SYNTHESIS OF LUPININE ESTERS AND THEIR INTERACTION WITH CHOLINESTERASES

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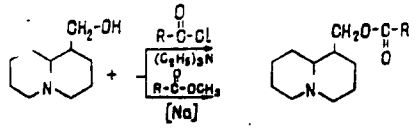
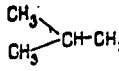
A number of lupinine esters of carboxylic acids and their hydrochlorides and methiodides have been synthesized. It has been shown that these compounds are reversible inhibitors of human blood erythrocyte acetylcholinesterase and horse blood serum butyryl cholinesterase. Noncompetitive inhibitors of these enzymes have been found among the compounds synthesized.

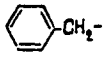
Many esters of lupinine and epilupinine, in interacting with acetylcholinesterase (ACE) and butyrylcholinesterase (BuCE), have proved to be substrates or inhibitors of these enzymes [1-4]. A number of benzoic acid esters possess well-defined local anesthetic properties [5, 6].

In order to search for biologically active substances derived from the alkaloid lupinine, we have performed the synthesis of a number of lupinine esters by the following scheme:



R = CH_3CH_2 - (I); CH_3-CH(-CH_3)- (II); $CH_3CH_2CH_2$ - (III);


(IV); C_2H_5-CH_2CH_2- (V); C_3H_7-CH_2CH_2 (VI);

$C_{13}H_{27}$-CH_2CH_2- (VII); CH_3-CH=CH- (VIII);  -CH_2- (IX).

The lupinine esters (I-VI, VIII) were obtained by reaction with the corresponding carboxylic acid chlorides in the presence of triethylamine in an absolute solvent, and the esters (VIII and IX) by the transesterification of the methyl esters of the corresponding acids with lupinine in the presence of a catalytic amount of metallic sodium in chlorobenzene solution. The lupinine esters formed oily products. Their individuality was checked by TLC and PC. Their structures were confirmed by IR and PMR spectroscopies.

The hydrochlorides and methiodides of all the compounds synthesized have been obtained. The physicochemical constants of the compounds synthesized are given in Table 1.

The interaction of compounds (I-IX) with ACE and BuCE has been studied (Table 2).

All the lupinine derivatives proved to be reversible inhibitors of both types of cholinesterase. However, depending on the structure of the inhibitor they exhibited different types of reversible inhibition (mixed and well-defined noncompetitive). With respect to their antichlinesterase properties, the compounds obtained can be divided arbitrarily into three groups. The first group of compounds is formed by the lupinine esters of propionic acid (I), isobutyric acid (II), butyric acid (III), and isovaleric acid (IV). Thus, on interaction with ACE the hydrochloride of the lupinine ester of propionic acid (I) has generalized reversible inhibition constants $\overline{K}_i$ showing, on an average, a 1.5-times more effective inhibiting activity than in the other esters (II-IV). A further lengthening of the acyl moiety of the molecule (V-VII) did not lead to any significant changes in the value of $\overline{K}_i$.

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TABLE 1. Physicochemical Constants of Lupinine Esters

Compound	n_D^{20}	d_4^{20}	R_f	Yield, %	MR_D, %		mp. of the methiodide, °C
					found	calc.	
I	1,4370	1,0317	0,60	86	63,42	62,80	115—118
II	1,4769	1,9871	0,56	85	68,65	68,04	154—156
III	1,4852	0,9961	0,68	78	68,04	68,87	131—132
IV	1,4677	0,9805	0,79	75	71,80	72,66	149—151
V	1,4833	0,9879	0,85	65	72,66	73,37	118—120
VI	1,4824	0,9816	0,75	83	77,88	77,72	126—128
VII	1,3580	0,7268	0,44	60	123,12	123,46	157—159
VIII	1,5350	1,0814	0,76	70	68,34	67,57	130—132
IX	1,6420	1,2423	0,67	80	83,58	82,91	Hygr.

Note. All the hydrochlorides obtained were hygroscopic apart from those of (VII) and (VIII), the melting points of which were 52-63°C and 142-143°C, respectively.

TABLE 2. Reversible Inhibition Constants [K_i, K_i', and $\overline{K}_i$ (M)] for ACE and BuCE of Lupinine Ester Hydrochlorides

Compound	ACE			BuCE		
	$K_i \cdot 10^{-5}$	$K_i' \cdot 10^{-5}$	$\overline{K}_i \cdot 10^{-5}$	$K_i \cdot 10^{-5}$	$K_i' \cdot 10^{-5}$	$\overline{K}_i \cdot 10^{-5}$
I	4,69	4,98	2,42	—	21,0	—
II	8,45	9,53	4,58	31,4	24,0	13,6
III	8,03	5,68	3,32	—	1,695	—
IV	8,58	9,66	4,54	—	0,31	—
V	5,56	11,6	3,76	47,6	10,4	7,50
VI	8,10	9,33	4,34	16,5	10,8	6,54
VII	6,09	7,96	3,45	13,5	10,3	5,83
VIII	29,1	0,71	0,69	3,46	4,05	1,98
IX	10,2	10,0	5,06	1,27	3,84	0,95

In interaction with BuCE, these compounds are reversible inhibitors of moderate strength and no definite dependence of the value of the generalized inhibition constant $\overline{K}_i$ on the structure of the inhibitor is observed, but a number of characteristic features of the fine mechanism of the interaction of the inhibitors with these enzymes have been recorded.

Thus, a comparison of the anticholinesterase efficiency of (I-IX) on the two enzymes — ACE and BuCE — presupposed, in our view, a predominant interaction between the acyl moiety of the effector and the esterase points of the two enzymes. In a number of compounds a qualitative jump from noncompetitive inhibition to competitive—noncompetitive was observed, which was well defined in the case of BuCE. The noncompetitive efficiency increased with a lengthening of the acyl chain for compounds (I), (III), and (IV) giving in the final account a separation into two components: competitive and noncompetitive [compound (II)].

In the case of ACE, this phenomenon was traced only from the decrease in noncompetitive efficiency, which is apparently connected with an increase in the role of the interaction with the broad hydrophobic region beyond the esterase point of the enzyme.

The three compounds (V-VII) confirm what has been said above, making it possible to assume that the region of sorption of the saturated aliphatic part of the acyl group lies within the region of the active site of the enzyme. The density of sorption and, possibly, the conformation of the active site of the enzyme, interfere with the access of the substrate to the active site of the enzyme and, in particular, to the esterase point.

In the case of compounds (VIII) and (IX) the anticholinesterase efficiency is due to a hydrogen bond between the esterase point of the enzyme and the center of conjugation arising through the unsaturated carbon-carbon bond. In this case, the absence of a strong hydrophobic interaction is compensated by the compactness of the center of the conjugation effect (VIII). In this respect, compound (IX) is less effective both for ACE and for BuCE.

EXPERIMENTAL

The thin-layer chromatography of the products was performed on Silufol UV-254 plates in the chloroform—ethanol (2:1) system with iodine vapor as the revealing agent. Paper chromatography was carried out on paper No. 2 of the Volodarskii

Leningrad mill in the butan-1-ol—hydrochloric acid—water (100:13.5:27) system with the Dragendorff reagent for revealing the spots. The products were purified on Al_2O_3 (activity grade II) and on silica gel LS 5/40. Refractive indices were determined on an IRF-454B refractometer. Melting points were established on a PTP melting-point instrument. IR spectra were recorded on a Specord-71-IR instrument. PMR spectra were taken on an XI 200 Varian instrument in carbon tetrachloride solution with HMDS as standard. Anticholinesterase efficiencies were determined on a Specol-221 instrument in the KIN regime.

Lupinine Ester of Propionic Acid (I). With stirring and cooling to 0°C, 2.3 g (0.025 mole) of propionyl chloride in 20 ml of absolute benzene was added over 30 min to a mixture of 2.4 g (0.42 mole) of lupinine and 2.5 g (0.025 mole) of triethylamine in 80 ml of absolute benzene. The reaction mixture was stirred with heating to 70-75°C for 2 h and was then cooled over 10-20 min.

The triethylamine hydrochloride that had deposited was filtered off and washed with benzene, and the unchanged lupinine was eliminated from the benzene solution by treatment with a 5% solution of boric acid. The benzene solution was washed with 10% Na_2CO_3 solution and was dried over calcined Na_2SO_4. The substance synthesized consisted of a claret-colored oil. Yield 1.93 g (86%).

PMR spectrum: 3.94-4.45 (2H, m, O—CH_2); 2.78 (2H, d, H_{2e} and H_{10e}, J = 10.6 Hz); 1.11-2.22 (14H, m, CH_2).

Compounds (II-VI and VIII) were synthesized similarly.

Lupinine Ester of Palmitic Acid (VII). A mixture of 3.4 g (0.02 mmole) of lupinine, 6.7 g (0.025 mole) of methyl palmitate, and 0.04 g (0.0017 mole) of metallic sodium was heated in the presence of chlorobenzene in an oil bath at 120-130°C in the vacuum of a water-jet pump. Then the methanol formed during the reaction and the chlorobenzene were distilled off to dryness. The residue was treated with a mixture of 50 ml of water and 10 ml of concentrated HCl. The base that separated out was extracted with benzene. The extracted solution was dried over calcined Na_2SO_4. After this, the final product was purified by column chromatography on Al_2O_3 (activity grade II). The solvent was distilled off in vacuum. The residue consisted of a light-brown oil. Yield 4.8 g (60%).

Its IR spectrum contained absorption bands in the 1760 cm^{-1} region that are characteristic for an OCO group and in the 2830 cm^{-1} region characteristic for a trans-quinolizidine system. PMR spectrum: 3.91-4.32 (2H, O—CH_2); 2.9 (2H, d, H_{2e} and H_{10e}, J = 10.6 Hz); 1.1-2.1 (42H, m, CH_2), 0.85 (3H, t, CH_3).

Compound (IX) was obtained in the same way as (VII).

To determine anticholinesterase properties by the principle of measuring a change in the rate of hydrolysis of acetylthiocholine we used Ellman's method [7]. The reaction mixture in the control sample corresponded to 0.04 ml of a 0.001 M solution of Ellman's reagent in 0.1 M phosphate buffer (pH 7.0), 0.7 ml of a 0.1 M phosphate buffer (pH 8.0), 0.4 ml of water, and 0.3 ml of an aqueous solution of the enzyme. The concentration of the enzyme was calculated so that 1 ml of reduction medium corresponded to 0.2 activity unit. In a sample where normal hydrolysis was performed, the volume of water was decreased to 0.2 ml, and 0.2 ml of a solution of the substrate in a concentration of from $1.25 \cdot 10^{-4}$ to $7.5 \cdot 10^{-5}$ M was added, which approximately corresponds to K_m-$2K_m$ for both enzymes.

The rate of hydrolysis was calculated from the change in the density of coloration of the reaction medium. The reversible inhibition constants were calculated from the following relations:

$$K_i = [I](VK^i/V^i K_m) - 1,$$

$$\overline{K}_i = [I](V/V^i)(K_m + K^i)/K_m - (K_m[S]^1) - 1,$$

$$K_i' = \overline{K}_i K_i/(K_i - \overline{K}_i),$$

where [I] is the concentration of inhibitor; V is the maximum rate in the norm; K_m is the Michaelis constant; V^i is the maximum rate in the presence of the inhibitor; K^i is a constant characterizing the rate of formation of the enzyme-substrate complex in the presence of the inhibitor; and K_i, K_i', and $\overline{K}_i$ are the competitive, noncompetitive, and generalized constants of reversible inhibition.

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ISOLATION, PURIFICATION, AND PROPERTIES OF THIOREDOXIN FROM *Ankistrodesmus braunii*

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Two forms of thioredoxin possessing the capacity for reactivating glutamine synthetase have been isolated from the cells of a green alga. Thioredoxins I and II are heat-stable proteins with molecular masses of 12 and 24 kDa, respectively. Analysis of the amino acid compositions of (I) and (II) have shown that they each contain two cysteine residues participating in the reduction of the oxidized form of glutamine synthetase.

Thioredoxins were initially found in extracts of *E. coli* as electron donors for the reduction of ribonucleotides to deoxyribonucleotides [1]. Thioredoxins are heat-stable low-molecular-mass proteins consisting of a single polypeptide chain containing two functionally active SH groups in the reduced state. NADPH-dependent thioredoxin reductase participates in the reduction of the disulfide form of thioredoxin in vivo. In plants a soluble low-molecular-mass protein — ferredoxin-dependent thioredoxin reductase — participates in the reduction of the disulfide groups of thioredoxin. In vitro, thioredoxin can be reduced nonenzymatically — by dithiothreitol [2, 3]. The thioredoxins of bacteria, yeasts, animals, and plants differ appreciably with respect to amino acid composition and have molecular masses of from 11 to 24 kDa [4, 5].

There are no reports in the literature on the properties and structures of the thioredoxins of algae or of their influence on the activity of the isoforms of glutamine synthetase (GS). The task of our investigations was to isolate, purify, and study some properties of the thioredoxin from the cells of *A. braunii*.

Electrophoretically homogeneous preparations of thioredoxins I and II were obtained with the aid of ion-exchange chromatography (Figs. 1 and 2), gel filtration, and ammonium-sulfate fractionation from the cells of the alga *A. braunii*. The UV spectra of preparations (I) and (II) showed their protein nature with an absorption maximum at 280 nm.

We have determined the molecular masses of (I) and (II). A graph of the dependence of the electrophoretic mobility on the molecular mass of the thioredoxins is shown in Fig. 3. The results obtained indicate that thioredoxins I and II each consists of a single polypeptide chain and they have molecular masses of 14 and 24 kDa, respectively.

The amino acid compositions of thioredoxins I and II have also been determined. As can be seen from Table 1, they differ in amino acid composition but have the same number of histidine residues which undergo reversible dithiol-disulfide transformations.

We have shown previously [8] that on the gel filtration of extracts of homogeneous preparation of GS there is a loss of activity of the enzyme. As can be seen from Table 2, the addition of 1 mM dithiothreitol to the enzyme solution partially restores the activity of the two forms of glutamine synthetase. The subsequent addition of 0.5 μg/ml of thioredoxin to the same enzyme solution leads to a rapid restoration of the activity of the enzyme to the initial level.

In contrast to dithiothreitol, monothiols — cysteine, mercaptoethanol, and reduced glutathione, which are stabilizers of the activity of GS [8] — do not possess the capacity for reactivating GS either in the presence or in the absence of thioredoxin.

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