Insecticidal, Anticholinesterase, and Hydrolytic Properties of *O,O*-Dialkyl *S*-Aryl Phosphorothiolates in Relation to Structure

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A series of *O,O*-dialkyl *S*-aryl phosphorothiolates was found to be generally powerful anticholinesterases, relatively labile to alkaline hydrolysis and moderately toxic to houseflies, the most active compounds being the diethyl *S*-(4-chloro- and 4-bromophenyl) phosphorothiolates. A positive correlation between in vitro inhibition of fly head cholinesterase and toxicity indicated their action to be similar to other organophosphorus anticholinesterases. The range of activity between the least and most active compounds was small in comparison to their phosphate analogs, indicating the restricted effect of aryl substitution on the P-S-phenyl linkage.

Organophosphorus compounds have been investigated extensively for insecticidal and other biological properties. The *O,O*-dialkyl *S*-aryl phosphorothiolates, however, have received only brief attention: The dimethyl and diethyl *S*-(4-nitrophenyl) isomers of methyl parathion and parathion have been studied in conjunction with their parent insecticides (Hecht and Wirth, 1950; Schrader, 1952). These compounds have a higher mammalian toxicity than the corresponding phosphorothionates or phosphates and are more potent inhibitors of cholinesterase (Aldridge and Barnes, 1952; Aldridge and Davison, 1952b; Diggle and Gage, 1951). Their insecticidal activity, however, is lower (Abdallah, 1963; Martin, 1950; Woodcock and Stringer, 1951). Since several of the *S*-aryl phosphorothiolates are isomeric with important phosphorothionate insecticides, they may be environmental degradation products or impurities in insecticide preparations.

We have synthesized and examined a series of dimethyl, diethyl, and diisopropyl *S*-(substituted-phenyl) phosphorothiolates for toxicity to houseflies, anticholinesterase activity, and hydrolytic stability to define further their biological properties and structure-activity relationships.

MATERIALS AND METHODS

O,O-dialkyl *S*-aryl phosphorothiolates were synthesized using the radical-chain transfer reaction of $BrCCl_3$, trialkyl phosphite, and substituted-benzenethiol according to a method described previously (Murdock and Hopkins, 1968). The only compound not recovered in high purity was *O,O*-diethyl *S*-(4-nitrophenyl) phosphorothiolate, which was substantially decomposed during purification on the Florisil column. One sample was partially purified on a deactivated column (purity >90%), and was used for all experiments.

Toxicity of the compounds to houseflies (*Musca domestica* L.) was determined by topical application of acetone solutions to the thorax of 4-day-old female insects. One-microliter drops of the solutions were administered with a microapplicator (ISCO, Lincoln, Neb.). The houseflies were a Beltsville susceptible strain never previously exposed to insecticides. After treatment the flies were held in pint jars with screen lids containing cotton saturated with water. Mortality counts were made at 24 hours. Treatments were replicated at least three times at each dosage level with 25 flies per replication. LD_{50} values were determined from plots of log dosage *vs.* the mean probit mortality.

Anticholinesterase activity was determined by incubating each compound with the housefly head enzyme preparation and assaying for cholinesterase activity by the method of Monroe and Robbins (1959). Housefly head enzyme was prepared from 4- to 5-day-old flies of both sexes. The heads were harvested by shaking frozen flies with dry ice chips in a 20-mesh standard sieve. The heads were homogenized in a glass tissue grinder with 25 heads to 5 ml. of ice-cold pH 7.2, 0.134*M* phosphate buffer. Thimerosal (Nutritional Biochemicals, Cleveland, Ohio) (0.075 ml. of a 0.01% solution) was added as a preservative to each 5 ml. and the homogenates were frozen until used.

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Before each run the homogenates were tested and adjusted to standard activity if necessary. Each inhibitor concentration was run in duplicate and replicated at least three times. I_{50} values were determined from plots of the mean log per cent inhibition *vs.* the log molar concentration of inhibitor.

The stability of the phosphorothiolates to basic hydrolysis was measured by incubating the compounds in 0.01*N* NaOH, adding sufficient HCl to stop the reaction, then extracting the unreacted compounds with chloroform (Heath, 1956), and measuring unreacted triesters by the method of Getz and Watts (1964). A standard curve was prepared for each compound.

In a typical kinetic run 2 ml. of 0.01*N* NaOH was added to each of six glass-stoppered 35-ml. centrifuge tubes. At intervals of one-half minute, 15 μl. of the acetone stock solution containing 15 μg. of the test compound was injected into the alkali, using a Hamilton 50-μl. syringe (Hamilton Co., Whittier, Calif.). An appropriate series of incubation times was determined in a preliminary run. After the solution incubated, 2 ml. of 0.01*N* HCl was added to the tube from a buret, and the tube was shaken. When all tubes had received the HCl, 3 ml. of distilled chloroform was added to each, and the tubes were reshaken. The chloroform layer was then drawn from the centrifuge tube, using a 5-ml. syringe, and was transferred to a Getz tube. The aqueous layer was washed twice more with 3 ml. of chloroform, and individual extracts were combined, evaporated in a stream of nitrogen, and analyzed by the triester method. Rates of disappearance were determined by taking an average of three replicate runs for each incubation time. Runs were made at 37° ± 0.5° in a water bath.

Under conditions of the method, hydrolysis of diethyl *S*-aryl phosphorothiolate follows pseudo-first-order kinetics. If a is the amount of phosphorothiolate placed in the tube and x units are hydrolyzed after time t, then

$$\ln \frac{a}{a - x} = kt$$

where k is the pseudo-first-order rate constant. k is determined by plotting the log term *vs.* t and algebraically estimating the slope of the resulting straight line.

The electron-withdrawing or donating effects of the substituents on hydrolysis were compared by plotting the Hammett sigma constants (Gould, 1959) *vs.* the log k_{hyd}.

RESULTS AND DISCUSSION

Toxicity to Houseflies. The toxicity of *S*-aryl phosphorothiolates to houseflies is given in Table I. The compounds were less toxic to houseflies than either the corresponding phosphates or phosphorothionates in the two series tested. In the diethyl 2,4,5-trichlorophenyl group, the phosphorothionate was more toxic than the phosphate, which again was more toxic than the phosphorothiolate. The thionate > phosphate > thiolate order of toxicity was also observed in the parathion series. Previous comparisons of the relative toxicity to insects of corresponding phosphorothionates, phosphates, and *S*-aryl phosphorothiolates have been limited to studies of parathion, paraoxon, and the parathion *S*-phenyl isomer (Abdallah, 1963; Martin, 1950; Schrader, 1952; Woodcock and Stringer, 1951). Both the phosphorothionate and phosphate were more toxic to insects than the parathion *S*-phenyl isomer, which our results confirm.

A striking characteristic of the *S*-aryl phosphorothiolates is the small range between the least and most toxic compounds. For example, considering all of the compounds regardless of the alkyl substituents, the diethyl 4-chloro compound was only about 20 times more toxic than the least toxic diisopropyl *S*-phenyl phosphorothiolate. In the diethyl series the most toxic 4-chloro derivative was only about seven times more toxic than the least active 4-methyl compound. Studies on a series of diethyl substituted-phenyl phosphates (Fukuto and Metcalf, 1956) provide a basis to compare the two classes of analogous compounds (Table II). Parallel results found in the two studies on LD_{50} and I_{50} values for paraoxon and ethyl ronoxon indicate such a comparison to be valid, particu-

Table I. Insect Toxicity, Anticholinesterase Properties, and Hydrolytic Stability of *O,O*-Dialkyl *S*-Aryl Phosphorothiolates and Some Related Compounds

Compound	LD_{50}-Housefly, μg./Fly	I_{50}-Housefly Head ChE (Molar Concn.)	Hydrolysis Constant, k_{hyd} Min.$^{-1}$
$(MeO)_2P(O)$ S Ph	2.0	2.0×10^{-6}	...
$(MeO)_2P(O)$ S Ph 4-Cl	0.4	8.6×10^{-8}	...
$(EtO)_2P(O)$ S Ph	0.51	2.8×10^{-7}	3.9×10^{-2}
$(EtO)_2P(O)$ S Ph 4-Cl	0.27	3.5×10^{-9}	7.9×10^{-2}
$(EtO)_2P(O)$ S Ph 2-Cl	1.0	1.5×10^{-8}	1.1×10^{-1}
$(EtO)_2P(O)$ S Ph 2,4,5-Cl_3	0.64	3.3×10^{-9}	3.9×10^{-1}
$(EtO)_2P(O)$ S Ph 4-Br	0.33	1.9×10^{-9}	5.1×10^{-2}
$(EtO)_2P(O)$ S Ph 4-F	1.3	5.2×10^{-7}	2.9×10^{-2}
$(EtO)_2P(O)$ S Ph 4-Me	1.9	2.0×10^{-6}	2.2×10^{-2}
$(EtO)_2P(O)$ S Ph 4-NO_2	0.42	9.0×10^{-9}	2.2×10^{-1}
$(Iso\text{-}PrO)_2P(O)$ S Ph	4–6	1.5×10^{-6}	...
$(Iso\text{-}PrO)_2P(O)$ S Ph 4-Cl	2.0	8.8×10^{-8}	...
$(Iso\text{-}PrO)_2P(O)$ S-Ph 2,4,5-Cl_3	1.8	1.8×10^{-7}	...
$(EtO)_2P(O)$ O Ph 4-NO_2 (Paraoxon)	0.05	5.5×10^{-8}	1.0×10^{-2}
$(EtO)_2P(O)$ O Ph 2,4,5-Cl_3 (Et Ronoxon)	0.17	2.2×10^{-9}	2.0×10^{-2}
$(EtO)_2P(S)$ O Ph 4-NO_2 (Parathion)	0.02	...	...
$(EtO)_2P(S)$ O Ph 2,4,5-Cl_3 (Et Ronnel)	0.10	...	...

Table II. LD_{50} and I_{50} Values of Diethyl-Substituted Phenyl Phosphates and Phosphorothiolates to Houseflies

Phenyl Substituent	LD_{50}, μg./G.		I_{50} (Molar Concn.)	
	Phosphate[a]	Phosphorothiolate[b]	Phosphate[a]	Phosphorothiolate[b]
H	>500	26	$>1.0 \times 10^{-3}$	2.8×10^{-7}
4-CH_3	>500	95	$>1.0 \times 10^{-3}$	2.0×10^{-8}
2-Cl	250	50	2.0×10^{-5}	1.5×10^{-8}
4-Cl	150	14	3.0×10^{-5}	3.5×10^{-9}
4-NO_2	0.5	21	2.6×10^{-8}	9.0×10^{-9}
	2.5[b]		5.5×10^{-8}[b]	
2,4,5-Cl_3	8.0	32	6.0×10^{-9}	3.3×10^{-9}
	8.5[b]		2.2×10^{-9}[b]	

[a] Data from Fukuto and Metcalf (1956). [b] Data from our study.

larly when noting wide differences in activity between other members of the two series. The most toxic phosphate, the 4-nitro compound (paraoxon), was over 1000 times more toxic than the 4-methylphenyl phosphate, whereas the widest range of the phosphorothiolates was only seven times. Such wide *vs.* narrow range in toxicity between the two groups shows that although the highly toxic 4-nitro- and 2,4,5-trichlorophenyl phosphates are more insecticidal than the corresponding phosphorothiolates, the reverse is true with the 2-chloro, 4-chloro, 4-methyl, and unsubstituted phenyl analogs. In the latter examples the phosphates are apparently much less toxic than the phosphorothiolates to houseflies.

The effect of the *O,O*-dialkyl ester substituents on the toxicity of *S*-aryl phosphorothiolates was compared in the *S*-phenyl and *S*-4-chlorophenyl series. In them, the diethyl compounds were most toxic, followed in order by the dimethyl derivatives and the diisopropyl *S*-aryl phosphorothiolates.

The effect of aryl substituents on toxicity is shown in the diethyl series. The order of decreasing toxicity was 4-chloro > 4-bromo > 4-nitro > unsubstituted phenyl > 2,4,5-trichloro > 2-chloro > 4-fluoro > 4-methyl. Phosphorothiolates with electron-withdrawing aryl substituents—i.e., 4-nitro, and 2,4,5-trichloro—were expected to be among the more toxic, since they would confer the greatest instability on the P-S-aryl bond and thereby increase the cholinesterase-inhibitory power of the compound and consequently, its toxicity. The substituent effect is seen in the diethyl substituted-phenyl phosphates, where the 4-nitro- and 2,4,5-trichloro compounds are among the more toxic (Fukuto and Metcalf, 1956). Such was not the case with the *S*-aryl phosphorothiolates. The explanation for the drop in toxicity with such substitution probably is the unstabilizing action of 4-nitro or 2,4,5-trichloro groups on an already labile P-S-aryl linkage. Less powerful electron-withdrawing substituents, such as 4-chloro or 4-bromo, apparently confer nearly "optimum instability" and maximum toxicity, while weakly electron-withdrawing or electron-donating substituents—i.e., 4-fluoro or 4-methyl—produce more stability, which again results in less toxicity.

Anticholinesterase Properties. The *O,O*-dialkyl *S*-aryl phosphorothiolates are powerful anticholinesterase agents, with inhibitory activity in the range of the more active phosphates (Table I). The *S*-phenyl parathion isomer and paraoxon were found approximately equipotent in that respect, agreeing with earlier studies of comparative inhibitory activity of the phosphorothiolate and phosphate (Aldridge and Davison, 1952b; Hecht and Wirth, 1950). Ethyl ronoxon and the diethyl *S*-(2,4,5-trichlorophenyl) phosphorothiolate are also approximately equal as cholinesterase inhibitors.

Derivatives containing electron-withdrawing ring substituents—i.e., 4-chloro, 4-bromo, 4-nitro, and 2,4,5-trichloro—were the more potent inhibitors, while the unsubstituted phenyl compounds were the least active.

The range between the most and least active inhibitors, like the LD_{50} values, was again narrow compared with the diethyl substituted-phenyl phosphates (Fukuto and Metcalf, 1956). Replacing the 4-nitro of paraoxon with hydrogen decreased anticholinesterase activity, to less than 1/40,000 its initial value, while a similar substitution in the *S*-aryl phosphorothiolates caused a decrease to only 1/70 (Table II). The relative independence of the phosphorothiolates from substituent effects is further supported by the uniformly low I_{50} values; all of these compounds inhibited cholinesterase below $10^{-5}M$ concentration.

The effect of the alkyl substituent can be compared directly in the *S*-phenyl and *S*-(4-chlorophenyl) series. The ethyl derivatives were better inhibitors than the methyl and isopropyl homologs; the latter two were approximately equal.

The order of decreasing anticholinesterase activity of the diethyl phosphorothiolates in relation to ring substituents was: 4-bromo > 2,4,5-trichloro > 4-chloro > 4-nitro > 2-chloro > 4-methyl > unsubstituted phenyl > 4-fluoro. The inhibitory activity of the diethyl *S*-(4-methylphenyl) derivative appears to be anomalous. It is a more powerful inhibitor than the diethyl *S*-phenyl or *S*-(4-fluorophenyl) derivative, even though, on the basis of the electron-donating influence of the methyl substituent on the phenyl ring, it would be expected to decrease the δ^+ charge on the phosphorus and hence be a less powerful phosphorylating agent than the other two compounds. The "methyl effect" also has appeared in the *O*-ethyl *S*-aryl ethylphosphonothiolates (Menn and Szabo, 1965). *O*-ethyl *S*-(4-methylphenyl) ethylphosphonothiolate was nearly ten times more powerful than the *O*-ethyl *S*-phenyl derivative in inhibiting cholinesterase.

Hydrolytic Stability. The only *S*-aryl phosphorothiolates examined for hydrolytic stability were the diethyl derivatives (Table I). Under the method used, methyl and isopropyl derivatives failed to follow first-order kinetics. The phosphorothiolates are hydrolyzed rapidly in 0.01*N* base. The effects of introducing a sulfur bridge between the aryl group and phosphorus can be estimated by comparing the hydrolytic rate constants of the two analogous pairs: paraoxon and diethyl *S*-(4-nitrophenyl) phosphorothiolate; ethyl ronoxon and diethyl *S*-(2,4,5-trichlorophenyl) phosphorothiolate. In both cases the sulfur-bridged compound hydrolyzes five to 10 times faster than the oxygen-bridged compound. Heath (1956) also observed rapid hydrolysis of organophosphorus

compounds containing the P-S-C linkage and attributed this to the ease of polarization of the sulfur atom.

The order of increasing stability of basic hydrolysis for the diethyl derivatives was: 2,4,5-trichloro < 4-nitro < 2-chloro < 4-chloro < 4-bromo < unsubstituted phenyl < 4-fluoro < 4-methyl. That order of hydrolytic stability essentially reflects the unstabilizing effect of various aryl substituents—i.e., 2,4,5-trichloro and 4-nitro—which were more susceptible to hydrolysis than derivatives with weak electron-withdrawing or electron-donating substituents—i.e., unsubstituted phenyl, 4-fluoro, or 4-methyl. In that respect the *S*-aryl phosphorothiolates resemble diethyl-substituted-phenyl phosphates (Aldridge and Davison, 1952a; Fukuto and Metcalf, 1956).

Fukuto and Metcalf (1956) showed a linear relationship between the electronic nature of the phenyl substituents (Hammett sigma constants) and hydrolytic stability of diethyl-substituted-phenyl phosphates. The linear relationship also holds with the diethyl *S*-substituted-phenyl phosphorothiolates where the phosphorus phenyl linkage is sulfur rather than oxygen (Figure 1). The strong electron-withdrawing effect of the 4-nitro group imparts the greatest instability on the P-S-phenyl linkage, while the electron-donating effect of the methyl group confers the greatest stability.

Structure-Activity Relationships. Aldridge and Davison (1952a) established that a direct relationship exists in most cases between the stability of organophosphorus compounds to aqueous hydrolysis and their effectiveness as cholinesterase inhibitors. The more stable the inhibitor is, the lower its anticholinesterase properties. A similar plot for the diethyl *S*-aryl phosphorothiolates shows that five of them closely approach a linear relationship, while the 4-chloro, 4-bromo, and 4-methyl show no close correlation (Figure 2). The latter three compounds were better anticholinesterases than their stability to hydrolysis indicated. Aldridge and Davison (1952a) have reported a

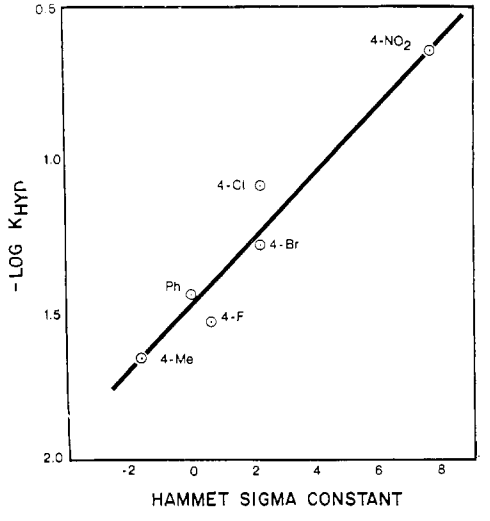

Figure 1. Relationship between decreasing hydrolytic stability (log k_{hyd}) and electronic properties (Hammett sigma constants) of phenyl substituents in diethyl *S*-(para-substituted-phenyl) phosphorothiolates (Ph-unsubstituted)

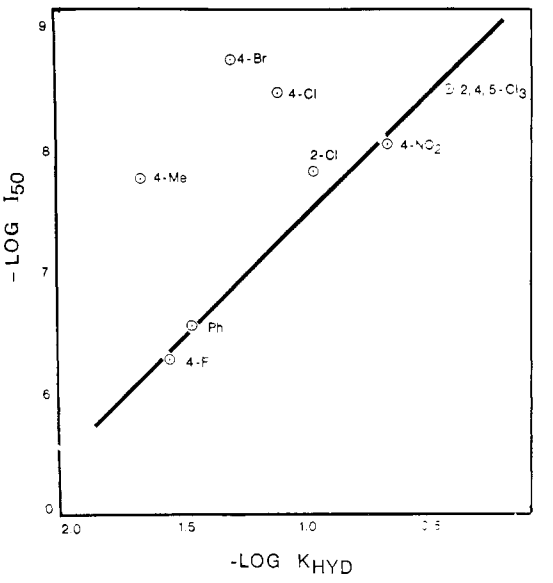

Figure 2. Relationship between increasing anticholinesterase potency (log I_{50}) and decreasing hydrolytic stability (log k_{hyd}) of diethyl *S*-(substituted-phenyl) phosphorothiolates (Ph-unsubstituted)

similar example with paraoxon. It inhibited cholinesterase much better than would be predicted from its hydrolytic rate, but no reason for this was apparent.

Toxicity of the organophosphorus insecticides is generally attributed to their anticholinesterase properties (O'Brien, 1960). Evidence for that hypothesis includes observations that in a series of related compounds, toxicity to organisms is roughly proportional to in vitro potency as inhibitors of cholinesterase. A plot of the log LD_{50} *vs.* log I_{50} shows a similar positive correlation existing between those two properties in the dialkyl *S*-aryl phosphorothiolates (Figure 3). The better in vitro inhibitors of housefly head cholinesterase were also the more toxic to houseflies. Several of the diethyl phosphorothiolates—i.e., 4-bromo, 2,4,5-trichloro, 2-chloro, and 4-methyl—were more active anticholinesterases in vitro than their toxicity to houseflies indicated. Conversely the unsubstituted phenyl and 4-fluoro were considerably more toxic than would be predicted from their inhibitory properties. Several factors undoubtedly are involved in preventing a closer correspondence between anticholinesterase activity and toxicity, including differing rates of cuticular penetration, detoxication, and transport to site of action.

The dialkyl *S*-aryl phosphorothiolates as a group exhibit properties generally consistent with other organophosphorus anticholinesterases. They are very active inhibitors of cholinesterase, and relatively labile to alkaline hydrolysis, although the correlation between the two is not always close. A general correlation exists between housefly toxicity and in vitro cholinesterase inhibition, indicating their mode of action to be similar to that of other such insecticides. The relatively narrow range of toxicity and inhibitory potency between the most and least active members of the group is strikingly different from their

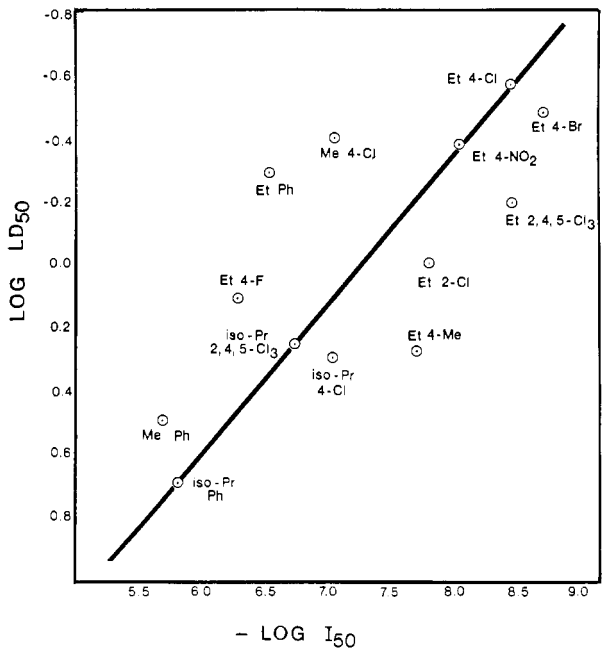

Figure 3. Relationship between increasing toxicity to houseflies (log LD_{50}) and increasing anticholinesterase potency (log I_{50}) in dialkyl *S*-(substituted-phenyl) phosphorothiolates

Compounds designated by alkyl group followed by phenyl substituent (Ph-unsubstituted)

phosphate analogs. Optimum insecticidal activity occurs when the alkyl ester substituents are ethyl and the aryl substitutes are 4-chloro or 4-bromo. Strong electron-withdrawing substituents such as 4-nitro or 2,4,5-trichloro decrease activity, as do weaker electron-withdrawing or electron-donating substituents.

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