Maleic Hydrazide Ribonucleoside: Its Synthesis and Its Effect on Fertilized Chick Eggs

Charles J. Thoman,*[1] Martin D. Appleton, Stanley W. Dziedzic, John P. Griffiths, and Lawrence C. Van Iseghem

The *N*-ribonucleoside and the *N,N'*-diribonucleoside of maleic hydrazide have been synthesized and tested to determine whether one of them might be used as an antitumor agent. A nontoxic dose of radioactively labeled mononucleoside was injected into fertilized chick eggs, but none was incorporated into the nucleic acids of the developed chick embryos. Rather, the radioactivity appeared in the initial droppings of the hatched chicks.

Maleic hydrazide (MH, 6-hydroxy-3(2*H*)-pyridazinone (**1**); Figure 1), an isomer of uracil, has found extensive use as a plant growth inhibitor. Its action has been shown (Callaghan et al., 1962) to involve incorporation of the MH into the RNA, especially the tRNA, of the plant nuclei, where it takes the place of cytosine rather than its isomer, uracil (Appleton et al., 1981).

The question arose as to whether MH might be incorporated into animal cells, resulting in a similar inhibiting effect on their growth. If so, it might be developed as a valuable therapeutic agent against tumorous cells, preventing or interfering with their proliferation.

Earlier work (Mays et al., 1968) had shown that MH itself was not incorporated into animal cells. After [^{14}C]MH was fed to rats over a 6-day period, 0.001% of it was found in the blood and tissues of the sacrificed animals, 0.2% in expired CO_2, 12% in the feces, and 77% in the urine, suggesting that most, if not all, of it had been absorbed and quickly eliminated without any significant incorporation into the animals' cells.

The present work was initiated to determine whether the ribofuranoside of MH might be incorporated into animal cells and inhibit their growth. Before this could be done, the previously unreported nucleoside had to be synthesized.

EXPERIMENTAL SECTION

All melting points, which are uncorrected, were determined with a Fisher-Johns melting point apparatus. Combustion analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Infrared spectra were obtained with a Perkin-Elmer 1310 spectrophotometer, and the nuclear magnetic resonance spectra, with a Varian T-60.

(1) Synthesis of Maleic Hydrazide Dinucleoside. (*A*) *2,3,5-Tri-O-benzoyl-D-ribosyl Chloride* (*2*). 1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribose (10.1 g, 0.02 mol) was added to 200 mL of anhydrous ether previously saturated with hydrogen chloride at 0 °C. The stoppered flask stood at 0–4 °C for 5 days. The solvent was removed in vacuo and the syrup treated three times with 50-mL portions of anhydrous benzene, each of which was removed under vacuum. The addition of 30 mL of anhydrous benzene afforded a solution of **2** that was used immediately. (If repeated, toluene should be substituted for benzene in these procedures.)

(*B*) *Mercury Salt of Maleic Hydrazide* (*3*). Maleic hydrazide (112 g, 1.00 mol; **1**) was heated to reflux with acetic anhydride (204 g, 2.00 mol) until all the solid had dissolved. Benzene (200 mL) was added to the cooled solution and then ligroin to the cloud point. The white needles that formed on standing were filtered, washed with ligroin, and dried to give acetyl-MH (79 g, 0.51 mol), mp 123 °C. Concentration of the mother liquor and treatment with ligroin gave a second crop, raising the total yield to 112.5 g (0.73 mol, 73%).

Acetyl-MH (36.8 g, 0.239 mol) was added to 500 mL of alcoholic sodium hydroxide (9.6 g, 0.24 mol) and stirred at temperatures below 35 °C until solution had occurred. Treatment of this solution with a solution of mercuric chloride (65.0 g, 0.24 mol) in 200 mL of ethanol produced a yellow precipitate. The neutral solution was warmed to 70 °C and cooled, and the product was filtered, washed with ethanol, and dried to give 49.3 g of **3** (0.234 mol, 98%).

(*C*) *1,2-Bis(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)maleic Hydrazide* (*4 or 5*). The benzene solution of 2,3,5-tri-*O*-benzoyl-D-ribosyl chloride made above (0.02 mol) was added to a stirred suspension of mercury MH (5.0 g, 0.024 mol) in hot xylene. After being refluxed for 1 h, the hot, turbid solution was filtered from crystalline material, the filtrate treated with low-boiling petroleum ether, and the resultant precipitate separated and dissolved in chloroform, washed with 30% aqueous potassium iodide and water, and dried over anhydrous sodium sulfate. Removal of the solvent on a water bath in vacuo left a syrup that was dissolved in 15 mL of ethyl acetate. Addition of low-boiling petroleum ether to cloudiness and cooling produced an oil that crystallized on overnight refrigeration. Washing of the precipitate with ether and recrystallization from ethyl acetate–petroleum ether gave pure **5** (5.50 g, 0.0055 mol, 55%), mp 182–184 °C. The IR and NMR spectra of this product were consistent with either structure **4** or **5**. Anal. Calcd for $C_{56}H_{44}N_2O_6$: C, 67.19; H, 4.44; N, 2.80. Found: C, 66.60, 66.82; H, 4.50, 4.69; N, 2.92, 2.96.

(*D*) *Proof of the Structure of 4*. 2,3-Dimethylbutadiene (1.0 g, 12 mmol) and the MH-dinucleotide (0.5 g, 0.5 mmol) were refluxed for 5 h in 30 mL of ethanol. The Diels–Alder adduct **7** obtained by removal of the solvent on the rotary evaporator was dissolved in 30 mL of 5% alcoholic potassium hydroxide in a 50-mL three-neck flask equipped with a reflux condenser and gas inlet tube, and air was bubbled through the solution for 24 h. The resulting yellow solid was filtered, washed with 10 mL of ether, and sublimed to give 0.04 g (0.23 mmol, 46%) of 4,5-dimethylphthalic anhydride (**8**) as white needles, mp 206–208 °C [lit. mp 206 °C (Sugasawa and Sugimoto, 1941)]. Anal. Calcd for $C_{10}H_8O_3$: C, 68.18; H, 4.58. Found: C, 68.24, 68.11; H, 4.92, 5.07.

Department of Chemistry, University of Scranton, Scranton, Pennsylvania 18510.

[1]Present address: Department of Chemistry, Stephen F. Austin State University, Nacogdoches, TX 75962.

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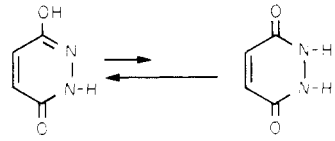

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Figure 1. Structure of maleic hydrazide (6-hydroxy-3-pyridazinone).

Recrystallization of **8** from hot ethanol gave 4,5-dimethylphthalic acid monoethyl ester (**9**): mp 119–121 °C; IR, C=O, 1675 cm^{-1}; NMR, 11.60 (s, 1 H), 7.77 (s, 1 H), 7.57 (s, 1 H), 4.43 (q, 2 H), 2.35 (s, 6 H), 1.37 ppm (t, 3 H).

(2) Synthesis of Maleic Hydrazide Ribofuranoside (13). *(A) 3,6-Bis[(trimethylsilyl)oxy]pyridazine (10).* MH (8.0 g, 0.0714 mol) was suspended in 40 mL of hexamethyldisilazane. Trimethylchlorosilane (0.8 mL) was added and the mixture allowed to reflux for 2 h. The solvent was evaporated under reduced pressure, leaving a white crystalline mass **10**, which was purified by sublimation: mp 73–74 °C; 17.2 g (0.0671 mol, 94%).

(B) 1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)MH (12). 3,6-Bis[(trimethylsilyl)oxy]pyridazine (**10**; 10.5 g, 0.0410 mol) was dissolved in 25 mL of dichloroethane and the resultant mixture added to a solution of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-furanose (**11**; 19.7 g, 0.391 mol) in 235 mL of dichloroethane. The mixture was cooled in an ice bath and $SnCl_4$ (3.38 mL) was added, after which stirring was continued at 22 °C for 4 h. The mixture was then diluted with 100 mL of dichloroethane and shaken with a saturated solution of $NaHCO_3$ (150 mL). An emulsion formed immediately, which was filtered over sand–cellite. The organic phase was separated, dried over Na_2SO_4, and evaporated to dryness under reduced pressure. Recrystallization from ethyl acetate–petroleum ether yielded 11.8 g of **12** (0.0212 mol, 52%) as white crystals, mp 202–205 °C.

(C) 1-(β-D-Ribofuranosyl)MH (13). 1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-3-hydroxypyridazine-6-one (**12**; 10 g, 0.0180 mol) was suspended in 250 mL of HPLC-grade methanol. Sodium methoxide (1.0 N) was added dropwise to achieve a pH of approximately 10 (universal test paper). The mixture was allowed to stir for 2 h or until dissolution was complete, after which water (10% of total volume) was added to enhance the ion exchange. Dowex 50W-X2 ion-exchange resin (10 g) was washed with distilled, deionized water to eliminate contaminating sulfates. This resin was added to the reaction flask and the mixture allowed to stir for 1 h. The resin was removed by filtration and washed with HPLC-grade methanol, which was added to the reaction solution. Impure product was precipitated by addition of ethyl ether (if no precipitation occurs, store overnight in a freezer at −15 °C), suction-filtered through sintered glass, and washed with ethyl ether. Recrystallization from a minimum volume of hot water produced 1.5 g of **13** (0.0056 mol, 31%) as fine, white crystals, mp 174–176 °C. The IR and NMR spectra were consistent with the structure proposed. Anal. Calcd for $C_9H_{14}N_2O_7$: C, 41.22; H, 5.38; N, 10.68. Found: C, 41.03, 41.15; H, 5.56, 5.53; N, 10.54, 10.67.

Radioactively labeled product was synthesized exactly as above with starting material containing [4-^{14}C]MH (1.11 mg, 50 μCi). To a scintillation cocktail made from diphenyloxazole (PPO; 400 mg), bis(phenyloxazolyl)benzene (POPOP; 50 mg), and M-5-grade Cab-O-Sil dissolved in 100 mL of sulfur-free toluene was added 3 mg of 1-(β-D-ribofuranosyl)[4-^{14}C]MH. The measured activity was 18 049 cpm/3 mg or 6016 cpm/mg.

Table I. Preliminary Study of MH-Ribofuranoside Dosage To Be Used in Fertilized Chick Eggs

mL stock soln[a]	mg nucleoside/ egg	% surviving (of 5 eggs)	mL stock soln[a]	mg nucleoside/ egg	% surviving (of 5 eggs)
0.51	8.5	0	0.18	3.0	40
0.48	8.0	0	0.15	2.5	60
0.45	7.5	20	0.12	2.0	60
0.42	7.0	20	0.09	1.5	40
0.39	6.5	40	0.06	1.0	40
0.36	6.0	0	0.03	0.5	20
0.33	5.5	0	0.00	0.0	40
0.30	5.0	20	drilled,		100
0.27	4.5	20	sealed		
0.24	4.0	20	control		100
0.21	3.5	20			

[a] Diluted to 0.5 mL with propylene glycol (except the first).

(3) Determination of the Amount of 1-(β-D-Ribofuranosyl)MH (13) To Be Used. A test solution was prepared by dissolving 1-(β-D-ribofuranosyl)MH (0.7 g, 0.0027 mol) in 7.00 mL of propylene glycol, known to be nontoxic in chicken embryos (McLaughlin et al., 1963, 1964).

Injections into the yolk sac were made with use of 1.00-mL tuberculin syringes with 1-in., 23-gauge needles. Each egg was drilled with a dentist's drill over the air cell to expose the shell membrane. Care must be taken not to rupture this membrane to avoid infection. Injected doses ranged from 0.00 to 8.50 mg in 0.50-mg increments (Table I). Five eggs were used for each dosage, and each was injected with 0.5 mL of solution. The 4-day-old eggs were candled to ensure fertility. After injection, the hole was sealed with sterile paraffin wax and the eggs were placed in an incubator equilibrated to 38 °C and an approximate humidity of 70%. They were turned mechanically three times a day up to day 18 and were candled once a day to discover dead eggs, which were then removed.

(4) Injection of 1-(β-D-Ribofuranosyl)[4-^{14}C]MH (13) into Chicken Embryos. *(A) Injection into the Yolk Sac.* A solution of 3 mg of labeled MH-nucleoside (**13**; 18 049 cpm) in 0.1 mL of propylene glycol was injected into the yolks of 30 fertile white leghorn chickens. The eggs were incubated at 38 °C and 70% humidity for 19 days. They were turned automatically every 2 h. Dead eggs were discarded on day 19.

(B) Injection into the Albumin. Thirty more fertile white leghorn chicken eggs were injected as above into the albumin. A hole was drilled through the shell approximately 5 mm from the margin of the air cell. The egg was then given a quick twist to allow the yolk sac to float free, and it was placed in a tray, small end upward at an angle of about 45°. This allows the yolk to float upward and away from the air cell. Injection was effected with a 0.5-in., 26-gauge needle. The hole was sealed with paraffin wax and incubation carried out as described above. Blanks were 10 fertile white leghorn chicken eggs injected with carrier only.

(5) Extraction and Counting of Genetic Material. Of the surviving 19-day-old eggs from each group (yolk-injected, albumin-injected, blank) 12 were sacrificed, the embryos decapitated, and the bodies frozen quickly and placed in a blender kept at 0 °C with 700–800 mL of a homogenizing solution containing 1% (w/v) NaCl, 0.5% (w/v) sodium lauryl sulfate, 0.5% (w/v) Na_2EDTA, 6.0% sodium *p*-aluminosalicylate, 0.5% (w/v) disodium naphthalene-1,5-disulfonate, and 3.0% (v/v) of a phenol mixture containing 70 mL of *m*-cresol, 55 mL of water, 0.5 g of 8-hydroxyquinoline, and 500 g of phenol. The homogenate was then filtered through gauze and added to an equal volume of the phenol mixture. This was shaken for

Table II. Liquid Scintillation Counts of Nucleic Acid Fractions and of Excretions

fraction counted[a]	counts/min test[b]	counts/min control
blank		73
total nucleic acid	64	68
DNA (yolk-injected eggs)	76	69
DNA (albumin-injected eggs)	65	69
high molecular weight RNA	78	68
low molecular weight RNA	78	74
excretions	270	71

[a] $N = 12$ eggs. [b] Only the DNA fraction of the albumin-injected eggs was counted.

20 min at room temperature and centrifuged at 5 °C for 30 min at 6000*g*. The top layer was separated, treated with solid sodium chloride (3 g/100 mL), and extracted with half its volume of the phenol mixture (which is already saturated with H_2O). This mixture was again shaken for 10 min at room temperature and centrifuged at 5 °C for 10 min at 8000*g*. The aqueous phase was separated, treated with two times its volume of ethanol–*m*-cresol (9:1, v/v), and allowed to stand for 1 h at 2 °C. The resultant precipitate of total nucleic acids was centrifuged at 5 °C for 5 min at 1000*g*. A quarter of this was set aside for counting. The rest was extracted twice with 25 mL of 3 M sodium acetate (pH 7.4). The top liquid fractions, containing DNA and low molecular weight RNA, were combined and set aside. The high molecular weight RNA precipitate was isolated and washed once with a mixture of 1 g of NaCl in 100 mL of 75% aqueous EtOH, once with 100 mL of 75% aqueous EtOH, and once with 100 mL of absolute EtOH. It was then dried and stored for counting. Addition of an equal volume of EtOH to the solution containing the DNA and low molecular weight RNA caused these to precipitate. The solid was extracted with a 2:1 mixture of 1 M NaCl and 2-ethoxyethanol (100 mL) and the residue of DNA separated by centrifugation at 5 °C for 3 min at 1000*g*. This was washed twice with EtOH, dried, and stored for counting. An equal volume of EtOH was added to the supernatant liquid to precipitate the low molecular weight RNA, which was washed twice with EtOH, dried, and stored for counting. The following amounts of each fraction for each type of egg were weighed out and placed in a vial with 20 mL of a scintillation cocktail containing 400 mg of PPO and 50 mg of POPOP/100 mL of sulfur-free toluene; the vials were then filled with M-5-grade Cab-O-Sil and counted: 0.190 50 g of total nucleic acid, 0.016 90 g of DNA, 0.285 85 g of high molecular weight RNA, and 0.055 50 g of low molecular weight RNA. Results of the counting of these fractions appear in Table II.

(6) Preparation and Counting of the Excretion Products of Newly Hatched Chicks. The droppings (0.1 g of feces and urine) of nine newly hatched chicks (three from each group) were placed in vials with the ingredients described above and counted. These results are also found in Table II.

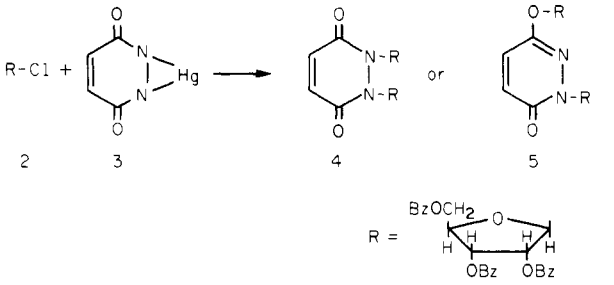
RESULTS AND DISCUSSION

Synthesis of Maleic Hydrazide Ribofuranoside (13). Though many derivatives of MH have been prepared and studied, no report of the preparation of a nucleoside has appeared. A product was extracted from excised wheat leaves from plants treated with ^{14}C-labeled MH which the authors (Towers et al., 1958) claimed was MH-β-glucoside, but no attempt was made to prove it.

Our first attempt at synthesis, using adaptations of the Hilbert–Johnson method (Kissman et al., 1955; Fox et al., 1956, 1957) gave a product whose combustion analysis was consistent with that of a dinucleoside of MH (Scheme I). In order to prove whether the *N,N'*- (**4**) or the *N,O*- (**5**) dinucleoside had been formed, a method (Scheme II) developed to prove 1,2-disubstitution in pyridazines was used (Druey et al., 1962). Our product was condensed with 2,3-dimethylbutadiene (**6**) and the resulting Diels–Alder adduct (**7**) treated in an attempt to aromatize it. The product proved to be 4,5-dimethylphthalic anhydride (**8**), which was converted to the monoethyl ester of 4,5-dimethylphthalic acid (**9**) by heating in ethyl alcohol. Thus, our sugar derivative is the MH-*N,N'*-dinucleoside (**4**).

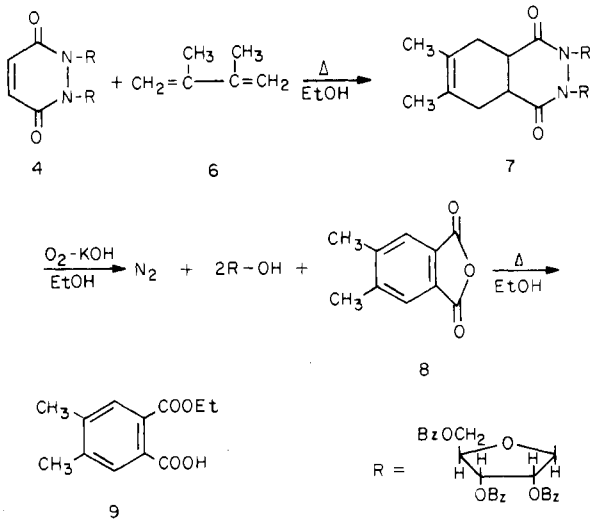
The ribofuranoside of MH was synthesized (Scheme III) by an adaptation (Niedbala and Vorbruggen, 1970, 1974) of the silyl method of Birkofer (1963). The initially formed

Scheme I. Synthesis of Maleic Hydrazide Diribofuranoside



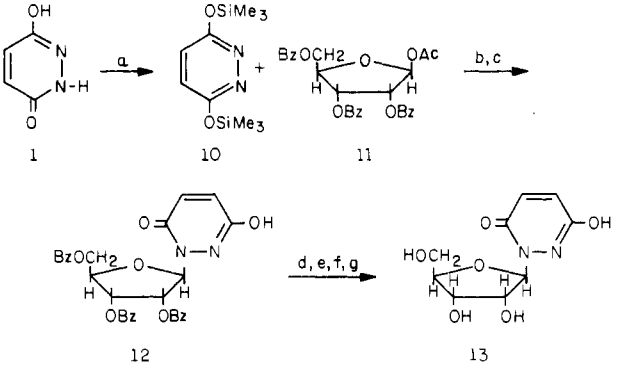
2 3 4 5

Scheme II. Proof of Structure of Maleic Hydrazide Diribofuranoside



4 6 7 8 9

Scheme III. Synthesis of Maleic Hydrazide Ribofuranoside[a]



1 10 11 12 13

[a] Key: (a) hexamethylsilazane, trimethylchlorosilane, reflux; (b) $SnCl_4$ in $ClCH_2CH_2Cl$; (c) $NaHCO_3$ in H_2O; (d) $NaOCH_3$ to pH 10; (e) Dowex 50W-X2, 10% H_2O; (f) Et_2O; (g) recrystallized from hot H_2O.

condensation product 12 was debenzoylated to give the ribofuranoside 13.

Determination of the Amount of MH-Ribofuranoside (13) To Be Used in This Study. In order to determine the dosage to be used in our incorporation studies, a test was conducted involving the injection of various amounts (0.0–8.5 mg, in increments of 0.5 mg) of 13 dissolved in propylene glycol (total volume 0.5 mL) into fertilized chick eggs. Since the injection of the propylene glycol itself (Table I) resulted, on average, in a 40% death rate (presumably due to defects in technique), a 60% survival rate with the 13 dissolved in it was considered the best level possible. Consequently, a dosage of 3.0 mg in propylene glycol (0.5 mL) was used for our incorporation studies.

Incorporation Studies of 13 in Fertilized Chick Eggs. The genetic material from the test eggs and the controls, which had been purified and separated into fractions, was counted for the presence of labeled MH-ribofuranoside in a Packard Tri-Carb 4530 scintillation counter. When none of the ^{14}C seemed to be present in any of the test material (Table II), the droppings of chicks that had been allowed to come to term and hatch and that had been isolated since birth were counted. The total weight of 3 days of droppings was about 40 g, indicating a total count of about 180 000 or about a 66.5% recovery of injected radioactivity.

Conclusions. It is clear that, as is the case with MH itself, MH-ribofuranoside (13) is not incorporated into the animal cells of developing chick embryos. Thus, it may not be able to be used as a therapeutic treatment for localized tumors. All of the radioactivity of the injected nucleoside was found in the excretion of the hatched eggs. As the embryo develops, it feeds off the yolk and this continues for several days after hatching. Since chicks have only one mode of excretion, we cannot say whether the nucleoside was broken down in the gastrointestinal tract and the MH absorbed and, with or without biotransformation, eliminated (as with the rats) or whether it was passed unchanged into the cloaca.

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Structural Studies of the Vinyltriazole Fungicide Diniconazole (ER Pure) Related to Its Binding to Cytochrome P-450

Toshiyuki Katagi

The conformation of diniconazole (ER pure) [(*R*)-(*E*)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol, Ia] in a lower energy state was optimized by semiempirical molecular orbital calculations. The optimized conformer was supported by the presence of a hydrogen bond between the hydroxy proton and the nitrogen atom at the 2-position of the 1,2,4-triazolyl moiety, as measured with infrared and nuclear magnetic resonance spectrometers. The substrate difference spectra with the rat liver microsomal enzymes indicated that a racemic compound of Ia was stoichiometrically bound to cytochrome P-450 enzymes. It was strongly suggested that Ia binds to cytochrome P-450 enzymes via the N4 lone pair of the 1,2,4-triazolyl moiety. The primary mode of action of Ia in fungi was discussed by the computer-aided superimposition of the optimized conformation of Ia on that of lanosterol, which was the intermediate in the ergosterol biosynthesis pathways.

Diniconazole (ER pure) [(*R*)-(*E*)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol, Ia] is a systemic fungicide showing fungitoxicity to a broad range of fungal species (Funaki et al., 1984). Among the possible four isomers, Ia shows the highest fungicidal activity (Sasaki, 1985). As with other fungicides possessing the triazolyl or imidazolyl group in the molecule (Gadher et al., 1983), Ia is known to inhibit the biosynthesis of ergosterol in fungal species (Takano et al., 1983).

The following studies have been performed to elucidate the mode of action of these fungicides: (1) GC–MS analysis of the intermediates of ergosterol biosynthesis (Aoyama and Yoshida, 1978); (2) substrate difference spectra of microsomal enzymes prepared from rat liver or yeast cells in the presence of a fungicide (Henry and Sisler, 1984); (3) computer graphics to visualize a structural similarity between a fungicide and lanosterol derivatives (Sugavanam, 1984; Marchington, 1983). The results of these studies strongly suggest that the primary mode of action of fun-

Laboratory of Biochemistry and Toxicology, Takarazuka Research Center, Sumitomo Chemical Company, Ltd., 4-2-1 Takatsukasa, Takarazuka, Hyogo 665, Japan.

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