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Toxic effect of the novel chiral insecticide IPP and its biodegradation intermediate in nematode Caenorhabditis elegans

Tingting Bian[a], Xiaolin Zhu[a], Jing Guo[a], Ziheng Zhuang[a], Zhiqiang Cai[a,*], Xiubo Zhao[a,b,*]

[a] Laboratory of Applied Microbiology and Biotechnology, School of Pharmaceutical Engineering & Life Science, Changzhou University, Changzhou, 213164, China

[b] Department of Chemical and Biological Engineering, University of Sheffield, Mappin Street, Sheffield S13JD, UK

* Corresponding author: Zhiqiang Cai, E-mail: zhqcai@cczu.edu.cn.. Xiubo Zhao, E-mail: Xiubo.Zhao@sheffield.ac.uk

ABSTRACT

Caenorhabditis elegans, a kind of model organism, was used to investigate biodegradation pathway of IPP and M1 in nematodes, in vivo toxicity from IPP and M1 and the possible underlying molecular mechanism. The results showed that both IPP and M1 could decrease lifespan, locomotion behavior, reproductive ability and AChE activity. During IPP biodegradation process, three intermediates (M1-M3) were monitored and identified. Based on the identified metabolites and their biodegradation courses, a possible biodegradation pathway was proposed. IPP was probably transformed to different three metabolites in nematodes through oxidation and

elimination of methyl and propyl etc. Under the same concentration, IPP had more severe toxicity than M1 on nematodes. IPP and M1 might reduce lifespan and decrease reproductive ability through influencing insulin/IGF signaling pathway and TOR signaling pathway. They could decrease expression levels of daf-16, sgk-1, aak-2, daf-15 and rict-1 genes, which involved in IGF and TOR signaling pathway.

KEYWORDS: Paichongding; Biodegradation intermediate; Toxic effect; Nematode; Caenorhabditis elegans

1. **Introduction**

Paichongding (IPP, 1-((6-chloropydidin-3-yl) methyl)-7-methyl-8-nitro-5 -propoxy-1,2,3,5,6,7-hexahydroimidazo[1,2-α-]-pyridine), is a novel chiral insecticide with independent intellectual property rights developed in China (Cai et al., 2015a, 2015b, 2016a; Fu et al. 2013). It has higher insecticidal activity (40-50 times) compared to imidacloprid-resistance pests, and also has low toxicity to human. In China IPP was used for pesticide control and sprayed for almost 3.3 million hectares (Cai et al. 2016a; Chen et al. 2017).

Insecticides residue in environment was accumulated and accelerated with the increase of insecticides application. Many studies reported that insecticides and their residue in soil environment have potential risk to the soil balance (soil biochemical properties and fertility, etc), they also can be transferred through food chain and make ecosystems deterioration (Zabaloy et al. 2012; Zhang et al. 2014). Previous studies have emphasized on the biodegradation pathway of IPP, its behavior in soils, and its effect on microbial community, soil enzyme activity etc. (Cai et al. 2016a, 2016b, 2016c). However, little information is available on the toxicity of IPP and its biodegradation intermediates on protozoan in soils.

The nematodes are a diverse animal phylum and live in complex microbial environments that present many potential challenges to their heath and viability. Caenorhabditis elegans is a kind of nematodes and the model organism, which has widely been used in drug discovery, drug toxicity, ageing mechanism research etc., due to its relatively short lifespan and conserved mechanisms for regulation of

antioxidant response (Avila et al. 2012). C. elegans has been widely accepted and utilized as an important alternative animal model for toxicity testing (Avila et al. 2012; Leung et al. 2008; Sprando et al. 2009). Many studies used C. elegant for toxicological research including organic compounds, drugs and nanomaterials etc., and found that toxicity is similar to that observed in mammals (Ju et al. 2013; Li et al. 2013; Zhao et al. 2013). In this study, we first studied and compared the toxicity of IPP and its metabolites, M1, to nematodes Caenorhabditis elegans, and IPP and M1 degradation pathway in nematodes were also studied and proposed. Moreover, there are limited information about toxicological mechanism for IPP and M1, we examined the relative gene expression for toxicity of IPP and M1. The results of this study will be useful for further understanding IPP degradation characteristics and its toxicity to animals.

2. Materials and methods

2.1. Chemicals and worm strain

Paichongding (IPP, 1-((6-chloropydidin-3-yl) methyl)-7-methyl-8-nitro-5-propoxy-1,2,3,5,6,7-hexahydroimidazo [1,2-α-]-pyridine, chemical purity 98.3%) was obtained from Jiangsu Kesheng Company Ltd.. The biodegradation intermediate of IPP, 1-((6-chloropydidin-3-yl) methyl)-7-methyl-8-nitro-5-hydroxyl-1,2,3,5,6,7-hexahydroimidazo [1,2-α-]-pyridine (M1), was synthesized in our lab and its chemical purity was 97.2%. M1 was prepared and stored in the lab using a protocol

described previously (Studzinski et al. 2017). Briefly, IPP was dissolved in dichloromethane and added boron tribromide at -78 ^{o}C, then reacted for overnight at room temperature. M1 was extracted by dichloromethane. Other chemical reagents are A.R. grade and purchased from Sinoreagent Company, China.

The wild type nematode, Caenorhabditis elegans, was used and maintained on nematode growth medium (NGM, pH 7.2) plates seeded with Escherichia coli OP50 at 20 ^{o}C as described previously (Sulston et al. 1974; Zhang et al. 2015; Tissenbaum et al. 2001). Age synchronous populations of C. elegans were obtained accrording to the previous reports (Zhao et al. 2013; Zhang et al. 2015). Different concentration of IPP and M1 (10mg L^{-1}, 5 mg L^{-1} and 2.5 mg L^{-1}) were added to the NGM plates just before inocubation.

2.2. Lifespan and reproduction

During the lifespan assay, the worms transferred daily for the first several days of adulthood. The surviving worms were measured, and recorded every day and would be scored as dead when they did not respond to the stimulation of a platinum wire. The results were treated with three replicates. The reproduction was assayed by the brood size, which was determined as the number of offspring at all stages beyond the egg. Twenty replicates were performed.

2.3. Locomotion behavior

For the locomotion behavior assay, different concentration of IPP and M1 treatment

were performed throughout the lifespan from L1-larvae. Head thrash and body bend were used as endpoints for locomotion behavior. Head thrashes are defined as a change in the direction of bending at the mid body. Body bends are defined as a change in the direction of the part of nematodes corresponding to the posterior bulb of the pharynx along they-axis, assuming that nematode was traveling along the x-axis.

During the locomotion behavior assay, the examined nematodes were transferred into the assay plate containing K medium on top of the agar. After a recovery time of 1 min, head thrashes, and body bends were counted for 1 min and 20 s respectively. Twenty replicates were performed for each experiment.

2.4. Acetylcholinesterase (AChE, EC 3.1.1.7) activity analysis

Inhibition of AChE in the LC50-treated worms was monitored after 24 and 48 h of exposure. Similarly, the normal worms were used to study the in vitro evaluation of AChE activity. 100 worms were chosen to estimate AChE activity (Ache, EC 3.1.1.7) and were ground with liquid nitrogen, then added 1.5ml of 0.1 M phosphate buffer (containing 0.1% of TritonX-100, pH 7.5) and mixed thoroughly. The mixture was centrifuged at 12,000 rpm for 30 min at 4 oC. The supernatant was used for estimation of AChE activity and protein content. Protein was estimated by the method of Lowry et al. (1951). AChE was assayed as described by Ellman et al. (1961). The procedures of enzyme assay were as follows: the blank consists of phosphate buffer (0.1 M, pH 8.0), substrate (0.075 M of acetylthiocholine iodide) and DTNB solutions (Dithiobisnitrobenzoic acid, 0.01M, 39.6mg of DTNB were dissolved in

10ml pH 7.0 phosphate buffer and 15 mg of sodium bicarbonate were added). 0.1 ml of supernatant and 0.5 ml of substrate were mixed together and kept at 37oC for 6 min, then 0.01 ml of DTNB solution and 4% of SDS solution were added in the reaction solution. The absorbance was measured at 421 nm. Enzyme activity was determined graphically using double-reciprocal plots of Leneweaver and Burk transformations

2.5. IPP/M1 degradation in nematodes and metabolite extraction

The wild type worms were grown for two generations on NGM plates seeded with OP50. The worms in six crowded plates were washed into 50 ml solution of NGM medium contained 10 mg L^{-1} of IPP or M1, grown at 22 ^{o}C and 220 rpm. Concentrated OP50 from 500 ml bacterial cultures were given on day 1, day 3 and day 5. The culture was harvested on day 7 by centrifugation at 5000 rpm. The supernatant was lyophilized and the residue was extracted with 95% ethanol for 10 h. the extraction was evaporated at RT to yield the crude extracts.

A Dionex U3000 HPLC system coupled with Bruker maXis 4G ion trap mass spectrometer with an electrospray ionization source (ESI) was used for LC-MS/MS analysis. The separate conditions were in accordance with those used for HPLC analysis. The ion source temperature was controlled at 250 °C, and the capillary voltage was -4.5 kV. The analysis mode of ionization was electrospray ionization (ESI, positive). The operation conditions were as follows: collision energy, 10.0 eV; ISCID energy and ion energy, 5.0 eV; dry gas, 6 $L \cdot min^{-1}$; dry temperature, 180 °C; gas pressure, 1.5 bar. The continuous full scanning from m/z 50 to 500 Da was performed

142 in positive ion mode.

143

144 2.6. RNA isolation and quantitative real-time PCR

145 Total RNA was isolated using Triozol RNA kit (Life technology) from worms
146 treated with or without 5 mg L^{-1} of IPP or M1 for 48 h. Total RNA was then
147 reverse-transcribed using PrimeScript 1st strand cDNA synthesis kit (Takara).
148 Quantitative real-time-polymerase chain reaction (RT-PCR) was used to determine the
149 relative quantification of the targeted genes (sod-2, daf-2, sgk-1, aak-2, age-1, daf-16
150 and aak-2 etc.) in comparison to the reference act-1 gene, and the results were
151 expressed as the relative expression ratio. The primers used in this study were
152 referenced the previous reports by Zhang et al and Zhuang et al. (Zhang et al. 2015;
153 Zhuang et al. 2014).

154

155 **3. Results and discussion**

156

157 3.1. Effect of IPP and M1 on lifespan of C. elegans.

158 Nematodes were treated with different concentration of IPP and M1 (2.5 mg L^{-1}, 5
159 mg L^{-1} and 10 mg L^{-1}) from L1-larvae stage in order to investigate IPP and M1's
160 effect on lifespan of C. elegans. The results were shown in Figure 1. Both IPP and M1
161 decreased nematodes lifespan significantly under the concentration of 10 mg L^{-1}, and
162 IPP has higher toxicity to nematodes than M1 under the same condition. Lifespan was
163 only 21 days in the 10 mg L^{-1} of IPP solution, while it reached 23 days in M1 solution.

IPP and M1 both had gentle toxicity to C. elegans under the lower concentration (2.5 mg L^{-1}).

3.2. Effect of IPP and M1 on the locomotion and reproduction of C. elegans.

C. elegans shows gradually impaired locomotion ability during its aging process. The effect of IPP and M1 on the locomotion ability of nematodes was investigated. Two important endpoints for locomotion ability, head thrash and body bend, were recorded every 1 minute during its lifespan after treated with different concentration of IPP or M1 solution for 48 hours. As shown in Figure 2a and 2b, higher concentration of IPP and M1 solution significantly decreased locomotion ability during its aging process compared with untreated nematodes, which indicated that IPP and M1 can decrease life quality of nematodes. The body bent frequency are 30 and 26 per minute after treated with 5 and 10 mg L^{-1} of IPP, while they are 39 and 36 per minute after treated with same concentration of M1, respectively. These results showed that under IPP has more toxicity than M1 under the same concentration.

Number of progeny of nematodes was used for investigating the effect of IPP and M1 on reproduction of C. elegans. Nematodes were pretreated with different concentration of IPP or M1 solution for 48 h, then moved to the plates at 35 ^{o}C. The number of progeny of nematodes were counted after 4 days and the results were showed in Figure 2c. There are significant difference on the numbers of progeny with or without IPP or M1 treatment, which indicated that the toxic effect of IPP and M1 on C. elegans existed by damaging the reproductive system of C. elegans. IPP shows

more toxicity to the reproductive system of C. elegans compared with M1 under the condition of same concentration.

3.3. Effect of IPP and M1 on AChE activity of C. elegans

Acetylcholinesterase (AChE) is the key enzyme in biological nerve conduction. Its enzymatic degradation of acetycholine could terminate the effects of neurotransmitters epicuticular on postsynaptic membrane excitation, for ensuring the neural transmission of nerve signals in vivo. It could be concluded from Figure 2d that higher concentration of IPP and M1 strongly inhibit AChE activity. 2.5 mg L^{-1} of IPP has almost no toxicity to AChE, however AChE activity was significantly inhibit by 5 mg L^{-1} of IPP. M1 has higher toxicity to AChE activity than IPP, especially under the condition of lower concentration.

IPP is a novel chiral neonicotinoid insecticide with four stereoisomers (RR, SS, RS and SR-IPP) and it was widely used in China for insecticide control in agriculture. Cai et al. reported that IPP could decrease soil enzyme activities, change soil microbial population, diversity and composition (Cai et al. 2015a, 2016b, 2016c). The activities of microbial protease, catalase, urease and dehydrogenase in soil are both impacted after IPP application in crop (Cai et al. 2015a, 2016b, 2016c). The relative abundance of genus of Bacillus, Pseudomonas, Azohydromonas and Paenibacillus in soils with IPP application increased, the genus of Brevundimonas, Xanthomonadaceae, Massilia, Pedobacter, and Hydrogenophaga were newly appearance. The bacterial species diversity and community structure in soils were remarkably different responding to

IPP-sprayed (Cai et al. 2016b, 2016c). Neonicotinoids have been considered to increase mortality in honey bees by impairing their homing ability and to reduce the reproductive success of bumble bees and solitary bees (Henry et al. 2012; Whitehorn et al. 2012; Woodcock et al. 2017). Until now, there was little information about neonicotinoids toxicity on other animals. In this study, Caenorhabditis elegans, regarded as the model organism, was used as an alternative animal model for IPP toxicity testing. The results showed that IPP and M1 can decrease nematodes lifespan, inhibit locomotion ability and reproductivity. Based on these results, it can be concluded that IPP had higher toxicity to C. elegans than M1 in nematodes. They also inhibit AChE activity significantly, AChE was more sensitive to M1. The toxicity differences between IPP and M1 at relatively high concentrations also occurred with the aid of head thrash, body bend, locomotion and lifespan. M1 is one of the intermediates of IPP biodegradation in environment, and also determined in soil after IPP application (Cai et al. 2015a, 2015b, 2016a, 2016b, 2016c; Chen et al. 2017; Fu et al. 2013). The toxicity of toxicants in C. elegans is similar to the toxic results in mammals, the results in this study imply the possible potential more severe toxicity of IPP than M1 in mammals. Woodcock et al. reported that reproduction in wild bees was negatively correlated with neonicotinoid residues and neonicotinoids can caused a reduced capacity of bee species to establish new populations (Woodcock et al. 2017). Neonicotinoid insecticides are high toxicity to vertebrates, and its use has been partly restricted for their effects on pollinators (Henry et al. 2012; Whitehorn et al. 2012). These researches are accordance with the results in our research.

230

231 3.4. IPP and M1 degradation characteristics by C. elegans.

232 During the degradation of IPP in nematode body, three degradation metabolites
233 were determined by HPLC analysis with retention times of 2.2 min (M1), 19.3 min
234 (M2) and 12.2 min (M3). These intermediates appeared with the decrease of IPP. And
235 these three biodegradation intermediates of IPP were analyzed and identified by
236 high-performance chromatography-time-of flight mass spectrometry and LC-MS/MS.

237 The ion fragments of IPP were at m/z 367.1534 ($C_{17}H_{23}ClN_4O_3$) $[M+H]^+$ and m/z
238 321.1578 (Figure 3a), and the ion fragment of m/z 367.1500 includes daughter ions of
239 306.1368 (100), 308.4442 (15) and 137.1069 (62), which were accordance with the
240 structure of standard IPP (Cai et al. 2015a,2015b; Chen et al. 2017). M1 showed m/z
241 325.1052 [M+H], and the daughter ion showed m/z 264.0904. Metabolite M1 was
242 identified as 1-((6-chloropyridin-3-yl) methyl)-7-methyl- 8-nitro-5-hydroxy-
243 1,2,3,5,6,7- hexahydroimidazo [1,2-α-] pyridine according to its LC-MS/MS and MS^2
244 spectra (Figure 3d), which indicated that M1 was generated through hydrolysis of
245 propoxyl group of IPP. M2 appeared after M1 and its m/z was 276.0887 (M+H, Figure
246 3c), the M2 ions matched the formula $C_{14}H_{14}ClN_3O$, M2 was identified as
247 1-((6-chloropyridin-3-yl)methyl)-2,3- dihydro-5-one-7- methylimidazo [1,2-α]
248 pyridine.

249 Figure 3d shows that the m/z of metabolite M3 was 297.1144, which should be
250 the transformation of the group of 8-nitroso on M1 to the group of 8-amino, and the
251 nitroso group on M1 transformed to hydroxyl. On the basis of these characteristics,

M3 was preliminarily identified as 1-((6-chloropyridin-3-yl)methyl)-5,7-diol-8-amino -1,2,3,5,6,7-hexahydroimidazo [1,2-α]pyridine.

During the degradation of M1 in nematode body, two degradation metabolites were determined by HPLC analysis with retention times of 19.3 min (M2) and 12.2 min (M3). M2 and M3 were same intermediates of IPP. The results showed that IPP and M1 have the same biodegradation intermediates and pathway in nematode.

In nematodes, there were three metabolites, M1, M2 and M3 were determined, M1 appeared first, and then M2 and M3 formed almost simultaneously. The propyl group of IPP was de-esterified and hydrolyzed to form M1, which was accordance with the previous study (Cai et al. 2015a,2015b; Chen et al. 2017). M1 was determined during the process of IPP biodegradation. The nitro group of M1 could be aminated to form amino, methyl was hydrolyzed to hydroxyl group, thus M3 was generated. M2 was formed through loss of nitro group from M1. Therefore the biodegradation pathway in nematodes was concluded in Figure 5. M1 biodegradation pattern was also described in Figure 5. IPP and M1 degradation pathway in nematodes is similar with IPP biodegradation in aquatic and soil environment (Cai et al. 2015a,2015b; Chen et al. 2017).

3.5. Effect of IPP and M1 on relative genes expression in nematodes

There are three major signaling pathways controlling nematodes' aging process, IGF (insulin/insulin-like growth factor), TOR (target of rapamycin) and germline signaling pathways. In order to illustrate the mechanism of IPP and M1 toxicity to the

nematodes lifespan, expression of several genes involved in three signaling pathways were investigated. After exposure in 5mg L^{-1} of IPP or M1, expression level of sod-2, daf-16, sgk-1 and aak-2 genes decreased, and daf-2 and age-1 genes increased (Figure 6). IPP has more toxicity for inhibiting the expression levels of daf-16, sgk-1 and aak-2 than M1. IPP and M1 did not increase expression level of some genes involved in TOR pathways, however, they both inhibited daf-15 and rict-1 gene expression (Fig. 6). Both IPP and M1 did not significantly influence expression level of some genes involved in germline signaling pathway. The results indicated that IPP and M1 should affect the lifespan through mixed IGF and TOF signaling pathways in nematodes.

The chemical structure of IPP and M1 is similar, the data of this study showed that IPP and M1 inhibit lifespan and reproduction of nematodes possibly through same molecular mechanism. They might reduce the lifespan and reproduction through affecting both the IGF signaling pathway and TOR signaling pathway. In C. elegans, insulin/IGF-1 receptor (DAF-2) can activate tyrosine kinase and initiates phosphorylation process, which make some kinase active, including phosphatidiylinositol 3-kinase (PI3K/AGE-1), 3-phosphoinositide-dependent kinase-1 (PDK-1) and serin/theonine-protein kinase (SGK-1) (Zhuang et al. 2014). SGK-1 phosphorylates and inactivates the FOXO transcription factor DAF-16, which can block the transcription of targeted genes (Zhuang et al. 2014). TOR C1 and TOR C2 are different complexes in TOR signaling pathway, and they have different coactivators, DAF-15 Raptor and RICT-1 Rictor. In this study, IPP and M1 can decrease lifespan and reproduction of nematodes, which probably due to the induction

of severe change of genes required for aging and reproductive control, and even alteration in signaling pathways. The results illustrated that daf-16, sgk-1, aak-2, daf-15 and rict-1 genes decreased after exposed to IPP and M1.

4. Conclusions

IPP and its metabolite, M1, were selected to study their toxicity to the model organism, Caenorhabditis elegans. The effect on lifespan, locomotion, reproduction and AChE activity were investigated using different concentration of IPP and M1. And the biodegradation pathway of IPP and M1 were also proposed through identifying their intermediates by LC/LC-MS. In order to discuss molecular mechanism of reduction of lifespan and reproduction in nematodes, the expression level of some genes related to three signaling pathways were illuminated. The data showed that both insulin/IGF-1 signaling pathway and TOR signaling pathway were involved in the regulation of toxicity induction from IPP or M1 in nematodes. We also hypothesized that IPP and M1 induced the toxicity on nematodes through same molecular mechanism, which will be helpful for better understanding the potential damage on health of animals.

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TABLE AND FIGURE CAPTIONS

Figure 1. Effect of IPP and M1 on lifespan of C. elegans. (A. 10 mg L^{-1}; B. 5 mg L^{-1}; C. 2.5 mg L^{-1}).

Figure 2. Effect of IPP and M1 on locomotion, reproduction and AChE activity of C. elegans. (A:Body bent frequency; B Head swing frequency; C: Number of progeny; D: AchE activity).

Figure 3. Mass spectra of IPP and metabolites (M1, M2 and M3) .(a:IPP; b:M1; c:M2; d:M3).

Figure 4. HPLC chromatograms with the retention time of degradation molecules

Figure 5. Proposed pathways of IPP and M1 degradation in nematode C. elegans.

Figure 6. Expression level of some genes involved in signaling pathways in nematode C. elegans.

Figure 1.

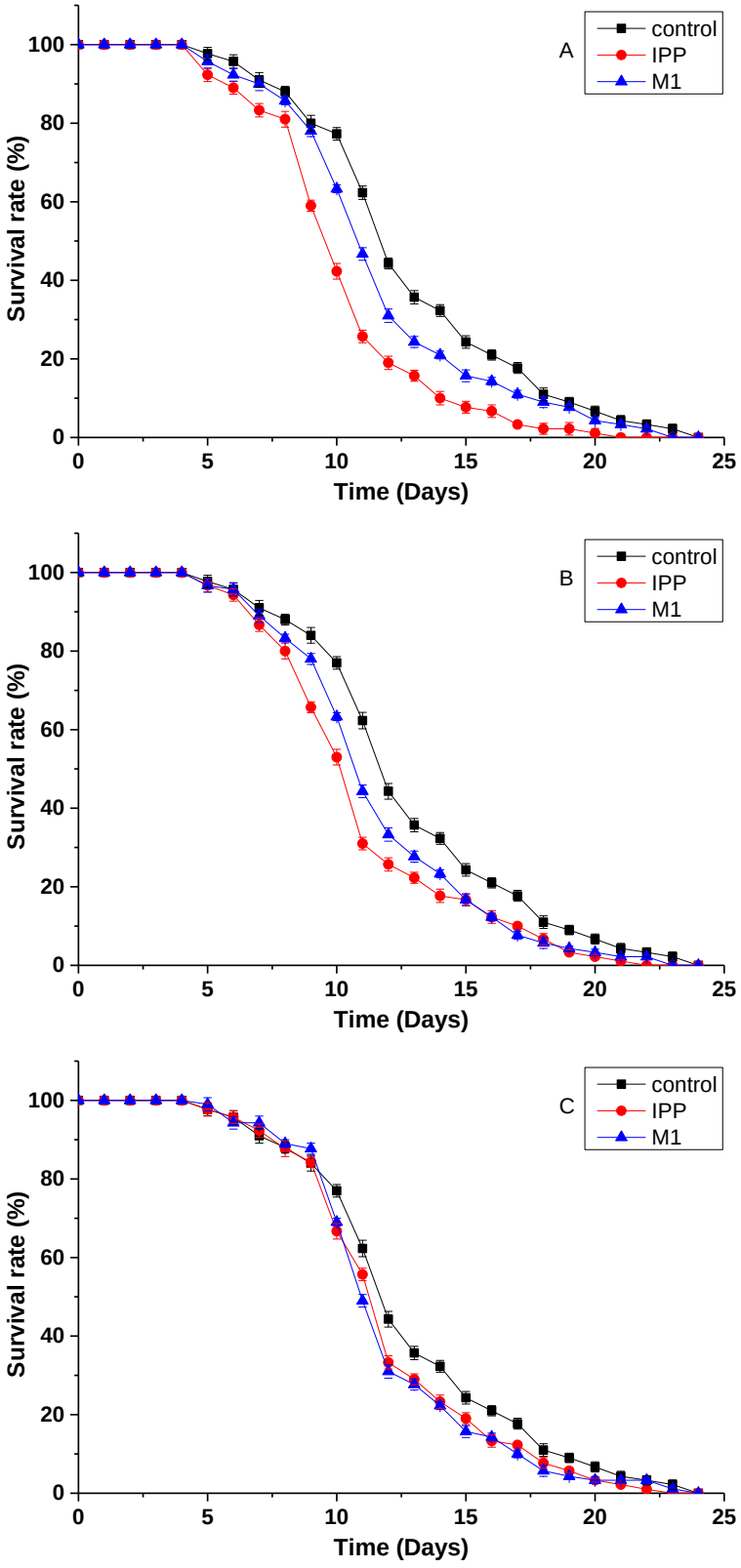

A
control
IPP
M1
Survival rate (%)
Time (Days)
B
control
IPP
M1
Survival rate (%)
Time (Days)
C
control
IPP
M1
Survival rate (%)
Time (Days)

Figure 2

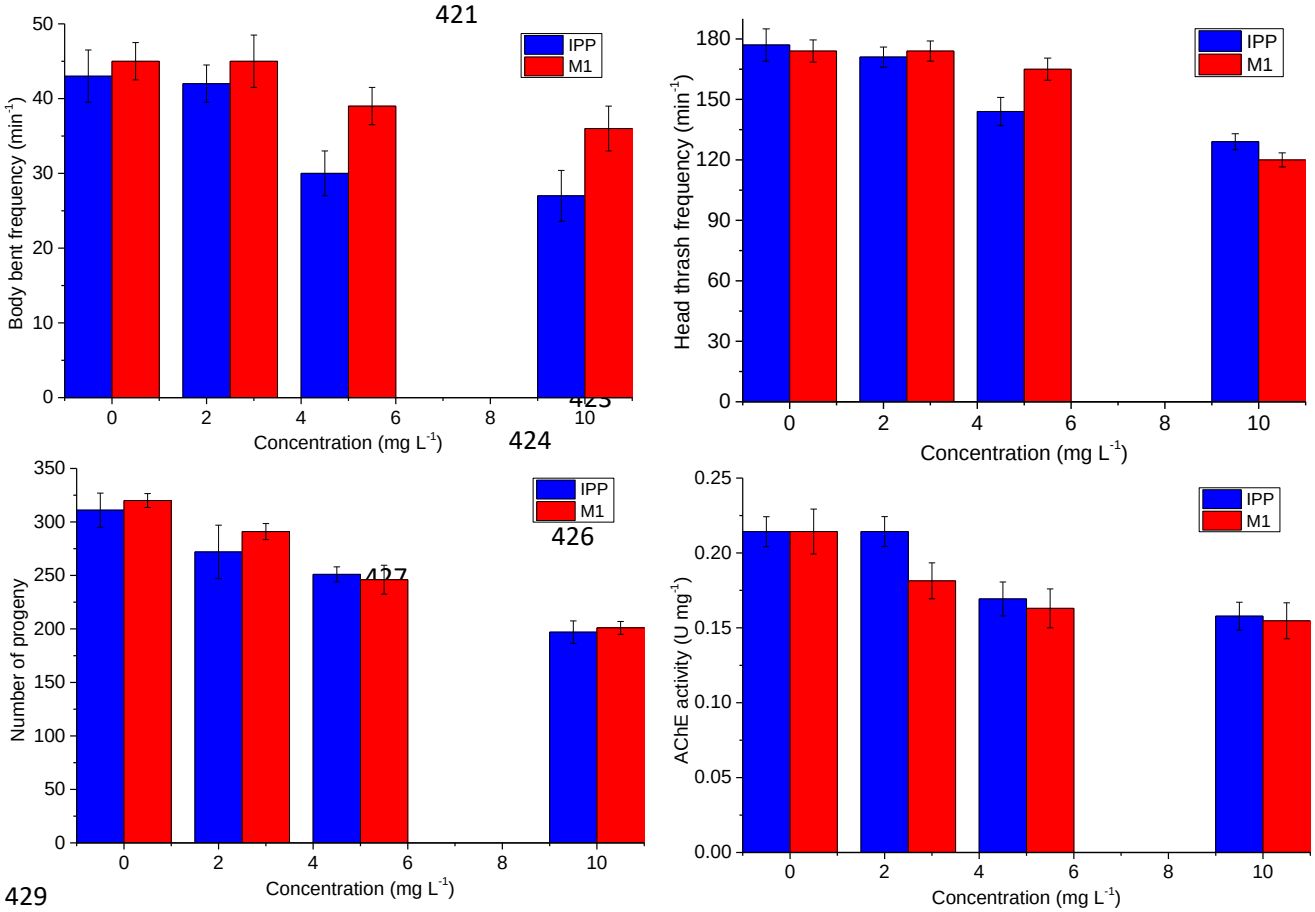
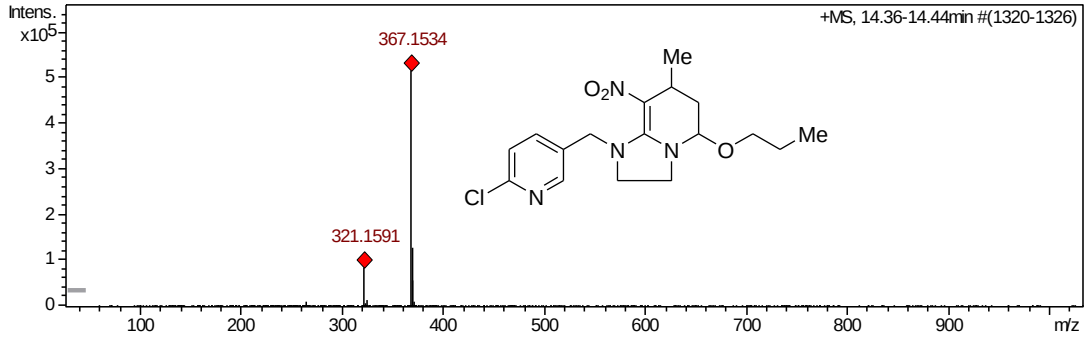
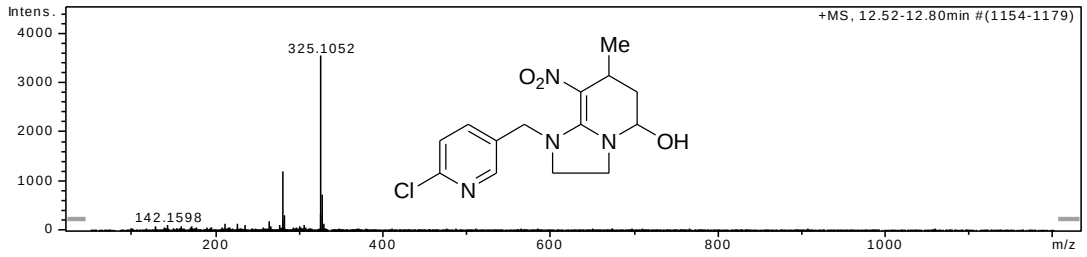
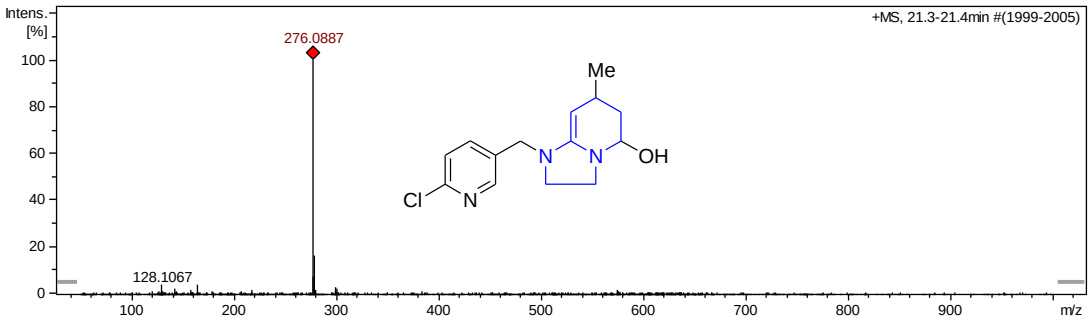

Body bent frequency (min-1)
Concentration (mg L-1)
IPP
M1
Head thrash frequency (min-1)
Concentration (mg L-1)
IPP
M1
Number of progeny
Concentration (mg L-1)
IPP
M1
AChE activity (U mg-1)
Concentration (mg L-1)
IPP
M1

Figure 3.


Intens.
x10^5
+MS, 14.36-14.44min #(1320-1326)
367.1534
321.1591
Me
O2N
N
N
O
Me
Cl
N
100
200
300
400
500
600
700
800
900
m/z


Intens.
+MS, 12.52-12.80min #(1154-1179)
325.1052
142.1598
Me
O2N
N
N
OH
Cl
N
200
400
600
800
1000
m/z


Intens.
[%]
+MS, 21.3-21.4min #(1999-2005)
276.0887
128.1067
Me
N
N
OH
Cl
N
100
200
300
400
500
600
700
800
900
m/z

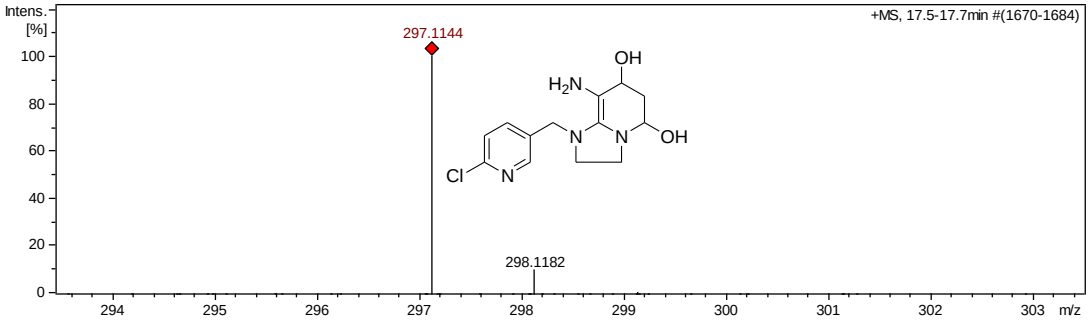

Intens.
[%]
+MS, 17.5-17.7min #(1670-1684)
297.1144
298.1182
OH
H2N
N
N
OH
Cl
N
294
295
296
297
298
299
300
301
302
303
m/z

Figure 4.

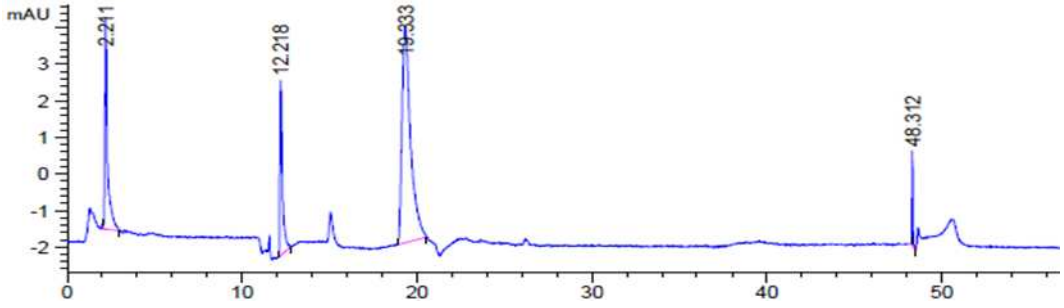


Figure 5.

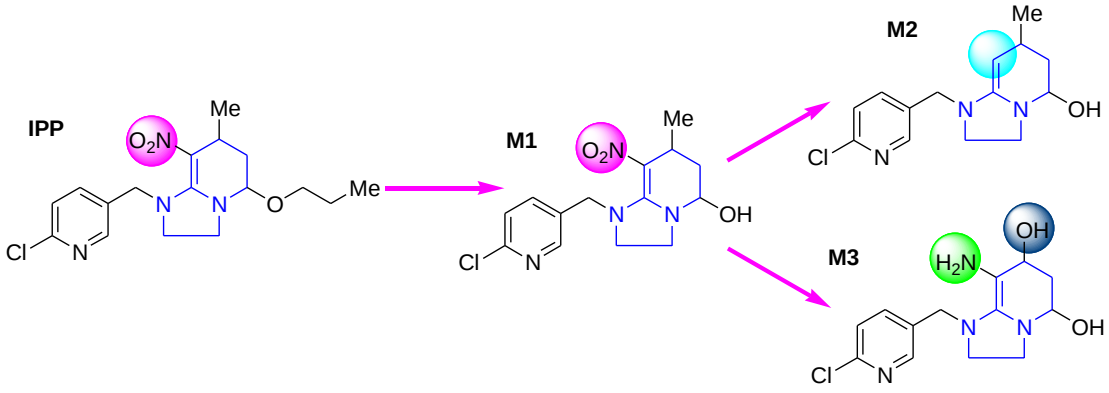
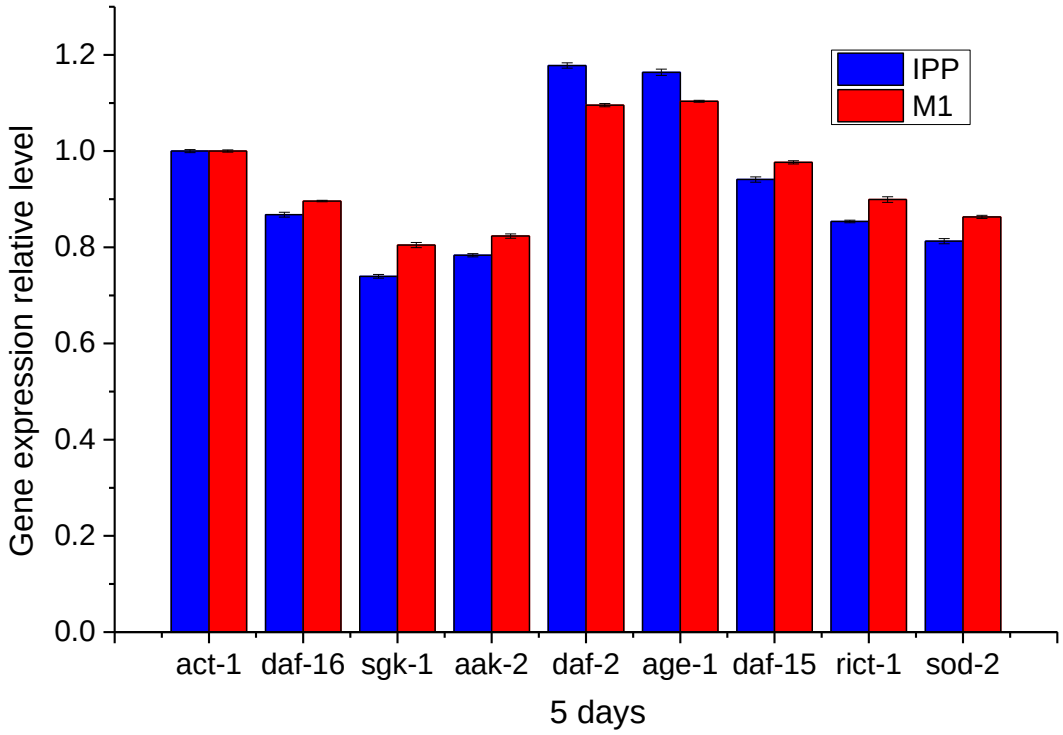
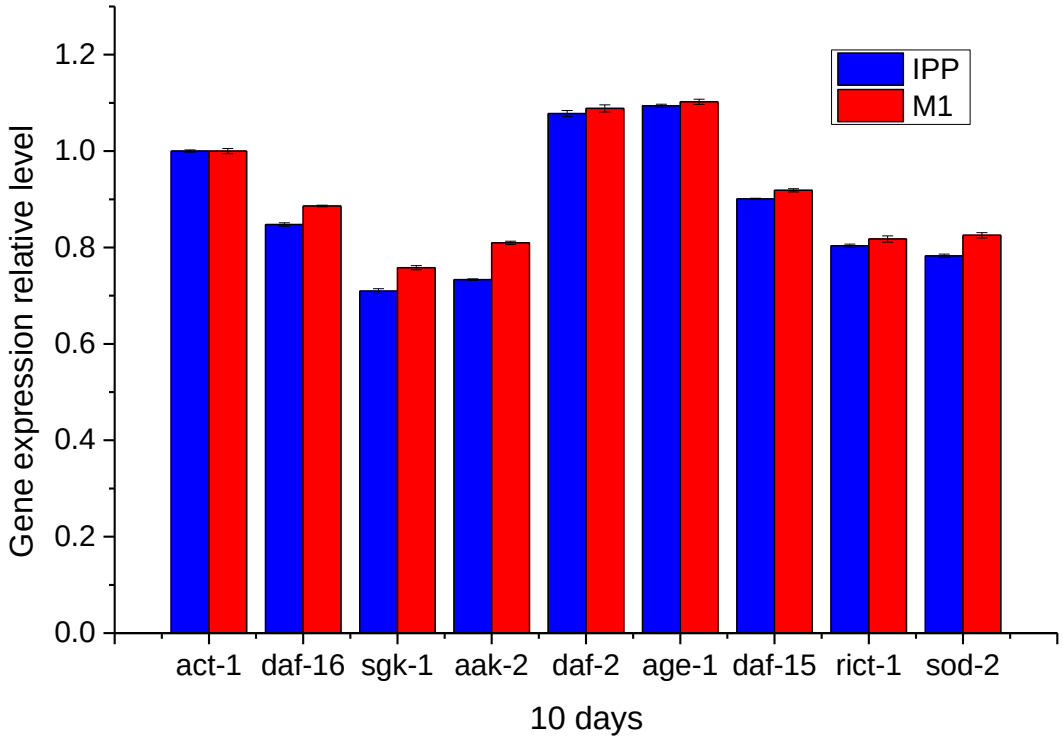


Figure 6


Gene expression relative level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
IPP
M1
act-1
daf-16
sgk-1
aak-2
daf-2
age-1
daf-15
rict-1
sod-2
5 days


Gene expression relative level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
IPP
M1
act-1
daf-16
sgk-1
aak-2
daf-2
age-1
daf-15
rict-1
sod-2
10 days