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SUBSTITUTED ETHYNYLPHOSPHONATES AND THEIR ANTICHOLINESTERASE ACTIVITY

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Esters of thiophosphoric acids $(RO)_2P(O)SC{\equiv}CX$ (I) have a strong ability to inhibit cholinesterases of various origins [1]. It has been suggested that the acetylene group of these organophosphorus compounds can be sorbed on some cholinesterase active-surface region which is in the region of the esterase center [2], as a result of which a high rate of the cholinesterase phosphorylation reaction (occurring, as is known, with cleavage of the P–S bond and formation of the phosphorylated enzyme) is ensured. It seemed of interest to study the reaction of cholinesterases with ethynylphosphonates with the general formula $(C_2H_5O)_2P(O)C{\equiv}CX$ (II), in which the acetylene group is bonded directly to the P atom. These substances were synthesized according to the Michaelis–Becker reaction or by the reaction of substituted ethynylmagnesium bromides with diethyl chlorophosphate in tetrahydrofuran (THF)

$$\left.\begin{array}{l}(C_2H_5O)_2PONa + BrC{\equiv}CX \\ (C_2H_5O)_2P(O)Cl + BrMgC{\equiv}CX\end{array}\right\} \longrightarrow \underset{\text{(IIa-c)}}{(C_2H_5O)_2P(O)C{\equiv}CX};$$

X = C_4H_9 (a), cyclo-C_6H_{11} (b), and $CH_2N(CH_3)_2$ (c).

Methiodide (IId) was obtained by the reaction of methyl iodide with (IIc).

$$(IIc) + CH_3I \rightarrow \underset{\text{(IId)}}{(C_2H_5O)_2P(O)C{\equiv}CCH_2N^+(CH_3)_3\bar{I}}.$$

For comparison of the reaction of sodium diethyl phosphite with alkyl bromides in THF, we obtained the corresponding saturated analogs (IIIa) and (IIIb).

$$(C_2H_5O)_2PONa + BrCH_2CH_2X \rightarrow \underset{\text{(IIIa, b)}}{(C_2H_5O)_2P(O)CH_2CH_2X}.$$

The saturated analog of (IId), namely, 3-(diethoxyphosphoryl)propyltrimethylamonium bromide (IIId), was obtained by the reaction of trimethylamine with diethyl 3-bromopropylphosphonate. The latter was synthesized from sodium diethyl phosphite and 1,3-dibromopropane in THF

$$(C_2H_5O)_2PONa \xrightarrow{Br(CH_2)_3Br} (C_2H_5O)_2P(O)(CH_2)_3Br \xrightarrow{N(CH_3)_3} \underset{\text{(IIId)}}{\rightarrow (C_2H_5O)_2P(O)CH_2CH_2CH_2N^+(CH_3)_3Br^-}.$$

For (IIa), its thione analog (IV) was synthesized by the reaction of hex-1-ynylmagnesium bromide with diethyl chlorophosphite with subsequent addition of sulfur

$$(C_2H_5O)_2PCl \xrightarrow{BrMgC{\equiv}CC_4H_9} (C_2H_5O)_2P{-}C{\equiv}CC_4H_9 \xrightarrow{S} \underset{\text{(IV)}}{(C_2H_5O)_2P(S)C{\equiv}CC_4H_9}.$$

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TABLE 1. Ethynylphosphonates and Their Saturated Analogs $(C_2H_5O)_2P(O)R$

Compound	R	d_4^{20}	n_D^{20}	MR found/ calculated	Empirical formula	Found/Calculated, %		
						C	H	P
(IIa)	$C\equiv CC_4H_9$	1,0175	1,4482 *	57,38/57,18	$C_{10}H_{19}O_3P$	–	–	–
(IIb)	$C\equiv CC_6H_{11}$-cyclo	1,0443	1,4716	65,12/64,77	$C_{12}H_{21}O_3P$	58,53/59,01	8,65/8,61	12,15/12,70
(IIc)	$C\equiv CCH_2N(CH_3)_2$	1,0486	1,4528	56,43/56,50	$C_9H_{18}NO_3P$	49,35/49,31	8,36/8,28	14,04/14,15
(IId)	$C\equiv CCH_2N^+(CH_3)_3I^-$	–	–	–	$C_{10}H_{21}INO_3P$	33,55/33,10	4,18/3,86	8,67/8,54
(IIIa)	$CH_2CH_2C_4H_9$	0,9704	1,4297 †	59,06/59,24	$C_{10}H_{23}O_3P$	–	–	–
(IIIb)	$CH_2CH_2C_6H_{11}$-cyclo	1,0111	1,4528	66,17/66,83	$C_{12}H_{25}O_3P$	57,94/57,84	10,04/9,85	12,45/13,06
(IIId)	$CH_2CH_2CH_2N(CH_3)_3Br^-$	–	–	–	$C_{10}H_{25}BrNO_3P$	36,65/36,97	8,05/7,86	9,18/9,74
(IV)	$(C_2H_5O)_2P(S)C\equiv CC_4H_9$	1,0626	1,4922	63,91/64,55	$C_{10}H_{19}O_2PS$	51,23/51,28	7,99/8,12	13,14/13,24

*n_D^{20} 1.4478 [3].
†n_D^{20} 1.4297 [4].

TABLE 2. Rate Constants of Inhibition k_{II} of Cholinesterases by $(C_2H_5O)_2P(X)R$ Compounds

Compound	X	R	k_{II}, liters/(mole·min)		
			human-erythrocyte AChE	horse blood-serum BuChE	fly-head FChE
(Ia) *	O	$SC{\equiv}CC_4H_9$	$9.6 \cdot 10^6$	$1.0 \cdot 10^8$	$1.5 \cdot 10^9$
(IIa)	O	$C{\equiv}CC_4H_9$	$0.81 \cdot 10^1$	$7.3 \cdot 10^1$	$7.0 \cdot 10^2$
(IIIa)	O	$CH_2CH_2C_4H_9$	Does not inhibit irreversibly		
(Ib)†	O	$SC{\equiv}CC_6H_{11}$-cyclo	$3.1 \cdot 10^5$	$1.2 \cdot 10^9$	$8.0 \cdot 10^8$
(IIb)	O	$C{\equiv}CC_6H_{11}$-cyclo	$0.89 \cdot 10^1$	$1.2 \cdot 10^2$	$1.0 \cdot 10^3$
(IIIb)	O	$CH_2CH_2C_6H_{11}$-cyclo	Does not inhibit irreversibly		
(IId)	O	$C{\equiv}CCH_2N^+(CH_3)_3I^-$	$2.1 \cdot 10^2$	$1.7 \cdot 10^3$	$1.2 \cdot 10^3$
(IIId)	O	$CH_2CH_2CH_2N^+(CH_3)_3Br^-$	Does not inhibit irreversibly		
(IV)	S	$C{\equiv}CC_4H_9$	»		»

*Data of [1].
†Data of [5].

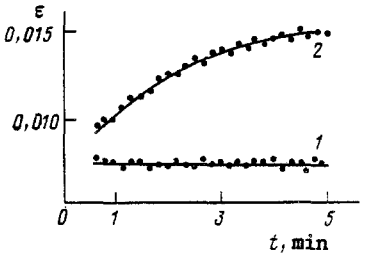

Fig. 1. Reactivation of AChE inhibited by $(C_2H_5O)_2P(O)C{\equiv}CC_4H_9$: 1) activity of AChE in the absence of the reactivator; 2) activity of AChE after the addition of TMB-4; ε) activity of inhibited AChE in arbitrary units.

The structure of the obtained substances was confirmed by IR-spectral data. Thus, for ethynylphosphonates, we observed absorption in the regions of 2200 cm^{-1} (C≡C) and 1270-1280 cm^{-1} (P=O).

The purity of the compounds was evaluated by thin-layer chromatography (TLC) using enzymatic and iodine development. The agreement of the results was proof of the absence of impurities. The obtained data are given in Table 1. For evaluation of the anticholinesterase activity of the obtained substances, we determined the bimolecular rate constants k_{II} of their reaction with human-erythrocyte acetylcholinesterase (AChE), horse blood-serum butyrylcholinesterase (BuChE), and fly (*Calliphora erytrocephola*) head cholinesterase (FChE).

For comparison, we give k_{II} for S-ethynyl thiophosphonates (Ia and Ib). From the data of Table 2, it is evident that ethynylphosphonates (IIa, IIb, and IId) are weak irreversible inhibitors of cholinesterases, whereas their saturated analogs (IIIa, IIIb, and IIId) do not possess such action. Compound (IId) exhibited the greatest inhibiting activity with respect to BuChE, and compounds (IIa) and (IIb) exhibited the greatest inhibiting activity with respect to FChE. It was found that AChE is less sensitive to the investigated ethynylphosphonates. Further, it was determined that O,O'-diethyl (hex-1-ynyl)thiophosphonate (IV), which is a thione analog (IIa), is unable to inhibit cholinesterases irreversibly. This fact is in good agreement with the generally accepted rule for typical organophosphorus inhibitors: replacement of the phosphoryl oxygen in their molecule by sulfur leads to the loss of anticholinesterase activity, which is related to the inability of the S atom to form H bonds [6].

In addition, we observed two additional facts that are also characteristic of irreversible inhibition of cholinesterases by typical organophosphorus inhibitors. Firstly, AChE, inhibited by ethynylphosphonates, is reactivated in the presence of TMB-4 (Fig. 1). Secondly, tetraalkylammonium ions act as concurrent reversible inhibitors in the reaction of

TABLE 3. Constants of Irreversible Inhibition K_i of the Reaction of AChE with $(C_2H_2O)_2P(O)R$ Compounds in the Presence of Tetraalkylammonium Ions

Compound	R	K_i of tetraalkylammonium ions, mole/liter		
		$(CH_3)_4N^+$	$(C_2H_5)_4N^+$	$(C_4H_9)_4N^+$
(Ia)	$SC{\equiv}CC_4H_9$	$3{,}2\cdot10^{-3}$	$1{,}4\cdot10^{-3}$	$4{,}6\cdot10^{-4}$
(IIa)	$C{\equiv}CC_4H_9$	$1{,}3\cdot10^{-2}$	$2{,}1\cdot10^{-3}$	$9{,}3\cdot10^{-5}$
(Ib)	$SC{\equiv}CC_6H_{11}$-cyclo	$1{,}1\cdot10^{-5}$	$1{,}0\cdot10^{-5}$	$4{,}1\cdot10^{-5}$
(IIb)	$C{\equiv}CC_6H_{11}$-cyclo	$6{,}9\cdot10^{-3}$	$1{,}5\cdot10^{-3}$	$2{,}2\cdot10^{-4}$

cholinesterases with ethynylphosphonates; i.e., just as in the case with typical organophosphorus inhibitors, they protect the enzyme from irreversible inhibition by these compounds (Table 3).

Thus, for the first time we have found irreversible inhibition, albeit weak inhibition, of cholinesterases by esters of phosphonic acids not containing electron-acceptor substituents at the C atoms.

EXPERIMENTAL

Ethynylphosphonates (IIa and IIb). Method A. To 0.1 mole of sodium diethyl phosphite [obtained from 13.8 g (0.1 mole) of diethyl phosphite and 2.3 g (0.1 mole) of Na] in 100 ml of THF at −30°C, 16.1 g (0.1 mole) of 1-bromo-1-hexyne was added slowly. After completion of the addition, the temperature was slowly raised to ~20°C, and the mixture was stirred for 3 h and then boiled for 1 h. The precipitate was filtered off, the solvent was evaporated, and the residue was chromatographed on SiO_2 L 100/160 (eluent acetone–benzene, 1:2). We obtained 8.24 g (37.8%) of diethyl (hex-1-ynyl)phosphonate.

We obtained similarly 5.91 g (27%) of diethyl 3-(dimethylamino)prop-1-ynylphosphonate.

Method B. To 0.1 mole of cyclohexylethynylmagnesium bromide [obtained from 10.8 g (0.1 mole) of cyclohexylacetylene, 2.4 g (0.1 mole) of Mg, and 11.99 g (0.11 mole) of ethyl bromide] in 100 ml of THF at 0°C, 17.25 g (0.1 mole) of diethyl chlorophosphate was added slowly. Then the temperature was slowly raised to ~20°C, and the mixture was stirred for 1 h. The reaction mixture was treated with 5% HCl at 0°C and extracted with ether, and the organic layer was washed with a saturated $NaHCO_3$ solution and water and dried with Na_2SO_4. The solvent was evaporated, and the residue was chromatographed on SiO_2 L 100/160 (eluent acetone–benzene, 1:2). We obtained 3.1 g (12.7%) of diethyl cyclohexylethynylphosphonate.

Diethyl Alkylphosphonates (IIIa and IIIb). To 0.1 mole of sodium diethyl phosphite in 100 ml of THF at −30°C was added slowly 15.6 g (0.1 mole) of 1-iodohexane. After completion of the addition, the temperature was slowly raised to ~20°C, and the mixture was stirred for 3 h and then boiled for 1 h. The solvent was evaporated, 100 ml of water was added to the residue, the whole was extracted with ether, and the extract was dried with Na_2SO_4. The solvent was evaporated, and the residue was chromatographed on SiO_2 L 100/160 (eluent benzene–ether, 1:1). We obtained 3.65 g (16.4%) of diethyl hexylphosphonate.

We obtained similarly 7.41 g (29.9%) of diethyl 2-cyclohexylethylphosphonate.

Methiodide (IId). To 0.657 g (0.03 mole) of (IIc) was added 38.1 g (0.3 mole) of MeI in 50 ml of ether, and the whole was left in the dark for 24 h. The precipitate was filtered off, washed with 50 ml of ether, and dried under vacuum. We obtained 0.87 g (81%) of 3-(diethoxyphosphoryl)prop-1-ynyltrimethylammonium iodide with mp 138°C.

Methiodide (IIId). To 24.24 g (0.12 mole) of 1,3-dibromopropane in 100 ml of THF was added with boiling for 4 h 0.03 mole of sodium diethyl phosphite [obtained from 4.14 g (0.03 mole) of diethyl phosphite and 0.69 g (0.03 mole) of Na] in 50 ml of THF. The mixture was boiled for 2 h, the precipitate was filtered off, and the solvent and the excess 1,3-dibromopropane were driven off under vacuum. The residue was chromatographed on SiO_2 L 100/160 (eluent benzene–acetone, 2:1). We obtained 10.26 g (33%) of diethyl 3-bromopropylphosphonate, d_4^{20} 1.3072, n_D^{20} 1.4516, found MR 53.41, calculated MR 53.16.

To 2.59 g (0.01 mole) of diethyl 3-bromopropylphosphonate in 50 ml of ether was added 5.9 g (0.1 mole) of Me_3N, and the whole was left in the dark for 24 h. The precipitate was

filtered off, washed with 50 ml of ether, and dried under vacuum. We obtained 2.34 g (73.5%) of 3-(diethoxyphosphoryl)propyltrimethylammonium bromide with mp 92°C.

Diethyl Hex-1-ynyl phosphonite. To 0.09 mole of hex-1-ynylmagnesium bromide [obtained from 2.18 g (0.09 mole) of Mg, 10.9 g (0.1 mole) of ethyl bromide, and 9.02 g (0.11 mole) of 1-hexyne] in 100 ml of THF at −60°C was added 14.1 g (0.09 mole) of diethyl chlorophosphite. The temperature was slowly raised to ~20°C, and the whole was stirred for 1 h. The solvent was evaporated, and 15.8 g (0.2 mole) of pyridine and 50 ml of hexane were added to the residue. The precipitate was filtered off and washed with 100 ml of hexane, the filtrate was evaporated, and the residue was distilled under vacuum in the presence of hydroquinone. We obtained 2.4 g (13.8%) of diethyl hex-1-ynylphosphonite, bp 72.5°C (1 mm), d_4^{20} 0.9603, n_D^{20} 1.4588, found MR 57.48, calculated MR 57.70.

O,O'-Diethyl (Hex-1-ynyl)thiophosphonate. To 0.38 g (0.19 mole) of sulfur in 50 ml of ether was added 2.6 g (0.0119 mole) of diethyl hex-1-ynylphosphonite in 25 ml of ether. The mixture was stirred for 1 h, and the excess sulfur was filtered off, washed with 25 ml of ether, and evaporated. The residue was chromatographed on SiO_2 L 100/160 (eluent benzene–hexane, 1:1). We obtained 1.35 g (52.75%) of O,O-diethyl (hex-1-ynyl)thiophosphonate, d_4^{20} 1.0626, n_D^{20} 1.4922, found MR 63.91, calculated MR 64.55.

Method for Enzymatic Development of Organophosphorus Compounds in TLC. A sample of the organophosphorus-compound solution being analyzed, in 10^{-3}-10^{-4} M concentration, was deposited (two spots) onto a Silufol plate by means of a thin capillary. After chromatography, the plate was dried in air and cut into two parts. One part was sprayed uniformly with an aqueous solution of butyrylcholinesterase (2.5 mg/ml) to a moist state and placed in a horizontal position for 15 min in a water-vapor-saturated thermostat at 37°C. The plate was dried slightly, sprayed uniformly with a solution of 5-bromoindoxyl acetate in 50% alcohol ($5 \cdot 10^{-3}$ mole/liter), and placed in a thermostat for 30 min. The organophosphorus compounds were detected in the form of white spots on a blue background, and the most distinct pattern was observed in the dry state. The other part was developed in iodine vapor.

Determination of the Anticholinesterase Activity of the Organophosphorus Compounds. The enzyme sources were partially purified, water-soluble preparations of human-erythrocyte AChE and horse blood-serum BuChE produced by the Perm Scientific-Research Institute of Vaccines and Serums and also a homogenate of Calliphora erytrocephola fly heads (FChE) prepared in a 0.01 M sodium phosphate buffer (SPB) with pH 7.5 in a 1:100 ratio.

The bimolecular rate constant k_{II} of the reaction of the enzyme with the organophosphorus compounds was calculated according to the equation

$$k_{II} = \frac{2.303}{t[I]} \ln \frac{V_{0,t}}{V_t}$$

where V_t is the rate of enzymatic hydrolysis of the substrate after t min of incubation of the enzyme with the inhibitor in concentration [I], exceeding many-fold the concentration of the enzyme active centers, and $V_{0,t}$ is the rate of enzymatic hydrolysis of the substrate without incubation with the inhibitor, i..e, at t = 0. The inhibition was carried out at 25°C and pH 7.5 in a reaction mixture containing 0.1 ml of a solution of the enzyme in 0.1 M SPB with pH 7.5 and 0.1 ml of an aqueous and sometimes aqueous alcoholic solution of the organophosphorus compound, in which the ethanol concentration did not exceed 10%. After 5 min of incubation of the enzyme with the inhibitor (t = 5 min), we added 1.5 ml of 0.05 M SPB with pH 7.5 containing acetylthiocholine (ATCh) in concentration $1.5 \cdot 10^{-3}$ mole/liter and bis[3-(bromocarbonyl)-4-nitrophenyl]disulfide in concentration $2 \cdot 10^{-4}$ mole/liter. After 15 min, we stopped the enzymatic hydrolysis of the substrate by adding proserine to final concentration 0.001% in runs with AChE and FChE and to 0.002% in runs with BuChE, and measuring the optical density of the solution at 412 nm, we determined the amount of thionitrobenzoic acid formed in the reaction of bis[3-(bromocarbonyl)-4-nitrophenyl]disulfide with thiocholine, a product of the enzymatic hydrolysis of ATCh (Ellmann's method).

To take into account the background coloration of the reagents and the nonenzymatic hydrolysis of the substrate, we carried out a similar measurement in a control run, adding proserine to the reaction mixture at the very beginning, before addition of the enzyme. The value of the V_t was calculated using the difference of the measurement in the experimental and control tests. The value of $V_{0,t}$ was determined similarly, the only difference being that the enzyme was added to a mixture of solutions of the substrate, the inhibitor, and bis[3-(bromocarbonyl)-4-nitrophenyl]disulfide. The determined value of $V_{0,t}$ was sometimes less than the one that was determined in the absence of the inhibitor V_0 because in some cases the inhibitor caused not

only irreversible, progressively developing inhibition of the enzyme, but also reversible inhibition that was exhibited practically instantaneously. If the substance exhibited only reversible action, then $V_{0,t} = V_0$; i.e., the degree of inhibition of the enzymatic reaction did not change with respect to time.

Reactivation of AChE Inhibited by Ethynylphosphonates. Inhibition of the enzyme was carried out at 25°C and pH 7.5 in a reaction mixture containing 0.05 ml of a solution of the enzyme in 0.1 M SPB with pH 7.5 and 0.05 ml of an aqueous or aqueous-alcoholic solution of the organophosphorus compound in which the ethanol concentration did not exceed 10%. After 5 min of incubation of the enzyme with the inhibitor, we added 1.5 ml of 0.05 M SPB with pH 7.5 containing $1.5 \cdot 10^{-3}$ M ATCh and bis[3-(bromocarbonyl)-4-nitrophenyl]disulfide in concentration $2.4 \cdot 10^{-4}$ mole/liter. Then 0.5 ml of the reaction mixture was withdrawn and placed in the cuvette of a Spekol-211 spectrophotometer, and the optical density of the solution was measured at 412 nm at 10-sec intervals for 33 min. Then 0.05 ml of an aqueous solution of TMB-4 ($5 \cdot 10^{-4}$ mole/liter) was added to the remaining reaction mixture, and the optical density was also measured at 412 nm at 10-sec intervals for 5 min. The activity of the inhibited AChE in arbitrary units was calculated according to the equation

$$\varepsilon = \varepsilon_{t+10''} - \varepsilon_t$$

where ε_t is the optical density of the solution at time t, and $\varepsilon_{t+10''}$ is the optical density of the solution at time t + 10 sec.

CONCLUSIONS

1. A series of substituted ethynylphosphonates was synthesized, and their anticholinesterase activity was studied.

2. The investigated compounds can inhibit cholinesterase of various origins irreversibly.

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