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# SYNTHESIS AND ANTIMONOAMINE OXIDASE ACTIVITY OF FLUORONITROCARBOXYLIC ACID HYDRAZIDES

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One of the fruitful and promising approaches in the discovery of new drug substances is the exploitation of the effective and specific inhibitors of key enzymes in the metabolism of biogenic amines, in particular the monoamine oxidases (MAO) [6, 8]. Among the MAO inhibitors are compounds possessing high antidepressant and psychostimulant activity [2]. The carboxylic acid hydrazides and similar compounds occupy an important position in this type of activity. In the past years a series of fluorocarboxylic acid hydrazides have given materials possessing significant psychotropic, antihypertensive, and antimicrobial activity [1]. It was thought that the physiological activity and the high reactivity of these compounds is connected with the electron-acceptor effects of the fluorine-containing group, lowering the electron density on the hydrazide fragment of the molecule. It would be expected that the introduction of an additional electron-acceptor grouping, for example a nitro group, into the structure of the molecule would show a corresponding influence on their physiological activity spectrum.

The literature describes different derivatives of difluoronitroacetic acid, but data on the synthesis of their hydrazides is extraordinarily limited, and the results of biochemical studies are completely absent.

The aim of the present work was the synthesis and study of the antimonoamineoxidase properties of a series of freshly synthesized difluoronitroacetic acid hydrazides (Table 1), which were prepared by acylation of substituted hydrazines with esters of difluoronitroacetic acid.

$$O_2NCF_2COOCH_3 + H_2NNRR^1 \longrightarrow \underset{I-XV}{O_2NCF_2CONHNRR^1}$$

R = H (I—IX, XI—XV), $CH_3$ (X); $R^1$ = $C_6H_5$ (I); p-tolyl (II), o-tolyl (III), m-$C_6H_4CF_3$ (IV); 2,5-$C_6H_3Cl_2$ (V); $CH_2C_6H_5$ (VI); p-$C_6H_4F$ (VII); p-$C_6H_4Br$ (VIII); p-$C_6H_4OCH_3$ (IX); $CH_3$ (X); $OCOC_2H_5$ (XI); $COCH_3$ (XII); $COC_6H_5$ (XIII); $SO_2C_6H_5$ (XIV); $COCH_3 \cdot N_2H_4$ (XV).

All of the synthesized compounds were colorless or slightly colored crystalline materials, easily soluble in organic solvents. The structure of the hydrazides was proven by IR, $^1H$ NMR, and $^{19}F$ NMR-spectroscopy and the composition by elemental analysis.

## EXPERIMENTAL (CHEMISTRY)

IR spectra were obtained with "Perkin-Elmer" (FRG) and "Specord IR-75" (GDR) spectrometers in mineral oil or in $CCl_4$ solution. NMR spectra were recorded on a Bruker CXP-200 instrument (GDR) at working frequencies of 200 MHz (NMR) and 188 MHz ($^{19}F$ NMR) in $CDCl_3$, with TMS ($^1H$ NMR) and $CF_3COOH$ ($^{19}F$ NMR) as internal standards.

1-Difluoronitroacetyl-2-phenylhydrazine (I). To 15.5 g (0.1 mole) of methyl difluoronitroacetate [5] dissolved in 50 ml of absolute methanol was added 10.8 g (0.1 mole) of phenyl-

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TABLE 1. Physicochemical Characteristics and Elemental Analysis Data for Derivatives of Difluoronitroacetic Acid

| Compound | Yield, % | mp, °C | Found, % | | Empirical formula | Calculated, % | |
|---|---|---|---|---|---|---|---|
| | | | C | N | | C | N |
| I | 47 | 95—7 | 41,08 | 18,80 | $C_8H_7F_2N_3O_3$ | 41,56 | 18,58 |
| II | 41 | 111 | 44,64 | 16,35 | $C_9H_9F_2N_3O_3$ | 44,08 | 17,14 |
| III | 45 | 91—3 | 44,46 | 16,83 | $C_9H_9F_2N_3O_3$ | 44,08 | 17,14 |
| IV | 48 | 85—7 | 36,86 | 13,59 | $C_9H_6F_5N_3O_3$ | 36,12 | 14,05 |
| V | 75 | 103—4 | 31,76 | 14,11 | $C_8H_5Cl_2F_2N_3O_3$ | 32,00 | 14,00 |
| VI | 42 | 95—7 | 44,50 | 16,85 | $C_9H_9F_2N_3O_3$ | 44,08 | 17,14 |
| VII | 62 | 62—4 | 35,81 | 17,36 | $C_8H_6F_3N_3O_3$ | 35,44 | 17,72 |
| VIII | 82 | 98—9 | 28,03 | 13,88 | $C_8H_6BrF_2N_3O_3$ | 28,19 | 14,09 |
| IX | 40 | 82—5 | 42,03 | 15,36 | $C_9H_9F_2N_3O_4$ | 41,38 | 16,09 |
| X | 37 | 147—8 | 26,84 | 22,85 | $C_4H_7F_2N_3O_3$ | 26,23 | 22,95 |
| XI | 70 | 130 (b.p.) | 29,54 | 19,77 | $C_5H_7F_2N_3O_5$ | 28,99 | 20,29 |
| XII | 65 | 103—4 | 25,01 | 20,00 | $C_4H_5F_2N_3O_4$ | 24,37 | 21,32 |
| XIII | 68 | 75—7 | 42,08 | 16,03 | $C_9H_7F_2N_3O_4$ | 41,70 | 16,22 |
| XIV | 73 | 92—4 | 36,14 | 14,04 | $C_8H_7F_2N_3O_5$ | 34,91 | 15,27 |
| XV | 20 | 140 | 20,31 | 30,02 | $C_4H_9F_2N_5O_4$ | 20,96 | 30,56 |

hydrazine in 30 ml of absolute methanol with stirring at 18-20°C. The reaction mass was kept for 6-8 h, then the methanol was removed at reduced pressure and the residue was dissolved in ether and precipitated with pentane to give 10.8 g (47%) of I. IR spectrum, $\nu_{max}$, $cm^{-1}$; 3426, 3340 (N–H), 1748 (C=O), 1602 ($NO_2$). $^{19}F$ NMR ($CDCl_3$), δ: 16.62 ppm. $^1H$ NMR ($CDCl_3$), δ, ppm: 7.28 ($C_6H_5$), 8.1 (N–H).

The remaining hydrazides were prepared analogously (II-XV).

For comparison of the physiological activity with the indicated compounds, trifluoroacetic acid hydrazide ($CF_3CONHNHC_6H_5$, XVI) was synthesized by known methods [4].

The physicochemical characteristics and elemental analysis data for compounds I-XV are presented in Table 1.

## EXPERIMENTAL (BIOLOGY)

Study Methods. The MAO preparations were obtained from the mitochondrial fraction of swine liver membrane. The membrane treatment included a stage of freeze-drying with subsequent solubilization in potassium phosphate buffer (0.01 M, pH 7.4) containing urea (1.3 M) and Triton X-100 (1.3%); the suspension was dewatered to a protein concentration of 40 mg/ml. The solubilizate was fractionated with a saturated ammonium sulfate solution, and the protein precipitate obtained in the 20-45% saturation interval was subjected to chromatography on ion-exchange cellulose DEAE-52 (Whatman), equilibrated with Tris-HCl buffer (0.02 M, pH 7.4). After preliminary washing with the same buffer, elution was carried out with a linear concentration gradient of NaCl (0-0.5 M) in Tris-HCl buffer (0.02 M, pH 7.4) containing Triton X-100 (1%). The fractions showing high monoamine oxidase activity were combined and subsequently worked up with 30 and 45% saturated ammonium sulfate solutions. The protein precipitate formed at 45% saturation was the final enzyme preparation used in the kinetic experiments. The MAO preparations obtained by this method showed by electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulfate one main band with a molecular weight ca. 60,000 D, which corresponds (according to the literature) to the molecular weight of a single subunit of MAO.

MAO activity was determined spectrophotometrically using benzylamine as substrate [2] and polarographically by the change in oxygen concentration in the solution under study, using tyramine, serotonin, and benzylamine as substrates [7]. The ratio of activities for these substrates (3 mM) was 100:12:88. The absolute value of the activity of the enzyme varied from 50 to 170 units/g of protein for different preparations. The irreversibility of the inhibition was shown by dialysis according to a known method [5].

The mono and dipotassium phosphate for the preparation of buffer solutions was "chemically pure" grade and the Tris-HCl buffer was "Reanal." The benzylamine, "chemically pure" grade, was distilled before use. Absorption spectra of the synthesized hydrazides and the enzyme preparations were recorded on an Aminco DW-2a UV-spectrophotometer (USA) and kinetic measurements were made with a Gilford UV-spectrophotometer (USA).

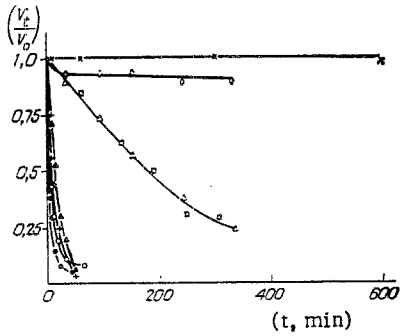


Fig. 1. Dependence of the stage of MAO inhibition on time of incubation with the subject compounds. ×) Control; ▲) III (0.1 mM); Δ) IV (0.1 mM); □) V (0.1 mM); +) VI (0.03 mM); •) VII (0.1 mM); ○) VIII (0.1 mM); ◇) IX (0.1 mM). Incubation was carried out at 25°C in Tris buffer (0.02 M, pH 7.4). For the control, MAO was incubated only with buffer. Abscissa: Time of incubation t (in min). Ordinate: $V_t/V_0$.

TABLE 2. Kinetic Parameters for the Inhibition of MAO by the Studied Compounds

| Compound | $k_i'$, $min^{-1}$ | $k_i''$, $min^{-1} \cdot M^{-1}$ | $k_1$, mM | $k_1^*$, mM | $\tau_{0,5}^{**}$, min |
|---|---|---|---|---|---|
| I | 0,15±0,05 | — | 0,39±0,05 | 0,31±0,05 | 22,6 |
| II | — | $2,27 \cdot 10^4$ | — | — | — |
| III | 0,25±0,05 | — | 0,08±0,01 | — | 5,0 |
| IV | 0,006±0,002 | — | 0,045±0,01 | — | 167,5 |
| V | 0,008±0,002 | — | 0,15±0,02 | — | 216,6 |
| VI | — | $1,25 \cdot 10^4$ | — | — | — |
| VII | 0,17±0,05 | — | 0,081±0,01 | — | 7,3 |
| VIII | 0,1±0,05 | — | 0,1±0,015 | — | 13,9 |
| IX | — | — | 0,08±0,01 | — | — |
| XVI | 0,2±0,03 | — | 0,22±0,02 | 0,20±0,01 | 11,1 |

*Determined under equilibrium conditions of inhibitor (conditions described in text).
**At inhibitor concentration of $10^{-4}$ M.

It was discovered that in the series of synthesized preparations the phenyl derivatives I-VIII showed significant antimonoamine oxidase activity (Fig. 1). The dialysis method established that for all of the substrates used (benzylamine, serotonin, tyramine), the enzyme was irreversibly inhibited. The kinetics of the MAO inhibition of the oxidation of benzylamine was studied in detail. It is known that the irreversible inhibition of enzymes takes place by two different mechanisms [9]: 1) through the formation of an intermediate reversible enzyme-inhibitor complex; in the simplest case through a single intermediate complex with an equilibrium established for the first step:

$$E_{act.} + I \underset{}{\overset{k_1}{\rightleftharpoons}} \underset{rev.}{EI_{inact.,}} \xrightarrow{k_i'} \underset{irrev.}{EI_{inact.,}} ; \quad (1)$$

2) as a result of a bimolecular reaction between the enzyme and the inhibitor:

$$E_{act.} + I \xrightarrow{k''} EI_{inact.} \quad (2)$$

These mechanisms may be differentiated by the character of the dependence of the apparent inactivation constants ($k_{in}$) for the active form of the enzyme on the concentration of the inhibitor. For the case when mechanism (1) applies

$$[E_{act.}] = \frac{[E_0]}{\left(1 + \frac{[I]}{k_1}\right)} e^{-k_{in} t}, \quad (3)$$

where $k_{in} = \dfrac{k_i'}{1 + \dfrac{k_1}{[I]}}$.

When mechanism (2) applies

$$[E_{act.}] = [E]_0 e^{-k_{in} t},$$

where $k_{in} = k_i''[I]$.
$E_0$ corresponds to the initial concentration of enzyme.

Thus, the curve for the dependence of the apparent inhibition constant $k_{in}$ on the concentration of the inhibitor is linear in the lower coordinates, and passes through the zero coordinate in the case of the bimolecular scheme for the inactivation of the enzyme (2) or intercepts the axial coordinate segment, corresponding to the first order rate constant for inhibition $k_i'$ and the equilibrium constant $k_1$ in the case of in the presence of a reversible enzyme complex (1).

The results of an analysis of the kinetics of enzyme inhibition by compounds I-VIII and XVI are presented in Table 2. MAO inhibition by compounds I, III-V, VII, and VIII, and also the hydrazide of trifluoroacetic acid XVI proceeds through the formation of an intermediate reversible enzyme complex and is characterized by a slight dependence on the kinetic and equilibrium parameters. In the case of compounds II and VI, the inhibition process is characterized by the second-order inhibition constant $k_i''$ and may be described by Scheme (2) as well as Scheme (1), assuming that even at the highest concentration of hydrazide, the equilibrium constant for dissociation of the reversible complex by far exceeds the concentration of inhibitor.

It is interesting to evaluate the time for quasireversible inhibition of the enzyme. From Eq. (3) it is shown that

$$t = \frac{1 + \frac{k_1}{[I]}}{k_i'} \ln \frac{[E_0]}{[E]}$$

where $[E] = [E_{act}] + [EI_{inact,rev}]$, i.e., even at the maximum level of inhibitor concentration ($10^{-4}$ M), the time beyond which the enzyme is irreversibly inactivated by 50% ($\tau_{0.5}$) exceeds 5 min (Table 2). Thus, a study of the kinetics of enzyme inhibition at time intervals of 5 min may define the mechanism of the reversible step of inhibition with a known degree of confidence. It was established that for both preparations I and XVI the present process concurrently inhibited MAO. The kinetic parameters of the inhibition process for the above compounds are presented in Table 2.

With the aim of establishing the structure and antienzymatic activity relationship between the studied compounds for phenylsubstituted hydrazides I-V, VII, and VIII the following correlation equation was found:

$$\lg k_i^* = 3.568 - 0.55\Sigma(\sigma^* - 0.49) - 3.6\Sigma\sigma_R^0 - 0.316(m-1),$$

where $k_i^*$ = effective second power inhibition constant corresponding to $k_i''$ corresponding to the enzyme inhibiting by mechanism (2), and equal to $k_i'/k_1$ for materials interacting with MAO according to Scheme (1). The influence of substituents was estimated by the induction constants $\sigma^*$ and the isomerization constants $\sigma_R^0$ [3].

To estimate the influence of the phenyl substituents on the neighboring carbon atom, the term 0.316 (m − 1) was introduced into the equation, where m = number of substituents. Thus, for compound V, m = 3, and for III, m = 2, and for the remaining compounds, m = 1. The value of the standard deviation $\lg k_i^*$ from experimental findings was ±0.021 (i.e., <0.5%).

Thus, in this series of difluoroacetic acid hydrazides the value and even the mechanism of inhibition depends to a significant degree on the position and nature of substituents on the aryl fragments of the molecules. In particular, for the N-phenyl derivatives, the logarithm of the inhibition constant linearly increases with the value of the donor properties of the substituent.

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