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Chemoenzymatic resolution of *rac*-malathion

David M. Hitt^{a,b}, Yamina Belabassi^a, Joyce Suhy^c, Clifford E. Berkman^d, and Charles M. Thompson^a

^aATERIS Technologies, 901 N Orange Street, Missoula MT 59802, USA

^bDepartment of Natural Sciences, Carroll College, 1601 N. Benton Ave., Helena, MT 59625, USA

Abstract

Malathion, diethyl 2-[(dimethoxyphosphorothioyl)sulfanyl]butanedioate, is an organophosphate used to control insect pests. Malathion contains a diethyl succinate moiety that is a known functional group susceptible to desymmetrizing enzymes such as esterases that selectively react with a single enantiomer. Purified *rac*-malathion was subjected to hydrolysis at the diethyl succinate moiety of malathion under various conditions using wild type pig liver esterase to form (*S*)-malathion (12 % ee) and ~ 3:2 mixture of α - and β -monoacids of (*R*)-malathion. Technical malathion could not be enriched due to the presence of esterase inhibitors. Further investigation of this resolution using a panel of six PLE isoenzymes also demonstrated formation of (*S*)-malathion, however, an improvement of up to 56 % ee was obtained.

1. Introduction

Malathion **1** (Scheme 1) is one of the most widely-recognized organophosphate (OP) insecticides. There has been a steady domestic and global demand for malathion covering four principle applications: (a) reduction or eradication of disease vectors (e.g., mosquitoes; malaria); (b) domestic or residential insect pest control including fleas; (c) in broadcast crop protection (e.g., cotton); and (d) as a pharmaceutical pediculicide (head lice, etc.).¹⁻⁶

Malathion is one of the few OP insecticides that is recommended by the World Health Organization (WHO) for indoor use for eradicating the mediterranean fruit fly ‘MedFly’, and mosquitoes carrying West Nile Virus. For decades, malathion and lindane (chlorinated hydrocarbon) were the only FDA-approved agents for pediculosis. For the most part, malathion is in continued use because it is relatively easy to prepare, inexpensive, minimally toxic to humans, and biodegradable.

Despite numerous advantages, the continued use of malathion is accompanied by concerns with the central nervous system (CNS) toxicity typically associated with OP compounds.

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^cCurrent Address: Synarc 7707 Gateway Boulevard, 3rd Floor, Newark, CA 94560, USA

^dCurrent Address: Department of Chemistry, Washington State University, Pullman, WA, USA

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rac-Malathion is relatively non-toxic to humans because it is preferentially hydrolyzed (detoxified) by carboxylesterases to form the innocuous malathion α - and β -monoacids (Scheme 1). However, malathion can be oxidatively converted into malaoxon **2**,⁷ which is a potent inhibitor of acetylcholinesterases (AChEs) and carboxylesterases (CaEs).^{8,9} Moreover, (*R*)-malaoxon (Scheme 1) had demonstrated up to 22-fold greater inhibition of mammalian AChE than the corresponding (*S*)-isomer,¹⁰⁻¹³ (*R*)-malaoxon (88% ee; LD₅₀ 23.1 mg/kg) is twice as toxic as the (*S*)-isomer (90% ee; LD₅₀ 48.0 mg/kg) to rats.^{13,14} Based on this data, (*S*)-malathion (*S*)-**1** is expected to be safer to humans since it metabolizes into (*S*)-malaoxon. The enantiomers of malathion and malaoxon (Scheme 1) were first synthesized in the author's lab^{12,15,16} although *O,O*-diethyl phosphate analogs of malathion and malaoxon have been previously prepared.¹⁷

The first synthetic approaches to asymmetric malathion provided 90% ee, but were lengthy, used expensive starting materials and highly reactive reagents, and were conducted at low temperature.^{12,16} Other groups have improved upon these synthetic approaches,^{13,14} and introduced chiral HPLC as a useful alternative for separating racemic malathion into the (*R*)- and (*S*)-isomers.^{18,19} However, these approaches may not be amenable for large scale preparation. The ease of preparation and very low cost of technical grade, racemic malathion suggest its possible use as a starting material.

As indicated, malathion is a substrate of mammalian esterases forming α - and β -monoacids although only the α -monoacid was initially found in the urine from rats²⁰ and sheep.^{21,22} Malathion resembles substrates of esterases that are known to stereospecifically hydrolyze one ester from a racemate,²³⁻²⁵ although the succinate diethyl ester may be less preferred as a substrate. Assuming malathion is a substrate, enzymatic resolution would form a separable, carboxylic acid of one enantiomer and an unreacted malathion enantiomer (Scheme 2). Although esterases have been used in organic synthesis for decades,²⁴ to the best of our knowledge, malathion has not been examined as a possible substrate. Thus, if racemic malathion can be resolved *via* enzymes, the individual enantiomers could be isolated following separation and esterification (Scheme 2).

As shown in Scheme 2, the (*R*)-(route 1) or (*S*)-malathion (route 2) could be directly obtained from an esterase^{26,27} along with the opposite enantiomer as the malathion monoacid. Using either route, the (*R*)- or (*S*)-malathion monoacid antipode could also be separated. Based on the *in vivo* data,^{20,28} malathion α -monoacid^{29,30} could be hypothesized to be the major or sole regioisomer formed.

Herein, we report the first preparation of enantiomerically enriched malathion and malathion monoacids *via* kinetic resolution using pig liver esterase (PLE). In addition to wild type PLE, a number of PLE isoforms have been engineered with distinctive ligand binding properties that have the potential to optimize the conversion. As noted in Scheme 2 (only the α -monoacid is shown), the resolution of malathion by PLE would produce the oppositely configured monoacid enantiomer and a malathion enantiomer that are easily separable by conventional extraction (sodium carbonate vs. ether). For validation, the spectroscopic and optical characteristics of the malathion products can be compared with known authentic

samples. Herein, the resolution of malathion was conducted by wild type and isoforms of PLE.

2. Results and discussion

2.1 Hydrolysis of malathion using wild type pig liver esterase

The resolution of racemic malathion into an enantiomer of malathion and a corresponding monoacid (Scheme 2) using wild-type pig liver esterase (PLE; Sigma-Aldrich) was examined. A solution of technical malathion (> 90% purity) in acetone was added to a solution of PLE (90 U/mmol) in phosphate buffered saline (PBS; 50 mM, pH 7.5) and when needed the reaction periodically adjusted to pH 7.8 using 0.01 M NaOH. Under these reaction conditions, form malathion was hydrolyzed to the monoacids as initially evidenced by thin layer chromatography (lower R_f on silica TLC).³¹ Unfortunately, only ~20 % of the malathion underwent conversion based on the isolation of monoacids and recovery of malathion. Suspecting possible inhibition for the low conversion, the enzyme activity was monitored as a function of time under the reaction conditions. In control studies (absence of malathion) a 16% reduction in PLE activity was observed over 24 h. However, when malathion was present at the onset of the reaction, the enzyme activity decreased by 70% of the initial rate within 1 h and the substrate turnover rate had diminished to slightly above background at 23 h ($A/t = 0.036 \text{ min}^{-1}$; Table 1). The enzyme activity was determined at various time points based on the hydrolysis of *p*-nitrophenylacetate (*p*NPA).³²

Based on these results, the observed reduction in enzyme activity was either due to malathion, the malathion monoacid products, and/or impurities in the malathion source. To resolve this issue, technical malathion was analyzed by ³¹P NMR. The spectrum contained the expected major peak at 96.2 ppm (malathion), but also a number of minor impurities including a peak at 28.3 ppm, which corresponds to malaaxon. As a result of this finding, technical malathion was chromatographed (95:5 hex/EtOAc) to afford > 99% purity, and resubjected to the same PLE-hydrolysis conditions. At 24 h, enzyme activity decreased to only 60% of the original level and by 43 h, residual activity remained but no additional conversion to the monoacid was observed. The improvement in retained PLE activity may be due to the removal of one or more minor amounts of the contaminants including malaaxon **2**, which is a known esterase inhibitor. However, the immediate inhibition of PLE suggests that malaaxon is likely to be an impurity in technical malathion rather than being produced from malathion under the reaction conditions. Therefore, the direct conversion of technical malathion into non-racemic malathion by PLE cannot be accomplished unless free of inhibitors.

After determining that PLE could remain active using purified malathion, the separation and analysis of the unreacted malathion from monoacid(s) were undertaken to determine the identity of the products and assess the recovery of malathion. The *rac*-malathion (20-200 mg) was subjected to the hydrolysis conditions with PLE and upon completion, the reaction mixture was diluted with an equal volume of ethyl acetate, adjusted to pH 9.0 with NaOH and separated. The ethyl acetate (EtOAc) fractions were re-extracted with saturated sodium carbonate to remove residual monoacids. The organic extract showed predominantly the malathion as determined by co-elution on TLC and ¹H and ³¹P NMR spectroscopic

comparison with authentic material.^{12,16} The unreacted/resolved malathion was recovered in 15-38% yield (n=12).

The aqueous solution containing the malathion monoacids was adjusted to pH 1-2 with 1.0 M HCl and extracted three times with ethyl acetate. Analysis showed the presence of two products by thin-layer chromatography³¹ and by ¹H decoupled-³¹P NMR with resonances at δ 95.6 and δ 95.5 (40:60 ratio) corresponding to the malathion β -monoacid (fast moving band on TLC) and the α -monoacid, respectively. Column chromatography could not separate the β - and α -monoacids, however, isolation of enriched amounts of β -monoacid correlated with the δ 95.5 signal and the slower moving α -monoacid showed a δ 95.6 signal thereby providing a relatively easy ³¹P NMR method to analyze reaction progress and hydrolysis regiochemistry.

Correspondingly, the ¹H NMR of the malathion monoacid mixture showed a discernible pair of doublet-of-doublets (dd) for the succinate methylene group (CH₂) of each isomer with the β -monoacid appearing slightly downfield (3.11 and 2.98 ppm) of the α -monoacid (3.05 and 2.92 ppm) consistent with that found by Chen et al.²⁰ In some experiments, ³¹P NMR analysis revealed a minor amount (0-3%) of an impurity at δ 66.7 ppm that correlates with dimethoxy phosphorothioic acid (MeO)₂P(O)SH likely formed from hydrolysis of the thiosuccinate leaving group.³⁴ The structures of the α - and β -malathion monoacids were confirmed by TLC³¹ correlation with authentic samples available by chemical hydrolysis, and through chemical shift data as previously reported.²⁰ The composite yield of the α - and β -malathion monoacids ranged from 6-48% (n=12).

2.2. Formation of non-racemic malathion and monoacid using wild type pig liver esterase

In order to assess the degree of enantiomeric enrichment by wild type PLE, the specific rotations were measured on the malathion-containing and the malathion monoacid-containing extracts. The malathion extract was a mixture of resolved and unresolved malathion with an $[\alpha]_D^{25} = -9.5$ (n = 6) representing an ee $\cong$ 12% enriched in the (*S*)-malathion stereoisomer.^{12,16} Additional support for the formation of the (*S*)-stereoisomer was acquired during analysis of the malathion monoacid extract that showed a range of $[\alpha]_D^{25} = +7.5$ to +12.0, which suggests the malathion-monoacid product is enriched with the (*R*)-stereoisomer (the specific rotation for either enantiomerically pure stereoisomer of α/β malathion-monoacid is unknown) (Scheme 3).

The poor conversion and enantiomeric enrichment of malathion by wild type PLE may be due to a number of factors including the reduced enantiomeric preference of succinate diesters, the distance of the bulky dimethoxy phosphorothionate from the asymmetric center, and the hydrolysis rate for ethyl esters. Overall, wild type PLE enriches *rac*-malathion into (*S*)-malathion and (*R*)-malathion monoacids (Scheme 3), but in relatively poor enantiomeric excess.

2.3. Resolution of malathion using pig liver esterase isoforms

Based on these preliminary findings showing that wild type PLE affords a modest enantioenrichment of (*S*)-malathion, six recombinant PLE isoforms²⁵ from *E. coli*

(Enzymicals Screening Kit™) were tested to find a suitable catalyst to optimize the resolution. In a typical experiment, 30 – 50 mg of purified *rac*-malathion (0.02 M) was dissolved in an 11:1 mixture of PBS (50 mM, pH 7.5) and acetone at room temperature.³⁵ Wild type or a single isoform of pig liver esterase (PLE1 – PLE6) was added (10 U/mmol) and the hydrolysis reaction conducted for > 40 h or until the reaction showed no additional evidence of product formation and/or loss of enzyme activity.

The initial enzyme activity ($t = 0$) was measured and represents an average value of three to five experiments (Table 2). The resolution reactions were worked-up as previously described to separate the non-racemic malathion from malathion monoacids. The percentage of malathion recovered was calculated, the specific rotation recorded, and converted into % ee and (*E*) for comparison (Table 2).

The initial hydrolysis rates ranged from 0.037 U/mg (PLE2) to 0.626 U/mg (PLE6) representing a 17-fold difference between the slowest and fastest isoforms. However, the fastest isoform, PLE6, was 27-fold slower than wild type PLE. Under these reaction conditions, each PLE isoform resolved malathion with concomitant conversion into the monoacids but, the efficiency and specificity varied greatly among the isoforms.

As indicated in Table 2, all of the isoforms performed similarly to the wild type by preferentially producing (*S*)-malathion, thus exhibiting a hydrolytic preference for the (*R*)-isomer. In general, most of the isoforms reacted with *rac*-malathion to produce modest ee (8-19%) and *E*-values (1.4-2.2) similar to those found with wild type PLE. Despite these disappointing results, the PLE3 isoform was a notable exception converting *rac*-malathion into (*S*)-malathion in 56% ee (*E* = 3.0). The percentage recovery of malathion from wild type and the PLE isoforms ranged from 62-70% except for the PLE3/PLE4 isoforms, which were 34-35%.

3. Conclusions

Herein we have reported the first enzymatic resolution of the organophosphate insecticide malathion. Based on preliminary studies with wild type PLE, it is doubtful that technical grade malathion can be enzymatically converted into a single enantiomer due to the presence of impurities such as malaoxon and isomalathion that inhibit esterases. As a result, purification of malathion is needed to conduct successful enzymatic resolution.

When relatively pure malathion was used as the substrate, it was found that PLE preferentially hydrolysed (*R*)-malathion into (*R*)-malathion monoacids. Isolation and characterization of the unreacted (*S*)-malathion further demonstrated this enantiopreference. These results are in contrast to mammalian-based hydrolyses where it has been demonstrated that (*S*)-malathion undergoes more rapid degradation in the environment than the (*R*)-isomer.¹⁹

Using wild type PLE, the recovery of (*S*)-malathion was good (~ 70%), although the 12% ee was poor (*E* = 2.0). The low ee value is thought to be indicative of a lack of discrimination between the succinate ester groups and/or poor discrimination of the

dimethoxythiophosphoryl moiety into the larger binding domain of the PLE model^{26,36,37} due to the sulfur spacer group.

A second interesting observation was that each of the PLE isoforms hydrolyzed *rac*-malathion to afford (*S*)-malathion. This result differs from previous reports showing that secondary alcohol esters form the (*S*)-alcohol from PLE3-6, but form the (*R*)-alcohol from PLE1 and PLE2.²⁵ Diesters also showed an enantiopreference toward the isoforms. PLE1-3 showed pro-(*R*) desymmetrization of cyclopentene-3,5-diacetates but PLE 4 and PLE5 yielded the pro-(*S*) alcohols.²⁵ The fact that no enantiopreference was found among the isoforms with malathion as the substrate cannot be readily explained, although malathion is not a symmetric diester. Given the poor enantioenrichment together with a lack of enantiopreference, suggests that malathion occupies the active sites of the PLE isoforms with near equal orientation.

The screening of six PLE isoforms revealed that five of these isoenzymes were similar to the wild type, converting malathion in very low enantiopreference. However, the isoenzyme PLE3 showed promise as a resolution catalyst resulting in 56% ee (*S*)-malathion. We are currently working to improve the recovery and ee for this process.

4. Experimental

4.1. General

Commercially available reagents and solvents were purchased from Sigma-Aldrich, and purified when necessary. Analytical thin-layer chromatography (TLC) was conducted on E. Merck aluminum-backed, 0.2 mm silica gel 60 F254, TLC plates. Column chromatography was conducted using Kieselgel 60, 230-400 mesh (Merck). Proton (¹H), carbon (¹³C), and phosphorus (³¹P) NMR spectra were recorded on a Bruker 400 MHz NMR instrument in deuterated chloroform (CDCl₃). Proton and carbon chemical shifts are relative to chloroform as the internal standard, and phosphorus chemical shifts are relative to phosphoric acid (H₃PO₄) in CDCl₃ as the external standard.

4.2. Pig liver esterase activity assay

Enzyme activity is expressed in units (U). One unit is equal to the formation of 1 μmol of p-nitrophenoxide per minute from the hydrolysis of p-nitrophenylacetate and calculated spectrophotometrically using: $U = [V/(e \times l)](-\Delta A/\Delta t)10^6$, where V = reaction volume (L), e = molar extinction coefficient (14760 M⁻¹cm⁻¹), l = pathlength (cm), and $-\Delta A/\Delta t$ = slope of best-fit line monitoring absorbance at 410 nm vs. time (min). Enzyme activities are normalized to the mg of enzyme used in the assay (U/mg). The hydrolysis assay was performed by mixing 100 μL pNPA (ca. 30 mM in DMSO) with enough PBS (50 mM, pH 7.5) and enzyme solution to bring the final assay volume to 1 mL and the final enzyme concentration to 5 μg/mL.

4.3. Representative example for the enzymatic resolution of *rac*-malathion into (*S*)-malathion and (*R*)-malathion monoacids using wild type pig liver esterase

To a stirred solution of wild type pig liver esterase (15.14 U) in PBS (5.4 mL, 50 mM, pH 7.5) at room temperature was added *rac*-malathion (50 mg, 0.151 mmol) in acetone (0.500 mL). After stirring for 5 h, the reaction mixture was brought to ~ pH 9 with 1 M NaOH and extracted with EtOAc (3 × 25 mL). The combined organic extracts were washed with sat. Na₂CO₃ (3 × 10 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure to afford malathion as a clear oil (19 mg, 0.058 mmol, 38 % recovery, [α]_D²¹ = −10.4). The ¹H and ³¹P NMR spectra for the isolated malathion were identical to an authentic sample (> 99%).^{12,16} ¹H NMR: δ 1.21 (t, *J* = 7.2 Hz, 3 H), 1.24 (t, *J* = 7.2 Hz, 3 H), 2.83 (dd, *J* = 5.2, 17.0 Hz, 1 H), 3.02 (dd, *J* = 9.0, 17.0 Hz, 1 H), 3.75 (d, *J* = 15.3 Hz, 3 H), 3.76 (d, *J* = 15.0 Hz, 3 H), 4.03-4.22 (m, 5 H). ³¹P NMR: δ 96.2.

The mixture of malathion monoacid was isolated by adjusting to pH ~ 2 with 1 M HCl and extracting with EtOAc (3 × 25 mL). The combined organic extracts were dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to afford a 60:40 mixture of α/β-monoacids as a pale yellow oil (12 mg, 0.042 mmol, 44 % based on recovery, [α]_D²¹ = +13.9). Partial separation of the β-monoacid: ¹H NMR: δ 1.29 (t, *J* = 7.5 Hz, 3 H), 2.98 (dd, *J* = 5.1, 17.1 Hz, 1 H), 3.11 (dd, *J* = 9.1, 17.1 Hz, 1 H), 3.83 (d, *J* = 15.3 Hz, 3 H), 3.82 (d, *J* = 15.3 Hz, 3 H), 4.25-4.10 (m, 3 H). ³¹P NMR: δ 95.61. Partial separation of the α-monoacid: ¹H NMR δ 1.27 (t, *J* = 7.1 Hz, 3 H), 2.92 (dd, *J* = 5.1, 17.3 Hz, 1 H), 3.05 (dd, *J* = 9.0, 17.1 Hz, 1 H), 3.825 (d, *J* = 15.3 Hz, 3 H), 3.82 (d, *J* = 15.3 Hz, 3 H), 4.25-4.10 (m, 3 H). ³¹P NMR: δ 95.50.

4.4. Enzymatic resolution of *rac*-malathion into (*S*)-malathion and (*R*)-malathion monoacid using isoforms of pig liver esterase

To a solution of pig liver esterase isoform (10 U/mmol of substrate) in PBS (5 mL, 50 mM, pH 7.5) at rt was added *rac*-malathion (50 mg, 0.151 mmol) in acetone (0.500 mL). After stirring for 43 h, the reaction mixture was brought to ~ pH 9 with 1 M NaOH, diluted with water (10 mL), and extracted with EtOAc (3 × 15 mL). The combined organic extracts were washed with saturated Na₂CO₃ (3 × 20 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure to afford enantiomerically enriched malathion. All isolated samples exhibited a single spot by silica TLC analysis (2,6-dibromoquinon-4-chloroimide visualization), and ¹H NMR and ³¹P spectra were identical to an authentic sample (>99 %).

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Scheme 1.

Hydrolysis of malathion to form non-toxic monoacids and oxidation of malathion stereoisomers to form the more toxic malaoxon stereoisomers.

Scheme 2.
Enzymatic resolution of malathion.

Scheme 3.

Wild type PLE forms (*S*)-malathion and (*R*)-malathion monoacids.

Table 1

Hydrolysis of *rac*-malathion by PLE (90 U/mmol) in acetone:PBS (pH 7.5; 50 mM) 1:11.

Time	A/ t (min ⁻¹)	U/mg	Activity (rel.)
0 ^a	1.301	17.3	1.00
1 h ^b	0.328	4.71	0.27
2 h ^b	0.195	2.80	0.16
23 h ^b	0.044	0.63	0.04

^a assay performed with stock enzyme solution.

^b assay performed with reaction aliquots.

Table 2Hydrolysis of *rac*-malathion by PLE isozymes (10 U/mmol) in 1:11, acetone:PBS (pH 7.5; 50 mM).

Enzyme	Initial activity (U·mg ⁻¹) ^a	Malathion recovery ^b	([α] _D) ^c	% ee (S)-malathion ^d	(E) ^e
Wild type- PLE	17.0 (2)	70	− 9.5	12	2.0
PLE1	0.074 (3)	62	− 6.5	8	1.4
PLE2	0.0372 (2)	68	− 11.6	15	2.2
PLE3	0.34 (2)	34	− 44.9	56	3.0
PLE4	0.131 (10)	35	− 15.5	19	1.4
PLE5	0.25 (2)	68	− 8.7	11	1.8
PLE6	0.626 (2)	62	− 9.5	12	1.7

^a Initial enzyme activities were measured by the pNPA hydrolysis assay (n = 3). Numbers in parentheses refer to the 95 % confidence interval for the mean.

^b Isolated at pH 9 – 10. All samples showed a single band by TLC and a single ³¹P NMR resonance (δ 95.6).

^c Measured in a 1 dm cell at 25 – 30 °C (c 0.55, CHCl₃).

^d Calculated using [α]_D of −80 for the enantiomerically pure (S)-isomer.^{12,16}

^e Calculated using: $E = \ln[(1-c)(1-ee)] / \ln[(1-c)(1+ee)]$, where c = malathion conversion, and ee = ee (S)-malathion.³³