Structure and Anticholinesterase Activity of Series of Ethyl Substituted Phenyl Methylphosphonates

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Thirty derivates from substituted-phenyl-ethyl methylphosphonates have been synthesized and their inhibiting power of acetyl-cholinesterase have been examined *in vitro* and *in vivo*. The correlation between inhibition of the enzyme and electrophylic power of the substituent of the phenyl group was excellent, but when this group contains two substituents, steric factors appear to operate. The activity of these compounds has been demonstrated to be higher than their phosphate analogs.

Introduction

The study of the organophosphorus compounds such as insecticides has been carried out by several groups of investigators [1–6] and the mechanisms of the reactions between these compounds and various esterases have been studied in detail, with the conclusion that the inhibition of these enzymes is brought about by the irreversible phosphorilation of the active center by these phosphorous compounds.

In previous papers of this laboratory [5, 6] several series of dialkyl-aryl phosphates were studied, and it was shown that the activity of these compounds was associated to their acyl-transferring ability which can be modified by changing the para position of the aryl group. The phosphonic acid derivates have been studied by Fukuto and Metcalf [3] who described a *p*-nitrophenyl methylphosphonate activity superior not only to the other alkylderivates but even to that of other more frequently used insecticides such as paraoxon (*p*-nitrophenyl-diethyl-phosphate).

The purpose of the present work is to determine if the higher anticholinesterase activity exhibited by the *p*-nitrophenyl derivative is a characteristic feature of this phosphorous compound or if it is a general norm in all methylphosphonates. For this reason several series of substituted phenyl-methylphosphonates have been synthesized and their anticholinesterase activities *in vitro* and *in vivo*, determined.

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Materials and Methods

The required compounds were synthetized by the method described hereunder. All the compounds gave satisfactory IR and NMR spectra.

Of the phenols employed some were obtained comercially while the others were synthetized by methods described in a previous paper [5] or by the method described herewith.

4-Bromo-3-methylphenol

m-Cresol (25 g), carbon tetrachloride (150 ml) were introduced into a three-necked flask provided with a stirrer, drying tube and dropping-funnels. All of these were then placed in a salt-ice bath. Bromine (40 g) dissolved in carbon tetrachloride (20 ml) was added drop by drop and then left to stand for half an hour, then washed with a 5% bisulphite solution, water, and finally dried over magnesium sulphate. On removing the solvent the residue was distilled *in vacuo* to give a boiling point fraction of 60 – 80 °C which crystallised from hexane at 60 °C giving a solid (33 g) m.p. 57 – 58 °C.

4-Bromo-3-ethylphenol

3-Ethylphenol (24.2g) and carbon tetrachloride (300 ml) were introduced into a three-necked round-bottomed flask provided with a drying tube, dropping-funnel, thermometer graduated from – 80 °C to – 20 °C and a magnetic stirring bar. The solution was chilled to – 20 °C and a cold solution of bromine (35 g) in carbon tetrachloride (150 ml) was dropped off. Throughout the whole dropping-process the temperature was maintained between – 5 °C and – 15 °C and the solution was then left at this temperature for one hour more; after which the preparation was allowed to warm to room temperature very slowly, permitting the

process to take place with an abundant but gradual release of gas. The solution was washed with a 5% bisulphite solution, water, and then dried. The solvent was removed and the residue distilled *in vacuo* (36 g) b.p. 77 – 78 °C/0.1 mm. Crystallization of the phenol from hexane at – 60 °C gave a solid (30 g) m.p. 43 – 44 °C.

Ethyl methylphosphonochloridate

Prepared according to Pelchowitz[7] b.p. 80 °C/20 mm. The diethyl-methylphosphonate required for this synthesis was prepared by the Ford-Moore method[8] b.p. 80 °C/12 mm.

Ethyl substituted phenyl methylphosphonate

The phenol (0.02 mol) in anhydric benzene or absolute ethanol (if the phenol is insoluble in benzene) was dropped off, into a magnetically stirred solution of sodium (0.022 mol) in absolute ethanol (15 ml). After shaking for 5 min the ethanol was distilled off by azeotropic distillation with benzene (60 ml) until the distillate reached a temperature of 79 – 80 °C The sodium salt of the phenol which remained in suspension was treated drop by drop with ethyl-methylphosphonochloridate (0.025 mol) in anhydric benzene (15 ml) and then refluxed for two hours. Sodium chloride was filtered off and the benzene solution was washed once with a 5% sodium carbonate solution, later with water, dried over magnesium sulphate, and finally distilled *in vacuo*. To remove the last trace of phenol a De-acidite G SRA-97 chromatography was necessary, and the elution was carried out with different anhydrous mixtures of benzene-hexane (Figs in Tables I – III). The yields were about 40 – 45%. The physical constants of the methylphosphonates are recorder in Tables I – III.

Inhibition in vitro

Determination of I_{50} values for the inhibition of fly-brain cholinesterase was carried out by the Warburg standard manometric technique[9, 10]. Houseflies were decapitated by the Morefield procedure[11]. A homogenous mixture containing a sufficient number of fly heads per milliliter to give an enzymatic activity of 6 ml CO_2/min after centrifuging for one hour, was used.

The pI_{50} were obtained graphycally, plotting negative logarithms of molar concentration against percentages of inhibition.

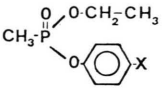
Toxicity test in vivo

Determinations of toxicity by contact were carried out by the Metcalf method[12]. *Musca domestica* L. were fed on a standard diet at 22 °C and 60% relative humidity for 3 or 4 days. The LD_{50} determinations were carried out by Probit analysis[13].

Results and Discussion

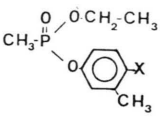
In order to study these compounds as inhibitors *in vivo*, we have taken into account that these products were phenol-free. When the methylphosphonates were obtained by the usual methods of purification they showed in their IR spectra, a band between 3,200 cm^{-1} and 3,400 cm^{-1} and their NMR spectra gave a typical sign of acidic H, indicating the presence of impurity. To remove this impurity it was necessary to do a chromatography with a

Table I. Physical constants for ethyl and phenyl 4-substituted methylphosphonates.



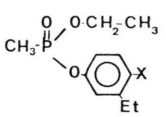
X	Formula	b. p. [°C]	[mm]	Eluent	Analysis [%]					
					Calculated			Found		
					C	H	P	C	H	P
H	$C_{10}H_{15}O_3P$	75	0.05	ben.-hex. 40/60	55.55	6.94	14.35	55.72	6.80	14.30
$-SO_2-CH_3$	$C_{11}H_{17}O_5PS$	182 – 4	0.05	benzene	45.20	5.82	10.61	44.70	5.53	10.60
$-SO-CH_3$	$C_{11}H_{17}O_4PS$	145	0.05	benzene	47.82	6.15	11.23	47.40	6.00	11.10
$-S-CH_3$	$C_{11}H_{17}O_3PS$	120	0.08	ben.-hex. 60/40	50.76	6.54	11.90	50.90	6.72	11.72
$-NO_2$	$C_{10}H_{14}O_5PN$	155	0.4	ben.-hex. 40/60	46.33	5.40	11.87	46.20	5.44	11.56
$-CN$	$C_{11}H_{14}O_3PN$	123 – 4	0.1	ben.-hex. 40/60	55.50	5.94	12.86	55.23	5.85	12.60
$-O-CH_3$	$C_{11}H_{17}O_4P$	104	0.1	ben.-hex. 40/60	54.09	6.96	12.70	54.13	6.91	12.60
Br	$C_{10}H_{14}O_3BrP$	106	0.03	ben.-hex. 40/60	40.67	4.74	10.50	40.50	4.80	10.20
Cl	$C_{10}H_{14}O_3PCl$	98	0.1	ben.-hex. 30/70	48.60	5.73	12.30	48.30	5.63	12.00
$-CH_3$	$C_{11}H_{17}O_3P$	91	0.1	ben.-hex. 30/70	57.90	7.45	13.59	58.10	7.70	13.00
$-N(-CH_3)_2$	$C_{12}H_{20}O_3PN$	106	0.03	ben.-hex. 40/70	56.03	7.78	12.06	55.80	7.57	11.90

Table II. Physical constants for ethyl and 3-methyl-phenyl 4-substituted methylphosphonates.



X	Formula	b. p. [°C]	[mm]	Eluent	Analysis [%]					
					Calculated			Found		
					C	H	P	C	H	P
H	$C_9H_{13}O_3P$	83	0.3	ben.-hex. 40/60	54.00	6.50	15.50	54.32	6.29	15.30
$-CH_3$	$C_{10}H_{15}O_3P$	82	0.1	ben.-hex. 40/60	56.07	7.00	14.48	56.20	7.10	14.10
$-Cl$	$C_9H_{12}O_3PCl$	90–2	0.1	ben.-hex. 40/60	46.00	5.11	13.21	46.30	5.30	13.00
$-Br$	$C_9H_{12}O_3PBr$	100	0.05	ben.-hex. 40/60	38.70	4.30	11.11	38.80	4.40	10.80
$-OCH_3$	$C_{10}H_{15}O_4P$	103	0.05	ben.-hex. 40/60	52.17	6.52	13.48	52.40	6.60	13.10
$-OEt$	$C_{11}H_{17}O_4P$	114	0.03	ben.-hex. 40/60	54.09	6.96	12.70	54.20	6.70	12.50
$-COOCH_3$	$C_{11}H_{15}O_5P$	113–5	0.05	ben.-hex. 40/60	51.16	5.81	12.01	51.30	5.60	12.10
$-CN$	$C_{10}H_{12}O_3PN$	112	0.05	ben.-hex. 80/20	53.33	5.33	13.77	53.10	5.00	13.50
$-NO_2$	$C_9H_{15}NO_5P$	137	0.3	ben.-hex. 40/60	44.08	4.90	12.65	44.38	5.21	12.40
$-S-CH_3$	$C_{10}H_{15}SO_3P$	105	0.05	ben.-hex. 80/20	48.78	6.09	12.60	48.60	6.20	12.50
$SOCH_3$	$C_{10}H_{15}SO_3P$	143–5	0.05	benzene	45.80	5.72	11.83	45.50	5.40	11.62
$-SO_2-CH_3$	$C_{10}H_{15}SO_5P$	177–9	0.03	benzene	43.16	5.39	11.15	43.10	5.40	10.05

Table III. Physical constants for ethyl and 3-ethyl-phenyl 4-substituted methylphosphonates.



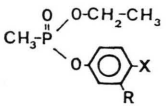
X	Formula	b. p. [°C]	[mm]	Eluent	Analysis [%]					
					Calculated			Found		
					C	H	P	C	H	P
H	$C_{11}H_{17}O_3P$	88–9	0.1	ben.-hex. 40/60	57.90	7.45	13.59	50.10	7.70	13.30
Cl	$C_1H_{16}O_3PCl$	108	0.2	ben.-hex. 40/60	50.30	6.09	11.80	50.30	6.21	11.50
Br	$C_{11}H_{16}O_3PBr$	125	0.5	ben.-hex. 40/60	42.99	5.21	10.09	42.65	5.05	10.00
$-CN$	$C_{11}H_{16}O_3PN$	106	0.05	benz.-hex. 30/70	56.91	6.32	12.25	56.80	6.17	12.00
$-NO_2$	$C_{11}H_{16}O_5PN$	141–2	0.1	ben.-hex. 40/60	48.35	5.85	11.35	48.42	6.07	11.10
$-S-CH_3$	$C_{12}H_{19}O_3PS$	123–5	0.08	benz.-hex. 60/40	52.55	6.93	11.31	52.40	6.00	11.00
$-SO-CH_3$	$C_{12}H_{19}O_4PS$	150–3	0.05	benz.-hex. 60/40	49.65	6.55	10.68	49.60	6.50	10.50
$-SO_2-CH_3$	$C_{12}H_{19}O_5PS$	175–7	0.01	benzene	47.05	6.20	10.13	47.00	6.10	9.80

styrene resin of quaternary ammonium groups using as eluent, anhydrous organic solvents. This purification gave satisfactory results.

Most of the compounds studied in this work are powerful inhibitors of acetylcholinesterase, when tested *in vivo* and *in vitro*. Table IV shows that the majority of I_{50} values range from 10^{-6} to 10^{-9} M. The inspection of these values when compared with those obtained from the literature for their phosphate and alkyl-phosphonate analogues indicated that the former are more powerful as inhibitors of these enzymes.

The activity *in vitro* of the different compounds of the methylphosphonate series is related to the electrophylic power of the substituent in para position: the most electronegative substituents give the highest activity thus confirming previous studies. A new substitution in metaposition increases this ac-

Table IV. Biological activity *in vitro* of ethyl-3-4-disubstituted phenyl methylphosphonates.



X	R	pI_{50}	I_{50}
H	H	4.24	5.75×10^{-5}
$-CH_3$	H	4.16	6.92×10^{-5}
Cl	H	5.43	3.71×10^{-6}
Br	H	5.58	2.63×10^{-6}
CH_3O-	H	3.88	1.32×10^{-4}
C_2H_5O-	H	3.97	1.07×10^{-4}
$-CN$	H	7.46	3.47×10^{-8}
CH_3-S-	H	5.89	1.23×10^{-6}
CH_3-SO_2-	H	7.07	8.51×10^{-8}
CH_3-SO_2-	H	8.02	9.55×10^{-9}
$-COOCH_3$	H	7.22	6.02×10^{-8}
Cl	CH_3	6.6	2.51×10^{-7}
$-CN$	CH_3	7.6	2.51×10^{-8}
Cl	C_2H_5	7.0	1.00×10^{-7}
$-CN$	C_2H_5	7.8	1.58×10^{-8}

Experimental error is ±0.05 for the 4-substituted series and ±0.20 for other compounds.

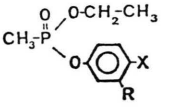
tivity, so *m*-ethyl substituted analogue is more active than the *m*-methyl substituted compound, and this more than a non-substituted one. This behaviour could be interpreted by accepting that the volume of the meta substituent facilitated the complementary between the compound and the enzyme. This effect is also shown by the *p*-substituents as can be observed on comparing substituents of similar electrophylic power and different radius (Cl and Br: CH_3O- and C_2H_5O-).

It has been described [14] that inhibition effect is related to the acyl-transferring ability of a given compound and this coincides with our results, but it has never been suspected that all methylphosphonates are more powerful inhibitors than their phosphate analogues because the methyl radical joined to the acyl-group has a positive inductive effect which does not help the necessary electrophylic capacity of the acyl group. For this reason we should take into account other factors as steric and resonance effects.

The study *in vivo* shows that the compounds with the most electronegative groups in para position, have the highest activities, which is in accordance with the *in vitro* studies, but in this case when the substituents in meta position increase, the activity *in vivo* decreases approximately threefold for the methyl series and sixfold for the ethyl series. All data appear in Table V.

The comparison of both studies indicates that although the activity is the result of the contributions of both substituents, they can act in a different way, since the electrophylic power is more important in the *p*-substituents than in the meta-substituents; on the other hand the size of the group has a major influence in the meta-substituents which is in accordince with the hypothesis of Metcalf that the meta-substituents fit into an enzymic pocket [15]. It is also apparent that the environment of the enzyme is very different in its physiological location than in the *in vitro* test location.

Table V. Biological activity *in vivo* of ethyl-3-4-disubstituted phenylmethylphosphonates.



X	R	LD_{50} [γ/g]	Limits
H	H	586	549–625
$-CH_3$	H	1.239	1174–1308
Cl	H	95	89–101
Br	H	63	58–68
CH_3O	H	260	245–276
C_2H_5O	H	2.458	2261–2672
$-CN$	H	1.83	1.77–2.09
CH_3-S-	H	1.44	1.36–1.53
CH_3-SO-	H	1.62	1.37–1.89
CH_3-SO_2-	H	1.63	1.52–1.75
$-NO_2$	H	1.08	0.94–1.24
H	H	1.006	8.50–1.229
$-CH_3$	$-CH_3$	3.210	2943–3500
$-OCH_3$	$-CH_3$	1.690	1568–1823
Cl	$-CH_3$	162	46–290
Br	$-CH_3$	340	293–397
$-N(CH_3)_2$	$-CH_3$	99	90–110
$-CN$	$-CH_3$	6.90	6.28–7.58
CH_3-S-	$-CH_3$	3.43	3.26–3.6
CH_3-SO-	$-CH_3$	5.41	5.08–5.75
CH_3-SO_2-	$-CH_3$	7.31	6.75–7.92
$-NO_2$	$-C_2H_5$	5.49	498–605
H	$-C_2H_5$	740	673–814
Cl	$-C_2H_5$	256	238–275
Br	$-C_2H_5$	192	10–11
$-CN$	$-C_2H_5$	11	178–207
CH_3-S-	$-C_2H_5$	6.11	5.7–6.5
CH_3-SO-	$-C_2H_5$	15.90	14–16
CH_3-SO_2-	$-C_2H_5$	19.40	17–21
$-NO_2$	$-C_2H_5$	11.80	10–12

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