Biotransformation of Vinclozolin by the Fungus *Cunninghamella elegans*

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This study investigated the biotransformation of the dicarboximide fungicide vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione] by the fungus *Cunninghamella elegans*. Experiments with phenyl-[U-*ring*-^{14}C]vinclozolin showed that after 96 h incubation, 93% had been transformed to four major metabolites. Metabolites were separated by HPLC and characterized by mass and NMR spectroscopy. Biotransformation occurred predominantly on the oxazolidine-2,4-dione portion of vinclozolin. The metabolites were identified as the 3R- and 3S- isomers of 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide, *N*-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3,5-dichlorophenyl-1-carbamic acid, and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide. The enanilide compound has been reported previously as a plant and mammalian metabolite and is implicated to contain antiandrogenic activity. The 3R- and 3S- isomers of 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide are novel metabolites.

Keywords: *Vinclozolin; dicarboximide fungicide; fungal biotransformation; Cunninghamella elegans*

INTRODUCTION

Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione] is a systemic dicarboximide fungicide used widely in Europe and the United States for control of diseases caused by *Botrytis cinerea, Sclerotinia sclerotiorum, and Monilinia* sp. in grapes, fruits, vegetables, ornamental plants, and turfgrass (Rankin et al., 1989).

The World Health Organization has previously evaluated the fungicide vinclozolin as a noncarcinogen (FAO/WHO, 1986), but the U.S. Environmental Protection Agency (U.S. EPA) has classified vinclozolin as a Group C chemical, which indicates a possible human carcinogen (U.S. EPA, 1997). Mutagenicity tests with vinclozolin using *Salmonella typhimurium* have shown negative results (U.S. EPA, 1985); however, some studies using *Schizosaccharomyces pombe* and diploid colonies of *Aspergillus nidulans* (mitotic recombination and mitotic nondisjunction) have given positive results (Chiesara et al., 1982; Vallini et al., 1993). Vinclozolin has been shown to alter morphological sex differentiation in male rats following perinatal exposure (Gray et al., 1994). Two antiandrogenic metabolites of vinclozolin (butenoic acid and enanilide) are potent inhibitors of rat androgen receptor binding (Kelce et al., 1994; Laws et al., 1996). Kelce et al. (1994) determined that vinclozolin itself had little affinity for the androgen receptor (K_i > 700 μM), whereas both metabolites, butenoic acid and enanilide, were effective antagonists (K_i's 92 and 9.7 μM, respectively). The higher antagonistic potency of enanilide relative to vinclozolin and butenoic acid is attributed to its structural resemblance to the potent antiandrogen, hydroxyflutamide (Wong, 1995). The cocarcinogenic properties of vinclozolin in liver, kidney, and lung microsomes of mice (Paolini et al., 1998) and in human peripheral blood lymphocytes cultured in vitro (Hrelia et al., 1996) have been reported. In general, vinclozolin showed a complex pattern of induction and suppression of cytochrome P450-dependent enzymes, as seen from the reduced expression of various monooxygenases depending upon dose, sex, and organ of the mammals studied. The findings indicated the clastogenic activity and the possible cotoxic, cocarcinogenic, and promoting potential of vinclozolin (Hrelia et al., 1996). The U.S. Environmental Protection Agency (1997) has established a reference dose (R_fD) of 12 μg/kg, and both EPA and the manufacturer (BASF Corporation) have estimated likely exposures of the U.S. population to not greater than 16% of the R_fD (BASF Corporation, 1997). However, occupational exposures may be greater. The potential for human exposure to vinclozolin in a greenhouse was estimated to be 25 mg after 8 h of work and the dermal exposure (on bare skin) to vinclozolin was estimated as 2.5 mg/day. This value is higher than the value permitted by the U.S. EPA (Nilsson and Papantoni, 1996).

Figure 1 illustrates several routes through which vinclozolin can undergo either chemical hydrolysis or metabolism by mammalian and bacterial systems. Vinclozolin also is unstable and undergoes hydrolysis of the 2,4-oxazolidinedione ring in methanolic and ethanolic solutions and water suspensions (Clark, 1983). Szeto et al. (1989a,c) isolated three hydrolytic degradation products from aqueous buffers which have been identified as 2-[[(3,5-dichlorophenyl)-carbamoxyl]oxy]-2-methyl-3-butenoic acid, 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide, and 3,5-dichloroaniline. They proposed that the degradation pathway of vinclozolin is via the open-

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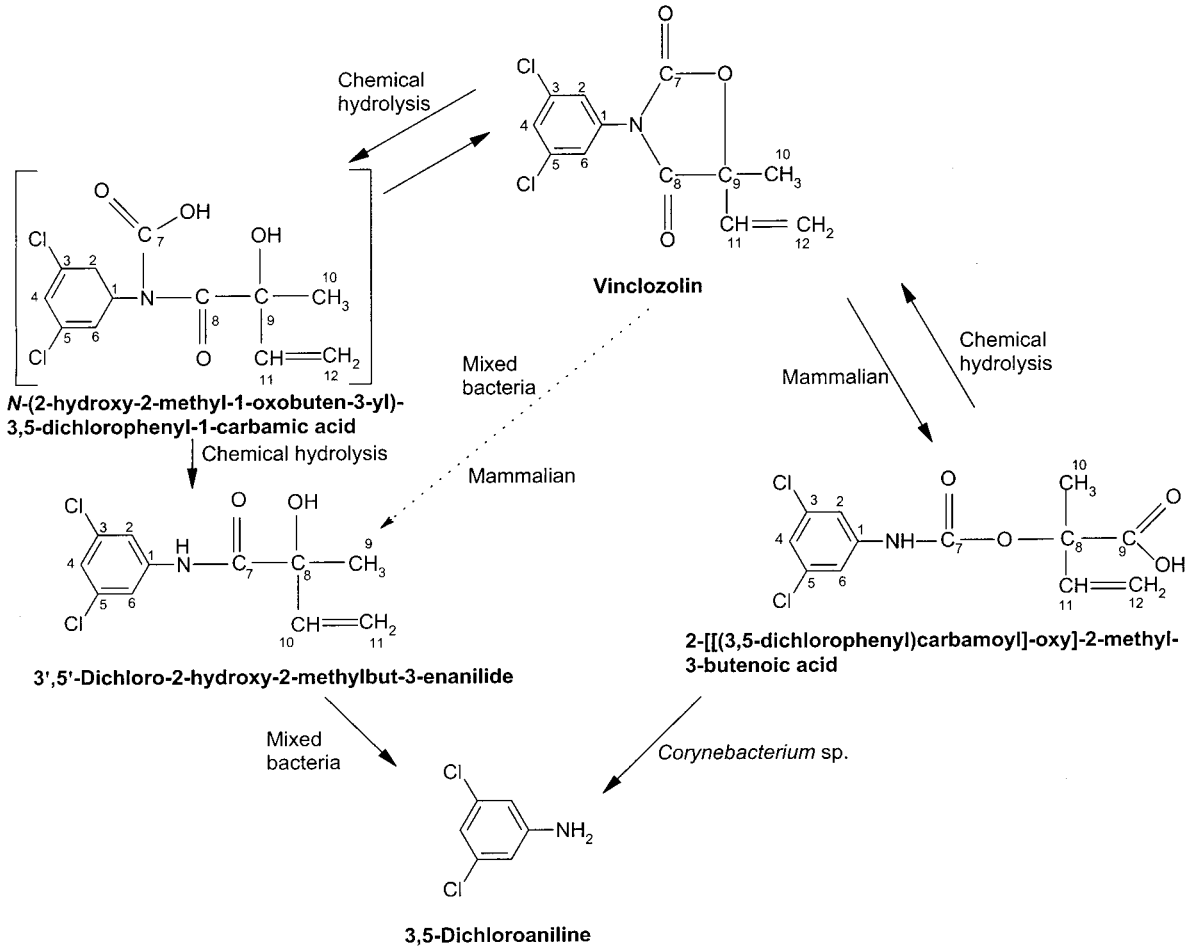

Figure 1. Conversion of vinclozolin by chemical hydrolysis and metabolism by bacterial and mammalian systems. Adapted from Mercadier et al. (1998) and Wong et al. (1995).

ing of the 2,4-oxazolidine ring of vinclozolin by hydrolysis to yield either the butenoic acid or the enanilide; however, the conversion of vinclozolin to butenoic acid is reversible. The rate of conversion of vinclozolin is pH dependent, favoring the forward reaction to 2-[[(3,5-dichlorophenyl)carbamoxyl]oxy]-2-methyl-3-butenoic acid at basic pH and the reverse reaction at acidic pH (Szeto et al., 1989a,c). Other researchers also reported similar transformation rates of vinclozolin; however, the transformation patterns varied (Melkebeke et al., 1986; Villedieu et al., 1994). The main difference is the breakdown of the ester bond (Clark, 1983; Melkebeke et al., 1986; Pirisi et al., 1986; Villedieu et al., 1994), but Szeto et al. (1989a) also reported the breakdown of the amide bond. Szeto et al. (1989c) also detected the two metabolites, butenoic acid and enanilide, in leaves of pea and bean plants grown in nutrient solutions containing vinclozolin. Vinclozolin and its butenoic acid and enanilide metabolites have been found within soils, plants, and animals exposed to vinclozolin, and may be potentially hazardous to health (Hawkins et al., 1990).

Vinclozolin undergoes photochemical reactions on plant surfaces when several organic solvents are used to simulate the plant cuticle environment. The main reaction is the photoaddition of solvent molecules to the vinclozolin vinyl group, followed by dechlorination of the photoproducts in further reactions (Schwack et al., 1995). Photolysis of vinclozolin in aqueous and methanol−water (50:50, v/v) solutions leads to the opening of the 2,4-oxazolidinedione ring, forming 3,5-dichlorophenyl isocyanate and 3,5-dichloroaniline (Schick et al., 1999).

In soils, under aerobic conditions, about 50% of vinclozolin is degraded within 22 days (Slade et al., 1992) and 23 days of application, but the rate of degradation is slower in acidic soils (Walker et al., 1986). Enhanced degradation in soils was observed in laboratory and field conditions after multiple (Frederick et al., 1994; Slade et al., 1992) and single applications (Mitchell and Cain, 1996; Walker, 1987) of vinclozolin. But in some nonflooded tropical rice soils, vinclozolin degradation is slower (Banerjee and Adhya, 1999). Golovleva et al. (1991) report residual amounts of vinclozolin remaining in soil after 12 months as 0.3 to 3.0 μg/g, when 5.0 μg/g was applied, and from 37.5 to 75.0 μg/g, when 625.0 μg/g was applied. Among several organic pesticides studied, vinclozolin was found to be the most persistent pesticide during the vinification process (Sala et al., 1996) but degraded rapidly in vineyard soils (Garcia-Cazorla and Xirau-Vayreda, 1998).

Mercadier et al. (1998) reported the transformation of vinclozolin by two mixed bacterial cultures isolated from a French soil adapted to the fungicide and found that this mixed culture was able to degrade vinclozolin via two pathways: (1) the formation of 2-[[(3,5-dichlorophenyl)-carbamoxyl]oxy]-2-methyl-3-butenoic acid and then 3,5-dichloroaniline or (2) the formation of 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide and then 3,5-dichloroaniline. Previously, Cain and Mitchell (1996)

isolated a *Pseudomonas putida* strain from soil that utilized vinclozolin as a source of carbon and energy, producing 3,5-dichloroaniline. Since dichloroanilines have been shown to be embryolethal, genotoxic, and teratogenic in animal studies (U.S. EPA, 1987) and 3,5-dichloroaniline is a chlorinated aromatic amine, human exposure to these compounds is considered a health hazard (Pothuluri et al., 1991; Szeto et al., 1989).

To our knowledge, no studies on the fungal metabolism of vinclozolin have appeared. Both ligninolytic and nonligninolytic fungi are being studied for their ability to degrade hazardous chemicals, including pesticides (Cerniglia, 1997). We have previously demonstrated the ability of the fungus *Cunninghamella elegans* to metabolize the herbicides alachlor and metolachlor to hydroxylated products that are generally considered less toxic than the parent compounds, whereas mammalian systems have shown a greater tendency toward bioactivation (Pothuluri et al., 1993; 1997).

In this study, we report in detail the first investigation of the fungal metabolism of vinclozolin and on the isolation and identification of the major metabolites of vinclozolin.

MATERIALS AND METHODS

Chemicals. Unlabeled vinclozolin (purity, >99%) was purchased from Radian International (Austin, TX). Phenyl-[U-*ring*-^{14}C]vinclozolin (specific activity, 1.0 mCi/mmol; radiochemical purity, >99%) and unlabeled 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide metabolite were gifts from BASF Corporation, Research Triangle Park, NC. High-performance liquid chromatography (HPLC)-grade solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA). All other chemicals were of reagent grade and the highest purity available.

Microorganism and Culture Conditions. The fungal incubation conditions reported previously (Pothuluri et al., 1993) were modified in this study. After the growth of *C. elegans* ATCC 36112 cultures in 30 mL of Sabouraud dextrose broth for 48 h, 10 mg of vinclozolin was dissolved in 0.5 mL of dimethyl sulfoxide (DMSO) and added to each culture. All cultures were incubated for an additional 96 h in the dark. After 96 h, the contents (50 mL) were pooled and filtered to separate the broth from the mycelia. The mycelia and the broth were then extracted with six equal volumes (300 mL total volume) of ethyl acetate. The extract was dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure at 38 °C. The residue was dissolved in methanol and analyzed by HPLC.

In separate experiments, duplicate flasks containing 30 mL of sterile Sabouraud dextrose broth were dosed with 10 mg of unlabeled vinclozolin dissolved in 0.5 mL of DMSO and incubated for 96 h. The broth was extracted and analyzed by HPLC as described above.

Kinetic experiments were also conducted as described above but with 2.28 μCi of [^{14}C]vinclozolin and 10 mg of unlabeled vinclozolin, dissolved in 0.5 mL of DMSO added to duplicate culture flasks. The percent metabolism to various products was quantified by liquid scintillation methods (Pothuluri et al., 1990). The percent recovery of vinclozolin and metabolites were corrected for extraction efficiency (80%) by adjusting the radioactivity recovered in the parent peak at zero time to 100 percent recovery. The variability of percent metabolism among the duplicate flasks was approximately ±4.0%.

Physical and Chemical Analyses. Vinclozolin and metabolites were separated on a Perkin-Elmer series 10 high-pressure liquid chromatograph (HPLC) equipped with an LC-95 UV−visible absorbance detector (Perkin-Elmer Corp., Norwalk, CT) operated at 254 nm. A Hewlett-Packard C18 column (25 cm by 4.6 mm; Hewlett-Packard, Palo Alto, CA) was used to separate the fungal metabolites. A 40-min linear gradient of methanol−water (from 30:70 to 95:5 [vol/vol]), at a flow rate of 1.0 mL/min, was used to elute the metabolites. Metabolites were collected from repeated HPLC injections. Fractions with similar retention times were pooled and concentrated in vacuo using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY). Two metabolites (compounds **II** and **III**) were further purified using the same HPLC solvent gradient conditions but at a flow rate of 0.8 mL/min.

In experiments with [^{14}C]vinclozolin, 0.5-mL fractions were collected every 0.5 min and added to scintillation vials containing 7.0 mL of Ultima Gold scintillation fluid (Packard Instrument Company, Meriden, CT). The radioactivity was determined on a Packard 2000CA Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

UV−visible absorption spectra of the metabolites were determined in methanol with a Hewlett-Packard (Wilmington, DE) 8453 UV−vis spectrophotometer equipped with a diode-array detector. Direct exposure probe (DEP) mass spectrometry (MS) analyses of vinclozolin and metabolites were performed on a TSQ 700 mass spectrometer (Finnigan Corp., San Jose, CA). Ionization techniques included electron ionization (EI) at 70 V electron energy and chemical ionization (CI) with 10% NH_3 in N_2 as the reagent gas at approximately 5 Torr ion source pressure. Both positive ion and negative ion chemical ionization (PICI and NICI, respectively) were performed. The ion source temperature was maintained at 150 °C (uncorrected) and the DEP wire was heated with a linear ramp of 5 mA/sec to approximately 750 mA for both EI and CI analyses. The analyzer quadrupole was scanned from *m*/*z* 50 to 550 with 0.5 s cycle time.

Liquid chromatography/mass spectral (LC/MS) analyses were performed using an HP 5989B mass spectrometer equipped with an HP 1090L/M HPLC and a photo diode-array detector (Hewlett-Packard, Palo Alto, CA). The mass spectrometer was operated in the positive electrospray ionization (ESI) mode with the capillary exit voltage at 100 V, high energy dynode at 10 kV, and multiplier at 2.1 kV. Full scans were acquired from *m*/*z* 150 to 350 at 1.67 scans/sec. HP Chemstation software was used to collect concurrent LC−PDA and positive ESI−MS data. Vinclozolin metabolites were resolved from impurities using a Prodigy ODS(3) 2.0 × 250 mm, 5 μm 100A HPLC column (Phenomenex, Torrance, CA). The mobile phase, delivered at 0.2 mL/min, was a 45-min linear gradient from 5% acetonitrile:95% water to 95% acetonitrile:5% water [vol:vol] with 0.1% formic acid.

The ^{1}H nuclear magnetic resonance (NMR) experiments were carried out on a Bruker AM 500 MHz spectrometer (Bruker Instruments, Billerica, MA) at 301 K. Vinclozolin and metabolites were dissolved in 0.6 mL of deuterated methanol (MeOD). The MeOD peak was assigned a resonance of 3.31 ppm. Typical NMR spectral acquisition parameters were data size, 32K; flip angle, 90; sweep width, 7000 Hz; and relaxation delay, 1 s. The number of scans used in each experiment ranged from 128 for metabolites **I** and **III** to 1024 for metabolites **II** and **IV**. Nuclear Overhauser experiment (NOE) difference and homonuclear coupling NMR experiments were run at every proton resonance for each metabolite.

RESULTS

Identification of Vinclozolin Metabolites. The HPLC elution profile of the ethyl acetate-extractable metabolites, formed during 96 h incubation of nonradioactive vinclozolin and [^{14}C]vinclozolin with *C. elegans* (Figure 1), show that vinclozolin was metabolized to four principal metabolites (**I**, **II**, **III**, and **IV**), which eluted at 14.9, 22.0, 22.4, and 28.1 min, respectively. Residual vinclozolin eluted at 30.2 min. The HPLC peaks eluting from 2 to 5 min were unlabeled and were not associated with vinclozolin metabolism (Figure 2). The HPLC elution profile from an experiment with sterile Sabouraud dextrose broth containing vinclozolin and incubated for 96 h did not show any transformation (not shown).

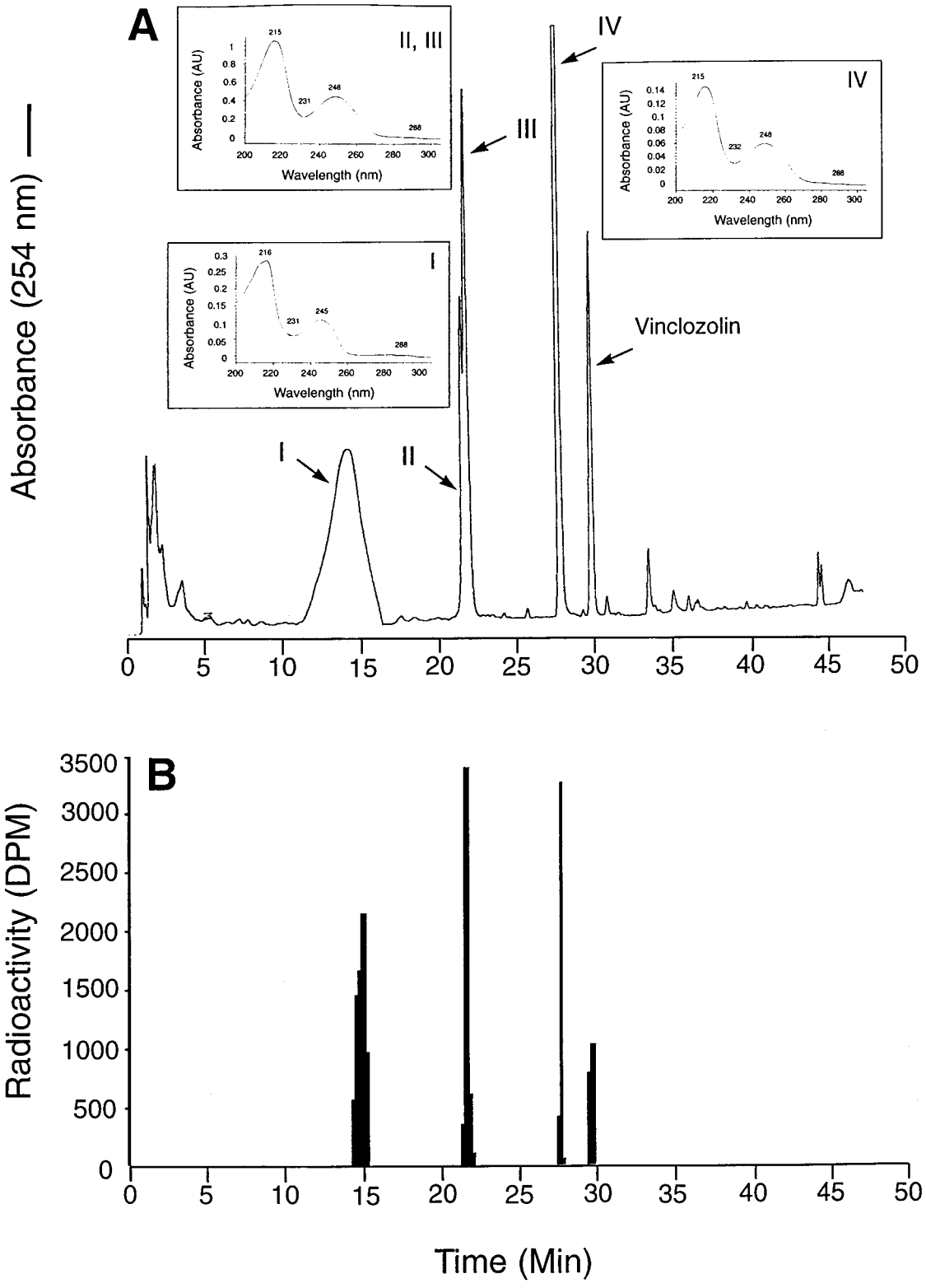

Figure 2. (A) HPLC elution profile of the ethyl acetate-soluble metabolites formed from [^{14}C]vinclozolin by *C. elegans*. Inset includes the UV−visible absorption spectra of metabolites **I**, **II** and **III**, and **IV**. (B) Radioactivity of fractions eluting from the column, collected at 0.5-min intervals, measured by liquid scintillation. The HPLC elution profile of a control incubation sample did not show any UV−visible absorbance after 5 min of the elution except for unmetabolized vinclozolin.

The UV−visible absorption spectrum for metabolite **I** showed absorption maxima at 216, 231, 245, and 288 nm (Figure 2 inset). The ESI data for metabolite **I** indicate a compound with a molecular weight of 303, which contains 2 chlorine atoms and an odd number of nitrogen atoms (Table 1). Fragment ions include *m/z* 286 and 260, which may arise from the loss of H_2O or CO_2 from the protonated molecule $[M+H]^+$. The ion at *m/z* 242 was from the loss of water $[MH-CO_2-H_2O]^+$ from *m/z* 260. The fragment ion at *m/z* 206 is presumed to form from the loss of the other side chain $[MH-C_5H_6O_2]^+$ (Table 1). The structure of metabolite **I** was further investigated by ^{1}H NMR spectroscopy. The aromatic, ethylene, and methyl protons of vinclozolin

Table 1. DEP/MS and LC/MS Data of Vinclozolin and Vinclozolin Metabolites Produced by *Cunninghamella elegans* and the Authentic 3′,5′-Dichloro-2-hydroxy-2-methylbut-3-enanilide Standard[a]

sample	EI [*m*/*z*(%RA[b])]	PICI [*m*/*z*(%RA)]	NICI [*m*/*z*(%RA)]	ESI $[M + Na]^+$	ESI $[M + H]^+$	fragment ions (*m*/*z*)
I	191(12), 189(68), 187(100), 161(18), 159(15), 126(11), 124(32)	305(2), 303(3), 287(2), 285(2), 282(1), 281(2), 189(26), 187(41), 165(11), 164(44), 163(60), 161(100), 116(70), 99(34)	352(4), 350(7), 348(5), 245(11), 244(9), 243(67), 242(13), 241(100)	326	304	286, 260, 242, 206
II	295(14), 293(20), 235(49), 233(72), 175(16), 164(21), 163(11), 162(42), 161(14), 127(15), 117(53), 106(12), 105(100), 87(27), 75(12), 73(19), 61(16), 59(84), 57(12)	313(12), 311(17), 298(10), 296(68), 295(18), 294(100)	297(18), 295(89), 294(38), 293(100), 289(10), 259(20), 257(60), 231(11)	316	294	276, 162
III	295(15), 293(25), 235(77), 233(99), 231(11), 164(17), 162(19), 117(18), 105(100), 87(39), 75(11), 71(14), 70(13), 61(12), 59(31)	313(10), 311(14), 298(12), 296(70), 295(17), 294(100), 260(12)	297(15), 296(22), 295(51), 294(38), 294(14), 293(100), 259(16), 257(43)	316	294	276, 162
IV	261(8), 259(9), 218(5), 216(9), 163(27), 161(39), 72(30), 71(100), 57(6), 54(5)	279(53), 278(10), 277(77), 264(13), 263(10), 262(65), 261(19), 260(100), 259(10), 246(4), 244(13), 242(14), 161(12), 71(10)	263(9), 262(8), 261(61), 260(16), 259(100), 223(6)		260	242
authentic enanilide standard[c]	261(4), 259(6), 163(13), 161(20), 72(33), 71(100), 57(6), 55(4), 54(5)			282	260	242
vinclozolin	287(56), 285(84), 241(10), 216(13), 215(45), 214(62), 213(73), 212(100), 202(11), 200(51), 198(75), 190(11), 189(48), 187(78), 186(14), 180(22), 178(69), 172(14), 161(10), 159(11), 147(10), 145(15), 126(15), 124(42), 55(10), 54(28), 53(24)					

[a] Most prominent ion of isotope cluster is listed. [b] %RA = relative abundance. [c] 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide.

Table 2. ^{1}H NMR Analysis of Vinclozolin and Vinclozolin Metabolites Produced by *Cunninghamella elegans* and Authentic 3′,5′-Dichloro-2-hydroxy-2-methylbut-3-enanilide

	chemical shifts (δ)					
proton[a]	vinclozolin	I	II	III	IV	authentic enanilide standard
H_2,H_6	7.56	7.47	7.67	7.66	7.68	7.68
H_4	7.08	7.00	7.14	7.15	7.14	7.14
ethylene CH	6.13	6.36	6.12	6.12		
ethylene CH_{21}	5.58	5.36			5.45	5.45
ethylene CH_{22}	5.44	5.11			5.17	5.17
ethyl CH			3.79	3.79		
ethyl CH_{21}			3.62	3.61		
ethyl CH_{22}			3.61	3.59		
methyl	1.59	1.67	1.45	1.45	1.50	1.50
J trans ethylene (Hz)	17.3	17.5			17.20	17.20
J cis ethylene (Hz)	10.8	11.0			10.90	10.90

[a] All experiments were determined on a 500 MHz Bruker NMR spectrometer with a MeOD deuterated solvent, and the MeOD peak was set to $\delta = 3.31$ ppm.

were observed. The NMR data were consistent with hydrolysis of the second ring system of vinclozolin at the ester linkage (Table 2, Figure 3a). On the basis of these spectroscopic analyses, metabolite **I** was identified as *N*-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3,5-dichlorophenyl-1-carbamic acid.

The UV−visible absorption spectra for metabolites **II** and **III** showed absorption maxima at 215, 231, 248, and 288, and 215, 232, 248, and 288 nm, respectively (Figure 2 insets). The ESI data for metabolites **II** and **III** did not show any distinguishing differences between the two metabolites in the retention times and mass spectra. The ESI mass spectra consisted of sodiated molecules $[M+Na]^+$ at *m*/*z* 316, protonated molecules at *m*/*z* 294, and fragment ions at *m*/*z* 276 $[MH-H_2O]^+$ and 162 (protonated dichloroaniline) (Table 1). The EI mass spectra of metabolites **II** and **III** consisted of molecular ions $[M^{+\cdot}]$ at *m*/*z* 293 and significant fragment ions at *m*/*z* 233, 162, 105 (base peak), and 59 (Table 1). The PICI mass spectra exhibited protonated molecules $[M+H]^+$ at *m*/*z* 294 and ammoniated adduct ions $[M+NH_4]^+$ at *m*/*z* 311 (Table 1). The NICI mass spectra showed molecular anions $[M^-]$ at *m*/*z* 293 and apparent fragment ions at *m*/*z* 257 $[M\text{-}HCl]^-$. All the DEP spectra were consistent with compounds containing 2 chlorine atoms and an odd number of nitrogen atoms. Metabolite **II**, an isomer of metabolite **III** separable by HPLC, showed a mass spectral fragmentation pattern similar to that of metabolite **III** except that the intensities of the peaks were different (Figure 4a and b). The NMR spectra of metabolites **II** and **III** indicated aromatic and methyl protons of vinclozolin. The ethylene protons of vinclozolin were not observed, but ethyl protons were observed (Table 2). The NMR results for metabolite **II**

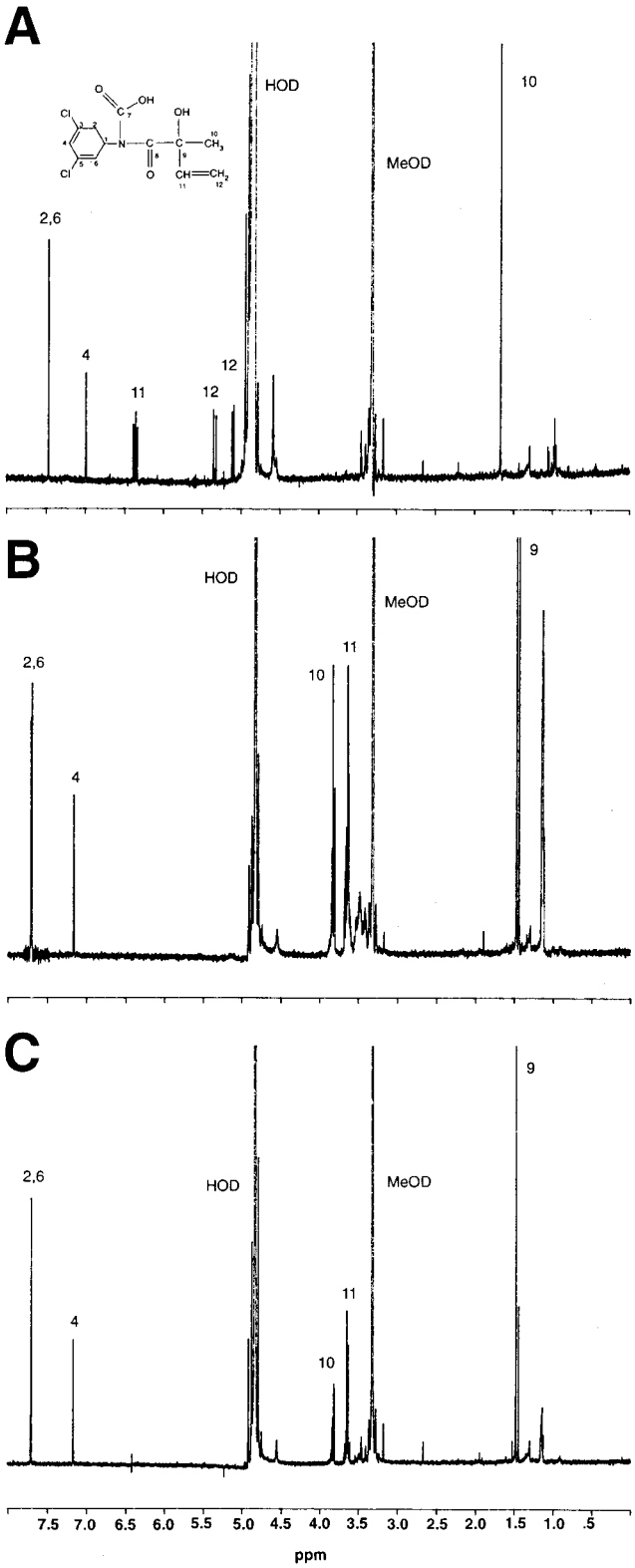

Figure 3. The 500 MHz ^{1}H NMR spectra of vinclozolin metabolites (A) *N*-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (**I**) and (B & C) R- and S-isomers of 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide (**II** and **III**, respectively) with the structure and resonance assignments. For metabolite **II**, the ratio of the two R- and S-isomeric components was approximately 1.00 to 0.30, whereas the ratio for metabolite **III** was 0.08 to 1.00. HOD and MeOD are the deuterated H_2O and methanol peaks, respectively.

were consistent with the addition of two hydroxy groups to the ethylene double bond to produce an ethyl diol side chain (Table 2). Similarly, the NMR results for metabolite **III** were also consistent with the addition of hydroxyl groups to the ethylene double bond to produce an ethyldiol side chain (Table 2). However, the difference between metabolites **II** and **III** is that they are stereoisomers with a chiral carbon on the ethyldiol group. Because metabolites **II** and **III** essentially coeluted (Figure 2) both NMR spectra contained the same two components, with the main difference between the spectra being the relative amounts of these two components (Figure 4 a and b). Based on both mass and NMR spectral data, metabolites **II** and **III** were identified as 3R- and 3S- isomers of 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide.

The UV−visible absorption spectrum of metabolite **IV** showed absorption maxima at 215, 232, 248, and 288 nm (Figure 2 inset). The ESI data indicated that metabolite **IV** and the authentic metabolite standard, 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide, were the same with regard to their retention times and mass spectra. The ESI mass spectra for metabolite **IV** showed protonated molecules at *m/z* 260 and fragment ions at *m/z* 242 $[MH-H_2O]^+$. However, the authentic standard, 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide, exhibited a very weak sodiated molecule $[M+Na]^+$ at *m/z* 282 (Table 1). The EI mass spectrum of metabolite **IV** contained a molecular ion at *m/z* 259 and significant fragment ions at *m/z* 161 and 71 (base peak) (Figure 4c). The PICI mass spectrum had a protonated molecule $[M+H]^+$ at *m/z* 260 and an ammonia adduct ion at *m/z* 277. Weak fragment ions at *m/z* 242 $[MH-H_2O]^+$, 161 $[C_6H_5N_1Cl_2]^+$, and 71 $[C_4H_7O]^+$ were consistent with the proposed structure. The NICI mass spectrum consisted of a molecular anion at *m/z* 259 and an apparent fragment ion at *m/z* 223 $[M\text{-}HCl]^-$. The DEP spectra were consistent with a compound containing two chlorine atoms and an odd number of nitrogen atoms (Table 1). The HPLC retention times, UV−visible spectra, and mass spectra of metabolite **IV** and the 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide standard were identical. The NMR analyses confirmed the identity of metabolite **IV** (Table 2), including the aromatic, ethylene, and methyl protons of vinclozolin. Metabolite **IV** was found to be the same as authentic 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide.

Kinetics of Vinclozolin Metabolite Formation. The rates of disappearance of [^{14}C]vinclozolin and the production of ethyl acetate-extractable metabolites **I**−**IV** by *C. elegans* at 24-h intervals during 96 h of incubation are shown in Figure 5. Initially, about 40% of the radioactivity was recovered in the organic phase at zero time incubation and the remainder was bound to the mycelia. At zero time, 80% (adjusted to 100% for extraction efficiency) of the radioactivity recovered in the organic phase was in the vinclozolin peak. The percent recoveries of vinclozolin and metabolites reported in Figure 5 have been corrected for extraction efficiency. The amount of vinclozolin had decreased to about 7% by 96 h, with 2.5% remaining at 168 h. Metabolite **I**, *N*-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3,5-dichlorophenyl-1-carbamic acid, accumulated to maximum levels at 96 h of incubation, accounting for about 50% of the total radioactivity recovered in ethyl acetate extracts. However, there was an 8-fold increase in the formation of metabolite **I** during the 24- to 72-h incubation period. Metabolites **II** and **III**, 3R- and 3S- isomers of 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide, together accounted for about 25% of the total recovered

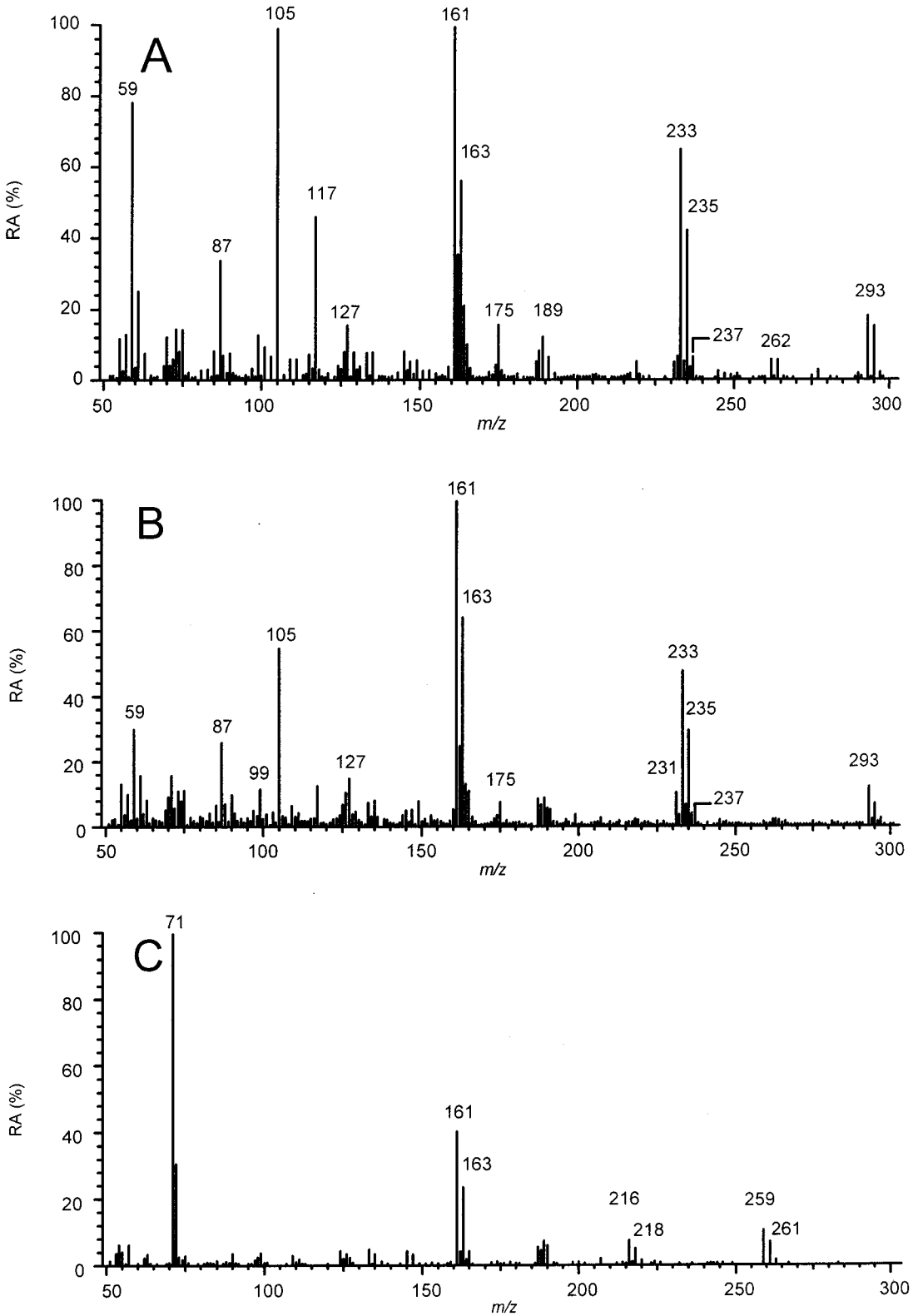

Figure 4. Electron-impact mass spectra of metabolites (A) **II**, (B) **III**, and (C) 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (**IV**) produced from *C. elegans* cultures exposed to vinclozolin. Percent RA is the relative abundance of ions.

radioactivity at 96 h incubation; however, they reached a maximum level of about 33% at 168 h. There was a 4-fold increase in the formation of metabolites **II** and **III** during 24-to 72-h incubation. At 96 h, the extractable fraction of metabolite **IV**, 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide, accounted for about 18% of the total recovered radioactivity, reaching a maximum level of about 20% at 168 h. However, there was an increase of almost eleven-fold in the formation of metabolite **IV** during 24- to 72-h incubation. Sterile control flasks with vinclozolin alone showed no metabolism.

DISCUSSION

Figure 6 illustrates the scheme of transformation of vinclozolin to several metabolites either by hydration

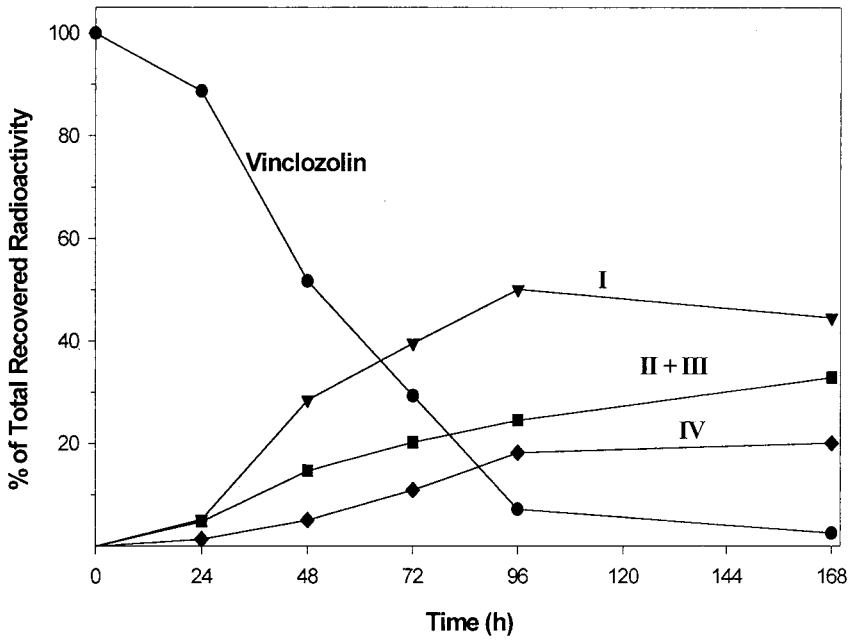

Figure 5. Biotransformation of [^{14}C]vinclozolin (●) and formation of metabolites **I** (▼), **II** and **III** (■), and **IV** (◆) over time by *C. elegans*.

and/or decarboxylation. The major metabolism of vinclozolin by *C. elegans* appears to be via the addition of a water molecule to the ester group to form *N*-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (**I**). The formation of metabolite **I** constituted the major initial transformation product of vinclozolin by *C. elegans*, as this metabolite accounted for 50% of the total metabolism at 96 h of incubation. *C. elegans* was able to open the oxazolidine ring of vinclozolin. The oxazolidine ring may also be opened in the presence of organic solvents and water; the two products from water suspension were identified as *N*-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3,5-dichlorophenyl-1-carbamic acid and its decarboxylation product, *N*-3,5-dichlorophenyl-2-hydroxy-2-methylbut-3-enanilide (Clark, 1983). Szeto et al. (1989) studied the kinetics of hydrolysis of vinclozolin and found that vinclozolin in phosphate buffer (pH 4.5) was hydrolyzed to the butenoic acid, which accounted for a maximum of 20.2% of the applied vinclozolin concentration at 170 h, whereas 79.5% of the enanilide had been formed at 1536 h. However, at pH 8.0, the butenoic acid and the enanilide were 80.8% and 19.2%, respectively, at 4300 h. Therefore, it was hypothesized that vinclozolin was converted to both the butenoic acid and the enanilide and the conversion from vinclozolin to butenoic acid was reversible and pH-dependent. Mercadier et al. (1998) also reported chemical hydrolysis of vinclozolin from aqueous buffer. Their findings showed that vinclozolin was transformed to a carbamic acid derivative that was an unstable intermediate, converting to an enanilide. In the present study, *C. elegans* formed both the carbamic and enanilide compounds (Figure 6). However, these two metabolites were mediated by enzymatic reaction, as shown by separate biological and abiotic control experiments. Mercadier et al. (1998) studied the biological degradation of vinclozolin by two different mixed bacterial cultures that degraded vinclozolin to butenoic acid or enanilide, leading to 3,5-dichloroaniline as the final product (Figure 1). They also isolated from soil *Corynebacterium sp*. which was able to degrade vinclozolin via formation of butenoic acid and 3,5-dichloroaniline as final products of transformation. Golovleva et al. (1991) also reported butenoic acid and enanilide as products of degradation by pure cultures of *P. fluorescens* 8/28, *Bacillus cereus* 625/1, and *B. brevis* 625/2; however, these transformation products appear to be more in agreement with chemical transformation, as seen in other studies (Clark, 1983; Melkebeke et al., 1986; Szeto et al., 1989; Villedieu et al., 1994). Other scientists using mixed bacterial cultures detected only 3,5-dichloroaniline (Cain and Mitchell, 1996) and an unidentified metabolite leading to the formation of 3,5-dichloroaniline (Head et al., 1988) as the major metabolic product.

Metabolite **IV**, 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide, was formed via decarboxylation of the oxazolidine portion of the vinclozolin molecule (Figure 6). This metabolite has also been previously identified as an irreversible hydrolysis product of vinclozolin from water suspension (Clark, 1983), aqueous buffer (Mercadier et al., 1998), plants and soil (Szeto et al., 1989a,b,c), and mammalian metabolism (Hawkins et al., 1990; Wong et al., 1995). However, in mammalian metabolism studies, when vinclozolin was administered to adult rats it was rapidly hydrolyzed to 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid and/or 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide products in rodent tissues; the major phase II metabolite was identified from urine and bile as a glucuronide conjugate of 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide following dihydroxylation of the vinyl group (Hawkins et al., 1990).

The 3R- and 3S- isomers of 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide (**II** and **III**, Figure 6) were presumably formed by an epoxide hydrolase reaction via ethylene dihydroxylation of the vinclozolin metabolite 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (**IV**). These two novel metabolites together accounted for about 33% of the total metabolism at 168 h incubation. The fungus *C. elegans* is known to transform polycyclic aromatic hydrocarbons (PAHs) by cytochrome P450 monooxygenase and epoxide hydrolase reactions to form *trans*-dihydrodiols (Pothuluri and Cerniglia, 1998). *C. elegans*, like most fungi, does not utilize PAHs as a

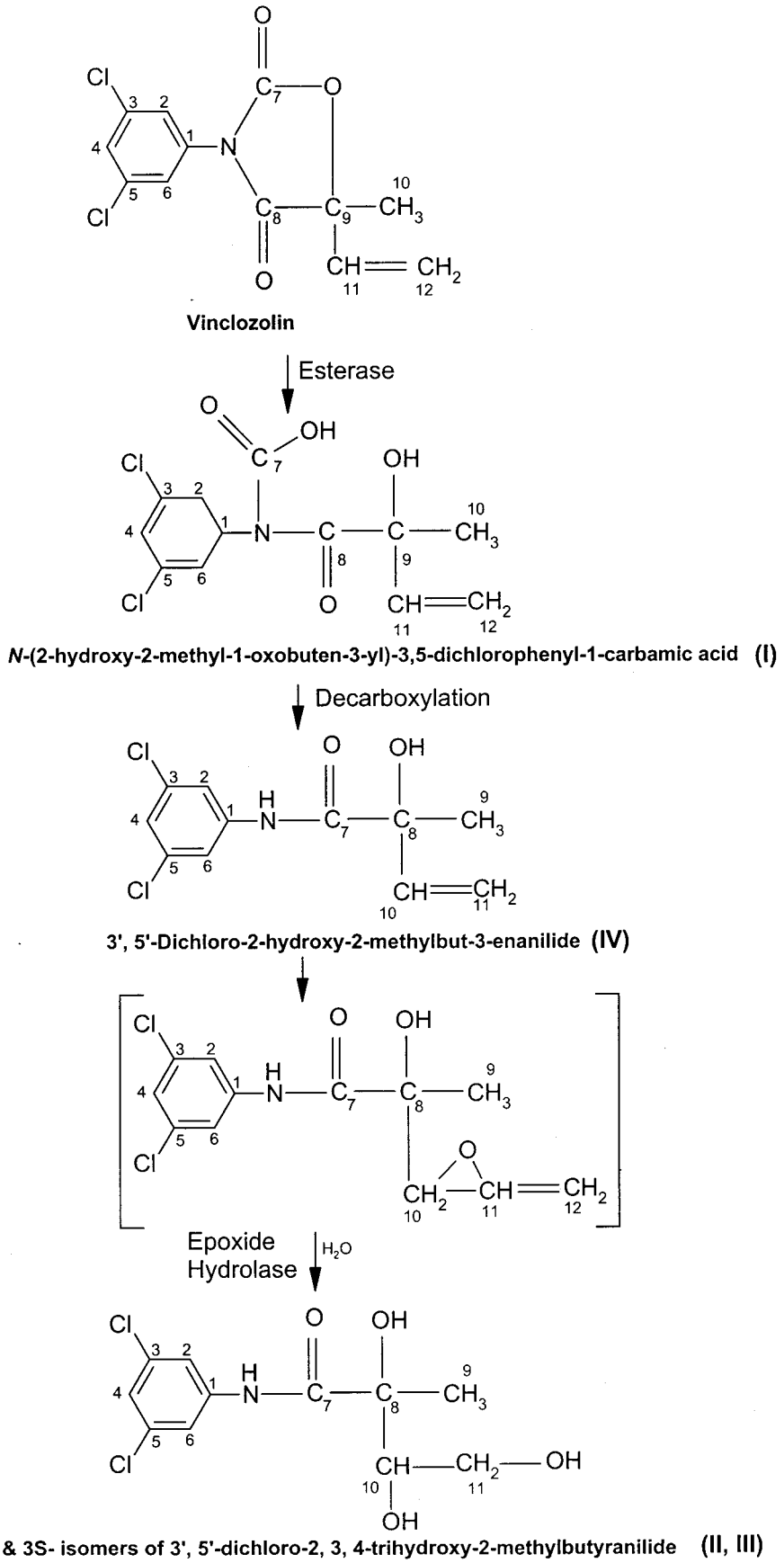

Figure 6. Proposed scheme of biotransformation of vinclozolin by *C. elegans*. Structure shown in brackets is a proposed intermediate that has not been characterized.

source of carbon and energy but biotransforms or co-metabolizes these recalcitrant compounds to detoxified products (Cerniglia, 1997). In previous studies, *C. elegans* was able to biotransform two chloracetamide herbicides, alachlor and metolachlor, via multiple site oxidation (Pothuluri et al., 1993, 1997).

Previous studies with several xenobiotics by *C. elegans* have shown the involvement of cytochrome P450 monooxygenase reactions followed by nonenzymatic rearrangement via an NIH shift mechanism (Cerniglia, 1997) to the phenol or enzymatic hydration of the corresponding epoxide to form the *trans*-dihydrodiol. Subsequent pathways involve sulfation and glycosylation (Zhang et al., 1996). In mammalian systems, hepatic microsomal cytochrome P450-dependent monooxygenase has been implied as the most important enzyme system involved in the phase I biotransformation of xenobiotics (Guengerich, 1987). Ronis et al. (1994) found induction of P450 isozymes in male rats when vinclozolin was administered. They postulated that the complex patterns of inhibition and induction of hepatic cytochrome P450 isozymes produced by ergosterol biosynthesis inhibiting fungicides, including vinclozolin, in mammals and birds could have potentially significant toxicological consequences. In vivo exposure of rodents to vinclozolin resulted in the ring-opening of vinclozolin to form two major metabolites, butenoic acid and the enanilide (Wong et al., 1995). Even though the biotransformation of vinclozolin occurs predominantly on the oxazolidine-2,4-dione portion of the vinclozolin molecule, in fungal as well as mammalian systems, our present study demonstrates multiple-site oxidation of vinclozolin by *C. elegans*. In contrast, bacterial transformation of vinclozolin (Figure 1) is mainly via the formation of 3,5-dichloroaniline (Cain and Mitchell, 1996; Head et al., 1988) with either butenoic acid or enanilide as intermediates (Mercadier et al., 1998).

Previous herbicide studies using microorganisms have shown formation of hydroxylated products, which have been implicated as detoxification products because these types of compounds are generally more soluble in water and therefore more amenable to biodegradation and accelerated degradation by organisms (Bailey and Coffey, 1986; Krause et al., 1985; Pothuluri et al., 1997). The formation of hydroxylated products by *C. elegans* may be useful in the bioremediation of vinclozolin.

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