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SYNTHESIS OF NEW RING SYSTEMS PYRROLIZINES AS POTENTIAL ANTITUBULIN AGENTS

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INDEX

Synthesis of new ring systems pyrrolizines as potential antitubulin agents

1. Introduction	pag. 1
2. Aim of the work	pag. 12
3. Molecular Docking Studies	pag. 13
4. Results and Discussion: Chemistry	pag. 23
5. Results and Discussion: Biology	pag. 38
6. Conclusion	pag. 45
7. Experimental Section	pag. 46
8. References	pag. 78

Synthesis of intracellular antagonists for CC Chemokine receptors CCR1 and CCR2

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1. Introduction	pag. 81
2. Results and Discussion: Chemistry	pag. 89
3. Results and Discussion: Biology	pag. 100
4. Experimental Section	pag. 103
5. References	pag. 122

INTRODUCTION

Cancer is still an important growing problem with twelve million of new cases in the world per year. For the majority of tumors prognosis is unfortunately relatively poor. Although in the last years there were several improvements in treatment and prevention with a consequent decrease in deaths, the number of new diagnoses continues to increase.

Alarming data from the World Health Organization (WHO) show that in 2012 cancer caused 8.2 million deaths. About half in the age group between 30 and 69 years and in particular, data from the Associazione Italiana di Oncologia Medica (AIOM) reported 365.500 new diagnoses in Italy in 2014.

This disease is characterized by uncontrolled cell growth with potential property to spread to other tissues or sites of the body via lymph or blood (this is known as *metastasis*). In normal conditions, human cells proliferate and divide to form new cells, and when old or damaged, they die, and new cells take their place. In tumor conditions, this orderly process breaks down, indeed old or damaged cells survive when they should die and cells grow and divide without any control (**Figure 1**).

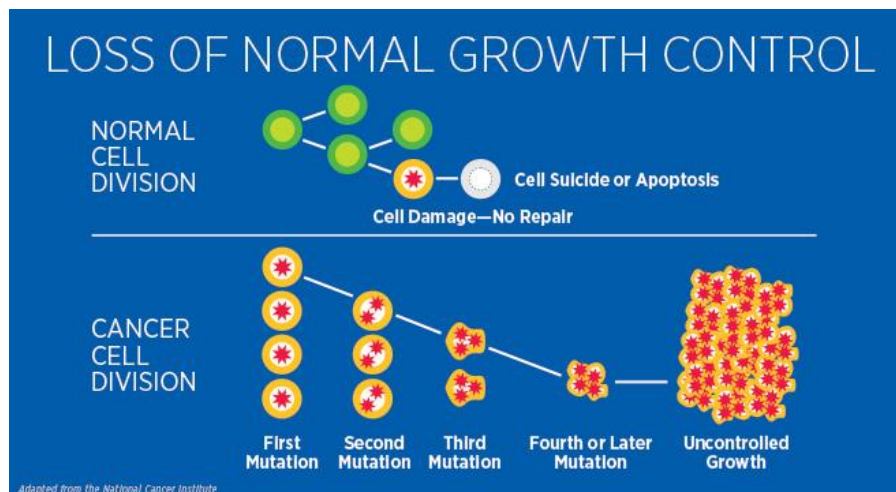


Figure 1

One of the most important differences between normal and cancer cells is that the latter are less specialized than normal ones; this is one reason that causes cancer cells dividing

without stopping. Furthermore, tumor-cells ignore signals to stop the proliferation or start apoptosis, also known as programmed cell death; these are normal “solutions” used by our body to remove unneeded cells.

Cancer cells are also able to influence the microenvironment formed by the surrounding normal cells and blood vessels. Cancer cells facilitate angiogenic processes that lead to the formation of new blood vessels through which nutrients and oxygen are supplied.

Other important property of cancer cells is the ability to evade the immune system that protects our body from dangerous conditions, removing abnormal or damage cells.

It is also important to discriminate cancers from benign tumors, the first ones are characterized by uncontrolled growth, invasion, infiltration and sometimes metastasis, whereas the second ones are self-limited, non-invasive do not metastasize, have slow growing and can be removed by surgery (**Figure 2**).

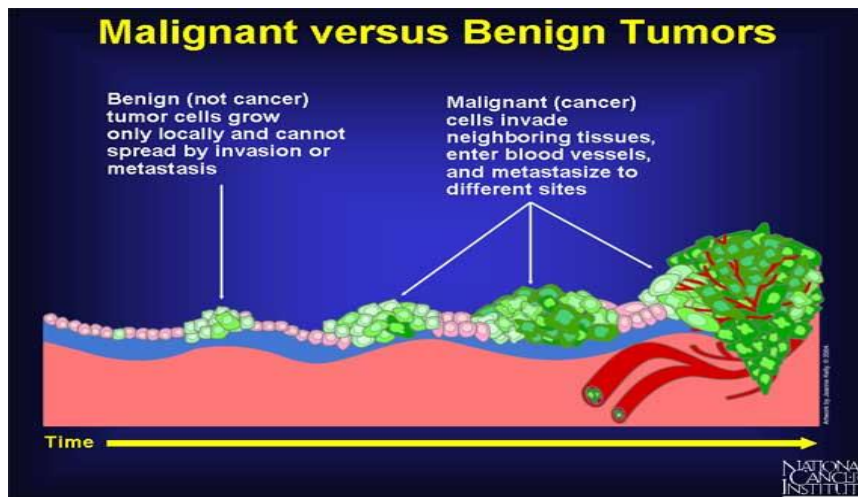


Figure 2

Cancer affects people at all ages with the risk of cancer increases with age and with the presence of some risk factors, such as tobacco smoke, obesity, radiation, chemicals, alcohol or infectious agents.

Cancers are caused by abnormalities present in the genetic material of the transformed cells.

The genetic changes that contribute to cancer affect three main types of genes:

- ❖ **proto-oncogenes**
- ❖ **tumor suppressor genes**
- ❖ **DNA repair genes**

Proto-oncogenes are normally involved in cell growth and division. When these genes are altered or more active than normal, they can become cancer-promoting genes or **oncogenes**. Oncogenes are typically activated in cancer cells, giving those cells new dangerous properties, such as protection against apoptosis, hyperactive growth and division, etc....

Tumor suppressor genes are also involved in cell growth and division. Alterations in these genes cause uncontrolled cell division. Tumor suppressor genes are inactivated in cancer cells, resulting in the loss of control over the cell cycle or DNA replication.

DNA repair genes are involved in fixing damaged present in the DNA, such as mutations. When these genes are altered, the mutations can accumulate and the cells become cancerous.

Unless error correction is properly carried out, the errors will survive, and might be passed along to daughter cells. Normally, the body use different methods to protect itself from cancer, for example apoptosis.

Moreover, it is possible that mutations affect one or more checkpoints of cell cycle, **G₁**, **G₂** and **M checkpoints (Figure 3)** causing uncontrolled cell cycle and uncontrolled mitosis.

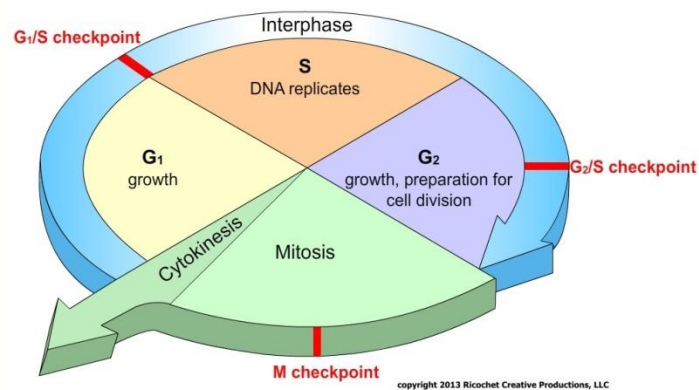


Figure 3

There are different types of cancer treatment. The treatment used depends on the type and status of the cancer and on the general state of person's health. Typical cancer treatments include surgery, chemotherapy, radiotherapy, immunotherapy, hormone therapy, monoclonal antibody therapy, targeted therapy and stem cell transplant. To obtain effective strategies these treatments might be also combined.

There are two big families of anticancer drugs: cytotoxic and cytostatic drugs. Cytotoxic drugs have a toxic effect on cells, indeed they are also known as "cell killing". Cytostatic drugs, also called "cell stopping", work suppressing cell growth, they stop the cancer cells from multiplying. The first category of medicines works by interfering with DNA replication, they stop cell duplication and the cells move to programmed cell death. In this group are included four different classes of drugs:

- ❖ Alkylating agents, which react covalently with DNA and distorting its shape;
- ❖ Intercalating agents, which insert between the base pairs of DNA, thereby unwinding the double helix;
- ❖ Antimetabolites, molecules with similar structure of natural nucleotides, that are incorporated into DNA, leading to non-functional DNA;
- ❖ Strand-breakers, which generate reactive radicals that produce cleavage of polynucleotide strands.

The second drug's family includes molecules that work by interfering with mitotic spindle. Generally, they are natural products with complex chemical structures that interfere with dynamic equilibrium of the microtubules, which are essential for cell division and cellular integrity. They cause mitotic arrest either by blocking microtubule disassembly or inhibiting tubulin polymerization¹.

Microtubules are long, cylindrical proteins, assembled from α and β tubulin heterodimers (**Figure 4**). They are important components of the cytoskeleton and are involved in many cellular processes, such as cell division, intracellular transport, chromosome movement and mitosis².

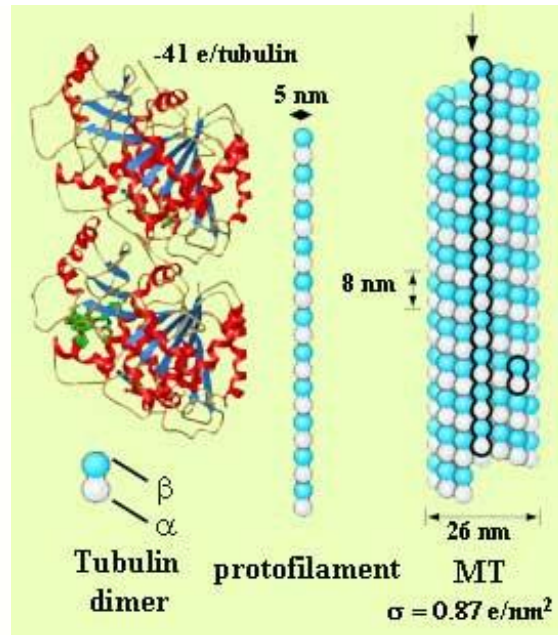


Figure 4

Tubulin's polymerization happens through longitudinal head-to-tail juxtaposition of heterodimers that form long and linear protofilaments. Thirteen aligned protofilaments form a microtubule within which the tubulin subunits interact through lateral and longitudinal bonds. When a cell begins to divide, the microtubules start assembling forming mitotic spindle which is really important for chromosome movement to the metaphase plate and for their correct segregation in sister chromatids during anaphase³. Therefore the tubulin is a really attractive target for the development of new anticancer compounds.

Many synthetic or natural molecules, interfering with the dynamic assembly of tubulin, prevent the formation of microtubules which are key cell structures.

Currently, the most known antitubulin agents are natural products, such as Taxanes, in particular Paclitaxel (Taxol®) and Docetaxel (Taxotere®)³, Colchicine⁴, Vinca Alkaloids, in particular Vinblastine (VBL) and Vincristine (VCR)⁵ and Combretastatin A4 (CA-4)⁶ (**Figure 5**).

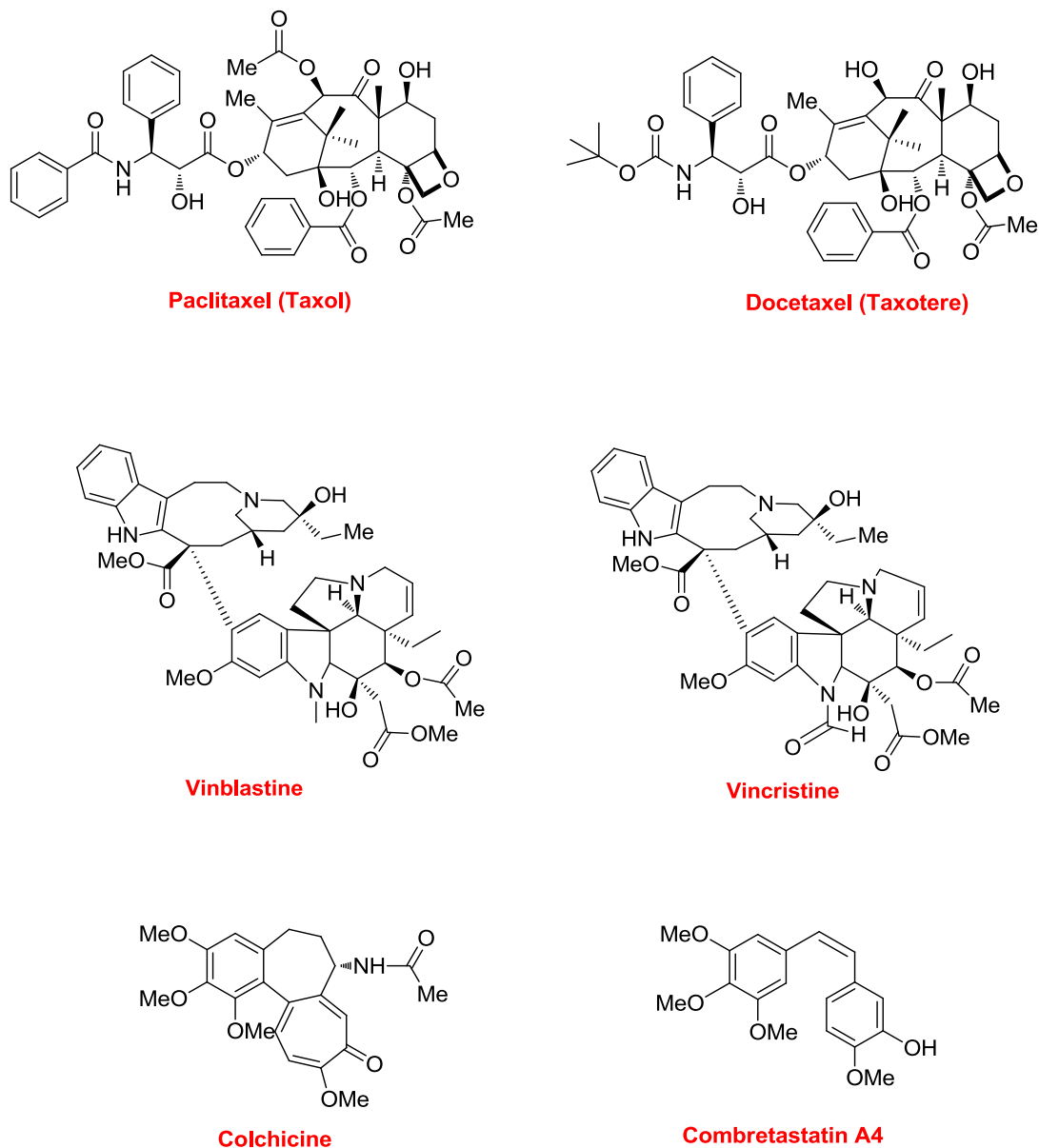


Figure 5

Taxanes are natural compounds extracted from *Taxus brevifolia* (Pacific yew tree) with a complex terpenoid structure. Most probably their anticancer activity is due to a lateral iso-serine side chain⁷. In 1993 **Paclitaxel** was FDA (Food and Drug Administration) approved to treat ovarian cancer's; but in general Paclitaxel and its semisynthetic derivate, **Docetaxel**, are clinically active against breast, ovarian, prostate and liver tumors^{3,8}. Their mechanism of action is different compared to Vinca alkaloid ones, in fact the Taxanes block

depolymerization of microtubules into tubulin whereas Vinca alkaloids inhibit polymerization of tubulin into microtubules³ (**Figure 6**).

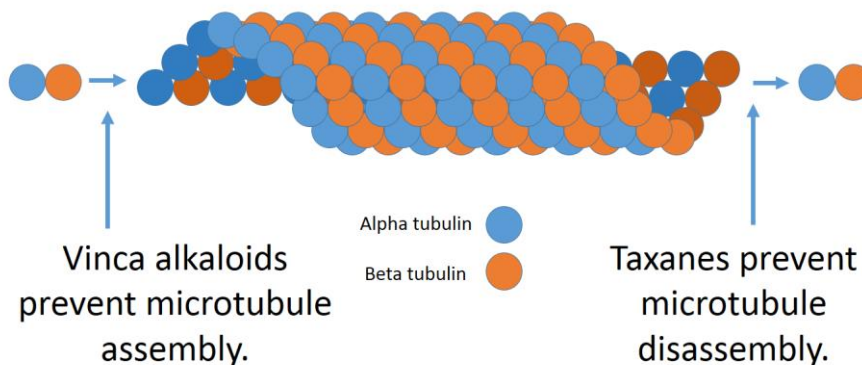


Figure 6

However, an important advantage is represented by their poor solubility, indeed lipidic emulsions based on castor oil or Polisorbate 80 are necessary for their clinical use. Instead, Vincristine and Vinblastine, extracted from *Catharanthus roseus* (Vincaroseae), Madagascar's plant, have hypoglycemic as well as cytotoxic activities. They are used for treating diabetes, high blood pressure and especially as antimitotic agents. They prevent microtubule assembly and block cells in phase G2/M. Vinblastine is used for breast cancer, Hodgkin and non-Hodgkin lymphomas' treatment; whereas Vincristine is used to treat Wilm's tumor, acute leukemia and other lymphomas⁹.

Colchicine, the most commonly antitubulin agent, is a natural compound, isolated from *Colchicum autumnale*. Colchicine binds with high affinity tubulin, resulting in a change of its secondary structure that blocks microtubules' formation.

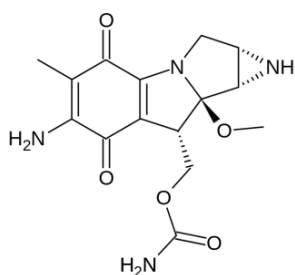
Finally, in 1989 Pettit *et al.* extracted from *Combretum caffrum* a highly active compound, **Combretastatin A-4** (CA-4)¹⁰.

Different studies were performed to evaluate its cytotoxic effects and in particular the National Cancer Institute (NCI) of Bethesda (USA) showed that Combretastatin A-4 has a really high activity with a GI₅₀ of 0.32×10⁻⁸. Furthermore, it inhibits tubulin polymerization binding the colchicine binding site¹¹. Its pro-drug, Combretastatin A-4 phosphate (CA-4P), is in phase II

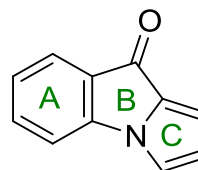
clinical trial in UK and USA. Moreover, from magnetic resonance' studies appear that CA-4P decreases bloodstream to cancer cells¹².

Although all these molecules have really interesting anticancer activity, they have also some restrictions such as neurotoxicity, development of drug-resistance and poor bioavailability after oral administration. Therefore, considering these side effects, the research of new antimitotic compounds is still in progress.

During the past years, after mitomycin discovery, a considerable interest for pyrrolo[1,2-*a*]indole system has arisen and many scientists developed several mitomycin analogues, also known as “mitosanes”. Particular importance has been given to a new class of compounds, 9*H*-pyrrolo[1,2-*a*]indol-9-one **1**¹³.

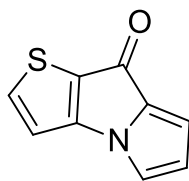


Mitomycin



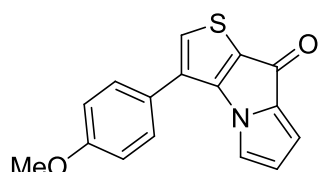
1

Different substitutions were studied to identify the key pharmacophoric moieties of these compounds. The first investigations, focused on the benzene ring **A**, led to the synthesis of analogues without this ring, or in which this ring was either changed with some bioisosteric heterocycles or decorated with different aryl or alkyl side chains. Furthermore, the carbonyl group was changed with hydroxyl group or oxyimino group. From these modifications, a wide range of different analogues have been synthesized. Among these, 8*H*-thieno[2,3-*b*]pyrrolizinones **2**, more recently named “tripentones”, represent an interesting class of anti-cancer agents, due to their cytotoxicity against tumor cells in the submicro-nanomolar range¹⁴⁻¹⁶.



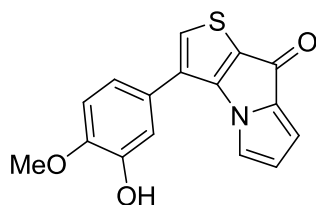
2

Different tripentones analogues have been synthesized to confirm the importance of thienopyrrolizinone core. The 3-aryl-8*H*-thieno[2,3-*b*]pyrrolizin-9-ones, tested on 60 tumor cell lines (NCI), have shown high cytotoxicity¹⁶. Many derivatives were selective against the leukemia subpanel and the most promising analogs were **2a**, **2b** and **2c**¹³.



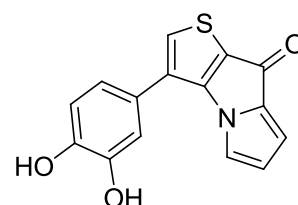
2a

$IC_{50} = 0.19 \mu M$



2b

$IC_{50} = 0.015 \mu M$



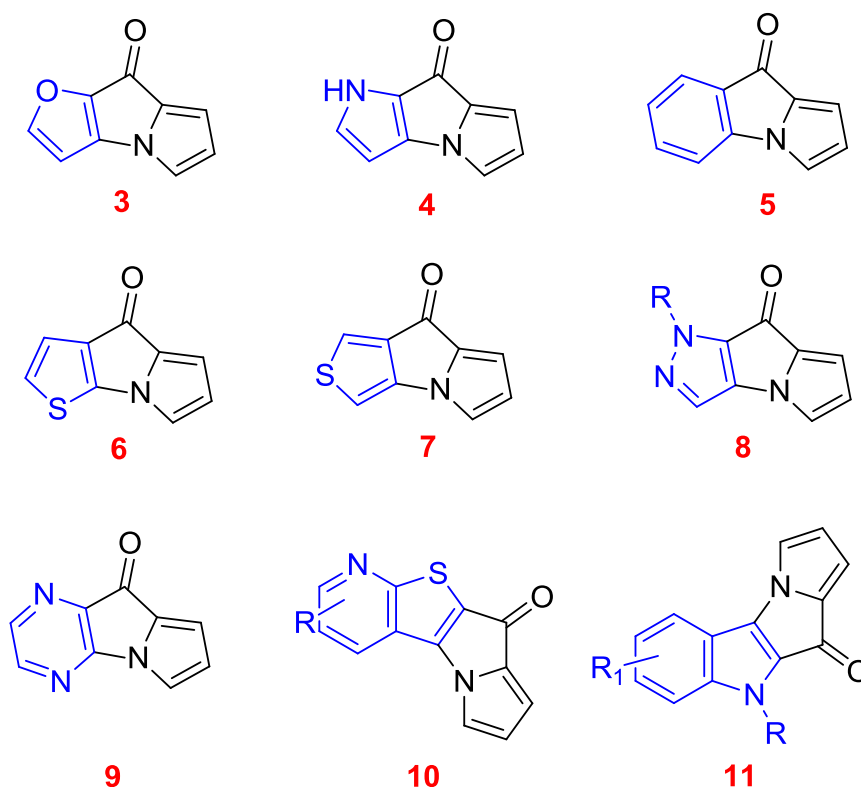
2c

$IC_{50} = 0.28 \mu M$

In particular, compound **2b** resulted the most active and characterized by high cytotoxicity ($IC_{50} = 0.015 \mu M$). Further studies on this derivative showed that it acts on tubulin polymerization ($IC_{50} = 2.9 \mu M$) causing cell cycle arrest in the G2/M phase. This effect was comparable to famous tubulin inhibitors such as Vinca alkaloids or Docetaxel. It was also able to induce apoptosis through the MAP kinase pathway. Derivative **2b** compared with a reference compound, Combretastatin A-4, shows an increase in a modest manner of the percentage of cells in the first phase of apoptosis. A dose-dependent activation of caspases **3** and **7** confirms the involvement of tripentone **2b** in programmed cell death's process. Moreover, this compound was also evaluated against a panel of several kinases and showed interesting inhibitory activity against Cdk1/cyclin B ($IC_{50} = 5.5 \mu M$) and GSK-3 β ($IC_{50} = 1.5 \mu M$)¹³. Strong inhibitor activity was showed against FLT3-ITD (Internal Tandem Duplication) ($K_d = 1.4 \mu M$). This derivative seems to be a promising hit compound for hematologic cancers' treatment due to its high selectivity towards this FLT3 tyrosine kinase's mutation that was the most commune genetic abnormality in Acute Myeloid Leukemias (AMLs)¹⁷.

Unfortunately *in vivo* studies showed an insufficient bioavailability due to a lack of solubility in physiological conditions¹³.

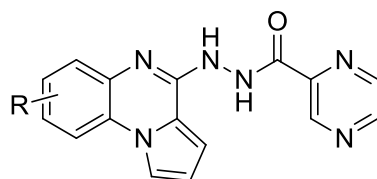
New tripentone systems were synthesized, in which the thiophene ring was replaced by a bioisosteric analogues such as furan **3**, pyrrole **4** and benzene **5**¹⁶, or by isomers as thiophenes **6** and **7**. Moreover, several heterocyclic tripentones such as pyrazole **8**, pyrazine **9**, pyridothieno- **10** and indole **11** pyrrolizinones were synthesized^{13,16,18}.



Unfortunately all new series of compounds appeared less active than the lead compound. Indeed some pyrrolo- and furo-pyrrolizinone derivatives exhibited a modest activity at micromolar concentrations ($IC_{50} = 8.9-64 \mu M$) with a weak inhibitory activity (micromolar range) against several cyclic-dependent kinases (CDKs1-5, GSK-3 β)¹⁹⁻²⁰. Several indole-pyrrolizinones showed modest activity against leukemia sub-panel, renal cancer sub-panel and non-small cell lung cancer sub-panel at micromolar concentrations¹⁸.

At the light of these data with the aim to improve pharmacokinetics properties of lead compounds, another series of pyrroloquinoxaline of type **12**, characterized by hydrazine side chains, have been synthesized. Pyrroloquinoxaline derivatives have shown an interesting cytotoxicity ($IC_{50} = 0.3-4 \mu M$) in a panel of four human cell lines, one breast cancer cells and three colon cancer cells.

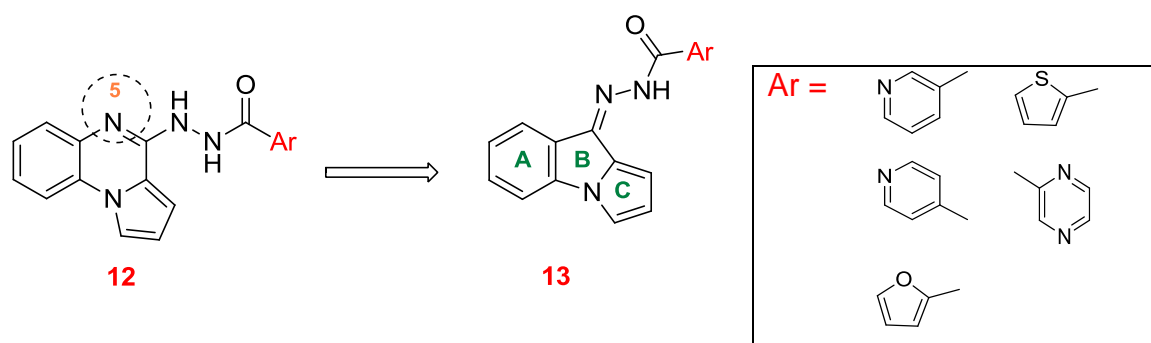
The presence of carbohydrazide linker and a planar arrangement seem necessary for their activity²¹.



12a: R=7-F

12b: R=9-F

Following series of N²-heteroaryl-9H-pyrrolo[1,2-*a*]indol-9-hydrazones **13** were planned by Grande *et al.*²², in which the central pyrazine ring was contracted from six to five terms by removal of N-5 atom, while the presence of heteroaryl carbohydrazide side chains was maintained. This new series of compounds was planned with the aim to improve pharmacokinetic properties and obviously the efficacy.

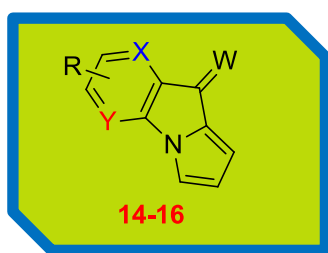


All new compounds were tested in a panel of six human cancer cell lines belong to breast cancer, colon cancer and cisplatin-resistant ovarian cancer. Although new derivatives weren't most active then lead compounds, the pharmacokinetics profile resulted of course improved²².

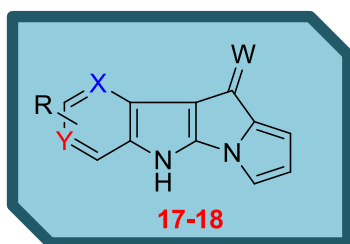
AIM OF THE WORK

From several years the research group where I carried out my PhD thesis has been interested in the synthesis and biological evaluation of polycondensed nitrogen heterocycles, containing pyridine or aza-indole moieties, endowed with antineoplastic activity.

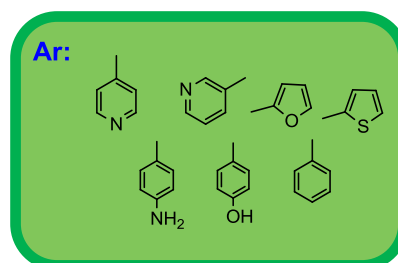
Considering the significant biological properties of tripentones, our aim was the synthesis of new analogues in which the thiophene ring was replaced by a pyridine ring **14-16** or by aza-indole ring **17-18** in order to assess how these structural modifications can influence the antitumor activity. Moreover, we decorated the new tripentones with heteroaryl carbohydrazone chains, offering a possibility of forming conjugate salts to improve water solubility, in order to improve their pharmacokinetic properties.



- 14:** R= H, 2-Br, X= N, Y= CH, W= O, N-NH-CO-Ar
15: R= NH-NH-CO-Ar, X= N, Y= CH, W= O, N-NH-CO-Ar
16: R= H, 3-Br, X= CH, Y= N, W= O, N-NH-CO-Ar



- 17:** R= H, OMe, X= N, Y= CH, W= O, N-NH-CO-Ar
18: R= H, X= CH, Y= N, W= O, N-NH-CO-Ar



MOLECULAR DOCKING STUDIES

Considering the interesting biological activities of our lead compounds and in particular against tubulin, with the aim of studying the potential interaction of our new compounds **14-18**, molecular modeling studies were performed on this target.

Tubulin-interacting agents (MDAs, *microtubule-targeted agents*), are compounds able to interfere with tubulin normal functions. They can act inhibiting its polymerization causing microtubule mass decrease, or they can increase the length of microtubules and consequently their stability. On these bases, they are divided in two main groups depending on their mode of action (**Figure 7**):

- Microtubule-destabilizing agents
- Microtubule-stabilizing agents

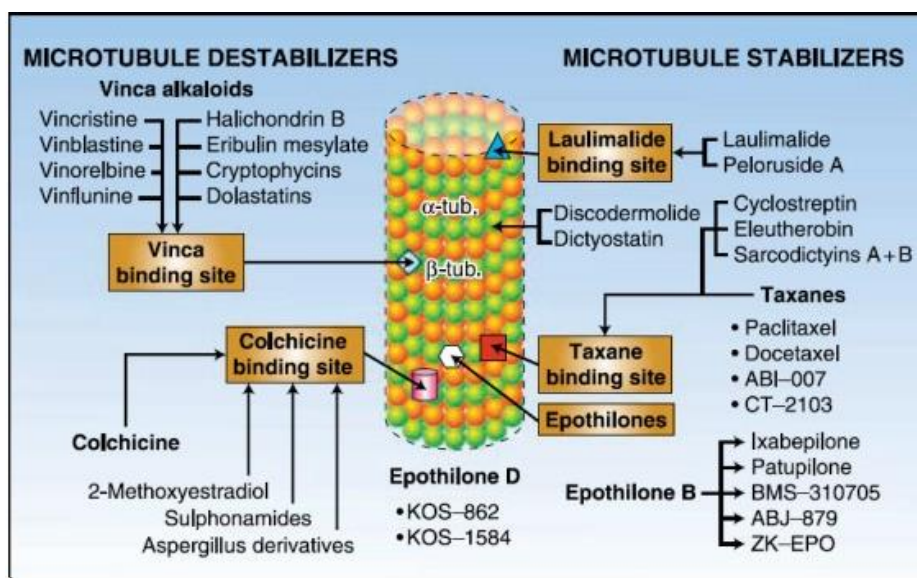
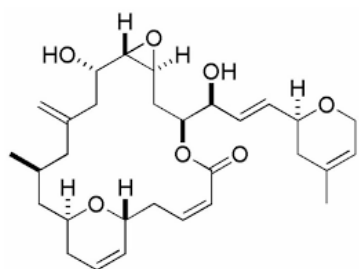


Figure 7

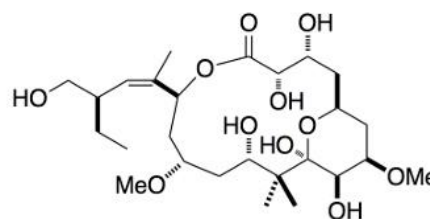
To Microtubule-destabilizing agents belong Vinca alkaloids and colchicine which binding sites are very close each other but they are not the same. Vinca alkaloids bind to β tubulin close to GTP binding site and they have two distinct sites on microtubules: binding site with high affinity at the microtubule end and one with low affinity along the sides of microtubule surface²³⁻²⁴. Colchicine binds to tubulin at the interface of α, β

subunits to give a soluble intermediate complex that undergoes to polymerization. The presence of colchicine causes obstruction of the lateral contacts between protofilaments inhibiting cell mitosis²⁵.

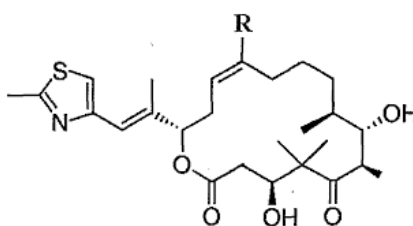
To Microtubule-stabilizing agents belong Taxanes, Laulilamide or Peroluside, and Epothilones (**Figure 8**).



Laulimalide



Peroluside



Epothilone A: R= H

Epothilone B: R= Me

Figure 8

Taxanes, such as Paclitaxel, bind to a pocket of the interior lumen of β tubulin facing the central cavity in the microtubule²⁶⁻²⁹. Paclitaxel at very beginning exerts its function binding a pocket located in the outer microtubule surface. It cause a β tubulin rearrangement followed by its penetration in the microtubule. This action finally results in stabilizing the GDP-bound- β -tubulin protofilaments giving a more stable structure³⁰. Paclitaxel shows different behaviors depending on its concentration. At low concentrations, it acts as Vinca alkaloids or colchicines, decreasing microtubule dynamicity, causing the arrest of the cell cicle, while at higher concentrations promotes tubulin polymerization.

Epothilones are macrolide antibiotics that stabilize microtubule in a way similar to that of taxanes³¹. They induce mitotic arrest in the G2/M phase of the cell cycle, causing apoptosis. Epothilones compete with taxanes for binding with tubulin and are able to displace them from microtubules. Although they are able to promote these events, electron crystallography studies showed that they interact with a binding site close to that of taxanes, both located in the β subunit of tubulin, with an independent molecular interactions³².

Laulilamide is a 20-membered ring isolated from a marine sponge. It binds a specific site of the β subunit and show antimitotic activity similar to that of taxanes³³⁻³⁶.

α , β Subunits of tubulin share about 50% sequence homology. Each monomer is constituted by a C-terminal domain (red), an N-terminal domain (blue) and an intermediate domain (orange) (**Figure 9**).

The C-terminal domain is formed by 2 antiparallel α helices (H11-H12) with an internal surface formed by different loops.

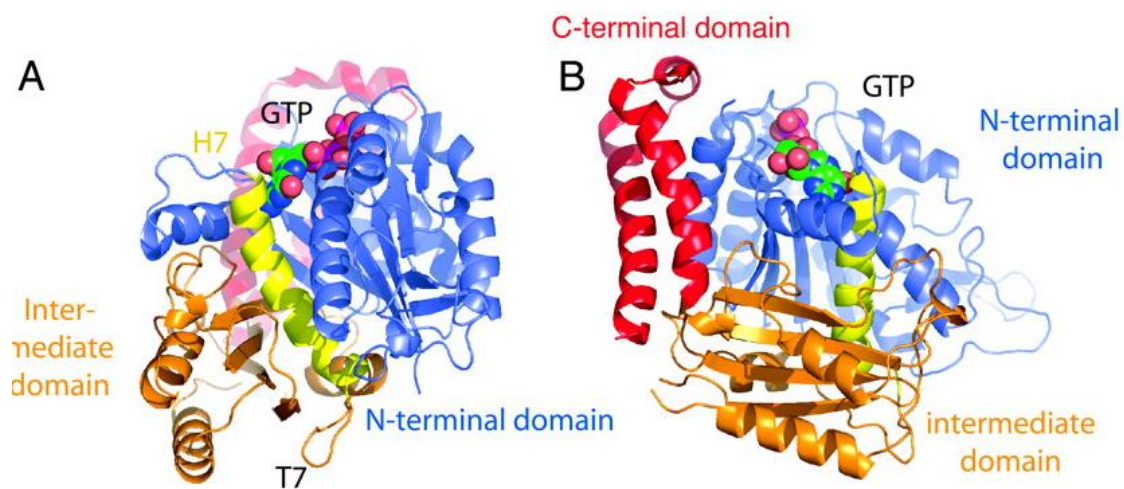


Figure 9

The N terminal domain is composed by 6 β sheets (S1-S6), 2 α helices (H1-H6), a nucleotide binding site (Loop T1-T6), and a binding site constituted by 3 α -helices (H8-H10) and 3 β -sheets (S7-S10). The intermediate domain provides loop T7 that activates

nucleotide hydrolysis in the protofilament when inserted into the active site of the next subunit³⁷.

MDAs can be classified also depending on their binding sites that have different locations as showed in **figure 10**.

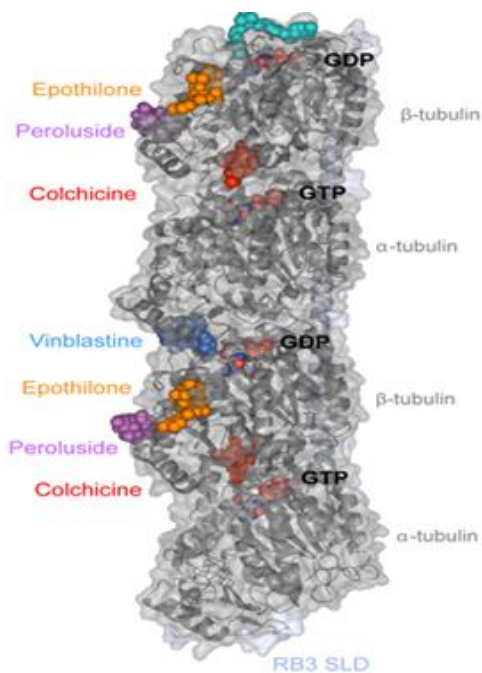


Figure 10

In particular, to date, four different binding sites are known:

- Colchicine binding site
- Vinca alkaloids binding site (vinblastine)
- Taxanes binding site (epothilone)
- Laulilamide binding site (peroluside).

The colchicine binding site was identified by Ravelli *et al.* in 2004 by the determination of a 3.5 Å X-ray structure of α , β -tubulin with pdb code 1SA0, complexed with N-deacetyl-N-(2-mercaptoacetyl) colchicine (DAMA-colchicine)³⁸. This structure also shows the the stathmin-like domain RB3. Experimental data showed that colchicine binds to β -tubulin at its interface with α -tubulin (**Figure 11**), resulting in inhibition of tubulin polymerization.

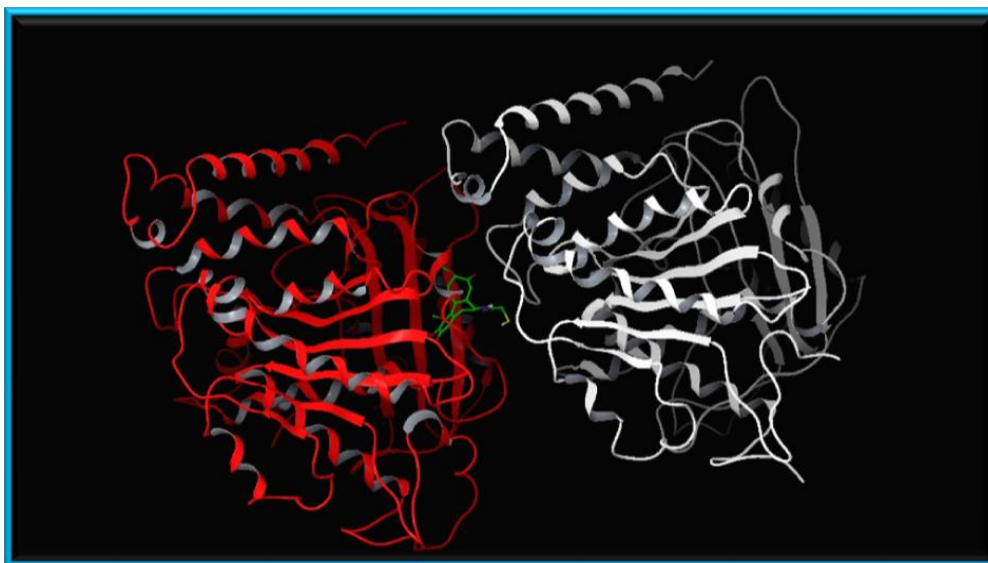


Figure 11 Structure 1SAO with DAMA-colchicine (green)

In particular, the trimethoxyphenyl groups of both DAMA-colchicine in the β -tubulin structure are in the vicinity of the amino acid residue Cys β 241. The width of the colchicine binding site is approximately 4–5 Å, and the volume of this site is confined in β -tubulin by helix 7 (H7) containing Cys β 241, loop 7 (T7) and helix 8 (H8).

Recently another structure was published by Porta *et al.* with a better resolution (2.3 Å) and with pdb code 4O2B³⁹ in which tubulin is co-crystallized with colchicine.

The Vinca alkaloids binding site is described in the X-ray structure, available with pdb code of 1Z2B⁴⁰, published by Giganti *et al.*. In this structure vinblastine is the co-crystallized ligand, located in the β subunit (**Figure 12**).

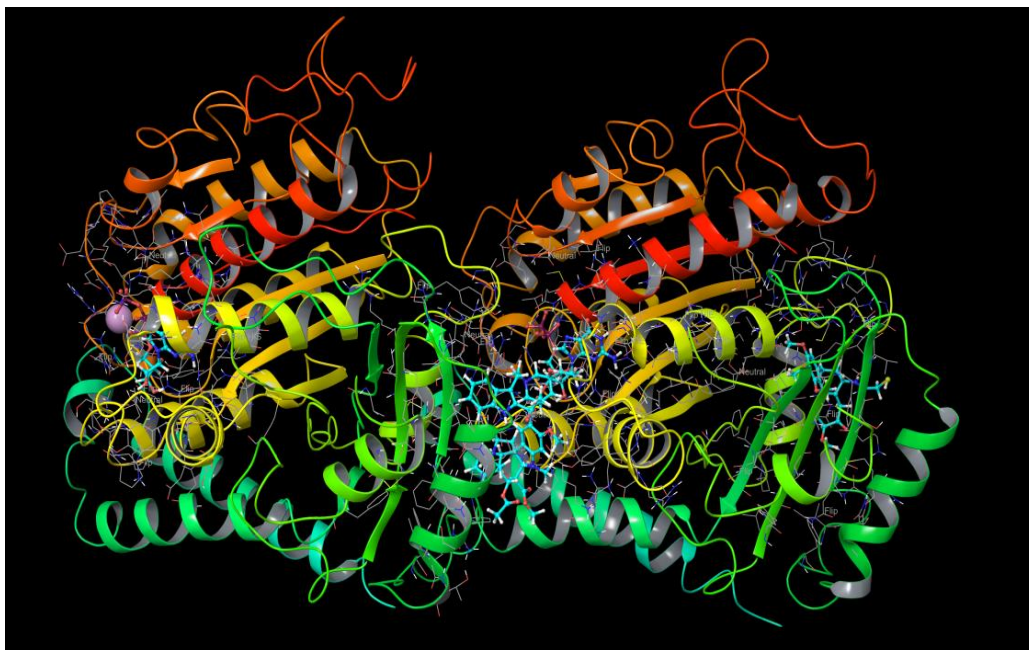


Figure 12 Structure 1Z2B with vinblastine (light blue)

The vinblastine site comprises sites located in the inner lumen of microtubules and involved in longitudinal protofilament contacts. This site is constituted by the area surrounded by loop T7, helix H10 and strand S9 in the α subunit and by the carboxy-terminal turn of helix H6 and loop T5 and H6-H7 in the β subunit.

There are not available structures with taxanes or paclitaxel as ligands. Recently it was published the tubulin structure with Etoposide A (pdb 4O4I)⁴¹. The same structure is also co-crystallized with Laulilamide (**Figure 14**).

The taxanes binding site is located on the luminal side of the microtubule and is formed by helix H7, β strand S7, the M-loop, loops H6-H7 and S9-S10.

Laulilamide binding site is located in a pocket on β tubulin that is formed by hydrophobic and polar residues of helices H9 and H10 and the loops H9-H9' and H10-S9.



Figure 13 Structure 4O4I with Epothilone A (orange) and Laulilamide (light blue)

Considering that any information about our lead compounds' binding site are available, different X-ray crystallographic structures were selected for our computational studies. In particular, three structures representative of the four known tubulin binding sites, were selected:

1. 1Z2B for Vinca alkaloids binding site;
2. 4O4I for both Taxanes and Laulilamide binding sites;
3. 4O2B for Colchicine binding site

For Colchicine binding site the most used structure is that with pdb code 1SA0³⁸ published by Ravelli *et al.*, but the X-ray structure 4O2B³⁹ was selected, considering the better results obtained in the re-docking and the better resolution.

All X-ray structures were downloaded from the Protein Data Bank (<http://www.rcsb.org>) and prepared using Schrodinger's Protein Preparation Wizard of Prime module⁴² for protein structure refinement, through which the stathmin-like domain and the subunits, no directly involved in the binding site, were removed.

Ligands preparation and docking studies were performed using Extra Precision mode (XP) of Glide module of Schrodinger. In order to evaluate the reliability of Glide

protocol, the re-docking of the co-crystallized ligand was performed and its pose was compared to that of the crystallographic structure.

In **table 1** are reported docking score values for all studied compounds and for each protein structure used. Results obtained for Vinca alkaloids binding site (pdb 1Z2B) were not shown because any possible pose was generated by Glide.

Compounds in table 1 were divided in four groups:

1. Compounds without side chains (yellow)
2. Compounds with side chains on the carbonyl group (green)
3. Compounds with side chains on the pyridine ring (blue)
4. Compounds with side chains on the pyridine ring and on the carbonyl group (ligh blue)

Cmp	4O4I Epot^a	4O4I Laul^a	4O2B^a
14a	-3.992	-4.808	-5.177
14i	-4.548	-4.911	-5.421
16a	-4.044	-5.028	-5.338
16i	-4.592	-5.007	-5.15
17a	-4.824	-4.941	-4.714
17b	-5.604	-4.597	-5.631
18a	-4.759	-4.899	-5.591
14b	-6.648	-5.357	-5.759
14c	-5.299	-5.843	-6.346
14d	-5.976	-5.918	-6.63
14e	-5.379	-5.783	-5.808
14f	-7.078	-6.511	-7.17
14g	-6.987	-5.777	-7.323
14h	-5.504	-3.721	-6.664
14j	-4.861	-5.111	-5.790
14k	-5.211	-4.993	-5.252
14l	-5.961	-5.582	-6.236
14m	-5.241	-4.542	-5.822
14n	-6.415	-5.266	-6.407
14o	-6.856	-5.313	-5.582
14p	-5.563	-5.633	-6.215
16b	-5.173	-6.153	-6.137
16c	-5.884	-6.838	-7.329
16d	-5.98	-7.025	-7.173
16e	-5.491	-7.061	-7.096

Cmp	4O4I Epot ^a	4O4I Laul ^a	4O2B ^a
16f	-6.61	-6.963	-7.25
16g	-5.351	-7.139	-7.565
16h	-5.783	-6.504	-4.694
16j	-6.123	-5.446	-6.575
16k	-5.915	-6.284	-6.977
16l	-5.847	-5.331	-6.636
16m	-5.122	-5.796	-6.316
16n	-5.856	-6.778	-4.614
16o	-6.982	-6.148	-6.303
16p	-5.7836	-5.067	-6.413
17c	-5.985	-5.571	-5.320
17d	-6.404	-6.153	-7.243
17e	-6.129	-4.932	-6.258
17f	-5.986	-5.846	-6.768
17g	-6.393	-5.673	-6.755
17h	-6.920	-6.297	-5.211
17i	-5.731	-4.484	-3.418
17j	-5.877	-4.692	-4.694
17k	-6.176	-4.742	-6.182
17l	-5.807	-4.818	-6.339
17m	-6.549	-3.674	-4.930
17n	-6.987	-5.333	-6.407
17o	-6.074	-5.777	-7.119
17p	-5.978	5.964	5.556
18b	-6.534	-4.894	-5.509
18c	-6.691	-5.652	-6.732
18d	-6.604	-5.687	-6.755
18e	-6.397	-5.754	-5.818
18f	-7.724	-4.773	-7.373
18g	-7.796	-4.607	-5.624
18h	-6.619	-5.971	-5.413

15a	-8.28	-6.097	-5.328
15b	-6.132	-6.483	-6.483
15c	-6.355	-5.888	-6.673
15d	-6.269	-7.275	-6.051
15e	-6.586	-6.432	nd ^b
15f	-6.666	-6.412	nd ^b
15g	-6.009	-7.131	-5.694

Cmp	4O4I Epot ^a	4O4I Laul ^a	4O2B ^a
15h	-6.735	-5.766	-5.619
15i	-6.405	-5.956	-5.259
15j	-6.111	-6.534	-3.355
15k	-6.42	-6.613	-5.353
15l	-6.893	-5.508	nd ^b
15m	-6.188	-6.069	-3.85
15n	-6.009	-7.131	-5.694

^a Glide Docking Score values

^b nd: not docked compounds

red: best docking score values

Table 1 Docking results of compounds **14-18**.

Analysing the docking score results is possible to notice that many compounds show good score values in particular for the Cochicine binding site (4O2B). Better results were obtained for compounds bearing side chains on the carbonyl group, in which a significant improvement is observed comparing docking score values of the first and second group.

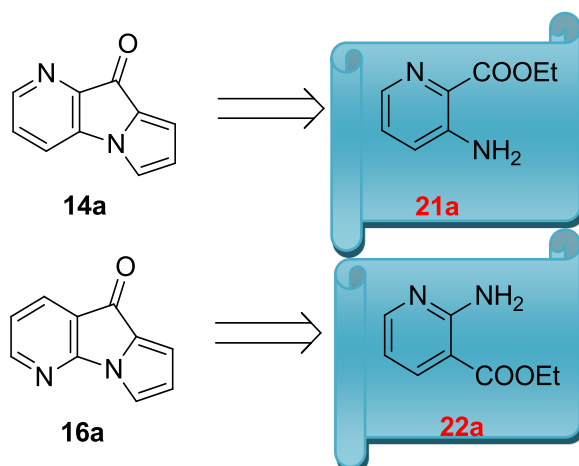
The best results are showed by compounds bearing side chains with hydrogen-acceptor groups (ex. compounds **14f** and **14g** with docking score values of -7.17 and -7.32 respectively; compounds **16f** and **16g** with docking score values of -7.25 and -7.56 respectively) responsible for specific interactions with Colchicine binding site, in particular with Asn350 residue.

Compounds of the third groups showed good docking score values but without selectivity towards a specific binding site.

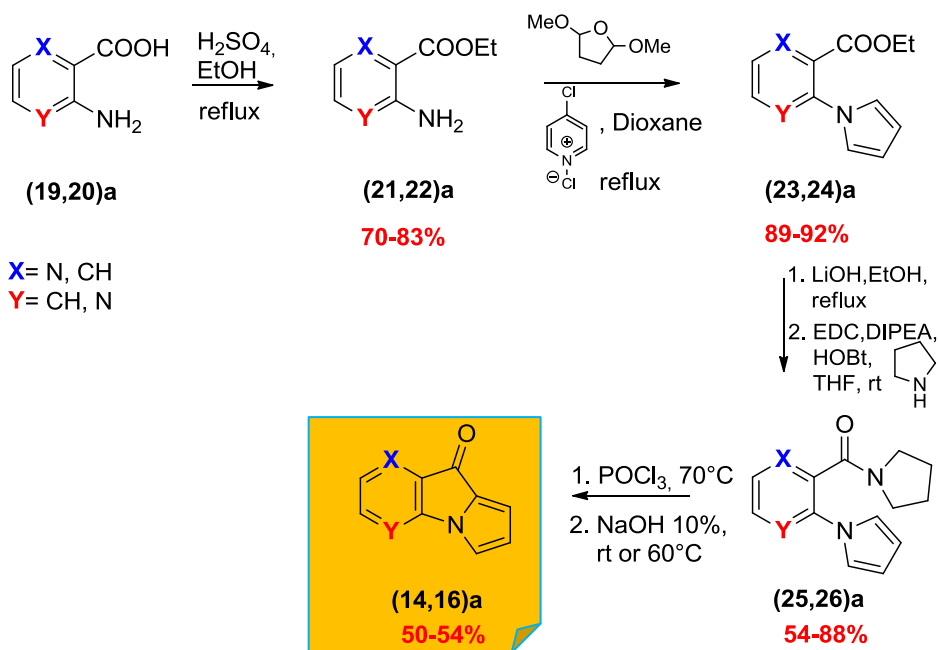
Compounds of the fourth group, bearing side chains on the pyridine ring and on the carbonyl group, show better docking score values for Taxanes binding site (4O4I) (wider than colchicine binding site), probably because of their steric hindrance caused by the presence of both side chains.

RESULTS AND DISCUSSIONS: CHEMISTRY

Through few steps it was possible to obtain the desired tripeptones (**14,16**)a in good yields (50-54%). The key intermediate for the synthesis of these pyrido-pyrrolizinones are respectively 3-aminoester **21a** and 2-aminoester **22a**.

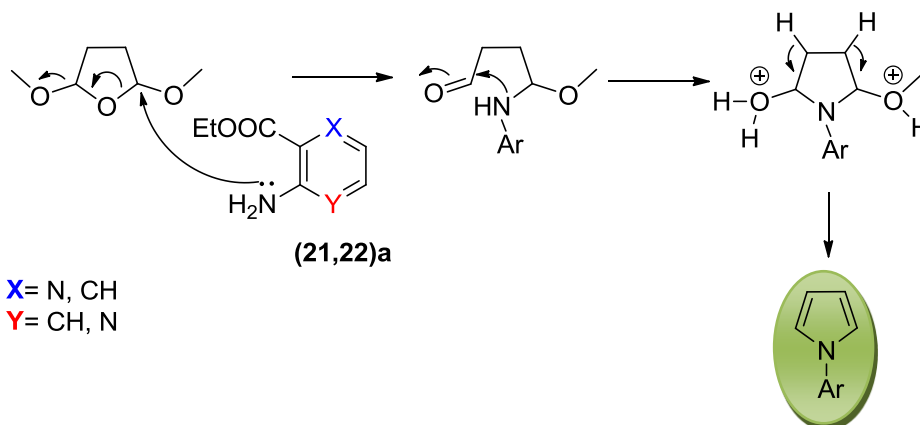


These key esters were obtained in good yields (70-83%) from commercial *ortho*-aminopyridine carboxylic acids of type (**19,20**)a, through Fischer esterification with concentrated sulfuric acid in absolute ethanol at reflux for 48 hours⁴³ (**Scheme 1**).



Scheme 1

The following pyrrolation on the amino group of amino-esters **(21,22)a** under Clauson-Kaas conditions in the presence of 2,5-dimethoxytetrahydrofuran, 4-chloropyridine hydrochloride in anhydrous 1,4-dioxane at reflux gave intermediates **(23,24)a** in excellent yields (89-92%).



Scheme 2

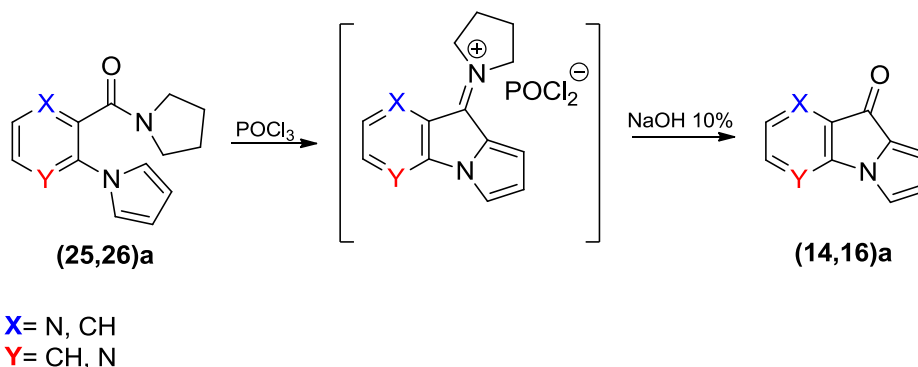
Through condensation of 2,5-dimethoxytetrahydrofuran on our primary amino group, following intermolecular attack on the carbonyl function and final dehydration, we obtained desired pyrrole in derivatives **(23,24)a** (Scheme 2).

These intermediates were later converted to the corresponding amides **(25,26)a**. The first attempt was made in refluxing pyrrolidine in ratio up to 1:42 for 36 hours. The reaction of amidation worked but the desired amides were obtained in low yields (38-42%). We decided to try a different method via carboxylic acid, by using activating agents of the carboxylic function such as *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and hydroxy-benzotriazole (HOBt). Indeed corresponding carboxylic acids were obtained by hydrolysis of the ester groups under basic condition, lithium hydroxide in ethanol at reflux for 4 hours. Following amidation with pyrrolidine, in presence of EDC and HOBt, at room temperature for one night, afforded to the desired amides **(25,26)a**. This “strategy” allowed us to isolate the desired derivatives from good to high yields (54-88%) and in short time (16 hours).

Cyclization of these latter was performed by acylation under Vilsmeier-Haack conditions followed by alkaline treatment. As difference of classic Vilsmeier-Haack

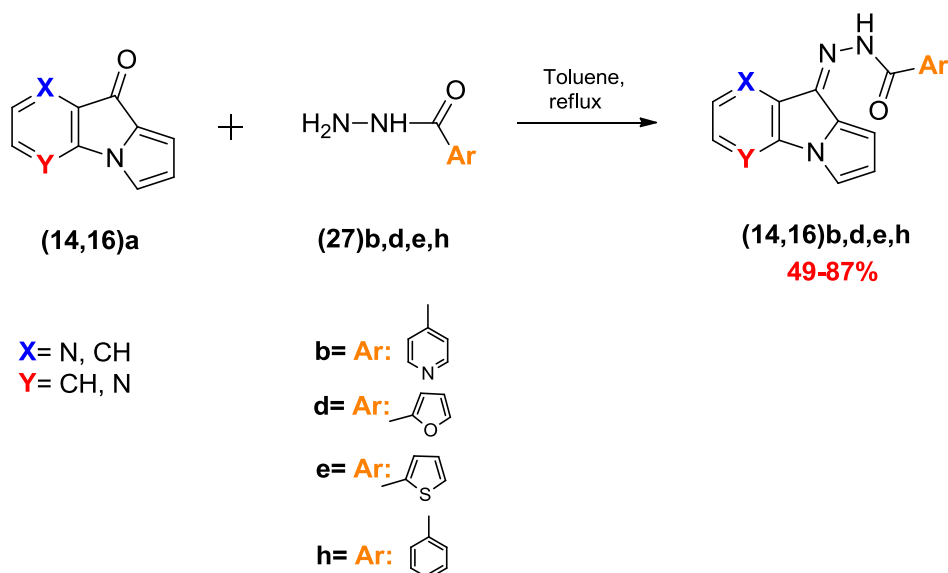
acylation, pyrrolidinecarboxamides (**25,26**)a take the place of dimethylformamide (DMF) that is the common formylating reagent used in this reaction¹³.

By treatment of these amides with phosphorous oxychloride (POCl₃) we obtained an intermediate iminium salt that was subsequently hydrolyzed in alkaline conditions (NaOH 10%) to give the triptones (**14,16**)a (Scheme 3).



Scheme 3

These latter, isolated in good yields (50-54%), were used for the following reaction of substitution with suitable heteroaryl carbohydrazide side chains to give derivatives of type (**14,16**)b-h (Scheme 4 and 5) (Table 2 and 3).



Scheme 4

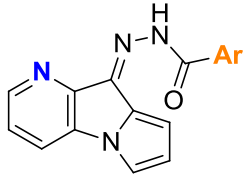
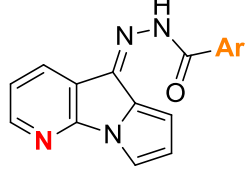
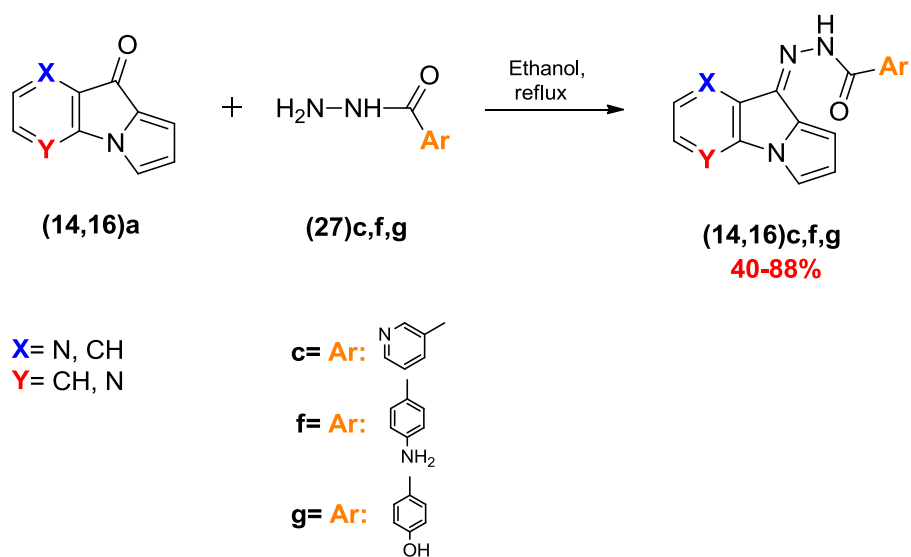
	Compound	Aryl	Yield
 <p>14</p>	14b	Pyridin4-yl	49%
	14d	Furan-2-yl	62%
	14e	Thiophen-2-yl	50%
	14h	Benzoyl	56%
 <p>16</p>	16b	Pyridin4-yl	51%
	16d	Furan-2-yl	87%
	16e	Thiophen-2-yl	49%
	16h	Benzoyl	71%

Table 2

In the first attempt we used the same conditions described from Grande *et al.* for the synthesis of N⁷-heteroacyl-9*H*-pyrrolo[1,2-*a*]indol-9-hydrazones **13**²². Therefore, tripentone **16a** was reacted with isonicotinohydrazide **27b** in a mixture of ethanol and toluene (1:3) at reflux for 36 hours. We isolated the desired compound **16b** but in too low yield (25%). We decided to try again this reaction, using only toluene as solvent of reaction and Dean-Stark apparatus to remove water that is produced during this reaction. This time we had got better yield (51%) and lower time of reaction (24 hours vs 36 hours) than the first attempt. This latter method was used also to prepare in moderate yield derivate **14b** (49%) by reaction between tripentone **14a** and isonicotinohydrazide **27b**. In the same conditions reaction between tripentones (**14,16a**) and 2-furan-2-carboxylic acid hydrazide **27d** gave the corresponding derivatives **14d** (62%) and **16d** (87%); reaction with 2-thiophenecarboxylic acid hydrazide **27e** gave the derivatives **14e** (50%) and **16e** (49%). Reaction from the same tripentones with benzhydrazide **27h** afforded the last final compounds **14h** (56%) and **16h** (71%).

Tripentones (**14,16c,f,g** (Table 3) (40-88%) were obtained by refluxing for 24 hours in ethanol of the tripentones (**14,16a**) with nicotinic hydrazide **27c**, 4-aminobenzhydrazide **27f** and 4-hydroxybenzhydrazide **27g** respectively (Scheme 5).

In this case we preferred to use ethanol instead of toluene, since we observed that when the reaction was run by using ethanol as solvent, a precipitation of the desired products occurred.

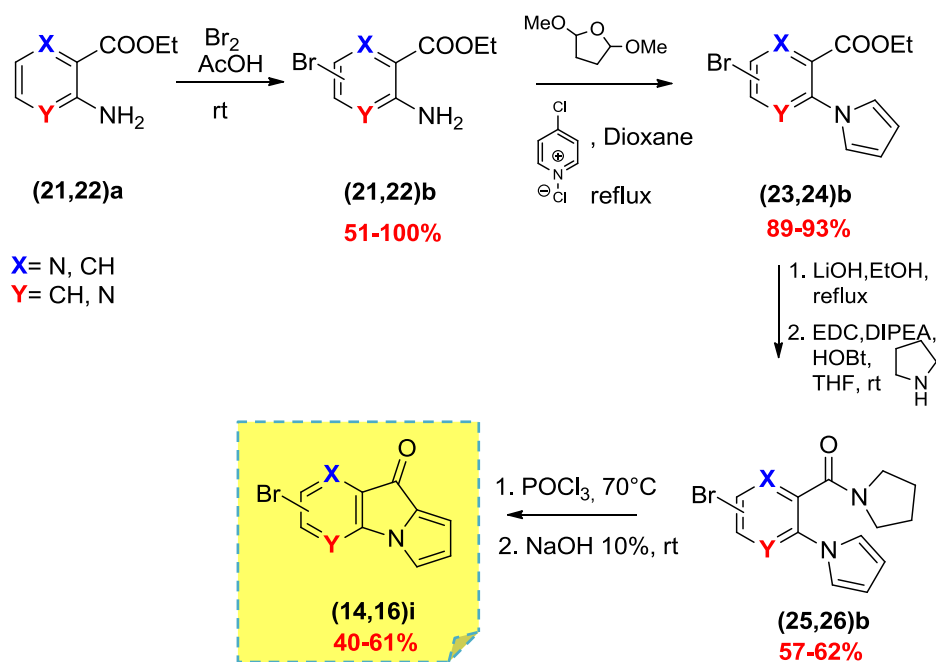


Scheme 5

	Compound	Aryl	Yield
14	14c	Pyridin-3-yl	88%
	14f	4-Aminobenzoyl	40%
	14g	4-Hydroxybenzoyl	80%
 16	16c	Pyridin-3-yl	60%
	16f	4-aminobenzoyl	45%
	16g	4-hydroxybenzoyl	85%

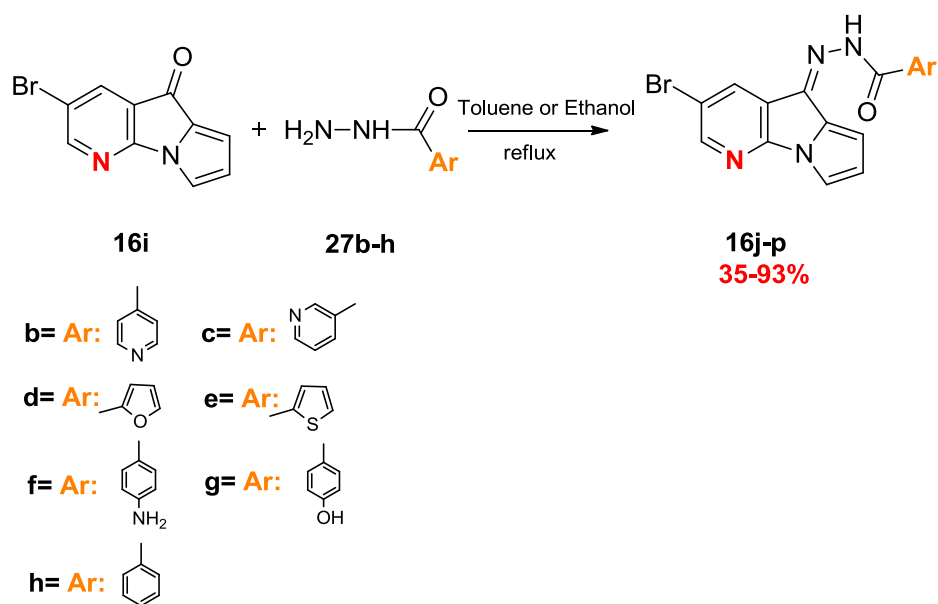
Table 3

In order to isolate tripentones **(14,16)i** first we used the same synthetic route reported for derivatives **(14,16)a** (Scheme 1), but this time we started from common intermediates amino-esters **(21,22)a** (Scheme 6).



Scheme 6

Bromuration of these amino-esters, performed with bromine in acetic acid at room temperature, afforded to the bromo-derivatives of type **(21,22)b** in good or excellent yields (51-100%). Next steps of pyrrolation, amidation and final cyclization were performed in the same conditions described above for the previous series. The new tripentone **16i** was reacted with heteroaryl carbohydrazides **27b-h** to give the derivatives **16j-p** (Table 4) in yields from 35% to 93%, using toluene as solvent of reaction for compounds **16j,l,m,p**, whereas ethanol for tripentones **16k,n,o** (Scheme 7).



Scheme 7

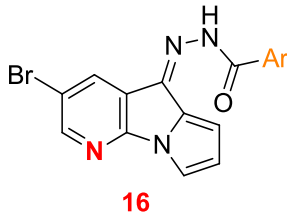
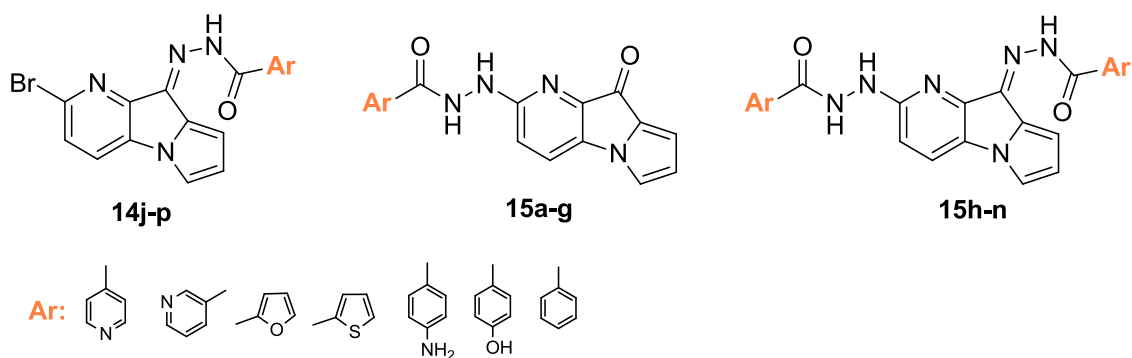
	Compound	Aryl	Yield
	16j	Pyridin-4-yl	40%
	16k	Pyridin-3-yl	63%
	16l	Furan-2-yl	45%
	16m	Thiophen-2-yl	74%
	16n	4-aminobenzoyl	52%
	16o	4-hydroxybenzoyl	93%
	16p	Benzoyl	35%

Table 4

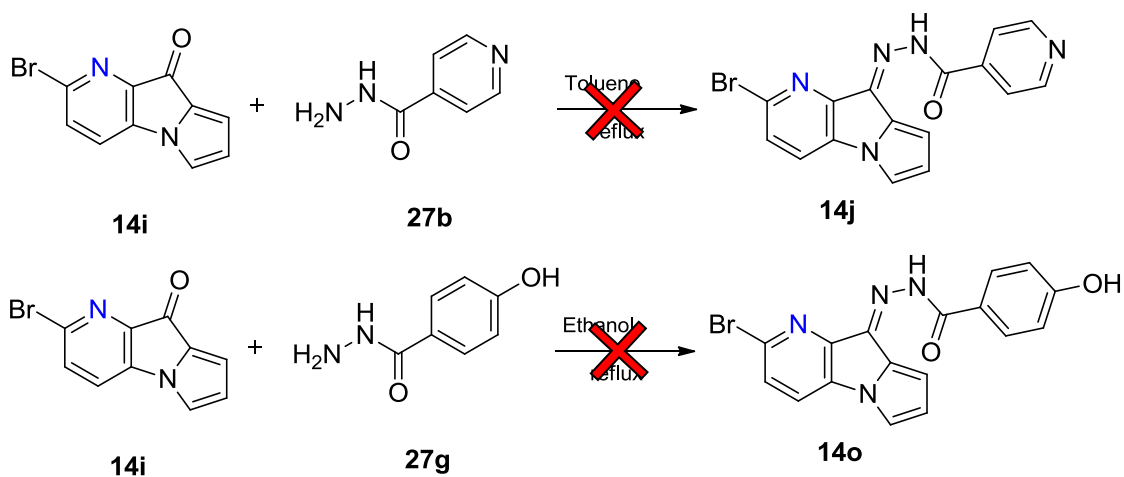
From the tripentone **14i** we could obtain different series of compounds, such as tripentones with heteroaryl carbohydrazide side chains on the carbonyl group (**14j-p**), tripentones with heteroaryl carbohydrazide side chains on the bromine (**15a-g**), because this position of halogen on pyridine ring is able to give $\text{S}_{\text{N}}\text{Ar}$, and finally tripentones with heteroaryl carbohydrazide side chains on both carbonyl group and bromine (**15h-n**) (**Scheme 8**).



Scheme 8

Unfortunately we couldn't isolate any compounds of these three series, despite we tried several times.

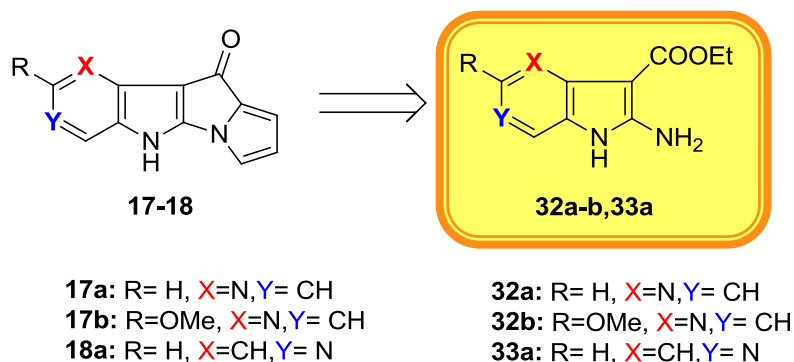
In the first attempt tripentone **14i** was reacted with isonicotinohydrazide **27b**, using toluene as solvent of reaction and Dean-Stark apparatus, at reflux for 24 hours (**Scheme 9**). At beginning we used 1eq of our starting material and another one of carbohydrazide chain, the same conditions used in the other series, but it wasn't enough. We decided to increase equivalents of chain from (1:1) to (1:3) until (1:5) but also in these cases we didn't obtain the desired compound. Indeed, the reaction mixture became really dirty and no priority spot was available to isolate.



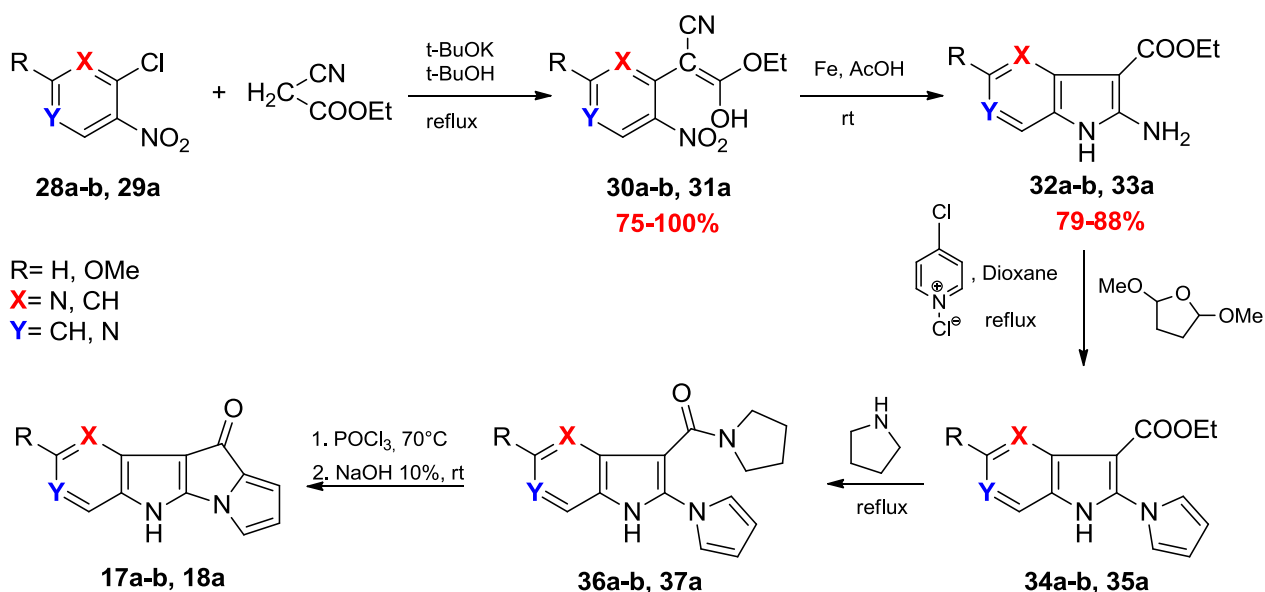
Scheme 9

Another attempt was conducted from tripentone **14i** and 4-hydroxybenzohydrazide **27g** (**Scheme 9**), but the reaction followed the same path of the one described above. Indeed, also in this case we didn't obtain the desired compound.

The synthetic route planned to obtain 4- or 6-azaindoles **17-18** identified the key intermediates in 4-azaindole-2-amino-3-esters **32a** or **32b** and 6-azaindole-2-amino-3-ester **33a**.



We started to synthesize intermediates **30a-b** and **31a** by reaction between the opportune commercial chloro-3-nitropyridines **28a-b**, **29a** with potassium enolate of ethyl cyanoacetate in *t*-butanol at reflux for 12 hours (**Scheme 10**)^{44,45}.

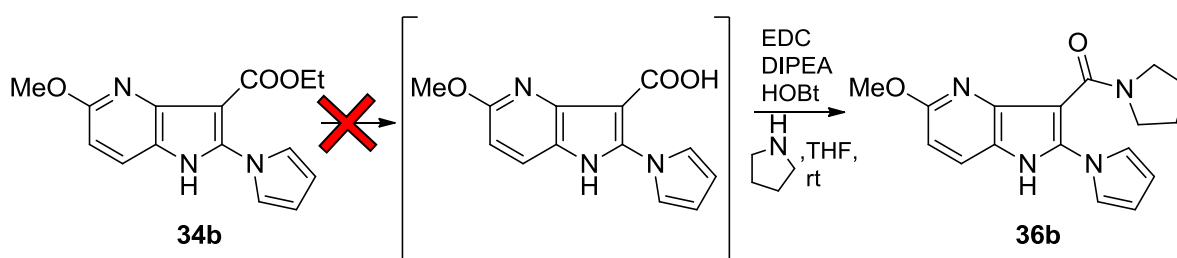


Scheme 10

These latter, isolated in high to excellent yields (75-100%), were reduced with iron in acetic acid at room temperature to the corresponding key intermediates **32a-b**, **33a** (79-88%).

We had several synthetic problems to afford derivatives **17a-b** and **18a** (Scheme 10). Different attempts were conducted to optimize this synthetic route.

Regarding the final triptone **17b** we could obtain intermediates **30b** (75%), **32b** (79%) and also, under Clauson-Kaas conditions, compound **34b** in good yield (65%). Following amidation reaction tried in refluxing pyrrolidine (1:42) for 24 hours, didn't allowed the isolation of desired product. The reaction mixture was really dirty without a priority spot to isolate and there was still starting material unreacted. We tried to obtain the corresponding amide, by hydrolysis of the ester group under basic condition, followed by amidation with pyrrolidine, using activating agents the carboxylic function such as EDC and HOBt (Scheme 11).



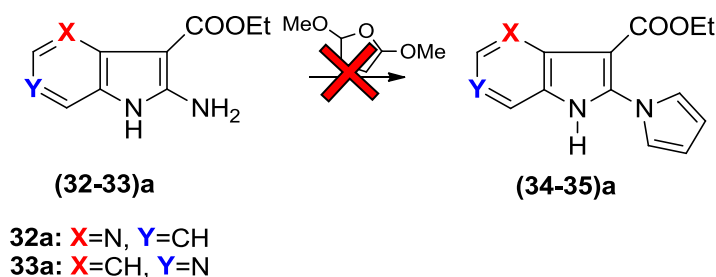
Scheme 11

Unfortunately, we found more complications to hydrolyze compound **34b**. In fact different attempts were made: using a 5% NaOH solution at reflux for 18 hours; 20% KOH solution at reflux for 24 hours; pellets of potassium hydroxide (1:11) in ethanol at reflux for 24 hours; or with lithium hydroxide (1:10) in ethanol at reflux for the same time. Finally, we used lithium hydroxide (1:10) in a mixture of methanol and water (4:1) at reflux for 30 hours. Even changing different conditions such as solvents, bases and times of reaction, none of these attempts worked and we couldn't obtain desired carboxylic acid.

Another idea was to obtain the corresponding acyl halide, function more reactive than ester (**34b**), but the problem was the same. We didn't obtain desired carboxylic acid to convert into corresponding acyl halide and then into amide **36b**.

Finally, we thought to invert reaction's order, because most likely pyrrole ring, formed during Clauson-Kaas reaction, inhibited the amidation of our ester for steric hindrance. Actually, we tried to carry out first amidation in refluxing pyrrolidine (1:42) for 24 hours and then to carry out Clauson-Kaas reaction. Unfortunately, also this attempt didn't work due to the amidation step.

Regarding final triptones **17a** and **18a**, we obtained in excellent yields (80-100%) derivatives **(30-31)a** and **(32-33)a** (85-88%) but we couldn't isolate following compounds **(34-35)a** (**Scheme 12**). Indeed, even changing different conditions such as amount of reagents, temperature, and times of reaction (**Table 5**), unfortunately, we didn't afford the desired intermediates.



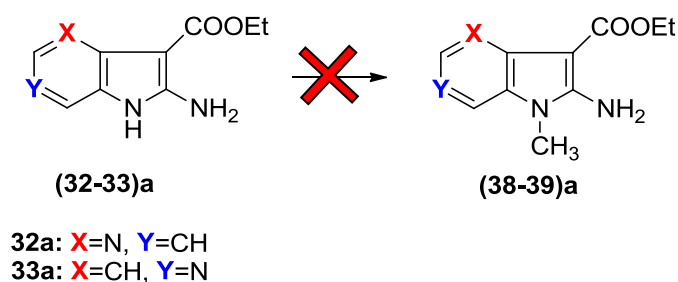
Scheme 12

Derivate	2,5-dimethoxy- THF	4-Chloropyridine hydrochloride	Temperature	Result
32a	1 eqv	1 eqv	Reflux, 4h	Side products
33a	1 eqv	1 eqv	Reflux, 4h	Side products
32a	2 eqv	2 eqv	Reflux, 5h	Side products
33a	2 eqv	2 eqv	Reflux, 5h	Side products
32a	1 eqv	1 eqv	Rt→50°C, 1h	Side products
33a	1 eqv	1 eqv	Rt→50°C, 1h	Side products

Table 5

From NMR data we understood that more probably Clauson-Kaas reaction didn't work for the presence of the free NH in the aza-indole ring. We decided to try a methylation in this position (**Scheme 13**), using potassium *t*-butoxide as base, tris[2-(2-methoxyethoxy)ethyl]amine (TDA-1) (1 or 2 drops) as phase transfer catalyst and

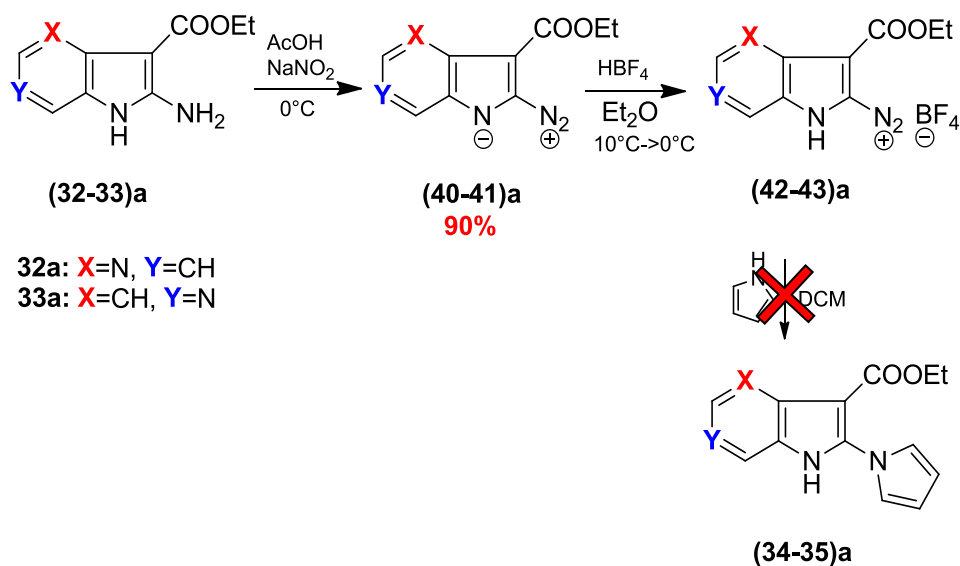
methyl iodide as methylating agent in toluene at room temperature. Unfortunately, this reaction didn't bring us to any result as there wasn't a priority spot to isolate in the reaction mixture. Therefore, we thought to carry on the methylation under nitrogen atmosphere and to use cesium carbonate as base and DMF as solvent of reaction. Also in this case we had the same result.



Scheme 13

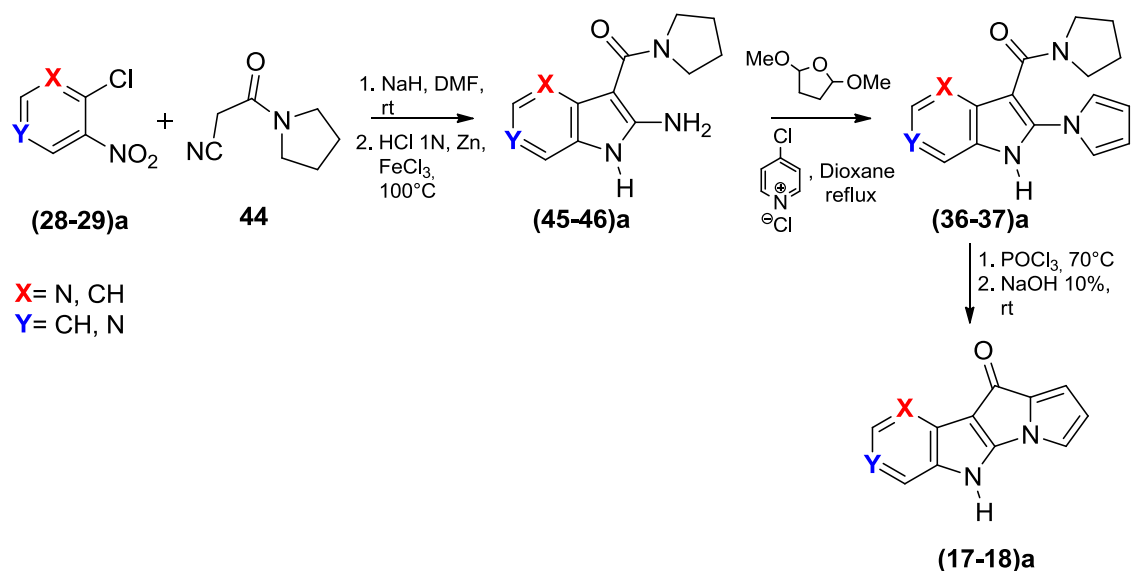
We also thought to try a diazotization of amino group in acetic acid with stoichiometric amount of sodium nitrite under nitrogen atmosphere in the dark, followed by addition of aqueous sodium carbonate^{44,45} (**Scheme 14**). In these reactions is crucial the strict control of temperature at 0°C both during diazotization and neutralization to obtain desired compounds in high yields.

Diazo compounds (**40-41**)a were treated with tetrafluoroboric acid in diethyl ether to give corresponding tetrafluoroborate derivatives (**42-43**)a. The crude of these latter intermediate compounds was taken up onto the next step of nucleophilic substitution with pyrrole, previously activated for treatment with a base, in dry DCM to afford the desired intermediates (**34-35**)a. Unfortunately, also this attempt failed in both series.



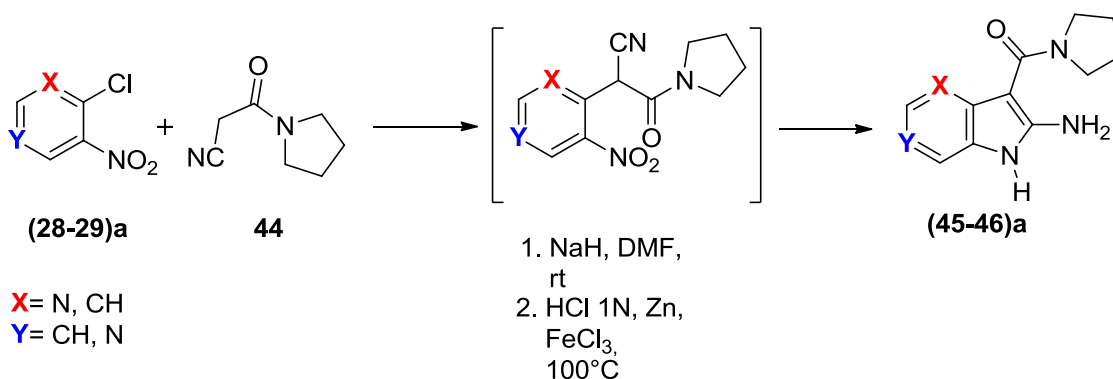
Scheme 14

Another synthetic route was planned starting from the same starting materials (**Scheme 15**). In the new route we tried to obtain 2-aminoazaindole-3-carboxamide (**45-46**)a by reaction between 1-(cianoacetyl)-pyrrolidine **44** and opportune 2- or 4-chloro-3-nitropyridines (**28-29**)a. Through following Clauson-Kaas reaction and final cyclization under Vilsmeier-Haack conditions we would have wanted to get final tripentones (**17-18**)a.



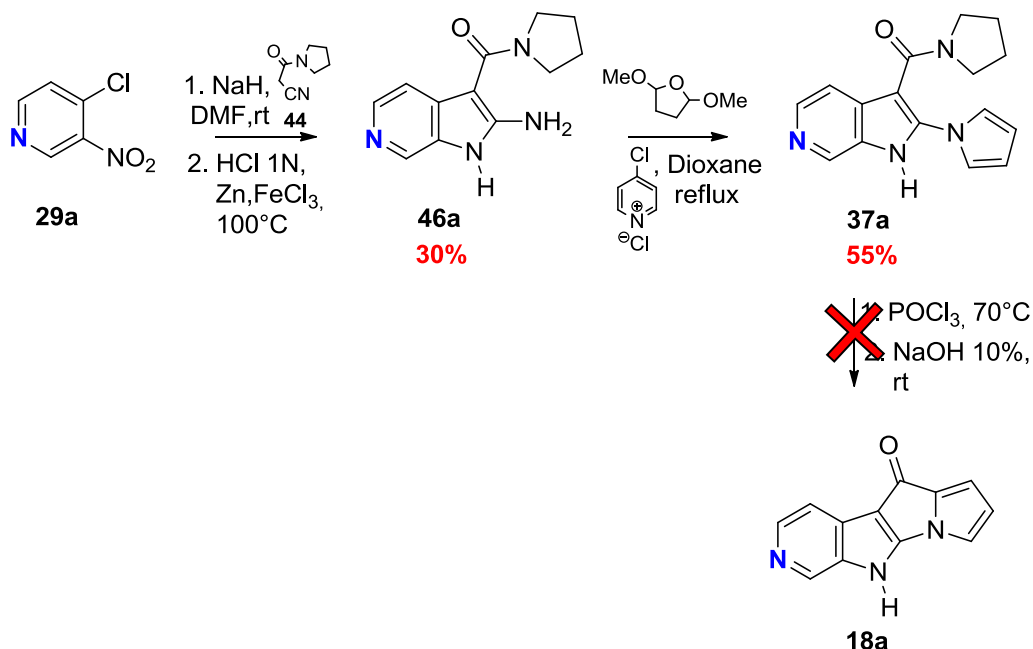
Scheme 15

In the first step, sodium hydride (in excess) was added to a solution of 1-(cianoacetyl)-pyrrolidine **44** in DMF and then opportune 2- or 4-Cl-3-nitropyridines (**28-29**)**a** was added. Only when the S_NAr reaction was completed, a solution 1 N HCl was added to neutralize the excess of sodium hydride, and the intermediate was reduced to desired amino-azaindole with FeCl₃ and Zn⁴⁶ (**Scheme 16**).



Scheme 16

We obtained only the 2-chloro-3-aminopyridine at place of 2-amino-azaindole-3-carboxamide **45a** because the reaction illustrated in scheme 16 didn't work for 4-azaindole's series.



Scheme 17

Regarding the 6-azaindole's series, we obtained the desired carboxamide **46a** (30%) and the Clauson-Kass's product **37a** (55%) as well. Unfortunately, the cyclization didn't work (**Scheme 17**). Even increasing amount of POCl₃ or changing temperature (reflux instead 70°C) we didn't isolate desired final tripentone **18a**.

RESULTS AND DISCUSSION: BIOLOGY

All tripentones synthesized **14a-i** and **16a-p** (Table 6) were submitted to biological laboratory of “Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche” (STEBICEF) University of Palermo in collaboration with Professor L. Tesoriere with the aim of evaluating their antitumoral activity.

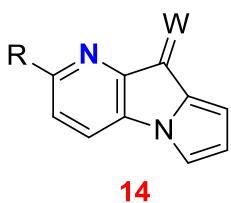
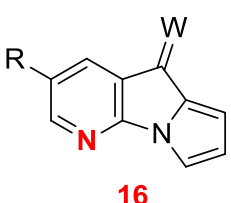
	Compound	R	W	Yield
 <p>14</p>	14a	H	O	50%
	14b	H	N-NH-CO-Pyridin-4-yl	49%
	14c	H	N-NH-CO-Pyridin-3-yl	88%
	14d	H	N-NH-CO-Furan-2-yl	62%
	14e	H	N-NH-CO-Thiophen-2-yl	50%
	14f	H	N-NH-CO-4-NH ₂ -C ₆ H ₄	40%
	14g	H	N-NH-CO-4-OH-C ₆ H ₄	80%
	14h	H	N-NH-CO-C ₆ H ₅	56%
	14i	Br	O	40%
	 <p>16</p>	16a	H	O
16b		H	N-NH-CO-Pyridin-4-yl	51%
16c		H	N-NH-CO-Pyridin-3-yl	60%
16d		H	N-NH-CO-Furan-2-yl	87%
16e		H	N-NH-CO-Thiophen-2-yl	49%
16f		H	N-NH-CO-4-NH ₂ -C ₆ H ₄	45%
16g		H	N-NH-CO-4-OH-C ₆ H ₄	85%
16h		H	N-NH-CO-C ₆ H ₅	71%
16i		Br	O	61%
16j		Br	N-NH-CO-Pyridin-4-yl	40%
16k		Br	N-NH-CO-Pyridin-3-yl	63%
16l		Br	N-NH-CO-Furan-2-yl	45%
16m		Br	N-NH-CO-Thiophen-2-yl	74%
16n		Br	N-NH-CO-4-NH ₂ -C ₆ H ₄	52%
16o		Br	N-NH-CO-4-OH-C ₆ H ₄	93%
16p		Br	N-NH-CO-C ₆ H ₅	35%

Table 6

Preliminary biological screening were performed on derivatives **14a-b,g,i** and **16a-p** on HCT116 colorectal carcinoma cells. Cell monolayers were incubated for 72 hours in the absence (control) or in the presence of the compounds at the indicated concentrations

and cell growth was assessed by MTT test as reported in the methods section. This assay is based on reduction of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into its insoluble formazan crystals (purple precipitate) by NADH-dependent cellular oxidoreductase enzymes (**Figure 13**). The absorbance of this colored solution can be quantified by spectrophotometry and it is directly proportional to cell viability. In fact, when the cells die, they lose the ability to convert MTT into purple colored Formazan product⁴⁷.

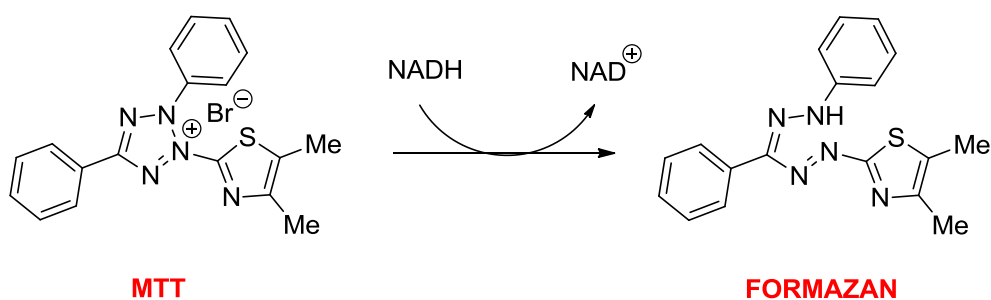


Figure 13 Structures of MTT and Formazan product

In the **figure 14** is possible to discern the effect of the different tripentone derivatives on the cell growth (tumor cells line HCT116). In greater detail it is showed the activity of not substituted tripentones (**14A**), tripentones substituted with isonicotinohydrazide and nicotinic hydrazide side chains (**14B**), tripentones substituted with 2-furan-2-carboxylic acid hydrazide and 2-thiophenecarboxylic acid hydrazide side chains (**14C**), tripentones substituted with 4-aminobenzhydrazide and 4-hydroxybenzhydrazide side chains (**14D**) and tripentones substituted with benzhydrazide side chain (**14E**) respectively (**Figure 14**). Results are indicated as the percentage of viable cells with respect to untreated controls. Values are the mean \pm SD of three separate experiments in triplicate.

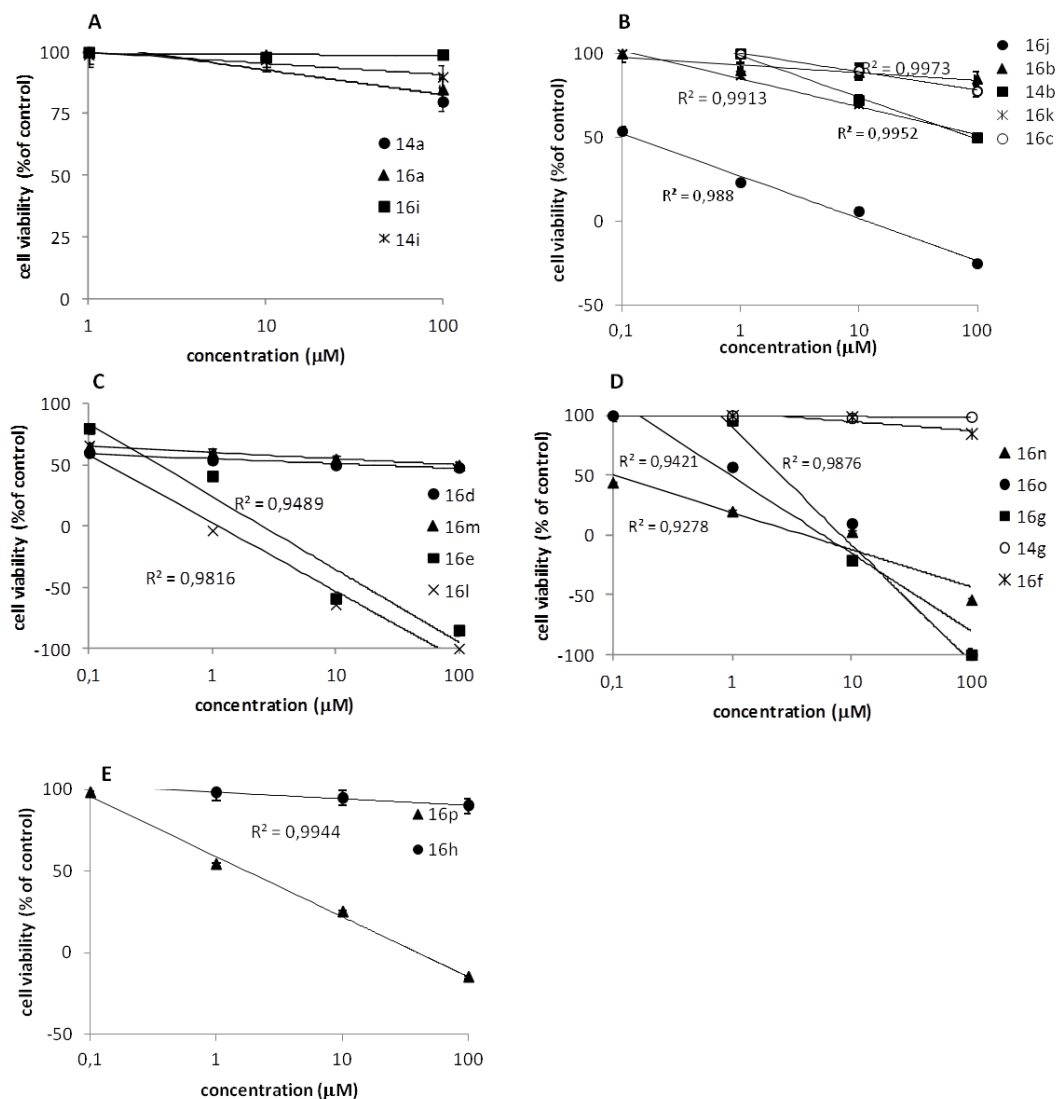


Figure 14 Effect of tripentone derivatives on the growth of human intestinal tumor cells line HCT116.

From these first results was possible to note that tripentones substituted result most active than not substituted ones in general. This data was in agreement with our original idea, indeed the introduction of heteroaryl carbohydrazone side chains increases anti-proliferative effect of our compounds and above all offers a possibility of forming conjugate salts to improve water solubility and thereby their pharmacokinetic properties.

Moreover, was interesting to note how the series **16** are better than **14** one in terms of activity. In fact, the most active compounds against HCT116 were **16e**, **16g**, **16j**, **16l** and **16n-p** with *in vitro* anti-proliferative activity in the micro-submicromolar range

(**Table 7**). In particular, in the environment of **16'** series the presence of bromine on the pyridine ring (position 3) appears crucial for conferring good activity to the 5*H*-pyrido[3,2-*b*]pyrrolizin-5-ones derivatives. Indeed, the best compounds were **16j**, **16l** and **16n**, having GI₅₀ values of 0.11±0.01 μM, 0.17±0.02 μM and 0.23±0.02 μM respectively. Cytotoxicity of **16e**, **16g**, **16j**, **16l** and **16n-p** was also assessed on MCF-7 cell line (human breast cancer) and the calculated GI₅₀ values are reported in **Table 7**.

	GI ₅₀ ±SD	
	HCT116	MCF7
16e	0.46±0.05	3.15±0.29
16g	2.54±0.16	16.11±1.45
16j	0.11±0.01	3.40±0.35
16l	0.17±0.02	1.61±0.98
16n	0.23±0.02	1.80±0.15
16o	1.66±0.18	15.62±1.11
16p	1.74±0.12	14.88±1.03

^aGI₅₀ is the molar concentration of the compound that inhibits 50% net cell growth. Values are the mean±SD of three separate experiments carried out in triplicate

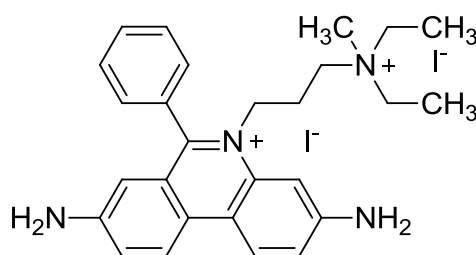
Table 7 GI₅₀ values (μM) for the *in vitro* anti-proliferative activity of the most active tripentone derivatives, determined by MTT test on HCT116 and MCF7 cells after 72 h treatment.

It can be observed that, although all tripentones inhibit the growth of tumor cells of breast cancer, their anti-proliferative efficacy appeared about one order of magnitude lower than that shown towards the cells of the colon rectal cancer HCT116.

We also investigated alterations in the cell cycle caused by tripentones **16e** (the most active compound without bromine on the pyridine ring) and **16j** (the most active compound with bromine on the pyridine ring) in colorectal cancer cells (**Figure 15**).

With the aim to discriminate cells in different phases of the cell cycle, flow cytometric analysis of nuclear DNA content after 24 hours treatment of HCT-116 cells was determined using propidium iodide (PI) **47**. The percentage of cells in the different phases of the cycle was calculated by Expo32 software. Values are the mean \pm SD of three separate experiments in triplicate.

Propidium iodide (PI) **47** is a DNA-binding dye by intercalating between the bases. It is the most commonly used dye to quantitatively assess DNA content as PI is stoichiometric, it binds in proportion to the amount of DNA present in the cell.



47

Differentiation between phases of the cell cycle is based on the content of genetic material, which in non-dividing cells is limited to one copy of DNA. The cell population in the S phase (DNA replication phase) is synthesizing genetic material, and thus contains more DNA than quiescent cells. The subsequent G2/M phase (interphase/mitosis) is characterized by the presence of two copies of DNA. At last, cells in the region subdiploid are considered as an index of apoptosis. Therefore, the alterations in these phases are used as a basis for the comparison of different treatments.

Both **16e** and **16j** caused a significant dose-dependent decrease in the percentage of cells in the G0/G1 and S phases, accompanied by a concomitant percentage increase of cells in the G2/M phase, and appearance of a subG1-cell population (**Figure 15**). These results indicated that antiproliferative mechanism induced by the compounds is in the process of separation of the chromatids.

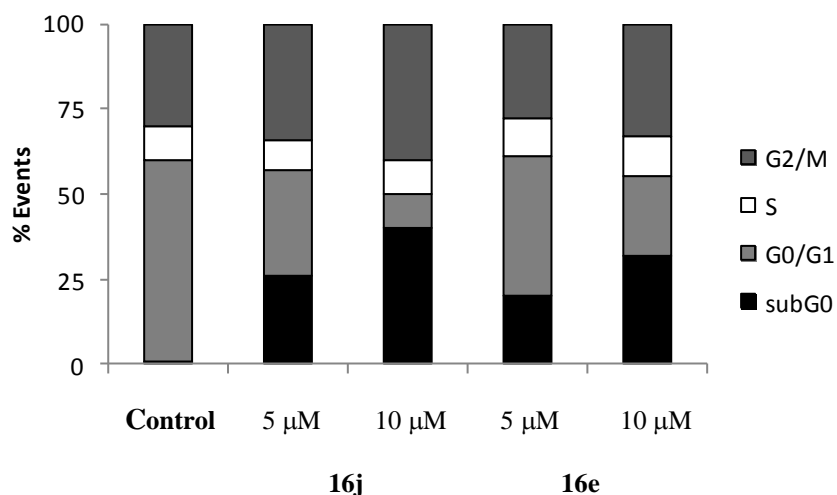


Figure 15 Effect of **16e** and **16j** on the cell cycle distribution of HCT116 colorectal cancer cells.

To determine whether HCT-116 cells undergo apoptosis upon treatment with the tripentone analogues, cells were treated with **16e** or **16j** for 24 hours, stained with both propidium iodide (PI) **47** and Annexin V-Fluorescein isothiocyanate (FITC), and analyzed by flow cytometry. Through this assay we aimed to evaluate the externalization of the Phosphatidylserine (PS) on the external cellular environment. In fact, during the earlier phases of apoptosis the phosphatidylserine is translocated from the inside to the outside of the cytoplasmic membrane. Annexin V has high selectivity toward PS and it can be conjugated with fluorochromes such as Fluorescein isothiocyanate (FITC). The complex Annexin V-FITC binds with high affinity PS. For this reason, the Annexin V-FITC assay can be used to notice cells that are undergoing apoptosis. In order to distinguish early, late apoptosis or necrosis staining with FITC Annexin V is usually used in combination with a vital dye such as propidium iodide (PI) **47**, indeed PI doesn't permeate inside of viable cells.

In particular, we can discern:

- ❖ viable cells in which Annexin V-FITC and PI are negative;
- ❖ early apoptosis cells in which Annexin V-FITC is positive instead PI is negative;
- ❖ late apoptosis cells in which annexin V-FITC and PI are positive;
- ❖ necrotic cells in which Annexin V-FITC is negative instead PI is positive.

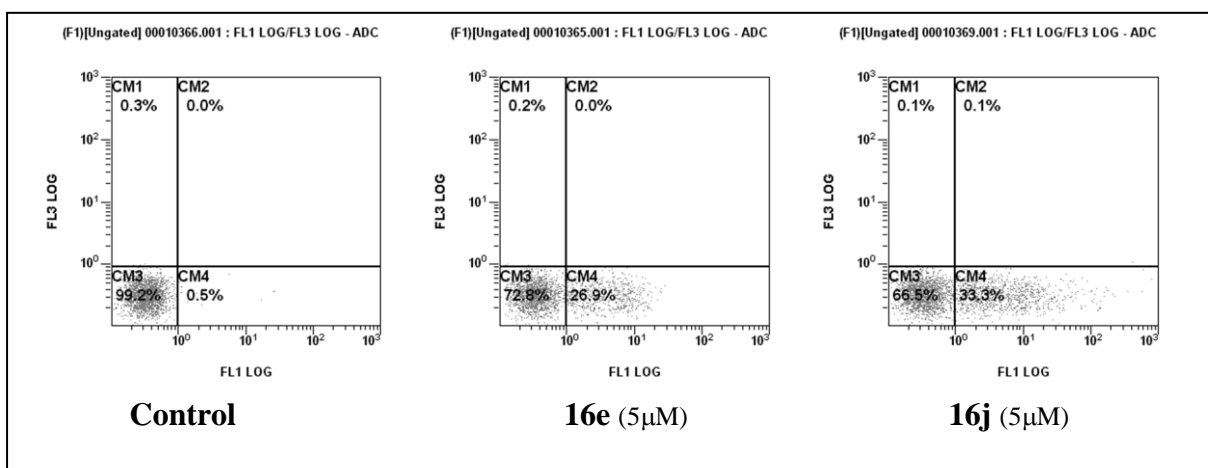


Figure 16 Cell apoptosis by **16e** and **16j** in HCT116 cells. Percentage of Annexin V/propidium iodide (PI) double-stained cells, as determined by flow cytometry. Control, cells treated with vehicle; cells were treated for 24 h with either tripentone derivatives. Representative images of three experiments with comparable results.

As shown in **figure 16** none of the compounds exerted necrotic effects on HCT116 cells, while inducing a clear pro-apoptotic effect with an high percentage of cells shifted towards early apoptosis.

CONCLUSION

In conclusion we reported:

- ❖ two series of new tripentones **14a-i** and **16a-p** were completed and synthesized;
- ❖ the new compounds showed anti-proliferative activity in micro or sub-micromolar range;
- ❖ the new tripentones showed the best activity against HCT-116 colorectal carcinoma cells;
- ❖ alterations in the cell cycle caused by tripentones **16e** (the most active compound without bromine on the pyridine ring) and **16j** (the most active compound with bromine on the pyridine ring) were investigated in colorectal cancer cells (HCT-116);
- ❖ both **16e** and **16j** caused a significant dose-dependent decrease in the percentage of cells in the G0/G1 and S phases, accompanied by a concomitant percentage increase of cells in the G2/M phase, and appearance of a subG1-cell population;
- ❖ Annexin V-FITC assay was assessed with the aim to determine whether HCT-116 cells undergo apoptosis upon treatment with the tripentones **16e** and **16j**;
- ❖ derivatives **16e** and **16j** induced a clear pro-apoptotic effect with a high percentage of cells shifted towards early apoptosis;
- ❖ all these data are in agreement with our original idea to synthesize potential antitubulin agents. However, other ongoing studies aim to better define the pharmacological profile and the mechanism of action of our tripentones.

- ❖ It might be interesting to perform a structure–activity relationship study (SAR) to evaluate the effect of different halogens on tripentone (position 3) with the aim to understand if the presence of bromine increases the activity of our compounds due either its lipophilicity (Br > Cl > F) or its electronic effects (F > Cl > Br). Moreover, since the most active compounds are decorated with isonicotinic hydrazide (**16j**), 2-furan-2-carboxylic acid hydrazide (**16l**) and 4-aminobenzhydrazide (**16n**) side chains it might be interesting also to evaluate the introduction of different substitutes (polar, apolar, small or big) on the terminal ring of the side chains.

EXPERIMENTAL SECTION

CHEMISTRY

General methods

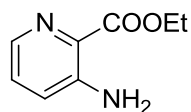
All melting points were taken on a Buchi-Tottoly capillary apparatus and were uncorrected. IR spectra were determined in bromoform with a Shimadzu FT / IR 8400S spectrophotometer. ^1H and ^{13}C NMR spectra were measured at 200 and 50 MHz, respectively, on DMSO- d_6 or CDCl_3 solution, using a Bruker Avance II series 200 MHz spectrometer. Chromatography column was performed with MERK silica gel 230-400 mesh ASTM or FLASH40i Biotage chromatography or with Buchi Sepacore chromatography module (prepacked cartridge reference). Elemental analyses (C, H, N) were within $\pm 0.4\%$ of the theoretical values.

Pyridines' series:

Synthesis of aminopyridine-carboxylates (21-22)a:

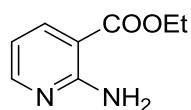
To a solution of suitable aminopyridine-carboxylic acid (**19-20**)a (1.0 g, 7.25 mmol) in absolute ethanol (4.07 mL), at 0°C , was slowly added concentrated H_2SO_4 (1.25 mL, 23.5 mmol) and the reaction mixture was heated to reflux for 48 hours. After cooling, the mixture was concentrated and poured into ice. The mixture was basified with concentrated aqueous ammonia to pH 8-9 and the white precipitate formed was collected by filtration. The filtrate was extracted with diethyl ether (4×10 mL), the organic layer was washed with brine (40 mL), dried over anhydrous Na_2SO_4 , filtered and evaporated. This crude was combined with that from the above filtration and the whole was purified by silica gel column chromatography (Petroleum Ether:Ethyl acetate, 8:2) to give the desired esters (**21-22**)a.

Ethyl 3-aminopyridine-2-carboxylate (21a)⁴³:



Yield: 70%, off-white solid; mp: 129.9-130.3°C; IR: 3491 – 3374 (NH₂), 1689 (CO) cm⁻¹; ¹H NMR (200 MHz, CDCl₃) δ: 8.08 (1H, dd, *J* = 4.2, 1.4 Hz, H-6), 7.27-7.19 (1H, dd, *J* = 8.3, 4.2 Hz, H-5), 7.04 (1H, dd, *J* = 8.4, 1.4 Hz, H-4), 5.76 (2H, bs, NH₂), 4.45 (2H, q, *J* = 7.1 Hz, CH₂), 1.45 (3H, t, *J* = 7.1 Hz, CH₃); ¹³C NMR (50 MHz, CDCl₃) δ: 167.6 (s), 146.9 (s), 138.7 (d), 128.1 (s), 127.9 (d), 124.8 (d), 61.3 (t), 14.4 (q). *Anal.* Calculated for C₈H₁₀N₂O₂ (MW: 166.18): C, 57.82; H, 6.07; N, 16.86%. Found: C, 57.75; H, 6.26; N, 16.82%.

Ethyl 2-aminopyridine-3-carboxylate (22a):

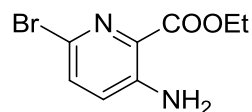


Yield: 83%, white solid; mp: 112.9-113.3°C; IR: 3501 – 3370 (NH₂), 1689 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO- *d*₆) δ: 8.20 (1H, dd, *J* = 5.2, 2.0 Hz, H-6), 8.05 (1H, dd, *J* = 8.2, 1.0 Hz, H-4), 7.17 (2H, bs, NH₂), 6.62 (1H, dd, *J* = 8.2, 5.2 Hz, H-5), 4.28 (2H, q, *J* = 6.8 Hz, CH₂), 1.32 (3H, t, *J* = 6.8 Hz, CH₃); ¹³C NMR (50 MHz, DMSO- *d*₆) δ: 166.5 (s), 159.4 (s), 153.9 (d), 139.6 (d), 111.8 (d), 104.6 (s), 60.4 (t), 14.1 (q). *Anal.* Calculated for C₈H₁₀N₂O₂ (MW: 166.18): C, 57.82; H, 6.07; N, 16.86%. Found: C, 57.92; H, 6.17; N, 16.66%.

Synthesis of amino-bromopyridine-carboxylates (21-22b):

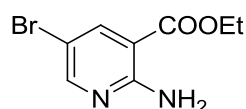
Bromine (0.08 mL, 1.56 mmol) solubilized in glacial acetic acid (0.33 mL) was added dropwise to a solution of suitable aminoester (**21-22**)**a** (0.2 g, 1.2 mmol) in glacial acetic acid (0.7 mL). The reaction mixture was vigorously stirred at room temperature for 1 hour. A small amount of water (3 mL) was added, and the precipitate formed **21b** was collected by filtration. Instead in the case of derivative **22b**, the solution was extracted with ethyl acetate (3 x 10 mL), dried over anhydrous Na₂SO₄, filtered and evaporated *in vacuo*. The crude was purified by silica gel column chromatography (DCM:Ethyl Acetate, 9:1) to afford compound **22b**.

Ethyl 3-amino-6-bromopyridine-2-carboxylate (**21b**):



Yield: 51%, yellow solid; mp: 131.0-131.3°C; IR: 3488 – 3375 (NH₂), 1689 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO- *d*₆) δ: 8.30 (1H, d, *J* = 2.6 Hz, H-4), 8.12 (1H, d, *J* = 2.6 Hz, H-5), 7.37 (2H, bs, NH₂), 4.28 (2H, q, *J* = 7.1 Hz, CH₂), 1.32 (3H, t, *J* = 7.1 Hz, CH₃); ¹³C NMR (50MHz, DMSO- *d*₆) δ: 165.9 (s), 147.6 (s), 132.4 (d), 128.7 (d), 125.5 (s), 124.3 (s), 60.5 (t), 14.2 (q). *Anal.* Calculated for C₈H₉ BrN₂O₂ (MW: 245.07): C, 39.21; H, 3.70; N, 11.43%. Found: C, 39.01; H, 3.57; N, 11.23%.

Ethyl 2-amino-5-bromopyridine-3-carboxylate (**22b**):



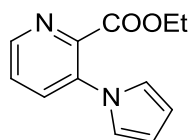
Quantitative yield, light yellow solid; mp: 140.0-140.5°C; IR: 3500 – 3374 (NH₂), 1689 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO- *d*₆) δ: 8.29 (1H, d, *J* = 2.5 Hz, H-6), 8.12 (1H, d, *J* = 2.5 Hz, H-4), 7.41 (2H, bs, NH₂), 4.28 (2H, q, *J* = 7.1 Hz, CH₂), 1.30 (3H, t, *J* = 7.1 Hz, CH₃); ¹³C NMR (50 MHz, DMSO- *d*₆) δ: 165.3 (s), 157.9 (s), 153.9 (d), 141.0 (d), 106.3 (s), 103.9 (s), 60.9 (t), 13.9 (q). *Anal.* Calculated for C₈H₉ BrN₂O₂ (MW: 245.07): C, 39.21; H, 3.70; N, 11.43%. Found: C, 39.31; H, 3.87; N, 11.54%.

Synthesis of (1*H*-pyrrol-1-yl)pyridine-carboxylates (**23-24**)a,b:

To a solution of 2,5-dimethoxytetrahydrofuran (0.23 mL, 1.81 mmol) in anhydrous 1,4-dioxane (21.4 mL), was added 4-chloropyridine hydrochloride (0.27 g, 1.81 mmol) and the reaction mixture was stirred at room temperature for 15 minutes. Appropriate derivative (**21-22**)a,b (1.81 mmol) was added and the reaction mixture was heated to reflux for 18 hours. Upon cooling a precipitate formed that was filtered. The solid was discarded, while the mother liquor containing the title compound was evaporated *in vacuo* and used into the next step without further purification for derivatives **23b** and

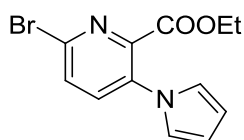
24b. In the case of compounds **(23-24)a**, the residue was purified by silica gel column chromatography, eluting by DCM:Ethyl Acetate, 98:2 (**23a**) or Petroleum Ether:Ethyl Acetate, 9:1 (**24a**) to give desired derivative.

Ethyl 3-(1H-pyrrol-1-yl)pyridine-2-carboxylate (23a):



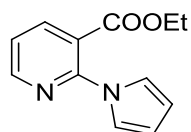
Yield: 92%, light yellow oil; IR: 1696 (CO) cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 8.47 (1H, dd, $J = 4.7, 1.5$ Hz, H-6), 7.58 (1H, dd, $J = 8.2, 1.5$ Hz, H-4), 7.35 (1H, dd, $J = 8.2, 4.7$ Hz, H-5), 6.71 (2H, s, H-2' and H-5'), 6.21 (2H, s, H-3' and H-4'), 4.14 (2H, q, $J = 7.1$ Hz, CH_2), 1.08 (3H, t, $J = 7.1$ Hz, CH_3); ^{13}C NMR (50 MHz, CDCl_3) δ : 165.5 (s), 147.6 (d), 146.0 (s), 136.1 (s), 134.0 (d), 125.9 (d), 121.6 (d x 2), 110.6 (d x 2), 62.1 (t), 13.8 (q). *Anal.* Calculated for $\text{C}_{12}\text{H}_{12}\text{N}_2\text{O}_2$ (MW: 216.24): C, 66.65; H, 5.59; N, 12.96%. Found: C, 66.79; H, 5.30; N, 12.76%.

Ethyl 6-bromo-3-(1H-pyrrol-1-yl)pyridine-2-carboxylate (23b):



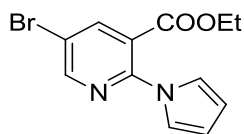
Yield: 93%, light brown solid; mp: 123.5-123.9°C; IR: 1734 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO}-d_6$) δ : 8.80 (1H, d, $J = 2.4$ Hz, H-4), 8.44 (1H, d, $J = 2.4$ Hz, H-5), 7.12 (2H, t, $J = 2.3$ Hz, H-1' and H-4'), 6.29 (2H, t, $J = 2.3$ Hz, H-2' and H-3'), 4.24 (2H, q, $J = 7.1$ Hz, CH_2), 1.18 (3H, t, $J = 7.1$ Hz, CH_3); ^{13}C NMR (50 MHz, $\text{DMSO}-d_6$) δ : 164.6 (s), 151.6 (d), 147.5 (s), 141.7 (d), 121.5 (s), 120.7 (d x 2), 116.3 (s), 110.8 (d x 2), 62.1 (t), 13.7 (q). *Anal.* Calculated for $\text{C}_{12}\text{H}_{11}\text{BrN}_2\text{O}_2$ (MW: 295.13): C, 48.84; H, 3.76; N, 9.49%. Found: C, 48.49; H, 3.85; N, 9.74%.

Ethyl 2-(1H-pyrrol-1-yl)pyridine-3-carboxylate (24a):



Yield: 89%, light yellow oil; IR: 1717 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO-}d_6$) δ : 8.64 (1H, dd, $J = 4.8, 1.5$ Hz, H-6), 8.20 (1H, dd, $J = 7.6, 1.5$ Hz, H-4), 7.48 (1H, dd, $J = 7.6, 4.8$ Hz, H-5), 7.14 (2H, s, H-2' and H-5'), 6.27 (2H, s, H-3' and H-4'), 4.22 (2H, q, $J = 7.0$ Hz, CH_2), 1.16 (3H, t, $J = 7.0$ Hz, CH_3); ^{13}C NMR (50 MHz, $\text{DMSO-}d_6$) δ : 166.0 (s), 151.0 (d), 148.9 (s), 139.9 (d), 121.6 (d), 120.6 (d x 2), 120.3 (s), 110.4 (d x 2), 61.6 (t), 13.7 (q). *Anal.* Calculated for $\text{C}_{12}\text{H}_{12}\text{N}_2\text{O}_2$ (MW: 216.24): C, 66.65; H, 5.59; N, 12.96%. Found: C, 66.51; H, 5.75; N, 13.06%.

Ethyl 5-bromo-2-(1H-pyrrol-1-yl)pyridine-3-carboxylate (24b):



Yield: 89%, brown solid; mp: 95.8-96.2°C; IR: 1689 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO-}d_6$) δ : 8.78 (1H, d, $J = 2.4$ Hz, H-6), 8.43 (1H, d, $J = 2.4$ Hz, H-4), 7.12 (2H, t, $J = 2.1$ Hz, H-2' and H-5'), 6.28 (2H, t, $J = 2.1$ Hz, H-3' and H-4'), 4.24 (2H, q, $J = 7.1$ Hz, CH_2), 1.18 (3H, t, $J = 7.1$ Hz, CH_3); ^{13}C NMR (50 MHz, $\text{DMSO-}d_6$) δ : 164.6 (s), 151.6 (d), 147.7 (s), 141.7 (d), 121.5 (s), 120.7 (d x 2), 116.3 (s), 110.8 (d x 2), 62.0 (t), 13.6 (q). *Anal.* Calculated for $\text{C}_{12}\text{H}_{11}\text{BrN}_2\text{O}_2$ (MW: 295.13): C, 48.84; H, 3.76; N, 9.49%. Found: C, 48.66; H, 3.95; N, 9.60%.

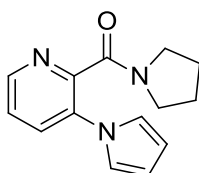
Synthesis of Pyrrolidinecarboxamides (25-26)a,b:

Method A: A solution of (23-24)a,b (1.39 mmol) in pyrrolidine (58.38 mmol, 4.8 mL) was heated to reflux for 36 hours. Upon cooling, the reaction mixture was concentrated under reduced pressure and the yellow oil was crystallized by diethyl ether and purified by silica gel column chromatography using ethyl acetate as eluent. Yield: 38-42%.

Method B: To a solution of **(23-24)a,b** (2.41 mmol) in ethanol (73.2 mL), was added lithium hydroxide (0.29 g, 12.0 mmol) and the reaction mixture was heated to reflux for 4 hours. After cooling to room temperature, the solvent was totally removed under reduced pressure. The crude, cooled by adding to ice, was acidified with 6N HCl and extracted with DCM (3 x 30 mL), dried over anhydrous Na₂SO₄, filtered and evaporated. The crude was taken up onto the next step.

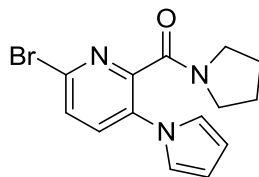
To a solution of the crude in THF (36.5 mL) were added hydroxy-benzotriazole (OHBT) (0.36 g, 2.64 mmol), *N,N*-diisopropylethylamine (DIPEA) (0.68 mL, 2.64 mmol) and *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) (0.34 g, 2.64 mmol) and the resulting reaction mixture was stirred at room temperature for 10 minutes. Pyrrolidine (1.0 mL, 12 mmol) was added and the mixture was stirred at room temperature for 12 hours. The solvent was removed under reduced pressure and to the residue was added an aqueous saturated NaHCO₃ solution (20.4 mL). The crude was extracted with ethyl acetate (3 x 30 mL), dried over anhydrous Na₂SO₄, filtered and evaporated *in vacuo*. It was purified by silica gel column chromatography eluting by DCM:Ethyl Acetate, 75:25 (**25a-b**) or Petroleum Ether:Ethyl Acetate, 6:4 (**26a-b**) to give desired amide. Yield on two steps: 54-88%.

2-(Pyrrolidin-1-yl)-3-(1*H*-pyrrol-1-yl)pyridine (**25a**):



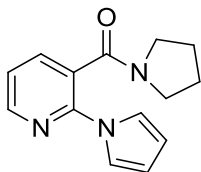
Yield on two steps: 88%, light yellow powder; mp: 117.5-118.0°C; IR: 1623 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO- *d*₆) δ: 8.56 (1H, d, *J* = 4.6 Hz, H-6), 7.99 (1H, d, *J* = 8.2 Hz, H-4), 7.60 (1H, dd, *J* = 8.2, 4.6 Hz, H-5), 7.04 (2H, s, H-2' and H-5'), 6.28 (2H, s, H-3' and H-4'), 3.41-3.34 (2H, m, CH₂), 2.96 (2H, t, *J* = 6.2 Hz, CH₂), 1.74 (4H, quint, *J* = 6.2 Hz, CH₂ x 2); ¹³C NMR (50 MHz, DMSO- *d*₆) δ: 146.6 (s), 148.6 (s), 146.9 (d), 133.2 (s), 132.5 (d), 124.9 (d), 120.9 (d x 2), 110.5 (d x 2), 46.6 (t), 44.9 (t), 25.2 (t), 23.7 (t). *Anal.* Calculated for C₁₄H₁₅N₃O (MW: 241.29): C, 69.69; H, 6.27; N, 17.41%. Found: C, 69.87; H, 6.07; N, 17.29%.

6-Bromo-3-(1*H*-pyrrol-1-yl)pyridin-2-yl](pyrrolidin-1-yl)methanone (25b):



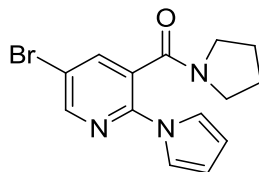
Yield on two steps: 57%, yellow oil; IR: 1640 (CO) cm^{-1} ; ^1H NMR (200 MHz, DMSO- d_6) δ : 8.75 (1H, d, $J = 2.4$ Hz, H-4), 8.27 (1H, d, $J = 2.4$ Hz, H-5), 7.27 (2H, t, $J = 2.2$ Hz, H-1' and H-4'), 6.36 (2H, t, $J = 2.2$ Hz, H-2' and H-3'), 3.05-2.98 (2H, m, CH_2), 2.33-2.27 (2H, m, CH_2), 1.78-1.72 (4H, m, $\text{CH}_2 \times 2$); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 163.5 (s), 149.9 (d), 145.4 (s), 140.2 (d), 125.5 (s), 119.6 (d $\times 2$), 116.3 (s), 111.2 (d $\times 2$), 45.5 (t), 45.0 (t), 25.2 (t), 23.8 (t). *Anal.* Calculated for $\text{C}_{14}\text{H}_{14}\text{BrN}_3\text{O}$ (MW: 320.18): C, 52.52; H, 4.41; N, 13.12%. Found: C, 52.35; H, 4.53; N, 13.19%.

3-(Pyrrolidin-1-yl)-2-(1*H*-pyrrol-1-yl)pyridine (26a):



Yield on two steps: 54%, light yellow oil; IR: 1616 (CO) cm^{-1} ; ^1H NMR (200 MHz, DMSO- d_6) δ : 8.56 (1H, dd, $J = 4.7, 1.5$ Hz, H-6), 7.92 (1H, dd, $J = 7.9, 1.5$ Hz, H-4), 7.42 (1H, dd, $J = 7.9, 4.7$ Hz, H-5), 7.23 (2H, s, H-2' and H-5'), 6.29 (2H, s, H-3' and H-4'), 3.41-3.35 (2H, m, CH_2), 2.88-2.70 (2H, m, CH_2), 1.75-1.67 (4H, m, $\text{CH}_2 \times 2$); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 165.2 (s), 149.4 (d), 146.4 (s), 138.2 (d), 124.2 (s), 121.5 (d), 119.6 (d $\times 2$), 110.8 (d $\times 2$), 46.8 (t), 45.37 (t), 25.2 (t), 23.8 (t). *Anal.* Calculated for $\text{C}_{14}\text{H}_{15}\text{N}_3\text{O}$ (MW: 241.29): C, 69.69; H, 6.27; N, 17.41%. Found: C, 69.53; H, 6.17; N, 17.69%.

5-bromo-2-(1*H*-pyrrol-1-yl)pyridin-3-yl](pyrrolidin-1-yl)methanone (26b):

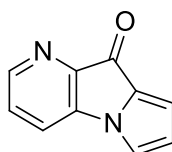


Yield on two steps: 62%, light yellow solid; mp: 119.9-120.2°C; IR: 1620 (CO) cm^{-1} ; ^1H NMR (200 MHz, DMSO- d_6) δ : 8.70 (1H, d, $J = 2.4$ Hz, H-6), 8.23 (1H, d, $J = 2.4$ Hz, H-4), 7.21 (2H, t, $J = 2.3$ Hz, H-2' and H-5'), 6.30 (2H, t, $J = 2.3$ Hz, H-3' and H-4'), 3.41 (2H, t, $J = 6.4$ Hz, CH_2), 2.94-2.85 (2H, m, CH_2), 1.72 (4H, quint, $J = 6.4$ Hz, $\text{CH}_2 \times 2$); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 163.5 (s), 149.9 (d), 145.4 (s), 140.2 (d), 125.5 (s), 119.6 (d $\times 2$), 116.3 (s), 111.2 (d $\times 2$), 46.9 (t), 45.5 (t), 25.2 (t), 23.8 (t). *Anal.* Calculated for $\text{C}_{14}\text{H}_{14}\text{BrN}_3\text{O}$ (MW: 320.18): C, 52.52; H, 4.41; N, 13.12%. Found: C, 52.71; H, 4.64; N, 13.32%.

Synthesis of -9*H*-pyrido[2,3-*b*]pyrrolizin-9-ones (14)a,i:

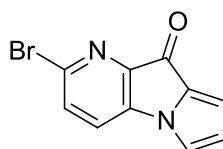
A solution of appropriate pyrrolidinecarboxamides (**25**)**a,b** (0.95 mmol) in phosphorus oxychloride (2.4 mL, 25.65 mmol) was stirred at 70°C for 6-24 hours. After cooling, the reaction mixture was concentrated to give iminium salt as black solid. A 10% aqueous NaOH solution (6.65 mL) was slowly added to the residue and the reaction mixture was stirred at room temperature for 30 minutes (**14i**) or heated to 65°C for 30 minutes (**14a**). Upon cooling, the dark crude was extracted with ethyl acetate (3 x 15 mL), dried over anhydrous Na_2SO_4 , filtered and evaporated *in vacuo*. It was purified by silica gel column chromatography eluting by DCM:Ethyl Acetate, 95:5 (**14a**) or Cyclohexane:Ethyl Acetate, 90:10 (**14i**) to give desired tripentone.

9*H*-Pyrido[2,3-*b*]pyrrolizin-9-one (14a):



Conditions: 70°C for 6 hours. Yield: 50%, yellow solid; mp: 187.4-188.4°C; IR: 1722 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO- *d*₆) δ: 8.39 (1H, d, *J* = 4.5 Hz, H-2), 7.98 (1H, d, *J* = 7.9 Hz, H-4), 7.73 (1H, m, H-6), 7.52 (1H, dd, *J* = 7.9, 4.6 Hz, H-3), 6.96 (1H, d, *J* = 2.5 Hz, H-8), 6.44 (1H, m, H-7); ¹³C NMR (50 MHz, DMSO- *d*₆) δ: 177.7 (s), 147.9 (s), 146.3 (d), 139.9 (s), 130.2 (s), 127.4 (d), 122.9 (d), 119.0 (d), 116.2 (d), 115.4 (d). *Anal.* Calculated for C₁₀H₆N₂O (MW: 170.17): C, 70.58; H, 3.55; N, 16.46%. Found: C, 70.78; H, 3.35; N, 16.16%.

2-Bromo-9H-pyrido[2,3-*b*]pyrrolizin-9-one (14i):



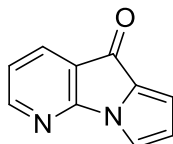
Conditions: 70°C for 24 hours. Yield: 40%, yellow powder; mp: 161.9-162.4°C; IR: 1700 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO- *d*₆) δ: 8.56 (1H, d, *J* = 2.2 Hz, H-4), 8.17 (1H, d, *J* = 2.2 Hz, H-3), 7.66 (1H, dd, *J* = 2.6, 0.7 Hz, H-6), 7.01 (1H, dd, *J* = 3.7, 0.7 Hz, H-8), 6.53 (1H, dd, *J* = 3.7, 2.6 Hz, H-7); ¹³C NMR (50 MHz, DMSO- *d*₆) δ: 175.0 (s), 154.8 (s), 152.2 (d), 135.1 (d), 132.0 (s), 125.1 (s), 121.1 (d), 117.8 (d), 116.5 (s), 115.9 (d). *Anal.* Calculated for C₁₀H₅BrN₂O (MW: 249.06): C, 48.22; H, 2.02; N, 11.25%. Found: C, 47.82; H, 2.34; N, 11.55%.

Synthesis of -5H-pyrido[3,2-*b*]pyrrolizin-5-ones (16)a,i:

A solution of suitable pyrrolidinecarboxamides (**26**)**a,b** (0.51 mmol) in phosphorus oxychloride (1.3 mL, 13.78 mmol) was stirred at 70°C for 18 hours. After cooling, the reaction mixture was concentrated to give iminium salt as black solid. A 10% aqueous NaOH solution (3.6 mL) was slowly added to the residue and the reaction mixture was stirred at room temperature for 30 min. Upon cooling, the dark crude was extracted with Ethyl Acetate (3 x 15 mL), dried over anhydrous Na₂SO₄, filtered and evaporated *in vacuo*. It was purified by silica gel column chromatography eluting by Petroleum

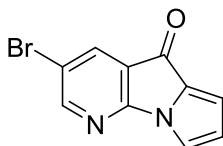
Ether:Ethyl Acetate, 85:15 (**16a**), or Cyclohexane:Ethyl Acetate, 99:1 (**16i**) to give desired triptentone.

5H-Pyrido[3,2-*b*]pyrrolizin-5-one (16a**)⁴⁸:**



Yield: 54%, yellow solid; mp: 132.0-132.3°C; IR: 1696 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO- *d*₆) δ: 8.40 (1H, dd, *J* = 5.2, 1.3 Hz, H-2), 7.95 (1H, dd, *J* = 7.3, 1.3 Hz, H-4), 7.63 (1H, dd, *J* = 2.8, 1.0 Hz, H-8), 7.25 (1H, dd, *J* = 7.3, 5.2 Hz, H-3), 6.96 (1H, dd, *J* = 3.8, 1.0 Hz, H-6), 6.48 (1H, dd, *J* = 3.8, 2.8 Hz, H-7); ¹³C NMR (50 MHz, DMSO- *d*₆) δ: 176.5 (s), 156.2 (d), 152.2 (s), 132.8 (d), 131.7 (s), 123.2 (s), 121.4 (d), 120.6 (d), 117.3 (d), 115.3 (d). *Anal.* Calculated for C₁₀H₆N₂O (MW: 170.17): C, 70.58; H, 3.55; N, 16.46%. Found: C, 70.36; H, 3.66; N, 16.35%.

3-Bromo-5H-pyrido[3,2-*b*]pyrrolizin-5-one (16i**):**

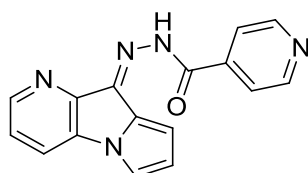


Yield: 61%, yellow solid; mp: 161.2-161.5°C; IR: 1700 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO- *d*₆) δ: 8.55 (1H, d, *J* = 2.2 Hz, H-2), 8.17 (1H, d, *J* = 2.2 Hz, H-4), 7.66 (1H, dd, *J* = 2.6, 0.7 Hz, H-8), 7.01 (1H, dd, *J* = 3.7, 0.7 Hz, H-6), 6.51 (1H, dd, *J* = 3.7, 2.6 Hz, H-7); ¹³C NMR (50 MHz, DMSO- *d*₆) δ: 175.0 (s), 154.8 (s), 152.2 (d), 153.1 (d), 132.0 (s), 125.1(s), 121.1 (d), 117.8 (d), 116.5 (s), 115.9 (d). *Anal.* Calculated for C₁₀H₅BrN₂O (MW: 249.06): C, 48.22; H, 2.02; N, 11.25%. Found: C, 47.99; H, 2.42; N, 11.16%.

Synthesis of substituted -9H-pyrido[2,3-b]pyrrolizin-9-ylidenes (14b,d,e,h):

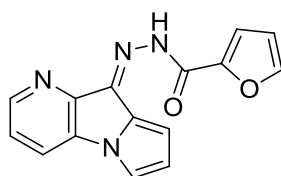
To a solution of 9H-pyrido[2,3-b]pyrrolizin-9-one **14a** (0.05 g, 0.29 mmol) in toluene (4 mL) was added opportune heteroaryl carbohydrazide **27b,d,e,h** (0.29 mmol). The resulting suspension was refluxed, using Dean-Stark apparatus, for 24-32 hours and then chilled overnight. The product was collected by filtration, washed with toluene and dried under vacuum to afford compound **14e**. In the case of derivatives **14b,d,h** the reaction mixture was quenched with a small amount of water, then extracted with DCM (3 x 10 mL), dried over anhydrous Na₂SO₄, filtered and evaporated *in vacuo*. It was purified by silica gel column chromatography eluting by DCM:MeOH, 97:3 (**14b**), DCM (**14d**) and DCM:Ethyl Acetate, 90:10 (**14h**) to give desired compound.

N'-[9H-Pyrido[2,3-b]pyrrolizin-9-ylidene]pyridine-4-carbohydrazide (**14b**):



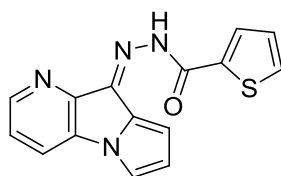
Conditions: reflux for 24 hours. Yield: 49%, light brown powder; mp: 244.0-244.4 °C; IR: 3402 (NH), 1683 (CO) cm⁻¹; ¹H NMR (200 MHz, CDCl₃) δ: 14.15 (1H, bs, NH), 8.85 (2H, dd, *J* = 4.4, 1.8 Hz, H-2' and H-6'), 8.38 (1H, dd, *J* = 5.0, 1.8 Hz, H-2), 7.88 (2H, dd, *J* = 4.4, 1.8 Hz, H-3' and H-5'), 7.57 (1H, dd, *J* = 7.9, 1.8 Hz, H-3), 7.38 (1H, dd, *J* = 7.9, 5.0 Hz, H-4), 7.10 (1H, d, *J* = 3.0 Hz, H-6), 6.89 (1H, d, *J* = 3.7 Hz, H-8), 6.41 (1H, t, *J* = 3.7 Hz, H-7); ¹³C NMR (50 MHz, CDCl₃) δ: 162.0 (s), 150.8 (d x 2), 147.5 (s), 143.5 (d), 140.1 (s), 139.7 (s), 136.2 (s), 130.6 (s), 125.1 (d), 121.3 (d x 2), 117.5 (d), 116.3 (d), 115.9 (d), 109.8 (d). *Anal.* Calculated for C₁₆H₁₁N₅O (MW: 289.29): C, 66.43; H, 3.83; N, 24.21%. Found: C, 66.21; H, 3.68; N, 24.36%.

N'-[9H-pyrido[2,3-b]pyrrolizin-9-ylidene]furan-2-carbohydrazide (**14d**):



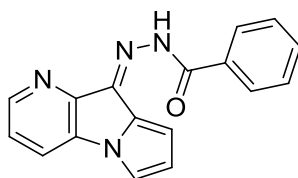
Conditions: reflux for 32 hours. Yield: 62%, yellow oil; IR: 3317 (NH), 1680 (CO) cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 13.95 (1H, bs, NH), 8.40 (1H, dd, $J = 3.4, 0.8$ Hz, H-2), 7.63 (1H, d, $J = 0.6$ Hz, H-4), 7.55 (1H, dd, $J = 8.0, 1.3$ Hz, H-4'), 7.36 (2H, t, $J = 8.0$ Hz, H-3' and H-5'), 7.08 (1H, dd, $J = 3.2, 0.6$ Hz, H-3), 6.85 (1H, d, $J = 3.1$ Hz, H-6), 6.60 (1H, d, $J = 3.6$ Hz, H-8), 6.39 (1H, t, $J = 3.6$ Hz, H-7); ^{13}C NMR (50 MHz, CDCl_3) δ : 155.2 (s), 147.4 (s), 147.1 (s), 145.0 (d), 143.5 (d), 138.6 (s), 135.9 (s), 130.9 (s), 124.6 (d), 117.1 (d), 116.1 (d), 115.9 (d), 115.4 (d), 112.3 (d), 109.0 (d). *Anal.* Calculated for $\text{C}_{15}\text{H}_{10}\text{N}_4\text{O}_2$ (MW: 278.27): C, 64.74; H, 3.62; N, 20.13%. Found: C, 64.89; H, 3.58; N, 20.07%.

***N'*-[9*H*-pyrido[2,3-*b*]pyrrolizin-9-ylidene]thiophene-2-carbohydrazide (14e):**



Conditions: reflux for 24 hours. Yield: 50%, dark yellow powder; mp: 163.0-163.7°C; IR: 3428 (NH), 1656 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO-}d_6$) δ : 13.93 (1H, bs, NH), 8.50 (1H, d, $J = 5.1, 1.2$ Hz, H-2), 8.12 (1H, dd, $J = 8.1, 1.2$ Hz, H-4), 8.00 (1H, dd, $J = 5.1, 1.2$ Hz, H-3), 7.90-7.84 (1H, m, H-4'), 7.64-7.58 (2H, m, H-3' and H-5'), 7.30 (1H, t, $J = 3.8$ Hz, H-6), 6.75 (1H, dd, $J = 3.6, 0.8$ Hz, H-8), 6.44 (1H, t, $J = 3.6$ Hz, H-7); ^{13}C NMR (50 MHz, $\text{DMSO-}d_6$) δ : 164.7 (s), 146.1 (s), 143.9 (d), 137.2 (s), 135.7 (s), 132.8 (s), 129.6 (d), 129.0 (d), 128.2 (d), 125.8 (d), 125.3 (s), 119.1 (d), 117.7 (d), 115.4 (d), 107.5 (d). *Anal.* Calculated for $\text{C}_{15}\text{H}_{10}\text{N}_4\text{OS}$ (MW: 294.33): C, 61.21; H, 3.42; N, 19.04%. Found: C, 61.35; H, 3.28; N, 19.14%.

***N'*-[9*H*-pyrido[2,3-*b*]pyrrolizin-9-ylidene]benzohydrazide (14h):**



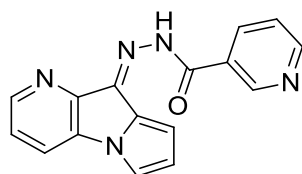
Conditions: reflux for 27 hours. Yield: 56%, yellow powder; mp: 177.2-178.0°C; IR: 3393 (NH), 1681 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO-}d_6$) δ : 13.90 (1H, bs, NH),

8.50 (1H, dd, $J = 4.8, 0.8$ Hz, H-2), 8.11 (1H, dd, $J = 8.2, 0.8$ Hz, H-4), 7.98 (2H, dd, $J = 6.2, 1.4$ Hz, H-2' and H-6'), 7.70-7.53 (5H, m, H-3', H-4', H-5', H-3 and H-6), 6.74 (1H, d, $J = 3.3$ Hz, H-8), 6.44 (1H, t, $J = 3.1$ Hz, H-7); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 162.3 (s), 146.2 (s), 143.9 (d), 138.3 (s), 135.7 (s), 133.2 (s), 132.6 (d), 129.7 (s), 129.1 (d x 2), 127.2 (d), 125.7 (d x 2), 119.1 (d), 117.7 (d), 115.4 (d), 107.7 (d). *Anal.* Calculated for $\text{C}_{17}\text{H}_{12}\text{N}_4\text{O}$ (MW: 288.30): C, 70.82; H, 4.20; N, 19.43%. Found: C, 70.75; H, 4.31; N, 19.29%.

Synthesis of substituted -9H-pyrido[2,3-b]pyrrolizin-9-ylidenes (14c,f,g):

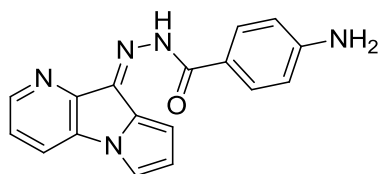
To a solution of 9H-pyrido[2,3-b]pyrrolizin-9-one **14a** (0.065 g, 0.38 mmol) in anhydrous ethanol (5 mL) was added opportune heteroaryl carbohydrazide **27c,f,g** (0.38 mmol). The resulting solution was heated to reflux for 24 hours and then chilled overnight. The product was collected by filtration, washed with cold ethanol and dried under vacuum to afford desired compound **14f-g**, or purified by silica gel column chromatography using DCM:Ethyl Acetate, 75:25 as eluent to give products **14c**.

N'-[9H-pyrido[2,3-b]pyrrolizin-9-ylidene]pyridine-3-carbohydrazide (14c):



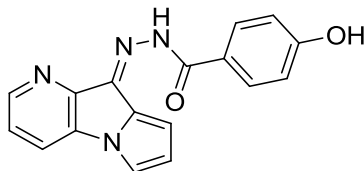
Yield: 88%, yellow powder; mp: 182.5-182.7°C; IR: 3385 (NH), 1684 (CO) cm^{-1} ; ^1H NMR (200 MHz, DMSO- d_6) δ : 13.92 (1H, bs, NH), 9.13 (1H, s, H-2'), 8.85 (1H, d, $J = 4.1$ Hz, H-6'), 8.49 (1H, dd, $J = 5.0, 1.1$ Hz, H-2), 8.32 (1H, d, $J = 8.0$ Hz, H-4), 8.11 (1H, dd, $J = 8.0, 1.2$ Hz, H-3), 7.70-7.57 (3H, m, H-4', H-5' and H-6), 6.77 (1H, d, $J = 3.2$ Hz, H-8), 6.44 (1H, t, $J = 3.1$ Hz, H-7); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 161.0 (s), 148.8 (d), 146.4 (s), 145.5 (s), 144.0 (d), 135.8 (s), 129.6 (s), 128.5 (s), 125.9 (d), 123.9 (d), 119.2 (d), 118.0 (d), 117.9 (d), 115.5 (d), 115.3 (d), 108.2 (d). *Anal.* Calculated for $\text{C}_{16}\text{H}_{11}\text{N}_5\text{O}$ (MW: 289.29): C, 66.43; H, 3.83; N, 24.21%. Found: C, 66.28; H, 3.76; N, 24.11%.

4-Amino-*N'*-[9*H*-pyrido[2,3-*b*]pyrrolizin-9-ylidene]benzohydrazide (14f):



Yield: 40%, yellow powder; mp: 276.1-277.0°C; IR: 3447 – 3337 (NH₂), 3227 (NH), 1662 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 13.73 (1H, bs, NH), 8.56 (1H, dd, *J* = 5.0, 1.0 Hz, H-2), 8.15 (1H, dd, *J* = 8.1, 1.0 Hz, H-4), 7.74 (2H, d, *J* = 8.6 Hz, H-2' and H-6'), 7.64 (2H, m, H-6 and H-8), 6.72 (3H, m, H-3', H-5' and H-3), 6.48 (1H, t, *J* = 3.4 Hz, H-7), 6.07 (2H, bs, NH₂); ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 162.3 (s), 152.9 (s), 146.2 (s), 143.9 (d), 136.5 (s), 135.4 (s), 130.1 (s), 129.1 (d), 125.3 (d), 118.9 (d), 118.5 (s), 117.0 (d x 2), 115.3 (d), 113.1 (d x 2), 106.7 (d). *Anal.* Calculated for C₁₇H₁₃N₅O (MW: 303.32): C, 67.32; H, 4.32; N, 23.09%. Found: C, 67.45; H, 4.15; N, 23.31%.

4-Hydroxy-*N'*-[9*H*-pyrido[2,3-*b*]pyrrolizin-9-ylidene]benzohydrazide (14g):



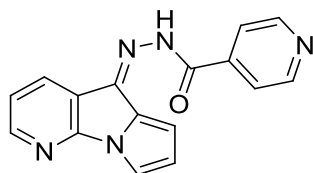
Yield: 80%, light yellow solid; mp: 294.5-295.3 °C; IR: 3415 (NH), 3201 (OH), 1675 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 13.78 (1H, bs, NH), 10.35 (1H, bs, OH), 8.51 (1H, dd, *J* = 5.0, 1.3 Hz, H-2), 8.08 (1H, dd, *J* = 8.1, 1.3 Hz, H-3), 7.85 (2H, d, *J* = 8.8 Hz, H-2' and H-6'), 7.60 (2H, m, H-4 and H-6), 6.95 (2H, d, *J* = 8.8 Hz, H-3' and H-5'), 6.70 (1H, d, *J* = 3.7 Hz, H-8), 6.43 (1H, t, *J* = 3.7 Hz, H-7); ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 162.0 (s), 161.3 (s), 146.2 (s), 143.9 (d), 137.4 (s), 135.5 (s), 129.9 (s), 129.4 (d), 125.5 (d x 2), 123.0 (s), 118.9 (d), 117.3 (d), 115.7 (d x 2), 115.3 (d), 107.2 (d). *Anal.* Calculated for C₁₇H₁₂N₄O₂ (MW: 304.30): C, 67.10; H, 3.97; N, 18.41%. Found: C, 67.43; H, 3.85; N, 18.08%.

Synthesis of substituted -5H-pyrido[3,2-*b*]pyrrolizin-5-ylidenes (16b,d,e,h) :

Method A: To a solution of 5H-pyrido[3,2-*b*]pyrrolizin-5-one **16a** (0.06 g, 0.35 mmol) in a mixture of ethanol:toluene (1:3, 5 mL) was added isonicotinic hydrazide **27b** (0.048 g, 0.35 mmol). The resulting solution was refluxed for 36 hours and then chilled overnight. The reaction mixture was quenched with a small amount of water, then extracted with ethyl acetate (3 x 10 mL), dried over anhydrous Na₂SO₄, filtered and evaporated *in vacuo*. It was purified by silica gel column chromatography eluting by DCM:MeOH, 9:1 to give desired compound **16b**. Yield: 25%.

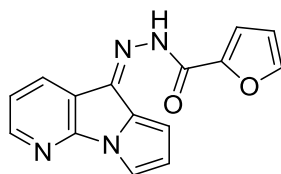
Method B: To a solution of 5H-pyrido[3,2-*b*]pyrrolizin-5-one **16a** (0.06 g, 0.35 mmol) in toluene (4 mL) was added opportune heteroaryl carbohydrazide **27b,d,e,h** (0.35 mmol). The resulting suspension was refluxed, using Dean-Stark apparatus, for 24-30 hours and then chilled overnight. The reaction mixture was quenched with a small amount of water, then extracted with ethyl acetate (3 x 10 mL), dried over anhydrous Na₂SO₄, filtered and evaporated *in vacuo*. It was purified by silica gel column chromatography eluting by Ethyl Acetate (**16b**), DCM:Ethyl Acetate, 9:1 (**16d**), DCM:Ethyl Acetate, 95:5 (**16e**) and DCM:Ethyl Acetate, 80:20 (**16h**) to give desired compound. Yield: 49-87%.

***N'*-[5H-Pyrido[3,2-*b*]pyrrolizin-5-ylidene]pyridine-4-carbohydrazide (16b):**



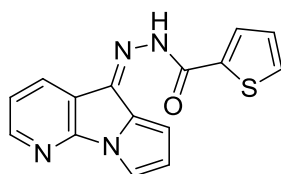
Conditions: reflux for 24 hours. Yield: 51%, light brown powder; mp: 236.3-236.8 °C; IR: 3353 (NH), 1702 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 11.50 (1H, bs, NH), 8.81 (2H, d, *J* = 5.4 Hz, H-2' and H-6'), 8.40 (1H, dd, *J* = 5.1, 1.4 Hz, H-2), 8.13 (1H, dd, *J* = 7.4, 1.4 Hz, H-4), 7.88 (2H, d, *J* = 5.4 Hz, H-3' and H-5'), 7.64 (1H, d, *J* = 2.6 Hz, H-6), 7.30 (1H, dd, *J* = 7.4, 5.1 Hz, H-3), 7.08 (1H, d, *J* = 2.6 Hz, H-8), 6.57 (1H, t, *J* = 2.6 Hz, H-7); ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 163.4 (s), 152.6 (s), 150.3 (d), 149.5 (d), 142.9 (s), 140.4 (s), 131.1 (d), 126.4 (s), 123.6 (s), 121.9 (d), 120.6 (d x 2), 116.4 (d x 2), 115.8 (d), 114.9 (d). *Anal.* Calculated for C₁₆H₁₁N₅O (MW: 289.29): C, 66.43; H, 3.83; N, 24.21%. Found: C, 66.03; H, 4.03; N, 24.09%.

***N'*-[5*H*-Pyrido[3,2-*b*]pyrrolizin-5-ylidene]furan-2-carbohydrazide (16d):**



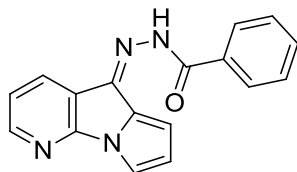
Conditions: reflux for 30 hours. Yield: 87%, dark yellow powder; mp: 247.2-247.8°C; IR: 3327 (NH), 1685 (CO) cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 9.71 (1H, bs, NH), 8.31 (1H, dd, $J = 5.2, 1.6$ Hz, H-2), 8.22 (1H, d, $J = 7.6$ Hz, H-4), 7.63 (1H, d, $J = 3.2$ Hz, H-3'), 7.48 (1H, dd, $J = 3.4, 1.0$ Hz, H-4'), 7.43 (1H, d, $J = 1.2$ Hz, H-5'), 7.14 (1H, dd, $J = 7.6, 5.2$ Hz, H-3), 6.87 (1H, d, $J = 3.6$ Hz, H-6), 6.64 (1H, dd, $J = 3.6, 1.8$ Hz, H-8), 6.57 (1H, dd, $J = 3.6, 2.9$ Hz, H-7); ^{13}C NMR (50 MHz, CDCl_3) δ : 154.3 (s), 153.1 (s), 149.2 (d), 146.6 (d), 145.0 (s), 139.3 (s), 131.5 (d), 126.0 (s), 123.9 (s), 120.3 (d), 117.5 (d), 116.4 (d), 116.3 (d), 112.9 (d), 111.8 (d). *Anal.* Calculated for $\text{C}_{15}\text{H}_{10}\text{N}_4\text{O}_2$ (MW: 278.27): C, 64.74; H, 3.62; N, 20.13%. Found: C, 64.59; H, 3.55; N, 20.24%.

***N'*-[5*H*-Pyrido[3,2-*b*]pyrrolizin-5-ylidene]thiophene-2-carbohydrazide (16e):**



Conditions: reflux for 24 hours. Yield: 49%, light yellow solid; mp: 231.7-232.2°C; IR: 3232 (NH), 1708 (CO) cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 8.96 (1H, bs, NH), 8.33 (2H, dd, $J = 5.2, 1.4$ Hz, H-2 and H-8), 8.16 (1H, d, $J = 7.4$ Hz, H-4), 7.63 (1H, dd, $J = 7.4, 1.2$ Hz, H-3), 7.49 (1H, d, $J = 2.6$ Hz, H-4'), 7.20-7.14 (m, 2H, H-3' and H-5'), 6.85 (1H, dd, $J = 3.4, 1.4$ Hz, H-6), 6.55 (1H, t, $J = 3.4$ Hz, H-7); ^{13}C NMR (50 MHz, CDCl_3) δ : 152.7 (s), 149.0 (d), 146.8 (d), 141.4 (d), 131.0 (s), 129.7 (d), 120.1 (s), 118.8 (s), 116.5 (s), 116.2 (d), 112.9 (d), 112.4 (d), 111.6 (d), 105.5 (d), 103.6 (s). *Anal.* Calculated for $\text{C}_{15}\text{H}_{10}\text{N}_4\text{OS}$ (MW: 294.33): C, 61.21; H, 3.42; N, 19.04%. Found: C, 60.91; H, 3.57; N, 19.19%.

***N'*-[5*H*-Pyrido[3,2-*b*]pyrrolizin-5-ylidene]benzohydrazide (16h):**

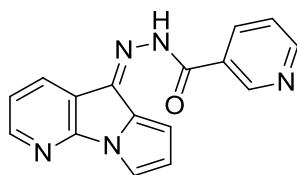


Conditions: reflux for 24 hours. Yield: 71%, light orange powder; mp: 239.6-240.2°C; IR: 3385 (NH), 1685 (CO) cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 9.36 (1H, bs, NH), 8.31 (1H, dd, $J = 5.2, 1.6$ Hz, H-2), 8.21 (1H, d, $J = 7.1$ Hz, H-4), 7.95 (2H, dd, $J = 6.4, 2.2$ Hz, H-6 and H-8), 7.63-7.48 (4H, m, H-2', H-3', H-5' and H-6'), 7.15 (1H, dd, $J = 7.5, 5.2$ Hz, H-3), 6.72 (1H, s, H-4'), 6.55 (1H, t, $J = 2.8$ Hz, H-7); ^{13}C NMR (50 MHz, CDCl_3) δ : 163.8 (s), 153.1 (s), 149.1 (d), 132.9 (s), 132.5 (d), 131.4 (d), 129.0 (d), 127.5 (d), 125.9 (s), 124.0 (s), 120.3 (d), 116.3 (d x 2), 116.2 (d), 115.7 (s), 111.6 (d x 2). *Anal.* Calculated for $\text{C}_{17}\text{H}_{12}\text{N}_4\text{O}$ (MW: 288.30): C, 70.82; H, 4.20; N, 19.43%. Found: C, 70.57; H, 4.35; N, 19.23%.

Synthesis of substituted -5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidenes (16c,f,g):

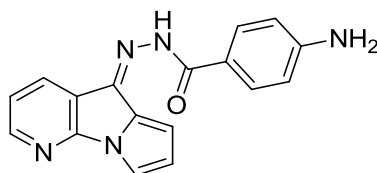
To a solution of 5*H*-pyrido[3,2-*b*]pyrrolizin-5-one **16a** (0.07 g, 0.41 mmol) in anhydrous ethanol (6 mL) was added opportune heteroaryl carbohydrazide **27c,f,g** (0.41 mmol). The resulting solution was heated to reflux for 24 hours and then chilled overnight. The precipitate formed was filtered, washed with cold ethanol and dried under vacuum to give derivative **16g**, or purified by silica gel column chromatography using DCM:MeOH, 99:1 as eluent to give products **16c,f**.

***N'*-[5*H*-Pyrido[3,2-*b*]pyrrolizin-5-ylidene]pyridine-3-carbohydrazide (16c):**



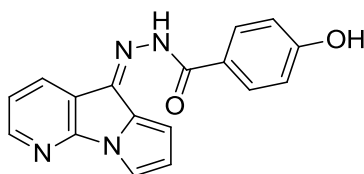
Yield: 60%, dark yellow powder; mp: 202.5-203.2°C; IR: 3379 (NH), 1674 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO-}d_6$) δ : 11.43 (1H, br s, NH), 9.11 (1H, s, H-2'), 8.80 (1H, dd, $J = 5.2, 1.6$ Hz, H-2), 8.37 (2H, dt, $J = 5.2, 3.2$ Hz, H-3 and H-7), 8.14-8.11 (1H, m, H-5'), 7.64-7.57 (2H, m, H-4' and H-6'), 7.28 (1H, dd, $J = 7.4, 5.2$ Hz, H-4), 7.13 (1H, s, H-8), 6.57 (1H, t, $J = 3.2$ Hz, H-6); ^{13}C NMR (50 MHz, $\text{DMSO-}d_6$) δ : 163.4 (s), 159.4 (s), 152.6 (s), 152.2 (d), 149.4 (d), 148.9 (d), 136.0 (d), 130.9 (d), 129.1 (s), 126.3 (s), 123.7 (s), 123.6 (d), 120.5 (d), 116.4 (d), 116.2 (d), 114.7 (d). *Anal.* Calculated for $\text{C}_{16}\text{H}_{11}\text{N}_5\text{O}$ (MW: 289.29): C, 66.43; H, 3.83; N, 24.21%. Found: C, 66.65; H, 3.68; N, 23.99%.

4-Amino-*N'*-[5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidene]benzohydrazide (16f):



Yield: 45%, yellow powder; mp: 265.4-266.3°C; IR: 3603 – 3559 (NH₂), 3359 (NH), 1725 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO-}d_6$) δ : 10.67 (1H, bs, NH), 8.36 (1H, dd, $J = 5.1, 1.4$ Hz, H-2), 8.12 (1H, dd, $J = 7.5, 1.4$ Hz, H-3), 7.73 (2H, d, $J = 8.6$ Hz, H-2' and H-6'), 7.60 (1H, d, $J = 2.8$ Hz, H-6), 7.30 (1H, dd, $J = 7.5, 5.1$ Hz, H-4), 6.99 (1H, d, $J = 3.4$ Hz, H-8), 6.65 (2H, d, $J = 8.6$ Hz, H-3' and H-5'), 6.57 (1H, t, $J = 3.4$ Hz, H-7), 5.91 (2H, bs, NH₂); ^{13}C NMR (50 MHz, $\text{DMSO-}d_6$) δ : 164.1 (s), 152.7 (s), 152.3 (s), 148.8 (d), 138.9 (s), 130.6 (d), 129.9 (d x 2), 126.5 (s), 123.9 (s), 120.4 (d), 118.9 (s), 116.3 (d), 115.5 (d), 113.3 (d), 112.7 (d x 2). *Anal.* Calculated for $\text{C}_{17}\text{H}_{13}\text{N}_5\text{O}$ (MW: 303.32): C, 67.32; H, 4.32; N, 23.09%. Found: C, 67.0; H, 4.15; N, 23.41%.

4-Hydroxy-*N'*-[5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidene]benzohydrazide (16g):



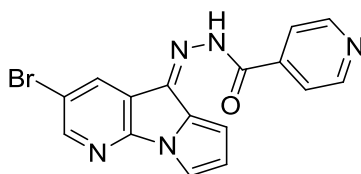
Yield: 85%, dark yellow powder; mp: 316.9-317.8°C; IR: 3390 (NH), 3198 (OH), 1675 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO-}d_6$) δ : 10.94 (1H, bs, NH), 10.24 (1H, bs, OH), 8.38 (1H, dd, $J = 5.2, 1.5$ Hz, H-2), 8.13 (1H, dd, $J = 7.6, 1.5$ Hz, H-4), 7.87 (2H, d, $J =$

8.7 Hz, H-2' and H-6'), 7.61 (1H, dd, $J = 2.8, 0.8$ Hz, H-8), 7.29 (1H, dd, $J = 7.6, 5.2$ Hz, H-3), 7.01 (1H, d, $J = 3.4$ Hz, H-6), 6.90 (2H, d, $J = 8.7$ Hz, H-3' and H-5'), 6.57 (1H, td, $J = 3.4, 0.8$ Hz, H-7); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 164.1 (s), 161.0 (s), 152.4 (s), 149.06 (d), 140.1 (s), 130.8 (d), 130.2 (d x 2), 126.5 (s), 123.8 (s), 123.5 (s), 120.5 (d), 116.3 (d), 115.7 (d), 115.1 (d x 2), 113.8 (d). *Anal.* Calculated for $\text{C}_{17}\text{H}_{12}\text{N}_4\text{O}_2$ (MW: 304.30): C, 67.10; H, 3.97; N, 18.41%. Found: C, 66.8; H, 3.67; N, 18.81%.

Synthesis of substituted -3-bromo-5H-pyrido[3,2-b]pyrrolizin-5-ylidenes (16j,l,m,p):

To a solution of 3-bromo-5H-pyrido[3,2-b]pyrrolizin-5-one **16i** (0.06 g, 0.24 mmol) in toluene (4 mL) was added opportune heteroaryl carbohydrazide **27b,d,e,h** (0.24 mmol). The resulting suspension was refluxed, using Dean-Stark apparatus, for 24-48 hours and then chilled overnight. The product was collected by filtration, washed with toluene and dried under vacuum to afford compound **16m**. In the case of derivatives **16j,l,p** the reaction mixture was quenched with a small amount of water, then extracted with ethyl acetate (3 x 10mL), dried over anhydrous Na_2SO_4 , filtered and evaporated *in vacuo*. The crude was purified by silica gel column chromatography eluting by DCM:Ethyl Acetate, 60:40 (**16j**), Cyclohexane:Ethyl Acetate, 80:20 (**16l**) and Petroleum Ether:Ethyl Acetate, 80:20 (**16p**) to give desired compound.

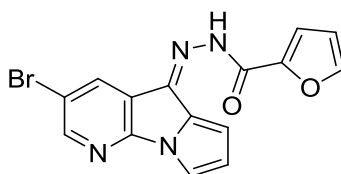
***N'*-[3-Bromo-5H-pyrido[3,2-b]pyrrolizin-5-ylidene]pyridine-4-carbohydrazide (**16j**):**



Conditions: reflux for 24 hours. Yield: 40%, yellow powder; mp: 250.0-250.6°C; IR: 3371 (NH), 1689 (CO) cm^{-1} ; ^1H NMR (200 MHz, DMSO- d_6) δ : 11.58 (1H, bs, NH), 8.82 (2H, d, $J = 5.0$ Hz, H-2' and H-6'), 8.53 (1H, d, $J = 1.8$ Hz, H-8), 8.27 (1H, s, H-

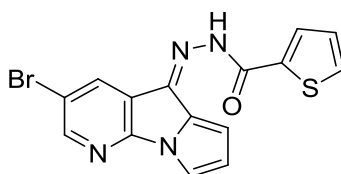
2), 7.88 (2H, d, $J = 5.0$ Hz, H-3' and H-5'), 7.64 (1H, d, $J = 2.8$ Hz, H-6), 7.13 (1H, s, H-4), 6.61 (1H, t, $J = 3.0$ Hz, H-7); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 165.1 (s), 151.3 (s), 150.3 (d), 149.8 (s), 149.6 (d), 140.4 (s), 133.1 (d), 126.8 (s), 125.7 (s), 122.0 (d), 121.5 (d), 116.9 (d x 2), 116.6 (d x 2), 115.5 (s). *Anal.* Calculated for $\text{C}_{16}\text{H}_{10}\text{BrN}_5\text{O}$ (MW:368.19): C, 52.19; H, 2.74; N, 19.02%. Found: C, 52.29; H, 3.04; N, 18.72%.

***N'*-[3-bromo-5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidene]furan-2-carbohydrazide (16l):**



Conditions: reflux for 27 hours. Yield: 45%, dark yellow solid; mp: 250.1-250.7°C; IR: 3372 (NH), 1681 (CO) cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 9.71 (1H, bs, NH), 8.36 (1H, s, H-2), 8.10 (1H, d, $J = 3.6$ Hz, H-6), 7.63 (1H, s, H-4), 7.48 (2H, dd, $J = 2.8, 0.6$ Hz, H-3' and H-5'), 6.88 (1H, d, $J = 3.4$ Hz, H-8), 6.66 (1H, dd, $J = 3.6, 1.8$ Hz, H-7), 6.58 (1H, dd, $J = 3.6, 2.8$ Hz, H-4'); ^{13}C NMR (50 MHz, CDCl_3) δ : 154.2 (s), 153.1 (s), 149.1 (d), 146.6 (s), 145.0 (d), 139.2 (s), 131.6 (d), 126.0 (s), 123.8 (s), 120.2 (d), 117.1 (d), 116.4 (d), 116.3 (s), 112.9 (d), 111.8 (d). *Anal.* Calculated for $\text{C}_{15}\text{H}_9\text{BrN}_4\text{O}$ (MW: 357.16): C, 50.44; H, 2.54; N, 15.69%. Found: C, 50.14; H, 2.69; N, 16.09%.

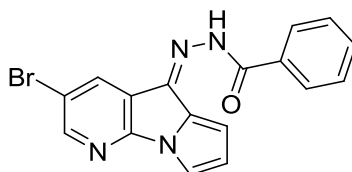
***N'*-[3-Bromo-5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidene]thiophene-2-carbohydrazide (16m):**



Conditions: reflux for 24 hours. The product was characterized only by ^1H NMR spectra, for its poor solubility, the ^{13}C spectra wasn't performed. Yield: 74%, yellow powder; mp: 276.4-277.0°C; IR: 3210 (NH), 1638 (CO) cm^{-1} ; ^1H NMR (200 MHz, DMSO- d_6) δ : 11.26 (1H, bs, NH), 8.51 (1H, d, $J = 2.1$ Hz, H-2), 8.27 (1H, d, $J = 2.1$ Hz, H-4), 8.12 (1H, dd, $J = 3.4, 0.8$ Hz, H-8), 8.03 (1H, dd, $J = 5.0, 1.0$ Hz, H-6), 7.63 (1H, d, $J = 2.8$ Hz, H-3'), 7.32 (1H, s, H-5'), 7.25 (1H, dd, $J = 5.0, 3.8$ Hz, H-7), 6.59

(1H, t, $J = 3.0$ Hz, H-4'). *Anal.* Calculated for $C_{15}H_9BrN_4OS$ (MW: 373.23): C, 48.27; H, 2.43; N, 15.01%. Found: C, 48.67; H, 2.58; N, 15.16%.

***N'*-[3-Bromo-5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidene]benzohydrazide (16p):**

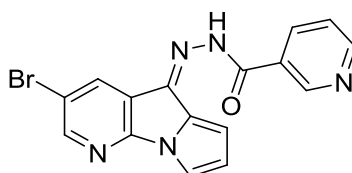


Conditions: reflux for 48 hours. Yield: 35%, dark yellow solid; mp: 227.6-227.9°C; IR: 3221 (NH), 1656 (CO) cm^{-1} ; 1H NMR (200 MHz, $CDCl_3$) δ : 9.32 (1H, bs, NH), 8.34 (2H, m, H-2 and H-4), 8.21 (2H, d, $J = 6.6$ Hz, H-6 and H-8), 7.64-7.45 (4H, m, H-2', H-3', H-5' and H-6'), 6.73 (1H, s, H-4'), 6.56 (1H, t, $J = 6.6$ Hz, H-7); ^{13}C NMR (50 MHz, $CDCl_3$) δ : 159.7 (s), 151.5 (s), 150.5 (s), 149.6 (d), 147.0 (s), 133.9 (d), 132.6 (d), 129.0 (d), 127.7 (d), 126.1 (s), 125.6 (s), 116.8 (d x 2), 116.4 (d), 116.1 (s), 111.8 (d x 2). *Anal.* Calculated for $C_{17}H_{11}BrN_4O$ (MW: 367.20): C, 55.61; H, 3.02; N, 15.26%. Found: C, 55.35; H, 3.15; N, 14.98%.

Synthesis of substituted -3-bromo-5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidenes (16k,n,o):

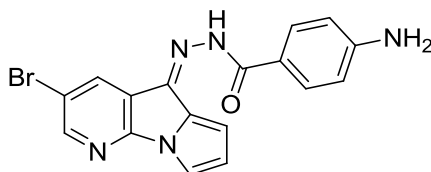
To a solution of 3-bromo-5*H*-pyrido[3,2-*b*]pyrrolizin-5-one **16i** (0.07 g, 0.28 mmol) in anhydrous ethanol (6 mL) was added opportune heteroaryl carbohydrazide **27c,f,g** (0.28 mmol). The resulting solution was heated to reflux for 24 hours and then chilled overnight. The precipitate formed was filtered, washed with cold ethanol and dried under vacuum to give derivatives **16k,o** or purified by silica gel column chromatography using DCM:Ethyl Acetate, 70:30 as eluent to afford product **16n**.

***N'*-[3-Bromo-5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidene]pyridine-3-carbohydrazide (16k):**



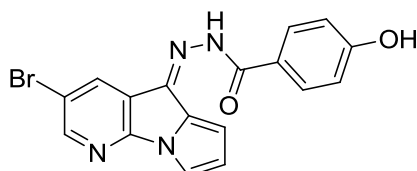
Yield: 63%, yellow solid; mp: 227.5-228.2°C; IR: 3187 (NH), 1652 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO-}d_6$) δ : 11.50 (1H, bs, NH), 9.11 (1H, s, H-2'), 8.80 (1H, dd, $J = 4.6, 1.0$ Hz, H-6), 8.53 (1H, d, $J = 2.0$ Hz, H-6'), 8.33 (1H, d, $J = 8.0$ Hz, H-8), 8.24 (1H, s, H-2), 7.65 (1H, d, $J = 2.2$ Hz, H-4'), 7.57 (1H, m, H-5'), 7.16 (1H, s, H-4), 6.60 (1H, t, $J = 3.2$ Hz, H-7); ^{13}C NMR (50 MHz, $\text{DMSO-}d_6$) δ : 164.2 (s), 152.4 (d), 151.2 (s), 149.5 (d), 149.0 (d), 135.2 (s), 132.0 (d), 129.1 (s), 126.7 (s), 125.8 (s), 123.6 (d), 121.1 (s), 116.9 (d), 116.5 (d), 115.9 (d), 115.0 (d). *Anal.* Calculated for $\text{C}_{16}\text{H}_{10}\text{BrN}_5\text{O}$ (MW: 368.19): C, 52.19; H, 2.74; N, 19.02%. Found: C 51.89; H, 2.92; N, 19.17%.

4-Amino-*N'*-[3-bromo-5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidene]benzohydrazide (16n):



Yield: 52%, dark yellow powder; mp: 289.1-290.0°C; IR: 3446 - 3340 (NH₂), 3219 (NH), 1662 (CO) cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 9.19 (1H, bs, NH), 8.27 (2H, m, H-2 and H-6), 8.04 (1H, s, H-4), 7.73 (2H, d, $J = 8.4$ Hz, H-2' and H-6'), 7.38 (1H, d, $J = 2.6$ Hz, H-8), 6.68 (2H, d, $J = 8.4$ Hz, H-3' and H-5'), 6.50 (1H, t, $J = 3.2$ Hz, H-7), 4.09 (2H, bs, NH₂); ^{13}C NMR (50 MHz, CDCl_3) δ : 162.0 (s), 150.8 (d x 2), 147.5 (s), 143.4 (d), 140.1 (s), 139.7 (s), 136.2 (s), 131.4 (s), 130.7 (s), 125.0 (s), 121.3 (d x 2), 117.6 (d), 116.3 (d), 116.0 (d), 109.9 (d). *Anal.* Calculated for $\text{C}_{17}\text{H}_{12}\text{BrN}_5\text{O}$ (MW: 382.21): C, 53.42; H, 3.16; N, 18.32%. Found: C 53.35; H, 3.23; N, 18.02%.

***N'*-[3-Bromo-5*H*-pyrido[3,2-*b*]pyrrolizin-5-ylidene]-4-hydroxybenzohydrazide (16o):**



Yield: 93%, yellow powder; mp: 288.6-289.1°C; IR: 3586 (NH), 3324 (OH), 1672 (CO) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO-}d_6$) δ : 11.03 (1H, bs, NH), 10.26 (1H, bs, OH), 8.51 (1H, d, $J = 2.1$ Hz, H-2), 8.26 (1H, d, $J = 2.1$ Hz, H-4), 7.87 (2H, d, $J = 8.7$ Hz, H-2' and H-6'), 7.64 (1H, d, $J = 3.0$ Hz, H-8), 7.04 (1H, d, $J = 3.5$ Hz, H-6), 6.93 (2H, d, $J =$

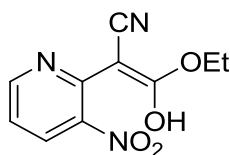
8.7 Hz, H-3' and H-5'), 6.60 (1H, t, $J = 3.5$ Hz, H-7); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 164.2 (s), 161.0 (s), 151.1 (s), 149.2 (d), 138.6 (s), 132.8 (d), 130.4 (d x 2), 126.9 (s), 125.9 (s), 123.4 (s), 116.8 (d), 116.0 (d), 115.4 (s), 115.1 (d x 2), 114.2 (d). *Anal.* Calculated for $\text{C}_{17}\text{H}_{11}\text{BrN}_4\text{O}_2$ (MW: 383.20): C, 53.28; H, 2.89; N, 14.62%. Found: C 53.40; H, 3.01; N, 14.87%.

Aza-indoles' series:

Synthesis of ethyl cyano(3-nitropyridin-yl)acetates (30a,b-31a):

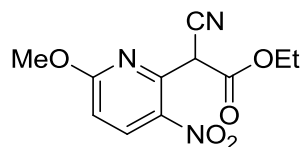
To a stirring solution of *t*-BuOK (1.7 g, 15.2 mmol) in *t*-BuOH (20 mL) ethyl cyanoacetate (1.8 mL, 16.56 mmol) was added. After 10 minutes, a solution of appropriate chloro-3-nitropyridine **28a-b**, **29a** (7.6 mmol) in *t*-BuOH was added and the resulting mixture heated to reflux for 12 hours. Upon cooling to room temperature, the reaction mixture was concentrated under reduced pressure and washed with 1M HCl, filtered and recrystallized from ethanol to give desired compound.

Ethyl cyano(3-nitropyridin-2-yl)acetate (30a)⁴⁴ :



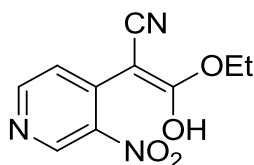
Yield: 80%, orange solid; mp: 136.4-136.9°C; IR: 3556 (OH), 2190 (CN) cm^{-1} ; ^1H NMR (200 MHz, DMSO- d_6) δ : 14.49 (1H, bs, OH), 8.46 (1H, d, $J = 8.0$ Hz, H-6), 8.36 (1H, d, $J = 4.0$ Hz, H-4), 7.05 (1H, t, $J = 8.0$ Hz, H-5), 4.23 (2H, q, $J = 7.0$ Hz, CH_2), 1.26 (3H, t, $J = 7.0$ Hz, CH_3); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 168.3 (s), 146.1 (s), 142.0 (d), 138.8 (d), 134.5 (s), 125.9 (s), 116.2 (s), 112.2 (d), 60.2 (t), 14.3 (q). *Anal.* Calculated for $\text{C}_{10}\text{H}_9\text{N}_3\text{O}_4$ (MW: 235.20): C, 51.07; H, 3.86; N, 17.87%. Found: C, 51.27; H, 3.69; N, 17.78%.

Ethyl cyano(6-methoxy-3-nitropyridin-2-yl)acetate (30b):



Yield: 75%, light yellow solid; mp: 72.9-73.4°C; IR: 2224 (CN), 1720 (CO) cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 8.46 (1H, d, $J = 9.1$ Hz, H-4), 6.93 (1H, d, $J = 9.1$ Hz, H-5), 5.86 (1H, s, CH), 4.34 (2H, q, $J = 7.1$ Hz, CH_2), 4.12 (3H, s, OCH_3), 1.35 (3H, t, $J = 7.1$ Hz, CH_3); ^{13}C NMR (50 MHz, CDCl_3) δ : 165.5 (s), 162.9 (s), 145.0 (s), 138.4 (s), 136.8 (d), 113.3 (s), 112.7 (d), 63.9 (t), 55.5 (q), 45.6 (d), 13.9 (q). *Anal.* Calculated for $\text{C}_{11}\text{H}_{11}\text{N}_3\text{O}_5$ (MW: 265.22): C, 49.81; H, 4.18; N, 15.84%. Found: C, 50.01, H, 4.29; N, 15.99%.

Ethyl cyano(3-nitropyridin-4-yl)acetate (31a)⁴⁵:



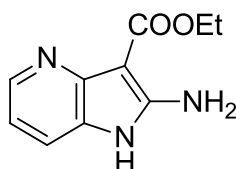
Quantitative yield, yellow powder; mp: 177.8-178.8°C; IR: 3558 (OH), 2200 (CN) cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO}-d_6$) δ : 13.39 (1H, bs, OH), 8.70 (1H, s, H-2), 7.87-7.90 (2H, m, H-5 and H-6), 4.07 (2H, q, $J = 7.0$ Hz, CH_2), 1.18 (3H, t, $J = 7.0$ Hz, CH_3); ^{13}C NMR (50 MHz, $\text{DMSO}-d_6$) δ : 164.8 (s), 145.5 (s), 137.9 (d), 136.6 (s), 135.7 (d), 119.0 (s), 117.6 (d), 70.7 (s), 59.5 (t), 14.4 (q). *Anal.* Calculated for $\text{C}_{10}\text{H}_9\text{N}_3\text{O}_4$ (MW: 235.20): C, 51.07; H, 3.86; N, 17.87%. Found: C, 51.24, H, 3.72; N, 17.76%.

Synthesis of ethyl 2-amino-1H-pyrrolo[3,2-b]pyridine-3-carboxylates, ethyl 2-amino-1H-pyrrolo[2,3-c]pyridine-3-carboxylate (32a-b, 33a):

To a stirring solution of opportune ethyl cyano(3-nitropyridin-yl)acetate **30a-b**, **31a** (2.12 mmol) in acetic acid (4 mL) iron powder (0.75 g, 13.44 mmol) was added. The resulting dark solution was stirred at room temperature for 4-5 hours. The reaction mixture, added an aqueous saturated Na_2CO_3 solution until pH 9-10, was extracted with

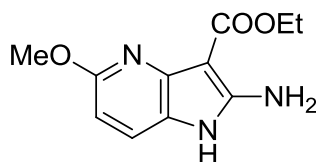
ethyl acetate (3 x 15 mL), dried over anhydrous Na₂SO₄, filtered and evaporated to give desired compound.

Ethyl 2-amino-1*H*-pyrrolo[3,2-*b*]pyridine-3-carboxylate (32a)⁴⁴:



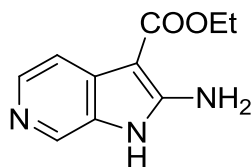
Conditions: room temperature for 5 hours. Yield: 85%, light green solid; mp: 110.8-111.5°C; IR: 3557 - 3534 (NH₂), 3279 (NH), 1720 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 10.85 (1H, bs, NH), 8.09 (1H, dd, *J* = 5.0, 1.4 Hz, H-5), 7.35 (1H, dd, *J* = 7.8, 1.4 Hz, H-7), 7.06 (2H, bs, NH₂), 6.85 (1H, dd, *J* = 7.8, 5.0 Hz, H-6), 4.25 (2H, q, *J* = 7.0 Hz, CH₂), 1.28 (3H, t, *J* = 7.0 Hz, CH₃); ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 165.7 (s), 156.1 (s), 145.5 (s), 141.3 (d), 127.1 (s), 115.9 (d), 114.8 (d), 84.5 (s), 58.6 (t), 15.3 (q). *Anal.* Calculated for C₁₀H₁₁N₃O₂ (MW: 205.21): C, 58.53; H, 5.40; N, 20.48%. Found: C, 58.64; H, 5.20; N, 20.22%.

Ethyl 2-amino-5-methoxy-1*H*-pyrrolo[3,2-*b*]pyridine-3-carboxylate (32b):



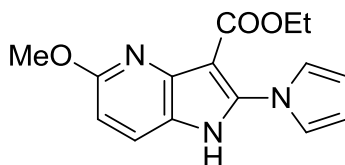
Conditions: room temperature for 4 hours. Yield: 79%, brown solid; mp: 229.2-230.0°C; IR: 3483 - 3395 (NH₂), 3267 (NH), 1651 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO- *d*₆) δ: 10.54 (1H, bs, NH), 7.32 (1H, d, *J* = 8.4 Hz, H-7), 6.84 (2H, bs, NH₂), 6.25 (1H, d, *J* = 8.4 Hz, H-6), 4.20 (2H, q, *J* = 6.9 Hz, CH₂), 3.84 (3H, s, OCH₃), 1.35 (3H, t, *J* = 6.9 Hz, CH₃); ¹³C NMR (50 MHz, DMSO- *d*₆) δ: 165.3 (s), 159.0 (s), 154.4 (s), 142.2 (s), 120.9 (s), 119.1 (d), 99.7 (d), 84.7 (s), 57.9 (t), 52.2 (q), 14.5 (q). *Anal.* Calculated for C₁₁H₁₃N₃O₃ (MW: 235.24): C, 56.16; H, 5.57; N, 17.86%. Found: C, 56.34; H, 5.69; N, 17.98%.

Ethyl 2-amino-1*H*-pyrrolo[2,3-*c*]pyridine-3-carboxylate (33a)⁴⁵:



Conditions: room temperature for 5 hours. Yield: 88%, light orange solid; mp: 151.6-152.6°C; IR: 3429 - 3396 (NH₂), 3315 (NH), 1678 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 10.35 (1H, bs, NH), 8.32 (1H, s, H-7), 8.03 (1H, d, *J* = 2.0 Hz, H-5), 7.42 (1H, d, *J* = 2.2 Hz, H-4), 7.21 (2H, bs, NH₂), 4.24 (2H, q, *J* = 7.0 Hz, CH₂), 1.34 (3H, t, *J* = 7.0 Hz, CH₃); ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 172.2 (s), 165.2 (s), 139.1 (d), 133.3 (s), 129.4 (s), 129.3 (d), 112.4 (d), 84.2 (s), 58.5 (t), 14.6 (q). *Anal.* Calculated for C₁₀H₁₁N₃O₂ (MW: 205.21): C, 58.53; H, 5.40; N, 20.48%. Found: C, 58.60; H, 5.24; N, 20.25%.

Synthesis of ethyl 5-methoxy-2-(1*H*-pyrrol-1-yl)-1*H*-pyrrolo[3,2-*b*]pyridine-3-carboxylate (34b):



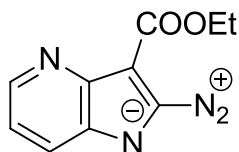
To a solution of 2,5-dimethoxytetrahydrofuran (0.14 mL, 1.10 mmol) in anhydrous 1,4-dioxane (13 mL), was added 4-chloropyridine hydrochloride (0.16 g, 1.10 mmol) and the reaction mixture was stirred at room temperature for 15 minutes. Derivate **32b** (0.26 g, 1.10 mmol) was added and the reaction mixture was heated to reflux for 18 hours. Upon cooling a precipitate formed, that was filtered and purified by silica gel column chromatography (DCM) to give desired compound **34b**. Yield: 65%, brown solid; mp: 195.5-196.2°C; IR: 3267 (NH), 1704 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 12.45 (1H, bs, NH), 7.68 (1H, d, *J* = 8.5 Hz, H-7), 7.27 (2H, s, H-2' and H-5'), 6.65 (1H, d, *J* = 8.5 Hz, H-6), 6.31 (2H, s, H-3' and H-4'), 4.20 (2H, q, *J* = 6.3 Hz, CH₂), 3.92 (3H, s, OCH₃), 1.24 (3H, t, *J* = 6.3 Hz, CH₃); ¹³C NMR (50 MHz,

DMSO- d_6) δ : 162.7 (s), 160.0 (s), 140.3 (s), 140.0 (s), 122.7 (d), 122.5 (d x 2), 121.5 (s), 109.8 (d x 2), 105.8 (d), 99.6 (s), 59.1 (t), 52.6 (q), 14.1 (q). *Anal.* Calculated for $C_{15}H_{15}N_3O_3$ (MW: 285.30): C, 63.15; H, 5.30; N, 14.73%. Found: C, 63.06; H, 5.16; N, 14.88%.

Synthesis of 2-diazo-3-ethoxycarbonyl-pyrrolopyridines (40-41)a:

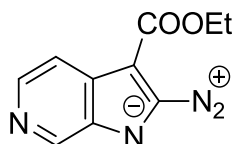
To a solution of opportune derivate (**32-33**)a (0.5 g, 2.44 mmol) in acetic acid (4.6 mL) at 0°C, under N_2 atmosphere, a solution of sodium nitrite ($NaNO_2$) (0.19 g, 2.75 mmol) in a small amount of water was slowly added. The mixture was stirred at 0°C for 8 hours. Upon neutralizing with 15% Na_2CO_3 solution, the reaction mixture was extracted with DCM (3 x 15 mL) dried over anhydrous Na_2SO_4 , filtered and evaporated to give desired compound.

2-Diazo-3-ethoxycarbonyl-pyrrolo[3,2-b]pyridine (40a)⁴⁴:



Yield: 90%, brown solid; mp: 162.0°C dec.; IR: 2198 (N_2^+), 1714 (CO) cm^{-1} ; 1H NMR (200 MHz, DMSO- d_6) δ : 6.99-8.72 (3H, m), 4.35 (2H, q, $J = 7.0$ Hz, CH_2), 1.34 (3H, t, $J = 7.0$ Hz, CH_3); ^{13}C NMR (50 MHz, DMSO- d_6) δ : 162.0 (s), 150.9 (d), 143.1 (s), 140.5 (d), 131.1 (s), 129.9 (s), 114.8 (d), 114.6 (s), 59.1 (t), 14.6 (q). *Anal.* Calculated for $C_{10}H_8N_4O_2$ (MW: 216.20): C, 55.55; H, 3.73; N, 25.91%. Found: C, 55.24; H, 3.96; N, 25.72%.

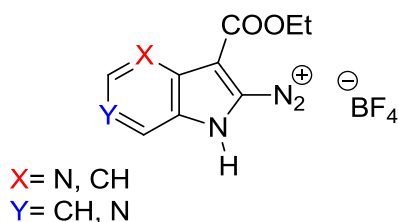
2-Diazo-3-ethoxycarbonyl-pyrrolo[2,3-c]pyridine (41a)⁴⁵:



Yield: 90%, red/orange solid; mp: 168.0°C dec.; IR: 2191 (N₂⁺), 1709 (CO) cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 9.06 (1H, s, H-7), 7.75-8.59 (2H, m, H-4 and H-5), 4.24 (2H, q, *J* = 7.0 Hz, CH₂), 1.38 (3H, t, *J* = 7.0 Hz, CH₃); ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 161.2 (s), 146.3 (d), 144.3 (s), 141.1 (d), 131.1 (s), 129.9 (s), 115.0 (d), 114.4 (s), 60.9 (t), 14.1 (q). *Anal.* Calculated for C₁₀H₈N₄O₂ (MW: 216.20): C, 55.55; H, 3.73; N, 25.91%. Found: C, 55.17; H, 3.92; N, 25.70%.

Synthesis of 3-(ethoxycarbonyl)-1*H*-pyrrolopyridine-2-diazonium tetrafluoroborates

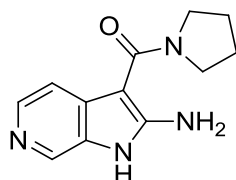
(42-43)a:



To a solution of opportune diazo (**40-41**)a (0.2 g, 0.92 mmol) in anhydrous diethyl ether (4 mL) at -10°C, under N₂ atmosphere, a solution of tetrafluoroboric acid (0.15 mL, 0.92 mmol) in a small amount of diethyl ether was slowly added. The mixture was warmed to 0°C and stirred overnight. A yellow solid was formed, filtered off, washed with water and dried under vacuum. The crude was taken up onto the next step that unfortunately didn't work.

Synthesis of (2-amino-1*H*-pyrrolo[2,3-*c*]pyridin-3-yl)(pyrrolidin-1-yl)methanone

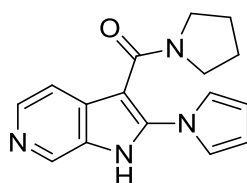
(46a):



To a stirred solution of 1-(cianoacetyl)-pyrrolidine **44** (0.35 g, 2.5 mmol) in anhydrous DMF (3 mL) was slowly added NaH (60% dispersion in mineral oil, 0.07 g, 2.75

mmol). After 10 minutes, 4-chloro-3-nitropyridine **29a** (0.4 g, 2.5 mmol) was added and the reaction mixture became deep purple. After 1 hour at room temperature, 1.0 N HCl (5.0 mmol) was added, following FeCl₃ (1.22 g, 7.5 mmol) and Zn dust (1.6 g, 25 mmol). The reaction mixture was heated to 100°C for 1 hour. Upon cooling, the crude, added of water (25 mL), was extracted with ethyl acetate (3 x 30 mL) and washed by an aqueous saturated NaHCO₃ solution (20 mL) and brine (20 mL). The organic layer was dried over anhydrous Na₂SO₄, filtered and evaporated *in vacuo*. The crude was purified by silica gel column chromatography using Petroleum Ether:Ethyl Acetate, 70:30 as eluent to give desired compound **46a**. Yield: 30%, uncolorless oil; IR: 3463 - 3388 (NH₂), 3192 (NH), 1615 (CO) cm⁻¹; ¹H NMR (200 MHz, CDCl₃) δ: 8.12 (1H, s, H-7), 7.87 (1H, d, *J* = 5.2 Hz, H-5), 7.16 (1H, d, *J* = 5.2 Hz, H-4), 4.33 (2H, bs, NH₂), 1.63-1.50 (2H, m, CH₂), 1.29-1.14 (4H, m, CH₂ x 2), 0.90-0.83 (2H, m, CH₂); ¹³C NMR (50 MHz, CDCl₃) δ: 171.0 (s), 140.6 (s), 139.2 (d), 137.9 (s), 135.5 (d), 127.6 (s), 124.2 (d), 82.4 (s), 60.7 (t), 41.3 (t), 28.6 (t), 17.3 (t). *Anal.* Calculated for C₁₀H₈N₄O₂ (MW: 230.27): C, 62.59; H, 6.13; N, 24.33%. Found: C, 62.74; H, 6.33; N, 24.47%.

Synthesis of pyrrolidin-1-yl[2-(1H-pyrrol-1-yl)-1H-pyrrolo[2,3-*c*]pyridin-3-yl]methanone (37a):



To a solution of 2,5-dimethoxytetrahydrofuran (0.14 mL, 1.09 mmol) in dry 1,4-dioxane (13.0 mL), was added 4-chloropyridine hydrochloride (0.16 g, 1.09 mmol) and the reaction mixture was stirred at room temperature for 15 min. (2-amino-1H-pyrrolo[2,3-*c*]pyridin-3-yl)(pyrrolidin-1-yl)methanone **46a** (0.25 g, 1.09 mmol) was added and the reaction mixture was heated to reflux for 90 minutes. Upon cooling, a precipitate formed, that was filtered. The solid was discarded, while the mother liquor containing the title compound was evaporated *in vacuo* and the residue was purified by silica gel column chromatography, using Petroleum Ether:Ethyl Acetate, 85:15 to give desired derivative **37a**. Yield: 55%, light yellow oil; IR: 3417 (NH), 1646 (CO) cm⁻¹; ¹H

NMR (200 MHz, CDCl₃) δ : 8.57 (1H, s, H-7), 8.44 (1H, d, J = 5.2 Hz, H-5), 7.44 (1H, d, J = 5.2 Hz, H-4), 6.91 (2H, t, J = 2.2 Hz, H-2' and H-5'), 6.37 (2H, t, J = 2.2 Hz, H-3' and H-4'), 1.5-1.14 (4H, m, CH₂ x 2), 1.03-0.85 (4H, m, CH₂ x 2); ¹³C NMR (50 MHz, CDCl₃) δ : 155.6 (s), 148.9 (d), 148.1 (d), 147.9 (s), 138.9 (s), 135.5 (s), 125.3 (d), 122.0 (d x 2), 110.4 (d x 2), 109.7 (s), 59.7 (t), 41.3 (t), 28.7 (t), 17.6 (t). *Anal.* Calculated for C₁₀H₈N₄O₂ (MW: 280.32): C, 68.55; H, 5.75; N, 19.99%. Found: C, 68.78; H, 5.55; N, 20.09%.

BIOLOGY

Drugs Preparation

To obtain stock solutions of compounds **14a-i**, **16a-p**, each compound was initially dissolved in DMSO in order to obtain 50mM solution, stored at +4°C, and diluted in complete culture medium immediately before use at the appropriate concentration.

Viability assay *in vitro*

Tripentone analogues, prepared as described above, were dissolved in dimethyl sulfoxide (DMSO) (all reagents and chemicals were from Sigma Chemical Co (St. Louis, MO), unless indicated) and then diluted in culture medium to have a DMSO concentration not exceeding 0.1%. HCT-116 and Caco-2 cell lines were purchased from American Type Culture Collection, Rockville, MD, USA and DMEM supplemented with 10% fetal, 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μ g/mL) and gentamicin (5 μ g/mL). Cells were maintained in log phase by seeding twice a week at a density of 3 x 10⁸ cells/L in humidified 5% CO₂ atmosphere, at 37 °C. In all experiments, HCT-116 and MCF-7 cells were made quiescent through overnight incubation before treatment with tested compounds or vehicle alone (control cells). No differences were found between cells treated with DMSO 0.1% and untreated cells in terms of cell number and viability. Cytotoxic activity of the tripentone derivatives **14a-i** and **14a-p** was determined by the colorimetric assay based on the reduction of 3-

(4,5-dimethyl-2-thiazolyl)bromide-2,5-diphenyl-2*H*-tetrazolium (MTT) to purple formazan by mitochondrial dehydrogenases. Briefly, HCT-116 and MCF-7 lines cells were seeded at 2×10^4 cells/well in 96-well plates containing 200 μ L RPMI. When appropriated, monolayer cultures were treated for 24 h with various concentrations (5-100 μ M) of the drugs. Then cells were washed with fresh medium and 50 μ L FBS-free medium containing 5 mg/mL MTT added. Cells were incubated 2 h at 37 °C, then medium was discarded by centrifugation, formazan blue formed in the cells dissolved in DMSO, and absorbance measured at 570 nm in a microplate reader (Bio-RAD, Hercules, CA). Formazan of control cells was taken as 100% viability. GI₅₀ was calculated by the curve of percent viability versus concentration. Each experiment was repeated at least three times in triplicate.

Cell cycle analysis

Cell cycle stage was analyzed by flow cytometry. HCT-116 cells (5.0×10^4 cells/cm²) were seeded in triplicate in 24-wells culture plates. After an overnight incubation, the cells were washed with fresh medium and incubated with compounds **16e** and **16j** in RPMI for 24 h. Then cells were harvested by trypsinization. Aliquots of 1×10^6 cells were washed with PBS and incubated in the dark in a PBS solution containing 20 μ g/ml propidium iodide (PI) and 200 μ g/ml RNase, for 30 min, at room temperature. Then samples of at least 1.0×10^4 cells were subjected to fluorescence-activated cell sorting (FACS) analysis by Epics XL™ flow cytometer using Expo32 software (Beckman Coulter, Fullerton, CA).

Measurement of phosphatidylserine (PS) exposure

The apoptosis-induced PS externalization to the cell surface was measured by flow cytometry by double staining with Annexin V-Fluorescein isothiocyanate (Annexin V-FITC)/propidium iodide (PI). Annexin V binding to phosphatidylserine is used to identify the earliest stage of apoptosis. PI, which does not enter cells with intact membranes, is used to distinguish between early apoptotic cells (Annexin V-FITC positive and PI negative), late apoptotic cells (Annexin V-FITC/PI-double positive) or

necrotic cells (annexin V-FITC negative and PI positive). After 24 h treatment, HCT-116 cells were harvested by trypsinization and adjusted at 1.0×10^6 cells/mL with combining buffer according to the manufacturer instructions (eBioscience, San Diego, CA). One hundred μL of cell suspensions were added to a new tube, and incubated with Annexin V-FITC and PI solution at room temperature in the dark for 15 min. Then samples of at least 1.0×10^4 cells were subjected to FACS analysis using appropriate 2-dimensional gating method.

Statistics

Results are given as means and their standard deviations. Three independent observations were carried out for each experiment, replicated three times. Statistical comparisons were made using a one-way ANOVA, followed by Bonferroni's test. $P < 0.05$ was considered statistically significant.

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SYNTHESIS OF INTRACELLULAR ANTAGONISTS FOR CC CHEMOKINE RECEPTORS CCR1 AND CCR2



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During my second PhD's year, I spent six months at the University of Leiden, Leiden Academic Centre for Drug Research (LACDR), under the supervision of Professor Ad IJzerman, where I carried out a project based on the synthesis of intracellular antagonists for CC chemokine receptors CCR1 and CCR2.

INTRODUCTION

Chemokines are small (8-12kDa), inducible, secreted chemotactic cytokines that have an highly variability in terms of homology sequence, indeed it is around 20-90%^{1,2}. They played a critical role in inflammation and immune-surveillance, as they are involved in the migration of white blood cells¹. Furthermore, they are associated to tissue repair process and abnormalities, unsuitable utilization or control can cause different diseases, such as inflammatory diseases and cancer²⁻³. They are constituted of a N terminus in which there is a chemokine domain, then a portion of 110 amino acids, especially wealthy in Threonine and Serine, followed by a transmembrane domain and a cytoplasmic tail². The chemokine superfamily can be classified, on the basis of the configuration of conserved cysteine residues, into four classes, CXC, CC, CX3C and C chemokines. Chemokine actions are mediated by specific cell membrane receptors that belong to the superfamily of G-protein-coupled receptors (GPCR). Basing on their

ligand preference, these receptors have been distinguished as CC, CXC, CX3C and C chemokine receptors¹.

GPCRs are the vastest family of cell membrane receptors and different kinds of ligands, such as neurotransmitters, ions, hormones, activate them, indeed they are involved in a large number of human diseases and disorders⁴. They contain three different subunits α , β and γ , knew also as heterotrimeric G proteins $\alpha\beta\gamma$, bind guanine nucleotides: GDP (Guanosine diphosphate), reversibly bound in inactivated form, while GTP (Guanosine triphosphate) in activated state. Specifically, in the inactivated state the subunits α binds GDP and the other two subunits forms a $\beta\gamma$ dimer; when a ligand binds the receptor, GTP substitutes GDP and binds G_α , followed from release of α -GTP and heterodimer $\beta\gamma$ and at the end physiological response. The process ends with the hydrolilysis of GTP to GDP from G_α , that has GTPase activity and with the regeneration of heterotrimeric $\alpha\beta\gamma$ ² (**Figure 1**).

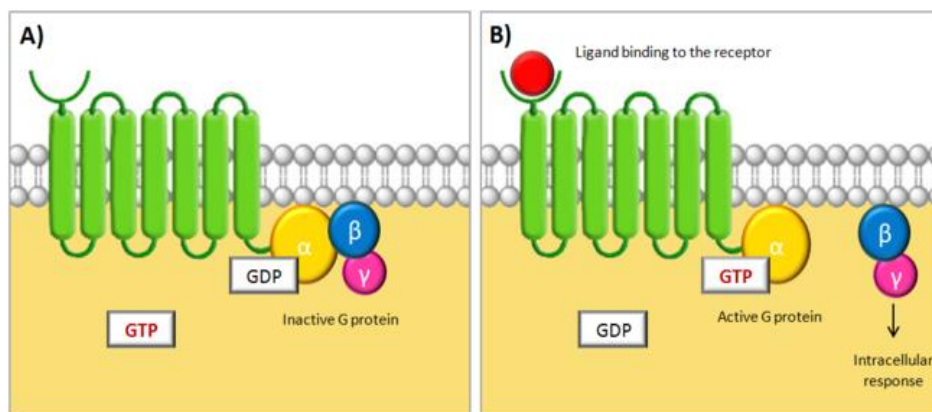


Figure 1

Furthermore GPCRs can be divided in three different families on the base of homology of the sequence and function:

1. Class A, rhodopsin-like;
2. Class B, secretin, glucagon, calcitonin receptors;
3. Class C, metabotropic glutamate receptors⁵.

In general there are three main G-protein-mediated signaling pathways, $G_{q/11}$, $G_{i/o}$ and G_s that have different effectors. For $G_{i/o}$ and G_s is cyclic-adenosine

monophosphate (cAMP), originated from ATP (cytosolic adenosine triphosphate) by action of adenylate cyclase (AC). G_s stimulates adenylate cyclase and increases cAMP, while in the opposite way acts $G_{i/o}$, indeed it inhibits AC and decreases cAMP. Instead, $G_{q/11}$ acts through phospholipase C (PLC) that catalyzes the transformation of PIP2 (phosphatidylinositol-4,5-biphosphate) into DAG (diacylglycerol) and IP3 (inositol-1,4,5-trisphosphate). These induce Ca^{2+} release, through activation of protein kinase K (PKC), and specific receptor binding on the endoplasmic reticulum, respectively (Figure 2).

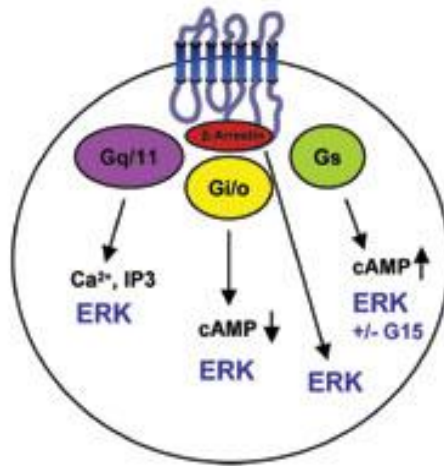


Figure 2

The CC-chemokine receptor CCR-2 is a G protein-coupled membrane receptor, class A, expressed on monocytes, dendritic cells and activated T lymphocytes and basophils. It consists of about 350 amino-acids with an extracellular N-terminal, necessary for ligand binding and specificity, seven helical transmembrane domains with intracellular and extracellular loops, and an intracellular C-terminal involved in the signal transduction⁶ (Figure 3).

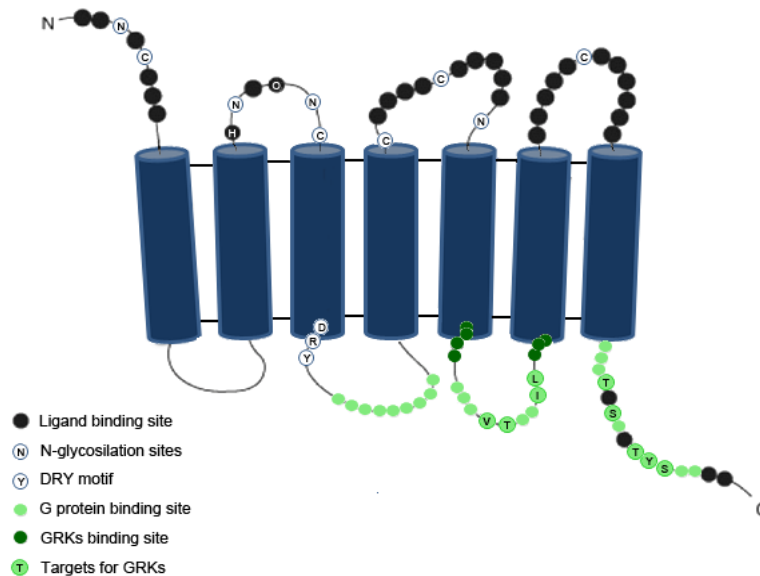
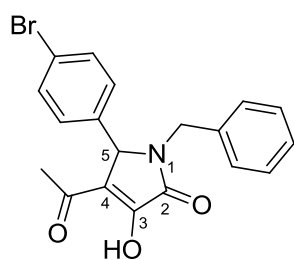


Figure 3

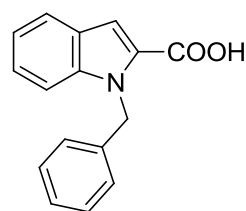
This receptor recognizes different ligands such as CCL2 (knew also as monocyte chemotactic protein-1, MCP-1), CCL7 (MCP-3), CCL8 (MCP-2), CCL11, CCL13 (MCP-4), CCL16 (HCC-4). Following interaction with their specific chemokine ligands, chemokine receptors trigger a flux in intracellular calcium (Ca^{+2}) ions (calcium signaling). This causes cell responses, including the onset of a process known as chemotaxis, that is the immune cells' migration towards increasing concentrations of chemokines at sites of inflammation. Over the past decade, much evidence has emerged suggesting a pathological role of CCL2 and CCR2 in diseases that are characterized by chronic inflammation such as atherosclerosis, rheumatoid arthritis, multiple sclerosis, allergic rhinitis, allergic asthma, uveitis and solid tumors⁷ but also in cardiovascular diseases, organ transplant rejection and diabetes⁸.

MCP-1 acts on monocytes, basophils and memory T cells by binding to CCR2 and generates a chemotactic gradient that triggers the migration of immune cells to the site of inflammation. In several inflammatory conditions is observed excessive monocyte recruitment and an overexpression of CCL2/CCR2 pair⁹. It has been pointed that, through animal model studies of chronic inflammatory diseases, inhibition of binding between MCP-1 and CCR-2 by an antagonist suppresses the inflammatory response. Furthermore, MCP-1 antagonists inhibit monocyte migration, blocking the development of asthma, uveitis and arthritis. MCP-1 has also been found in the nasal mucosa of most

patients with dust mite allergies and it induces histamine release from basophils, *in vitro*. Histamines and allergens trigger the expression of MCP-1 and other chemokines in the nasal mucosa of people with allergic rhinitis, suggesting the presence of a positive feedback loop in such patients⁷. So it's evident that chemokines and their receptors are key targets to find a cure for different kinds of disease. In the literature there are various inhibitors of CCR2 and especially we have focused our attention on two different classes of chemicals, pyrrolidinone as **1**^{10,11} and N-benzylindole-2-carboxylic acid as **2**¹².

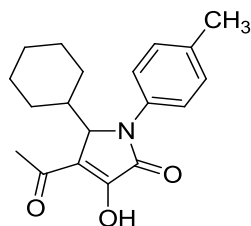


1
IC₅₀=8μM



2
IC₅₀ = 1.7 μM

The class of pyrrolidinone **1** is an interesting group of acidic CCR2 antagonists with values of IC₅₀ in nanomolar range, so for these compounds, an important SAR study was made to better understand the moieties involved in the binding with the receptor. Indeed the first investigations were focused on the decoration of C-5 and of amidic nitrogen: their removals showed a total decrease of activity. Changing the C-5 aryl group with a cyclohexyl group, a great result was obtained with better value of IC₅₀ than their lead compound. Was also tried the conversion of the acetyl group with a bigger substituent (*t*-butyl) obtaining worse result in terms of activity. Furthermore, a modification of enolic moiety with a methyl group generates a totally inactive compounds¹⁰. Dasse *et al.* in a further study, describe a new really active pyrrolidinone **3** obtained from the pyrrolidinone-based CCR2 antagonist **1**¹¹.

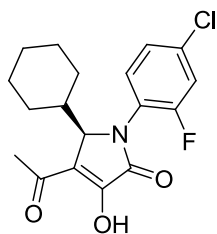


3

IC₅₀ = 498 nM

They developed a new interesting class of pyrrolidinones through modifications of this lead compound **3**, identifying the key pharmacophoric moieties of this scaffold, that are an hydrophobic alkyl portion, an aromatic group and an ionizable acidic feature. Structure-activity relationship study with the aim to investigate this new lead compound showed that attempt of methylation or conversion to carboxamide of the hydroxylic group, afford new compounds with removal or lowered affinity (respectively IC₅₀ >25 μM and 2.5 μM). A 30-fold loss of binding affinity was registered with the conversion of the acetyl group to ester, while the conversion to amides allow to obtain analogs with IC₅₀ values around 1.4-2.0 μM. Instead the removal of acetyl group underlines the importance of 1,3-dicarbonyl feature to interact with CCR2, indeed attempts of these modifications led to inactive compounds. Really interesting are the introduction of halogens such as chlorine, fluorine or bromine on the phenyl group at place of methyl or in different positions of the same ring, but keeping in all these cases a substituent in four position. Furthermore, it was possible to explore the influence of the cyclohexyl functionality to CCR2 binding, substituting it with smaller or larger rings or with allyl chains, obtaining only in some case interesting molecules in terms of IC₅₀¹¹.

An important aspect of these compounds is their pharmacokinetics parameters, indeed *in vivo* they show good oral availability and lessened metabolic clearance¹¹. They are especially indicated for treating of different pathological conditions characterized from involvement of MCP-1, CCR2 or both, such as asthma, food allergy, allergic rhinitis, multiple sclerosis, atherosclerosis, cardiac diseases, pulmonary fibrosis, cancer, autoimmune diseases, etc...¹³. Another interesting aspect is that pyrrolidinone type **4** acts with an allosteric or noncompetitive CCR2 antagonism with respect to CCL2, through interaction with an intracellular binding pocket¹⁴⁻¹⁵.

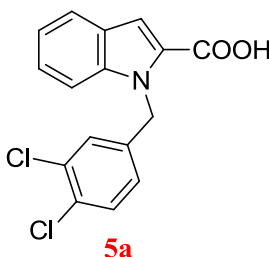


4

$$IC_{50} = 0.062 \mu\text{M}$$

This aspect is really significant in terms of pharmacological properties because allosteric modulation displays several advantages from orthosteric one. After interaction of allosteric modulators with GPCRs at different sites from the orthosteric one, a conformational change in the receptor occurs and this can modify the orthosteric ligand's association/dissociation rate or both in its binding site. Allosteric modulators show their effect only in presence of endogenous ligand, modulating his activity in a positive or negative mode, furthermore another advantage is that allosteric site's sequence is poorly conserved and in this leads to a higher selectivity⁴.

Relative to N-benzylindole-2-carboxylic acids of type **2** different patents describe indole compounds with N-benzyl moiety as leukotriene biosynthesis's inhibitors, others as allosteric modulators at muscarinic receptors, or anti-allergy or for head injury but also as MCP-1 inhibitors¹⁶. In this case through SAR studies, was possible understand the principal moiety responsible of this effect. The benzyl group is essential for activity, indeed modification with aliphatic group or the total removal, led to total inactive molecules. Instead small, lipophilic substituents are well tolerated and also 3,4-disubstitution led to compounds with higher binding affinity. Indeed, in further studies investigating the role of carboxylic acid the 3,4-dichlorobenzyl analogue **5a** has been selected as lead compound.

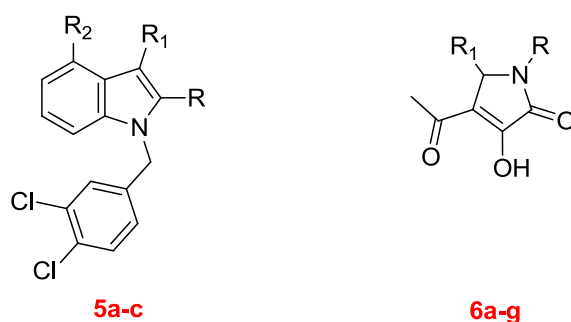


5a

$$IC_{50} = 0.23 \mu\text{M}$$

The modification of carboxylic acid with tetrazole or acylsulfonamides or amide doesn't lead to better compounds. Finally, substitutions on the indole ring show that strong electron withdrawing are generally poorly tolerated save in 3- and 5-positions. Electron donating substituents don't show big differences from the lead compound **5a** in terms of affinity, instead halogens, especially fluorine display interesting results with IC₅₀ of 56 nM. Furthermore, representative N-benzylindole-2-carboxylic acids are tested on 40 seven-transmembrane G-protein coupled receptor binding assays and all show an IC₅₀ higher than 10 μM¹².

At light of these interesting data, the aim of my project was to prepare the most active compounds belong to the pyrrolidinone's and N-benzylindole-2-carboxylic acid's classes (**5-6**)^{16,17} and also to synthesize new compounds bearing a proper decoration to evaluate their activity against CCR2, but also against CCR1 as high sequence similarity of these receptors. The compounds synthesized are indicated below (**Table 1**):

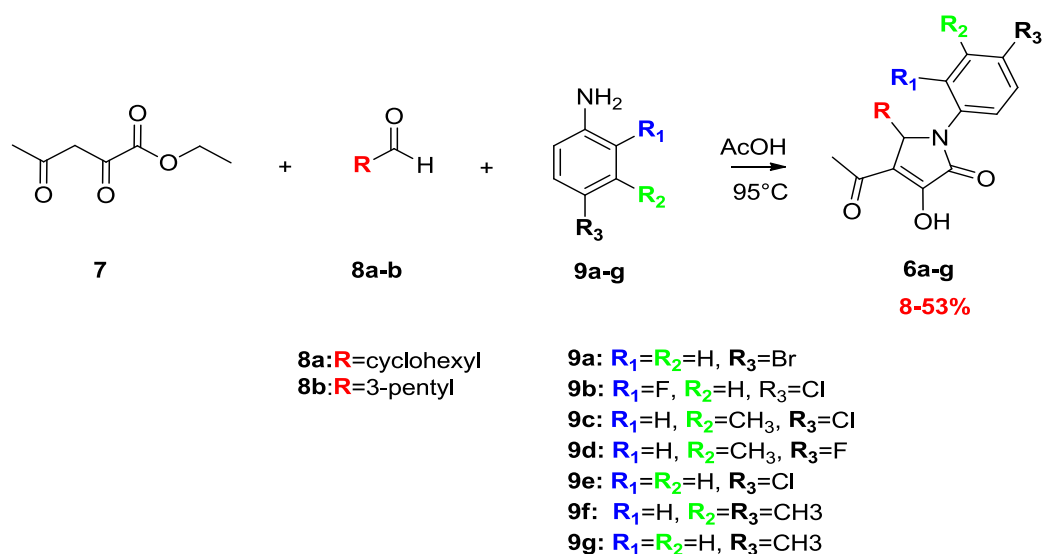


Compound	R	R ₁	R ₂
5a	COOH	H	H
5b	H	COOH	H
5c	COOH	COOH	H
6a	4-bromophenyl	Cyclohexyl	-
6b	4-chloro-2-fluorophenyl	Cyclohexyl	-
6c	4-chloro-3-methylphenyl	Cyclohexyl	-
6d	2-fluoro-4-methylphenyl	Cyclohexyl	-
6e	4-chlorophenyl	Cyclohexyl	-
6f	3,4-dimethylphenyl	3-pentyl	-
6g	4-methylphenyl	3-pentyl	-

Table 1

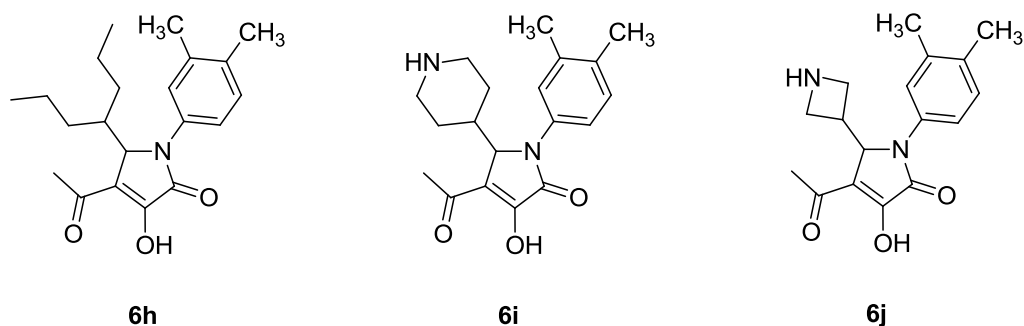
RESULTS AND DISCUSSION: CHEMISTRY

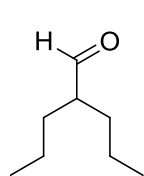
Through one-pot reaction was possible to obtain the desired pyrrolidinones **6a-g** from low to good yields (8-53%). Indeed ethyl-2,4-dioxovalerate **7** was put to react with appropriate aldehydes (**8a-b**) and anilines (**9a-g**) in acetic acid at 95°C for 2 hours¹⁷ and later through easy filtration or purification by silica gel column chromatography was possible to isolate the desired compounds **6a-g** (Scheme 1).



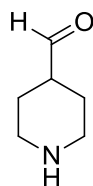
Scheme 1

It was also tried the synthesis of new pyrrolidinones **6h-j** starting from the synthesis of their appropriate aldehydes **12**, **17**, **20**.

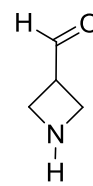




12

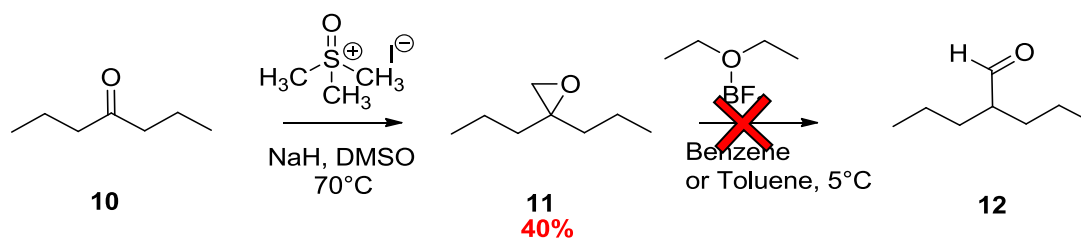


17



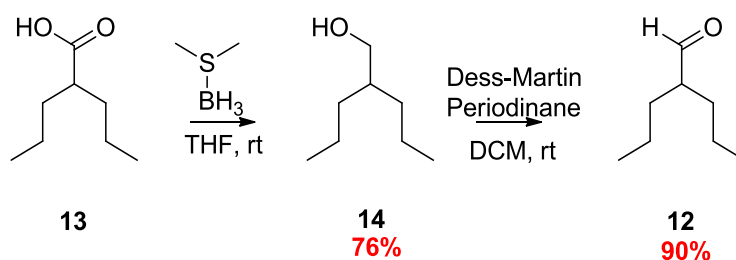
20

To obtain 2-propylpentanal **12** the first attempt was to synthesize 2-propyl-1,2-epoxypentane **11** by reacting 4-heptanone **10** with sodium hydride and trimethylsulfoxonium iodide in DMSO under inert atmosphere at 70°C for 5 hours¹⁸. The epoxide **11**, obtained in good yields (40%), was reacted with boron trifluoride etherate using benzene¹⁸ or toluene as solvents (**Scheme 2**), but unfortunately in both cases this reaction didn't work.



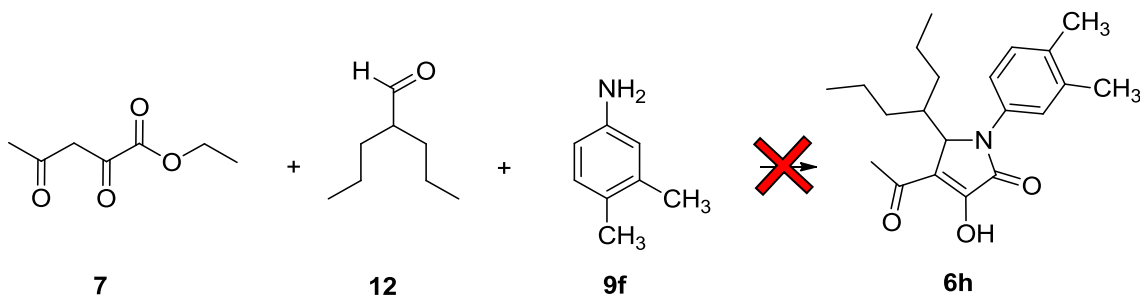
Scheme 2

We tried another synthetic route starting from 2-propyl pentanoic acid **13** that was reduced to the corresponding alcohol **14** (76% yield) with dimethyl sulphide borane complex in THF at room temperature for 4 hours¹⁹ and directly oxidized to desired aldehyde **12** in high yields (90%), by using Dess-Martin Periodinane reagent in DCM at room temperature in 2 hours (**Scheme 3**).



Scheme 3

Thus, it was tried the final step with 3,4-dimethylaniline **9f** and ethyl-2,4-dioxovalerate **7** to obtain the corresponding pyrrolidinone **6h** but without success. Even changing different conditions such as solvents, temperatures, and times of reaction¹⁷ (**Table 2**), unfortunately we couldn't isolate the desired product (**Scheme 4**).

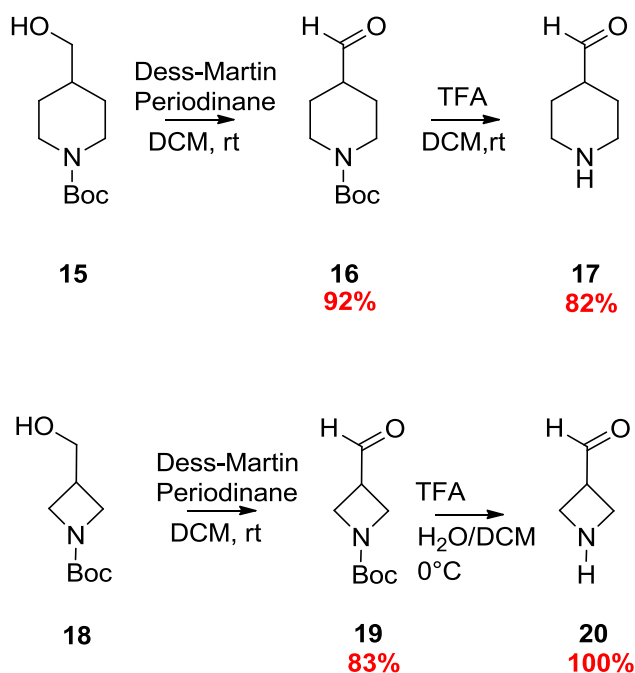


Scheme 4

Solvent	Temperature	Time of reaction	Result
Acetic acid glacial	95°C	2 h	Side products
Acetic acid glacial	95°C	3h 30min	Side products
Acetic acid glacial	105°C	2h 30min	Side products
THF	room temperature	24h	Side products

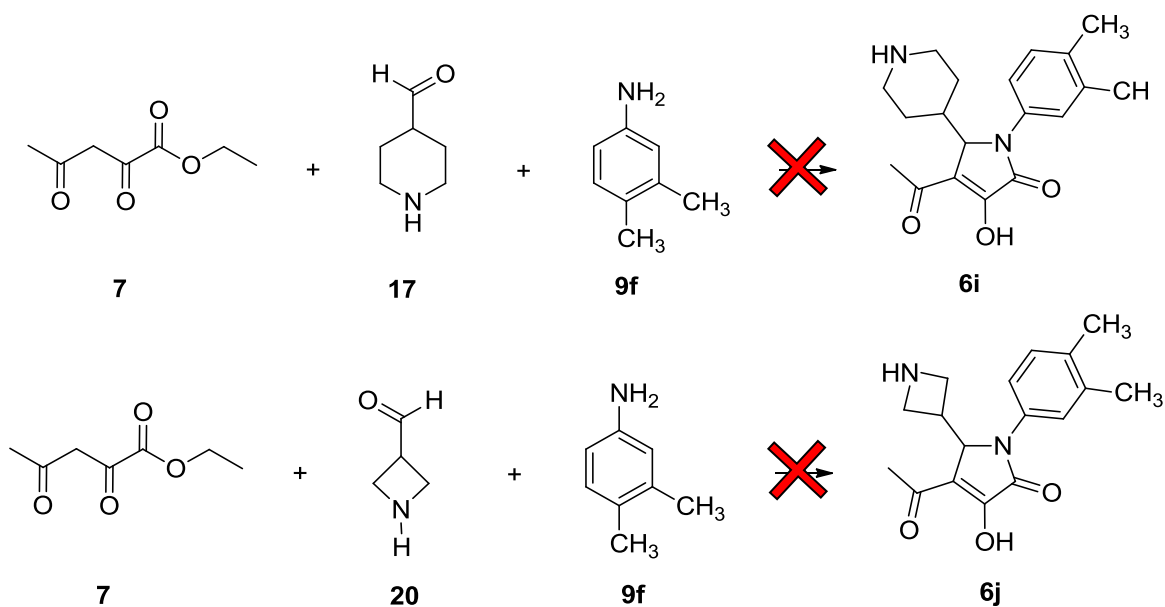
Table 2

To synthesize aldehydes **17** and **20** we started from the oxidation, respectively of 4-hydroxymethyl-piperidine, N-Boc **15** and *t*-butyl-3-(hydroxymethyl)azetidine-1-carboxylate **18** by using Dess-Martin Periodinane reagent as oxidative agent in DCM at room temperature 1-2 hours to obtain corresponding aldehydes N-Boc **16** and **19** in high yields (83-92%). The protective group N-Boc was removed for reaction with trifluoroacetic acid (TFA) in DCM at room temperature²⁰ or TFA in a mixture of DCM/Water at 0°C²¹. In both cases the reaction was complete in 1 hour (**Scheme 5**).



Scheme 5

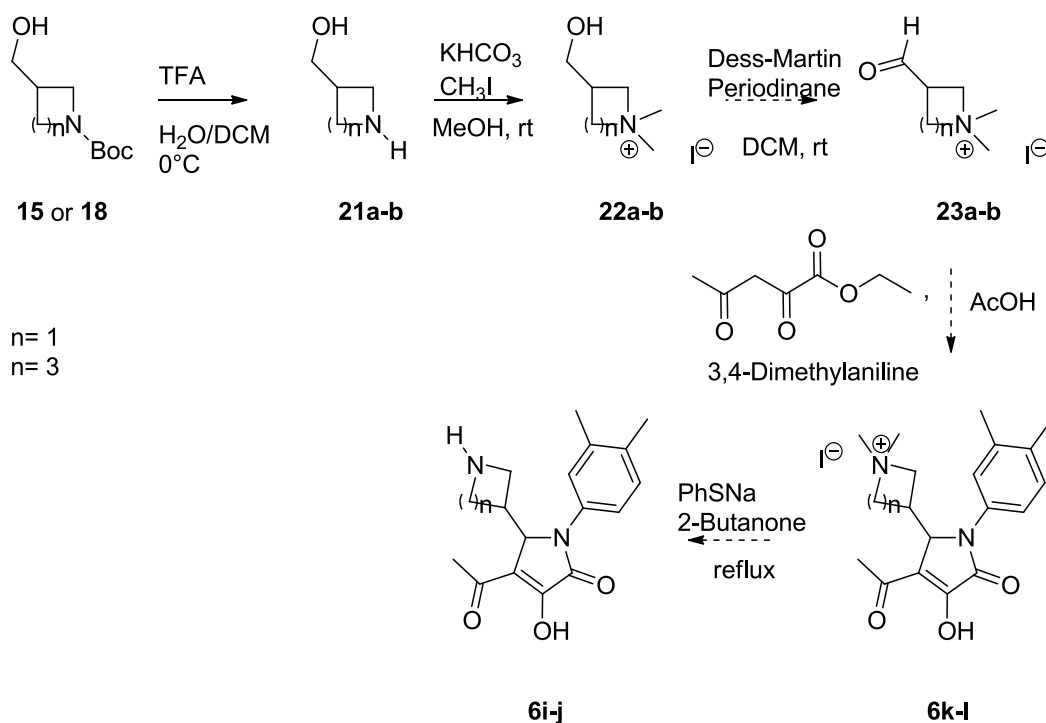
Also in both cases were tried the final step in glacial acetic acid with ethyl-2,4-dioxovalerate **7** and 3,4-dimethylaniline **9f** but we didn't isolate the desired products (**Scheme 6**). We also tried to carry out the reaction by using the intermediate products, **16** and **19** respectively, but unfortunately the result was the same.



Scheme 6

We through to change the protective group, quaternizing amines in an easy way with methyl iodide, potassium bicarbonate in methanol at 20°C for 24 hours. This method is mild and selective and doesn't attack hydroxyl group²². Furthermore by literature research these salts are common used during oxidation reactions. These properties are really important in our case.

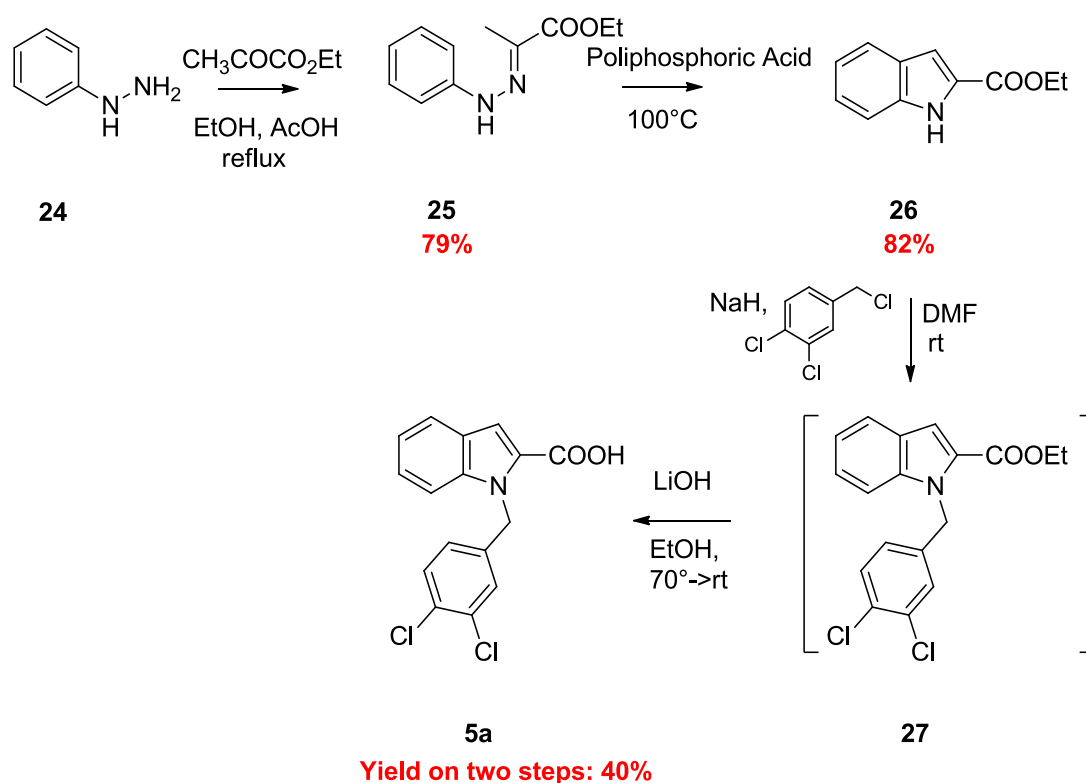
The idea was to oxidize the quaternary salts **22a-b** with Dess-Martin Periodinane reagent in DCM, after that trying to obtain pyrrolidinones **6k-l** and finally trough cleavage with sodium thiophenolate and 2-butanone at reflux²³ to isolate the final compounds **6i-j** (**Scheme 7**). We decided to start with the six members ring, 4-(hydroxymethyl)-piperidine, N-Boc **15**, that trough the acid catalyzed removal of the protective group *t*-Boc, using TFA and water in DCM at 0°C²¹, gave us amine **21a** in 65% of yield. Unfortunately, the next attempt of quaternization didn't work and for this reason this route was stopped and it wasn't tried for the corresponding four member ring **18**.



Scheme 7

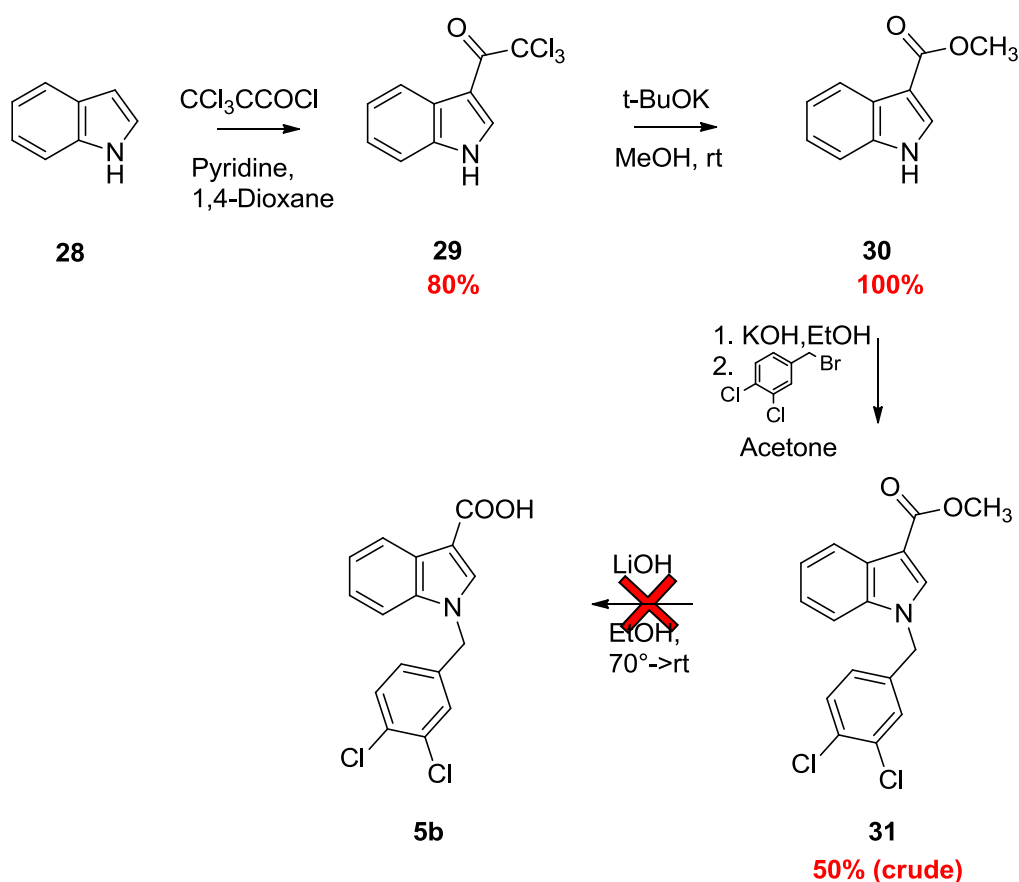
To synthesize N-benzylindole-carboxylic acid **5a-c** were planned different routes. 1-(3,4-Dichlorobenzyl)-1*H*-indole-2-carboxylic acid **5a** was obtained starting from phenylhydrazine **24** that was reacted with ethylpyruvate in acetic acid and ethanol at reflux for 3 hours to give in good yields (79%) the intermediate product **25**. This one by Fischer reaction with polyphosphoric acid at 100°C overnight²⁴ gave the indole **26** (82%), that was reacted with sodium hydride and 3,4-dichlorobenzylchloride in DMF, giving the intermediate **27** that was used as crude for the final step.

The first attempt of saponification was made by using a 12% KOH solution at reflux for 3 hours but it wasn't enough to obtain the carboxylic acid. We tried with lithium hydroxide in ethanol at 70°C for 3 hours and then at room temperature overnight. This time we isolated the desired 1-(3,4-dichlorobenzyl)-1*H*-indole-2-carboxylic acid **5a**^{12,16} (Scheme 8).



Scheme 8

In order to isolate 1-(3,4-dichlorobenzyl)-1*H*-indole-3-carboxylic acid **5b**²⁵ was planned a first synthetic route starting from indole **28**. This compound, in presence of trichloroacetyl chloride and pyridine in 1,4-dioxane at reflux for 1 hour and 20 minutes²⁶, was converted in the intermediate compound **29** and than to the corresponding ester **30**, in quantitative yields, by using potassium *t*-butoxide as base and methanol as solvent. Different methods were tried to obtain compound **31**, indeed the same method used for other derivate **27** (NaH in DMF with 3,4-dichlorobenzylchloride) didn't work. We tried to change the 3,4-dichlorobenzylchloride with more reactive 3,4-dichlorobenzylbromide in the same conditions but also in this case the reaction didn't work. Finally we obtained the compound **31**, working in two steps. In the first one, the starting material **30** was reacted with potassium hydroxide in ethanol and after the removal of solvent under reduced pressure, the residue was dissolved in acetone and reacted with the 3,4-dichlorobenzylbromide²⁷. The crude intermediate compound **31** was taken up onto the final step of saponification that unfortunately didn't work, also using longer time than the derivate **5a** (Scheme 9).

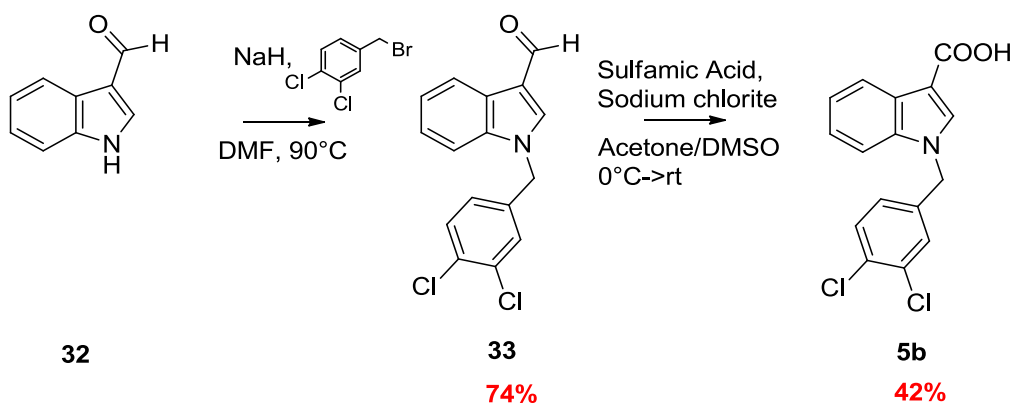


Scheme 9

Therefore, we decided to continue the synthesis of indole **5b** through another synthetic route that, at the end, was faster and luckier than the first one.

We started from the reaction of indole-3-carboxaldehyde **32** with sodium hydride and 3,4-dichlorobenzylbromide in DMF at 90°C for 3 hours and half²⁵. We obtained a good final result, isolating compound **33** in high yields (74%).

Later the oxidation reaction was made using sulfamic acid and sodium chlorite in a mixture of acetone and DMSO at 0°C for half hour and then at room temperature overnight. In this way, it was possible to obtain the final desired compound **5b** in good yields (42%) (**Scheme 10**).



Scheme 10

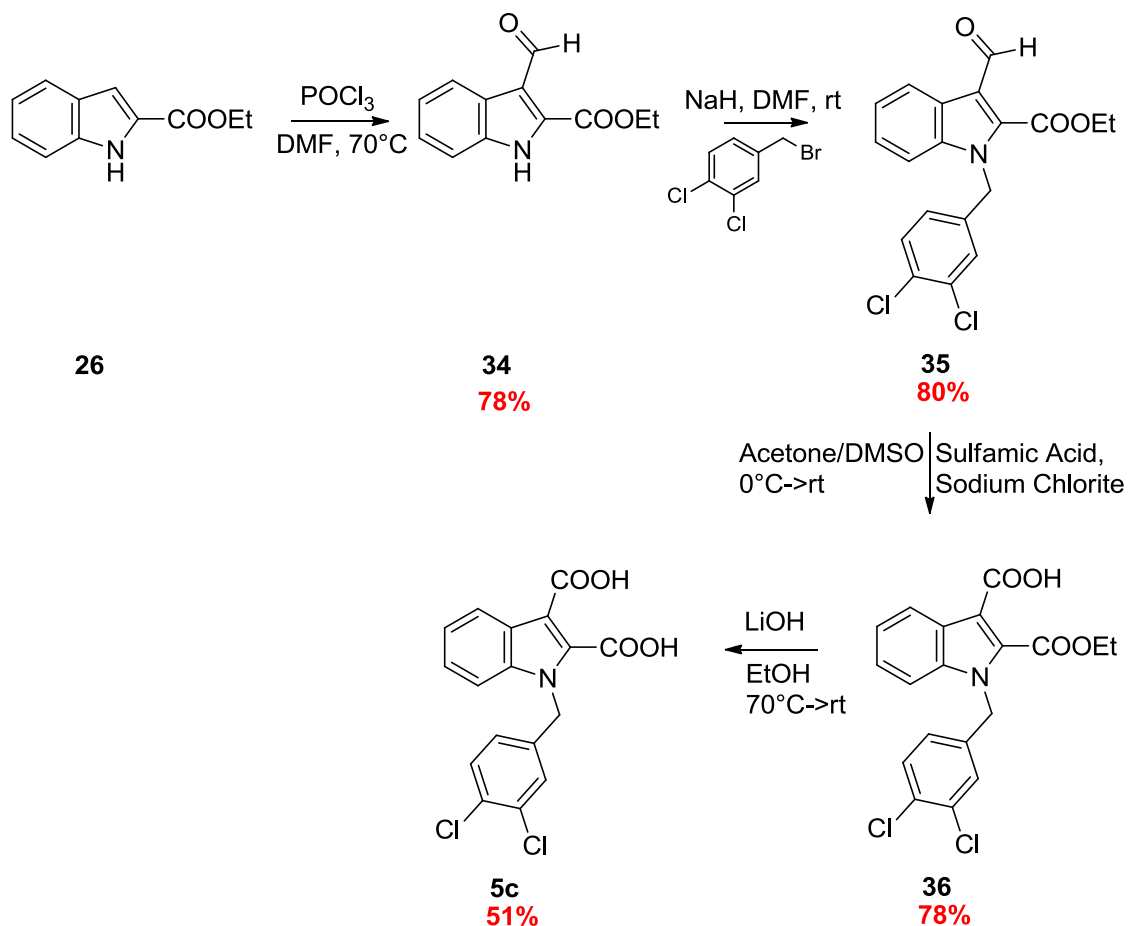
Another synthetic route was undertaken to isolate 1-(3,4-dichlorobenzyl)-1H-indole-2,3-dicarboxylic acid **5c**^{12,16}.

A first attempt consisted in a Vilsmeier-Haack reaction to insert an aldehyde function on the previous intermediate **27** (scheme 8), using phosphoryl chloride and DMF but with low results.

Therefore, we decided to start from another common intermediate **26** and try again Vilsmeier-Haack reaction²⁸, obtaining in this case the desired result in high yields (78%).

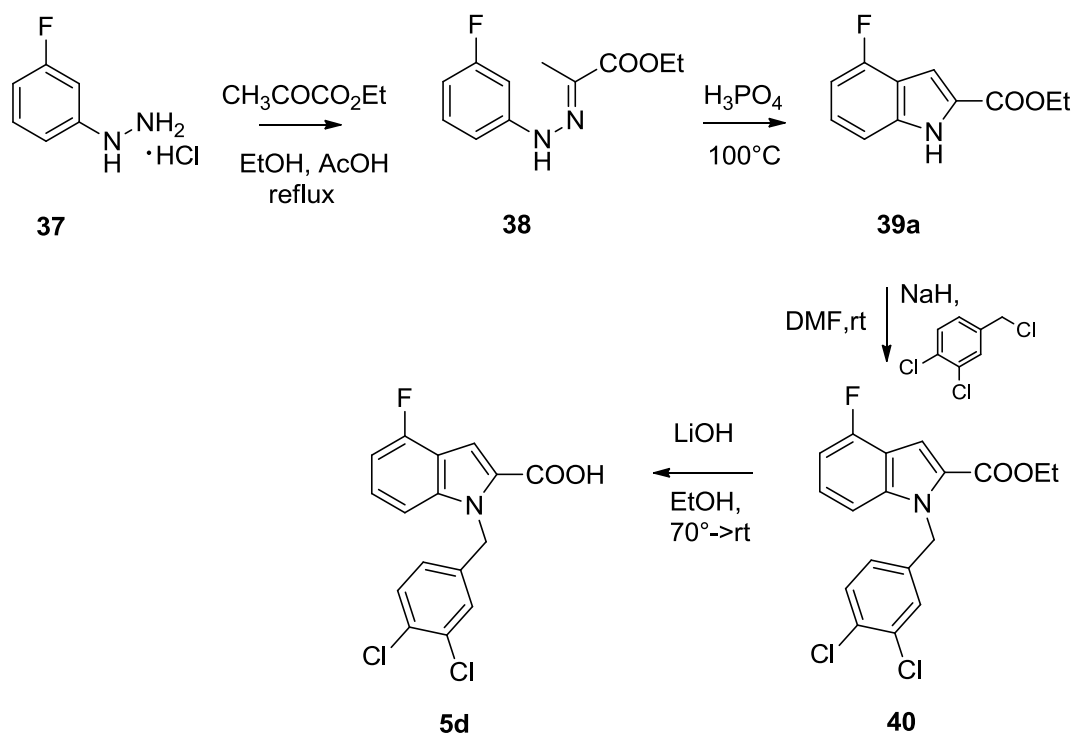
The further attack of 3,4-dichlorobenzylbromide to NH of indole **34**, was conducted using the same method described early. Intermediate compound **35** was later oxidised to the corresponding carboxylic acid **36** with sulfamic acid and sodium chlorite in a mixture of acetone and DMSO at 0°C for half hour and then at room temperature

overnight. The final saponification of compound **36** using lithium hydroxide in ethanol at 70°C lead to the desired 1-(3,4-dichlorobenzyl)-1H-indole-2,3-dicarboxylic acid **5c**^{12,16} (51%) (Scheme 11).



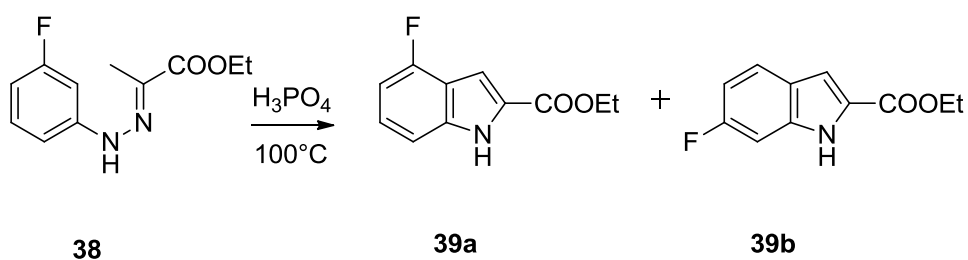
Scheme 11

Finally we through to synthesize also the 4F-(3,4-dichlorobenzyl)-1H-indole-2-carboxylic acid **5d**^{12,16} planning a similar route used for previous 1-(3,4-dichlorobenzyl)-1H-indole-2-carboxylic acid **5a** (scheme 8), starting from 3-Fluorophenylhydrazine hydrochloride **37** (Scheme12).



Scheme 12

Unfortunately in this case the presence of fluorine was a problem for the Fischer reaction (**Scheme 13**), in fact we obtained a mixture of the two possible isomers of indole **5d** of difficult separation.

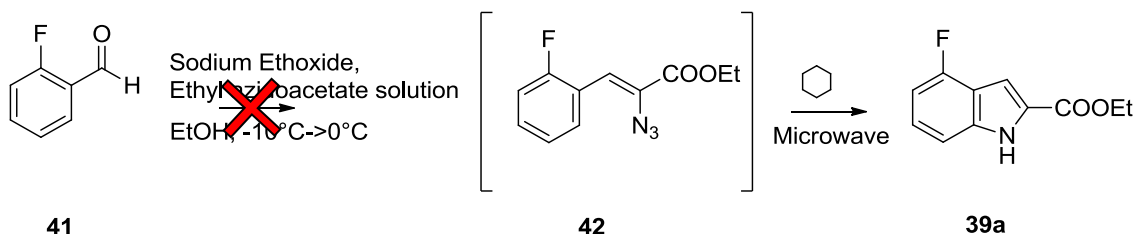


Scheme 13

We decided to try another synthetic route changing the steps to isolate the ethyl 4-fluoroindole-2-carboxylate **39a**.

The first try was made starting from 2-fluorobenzaldehyde **41**, dissolved in a commercial 25% solution of ethyl azidoacetate in ethanol, that in presence of sodium

ethoxide in absolute ethanol would bring to isolate the corresponding azidoacrylic ester **42** that directly as crude would be dissolved in cyclohexane and subjected to microwave heating to give the desired indole **39a** (Scheme 14).



Scheme 14

Unfortunately, these reactions didn't work and we thought to create directly *in situ* sodium ethoxide dissolving sodium in absolute ethanol, and adding at -10°C the solution of 2-fluorobenzaldehyde **41** in ethyl azidoacetate. The resulting yellow suspension was stirred at -10°C for 1 hour and half and for another 1 hour and half at 0°C ²⁹, but also this attempt didn't work.

In the last attempt we changed the times of reaction, indeed we kept the stirring suspension at -10°C for two hours and overnight at 0°C , but unfortunately the result was the same also this time.

Other attempts to obtain indole **5d** will be tried since the really interesting activity against CCR2 from literature research.

RESULTS AND DISCUSSION: BIOLOGY

All compounds synthesized belong to the N-benzylindole-2-carboxylic acid's and pyrrolidinone's classes (**5-6**) were submitted to evaluate their activity against CCR2, and also against CCR1 as high sequence similarity of these receptors. CCR1 was involved in different biological processes such as leukocyte trafficking, gene transcription, apoptosis and also the binding of chemokines CCL3, CCL5 and CCL7 to CCR1 plays an important role in inflammatory diseases as multiple sclerosis or rheumatoid arthritis for the monocytes', macrophages' and TH1 cells' trafficking. Indeed antagonism of the binding CCR1-its chemokines could blocks chemotaxis of these immune cells³⁰.

The biological screening were assessed in the biological laboratories of Professor IJerman and are indicated in **table 3**. [³H]-CCR2-RA-[R] binding assays were performed as reported in the methods section.

It was very interesting to note as compounds highly active against CCR2 receptor were also active against CCR1 receptor. This is a really interesting as these molecules were tested, for the first time, also against CCR1 receptor. These molecules seem dual antagonists and some with very good affinity actually, as **6a-e** and **5a,c**.

All the molecules showed interesting value of affinity with the two receptors at the tested concentrations. Regarding pyrrolidinone scaffold we understood that modifications in R₂ weren't really important in terms of selectivity, in opposite to other ones in R₁ and R₃. Furthermore the presence of cyclohexyl group confirms its importance for the binding to the receptors CCR2 and CCR1, while the attempt of its substitution with an open chain, decreases the affinity values. In addition the presence of halogens on the aromatic ring (bromine, fluorine, chlorine) increases the binding affinity, indeed compounds **6a-e** are the most active compounds of all. Compound **6b**, bearing fluorine and chlorine on the aromatic ring, shows the best results against both receptors, reaching value of 15 nM and 10 nM of K_i against CCR1 and CCR2 respectively.

Regarding benzyl-indoles **5a-c** was interesting to investigate how different positions of the carboxylic group on the indole ring can influence their activity. Indeed, compounds with carboxylic group in position 2 (**5a,c**) were resulted better than other one with COOH in position 3 (**5b**), but there isn't a clear trend of selectivity.

					CCR1	CCR2
Cmp	Scaffold	R ₁	R ₂	R ₃	pK _i ± SEM (K _i , nM)	pK _i ± SEM (K _i , nM)
6a		<i>c-hexyl</i>	<i>4-Br Ph</i>	<i>-Me</i>	7,6 ± 0,0 (24)	7,8 ± 0,1 (17)
6b		<i>c-hexyl</i>	<i>2-F, 4-Cl Ph</i>	<i>-Me</i>	7,8 ± 0,1 (15)	8,0 ± 0,1 (10)
6c		<i>c-hexyl</i>	<i>3-Me, 4-Cl Ph</i>	<i>-Me</i>	7,5 ± 0,0 (31)	8,1 ± 0,2 (8)
6d		<i>c-hexyl</i>	<i>2-F, 4-Me Ph</i>	<i>-Me</i>	7,6 ± 0,1 (29)	7,5 ± 0,1 (34)
6e		<i>c-hexyl</i>	<i>4-Cl Ph</i>	<i>-Me</i>	7,4 ± 0,1 (40)	7,8 ± 0,1 (18)
6f		<i>3-pentyl</i>	<i>3,4-diMe Ph</i>	<i>-Me</i>	6,9 ± 0,1 (117)	7,0 ± 0,1 (108)
6g		<i>3-pentyl</i>	<i>4-Me Ph</i>	<i>-Me</i>	6,9 ± 0,1 (120)	6,8 ± 0,0 (164)
5a					7,2 ± 0,1 (66)	7,7 ± 0,0 (18)
5b					7,42% ^a	23,47% ^a
5c					7,1 ± 0,0 (74)	7,5 ± 0,1 (36)

Values are means ± SEM. of at least three independent experiments performed in duplicate.

^a % of displacement at 1 μM, representing the mean of two independent experiments

Table 3

In conclusion most of our compounds showed good affinity against the two receptors tested but they aren't very selective against CCR1 and CCR2. It was possible that dual antagonists might be better than just one for diseases in which both receptors are involved, using only one drug than two.

EXPERIMENTAL SECTION

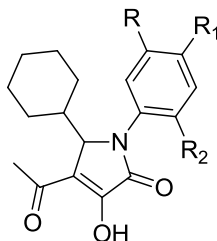
CHEMISTRY

General Methods

^1H and ^{13}C NMR spectra were recorded on a Bruker AV 400 liquid spectrometer (^1H NMR, 400 MHz; ^{13}C NMR 100 MHz). All melting points were taken on a Büchi-Tottoli apparatus and are uncorrected. Analytical purity of the final compounds was determined by high-performance liquid chromatography (HPLC) with a Phenomenex Gemini 3 μm C18 110A column (50 x 4.6 mm, 3 μm), measuring UV absorbance at 254 nm. All compounds showed a single peak at the designated retention time and are at least 95% pure. IR spectra were determined with a Shimadzu FT-IR/ATR spectrophotometer. Microwave reactions were carried out in a Biotage Emrys™ Optimizer using sealed tubes and at a set reaction temperature. Liquid chromatography–mass spectrometry (LC–MS) analyses were performed using Thermo Finnigan Surveyor—LCQ Advantage Max LC–MS system and a Gemini C18 Phenomenex column (50 x 4.6 mm, 3 μm). Purification by column chromatography was achieved by use of Grace Davison Davisil silica column material (LC60A, 30–200 μm).

Pyrrolidinones' series

Synthesis of pyrrolidinones (6a-e)¹⁷:



To a stirring solution of appropriate substituted aniline **9a-g** (4.42 mmol) in acetic acid (4.2 mL) at room temperature were added cyclohexane carboxaldehyde **8a** (4.42 mmol)

and ethyl-2,4-dioxovalerate **7** (4.42 mmol). The reaction mixture was heated to 95°C for 2 hours. Upon cooling to room temperature it was concentrated and diethyl ether (10.5 mL) was added. The resulting mixture was stirred for half an hour, whereupon a precipitate forms that was collected by filtration.

4-Acetyl-1-(4-bromophenyl)-5-cyclohexyl-3-hydroxy-1,5-dihydro-2H-pyrrol-2-one (6a):

This compound was obtained from reaction of 4-bromoaniline **9a** with cyclohexane carboxaldehyde **8a** and ethyl-2,4-dioxovalerate **7** in acetic acid in 2 hours at 95°C. Yield: 53%, white solid; mp: 228-230°C; FT-IR/ATR: 3178, 2926, 2850, 1674, 1651, 1492, 1446, 1406, 1392, 1217, 1197 cm⁻¹; ¹H NMR (400 MHz, DMSO- *d*₆) δ: 7.63 (d, *J* = 8.7 Hz, 2H), 7.52 (d, *J* = 8.7 Hz, 2H), 5.07 (d, *J* = 1.6 Hz, 1H), 2.44 (s, 3H), 1.83 (t, *J* = 10.8 Hz, 1H), 1.59 (d, *J* = 12.0 Hz, 1H), 1.52-1.34 (m, 4H), 1.03-0.77 (m, 4H), 0.53 (q, *J* = 12.4 Hz, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 195.81 (s), 164.87 (s), 147.40 (s), 137.21 (s), 133.01 (s), 132.26 (d x 2), 126.68 (d x 2), 119.03 (s), 61.86 (q), 40.71 (d), 30.95 (t), 30.03 (t), 27.04 (t), 26.84 (t), 26.45 (t); MS: 380.00; HPLC purity: 98,7% ; t_R: 10.18 min.

4-Acetyl-1-(4-chloro-2-fluorophenyl)-5-cyclohexyl-3-hydroxy-1,5-dihydro-2H-pyrrol-2-one (6b):

This compound was obtained from reaction of 4-chloro-2-fluoroaniline **9b** with cyclohexane carboxaldehyde **8a** and ethyl-2,4-dioxovalerate **7** in acetic acid in 2 hours at 95°C. Yield: 12%, light yellow solid; mp: 195-198°C; FT-IR/ATR: 3151, 2929, 1689, 1643, 1501, 1220 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ: 9.10 (bs, 1H), 7.39 (t, *J* = 8.2 Hz, 1H), 7.28-7.27 (m, 1H), 7.25 (s, 1H), 4.98 (d, *J* = 1.7 Hz, 1H), 2.55 (s, 3H), 1.97 (td, *J* = 12.4, 2.1 Hz, 1H), 1.71 (d, *J* = 13.4 Hz, 1H), 1.60-1.47 (m, 4H), 1.17-0.85 (m, 4H), 0.65 (qd, *J* = 12.4, 3.0 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ: 194.35 (s), 165.03 (s), 156.24 (d, *J*_{C-F} = 253.0 Hz), 153.07 (s), 134.38 (d, *J*_{C-F} = 9.0 Hz), 128.87 (d), 125.31 (d), 123.51 (s), 120.15 (s), 117.85 (d, *J*_{C-F} = 23.0 Hz), 64.04 (d), 40.76 (q), 30.24 (t), 30.13 (d), 26.86 (t), 26.50 (t), 26.35 (t), 26.30 (t); MS: 352.13 ; HPLC purity: 95,0% ; t_R: 10.02 min

4-Acetyl-1-(4-chloro-3-methylphenyl)-5-cyclohexyl-3-hydroxy-1,5-dihydro-2H-pyrrol-2-one (6c):

This compound was obtained from reaction of 4-chloro-3-methylaniline **9c** with cyclohexane carboxaldehyde **8a** and ethyl-2,4-dioxovalerate **7** in acetic acid in 2 hours at 95°C. Yield: 33%, white solid; mp: 217-218°C; FT-IR/ATR: 3076, 2931, 2852, 1687, 1643, 1483, 1415, 1276, 1224, 1199 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ: 7.42 (d, *J* = 8.4 Hz, 1H), 7.35 (d, *J* = 2.4 Hz, 1H), 7.19 (dd, *J* = 8.4, 2.4 Hz, 1H), 4.96 (d, *J* = 2.0 Hz, 1H), 2.57 (s, 3H), 2.44 (s, 3H), 1.95 (td, *J* = 12.6, 2.0 Hz, 1H), 1.70 (d, *J* = 12.6 Hz, 1H), 1.62-1.48 (m, 3H), 1.44 (d, *J* = 12.6 Hz, 1H), 1.14-0.87 (m, 4H), 0.68 (qd, *J* = 12.6, 3.6 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ: 194.20 (s), 176.53 (s), 153.27 (s), 137.45 (s), 135.31 (s), 132.78 (s), 129.91 (d), 126.52 (d), 122.83 (d), 119.50 (s), 63.44 (q), 40.70 (q), 30.32 (t), 29.95 (d), 27.48 (t), 26.75 (t), 26.49 (t), 26.29 (t), 20.53 (d); MS: 348.07; HPLC purity: 99,2% ; t_R: 10.45 min.

4-Acetyl-5-cyclohexyl-1-(2-fluoro-4-methylphenyl)-3-hydroxy-1,5-dihydro-2H-pyrrol-2-one (6d):

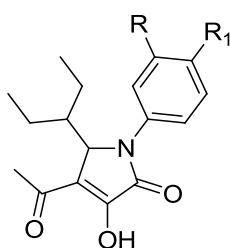
This compound was obtained from reaction of 4-fluoro-3-methylaniline **9d** with cyclohexane carboxaldehyde **8a** and ethyl-2,4-dioxovalerate **7** in acetic acid in 2 hours at 95°C. Yield: 35%, white solid; mp: 204-207°C; FT-IR/ATR: 3190, 2926, 2851, 1681, 1651, 1519, 1402, 1305, 1220 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ: 7.25 (t, *J* = 8.3 Hz, 1H), 7.04-6.99 (m, 2H), 4.93 (d, *J* = 1.6 Hz, 1H), 2.55 (s, 3H), 2.42 (s, 3H), 1.97 (td, *J* = 12.5, 2.0 Hz, 1H), 1.70 (d, *J* = 12.5 Hz, 1H), 1.58-1.45 (m, 4H), 1.16-0.86 (m, 4H), 0.65 (qd, *J* = 12.5, 3.4 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ: 194.42 (s), 165.19 (s), 156.98 (d, *J*_{C-F} = 250.0 Hz), 153.47 (s), 140.31 (s), 127.85 (d), 125.53 (d), 121.94 (s), 120.09 (s), 117.55 (d, *J*_{C-F} = 19.0 Hz), 64.17 (q), 40.59 (q), 30.34 (d), 30.05 (t), 26.91 (t), 26.50 (t), 26.43 (t), 26.35 (t), 21.36 (d); MS: 332.07; HPLC purity: 95,5% ; t_R: 9.75 min.

4-Acetyl-1-(4-chlorophenyl)-5-cyclohexyl-3-hydroxy-1,5-dihydro-2H-pyrrol-2-one (6e):

This compound was obtained from reaction of 4-chloroaniline **9e** with cyclohexane carboxaldehyde **8a** and ethyl-2,4-dioxovalerate **7** in acetic acid in 2 hours at 95°C. Yield: 48%, yellow solid; mp: 229-231°C; FT-IR/ATR: 3070, 2983, 2926, 1708, 1645,

1458, 1394, 1236, 1163, 1139, 1122 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ : 8.70 (bs, 1H), 7.41 (d, $J = 4.3\text{Hz}$, 4H), 4.95 (d, $J = 2.5\text{ Hz}$, 1H), 2.53 (s, 3H), 1.93 (td, $J = 12.3, 2.7\text{ Hz}$, 1H), 1.68 (d, $J = 12.3\text{ Hz}$, 1H), 1.60-1.52 (m, 3H), 1.42 (d, $J = 12.3\text{ Hz}$, 1H), 1.15-0.84 (m, 4H), 0.66 (qd, $J = 12.3, 3.5\text{ Hz}$, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ : 194.07 (s), 164.89 (s), 143.7 (s), 135.42 (s), 132.36 (s), 129.54 (d x 2), 125.21 (d x 2), 119.03 (s), 63.11 (d), 40.67 (d), 30.06 (q), 29.93 (t), 27.38 (t), 26.65 (t), 26.38 (t), 26.17 (t); MS: 334.00; HPLC purity: 97,9% ; t_{R} : 10.03 min.

Synthesis of Pyrrolidinones (6f-g)¹⁷:



To a stirring solution of appropriate substituted aniline **9f-g** (5.77 mmol) in acetic acid (4.2 mL) at room temperature were added 2-ethylbutyraldehyde **8b** (5.77 mmol) and ethyl-2,4-dioxoalate **7** (5.77 mmol). The reaction mixture was heated to 95°C for 2 hours. Upon cooling to room temperature it was concentrated and diethyl ether (10.5 mL) was added. The resulting mixture was stirred for half an hour, whereupon a precipitate forms that was collected by filtration. In absence of precipitate, diethyl ether was removed under reduced pressure and the crude was dissolved in few ml of water and extracted with ethyl acetate (3 x 10 mL), dried over anhydrous MgSO_4 , filtered and evaporated. It was purified by silica gel column chromatography (DCM:Ethyl Acetate, 7:3) to give the desired product.

4-Acetyl-1-(3,4-dimethylphenyl)-3-hydroxy-5-(pentan-3-yl)-1,5-dihydro-2H-pyrrol-2-one (6f):

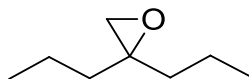
This compound was obtained from reaction of 3,4-dimethylaniline **9f** with 2-ethylbutyraldehyde **8b** and ethyl-2,4-dioxoalate **7** in acetic acid in 2 hours at 95°C. Yield: 9%, white solid; mp: 198-200°C; FT-IR/ATR: 3097, 2960, 2903, 1685, 1647, 1506, 1456, 1417, 1394, 1284, 1219 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ : 9.23 (bs, 1H),

7.20 (d, $J = 8.0$ Hz, 2H), 7.17 (d, $J = 2.0$ Hz, 1H), 7.10 (dd, $J = 8.0, 2.0$ Hz, 1H) 5.13 (d, $J = 1.2$ Hz, 1H), 2.55 (s, 3H), 2.31 (s, 3H), 2.30 (s, 3H), 1.70 (quint, $J = 6.8$ Hz, 1H), 1.37-1.26 (m, 1H), 1.23-1.12 (m, 1H), 1.08-0.98 (m, 1H), 0.93-0.89 (m, 4H), 0.84 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ : 194.33 (s), 166.17 (s), 153.07 (s), 137.72 (s), 135.99 (s), 134.12 (s), 130.33 (d), 125.76 (d), 122.02 (d), 120.19 (s), 60.38 (q), 43.74 (q), 30.47 (q), 23.18 (t), 21.52 (t), 20.02 (d), 19.53 (d), 12.90 (q), 12.28 (q); MS: 316.00; HPLC purity: 99,8% ; t_{R} : 10.06 min.

4-Acetyl-3-hydroxy-1-(4-methylphenyl)-5-(pentan-3-yl)-1,5-dihydro-2H-pyrrol-2-one (6g):

This compound was obtained from reaction of *p*-toluidine **9g** with 2-ethylbutyraldehyde **8b** and ethyl-2,4-dioxovalerate **7** in acetic acid in 2 hours at 95°C. Yield: 39%, yellow solid; mp: 138-139°C; FT-IR/ATR: 3103, 2962, 2931, 1683, 1647, 1517, 1454, 1400, 1276, 1220, 1193 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ : 9.53 (bs, 1H), 7.30-7.25 (m, 4H), 5.14 (d, $J = 1.6$ Hz, 1H), 2.56 (s, 3H), 2.40 (s, 3H), 1.70 (quint, $J = 6.8$ Hz, 1H), 1.36-1.25 (m, 1H), 1.22-1.11 (m, 1H), 1.06-0.97 (m, 1H), 0.93-0.89 (m, 4H), 0.83 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ : 194.36 (s), 165.39 (s), 152.85 (s), 137.26 (s), 133.87 (s), 129.90 (d x 2), 124.56 (d x 2), 120.46 (s), 60.46 (q), 43.74 (q), 30.60 (d), 23.21 (t), 21.52 (t), 21.19 (d), 12.89 (q), 12.31 (q); MS: 302.03; HPLC purity: 99,9% ; t_{R} : 9.69 min.

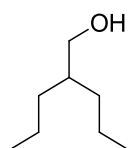
Synthesis of 2-propyl-1,2-epoxypentane (11)¹⁸:



To a stirring mixture of sodium hydride (0.33 g, 14 mmol) and trimethylsulfoxonium iodide (2.9 g, 13.12 mmol) under N_2 atmosphere DMSO (10.6 mL) was slowly added. Hydrogen evolution caused and when the gas evolution was complete, 4-heptanone **10** (1 g, 8.75 mmol) was added and the reaction mixture was heated at 70°C for 5 hours. After cooling to room temperature, the mixture reaction was poured into water (15 mL) and extracted with ethyl acetate (3 x 15 mL). The organic layer was washed with water

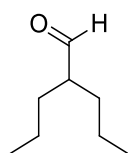
(20 mL) and brine (20 mL), dried over anhydrous MgSO_4 , filtered and evaporated to give the desired compound **12**. The epoxide was used for the next step without further purification that unfortunately didn't work. Yield: 40%. The spectroscopic data are in agreement with the literature¹⁸.

Synthesis of 2-propylpentan-1-ol (14):



To a solution of 2-propyl-pentanoic acid **13** (1 g, 6.93 mmol) in THF (8.15 mL) at 0°C was added slowly borane dimethyl sulfide complex (2.8 mL, 2.77 mmol). The reaction mixture was stirred with gradual warming to room temperature for 4 hours, recooled to 0°C and quenched with MeOH (8-10 mL). It was concentrated under reduced pressure and the resulting crude was diluted with water (10 mL) and extracted with ethyl acetate (3 x 10 mL). The organic extract was washed with brine, dried over anhydrous MgSO_4 , filtered and evaporated *in vacuo* to give the desired compound **14**, which was used without further purification. Yield: 76%, colorless oil. ^1H NMR (400 MHz, CDCl_3) δ : 0.85 (t, $J = 7.2$ Hz, 6H), 1.18-1.31 (m, 8H), 1.42-1.44 (m, 1H), 3.09 (s, 1H), 3.43 (d, $J = 5.6$ Hz, 2H).

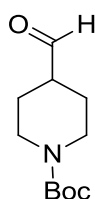
Synthesis of 2-propylpentanal (12):



2-Propylpentan-1-ol **14** (0.3 g, 2.3 mmol) was dissolved in DCM (15 mL) and under N_2 atmosphere was added Dess-Martin Periodinane reagent (1.4 g, 3.22 mmol) and the reaction mixture was stirred at room temperature for 2 hours. The solution was diluted with water (25 mL) and extracted with diethyl ether (3 x 15 mL). The organic layer was

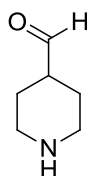
washed with an aqueous 5% $\text{Na}_2\text{S}_2\text{O}_3$ solution (30 mL) and an aqueous saturated NaHCO_3 solution (30 mL), dried over anhydrous MgSO_4 , filtered and concentrated *in vacuo* to give desired aldehyde **12**. Yield: 90%, white solid. The spectroscopic data are in agreement with the literature¹⁸.

Synthesis of 1-*t*-butoxycarbonyl-4-formylpiperidine (16):



4-(Hydroxymethyl)-piperidine, N-Boc **15** (0.2 g, 0.93 mmol) was dissolved in DCM (5 mL) and under N_2 atmosphere was added Dess-Martin Periodinane reagent (0.55 g, 1.30 mmol) and the reaction mixture was stirred at room temperature for 1 hour. The solution was diluted with water (20 mL) and extracted with diethyl ether (3 x 5 mL). The organic layer was washed with an aqueous 5% $\text{Na}_2\text{S}_2\text{O}_3$ solution (20 mL) and an aqueous saturated NaHCO_3 solution (20 mL), dried over anhydrous MgSO_4 , filtered and evaporated. It was purified by silica gel column chromatography (DCM:Ethyl Acetate, 8:2) to give the desired product **16**. Yield: 92%, colorless oil. The spectroscopic data are in agreement with the literature³¹.

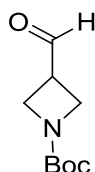
Synthesis of 4-formylpiperidine (17):



To a solution of 1-*t*-butoxycarbonyl-4-formylpiperidine **16** (0.5 g, 2.34 mmol) in DCM (2 mL) was added TFA (1.65 g, 14.51 mmol), and the resulting solution was stirred at room temperature for 2 hours. Concentration of the solution and the concentrate was poured into an aqueous saturated NaHCO_3 solution (10 mL) and extracted with ethyl

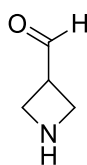
acetate (3 x 10 mL). The organic layer was dried over anhydrous MgSO₄, filtered and concentrated *in vacuo*. The crude was taken up onto the final step that unfortunately didn't work.

Synthesis of tert-butyl 3-formylazetidine-1-carboxylate (19):



t-Butyl-3-(hydroxymethyl)azetidine-1-carboxylate **18** (1.0 g, 5.34 mmol) was dissolved in DCM (25 mL) and under N₂ atmosphere was added Dess-Martin Periodinane reagent (3.2 g, 7.48 mmol) and the reaction mixture was stirred at room temperature for 3 hours. The solution was diluted with water (40 mL) and extracted with diethyl ether (3 x 25 mL). The organic layer was washed with an aqueous 5% Na₂S₂O₃ solution (50 mL) and an aqueous saturated NaHCO₃ solution (50 mL), dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (DCM:Ethyl Acetate, 75:25) to give the desired aldehyde **19**. Yield: 83%, colorless oil. The spectroscopic data are in agreement with the literature³².

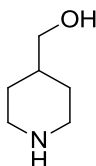
Synthesis of azetidine-3-carbaldehyde (20):



To a solution of *t*-butyl-3-formylazetidine-1-carboxylate **19** (0.32 g, 1.75 mmol) in DCM (7.3 mL) were added TFA (7.3 mL) and water (0.75 mL) and the resulting solution was stirred at 0°C for 1 hour. After concentration the crude was dissolved in a mixture of MeOH:H₂O (1:3, 29.21 mL) and neutralized with 2M NaOH solution. The reaction mixture was stirred at 0°C for 12 hours. The most of solvent was removed

under reduced pressure and the concentrate was extracted with ethyl acetate (3 x 20 mL). The crude was taken up onto the final step that unfortunately didn't work.

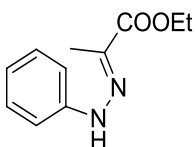
Synthesis of (piperidin-4yl)methanol (21a):



To a solution of 4-(hydroxymethyl)-piperidine, N-Boc **15** (0.3 g, 1.39 mmol) in DCM (5.8 mL) were added TFA (5.8 mL) and water (0.6 mL) and the resulting solution was stirred at 0°C for 1 hour. After concentration the crude was dissolved in a mixture of MeOH:H₂O (1:3, 23.2 mL) and neutralized with 2M NaOH solution. The reaction mixture was stirred at 0°C for 12 hours. The most of solvent was removed under reduced pressure and the concentrate was extracted with ethyl acetate (3 x 15 mL). Yield: 65%, light yellow oil. The spectroscopic data are in agreement with the literature³³.

Indoles' series

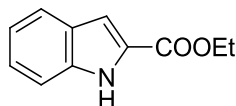
Synthesis of ethyl 2-(2-phenylhydrazinylidene)propanoate (25):



A mixture of phenylhydrazine **24** (1.0 g, 9.2 mmol), ethyl pyruvate (1.74 g, 15 mmol), and glacial acetic acid (0.10 mL) in anhydrous ethanol (9.92 mL) was heated to reflux for 3 hours. Upon cooling to room temperature, the most of solvent was removed under reduced pressure, and the concentrate was extracted with ethyl acetate (3 x 10 mL), dried over anhydrous MgSO₄, filtered and evaporated to give the crude product. It was purified by silica gel column chromatography (DCM) to afford the desired product **25**. Yield: 79%, yellow solid; ¹H NMR (400 MHz, CDCl₃) δ: 7.67 (s, 1H), 7.31 (t, *J* = 7.2

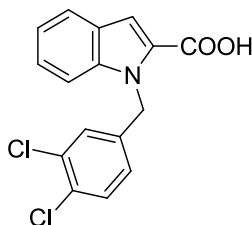
Hz, 2H), 7.21 (d, $J = 1.2$ Hz, 2H), 6.97 (t, $J = 7.2$ Hz, 2H), 4.32 (q, $J = 7.2$ Hz, 2H), 2.11 (s, 3H), 1.38 (t, $J = 7.2$ Hz, 3H). The data are in agreement with the literature. Other spectroscopic data (mp, IR, ^{13}C -NMR) are reported as well³⁴.

Synthesis of ethyl 1*H*-indole-2-carboxylate (26):



A mixture of ethyl 2-(2-phenylhydrazinylidene)propanoate **25** (0.8 g, 3.87 mmol) and polysphoric acid (9.3 g, 95.03 mmol) was heated to 100°C overnight, then it was poured into crush ice with acutely stirring, extracted with ethyl acetate (3 x 15 mL), dried over anhydrous MgSO_4 , filtered and concentrated to give the crude product, that was used directly for the next step without purification. Yield: 82%, brown solid; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ : 11.87 (s, 1H), 7.65 (d, $J = 8.0$ Hz, 1H), 7.45 (d, $J = 8.4$ Hz, 1H), 7.25 (t, $J = 7.6$ Hz, 1H), 7.14 (d, $J = 1.6$, 1H), 7.07 (t, $J = 7.6$ Hz, 1H), 4.33 (q, $J = 7.2$ Hz, 2H), 1.33 (t, $J = 7.2$ Hz, 3H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ : 161.39 (s), 137.52 (s), 127.41 (s), 126.8 (s), 124.68 (d), 122.10 (d), 120.18 (d), 112.70 (d), 107.73 (d), 60.52 (t), 14.33 (q). The data are in agreement with the literature. Other spectroscopic data (mp, IR) are reported as well^{28,35}.

Synthesis of 1-(3,4-dichlorobenzyl)-1*H*-indole-2-carboxylic acid (5a):

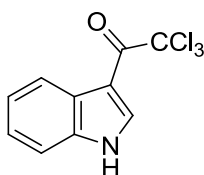


To a solution of ethyl 1*H*-indole-2-carboxylate **26** (0.35 g, 1.85 mmol) in dry DMF (4 mL), sodium hydride (0.053 g, 2.22 mmol) was added at 0°C and the reaction mixture was stirred for 1 hour and half at room temperature. 3,4-dichlorobenzyl chloride (0.54 g,

2.27 mmol) was added and the reaction mixture was stirred at room temperature for 1 hour and half. Then the reaction was poured into ice and brine (10 mL) and the aqueous solution was extracted with ethyl acetate (3 x 10 mL), dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure. The crude was taken up onto the next step.

To a suspension of crude (0.2 g, 0.5 mmol) in ethanol (5 mL) was added lithium hydroxide (0.07 g, 2.85 mmol) and the reaction mixture was heated to 70°C for 3 hours, and then at room temperature overnight. It was poured into ice water and acidified with 1M HCl. A light yellow precipitate was filtered off. Yield on two step: 40%, light yellow solid; mp: 189-190°C; FT-IR/ATR: 2983, 2870, 1708, 1645, 1506, 1458, 1394, 1238, 1122 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ: 7.75 (d, *J* = 8.0 Hz, 1H), 7.56 (s, 1H), 7.36 (d, *J* = 7.6 Hz, 1H), 7.31 (t, *J* = 8.0 Hz, 2H), 7.21 (t, *J* = 7.6 Hz, 1H), 7.17 (s, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 5.78 (s, 2H); ¹³C NMR (100 MHz, CDCl₃) δ: 166.06 (s), 139.94 (s), 138.35 (s), 132.84 (s), 131.43 (s), 130.70 (d), 128.39 (d), 126.48 (d), 126.17 (s), 126.03 (s), 125.76 (d), 123.29 (d), 121.50 (d), 113.83 (d), 110.60 (d), 47.03 (t); MS: no signal in ms, but previous reaction (ethylester) showed correct ms; HPLC purity: 98,2%; t_R: 10.28 min. The spectroscopic data are in agreement with the literature^{12,16}.

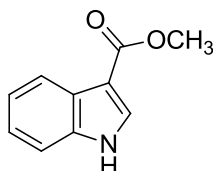
Synthesis of 2,2,2-trichloro-1-(1*H*-indol-3-yl)ethanone (29)²⁶:



A solution of indole **28** (1.0 g, 8.53 mmol) in dry dioxane (3,5 mL) was dropped into a mixture of trichloro-acetyl chloride (1.86 g, 10.22 mmol), pyridine (0.84 mL), and dry 1,4-dioxane (13.4 mL). After 80 minutes the reaction mixture was poured into 50 mL of water and the precipitate filtered, washed with water and pentane to give the desired product **29**. Yield: 80%, light brown solid; mp: 231-234°C (decomp.); FT-IR/ATR: 3248, 3201, 1639, 1510, 1429, 1236, 1153 cm⁻¹; ¹H-NMR (400 MHz, DMSO-d₆) δ: 12.55 (s, 1H), 8.60 (d, *J* = 3.4 Hz, 1H), 8.20 (sest, *J* = 3.2 Hz, 1H), 7.59 (sest, *J* = 2.8

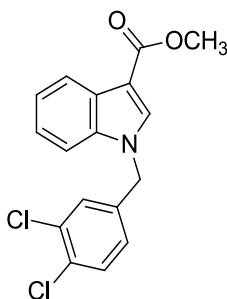
Hz, 1H), 7.32 (sest, $J = 3.2$ Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): 177.23 (s), 137.18 (d), 136.62 (d), 127.57 (s), 124.29 (d), 123.62 (d), 121.73 (d), 113.42 (d), 105.22 (s), 66.86 (s). The spectroscopic data are in agreement with the literature²⁶.

Synthesis of methyl 1H-indole-3-carboxylate (30):



To a solution of potassium *t*-butoxide (0.042 g, 0.38 mmol) and methanol (5 mL) was added 2,2,2-trichloro-1-(1H-indol-3-yl)ethanone **29** (0.1 g, 0.38 mmol) and the reaction mixture was stirred at room temperature for 1 hour. The solvent was removed under reduced pressure and the residue was purified by column chromatography (DCM) to afford the corresponding product **30**. Yield: 100%, white solid; mp: 146-148°C (decomp.); FT-IR/ATR: 3232, 3199, 1662, 1525, 1442, 1195 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ : 8.81 (s, 1H), 8.24-8.22 (m, 1H), 7.94 (d, $J = 2.8$ Hz, 1H), 7.42-7.39 (m, 1H), 7.28-7.25 (m, 2H), 3.93 (s, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ : 165.9, 136.1, 131.2, 125.7, 123.2, 121.4, 111.6, 108.6, 51.1. The data are in agreement with the literature. Other spectroscopic data (mp, ^{13}C NMR) are reported as well³⁶.

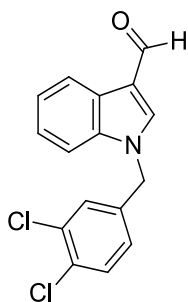
Synthesis of methyl 1-(3,4-dichlorobenzyl)-1H-indole-3-carboxylate (31):



Potassium hydroxide (0.062 g, 0.89 mmol) was added at room temperature to a solution of methyl 1H-indole-3-carboxylate **30** (0.16 g, 0.89 mmol) in ethanol (7.42 mL). After

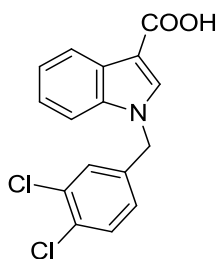
stirring until total solubilization, the ethanol was totally removed under reduced pressure and acetone (7.42 mL) was added followed by 3,4-dichlorobenzylbromide (0.21 g, 0.89 mmol). Immediately a precipitate was formed that was filtered and the filtrate concentrated to give the crude of desired product. The crude was taken up onto the final step of saponification that unfortunately didn't work.

Synthesis of 1-(3,4-dichlorobenzyl)-indole-3-carboxaldehyde (33):



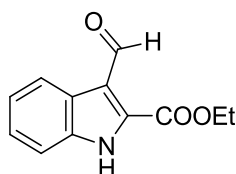
The indole-3-carboxaldehyde **32** (1.0 g, 6.89 mmol) was dissolved in dry DMF (8.3 mL) and the stirred solution was treated with sodium hydride (0.2 g, 8.27 mmol). The reaction mixture was stirred at room temperature for 15 minutes. 3,4-Dichlorobenzyl bromide (2.0 g, 8.27 mmol) was added and the reaction mixture was maintained at 90 °C for 3 hours and half. Upon cooling the crude was poured onto ice and acidified with 2N-HCl, whereupon a precipitate forms and it was collected by filtration. Yield: 74%, white solid; ¹H NMR (400 MHz, CDCl₃) δ: 10.03 (s, 1H), 8.35-8.30 (m, 1H), 7.74 (s, 1H), 7.42 (d, *J* = 8.3 Hz, 1H), 7.36-7.32 (m, 2H), 7.29-7.26 (m, 2H), 6.98 (dd, *J* = 8.3, 2.2 Hz, 1H), 5.33 (s, 2H) ppm; ¹³C NMR (100 MHz, CDCl₃) δ: 184.68 (d), 138.19 (d), 137.22 (s), 135.72 (s), 133.45 (s), 132.72 (s), 131.21 (d), 129.01 (d), 126.30 (d), 125.57 (s), 124.54 (d), 123.38 (d), 122.40 (d), 118.95 (s), 110.19 (d), 49.89 (t) ppm. The data are in agreement with the literature. Other spectroscopic data (mp and IR) are reported as well³⁷.

Synthesis of 1-(3,4-dichlorobenzyl)-1H-indole-3-carboxylic acid (5b):



To a solution of 1-(3,4-dichlorobenzyl)-indole-3-carboxaldehyde **33** (0.6 g, 1.97 mmol) in a mixture of DMSO (8.6 mL) and acetone (21.3 mL) was added an aqueous solution (6.5 mL) of sulfamic acid (0.33 g, 3.37 mmol). The solution was cooled at 0°C and an aqueous solution (14.7 mL) of sodium chlorite (0.36 g, 3.94 mmol) was slowly added. The reaction was stirred at 0°C for 30 minutes and then at room temperature overnight. Water (40.5 mL) was added and the precipitate was filtered off. It was purified by silica gel column chromatography (DCM). Yield: 42%, white solid; mp: 210-213°C; IR (Perkin-Elmer 298 Nujol): 1650, 1525, 1275, 1190 cm^{-1} ; ^1H NMR (400 MHz, DMSO- d_6) δ : 12.09 (bs, 1H), 8.29 (s, 1H), 8.03 (dd, $J = 6.6, 3.2$ Hz, 1H), 7.63 (d, $J = 1.7$ Hz, 1H), 7.60 (d, $J = 8.4$ Hz, 1H), 7.55 (dd, $J = 6.8, 3.2$ Hz, 1H), 7.24-7.20 (m, 3H), 5.52 (s, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ : 166.05 (s), 138.92 (s), 136.64 (s), 136.05 (d), 131.74 (s), 131.45 (d), 130.85 (s), 129.92 (d), 128.15 (d), 127.12 (s), 123.08 (d), 122.09 (d), 121.48 (d), 111.53 (d), 107.75 (s), 48.74 (t); MS: 319.87; HPLC purity: 99,9%; t_R : 9.60 min. The data are in agreement with the literature. Other spectroscopic data (mp and IR) are reported as well²⁵.

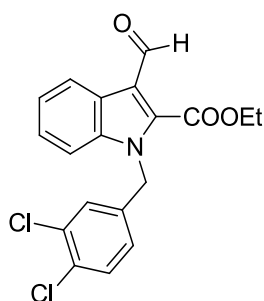
Synthesis of ethyl-3-formyl-1H-indole-2-carboxylate (34)²⁸:



Phosphorus oxychloride (POCl_3) (0.44 g, 2.9 mmol) was added dropwise to dry DMF (1 mL) at 0°C, after 5 minutes a solution of ethyl-1H-indole-2-carboxylate **26** (0.5 g, 2.64

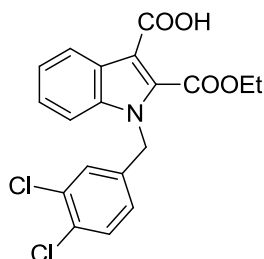
mmol) in dry DMF (3 mL) was added and the resulting mixture was stirred at room temperature for 1 hour and at 70°C for 6 hours. The reaction mixture was poured into cold water and neutralized with 2M NaOH. The yellow precipitate was collected by filtration to give the desired product. Yield: 78%, light brown powder; ¹H NMR (400 MHz, DMSO- *d*₆) δ: 12.84 (s, 1H), 10.63 (s, 1H), 8.26 (d, *J* = 8.0 Hz, 1H), 7.41 (dt, *J* = 7.2, 1.2 Hz, 1H), 7.31 (dt, *J* = 7.2, 1.2 Hz, 1H), 4.47 (q, *J* = 7.2 Hz, 2H), 1.41 (t, *J* = 7.2 Hz, 3H). The data are in agreement with the literature. Other spectroscopic data (mp, IR, ¹³C-NMR) are reported as well^{28,38}.

Synthesis of ethyl-1-(3,4-dichlorobenzyl)-3-formyl-1H-indole-2-carboxylate (35):



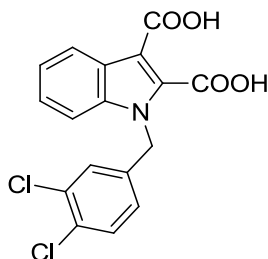
A solution of ethyl-3-formyl-1H-indole-2-carboxylate **34** (0.2 g, 0.92 mmol) in dry DMF (3 mL) was added dropwise under N₂ atmosphere to an ice-cooled suspension of sodium hydride (0.033 g, 1.38 mmol) in dry DMF (2 mL). After stirring at 0 °C for 20 minutes, 3,4-dichlorobenzylbromide (0.26 g, 1.10 mmol) was added dropwise and the reaction mixture was warmed to room temperature and stirred overnight. It was poured into ice-water (10 mL) and then extracted with ethyl acetate (3 x 15 mL), dried over anhydrous MgSO₄, filtered and evaporated *in vacuo*. It was purified by silica gel column chromatography (Petroleum Ether:DCM, 8:2) to give the desired product **35**. Yield: 80%, yellow oil; FT-IR/ATR: 2983, 2870, 1708, 1645, 1458, 1394, 1236, 1139, 1122 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ: 10.37 (s, 1H), 7.19-7.02 (m, 7H), 5.49 (s, 2H), 4.20 (q, *J* = 7.2 Hz, 2H), 1.13 (t, *J* = 7.2 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ: 188.54 (d), 160.84 (s), 138.16 (s), 137.09 (s), 133.07 (s), 132.86 (s), 131.89 (s), 130.85 (d), 128.40 (d), 126.91 (d), 125.70 (d), 124.72 (s), 124.55 (d), 124.15 (d), 120.69 (s), 110.54 (d), 62.48 (t), 47.70 (t), 14.24 (q).

Synthesis of 1-(3,4-dichlorobenzyl)-2-(ethoxycarbonyl)-1H-indole-3-carboxylic acid (36):



To a solution of ethyl 1-(3,4-dichlorobenzyl)-3-formyl-1H-indole-2-carboxylate **35** (0.26 g, 0.69 mmol) in a mixture of DMSO (3 mL) and acetone (7.5 mL) was added an aqueous solution (2.5 mL) of sulfamic acid (0.11 g, 1.18 mmol). The solution was cooled at 0°C and an aqueous solution (5 mL) of sodium chlorite (0.12 g, 1.38 mmol) was added slowly. The reaction was stirred at 0°C for 30 minutes and then at room temperature overnight. Water (14.2 mL) was added and extracted with ethyl acetate (3 x 15 mL), dried over anhydrous MgSO₄, filtered and evaporated *in vacuo*. It was purified by silica gel column chromatography (DCM:Ethyl Acetate, 95:5) to give the desired product **36**. Yield: 78%, light orange solid; mp: 165-166°C; FT-IR/ATR: 2922, 2850, 1716, 1660, 1541, 1435, 1398, 1236, 1192 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ: 8.38 (dd, *J* = 6.8, 2.0 Hz, 1H), 7.41-7.35 (m, 3H), 7.33-7.29 (m, 1H), 7.14 (d, *J* = 2.0 Hz, 1H), 6.90 (dd, *J* = 8.0, 2.0 Hz, 1H), 5.5 (s, 2H), 4.42 (q, *J* = 7.2 Hz, 2H), 1.31 (t, *J* = 7.2 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ: 166.21 (s), 163.49 (s), 144.52 (s), 137.11 (s), 136.80 (s), 136.59 (s), 133.22 (s), 132.16 (s), 130.98 (d), 128.32 (d), 126.62 (s), 126.35 (d), 125.56 (d), 123.93 (d), 123.75 (d), 110.44 (d), 63.41 (t), 48.26 (t), 13.83 (q).

Synthesis of 1-(3,4-dichlorobenzyl)-1H-indole-2,3-dicarboxylic acid (5c):



To a solution of 1-(3,4-dichlorobenzyl)-2-(ethoxycarbonyl)-1*H*-indole-3-carboxylic acid **36** (0.21 g, 0.54 mmol) in ethanol (5 mL) at room temperature was added lithium hydroxide (0.070 g, 2.85 mmol) and the mixture reaction was heated at 70°C for 2 hours and then at room temperature overnight. It was poured into ice water (10 mL) and acidified with 2M HCl. A yellow precipitate was filtered off. Yield: 51%, yellow powder; mp: 252-255°C; FT-IR/ATR: 2983, 2870, 1708, 1645, 1458, 1394, 1236, 1139, 1122 cm⁻¹; ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.50 (d, *J* = 7.8 Hz, 1H), 7.50 (q, *J* = 8.3 Hz, 2H), 7.38 (d, *J* = 1.9 Hz, 1H), 7.26 (dt, *J* = 7.0, 1.2 Hz, 1H), 7.18 (t, *J* = 7.0 Hz, 1H), 6.99 (dd, *J* = 8.4, 2.0 Hz, 1H), 6.11 (s, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 166.42 (s), 163.69 (s), 146.08 (s), 139.12 (s), 136.42 (s), 136.29 (s), 131.63 (s), 131.32 (d), 130.49 (s), 129.24 (d), 127.43 (d), 126.05 (s), 125.01 (d), 123.07 (d), 122.80 (d), 111.87 (d), 47.19 (t); MS: 363.80; HPLC purity: 97,5%; t_R: 9.83 min. The spectroscopic data are in agreement with the literature^{12,16}.

BIOLOGY

Chemicals and Reagents

CCR2-RA-[R] was synthesized in-house in agreement with the literature¹⁰. [³H]-CCR2-RA-[R] (specific activity 59,6 Ci mmol⁻¹) was custom-labeled by Vitrax (Placentia, CA), for which a dehydrogenated precursor was provided ((*R*)-4-acetyl-1-(4-chloro-2-fluorophenyl)-5-cyclohexyl-3-hydroxy-1,5-dihydro-2*H*-pyrrol-2-one). Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, USA). Bicinchoninic acid (BCA) and BCA protein assay reagent were purchased from Pierce Chemical Company (Rockford, IL, USA). Tango™ CCR1-*bla* and CCR2-*bla* U2OS cells stably expressing the human CCR1 or human CCR2 (U2OS-CCR1 or U2OS-CCR2) were obtained from Invitrogen (Carlsbad, CA). All other chemicals were obtained from standard commercial sources.

Cell culture

Human osteosarcoma (U2OS) cells stably expressing the human CCR1 or CCR2 (Invitrogen, Carlsbad, CA) were cultured in McCoy's 5A medium supplemented with

10% (v/v) fetal calf serum, 2 mM glutamine, 0,1 mM nonessential amino acids (NEAAs), 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml G418, 50 µg/ml hygromycin, and 125 µg/ml zeocin (200 µg/ml zeocin for U2OS-CCR1) in a humidified atmosphere at 37°C and 5% CO₂. Cells were subcultured twice weekly at a ratio of 1:4 to 1:6 on 10-cm Ø and 15-cm Ø plates by trypsinization.

Cell membrane preparation

Membranes were prepared as described in the literature¹⁴. Briefly, cells were detached from 15-cm Ø plates by scraping into 5 ml of phosphate-buffered saline (PBS) and subsequently centrifuged for 5 minutes at 3000 rpm (200 x g). The pellets were resuspended in ice-cold 50 mM Tris-HCl buffer and 5 mM MgCl₂, pH 7.4, and homogenized with an Ultra Turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). Membranes and the cytosolic fraction were separated by several centrifugation steps at 31,000 rpm in an Optima LE-80 K ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) at 4°C for 20 minutes. Finally, the membrane pellet was resuspended in 50 mM Tris-HCl buffer and 5 mM MgCl₂, pH 7.4, and aliquots of 100 µl and 250 µl were stored at -80°C. Membrane protein concentrations were measured using a BCA protein determination with BSA as a standard³⁹.

[³H]-CCR2-RA-[R] binding assays

[³H]-CCR2-RA-[R] binding assays were performed in a 100 µL reaction volume containing 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl₂, 0.1% CHAPS and 5 to 15 µg of membrane protein at 25 °C. Displacement assays with U2OS-CCR1 or -CCR2 were carried out with an average concentration of 6.0 nM [³H]-CCR2-RA-[R]. In all cases, at least six concentrations of competing ligand were incubated for 120 min at 25 °C. Non-specific binding was determined in the presence of 10 µM CCR2-RA-[R]. In all cases, total radioligand binding did not exceed 10% of the amount added to prevent ligand depletion. For all experiments incubations were terminated by dilution with ice-cold 50 mM Tris-HCl buffer supplemented with 0.05% CHAPS. Separation of bound from free radioligand was performed by rapid filtration through a 96-well GF/B filter plate using a Perkin Elmer Filtermate harvester (Perkin Elmer, Groningen, the

Netherlands). Filters were washed 10 times with ice-cold wash buffer. 25 μ L of Microscint scintillation cocktail (Perkin-Elmer, Groningen, the Netherlands) was added to each well and the filter-bound radioactivity was determined by scintillation spectrometry using the P-E 2450 Microbeta¹⁴ scintillation plate-counter (Perkin Elmer, Groningen, The Netherlands).

Data analysis

All experiments were analyzed using the non-linear regression curve fitting program Prism 6 (GraphPad, San Diego, CA, U.S.A.). The IC₅₀ values of CCR2-RA-[R] were obtained by non-linear regression analysis of the displacement curves. The IC₅₀ values of the unlabeled ligands obtained from radioligand displacement experiments were converted into K_i values using the Cheng-Prusoff equation⁴⁰. All values obtained are means of at least three independent experiments performed in triplicate, unless stated otherwise.

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