J. Pesticide Sci. **8**, 115–120 (1983)

Note

Enantiotopic Demethylation of Fenitrothion into Partially Racemized $(R)_p$-(+)-Desmethylfenitrothion by Mouse Liver Homogenate and Mice

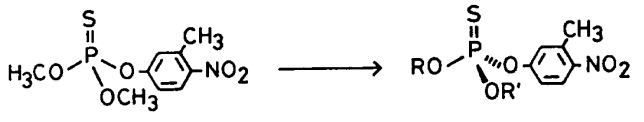
Akio MIYAZAKI, Mitsuo KAWARADANI, Shingo MARUMO* and Chojiro TOMIZAWA**

Osaka Agricultural Research Center, Habikino, Osaka 583, Japan
**Department of Agricultural Chemistry, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464, Japan*
***National Institute of Agricultural Sciences, Tsukuba-gun, Ibaraki 305, Japan*

(Received August 19, 1982)

Metabolic experiments of fenitrothion with mouse liver homogenate and mice were conducted, in order to examine whether an enantiotopic demethylation reaction occurs or not during the metabolism. Metabolic desmethylfenitrothion extracted was chemically derivatized into the diastereomeric phosphoramidothioate with (*S*)-(−)-α-phenylethylamine, which was analyzed by HPLC to determine their diastereomeric composition. The results showed that a larger amount of $(R)_p$-(+)-desmethylfenitrothion was produced with a lesser amount of a $(S)_p$-(−) enantiomer in both *in vitro* and *in vivo* metabolism, whose absolute stereochemistry was determined by comparative analysis of ^{1}H-NMR spectra of the diastereomers.

Fenitrothion, *O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl)phosphorothioate (**1**), is a pesticide practically used for protection of agricultural crops from various insect damage. Its metabolism in mammals,[1–11] fish,[12] plants,[1,2,13,14] and soils[15,16] was extensively investigated, and its major degradation pathway in the environment as well as the biological activity of degradation products have been clarified.[17] Desmethylfenitrothion ((±)-**2**) was identified as one of the major degradation products of fenitrothion, when the pesticide was metabolized by mammals,[1,2,4–9,11] insects[1–3] and soil microorganisms.[15] Desmethylfenitrothion was non-toxic to animals, and the metabolism of fenitrothion to this compound was considered to be one of the detoxication reactions.[18] The enzyme mediating this reaction was investigated in mammals,[2,3,6,11] insects,[2,3,11] and plants,[2,11] and glutathione was found to be required as a cofactor of this reaction.[11,19] One of the interesting problems which still remains unclear in this reaction is that achiral fenitrothion is converted into chiral desmethylfenitrothion, in which an asymmetric phosphorus atom is newly produced, but whether the metabolic product is either racemic or optically active has not yet been determined. Since several enantiotopic biological reactions which transform achiral substrates into chiral products have been reported,[20,21] there is a possibility that metabolic desmethylfenitrothion may be optically active. We wish now to describe that an enantiotopic demethylation reaction occurs actually in the metabolism of fenitrothion by mouse liver homogenate and mice, producing



1

$(R)_p$-(+)-**2**: R=H, R′=CH_3
$(S)_p$-(−)-**2**: R=CH_3, R′=H

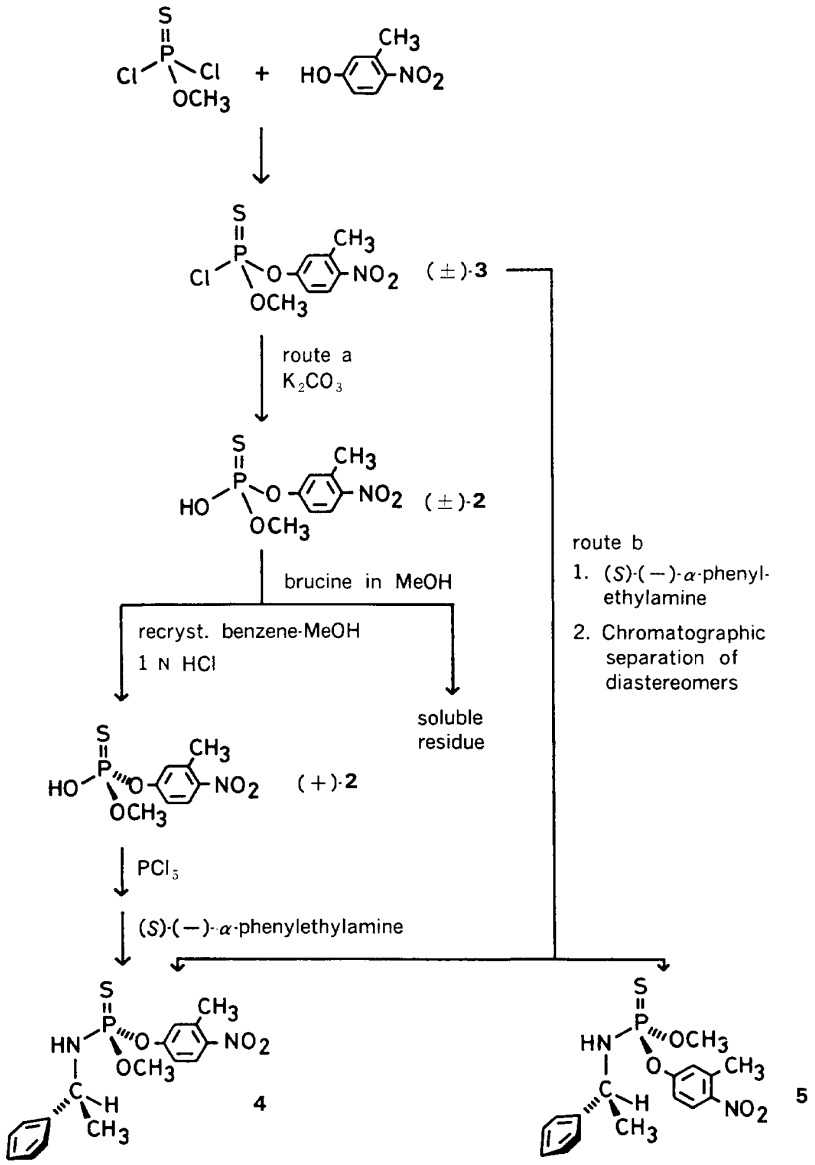
optically active $(R)_D$-(+)-desmethylfenitrothion, although the product is not optically pure but partially racemized.

EXPERIMENTAL SECTION

1. Chemicals

A pair of diastereomers, **4** and **5**, was synthesized as reference compounds to be used for chromatographic determination of the absolute stereochemistry of metabolic desmethylfenitrothion. Namely, recemic *O*-methyl *O*-(3-methyl-4-nitrophenyl)phosphorochloridothioate ((±)-**3**), prepared from *O*-methyl dichlorothiophosphate and 3-methyl-4-nitrophenol (yield 20%) (Scheme 1), was reacted with (*S*)-(−)-α-phenylethylamine and pyridine (route b) to give a diasteromeric mixture of phosphoramidothioate derivatives (**4** and **5**), which were separated through column chromatography on silicic acid (solvent: 10% ethyl acetate in *n*-hexane) into optically pure diastereomers, i.e., **4**: mass *m/z* 366 (M^+); $[\alpha]_D$ +13.9±0.3° (*c* 1.00, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ1.51 (d, *J*=7.0, 3H), 2.45 (s, 3H), 3.78 (d, *J*=14.3, 3H), 4.59 (tq, *J*=10.0, 7.0, 1H), 6.75–8.00 (m, 8H), and **5**: mass *m/z* 366 (M^+); $[\alpha]_D$ −69.8±0.4° (*c* 0.90, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ1.52 (d, *J*=7.0, 3H), 2.58 (s, 3H), 3.53 (d, *J*=14.3, 3H), 4.59 (tq, *J*=10.0, 7.0, 1H), 7.00–8.10 (m, 8H).

In order to clarify the optical rotation of chiral thiophosphorus moiety in **4**, and also to examine the extent of racemization during chemical derivatization of (+)-**2** to **4**, optically



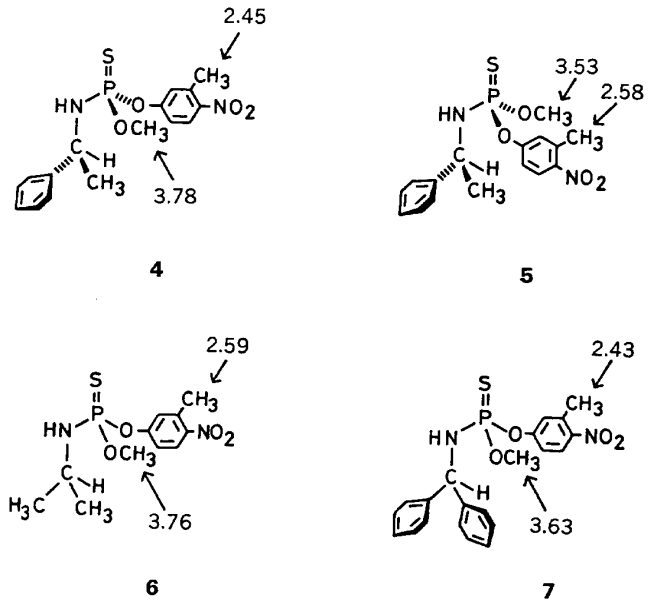
Scheme 1

pure (+)-**2** was prepared as follows. Racemic **2**, prepared from (±)-**3** by alkaline hydrolysis (K_2CO_3 in dioxane-H_2O) (route a), was treated with an equimolar amount of brucine in methanol to give a precipitate of brucine salt, which was recrystallized repeatedly (6 times) from benzene-methanol until constant values of both mp 209°C and $[\alpha]_D+2.2\pm0.3°$ (*c* 1.30, $CHCl_3$) were obtained (yield 20%). The pure crystals were treated with 1 N HCl, liberating (+)-**2**, $[\alpha]_D+4.6\pm0.4°$ (*c* 1.00, $CHCl_3$), which should be optically pure. (+)-**2** was then converted by successive reactions of chlorination (PCl_5 in $CHCl_3$) and amidation ((*S*)-(−)-α-phenylethylamine and pyridine) into **4**. Since both reactions proceed via stereochemical inversion,[22] the stereochemistry of the thiophosphorus atom in **4** should retain that of (+)-**2**, and the extent of racemization during these reactions was determined by chromatographic analysis of product **4** to contain 4.2% of **5**, thus, 8.4% racemization occurred during chemical conversion of **2** into **4**. Two racemates, **6** and **7** (Scheme 2), were also synthesized from (±)-**3**, by reacting it with either isopropylamine or diphenylmethylamine. These were used as reference compounds for determining the absolute stereochemistry of **4** and **5** by ^{1}H-NMR analysis.

2. Metabolism in vitro

Crude liver enzyme of JCL:ICR mice was prepared by the method of Fukami and Shishido.[19] Mouse liver was homogenized in ice-cold 0.25 M sucrose–0.05 M potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at $105{,}000\times g$ for 60 min to separate the supernatant fraction, which was used as the crude enzyme solution. The incubation mixture contained the crude enzyme solution (10 ml), reduced glutathione (GSH) (45 mg), and fenitrothion (0.6 and 4.0 mg) in the above buffer solution (2 ml), and incubation took place at 37°C for 3 hr. After incubation, the reaction mixture was heated at 100°C for 2 min and washed with $CHCl_3$ to remove neutral and basic substances. The residual enzyme solution was then acidified with 1 N HCl (pH 1.0) and extracted with benzene. The benzene solution was evaporated to dryness, and the residue which contained acidic metabolites of fenitrothion was dissolved in $CHCl_3$ and was subjected to successive chlorination and amidation reactions as described above. Thus, the phosphoramido derivatives of metabolic desmethylfenitrothion were prepared.

The experimental conditions of high performance liquid chromatography (HPLC) employed for analysis of diastereomeric composition of phosphoramido derivatives are described in the footnotes of Fig. 1.



4 **5** **6** **7**

Scheme 2

3. Metabolism in vivo

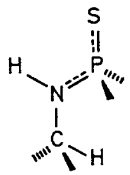
Two groups of mice each containing five JCL : ICR males, whose summed weight was ca. 150 g, were injected intraperitoneally with 2.5 and 5.0 mg (16.7 and 33.3 mg/kg) of fenitrothion dissolved in H_2O containing Triton X100. After administration, diet and water were given *ad libitum* to the mice. The urine excreted for 7 days was collected and washed at pH 10.0 with ethyl acetate to remove neutral and basic substances. Subsequent experimental procedures for converting the metabolic products into the phosphoramido derivatives were the same as those described in the *in vitro* metabolism.

RESULTS AND DISCUSSION

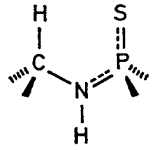
The absolute stereochemistry of desmethylfenitrothion was determined by comparative analysis of ^{1}H-NMR spectra of **4** and **5**, com-

bined with the conclusion on their most stable conformation. Namely, the proton signals whose chemical shifts were significantly different between **4** and **5** were those of the *O*-methyls, appearing at δ 3.78 in **4** and δ 3.53 in **5**. This difference of chemical shift (0.25 ppm) should be attributed to an anisotropic effect of the benzene ring of (*S*)-(—)-α-phenylethylamido moiety which shifted neighboring protons to the higher field; thus, the *O*-methyl group in **5** should be oriented to the same side as the benzene ring and hence the *O*-methyl in **4** to the opposite side.

A stable conformation of the phosphoramidothioate bond in both **4** and **5** could be assigned as a transoid form (a), rather than its cisoid form (b), because of the less steric



transoid form (a)



cisoid form (b)

hindrance between P=S and N–H bonds in the former form. A severe steric interaction between the P=S bond and phenylethyl group in the latter, however, made it impossible to take the cisoid conformation. Next, the (*S*)-α-phenylethyl moiety might be favored in such conformation where the methin proton attached to the asymmetric carbon as the smallest group flanked between the *O*-methyl and *O*-phenyl groups of the thiophosphorus moiety. Thus, the most stable conformation of **4** and **5** was postulated as drawn in Scheme 2. This conclusion was further confirmed by the facts that the *O*-methyls in the reference compounds **6** and **7** appeared at δ 3.76 and 3.63, respectively, whose chemical shifts were close to those of **4** and **5**, respectively; the methyl signals of the methyl-nitrophenyl group in **6** and **7** which appeared at δ 2.59 and 2.43, respectively, were also well in agreement with δ 2.58 in **5** and δ 2.45 in **4**, respectively.[23] Since **4** was synthesized from the (*S*)-(—) amine and (+)-**2** and the stereochemistry of the latter compound was retained in **4**, the absolute stereochemistry of (+)-**2** was concluded to be $(R)_p$, hence its antipodal $(S)_p$-(—)-**2** was contained in **5**.

Metabolic experiments of fenitrothion with mouse liver homogenate as well as mice were conducted in order to examine whether or not an enantiotopic demethylation reaction occurs during the metabolism. Metabolic desmethylfenitrothion was extracted from the incubated enzyme solution or the urine of treated mice and was chemically derivatized into the phosphoramidothioate derivative, which was then subjected to HPLC analysis to determine the diastereomeric composition. A chromatographic result obtained on the sample prepared from the incubated enzyme solution is shown in Fig. 1. Peak A was identified as **4**, on the basis of the complete coincidence of their retention time (6.1 min) and the mass spectra, and peak B was assigned to **5** through similar evidence. Since peaks A and B are recognized in the figure, both enantiomers of desmethylfenitrothion were produced enzymatically from fenitrothion, and from their peak area an enantiomeric composition of the metabolic product was calculated. Thus, the results obtained from *in vitro* and *in vivo* experiments are summarized in Table 1. This table shows that an enantiotopic demethylation reaction cleaıly occurs in both *in vitro* and *in*

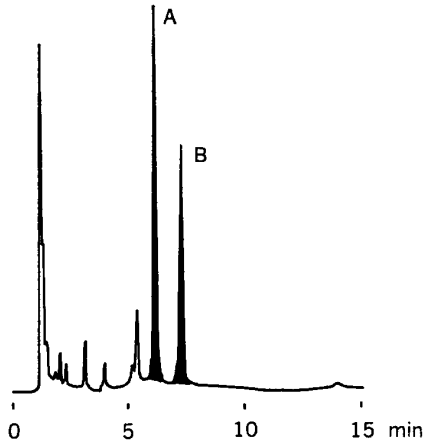


Fig. 1 High performance liquid chromatogram of (*S*)-(—)-α-phenylethylamido derivatives of metabolic desmethylfenitrothion extracted from incubated enzyme solution (*in vitro*).

Peak A: compound **4**, B: compound **5** in Scheme 1.

Column: Zorbax SIL, 25 cm × 4.6 mm o.d., Mobile phase: ethyl acetate-*n*-hexane (5 : 95), Flow rate: 1.0 ml/min, Detector: UV 254 nm.

Table 1 Yields and enantiomeric ratios of desmethylfenitrothion metabolized *in vitro* (mouse liver homogenate) and *in vivo* (mice) experiments.

	Administered fenitrothion (mg)	Yields[a] (μg) of desmethylfenitrothion		Enantiomeric ratio of (+)/(−)
		(+)-	(−)-	
In vitro	0.6	39.5	14.0	74/26
	4.0	65.4	42.0	61/39
In vivo	2.5[b]	4.54	2.73	62/38
	5.0[b]	12.3	6.00	67/33

[a] Yields and hence enantiomeric ratios were corrected by considering the partial racemization (8.4%) occurring during chemical derivatization.

[b] Administered dose of fenitrothion was 0.5 and 1.0 mg/mouse, respectively.

vivo metabolism of fenitrothion, producing a larger amount of $(R)_P$-(+)-desmethylfenitrothion contaminated by a lesser amount of a $(S)_P$-(−) enantiomer. The enantiomeric composition of desmethylfenitrothion obtained from *in vitro* and *in vivo* experiments is rather similar, and a dose of administered fenitrothion does not seem to affect the enantiomeric ratio of the product significantly.

Donninger[24] studied the metabolism of *O,O*-dimethyl *O*-1-naphthylphosphorothioate using pig liver enzyme and found that a reaction of about 90% enantiotopic demethylation occurred in that case, however, the absolute stereochemistry of the chiral demethylated product could not be determined. Our present study resulted in a lower stereoselectivity in an enantiotopic demethylation reaction of fenitrothion compared with Donninger's. The reason might be due to the metabolism of different chemicals by different animal enzymes. However, the present study first clarified the absolute stereochemistry of desmethylfenitrothion produced as a biodegradation product of fenitrothion, although the enantioselectivity of the demethylating enzyme was rather low. Further metabolic study on chiral and/or achiral pesticides from a stereochemical approach is now being done.

REFERENCES

1) J. Miyamoto, Y. Sato, T. Kadota, A. Fujinami & M. Endo: *Agric. Biol. Chem.* **27**, 381 (1963)
2) T. Shishido & J. Fukami: *Botyu-Kagaku* **28**, 69 (1963)
3) A. Vardanis & L. G. Crawford: *J. Econ. Entomol.* **57**, 136 (1964)
4) J. Miyamoto: *Agric. Biol. Chem.* **28**, 411 (1964)
5) R. M. Hollingworth, R. L. Metcalf & T. R. Fukuto: *J. Agric. Food Chem.* **15**, 242 (1967)
6) J. Miyamoto, Y. Sato, K. Yamamoto & S. Suzuki; *Botyu-Kagaku* **33**, 1 (1968)
7) P. G. C. Douche, C. E. R. Hook & J. N. Smith: *Aust. J. Pharm.* **49**, s70 (1968)
8) J. Miyamoto: *Residue Rev.* **25**, 251 (1969)
9) J. Miyamoto, K. Mihara & S. Hosokawa: *J. Pesticide Sci.* **1**, 9 (1976)
10) K. Mihara, Y. Okuno, Y. Misaki & J. Miyamoto: *J. Pesticide Sci.* **3**, 233 (1978)
11) T. Shishido & J. Fukami: *Bull. Natl. Inst. Agric. Sci., Ser. C* **32**, 1 (1978)
12) Y. Takimoto & J. Miyamoto: *J. Pesticide Sci.* **1**, 261 (1976)
13) J. Miyamoto & Y. Sato: *Botyu-Kagaku* **30**, 45 (1965)
14) S. Hosokawa & J. Miyamoto: *Botyu-Kagaku* **39**, 49 (1974)
15) Y. Takimoto, M. Hirota, H. Inui & J. Miyamoto: *J. Pesticide Sci.* **1**, 131 (1976)
16) C. J. Spillner, Jr., J. R. Debaun & J. J. Menn: *J. Agric. Food Chem.* **27**, 1054 (1979)
17) J. Miyamoto, N. Mikami, K. Mihara, Y. Takimoto, H. Kohda & H. Suzuki: *J. Pesticide Sci.* **3**, 35 (1978)
18) T. Shishido: *J. Pesticide Sci.* **3**, 465 (1978)
19) J. Fukami & T. Shishido: *J. Econ. Entomol.* **59**, 1338 (1966)
20) F. Korte & H. Arent: *Life Sci.* **4**, 2017 (1965)
21) K. Imai & S. Marumo: *Tetrahedron Lett.* **1974**, 4401
22) M. Mikolajczyk: *Tetrahedron* **23**, 1543 (1967)
23) The postulated conformation of **4** and **5** was further confirmed by the unambiguous evidence that (*S*)- and (*R*)-α-phenylethylamido derivatives of *O*-ethyl *O*-hydrogen phenylphosphonothioate, whose absolute stereochemistry was

established by X-ray analysis (S. Kogiso, H. Ohkawa & J. Miyamoto: Lecture delivered at the Annual Meeting of the Agricultural Chemical Society of Japan, April, 1981), showed quite similar ^{1}H-NMR spectra to those of **4** and **5**, details of which will be published in our forthcoming paper.

24) C. Donninger: *Bull. W. H. O.* **44**, 265 (1971)

要　　約

フェニトロチオンのマウス肝臓ホモジネートおよびマウスによる $(R)_p$-(+)-デスメチルフェニトロチオンへのエナンチオトピック脱メチル化反応

宮崎昭雄，瓦谷光男，丸茂晋吾，富澤長次郎

フェニトロチオンが動物体内でエナンチオトピック脱メチル化反応を起こす際，光学活性デスメチルフェニトロチオンが生成するかどうかを知るためマウス肝臓ホモゲネートおよびマウスにフェニトロチオンを投与し，代謝されたデスメチルフェニトロチオンを(*S*)-(−)-*α*-フェニルエチルアミンとのジアステレオマーに誘導し，液体クロマトグラフィーによって分離定量した．その結果，$(R)_p$ 体が優先的に生成したが，$(S)_p$ 体もある程度代謝されることが明らかとなった．また，デスメチルフェニトロチオンの不斉リンの絶対立体化学は合成によって得られた上記ジアステレオマーのプロトン NMR を比較検討することにより，絶対構造と旋光性を $(R)_p$-(+)，$(S)_p$-(−)と決定した．