UNIVERSITY OF MEDICINE AND PHARMACY

„CAROL DAVILA" BUCHAREST

DOCTORAL SCHOOL

PHARMACY FIELD

SYNTHESIS AND CHARACTERIZATION OF SOME NEW HETEROCYCLIC COMPOUNDS WITH PYRROLE OR FUSED PYRROLE CORE WITH POTENTIAL BIOLOGICAL ACTIVITY

PHD THESIS SUMMARY

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Introduction

The resistance of cancer cells and microorganisms to the action of current treatments or the unavailability of some therapies for certain serious diseases would be some of the reasons why the need for synthesis from organic chemistry is a real and well-founded one. The chemistry of heterocycles, a branch of organic chemistry, is an intensively studied research field that can offer solutions in the discovery of new molecules with therapeutic action.

The heterocyclic compounds are of particular importance, both from the physico-chemical point of view, and especially from a biological perspective, a large number of drugs with a wide spectrum of action, used to treat various ailments, contain such molecules as active substances. The statistical data indicated that more than 85% of the total number of bioactive molecules are heterocycles that contain nitrogen as a heteroatom in the molecule [1-3]. From the five-membered heterocyclic compounds class, the pyrrole, a heterocycle with one nitrogen atom, is one of the most widespread compounds of vital importance to living organisms, given that it is found in the structure of numerous biomolecules, including heme B, chlorophyll, vitamin B12, bile pigments (bilirubin and biliverdin), myoglobin, cytochromes, cofactor F430. A rich source of biocompounds with pyrrole structure are alkaloids extracted from marine organisms, bacteria, fungi, plants, vertebrates and invertebrates [4-6]. The literature data has shown that different synthesis derivatives with a pyrrole core display numerous biological properties, such as antitumoral, antimicrobial, analgesic, anti-inflammatory, antiviral, hypoglycemic, antidepressant actions [3-8]. Moreover, this core is present in the structure of some drugs, the most known being atorvastatin, sunitinib, pemetrexed, tolmetin, zomepirac, ketorolac, pirvinium, tropisetron, ruxolitinib, remdesivir, ondansetron [3-8].

On the other hand, studying the literature data related to the compounds of the pyrrole class, the compounds with a fused pyrrole core also caught my attention. Although the condensing possibilities of this core with another heterocycle are numerous, I directed my research to those containing a pyridazine core, the aromatic six-membered heterocycle containing two nitrogen atoms in positions 1 and 2, namely on heterocyclic compounds fused with pyrrolo[1,2-*b*]pyridazine structure. The synthetic derivatives of pyridazine are known for their wide spectrum of biological properties, for example antitumor, analgesic, anti-inflammatory, antimicrobial, antidiabetic, anticonvulsant, antidepressant, hypotensive, etc

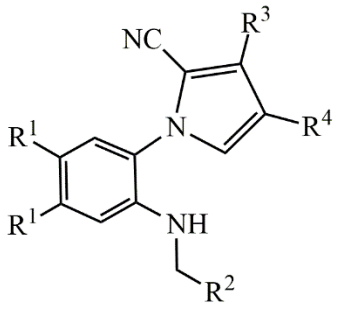
[9-16]. However, compared to pyrrole derivatives, the number of pyridazine derivatives biosynthesized by different organisms is much lower [9]. Another encouraging aspect regarding the importance of pyridazine is its presence in the structure of some drugs: tepotinib, pildralazine, quadralazine, emorphazone, minaprine [9-16]. These fused heterocyclic systems, pyrrolo[1,2-*b*]pyridazines, are known for biological properties such as antitumor, antimicrobial, anti-inflammatory, hypolipidemic and anxiolytic [17-25], and also for their remarkable fluorescent properties [26-33]. However, compared to the potential of these two heterocyclic cores present in the molecule, the physico-chemical and biological properties of pyrrolo[1,2-*b*]pyridazines are little studied.

Considering these multiple properties and applications of heterocycles from the pyrrole, pyridazine and pyrrolo[1,2-*b*]pyridazine class, we considered it interesting to synthesize new compounds with an isolated tri- or tetrasubstituted and fused pyrrole core, from the pyrrolo[1,2-*b*]pyridazines, in order to identify new biologically active derivatives with therapeutic potential.

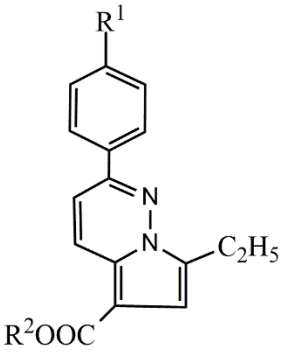
Furthermore, knowing that indole, a heterocycle containing a pyrrole core fused with a benzene ring, is found in the structure of some natural compounds (tryptophan, serotonin, melatonin and in some alkaloids), and also in the structure of some drugs (vincristine, vinblastine, osimertinib, panobinostat, indomethacin, acemethacin, arbidol, sumatriptan, fluvastatin, etc.) [34-36], another distinct objective of this research was the study of the Nenitescu synthesis [37].

The obtaining of the new compounds was achieved through the development of synthesis methods established by the research group led by PhD Florea Dumitrascu from the "Costin D. Nenitescu" Institute of Organic and Supramolecular Chemistry, Romanian Academy [38-40]. Tri- or tetra-substituted pyrrole derivatives were obtained from *N*-ylides generated *in situ* from various 3-cyanomethyl-benzimidazolium bromides in 1,2-epoxybutane medium, with dipolarophile alkynes via the 1,3-dipolar cycloaddition reaction (Fig. 1.). The pyrrolo[1,2-*b*]pyridazine derivatives were obtained by the cycloaddition reaction of mesoionic oxazolones, generated *in situ* from the corresponding pyridazinone acids in acetic anhydride, with dipolarophile alkynes (Fig. 1.). Moreover, by reinvestigating Nentescu synthesis of 5-hydroxyindole in which a compound was isolated and was attributed a bisazepine structure

[37], the new X-ray diffraction investigations demonstrated a pyrrolilidene-azepine structure, different from the previously formulated one (bisazepine) (Fig. 2).



R^1, R^2, R^3, R^4: H, alchil, aril, benzoil, alchiloxicarbonil



R^1: H, X, R^2: CH_3, C_2H_5

Figure 1. Structure of new heterocyclic compounds with pyrrole core

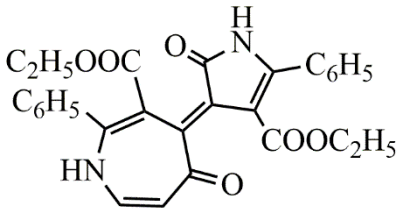


Figure 2. Structure of the new pyrrolilidene-azepine compound

The structures of the new compounds were confirmed by elemental analysis, infrared spectrometry (IR), nuclear magnetic resonance spectrometry (^{1}H-NMR and ^{13}C-NMR), and in the case of some of them by X-ray diffraction.

In order to identify new compounds with biological potential, cytotoxicity was investigated on plant cells of *Triticum aestivum* L., animal cells of some crustaceans (*Artemia franciscana* Kellogg and *Daphnia magna* Straus), standardized human tumor cell lines (LoVo, MCF-7, SK-OV-3) and normal HUVEC cells.

The present work is structured in two parts: The General part and Personal contributions, to which are added the List of published works, List of abbreviations and symbols, Introduction, Conclusions and personal contributions, References and Appendices. In the first part (General Part), I highlighted the most important research regarding the synthesis and biological properties of these compounds, while in the second part (Personal Contributions), I presented the synthesis of the new compounds, the physico-chemical characterization and their cytotoxicity evaluation.

I. GENERAL PART

Chapter 1. Heterocyclic compounds with pyrrole core

The pyrrole is an aromatic five-membered heterocyclic compound, containing the nitrogen as a heteroatom, being the structural unit in the composition of various biomolecules and drugs. The presence of the pyrrole in the structure of porphyrins gives it a special importance, the best known being heme B and chlorophyll. Other biomolecules containing the pyrrole core are vitamin B12, bilirubin, biliverdin, cofactor F430 and numerous alkaloids. Among the drugs containing the pyrrole core, the following stand out: atorvastatin (HMG-CoA reductase inhibitor), sunitinib (antitumor by inhibiting protein kinases), tolmetin and zomepirac (non-steroidal anti-inflammatory drugs), pirvinium (anthelmintic) [4-8].

In this chapter is presented the main synthesis methods and biological properties of pyrrole core derivatives. One of the most important synthesis methods is the Huisgen reaction, that consists of the [3+2] cycloaddition reaction between 1,3-dipoles and alkenes or alkynes dipolarophiles [41, 42], synthesis on which I focused my research within these doctoral studies. Afterward, the literature data on the biological properties of numerous heterocyclic compounds with a pyrrole core are presented: antitumoral, antimicrobial, antiviral, analgesic, anti-inflammatory, hypoglycemic, antidepressant [3-8].

Chapter 2. Heterocyclic compounds with fused pyrrole core

Fused heterocyclic compounds of the pyrrolo[1,2-*b*]pyridazines class, obtained by the formal condensation of two heterocyclic core, pyrrole and pyridazine, are known for their biological potential. In this chapter, similar to the previous chapter, the main method of obtaining and biological properties of these compounds are presented [17-25, 39, 40]. The methods of obtaining of these fused compounds can be classified, depending on the used precursors, as follows: from pyridazine or its derivatives or from derivatives with a pyrrole core. Among these, the research were focused on the methods of obtaining from pyridazine derivatives, being presented literature data related to their reactions with alkynes dipolarophiles by 1,3-dipolar cycloaddition. Furthermore, updated literature data on the antitumoral, antimicrobial and anti-inflammatory properties of pyrrolo[1,2-*b*]pyridazine derivatives was introduced.

II. PERSONAL CONTRIBUTIONS

Chapter 3. Working hypothesis and general objectives

Starting from the literature data in which the importance of heterocyclic compounds with a pyrrole core is presented, in order to identify new biological agents more active with low toxicity compared to the existing ones, we proposed the synthesis of new derivatives with a pyrrole core and fused pyrrole from pyrrolo[1,2-*b*]pyridazines class, by the 1,3-dipolar cycloaddition reaction between heteroaromatic *N*-ylides or mesoionic oxazolones and alkynes dipolarophiles.

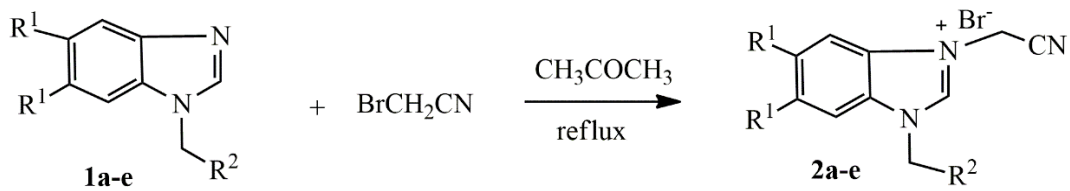
The general objectives proposed for these doctoral studies were:

- synthesis of new tri- or tetrasubstituted pyrrole derivatives;
- synthesis of new fused pyrrole derivatives from the pyrrolo[1,2-*b*]pyridazine class;
- the confirmation of the chemical structure of the new synthesized compounds by elemental analysis, spectral methods (^{1}H-NMR, ^{13}C-NMR, IR) and X-ray diffraction;
- the assesment of *in vivo* cytotoxicity on plant cells of *Triticum aestivum* L. and animal cells (*Artemia franciscana* Kellogg *and Daphnia magna* Straus);
- the assesment of *in vitro* cytotoxicity on tumor cell lines (LoVo, MCF-7, SK-OV-3).

Chapter 4. Synthesis and characterization of some new heterocyclic compounds with pyrrole core

For the obtaining of the new tri- or tetrasubstituted pyrroles compounds a two-step synthesis method was used. The benzimidazolium salts were synthesized in the first step, and in the second, by their reaction with the alkynes dipolarophiles, the new pyrroles were obtained.

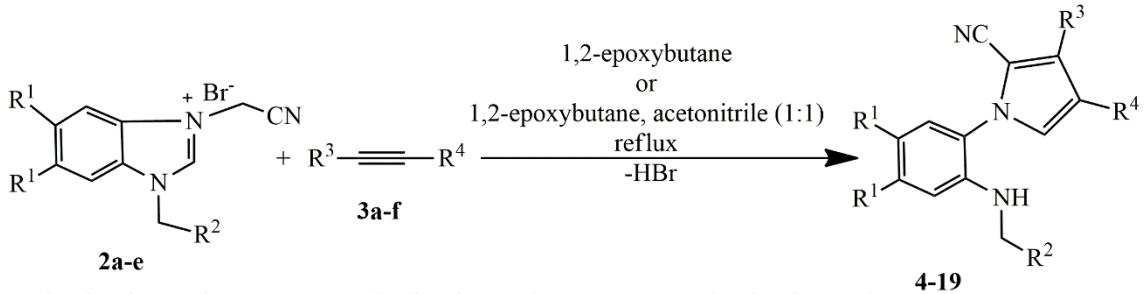
The benzimidazolium salts intermediates **2a-e** were obtained by the reaction of the N-substituted benzimidazole derivative **1a-e** with bromoacetonitrile (Scheme 4.1), with yields of 75–87%. Among them, only derivatives **2d** and **2e** are new compounds [38, 43, 44].



a: R^1=R^2=H; **b**: R^1=H, R^2=C_6H_5; **c**: R^1=H, R^2=4-$CH_3C_6H_4$; **d**: R^1=CH_3, R^2=C_6H_5; **e**: R^1=H, R^2=4-FC_6H_4

Scheme 4.1. Synthesis of benzimidazolium salts intermediates **2a-e**

Regarding the new heterocyclic pyrrole compounds, the synthesis of derivatives **4-13** was carried out in 1,2-epoxybutane medium, at reflux for 48 h, with yields of 35-60%. In contrast, the fluorinated derivatives **14-19** were obtained by an improved method, in 1,2-epoxybutane and acetonitrile (v:v=1:1) under reflux for 6 h, with yields of 46-61% (Scheme 4.2.) [45-47].



4: R^1=R^2=R^3=H, R^4=COC_6H_5; **5**: R^1=R^2=R^3=H, R^4=$COOCH_3$; **6**: R^1=R^2=R^3=H, R^4=$COOC_2H_5$;
7: R^1=R^2=R^3=H, R^4=$COOCH(CH_3)_2$; **8**: R^1=R^2=H, R^3=R^4=$COOCH_3$; **9**: R^1=R^2=H, R^3=R^4=$COOC_2H_5$;
10: R^1=R^3=H, R^2=C_6H_5, R^4=COC_6H_5; **11**: R^1=R^3=H, R^2=4-$CH_3C_6H_4$, R^4=COC_6H_5; **12**: R^1=CH_3, R^2=C_6H_5,
R^3=H, R^4=$COOCH_3$; **13**: R^1=CH_3, R^2=C_6H_5, R^3=H, R_4=$COOC_2H_5$; **14**: R^1=R^3=H, R^2=4-FC_6H_4, R^4=COC_6H_5;
15: R^1=R^3=H, R^2=4-FC_6H_4, R^4=$COOCH_3$; **16**: R^1=R^3=H, R^2=4-FC_6H_4, R^4=$COOC_2H_5$; **17**: R^1=R^3=H, R^2=4-FC_6H_4,
R^4=$COOCH(CH_3)_2$; **18**: R^1=H, R^2=4-FC_6H_4, R^3=R^4=$COOCH_3$; **19**: R^1=H, R^2=4-FC_6H_4, R^3=R^4=$COOC_2H_5$

Scheme 4.2. Synthesis of new tri- or tetrasubstituted pyrrole derivatives **4-19**

The structural confirmation of the new pyrrole derivatives **4-19**, purified by column chromatography and recrystallized from alcohol, was carried out by elemental analysis and spectral methods (IR, ^{1}H-NMR and ^{13}C-NMR), and in the case of derivatives **6**, **13** and **19**, by X-ray diffraction. Moreover, the structure of the new salts **2d** and **2e** was confirmed by elemental analysis, ^{1}H- and ^{13}C-NMR spectrometry.

Structure determination of the benzimidazolium salts 2a-e

In the proton spectra, the most important evidence for the structural confirmation of the benzimidazolium salts was the presence of the new singlet signal of the methylene protons bound to the cyano group, at the chemical shift of 5.80 ppm (for **2d**) and 5.83 ppm (**2e**).

The ^{13}C-NMR spectra confirmed the structure of the bromides through the presence of two new signals corresponding to the two carbons of the cyanomethylene radical, the carbon atom of the cyan group being present at 114.2 ppm (**2d**) and 114.3 ppm (**2e**), and the methylene one at 35.0 ppm (**2d**) and 35.2 ppm (**2e**).

Structure determination of new heterocyclic compounds with pyrrole core 4-19

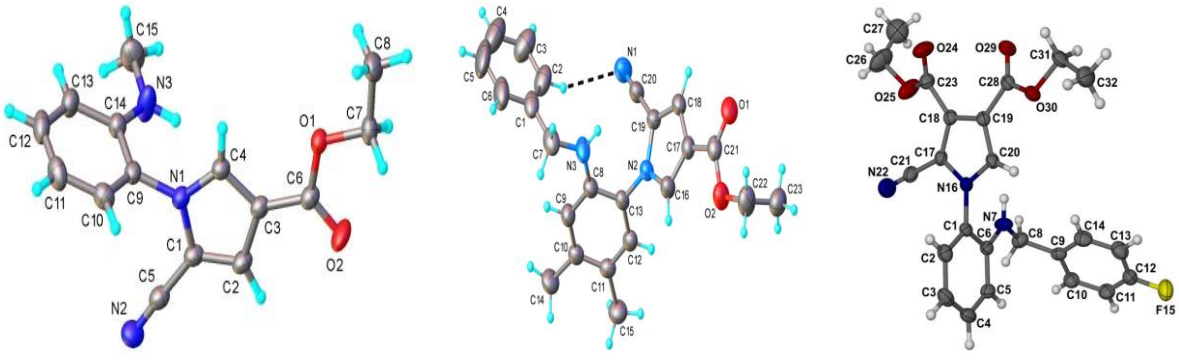
In the infrared spectra of the new compounds **4-19**, a new absorption band characteristic of the stretching vibration of the amino group is present in the region 3341-3427 cm^{-1},

confirming that the reaction took place by breaking the imidazolic ring with the formation of the new pyrrole core. Another evidence confirming the cycloaddition reaction is the presence of the new $\nu_{C=O}$ absorption band from the benzoyl group (1632-1637cm^{-1}) or from the alkyloxycarbonyl fragment (1699-1752 cm^{-1}).

In the proton spectra of the new compounds **4-19**, the appearance of the new signal of the proton from the amino group at values of the chemical shift δ in the range 3.38-4.01 ppm as triplet (N**H**-CH_2-C_6H_5), with coupling constant J=5.2-5.5 Hz, or as a quartet (N**H**-CH_3), with J=4.7-4.9 Hz confirmed that the reaction occurred. In the case of the trisubstituted derivatives **4-7**, **10-17**, by the coupling of the two protons from the pyrrole core, H-3 and H-5, two doublet signals appeared with J=1.6-1.7 Hz, at δ=7.29-7.47 ppm for H-3 and at δ=7.45-7.52 ppm for H-5. The proton H-5 of the pyrrole core of the tetrasubstituted derivatives **8**, **9**, **18** and **19** appeared as singlet signal (7.36-7.41 ppm). The proton signals from the ester group, grafted at positions 4, or 3 and 4, appeared at corresponding values of the chemical shifts.

In the ^{13}C-NMR spectra, the signals of the carbon atoms from the pyrrole core appeared at chemical shifts ranging from 121.6-122.6 ppm for C-3, 118.0-126.3 ppm for C-4, and 131.6-132.8 ppm for C-5. The signals of all carbons in the benzoyl, methyl- ethyloxycarbonyl fragments appeared at the corresponding δ values.

X-ray diffraction data for pyrroles **6**, **13** and **19** unequivocally confirmed the structure of the new compounds. The molecules crystallized in the monoclinic system in P21/c space group as two independent molecules (compound **6**, Fig. 4.1.a) or in the triclinic system in P-1 space group as two chemically equivalent but crystallographically independent molecules (**13**, **19**, Fig. 4.1.b-c).



a) 6 **b) 13** **c) 19**

Figure 4.1. ORTEP diagrams of pyrroles **6**, **13** and **19**

In the crystal, the molecules interacted through hydrogen bonds of the N-H···O or C-N···H type, forming two similar but crystallographically independent supramolecular chains (Fig. 4.2.a.). In the case of pyrrole **19**, it could be observed that the structure of the other dimer was chemically identical, forming the same hydrogen bonds (Fig. 4.2.b.).

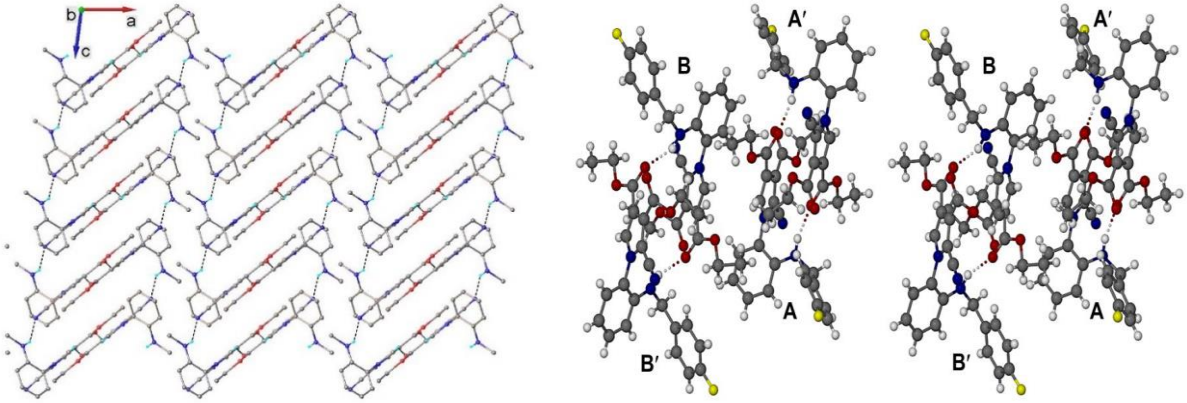
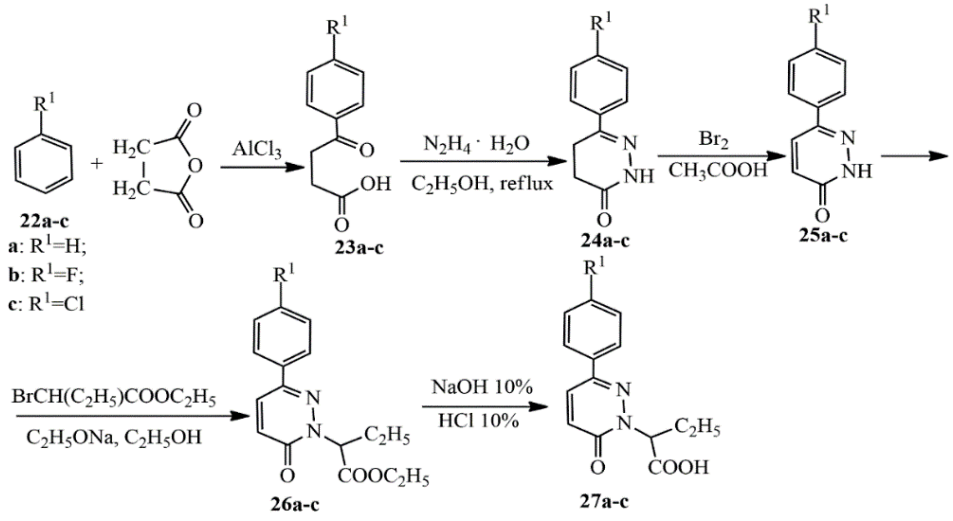


Figure 4.2. a) Crystal packing of pyrrole **6; b)** Structure of pyrrole dimer **19**

Chapter 5. Synthesis and characterization of some new heterocyclic compounds with fused pyrrole core from the pyrrolo[1,2-*b*]pyridazines class

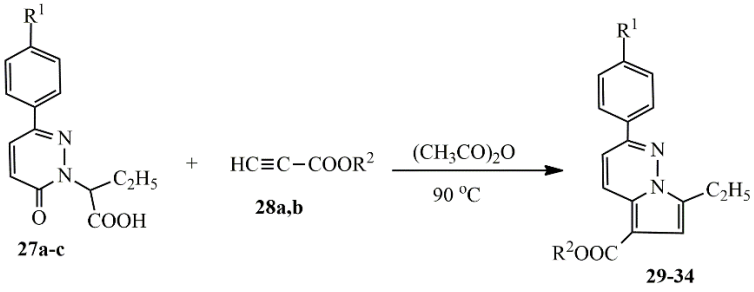
The synthesis of fused heterocyclic compounds from the pyrrolo[1,2-*b*]pyridazines class was carried out in several steps, the pyridazinone esters **26a-c** being obtained with yields of 67-74%, and the key intermediates, acids **27a-c**, with yields of 87-90% (Scheme 5.1.).



Scheme 5.1. Synthesis of pyridazinone esters **26a-c** and pyridazinone acids **27a-c**

The synthesis of new pyrrolo[1,2-*b*]pyridazines **29-34** took place by the reaction of pyridazinone acids **27a-c** with unsymmetrical alkynes **28a**,**b** (41-52% yields; Scheme 5.2.) [48].

The confirmation of the structures of esters **26a-c**, acids **27a-c** and new pyrrolo[1,2-*b*]pyridazines **29-34** was carried out by elemental analysis, IR, ^{1}H-, ^{13}C-NMR spectrometry, and also by X-ray diffraction in the case of ester **26c**, acid **27b** and pyrrolo[1,2-*b*]pyridazine **29**.



27a: R^1=H; **27b**: R^1=F; **27c**: R^1=Cl

28a: R^2=CH_3; **28b**: R^2=C_2H_5

29: R^1=H, R^2=CH_3; **30:** R^1=F, R^2=CH_3; **31:** R^1=Cl, R^2=CH_3; **32**: R^1=H, R^2=C_2H_5; **33**: R^1=F, R^2=C_2H_5; **34**: R^1=Cl, R^2=C_2H_5

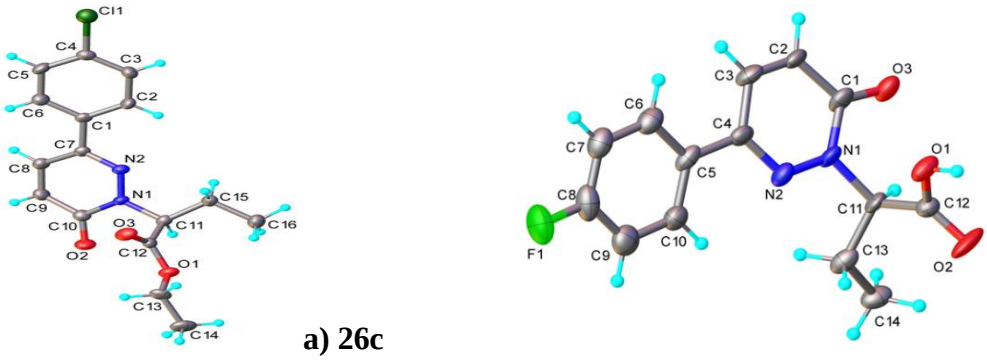
Scheme 5.2. Synthesis of pyrrolo[1,2-*b*]pyridazine derivatives **29-34**

Structure determination of pyridazinone esters 26a-c and pyridazinone acids 27a-c

The structure of esters and acids intermediates was confirmed by the $\nu_{C=O\ ester/acid}$ absorption bands (1734-1738/1708 cm^{-1}) and the characteristic $\nu_{C=O\ lactam}$ bands (1631-1665 cm^{-1}). In the spectra of acids **27a-c**, the associated ν_{OH} band (2931-2980 cm^{-1}) was also observed.

The ^{1}H-NMR and ^{13}C-NMR spectra of compounds **26a-c** and **27a-c** confirmed their structure by the presence of proton and carbon signals from the CH_3-CH_2-CH-COO fragment. Also, in the spectra of esters **26a-c**, the protons and carbons signals from the ethyloxycarbonyl fragment were highlighted, and in **27a**, the carboxyl proton was observed at 9.65 ppm.

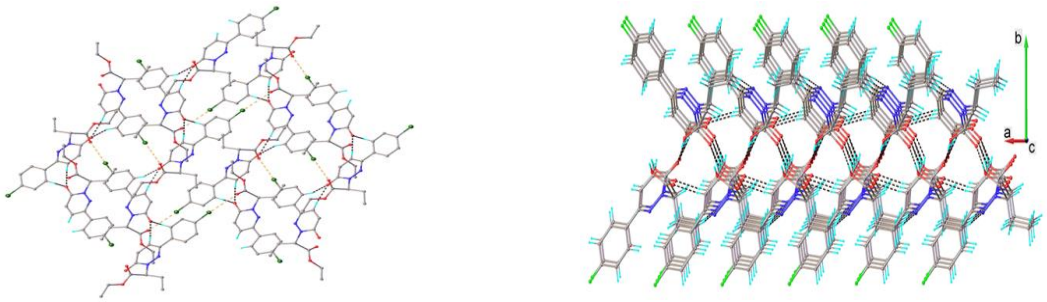
The X-ray data confirmed the structures of ester **26c** and acid **27b** (Fig. 5.1.).



a) 26c **b) 27b**

Figure 5.1. ORTEP diagrams of compounds **26c** and **27b**

In the fig. 5.2. are presented the supramolecular structures of these intermediates.



a) 26c **b) 27b**

Figure 5.2. a) 3D supramolecular network of ester **26c**; **b)** 2D structure of the supramolecular chains of acid **27b**

Structure determination of the pyrrolo[1,2-*b*]pyridazines 29-34

The IR spectra of the new compounds **29-34** confirmed the presence of the ester-type carbonyl functional group through the $\nu_{C=O\ ester}$ band in the region 1666-1688cm^{-1}. The absence of the $\nu_{C=O\ lactam}$ band from the acids precursors confirmed the 1,3-dipolar cycloaddition reaction.

In the ^{1}H-NMR spectra, a new characteristic signal was that of the proton from 6 position of the pyrrolo[1,2-*b*]pyridazine ring, present as singlet in the case of derivative **31** at δ=7.13 ppm. The spectra of derivatives **29**, **32** and **34**, recorded in diluted solution, highlighted a triplet signal for this proton due to the coupling with the protons of the methylene group from the 7 position of the ethyl radical, at δ=7.11-7.15 ppm (J= 0.9Hz). The ^{13}C-NMR spectra confirmed the formation of the new pyrrole ring through the presence of the two signals of the C-5 carbon (δ=103.0-103.6 ppm) and C-6 (δ=112.6-112.8 ppm) atoms, the signals of the other atoms being present at corresponding δ values.

By X-ray diffraction it was observed that the structural unit of pyrrolo[1,2-*b*]pyridazine **29** was formed by two chemically identical, crystallographically independent molecules (Fig. 5.3.a) and presented a parallel crystallographic packing according to 1D structure (Fig. 5.3.b).

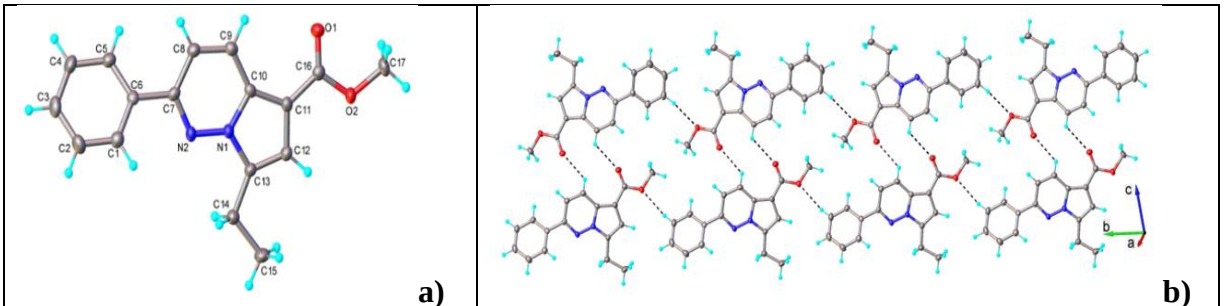
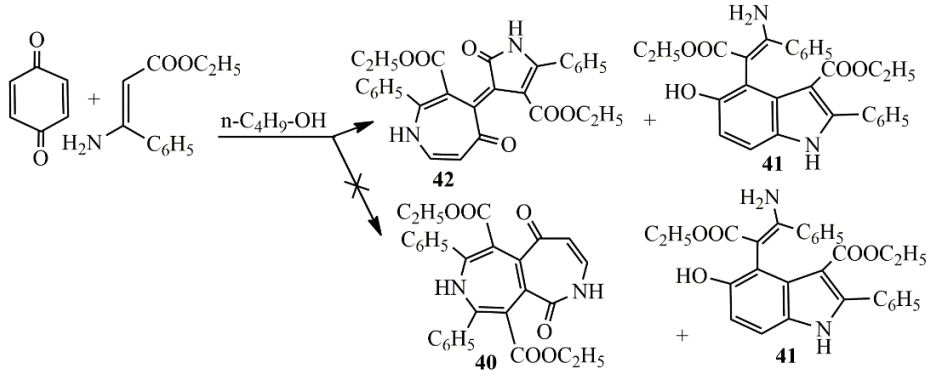


Figure 5.3. a) Structure of pyrrolo[1,2-*b*]pyridazine **29**; **b)** 1D supramolecular structure

Chapter 6. Synthesis and characterization of some heterocyclic compounds with pyrrole core obtained by the reinvestigating Nenitescu reaction

Through the reaction between *p*-benzoquinone and ethyl 3-aminocinnamate in 1-butanol medium, in 1988 Palaghiță M. and Răileanu D. [37] obtained a mixture consisting of two products, a bisazepine derivative **40** and a 4-enamino-5-hydroxyindole **41** (Scheme 6.1.), whose structures were confirmed by IR and NMR spectrometry [37]. In the ^{1}H-NMR spectrum of compound **40**, the authors [37] reported the nonequivalence of two methylene protons in ester group.

Through the X-ray diffraction reinvestigation of the single-crystal structure of compound **40**, the bisazepine structure was disproved, revealing an isomer of it, type **42** (Scheme 6.1.) [49].



Scheme 6.1. Synthesis of the pyrrolilidene-azepine derivative **42** and the 5-hydroxyindole derivative **41** by reinvestigation of the Nenitescu reaction

The analysis of the single-crystal structure revelead, that the molecule did not present two fused azepinie rings **40** [37], but that it contains an azepine and a pyrrole core joined by a C5=C20 double bond, compound **42** (Fig. 6.1.). The X-ray diffraction data explained the magnetic nonequivalence of the methylene protons by the values of the intra- and intermolecular hydrogen bond lengths.

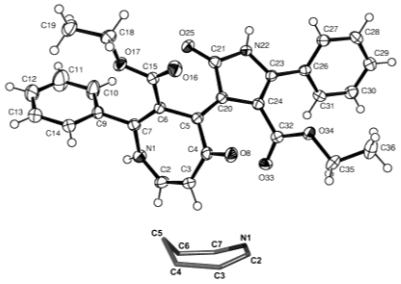


Figure 6.1. Molecular structure of pyrrolilidene-azepine **42** obtained by X-ray diffraction.

Chapter 7. Cytotoxicity evaluation of the new compounds with pyrrole core and fused pyrrole from pyrrolo[1,2-*b*]pyridazines class

The evaluation of the cytotoxicity of some of the new synthesized compounds (pyrroles **4-13**, benzimidazolium salts **2a-d**, pyrrolo[1,2-*b*]pyridazines **29-31**, **33**, **34**, pyridazinone acids **27a-c** and pyrrolilidene-azepine **42**) was carried out on the plant cell of *Triticum aestivum* L., on the animal cells of the crustaceans *Artemia franciscana* Kellogg and *Daphnia magna* Straus and/or on the three standardized cell lines derived from human adenocarcinomas colon (LoVo), breast (MCF-7), ovary (SK-OV-3) and human umbilical vein endothelial cells (HUVEC).

Cytotoxicity evaluation on the plant cell of *Triticum aestivum* L.

The cytotoxicity evaluation of the studied compounds on the plant cell of *Triticum aestivum* L. by the Constantinescu method, indicated that among the pyrroles **4-13** and the bromides **2a-d**, the trisubstituted pyrrole compounds **12**, **13**, **10** and **5** presented the highest percentages of inhibition at 72 h and at the concentration of 1000 μM (69.5%, 60.6%, 44.0% and 29.7%), these having a weaker inhibitory effect compared to intermediate bromides **2d** – 97.0%, **2c** – 95.2%, **2b** – 91.5%, except for compound **5** (29.7% vs. bromide **2a** – 16.0%). It was observed that the trisubstituted pyrroles showed a more pronounced inhibitory effect compared to the tetrasubstituted ones and that the grafting of two methyl fragment in the 4 and 5 positions on the benzene ring enhanced the inhibitory effect.

In the case of fused pyrrole compounds, the pyrrolo[1,2-*b*]pyridazine **33** obtained from pyridazinone acid **27b** had the most pronounced inhibitory effect, presenting an inhibition percentage higher than 35% for all tested concentrations (98.3%-36.5%). It was observed that the grafting of the fluorine atom improved their inhibitory effect.

Microscopic analysis confirmed the inhibitory effect of these compounds by visualizing changes in the mitotic film and mitoinhibition, examples being: metaphases and telophases in tropokinesis, disorganized metaphases, anaphases in tropokinesis, telophase bridges, etc.

Cytotoxicity evaluation on the animal cells of *Artemia franciscana* Kellogg and *Daphnia magna* Straus

The *in vivo* cytotoxicity evaluation on *Artemia franciscana* crustaceans of the new pyrroles **4–13** and bezimidazolium salts **2a–d** indicated that they were not toxic, except for pyrrole **12** containing a benzylamino moiety and two methyl groups on the benzene ring linked

to the pyrrole nitrogen atom and its precursor bromide **2d**, that at 48 h showed LC_{25} values of 168.8 μM and 793.2 μM, respectively. In the case of *Daphnia magna* Straus, these crustaceans showed greater sensitivity to the new compounds evaluated; except for pyrroles **7**, **8**, **10** and **13**, all compounds had significant percentages of lethality, the most toxic being pyrroles **5**, **6**, **11** and **12** (LC_{50}=4.58 μM), and among the salts, the derivative **2d** (LC_{50}=157.0 μM).

Pyrrolo[1,2-*b*]pyridazines and acids intermediates were found to be non-toxic to Artemia nauplii. However, the invertebrates from the *Daphna magna* species were more sensitive to their action, highlighting pyrrolo[1,2-*b*]pyridazine **29** (LC_{50}=46.12 μM), and among the acids, derivative **27a** (LC_{50}=521 μM).

Cytotoxicity evaluation on tumor cell lines

Cytotoxicity evaluation of the new heterocyclic compounds with an isolated pyrrole core **4-11** and **13**, bromides **2a,d** precursors, pyrrolo[1,2-*b*]pyridazines **29-31**, **33**, **34**, acids **27a-c** intermediate and of pyrrolilidene-azepine **42** on the three human tumor cell lines LoVo, MCF-7, SK-OV-3 and on normal endothelial cells HUVEC was compared with the drugs 5-fluorouracil (5-FU) and cisplatin (Cis-Pt) [45, 48].

The results obtained by investigating of the cytotoxic activity of the isolated pyrrole core derivatives **4-11** and **13** and of the precursor bromides **2a,d** on the human colon adenocarcinoma cell line (LoVo) indicated that they exhibit a concentration- and time-dependent cytotoxic effect. Thus, all compounds decreased cell viability below 70% at the concentration of 400 μM at 24 h, and they were below 55% at 48 h. Among the tested compounds, the lowest values of cell viability were presented by the new pyrrole **13** (3.93% – 400 μM) containing a benzylamino fragment and two methyl groups on the benzene ring linked to the nitrogen atom and a radical 4-ethyloxycarbonyl, lower values (19.06-61.79%) at the concentrations of 200-25 μM, at 24 h, than 5-FU (39.27-76.22%) and Cis-Pt (54.95 -76.03%). By contrast, at 48 h, its cytotoxic effect was weaker compared to the references. Among the tested compounds, at the highest concentrations, especially at 400 μM, the pyrrole derivative **4** (24.02%), followed by homologues **9** (31.18%) and **11** (30.72%) stood out (Fig. 7.1.).

In the case of the MCF-7 cell line, the pyrrole derivative **13** (16.67% - 400 μM) showed the best inhibitory effect on cell proliferation, with cell viability percentages (41.92-85.23%) at 24 h, lower than 5-FU (45.47-90.65%) at 200-12.5 μM. Another compound with a more pronounced action was derivative **10** containing the benzylamino and benzoyl fragments

(23.54% – 400 µM). Other compounds that showed cell viability below 70% at 400 µM were pyrroles **5-9**. In contrast to them, derivative **11** had a stimulatory effect on cell proliferation at the first tested concentrations (Fig. 7.2.). At 48 h, the same derivative **13** followed by **10** showed cytotoxic effect at 400 µM (12.82% and 15.12%) and 200 µM (44.77% and 52.07%).

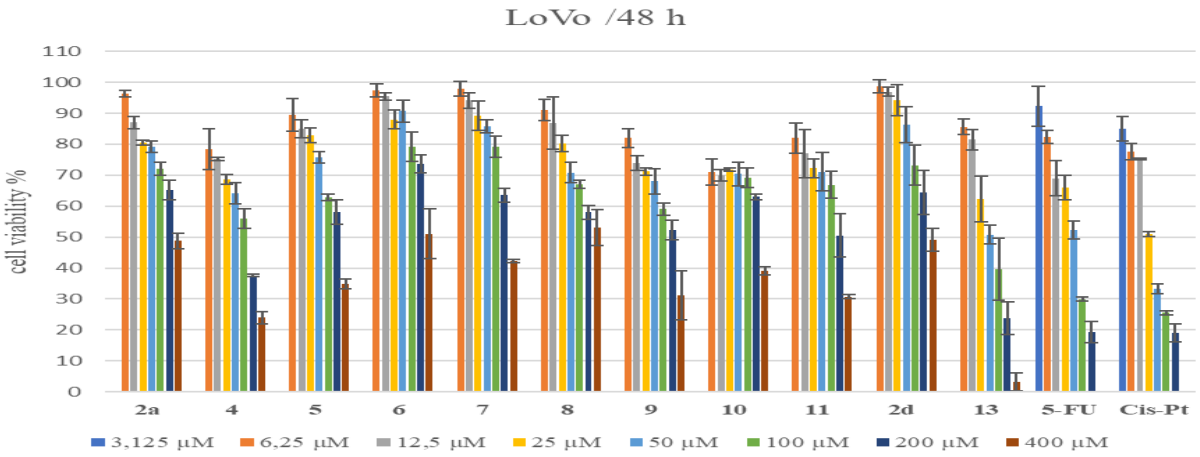


Figure 7.1. Cell viability (expressed as percentage) of LoVo cells treated with pyrroles **4-11**, **13**, salts **2a,d** intermediates and the references at 48 h

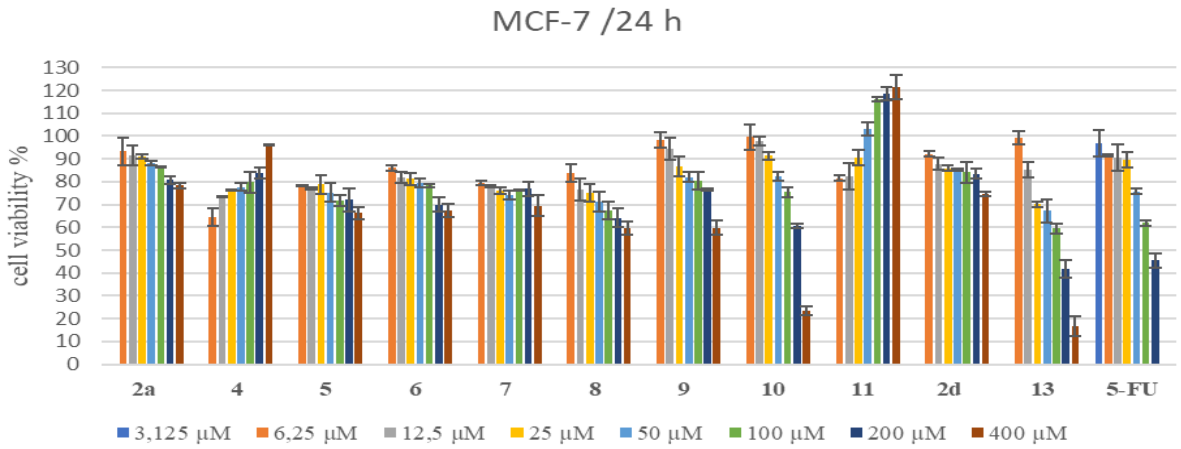


Figure 7.2. Cell viability (expressed as percentage) of MCF-7 cells treated with pyrroles **4-11**, **13**, salts **2a**,**d** intermediates and reference at 24 h

On the SK-OV-3 cell line, at 24 h, the lowest values of cell viabilities, 400-25 µM, were presented by pyrrole **13** (35.27-80.27%), followed by homologue **10** (54.93-81.88%), the first one having a comparable effect to Cis-Pt at 200-6.25 µM. On the other hand, at 48 h, pyrrole **10** had the lowest values of cell viability, 400-25 µM, the cytotoxic effect being much lower than that of the reference. Also in the case of this cell line, a stimulatory effect of cell proliferation

was registered when they were treated with pyrroles **8** or **11**, both at 24 h and at 48 h, at the highest concentrations. The results of the cytotoxicity evaluation on HUVEC cells indicated that the compounds with an isolated pyrrole core and the corresponding precursors tested were not toxic, showing viabilities above 80%, except pyrrole **13** at 24 h, at 400 µM (55.10%) and 200 µM (71.80%) and at 48 h at 400-25 µM (29.01-79.43%) and of pyrrole **10** at 48 h, 400 µM (60.41%).

The cytotoxic effect of pyrrolo[1,2-*b*]pyridazines **29-31**, **33** and **34** and acids **27a-c** was lower than that of compounds with isolated pyrrole core. The antiproliferative effect on LoVo cell line at 24 h indicated that derivatives **27a,c** and pyrrolo[1,2-*b*]pyridazines **29**, **31**, **34** showed cell viabilities below 80% at the highest concentration. At 48 h and at the highest concentration, all tested compounds, exception of acid **27b**, showed values of cell viabilities below 70%, highlighting the derivative **34** (41.82%) containing the ethyloxycarbonyl fragment and a chlorine atom and the homologue **29** (48.47%) with a methyloxycarbonyl fragment (Fig. 7.3.).

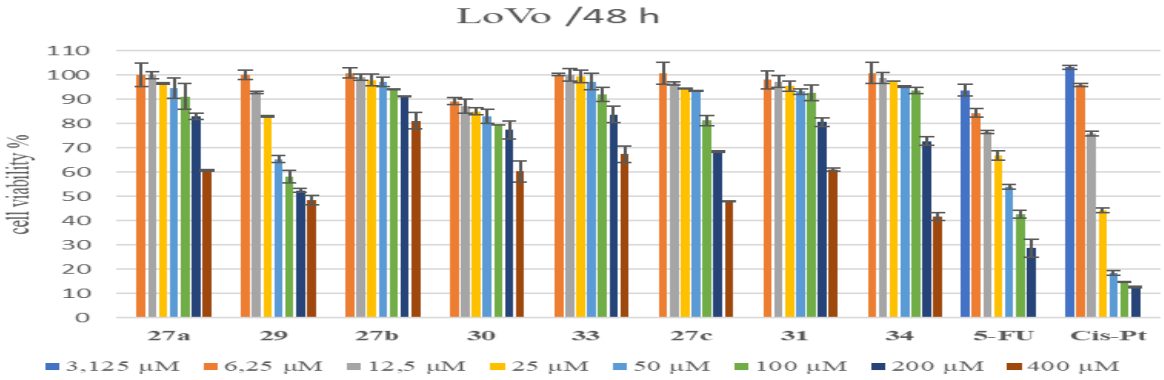


Figure 7.3. Cell viability (expressed as percentage) of LoVo cells treated with pyrrolo[1,2-*b*]pyridazines **29-31**, **33**, **34**, acids **27a-c** intermediates and the references at 48 h

Their cytotoxic effect was further reduced on MCF-7 and SK-OV-3 cell lines, the lowest values of cell viability being observed at 400 µM and at 48 h in the case of those treated with pyrrolo[1,2-*b*]pyridazines containing a fluorine atom into the molecule, compound **33** for MCF-7 cells (73.13%) and compound **30** for SK-OV-3 cells (79.76%). By treating HUVEC cells with these compounds, the absence of cytotoxic effect was observed, the values of viabilities, at both time intervals, being greater than 90%. Similarly, in the case of pyrrolilidene-azepine **42** the lowest values of cell viabilities (28.79%) were obtained on the LoVo cell line, at 48 h and at 400 µM.

Chapter 8. Conclusions and personal contributions

The synthesis of new heterocyclic compounds is one of the indisputable ways to discover new molecules with biological activity, considering that a very large percentage of the total number of drugs contain a heterocyclic core in the molecule, most often with at least one atom of nitrogen as a heteroatom. The general objective of this study was the synthesis and characterization of new heterocyclic compounds with a pyrrole core, in order to identify new molecules with possible therapeutic activity. For this purpose, an important, preliminary stage consisted in the study of the literature data regarding the chemistry of some heterocyclic compounds with an isolated and fused pyrrole core from the pyrrolopyridazines class, regarding to the synthesis, but also their biological properties that was presented in the general part.

Based on these data, the objectives pursued in the synthesis, physico-chemical characterization and evaluation of the cytotoxic effect of some new heterocyclic compounds with tri- or tetrasubstituted pyrrole core and of some compounds with fused pyrrole core from the pyrrolo[1,2-*b*]pyridazines class I consider them fulfilled.

The synthesis of the new isolated pyrrole core compounds **4-19** took place in two steps, the first consisting in obtaining benzimidazolium bromides **2a-e** by the reaction of benzimidazole derivatives and bromoacetonitrile, in acetone, at reflux, two of them, **2d** and **2e**, being uncited in the literature. In the next step, by treating the benzimidazolium salts with various alkynes dipolarophiles (1-phenyl-2-propyn-1-one, methyl propiolate, ethyl propiolate, isopropyl propiolate, dimethyl acetylenedicarboxylate, diethyl acetylenedicarboxylate) in 1,2-epoxybutane, at reflux, for 48 h, the new tri- or tetrasubstituted pyrrole compounds **4-13** were obtained with moderate yields (35-60%). An element of originality consisted in the modification of the reaction medium by adding acetonitrile along with 1,2-epoxybutane in a volumetric ratio of 1:1, in the case of fluorinated compounds **14-19**, decreasing substantially the reaction time, at 6 h, the reaction yields being slightly increased, 46-61%. The reaction involves the *in situ* generation of N-ylides which, through a 1,3-dipolar cycloaddition with alkynes dipolarophiles, finally led to the new pyrrole compounds **4-19**. By introducing unsymmetrical alkynes (1-phenyl-2-propyn-1-one, methyl-, ethyl- or isopropyl propiolate) the pyrrole compounds **4-7** and **10-17** substituted in 4 position with alkyloxycarbo-nyl radical were obtained and not in the 5 position, indicating the regioselectivity of the reaction (chapter 4).

The new compounds with fused pyrrole core, pyrrolo[1,2-*b*]pyridazines **29-34**, were synthesized from pyridazinone acids **27a-c** with unsymmetrical alkynes dipolarophiles (methyl or ethyl propiolate) in acetic anhydride at 90 ^{o}C, with yields of 41-52%. The reaction was regioselective, obtaining pyrrolo[1,2-*b*]pyridazines **29-34** substituted in 5 position and not 6, occurring through the *in situ* generation of some mesoionic oxazolones. The choice of unsymmetrical alkynes and identification of the regioselectivity of the reaction represents a novelty and adds a significant contribution to the synthesis and reaction mechanisms. The acid **27a-c** precursors were obtained by hydrolysis of the corresponding esters **26a-c**, the latter being synthesized in four successive steps, starting from benzene or its halogenated derivatives with succinic anhydride through a Friedel-Crafts acylation reaction, followed by reaction with hydrazine hydrate, then dehydrogenation and alkylation with the ethyl ester of 2-bromo-butanoic acid (chapter 5).

The structural confirmation of the new compounds was carried out by elemental analysis, IR, ^{1}H-NMR and ^{13}C-NMR spectral methods and in the case of some of them by X-ray diffraction. The results confirmed the proposed structures and highlighted the regioselective nature of the 1,3-dipolar cycloaddition reaction.

Considering the importance of the fused pyrrole core, an important part of this thesis was the reinvestigation of the structure of a bisazepine compound obtained by the Nenitescu synthesis, by the reaction of *p*-benzoquinone with ethyl 3-aminocinnamate, in n-butanol medium [37]. The analysis of the structure by X-ray diffraction, resulted a new compound with pyrrolilidene-azepine structure, the two cores being linked by a C=C double bond. The obtaining of such a compound with a pyrrolilidene-azepine structure through the Nenitescu reaction has not been reported in the literature before, reflecting a significant contribution to scientific research in the field (chapter 6).

In order to identify new molecules with biological potential, new heterocyclic pyrrole compounds **4-13**, pyrrolo[1,2-*b*]pyridazines **29-31**, **33**, **34**, pyrrolilidene-azepine **42** and **2a-d**, **27a-c** intermediates were tested for *in vivo* and/or *in vitro* cytotoxicity. The *in vivo* cytotoxicity studies of the compounds were carried out on the plant cell of *Titicum aestivum* L. and on two crustaceans *Artemia franciscana* Kellogg and *Daphnia magna* Straus, and the *in vitro* studies on three cell lines derived from human adenocarcinomas colon (LoVo), breast (MCF-7) and ovary (SK-OV-3) and on normal cells HUVEC, used as control.

The results obtained by the cytotoxicity evaluating of the compounds tested on the plant cell, in the range of concentrations 1000-10 μM, indicated low toxicity in general. Among the pyrrole derivatives, the trisubstituted ones **12** (69.5%), **13** (60.6%), **10** (44.0%) and **5** (29.7%) showed the highest inhibition percentages of root elongation at 72 h, at 1000 μM. In the case of pyrrolo[1,2-*b*]pyridazines, derivative **33** containing a fluorine atom was highlighted, with root elongation inhibition values of 98.3%-36.5% at 72 h, in the range of concentrations used. The results observed macroscopically were supported by the microscopic observations, visualizing different changes in the mitotic film or some cellular structures.

By evaluating the cytotoxicity of the compounds tested on the animal cell, on the crustaceans *Artemia franciscana* Kellogg, it was found that these were generally devoid of toxicity, except for pyrrole **12** containing a benzylamino fragment and two methyl groups on the benzene ring linked to the nitrogen atom and a 4-methyloxycarbonyl radical and its precursor benzimidazolium bromide **2d** that, at 48 h induced lethality, with LC_{25} values of 168.8 μM and 793.2 μM, respectively. Nevertheless, *Daphnia magna* Straus crustaceans were more sensitive to the action of the tested compounds, the results indicating that the most toxic were pyrroles **5**, **6**, **11** and **12**, and among the salts the **2d** derivative, the LC_{50} values, at 24 h, which were calculated for the latter two derivatives being 4.58 μM and 157.0 μM, respectively. Compounds with fused pyrrole core did not show toxicity to Artemia nauplii, instead, daphnids were more sensitive to their action, highlighting pyrrolo[1,2-*b*]pyridazine **29** and the corresponding precursor acid, **27a** which, at 48 h, showed LC50 values of 46.12 μM and 521.3 μM, respectively.

The screening of the antitumor potential of the new compounds by determining the viability of tumor cells by the MTS colorimetric method, indicated that they had a dose- and time-dependent cytotoxic effect, the most pronounced being on the LoVo cell line. Among the derivatives with an isolated pyrrole core, compound **13**, homologous to pyrrole **12**, containing a benzylamino fragment and two methyl groups on the benzene ring linked to the nitrogen atom and a 4-ethyloxycarbonyl radical, stood out with the most pronounced antitumor effect on all tumor cell lines studied. The lowest percentage of viability was observed on the LoVo cell line, at 400 μM (3.93%), even lower values (19.06-61.79%) than the reference drugs, 5-FU (39.27-76.22%) and Cis-Pt (54.95-76.03%), in the concentration range 200-25 μM and at 24 h. The same compound showed more intense cytotoxic effect than the reference 5-FU on the MCF-7

cell line and at 24 h, but the cell viability percentages were higher than on LoVo. The evaluation of the cytotoxic effect on HUVEC endothelial cells indicated the lack of toxicity of the tested compounds, except for compound **13** that presented percentages of cell viability below 80% both at 24 h at the first two concentrations (400 μM – 55.10 %, 200 μM – 71.80%), and at 48 h in the concentration range 400-25 μM (29.01%-79.43%). The results obtained in the case of compounds with fused pyrrole core indicated a weaker cell proliferation inhibitory effect than those with an isolated pyrrole core. The lowest cell viability values were also obtained on the LoVo cell line, being devoid of cytotoxicity on HUVEC cells. Among these compounds, pyrrolo[1,2-*b*]pyridazine **34** with a chlorine atom and an ethyloxycarbonyl group showed a more pronounced cytotoxic effect on the LoVo cell line at 48 h, the percentage of cell viability at 400 μM being 41.82%, but higher than the reference drugs. In the case of pyrrolilidene-azepine, the most pronounced cytotoxic effect was on the LoVo cell line, the lowest percentage of cell viability at 48 h and at 400 μM being 28.79%, a lower effect but comparable to the references, encouraging being the lack of cytotoxicity on normal cells (chapter 7).

The originality of this work consists in the synthesis of a number of 23 new heterocyclic compounds with pyrrole core, 16 with a tri- or tetrasubstituted pyrroles and 6 pyrrolo[1,2-*b*]pyridazines, a pyrrolilidene-azepine, uncited in the literature, but also of 8 new intermediates, 2 benzimidazolium salts, 3 pyridazinone esters and 3 pyridazinone acids that were fully physico-chemical characterized, with the structure confirmed by elemental analysis, spectral methods IR, ^{1}H-NMR and ^{13}C-NMR, and in the case of some by X-ray diffraction.

The results obtained by the cytotoxicity evaluation of the new compounds allowed the identification of the antitumor potential of some new molecules, opening perspectives for research in the medical field.

Based on these results, we propose the following perspectives for future research: the evaluation of antimicrobial activity of new synthesized compounds, acute toxicity evaluation, analgesic and anti-inflammatory activity on a murine model, continuation of the evaluation of the antitumor activity of the synthesized compounds on other tumor cell lines, evaluation of the cell cycle, the modulation of apoptosis, and also the synthesis of new compounds from these classes with improved yields by changing the reaction conditions and grafting new substituents as promising pharmacophore centers for improving the biological activity in general and the antitumor in particular, with the ability to differentiate between tumor and normal cells.

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List of published scientific papers

ISI indexed papers

1. **Ivan BC**, Barbuceanu SF, Hotnog CM, Olaru OT, Anghel AI, Ancuceanu RV, Mihaila MA, Brasoveanu LI, Shova S, Draghici C, Nitulescu GM, Dumitrascu F. Synthesis, characterization and cytotoxic evaluation of new pyrrolo[1,2-*b*]pyridazines obtained via mesoionic oxazolo-pyridazinones. *Int. J. Mol. Sci.*, 24 (14), 11642, 2023. IF (2022)=5.6; https://doi.org/10.3390/ijms241411642 (chapters 5 and 7).

2. **Ivan BC**, Barbuceanu SF, Hotnog CM, Anghel AI, Ancuceanu RV, Mihaila MA, Brasoveanu LI, Shova S, Draghici C, Olaru OT, Nitulescu GM, Dinu M, Dumitrascu, F. New pyrrole derivatives as promising biological agents: design, synthesis, characterization, in silico, and cytotoxicity evaluation. *Int. J. Mol. Sci.*, 23 (16), 8854, 2022. IF (2022)=5.6; https://doi.org/10.3390/ijms23168854 (chapters 4 and 7).

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4. **Ivan BC**, Dumitrascu F, Anghel AI, Ancuceanu RV, Shova S, Dumitrescu D, Draghici C, Olaru OT, Nitulescu GM, Dinu M, Barbuceanu SF. Synthesis and toxicity evaluation of new pyrroles obtained by the reaction of activated alkynes with 1-methyl-3-(cyanomethyl)benzimidazolium bromide. *Molecules*, 26 (21), 6435, 2021. IF (2021)=4.927; https://doi.org/10.3390/molecules26216435 (chapters 4 and 7).

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1. **Ivan BC**, Caira MR, Dumitrascu F. Nenitzescu indole synthesis: 1929-2019 unexpected formation of a pyrrole-azepine hybrid in the Nenitzescu indole synthesis: A reinvestigation. *Rev. Chim.*, 71 (5), 51-57, 2020; https://doi.org/10.37358/RC.20.5.8112 (chapter 6).

Papers published in journals and conference volumes

1. **Ivan BC**, Dumitrascu F, Draghici C, Hotnog CM, Mihăilă MA, Brașoveanu LI, Shova S, Bărbuceanu ȘF. Synthesys of some new pyridazines derivatives with potential antitumoral activity. Congress of "Carol Davila" University of Medicine and Pharmacy, Bucharest, 10th

Edition, November 10th-12th 2022, oral presentation (ID: 472), Young Investigators' Award – Pharmacy, abstract published in *Maedica - a Journal of Clinical Medicine*, 17, Supplement, pg. 89, ISSN: 2501-6903, 2022.

2. **Ivan BC**, Dumitrascu F, Ancuceanu RV, Anghel AI, Draghici C, Barbuceanu ȘF. Synthesys and toxicity evaluation of some new pyrrole derivatives with potential biological activity. Congress of "Carol Davila" University of Medicine and Pharmacy in Bucharest, 9th Edition, November 25th-27th, 2021, oral presentation (ID: 377), Young Investigators' Award – Pharmacy, First Prize in the field of Pharmacy, abstract published in *Maedica - a Journal of Clinical Medicine*, 16, Supplement, pg. 96, ISSN: 2501-6903, 2021.

3. **Ivan BC**, Dumitrascu F, Anghel AI, Ancuceanu RV, Dumitrescu D, Draghici C, Dinu M, Barbuceanu ȘF. 'Synthesis and toxicity evaluation of some new compounds from pyrrole class. National Congress of Pharmacy, 18th edition, September 15th-17th, 2021, e-poster (ID: 139), abstract published in the collection of abstracts, pg. 29, I-SBN 978-606-10-2144-4 .

Projects participation

PhD student in the "Net4SCIENCE: Applied doctoral and postdoctoral research network in the fields of smart specialization Health and Bioeconomy, project code POCU/993/6/13/154722", period December 2022 - September 2023. The project was organized by the University of Medicine and Pharmacy "Carol Davila", Bucharest, the internship partner being the Institute of Virology "Stefan S. Nicolau" Romanian Academy.