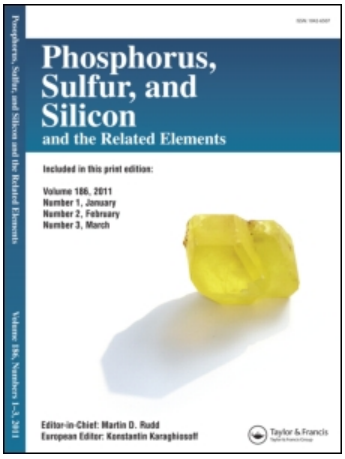

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SELECTIVE BUTYRYLCHOLINESTERASE INHIBITORS AMONG DIALKYLPHOSPHORAMIDOFLUORIDATES

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SELECTIVE BUTYRYLCHOLINESTERASE INHIBITORS AMONG DIALKYLPHOSPHORAMIDOFLUORIDATES

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Supposition of a relationship between blood butyrylcholinesterase (BuChE) activity and its lipid composition was reported in a number of works [1–7]†. To check this hypothesis, a search of selective BuChE inhibitors among substituted cyclohexyl ester N,N-dialkylphosphoramidofluoridic acids has been accomplished by us. For synthesis of substances to be investigated, interaction of N,N- dialkylphosphoramidic difluorides with Na and Al derivatives of corresponding alcohols was used. Investigation of purified, water soluble preparations of BuChE and acetylcholinesterase (AChE) indicated that there were high selective BuChE inhibitors among the investigated compounds. In particular, for N,N-diethyl-O-(trans-2-piperidinocyclohexyl)phosphoramidofluoridate, the reaction constants for cholinesterase inhibition were: $k^0_{BuChE} = 1{,}4 \times 10^8\ M^{-1} \times min^{-1}$, $k^0_{AChE} = 2{,}1 \times 10^4\ M^{-1} \times min^{-1}$ and for its derivative, 1-(trans-2-N,N-diethylamidofluorophosphoryloxycyclohexyl)-1-methylpiperidinium iodide $k^0_{BuChE} = 2{,}6 \times 10^7\ M^{-1} \times min^{-1}$, while the AChE inhibition by that compound was reversible ($K_i = 9{,}8 \times 10^{-6}$ M).

Keywords: Acetylcholinesterase; butyrylcholinesterase; O-alkyl-N,N-dialkylphosphoramidofluoridates; inhibitor

INTRODUCTION

In the past time there is an interest to the substances which selectively interact with butyrylcholinesterase (BuChE EC 3.1.1.8), or pseudocholinesterase—an enzyme, contained mainly in blood plasma of the mammals. Perhaps, this interest

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†Here you have only part of reports which are devoted to the abovementioned theme.

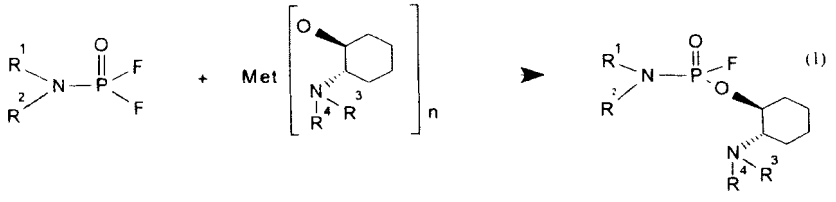
is associated with the fact that a number of papers pointed to an existing relationship between human blood BuChE activity and lipid composition of the blood.[1–4] In particular, as for patients poisoned by parathion,[4] β-lipoprotein level in their blood was revealed to decrease together with decreasing BuChE activity. The same relationship was also observed in experiments with animals.[4–7] For example, parallel to decreasing BuChE activity in the rat blood plasma due to administration of neostigmin two times, the concentration of β-lipoproteins decreased threefold, while the level of α-lipoproteins increased.[7] Obviously, in order to substantiate the above mention relationship, it was necessary to exclude an influence of other processes, except changing the BuChE activity on blood lipid composition. For instance, changing the activity of acetylcholinesterase (AChE, EC 3.1.1.7) must be excluded. Therefore, powerful and selective BuChE inhibitors should be used in this experiments.

This work is focused on the search for selective BuChE inhibitors among the cyclohexyl esters of dialkylphosphoramidofluoridic acid, which contain a ternary or quaternary nitrogen atom at the 2-trans position. Selection of the inhibitors of this type was determined by the following reasons:

1 O-alkyl-N,N-dialkylphosphoramidofluoridates, as a rule, more intensively inhibit BuChE than AChE;
2 Conformation of the fragment N-C-C-O in trans-2-dialkylaminocyclohexanol derivatives is unfavourable for interaction with AChE.

RESULTS

To synthesize final N,N-dialkylphosphoramidofluoridates, we used a method involving the interaction of N,N-dialkylphosphoramidic difluorides with metal derivatives of trans-2-N,N-dialkylaminocyclohexanols (1)



where Met = Al, Na; n = 1,3.

In most cases the final substances were produced with yields of 50–70% if sodium derivatives of aminoalcohols were used.

Quaternary ammonia derivatives of the final compounds were produced by interaction of bases with iodomethan.

Selectivity of synthesized substances with respect to BuChE was quantitatively estimated by observed second-order rate constants that characterize their inhibitory effect on the two studied cholinesterases, AChE(k^0_{AChE}) and BuChE(k^0_{BuChE}). A ratio $k_R = k^0_{BuChE}/k^0_{AChE}$ was used as a measure of the selectivity of phosphorus-containing (PC) inhibitors The values of k_R, k^0_{BuChE} and k^0_{AChE} are showed in the Table I.

As demonstrated in our experiments, all the studied substances displayed higher inhibitory effect on BuChE, rather than AChE. It should be stressed here that BuChE inhibition was characterized by elevated values of the rate constants ($10^6 \div 10^8$ $M^{-1} \times min^{-1}$), while as for AChE inactivation, these constants were equal to $10^3 \div 10^4$ $M^{-1} \times min^{-1}$. Among the studied inhibitors, substance 9 had the highest value of K_R equal to 6670.

Of special interest was substance 10 which may be referred to the most powerful BuChE inhibitors. The extent of BuChE inhibition induced by this substance increased with time up to a completely irreversible loss of the enzymatic activity under conditions of kinetic experiments at least. However, substance 10 behaved as a typical reversible inhibitor of AChE. Some possible explanations of its differing inhibitory effects on both BuChE and AChE will be considered below. Note also that substances 13 and 14 were reversible inhibitors of cholinesterases.

In addition to anticholinesterase effects, we studied some physicochemical properties of PC inhibitors which may be essential for their biological effects. As it followed from our data, dialkylamides displayed a very strong resistance to hydrolysis in alkaline media. Thus, dimethylamide (substance 2) possessed the stability of four orders higher (the constant of alkaline hydrolysis, k_{ah} = 4.7 $M^{-1} \times min^{-1}$) than methylphosphonate containing the same alkoxyl group (k_{ah} = 5.5 10^4 $M^{-1} \times min^{-1}$); as for substance 15, this constant was equal to 0.19 $M^{-1} \times min^{-1}$.

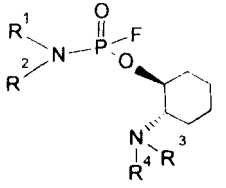
pK_a values of acids conjugated with the studied PC inhibitors (nitrogen - containing bases) were the following: 9.3 (substance 3); 8.51 (substance 7); 8.85 or 8.3 in 40% H_2O/C_2H_5OH (substance 9); 9.04 or 8.22 in 40% H_2O/C_2H_5OH (substance 13). Therefore, all these substances that contain trialkylamino[‡] groups in the alkoxyl radical may be referred to medium - strong organic bases.

The studied phosphoramidofluoridates (free bases) were hydrophobic as judged by coefficients of their partition (P) in octanol-borate buffer (pH = 8.0): P values were of 77.8 ± 11.4 (substance 7), 550 ± 105 (substance 9), and 180 ± 20 (substance 15 without dialkylamino group).

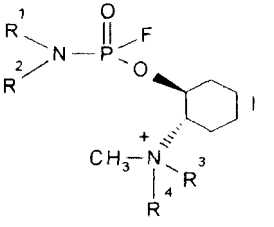
[‡]cyclohexyl was the third alkyl group at the nitrogen atom of trialkylamine

TABLE I INHIBITORY PROPERTIES OF FINAL COMPOUNDS

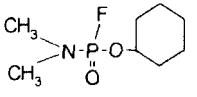
Substances of Type I



Substances of Type II



Substance N 15



SUBSTANCES OF TYPE I								*SUBSTANCES OF TYPE II*			
N	*K*$^{-1}$ $M^{-1} \times min^{-1}$		k_R	R_1	R_2	R_3	R_4	*N*	*K*$^{-1}$ $M^{-1} \times min^{-1}$		k_R
	BuChE	*AChE*							*BuChE*	*AChE*	
1	$1{,}3 \times 10^6$	$1{,}1 \times 10^5$	11.8	CH_3	CH_3	CH_3	CH_3	2	$2{,}6 \times 10^6$	$3{,}0 \times 10^3$	867
3	$8{,}7 \times 10^6$	$8{,}6 \times 10^4$	101	CH_3	CH_3	C_2H_5	C_2H_5	4	$3{,}1 \times 10^6$	$1{,}1 \times 10^4$	281
5	$3{,}5 \times 10^6$	$5{,}9 \times 10^3$	593	C_2H_5	C_2H_5	CH_3	CH_3	6	$3{,}8 \times 10^6$	$2{,}3 \times 10^3$	1652
7	$2{,}4 \times 10^7$	$6{,}1 \times 10^3$	4000	C_2H_5	C_2H_5	$R_3 + R_4 =$ $-(CH_2)_4-$		8	$3{,}5 \times 10^6$	$3{,}8 \times 10^3$	921
9	$1{,}4 \times 10^8$	$2{,}1 \times 10^4$	6667	C_2H_5	C_2H_5	$R_3 + R_4 =$ $-(CH_2)_5-$		10	$2{,}6 \times 10^7$	pI_{50} 4,25 human pI_{50} 4,14	–
11	$2{,}0 \times 10^6$	$6{,}5 \times 10^3$	308	C_2H_5	C_3H_7	CH_3	CH_3	12	$5{,}1 \times 10^6$	$8{,}4 \times 10^3$	607

TABLE I *continued*

SUBSTANCES OF TYPE I								*SUBSTANCES OF TYPE II*			
N	K^o $M^{-1} \times min^{-1}$		k_R	R_1	R_2	R_3	R_4	*N*	K^o $M^{-1} \times min^{-1}$		k_R
	BuChE	*AChE*							*BuChE*	*AChE*	
13	pI_{50} 4,82	pI_{50} 3,15	–	iC_3H_7	iC_3H_7	CH_3	CH_3	14	pI_{50} 4,33	pI_{50} 2,4	–
15	$7{,}4 \times 10^6$	$1{,}7 \times 10^5$	43	CH_3	CH_3	–	–	–	–	–	–

DISCUSSION

In this part, we consider in more details the influence of the PC inhibitor structures on their ability of irreversible BuChE inhibition.

First of all, replacement of a methyl group joined to the P atom in the methylphosphonate molecule for a dimethylamide group resulted in a pronounced drop of the anticholinesterase activity of inhibitors. However, while the rate constant of BuChE inhibition decreased by ca. one order, this value was drastically reduced by three orders in the case of AChE. For example, methylphosphonate (N-[trans-2-fluoro(methyl)phosphoryloxycyclohexyl]-N,N,N-trimethylammonium iodide) and dimethylamide containing the same alkoxyl radical (substance 2) were characterized by $k^0_{BuChE} = 4.6 \times 10^7 M^{-1} \times min^{-1}$; $k^0_{AChE} = 3.5 \times 10^6 M^{-1} \times min^{-1}$ and $k^0_{BuChE} = 2.9 \times 10^6 M^{-1} \times min^{-1}$; $k^0_{AChE} = 4.8 \times 10^3 M^{-1} \times min^{-1}$, respectively. With the following increase in the size of the dialkylamide groups in the order: diethylphosphoramidofluoridate (substance 6)—N-propyl-N-ethylphosphoramidofluoridate (substance 12), the rate constants were virtually unchanged, fluctuating about values of rate constants typical of dimethylamide (substances 2). Thus, basic differences in the size and spatial structure of methyl and dialkylamido groups were obvious. These data pointed to the fact that steric hindrances caused by specific structures of PC inhibitors influence to a much greater extent the binding of ligands to AChE's active surface than to BuChE.

Some qualitative changes occurred in the case of cholinesterase inhibition by N,N-diisopropylphosphoramidofluoridates (substances 13, 14) which were incapable to induce time-progressing inactivation of both AChE and BuChE. Hence, this substance behaved as a typical reversible inhibitor. Probably, a large diisopropylamide group could exert strong steric hindrances to the proper orientation of a phosphoryl group relative to proton donor groups in the site of binding with acyl moieties of substrate or inhibitor.[8,9] This may preclude the formation of a "correct" enzyme-substrate complex, necessary for phosphorylation of a hydroxyl group of serine—198.[10] In this case, an inhibitor could bind with cholinesterases only in a region of anionic sites of the active centers. In so doing, inactivation of cholinesterases is only provided by much higher concentrations of PC inhibitors (see pI_{50} values for substances 13,14).

Let us consider the question how the structure of O-2-dialkylamino- and trialkylammoniumcyclohexyl ester groups of the studied inhibitors influences their antienzymatic activity.

The data on the inhibitory effects of substances 1,3 and 5,7,9 suggests that increasing the size of the dialkylamino groups of alkoxy radicals is accompanied

by enhancing affinity of PC inhibitors for BuChE. The recent studies have shown that amino acid sequences of the site responsible for binding to substrate's acyl residues in BuChE active center are unique and strongly different from the homologous site of AChE.[11,12]

In particular, it has been established that BuChE and AChE active centers are located on a bottom of a deep and narrow gorge of a protein globule framed with hydrophobic residues and the amino acid chains lying along this gorge are different for BuChE and AChE.[12,13]

Based on these data, one can assume that the surfaces of active BuChE and AChE sites, which are in contact with inhibitor molecules, have different spatial structure and physicochemical properties, e.g. hydrophobicity.

An increase in the anti-BuChE activity in this case may be assigned to hydrophobic interactions between enlarged hydrocarbon radicals of dialkylamine groups with the hydrophobic residues upon the anionic site of BuChE's active center.[14,15] At least, comparison of partition coefficients of substance 7 (P = 78) and substance 9 (P = 550) allows one to assume that the observed increase in PC inhibitory effects on BuChE may be due to such interactions, unlike AChE which was less responsive to changing structure of dialkylamine groups. Hence, changing the structure of dialkylamino groups of alkoxyl radicals in PC inhibitors is one of factors responsible for the selectivity with respect to BuChE.

As judged by comparison of K^0_{BuChE} in pairs of the studied inhibitors (e.g. substances 1 and 2; 3 and 4; 5 and 6; 7 and 8; 11 and 12), there were not marked differences in anti-BuChE activity between dialkylamino and trialkylammonium derivatives.

It should be stressed here that the division of PC in two groups—free N-containing bases and tetraalkylammonium salts, is rather conditional: at least 75% of the trialkylamino groups are transformed after protonation into trialkylammonium salts under the given experimental conditions (pH = 8):

In our experiments, PC inhibitors were used in concentrations exceeding those of active BuChE's centers by several orders of magnitude; hence, trialkylammonium salt may be an active principle of the studied substances. Presumably, all the above facts may explain the similar inhibitory activity of both dialkylamino and trialkyammonium derivatives.

However, the pattern of AChE inactivation by the studied PC substances has certain specific features. Thus, comparison of K^0_{AChE} in pairs of the inhibitors (substances 1 and 2, 3 and 4, 5 and 6, 7 and 8, 13 and 14) showed that, excluding substances 11 and 12, the anti-AChE activity of iodomethylated derivatives was always lower than that of free bases. After methylation of the piperidine group of substance 9 (irreversible AChE inhibitor), its inhibitory properties changed. Substance 10, an iodomethylated derivative of substance 9, lost the ability to

induce irreversible inhibition of AChE, being transformed into a reversible inhibitor with $K_i = 9{,}8 \times 10^{-6}$ M, similar to decamethonium ($K_i = 5.8 \times 10^{-6}$ M).

CONCLUSIONS

Based on these results, we are thus able to conclude that the selectivity of PC inhibitors with respect to BuChE, as a rule, increases from O-2-dialkylaminocyclohexyl—containing substances to the corresponding iodomethylated derivatives.

Our experimental data led us to the following suggestion. The active BuChE's surface is constructed so to exert relatively weak steric hindrances to ligands during formation of an enzyme—inhibitor complex, while the structure of AChE's active surface strongly limits this process. In our view, the data on BuChE and AChE inhibition by the studied N,N-dialkylphosphoramidofluoridates prove the above suggestion. The revealed differences in inhibitory effects on both BuChE and AChE are of importance for further design of new selective BuChE inhibitors.

It's commonly accepted now that the mechanisms of time—progressing irreversible inhibition by PC substances are virtually identical for both BuChE and AChE and involve the formation of an enzyme—inhibitor complexes followed by phosphorylation of cholinesterases resulting in their irreversible inactivation.[9,10] The study of the properties of substance 10 showed that the capability of PC inhibitors to phosphorylation was a necessary but not sufficient condition for irreversible cholinesterases inhibition to develop in time. The irreversible inhibition of BuChE proceeded readily with high rates, being reversible in the case of AChE, nevertheless. Thus, results of our experiments point to an important functional role of enzyme—inhibitor complexes in the irreversible inactivation of BuChE.

Possibly, after interaction of a large-size tetraalkylammonium group (substance 10) with the cation - binding site of AChE's active surface, the functional groups of an acidic part of the inhibitor molecule can't come closer together with acyl-binding AChE's site. The impossibility of required enzyme—inhibitor contact may be due to steric obstacles in the active AChE's surface and, possibly, to the changes of enzyme conformation (in this case at least), which are associated with the effect of tetraalkylammonium salts on AChE.[16–18] These facts need further corroboration.

Among the studied dialkylphosphoramidofluoridates which contain dialkylamino or trialkylamino groups in the alkoxyl radical, we found powerful highly selective inhibitors of BuChE.

EXPERIMENTAL

General

The ^{31}P NMR spectroscopy was perfomed on a "Varian—HA—100D" spectrometer (40.5 Mhz, 85% H_3PO_4 as standard), ^{19}F spectra were recorded by using a "Varian—EM—390"—spectrometer (84.7 Mhz, freon-12 as standard), ^1H spectra were obtained by using a "Varian—EM—390" spectrometer (90 Mhz, $(CH_3)_4Si$ as standard). IR spectroscopy was perfomed on "Perkin-Elmer" M-580, M-283 spectrophotometers.

Values of the rate and equilibrium constants that characterize the enzyme inhibition by studied substances were determined by potentiometric titration with the use of an automatic titrator RTS-822 (Radiometer) in pH-state mode. pKa values were determined by procedure[19] with the using autotitration system DTS-833 (Radiometer) having the 2,5 ml burette filled with 0.1M HCl.

Materials

We synthesized the following starting compounds by using the corresponding techniques, namely for: N,N-dialkylphosphoramidic difluoride[20] and trans-2-N,N-dialkylaminocyclohexanols.[21] The other starting reagents of various chemical companies were purified before use.

Preparation of N,N-diethyl-O-(trans-2-piperidinocyclohexyl)phosphoramidofluoridate (substance 9, Table I)

Suspension of 0,1M trans-2-piperidinocyclohexyloxy sodium in 100 ml of benzene is added to a solution of 0.11M of N,N-diethylphosphoramidic difluoride in 100 ml of benzene. The precipitate is filtered off, the solvent is evaporated and the residue is distilled. The yield is 72%.

Substances 3,5,7,11, 13 listed in Table I were obtained in a similar way.

Preparation of N,N-dimethyl-O-(trans-2-N,N-dimethylaminocyclohexyl)phosphoramidofluoridate (substance 1, Table I)

Tris(trans-2-N,N-dimethylaminocyclohexyloxy)aluminum (0,013M) in absolute benzene (50 ml) was added to N,N-dimethylphosphoramidic difluoride (0.039M) dissolved in absolute toluene (50 ml). The mixture was stirred for 8 hours, the precipitate was filtered off and the residue was distilled.

Substance 15 from the Table I was obtained in a similar way.

Preparation of N-(trans-2-N',N'-dimethylamidofluorophosporyloxycyclohexyl)-N,N,N-Trimethylammonium iodide (substance 2, Table I)

0.03 M Iodomethane are added to a solution of 0,015 M trans-2-N,N-dimethylaminocyclohexyl ester of N,N-dimethylphosphoramidofluoric acid in 30 ml of benzene. The precipitate is filtered off, washed three times with 100 ml portions of benzene and dried in vacuum The yield is 93%.

Substances № 4, 6, 8, 10,12,14 were obtained in a similar way (Table I).

Preparation of tris(trans-2-N,N-dimethylaminocyclohexyloxy)aluminium

Trans-2-N,N-dimethylaminocyclohexanol (0,05M) and tris(isopropoxy)-aluminium (0,016M) were placed in a distillation flask and were heated. After isopropanol (2.5 ml) had distilled off, the residue was kept under oil pump vacuum and distilled. B.P. = 195–7/0,1 tort Yield = 75%. ^{1}H NMR δ: 1,2 and 1,7, m, 8H [$(CH_2)_4$]; 2,2, c, 6H [$N(CH_3)_2$]; 2,3, m, 1H [NCH]; 3,3, m, 1H (OCH).

The data of the elementary analysis for the synthesized compounds are in accord with the calculated values. The NMR data and physical properties are given in Table II and the IR data are listed in Table III.

Determination of rate constants of BuChE and AChE inhibition (k^0_{BuChE} and k^0_{AChE})

Solutions of AChE and BuChE (0,1–0,4ml) were added to 35 ml (AChE) or 25 ml (BuChE) of a salt solution, containing 0,15M NaCl, 3,2mM KCl, 1,2mM $CaCl_2$. The mixture was thermostated in a titrator cell at 25°C (pH 8,0) for 10 min. Aliquots of inhibitor solution (0,05–0,5 ml) were added and the mixture was incubated for 45 sec. Inhibitors were added in such concentrations to reach the residual enzymatic activity of 20–80% of the initial. An interaction between

TABLE II Data of NMR spectroscopy and BP/MP of final substances.

#	BP°C/mm Hg MP°C	$\delta(^{19}F)$ $J_{F\text{-}P}Hz$	$\delta(^{31}P)$ $J_{P\text{-}F}Hz$	$\delta(^{1}H)$
1	*2*	*3*	*4*	*5*
		74, d		1.3, 1.8 m., 8H, $[(CH_2)_4]$;
1	86–7/0,2	931	4.1, d	2.4, s, 6H, $[N(CH_3)_2]$; 2.5, m., 1H, [NCH];
	–	76, d	931	2.7, d, 6H, $[N(CH_3)_2]$; $J_{H\text{-}P}$ = 10 Hz,
		931		5.2, m., 1H, [OCH].
		75, d		1.4, 1.9 m., 8H, $[(CH_2)_4]$;
2	–	930	5.3,d	2.7, d, 6H, $[N(CH_3)_2]$; $J_{H\text{-}P}$ = 10 Hz,
	135–6	77, d	930	3.4, s, 6H, $[N(CH_3)_3]$; 4.2, m., 1H, [NCH];
		930		5.4, m., 1H, [OCH].
		74, d		1.3, t, 6H, $[(CH_3)_2]$; $J_{H\text{-}H}$ = 6 Hz,
3	86–8/0,8	930	4.1, d	1.4, 1.9 m., 8H, $[(CH_2)_4]$; 2,7, m, 4H, $[N(CH_2)_2]$;
	–	76, d	930	2.8, m., 1H, [NCH];
		930		2.9, d, 6H, $[N(CH_3)_2]$; $J_{H\text{-}P}$ = 10 Hz, 5.3, m., 1H, [OCH].
		71, d		1.3, t, 6H, $[(CH_3)_2]$; J = $6_{H\text{-}P}$ Hz,
4	–	959	3.8,d	1.4, 1.9 m., 8H, $[(CH_2)_4]$; 2.9, d, 6H, $[N(CH_3)_2]$; $J_{H\text{-}P}$ = 10 Hz,
	102–103	73, d	959	4.1, m, 4H, $[N(CH_2)_2]$;
		959		4.5, m., 1H, [NCH]; 5.3, m., 1H, [OCH].
		68, d		1.1, t, 6H, $[(CH_3)_2]$; $J_{H\text{-}P}$ = 6 Hz,
5	111–2/0,3	931	4.7, d	1.2, 1.7 m., 8H, $[(CH_2)_4]$; 2.2, s, 6H, $[N(CH_3)_2]$;
	–	73, d	931	2,3, m., 1H, [NCH];
		931		2.8, m, 4H, $[N(CH_2)_2]$; 4.3, m., 1H, [OCH].
		67, d		1.4, t, 6H, $[(CH_3)_2]$; $J_{H\text{-}P}$ = 6 Hz,
6	–	960	3.1, d	1.8, 2.5 m., 8H, $[(CH_2)_4]$; 3.3, m, 4H, $[N(CH_2)_2]$;
	96–7	69, d	960	3.5, s, 9H, $[N(CH_3)_3]$;
		960		4.4, m., 1H, [NCH]; 5.2, m., 1H, [OCH].
		70, d		1.1, t, 6H, $[(CH_3)_2]$, $J_{H\text{-}P}$=7 Hz;
7	117–8/0,1	5.5, d		1.3, m., 12H, $[(CH_2)_4]$; $[(CH_2)_2]$; 2.4, m., 4H, $[N(CH_2)_2]$;
	–	72, d	960	2.6, m., 1H, [NCH];
		960		3.1, m., 4H, $[N(CH_2)_2]$; 4.2, m., 1H, [OCH].
		67, d		1.2, t, J = 7Hz, 6H, $[(CH_3)_2]$;
8	123–5	960	5.0, d	1.4, m., 12H, $[(CH_2)_4]$; $[(CH_2)_2]$; 3.3, m., 4H, $[N(CH_2)_2]$;
	–	69, d	960	3.9, s., 3H, $[NCH_3]$;
		960		4.1, m., 4H, $[N(CH_2)_2]$; 4.3, m., 1H, [NCH]; 5.0, m., 1H, [OCH].
		67, d		1.1, t, 6H,$[(CH_3)_2]$, $J_{H\text{-}P}$ = 7Hz;
9	126–7	958	4.1, d	1.4, 1,7, m, 14H, $[(CH_2)_4]$; $[(CH_2)_3]$;
		73 d		2.4, m, 4H, $[N(CH_2)_2]$;
	0,1	958	958	2.6, m, 1H, [NCH]; 3.1, m, 4H,$[N(CH_2)_2]$; 4,3, m, 1H,[OCH].
		68, d		1.2, t, 6H, $[(CH_3)_2]$, $J_{H\text{-}P}$ = 7Hz;
10	—	958	4.3, d	2.0, m, 14H, $[(CH_2)_4]$; $[(CH_2)_3]$; 3.0, m, 4H,$[N(CH_2)_2]$;
	140–2	75, d	958	3.2, s, 3H, $[NCH_3]$;

TABLE II *continued*

#	*BP°C/mm Hg MP°C*	$\delta(^{19}F)$ $J_{F-P}Hz$	$\delta(^{31}P)$ $J_{P-F}Hz$	$\delta(^{1}H)$
1	*2*	*3*	*4*	*5*
		958		3.7, m, 9H,[NCH,N(CH_2)$_2$)]; 4.9, m, 1H, [OCH].
		71, d		0.9, t, 3H,[CH_3], J_{P-H} = 7 Hz;
11	110–2	960	4.3, d	1.1, t, 3H,[CH_3]; J_{P-H} = 7Hz 1.3, 1.7, m, 8H, [(CH_2)$_4$];
	0,1	73, d	960	1.4, m, [CH_2]
		960		2.3, s, 6H,[N(CH_3)$_2$]; 2.5 m, 1H, [NCH] 3.0, m, 4H, [NCH_2,(NCH)$_2$]; 4.3, m, 1H,[OCH].
		72, d		1,0, t, 3H, [(CH_3)$_2$], J_{P-H} = 7Hz;
12	–	964	4.5, d	1.2, t, 3H,[CH_3] J_{P-H} = 7Hz; 1.6, m, 8H, [(CH_2)$_4$];
	55–7	74, d	964	3.2, m, 4H,[NCH_2,(NCH)$_2$];
		964		3.7, s, 9H,[N(CH_3)$_3$]; 4.0, m, 1H, [NCH] 4.8, m, 1H,[OCH].
		61, d		1.2, d, 12H,[(CH_3)$_4$], J_{P-H} = 7Hz;
13	112/0.1	960	4.1, d	1.3, 1.7, m, 8H, [(CH_2)$_4$]; 2.2, s, 6H,[N(CH_3)$_2$];
	-	67, d	960	2.3, m, 1H,[NCH];
		960		3.3, m, 2H,[N($CHMe_2$)$_2$]; 4.3, m, 1H, [OCH].
		63, d		1.2, d, J = 7Hz, 12H,[(CH_3)$_4$];
14	–	960	4.6, d	1.3, 1.7, m, 8H, [(CH_2)$_4$]; 3.3, m, 2H, [N($CHMe_2$)$_2$];
	175–6	69, d	960	3.5, s,9H,[N(CH_3)$_3$];
		960		3.7, m, 1H,[NCH]; 4,9, m, 1H, [OCH].
15	56/0.1	73, d	4.3, d	1.2, 1.8 m, 10H, [(CH_2)$_5$]; 2.9, d, 6H, [N(CH_3)$_2$]; J_{H-P} = 10 Hz, 5.0, m, 1H, [OCH].
	-	941	941	

the enzyme and inhibitor was stopped by adding 5ml (for AChE) or 15ml (for BuChE) of 0,018M substrate solution. Acetylcholine iodide was used by us as a substrate in assays of both enzymes. The progress of the enzymatic reaction was followed by the uptake of 0,02M NaOH. Values of enzymatic activity were derived from the slopes of plotted kinetic curves in the presence or absence of the inhibitor. Bimolecular rate constants k^0 were calculated by the following equation:

$$k^0 = \frac{1}{t \cdot [i]} \ln \frac{V_0}{V_i}$$

where v_i and v_0 are reaction rates in the presence and absence of the inhibitor respectively, [i]—concentration of inhibitor before addition of the substrate, M, t—time of incubation, min.

TABLE III Data of IR spectroscopy of final substanses

#	*Frequency of absorption bands* cm^{-1}				
	P-F	*P-N(CH₃)₂*	*P-O-C*	*P=O*	*Bohlmann band*
1	856	1317	1004	1284	2820
2	850	1327	1015	1279	–
3	857	1324	1006	1283	2814
4	850	1327	1015	1279	–
5	856	–	1030	1286	2782
6	865	–	1030	1286	–
7	845	–	1029	1285	2802
8	852	–	1025	1278	–
9	862	–	1029	1285	2802
10	863	–	1004	1278	–
11	864	–	1022	1288	2782
12	852	–	1020	1280	–
13	833	–	1008	1281	2783
14	850	–	1020	1279	–
15	856	1317	1004	1284	–

Determination of the constant of the reversible AChE inhibition

To determine the constant of reversible AChE inhibition by 1-(trans-2-N,N-diethylamidofluorophosphoryloxycyclohexyl)-1-methylpiperidinium iodide (substance 10 Table I), initial rates of acetylcholine hydrolysis were measured for different concentrations of the substrate with and without the inhibitor. This reaction was initiated by adding 0,3 ml of the enzyme solution to 40 ml of the salt solution which contained a certain concentration of the substrate and was previously thermostated in the titrator cell at 25°C (pH 8,0) for 10 min. The progress of the enzymatic reaction was monitored by the 0,02M NaOH uptake. Within 1–2 min of the reaction onset, 0,05 ml of the inhibitor solution was introduced into the reaction mixture. The most preferential inhibitor concentration (in our case 0,017 mM) was enough to record sharp changes in the kinetic curve slopes for all the substrate concentrations ranging from 0,25–0,75 mM. Values of K_i—were calculated by the Hunter-Downs graphic method.[22]

Determination of pI_{50}—Values

Often it seems convenient to use a parameter pI_{50} that is a reciprocal logarithm of the concentration at which a half inhibition of enzymes is attainable under experimental conditions. Values of pI_{50} were determined by the aforementioned procedure of reversible inhibition rate constants measuring only for those substances that displayed the reversible inhibitory effect. Experiments were performed at the constant substrate concentration equal to 2,3 mM (for AChE) or 7,0 mM (for BuChE). Each of the studied substances was characterized in 4–5

independed experiments with the use of those inhibitor concentrations at which the residual enzymatic activity ranged from 20% to 80% of the initial. Calculation of pI_{50} values were derived from a plotted dependence of the residual enzymatic activity (expressed as percentage of the initial) versus a logarithm of the inhibitor concentration in the titrator cell.

Determination of "water—n-octanol" partition coefficients

Determination of partition coefficients were carried out in accordance with the recommendations written in the monograph;[123] 0,05 M Borate buffer (pH 8,0) was used as the water phase. For determining the concentration of studied substances in water phase the method based on their capability of inhibiting BuChE was used. Experimental conditions were the same as described for determination of BuChE inhibition rate constant. Working solutions were prepared by diluting the stock solution of the known concentration. The residual enzyme activity was assessed for 4–5 concentrations of this substance and the calibration curve "percentage of residual activity" versus "logarithm of inhibitor concentration" was plotted. Aliquots of water phase or its diluted samples were then incubated with the enzyme in titrator cell as described above. Concentrations of the substance were derived from the calibration curve.

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