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ORIGINAL PAPER

Hydroxylation of thiacloprid by bacterium *Stenotrophomonas maltophilia* CGMCC1.1788

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Abstract Chloropyridinyl neonicotinoid insecticides play a major role in crop protection and flea control on cats and dogs. Imidacloprid, thiacloprid and acetamiprid have in common the 6-chloro-3-pyridinylmethyl group but differ in the nitroguanidine or cyanoamidine substituent on an acyclic or cyclic moiety. Our previous study found that *Stenotrophomonas maltophilia* CGMCC 1.1788 could hydroxylate imidacloprid to 5-hydroxy imidacloprid, and 5-hydroxy imidacloprid was easily converted to 10–19 times higher insecticidal olefin imidacloprid against aphid or whitefly. Acetamiprid could be transformed by *S. maltophilia* to form *N*-demethylation product(IM 2-1). In this paper, we examined *S. maltophilia* CGMCC 1.1788's ability of transformation of thiacloprid. *S. maltophilia* CGMCC 1.1788 can hydroxylate thiacloprid to 4-hydroxy thiacloprid characterized by HPLC-MS/MS and NMR analysis, however 4-hydroxy thiacloprid could not be converted to olefin thiacloprid under acid conditions like imidacloprid, whereas oxidized and decyonated simultaneously to form 4-ketone thiacloprid imine in alkaline solution. Bioassays indicated that 4-hydroxy thiacloprid had 156 times lower insecticidal activity than thiacloprid, and the ketone-imine derivative almost had no toxicity towards aphid. Though both imidacloprid and thiacloprid are hydroxylated by *S. maltophilia* CGMCC 1.1788 at the same carbon atom position, however the structural difference between in imidacloprid and thiacloprid, originate the entire discrepancy in bioefficacy of metabolite and its further degrading pathway. These results explain that why thiacloprid is classified as not relevant grade for soil and seed applications, whereas imidacloprid is recommended and acetamiprid is limited.

Keywords Hydroxylation · Imidacloprid · Thiacloprid · Acetamiprid · *Stenotrophomonas maltophilia*

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Introduction

Neonicotinoid insecticides are now becoming the most successful, highly effective and largest selling insecticide worldwide for crop protection and veterinary pest control. They are active against numerous sucking and biting insect pests, including aphids, whiteflies, thrips, leaf miners, beetles, and some lepidopteran species (Nauen et al. 2003; Rouchaud

et al. 1994; Elbert et al. 2000). In this chemical class, imidacloprid, thiacloprid and acetamiprid belong to the chloropyridinyl class. The technical profiles and main differences between the three insecticides include their spectrum of efficacy, and versatile application forms, especially soil and seed treatment. Uses in soils are classified as follows: + + +, broad for imidacloprid; +, limited for acetamiprid; −, no relevant for thiacloprid (Elbert et al. 2008). Horowitz et al. (1998) revealed that acetamiprid was superior after foliar application, whereas imidacloprid was more effective after soil application. It is known that microbial activity is one of the most important factors in degradation of insecticide in soil, so the discrepancy in soil application between those three insecticides must be affected by microbial transformation.

Previously we reported that three bacterium *S. maltophilia* strains isolated from soils and screened from stock culture were able to hydroxylate imidacloprid to form 5-hydroxy imidacloprid, which was easily converted to olefin imidacloprid under acid condition. The insecticidal activity of olefin imidacloprid was 19 times higher than that of imidacloprid against horsebean aphid imago (Dai et al. 2006). *S. maltophilia* also transformed acetamiprid to form the 10 times lower insecticidal metabolite, *N*-demethylated(IM 2-1) (Chen et al. 2008). These results lead a new investigation on the microbial transformation to understand why longer time and better control are achieved when imidacloprid is applied as a soil drench application than the other methods, and acetamiprid shows weaker bioefficacy after soil application. In the present study, we examined biotransformation of thiacloprid registered for foliar application only, by the same *S. maltophilia* strain CGMCC 1.1788, which had the highest hydroxylation activity of acetamiprid. The results show that the hydroxylation appeared at imidazolidine cycle in imidacloprid, also arise at the same site in the thiazolidine cycle in thiacloprid to form 4-hydroxy thiacloprid. Bioassay indicates 4-hydroxyl thiacloprid is 156 fold lower activities than the parent compound thiacloprid against horsebean aphid. At acid condition, unlike the formation of olefin imidacloprid from 5-hydroxy imidacloprid, 4-Hydroxyl thiacloprid is further oxidized and decyonated simultaneously to form 4-ketone thiacloprid imine (Fig. 1). Bioassay showed the 4-ketone thiacloprid imine had no toxicity against horsebean aphid. Our results present that the minor molecular difference in neonicotinoid insecticides lead to different bioefficacy and subsequent degrading pathway of metabolites. Combination with the lower bioefficacy of 4-hydroxy thiacloprid and 4-ketone thiacloprid imine, it seems that thiacloprid is indeed not adapt for soil use, and only registered for foliar application. The hydroxylation by cytochrome P450 enzyme of *S. maltophilia* is always located at the carbon atom neighboring the nitrogen atom attached 6-chloro-3-pyridinylmethyl moiety.

Materials and methods

Bacterium cultivation and biotransformation of thiacloprid

The bacterium *S. maltophilia* CGMCC 1.1788 was obtained from the China General Microbiological Culture Collection Center. For assays of the biotransformation of thiacloprid, *S. maltophilia* was grown in 100 ml Erlenmeyer flasks containing 30 ml LB on shakers with 220 rpm at 30°C for 24 h. Cells were harvested by centrifugation at 6,000×*g* for 5 min, washed twice with 1/15 M Na_2HPO_4/KH_2PO_4 buffer (pH 7.2) and re-suspended in 10 ml of the same buffer containing 500 mg sucrose, and either 1.6 mg thiacloprid for the biotransformation. Reactions were carried out in 100-ml Erlenmeyer flasks on an orbital shaker at 220 rpm for 48 h at 30°C. After 48 h, cells were removed by centrifugation at 12,000×g for 10 min and the supernatant was filtered through a 0.22 μm filter membrane prior to HPLC analysis. The results are obtained at least by three independent experiments with three replicates.

Preparation of the metabolites of thiacloprid in 5 l fermentor

A single colony of *S. maltophilia* was used to inoculate a 1-l flask containing 300 ml of LB broth which was then incubated at 30°C on an orbital shaker at 220 rpm for 10 h. The culture broth was then transferred to a 5-l fermenter (Eastbiotech Co., Zhenjiang, China) containing 3.0 l LB broth. During cultivation, the fermenter was constantly aerated and stirred at 500 rpm at 30°C. After 8 h, cells were harvested from the broth by centrifugation at 6,000×*g* for 20 min and suspended in 3.0 l of

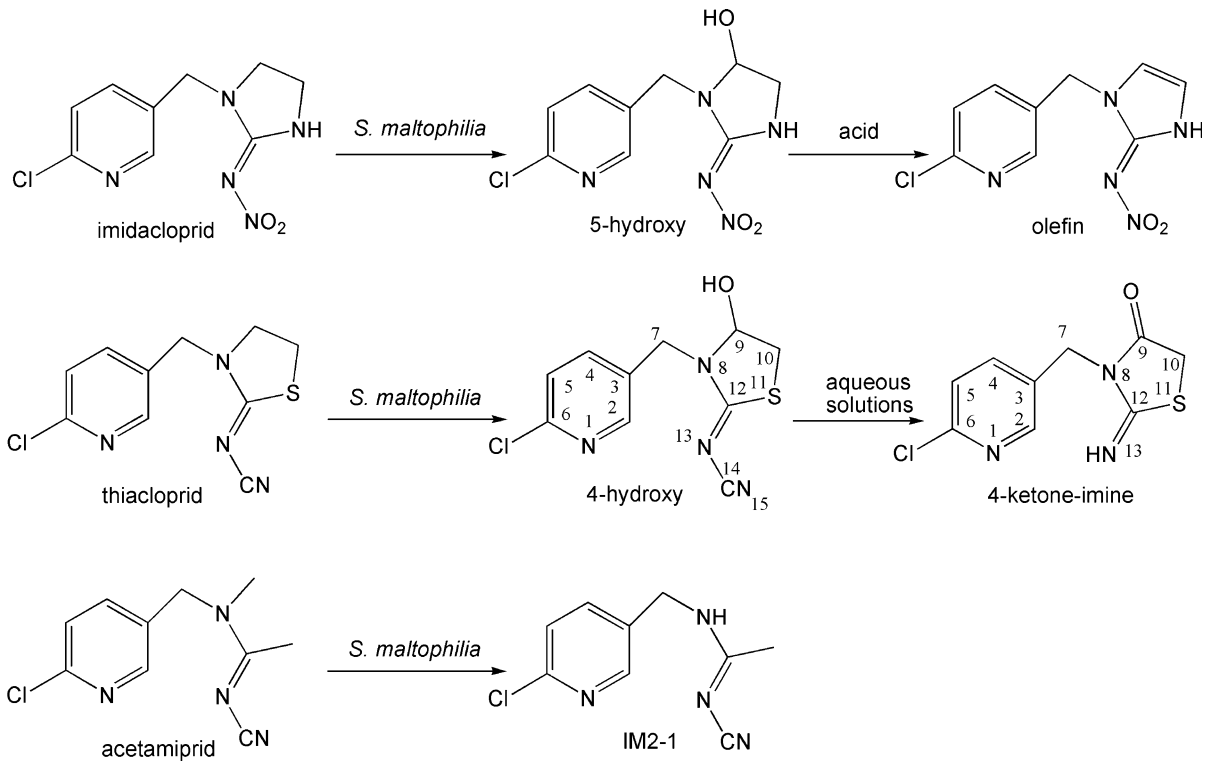

Fig. 1 Molecular structure of imidacloprid, thiacloprid, acetamiprid and its metabolites in this study

0.2 M Na_2HPO_4/KH_2PO_4 buffer (pH 7.2) containing 150 g sucrose and either 0.6 g thiacloprid. This mixture was then transferred to a 5-l fermenter for transformation. During biotransformation, the fermenter was maintained at 30°C and was constantly aerated and stirred at 500 rpm. After 48 h, cells were removed from the broth by centrifugation at 6,000×*g* for 20 min, and the supernatant was collected. The supernatant was extracted twice over a 1/10 volume of dichloromethane and an equal volume of acetoacetate. The organic extraction was concentrated to an appropriate volume using a vacuum rotary evaporator. The concentrated extraction was first purified by TLC (silica gel GF250, 20× 20 cm, dichloromethane: acetoacetate = 5:1) and further purified using a Shimadzu SPD-6AV preparative HPLC system with a shimpack prep ODS (10 μm, 250 × 30 mm) column. The column was eluted with methanol/water (28/72, v/v) at a flow rate of 15 ml/min, and monitored at 242 nm for thiacloprid with a Shimadzu SPD-10A UV detector. The elution was subsequently concentrated using a vacuum rotary evaporator until crystal formation.

Identification of the metabolite of thiacloprid

Mass spectrum analyses were conducted using an Agilent 1100 LC-MSD mass spectrometer equipped with an electrospray interface that was operated in either the positive or negative ion mode. IR spectra were recorded using KBr pellets on a Nicolet FTIR 670 IR spectrophotometer. ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectra of the biotransformation products, dissolved in DMSO-d6, were obtained using Bruker AV-400 spectrometer (Switzerland) operating at 100 and 400 MHz, respectively. Chemical shifts were referenced against an internal TMS reference standard. Several kinds of NMR techniques were used, including ^{1}H, ^{13}C NMR, DEPT, ^{1}H–^{13}C, ^{1}H–^{1}H COSY to assign chemical shifts to proton and carbon atoms (*d*).

Bioassays of metabolites of thiacloprid

The feeding and contact bioassay against horsebean aphid *Aphis craccivora* was tested by the method described by Nauen (1999) and our previous study

(Chen et al. 2008) at the National Pesticide Research & Development South Centre, Nanjing. Bioassay was replicated three times, the number of pest exceeded 30 in each dependant experiment, and the total number of pest was more than 100.

Inhibition of hydroxylation of thiacloprid by piperonyl butoxide (PBO)

The inhibition of imidacloprid hydroxylation activity by PBO was examined using the method of Matsuzaki and Wariishi (2004). The detail procedures have been described in our previous report (Dai et al. 2007).

HPLC analysis

The HPLC analysis was conducted using an Agilent 1100 HPLC system containing a Zorbax octadecylsilyl silicagel (ODS) column (250 × 4.6 mm, 5 μm). The elution was carried out at a flow rate of 1 ml/min with a mobile phase of water with 0.01% acetic acid: acetonitrile = 65:35 (v/v). The elution was monitored at 242 nm for thiacloprid using an Agilent G1314A UV detector. 20 μl of samples were injected for analysis after centrifugation (12,000×*g*) and deproteinization by heating.

Chemicals

Thiacloprid was provided by the Tianjin Rennong pesticide limited-liability company, China (>97% purity). Solvents used for the HPLC analysis were all of HPLC grade; all other solvents and inorganic reagents were of analytical grade and obtained from commercial sources.

Results

Microbial conversion of thiacloprid and the identification of metabolites

Thiacloprid was transformed into a single polar metabolite (P1) by *S. maltophilia* compared to culture control and substrate control, as determined by HPLC (Fig. 2). P1 and thiacloprid exhibited [*M* + H] peaks at *m*/*z* 268 and 252, respectively, in the MS data. This suggested that a hydroxyl group was introduced to

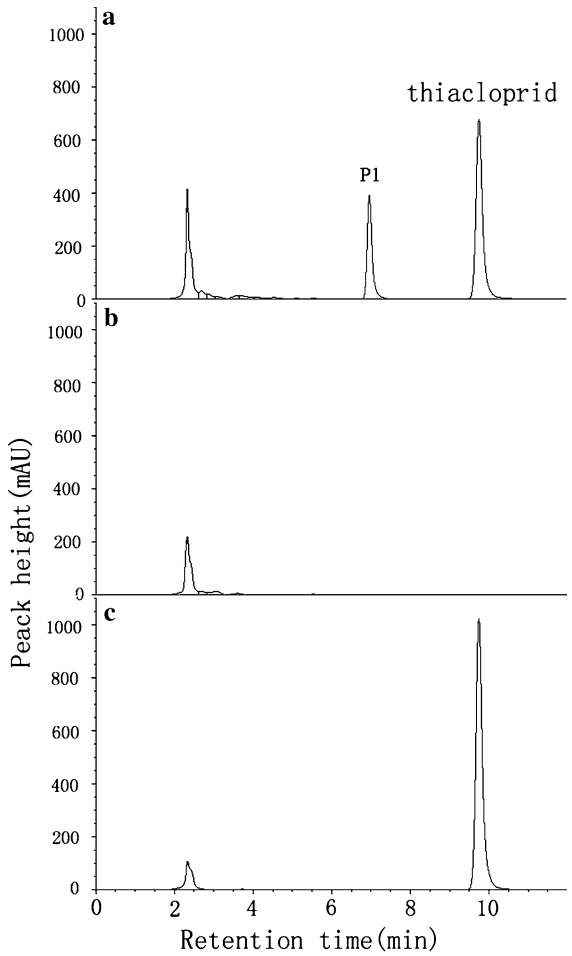

Fig. 2 HPLC spectrum of the metabolism of thiacloprid by *S. maltophilia*. **a** Thiacloprid and inoculated bacterium; **b** bacterium alone; **c** thiacloprid alone

thiacloprid. P1 was further identified using NMR analysis. ^{13}C NMR and ^{1}H NMR spectral data were listed in Table 1. ^{1}H and ^{13}C NMR data show there are nine protons and ten carbon atoms in metabolite P1, which is identical with parent thiacloprid. However, two new protons with chemical shift at δ 5.6 and δ 7.3 respectively, were observed in ^{1}H NMR analysis. The proton at δ 7.3 is uncoupled with carbon atom by HSQC analysis, so it can be assigned as the proton of hydroxyl moiety. The proton at δ 5.6 can be assigned the proton at C9 position. Based on the COSY spectra, it was observed that the proton δ 7.3 correlated with the proton (δ 5.6) at C9 position also was correlated to proton at C7 position and C10 position. Therefore, the P1 metabolite transformed by *S. maltophilia* CGMCC 1.1788 was identified as

Table 1 NMR spectral data for 4-hydroxy thiacloprid and 4-ketone thiacloprid imine (DMSO-d_6) (δ in ppm, J in Hz)

Number of atoms	4-Hydroxy thiacloprid (P1)		4-Ketone thiacloprid imine (P2)	
	^{13}C NMR (δ_C)	^{1}H NMR δ_H(J/Hz)	^{13}C NMR (δ_C)	^{1}H NMR δ_H(J/Hz)
2	149.9	8.4(d, 2.4)1H	149.5	8.4(d, 2.0)1H
3	131.7	–	133.4	–
4	124.6	7.5(d, 8.2)1H	124.7	7.5(ddd, 8.0, 4.9, 4.5) 1H
5	140.0	7.8(dd, 8.3, 2.6)1H	139.7	7.8(ddd, 9.6, 8.3, 2.5) 1H
6	149.9	–	149.9	–
7	44.7	4.7(d, 15.6)1H, 4.5(d, 15.6) 1H	47.4	4.6(s) 1H, 4.5(s) 1H
9	87.1	5.6(s)1H	187.4	
10	36.0	3.8(dd, 12.1, 6.3)1H, 3.3(dd, 12.1, 2.3)1H	45.1	3.9(s) 2H
12	174.9	–	181.5	–
14	117.1	–	–	9.6(s)1H
H-(OC9)	–	7.3(s)1H, 9-OH		

hydroxylation of thiacloprid at the C12 position, which often named as 4-hydroxy thiacloprid.

Time course of hydroxylation of thiacloprid and PBO Inhibition

Time course of the biotransformation of thiacloprid by the resting cells of *S. maltophilia* (Fig. 3) showed that the hydroxylation of thiacloprid had a high molar transformation ratio. After transformation for 60 h, almost 100% reduced substrate (0.10 mmol/l) was converted to 4-hydroxy thiacloprid (0.10 mmol/l). However, the transformation rate is only 24.2% (initial 0.46 mmol/l) after transformed for 108 h. The low transformation rate may be concerned with low water solubility of thiacloprid with 0.18 g/l.

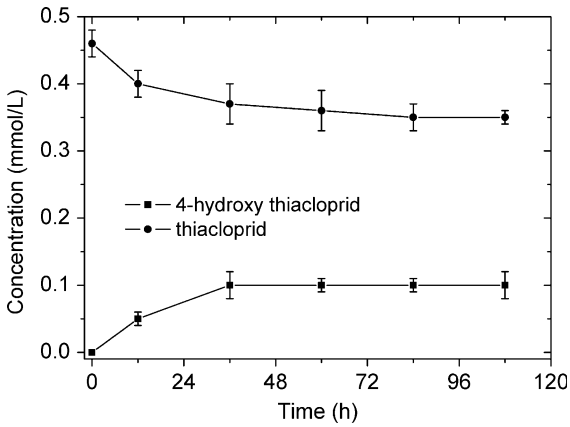

Fig. 3 Time course of the transformation of thiacloprid by resting cells of *S. maltophilia*. Transformations were conducted in 100-ml Erlenmeyer flasks with 10 ml of cell suspension containing 500 mg sucrose and 1.6 mg thiacloprid. Samples were tested by HPLC at indicated time. Data are presented as the mean ± SD ($n = 9$) from three independent experiments with three replicates

PBO is a common inhibitor of cytochrome P450, and is often used for demonstrating whether the reaction is catalyzed by cytochrome P450 enzymes (Sato et al. 2002). Previously we hypothesized that cytochrome P450 was involved in hydroxylation of imidacloprid by PBO inhibition (Dai et al. 2007). In this study, we demonstrated that the hydroxylation of thiacloprid was also inhibited by PBO, and the relative activity was reduced by increasing PBO concentration (Fig. 4). So both hydroxylation of imidacloprid and thiacloprid may be catalyzed by the same cytochrome P450 enzymes.

Conversion of 4-hydroxy thiacloprid in aqueous solutions

In previous study, we reported that 5-hydroxy imidacloprid dealt with hydrochloric acid, was easily converted to form olefin imidacloprid with high molar transformation ratio. The olefin imidacloprid is 19-fold higher activities than parent imidacloprid against horsebean aphid imago (Dai et al. 2006). At the same condition, 4-hydroxy thiacloprid was converted to a new product 2 (P2), and the conversion ratio enhanced by increasing pH value and weak acid (Fig. 5). The purified metabolite P2 was further characterized using NMR and infrared spectrum

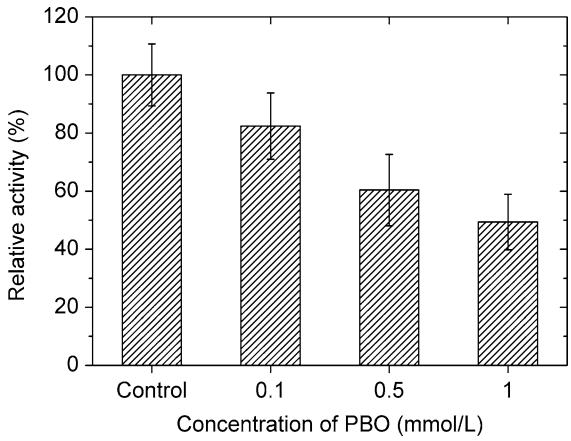

Fig. 4 Inhibition of PBO on the hydroxylation of thiacloprid by resting cells of *S. maltophilia*. Reactions were conducted in 100-ml flasks with 10 ml of transformation broth containing 500 mg sucrose and 1.6 mg thiacloprid. Data presented are the mean ± SD ($n = 9$) from three independent experiments with three replicates

analysis. ^{13}C NMR and ^{1}H NMR spectral data of product P2 were listed in Table 1. In comparison with P1, however, both signals of the reactive hydrogen (OH δ 7.3) and the proton at C9 (δ 5.6) were not present, and a new proton uncoupled with carbon atom appeared at δ 9.6 in ^{1}H NMR spectra. The chemical shift at δ117.1 (C14) in ^{13}C NMR was not observed. The infrared spectra showed characteristic absorption bands at 1,618 cm^{-1}(C = NH) and 1,683 cm^{-1}(C = O), and none at 3,362 cm^{-1} (OH) or 2,180 cm^{-1}(CN). These data suggested that the cyano group was released and the hydroxyl group was oxidized to carbonyl group. The MS data further confirmed that thiacloprid lost its cyano and hydroxyl groups. Therefore, metabolite P2 was determined to be 4-ketone-thiacloprid imine (Fig. 1). Unlike 5-hydroxy imidacloprid to form olefin imidacloprid, 4-hydroxy thiacloprid was failed to convert to olefin thiacloprid, whereas to 4-ketone-thiacloprid imine. It may be due to the cyanoimine in thiacloprid is more unstable than nitroimine in imidacloprid, as well as perhaps affected by sulfur atom in thiazolidine cycle, the hydroxyl moiety is oxidative to ketone, not olefin formation (Tomizawa et al. 2000).

The 4-hydroxy thiacloprid can not be converted to olefin thiacloprid. It means that formation of olefin thiacloprid (M38) appeared in plants metabolism (Klein 2001), is confirmed as biological process, whereas due to the easily conversion of 5-hydroxy imidacloprid to olefin imidacloprid, the formation of olefin imidacloprid in animals by biotic or abiotic action is still unclear (Schulz-Jander and Casida 2002).

Bioassay of the metabolites of thiacloprid

In bioassays against Aphid *Aphis craccivora*, 4-hydroxy thiacloprid exhibited considerable activity with LC_{50} value as 1.56 mg/l, however, compared with substrate thiacloprid, 4-hydroxy thiacloprid showed 156 times lower insecticidal efficacy. The 4-ketone thiacloprid imine almost lost its toxicity, having a LC_{50} value more than 4 mg/l (Table 2). It is well known that cyanoimine is an electron withdrawing group which has an influence on the insecticidal activity, and this group is the most important pharmacophore of neonicotinoid insecticides. So the hydroxylation as well as decyanotation is both detoxic process.

Discussion

Metabolism of thiacloprid has been extensively studied in plants, animals and soil (Klein 2001; Krohn 2001). One of the main degradation pathways in animals such as goats, hens, rats and plants, is via hydroxylation to form 4-hydroxy thiacloprid (M01). In rats, 4-hydroxy thiacloprid (M01) could be further oxidized in hydroxyl group to form 4-ketone thiacloprid (M27). In plants, 4-hydroxy thiacloprid was found to be further transformed to olefin thiacloprid (M38) (Klein 2001). However, the hydroxylation of thiacloprid was not found in soil metabolism (krohn 2001), while that of imidacloprid did (Krohn 2001; Krohn and Hellpointner 2002). We speculate that the hydroxylation of thiacloprid has lower transformation efficient by microbes than that of imidacloprid, and because 4-hydroxy thiacloprid is unstable and easily converted to form 4-ketone thiacloprid imine. Therefore, the level of 4-hydroxy thiacloprid is too low to be detected in soils.

Bioassay showed that the hydroxylation by *S. maltophilia* and decyanotation of 4-hydroxy thiacloprid are dramatic detoxic process, and the *N*-demethylation of acetamiprid is ten times lower detoxicity (Chen et al. 2008), while the hydroxylation of imidacloprid and subsequent elimination of water to form olefin imidacloprid are ten times increasing

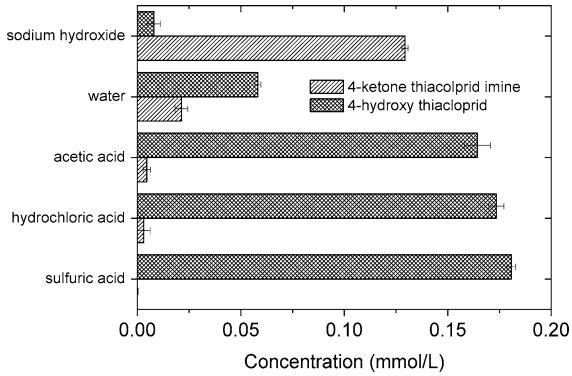

Fig. 5 4-Hydroxy thiacloprid (0.2 mmol/l) was converted to 4-ketone thiacloprid imine in 0.5 mmol/l sulfuric acid, hydrochloric acid, acetic acid or sodium hydroxide respectively, at 50°C for 66 h. Control was the redistilled water

toxic process. That is reasonable for explaining why in most cases soil application is recommended with imidacloprid, limited with acetamiprid and no relevant with thiacloprid (Elbert et al. 2008).

Interestingly, microbial hydroxylation of imidacloprid and thiacloprid by *S. maltophilia* CGMCC 1.1788 administer the same hydroxylation site and the same cytochrome P450 enzyme mechanism, in despite the varying molecular structure in five-member ring between thiacloprid and imidacloprid (sulfur atom replace amine group). But the subsequent metabolite degradation disperses, which concerned with the molecular structure of substrate and metabolite. It seems that substrate selectivity of hydroxylase of *S. maltophilia* is diverse with hydroxylation at imidazolidine or thiazolidine cycle, even or *N*-demethylation of acetamiprid. Compared with the molecular structure of imidacloprid, thiacloprid and acetamiprid, they have in common the 6-chloro-3-pyridinylmethyl group, but differ in the cyclic nitroguanidine, cyanoamidine or acyclic moiety. The hydroxylation or demethylation by *S. maltophilia* always site on the carbon atom neighbor nitrogen atom attached 6-chloro-3-pyridinylmethyl moiety (see Fig. 1). Another case is that replacement of 6-chloro-3-pyridinylmethyl moiety in imidacloprid with 2-chloro-5-thiazolmethyl, *S. maltophilia* still hydroxylate at the same site in imidazolidine cycle (unpublished data). So that the carbon atom attached with methylene-amide moiety is a key and important site for microbial P450 enzyme action. Thus our work is helpful to realize the metabolism of neonicotinoid insecticide. In addition, since the insect resistance to neonicotinoid insecticides is relative with cytochrome P450 enzymes (Jouben et al. 2008), someone may aim at the active site hydroxylated by P450 enzyme to design new neonicotinoid insecticide to avoid the detoxicity caused by insect P450 enzyme.

Table 2 Efficacy of thiacloprid and its derivatives against the aphid *Aphis craccivora* using the systemic bioassay method (24 h)

Reagents	LC_{50} (mg/l)[a]	FL 95%[b]	Slope
Thiacloprid	0.01	0.007–0.286	0.95
4-Hydroxy thiacloprid	1.56	1.27–1.93	1.64
4-Ketone thiacloprid imine	>4	–	>4

Bioassay was replicated three times, the number of pest exceeded 30 in each dependant experiment, and the total number of pest was more than 100

[a] LC_{50} is 50% lethal concentration

[b] FL 95% is 95% fiducial limits

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