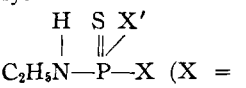
Thiophosphoric Acid Derivatives of Ethylamine, DL-Methionine, and L-Proline Ethyl Esters I

Synthesis

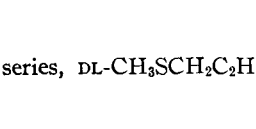
By FLORENCE C. KLEE† and ERNST R. KIRCH

Thiophosphoric acid derivatives having structures similar to known anticholinesterases, were synthesized for the investigation of their possible enzyme inhibition *in vivo*. Based on theoretical considerations concerning structures of known chemotherapeutic agents, these compounds may possess antitumor activity. The phenyl ester was found to be white needle-like crystals rather than the liquid reported by Michaelis. Procedures for obtaining good yields of the ethyl esters of L-proline and DL-methionine are given. A modified method of King's colorimetric determination of phosphorus is presented.

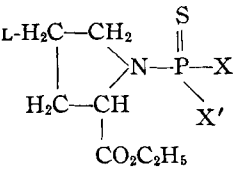
THE PURPOSE of this investigation was to prepare some thiophosphoric acid derivatives which have structures similar to known anticholinesterases, and to investigate their biological action *in vivo*. The three series of compounds synthesized are the following: ethylamine series,



C_2H_5N—P—X (X = X′ = Cl; X = X′ = OR where R is an ethyl, *n*-propyl, isopropyl, *n*-, *sec*-, *tert*- or iso-butyl, or a phenyl group; X = OC_2H_5, X′ = Cl); DL-methionine ethyl ester



series, DL-$CH_3SCH_2C_2H_5O_2C$—P—X, and L-proline ethyl ester series,



where

X = X′ = OC_2H_5

X = X′ = Cl

X = OC_2H_5, X′ = Cl.

These derivatives were selected with the intention of synthesizing anticholinesterases with potential antitumor activity. The rationale for the synthesis of these derivatives is as follows: (*a*) The phosphate radical is very important in biological systems and these organothiophosphorus derivatives may interfere with metabolic pathways providing the energy for cell metabolism of cancer cells. There is a possibility that the P=S group is converted to P=O *in vivo*, but this conversion would not be undesirable, since the anticholinesterase activity of these compounds would be enhanced (1).

(*b*) The amino acids, methionine and proline, were bound to the thiophosphorus radical in an attempt to produce potential amino acid antagonists since the amino acid antagonists DL-ethionine (2) and the bis-chloroethyl derivative of carbamoylserine (3) have been reported as tumor inhibitors. S-Carbamylcysteine has been observed to exhibit antitumor action (4). Methionine has been reported to reduce the growth of a rat sarcoma (5). Certain derivatives of proline have insecticidal activity (6).

(*c*) Certain phosphoric acid ester amides (7) and certain ethylenephosphoramides (8) have carcinostatic activity. A thiophosphoramide (thio-TEPA) (9, 10) is said to be curative and widely used.

(*d*) In the literature, anticholinesterase activity by alkyl phosphate compounds (11 – 13) and antitumor activity by certain chemotherapeutic agents (9) have been explained by proposed mechanisms involving an alkylation. The former activity is due to a dialkylphosphorylation of the enzyme.

The monosubstituted amides of dialkoxy(or diphenoxy)thiophosphoric acids were prepared according to a method similar to that reported by Michaelis (14) for the synthesis of dimethylamidodiethoxyphosphate.

The use of triethylamine in N-phosphoryl amino ester synthesis has been reported by Li (15). This tertiary amine was used in the synthesis of the thiophosphoric acid derivatives of DL-methionine and L-proline esters and in the preparation of ethylamidoethoxythionophosphoryl chloride.

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EXPERIMENTAL

Thiophosphoryl chloride was prepared by the Knotz method (16) from 24 Gm. (0.75 mole) of powdered sulfur, 100 Gm. (0.73 mole) of phosphorus trichloride, and 2 Gm. (0.015 mole) of aluminum chloride as the catalyst. Reported b.p. 125–126°; yield, 120 Gm. (97.6%). Observed b.p. 124° (uncorr.); yield, 88.5 Gm. (71.8%).

Ethylamidothiophosphoryl chloride, $C_2H_5NHP(S)Cl_2$, was prepared from 32.6 Gm. (0.4 mole) of ethylamine hydrochloride and 136 Gm. (0.8 mole) of thiophosphoryl chloride according to the procedure of Michaelis (14). Reported b.p. 105°/9 mm.; yield is very good. Observed b.p. (uncorr.) 98–102°/8 mm.; yield, 58.0 Gm. (81.5%); n_D^{20} 1.5432.

Anal.—Calcd. for $C_2H_5Cl_2NPS$: N, 7.87; P, 17.40; S, 18.02. Found: N, 7.69; P, 17.49; S, 17.91. Qualitative chemical test for halogen.

Dialkoxyethylamidothiophosphates, $C_2H_5NHP(S)(O\text{-}Alkyl)_2$.—A solution of ethylamidothiophosphoryl chloride (8.90 Gm., 0.05 mole)[1] in anhydrous ether was slowly introduced into a warm sodium alkoxide solution prepared from 2.3 Gm. (0.1 mole) of sodium[2] dissolved in an excess of absolute alcohol. The ester formed upon heating for one-half hour and sodium chloride separated out on cooling. Distilled water was added dropwise to the mixture until the sodium chloride was completely dissolved, and the two layers were transferred to a separator and additional ether was introduced. The ethereal layer was removed, the aqueous layer was extracted again with ether, and the combined ether extracts were dried over anhydrous potassium carbonate. The ether was distilled and the remaining oil was subjected to vacuum distillation. The physical constants, yields, and analyses of the synthesized compounds are shown in Table I.

Diphenoxyethylamidothiophosphate, $C_2H_5N(H)-P(=S)(OC_6H_5)OC_6H_5$.—In 1903, Michaelis (14) reported that the phenyl ester analog of $C_2H_5N(H)-P(=S)(OC_2H_5)OC_2H_5$ is a liquid, but failed to report its method of preparation, analysis, or physical constants. The phenyl ester used in this investigation was prepared in the following manner: sodium phenoxide was prepared from 2.3 Gm. (0.1 mole) sodium in 10 ml. (10.7 Gm., 0.11 mole) of phenol. A small volume of phenol was added to cover the sodium phenoxide. A large excess of phenol was avoided in order to facilitate the isolation of the ester. Ethylamidothiophosphoryl chloride (8.9 Gm., 0.05 mole) in anhydrous ether was added to the cooled sodium phenoxide mixture and warmed on a steam bath for 10 minutes. The cooled mixture was treated with distilled water, extracted, and dried as given in the procedure for the dialkyl esters. After removal of the ether and excess phenol by distillation, the residue decomposed in part when subjected to vacuum distillation, as evidenced by the dark colored residue and evolution of gaseous products. The residue was dissolved in ether and, upon evaporation of the solvent, a crystalline tarry mass resulted. The mass was dissolved in warm 95% ethanol and filtered. Distilled water was added dropwise into the filtrate at 0° until precipitation began to take place. After repeated recrystallization, slow evaporation of the alcoholic solution produced white needle-like crystals rather than the liquid reported by Michaelis. Caution was used in the recrystallization of the product because an oil would form if the proper amount of water was not used. The melting point of the product was 64–64.5° (uncorr.) and the yield was 1.49 Gm. (10.2%).

Anal.—Calcd. for $C_{14}H_{16}NO_2PS$: N, 4.78; P, 10.56; S, 10.93. Found: N, 4.92; P, 10.56; S, 10.68. Quantitative chemical analysis and infrared data are consistent with the desired phenyl ester.

Ethylamidoethoxythionophosphoryl Chloride, $C_2H_5N(H)-P(=S)(OC_2H_5)Cl$.—Ethylthiophosphoryl dichloride ($C_2H_5OP(S)Cl_2$) was prepared according to the method of Bakanova, *et al.* (17), from 17 Gm. (0.1 mole) of thiophosphoryl chloride, 3 Gm. (0.065 mole) of absolute ethanol, and 0.5 Gm. (0.0185 mole) of fine aluminum wire. Reported b.p. 68°/20 mm., yield, 40%. Observed b.p. (uncorr.) 67°/20 mm.; yield, 3.50 Gm. (30%).

A solution of 4.48 Gm. (0.025 mole) of $C_2H_5OP(S)Cl_2$ and 5.06 Gm. (0.05 mole) of triethylamine in 30 ml. of dry chloroform was added dropwise (20 minutes) to a constantly stirred suspension of 2.04 Gm. (0.025 mole) of ethylamine hydrochloride in 25 ml. of dry chloroform at 0°. Stirring was continued at 25° for 40 minutes. A minute quantity of precipitate formed which did not increase in amount even after a 5-day period at 25° followed by heating at 45–50° for 2½ hours. After filtration, the chloroform solution was washed with two 25-ml. portions of distilled water, 1 *N* hydrochloric acid, 10% sodium bicarbonate solution, three 25-ml. portions of distilled water, and then dried over anhydrous sodium sulfate. Removal of the solvent by means of flash evaporation left a residue which distilled as a clear, colorless oil. Observed b.p. (uncorr.) 65–67°/0.25 mm.; n_D^{20} 1.4999; yield, 2.84 Gm. (60.6%).

Anal.—Calcd. for $C_4H_{11}ClNOPS$: N, 7.47; P, 16.51; S, 17.10. Found: N, 7.34; P, 16.59; S, 16.89. Qualitative chemical test for halogen.

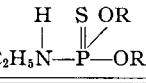
Preparation of Thiophosphoric Acid Derivatives of DL-Methionine Ethyl Ester.

Synthesis of the Ethyl Ester of DL-Methionine.—It was found that the highest yield of ester was obtained when a suspension of 14.92 Gm. (0.1 mole) DL-methionine in 150 ml. of absolute ethanol was saturated at 0° with hydrogen chloride and refluxed for 1 hour. After a 48-hour period, most of the solvent was removed by use of a flash evaporator. The remaining viscous yellow liquid was dissolved in 40 ml. of absolute ethanol, 50 ml. of anhy-

[1] The ethyl ester was prepared from 0.2 mole (35.6 Gm.) and the isobutyl ester from 0.08 mole (14.2 Gm.) of ethylamidothiophosphoryl chloride.

[2] Potassium was used to make the alkoxide of tertiary butyl alcohol since the reaction was too sluggish with sodium and the yield was less than that obtained with the more active metal.

TABLE I.—DIALKOXYETHYLAMIDOTHIOPHOSPHATES



R	B.p.,[a] °C.	n_D^{20}	Yield, Gm.	Yield, %	Analyses, % Calcd.[b] N	Calcd. P	Calcd. S	Found N	Found P	Found S
C_2H_5[c]	67–70	1.4716	26.6	67.4	7.10	15.71	16.26	6.99	15.71	16.21
n-C_3H_7	84–87	1.4695	7.9	70.1	6.22	13.75	14.23	6.06	13.84	14.00
iso-C_3H_7	72–75	1.4617	8.3	73.7	6.22	13.75	14.23	6.10	13.92	14.14
n-C_4H_9	112–116	1.4690	9.0	70.6	5.53	12.23	12.66	5.47	12.14	12.48
sec-C_4H_9	87–90	1.4658	5.8	45.8	5.53	12.23	12.66	5.68	12.14	12.65
tert-C_4H_9	78–81	1.4662	4.9	38.7	5.53	12.23	12.66	5.48	12.04	12.47
iso-C_4H_9	90–92	1.4640	14.6	71.8	5.53	12.23	12.66	5.38	11.95	12.51

[a] Uncorrected b.p. at 0.25 mm. [b] Calculated for $C_6H_{16}NO_2PS$ (ethyl ester); $C_8H_{20}NO_2PS$ (propyl esters); $C_{10}H_{24}NO_2PS$ (butyl esters). [c] Reported in literature (14), b.p. 94°/12 mm.; yield, none reported.

drous ether was added, and anhydrous ammonia gas was bubbled through the two phase system at 0° for 1 hour. After 66 hours, the ammonium chloride and any unreacted amino acid were removed by filtration. The solvent was removed by flash evaporation and the residue subjected to vacuum distillation, giving 12.5 Gm. (70.3%) of the ethyl ester as a clear, colorless, oil. Observed b.p. (uncorr.) 84–86°/0.25 mm. Reported (18) b.p. 112°/2.5 mm.; yield, 88%.

Synthesis of N-(Diethoxyphosphinothioyl)-DL-methionine Ethyl Ester.—A dry chloroform solution (25 ml.) of O,O-diethyl phosphorochloridothioate (Ethyl PCT-Monsanto) (2.36 Gm., 0.0125 mole) was added dropwise within 60 minutes to a constantly stirred dry chloroform solution (25 ml.) of DL-methionine ethyl ester (2.19 Gm., 0.0124 mole) and triethylamine (1.25 Gm., 0.0125 mole) at 0–5°. Stirring was continued for 30 minutes at room temperature and after 18 hours the chloroform solution was washed and dried in the same manner as ethylamidoethoxythionophosphoryl chloride. A flash evaporator was used to remove chloroform and any remaining halogen-containing reactant. The ester distilled as a clear, colorless oil. Observed b.p. (uncorr.) 145–147°/0.25 mm.; n_D^{20} 1.4950; yield, 2.31 Gm. (56.8%).

Anal.—Calcd. for $C_{11}H_{24}NO_4PS_2$: N, 4.25; P, 9.40; S, 19.47. Found: N, 4.23; P, 9.47; S, 19.57.

Synthesis of N-(Ethoxychlorophosphinothioyl)-DL-methionine Ethyl Ester.—This ester was prepared according to the directions given for N-(diethoxyphosphinothioyl)-DL-methionine ethyl ester. The ethylthiophosphoryl dichloride (4.48 Gm., 0.025 mole) was added within 70 minutes to 4.43 Gm. (0.025 mole) of DL-methionine ethyl ester and 2.53 Gm. (0.025 mole) of triethylamine. This ester, a clear yellow oil, underwent complete decomposition when high vacuum distillation was attempted. n_D^{20} 1.4962; yield, 6.18 Gm. (77.3%).

Anal.—Calcd. for $C_9H_{19}ClNO_3PS_2$: N, 4.38; P, 9.69; S, 20.06. Found: N, 4.22; P, 9.65; S, 19.83. Qualitative chemical test for halogen.

Synthesis of N-(Dichlorophosphinothioyl)-DL-methionine Ethyl Ester.—This derivative was prepared in the same manner as the N-(diethoxyphosphinothioyl)-DL-methionine ethyl ester. Thiophosphoryl chloride (2.99 Gm., 0.0176 mole) was added within 180 minutes to 3.13 Gm. (0.0176 mole) of DL-methionine ethyl ester and 1.79 Gm. (0.0176 mole) of triethylamine. The product was washed with four 25-ml. portions of each solution instead of the indicated number. This derivative, a clear yellow oil, underwent complete decomposition when high vacuum distillation was attempted. Yield, 3.76 Gm. (68.6%). It was observed that the dichloro derivative was unstable at room temperature, since approximately 10 days after its synthesis, the yellow oil started to decompose.

Preparation of the Thiophosphoric Acid Derivatives of L-Proline Ethyl Ester

Synthesis of the Ethyl Ester of L-Proline.—It was found that the following procedure gives a 65% yield of the ester from 0.109 mole of L-proline. Dry hydrogen chloride gas was introduced very slowly (4 hours) into a constantly stirred suspension of L-proline (12.58 Gm., 0.109 mole) in absolute ethanol (150 ml.) at 0°. After 2 days at 25°, the solvent was removed by flash evaporation and the residue dissolved in absolute ethanol (40 ml.). After the addition of anhydrous ether (50 ml.), dry ammonia gas was introduced slowly at 0° until saturation occurred (1 hour). After an 18-hour period the ammonium chloride was removed by filtration and washed with anhydrous ether. The ether of the combined ethereal solutions was removed by flash evaporation and the residue was subjected to vacuum distillation. Reported (19) b.p. 78°/12–14 mm.; yield, 40.5 Gm. (66%). Observed b.p. (uncorr.) 77–78°/12 mm.; yield, 10.1 Gm. (64.5%).

Synthesis of N-(Diethoxyphosphinothioyl)-L-proline Ethyl Ester.—A dry chloroform solution (25 ml.) of O,O-diethyl phosphorochloridothioate (4.72 Gm., 0.025 mole) (Ethyl PCT-Monsanto) was introduced within 30 minutes into a constantly stirred dry chloroform solution (25 ml.) of proline ethyl ester (3.58 Gm., 0.025 mole) and triethylamine (2.53 Gm., 0.025 mole) at 0°. Stirring was continued for 30 minutes and the solution was allowed to stand at 25° for 18 hours.[3] The chloroform solution was washed, dried, and distilled in the same manner as the ethylamidoethoxythionophosphoryl chloride. This ester is a clear, colorless, distillable oil. Observed b.p. (uncorr.) 118–120°/0.25 mm.; n_D^{20} 1.4810; yield, 4.65 Gm. (63.0%).

[3] This derivative was heated for 1 hour at 55–60° on 2 successive days to insure completeness of reaction. It was found that the quantity of white precipitate that had originally formed did not increase in amount upon further heating.

Anal.—Calcd. for $C_{11}H_{22}NO_4PS$: N, 4.74; P, 10.49; S, 10.86. Found: N, 4.68; P, 10.47; S, 10.86.

Synthesis of N-(Ethoxychlorophosphinothioyl)-L-proline Ethyl Ester.—This ester was prepared in a similar manner as the N-(diethoxyphosphinothioyl)-L-proline ethyl ester. Ethylthiophosphoryl dichloride (3.97 Gm., 0.022 mole) was introduced within 120 minutes into the proline ethyl ester (3.18 Gm., 0.022 mole) and triethylamine (2.24 Gm., 0.022 mole). No additional heating was applied after the solution was allowed to stand at 25° for 18 hours. The ester is a clear, yellow, undistillable oil. n_D^{25} 1.5088; yield, 4.81 Gm. (75.9%).

Anal.—Calcd. for $C_9H_{17}ClNO_3PS$: N, 4.90; P, 10.84; S, 11.23. Found: N, 4.83; P, 10.86; S, 10.99. Qualitative chemical test for halogen.

Synthesis of N-(Dichlorophosphinothioyl)-L-proline Ethyl Ester.—This derivative was prepared according to the procedure given for the N-(diethoxyphosphinothioyl)-L-proline ethyl ester. Thiophosphoryl chloride (4.24 Gm., 0.025 mole) was introduced within 120 minutes into the proline ethyl ester (3.58 Gm., 0.025 mole) and triethylamine (2.53 Gm., 0.025 mole). No additional heating was applied after the solution was allowed to stand at 25° for 18 hours. The ester is a clear, yellow, undistillable oil. Yield, 5.67 Gm. (82.2%). As was found in the methionine series, this dichloro derivative is not stable at room temperature.

Colorimetric Determination of Phosphorus.—The thiophosphoric acid derivatives of ethylamine, methionine, and proline were analyzed for phosphorus by the method of King (20). The following modifications were introduced: A standard curve was made up from aliquots (containing 0.01 to 0.15 mg. of phosphorus) of a standard phosphate solution. Readings were obtained in a Coleman spectrophotometer at 650 mμ. King compares the color of a standard phosphate solution with that of the unknown solution in a Duboscq colorimeter.

Double quantities of 72% perchloric acid, 5% ammonium molybdate, and sulfonic acid were used and diluted to 100 ml. instead of 15 ml.

The sample (approximately 40 mg.) was digested with 1 ml. of perchloric acid,[4] diluted to 100 ml. with distilled water and an aliquot (usually 1 ml.), containing between 0.05 and 0.1 mg. phosphorus, was used for the colorimetric determination. Results were very satisfactory.

DISCUSSION

Since the yield of DL-methionine ethyl ester when prepared by the method of Booth, *et al.* (18), did not furnish enough starting material for further syntheses, the method of preparation was investigated to improve the yield. The highest yield of ester was obtained by the procedure given in the experimental section.

Kapfhammer and Matthes (19) reported the synthesis of the ethyl ester of L-proline from 50 Gm. of L-proline with no mention of the amount of ethanol, rate of hydrogen chloride introduction, time, and temperature at which the reaction took place. It was found in this investigation that a 65% yield of the ester could be obtained from 12.58 Gm. of L-proline according to the directions given in the experimental part. Freshly prepared ester was used in the syntheses of the thiophosphoramides, since this ester loses ethanol spontaneously at room temperature, to form the solid anhydride or diketopiperazine derivative.

The thiophosphoric acid derivatives of both amino acid ethyl esters were prepared from the amino acid ester and an appropriate thiophosphoryl chloride (1:1 molar ratio) in the presence of triethylamine. The tertiary amine reacted with the liberated hydrogen chloride gas and allowed most of the amino acid ester to form the desired product instead of the amino acid ester hydrochloride.

Analytical Methods.—Nitrogen analyses were by a semimicro Kjeldahl method and phosphorus analyses were determined by a modified King method. With the exception of the methionine series compounds, sulfur was determined gravimetrically after a perchloric-nitric acid oxidation method of Webster and Powers (21). Callan and Toennies (22) have reported the incomplete oxidation of sulfur in methionine when wet-oxidative methods were used. In this investigation it was found that the methionine derivatives were incompletely oxidized by perchloric-nitric acid, but completely oxidized when they were burned in the Parr bomb. The sulfur was then determined gravimetrically as barium sulfate. Halogen was detected by a qualitative chemical test after a sodium fusion of the halogen-containing compounds in the three series.

SUMMARY

The thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters of the general formula, $-\overset{|}{N}-\overset{\overset{S}{\|}}{P}(X)_2$ where $(X)_2$ is $(O\text{-alkyl})_2$, $(O\text{-phenyl})_2$, $(C_2H_5O)Cl$, or $(Cl)_2$, were synthesized for the investigation of their possible enzyme inhibition *in vivo*.

It was found that the phenyl ester is crystalline rather than liquid as reported by Michaelis who failed to report its method of preparation, analysis, and physical constants. The procedures for obtaining good yields of the ethyl esters of these two amino acids from 0.1 mole of the amino acid are given.

All of the compounds, with the exception of the methionine derivatives, were oxidized by the wet-oxidative perchloric-nitric acid method of Webster and Powers. The methionine derivatives were completely oxidized in the Parr bomb.

It was found that a modified King method gave very satisfactory results for the colorimetric determination of phosphorus.

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[4] In some cases where oxidation was slow, a drop or two of nitric acid was added. Heating was continued for 3 or 4 minutes after the mixture had been colorless to drive off the excess of nitric acid.

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Thiophosphoric Acid Derivatives of Ethylamine, DL-Methionine, and L-Proline Ethyl Esters II

Biological Activities in Guppies

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Observed biological responses of guppies in 0.01% w/v aqueous solutions of the thiophosphoric acid derivatives indicate that the compounds are acetylcholinesterase inhibitors. The 0.001% solutions furnished additional information concerning the biological activity of these compounds. Structure-toxicity relationship is discussed.

THE SYNTHESIS of thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters has recently been published (1).

The thiophosphoric acid derivatives used in this investigation have the general formula

—N—P(X)$_2$, where (X)$_2$ = (O-alkyl)$_2$, (O-C$_6$H$_5$)$_2$, (C$_2$H$_5$O)Cl, or (Cl)$_2$, and N represents the amine or amino acid ester moiety. These structures are similar to those of known anticholinesterases, thus the thiophosphoric acid derivatives may be acetylcholinesterase inhibitors. If so, then the following types of biological responses may be visualized in guppies (*Lebistes Reticulatus*): difficulty in breathing, dizziness, evacuation of rectum and bladder, convulsions, and total paralysis (death).

The purpose of this investigation was to observe the biological responses of baby guppies which have been immersed for 24 hours in 0.01 and 0.001% w/v aqueous solutions of each compound.

EXPERIMENTAL

Table I lists the compounds used in this study. Stock aqueous solutions or aqueous suspensions (0.1% w/v) were prepared from which 0.01 and 0.001% w/v solutions were obtained after making a tenfold dilution of the stock and 0.01% w/v solution.

An alcoholic stock solution and alcoholic dilutions were made of diphenoxyethylamidothiophosphate, since it was not possible to obtain a uniform 0.1% w/v aqueous suspension. Aliquots of the alcoholic solutions were allowed to evaporate to dryness and a volume of distilled water equal to the volume of alcohol was introduced before testing the biological activity of the compound.

Ten milliliters of 0.01 and 0.001% w/v aqueous solutions of each compound were placed in separate vials (2.4 cm. diameter and 5.5 cm. in height) and 10 young guppies were placed in each vial. For control purposes, distilled water was used instead of an aqueous solution of a compound under test and it was observed that all of the guppies survived after 24 hours. The O,O-diethyl phosphorochloridothioate (Ethyl PCT-Monsanto), C$_2$H$_5$O—P(S)—Cl,

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