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Herbicidal Diphenyl Ethers: Stereochemical Studies Using Enantiomers of a Novel Diphenyl Ether Phthalide

Patrick Camilleri,* Alan Gray, Karen Weaver, John R. Bowyer, and David J. Williams

Nitrodiphenyl ethers are a highly active class of herbicides that cause light-dependent membrane lipid peroxidation, but the molecular basis of their herbicidal effects is not known. As part of a program to elucidate their primary mode of action, small quantities of the enantiomers of a novel diphenyl ether phthalide have been isolated by high-performance liquid chromatography, using a chiral stationary-phase column, and their relative biological activities have been evaluated. The characteristics of the herbicidal effects of the phthalide diphenyl ether appear to be identical with those of nitrodiphenyl ether herbicides. The *S*-(−) isomer was found to be substantially more active than the *R*-(+) isomer in a test designed to monitor plant membrane breakdown by following ethane production. This selectivity was confirmed in a glasshouse bioassay when the activity of the two isomers on six plant species was determined. The information detailed in this report is the first evidence for the likely involvement of an enzymatic binding process in the mode of action of peroxidizing diphenyl ether herbicides.

Nitrodiphenyl ethers (NDPE's) are highly effective and fast-acting herbicides that are primarily used in the selective control of broadleaf weeds in a variety of crops such as soyabean, barley, wheat, and rice. The effectiveness of this class of herbicides depends on the presence of light (Fadayomi and Warren, 1976; Matsunaka, 1969) and oxygen (Kunert and Böger, 1984; Orr and Hess, 1982; Kenyon and Duke, 1985). Several studies (Vanstone and Stobbe, 1979; Bowyer et al., 1987) have shown that treatment of plants with NDPE's in the light leads to considerable ultrastructural damage after a relatively short time (<12 h after application). The usual symptoms are the initial disruption of the tonoplast membrane and the chloroplast envelope, followed by movement of the ruptured chloroplasts and cytoplasm away from the cell wall, and finally destruction of the thylakoid system. The result of this damage is the evolution of hydrocarbons, predominantly ethane, which are products of decomposition of ω^3-unsaturated fatty acid hydroperoxides. It has been reported that the oxidative damage to plants by NDPE's can be reduced by the presence of antioxidants such as vitamins C and E (Kunert and Boger, 1984; Kenyon and Duke, 1985).

Studies to date have not allowed a distinction to be made between primary and secondary modes of action of NDPE's. A great deal of controversy exists in the literature about the primary mode of action of this class of compounds. From a study (Lambert et al., 1983, 1984) of the mode of action of NDPE's on the green alga *Scenedesmus obliquus* it was stated that photosynthetic electron transport is involved in a manner similar to that in the case of paraquat. This acts as a photosystem I electron acceptor, leading to the production of toxic oxygen species. However, these results are at variance with studies carried out on another green alga *Chlamydomonas eugametos* (Ensminger and Hess, 1985a) and on plants (Fadayomi and Warren, 1976; Matsunaka, 1969, Orr and Hess, 1982; Bowyer et al., 1987; Camilleri et al., 1988) and flower petals

Sittingbourne Research Centre, Shell Research Limited, Sittingbourne, Kent ME9 8AG, U.K. (P.C., A.G., K.W.), Department of Biochemistry, Royal Holloway and Bedford New College, Egham Hill, Egham, Surrey TW20 0EX, U.K. (J.R.B.), and Department of Chemistry, Imperial College, London SW7 2AY, U.K. (D.J.W.).

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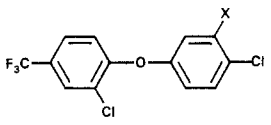
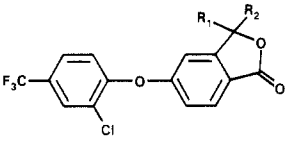
(Bowyer et al., 1987). The latter studies strongly indicate that neither photosystem I nor photosystem II is directly involved in the primary action of NDPE's. Moreover, some diphenyl ether molecules with redox potentials that, unlike those of NDPE's, are well beyond the range likely to be reduced by the plant's photosynthetic apparatus (Ensminger and Hess, 1985b; Ensminger et al., 1985; Ridley, 1983) show the same effects on the ultrastructure of treated leaves and on ethane evolution. Examples of such molecules are those in which the nitro group has been replaced by a halogen substituent (I) or the phthalide derivatives (II) introduced by us recently (Camilleri et al., 1988).



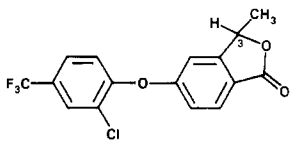
(X = CH_3, OCH_3 and NO_2)
(I)



(R_1 and R_2 are generally H or alkyl)
(II)

These observations suggest that DPE's may act at a site outside the thylakoid system but still containing carotenoid, e.g. the chloroplast (or chromoplast) envelope.

In an effort to increase our understanding of the chemical nature of the effect of DPE's, we have carried out detailed studies on the phthalide diphenyl ether, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-3-methylphthalide (III). This compound contains two enantiomeric forms,



(III)

differing only by their absolute configuration at the 3-position of the phthalide ring. In this report the analytical separation, the determination of the absolute configuration, and the herbicidal activities of the two enantiomers are described.

MATERIALS AND METHODS

Chromatographic Isolation of the Enantiomers of III. The chromatographic resolution was carried out on a sample of III that had an assigned purity of 98 ± 2%. The high-performance liquid chromatographic conditions used to isolate milligram quantities of the individual enantiomers are summarized. Column: 0.25 m × 20 mm (i.d.) stainless steel packed with the stationary phase S5 CHIRAL (UMIST Batch), 5-μm particle size, supplied by Phase Separations Ltd. Mobile phase: propan-2-ol plus hexane (0.5 × 99.5, v/v) at a flow rate of 10 mL/min. Temperature: ambient (about 18 °C). Detection: UV absorbance at 240 nm in a 0.8-μL-volume flow cell. Sample injection: 100 μL at 8 mg/mL III in chloroform + hexane, (3 + 97, v/v). Retention times: (−) enantiomer 28.6 min; (+) enantiomer 29.1 min.

Under the above conditions the two enantiomers were not completely resolved (Figure 1). High-purity samples (ca. 95%) of the individual enantiomers were therefore obtained by collecting only the front part of the first eluting peak and the back part of the second peak. A total of 90 sample injections were made, and about 12 mg of each fraction was collected.

Solutions of the isolated enantiomers were analyzed with the same HPLC conditions (Figure 1). The purity of each isomer was determined as 95 (±2)%. These samples were used for the determination of the absolute configuration and the herbicidal activity of the two enantiomers.

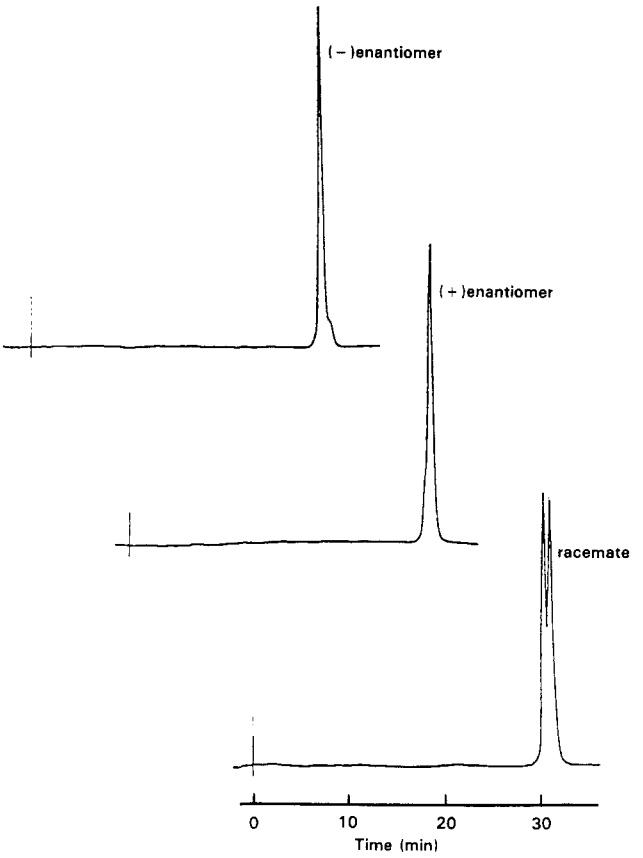


Figure 1. HPLC analysis of isolated enantiomers of III.

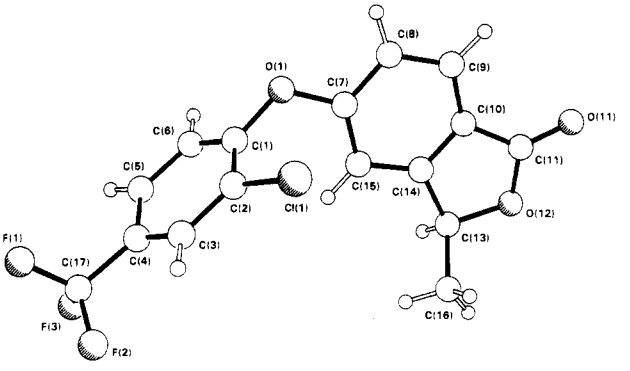


Figure 2. X-ray crystal structure of the *S*-(−) enantiomer of III.

Characterization. The chemical identity of the two enantiomers was confirmed by chemical ionization mass spectrometry (Finnigan 4500 mass spectrometer) with methane reagent gas. A parent ion was observed at m/z 343 ($(M + 1)^+$, 1 chlorine pattern) together with m/z 383 (C_3H_5 addition), m/z 371 (C_2H_5 addition), m/z 323 (HF elimination), m/z 307 (HCl elimination), and m/z 287 (HF and HCl elimination).

Optical rotation of isomers was determined with a Perkin-Elmer 141 polarimeter at the sodium D line (589 nm). Specific rotations for the (−) and the (+) isomers were determined approximately at −33° and +27°, respectively. The absolute configuration of the (−) enantiomer was determined by X-ray crystallography (Figure 2). This result showed that the (−) isomer has the *S* configuration at C-3. This is in accord with the absolute configuration determined for (−)-3-methylphthalide, which was found to have the same configuration on the basis of the known absolute configuration of methylphenylcarbinol (Nagai et al., 1965).

Biological Testing of the Separated Enantiomers of III. *Ethane Measurement.* French beans (*Vicia faba*)

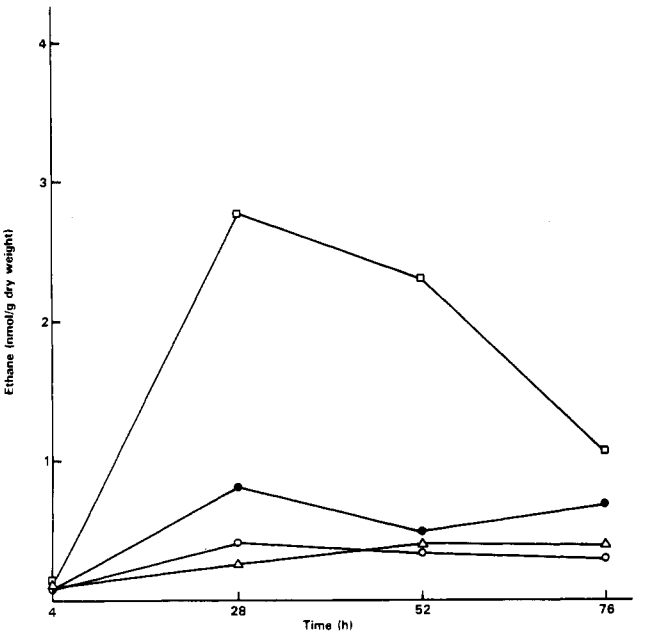


Figure 3. Production of ethane from barley leaves treated with 10^{-5} mol dm^{-3}: (□) *S*-(−) enantiomer; (○) *R*-(+) enantiomer; (●), *R*/*S* enantiomer mixtures; (△) control.

and barley (*Hordeum vulgare*) were grown for 8 days in a growth cabinet (light intensity about 35 W m^{-2}) before treatment. Stock solutions of the separated enantiomers and the unresolved mixture of III were prepared in dimethyl sulfoxide at a concentration of 10^{-2} mol dm^{-3}. For the biological testing, aliquots from the stock solutions were diluted with 0.01% Triton X100 in water to yield concentrations of 5×10^{-5}, 10^{-5}, 5×10^{-6}, and 10^{-6} mol dm^{-3}.

Barley stems were painted with 5×10^{-5} and 10^{-5} mol dm^{-3} concentrations whereas French bean plants were treated with the lower concentrations of 5×10^{-6} and 10^{-6} mol dm^{-3} by complete immersion of the two primary leaves of each plant. This concentration difference allowed for differences in surface area and the physical characteristics of the two plants. Sufficient numbers of plants were treated to allow sampling in triplicate at 0, 24, 48, and 72 h after treatment. Control plants were treated in a similar manner with aqueous 0.01% Triton X100 solution. After the plants had dried, they were returned to the growth cabinet for periods of 0, 24, 48, and 72 h. The barley leaves were cut and placed in glass test tubes (four leaves per tube) that were then sealed with rubber suba seals. The french bean leaves were placed in 25-mL conical flasks (two leaves per flask) and again sealed with rubber suba seals. The tubes and flasks were then exposed to light of intensity 330 W m^{-2}, for a further 4 h.

For analysis, a 1-mL volume of headspace gas was removed and injected into a Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector. The chromatographic conditions for the quantitative estimation of ethane were as follows: glass column (1.5 m × 4 mm (i.d.)) packed with Silica F-1 support; oven temperature, 125 °C; injection port temperature, 150 °C; detector temperature, 200 °C; carrier gas, nitrogen at 50 cm^3 min^{-1}. Under these conditions ethane had a retention time of 48 s. The concentration (ppm) of ethane in the headspace was calculated by peak height measurements relative to a standard. This was divided by the dry weight of the plant material (obtained by desiccating leaves in a freeze drier) to give the results presented in Figures 3 and 4, expressed as parts per million of hydrocarbon per gram dry weight of plant material.

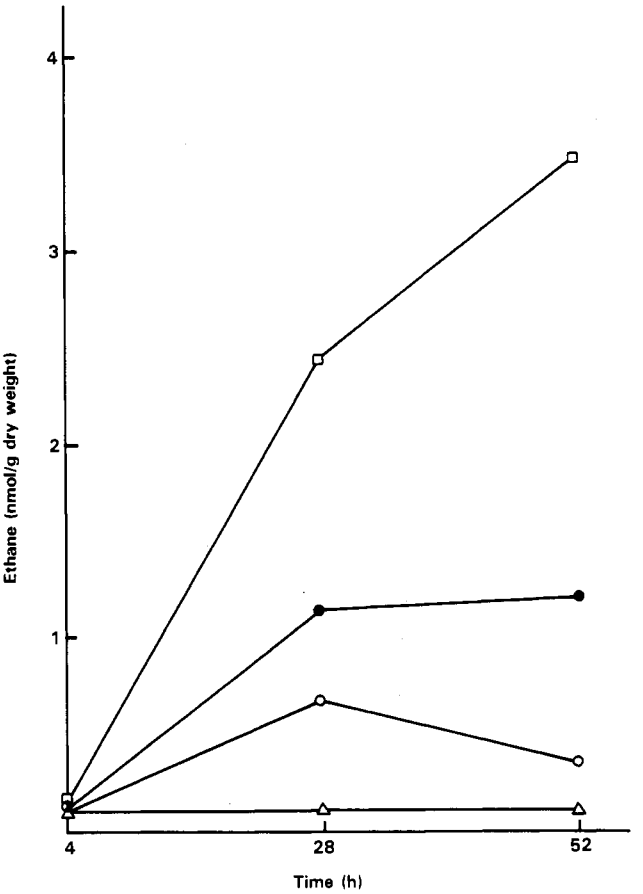


Figure 4. Production of ethane from french bean leaves treated with 10^{-6} mol dm^{-3}: (□) *S*-(−) enantiomer; (○) *R*-(+) enantiomer; (●) *R*/*S* enantiomer mixture; (△) control.

Table I. Glasshouse Bioassay

species[a]	GID_{90},[b] kg ha^{-1}		
	(−) isomer	racemic mixture	(+) isomer
WH	0.50	0.60	1.50
GG	0.50	0.60	1.50
BA	0.20	0.30	1.00
ST	0.10	0.20	0.60
FB	0.06	0.08	0.30
SW	0.02	0.04	0.07

[a] Symbols for each species are explained in Materials and Methods. [b] Dosage required to give 90% effect. GID_{90} values were assessed 21 days after treatment.

Glasshouse Bioassay. The plant species in the glasshouse bioassay were wheat (WH, *Triticum aestivum*), barley (BA, *H. vulgare*), french bean (FB, *V. faba*), cleavers (GG, *Galium aparine*), chickweed (ST, *Stellaria media*), and speedwell (SW, *Veronica persica*). Compounds were applied at doses of 0.06, 0.02, 0.006, and 0.002 kg ha^{-1} to all species and were formulated in a 1:1 acetone–water mixture containing about 0.2% Triton X155.

Phytotoxicity was assessed visually on a 0–9 scale (0 = no effect; 9 = dead) at 2, 8, 14, and 21 days after treatment. The dose required to give a 90% level of effect (GID_{90}) for each species, 21 days after treatment, was plotted for each compound to identify any pattern of selectivity (Table I).

RESULTS AND DISCUSSION

Ethane production is a good measure of the level of plant membrane damage after chemical treatment (Bowyer et al., 1987). The gas is produced via the peroxidation of membrane lipids in a free-radical mechanism. Figures 3 and 4 show that the amount of ethane produced by plants treated with the *S*-(−) isomer of III is substantially higher

than that from plants treated with the *R*-(+) isomer. As expected, the activity of the racemate lies in between that of the separate enantiomers. The decrease in hydrocarbon production after 24-h treatment of barley (Figure 3) was due to recovery of the plants; this phenomenon was also evident from visual inspection. French bean is more sensitive to herbicide treatment than barley, and concentrations as low as 10^{-6} mol dm^{-3} had to be applied so that differences could be observed between plants treated with the enantiomers and the racemate (Figure 4).

The difference in activity between the enantiomeric forms of III was further confirmed in a glasshouse bioassay using wheat, barley, french bean, and three weed species, cleavers, chickweed, and speedwell (Table I). All treatments demonstrated a similar spectrum of activity. As in the ethane test, barley damage declined with time, while french bean was much more sensitive to treatment. Wheat behaved in a manner similar to that of barley. The remaining species (with the exception of cleavers) were generally more susceptible to treatment, and in these cases activity was found to peak at the 8-day visual assessment. Statistical analysis of the results obtained from this glasshouse bioassay broadly confirmed the data from the ethane bioassay experiments. The activity of the *S*-(−) isomer was calculated as being 4 times more active than that of the *R*-(+) isomer, while the racemic mixture was found to be about 1.5 times less active than the *S*-(−) isomer. Unfortunately, neither of the two isomers showed any clear-cut evidence of cereal selectivity.

The observed differences in biological activity of the enantiomers of III may indicate either that the initial step in the action of herbicidal DPE's involves the interaction of these molecules with an enantiotopically discrete active site or that the two enantiomers are metabolized at different rates.

In studies on the metabolism of NDPE herbicides, it has been shown that, in susceptible species, the half-life of fomesafen [5-[2-chloro-4-(trifluoromethyl)phenoxy]-*N*-(methylsulfonyl)-2-nitrobenzamide] is greater than 1 week (Cavell, 1985). In soybean, which is one of the few dicotyledonous plants known to be tolerant of NDPE herbicides, fomesafen and acifluorfen [sodium-5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate] are metabolized to nontoxic products in less than 24 h (Frear et al., 1983). The tolerance of soybean to NDPE's presumably reflects the presence of a metabolizing enzyme which is, however, absent in susceptible species. Since French bean is very susceptible to both NDPE's (Camilleri, P., Weaver, K., Bowyer, J. R., Hallahan, B. J., unpublished observations) and the *S*-(−) isomer of III, it seems likely that this tissue also lacks a metabilizing enzyme and it would be very unlikely that an enzyme showing a uniquely high specificity for the *R*-(+) isomer of III existed.

Thus, although we have not yet rigorously ruled out the possibility that the differential efficacy of the two enantiomers of III is due to differential metabolism, it seems far more likely that it reflects the interaction of these molecules with an enantiotopically specific active site. The recent report by Matringe and Scalla (1987) that NDPE's and their analogues act by enhancing the level of protoprophyrin IX implies that NDPE's and phthalide diphenyl ethers may act by inhibiting an enzyme on the biosynthetic path between protoporphyrin IX and protochlorophyllide. We intend to test these possibilities and in particular to compare the efficacy of the two enantiomers of III using isolated cucumber etioplasts capable of both protoporphyrin IX and protochlorophyllide accumulation (Daniell and Rebeiz, 1982).

The higher phytotoxicity of the *S*-(−) isomer of III and a comparison of the structural features of phthalide diphenyl ethers with those of the more active nitrodiphenyl ether herbicides permit speculation as to the molecular requirements for optimal fit of DPE's to a putative "enzyme active site". In a recent publication (Bowyer et al., 1987) we had introduced the potent nitrodiphenyl ether 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitroacetophenone oxime *O*-(acetic acid, methyl ester), which we called DPEI. The X-ray structure of this compound reveals (results not shown) that the nitro group lies in the plane of the phenyl ring, in the same way as the carbonyl group in the enantiomers of III. Moreover, MNDO calculations, carried out on the X-ray structures, reveal that the residual charges on the nitro and carbonyl groups are of the same order of magnitude. Therefore, these two groups can "mimic" each other and probably occupy a common hydrophilic binding site on the enzyme. Another structural property common to both DPEI and the two isomers of III is the halogenated phenyl entity that is expected to occupy a hydrophobic "cleft".

Previous evidence suggests (Camilleri et al., 1988) that the phthalide DPE moiety binds tightly to the site of action. Stereoselective replacement of one of the hydrogens at C-13 (Figure 2) by a methyl group results in one of the enantiomers binding more strongly than the other enantiomer and is evidence that the "active site" of DPE's contains a binding niche that is specific to accommodating a methyl in preference to a hydrogen.

In conclusion, the difference in the biological activity of the *S*-(−) and *R*-(+) isomers of III tends to point for the first time to the likely involvement of an enzymic binding process in the mode of action of peroxidizing diphenyl ether herbicides.

ACKNOWLEDGMENT

We thank I. G. Blackwell for mass spectral analysis and C. A. Jovanovic for the biological testing.

Registry No. (*S*)-III, 118400-08-1; (*R*)-III, 118400-09-2.

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Kinetics of Hydrolysis of the Dicarboximide Fungicide Vinclozolin

Sunny Y. Szeto,* Nick E. Burlinson, James E. Rahe, and Peter C. Oloffs[1]

The hydrolysis of the dicarboximide fungicide vinclozolin was studied in several aqueous buffers of pH 4.5–8.3 at 13–35 °C. The reaction was base-catalyzed and the rate proportional to pH. At 35 °C the pseudo-first-order rate constants ranged from 1.30×10^{-3} to 1.11 h^{-1}, and the second-order rate constants, from 4.10×10^{6} to 5.56×10^{5} M^{-1} h^{-1}. The energy of activation for the hydrolysis of vinclozolin at pH 7.0 was calculated from the Arrhenius plot to be 97.2 kJ mol^{-1}. On the basis of kinetics, a degradation pathway was proposed. On hydrolysis the 2,4-oxazolidinedione ring opens to yield 2-[[(3,5-dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) independently. The conversion to M1 is reversible, which leads to the formation of vinclozolin by recyclization. At basic pH the forward reaction to yield M1 is favored whereas at acidic pH the reverse reaction to yield vinclozolin is favored. 3,5-Dichloroaniline (M3) was detected as a minor degradation product.

Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione] (Figure 1) is a protectant fungicide marketed by BASF AG. It is effective for controlling fungal diseases caused by *Botrytis* spp., *Sclerotinia* spp., and *Monilinia* spp. in grapes, fruits, vegetables, ornamentals, hops, rapeseed, and turfgrass (Spencer, 1982). Since its introduction, vinclozolin has been widely used in Europe for the control of fungal diseases. Vinclozolin is currently registered in the United States, but not in Canada.

Vinclozolin is unstable in methanolic and ethanolic solutions and water suspension (Clark, 1983). Three hydrolytic degradation products of vinclozolin have been isolated and identified by us, namely 2-[[(3,5-dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid (M1), 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) (Figure 1), and 3,5-dichloroaniline (M3) (unpublished data). Vinclozolin was more susceptible to hydrolysis at basic than acidic pH (Melkebeke et al., 1986). Melkebeke et al. (1986) studied the chemical hydrolysis of vinclozolin in the range pH 3.0–11.0 and reported the half-lives and rate constants for the disappearance of the parent compound. However, they did not study the mechanism of degradation. Considering the structures of the two degradation products, the butenoic acid and the enanilide, it is highly unlikely that the enanilide is formed via the butenoic acid as an intermediate because conversion from the butenoic acid to the enanilide would require complicated rearrangement of the molecule.

A comprehensive study is required to investigate the disappearance of vinclozolin and the production of degradation products, so that the mechanism of hydrolytic degradation can be better understood. The present study describes the kinetics of hydrolysis of vinclozolin in several aqueous buffers in the range pH 4.5–8.3 and in the temperature range 13–35 °C, and on the basis of these data, a degradation pathway is proposed.

EXPERIMENTAL SECTION

Preparation of Vinclozolin and Its Hydrolytic Degradation Products. Vinclozolin, 2-[[(3,5-dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid, and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide were obtained from BASF Aktiengesellschaft, and their purities were, respectively, 99%, 98%, and 96%. 3,5-Dichloroaniline (98%) was obtained from the Laboratory Services Division of Agriculture Canada in Ottawa.

Preparation of Aqueous Buffers. Buffered solutions of 0.01 M were prepared with sterilized deionized water by adjusting the pH with the following chemicals: NaH_2PO_4/H_3PO_4 and NaOAc/HOAc for pH 4.5;

Agriculture Canada Research Station, 6660 N.W. Marine Drive, Vancouver, B.C., Canada V6T 1X2 (S.Y.S.), Department of Chemistry, University of British Columbia, Vancouver, B.C., Canada V6T 1Y6 (N.E.B.), and Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., Canada V5A 1S6 (J.E.R., P.C.O.).
[1] Deceased, June 19, 1987.

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