Mode of Action of Oxathiin Systemic Fungicides

Structure–Activity Relationships

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The toxicities of the oxathiin systemic fungicide carboxin and eight structurally related compounds were compared with regard to their ability to affect various metabolic pathways in sensitive fungi. Those compounds that were toxic also strongly inhibited the metabolism of acetate and the synthesis of ribonucleic acid. This indicates a similar mode of action for the toxic compounds even though two of them are thiazoles and not oxathiins. Oxidation of the sulfur atom of the carboxin molecule greatly reduced the toxicity of the compound, particularly if one oxygen atom were added rather than two. Elimination of the benzene ring from the carboxin molecule also destroyed all activity. When the two sulfoxide compounds are excluded, the toxicity of the compounds to Rhizoctonia solani at 10^{-5} M is correlated with their partition coefficient. A parabolic equation, derived by the method of least squares, accounts for more than 86% of the variance in the data.

The use of systemic pesticides for control of insects and weeds has been common for a number of years. Only recently, however, have systemic fungicides been developed, and many are still in the experimental stage. von Schmeling and Kulka (1966) first reported on the systemic fungicidal nature of a new group of organic compounds, the oxathiins, which was developed by Uniroyal Chemical. Since then numerous reports have appeared concerning the potential value of the oxathiins for controlling various plant diseases including the rusts and smuts (Borum and Sinclair, 1967; Edgington and Kelly, 1966; Hardison, 1967; Powelson and Shaner, 1966; Venkata Ram, 1969). A unique feature of several of the oxathiins is their specificity for many members of the fungal class Basidiomycetes (Edgington et al., 1966) with a wider spectrum of activity being reported for various isomers of the oxathiin D735 (Carboxin = 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) (Edgington and Barron, 1967; Snel et al., 1970). The specificity of D735 was shown by Mathre (1968) to be related to the amount of fungicide absorbed and bound to cell organelles. In addition, the mechanism of action of D735 appears to be associated with its ability to inhibit the tricarboxylic acid cycle (TCA cycle) in sensitive organisms (Mathre, 1970; Ragsdale and Sisler, 1970).

Since the oxathiins are a new group of organic chemicals that have pesticidal properties, this study was made to determine the effect of chemical structure of the oxathiins and related compounds on their toxicity to fungi sensitive to D735. In addition, the effect of these compounds on the metabolism of sensitive and resistant fungi was also investigated. Some of the compounds tested appear in plants and soil that has been treated with D735 (Chin et al., 1970a,b).

MATERIALS AND METHODS

Chemicals. The following technical grade chemicals were obtained from Uniroyal Chemical, Bethany, Conn., with their structures shown in Figure 1.

M223 = 5,6-dihydro-2-methyl-1,4-oxathiin
F362 = 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxylic acid
F416 = 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxamide
D735 = Carboxin = 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide

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F831 = 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide 4-oxide
F461 = Oxycarboxin = 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide 4,4-dioxide
F427 = 5,6-dihydro-2-methyl-N-(2-biphenylyl)-1,4-oxathiin-3-carboxamide
G696 = 2,4-dimethyl-5-thiazolecarboxanilide
F849 = 2-amino-4-methyl-5-thiazolecarboxanilide.

The purity of these chemicals was verified by thin-layer chromatography using Eastman Chromagram silica gel G sheets developed in chloroform. Spots were detected under ultraviolet light after the chromatograms were sprayed with fluoroscein.

Acetate-1-^{14}C (sp. act. 52.9 mCi/mM), L-phenylalanine-U-^{14}C (sp. act. 10 mCi/mmol), and uracil-2-^{14}C (sp. act. 52.5 mCi/mmol) were purchased from Amersham/Searle, Arlington Heights, Ill.

Test Organisms. The following Basidiomycetous· fungi were used: Ustilago nuda teliospores and Rhizoctonia solani mycelium. In some tests, cells of the Ascomycetous yeast Saccharomyces cerevisiae and mycelium and spores of the imperfect fungus Fusarium oxysporum f. sp. lycopersici were also used. The growth conditions were as previously described (Mathre, 1968).

Toxicity Tests. The ability of the various compounds to inhibit growth of R. solani mycelium or to inhibit germination of U. nuda teliospores was determined by dissolving the test compound in acetone or methanol and then incorporating this solution into warm potato–dextrose agar. Appropriate checks for the effect of the solvent were run. Growth of R. solani was measured after 3 days and the percent germination of the teliospores determined after 16 hr incubation at room temperature.

Partition Coefficients. The oil–water partition coefficient of each compound was determined in the manner described by Lukens and Horsfall (1967), but octanol was used instead of oleyl alcohol.

Effects on Metabolism. Respiration, as affected by the various compounds, was determined by conventional manometric techniques using a Gilson differential respirometer. For radioactive tracer studies, ^{14}C-labeled metabolites were administered to the test organism after it had been in contact with the test chemical for 45–60 min, since this is the time required for maximum uptake of D735 (Mathre, 1968). ^{14}C-Acetate, ^{14}C-uracil, and ^{14}C-phenylalanine were used to

study the effects on the TCA cycle, RNA synthesis, and protein synthesis, respectively. To determine if any uracil was incorporated into DNA, the method of Hock and Sisler (1969) was used to separate the RNA and DNA fractions. More than 95% of the activity incorporated into the nucleic acid fraction following administration of ^{14}C-uracil was found in the RNA fraction, thus indicating that use of this technique actually measured RNA synthesis. Respired $^{14}CO_2$ was trapped and counted by the method of Buhler (1962). Extractions of the tissue were as described by Mathre (1970). Samples which had been exposed to phenylalanine-^{14}C were extracted five times with ethanol, washed twice with water, and then dissolved in NCS solubilizer (Amersham/Searle, Arlington Heights, Ill.). Radioactivity of the various fractions was determined by adding 0.1 ml to a scintilation solution as described by Strobel (1967). Samples were counted in a Model 6804 Nuclear Chicago scintillation counter for a minimum of 10,000 counts. All counts were corrected for background and for quenching by use of the channels–ratio method.

RESULTS

Toxicity. The ability of the various compounds to inhibit growth or spore germination is shown in Table I. The most toxic compound was D735 since it inhibited growth or spore germination over 50% at a level of 1 μM (ca. 0.2 ppm). The closely related compound F427 was almost as effective. The thiazoles G696 and F849 were also quite effective at levels above 1 μM. The oxide compounds F831 and F461 had very low toxicities compared to D735.

Respiration. The effects of the compounds on respiration are shown in Table II. Most had little effect on oxygen uptake and in some cases stimulated it slightly. The compounds that inhibited growth or spore germination also inhibited respiration but only at the higher concentrations. Furthermore, the inhibition of respiration was not nearly as great as the inhibition of growth or germination. These compounds were also tested for their effect on respiration of *S. cerevisiae* and *F. oxysporum*, both of which are insensitive to the most toxic compound D735. Even at $10^{-4} M$, none of the compounds inhibited respiration of these fungi more than 8%.

Metabolism of Acetate. Since previous studies showed that D735 is a potent inhibitor of the metabolism of acetate (Mathre, 1970), thus indicating an inhibition of the TCA cycle, acetate-1-^{14}C was used to study the effect of the various test chemicals on this aspect of metabolism. Their effect on the amount of $^{14}CO_2$ released during a 1-hr incubation with the acetate is shown in Table II. If an inhibitory effect was
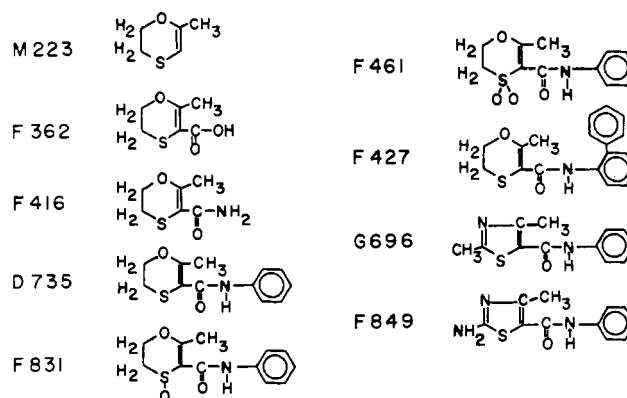

Figure 1. Chemical structure of oxathiin fungicides and related compounds

Table I. Toxicity of Oxathiins and Related Compounds to the Growth of *Rhizoctonia solani* or the Germination of *Ustilago nuda* Teliospores

	Molar concentration					
	R. solani			*U. nuda*		
Compound	10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-6}
M223	21[a]	13	6	0	0	0
F362	13	11	13	0	0	0
F416	21	17	6	0	0	0
D735	92	88	53	100	100	73
F831	10	15	10	0	0	0
F461	47	8	6	0	0	0
F427	84	72	39	100	100	53
G696	89	74	10	100	100	34
F849	87	50	13	100	66	46

[a] Percent inhibition when compared to the solvent check.

observed on the release of $^{14}CO_2$, there was also an inhibition of the uptake of acetate and the incorporation of label into the hot trichloroacetic acid soluble fraction and the residue remaining after the trichloroacetic acid extraction. The compounds D735, F461, and F427 were also tested for their effect on the metabolism of acetate in *S. cerevisiae* and *F. oxysporum*. Even at concentrations of $10^{-4} M$, no inhibition of the uptake of acetate or the release of $^{14}CO_2$ was observed using these fungi. The oxides of D735 again failed to greatly inhibit acetate metabolism and, in fact, stimulated the release of $^{14}CO_2$ somewhat.

Nucleic Acid Synthesis. The uptake and incorporation of ^{14}C-uracil into a hot trichloroacetic acid soluble fraction was used to determine the effect of the test compounds on RNA synthesis. *R. solani* was used because it could be kept com-

Table II. The Effect of Oxathiins and Related Compounds on Various Metabolic Activities of *Rhizoctonia solani* and *Ustilago nuda*

	Respiration				Acetate metabolism				RNA synthesis	Protein synthesis
	R. solani		*U. nuda*		*R. solani*		*U. nuda*		*R. solani*	*R. solani*
Compound	$10^{-4} M$	$10^{-5} M$	$10^{-4} M$	$10^{-5} M$	$10^{-4} M$	$10^{-5} M$	$10^{-4} M$	$10^{-5} M$	$10^{-4} M$	$10^{-4} M$
M223	103[a]	92	103	102	103	90	98	91	...[b]	...
F362	87	107	98	100	100	103	99	92	109	102
F416	93	96	103	106	101	113	99	90	95	97
D735	74	67	80	68	27	36	8	49	32	62
F831	100	96	103	102	115	108	110	100	92	101
F461	71	102	81	98	52	103	110	100	47	80
F427	75	78	63	85	3	22	8	75	5	39
G696	60	73	71	102	18	36	7	127	12	62
F849	73	69	67	94	25	40	4	95	35	50

[a] Percent of the control. [b] Not tested.

Table III. Oil–Water Partition Coefficients of Oxathiins and Related Compounds

Compound	Partition coefficient
F362	1.1
F416	0.6
D735	54.2
F831	4.4
F461	5.5
F427	343.8
G696	12.1
F849	3.2

pletely free of contaminating microorganisms at all times throughout the tests. Those compounds strongly inhibiting the metabolism of acetate also inhibited RNA synthesis, i.e., D735, F427, G696, and F849 (Table II). F461 was moderately inhibitory. In all cases the uptake of uracil was not inhibited as much as was the incorporation of label into nucleic acid.

Protein Synthesis. The uptake and incorporation of ^{14}C-phenylalanine into ethanol insoluble materials was used as a measure of protein synthesis in R. solani (Table II). F427 was the most inhibitory compound, but none of the test chemicals inhibited protein synthesis to the degree that they inhibited nucleic acid synthesis or acetate metabolism. Uptake of phenylalanine was not inhibited by any of the compounds except F427, which inhibited uptake by 15% at $10^{-4}M$ but not at lower concentrations.

Oil–Water Partition Coefficients. The partition coefficients of the various compounds were determined to see if lipid solubility was related to toxicity as has been shown for imide–SCCl$_3$ compounds (Lukens and Horsfall, 1967; Lien, 1969). As expected, F427 had the highest coefficient followed by D735, while the thiazoles F849 and G696 had coefficients somewhat lower (Table III). When a regression analysis of the data for the toxicity of the compounds at 10^{-5} M to R. solani was made, a fair correlation was obtained if the sulfoxide compounds F461 and F831 were excluded. Equation 1 describes this parabolic correlation.

$$\log\% \text{ inhibition} = -0.209 (\log P)^2 + 0.766 \log P + 1.262 \quad (1)$$

$$n = 6, r = 0.930, s = 0.179$$

More than 86% ($r^2 = 0.865$) of the variance in the data can be explained by eq 1. A similar equation is obtained describing the relation between partition coefficient and RNA synthesis in R. solani at 10^{-5} M concentration of chemical.

$$\log\% \text{ inhibition} = -0.380 (\log P)^2 + 1.525 \log P + 0.315 \quad (2)$$

$$n = 4, r = 0.9995, s = 0.014$$

The optimum log P values for the maximum activity for eq 1 and 2 are 1.83 and 2.01, respectively.

DISCUSSION

This study and that of Snel et al. (1970) provide evidence that compounds having a carboxanilide structure similar to D735 are toxic to various fungi even though they are not oxathiins (e.g., G696 and F849). Based on their inhibition of the metabolism of acetate and their inhibition of RNA synthesis, these compounds appear to have the same mode of action as D735. Thus, the oxathiin structure per se is not

necessary for toxicity. Snel et al. (1970) have also shown that o-toluanilide is nearly as toxic to R. solani as D735. Butyranilide is about $^1/_{10}$ as toxic. In addition, my results indicate that the oxathiin moiety by itself, i.e., M223, and M223 with several added structures, i.e., F362 and F416, are also nontoxic and have little or no effect on the various aspects of metabolism that were studied. Therefore, my results would substantiate the suggestion made by Snel et al. (1970) that the carboxamide moiety is integral for fungi toxicity. The reason that the compounds F362 and F416 are nontoxic may be due in part to their low lipid solubility, since the oil–water partition coefficient of the various compounds was related to toxicity if the sulfoxide compounds were excluded.

It is interesting to note that oxidation of the sulfur atom, i.e. F831, greatly decreases the toxicity of the compound and its ability to inhibit any of the major metabolic pathways. In some cases, metabolism is stimulated (Table II). Since the lack of toxicity of F831 and F461 was not correlated to their partition coefficients in the same manner as described for the other compounds by eq 1, the decreased activity of these compounds may be more directly related to another mechanism such as not binding to an organelle or the failure to inhibit one or more enzymes.

Various reports have appeared that indicate that D735 is oxidized very rapidly in plants and soil to F831 (Chin et al., 1970a,b; Snel and Edgington, 1970). Since F831 is nontoxic, the rapid oxidation of D735 in plants to F831 could explain why treatment with D735 provided poor control of bean rust (Snel and Edgington, 1970). Rapid disappearance in plants and soil may minimize the persistence of D735 per se in the environment. Furthermore, the decomposition products of D735, e.g., F831, F461, and butyranilide (Snel et al., 1970; Wallnöfer, 1969) are substantially less toxic to fungi and perhaps other organisms.

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