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THE REACTION OF ACETYLCHOLINESTERASE WITH *O*-DIMETHYLCARBAMYL ESTERS OF QUATERNARY QUINOLINIUM COMPOUNDS*

RICHARD J. KITZ, SARA GINSBURG and IRWIN B. WILSON†

Departments of Anesthesiology, Biochemistry, and Neurology, College of Physicians and Surgeons, Columbia University, New York, N.Y., U.S.A.

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Abstract—The dimethylcarbamate esters of some mono- and diquaternary quinolinols were prepared and their anticholinesterase properties investigated by using acetylcholinesterase from electric eel. The I_{50} values in the steady state of equal rates of carbamylation and decarbamylation were measured and the values of the second-order rate constants for carbamylation were evaluated. Compounds containing the dimethylcarbamyl function substituted in the 5 and 7 positions were of the same order of activity as neostigmine to which they are structurally related. When substituted in the 3 position, the dimethylcarbamyl derivative was about as active as pyridostigmine which is structurally similar. It was concluded that the structure of the leaving group is important in determining the activity of these compounds and the pK_a value of the leaving group appears to be relatively unimportant.

SEVERAL carbamates containing catonic ammonium functions, such as physostigmine, neostigmine, and pyridostigmine, are potent inhibitors of acetylcholinesterase and are useful in the treatment of some diseases.[1] Other carbamates are important insecticides.[2] Although usually considered reversible inhibitors, it is known that many carbamates react with cholinesterase to produce relatively stable carbamyl enzyme derivatives which hydrolyze, often within an hour, to restore the free and active enzyme.[3–5] The carbamyl enzyme derivatives are analogous to the normal acetyl enzyme formed during acetylcholine hydrolysis. But the acetyl enzyme requires only 100 μsec to hydrolyze. Thus these inhibitors are extremely poor substrates, as was recognized by Goldstein and Hamlesch.[6] The mechanism of inhibition by carbamates is similar to the mechanism of inhibition by irreversible inhibitors, the organophosphate and the methanesulfonate compounds. It has been suggested that these three groups of inhibitors be called acid-transferring inhibitors to denote that the inhibited enzyme is a covalent derivative formed by the transfer of an acid group, viz. a phosphoryl, carbamyl or sulfonyl function to the enzyme. The term "leaving group" refers to that portion of the inhibitor which is split out as the first product of the hydrolytic reaction. The remaining portion of the inhibitor, the acyl group, is transferred to the active site forming the covalent, acyl enzyme derivative.

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† Present address, Chemistry Department, University of Colorado, Boulder, Colo., U.S.A.

Although many carbamate inhibitors have been synthesized, those containing a quaternary quinolinium function in the leaving group have not been studied. It appeared of some interest to study such compounds because they have a structural similarity to neostigmine and pyridostigmine and because some of the corresponding *o*-diethylphosphoryl esters are extremely potent inhibitors.[7, 8]

In this paper we report the synthesis of a series of dimethylcarbamyl esters of some mono- and diquaternary quinolinols and their reaction with acetylcholinesterase (E.C. 3.1.1.7). We hoped that the comparison of the anticholinesterase activity of these compounds with others might enable us to discern the relative importance of molecular complementarity as against the intrinsic ability of the hydroxy compound to serve as a leaving group. This latter quality is related to the ease with which the compound undergoes hydrolysis; complementarity includes all factors which allow for appropriateness of "fit" between enzyme and inhibitor.

Carbamylation of the enzyme was carried out in the absence of acetylcholine and acetylcholine was added after suitable times only to measure the amount of enzyme which had not carbamylated.

EXPERIMENTAL PROCEDURE

Synthesis

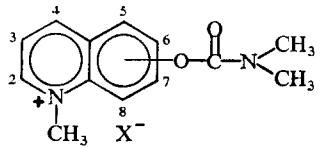
Dimethylcarbamyl quinolinols. Quinolinols were dissolved in a 1 N methanolic solution of sodium methylate (1·1 M for each M of quinolinol), dimethylcarbamyl-chloride (1·2 M) was added, and the mixture was heated on a steam bath for 5 min. After 10 min at room temperature the reaction mixture was diluted with water and extracted with chloroform. After washing with a 0·1 N solution of NaOH and then with water, the chloroform layer was decolorized and the solvent evaporated. The crude product (low melting solids) was sufficiently pure to be used for subsequent preparations without distillation. Yields varied from 30–40 per cent, except for the 8 quinolinol, which was 80 per cent. Up to 50 per cent of starting material was recovered from the mother liquors.

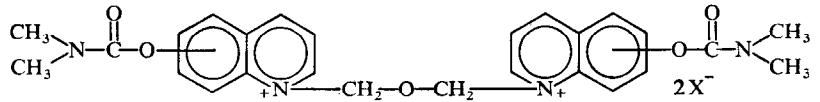
Quaternization. The *N*-methyl quaternary salts were prepared in the usual manner by heating the carbamyl esters (dissolved in dimethylformamide) with an excess of methyl iodide on a steam bath for 15–20 min. The product was precipated with ether and purified by recrystallization from methanol, in some cases with added ether. Yields were about 50 per cent. Microanalysis data appear in Table 1.

Diquaternary compounds containing an N,N′*dimethylene ether bridge. Bis* (chloromethyl) ether was transformed to the iodo-derivative with excess sodium iodide in acetone. Sodium chloride was filtered off. The dimethylcarbamylated quinolinol in acetone was added to the diiodoether and the mixture was left at room temperature for 1–2 hr. The precipitated product was collected and, if relatively high melting, it was purified by recrystallization from methanol. However, if purification was difficult, it was dissolved in hot water or aqueous methanol and transformed into the picrate by addition of an aqueous solution of sodium picrate. The picrate compounds were recrystallized from a mixture of methanol and acetone. Yields averaged 20 per cent. Microanalysis data appear in Table 1.

Picrate analysis. The formula weight of the picrate compounds was correct to ±1·5 per cent when analyzed by the picrate method previously described.[7]

TABLE 1. MONO- AND DIQUATERNARY DIMETHYLCARBAMYL ESTERS OF QUINOLINOLS





Cmpd. no.	†	X	m.p.*	Formula	Group A Calcd. (%) C	H	N	I	Group B Found (%) C	H	N	I
				Group A								
1	3	Picrate§	148°	$C_{19}H_{17}N_5O_9$	49·67	3·73	15·25		49·84	4·00	15·11	
2	5	I	192°	$C_{13}H_{15}N_2O_2I$				35·43				36·05
3	6	I	144°	$C_{13}H_{15}N_2O_2I$				35·43				35·16
4	7	I	197°	$C_{13}H_{15}N_2O_2I$				35·43				34·95
5	8	Picrate	232°[16]	$C_{19}H_{17}N_5O_9$	49·67	3·73	15·25		49·26	3·62	14·45	
6	5 iso	I	212°	$C_{13}H_{15}N_2O_2I$	43·59	4·22	7·82	35·43	43·81	4·43	7·46	35·06
				Group B								
7	5	Picrate	195°	$C_{38}H_{32}N_{10}O_{19}$								
8	6	Picrate	171°	$C_{38}H_{32}N_{10}O_{19}$								
9	7	Picrate	184°	$C_{38}H_{32}N_{10}O_{19}$								
10	5 iso	I	221°	$C_{22}H_{28}N_4O_5I_2$	42·76	3·86	7·67	34·75	42·32	3·96	7·65	34·41
11	‡	I	183°	$C_{18}H_{24}N_4O_5I_2$				40·28				40·47

* Melting points were taken on a Uni-Melt apparatus.

† Number refers to the position of substitution on the ring.

‡ Hydroxy-1-methylquinolinium by 3-hydroxy-1-methyl pyridinium.

§ For picrate analysis, see text.

Enzyme

Acetylcholinesterase (EC 3.1.1.7) was prepared from *Electrophorus electricus*.[9] The preparation had an activity of 90 m-mole of acetylcholine (ACh) hydrolyzed/min/mg protein when measured by automatic titration at 25°, pH 7, in a medium consisting of 0·1 M NaCl, 0·02 M $MgCl_2$, 0·005% gelatin, 1×10^{-5} EDTA, and 1×10^{-3} ACh. A K_m value of $9{\cdot}1 \times 10^{-5}$ mole/l. was measured.

Assay technique

The carbamylation reaction was initiated by the addition of appropriate amounts of inhibitor to a solution of enzyme. The rate of progressive inhibition was followed by withdrawing samples at some 7–10 time intervals and assaying in the presence of

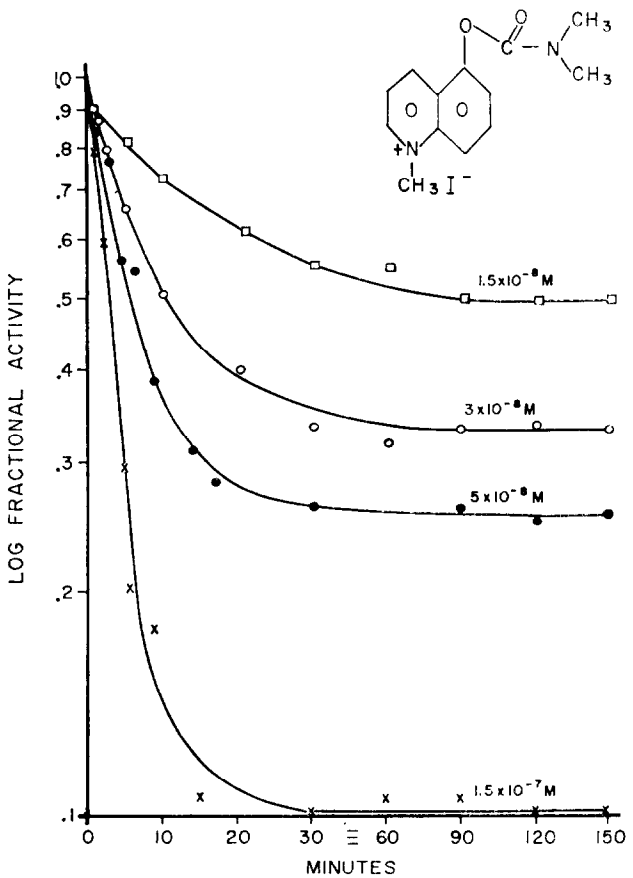


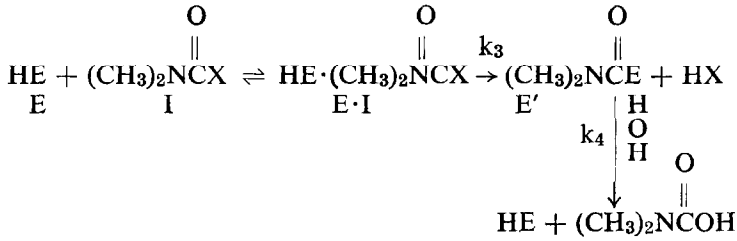
FIG. 1. Acetylcholinesterase at pH 7, 25°, was allowed to react with different concentrations of compound 2, 5-dimethylcarbamyloxy-*N*-methyl quinolinium iodide. A steady state was reached in which the rate of inhibition (k'_3) was equal to the rate of spontaneous recovery (k_4). The value of the ratio of inhibited enzyme to free enzyme, $(E'/E)_{ss}$ was obtained from the horizontal part of the curve and used to calculate k'_3/k_4 (see text). The value of the reciprocal k_4/k'_3 is equal to $(I_{50})_{ss}$, the concentration of inhibitor necessary to carbamylate 50 per cent of the enzyme in the steady state. Note the change in time scale at 30 min.

ACh. The activity was measured by the decrease in the substrate concentration in 2 min. Acetylcholine was determined by the hydroxamic method described by Hestrin.[10] The medium was $1{\cdot}8 \times 10^{-3}$ M ACh (initial concentration), 0·1 M NaCl, 0·01 M $MgCl_2$, 0·02 M sodium phosphate, 0·005% gelatin, 1×10^{-5} M EDTA at 25°, pH 7.

The relative amounts of enzyme activity so determined were plotted as a function of time. Typical data are plotted in Fig. 1. The reaction was followed until a steady state was maintained for 1 hr. The values for $(I_{50})_{ss}$ and for the second-order rate constants could then be evaluated (see Results). Measurements were made at three or four different inhibitor concentrations varied over a 4- to 10-fold range.

RESULTS AND DISCUSSION

The reaction of acetylcholinesterase with dimethylcarbamate inhibitors is given by the formal scheme:



where E is the enzyme, I the inhibitor, E·I the enzyme inhibitor complex, E′ the carbamyl enzyme, and X is the leaving group. A steady state is approached (Fig. 1) in which the rate of carbamylation equals the rate of decarbamylation and

$$\left(\frac{\mathscr{E}}{E'}\right)_{ss} = \frac{k_4}{k_3}\left(1 + \frac{K}{I}\right)$$

where

$$(\mathscr{E}) = (E) + (E{\cdot}I)$$

The measured enzyme activity is proportional to $(\mathscr{E})$ because in the assay the enzyme solution is diluted into acetylcholine solution and E·I almost completely dissociates. The value of k_4, $2{\cdot}6 \times 10^{-2}$ min^{-1} (including the concentration of water), is the same for all dimethylcarbamates and was previously evaluated by diluting a solution of inhibited enzyme and measuring the rate of return of enzyme activity.[4]

Under our conditions of measurement, (I) was considerably less than the value of K and the steady state equation reduces to

$$\left(\frac{E'}{\mathscr{E}}\right)_{ss} = \frac{k_3/K}{k_4}(I) = \frac{k'_3}{k_4}(I)$$

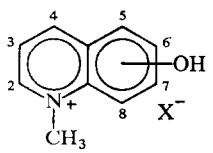
$$(I_{88})_{ss} = \frac{k_4}{k'_3}$$

where k'_3 is the second-order rate constant for carbamylation.

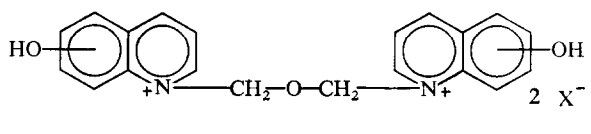
By measuring $(E'/\mathscr{E})_{ss}$ at several values of (I), the value of $(I_{50})_{ss}$ was obtained and since k_4 is known k'_3 was also obtained.

All compounds tested showed progressive development of inhibition and spontaneous recovery of activity upon dilution, as expected for a steady state in whcih the rates of carbamylation and decarbamylation are equal. Typical data showing the approach to the steady state are presented in Fig. 1. The concentrations of inhibitor that produce 50 per cent carbamylation in the steady state, $(I_{50})_{ss}$, and k'_3 values are given in Table 2. Second-order rate constant values for phosphorylation of the enzyme by analogous *o*-diethylphosphoryl derivatives and the pK_a values of the leaving groups were previously measured[8] but are included to facilitate comparisons.

TABLE 2. STRUCTURAL RELATIONSHIPS AND RATE CONSTANT VALUES FOR THE CARBAMYLATION OF ACETYLCHOLINESTERASE BY DIMETHYLCARMATES



Group A



Group B

Cmpd. no.	Position of substitution on ring	Leaving group	pK_a of leaving group	$(I_{50})_{ss}$	k'_3 carbamylation	k'_3 phosphorylation
1	3	A	5·2	$6{\cdot}8 \times 10^{-6}$	$3{\cdot}8 \times 10^{3}$	$1{\cdot}2 \times 10^{8}$
2	5	A	6·1	$1{\cdot}6 \times 10^{-8}$	$1{\cdot}6 \times 10^{6}$	$2{\cdot}4 \times 10^{6}$
3	6	A	7·0	$7{\cdot}7 \times 10^{-6}$	$3{\cdot}4 \times 10^{3}$	$9{\cdot}3 \times 10^{6}$
4	7	A	5·7	$6{\cdot}1 \times 10^{-8}$	$4{\cdot}2 \times 10^{5}$	$1{\cdot}2 \times 10^{8}$
5	8	A	6·3	$6{\cdot}8 \times 10^{-4}$	$3{\cdot}8 \times 10$	$5{\cdot}3 \times 10^{2}$
6	5*	A	6·8	$7{\cdot}5 \times 10^{-8}$	$3{\cdot}5 \times 10^{5}$	$6{\cdot}5 \times 10^{4}$
7	5	B		$1{\cdot}3 \times 10^{-6}$	$2{\cdot}1 \times 10^{4}$	
8	6	B		$1{\cdot}1 \times 10^{-6}$	$2{\cdot}3 \times 10^{4}$	$2{\cdot}7 \times 10^{8}$
9	7	B		$5{\cdot}1 \times 10^{-7}$	$5{\cdot}1 \times 10^{4}$	
10	5*	B		$3{\cdot}8 \times 10^{-8}$	$6{\cdot}8 \times 10^{5}$	$8{\cdot}1 \times 10^{5}$
11	†	B		$2{\cdot}4 \times 10^{-6}$	$1{\cdot}1 \times 10^{4}$	
12	†	A	5·0	$1{\cdot}6 \times 10^{-6}$	$1{\cdot}6 \times 10^{4}$	
13	‡	A	8·0	$2{\cdot}7 \times 10^{-8}$	$9{\cdot}5 \times 10^{5}$	4×10^{6}¶
14	§		8·9	$7{\cdot}6 \times 10^{-10}$	$4{\cdot}0 \times 10^{6}$	
15	$(CH_3)_3N^+CH_2CH_2OH$		14·0‖	$8{\cdot}0 \times 10^{-5}$	$3{\cdot}2 \times 10^{2}$	0
16	HF		3·5	$1{\cdot}4 \times 10^{-5}$	$1{\cdot}8 \times 10^{3}$	$2{\cdot}5 \times 10^{5}$

The $(I_{50})_{ss}$ values are the concentrations of inhibitor that produce 50 per cent carbamylation of the enzyme in the steady state in the absence of substrate. The k'_3 values are the second-order rate constants for carbamylation or phosphorylation of the enzyme in the absence of substrate. Compound 14 has a different carbamyl group; the general formula is X $CONH(CH_2)_{10}NH$ CO X where HX is 3-hydroxy-*N,N,N*-trimethylanilinium bromide. The $(I_{50})_{ss}$ value for this compound is calculated from directly measured k'_3 value. In the other cases, the value of $(I_{50})_{ss}$ was measured and k'_3 calculated by using a k_4 value of $2{\cdot}6 \times 10^{-2}$ min^{-1} (see text). Compound 11 is pyridostigmine and compound 13 is neostigmine. Dimensions of $(I_{50})_{ss}$ are mole $liter^{-1}$ and k'^3 liter · $mole^{-1}$ min^{-1}. The phosphorylation data were taken from Reference 8 and are the second-order rate constant values for inhibition by the diethylphosphoryl analogues of the appropriate carbamates. Carbamylation data for compounds 12, 13, 15, and 16 are from Reference 4.

* Hydroxy-1-methylquinolinium replaced by 5-hydroxy-2-methylisoquinolinium.

† Hydroxy-1-methylquinolinium replaced by 3-hydroxy-1-methylpyridinium.

‡ Hydroxy-1-methylquinolinium replaced by 3-hydroxy-N,N,N-trimethylanilinium.

§ Compound 14 is BC-48,[14, 15] see text.

‖ Estimated value.

¶ Value calculated from data in reference 18.

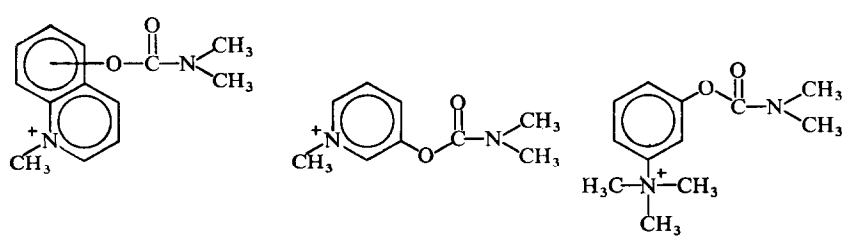
A priori, the rate of carbamylation might be expected to depend principally upon two factors: (1) The intrinsic ability of the compound to act as a carbamylating agent. The rate of hydrolysis of the compound or the pK_a of the leaving group might be taken as an approximate measure of this characteristic. (2). Molecular complementarity between enzyme and inhibitor.

A sensitive direct relationship between the rate of inhibition and the rate of hydrolysis[11] or the acidity of the leaving group[8] was found for a group of diethylphosphoryl esters which do not contain a cationic nitrogen function. However, even in those compounds containing a quaternary nitrogen function, the acidity of the leaving group appears to be important.[8] On the other hand, the more potent uncharged carbamate inhibitors are those that are more difficult to hydrolyze.[12]

In this study no relationship between the pK_a value of the leaving group and the inhibitory strength of the compound is apparent. The quaternary compounds studied, except for the choline derivative, had leaving groups with relatively similar pK_a values varying in the relatively narrow range of less than 3 orders of magnitude. It is possible that a relationship would emerge if the compounds contained leaving groups with widely varying values of pK_a. The dominance of molecular complementarity revealed by this study might possibly mask such a relationship of secondary importance.

The greater importance of molecular complementarity in the carbamate series as compared with the organophosphates is suggested by the fact that the choline derivatives of the organophosphates are not active. The organophosphate derivative of the much more acidic thiocholine is a very potent inhibitor,[13] but the carbamate derivative of thiocholine is a poorer inhibitor than the corresponding choline compound.[4] Although other features may be involved, the reaction of an organophosphate with the enzyme seems to be primarily a nucleophilic reaction that can be facilitated by the enzymic binding of a cationic ammonium function contained in the leaving group. But this description does not seem satisfactory for the reaction of a carbamate with the enzyme, for here the precise structure of the inhibitor is important as is shown by the wide range of 3–5 orders of magnitude in the values of the second-order rate constants. However, no trend is noted within a relatively narrow range of pK_a values. The interaction of carbamate inhibitors and the enzyme must be much more specific than is the case with phosphate inhibitors.

The relationship between the quinoline derivatives and neostigmine and pyridostigmine is shown in Fig. 2. If we consider that the quaternary ammonium



Hydroxy-1-methylquinolimium dimethylcarbamate | Pyridostigmine | Neostigmine

FIG. 2. The spatial relationship of hydroxyquinolinium dimethylcarbamates to pyridostigmine and neostigmine. The carbamate ester function in the 3 position of *N*-methyl quinolinium corresponds to pyridostigmine; in the 5 and 7 positions it is similar to neostigmine.

structure is bound at the anionic site of the enzyme, the carbamate ester function in the 3 position of quinolinium corresponds to pyridostigmine and in the 5 and 7 positions to neostigmine. Data in Table 2 indicate that the rates of inhibition are reasonably similar for the 3 ester of quinoline and pyridostigmine, and for the 5 and 7 quinoline esters and neostigmine. The very low activity of the 8 ester is probably caused by steric hindrance.

In the case of diethylphosphate inhibitors previously reported,[8] it was found that all of 6 diquaternary compounds bridged through the quaternary nitrogen atoms were at least 10 times more active than the parent monoquaternary compounds. Two of these compounds are listed in Table 2. In the case of the carbamates, only 1 of 6 *bis* compounds is distinctly more active than the parent compound and 2 are distinctly less active. Five of the 6 *bis* compounds have similar activities which vary within a factor of 5, although the parent monoquaternary analogues differ in activity by a factor of 500.

Compound 14, BC 48[14, 15] is a *bis* compound bridged through the carbamate function and therefore entirely different from those we have been discussing. The carbamyl enzyme derived from this compound is very different from the dimethylcarbamyl enzyme derived from the other inhibitors and hydrolyzes only very slowly.[4] This inhibitor behaves therefore like the organophosphates and may be thought of as an "irreversible" inhibitor in this regard. Therefore only the rate of inhibition, the k'_3 value, is of interest. As in the case of an organophosphate compound, the steady state value, $(I_{50})_{ss}$, which has been calculated for a negligible enzyme concentration, is given in Table 2. It is of minor significance because many days would be required to reach this state. The value of the second-order rate constant is quite high, about half as great as the rate for neostigmine to which it is clearly structurally related. Other BC compounds (BC 40, BC 47, and BC 51) reacted very slowly and the k'_3 values were not measured.

Compound 8 was previoulsy prepared by Aeschlimann and Reinert.[16] They tested its miotic action in the cat's eye and its action on the isolated rabbit intestine. Blaschko *et al.*[17] reported a pI_{50} value of 7·1 with acetylcholinesterase from dog caudate nucleus and of 7·6 with serum cholinesterase from horse. These values are very different from what we have found with acetylcholinesterase from eel.

In summary, the structure of the leaving group is very important in determining the activity of carbamate anticholinesterase agents and the pK_a value of the leaving group is relatively unimportant. This conclusion is just the opposite to that reached for diethylphosphoryl anticholinesterases.

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