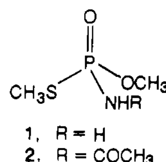
Resolution and Biological Activity of Both Enantiomers of Methamidophos and Acephate

Akio Miyazaki,* Takashi Nakamura, Mitsuo Kawaradani, and Shingo Marumo[1]

Both enantiomers, optically pure, of methamidophos, *O,S*-dimethyl phosphoramidothioate (1), and acephate, *O,S*-dimethyl *N*-acetylphosphoramidothioate (2), were prepared by optical resolution via diastereomers with L-proline ethyl ester and by HPLC using a chiral adsorbent, respectively. The $(R)_P$-(+) enantiomers were more potent to houseflies than the optical antipodes and racemates. In contrast the $(S)_P$-(−) enantiomers were more toxic within 12 h after application against German cockroaches, although the 24-h LD_{50} values were not significantly different among both enantiomers and racemate of 1, as well as 2.

Methamidophos, *O,S*-dimethyl phosphoramidothioate (1), and acephate, *O,S*-dimethyl *N*-acetylphosphoramidothioate (2), are broad-spectrum insecticides. Acephate does



not inhibit acetylcholinesterase (AChE) directly and is converted in insects to methamidophos with intoxication (Magee, 1982). These compounds have asymmetric phosphorus atoms in the molecules, but synthesis of both the enantiomers has yet to be done. This paper describes the resolution and biological activity of chiral methamidophos and acephate.

MATERIALS AND METHODS

General Procedures. Melting points (mp) were determined by the micro hot-plate method and were uncorrected. Proton (^{1}H) NMR spectra were recorded at 360 MHz with a Bruker WM 360 NMR spectrometer. Tetramethylsilane and deuteriochloroform were used as an internal standard and solvent, respectively. Abbreviations used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; bs, broad singlet. Mass spectrometry (MS) was measured on a Shimadzu LKB-9000 mass spectrometer at 70 eV. Optical rotations were measured at 25 ± 2 °C with a cell pass length of 100 mm by using a Jasco DIP-4 digital polarimeter.

Chemicals. Chemicals were from the following sources: racemic methamidophos and acephate, Wako Pure Chemical Industries, Ltd.; *S*-methyl phosphorodichloridothioate (3), prepared according to Labkowitz et al. (1974); L-proline ethyl ester, from treatment of L-proline hydrochloride with thionyl chloride and ethanol [general procedure of Patel and Price (1965)].

Synthesis of Both Enantiomers of Methamidophos [(+)- and (−)-1]. A solution of L-proline ethyl ester (13 g) in dry pyridine (60 mL) was added dropwise to a cooled solution of 3 (15 g) in dry benzene (50 mL). Stirring was continued for 4 h followed by addition of 14 mL of ammonia water (25%). After being stirred overnight, the mixture was extracted with ethyl acetate (EtOAc), washed with H_2O, and dried over anhydrous $MgSO_4$, and then the solvent was removed under reduced pressure. The residues were separated through column chromatography on silica gel (Wakogel C-200, Wako) by eluting with benzene–ethanol (95:5) and by subsequent recrystallization (EtOAc) into diastereomerically pure 4a and 4b. 4a was obtained (1.32 g) as colorless plates: mp 87 °C; MS, m/z 252 (M^+), 205, 179, 70; $[\alpha]_D$ −41.1° (*c* 0.90, $CHCl_3$); NMR δ 1.28 (t, J = 7.2 Hz, OCH_2CH_3), 1.80–2.20 (m, $NCH_2(CH_2)_2C$), 2.26 (d, J = 13.4 Hz, SCH_3), 3.32 (bs, NH_2), 3.37 (m, NCH_2CH_2), 4.18 (q, J = 7.2 Hz, OCH_2CH_3), 4.41 (m, $NCH(COOEt)CH_2$). 4b was obtained (0.96 g) as colorless plates: mp 62 °C; MS, m/z 252 (M^+), 205, 179, 70; $[\alpha]_D$ −143° (*c* 0.63, $CHCl_3$); NMR δ 1.28 (t, J = 7.1 Hz, OCH_2CH_3), 1.80–2.20 (m, $NCH_2(CH_2)_2C$), 2.29 (d, J = 13.4 Hz, SCH_3), 3.45 (bs, NH_2), 3.45 (m, NCH_2CH_2), 4.18 (q, J = 7.1 Hz, OCH_2CH_3), 4.28 (m, $NCH(COOEt)CH_2$).

4a (740 mg) was treated with anhydrous HCl–methanol (10%, 10 mL) at room temperature with stirring overnight, and then most of the methanol was removed under reduced pressure. The residues were purified through column chromatography on silica gel (Wakogel C-200) by eluting with $CHCl_3$–methanol (95:5) to give 120 mg of (+)-1 (29%): mp 68 °C (acetone–EtOAc); $[\alpha]_D$ +55.0° (*c* 0.45, $CHCl_3$). By the same way, (−)-1 was afforded from 4b (30%): mp 68 °C (acetone–EtOAc); $[\alpha]_D$ −53.9° (*c* 0.36, $CHCl_3$).

Resolution of Both Enantiomers of Acephate [(+)- and (−)-2]. Racemic 2 was resolved with high-performance liquid chromatography (HPLC; Figure 1) into optically active (+)-2 [$[\alpha]_D$ +64° (*c* 0.10, $CHCl_3$)] and (−)-2 [$[\alpha]_D$ −62° (*c* 0.10, $CHCl_3$)]. HPLC was carried out with a Shimadzu LC-3A liquid chromatograph, and chromatographic conditions were as follows: column, CHIRALCEL OC (4.6 × 250 mm, Daicel Chemical Industries, Ltd.); solvent, hexane–propan-2-ol (4:1), flow rate, 1.0 mL/min; detection, UV 235 nm.

Bioassay. Insecticidal activity was determined by topically applying the test compounds in acetone (1 μL) to the abdomen of adult female houseflies (*Musca domestica* L.; Takatsuki strain) and to adult male German cockroahces (*Blatella germanica* L.). The treated insects were held at 26 °C, and mortalities were measured over a 24-h time course. Each 24-h LD_{50} value is the mean of two or four experiments.

RESULTS AND DISCUSSION

The synthesis of both enantiomers of 1 is shown in Scheme I. The resolution was achieved by procedures based on Koizumi et al. (1974) and Leader and Casida (1982). The phosphorodiamidothioate diastereomeric mixture, 4a and 4b, which was prepared with *S*-methyl phosphorodichloridothioate (3) and L-proline ethyl ester, was easily separated with silica gel column chromatography. Alcoholysis of the diastereomers with 10% HCl–

Osaka Agricultural Research Center, Shakudo, Habikino-shi, Osaka 583, Japan.

[1] Present address: Department of Agricultural Chemistry, Nagoya University, Nagoya 464, Japan.

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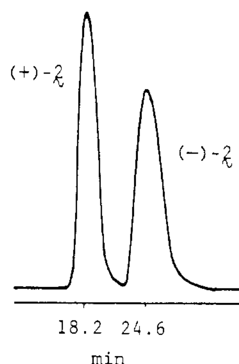
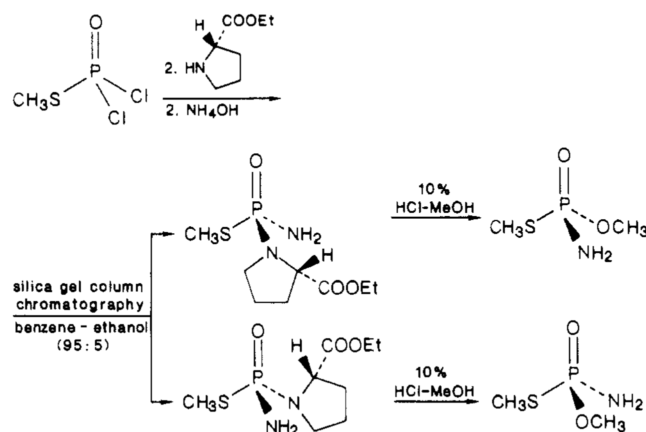


Figure 1. Chiral HPLC chromatogram (CHIRALCEL OC; hexane–propan-2-ol, 4:1) of racemic acephate (2).

Scheme I



methanol proceeded with predominant inversion of the stereochemistry (Leader and Casida, 1982). Both enantiomers of 1, upon repeated recrystallization, gave the nearly equal optical rotation except the signs were opposite ($[\alpha]_D$ +55.0° and −53.9°). The chiral HPLC (column, the same adsorbent as acephate; solvent, hexane–propan-2-ol (9:1); flow rate, 1.5 mL/min; retention times of (+)- and (−)-1, 25.0 and 29.2 min, respectively) showed that each of them did not contain any other enantiomer.

The (+) enantiomer of methamidophos has been already synthesized by Hall and Inch (1979). The preparation was based on using (−)-ephedrine as a resolving reagent to form the cyclic diastereomers and then converting one diastereomer into (+)-1 ($[\alpha]_D$ +24°), the optical purity of which was measured with ^{1}H NMR on addition of Eu(hfc)$_3$ to be

Table I. Insecticidal Activity of the Enantiomers of Methamidophos and Acephate against Houseflies and German Cockroaches[a]

	LD_{50},[b] μg/g	
	houseflies	German cockroaches
(+)-methamidophos ((+)-1)	2.4	2.2
racemic methamidophos ((±)-1)	2.8	2.2
(−)-methamidophos ((−)-1)	15.0	2.3
(+)-acephate ((+)-2)	3.0	4.2
racemic acephate ((±)-2)	3.4	4.3
(−)-acephate ((−)-2)	15.0	4.5

[a] Topical application: 1 μL of acetone solution. [b] 24 h after application.

ca 97% of the enantiomer. But the optical antipode was not prepared. The optical rotation of the (+) enantiomer was much lower than that synthesized by us.

The absolute stereochemistry of (+)-1 has been determined to be of $(R)_P$ configuration by ^{1}H NMR with Eu(hfc)$_3$ (Hall and Inch, 1979). (+)-Acephate [(+)-2] has the same absolute stereochemistry as (+)-1, because (+)-1 was converted with acetyl chloride and sodium hydride in ethyl ether into partially racemized (+)-2, $[\alpha]_D$ +20°.

The insecticidal activity of optically active and racemic methamidophos and acephate to the houseflies is shown in Table I. (+)-Methamidophos was approximately 6-fold more toxic than the (−) enantiomer. The toxicity of the racemate was intermediate between both enantiomers, and the LD_{50} showed to be close to that of the (+) enantiomer. In the case of acephate, the (+) enantiomer, which has the same absolute stereochemistry as (+)-methamidophos, was more potent than the (−) enantiomer and racemate. There is no difference in insecticidal activity between methamidophos and acephate with the same absolute stereochemistry, as well as between the racemates.

Against German cockroaches, however, both (−) enantiomers had stronger activity than the antipodes in a short time. At the dose of 3.1 μg/g of methamidophos, the (−) enantiomer showed 10, 63, and 75% of the mortality 1.5, 3, and 5 h after appication, respectively (Figure 2A). On the other hand, (+) enantiomer revealed almost no toxicity at 3 h and less, and 50% at 9 h. Similar results were also obtained with acephate (Figure 2B). These suggest that the (−) enantiomers might be transported more rapidly to the active site and/or have the stronger affinity for AChE than the (+) enantiomers. The 24-h LD_{50} values of methamidophos are not significantly different among both

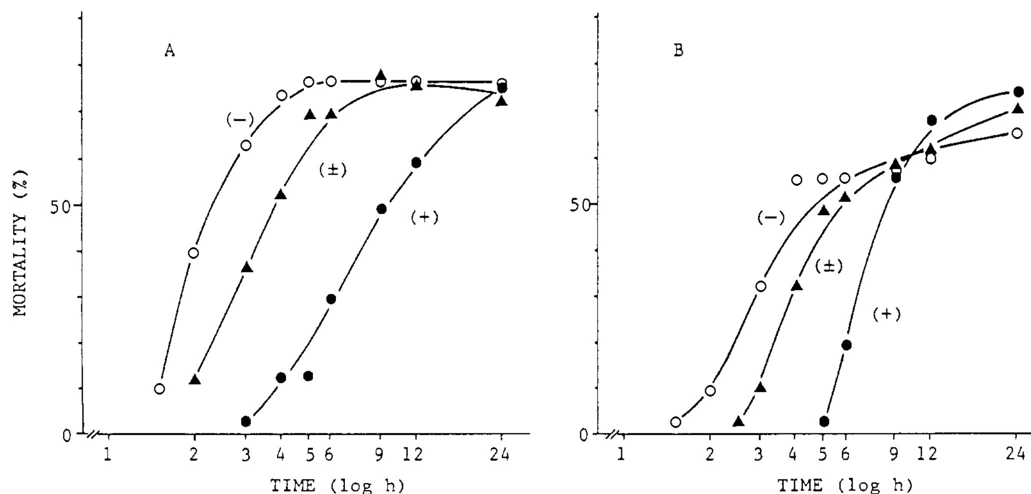


Figure 2. Insecticidal activity of both enantiomers and racemates of methamidophos (A, 3.1 μg/g of 1) and acephate (B, 5.4 μg/g of 2) against German cockroaches.

enantiomers and racemate, and also those of acephate, except that the former was 2-fold more potent than the latter (Table I). This is different from housefly data. The present study clarified the biological activity of both enantiomers of methamidophos and acephate, which were first resolved. Further experiments on metabolic activity using stereochemistry are now being done.

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[^{14}C]Virginiamycin Residues in Eggs

Denis E. Corpet,* Maryse Baradat, and Georges F. Bories

Laying hens were fed ab libitum a diet supplemented at 40, 10, and 0 mg/kg with [^{14}C]virginiamycin, to determine whether or not virginiamycin or its metabolites were transferred to the eggs. About 0.05% of the ingested ^{14}C dose was recovered in the eggs. Radiolabeled residues expressed in terms of virginiamycin equivalents were found to be 5.1 ng/g in the albumen and 31.8 ng/g in the yolk from hens fed a 10 mg/kg diet. No antibiotic activity could be detected in the eggs. Tentative identifications showed that in the albumen about 17% of the ^{14}C behaved chromatographically like virginiamycin and 18% was associated to the ovalbumin. In the yolk 31% of the ^{14}C was associated to proteins, 58% to fatty acids, and 4% to unsaponifiable matter.

Virginiamycin is an antibiotic used in poultry husbandry at levels of 7.5–40 mg/kg to improve poultry performance. Virginiamycin-supplemented diet stimulates growth and improves feed efficiency in broilers. It increases egg production in laying hens (Keppens et al., 1981; Miles et al., 1985).

Numerous studies have shown that detectable levels of antimicrobial residues can occur in eggs following the administration to hens of antibiotics at therapeutic levels (Petz, 1984). However, residues could not be detected in eggs by microbiological methods, after the administration of antibiotics at growth-promoter levels (Katz et al., 1974).

Virginiamycin seems to be absorbed in the rat, since 17% of the administered ^{3}H disappeared from the ligated stomach after an oral dose of virginiamycin [^{3}H] factor M_1 (Roberfroid and Dumont, 1972) and since about 88% of an oral dose of [^{14}C]virginiamycin was recovered in feces of rats and cattle (Gottschall et al., 1987). Virginiamycin seems to be less absorbed in poultry, since the plasma concentration of radioactive residues was found to be 0.04 μg/g in broilers given for 12 days a diet supplemented at 44 ppm (activity) (Miller, J. A., personal communication). Microbiological assays of eggs from hens fed a diet containing 40 ppm (activity) virginiamycin for 6 months showed no detectable antibiotic residues, the detection limits being 20 ng/g in the albumen and 50 ng/g in the yolk (Di Cuollo, 1980).

Since in European countries virginiamycin is currently being approved for use in laying hens at 10–40 ppm (activity) inclusion level, the objective of the present study was to determine the amount and the nature of residual radioactive material in eggs from hens fed diets supplemented with 10 and 40 mg/kg of [^{14}C]virginiamycin. According to Smith Kline Corp., which provided us with the drug, and due to improvements in the synthesis procedure, the microbiological activity of the drug we used was 200%. This means that the 10 and 40 mg (weight) of drug we added per kilogram of diet corresponded to commercial feedstuffs containing 20 and 80 ppm (activity) of virginiamycin, respectively.

Xênobiotiques Laboratory, INRA, BP. 3, F-31931 Toulouse Cedex, France.

MATERIALS AND METHODS

[^{14}C]Virginiamycin. Generally labeled [^{14}C]virginiamycin was provided by Smith Kline Corp. It was prepared by fermentation from [^{14}C]acetate and different ^{14}C amino acid precursors used simultaneously (Gottschall et al., 1987). The radiochemical purity of the drug was checked by thin-layer chromatography (TLC), using two solvent systems, followed by radio-TLC scanning. Two radioactive areas were detected, which corresponded to factors M (66–68%) and S (32–34%) of virginiamycin. This preparation of virginiamycin was assayed vs the original activity standard and was twice as active as the original standard, for an equivalent sample weight (Gottschall et al., 1987).

Preparation of Experimental Feed. Usual precautionary handling procedures were followed each time radioactive material was used. [^{14}C]Virginiamycin, 30.4 kBq/mg, was dissolved in ethanol, and a known volume was adsorbed onto the feed pellets of a standard laying hen diet, so that the diets contained either 10 mg/kg of virginiamycin and 304 Bq/g or 40 mg/kg and 1215 Bq/g. Due to the 200% microbiological activity of the product, these actual concentrations (weight basis) corresponded to 20 and 80 g/ton supplemented feed, respectively, ac-

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