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Effects of Miconazole and Dodecylimidazole on Sterol Biosynthesis in *Ustilago maydis*

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Miconazole at minimal fungitoxic concentrations inhibits ergosterol biosynthesis in sporidia of *Ustilago maydis* by interference with sterol C14 demethylation. The action is analogous to that of the fungicides triarimol and fenarimol. The fungicide 1-dodecylimidazole at low concentrations (0.1 to 0.25 μg/ml) inhibits sterol C14 demethylation; however, at higher concentrations (1.0 μg/ml or greater) it also inhibits 2,3-oxidosqualene cyclization and subsequent transmethylation. It is postulated that this diversity of effects of 1-dodecylimidazole results from binding of the inhibitor to sterol carrier protein(s).

Miconazole is a fungitoxic agent with a wide antifungal spectrum used medically to control mycotic infections (27). Various observations with *Candida albicans* after exposure times ranging from 3 to 24 h indicated that miconazole affects membrane permeability and transport as well as cell morphology (8-10, 26, 28).

In studies with imazalil, a miconazole analog, Van Tuyl observed that cross-resistance invariably occured between the former compound and fenarimol in mutants of *Aspergillus nidulans* (30, 31). Fenarimol, like triarimol, is an inhibitor of ergosterol biosynthesis (3, 19). These results suggested that the mode of action of miconazole was possibly analogous to that of the sterol C14 demethylation inhibitors triarimol (18, 21), fenarimol, imazalil (16, 24), triadimefon (2), triforine (23), and Denmert (14). Recent studies show, in fact, that miconazole is an inhibitor of ergosterol biosynthesis in *Ustilago maydis* (12) and *C. albicans* (29).

N-dodecylimidazole, like miconazole, is a fungitoxic 1-alkylimidazole. This compound is reported to inhibit cholesterol biosynthesis in rat livers (K. H. Baggaley, R. M. Hindley, J. L. Tee, and G. S. Brooks, British Patent 1,364,312, January 1971). Atkin et al. concluded that it is a specific inhibitor of 2,3-oxidosqualene cyclase, the enzyme which converts 2,3-oxidosqualene to lanosterol (1). It was of interest to compare the effects of these two inhibitors on the sterol biosynthetic pathway in *U. maydis*, an organism in which the action of triarimol has been thoroughly investigated (12, 18, 20, 21). This report presents the results of these investigations.

MATERIALS AND METHODS

Sporidia of *U. maydis* (DC) Cda. ATCC 14826 were cultured as previously described (5, 20), in a defined nutrient medium at an initial concentration of 6.9×10^6 sporidia per ml (0.2 mg [dry wt] per ml). Inhibitors were added as methanolic solutions so that the methanol did not exceed 1% in the culture media. After centrifugation from culture media, sporidia were lyophilized, and crude lipids were extracted with chloroform-methanol (2:1, vol/vol) (11, 18, 20). Saponification was performed by the method of Clayton et al. (4) with mild reflux for 45 min at 80 to 90°C. Thin-layer chromatography (TLC) was performed as described elsewhere (21) by using a solvent system of hexane-diethyl ether-acetic acid (70:30:1 or 70:50:1). Sterols of unsaponified lipid samples were analyzed by gas-liquid chromatography after separation by TLC, whereas saponified lipid samples were analyzed directly. Analyses were performed on a Glowall Chromalab 310 chromatograph by using a column (1.8 m by 3.4 mm) packed with 3% SE-30 on Gas Chrom Q (100 to 120 mesh) at 255°C. Compounds were detected by an argon ionization detector and quantified in relation to a cholesterol standard. Mass spectroscopy (MS) was performed on an LKB model 9000 gas-liquid chromatograph-mass spectrometer. Compounds were introduced through a 0.75% SE-30 column. The ionization energy was 70 eV, and the temperature of the ion source was 288°C. Spectra were interpreted with a Varian SS100 data system. Synthesis of 2,3-oxidosqualene was performed as previously described (17), and confirmation of the product was by TLC and infrared spectroscopy.

RESULTS AND DISCUSSION

Miconazole is extremely toxic to *U. maydis* (Table 1). A concentration of 0.25 μg/ml permitted approximately one doubling of sporidial number with little delay, but strongly suppressed multiplication thereafter. There was essentially no effect on dry weight increase during the first doubling, but it was markedly inhibited thereafter, although less so than was sporidial multiplication. A concentration of 5.0 μg/ml

completely prevented both sporidial multiplication and dry weight increase, indicating possible involvement of a secondary toxicity mechanism which is nonoperative at lower concentrations. The effects of 0.025 and 0.1 μg of miconazole per ml resembled those produced by 0.25 μg/ml; however, there was nearly total recovery from the toxic effects of the two lower concentrations after 12 to 24 h.

Concentrations of 0.25 and 1.0 μg of dodecylimidazole per ml produced initial effects on sporidial multiplication and dry weight increase more severe than those of 0.25 μg of miconazole per ml, but there was a greater tendency for recovery after the first sporidial division than there was with miconazole (Table 1). Concentrations of 2.5 and 5.0 μg/ml completely prevented increase in cell number and dry weight.

Analyses of sterols showed patterns of effects by miconazole essentially identical to those produced by triarimol (Table 2). Ergosterol biosynthesis was inhibited by both compounds, and two other sterols accumulated concurrently. These sterols, which had retention times relative to cholesterol of 1.89 and 1.52, were previously identified as 24-methylenedihydrolanosterol and obtusifoliol in triarimol-treated cells of *U. maydis* (18). A mass spectrum recorded for the former sterol was consistent with that reported by Ragsdale (18). Exposure to either inhibitor for 6 h resulted in a small accumulation of 14-α-methyl-$\Delta^{8,24(28)}$-ergostadienol, a sterol previously reported by Ragsdale (18) to accumulate in triarimol-treated sporidia.

The effect of 0.1 and 0.25 μg of dodecylimidazole per ml on sterol biosynthesis was nearly identical to that produced by 2 μg of triarimol per ml or 0.25 μg of miconazole per ml. However, the effects of 1.0 μg of dodecylimidazole per ml differed markedly in some respects from those of the above concentrations of triarimol and miconazole (Table 3). Although there were sim-

TABLE 1. *Toxicity of miconazole and dodecylimidazole to growth of U. maydis as measured by increase in sporidial number*

Treatment	Concn (μg/ml)	No. of sporidia (× 10^6) per ml at:					
		0 h	2 h	4 h	6 h	8 h	12 h
Control		6.9	12.3	18.5	28.2	39.8	101
Miconazole	0.025	6.9	11.0	16.0	17.0		43.2
	0.10	6.9	11.9	17.0	18.5	25.2	
	0.25	6.9	11.8	16.9	17.1	19.8	26.0
	1.0	6.9	11.0	16.1	17.6	19.6	
	2.5	6.9	6.0	6.2	10.0		27.0
	5.0	6.9	6.8	7.9	7.8		4.4
Dodecylimidazole	0.010	6.9	9.8	18.0	23.0		120
	0.10	6.9	11.1	13.2	23.3	32.2	83.2
	0.25	6.9	10.4	13.8	17.3	23.7	59.5
	1.0	6.9	8.0	11.0	11.4	15.6	28.8
	2.5	6.9	6.0	5.5	7.2		7.0
	5.0	6.9	7.6	8.6	7.9		4.3

TABLE 2. *Effect of miconazole and triarimol on the quantity of various sterols in sporidia of U. maydis*[a]

Treatment	Time (h)	Amt (μg/500 ml of culture) of the following in sporidia of *U. maydis*:					Sporidial dry wt mg/culture
		24-Methylene-dihydro-lanosterol	Obtusi-foliol	14-α-Methyl-$\Delta^{8,24(28)}$-ergo-stadienol	Desmethyl-sterols[b]	Ergos-terol	
Control	0	37	11	ND[c]	46	240	109
	4	48	Trace[d]	ND	197	655	237
	6	59	ND	ND	143	1,897	405
Miconazole	4	1,465	128	ND	Trace	401	239
(0.25 μg/ml)	6	2,237	339	12	ND	163	285
Triarimol	4	1,327	84	ND	6	222	197
(2.0 μg/ml)	6	1,562	227	10	Trace	170	278

[a] Sterols were isolated by TLC and analyzed by gas-liquid chromatography.
[b] Desmethyl sterols excluding ergosterol.
[c] ND, Not detected.
[d] Trace: detected but too small to quantify.

TABLE 3. *Effect of dodecylimidazole on the quantity of sterols and sterol precursors in saponified lipid samples of U. maydis at 4 h after treatment*

Treatment	Concn (μg/ml)	Amt (μg/500 ml of culture) of the following at 4 h after treatment:						Sporidial dry wt mg/culture
		Squalene	2,3-Oxido-squalene	Lanosterol	24-Methylene-dihydro-lanosterol	Obtusi-foliol	Ergos-terol	
Control		17 (4)[a]	17 (ND)[b]	ND (ND)	40 (10)	ND (ND)	1,876 (292)	340 (110)
Dodecylimi-dazole	0.1	15	6.2	ND	638	189	1,206	334
	0.25	17	60	2.4	831	147	524	304
	1.0	114	222	149	249	4.9	430	222

[a] Values in parentheses represent zero-time measurements.
[b] ND, Not detected.

ilarities with respect to inhibition of ergosterol biosynthesis and accumulation of 24-methylenedihydrolanosterol, appreciable quantities of another sterol and a nonsterol component not normally detectable in control or miconazole or triarimol-treated sporidia were present.

The sterol was identified as lanosterol on the basis of the same gas-liquid chromatography retention time and TLC mobility as a lanosterol standard (Sigma) as well as by MS analysis. The sterol had a retention time ratio relative to cholesterol of 1.62 and TLC R_f of approximately 0.35 to 0.40 in hexane–diethyl ether–acetic acid (70:50:1). MS analysis indicated the following: m/e 426 (M^+; 25%), 411 ($M-CH_3$; 56%), 408 ($M-H_2O$; 22%), 393 ($M-CH_3-H_2O$; 100%), 339 (10%), 311 (8%), 297 (7%), and 69 (64%). The MS data are consistent with those reported by Smith et al. (25) for lanosterol.

The level of nonsterol component was strongly enhanced by treatment with 1 μg of dodecylimidazole per ml. This component was identified as 2,3-oxidosqualene on the basis of an identical TLC R_f (0.7 to 0.8), an identical gas-liquid chromatography retention time relative to cholesterol (0.70) as a synthetic standard, and the following MS analysis: m/e 426 (M^+; 3%), 408 ($M-H_2O$; 2%), 357 (M-1 isoprene unit; 2%), 289 (M-2 isoprene units; 1%), 221 (M-3 isoprene units; 1%), 203 (5%), 153 (10%), 135 (14%), 81 (82%), and 69 (100%). The MS data are consistent with those reported by Katayama and Marumo (13) and Atkin et al. (1) for 2,3-oxidosqualene. Accumulation of 2,3-oxidosqualene was accompanied by an increase in squalene, a compound detectable in small quantities in untreated sporidia. Traces of lanosterol together with an increased level of 2,3-oxidosqualene were also detectable in sporidia treated with 0.25 μg of dodecylimidazole per ml.

The present study demonstrates that triarimol and miconazole, at their lowest effective fungitoxic levels, inhibit ergosterol biosynthesis by analogous mechanisms in *U. maydis*, i.e., by inhibition of sterol C14 demethylation. This inhibition appears to be a primary biochemical lesion leading to fungitoxicity of both compounds. Membrane damage caused by low concentrations of miconazole in *C. albicans* (26, 28) undoubtedly is due to an ergosterol deficiency resulting from inhibition of sterol C14 demethylation. Recent studies of the action of miconazole in *C. albicans* by Van den Bossche (29) also indicate the involvement of such a mechanism leading to membrane damage. Although C14 methyl sterols accumulate, it has been shown that these do not function effectively as membrane components (32).

With respect to the mode of action of dodecylimidazole in *U. maydis*, our data indicate that the compound is not a specific inhibitor of 2,3-oxidosqualene cyclase in the sterol biosynthetic pathway, as was reported to be the case in rat livers (1). The cyclase reaction is, in fact, of secondary sensitivity in comparison with that of sterol C14 demethylation. This was suggested by the data of a previous study with rat livers which reported a threefold elevation of "lanosterol-containing" lipids after treatment with dodecylimidazole (1). The sterol C14 demethylation reaction appears to be the rate-limiting step in cells of *U. maydis* treated with 0.1 and 0.25 μg of dodecylimidazole per ml, as is indicated by suppression of ergosterol synthesis and accumulation of 24-methylenedihydrolanosterol and obtusifoliol as previously observed with the C14 demethylation inhibitors miconazole and triarimol. At 1.0 μg of dodecylimidazole per ml, accumulation of these two ergosterol precursors is reduced because of interference with the preceding cyclase and transmethylation reactions which lead, respectively, to accumulations of 2,3-oxidosqualene, squalene, and lanosterol. The contrasts in effects produced by 0.1 and 0.25 μg of dodecylimidazole per ml as compared with 1.0 μg/ml were not evident with miconazole. All concentrations of miconazole (0.1 to 2.5 μg/ml) produced similar effects on sterol biosynthesis;

in no case was there an acumulation of 2,3-oxidosqualene or lanosterol. At 1 μg of dodecylimidazole per ml there is evidence for concomitant inhibition of three types of enzymatic reactions; a cyclase reaction, a transmethylation reaction, and a demethylation reaction. It seems quite unusual that lanosterol should accumulate concurrently with 2,3-oxidosqualene in sporidia treated with 1 μg of dodecylimidazole per ml since the former compound was not detected in control sporidia. These results indicate that transmethylation is of equal or greater sensitivity to the inhibitor as the cyclase reaction.

Sterol carrier protein(s) are involved in all steps of sterol biosynthesis from squalene to cholesterol (7, 22). In view of the diversity of reaction types affected by low concentrations of dodecylimidazole, the action of the compound is best explained by assuming that it binds to the carrier protein(s) and thus interferes with the interaction of several types of enzymes with the sterol carrier protein-sterol (2,3-oxidosqualene) complex. The binding of AY-9944 to sterol carrier protein has been described as the mechanism of inhibiting sterol biosynthesis (6), and such a mechanism of interference has also been suggested for triarimol (18) and triforine (23). This type of interference may be the common basis of action for the fungitoxic compounds imazalil (16, 24), triarimol (18), fenarimol (3, 19), triforine (23), triadimefon (2), and Denmert (14, 15).

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