
3	6.80		1.93
4			2.30
5	6.80	6.55 (J = 8.0)	[d]
6	7.22	6.75 (J = 8.0, 2.0)	[d]
2′	3.40	3.32	3.41
3′			2.06
5′	3.70	3.66	3.80
6′	6.39	6.36	6.98
2″	7.45	7.45	7.67
3″	6.85	6.85	[d]
4″			2.30
5″	6.85	6.85	[d]
6″	7.45	7.45	7.67

[a] In ppm downfield from internal Me_4Si. [b] In Me_2SO-d_6. [c] In acetone-d_6. Shifts of the acetate methyls. [d] Multiplet centered at 7.23 ppm.

analyses for the three compounds were λ_{max}^{EtOH} 228 (shoulder) and 278 nm (log ϵ = 4.64 and 4.62, respectively) for I; λ_{max}^{EtOH} 228 (shoulder) and 280 nm (log ϵ = 4.71 and 4.66, respectively) for II; and λ_{max}^{EtOH} 230 (shoulder) 263 and 303 nm (log ϵ = 4.66, 4.62 and 4.38, respectively) for III. The IR spectra for I gave the following values: 3300–3400 (broad, strong) 1600, 1520, 1485, 1410, 1360, 1300, 1220, 1105, 1062, 1002, 830, 775 cm^{-1}. Values for II were: 3350 (broad, medium) 1600, 1570, 1485, 1442, 1405, 1362, 1320, 1225, 1120, 1070, 1000, 895, 822, and 790 cm^{-1}. Both spectra were highly complex.

High-resolution mass spectral analyses gave a molecular ion peak (M^+) at m/e 338.1158 for I (calculated mass 338.1153) and molecular formula $C_{20}H_{18}O_5$. Prominent ion fragment peaks were at m/e 323 ($M^+ - CH_3$), 308 ($M^+ - 2CH_3$), and 292 ($M^+ - CH_3 - OCH_3$). Elemental analysis of I gave the following: C, 71.00; H, 5.40; O, 23.72% (calculated: C, 71.03; H, 5.36; O, 23.66%). For II, HRP analyses showed M^+ at m/e 354.1120 (calculated mass 354.1103) and molecular formula $C_{20}H_{18}O_6$. Other significant ion fragment peaks were at m/e 338 ($M^+ - O$), 323 ($M^+ - OCH_3$), 308 ($M^+ - O, -2CH_3$), and 292 ($M^+ - (OCH_3)_2$). Elemental analysis of II yielded the following: C, 67.64; H, 5.17; O, 27.08% (calculated: C, 67.83; H, 5.12; O, 27.11%). For III, HRP analyses showed M^+ at m/e 522.116 and molecular formula $C_{28}H_{26}O_{10}$. Other significant peaks were at m/e 480 ($M^+ - CH_2CO$), 438 ($M^+ - 2CH_2CO$), 396 ($M^+ - 3CH_2CO$), and 354 ($M^+ - 4CH_2CO$).

The ^{1}H NMR spectrum of compound I in Me_2SO-d_6 (Table I) shows the presence of two para-substituted phenyl rings, two methoxy groups, and a broadened singlet for an aromatic proton coupled to the lower field methoxy group. The ^{13}C NMR spectrum of compound I (Table II) exhibits 16 peaks. Intensity considerations suggest that four of the peaks are due to two carbons each, whereas the remainder are due to single carbons. The multiplicity of the peaks obtained from the sford spectrum establishes that compound I contains two methyl, nine methine, and nine quaternary carbons.

All the data for compound I indicate a substituted terphenyl. Allowing for a difference in solvents, the ^{1}H NMR and mass spectra of compound I are identical with those reported for terphenyllin (2′,5′-dimethoxy-4,3′,4″-trihydroxy-p-terphenyl), a previously reported metabolite isolated from *Aspergillus candidus* grown on rice (Takahashi et al., 1976).

Elemental analysis and the mass spectrum suggest that compound II contains an additional oxygen atom compared to compound I. The fact that II forms a tetraacetate

Table II. ^{13}C Chemical Shifts for Some Terphenyl Derivatives[a]

Carbon	1[b]	2[b]
1	128.7 s	130.6 s
2	131.6 d	115.5 d
3	114.3 d	144.5 s
4	155.6 s	144.8 s
5	114.3 d	119.1 d
6	131.6 d	122.6 d
1′	124.2 s	125.6 s
2′	147.6 s	148.7 s
3′	138.9 s	139.9 s
4′	116.5 s	117.9 s
5′	156.4 s	157.3 s
6′	102.9 d	103.7 d
1″	132.1 s	132.8 s
2″	129.3 d	129.4 d
3″	114.9 d	115.8 d
4″	152.8 s	153.7 s
5″	114.9 d	115.8 d
6″	129.3 d	129.4 d
2′ CH_3	55.5 q	56.4 q
3′ CH_3	59.9 q	60.8 q

[a] In ppm downfield from internal Me_4Si. [b] In Me_2SO-d_6.

derivative indicates that II contains four hydroxyl groups. The ^{1}H NMR spectrum of compound II in Me_2SO-d_6 shows the presence of two methoxy groups, a para-substituted phenyl ring, a broadened singlet for an aromatic proton coupled to the lower-field methoxy group, and an ABX pattern for three aromatic protons (Table I). The coupling constants for the ABX pattern clearly establishes that it arises from a 1,2,4-trisubstituted aromatic ring. The proton decoupled ^{13}C NMR spectrum of II exhibits 18 peaks, two of which are due to two carbons each (Table II). Results from the sford ^{13}C spectrum show that II contains two methyl, eight methine, and ten quaternary carbons.

Comparison of the NMR data of II with those of terphenyllin (I) suggests that II is also a terphenyl and contains an additional hydroxyl group on one of the terminal phenyl rings. Close inspection of the ^{1}H chemical shifts of I and II indicates that the additional hydroxyl group is located on the phenyl ring ortho to the hydroxyl group on the center ring. The remaining question concerning the structure of II is the location of the extra hydroxyl group. Substitution of a hydroxyl group on either carbon 2 or 3 of I would give rise to the coupling pattern observed for the ABX pattern in II. A comparison of the ^{1}H and ^{13}C chemical shifts expected on going from compound I to either a 2- or 3-hydroxy-substituted terphenyllin utilizing the additivity of substituent effects, and the ^{1}H chemical shift differences observed between p-cresol and 4-methylcatechol (Pouchert and Campbell, 1974) clearly indicates that the additional hydroxyl group is located on carbon 3. Thus, the spectral data establish the structure of compound II as 2′,5′-dimethoxy-3,4,3′,4″-tetrahydroxy-p-terphenyl.

If the biological activity of the terphenyllins is directly related to the placement and number of hydroxyl groups on either, or all, of the three phenyl rings, the basic template offers an interesting starting point for further production of medicinal and agricultural chemicals of significant value.

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Gas Chromatographic–Mass Spectral Analyses of *s*-Triazine Metabolites

William R. Lusby* and Philip C. Kearney

Trimethylsilyl (Me_3Si) derivatives of six suspected metabolites of 2-chloro-4,6-bis(ethylamino)-*s*-triazine (simazine) were prepared by reaction with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 150 °C for 15 min. Three of the suspected metabolites, 2,4,6-trihydroxy-*s*-triazine (cyanuric acid), 2,4-dihydroxy-6-amino-*s*-triazine (ammelide), and 2,4-diamino-6-hydroxy-*s*-triazine (ammeline), yielded $(Me_3Si)_3$ derivatives. 2-Amino-4-ethylamino-6-hydroxy-*s*-triazine (*N*-ethylammeline) and 2,4-dihydroxy-6-ethylamino-*s*-triazine (*N*-ethylammelide) gave predominately the $(Me_3Si)_2$ derivitives. 2-Hydroxy-4,6-bis(ethylamino)-*s*-triazine (hydroxysimazine) yielded the Me_3Si product plus a lesser amount of the $(Me_3Si)_2$ derivative. The derivatives were analyzed by gas chromatography–mass spectrometry, with electron impact ionization (70 eV) and chemical ionization (isobutane). Peaks for the parent M, M – 15 (minus methyl), and trimethylsilyl (*m/e* 73) ions were among the largest in the electron impact spectra for all derivatives. The chemical ionization spectra confirmed the molecular weights of the derivatives.

s-Triazine herbicides rank number one in production of all herbicides. Their principal use is on corn, sorghum, and sugar cane. In 1974, 95 million pounds of atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) were produced in the U.S., and 75 million pounds were used domestically (von Rumker et al., (1974).

Metabolites of *s*-triazine herbicides have been isolated from many sources. In 1969, Montgomery et al. isolated *s*-triazine metabolites from corn, and hydroxysimazine [2-hydroxy-4,6-bis(ethylamino)-*s*-triazine] was identified as a major product from simazine. Dehalogenated, dealkylated, and deaminated derivatives of the *s*-triazine herbicides have been isolated from several microbial systems (Kaufman and Kearney, 1970). In 1972, Bakke et al. isolated metabolites from the rat. Many other investigators have isolated *s*-triazine metabolites from other sources. Most *s*-triazine metabolites are highly polar and often cannot be analyzed directly by gas chromatography. Their polarity usually renders mass spectral analysis difficult, even by direct probe introduction.

For our investigation of the gas chromatographic–mass spectral analysis (GC–MS) of trimethylsilyl derivatives of *s*-triazine metabolites, we chose six compounds reported to be *s*-triazine herbicide metabolites or likely to be isolated in metabolic studies. The compounds were 2-hydroxy-4,6-bis(ethylamino)-*s*-triazine(hydroxysimazine), 2-amino-4-ethylamino-6-hydroxy-*s*-triazine (*N*-ethylammeline), 2,4-dihydroxy-6-ethylamino-*s*-triazine (*N*-ethylammelide), 2,4-diamino-6-hydroxy-*s*-triazine (ammeline), 2,4-dihydroxy-6-amino-*s*-triazine (ammelide), and 2,4,6-trihydroxy-*s*-triazine (cyanuric acid). The compounds were either supplied by CIBA-Geigy Corporation or obtained from commercial chemical supply houses.

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METHODS AND MATERIALS

The trimethylsilyl (Me_3Si) derivatives were prepared essentially by the method of Flint and Aue (1970). About 100 μg of the compound to be derivatized was placed into a 1-mL micro-reaction vial, and while the vial was swept with dry nitrogen, 100 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added. The vial was capped and heated at 150 °C for 15 min; excess BSTFA reagent was evaporated with a stream of dry nitrogen; then 100 μL of hexane was added. A portion of the hexane solution was analyzed by use of a Dupont 491B gas chromatograph–mass spectrometer which was fitted with a 6-ft by 2 mm i.d. 3% OV-17 column, temperature programmed from 155 to 235 °C at 4 °C/min. For electron impact spectra an ionizing voltage of 70 eV and a source temperature of 220 °C were used. For chemical ionization spectra, isobutane was used as the reagent gas and the source temperature was 170 °C.

RESULTS AND DISCUSSION

A gas chromatogram of Me_3Si derivatives of cyanuric acid, ammelide, ammeline, *N*-ethylammelide, *N*-ethylammeline, and hydroxysimazine is shown in Figure 1. Under our reaction conditions both the Me_3Si and $(Me_3Si)_2$ derivatives of hydroxysimazine were produced. Two derivatives, $(Me_3Si)_3$ and $(Me_3Si)_2$ were also formed from *N*-ethylammelide. For cyanuric acid, ammeline, and ammelide, the $(Me_3Si)_3$ derivatives were the predominant products. The reaction yielded the $(Me_3Si)_2$ derivative of