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PhD thesis summary

**MAJOR ENVIRONMENTAL POLLUTANTS:
DINITROPHENOLS AND DINITROPHENYL
ETHERS, SYNTHESIS, CHEMICAL
CHARACTERIZATION AND BIOLOGICAL
PROPERTIES**

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Major environmental pollutants: Dinitrophenols and dinitrophenyl ethers, synthesis, chemical characterization and biological properties

Dinitrophenols and their derivatives are used in medicine, in chemical syntheses, in explosive and dye industry and especially in agriculture as insecticides, herbicides, acaricides and fungicides (Coman et al., 1986; Sebollela et al., 2010; Mu et al., 2012). These compounds are great environmental pollutants since they are usually highly toxic, they accumulate in the body or turn into other compounds with greater toxicity, numerous fatalities through their intake being described (Takahashi et al., 2003; Harvey et al., 2004; Whang et al., 2005 De La Torre-Sanchez et al., 2006, Harper et al., 2001, Pace et al., 2002, Barker et al., 2006). Their doses accumulate, their remanence in the body being approximately 38 days long. In soil and water they are microbiologically but not chemically degradable, their life span ranging from 8 to 120 days (Hirooka et al., 2006).

Recently, dinitrophenol and its derivatives have been used to reduce the symptoms in Alzheimer's, heart and neurodegenerative diseases (Korda et al., 2005, The Felice et al., 2007, Han et al., 2008). Dinitrophenols decouple oxidative phosphorylation from respiratory processes. It is assumed that they prevent translocation of protons through the mitochondrial membrane, which leads to the inhibition of oxidative phosphorylation. There are two main theories that try to explain the uncoupling of oxidative phosphorylation from breathing: the chemiosmotic theory (Peter Mitchell, Nobel Prize, 1978) and the theory according to which dinitrophenols manifest their harmful action due to their capacity to prevent the formation of unstable triplet states (Szent-Gyorgyi, 1956).

In order to determine organic pollutants such as dinitrophenol pesticides were used: voltammetry, spectral techniques, etc.. (Osselton et al., 1986, Lu et al., 1999; Shormanov et al., 1995, 1996, 1998, 2003, Berhanu et al., 2006).

Given the data in the specialized literature, it can be said that the physical and chemical properties of dinitrophenols and their derivatives are known. However, their biological and biomedical properties are less known.

Therefore, the thesis "**Major environmental pollutants: Dinitrophenols and dinitrophenyl ethers, synthesis, chemical characterization and biological activity**" aims at a complex study of dinitrophenols and other related compounds, using modern chemical, biochemical and toxicological methods, so as to highlight new aspects of their biological and biomedical activity and obtain new experimental data intended to contribute to the elaboration of the action mechanisms of these highly toxic, antidote-lacking compounds. Thus, the research presented in this paper had the following objectives: the analysis of the biological activity of dinitrophenols compared to that of other inhibitors of oxidative phosphorylation or to that of

some stimulants; the synthesis of ethers derived from dinitrophenol; the comparative study of the structure, stability and reactivity of dinitro-derivatives; determining the structure of the compounds obtained by spectral and chemical elemental analysis methods; testing the properties of fluorescence quenching using nitro-derivatives; the theoretical study of the physicochemical properties of the studied compounds.

The first part of the thesis, **Studies in the specialized literature**, summarizes the key data in the literature in the field of dinitrophenols and their ethers with respect to the methods of synthesis, physical and chemical properties, biological activity, toxicity, possible toxicity mechanisms and methods of decontamination up to the year 2012.

The original results of this thesis appear in the second part of the paper, starting with Chapter II.1, which presents the research on the synthesis and chemical characterization of some dinitrophenols and of their derivatives. Some compounds were synthesized, compounds known but poorly characterized through modern methods. Various compounds were characterized, needed to produce the probable action mechanism of dinitrophenols and their derivatives.

While analyzing the IR spectrum of 2,4-DNF, signals were observed at 1300-1000 cm^{-1} specific to ethers, at 1470-1350 cm^{-1} specific to aliphatic C-H bonds, at 3100 cm^{-1} specific to aromatic C-H bonds, in 1607 -1546 cm^{-1} , 1344 cm^{-1} specific to the NO_2 group. In the spectrum of 3-(2,4-dinitrophenoxi) propane)-1,2-diol were observed a signal at 1076 cm^{-1} characteristic to the ether bond, a broad signal around 3352 cm^{-1} characteristic of OH groups as well as of characteristic absorption at 2942.8 cm^{-1} , 1417.4 cm^{-1} , 1288.2 cm^{-1} , 1114 cm^{-1} , 1076 cm^{-1} , 943 cm^{-1} , 874 cm^{-1} and 724 cm^{-1} . Nitro groups showed intense signal at 1530-1540 cm^{-1} .

Chapter II.2 includes spectroscopic studies on some dinitrophenols but also of some representative nitro-derivatives. Their absorption spectra and calibration curves were realized. In an aqueous solution of 2,4-DNP of 10 μM concentration: the UV-VIS absorption spectrum presented four absorption maximums at 212 nm ($A = 2.524$), 228 nm ($A = 2.127$), 259 nm ($A = 2.413$), 356 nm ($A = 1.962$) and a shoulder at 393 nm ($A = 1.337$); the derived spectrum intersected the wavelength axis at 212 nm, 228 nm, 259 nm, 359 nm, and the derived spectrum of second order presented the most significant minimums at 212 nm, 259 nm and 366 nm.

In the study of the analytical reactions of some di- and tri-nitro-derivatives, were created the UV-VIS absorption spectra corresponding to the monitoring of picric acid reaction with potassium cyanide, using ultrasounds in the reaction (maximum absorbance was recorded at 30 s at 360 nm ($A = 1145$)) and were proposed 4 new methods for the determination of cyanide with 2,4-DNP and DNOC in the absence or presence of resorcinol. The studied methods are very simple, accurate, rapid, selective and sensitive and could be applied to the determination of cyanide in the environment and in forensic studies which trace cyanide accumulation in blood, in smokers' body (Dumitraş-Huţanu et al., 2010a; 2010b).

Chapter II.3 assesses the effects of fluorescence quenching in the case of tryptophan, E376H-MCAD enzyme or fluorescein with the help of some nitro-derivatives. Their interaction with molecules in an excited state was used for understanding the transfer of energy and electrons from excited molecules to dinitrophenols (Dumitraș-Huțanu et al., 2008b; Drochioiu, Dumitraș-Huțanu et al., 2009).

We studied the effect of tryptophan fluorescence quenching using nitro-derivatives such as 2-((2,4-dinitrophenyl)amino)acetic acid (Gli DNP), 4,6-dinitro-ortho-cresol (DNOC), 2-amino-5-((1-((carboxymethyl)amino)-3-((2,4-dinitrophenyl)tio)-1-oxo-propane-2-il)amino)-5-oxopentanoic acid (SDN GSH), 2-(1-metilheptil)-4,6-dinitrophenyl crotonate (Karathan), 2,4-dinitroanisol (2,4-DNA), 2,4-dinitrobenzoic acid (2,4-DNB), 4-nitrobenzophenone (4-NBF), etc.. In the spectrum of tryptophan fluorescence quenching in response to the addition of 2,4-DNP of 30.00 μM concentration, the intensity decreased remarkably and was accompanied by a major shift of maximum emission wavelength from 357 nm to 380 nm, and 323 * nm respectively. 2,4-DNP has a constant fluorescence quenching by static mechanism: 3.09 times higher than Karathan, 2.03 times higher than 2,4-DNA and 1.51 times higher than GSH SDN.

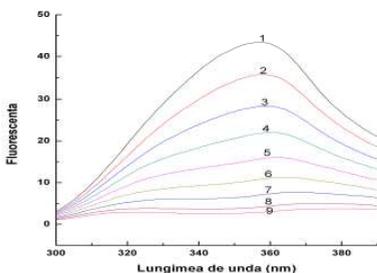


Figure II.3.5. Fluorescence spectra of tryptophan (1.0 $\mu\text{g/mL}$) in the absence (black, spectrum 1) or presence of 2,4-DNP (6.65; 10; 13.33; 16.66; 20.00; 23.33; 26.66 respectively 30.00 μM , spectra 2-9) in Tris buffer 20 mM at pH 7.0.

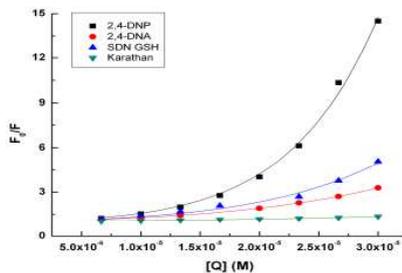


Figure II.3.20. Modified Stern-Volmer plot for the binding of 2,4-DNP, 2,4-DNA, SDN GSH and Karathan to the tryptophan conditions: Tris buffer 20 mM.

The study of fluorescein fluorescence quenching of was carried out using 2,4-DNP and 3,5-dinitrosalicilic acid (DNSA) at different concentrations ($\lambda_{\text{ex}}=490 \text{ nm}$, $\lambda_{\text{em}}=511 \text{ nm}$).

From experiments it was observed that the analytical methods based on fluorescence are very specific and very sensitive, therefore these methods can be extremely useful in the analysis and monitoring of certain environmental pollutants.

Chapter II.4 presents the results obtained regarding the determination of some molecular parameters of the studied molecules by computational modeling, by applying HyperChem 7.5 specialized software (Stănculescu et al., 2006; Jayanthi et al., 2011, Nădejde, 2010). They sought possible correlations between these parameters and the optical or biological

properties of the investigated compounds. Thus, variation in toxicity was observed in the following order: 2,4-DNP > 2,6-DNP > 2,5-DNP, similar to the length of O-H bonds.

Spatial structures and physicochemical parameters were obtained after performing geometry optimization for the following compounds. There was noted increase in hydration energy, surface area, volume, LogP, refractive and polarization ability in the following order: 2,4-DNP < 2,4-DNA < 2,4-DNF < 2,4-DNBu, reversed than the toxicity variation of these compounds.

Analysis was carried out about the physicochemical properties of mono-complexes and di-nitro-derivatives with tryptophan using Pearson's reactions. Electronic chemical potential could make important contributions to the elucidation of the toxicity mechanism of 2,4-DNP and its derivatives.

Chapter II.5 includes toxicological research. We surveyed superior as well as inferior organisms. The results were statistically interpreted. We studied the biological activity of some dinitrophenols on the germination of wheat seeds.

Table 10 The toxicity of 2,4-DNP; 2,5-DNP; 2,6-DNP and DNOC in germination experiments.

Treatment *)	Faculty of germination (F.G.)	Number of plantlets in batch (N)	Total mass of plantlets in batch (M, g)	Mass of plantlets (m, mg)
1. Control sample, H ₂ O	94%	46	2,61±0,1	55,3±7,1
2. 2,4-DNP, 4x10 ⁻³ M	0%	0	0	0
3. 2,5-DNP, 4x10 ⁻³ M	13%	5	0,16±0,1	24,7±4,4
4. 2,6-DNP, 4x10 ⁻³ M	1%	0	0	0
5. DNOC, 4x10 ⁻³ M	0%	0	0	0
D (Test Tukey)	7,9	7	0,12	2,7

*) Average of three independent values

We compared the biological effects of some decoupling agents of oxidative phosphorylation with different stimulators, also involving biometric studies. The big differences in the length of plantlets, except control samples, demonstrate once again that the presence of chemicals, whether stimulating or inhibiting, has a disruptive effect on the development of plantlets (Dumitraş-Huţanu, 2010c).

Within the study of the effects of nitrophenyl derivatives and other compounds on wheat seed germination, several samples were contaminated with fungi, except those treated with 2-oxo-2-(4-nitrophenyl) acetic acid and 4-nitrobenzoic acid, probably due to the reduction of nitro groups to amino groups. The size of plantlets was stimulated the most by phenylalanine, by 8%. The mass of plantlets was inhibited in the highest degree by picric acid 30.6 mg/plantlet, but stimulated by 4-nitrobenzoic acid 60.8 mg/plantlet (Drochioiu, Molnar, Grebinişan, Dumitraş-Huţanu et al., 2008).

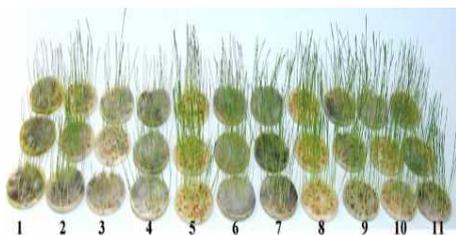


Figure II.5.11. The effect of nitrophenyl derivatives and other compounds at treatment solution concentrations of 5×10^{-3} M.

1. picric acid, 2. p-nitroacetophenone 3. 2,2'-bipyridin-3,3',6,6-tetra-carboxylic acid 4. 3,5-dinitrosalicilic acid 5. 2-oxo-2-(4-nitrophenyl) acetic acid 6. 2,4-dinitrobenzoic acid 7. 2-oxo-glutaric acid; 8. 4-nitrobenzoic acid 9. L-phenylalanine; 10. resorcinol; 11. Control sample.

We studied the effect of 2,4-DNP and magnesium sulphate on wheat seed germination. Given the number of sprouted seeds, the difference between samples treated with 2,4-DNP and those treated with a mixture of 2,4-DNP and $MgSO_4$, is significant. Perhaps $MgSO_4$ could be used to treat poisoning with 2,4-DNP (Dumitraş-Huţanu, 2010b, 2010d).

We decided to test dinitrophenol compounds on yeasts as well since these can provide more information about the action course of dinitrophenols at cellular level. For example, the biological effect of 2,4-dinitrophenol (2,4-DNF), of 2,4-dinitrophenoxiacetic acid (2,4-DNPac acid) and of 6-methyl-2,4-dinitrophenoxiacetic acid (6-methyl-2,4-DNPac acid) was studied at $22^\circ C$. The samples had the same concentration of: yeast, *Saccharomyces cerevisiae*, 10 g/L, sucrose 50 g/L and mono-potassium phosphate, KH_2PO_4 , 0.4 g/L. 2,4-DNPac acid stimulated the production of alcohol by 10.2%, 2,4-DNF by 20.3% and 6-methyl-2,4-DNPac acid by 27.6%.

In Chapter II.6, **The experimental part**, are presented: the materials, the procedures for obtaining 2,4-dinitrophenyl ethers, 2,4-dinitrophenoxiacetic acids, the spectral methods of analysis of nitrated derivatives and also the methods used in toxicological research.

The paper ends with **General conclusions**, **Bibliography** and **Appendices**.

The research conducted in this paper allows us to draw the following general conclusions:

I. With conventional heating and/or under the action of ultrasounds, new methods for the synthesis of dinitrophenyl ethers were found, methods which are faster and more efficient (short period of time, low energy consumption) than that provided by conventional heating.

II. The use of ultrasounds in the synthesis of the studied ethers resulted in increased yield and reduced reaction time.

III. The spectroscopic studies of some nitro-derivatives led to the conclusion that the highest value of the radiant energy absorbed by one mole of substance, $E=26835/\lambda_{max}$ (kcal/mol) was obtained in the case of 2,4-DNP.

IV. The spectroscopic studies regarding the use of some nitro-derivatives in the determining of cyanide led to the following conclusions:

a. Under the action of ultrasounds, a new method for the determination of cyanide was proposed, method which is faster and more efficient (short period of time, low energy consumption) than that provided by conventional heating;

b. We also proposed four new methods for the determination of cyanide with 2,4-DNP, DNOC and 2,4-DNP or DNOC and resorcinol.

V. The following considerations can be made about the ability of fluorescence quenching of dinitrophenols and their derivatives:

a. The high values of the quenching constants provide us a proof of the high efficiency of dinitrophenols and their derivatives as quenchers of fluorescence;

b. Out of the studied compounds, 2,4-DNP and DNOC showed the highest values of the constants of tryptophan fluorescence quenching, these being also the most toxic compounds.

c. Decreased tryptophan fluorescence intensity in the presence of mono- or dinitro-derivatives is generally accompanied by a shift in the maximum emission wavelength. 2,4-DNP and DNOC, which are the most powerful quenchers of tryptophan fluorescence, produced the biggest variations in maximum emission wavelengths.

d. The lock of -OH phenolic group or its replacement decreased tryptophan fluorescence quenching ability by dinitro-derivatives;

e. The change of NO₂ group from position 4 also led to the decrease in the values of Stern-Volmer constants;

f. Nitro-derivatives acted through the dynamic mechanism as well as through the static mechanism of tryptophan fluorescence quenching;

g. According to the linear Stern-Volmer representations, DNSA acid quenches fluorescein fluorescence more than 2,4-DNP;

h. Decrease in fluorescein fluorescence intensity in the presence of DNSA acid or 2,4-DNP is not accompanied by a shift in the maximum emission wavelength.

VI. The theoretical studies of some dinitro-derivatives led to the following conclusions:

a. The modeling of the molecular structures of dinitrophenols, dinitrophenyl ethers and other dinitro-derivatives - by three calculation methods: one of molecular mechanics, MM+ and two semi-empirical methods, PM3 and AM1, within the HyperChem program, permitted the calculation of some parameters specific to the ground state (dipole time, polarization, energy, etc.);

b. Within the theoretical study of the interaction of dinitro-derivatives with tryptophan by MM+, PM3 and AM1 methods, we obtained the values of QSAR properties (the accessible surface of the solvent, the molecular volume, logP, refractive and polarization ability) for complexes;

c. The interaction energy was calculated using the MM+ method, and $\Delta H_{\text{association}}$ and the dipole moment for the studied complexes were calculated using the PM3 method.

d. The value of the electronic chemical potential of the most stable complex of 2,4-DNP-with tryptophan is higher than the values of the electronic chemical potentials of complexes corresponding to derivatives.

VII. The studies on the toxicity, degradation and action mechanism of dinitrophenols and their derivatives led to the following conclusions:

a. Tests were performed on yeasts and wheat seeds, which showed a different behavior of superior plants in comparison to that of *Saccharomyces cerevisiae*;

b. The experiments with wheat seeds subjected to germination demonstrated their sensitivity to dinitrophenols and dinitrophenyl ethers, in the concentration range 10^{-4} M, 10^{-2} M;

c. Dinitrophenols are highly toxic compounds that do not allow the germination of wheat seeds starting from the concentration of 5×10^{-3} M;

d. The experimental results obtained were accurate and statistically secured;

e. The present research showed that dinitrophenyl ethers are likely to act in a non-chemical way, perhaps by forming radicals and triplet states, and that proton translocation could be a secondary phenomenon in the process of oxidative phosphorylation;

f. The action mechanism of the investigated compounds appears to be consistent with the well-known bio-structural theory developed by Eugen Macovschi, while the data obtained indicate the need for reassessing Peter Mitchell's chemiosmotic hypothesis on ATP synthesis during the breathing process as a result of proton translocation through mitochondrial membranes;

g. The obtained data open the way for further research intended to evaluate the relationship between the chemical structure of dinitrophenols as well as of other derivatives, and their properties as decoupling agents. Further investigations are needed to bring further explanations and details of decoupling mechanisms.

The results of the research are recorded in 146 tables, 104 figures and 16 diagrams, and form the subject of 6 original published papers and of 2 papers to be published. Thus, some of the results contained in this paper are subject to several scientific papers published or in press (a paper published in a journal impact factor and 2 articles for publication). However, two papers were submitted for publication in foreign journals with impact factor, 3 scientific papers published in journals of category B+, 2 scientific papers published in CNCSIS journals, 2 papers presented at international conferences and 9 papers presented at national conferences. The numbering of chapters, figures, tables and diagrams is the same as in the thesis.