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Oxazolidinones: a new chemical class of fungicides and inhibitors of mitochondrial cytochrome bc_1 function

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Abstract: Famoxadone is a preventative and curative fungicide recently commercialized for plant-disease control. The molecule and its oxazolidinone analogs are potent inhibitors of mitochondrial ubiquinol : cytochrome c oxidoreductase (cytochrome bc_1) and they bind in the Q_0 site of the enzyme near the low potential heme of cytochrome b. Inhibitor binding constants for five mutant cytochrome bc_1 enzymes from *Saccharomyces cerevisiae* having single amino acid changes in their apocytochrome b located near the low potential heme were compared with their two parental wild-type enzymes. The five individual amino acid changes altered the inhibition constants for the inhibitors famoxadone, myxothiazol, azoxystrobin, and kresoxim-methyl in dissimilar fashion. The log scale differences in binding constants relative to those of their parentals provide fingerprints for the effects of the amino acid changes on binding of the individual inhibitors, thus reflecting the structural diversity of the inhibitors.

Keywords: famoxadone; mode of action; fungicide; plant disease; cytochrome bc_1; enzyme inhibitors; oxazolidinones

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1 INTRODUCTION

Famoxadone (3-anilino-5-methyl-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione; Famoxate®; DPX-JE874) is a newly developed fungicide useful for preventative and curative control of fungal diseases in crops.[1] The fungicidal properties of this molecule were discovered through a chemical scouting and optimization methodology using enzyme and whole-plant data rather than through an analog program against natural products or other known fungicides.[2] From results of independent experimental methods, we have determined that famoxadone and its analogs bind to the Q_0 site of cytochrome bc_1 and thereby inhibit the enzyme's catalytic functions. Further, we have concluded that inhibition of cytochrome bc_1 function is the primary cause of famoxadone's physiological and fungicidal properties. In this report we summarize studies which indicate that famoxadone has a different binding mode to the enzyme than other inhibitors of cytochrome bc_1, which are also known to bind to the enzyme's Q_0 site.

2 EXPERIMENTAL

Saccharomyces cerevisiae Meyer ex Hansen parentals and their isolates having single amino acid changes in their apocytochrome b were a kind gift from Dr A-M Colson and they have been described in detail elsewhere.[3] All other experimental details will be presented elsewhere.

3 RESULTS AND DISCUSSION

Submitochondria were isolated from two wild-type parentals of *S. cerevisiae* and five of their isolates, which have been characterized as having single amino acid changes in their apocytochrome b.[3] IC_{50} values of four Q_0 center inhibitors of mitochondrial cytochrome bc_1, famoxadone,[1,2] azoxystrobin,[4,5] kresoxim-methyl[6] and myxothiazol[7] (Fig. 1), were determined against each of the mitochondrial preparations by measuring inhibition of the overall reaction NADH to O_2 by following the oxidation of NADH spectrophotometrically. The IC_{50} values of the five isolates, compared to those of their respective wild-type parentals, gave the relative values shown in log scale in Fig 1 for the individual inhibitors.

Inspection of the fingerprint profiles presented in Fig. 1 indicates considerable differences between the four Q_0 center inhibitors with respect to the influences of individual amino acid changes in apocytochrome b and their relative IC_{50} values. The G137R, L275S, and F129L changes were detrimental to the binding of all four inhibitors by factors of 3.8–180, 1.5–24, ad 2.2–64, respectively. The L275F change enhanced the binding of famoxadone by a factor of 20 and that of azoxystrobin by a factor of 2.5, whereas the L275F change decreased the affinity for kresoxim-methyl by a factor of 1.8 and that of

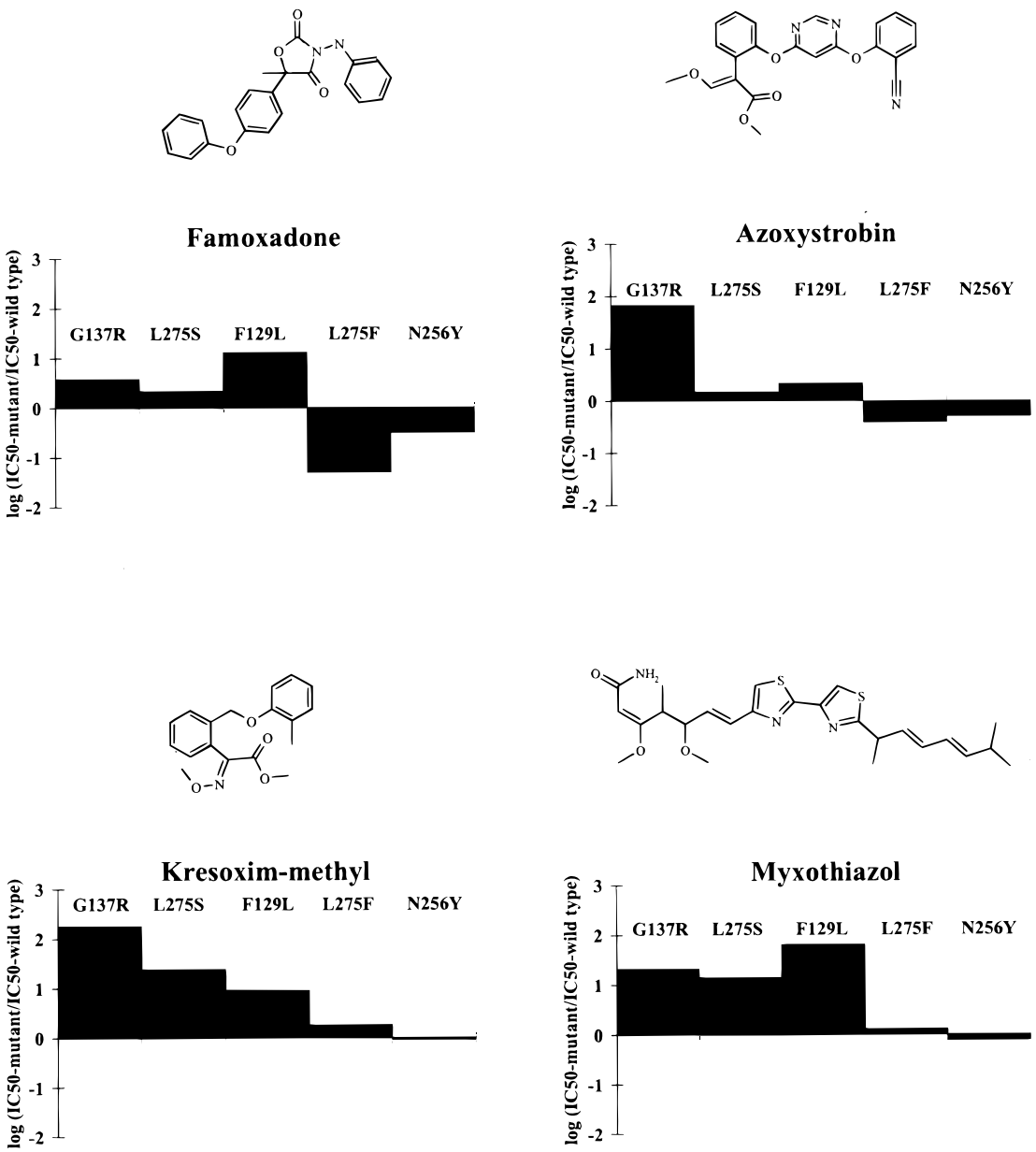

Figure 1. Comparisons of IC_{50} values for Q_0 site inhibitors versus submitochondria from *Saccharomyces cerevisiae* isolates relative to their wild-type parentals. Isolates MUC-771 and MUC-772 equate to mutations in apocytochrome b of G137R and L275S, respectively, and their IC_{50} values were divided by the IC_{50} value of their parental, D225-5A.[3] Isolates MYX1-103, MYX2-124, and MYX3-119 equate to mutations in apocytochrome b of F129L, L275F, and N256Y, respectively, and their IC_{50} values were divided by the IC_{50} value of their parental, GM50-3C.[3] Note the log scale.

myxothiazol by a factor of 1.3. The N256 change enhanced the binding of all four inhibitors by factors of 1.1–3.2. As a benchmark, IC_{50} values were measured against all mitochondria from isolates and parentals for antimycin A (a Q_i center inhibitor, whose IC_{50} values should not change in the isolates in comparison to their parentals) and it was found that the relative values for antimycin A (isolates/parentals) ranged from 0.8 to 1.8. Thus, relative IC_{50} values within a factor of two should be considered cautiously.

Three of the four Q_0 center inhibitors examined in this work are recently commercialized fungicides useful for protecting plants from fungal diseases.[1,2,4–6] The other Q_0 center inhibitor, myxothiazol, is a natural product.[7] Azoxystrobin and kresoxim-methyl may be considered as strobilurin A analogs, as this was the template from which they were derived.[4–6] Famoxadone and myxothiazol have completely different origins.[1,2] The fingerprints of relative IC_{50} values of Fig. 1 indicate that amino acid changes in apocytochrome b have differential effects on the binding properties of the four inhibitors and these differences are inherent in the chemical diversity of the four inhibitors (Fig 1). A more exacting profile of the binding mode of these four inhibitors and others may become available through X-ray crystallographic studies of the enzyme in follow-up studies to the recently reported X-ray structure of the bovine cytochrome bc_1 complexed with inhibitors.[8] Recently, the first sequence of apocytochrome b from a plant pathogen was reported and it had the

same amino acid composition as the wild-type parentals of *S. cerevisiae* studied in this work.[9]

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Isotope-edited nuclear magnetic resonance: novel methodologies for investigating metabolism

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Abstract: While the use of NMR and stable isotopes in metabolism studies is hardly new, it is only recently that isotope-edited NMR spectroscopy has been applied in kinetic studies of glyphosate metabolism of soil microbes. NMR can detect multiple species simultaneously and non-destructively, yielding valuable information on structural identification of metabolites.

Triple Resonance Isotope EDited spectroscopy (TRIED), [^{2}H]NMR, and [^{2}H–^{13}C] INEPT (Insensitive Nucleus Enhancement through Polarization Transfer) are three isotope-edited techniques which have been used in combination to examine the microbial degradation of glyphosate (*N*-phosphonomethylglycine). Using ^{13}C- and ^{15}N-labeled glyphosate, TRIED can detect multiple metabolites in crude matrices at submicrogram levels, an improvement over earlier techniques where milligrams were needed. It can detect 500 nanograms of ^{13}C–^{15}N-labeled compound in a crude sample (1 : 1400 mass ratio), only a few hours work being required. [^{2}H]NMR and [^{2}H–^{13}C]INEPT were also used as complementary techniques to further examine metabolites whose ^{13}C–^{15}N bond has been cleaved.

The three-isotope-edited methods produced results consistent with both radioactivity and HPLC analyses. Accordingly, we are able to detect minute levels of metabolites in the presence of complex mixtures, minimizing the costs and time of sample purification.

Keywords: NMR; isotope-edited NMR; herbicide metabolism; microbial soil isolates

Triple Resonance Isotope EDited spectroscopy (TRIED), [^{2}H]NMR, and [^{2}H–^{13}C]INEPT (Insensitive Nucleus Enhancement through Polarization Transfer) form a powerful arsenal of isotope-edited techniques that can be used to study metabolism in extracts from biological systems. All three methods circumvent the use of radioactive labels. TRIED was developed to detect and examine minute levels of glyphosate (*N*-phosphonomethylglycine) metabolites in microbial soil isolates.[1] It uses stable isotopic labeling (^{13}C and ^{15}N), and allows the simultaneous detection of multiple metabolites in crude matrices at submicrogram levels with high signal-to-noise (s/n) ratios and nearly complete suppression of unwanted signals. Both [^{2}H]NMR and [^{2}H–^{13}C]INEPT allow continued metabolic studies of labeled compounds beyond the point where the ^{13}C–^{15}N bond has been broken. The pathways of glyphosate metabolism by LBAA, one strain of *Ochrobactrum anthropi*, have been studied using isotope-edited experiments. No detectable levels of glycine or sarcosine were observed in media or lysates, leaving the aminomethylphosphonate (AMPA) pathway as the means of glyphosate metabolism. Other metabolites produced included *N*-methylAMPA, acetylAMPA, glyphosate esters, and *N*-methylacetamide.[2]

TRIED is patterned after triple-resonance techniques used in NMR measurements of ^{13}C–^{15}N labeled proteins. Coherence originates as proton magnetization, which is transferred from the protons to their adjacent carbons, and subsequently to their adjacent nitrogens. The signal transfer stops in the absence of a ^{13}C or ^{15}N label. Prior to high-sensitivity proton detection, the pathway of the magnetization transfer is reversed.

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