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VARIA

The synthesis and in vitro testing of symmetric bisquaternary salts with pyridin-isonitrosoacetanilide structure, active in the exposure to neurotoxic chemical agents

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Abstract: *Neurotoxic chemical agents induce complex toxicological effects with major adverse effects for those who are exposed to them. Medical countermeasures include administering atropine, a cholinesterase reactivating oxime and an anticonvulsant. The purpose of this reseach is the optimization of an antidote-based treatment in the intoxication with organophosphates through the synthesis and toxicological testing of new compounds with a pyridin-isonitrosoacetanilide structure and their comparison with obidoxime as reference.*

The objective of this study was the in vitro/in vivo assessment of the antidote properties and acute toxicity of synthethic oximes active in the exposure to neuroparalytic organophosphates.

The CT50 value for obidoxime was estimated, through calculus, to be 3.24 mg/ml and 9.33 ×10 -6 M respectively. The CT50 value for Bis1,3[2-hydroxyimino-N-(pyridyl)acetamide]oxapropandichloride (HIN 2) was estimated to be 2.72 mg/ml and 6.29 ×10 -6 M respectively.

The studied compounds showed values close to cellular viability at low and moderate equimolecular concentrations, administered in vitro to a cellular culture of fibroblasts. Bis1,3[2-hydroxyimino-N-(piridyl)acetamide]oxapropandichloride (HIN 2) showed an in vitro cellular viability value smalled than obidoxime at higher equimolecular concentrations, proving itself to be more toxic than obidoxime at high concentrations. Taking into account the close values of CT50, the newly synthethized compound, having this particular characterization from a toxicological standpoint, qualifies for further studies as an antidote.

Keywords: *paraoxon, acetylcholinesterase, obidoxime, N-acetyl-2-hydroxyiminomethylpyridinbromide (PYC8); 1,3(2-hydroxyimino-N-pyridin-3-propan bromide (HIN1); 1,3(2-hydroxyimino-N-pyridin-3-oxapropanchloride (HIN2)*

INTRODUCTION

High risk organophosphates (sarin, soman, tabun) are extremely toxic substances with a phosphoric acid esther structure. Due to their extremely high toxicity, they were registered on the list of substances that require destruction according to international regulations (Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction – Geneva). Their production, stockpiling and use are monitored by international organizations (Organization for prohibition of chemical weapons – OPCW). [4, 7, 23].

Neuroparalytic chemical agents have the property of irreversibly inhibiting erythrocyte acetylcholinesterase (AChE). The coupling reaction of the organophosphate at the level of the active site of the enzyme is shown in Figures 1, 2. [23]

The inhibition of AChE leads to the acummulation of acetylcholine (ACh) at the level of the synaptic cleft and the hyperstimulation of muscarinic and nicotinic receptors. The

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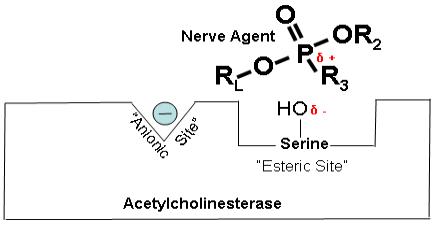
clinical signs of mild intoxication are pupil constriction, sweating, muscular fasciculation, and the decrease of cardiac frequency and of arterial tension.

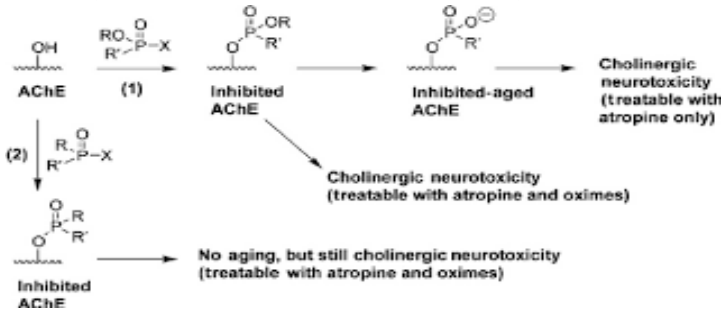
Figure 1: The coupling mechanism of the organophosphate at the level of the active site of AchE



In high concentrations of the toxic compounds, convulsions occur and death takes place through respiratory insufficiency caused by the paralysis of respiratory centers. Cholinesterase-inhibiting neurotoxic organophospates possess, in their molecule, two organic groups, identical or different, and an active group that is inorganic or organic, bound to a pentavalent phosphorus atom. This atom can be bound to an oxygen or sulphur atom (phosphates, phosphonates, tiophosphates, tiophosphonates). [1, 4, 9, 23] In scientific literature, they were presented as potential simulants for each class or warfare chemical agents [4, 16, 22].

The laboratory use of neurotoxic chemical agents is restricted, as they are extremely toxic substances. Thus, antidote studies and those refering to their pharmacotoxicological properties are performed utilizing simulants of the paraoxon type. A simulant is defined as a compound possessing all relevant physical and chemical properties of the corresponding neurotoxic chemical agent, but with lower intrinsical toxicity; The utilization of simlants in research and testing activities contributes to the reduction of the stress and risk the personnel involved in the testing procedures is subjected to by handling chemical agents with high toxicity. [1, 23]

Figure 2: The interaction between AchE and the organophosphate



The general emergency measures necessary to stop the exposure are masks, decontamination and the evacuation of the individual from a contaminated environment. Medical countermeasures are the administration of atropine, of a cholinesterase reactivating oxime and of an anticonvulsant. The increase of antidote treatment efficiency in the intoxication with organophosphates is one of the main objectives of research activity at national and international level, taking into account both aspects of clinical toxicology of organophosphates used as pesticides and of experimental toxicological research of neuroparalytic war chemical agents. [4, 8, 12, 16, 19] Cholinesterase-reactivating compounds used in the UN member states (pralidoxime, obidoxime, HI6) possess therapeutic efficiency for different neuroparalytic chemical agents [1, 2, 3, 5, 11, 15, 17, 22]. Having quaternary and bisquaternary salts chemical structures with pyridinic rings, this substances are known and studied as efficient antidotes in the processes of activation-reactivation of acetylcholinesterase in the intoxication with organophosphates [6, 10, 22].

Bisquaternary compounds such as Hagedorn, HI-6, Obidoxime, are obtained through the quaternization of pyridine bases substituted (oximes, carboxamides) with reactive dihalogenated derivatives such as symmetric dichloromethylether (DCDMS) or 1,3-dibromopropane [6, 14, 22]. Symmetric quaternary salts obtained through the quaternization of pyridine substituted with oxime or amide groups in a 2:1 molar ratio to the dihalogenated derivate are intensely studied. The typical example is represented by obidoxime, the product of 4-pyridinaldoxime bisquaternization with exces symmetric dichlorodimethylether. This compound is easily obtained and has good stability. [6, 7, 10, 12, 14, 22].

Through the monoquaternization of a pyridine base or of another cyclic tertiary amine with DCDMS or 1,3-dibromopropane in excess, the monoquaternary salt is obtained, usually unisolable that, in the next step, can be quaternized with another different base, obtaining mixed products with an antidote efficiency that is higher and a

reduced stability. In (bis)quaternization, tertiary bases that can be utilized are pyridines which are monosubstituted with aldoxime or carboxamide groups in all three positions, 1,3-N-methyl-2-imidazolilaldoxime or quinuclidinic bases: 3-quinuclidinonoxime, 3-quinuclidinyl-N,N-dimethylaminocarbamate etc, which, theoretically, through combination with dihalogenate derivatives cand lead to a large variety of antidotes [6, 14, 19, 20].

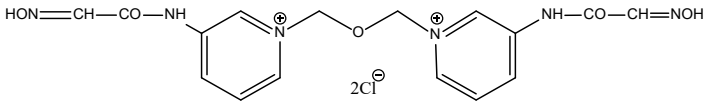
The present paper proposes the synthesis and characterization of new symmetric bisquaternization compounds with 3-pyridinylisonitroso-acetanilide as a precursor, the quaternization agents being symmetric dichlorodimethylether and 1,3-dibromopropane. These compounds are PAM-2 analoges, N-Methyl-2-hydroximinomethyl pyridine chloride being utilized for the first time as an antidote in the intoxications with neurotoxic organophosphates. [14, 19, 21]

MATERIAL AND METHOD

The reactivating potential of two oxime compounds with pyridin-isonitrosoacetanilide structure was tested in vitro: 1,3(2-hydroxyimino-N-pyridin-3-propane bromide (HIN1); 1,3(2-hydroxyimino-N-pyridin-3-oxapropanchloride (HIN2). The HIN 1 compound did not disolve in the culture medium. The acute toxicity of obidoxime and of HIN 2 was assessed through the determination of cellular viability the two produced on cell cultures from 3T3 fibroblast lines. The cells were incubated for 24 hours at concentrations of 1 mg/ml, 3 mg/ml, and 5 mg/ml and optical density was read at 490 nm.

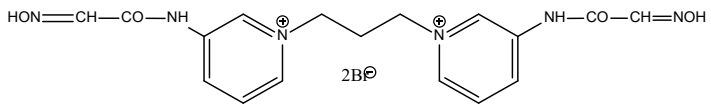
For this purpose, two compounds with pyridinisonitroso-acetanilide structure were tested, compounds considered active in the intoxication with neuroparalytic chemical agents due to the presence in their molecule of the oxime group R=N-OH, as well as of the pyridine structure resembling obidoxime. (Figure 3)

Figure 3: Chemical structures of newly synthethized compounds



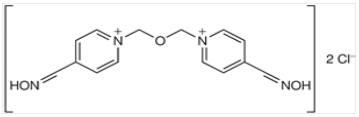
$C_{16}H_{18}Cl_2N_6O_5$
Masa Moleculara; 445,26

bis 1,3[2-hidroxiimino-N-(piridin-3-il)] oxapropandiclorura



$C_{17}H_{20}Br_2N_6O_4$
Masa Moleculara: 532,19

bis 1,3[2-hidroximino-N(piridin-3-il)]propandiona



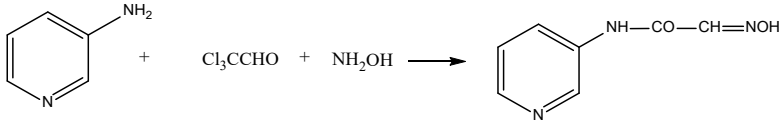
$C_{14}H_{16}N_4O_3Cl_2$
1,1-bis-metilen-bis-hidroxiiminometilpiridinium
Molecular mass 358,75

The synthesis of new symmetric pyridin-isonitroso-acetanilide bisquaternary salts with cholinesterase reactivation properties

The sythesis of the isonitrosoacetanilide of 3-aminopyridine named 2-(hydroxyimino)-N-(pyridin-3-yl) acetamide.

The isonitrosoacetanilide of 3-aminopyridine is obtained from 3-aminopyridine, chloral hydrate and hydroxylamine chlorohydrate in a saturated solution of sodium sulphate according to the following reaction in Figure4.

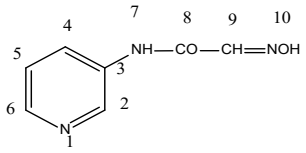
Figure 4: The synthesis of 2-(hydroxyimino)-N-(pyridin-3-yl) acetamide.



In a 2L balloon with efficient mechanical agitation, around 400ml of water are introduced. Under agitation, the sodium sulphate is brought in portions, and the chloral hydrate is added to the saturated solution within 2-5m. Seperately, the amine chlorhydrate is prepared in an aqueous solution of amine, 10ml HCl 35 and 20ml water. It is added under agitation to the previously prepared solution within 5m. Then, it is heated at 30-40^{0}C, and the hydroxylamine chlorhydrate is added in small portions after being dissolved in 50ml of water. The temperature is steadily increased to 80-90^{0}C within 1h, followed by boiling for another 15m. Then it is left to cool down at room temperature overnight and the sodium sulphate is filtered. The sodium sulphate is then washed twice with 50ml of water each time, and the filtered solution is added to the previous one. The reunited filtered solutions are neutralized with saturated ammonia solutions and are cooled down to 0-5 degrees. The precipitate is filtered, and the resulted solution undergoes extraction three times with 150ml methylen chloride each time. Through distillation, the solvent is removed, and the residue is recrystallized from methanol (through boiling with animal charcoal). The isonitrosoderivative is obtained with a yield of 65-70 %.

The compound was characterized through spectral RMN analyses, hydrogen and carbon spectrums which confirm the structure(Figure 5).

Figure 5



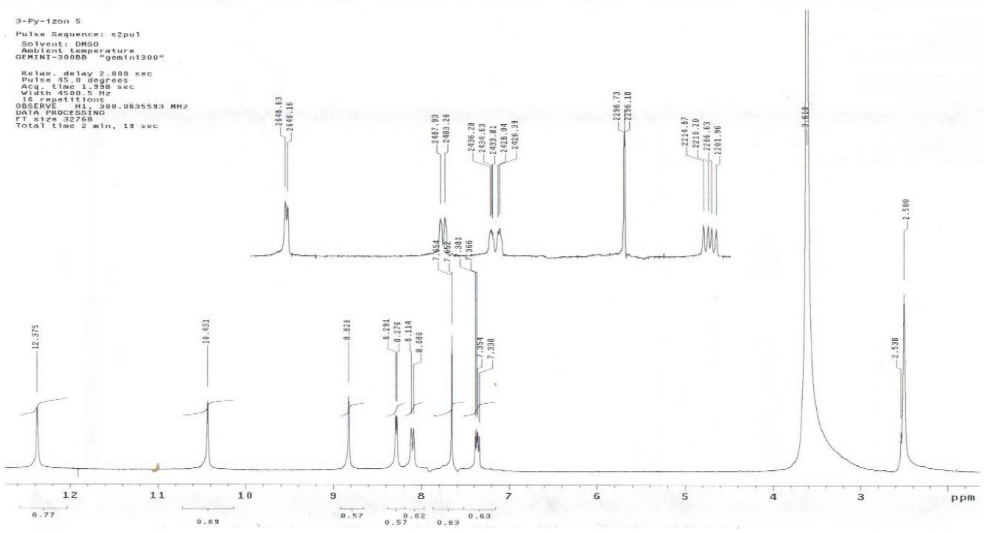
RMN characterization

1H-RMN – 1H-RMN (DMSO-d6, δ ppm, J, Hz) spectrum: 10.32(s, 1H, H-10); 8.85(dl, 2.6, 1H, H2); 8.29(dd, 4.4, 1.3, 1H, H-6); 8.12(dl, 4.3, 1H, H-4) (Figure 13).

Quaternization with symmetric dichlorodimethylether (DCMS) to bis1,3[2-hydroxyimino-N-(pyridin-3-yl)acetamidî oxapropandichloride

The symmetric quaternization reaction of the isonitrosoacetanilide of aminopyridine with dichloroether in a 2:1 ratio is performed in anhydrous dimethylformamide at temperatures of 30-40^{0}C. The quaternization agent, symmetric dichlorodimethylether, is obtained from paraformaldehide and thionyl chloride (Figure 6).

Figure 6: Isonitrosoacetanilide 3-aminopyridine –H-RMN spectrum



13C-RMN – 13C-RMN (DMSO-d6, δ ppm) spectrum: 160.91(C-8); 144.77(C-9); 143.80(C-6); 141.60(C-2); 135.22_C-3); 127.04(C-4); 123.69(C-5).

In an Erlenmayer, dimethylformamide and isonitroso-acetanilide are introduced. They are magnetically agitated to complete dissolution, and then dichlorodimethylether that was previously dissolved in 5ml of anhydrous

dimethylformamide is added. The recipient is then sealed and agitated for 10h at room temperature and, finally, another 5h at 30-40^{0}C. It is cooled at 0-5^{0}C for 3h and is then filtered through vacuum filtering. The precipitate is repeteadly washed with anhydrous acetone and then dried in a vacuum, in the desiccator. 2.3 g of white product are obtained with m.p=145-150^{0}C, which corresponds to a yield of 52%. The synthethized compound, the bisquaternary salt, was characterized through spectral RMN and proton and carbon spectrum analyses which confirm its structure.

Figure 7: Isonitrosoacetanilide 3-aminopyridine - C- RMN spectrum

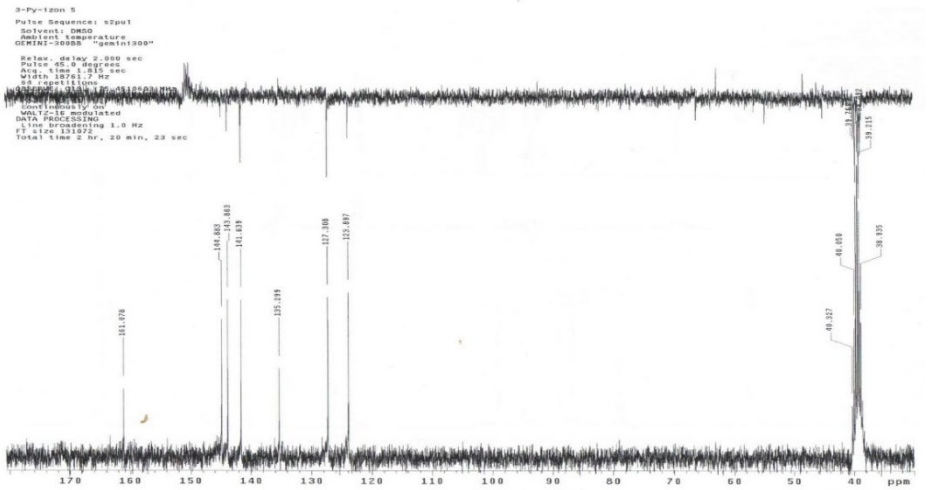
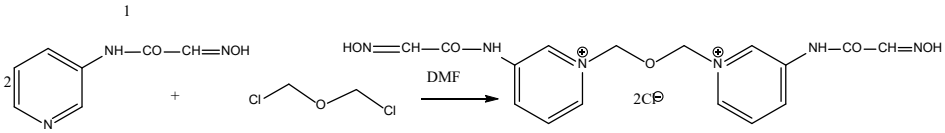
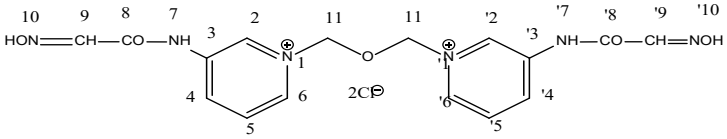


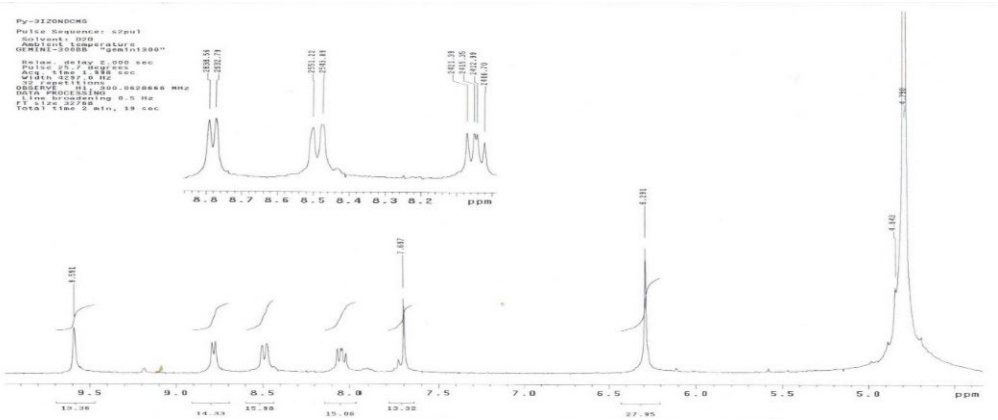
Figure 8: The synthesis of bis1,3[2-hydroxyimino-N-(pyridin-3-yl)acetamid]oxapropandichloride



RMN characterization of the 1H-RMN hydrogen spectrum is shown in Figure 9 and the13C-RMN carbon spectrum is shown in Figure 10.

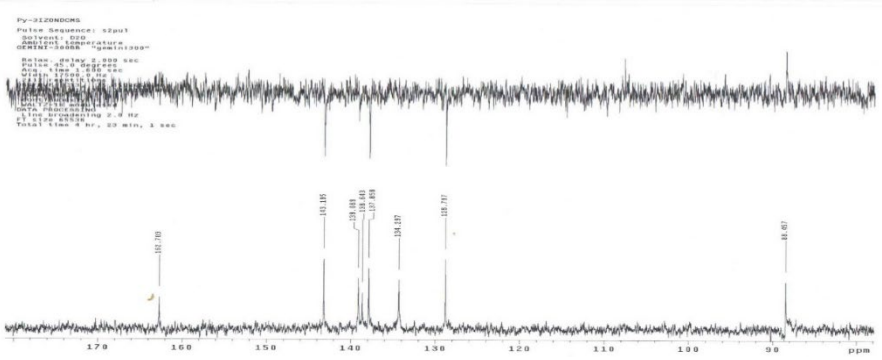
Figure 9: Bis 1,3[2-hydroxyimino-N-(pyridin-3-yl)acetamid]oxapropandichloride compounds - 1H-RMN spectrum





1H-RMN (D2O, δ ppm, J, Hz) hydrogen spectrum: 9.58(sl, 2H, H-2, H-2'), 8.77(dl, 6.0, 2H, H-6, H-6'), 8.48(dl, 9.7); 8.04(dd, 8.7, 6.3, 2H, H-6, H-6'), 6.28 (s, 4H, H-11, H-11')

Figure 10: Bis 1,3[2-hydroxyimino-N-(pyridin-3-yl)acetamid]oxapropandichloride compound - C-RMN spectrum

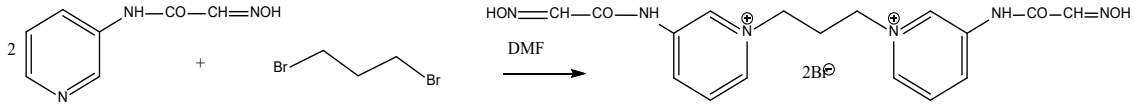


3C-RMN (D2O, δ ppm) carbon spectrum: 162.69(C-8, C-8'), 143.18(C-9, C-9'), 139.08(C-6, C-6'), 138.69(C-2, C-2'), 137.84 (C-4, C-4'); 134.30(C-3, C-3'); 128.79(C-5, C-5'); 88.48(C-11, C-11')

Quaternization with 1,3 dibromopropane to bis 1,3[2-hydroxyimino-N-(pyridin-3-yl)acetamid]propan dibromide.

The symmetric quaternization reaction of 3-pyridyliso-nitrosoacetanilide with 1,3-dibromopropane in a 2:1 ratio goes as described below and is also performed in anhydrous DMF DMF, at a temperature of 40-55^{0}C (Figure 11).

Figure 11: The synthesis of bis 1,3[2-hydroxyimino-N-(pyridin-3-yl)acetamid]propane dibromide



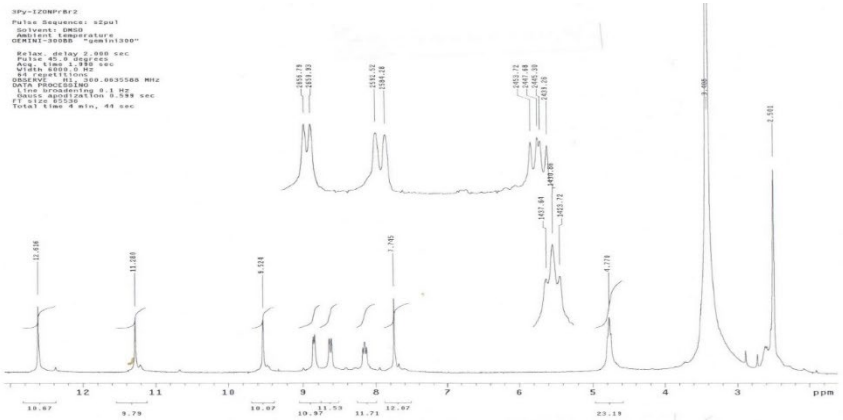
In an Erlenmayer glass, dimethylformamide and isonitrosoacetanilide are introduced. They are magnetically agitated to complete dissolution, then 1,3-dibromopropane that was previously dissolved in 5ml of anhydrous dimethylformamide is added. The recipient is sealed and agitated for 4h at room temperature and for another 10h at 40-60^{0}C. It is cooled down to 0-5^{0}C, and then vacuum filtered. The precipitate is repeteadly washed on the filter, with anhydrous acetone. It is dried in a desiccator in a vacuum overnight. 3.1 g of white crystal product are obtained with mp.180-182^{0}C and a yield of 59.0%.

The synthethized compound, the bisquaternary salt, was characterized through spectral RMN analyses, which confirm its chemical structure. (Figures 12, 13)

RMN characterization

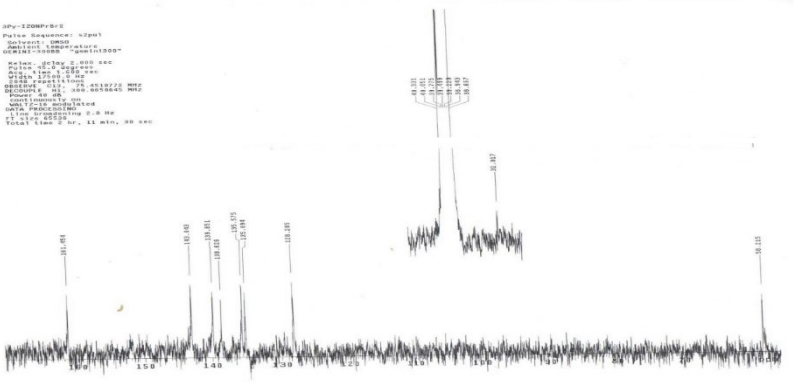
The 1H-RMN hydrogen spectrum is shownin Figure 12and the 13C-RMN carbon spectrum is shownin Figure 18.

Figure 12: 1,3 dibromopropane to bis 1,3[2-hydroxyimino-N-(pyridin-3-yl)acetamid]propan dibromide - H-RMN spectrum



1H-RMN (DMSO-d6, δ ppm, J Hz) spectrum: 10.52 (s, 2H, H-7, H-7); 8.8 (dl, 2.4, 2H, H'-2, H-2); 8.3(dd, 5.0; 1.6, 2H, H-6, H-6); 8.1 (dl, 8.4, 2H, H-4,H-4)', 7.68(s, 2H, H-9, H-9'); 7.43(dd, 8.4, 4.7, 2H, H-5, H-5'), 4.71(t, 7.1, 4H, H-11, H-11'); 2.48(t, 7.1, 2H, H-12)

Figure 13: 1,3 dibromopropane to bis 1,3[2-hydroxyimino-N-(pyridin-3-yl)acetamid]propan dibromide - C-RMN spectrum



13C-RMN (DMSO-d6, δ ppm) carbon spectrum: 160.84 (C-8, C-8'); 143.97 (C-6, C-6'); 143.64 (C-2, C-2'); 140.70(C-9, C-9'), 135.36 (C-3, C-3'); 127.69(C-4, C-4'); 123.95(C-5, C-5'), 59.11(C-11, C-11'); 33.13(C12)

In vitro testing and toxicological characterization of symmetric bisquaternar compounds with a pyridin-isonitrosoacetanilide structure

The experiment was performed in sterile conditions in a microbiological niche.

In vitro testing incldued the two synthethized compounds: bis 1,3[2-hydroxyimino-N-(pyridin-3-yl)acetamid]propane dibromide (coded under the name of HIN 1) and bis 1,3[2-hydroxyimino-N-(pyridyl)acetamid]oxapropandichloride (HIN 2), utilizing obidoxime as reference. Each of these compounds was dissolved in a culture medium, and 3 solutions were obtained for each, with the following concentration: 5mg/ml; 3 mg/ml and 1 mg/ml. HIN 1 was insoluble in the culture medium at the above mentioned concentration.

On a 3T3 cellular line (fibroblasts) from Lonza from which a cellular subculture with a concentration of 1×10^5 cells /ml was obtained as follows:

- the cells were counted initially with the Burger Turck hemacytometer to establish the initial concentration of cells per ml;

- the dilution was performed so that a final concentration of 105 cells/ml would be obtained;

- the cells were cultivated with a proper medium (supplemented DMEM, Lonza reactive) for 24 hours at a temperature of 37 degree Celsius in a CO_2 atmosphere;

- 2 ml of tripsine solution are added (Sigma reactive);

- 5 minutes of centrifugation at 500 G at a temperature of 5-8 degrees Celsius;

- the supernatant is removed;

- resuspension in 5 ml of culture medium (DMEM);

- a volume of 200 microliters is placed in a plate with 96 wells;

- 6 h of incubation in CO_2 atmosphere for adherence;

- after 6 h of incubation for adherence, obidoxime and HIN2 are added after dissolution in the culture medium, equimolecularly, in 3 concentrations: 5 mg/ml, 3 mg/ml and 1 mg/ml;

- 24 h of incubation;

- after 24 h of incubation, 20 microliters of MTS reactive (Lonza) are added to each well;

- 4 h of incubation at 37 degrees Celsius in a CO_2 atmosphere.

The samples were read at 490 nm with a Biorad microplate reader and compared to the control sample represented by the culture medium.

RESULTS

The in vitro toxicity of HIN 2 and obidoxime was assessed through the values of their optical densities corresponding to different molar concentrations. The results are shown in Table 1 and in Figures 14 and 15.

The molar concentrations of studied compounds can be correlated with the cellular viability percentage.

The results are shown in Table 2 and in Figures 16 and 17.

In lower concentrations of 1 mg/ml, values that were close to the in vitro cellular viability were recorded (73.43% for obidoxime, and 82.42% for HIN 2).

For medium molar concentrations (3 mg/ml), cellular

viability values were 67.75% for HIN 2 and 67.36% for obidoxime.

Table 1: The variations of optical density values of obidoxime and HIN 2

	Well1	Well2	Well3	Mean	Substraction blank
Untreated CTRL	1.068	1.112	1.67	1.283333±0.174	1.16083
Obi 5mg/ml	0.362	0.353	0.449	0.388±0.02	0.2655
Obi 1mg/ml	0.875	1.075		0.975±0.025	0.8525
Obi 3mg/ml	0.837	0.972		0.904 ±0.033	0.782
HIN 2 5mg/ml	0.141	0.149	0.152	0.147±0.012	0.0248
HIN2 1mg/ml	0.895	1.078	1.265	1.079±0.030	0.9568
HIN2 3mg/ml	0.849	0.969		0.9090±021	0.7865
CTRL BLANK	0.122	0.123		0.1225	0

Figure 14: Variation of optical density (DO) correlated with concentrations of obidoxime

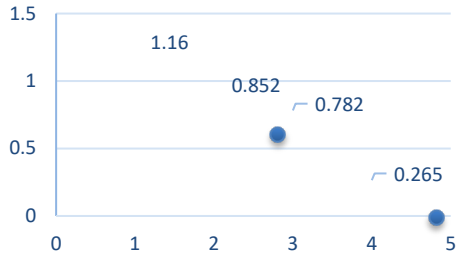


Figure 15: Variation of optical density (DO) correlated with concentrations of HIN 2

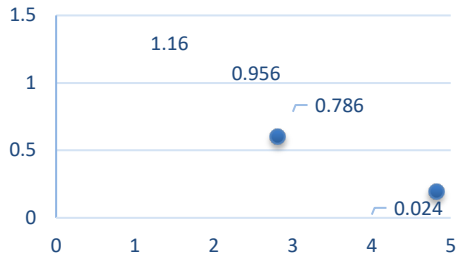


Table 2: Percetange variation of cellular viability for obidoxime and HIN 2

Dose mg/ml	DO Obidox	Cellular viability vs control (%)	DO HIN2	Cellular viability percentage vs control (%)
0	1.160833	100	1.160833	100
5	0.2655	22.8715	0.024833	2.139268
1	0.8525	73.43862	0.956833	82.42642
3	0.782	67.3654	0.7865	67.75305

For high doses, (5 mg/ml) HIN 2 showed a cellular viability value of 2.13%, much lower than that of obidoxime, and 22.87% respectively. Thus, HIN2 is more toxic than obidoxime.

Figure 16: Cellular viability percentage corresponding to different concentrations of obidoxime (y = -13.873 + 97.379)

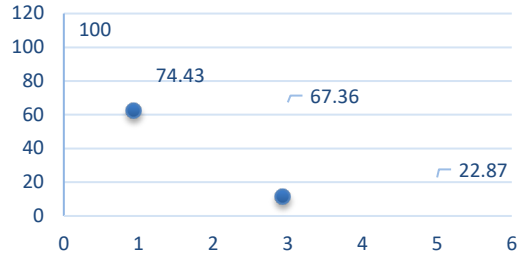
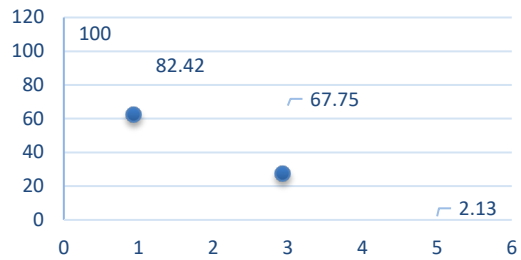


Figure 17: Cellular viability percentage corresponding to different concentrations of HIN 2 (y = -18.397 + 104.47)



The assessment of CT50 was performed according to the working protocol described in the Romanian Pharmacopoeia.

In the regression line ecuations shown in Figures 14 and 15, the y value was replaced with the probit corresponding to the ideal mortality of 50 %. The probit is a statistical measure resulted from the transformation of the percentage of positive answer into a numeric value and is linearly dependent on the logarithm of the concentration. Thus, the x value in the ecuation of the regression line represents the logarithm of CT50. Through calculus, we could estimate CT50 for obidoxime at a value of 3.24 ± 0.005 mg/ml. The CT50 value for HIN 2 was estimated at 2.72 ± 0.009 mg/ml. The CT50 value being inversely proportional to the toxicity of the

substance, it can be understood that the CT50 values are comparable (around the value of 3 mg/ml), HIN 2 being more toxic than obidoxime.

The statistical analysis of experimental results obtained showed statistically significant variations between the determined cellular viabilities of the two compounds in equimolecular concentrations only for high doses. The experimental results are represented in Tables 3-5.

Table 3: The representation of probability P associated with mean values of optical density (OD) correlated with cellular viability corresponding to a concentration of 1 mg/ml (2.29×10^{-6} M)

No.	Compound	Adm. Molar Conc.	Mean value of OD	Probability (P)	Observations
1	Obidoxime	2.29×10^{-6} M;	1.160833333	0.276632	P ≥0,05 – mean values do not significantly differ
2	HIN 2	2.29×10^{-6} M;	0.956833333		

Table 4: The representation of probability P associated with mean values of optical density (OD) correlated with cellular viability corresponding to a concentration of 3 mg/ml (6.94×10^{-6} M)

No.	Compound	Adm. Molar Conc.	Mean value of OD	Probability (P)	Observations
1	Obidoxime	6.94×10^{-6} M;	0.7820	0.4820	P ≥0,05 - mean values do not significantly differ
2	HIN 2	6.94×10^{-6} M;	0.7865		

Table 5: The representation of probability P associated with mean values of optical density (OD) correlated with cellular viability corresponding to a concentration of 5 mg/ml (11.57×10^{-6} M)

No.	Compound	Adm. Molar Conc.	Mean value of OD	Probability (P)	Observations
1	Obidoxime	11.57×10^{-6} M;	0.2655	0.000723	P≤0,05 – mean values significantly differ
2	HIN 2	11.57×10^{-6} M;	0.0248		

CONCLUSIONS

The syntheses took into consideration the principle of obtaining a cholinesterase reactivating chemical compound that is stable, with a possible action at central nervous level, which could prove to be an alternative therapy to obidoxime, being usable in antidote studies.

Following solubility tests, only one compound, bis1,3[2-hydroxyimino-N-(pyridyl)acetamid]oxapropandichloride (HIN 2) qualified for in vitro testing, the other being insoluble.

The CT50 value for obidoxime was estimated through calculus to be 3.24 ± 0.005 mg/ml, or 9.33×10^{-6} M;

The CT50 value for bis1,3[2-hydroxyimino-N-(pyridyl)acetamid]oxapropandichloride (HIN 2) was estimated to be 2.72± 0.009 mg/ml or 6.29×10^{-6} M;

The in vitro tested compounds showed values close to cellular viability in low and moderate equimolecular concentrations in the fibroblast cellular culture (73.43% for obidoxime, and 82.42% for HIN 2).

The bis1,3[2-hydroxyimino-N-(pyridyl)acetamid]oxapropandichloride compounds (HIN 2) showed a value of cellular viability in vitro lower than obidoxime at high equimolecular concentrations, proving to be more toxic. (2.13%, for HIN2, and 22.87% for obidoxime)

Taking into account the close CT50 values, the newly synthethized compound that is as such characterized from a toxicological standpoint can qualify for further studies as an antidote.

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