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OCHRINDOLES A–D: NEW BIS-INDOLYL BENZENOIDS FROM THE SCLEROTIA OF *ASPERGILLUS OCHRACEUS* NRRL 3519

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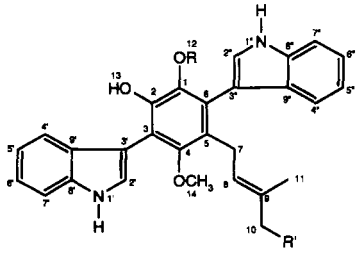
ABSTRACT.—Four new prenylated bis-indolyl benzenoid metabolites (ochrindoles A–D; **1–4**) were isolated from antiinsectan organic extracts of the sclerotia of *Aspergillus ochraceus* (NRRL 3519). The structures of these compounds were determined primarily through HMBC, selective INEPT, and NOESY experiments. These compounds displayed moderate activity in feeding assays against the corn earworm *Helicoverpa zea* and the driedfruit beetle *Carpophilus hemipterus*. Compounds **1–4** also exhibited activity against Gram-positive bacteria.

Our continuing interest in fungal sclerotia as sources of new anti-insectan metabolites (1–4) prompted us to investigate the chemistry of several sclerotium-producing isolates of *Aspergillus ochraceus* Wilhelm. Earlier studies of the sclerotia of *A. ochraceus* (NRRL 3519) afforded three new diketopiperazine metabolites with moderate activity against the corn earworm *Helicoverpa zea* (4), but these compounds did not account for all of the activity of the sclerotial extracts. Further studies of these extracts have resulted in the isolation of three new bis-indolyl benzenoids [**1–3**] and one new bis-indolyl quinone [**4**] which we have named ochrindoles A–D. Details of this work are described here.

RESULTS AND DISCUSSION

Ochrindole A [**1**], the most abundant of the four compounds, has the molecular formula $C_{29}H_{28}N_2O_3$, as deduced from hreims data (M^+ 452.2122, Δ −2.2 mmu). Decoupling, HMQC, and HMBC results revealed the presence of two 3-substituted indoles, two MeO groups, one exchangeable phenolic OH, and a 3-methyl-2-butenyl side-chain, and permitted the corresponding ^{1}H- and ^{13}C-nmr assignments to be made (Table 1). These features accounted for all but four of the necessary unsaturations, six



1 R=CH_3; R′=H
2 R=CH_3; R′=OH
3 R=R′=H

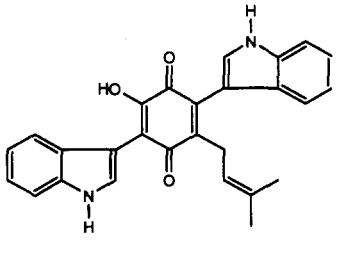


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TABLE 1. Nmr Data for Ochrindoles A [**1**], B [**2**], and D [**4**].

Position	Compound					
	1		**2**		**4**	
	δ_C[a]	δ_H[b] (Multiplicity, J_{HH})	δ_C[a]	δ_H[c] (Multiplicity, J_{HH})	δ_C[d]	δ_H[e] (Multiplicity, J_{HH})
1	142.5	—	142.7	—	184.2	—
2	145.7	—	145.9	—	150.9	—
3	114.6	—	114.7	—	137.3	—
4	153.4	—	153.3	—	187.8	—
5	127.1	—	126.8[f]	—	146.4	—
6	126.6	—	126.3[f]	—	135.1	—
7	27.0	3.08 (br dd, 14.6, 6.4) 3.34 (br dd, 14.6, 6.8)	26.5	3.21 (dd, 15.4, 6.1) 3.35 (dd, 15.9, 7.4)	28.8	3.32 (d, 6.5)
8	125.1	5.07 (m)	126.8	5.25 (m)	122.4	5.08 (m)
9	130.0	—	133.4	—	133.4	—
10	25.6	1.52 (s)	69.1	3.72 (s)	25.8	1.55 (s)
11	17.4	1.24 (s)	13.1	1.20 (s)	17.8	1.29 (s)
12	60.8	3.35 (s)	60.9	3.37 (s)	—	—
13	—	5.79 (br s; OH)	—	5.79 (br s; OH)	—	10.75 (br s)
14	60.2	3.26 (s)	60.3	3.26 (s)	—	—
1′	—	8.38 (br s)	—	8.45 (br s)	—	—
2′	124.7	7.50 (d, 2.2)	124.7	7.49 (d, 2.5)	129.1	7.67 (d, 1.5)
3′	108.5	—	108.3	—	106.6	—
4′	121.2	7.69 (br d, 7.9)	121.2	7.66 (br d, 7.9)	122.9	7.56 (d, 8.0)
5′	119.9	7.17 (br dd, 8, 8)	120.0	7.17 (ddd, 9.6, 7.2, 1.1)	119.9	7.03 (ddd, 7.7, 6.2, 1.0)
6′	122.2	7.24 (m)	122.2	7.24 (m)	122.1	7.11 (ddd, 7.8, 6.2, 1.0)
7′	110.9	7.44 (d, 8.2)	111.1	7.46 (m)	112.1	7.45 (d, 8.1)
8′	136.1	—	136.1	—	137.1	—
9′	126.9	—	126.8	—	127.9	—
1″	—	8.27 (br s)	—	8.34 (br s)	—	10.81 (br s)
2″	123.5	7.21 (d, 2.4)	123.3	7.20 (d, 2.3)	127.6	7.46 (d, 1.5)
3″	111.9	—	112.0	—	108.2	—
4″	120.4	7.46 (d, 7.9)	120.3	7.43 (m)	120.8	7.41 (d, 7.9)
5″	119.8	7.12 (br dd, 8, 8)	119.8	7.12 (ddd, 9.6, 7.2, 1.0)	120.5	7.07 (ddd, 7.7, 6.2, 1.0)
6″	122.1	7.22 (m)	122.6	7.23 (m)	122.6	7.16 (ddd, 7.8, 6.2, 1.0)
7″	111.1	7.42 (d, 8.2)	111.1	7.44 (m)	112.5	7.50 (d, 8.1)
8″	135.9	—	135.9	—	137.2	—
9″	127.9	—	128.0	—	128.0	—

[a]75 MHz, in $CDCl_3$.
[b]600 MHz, in $CDCl_3$.
[c]300 MHz, in $CDCl_3$.
[d]75 MHz, in Me_2CO-d_6.
[e]600 MHz, in Me_2CO-d_6.
[f]These assignments may be interchanged.

carbons of the molecular formula, and six non-protonated aromatic/vinylic carbon resonances, three of which are oxygenated. Thus, ochrindole A appeared to contain a hexasubstituted benzene ring with the six units listed above as substituents.

The substitution pattern of the benzene ring was assigned primarily on the basis of HMBC and selective INEPT correlations (Figure 1). The vinylic proton of the prenyl side-chain (5.07 ppm) showed an HMBC correlation with the non-indole aryl carbon signal at 127.1 ppm (C-5), thereby indicating linkage of the prenyl group to the benzene ring at the corresponding carbon. The remaining non-oxygenated aryl carbon signals (114.6, 126.6 ppm) must be the points of attachment for the indolyl substituents. One of the methylene proton signals of the prenyl group (3.08 ppm) also showed HMBC correlations to the signal at 127.1 ppm and to a downfield resonance (153.4 ppm; C-4) that was itself correlated to one of the MeO proton resonances (3.26 ppm), placing this MeO group ortho to the prenyl substituent. A selective INEPT experiment optimized for $^{n}J_{CH}=7$ Hz verified these assignments and showed an additional correlation between the methylene proton and the carbon signal at 126.6 ppm (C-6), thereby placing one of the indole substituents at the other ortho position relative to the prenyl group. The second MeO group (3.35 ppm) was correlated with the aryl carbon at 142.5 ppm (C-1). The phenolic OH proton at 5.79 ppm was also correlated with this signal, and with other aryl resonances at 145.7 and 114.6 ppm (C-2 and C-3). These data place the second MeO group and the OH group ortho to each other, and require connection of the second indole substituent at a position ortho to the OH group. None of the indole proton signals showed HMBC or selective INEPT correlations with any of the six benzenoid carbon resonances. However, NOESY data showed cross-peaks correlating H-2″ of one of the indole subunits with the MeO signal at 3.35 ppm and the vinylic proton of the prenyl group, placing the corresponding indole subunit between these groups on the benzenoid ring. Based on these results, the positions of the indole subunits were established, leading to assignment of the structure of ochrindole A as **1**.

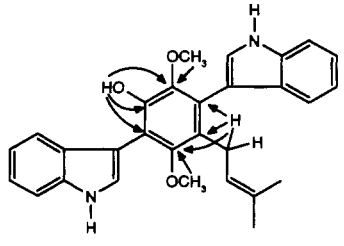

FIGURE 1. Selected HMBC/Selective INEPT Correlations for Ochrindole A [**1**].

The ^{1}H-nmr spectrum of ochrindole B (**2**; Table 1) was nearly identical to that of **1**, except for the absence of one of the prenyl Me group signals and the appearance of an oxymethylene singlet at 3.72 ppm. The eims contained a molecular ion at *m/z* 468, suggesting the presence of one additional oxygen atom. These data, plus HMQC, HMBC, and NOESY results, indicated that **2** differs from **1** by replacement of the prenyl Me group cis to the vinylic proton with a CH_2OH group. NOESY correlations between indole ring protons and the MeO groups permitted differentiation of the two sets of indole nmr signals. The signal at 3.26 ppm (H_3-14) showed cross-peaks with signals at 7.49 and 7.66 ppm (H-2′ and H-4′), while the H_3-12 MeO signal at 3.37 ppm correlated with signals at 7.20 and 7.44 ppm (H-2″, H-4″, and H-7″).

The ^{1}H-nmr spectrum of ochrindole C [**3**] was also similar to that of **1** except for the absence of the MeO singlet at 3.35 ppm and the appearance of an additional

exchangeable (phenolic) proton at 5.04 ppm, suggesting that **3** is a desmethyl analog of **1**. This proposal was consistent with the eims of **3**, which showed a molecular ion 14 mass units lower than that of **1**. HMBC results showed a set of correlations analogous to those described above, with the remaining MeO group correlated to C-4. Thus, compounds **1** and **3** differ only in the replacement of the C-1 MeO group in **1** with an OH group. Unlike **1** and **2**, ochrindole C readily decomposed, and was characterized only by nmr and ms. However, on the basis of the data obtained, and by analogy to **1** and **2**, the structure was proposed as shown in **3**.

Like compounds **1–3**, the nmr data for ochrindole D (**4**; Table 1) contained signals corresponding to two 3-substituted indoles and a prenyl side-chain, indicating a similar structure. However, the presence of two carbonyl groups (^{13}C-nmr signals at 184.2 and 187.8 ppm; ir band at 1660 cm^{-1}) and the uv spectrum suggested that the central ring was a benzoquinone unit. The eims showed a molecular ion at *m/z* 422, as well as a relatively intense $[M+2H]^+$ ion, further indicating a quinone moiety (5). The nmr assignments for **4** were established based on HMQC and HMBC data. The indole rings were distinguished on the basis of a weak HMBC correlation between H-2″ and C-6, along with NOESY correlations between H-8 and H-2″ and between H-7 and H-4″.

Interestingly, the nmr signals for the methylene protons at C-7 in compounds **1–3** appeared as doublets of doublets when the spectra were measured in $CDCl_3$, Me_2CO-d_6, DMSO-d_6, CD_3OD, or C_6D_6, while the H_2-7 signal in the spectrum of **4** appeared as a doublet (in Me_2CO-d_6). These observations, together with optical rotations measured for **1** and **2**, suggest that some restriction to free rotation for the indole rings exists for **1–3**, presumably caused by steric interactions with the substituents on the central aromatic ring. In order to determine whether these signals are influenced by increased temperatures, the ^{1}H-nmr spectrum of **1** was recorded at temperatures up to 70°. C_6D_6 was employed as the solvent to avoid overlap of the H_2-7 multiplets with solvent signals. Incremental increases in probe temperature caused gradual coalescence and a loss in definition of the multiplets. At 70°, a single, broad distorted methylene signal was observed at 3.50 ppm. Upon cooling to room temperature, the spectrum returned to its original appearance. This process did not cause a change in the optical rotation.

Members of this general structural class have been previously reported as fungal metabolites (e.g., the asterriquinones and asterriquinols from *A. terreus*) (6–10). The known compounds typically contain prenyl group(s) at various positions on the indole moieties. Ochrindoles A–D [**1–4**] are unique in that the prenyl side-chain is located on the central benzenoid ring. These new compounds showed moderate activity in assays against the corn earworm, *Helicoverpa zea*, and the fungivorous beetle, *Carpophilus hemipterus*. For example, relative to controls, ochrindole A [**1**] caused a 30% reduction in weight gain in tests against *H. zea* larvae and a 20% feeding reduction in assays against *C. hemipterus* adults and larvae at a dietary concentration of 200 ppm (wet wt). Compounds **1–4** also showed activity against *Bacillus subtilis* in standard disk assays, affording zone sizes ranging from 15–18 mm at 100 μg/disk. Although ochrindoles A–D are not especially potent by comparison to metabolites previously isolated from the sclerotia of other Aspergilli, they are major components of *A. ochraceus* NRRL 3519 sclerotia, and are therefore responsible for a significant portion of the antiinsectan activity of the extracts.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—HMQC, HMBC, COSY, and NOESY data were obtained using a Bruker AMX-600 spectrometer. HMQC and HMBC experiments were optimized for *J* values of 152 and 8 Hz, respectively. All other ^{1}H- and ^{13}C-nmr data were obtained using a Bruker AC-300 instrument. Selective INEPT experiments were performed at 75 MHz, optimizing for $^nJ_{CH}$=4, 7, or 10 Hz. Hreims data

were recorded on a VG ZAB-HF mass spectrometer, while low-resolution eims data were recorded at 50 or 70 eV using a VG Trio 1 quadrupole mass spectrometer. A Beckman Ultrasphere C_{18} column (5 μm, 10 mm×25 cm, flow rate=2.0 ml/min) was used in all hplc separations. Details of other general experimental procedures and insect assays have been described elsewhere (1–4, 11, 12).

FUNGAL MATERIAL.—The strain employed in these studies (NRRL 3519) was originally deposited in the ARS culture collection in 1948 by J. Winitsky of Buenos Aires, Argentina, and identified as *A. melleus* Yukawa. The strain was reported to produce ochratoxin A (13), and was deposited in the American Type Culture Collection as *A. melleus* ATCC 34443. However, the culture was later re-examined and identified as *A. ochraceus* Wilhelm (14) by D. Fennell. Sclerotia of *A. ochraceus* NRRL 3519 were produced by solid substrate fermentation on autoclaved corn kernels and harvested using procedures described previously (11).

EXTRACTION AND ISOLATION.—A sample of ground sclerotia (189 g) was extracted sequentially with 1200 ml each of hexane, $CHCl_3$, EtOAc, and MeOH. After evaporation of the solvent, the $CHCl_3$ extract (951 mg) was subjected to vacuum-flash chromatography on Si gel using CH_2Cl_2, followed by elution with increasing percentages of MeOH. Based on tlc [CH_2Cl_2-MeOH (9:1)], the vlc fractions eluting at 1% and 2% MeOH were combined to yield a 385 mg sample. Further purification was carried out using continuous gradient chromatography [100% CH_2Cl_2 to CH_2Cl_2-MeOH (95:5)] on a column containing 38 g of Si gel. Subsequent chromatography [100% hexane to hexane-EtOAc (60:40)] on a column containing 28 g of Si gel afforded ochrindoles A (**1**; 64 mg) and D (**4**; 23 mg).

The EtOAc extract (260 mg) was subjected to partitioning on a Sephadex LH-20 column (2×41.5 cm) eluting with a 3:1:1 mixture of hexane-toluene-MeOH at a flow rate of 0.5 ml/min to yield 124 fractions. Fractions were pooled based on tlc to afford ochrindoles A (**1**; 18 mg) and B (**2**; 4.4 mg). Ochrindole C [**3**] was obtained from a separate EtOAc extraction using a slightly different isolation procedure. In this case, the EtOAc extract obtained from 79 g ground sclerotia was subjected to Sephadex LH-20 fractionation (2×12.5 cm column) using CH_2Cl_2-hexane (1:1), followed by CH_2Cl_2-MeOH (1:1) as eluents. The first fraction to elute with CH_2Cl_2/MeOH was further processed by reversed-phase hplc to afford **3** (1.4 mg), as well as **1** and **4**. It is unlikely that any of these compounds are artifacts of the isolation process because all could be isolated under mild chromatographic conditions (LH-20/reversed-phase hplc), and because peaks corresponding to all four compounds could be observed by hplc in the EtOAc extract.

Ochrindole A [**1**].—Compound **1** exhibited: R_f 17.8 min [MeOH-H_2O (8:2)]; $[\alpha]_D$ +9.2° (*c*=0.0013 g/ml, MeOH); uv (MeOH) λ max 231 (ε 14000), 281 (ε 9700) nm; ir ν max (neat) 3400, 2930, 2850, 1460 cm^{-1}; eims *m/z* 452 ($[M]^+$, 100), 437 (14), 409 (53), 379 (46), 349 (28), 334 (17), 194 (17), 117 (15); ^1H and ^{13}C nmr, see Table 1; HMBC correlations ($CDCl_3$; H#=>C#) H_2-7=>C-4, 5, 6*, 8, 9; H-8=>C-5, 7, 10, 11; H_3-10=>C-8, 9; H_3-11=>C-8, 9; H_3-12=>C-1; H-13=>C-1, 2, 3; H_3-14=>C-4; H-1′=>C-3′, 9′; H-2′=>C-3′, 8′, 9′; H-4′=>C-3′, 6′, 8′; H-5′=>C-7′, 9′; H-6′=>C-4′, 8′; H-7′=>C-5′, 9′; H-1″=>C-2″, 3″, 9″; H-2″=>C-3″, 8″, 9″; H-4″=>C-3″, 6″, 8″; H-5″=>C-7″, 9″; H-6″=>C-4″, 8″; H-7″=>C-5″, 9″ (*indicates selective INEPT result); hreims found 452.2122, calcd for $C_{29}H_{28}O_3N_2$, 452.2100.

Ochrindole B [**2**].—Compound **2** exhibited: R_f 20.3 min [MeOH-H_2O (7:3)]; $[\alpha]_D$ = +10° (*c*=0.001 g/ml, MeOH); uv λ max (MeOH) 225 (ε 25000), 284 (ε 9400) nm; ir ν max (neat) 3380, 2930, 2860, 1460 cm^{-1}; eims *m/z* 468 ($[M]^+$, 4), 450 (100), 435 (7), 407 (23), 379 (6), 349 (9), 334 (5), 293 (5), 194 (17), 181 (27), 153 (33); ^1H and ^{13}C nmr, see Table 1; HMBC correlations ($CDCl_3$) H_2-7=>C-4, 5/8, 9; H-8=>C-10, 11; H_2-10=>C-8, 9, 11; H_3-11=>C-8, 9, 10; H_3-12=>C-1; H-13=>C-1, 2, 3; H_3-14=>C-4; H-2′=>C-3′, 8′, 9′; H-4′=>C-3′, 6′, 8′; H-5′=>C-7′, 9′; H-6′=>C-5′; H-7′=>C-9′; H-2″=>C-3″, 9″; H-4″=>C-5″, 8″, 9″; H-5″=>C-7″, 9″; H-6″=>C-5″, 8″; H-7″=>C-5″, 6″, 8″.

Ochrindole C [**3**].—Compound **3** exhibited: R_f 19.2 min [MeOH-H_2O (78:22)]; eims (50 eV) *m/z* 438 ($[M]^+$, 5), 395 (5), 380 (2), 351 (4), 281 (4), 207 (9), 117 (5); ^1H nmr ($CDCl_3$) δ 8.43 (1H, br s, H-1′), 8.39 (1H, br s, H-1″), 7.67 (1H, br d, *J*=7.7 Hz, H-4′), 7.49 (1H, d, *J*=2.2 Hz, H-2′), 7.46 (1H, br d, *J*=7.7 Hz, H-7′), 7.45 (2H, br d, *J*=8.5 Hz, H-4″, H-7″), 7.25 (3H, m, H-2″, H-6′, H-6″), 7.17 (1H, br dd, *J*=7.7 and 7.7 Hz, H-5′), 7.14 (1H, br dd, *J*=7.5 and 7.5 Hz, H-5″), 5.36 (1H, br s, OH-13), 5.04 (1H, br s, OH-12), 5.04 (1H, m, H-8), 3.35 (1H, br dd, *J*=15 and 6.8 Hz, H-7), 3.25 (3H, s, H_3-14), 3.10 (1H, br dd, *J*=14.6 and 6.8 Hz, H-7), 1.50 (3H, s, H_3-10), 1.20 ppm (3H, s, H_3-11); ^{13}C nmr ($CDCl_3$) δ 150.4 (C-4), 140.2 (C-1), 138.5 (C-2), 136.3 (C-8″), 136.2 (C-8′), 130.0 (C-9), 127.3 (C-9″), 126.6 (C-5*, C-9′), 125.0 (C-2′), 124.7 (C-8), 123.9 (C-2″), 122.8 (C-6″), 122.4 (C-6′), 121.1 (C-4′), 120.3 (C-5″), 120.2 (C-5′, C-4″), 119.6 (C-6*), 114.7 (C-3), 111.3 (C-7″), 111.2 (C-7′), 110.0 (C-3″), 108.0 (C-3′), 60.5 (C-14), 27.1 (C-7), 25.6 (C-10), 17.7 (C-11); HMBC correlations ($CDCl_3$) H_2-7=>C-4, 5*, 6*, 8, 9; H-8=>C-5*, 7, 10, 11; H_3-10=>C-8, 9, 11; H_3-11=>C-8, 9, 10; H-13=>C-2, 3; H_3-14=>C-4; H-1′=>C-3′, 9′; H-2′=>C-3′, 8′, 9′; H-4′=>C-3′, 6′, 8′, 9′; H-5′=>C-4′, 7′, 9′; H-6′=>C-4′, 5′, 7′, 8′; H-7′=>C-5′, 9′; H-1″=>C-3″; H-2″=>C-3″, 8″, 9″; H-4″=>C-3″, 6″, 8″, 9″; H-5″=>C-7″, 9″; H-6″=>C-7″; H-

7″=>C-5″, 6″, 8″, 9″; (*indicates that assignments for these carbon atoms may be interchanged. Nmr assignments for the indole subunits may also be interchanged in this case.).

Ochrindole D [**4**].—Compound **4** exhibited: *R*, 22.8 min [MeOH-H_2O (8:2)]; uv λ max (MeOH) 226 (ε 27000), 272 (ε 20000) nm; ir ν max (neat) 3310, 2920, 1660, 1440, 1070 cm^{-1}; eims *m/z* 424 ($[M+2H]^+$, 13) 422 ($[M]^+$, 46), 407 (100), 351 (7), 305 (5), 234 (5), 211 (13), 203 (17), 194 (20), 183 (19), 167 (16), 144 (23), 130 (39), 117 (21); ^1H and ^{13}C nmr, see Table 1; HMBC correlations (Me_2CO-d_6) H_2-7 =>C-4, 5*, 6*, 8, 9; H-8=>C-7, 10, 11; H_3-10=>C-8, 9, 11; H_3-11=>C-8, 9, 10; H-2′=>C-3′, 8′, 9′; H-4′=>C-3′, 6′, 8′; H-5′=>C-7′, 9′; H-6′=>C-4′, 8′; H-7′=>C-5′, 9′; H-2″=>C-6, 3″, 8″, 9″; H-4″=>C-3″, 6″, 8″, 9″; H-5″=>C-7″, 9″; H-6″=>C-4″, 8″; H-7″=>C-5″, 9″; (*indicates that assignments for these carbon atoms may be interchanged).

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