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SYNTHESIS AND STRUCTURE OF NEW PHENYL- AND DIPHENYLPHOSPHINE DERIVATIVES OF ANABASINE AND THEIR INFLUENCE ON THE ACTIVITY OF MOUSE LIVER MICROSOMAL CYTOCHROME-P-450-DEPENDENT MONOOXYGENASES

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A series of alkyl N-β-hydroxypropylanabasinyl and alkyl N-but-2-ynylanabasinyl phenylphosphonates and also N-β-hydroxypropylanabasinyl and N-but-2-enylanabasinyl diphenylphosphinates possessing considerable antimonooxygenase activity, exceeding that of the standard inhibitor SKF-525A with respect both to an insecticide (paraoxon) and to a medicinal drug (aminopyrine), have been synthesized.

Microsomal (M) cytochrome-P-450-dependent monoxygenases (MMs) take an active part in the mechanism of xenobiotics, especially pesticides and drugs. MM inhibitors can be used as insecticide synergists and for prolonging the action of drugs.

Many groups of compounds capable of suppressing the activity of MMs are known – in particular, pyridine derivatives, tertiary amines, and acetylene-containing compounds [1]. In order to find effective MM inhibitors we have synthesized phosphorylated derivatives of anabasine and have investigated their influence on the rate of oxidation of various compounds under the action of mouse liver MMs in vitro: standard MM substrates – p-nitroanisole and aminopyrine [2] – and also paraoxon, which we selected as a model insecticide. The efficacy of the compounds investigated was also evaluated in vivo from their influence on the duration of hexobarbital soluble sleep of mice. The lowering of the rate of decomposition of hexobarbital soluble by the MM system of the liver leads to a prolongation of its action.

All stages in the prepration of alkyl phenylphosphonochloridates and diphenylphosphinic chloride have been described in the literature [3-6]. N-β-Hydroxypropylanabasine was obtained by condensing anabasine with propylene oxide in ethanol [7]. N-(4-Hydroxybut-2-ynyl)-anabasine was synthesized by the interaction of anabasine, propargyl alcohol, and paraformaldehyde in dioxane [8]. The final substances, alkyl N-β-hydroxypropylanabasinyl phenylphosphonates, alkyl N-but-2-ynylanabasinyl phenylphosphonates, N-β-hydroxypropylanabasinyl diphenylphosphinate, and N-but-2-ynylanabasinyl diphenylphosphinate were obtained by the reactions of alkyl phenylphosphonochloridates or diphenylphosphinic chloride with the appropriate amino alcohols in absolute ether in accordance with the scheme given below.

The structures of the compounds were confirmed by the results of elementary analysis and by IR and PMR spectroscopy.

In the IR spectrum of pentyl N-β-hydroxypropylanabasinyl phenylphosphonate chracteristic bands of the following functional groups were observed (ν, cm^{-1}): (P–OC_5H_{11}) 980-1000, (P=O) 1250, (C–H) 1450, (N–C) 1540, (P–C_6H_5) 1640.

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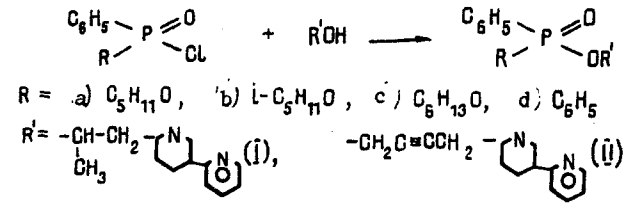

In the PMR spectrum of N-but-2-ylanabasinyl diphenylphosphinate there were signals in the weak field not only of the two phenyl radicals (7.15-7.8 ppm) but also those of a β-substituted pyridine, which are characteristic for the anabasine molecule [9]: $H_{\alpha'}$ – 8.46 ppm, H_{α} – 8.41 ppm, H_{γ} – 7.60 ppm, and H_{β} – 7.15 ppm. A double triplet at 4.70 ppm and a triplet at 3.05 ppm belonged, respectively, to the signals of the protons of the OCH_2 and $N{-}CH_2$ groups separated by a triple bond. The signals of the pyridine ring of the anabasine moiety had the following chemical shifts: $H_{2\alpha}$ – 3.27 ppm, H_{6e} – 2.76 ppm, and H_{6b} – 2.45 ppm, while the other protons resonated in the 1.1-1.9 ppm region (6H, m, CH_2).

It can be seen from Table 1 that the phosphorylated anabasine derivatives investigated possessed a high biological activity. With respect to their capacity for inhibiting MMs in vitro they considerably exceeded anabasine and the standard MM inhibitor – SKF-525A. Differences were observed in their influences on the rates of oxidation of various substrates. The O-demethylation of p-nitroanisole was considerably less sensitive than the O-deacylation of paraoxon and the N-demethylation of aminopyrine. This is apparently due to the fact that the inhibitors investigated had greater structural similarity to the latter two substrates.

The dependence of the inhibiting activities of the compounds on their structure was slight. On the whole, the substances containing a butynyl fragment (2a-d) were somewhat more powerful than their β-hydroxypropyl analogs (Ia-d), with the exception of the experiment with aminopyrine. An elongation of the alkyl radicals from amyl to hexyl in the alkyl phenylphosphonate derivatives led to an enhancement of the inhibiting action, particularly in the experiments with paraoxon. The isoamyl derivatives scarcely differed in the strength of their action from the amyl derivatives. The diphenylphosphinic acid derivatives (Ia and IId) were weaker than the hexyl phenylphosphonate derivatives (experiments with p-nitroanisole) but were scarcely inferior to them in the experiments with paraoxon and aminopyrine.

The compounds investigated significantly extended the hexobarbital soluble sleep of mice, but no clear correlation was observed between the activity in relation to hexobarbital soluble in vivo and the I_{50} values for the oxidation reactions of the substrates investigated in vitro. Apparently the structure of the substance being oxidized played a role here, as well.

Thus, phosphorylated derivatives of anabasine possess a considerable antimonooxygenase activity, exceeding the activity of the standard inhibitor SKF-525A, in relation both to an insecticide (paraoxon) and in relation to a drug (aminopyrine). Further investigations of

TABLE 1. Physicochemical Constants and Inhibiting Activities of Phosphorylated Anabasine Derivatives in Relation to the Oxidation of Various Substrates ($I_{50} \cdot 10^{-5}$ M) under the Action of Mouse Liver Microsomal Monooxygenases and their Influence on the Duration of Hexobarbital Soluble Sleep Mice

Compound	Yield, %	n_D^{20}	Substrate			Lengthening of hexobarbital soluble sleep, times
			p-nitro-	paraoxon	aminopyrine	
Ia	41,4	1,5117	0,10±0,12	4,1±0,5	—	—
b	43,8	1,5112	0,13±0,2	4,3±0,6	—	—
c	34,1	1,5128	8,2±1,2	77±1,1	30,0±0,7	3,5±0,8
d	48,0	1,5599	0,30±0,7	82±1,1	95,0±0,6	1,9±0,4
IIa	43,7	1,5365	8,9±1,5	1,4±0,1	—	—
b	46,2	1,5165	8,5±1,4	1,7±0,2	—	—
c	45,5	1,5359	6,0±1,0	67±0,4	47,0±0,6	2,2±0,4
d	53,7	1,5692	0,24±0,3	62±0,8	85,0±0,1	2,1±0,4
$C_3H_7C(C_6H_5)_2C(O)C_2H_5N(C_2H_5)_3$ SKF-525A			— 0,25±0,7	$2 \cdot 10^{-4}$ M 0,08±0,0 3,0±0,3	32±2% 3,6±0,3	— 3,9±0,7

this group of compounds may serve as a basis for the creation of new and effective synergists of drugs and agents prolonging their action.

EXPERIMENTAL

The IR spectra of the compounds synthesized were taken on a Specord IR-71 instrument (Germany) in paraffin oil and in KBr tablets.

PMR spectra were taken on a Varian XL-200 instrument (USA) with a working frequency of 200 MHz, using CCl_4 as solvent.

For column chromatography we used Al_2O_3 (activity grade II) with absolute ether as eluent. The solvent system for TLC (unfixed layer) was benzene–ether–ethanol (10:5:2).

Synthesis of N-β-Hydroxypropylanabasinyl Pentyl Phenylphosphonate. A three-necked flask fitted with the reflux condenser and dropping funnel was charged with 7.30 g (0.03 mole) of pentyl phenylphosphonochloridate dissolved in 70 ml of absolute ether. With intensive stirring and cooling, a mixture of 6.60 g (0.03 mole) of N-β-hydroxypropylanabasine and 3.0 g (0.03 mole) of triethylamine dissolved in 70-80 ml of absolute ether was added to the reaction flask dropwise. After the addition of the reagents, the reaction mixture was stirred at the boiling point of the solvent for 1-2 h. The precipitate of triethylamine hydrochlorate that had deposited was separated off, the solvent was eliminated by distillation, and the final product was purified by column chromatography. The other compounds were synthesized similarly.

The inhibiting activities of the compounds in the in vitro experiments were evaluated from the concentrations of the inhibitors lowering the rate of oxidation of the substrate by 50% (I_{50}, M) at a concentration of the substrate equal to K_M.

The enzymatic O-demethylation of p-nitroanisole was performed in a reaction medium (1 ml) containing 0.05 M Na phosphate buffer, pH 7.5, $2.5 \cdot 10^{-4}$ M $NADPH_2$, $4 \cdot 10^{-5}$ M p-nitroanisole, inhibitors in various concentrations, and a suspension of mouse liver microsomes prepared by the method of Omura and Sata [10]. The control samples contained no $NADPH_2$. After incubation at 37°C for 20 min with constant shaking, the reaction was stopped by the addition of a solution of Triton X-100, EDTA and NaOH to final concentrations of 1%, 0.1%, and 0.1 N. The optical density of the medium at 400 nm was measured and the rates of formation of the product – p-nitrophenol – in the presence and in the absence of the inhibitor were calculated.

The enzymatic oxidation of paraoxon (diethyl p-nitrophenyl phosphate) was conducted in the same reaction medium (0.4 ml) containing as substrate $2.5 \cdot 10^{-6}$ M paraoxon. Control samples contained no $NADPH_2$. After incubation for 20 min, 0.025-ml aliquots were transferred into 0.725-ml portions of a solution of acetylcholinesterase (ACE) from human erythrocytes in 0.05 M Na phosphate buffer, pH 7.5, and after 10 min, in each case, 0.25 ml of a $4 \cdot 10^{-3}$ M solution of acetylthiocholine (ATC) and $1 \cdot 10^{-3}$ M 5,5-dithiobis(2-nitrobenzoic acid) in 0.05 M Na phosphate buffer, pH 7.5, was added. After 15 min, the enzymatic hydrolysis of the ATC was stopped by the addition of a solution of proserine to a final concentration of 0.002%, and the optical density of the solution at 412 nm was measured. Concentrations of paraoxon in solutions containing and not containing $NADPH_2$ and the inhibitor were calculated from the degree of suppression of the activity of the ACE, and the rates of its oxidative decomposition under the action of the preparation of microsomes in the presence and in the absence of the inhibitor were also calculated.

The enzymatic N-demethylation of aminopyrine was conducted in a reaction mixture (1 ml) containing 0.05 M K-Na phosphate buffer, pH 7.4, $4 \cdot 10^{-3}$ M $MgCl_2$, $5 \cdot 10^{-4}$ M $NADPH_2$, $5 \cdot 10^{-3}$ M glucose 6-phosphate, $8 \cdot 10^{-3}$ M nicotinamide, $7.5 \cdot 10^{-3}$ semicarbazide, $5 \cdot 10^{-3}$ M aminopyrine, and the supernatant liquid obtained on centrifuging a mouse liver homogenate at 10,000 g for 10 min. After incubation for 30 min, the reaction was stopped by the addition of a solution of $ZnSO_4$ to a final concentration of 3.5%. The samples were well stirred and, after 10 min, 0.3 ml of a saturated solution of $Ba(OH)_2$ was added to each of them and they were again well stirred. After another 10 min, the samples were centrifuged at 3000 rpm for 20 min in a T-52 centrifuge.

To aliquots with a volume of 0.75 ml were added 0.3-ml portions of the Nash reagent (30 g of CH_3COONH_4, 0.4 ml of acetylacetone, and water to 100 ml). Control samples contained no substrate. The samples were incubated at 60°C for 30 min and then the optical

densities of the media were measured at 415 nm, and the rates of formation of the oxidation product – formaldehyde – in the presence and in the absence of the inhibitor were calculated.

In the in vivo experiments, 0.1% solutions of the substrates in 20-50% dimethyl sulfoxide were injected intraperitoneally into mice in doses of 10 mg/kg 30 min before the administration of hexobarbital soluble (40 mg/kg subcutaneously). The prolongation of the sleep of the mice as compared with the control amounted to 20 ± 3.6 min.

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IMMUNOAFFINITY FRACTIONATION OF NEUTRALIZING POLYCLONAL ANTIBODIES TO NERVE GROWTH FACTORS

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A method of fractionation according to affinity and specificity of polyclonal antibodies to murine and ovine nerve growth factors (NGFs) has been developed. The antibody preparations obtained neutralize the biological activity of β-NGF at a molar ratio of about 1 and recognize the species-specific and conservative epitopes of these NGFs, being valuable probes for two-site immunoanalyses.

Nerve growth factors (NGFs) are polypeptide growth factors with a broad spectrum of biological action which regulate the establishment, development, and functioning of individual populations of neurons of the peripheral and central nervous systems [1-4]. A considerable part of the investigations of NGFs is based on the use of antibodies, obtained most frequently against NGFs isolated from classical sources (mouse submaxillary salivary glands [5]. the venoms of certain snakes [6-8], and bovine seminal plasma [9]).

In spite of the wide use of monoclonal technology, polyclonal antisera are continuing to be widely used, their advantages being the effective neutralization of biological activity and also a usually higher affinity of the specific antibodies. In the present paper we describe a method for fractionating clonal antibodies with respect to affinity and specificity which permits high-affinity fractions of antibodies to species-specific and conservative epitopes of NGFs to be obtained.

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