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Synthesis of O,O-dialkyl-S-[benzoyl-(benzylidene amino)] dithiophosphorates and their inhibitory effect on acetylcholinesterase activity

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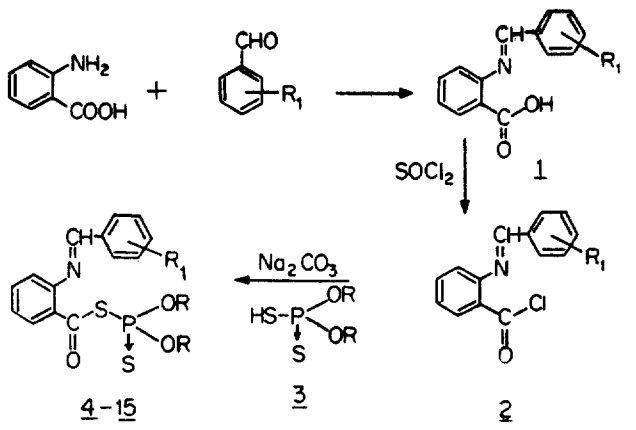
Abstract. A new series of O,O-dialkyl-S-[benzoyl-(*o*/*p*-benzylidene amino)] dithiophosphorates have been synthesised and their anti-acetylcholinesterase activity using rat brain homogenate was investigated. The maximum inhibitory activity was exhibited in compound 29 and the minimum in compound 12. These compounds were also evaluated for their antihookworm activity against *Nippostrongylus brasiliensis* in rats and *Nematospiroids dubius* in mice but none of the compounds showed any activity.

Keywords. O,O-Dialkyl dithiophosphoric acid ; *o*/*p*-benzylidene amino benzoic acid; anti-acetylcholinesterase activity ; antihookworm activity.

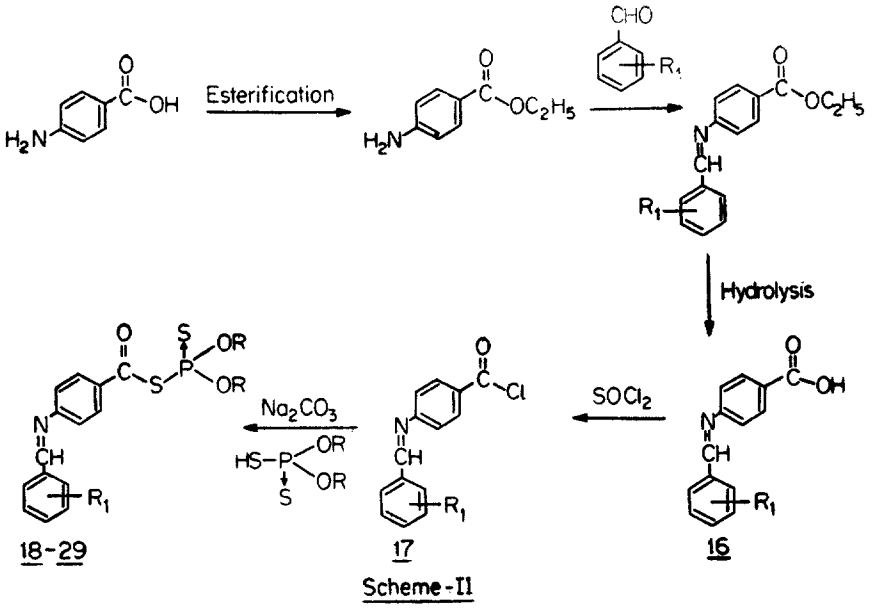
1. Introduction

A large number of organophosphorous compounds are known to possess anthelmintic (Hass 1970), antimicrobial (Jamison 1967), insecticidal (Casida 1956; Trueb and Reisser 1970) and rodenticidal (Krenzer and Richter 1971) activities but a majority of them are toxic and find use in veterinary practice. However, the clinical efficacy of several organophosphorous compounds as acetylcholinesterase inhibitor has opened newer avenues in the medicinal chemistry of organophosphates. Based on these observations it was considered worthwhile to synthesise a series of O,O-dialkyl-S-[benzoyl-(*o*/*p*-benzylidene amino) dithiophosphorates] as potential acetylcholinesterase inhibitors.

The Schiff's bases *o*/*p*-arylidene amino benzoic acid (Ekeley and Dean 1912; Ekeley and Slater 1914; Senior and Forster 1914) were converted into corresponding acid chlorides (2 and 17) by the reaction of thionyl chloride. The O,O-dialkyl-dithiophosphoric acids (3) were prepared by the reaction of phosphorous pentasulfide in anhydrous benzene with appropriate alcohol. Reaction of 2 and 17 with sodium salt of 3 yielded O,O-dialkyl-S-[benzoyl-(*o*/*p*-benzylidene amino)] dithiophosphorates (4–15 and 18–29).



Scheme-I



Scheme-II

2. Biological Assay

2.1. *Enzyme preparation*

Adult rats weighing approximately 150 g were decapitated and the brains were removed quickly, weighed and homogenised in ice cold 0·25 M sucrose solution in a motor-driven teflon-pyrex homogeniser. The final concentration of the crude homogenate was 10% (w/v).

2.2. *Determination of acetylcholinesterase activity*

Acetylcholinesterase activity was determined colorimetrically, with acetylthiocholine as substrate (Parmar *et al* 1961). The reaction mixture in final concentration, consisted of 43 mM Tris buffer, pH 7·4, 350 mM sodium chloride and 0·3 ml of brain homogenate. Acetylthiocholine was used at a final concentration of 1·5 mM. Water was added to adjust the final volume to a total of 2·0 ml. The reaction mixture with or without inhibitor was preincubated at a constant temperature (37° C) for 10 min in the absence of acetylthiocholine. Acetylthiocholine was then added and the reaction was allowed to continue for an additional 10 min at 37° C, with occasional stirring. Trichloroacetic acid (0·5 ml of 25% (w/v)) was then added and the resultant solution was centrifuged for 5 min at 500 g. An aliquot of the clear supernatant liquid was withdrawn and the enzymatically formed thiocholine was determined colorimetrically. Each assay was done in triplicate where tissue and substrate blanks were substracted to give the actual value for the hydrolysis of acetylthiocholine. Results are expressed as change in extinction per 100 mg fresh tissue (Parmar *et al* 1966).

2.3. *Determination of Michaelis constant and the inhibitor constant*

A graphical plot of the enzyme activity against different concentrations of the substrate was made according to the method of Lineweaver and Burk (1934) and Dixon (1953). The inhibitor constant (K_i) of the newly synthesised anti-acetylcholinesterase agents was calculated by the graphic method of Dixon (1953).

2.4. *Determination of anthelmintic activity*

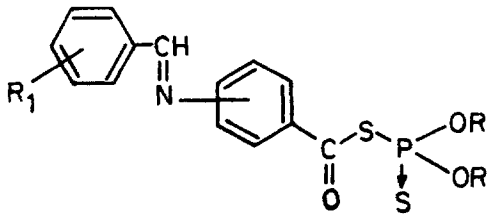
All the compounds were evaluated for their anthelmintic activity against *Nippostrongylus brasiliensis* in rats and *Nematospiroides dubius* in mice by standard methods (Standen 1963; Howes and Lynch 1967).

2.5. *Results and discussion*

The results of the study of inhibition of acetylcholinesterase by the O,O-dialkyl-S-[benzoyl-(*o*/*p*-benzylidene amino)] dithiophosphorates at a final concentration of 1×10^{-3} and their I_{50} values (as determined by the decrease in the hydrolysis of acetylthiocholine by rat brain homogenate) are shown in table 1. It was found that the diethyl, di-*i*-propyl and di-*n*-propyl dithiophosphoric acid esters inhibit the acetylcholinesterase whereas the dimethyl esters were inactive. In general, the O,O-dialkyl-S-[benzoyl-(*o*-benzylidene amino)] dithiophosphorates have weaker inhibitory activity than O,O-dialkyl-S-[benzoyl-(*p*-benzylidene amino)] dithiophosphorates.

In case of O,O-dialkyl-S-[benzoyl-(*p*-benzylidene amino)] dithiophosphorates, the O,O-di-*n*-propyl-S-[benzoyl-(*p*-benzylidene amino)] dithiophosphorates have greater inhibitory activity than O,O-diethyl and O,O-di-*i*-propyl-S-[benzoyl-(*p*-benzylidene amino)] dithiophosphorates. It is difficult at present to suggest a suitable explanation for the weak inhibitory activity observed with O,O-di-*i*-propyl-S-[benzoyl-(*p*-benzylidene amino)] dithiophosphorates. The O, O-di-*n*-propyl-S-{benzoyl-[*p*-(*p'* chlorobenzylidene amino)]} dithiophosphorate show maximum

Table 1. O,O-dialkyl-S-[benzoyl-(*o*/*p*-benzylidene amido)] dithiophosphorates.



Sl. No.	*R*	*R*[a]	m.p. ° C	Molecular formula[b]	Yield (%)	Anti-acetylcholinesterase activity[c]	
						Inhibition(%)	I_{50} 1×10^{-4} M
4.	CH_3	*p*-OH	234	$C_{16}H_{16}NO_4PS_2$	60	Nil	..
5.	CH_3	*p*-Cl	108	$C_{16}H_{15}ClNO_3PS_2$	70	Nil	..
6.	CH_3	*p*-CH_3CONH	175	$C_{18}H_{19}N_2O_4PS_2$	72	Nil	..
7.	CH_3	*p* $(CH_3)_2$ N	145	$C_{18}H_{21}N_2O_3PS_2$	76	Nil	..
8.	C_2H_5	*p*-OH	95	$C_{17}H_{18}NO_4PS_2$	55	20·0	9·24
9.	C_2H_5	*p*-Cl	100	$C_{17}H_{17}ClNO_3PS_2$	70	50·8	3·0
10.	C_2H_5	*p*-CH_3CONH	200°d	$C_{19}H_{21}N_2O_4PS_2$	65	36·0	4·5
11.	C_2H_5	*p*-$(CH_3)_2N$	180	$C_{19}H_{23}N_2O_3PS_2$	72	25·0	7·2
12.	*i*-C_3H_7	*p*-OH	140	$C_{18}H_{20}NO_4PS_2$	75	18·7	10·0
13.	*i*-C_3H_7	*p*-Cl	116	$C_{18}H_{19}ClNO_3PS_2$	78	29·2	6·0
14.	*i*-C_3H_7	*p*-CH_3CONH	185	$C_{20}H_{23}N_2O_4PS_2$	68	22·0	8·4
15.	*i*-C_3H_7	*p*-$(CH_3)_2$ N	148	$C_{20}H_{25}N_2O_3PS_2$	63	22·5	7·6
18.	CH_3	*p*-OH	155	$C_{16}H_{16}NO_4PS_2$	55	Nil	..
19.	CH_3	*p*-Cl	165	$C_{16}H_{15}ClNO_3PS_2$	60	Nil	..
20.	CH_3	*p*-CH_3CONH	236	$C_{18}H_{19}N_2O_4PS_2$	62	Nil	..
21.	CH_3	*p*-$(CH_3)_2$ N	110	$C_{18}H_{21}N_2O_3PS_2$	60	Nil	..
22.	C_2H_5	*p*-OH	135	$C_{17}H_{18}NO_4PS_2$	53	55·0	2·80
23.	C_2H_5	*p*-Cl	116	$C_{17}H_{17}ClNO_3PS_2$	65	60·5	2·28
24.	C_2H_5	*p*-CH_3CONH	130	$C_{19}H_{21}N_2O_4PS_2$	68	50·0	3·0
25.	C_2H_5	*p*-$(CH_3)_2N$	150	$C_{19}H_{23}N_2O_3PS_2$	64	45·0	3·4
26.	*i*-C_3H_7	*p*-OH	168	$C_{18}H_{20}NO_4PS_2$	70	28·0	6·3
27.	*i*-C_3H_7	*p*-Cl	188	$C_{18}H_{19}ClNO_3PS_2$	68	35·2	4·8
28.	*n*-C_3H_7	*p*-OH	150	$C_{18}H_{20}NO_4PS_2$	60	63·5	2·20
29.	*n*-C_3H_7	*p*-Cl	172	$C_{18}H_{19}ClNO_3PS_2$	54	80·0	1·54

[a] In compound 4–15 the benzylidene amino group is at *o*-position while in compounds 18–20 it is at *p*-position.

[b] All the compounds were analysed for C, H and N were found within the range of ±·4%.

[c] The enzyme activity was determined by measuring the change in extinction per 100 mg wet tissue weight during hydrolysis of acetylthiocholine for 10 min. The % inhibition was calculated on the basis of decrease in enzyme activity, using esters at a final concentration of 1×10^{-3} M. The I_{50} values indicate the concentration required to produce 50% inhibition. Eserine was used as standard acetylcholinesterase inhibitor. The I_{50} values for eserine was $5{\cdot}49 \times 10^{-7}$ M under similar experimental conditions.

enzyme inhibition which reduces selectively by the introduction of *p*-hydroxy group in place of *p*-chloro group.

A similar trend of enzyme inhibition was observed in O,O-dialkyl-S-[benzoyl-(*o*-benzylidene amino)] dithiophosphorates.

The nature of the enzyme inhibition of the synthesised O,O-dialkyl-S-[benzoyl-(*o*/*p*-benzylidene amino)] dithiophosphorates was evaluated by the conventional reciprocal plots and are shown in figure 1 for compound 15 and in figure 2 for compound 29. From the intercept at the $1/[S]$ axis a K_m (Michaelis constant) of $3{\cdot}3 \times 10^{-3}$ M was obtained for rat brain homogenate, with acetylthiocholine as substrate. Furthermore, in plots of $1/[v]$ with respect to inhibitor concentration $[I]$ at different substrate concentrations, the intercept is at a point where $I = K_i$ (inhibitor constant). The K_i values for some of the compounds were determined graphically and summarised in table 2. As indicated in figure 1 truly non-competitive nature of inactivation was found with compound 15. Recently the tertiary mono and diquaternary diethyl phosphoryl and dimethyl carbamyl derivatives of the quinolinols and isoquinolinols were synthesised and the irreversible non-competitive nature of enzyme inhibition was indicated (Ginsburg *et al* 1966; Kitz *et al* 1967). The inhibition produced by the compound 29 was of a similar nature. These compounds were also called as acid-transferring inhibitors indicating that the mechanism of inhibition involves the formation of a covalent

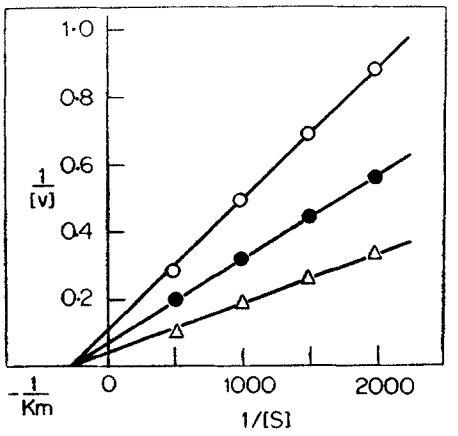


Figure 1

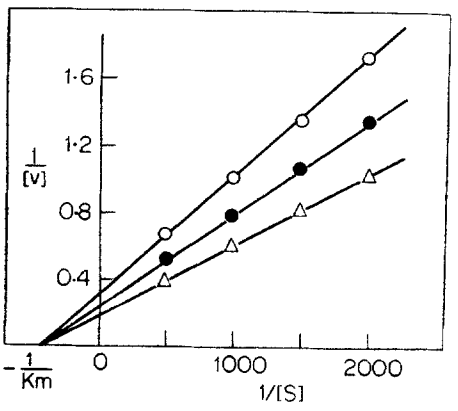


Figure 2

Figure 1. Kinetic study showing non-competitive inhibition of rat brain acetylcholinesterase by compound 15, table 1. All reactions were carried out as described in the text $1/v$ denotes reciprocal of $\Delta E/100$ mg fresh tissue/10 min ; $[S]$ denotes molar concentration of acetylthiocholine ; Δ = control ; ● = 1×10^{-3} M and ○ = 2×10^{-3} M of the inhibitor (compound 15). Km = $3{\cdot}3 \times 10^{-3}$M.

Figure 2. Non-competitive inhibition of acetyl-cholinesterase by compound 29, Table 1. $[S]$ denotes molar concentration of acetylthiocholine and $1/v$, reciprocal of $\Delta E/100$ mg fresh tissue/10 min. Km = $3{\cdot}3 \times 10^{-3}$ M ; Δ = control ; ● = $1{\cdot}5 \times 10^{-1}$ M and ○ = 3×10^{-1} M concentrations, respectively of compound 29.

Table 2. Inhibitor constant (K_i) of O, O-dialkyl-S-[Benzoyl-(*o*/*p*-benzylidene amino)] dithiophosphorates.

Compound No.	Inhibitor constant (K_i) $\times 10^{-3}$ M
8	2·55
11	2·70
12	2·50
13	2·80
14	2·70
15	2·72
26	2·60

Assay procedure and the components of the reaction are described in the text. In all these experiments Michaelis constant was 3.2×10^{-3}. Compound numbers as in table 1.

enzyme derivative by the transfer of the inhibitor's acid groups to the enzyme active site (Alexander *et al* 1963).

It has also been demonstrated that the inhibition of acetylcholinesterase by organophosphates is believed to depend on the affinity of the compound for the active site of esterase; this has given rise to the hypothesis that inhibitory power depends, at least in part, on the electron density in the region of phosphorous atom (O'Brien 1960). In the studies described, evidence is presented for an increased inhibition of acetylcholinesterase depending on the ester groups attached to the phosphorous atom.

All the compounds listed in table 1 were evaluated for their anthelmintic activity against *Nippostrogylus brasiliensis* in rats and *Nematospiroides dubis* in mice but none showed any activity upto an oral dose of 500 mg/kg.

3. Experimental

Melting points were recorded in an open capillary tube and are uncorrected. The IR spectra of the compounds were taken in a Perking-Elmer 137 and 177 spectrophotometers in KBr pellets and the mass spectra of the compounds were taken on JEOL JMS-D-300 instrument.

3.1. *Synthesis of o/p benzylideneaminobenzoic acid*

The aminobenzoic acid has been prepared according to the method of Ekeley and Dean (1912) and Ekeley and Slater (1914). The condensed products showed IR stretching absorption band in the region of $1660 \sim 1640\ cm^{-1}$ due to the presence of $-C=N-$. They were converted into corresponding acid chlorides by the reaction of thionyl chloride.

3.2. *Synthesis of* O,O-*dialkyldithiophosphoric acid*

O,O-Dialkyldithiophosphoric acid were prepared according to the known method (Kabachnik and Mastryukova 1954).

3.3. *Synthesis of* O,O-*dimethyl-S-*{*benzoyl-*[*o-*(*p'-chlorobenzylidene amino*)]} *dithiophosphorates* (5)

O,O-Dimethyl dithiophosphoric acid (4 g, 0·025 mol) was gradually added with stirring to a suspension of anhydrous sodium carbonate (2·7 g, 0·025 mol) in 200 ml of benzene. The *o* (*p'*-chlorobenzylidene amino) benzoyl chloride (6·90 g, 0·05 mol) was then added and the mixture was refluxed at 80° C for 18 hr. The mixture was cooled, sodium carbonate and sodium chloride were removed by filtration and benzene was distilled from filtrate under reduced pressure The solid mass which separated was collected by filtration and washed with water The crude ester thus obtained was recrystallised from alcohol, m.p. 108° C. Analysis : Found : C, 47·98; H, 3·50; N 3·00, $C_{16}H_{15}ClNO_3PS_2$. Calc. : C, 48·06; H 3·75; N, 3·50; I.R. (KBr) ν_{max} cm^{-1} : 700($>\overset{|}{p}=S$) 650 ($-C-S-$) and 1600 ($-N=CH-$), Mass : M^+ at m/e = 399.

The other compounds of the series were prepared in a similar manner. The results are listed in table 1.

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