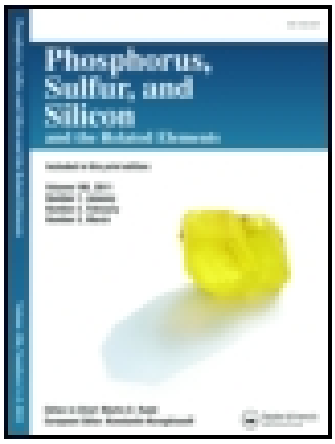

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A CONVENIENT METHOD FOR THE SYNTHESIS OF PHOSPHOROTHIOATES AND THEIR ANTICHOLINESTERASE ACTIVITIES

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A CONVENIENT METHOD FOR THE SYNTHESIS OF PHOSPHOROTHIOATES AND THEIR ANTICHOLINESTERASE ACTIVITIES

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A simple, efficient, and general method has been developed for the synthesis of phosphorothioates through a one-pot reaction of alkyl halides with the mixture of diethyl phosphite in the presence of $NH_4OAc/S/CaO$ under solvent–free conditions. The anticholinesterase activities of eight different phosphorothioates were investigated on acetylcholinesterase from electric eel.

Keywords: Ammonium acetate; anticholinesterase activity; calcium oxide; phosphorothioate; sulfur

Phosphorothioates have found a wide range of application in the areas of industrial, agricultural, and medicinal chemistry owing to their biological and physical properties as well as their utility as synthetic intermediates.[1] One of the useful properties of phosphorothioate compounds is their relatively low stability and rapid metabolic breakdown in plants, in animal organism, in soil, and in other components of the environment with the formation of products that are safe for human beings and domestic animals. Phosphorothioates have been prepared as pesticides and thio-analogues of biologically active phosphoric mono- and diesters.[2] In recent years a number of phosphorothioates have been introduced as potential chemotherapeutic agent[3,4] and inhibitors of different enzymes.[5] Despite their wide range of pharmacological activity, industrial and synthetic applications, the synthesis of phosphorothioates has received little attention. The following methods,

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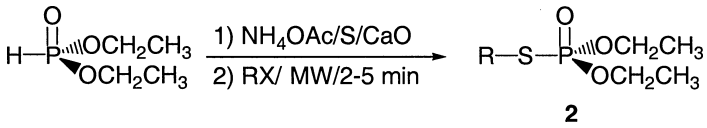
not generally applicable, have been reported in the literature: (i) reaction of dialkyl phosphites with sulfenyl chlorides,[6] sulfenyl cyanides,[7] thiosulfonates,[8] disulfides,[9] and sulfur,[10] (ii) condensation of phosphorchloridate with thiols,[11] and (iii) treatment of phosphites with thiols in the presence of tellurium chloride in a redox-type reaction. However, all of these methods have problems, including drastic reaction conditions and also some severe side reactions. In recent years the use of reagents and catalysts immobilised on solid supports has received considerable attention. Such reagents not only simplify purification processes but also help prevent release of reaction residues into the environment.[12] We recently reported a novel synthetic preparation of phosphorothioates by reaction of diethyl phosphite with alkyl halides in the presence of a mixture of ammonium acetate/sulfur/alumina under solvent-free conditions using microwave irradiation.[13] This methodology prompted us to try the use of other metal oxides in place of the acidic alumina in the above reaction for the preparation of phosphorothioates. We found that replacing of alumina with calcium oxide is a convenient solid media that does not need microwave irradiation in first step of above reaction. We here report that diethyl phosphite in the presence of ammonium acetate/sulfur/calcium oxide under solvent–free conditions is a convenient reagent for the preparation of phosphorothioates (Scheme 1). The anticholinesterase activities of eight different phosphorothioates were investigated on acetylcholinesterase from electric eel.



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SCHEME 1

As shown in Table I, a wide range of alkyl halides in the presence of a mixture of ammonium acetate/sulfur/calcium oxide and diethyl phosphite, gives the required products 2 in excellent yields. The reactions were clean with no tar formation. Ammonium formate (NH_4O_2CH) is not as effective as ammonium acetate and gave low yields of the required product. Other ammonium salts (NH_4Cl, NH_4Br, NH_4PF_6) are not effective and did not give any product.

In summary, a simple work-up, low consumption of solvent, fast reaction rates, mild reaction condition, good yields, relatively clean with no tar formation in the course of the reaction make this method an attractive and a useful contribution to present methodologies. Indeed, a

TABLE I Reaction of Alkyl Halides in the Presence of a Mixture of Ammonium Acetate/Sulfur/Calcium Oxide and diethyl Phosphite, under Solvent-Free Conditions Using Microwave Irradiation

2	R	X	Reaction time (min)	Yield %[a]
a	$PhCH_2$—	Br	2	80
a	$PhCH_2$—	Cl	2	83
b	Ph_2CH—	Br	3	73
c	$PhCH_2CH_2$—	Br	2	65
d	*p*-$NO_2C_6H_4CH_2$—	Br	2	80
e	*p*-$MeC_6H_4CH_2$—	Cl	2	78
f	*o*-$MeC_6H_4CH_2$—	Cl	5	76
g	*m*-$MeC_6H_4CH_2$—	Cl	3	60
h	1-$C_{10}H_7CH_2$—	Cl	2	73

[a]Isolated yields.

wide range of alkyl halides was converted to the corresponding phosphorothioates using this method.

ANTICHOLINESTEREASE PROPERTIES

Acetylcholinesterase is the most widely used target enzyme in studies with the purpose to synthesize new and more effective therapeutic agents to treat patients with diseases such as Alzheimer's disease or myasthenia gravis.[14–16] Also, acetylcholinesterase is the most widely used target in the studies of compounds with insecticidal activities, as all commercial organophosphate compounds are believed to exert their effects through inhibition of the enzyme.[2b] Assays showed that all compounds inhibited the enzyme to different degrees and these results enhanced the importance of the introduced protocol for the synthesis of new phosphorothioates.

Results of the anticholinesterase assay of eight phosphorothioate derivatives are summarized in Table II. The range of inhibitory activities varies from 30.2–77.5% for **2a–2h**. The results show that compounds containing two aromatic rings in their structure (**2b** and **2h**) have greatest inhibition in comparison with other compounds listed in Table I. This occurs through favorable binding of these compounds with the active site residues of the enzyme through π-π interactions.[17] Other derivatives of phosphorothioates displayed different levels of inhibition of acetylcholinesterase at the same concentrations. The minimum inhibitory property was recorded with compound **2f** having a nitro group

TABLE II Anticholinesterase Activity of Some Phosphorothioates **2a–h**

2	R	Activity remained (%)[a]	Inhibition (%)[b]
a	$PhCH_2$—	62.6	37.4
b	Ph_2CH—	22.5	77.5
c	$PhCH_2CH_2$—	65.6	34.4
d	*p*-$NO_2C_6H_4CH_2$—	69.8	30.2
e	*p*-$MeC_6H_4CH_2$—	52.3	47.7
f	*o*-$MeC_6H_4CH_2$—	55.5	44.5
g	*m*-$MeC_6H_4CH_2$—	58.5	41.5
h	1-$C_{10}H_7CH_2$—	41.4	58.6

[a]The activities were compared to the activity of a control and expressed as percent inhibition.

[b]Results shown are averages of triplicate experiments.

in the *para* position of the aromatic ring (30.2%). Other compounds inhibit the enzyme in ranges between compounds having two aromatic ring structures and compound **2d** having one aromatic ring with a *para* nitro group on it.

DATA CONCLUSIONS

With the use of the proposed method, we have synthesized phosphorothioate compounds shown to have inhibitory effects on acetylcholinesterase. It seems that hydrophobic π-π interactions play the central role in showing the highest inhibitory activities of the compounds synthesized in this study.

EXPERMENTAL

All chemicals were commercial products and distilled or recrystallized before use. Acetylcholinesterase from electric eel (EC 3.1.1.7), actetylcholine chloride and gelatin were from Sigma and were used without further treatment. A commercially available pulse microwave at 2450 MHz (900 W) was used in all experiments. The infrared (IR) spectra were determined using a FT-IR Brucker-Vector 22. NMR spectra were taken with a DMX-500 Bruker Advance instrument with the chemical shifts being reported as δ ppm and couplings expressed in Hertz. Silica gel column chromatography was carried out with Silica gel 100 (Merck No. 10184). Merck Silica-gel 60 F254 plates (No. 5744) were used for the preparative TLC. Acetylcholinesterase activity was

measured titrimetrically[18] using an automatic dispenser (765 Dosimat, Metrohm), a pH meter (713 Metrohm) and at controlled temperature (25°C), by the use of a high-precision temperature-controlled water pump (Multi Temp III Pharmacia).

General Procedure for the Synthesis of Phosphorothioates

This solvent-free reaction method is operationally simple. 10 mmol of the reagent were prepared by the combination of ammonium acetate (10 mmol, finely ground), sulfur (10 mmol) and calcium oxide (CaO, 2.5 g) in a mortar and pestle by grinding them together until a fine, homogeneous, powder was obtained (5–10 min). Diethyl phosphite (10 mmol) was added to this mixture and the mixture was shaken for 10 min. The alkyl halide (9 mmol) was added to this reagent (solid alkyl halides need to be grinded) and was irradiated by microwave for 2–5 min using 600 W (A kitchen-type microwave was used in all experiments). Chromatography through a plug of silica gel with EtOAc/*n*-hexane (1:9 to 5:5) and evaporation of the solvent under reduced pressure gave the pure products as oils in 60–83% yields. All products gave satisfactory spectral data in accord with the assigned structures. For **2a** (*S*-Benzyl *O*, *O*-Diethyl Phosphorothioate) as an example ^{1}H-NMR ($CDCl_3$/TMS-500 MHz): 1.31 (6H, t, $J = 7.1$ Hz), 4.01–4.25 (6H, m), 7.27–7.29 (5H, m); ^{31}P-NMR ($CDCl_3$/H_3PO_4-85%): 27.05 ppm; ^{13}C-NMR: 16.39, 35.4, 63.9, 128.0, 129.1, 137.9; IR (neat): 1260 (P═O), 1162(P—O—Et) cm^{-1}.

ANTICHOLINESTEREASE ACTIVITIES

Acetylcholinesterease activity was measured titrimetrically.[18] The assay was carried out in potassium phosphate buffer (pH 7.0). A solution of 7.5 mL of acetylcholine chloride (0.005 M), 7.5 mL of salt-gelatin mixture [containing sodium chloride (0.2 M) and magnesium chloride (0.04 M) in aqueous 0.01% gelatin] was added to a dual wall cell at 25°C. This solution was stirred continuously to ensure complete mixing and thermal exchange. After this step, the enzyme (100 μL, 7.7×10^{-9} M) was added to the mixture. In order to titrate the acetic acid produced by enzymatic reaction, NaOH (0.15 M) was added in micro liter amounts with Dosimat to maintain the pH of the mixture at 7.0 for 5 min. Activities were then measured according to the following formula:

Units/mg = [mL base added/min × base molarity × 1000]/[mg enzyme in reaction mixture]

Phosphorothioates were dissolved in methanol and then added as the final ingredients before addition of the enzyme. The maximum concentration of methanol in the reaction mixture was 1%.

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