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Degradation of the insecticide Hydramethylnon by *Phanerochaete chrysosporium*

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Abstract

The decomposition of the amidinohydrazone-type insecticide Hydramethylnon (HMN) by soil fungi has been investigated. A simple spectrophotometric method was developed for the estimation of HMN in soil and fungal culture media. HMN was found to be degraded in soil with a half life of 14 to 25 days.

Degradation of HMN by the lignolytic fungus, *Phanerochaete chrysosporium* yielded two major breakdown products; *p*-(trifluoromethyl)-cinnamic acid (TFCA) and *p*-(trifluoromethyl)-benzoic acid (TFBA). TFCA was converted to TFBA which was subsequently metabolised via the *meta*-fission pathway. Fluoride release from HMN could not be detected.

Abbreviations: BzDAc – benzene, dioxane, acetic acid (60: 36: 4); DCM – dichloroethane; DNPH – 2,4-dinitro-phenylhydrazine; HMN – Hydramethylnon; TDAc – toluene, dioxane, acetic acid (90: 30: 1); TFCA – *p*-(trifluoromethyl)-cinnamic acid; TFBA – *p*-(trifluoromethyl)-benzoic acid; TFP 1,5-bis(trifluoro-*p*-tolyl)-1,4-pentadien-3-one; VA – veratryl alcohol

Introduction

In 1987 the amidinohydrazone class of insect toxicants was discovered (Lovell 1979) and one of these, Hydramethylnon (HMN), is one of the few compounds registered for the control of Red Introduced Fire Ants (RIFA). This task requires a slow-acting insecticide so as to allow a foraging ant to distribute the toxicant to other members of the colony, thereby poisoning the queen and larvae as well as worker ants (Vander-Meer et al. 1982).

Commercially, HMN (as 0.88% active ingredient in a soybean oil attractant) is currently marketed by the American Cyanamid Co. as AMDRO™ fire ant insecticide, and Combat™ and Maxforce™ preparations for the control of cockroaches (Mallipudi et al. 1986). In New Zealand, two species of wasps, *Vespula vulgaris* and *V. germanica* have become an increasing problem and HMN is currently being evaluated for controlling wasps in New Zealand (Spurr 1991). This prompted the present investigation into the biodegradation of HMN.

Properties of Hydramethylnon

HMN is a yellow-orange crystalline, odourless, powder. Its structure and formal name is: 2-(1H)tetrahydro-5,5-dimethyl-pyrimidinone [3-[4-(trifluoromethyl) phenyl]-1-[2-[4-(trifluoromethyl)-phen-

yl] ethenyl]-2-propenylidene] hydrazone. Molecular weight ($C_{25}H_{24}N_4F_6$): 494.19 (See Fig. 3 for structure).

The dry powder form is stable for long periods, even in sunlight (Wayne et al. 1988). However, in the presence of water, HMN undergoes rapid photolysis to produce four major breakdown products; *p*-trifluoromethyl-benzoic acid (TFBA), *p*-trifluoromethyl-cinnamic acid (TFCA), 1,5-bis(trifluoro-*p*-tolyl)-1,4-pentadien-3-one (TFP) and 6,7,8,9-tetrahydro-7,7-dimethyl-3-[*p*-(trifluoromethyl) styryl]-4*H*-pyrimido-[2,1-c]-triazin-4-one (TFPT) (Mallipudi et al. 1986). Under field conditions HMN degradation occurred only during daylight hours (Vander-Meer et al. 1982).

Little is known of the biotranformation of HMN; Hollingshaus & Little (1984b) reported that the tobacco budworm metabolised HMN to at least six major breakdown products which were similar to the photo-oxidation products of HMN.

Degradation of lignin and xenobiotics by Phanerochaete chrysosporium

Phanerochaete chrysosporium is one of several white rot fungi able to degrade lignin and other recalcitrant compounds (Eggeling 1983). This ability is due, in part, to the secretion of unique peroxidases which catalyse a free-radical-dependent oxidation and depolymerisation of lignin (Tien & Kirk 1984). These peroxidases are collectively referred to as lignin peroxidases (LiPs) or Mn-peroxidases. Like other peroxidases, these enzymes can carry out single electron oxidations of substrates at the expense of hydrogen peroxide. However, under the acidic conditions prevailing during lignin breakdown, LiP can oxidise nonphenolic aromatic compounds, an activity not displayed by most other peroxidases (Harvey 1986).

LiP is produced by cultures of *P. chrysosporium* when nutrient nitrogen, carbon or sulphur becomes limiting. The enzyme usually appears after three days of growth in Kirk's defined medium (Kirk et al. 1978), reaches a peak after six days and subsequently declines in activity correlating with the appearance of extra-cellular proteases (Dosoretz et al. 1990). Although molecular oxygen is not a requirement for LiP or manganese peroxidase enzymes, lignin degradation has been reported to be greatly enhanced by increased availability of oxygen (Kirk et al. 1978; Dosoretz & Grethlein 1991).

The LiP from *P. chrysosporium* has also been found to be able to catalyse the degradation of a diverse range of environmental pollutants including quinones (Momohara et al. 1991), polymethoxybenzenes (Ageorges et al. 1991) and polycyclic aromatic hydrocarbons (Bumpus 1989). Many of the xenobiotic compounds degraded are aromatic, conjugated nitrogen (azo) or halogenated compounds (Bumpus & Aust 1987). Loss of chlorine was observed from pollutants such as chlorolignins (Pellinen et al. 1988), DDT (Bumpus & Aust 1987; Fernando et al. 1989), TCDD (Bumpus et al. 1985), 2,4,5-T (Ryan & Bumpus 1989) and PCPs (Lamar et al. 1990a, b) when incubated with *P. chrysosporium.* Dehalogenation of the non-aromatic insecticides aldrin, dieldrin, heptachlor, chlordane, mirex and lindane has also been demonstrated (Kennedy et al. 1990). Similarly the recalcitrant azo, polymeric (Glenn & Gold 1983; Platt et al. 1985; Paszczynski et al. 1991) and heterocyclic dyes (Cripps et al. 1990) are also susceptible to lignolytic degradation.

In most cases, the degradation of pollutants by *P. chrysosporium* was attributed to the LiP enzyme system and pure enzyme preparations have been used in some studies (Aitken et al. 1989; Bumpus & Brock 1988). However, DDT (Kohler et al. 1988) and crystal violet (Bumpus & Brock 1988) have also been reported to be metabolised in the absence of ligninolytic conditions.

The ability of *P. chrysosporium* to degrade such a diverse range of xenobiotic compounds has many advantages for biodegradation of environmental pollutants. Firstly, white rot fungi are ubiquitous. Secondly, the peroxidases are extracellular, which is an advantage since many pollutants bind to soil particles or are only slightly soluble in water and therefore less accessible to microbes. Thirdly, the reactions are free radical in nature and therefore not limited by the constraints of enzymatic reactions; thus a wide range of substrates can be dealt with simultaneously and at low concentration. Lastly, the enzyme is regulated by environmental factors, not

by substrate levels, and the enzyme is synthesised irrespective of substrate availability.

In this paper we wish to report the breakdown of HMN by LiP from *P. chrysosporium.*

Materials and methods

Hydramethylnon: technical grade HMN (97% purity) was kindly donated by American Cyanamid Co., Agricultural Research Division, Princetown, NJ. *p*-Trifluoromethyl-cinnamic acid (TFCA), *p*-trifluoromethyl-benzoic acid (TFBA) and veratryl alcohol (VA) were obtained from Aldrich Chemical Co. (USA). All other reagents used were reagent grade or better.

UV spectra were determined by means of a Hewlett Packard Model HP8452A diode array spectrophotometer.

Fluoride was measured with a F^- selective electrode (model ISE333, EDT Research, UK). Samples were assayed by mixing equal volumes of sample and total ionic strength adjustment buffer (TISAB; 57 ml of glacial acetic acid, 58 g of NaCl and 2 g of 1,2-diamino cyclohexane-N,N,N,N-tetraacetic acid (CDTA) in 1 l of water, pH adjusted to 5.5 with 4 M NaOH) (Walker & Bong 1981). A fresh calibration curve was prepared each day.

Estimation of Hydramethylnon

Existing methods for the estimation of HMN required a tedious sample separation followed by analysis by GLC or HPLC (Vander-Meer et al. 1982; Wayne et al. 1988; Mallipudi et al. 1986). These were considered too time-consuming for this project so a simple and rapid UV spectrophotometric assay was developed to monitor the rate of decomposition of HMN.

The UV absorption spectrum of HMN shows a broad peak at 295 nm but this wavelength was unsuitable for an assay since many other compounds, including possible HMN breakdown products, absorbed strongly in this region. However, above pH 10, the spectrum of HMN undergoes a marked bathochromic shift to 395 nm (Fig. 1) and this was utilised for its assay. Because HMN is only sparingly soluble in water (6×10^{-9} g l^{-1}) there was need to add

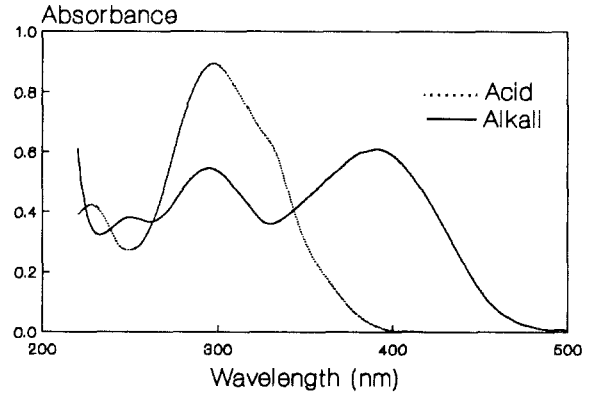

Fig. 1. UV spectra of HMN in presence of acid (pH 3) or alkali (pH 10).

a water-miscible solvent to solublise any HMN in samples. Basification of samples could lead to the precipitation of Ca^{2+} and Mg^{2+} hydroxides and turbid solutions; this was prevented by addition of EDTA to complex these ions.

Acetone (10 ml) was added to the sample extract (10 ml) or culture filtrate (10 ml) to dissolve any HMN. Next, 1 ml of 0.4 M NaOH containing 10 mM EDTA was added to this extract and the alkali-induced bathochromic shift measured at 395 nm ($\varepsilon_{395} = 2.69\times10^4$). Beer's law was obeyed (±4%) up to 75 µM HMN.

Because HMN is susceptible to photolytic degradation (up to 7% loss after 1.5 hr) all procedures were carried out in reduced light. However there was negligible loss of absorbence (less than 2% after 45 min) for samples held in the spectrophotometer at 295 nm.

For measurements of HMN degradation in test soils 3 g samples were extracted with 10 ml ethanol or acetone in an amber glass vial. After standing for 5 min the suspension was clarified by centrifugation at 4000*g*. Next, the absorbance was recorded at 395 nm for a 2 ml aliquot of the supernatant mixed with 0.1 ml NaOH/EDTA using a pure solvent blank. The mean absorbence (A_{395} CONTROL) of several extracts of HMN-free soil was used to correct the sample (A_{395} SAMPLE) readings.

Thin layer chromatography

HMN and aromatic compounds, extracted from culture filtrates with dichloromethane (DCM),

were separated by TLC on silica gel plates impregnated with fluorescent indicator (254nm) and developed with toluene, dioxane, acetic acid (90: 30: 1) (TDAc) (Mallipudi et al. 1986).

Compounds were located under UV light and the zone eluted with 300µl methanol for subsequent spectral analysis. Various chromogenic spray reagents were also used to attempt to locate ketones, nitrogen- and fluorine-containing compounds.

Keto-acids were identified by derivatizing with 2,4-dinitrophenylhydrazine (DNPH); these were extracted into ethyl acetate and then reextracted into dilute (10% v/v) Na_2CO_3. The keto-acid DNPHs were separated by TLC on silica gel plates developed with benzene, dioxane, acetic acid (60: 36: 4) (BzDAc) (Walker & Taylor 1983).

High performance liquid chromatography (HPLC)
Analyses were performed on a Varian 5000 Liquid Chromatograph equipped with an UV detector set at 280nm. A C18 (MCH-5-NCAP) reverse phase column (150mm ×4 mm, with a 10mm guard column) was used with acetic acid (0.1%) in water/methanol mixture (40: 60), at a flow rate of 1.5ml/min, as the mobile phase. TFCA and TFBA solutions (5mM and 50mM), were used as calibration standards; HMN did not elute under the above conditions.

Photolysis of HMN
Sealed vials containing HMN (35µM or 70µM) in 5ml water were incubated under constant illumination from both tungsten and fluorescent lamps. Control vials were kept in the dark. Loss of HMN was assayed spectrophotometrically and the degradation products analysed by TLC.

Isolation of HMN degrading organisms
Selective enrichment culture in a simple mineral salts medium (MSM) (Kirk et al. 1978) containing 70µM HMN (final concentration) was used to attempt to isolate HMN-degrading organisms from soil. Fungi were grown on potato dextrose agar (PDA) or malt extract agar (MEA) and incubated at 26°C, in darkness to avoid risk of photolysis of HMN.

Cultivation of P. chrysosporium
P. chrysosporium Burds (strain 24725, University of Canterbury) was cultured on PDA for 3–4 days at 37°C, then maintained at 26°C and subcultured every 4 to 5 weeks. A spore suspension was used to inoculate batch cultures grown on mineral salt medium (Kirk et al. 1978) and incubated at 37°C without shaking.

The extracellular LiP from 6-day culture filtrates of *P. chrysosporium* was concentrated and partially purified by precipitation with three volumes of cold (−18°C) acetone followed by resuspension in 0.1M-phosphate buffer (pH 3.5). This gave a five fold increase in LiP activity and also removed many endogenous DCM-extractible metabolites.

Degradation of HMN by P. chrysosporium; *isolation of degradation products*
P. chrysosporium was grown for four days in liquid culture and then HMN (70µM HMN final concentration) was introduced to coincide with the onset of lignolytic activity. To minimise the risk of photodegradation of HMN, cultures were incubated in the dark at 26°C and assayed for HMN daily. This experiment was repeated under both nitrogen sufficient and deficient conditions (24mM and 2.4mM NH_4^+, respectively).

In similar experiments using the acetone-ppt LiP preparation 5ml enzyme suspension was incubated with 10% (v/v) Tween 20 (50µl), 10mM-HMN in ethanol (100µl) and 25mM-H_2O_2) (100µl) at 37°C.

Spectrophotometric assay of lignin peroxidase activity
Fungal cultures were assayed for extracellular LiP activity by the veratryl alcohol (VA) oxidase assay method of Tien & Kirk (1984) which measured the rate of conversion of VA to veratrylaldehyde.

The oxidation of HMN, TFCA, TFBA and veratryl alcohol (VA) by extra-cellular LiPs secreted by *P. chrysosporium* was monitored using a diode array spectrophotometer. Reaction cuvettes contained the following; enzyme (1.4ml), 0.36M – Na 2,2-dimethylsuccinate buffer (pH 3.0) (0.6ml), plus substrate and inhibitor in a total volume of 3ml. The spectrophotometer was blanked on this mixture and then the substrate introduced and scanning ini-

tiated. Multiple scans from 200–500 nm were made at 20 s or 30 s intervals for 5–15 min and experiments were conducted at room temperature. At the end of experiments with HMN the assay mixture was adjusted to pH 10 to check if any HMN remained in the sample.

Results

Initial attempts to isolate microbes capable of growth in the presence of HMN were inconclusive. A fungus, isolated from river bank soil, grew as a suspended pellet in MSM containing 2.5 mM HMN and was identified as *Fusarium solani*. However, although *F. solani* grew in the presence of HMN, the metabolism of HMN by this isolate could not be confirmed.

In experiments with cultures of *P. chrysosporium* disappearance of HMN began after a lag phase of three days (Fig. 2) which coincided with the onset of the idiophase when ligninolysis is likely to begin (Aust 1990). Disappearance of HMN correlated with the LiP activity of the cultures; both HMN disappearance and LiP activity were suppressed when nutrient nitrogen (as 24 mM NH_4^+) was added and both exhibited a 3–4 day lag period prior to onset. This is consistent with many reports (for a summary see Bumpus & Aust 1990), where the mineralisation of xenobiotic compounds was found to be related to lignin-degrading ability and nitrogen limitation (Aust 1990).

Experiments with partially purified lignin peroxidase

Preliminary TLC examination of DCM extracts of HMN-containing cultures revealed many spots from both control and HMN culture filtrates. Therefore, in order to minimise the number extraneous spots on TLC plates, these experiments were repeated using a partially-purified LiP fraction (acetone ppt.) from culture filtrates of *P. chrysosporium*. TLC of DCM extracts from these experiments yielded several degradation products and each spot (unknowns A–H) was eluted from the TLC plate and its UV spectrum recorded.

Unknown C co-chromatographed with the

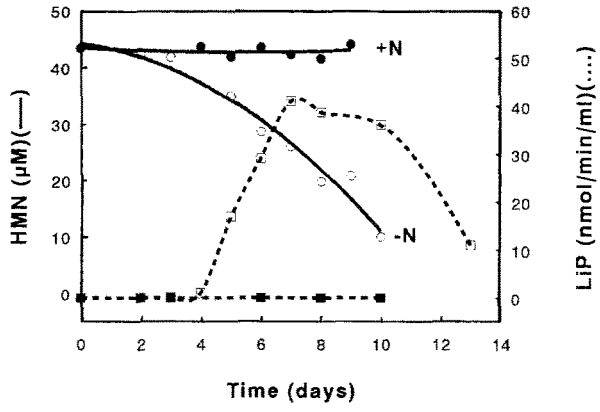

Fig. 2. Lignin peroxidase (LiP) activity and HMN degradation by *P. chrysosporium* in liquid culture; with (O, [X]) and without (O, []) nutrient nitrogen.

TFCA and TFBA standards, gave the same UV fluorescence colour and a positive test for organic fluorine with diphenylamine spray reagent. Its UV spectrum showed a λ_{max} at 262 nm which shifted to 266 nm (+4 nm) with $AlCl_3$. This correlated with authentic TFCA whereas TFBA had spectral maxima at 274 nm and 282 nm and exhibited no shift on addition of $AlCl_3$. Therefore unknown C was tentatively identified as TFCA.

Unknown E showed spectral maxima at 280 nm and 320 nm whilst unknown H had one peak at 310 nm; both spectra exhibited bathochromic shifts with alkali. Therefore unknowns E and H probably still retained the parent guanidine and hydrazone groups, as well as at least one aromatic function. Neither of these reacted with any of the spray reagents tested.

Unknown G co-chromatographed with HMN, exhibited the same UV fluorescence and gave the same colour with nitroprusside spray reagent. Its UV spectrum was similar to that of authentic HMN and exhibited the same characteristic bathochromic shift (Fig. 1). It was therefore assumed to be unmetabolised HMN.

The remaining spots were too weak to allow further identification.

HPLC analysis; further metabolism of TFCA

HPLC analysis of the HMN replacement culture medium yielded two major peaks which were ab-

sent from the control. These peaks were subsequently identified as TFCA and TFBA.

TFCA and TFBA having been tentatively identified as degradation products of HMN, evidence for their further metabolism by *P. chrysosporium* was sought. When TFCA was incubated with fungal cultures, four peaks, absent in the control, appeared in the HPLC trace. One of these was identified as TFBA, the other as unmetabolised TFCA; the other two could not be identified. It was concluded therefore that *P. chrysosporium* converted TFCA to TFBA.

Role of lignin peroxidase

Further evidence that the lignin degrading system of *P. chrysosporium* was involved in the degradation of HMN came from spectrophotomeric observations of the reactivity of the culture fluid from lignolytic fungal cultures. Triplicate cuvettes were set up with the standard LiP assay system plus 1mM-HMN (5µl), 10% (v/v) Tween 20 (10µl) to disperse the HMN. The reaction was started by the addition of H_2O_2 and monitored spectrophotometrically for 500s, at which time the reaction was stopped by the addition of 2.5M NaOH (0.5ml) and the absorbance measured at 396nm. The characteristic HMN spectrum displayed a marked change from one major peak at 305nm to three peaks at 280nm, 290nm and 310nm. When H_2O_2 or enzyme were omitted no change in the spectrum of HMN was observed. A control tube had NaOH added directly without an incubation period. An additional experiment showed that loss of HMN was enhanced by the addition of 24mM VA (20µl) to the above system.

The effect of culture fluid LiP on the putative metabolites TFCA and TFBA was investigated in a similar manner. TFBA was oxidised rapidly as evidenced by an increase in absorbence at 280nm and the appearance of a new peak at 310nm. TFCA reacted similarly to TFBA but with a less dramatic spectral change. Both of these reactions required H_2O_2 and enzyme.

The effect of metabolic inhibitors on the above reactions was similar in all cases (Table 1). All reactions were inhibited by 10mM EDTA and 10mM KCN, suggesting that a metal ion was required by the degradative system. The level of H_2O_2 was found to be important; H_2O_2 was required to start the reaction but at elevated levels (12mM) was inhibitory. These results are consistent with the properties of LiP.

The above results, together with other evidence such as that the reactions proceeded at pH 3 and that the degradation of HMN was increased in the presence of VA, support the hypothesis that LiP was active against HMN.

Ring fission of TFCA and TFBA

Fungal cultures (100ml) were grown for six days and their LiP activity confirmed by the VA oxidase assay. TFCA or TFBA (0.4ml 0.05M in ethanol) was then added and, after incubation, samples were analysed by HPLC or TLC (TDAc solvent) for breakdown products, keto acids, or F^-.

In an attempt to increase keto acid accumulation some TFCA or TFBA replacement cultures also included 4mM arsenite, an inhibitor of oxidative decarboxylation (Walker & Taylor 1983).

Identification of ring-fission products; keto acids

Cultures of *P. chrysosporium* incubated with TFBA or TFCA accumulated several ketoacids. Pyruvate was identified in both control and HMN degradation experiments, but concentrations were always higher in the latter. Two additional keto-acids accumulated in the HMN-containing cultures; the DNPH-derivative one of these co-chromatographed with authentic 2-oxoglutarate-DNPH in TDAc and BzDAc. The second DNPH-derivative was bright orange whilst all of the other DNPH de-

Table I. Inhibition (as % loss) of enzyme activity by selected reagents.

		Substrate			
		VA	HMN	TFBA	TFCA
Assay wavelength*		310	280	310	310
Inhibitor					
KCN	10mM	83.4	77.7	nt	nt
EDTA	10mM	98	95.4	98	100
H_2O_2	12mM	77.0	71.4	nt	nt

* Wavelength at which change in absorbance was measured. (nt = not tested).

rivatives were a bright canary-yellow in colour. This compound was designated unknown Q; TFCA cultures accumulated more of this compound than did TFBA cultures.

Accumulation of ketoacids was enhanced by the addition of 4 mM arsenite to fungal cultures. In this case pyruvate was absent from the controls but present in high concentration in the TFCA degradation experiment which suggested that in this case aromatic ring cleavage had occurred via the *meta*-fission pathway (Dagley 1971). Accumulation of Unknown Q and 2-oxoglutarate also increased and a new compound, with an R_f higher than that of pyruvate, appeared.

Unknown Q could not be identified. The DNPH extraction procedure favoured acidic carbonyl compounds and the low R_f of Q-DNPH suggests that it is a dicarboxylic keto acid; probably an intermediate in the *meta*-fission pathway. However it is unlikely that Q is a β-keto acid because such compounds are easily decarboxylated and heating had no effect on the R_f of this compound.

Photolytic decomposition of HMN

HMN was degraded under high light intensity, with a half life of approximately 180 minutes. TLC analyses of the photodegradation products showed an initial rapid increase in the number of DCM-extractible compounds which then declined to only five major products; no trace of HMN remained at this stage.

One of the photodegradation products possessed an R*f* value, UV spectrum and $AlCl_3$ shift similar to that of authentic TFCA. This agrees with Mallipudi et al. (1986) who reported TFCA and TFBA as major photo-degradation products of HMN.

Removal of HMN from soil

When HMN was incubated with soil samples its concentration diminished steadily with a half life of approximately 14 days at 38° C and 25 days at 26° C. Disappearance of HMN was attributed to microbial action since the samples were incubated in darkness and no loss of HMN occurred in sterilised controls.

Analysis for fluoride liberation

Using the F^- selective electrode liberation of F^- from the trifluoromethyl moiety could not be detected when HMN was incubated with *P. chrysosporium*. Control experiments with added F^- confirmed that the loss of F^- by adsorption was negligible. Similarly, cultures incubated with TFCA and TFBA (both 2 mM) also failed to liberate F^-.

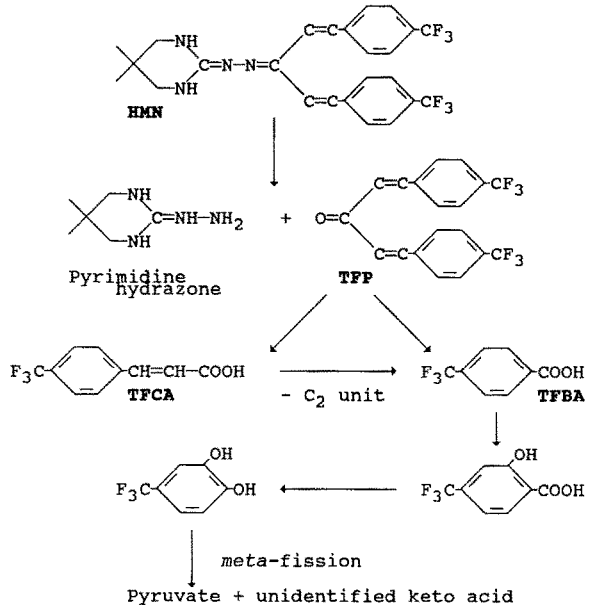

Fig. 3. Tentative pathway for the breakdown of HMN by *P. chrysosporium*.

Discussion

HMN was degraded by the extracellular LiP enzyme system from *P. chrysosporium*. Two of the HMN-decomposition products, TFCA and TFBA, were provisionally identified on the basis of TLC, UV spectroscopy, HPLC and chromogenic reactions. Both TFCA and TFBA have been reported previously as photo-degradation products and as metabolites of HMN in the tobacco budworm. This pathway is probably initiated by the hydrolysis of HMN to TFP and a pyrimidine hydrazone (Fig. 3).

TFP could not be confirmed in this study because of lack of an authentic reference compound. Two other HMN degradation products which retained the alkali-induced bathochromic shift were located by TLC. This suggests that hydrolysis was not the

only reaction to take place durig initial transformation.

Further metabolism of TFCA to TFBA, presumably via β-oxidation of the side chain, was demonstrated and both compounds were degraded by the LiP system. Since the carboxyl group is already fully oxidised. and the trifluoromethyl group maybe considered to be unreactive, it seems likely that the aromatic ring nucleus was cleaved. Because carboxyl groups are *ortho/para* directing towards incoming nucleophiles, whilst alkyl are *meta*-directing (Fessenden & Fessenden 1986), it is likely that a hydroxyl group would attach adjacent to the carboxyl group. Thus this reaction could fulfill the usual requirement for *vicinal* phenolic hydroxyl groups prior to ring fission.

Two possibilities exist for aromatic ring cleavage during the metabolism of TFBA and TFCA. Cultures incubated with TFBA and TFCA accumulated pyruvate, suggesting that these compounds were broken down by *meta,* rather than *ortho,* ring-fission (Dagley 1971). Failure to detect β-keto-adipate (an indicator of *ortho*-fission), and the enhanced accumulation of keto-acids in the presence of arsenite, support this view. Furthermore, these results agree with the route for *p*-toluic (4-methyl-benzoic) acid which undergoes ring hydroxylation and reduction of the carboxyl group to form 4-methyl catechol prior to ring cleavage by the *meta*-pathway (Smith 1991).

Several hypotheses could explain the failure to detect release of fluoride:

1. Trifluoromethyl aromatic compounds may be recalcitrant and mineralised slowly. Although the mineralisation of fluorobenzene type compounds has been well documented (Hardman 1991), information on the fate of halomethylbenzenes is scarce.
2. Trifluoromethyl-benzene compounds, or their ring-fission products, may inhibit key enzymes in the degradative pathway.
3. These compounds may not be completely mineralised.

The evidence from this study, suggests that HMN could be removed from soil by the combined actions of photolysis (at the surface) and degradation by soil microorganisms and *P. chrysosporium*; therefore it should not be a too recalcitrant compound. It seems unlikely that HMN would bioaccumulate or biomagnify through food chains to any great extent and that HMN will be short-lived followed application. Nevertheless the possibility that some of its breakdown products may be toxic cannot yet be eliminated.

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