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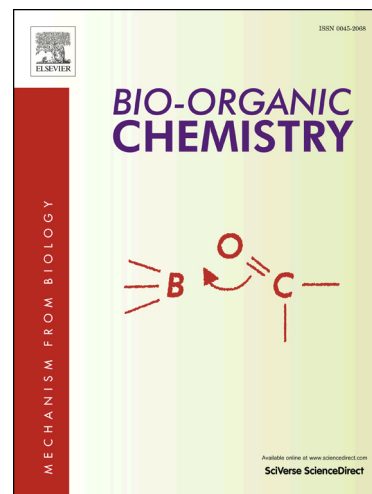
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Efficient synthesis of novel dialkyl-3-cyanopropylphosphate derivatives and evaluation of their anticholinesterase activity

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Abstract:

Based on the broad spectrum of biological activities associated with organophosphates, a novel type of this class of compounds was synthesized, bearing a nitrile group, from the sodium alkoxide-catalyzed reaction of dialkylphosphites with γ -ketonitriles at 80 °C under solvent-free conditions. A reaction mechanism involving a phospho-Brook type rearrangement is proposed. Eight title compounds were investigated for their *in vitro* inhibitory potency and selectivity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) using Ellman's spectrophotometric method. The synthesized derivatives exhibited mostly a moderate activity against both cholinesterases. The IC₅₀ values for BChE were in a smaller concentration range (5.96–23.35 μ M) compared to those for AChE inhibition (9.61–53.74 μ M). The diethyl-3-cyano-1-*p*-tolylpropylphosphate which displayed the higher dual inhibitory potency towards both cholinesterases could be considered as a potential candidate for developing new drugs to treat Alzheimer's disease.

Keywords: Organophosphates; γ -Ketonitriles; Dialkyl-3-cyanopropylphosphates; Phospho-Brook rearrangement; Cholinesterase inhibitors.

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1. Introduction

Alzheimer's disease, the most common form of neurodegenerative senile dementia, is associated with the selective loss of cholinergic neurons and a reduced level of the acetylcholine (ACh) neurotransmitter. The cholinergic hypothesis postulates that Alzheimer's disease (AD) is caused by a decrease in ACh levels in the brain, leading to gradual neurodegeneration [1]. In normal brain signaling, ACh is related to the preservation and access to memory as well as function [1]. Acetylcholinesterase (AChE), a drug target for Alzheimer's disease, is an enzyme that belongs to the very large serine hydrolase class of enzymes [2]. AChE catalyzes the hydrolysis of ACh to choline and acetic acid which allows the control of the ACh level and to regulate its action as a cholinergic neurotransmitter interacting with postsynaptic cell receptors. This enzyme is widely distributed throughout the body and is the main cholinesterase in the human brain [3,4]. Additionally, Butyrylcholinesterase (BChE) plays a secondary role in the regulation of the ACh level and compensates for the lack of AChE, allowing the cholinergic neurotransmission to continue [5-7].

Inhibitors of ChE have been used in the treatment of various diseases such as Alzheimer's, myasthenia gravis and some other dementias [8], parasitic infections [9], glaucoma and obstipation [10].

Organophosphates are well-known anticholinesterase agents [11,12]. They cause irreversible inhibition of both AChE and BChE by phosphorylation of the active site catalytic serine hydroxyl group [13], thus inhibiting its physiologic action of hydrolyzing the ACh neurotransmitter at the central and peripheral synapses [8, 14]. This results in ACh accumulation and in thus an over-stimulation of cholinergic receptors [15]. For instance, salicylanilide diethyl phosphates were found to be excellent inhibitors of both AChE and BChE with IC_{50} values in the micromolar range, and in some cases they exhibited even better inhibitory activity than the current AD medications Rivastigmine and Galantamine [11,16].

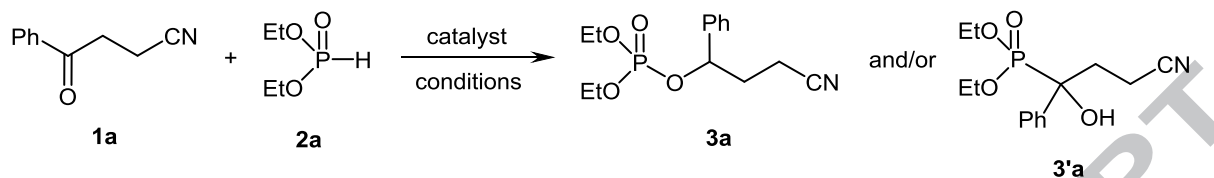
In the present study, we report a simple synthesis of the novel dialkyl-3-cyanopropylphosphates starting from dialkyl phosphites and γ -ketonitriles. A preliminary evaluation of the inhibitory activities of AChE and BChE is also reported for eight dialkyl-3-cyanopropylphosphate derivatives. It should be noted that the presence of the nitrile unit in these molecules might regulate important biological functions and could improve the biological

activity of such compounds by accentuating the binding to receptor targets, in a similar way to that reported for other pharmaceuticals [17].

2. Results and discussion

2.1. Chemistry

We first aimed at optimizing the reaction conditions for the formation of the dialkyl-3-cyanopropylphosphate target compounds, by using γ -ketonitrile **1a** and diethyl phosphite as model substrates. The reaction was studied with various basic catalysts under different conditions. The results of these comparative experiments are summarized in Table 1. Inspired by our previous work regarding the synthesis of γ -cyano- α -hydroxyphosphonates *via* the reaction of γ -ketonitriles with dialkyl phosphites on magnesia support at room temperature and in solvent-free conditions [18], we thought that conducting the same reaction at a higher temperature may lead, after a phospho-Brook type rearrangement [19] promoted by heat, to the corresponding dialkyl-3-cyanopropylphosphate **3a**. Contrary to our expectations, the reaction did not yield the desired product but gave the corresponding α -hydroxyphosphonate **3'a** with less than 30% yields (Table 1, entries 1 and 2). The use of K_2CO_3 and amines as basic catalysts was also tested, but this left the starting materials intact even after prolonged heating (Table 1, entries 3-7). An improvement in the yield of product **3a** was however observed when employing sodium ethoxide as catalyst under solvent-free conditions (Table 1, entry 9). Finally, under the same reaction conditions and by increasing the amount of diethyl phosphite up to 5 molar equivalents, it was gratifying to obtain the desired product **3a** with 85% yield (Table 1, entry 10).

Table 1. Optimization of the reaction conditions.

Entry	Catalyst	Ratio (1/2/catalyst)	Solvent	Temp. (°C)	Time (h)	Yield ^a
1	MgO	1/2/2	-	80	48	26% 3'a
2	MgO	1/2/2	-	140	48	15% 3'a
3	K ₂ CO ₃	1/2/1	-	80	48	ND ^b
4	Et ₂ NH	1/2/0.2	toluene	111	48	ND
5	Et ₂ NH	1/2/0.2	-	80	48	ND
6	Et ₃ N	1/2/0.2	-	80	48	ND
7	piperazine	1/2/0.2	-	80	48	ND
8	EtONa	1/2/0.2	ethanol	78	48	ND
9	EtONa	1/2/0.2	-	80	48	32% 3a
10	EtONa	1/5/0.2	-	80	24	85% 3a

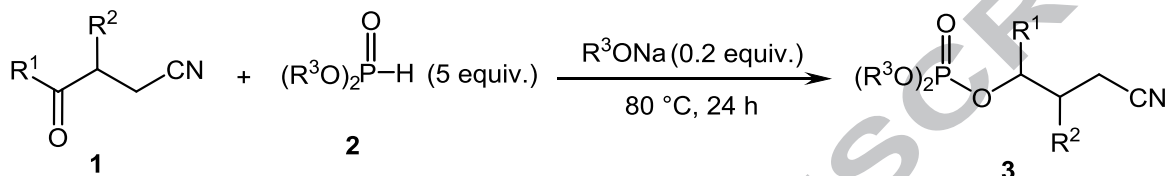
^a Isolated yield.^b No products detected.

Once the optimized reaction conditions were obtained, the scope of this methodology was studied. A variety of structurally diverse γ -ketonitriles and dialkyl phosphites were investigated and a series of dialkyl-3-cyanopropylphosphates of type **3** were obtained in good yields (Table 2). Compounds **3g** and **3h**, presenting two stereocenters, were obtained as a mixture of two unseparable diastereoisomers in an approximate 3:2 ratio, as evidenced by their NMR spectral data. The relative proportions of these diastereoisomers were estimated from the ³¹P NMR spectra where a singlet for each one was found (see experimental section).

A mechanistic rationalization of this reaction is depicted in Scheme 1. In this proposed mechanism, the role of sodium alkoxide is to promote the deprotonation of dialkyl H-phosphonate **2** giving rise to the more nucleophilic dialkylphosphite anion which can react with the carbonyl of the γ -ketonitrile **1** to supply an α -hydroxyphosphonate **I₂** as intermediate, in equilibrium with the alkoxide form **I₁**. When heating, the latter undergoes a phospho-Brook type

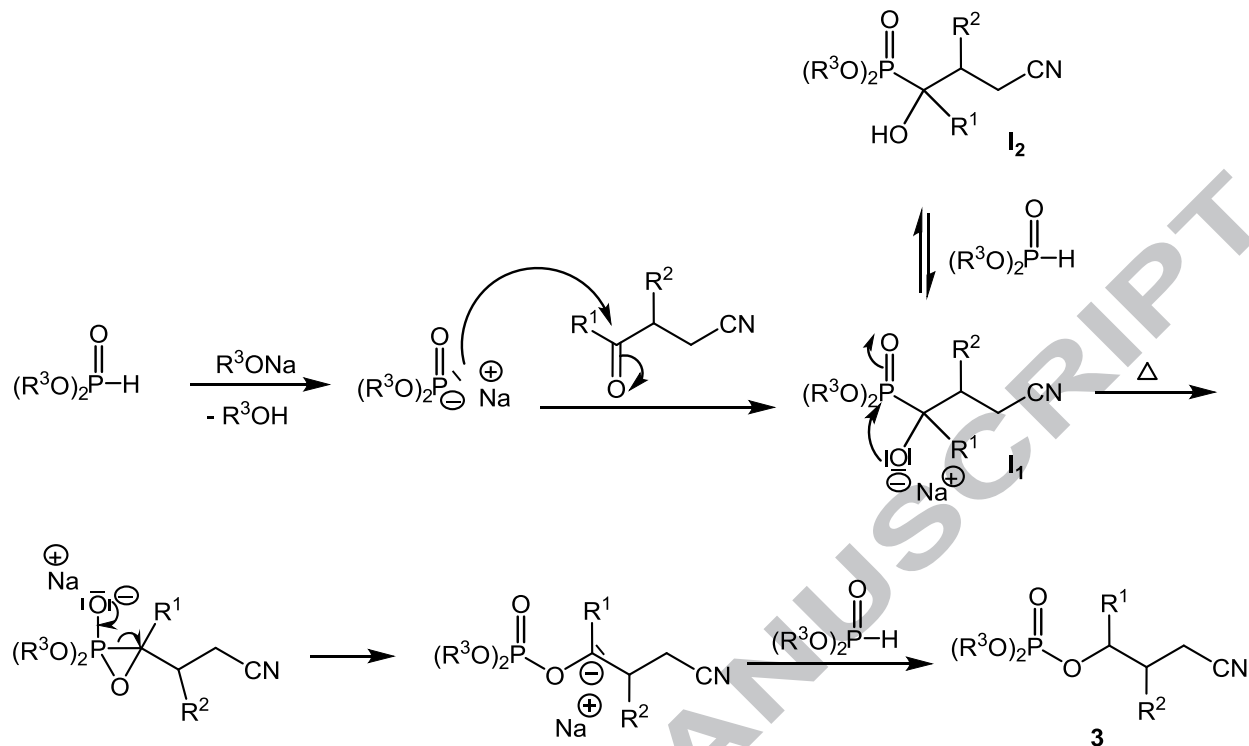
rearrangement [19] that consists in an intramolecular 1,2-migration of the phosphoryl group from carbon to oxygen. This includes the formation of a three-membered cyclic species from intermediate **I**₁. Subsequent ring opening and irreversible fast protonation of the carbanion by the dialkyl phosphite, lead to the final dialkyl-3-cyanopropylphosphate **3**.

Table 2. Substrate scope studies.



Entry	R ¹	R ²	R ³	Product	Yield (%) ^a
1	Ph	H	Et	3a	85
2	Ph	H	Me	3b	83
3	<i>p</i> -Me-C ₆ H ₄	H	Et	3c	80
4	<i>p</i> -Me-C ₆ H ₄	H	Me	3d	81
5	<i>p</i> -MeO-C ₆ H ₄	H	Et	3e	76
6	<i>p</i> -MeO-C ₆ H ₄	H	Me	3f	78
7	Ph	Me	Et	3g	72
8	Ph	Me	Me	3h	75

^a Isolated yield



Scheme 1. Proposed mechanism for the formation of dialkyl-3-cyanopropylphosphates **3**.

2.2. Anticholinesterase activity

The inhibitory potency of the eight newly synthesized dialkyl-3-cyanopropylphosphates against AChE and BChE were evaluated *in vitro* using modified Ellman's method [20]. The efficiency of these inhibitors is expressed as IC₅₀, representing the concentration of an inhibitor leading to 50% inhibition of the enzyme. The obtained results were compared with Rivastigmine, an acylating pseudo-irreversible carbamate inhibitor that is able to inhibit both AChE and BChE.

With respect to the inhibition of cholinesterases, all tested dialkyl-3-cyanopropylphosphates exhibited a significant inhibitory power towards AChE and BChE, with IC₅₀ values ranging from 5.96 to 53.74 μM (Table 3). In addition, and as awaited from previous studies with phosphate inhibitors [11,13b], compounds **3a-h** showed greater inhibition of both enzymes than Rivastigmine. This result is probably related to the chemical structure of the **3a-h** molecules which contains a good leaving group (-O-CH(R¹)-CH(R²)-CH₂-CN) enabling them to perform the phosphorylation of the nucleophilic serine hydroxyl group in the active site of the ChE enzymes. In addition, the cyano group present in these molecules could contribute to this

activity by its electron-withdrawing inductive effect which increases the leaving group ability of the 3-cyanopropoxy moiety.

Moreover, in agreement with previous studies showing that organophosphorus molecules behave more selectively towards BChE [11,21,22], we also demonstrated that our dialkyl-3-cyanopropylphosphates **3a-h** inhibited BChE more effectively than AChE, by 1.43- to 2.59-fold for **3h** and **3e**, respectively. Only compound **3d** exhibited comparable IC₅₀s for both enzymes.

Among the eight tested compounds, the most potent inhibitor of AChE was found to be the diethyl-3-cyano-1-*p*-tolylpropylphosphate (**3c**) with an IC₅₀ value of 9.61 μM, which is also the best inhibitor of BChE (IC₅₀ = 5.96 μM). In contrast, the diethyl-3-cyano-1-(4-methoxyphenyl)propylphosphate (**3e**) was the weakest inhibitor of AChE (IC₅₀ = 53.74 μM); while the dimethyl-3-cyano-1-(4-methoxyphenyl)propylphosphate **3f** (IC₅₀ = 23.35 μM) was assayed as the least effective inhibitor of BChE.

Despite the low number of compounds tested, some trends in terms of structure-activity relationships have however emerged based on the substitution and activity of our dialkyl-3-cyanopropylphosphates **3a-h**. First, whatever the substitution at R² and the R³ chain at the phosphorus atom, compounds **3a-b,g-h** bearing a phenyl group in R¹ position displayed the same medium inhibitory activity towards either AChE (mean IC₅₀ = 32.56 ± 2.37 μM), or BChE (mean IC₅₀ = 19.73 ± 0.98 μM). This finding suggests that the nature of the R¹ substitution drives the level of inhibition probably by increasing/reducing the (R³O)₂P(O)-X lability; or in other words by favoring the release of the 3-cyanopropoxy group upon catalytic serine-hydroxyl nucleophilic attack.

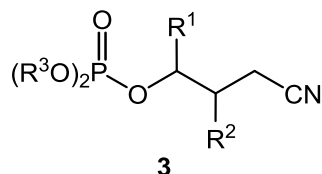
When a bulky *p*-tolyl group was introduced in R¹ position, the corresponding compounds **3c** and **3d** were found to strongly impair the activity of both enzymes. A clear difference was however observed between both compounds depending on the (R³O)₂P(O) phosphonic ester moiety. In each case, compound **3d** in which R³ = Me, exhibited a 1.8- and 2.7-fold lower anticholinesterase activity than **3c** (R³ = Et) towards AChE and BChE, respectively.

This variation in activity could be attributed to the respective hydrophobicity of the R³ chain, *i.e.* Methyl *vs.* Ethyl. It is indeed well known that increasing the length of the *n*-alkyl moiety of R³-groups for dialkylphosphates produces an increase in their reactivity with ChE [23].

Such assumption regarding hydrophobicity effects of our compounds seems, however, necessary but not sufficient to obtain a good inhibition. Replacing the *p*-Me-C₆H₄ group by the

less hydrophobic *p*-MeO-C₆H₄ group resulted in exactly the reverse reactivity of **3e** vs. **3f** towards AChE; while no significant difference in IC₅₀s were observed for the two compounds towards BChE.

Table 3: IC₅₀ values for AChE and BChE inhibition.



Tested compound	R ¹	R ²	R ³	IC ₅₀ for AChE [μM] ^a	IC ₅₀ for BChE [μM] ^a	Selectivity to BChE ^b
3a	Ph	H	Et	33.64 ± 0.52	18.13 ± 0.99	1.85
3b	Ph	H	Me	31.39 ± 1.23	19.25 ± 0.71	1.63
3c	<i>p</i> -Me-C ₆ H ₄	H	Et	9.61 ± 0.90	5.96 ± 0.35	1.61
3d	<i>p</i> -Me-C ₆ H ₄	H	Me	17.27 ± 1.05	16.01 ± 0.40	1.08
3e	<i>p</i> -MeO-C ₆ H ₄	H	Et	53.74 ± 0.51	20.75 ± 0.91	2.59
3f	<i>p</i> -MeO-C ₆ H ₄	H	Me	36.83 ± 0.64	23.35 ± 0.89	1.58
3g	Ph	Me	Et	35.75 ± 0.55	17.83 ± 0.78	2.00
3h	Ph	Me	Me	29.48 ± 0.68	20.57 ± 0.64	1.43
Rivastigmine				65.24 ± 0.63	32.47 ± 0.51	2.01

^a AChE and BChE inhibition is expressed as the mean ± SD (n = 3 experiments). The best results for each enzyme are shown in bold.

^b Selectivity to BChE = IC₅₀ for AChE/IC₅₀ for BChE.

2.3. *In silico* molecular docking

To further investigate the interactions occurring at the enzyme active site during the binding of the dialkyl-3-cyanopropylphosphate derivatives, *in silico* molecular docking experiments were conducted with compound **3c** using the reported crystal structures of AChE (PDB entry code: 5HF5 [24]) and BChE (PDB entry code: 1P0I [25]) as described previously

[26]. In both cases, the best scoring positions obtained (*i.e.* lowest energy complex) showed that phosphate moiety adopted a productive orientation (Fig. 1). The reactive phosphorus atom of **3c** was indeed found in a favorable position with the 3-cyano-1-*p*-tolylpropyloxy group in apical position and opposite to the catalytic serine, thus allowing the occurrence of a nucleophilic attack by this catalytic residue ($d[\text{Ser-O}\gamma/\text{P}=\text{O}]$ distance $< 2.5 \text{ \AA}$) and thus the probable formation of a covalent bond.

The docked **3c**-ChE complex was then subjected to interactions analysis using Ligplot+ v.1.4 [27] (Fig. 1C-D) to identify the potential hydrogen bonds and hydrophobic interactions between the ligand (*i.e.*, **3c**) and the active site residues of each protein during the binding process. As depicted in Fig. 1C-D, the Ligplot+ analysis shows that the reactive phosphorus atom is stabilized by H-bonding with His447 and Gly116 residues in the case of AChE (Fig. 1C) and BChE (Fig. 1D), respectively. The cyano group may also form hydrogen bond with either Tyr133 (Fig. 1C – AChE), or Leu286 (Fig. 1D – BChE). Moreover, several hydrophobic contacts can be detected that are important to stabilize the inhibitor inside each active site (Fig. 1C-D). Besides all these observations, the main differences between both enzymes resides in their respective active site configuration: a narrow tunnel for AChE, and on the contrary a large open cavity in the case of BChE. Such strong dissimilarities may explain in part the observed selectivity in inhibition towards BChE *vs.* AChE. Taken together, all these results therefore might provide a good picture of the inhibition of both enzymes by compound **3c**.

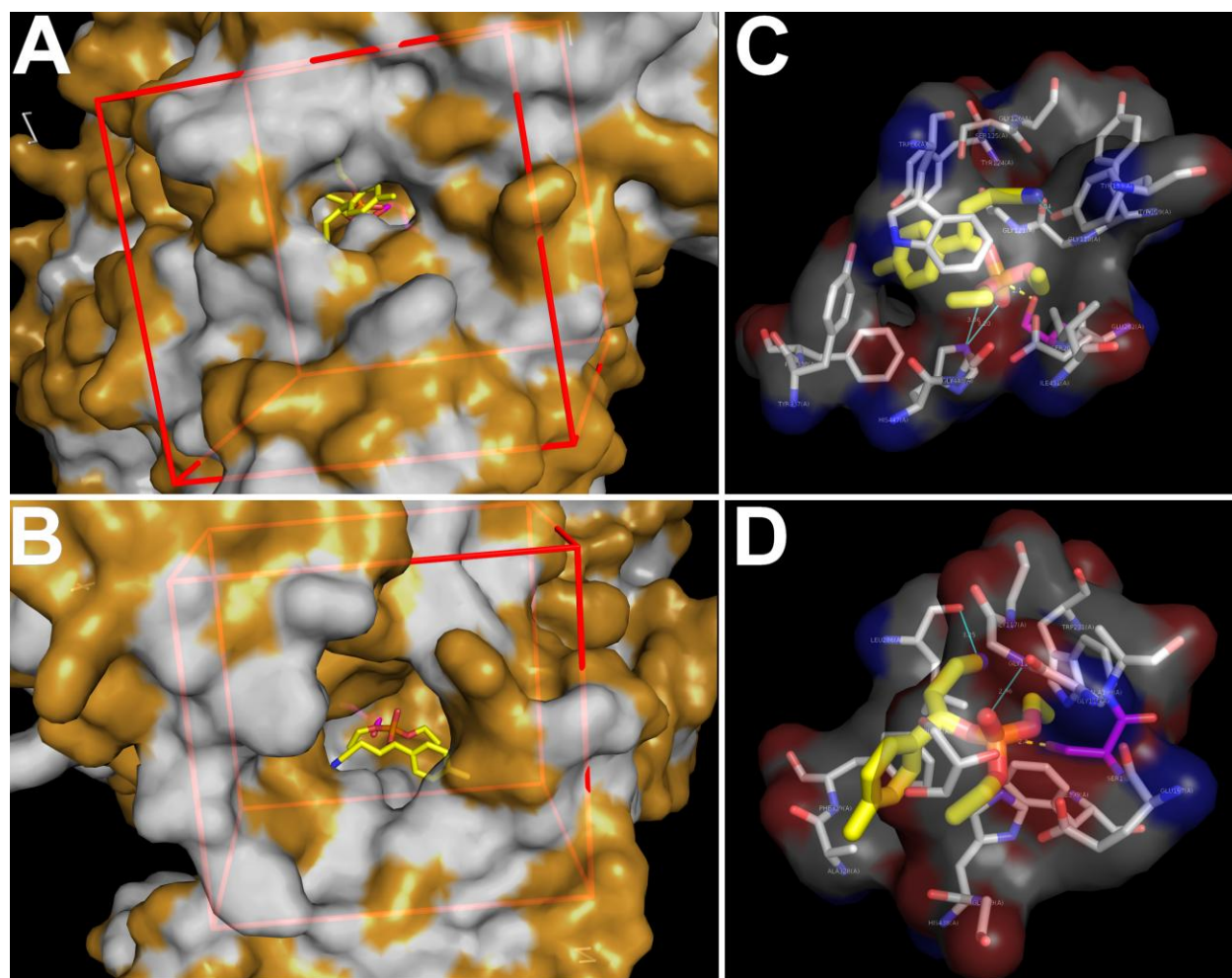


Figure 1. (A-B) *In silico* molecular docking of **3c** into the crystallographic structures of (A) AChE and (B) BChE in a van der Waals surface representation. Hydrophobic residues (alanine, leucine, isoleucine, valine, tryptophan, tyrosine, phenylalanine, proline and methionine) are highlighted in white. (C-D) Ligplot+ analyses results: 3D representation of schematic ligand-protein interactions of **3c** in (C) AChE or (D) BChE active site showing both hydrogen-bonds (cyan lines) and hydrophobic interactions. **3c** is in stick representation with the following atom color-code: oxygen, red; phosphorus, orange; carbon, yellow. The catalytic Serine residues are colored in magenta and are positioned at 2.1 Å (panel C: Ser203 – AChE) and 2.4 Å (panel D: Ser198 – BChE) from the phosphorus atom of **3c**. Structures were drawn with PyMOL Molecular Graphics System (Version 1.4, Schrödinger, LLC) using the PDB files 5HF5 and 1P0I for AChE and BChE, respectively.

3. Conclusions

An efficient way to prepare previously unreported dialkyl-3-cyanopropylphosphates *via* the sodium alkoxide-catalysed reaction of dialkyl phosphites with γ -ketonitriles at 80 °C under solvent-free conditions has been developed. A possible reaction mechanism, involving a phospho-Brook type rearrangement, has been proposed. This synthetic strategy offers significant advantages such as efficiency, high yields, versatile substituents and mild reaction conditions, making this type of reaction an enhancement for the organic synthesis bibliography. All the title compounds thus synthesized were evaluated against acetylcholinesterase and butyrylcholinesterase showing a significant and higher inhibition of both enzymes than the standard Rivastigmine, with IC₅₀ values in the micromolar range. It was also observed that the activity against butyrylcholinesterase was comparatively higher than that obtained for acetylcholinesterase. Among the synthesized compounds, derivative **3c**, which contains the most hydrophobic substituents and displayed the highest inhibitory activity against AChE and BChE was further used for *in silico* molecular docking in both enzymes active site, giving information regarding its binding mode. Anyway, other studies to enrich this first series of promising products and on the modification of the nitrile functionality in **3**-compounds to obtain additional dialkyl propylphosphate derivatives with amino or carboxyl units, are ongoing in our laboratory and will be reported in due course. This might further enhance the biological activity of these pharmacophores.

4. Experimental

¹H, ³¹P and ¹³C NMR spectra were recorded using CDCl₃ as the solvent on a Bruker AC-300 spectrometer operating at 300.1MHz for ¹H, 121.5 MHz for ³¹P and 75.5 MHz for ¹³C. The chemical shifts are reported in ppm relative to TMS (internal reference) for ¹H and ¹³C NMR and relative to 85% H₃PO₄ (external reference) for ³¹P NMR. The coupling constants are reported in Hz. The multiplicity of signals is indicated by the following abbreviations: s: singlet; d: doublet; t: triplet; q: quartet; quint: quintet; m: multiplet. Mass spectra were determined on an Agilent 5975B spectrometer under electronic impact (EI) conditions. IR spectra were recorded on a

Nicolet IR200 spectrometer. The progress of the reactions was monitored by TLC. The purification of the product was performed by column chromatography using silica gel (Fluka).

4.1. General procedure for the synthesis of dialkyl-3-cyanopropylphosphates 3

A solution of 0.05 g of sodium in 1 mL of dry ethanol (or methanol when using dimethyl phosphite) was added dropwise while stirring at 25 °C to a mixture of γ -ketonitrile (0.01 mol) and dialkylphosphite (0.05 mol). The reaction mixture was then heated to 80 °C with constant stirring for 24 h. The excess dialkylphosphite was removed under reduced pressure and then CHCl_3 (50 mL) was added. The organic phase was washed with water (2 x 25 mL), dried over Na_2SO_4 and concentrated under vacuum. The crude product obtained was chromatographed in a silica gel column using a mixture of ether and hexane (7:3) as an eluent.

Diethyl-3-cyano-1-phenylpropylphosphate (3a): Yellow oil; ^{31}P NMR: $\delta = -3.51$; ^1H NMR: $\delta = 1.23$ (t, 3H, $^3J_{\text{HH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 1.34 (t, 3H, $^3J_{\text{HH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 2.14-2.30 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 2.37-2.54 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 3.93 (quint, 2H, $^3J_{\text{HH}} = ^3J_{\text{PH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 4.09 (quint, 2H, $^3J_{\text{HH}} = ^3J_{\text{PH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 5.34-5.37 (m, 1H, O-CH-CH_2); 7.35-7.38 (m, 5H, arom-H); ^{13}C NMR: $\delta = 13.2$ (s, $\text{CH}_2\text{-CH}_2\text{-CN}$); 15.7 (d, $^3J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 16.1 (d, $^3J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 33.5 (d, $^3J_{\text{CP}} = 6.7$, $\text{CH}_2\text{-CH}_2\text{-CN}$); 61.5 (d, $^2J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 63.7 (d, $^2J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 78.1 (d, $^2J_{\text{CP}} = 6.0$, O-CH-CH_2); 118.8 (s, CN); phenyl carbons: $\delta = 126.02$, 128.59, 128.65, 138.62, 138.65; IR (neat): $\nu_{\text{P-O-C}} = 1037\text{ cm}^{-1}$; $\nu_{\text{P=O}} = 1271\text{ cm}^{-1}$; $\nu_{\text{C=N}} = 2268\text{ cm}^{-1}$; EI-HRMS: calculated for $\text{C}_{14}\text{H}_{20}\text{NO}_4\text{P}$: 297.1130 (M^+); found: 297.1126.

Dimethyl-3-cyano-1-phenylpropylphosphate (3b): Yellow oil; ^{31}P NMR: $\delta = 0.25$; ^1H NMR: $\delta = 1.89$ -2.14 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 2.16-2.34 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 3.45 (d, 3H, $^3J_{\text{HP}} = 12.0$, $\text{CH}_3\text{-O}$); 3.53 (d, 3H, $^3J_{\text{HP}} = 12.0$, $\text{CH}_3\text{-O}$); 5.11-5.18 (m, 1H, O-CH-CH_2); 7.14-7.17 (m, 5H, arom-H); ^{13}C NMR: $\delta = 13.2$ (s, $\text{CH}_2\text{-CH}_2\text{-CN}$); 33.2 (d, $^3J_{\text{CP}} = 7.5$, $\text{CH}_2\text{-CH}_2\text{-CN}$); 54.1 (d, $^2J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-O}$); 54.2 (d, $^2J_{\text{CP}} = 5.3$, $\text{CH}_3\text{-O}$); 78.4 (d, $^2J_{\text{CP}} = 5.2$, O-CH-CH_2); 118.8 (s, CN); phenyl carbons: $\delta = 126.01$, 128.64, 128.75, 138.38, 138.41; IR (neat): $\nu_{\text{P-O-C}} = 1041\text{ cm}^{-1}$; $\nu_{\text{P=O}} = 1263\text{ cm}^{-1}$; $\nu_{\text{C=N}} = 2241\text{ cm}^{-1}$; EI-HRMS: calculated for $\text{C}_{12}\text{H}_{16}\text{NO}_4\text{P}$: 269.0817 (M^+); found: 269.0823.

Diethyl-3-cyano-1-p-tolylpropylphosphate (3c): Yellow oil; ^{31}P NMR: $\delta = -3.27$; ^1H NMR: $\delta = 1.13$ (t, 3H, $^3J_{\text{HH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 1.28 (t, 3H, $^3J_{\text{HH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 2.06-2.23 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 2.26 (s, $\text{CH}_3\text{-C}_6\text{H}_4$); 2.31-2.40 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 3.88 (quint, 2H, $^3J_{\text{HH}} = ^3J_{\text{PH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 4.14 (quint, 2H, $^3J_{\text{HH}} = ^3J_{\text{PH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 5.19-5.26 (m, 1H, O-CH-CH_2); 7.01-7.24 (m, 4H, arom-H); ^{13}C NMR: $\delta = 12.4$ (s, $\text{CH}_2\text{-CH}_2\text{-CN}$); 15.2 (d, $^3J_{\text{CP}} = 6.7$, $\text{CH}_3\text{-CH}_2\text{-O}$); 15.5 (d, $^3J_{\text{CP}} = 5.2$, $\text{CH}_3\text{-CH}_2\text{-O}$); 19.6 (s, $\text{CH}_3\text{-C}_6\text{H}_4$); 32.6 (d, $^3J_{\text{CP}} = 6.7$, $\text{CH}_2\text{-CH}_2\text{-CN}$); 60.7 (d, $^2J_{\text{CP}} = 5.2$, $\text{CH}_3\text{-CH}_2\text{-O}$); 61.7 (d, $^2J_{\text{CP}} = 6.7$, $\text{CH}_3\text{-CH}_2\text{-O}$); 77.3 (d, $^2J_{\text{CP}} = 5.3$, O-CH-CH_2); 117.9 (s, CN); phenyl carbons: $\delta = 125.15, 128.59, 134.69, 134.73, 137.69$; IR (neat): $\nu_{\text{P-O-C}} = 1052\text{ cm}^{-1}$; $\nu_{\text{P=O}} = 1267\text{ cm}^{-1}$; $\nu_{\text{C}\equiv\text{N}} = 2271\text{ cm}^{-1}$; EI-HRMS: calculated for $\text{C}_{15}\text{H}_{22}\text{NO}_4\text{P}$: 311.1286 (M^+); found: 311.1284.

Dimethyl-3-cyano-1-p-tolylpropylphosphate (3d): Yellow oil; ^{31}P NMR: $\delta = 0.17$; ^1H NMR: $\delta = 1.96\text{-}2.13$ (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 2.17 (s, 3H, $\text{CH}_3\text{-C}_6\text{H}_4$); 2.22-2.59 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 3.58 (d, 3H, $^3J_{\text{HP}} = 12.0$, $\text{CH}_3\text{-O}$); 3.60 (d, 3H, $^3J_{\text{HP}} = 12.0$, $\text{CH}_3\text{-O}$); 5.13-5.15 (m, 1H, O-CH-CH_2); 6.98-7.29 (m, 4H, arom-H); ^{13}C NMR: $\delta = 13.3$ (s, $\text{CH}_2\text{-CH}_2\text{-CN}$); 21.4 (s, $\text{CH}_3\text{-C}_6\text{H}_4$); 33.0 (d, $^2J_{\text{CP}} = 7.5$, $\text{CH}_2\text{-CH}_2\text{-CN}$); 51.8 (d, $^2J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-O}$); 53.6 (d, $^2J_{\text{CP}} = 6.7$, $\text{CH}_3\text{-O}$); 78.5 (d, $^2J_{\text{CP}} = 5.3$, O-CH-CH_2); 118.7 (s, CN); phenyl carbons: $\delta = 125.49, 128.93, 135.29, 135.33, 138.66$; IR (neat): $\nu_{\text{P-O-C}} = 1054\text{ cm}^{-1}$; $\nu_{\text{P=O}} = 1263\text{ cm}^{-1}$; $\nu_{\text{C}\equiv\text{N}} = 2263\text{ cm}^{-1}$; EI-HRMS: calculated for $\text{C}_{13}\text{H}_{18}\text{NO}_4\text{P}$: 283.0973 (M^+); found: 283.0968.

Diethyl-3-cyano-1-(4-methoxyphenyl)propylphosphate (3e): Yellow oil; ^{31}P NMR: $\delta = -3.59$; ^1H NMR: $\delta = 1.08$ (t, 3H, $^3J_{\text{HH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 1.22 (t, 3H, $^3J_{\text{HH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 1.75-1.90 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 2.19-2.44 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 3.66 (s, 3H, $\text{CH}_3\text{O-C}_6\text{H}_4$); 4.00 (quint, 2H, $^3J_{\text{HH}} = ^3J_{\text{PH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 4.12 (quint, 2H, $^3J_{\text{HH}} = ^3J_{\text{PH}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 5.71-5.82 (m, 1H, O-CH-CH_2); 6.73-7.08 (m, 4H, arom-H); ^{13}C NMR: $\delta = 13.6$ (s, $\text{CH}_2\text{-CH}_2\text{-CN}$); 15.9 (d, $^3J_{\text{CP}} = 6.7$, $\text{CH}_3\text{-CH}_2\text{-O}$); 16.1 (d, $^3J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 33.5 (d, $^3J_{\text{CP}} = 6.0$, $\text{CH}_2\text{-CH}_2\text{-CN}$); 55.0 (s, $\text{CH}_3\text{O-C}_6\text{H}_4$); 61.2 (d, $^2J_{\text{CP}} = 7.5$, $\text{CH}_3\text{-CH}_2\text{-O}$); 61.5 (d, $^2J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 79.0 (s, O-CH-CH_2); 119.4 (s, CN); phenyl carbons: $\delta = 127.41, 128.35, 132.92, 159.26$; IR (neat): $\nu_{\text{P-O-C}} = 1067\text{ cm}^{-1}$; $\nu_{\text{P=O}} = 1268\text{ cm}^{-1}$; $\nu_{\text{C}\equiv\text{N}} = 2259\text{ cm}^{-1}$; EI-HRMS: calculated for $\text{C}_{15}\text{H}_{22}\text{NO}_5\text{P}$: 327.1236 (M^+); found: 327.1239.

Dimethyl-3-cyano-1-(4-methoxyphenyl)propylphosphate (3f): Yellow oil; ^{31}P NMR: $\delta = 0.77$; ^1H NMR: $\delta = 1.71$ -1.88 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 2.25-2.36 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CN}$); 3.52 (s, 3H, $\text{CH}_3\text{-O-C}_6\text{H}_4$); 3.55 (d, 3H, $^3J_{\text{HP}} = 12.0$, $\text{CH}_3\text{-O-P}$); 3.58 (d, 3H, $^3J_{\text{HP}} = 12.0$, $\text{CH}_3\text{-O-P}$); 5.49-5.54 (m, 1H, O-CH-CH_2); 7.26-7.30 (m, 4H, arom-H); ^{13}C NMR: $\delta = 11.5$ (s, $\text{CH}_2\text{-CH}_2\text{-CN}$); 33.0 (d, $^3J_{\text{CP}} = 8.2$, $\text{CH}_2\text{-CH}_2\text{-CN}$); 51.7 (s, $\text{CH}_3\text{-O-C}_6\text{H}_4$); 53.7 (d, $^2J_{\text{CP}} = 7.5$, $\text{CH}_3\text{-O-P}$); 53.8 (d, $^2J_{\text{CP}} = 7.5$, $\text{CH}_3\text{-O-P}$); 77.6 (s, O-CH-CH_2); 119.7 (s, CN); phenyl carbons: $\delta = 127.28$, 129.16, 130.11, 159.07; IR (neat): $\nu_{\text{P-O-C}} = 1062\text{ cm}^{-1}$; $\nu_{\text{P=O}} = 1273\text{ cm}^{-1}$; $\nu_{\text{C=N}} = 2258\text{ cm}^{-1}$; EI-HRMS: calculated for $\text{C}_{13}\text{H}_{18}\text{NO}_5\text{P}$: 299.0923 (M^+); found: 299.0916.

Diethyl-3-cyano-2-methyl-1-phenylpropylphosphate (3g): Yellow oil; ^{31}P NMR: $\delta = -3.3$ (min), -2.4 (maj); % maj/min: 58/42; ^1H NMR: $\delta = 1.12$ -1.28 (m, 9H, 2 $\text{CH}_3\text{-CH}_2\text{-O}$ and $\text{CH}_3\text{-CH}$); 2.40-2.54 (m, $\text{CH-CH}_2\text{-CN}$); 2.56-2.72 (m, 1H, $\text{CH-CH}_2\text{-CN}$); 3.86-4.12 (m, 4H, $\text{CH}_3\text{-CH}_2\text{-O}$); 5.72-5.78 (m, 1H, O-CH-CH); 7.22-7.58 (m, 5H, arom-H); ^{13}C NMR: $\delta = 15.9$ (d, $^3J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 16.2 (d, $^3J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 17.8 (s, $\text{CH-CH}_2\text{-CN}$); 18.3 (s, $\text{CH}_3\text{-CH}$, min); 20.0 (s, $\text{CH}_3\text{-CH}$, maj); 37.8 (d, $^3J_{\text{CP}} = 6.0$, $\text{CH-CH}_2\text{-CN}$); 61.5 (d, $^2J_{\text{CP}} = 5.2$, $\text{CH}_3\text{-CH}_2\text{-O}$); 63.4 (d, $^2J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-CH}_2\text{-O}$); 80.2 (d, $^2J_{\text{CP}} = 6.0$, O-CH-CH); 118.4 (s, CN, maj); 118.5 (s, CN, min); phenyl carbons: $\delta = 126.93$, 127.74, 128.29, 128.37, 128.75, 129.16, 134.58, 134.61, 137.50, 137.53; IR (neat): $\nu_{\text{P-O-C}} = 1088\text{ cm}^{-1}$; $\nu_{\text{P=O}} = 1268\text{ cm}^{-1}$; $\nu_{\text{C=N}} = 2241\text{ cm}^{-1}$; EI-HRMS: calculated for $\text{C}_{15}\text{H}_{22}\text{NO}_4\text{P}$: 311.1286 (M^+); found: 311.1281.

Dimethyl-3-cyano-2-methyl-1-phenylpropylphosphate (3h): Yellow oil; ^{31}P NMR: $\delta = 0.20$ (min), 0.37 (maj); % maj/min: 63/37; ^1H NMR: $\delta = 1.05$ (d, 3H, $^3J_{\text{HH}} = 6.0$, $\text{CH}_3\text{-CH}$, min); 1.12 (d, 3H, $^3J_{\text{HH}} = 6.0$, $\text{CH}_3\text{-CH}$, maj); 1.90-2.14 (m, 2H, $\text{CH-CH}_2\text{-CN}$); 2.21-2.60 (m, 1H, $\text{CH-CH}_2\text{-CN}$); 3.62 (d, 3H, $^3J_{\text{HP}} = 12.0$, $\text{CH}_3\text{-O}$); 3.68 (d, 3H, $^3J_{\text{HP}} = 12.0$, $\text{CH}_3\text{-O}$); 5.12-5.18 (m, 1H, O-CH-CH); 7.12-7.64 (m, 5H, arom-H); ^{13}C NMR: $\delta = 15.7$ (s, $\text{CH-CH}_2\text{-CN}$); 18.5 (s, $\text{CH}_3\text{-CH}$, maj); 20.6 (s, $\text{CH}_3\text{-CH}$, min); 37.5 (d, $^3J_{\text{CP}} = 6.7$, $\text{CH-CH}_2\text{-CN}$); 51.9 (d, $^2J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-O}$); 52.1 (d, $^2J_{\text{CP}} = 6.0$, $\text{CH}_3\text{-O}$); 81.0 (d, $^2J_{\text{CP}} = 6.0$, O-CH-CH); 118.2 (s, CN, maj); 118.6 (s, CN, min); phenyl carbons: $\delta = 126.41$, 126.79, 128.08, 128.26, 128.70, 129.29, 137.32, 137.35, 137.60, 137.72; IR (neat): $\nu_{\text{P-O-C}} = 1066\text{ cm}^{-1}$; $\nu_{\text{P=O}} = 1274\text{ cm}^{-1}$; $\nu_{\text{C=N}} = 2271\text{ cm}^{-1}$; EI-HRMS: calculated for $\text{C}_{13}\text{H}_{18}\text{NO}_4\text{P}$: 283.0973 (M^+); found: 283.0965.

4.2. Determination of IC_{50} for cholinesterases

The IC_{50} values were determined using the spectrophotometric Ellman's method [20], widely used for evaluating cholinesterase activity and screening potential inhibitors. Cholinesterase activity is measured indirectly by quantifying the concentration of the 5-thio-2-nitrobenzoic acid (TNB) ion formed in the reaction between the thiol reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and thiocholine, a product of substrate hydrolysis (*i.e.*, acetylthiocholine, ATCh) by cholinesterases [28]. The eight synthesized dialkyl-3-cyanopropylphosphates (**3a-h**) were dissolved in 0.01 M dimethyl sulphoxide and then diluted in demineralized water to 1 mM and 0.1 mM. Pursuing the procedure described in [21], the activities were determined as follows: the reaction mixture containing the phosphate buffer, AChE or BChE and the chosen dialkyl-3-cyanopropylphosphate, was prepared and intensively stirred. DTNB and ATCh were then added to the sample and mixed, and the absorbance was then measured at 405 nm using a Thermo Scientific Multiskan FC Microplate Photometer.

Electric eel AChE (Type-VI-S, EC 3.1.1.7; Sigma, St. Louis, USA) and horse serum BChE (EC 3.1.1.8; Sigma) were used.

Rivastigmine (Sigma) was used as reference drug.

4.3. Molecular docking experiments

In silico molecular docking of compound **3c** present in the active site of AChE and BChE was performed with the Autodock Vina program [29] as previously described [26]. The PyMOL Molecular Graphics System (version 1.4, Schrödinger, LLC) was used as working environment with an in-house version of the AutoDock/Vina PyMOL plugin [30]. The X-ray crystallographic structures of AChE (PDB entry code: 5HF5; 2.15 Å resolution [24]), and BChE (PDB entry code: 1P0I; 2.00 Å resolution [25]) available at the Protein Data Bank were used as receptors. Docking runs were performed after replacing the catalytic serine by a glycine residue to enable the ligand to adopt a suitable position corresponding to the pre-bound intermediate before the nucleophilic attack in the enzyme active site. The box size used for the various receptors was chosen to fit the whole active site cleft and to allow non constructive binding positions. A model structure file was generated for the inhibitor molecule, and its geometry was refined using the Avogadro open-source program (version 1.1.1. <http://avogadro.openmolecules.net/>).

Conflict of interest

The authors declare no conflict of interest.

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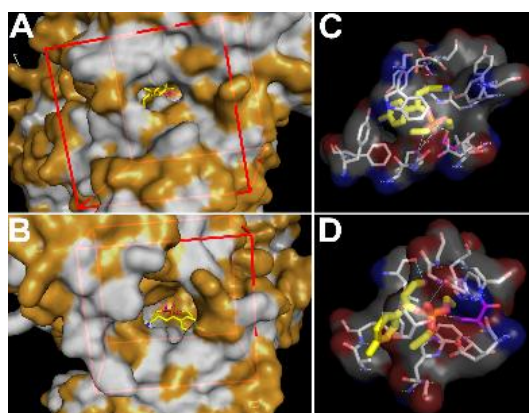
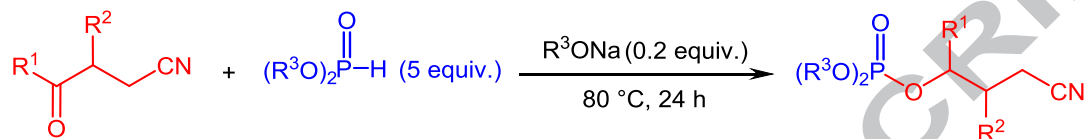
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ACCEPTED MANUSCRIPT

Graphical abstract



72-85% yield

Promising
anticholinesterase
activity

Highlights

- First synthesis of dialkyl-3-cyanopropylphosphate derivatives.
- Possible reaction mechanism, involving a phospha-Brook type rearrangement.
- *In vitro* studies of the synthesized compounds as possible inhibitors of acetylcholinesterase and butyrylcholinesterase.
- They showed higher inhibition of both enzymes than the standard Rivastigmine.
- They behave more selectively towards butyrylcholinesterase.
- *In silico* molecular docking in both enzymes active site, gave information regarding the binding mode.