resented 1–2% of the applied dose.

Norflurazon is metabolized in rats by two major pathways: N-demethylation and probably reaction with glutathione. The central role of these pathways in the metabolism of norflurazon by rats is demonstrated by our finding that eight of the nine metabolites we identified are produced by these routes. All metabolites except **5** arose after N-demethylation. As with other aromatic xenobiotic substrates, we suspect that sulfur is added to norflurazon via initial conjugation with glutathione (Bakke, 1986). The glutathione adduct then can be converted to thiol, methyl sulfide, sulfoxide, and sulfone after β-lyase cleavage. Glutathione appears both to displace the chlorine and to attack the aromatic ring in norflurazon. Secondary glutathione metabolites (i.e., mercapturic acid **4**, thiol **5**, methyl sulfide **6**, sulfoxides **3** and **9**, and sulfones **11** and **12**) were the major metabolites of norflurazon. A minor metabolic pathway involved replacement of the chlorine in norflurazon with hydrogen.

Registry No. **1**, 27314-13-2; **2**, 23576-24-1; **3**, 121442-73-7; **4**, 121442-69-1; **5**, 121442-70-4; **6**, 121442-74-8; **9**, 121442-71-5; **10**, 121442-72-6; **11**, 121442-75-9; **12**, 121442-76-0.

LITERATURE CITED

Bakke, J. E. *Xenobiotic Conjugation Chemistry*; Paulson, G. D., Caldwell, J., Hutson, D. H., Menn, J. J., Eds.; ACS Symposium Series 299; American Chemical Society: Washington, DC, 1986; pp 301–321.

Eder, F. A.; Sauer, H. H.; Wisson, M. L. Behavior of Norflurazon in the Soil Environment. *Proc. Eur. Weed Res. Com. Symp. Herbicides-Soil* **1973**, *73*, 202.

Hagve, T.-A.; Christopherson, B. O.; Böger, P. Norflurazon - An Inhibitor of Essential Fatty Acid Desaturation in Isolated Liver Cells. *Lipids* **1985**, *20*, 719–722.

Karapally, J. C., Sandoz Ltd. Unpublished results, 1974.

Numata, T.; Oae, S. Acetyl Chloride as Reducing Agent: A Facile Reduction of Sulphoxides, Sulphilimines and Sulphonium Ylids. *Chem. Ind.* **1973**, 277.

Sandmann, G.; Böger, P. *Biochemical Responses Induced by Herbicides*; Moreland, D. E., St. John, J. B., Hess, F. D., Eds.; ACS Symposium Series 181; American Chemical Society: Washington, DC, 1982; pp 111–130.

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Stability of Benomyl Homologues and Their Efficacy against Sensitive and Benomyl-Resistant *Botrytis cinerea*

John Northover* and Mikio Chiba

Methyl, ethyl, and hexyl isocyanate homologues of benomyl (MBC-MIC, MBC-EIC, and MBC-HIC, respectively) were prepared and compared with benomyl (MBC-BIC). In water (pH 6.2), the half-lives of these compounds were 2.7–7.7 times greater at 178 μM than at 1.78 μM. They were less stable at 10 and 25 °C than at 1 °C. These compounds were tested for the protection of apples wound-inoculated with sensitive (S) and benomyl-resistant (R) isolates of *Botrytis cinerea* and stored at 1 and/or 20 °C. In the 20 and 1 °C/20 °C programs, MBC-EIC was comparable to benomyl and superior to MBC-MIC against S. Against R, MBC-EIC and MBC-MIC were cross-resistant to benomyl but much more active than benomyl. At 1 °C, MBC-MIC was negatively cross-resistant to R. MBC-HIC had slight activity to S but no activity to R.

Benzimidazole fungicides were very effective for plant protection when they were first introduced commercially in the early 1970s. Two of the widely used compounds were carbendazim, methyl 1*H*-benzimidazol-2-ylcarbamate (MBC), and its butyl isocyanate derivative benomyl, methyl [1-(butylcarbamoyl)-1*H*-benzimidazol-2-yl]carbamate (MBC-BIC). Within 2–3 years of intensive fungicide use, many fungal populations became benzimidazole-resistant and the fungicides became ineffective (Bollen and Scholten, 1971; Dekker, 1976; Delp, 1980; Elad et al., 1988; Northover, 1986; Northover and Matteoni, 1986). We found that the methyl and ethyl isocyanate homologues of benomyl (MBC-MIC and MBC-EIC, respectively) were fungitoxic to both the sensitive and the benomyl-resistant spores of *Botrytis cinerea* Pers: Nocca & Balbis (Chiba and Northover, 1988). Furthermore, MBC-EIC was effective for the protection of wounded apples inoculated with benomyl-resistant *B. cinerea* and stored briefly at 20 °C, but its efficacy under commercial cold storage was not known. Hence, the present studies were undertaken to examine the effects of temperature upon the rate of in vitro degradation of benomyl and three isocyanate homologues. The activities of these compounds for the protection of wounded apples against *B. cinerea* both sensitive and resistant to benomyl were determined under conditions that simulated commercial cold storage of apple fruit.

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MATERIALS AND METHODS

Synthesis of Compounds. Benomyl was of analytical grade (99% purity) provided by E. I. du Pont de Nemours & Co. Inc. (Wilmington, DE). The methyl, ethyl, and hexyl isocyanate homologues of benomyl (MBC-MIC, MBC-EIC, and MBC-HIC, respectively) were prepared by the reaction of MBC (99% purity) (Du Pont) with, respectively, methyl isocyanate (MIC) (Eastman Kodak Co., Rochester, NY), ethyl isocyanate (EIC) (Aldrich Chemical Co., Milwaukee, WI), and hexyl isocyanate (HIC) (Eastman Kodak Co.). The procedures were described previously (Chiba and Northover, 1988).

Identity of Compounds. The identities of benomyl and the three synthesized compounds were established by elemental analysis, which was reported earlier (Chiba and Northover, 1988), and by proton nuclear magnetic resonance (NMR), mass spectrometry (MS), and high-performance liquid chromatography (HPLC).

The NMR analyses were made with a Bruker AC-200 multinuclear FT-NMR equipped with an Aspect 3000 computer and array processor. The spectra were run in 5-mm-o.d. NMR tubes with deuterated dimethyl sulfoxide as the solvent and tetramethylsilane as the internal standard. Sample concentrations were 15–20 mg/2 mL of solvent. Spectra were collected at ambient

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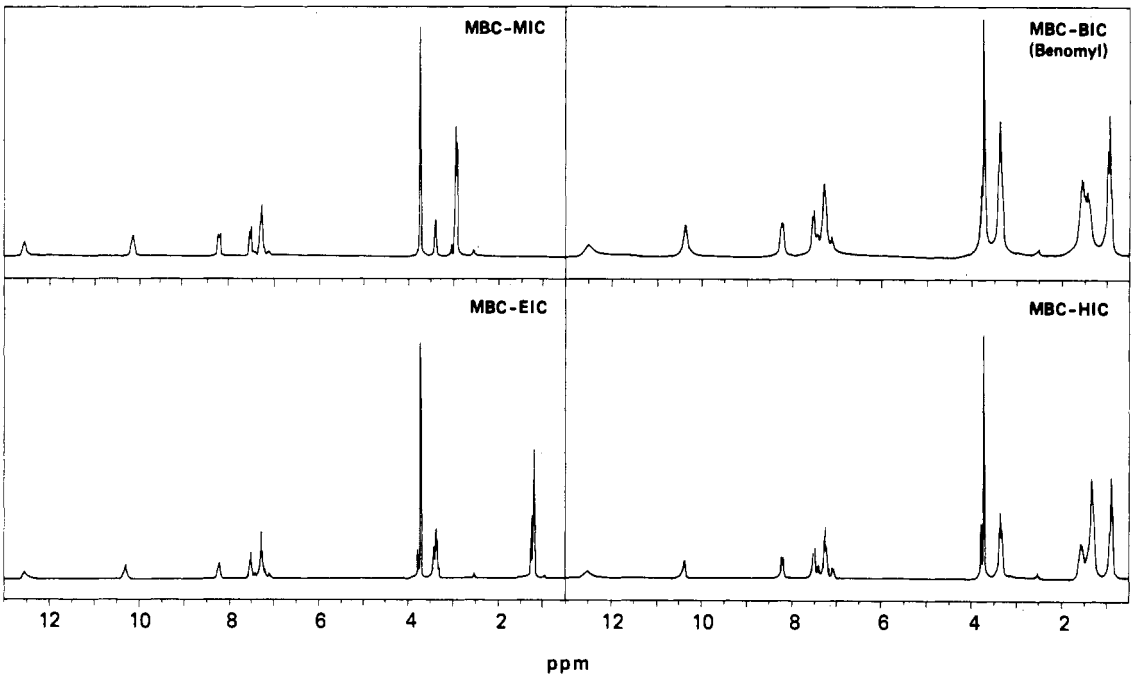

Figure 1. Proton NMR spectra of MBC-MIC, MBC-EIC, and MBC-HIC homologues of MBC-BIC (benomyl).

temperature with 16K data points and 64 averaged transients.

MS analyses were performed with a Kratos/AEI MS 30 mass spectrometer equipped with a DS55 data system using 4 kV at an ionization potential of 70 eV and source temperatures of 180 and 190 °C.

HPLC analyses were conducted with a Hewlett-Packard HP-1090 system equipped with a HP-1040A diode array UV absorption detector at 286 and 294 nm. A mobile phase solvent consisting of CH_3OH and 0.034 M phosphate buffer solution (pH 7.0) at 40:60 (v/v) was used at a flow rate of 0.5 mL/min to check the purity of these compounds and their retention times relative to that of MBC-MIC. The column used was a Regis Hi-Chrom reversible ODS-1, 5 μm, 4.6 mm (i.d.) × 15 cm.

Stability of Compounds in Water. Suspensions of each of the four compounds were prepared in distilled water (pH 6.2) containing dextrose (20 mg/mL) and Tween 20 (polyoxyethylene sorbitan monolaurate; Atlas Chemical Industries, Brantford, Ont.) (0.05 mg/mL) at 1.78, 17.8, and 178 μM and were dispensed into 2-mL HPLC vials and stored at 1, 10, and 25 °C. Replicated samples were analyzed at intervals of approximately 0, 1, 2, 5, 10, 14, 21, 28, 35, and 42 days after commencement of the temperature treatment. HPLC analyses were conducted with a fresh vial of sample at each analysis time. Mobile phase solvents consisting of CH_3OH and 0.034 M phosphate buffer solution (pH 7.0) at volume ratios best suited to the individual compounds: MBC-MIC, 50:50; MBC-EIC, 60:40; MBC-BIC, 65:35; MBC-HIC, 70:30. The flow rate was set at 1 mL/min, and the injection volume was 20 μL.

The data were linearized and examined by regression analyses of the generalized form

$$\ln Y = a + bX$$

where Y was the concentration of the parent compound (μM), X was time (days), a was the calculated initial concentration, and b was the regression coefficient, or negative slope of the degradation curve. Values for the half-lives of each of the four compounds at three concentrations and three temperatures were calculated from the respective regression equations.

Protection of Wounded Apples. The procedures used were similar to those described previously (Chiba and Northover, 1988). McIntosh apples from cold storage (1 °C, air) were surface-sterilized in sodium hypochlorite solution (15 mg/mL) for 3 min, rinsed with sterile water, and dried at 23 °C. Twenty apples were supported on their sides on inverted metal jar lids (5-cm diameter) arranged in a paper-lined, wooded tray (30 × 42 × 8.5 cm), with each tray constituting a treatment unit. Fruits were stored overnight at 10 °C, and then each fruit was punctured once on the upper side to a depth of 4 mm, with a 4-mm-diameter sterilized nail. Each wound was immediately inoculated with a 30-μL drop of freshly prepared spore suspension of either a sensitive (S) or a benomyl-resistant (R) monoconidial isolate of *B. cinerea*. The suspension contained 1×10^4 conidia/mL in a dilute salts solution containing 0.02 mg/mL of sodium citrate and 0.02 mg/mL of potassium citrate. Four hours later when the drop of inoculum had been absorbed, a 30-μL drop of fungicide suspension was added to each wound. The drop contained dextrose (10 mg/mL), Tween 20 (0.05 mg/mL), and test compound at concentrations in the range of 0–3170 μM (Table III), with appropriate concentrations chosen on the basis of previous studies (Chiba and Northover, 1988). A wounded, noninoculated check was also included. Two trays of fruits were treated similarly for each combination of fungicide concentration and S or R inoculum. One tray was incubated at 20 °C for 7 days and evaluated, to simulate the shelf storage of commercial fruit. The second tray was incubated at 1 °C for 42 days, evaluated, and incubated at 20 °C for a further 7 days and evaluated, to simulate commercial cold storage followed by shelf storage. During incubation the trays of apples were stored at a relative humidity >90% and were shrouded in large, dark polyethylene bags to minimize aerial contamination.

Each fruit was evaluated for the presence of a soft brown lesion centered on the wound and for *B. cinerea* sporulation on the larger lesions. Isolations were made from the margins of most of the lesions onto potato dextrose agar (PDA; Difco) unamended, or amended with benomyl at 1 μg/mL prepared by incorporating aqueous suspension of Benlate (Du Pont) into PDA at 50 °C immediately prior to pouring into 9.0-cm Petri dishes. This was to confirm the identity of the pathogen and its sensitivity or resistance to benomyl and its similarity to the inoculum applied to the wound. The data were expressed as percentage inhibition of infection in relation to concentration of the test compound and examined by Probit analysis. The relative efficacies of the four compounds were judged from the computed concentrations giving 50% (EC_{50}) and 95% (EC_{95}) inhibition of the deliberate inoculations.

RESULTS

Identity of the Compounds. The proton NMR spectrum of benomyl (Figure 1) was similar to that reported previously (White et al., 1973), and the spectra of the three benomyl homologues represent the following chemical shifts for resonance groups: at 12.52–12.53 ppm for amide in amido methyl ester, at 10.11–10.37 ppm for amide in side chains, at 7.0–8.2 ppm for ring system, at 3.69–3.71

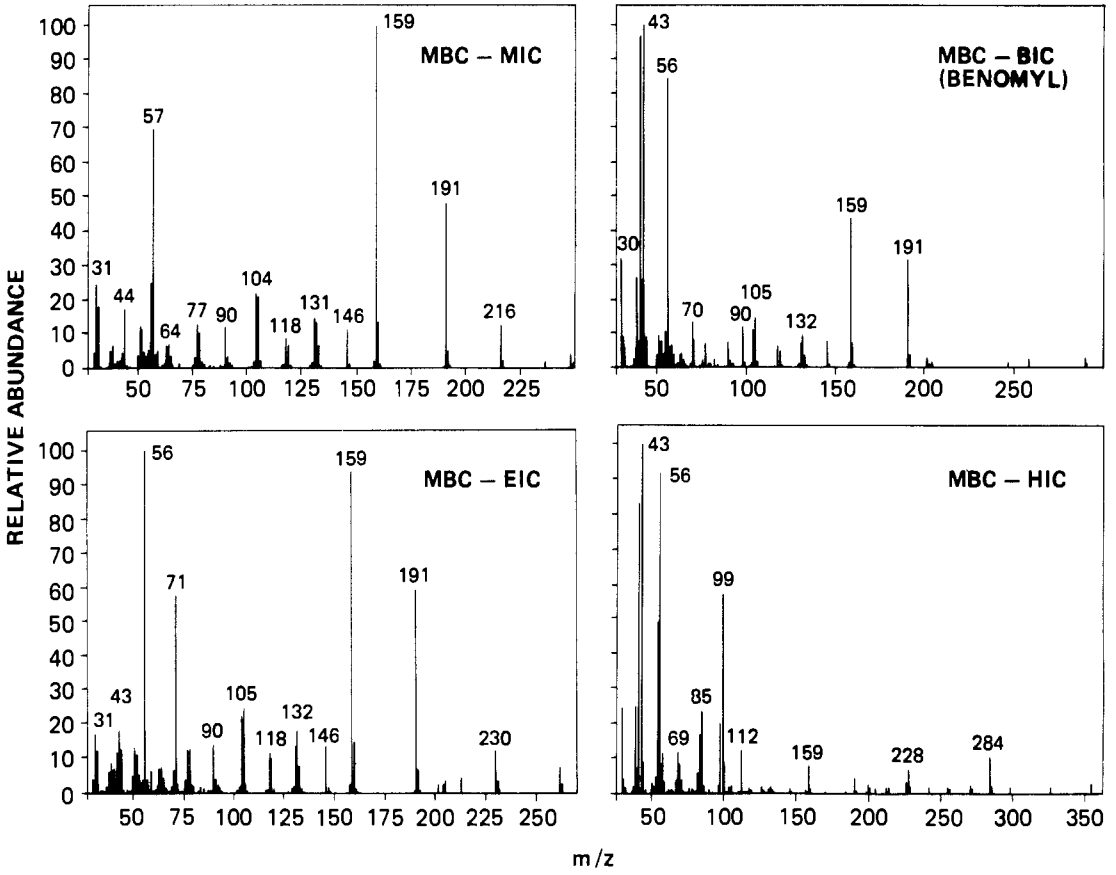

Figure 2. Mass spectra of MBC-MIC, MBC-EIC, and MBC-HIC homologues of MBC-BIC (benomyl).

ppm for methyl in methyl ester, and at 0.88–3.35 ppm for resonances due to side chains (Figure 1).

The MS results show molecular ions of MBC-MIC, MBC-EIC, and MBC-BIC (benomyl) at m/z 248, 262, and 290, respectively (Figure 2). MBC-HIC was the exception and did not show a molecular ion at m/z 318. Probably it had decomposed thermally at 180 °C, because at a slightly higher temperature of 190 °C the molecular ions of MBC-EIC and MBC-BIC also were not observed. MBC-MIC was the only compound showing a molecular ion at 190 °C. The general fragmentation trend for these compounds was toward immediate loss of the isocyanate (RNCO) moiety as demonstrated by CH_3NCO (m/z 57) in MBC-MIC and C_2H_5NCO (m/z 71) in MBC-EIC. MBC was well represented at m/z 191. The peak at m/z 159 signifies the loss of CH_3OH from MBC.

HPLC results proved the high purity of these compounds, and there were no signs of impurities except for a small quantity of MBC with each compound. The presence of MBC in aqueous suspensions of these compounds in the HPLC procedure is unavoidable because of their rapid degradation (White et al., 1973). The retention times of these compounds relative to MBC-MIC are listed in Table I. Also included in Table I are the retention times of MBC and the four test compounds using the different mobile phase solvents best suited to these individual compounds.

Stability of Compounds in Water. The half-life values for benomyl and the three homologues were approximately 2.7–7.7 times greater at a concentration of 178 μM than at 1.78 μM (Table II). Chemical stability was also greatly reduced at higher temperatures, and it varied greatly among the four compounds. The half-life values for benomyl at 178 μM, at 1 and 10 °C, were >42 days, but at 25 °C it was 7.7 days. MBC-HIC at 178 μM showed a moderately flat response to temperature with half-life values at 1, 10, and 25 °C of 33, 15, and 12 days, respectively. MBC-MIC and MBC-EIC were appreciably less stable than benomyl. At 1, 10, and 25 °C, MBC-EIC with

Table I. Retention Time of Tested Compounds in HPLC Analysis

compd	mobile phase CH_3OH–buffer,[a] v/v %	retention time, min		
		MBC	parent	rel to MBC-MIC[b]
MBC-MIC	50:50	3.1	6.5	1.0
MBC-EIC	60:40	2.7	4.6	1.4
MBC-BIC (benomyl)	65:35	2.5	6.9	3.2
MBC-HIC	70:30	2.3	8.0	9.7

[a] 0.034 M phosphate buffer (pH 7.0). [b] A common mobile phase of CH_3OH–buffer (40:60, v/v, %) was used.

Table II. Half-Life Values (Days) of Benomyl and Benomyl Homologues at Three Initial Concentrations in Water at Three Temperatures

compd	temp, °C	concentration, μM		
		1.78	17.8	178
MBC-MIC	1	10.3	6.5	27.4
	10	1.9	1.6	6.1
	25	0.4	0.2	1.2
MBC-EIC	1	18.0	17.3	>42
	10	1.5	3.3	8.4
	25	NA[a]	0.9	3.9
MBC-BIC (benomyl)	1	>42	>42	>42
	10	19.6	12.6	>42
	25	1.0	2.1	7.7
MBC-HIC	1	NC[b]	18.5	33.1
	10	NC	12.4	15.0
	25	NC	6.2	12.2

[a] Insufficient data were available to calculate the half-life. [b] Concentrations were too low for detection, and the half-life values were not calculated.

Table III. Efficacy of Topical Applications of Benomyl and Three Benomyl Homologues, Made to Wounded and Inoculated Apples, for Reducing Percentage Fruit Infection by Sensitive (S) and Benomyl-Resistant (R) Isolates of *B. cinerea*, after Storage at 1 °C for 42 Days Followed by 20 °C for 7 Days

	MBC-MIC		MBC-EIC		MBC-BIC		MBC-HIC	
concn, μM	S	R	S	R	S	R	S	R
0	100[a]	100	100	100	100	100	100	100
10	–[b]	–	–	–	100	–	–	–
18	100	–	100	–	100	–	–	–
24	–	–	–	–	100	–	–	–
32	100	–	100	–	90	–	100	–
42	–	–	–	–	80	–	–	–
56	100	100	95	100	65	–	100	–
75	–	–	–	–	68	–	–	–
100	95	95	70	100	30	–	100	100
178	50	100	28	85	26	–	100	100
317	40	84	11	45	–	–	100	100
564	16	65	0	15	–	–	95	100
1000	–	15	–	11	–	–	100	100
1787	–	5	–	0	–	100	85	100
3170	–	–	–	–	–	100	–	100
noninoculated check	5	–	0	–	0	–	0	–

[a] Percentage of infected fruits of the 20 wounded and inoculated apples per treatment. [b] Treatment not conducted; data not available.

Table IV. Concentrations of Benomyl and Benomyl Homologues Giving 50% (EC_{50}) and 95% (EC_{95}) Inhibitions of Infections of Apples by Sensitive (S) and Benomyl-Resistant (R) *B. cinerea*, As Affected by Posttreatment Storage at 1 and/or 20 °C

storage period (days) at			EC_{50}, μM		Rf of EC_{50}	EC_{95}, μM	
1 °C	20 °C	compd	S	R	values[a]	S	R
0	7	MBC-MIC	146 BC[b]	292 B*[c]	2.0	482 A	831 A
		MBC-EIC	76 A	278 B*	3.7	253 A	789 A*
		MBC-BIC	68 A	>3170*	>46.6	E 342 AB	>3170*
		MBC-HIC	>1787	>3170	>1.8	>1787	>3170
42	0	MBC-MIC	166 BC	79 A*	0.48	E 845 B	409 A
		MBC-EIC	92 A	64 A	0.70	305 A	433 A
		MBC-BIC	73 A	>3170	>43.4	273 AB	>3170*
		MBC-HIC	>1787	>3170	>1.8	>1787	>3170
42	7	MBC-MIC	245 C	631 C*	2.6	E 796 BC	1601 A
		MBC-EIC	137 B	333 B*	2.4	348 A	943 A*
		MBC-BIC	89 AB	>3170*	>35.6	E 324 AB	>3170
		MBC-HIC	>1787	>3170	>1.8	>1787	>3170

[a] The resistance factor was calculated by dividing the EC_{50} value for R by the EC_{50} value for S in the corresponding treatment. [b] Values in the same column followed by dissimilar letters are significantly different ($P = 0.05$) with nonoverlapping 95% confidence limits. Values with the prefix E are calculated values greater than the highest concentration tested. Values without letters were the highest concentrations tested, but at which efficacy was less than 50% or 95% in the appropriate columns. The EC_{50} and EC_{95} values could not be calculated but were expected to exceed the highest concentration tested, hence the ">" symbol. [c] The asterisk indicates that the value for the resistant isolate is significantly different ($P = 0.05$) from that for the sensitive isolate in the respective EC_{50} or EC_{95} comparison.

half-life values of >42, 8.4, and 3.9 days, respectively, was slightly more stable than MBC-MIC with corresponding half-life values of 27.4, 6.1, and 1.2 days at 178 μM (Table II). An unidentified compound with a shorter retention time than MBC was formed from MBC-MIC and MBC-EIC.

Protection of Wounded Apples. There was virtually no infection of the noninoculated wounded apples by *B. cinerea*, but there was a very low incidence of infection caused by *Penicillium spp.* In a few trays it was necessary to discount one or two apples per treatment, because of infections unrelated to the inoculation that encroached upon the inoculation site. In the check treatments, inoculated with S or R, 100% of the apples was infected at 20 °C after 7 days, or at 1 °C after 42 days. The lesions were about 50 mm in diameter and were similar in appearance. For check-inoculated apples stored at 1 °C for 42 days and then incubated at 20 °C for 7 days, the lesions were greater than 50 mm in diameter and often enveloped individual apples.

Systematic testing of fungal isolates from apple lesions on PDA and PDA amended with 1 μg/mL of benomyl confirmed that there was no measurable selection of benomyl-resistant *B. cinerea* in the chemically treated apples inoculated with the sensitive isolate. Nor were sensitive isolates recovered from apples inoculated with the R isolate.

The incidence of infection in apples stored at 1 °C for 42 days followed by 20 °C for 7 days (1/20 °C program) is shown in Table III. Similar bodies of data were obtained from apples stored at 20 °C for 7 days (20 °C program) and for those that were evaluated after storage at 1 °C for 42 days (1 °C program). The EC_{50} and EC_{95} values were calculated for each of the 24 groups of data, by Probit analysis, and these values are displayed in Table IV. In a comparison of the EC_{50} values for the S isolate, MBC-EIC was as effective as MBC-BIC (benomyl) and was more effective than MBC-MIC, in each of the three storage programs. MBC-HIC showed little activity with an EC_{50} > 1787 μM. Against the R isolate, in the 1/20 °C program, MBC-EIC (EC_{50} 333 μM) was more effective than MBC-MIC (EC_{50} 631 μM), but in the 20 °C storage study the activities were similar and were superior to those of MBC-BIC (benomyl) and MBC-HIC, both of which gave no protection against R at 3170 μM.

The calculated EC_{95} values for S were generally about 3 times greater than the corresponding EC_{50} values. MBC-MIC was comparable to MBC-BIC but was less effective than MBC-EIC in the 1 °C and 1/20 °C programs (Table IV). The higher EC_{95} values needed to provide

protection against R in the 1/20 °C program were 943 μM for MBC-EIC and 1601 μM for MBC-MIC. In contrast, the two remaining compounds were inactive against R even at 3170 μM.

The effect of temperature upon fungitoxicity was judged from the EC_{50} values for the 20 and 1 °C programs. Against S, the EC_{50} values for MBC-MIC, MBC-EIC, and MBC-BIC (benomyl) were similar in both programs. Against R, the EC_{50} values were significantly lower for MBC-MIC and MBC-EIC at 1 °C than at 20 °C. However, when the cold-stored apples were incubated at 20 °C for a further 7 days, many more R lesions developed and the EC_{50} values against R in the 1/20 °C program became comparable to or higher than those for the 20 °C program.

In the 42-day cold-storage study at 1 °C, with the EC_{50} values, MBC-BIC (benomyl) was ineffective against the R isolate with a resistance factor (Rf) greater than 43 (Table IV). In sharp contrast, MBC-EIC was equally effective against R and S (Rf = 0.70) and MBC-MIC was significantly more effective against R than S (Rf = 0.48). However, with subsequent incubation of these fruits at 20 °C for 7 days the differential activity of these compounds changed. In the 1/20 °C program the Rf values for MBC-MIC and MBC-EIC were 2.6 and 2.4, respectively, resembling comparable Rf values of 2.0 and 3.7, respectively, in the 20 °C program.

DISCUSSION

The identities of the three synthesized alkyl isocyanate homologues of benomyl were established convincingly by NMR, MS, and HPLC. The supporting elemental analyses of these compounds were reported earlier (Chiba and Northover, 1988). In the studies of chemical stability in aqueous suspension, the principal degradation product was MBC, although smaller amounts of a unidentified compound, other than the corresponding alkyl isocyanate, and having a shorter retention time than MBC, were formed from MBC-MIC and MBC-EIC.

The rate of degradation of the homologues appeared slower at the higher concentrations of each of the parent compounds. This was anticipated because these compounds had low aqueous solubilities, possibly similar to that of benomyl (<4 μg/mL at pH 3–10) (Singh and Chiba, 1985). At 178 μM, most of the parent compound remained insoluble in a saturated solution and therefore chemically stable. It is supposed that only those parent molecules entering solution could degrade, so that the rate of degradation would have been influenced strongly by the aqueous solubility of the compounds.

The four parent compounds in aqueous suspension at 178 μM had half-life values of 27–>42 days at 1 °C. However, they were increasingly unstable at 10 and 25 °C. Agricultural conditions best suited to the use of these compounds as fungicides would involve low temperatures such as hydrocooling and the dipping or spraying of produce prior to cold storage. The low temperature of the suspension would delay the degradation of the fungicide in the dipping tank. Also, the period of protection of the treated produce would be longer at lower storage temperatures, because of the greater persistence of the residues.

In the apple protection study involving storage at 20 °C for 7 days, the performance of MBC-BIC (benomyl) and MBC-EIC was in good agreement with those reported earlier (Chiba and Northover, 1988). The calculated EC_{50} values were slightly higher for MBC-EIC against the sensitive isolate (S) (76 versus 39 μM), but again MBC-EIC was dramatically effective (EC_{50} = 278 μM) against benomyl-resistant *B. cinerea*, in contrast to the inefficacy of MBC-BIC (benomyl) (EC_{50} > 3170 μM).

In the 1/20 °C program involving cold storage at 1 °C for 42 days followed by 20 °C for 7 days, the EC_{50} values in some cases were up to double those for the 20 °C program, and the difference was significant for MBC-EIC against S. Doubt was expressed earlier (Chiba and Northover, 1988) that the performance of these fungicides in the short-term 20 °C program could be extrapolated to long-term commercial cold storage. Indeed, the results of the 20 and the 1/20 °C programs were qualitatively similar and differed quantitatively by not more than a factor of 2, in some cases. Nevertheless, this concern was vindicated by the current findings where MBC-MIC and MBC-EIC were more effective against R at 1 than 20 °C.

The extrapolated EC_{95} value for MBC-BIC (benomyl) against S in the 1/20 °C program was 324 μM. The concentration of benomyl recommended (OMAF, 1986) for dipping apples for protection of wounds against *B. cinerea* and *Penicillium expansum* of 250 μg/mL is equivalent to 862 μM or 2.7 times the EC_{95} value for S. However, this treatment is ineffective against benomyl-resistant isolates. By comparison, the EC_{95} values of MBC-EIC against S and R in the 1/20 °C program were 348 and 943 μM, respectively. Utilizing the conversion factor of 2.7, the commercial concentration of active ingredient, would approximate 940 μM against S and 2500 μM against both S and R. These concentrations are high but should not be too high to preclude the use of MBC-EIC in a dip or spray application.

MBC-MIC and MBC-EIC exhibited cross-resistance (CR) to MBC-BIC in the 20 and 1/20 °C programs with Rf values between 2.0 and 3.7. In sharp contrast, in the 1 °C program MBC-MIC was more active against R than S (Rf 0.48), constituting an example of negatively correlated cross-resistance (NCCR) that was also temperature-dependent (TD), because the response at 20 °C was only of cross-resistance (CR). A similar TD-NCCR response was reported for diphenylamine (DPA) against apple infections by benomyl-resistant *Penicillium expansum* (Rosenberger and Meyer, 1985) with activity at 2.2 °C but not at 16–22 °C. However, the response obtained with DPA was different from that obtained here with MBC-MIC, because with DPA NCCR occurred in mycelial growth studies at 20 °C for *P. expansum* (Rosenberger and Meyer, 1985) and for *B. cinerea* (Sharom and Edgington, 1985). In our germ tube studies conducted at 20 °C, MBC-MIC and MBC-EIC showed only CR with Rf values of 4 and 3, respectively (Chiba and Northover, 1988), closely resembling the Rf values for the apple infection studies in the 20 and 1/20 °C programs reported here.

It was surmised earlier (Chiba and Northover, 1988) that these benomyl homologues might have a dual mode of action similar to that of benomyl (Hammerschlag and Sisler 1973). Upon degradation, the MBC moiety interferes with spindle microtubule assembly and nuclear division of S isolates (Davidse, 1986), whereas the alkyl isocyanate moiety might be biologically active against both the S and R isolates. MIC, BIC, and HIC were shown to inhibit respiration and fermentation of yeast (Chiba et al., 1987). However, MIC and EIC are very reactive and would hydrolyze rapidly to the respective amine and carbon dioxide.

When the apples that had been stored at 1 °C for 42 days were incubated at 20 °C for 7 days, a substantial number of lesions developed from sites where *B. cinerea* had remained quiescent at the lower temperature. This resulted in a worsened assessment of MBC-MIC and

MBC-EIC especially against the R isolate. The fungitoxicity at 1 °C may be described as having been partially fungistatic rather than entirely fungicidal. The fungistatic effect persisted at 1 °C but was lost quickly at 20 °C, possibly because the MBC-MIC and MBC-EIC degraded more rapidly at the higher temperature.

Fungistasis was also a characteristic of alkylamines (Eckert and Kolbezen, 1963). Sharom and Edgington (1985) showed that, with prolonged storage of inoculated grapes, most of the diphenylamine treatments ultimately failed, and we interpret this as evidence of fungistatic activity. These observations strengthen the hypothesis that amines rather than isocyanates may be important fungitoxic degradation products of MBC-MIC and MBC-EIC.

However, contrary to this hypothesis of the involvement of amines is the evidence that the lower alkyl amines show only slight fungitoxicity (Eckert and Kolbezen, 1963). Ethylamine hydrochloride and *n*-butylamine hydrochloride had very high EC_{50} values of 30 000 μM, which contrasted with the more active *sec*-butylamine hydrochloride with an EC_{50} value of 300 μM, against spore germination of *Penicillium digitatum*. The toxicity of methylamine hydrochloride was not examined; therefore, the possible involvement of methylamine in the toxicity of MBC-MIC remains speculative. During the degradation of MBC-MIC or MBC-EIC in apple tissue, it is anticipated that several potentially fungitoxic compounds could be present simultaneously, including the parent compound, MBC, the corresponding isocyanate and amine, and possibly the unidentified compound detected in the in vitro degradation studies. With the exception of MBC, any of these materials either by themselves or in combination, could have contributed to the fungitoxicity of MBC-MIC and MBC-EIC, and particularly to the TD-NCCR activity against benomyl-resistant *B. cinerea*.

N-Phenylcarbamates are also of particular interest for the control of benzimidazole-resistant plant pathogens because they exhibit marked NCCR activity at temperatures of 18–20 °C (Elad et al., 1988; Kato et al., 1984; Takahashi et al., 1988). The mode of action of the *N*-phenylcarbamates and of diphenylamine upon benzimidazole-resistant pathogens has not been fully elucidated (Suzuki et al., 1984; Davidse, 1986).

The methyl and ethyl isocyanate homolgues of benomyl appear to offer a novel mechanism for the circumvention of benzimidazole resistance in *B. cinerea*. The fungicidal activity is most pronounced at low temperatures, at which the chemicals are most stable, indicating a possible use for these chemicals as fungicides for hydrocooling and for the protection of cold-stored fruits and vegetables.

ABBREVIATIONS USED

MIC, methyl isocyanate; EIC, ethyl isocyanate; BIC, butyl isocyanate; HIC, hexyl isocyanate; MBC, carbendazim, methyl 1*H*-benzimidazol-2-ylcarbamate; MBC-MIC, methyl [1-(methylcarbamoyl)-1*H*-benzimidazol-2-yl]carbamate; MBC-EIC, methyl [1-(ethylcarbamoyl)-1*H*-benzimidazol-2-yl]carbamate; MBC-BIC, benomyl, methyl [1-(butylcarbamoyl)-1*H*-benzimidazol-2-yl]carbamate; MBC-HIC, methyl [1-(hexylcarbamoyl)-1*H*-benzimidazol-2-yl]carbamate.

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LITERATURE CITED

Bollen, G. J.; Scholten, G. Acquired resistance to benomyl and some other systemic fungicides in a strain of *Botrytis cinerea* in cyclamen. *Neth. J. Plant Pathol.* **1971**, *77*, 83–90.

Chiba, M.; Northover, J. Efficacy of new benzimidazole fungicides against sensitive and benomyl-resistant *Botrytis cinerea*. *Phytopathology* **1988**, *78*, 613–618.

Chiba, M.; Bown, A. W.; Danic, D. Inhibition of yeast respiration and fermentation by benomyl, carbendazim, isocyanates, and other fungicidal chemicals. *Can. J. Microbiol.* **1987**, *33*, 157–161.

Davidse, L. C. Benzimidazole fungicides: mechanism of action and biological impact. *Annu. Rev. Phytopathol.* **1986**, *24*, 43–65.

Dekker, J. Acquired resistance to fungicides. *Annu. Rev. Phytopathol.* **1976**, *14*, 405–428.

Delp, C. Coping with resistance to plant disease control agents. *Plant Dis.* **1980**, *64*, 652–657.

Eckert, J. W.; Kolbezen, M. J. Control of penicillium decay of oranges with certain volatile aliphatic amines. *Phytopathology* **1963**, *53*, 1053–1059.

Elad, Y.; Shabi, E.; Katan, T. Negative cross resistance between benzimidazole and *N*-phenylcarbamate fungicides and control of *Botrytis cinerea* on grapes. *Plant Pathol.* **1988**, *37*, 141–147.

Hammerschlag, R. S.; Sisler, H. D. Benomyl and methyl-2-benzimidazolecarbamate (MBC): biochemical, cytological and chemical aspects of toxicity to *Ustilago maydis* and *Saccharomyes cerevesiae*. *Pestic. Biochem. Physiol.* **1973**, *3*, 42–54.

Kato, T.; Suzuki, K.; Takahashi, J.; Kamoshita, K. Negatively correlated cross-resistance between benzimidazole fungicides and methyl *N*-(3,5-dichlorophenyl) carbamate. *J. Pestic. Sci.* **1984**, *9*, 489–495.

Northover, J. Characterization and detection of benomyl resistant *Venturia inaequalis* in Ontario apple orchards. *Can. J. Plant Pathol.* **1986**, *8*, 117–122.

Northover, J.; Matteoni, J. A. Resistance of *Botrytis cinerea* to benomyl and iprodione in vineyards and greenhouses after exposure to the fungicides alone or mixed with captan. *Plant Dis.* **1986**, *70*, 398–402.

Ontario Ministry of Agriculture and Food. *Fruit Production Recommendations*; Publication 360; Legislative Buildings: Toronto, Ontario, Canada M7A 1A2, 1986; p 78.

Rosenberger, D. A.; Meyer, F. W. Negatively correlated cross-resistance to diphenylamine in benomyl-resistant *Pencillium expansum*. *Phytopathology* **1985**, *75*, 74–79.

Sharom, M. S.; Edgington, L. V. Temperature dependent negatively correlated cross-resistance between benomyl and diphenylamine for *Botrytis cinerea*, *Gerlachia nivalis*, and *Monilinia fructicola*. *Can. J. Plant Pathol.* **1985**, *7*, 389–394.

Singh, R. P.; Chiba, M. Solubility of benomyl in water at different pHs and its conversion to methyl 2-benzimidazole carbamate, 3-butyl-2,4-dioxo(1,2-*a*)-*s*-triazinobenzimidazole, and 1-(2-benzimidazolyl)-3-*n*-butylurea. *J. Agric. Food Chem.* **1985**, *33*, 63–67.

Suzuki, K.; Kato, T.; Takahashi, J.; Kamoshita, K. Mode of action of methyl *N*-(3,5-dichlorophenyl)-carbamate in the benzimidazole-resistant isolate of *Botrytis cinerea*. *J. Pestic. Sci.* **1984**, *9*, 497–501.

Takahashi, J.; Nakamura, S.; Noguchi, H.; Kato, T.; Kamoshita, K. Fungicidal activity of *N*-phenylcarbamates against benzimidazole resistant fungi. *J. Pestic. Sci.* **1988**, *13*, 63–69.

White, E. R.; Bose, E. A.; Ogawa, J. M.; Manji, B. T.; Kilgore, W. W. Thermal and base-catalyzed hydrolysis products of the systemic fungicide, benomyl. *J. Agric. Food Chem.* **1973**, *21*, 616–618.

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