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Computer-assisted synthesis and in-vitro cytotoxic evaluation of new pyrazole-fused isoquinolinoquinones derivatives as PI3K receptor antagonist with promising antitumoral activity

Tesi di laurea sperimentale

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ABSTRACT

The importance of pyrazole and isoquinoline-5,8-dione scaffolds in medical chemistry is underlined by the high number of drug currently on trading that contains these active ingredients. Due to their cytotoxic capability, the interest of medicinal chemists in these heterocyclic rings has grown exponentially especially, for cancer therapy. In this project, the first synthesis of pyrazole-fused isoquinoline-5,8-diones has been developed. 1,3-Dipolar cycloaddition followed by oxidative aromatization, established by our research group, has been employed. Screening of reaction conditions and characterization studies about the regioselectivity have been successfully performed. A remote control of regioselectivity, to achieve the two possible regioisomers has been accomplished. Through *Molecular Docking* studies, Structure-Activity relationship of differently substituted scaffolds containing our central *core* proved that a family of PI3K inhibitors have been discovered. Finally, in order to verify the promising antitumor activity, a first test of cell viability *in vitro* on T98G cell line of a solid brain tumor, the Glioblastoma Multiforme, showed cytotoxic inhibition comparable to currently trade anticancer drugs.

RIASSUNTO

L'importanza degli *scaffolds* farmaceutici contenenti nuclei pirazolici e isochinolino-chinonici è evidenziata dall' elevato numero di farmaci, attualmente in commercio, che presentano questi nuclei all' interno dei loro principi attivi. Grazie alla loro elevata capacità citotossica, l'interesse in questi eterocicli è cresciuto esponenzialmente, in particolar modo per quanto riguarda la loro attività anticancerogena. Per queste ragioni, in questo progetto, è stata sviluppata per la prima volta, una sintesi di pirazoli-isochinolino-chinoni tricicli fusi; la metodologia utilizzata è stata una cicloaddizione 1,3 dipolare con successiva aromatizzazione ossidativa, già ampiamente sviluppata in precedenza dal gruppo di ricerca. Si è studiata, quindi, l'ottimizzazione delle condizioni della reazione di cicloaddizione così come la sua regiochimica. Da questi studi è risultato possibile, attraverso il controllo di un gruppo remoto al sito di attacco, ottenerne l'inversione di regiochimica. Attraverso studi di *Molecular Docking*, poi, si è evidenziata l'interazione di *scaffolds* diversamente funzionalizzati, contenenti il *core* da noi sintetizzato, all'interno del sito attivo della proteina PI3K, dimostrandosi una serie di promettenti inibitori. Infine, per verificare la loro attività antitumorale, test *in vit*ro

preliminari sulla linea cellulare T98G hanno mostrato un'attività citotossica per il Glioblastoma Multiforme comparabile a farmaci ad oggi in commercio.

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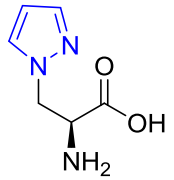
1. Introduction

1.1. Pyrazole in Medicinal Chemistry

Pyrazole, term given by Ludwig Knorr in 1883, refers to a class of simple aromatic organic compounds of the heterocyclic series, characterized by a 5-membered ring structure containing two nitrogen atoms in adjacent positions. In 1959, the first natural pyrazole, 1- pyrazolyl-alanine, was isolated from the seeds of watermelon.[1]



3-(1-pyrazolyl)-L-alanine

Fig.1 Structure of first isolated natural pyrazole

Pyrazole derivatives have been used for long time in agrochemicals and pharmaceutical industry as herbicides and active pharmacuticals ingredients (APIs)s. After the success of pyrazole-containing drugs, due to their inhibitory capacities, the importance of these heterocyclic rings has exponentially grown in medicinal chemistry.

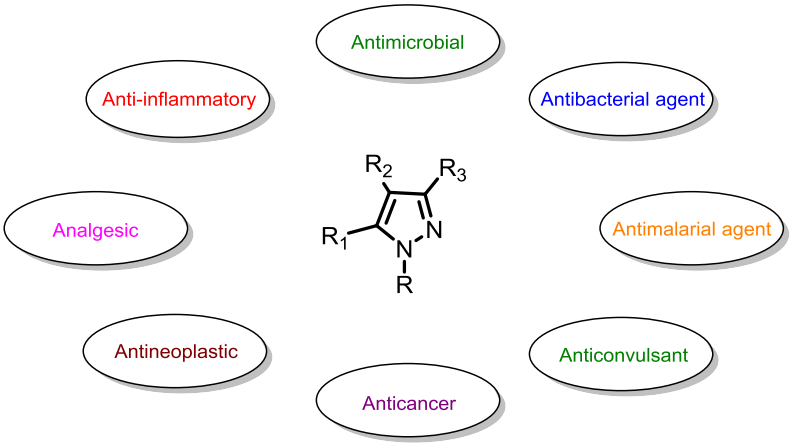
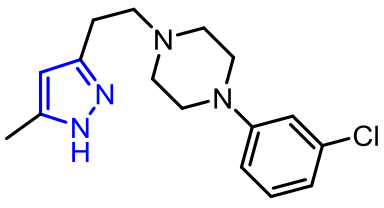


Fig.2 Biological activity of pirazole-containing drugs

Even if they are rarely found in nature, probably due to difficulty encountered in the formation of N-N bonds by the living organisms, still plenty of research publications during last decade showed an increasing interest of synthetic as well as medicinal chemists in pyrazole containing compounds, in view of their wide applications in different fields.[2]

1.1.1 Approved drug containing pyrazole mojeties

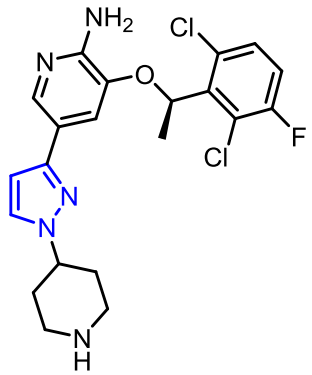
A large number of commercially available top selling drugs containing pyrazoles can be found, exhibiting antispasmodic, anti-inflammatory, antibacterial, antihyperglicemic, antidepressive and, more recently, antitumor activities.[3] In order to underline the variety of simple substituted pyrazoles that can be found on the market of pharmaceuticals, few examples of commercially available drugs are shown below. Mepiprazole, main active ingredient of Psigodal, is an anxiolytic and antidepressant drug of the phenylpiperazine group, commercially available in Spain. It's an α_1-adrenergic receptor antagonist, producing mCPP (*meta*-Chlorophenylpiperazine) as active metabolite, and has been found that, unlike other psycho-pharmaceuticals, has specific dopamine- and serotonin-potentiating activity and simultaneously has noradrenaline-blocking activity in the central nervous system. Thus, for example, dopamine reuptake was inhibited by 50-70% in rats by administration of 20 mg/kg; it showed a reduction in serotonin turnover of about 50% and an increase in noradrenaline turnover by 60%. This combination of effects has not been observed heretofore in psycho-pharmaceuticals, allowing normalization of noradrenaline and serotonin transmitter metabolism.[4,5]



Mepiprazole

Fig.3 Simple pyrazole use as anxiolytic and antidepressant drug

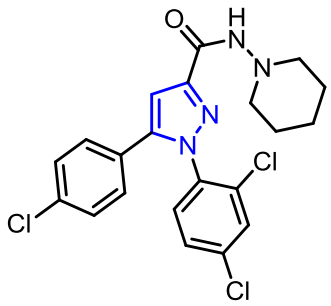
Secondly, Crizotinib, sold by Pfizer under the trade name of Xalkori, is a disubstituted simple pyrazole with antitumor properties. It acts as a ROS-1 gene (proto-oncogene-1) inhibitor and has obtained excellent results in clinical trials against anaplastic large cell lymphoma, neuroblastoma and other advanced state solid tumors.[6]



Crizotinib

Fig.4 Anticancer commercially approved by FDA drug based on pyrazole moiety

Rimonabant is a cannabinoid receptor antagonist that reached the market in several countries as anorectic and anti-obesity drug, with a potential for aiding smoking cessation. CB1 receptors antagonists have been investigated mainly as a potential strategy for treating obesity and associated metabolic disorders. Despite their putative clinical applications, the experimental evidence heralded the notion that Rimonabant could induce psychiatric side effects.[7]



Rimonabant

Fig.5 Trisubstitued carboxamide pyrazole with multiple biological activity

1.1.2 Ring-fused pyrazole

Ring-fused pyrazoles are, if possible, even more important than simple rings in the above mentioned applicative categories. Allopurinol, for example, is a generic drug used for treatment of hyperuricemia, i.e. an excess of uric acid in blood, playing a crucial role in the inhibition of xanthine-oxidase, an enzyme involved in the purinic metabolism. This molecule showed a better binding affinity to the target-protein compared to the corresponding Xanthine, but, due to the presence of the pyrazole ring, instead of the imidazole one, was not affected by the oxidative circle. It was also included in the World Health Organization's List of Essential Medicine.[8]

Fig 6. Allopurinol and Xanthine

Another biologically active ring-fused pyrazole is Apixaban: developed by Bristol-Myers Squibb in collaboration with Pfizer, actually in phase III of clinical trials for the excellent anticoagulant properties, it contains a N-substituted pyrazole-fused δ-lactam. This drug acts as an inhibitor of the Xa factor, a serine protease that plays an important role in the coagulation cascade.[9] Compared to Warfarin (known under the trade name of Coumadin), currently the most common drug for the treatment of this disease, Apixaban has been found to be "not inferior" in ictus prevention and superior in secondary hemorrhaging's prevention, despite some side effects.[10]

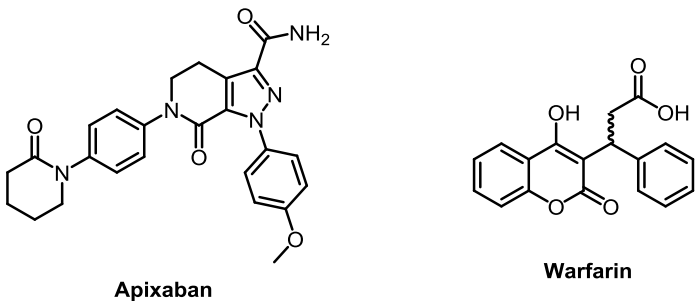


Fig.7. Apixaban and Warfarin, two anticoagulant drugs

Moreover, condensed pyrazole derivatives have often shown antiproliferative activities towards a wide number of cellular lines and are often employed in anticancer drug discovery screening. For example, a number of reports have suggested that CK2 is a potential target for cancer treatment due to its overexpression in a wide variety of tumours, and various small-molecule CK2 inhibitors have been developed. Recently, based on our previous SAR (Structure-Activity Realationship) studies, benzo[g]indazole derivatives were synthesized, through a tricomponent cascade reaction, as novel CK2 inhibitor candidates. In **Fig. 8**, the most active compounds of this class **I** and **II** has been reported.[11]

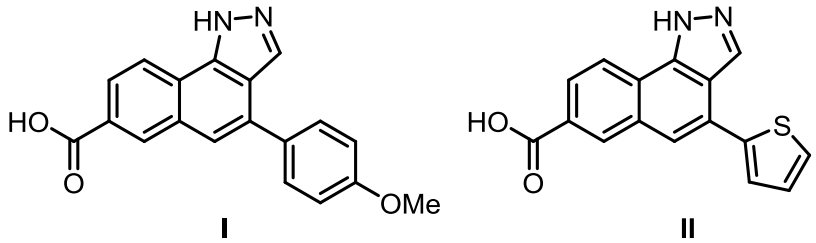


Fig.8 Ring-fused pyrazoles exhibiting promising anticancer activity

Finally, as another example, pyrrolo-[3,4-c]-pyrazole **III** (Danusertib), an Aurora kinase inhibitor, has advanced in phase II clinical trials for the treatment of Bcr-Abl positive leukemia, due to the good pharmacokinetic properties as inhibitor of Aurora kinases and Bcr-Abl tyrosine kinase (mitosis regulators aberrantly overexpressed in cancer cells), along with general safety profiles shown in phase I clinical studies. Compared with other hinge binder templates, its double heterocyclic core offers efficient hydrogen bonds interactions, improving greatly its physiochemical properties.[12]

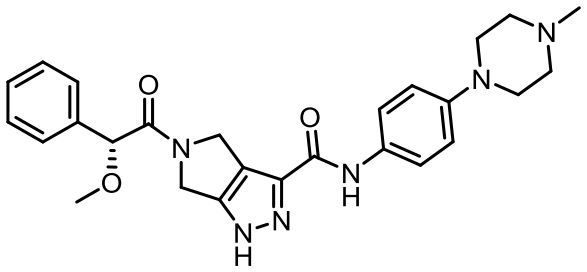


Fig.9 . Danusertib, a ring-fused pyrazole in clinical trials for the treatment of Bcr-Abl positive leukemias.

1.1.3 Pyrazole for Cancer Therapy

Pyrazole and its derivatives have been widely studied for the development of new therapeutics for various diseases, including cancer. In the last decade, many researchers have reported a large series of pyrazole derivatives having promising anticancer activities, indicating the use of pyrazole motif as a powerful tool for novel anticancer drugs development.[13] Recent studies have shown several diarylpyrazole derivatives possessing ALK5, FLT3, ERK and B-raf kinase inhibitory activities. This clearly suggests that the kinase inhibition may play a role for the observed cytotoxicity. In a 2014 work of Banoglu et al.[14], in order to get a wide library of biologically active, pyrazole containing compounds, a quinolinyl pyrazole carboxylic acid general scaffold, with a well-known cytotoxic activity against several human cancer cell lines, was substituted with different moieties through amidation reactions with aryl- and heteroaryl- substituted primary amines. Among the large number of synthesized molecules, compound **IV** was found to be the most active in sulfohodamine B assay with IC_{50} (half maximal Inhibitory Concentration) values of 1.1 μM and produced dramatic cell cycle arrest at G1 phase as an indicator of apoptotic cell death induction.

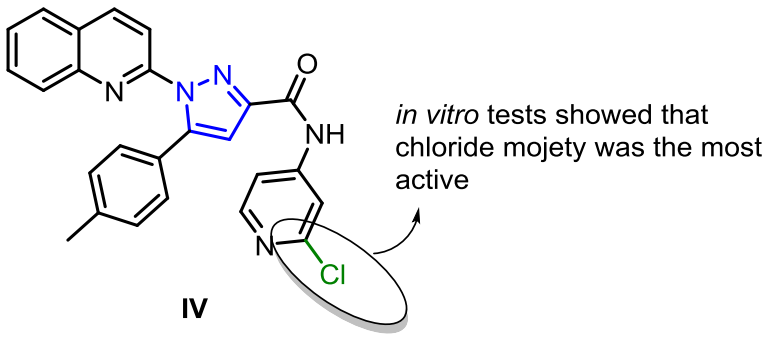


Fig.10 N-(2-Chloropyridin-4-yl)-5-(4-methylphenyl)-1-(quinolin- 2-yl)-1H-pyrazol-3-carboxamide

Phosphatidylinositol 3-kinases (PI3Ks) are crucial coordinators of intracellular signaling in response to extracellular stimuli. Due to the central role of this class of protein in various signal transduction-mediated events, they have been an area of intense investigation in the last decade. The discovery of PI 3-kinases by Lewis Cantley and colleagues began with their identification of a previously unknown phosphoinositide kinase associated with the polyoma middle T protein.[15] They observed unique substrate specificity and through ion-exchange chromatographic separation of

the products of the lipid kinase, they discovered that this phosphoinositide kinase has the unprecedented ability to phosphorylate phosphoinositides on the 3' position of the inositol ring (**Fig.11**).

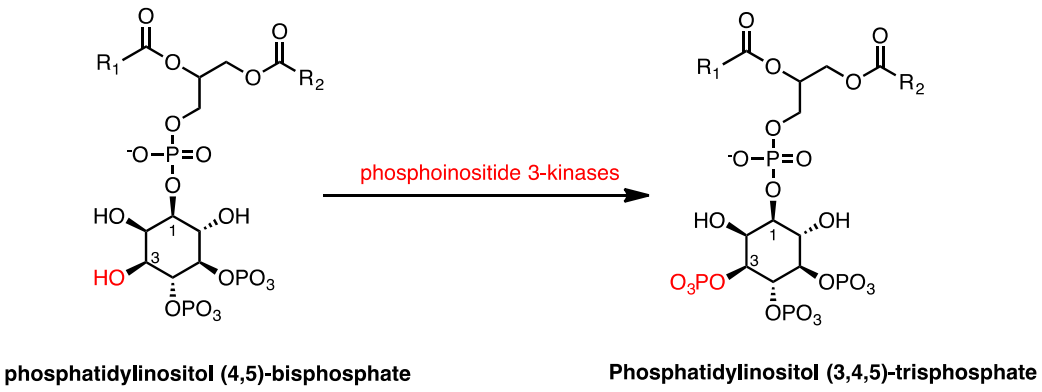


Fig. 11 Example of phosphorylation by PI3K

Hyperactivation of PI3K signaling cascades seems to be one of the most common events in human cancers. As the basic biology of the PIKs has become clearer, their roles in other various pathologies as inflammation or cardiovascular diseases was better understood. This led to the synthesis of new agents that weaved in this complex tree of signals. The central role of phosphoinositide 3-kinase activation in tumor cell biology has prompted a sizeable effort to target PI3K and/or downstream kinases such as AKT and mammalian Target Of Rapamycin (mTOR) in cancer treatment.[16] Quinoxaline derivatives **V** were found to be a family of inhibitors of PI3K that worked very well for a variety of substituents (R1 and R2), with an IC_{50} values < 0.1 μM. Moreover, as part of series of pyrazoles, disclosed in a patent application as AKT inhibitors, C-aryl-pyrazole **VII** showed an AKT inhibition activity with an IC_{50} value < 0.1 μM.[17] As another example, pyrazole **VI** was disclosed in a patent as a mixed inhibitor of mTOR (mammalian Target Of Rapamycin) and PI3K, with potentially uses in oncology.[18]

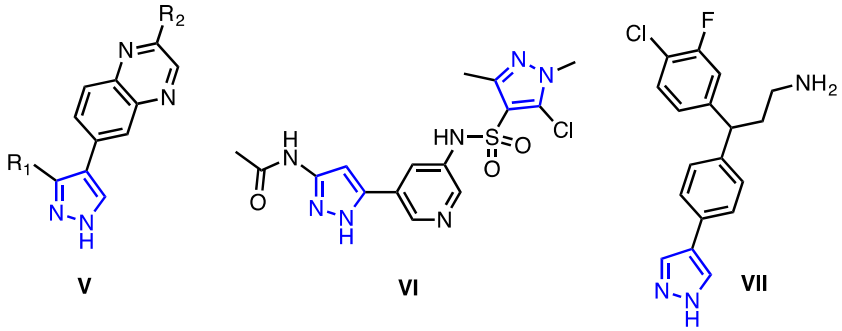


Fig. 12 Structure of pyrazole-based drugs that showed antitumoral activity

1.2. Biological activity of isoquinoline-5,8-dione

Isoquinoline-5,8-diones have a wide range of biological activities as antibacterial, antifungal, antimalarial and antitumoral agents.

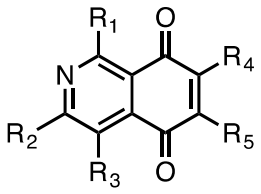


Fig.13 Structure of polysubstitued isoquinoline-5,8-dione

This moiety is present as an important *core* in a number of cytotoxic agents, such as naturally occurring mimosamycines and cribrostatines. To underline the efficacy of the substrates belonging to these families as antitumoral agents, naturally occurring cribrostatin 7 **IX**, renierone **X** and *O*-demethylrenierone **XI** showed remarkable cytotoxic activities against human colon carcinoma cell line, with IC_{50} values respectively of 45, 24 and 34 nM.

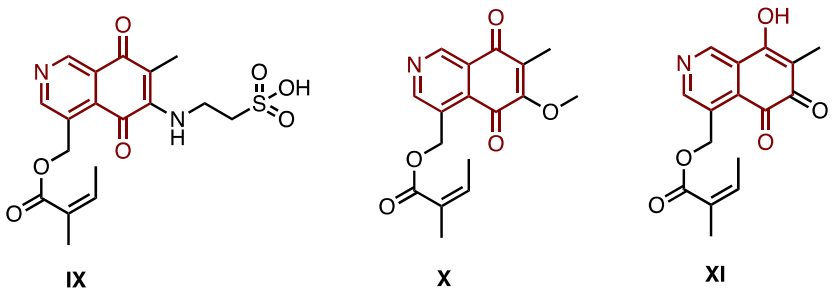
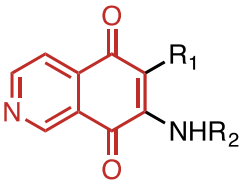


Fig. 14 Structure of active isoquinoline-5,8-diones against colon carcinoma

Gustafson group at the National Cancer Institute (NCI) reported the isolation of caulibugulones A–F, a series of novel cytotoxic isoquinoline quinones (showed in **Fig. 15**) and iminoquinones, from an extract of the marine bryozoan *Caulibugula intermis.*[19] They also disclosed that caulibugulones A–F exhibited IC_{50}values of 0.03–1.67 μM against murine tumor cells based on an *in vitro* cytotoxicity assay.



Caulibugulone

A: R_1= H R_2= Me
B: R_1= Br R_2= Me
C: R_1= Cl R_2= Me
D: R_1= H R_2= CH2CH2OH

Fig. 15 Natural isoquinolinequinone-core Caulibugulones

Anticancer quinones are currently the focus of intensive research because of their biological activity and complex modes of action, which differ depending on their particular structure. They represent a class of toxicological compounds that can generate a variety of critical effects including acute cytotoxicity, immunotoxicity and carcinogenesis with a complex pathway of action not always completely explained at

present time. Quinones moiety are Michael acceptors, and cellular damage can occur through alkylation of particular cellular proteins or DNA. On the other hand, quinones are highly redox-active agents that can undergo either an intracellular two-electron reduction to hydroquinones or one-electron to semiquinone, leading to the formation of reactive oxygen species (ROS) like peroxide, superoxide and hydroxyl radicals that have been associated with aging and carcinogenesis. Pixandrone, an aza-anthraquinone with a isoquinolinequinone moiety, commercialized under the trade name Pixuvri, is an antineoplastic drug, that showed fewer toxic effects on cardiac tissues compared to its non-aza analogues anthracyclines, such as mitoxantrone[20]. Anthracyclines are indeed important chemothepy agents, however their use is often associated with irreversible heart damage; thus some aza-derivates were developed in order to reduce heart damages, maintaining the same antitumor efficacy.

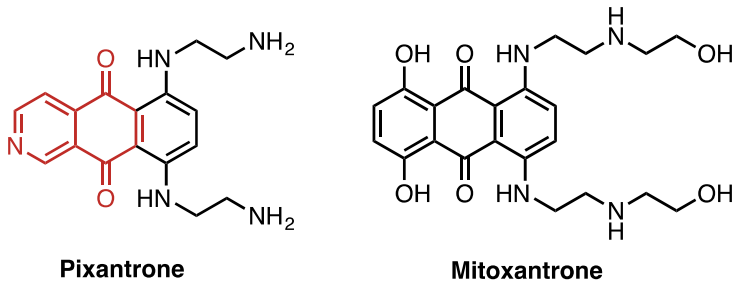


Fig. 16 Antitumoral Pixantrone and non-aza derivate Mitoxantrone

Pixantrone stands indeed in the family of antitumor antibiotics for the treatment of different type of cancers, including solid tumors and hematological cancers, such as non-Hodgkin lymphomas, that has completed phase III clinical trials.

1.3. Halogen compounds in Medicinal Chemistry

A significant number of drugs and drug candidates in clinical development present halogenated structures. For a long time, insertion of halogen atoms on hit or lead compounds was predominantly performed to exploit their steric effects, through the ability of these bulky atoms to occupy the binding site of molecular targets. However, halogens in ligand-active site complexes influence several processes rather than steric

aspects alone. For example, the formation of halogen bonds in ligand-target complexes is now recognized as a kind of intermolecular interaction that favorably contributes to the stability of protein-ligand complexes.

Due to their molecular complexity and diversity, secondary metabolites from natural sources, mainly plants, still inspire the design of drugs. Although in recent years marine animals have demonstrated to be rich sources of halogenated metabolites,[21] the occurrence of halogenated natural products in plants is rare.[22] Thus, it would be expected that halogenated drugs would have little importance in the drugs scenario, but this is not the case, since this class of compounds has a prominent position. Of course they are, in their majority, of synthetic origin. In the diagram is showed the relative abundance of halogenated drug classified, according to the kind of halogen by the FDA from 1988 to 2010.[23]

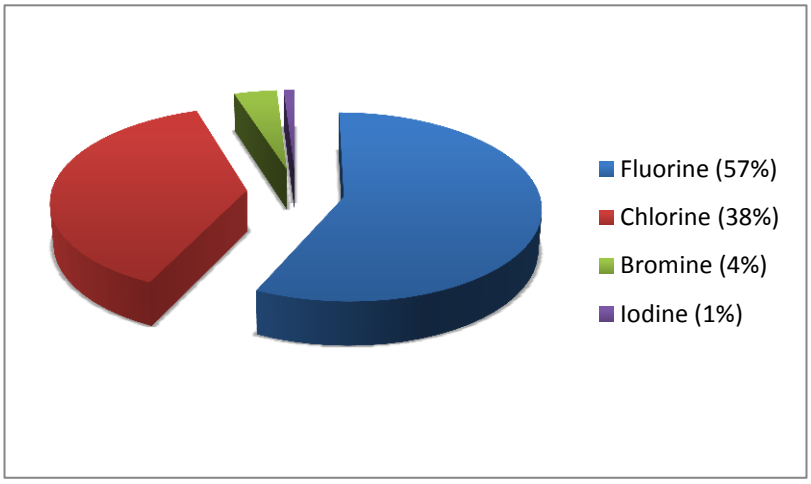


Table 1. Abundance of halogenated drug approved by FDA; salts and metal complexes are excluded from this diagram.

Halogen bonding refers to non-covalent weak interaction between halogen-bearing compounds and nucleophiles or electron rich species. With the aid of computational tools combined with experimental observation it was clarified that the halogen bond has to respect some rigorous requirements to be of some importance: distance between the two active species has to be minor than the sum of van der Waals radii, and the optimal angle has to range from 160° to 180°. Another feature is the strength of the bond, increasing proportionally with the weight of the halide substituent.[24] The first observation was made in cocrystal structures of 1,4-dioxane and Br_2 by Hassel and

Hvoslef in 1954. One of the first systematic investigation of halogen bonding in protein-ligand interactions, carried out by Diederich group of ETH Zurig in support with Novartis and Basell, focused on rationalizing the role of a halogen atom, present in an inhibitor of Cathepsin L, an important lysosomal endopeptidase enzyme involved in the initiation of protein degradation.

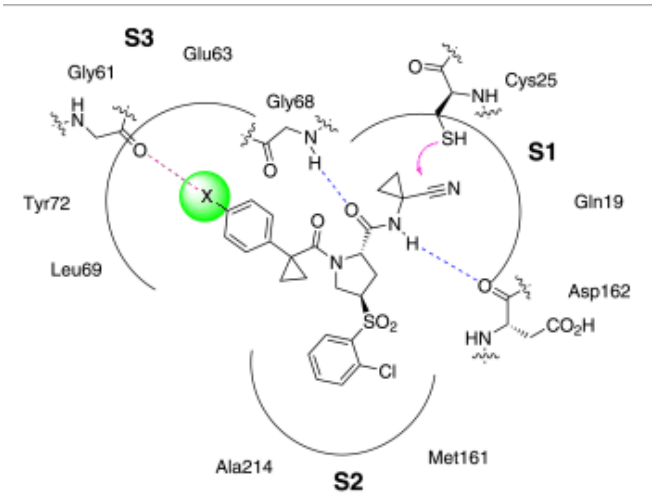
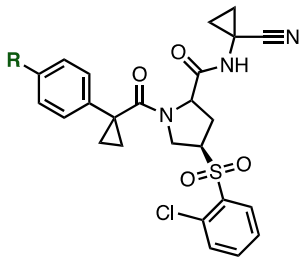


Fig. 17 Interaction of aryl-halogenated portion in active site

IC_{50} values in 4-substituted phenyl derivatives were determined in a fluorescence assay, leading to the discovery that IC_{50} values were directly proportional to the strength of the halogen-bond.



R	IC_{50} (nM)
H	290
Cl	22
F	340
Br	12
I	6
Me	130

Fig.18 Effect of para-substitued aryl group shows that halogen substitutions give better inhibition of the protein

For these reasons the halogen-bond seems to be a powerful tool, comparable to the more investigated hydrogen bond, capable to increase the binding affinity in protein-drug interactions, making halogenated moieties promising candidates for drug-design and structure-activity relationship studies.

1.3.1 Fluorine in medicinal chemistry

In medicinal chemistry, fluorine is generally viewed as a classical bioisoster of hydrogen. One example of this statement is the classical exachanging of hydrogen with fluorine on the purine base uracile, resulting in the anticancer drug 5-fluorouracil (5-FU) (**Fig. 19**). Thereby, the fluorine chemistry provides good opportunities for enhancing the binding affinity of potential drug candidates.

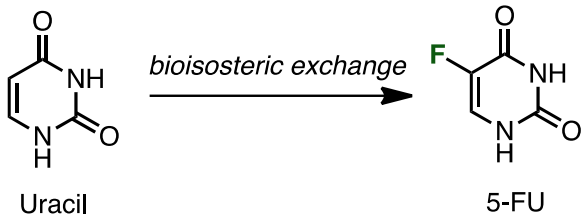
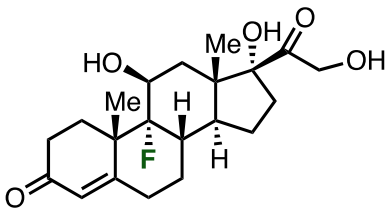


Fig.19 Bioisosteric exchange to give a fluorinated antitumor drug from the purine base uracile

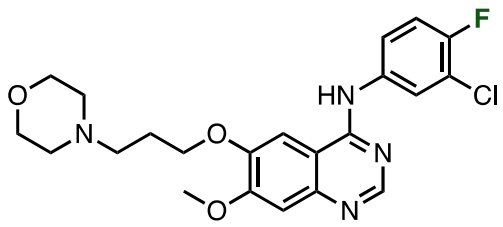
As expected from the fluorine position on the periodic table of elements, it possesses some extreme properties, in particular, ultimate electronegativity and oxidation potential. However, in the late 1940−1950s the idea of introducing fluorine into molecules of natural products was rather unconceivable. The prevailing wisdom of that time clearly suggested that fluorine is an abiotic element, and its applications were limited to military and special materials needs. Furthermore, quite poisonous properties of a few naturally occurring fluoroorganic compounds were very well known. It is interesting to note that discovery of fludrocortisone (**XII**), the first fluorine-containing pharmaceutical product, was a result of a systematic study on a series of 9α-halogenated cortisone derivatives, and the fluorinated compound was not included in the original study. After trying all the other halogen derivatives, they tried, just to complete the series, to synthetize the fluorinated compound, discovering its remarkable glucocorticoid activity, compared to the others.[25]



XII

Fig. 20 Structure of Fludrocortisone

There are many effects that fluorine and fluorine-containing substituents can impart to organic compounds. It is well known that fluorine electronegativity, size, omniphobicity-lipophilicity, and electrostatic interactions can dramatically influence chemical reactivity. The strategic use of fluorine substitution in drug design has culminated with the production of some of the key drugs available on the market. These include Fluoxetine[26] (antidepressant, commercialized as Prozac), Faslodex[27] (anticancer), and Efavirenz[28] (antiviral). Generally, the effect of fluorine on the biological activity of organic compounds is rather subtle and difficult to predict. Accordingly, quite intense structure-activity relationship studies are usually necessary to pinpoint the correct position of fluorine in the target molecule. Gefitinib (ZD-1839), developed and launched by Astra-Zeneca, is an oral epidermal growth factor receptor (EGFR) inhibitor used for the treatment of certain breast and lung cancers. It was first launched in Japan in July 2002 for the treatment of inoperable or recurrent non-small cell lung cancer (NSCLC), and then, it was launched in the United States as a third-line monotherapy.[29]

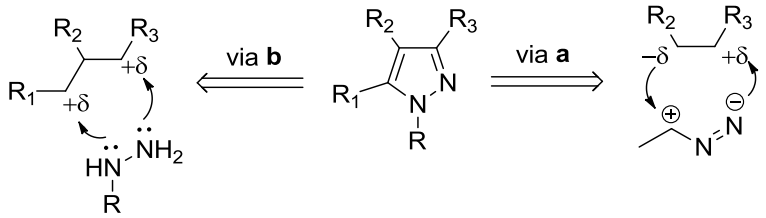


Gefitinib

Fig.21 Structure of EGFR-inhibitor for the treatment of breast and lung cancer

1.4. Pyrazole synthesis

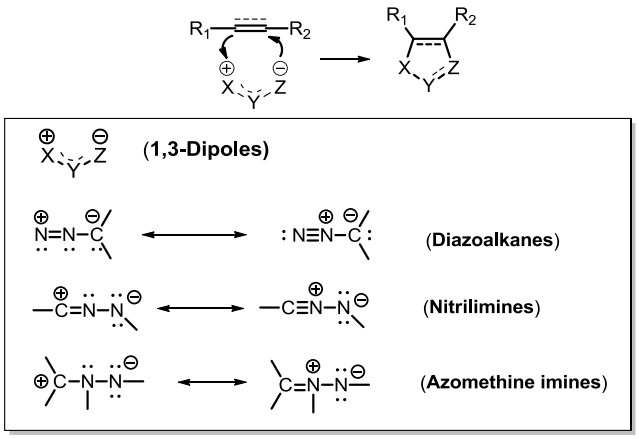
Conventional approaches for the preparation of substituted pyrazoles involve either the construction of two C-N bonds by cyclocondensation of hydrazines with 1,3-dielectrophilic compounds (**Scheme 1**, via **b**) or the generation of one C-N and one C-C bond by 1,3-dipolar [3+2] cycloaddition (**Scheme 1**, via **a**). Each method has its own scope and efficiency limitations, however, general and efficient methodologies have been developed, following these general strategies, with the aim of increasing the yield and the regioselectivity in the preparation of substituted pyrazoles.



Scheme 1. General approaches for the synthesis of pyrazole

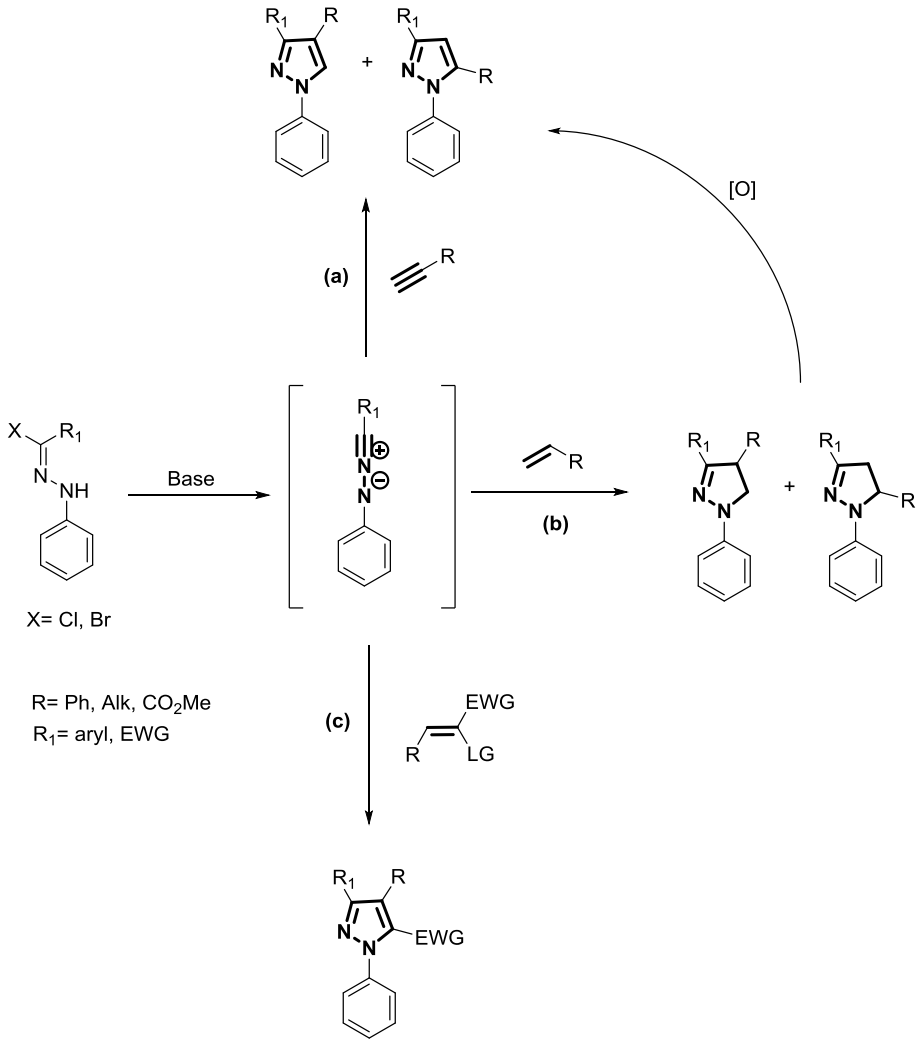
1.4.1 1,3-Dipolar cycloadditions

The 1,3-dipolar cycloaddition reaction has been employed as one of the most powerful synthetic tools to provide substituted pyrazoles. Three main classes of 1,3-dipoles have been used as [C,N,N] synthons, namely, diazoalkanes, azomethine imines and nitrilimines; the [C,C] fragment usually comes from activated π-bonds of alkanes and alkynes.



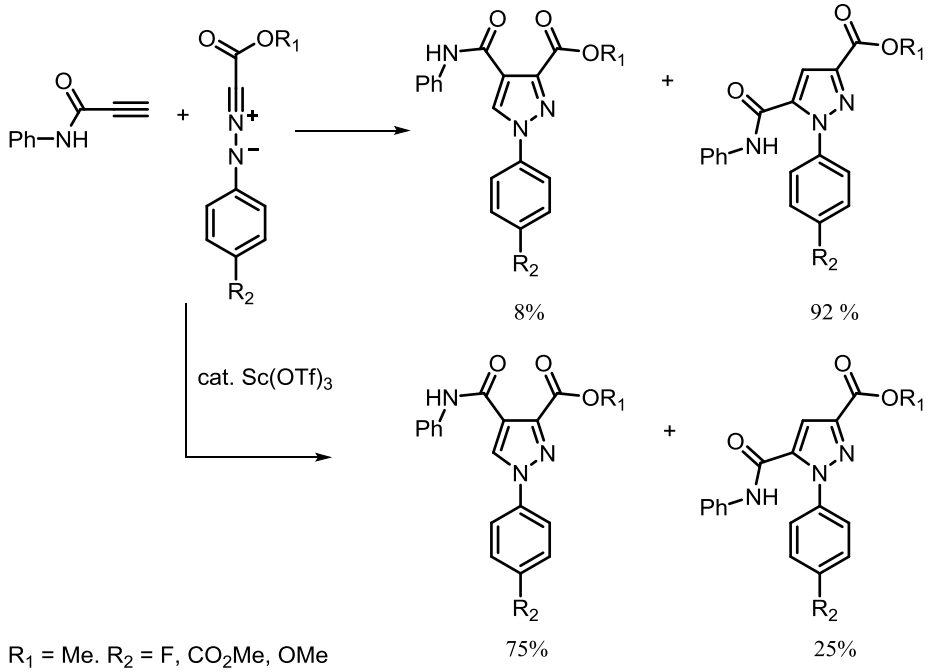
Scheme 2. Most common dipoles for 1,3-dipolar cycloadditions leading to pyrazoles

Compared to cyclocondensations between hydrazines and 1,3-dielectrophiles, 1,3-dipolar cycloadditions are intrinsically more highly regioselective owing to the significant electronegative difference between the N and the C atom of the substrate. Nitrilimines are generated in situ by treatment of hydrazonoyl halides with a base. Their 1,3-dipolar cycloaddition to alkynes (**Scheme 3,** via **a**[30]) or alkenes bearing a leaving group (**Scheme 3,** via **c**[31]) leads directly to pyrazoles, while addition to simple activated double bonds produces pyrazolines (**Scheme 3,** via **b**[30]) that must be subsequently oxidized to the desired aromatic pyrazole.



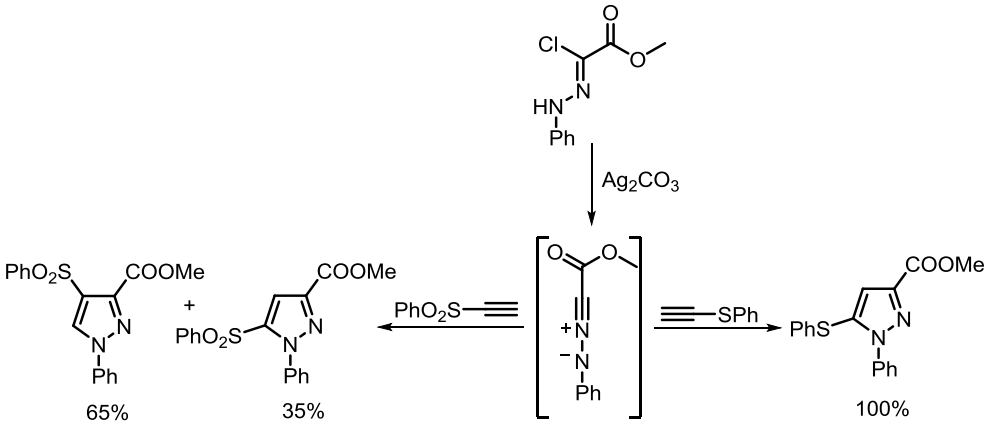
Scheme 3. Nitrilimine formation and cycloaddition with alkynes (a), alkenes followed by oxidation (b) and alkene bearing a leaving group (c)

A very convenient prerogative of this synthetic strategy relies on the facile control and modification of the regiochemistry, as proved in our laboratories. For example, a catalytic amount of scandium triflate, $Sc(OTf)_3$, as Lewis acid, is responsible for the inversion of the regioisomeric ratio in the cycloaddition of N-aryl-C-carboxyalkyl nitrilimine and an activated acetylene, such as N-phenyl-propiolamide.[32]



Scheme 4. Regioselectivity reversion with $Sc(OTf)_3$

Moreover, the electronic nature of the activating substituent of the multiple bond acts as a director of regiochemistry. In particular, electron donating groups (EDG), such as sulfide in **Scheme 5**, lead to 5-substitued pyrazoles, while electron-withdrawing groups (EWG) such as sulfone in **Scheme 5**, lead to 4-substitued pyrazoles as major products. These experimental results were broadly investigated by our research group and were confirmed by computational calculations as an interaction between distorted frontier molecular orbitals (HOMO-LUMO) both on the dipolarophile and on the nitrilimine dipole.[33]



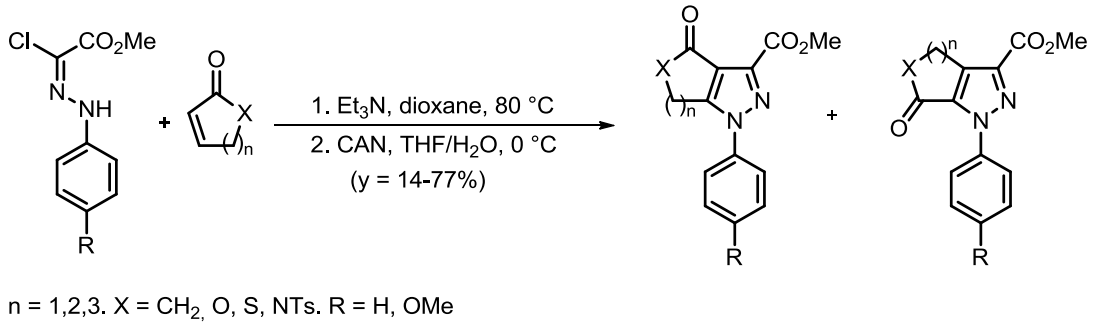
Scheme 5. Regioselectivity directed by acetylene substituents

1.4.2 Synthesis of ring-fused pyrazoles

Because of the excellent bioactivity and the wide range of application, thousands of papers concerning the synthesis of condensed pyrazole derivatives have been published. However, it is possible to summarize all these strategies into three main classes: methods starting from a pre-formed ring to which the pyrazole is subsequently fused, methods starting from a pre-formed pyrazole to which a new ring is subsequently fused and, finally, methods generating both pyrazole and its fused ring at once, following one-pot reaction procedures. Multicomponent one-pot syntheses are well known to produce highly functionalized, complex molecular scaffolds in very convenient, step- and atom-economical procedures. Nevertheless, the outcome of these reactions is very often difficult to predict and control, rendering this strategy quite unappealing for the synthesis of a precise target compound. On the other hand, methods condensing a new cycle on the pyrazole ring suffer from the complexity of the direct functionalization of an heterocycle of hybrid electronic characteristics, not yet completely investigated and understood.

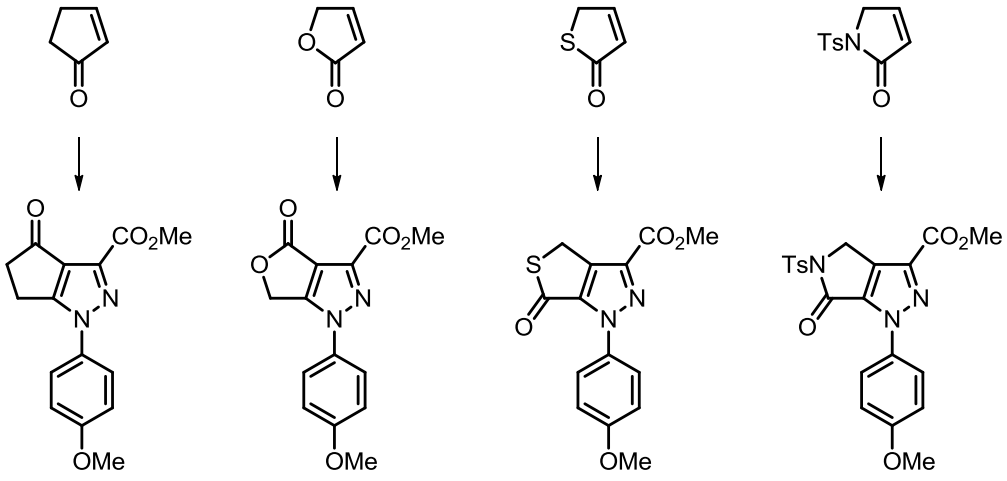
Hence, strategies constructing the pyrazole on pre-existing rings seem to be the more convenient pathway to condensed pyrazole derivatives, due to the wide spectrum of synthetic methods for the obtainment of the heterocycle, also from very different

starting materials. For these reasons, our research group has quite recently reported a new synthetic procedure leading to cycloalkenone, lactone, thiolactone and lactam-fused pyrazoles, employing the 1,3-dipolar cycloaddition of various nitrilimines with α,β-unsaturated cyclic systems (**Scheme 6**)[34]



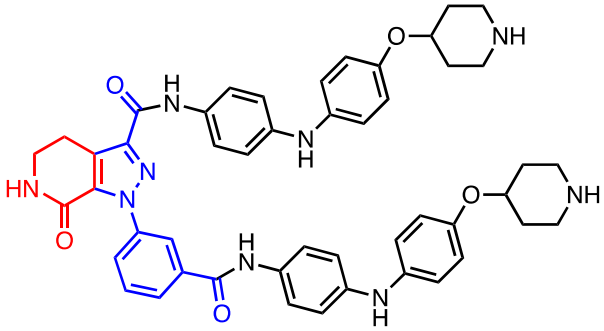
Scheme 6. Cycloaddition of nitrilimines with various α,β-unsaturated cyclic systems

Finally, the regiochemistry of the reaction was broadly investigated and the experimental results were justified through theoretical and computational studies. In most cases, 5-acyl-pyrazole derivatives were found to be the major products; although, only small and hard dipolarophiles (cyclopentanone and α,β-unsaturated γ-butyrolactone) reacted with electron-rich nitrilimines (such as N-p-methoxyphenyl, C-carboxymethyl nitrilimine) under the mandatory presence of Et_3N as a base, giving an inversion in the selectivity, in favor of 4-acyl-pyrazoles.[34]



Scheme 7. Major product arising from cycloaddition-oxidation of N-p-methoxyphenyl, C-carboxymethyl- nitrilimine and each of the shown dipolarophiles

To prove the applicability of this approach in the obtainment of complex structures for medicinal chemistry applications, an highly convergent synthesis of a novel lactam-fused pyrazole was reported by our research group. The structure (**XIII** in **Fig.22**) showed interesting IC_{50} values in the range of 15-60 μM, results comparable with nowadays commercially available anticancer compounds.[35]



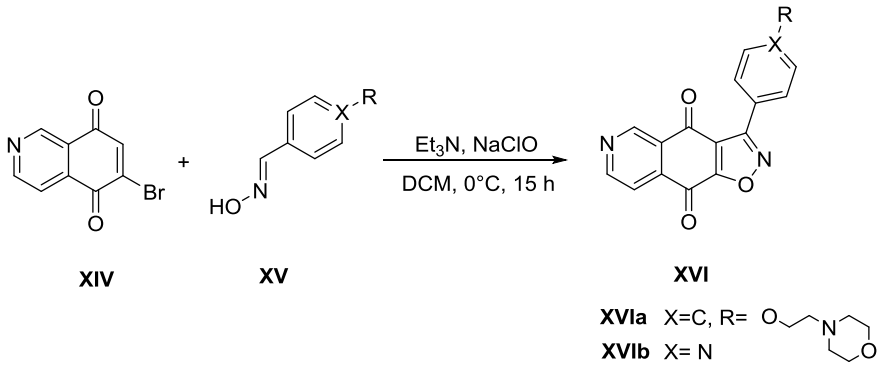
XIII

Fig. 22 Target molecule recently synthesized by research group

1.5. Ring-fused quinones

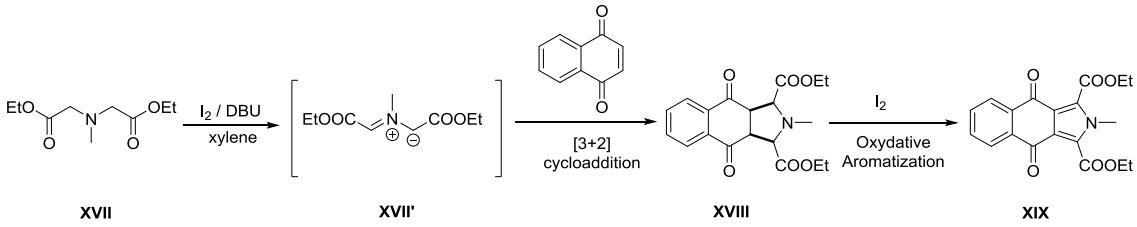
As already envisioned in previous chapters quinones are a class of organic compounds endowed with a variety of biological activities, mostly connected with their redox properties. A number of natural and synthetic ring-fused quinones show remarkable anticancer activity, and a series of synthetic 1,4-naphtoquinones have recently been identified as Hsp90 inhibitors. Multiple signal transduction pathways implicated in the regulation of cell prolification and survival are Hsp90 dependent. In a recent publication also ring-fused isoquinoline-5,8-diones have been identified as higly specific Hsp90 inhibitors. In this work 1,3-dipolar cycloaddition between *in situ* generated nitriloxides from **XV** and 6-bromo-isoquinoline quinones **XIV** afforded isoxazole ring-fused products **XVI** with good regioselectivities, mediated by the presence of the bromide in the electrophilic site (**Scheme 3**, via **c**). The isoquinoline quinone nucleus, compared to the naphtoquinonic *core*, led to an improvement of both the binding affinity and the antiproliferative activity compared to the corresponding naphthoquinone derivatives. The most potent compounds of the whole series, **XVIa** and **XVIb**, were tested for their

ability to downregulate the expression of Hsp90 client proteins in the squamous cell carcinoma A431 and the mesothelioma STO cells and showed nanomolar concentration cytotoxic activity.[36]



Scheme 8. 1,3-Dipolar cycloaddition affords isoxazole-ring-fused isoquinoline-5,8-diones

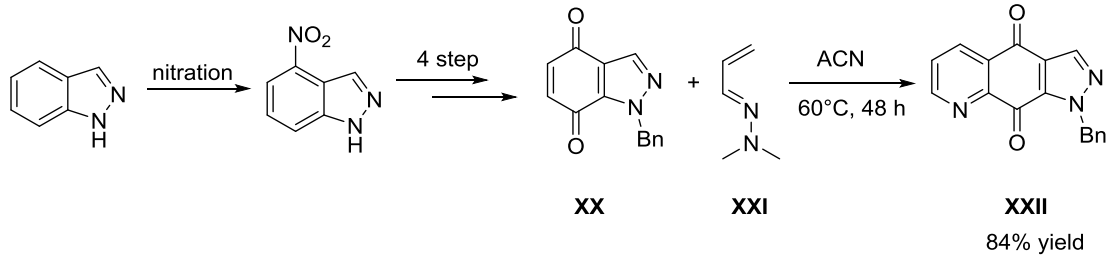
In 2014 a research group developed a one-pot, molecular iodine-induced, 1,3-dipolar cycloaddition/oxidative aromatization sequence to construct 2-substituted benzo[f]isoindole-1,3-dicarboxylates: a rapid and efficient strategy to construct biologically important compounds containing the quinone structure. Azomethine ylide **XVII'** was formed *in situ* by reaction of *N*-methyliminodiacetate **XVII** with molecular iodine and showed that simple naphtoquinone reacted well generating the pyrroline-derivate **XVIII.** After iodine-induced oxidative aromatization the tricyclic-fused system was obtained in good yield[37].



Scheme 9. One-pot, molecular iodine-induced, 1,3-dipolar cycloaddition/oxidative aromatization

Even if 1,3-dipolar cycloaddition reactions between quinolinequinone and isoquinoline-5,8-diones could provide a convenient and straighforward synthesis, leading to bioactive compounds, literature in this area is still vacant. Pyridine-fused indazol-4,7-diones has been synthesized through aza-Diels Alder reaction (also called Ghosez-type reaction) between indazol-4,7-diones and acrolein *N*,*N*-dimethyl hydrazine[38]. Synthesis

of indazole derivates is by the way not trivial. Direct synthesis of simple quinone-fused pyrazoles had proved not to be achievable through 1,3-dipolar cycloaddition between nitrile-imines and *p*-benzoquinone[39]. Synthesis of the dienophiles had to involve oxidation to quinone moiety of the benzene ring of indazole itself. After nitration reaction that lead to 7-nitro indazole a 4 step synthesis afforded the desired indazol-4,7-diones (**Scheme 10**). The high number of steps, the use of toxic reagents and the difficulty encountered in the functionalisation of the dienophile, such as *C*-subtitution on the pyrazole moiety, made the reaction uneasy for further investigations.

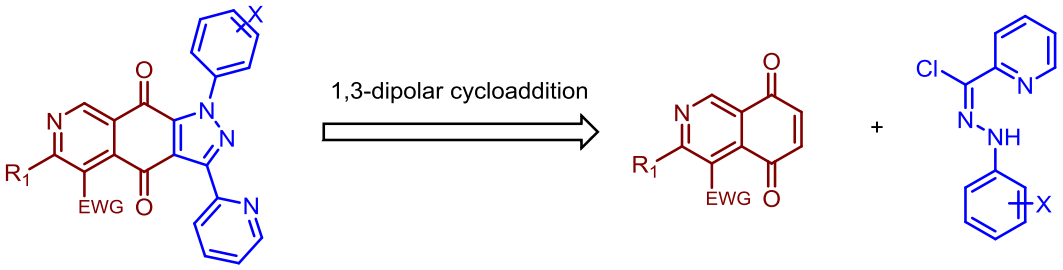


Scheme 10. Synthesis of tricyclic pyrazole-fused quinoline quinone

Above all, this reaction approach is useless in synthesis of isoquinoline quinone derivate, because it concerns the use of N-vinyl imines that would required an inverse electron demand Diels Alder reaction, unfittable with the use of indazol-4,7-diones. Maybe for all these reasons a precise methodology and scope in the synthesis of tricyclic pyrazole-fused isoquinoline quinones is still yet unexplored.

2. Aim of the Thesis

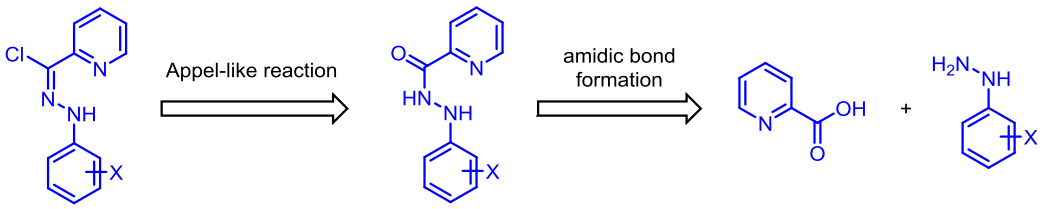
The importance of pyrazoles and isoquinoline-5,8-diones as potent biological scaffolds in medicinal chemistry has been widely underlined in the previous chapters. The aim of this thesis project is to synthetize a novel class of tricyclic polysubstitued pyrazole-fused isoquinoline-5,8-diones and to investigate their biological potential against malignal tumor, acting as PI3K inhibitors. A highly convergent synthetic pathway to achieve these new polycyclic rings, involving a 1,3-dipolar cycloaddition with isoquinoline-quinones as dipolarophiles will be investigated for the first time. Moreover, in order to ensure a suitable and scalable process, reaction conditions will be optimized for every step and the robustness of the method will be tested in synthesizing differently substituted compounds, sharing the same central scaffold. Finally, with the help of *Molecular Docking*, the most suitable scaffolds will be chosen to check the potential inhibition of this newly synthetized compounds in preliminary *in-vitro* tests. Retrosynthetic analysis, starting from the final products, may help in recognizing the different intermediate scaffolds employed and foreseeing the synthetic strategy adopted.



Following the most suitable retrosynthetic approach, due to the experience gained from our laboratory in [3+2] cycloaddition reactions, the central core will be formed through a telescopic two-step 1,3-dipolar cycloadditon followed by oxidation, between the ring-fused quinone (dipolarophile, in red in retrosynthetic scheme) and the nitrilimine (dipole) derived from the hydrazonoyl chloride (in blue in retrosynthetic scheme). Optimization of reaction conditions, such as temperature, concentration, reaction initiators will be broadly studied in terms of yield and regioselectivity. Since none of the

two building blocks is commercially available, the synthesis of these two precursors will be widely investigated.

Even if the synthesis of aryl-substituted hydrazonoyl chlorides, starting from the corresponding hydrazide, is already present in literature, an heteroaromatic variant, as far as we know, is still vacant; therefore a precise methodology will be defined.

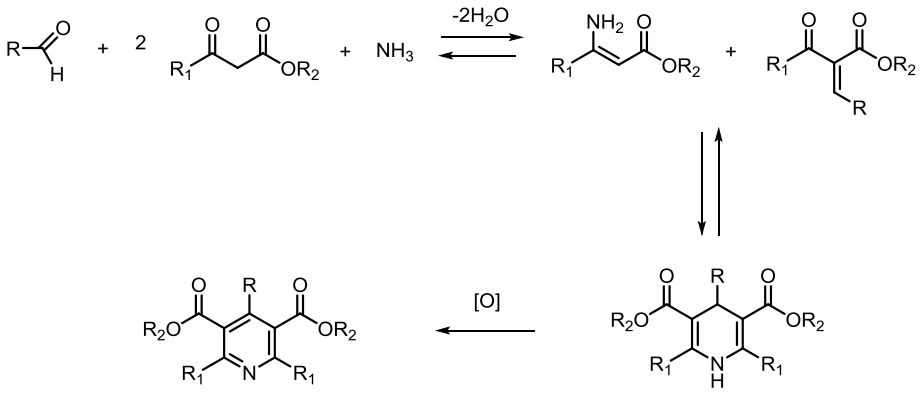


For the dipolarophile, literature presents many ways to obtain isoquinoline-quinones; however, sometimes the scope is restricted to a small range of amenable functionalization. Moreover, unsubstitued quinone moiety, needed in the subsequent 1,3-dipolar cycloaddition step, is most often impossible to achieve. In addition, *taylor made* synthesis, designed to reach precise scaffolds, tunable in base of their bioactive capability, often require an inaccessible number of steps. This considered, our aim will be to find a step-economical and versatile synthetic pathway, in order to obtain polysubstitued isoquinoline-5,8-diones starting from commercially available and relatively cheap materials.

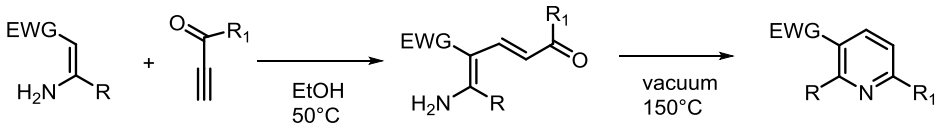
3. Results and Discussions

3.1. Synthetic methology for the dipolarophile

For the synthesis of isoquinoline-5,8-diones, a modification of a very well-known literature procedure seemed to be the most suitable starting point for our purpose. The reaction employed could be seen as a combination between the Hantzsch dihydropyridine synthesis, followed by oxydation, in which a cyclocondensation of *in situ*-generated enone and enamine species gives dihydropyridines (**Scheme 11**), and the Bolmann-Rahtz pyridine synthesis, in which the direct use of ynones, instead of enones, obviates the need for an aromatizing oxidation step to get the target pyridines (**Scheme 12**). Although the Bohlmann-Rahtz Synthesis is more versatile, purification of the intermediate and the high temperatures required for the cyclodehydration are significant drawbacks that have limited its synthetic utility.



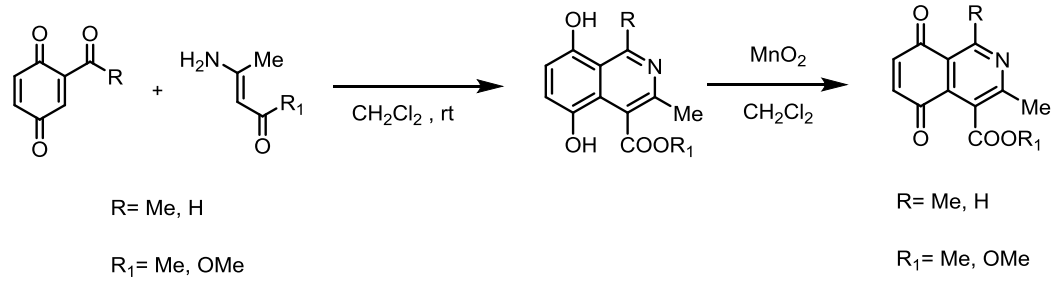
Scheme 11. Hantzsch Dyhydropyridine synthesis followed by oxidation in nitric acid



Scheme 12. Bolhmann-Ratz pyridine synthesis that afford substitued pyridine without oxidation needed

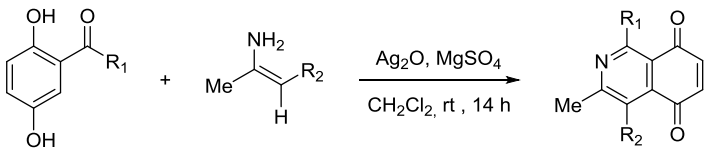
In 2006, Valderrama et al.[40] synthetized a series of 1-Aryl-substitued aminoisoquinolinequinones, studying their antitumoral activity. Starting with a

preformed 3-aminocrotonate, as enone, bis-conjugated acetyl-1,4-benzoquinones, as Micheal acceptors, and MnO_2 as the oxidant, they obtained substituted isoquinoline-5,8-diones with acceptable yields (**Scheme 13**).



Scheme 13. Cyclocondensation reaction between enaminone and quinone

Interestingly, they observed that the reaction could be carried out in a one-pot procedure, starting directly from a suitable 2-carboxy hydroquinone, exploiting the fast redox equilibrium of this species with the oxidizing agent used for the final irreversible step. After some screening, they found that the use of 2 equivalents of Ag_2O acting as an oxidant for both the initial formation of the Micheal acceptor and for the final step regeneration of the quinone moiety, gave the desired products in good yields (**Scheme 14**)[41].



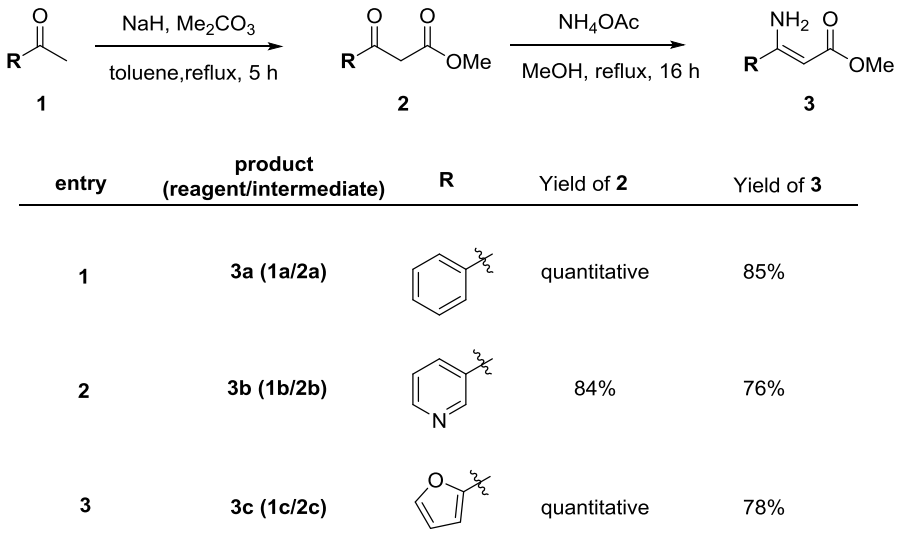
R^1	R^2	Yield (%)
H	CO_2Me	86
phenyl	CO_2Me	60
2-thienyl	CO_2Me	67
2-furyl	CO_2Me	56
H	COMe	74
phenyl	COMe	53
2-thienyl	COMe	72
2-furyl	COMe	56

Scheme 14. One-pot Micheal addition-cyclocondensation-oxydation reaction

In this work, Valderrama's group explored mainly the screening of possible substitutions in the 1-position of the quinolinic ring (**Scheme 14**, column R^1) and, partially, in the electron-withdrawing group, employing methyl 3-aminocrotonate to afford a carboxylated substitution, or 3-amino-penten-2-one for an acetyl-substituted isoquinolinequinone (**Scheme 14** column R^2).

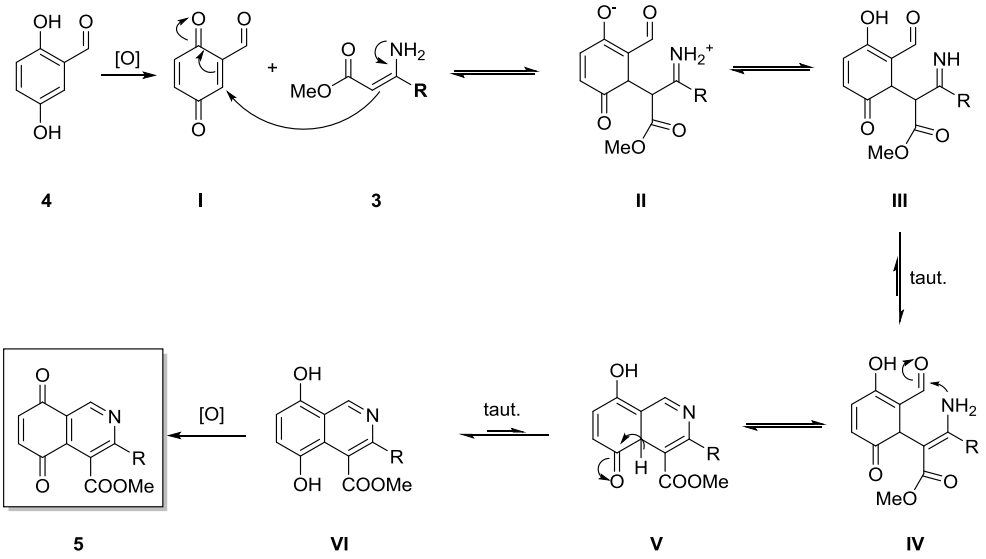
3.1.1 Synthesis of Isoquinoline-5,8-diones

Considered the accessible starting material, the good yields and the mild conditions, we thought that the previously described pathway could be a simple and straightforward method to synthesize the dipolarophiles of interest, but the lack of a consistent scope on the 3-position of the ring, with more bioactive and challenging moieties, rather than a simple methyl substituent, was undoubtedly a drawback for our purpose. For this reason, we decided, initially, to maintain the same EWG (Electron Withdrawing Group) employed by Valderrama (COOMe) and expand the outlook on the substitution of the 3-position. As shown in **Scheme 14**, for the synthesis of the previously mentioned isoquinoline-5,8-diones a cyclocondensation reaction between 2,5-dihydroxybenzaldheyde and enaminones has to be employed. While 2,5-dihydoxybenzaldheyde is commercially avaible, enaminones were synthetized, following a literature procedure, from β-keto esters. These 1,3-dicarbonilic compounds were also synthetized according to a reported procedure.[42] For the synthesis of β-keto esters, well-established procedures are Claisen condensation and Blaise reaction, but major drawbacks such as use of toxic reagents and formation of side products are involved. So, we decided to use a methodology involving the formation of the enolate of a substitued methyl ketone (by reaction with NaH in boiling toluene), followed by its nucleophilic attack on dimethyl carbonate. β-Keto esters **2** were obtained with excellent yields and often with no need for further purification. Substrates employed are shown in **Scheme 15**.



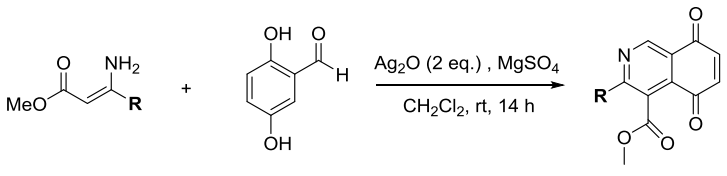
Scheme 15. Synthesis of substituted enaminones

The second step consisted in an amination reaction using ammonium acetate (NH_4OAc) as the source of ammonia in a condensation-tautomerization process involving the ketonic carbonyl of keto esters **2**. Enaminones **3a**, **3b** and **3c** were easily obtained after purification by column chromatography on silica-gel. Subsequently, the reactivity of the phenyl, 2-furyl and 3-pyridyl derivates **3a**, **3b** and **3c** was tested to see if aryl and heteroaryl substituents on the 3-aminoacrylate were well tolerated in the synthesis of the desired isoquinoline-5,8-quinones. The cyclocondensation reaction was carried out using the previously described method. As it is possible to see in the proposed mechanism showed in **Scheme 16**, according to the literature, the first step is the formation of the Micheal acceptor (quinone **I)** from the oxidation of the starting material performed by the first equivalent of Ag_2O, followed by the attack of the enamine in the most reactive position. Tautomerism of the imine **III** to a more stable conjugated enamine **IV** triggers the 6-exo-trig cyclocondensation. Tautomerization of ketone **V** leads then to a more stable 5,8-dihydroxyisoquinoline, due to aromatization of the two condensed rings. As the last step, the second equivalent of Ag_2O affords the oxidized form **5**, as the reaction product.



Scheme 16. Proposed mechanism for the synthesis of isoquinoline-5,8-diones

Scheme 17 shows the results for the isoquinoline-5,8-diones formation. Initially, monitoring the reaction by TLC, we found a plateuax in the conversion after 6 hours, in which both hydroxyquinone and enaminone were still observable. After the addition of further 0.5 equivalents of Ag_2O the reagents were no longer detectable by TLC analysis. This behavior was imputable to an *in situ* degradation of silver oxide; thus, we decided to add the oxidizing agent portionwise, 0.4 equivalent hourly in 5 hours and then to stir overnight.

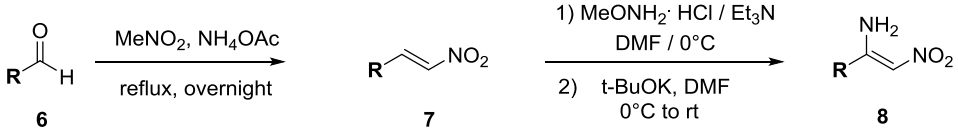


entry	product (reagent)	R	Yield of **5**
1	5a (3a)		90 %
2	5b (3b)		71 %
3	5c (3c)		82 %

Scheme 17. Reaction to afford different substituted isoquinoline-5,8-diones

The reaction afforded almost pure products **5**, that could be purified by a short plug of silica gel in the case of phenyl **5a** and 2-furyl **5c** substituted isoquinoline-5,8-diones and by column chromatography on silica gel with the 3-pyridyl substituent **5b**.

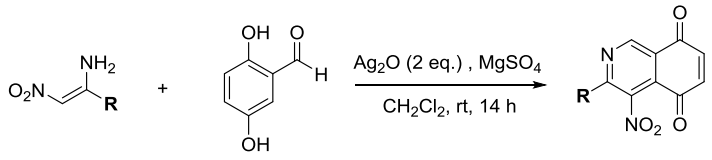
After the good results obtained with the screening of different substitutions on the 3-position of the isoquinoline-5,8-dione substrates, we investigated the possibility of enhancing the scope of the Electron Withdrawing Group present at the 4-position, deriving from the enaminone. For its enhanced EWG potential, the possibility for further functionalizations substitution of methyl ester with a nitro group seemed the most attractive idea. Using procedures previously described in literature[43] we decided to synthetize β-amino nitrolefins, laying our attention on the maintenance of the same substitution pattern of the previously synthesized products **5**. Except for commercialy avaible β-nitrostyrene **7a**, nitroolefins **7b** and **7c** had to be synthetized. Thus, starting from commercially available 2-furfural **6c** and 3-pyridin carboxaldheyde **6b** we obtained the correspondent nitroolefin **7b** and **7c** through Henry reaction, followed by heat-mediated dehydration. Sometimes, especially for pyridine derivative **7b**, the intermediate aminoalchol was still observable in the crude mixture (by means of ^{1}H NMR analysis) and an acidic work-up with TFAA (trifluoroacetic acid) was required, in order to favor water elimination.



entry	product reagent/ intermediate	R	Yield of **7**	Yield of **8**
1	**8a (7a)**			90%
2	**8b (6b/7b)**		55%	43 %
3	**8c (6c/7c)**		92 %	75%

Scheme 18. Pathway through the synthesis of β-amino nitrolefins

The second step of the process is an aza-nitro-Micheal addition of pre activated methoxylamine on the nitroolefin. The addition intermediated was filtered and added dropwise to a solution of potassium *tert*-butoxyde, to give elimination of the methoxide ion to form a stable conjugated β-amino nitroolefins **8**. This synthesis was performed in multiple gram scale with non-consistent loss of yield. Despite the good yield of phenyl and furyl derivatives **8a** and **8c**, some difficulties were encountered in the purification of the substrate containing 3-pyridyl moiety **8b** due to the extreme insolubility of the desired product. As anticipated before, we then tested the efficacy of the β-amino nitroolefins in the cyclocondensation reaction for the synthesis of the isoquinoline-5,8-dione scaffolds. The reaction was carried out in the same conditions as before (**Scheme 17**). We thought that, due to a less nucleophilic enaminone, both on the carbon that undergoes the Micheal addition and on the amine, coordinated through hydrogen bonding to the nitro group (NMR analysis shows two different aminic hydrogen, one in a very deshielded region), we would notice a dramatic decrease of reactivity. Interestingly, as it is possible to see in **Scheme 19**, even if a lower degree of reactivity was observed, we were able to achieve good yields in every case. Moreover, the decrease in the yield, compared to products **5**, can be attributed to the fact that nitro-isoquinoline-5,8-diones **9** needed always to be purified by column chromatography, in order to obtain a product of suitable purity (see **Scheme 10** for comparison).



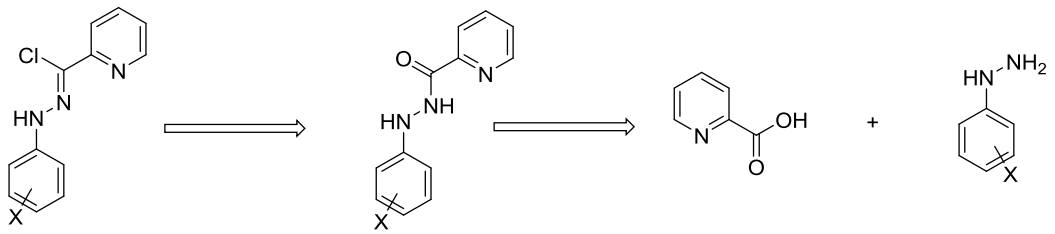
8 **4** **9**

entry	product (reagent)	R	Yield of **5**
1	9a (8a)		90 %
2	9b (8b)		71 %
3	9c (8c)		82 %

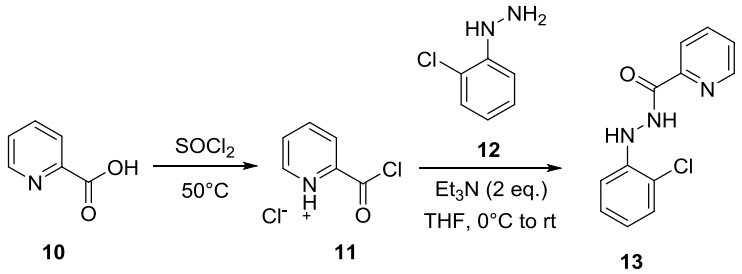
Scheme 19. Screening of feseability of the reaction to afford different substituted 4-nitro-isoquinoline-5,8-diones

3.2. Synthesis of the hydrazonoyl chloride, precursor of the 1,3-dipole

Afterwards, we moved to the synthesis of the precursor of the nitril-imine. A well-established procedure, previously employed by our research group for the synthesis of *C*-aryl hydrazonoyl chlorides, was optimized for an hetero-aromatic ring substitution. As seen in the previous chapter, the disconnection approach leading to the precursor, started with the synthesis of the hydrazide by nucleophilic acyl substitution of a suitable hydrazine on a previously activated carboxylic acid.

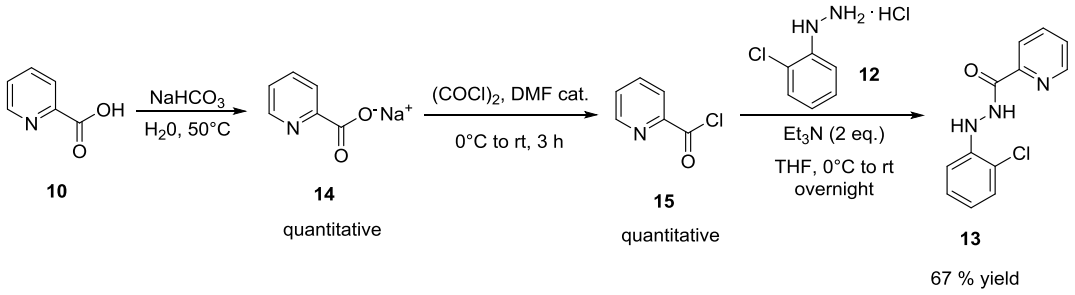


The 2-pyridyl and halogen-containing aryl moieties were chosen both to investigate for the first time an heteroaryl ring placed on a nitril-imine and for their remarkable biological properties, as already established in previous chapters (***1.3***). Initially, activation of the carboxylic group of picolinic acid **10** was accomplished via acyl-chloride formation. The first attempt was a direct chlorination of picolinic acid (3-pyridyl-carboxylic acid, **10**) with thionyl chloride ($SOCl_2$) to form intermediate picolinoyl chloride (hydrochloric salt) **11**, followed by nucleophilic substitution of 2-chloro-phenyl hydrazine **12**, with 2 eq. of triethylamine (**Scheme 20**). The reaction afforded the desired hydrazide **13** in poor yield (34 %) due to difficulties encountered in handling the unstable and poorly soluble intermediate **11**.



Scheme 20. Hydrazide formation by activation through acyl chloride formation

Another activation exploiting the acyl chloride was then tested. The formation of picolinoyl chloride **15** was achieved by activation of sodium picolinate **14** with a large excess of $(COCl)_2$ (oxalyl chloride) and catalytic DMF (dimethylformamide). The formation of NaCl, instead of HCl in the chlorination process, avoids the formation of insoluble **11** in favor of the corresponding free, soluble form **15**. Synthesis of hydrazide **13** in the same abovementioned conditions afforded the desired product in improved 67% yield after biphasic trituration in water- EtOAc/Hexane (4:3) and filtration (the high insolubility of **13** was exploited).

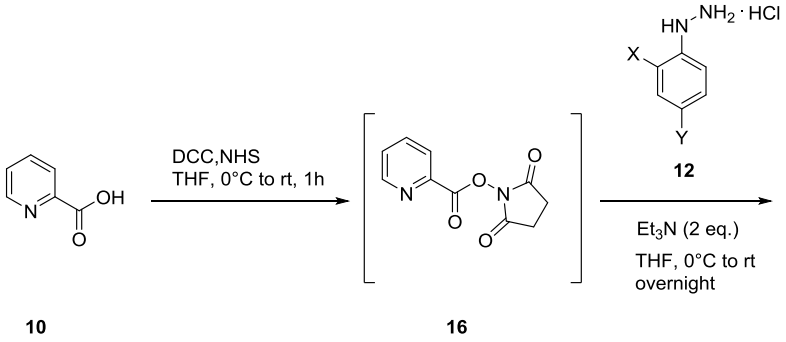


Scheme 21. Formation of picolinoyl chloride with oxalyl chloride and nucleophilic substitution with hydrazine

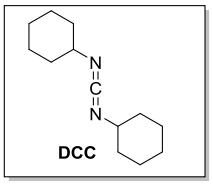
Due to the uneasy work-up for the removal of excess of oxalyl chloride, and to the need for improving the step-economy and the scalability of the substrate preparation (employing a high amount of $(COCl)_2$ also poses safety problems), we explored a direct activation of picolinic acid **10**. Pyridine-2-carboxylic acid **10** was reacted with DCC (dicyclohexylcarbodiimide) and NHS (*N*-hydroxysuccinimide), in anhydrous THF, to form *in situ* an activated NHS-ester **16** that can undergo direct substitution by pre-activated hydrazine **12**. After biphasic reslurry in water-EtOAc/Hexane (4:3), filtration and recrystallization from ethanol to remove the DCU (dicyclohexylurea) coproduct, desired product **13** was obtained in quantitative yield.

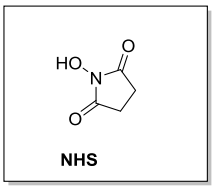
As mentioned in the previous chapters, halogen-containing drugs are an important family of bioactive pharmaceutical compounds and the possibility to *fine tune* an halogen substitution in different ways is an important tool in synthetic organic chemistry. For this purpose, the shown process is to be considered a highly efficient

methodology, due to the large number of cheap, commercially available halogen-substitued phenyhydrazines. For these reasons hydrazides **13a**, **13b** and **13c** derived from differently halogen-substituted hydrazines **12a**, **12b** and **12c** were prepared. The desired product was always achieved with good yields. The minor yield obtained for the the bis-fluorinated hydrazide **13c** can be imputable to a different purification process: in fact, due to the higher solubility often showed by fluorinated molecules, the trituration-recrystallization process was no longer exploitable and separation from DCU by column chromatography on silica gel was necessary.



entry	product (reagent)	X	Y	Yield
1	13a (12a)	Cl	H	quantitative
2	13b (12b)	Br	H	quantitative
3	13c (12c)	F	F	77 %

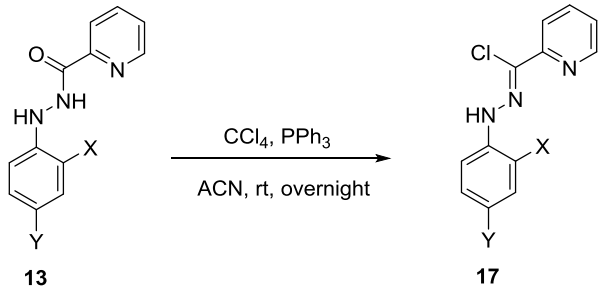




Scheme 22. Synthesis of halogen-substituted hydrazides through activation of carboxylic acid with DCC/NHS

The last step of the synthesis was the formation of hydrazonoyl chlorides bearing an hetero-aromatic ring such as 2-pyridyl. Even if the procedure adopted was identical to the one reported for aryl-substituted hydrazide, the formation of heteroaryl-hydrazonoyl chlorides is yet unexplored. Reaction was performed in Appel-like conditions: CCl_4 (methane tetrachloride) and PPh_3 (triphenylphosphine) were employed as chlorinating agents, in anhydrous acetonitrile (ACN). In the three cases we observed a very clean

reaction. TLC and ^{1}H NMR on the crude product showed only the presence of hydrazonoyl chloride and triphenylphopshine oxide. After purification on short column chromatography we obtained the desired products **17** with comparable literature yields (**Scheme 23**).



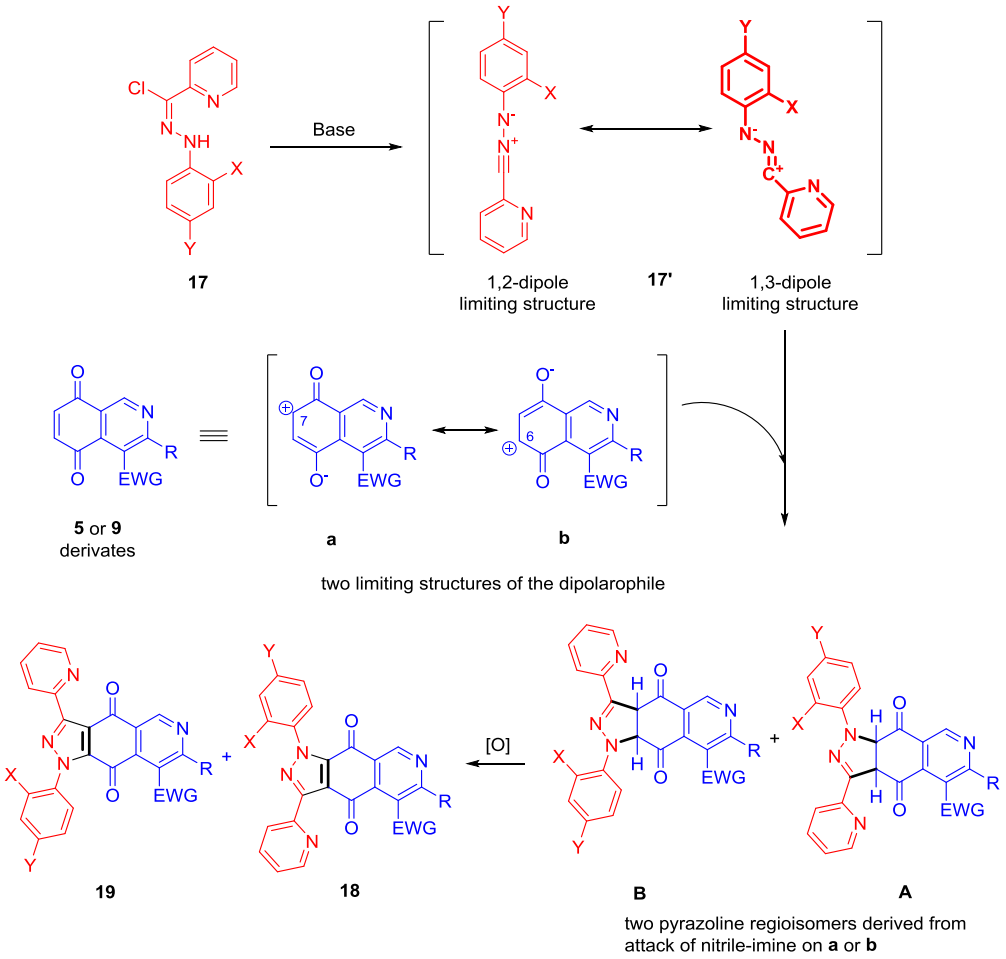
entry	product (reagent)	X	Y	Yield
1	17a (13a)	Cl	H	80%
2	17b (13b)	Br	H	74%
3	17c (13c)	F	F	62%

Scheme 23. Synthesis of hydrazonoyl chloride starting from hydrazide in Appel-like condition

3.3. 1,3-Dipolar cycloaddition of the nitril-imine with the isoquinoline-5,8-diones followed by oxidation: synthesis of tricyclic ring-fused pyrazoles

With the dipolarophiles (**5** and **9**) and the precursor of the 1,3-dipole **17** in hand, the feasibility, the regiochemistry and the best reaction conditions for the [3+2] dipolar cycloaddition-oxydation were then investigated. 1,3-Dipolar cycloaddition between isoquinoline-5,8-diones and nitrile-imines has never been explored and hides problems of controlling both reactivity and regioselectivity. Base-mediated *in situ* formation of nitrile-imine **17'** from hydrazonoyl chloride **17,** affords the 1,3-dipole, that captures the dipolarophile (isoquinoline-5,8-dione derivates **5** or **9**) to give non-aromatic pyrazolines

A and **B**. In the first step the rate of formation of the reactive species is fundamental and good reaction outcomes depend on the stability of the nitrile-imine, that could undergo unwanted dimerization or degradation processes, compared to the capability of the dipolarophile to perform the cycloaddition reaction. On the other hand, a regio-selectivity problem is present. Isoquinoline-5,8-diones bear an unsymmetrical di-α,β-unsatured ketone portion that has two resonance forms **a** and **b** that share the characteristics to react with nitrile-imine **17'** to afford regioisomeric ring-fused pyrazoline derivates **A** and **B** respectively**.**



Scheme 24. 1,3-Dipolar cycloaddition pathway between *in situ* generated nitrilimine and isoquinoline-5,8-diones

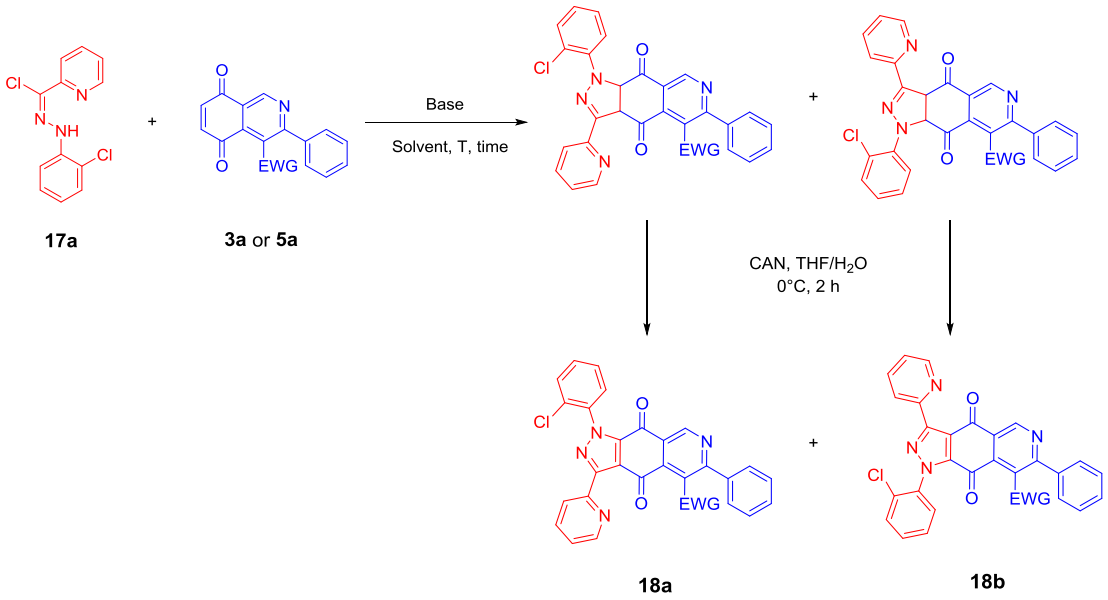
The previously cited Valderrama's work shows that nucleophlic attack on substrates like **5** or **9**, happens at the 7-position, clarifying the preferential location of the LUMO site. Therefore the HOMO site of the nitrile-imine (*N* atom of the 1,3 dipole moiety)

should attack the C-7 position rendering regioisomer **A** as the major product. Nevertheless, cycloaddition reactions are often much more complicated and sometimes unpredictable. Moreover, if it is the EWG group on the adjacent ring that guides the regiochemistry, a remote and tunable control of regioselectivity can be achieved.

At the end, the oxydation step, affording aromatization of pyrazolines **A** and **B** into pyrazoles **18** and **19** proved to be a rather trivial and well-established process, borrowed from previous works of the research group. The whole reaction pathway is summarized in **Scheme 24**.

3.3.1 Optimization of reaction condition

We therefore started to investigate the reaction conditions to perform the 1,3-dipolar cycloaddition reaction. Employing isoquinoline-5,8-diones **5a** or **9a** (dipolarophiles) and hydrazonoyl chloride **17a** (precursor of the 1,3-dipole, nitrile-imine) as model substrates, we tested the effect of different bases, as initiators for the formation of the nitrile-imine, different temperatures, solvents and reaction times on the yield of the reaction and the regioisomeric ratio. The oxidation step that followed the cycloaddition reactions, involved always suspension of the cycloaddition crude product in a mixture of THF and water and treatment with cerium ammonium nitrate (CAN). This proved to be efficient enough and did not need any optimization. Results are summarized in **Table 2**.



17a **3a** or **5a** **18a** **18b**

entry	EWG	solvent	time (h)	T (°C)	Base	eq. of 17	Yield (%)[b]	18a:18b ratio
1	$-NO_2$	toluene	12	50	Et_3N	1	no reaction	
2	$-NO_2$	toluene	12	reflux	Et_3N	1	decomp.	
3	$-NO_2$	dioxane	2.5	40	Et_3N	0.8	7	5:1
4	$-NO_2$	dioxane	12	40	Et_3N	1.3	11	5:1
5	$-NO_2$	dioxane	2.5	60	Et_3N	1.3	decomp.	
6	$-NO_2$	dioxane	2	rt	Et_3N	1.3	no reaction	
7	$-NO_2$	dioxane	14	rt	Et_3N	1.3	35	5:1
8	$-NO_2$	dioxane	14	rt	AgOAc	1.3	decomp.	
9	$-NO_2$	dioxane	14	rt	Ag_2CO_3	1.3	46	4:1
10	$-NO_2$	dioxane	6 h	50	Ag_2CO_3	1.3	50	4:1
11[c]	**$-NO_2$**	**dioxane**	**6 h**	**50**	**Ag_2CO_3**	**1.3**	**67**	**4:1**
12[c]	-COOMe	dioxane	6 h	50	Ag_2CO_3	1.3	51	6:1
13[c]	**-COOMe**	**dioxane**	**6 h**	**reflux**	**Ag2CO3**	**1.3**	**64**	**6:1**
14[c,d]	**$-NO_2$**	**dioxane**	**6 h**	**50**	**Ag_2CO_3**	**1.3**	**66**	**7:1**

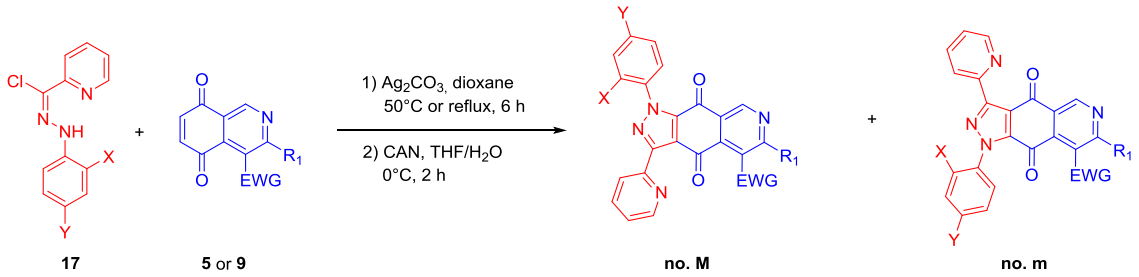
(**a**) Reaction conditions: Sealed tube **5a/9a** (0.1 mmol), base (2.5 of **17a**), solvent (0.5 µL); then CAN (2.5 eq), THF/H_2O 8:6 (3.5 mL), 0 °C, 2 h. (**b**) Yield of **18a** isolated after column chromatography, referred to **5a/9a**. (**c**) 0.021 mmol **17a** was added hourly in 6 h until the necessary amount. (**d**) in presence of 0.1 mmol of Schreiner's thiourea.

Table 2. Screening of 1,3-dipolar reaction conditions

Triethylamine, the most employed base for the formation of nitrile-imines, was found to be too reactive, leading almost instantaneously to the formation of tar-like decomposition products. Solvents and reaction temperatures did not improve much the reaction outcomes in these cases (entries **4**, **5** and **6**). Nevertheless, the process seemed to be promisingly feasible and quite regioselective. With our pleasure, we found that silver carbonate, which is reported to react differently from TEA in the formation of the nitrilimine,[44] improved greatly the conversion of the reagents into the desired products although with a low detriment in the regioisomeric ratio. With small case-by-case adaptations we were able to obtain clean and decomposition products-free crudes (by ^{1}H-NMR) and greater yields after purifications (entries **9**, **10** and **11**). Interestingly, we found that when 1 equivalent of Schreiner's thiourea was added in the reaction mixture, we had a better regioselectivity, maybe imputable to the coordination of the thiourea to the nitro-group that enhanced its electron-withdrawing ability and presumably confirming the previously reported hypothesis concerning the regiocontrol.

3.3.2 Screening of the substitution on both the cycloadditition reaction partners: synthesis of a class of triclycic ring-fused pyrazoles

Thereafter, a series of substituted pyrazole-fused isoquinoline-5,8-diones were synthetized, starting from differently halogen-substituted hydrazonoyl chlorides **17a**, **17b**, **17c** and aryl- or heteroaryl-substituetd isoquinoline-5,8-diones (**5a**, **5b, 5c** and **9a**, **9b**, **9c**).



Entry[c]	no. (17/5 or 9)	X	Y	EWG	R_1	Yield (%)[b]	M/m
1	18	Cl	H	$-NO_2$	phenyl	67	5:1
2	19	Cl	H	$-NO_2$	2-furyl	72	5:1
3	20	Cl	H	$-NO_2$	3-pyridyl	55	4:1
4	21	Cl	H	-COOMe	phenyl	64	5:1
5	22	Cl	H	-COOMe	3-pyridyl	35	4:1
6	23	Cl	H	-COOMe	2-furyl	61	6:1
7	24	Br	H	$-NO_2$	2-furyl	69	4:1
8	25	F	F	$-NO_2$	2-furyl	61	5:1
9	26	F	F	$-NO_2$	phenyl	58	3:1

(**a**) Reaction conditions: Sealed tube **5a/9a** (0.1 mmol), base (2.5 of **17a**), dioxane (0.5 μL); then CAN (2.5 eq), THF/H_2O 8:6 (3.5 mL), 0 °C, 2 h. (**b**) Yield of **no. M** isolated after column chromatography, referred to **5a/9a**. (**c**) 0.021 mmol **17a** was added hourly in 6 h until the necessary amount.

Table 3. Synthesis of different substituted pyrazole-fused isoquinoline-5,8-diones

Subjecting to the optimized reaction conditions the previously mentioned substrates, we found that aryl and heteroaryl substituents on the isoquinoline-5,8-dione scaffold were well tolerated and afforded good yields and almost pure crude products in both the regioisomeric forms. Decreased yield observed with the 2-pyridyl moiety were not attributed to a different reactivity, but to a more insoluble reagent that did not undergo full conversion with the applied reaction conditions.

These good results considered, this method represents, to our knowledge, the only straightforward synthetic strategy to afford triclyclic ring-fused pyrazoles. Moreover, it also proved to be better performing, in terms of overall yield, compared to other very similar synthetic sequences.[45]

In order to attribute the exact structure of the major regioisomer, X-Ray diffraction analysis was performed on a single crystal of product **26**. The structure shown is in

complete agreement with the theoretical and experimental studies conducted by our research group and reported in the introduction and with the previously formulated hypothesis (*vide supra*).

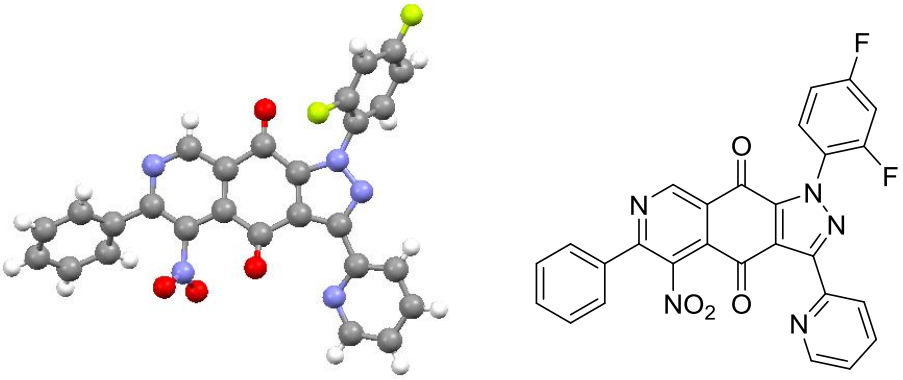
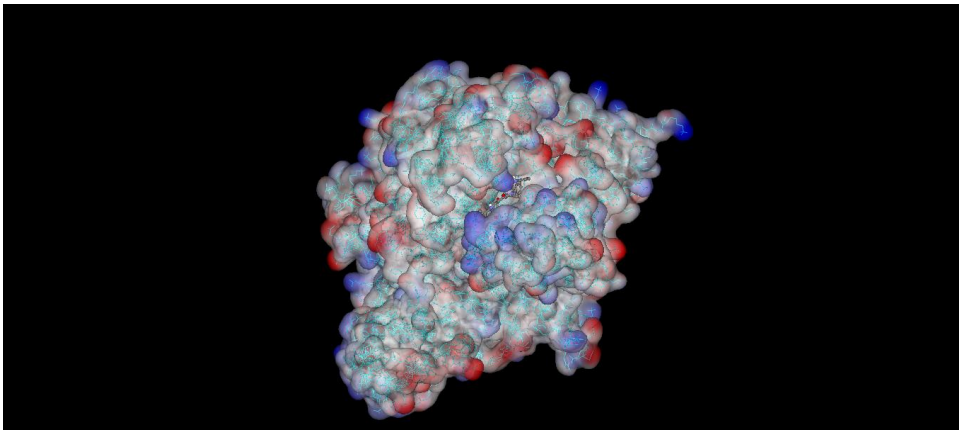


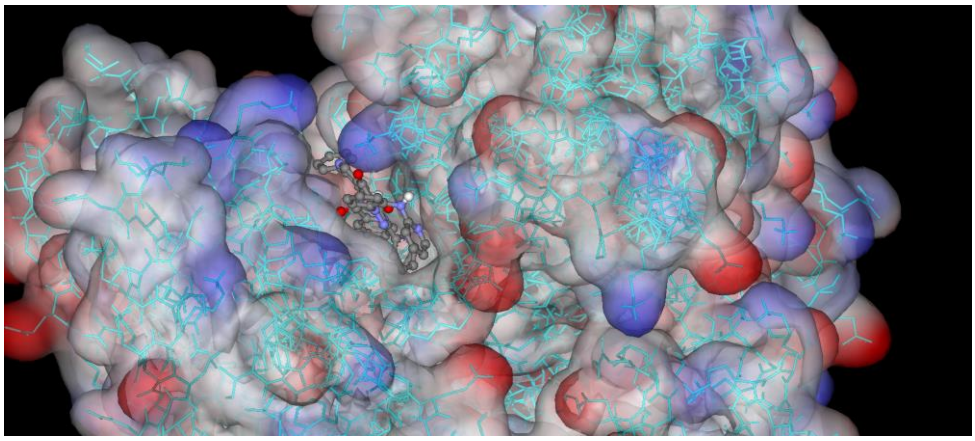
Fig. 23 X-ray structure of the major regioisomer (Blue= Nitrogen, Red= Oxygen, Grey=Carbon, White= Hydrogen, Yellow=Fluorine)

3.4. Molecular Docking and *in vitro* cytotoxic evaluation: pyrazole-fused isoquinoline-5,8-diones as a class of promising antitumoral agent

As already been said in previous chapters, Phosphatidylinositol-4,5-bisphosphate 3-kinase (also called phosphatidylinositide 3-kinases, phosphatidylinositol-3-kinases, PI 3-kinases, PI(3)Ks, PI-3Ks or by the HUGO (Human Genome Organization) official stem symbol for the gene family, PI3K(s)) are a family of volved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking, which in turn are involved in cancer. Since there are a number of different classes and isoforms of PI3Ks, Structure-Activity Relationship (SAR) studies were carried from collaborators of our research group to test the potential anticancer activity against PI3Kγ isoform of the same protein, that is thought to be an important modulator of extracellular signals, including those elicited by E-cadherin-mediated cell-cell

adhesion, which plays an important role in maintenance of the structural and functional integrity of epithelia. In addition to its role in promoting assembly of adherens junctions, the protein is thought to play a pivotal role in the regulation of cytotoxicity in cancer cells. We wanted to investigate either some already synthesized molecules or other ones bearing possible functionalization that could be made thinking about the reactivity of our building blocks, in order to prove, not just the efficacy of a singular candidate, but of a whole class of biologically active, easily obtainable and fine tunable molecules. **Fig. 24** shows protein PI3Kγ taken from RCBS Protein Data Bank. The active site is clearly visible on figures underling also the "small entrance" of the site, that justifies our





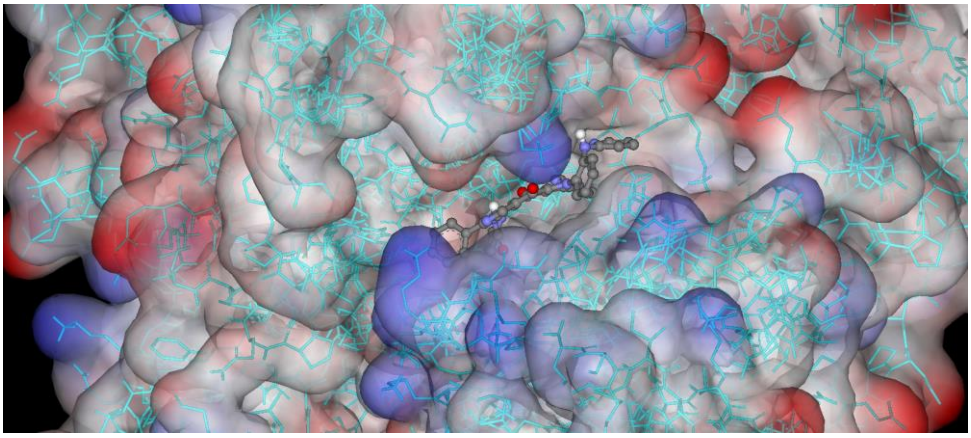
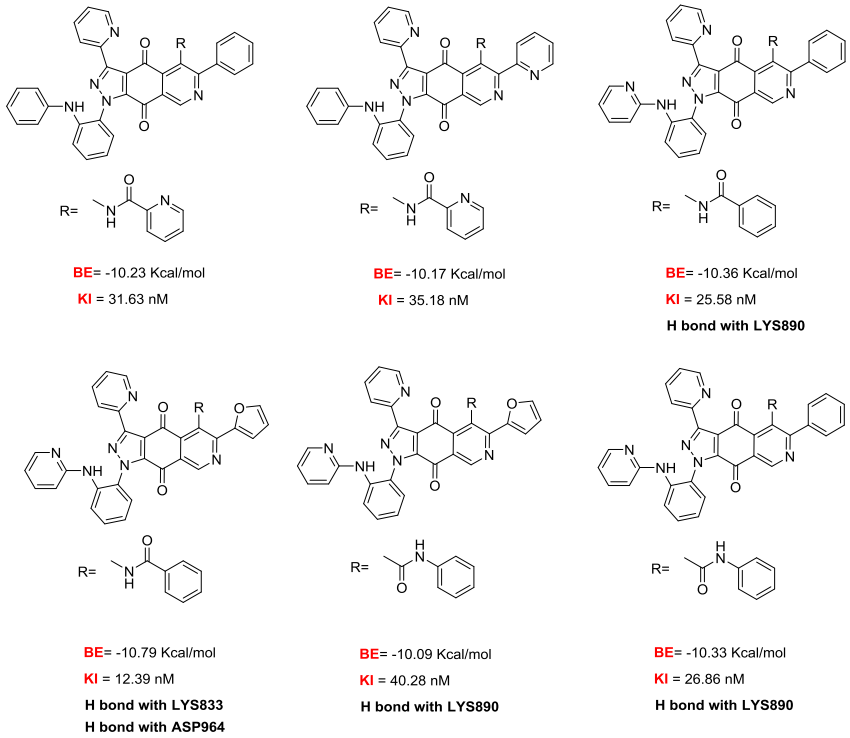


Fig. 24 Computational modelling of ligand-PI3K's active interaction

choice of the ligand with flat aryl and heteroaryl moieties that allows a favorable conformation to slip into the pocket. However, it seems remarkable the ability of all the docked ligands to fit in such an uneasy opening space. To test the Binding Energy and calculate the theoretical $IC_{50,}$ all the molecules docked were first optimized geometrically at physiological pH and Temperature through mechanical calculation in Gaussian, then the output was launched in AutodockTools, in a pre-optimized protein, that gave the results showed in **Table 4**.



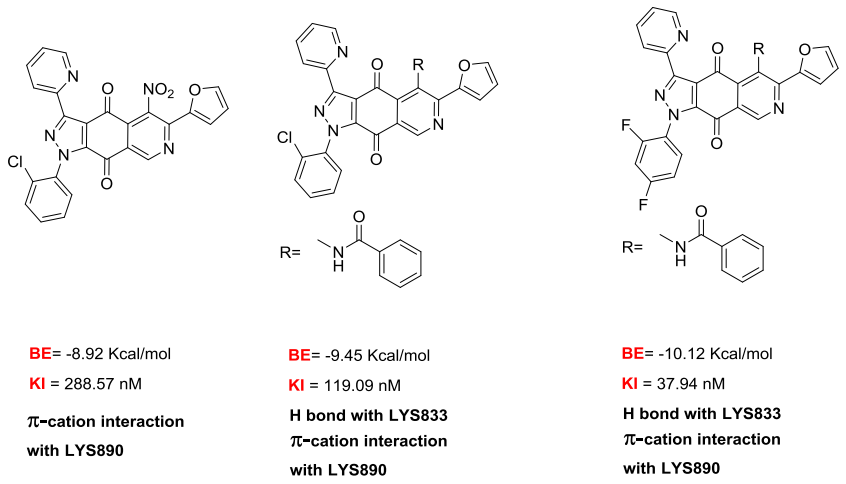
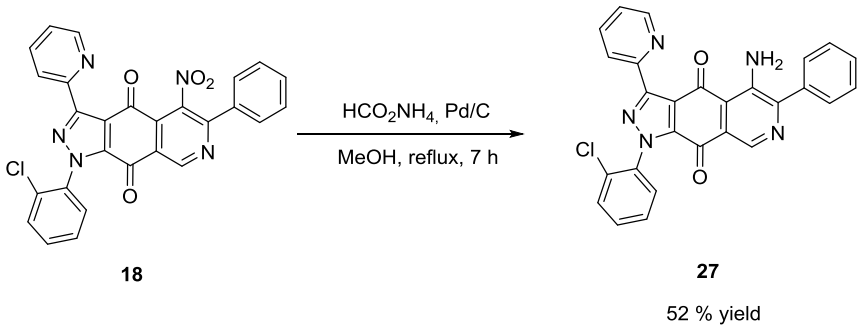


Table 4. Docking screening on target molecules containing tricyclic pyrazole-fused isoquinoline-5,8-diones *core*

All the molecules tested were thought on the basis of the reactivity of our precursors by adding typical moieties found in several bioactive compounds such as amide group, aryl or heteroaryl substituents. Substitution on the *N*-aryl ring, for example, with aniline or 2-aminopyridine could be synthetized by a metal-mediated amination reaction, like direct Buchwald-Hartwig coupling on bromine derivative **24**, or Cham-Lam coupling[46] on a pre-formed boronic acid. Amide formation could be carried out with a series of steps, involving saponification, activation of the acid and amination, for compounds **21**, **22** and **23,** deriving from –COOMe substituted cycloadducts, or by reduction of nitro-group and amidation with anhydrides of acyl chlorides for nitro-derivates **18, 19, 20, 24, 25** and **26**.

This theoretical investigation with different functionalizations showed pyrazole-fused isoquinoline-5,8-diones as a promising class of phosphoinositide 3-kinase inhibitor (PI3K inhibitor) by inhibiting one or more of the phosphoinositide 3-kinase enzymes, which are part of the PI3K/AKT/mTOR pathway, an important signaling pathway for many cellular functions such as growth control, metabolism and translation initiation which may result in tumor suppression. Interestingly, our pre-functionalized scaffolds, bearing a chlorine atom in the ortho position and two fluorine atoms in the ortho and para positions resulted in good binding energy values, demonstrating the capability of the central tricyclic scaffold alone in stabilizing the ligand-protein system. Moreover,

these substrates were found to be peculiar for two main reasons. One, in the conformational optimization analysis with Gaussian interface, they showed only one energetic cluster of population at the minimum binding energy, meaning that just one conformation was found inside the protein. Second, a new π-cation interaction was found between one hydrogen of a protonated primary amine of LYS890 and the delocalized π system of halogen-substituted aryl group. These two characteristics were enhanced with the ortho-para fluoride substitution. For these reasons, we decided to functionalize compounds **18** and **25** to effectuate preliminary *in vitro* studies on the potential citotoxycity of these candidates. We then envisaged to reduce the nitro-group of our products in order to get a compound that would be more suitable for *in vitro* tests (more water-soluble, for instance) and to show the potential of the scaffold itself by keeping a relative low number of elaboration steps. Reduction of the nitro group of **18** into arylamine **27** was achieved by catalytic hydrogenation using $HCOONH_4$ (ammonium formate) as hydrogen source, 10% Pd on activated carbon as catalyst and degassed MeOH as solvent. Product **9** was thus purified by column chromatography and obtained in 52% yield. (**Scheme 25**)

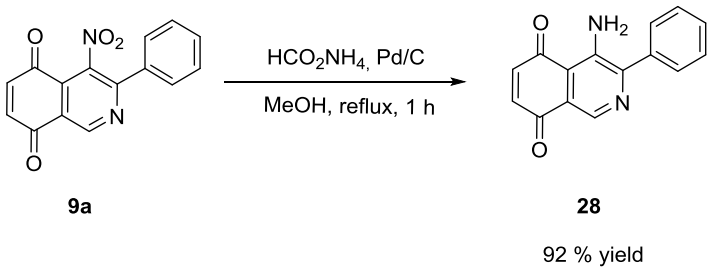


18 **27**

52 % yield

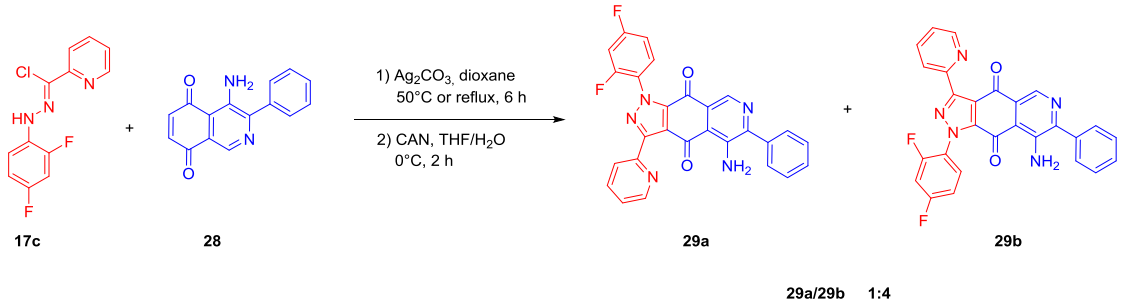
Scheme 25. Reduction of **18** with ammonium formate as a source of hydrogen to afford 1-amine moiety

We then thought that reducing the nitro group of the isoquinoline-5,8-dione before the cycloaddition, might have enhanced the reaction yield due to a more simple substrate. That was confirmed by conducting the reaction in the same conditions. TLC analysis showed a complete conversion after 1 hour and afforded 3-phenyl-4-amino isoquinoline-5,8-dione **28** in 92 % yield with no need for further purifications.



Scheme 26. Reduction done directly on the nitro-isoquinoline-5,8-dione

We then tried the 1,3-dipolar cycloaddition reaction on compound **28** in the same conditions as shown in **Scheme 26** and, interestingly, we envisioned an inversion of regioselectivity that lead to the preferential formation of **29b** in **4:1** ratio.

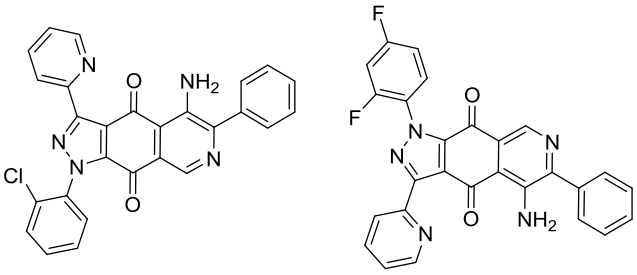


Scheme 27. Cycloaddition reaction between pre-reduced amino-isoquinolinequinone

Finally, in the last part of this work, we choose two (**27** and **30**) candidates that have been sent to the Biology Laboratory under the supervision of Doctor Mario Chiariello, Istituto Toscano Tumori, Siena. This collaborating group performed a first test of cell viability *in vitro* on T98G cell line of a solid brain tumor, the Glioblastoma Multiforme.

As it can be seen in **Fig. 24**, high cell mortality with very interesting IC_{50}'s have been obtained with both molecules. Indeed, the range 7-50 micromolar represent promising values which are comparable with already marketed drugs as reported in the literature[47].

These compounds represent preliminary and interesting examples of the final molecules we wish to test once the amidation reactions together with a complete set of docking studies will be available.



27 **30**

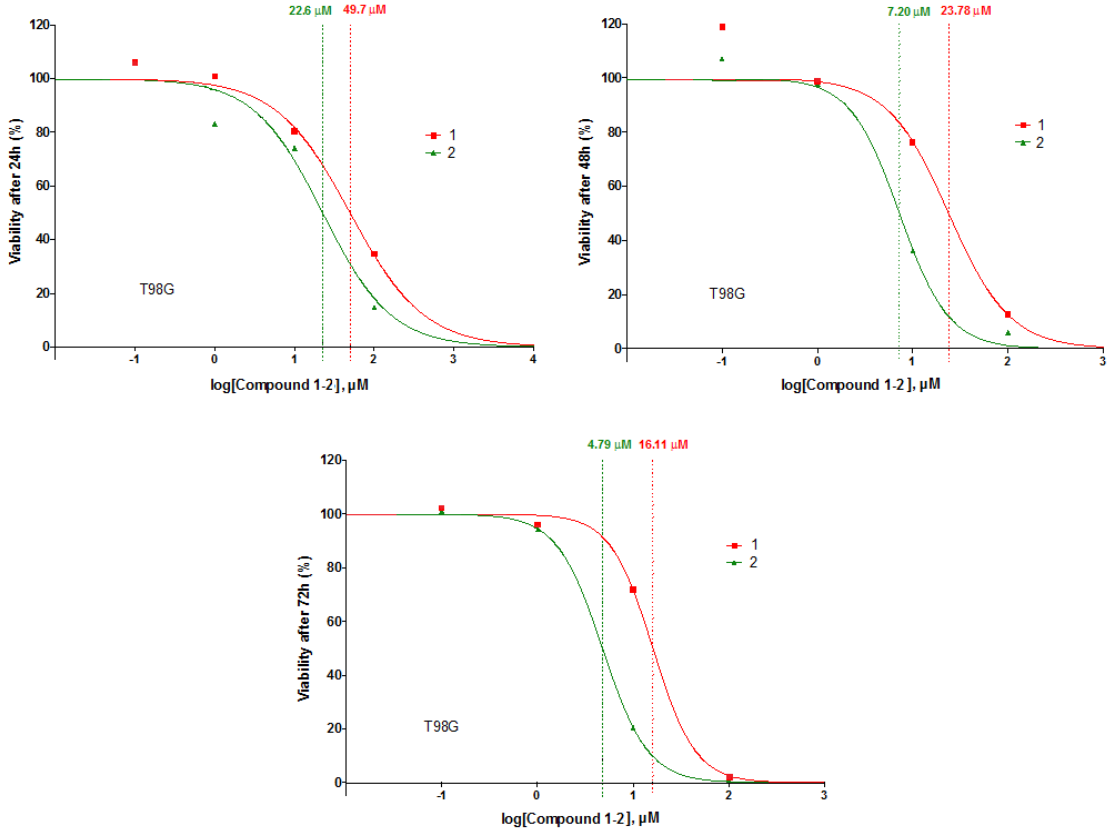


Fig. 24 IC_{50} values calculated through *in vitro* test on T98G cell. Red curve (1) corresponds to compound **27**, green curve corresponds to compound **30**.

4. Conclusions

In conclusion, a class of tricyclic polysubstitued pyrazole-fused isoquinoline-5,8-diones has been synthetized for the first time.

The robustness of the one-pot oxidation-Micheal addition-cyclocondensation reaction has been tested chequering different potentially biological active aryl- and heteroaryl-substituents in 3-position, filling up the lack of investigation in these interesting medicinal chemistry building blocks.

We synthetized, to our knowledge, the first example of *N*-heteroaryl nitrile-imine and, starting from different halogen-substituted hydrazines, we synthetized a series of hydrazonoyl chlorides optimizing the reaction conditions in order to get a scalable, good-yielded and step-economical two-passage synthesis for the nitrile-imine precursor from relatively cheap and commercially available starting materials.

The synthesis of yet unexplored tricyclic-fused pyridine-quinone-pyrazole *core* has been our main issue. A straightforward procedure, namely 1,3-dipolar cycloaddition followed by oxidative aromatization, established by our research group, has been employed. Screening of reaction conditions and characterization studies about the regioselectivity have been successfully performed and a class of complexes and polysubstituted scaffolds were synthetized with 36 % global yield in the major product (calculated for compound **18**), which is comparable or, in some cases, much higher than currently commercially available pyrazole-containing drugs.

Interestingly, a way to afford both the two regioisomers, by reducing before or after the cycloaddition reaction, the $–NO_2$ moiety on the isoquinoline-5,8-dione ring, has been developed.

Through *Molecular Docking* calculations we envisioned that our scaffolds stand as a whole new class of promising candidates as PI3K inhibitors for anticancer therapy. Finally, high purity samples of target compounds **27** and **30** have been obtained. They showed a good result of IC_{50} towards one type of brain cancer cells in a first test *in vitro* performed by a collaborating group.

A first aim of future studies, the inversion of regioselectivity observed considered, will be to test the cytotoxic activity of both regioisomers of the same molecule on different

cell lines. A second objective is to test whether our molecules bring a real interaction with PI3K, from the biochemical point of view.

However, with the results obtained, we have shown that the planned backbone is well suited to act as a cytotoxic agent on cancer cell lines, and for this reason further studies are ongoing in our research group.

5. Experimental section

General Methods. ^{1}H, ^{13}C NMR spectra were recorded on a Varian AS 300, 400 or 600 spectrometer. Chemical shifts (δ) are reported in ppm relative to residual solvent signals for ^{1}H and ^{13}C NMR. Multiplicity is explained in brackets as follow: “s”, singlet; “d”, doublet; “t”, triplet; “q”, quadruplet; “sept”, septuplet; “m”, multiplet; a “b” before the letter means “broad”.

Materials. Analytical grade solvents and commercially available reagents were used as received. Anhydrous THF was obtained by standing overnight on KOH, filtration through a short pad of basic alumina and distilled over Na/benzophenone. All the reactions demanding anhydrous conditions were performed in nitrogen atmosphere, passed through $CaCl_2$ and silica gel with indicator. Anhydrous toluene was obtained by distillation on Na. Dry DMF and ACN was obtained by overnight standing on activated 4 Å molecular sieves. Degassed MeOH was obtained by bubbling a nitrogen flux in an ice bath.

5.1.Synthesis of the dipolarophile

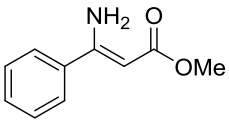
5.1.1 General method for the synthesis of β-ketoesters

To a dried 100 ml RBF (round bottom flask) were added NaH (2.2 g, 60% w/w, 56 mmol), dimethyl carbonate (3.6 g, 40 mmol) and toluene (20 mL) under nitrogen. After the mixture was heated to reflux, a solution of **3** (20 mmol) in toluene (10 mL) was added dropwise in 30 minutes. After the evolution of hydrogen ceased, the reaction was cooled down to room temperature. The reaction system was then diluted with 200 mL of EtOAc. The organic layer was separated, washed with brine (20 mL) and dried over $MgSO_4$. The crude product was evaporated *in vacuo* and the residue was purified by column chromatography on silica gel with EtOAc/Hexane (1:3) to give the desired product. **^{1}H NMR** analysis were comparable to the one already present in literature[42].

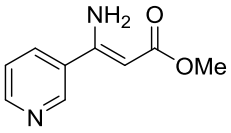
5.1.2 General method for the synthesis of enaminones

A solution of β-ketoesters (12 mmol) and NH_4OAc (1.9 g, 24 mmol) in methanol (12 mL), under nitogen atmosphere, was stirred under reflex overnight. The solvent was removed under reduced pressure and the residue was suspended in EtOAc (30 mL). The insoluble solid was filtered off and washed with EtOAc. The combined filtrate was washed with water and brine and dried over $MgSO_4$. The crude product was evaporated *in vacuo* and the residue was purified by column chromatography on silica gel with EtOAc/Hexane (1:1).

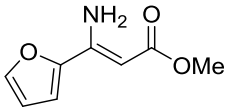
Experimental data:



Methyl (Z)-3-amino-3-phenylacrylate (**3a**); **^{1}H NMR** (300 MHz, $CDCl_3$) δ 7.55-7.51 (m, 2H), 7.47-7.36 (m, 3H), 4.97 (t, J = 0.7, 1H), 3.70 (s, 1H) ppm.



Methyl (Z)-3-amino-3-(pyridin-3-yl)acrylate **(3b)**; **^{1}H NMR** (300 MHz, $CDCl_3$) δ 8.80 (dd, J_1=2.4, J_2=0.7, 1H), 8.68 (dd, J_1=4.8, J_2=1.6, 1H), 7.83 (dt, J_1=8.0, J_2=1.9, 1H), 7.36 (ddd, J_1=7.9, J_2=4.9, J_3= 0.7, 1H), 4.97 (s, 1H), 3.73 (s, 1H) ppm.



Methyl (Z)-3-amino-3-(furan-2-yl)acrylate **(3c)**; **^{1}H NMR** (300 MHz, $CDCl_3$) δ 7.48 (dd, J_1=1.8, J_2=0.8, 1H), 6.75 (dd, J_1=3.5, J_2=0.7, 1H), 6.42 (dd, J_1=3.5, J_2=1.8, 1H), 5.14 (s, 1H), 3.70 (s, 3H) ppm.

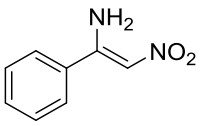
5.1.3 General method for the synthesis of β-nitroolefins

Freshly distilled aldehyde (50 mmol) was added to a stirred solution of ammonium acetate (0.96 g, 0.25 eq.) in dry nitromethane (50 mL) at 90^{o}C. The mixture was heated at reflux overnight, cooled to room temperature, poured into water and extracted with diethyl ether (3 × 50 mL). The extract was washed with brine, dried over $MgSO_4$, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc/Hex 1:2) to give the desired product. **^{1}H NMR** analysis were comparable to the one already present in literature[43].

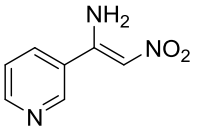
5.1.4 General method for the synthesis of β-amino nitroolefins

To a solution of methoxylamine-HCl (4.26 g, 1.1 eq.) in dimethylformamide (75 mL) was added Triethylamine (6.85 mL, 1.1 eq.) at 0^oC in an ice bath. Nitroolefin (43.5 mol) was added and stirred at 0^oC for 15 min then at rt for 5 min. The precipitate was removed by filtration and wash the solid with a small amount of DMF. The combined filtrate were placed into an addition funnel and add dropwise over 30 min to potassium t-butoxide (10.15 g, 2 eq.) in DMF (100 mL) at 0^oC. The bath was removed and the mixture was stirred at rt for 30 min. After the quenching of the reaction with sat. NH_4Cl (30 mL). the volume was reduced in half *in vacuo* and then extracted with CH_2Cl_2. Wash with water, brine, and dried over anhydrous $MgSO_4$, filter and concentrate in vacuo to give the desired amino-nitroolefin..

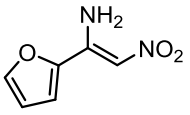
Experimental data:



(Z)-2-nitro-1-phenylethen-1-amine **(8a) ^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.07 (bs, 1H), 8.81 (dd, J_1=2.4, J_2=0.7, 1H), 8.79 (dd, J_1=4.8, J_2=1.6, 1H), 7.78 (dt, J_1=8.0, J_2=1.9, 1H), 7.42 (ddd, J_1=7.9, J_2=4.9, J_3= 0.7, 1H), 6.82 (s, 1H) ppm.



(Z)-2-nitro-1-(pyridin-3-yl)ethen-1-amine **(8b) ^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.07 (bs, 1H), 8.81 (dd, J_1=2.4, J_2=0.7, 1H), 8.79 (dd, J_1=4.8, J_2=1.6, 1H), 7.78 (dt, J_1=8.0, J_2=1.9, 1H), 7.42 (ddd, J_1=7.9, J_2=4.9, J_3= 0.7, 1H), 6.82 (s, 1H), 5.81 (bs, 1H) ppm.

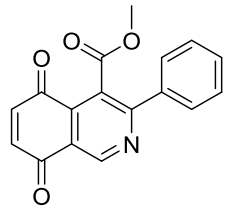


3-(Furan-2-yl)-4-nitroisoquinoline-5,8-dione **(8c) ^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.44 (s, 1H) 7.67 (dd, J_1=1.8, J_2=0.8, 1H), 7.41 (dd, J_1=3.5, J_2=0.7, 1H), 7.06 (s, 2H), 6.83 (dd, J_1=3.5, J_2=1.8, 1H) ppm

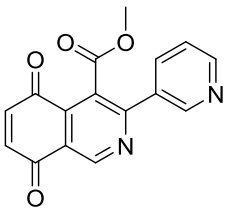
5.1.5 General procedure for the synthesis of isoquinoline-5,8-diones

A suspension of 2,5-dihydroxybenzaldehyde (1.6 g, 10.1 mmol), enamine (10 mmol), Ag_2O (4.63 g, 20.2 mmol, stepwise hourly for 5 hours) and $MgSO_4$ (5.5 g) in CH_2Cl_2 (150 mL) was stirred at room temperature overnight. The mixture was filtered, the solids were washed with CH_2Cl_2 and the solvent removed under reduced pressure. When necessary, the residue was purify by column chromatography over silica gel (90:10 CH_2Cl_2/AcOEt) to yield pure isoquinolinequinone.

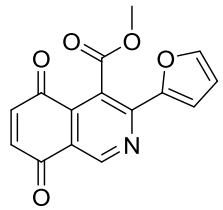
Experimental data:



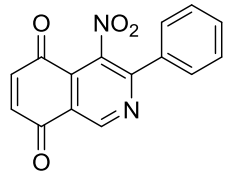
Methyl 5,8-dioxo-3-phenyl-5,8-dihydroisoquinoline-4-carboxylate **(5a)**; **^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.47 (s, 1H), 7.71-7.68 (m, 2H), 7.50-7.47 (m, 3H), 7.0 (s, 2H), 3.48 (s, 3H) ppm.



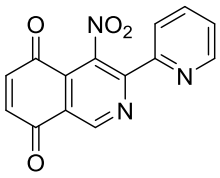
Methyl 5,8-dioxo-3-(pyridin-3-yl)-5,8-dihydroisoquinoline-4-carboxylate **(5b)**; **^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.67 (s, 1H), 8.94 (dd, J_1=2.4, J_2=0.7, 1H), 8.74 (dd, J_1=4.8, J_2=1.6, 1H), 8.04 (dt, J_1=8.0, J_2=1.9, 1H), 7.43 (ddd, J_1=7.9, J_2=4.9, J_3= 0.7, 1H), 7.10 (s, 2H), 3.93 (s, 3H) ppm.



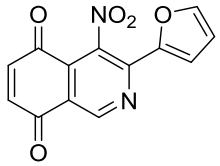
Methyl 3-(furan-2-yl)-5,8-dioxo-5,8-dihydroisoquinoline-4-carboxylate **(5c)**; **^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.35 (s, 1H) 7.63 (dd, J_1=1.8, J_2=0.8, 1H), 7.37 (dd, J_1=3.5, J_2=0.7, 1H), 7.03 (s, 2H), 6.61 (dd, J_1=3.5, J_2=1.8, 1H), 4.10 (s, 3H) ppm.



4-Nitro-3-phenylisoquinoline-5,8-dione **(9a)** **^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.56 (s, 1H), 7.71-7.65 (m, 2H), 7.56-7.50 (m, 3H), 7.12 (s, 2H) ppm.



4-Nitro-3-(pyridin-2-yl)isoquinoline-5,8-dione **(9b) ^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.81 (s, 1H), 8.98 (dd, J_1=2.4, J_2=0.7, 1H), 8.80 (dd, J_1=4.8, J_2=1.6, 1H), 8.05 (dt, J_1=8.0, J_2=1.9, 1H), 7.46 (ddd, J_1=7.9, J_2=4.9, J_3= 0.7, 1H), 7.13 (s, 2H) ppm.



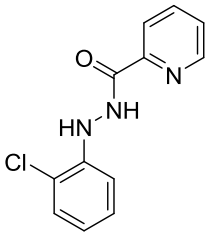
3-(Furan-2-yl)-4-nitroisoquinoline-5,8-dione **(9c) ^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.44 (s, 1H) 7.67 (dd, J_1=1.8, J_2=0.8, 1H), 7.41 (dd, J_1=3.5, J_2=0.7, 1H), 7.06 (s, 2H), 6.83 (dd, J_1=3.5, J_2=1.8, 1H) ppm**.**

5.2. Synthesis of the 1,3-dipole

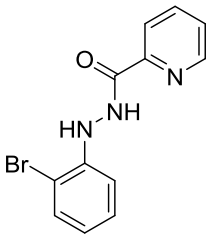
5.2.1 General synthesis of hydrazide

To a solution of picolinic acid (30 mmol) and *N*-hydroxysuccinimide (30 mmol) in anydrous THF, *N*-dicyclohexylcarbodiimide (33 mmol) was slowly added under a nitrogen atmosphere at 0°C. The ice bath was then removed and the reaction mixture was stirred for 1h at rt. After that the reaction mixture was cooled again to 0°C and a previously formed suspension of 2,4-difluorophenylhydrazine hydrochloride (33 mmol, 1.1 eq.) and triethylamine (1.1 eq) in anhydrous THF was added portionwise. The ice bath was removed and the reaction was stirred overnight at rt. The reaction mixture was dried *in vacuo* and then washed with H_2O and EtOAc/EtPet 4:3 for 30 min. For compound **17a** and **17b** the reaction mixture was filtrated and the filtrate was recrystallized in EtOH (ca. 33 mL). **17c** was, instead, directly washed with EtOAc and brine and purified by column chromatography on silica gel (EtOAc/Hex 1:2) to afford pure hydrazide.

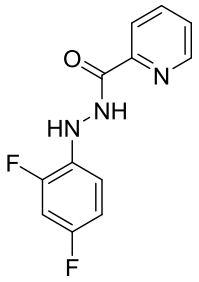
Experimental data:



N'-(2-chlorophenyl)picolinohydrazide **(16a)** **^{1}H NMR** (300 MHz, $CDCl_3$) δ 10.78 (bs, 1H) 8.78 (dd, J_1=2.4, J_2=0.7, 1H), 8.05 (m, 2 H), 7.68 (dt, J_1=8.0, J_2=1.9, 1H), 7.62 (s, 1H), 7.36 (d, J=4.9, 1H), 7.18 (t, J=7.9, 1H), 6.8 (m, 2H) ppm.



N'-(2-bromophenyl)picolinohydrazide **(16b)** **^{1}H NMR** (300 MHz, $CDCl_3$) δ 10.78 (bs, 1H),), 8.71 (dd, J_1=2.4, J_2=0.7, 1H), 8.02 (m, 2 H), 7.63 (dt, J_1=8.0, J_2=1.9, 1H), 7.42 (d, J=4.9, 1H), 7.38 (s, 1H), 7.18 (t, J=7.9, 1H), 6.80-6.62 (m, 2H) ppm.

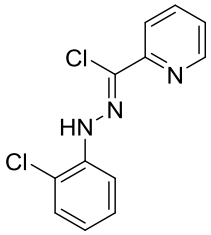


N'-(2,4-difluorophenyl)picolinohydrazide **(16c) ^{1}H NMR** (300 MHz, $CDCl_3$) δ 9.57 (d, J=3.6, 1H), 8.61 (ddd, J_1= 5.0, J_2=2.7, J_3= 1.0, 1H), 8.17 (dt, J_1= 7.6 , J_2=1.1), 7.88 (dt, J_1= 7.6, J_2= 1.6, 1H), 7.51-7.48 (m, 1H), 7.0-6.7 (m, 3H), 6.34 (d, J= 3.6, 1H).

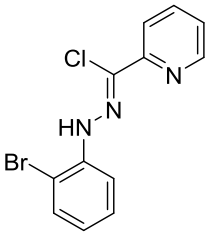
5.2.2 General synthesis of hydrazonoyl chloride

In a round bottom flask, under nitrogen atmosphere, hydrazide (16 mmol), triphenylphospine (5.25 g, 20 mmol), tetrachloro methane (2 mL, 20 mmol) and acetonitrile (30 mL, passed overnight on 3 angstrom molecular sieves) were stirred overnight at room temperature. The solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (Hexane/EtOAc 5:1) to afford the desired hydrazonoyl chloride.

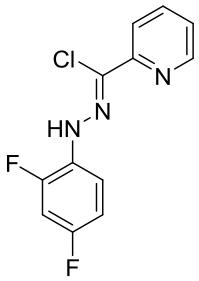
Experimental data:



(Z)-N-(2-chlorophenyl)picolinohydrazonoyl chloride **(17a) ^{1}H NMR** (300 MHz, $CDCl_3$) δ 8.84 (bs, 1H) 8.72 (dd, J_1=2.4, J_2=0.7, 1H), 8.19 (d, J=8.0 1H), 7.97 (dt, J_1=8.0, J_2=1.9, 1H), 7.72 (d, J=4.9 1H), 7.53 (m, 2H), 7.18 (t, J=7.9, 1H), 7.05 (t, J=7.9, 1H) ppm.



(Z)-N-(2-bromophenyl)picolinohydrazonoyl chloride **(17b) ^{1}H NMR** (300 MHz, $CDCl_3$) δ 8.81 (bs, 1H) 8.67 (dd, J_1=2.4, J_2=0.7, 1H), 8.09 (d, J=8.0 1H), 7.89 (dt, J_1=8.0, J_2=1.9, 1H), 7.62 (d, J=4.9 1H), 7.23 (m, 2H), 7.12 (t, J=7.9, 1H), 7.05 (t, J=7.9, 1H) ppm.

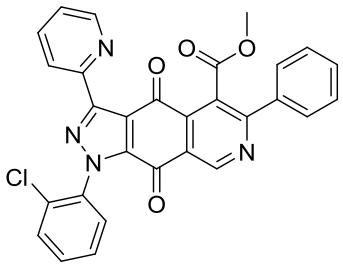


(Z)-N-(2,4-difluorophenyl)picolinohydrazonoyl chloride **(17c)** **^{1}H NMR** (300 MHz, $CDCl_3$) δ 8.89 (d, J=3.6, 1H), 8. 70 (ddd, J_1= 5.0, J_2=2.7, J_3= 1.0, 1H), 8.09 (dt, J_1= 7.6 , J_2=1.1), 7.61 (dt, J_1= 7.6, J_2= 1.6, 1H), 7.51-7.48 (m, 1H), 7.0-6.7 (m, 3H), 6.34 (d, J= 3.6, 1H).

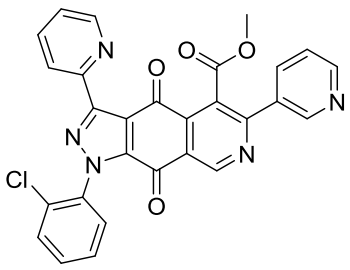
5.3. General method for 1,3-dipolar Cycloaddition reaction

To a stirred suspension of isoquinoline-5,8-dione (1 mmol) and Ag_2CO_3 (1.3 mmol) in anhydrous dioxane (4 mL), under nitrogen atmosphere at 50°C, was added hourly hydrozonoyl chloride (0.2 eq x 6) as solid until after 6 h whole 1.2 eq. were reached. The reaction mixture was stirred overnight. After the reaction was complete the mixture was cooled to room temperature, filtered through a plug of celite and evaporate in vacuo. The crude product was directly suspended in 100 mL 4:3 THF/water and cooled to 0°C. Cerium ammonium nitrate (CAN, 2.5 mmol) was added portionwise and the reaction was stirred for 2 h. After the removal of THF in vacuo, the mixture was was extracted with EtOAc (3x75 mL) and brine (75 mL), dried over $MgSO_4$ and evaporated in vacuo. The crude product was purified by column chromatography (EtOAc/EtPet 2:1) to afford cycloaddition product.

Experimental data:

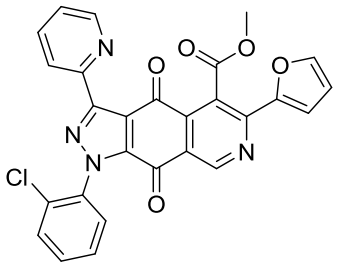


Methyl 1-(2-chlorophenyl)-4,9-dioxo-6-phenyl-3-(pyridin-2-yl)-4,9-dihydro-1H-pyrazolo[4,3-g]isoquinoline-5-carboxylate **(18)** **^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.58 (s, 1H), 8.81 (dd, J_1=2.4, J_2=0.7, 1H), 8.33 (dd, J_1=4.8, J_2=1.6, 1H), 7.90 (dt, J_1=8.0, J_2=1.9, 1H), 7.59 (m, 10H), 7.43 (ddd, J_1=7.9, J_2=4.9, J_3= 0.7, 1H), 4.03 (s, 3H) ppm.

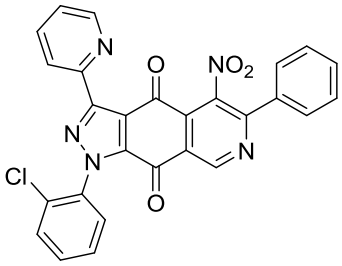


Methyl 1-(2-chlorophenyl)-4,9-dioxo-3-(pyridin-2-yl)-6-(pyridin-3-yl)-4,9-dihydro-1H-pyrazolo[4,3-g]isoquinoline-5-carboxylate **(19)** **^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.53 (s, 1H), 8.96 (d, J=1.8, 1H), 8.82 (dd, J_1=4.8, J_2=1.6, 1H), 8.75 (dd, J_1=4.8, J_2=1.6, 1H), 8.37 (dt, J_1=8.0,

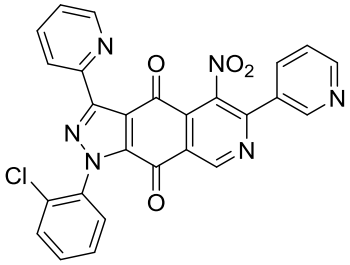
J_2=1.9, 1H), 8.07 (dt, J_1=8.0, J_2=1.9, 1H), 7.91 (dt, J_1=8.0, J_2=1.9, 1H), 7.45 (m, 10H), 3.92 (s, 3H) ppm.



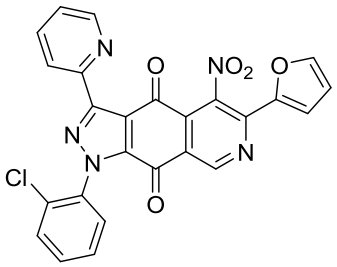
Methyl 1-(2-chlorophenyl)-6-(furan-2-yl)-4,9-dioxo-3-(pyridin-2-yl)-4,9-dihydro-1H-pyrazolo[4,3-g]isoquinoline-5-carboxylate **(20) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.39 (s, 1H), 8.82 (dd, J_1=4.8, J_2=1.6, 1H), 8.40 (dt, J_1=8.0, J_2=1.9, 1H), 7.92 (dt, J_1=8.0, J_2=1.9, 1H), 7.68-7.34 (m, 7H), 6.62 (dd, J_1=3.5, J_2=1.8, 1H) 4.14 (s, 3H) ppm.



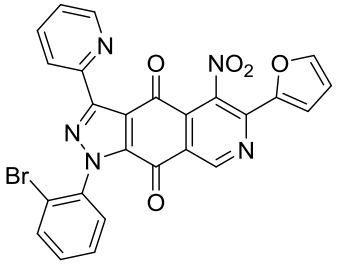
1-(2-Chlorophenyl)-5-nitro-6-phenyl-3-(pyridin-2-yl)-1H-pyrazolo[4,3-g]isoquinoline-4,9-dione **(21) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.59 (s, 1H), 8.81 (dd, J_1=2.4, J_2=0.7, 1H), 8.35 (dd, J_1=4.8, J_2=1.6, 1H), 7.91 (dt, J_1=8.0, J_2=1.9, 1H), 7.75-7.50 (m, 9H), 7.43 (ddd, J_1=7.9, J_2=4.9, J_3= 0.7, 1H) ppm.



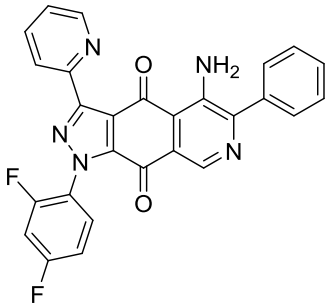
1-(2-Chlorophenyl)-5-nitro-3,6-di(pyridin-2-yl)-1H-pyrazolo[4,3-g]isoquinoline-4,9-dione **(22) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.52 (s, 1H), 8.96 (d, J=1.8, 1H), 8.81 (dd, J_1=4.8, J_2=1.6, 1H), 8.73 (dd, J_1=4.8, J_2=1.6, 1H), 8.33 (dt, J_1=8.0, J_2=1.9, 1H), 8.07 (dt, J_1=8.0, J_2=1.9, 1H), 7.94 (dt, J_1=8.0, J_2=1.9, 1H), 7.45 (m, 10H) ppm.



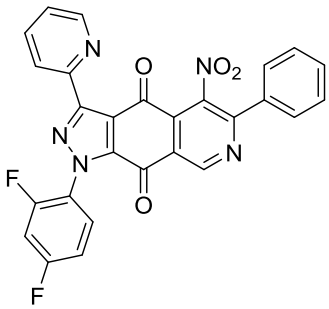
1-(2-chlorophenyl)-6-(furan-2-yl)-5-nitro-3-(pyridin-2-yl)-1H-pyrazolo[4,3-g]isoquinoline-4,9-dione **(23) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.44 (s, 1H), 8.81 (dd, J_1=4.8, J_2=1.6, 1H), 8.33 (dt, J_1=8.0, J_2=1.9, 1H), 7.90 (dt, J_1=8.0, J_2=1.9, 1H), 7.68-7.34 (m, 7H), 6.62 (dd, J_1=3.5, J_2=1.8, 1H) ppm.



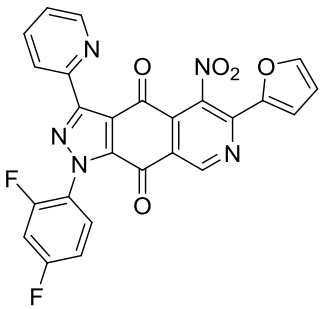
1-(2-Bromophenyl)-6-(furan-2-yl)-5-nitro-3-(pyridin-2-yl)-1H-pyrazolo[4,3-g]isoquinoline-4,9-dione **(24) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.45 (s, 1H), 8.81 (dd, J_1=4.8, J_2=1.6, 1H), 8.33 (dt, J_1=8.0, J_2=1.9, 1H), 7.91 (dt, J_1=8.0, J_2=1.9, 1H), 7.64-7.31 (m, 7H), 6.62 (dd, J_1=3.5, J_2=1.8, 1H) ppm.



5-amino-1-(2,4-difluorophenyl)-6-phenyl-3-(pyridin-2-yl)-1H-pyrazolo[4,3-g]isoquinoline-4,9-dione **(30) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 8.73 (s, 1H), 8.71 (d, J= 1.9, 1H) 8.5 (d, J=4.8, 1H), 8.33 (dt, J_1=8.0, J_2=1.9, 1H), 7.90 (dt, J_1=8.0, J_2=1.9, 1H), 7.68-7.34 (m, 6H), 6.62 (dd, J_1=3.5, J_2=1.8, 1H) ppm.



1-(2,4-difluorophenyl)-5-nitro-6-phenyl-3-(pyridin-2-yl)-1H-pyrazolo[4,3-g]isoquinoline-4,9-dione **(26) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.60 (s, 1H), 8.81 (ddd, J_1= 5.1, J_2= 2.6, J_3= 1.2 1H) 8.33 (dt, J_1=8.0, J_2=1.9, 1H), 7.91 (dt, J_1=8.0, J_2=1.9, 1H), 7.68-7.34 (m, 9H), ppm.



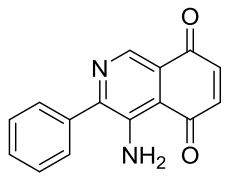
1-(2,4-difluorophenyl)-6-(furan-2-yl)-5-nitro-3-(pyridin-2-yl)-1H-pyrazolo[4,3-g]isoquinoline-4,9-dione **(25) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.46 (s, 1H), 8.81 (ddd, J_1= 4.8, J_2= 2.6, J_3= 1.2 1H) 8.32 (dt, J_1=8.0, J_2=1.9, 1H), 7.88 (dt, J_1=8.0, J_2=1.9, 1H), 7.61-7.30 (m, 7H), 6.62 (dd, J_1=3.5, J_2=1.8, 1H) ppm.

5.4. General method for reduction

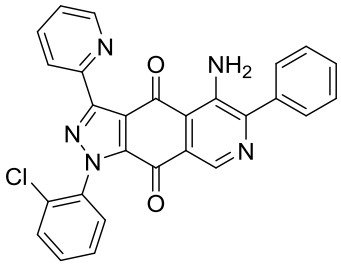
In a round bottom flask equipped with a magnetic stirring bar and under nitrogen atmosphere, product (6.0 mmol) and ammonium formate (3.43 g, 54.5 mmol) were dissolved in 60 mL of degassed MeOH and the solution was heated to 50 °C. 10% w/w Palladium on activated charcoal (0.23 g) was added and the reaction mixture was stirred at 90 °C for 5 h.

Hereafter, the reaction mixture was filtered through a short plug of celite, washed with DCM (40mL) and evaporated *in vacuo*. The crude product was suspended in DCM (50 mL), filtered again on celite and washed with DCM (50 mL) to give the amino-substituted product.

Experimental data:



4-Amino-3-phenylisoquinoline-5,8-dione **(28) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 8.69 (s, 1H), 7.70-7.66 (m, 2H), 7.59-7.50 (m, 3H), 6.98 (d, J= 9.9, 1H), 6.93 (d, J=9.9, 1H) ppm.



5-amino-1-(2-chlorophenyl)-6-phenyl-3-(pyridin-2-yl)-1H-pyrazolo[4,3-g]isoquinoline-4,9-dione **(27) ^{1}H NMR (300 MHz, $CDCl_3$)** δ 8.70 (s, 1H), 8.80 (dd, J_1=2.4, J_2=0.7, 1H), 8.35 (dd, J_1=4.8, J_2=1.6, 1H), 7.98 (dt, J_1=8.0, J_2=1.9, 1H), 7.75-7.50 (m, 9H), 7.43 (ddd, J_1=7.9, J_2=4.9, J_3= 0.7, 1H) ppm.

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