Acta Chemica Scandinavica B 38 (1984) 641–645

Effect of *para* Substituents of 2-(*N*,*N*-Dimethylamino)ethyl Phenyl Phenylphosphonates on Inhibition of Human Pseudocholinesterase

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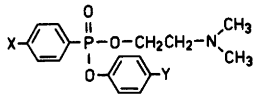
Human serum pseudocholinesterase inhibition by nineteen of the title phosphonates was studied and the effect of *para* substituents of the phenoxy and phenyl groups on inhibition rates was compared. The effect of substituents of the phenoxy group on the reaction rate is in some cases even 60 times as great as the effect of substituents of the phenyl group. Logarithms of rate constants correlate linearly with σ_p^o substituent constants of the phenoxy group, but no linear dependence is observed for *para* substituents of the phenyl group. The role of the apicophilicity of substituents of phosphorus in the enzyme–inhibitor complex is discussed.

This study forms part of a broader investigation into the effects of substituents on the chemical and physical properties of organic phosphorus compounds. Earlier results from NMR and decomposition studies on 2-(*N*,*N*-dimethylamino)ethyl phenyl phenylphosphonates (Scheme 1) have revealed linear changes in chemical shifts and logarithms of decomposition rates plotted against σ_p^o parameters of *para* substituents of the phenyl group,[1,2] but the changes in these quantities are less pronounced and more irregular for *para* substituents of the phenoxy group.



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Scheme 1. 2-(*N*,*N*-Dimethylamino)ethyl phenyl phenylphosphonates, X=Me_2N, MeO, Me, H, F, Br and Y=MeO, Me, H, F, Cl, Br.

0302-4369/84 $2.50

In the present work we have studied the effect of *para* substituents of phenoxy and phenyl groups on inhibition of human serum pseudocholinesterase. The inhibition of cholinesterase enzymes by phosphorus compounds having a phenoxy leaving group has been studied extensively.[3,4,5] Potent inhibitors usually have a good leaving group: for instance, methyl 4-nitrophenyl ethylphosphonate, which has an easily removable 4-nitrophenoxy group, posses an inhibition ability against fly cholinesterase enzymes about 40.000 times as great as its 4-methoxy derivative (measured as I_{50} values).[5]

In studies of the effect of the non-leaving group on the inhibition of cholinesterase enzymes, alkyl or alkoxy groups are often used as substituents of phosphorus. Lengthening of the alkyl group decreases the inhibition ability of phosphorus compounds owing to the increase in steric requirements of the alkyl groups.[6] By contrast, when the alkyl group is attached as *para* substituent to a phenyl group of phenylphosphonate rather than directly to phosphorus, lengthening of the chain seems to enhance the inhibition ability slightly, as shown by Becker *et al.*[6] for 4-nitrophenyl ethyl 4-alkylphenylphosphonates. This effect is probably due to the stronger interaction between the inhibitor and the hydrophobic site or sites on the enzyme.

To avoid the effects of size or hydrophobicity of substituents, we have studied compounds whose structures allow us to suppose that only electronic effects are relevant to the substituent effects, because substituents are distant from the reaction centre, chargeless and small relative to whole molecule (Scheme 1).

RESULTS AND DISCUSSION

As a measure of the inhibition ability of the studied phosphonates against human serum pseudocholinesterase we have used the bimolecular rate constant determined by equation[7]

$$\log\left(100\,\frac{E_t}{E_o}\right) = -\frac{t}{2.303}\,k_i i \qquad (1)$$

where i is the concentration of the inhibitor, t is the time of preincubation of the enzyme and inhibitor before addition of substrate and E_t/E_o is the relative residual activity of an the enzyme after inhibition.

The dependence of the logarithm of the residual activity on concentration of the inhibitor was not linear in the manual method when higher concentration was used (Fig. 1), and therefore the slope of the curve at the beginning of the plot was used for determination of all rate constants.

The Y substituent was found to have a strong effect on the inhibition. Thus the derivative X=MeO, Y=Br is about 200 times as active as the derivative X=MeO, Y=MeO (Table 1).

The effect of X is small and especially the derivatives X=Br possess unexpectedly low reaction rates as evaluated from earlier results of spontaneous decomposition reactions and of NMR studies.[1,2]

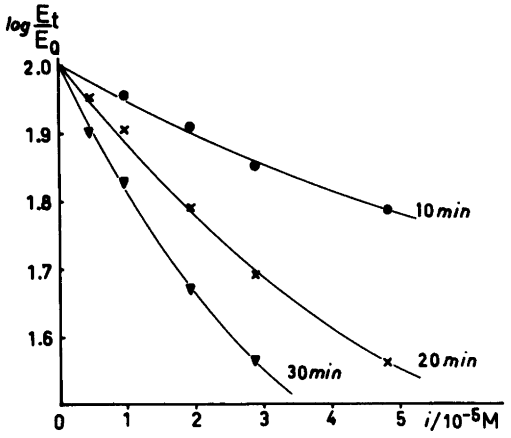


Fig. 1. Dependence of the logarithm of residual activity of inhibition of serum pseudocholinesterase on concentration of the inhibitor (X=Br, Y=H), using different incubation times.

Table 1. The rate constants of the inhibition reaction and their logarithms for substituted phenyl phenylphosphonates (Scheme 1) determined by eqn. (1), using the manual method.

X	Y	$k_i/10^3$ $M^{-1}min^{-1}$	log k_i
Me	MeO	92.9(177)[a]	4.968(83)
	Me	229.7(87)[a]	5.361(16)
	H	57.6(33)	4.760(25)
	Br	1480(74)	6.170(22)
MeO	MeO	6.79(36)	3.832(23)
	Me	12.19(53)	4.086(19)
	H	19.6(12)	4.292(26)
	Br	1385(73)	6.142(23)
H	MeO	34.0(21)	4.532(26)
	Me	32.2(20)	4.508(27)
	H	119.1(45)	5.076(16)
	Br	2084(74)	6.319(15)
Br	MeO	22.3(12)	4.348(24)
	Me	49.6(24)	4.696(21)
	H	120.3(73)	5.080(27)

[a] Samples probably contain the ortho isomer as an impurity.

Plots of log (k_i) against σ_p^o constants of Y in Fig. 2 show an approximately linear dependence when X=MeO, H or Br. Analogous effects of para substituents of a phenoxy leaving group on inhibition of cholinesterase enzymes from different sources have been presented in the literature for several organophosphorus inhibitors.[4,5,8]

A more interesting observation here than the expected effect of Y is the small effect of X. Thus the good leaving group p-bromophenoxy group alone seems to determine the reaction rate and X has no effect at all (Fig. 2h). As the leaving group becomes poorer the effect of X increases but is by no means proportional to the electronic properties of the substituents. The concentration of the studied phosphonates in polar solutions decreases by spontaneous decomposition only slightly when an incubation time of 20 min is used.[2,9] Dependence of the decomposition of dialkyl 2-(*N,N*-dimethyl-amino)ethyl phosphates on solvent polarity, was determined using the Grunwaldt-Winstein equation and this relationship between reaction rates and polarity of the medium was applied for the phenylphosphonate.[2,9]

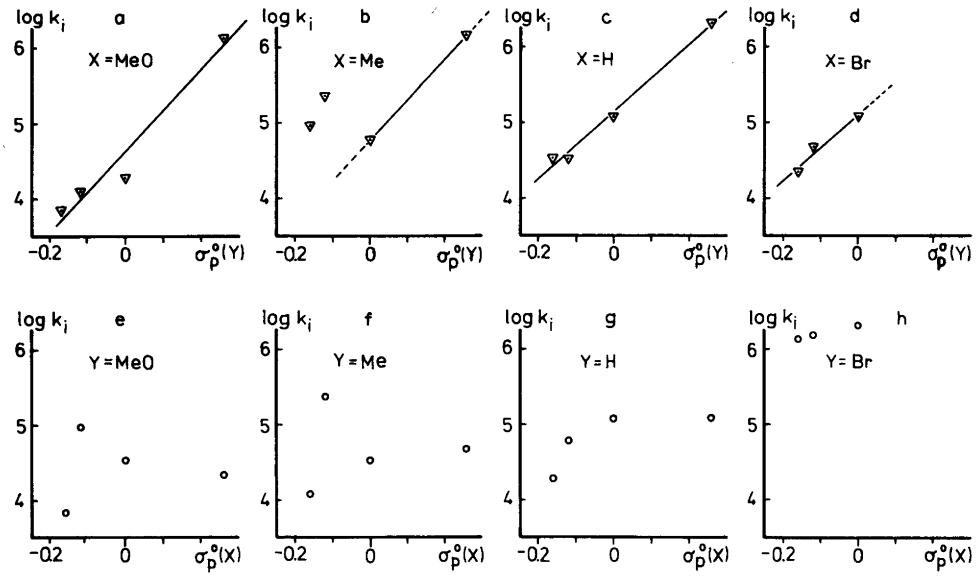


Fig. 2. Relationship between log k_i (Table 1) and the σ_p^o substituent constant for changes of Y (a–d) and changes of X (e–f). Points obtained for X=Me, Y=MeO or Me are neglected in b.

Though the possible catalytic effects of buffer and pH of solution have not been considered, we would conclude that the unexpected and non-regular effect of X is most probably inherent in the inhibition reaction itself.

The rate constants of the derivatives X=Me, Y=Me and X=Me, Y=MeO (Fig. 2b) are abnormally large (Table 1), probably because of the presence of small amounts of the ortho isomer of 4-methylphenylphosphonate, owing to the synthetic route used.[1] When a different preparation pathway was chosen[2] pure *para* isomers were obtained. These were used in all later measurements by the automated method (Table 2).

Table 2. The rate constants of the inhibition reaction and their logarithms for substituted phenyl phenylphosphonates (Scheme 1) determined by eqn. (1) using the automatic method. Respective substituent constants are shown.[10]

X	Y	$k_i/10^3$ $M^{-1}min^{-1}$	log k_i	σ_p^o
Me	MeO	21.1(5)	4.325	−0.16
	Me	42.2(6)	4.626	−0.12
	H	49.7(8)	4.696	0.00
	F	111.5(33)	5.047	0.17
	Cl	778(38)	5.891	0.27
	Br	729(20)	5.863	0.26
Me_2N	Me	13.2(6)	4.121	−0.44
MeO		17.8(4)	4.251	−0.16
Me		42.2(6)	4.626	−0.12
H		54.8(10)	4.739	0.00
F		14.9(4)	4.172	0.17
Br		21.8(4)	4.339	0.26

The effect of X and Y on inhibition rates is seen only roughly from Fig. 2. To obtain a more complete picture of the substituent effects several additional compounds were synthesized and the inhibition abilities for one series with variation of X (Y=Me) and another with variation of Y (X=Me) were measured by an automated method based on a continuous-flow system. The k_i values differed in absolute value from those determined manually, but the two series displayed the same overall effects as before. The small effect of X and especially the low values for X=F and X=Br when Y=Me were again evident (Fig. 3b).

The wider scale of substituents shows that the effect of the electron donating substituents (MeO, Me, H) is only slightly greater for X than for Y, and a significant reduction in inhibition ability occurs only when X is a strongly electron attracting atom (F, Br) (Fig. 3).

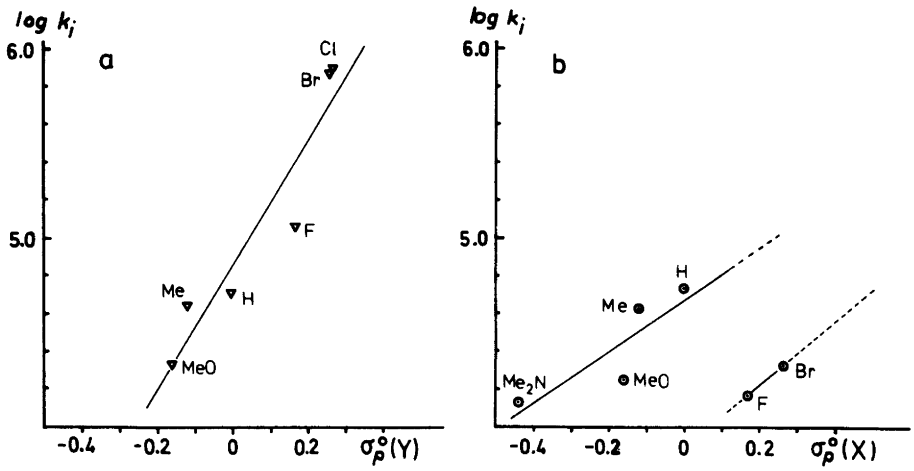
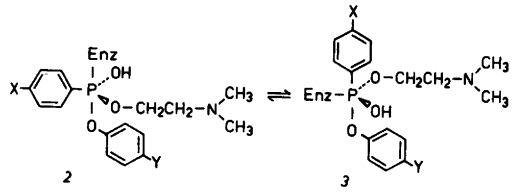


Fig. 3. Effects of substituents on inhibition rates of (a) X=Me, Y varies and (b) X varies, Y=Me. The numerical values are shown in Table 2.

One possible reason for the low inhibition ability of the X=F and X=Br derivatives could be the different stereochemical configuration of the pentacoordinated enzyme-inhibitor complex. The preference of substituents of phosphorus to occupy apical or equatorial positions (apicophilicity *vs.* equatophilicity) depends on the steric and electronic properties of the substituent.[11] Leaving groups tend to occupy apical position, as do other electron attracting and small groups (and atoms). Phenyl group containing the substituent X should then lie in an equatorial position until X becomes electron attracting enough that it can occupy the apical position (Scheme 2). Electron accepting effect of X is now weaker due to a longer bond between the benzene ring and the phosphorus atom and thereby inhibition rates for X=F and X=Br are lowered. The rate of inhibition increases again, however, when changing from fluorine to bromine, meaning that only the level of inhibition has changed and not the tendency to increase with increasing σ_p^o values.



Scheme 2. Pseudorotation of the enzyme-inhibitor complex.

To enable comparisons between different types of cholinesterase enzymes we determined the inhibition ability of a few of the studied compounds against electric eel acetylcholinesterase. To accomplish measurable changes in the enzyme activity, however, concentrations needed to be greater than 10^{-3} M. And since these phosphonates do not dissolve well in water further investigations with this enzyme were not undertaken.

The proposed pseudorotation of the enzyme–inhibitor complex introduced to explain the exceptional results for X=F, Br needs to be tested using electron acceptors as substituents and with other inhibitors, *e.g.* choline derivatives of the present compounds.

EXPERIMENTAL

Preparation of the oxalates of 2-(*N,N*-dimethylamino)ethyl phenyl phenylphosphonates has been described elsewhere.[1,2]

The inhibition of human serum pseudocholinesterase by the title phosphonates was studied by the Ellman method[12] using both manual and automatic analysis techniques.

In the manual method inhibitors were dissolved as oxalates in pure water. The incubation time was 20 min, temperature 22 °C and colour formation time was 3 min. Enzyme activity measurements were carried out with a nine-channel cuvette spectrophotometer (FP-9 Analyzer, Finnpipette).

In the automatic method the sample was dissolved in methanol and then diluted with the appropriate amount of water. Enzyme solution was pumped to the sample flow and after 12 min of incubation time at 37 °C the substrate was pumped to the reaction mixture. The activity of the enzyme was measured after three minutes and recorded (instrument: Automatic Chemical Analysis System (AKEA), Datex).

Enzyme/buffer/colour reagent solution: Human serum pseudocholinesterase was diluted in buffer solution to achieve the final enzyme activity 0.015 U/ml. Phosphate buffer, pH 7.2, 0.052 M. Gelatine 0.1 % (w/v). Sodium chloride 0.1 M. 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) 0.26 M.

Substrate solution: Acetylthiocholine iodide 0.010 M. The surfactant Brij 35 was added in all solutions used in the automatic method.

For determination of k_i-values five or six different inhibitor concentrations and at least two parallel samples of each were used in both methods. In the automatic method the height of peaks *vs.* base lines were used for activity determinations.

REFERENCES

1. Manninen, P. A. *Acta Chem. Scand. B 35* (1981) 13.
2. Manninen, P. A. *Acta Chem. Scand. B 38* (1984) 635.
3. Brestkin, A. P. and Godovikov, N. N. *Russ. Chem. Rev. 47* (1978) 859.
4. Fukuto, T. R. *Residue Rev. 25* (1969) 327.
5. Darlington, W. A., Partos, R. D. and Ratts, K. W. *Toxicol. Appl. Pharmacol. 18* (1971) 542.
6. Becker, E. L., Punte, C. L. and Barbaro, J. F. *Biochem. Pharmac. 13* (1964) 1229.
7. Aldridge, W. N. and Reiner, E. In Ariens, G., Ed., *Frontiers of Biology,* North-Holland, Academic, New York 1971, Vol. 26, p. 161.
8. Remplio, A. M. *Z. Naturforsch. 32* (1977) 760.
9. Manninen, P. A., Sipponen, K. and Zukale, T. *Unpublished results.*
10. Exner, O. In Chapman, N. B. and Shorter, J., Eds., *Advances in Linear Free Energy Relationships,* Plenum, London 1972, p. 1.
11. Emsley, J. and Hall, D. *The Chemistry of Phosphorus,* Harper & Row, London 1976, pp. 65 and 211.
12. Ellman, G. L., Courtney, K. D., Andres, V., Jr. and Featherstone, R. M. *Biochem. Pharmac. 7* (1961) 88.

Received October 20, 1983.