Journal of Natural Products
Vol. 49, No. 5, pp. 866-871, Sep-Oct 1986

MUTAGENIC PERYLENEQUINONE METABOLITES OF *ALTERNARIA ALTERNATA*: ALTERTOXINS I, II, AND III[1]

MICHAEL E. STACK, EUGENE P. MAZZOLA,* SAMUEL W. PAGE, ALBERT E. POHLAND,

Division of Chemistry and Physics, Food and Drug Administration, Washington, DC 20204

ROBERT J. HIGHET,

National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

MICHAEL S. TEMPESTA, and DAVID G. CORLEY

Department of Chemistry, University of Missouri, Columbia, Missouri 65211

ABSTRACT.—The mold genus *Alternaria* is a widely distributed plant pathogen. Some of these species, e.g., *A. alternata*, are common decay organisms of fruits and vegetables. Two novel perylene oxide metabolites, altertoxins II and III, have been identified in extracts of *A. alternata* isolates that exhibit mutagenic responses in the Ames *Salmonella typhimurium* assay. These identifications were based on mass, optical rotational, and ^{1}H- and ^{13}C-nmr spectral studies. Previous reports of related perylene dione mycotoxins have been clarified.

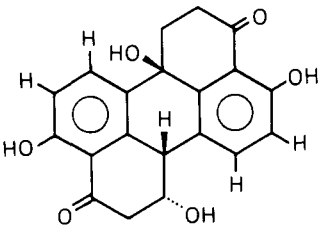
Species of *Alternaria* are widely distributed plant pathogens, and some species are common decay organisms of mature fruits and vegetables. These molds produce a large variety of toxic and nontoxic secondary metabolites (1-3). In a recent survey of moldy tomatoes collected from processing lines, almost half contained a major *Alternaria* metabolite, tenuazonic acid (4). This finding supports the concern of the possible contamination of food products by *Alternaria* mycotoxins. In Ames *Salmonella* assays of extracts of isolates and purified components of *Alternaria alternata*, the major portion of total mutagenic activity has been attributed to the altertoxins (5-7). Altertoxins I and II were first reported in 1972 (8). Chu (9) reported additional studies of these toxins, but a structure was not assigned to altertoxin I until 1982 (10). This assignment (**1**), which proved to be incorrect, was based on nmr spectroscopic studies. However, in 1983, Japanese researchers (11) reported studies of the pigments of an unidentified *Alternaria* species. They determined the structures of two of these compounds, which they designated as alterperylenol (**2**) and dihydroalterperylenol (**3**). The structure for **2** was based on X-ray crystallographic analysis, while the structure for **3** was assigned from nmr spectral comparisons with **2**. The physical and spectral data given for **3** are identical with those reported for altertoxin I, but no reference was given to the previous reports on the altertoxins. Shortly after the report on the "alterperylenols" appeared, Robeson and co-workers (12) reported a "novel" phytotoxin, altheichin (**2**), which had been isolated from *Alternaria eichorniae*. This structure was based on an X-ray crystallographic determination and, given that no absolute stereochemistry was implied, corresponds to that for alterperylenol. The work of Okuno *et al.* (11) was cited in a note added in proof; however, again no reference to the previous altertoxins work was given.

We report here our studies on the isolation and structure determination of altertoxins I (**3**) and II (**4**) and a new related metabolite, altertoxin III (**5**). A correlation with the earlier reports is presented to clarify some of the confusion in the literature regarding the perylenequinone derivatives.

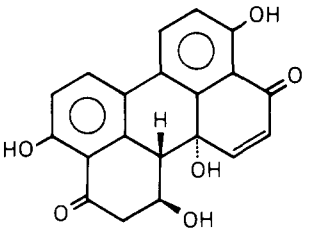
---

[1]Preliminary report: International Research Congress on Natural Products, University of North Carolina, Chapel Hill, NC, July 7-12, 1985.

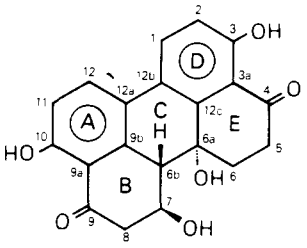
NOTE ADDED IN PROOF: The authors are indebted to Dr. G. Nasini for prior communication of his finding of a dihydroanthracenyl structure for a metabolite of *S. botryosum* that is similar to **5**.



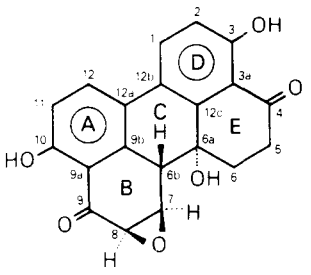
1



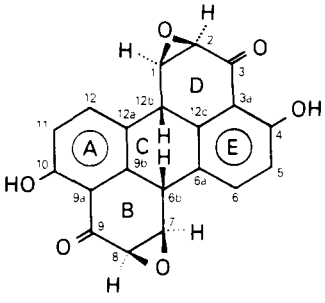
2



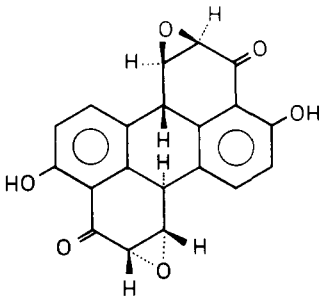
3



4



5



6

RESULTS AND DISCUSSION

Comparison of the ^{1}H-nmr spectra and carbon chemical shifts of **3** and **4** (Table 1) suggested a common carbon skeleton. Due to the similarity of its A/B- and D/E-ring systems, **3** possesses three pairs of protons (1/12, 2/11, and 3/10-OH) and eight pairs of carbon resonances (1/12, 2/11, 3/10, 3a/9a, 4/9, 5/6, 9b/12c, and 12a/b) whose assignments are interchangeable. In fact, at a field strength of 1.9 T (20 MHz, C-13), only 12 carbon signals are evident. This observation caused considerable delays in our original attempts at the structure elucidation of **3**. However, the selective INEPT (13) or INAPT (14) experiment permitted differentiation among all of these pairs of signals in DMSO-d_6. In general, protons whose assignments are known were employed to assign carbon resonances. Specifically, in an experiment designed to transfer magnetization via proton-carbon couplings of two bonds or more, irradiation of H-7 at 4.5 ppm allowed C-9b (three bonds distant) to be distinguished from C-12c (four bonds away). In a second experiment, designed to transfer magnetization via one-bond proton-carbon coupling, irradiation of the more-shielded 6-methylene proton (2.30 ppm) resulted in observation of C-6, thus differentiating it from C-5. The irradiated proton signal was, moreover, assigned to the axial position on the basis of its two large couplings (geminal and *trans*-vicinal). Irradiation of the proton resonance at 2.59 ppm resulted in observation in one experiment of C-5 and in another, of C-6a. Appearance of these two carbon signals identified the irradiated resonance as one of the 5-methylene protons. It was further determined to be H-5eq from the one large geminal coupling which it exhibits. Assignment of this proton signal also permitted C-3a to be distinguished from C-9a

TABLE 1. ^{1}H- and ^{13}C-nmr Data for Compounds **3**, **4**, and **5**

Position	Compounds							
	3[a]		**3**[b,c]		**4**[a]		**5**	
	H-1	C-13	H-1	C-13	H-1	C-13	H-1[a]	C-13[b]
1	7.87, d (8.8)[d]	132.7, d	8.1, d	132.9, d	7.91, d (8.8)[d]	133.0, d	4.60, d (3.4)	56.0, d
2	7.01, d (8.8)	119.5, d	7.1, d	117.8, d	7.12, d (8.8)[e]	119.9, d	3.86, dd (3.4, 1)	53.6, d
3	—	162.3[e]	—	161.0	—	163.3[f]	—	196.8
3a	—	116.9[f]	—	113.8	—	113.5[g]	—	112.3
4	—	205	—	206	—	204.1	—	159.6
5 eq	2.65, dt (15, 3)	34.0, t[g]	2.59, dt	33.5, t	2.83, ddd (17, 3, 2)	32.1, t[h]	6.90, d (8.6)	114.5, d
5 ax	3.17, m	—	ca. 3, m	—	3.26, ddd (17, 14, 4)	—	—	—
6 ax	2.43, dt (13, 3)	34.5, t[g]	2.30, dt	34.8, t	2.41, dt (14, 3)	33.3, t[h]	7.60, d (8.6)	132.1, d
6 eq	3.17, m	—	ca. 3, m	—	2.89, ddd (14, 4, 2)	—	—	—
6a	—	69.2	—	68.0	—	68.3	—	128.8
6b	3.09, d (9)	51.9, d	2.86, m	51.4, d	3.55, d (0.5)	45.1, d	4.2, br s	37.5, d
7	4.78, ddd (12, 9, 5)	66.1, d	4.50, d	64.7, d	4.23, d (3)	55.7, d	4.60, d (3.4)	56.0, d
8 ax	2.94, dd (16, 12)	47.7, t	ca. 3, m	47.5, t	3.71, dd (3, 0.5)	52.8, d	3.86, dd (3.4, 1)	53.6, d
8 eq	3.07, dd (16, 5)	—	2.86, m	—	—	—	—	—
9	—	202	—	204.2	—	196.6	—	196.8
9a	—	117.4[f]	—	116.5	—	114.6[g]	—	112.3
9b	—	139.1	—	140.7	—	138.8	—	143.0
10	—	162.0[e]	—	160.4	—	162.6[f]	—	159.6
11	6.92, d (8.8)	117.5, d	6.9, d	115.5, d	7.07, d (8.8)[e]	118.0, d	6.90, d (8.6)	114.5, d
12	7.82, d (8.8)[d]	132.4, d	8.0, d	132.5, d	7.86, d (8.8)[d]	132.6, d	7.60, d (8.6)	132.1, d
12a	—	124.1	—	124.8	—	124.0	—	128.8
12b	—	122.7	—	123.5	—	122.4	4.2, br s	37.5, d
12c	—	135.5	—	138.4	—	133.5	—	143.0
3-OH	12.7	—	12.7	—	12.7	—	11.5	—
10-OH	12.4	—	12.3	—	12.1	—	11.5	—

[a]In $CDCl_3$.
[b]In DMSO-d_6.
[c]Assignments made by INAPT experiments.
[d-h]Assignments in vertical columns may be interchanged.

and C-4 from C-9. Irradiation at 2.86 ppm resulted in observation, via one-bond couplings, of C-6b and C-8, indicating coincident proton chemical shifts. Experiments exploiting long-range C-H couplings, moreover, allowed C-12a (three bonds distant from H-6b) to be differentiated from C-12b.

When the strongly hydrogen-bonded resonance at 12.7 ppm was irradiated, C-3a again appeared together with signals for C-2 and C-3, which were thus distinguished from C-11 and C-10, respectively. This experiment also established the chemical shift of 3-OH as 12.7 ppm, differentiating it from 10-OH. Conversely, irradiation of 10-OH (12.3 ppm) confirmed the previous assignments by producing resonances for C-9a, C-10, and C-11. Irradiation of the proton signal at 6.9 ppm again resulted in observation of C-11, thereby establishing the former resonance as that of H-11 and distinguishing it from H-2. Furthermore, selective homonuclear decoupling of this proton signal collapsed the doublet at 8.0 ppm, thus establishing the latter resonance as H-12 and differentiating it from H-1. Lastly, irradiation of H-12 resulted in observation of C-12, hence distinguishing it from C-1.

Compound **4** is two mass units less than **3**, but unlike **2**, lacked olefinic proton nmr signals. The presence of methine carbons at 52.8 and 55.7 ppm, which exhibit 185-Hz one-bond proton couplings, and absence of both the 8-methylene and 7-methine carbons, indicated that in **4** an epoxide group had replaced the secondary 7-β-OH function of **3**. The absence of vicinal coupling between H-6b and H-7 suggests that the dihedral angle between these protons is approximately 90°and, therefore, requires a similar β-configuration for the epoxide group in **4**.

The situation of interchangeable proton and carbon chemical shift assignments encountered with **3** is also found in **4**. However, INAPT experiments could not be employed in this case due to both poor solubility and stability of **4** in a variety of solvents. Both the 7-proton and 7-carbon of the epoxide group resemble the β-substituents of an α,β-unsaturated carbonyl systems, being more deshielded than their 8-counterparts. Similarly, allylic coupling is observed between H-6b and H-8, whereas vicinal coupling between H-6b and H-7 is not. Both dihedral angles are thus concluded to be approaching 90°.

Comparison of the ^{1}H-nmr spectra and carbon chemical shift data of **4** and **5** (Table 1) likewise revealed considerable, although deceptive, similarity (see below). Compound **5** is two mass units less than **4**, suggesting that an epoxide group had again replaced an alcohol function. The appearance of methine carbons at 53.6 and 56 ppm, which exhibit 185-Hz one-bond proton couplings, once more confirmed the presence of epoxide functionalities. As in **4**, these groups are assigned a β-configuration on the basis of the very small vicinal coupling between H-1 and H-12b (also H-7 and H-6b).

However, **5** represents a considerable departure from known perylenequinone compounds (10-12, 15, 16) in two respects: (a) it possesses an element of symmetry as evidenced by its ^{1}H-nmr and ^{13}C-nmr spectra, which exhibit signals corresponding to half of the nuclei present, and (b) its two aromatic rings are part of an unconjugated dihydroanthracenyl, rather than a biphenyl, system. Several lines of evidence support the latter conclusion. First, reduction of **5** with $NaBH_4$ provided a product whose uv spectrum resembles that of a phenol anion [234 (14,000) and 287 nm (5,200) compared with 235 (9,300) and 287 nm (2,600), respectively] while the corresponding reduction product of **3** exhibits more extensive conjugation, 214 (31,000) and 299 nm (24,000). Second, irradiation of epoxide protons-1(7) resulted in an nOe at the aromatic protons-6(12). The reverse experiment, in which H-6(12) was irradiated, likewise produced enhancement in the integrated intensity of H-1(7).

Compound **5** is readily seen (see above) to be a symmetrical molecule. Moreover, exhibition of optical rotation and observation of a cd spectrum require the presence of a

C_2-axis (perpendicular to the plane of the molecule through the middle of the C-ring) and structure **5** rather than a center of symmetry. The latter would, like a mirror plane, give rise to the *meso*-molecule (**6**).

Altertoxins I, II, and III were shown to be mutagenic using the Ames test (6). Compound **5** with two epoxide groups is 1.5 times more potent a mutagen than **4**, with one epoxide group, and 23 times more potent than **3**, which has no epoxide groups. The analogies with the arene oxide mechanisms in the metabolic activation in carcinogenesis of a polynuclear aromatic system are obvious (17).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Kofler apparatus and are uncorrected. Uv spectra were recorded on a Beckman DU-7 spectrophotometer and ir spectra on a Digilab-FTS-10X instrument. Optical rotation measurements were made on a Perkin-Elmer 241 MC polarimeter, and cd spectra were recorded on a JASCO J-500A spectropolarimeter. Mass spectra were obtained on a VG-ZAB 2F mass spectrometer at 70 eV. ^{1}H- and ^{13}C-nmr spectra were determined at 360 and 90 MHz, respectively, in $CDCl_3$ on a Nicolet NT-360 spectrometer and obtained at 300 and 75 MHz, respectively, in DMSO-d_6 on a Nicolet NT-300 WB spectrometer. Chemical shifts were referenced to internal TMS (0.0 ppm) for ^{1}H spectra and the appropriate deuterated solvent (77.0 ppm, $CDCl_3$ or 39.5 ppm, DMSO-d_6) for ^{13}C spectra. The nOe, obtained as nOe-difference spectra, were determined by subtracting off-resonance free induction decays (fids) from on-resonance fids on a Varian Associates XL-200 spectrometer at 200 MHz. Selective INEPT experiments employed the pulse sequence of Bax (13). A decoupler field strength ($\gamma H_2 = 25$ Hz) was used to generate a selective 90° proton pulse = 10 msec. The polarization transfer delays, Δ_1 and Δ_2, were optimized for 125- and 6-Hz proton-carbon couplings in experiments designed to enhance one-bond and long-range couplings, respectively. Between 6,000 and 40,000 16K transients were signal-averaged in double-precision acquisition mode and processed in floating-point mode with standard Nicolet software.

A. alternata, isolated from cherries and designated strain No. 42, is deposited with the Division of Microbiology, Food and Drug Administration, Washington, DC.

CULTURE CONDITIONS.—Rice (250 g) and 250 ml H_2O were autoclaved in 2,800-ml Fernbach flasks. They were then inoculated with a fresh culture of *A. alternata* and incubated for 20 days at 22°.

ISOLATION PROCEDURE.—The moldy rice was broken up with a spatula, blended with 1 liter $CHCl_3$, and filtered. The filter cake was reblended with an additional 750 ml $CHCl_3$ and filtered. The combined filtrates from ten Fernbach flask cultures were concentrated to 400 ml using a rotary evaporator.

PURIFICATION.—The concentrated extracts were added to a 150-g silica gel column (E. Merck silica gel 60, 0.06-0.2 mm) and eluted with 3 liters CH_2Cl_2 to obtain **5**, then 3 liters $CHCl_3$ for **4**, and finally with 3 liters $CHCl_3$-MeOH (98:2) to obtain **3**. Fractions were collected and analyzed for **3**, **4**, and **5** by hplc (Waters 6000A pump, Waters 216K injector, Waters 5-μm silica gel column, Waters Model 450 absorbance detector set at 355 nm, Hewlett-Packard 7130A recorder; mobile phase $CHCl_3$-HOAc-MeOH (98:1:1), flow rate 1 ml/min; elution times: **5**, 1.4 min; **4**, 3.0 min; and **3**, 8.5 min with CH_2Cl_2. Fractions containing mixtures were rechromatographed on smaller columns. Repeated chromatography and crystallization from $CHCl_3$/hexane yielded 150 mg **3**, 317 mg **4**, and 451 mg **5**.

$NaBH_4$ REDUCTIONS.—Compound **3**, 1.34 mg, was dissolved in 10 ml MeOH and treated repeatedly with milligram quantities of $NaBH_4$, uv spectrum: 299 (24,000) and 214 (31,000) nm.

Compound **5**, 0.940 mg, was dissolved in 50 ml MeOH, treated repeatedly with $NaBH_4$, and allowed to stand overnight, uv spectrum: 287 (5,200) and 234 (14,000) nm.

Altertoxin I (**3**).—Mp > 180° (dec.); $[\alpha]_D$ +484° (*c* 0.002 in $CHCl_3$); cd (*c* 2×10^{-4} in $CHCl_3$) +391, −351, +313 nm ($\Delta\epsilon$ +19.7, −8.8, +7.3); ir ν max (KBr) 3450, 1641, 1598, 1489, 1465, 1368, 1337, 1234, 1170, 1062, 951, 829 cm^{-1}; uv λ max (MeOH) 215 (25,500), 256 (34,600), 285 (16,300), 296sh (13,300), 356 nm (6,000); ms *m/z* 352.0948 (M^+, calcd for $C_{20}H_{16}O_6$, 352.0946), 334 (M-H_2O), 316 (M-$2H_2O$), 314 (M-$2H_2O$-2H), 291 (M-H_2O-C_2H_3O), 290 (M-$C_2H_6O_2$), 275 (M-$2H_2O$-CHO).

Altertoxin II (**4**).—Mp 245-250°; $[\alpha]_D$ +636° (*c* 0.001 in $CHCl_3$); cd (*c* 10^{-4} in $CHCl_3$) 395, 327 nm ($\Delta\epsilon$ +21, 8.8); ir ν max (KBr) 3462, 1643, 1598, 1485, 1384, 1342, 1321, 1296sh, 1223, 1184, 1138, 1103, 1095, 1061, 1041, 952, 833, 774 cm^{-1}; uv λ max (MeOH) 215 (27,000), 258 (31,700), 286sh (17,000), 297sh (13,500), 358 nm (5,300); ms *m/z* 350.0784 (M^+, calcd for $C_{20}H_{14}O_6$, 350.0790), 332 (M-H_2O), 321 (M-CHO), 305 (M-CHO_2), 291 (M-2CHO-H).

Altertoxin III (**5**).—Mp 175-230° (subl.); $[\alpha]_D$ +845° (*c* 4×10^{-4} in $CHCl_3$); cd (*c* 4×10^{-5} in $CHCl_3$) 344 nm ($\Delta\epsilon$ +39.2); ir ν max (KBr) 3452, 1650, 1620, 1473, 1440, 1382, 1342, 1249, 1234, 1198, 1045, 1003, 806, 746, 715 cm^{-1}; uv λ max (MeOH) 210 (19,500), 265 (14,500), 352 nm (5,200); ms *m/z* 348.0633 (M^+, calcd for $C_{20}H_{12}O_6$, 348.0634), 346 (M-2H), 331 (M-OH), 329 (M-H_2O-H), 319 (M-CHO), 314 (M-2OH), 291 (M-CHO-CO), 263, 250.

ACKNOWLEDGMENTS

We thank J.T. Chen (FDA) for the ir, Herman Ziffer (NIH) for the cd spectral analyses, and D. Andrzejewski (FDA) for the ms data. MST is grateful for partial financial support from the Petroleum Research Fund (16124-G1) and to the National Science Foundation for partial support of the nmr (PCM-8115599) facility at the University of Missouri.

LITERATURE CITED

1. E.E. Stinson, *J. Food Prot.*, **48**, 80 (1985).
2. A.D. King and J.E. Schade, *J. Food Prot.*, **47**, 886 (1984).
3. J.E. Schade and A.D. King, *J. Food Prot.*, **47**, 978 (1984).
4. J. Harwig, P.M. Scott, D.R. Stoltz, and B.J. Blanchfield, *Appl. Environ. Microbiol.*, **38**, 267 (1979).
5. P.M. Scott and D.R. Stoltz, *Mutat. Res.*, **78**, 33 (1980).
6. M.E. Stack, M.S. Thesis, American University, Washington, DC, 1984.
7. M.E. Stack, P.B. Mislivec, J.A.G. Roach, and A.E. Pohland, *J. Assoc. Off. Anal. Chem.*, **68**, 640 (1985).
8. R.W. Pero, H. Posner, M. Blois, D. Harvan, and J.W. Spalding, *Environ. Health Perspect.*, **4**, 87 (1973).
9. F.S. Chu, *J. Am. Oil Chem. Soc.*, **58**, 1006A (1981).
10. E.E. Stinson, S.F. Osman, and P.E. Pfeffer, *J. Org. Chem.*, **47**, 4110 (1982).
11. T. Okuno, I. Natsume, K. Sawai, K. Sawamura, A. Furusaki, and T. Matsumoto, *Tetrahedron Lett.*, **24**, 5653 (1983).
12. D. Robeson, G. Strobel, G.K. Matsumoto, E.L. Fisher, M.H. Chen, and J. Clardy, *Experientia*, **40**, 1248 (1984).
13. A. Bax, *J. Magn. Reson.*, **57**, 314 (1984).
14. A. Bax, J.A. Ferretti, N. Nashed, and D.M. Jerina, *J. Org. Chem.*, **50**, 3029 (1985).
15. R.J. Lousberg, G.A. Salemink, U. Weiss, and T.J. Batterham, *J. Chem. Soc. C*, 1219 (1969).
16. L. Merlini and G. Nasini, in: "Chemistry and Biotechnology of Biologically Active Natural Products." Ed. by C. Szantay, Akademiai Kiado, Budapest, 1984, p. 121.
17. A. Dipple, R.C. Moschel, and C.A.H. Bigger, in: "Chemical Carcinogens." Ed. by C.E. Searle, 2nd ed., vol. 1, American Chemical Society, Washington, DC, 1984.

Received 13 March 1986