Journal of Chemical Ecology, Vol. 18, No. 10, 1992

SOIL TRANSFORMATION OF 2(3H)-BENZOXAZOLONE OF RYE INTO PHYTOTOXIC 2-AMINO-3H-PHENOXAZIN-3-ONE

RONALD W. GAGLIARDO and WILLIAM SCOTT CHILTON*

Department of Botany, Box 7612
North Carolina State University
Raleigh, North Carolina 27695-7612

(Received March 19, 1992; accepted May 18, 1992)

Abstract—Nonsterile soil transforms the rye metabolite 2(3H)-benzoxazolone (BOA) into 2-amino-3H-phenoxazin-3-one, which is an order of magnitude more toxic to barnyard grass than benzoxazolone. Benzoxazolone was recovered unchanged from sterile soil. However, *o*-aminophenol is converted to aminophenoxazinone by both sterile and nonsterile soil in the presence of air. Aminophenoxazinone is probably produced by microbial hydrolysis of benzoxazolone into *o*-aminophenol, which is oxidized to aminophenoxazinone in both sterile and nonsterile soil. No 2,2′-oxo-1,1′-azobenzene was found in any incubations of soil with benzoxazolone, *o*-aminophenol, or *o*-azophenol.

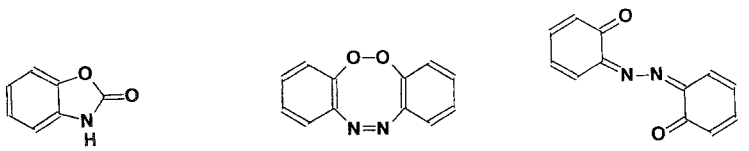
Key Words—2-Amino-3H-phenoxazin-3-one, 2(3H)-benzoxazolone, BOA, *o*-aminophenol, rye, *Secale cereale*, soil transformation, phytotoxicity, allelopathy, barnyard grass, *Echinocloa crus-galli*.

INTRODUCTION

The use of allelopathic mulches in reduced or no-till cropping systems has received attention in recent years. The practical and chemical aspects of using winter rye, *Secale cereale* L., mulch are perhaps the best studied (Worsham, 1989). Phytotoxic activity of rye mulch has been attributed largely to the cyclic hydroxamic acid 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and its decomposition product 2(3H)-benzoxazolone (BOA) (**1**, Figure 1) (Barnes et al., 1987). A crystalline, red transformation product of BOA has recently been isolated from Michigan soil, and the structure 2,2′-oxo-1,1′-azobenzene (**2**) has

*To whom correspondence should be addressed.

0098-0331/92/1000-1683$06.50/0



1 2 3

FIG. 1. Structures of 2(3H)-benzoxazolone **(1)**, 2,2′-oxo-1,1′-azobenzene **(2)**, and *o*-benzoquinone azine **(3)**.

been proposed for this pigment (Nair et al., 1990). This red transformation product proved more phytotoxic than BOA in vitro (Chase et al., 1991a) and is therefore a further possible allelochemical of rye mulch. The soil transformation of BOA was later determined to be mediated by the microorganism *Acinetobacter calcoaceticus* (Chase et al., 1991b).

Soil microorganisms are known to transform substituted anilines into azo compounds (Bartha and Pramer, 1972). However, microbial formation of an oxygen–oxygen bond between phenols is unprecedented. In fact, chemical attempts to generate phenolic peroxides by dimerization of ortho-, para-blocked phenolic radicals does not lead to stable phenolic peroxides (Pummerer et al., 1952), and yet the red soil transformation product appeared to be stable and to have a very high melting point with no decomposition reported. We have considered possible alternative structural interpretations of the data used to arrive at the 2,2′-oxo-1,1′-azobenzene structure. The published [^{1}H]- and [^{13}C]NMR data indicated an element of symmetry in the pigment leading to six carbon and four hydrogen signals for $C_{12}H_8N_2O_2$. All isomeric structures with the required symmetry can be rejected based on published melting point and spectral data except one, *o*-benzoquinone azine **(3)** reported to be the product obtained by silver(II) oxide oxidation of *o*-aminophenol (Ortiz et al., 1972). The ultraviolet–visible spectrum reported for this red oxidation product is the same as that reported for 2,2′-oxo-1,1′-azobenzene, and its high melting point is similar to that reported for the soil transformation pigment. It appeared that the red soil transformation product and the product of silver(II) oxide oxidation of *o*-aminophenol might be identical. Therefore we reinvestigated the properties of these red pigments.

METHODS AND MATERIALS

Soil Sample. Cecil and Appling gravelly sandy loam (Typic Kanhaludult), was collected from a fallow corn field at the North Carolina State University research farm at the Dorothea Dix property (plot number 163/H-2). Soil was stored in a sealed plastic bag at 4°C until needed.

Soil Transformation of BOA. A 2-g sample of BOA **(1)** was mixed into 400 g soil and incubated in the dark at 28°C. After 10 days, the soil was extracted twice with 400 ml methanol. The extract was concentrated to dryness (1.2 g), and the residue was redissolved in a small amount of ethyl acetate. After two days, crystals were collected from this dark red solution, which was allowed to stand for further crystallization. A final yield of 311 mg of red crystals was obtained.

Effect of Sterile and Septic Soil. To test whether soil microorganisms are required for transformation of BOA into the red pigment, sterile and nonsterile soil was supplemented with BOA and two potential intermediates, *o*-aminophenol and *o*-azophenol. A 50-g portion of soil was mixed with 50 mg of the test compound and 5 ml deionized water. The mixture was transferred to an Erlenmeyer flask, stoppered with a foam plug, and incubated in the dark at 25–28°C. In cases where sterile soil was used, the test compound was added aseptically to double-autoclaved soil. After 10 days the soil was extracted by stirring twice with 100 ml of methanol. The extracts were concentrated under vacuum and compared to the silver oxide oxidation product by thin-layer chromatography (TLC) in two solvent systems: toluene–ethyl acetate (50:40) and chloroform–methanol (95:5).

Silver(II) Oxide Oxidation of o- *Aminophenol.* Silver(II) oxide, AgO, was prepared according to the method of Hammer and Kleinberg (1953). The silver(II) oxide oxidation of *o*-aminophenol was carried out according to the method of Ortiz et al. (1972). A mixture of 6 g *o*-aminophenol and 31 g freshly prepared silver(II) oxide was stirred in benzene for 4 h. Insoluble silver oxides were removed by vacuum filtration. The dark red pigment in the filtrate was purified by flash column chromatography (chloroform–methanol 80:20). Preparative TLC (chloroform–methanol 9:1) yielded a dark red product (R_f 0.65 in chloroform–methanol 95:5), which was triturated with water to remove traces of *o*-aminophenol. The red solid was dissolved in the minimal amount of dimethylsulfoxide. Slow evaporation of some of the solvent from an open beaker produced fine, dark red crystals which were homogeneous by TLC in three solvent systems: chloroform–methanol 95:5 (R_f 0.6), hexane–acetone 70:30 (R_f 0.7), and toluene–ethyl acetate 50:40 (R_f 0.5). The dark red product diluted for TLC appeared as a bright orange spot, which was the only spot detected visually, by fluorescence quenching or by treatment with phosphomolybdic acid (Peña-Rodriguez et al., 1988).

Synthesis of Aminophenoxazinone. 2-Amino-3H-phenoxazin-3-one **(4)** was synthesized by the air oxidation of *o*-aminophenol as described previously (Kehrmann, 1906). In this procedure, 4 g of *o*-aminophenol were stirred in aqueous methanol at room temperature for three weeks. Production of the dark red aminophenoxazinone was monitored by TLC (chloroform–methanol 95:5). Solids that precipitated from the solution were collected and recrystallized from meth-

anol to give clusters of reddish black needlelike crystals. The aminophenoxazinone was further purified by washing with hot water and recrystallizing from methanol. The crystallized product was analyzed by TLC, UV, NMR, and MS. The yield of aminophenoxazinone after three weeks was 1.1 g. There was still some unoxidized *o*-aminophenol in the original methanolic solution after three weeks.

Spectroscopy. Ultraviolet spectra were measured on a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer. [^{1}H]NMR was measured in DMSO-d_6 at 300 and 500 MHz on a General Electric Omega spectrometer. Proton-coupled and proton-decoupled [^{13}C]NMR spectra were measured at 125 MHz. Low-resolution mass spectral analysis was performed using a Hewlet-Packard 5985-B spectrometer.

Bioassay. Phytotoxicity was tested by a radicle elongation assay. A 1 mg/ml stock solution of the test compound was prepared in methanol. Volumes corresponding to 12.5, 25, 50, 100, 175, and 250 mg of test compound were applied evenly to 6-cm filter paper circles, and the solvent was allowed to evaporate completely. Filter paper wet with methanol and dried was used as a control. The dried paper impregnated with test compound was wet with 1.5 ml water in a 60 × 15-mm Petri dish, and 10 seeds of barnyardgrass (*Echinocloa crus-galli* L.) were added. After incubation in the dark for 72 h, the radicle lengths (mm) were measured and expressed as a percentage of the control radicle length.

RESULTS

Transformation of BOA and Other Compounds by Soil. BOA incubated with moist, nonsterile soil at 28°C was converted within 10 days into a dark red substance extractable by methanol. TLC showed the presence of a major orange pigment (red on concentration), higher R_f, very weak yellow and brown pigments, and a very small amount of unreacted, colorless BOA, detected by ultraviolet fluorescence quenching and by development with phosphomolybdic acid. Soil that had not been treated with BOA contained no extractable pigments.

The red pigment, recrystallized from methanol, had mp 252–258°C; UV maxima in methanol at 236 (31,000) and 432 nm (26,000); EIMS *m/z* 212 (100%, M^+), 185 (58%), 184 (28%); [^{1}H]NMR in DMSO-d_6 at 300 MHz: 6.34 ppm (s, 1H), 6.35 (s, 1H), 6.81 (broad, NH_2), 7.48 (m, 3H), 7.71 (dd, J = 1, 8 Hz, 1H). The singlets at 6.34 and 6.35 ppm were not resolved in pure DMSO but did resolve after addition of a drop of D_2O. The three-proton multiplet at 7.48 ppm was resolved at 500 MHz: 7.38 (dt, J = 1, 8 Hz,), 7.45 (dt, J = 1, 8 Hz,), and 7.48, (dd, J = 1, 8 Hz). Carbon resonances were assigned in the [^{13}C]NMR spectrum (Table 1) consistent with heteronuclear multiple bond

correlation spectra, multiplicities observed in [^{1}H]- and [^{13}C]NMR spectra, and model compounds.

Ten days after addition of BOA to doubly autoclaved soil, unreacted BOA was detectable in the methanolic extract, but no pigment was present. The concentration of BOA in sterile soil was undiminished judging from TLC spot intensity. On the other hand, TLC analysis showed that *o*-aminophenol was completely converted to the red pigment and no other product within 10 days in both sterile and non-sterile soil. *o*-Azophenol, a conceivable precursor to 2,2′-oxo-1,1′-azobenzene, or *o*-benzoquinone azine, was unaltered after 10 days of incubation in nonsterile soil.

Red Pigment Produced by Silver(II) Oxidation. The pigment produced by silver(II) oxide oxidation of *o*-aminophenol was purified by column chromatography and preparative TLC, followed by crystallization from dimethylsulfoxide. The red crystals had mp 268°C; UV maxima in methanol at 236 (23,000) and 432 nm (20,000); EIMS *m/z* 212 (100%, M^+), 185 (50%), 184 (21%); [^{1}H]NMR in DMSO-d_6 at 300 MHz: 6.42 ppm (s, 1H), 6.44 (s, 1H), 6.87 (broad, NH_2), 7.54 (m, 3H), 7.78 (dd, J = 1, 8 Hz, 1H); [^{13}C]NMR as in Table 1.

Comparison of Pigments to Aminophenoxazinone. 2-Amino-3H-phenoxazin-3-one **(4)** (Scheme 1) was prepared by air oxidation of *o*-aminophenol (Kehrmann, 1906) and recrystallized twice from methanol, mp 253–267°C (lit. mp 255–257°C). The red crystals had visible–ultraviolet maxima in methanol at 237 nm (22,000), 432 (19,600); EIMS *m/z* 212 (100%, M^+), 185 (53%),

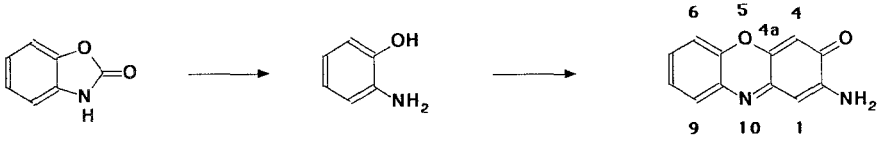
TABLE 1. [^{13}C] NMR SPECTRUM OF SOIL TRANSFORMATION PRODUCTS FROM BENZOXAZOLINONE

C, H#	Aminophenoxazinone from soil transformation	2,2′-Oxo-1,1′-azobenzene (Nair et al., 1990)
1	103.43, doublet[a]	104.86, doublet[b]
2	147.38, singlet	
3	180.24, singlet	
4	98.34, doublet	99.87, doublet[b]
4a	148.88, singlet	
5a	141.91, singlet	
6	115.93, doublet	117.25, doublet[b]
7	128.81, doublet	130.34, doublet[b]
8	125.28, doublet	126.60, singlet[b]
9	127.96, doublet	129.29, singlet[b]
9a	133.73, singlet	
10a	148.23, singlet	

[a] Proton decoupled [^{13}C]NMR spectra were measured in DMSO-d_6 at 125 MHz.
[b] Spectrum measured in DMSO-d_6; No C# assignments intended.

184 (22%). The [^{1}H]- and [^{13}C]NMR agreed with spectra previously reported for 2-amino-3H-phenoxazin-3-one (Hasegawa and Ueno, 1985). The red pigments from soil transformation of BOA and from silver(II) oxidation of *o*-aminophenol were indistinguishable from 2-amino-3H-phenoxazin-3-one by UV, EIMS, and [^{1}H]- and [^{13}C]NMR. The red pigments prepared by soil transformation and by Ag(II) oxide oxidation also comigrated with aminophenoxazinone on silica gel TLC at R_f 0.5 in toluene–ethyl acetate, 50:40 and at R_f 0.6 in chloroform–methanol (95:5).

Phytotoxicity of Aminophenoxazinone. The radicle elongation assay on barnyard grass showed that aminophenoxazinone was more phytotoxic than its precursor BOA (Figure 2). Root elongation was inhibited 50% by 0.1 mM aminophenoxazinone, 0.7 mM BOA, and 1.2 mM *o*-aminophenol, the probable hydrolytic intermediate between BOA and aminophenoxazinone.



4

SCHEME 1. Soil transformation of benzoxazolone into 2-amino-3H-phenoxazin-3-one (4).

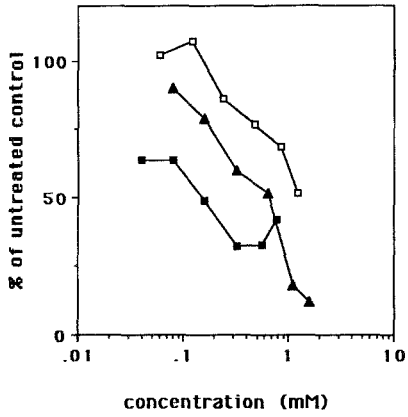


FIG. 2. Effects of aminophenoxazinone (—■—), *o*-aminophenol (—▲—), and benzoxazolone (—□—) on radicle elongation of barnyard grass.

DISCUSSION

In an earlier study silver(II) oxide was found to oxidize 7 substituted anilines to azoarenes (Ortiz et al., 1972). Based on this analogy and elemental composition, but no NMR data, the structure *o*-benzoquinone azine **(3)** was proposed for the red crystals produced by oxidation of *o*-aminophenol with silver(II) oxide. We synthesized and characterized this compound by NMR and other spectroscopic techniques because this product has a similar high melting point, the identical elemental composition and ultraviolet spectrum, and the symmetry of the reported soil transformation product of 2(3H)-benzoxazolone (BOA) **(1)** previously assigned the structure 2,2′-oxo-1,1′-azobenzene **(2)** (Nair et al., 1990). We found that the product of silver(II) oxidation of *o*-aminophenol is in fact not *o*-benzoquinone azine, but rather 2-amino-3H-phenoxazin-3-one **(4),** from which it is indistinguishable by thin-layer chromatography, mass spectral fragmentation, [^{1}H]- and [^{13}C]NMR, and UV spectra. Aminophenoxazinone and its derivatives are the only known products of oxidation of *o*-aminophenol by air (Kehrmann, 1906), photoirradiation (Ikekawa et al., 1968), inorganic oxidants (Ikekawa et al., 1968; Hishida et al., 1974), mammalian tissue (Tomoda, 1986), and purified enzymes (Nagasawa, 1959; Barry et al., 1989). Aminophenoxazinone, also known as the antibiotic questiomycin A from *Streptomyces thioluteus* (Gerber, 1967), is produced by a number of fungi (Turner and Aldridge, 1983) and bacteria (Gerber and Lechevalier, 1964).

We have also identified the red pigment produced from BOA by nonsterile soil as 2-amino-3H-phenoxazin-3-one. We note the identity of the ultraviolet and mass spectra and near identity of the proton and [^{13}C]NMR spectra of aminophenoxazinone obtained from transformation of BOA by North Carolina soil to that of the red pigment obtained by transformation with Michigan soil. Aminophenoxazinone contains all of the ^{13}C signals reported for the Michigan transformation product plus six low-intensity quaternary carbon signals not previously reported (Table 1). The [^{1}H]NMR of aminophenoxazinone contains all of the signals reported for 2,2′-oxo-1,1′-azobenzene **(2),** with the same chemical shifts and multiplicities, but, in addition, contains two singlets (6.34 and 6.35 ppm) and a broad singlet due to an NH_2 (6.81 ppm) not reported for the Michigan soil transformation product.

Although nonsterile soil converts the allelochemical BOA into the phytotoxic pigment aminophenoxazinone, sterile soil does not. The probable route of transformation of BOA is its hydrolysis to *o*-aminophenol, followed by oxidation to aminophenoxazinone (Scheme 1). Since hydrolysis of BOA in the laboratory requires prolonged treatment with aqueous alkali or acid at 100°C or higher (Graebe and Rostovzeff, 1902) and does not occur in sterile soil, the hydrolysis of BOA to *o*-aminophenol must be a step requiring soil microorganisms. Sterile soil in contact with air is capable of accomplishing the subsequent oxidation of

o-aminophenol into aminophenoxazinone. Some microorganisms are also known to oxidize *o*-aminophenol to aminophenoxazinone, but their presence has not been demonstrated in soil samples capable of converting BOA or *o*-aminophenol into aminophenoxazinone. The present study does not exclude the possibility that microorganisms contribute to the rate of oxidation of aminophenol to aminophenoxazinone. The transformation product, aminophenoxazinone, is an order of magnitude more phytotoxic than BOA. It is significant that this compound has recently been identified as the phytotoxin of the plant pathogen *Acrospermum viticola* (Kinjo et al., 1987). Thus, the microbially produced aminophenoxazinone has the potential for increasing the allelopathic effect of rye mulch.

Acknowledgments—This study was supported in part by U.S. Department of Agriculture grant 91-37303-6433.

REFERENCES

BARNES, J.P., PUTNAM, A.R., BURKE, B.A., and AASEN, A.J. 1987. Isolation and characterization of allelochemicals in rye herbage. *Phytochemistry* 26:1385–1390.

BARRY, C.E., NAYAR, P.G., and BEGLEY, T.P. 1989. Phenoxazine synthase: mechanism for the formation of the phenoxazinone chromophore of actinomycin. *Biochemistry* 28:6323–6333.

BARTHA, R., and PRAMER, D. 1972. Biochemical transformation of herbicide-derived anilines: Requirements of molecular configuration. *Can. J. Microbiol.* 161:1617–1622.

CHASE, W.R., NAIR, M.G., and PUTNAM, A.R. 1991a. 2,2′-Oxo-1,1′-azobenzene: Selective toxicity of rye (*Secale cereale* L.) allelochemicals to weed and crop species: II. *J. Chem. Ecol.* 17:9–19.

CHASE, W.R., NAIR, M.G., PUTNAM, A.R., and MISHRA, S.K. 1991b. 2,2′-Oxo-1,1′-azobenzene: Microbial transformation of rye (*Secale cereale* L.) allelochemical in field soils by *Acinetobacter calcoaceticus*: III. *J. Chem. Ecol.* 17:1575–1584.

GERBER, N.N. 1967. Phenazines, phenoxazinones, and dioxopiperazines from *Streptomyces thioluteus*. *J. Org. Chem.* 32:4055–4057.

GERBER, N.N., and LECHEVALIER, M.P. 1964. Phenazines and phenoxazines from *Waksmania aerata* sp. *nov.* and *Pseudomonas iodina*. *Biochemistry* 3:598–602.

GRAEBE, C., and ROSTOVZEFF, S. 1902. Ueber die Hofmann'sche Reaction. *Chem. Ber.* 35:2747–2752.

HASEGAWA, K., and UENO, Y. 1985. The carbon-13 NMR spectra and electronic structure of 3H-phenoxazin-3-ones. *Bull. Chem. Soc. Jpn.* 58:2832–2839.

HAMMER, R.N., and KLEINBERG, J. 1953. Silver(II) oxide. *Inorg. Synth.* 4:12–13.

HISHIDA, T., NOGAMI, T., SHIROTA, Y., and MIKAWA, H. 1974. Reaction of *o*-benzoquinone monoimine with *o*-aminophenol. *Chem. Lett.* 293–296.

IKEKAWA, T., UEHARA, N., and OKUDA, T. 1968. Photochemistry of antibiotics I. Oxidative coupling of *o*-aminophenol by photoirradiation. *Chem. Pharm. Bull.* 16:1705–1708.

KEHRMANN, F. 1906. Ueber Oxydationsproducte von *o*-aminophenolen. *Chem. Ber.* 39:134–138.

KINJO, J., YOKOMIZO, K., AWATA, Y. SHIBATA, M., and NOHARA, T. 1987. Structures of phytotoxins, AV-toxins C, D and E produced by zonate leaf spot fungus of mulberry. *Tetrahedron Lett.* 28:3697–3698.

NAGASAWA, H.T., GUTMAN, H.R., and MORGAN, M.A. 1959. The oxidation of *o*-aminophenols by cytochrome *c* and cytochrome oxidase. *J. Biol. Chem.* 234:1600–1604.

NAIR, M.G., WHITENACK, C.J., and PUTNAM, A.R. 1990. 2,2′-Oxo-1,1′-azobenzene, a microbially transformed allelochemical from 2,3-benzoxazolinone: I. *J. Chem. Ecol.* 16:353–364.

ORTIZ, B., VILLANUEVA, P., and WALLS, F. 1972. Silver(II) oxide as a reagent. Reaction with aromatic amines and miscellaneous related compounds. *J. Org. Chem.* 37:2748–2750.

PEÑA-RODRIGUEZ, L.M., ARMINGEON, N.A., and CHILTON, W.S. 1988. Toxins from weed pathogens, I. Phytotoxins from a *Bipolaris* pathogen of Johnson grass. *J. Nat. Prod.* 51:821–828.

PUMMERER, R., SCHMIDUTZ, G., and SEIFERT, H. 1952. Ueber Dehydro-tetrachlor-*p*-kresol, ein Radikal mit einwertigem Sauerstoff; XI. Mitteil. ueber die Oxydation der Phenole. *Chem Ber.* 85:535–555.

TOMODA, A., YAMAGUCHI, J., KOJIMA, H., AMEMIYS, H., and YONEYAMA, Y. 1986. Mechanism of *o*-aminophenol metabolism in human erythrocytes. *FEBS Lett.* 196:44–48.

TURNER, W.B., and ALDRIDGE, D.C. 1983. Fungal Metabolites II, p. 397, Academic Press, New York.

WORSHAM, A.D. 1989. Current and potential techniques using allelopathy as an aid in weed management. *Acad. Sin. Monogr. Ser.* 9:275–291.