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Monopharmacology, polypharmacology and PROTAC approaches in drug discovery: investigation of enzyme inhibitors and antioxidant compounds

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Abstract

Nowadays the better understanding of pathological mechanisms is strongly correlated with the evolution of drug discovery: new targets have been validated and new strategies have been performed. The approach based on the direct relationship between a specific protein and the resulting phenotype, called "monopharmacology" resulted fundamental over the years for the developing of commercial drugs and it is still used in drug discovery to develop selective enzymatic inhibitors. Progress in biology studies demonstrated that the traditional approach is limited, so that designing a single drug acting simultaneously on different targets may be a better solution. This approach called "polypharmacology" refers to a single drug which acts simultaneously on two or more targets, achieving additively or synergistically activity. A further evolution of drug discovery is the Proteolysis-Targeting Chimeras (PROTACs), heterobifunctional molecules, constituted by a small molecule inhibitor linked to a ligand for an E3 ligase and offer a different method in drug discovery: the protein target is not just "inhibited", but it is eliminated via the cells own proteasomal machinery. This thesis describes the design, synthesis and biological activity of compounds using the above described three different approaches in drug discovery.

- In the first chapter three classes of compounds have been designed as selective Carbonic Anhydrase inhibitors. The first class of compounds, thiazolin-4-one sulfamates, was designed starting from SLC-0111, a selective hCAIX inhibitor currently in Phase Ib/II clinical trials. The thiazolinone ring was introduced as ureido-analog of SLC-011 and decorated with arylsulfamate and arylidene rings. The new sulfamates were tested against hCAI, II, IV and IX, showing interesting activity against cancer-related isoform hCAIX. Among them the 3,4,5-trimethoxyphenyl derivative and the naphthyl derivative resulted the best compounds of the series with Ki 17.6 nM and 20.9 nM respectively.

  N^1-acetyl-3,5-diarylpyrazoline sulfamates were the second class of CA inhibitors studied. The new series of pyrazoline are endowed with arylsulfamate moieties at the 3- or 5-position of the 4,5-dihydropyrazole ring. All pyrazoline sulfamates resulted weak inhibitors of the off-target hCAI isoform while many of them showed activity at low nanomolar levels against hCAII, hCAIX and hCAXII. From biological results emerged a clear correlation between the position of sulfamate moiety on 5 or 3-aryl and the different inhibitory profile of sulfamates. For what concern cancer-related isoforms hCAIX and hCAXII the sulfamic group on the 3- or 4- position of the 5-aryl is necessary, as well as an electron-withdrawing group on the 4-position of the 3-aryl ring.

The third class of compounds studied is constituted by 4-sulfamoylbenzoyl-piperidine derivatives, bearing carbonyl ureido or thioureido moieties as the tail of inhibitor. These series were designed modifying SLC-0111 structure with the incorporation of the *N*-substituted piperazine ring and carbonyl ureido and thioureido moieties. All ureido/thioureido compounds were tested against hCAI, hCAII, hCAIX and hCAXII and among them some compounds resulted extremely selective against cancer-related isoform hCAIX and hCAXII. The 4-methoxybenzyl ureido derivative displayed the best inhibitory activity on hCAXII (6.4 nM), resulting about 7-fold more selective as compared to both hCAII and hCAIX. Interestingly, the 2,6-difluoro and the 2,6-dimethylphenyl thioureido derivatives showed inhibitory activity at low nanomolar range and high selectivity against both hCAIX and hCAXII as compared to hCAII.

- In the second chapter the multitarget approach was used to obtain multitarget compounds.
  The first series of compounds are hybrid molecules endowed with an active portion, the arylsulfamate group, which can bind two different enzymes, Carbonic Anhydrase and Steroid Sulfatase (STS). All sulfamates were tested against hCAI, hCAII, hCAXI and hCAXII and using a JEG-3 cell lysate for what concerns STS inhibition. Among them the 3-methylphenyl derivative showed inhibitory activity against hCAIX at sub-nanomolar levels, with Ki 0.91 nM, and a good STS residual activity (10.4% ± 1.9). Furthermore, the benzofuran-2-yl derivative showed inhibitory activity at low nanomolar range against both hCAIX (6.7 nM) and hCAXII (1.0 nM) and STS residual activity of 4.7% ± 0.7. These dual CA/STS inhibitors may be attractive for further development and *in vivo* evaluation.
  In the second part, four series of compounds endowed with antioxidant, photoprotective and antiproliferative activity were investigated. The first three series are arylhydrazones bearing benzofuran, indole and benzimidazole scaffolds, concluding with 2-arylbenzimidazoles as the last series. Concerning arylhydrazones derivatives, the SAR data obtained from antioxidant assays showed an interesting correlation between the number and the position of hydroxy groups on arylidene moiety and the antioxidant activity. High antioxidant activity is showed by hydrazones bearing a 2-hydroxy-4-diethylaminoarylidene group. On the contrary, the presence of electron withdrawing groups, such as chlorine or bromine atoms reduced the antioxidant activity. A significant photoprotective activity emerged for the mono-hydroxylated compounds, as well as for the 2-hydroxynaphtyl and 2-hydroxy-4-diethylamino compounds. The compounds showing high antioxidant activity were also tested *in vitro* on human melanoma Colo38 and erythroleukemic K562 cell lines displaying interesting antiproliferative activity. In the 2-arylbenzimidazole series the presence of a

sulfonic acid at 5-position of benzimidazole scaffold is the least favorable while the substitution with carboxyl or cyano groups in the same position is better tolerated. Among them high antioxidant activity is showed by the 2-dihydroxyaryl and 2-trihydroxyaryl derivatives as well as by the 2-hydroxy-4-diethylamino substituted compounds. The compounds with the best antioxidant profile were investigated for their photoprotective activity showing broad-spectrum filtering activity. Furthermore, the compounds with the best dual activity were tested against Colo38 cell line and normal HaCat keratinocyte cells, demonstrating selectivity against cancer cells.

- The last chapter on PROTAC technology describes a new series of PROTAC-based TubastatinA designed to promote the degradation of Histone Deacetilase10. The three components, the E3 ligase, the linker and the small molecule inhibitor were performed to find the best combination between the linker and the two active parts of the molecules, using a cell-based target occupancy (BRET) assay. The preliminary assays indicated four compounds as good candidates for the *in-vitro* tests to evaluate their ability to promote the degradation of HDAC10, and ultimately their ability to promote HDAC10-associated phenotypes.

In this thesis new selective hCAIX and hCAXII inhibitor compounds were prepared, followed by the development of dual CA/STS inhibitors, which are interesting targets in the anticancer field. Furthermore, polyphenols-based compounds endowed with antioxidant and photoprotective activity were studied showing promising antiproliferative activity due to *in vitro* results on human melanoma Colo38 and erythroleukemic K562 cell lines. To conclude, Tubastatin-based PROTACs were prepared with the aim of promoting degradation of HDAC10, a new important target in Neuroblastoma treatment.

Index

Abbreviation

AAZ: Acetozolamide
AcOEt: Ethyl Acetate
AcOH: Acetic acid
AcONa: Sodium acetate
AE: Anion Exchanger
AML: Acute myeloid lymphoma
AR: Androgen Receptor
ATG: Autophagy related Genes
BL: Burkitt's Lymphoma
Boc_2O: Di-tert-butyl dicarbonate
BRD4: Bromodomain-Containing Protein 4
BRET: Cell-based target occupancy
CA: Carbonic Anhydrase
CARPs: Carbonic Anhydrase Related Proteins
CIAP1: Cellular Inhibitor of Apoptosis Protein-1
CNS: Central Nervous System
CRABP: Retinoic acid binding proteins
CuAAC: Cycloaddiction catalysed by Copper(I)
DCM: Dichlorometane
DIPEA: N,N-Diisopropyletyilamine
DKFZ: German Cancer Research Centre
DMA: N,N-Dimethylacetamide
DMF: N,N-Dimethylformamide
DMSO: Dimethylsulphoxide
DPPH: 1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity
ECD: Extracellular Domain
EDCI: 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EGF/EGFR: Epidermal Growth Factor/Epidermal Growth Factor Receptor
EMBL: European Molecular Biology Laboratory
ERRα: Estrogen-related receptor
ESI: Positive-ion electrospray ionization
Et_2O: Diethyl Ether
EtOH: Ethanol
FDA: Food and Drug Administration
FRAP: Ferric Reducing Antioxidant Power
HATs: Histone acetyltransferases
HDAC: Histone deacetylases
HER2: Human Epidermal Growth Factor Receptor 2
HIF-1α: Hypoxia-Inducible Transcription Factor
HOBt: 1-Hydroxybenzotriazole hydrate
iPr_2O: isopropyl ether
IT: Intracellular Tail
M.p.: Melting Point
MCL1: Induced Myeloid Leukaemia Cell Differentiation Protein 1
MDM2: P53-degrading Mouse Double Minute 2

MeCN: Acetonitrile
MeOH: Methanol
Na_2SO_4: Sodium Sulfate
NaOH: Sodium Hydroxide
NBC: Na^+/HCO_3^- Cotransporter
ORAC: Oxygen radical absorbance capacity
PBSA: 2-Phenyl-1*H*-benzo[*d*]imidazole-5-sulfonic acid
PI-3K/Akt: Phosphatidylinositol-3 Kinase/Akt Kinase
PROTAC: Proteolysis-Targeting Chimeras
RAR: Retinoic acid receptor
ROS: Reactive Oxygen Species
SAHA: Suberoylanilide hydroxamic acid
SAR: Structure Activity Relationship
SiO_2: Silico Dioxide
SPF: Solar Protection Factor
STS: Steroid Sulfatase
TEA: Triethylamine
TFA: Trifluoroacetic acid
THF: Tetrahydrofuran
TM: Transmembrane Region
TTA: tris((1-(tert-Butyl)-1*H*-1,2,3-triazol-4-yl)methyl)amine
UVAPF: UVA Protection Factor
VHL: Von Hippel Lindau

1. Introduction

1.1 History

In 1908 drug discovery research drastically changed due to the innovative work of the Nobel prize Paul Ehrlich that postulated the concept of "magic bullet: drugs that go straight to their intended cell-structural targets"[1]. He also postulated that "the key for the synthetic chemistry in drug discovery is to modify a lead compounds in different ways and evaluate the different activity of final products".[1]The analysis of the structure-activity relationship (SAR) to optimize a lead compound through systematic chemical modification, completely changed the drug discovery approach. The increasing power medicinal chemistry due to SAR analysis was helpful in producing compounds endowed with desirable properties such as solubility, distribution, low toxicity and resistance to metabolism. [2-5] Furthermore, in the 1980s and 1990s, during early genomics age, the characterization of molecular physio-pathological mechanisms become the central issue of drug discovery.[5,6] This scientific and technological revolution, outcomes in the modern drug discovery approach: the development of molecules acting on a specific target with high potency and selectivity via structural optimization and the SAR analysis. Indeed, from a lead compound some common modifications are possible: removing chiral centers retaining the key pharmacophores and reducing the scaffold complexity. One of the most successful examples of scaffold simplification is the development of simplified morphine-derived analgesics, in which the complexity of morphine pentacyclic system was systemically reduced.

1.2 Traditional drug discovery approach

This traditional approach is based on the direct relationship between a specific protein and the resulting phenotype so that a compound able to modulate the pathological activity of an enzyme may be able to revert the correspondent phenotype.[7]This type of method has proved to be fundamental over the years, for the development of commercial drugs and is also crucial for design selective enzymatic inhibitors. Indeed, advances in molecular biology showed new targets but also the presence of different isoforms of the same enzyme. For example, in recent years, the enzyme Carbonic Anhydrase (CA) was intensively studied, finding different isoforms with several biological effects. Acetazolamide is a carbonic anhydrase inhibitor approved by the Food and Drug Administration (FDA) for the treatment of epilepsy, glaucoma, and edema[8]. In addition to these therapeutic indications, acetazolamide has also a broad range of side effects. Understanding the structural features of Acetazolamide and the structure of CA isoforms is crucial to design highly selective inhibitors.

1.3 Multitarget approach

Progress in cellular machinery knowledge also revealed the involvement of different pathways in numerous diseases such as cancer and CNS diseases, so the mono-pharmacology approach is limited.[9] Nowadays, designing a single drug acting simultaneously on different targets, increase the possibility to control complex diseases, and may reduce disadvantages derived from single-target drug or a combination of two or more drugs. This approach is called "polypharmacology", different from "compound promiscuity" which refers to nonspecific binding events due to compound liabilities.[10] A single drug acting simultaneously on two or more targets may have an improved efficacy due to additively or synergistically activity and may be less susceptible to the insurgence of drug resistance mutations. The modulation of different targets is achieved also using combination of two or more drugs, especially in complex diseases such as cancer and CNS diseases.[10] The goal of polypharmacology is to obtain the same relief of combine therapy but using a single drug molecule. In the mono-pharmacology approach the therapeutic agents, especially in chronic diseases, may be affected by multiple side effects and toxicity which result in reduced efficacy, drug resistance and reduced compliance. In order to reduce all these disadvantages, specific drugs combination is used to act simultaneously in properly biological targets. Combination drugs are designed to obtain synergistic effects by the different mechanisms of action against different targets in the same pathological pathways.[11] This is crucial for example, in cancer therapy where the biological and genetic complexity of tumor cells suggests that targeting a multiple oncogenic pathway is necessary to achieve patient remission. One of the most common way to obtain compounds endowed with multiple biological effects is the analysis of natural products. Natural products can be defined as chemical entities with multiple biological functions derived from plants.[12] Polyphenols are a class of natural products present in vegetables, fruits, seeds, legumes etc., classified in flavonoids, phenolic acids, and other polyphenols including stilbenes and lignans.[13] Polyphenols have been intensively studied proving their potential activity against cancers and cardiovascular, metabolic,[14] and neurodegenerative diseases[15] due to their antioxidant and antimutation properties. In metabolic pathways polyphenols can neutralize free radicals by donating an electron or hydrogen atom to suppress the generation of free radicals or deactivate the active species and precursors of free radicals. Polyphenols may act also as metal chelators, they chelate transition metals such as Fe^{2+} and directly reduce the rate of Fenton reaction, thus preventing oxidation caused by highly reactive hydroxyl radicals (•OH).[16] For all these reasons, polyphenols and, in general, natural products, are great starting points for design multitarget compounds.

The poly-pharmacology approach showed multiple advantages:

- A multitarget drug may offer better efficacy against complex and advanced stage diseases compared to high specificity single-target drugs;
- The pharmacokinetic of a multitarget drug is more predictable than a combination of two or more drugs;[17]
- A multitarget drug may have a superior safety profile, especially if side effects are molecule based whereas no clear advantages are obtained if side effects are target-based;
- Acute and delayed toxicity is generally higher in drug combination, especially when the two or more drugs in the combinations have poor selectivity. For this reason, the use of "drug cocktails" results in a negative patient compliance;[18]
- Studies showed that the probability of target-based resistance is lower for multitarget compounds compared to single-target compounds. Furthermore, drug-drug interactions are lower in multitarget compounds than single-target compounds.[19]

For these reasons, the development of a single compound with multitarget profile may be a better alternative to drug combinations as well as compounds with mono-target profile.

In order to design a multitarget compound is crucial to consider the structure-activity relationship profiles of these compounds when interact with two or more targets, especially when these are distantly related or unrelated.[20] There are different approaches to obtain multitarget compounds: molecules containing different pharmacophores related to different targets,[21] molecules resulting by the combination of entire drugs,[22] hybrid molecules endowed with an active portion which can potentially bind two or more targets[23] and molecules based on natural compounds with a well-known activity.[24]

1.4 The PROTAC approach

A relatively new technique to overcome the traditional drug discovery limitations is the PROTAC approach. In general, to maintain a good level of inhibition, high concentration of inhibitor is required, which results in side-effects. In this context, the PROTAC concept offers a novel modality for drug discovery: the protein target is not just "inhibited", but it is eliminated via the cells own proteasomal machinery, resulting in a so-called "chemical knockdown".[25] Proteolysis-Targeting Chimeras (PROTACs) are heterobifunctional molecules, constituted by a small molecule inhibitor linked to a ligand for an E3 ligase.[26] PROTACs simultaneously bind the E3 ubiquitin ligase and the target protein and in this way lysines on the target protein are exposed to be poly-ubiquitinated by the E3-ubiquitin ligase complex. After the poly-ubiquitylation, the target protein is recognized by the proteasome and degraded.[27]

The PROTAC approach is unique, compared with other therapeutic interventions so it may have some important advantages:

- PROTACs may have the capacity to target "undruggable proteins". FDA shows that more than 85% of proteins associated with disease do not have an associated therapy, because of the incapacity of traditional approaches to target these proteins. Traditional drugs need to affect the function of an enzyme or receptor upon binding for a therapeutic effect, but there are some enzymes that do not have a classic drug binding site, so it is not possible to develop a traditional drug.[27] PROTACs need to bind the target protein, regardless of whether this binding has a functional effect, so they potentially can be used to promote the degradation of a bigger amount of protein. They can also be used for the treatment of neurodegenerative diseases which depend of protein aggregation, such as Huntington's disease;[28]
- PROTACs can overcome the accumulation or upregulation of target proteins. A traditional drug can stabilize the target protein or can lead to compensatory upregulation and, consequently, an accumulation of the target protein. The stabilization of target protein is a well-known phenomenon and it has been observed with HER2 inhibitors, BRD4 inhibitors and MCL1 inhibitors.[29-31] Furthermore, in some cases the inhibition of the target by a traditional drug can lead to a compensatory upregulation, for example in Androgen Receptor (AR)[31] or in Bromodomain-containing protein 4 (BRD4). It has been proved that traditional BRD4 inhibitors quickly lose efficacy due to upregulation, while BRD4-PROTACs maintain low levels of the target protein.[30]
- PROTACs could be a solution for drug resistance because the elimination of target protein prevents mutations, or the possible complex between the target protein and an auxiliary protein that is resistant to traditional inhibitors. PROTACs can also prevent resistance mechanisms that cannot be predicted until clinical trials.[27] The complete elimination of the target protein makes also possible to achieve an optimal pharmacodynamic profile, as covalent inhibitors. Due to their ability to promote the protein degradation, the organism takes long time to re-synthetize the required protein in the cell, so PROTACs do not need a continue cellular exposure to maintain the pharmacological effect.[27]

Starting from the above consideration, in this Ph.D. thesis I have investigated the three different approaches in drug discovery: the traditional single-target approach, the multi-target approach and the PROTAC approach. For what concerns the single target approach, I developed new selective Carbonic Anhydrase inhibitors. Speaking about multitarget approach, I developed compounds with Carbonic Anhydrase and Steroid Sulfatase inhibitory efficacy and compounds

endowed with antioxidant, photoprotective and antiproliferative activity. In the last part of this thesis, a new approach in drug discovery has been described: the degradation of Histone Deacetilase10 using the PROTAC technology.

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2. Carbonic Anhydrase inhibitors

2.1 Introduction

Carbonic anhydrase (CA) is a superfamily of metalloenzymes that catalyses the reversible conversion of CO_2 into hydrogen carbonate ions and protons,[1] water-soluble products that play an important role in pH regulation. Currently five different families of CA have been recognized: α, β, γ, δ, ζ of which αCAs are present in humans with implication in regulation of biosynthetic reactions, physiological and physio-pathological processes.[2] αCAs are also present in different organisms such as fungi, protozoa, corals, algae and in some bacteria.[3-5] βCAs have been described in some bacteria, algae, fungi and archaea[6-9] whereas γCAs have been found in archaea, bacteria and plants.[10,11] The δCAs are present in the marine phytoplankton, where contribute to the CO_2 fixation, while ζCAs are present only in diatomee.[12-14] The CA activity is connected with widespread biological processes: in vertebrates this enzyme relates to gluconeogenesis, lipogenesis, ureagenesis, bone resorption, calcification and tumorigenicity;[15-21] in algae, plants and some cyanobacteria it has been demonstrated the important role of CA in photosynthesis and other biosynthetic reaction;[2,3,22] in diatomee CAs are implicated in CO_2 fixation and in SiO_2 cycle.[14]. CAs were defined as metalloenzymes, so the metal ion in the active site is crucial for the activity and Zn(II) is the most used by all classes[23] with some differences. For example, the γCAs are probably Fe(II) enzymes, whereas ζCAs use Cd(II) for the catalytic reaction. It has been also clarified the aminoacidic environment close to the metal ion, which is important in the catalytic process. In α, γ, and δ CAs the metal ion ligands are three Histidine residues[23-25] (Figure 2.1, A) while the βCAs have four ligands: one Histidine, two Cysteine and one Asparagine coordinated the metal ion (Figure 2.1, C). Due to these four ligands, at pH < 8 no water molecule is coordinated with the metal ion. At pH > 8 an Arginine, belonging to the called catalytic dyad, makes a salt bridge with the Asparagine coordinated with the metal ion, liberating the position which is now occupied by the water molecule/hydroxide ion[13-26] (Figure 2.1, B).

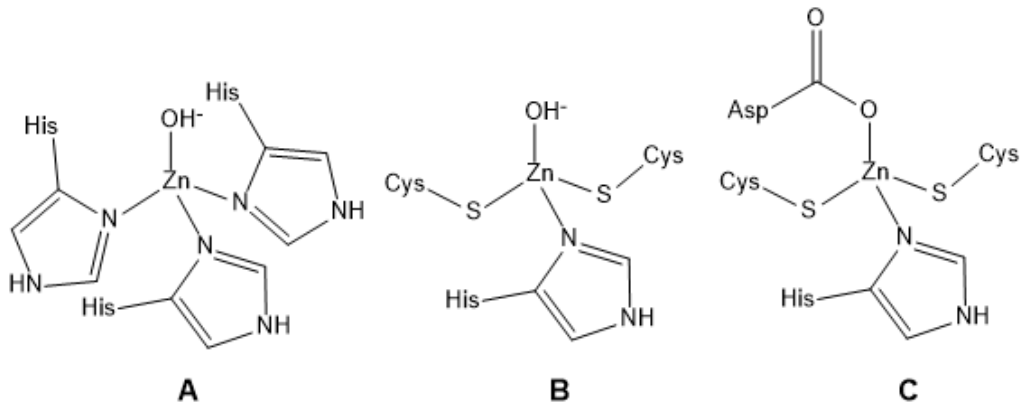


Figure 2.1. Metal ion coordination in: A) α, γ, and δ CAs; B) β CA at pH > 8; C) β CA at pH < 8

Although there are some differences in the aminoacidic residues that bind the metal in the different families of CA, there is a remarkable characteristic that all of them have in common: the presence of two different environments in the active site. In all CAs there is a hydrophobic environment, important for the CO_2 and a hydrophilic environment necessary for the products, protons and hydrogen carbonate.[27].

2.2 Human CAs

Currently 15 different αCAs isoforms have been recognized and described in humans, which 12 are catalytically active: CAs I-IV, CA VA-VB, CA VI, CA VII, CA IX and CAs XII-XIV. The three isoforms VIII, X and XI are called CA-related proteins CARPs.[1] The active hCAs have different catalytic efficiencies and different cellular localization. These isoforms have been grouped in four different classes depending on localization: hCAs I, II, III, VII, and XIII are in the cytosol, hCAs IV, IX, XII, and XIV are membrane-associated, and hCAs VA and VB are present in mitochondria, whereas hCAVI is present in saliva and milk.[28] hCAs are distributed in several tissues and organs and are implicated in various biological and a dysregulation of hCAs may promote important pathological processes.

The structure of hCAs has been intensively studied and x-ray crystallography analysis proved that all the isoforms have a common three-dimensional structure. The structure of hCAs consist of a central twisted β-sheet, surrounded by helical connection and supplementary β-strands[29] (Figure 2.2).

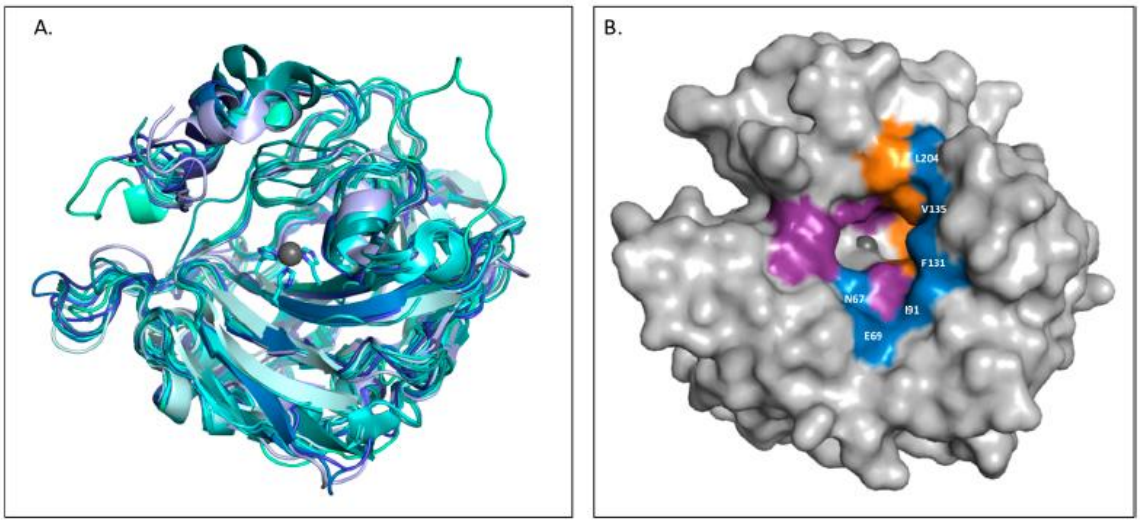


Figure 2.2. A) Overlay of hCAs; B) Surface representation of hCAII, with the hydrophobic region in purple, the hydrophilic region in orange and the isoforms-related residues close to the active site in blue[30].

Additionally, all isoforms are monomeric with three exceptions: the hCAIX can exist as a dimer with the same membrane orientation of both monomers or as a trimer,[31,32] hCAXII,[33] and hCAIV[34] which is the secreted isoform. Despite that difference, all isoforms have the active site positioned in

a conical space which is extended from the surface of the protein to the center. At the bottom of this cavity there is the Zn(II)ion, coordinated by His94, His96 and His119. The Zn(II) ion is also coordinated by a water molecule, essential for the catalytic activity.[35] (Figure 2.3). In fact it has been demonstrated that the water molecule coordinates with Zn(II) ion and forms a network of hydrogen bonds with two supplemental water molecules. The first one, called "deep water" is positioned in a hydrophobic pocket and the second one is on the opposite sides of the cavity, toward the entrance of the active site.[29]

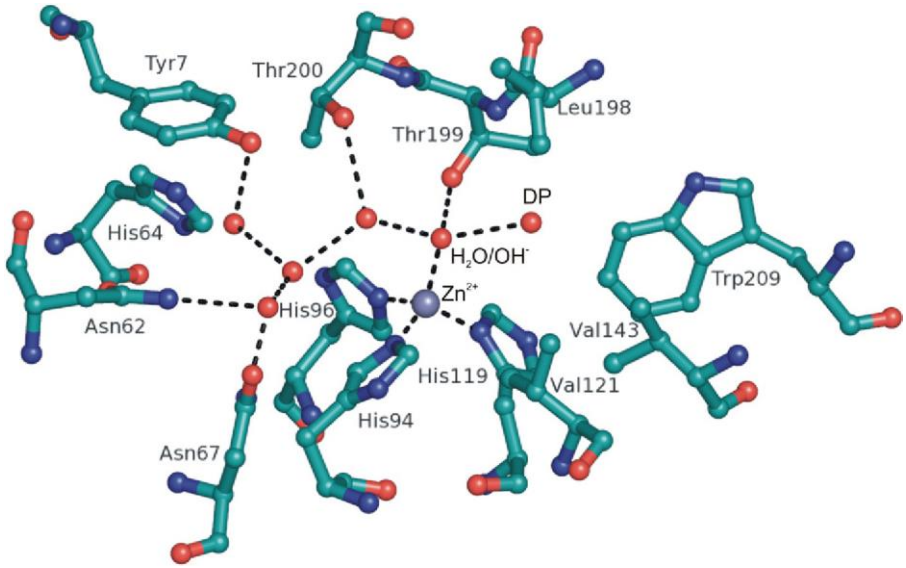


Figure 2.3. The active site of hCAII[29]

There is a strong connection between this network and the activity of the enzyme because it improves the nucleophilicity of the Zn(II) ion in the first step of the catalytic reaction. Different studies on the catalytic process demonstrated the important role of the His64, placed in the middle of the active site. This specific histidine acts as a "proton shuttle" during the reaction, from the zinc-bound water molecule to the external environment.[36] The same studies proved that the absence of this residue, such as in hCAIII, where the histidine is substituted by a lysine, is strictly correlated to a low catalytically efficiency of the enzyme.[36] For all the classes, a two-step reaction mechanism has been proposed (Figure 2.4). In this mechanism a metal hydroxide species of the enzyme, the catalytically active species, act as a nucleophile on the CO_2 in the hydrophobic pocket of the active site with formation of HCO_3^-, coordinated to the zinc. The ion-zinc binding is rather labile so that the hydrogen carbonate ion is displaced by a water molecule, generating the catalytically inactive form of the enzyme. In the second step there is the regeneration of the metal hydroxide species: a proton from the metal-bound water molecule is transferred to an acceptor in the active site of the enzyme.[23]

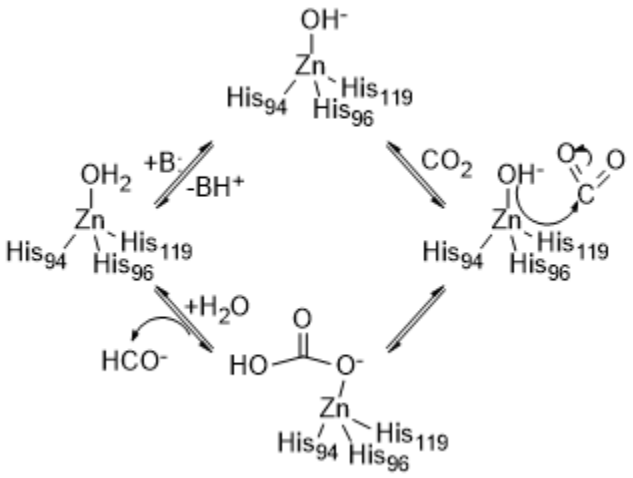


Figure 2.4. Catalytic mechanism of hCA.

Recent studies on the crystal structure of hCAII showed that in the first step of the reaction, the CO_2 is bound in a small hydrophobic pocket delimited by Val121, Val143m Leu198 and Trp209, in the same position of the "deep water".[37,38] Furthermore, these studies showed that the formed hydrogen carbonate ion stays in the same plane of the CO_2 and is tetrahedrally coordinated to the metal ion.

hCAs are largely distributed in the organism and consequently are implicated in physiological activity. Some hCAs are ubiquitous, such as hCAI and hCAII and, although they should be an interesting target for drug discovery, at the same time they are important for the off-target effects. hCAI has been found in the eyes and in the gastrointestinal tract and is associated with retinal and cerebral edema.[40] hCAII, is the most diffused isoform in the organism and it has been correlated with glaucoma, edema, epilepsy and altitude sickness.[42,43] hCAIV is broadly distributed in the organism and it is a relevant target for the treatment of glaucoma, retinitis pigmentosa and stroke.[44] hCAVI is secreted in saliva and milk and may be implicated in cariogenesis.[45] hCAVII is one of the most active isoforms distributed in brain tissues and with implication in epilepsy and cells protection from Reactive Oxygen Species (ROS).[46,47] hCAIX and XII have been found in cancer cells and it has been clarified their role in tumor growing and survival, although hCAXII is also present in normal tissues.[48]. Recent studies demonstrated the presence of hCAXIII in thymus, intestine and colon, as well as in sperm cells, in the uterine cervix and in endometrial glands.[49] hCAXIV is present in eyes and in brain tissues and it is involved in retinopathies and epilepsy genesis.[50,51]. All these studies indicate that hCAs are interesting targets in drug discovery but is also clear that it is necessary to develop isozyme-selective inhibitors to avoid important side-effects.

2.3 CAIX and XII

Cancer is generally characterized by an abnormal cell growth and spreading into surrounding tissues, but typically this overgrowing is not followed by an adequate oxygen and nourishment delivery due to the poor tumor vasculature. Consequentially, this condition leads to the

development of hypoxic regions which could limit the tumor progression. Several adaptive processes are necessary for the continual progression and metastasis, such as metabolic changes, angiogenesis, cell migration and reduced cell death. The hypoxia situation leads to important changes in gene expressions, mediated by hypoxia-inducible transcription factor (HIF-1α).[52] HIF-1α is immediately degraded under physiological oxygen level, but in hypoxia condition promotes glycolysis to enhance the cell survival.[48] Due to glycolysis, tumor cells produce a huge amount of lactic acid with consequent decrease of cytosolic pH, so that cancer cells need to control the intracellular pH. The extrusion of acidic substances is also responsible of extracellular acidification which facilitates tumor expansion and aggressiveness.[53] In the last years hCAIX and XII become an interesting target for the development of new antiproliferative compounds, due to their important role in the cancer cell survival. The environment changes, such as the metabolic acidosis, are better tolerated in cancer cells, but the control of the cytosolic pH is necessary for their survival and proliferation, so that to regulate intracellular pH, different transporters and exchangers are required. Hydrogen carbonate ions, which are important pH buffers, are imported by transporters such as Cl^-/HCO_3^- anion exchanger (AE) and Na^+/HCO_3^- cotransporter (NBC) and in this way they consume cytosolic protons to give a new molecule of CO_2. The new molecule of CO_2 leaves the cytoplasm to be hydrated by hCAIX and XII. In this way these two CA isoforms lead to the control of intracellular pH and participate in the extracellular acidification which supports the tumor aggressiveness.[54] The reversible hydration of CO_2 to proton and hydrogen carbonate ion mediated by hCAIX and CA XII regulates pH and promote tumor progression, so that a selective inhibition of these isoforms may have interesting clinical implication.[55] hCAIX is a transmembrane glycoprotein and consists of three important regions: the extracellular domain (ECD) where the catalytic site is located, the transmembrane region (TM) and the intracellular tail (IT).[56] In this monomeric form CA IX is 54-58 kDa but it has been proposed that in physiological condition, CAIX is present as a trimer of 153 kDa. Recent studies focused on the interaction between the two monomers, showed that the two catalytic domains are associated to form a dimer by an intermolecular disulphide bond.[57] It has been proposed an interaction between CAIX and the component of extracellular matrix, consequentially CAIX is involved in cell adhesion and spreading.[58,59] The enzymatic activity of hCAIX is controlled by three phosphorylation sites in the IT tail: Thr443, Ser448 and Tyr449. The phosphorylation of Thr443 by protein kinase A and the dephosphorylation of Ser448 are mandatory for the enzymatic activity. Furthermore, Tyr449 is implicated in signal transduction by epidermal growth factor/epidermal growth factor receptor (EGF/EGFR) and phosphatidylinositol-3 kinase/Akt kinase pathway (PI-3K/Akt).[60,61] hCA XII is a 40-45 kDa transmembrane protein, with a N-terminal domain, a TM region and an IT tail. As in CA IX, the

active site is in the extracellular domain, which is orientated by two glycosylation sites. The IT domain contains two phosphorylation sites that may have implications on the enzymatic activity and signalling.[62]

2.4 Development of CA inhibitors

Due to the important role of CAIX and CAXII in cancer cells, the overexpression of these two isoforms is present in a wide variety of solid tumors, such as in uterine cervix, colon, lung, breast, brain, pancreas cancers.[63-67] In breast cancer hCAIX is associated with tumor necrosis and worse overall survival,[68] but it was also related to chemotherapy and endocrine therapy resistance.[69] Furthermore, CAIX overexpression is related to poor prognosis in ovarian carcinoma and in non-small cell lung cancer.[70,71] The aggressive phenotype and the poor prognosis related to the overexpression of these isoforms, make them interesting targets in anticancer therapy. Due to the clinical relevance of hCA, the processes of inhibition and activation have been exhaustively studied. Four classes of CA inhibitors differing in their mechanism of inhibition have been recognized;[72]

Metal Ions Binders. The Zn(II) ion is coordinated in tetrahedral or bipyramidal way by sulfonamides, sulfamates, dithiocarbamates and hydroxamates.[15,25,73-75] All of these compounds act as anions with the nitrogen or the sulphur atoms coordinating tetrahedrally the Zn(II) ion. The scaffold of the inhibitor is also important due to the possible interactions with the regions close to the active site to improve the potency or the selectivity of the inhibitor (Figure 2.5, A).[76]

Zinc-coordinated water molecule/hydroxide ion binders. This class of inhibitors includes phenols, polyamine, and sulfocoumarines, that act as prodrug inhibitors. These compounds are hydrolized by the sulfatase-CA activity to the corresponding hydroxyphenyl-ω-ethenylsulfonic acid, which is the real ion binder.[77-79].The scaffold of the inhibitor is again important for the interaction with the residues in the active site (Figure 2.5, B).

Compounds that occlude the active site. This class of inhibitors acts with a different mechanism of action, and includes coumarins and their isosteres, such as thiocumarins, thiolactones and five-six membered lactones. As the compounds from the second class, these inhibitors act as prodrugs, being hydrolized to substituted 2-hydroxycinnamic acids which bind the residues at the entry of the active site, occluding it.[80,81].The structure of the inhibitors for this class has a different importance, because in this case the entrance of the active site is different for each isoform, so that is theoretically possible the development of selective inhibitors (Figure 2.5, C).

Undefined binders. This class of inhibitors is composed by secondary and tertiary sulfonamides and some tyrosine kinase inhibitors such as imatinib and nilotinib (Figure 2.5, D). Secondary and tertiary sulfonamides cannot bind the Zn(II) ion because of the bulky groups present at the sulfonamide moiety, but they can potentially interact with the zinc coordinated water

molecule/hydroxide ion.[82] An interesting activity against some CA isoforms, such as CA II, IX and XII was observed for imatinib and nilotinib, but their mechanism of inhibition is unknown at the moment.[83]

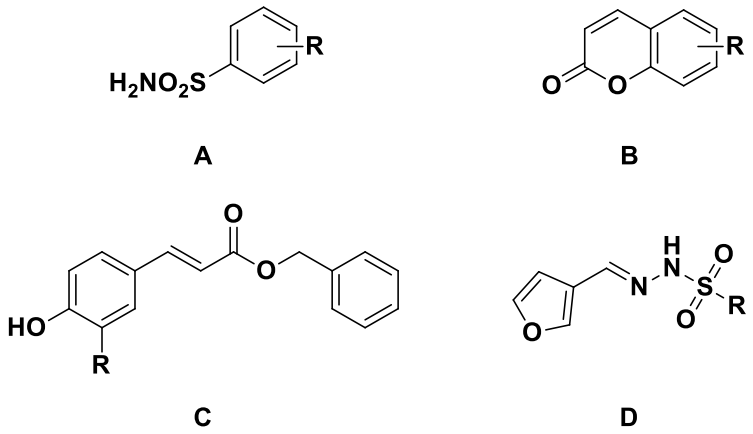


Figure 2.5. General structures of: A) Metal Ions Binders; B) Zinc-coordinated water molecule/hydroxide ion binders; C) Compounds that occlude the active site; D) Undefined binders.

During last years several studies were focused on the development of selective hCA IX and XII inhibitors in anticancer field.[73,74,79,80] Currently the most common classes of hCA inhibitors are sulfonamides and their isosteres, due to the good stability and low toxicity, whereas the most important problem is the lack of selectivity. Sulfonamide group is the active part of the molecule so that, to improve the specificity against the tumour-associated isoform, different modifications at the scaffold are necessary. One of the most successful approaches is the decrease of membrane permeability by the introduction of aromatic and heteroaromatic rings[84] or ureido and thioureido moieties,[85] coumarins and thiocoumarins.[86] Based on these considerations, the purpose of this thesis is to design, synthesize and biological evaluate new series of carbonic anhydrase inhibitors. All the new inhibitors have been designed as metal ion binders, such as sulfonamides and sulfamates and to improve the selectivity against tumor-associate carbonic anhydrase isoforms these moieties have been inserted in different scaffolds. All series have been tested for their CA inhibitory activity by a stopped flow CO_2 hydrase assay in the presence of acetazolamide as standard inhibitor[87] at the Department of NEUROFARBA, Section of Pharmaceutical and Nutraceutical Sciences, Laboratory of Molecular Modeling Cheminformatics & QSAR, University of Florence, Italy.

2.5 Thiazolin-4-one series

SLC-0111, a selective CAIX inhibitor, completed Phase I clinical trials for the treatment of advanced, metastatic hypoxic tumors over-expressing hCA IX, and is currently in Phase Ib/II clinical trials in a multi-center, open-label study in combination with gemcitabine (administered i.v.) in subjects affected by metastatic pancreatic ductal adenocarcinoma. [88-92] In the first series of new CA inhibitors designed in this thesis a thiazolinone ring moiety has been introduced as ureido-analog of SLC-0111 (Figure 2.6). The thiazolinone ring has been decorated with arylsulfamate and arylidene rings. The thiazolin-4-one is also a well-known scaffold with interesting antiproliferative effects.[93]

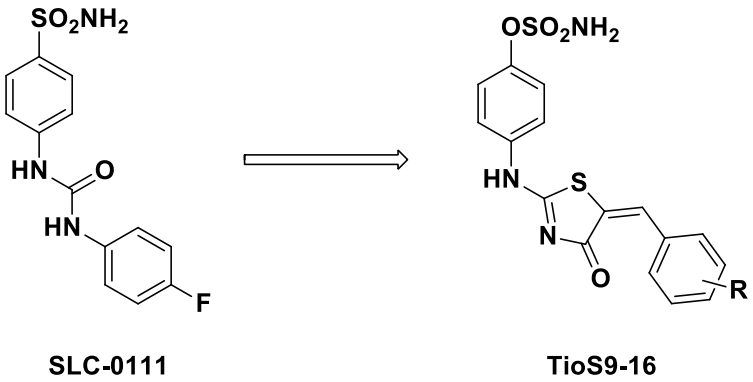
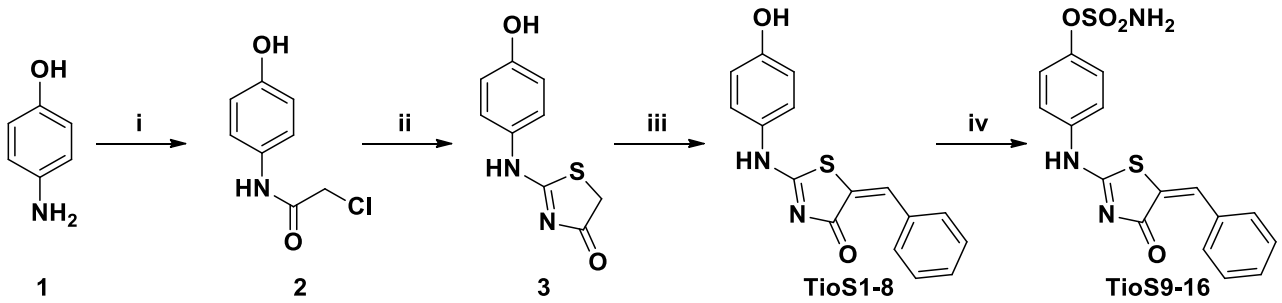


Figure 2.6. Structures of SLC-0111 and thiazolin-4-one aryl sulfamates **TioS9-16** here reported**.**

Sulfamates are congeners and bioisoster of sulfonamides and possess an additional electron-withdrawing oxygen atom. This oxygen atom is able to form a more complex hydrogen network, compared to sulfonamide group, nearby the zinc ion in the active site so that sulfamates were reported to possess highly inhibitory properties against hCAs.[73] Furthermore, phenols should act as zinc-coordinated water molecule/hydroxide ion binders,[77] so in this series the potentially carbonic anhydrase inhibition of phenolic derivatives, precursors of sulfamates were evaluated as CA inhibitors. The synthetic pathway to obtain sulfamates **TioS9-16**[94] (Scheme 2.1) started from the condensation of 4-aminophenol **1** with chloroacetyl chloride in the presence of sodium acetate (AcONa) using acetic acid (AcOH) as solvent. The resulting 2-chloro-N-(4-hydroxyphenyl)acetamide **2** was condensed with ammonium thiocyanate in dry ethanol (EtOH) to obtain the substituted thiazolin-4-one **3**. The intermediate **3** was then condensed with various aromatic aldehydes, in the presence of anhydrous AcONa, in AcOH to obtain the phenols **TioS1-8**. The desired compounds **TioS9-16** were finally obtained by sulfamoylation of the phenol group of **TioS1-8** by treatment with freshly prepared sulfamoyl chloride. This last was obtained by reacting chlorosulfonyl isocyanate and formic acid, in N,N-dimethylacetamide (DMA) solution.



Scheme 2.1. General synthetic procedure for thiazolin-4-one derivatives **TioS1**–**16**. Reagents and conditions: (i) chloroacetyl chloride, AcONa, AcOH, 5°C r.t., 45 min; (ii) ammonium thiocyanate, EtOH, 4 h, reflux; (iii) ArCHO, AcONa, AcOH, reflux, 12 h; (iv) $ClSO_2NH_2$, DMA, r.t. 12 h.

The structures of all the new compounds were assigned based on their analytical and spectral data. The prototropic tautomerism and stereoisomerism of arylthiazolin-4-one were previously studied,[94-96] showing different conformation. ^{1}H NMR spectra showed diagnostic multiplication for C=H (7.60-7.80 ppm) and for N-H (11.60-12.20 ppm) signals, due to co-existence of the two different tautomeric forms. Furthermore, the tautomer with exocyclic double bond C=N is present as a mixture of *E* and *Z* stereoisomers which is consistent with results of reported studies[97,98] (Figure 2.7).

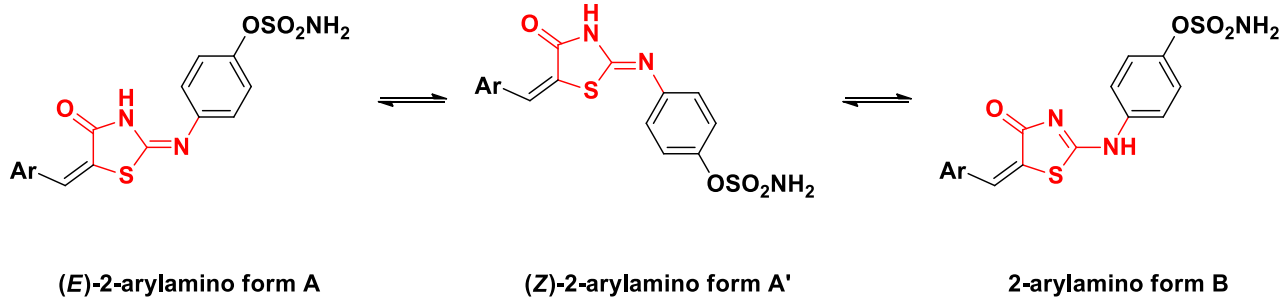
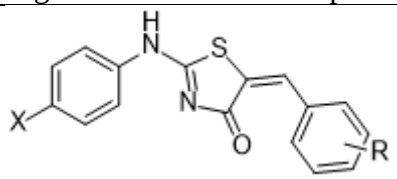


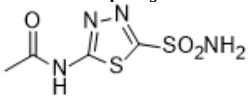
Figure 2.7. Different isomers and tautomers of arylthiazolin-4-one derivatives.

The compounds **TioS1-16** as well as their synthetic precursors **Tio1-9** were assayed for their inhibitory activity on hCA I, II, IV and IX. The inhibition data expressed as Ki at nanomolar concentrations are showed in Table 2.1. The first analysis of the inhibitory profile showed an unexpected low activity of derivatives **TioS1-9** against all the four isoforms, incompatible with the binding mode proposed for phenols.[98] None of compounds **TioS1-9** inhibited the cytosolic hCAI and the hCAIV at concentrations below 100μM while derivatives **Tios1**, **2**, **3**, **5**, **6** which are endowed with a 4-substituted arylidene moiety acted as high micromolar inhibitor against hCAII an

hCAIX. Compounds **TioS-1**, **TioS-2** and **TioS-3**, bearing 4-nitro, 4-chloro and 4-fluorophenyl moieties, inhibited both hCAII and hCAIX with K_i in the 76-92 μM range for hCAII and in the 11-32 μM range for hCAIX with a four to nine-fold greater extent toward hCAIX.

Table 2.1. Inhibition data of human CA isoforms hCA I, II, IV and IX with derivatives **TioS1–16** reported here and the standard sulfonamide inhibitor acetazolamide (AAZ) by a stopped flow CO_2 hydrase assay (errors were in the range of ±5–10% of the reported values).



Compound	X	R	K_i (nM)			
			hCAI	hCAII	hCAIV	hCAIX
TioS-1	OH	4-NO_2	>100000	86967	>100000	27719
TioS-2	OH	4-Cl	>100000	92295	>100000	11251
TioS-3	OH	4-F	>100000	75990	>100000	32106
TioS-4	OH	4-CF_3	>100000	>100000	>100000	22094
TioS-5	OH	4-CH_3	>100000	76119	>100000	>100000
TioS-6	OH	4-OCH_3	>100000	90189	>100000	>100000
TioS-7	OH	3,4,5-$(OCH_3)_3$	>100000	>100000	>100000	>100000
TioS-8	OH	naphtyl	>100000	>100000	>100000	>100000
TioS-9	OSO_2NH_2	4-NO_2	388.6	41.5	343.1	29.8
TioS-10	OSO_2NH_2	4-Cl	929.4	62.0	330.5	28.8
TioS-11	OSO_2NH_2	4-F	311.6	72.1	395.1	29.5
TioS-12	OSO_2NH_2	4-CF_3	158.1	34.3	220.4	26.9
TioS-13	OSO_2NH_2	4-CH_3	609.1	84.3	313.2	63.1
TioS-14	OSO_2NH_2	4-OCH_3	503.6	28.6	299.2	33.0
TioS-15	OSO_2NH_2	3,4,5-$(OCH_3)_3$	3239.8	50.9	42.3	17.6
TioS-16	OSO_2NH_2	naphtyl	940.2	62.7	405.1	20.9
AAZ			250	12	74	25

The 4-(trifluoromethyl)phenyl derivative (**TioS-4**) only inhibited hCAIX with a K_i of 22 μM whereas the 4-methylphenyl and the 4-methoxyphenyl derivatives only inhibited hCAII whit K_i of 76 and 90 μM respectively. The compounds **TioS-7** and **TioS-8** were completely inactive against all the isoforms with a K_i>100 μM. As expected, the introduction of the sulfamate moiety improved the inhibitory activity against the four isoforms. All sulfamates **TioS9-16** moderately inhibit hCAI with K_i values in the 158 - 3239 nM range. Compounds **TioS-9, TioS-11** and **TioS-12** exhibited higher hCAI inhibitory efficacy (K_i values 338.6, 311.6 and 158.1 nM respectively) due to the presence of strong electron-withdrawing and H-bond acceptor at the para position of the phenyl ring (4-NO_2, 4-F and 4-CF_3 respectively). The presence of a bulky substitution such as in compounds

TioS-15 and **TioS-16** (3,4,5-trimethoxyphenyl and 1-naphthyl derivatives) results in reduction of hCAI inhibition to a micromolar range, with K_i values 3239.8 and 940.2 nM respectively.

All sulfamates **TioS9-16** inhibited the ubiquitous hCAII in the nanomolar range being 4-methoxyphenyl derivative **TioS-14** the most active of the series. (K_i 28.6 nM). The substitution of the 4-methoxy group with a 4-methyl group (**TioS-13,** K_I 84.3 nM) or with a fluorine atom (**TioS-11**, K_I 72.1 nM). resulted in a reduction of activity. The presence of a bulky 3,4,5-trimethoxyphenyl (**TioS-15** K_i 50.9 nM) or naphthyl (**TioS-16** K_i 62.7 nM) substituents slightly decreased the activity against hCAII, as compared to **TioS-14**. The 3,4,5-trimethoxyphenyl derivative **TioS-15** displayed the best inhibitory activity against hCAIV with Ki 42.3 nM. **TioS-15** possess the bulkiest substitution at the phenyl ring, so it is possible that this huge portion result in a torsion of the molecule which determined more favourable interactions with the bind site cavity.

Sulfamates **TioS9-16** acted as inhibitors of the tumor-associated isoform CAIX in the low nanomolar range (K_is 63.1-17.6 nM). All the compounds acted as two to four-fold more potent hCAIX inhibitors compared to the off-target isoforms hCAI and hCAII, with the only exception for **TioS-13** bearing the 4-methylphenyl group (K_i 63.1 nM). As previous discussed, **TioS-15** (3,4,5-trimethoxyphenyl derivative, K_i 17.6) was the best inhibitor followed by **TioS-16** (naphthyl derivative, K_I 20.3 nM). Except for **TioS-14** (4-methoxyphenyl substituted), all studied sulfamates showed a hCAIX/II selectivity ratio 1.2 and 3, being **TioS-15** (Ki 17.6 nM) and **TioS-16** (Ki 20.9 nM) the most selective compounds.

2.5.1 Conclusion

Taken together the results indicated that the conversion of the low active CA inhibitors **TioS1-8** in their sulfamate derivatives **TioS9-16** afforded different nanomolar inhibitors of the tumor associated CAIX isoform. The sulfamates inhibited hCAI and hCAIV in the medium-high nanomolar range, except for the 3,4,5-trimethoxyphenyl derivative **TioS15** (hCAIV Ki 42.3 nM). Low nanomolar inhibitors were evidenced against hCA II (KIs in the range of 28.7–84.3nM) and IX (KIs in the range of 17.6–73.3 nM). The 3,4,5-trimethoxyphenyl derivative **TioS15** and the naphtyl derivative **TioS16** resulted the best compounds of the series against the tumour related isoform hCAIX, with Ki 17.6 nM and 20.9 nM respectively.

2.6 *N*[1]-acetyl-3,5-diarylpyrazoline series.

To further investigate different CAIX selective inhibitors, we have designed four new series of N[1]-acetyl pyrazoline derivatives bearing phenylsulfamate moiety. Pyrazoline scaffold is a five-member heterocycle which a wide range of pharmacological activities, such as cytotoxic,[99] antimicrobial,[100] antimalarial, anti-inflammatory[101] and neuroprotective activity.[102] Additionally, in the last years several series of pyrazolines bearing benzenesolphonamide moiety were reported to act as CA inhibitors.[103-106] These considerations let us to design a new series of *N*[1]-acetylpyrazolines[73,106] bearing phenylsulfamate moieties at the 3- or 5-position of the 4,5-dihydropyrazole ring (Figure 2.8).

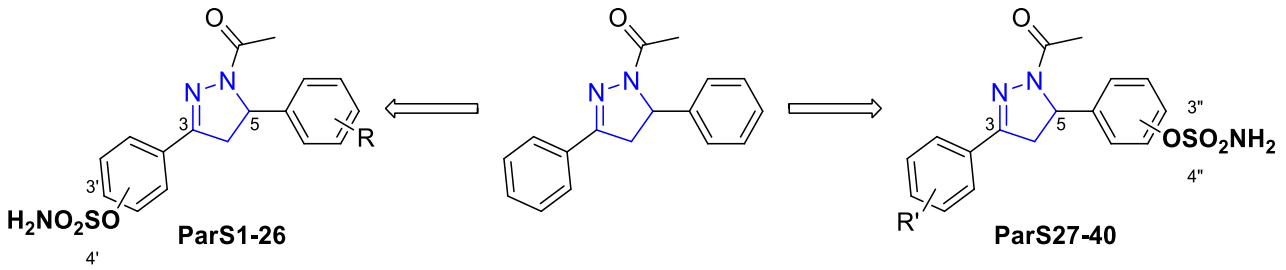
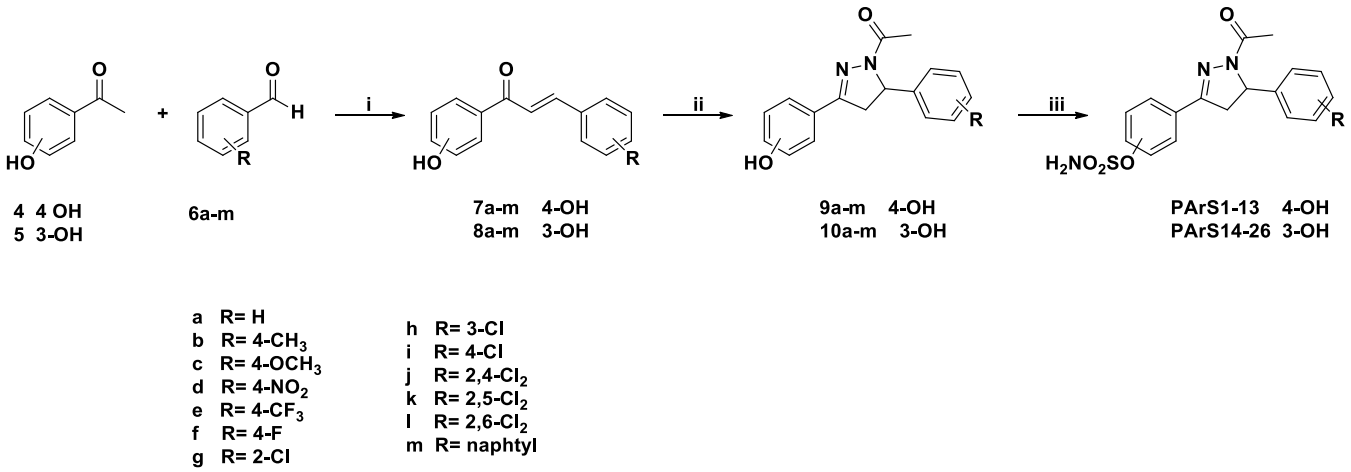
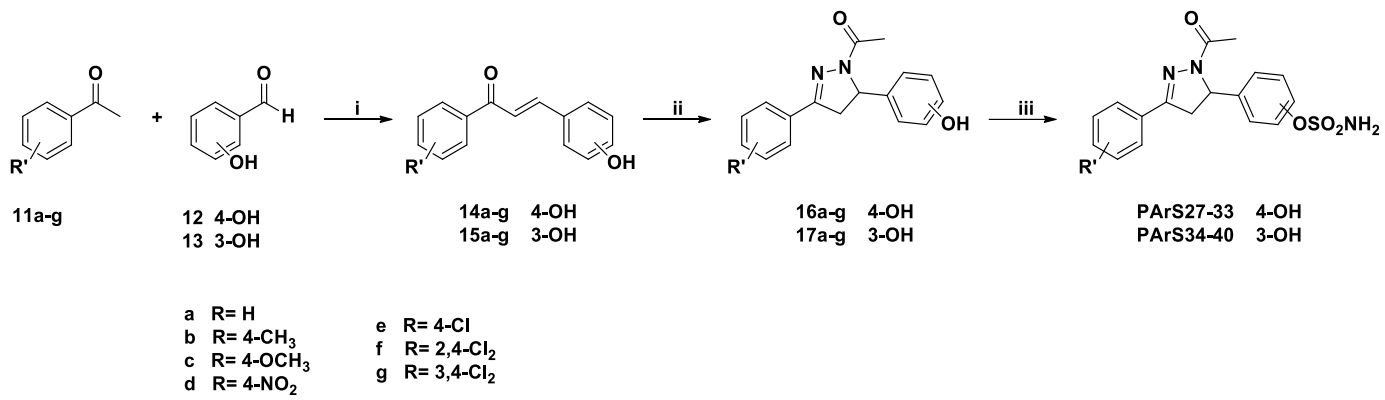


Figure 2.8. Proposed design for the N[1]-acetylpyrazolines **PArS 1-40**

The synthetic pathway to obtain these *N*[1]-acetyl-3,5-diaryl-4,5-dihydropyrazole sulfamates **PArS 1-40** started with the preparation of chalcones **4a-m**, **5a-m**, **13a-g**, **14a-g** through the Claisen-Schmidt condensation between substituted acetophenones and substituted benzaldehydes in methanol, in the presence of 50% NaOH solution (Schemes 2.2 and 2.3). Chalcones **7a-m**, **8a-m**, **14a-g**, **15a-g** were reacted with hydrazine hydrate in boiling AcOH to afford the cyclization into 4,5-dihydropyrazole derivatives **9a-m**, **10a-m**, **16a-g**, **17a-g**.



Scheme 2.2. General synthetic procedure for PArS **1-26**. Reagents and conditions: (i) MeOH, 50% aqueous NaOH, r.t., 12 h; (ii) NH_2NH_2 H_2O, AcOH, 3 h, reflux; (iii) $ClSO_2NH_2$, DMA, r.t. 12 h.



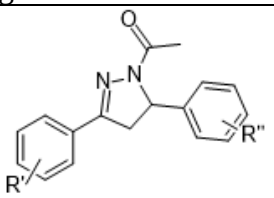
Scheme 2.3. General synthetic procedure for PArS **27-40**. Reagents and conditions: (i) MeOH, 50% aqueous NaOH, r.t., 12 h; (ii) NH_2NH_2 H_2O, AcOH, 3 h, reflux; (iii) $ClSO_2NH_2$, DMA, r.t. 12 h

.

Finally, the desired compounds **ParS1-40** were obtained by sulfamoylation of the phenol group by treatment with freshly prepared sulfamoyl chloride in N,N-dimethylacetamide solution. Structures were confirmed on the basis of analytical and spectral data and are consistent with results of reported studies. Indeed, ^{1}H NMR spectra showed diagnostic double doublet for the two protons at 3-position (3.10-3.90 ppm) and for the proton at 5-position (5.40-5.70 ppm).[106-108]

The inhibitory activity of sulfamates **PArS 1-40** against CAI, CAII, CAIX and CAXII are showed in Table 2.2. Concerning the activity against hCAI, all sulfamates showed low inhibitory activity with high nM IC_{50} values except for **PArS14, PArS16** and **PArS19** endowed with a better inhibitory activity as compared to AAZ. All sulfamates **PArS 1-40** inhibited the ubiquitous hCAII in the nanomolar range, in particular the 5-aryl substituted sulfamates **PArS1-12** displayed the highest activity (Ki between 0.8-13.2 nM). The 4-(trifluoromethyl)phenyl derivative **PArS-5** showed the best activity of the series (Ki 0.8 nM). The replacement of the 4-trifluoromethyl group with a fluorine or a chlorine atom (**PArS6**, Ki 1.5 nM; **PArS9** Ki 6.0 nM) led to a reduction of activity. The introduction of a chlorine atom in 3-position repristinated the activity (**PArS8** Ki 0.87 nM) whereas the displacement of the chlorine into 2-position (**PArS7** Ki 9.0 nM) or the introduction of two chlorine atoms (**PArS10-12**) led to a reduction of inhibitory activity. The displacement of the sulfamate group from 4-position to 3-position resulted in a general reduction of the activity against hCAII. Sulfamates **PArS18** and **PArS22** bearing 4-trifluoromethyl and 4-chlorine moieties respectively, maintained good activity (**PArS18** Ki 22.2 nM; **PArS22** Ki 14.7 nM). Switching the sulfamate group to 4-position on 5-aryl ring the activity is strictly related to the presence of substituents on the 3-aryl ring, as confirmed by the unsubstituted compound **PArS27** (Ki 44.8 nM) which is the compound with the lowest activity of this series. The best compound is **PArS29** bearing 4-methoxyphenyl group (Ki 0.42 nM) and it is interesting to note the different

Table 2.2. Inhibition data of human CA isoforms hCA I, II, IX and XII with derivatives **PArS1-40** the standard AAZ (errors were in the range of ±5–10% of the reported values).



Compound	R'	R''	K_i (nM)			
			hCAI	hCAII	hCAIX	hCAXII
PArS-1	4-OSO_2NH_2	H	1308.4	8.9	34.1	n.d.
PArS-2	4-OSO_2NH_2	4-CH_3	817.1	6.5	7.1	22.3
PArS-3	4-OSO_2NH_2	4-OCH_3	1607.4	5.3	25.0	42.6
PArS-4	4-OSO_2NH_2	4-NO_2	3208.5	1.2	74.3	n.d.
PArS-5	4-OSO_2NH_2	4-CF_3	2741.9	0.8	29.3	n.d.
PArS-6	4-OSO_2NH_2	4-F	666.5	1.5	5.9	11.8
PArS-7	4-OSO_2NH_2	2-Cl	8253.1	9.0	25.9	n.d.
PArS-8	4-OSO_2NH_2	3-Cl	2313.3	0.87	0.72	9.8
PArS-9	4-OSO_2NH_2	4-Cl	1137.1	6.0	6.9	27.3
PArS-10	4-OSO_2NH_2	2,4-Cl_2	3752.5	9.3	11.2	26.2
PArS-11	4-OSO_2NH_2	2,5-Cl_2	4752.2	12.4	25.5	n.d.
PArS-12	4-OSO_2NH_2	2,6-Cl_2	4083.0	13.2	33.4	43.7
PArS-13	4-OSO_2NH_2	naphtyl	3298.5	27.5	49.0	56.4
PArS-14	3-OSO_2NH_2	H	188.4	39.4	29.9	55.1
PArS-15	3-OSO_2NH_2	4-CH_3	558.2	71.2	29.4	55.9
PArS-16	3-OSO_2NH_2	4-OCH_3	172.2	52.4	8.7	48.6
PArS-17	3-OSO_2NH_2	4-NO_2	1458.2	133.9	10.1	20.8
PArS-18	3-OSO_2NH_2	4-CF_3	951.8	22.2	3.7	8.9
PArS-19	3-OSO_2NH_2	4-F	67.4	38.0	8.4	13.5
PArS-20	3-OSO_2NH_2	2-Cl	939.5	90.1	43.6	74.4
PArS-21	3-OSO_2NH_2	3-Cl	805.2	56.9	7.3	44.6
PArS-22	3-OSO_2NH_2	4-Cl	437.7	14.7	6.9	9.3
PArS-23	3-OSO_2NH_2	2,4-Cl_2	1413.2	194.7	13.8	33.8
PArS-24	3-OSO_2NH_2	2,5-Cl_2	2035.7	92.6	58.9	34.2
PArS-25	3-OSO_2NH_2	2,6-Cl_2	1782.5	223.4	63.6	61.3
PArS-26	3-OSO_2NH_2	naphtyl	2244.1	215.7	51.1	462.6
PArS-27	H	4-OSO_2NH_2	644.7	44.8	45.1	10.6
PArS-28	4-CH_3	4-OSO_2NH_2	2338.4	9.9	22.4	7.7
PArS-29	4-OCH_3	4-OSO_2NH_2	2977.2	0.42	22.8	9.5
PArS-30	4-NO_2	4-OSO_2NH_2	3818.1	9.5	15.0	0.88
PArS-31	4-Cl	4-OSO_2NH_2	970.2	19.5	8.3	29.5
PArS-32	2,4-Cl_2	4-OSO_2NH_2	3518.6	5.3	49.9	42.0
PArS-33	3,4-Cl_2	4-OSO_2NH_2	2898.4	27.6	7.7	12.9
PArS-34	H	3-OSO_2NH_2	755.0	16.5	13.1	22.9
PArS-35	4-CH_3	3-OSO_2NH_2	1539.2	25.4	8.9	49.8
PArS-36	4-NO_2	3-OSO_2NH_2	2546.8	22.9	9.4	20.5
PArS-37	4-F	3-OSO_2NH_2	5225.3	6.5	15.8	n.d.
PArS-38	4-Cl	3-OSO_2NH_2	2140.9	32.7	0.81	22.3
PArS-39	2,4-Cl_2	3-OSO_2NH_2	6936.0	12.0	48.7	30.8
PArS-40	3,4-Cl_2	3-OSO_2NH_2	8174.1	54.8	15.5	85.2
AAZ	-		250	12.5	25	5.7

inhibition profile as comparing **PArS29** and **PArS3**, the analog with the sulfamate group in 4-position on 3-aryl ring. In fact, **PArS29** (Ki 0.42 nM) resulted about 13-fold more potent as compared with the analog **PArS3** (Ki 5.3 nM) and this tendency is in general confirmed by other compounds bearing the sulfamate group in 4-position of 5-aryl ring.

The introduction of 4-methylphenyl (**PArS28** Ki 9.9 nM), 4-nitrophenyl (**PArS30** Ki 9.5 nM) and 2,4-dichlorophenyl (**PArS32** Ki 5.3 nM) substituents resulted in a good activity. Shifting the sulfamate group into 3 position on 5-aryl ring resulted in a slight reduction of the activity, being the 4-fluorophenyl derivative the compound with the best activity (**PArS 37** Ki 6.5 nM).

On the CA isoform expressed in hypoxic tumoral cells, the CAIX, the 3-chlorophenyl derivative **PArS8** (Ki 0.72 nM) showed the best activity of the series. Displacing the chlorine atom into 4-position (**PArS9** Ki 6.9 nM) led to about 10-fold reduction in activity as well as the substitution of the 3-chlorine with the 4-methyl group (**PArS2** Ki 7.1 nM). The replacement of 4-chlorine with 4-fluorine (**PArS6** Ki 5.9) slightly improved the activity while the presence of two chlorine atom in the phenyl ring (**PArS10-12**) resulted in a decrease of activity. The displacement of the 4-sulfamate group into 3-position on the 3-aryl ring, to give the isomeric pyrazolines **PArS 14-26** induced similar or better activity as compared with the corresponding analogs **PArS 1-13**, except for **PArS20** (Ki 43.6 nM), **PArS24** (Ki 58.9 nM) and **PArS25** (Ki 63.6 nM). Shifting the 4-sulfamate group from the 3-aryl ring to the 5-aryl ring resulted in a similar inhibitory profile compared to the analogs compounds. Interestingly, the transposal of the 4-nitrophenyl and the phenyl sulfamate substituents of **PArS4** to give the isomeric **PArS30** produced about 5-fold increase in activity. The shift of the sulfamate moiety form 4-position to 3-position on the 5-aryl ring to give **PArS34-40** produced and improvement of activity, especially for the 4-chlorophenyl derivative **PArS38** (Ki 0.81 nM), that resulted about 8.5-fold more active of the analog **PArS22** (Ki 6.9 nM) bearing the sulfamate group in 3-position on the 3-aryl ring. **PArS38** resulted also 10-fold more active as compared to the analog **PArS31** (Ki 8.3 nM) bearing the sulfamate moiety in 4-position into the 5-aryl ring. Similar activity trend is showed by the unsubstituted derivative **PArS34** (Ki 13.1 nM) which is about 3-fold more active than **PArS27** (Ki 45.1 nM), the analog with the sulfamate group in 4-position on the 5-aryl ring.

On the second cancer-related isoform the CAXII, **PArS6** (Ki 11.8 nM), **PArS8** (Ki 9.8 nM), **PArS18** (Ki 8.9 nM), **PArS19** (Ki 13.5 nM) and **PArS22** (Ki 9.3 nM) showed good activity, confirming also the activity showed against CAIX. The best compound of the series is the 4-nitrophenyl derivative **PArS30** (Ki 0.88 nM). The removal of the 4-nitrophenyl group (**PArS27** Ki 10.6 nM) or its replacement with a 4-methoxyphenyl group (**PArS-29** Ki 9.5 nM) led to a

consistent of the activity. The displacement of the 4-sulfamate into 3 position on 5-aryl ring (**PArS34-40**) led to a general reduction of the activity against CAXII.

The most interesting compounds of the series (**PArS6, PArS8, PArS18, PArS29, PArS30, PArS32, PArS38**) were selected to better understand the binding patterns, by docking studies, using the four isoforms evaluated in this study, CA I (PDBID: 3w6h), CA II (PDBID: 4g0c), CA IX (PDBID: 3iai), CA XII (PDBID: 1jd0). These crystal structures were selected for the presence of AAZ as co-crystalized ligand. From the first analysis of the binding site, it is possible to find some interesting informations: the main difference among the four isoforms is the size of the binding site. For example, the smaller binding site of CAI can explain the lower activity of the sulfamates on this isoform. Furthermore, another important information is related to the hydrophobic properties of the different isoforms, such as CAXII endowed with a more polar binding site than the other isoforms (Figure 2.9). Another important difference between CAII and the other isoforms relates residue 200, a histidine in CAI whereas a threonine in CAII, IX and XII. As in most of the pose of docked compounds, there is an interaction between the ligand and Thr200. The Thr/His mutation is likely to induce the ligands lower activity against CAI.

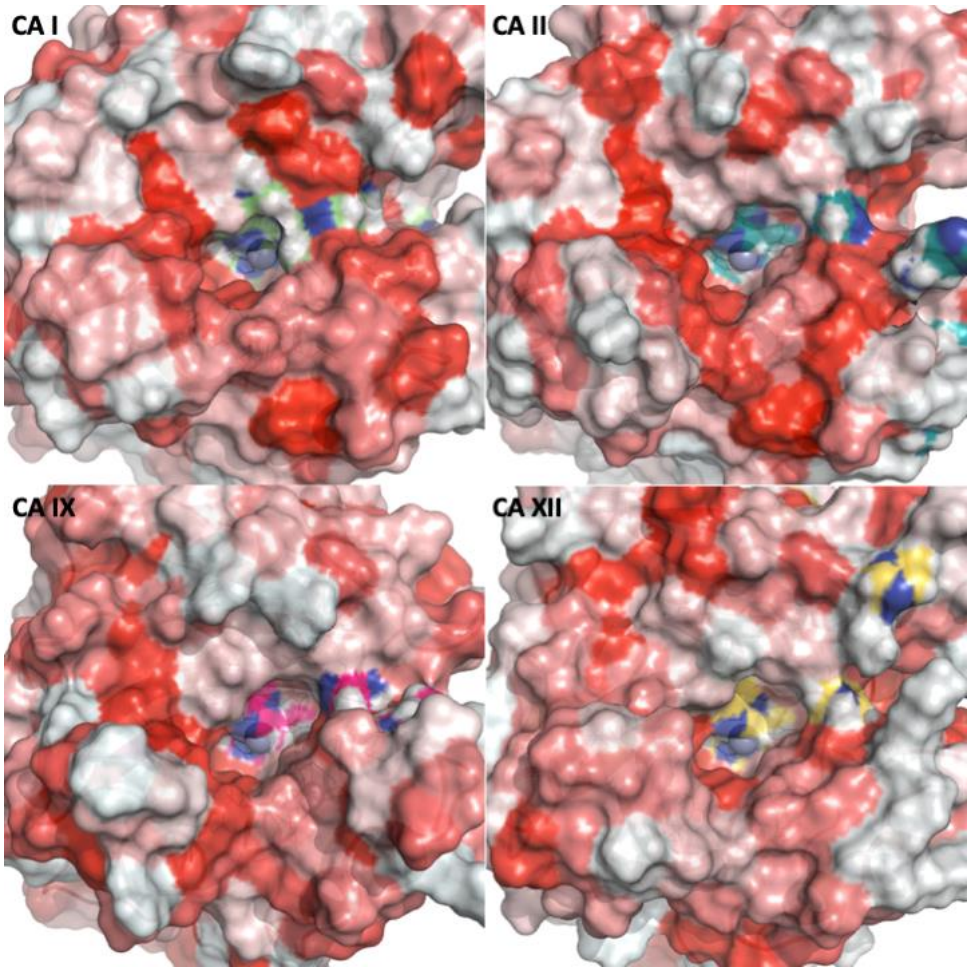


Figure 2.9. hCA Binding sites comparison. Red: Hydrophobic surface

Concerning the docking score, it was not possible to find a clear correlation with *in-vitro* inhibition results, but some useful informations to better understand the activity and selectivity of sulfamates derivatives can be obtained looking at the best scored poses (Table 2.3).

Table 2.3. hCA docking score and experimental inhibition values

Compound	CAI Score	CAI Exp	CAII Score	CAII Exp	CAIX Score	CAIX Exp	CAXII Score	CAXII Exp
PArS6	-16.38	666.5	-22.03	1.5	-13.47	5.9	-18.50	11.8
PArS8	-17.67	2313.3	-16.16	0.87	-14.90	0.72	-10.20	9.8
PArS18	-9.79	951.8	-17.71	22.2	-10.38	3.7	-18.57	8.9
PArS29	-21.05	2977.2	-22.49	0.42	-25.57	22.8	-24.17	9.5
PArS30	-13.32	3818.1	-18.22	9.5	-17.92	15.0	-13.83	0.88
PArS32	-17.19	3518.6	-21.07	5.3	-21.86	49.9	-17.16	42.0
PArS38	-18.12	2140.9	-18.36	32.7	-9.55	0.81	-14.51	22.3

The sulfamate group of all compounds reproduced almost the same interactions with the enzyme, compared with the sulfonamide group of AAZ. The other portion of the molecules is related to the different activity and selectivity of the selected compounds. Concerning hCAI, no other important interaction was found except for **PArS18**, which established an H-bond between one fluorine atom of trifluoromethyl group and Asn69 side chain (Figure 2.10).

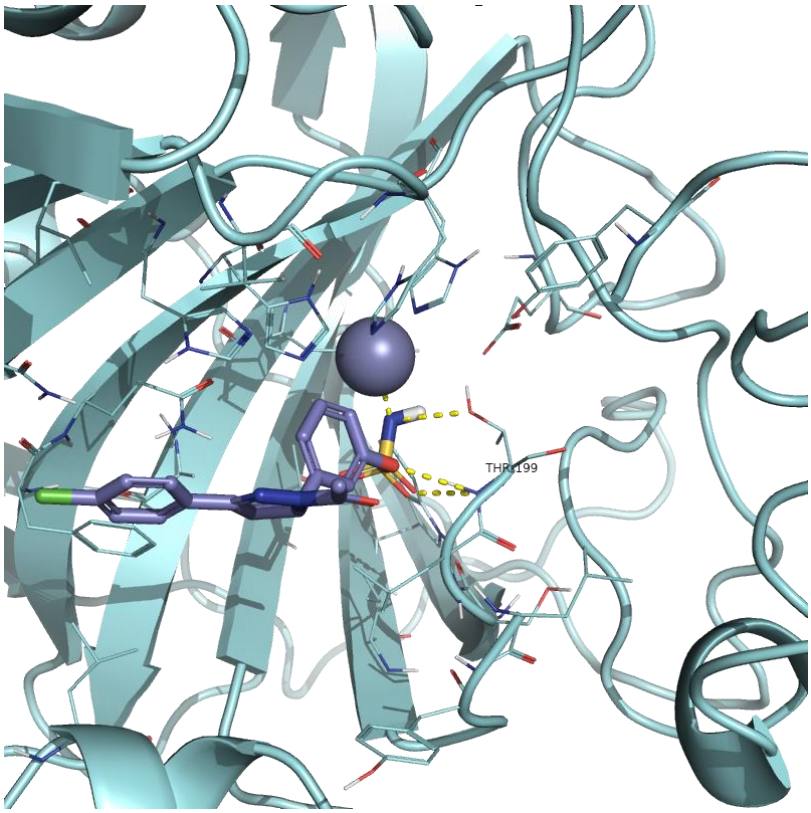


Figure 2.10. **PArS18** best pose in hCAI, focus on the sulfamate interactions.

Regarding hCAII, all selected compounds performed a π-π stacking with Phe131. Further H-bonds performed by the compounds are with Asn62 and Asn67 (**PArS6, PArS8, PArS38**). Moreover, for the most active compounds of the series **PArS29**, as well as **PArS30** and **PArS38,** a H-bond exists between their carbonyl moiety and the hydroxyl group of Thr200 (Figure 2.11).

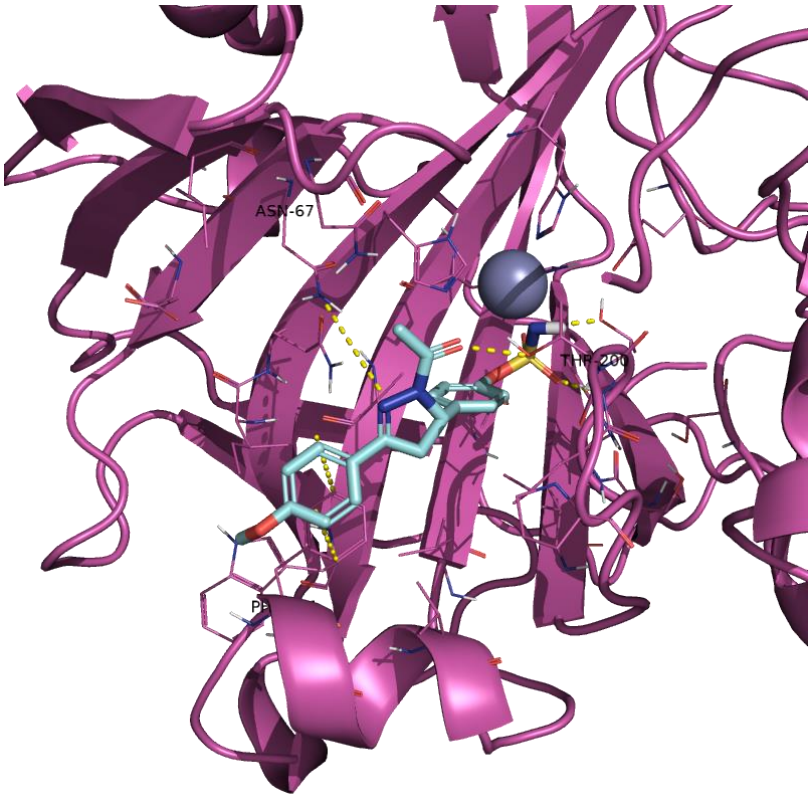


Figure 2.11. **PArS29** best pose in CA II

Speaking about hCAIX active site, **PArS6, PArS8, PArS18** and **PArS38** showed potential H-bond with Asn67 and Gln92 while **PArS29, PArS30** and **PArS32** established H-bond with Thr200. Furthermore, the nitro group in **PArS30** was in a good position to form an additional H-bond with Asp132 (Figure 2.12).

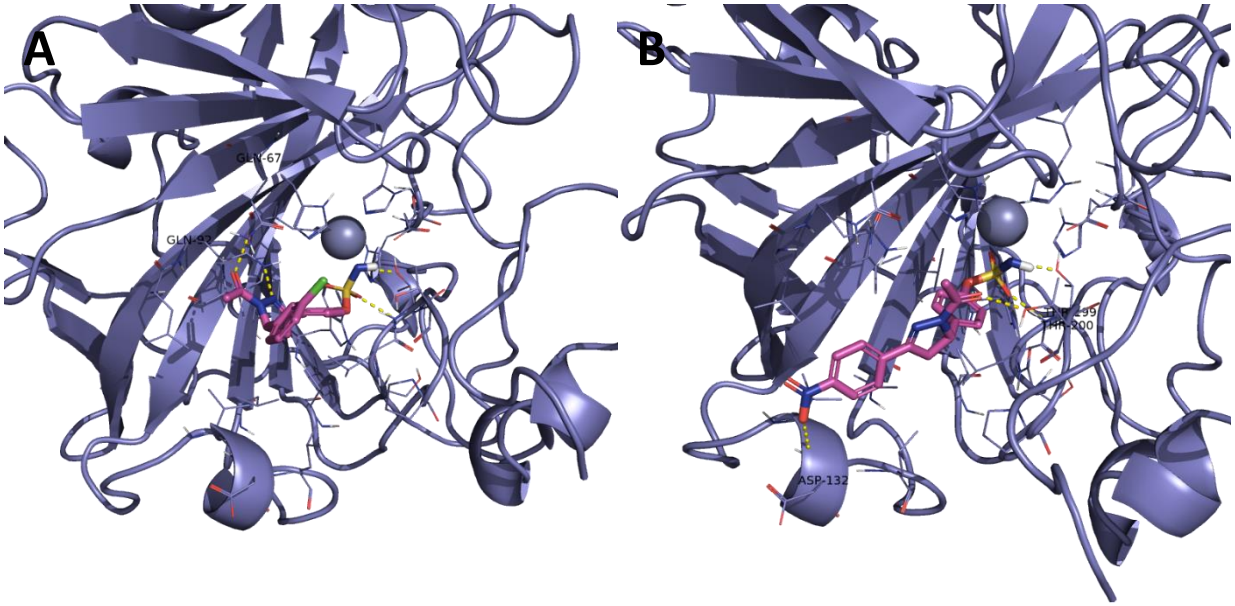


Figure 2.12. Best poses of **PArS8** (A) and **PArS30** (B) in hCAIX

In hCAXII all compounds except **PArS6** and **PArS18** established H-bonds with Lys67 as well as with Thr91 and/or Gln92. Moreover, **PArS18, PArS29, PArS30** and **PArS38** established H-bonds by the carbonyl group or the pyrazoline nitrogen with Thr200. PArS18, **PArS29** and **PArS30** bearing an H-bond acceptor group in para-position on the non-sulfamate ring, established interactions with Ser132 (Figure 2.13).

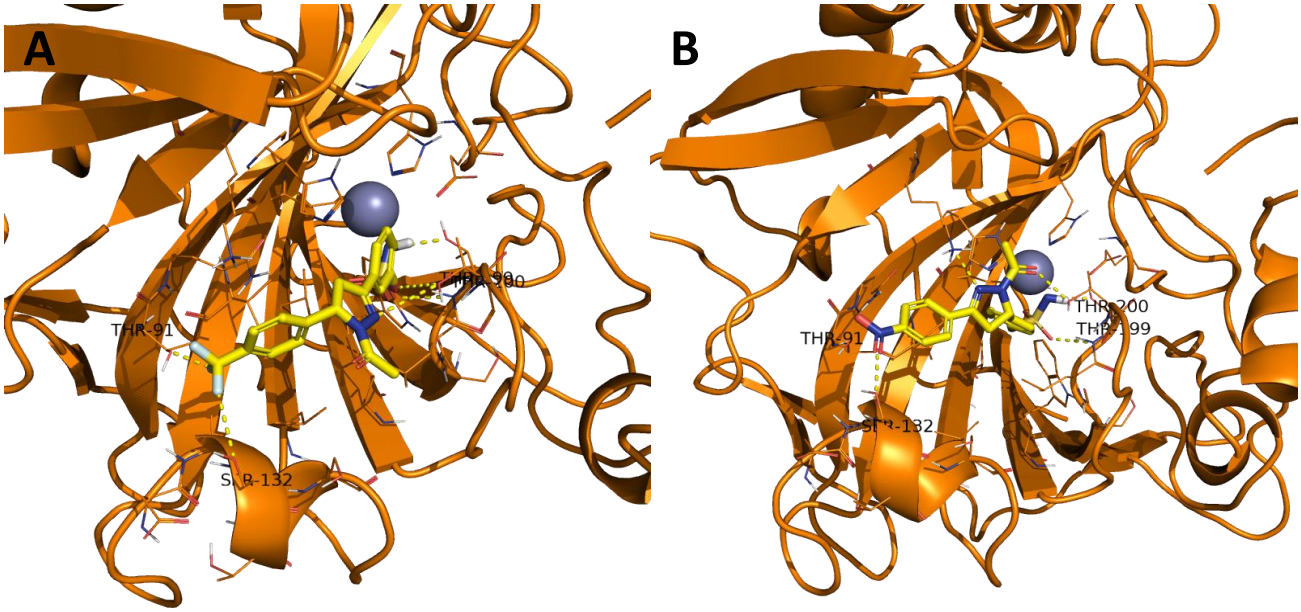


Figure 2.13. *Best poses of **PArS18** (A) and **PArS30** (B) in hCAXII.*

2.6.1 Conclusions

The pyrazoline sulfamates showed weak inhibitory activity against the off-target hCA I, while many sulfamates showed activity at low nanomolar levels against hCA II (Kis in the range of 0.42-90.1 nM), IX (Kis in the range of 0.72-63.6 nM), and XII (KIs in the range of 0.88 e 85.2 nM) being the position of sulfamate moiety on 5 or 3-aryl ring is strictly correlated with the inhibitory activity. For what concerns the activity against hCAII, the best substitution fragments at the pyrazoline ring included the sulfamic group on the 3-aryl, with halogens on the 5-aryl, a methoxy group on the 3-aryl and a 4-sulfamate group on the 5-aryl. Speaking about the cancer related isoforms hCAIX and hCAXII the sulfamic group on the 3- or 4- position of the 5-aryl is necessary, with an electron-withdrawing group on the 4-postion of the 3-aryl ring.

2.7 Ureido and thioureido derivatives

Two different series of substituted benzensulfonamides were designed based on SLC-0111 scaffold. SLC-0111 is endowed with ureido moiety connected with the benzene sulfonamide which improves flexibility of the tail in order to adopt different orientation to better interact with the amino acid residues at the active site of the enzyme.[109-111] In 2015 Congiu et al. reported on a new series of carbonic anhydrase inhibitors based on SLC-0111 scaffold, bearing piperazinyl-ureido moiety.[85] The most important modification at the SLC-0111 scaffold was the incorporation of the ureido moiety into a rigid heterocyclic system, the *N*-substituted piperazine ring (Figure 2.14, A). Piperazine ring is broadly used in drug discovery and piperazine derivatives are well-known to produce compounds with different pharmacological activities, and recently it has been used in the development of CA inhibitors.[112] In 2010 Liu L. et al. proved the importance of a carbonyl thioureido moiety to improve the selectivity against the cancer related isoform hCAIX (Figure 2.14, B). In their work, the docking analysis showed that the polar sulfur atom and of thioureido group and the oxygen from the carbonyl group can form hydrogen bonds with Asn62 and Gln67 of hCAIX.[113] Starting from these considerations in this thesis a new series of benzensulfonamide derivatives were designed (Figure 2.14) and tested for their inhibitory activity against hCAs. To better understand the importance of rigid heterocyclic scaffold, the piperazine ring was substituted with a piperidine moiety. Furthermore, carbonyl ureido and thioureido moieties were introduced as tail of inhibitors.

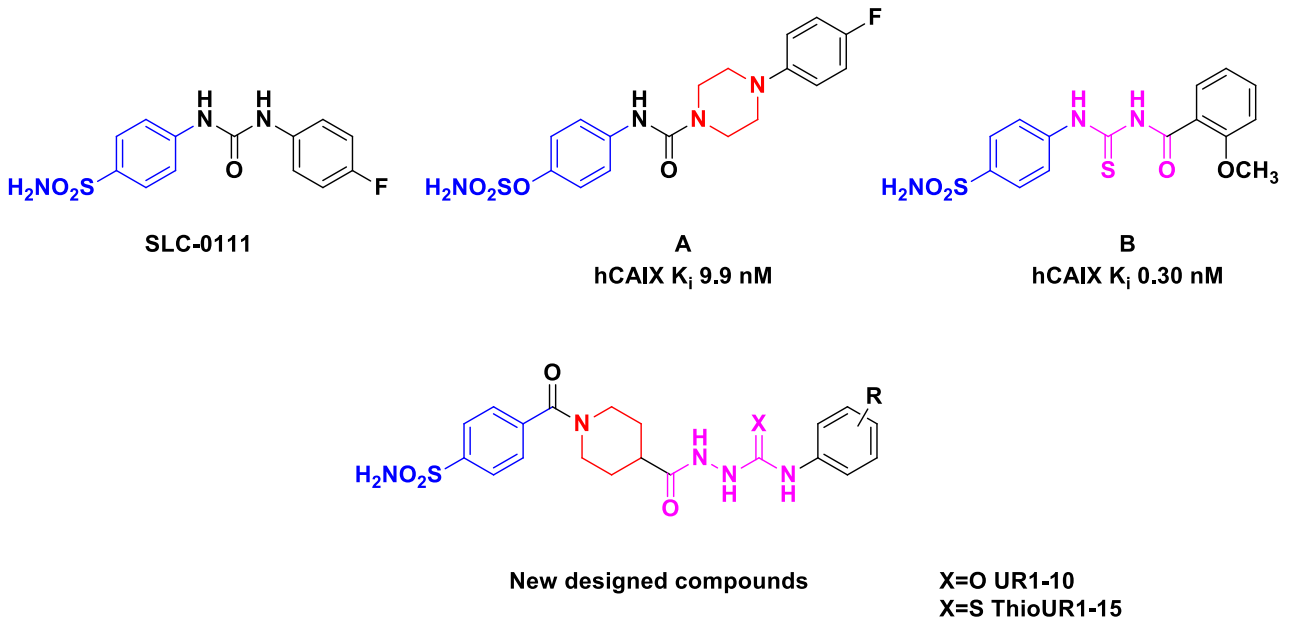
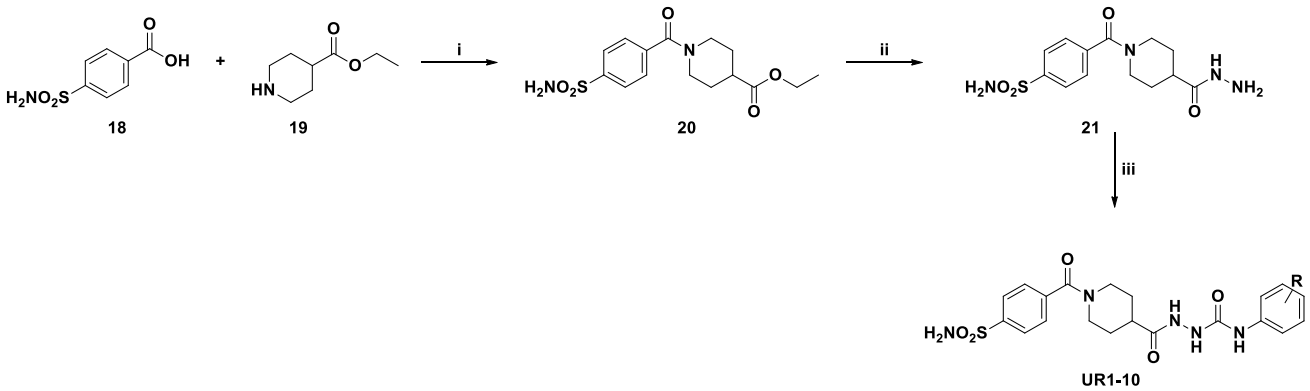


Fig 2.14. Design of new 4-sulfamoylbenzoyl-piperidine **UR1-10**, **ThioUR1-15** starting from SLC-011, A[85] and B[113]

The synthetic pathway to obtain these 4-sulfamoylbenzoyl-piperidine derivatives **UR1-10**[114] started with the preparation of ethyl 1-(4-sulfamoylbenzoyl)piperidine-4-carboxylate **3** by amide coupling

of ethyl piperidine-4-carboxylate **2** and 4-sulfamoylbenzoic acid **1** using as coupling agent 1-(3-dimthylaminopropyl)3-ethylcarbodiimide hydrochloride (EDCI) (Scheme 2.4). The reaction was performed in dry acetonitrile solution (MeCN), in the presence of 1-hydroxybenzotriazole hydrate (HOBt). The resulting ethyl 1-(4-sulfamoylbenzoyl)piperidine-4-carboxylate **3** was treated with hydrazine hydrate in absolute ethanol to obtain the corrisponding hydrazide **4**. The target ureas **UR1-10** were obtained by reaction between the 4-(4-(hydrazinecarbonyl)piperidine-1-carbonyl)benzenesulfonamide **4** and substituted isocyanates. Structures of **UR1-10** were assigned on the basis of analytical and spectral data and are consistent with results of reported studies.[85,113] ^{1}H NMR spectra showed some diagnostic signals: a singlet for the proton at 4-position of piperidine ring (4.30-4.50 ppm) and three NH signals of the hydrazinocarboxamide moiety at 7.80-8.30 ppm, 9.00-9.40 ppm and 9.60-9.90 ppm.[85,113]

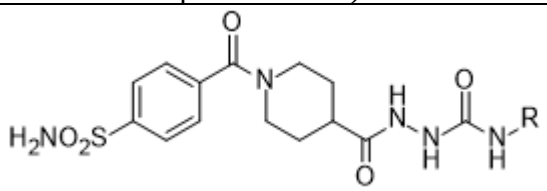


Scheme 2.4. General synthetic procedure for **UR1-10**. Reagents and conditions: (i) EDCI, HOBt, dry CH_3CN, r.t. 24h; (ii) NH_2NH_2 H_2O, EtOH, 3 h, reflux; (iii) Substituted Isocyanates, EtOH, 160°C, 12 h.

As reported in Table 2.4 the off-target isoform hCAI was inhibited by all the derivatives **UR1-10** with a variety of potency. The 4-fluorophenyl derivative **UR6** resulted to be the best compound of the series with a K_i of 60.6 nM. The substitution of the 4-fluorine group with a methyl group slightly reduced the inhibitory activity (**UR7** Ki 81.7 nM). the introduction of one or two chlorine groups, such as in compounds **UR2** and **UR10** led to reduction of activity (**UR2** Ki 259.4 nM, **UR10** Ki 217 nM) while the absence of substituent on the aryl-ureido ring restored the activity (**UR1** Ki 93.3 nM). Furthermore, the introduction of benzyl and 4-(methoxy)benzyl groups such as in derivative **UR8** (Ki 77.8 nM) and derivative **UR3** (Ki 129.8 nM) partially restored the activity.
The cytosolic isoform hCAII was inhibited by all ureido derivatives with Ki in the range between 5.1-101.3 nM. The phenyl derivative **UR1** showed Ki of 29.3 nM and the introduction of a substituent in 4-position, such as a fluorine (**UR6** Ki 12.1 nM) or a methyl group (**UR7** Ki 22.0 nM) led to increase in activity. The introduction of a second methyl group to give the 2,6-

dimethylphenyl derivative **UR9** reduced the inhibitory activity (Ki 62.1 nM), while the introduction of a chlorine in 3-position (**UR10** Ki 5.1 nM) led to increase in activity.

Table 2.4. Inhibition data of human CA isoforms hCA I, II, IX and XII with derivatives **UR1-10** reported here and the standard sulfonamide inhibitor acetazolamide (AAZ) by a stopped flow CO_2 hydrase assay (errors were in the range of ±5–10% of the reported values).



Compound	R	K_i (nM)			
		hCAI	hCAII	hCAIX	hCAXII
UR1	phenyl	93.3	29.3	16.5	22.8
UR2	2,6-dichlorophenyl	259.4	68.8	28.9	16.4
UR3	4-methoxybenxyl	129.8	40.7	45.3	6.4
UR4	naphtyl	521.5	101.3	26.7	60.6
UR5	2,4-dimethoxyphenyl	321.8	19.8	23.2	15.6
UR6	4-fluorophenyl	60.6	12.1	2.1	24.0
UR7	4-methylphenyl	81.7	22.0	8.1	36.7
UR8	benzyl	77.8	48.2	40.0	20.1
UR9	2,6-dimethylphenyl	363.6	62.1	25.1	51.2
UR10	3-chlorophenyl	217.9	5.1	37.8	44.6
AAZ	-	250	12.5	25	5.7

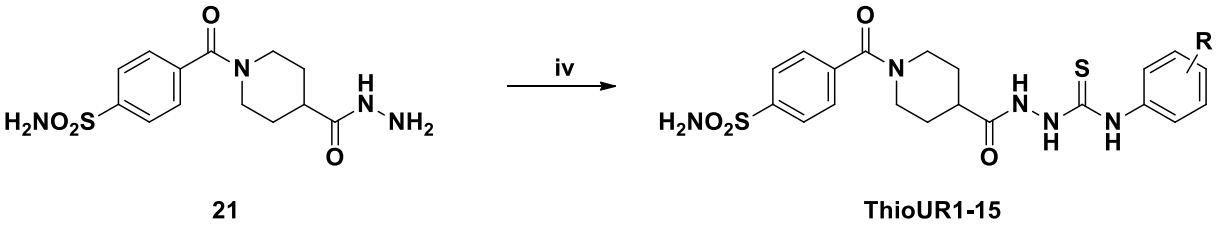
The introduction of a second chlorine atom to give the 2,6-dichlorophenyl derivative **UR2** led to considerable decrease in activity (Ki 68.8 nM). Furthermore, the substitution of the phenyl group with a benzyl group (**UR8** Ki 48.2 nM) or with a naphtyl group (**UR4** Ki 101.3 nM) reduced the inhibitory activity.

Concerning the first cancer-related isoform hCAIX, all the compounds showed good inhibitory activity. The phenyl derivative **UR1** showed a Ki of 16.5 nM while the substitution of the phenyl ring with a benzyl group (**UR8** Ki 40.0 nM) or with a 4-methoxybenzyl group (**UR3** Ki 40.7 nM) resulted in about 4-fold reduction of activity. Furthermore, the substitution of the phenyl group with a naphthyl group also reduced the activity **(UR4** Ki 26.7 nM). Remarkably, the substitution of the 4-position led to increased activity. For example, the 4-fluorophenyl derivative **UR6** (Ki 2.1 nM) resulted to be 8-fold more active than **UR1** as well as the 4-methylphenyl derivative **UR7** (Ki 8.1 nM) was 2-fold more active than **UR1**. The introduction of two chlorine atoms to obtain the 2,6-dichlorophenyl derivative **UR2** led to reduction in activity (Ki 28.9 nM) as well as the replacement of the chlorine atoms with methyl groups (**UR9** Ki 25.1 nM).

Concerning hCAXII, this isoform was inhibited by ureido derivatives with Ki in the 6.4-60.6 nM range. The 4-methoxybenzyl derivative **UR3** resulted the best compound of the series, with a Ki of 6.4 nM and it was about 7-fold more selective against hCAXII respect to both hCAII and hCAIX.

The replacement of the 4-methoxyphenyl group with a benzyl moiety to give **UR8** (Ki 20.1 nM) led to reduction in activity. The replacement of the benzyl group with the phenyl group to give **UR1** (Ki 22.8 nM) did not change the activity, whereas the introduction of a naphthyl group led to a 3-fold reduction of activity (**UR4** Ki 60.6 nM). The introduction of two chlorine atoms on the aryl ring of **UR1** to give the 2,6-dichlorophenyl derivative **UR2** increased the activity (Ki 16.4 nM). On the contrary the replacement of the chlorine groups with methyl groups (**UR9** Ki 51.2 nM) resulted in decrease of activity. Unlike the inhibitory activity showed against hCAIX, 4-fluorophenyl derivative (**UR6** Ki 24.0 nM) and 4-methylphenyl (**UR7** Ki 36.7 nM) resulted about 11-fold and 4-fold less active against hCAXII respectively.

To further investigate the potential activity of the piperidine-4-carbohydrazide derivatives, a second series of thioureido derivatives **ThioUR1-15**[114] was designed and synthetized (Figure 2.14 and Scheme 2.5). In the second series of 1-(4-sulfamoylbenzoyl)piperidine derivatives, the benzylureido moiety was substituted with the analog benzylthioureido moiety. The desired compounds **ThioUR1-15** were obtained by reaction between the 4-(4-(hydrazinecarbonyl)piperidine-1-carbonyl)benzenesulfonamide **4** and substituted isothiocyanates. ^{1}H NMR spectra of compounds **ThioUR1-15** were consistent with **UR1-10** chemical shifts.



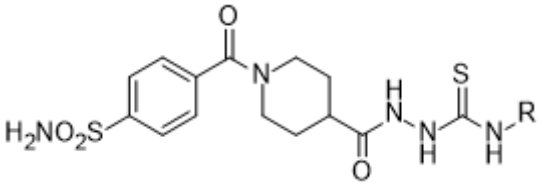
Scheme 2.5. General synthetic procedure for **ThioUR1-10**. Reagents and conditions: (i) Substituted Isothiocyanates, EtOH, 160°C, 12 h.

The CA inhibition data of compounds **ThioUR1-15** are displayed in Table 2.5. Concerning the activity against hCAI, all thioureas showed inhibitory activity at high nanomolar levels, with the exception for the 2,6-difluorophenyl derivative **ThioUR9** (Ki 65.4 nM), the phenylethyl derivative **ThioUR11** (Ki 76.6 nM) and the 4-fluorophenyl derivative **ThioUR13** (Ki 79.6 nM).

Speaking about the off-target hCAII, the compounds of the series showed Ki values between 5.7 and 71.4 nM. The 3-chlorophenyl derivative **ThioUR1** displayed good inhibitory activity, with Ki of 16.6 nM. The results proved the strictly correlation between the number and the position of chlorine atoms and the activity against hCAII. Displacing the chlorine atom into 4-position led to about 3-fold decrease of activity (**ThioUR2** Ki 41.6 nM) as compared to **ThioUR1**, while the

introduction of a second chlorine atom to give the 3,4-dichlorophenyl derivative **ThioUR3** (Ki 22.4 nM) restored the activity.

Table 2.5. Inhibition data of human CA isoforms hCA I, II, IX and XII with derivatives **ThioUR1-15** reported here and the standard sulfonamide inhibitor AAZ by a stopped flow CO_2 hydrase assay (errors were in the range of ±5–10% of the reported values).



Compound	Formula	K_i (nM)			
		hCAI	**hCAII**	**hCAIX**	**hCAXII**
ThioUR1	3-chlorophenyl	322.8	16.6	18.0	9.7
ThioUR2	4-chlorophenyl	130.9	41.6	26.1	17.7
ThioUR3	3,4-dichlorophenyl	442.3	22.4	4.7	26.9
ThioUR4	2,4-dichlorophenyl	228.6	53.4	18.0	2.6
ThioUR5	cyclohexyl	117.6	5.7	1.7	19.6
ThioUR6	4-methoxybenzyl	178.3	29.3	31.7	29.9
ThioUR7	4-nitrophenyl	297.9	60.4	13.6	45.0
ThioUR8	3,4,5-trimethoxyphenyl	428.1	36.8	16.6	27.3
ThioUR9	2,6-difluorophenyl	65.4	28.2	5.9	5.6
ThioUR10	2,5-dimethylphenyl	165.4	47.8	34.4	44.7
ThioUR11	phenylethyl	76.6	7.6	2.9	10.4
ThioUR12	4-methoxyphenyl	211.4	15.1	22.1	27.8
ThioUR13	4-fluorophenyl	79.6	23.0	20.1	15.9
ThioUR14	2,6-dimethylphenyl	307.9	89.6	4.7	9.5
ThioUR15	benzyl	198.1	71.4	24.2	25.0
AAZ	-	250	12.5	25	5.7

Furthermore, moving the chlorine atom from 3- to 2- position to give the 2,4-dichlorophenyl derivative **ThioUR4** (Ki 53.4 nM) reduced the activity, so we can assume that the substitution at 3-position is necessary for the inhibitory activity against hCAII. The replacement of the 4-chlorine atom with a fluorine atom **ThioUR13**, increased the activity (Ki 23.0 nM). The replacement of the fluorine atom with a nitro group resulted in a 4-fold reduction of activity (**TioUR7** Ki 60.4 nM) whereas the 4-methoxyphenyl derivative showed high inhibitory activity (**ThioUR12** Ki 15.1 nM). The introduction of two fluorine atoms into 2- and 6-position to give **ThioUR9** slightly reduced the activity (Ki 28.2 nM) while the replacement of the fluorine atoms with a methyl group resulted in 3-fold reduction of activity (**ThioUR14** Ki 89.6 nM). The benzyl derivative **ThioUR15** resulted to be active at high nanomolar range (Ki 71.4 nM). The activity is restored with the introduction of methoxy group at 4-position of the phenylureido ring (**ThioUR6** Ki 29.3 nM). Among all the tested

compounds, the cyclohexyl derivative **ThioUR5** and the phenylethyl derivative **ThioUR11** showed activity at low nanomolar levels, with Ki values of 5.7 nM and 7.6 nM respectively.

On the hCAIX the cyclohexyl derivative**ThioUR5** showed the best activity with a Ki 1.7 nM. As showed for hCAII also the phenylethyl derivative **ThioUR11** displayed activity against hCAIX at low nanomolar level with Ki 2.9 nM. The 3-chlorophenyl derivative **ThioUR1** was endowed with high activity (Ki 18.0 nM) and the introduction of a second chlorine atom in 4-position to give the 3,4-dichlorophenyl derivative **ThioUR3** increased the activity. Indeed, **ThioUR3** with a Ki of 4.7 nM resulted to be about 3-fold more active than **ThioUR1** and about 5-fold more selective against the cancer related isoform hCAIX compared with hCAII. The displacing of the chlorine atom from 3- into 4-position led to a decrease in activity (**ThioUR2** Ki 26.1 nM) while the introduction of a second chlorine atom in 2-position to give the 2,4-dichlorophenyl derivative **TioUR4** restored the activity (Ki 18.0 nM). The substitution of the chlorine atom in 4-position with a fluorine atom (**ThioUR13** Ki 20.1 nM) or with a methoxy group did not change the activity (**ThioUR12** Ki 22.1 nM) while the introduction of a 4-nitro group slightly improved the activity (**ThioUR7** Ki 13.6 nM). The 2,6-difluorophenyl derivative **ThioUR9** showed inhibitory activity at low nanomolar level, with Ki 5.9 nM. A comparable inhibitory activity is showed by the 2,6-dimethylphenyl derivative **ThioUR 14** with a Ki of 4.7 nM. The 3,4,5-trimethoxyphenyl derivative **ThioUR8** is endowed with high activity (Ki 16.6 nM) whereas the benzyl derivative (**ThioUR15** K_i 24.2 nM) and the 4-methoxybenzyl derivative (**ThioUR6** K_i 31.7 nM) showed lower activity.

On hCAXII the 2,4-dichlorophenyl derivative **ThioUR4** showed the best activity of the series with Ki of 2.6 nM. Displacing the chlorine atom from 2- to 3-position gave reduction of the activity (**ThioUR3** Ki 26.9 nM). To what concern the 3-chlorophenyl derivative **ThioUR1** (Ki 9.7 nM) and the 4-chlorophenyl derivative **ThioUR2** (17.7 nM), displacing the chlorine from 3-position to 4-position did not make significant changes in activity. It is interesting to note the inhibitory profile of the 2,6-difluorophenyl derivative **ThioUR9** and the analog 2,6-dimethylphenyl derivative **ThioUR14**. **ThioUR9** is endowed with high inhibitory activity, with Ki 5.6 nM, comparable with the activity against hCAIX (Ki 5.9 nM.) Furthermore, **ThioUR9** resulted about 5-fold more selective for both the cancer related isoforms hCAIX and hCAXII than the off-target hCAII. The 2,6-dimethylphenyl analog **ThioUR14** showed high activity against hCAXII with a Ki of 9.5 nM, resulting about 20-fold more selective against hCAIX and about 10-fold more selective against hCAXII as compared with the inhibitory activity against the off-target isoform hCAII.

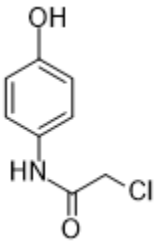
2.7.1 Conclusion

The benzenesulfonamide derivatives **UR1-10** and **ThioUR1-15** inhibited the cytosolic hCAI in the range of 60.6-363.6 nM. These compounds displayed high activity against hCAII, being the 3-chlorophenyl derivative **UR10** the best compound of the series (Ki 5.1 nM). Speaking about hCAIX, the presence at 4-position of fluorine atom (**UR6** Ki 2.1 nM) or methyl group (**UR7** Ki 8.1 nM) gave the most potent inhibitors. The 4-methoxybenzyl derivative **UR3** displayed the best inhibitory activity on hCAXII (6.4 nM), resulting about 7-fold more selective as compared to both hCAII and hCAIX inhibitory activity. The second series of benzenesulfonamide derivatives **ThioUR1-15** displayed inhibitory activity on hCAI at high nanomolar levels while their activity against hCAII resulted in the 5.7-89.6 nM range. The cyclohexyl derivative **ThioUR5** showed the best activity against both hCAII and hCAIX with Ki 5.7 and 1.7 nM respectively. Compounds **ThioUR9** and **ThioUR14** showed interesting inhibitory profile and selectivity against both the cancer related isoform as compared to hCAII inhibitory activity.

2.8 Experimental

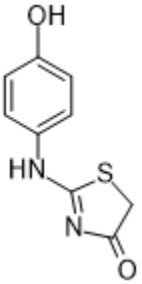
All commercially available solvents and reagents were used without further purification. ^{1}H NMR spectra were recorded on an Inova 500 spectrometer (Varian, Palo Alto, CA, USA). The chemical shifts (δ) are reported in part per million downfield from tetramethylsilane (TMS), which was used as internal standard. The spectra were recorded in hexadeuteriodimethylsulphoxide (DMSO-d_6). Infrared spectra were recorded on a Vector 22 spectrometer (Bruker, Bremen, Germany) in Nujol mulls. The main bands are given in cm^{-1}. Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing MAT 95 instrument (Finnigan, Waltham, MA, USA) with BE geometry. Melting points (mp) were determined with a SMP1 Melting Point apparatus (Stuart Scientific, Stone, UK) and are uncorrected. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. The purity of the tested compounds was determined by combustion elemental analyses conducted by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara with a MT-5 CHN recorder elemental analyser (Yanagimoto, Kyoto, Japan) and the values found were within 0.4% of theoretical values.

2-Chloro-N-(4-hydroxyphenyl)acetamide (2)



To a solution of 4-aminophenol (1) (50 mmol, 5.45g) in AcOH (25 mL), a saturated AcONa solution AcOH (25 mL) was added. The mixture was cooled to 0°C then chloroacetylchloride (100 mmol, 7.9 mL) was added dropwise with continuous stirring for half an hour. The formed solid was filtered and washed with 50% aqueous acetic acid and water. The crude compound was purified by crystallization from EtOH[115].

2-((4-Hydroxyphenyl)amino)thiazol-4(5*H*)-one (3)

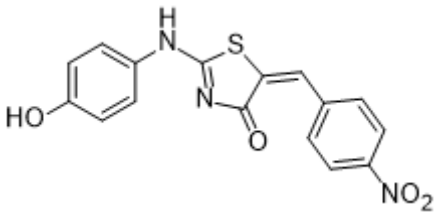


A mixture of 2-chloro-N-(4-hydroxyphenyl)acetamide (2) (1.19 g, 10 mmol) and ammonium thiocyanate (0.84 g, 11 mmol in EtOH (5 mL) was heated under reflux for 4 h. After cooling, the

obtained precipitate was filtered off and washed with water[97]. ^{1}H NMR (DMSO-d_6): δ 3.90-3.96 (s, 2H, CH_2), 6.76 (d, *J* = 8.5 Hz, 2H, Ar), 6.92, (d, *J* = 7.5 Hz, 2H, Ar), 9.42-9.45 (s, 1H, OH), 11.42 (s, 1H, NH). IR (Nujol) 3138, 1661, 1603, 1574 cm^{-1}. Elemental analysis: calculated for $C_9H_8N_2O_2S$ (208.03) %C 51.91, %H 3.87, %N 13.45, found %C 51.95, %H 3.86, %N 13.48. M/z 209.

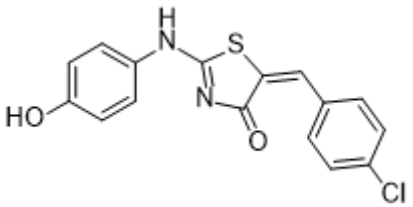
General procedure for the synthesis of 5-arylidene-2- arylaminothiazol-4(5*H*)-ones (TioS 1-8). A mixture of 2-((4-hydroxyphenyl)amino)thiazol-4(5*H*)-one (3) (0.42 g, 2 mmol), the appropriate aldehyde (2 mmol), dry AcONa (1.6 g, 2 mmol) in AcOH (5 mL) was heated under reflux for 12 h. The formed precipitate was filtered in vacuo, washed with water and diethyl ether (Et_2O) to afford the title compounds **TioS 1-8**.

2-((4-hydroxyphenyl)amino)-5-(4-nitrobenzylidene)thiazol-4(5*H*)-one (TioS1)



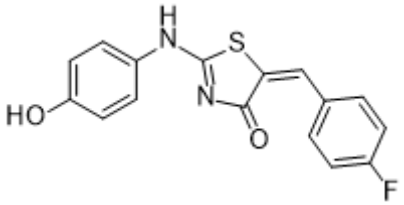
Following the general procedure, the title compound was prepared starting from 4-nitrobenzaldehyde. Yield: 65%, M.p. > 250 °C. ^{1}H NMR (DMSO-d_6): δ 6.82-6.83 (d, 2H, *J* = 6.5 Hz, Ar), 6.97-7.58 (d, 2H, *J* = 8.5 Hz, Ar), 7.78-7.87 (d, 2H, *J* = 8.0 Hz, Ar), 8.30-8.37 (d, *J* = 8.0 Hz, 2H, Ar), 7.70-7.72 (s, 1H, CH), 9.53-9.57 (s, 1H, OH), 11.90-12.15 (s, 1H, NH). IR (Nujol) 3249, 1604, 1519 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{11}N_3O_4S$ (341.34) %C 56.30, %H 3.25, %N 12.31, found %C 56.26, % H 3.26, %N 12.28. M/z 342.

5-(4-Chlorobenzylidene)-2-(4-hydroxyphenylamino)thiazol-4(5*H*)-one (TioS2)



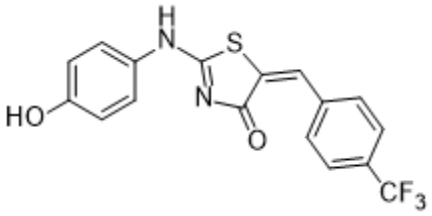
Following the general procedure, the title compound was prepared starting from 4-chlorobenzaldehyde Yield: 66%. mp > 250 °C[94]. ^{1}H NMR (DMSO-d_6): δ 6.80-6.81 (d, 2H, *J* = 8.1 Hz, Ar), 6.97-7.58 (d, 4H, *J* = 8.0 Hz, Ar), 7.71-7.77 (s, 1H, CH), 7.80-7.83 (d, 2H, *J* = 8.1 Hz, Ar), 9.56-9.62 (s, 1H, OH), 11.58-12.17 (s, 1H, NH). IR (Nujol) 3233, 1670, 1621, 1601 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{11}ClN_2O_2S$ (330.79) %C 58.09, %H 3.35, %N 8.47, found %C 58.05, %H 3.36, %N 8.44. M/z 331.

5-(4-Fluorobenzylidene)-2-((4-hydroxyphenyl)amino)thiazol- 4(5*H*)-one (TioS3)



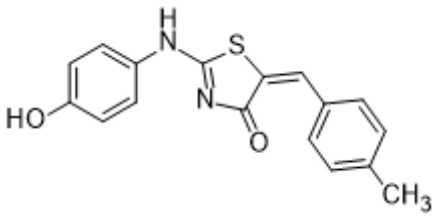
Following the general procedure, the title compound was prepared starting from 4-fluorobenzaldehyde. Yield: 60% M.p. > 250 °C[94]. ^{1}H NMR (DMSO-d_6): δ 6.82 (m, 2H, Ar), 6.94-7.58 (s, 1H, CH), 7.33-7.40 (t, *J* = 8.0 Hz, 1H, Ar), 7.39 (d, *J* = 8.0 Hz, 1H, Ar), 7.60 (d, *J* = 8.0 H z, 2H, Ar), 7.68 (d, *J* = 8.5 Hz, 2H, Ar), 9.64-9.66 (s, 1H, OH), 12.14 (s, 1H, NH). IR (Nujol) 3200, 1655, 1620, 1574 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{11}FN_2O_2S$ (314.33) %C 61.14, %H 3.53, %N 8.91, found %C 61.09, %H 3.54, %N 8.94. M/z 315.

2-((4-Hydroxyphenyl)amino)-5-(4-(trifluoromethyl)benzylidene)thiazol-4(5*H*)-one (TioS4)



Following the general procedure, the title compound was prepared starting from 4-trifluoromethylbenzaldehyde. Yield: 73%. M.p. > 250 °C. ^{1}H NMR (DMSO-d6): δ 6.83 (m, 2H, Ar), 6.85 (s, 1H, CH), 7.65 (d, *J* = 7.5 Hz, 2H, Ar), 7.70 (m, 1H, Ar), 7.85 (m, 3H, Ar), 9.64 (s, 1H, OH), 12.11 (s, 1H, NH). IR (Nujol) 3249, 1673, 1601, 1548 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{11}F_3N_2O_2S$ (364.34) %C 56.04, %H 3.04, %N 7.69, found %C 55.98, %H 3.03, %N 7.73. M/z 365.

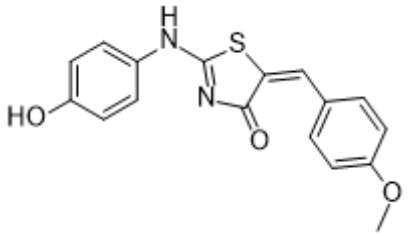
2-((4-Hydroxyphenyl)amino)-5-(4-methylbenzylidene)thiazol-4(5*H*)-one (TioS5)



Following the general procedure, the title compound was prepared starting from 4-methylbenzaldehyde. Yield: 57%. M.p. > 250 °C. ^{1}H NMR (DMSO-d_6): δ 2.33-2.38 (s, 3H, CH_3), 6.84 (d, *J* = 8.5 Hz, 2H, Ar), 6.96-7.59 (s, 1H, CH), 7.30 (d, *J* = 6.5 Hz, 1H, Ar), 7.36 (d, *J* = 7.5 Hz, 1H, Ar), 7.42 (d, *J* = 7.5 Hz, 1H, Ar), 7.51 (d, J = 7.0 Hz, 1H, Ar), 7.67 (d, *J* = 8.5 Hz, 1H, Ar), 7.61 (m, 1H, Ar), 9.65 (s, 1H, OH), 12.12 (s, 1H, NH). IR (Nujol) 3200, 1664, 1574 cm^{-1}.

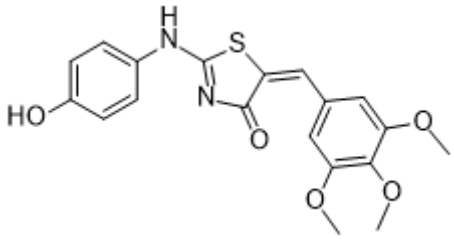
Elemental analysis: calculated for $C_{17}H_{14}N_3O_2S$ (310.37) %C 65.79, %H 4.55, %N 9.03, found %C 65.71, %H 4.53, %N 9.00. M/z 311.

2-((4-Hydroxyphenyl)amino)-5-(4-methoxybenzylidene)thiazol-4(5*H*)-one (TioS6)



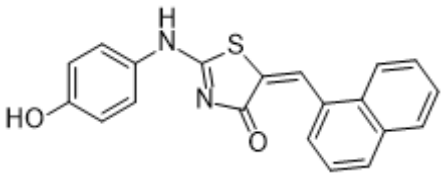
Following the general procedure, the title compound was prepared starting from 4-methoxylbenzaldehyde. Yield: 56%. M.p. > 250 °C ^{1}H NMR (DMSO-d_6): δ 3.79-3.84 (s, 3H, OCH_3) 6.82 (m, 2H, Ar), 6.96 (s, 1H, CH), 7.06 (d, *J* = 8.0 Hz, 2H, Ar), 7.12 (d, *J* = 8.5 Hz, 1H, Ar), 7.49 (d, *J* = 8.5 Hz, 1H, Ar), 7.58-7.61 (d, *J* = 7.5 Hz, 2H, Ar), 9.56 (s, 1H, OH), 11.87 (s, 1H, NH). IR (Nujol) 3187, 1651, 1620, 1574 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{14}N_2O_3S$ (326.37) %C 62.56, %H 4.32, %N 8.58, found %C 62.61, %H 4.34, %N 8.55. M/z 327.

2-((4-Hydroxyphenyl)amino)-5-(3,4,5-trimethoxybenzylidene)thiazol-4(5*H*)-one (TioS7)



Following the general procedure, the title compound was prepared starting from 3,4,5-trimethoxylbenzaldehyde. Yield: 54%. mp > 250 °C. ^{1}H NMR (DMSO-d_6): δ 3.73, 3.74, 3.76, 3.77, 3.85, 3.86 (s, 9H, OCH_3), 6.81-7.52 (s, 1H, CH), 6.84 (d, *J* = 7.5 Hz, 2H, Ar), 6.95 (d, *J* = 7.5 Hz, 2H, Ar), 7.59 (s, 2H, Ar), 9.54-9.59 (s, 1H, OH), 11.91 (s, 1H, NH). IR (Nujol) 3200, 1664, 1598 cm^{-1}. Elemental analysis: calculated for $C_{19}H_{18}N_2O_5S$ (386.42) %C 59.06, %H 4.70, %N 7.25, found %C 59.11, %H 4.68, %N 7.25. M/z 387.

2-((4-Hydroxyphenyl)amino)-5-(naphthalen-1-ylmethylene)thiazol-4(5*H*)-one (TioS8)



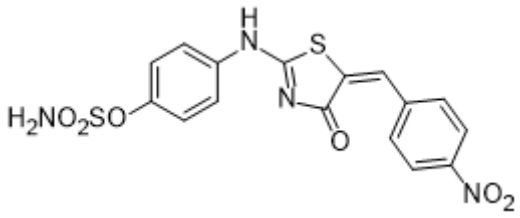
Following the general procedure, the title compound was prepared starting from 3,4,5-napfthaldehyde. Yield: 56%. mp > 250 °C. ^{1}H NMR (DMSO-d_6): δ 6.80 (m, 4H, Ar and CH), 7.66 (m, 5H, Ar), 7.96 (m, 1H, Ar), 8.11 (m, 2H, Ar), 9.53 (s, 1H, OH), 12.09 (s, 1H, NH). IR (Nujol)

3424, 3226, 3058, 1688, 1666, 1615, 1597 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{14}N_2O_2S$ (346.40) %C 69.35; %H 4.07; %N 8.09. Found %C 69.41; %H 4.09; %N 8.06. M/z 347.

General procedure for the preparation of 5-((arylidene-4-oxo- 4,5-dihydrothiazol-2-yl)amino)phenyl sulfamates (TioS 9-16)

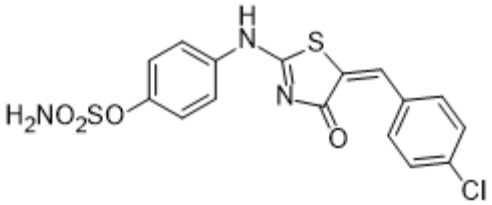
To a stirred solution of 5-arylidene-2-((4-hydroxyphenyl)amino)thiazol-4(5*H*)-one (1 mmol) in anhydrous DMA (10 mL), freshly prepared sulfamoyl chloride (0.81 g, 7 mmol) in dry DMA (5 mL) was added dropwise in 30 min. The obtained mixture was stirred at room temperature overnight, then water (30 mL) was added. The mixture was stirred for additional 2 h, then the formed precipitate was filtered off, washed with water and dried.

4-((5-(4-Nitrobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)amino)phenylsulfamate (TioS9)



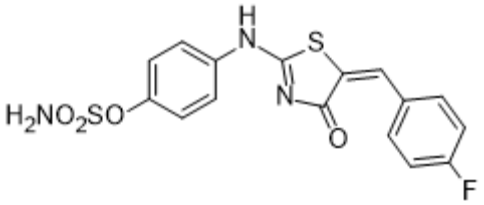
Following the general procedure, the title compound was prepared starting from 2-((4-hydroxyphenyl)amino)-5-(4-nitrobenzylidene)thiazol-4(5*H*)-one. Yield: 41% M.p. > 250 °C. ^{1}H NMR: (DMSO-d_6): δ 6.83 (d, 2H, *J* = 6.5 Hz, Ar), 6.94-7.59 (d, 2H, *J* = 8.5 Hz, Ar), 7.60 (s, 1H, CH), 7.78-7.86 (d, 2H, *J* = 8.0 Hz, Ar), 8.04 (s, 2H, NH_2), 8.30-8.38 (d, *J* = 8.0 Hz, 2H, Ar), 11.60 (s, 1H, NH). IR (Nujol) 3404, 3320, 3254, 3214, 1766, 1671, 1610 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}N_4O_6S_2$ (420.42) %C 45.71, %H 2.88, %N 13.33, found %C 45.66, %H 2.87, %N 13.37. M/z 421.

4-((5-(4-Chlorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)amino)phenylsulfamate (TioS10)



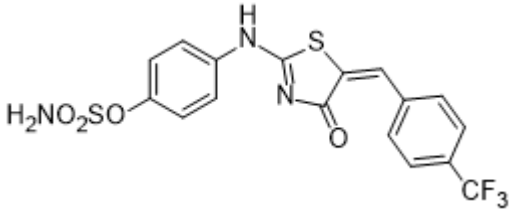
Following the general procedure, the title compound was prepared starting from 5-(4-chlorobenzylidene)-2-(4-hydroxyphenylamino)thiazol-4(5*H*)-one. Yield: 22% M.p. > 250 °C. ^{1}H NMR (DMSO-d_6): δ 6.80-6.81 (d, *J* = 8.1 Hz, 2H, Ar), 6.97-7.58 (d, *J* = 8.0 Hz, 4H, Ar), 7.72-7.79 (s, 1H, CH), 7.83-7.85 (d, *J* = 8.0 Hz, 2H, Ar), 8.02 (s, 2H, NH_2), 11.52, 11.76 (s, 1H, NH). IR (Nujol) 3200, 1724, 1669, 1640 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}ClN_3O_4S_2$ (409.87) %C 46.89, %H 2.95, %N 10.25, found %C 46.93, %H 2.96, %N 10.21. M/z 410.

4-((5-(4-Fluorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)amino)phenylsulfamate (TioS11)



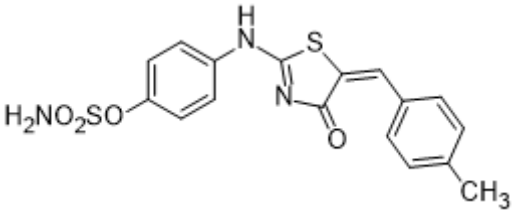
Following the general procedure, the title compound was prepared starting from 5-(4-fluorobenzylidene)-2-((4-hydroxyphenyl)amino)thiazol-4(5*H*)-one. Yield: 20% M.p. 233–235 °C. ^{1}H NMR (DMSO-d_6): δ 6.82 (d, *J* = 7.0 Hz, 2H, Ar), 6.96-7.59 (s, 1H, CH), 7.32-7.40 (d, *J* = 8.5 Hz, 2H, Ar), 7.45-7.73 (m, 4H, Ar), 8.02 (s, 2H, NH_2), 11.42 (s, 1H, NH). IR (Nujol) 3423, 3230, 1675, 1638 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}FN_3O_4S_2$ (393.41) %C 48.85, %H 3.07, %N 10.68, found %C 48.80, %H 3.08, %N 10.70. M/z 394.

4-((4-Oxo-5-(4-(trifluoromethyl)benzylidene)-4,5-dihydrothiazol-2-yl)amino)phenylsulfamate (TioS12)



Following the general procedure, the title compound was prepared starting from 2-((4-hydroxyphenyl)amino)-5-(4-(trifluoromethyl)benzylidene)thiazol-4(5*H*)-one. Yield: 39% M.p. > 250 °C. ^{1}H NMR (DMSO-d_6): δ 6.80 (m, 2H, Ar), 6.83-7.68 (s, 1H, CH), 7.58 (d, *J* = 7.5 Hz, 2H, Ar), 7.73 (d, *J* = 8.0 Hz, 2H, Ar), 7.90 (d, *J* = 7.5 Hz, 2H, Ar), 8.02 (s, 2H, NH_2), 11.54 (s, 1H, NH). IR (Nujol) 3378, 3217, 1673, 1637, 1580 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{12}F_3N_4O_2S_2$ (443.42) %C 46.05, %H 2.73, %N 9.48, found %C 46.10, %H 2.72, %N 9.45. M/z 444.

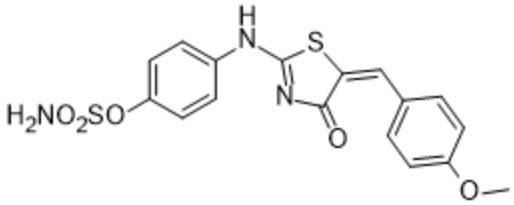
4-((5-(4-Methylbenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)amino)phenylsulfamate (TioS13)



Following the general procedure, the title compound was prepared starting from 2-((4-Hydroxyphenyl)amino)-5-(4-methylbenzylidene)thiazol-4(5*H*)-one. Yield: 56% M.p. 198–200 °C. ^{1}H NMR (DMSO-d_6): δ 2.32-2.37 (s, 3H, CH_3), 6.81 (d, *J* = 8.5 Hz, 2H, Ar), 6.95-7.59 (s, 1H, CH), 7.29 (d, *J* = 7.5 Hz, 1H, Ar), 7.36 (d, *J* = 6.5 Hz, 1H, Ar), 7.41 (d, *J* = 7.5 Hz, 1H, Ar), 7.50 (d, *J* = 7.5 Hz, 1H, Ar), 7.65 (d, *J* = 8.5 Hz, 1H, Ar), 7.65 (m, 1H, Ar), 8.02 (s, 2H, NH_2), 11.60 (s, 1H,

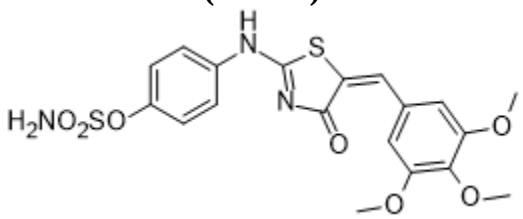
NH). IR (Nujol) 3418, 3232, 1675, 1643 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}N_3O_4S_2$ (389.45) %C 52.43, %H 3.88, %N 10.79, found %C 52.37, %H 3.89, %N 10.82. M/z 390.

4-((5-(4-Methoxybenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)amino)phenylsulfamate (TioS14)



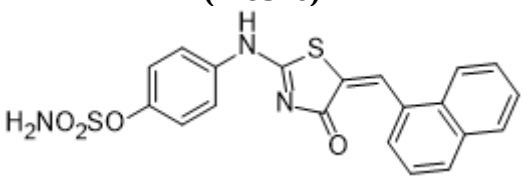
Following the general procedure, the title compound was prepared starting from 2-((4-hydroxyphenyl)amino)-5-(4-methoxybenzylidene)thiazol-4(5*H*)-one. Yield: 35% M.p. > 250 °C. ^{1}H NMR (DMSO-d_6): δ 3.79-3.83 (s, 3H, OCH_3), 6.81 (d, *J* = 8.0 Hz, 2H, Ar), 6.95 (d, *J* = 7.0 Hz, 2H, Ar), 7.12-7.57 (s, 1H, CH), 7.32-7.47 (d, *J* = 7.5 Hz, 4H, Ar), 8.02 (s, 2H, NH_2), 11.70 (s, 1H, NH). IR (Nujol) 3415, 1674, 1645 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}N_3O_3S_2$ (405.45) %C 50.36, %H 3.73, %N 10.36, found %C 50.41, %H 3.72, %N 10.38. M/z 406.

4-((4-Oxo-5-(3,4,5-trimethoxybenzylidene)-4,5-dihydrothiazol-2-yl)amino)phenylsulfamate (TioS15)



Following the general procedure, the title compound was prepared starting from 2-((4-hydroxyphenyl)amino)-5-(3,4,5-trimethoxybenzylidene)thiazol-4(5*H*)-one. Yield: 13%. M.p. 176–177 °C. ^{1}H NMR (DMSO-d_6): δ 3.70, 3.74, 3.75, 3.82, 3.86, 3.92 (s, 9H, OCH_3), 6.84-7.61 (s, 1H, CH), 6.99 (d, *J* = 7.5 Hz, 2H, Ar), 7.30 (d, *J* = 7.5 Hz, 2H, Ar), 7.71 (s, 2H, Ar), 8.00 (s, 2H, NH_2), 11.68 (s, 1H, NH). IR (Nujol) 3420, 3208, 1670, 1633, 1600, 1579 cm^{-1}. Elemental analysis: calculated for $C_{19}H_{19}N_3O_7S_2$ (465.50) %C 49.02, %H 4.11, %N 9.03, found %C 48.97, %H 4.12, %N 9.07. M/z 466.

4-((5-(Naphthalen-1-ylmethylene)-4-oxo-4,5-dihydrothiazol-2-yl)amino)phenyl sulfamate (TioS16)



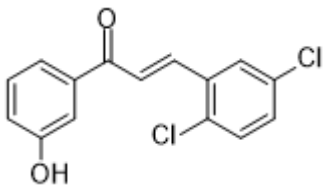
Following the general procedure, the title compound was prepared starting from 2-((4-hydroxyphenyl)amino)-5-(naphthalen-1-ylmethylene)thiazol-4(5*H*)-one. Yield: 24%. M.p. 168–169 °C. ^{1}H NMR (DMSO-d_6): δ 6.77–7.37 (m, 4H, Ar and CH), 7.61–7.78 (m, 6H, Ar), 8.00 (s, 2H, NH_2), 8.26 (m, 2H, Ar), 11.70 (s, 1H, NH). IR (Nujol) 3367, 3230, 1686, 1634, 1574 cm^{-1}.

Elemental analysis: calculated for $C_{20}H_{15}N_3O_4S_2$ (425.05) %C 56.46, %H 3.55, %N 9.88, found %C 56.51, %H 3.57, %N 9.85. M/z 426.

General procedure for the preparation of chalcones 7a-m, 8a-m, 14a-g, 15a-g.

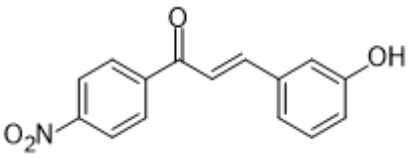
To a solution of substituted acetophenone (4 mmol) in MeOH (10 mL) 50% NaOH solution (1.6 mL) was added. The resulting mixture was stirred rt for 10 minutes than substituted benzaldehyde was added. The mixture was stirred rt and monitored by TLC, then the methanol was removed under reduced pressure and the resulting water solution was neutralized with HCl 1N. The formed solid was filtered off, washed with water, dried and used in the next step without further purification. Chalcones **7a**[116], **7b**[117], **7c**[118], **7d**[119], **7e**[119], **7f**[116], **7g**[120], **7h**[121], **7i**[118], **7j**[117], **7k**[122], **7l**[116], **7m**[123], **8a**[123], **8b**[118], **8c**[124], **8d**[116], **8e**[116], **8f**[125], **8g**[121], **8h**[119], **8i**[125], **8j**[126], **8l**[125], **8m**[122], **14a**[121], **14b**[121], **14c**[127], **14d**[119], **14e**[121], **14f**[128], **14g**[129], **15a**[127], **15b**[118], **15c**[118] and **15e**[118] have been prepared as previously described.

(*E*)-3-(2,5-Dichlorophenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one (8k)



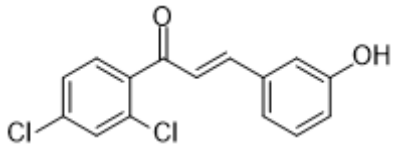
Following the general procedure, the title compound was prepared starting from 2,4-dichloro benzaldehyde. Yield 72% M.p. 56-57 °C. ^{1}H NMR (DMSO-d_6): δ 7.22 (d, *J* = 14.5 Hz, 1H, CH), 7.26 (d, *J* = 7.0 Hz , 1H, Ar), 7.28 (m, 2H, Ar), 7.30 (d, *J* = 7.5 Hz, 1H, Ar), 7.36 (d, *J* = 15.0, 1H, CH), 7.41 (m, 3H, Ar), 9.24 (s, 1H, OH). IR (Nujol) 3123, 1689, 1572 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{10}Cl_2O_2$ (292.01) %C 61.46, %H 3.44, found %C 61.50, %H 3.41. M/z 293.

(*E*)-3-(3-Hydroxyphenyl)-1-(4-nitrophenyl)prop-2-en-1-one (15d)



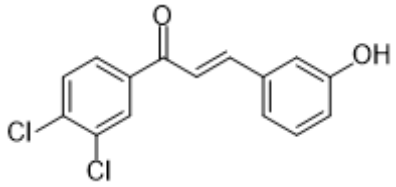
Following the general procedure, the title compound was prepared starting from 4-nitroacetophenone. Yield 56% M.p. 71-72 °C. ^{1}H NMR (DMSO-d_6): δ 7.16 (d, *J* = 15.5 Hz, 1H, CH), 7.23 (d, *J* = 7.5 Hz, 1H, Ar), 7.28 (d, *J* = 8.0 Hz, 2H, Ar), 7.32 (d, *J* = 6.5 Hz, 1H, Ar), 7.38 (d, *J* = 15.0 Hz, 1H, CH), 7.52 (d, *J* = 8.5 Hz, 2H, Ar), 7.77 (m, 1H, Ar), 9.32 (s, 1H, OH). IR (Nujol) 3111, 1679, 1554 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{11}NO_4$ (269.25) %C 66.91, %H 4.12, %N 5.20 found %C 66.96, %H 4.10, %N 5.26. M/z 270.

(*E*)-1-(2,4-Dichlorophenyl)-3-(3-hydroxyphenyl)prop-2-en-1-one (15f)



Following the general procedure, the title compound was prepared starting from 2,4-dichloroacetophenone. Yield 74% M.p. 48-50 °C. ^{1}H NMR (DMSO-d_6): δ 6.96 (m, 1H, Ar), 7.12 (d, *J* = 15.5 Hz, 1H, CH), 7.20 (d, *J* = 8.5 Hz, 1H, Ar), 7.25 (m, 1H, Ar), 7.29 (d, *J* = 7.5 Hz, 1H, Ar), 7.33 (d, *J* = 14.0 Hz, 1H, CH), 7.58 (m, 3H, Ar), 9.27 (s, 1H, OH). IR (Nujol) 3118, 1620, 1547 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{10}Cl_2O_2$ (292.01) %C 61.46, %H 3.44, found %C 61.51, %H 3.42. M/z 293.

(*E*)-1-(3,4-Dichlorophenyl)-3-(3-hydroxyphenyl)prop-2-en-1-one (15g)

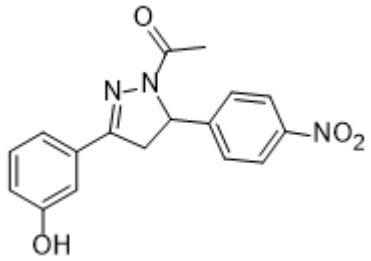


Following the general procedure, the title compound was prepared starting from 3,4-dichloroacetophenone. Yield 53% M.p. 60-62°C. ^{1}H NMR (DMSO-d_6): δ 7.03 (m, 2H, Ar), 7.16 (d, *J* = 15.0 Hz, 1H, CH), 7.19 (m, 1H, Ar), 7.22 (m, 1H, Ar), 7.39 (d, *J* = 15.5 Hz, 1H, CH), 7.89 (m, 3H, Ar), 9.33 (s, 1H, OH). IR (Nujol) 3203, 1687, 1553 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{10}Cl_2O_2$ (292.01) %C 61.46, %H 3.44, found %C 61.51, %H 3.46. M/z 293.

General procedure for the preparation of 1-acetyl-3,5-diaryl-4,5-dihydro-1*H*-pyrazoles.

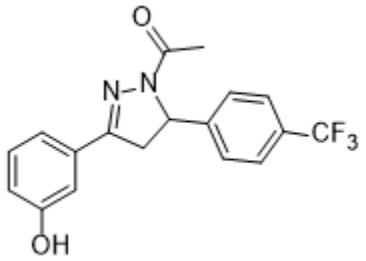
To a solution of chalcone derivative (1 mmol) in AcOH (3 mL) hydrazine hydrate (0.3 mL, 6 mmol) was added. The mixture was refluxed under stirring for 3 h, and then poured onto crushed ice. The formed precipitate was filtered off, washed with cold water, and crystallized from MeOH to give the titled pyrazolines **9a-m**, **10a-m**, **16a-g**, **17a-g**. Pyrazolines **9a-c**[130], **9d-h**[131], **9i**[130], **9j-l**[130], **9m**[131], **10a-c**[132], **10g**[132], **10l**[130], **16a-c**[131], **16e**[132], **17a**[130] have been prepared as previously described.

1-(3-(3-Hydroxyphenyl)-5-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (10d)



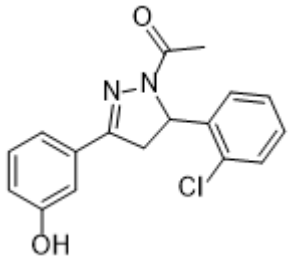
Following the general procedure, the title compound was prepared starting from (*E*)-3-(4-nitrophenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one. Yield 88% M.p. 172-173 °C. ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3), 3.13 (dd, *J* = 3.5, 13.0 Hz, 1H), 3.87 (dd, *J* = 13.5, 3.5 Hz, CH), 5.66 (dd, *J* = 14.5, 3.0 Hz, 1H), 6.87 (d, *J* = 8.5 Hz, 1H, Ar), 7.18 (d, *J* = 8.5 Hz, 1H, Ar), 7.23 (m, 1H, Ar), 7.28 (s, 1H, Ar), 7.49 (d, *J* = 7.0 Hz, 2H, Ar), 7.57 (d, *J* = 7.0 Hz, 2H, Ar), 8.21 (s, 1H, OH). IR (Nujol) 3236, 1633 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}N_3O_4$ (325.32) %C 62.76, %H 4.65, %N 12.92, found %C 62.80, %H 4.67, %N 12.88. M/z 326.

1-(3-(3-Hydroxyphenyl)-5-(4-(trifluoromethyl)phenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (10e)



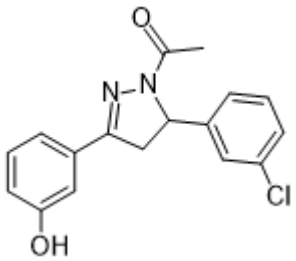
Following the general procedure, the title compound was prepared starting from (*E*)-3-(4-trifluoromethylphenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one. Yield 76% M.p. 104-105°C. ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3), 2.89 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.85 (dd, *J* = 14.5, 3.5 Hz, CH), 5.63 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.63 (d, *J* = 7.5 Hz, 1H, Ar), 6.88 (d, *J* = 7.0 Hz, 1H, Ar), 7.18 (m, 1H, Ar), 7.48 (d, *J* = 8.5 Hz, 2H, Ar), 7.89 (d, *J* = 8.0 Hz, 2H, Ar), 8.16 (s, 1H, Ar), 9.67 (s, 1H, OH). IR (Nujol) 3227, 1635 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{15}F_3N_2O_2$ (348.32) %C 62.07, %H 4.34, %N 8.04, found %C 62.12, %H 4.32, %N 8.09. M/z 349.

1-(5-(2-Chlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (10g)



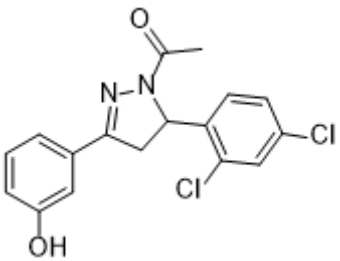
Following the general procedure, the title compound was prepared starting from (*E*)-3-(2-chlorophenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one. Yield 84% M.p. 214-215 °C. ^{1}H NMR (DMSO-d_6): δ 2.36 (s, 3H, CH_3), 3.01 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.94 (dd, *J* = 14.5, 3.5 Hz, CH), 5.76 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.87 (d, *J* = 7.5 Hz, 1H, Ar), 7.07 (d, *J* = 7.0 Hz, 1H, Ar), 7.16 (m, 1H, Ar), 7.23 (d, *J* = 7.5 Hz, 2H, Ar), 7.31 (d, *J* = 8.0 Hz, 2H, Ar), 7.49 (s, 1H, Ar), 9.64 (s, 1H, OH). IR (Nujol) 3231, 1643, 1573 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}ClN_2O_2$ (314.77) %C 64.87, %H 4.80, %N 8.90, found %C 64.82, %H 4.82, %N 8.94. M/z 315.

1-(5-(3-Chlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (10h)



Following the general procedure, the title compound was prepared starting from (*E*)-3-(3-chlorophenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one. Yield 92% M.p. 128-129 °C. ^{1}H NMR (DMSO-d_6): δ 2.31 (s, 3H, CH_3), 3.32 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.84 (dd, *J* = 14.5, 3.5 Hz, CH), 5.54 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.87 (d, *J* = 7.5 Hz, 1H, Ar), 7.18 (d, *J* = 7.0 Hz, 1H, Ar), 7.23 (m, 1H, Ar), 7.27 (d, *J* = 7.5, 2H, Ar), 7.31 (d, *J* = 8.0, 2H, Ar), 7.37 (s, 1H, Ar), 9.66 (s, 1H, OH). IR (Nujol) 3172, 1640, 1574 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}ClN_2O_2$ (314.77) %C 64.87, %H 4.80, %N 8.90, found %C 64.91, %H 4.82, %N 8.95. M/z 315.

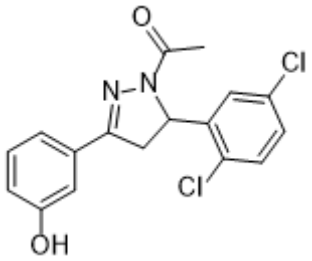
1-(5-(2,4-Dichlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (10j)



Following the general procedure, the title compound was prepared starting from (*E*)-3-(2,4-dichlorophenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one. Yield 92% M.p. 163-164 °C. ^{1}H NMR

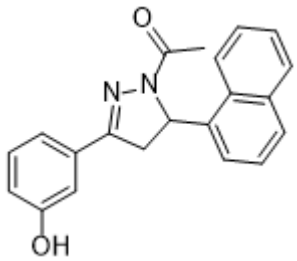
(DMSO-d_6): δ 2.34 (s, 3H, CH_3), 3.02 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.90 (dd, *J* = 13.5, 3.5 Hz, CH), 5.71 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.87 (d, *J* = 7.5 Hz, 1H, Ar), 7.08 (d, *J* = 7.0 Hz, 1H, Ar), 7.22 (m, 2H, Ar), 7.66 (m, 2H, Ar), 7.72 (s, 1H, Ar), 9.65 (s, 1H, OH). IR (Nujol) 3264, 1644, 1574 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{14}Cl_2N_2O_2$ (349.21) %C 58.47, %H 4.04, %N 8.02, found %C 58.50, %H 4.02, %N 8.06. M/z 349.

1-(5-(2,5-Dichlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (10k)



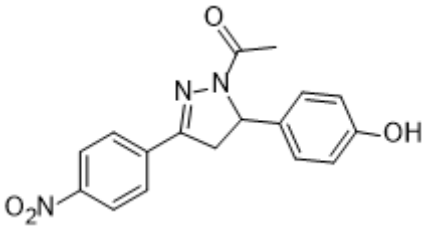
Following the general procedure, the title compound was prepared starting from (*E*)-3-(2,5-dichlorophenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one. Yield 91% M.p. 233-234 °C. ^{1}H NMR (DMSO-d_6): δ 2.35 (s, 3H, CH_3), 3.25 (dd, *J* = 4.0, 14.0 Hz, 1H), 4.07 (dd, *J* = 13.5, 3.5 Hz, CH), 5.68 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.93 (d, *J* = 7.5 Hz, 1H, Ar), 7.35 (d, *J* = 7.0 Hz, 1H, Ar), 7.56 (m, 2H, Ar), 7.73 (m, 2H, Ar), 8.06 (s, 1H, Ar), 10.10 (s, 1H, OH). IR (Nujol) 3320, 1636, 1571 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{14}Cl_2N_2O_2$ (349.21) %C 58.47, %H 4.04, %N 8.02, found %C 58.41, %H 4.06, %N 7.97. M/z 350.

1-(3-(3-Hydroxyphenyl)-5-(naphthalen-1-yl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (10m)



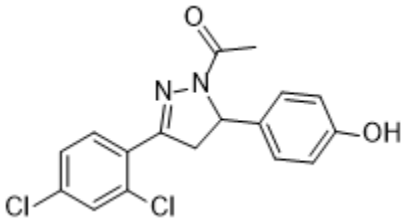
Following the general procedure, the title compound was prepared starting from (*E*)-1-(3-hydroxyphenyl)-3-(naphthalen-2-yl)prop-2-en-1-one. Yield 71% M.p. 181-182 °C. ^{1}H NMR (DMSO-d_6): δ 2.44 (s, 3H, CH_3), 2.98 (dd, *J* = 4.0, 14.0 Hz, 1H), 4.22 (dd, *J* = 13.5, 4 Hz, CH), 6.17 (dd, *J* = 13.0, 3.5 Hz, 1H), 6.62 (d, *J* = 8.0 Hz, 2H, Ar), 7.08 (d, *J* = 8.5 Hz, 2H, Ar), 7.62 (m, 3H, Ar), 7.70 (m, 2H, Ar), 7.92 (m, 2H, Ar), 8.15 (s, 1H, Ar), 9.42 (s, 1H, OH). IR (Nujol) 3212, 1643 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{18}N_2O_4$ (330.38) %C 76.34, %H 5.49, %N 8.48, found %C 76.29, %H 5.48, %N 8.52. M/z 331.

1-(5-(4-Hydroxyphenyl)-3-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (16d)



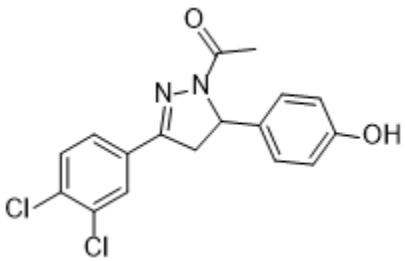
Following the general procedure, the title compound was prepared starting from (*E*)-3-(4-hydroxyphenyl)-1-(4-nitro)prop-2-en-1-one. Yield 41% M.p. 131-132 °C. ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, CH_3), 3.11 (dd, *J* = 3.0, 13.0 Hz, 1H), 3.88 (dd, *J* = 14.0, 4.0 Hz, CH), 5.61 (dd, *J* = 13.0, 3.5 Hz, 1H), 6.77 (d, *J* = 8.5 Hz, 2H, Ar), 7.14 (d, *J* = 8.0 Hz, 2H, Ar), 8.22 (d, *J* = 7.0 Hz, 2H, Ar), 8.30 (d, *J* = 7.5 Hz, 2H, Ar), 9.66 (s, 1H, OH). IR (Nujol) 3259, 1702, 1606cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}N_3O_4$ (325.32) %C 62.76, %H 4.65, %N 12.92, found %C 62.80, %H 4.63, %N 12.96. M/z 326.

1-(3-(2,4-Dichlorophenyl)-5-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (16f)



Following the general procedure, the title compound was prepared starting from (*E*)-3-(4-hydroxyphenyl)-1-(2,4-dichloro)prop-2-en-1-one. Yield 62% M.p. 128-129 °C. ^{1}H NMR ^{1}H NMR (DMSO-d_6): δ 22.28 (s, 3H, CH_3), 3.13 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.81 (s, 2H, NH_2), 3.86 (dd, *J* = 13.5, 3.5 Hz, CH), 5.44 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.73 (d, *J* = 8.5 Hz, 2H, Ar), 7.02 (d, *J* = 8.0 Hz, 2H, Ar), 7.61 (d, *J* = 7.5 Hz, 1H, Ar), 7.80 (d, *J* = 8.0 Hz, 1H, Ar), 7.81 (s, 1H, Ar), 9.35 (s, 1H, OH). IR (Nujol) 3318, 1645, 1587 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{14}Cl_2N_2O_2$ (349.21) %C 58.47, %H 4.04, %N 8.02, found %C 58.52, %H 4.05, %N 7.97. M/z 350.

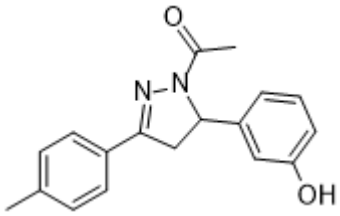
1-(3-(3,4-Dichlorophenyl)-5-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (16g)



Following the general procedure, the title compound was prepared starting from (*E*)-3-(4-hydroxyphenyl)-1-(3,4-dichloro)prop-2-en-1-one. Yield 84% M.p. 123-124 °C. ^{1}H NMR (DMSO-d_6): δ 2.31 (s, 3H, CH_3), 3.09 (dd, *J* = 4.5, 14.0 Hz, 1H), 3.88 (dd, *J* = 13.5, 3.5 Hz, CH), 5.52 (dd, *J*

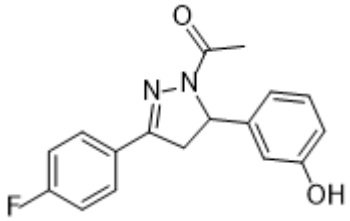
= 13.0, 3.0 Hz, 1H), 6.73 (d, *J* = 8.0 Hz, 2H, Ar), 7.01 (d, *J* = 8.0 Hz, 2H, Ar), 7.76 (d, *J* = 7.5 Hz, 1H, Ar), 7.89 (d, *J* = 8.0 Hz, 1H, Ar), 7.94 (s, 1H, Ar), 9.33 (s, 1H, OH). IR (Nujol) 3250, 1646, 1595cm^{-1}. Elemental analysis: calculated for $C_{17}H_{14}Cl_2N_2O_2$ (349.21) %C 58.47, %H 4.04, %N 8.02, found %C 58.51, %H 4.02, %N 8.07. M/z 350.

1-(5-(3-Hydroxyphenyl)-3-(p-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (17b)



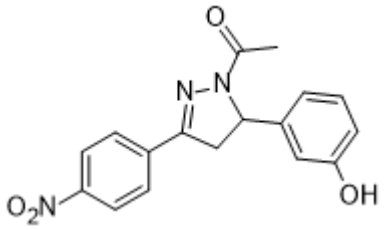
Following the general procedure, the title compound was prepared starting from (*E*)-3-(3-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one. Yield 83% M.p. 100-101 °C. ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 2.80 (dd, *J* = 3.5, 13.0 Hz, 1H), 3.41 (dd, *J* = 13.5, 3.5 Hz, CH), 4.73 (dd, *J* = 14.5, 3.0 Hz, 1H), 6.65 (d, *J* = 8.5 Hz, 1H, Ar), 6.79 (d, *J* = 8.5 Hz, 1H, Ar), 7.20 (m, 1H, Ar), 7.52 (d, *J* = 7.0 Hz, 2H, Ar), 7.68 (d, *J* = 7.0 Hz, 2H, Ar), 9.32 (s, 1H, OH). IR (Nujol) 3328, 1595 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{18}N_2O_2$ (294.35) %C 73.45, %H 6.16, %N 9.52, found %C 73.50, %H 6.18, %N 9.48. M/z 295.

1-(3-(4-Fluorophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (17c)



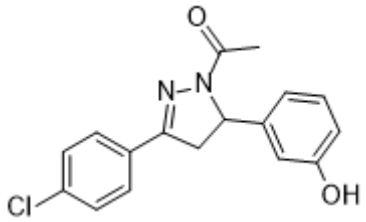
Following the general procedure, the title compound was prepared starting from (*E*)-3-(3-hydroxyphenyl)-1-(4-fluoro)prop-2-en-1-one. Yield 75% M.p. 184-185 °C. ^{1}H NMR (DMSO-d_6): δ 2.31 (s, 3H, CH_3), 3.13 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.82 (dd, *J* = 13.5, 3.5 Hz, CH), 5.45 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.56 (d, *J* = 7.5 Hz, 1H, Ar), 6.63 (d, *J* = 7.0 Hz, 1H, Ar), 7.11 (m, 1H, Ar), 7.32 (d, J = 7.5 Hz, 2H, Ar), 7.83 (m, 2H, Ar), 9.36 (s, 1H, OH). IR (Nujol) 3072, 2783, 1663, 1583cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}FN_2O_2$ (298.31) %C 68.45, %H 5.07, %N 9.39, found %C 68.49, %H 5.05, %N 9.32. M/z 299.

1-(5-(3-Hydroxyphenyl)-3-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (17d)



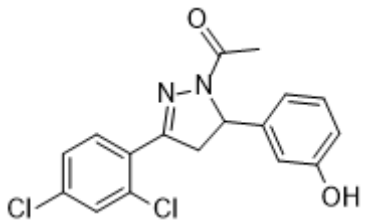
Following the general procedure, the title compound was prepared starting from (*E*)-3-(3-hydroxyphenyl)-1-(4-nitro)prop-2-en-1-one. Yield 71% M.p. 140-141 °C. ^{1}H NMR (DMSO-d_6): δ 2.19 (s, 3H, CH_3), 3.15 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.98 (dd, *J* = 14.5, 3.5 Hz, CH), 5.63 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.89 (d, *J* = 7.5 Hz, 1H, Ar), 7.12 (d, *J* = 7.0 Hz, 1H, Ar), 7.21 (m, 1H, Ar), 7.54 (d, *J* = 8.5 Hz, 2H, Ar), 7.69 (d, *J* = 8.0 Hz, 2H, Ar), 9.33 (s, 1H, OH). IR (Nujol) 3347, 1604, 1522 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}N_3O_4$ (325.32) %C 62.76, %H 4.65, %N 12.92, found %C 62.81, %H 4.64, %N 12.98. M/z 326.

1-(3-(4-Chlorophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (17e)



Following the general procedure, the title compound was prepared starting from (*E*)-3-(3-hydroxyphenyl)-1-(4-chloro)prop-2-en-1-one. Yield 67% M.p. 110-111 °C. ^{1}H NMR (DMSO-d_6): δ 2.22 (s, 3H, CH_3), 3.13 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.96 (dd, *J* = 14.5, 3.5 Hz, CH), 5.01 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.81 (d, *J* = 7.5 Hz, 1H, Ar), 6.98 (d, *J* = 7.0 Hz, 1H, Ar), 7.22 (m, 1H, Ar), 7.46 (d, J = 7.5 Hz, 2H, Ar), 7.58 (d, *J* = 8.0 Hz, 2H, Ar), 9.36 (s, 1H, OH). IR (Nujol) 3322, 1652, 1591cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}ClN_2O_4$ (314.77) %C 64.87, %H 4.80, %N 8.90, found %C 64.81, %H 4.82, %N 8.95. M/z 315.

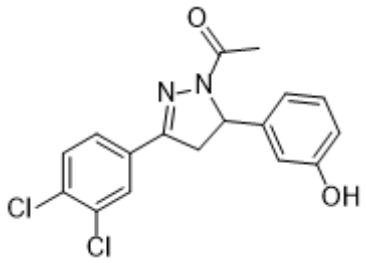
1-(3-(2,4-Dichlorophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (17f)



Following the general procedure, the title compound was prepared starting from (*E*)-3-(3-hydroxyphenyl)-1-(2,4-dichloro)prop-2-en-1-one. Yield 51% M.p. 125-126 °C. ^{1}H NMR (DMSO-d_6): δ 2.31 (s, 3H, CH_3), 3.17 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.97 (dd, *J* = 13.5, 3.5 Hz, CH), 5.03 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.67 (d, *J* = 7.5 Hz, 1H, Ar), 6.91 (d, *J* = 7.0 Hz, 1H, Ar), 7.11 (m, 1H, Ar),

7.19 (d, J = 7.5 Hz, 1H, Ar), 7.53 (d, *J* = 8.0 Hz, 1H, Ar), 7.79 (s, 1H, Ar), 9.12 (s, 1H, OH). IR (Nujol) 3330, 1672, 1585 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{14}Cl_2N_2O_2$ (349.21) %C 58.47, %H 4.04, %N 8.02, found %C 58.41, %H 4.05, %N 7.96. M/z 350.

1-(3-(3,4-Dichlorophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone (17g)

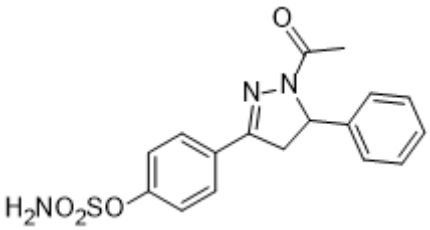


Following the general procedure, the title compound was prepared starting from (*E*)-3-(3-hydroxyphenyl)-1-(3,4-dichloro)prop-2-en-1-one. Yield 73% M.p. 116-117 °C. ^{1}H NMR (DMSO-d_6): δ 2.33 (s, 3H, CH_3), 3.18 (dd, *J* = 4.0, 14.0 Hz, 1H), 4.01 (dd, *J* = 13.5, 3.5 Hz, CH), 5.13 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.52 (d, *J* = 7.5 Hz, 1H, Ar), 6.81 (d, *J* = 7.0 Hz, 1H, Ar), 7.31 (m, 1H, Ar), 7.44 (d, *J* = 7.5 Hz, 1H, Ar), 7.58 (d, *J* = 8.0 Hz, 1H, Ar), 7.89 (s, 1H, Ar), 9.12 (s, 1H, OH). IR (Nujol) 3407, 1669, 1590 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{14}Cl_2N_2O_2$ (349.21) %C 58.47, %H 4.04, %N 8.02, found %C 58.52, %H 3.98, %N 7.98. M/z 350.

General procedure for the preparation of 1-acetyl-3,5-diaryl-4,5-dihydro-1*H*-pyrazole sulfamates (PArS 1-40)

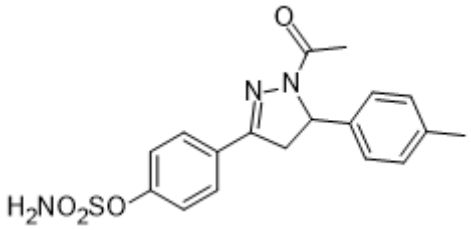
To an ice-cooled stirred solution of pyrazolines (1 mmol) in anhydrous DMA (10 mL), freshly prepared sulfamoyl chloride (0.81 g, 7 mmol) in DMA (5 mL) was added dropwise in 30 min. The obtained mixture was stirred at room temperature for 12 hours, then water (30 mL) was added. The mixture was stirred for additional 2 h, then the formed precipitate was filtered off and dried.

4-(1-Acetyl-5-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-1)



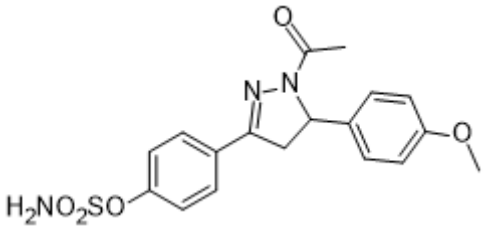
Following the general procedure, the title compound was prepared starting from 1-(3-(4-hydroxyphenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone. Yield 96%. M.p. 186–187 °C. ^{1}H NMR (DMSO-d_6): δ 2.31 (s, 3H, CH_3), 3.13 (dd, *J* = 5.5, 19.0 Hz, 1H, CH), 3.87 (m, 1H, CH), 5.59 (m, 1H, CH), 7.28 (m, 5H, Ar), 7.70 (m, 2H, Ar), 7.88 (d, *J* = 8.0 Hz, 2H, Ar), 7.97 (s, 2H, NH_2). IR (Nujol) 3310, 3182, 1640, 1595 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{17}N_3O_4S$ (359.40) %C 56.81; %H 4.77; %N 11.69. Found %C 56.87; %H 4.76; %N 11.66. M/z 360.

4-(1-Acetyl-5-(p-tolyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-2)



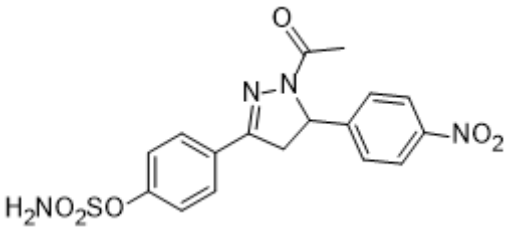
Following the general procedure, the title compound was prepared starting from 1-(3-(4-hydroxyphenyl)-5-(p-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 22% M.p. 142-143 °C. ^{1}H NMR (DMSO-d_6): δ 2.15 (s, 3H, CH_3), 3.11 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.36 (dd, *J* = 13.0, 3.5 Hz, CH), 3.61 (s, 3H, CH_3), 4.22 (s, 2H, NH_2), 5.47 (dd, *J* = 4.0 13.5, Hz, 1H), 6.83 (d, *J* = 8.0, 2H, Ar), 7.09 (d, J = 8.5 Hz, 2H, Ar), 7.62 (d, J = 7.0 Hz, 2H, Ar), 7.87 (d, J = 7.5 Hz, 2H, Ar). IR (Nujol) 3155, 1629, 1516 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{19}N_3O_4S$ (373.43) %C 57.89, %H 5.13, %N 11.25, found %C 57.93, %H 5.15, %N 11.21. M/z 374.

4-(1-Acetyl-5-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-3)



Following the general procedure, the title compound was prepared starting from 1-(3-(4-hydroxyphenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 61% M.p. 119-120°C. ^{1}H NMR (DMSO-d_6): δ 2.15 (s, 3H, CH_3), 3.09 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.68 (dd, *J* = 13.0, 3.5 Hz, CH), 3.72 (s, 3H, OCH_3), 3.87 (s, 2H, NH_2), 5.44 (dd, *J* = 13.5, 4.0 Hz, 1H), 6.59 (d, *J* = 8.0 Hz, 2H, Ar), 6.84 (d, *J* = 8.5 Hz, 2H, Ar), 7.66 (d, *J* = 7.0 Hz, 2H, Ar), 7.85 (d, *J* = 7.5 Hz, 2H, Ar). IR (Nujol) 3198, 1611 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{19}N_3O_5S$ (389.43) %C 55.52, %H 4.92, %N 10.79, found %C 55.48, %H 4.93, %N 10.83. M/z 390.

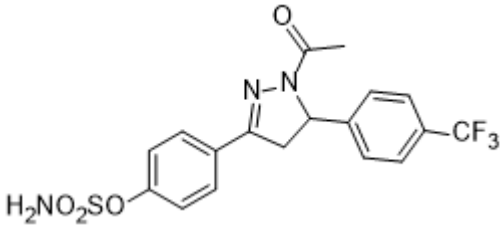
4-(1-Acetyl-5-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-4)



Following the general procedure, the title compound was prepared starting from 1-(3-(4-Hydroxyphenyl)-5-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone. Yield 25% M.p. 169–170 °C. ^{1}H NMR (DMSO-d_6): δ 2.34 (s, 3H, CH_3), 3.24 (dd, *J* = 4.5, 18.0 Hz, 1H, CH), 3.95 (m,

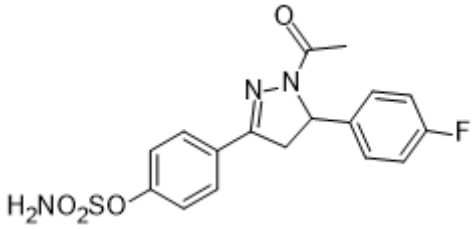
1H, CH), 5.70 (dd, *J* = 4.5, 12.0 Hz, 1H, CH), 7.38 (d, *J* = 8.0 Hz, 2H, Ar), 7.50 (d, *J* = 8.5 Hz, 2H, Ar), 7.88 (d, *J* = 8.0 Hz, 2H, Ar), 8.11 (s, 2H, NH_2), 8.22 (d, *J* = 6.5 Hz, 2H, Ar). IR (Nujol) 3311, 3039 1641, 1599 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}N_4O_6S$ (404.40) %C 50.49; %H 3.99; %N 13.85. Found %C 50.54; %H 3.98; %N 13.88. M/z 405.

4-(1-Acetyl-5-(4-(trifluoromethyl)phenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-5)



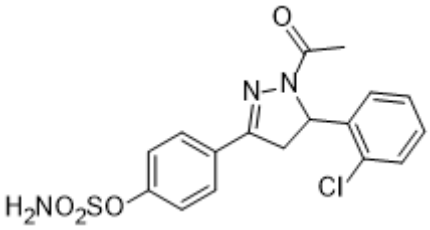
Following the general procedure, the title compound was prepared starting from 1-(3-(4-Hydroxyphenyl)-5-(4-(trifluoromethyl)phenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone. Yield 50% M.p. 194–195 °C. ^{1}H NMR (DMSO-d_6): δ 2.33 (s, 3H, CH_3), 3.23 (dd, *J* = 5.0, 18.0 Hz, 1H, CH), 3.92 (dd, *J* = 12.0, 18.0 Hz, 1H, CH), 5.66 (dd, *J* = 5.0, 12.0 Hz, 1H, CH), 7.37 (d, *J* = 8.5 Hz, 2H, Ar), 7.43 (d, *J* = 7.5 Hz, 2H, Ar), 7.71 (d, *J* = 8.0 Hz, 2H, Ar), 7.87 (d, *J* = 9.0 Hz, 2H, Ar), 8.09 (s, 2H, NH_2). IR (Nujol) 3301, 1636, 1600 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{16}F_3N_3O_4S$ (427.40) %C 50.58; %H 3.77; %N 9.83. Found %C 50.63; %H 3.76; %N 9.86. M/z 428.

4-(1-Acetyl-5-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-6)



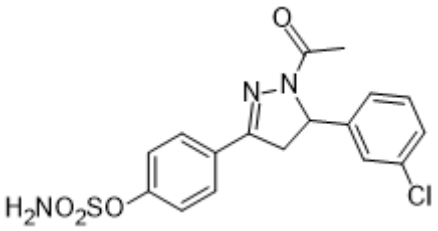
Following the general procedure, the title compound was prepared starting from 1-(5-(4-fluorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 95% M.p. 135-136 °C. ^{1}H NMR (DMSO-d_6): δ 2.33 (s, 3H, CH_3), 3.23 (dd, *J* = 5.0, 13.0 Hz, 1H, CH), 3.92 (dd, *J* = 4.5, 12.0 Hz, 1H, CH), 4.16 (s, 2H, NH_2), 5.66 (dd, *J* = 5.0, 13.0 Hz, 1H, CH), 7.37 (d, *J* = 8.5 Hz, 2H, Ar), 7.43 (d, *J* = 7.5 Hz, 2H, Ar), 7.71 (d, *J* = 8.0 Hz, 2H, Ar), 7.87 (d, *J* = 9.0 Hz, 2H, Ar). IR (Nujol) 3265, 1652, 1513 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}FN_3O_4S$ (377.39) %C 54.10, %H 4.27, %N 11.37, found %C 54.15, %H 4.26, %N 11.34. M/z 378.

3-(1-Acetyl-5-(2-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-7)



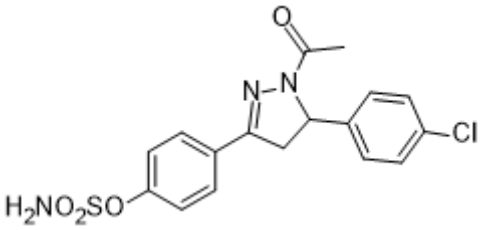
Following the general procedure, the title compound was prepared starting from 1-(5-(2-chlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 54% M.p. 123-124 °C. ^{1}H NMR (DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.96 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.93 (dd, *J* = 14.5, 3.5 Hz, CH), 4.88 (s, 2H, NH_2), 5.77 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.87 (d, *J* = 7.5 Hz, 1H, Ar), 7.08 (d, *J* = 7.0 Hz, 1H, Ar), 7.22 (m, 1H, Ar), 7.38 (d, *J* = 7.5 Hz, 2H, Ar), 7.72 (d, *J* = 8.0 Hz, 2H, Ar), 8.08 (s, 1H, Ar). IR (Nujol) 3237, 1642, 1574 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}ClN_3O_4S$ (393.84) %C 51.84, %H 4.09, %N 10.67, found %C 51.78, %H 4.08, %N 10.72. M/z 394.

4-(1-Acetyl-5-(3-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-8)



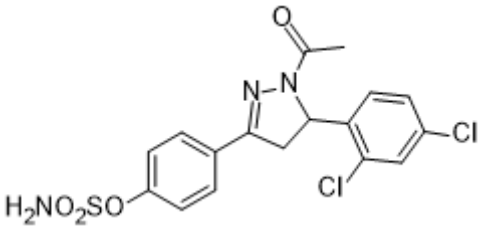
Following the general procedure, the title compound was prepared starting from 1-(5-(3-chlorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 91% M.p. 120-121 °C. ^{1}H NMR (DMSO-d_6): δ 2.28 (s, 3H, CH_3), 3.13 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.76 (dd, *J* = 14.5, 3.5 Hz, CH), 3.82 (s, 2H, NH_2), 5.51 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.82 (d, *J* = 7.5 Hz, 1H, Ar), 6.86 (d, *J* = 7.0 Hz, 1H, Ar), 7.32 (m, 1H, Ar), 7.46 (s, 1H, Ar), 7.62 (d, *J* = 7.5 Hz, 2H, Ar), 7.88 (d, *J* = 8.0 Hz, 2H, Ar). IR (Nujol) 3222, 1610 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}ClN_3O_4S$ (393.84) %C 51.84, %H 4.09, %N 10.67, found %C 51.89, %H 4.07, %N 10.63. M/z 394.

4-(1-Acetyl-5-(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-9)



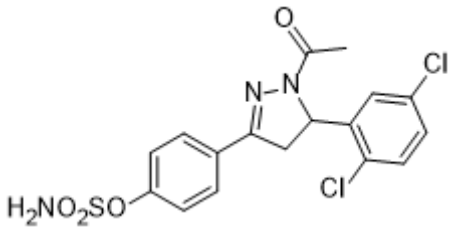
Following the general procedure, the title compound was prepared starting from 1-(5-(4-chlorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 31% M.p. 124-125 °C. ^{1}H NMR (DMSO-d_6): δ 2.28 (s, 3H, CH_3), 3.15 (dd, *J* = 3.5, 14.0 Hz, 1H), 3.82 (dd, *J* = 13.0, 3.5 Hz, CH), 4.67 (s, 2H, NH_2), 5.55 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.85 (d, *J* = 8.0, 2H, Ar), 7.21 (d, *J* = 8.0, 2H, Ar), 7.63 (d, *J* = 7.5, 2H, Ar), 7.88 (d, *J* = 8.0, 2H, Ar). IR (Nujol) 3329, 1634, 1609cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}ClN_3O_4S$ (393.84) %C 51.84, %H 4.09, %N 10.67, found %C 51.79, %H 4.10, %N 10.70. m/z 394.

4-(1-Acetyl-5-(2,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenyl sulfamate (PArS10)



Following the general procedure, the title compound was prepared starting from 1-(5-(2,4-dichlorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 49% M.p. 184-185 °C. ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3), 3.14 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.88 (s, 2H, NH_2), 3.95 (dd, *J* = 13.5, 3.5 Hz, CH), 5.68 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.83 (d, *J* = 8.5 Hz, 2H, Ar), 7.07 (d, *J* = 8.0 Hz, 2H, Ar), 7.62 (d, *J* = 7.5 Hz, 1H, Ar), 7.85 (d, *J* = 8.0 Hz, 1H, Ar), 8.09 (s, 1H, Ar). IR (Nujol) 3296, 1630, 1599 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) %C 47.67, %H 3.53, %N 9.81, found %C 47.71, %H 3.52, %N 9.84. M/z 429.

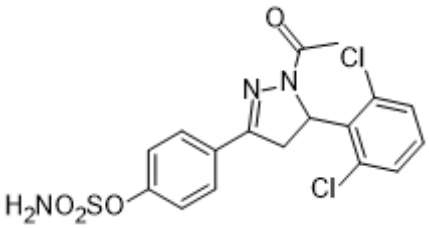
4-(1-Acetyl-5-(2,5-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-11)



Following the general procedure, the title compound was prepared starting from 1-(3-(4-Hydroxyphenyl)-5-(2,5-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethenone. Yield 50% , M.p. 174–176 °C. ^{1}H NMR (DMSO-d_6): δ 2.31 (dd, *J* = 4.5, 16.0 Hz, 1H, CH), 2.35 (s, 3H, CH_3), 3.94

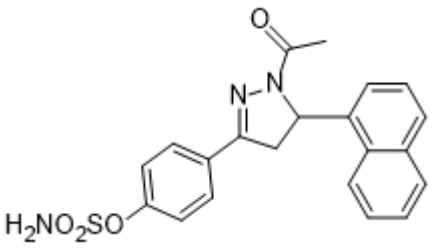
(m, 1H, CH), 5.74 (dd, *J* = 4.5, 12.5 Hz, 1H, CH), 7.11 (m, 1H, Ar), 7.36 (m, 3H, Ar), 7.66 (s, 1H, Ar), 7.85 (m, 2H, Ar), 8.09 (s, 2H, NH_2). IR (Nujol) 3308, 1637, 1603 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) % 47.67; %H 3.53; %N 9.81. Found %C 47.62; %H 3.54; %N 9.78. m/z 429.

4-(1-Acetyl-5-(2,6-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS12)



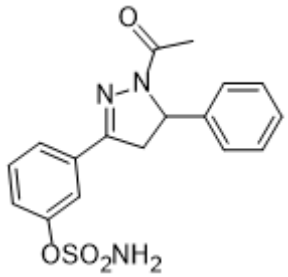
Following the general procedure, the title compound was prepared starting from 1-(5-(2,6-dichlorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 75% M.p. 140-141 °C. ^{1}H NMR (DMSO-d_6): δ 2.24 (s, 3H, CH_3), 3.16 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.78 (dd, *J* = 13.0, 3.5 Hz, CH), 3.88 (s, 2H, NH_2), 6.05 (dd, *J* = 13.5, 4.0 Hz, 1H), 6.85 (d, *J* = 8.0 Hz , 2H, Ar), 7.31 (m, 2H, Ar), 7.39 (m, 2H, Ar), 7.88 (d, *J* =7.5 Hz, 2H, Ar). IR (Nujol) 3387, 1638, 1602 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) %C 47.67, %H 3.53, %N 9.81, found %C 47.70, %H 3.54, %N 9.83. M/z 429.

4-(1-Acetyl-5-(naphthalen-1-yl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenyl sulfamate (PArS13)



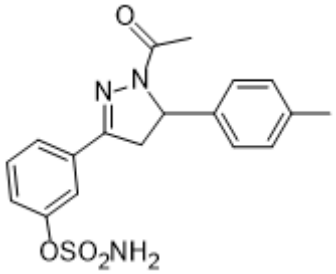
Following the general procedure, the title compound was prepared starting from 1-(3-(4-hydroxyphenyl)-5-(naphthalen-1-yl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 71% M.p. 118-119 °C. ^{1}H NMR (DMSO-d_6): δ 2.39 (s, 3H, CH_3), 3.10 (dd, *J* = 4.0 Hz, 14.0 Hz, 1H), 3.44 (s, 2H, NH_2), 4.13 (dd, *J* = 13.5, 4.0 Hz, CH), 6.30 (dd, *J* = 13.0, 3.5 Hz, 1H), 7.14 (d, *J* = 8.0, 2H, Ar), 7.32 (d, *J* = 8.5 Hz, 2H, Ar), 7.44 (m, 3H, Ar), 7.62 (m, 2H, Ar), 7.87 (m, 2H, Ar). IR (Nujol) 3285, 1646 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{19}N_3O_4S$ (409.46) %C 61.60, %H 4.68, %N 10.26, found %C 61.55, %H 4.69, %N 10.29. M/z 410.

3-(1-Acetyl-5-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-14)



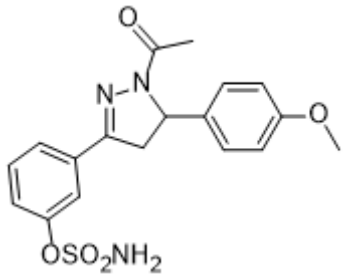
Following the general procedure, the title compound was prepared starting from 1-(3-(3-hydroxyphenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 28% M.p. 105-106 °C. ^{1}H NMR (DMSO-d_6): δ 2.33 (s, 3H, CH_3), 3.08 (dd, *J* = 3.5, 13.0 Hz, 1H), 3.88 (dd, *J* = 14.5, 3.5 Hz, CH), 5.54 (s, 2H, NH_2), 5.57 (dd, *J* = 14.0, 3.0 Hz, 1H), 6.88 (d, *J* = 8.0, 1H, Ar), 6.83 (d, *J* = 8.5 Hz, 1H, Ar), 7.26 (m, 1H, Ar), 7.55 (m, 3H, Ar), 7.88 (m, 2H, Ar), 8.05 (s, 1H, Ar). IR (Nujol) 3261, 1615 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{17}N_3O_4S$ (359.40) %C 56.81, %H 4.77, %N 11.69, found %C 56.86, %H 4.75, %N 11.72. M/z 360.

3-(1-Acetyl-5-(p-tolyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-15)



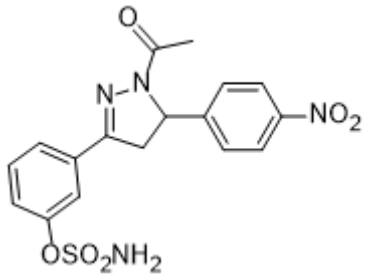
Following the general procedure, the title compound was prepared starting from 1-(3-(3-hydroxyphenyl)-5-(p-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 88% M.p. 118-119 °C. ^{1}H NMR (DMSO-d_6): δ 2.17 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 3.17 (dd, *J* = 3.5, 13.0 Hz, 1H), 3.89 (dd, *J* = 13.5, 3.5 Hz, CH), 5.21 (s, 2H, NH_2), 5.33 (dd, *J* = 14.5, 3.0 Hz, 1H), 6.70 (d, *J* = 8.5 Hz, 1H, Ar), 6.88 (d, *J* = 8.5 Hz, 1H, Ar), 7.12 (m, 1H, Ar), 7.41 (d, *J* = 7.0 Hz, 2H, Ar), 7.84 (d, *J* = 7.0 Hz, 2H, Ar), 8.09 (s, 1H, Ar). IR (Nujol) 3246, 1614cm^{-1}. Elemental analysis: calculated for $C_{18}H_{19}N_3O_4S$ (373.43) %C 57.89, %H 5.13, %N 11.25, found %C 57.93, %H 5.11, %N 11.22. M/z 374.

3-(1-Acetyl-5-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-16)



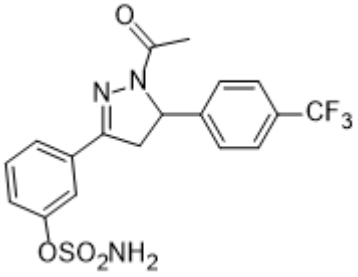
Following the general procedure, the title compound was prepared starting from 1-(5-(4-methoxyphenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 42% M.p. 118-119 °C. ^{1}H NMR (DMSO-d_6): δ 2.28 (s, 3H, CH_3), 3.12 (dd, *J* = 3.5, 13.0 Hz, 1H), 3.72 (s, 3H, OCH_3), 3.79 (dd, *J* = 13.5, 3.5 Hz, CH), 3.90 (s, 2H, NH_2), 5.49 (dd, *J* = 14.5, 3.0 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 1H, Ar), 7.04 (d, *J* = 8.5 Hz, 1H, Ar), 7.12 (m, 1H, Ar), 7.27 (d, *J* = 7.0 Hz, 2H, Ar), 7.71 (d, *J* = 7.0 Hz, 2H, Ar), 8.06 (s, 1H, Ar). IR (Nujol) 3246, 1614 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{19}N_3O_5S$ (389.43) %C 55.52, %H 4.92, %N 10.79, found %C 55.59, %H 4.91, %N 10.77. M/z 390.

3-(1-Acetyl-5-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-17)



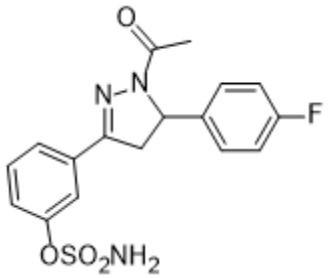
Following the general procedure, the title compound was prepared starting from 1-(5-(4-nitrophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 47% M.p. 205-206 °C. ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3), 3.11 (dd, *J* = 3.5, 13.0 Hz, 1H), 3.17 (dd, *J* = 13.5, 3.5 Hz, CH), 3.87 (s, 2H, NH_2), 5.68 (dd, *J* = 14.5, 3.0 Hz, 1H), 6.89 (d, *J* = 8.5 Hz, 1H, Ar), 6.71 (d, *J* = 8.5, 1H, Ar), 7.25 (m, 1H, Ar), 7.49 (d, *J* = 7.0, 2H, Ar), 7.57 (d, *J* = 7.0, 2H, Ar), 8.20 (s, 1H, Ar). IR (Nujol) 3239, 1634 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}N_4O_6S$ (404.40) %C 50.49, %H 3.99, %N 13.85, found %C 50.56, %H 4.01, %N 13.81. M/z 405.

3-(1-Acetyl-5-(4-(trifluoromethyl)phenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-18)



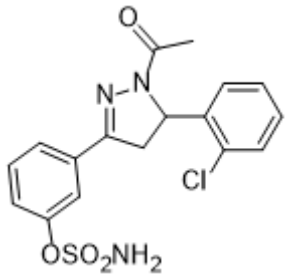
Following the general procedure, the title compound was prepared starting from 1-(5-(4-trifluoromethylphenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 40% M.p. 115-116 °C. ^{1}H NMR (DMSO-d_6): δ 2.34 (s, 3H, CH_3), 3.04 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.59 (dd, *J* = 14.5, 3.5 Hz, CH), 3.98 (s, 2H, NH_2), 5.69 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.88 (d, *J* = 7.5 Hz, 1H, Ar), 7.17 (d, *J* = 7.0 Hz, 1H, Ar), 7.28 (m, 1H, Ar), 7.45 (d, *J* = 8.5 Hz, 2H, Ar), 7.88 (d, *J* = 8.0 Hz, 2H, Ar), 7.98 (s, 1H, Ar). IR (Nujol) 3209, 1642, 1584 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{16}F_3N_3O_4S$ (427.40) %C 50.58, %H 3.77, %N 9.83, found %C 50.64, %H 3.76, %N 9.80. M/z 428.

3-(1-Acetyl-5-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-19)



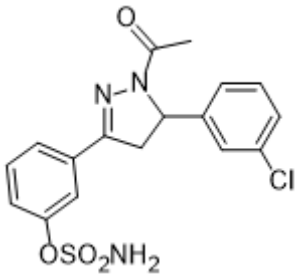
Following the general procedure, the title compound was prepared starting from 1-(5-(4-fluorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 27% M.p. 95-96 °C. ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, CH_3), 3.12 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.74 (dd, *J* = 14.5, 3.5 Hz, CH), 3.88 (s, 2H, NH_2), 5.54 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.87 (d, *J* = 7.5 Hz, 1H, Ar), 7.15 (d, *J* = 7.0, 1H, Ar), 7.22 (m, 1H, Ar), 7.39 (d, *J* = 8.5 Hz, 2H, Ar), 7.55 (d, *J* = 8.0 Hz, 2H, Ar), 8.06 (s, 1H, Ar). IR (Nujol) 3264, 1607 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}FN_3O_4S$ (377.39) %C 54.10, %H 4.27, %N 11.13, found %C 54.04, %H 4.28, %N 11.17. M/z 378.

3-(1-Acetyl-5-(2-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-20)



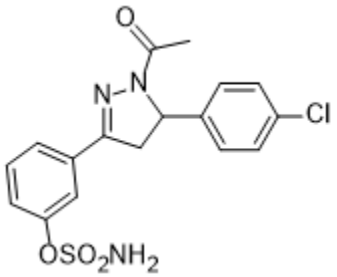
Following the general procedure, the title compound was prepared starting from 1-(5-(2-chlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 54% M.p. 123-124 °C. ^{1}H NMR (DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.96 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.93 (dd, *J* = 14.5, 3.5 Hz, CH), 4.88 (s, 2H, NH_2), 5.77 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.87 (d, *J* = 7.5 Hz, 1H, Ar), 7.08 (d, *J* = 7.0 Hz, 1H, Ar), 7.22 (m, 1H, Ar), 7.38 (d, *J* = 7.5 Hz, 2H, Ar), 7.72 (d, *J* = 8.0 Hz, 2H, Ar), 8.08 (s, 1H, Ar). IR (Nujol) 3237, 1642, 1574 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}ClN_3O_4S$ (393.84) %C 51.84, %H 4.09, %N 10.67, found %C 61.79, %H 4.11, %N 10.69. M/z 394.

3-(1-Acetyl-5-(3-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-21)



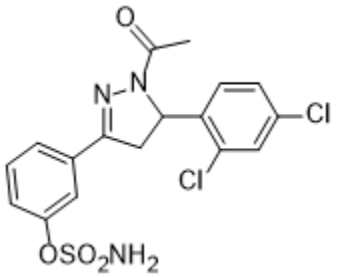
Following the general procedure, the title compound was prepared starting from 1-(5-(3-chlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 44% M.p. 98-99 °C. ^{1}H NMR (DMSO-d_6): δ 2.34 (s, 3H, CH_3), 3.14 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.64 (dd, *J* = 14.5, 3.5 Hz, CH), 3.82 (s, 2H, NH_2), 5.58 (dd, *J* = 13.5, 3.0 Hz, 1H), 7.16 (d, *J* = 7.5 Hz, 1H, Ar), 7.27 (d, *J* = 7.0 Hz, 1H, Ar), 7.39 (s, 1H, Ar), 7.55 (d, *J* = 7.5 Hz, 2H, Ar), 7.72 (d, *J* = 8.0 Hz, 2H, Ar), 8.05 (s, 1H, Ar). IR (Nujol) 3262, 1614, 1574 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}ClN_3O_4S$ (393.84) %C 51.84, %H 4.09, %N 10.67, found %C 51.88, %H 4.08, %N 10.71. M/z 394.

3-(1-Acetyl-5-(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-22)



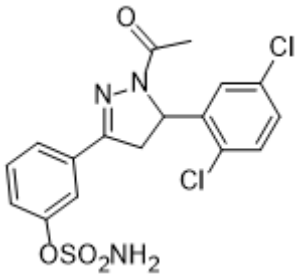
Following the general procedure, the title compound was prepared starting from 1-(5-(4-chlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 72% M.p. 110-111 °C. ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, CH_3), 2.95 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.80 (dd, *J* = 14.5, 3.5 Hz, CH), 3.91 (s, 2H, NH_2), 5.54 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.88 (d, *J* = 7.5 Hz, 1H, Ar), 7.16 (d, *J* = 7.0 Hz, 1H, Ar), 7.39 (m, 1H, Ar), 7.55 (d, *J* = 7.5 Hz, 2H, Ar), 7.73 (d, *J* = 8.0 Hz, 2H, Ar), 8.06 (s, 1H, Ar). IR (Nujol) 3265, 1615, 1543 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}ClN_3O_4S$ (393.84) %C 51.84, %H 4.09, %N 10.67, found %C 51.79, %H 4.08, %N 10.70. M/z 394.

3-(1-Acetyl-5-(2,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-23)



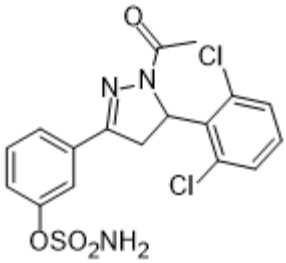
Following the general procedure, the title compound was prepared starting from 1-(5-(2,4-dichlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 22% M.p. 107-108 °C. ^{1}H NMR (DMSO-d_6): δ 2.33 (s, 3H, CH_3), 3.14 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.88 (dd, *J* = 13.5, 3.5 Hz, CH), 4.01 (s, 2H, NH_2), 5.78 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.87 (d, *J* = 7.5 Hz, 1H, Ar), 7.11 (d, *J* = 7.0 Hz, 1H, Ar), 7.38 (m, 2H, Ar), 7.67 (m, 2H, Ar), 8.05 (s, 1H, Ar). IR (Nujol) 3220, 1640, 1584 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) %C 47.67, %H 3.53, %N 9.81, found %C 47.71, %H 3.52, %N 9.78. M/z 429.

3-(1-Acetyl-5-(2,5-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-24)



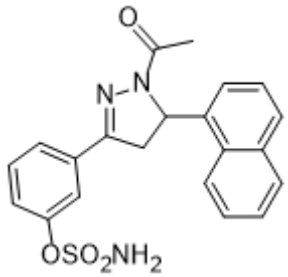
Following the general procedure, the title compound was prepared starting from 1-(5-(2,5-dichlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 88% M.p. 161-162 °C. ^{1}H NMR (DMSO-d_6): δ 2.37 (s, 3H, CH_3), 3.18 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.97 (dd, *J* = 13.5, 3.5 Hz, CH), 4.57 (s, 2H, NH_2), 5.77 (dd, *J* = 13.5, 3.0 Hz, 1H), 7.11 (d, *J* = 7.5 Hz, 1H, Ar), 7.55 (d, *J* = 7.0 Hz, 1H, Ar), 7.69 (m, 2H, Ar), 7.73 (m, 2H, Ar), 8.06 (s, 1H, Ar). IR (Nujol) 3190, 1647, 1572 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) %C 47.67, %H 3.53, %N 9.81, found %C 47.62, %H 3.54, %N 9.77. M/z 429.

3-(1-Acetyl-5-(2,6-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenyl sulfamate (PArS-25)



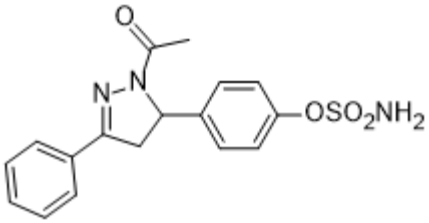
Following the general procedure, the title compound was prepared starting from 1-(5-(2,6-dichlorophenyl)-3-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 41% M.p. 130-131 °C. ^{1}H NMR (DMSO-d_6): δ 2.25 (s, 3H, CH_3), 3.22 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.72 (dd, *J* = 13.5, 3.5 Hz, CH), 3.88 (s, 2H, NH_2), 6.07 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.14 (d, *J* = 7.5 Hz, 1H, Ar), 6.89 (d, *J* = 7.0 Hz, 1H, Ar), 7.28 (m, 1H, Ar), 7.33 (d, *J* = 7.5 Hz, 1H, Ar), 7.71 (d, *J* = 8.0 Hz, 1H, Ar), 8.09 (s, 1H, Ar). IR (Nujol) 3230, 1642, 1576 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) %C 47.67, %H 3.53, %N 9.81, found %C 47.72, %H 3.54, %N 9.78. M/z 429.

3-(1-Acetyl-5-(naphthalen-1-yl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenylsulfamate (PArS-26)



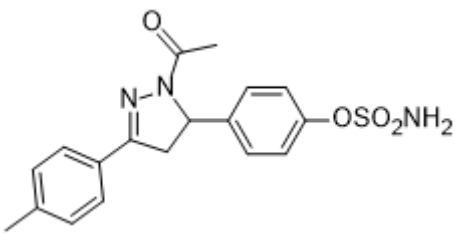
Following the general procedure, the title compound was prepared starting from 1-(3-(3-hydroxyphenyl)-5-(naphthalen-1-yl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 79% M.p. 120-121 °C. ^{1}H NMR (DMSO-d_6): δ 2.42 (s, 3H, CH_3), 2.96 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.04 (s, 2H, NH_2), 4.12 (dd, *J* = 13.5, 4.0 Hz, CH), 6.27 (dd, *J* = 13.0, 3.5 Hz, 1H), 6.87 (d, *J* = 8.0 Hz, 2H, Ar), 7.22 (d, *J* = 8.5 Hz, 2H, Ar), 7.45 (m, 3H, Ar), 7.64 (m, 2H, Ar), 7.87 (m, 2H, Ar), 8.16 (s, 1H, Ar). IR (Nujol) 3213, 1640, cm^{-1}. Elemental analysis: calculated for $C_{21}H_{19}N_3O_4S$ (409.46) %C 61.60, %H 4.68, %N 10.26, found %C 61.67, %H 4.69, %N 10.23. M/z 410.

4-(1-Acetyl-3-phenyl-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-27)



Following the general procedure, the title compound was prepared starting from 1-(5-(4-hydroxyphenyl)-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 19% M.p. 125-126 °C. ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, CH_3), 3.13 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.87 (dd, *J* = 13.5, 4.0 Hz, CH), 4.62 (s, 2H, NH_2), 5.61 (dd, *J* = 13.0, 3.5 Hz, 1H), 7.07 (d, *J* = 8.0 Hz, 2H, Ar), 7.23 (d, *J* = 8.5 Hz, 2H, Ar), 7.48 (m, 3H, Ar), 7.98 (m, 2H, Ar). IR (Nujol) 3196, 1596 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{17}N_3O_4S$ (359.40) %C 56.81, %H 4.77, %N 11.69, found %C 56.76, %H 4.76, %N 11.73. M/z 360.

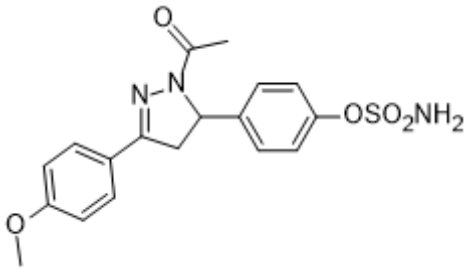
4-(1-Acetyl-3-(p-tolyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-28)



Following the general procedure, the title compound was prepared starting from 1-(5-(4-hydroxyphenyl)-3-(p-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 47% M.p. 127-128 °C. ^{1}H NMR (DMSO-d_6): δ 2.27 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 3.14 (dd, *J* = 3.5, 14.0 Hz, 1H), 3.87 (dd,

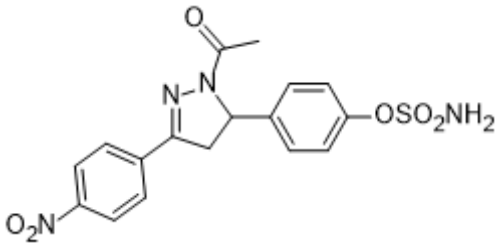
J = 13.0, 3.5 Hz, CH), 4.23 (s, 2H, NH_2), 5.57 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.69 (d, *J* = 8.0 Hz, 2H, Ar), 6.98 (d, *J* = 8.0 Hz, 2H, Ar), 7.37 (d, *J* = 7.5 Hz, 2H, Ar), 7.90 (d, *J* = 7.0 Hz, 2H, Ar). IR (Nujol) 3346, 1637, 1607 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{19}N_3O_4S$ (373.43) %C 57.89, %H 5.13, %N 11.25, found %C 57.96, %H 5.11, %N 11.22. M/z 374.

4-(1-Acetyl-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-29)



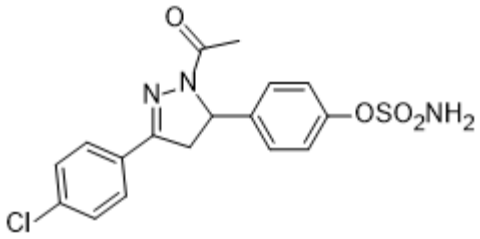
Following the general procedure, the title compound was prepared starting from 1-(5-(4-hydroxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 30% M.p. 177-178 °C. ^{1}H NMR (DMSO-d_6): δ 2.31 (s, 3H, CH_3), 3.11 (dd, *J* = 4.5, 17.5 Hz, 1H, CH), 3.84 (m, 4H, CH and OCH_3), 5.56 (d, *J* = 4.5, 11.5 Hz, 1H, CH), 7.02 (d, *J* = 8.5 Hz, 2H, Ar), 7.23 (d, *J* = 9.0 Hz, 2H, Ar), 7.27 (d, *J* = 8.5 Hz, 2H, Ar), 7.73 (d, *J* = 9.0 Hz, 2H, Ar), 7.98 (s, 2H, NH_2). IR (Nujol) 3321, 3167, 1637, 1568 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{19}N_3O_5S$ (389.10) %C 55.52; %H 4.92; %N 10.79. Found %C 55.58; %H 4.90; %N 10.76. M/z 390.

4-(1-Acetyl-3-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-30)



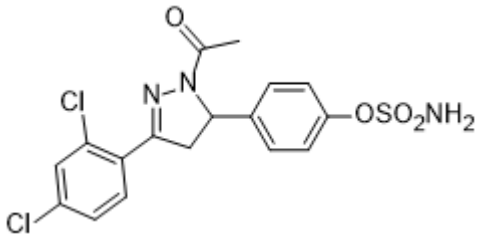
Following the general procedure, the title compound was prepared starting from 1-(5-(4-hydroxyphenyl)-3-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 60% M.p. 135-136 °C. ^{1}H NMR (DMSO-d_6): δ 2.31 (s, 3H, CH_3), 2.95 (s, 2H, NH_2), 3.13 (dd, *J* = 3.0, 13.0 Hz, 1H), 3.86 (dd, *J* = 14.0, 4.0 Hz, CH), 5.57 (dd, *J* = 13.0, 3.5 Hz, 1H), 6.72 (d, *J* = 8.5 Hz, 2H, Ar), 7.19 (d, *J* = 8.0 Hz, 2H, Ar), 8.13 (d, *J* = 7.0 Hz, 2H, Ar), 8.33 (d, *J* = 7.5 Hz, 2H, Ar). IR (Nujol) 3203, 1643, 1605 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}N_4O_6S$ (404.40) %C 50.49, %H 3.99, %N 13.85, found %C 50.53, %H 4.01, %N 13.81. M/z 405.

4-(1-Acetyl-3-(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-31)



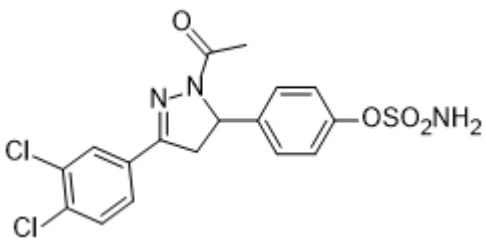
Following the general procedure, the title compound was prepared starting from 1-(3-(4-chlorophenyl)-5-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 61% M.p. 120-121 °C. ^{1}H NMR (DMSO-d_6): δ 2.27 (s, 3H, CH_3), 3.14 (dd, *J* = 3.5, 14.0 Hz, 1H), 3.83 (dd, *J* = 13.0, 3.5 Hz, CH), 4.47 (s, 2H, NH_2), 5.57 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.71 (d, *J* = 8.0, 2H, Ar), 7.09 (d, *J* = 8.0, 2H, Ar), 7.54 (d, *J* = 7.5 Hz, 2H, Ar), 7.93 (d, *J* = 8.0 Hz, 2H, Ar). IR (Nujol) 3187, 1640, 1592 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}ClN_3O_4S$ (393.84) %C 51.84, %H 4.09, %N 9.00, found %C 51.79, %H 4.08, %N 9.04. M/z 394.

4-(1-Acetyl-3-(2,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-32)



Following the general procedure, the title compound was prepared starting from 1-(3-(2,4-dichlorophenyl)-5-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 77% M.p. 115-116 °C. ^{1}H NMR (DMSO-d_6): δ 2.26 (s, 3H, CH_3), 3.17 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.76 (s, 2H, NH_2), 3.89 (dd, *J* = 13.5, 3.5 Hz, CH), 5.45 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.71 (d, *J* = 8.5 Hz, 2H, Ar), 7.02 (d, *J* = 8.0 Hz, 2H, Ar), 7.62 (d, *J* = 7.5 Hz, 1H, Ar), 7.78 (d, *J* = 8.0 Hz, 1H, Ar), 7.82 (s, 1H, Ar). IR (Nujol) 3271, 1646, 1587 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) %C 47.67, %H 3.53, %N 9.81, found %C 47.63, %H 3.52, %N 9.84. M/z 429.

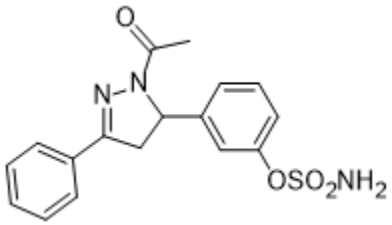
4-(1-Acetyl-3-(3,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-33)



Following the general procedure, the title compound was prepared starting from 1-(3-(3,4-dichlorophenyl)-5-(4-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 66% M.p. 125-126 °C. ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, CH_3), 3.14 (dd, *J* = 4.5, 14.0 Hz, 1H), 3.81 (dd, *J* =

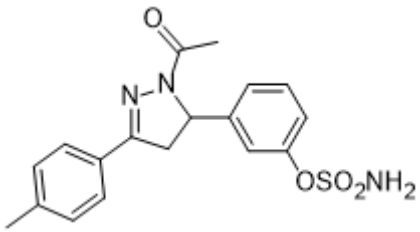
13.5, 3.5 Hz, CH), 4.21 (s, 2H, NH_2), 5.47 (dd, *J* = 13, 3.0 Hz, 1H), 6.69 (d, *J* = 8.0, 2H, Ar), 7.08 (d, *J* = 8.0 Hz, 2H, Ar), 7.73 (d, *J* = 7.5 Hz, 1H, Ar), 7.81 (d, *J* = 8.0 Hz, 1H, Ar), 7.98 (s, 1H, Ar). IR (Nujol) 3250, 1645, 1594 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) %C 47.67, %H 3.53, %N 9.81, found %C 47.73, %H 3.54, %N 9.78. M/z 428.

3-(1-Acetyl-3-phenyl-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-34)



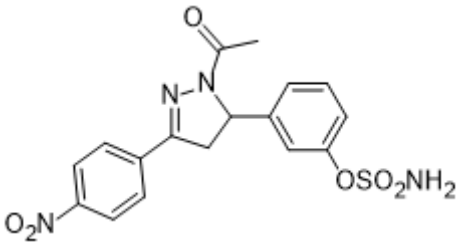
Following the general procedure, the title compound was prepared starting from 1-(5-(3-hydroxyphenyl)-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 51% M.p. 94-95 °C. ^1H NMR (DMSO-d_6): δ 2.22 (s, 3H, CH_3), 3.11 (dd, *J* = 3.5, 13.0 Hz, 1H), 3.87 (dd, *J* = 14.5, 3.5 Hz, CH), 5.01 (s, 2H, NH_2), 5.12 (dd, *J* = 14.0, 3.0 Hz, 1H), 6.71 (d, *J* = 8.0 Hz, 1H, Ar), 6.83 (d, *J* = 8.5 Hz, 1H, Ar), 7.26 (m, 1H, Ar), 7.55 (m, 3H, Ar), 7.88 (m, 2H, Ar). IR (Nujol) 3200, 1598 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{17}N_3O_4S$ (359.40) %C 56.81, %H 4.77, %N 11.69, found %C 56.86, %H 4.76, %N 10.65. M/z 360.

3-(1-Acetyl-3-(p-tolyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-35)



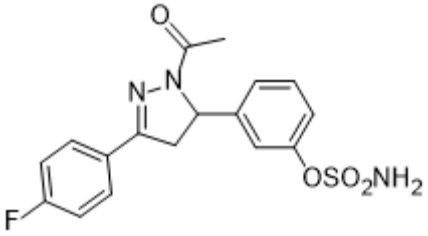
Following the general procedure, the title compound was prepared starting from 1-(5-(3-hydroxyphenyl)-3-(p-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 88% M.p. 108-110 °C. ^1H NMR (DMSO-d_6): δ 2.17 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 3.17 (dd, *J* = 3.5, 13.0 Hz, 1H), 3.89 (dd, *J* = 13.5, 3.5 Hz, CH), 5.21 (s, 2H, NH_2), 5.33 (dd, *J* = 14.5, 3.0 Hz, 1H), 6.70 (d, *J* = 8.5 Hz, 1H, Ar), 6.88 (d, *J* = 8.5 Hz, 1H, Ar), 7.12 (m, 1H, Ar), 7.41 (d, *J* = 7.0 Hz, 2H, Ar), 7.84 (d, *J* = 7.0 Hz, 2H, Ar). IR (Nujol) 3250, 1609 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{19}N_3O_4S$ (373.43) %C 57.89, %H 5.13, %N 11.25, found %C 57.84, %H 5.15, %N 11.29. M/z 374.

3-(1-Acetyl-3-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-36)



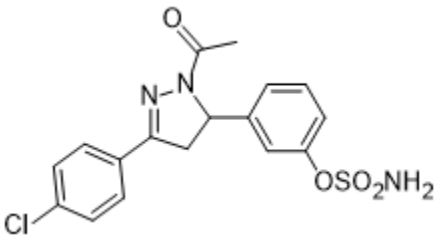
Following the general procedure, the title compound was prepared starting from 1-(5-(3-hydroxyphenyl)-3-(4-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 47% M.p. 175-176 °C. ^{1}H NMR (DMSO-d_6): δ 2.23 (s, 3H, CH_3), 3.04 (dd, *J* = 3.0, 14.0 Hz, 1H), 3.88 (dd, *J* = 14.5, 3.5 Hz, CH), 4.58 (s, 2H, NH_2), 5.44 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.79 (d, *J* = 7.5, 1H, Ar), 7.09 (d, *J* = 7.0 Hz, 1H, Ar), 7.23 (m, 1H, Ar), 7.47 (d, *J* = 8.5 Hz, 2H, Ar), 7.90 (d, *J* = 8.0 Hz, 2H, Ar). IR (Nujol) 3252, 1650, 1605 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}N_4O_6S$ (404.08) %C 50.49, %H 3.99, %N 13.85, found %C 50.55, %H 4.01, %N 13.81. M/z 405.

4-(1-Acetyl-3-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-37)



Following the general procedure, the title compound was prepared starting from 1-(3-(4-fluorophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 40% M.p. 190-191 °C. ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3), 3.18 (dd, *J* = 4.0, 18.0 Hz, 1H, CH), 3.88 (dd, *J* = 13.5, 16.5 Hz, 1H, CH), 5.59 (m, 1H, CH), 7.11 (s, 1H, Ar),7.16 (t, *J* = 7.5 Hz, 2H, Ar), 7.32 (t, *J* = 7.5 Hz, 1H, Ar), 7.42 (t, *J* = 7.5 Hz, 2H, Ar), 7.85 (m, 2H, Ar), 7.99 (s, 2H, NH_2). IR (Nujol) 3325, 1638, 1608 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}FN_3O_4S$ (377.39) %C 54.10, %H 4.27; %N 11.13. Found %C 54.05; %H 4.28; %N 11.17. M/z 378.

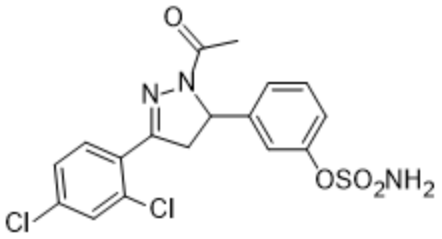
3-(1-Acetyl-3-(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-38)



Following the general procedure, the title compound was prepared starting from 1-(3-(4-chlorophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 69% M.p. 95-96 °C. ^{1}H NMR (DMSO-d_6): δ 2.18 (s, 3H, CH_3), 3.08 (dd, *J* = 3.0, 14.0 Hz, 1H), 4.01 (dd, *J* = 14.5,

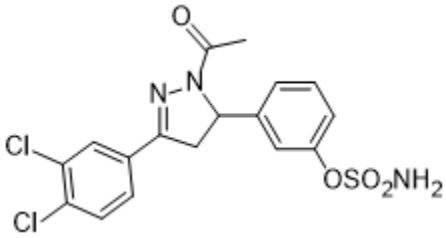
3.5 Hz, CH), 4.69 (s, 2H, NH_2), 5.08 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.71 (d, *J* = 7.5 Hz, 1H, Ar), 6.78 (d, *J* = 7.0 Hz, 1H, Ar), 7.22 (m, 1H, Ar), 7.36 (d, *J* = 7.5 Hz, 2H, Ar), 7.68 (d, *J* = 8.0 Hz, 2H, Ar), 7.88 (s, 1H, Ar). IR (Nujol) 3222, 1673, 1589 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{16}ClN_3O_4S$ (393.84) %C 51.84, %H 4.09, %N 10.67, found %C 51.80, %H 4.11, %N 10.70. M/z 394.

3-(1-Acetyl-3-(2,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-39)



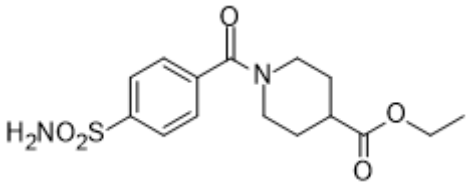
Following the general procedure, the title compound was prepared starting from 1-(3-(2,4-dichlorophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 77% M.p. 125-126 °C. ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, CH_3), 3.18 (dd, *J* = 4.0, 14.0 Hz, 1H), 3.96 (dd, *J* = 13.5, 3.5 Hz, CH), 4.57 (s, 2H, NH_2), 5.03 (dd, *J* = 13.5, 3.0 Hz, 1H), 6.64 (d, *J* = 7.5 Hz, 1H, Ar), 6.88 (d, *J* = 7.0 Hz, 1H, Ar), 7.13 (m, 1H, Ar), 7.19 (d, *J* = 7.5 Hz, 1H, Ar), 7.53 (d, *J* = 8.0 Hz, 1H, Ar), 7.77 (s, 1H, Ar). IR (Nujol) 3238, 1684, 1583 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) %C 47.67, %H 3.53, %N 9.81, found %C 47.71, %H 3.54, %N 9.79. M/z 429.

3-(1-Acetyl-3-(3,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl)phenylsulfamate (PArS-40)



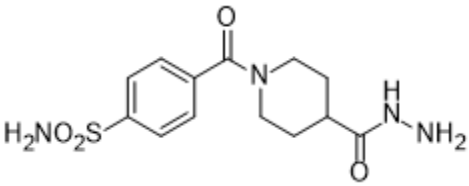
Following the general procedure, the title compound was prepared starting from 1-(3-(3,4-dichlorophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone. Yield 70% M.p. 110-111 °C. ^{1}H NMR (DMSO-d_6): δ 2.23 (s, 3H, CH_3), 3.22 (dd, *J* = 4.0, 14.0 Hz, 1H), 4.01 (dd, *J* = 13.5, 3.5 Hz, CH), 5.13 (dd, *J* = 13.5, 3.0 Hz, 1H), 5.36 (s, 2H, NH_2), 6.46 (d, *J* = 7.0, 1H, Ar), 6.72 (d, J = 7.0, 1H, Ar), 7.28 (m, 1H, Ar), 7.49 (d, *J* = 7.5, 1H, Ar), 7.75 (d, *J* = 8.0, 1H, Ar), 7.95 (s, 1H, Ar). IR (Nujol) 3241, 1679, 1584 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}Cl_2N_3O_4S$ (428.29) %C 47.67, %H 3.53, %N 9.81, found %C 47.62, %H 3.52, %N 9.85. M/z 428.

Ethyl 1-(4-sulfamoylbenzoyl)piperidine-4-carboxylate (20)



4-(Aminosulfonil)-benzoic acid (18) (4.2 g, 20 mmol), EDCI (3.9g, 22 mmol) and HOBt (2.7 g, 20 mmol) were dissolved in anhydrous MeCN (100 mL). The resulting mixture was stirred at rt for 30 minutes, then ethyl isonipecotate (19) (3.1 g, 20 mmol) was added. The mixture was stirred at rt for 12 hours. The solvent was removed in vacuo and the residue was dissolved in ethyl acetate (30 mL) and washed sequentially with water (2 x 30 mL), saturated $NaHCO_3$ aqueous solution (2 x 30 mL), 10% aqueous citric acid (2 x 30 mL) and brine (2 x 20 mL). The organic layer was dried over anhydrous sodium sulfate (Na_2SO_4), filtered and evaporated under reduced pressure. The residue was treated with isopropyl ether (iPr_2O) and the formed solid was filtered off, dried and used in the next step without further purification. Yield 77% M.p. 155-156 °C. ^{1}H NMR (DMSO-d_6): δ 1.17 (t, *J* = 7.5 Hz, 3H CH_3), 1.52 (m, 2H, CH_2), 1.78-1.92 (m, 2H, CH_2), 2.63-2.95 (m, 2H, CH_2), 3.09-3.43 (m, 2H, CH_2), 4.07 (q, *J* = 7.5 Hz, 2H, CH_2), 4.32 (m, 1H, CH), 7.42 (s, 2H, NH_2), 7.55 (d, *J* = 8.0 Hz, 2H, Ar), 7.85 (d, *J* = 8.0 Hz, 2H, Ar). IR (Nujol) 3328, 3225, 1732, 1596 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{20}N_2O_5S$ (340.39) %C 52.93, %H 5.92, %N 8.23, found %C 52.9, %H 5.90, %N 8.28. m/z 341.

4-(4-(Hydrazinecarbonyl)piperidine-1-carbonyl)benzenesulfonamide (21)

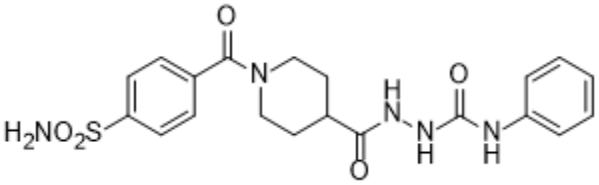


A mixture of ethyl 1-(4-sulfamoylbenzoyl)piperidine-4-carboxylate (20) (4.9 g, 15 mmol) and hydrazine monohydrate (2.5 mL, 45 mmol) in EtOH was refluxed overnight. After cooling, the formed precipitate was filtered off, washed with water (3 x 10 mL), dried and used in the next step without further purification. Yield 78% M.p. 191-192 °C. ^{1}H NMR (DMSO-d_6): δ 1.54 (m, 2H, CH_2), 1.73-1.84 (m, 2H, CH_2), 2.35-2.81 (m, 2H, CH_2), 3.04-3.48 (m, 2H, CH_2), 4.14 (s, 2H, NH_2) 4.44 (m, 1H, CH), 7.42 (s, 2H, NH_2), 7.55 (d, *J* = 8.5 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 8.99 (s, 1H, NH). IR (Nujol) 3309, 3208, 1614 cm^{-1}. Elemental analysis: calculated for $C_{13}H_{18}N_4O_4S$ (326.37) %C 47.84, %H 5.56, %N 17.17, found %C 47.90, %H 5.54, %N 17.21. m/z 327.

General procedure for the preparation of 4-sulfamoylbenzoyl-piperidine-4-carbonyl-hydrazin-amides (UR1-10)

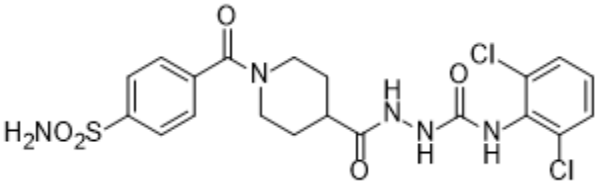
A mixture of 4-(4-(hydrazinecarbonyl)piperidine-1-carbonyl)benzenesulfonamide (0.326 g, 1 mmol) and the appropriate isocyanate (1 mmol) in EtOH (5 mL) was refluxed overnight. After cooling, the formed precipitate was filtered off, washed with Et_2O (2 x 5 mL) and recrystalized from EtOH.

***N*-Phenyl-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (UR1)**



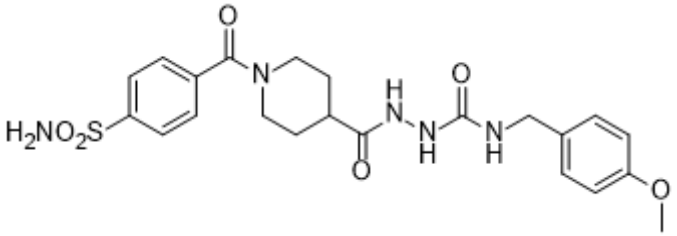
Following the general procedure, the title compound was prepared starting from phenylisocyanate. Yield 72% M.p. >250 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (m, 2H, CH_2), 1.71-1.84 (m, 2H, CH_2), 2.53-2.88 (m, 2H, CH_2), 3.08-3.49 (m, 2H, CH_2), 4.43 (m, 1H, CH), 6.93 (d, *J* = 7.0 Hz, 1H, Ar), 7.23 (m, 2H, Ar), 7.40 (m, 1H, Ar), 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 7.0 Hz, 2H, Ar), 7.85 (m, 1H, Ar), 7.87 (d, *J* = 7.5 Hz, 2H, Ar), 7.96 (s, 1H, NH), 8.67 (s, 1H, NH), 9.65 (s, 1H, NH). IR (Nujol) 3265, 1667, 1563 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{23}N_5O_5S$ (445.49) %C 53.92, %H 5.20, %N 15.72, found %C 53.88, %H 5.19, %N 15.76. m/z 446.

***N*-(2,6-Dichlorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (UR2)**



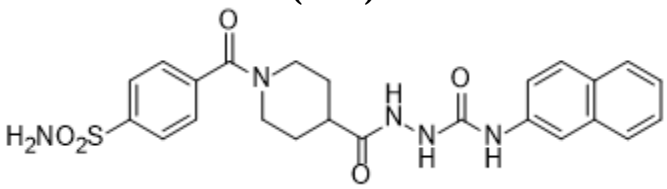
Following the general procedure, the title compound was prepared starting from 2,6-dichlorophenylisocyanate. Yield 78% M.p. 220-221 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (m, 2H, CH_2), 1.72-1.83 (m, 2H, CH_2), 2.84-3.05 (m, 2H, CH_2), 3.48-4.33 (m, 2H, CH_2), 4.43 (m, 1H, CH), 7.27 (d, *J* = 8.0 Hz, 1H, Ar), 7.42 (s, 1H, NH_2), 7.47 (d, *J* = 8.0 Hz, 2H, Ar), 7.56 (d, *J* = 7.0 Hz, 2H, Ar), 7.87 (d, *J* = 8.0 Hz, 2H, Ar), 8.29 (s, 1H, NH), 9.03 (s, 1H, NH), 9.73 (s, 1H, NH). IR (Nujol) 3316, 3216, 1614, 1534 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{21}Cl_2N_5O_5S$ (514.38) %C 46.70, %H 4.11, %N 13.62, found %C 46.75, %H 4.10, %N 13.66. m/z 514.

***N*-(4-Methoxybenzyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (UR3)**



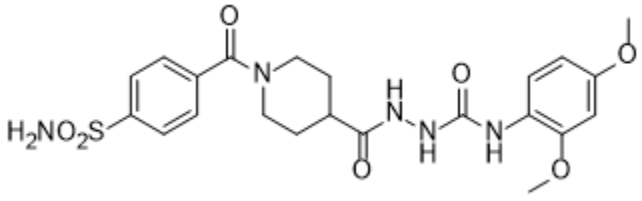
Following the general procedure, the title compound was prepared starting from 4-methoxybenzilisocyanate. Yield 66% M.p. > 250 °C. ^{1}H NMR (DMSO-d_6): δ 1.52 (m, 2H, CH_2), 1.67-1.82 (m, 2H, CH_2), 2.44-2.85 (m, 2H, CH_2), 3.05-3.48 (m, 2H, CH_2), 3.70 (s, 3H, OCH_3), 4.12 (s, 2H, CH_2), 4.43 (m, 1H, CH), 6.71 (s, 1H, NH), 6.84 (d, *J* = 8.5 Hz, 2H, Ar), 7.14 (d, *J* = 8.5 Hz, 2H, Ar), 7.42 (s, 2H, NH_2), 7.55 (d, *J* = 8.5 Hz, 2H, Ar), 7.72 (s, 1H, NH), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 9.49 (s, 1H, NH). IR (Nujol) 3383, 3306, 3214, 1613 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{27}N_5O_6S$ (489.54) %C 53.98, %H 5.56, %N 14.33, found %C 54.03, %H 5.58, %N 14.28. m/z 490.

***N*-(Naphthalen-2-yl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (UR4)**



Following the general procedure, the title compound was prepared starting from naphtylisocyanate. Yield 71% M.p. 233-234 °C. ^{1}H NMR (DMSO-d_6): δ 1.59 (m, 2H, CH_2), 1.73-1.88 (m, 2H, CH_2), 2.56-2.90 (m, 2H, CH_2), 3.09-3.52 (m, 2H, CH_2), 4.46 (m, 1H, CH), 7.44 (s, 2H, NH_2), 7.55 (m, 5H, Ar), 7.80 (m, 1H, Ar), 7.88 (d, *J* = 8.5 Hz, 2H, Ar), 7.91 (m, 1H, Ar), 8.05 (d, *J* = 8 Hz, 2H, Ar), 8.32 (s, 1H, NH), 8.75 (s, 1H, NH), 9.80 (s, 1H, NH). IR (Nujol) 3222, 1611, 1538 cm^{-1}. Elemental analysis: calculated for $C_{24}H_{25}N_5O_5S$ (495.55) %C 58.17, %H 5.08, %N 14.13, found %C 58.22, %H 5.10, %N 14.06. m/z 496.

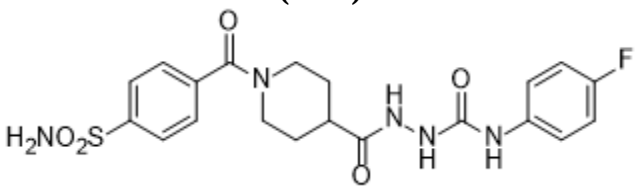
(2,4-Dimethoxyphenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (UR5)



Following the general procedure, the title compound was prepared starting from 2,4-dimethoxyphenylisocyanate. Yield 84% M.p. >250 °C. ^{1}H NMR (DMSO-d_6): δ 1.56 (m, 2H, CH_2), 1.70-1.86 (m, 2H, CH_2), 2.54-2.84 (m, 2H, CH_2), 3.07-3.50 (m, 2H, CH_2), 3.71 (s, 3H, OCH_3), 3.81

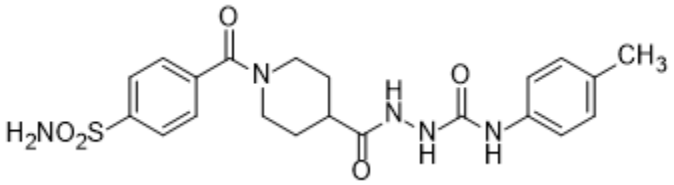
(s, 3H, OCH_3), 4.44 (m, 1H, CH), 6.44 (m, 1H, Ar), 6.58 (s, 1H, NH), 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.82 (d, *J* = 8.0 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 8.38 (s, 1H, NH), 9.71 (s, 1H, NH). IR (Nujol) 3332, 3212, 1615 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{27}N_5O_7S$ (505.54) %C 52.27, %H 5.38, %N 13.85, found %C 52.20, %H 5.40, %N 13.90. m/z 506.

_N_-(4-Fluorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (UR6)



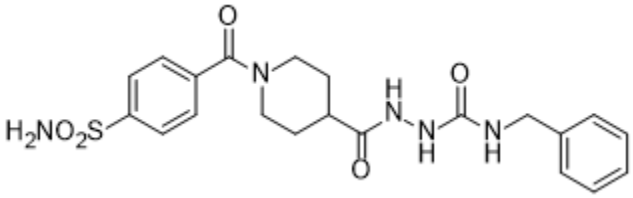
Following the general procedure, the title compound was prepared starting from 4-fluorophenylisocyanate. Yield 65% M.p. 209-210 °C. ^{1}H NMR (DMSO-d_6): δ 1.54 (m, 2H, CH_2) 1.72-1.81 (m, 2H, CH_2), 2.55-2.86 (m, 2H, CH_2), 3.08-3.51 (m, 2H, CH_2), 4.43 (m, 1H, CH), 7.11 (d, *J* = 8.5 Hz, 2H, Ar), 7.36 (d, *J* = 8.5 Hz, 2H, Ar), 7.42 (s, 2H, NH_2), 7.57 (d, *J* = 8.0 Hz, 2H, Ar), 7.88 (d, *J* = 8.5 Hz, 2H, Ar), 7.92 (s, 1H, NH), 8.63 (s, 1H, NH), 9.58 (s, 1H, NH). IR (Nujol) 3311, 1612, 1508 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{22}FN_5O_5S$ (463.48) %C 51.83, %H 4.78, %N 15.11, found %C 51.87, %H 4.79, %N 15.05. m/z 464.

2-(1-(4-Sulfamoylbenzoyl)piperidine-4-carbonyl)-_N_-(p-tolyl)hydrazinecarboxamide (UR7)



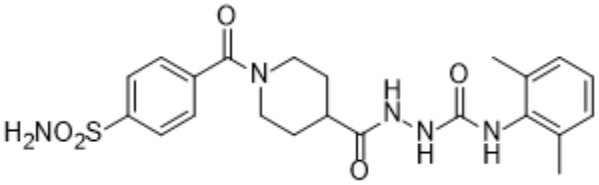
Following the general procedure, the title compound was prepared starting from 4-methylphenylisocyanate. Yield 82% M.p. 233 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (m, 2H, CH_2), 1.69-1.84 (m, 2H, CH_2), 2.21 (s, 3H, CH_3), 2.51-2.88 (m, 2H, CH_2), 3.08-3.49 (m, 2H, CH_2), 4.44 (m, 1H, CH), 7.03 (d, *J* = 8.5 Hz, 2H, Ar), 7.29 (d, *J* = 8.5 Hz, 2H, Ar), 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 7.90 (s, 1H, NH), 8.56 (s, 1H, NH), 9.65 (s, 1H, NH). IR (Nujol) 3332, 3228, 1599, 1536 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{25}N_5O_5S$ (459.52) %C 54.89, %H 5.48, %N 15.24, found %C 54.94, %H 5.46, %N 15.18. m/z 460.

***N*-Benzyl-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (UR8)**



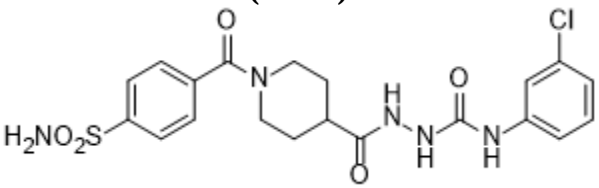
Following the general procedure, the title compound was prepared starting from benzylisocyanate. Yield 70% M.p. 246-247 °C. ^{1}H NMR (DMSO-d_6): δ 1.52 (m, 2H, CH_2), 1.72-1.89 (m, 2H, CH_2), 2.45-2.85 (m, 2H, CH_2), 3.06-3.49 (m, 2H, CH_2), 4.43 (m, 1H, CH), 4.72 (s, 2H, CH_2), 7.19 (m, 1H, Ar), 7.22 (m, 4H, Ar), 7.43 (s, 2H, NH_2), 7.55 (d, *J* = 8.5 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 8.35 (s, 1H, NH), 9.25 (s, 1H, NH), 9.78 (s, 1H, NH). IR (Nujol) 3225, 1659, 1539 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{25}N_5O_5S$ (459.52) %C 53.92, %H 5.20, %N 15.72, found %C 53.95, %H 5.18, %N 15.68. m/z 446.

***N*-(2,6-Dimethylphenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (UR9)**



Following the general procedure, the title compound was prepared starting from 2,6-dimethylphenylisocyanate. Yield 85% M.p. 212-213 °C. ^{1}H NMR (DMSO-d_6): δ 1.56 (m, 2H, CH_2), 1.70-1.86 (m, 2H, CH_2), 2.14 (s, 6H, CH_3), 2.54-2.84 (m, 2H, CH_2), 3.07-3.50 (m, 2H, CH_2), 4.44 (m, 1H, CH), 7.02 (m, 3H, Ar), 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 7.5 Hz, 2H, Ar), 7.81 (s, 1H, NH), 7.85 (s, 1H, NH), 7.87 (d, *J* = 8.0 Hz, 2H, Ar), 9.65 (s, 1H, NH). IR (Nujol) 3323, 3224, 1611 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{27}N_5O_5S$ (473.55) %C 55.80, %H 5.75, %N 14.79, found %C 55.85, %H 5.76, %N 14.72. M/z 474.

***N*-(3-Chlorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (UR10)**



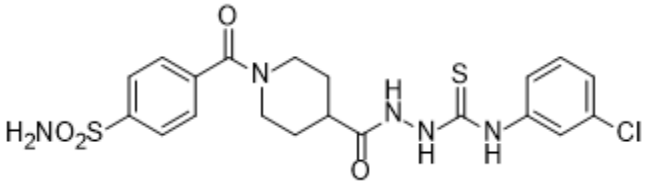
Following the general procedure, the title compound was prepared starting from 3-chlorophenylisocyanate. Yield 79% M.p. 231-232 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (m, 2H, CH_2), 1.76-1.91 (m, 2H, CH_2), 2.53-2.88 (m, 2H, CH_2), 3.08-3.51 (m, 2H, CH_2), 4.44 (m, 1H, CH), 7.20 (s, 1H, Ar), 7.34 (m, 3H, Ar) 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 7.5 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 9.59 (s, 1H, NH), 9.67 (s, 1H, NH), 9.93 (s, 1H, NH). IR (Nujol) 3309, 3183, 3094, 1680,

1546 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{22}ClN_5O_5S$ (479.94) %C 50.05, %H 4.62, %N 14.59, found %C 50.09, %H 4.60 %N 14.64. M/z 480.

General procedure for the preparation of N-aryl-4-sulfamoylbenzoyl-piperidine-4-carbonyl-hydrazincarbothioamides (ThioUR1-13)

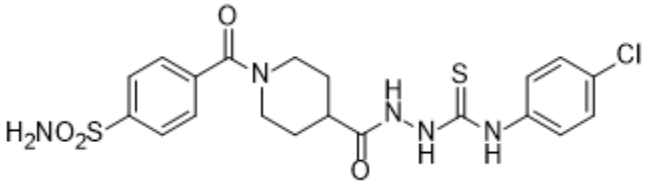
A mixture of 4-(4-(hydrazinecarbonyl)piperidine-1-carbonyl)benzenesulfonamide (0.326 g, 1 mmol) and the appropriate isothiocyanate (1 mmol) in absolute EtOH (5 mL) was refluxed overnight. After cooling, the formed precipitate was filtered off, washed with Et_2O (2 x 5 mL) and recrystalized from EtOH.

***N*-(3-Chlorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR1)**



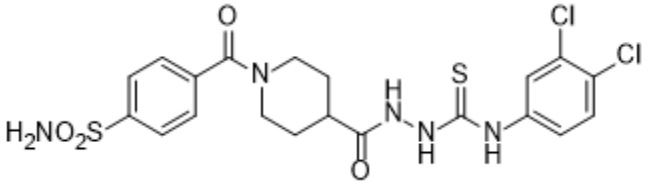
Following the general procedure, the title compound was prepared starting from 3-chlorophenylisothiocyanate. Yield 61% M.p. 226-227 °C. ^{1}H NMR (DMSO-d_6): δ 1.52 (m, 2H, CH_2), 1.72-1.89 (m, 2H, CH_2), 2.53-2.85 (m, 2H, CH_2), 3.05-3.49 (m, 2H, CH_2), 4.43 (m, 1H, CH), 7.19 (d, *J* = 7.0 Hz, 1H, Ar), 7.25 (m, 3H, Ar), 7.42 (s, 2H, NH_2), 7.55 (d, *J* = 8.0 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 8.33 (s, 1H, NH), 9.24 (s, 1H, NH), 9.77 (s, 1H, NH). IR (Nujol) 3277, 3175, 3087, 1677, 1544 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{22}ClN_5O_4S_2$ (496.00) %C 48.43, %H 4.47, %N 14.12, found %C 48.38, %H 4.45, %N 14.17. M/z 497.

***N*-(4-Chlorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR2)**



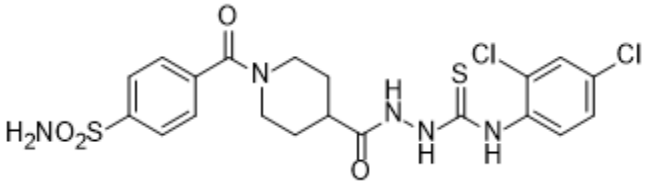
Following the general procedure, the title compound was prepared starting from 4-chlorophenylisothiocyanate. Yield 79% M.p. 222-223 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (s, 2H, CH_2), 1.74-1.91 (m, 2H, CH_2), 2.63-2.88 (m, 2H, CH_2), 3.08-3.51 (m, 2H, CH_2), 4.46 (m, 1H, CH), 7.36 (d, *J* = 8.5 Hz, 2H, Ar), 7.42 (s, 2H, NH_2), 7.46 (m, 2H, Ar), 7.56 (d, *J* = 7.5 Hz, 2H, Ar), 7.86 (d, *J* = 8.0 Hz, 2H, Ar), 9.59 (s, 2H, NH), 9.91 (s, 1H, NH). IR (Nujol) 3322, 3290, 3185, 1686, 1590 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{22}ClN_5O_4S_2$ (596.00) %C 48.43, %H 4.47, %N 14.12, found %C 48.39, %H 4.48, %N 14.11. m/z 597.

***N*-(3,4-Dichlorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR3)**



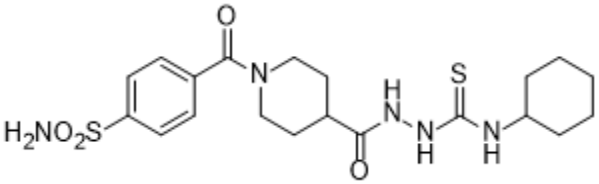
Following the general procedure, the title compound was prepared starting from 3,4-dichlorophenylisothiocyanate. Yield 97% M.p. 232-233 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (m, 2H, CH_2), 1.76-1.91 (m, 2H, CH_2), 1.53-2.88 (m, 2H, CH_2), 3.08-3.52 (m, 2H, CH_2), 4.45 (m, 1H, CH), 7.43 (s, 2H, NH_2), 7.47 (d, *J* = 8.0 Hz, 2H, Ar), 7.56 (d, *J* = 7.0 Hz, 2H, Ar), 7.82 (s, 1H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 9.64 (s, 1H, NH), 9.76 (s, 1H, NH), 9.95 (s, 1H, NH). IR (Nujol) 3343, 3271, 3149, 1683, 1540 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{21}Cl_2N_5O_4S_2$ (530.45) %C 45.29, %H 3.99, %N 13.20, found %C 45.33, %H 3.97, %N 13.25. M/z 531.

***N*-(2,4-Dichlorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR4)**



Following the general procedure, the title compound was prepared starting from 2,4-dichlorophenylisothiocyanate. Yield 81% M.p. 220-221 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (m, 2H, CH_2); 1.74-1.89 (m, 2H, CH_2), 2.53-2.87 (m, 2H, CH_2), 3.07-3.50 (m, 2H, CH_2), 4.44 (m, 1H, CH), 7.39 (m, 2H, Ar), 7.42 (s, 2H, NH_2), 7.54 (s, 1H, Ar), 7.55 (d, *J* = 8.0 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 9.35 (s, 1H, NH), 9.74 (s, 1H, NH), 9.99 (s, 1H, NH). IR (Nujol) 3243, 1681, 1532 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{21}N_5O_5S_2$ (530.45) %C 45.29, %H 3.99, %N 13.20, found %C 45.24, %H 4.02, %N 13.17. M/z 531.

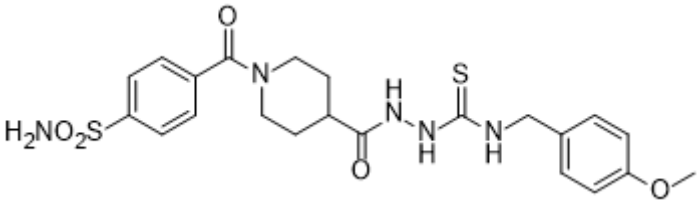
***N*-Cyclohexyl-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR5)**



Following the general procedure, the title compound was prepared starting from ciclohexylisothiocyanate. Yield 67% M.p. 183-184 °C. ^{1}H NMR (DMSO-d6) δ 1.06 (m, 2H, CH_2), 1.22 (m, 4H, CH_2), 1.54 (m, 4H, CH_2), 1.56 (m, 2H, CH_2), 1.77-1.86 (m, 2H, CH_2), 2.53-2.86 (m, 2H, CH_2), 3.06-3.50 (m, 2H, CH_2), 4.03 (m, 1H, CH), 4.43 (m, 1H, CH), 7.36 (s, 1H, NH), 7.43 (s, 2H, NH_2), 7.55 (d, *J* = 7.5 Hz, 2H, Ar), 7.86 (d, *J* = 8.0 Hz, 2H, Ar), 8.99 (s, 1H, NH), 9.64 (s, 1H,

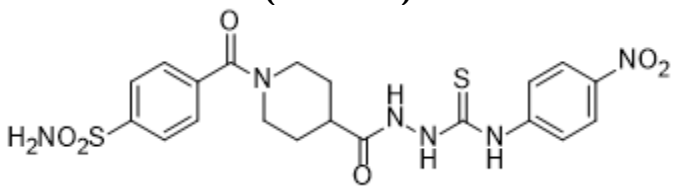
NH). IR (Nujol) 3324, 3177, 1672, 1555 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{29}N_5O_4S_2$ (467.61) %C 51.37, %H 6.25, %N 14.98, found %C 51.42, %H 6.26, %N 14.92. M/z 468.

_N_-(4-Methoxybenzyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR6)



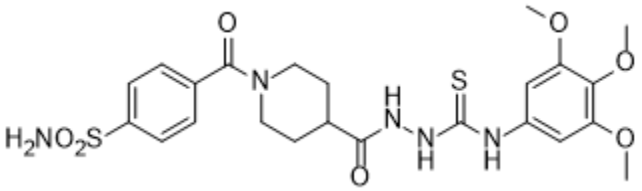
Following the general procedure, the title compound was prepared starting from 4-methoxbenzylisothiocyanate. Yield 64% M.p. 240-241 °C. ^{1}H NMR (DMSO-d_6): δ 1.51 (m, 2H, CH_2), 1.72-1.88 (m, 2H, CH_2), 2.54-2.85 (m, 2H, CH_2), 3.04-3.49 (m, 2H, CH_2), 3.71 (s, 3H, OCH_3), 4.42 (m, 1H, CH), 4.62 (s, 2H, CH_2), 6.84 (d, *J* = 8.5 Hz, 2H, Ar), 7.19 (d, *J* = 8.5 Hz, 2H, Ar), 7.42 (s, 2H, NH_2), 7.54 (d, *J* = 8.5 Hz, 2H, Ar), 7.85 (d, *J* = 8 Hz, 2H, Ar), 8.23 (s, 1H, NH), 9.18 (s, 1H, NH), 9.73 (s, 1H, NH). IR (Nujol) 3347, 3249, 3150, 1669, 1548 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{27}N_5O_5S_2$ (505.61) %C 52.26, %H 5.38, %N 13.85, found %C 52.31, %H 5.36, %N 13.90. M/z 506.

_N_-(4-Nitrophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR7)



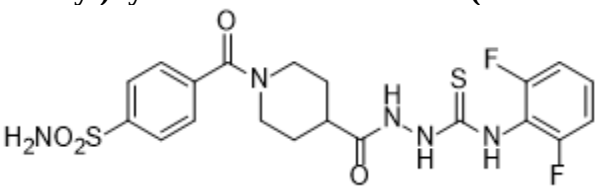
Following the general procedure, the title compound was prepared starting from 4-nitrophenylisothiocyanate. Yield 85% M.p. 236-237 °C. ^{1}H NMR (DMSO-d6) δ 1.56 (m, 2H, CH_2), 1.76-1.91 (m, 2H, CH_2), 2.56-2.89 (m, 2H, CH_2), 3.09-3.52 (m, 2H, CH_2), 4.45 (m, 1H, CH), 7.43 (s, 2H, NH_2), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.87 (m, 4H, Ar), 8.19 (d, *J* = 9.0 Hz, 2H, Ar), 9.86 (s, 1H, NH), 9.93 (s, 1H, NH), 9.97 (s, 1H, NH). IR (Nujol) 3317, 3220, 3137, 1678, 1598 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{22}N_6O_6S_2$ (506.46) %C 47.42, %H 4.38, %N 16.59, found %C 47.47, %H 4.39, %N 16.63. M/z 507.

2-(1-(4-Sulfamoylbenzoyl)piperidine-4-carbonyl)-N-(3,4,5-trimethoxyphenyl)hydrazinecarbothioamide (ThioUR8)



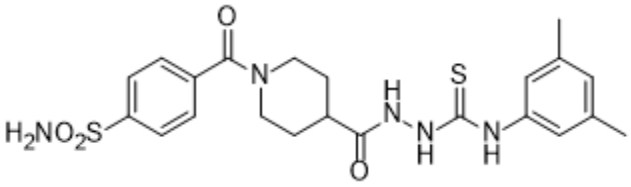
Following the general procedure, the title compound was prepared starting from 2,6-difluorophenylisothiocyanate. Yield 44% M.p. >250 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (m, 2H, CH_2), 1.75-1.90 (m, 2H, CH_2), 2.53-2.87 (m, 2H, CH_2), 3.08-3.52 (m, 2H, CH_2), 3.63 (s, 3H, OCH_3), 3.73 (s, 6H, OCH_3), 4.44 (m, 1H, CH), 6.82 (s, 2H, Ar), 7.43 (s, 2H, NH_2), 7.56 (d, *J* = 8 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 9.48 (s, 2H, NH), 9.88 (s, 1H, NH). IR (Nujol) 3533, 3284, 3168, 1692, 1565 cm^{-1}. Elemental analysis: calculated for $C_{23}H_{29}N_5O_7S_2$ (551.64) %C 50.08, %H 5.30, %N 12.70, found %C 50.01, %H 5.32, %N 12.66. M/z 552.

***N*-(2,6-Difluorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR9)**



Following the general procedure, the title compound was prepared starting from 2,6-difluorophenylisothiocyanate. Yield 77% M.p. 242-243 °C. ^{1}H NMR (DMSO-d_6) δ 1.55 (m, 2H, CH_2), 1.76-1.92 (m, 2H, CH_2), 2.55-2.86 (m, 2H, CH_2), 3.07-3.38 (m, 2H, CH_2), 4.45 (m, 1H, CH), 7.11 (d, *J* = 8.0 Hz, 2H, Ar), 7.33 (d, *J* = 7.0 Hz, 1H, Ar), 7.43 (s, 2H, NH_2), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 9.15 (s, 1H, NH), 9.83 (s, 1H, NH), 10.02 (s, 1H, NH). IR (Nujol) 3314, 3279, 3201, 1658, 1566 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{21}F_2N_5O_4S_2$ (497.54) %C 48.28, %H 4.25, %N 14.08, found %C 48.32, %H 4.27, %N 14.12. M/z 498.

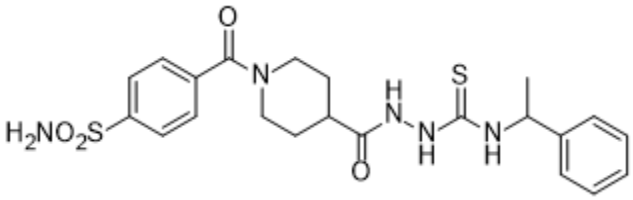
***N*-(3,5-Dimethylphenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR10)**



Following the general procedure, the title compound was prepared starting from 3,5-dimethylphenylisothiocyanate. Yield 90% M.p. 216-217 °C. ^{1}H NMR (DMSO-d_6): δ 1.56 (m, 2H, CH_2), 1.70-1.84 (m, 2H, CH_2), 2.19 (s, 6H, CH_3), 2.53-2.89 (m, 2H, CH_2), 3.09-3.50 (m, 2H, CH_2), 4.45 (m, 1H, CH), 6.58 (s, 2H, Ar), 7.04 (s, 2H, Ar), 7.43 (s, 2H, NH_2), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.87 (d, *J* = 8.5 Hz, 2H, Ar), 7.91 (s, 1H, NH), 8.51 (s, 1H, NH), 9.65 (s, 1H, NH). IR (Nujol) 3289,

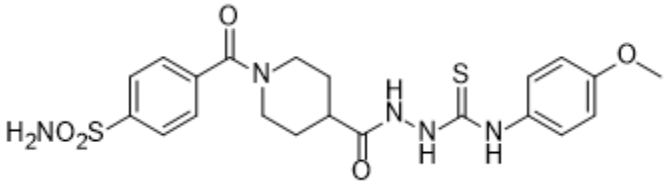
3068, 1644, 1556 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{27}N_5O_4S_2$ (489.61) %C 53.97, %H 5.56, %N 14.30, found %C 53.94, %H 5.61, %N 14.35. M/z 490.

_N_-(1-Phenylethyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR11)



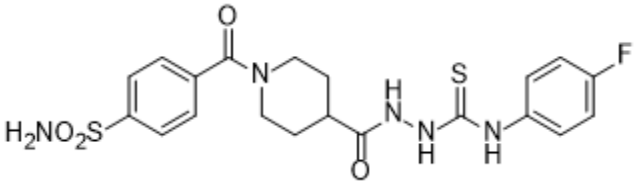
Following the general procedure, the title compound was prepared starting from phenylethylisothiocyanate. Yield 49% M.p. 196-197 °C. ^{1}H NMR (DMSO-d_6): δ 1.41 (d, *J* = 7.0, 3H, CH_3), 1.53 (m, 2H, CH_2), 1.72-1.88 (m, 2H, CH_2), 2.62-2.86 (m, 2H, CH_2), 3.06-3.49 (m, 2H, CH_2), 4.43 (s, 1H, CH), 5.57 (m, 1H, CH), 7.20 (m, 1H, Ar), 7.29 (m, 4H, Ar), 7.42 (s, 2H, NH_2), 7.55 (d, *J* = 8.5 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 7.99 (s, 1H, NH), 9.14 (s, 1H, NH), 9.71 (s, 1H, NH). IR (Nujol) 3335, 3230, 3087, 1688, 1597 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{27}N_5O_4S_2$ (489.61) %C 53.97, %H 5.56, %N 14.30, found %C 53.91, %H 5.58, %N 14.22. M/z 490.

_N_-(4-Methoxyphenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR12)



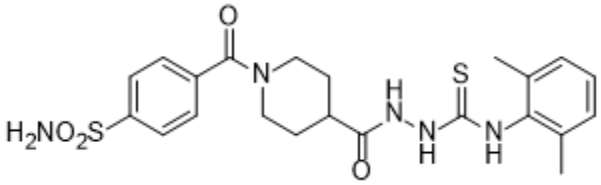
Following the general procedure, the title compound was prepared starting from 4-methoxyphenylisothiocyanate. Yield 84% M.p. >250 °C. ^{1}H NMR (DMSO-d6) δ 1.54 (m, 2H, CH_2), 1.75-1.91 (m, 2H, CH_2), 1.54-1.87 (m, 2H, CH_2), 3.07-3.51 (m, 2H, CH_2), 3.73 (s, 3H, OCH_3), 4.44 (m, 1H, CH), 6.87 (d, *J* = 8.0 Hz, 2H, Ar), 7.24 (d, *J* = 7.5 Hz, 2H, Ar), 7.43 (s, 2H, NH_2), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 9.38 (s, 1H, NH), 9.43 (s, 1H, NH), 9.86 (s, 1H, NH). IR (Nujol) 3335, 323, 1680, 1543cm^{-1}. Elemental analysis: calculated for $C_{21}H_{25}N_5O_5S_2$ (491.58) %C 51.31, %H 5.13, %N 13.05, found %C 51.35, %H 5.11, %N 13.11. M/z 492.

***N*-(4-Fluorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR13)**



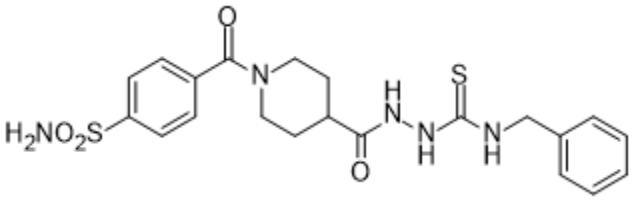
Following the general procedure, the title compound was prepared starting from 4-fluorophenylisothiocyanate. Yield 85% M.p. 215-216 °C. ^{1}H NMR (DMSO-d6) δ 1.55 (m, 2H, CH_2), 1.76-1.91 (m, 2H, CH_2), 2.53-2.87 (m, 2H, CH_2), 3.08-3.51 (m, 2H, CH_2), 4.45 (m, 1H, CH), 7.14 (d, *J* = 8.5 Hz, 2H, Ar), 7.39 (m, 2H, Ar), 7.45 (s, 2H, NH_2), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.87 (d, *J* = 8.0 Hz, 2H, Ar), 9.54 (s, 2H, NH), 9.90 (s, 1H, NH). IR (Nujol) 3320, 3175, 1685, 1563cm^{-1}. Elemental analysis: calculated for $C_{20}H_{22}FN_5O_4S_2$ (479.55) %C 50.09, %H 4.62, %N 14.60, found %C 50.01, %H 4.60, %N 14.54. M/z 480.

***N*-(2,6-Dimethylphenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR14)**



Following the general procedure, the title compound was prepared starting from 2,6-dimhetylphenylisothiocyanate. Yield 64% M.p. 233-234 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (m, 2H, CH), 1.70-1.86 (m, 2H, CH_2), 2.14 (s, 6H, $2CH_3$), 2.52-2.86 (m, 2H, CH_2), 3.07-3.36 (m, 2H, CH_2), 4.45 (m, 1H, CH), 7.16 (d, *J* = 8.0 Hz, 2H, Ar), 7.39 (d, *J* = 7.0 Hz, 1H, Ar), 7.43 (s, 2H, NH_2), 7.58 (d, *J* = 8.0 Hz, 2H, Ar), 7.89 (d, *J* = 8.5 Hz, 2H, Ar), 9.11 (s, 1H, NH), 9.86 (s, 1H, NH), 10.05 (s, 1H, NH). IR (Nujol) 3330, 3238, 1689, 1518cm^{-1}. Elemental analysis: calculated for $C_{22}H_{27}N_5O_4S_2$ (489.61) %C 53.97, %H 5.56, %N 14.30, found %C 54.04, %H 5.54, %N 14.26. M/z 490.

***N*-Benzyl-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarbothioamide (ThioUR15)**



Following the general procedure, the title compound was prepared starting from benzylisothiocyanate. Yield 83% M.p. >250 °C. ^{1}H NMR (DMSO-d_6): δ 1.52 (m, 2H, CH_2); 1.73-1.88 (m, 2H, CH_2), 2.45-2.84 (m, 2H, CH_2) 3.10-3.49 (s, 2H, CH_2), 4.42 (m, 1H, CH), 4.70 (s, 2H, CH_2), 7.19 (m, 1H, Ar), 7.24 (m, 4H, Ar), 7.46 (s, 2H, NH_2), 7.59 (d, *J* = 8.0 Hz, 2H, Ar), 7.88 (d, *J* = 8.5 Hz, 2H, Ar), 8.33 (s, 1H, NH), 9.24 (s, 1H, NH), 9.77 (s, 1H, NH). IR (Nujol) 3333, 3244,

1688, 1560 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{25}N_5O_4S_2$ (475.58) %C 53.03, %H 5.30, %N 14.73, found %C 52.96, %H 5.29, %N 14.78. M/z 476.

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3.0 Multitarget compounds

3.1 Dual Carbonic Anhydrase (CA)-Steroid Sulfatase (STS) inhibitors

As mentioned above, CA is a well-established target in drug discovery due to the implication of some isoforms in different diseases, such as glaucoma and cancer. It has already proved the importance of sulfamate group as a zinc binder in the development of CA inhibitors, but sulfamate group is also crucial for the inhibition of Steroid Sulfatase (STS). STS allows the conversion of inactive sulfated steroids into active non-sulfated steroid and catalyse the conversion of dehydroepiandrosterone sulfate to dehydroepiandrosterone by hydrolysis[1] (Figure 3.1).

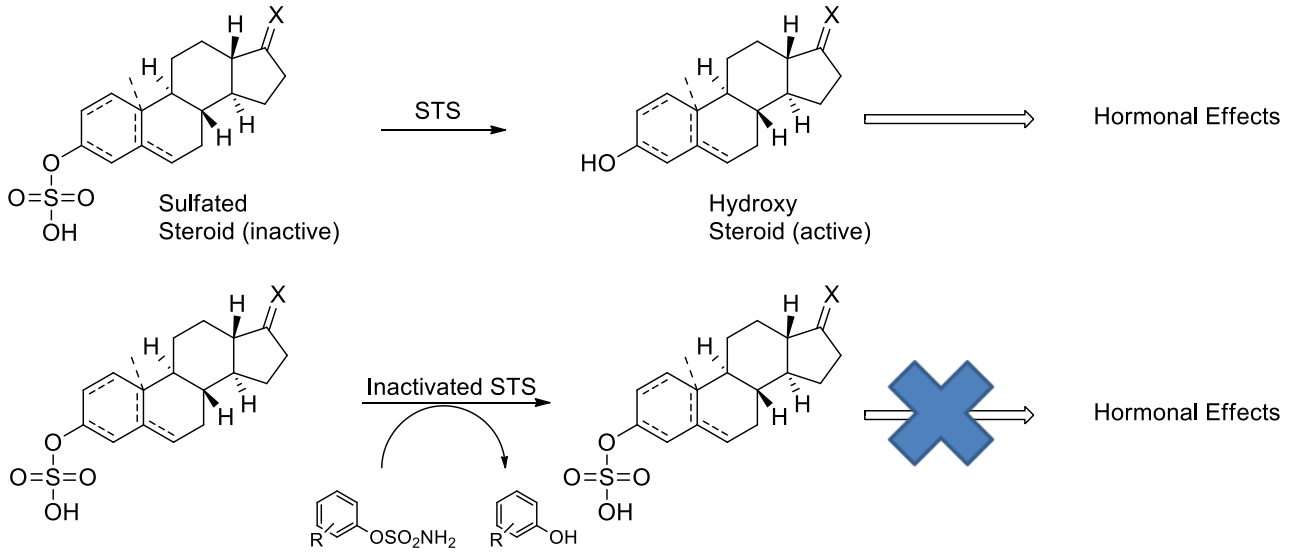


Figure 3.1. Mechanism of action of STS and STS inhibitors.

STS is widely distributed in all the human body and due to its implication in steroid hormones pathways, is related to hormone dependent cancers[2] such as breast cancer. Different STS inhibitors have been described in literature and the first generation of STS inhibitors was steroid based. For instance, estrone and estradiol-based sulfamate have been described as irreversible active-site directed STS-inhibitors, but due to their potent estrogenic activity they were not considered suitable for clinical studies as anticancer drug candidates.[3] Among them, estradiol-3-O-sulfamate is currently in phase II human clinical trials as a prodrug of estradiol in hormone replacement therapy.[4,5] To overcome the limitations from steroidal based STS inhibitors, several structurally diverse inhibitors bearing sulfamate moiety have been reported.[4] The first non-steroidal STS inhibitors were coumarin-7-O-sulfamate derivatives which showed high STS inhibitory activity and no significant estrogenicity[6] (Figure 3.2). Irosustat/STX64 displayed potent STS inhibitory activity and reached clinical trials[4,5]. Recently, clinical report relates to Irosustat in combination with aromatase inhibitor[7] demonstrated the interesting antiproliferative effect of Irustat in early breast cancer treatment-naïve patients. All the arylsulfamate based drugs led to irreversible inactivation of

the enzyme by sulfamoyl group transfer to the hydrated formyl glycine residue in the active site.[5] Different studies have been focused on the development of reversible inhibitors without sulfamate moiety and all reported inactive compounds.[8-10] Starting from these considerations, in this thesis the development of non-steroidal STS/CA inhibitors bearing sulfamate moiety was studied. Non-steroidal STS inhibitors often present a sulfamoylated fused AB phenolic ring steroid surrogate motif, although compounds endowed with single ring showed good potency.[11] Recently, El-Gamal et al.[11] developed a series of arylamide derivatives bearing the arylsulfamate moiety linked to an aliphatic region by amide bond. The compound A (Figure 3.2) bearing a cyclohexyl ring had an IC_{50} of 421 nM and an interesting STS inhibitory activity in whole cells, showing potential for further optimization.

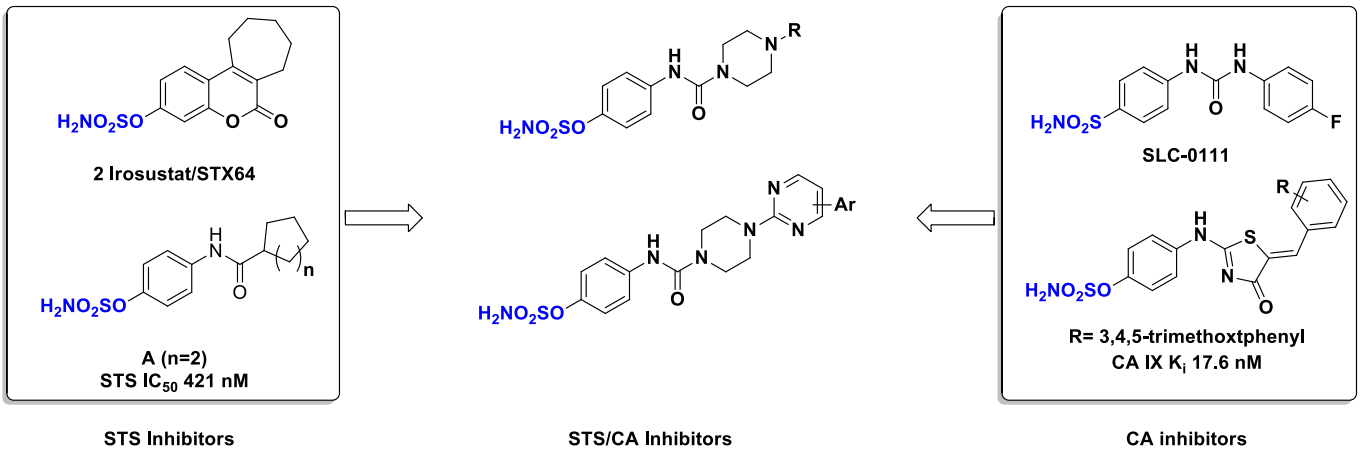
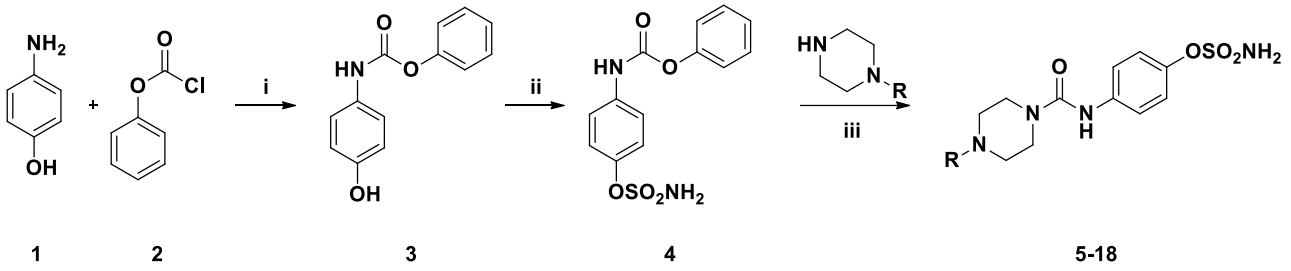


Figure 3.2. *Design of new CA/STS inhibitors.*

On the other hand, it has already reported the importance of SLC-0111 and its analogs as selective tumor associated hCA IX/XII inhibitors. Starting from the structures of STS inhibitors and CA inhibitors, two series of urea derivatives have been designed as dual STS/CA inhibitors. The solfonamide group of SLC-0111 was replaced by a sulfamate group, in order to obtain the dual CA/STS activity.[12] The arylamide group was changed into an arylureido group, linked to a substituted piperazine to generate chemical diversity and to improve physicochemical properties.

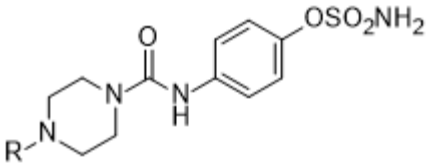
The synthesis of the first series is reported in scheme 1. The hydroxylarylcarbamate **3** was obtained in good yield by reacting the 4-aminophenol **1** with phenylchloroformate **2** in the presence of N,N-diisopropyletyilamine (DIPEA) in dry tetrahydrofuran (THF). The next step was the sulfamoylation of aryl carbamate **3** upon treatment with freshly prepared sulfamoyl chloride[13] in DMA solution to obtain the key intermediate **4**. Coupling of **4** with 1-substituted piperazines in dimethylsulphoxide (DMSO) gave the desired piperazinyl urea derivatives **5-18**.



Scheme 3.1. Reagents and conditions: (i) DIPEA, THF, r.t. 24 h; (ii) $ClSO_2NH_2$, DMA, r.t. 12 h; (iii) Substituted piperazine, DMSO, r.t. 24 h.

The inhibitory activity against hCAI, hCAII, hCAIX and hCAXII of the sulfamate derivatives was tested as above described[12] and it is shown in Table 3.1.

Table 3.1. Inhibition data of human CA isoforms hCA I, II, IX and XII with derivatives **5–18** reported here and the standard sulfonamide inhibitor AAZ by a stopped flow CO_2 hydrase assay (errors were in the range of ±5–10% of the reported values).



Compound	R	K_i (nM)			
		hCAI	**hCAII**	**hCAIX**	**hCAXII**
5	Benzyl	316.5	11.7	10.5	1.0
6	Phenyl	896.8	71.9	11.1	1.0
7	3-methylphenyl	851.5	15.9	0.91	35.8
8	4-chlorophenyl	581.3	16.9	10.4	84.5
9	2-methylphenyl	692.0	56.9	29.7	-
10	4-fluorophenyl	9.4	18.2	61.5	64.7
11	4-methoxyphenyl	282.4	9.1	114.1	1.1
12	3-methoxyphenyl	88.1	11.2	34.1	37.0
13	3,4-dichlorophenyl	918.8	45.4	90.3	-
14	2,3-dimethylphenyl	63.5	11.1	32.3	1.0
15	benzofuran-2-ylmethyl	760.3	36.2	32.7	-
16	n-heptyl	637.3	31.1	61.0	-
17	n-octyl	752.3	15.9	31.4	-
18	n-decyl	680.1	21.0	32.2	-
AAZ	-	250	12.5	25	5.7

The 4-fluorophenyl substituted sulfamate **10** was a potent hCAI inhibitor, with an inhibition constant of 9.4 nM, whereas the replacement of the fluorine with a chlorine atom led to a reduction in activity (**8** and **13**). Likewise, the unsubstituted compound **6** and methyl substituted compounds **7** and **9** showed weak efficacy against hCAI with inhibition constants (K_is) ranging between 692 and 897 nM.

On the contrary, the presence of a 2,3-dimethylphenyl group (compound **14**) or 3-methoxyphenyl group (compound **12**) restored the activity. The displacement of the methoxy group in 4-position produced about a third reduction in activity (**11**, Ki value 292 nM). The replacement of the aryl ring with an heteroaryl as in compound **15** or with alkyl chains as in compounds **16**-**18** also afforded weak inhibitors of this slow cytosolic hCA isoform.

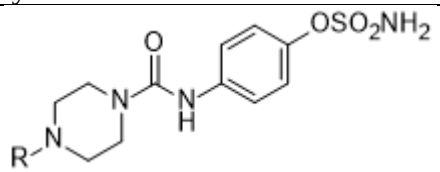
Speaking about hCAII, the unsubstituted compound **6** showed both the worse K_I against CA II and an approximately 7-fold II/IX selectivity. The 3-methylphenyl compound **7** showed a very potency to AAZ against CA II and a 16-fold II/IX selectivity. The shift of the methyl in the 2-position to give the analog **9** produced about a 4-fold reduction of activity against CA II as compared to compound **7**, while the selectivity toward CAIX was worsened. The alkyl substituted compound **18** and 4-chlorine derivative **8** showed about the same KIs values against CA II and CA IX (21.2 and 32.2 nM; 16.9 and 10.4 nM, respectively). The introduction of a second chlorine atom as in compound **13** reduced the activity against both isoforms (45.4 and 90.3 nM, respectively).

Concerning the activity against the cancer related isoform hCAIX, all the sulfamates showed activity in the range of 0.91 and 61.5 nM except for sulfamate **11** (Ki 114.1 nM) and sulfamate **13** (K_i 90.3 nM). The 3-methylphenyl derivative **7** showed the best activity of the series (K_i 0.91 nM). Displacing the methyl group into 2-position, as in sulfamate **9**, led to about 20-fold reduction in activity (Ki 29.7 nM) as well as the introduction of a second methyl group as in sulfamate **14** (Ki 34.1 nM). The replacement of the methyl group in 4-position with a methoxy group to give sulfamate **11** decreased the activity (Ki 114.1 nM), while the shift of methoxy group into 3-position partially restored the activity (sulfamate **12** Ki 34.1 nM). Remarkably, the phenyl (sulfamate **6**) and the benzyl (sulfamate **5**) derivatives showed good activity, with a Ki 11.1 nM and 1.05 nM respectively. Furthermore, the introduction of a chlorine atom in 4-position (sulfamate **8**) produced high inhibitory activity (Ki 10.4 nM), similar to sulfamate **5** and **6**. The introduction of a second chlorine in 3-position to give sulfamate **13** (Ki 90.3 nM) that resulted about 9-fold less potent than the mono-substituted analog **8**.

On the second cancer-related isoform hCAXII, sulfamates **5** (benzyl derivative), **6** (phenyl derivative), **11** (4-methoxyphenyl derivative) and **14** (2,3-dimethylphenyl derivative) showed potent activity, with Ki values in the 1.0 and 1.1 nM range, also confirming the good activity showed against CAIX. Displacing the methoxy group from 4- to 3-position (sulfamate **12** Ki 37.0 nM) resulted in about 37-fold reduced potency. Furthermore, the replacement of the 4-methoxy group with a chlorine atom (sulfamate **8** Ki 84.5 nM), or with a fluorine atom (sulfamate **10** Ki 64.7 nM) decreased the inhibitory activity.

The *in vitro* STS inhibition of activity of all the sulfamates was measured in an assay using a JEG-3 cell lysate and results are shown in Table 3.2. The *in vitro* inhibition results are reported as % of residual STS at 10μM inhibitor concentration and IC_{50} values were determined in the relevant cases. Compounds showing strong STS inhibition were selected for whole cell experiments to assess the ability to cross a lipid bilayer using intact monolayers of JEG-3 cells (Figure 3.3). All the compounds were tested by the Medicinal Chemistry & Drug Discovery group, Department of Pharmacology, University of Oxford.

Table 3.2. STS inhibitory activity of sulfamates **5-18**.



Compound	R	Residual STS % activity ± SD[a]
5	benzyl	18.7 ± 1.6
6	phenyl	16.9 ± 3.3
7	3-methylphenyl	10.4 ± 1.9
8	4-chlorophenyl	14.9 ± 1.6
9	2-methylphenyl	47.9 ± 6.3
10	4-fluorophenyl	93.5 ± 6.5
11	4-methoxyphenyl	37.0 ± 2.4
12	3-methoxyphenyl	26.3 ± 1.9
13	3,4-dichlorophenyl	23.0 ± 3.4
14	2,3-dimethylphenyl	5.9 ± 0.8
15	benzofuran-2-ylmethyl	20.2 ± 0.7
16	n-heptyl	27.7 ± 1.6
17	n-octyl	12.4 ± 2.7
18	n-decyl	3.5 ± 0.7

. [a]Residual activity after JEG-3 cell lysate treatment with 10μM inhibitor is shown.

Sulfamate **5** showed a good STS residual activity in both isolated enzyme assay (18.7% ± 1.6) and on intact JEG-3 cells (19.5% ± 1.3). The removal of the methylene group to obtain the sulfamate **6** did not have a significant influence in the inhibitory activity against the isolated enzyme (16.9% ± 3.3) whereas the activity on JEG-3 was about a half as compared to sulfamate **5** (32.8% ± 6.2). The introduction of a methyl group into 3-position of aromatic ring (sulfamate **7**) led to an increase of activity against the isolated enzyme (10.4% ± 1.9) and on intact JEG-3 cells (23.4% ± 1.1) as compared with sulfamate **6**. The shifting of the methyl group into the 2-position to give sulfamate **9** led to a reduction of activity (47.9% ± 6.3) while the introduction of a second methyl group (sulfamate **14**) resulted in a high activity on the isolated enzyme (5.9% ± 0.8).

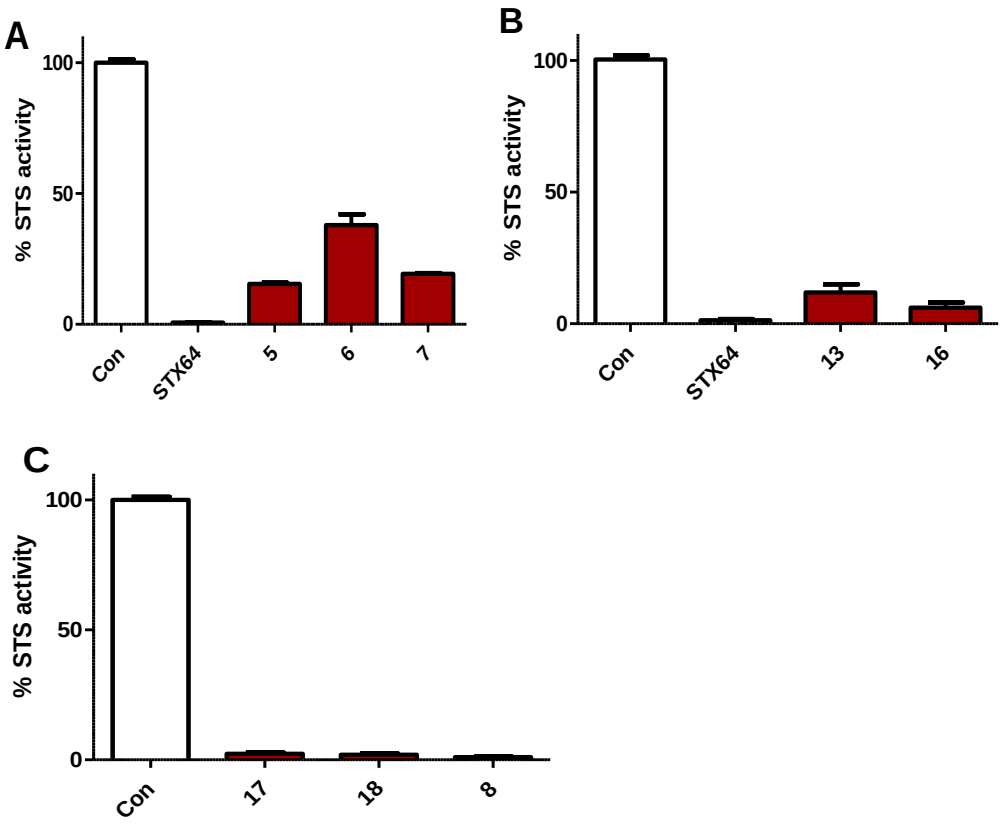


Figure 3.3. Evaluation of sulfamates **5-7** (A), **13** and **16** (B), **8**, **17** and **18** (C) in whole cell JEG-3. All compounds were tested at 10 µM, the reference inhibitor STX64 was used as positive control. All data represents mean ± S.D., n = 3.

The replacement of the 3-methyl group with a 3-methoxy group (sulfamate **12**) resulted in a reduction of activity (26.3% ± 1.9), as well as the shift of the methoxy group into 4-position to give sulfamate **11** (37.0% ± 2.4). The introduction of 4-fluorine (sulfamate **10**) led to an impressive reduction of activity (93.5% ± 6.5). Whereas the replacement of the fluorine atom with a chlorine in the same position to give sulfamate **8**, resulted in an increase of activity (14.9% ± 1.6). The introduction of a second chlorine atom (sulfamate **13**) in 3-position led to a reduction of activity on the isolated enzyme (23.0% ± 3.4). The replacement of the benzyl group with a benzofurylmethyl group (sulfamate **15**) did not afford significant change in inhibitory activity (20.2% ± 0.7) as compared to sulfamate **5**. The introduction of aliphatic chains to give sulfamates **16-18** produced good inhibitory activity and a correlation between the chain carbon atom number and the inhibitory activity emerged. Sulfamate **18** bearing N-decylpiperazine group resulted to be the best compound of the series (3.5% ± 0.7).

The compounds showing the best inhibitory activity were evaluated for their IC_{50} in STS inhibition (Figure 3.4). Sulfamates **13** and **16** showed IC_{50} of 1.23 µM and 1.69 µM whereas sulfamates **8**, **14**, **17** and **18** showed IC_{50} values in the nM range: 94.0 nM, 66.0 nM, 43.7 nM and 33.2 nM respectively.

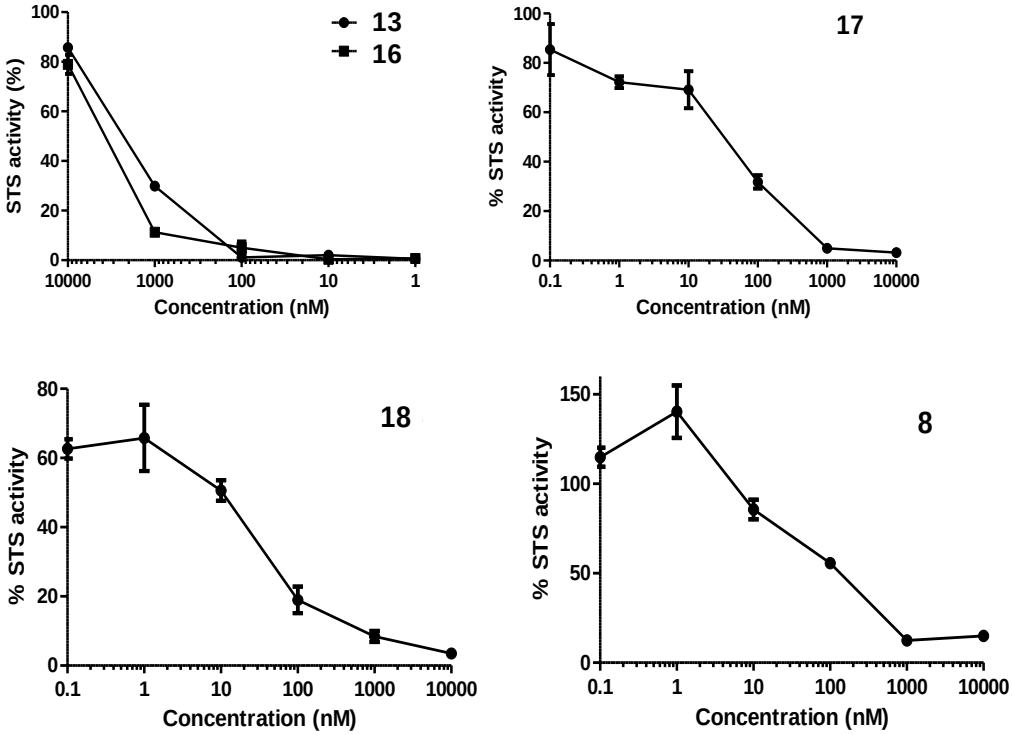
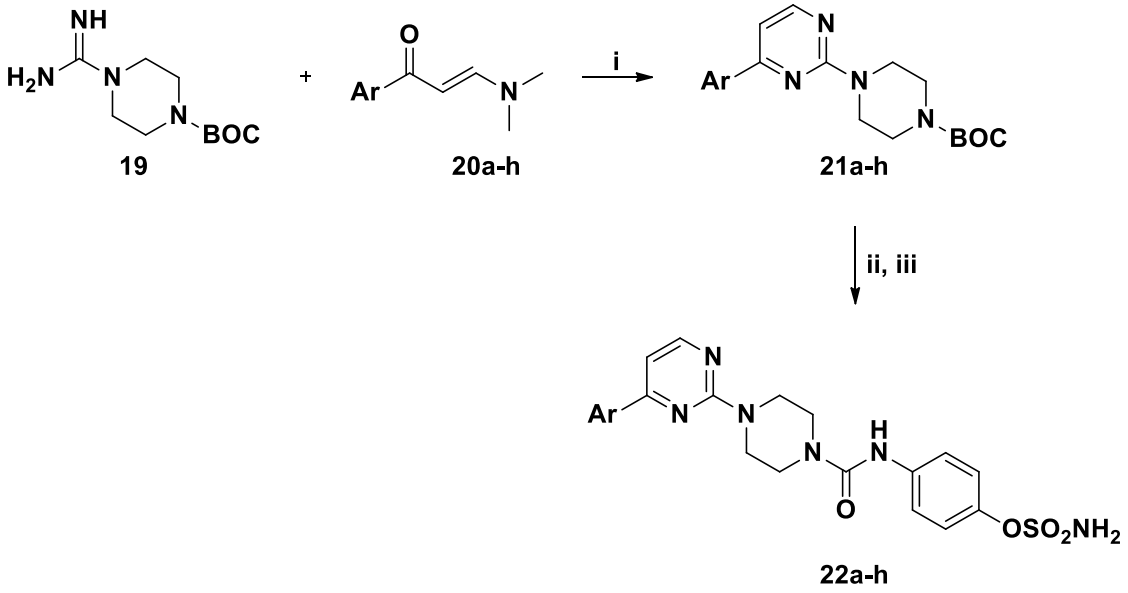


Figure 3.4. IC_{50} of STS inhibition determined for sulfamates **8**, **13**, **16**, **17**, and **18**, using JEG-3 protein. All data represents mean ± S.D., n = 3

Starting from the interesting results of compounds **5-18**, a second series of ureido sulfamates was prepared. In the second series a pyrimidine ring was incorporated between the piperazine ring and the substituted phenyl ring.



Scheme 3.2. Reagents and conditions: (i) 1-propanol, reflux 12 h; (ii) TFA, DCM, r.t. 24 h; (iii) 4, DIPEA, DMSO, r.t. 24 h.

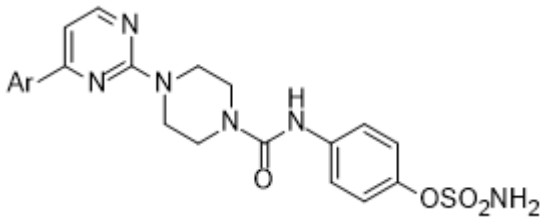
The pyrimidinyl-piperazinourea compounds **22a-h** were synthesized by heterocyclization of 4-Boc-piperazine-1-carboxamidine (**19**) with substituted 3-(dimethylamino)propenones **20a-h** in boiling 1-propanol, followed by trifluoroacetic acid (TFA)-mediated deprotection in dichlorometane (DCM) solution (Scheme 3.2). The resulting intermediates **21a-h** are coupled with 4-((phenoxycarbonyl)amino)phenyl sulfamate to obtain the desired compounds **22a-h**.

The analysis of the hCA inhibitory profile of sulfamates **22a-h** showed the presence of the pyrimidine ring generally afforded poor hCAI inhibitors except for compound **22h**. Interestingly, the presence of a 3-trifluoromethylphenyl and 3-bromophenyl as in compounds **22e** and **22d** led drop in activity against hCAI isoform maintaining at the same time high potency on tumor expressed CA IX isoform. As hCAI is basically an off- target isoform, its weak inhibition with sulfamates **22d** and **22e** might be considered of interest for the development of anticancers based on CA inhibition.

The presence on the aryl ring of three methoxy groups (sulfamate **22g**, K_I of 6.8 nM) led to about a 4-fold increase in activity against CA II as compared with the 4-methoxy analog **22b**.

Speaking about hCAII, sulfamate **22h** resulted the best compound of the series, with a Ki of 1.0 nM. The 4-methoxyphenyl derivative showed potent inhibitory activity (Ki 9.1 nM) while introduction of a second methoxy group in 3-position to give sulfamate **22f**, led to a reduction of activity (Ki 36.2 nM). The introduction of a third methoxy group to give the 3,4,5-trimethoxy derivative **22g** restored the inhibitory activity (Ki 6.8 nM). On the contrary, both compounds **22b** and **22g** showed about the same Ki_s against the CA IX (31.8 and 24.2 nM). The presence of a substituent at the 3-position of the phenyl ring, such as methyl (sulfamate **22c**), bromine (sulfamate **22d**), trifluoromethyl (sulfamate **22e**) groups led to a reduction of activity as well as the absence of substituents on the phenyl ring.

Table 3.3. Inhibition data of human CA isoforms hCA I, II, IX and XII with derivatives **22a-h** and the standard AAZ by a stopped flow CO_2 hydrase assay (errors were in the range of ±5–10% of the reported values).

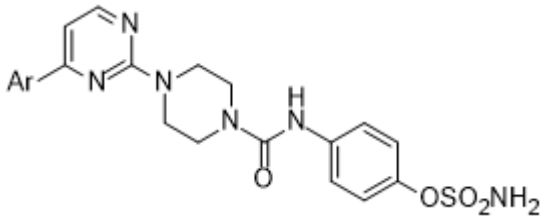


Compound	Ar	K_i (nM)			
		hCAI	hCAII	hCAIX	hCAXII
22a	phenyl	2370	705.2	155.9	-
22b	4-methoxyphenyl	282.4	9.1	114.1	1.1
22c	3-methylphenyl	4069.9	206.9	93.3	-
22d	3-bromophenyl	7769.1	124.2	30.5	-
22e	3-trifluorometylphenyl	3472.9	58.7	27.1	-
22f	3,4-dimethoxyphenyl	760.3	36.2	32.7	-
22g	3,4,5-trimethoxyphenyl	5284.0	6.8	24.2	-
22h	benzofuran-2-yl	70.2	1.0	6.7	1.0
AAZ	-	250	12.5	25	5.7

All the sulfamates **22** showed good inhibitory activity against the cancer related isoform CAIX, with the exception for sulfamates **22a** and sulfamate **22b** (Ki 155.9 nM and Ki 114.1 nM respectively). Sulfamate of **22c** showed inhibitory activity at high nanomolar value (Ki 93.3 nM). The replacement of the 3-methyl group with a bromine atom or a trifluoromethyl group led to increase in activity (sulfamate **22d** Ki 30.5 nM, sulfamate **22e** Ki 27.1 nM). Furthermore, the replacement of the phenyl ring with a benzofuran ring to give sulfamate **22h** increased the activity (Ki 6.7 nM).

Concerning the sulfamates **22a-h** STS inhibitory activity (Table 3.4), the 4-methoxyphenyl derivative **22b** was the most potent, on both isolated enzyme (7.9% ± 1.3) and whole cells (13.7% ± 2.6) (Figure 3.5 A). The presence of three methoxy groups on the phenyl ring as in sulfamate **22g** led to a slight reduction of activity (19.0% ±3.1) while the presence of two methoxy groups as in sulfamate **22f** led to significant activity reduction (60.7% ±9.4).

Table 3.4. STS inhibitory activity of sulfamates **22a-h**



Compound	Ar	Residual STS % activity ± SD[a]
22a	phenyl	65.9 ± 5.4
22b	4-methoxyphenyl	7.9 ± 1.3
22c	3-methylphenyl	26.7 ± 8.5
22d	3-bromophenyl	39.4 ± 9.9
22e	3-trifluorometylphenyl	44.3 ± 1.0
22f	3,4-dimethoxyphenyl	60.7 ± 9.4
22g	3,4,5-trimethoxyphenyl	19.0 ±3.1
22h	benzofuran-2-yl	4.7 ± 0.7

[a]Residual activity after JEG-3 cell lysate treatment with 10μM inhibitor is shown.

The phenyl unsubstituted sulfamate **22a** or its analogs bearing substituent at 3-position (sulfamates **22c**, **22d** and **22e)** showed reduction in activity as compared to **22b**. The replacement of the aryl ring with a benzofuran ring (sulfamate **22h**) produced a high increase in activity in the isolated enzyme (4.7% ± 0.7) as well as in JEG-3 cells (4.1% ± 0.9).

Due to its high potency, sulfamate **22h** was chosen for IC_{50} evaluation and it exhibited a value of 139 nM (Figure 3.5).

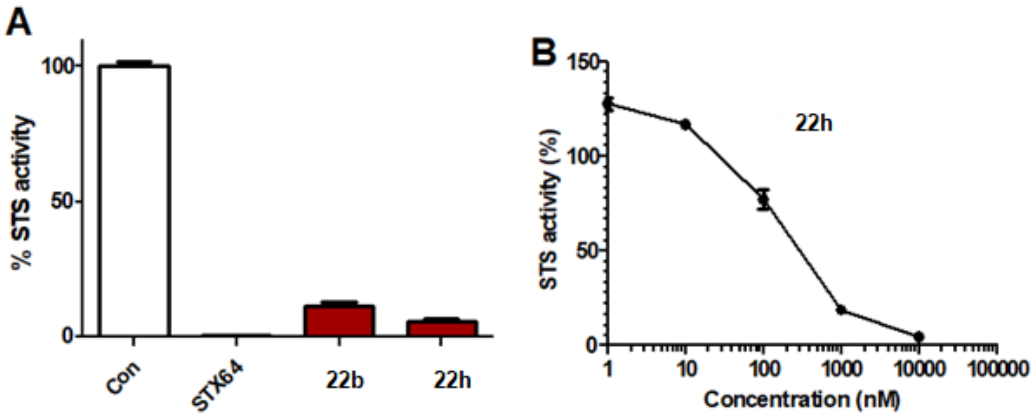


Figure 3.5. A) Evaluation of the STS inhibitory activity of sulfamate compounds **22b** and **22h** in whole cell JEG-3 tested at 10 μM, the reference inhibitor STX64 was used as positive control. B) STS inhibition IC_{50} value of compound **22h** determined using JEG-3 protein. All data represents mean ± S.D., n = 3.

3.1.1 Conclusions

Starting from SLC-0111 and Irosustat/STX64 as lead compounds, two new series of ureido-aryl sulfamates were designed and synthetized to obtain dual CA/STS inhibitors. For what concern the first series, sulfamates **7** and **14** showed interesting dualistic activity. The 3-methylphenyl derivative **7** showed inhibitory activity against hCA IX at sub nanomolar levels, with Ki 0.91 nM, and a good STS residual activity (10.4% ± 1.9). The 2,3-dimethylphneyl derivative **14** displayed STS residual activity better than **7** (5.9% ± 0.8) and it is also endowed with inhibitory activity against hCAXII at low nanomolar levels (Ki 1.0 nM). Moving to the second series, compounds **22g** and **22h** also showed good inhibitory against both CA and STS. The 3,4-trimethoxyphenyl derivative **22g** displayed Ki 24.2 nM against hCAIX and STS residual activity of 19.0% ± 3.1, while the benzofuran-2-yl derivative **22h** showed inhibitory activity at low nanomolar range against both hCA IX (6.7 nM) and hCA XII (1.0 nM) and STS residual activity of 4.7% ± 0.7. Therefore, sulfamates **7**, **14**, **22g** and **22h** are endowed with dualistic CA/STS inhibitory activity and may be attractive for further development and potential *in vivo* evaluation.

3.2 Arylhydrazones derivatives

The sun UV radiation is composed of UVA, UVB and UVC rays based on photon wavelength with UVA having the longest wavelengths (315–400 nm), UVB being mid-range (290–320 nm) and UVC being the shortest wavelengths (100–280 nm). Generally, UVC radiation is absorbed by ozone while UVA and UVB can potentially interact with the human body. (figure 3.6) The uncontrolled and unprotected exposure to UV radiation can result in mutagenesis in skin cells. Indeed, UVB can cause direct damage to DNA and reach the epidermis whereas UVA can penetrate the dermis and increase levels of Reactive Oxygen Species (ROS) that indirectly induce DNA mutagenesis.[15]

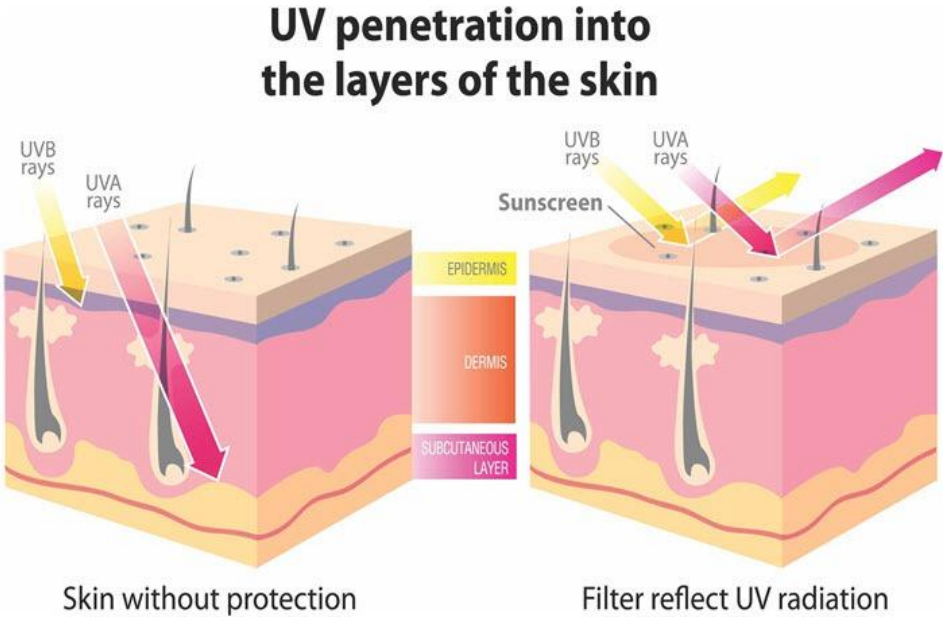


Figure 3.6. UV rays skin penetration without and with UV protection[14].

UVB rays are a well-known mutagen, but recently different studies have defined the role of UVA in cancerogenesis through promotion of ROS production.[15] Generally, ROS are produced by cells during normal metabolic activity and, under physiological conditions, different antioxidant enzymes mediated their removal, such as Superoxide Dismutase (SODs). Without a correct inactivation or in case of over-exposure of ROS inducer factor such as UVA, oxidative stress is closely related skin photo-aging process, to skin inflammation and skin diseases, such as erythema and hyperpigmentation, and from the process of carcinogenesis, including the onset of melanoma.[16,17] Overexposure of UV radiation, such as in the case of sunburn, is a key factor in development of melanoma, a malignant and aggressive skin tumoral form. In fact, it has been demonstrated that more than five sunburns in lifetime, double the risk to develop melanoma.[18] Polyphenols were intensively studied due to their antioxidant properties and between them flavonoids are well known for their antioxidant and chelating properties.[19] Some flavonoids have further demonstrated to

absorb UVB rays, hence contributing to their photoprotective effect in plants, by behaving as UV filters and protecting underlying elements. This flavonoids photoprotective property has been adapted and investigated in human cells and in mice models, to determine whether flavonoids and their derivatives could be used as photoprotective agents in humans.[20] Currently, several studies are focused on the development of both antioxidant and photoprotective compounds.

In this context, in this thesis we have designed, synthesized and characterized new benzofuran, indole and benzimidazole hydrazones bearing in the arylidene moiety phenol or polyphenol moieties with the aim to obtain multifunctional compounds endowed both antioxidant and photoprotective capabilities and with potentially antiproliferative activity.

All series have been tested for their antioxidant, photoprotective and antiproliferative activity at the Department of Life Sciences and Biotechnology, University of Ferrara, Italy.

3.2.1 Benzofuran hydrazones

Benzofuran is an important oxygen containing heterocycle used in the drug design due to the biological properties of its derivatives. Benzofuran core is present in various natural compounds deriving from secondary plant metabolism and it has been demonstrated that benzofuran derivatives possess broad spectrum biological activity, such as antiviral,[21] anticancer,[22,23] antimicrobial,[24,25] anti-Alzheimer,[26,27] antinflammatory and anticonvulsivant[28] activity. Furthermore, natural benzofuran derivatives have been studied for their important antioxidant activity (Figure 3.7). For example, Vitiferin, a stilbene derivative from Vitis Vinifera, is a well-known high potent antioxidant as well as metoxalene, used for the treatment of different skin diseases due to its dermo-protective effect.[29].

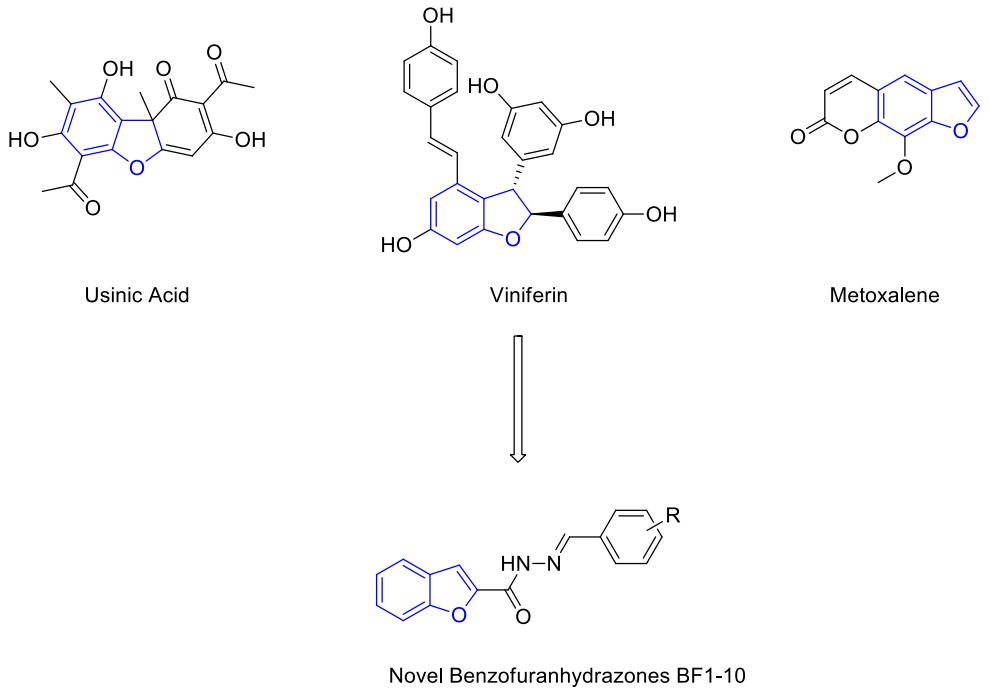
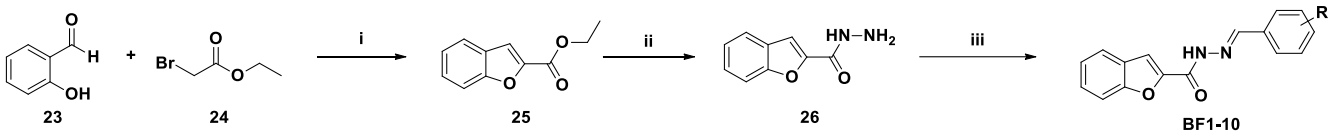


Figure 3.7. Design of new hydrazones **BF1-10** starting from Usinic Acid, Viniferin and Metoxalene structure.

Furthermore, usnic acid, present in lichens, has been studied for its antioxidant and photoprotective action against UV rays so that it is currently used in cosmetic field to produce sunscreens.[30] Starting from the interesting activity of these natural compounds, it has been investigated the development of compounds bearing benzofuran ring having dual antioxidant and photoprotective activity. The synergistic action of these two properties it has been reported to prevent skin cancer such as melanoma.[31] On the other hand, hydrazone derivatives have been broadly studied in drug discovery to develop antiproliferative and antioxidant compounds.[32,33] Thus starting from these considerations we designed, synthetized and tested a new series of benzofuran hydrazones with dualistic antioxidant and photoprotective activity.[33]

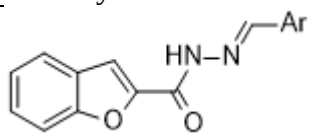
As reported in Scheme 3.3 the target hydrazones **BF1-10**[34] were synthetized starting from the reaction of salicylaldehyde **23** with ethyl bromoacetate **24** in the presence of potassium carbonate (K_2CO_3) in MeCN to achieve the desired ethyl benzofuran-2-carboxylate **25**. The ester **25** was treated with hydrazine hydrate in absolute ethanol to obtain the corresponding hydrazide **26**. Finally, the desired hydrazones **BF1-10** were obtained by coupling hydrazide **26** with the appropriate hydroxyarylaldehyde in absolute ethanol. All the newly benzofuranhydrazones were in agreement with expected analytical data. The IR and NMR spectral data are consistent with the assigned structure. According to the literature, the presence of a singlet downfield resonating (8.27-8.87 ppm) CH=N signal, exclusively accounts for formation of *E*-isomers.[35]



Scheme 3.3. General synthetic procedure for BF1-10. Reagents and conditions: (i) MeCN, K_2CO_3, reflux 1.5 h; (ii) $NH_2NH_2 \cdot H_2O$, EtOH, reflux 3h; (iii) ArCHO, EtOH, reflux 18h.

The evaluation of the antioxidant properties of phenylhydrazones derivatives was performed by 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activity (DPPH), Ferric Reducing Antioxidant Power (FRAP), and Oxygen radical absorbance capacity (ORAC) methods. Results are expressed as mmolTE/g for DPPH, FRAP, and ORAC tests (Table 3.5). For the best interpretation of the results of the DPPH, for each compound the concentration was sought in order to inhibit 50% of the radical. The analysis of DPPH test results showed that the antioxidant activity is related to the number and position of hydroxy groups on the arylidene ring. The best compound of the series is **BF5**, the 2,3,4 trihydroxybenzylidene derivative while the 2,4,6 trihydroxybenzylidene **BF6** analog showed a small reduction of activity. On the other hand, the mono-hydroxy derivatives, such as **BF1** and **BF2** (4-hydroxybenzylidene and 3-hydroxybenzylidene derivatives) showed weak antioxidant activity. The introduction of a second hydroxy group into the arylidene ring to give **BF3** (2,4-dihydroxybenzylidene derivative) improved the activity, whereas the 2,5-dihydroxybenzylidene analog **BF4** showed further increase in activity.

Table 3.5. Evaluation of Antioxidant activity.



Compound	Ar	DPPH[a] (µmolTE/g)	FRAP[a] (µmolTE/g)	ORAC[a] (µmolTE/g)
BF1	4-hydroxyphenyl	45.80±2.97	2293.83±20.62	26059.89±46.23
BF2	3-hydroxyphenyl	76.60±2.45	1667.38±13.04	6786.29±16.89
BF3	2,4-dihydroxyphenyl	148.51±4.92	1212.75±6.45	9375.99±26.70
BF4	2,5-dihydroxyphenyl	6202.86±30.09	6966.77±28.25	19003.78±26.13
BF5	2,3,4-trihydroxyphenyl	9210.10±34.85	13248.57±35.89	5257.42±17.21
BF6	2,4,6-trihydroxyphenyl	1140.96±6.20	4023.34±8.63	3560.49±6.72
BF7	3-methoxy-4-hydroxyphenyl	101.13±0.72	3102.81±4.29	10888.11±10.98
BF8	2-hydroxy-5-clorophenyl	<LOQ[b]	46.29±1.49	-
BF9	2-hydroxy-4-(diethylamino)phenyl	1006.62±14.19	4150.23±7.53	22119.18±16.89
BF10	naphtyl	131.57±7.09	132.18±4.12	-

[a]Each value was obtained from 3 experiments (mean±SE); [b] LOQ limit of quantification, - not tested.

The presence of electron withdrawing groups on the benzylidene ring, such as a 5-chlorine atom (**BF8** 2-hydroxy-5-chlorobenzylidene derivative), resulted in depletion of antioxidant activity, even if accompanied by the hydroxy group. The introduction of a diethylamino group at 4-postion (**BF10** 2-hydroxy-4-(diethylamino)benzylidene derivative) produced good antioxidant activity, comparable with the activity showed by the 2,4,6-trihydroxyatylidene derivative **BF6**. According to the FRAP analysis results, all the hydrazones were powerful antioxidants, except for **BF8** and **BF10**. As in the DPPH test, the hydrazone endowed with the best antioxidant activity was the 2,3,4 trihydroxybenzylidene derivative **BF5** followed by the 2,5-dihydroxybenzylidene derivative and by the 2,4,6-trihydroxybenzylidene derivative (**BF4** and **BF6** respectively). Interesting antioxidant activity was shown by **BF7** and **BF9** that are characterized by the presence at 4-postion of the arylidene ring of an electron donor group, the methoxy group in **BF7** and the diethylamino group in **BF9**. The ORAC test was used to investigate the antioxidant activity of the compounds with a satisfactory antioxidant activity in the DPPH and FRAP tests, to confirm the results obtained with the two previous tests. Surprisingly, **BF1** resulted the best compound of the series while in the ORAC and FRAP tests showed low antioxidant activity. Hydrazones **BF4**, **BF7** and **BF9** presented a high ORAC antioxidant power whereas the trihydroxybenzylidene derivatives (which resulted the best derivatives of the series in DPPH and FRAP tests) demonstrated the weakest activity among all the hydrazones.

The hydrazones showing good antioxidant properties were evaluated for their photoprotective activity. The Solar Protection Factor (SPF) is used for the evaluation of compounds with potential use as sunscreen. The selected hydrazones were tested using the *in vitro* method of Diffey and Robson[36] using 2-phenyl-1H-benzo[*d*]imidazole-5-sulfonic acid (PBSA), one of the UVB filters mostly used in cosmetics for sun protection, characterized by high water solubility and excellent safety profile, as a reference (Table 3.6).

SPF is also related to the UV absorption, so that the maximum absorption wavelength (λ max) and the molar extinction coefficient (ε) were evaluated. To better define the protection capacity of a solar filter to provide against UV radiation, the FDA, classified the critical wavelength in five numerical categories: 0 ($\lambda c<325nm$); 1 ($325\leq\lambda c\leq335$); 2 ($335\leq\lambda c\leq350$); 3 ($350\leq\lambda c\leq370$) 4 ($\lambda c\geq370$)[37]. A widespectrum solar filter able to protect the skin from both UVB and UVA may have a value of $\lambda c\geq370$, placed in category 4.[38]

Table 3.6. UV-filtering activity of benzofurane hydrazones **BF1-10** and the reference sun filter PBSA in solution

Compound	SPF (P ≤0.05)	UVA/UVB (P ≤0.05)	UVAPF (P ≤0.05)	λc[a] (nm)
BF1	3.40	0.29	1.03	322
BF2	7.58	0.85	2.45	353
BF3	10.23	0.26	1.50	342
BF4	2.18	1.33	2.38	366
BF5	7.58	0.85	2.45	353
BF6	1.99	1.32	2.02	366
BF7	3.36	2.33	5.57	371
BF8	5.78	1.14	2.89	357
BF9	2.59	1.29	3.60	387
BF10	8.82	0.39	2.85	359
PBSA	1.68	1.27	5.10	394

[a]Wavelength at which the integral of the spectral absorbance curve reaches 90% of the area under the curve from 290 to 400 nm.

The SPF value was obtained starting from the transmittance spectra of **BF1-10**. All hydrazones showed better SPF value than PBSA and in general the values obtained were in agreement with literature data of sunscreen filters bearing substituted phenyl rings: the presence of 4-methoxy, 2-hydroxy or 4-hydroxy groups, increase the filtering capacity.[39] Hydrazones **BF1, 4, 6, 7** showed comparable protection value despite differences in number and positions of substituents in the benzylidene ring. In the case of the mono-hydroxy derivatives, the displacement of 4-hydroxy group of **BF1** into 3-position (**BF2)**, produced an increase in filtering capability. A similar effect has been observed for the di-hydroxy derivatives: displacing the 5-hydroxy group of the 2,5-dihydroxybenzylidene derivative (**BF4**) into 4-position to give the hydrazone **BF3** increased the SPF value. The compound endowed with the best filtering capability was the 2-hydroxynaphtyl derivative **BF10**.

According to the FDA classification of critical wavelength, **BF1** was classified as 0; **BF3** was classified as 2; **BF2, BF4, BF5, BF6, BF8** and **BF10** were classified as 3; **BF7** and **BF9** were classified as 4. Compounds **BF7** and **BF9** were the only interesting benzofuran hydrazones of the series, with a λc comparable with PBSA.

Another important parameter for evaluating a sunscreen compound, is the UVA/UVB absorbance ratio. According to the latest UE recommendation (2006/247/EC), this ratio should be worth at least 0.33 so that, excluding **BF1** and **BF3**, all the benzofuran hydrazones of the series displayed good values. The best compound of the series **BF7**, showed an UVA/UVB absorbance ratio value of 2.33.

The UVA Protection Factor (UVAPF) (according to the ISO-24443 guidelines) showed that only **BF7** have a better UVAPF then PBSA (UVAPF-**BF7** 5.57, UVAPF-PBSA 5.10).

All the compounds **BF1-10** were also tested on human melanoma Colo38 cell line and human erythroleukemic K562 to evaluate their potential antiproliferative and differentiating effects.[40] In Table 3.7 IC_{50} values, expressed in μM concentration, against both the cell lines are displayed.

Table 3.7. Effects of the benzofuran derivatives **BF1-10** on the proliferation of Colo38 and K562 cells

Compound	**IC_{50} (μM)**	
	Colo38	**K562**
BF1	39.44±2.45	79.9±6.05
BF2	6.2±0.47	52.4±6.43
BF3	0.57±0.12	4.3±0.12
BF4	6.26±1.63	36.8±2.81
BF5	6.48±0.75	6.0±0.28
BF6	24.41±2.94	64.0±5.13
BF7	48.96±3.94	5.6±0.21
BF8	2.02±0.03	3.7±0.14
BF9	0.44±0.06	0.52±0.07
BF10	3.01±0.19	4.5±0.78

The best compound of the series, the 2-hydroxy-4-(diethylamino)benzylidene derivative (**BF9**), displayed antiproliferative effect at submicromolar concentrations on both Colo38 (IC_{50} value 0.44±0.06 μM) and K562 (IC_{50} value 0.52±0.07 μM) whereas the 2,4-dihydroxybenzylidene derivative (**BF3**) selectively inhibited the Colo38 cell growth (IC_{50} value 0.57±0.12 μM). The number and the position of hydroxy groups on arylidene ring resulted crucial for the antiproliferative activity. For example, the 4-hydroxybenzylidene derivative (**BF1**) showed antiproliferative activity at high micromolar range against both cell lines (Colo38 IC_{50} value 39.44±2.45 μM, K562 IC_{50} value 79.9±6.05 μM) while the corresponding 3-hydroxybenzylidene derivative (**BF2**) showed high potency on Colo38 cell line (IC_{50} value 6.2±0.47 μM). As mentioned above, 2,4-dihydroxybenzylidene derivative (**BF3**) showed an interesting activity against both Colo38 and K562 while the presence of 2,5-dihydroxybenzylidene group (**BF4**) led to reduction of the inhibitory activity (Colo38 IC_{50} value 6.26±1.63 μM, K562 IC_{50} value 36.8±2.81 μM). The presence of a third hydroxy group on the arylidene ring (**BF5** and **BF6**) resulted in a decrease of activity as compared to **BF3**. The introduction of a 2-hydroxy-5-chlorobenzylidene moiety (**BF8**) led to an improvement of antiproliferative effect (Colo38 IC_{50} value 2.02±0.03 μM, K562 IC_{50} value 3.7±0.14 μM) as compared to the 2,5-dihydroxybenzylidene analog **BF4**. The replacement of the benzylidene group with a 2-hydroxynaphtylidene (**BF10**) produced an improvement of antiproliferative activity against both Colo38 (IC_{50} 3.01±0.19 μM) and K562 (IC_{50} 4.5±0.78 μM).

3.2.2 Indole hydrazones

Starting from the good results of benzofuranehydrazone series, to further investigate the antioxidant and photoprotective activity of hydrazones derivatives, we have designed, synthetized and characterized a series of indole hydrazones **IND1-14**.

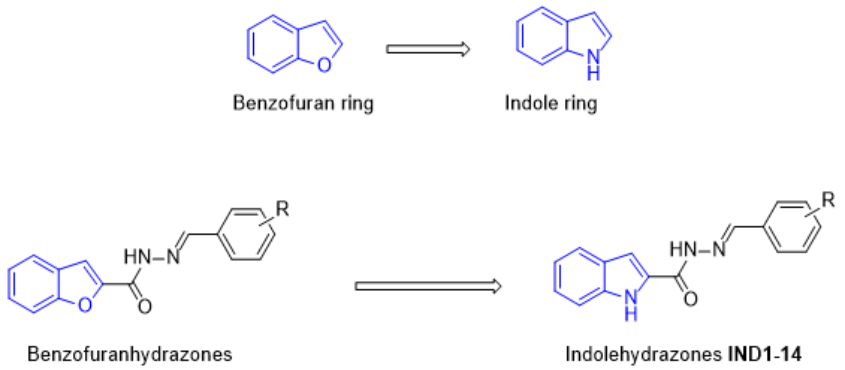


Figure 3.8. Design of new indolehydrazones

Indole ring, a bioisoster of benzofuran ring, is a well-known scaffold, present in biologically active products derived from plant and animal organisms (Figure 3.8). Several studies demonstrated the importance of indole scaffold in medicinal chemistry for the development of anticancer agents[41,42]. Different alkaloids bearing indole ring were extensively studied for their pharmacological activity (Figure 3.9): for example, Raubasine and Reserpine are active on circulatory system while different studies conducted on indole-3-carbinole revealed interesting antioxidant, antiproliferative and anti-atherogenic effects.[43,44]

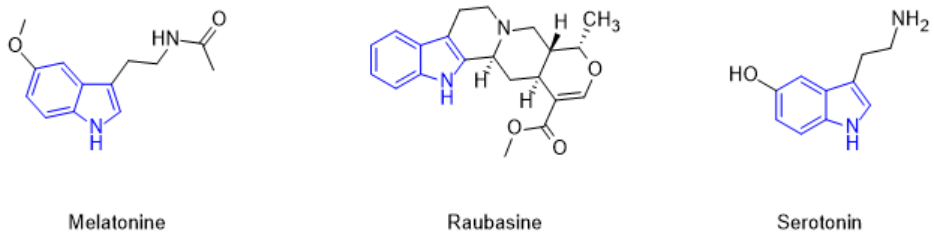
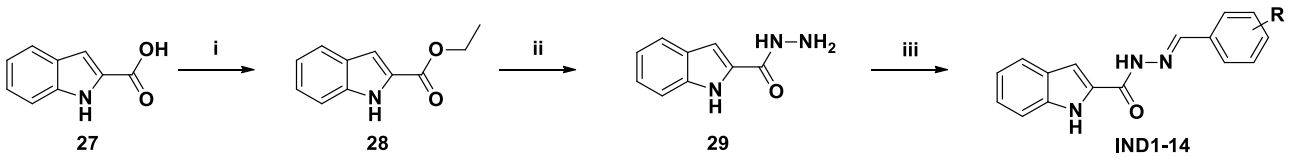


Figure 3.9. Bioactive compounds bearing indole ring.

The indole ring is also present in serotonin, a neurotransmitter implicated in different diseases such as depression, Alzheimer's disease, anxiety etc.[45] Together with the homolog melatonin, serotonin is a powerful scavenger of free radicals and the indole ring was indicated as responsible for antioxidant properties due to its high resonance stability and low activation energy barrier in direct reactions against ROS and free radicals.[46,47] Furthermore, indole-hydrazone derivatives have been reported to induce apoptosis and to interact with tubulin.[48-50]

The target hydrazones **IND1-14**[51] were synthetized as shown in Scheme 3.4. The 1(*H*)-indole-2-carboxylic acid **27** was converted into the corresponding ethyl ester **28** by reaction with an excess of

absolute EtOH in the presence of concentrated H_2SO_4. Then, the ethyl carboxylate **28** was treated with hydrazine hydrate in ethanol to obtain the corresponding hydrazide **29**. Finally, the desired hydrazones **IND1-14** were obtained by coupling the hydrazide with the appropriate hydroxyarylaldehyde in EtOH.

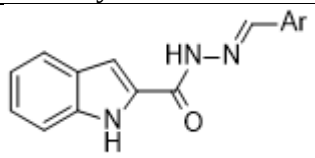


Scheme 3.4. General synthetic procedure for **IND1-14**. Reagents and conditions: (i) Ethanol, H_2SO_4, reflux, 6h; (ii) $NH_2NH_2 \cdot H_2O$, EtOH, reflux 3h; (iii) ArCHO, EtOH, reflux, 18h.

All indolehydrazones analytical and spectral data were consistent with the assigned structure. According to the literature and **BF1-10** spectral data, the presence of a singlet downfield resonating (8.27-8.87 ppm) CH=N signal, exclusively accounts for formation of *E*-isomers.[51]

The analysis of DPPH results confirmed the SAR formulated for the benzofuranhydrazones: high antioxidant capacity was correlated to high number of hydroxy groups on the arylidene ring and to their position. The 2-hydroxy, 3-hydroxy and 4-hydroxybenzylidene derivatives (**IND1, IND2, IND3**) showed weak activity, while the introduction of a second (**IND4, IND5**) or a third hydroxy group (**IND6, IND7**) resulted in an enhancement of the antioxidant activity. Furthermore, the position of the hydroxy group is strictly connected to the antioxidant activity. The 2,5-dihydroxyarylidene derivative (**IND4**) was 4.5-fold more active than the 2,4-dihydroxybenzylidene analog (**IND5**) and the 2,3,4-trihydroxybenzylidene derivative (**IND6**) was 3-fold more active than the 2,4,6-trihydroxy analog (**IND7**). The replacement of one hydroxy group with a methoxy group (**IND8, IND9**), an ethoxy group (**IND10**) or halogen atoms (**IND11, IND12**) led to a drastic reduction of the activity. As observed in the benzofuranehydrazone series, the presence of a 4-diethylamino group (**IND13**) is correlated with high antioxidant activity.

Table 3.8. Evaluation of Antioxidant activity of **IND1-14**



Compound	Ar	DPPH[a] (µmolTE/g)	FRAP[a] (µmolTE/g)	ORAC[a] (µmolTE/g)
IND1	2-hydroxyphenyl	8.71±1.85	34.56±1.2	-
IND2	3-hydroxyphenyl	59.21±1.0	960.94±11.5	21031.02±31.03
IND3	4-hydroxyphenyl	233.16±5.7	1559.13±11.5	35124.02±64.55
IND4	2,5-dihydroxyphenyl	9958.30±13.6	4378.91±14.7	21700.40±28.73
IND5	2,4-dihydroxyphenyl	2004.80±6.0	873.06±1.9	13014.11±25.10
IND6	2,3,4-trihydroxyphenyl	12846.01±16.8	10655.78±25.1	16621.77±34.41
IND7	2,4,6-trihydroxyphenyl	4187.20±7.4	4137.42±8.8	4505.29±49.27
IND8	3-hydroxy-4-methoxyphenyl	205.65±3.7	2667.35±6.5	16838.33±41.07
IND9	2-hydroxy-4-methoxyphenyl	221.9±2.3	43.04±2.1	-
IND10	2-hydroxy-3-ethylphenyl	38.66±0.1	115.78±2.2	-
IND11	2-hydroxy-5-chlorophenyl	<<19.1[b]	97.04±1.9	-
IND12	2-hydroxy-5-bromophenyl	14.15±0.12	25.89±1.0	-
IND13	2-hydroxy-4-(diethylamino)phenyl	2436.60±15.29	3760.80±8.07	22761.19±18.48
IND14	2-hydroxynaphtyl	1783.54±13.1	97.4±1.1	9398.69±14.92

[a]Each value was obtained from three experiments (mean ± SE). [b] LOQ limit of quantification; – not tested.

Results from the FRAP analysis, displayed that the best derivatives were **IND2, IND3, IND4, IND5, IND6, IND7, IND8** and **IND13** and according with the results of DPPH test, there is a correlation between the activity and the number and the position of hydroxy groups on the arylidene ring. For example, the 2,5-dihydroxybenzylidene derivative (**IND4**), showed high antioxidant activity while the 2,4-dihydroxybenzylidene analog (**IND5**) was about 5-fold less active. The introduction of a third hydroxy group led to an improvement of the activity, but also in this case the variation of the hydroxy groups position affected the activity: the 2,3,4-trihydroxybenzylidene derivative (**IND6**) was about 2-fold more active than the 2,4,6-trihydroxybenzylidene analog (**IND7**). As a confirm of DPPH results, the 2-hydroxy-4-diethylamino derivative (**IND13**) is endowed with high antioxidant activity.

Compounds **IND2-8** and **IND3-14** were further investigated for their antioxidant activity, by the ORAC test. The 4-hydroxybenzylidene derivative (**IND3**) showed the best antioxidant activity, while the shift of the hydroxy group into 3-position to give the analog **IND2** was related to a reduction of the activity. The 2,5-dihydroxybenzylidene derivative (**IND4**) demonstrated a good antioxidant activity, while the shift of the 5-hydroxy group into the 4-position to give the analog **IND5** led to a decrease of the activity. The replacement of the 4-hydroxy group with a 4-diethylamino group as in compound **IND13** restored the antioxidant properties. The introduction of

a third hydroxy group afforded drop in the activity, however the 2,3,4-trihydroxybenzylidene derivative (**IND6**) showed better activity than the 2,4,6-trihydroxybenzylidene analog (**IND7**). The 3-hydroxy-4-methoxybenzylidene derivative (**IND8**) showed antioxidant activity similar to the 2,3,4-trihydroxybenzyl derivative.

Considering their high antioxidant activity, the hydrazones **IND2-8** and **IND13-14** were evaluated for their photoprotective activity, determining the SPF and evaluating the critical wavelength (λc) (Table 3.9).

Table 3.9. UV-filtering activity of selected indoles in solution

Compound	SPF (P ≤0.05)	UVA/UVB (P ≤0.05)	UVAPF (P ≤0.05)	λc[a] (nm)
IND2	10.42	0.43	1.77	346
IND3	10.05	0.81	2.56	352
IND4	5.13	1.96	4.60	363
IND5	5.78	1.93	4.97	363
IND6	5.01	2.09	4.32	362
IND7	4.34	2.66	6.19	367
IND8	5.75	1.34	2.87	356
IND13	2.21	1.39	9.55	392
IND14	4.81	1.71	8.55	385
PBSA	3.40	0.29	1.03	322

[a]Wavelength at which the integral of the spectral absorbance curve reaches 90% of the area under the curve from 290 to 400 nm.

In general, based on SPF values the tested compounds showed better filtering capability than PBSA. **IND4, 5, 6, 7, 8** and **14** showed comparable SPF value, while the 2-hydroxy-4-diethylamino derivative **IND13** resulted the worst compound of the series with SPF value 2.21. Surprisingly, the compounds with the best filtering capabilities were the 3-hydroxybenzylidene (**IND2**) and 4-hydroxybenzylidene (**IND3**) derivatives with SPF value 10.42 and 10.05 respectively.

According with the FDA classification for the λc (0 (λc<325nm); 1 (325≤λc≤335); 2 (335≤λc≤350); 3 (350≤λc≤370) 4 (λc≥370)), **IND2** was classified as 2, **IND3, IND4, IND5, IND6, IND7** and **IND8** were classified as 3, **IND13** and **IND14** were classified as 4. As described above, a good solar filter may have a value of λc≥370 so that **IND13** and **IND14** were the best hydrazones of this series, with a λc value comparable with PBSA.

Following the UE recommendation for the UVA/UVB absorbance ratio, all the tested indolehydrazones displayed good values. The best compound of the series, **IND7** showed an UVA/UVB absorbance ratio value of 2.66.

The UVAPF of the tested indolehydrazones revealed, according to the ISO-24443 guidelines, that all the tested hydrazones displayed better UVAPF than PBSA. Among them, the best compound resulted again the 2,4,6-trihydroxybenzylidene derivative **IND7**, with UVAPF value of 6.19.

The indolehydrazones **IND1-14** were tested on human melanoma Colo38 and erytroleukemic K565 cells to evaluate their antiproliferative activity. The hydrazones were tested on both cell lines to determine the relative IC_{50} values expressed in μM concentration (Table 3.10).

Table 3.10. Effects of indole derivatives **IND1-14** on the proliferation of Colo38 and K562 cells

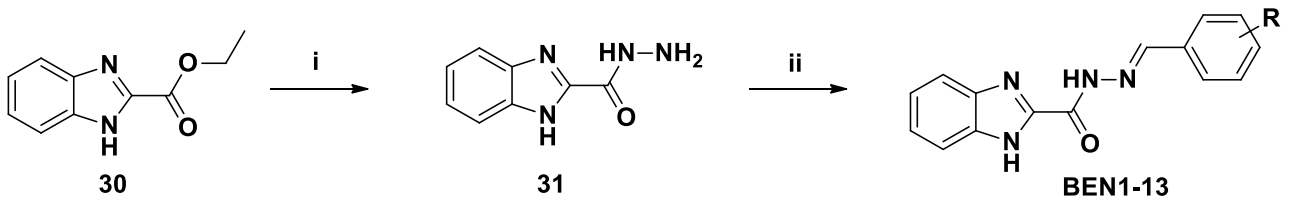
Compound	**IC_{50} (μM)**	
	Colo38	**K562**
IND1	>100	>100
IND2	10.02±0.91	47.80±7.80
IND3	0.73±0.05	46.11±4.30
IND4	2.96±0.13	8.60±0.60
IND5	0.57±0.05	4.31±0.40
IND6	6.13±0.26	5.39±0.01
IND7	8.94±0.13	54.52±0.11
IND8	0.59±0.03	0.067±0.001
IND9	>100	100
IND10	>100	>100
IND11	>100	>100
IND12	>100	>100
IND13	0.54±0.15	0.63±0.05
IND14	0.83±0.09	0.63±0.04

The 3-hydroxy-4-methoxybenzylidene derivative (**IND8**) showed the best activity of the series with an antiproliferative effect on both cell lines at nanomolar concentrations (Colo38 IC_{50} 0.59±0.03 μM, K562 IC_{50} 0.067±0.001 μM). While the removal of the 4-methoxy group to give the 3-hydroxybenzylidene derivative (**IND2)** decreased the antiproliferative activity (Colo38 IC_{50} 10.02±0.91 μM, K562 IC_{50} 47.80±7.80 μM). Shifting of the hydroxy group from 3-position to 4-position (**IND3**) restored the antiproliferative activity against Colo38 (IC_{50} 0.73±0.05 μM) without modifying the activity against K562 (IC_{50} 46.11±4.30 μM). The introduction of a second hydroxy group on the benzylidene ring to give the 2,5-dihydroxybenzylidene compound (**IND4)** reduced the activity against Kolo38 (IC_{50} 2.96±0.13 μM) compared with **IND3** but restored the activity against K562 (IC_{50} 8.60±0.60 μM). Displacing the hydroxyl group from 5- to 4-position (**IND5**) resulted about 5-fold more active against Kolo38 (IC_{50} 0.57±0.05 μM) and about 2-fold more active against K562 (IC_{50} 4.31±0.40 μM) cell lines. On the contrary the introduction of a third hydroxyl group (**IND6, IND7**) determined a reduction of activity, especially for the 2,4,6-trihydroxybenzylidene derivative against K562 cells (IC_{50} 54.52±0.11 μM). As showed above for the analog **BF9**, the replacement of the 4-hydroxy group with a 4-diethylamino group (**IND13**), maintained the

antiproliferative activity against Colo38 cells (IC_{50} 0.54±0.15 μM) and at the same time led to increase in activity against K562 cells (IC_{50} 0.63±0.05 μM). The 2-hydroxynaphtylidene derivative (**IND14**) showed high antiproliferative activity against both Colo38 (IC_{50} 0.83±0.09 μM) and K562 (IC_{50} 0.63±0.04 μM).

3.2.3 Benzimidazohydrazones

The third series of hydrazones **BEN1-13** is characterized by a benzimidazole core. Benzimidazole is a privileged structure in drug discovery and it has been found in several natural compounds, such as the 5,6-dimethyl-1-(D-ribofuranosil)-benzimidazole, as an integral part of vitamin B12.[52] Benzimidazole scaffold can be considered as isostere of indoles, purine and other natural compounds endowed with the capability to interact with proteins and nucleic acids.[53] Benzimidazole ring system is present in different commercial drugs, such as Candesartan, Pantoprazole, Astemizole and in analgesic, anti-diabetic, anthelmintic and antifungals drugs.[32,54] It has also reported the interesting antiproliferative, antioxidant and photoprotective activity of benzimidazole derivatives.[55-59] Indeed, benzimidazole ring is present in some commercial sunscreens, such as in PBSA. Another interesting compound is the disodium salt of phenyl-dibenzimidazole-tetrasulfonate (known as Neo Heliopan® AP). This compound absorbs mainly in the UVA range, is well soluble in aqueous phase when added with a base, it is stable and safe with a low degree of penetration of the skin. Starting from these considerations, a new series of compounds was designed and synthetized to combine the properties of PBSA and the strong antioxidant properties of polyphenols to obtain dualistic molecules.[60] The target benzimidazolehydazones were synthetized as shown in scheme 3.5. The ethanolic solution of ethyl 1*H*-benzo[*d*]imidazole-2-carboxylate **30** was treated with hydrazine hydrate and refluxed for three hours to afford the hydrazide intermediate **31**. The desired hydrazones **BEN1-13** were obtained by coupling the hydrazide **31** with the appropriate hydroxyarylaldehyde in ethanol.

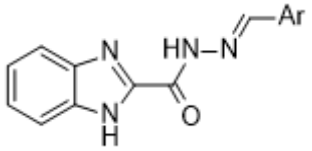


Scheme 3.5. General synthetic procedure for **BEN1-13**. Reagents and conditions: (i) $NH_2NH_2 \cdot H_2O$, EtOH, reflux 3h; (ii) ArCHO, EtOH, reflux 18h.

All the new compounds gave corrected analytic data. The IR and NMR data were consistent with the assigned structure. According to the literature and the spectral data of **BF1-10** and **IND1-14** the presence of a single downfield resonanting (8.49-9.79 ppm) CH=N signal indicates the exclusive formation of *E*-isomers.

As observed for benzofuranehydrazones and indolehydrazones, the DPPH test showed a clear connection between the antioxidant activity and both number and position of hydroxy groups on the arylidene ring (figure 3.11). The best compound of the series resulted the 2,3,4-trihydroxybenzylidene derivative **BEN6**, closely followed by the 2,5-dihydroxybenzylidene derivative **BEN5**. The 2,4,6-trihydroxybenzylidene derivative **BEN8** showed reduced antioxidant activity as well as the 2,4-dihydroxybenzylidene derivative **BEN3**.

Table 3.11. Evaluation of antioxidant activity of compounds **BEN1-13**.



Compound	Ar	DPPH[a] (µmolTE/g)	FRAP[a] (µmolTE/g)	ORAC[a] (µmolTE/g)
BEN1	4-hydroxyphenyl	29,63 ± 0,1	1040,15 ± 11,2	30911,28± 36,4
BEN2	3-hydroxyphenyl	13,00 ± 1	1023,28 ± 9,4	21807,97 ± 99,14
BEN3	2,4-dihydroxyphenyl	200,56 ± 1,5	286,69 ± 10,3	9296,77 ± 72,29
BEN4	2-hydroxyphenyl	23,20 ± 0,1	48,05 ± 2,6	-
BEN5	2,5-dihydroxyphenyl	9387,86 ± 13,8	5330,55 ± 29,6	17856,91 ± 35,1
BEN6	2,3,4-trihydroxyphenyl	10945,20 ± 38,5	10064,57 ± 24,6	4221,50 ± 10,5
BEN7	2-hydroxy-4-methoxyphenyl	201,17 ± 4.9	231,90 ± 5,5	-
BEN8	2,4,6-trihydroxyphenyl	192,55 ± 3,5	4071,740 ± 10	4098,619 ± 39,1
BEN9	2-hydroxy-4-(diethylamino)phenyl	1065,00 ± 5,9	3525,37 ± 13,4	754,4 ± 19,0
BEN10	2-hydroxy-3-ethoxyphenyl	38,12 ± 1,6	104,50 ± 2,1	-
BEN11	2-hydroxy-5-chlorophenyl	13,286	59,951	-
BEN12	2-hydroxy-5-bromophenyl	28,83 ± 1,2	81,69 ±2,7	-
BEN13	2-hydroxynaphtyl	123,38 ± 2,6	99,52 ± 3,2	-

[a]Each value was obtained from three experiments (mean ± SE). [b] LOQ limit of quantification; – not tested.

Concerning the mono-hydroxy derivatives **BEN1, BEN2** and **BEN4** (4-hydroxybenzylidene, 3-hydroxybenzylidene and 2-hydroxybenzylidene derivatives) showed weak antioxidant activity. The introduction of electron withdrawing groups such as chlorine or bromine atoms in 5-positions (**BEN11** 2-hydroxy-5-chlorobenzylidene and **BEN12** 2-hydroxy-5-bromobenzylidene derivatives) led to a depletion of activity, as well as the introduction of a methoxy group in 4-position (**BEN7** 2-hydroxy-4-methoxybenzylidene derivative) or an ethoxy group in 3-position (**BEN10** 2-hydroxy-3-ethoxybenzylidene derivative). The introduction of diethylamino group in 4-position such as in **BEN9** (2-hydroxy-4-diethylamino derivative) partially restored the activity while the 2-hydroxynaphtyl derivative **BEN13** showed weak antioxidant activity. The FRAP analysis confirmed the good antioxidant activity of **BEN5** and **BEN6**. In contrast with DPPH results, the

2,4,6-trihydroxybenzylidene derivative **BEN8** showed good antioxidant activity, comparable to **BEN5**. According to DPPH test **BEN1, BEN3, BEN4, BEN7, BEN10, BEN11-13** showed weak antioxidant activity, while **BEN9** was endowed with good activity. Furthermore, according to FRAP test the 2-hydroxy-4-diethylaminobenzylidene derivative **BEN9** showed good antioxidant activity.

Lastly, the compounds with the best antioxidant activity in DPPH and FRAP tests were also tested for the evaluation of free radical scavenger activity using the ORAC test. The 2,5-dihydroxybenzylidene derivative **BEN5** resulted one of the best compounds of the series, confirming the good antioxidant activity showed in DPPH and FRAP tests, as well as **BEN6** and **BEN8.** According to ORAC test, **BEN3** displayed good antioxidant potency while the 2-hydroxy-4-diethylamino derivative **BEN9** showed low antioxidant activity.

BEN1-3, **BEN5-6** and **BEN8-9** showing the best antioxidant profile were also evaluated for their photoprotective activity (Table 3.12). The analysis of SPF value showed that all tested hydrazones displayed better values than the reference PBSA with the only exception for the 2-hydroxy-4-diethylaminobenzylidene derivative **BEN9**, showing SPF value of 1.57. Hydrazones **BEN1**, **BEN2** and **BEN5**, exhibited similar protection value, with SPF>10. In the case of mono-hydroxy derivatives **BEN1** and **BEN2** the position of the hydroxy group did not result in significant variation of filter capability. On the other hand, in the di-hydroxy derivatives the SPF was related to their position being the 2,5-dihydroxybenzylidene derivative (**BEN5**) SPF value better than 2,4-dihydroxybenzylidene analog (**BEN3**).

Table 3.12. UV-filtering activity of selected benzimidazoles in solution

Compound	SPF (P ≤0.05)	UVA/UVB (P ≤0.05)	UVAPF (P ≤0.05)	λc[a] (nm)
BEN1	12.32	0.75	2.95	353
BEN2	11.54	0.27	1.60	343
BEN3	4.81	1.26	4.84	366
BEN5	11.23	0.32	3.24	377
BEN6	8.12	2.23	6.79	366
BEN8	8.34	2.27	10.65	370
BEN9	1.57	1.18	3.99	394
PBSA	3.40	0.29	1.03	322

[a]Wavelength at which the integral of the spectral absorbance curve reaches 90% of the area under the curve from 250 to 500 nm.

According with the FDA classification for the λc (0 (λc<325nm); 1 (325≤λc≤335); 2 (335≤λc≤350); 3 (350≤λc≤370) 4 (λc≥370)), **BEN2** was classified as 2, **BEN1, BEN3, BEN6,** and **BEN8** were classified as 3, **BEN5** and **BEN9** were classified as 4. A good solar filter should have a value of λc≥370 so that **BEN5** and **BEN9** showed the best results.

Considering the UE recommendation for the UVA/UVB absorbance ratio, **BEN6** and **BEN8** displayed better values than the reference PBSA, while **IND2** showed UVA/UVB absorbance ratio similar to PBSA value. All the tested hydrazones showed better UVAPF value than PBSA and the 2,3,4-trihydroxybenzilidene derivative **BEN8** resulted the best compound of the series, with UVAPF value 10.65.

The benzimidazole derivatives **BEN1-3**, **5**, **6**, **8-10** were tested on human melanoma Colo38 and erythroleukemic K565 cells to evaluate their antiproliferative activity and resulting IC_{50} values are reported in Table 3.13. The 2-hydroxy-4-diethylamino derivative **BEN9** resulted the best compound of the series against Colo38 cell line, with IC_{50} 0,50±0,12 μM. **BEN9** also showed good activity against K562 cell line, with IC_{50} 5.81±0.5 μM. The mono-hydroxy derivatives **BEN1** and **BEN2** displayed antiproliferative activity at high μM levels as well as the di- and tri-hydroxy derivatives. Among them, only **BEN6** showed good activity against K562 cell line, with IC_{50} 7.5±1.13 μM. The 2-hydroxy-3-ethoxybenzylidene derivative **BEN10** is endowed with antiproliferative activity at high μM levels against Colo38 cell line (IC_{50} 55,90±1,27 μM) while it resulted the best compound of the series against K562 cell line, with IC_{50} 0,84±0,03 μM.

Table 3.13. Effect of selected benzimidazole hydrazones on the proliferation of Colo38 and K562 cells.

Compound	IC_{50} (μM)	
	Colo38	**K562**
BEN1	216.16±40.21 μM	91.85±4.0 μM
BEN2	362.63±15.05 μM	-
BEN3	46.62±0.82 μM	-
BEN5	35.49±0.42 μM	48.9±9,3 μM
BEN6	46.62±3.57 μM	7.5±1.13 μM
BEN8	459.59±103.87 μM	68.20±4.2 μM
BEN9	0.50±0.12 μM	5.81±0.5 μM
BEN10	55.90±1.27 μM	0.84±0,03 μM

3.2.4 Conclusions

For the investigation of compounds endowed with antioxidant and photoprotective compounds, three scaffolds have been selected due to their well-known biological effects: benzofuran, indole and benzimidazole, decorated with various arylhydrazones. The three series showed different extent of radical-scavenging ability towards the nitrogen radical by the DPPH test, to reduce ferric ion by the FRAP test and to inhibit the oxidative degradation caused by peroxyl radicals using the ORAC test. The SAR data obtained from DPPH, FRAP and ORAC assays, showed an interesting correlation between the number and the position of hydroxyl groups on arylidene moiety and the antioxidant activity, as well as the presence of 2-hydroxy-4-diethylamino group. On the contrary,

the presence of electron attractor groups, such as chlorine or bromine atoms reduced the antioxidant activity. The hydrazones were further tested *in vitro* to evaluate their filtering power by the analysis of important parameters such as SPF, λc and UVAPF values. From these results emerged the significant photoprotective activity of the mono-hydroxylated compounds, as well as the activity of the 2-hydroxynaphtyl and 2-hydroxy-4-diethylamino compounds, comparable with the photoprotective capabilities of commercial PBSA sunscreen filter. The best compounds from antioxidant and photoprotective assays were also tested *in vitro* on Human melanoma Colo38 and erythroleukemic K562 cell lines to evaluate their potential antiproliferative activity. Interestingly, **BF9**, **IND13** and **BEN9** bearing the 2-hydroxy-4-diethylamino group also showed the best antiproliferative activity against both cell lines. Taken together all these results, benzofuran, indole and benzimidazole hydrazones displayed multifunctional properties and indicated these compounds in the possible treatment of neoplastic diseases due to the good antioxidant properties correlated to their high antiproliferative activity.

3.3 2-Arylbenzimidazoles

To further investigate new molecules endowed with antioxidant, photoprotective and antiproliferative activity, in this thesis a new series of benzimidazole derivative has been developed. The new benzimidazole series was designed as analog of commercial compounds, such as PBSA, Oxisol and Neo Heliopan (Figure 3.10). Starting from **BEN1-10** scaffold, in the new series an hydroxylaryl moiety is directly bound at 2-position of benzimidazole ring, and three different groups, CN, COOH and SO_3H, were introduced at 5-position of benzimidazole ring.[61]

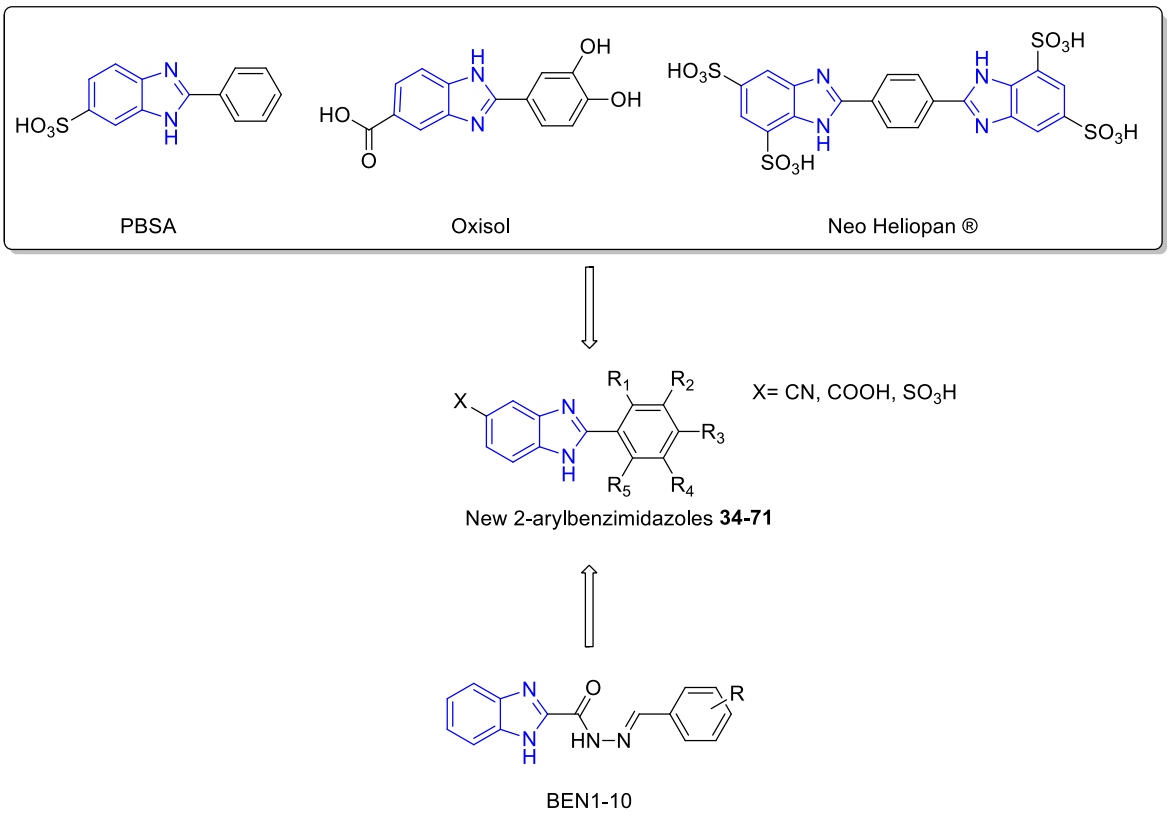
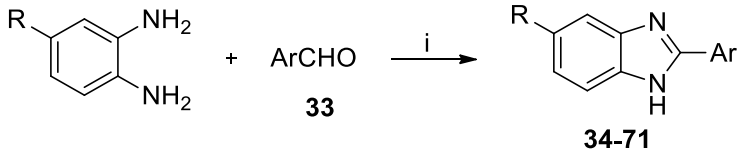


Figure 3.10. Commercial photoprotective benzimidazole derivatives and design of new 2-arylbenzimidazoles **34-71**.

The target benzimidazoles **34–71** were easily synthesized by the condensation between 5-substituted diaminobenzenes **32a-d**, and the appropriate aldehyde **33** in EtOH and in the presence of sodium metabisulphite in aqueous solution. The resulting mixture was refluxed to achieve benzimidazole **34-71** in good yields (Scheme 3.5). Structures were confirmed based on analytical and spectral data and are consistent with results of reported studies[62,63].



32a R = H
32b R = CN
32c R = COOH
32d R = SO_3H

Scheme 3.5. General synthetic procedure for benzimidazoles **3-40**. Reagents and conditions: EtOH, 2.5 N sodium metabisulphite, reflux 24 h.

As for the previously described series, the new 2-arylbenzimidazoles **34-71** were evaluated for their antioxidant (Table 3.14), photoprotective (Table 3.15) and antiproliferative (Table 3.16) activity.

As observed for the arylhydrazones described above, the antioxidant activity of 2-arylbenzimidazoles is correlated with the number of hydroxy groups on the aryl ring (Table 3.14). The mono-hydroxylated benzimidazoles **34-71** showed weak activity while the introduction of a second (benzimidazoles **44-48**) or a third hydroxyl group (benzimidazoles **62-67**) led to the best compounds of the series. Furthermore, the position of the hydroxy groups had an impact on antioxidant activity. For example, the 2,5-dyhydroxybenzimidazole derivatives **44**, **45** resulted about 2-fold more active than the 3,4-dihydroxyphenyl analogs **47**, **48**, while shifting the 3-hydroxy group of **47** into 2-position to give the analog **43** produced drop in activity. Additionally, the 2,3,4-trihydroxphenyl derivatives **62** and **63** showed better antioxidant activity than the 2,4,6-trihydroxyphenyl analogs **65** and **66.** In contrast, the 2,4,6-trihydroxyphenyl analog **67** was characterized by better antioxidant activity than the corresponding 2,3,4-trihydroxyphenyl derivative **64**. Replacing one hydroxy group with an alkoxy group (compounds **49-55**) or halogen atoms (compounds **56-61**) led to reduction in activity while the introduction of a 4-diethylamino group (compounds **68** and **69**) increased the activity.

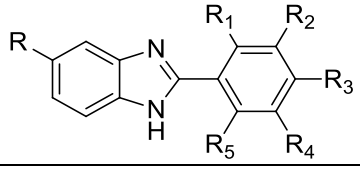
FRAP analysis revealed that the compounds showing high antioxidant activity were **44-48** and **62-68**. Remarkably, the 2-hydroxy-3-ethoxyphenyl derivatives **52**, **53** and the 2-hydroxy-4-(diethyl)amino derivative **69** displayed good antioxidant activity. On the base of the DPPH and FRAP analysis, the most powerful compounds were tested to determine their capability as radical scavenger by ORAC test. Among them, compound **44** showed the best antioxidant activity.

The 2-hydroxy-4-(diethyl)aminophenyl derivatives **68** and **69** displayed high antioxidant capacity while the 2,3,4-trihydroxyphenyl derivative **62** showed better activity as compared to the corresponding 2,4,6-trihydroxyphenyl analog **65**, in contrast with DPPH and FRAP analysis.

The compounds **44-48**, **52**, **53**, **61-69** and **71** endowed with the best antioxidant activity were tested to evaluate their photoprotective properties (Table 3.15).

Benzimidazoles **47**, **48**, **52**, **53** and **67** showed better SPF value than PBSA and the 2-hydroxy-3-ethoxyphenyl derivatives **52** and **53** displayed the highest values, 6.03 and 6.25 respectively. Speaking about the 2,4,6-trihydroxyphenyl derivative **65-66**, they showed SPF value 3.34 and 3.20 respectively, similar to PBSA. Shifting the hydroxy group from 5-position to 4-position as in benzimidazole **47** and **48**, increased the protection value.

Table 3.14. Evaluation of Antioxidant activity.



Compd.	R	R_1	R_2	R_3	R_4	R_5	DPPH[a] µmolTE/g	FRAP[a] µmolTE/g	ORAC[a] µmolTE/g
34	H	H	H	OH	H	H	10.9 ±1.5	15.1 ± 0.4	-
35	CN	H	H	OH	H	H	<15.8 [b]	30.3 ± 2.7	-
36	COOH	H	H	OH	H	H	< 39.2 [b]	94.2 ± 2.5	-
37	SO_3H	H	H	OH	H	H	na[c]	24.6 ± 1.8	-
38	COOH	OH	H	H	H	H	51.8 ± 0.6	103.3 ± 3.7	-
39	SO_3H	OH	H	H	H	H	na[c]	187.4 ± 4.9	-
40	CN	H	OH	H	H	H	<18.3[b]	29.0 ± 2.5	-
41	COOH	H	OH	H	H	H	87.4 ± 1.2	223.4 ± 3.8	-
42	SO_3H	H	OH	H	H	H	na[c]	56.5 ± 1.2	-
43	CN	OH	H	OH	H	H	<27.5[b]	209.2 ± 1.8	-
44	CN	OH	H	H	OH	H	4747.2 ± 19.9	10109.0 ± 13.7	20827.9 ± 29.5
45	COOH	OH	H	H	OH	H	4824.7 ± 11.6	10318.2 ± 15.8	18378.7 ± 18.4
46	SO_3H	OH	H	H	OH	H	515.7 ± 1.8	1562.7 ± 13.1	16014.2 ± 67.4
47	CN	H	OH	OH	H	H	2042.1 ± 7.3	6353.8 ± 11.6	8210.2 ± 25.0
48	COOH	H	OH	OH	H	H	1946.8 ± 8.1	5502.3 ± 13.2	13536.6± 15.6
49	CN	H	OH	OMe	H	H	39.9 ± 0.9	72.7 ± 3.3	-
50	COOH	H	OH	OMe	H	H	48.4 ± 3.5	70.1 ± 2.3	-
51	SO_3H	H	OH	OMe	H	H	na[c]	814.9 ± 5.8	-
52	COOH	OH	OEt	H	H	H	109.7 ± 0.4	4098.7 ± 16.3	7639.9 ± 25.9
53	SO_3H	OH	OEt	H	H	H	< 50.9 [b]	2556.6 ± 17.6	13900.6 ± 61.4
54	COOH	OH	H	OMe	H	H	41.2 ± 2.4	113.5 ± 5.1	-
55	SO_3H	OH	H	OMe	H	H	na[c]	27.7 ± 2.4	-
56	CN	OH	H	H	Cl	H	45.0 ± 1.8	64.7 ± 3.9	-
57	COOH	OH	H	H	Cl	H	81.6 ± 3.4	94.2 ± 2.5	-
58	SO_3H	OH	H	H	Cl	H	<< 59.6 [b]	174.7 ± 6.2	-
59	CN	OH	H	H	Br	H	48.7 ± 1.7	48.5 ± 1.5	-
60	COOH	OH	H	H	Br	H	81.3 ± 0.9	117.3 ± 4.1	-
61	SO_3H	OH	H	H	Br	H	<< 50.0 [b]	63.4 ± 2.7	-
62	CN	OH	OH	OH	H	H	7112.7 ± 15.1	12049.2 ± 19.2	8312.9 ± 31.8
63	COOH	OH	OH	OH	H	H	5026.6 ± 13.9	11375. ± 19.6	7321. ± 17.8
64	SO_3H	OH	OH	OH	H	H	1324.1 ± 14.2	9097.5 ± 16.7	6726.9 ± 17.4
65	CN	OH	H	OH	H	OH	1473.0 ± 10.3	7138.2 ± 12.4	4758.0 ± 13.7
66	COOH	OH	H	OH	H	OH	897.3 ± 5.6	2334.8 ± 7.5	9467.8 ± 63.2
67	SO_3H	OH	H	OH	H	OH	5026.6 ± 13.8	1970.3 ± 3	8679.5 ± 28.0
68	COOH	OH	H	$N(Et)_2$	H	H	165.1 ± 7.5	5233.1 ± 6.2	14583.9 ± 11.0
69	SO_3H	OH	H	$N(Et)_2$	H	H	84.9 ± 0.7	925.7 ± 8.8	12871.7 ± 35.6
70	COOH	2-OH-naphthyl					< 27.6 [b]	187.7 ± 3.1	-
71	SO_3H	2-OH-naphthyl					4.9 ± 0.7	527.7± 8.8	4297.5 ± 41.5

[a] Each value was obtained from three experiments (mean ± SE).[b] LOQ limit of quantification; – not tested. [c] precipitation of the compound is observed.

Table 3.15. UV-filtering activity of selected benzimidazoles in solution.

Compound	SPF (P ≤0.05)	UVA/UVB (P ≤0.05)	UVAPF (P ≤0.05)	λc[a] (nm)
44	2,93	0,73	2,28	369
45	2,79	0,75	2,19	368
46	2,55	0,70	2,06	373
47	4,63	0,86	1,79	350
48	4,26	0,85	1,71	349
52	6,03	0,37	1,39	342
53	6,25	0,29	1,32	340
62	2,56	1,10	1,48	347
63	2,71	1,04	1,51	348
64	1,70	0,81	1,10	343
65	3,34	1,27	1,78	349
66	3,20	0,90	1,49	355
67	4,96	0,67	1,58	350
68	1,79	2,10	14,30	387
69	1,71	2,20	15,77	386
71	2,3	0,77	1,62	362
PBSA	3.40	0.29	1.03	322

According to the FDA classification of critical wavelength, compounds **47**, **48**, **52**, **53**, **62-65** and **67** were classified as 2; compounds **66** and **71** were classified as 3 and compounds **68** and **69** were classified as 4. According to the EU recommendation on the efficacy of sun protection products, benzimidazoles **68** and **69** were the only interesting benzimidazoles of the series, with a λc of 387 and 386 respectively.

Another important parameter for evaluating a sunscreen compound, is the UVA/UVB absorbance ratio. According with the latest UE recommendation (2006/247/EC), this ratio should be worth at least 0.33 so that, excluding **53**, all the tested compounds showed UVA/UVB ratio >0.33. The best compound of the series **68** showed UVA/UVB absorbance ratio value of 2.10 and 2.20 respectively. Furthermore, the UVAPF showed that compounds **68** and **69** were also endowed with better UVAPF than PBSA (UVAPF **68** 14.30, UVAPF **69** 15.77), confirming their good photoprotective activity.

The benzimidazoles **44-48**, **52**, **53**, **62-69** and **71** and were also tested on human melanoma Colo38 cell line to determine their antiproliferative activity (Table 3.16).

Benzimidazole **44** exhibited the best activity of the series, displaying IC_{50} values 50.14±2.41 μM. The 2,3,4-trihydroxyphenyl derivatives **62-64** showed similar antiproliferative activity at micromolar concentration, while their 2,4,6-trihydroxyphenyl analogs (**65-67**) showed poor antiproliferative activity. Benzimidazole **44**, demonstrating the best antiproliferative activity, was also tested on normal human keratinocyte HaCat cell line to preliminary evaluate their selectivity against cancer cells. The compound **44** showed low activity against HaCat cells (IC_{50} value

278.97±48.01 μM) being about 5-fold more active against Colo38 cell line. This result suggests a preferential activity of tested compounds against cancer cells.

Table 3.16. Effects of selected benzimidazole derivatives on the proliferation of Colo38 cells.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
44	50.14±2.41	**63**	65.07±0.18
45	409.31±4.44	**64**	74.4±6.27
46	79.13±10.21	**65**	446.96±33.71
47	62.02±7.76	**66**	> 500
48	424.53±44.47	**67**	> 500
52	318.06±40.49	**68**	171.84±38.24
53	> 500	**69**	450.79±41.32
62	96.23±10.25	**71**	323.78±22

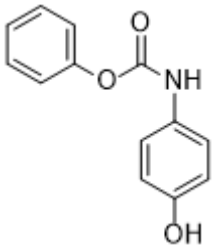
3.3.1 Conclusions

Starting from the good results of arylhydrazones compounds, a new series of 2-arylbenzimidazole derivatives **34-71** were synthesized and tested to evaluate antioxidant and photoprotective properties in the context multitarget study. The antioxidant capability of derivatives **34-71** were evaluated by DPPH, FRAP and ORAC assays, showing that the presence of a sulfonic acid at 5-position of benzimidazole scaffold, is the least favourable whereas benzimidazole bearing carboxyl or cyano groups in the same position showed various antioxidant activity. The compounds with the best antioxidant profile were investigated for their photoprotective activity and among them, compounds **46**, **47**, **48** and **69** were the best in the terms of broad-spectrum filtering activity. Selected compounds were also tested against Colo38 cell line and among them, benzimidazole **44** resulted the best compound of the series, with IC_{50} 50.14±2.41 μM. Furthermore, benzimidazole **44** showed weak activity against the normal HaCat keratinocyte cells, demonstrating selectivity against cancer cells.

3.4 Experimental

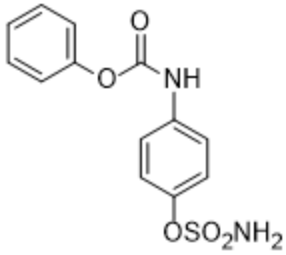
All commercially available solvents and reagents were used without further purification. ^{1}H NMR spectra were recorded on an Inova 500 spectrometer (Varian, Palo Alto, CA, USA). The chemical shifts (δ) are reported in part per million downfield from tetramethylsilane (TMS), which was used as internal standard. The spectra were recorded in hexadeuteriodimethylsulphoxide (DMSO-d_6). Infrared spectra were recorded on a Vector 22 spectrometer (Bruker, Bremen, Germany) in Nujol mulls. The main bands are given in cm^{-1}. Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing MAT 95 instrument (Finnigan, Waltham, MA, USA) with BE geometry. Melting points (mp) were determined with a SMP1 Melting Point apparatus (Stuart Scientific, Stone, UK) and are uncorrected. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. The purity of the tested compounds was determined by combustion elemental analyses conducted by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara with a MT-5 CHN recorder elemental analyser (Yanagimoto, Kyoto, Japan) and the values found were within 0.4% of theoretical values.

Phenyl (4-hydroxyphenyl)carbamate (3)



To an ice-cooled stirred solution of 4-aminopheol (0.545 g, 5 mmol) and DIPEA (0.69 mL, 4 mmol) in anhydrous THF (10 ml) phenylchloroformiate (0.5 mL, 4 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 24 hours, then water (100 mL) was added; the mixture was stirred for additional 2 h, the formed solid filtered off, and vacuum dried to give the desired compound. Yield 90%. M.p. 126-127 °C. ^{1}H NMR (DMSO-d_6) δ 6.62 (m, 2H, Ar), 7.19 (m, 2H, Ar), 7.26 (m, 2H, Ar), 7.41 (m, 3H, Ar), 9.52 (s, 1H, NH), 9.82 (s, 1H, OH). IR (Nujol) 3425, 3403, 1730, 1639, 1610 cm^{-1}. Elemental analysis: calculated for $C_{13}H_{11}NO_3$ (229.07) %C 68.11, %H 4.84, %N 6.11, found %C 68.07, %H 4.81, %N 6.15. M/z 230.

4-((phenoxycarbonyl)amino)phenylsulfamate (4)

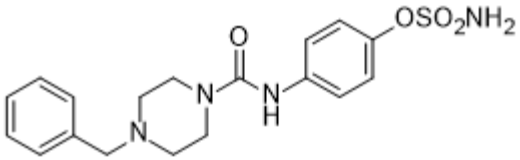


To a stirred solution of phenyl (4-hydroxyphenyl)carbamate (1.145 g, 5 mmol) in anhydrous DMA (10 mL), freshly prepared sulfamoyl chloride (0.81 g, 7 mmol) in DMA (5 mL) was added dropwise in 30 min. The mixture was stirred at room temperature overnight, then water (20 mL) was added. The mixture was stirred for an additional 2h, then the formed white solid was filtered off and dried to give sulfamate in good purity to be used in the next step without further purification. Yield 82%. M.p. 165-166 °C. ^{1}H NMR (DMSO-d_6): δ 7.23 (m, 4H, Ar), 7.43 (m, 3H, Ar), 7.55 (m, 2H, Ar), 7.91 (s, 2H, NH_2), 10.32 (s, 1H, NH). IR (Nujol) 3383, 3336, 3242, 1726 cm^{-1}. Elemental analysis: calculated for $C_{13}H_{12}NO_3$ (308.31) %C 50.64, %H 3.92, %N 9.09, found %C 50.69, %H 3.90, %N 9.12. M/z 309.

General procedure for the synthesis of 4-(piperazinocarbonyl)aminosulfamates (5-18)

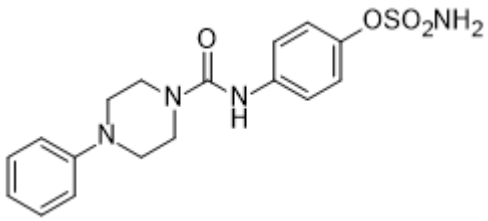
A mixture of 4-((phenoxycarbonyl)amino)phenyl sulfamate (1 mmol) and substituted piperazine (1 mmol) and DIPEA (0.5 mmol), in anhydrous DMSO (3 mL) was stirred at room temperature for 24 h. Then, water (10 mL) was added and the mixture was stirred at room temperature until a solid is formed. The solid formed was filtered off, washed with water and air dried to give the title aminosulfamates.

4-(4-Benzylpiperazine-1-carboxamido)phenylsulfamate (5)



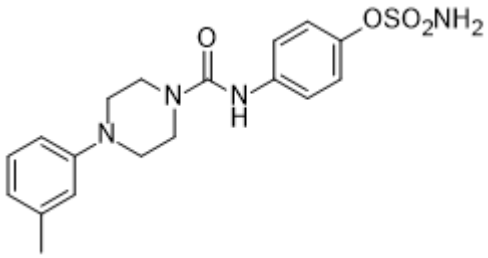
Following the general procedure, the title compound was prepared starting from benzylpiperazine. Yield 73%. M.p. 168-170 °C. ^{1}H NMR (DMSO-d_6): δ 2.37 (m, 4H, CH_2), 3.43 (m, 4H, CH_2), 3.50 (s, 2H, CH_2), 7.13 (d, *J* = 7.5, 2H, Ar), 7.25 (m, 2H, Ar), 7.31 (m, 3H, Ar), 7.47 (d, *J* = 7.5 Hz, 2H, Ar), 7.73 (s, 2H, NH_2), 8.58 (s, 1H, NH). IR (Nujol) 3419, 3323, 1649 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{22}N_4O_4S$ (390.46) %C 55.37, %H 5.68, %N 14.35, found %C 55.30, %H 5.65, %N 14.31. M/z 391.

4-(4-Phenylpiperazine-1-carboxamido)phenylsulfamate (6)



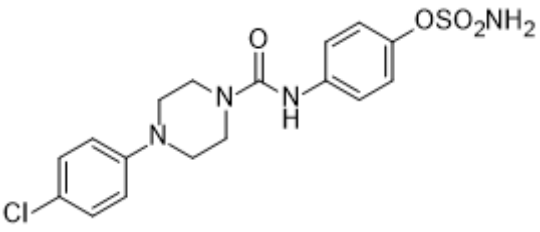
Following the general procedure, the title compound was prepared starting from phenylpiperazine. Yield 83%. M.p. 196-198 °C. ^{1}H NMR (DMSO-d_6): δ 3.15 (m, 4H, CH_2), 3.59 (m, 4H, CH_2), 6.80 (m, 2H, Ar), 6.98 (d, *J* = 7.0 Hz, 2H, Ar), 7.15 (m, 2H, Ar), 7.23 (m, 3H, Ar), 7.84 (s, 2H, NH_2), 8.71 (s, 1H, NH). IR (Nujol) 3411, 3306, 1642 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{20}N_4O_4S$ (376.43) %C 54.24, %H 5.36, %N 14.88, found %C 54.19, %H 5.34, %N 14.81. M/z 377.

4-(4-(m-Tolyl)piperazine-1-carboxamido)phenylsulfamate (7)



Following the general procedure, the title compound was prepared starting from m-tolylpiperazine. Yield 78%. M.p. 202-204 °C. ^{1}H NMR (DMSO-d_6): δ 2.26 (s, 3H, CH_3), 3.14 (m, 4H, CH_2), 3.58 (m, 4H, CH_2), 6.63 (m, 1H, Ar), 6.98 (m, 2H, Ar), 7.14 (m, 3H, Ar), 7.51 (d, *J* = 6.5 Hz, 2H, Ar), 7.72 (s, 2H, NH_2), 8.71 (s, 1H, NH). IR (Nujol) 3373, 3208, 1658 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{22}N_4O_4S$ (390.14) %C 55.37, %H 5.68, %N 14.35, found %C 55.32, %H 5.70, %N 14.30. M/z 391.

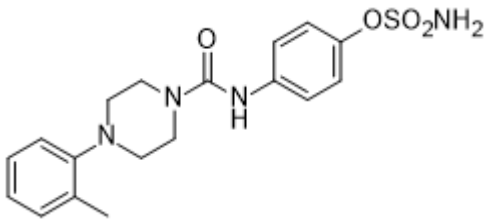
4-(4-(4-Chlorophenyl)piperazine-1-carboxamido)phenylsulfamate (8)



Following the general procedure, the title compound was prepared starting from 4-chlorophenylpiperazine. Yield 93%. M.p. 192-194 °C. ^{1}H NMR (DMSO-d_6): δ 3.16 (m, 4H, CH_2), 3.58 (m, 4H, CH_2), 6.98 (m, 2H, Ar), 7.14 (d, *J* = 7.5 Hz, 2H, Ar), 7.23 (m, 2H, Ar), 7.50 (d, *J* = 7.5 Hz, 2H), 7.86 (s, 2H, NH_2), 8.70 (s, 1H, NH). IR (Nujol) 3418, 3256, 1640 cm^{-1}. Elemental

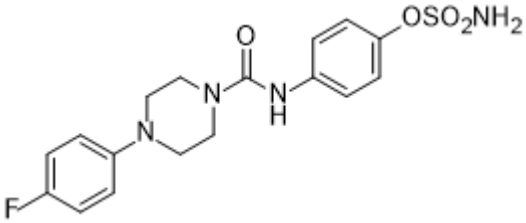
analysis: calculated for $C_{17}H_{19}ClN_4O_4S$ (410.88) %C 49.69, %H 4.66, %N 13.64, found %C 49.61, %H 4.64, %N 13.59. M/z 411.

4-(4-(o-Tolyl)piperazine-1-carboxamido)phenylsulfamate (9)



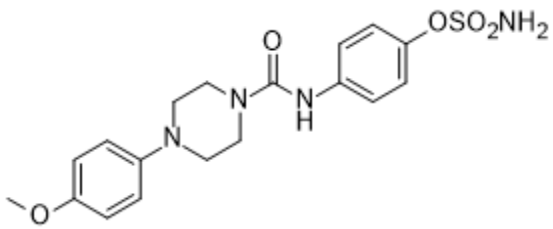
Following the general procedure, the title compound was prepared starting from o-tolylpiperazine. Yield 72%. M.p. 170-171 °C. ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, CH_3), 2.85 (m, 4H, CH_2), 3.60 (m, 4H, CH_2), 6.98 (d, *J* = 7.0 Hz, 1H, Ar), 7.04 (d, *J* = 8.5 Hz, 1H, Ar), 7.15 (d, *J* = 8.0 Hz, 2H, Ar), 7.18 (d, *J* = 7.0 Hz, 2H, Ar), 7.52 (d, *J* = 8.0 Hz, 2H, Ar), 7.86 (s, 2H, NH_2), 8.70 (s, 1H, NH). IR (Nujol) 3329, 1647, 1535 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{22}N_4O_4S$ (390.14) %C 55.37, %H 5.68, %N 14.35, found %C 55.30, %H 5.66, %N 14.39. M/z 391.

4-(4-(4-Fluorophenyl)piperazine-1-carboxamido)phenylsulfamate (10)



Following the general procedure, the title compound was prepared starting from 4-fluorophenylpiperazine. Yield 68%. M.p. 188-190 °C. ^{1}H NMR (DMSO-d_6): δ 3.10 (m, 4H, CH_2), 3.59 (m, 4H, CH_2), 7.00 (m, 2H, CH_2), 7.06 (m, 2H, Ar), 7.14 (d, *J* = 8.5 Hz, 2H, Ar), 7.51 (d, *J* = 8.5 Hz, 2H, Ar), 7.86 (s, 2H, NH_2), 8.70 (s, 1H, NH). IR (Nujol) 3368, 3188, 1661 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{19}FN_4O_4S$ (394.42) %C 51.77, %H 4.86, %N 14.20, found %C 51.72, %H 4.85, %N 14.14. M/z 395.

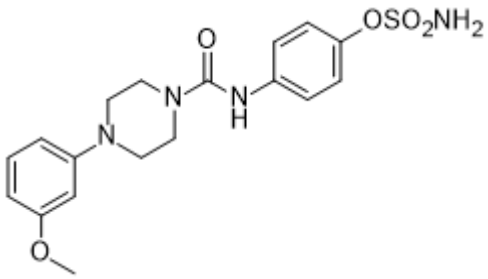
4-(4-(4-Methoxyphenyl)piperazine-1-carboxamido)phenylsulfamate (11)



Following the general procedure, the title compound was prepared starting from 4-methoxyphenyllpiperazine. Yield 80%. M.p. 190-192 °C. ^{1}H NMR (DMSO-d_6): δ 3.02 (m, 4H, CH_2), 3.58 (m, 4H, CH_2), 3.68 (s, 3H, OCH_3), 6.83 (d, *J* = 8.5 Hz, 2H, Ar), 6.93 (s, 2H, Ar); 7.14 (d, *J* = 8.5 Hz, 2H, Ar),7.50 (d, *J* = 7.5 Hz, 2H, Ar), 7.86 (s, 2H, NH_2), 8.69 (s, 1H, NH). IR (Nujol)

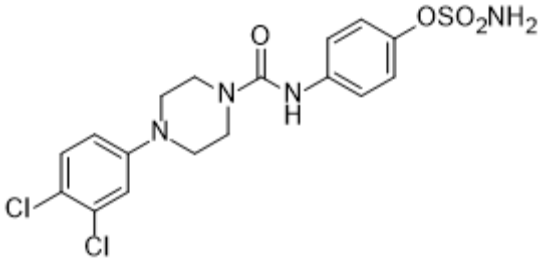
3406, 3284, 1638 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{22}N_4O_5S$ (406.46) %C 53.19, %H 5.46, %N 13.78, found %C 53.12, %H 5.44, %N 13.83. M/z 407.

4-(4-(3-Methoxyphenyl)piperazine-1-carboxamido)phenylsulfamate (12)



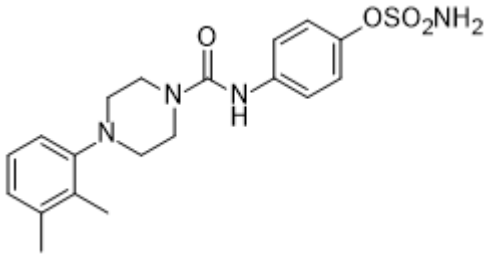
Following the general procedure, the title compound was prepared starting from 3-methoxyphenylpiperazine. Yield 59%. M.p. 193-195 °C. ^{1}H NMR (DMSO-d_6): δ 3.15 (m, 4H, CH_2), 3.57 (m, 4H, CH_2), 3.71 (s, 3H, OCH_3), 6.39 (m, 1H, Ar), 6.49 (s, 1H, Ar), 6.56 (m, 1H, Ar), 7.14 (m, 3H, Ar), 7.51 (d, *J* = 8.0 Hz, 2H, Ar), 7.80 (s, 2H, NH_2), 8.70 (s, 1H, NH). IR (Nujol) 3383, 3179, 1657 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{22}N_4O_5S$ (406.46) %C 53.19, %H 5.46, %N 13.78, found %C 53.14, %H 5.48, %N 13.72. M/z 407.

4-(4-(3,4-Dichlorophenyl)piperazine-1-carboxamido)phenylsulfamate (13)



Following the general procedure, the title compound was prepared starting from 3,4-dichlorophenylpiperazine. Yield 98%. M.p. 144-145°C. ^{1}H NMR (DMSO-d_6): δ 3.23 (m, 4H, CH_2), 3.58 (m, 4H, CH_2), 6,97 (s, 1H, Ar), 7.14 (d, *J* = 8.5 Hz, 2H, Ar), 7.20 (m, 2H, Ar), 7.52 (d, *J* = 8.5 Hz, 2H, Ar), 7.87 (s, 2H, NH_2), 8.72 (s, 1H, NH). IR (Nujol) 3341, 1649 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{18}Cl_2N_4O_4S$ (445,32) %C 45.85, %H 4.07, %N 12.58, found %C 45.92, %H 4.48, %N 12.62. M/z 446.

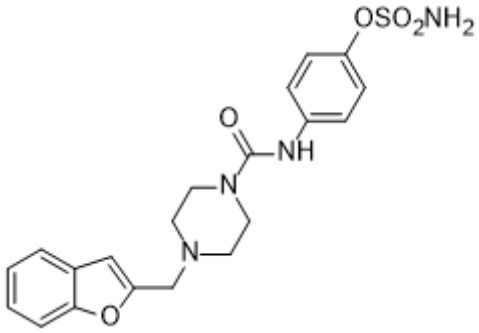
4-(4-(2,3-Dimethylphenyl)piperazine-1-carboxamido)phenyl sulfamate (14)



Following the general procedure, the title compound was prepared starting from 2,3-dimethylphenylpiperazine. Yield 42%. M.p. 176-178 °C. ^{1}H NMR (DMSO-d_6): δ 2.19 (s, 3H, CH_3),

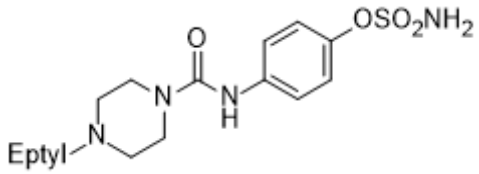
2.21 (s, 3H, CH_3), 2.80 (m, 4H, CH_2), 3.60 (m, 4H, CH_2), 6.89 (m, 2H, CH_2), 7.04 (m, 1H, Ar), 7.14 (d, *J* = 8.5 Hz, 2H, Ar), 7.51 (d, *J* = 7.5 Hz, 2H, Ar), 7.86 (s, 2H, NH_2), 8.68 (s, 1H, NH). IR (Nujol) 3353, 3196, 3097, 1648 cm^{-1}. Elemental analysis: calculated for $C_{19}H_{24}N_4O_4S$ (404.48) %C 56.42, %H 5.94, %N 13.85, found %C 56.37, %H 5.99, %N 13.77. M/z 407.

4-(4-(Benzofuran-2-ylmethyl)piperazine-1-carboxamido)phenylsulfamate (15)



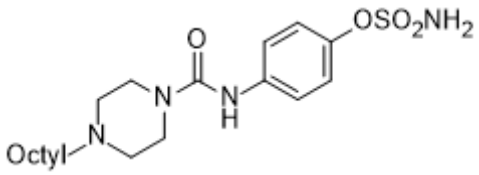
Following the general procedure, the title compound was prepared starting from 1-(benzofuran-2-ylmethyl)piperazine. Yield 40%. M.p. 140-141°C. ^1H NMR (DMSO-d_6) δ 2.54 (s, 2H, CH_2), 3.30 (m, 4H, CH_2), 3.51 (m, 4H, CH_2), 6.81 (s, 1H, Ar), 7.13 (d, *J* = 9.0 Hz, 1H, Ar), 7.16 (d, *J* = 9.0 Hz, 1H, Ar), 7.26 (d, *J* = 9.5 Hz, 1H, Ar), 7.43 (d, *J* = 7.0 Hz, 1H, Ar), 7.46 (d, *J* = 7.0 Hz, 1H, Ar), 7.49 (d, *J* = 9.5 Hz, 1H, Ar), 7.47 (m, 2H, Ar), 7.88 (s, 2H, NH_2), 8.61 (s, 1H, NH). IR (Nujol) 3386, 1645 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{22}N_4O_5S$ (430,13) %C 55.80, %H 5.15, %N 13.02, found %C 55.73, %H 5.13, %N 12.98. M/z 431.

4-(4-Heptylpiperazine-1-carboxamido)phenylsulfamate (16)



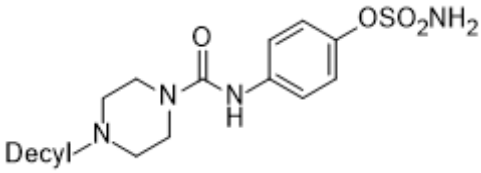
Following the general procedure, the title compound was prepared starting from *n*-heptylpiperazine. Yield 42%. M.p. 114-115 °C. ^1H NMR (DMSO d_6): δ 0.87 (m, 3H, CH_3), 1.28 (m, 12H, CH_2), 1.48 (m, 2H, CH_2), 3.29 (m, 4H, CH_2), 3.48 (m, 2H, CH_2), 7.14 (s, 2H, Ar), 7.48 (s, 2H, Ar), 7.86 (s, 2H, NH_2), 8.63 (s, 1H, NH). IR (Nujol) 3348, 1642, 1538 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{30}N_4O_4S$ (398.52) %C 54.25, %H 7.59, %N 14.06, found %C 54.31, %H 7.57, %N 14.09. M/z 399.

4-(4-Octylpiperazine-1-carboxamido)phenylsulfamate (17)



Following the general procedure, the title compound was prepared starting from *n*-octylpiperazine. Yield 38%. M.p. 154-155 °C. ^{1}H NMR (DMSO d_6): δ 0.87 (m, 3H, CH_3), 1.27 (m, 14H, CH_2), 2.36 (s, 4H, CH_2), 3.43 (s, 4H, CH_2), 7.14 (d, *J* = 7.0 Hz, 2H, Ar), 7.49 (d, *J* = 7.5 Hz, 2H, Ar), 7.86 (s, 2H, NH_2), 8.59 (s, 1H, NH). IR (Nujol) 3373, 1642 cm^{-1}. Elemental analysis: calculated for $C_{19}H_{32}N_4O_4S$ (412.55) C, 55.32; H, 7.82; N, 13.58. Found C, 55.27; H, 7.98; N, 13.62. M/z 413.

4-(4-Decylpiperazine-1-carboxamido)phenyl sulfamate (18)

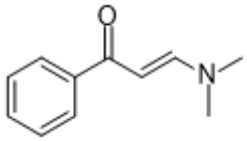


Following the general procedure, the title compound was prepared starting from *n*-decylpiperazine. Yield 51%. M.p. 159-160°C. ^{1}H NMR (DMSO d_6): δ 0.86 (m, 3H, CH_3), 1.26 (m, 18H, CH_2), 2.38 (s, 4H, CH_2), 3.44 (s, 4H, CH_2), 7.13 (d, *J* = 9.0 Hz, 2H, Ar), 7.50 (d, *J* = 9.0 Hz, 2H, Ar), 7.86 (s, 2H, NH_2), 8.60 (s, 1H, NH). IR (Nujol) 3388, 1642 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{36}N_4O_4S$ (440.60) %C 57.25, %H 8.24, %N 12.72, found %C 55.19, %H 8.21, %N 12.76. M/z 441.

General procedure for the synthesis of phenylprop-2-en-1-ones (20a-h)

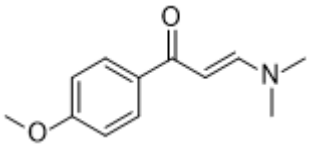
A mixture of substituted acetophenone (0.9 g, 5 mmol) and DMF-DMA (1.79 g, 15 mmol) in anhydrous toluene (10 mL) was refluxed for 1 h, then was allowed to reach the room temperature and stirred for additional 24 h. The mixture was carefully concentrated in vacuum to give the title compounds.

(*E*)-3-(Dimethylamino)-1-phenylprop-2-en-1-one (20a)



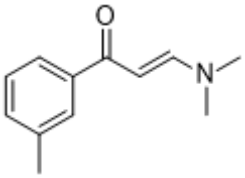
Following the general procedure, the title compound was prepared starting from acetophenone. Yield 87%. M.p. 89-90 °C. ^{1}H NMR (DMSO d_6): δ 2.91 (s, 3H, CH_3), 3.12 (s, 3H, CH_3), 5.71 (d, *J* = 12.5 Hz, 1H, CH), 7.49 (m, 3H, Ar), 7.79 (d, *J* = 12.5 Hz, CH), 7.93 (m, 2H, Ar). IR (Nujol) 3583, 1643 cm^{-1}. Elemental analysis: calculated for $C_{11}H_{13}NO$ (175.23) %C 75.40, %H 7.48, %N 7.99, found %C 75.34, %H 7.51, %N 7.95. M/z 176.

(*E*)-3-(Dimethylamino)-1-(4-methoxyphenyl)prop-2-en-1-one (20b)



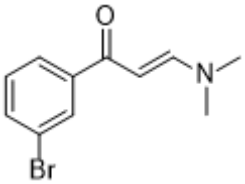
Following the general procedure, the title compound was prepared starting from 4-methoxyacetophenone. Yield 83%. M.p. 84-85 °C. ^{1}H NMR (DMSO d_6): δ 2.99 (m, 6H, CH_3), 3.82 (s, 3H, OCH_3), 5.69 (d, *J* = 12.0 Hz, 1H, CH), 6.89 (d, *J* = 8 Hz, 2H, Ar), 7.76 (d, *J* = 12.5 Hz, 1H, Ar), 7.89 (d, *J* = 7.5 Hz, 2H, Ar). IR (Nujol) 3567, 1638 cm^{-1}. Elemental analysis: calculated for $C_{12}H_{15}NO_2$ (205,25) %C 70.22, %H 7.37, %N 6.86, found %C 70.15, %H 7.40, %N 7.80. M/z 206.

(*E*)-3-(Dimethylamino)-1-(m-tolyl)prop-2-en-1-one (20c)



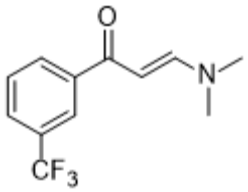
Following the general procedure, the title compound was prepared starting from m-tolylacetophenone. Yield 92%. Oil. ^{1}H NMR (DMSO d_6): δ 2.36 (s, 3H, CH_3), 2.90 (s, 3H, CH_3) 3.12 (s, 3H, CH_3), 5.79 (d, *J* = 12.5 Hz, 1H, CH), 7.29 (m, 3H, Ar), 7.79 (d, *J* = 12.5 Hz, 1H, Ar), 7.96 (m, 1H, Ar). IR (Nujol) 3583, 1638 cm^{-1}. Elemental analysis: calculated for $C_{12}H_{15}NO$ (189,25) %C 76.16, %H 7.99, N 7.40, found %C 76.09, %H 8.03, %N 7.44. M/z 190.

(*E*)-1-(3-Bromophenyl)-3-(dimethylamino)prop-2-en-1-one (20d)



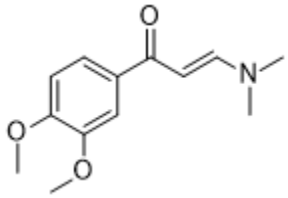
Following the general procedure, the title compound was prepared starting from 3-bromoacetophenone. Yield 90%. M.p. 53-54 °C. ^{1}H NMR (DMSO d_6): δ 2.94 (s, 3H, CH_3), 3.16 (s, 3H, CH_3), 5.82 (d, *J* = 12.0 Hz, 1H, CH), 7.40 (d, *J* = 7.5 Hz, 1H, Ar), 7.67 (d, *J* = 8 Hz, 1H, Ar), 7.72 (d, *J* = 12.5 Hz, 1H, Ar), 7.90 (d, *J* = 7.5 Hz, 1H, Ar), 8.02 (m, 1H, Ar). IR (Nujol) 3539, 1641 cm^{-1}. Elemental analysis: calculated for $C_{11}H_{12}BrNO$ (254,12) C, 51.99; H, 4.76; N, 5.51. Found C, 52.05; H, 4.77; N, 5.48. M/z 255.

(*E*)-3-(Dimethylamino)-1-(3-(trifluoromethyl)phenyl)prop-2-en-1-one (20e)



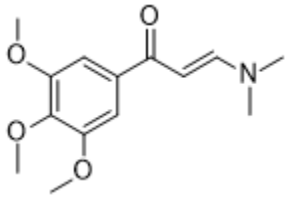
Following the general procedure, the title compound was prepared starting from 3-trifluoromethylacetophenone. Yield 80%. M.p. 53-54 °C. ^{1}H NMR (DMSO d_6): δ 2.84 (s, 3H, CH_3), 3.17 (s, 3H, CH_3), 5.64 (d, *J* = 12.5, 1H, CH), 7.42 (d, *J* = 8.5 Hz, 1H, Ar), 7.77 (d, *J* = 8.0 Hz, 2H, Ar), 7.84 (d, *J* = 12.5 Hz, 1H, Ar), 7.98 (s, 1H, Ar). IR (Nujol) 3541, 1642 cm^{-1}. Elemental analysis: calculated for $C_{12}H_{12}F_3NO$ (243,22) %C 59.26, %H 4.97, %N 5.76, found %C 59.32, %H 4.95, %N 5.74. M/z 244.

(*E*)-3-(Dimethylamino)-1-(3,4-dimethoxyphenyl)prop-2-en-1-one (20f)



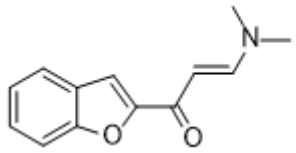
Following the general procedure, the title compound was prepared starting from 3,4-dimethoxyacetophenone. Yield 77%. M.p. 113-114 °C. ^{1}H NMR (DMSO-d_6): δ 2.91 (s, 3H, CH_3), 3.12 (s, 3H, CH_3), 3.81 (s, 6H, OCH_3), 5.81 (d, *J* = 12.0 Hz, 1H, CH), 6.97 (d, *J* = 8.0 Hz, 1H, Ar), 7.45 (s, 1H, Ar), 7.53 (d, *J* = 8.5 Hz, 1H, Ar), 7.65 (d, *J* = 12.0 Hz, 1H, CH). IR (Nujol) 3583, 1636 cm^{-1}. Elemental analysis: calculated for $C_{13}H_{17}NO_3$ (253.28) %C 66.36, %H 7.28, %N 5.95, found %C 66.27, %H 7.33, %N 5.91. M/z 254.

(*E*)-3-(Dimethylamino)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (20g)



Following the general procedure, the title compound was prepared starting from 3,4-dimethoxyacetophenone. Yield 75%. M.p. 114-115 °C. ^{1}H NMR (DMSO-d6): δ 2.94 (s, 3H, CH_3), 3.15 (s, 3H, CH_3), 3.71 (s, 3H, OCH_3), 3.85 (s, 6H, OCH_3), 5.82 (d, *J* = 12.0 Hz, 1H, CH), 7.17 (s, 2H, Ar), 7.68 (d, *J* = 12.0 Hz, 1H, CH). IR (Nujol) 3583, 1637 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{19}NO_4$ (265.28) %C 63.38, %H 7.22, %N 5.28, found %C 63.43, %H 7.20, %N 5.31. M/z 266.

(*E*)-1-(Benzofuran-2-yl)-3-(dimethylamino)prop-2-en-1-one (20h)

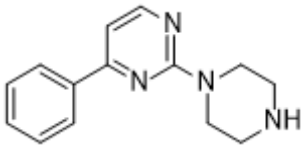


Following the general procedure, the title compound was prepared starting from 3,4-dimethoxyacetophenone. Yield 66%[64].

General procedure for the synthesis of phenyl-2-(piperazin-1-yl)pyrimidine

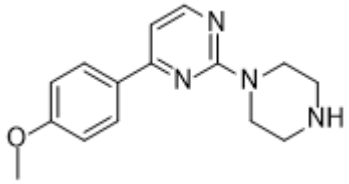
A solution of substituted phenylprop-2-en-1-ones (2 mmol), 4-(tert-butoxycarbonyl)piperazine-1-carboxamidine (1.02 g, 2.2 mmol) and sodium methylate 30% MeOH solution (0.8 ml, 4 mmol) in anhydrous EtOH (5 mL) was refluxed 8 h. After cooling to room temperature, the solvent was removed under reduced pressure. The residue was treated with AcOEt (20 mL) and washed with water (3 x 10 mL) and brine (10 mL). After drying over Na_2SO_4 the solvent was removed under reduced pressure. Then the residue was dissolved in anhydrous DCM (10 mL) and TFA (5 mL) was added. The mixture was stirred at room temperature overnight and after evaporation of the solvent, the residue was treated with a Et_2O (20 mL) to obtain a solid that was filtered off and dried. The formed solid was used in the next step without further purification.

4-Phenyl-2-(piperazin-1-yl)pyrimidine (21a)



Following the general procedure, the title compound was prepared starting from (*E*)-3-(dimethylamino)-1-phenylprop-2-en-1-one. Yield 42%. M.p. 119-120 °C. ^{1}H NMR (DMSO-d_6): δ 3.22 (s, 4H, CH_2), 4.04 (s, 4H, CH_2), 7.31 (d, *J* = 5.5 Hz, 2H, Ar), 7.53 (m, 2H, Ar), 8.15 (d, *J* = 5 Hz, 2H, Ar), 8.49 (m, 1H, Ar), 8.93 (s, 1H, NH). IR (Nujol) 3367, 1687 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{16}N_4$ (240.30) %C 69.97, %H 6.71 %N 23.32, found %C 70.01, %H 6.75, %N 23.36. M/z 241.

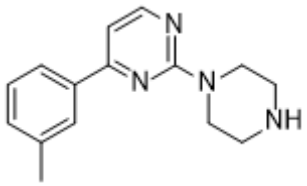
4-(4-Methoxyphenyl)-2-(piperazin-1-yl)pyrimidine (21b)



Following the general procedure, the title compound was prepared starting from (*E*)-3-(dimethylamino)-1-(4-methoxyphenyl)prop-2-en-1-one. Yield 52%. M.p. <240 °C. ^{1}H NMR (DMSO-d_6) δ 2.81 (s, 4H, CH_2), 3.21 (s, 4H, CH_2), 4.01 (s, 3H, OCH_3), 7.27 (d, *J* = 8 Hz, 1H, Ar),

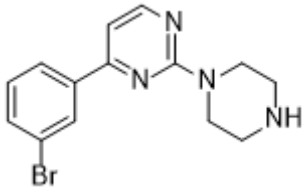
7.52 (d, *J* = 7.5 Hz, 2H, Ar), 7.74 (d, *J* = 7.5 Hz, 2H, Ar), 8.22 (d, *J* = 5.0 Hz, 1H, Ar), 8.89 (s, 1H, NH). IR (Nujol) 3583, 1665 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{18}N_4O$ (270.33) %C 66.64, %H 6.71, %N 20.73, found %C 66.69, %H 6.67, %N, 20.78. M/z 271.

2-(Piperazin-1-yl)-4-(m-tolyl)pyrimidine (21c)



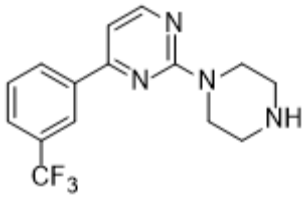
Following the general procedure, the title compound was prepared starting from (*E*)-3-(dimethylamino)-1-(m-tolyl)prop-2-en-1-one. Yield 58%. M.p. 113-114 °C. ^1H NMR (DMSO-d_6): δ 2.41 (s, 3H, CH_3), 3.23 (s, 4H, CH_2), 4.05 (s, 4H, CH_2), 7.31 (d, *J* = 5.0 Hz, 1H, Ar), 7.35 (d, *J* = 7 Hz, 1H, Ar), 7.40 (d, *J* = 7.5 Hz, 1H, Ar), 7.95 (d, *J* = 7.5 Hz, 2H, Ar), 8.49 (d, *J* = 5.0, 1H, Ar), 9.08 (s, 1H, NH). IR (Nujol) 3374, 1673 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{18}N_4$ (254.33) %C 70.84, %H 7.13, %N 22.03, found %C 70.89, %H 7.16, %N 22.07. M/z 275.

4-(3-Bromophenyl)-2-(piperazin-1-yl)pyrimidine (21d)



Following the general procedure, the title compound was prepared starting from (*E*)-1-(3-bromophenyl)-3-(dimethylamino)prop-2-en-1-one. Yield 40%. M.p. 108-109 °C. ^1H NMR (DMSO-d*6*): δ 3.26 (s, 4H, CH_2), 4.11 (s, 4H, CH_2), 7.38 (d, *J* = 5.0 Hz, 1H, Ar), 7.49 (d, *J* = 7.0 Hz, 1H, Ar), 7.74 (d, *J* = 7.5 Hz, 1H, Ar), 8.16 (d, *J* = 6.5 Hz, 1H, Ar), 8.33 (m, 1H, Ar), 8.54 (d, *J* = 5.0, 1H, Ar), 8.88 (s, 1H, NH). IR (Nujol) 3368, 1671 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{15}BrN_4$ (319.20) %C 70.84, %H 7.13, %N 22.03, found %C 70.89, %H 7.17, %N 22.08. M/z 320.

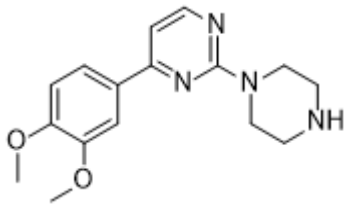
2-(Piperazin-1-yl)-4-(3-(trifluoromethyl)phenyl)pyrimidine (21e)



Following the general procedure, the title compound was prepared starting from (*E*)-1-(3-(trifluoromethyl)phenyl)-3-(dimethylamino)prop-2-en-1-one. Yield 60%. M.p. 105-106 °C. ^1H NMR (DMSO-d_6): δ 3.24 (s, 4H, CH_2), 4.06 (s, 4H, CH_2), 7.46 (d, *J* = 5.0 Hz, 1H, Ar), 7.77 (d, *J* =

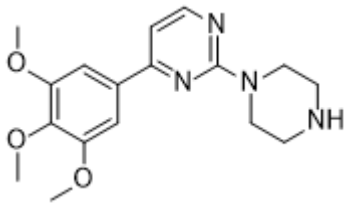
7.5 Hz, 1H, Ar), 7.91 (d, *J* = 7.5 Hz, 1H, Ar), 8.06 (d, *J* = 8.0 Hz, 2H, Ar), 8.57 (d, 1H, *J* = 5.0, 1H, Ar), 8.92 (s, 1H, NH). IR (Nujol) 3371, 1668 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{15}F_3N_4$ (308.30) %C 58.44, %H, 4.90, %N 18.17, found %C 58.48, %H 4.94, %N 18.13. M/z 309.

4-(3,4-Dimethoxyphenyl)-2-(piperazin-1-yl)pyrimidine (21f)



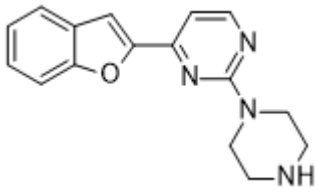
Following the general procedure, the title compound was prepared starting from (E)-3,4-(dimethoxylamino)-1-(3,4-dimethoxyphenyl)prop-2-en-1-one. Yield 48%. M.p. >240 °C. ^{1}H NMR (DMSO-d_6): δ 2.79 (s, 4H, CH_2), 3.33 (s, 4H, CH_2), 4.31 (s, 6H, OCH_3), 6.97 (d, *J* = 7.0 Hz, 1H, Ar), 7.25 (s, 1H, Ar), 7.54 (d, *J* = 7.5 Hz, 1H, Ar), 7.85 (m, 2H, Ar), 8.79 (s, 1H, NH). IR (Nujol) 3583, 1636 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{20}N_4O_2$ (300.36) %C 63.98, %H 6.71, %N 18.65, found %C 63.87, %H 6.74, %N 18.70. M/z 301.

2-(Piperazin-1-yl)-4-(3,4,5-trimethoxyphenyl)pyrimidine (21g)



Following the general procedure, the title compound was prepared starting from (*E*)-3-(dimethylamino)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one. Yield 36%. M.p. >240 °C. ^{1}H NMR (DMSO-d_6): δ 3.73 (s, 4H, CH_2), 3.79 (s, 4H, CH_2), 3.88 (s, 9H, OCH_3), 7.41 (d, *J* = 1H, Ar), 7.44 (s, 2H, Ar), 8.35 (d, *J* = 6.0 Hz, 1H, Ar), 9.02 (s, 1H, NH). IR (Nujol) 3583, 1637 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{22}N_4O_3$ (330.28) %C 61.80, %H 6.71, %N 16.96, found %C 61.87, %H 6.76, %N 17.02. M/z 331.

1-(4-(Benzofuran-2-yl)pyrimidin-2-yl)piperazine (21h)



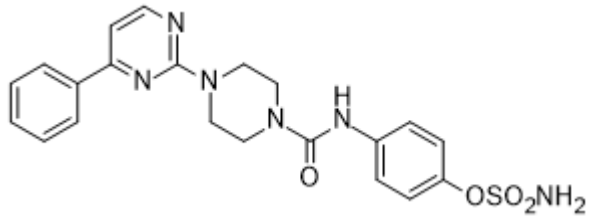
Following the general procedure, the title compound was prepared starting from (*E*)-benzofuran-2-yl-3-(dimethylamino)-prop-2-en-1-one. Yield 84%. M.p. 176-177 °C. ^{1}H NMR (DMSO-d_6): δ 3.21 (s, 4H, CH_2), 4.07 (s, 4H, CH_2), 7.24 (d, *J* = 4.9 Hz, 1H), 7.34 (m, 1H, Ar), 7.45 (m, 1H, Ar), 7.70 (m, 1H, Ar), 7.78 (m, 1H, Ar), 7.82 (s, 1H, Ar), 8.58 (d, *J* = 4.9 Hz, 1H, Ar), 9.33 (s, 1H, NH). IR

(Nujol) 2886, 1678, 1575 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{16}N_4O$ (280.32) %C 68.55, %H 5.75, %N 19.99, found %C 68.58, %H 5.77, %N 20.04. M/z 281.

General procedure for the synthesis of 4-(4-(4-aryl)pyrimidin-2-yl)piperazinocarbonyl)aminophenyl sulfamates (22a-h)

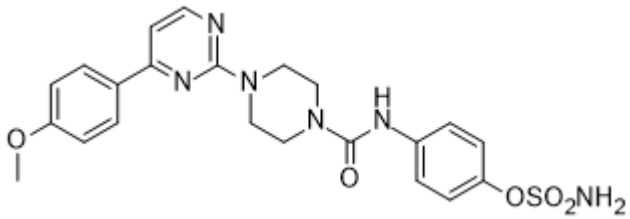
A mixture of 4-(phenoxycarbonyl)aminophenylsulfamate (0,31 g, 1 mmol) and the appropriate substituted pyrimidine (1 mmol), in anhydrous DMSO (3 mL) was stirred at room temperature for 24 h. Then, water (10 mL) was added and the mixture was stirred at room temperature until a solid precipitated. The formed solid was filtered off, washed with water, air dried and recrystallized from EtOH to give the title sulfamates.

4-(4-(4-Phenylpyrimidin-2-yl)piperazine-1-carboxamido)phenylsulfamate (22a)



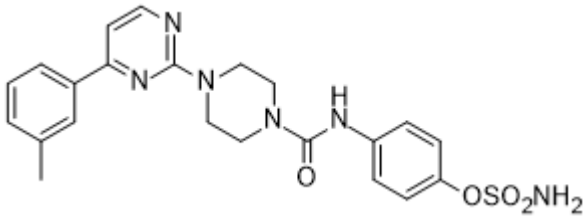
Following the general procedure, the title compound was prepared starting from 4-phenyl-2-(piperazin-1-yl)pyrimidine. Yield 25%. M.p. 134-135 °C. ^{1}H NMR (DMSO-d_6): δ 3.55 (m, 4H, CH_2), 3.87 (m, 4H, CH_2), 6.65 (s, 1H, CH), 7.22 (m, 4H, Ar), 7.25 (d, *J* = 5.0 Hz, 1H, Ar), 7.27 (d, *J* = 5.0 Hz, 1H, Ar), 7.51 (m, 4H, Ar), 8.15 (s, 2H, NH_2) 8.47 (s, 1H, NH). IR (Nujol) 3336, 1634, 1567 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{22}N_6O_4S$ (454.50) %C 55.49, %H 4.88, %N 18.49, found %C 55.55, %H 4.87, %N 18.52. M/z 455.

4-(4-(4-(4-Methoxyphenyl)pyrimidin-2-yl)piperazine-1-carboxamido)phenylsulfamate (22b)



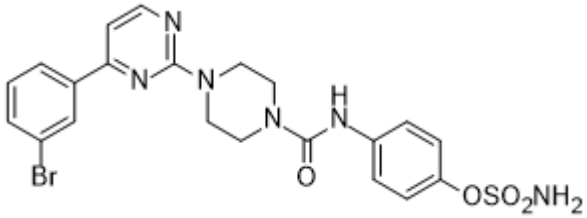
Following the general procedure, the title compound was prepared starting from 4-(4-methoxyphenyl)-2-(piperazin-1-yl)pyrimidine. Yield 45%. M.p. 134-135 °C. ^{1}H NMR (DMSO-d_6): δ 3.59 (s, 3H, OCH_3), 3.84 (m, 4H, CH_2), 3.88 (m, 4H, CH_2), 7.07 (d, *J* = 7.5, 2H, Ar), 7.16 (d, *J* = 9.0 Hz, 2H, Ar), 7.20 (m, 2H, Ar), 7.53 (d, *J* = 9.0 Hz, 2H, Ar), 7.88 (s, 2H, NH_2) 8.13 (d, *J* = 7.5 Hz, 2H, Ar), 8.73 (s, 1H, NH). IR (Nujol) 3316, 1650, 1568 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{24}N_6O_5S$ (484.53) %C 54.53, %H 4.99, %N 17.34, found %C 54.47, %H 5.01, %N 17.38. M/z 485.

4-(4-(4-(m-Tolyl)pyrimidin-2-yl)piperazine-1-carboxamido)phenylsulfamate (22c)



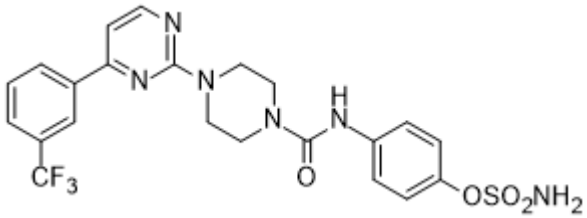
Following the general procedure, the title compound was prepared starting from 2-(piperazin-1-yl)-4-(m-tolyl)pyrimidine. Yield 31%. M.p. 164-165 °C. ^{1}H NMR (DMSO-d_6): δ 2.42 (s, 3H, CH_3), 3.60 (m, 4H, CH_2), 3.89 (m, 4H, CH_2), 7.16 (d, *J* = 6.0 Hz, 1H, Ar), 7.18 (d, *J* = 6.0 Hz, 1H, Ar), 7.37 (m, 4H, Ar), 7.51 (d, *J* = 8.0 Hz, 2H, Ar), 7.53 (d, *J* = 8.0 Hz, 2H, Ar), 7.88 (s, 2H, NH_2), 8.71 (s, 1H, NH). IR (Nujol) 3295, 1644, 1538 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{24}N_6O_4S$ (468.53) %C 56.40, %H 5.16, %N 17.94, found %C 56.34, %H 5.18, %N 17.97. M/z 469.

4-(4-(4-(3-Bromophenyl)pyrimidin-2-yl)piperazine-1-carboxamido)phenylsulfamate (22d)



Following the general procedure, the title compound was prepared starting from 4-(3-bromophenyl)-2-(piperazin-1-yl)pyrimidine. Yield 26%. M.p. 164-165 °C. ^{1}H NMR (DMSO-d_6): δ 3.59 (m, 4H, CH_2), 3.89 (m, 4H, CH_2), 7.17 (m, 2H, Ar), 7.31 (d, *J* = 5.0 Hz, 1H, Ar), 7.53 (m, 3H, Ar), 7.73 (d, *J* = 7.5 Hz, 1H, Ar), 7.87 (s, 2H, NH_2), 8.16 (d, *J* = 7.5, 1H, Ar), 8.31 (s, 1H, Ar), 8.52 (d, J = 5.0 Hz, 1H, Ar), 8.72 (s, 1H, NH). IR (Nujol) 3323, 1646, 1580 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{24}N_6O_4S$ (532.05) %C 47.29, %H 3.97, %N 15.76, found %C 47.22, %H 3.98, %N 15.80. M/z 534.

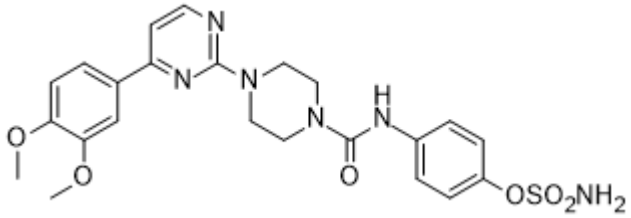
4-(4-(4-(3-(Trifluoromethyl)phenyl)pyrimidin-2-yl)piperazine-1-carboxamido)phenylsulfamate (22e)



Following the general procedure, the title compound was prepared starting from 2-(piperazin-1-yl)-4-(3-(trifluoromethyl)phenyl)pyrimidine. Yield 41%. M.p. 176-177 °C. ^{1}H NMR (DMSO-d_6): δ 3.60 (m, 4H, CH_2), 3.90 (m, 4H, CH_2), 7.16 (d, *J* = 7.0 Hz, 2H, Ar), 7.40 (d, *J* = 5.0 Hz, 1H, Ar), 7.53 (m, 3H, Ar), 7.78 (d, *J* = 7.0 Hz, 2H, Ar), 7.90 (s, 2H, NH_2), 8.44 (s, 1H, Ar), 8.55 (d, *J* = 5.0

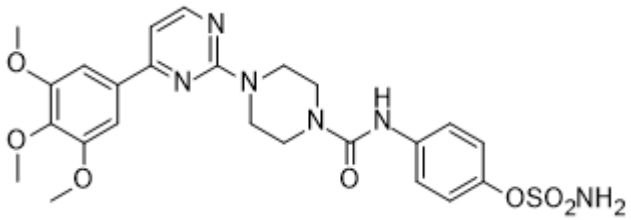
Hz, 1H, Ar), 8.72 (s, 1H, NH). IR (Nujol) 3307, 1642, 1571 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{24}N_6O_4S$ (522.50) %C 50.57, %H 4.05, %N 16.08, found %C 50.66, %H 4.02, %N 16.12. M/z 523.

4-(4-(4-(3,4-Dimethoxyphenyl)pyrimidin-2-yl)piperazine-1-carboxamido)phenylsulfamate (22f)



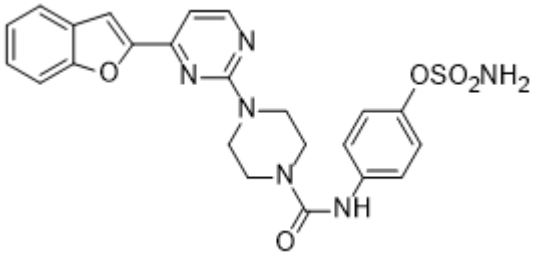
Following the general procedure, the title compound was prepared starting from 4-(3,4-dimethoxyphenyl)-2-(piperazin-1-yl)pyrimidine. Yield 27%. Oil. ^{1}H NMR (DMSO-d_6): δ 3.56 (s, 4H, CH_2), 3.83 (s, 4H, CH_2), 4.42 (s, 6H, OCH_3), 7.07 (m, 2H, Ar), 7.19 (s, 1H, Ar), 7.45 (d, *J* = 7.5 Hz, 1H, Ar), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.77 (d, *J* = 7.0 Hz, 1H, Ar), 7.84 (s, 1H, NH), 8.33 (s, 2H, NH_2), 8.61 (d, *J* = 8.5 Hz, 2H, Ar). IR (Nujol) 3328, 1634, 1516 cm^{-1}. Elemental analysis: calculated for $C_{23}H_{26}N_6O_6S$ (514.20) %C 53.69, %H 5.09, %N 16.33, found %C 53.62, %H 5.11, %N 16.37. M/z 515.

4-(4-(4-(3,4,5-Trimethoxyphenyl)pyrimidin-2-yl)piperazine-1-carboxamido)phenylsulfamate (22g)



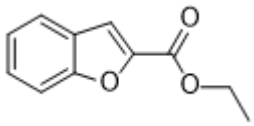
Following the general procedure, the title compound was prepared starting from 4-(3,4,5-trimethoxyphenyl)-2-(piperazin-1-yl)pyrimidine. Yield 31%. M.p 119-120 °C. ^{1}H NMR (DMSO-d_6): δ 3.59 (s, 4H, CH_2), 3.74 (s, 4H, CH_2), 3.90 (s, 9H, OCH_3), 7.16 (d, *J* = 7.5 Hz, 2H, Ar), 7.29 (m, 1H, Ar), 7.45 (m, 2H, Ar), 7.53 (d, *J* = 7.5 Hz, 2H, Ar), 7.88 (s, 2H, NH_2), 8.45 (s, 1H, NH), 8.72 (m, 1H, Ar). IR (Nujol) 3330, 1632, 1552 cm^{-1}. Elemental analysis: calculated for $C_{24}H_{28}N_6O_7S$ (544.58) %C 52.93, %H 5.18, %N 15.43, found %C 52.97, %H 5.20, %N 15.40. M/z 545.

4-(4-(4-(Benzofuran-2-yl)pyrimidin-2-yl)piperazine-1-carboxamido)phenyl sulfamate (22h)



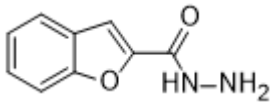
Following the general procedure, the title compound was prepared starting from 4-(benzofuran-2-yl)-2-(piperazin-1-yl)pyrimidine. Yield 73%. M.p 146-148 °C. ^{1}H NMR (DMSO-d_6) δ 3.59 (m, 4H, CH_2), 3.89 (m, 4H, CH_2), 7.17 (m, 3H, Ar), 7.33 (m, 1H, Ar), 7.44 (m, 1H, Ar), 7.52 (d, *J* = 8.8 Hz, 2H, Ar), 7.70 (m, 1H, Ar), 7.78 (m, 2H, Ar), 7.87 (s, 2H, NH_2), 8.54 (d, *J* = 4.9 Hz, 1H, Ar), 8.74 (s, 1H, NH). IR (Nujol) 3410, 3321, 1648 cm^{-1}. Elemental analysis: calculated for $C_{23}H_{22}N_6O_5S$ (494.52) %C 55.86, %H 4.48, %N 16.99, found %C 55.79, %H 4.45, %N 16.95. M/z 495.

Ethyl benzofuran-2-carboxylate (25)



To a solution of salicylaldehyde (23) (1.22 g, 0.01 mol) in MeCN (10 mL) ethyl bromoacetate (24) (1.1 mL, 0.01 mol) and K_2CO_3 (2.76 g, 0.02 mol) were added. The reaction mixture was refluxed for 1.5 h. After cooling to r.t. the reaction mixture was poured into crushed ice. Then the residue was extracted using AcOEt (50 mL), the combined organic layer was washed using brine (20 mL). The organic layer was dried using anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure to afford the title ester as oil. Yield 80%. ^{1}H NMR (DMSO-d_6) δ 1.33 (t, *J* = 7.5 Hz, 3H, CH_3); 4.37 (q, *J* = 7.5 Hz, 2H, CH_2); 7.35 (m, 1H, Ar), 7.50 (m, 1H, Ar), 7.71-7.80 (m, 3H, Ar). IR (Nujol) 2984, 1731, 1614, 1563 cm^{-1}. Elemental analysis: calculated for $C_{11}H_{11}O_3$ (190.20) %C 69.46, %H 5.30, found %C 69.39, %H 5.32. M/z 192.

Benzofuran-2-carbohydrazide (26)



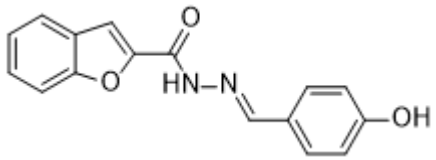
A mixture of ethyl benzofuran-2-carboxylate (25) (3.80 g,20 mmol) and hydrazine monohydrate (3 mL, 61.5 mmol) in EtOH (5 mL) was refluxed for 3 h. After cooling the formed precipitate was filtered off, washed with water (5x10 mL) dried and used in the next step without further purification. Yield 85%, Mp 190-191 °C. ^{1}H NMR (DMSO-d_6): δ 4.65 (s, 2H, NH_2), 7.31 (m, 1H, Ar), 7.42 (m, 1H, Ar), 7.50 (s, 1H, Ar), 7.63 (d, *J* = 8.5 Hz, 1H, Ar), 7.74 (d, *J* = 8.0 Hz, 1H, Ar),

10.00 (s, 1H, NH). IR (Nujol) 3322, 3184, 1661, 1601 cm^{-1}. Elemental analysis: calculated for $C_9H_8N_2O_2$ (176.17) %C 61.36, %H 4.58, %N 15.90 found %C 61.29, %H 4.59 %N 15.93. M/z 192.

General procedure for the synthesis of hydrazones (BF1-10)

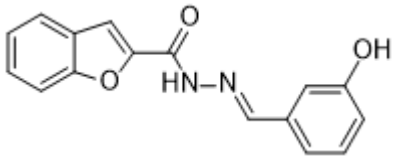
A mixture of benzofuran-2-carbohydrazide (26) (0.176 g, 1 mmol) and the appropriate aldehyde (1 mmol) in EtOH (10 mL) was refluxed for 18 h. After cooling the formed precipitate was filtered off and purified by crystallization from EtOH to give the hydrazone derivatives.

(*E*)-*N'*-(4-hydroxybenzylidene)benzofuran-2-carbohydrazide (BF1)



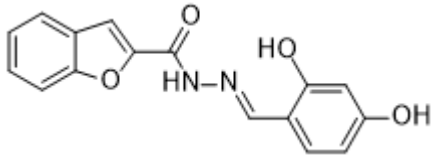
Following the general procedure, the title compound was prepared starting from 4-hydroxybenzaldehyde. Yield 79% M.p. >250°C. ^{1}H NMR (DMSO-d_6): δ 6.83 (d, *J* = 8.0 Hz, 1H, Ar), 6.85 (d, *J* = 8.0 Hz, 1H, Ar), 7.11-7.75 (m, 4H, Ar), 7.81 (d, *J* = 8.0 Hz, 1H, Ar), 7.68 (d, *J* = 8.0 Hz, 1H, Ar), 7.81 (s, 1H, Ar) 8.43 (s, 1H, CH), 9.62 (s, 1H, OH), 12.09 (s, 1H, NH). IR (Nujol) 3265, 1662, 1612, 1580 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}N_2O_3$ (280.28) %C 68.56, %H 4.32, %N 9.99, found %C 68.61, %H 4.33, %N 10.03. M/z 281.

(*E*)-*N'*-(3-hydroxybenzylidene)benzofuran-2-carbohydrazide (BF2)



Following the general procedure, the title compound was prepared starting from 3-hydroxybenzaldehyde. Yield 93% M.p. 221-222 °C. ^{1}H NMR (DMSO-d6): δ 6.83 (d, *J* = 8.0 Hz, 1H, Ar), 6.85 (d, *J* = 8.0 Hz, 1H, Ar), 7.11-7.75 (m, 4H, Ar), 7.81 (d, *J* = 8.0 Hz, 1H, Ar), 7.68 (d, J = 8.0 Hz, 1H, Ar), 7.81 (s, 1H, Ar) 8.43 (s, 1H, CH), 9.62 (s, 1H, OH), 12.09 (s, 1H, NH) IR (Nujol) 3265, 1662, 1612, 1580cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}N_2O_3$ (280.28) %C 68.56, %H 4.32, %N 9.99, found %C 68.61, %H 4.33, %N 9.96. M/z 281.

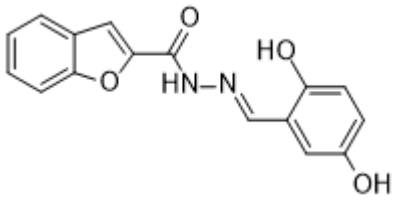
(*E*)-*N'*-(2,4-dihydroxybenzylidene)benzofuran-2-carbohydrazide (BF3)



Following the general procedure, the title compound was prepared starting from 2,4-dihydroxybenzaldehyde. Yield 74% M.p. 234-235 °C. ^{1}H NMR (DMSO-d_6): δ 6.32-6.38 (m, 2H, Ar), 7.32-7.38 (m, 2H, Ar), 7.51 (s, 1H, Ar), 7.70-7.81 (m, 3H, Ar), 8.59 (s, 1H, CH), 9.91 (s, 1H,

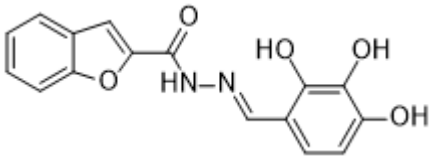
OH), 11.20 (s, 1H, OH), 12.20 (s, 1H, NH). IR (Nujol) 1627, 1610cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}N_2O_4$ (296.28) %C 64.86, %H 4.08, %N 9.46, found %C 64.91, %H 4.06, %N 9.50. M/z 297.

(*E*)-*N'*-(2,5-dihydroxybenzylidene)benzofuran-2-carbohydrazide (BF4)



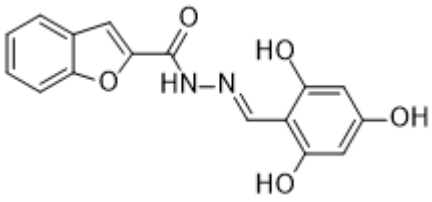
Following the general procedure, the title compound was prepared starting from 2,5-dihydroxybenzaldehyde. Yield 54% M.p. >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.72 (d, *J* = 7.0 Hz, 2H, Ar), 7.01 (s, 1H, Ar), 7.35-7.38 (m, 2H, Ar), 7.70-7.83 (m, 3H, Ar), 8.66 (s, 1H, CH), 9.00 (s, 1H, OH), 10.20 (s, 1H, OH), 12.29 (s, 1H, NH). IR (Nujol) 1661, 1597cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}N_2O_4$ (296.28) %C 64.86, %H 4.08, %N 9.46, found %C 64.91, %H 4.06, %N 9.50. M/z 297.

(*E*)-*N'*-(2,3,4-trihydroxybenzylidene)benzofuran-2-carbohydrazide (BF5)



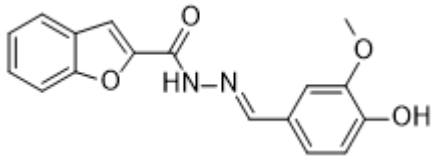
Following the general procedure, the title compound was prepared starting from 2,3,4-trihydroxybenzaldehyde. Yield 53% M.p. >250 °C. ^{1}H NMR (DMSO-d6): δ 6.40 (d, J = 8.5 Hz, 1H, Ar), 6.80 (d, *J* = 8.5 Hz, 1H, Ar), 7.35-7.81 (m, 5H, Ar), 8.55 (s, 1H, CH), 9.82 (s, 1H, OH), 11.20 (s, 1H, OH), 12.15 (s, 1H, NH), 13.10 (s, 1H, OH). IR (Nujol) 3341, 1655, 1595cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}N_2O_5$ (312.28) %C 61.54, %H 3.87, %N 8.97, found %C 61.59, %H 3.86, %N 9.01. M/z 313.

(*E*)-*N'*-(2,4,6-trihydroxybenzylidene)benzofuran-2-carbohydrazide (BF6)



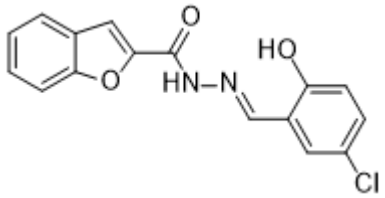
Following the general procedure, the title compound was prepared starting from 2,4,6-trihydroxybenzaldehyde. Yield 60% M.p. 248-249 °C. ^{1}H NMR (DMSO-d_6): δ 5.85 (s, 2H, Ar), 7.34-7.80 (m, 5H, Ar), 8.87 (s, 1H, CH), 9.83 (s, 1H, OH), 11.03 (s, 2H, OH), 12.24 (s, 1H, NH). IR (Nujol) 3341, 1655, 1596cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}N_2O_5$ (312.28) %C 61.54, %H 3.87, %N 8.97, found %C 61.49, %H 3.86, %N 8.94. M/z 313.

(*E*)-*N'*-(4-hydroxy-3-methoxybenzylidene)benzofuran-2-carbohydrazide (BF7)



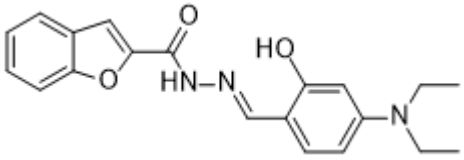
Following the general procedure, the title compound was prepared starting from 3-hydroxy-4-methoxybenzaldehyde. Yield 84% M.p. 192-193 °C. ^{1}H NMR (DMSO-d_6): δ 3.81 (s, 3H, OCH_3), 6.99 (d, *J* = 8.0 Hz, 1H, Ar), 7.07 (d, *J* = 8.0 Hz, 1H, Ar), 7.30-7.81 (m, 6H, Ar), 8.38 (s, 1H, CH), 9.30 (s, 1H, OH), 12.01 (s, 1H, NH). IR (Nujol) 3230, 1643, 1609 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{14}N_2O_4$ (310.30) %C 65.80, %H 4.55, %N 9.03, found %C 65.89, %H 4.54, %N 8.99. M/z 311.

(*E*)-*N'*-(5-chloro-2-hydroxybenzylidene)benzofuran-2-carbohydrazide (BF8)



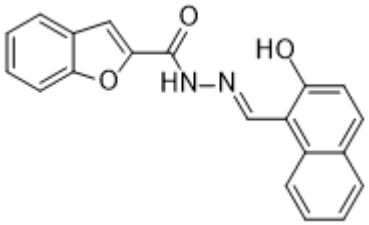
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-chlorobenzaldehyde. Yield 76% M.p. >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.95 (d, J = 9.0 Hz, 1H, Ar), 7.31 (m, 2H, Ar), 7.49 (m, 1H, Ar), 7.53-7.83 (m, 4H, Ar), 8.71 (s, 1H, CH), 11.11 (s, 1H, OH), 12.48 (s, 1H, NH). IR (Nujol) 3177, 1657, 1605 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{11}ClN_2O_3$ (314.72) %C 61.06, %H 3.52, %N 8.90, found %C 61.00, %H 3.53, %N 8.94. M/z 315.

(*E*)-*N'*-(4-(diethylamino)-2-hydroxybenzylidene)benzofuran-2-carbohydrazide (BF9)



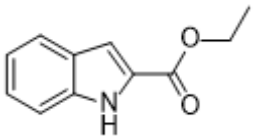
Following the general procedure, the title compound was prepared starting from 2-hydroxy-4-diethylaminobenzaldehyde. Yield 74% M.p. 105-106 °C. ^{1}H NMR (DMSO-d_6): δ 1.10 (t, *J* = 7.0 Hz, 6H, CH_3), 3.34 (d, *J* = 7.0 Hz, 4H, CH_2), 6.12 (s, 1H, Ar), 6.26 (d, *J* = 6.0 Hz, 1H, Ar), 7.19 (d, *J* = 6.0 Hz, 1H, Ar), 7.34 (m, 2H, Ar), 7.50-7.81 (m, 3H, Ar), 8.50 (s, 1H, CH), 11.31 (s, 1H, OH), 12.14 (s, 1H, NH). IR (Nujol) 1628, 1602 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{21}N_3O_3$ (351.40) %C 61.06, %H 3.52, %N 8.90, found %C 61.00, %H 3.53, %N 8.94. M/z 352.

(*E*)-*N'*-((2-hydroxynaphthalen-1-yl)methylene)benzofuran-2-carbohydrazide (BF10)



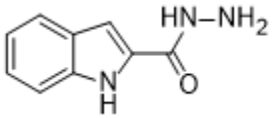
Following the general procedure, the title compound was prepared starting from 2-hydroxynaphtaldehyde. Yield 73% M.p. 238-239°C. ^{1}H NMR (DMSO-d_6): δ 7.24 (d, J = 9.0 Hz, 1H, Ar), 7.36 (d, *J* = 7.5 Hz, 1H, Ar), 7.38 (d, *J* = 7.5 Hz, 1H, Ar), 7.40-7.64 (m, 3H, Ar), 7.73-7.95 (m, 5H, Ar), 8.27 (s, 1H, CH), 9.59 (s, 1H, OH), 12.52 (s, 1H, NH). IR (Nujol) 3324, 1674, 1583 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{14}N_2O_3$ (330.34) %C 72.72, %H 4.27, %N 8.48, found %C 72.67, %H 4.28, %N 8.52. M/z 331.

Ethyl 1*H*-indole-2-carboxylate (28)



To a stirred solution of 1*H*-indole-2-carboxylic acid (27) (30 g, 186.1 mmol) in EtOH (100 mL), H_2SO_4 (10 mL) was added. The solution was heated at reflux temperature for 24 h, then water (10 mL) was added; the resulting mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic phase was washed with brine and water and dried over MgSO4. Then, the solvent was evaporated under vacuum to give to afford the title ester as a white powder[65].

1*H*-indole-2-carbohydrazide (29)

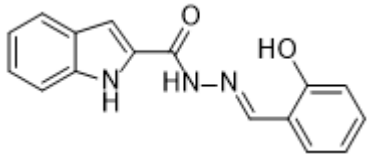


A mixture of ethyl-1*H*-indole-2-carboxylate (28) (3.80 g, 20 mmol) and hydrazine monohydrate (3 mL, 61.5 mmol) in EtOH (5 mL) was refluxed for 3 h. After cooling the formed precipitate was filtered off, washed with water (5x10 mL) dried and used in the next step without further purification[66].

General procedure for the synthesis of hydrazones (IND1-14)

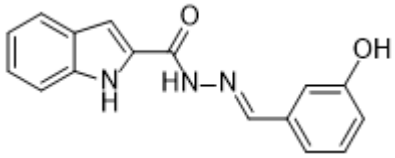
A mixture of 1*H*-indole-2-carbohydrazide (29) (0.175 g, 1mmol) and the appropriate aldehyde (1mmol) in EtOH (10mL) was refluxed for 18h. After cooling the formed precipitate was filtered off and purified by crystallization from EtOH to give the hydrazone derivatives.

(*E*)-*N'*-(2-hydroxybenzylidene)-1*H*-indole-2-carbohydrazide (IND1)



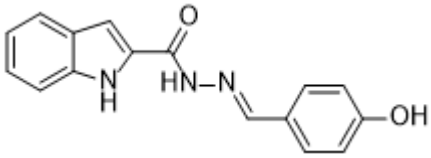
Following the general procedure, the title compound was prepared starting from 2-hydroxybenzaldehyde. Yield 64% M.p. >250 °C. ^{1}H NMR (DMSO-d_6) δ 6.92 (m, 2H, Ar), 7.05 (m, 1H, Ar), 7.21 (m, 1H, Ar), 7.28 (m, 2H, Ar), 7.45 (d, *J* = 8.0 Hz, 1H, Ar), 7.56 (m, 1H, Ar), 7.66 (d, *J* = 8.0 Hz, 1H, Ar), 8.63 (s, 1H, CH), 11.16 (s, 1H, OH), 11.79 (s, 1H, NH), 12.09 (s, 1H, NH). IR (Nujol) 3294, 1654, 1617 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{13}N_3O_2$ (279.29) %C 68.81, %H 4.69, %N 15.05, found %C 68.89, %H 4.70, %N 15.02. M/z 280.

(E)-*N'*-(3-hydroxybenzylidene)-1H-indole-2-carbohydrazide (IND2)



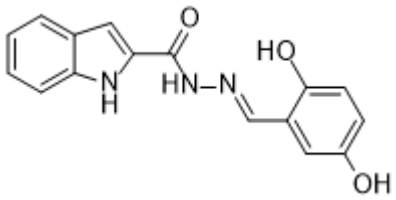
Following the general procedure, the title compound was prepared starting from 3-hydroxybenzaldehyde. Yield 84% M.p. >250 °C. ^{1}H NMR (DMSO-d_6) δ 6.81 (m, 1H, Ar), 7.04 (m, 1H, Ar), 7.10 (d, *J* = 7.5 Hz, 1H, Ar), 7.18–7.27 (m, 4H, Ar), 7.43 (d, *J* = 8.5 Hz, 1H, Ar), 7.65 (d, *J* = 8.0 Hz, 1H, Ar), 8.34 (s, 1H, CH), 9.58 (s, 1H, OH), 11.75 (s, 1H, NH), 11.79 (s, 1H, NH). IR (Nujol) 3255, 1638, 1580 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{13}N_3O_2$ (279.29) %C 68.81, %H 4.69, %N 15.05, found %C 68.73, %H 4.68, %N 15.08. M/z 280.

(*E*)-*N'*-(4-hydroxybenzylidene)-1*H*-indole-2-carbohydrazide (IND3)



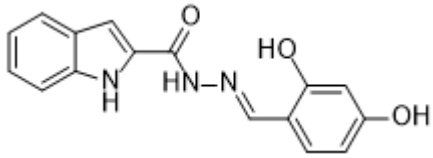
Following the general procedure, the title compound was prepared starting from 4-hydroxybenzaldehyde. Yield 80% M.p. >250°C. ^{1}H NMR (DMSO-d_6) δ 6.82 (d, *J* = 8.0 Hz, 2H, Ar), 7.03 (m, 1H, Ar), 7.19 (m, 1H, Ar), 7.43 (d, *J* = 8.0 Hz, 2H, Ar), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.65 (d, *J* = 7.5 Hz, 1H, Ar), 8.33 (s, 1H, CH), 9.88 (s, 1H, OH), 11.64 (s, 1H, NH), 11.71 (s, 1H, NH). IR (Nujol) 3223, 1608 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{13}N_3O_2$ (279.29) %C 68.81, %H 4.69, %N 15.05, found %C 68.74, %H 4.70, %N 15.07. M/z 280.

(*E*)-*N'*-(2,5-dihydroxybenzylidene)-1*H*-indole-2-carbohydrazide (IND4)



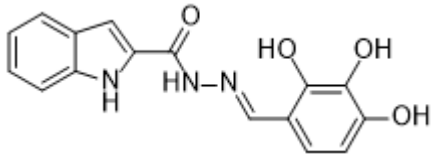
Following the general procedure, the title compound was prepared starting from 2,5-dihydroxybenzaldehyde. Yield 80% M.p. >250°C. ^{1}H NMR (DMSO-d6) δ 6.74 (m, 2H, Ar), 7.05 (m, 2H, Ar), 7.22 (m, 1H, Ar), 7.31 (s, 1H, Ar), 7.46 (d, *J* = 8.0 Hz, 1H, Ar), 7.67 (d, *J* = 8.0 Hz, 1H, Ar), 8.57 (s, 1H, CH), 8.95 (s, 1H, OH), 10.26 (s, 1H, OH), 11.78 (s, 1H, NH), 11.99 (s, 1H, NH). IR (Nujol) 3507, 3345, 3266, 1662 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{13}N_3O_3$ (295.29) %C 65.08, %H 4.44, %N 14.23, found %C 65.15, %H 4.43, %N 14.20. M/z 296.

(*E*)-*N'*-(2,4-dihydroxybenzylidene)-1*H*-indole-2-carbohydrazide (IND5)



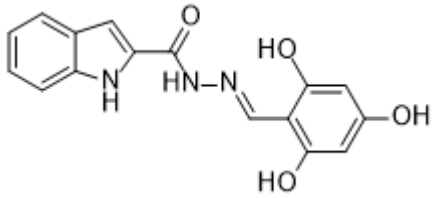
Following the general procedure, the title compound was prepared starting from 2,4-dihydroxybenzaldehyde. Yield 86% M.p. >250 °C. ^{1}H NMR (DMSO-d_6) δ 6.35 (m, 2H, Ar), 7.06 (m, 1H, Ar), 7.21 (m, 1H, Ar), 7.33 (m, 2H, Ar), 7.46 (d, *J* = 8.0 Hz, 1H, Ar), 7.66 (d, *J* = 8.0 Hz, 1H, Ar), 8.50 (s, 1H, CH), 9.92 (s, 1H, OH), 11.35 (s, 1H, OH), 11.76 (s, 1H, NH), 11.92 (s, 1H, NH). IR (Nujol) 3417, 1607, 1554 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{13}N_3O_3$ (295.29) %C 65.08, %H 4.44, %N 14.23, found %C 65.00, %H 4.43, %N 14.28. M/z 296.

(*E*)-*N'*-(2,3,4-trihydroxybenzylidene)-1*H*-indole-2-carbohydrazide (IND6)



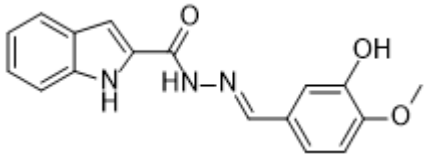
Following the general procedure, the title compound was prepared starting from 2,3,4-trihydroxybenzaldehyde. Yield 86% M.p. >250 °C. ^{1}H NMR (DMSO-d_6) δ 6.35 (m, 2H, Ar), 7.06 (m, 1H, Ar), 7.21 (m, 1H, Ar), 7.33 (m, 2H, Ar), 7.46 (d, *J* = 8.0 Hz, 1H, Ar), 7.66 (d, *J* = 8.0 Hz, 1H, Ar), 8.50 (s, 1H, CH), 9.92 (s, 1H, OH), 11.35 (s, 1H, OH), 11.76 (s, 1H, NH), 11.92 (s, 1H, NH). IR (Nujol) 3417, 1607, 1554 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{13}N_3O_4$ (311.29) %C 61.73, %H 4.21, %N 13.50, found %C 61.79, %H 4.23, %N 13.47. M/z 312.

(*E*)-*N'*-(2,4,6-trihydroxybenzylidene)-1*H*-indole-2-carbohydrazide (IND7)



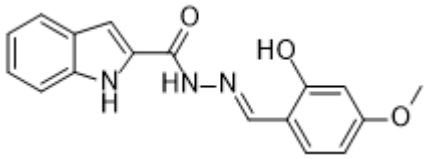
Following the general procedure, the title compound was prepared starting from 2,4,6-trihydroxybenzaldehyde. Yield 77% M.p. >250 °C. ^{1}H NMR (DMSO-d6): δ 5.85 (s, 2H, Ar), 7.05 (m, 1H, Ar), 7.22 (m, 2H, Ar), 7.45 (d, *J* = 8.5 Hz, 1H, Ar), 7.66 (d, *J* = 8.0 Hz, 1H, Ar), 8.79 (s, 1H, CH), 9.79 (s, 2H, OH), 11.06 (s, 1H, OH), 11.74 (s, 1H, NH), 11.92 (s, 1H, NH). IR (Nujol) 3347, 1642, 1611 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{13}N_3O_4$ (311.29) %C 61.73, %H 4.21, %N 13.50, found %C 61.68, %H 4.22, %N 13.54. M/z 312.

(*E*)-*N'*-(3-hydroxy-4-methoxybenzylidene)-1*H*-indole-2-carbohydrazide (IND8)



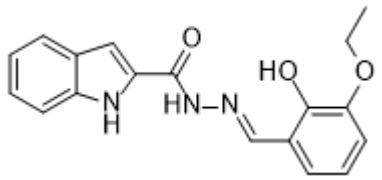
Following the general procedure, the title compound was prepared starting from 3-hydroxy-4-methoxybenzaldehyde. Yield 90% M.p. 158-159°C. ^{1}H NMR (DMSO-d_6): δ 3.81 (s, 3H, CH_3), 6.98 (d, *J* = 8.0 Hz, 1H, Ar), 7.10 (m, 2H, Ar), 7.24 (m, 3H, Ar), 7.45 (d, *J* = 8.0 Hz, 1H, Ar), 7.66 (d, *J* = 7.5 Hz, 1H, Ar), 8.30 (s, 1H, CH), 9.31 (s, 1H, OH), 11.71 (s, 1H, NH), 11.77 (s, 1H, NH). IR (Nujol) 3300, 1621, 1563 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}N_3O_3$ (309.32) %C 66.01, %H 4.89, %N 13.58, found %C 66.07, %H 4.90, %N 13.54. M/z 310.

(*E*)-*N'*-(2-hydroxy-4-methoxybenzylidene)-1*H*-indole-2-carbohydrazide (IND9)



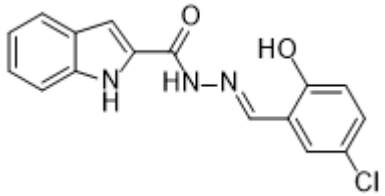
Following the general procedure, the title compound was prepared starting from 2-hydroxy-4-methoxybenzaldehyde. Yield 74% M.p. >250 °C. ^{1}H NMR (DMSO-d_6): δ 3.77 (s, 3H, OCH_3), 6.49–6.53 (m, 2H, Ar), 7.34–7.68 (m, 6H, Ar), 8.54 (s, 1H, CH), 11.49 (s, 1H, OH), 11.76 (s, 1H, NH), 12.0 (s, 1H, NH). IR (Nujol) 3315, 3241, 1651, 1630, 1606 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{15}N_3O_3$ (309.32) %C 66.01, %H 4.89, %N 13.58, found %C 65.95, %H 4.91, %N 13.62. M/z 310.

(*E*)-*N'*-(3-ethoxy-2-hydroxybenzylidene)-1*H*-indole-2-carbohydrazide IND10



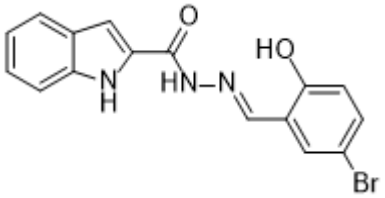
Following the general procedure, the title compound was prepared starting from 2-hydroxy-3-ethoxybenzaldehyde. Yield 87% M.p. 214-215 °C. ^{1}H NMR (DMSO-d_6): δ 1.35 (t, *J* = 7.0 Hz, 3H, CH_3), 4.06 (q, *J* = 7.0 Hz, 2H, CH_2), 6.85 (t, *J* = 8.0 Hz, 1H, Ar), 7.04 (d, *J* = 8.0 Hz, 1H, Ar), 7.16 (d, *J* = 8.0 Hz, 1H, Ar), 7.20–7.68 (m, 5H, Ar), 8.64 (s, 1H, CH), 10.85 (s, 1H, OH), 11.81 (s, 1H, NH), 12.1 (s, 1H, NH). IR (Nujol) 3320, 1655, 1621, 1605 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{17}N_3O_3$ (323.35) %C 66.86, %H 5.30, %N 13.00, found %C 66.80, %H 5.32, %N 13.03. M/z 324.

(*E*)-*N'*-(5-chloro-2-hydroxybenzylidene)-1*H*-indole-2-carbohydrazide (IND11)



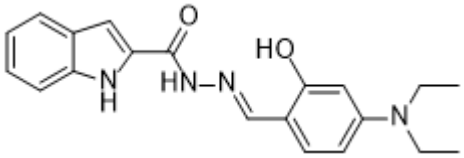
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-chlorobenzaldehyde. Yield 81% M.p. >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.95 (d, *J* = 8.0Hz, 1H, Ar), 7.07 (m, 1H, Ar), 7.23 (m, 1H, Ar), 7.33 (s, 1H, Ar), 7.44 (m, 2H, Ar), 7.46 (d, *J* = 8.5 Hz, 1H, Ar), 7.68 (d, *J* = 8.0 Hz, 1H, Ar), 8.62 (s, 1H, CH), 11.18 (s, 1H, OH), 11.80 (s, 1H, NH), 12.18 (s, 1H, NH). IR (Nujol) 3342, 3325, 1666 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}ClN_3O_3$ (313.74) %C 61.25, %H 3.86, %N 13.39, found %C 61.19, %H 3.87, %N 13.42. M/z 314.

(*E*)-*N'*-(5-bromo-2-hydroxybenzylidene)-1*H*-indole-2-carbohydrazide IND12



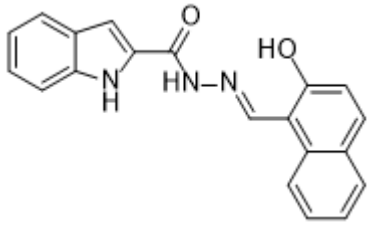
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-bromobenzaldehyde. Yield 81% M.p. >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.98 (d, *J* = 7.0 Hz, 1H, Ar), 7.07 (m, 1H, Ar), 7.23 (m, 1H, Ar), 7.33 (s, 1H, Ar), 7.44 (m, 2H, Ar), 7.68 (d, *J* = 8.5 Hz, 1H, Ar), 7.81 (m, 1H, Ar), 8.61 (s, 1H, CH), 11.19 (s, 1H, OH), 11.81 (s, 1H, NH), 12.18 (s, 1H, NH). IR (Nujol) 3312, 1668, 1605 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{12}BrN_3O_3$ (358.19) %C 53.65, %H 3.38, %N 11.73, found %C 53.70, %H 3.37, %N 11.70. M/z 358.

(*E*)-*N'*-(4-(diethylamino)-2-hydroxybenzylidene)-1*H*-indole-2-carbohydrazide (IND13)



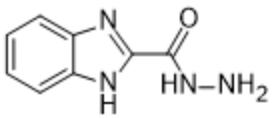
Following the general procedure, the title compound was prepared starting from 2-hydroxy-4-diethylaminobenzaldehyde. Yield 80% M.p. 200-201 °C. ^{1}H NMR (DMSO-d_6): δ 1.11 (t, *J* = 7.5 Hz, 6H, CH_3), 3.35 (q, *J* = 7.5 Hz, 4H, CH_2), 6.13 (s, 1H, Ar), 6.28 (d, *J* = 8.0 Hz, 1H, Ar), 7.06 (m, 1H, Ar), 7.21 (m, 3H, Ar), 7.45 (d, *J* = 8.0 Hz, 1H, Ar), 7.66 (s, 1H, Ar), 8.42 (s, 1H, CH), 11.35 (s, 1H, OH), 11.74 (s, 1H, NH), 11.83 (s, 1H, NH). IR (Nujol) 3295, 1635, 1592 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{22}N_4O_2$ (350.22) %C 68.55, %H 6.33, %N 15.99, found %C 68.49, %H 6.32, %N 16.03. m/z 351.

(*E*)-*N'*-((2-hydroxynaphthalen-1-yl)methylene)-1*H*-indole-2-carbohydrazide IND14



Following the general procedure, the title compound was prepared starting from 2-hydroxy-naphtaldehyde. Yield 85% M.p. >250 °C. ^{1}H NMR (DMSO-d_6) δ 7.08 (d, *J* = 7.5 Hz, 1H, Ar), 7.24 (d, *J* = 7.5 Hz, 1H, Ar), 7.42 (d, *J* = 7.5 Hz, 1H, Ar), 7.48–7.60 (m, 3H, Ar), 7.64 (d, *J* = 8.0 Hz, 1H, Ar), 7.71 (d, *J* = 8.0 Hz, 1H, Ar), 7.89–8.27 (m, 3H, Ar), 9.46 (s, 1H, CH), 11.86 (s, 1H, OH), 12.22 (s, 1H, NH), 12.69 (s, 1H, NH). IR (Nujol) 3322, 1673, 1620, 1571 cm^{-1}. Elemental analysis: calculated for $C_{20}H_{15}N_3O_2$ (329.35) %C 72.94, %H 4.59, %N 12.76, found %C 72.99, %H 4.61, %N 12.71. m/z 330.

1*H*-benzo[*d*]imidazole-2-carbohydrazide (31)

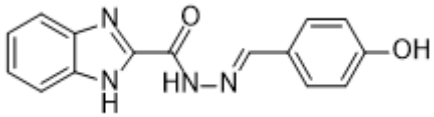


A mixture of ethyl 1*H*-benzo[d]imidazole-2-carboxylate (30) (**1**, 3.80 g, 20 mmol), and hydrazine monohydrate (3 mL, 61.5 mmol) in EtOH (5 mL) was refluxed for 3 h. After cooling the formed precipitate was filtered off, washed with water (5 x 10 mL) dried and used without further purification. Yield 80%. Mp 240–242 °C. ^{1}H NMR (DMSO-d_6): δ 4.62 (s, 2H, NH2), 7.28 (m, 2H, Ar), 7.53 (d, *J* = 8.0 Hz, 1H, Ar), 7.70 (d, *J* = 8.0 Hz, 1H, Ar), 10.14 (s, 1H, NH), 13.20 (s, 1H, NH). IR (Nujol) 3321, 3265, 3066, 1661, 1609 cm^{-1}. Elemental analysis: calculated for $C_8H_8N_4O$ (176.19) %C, 54.54; %H, 4.58; N, 31.80. Found: C, 54.57; H, 4.57; N, 31.76. M/z 177.

General Procedure for the Synthesis of Hydrazones (BEN1-13)

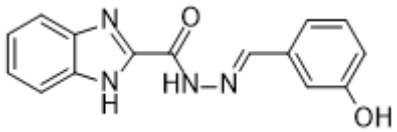
A mixture of hydrazide (32) (1 mmol) and the appropriate aldehyde (1 mmol) in EtOH (10 mL) was refluxed for 5 h. After cooling the formed precipitate was filtered off and purified by crystallization from the adequate solvent to give the hydrazone derivatives.

(*E*)-*N'*-(4-Hydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN1)



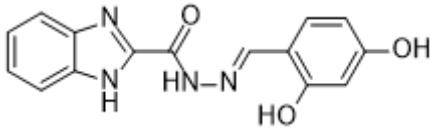
Following the general procedure, the title compound was prepared starting from 4-hydroxybenzaldeyde. Yield 80%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.86 (d, J = 8.5 Hz, 2H, Ar), 7.34 (d, J = 7.0 Hz, 2H, Ar), 7.57–7.59 (m, 3H, Ar), 7.77 (d, J = 7.0 Hz, 1H, Ar), 8.53 (s, 1H, CH), 10.03 (s, 1H, OH), 12.23 (s, 1H, NH), 13.43 (s, 1H, NH). IR (Nujol) 3289, 1668,1609, 1584 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{12}N_4O_2$ (280.28) %C, 64.28; %H, 4.32; %N, 19.99. Found: %C, 64.33; %H, 4.31; %N, 20.04. M/z 281.

(*E*)-*N'*-(3-hydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN2)



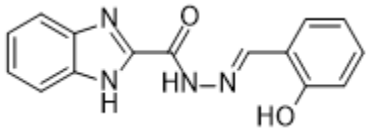
Following the general procedure, the title compound was prepared starting from 4-hydroxybenzaldeyde. Yield 78%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.85 (d, *J* = 8.0 Hz, 1H, Ar), 7.11 (d, *J* = 7.5 Hz, 1H, Ar), 7.21 (s, 1H, Ar), 7.26 (d, *J* = 7.5 Hz, 1H, Ar), 7.29–7.78 (m, 4H, Ar), 8.56 (s, 1H, CH), 9.69 (s, 1H, OH), 12.40 (s, 1H, NH), 13.47 (s, 1H, NH). IR (Nujol) 3221, 1677, 1610, 1576 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{12}N_4O_2$ (280.28) %C, 64.28; %H, 4.32; %N, 19.99. Found: %C, 64.34; %H, 4.31; %N, 20.03. M/z 281.

(*E*)-*N'*-(2,4-dihydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN3)



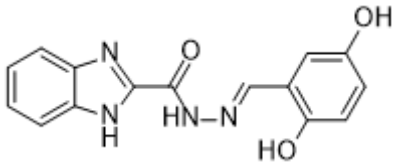
Following the general procedure, the title compound was prepared starting from 2,4-dihydroxybenzaldeyde. Yield 75%. Mp >250 °C. ^{1}H NMR (DMSO-d_6) δ 6.34 (m, 2H, Ar), 6.38 (d, *J* = 6.5 Hz, 1H, Ar), 7.57 (m, 4H, Ar), 8.68 (s, 1H, CH), 10.09 (s, 1H, OH), 11.43 (s, 1H, OH), 12.59 (s, 1H, NH), 13.45 (s, 1H, NH). IR (Nujol) 3227, 1673, 1638, 1587 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{12}N_4O_3$ (296.28) %C, 60.81; %H, 4.08; %N, 18.91. Found: %C, 60.76; %H, 4.10; %N, 18.94. M/z 297.

(*E*)-*N'*-(2-hydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN4)



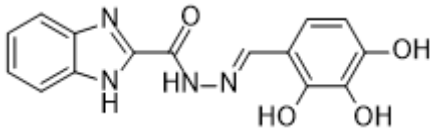
Following the general procedure, the title compound was prepared starting from 2-hydroxybenzhaldeyde. Yield 75%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.94 (m, 2H, Ar), 7.31 (m, 3H, Ar) 7.67 (m, 3H, Ar), 8.83 (s, 1H, CH), 11.23 (s, 1H, OH), 12.78 (s, 1H, NH), 13.51 (s, 1H, NH). IR (Nujol) 3191, 1664, 1614, 1556cm^{-1}. Elemental analysis: calculated for $C_{15}H_{12}N_4O_2$ (280.20) %C, 64.28; %H, 4.32; %N, 19.99. Found: %C, 64.22; %H, 4.33; %N, 20.05. m/z 281.

(*E*)-*N'*-(2,5-dihydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN5)



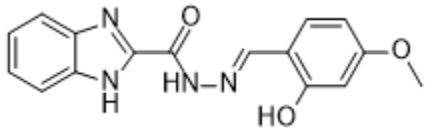
Following the general procedure, the title compound was prepared starting from 2,5-dihydroxybenzaldeyde. Yield 81%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.77 (d, *J* = 9.0 Hz, 1H, Ar), 6.79 (d, *J* = 9.0 Hz, 1H, Ar), 6.96 (s, 1H, Ar), 7.59 (m, 4H, Ar), 8.75 (s, 1H, CH) 9.03 (s, 1H, OH), 10.38 (s, 1H, OH), 12.67 (s, 1H, NH), 13.48 (s, 1H, NH). IR (Nujol) 3238, 1681, 1620, 1586 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{12}N_4O_3$ (296.28) %C 60.81; %H, 4.08; %N, 18.91. Found: %C, 60.86; %H, 4.09; %N, 18.87. M/z 297.

(*E*)-*N'*-(2,3,4-trihydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN6)



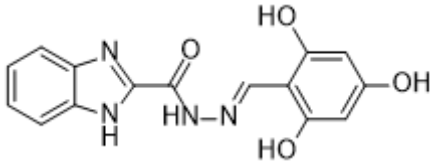
Following the general procedure, the title compound was prepared starting from 2,3,4-trihydroxybenzaldeyde. Yield 62%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.42 (d, *J* = 8.5 Hz, 1H, Ar), 6.76 (d, *J* = 8.5 Hz, 1H, Ar), 7.64 (m, 4H, Ar), 8.65 (s, 1H, CH), 9.57 (s, 2H, OH), 11.49 (s, 1H, OH), 12.65 (s, 1H, NH), 13.48 (s, 1H, NH). IR (Nujol) 3228, 3127, 3061, 1672, 1644 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{12}N_4O_4$ (312.09) %C 57.69; %H, 3.87; %N, 17.94. Found: %C, 57.64; %H, 3.88; %N, 17.91. M/z 313.

(*E*)-*N'*-(2-hydroxy-4-methoxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN7)



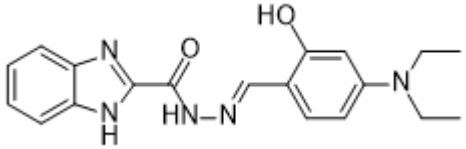
Following the general procedure, the title compound was prepared starting from 2-hydroxy-4-methoxybenzhaldeyde. Yield 79%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 3.79 (s, 3H, OCH_3), 6.54 (m, 2H, Ar), 7.48 (m, 5H, Ar), 8.74 (s, 1H, CH), 11.55 (s, 1H, NH), 12.69 (s, 1H, OH), 13.47 (s, 1H, NH). IR (Nujol) 3214, 1665, 1633, 1607, 1567 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{14}N_4O_3$ (310.31) % 61.93; %H, 4.55; %N, 18.06. Found: %C, 61.99; %H, 4.53; %N, 18.02. M/z 311.

(*E*)-*N'*-(2,4,6-trihydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN8)



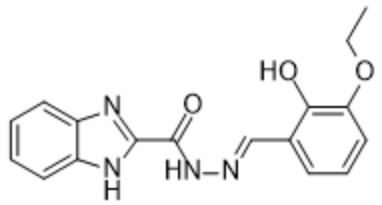
Following the general procedure, the title compound was prepared starting from 2,4,6-trihydroxybenzaldeyde. Yield 60%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 5.80 (s, 1H, Ar) 5.86 (s, 1H, Ar), 7.26 (d, *J* = 7.0 Hz, 1H, Ar), 7.30–7.78 (m, 3H, Ar), 8.98 (s, 1H, CH), 10.14 (s, 1H, OH), 11.14 (s, 2H, OH), 12.68 (s, 1H, NH), 13.43 (s, 1H, NH). IR (Nujol) 3225, 1672, 1592 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{12}N_4O_4$ (312.09) %C, 57.69; %H, 3.87; %N, 17.94. Found: %C, 57.73; %H, 3.86; %N, 17.92. M/z 313.

(E)-*N'*-(4-(diethylamino)-2-hydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN9)



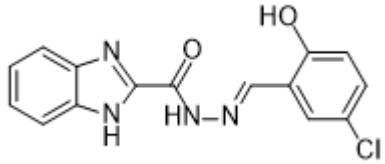
Following the general procedure, the title compound was prepared starting from 2-hydroxy-4-diethylaminobenzaldeyde. Yield 78%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 1.10 (t, *J* = 7.0 Hz, 6H, CH_3), 3.35 (q, *J* = 7.0 Hz, 4H, CH_2), 6.12 (s, 1H, Ar), 6.27 (d, *J* = 6.0 Hz, 1H, Ar), 7.14 (d, *J* = 6.0 Hz, 1H, Ar), 7.32 (m, 2H, Ar), 7.57 (m, 1H, Ar), 7.77 (m, 1H, Ar), 8.60 (s, 1H, CH), 11.42 (s, 1H, OH), 12.52 (s, 1H, NH), 13.42 (s, 1H, NH). IR (Nujol) 1668, 1631, 1586 cm^{-1}. Elemental analysis: calculated for $C_{19}H_{21}N_5O_2$ (351.40) %C 64.94; %H, 6.02; %N, 19.93. Found: %C, 65.01; %H, 5.99; %N, 19.97. M/z 352.

(*E*)-*N'*-(4-ethoxy-2-hydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN10)



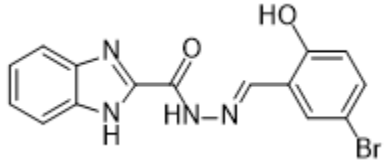
Following the general procedure, the title compound was prepared starting from 2-hydroxy-3-ethoxybenzaldeyde. Yield 71%. Mp >250 °C. ^{1}H NMR (DMSO-d$_6$): δ 1.36 (t, *J* = 7.0 Hz, 3H, CH_3), 4.07 (q, *J* = 7.0 Hz, 2H, CH_2), 6.86 (m, 1H, Ar), 7.03 (d, *J* = 8.0 Hz, 1H, Ar), (d, *J* = 8.0 Hz, 1H, Ar), 7.59 (m, 4H, Ar), 8.84 (s, 1H, CH), 10.99 (s, 1H, NH), 12.81 (s, 1H, OH), 13.53 (s, 1H, NH). IR (Nujol) 3322, 1694, 1610, 1583 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{13}N_4O_3$ (324.33) %C 62.95; %H, 4.97; %N, 17.27. Found: %C, 63.01; %H, 4.99; %N, 17.23. M/z 325.

(*E*)-*N'*-(5-chloro-2-hydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN11)



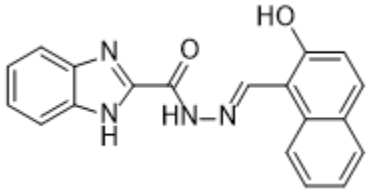
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-chlorobenzhaldeyde. Yield 85%. Mp >250 °C. ^{1}H NMR (DMSO-d$_6$): δ 6.96 (d, *J* = 8.0 Hz, 1H, Ar), 7.33–7.65 (m, 6H, Ar), 8.82 (s, 1H, CH), 11.15 (br s, 1H, OH), 12.90 (br s, 1H, NH), 13.30 (br s, 1H, NH). IR (Nujol) 3215, 1680, 1605, 1591 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{11}ClN_4O_2$ (314.73) %C, 57.24; %H, 3.52; %N, 17.80. Found: %C, 57.30; %H, 3.51; %N, 17.83. M/z 315.

(*E*)-*N'*-(5-bromo-2-hydroxybenzylidene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN12)



Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-bromobenzaldeyde. Yield 82%. Mp >250 °C. ^{1}H NMR (DMSO-d$_6$): δ 6.91 (d, *J* = 9.0 Hz, 1H, Ar), 7.33 (d, *J* = 6.0 Hz, 1H, Ar), 7.35 (d, *J* = 6.0 Hz, 1H, Ar), 7.60 (m, 4H, Ar), 8.81 (s, 1H, CH), 11.15 (s, 1H, OH), 12.85 (s,1H, NH), 13.50 (s, 1H, NH). IR (Nujol) 3200, 1680, 1602, 1588 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{11}BrN_4O_2$ (359.18) %C, 50.16; %H, 3.09; %N, 15.60. Found: %C, 50.22; %H, 3.11; %N, 15.57. M/z 360.

(*E*)-*N'*-((2-hydroxynaphthalen-1-yl)methylene)-1*H*-benzo[*d*]imidazole-2-carbohydrazide (BEN13)

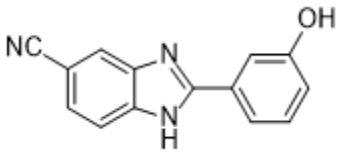


Following the general procedure, the title compound was prepared starting from 2-hydroxy-naphtaldeyde. Yield 82%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 7.26 (d, *J* = 9.0 Hz, 1H, Ar), 7.35 (d, *J* = 6.5 Hz, 1H, Ar), 7.38 (d, *J* = 6.5 Hz, 1H, Ar), 7.54 (m, 3H, Ar), 7.82 (d, *J* = 7.0 Hz, 1H, Ar), 8.11 (m, 3H, Ar), 9.79 (s, 1H, CH), 12.77 (s, 1H, NH), 12.86 (s, 1H, OH), 13.58 (s, 1H, NH). IR (Nujol) 3254, 1679, 1625, 1576 cm^{-1}. Elemental analysis: calculated for $C_{19}H_{14}N_4O_2$ (330.34) % C, 69.08; %H, 4.27; %N, %16.96. Found: %C, 69.14; %H, 4.29; %N, 17.01. M/z 360.

General procedure for the synthesis of benzimidazoles (34-71)

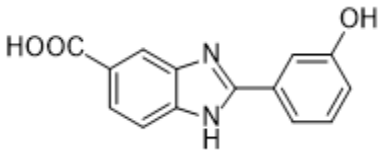
To a solution of the appropriate 3,4-diaminobenzene derivative (32a-d) (2 mmol) in EtOH (15 mL) 2.85 N aqueous solution of sodium metabisulphite (1.6 mL) and the appropriate substituted arylaldehyde (2 mmol) were added. The reaction mixture was heated at reflux for 24 h. The solvent was then evaporated under reduced pressure. The residue was added with aqueous HCl 1N (10 mL), the formed precipitate was filtered off, washed with water (3 x 10 mL) and purified by crystallization from EtOH to give the title compounds. Following the general procedure benzimidazoles **34**[62], **35**[67], **36**[68], **37, 38, 39 63** and **64**[69] were prepared and their analytical and spectral data are in agreement with those reported in literature.

2-(3-Hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carbonitrile (40)



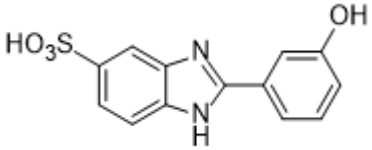
Following the general procedure, the title compound was prepared starting from 3-hydroxybenzaldehyde. Yield 80%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 7.08 (d, *J* = 8.0 Hz, 1H, Ar), 7.42 (t, *J* = 8.0 Hz, 1H, Ar), 7.64-7.70 (m, 3H, Ar), 7.81 (d, *J* = 8.5 Hz, 1H, Ar), 8.19 (s, 1H, Ar). IR (Nujol) 2227, 1589 cm^{-1}. Elemental analysis: calculated for $C_{14}H_9N_3O$ (235.15) %C 71.48, %H 3.86, %N 17.86, found %C 71.41, %H 3.87, %N 17.90. m/z 236.

2-(3-Hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (41)



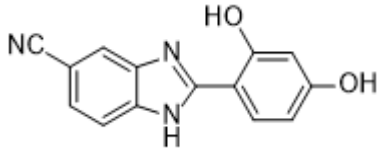
Following the general procedure, the title compound was prepared starting from 3-hydroxybenzaldehyde. Yield 62%. Mp 208-210 °C. ^{1}H NMR (DMSO-d_6): δ 7.03 (d, *J* = 7.5 Hz, 1H, Ar), 7.44 (t, *J* = 8.0 Hz, 1H, Ar), 7.64 (m, 2H, Ar), 7.74 (d, *J* = 8.5 Hz, 1H, Ar), 7.94 (d, *J* = 8.0 Hz, 1H, Ar), 8.23 (s, 1H, Ar), 9.96 (s, 1H, OH). IR (Nujol) 3360, 2726, 1693, 1569 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{10}N_2O_3$ (254.53) %C 66.14, %H 3.96, %N 11.02, found %C 66.09, %H 3.98, %N 11.05. m/z 255.

2-(3-Hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (42)



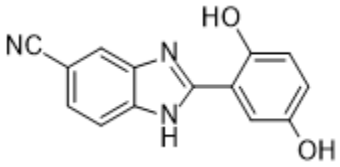
Following the general procedure, the title compound was prepared starting from 3-hydroxybenzaldehyde. Yield 69%. Mp 219-220 °C. ^{1}H NMR (DMSO-d_6): δ 7.16 (d, *J* = 8.0 Hz, 1H, Ar), 7.53 (d, *J* = 8.0 Hz, 1H, Ar), 7.57 (s, 1H, Ar), 7.62 (d, *J* = 8.0 Hz, 1H, Ar), 7.77 (d, *J* = 8.0 Hz, 1H, Ar), 7.82 (d, *J* = 8.5 Hz, 1H, Ar), 9.96 (s, 1H, Ar), 10.23 (s, 1H, OH). IR (Nujol) 3397, 3274, 1631, 1588 cm^{-1}. Elemental analysis: calculated for $C_{13}H_{10}N_2O_4S$ (290.06) %C 53.79, %H 3.47, %N 9.65, found %C 53.84, %H 3.46, %N 9.68. m/z 291.

2-(2,4-Dihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carbonitrile (43)



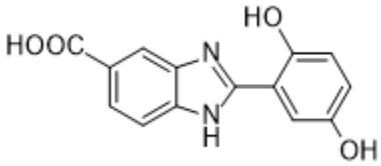
Following the general procedure, the title compound was prepared starting from 2,4-dihydroxybenzaldehyde. Yield 63%. Mp 233-234 °C. ^{1}H NMR (DMSO-d_6): δ 6.56 (d, *J* = 8.5 Hz, 1H, Ar), 6.68 (s, 1H, Ar), 7.81 (d, *J* = 8.0 Hz, 1H, Ar), 7.89 (d, *J* = 8.0 Hz, 1H, Ar), 8.05 (d, *J* = 8.5 Hz, 1H, Ar), 8.20 (s, 1H, Ar). IR (Nujol) 3348, 3211, 3086, 2242, 1611 cm^{-1}. Elemental analysis: calculated for $C_{14}H_9N_3O_2$ (251.28) %C 66.93, %H 3.61, %N 16.73, found %C 66.99, %H 3.60, %N 16.70. m/z 252.

2-(2,5-Dihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carbonitrile (44)



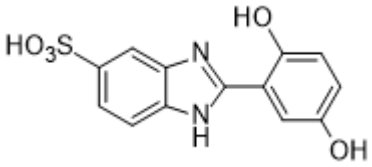
Following the general procedure, the title compound was prepared starting from 2,5-dihydroxybenzaldehyde. Yield 78%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.98 (s, 2H, Ar), 7.50 (s, 1H, Ar), 7.73 (d, *J* = 8.0 Hz, 1H, Ar), 7.86 (d, *J* = 8.5 Hz, 1H, Ar), 8.21 (s, 1H, Ar). IR (Nujol) 2230, 1617, 1564 cm^{-1}. Elemental analysis: calculated for $C_{14}H_9N_3O_2$ (251.28) %C 66.93, %H 3.61, %N 16.73, found %C 66.87, %H 3.63 %N 16.77. m/z 252.

2-(2,5-Dihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (45)



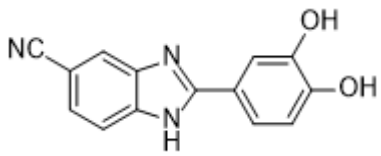
Following the general procedure, the title compound was prepared starting from 2,5-dihydroxybenzaldehyde. Yield 65%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 6.99 (s, 2H, Ar), 7.47 (s, 1H, Ar), 7.81 (d, *J* = 8.0 Hz, 1H, Ar), 7.99 (d, *J* = 8.5 Hz, 1H, Ar), 8.31 (s, 1H, Ar), 9.40 (1H, OH). IR (Nujol) 3184, 2720, 1718, 1620 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{10}N_2O_4$ (270.33) %C 62.22, %H 3.73, %N 10.37, found %C 62.28, %H 3.73 %N 10.34. m/z 271.

2-(2,5-Dihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (46)



Following the general procedure, the title compound was prepared starting from 2,5-dihydroxybenzaldehyde. Yield 71%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 7.04 (s, 2H, Ar), 7.43 (s, 1H, Ar), 7.75 (s, 2H, Ar), 8.02 (s, 1H, Ar), 9.41 (s, 1H, NH). IR (Nujol) 3392, 3225, 3097, 1633, 1569 cm^{-1}. Elemental analysis: calculated for $C_{13}H_{10}N_2O_5S$ (306.14) %C 50.98, %H 3.29, %N 9.15, found %C 50.94, %H 3.31 %N 9.18. m/z 307.

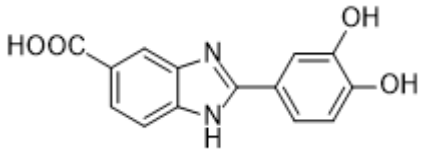
2-(3,4-Dihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carbonitrile (47)



Following the general procedure, the title compound was prepared starting from 3,4-dihydroxybenzaldehyde. Yield 75%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 7.04 (d, *J* = 8.5 Hz, 1H,

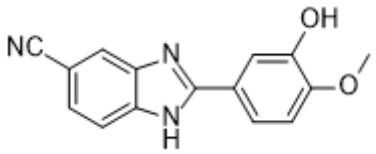
Ar), 7.65 (s, 1H, Ar), 7.67 (d, *J* = 8.5 Hz, 1H, Ar), 7.79 (d, *J* = 8.5 Hz, 1H, Ar), 7.85 (d, *J* = 8.0 Hz, 1H, Ar), 8.21 (s, 1H, NH). IR (Nujol) 3428, 3331, 3105, 2242, 1611 cm^{-1}. Elemental analysis: calculated for $C_{13}H_{10}N_2O_5S$ (251.38) %C 66.93, %H 3.61, %N 16.73, found %C 66.85, %H 3.59 %N 16.70. m/z 252.

2-(3,4-Dihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (48)



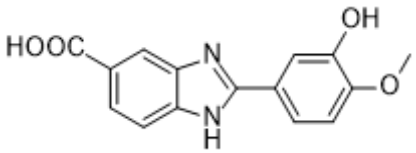
Following the general procedure, the title compound was prepared starting from 3,4-dihydroxybenzaldehyde. Yield 81%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 7.04 (d, *J* = 8.5 Hz, 1H, Ar), 7.67 (d, *J* = 9.0 Hz, 1H, Ar), 7.69 (s, 1H, Ar), 7.78 (d, *J* = 8.5 Hz, 1H, Ar), 8.01 (d, *J* = 8.0 Hz, 1H, Ar), 8.23 (s, 1H, NH), 9.50 (1H, OH). IR (Nujol) 3317, 2760, 1694, 1603, 1524 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{10}N_2O_4$ (270.44) %C 62.22, %H 3.73, %N 10.37, found %C 62.16, %H 3.74 %N 10.40. m/z 271.

2-(3-Hydroxy-4-methoxyphenyl)-1*H*-benzo[*d*]imidazole-5-carbonitrile (49)



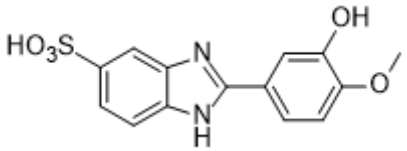
Following the general procedure, the title compound was prepared starting from 3,4-dihydroxybenzaldehyde. Yield 58%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 3.81 (s, 3H, OCH_3), 6.66-6.68 (m, 2H, Ar), 7.73 (d, *J* = 8.0 Hz, 1H, Ar), 7.83 (d, *J* = 8.5 Hz, 1H, Ar), 8.06 (d, *J* = 8.5 Hz, 1H, Ar), 8.15 (s, 1H, NH). IR (Nujol) 2228, 1618 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{10}N_2O_4$ (265.29) %C 67.92, %H 4.18, %N 15.84, found %C 67.98, %H 4.20 %N 15.80. m/z 266.

2-(3-Hydroxy-4-methoxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (50)



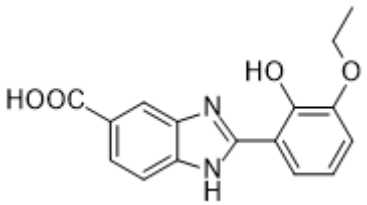
Following the general procedure, the title compound was prepared starting from 3-hydroxy-4-methoxybenzaldehyde. Yield 60%. Mp 223-224 °C. ^{1}H NMR (DMSO-d_6): δ 3.83 (s, 3H, OCH_3), 6.72 (s, 2H, Ar), 7.81 (d, *J* = 8.0 Hz, 1H, Ar), 8.00 (d, *J* = 8.5 Hz, 1H, Ar), 8.08 (d, *J* = 9.5 Hz, 1H, Ar), 8.30 (s, 1H, NH), 9.70 (1H, OH). IR (Nujol) 3293, 2725, 1697, 1626 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{10}N_2O_4$ (284.10) %C 63.38, %H 4.25, %N 9.85, found %C 63.33, %H 4.27 %N 9.88. m/z 285.

2-(3-Hydroxy-4-methoxyphenyl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (51)



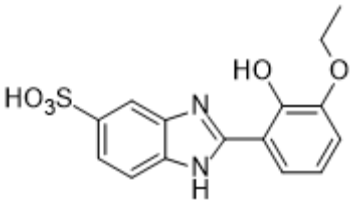
Following the general procedure, the title compound was prepared starting from 3-hydroxy-4-methoxybenzaldehyde. Yield 69%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 3.93 (s, 3H, OCH_3), 7.30 (d, *J* = 8.5 Hz, 1H, Ar), 7.60 (s, 1H, Ar), 7.68-7.68 (m, 2H, Ar), 7.79 (d, *J* = 9.0 Hz, 1H, Ar), 7.93 (s, 1H, NH), 9.78 (s, 1H, OH). IR (Nujol) 3367, 3149, 3089, 2781, 1634, 1610, 1583 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{12}N_2O_5S$ (320.33) %C 52.49, %H 3.78, %N 8.75, found %C 52.54, %H 3.77 %N 8.72. m/z 321.

2-(3-Ethoxy-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (52)



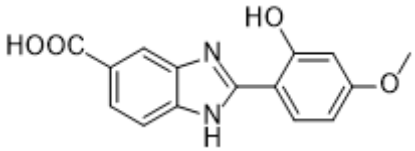
Following the general procedure, the title compound was prepared starting from 3-ethoxy-2-hydroxyphenylbenzaldehyde. Yield 52%. Mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 1.37 (t, *J* = 7.0 Hz, 3H, CH_3), 4.11 (q, *J* = 7.0 Hz, 2H, CH_2), 6.98 (t, *J* = 8.0 Hz, 1H, Ar), 7.15 (d, *J* = 8.0 Hz, 1H, Ar), 7.64 (d, *J* = 7.5 Hz, 1H, Ar), 7.77 (d, *J* = 8.0 Hz, 1H, Ar), 7.95 (d, *J* = 9.0 Hz, 1H, Ar), 8.27 (s, 1H, NH). IR (Nujol) 3463, 3030, 2680, 1682, 1624, 1594 cm^{-1}. Elemental analysis: calculated for $C_{16}H_{14}N_2O_4$ (298.20) %C 64.42, %H 4.73, %N 9.39, found %C 64.37, %H 4.74 %N 9.43. m/z 299.

2-(3-Ethoxy-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (53)



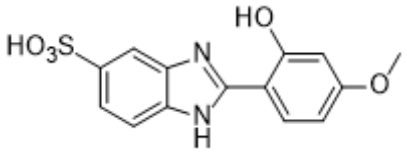
Following the general procedure, the title compound was prepared starting from 3-ethoxy-2-hydroxyphenylbenzaldehyde. Yield 56%. Mp 236-237 °C. ^{1}H NMR (DMSO-d_6): δ 1.40 (t, *J* = 6.5 Hz, 3H, CH_3), 4.21 (q, *J* = 6.5 Hz, 2H, CH_2), 7.08 (t, *J* = 8.0 Hz, 1H, Ar), 7.31 (d, *J* = 8.0 Hz, 1H, Ar), 7.59 (d, *J* = 8.0 Hz, 1H, Ar), 7.78 (d, *J* = 9.0 Hz, 1H, Ar), 7.80 (d, *J* = 8.5 Hz, 1H, Ar), 8.06 (s, 1H, NH). IR (Nujol) 3448, 3227, 1625, 1561 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{14}N_2O_5S$ (334.32) %C 53.88, %H 4.22, %N 8.38, found %C 53.94, %H 4.24 %N 8.34. m/z 335.

2-(2-Hydroxy-4-methoxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (54)



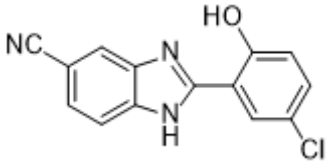
Following the general procedure, the title compound was prepared starting from 2-hydroxy-4-methoxyphenylbenzaldehyde. Yield 58%. Mp 219-220 °C. ^{1}H NMR (DMSO-d_6): δ 3.80 (s, 3H, OCH_3), 7.22 (d, *J* = 8.5 Hz, 1H, Ar), 7.65 (s, 1H, Ar), 7.70 (d, *J* = 8.5 Hz, 1H, Ar), 7.76 (d, *J* = 8.0 Hz, 1H, Ar), 7.98 (d, *J* = 8.5 Hz, 1H, Ar), 8.22 (s, 1H, NH). IR (Nujol) 3447, 3130, 2766, 1713, 1615 cm^{-1}. Elemental analysis: calculated for $C_{15}H_{12}N_2O_4$ (284.17) %C 63.38, %H 4.25, %N 9.85, found %C 63.45, %H 4.23 %N 9.89. m/z 285.

2-(2-Hydroxy-4-methoxyphenyl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (55)



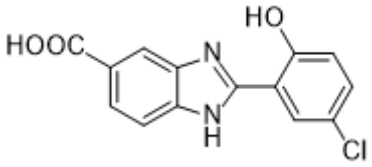
Following the general procedure, the title compound was prepared starting from 2-hydroxy-4-methoxyphenylbenzaldehyde. Yield 59%. Mp 219-220 °C. ^{1}H NMR (DMSO-d_6): δ 3.87 (s, 3H, OCH_3), 6.69 (s, 1H, Ar), 6.78 (m, 1H, Ar), 7.71 (m, 2H, Ar), 7.98 (m, 1H, Ar), 8.02 (s, 1H, Ar). IR (Nujol) 3163, 1621, 1572 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{12}N_2O_5S$ (320.05) %C 52.49, %H 3.78, %N 8.75, found %C 52.44, %H 3.80 %N 8.78. m/z 321.

2-(5-Chloro-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carbonitrile (56)



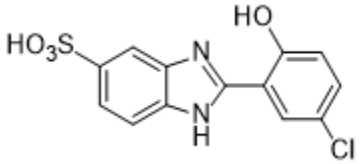
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-chlorophenylbenzaldehyde. Yield 83%. Mp 200-202 °C. ^{1}H NMR (DMSO-d_6): δ 7.16 (d, *J* = 9.0 Hz, 1H, Ar), 7.47 (d, *J* = 9.0 Hz, 1H, Ar), 7.71 (d, *J* = 8.5 Hz, 1H, Ar), 7.86 (d, *J* = 8.5 Hz, 1H, Ar), 8.19 (s, 1H, Ar), 8.20 (s, 1H, NH). IR (Nujol) 3340, 3059, 2727, 2232, 1615 cm^{-1}. Elemental analysis: calculated for $C_{14}H_8ClN_3O$ (269.29) %C 62.35, %H 2.99, %N 15.58, found %C 62.42, %H 2.98 %N 15.55. m/z 270.

2-(5-Chloro-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (57)



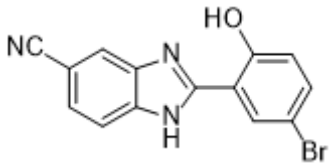
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-chlorophenylbenzaldehyde. Yield 82%. Mp 203-204 °C. ^{1}H NMR (DMSO-d_6): δ 7.14 (d, *J* = 8.5 Hz, 1H, Ar), 7.45 (d, *J* = 8.0 Hz, 1H, Ar), 7.78 (d, *J* = 8.5 Hz, 1H, Ar), 7.95 (d, *J* = 8.5 Hz, 1H, Ar), 8.19 (s, 1H, Ar), 8.28 (s, 1H, NH). IR (Nujol) 3320, 3225, 3070, 2688, 1685, 1632, 1558 cm^{-1}. Elemental analysis: calculated for $C_{14}H_9ClN_2O_3$ (288.15) %C 58.25, %H 3.14, %N 9.70, found %C 58.16, %H 3.15 %N 9.74. m/z 289.

2-(5-Chloro-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (58)



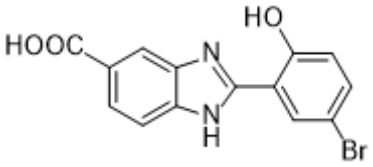
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-chlorophenylbenzaldehyde. Yield 85%. Mp 226-227 °C. ^{1}H NMR (DMSO-d_6): δ 7.25 (d, *J* = 8.0 Hz, 1H, Ar), 7.61 (d, *J* = 8.0 Hz, 1H, Ar), 7.80 (m, 2H, Ar), 8.06 (s, 1H, Ar), 8.20 (s, 1H, NH). IR (Nujol) 3582, 3375, 3060, 2720, 1624, 1556 cm^{-1}. Elemental analysis: calculated for $C_{13}H_9ClN_2O_4S$ (288.15) %C 48.08, %H 2.79, %N 8.63, found %C 48.03, %H 2.81 %N 8.67. m/z 325.

2-(5-Bromo-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carbonitrile (59)



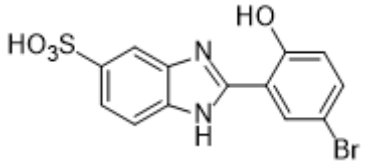
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-bromophenylbenzaldehyde. Yield 85%. Mp 215-216 °C. ^{1}H NMR (DMSO-d_6): δ 7.12 (d, *J* = 9.0 Hz, 1H, Ar), 7.58 (d, *J* = 9.0 Hz, 1H, Ar), 7.72 (d, *J* = 8.0 Hz, 1H, Ar), 7.86 (d, *J* = 8.5 Hz, 1H, Ar), 8.20 (s, 1H, Ar), 8.31 (s, 1H, NH). IR (Nujol) 2220, 1607, 1552 cm^{-1}. Elemental analysis: calculated for $C_{13}H_8BrN_3O$ (313.31) %C 53.53, %H 2.57, %N 13.38, found %C 53.49, %H 2.58 %N 13.41. m/z 314.

2-(5-Bromo-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (60)



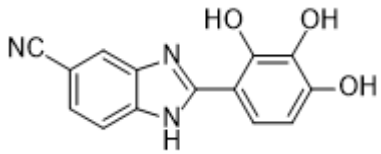
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-bromophenylbenzaldehyde. Yield 84%. Mp 233-235 °C. ^{1}H NMR (DMSO-d_6): δ 7.09 (d, *J* = 8.5 Hz, 1H, Ar), 7.60 (d, *J* = 8.5 Hz, 1H, Ar), 7.78 (d, *J* = 8.5 Hz, 1H, Ar), 7.95 (d, *J* = 8.5 Hz, 1H, Ar), 8.28 (s, 1H, Ar), 8.31 (s, 1H, NH). IR (Nujol) 3320, 3170, 2670, 1638, 1624, 1555 cm^{-1}. Elemental analysis: calculated for $C_{14}H_9BrN_2O_3$ (331.08) %C 50.47, %H 2.72, %N 8.41, found %C 50.53, %H 2.71 %N 8.44. m/z 332.

2-(5-Bromo-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (61)



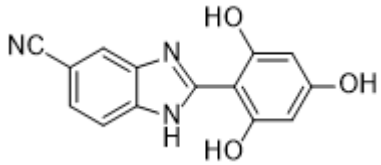
Following the general procedure, the title compound was prepared starting from 2-hydroxy-5-bromophenylbenzaldehyde. Yield 88%. Mp 240-241 °C. ^{1}H NMR (DMSO-d_6): δ 7.16 (d, *J* = 9.0 Hz, 1H, Ar), 7.72 (d, *J* = 9.0 Hz, 1H, Ar), 7.79 (m, 2H, Ar), 8.04 (s, 1H, Ar), 8.27 (s, 1H, NH). IR (Nujol) 3350, 3264, 3199, 2722, 2670, 1625, 1605, 1556 cm^{-1}. Elemental analysis: calculated for $C_{13}H_9BrN_2O_4S$ (367.11) %C 42.29, %H 2.46, %N 7.59, found %C 42.24, %H 2.45 %N 7.62. m/z 368.

2-(2,3,4-Trihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carbonitrile (62)



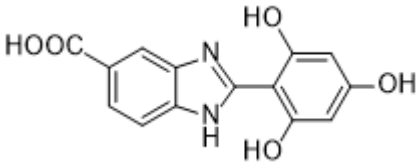
Following the general procedure, the title compound was prepared starting from 2,3,4-trihydroxybenzaldehyde. Yield 72%. Mp 232-233 °C. ^{1}H NMR (DMSO-d_6): δ 6.65 (d, *J* = 9.0 Hz, 1H, Ar), 7.60 (d, *J* = 8.5 Hz, 1H, Ar), 7.80 (d, *J* = 8.0 Hz, 1H, Ar), 7.88 (d, *J* = 8.0 Hz, 1H, Ar), 8.20 (s, 1H, NH). IR (Nujol) 2228, 1619, 1571 cm^{-1}. Elemental analysis: calculated for $C_{14}H_9N_3O_3$ (266.22) %C 62.92, %H 3.39, %N 15.72, found %C 62.85, %H 3.41 %N 15.76. m/z 267.

2-(2,4,6-Trihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carbonitrile (65)



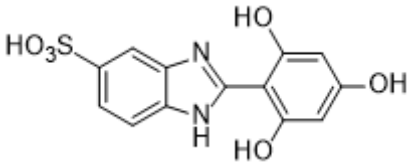
Following the general procedure, the title compound was prepared starting from 2,4,6-trihydroxybenzaldehyde. Yield 75%. Mp >250°C. ^{1}H NMR (DMSO-d_6): δ 6.22 (s, 1H, Ar), 6.22 (s, 1H, Ar), 7.87 (d, *J* = 8.5 Hz, 1H, Ar), 7.95 (d, *J* = 8.5 Hz, 1H, Ar), 8.21 (s, 1H, NH). IR (Nujol) 2230, 1625 cm^{-1}. Elemental analysis: calculated for $C_{14}H_9N_3O_3$ (266.22) %C 62.92, %H 3.39, %N 15.72, found %C 62.99, %H 3.38 %N 15.69. m/z 267.

2-(2,4,6-Trihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (66)



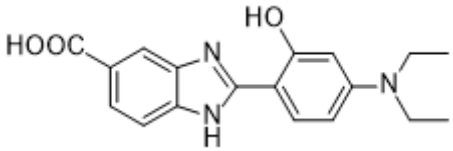
Following the general procedure, the title compound was prepared starting from 2,4,6-trihydroxybenzaldehyde. Yield 75%. Mp >250°C. ^{1}H NMR (DMSO-d_6): δ 6.01 (s, 2H, Ar), 7.69 (d, *J* = 8.0 Hz, 1H, Ar), 7.83 (d, *J* = 8.5 Hz, 1H, Ar), 8.26 (s, 1H, NH), 13.19 (s, 1H, OH), 13.24 (s, 1H, OH). IR (Nujol) 3404, 3233, 2718, 1704, 1614, 1565 cm^{-1}. Elemental analysis: calculated for $C_{14}H_{10}N_2O_5$ (286.07) %C 58.74, %H 3.52, %N 9.79, found %C 58.69, %H 3.50 %N 9.83. m/z 267.

2-(2,4,6-Trihydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (67)



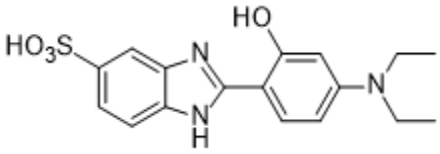
Following the general procedure, the title compound was prepared starting from 2,4,6-trihydroxybenzaldehyde. Yield 75%. Mp >250°C. ^{1}H NMR (DMSO-d_6): δ 6.14 (s, 2H, Ar), 7.70 (d, *J* = 8.5 Hz, 1H, Ar), 7.74 (d, *J* = 8.5 Hz, 1H, Ar), 8.11 (s, 1H, NH), 10.45 (s, 1H, NH), 11, 80 (s, 2H, OH), 13,20 (s, 1H, OH). IR (Nujol) 3489, 3216, 1629 cm^{-1}. Elemental analysis: calculated for $C_{13}H_{10}N_2O_6S$ (286.07) %C 48.45, %H 3.13, %N 8.69, found %C 48.49, %H 3.11 %N 8.72. m/z 267.

2-(4-(Diethylamino)-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (68)



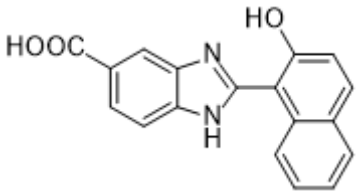
Following the general procedure, the title compound was prepared starting from 2-hydroxy-4-(diethylamino)benzaldehyde. Yield 59%. Mp 212-213°C. ^{1}H NMR (DMSO-d_6): δ 1.14 (t, *J* = 7.0 Hz, 6H, CH_3), 3.41 (q, *J* = 7.0 Hz, 4H, CH_2), 6.30 (s, 1H, Ar), 6.47 (d, *J* = 9.0 Hz, 1H, Ar), 7.70 (d, *J* = 8.5 Hz, 1H, Ar), 7.85 (d, *J* = 8.5 Hz, 1H, Ar), 7.94 (d, *J* = 8.0 Hz, 1H, Ar), 8.20 (s, 1H, NH). IR (Nujol) 3446, 3181, 2681, 1691, 1615 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{19}N_3O_3$ (325.20) %C 66.45, %H 5.89, %N 12.91, found %C 66.52, %H 5.86 %N 12.94. m/z 326.

2-(4-(Diethylamino)-2-hydroxyphenyl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (69)



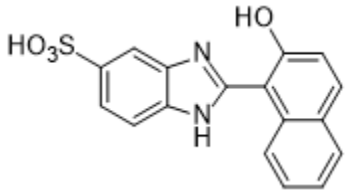
Following the general procedure, the title compound was prepared starting from 2-ydroxy-4-diethylaminobenzaldehyde. Yield 63%. Mp 221-222°C. ^{1}H NMR (DMSO-d_6): δ 1.16 (t, *J* = 7.0 Hz, 6H, CH_3), 3.43 (q, *J* = 7.0 Hz, 4H, CH_2), 6.36 (s, 1H, Ar), 6.53 (d, *J* = 8.5 Hz, 1H, Ar), 7.65 (d, *J* = 8.5 Hz, 1H, Ar), 7.71 (d, *J* = 8.5 Hz, 1H, Ar), 7.85 (d, *J* = 8.5 Hz, 1H, Ar), 7.97 (s, 1H, Ar), 11.42 (s, 1H, NH), 13.55 (s, 1H, OH). IR (Nujol) 3442, 3321, 1613 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{19}N_3O_4S$ (361.11) %C 56.50, %H 5.30, %N 11.63, found %C 56.45, %H 5.28 %N 11.67. m/z 362.

2-(2-Hydroxynaphthalen-1-yl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (70)



Following the general procedure, the title compound was prepared starting from 2-hydroxy-naphtaldehyde. Yield 58%. Mp >250°C. ^{1}H NMR (DMSO-d_6): δ 7.42 (m, 2H, Ar), 7.54 (t, *J* = 8.5 Hz, 1H, Ar), 7.87 (d, *J* = 9.0 Hz, 1H, Ar), 7.92-7.96 (m, 2H, Ar), 8.05 (d, *J* = 8.5 Hz, 1H, Ar), 8.10 (d, *J* = 8.5 Hz, 1H, Ar), 8.35 (s, 1H, NH). IR (Nujol) 3463, 2680, 1682, 1624, 1594 cm^{-1}. Elemental analysis: calculated for $C_{18}H_{12}N_2O_3$ (304.05) %C 71.50, %H 3.97, %N 9.21, found %C 70.98, %H 3.98 %N 9.25. m/z 305.

2-(2-Hydroxynaphthalen-1-yl)-1*H*-benzo[*d*]imidazole-5-sulfonic acid (71)



Following the general procedure, the title compound was prepared starting from 2-hydroxy2-ydroxy-naphtaldehyde. Yield 61%. Mp >250°C. ^{1}H NMR (DMSO-d_6): δ 7.43 (d, *J* = 9.0 Hz, 1H, Ar), 7.47 (t, *J* = 7.5 Hz, 1H, Ar), 7.57 (t, *J* = 8.0 Hz, 7.5 Hz, 1H, Ar), 7.72 (d, *J* = 8.0 Hz, 1H, Ar), 7.83 (d, *J* = 9.0 Hz, 1H, Ar), 7.89 (d, *J* = 8.0 Hz, 1H, Ar) 8.01 (d, *J* = 8.0 Hz, 1H, Ar), 8.04 (s, 1H, Ar), 8.18 (d, *J* = 8.5 Hz, 1H, Ar), 10.81 (s. 1H, OH). IR (Nujol) 3423, 1621, 1588 cm^{-1}. Elemental analysis: calculated for $C_{17}H_{12}N_2O_4S$ (340.33) %C 59.99, %H 3.55, %N 8.23, found %C 60.05, %H 3.53 %N 8.26. m/z 341.

3.5 References

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4. The PROTAC technology

4.1 Histone Deacetylase

Epigenetic is defined as a change in gene expression without any modification in the DNA sequence, through acetylation, deacetylation, methylation and hydroxymetylation.[1] Histones are important components of chromosomes in eukaryotic cells and their acetylation and deacetylation regulate gene expression. Two important classes of enzymes regulate these modifications: Histone acetyltransferases (HATs) and histone deacetylases (HDACs). These enzymes act by the acetylation and the deacetylation of the amino group of lysine residues in histone tails and some non-histone proteins.[2] Up to 1750 proteins have been identified as HDAC target, including both histone and nonhistone proteins, so that dysregulation of HDACs activity or expression is related to different diseases such as cancer.[3] Currently, 18 HDACs have been identified and grouped into 4 different classes, based on their cellular localization and enzymatic activity. Class I consists of four isoforms (HDAC1, 2, 3, 8) that are primarily localized in the nucleus and act on histones and transcription factors. Class II consists of two subclasses: IIa with four isoforms (HDAC 4, 5, 7, 9) and IIb with two isoforms (HDAC 6, 10). These isoforms move between the nucleus and cytoplasm, where they act on non-histone proteins. Class IV contains only one member, the HDAC11. Class III consists of seven isoforms named sirtuins (sirt1-7). Classes I, II, IV and Class III differ in their catalytic mechanism: a Zn^{2+} dependent mechanism in class I, II, and IV and an NAD^{+} dependent mechanism in class III. Recent studies showed that HDACs, especially Class I and Class IIb are overexpressed in several cancer forms, such as breast cancer, liver cancer, multiple myeloma, neuroblastoma etc. In this context, HDAC6 and HDAC8 play an essential role in tumour genesis and survival, so they are important targets for the design of selective inhibitors.[4] HDAC inhibitors bind the catalytic domains of their enzyme target, decreasing its enzymatic activity, which can phenotypically result in diminished cell survival and proliferation.[5] Class I HDAC inhibitors increase levels of acetylation on histone lysine residues, which results in a more “open” chromatin conformation that is more available for gene expression. Agents that inhibit HDAC6, for example in multiple myeloma, increase acetylation of tubulin, which leads to accumulation of protein aggregates and cell death.[5] Non-selective HDAC inhibitors, that inhibit many HDAC subtypes, called pan-HDACi, showed important clinical benefits in some cancer forms but their toxicity, which may be a result of their poor selectivity. This is a limit on clinical treatment, particularly when used in combination with other chemotherapeutic agents. Thus, the discovery of effective and specific HDAC inhibitors that are safe and well tolerated is necessary. Several natural compounds such as trapoxin, herbimycin, depudecin, apicidin, and synthetic small molecules, such as suberoylanilide hydroxamic acid (SAHA) (Figure 4.1) act as non-selective HDAC inhibitors.[6] In 2006, SAHA became the first FDA-

approved HDAC inhibitor, but it shows several limitations, including low potency, cardiovascular concerns and drug-drug interactions.[6] Starting from SAHA, and a host of other HDAC inhibitors, scientists have completely explained the pharmacophore model for HDAC inhibitors. In fact, HDAC inhibitors are endowed with a chelating group, such as the hydroxamic acid moiety, that forms a complex with the a Zn^{2+} and a hydrophobic spacer linked to the chelating group that fits into the pocket of the active site.[6]

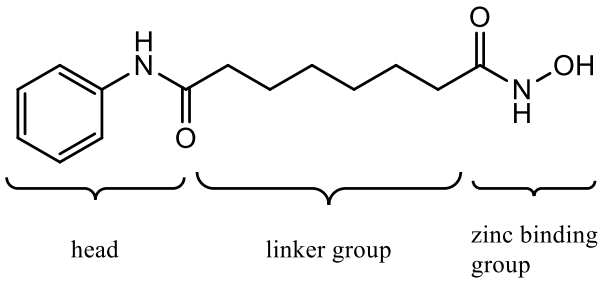


Figure 4.1. Structure of SAHA.

Different studies on HDAC10, a class IIB member, demonstrated an important correlation between overexpression of HDAC10 and poor clinical outcome for advanced stage neuroblastoma patients.[7] Indeed, it has been demonstrated an interesting correlation between HDAC10 expression and autophagy. In cancer cells, autophagy represents in the most of cases a survival mechanism, helping the cell to overcome cellular stress induced by traditional chemotherapeutic drugs such as Doxorubicin.[8] In neuroblastoma cell lines, HDAC10 interacts with Hsp70 family proteins Hsc70 that is implicated in several cellular functions such as protein folding, ubiquitin mediated protein degradation, lysosomal protein degradation, and lysosomal membrane integrity.[9] HDAC10 expression is also correlated with Autophagy related Genes (ATG), which regulates autophagosome biogenesis. Therefore, the selective inhibition of HDAC10 or the selective degradation mediated by PROTAC might represent a promising approach to sensitize aggressive neuroblastoma to traditional drugs.

4.2 Design of HDAC10-PROTAC

To obtain drug-like PROTACs it is necessary to perform all the three components: the E3 ligase, the linker and the small molecule inhibitor. A few E3 ligases are now available for the PROTAC technology, whereas the most common are: MDM2, clAP1, VHL and CEREBLON where VHL and CEREBLON are the most successful.[10] The p53-degrading Mouse Double Minute 2 (MDM2) ubiquitin ligase was used in the first small-molecule PROTAC designed by Schneekloth et al.[11] as an AR degrader. This compound promoted the degradation of the target protein at concentration >10μM in HeLa cells. The cellular inhibitor of apoptosis protein-1 (cIAP1) E3 ubiquitin ligase was used for the first time by Ishikawa and Hashimoto[12] to promote the degradation of the retinoic acid

binding proteins CRABP I and II. Further PROTACs with cIAP1 have been prepared to promote degradation of retinoic acid receptor (RAR), ERα, and AR.[13-15] Recently Thalidomide, Lenalidomide and Pomalidomide have been described as binders for the Cul4-Rbx1-DDB1-CEREBLON E3 ubiquitin ligase and successfully incorporated into PROTAC. Two studies described the development of BRD4-PROTACs using thalidomide and its derivatives, demonstrating significant BRD4 degradation in Burkitt's Lymphoma (BL)[16] and in acute myeloid lymphoma (AML).[17] The Von Hippel Lindau (VHL) was used for the first time in a peptide-like PROTAC to replace the IκBα-phosphopeptide[18] with the aim to the target estrogen-related receptor (ERRα) and the serine/threonine kinase.[10].

The linker is another crucial component of PROTACs because it connects the E3 ligase portion to the small molecule inhibitor, so that a correct optimization of the linker promotes favourable interactions between the two active moieties which result in an efficient ubiquitination of the target protein.[19] Generally, the length of the linker is randomly selected, with a variation from 15 to over 20 carbon chain where the best length needs to be determined on a case by case basis.[20] It is also important to find the correct linkage between the two active part and the linker, to modify the small molecule inhibitor and the E3 ligase portion as less as possible.

The small molecule inhibitor is the most various component of the PROTAC because it depends on the target protein. During the early stage of the PROTAC design, it is crucial to identify a well-validated, selective and potent small molecule inhibitor, to avoid the non-selective protein degradation. During the first stage of the PROTAC design it is also necessary to find a suitable position to introduce the linker to maintain the activity against the target protein. Several HDAC6 selective inhibitors have been studied and characterized due to the importance of this isoform in cancer, and among these TubastatinA is a potent and high selective HDAC6 inhibitor.[21,22] M. Géraly et al.[23]demonstrated that Tubastatin A and its analogs were active in low nanomolar range against HDAC10. Starting from these considerations we have designed a new series of PROTACs based on Tubastatin A scaffold. This project has been developed during my Erasmus stay in collaboration with the Cancer Drug Development research group of the German Cancer Research Centre (DKFZ) and the European Molecular Biology Laboratory (EMBL) in Heidelberg.

As mentioned above, the hydroxamic acid moiety is the zinc binder group so it is important to not modify the portion of the molecule nearby to the binder group. In the figure 4.2 two possible modifications at the Tubastatin scaffold are shown: one at the aromatic ring of the indole and the other one at the nitrogen group of the carboline ring.

Different studies[22-24] verified that the substitution at the nitrogen of the carboline group is well tolerated, so that this position has been used for the introduction of the linker.

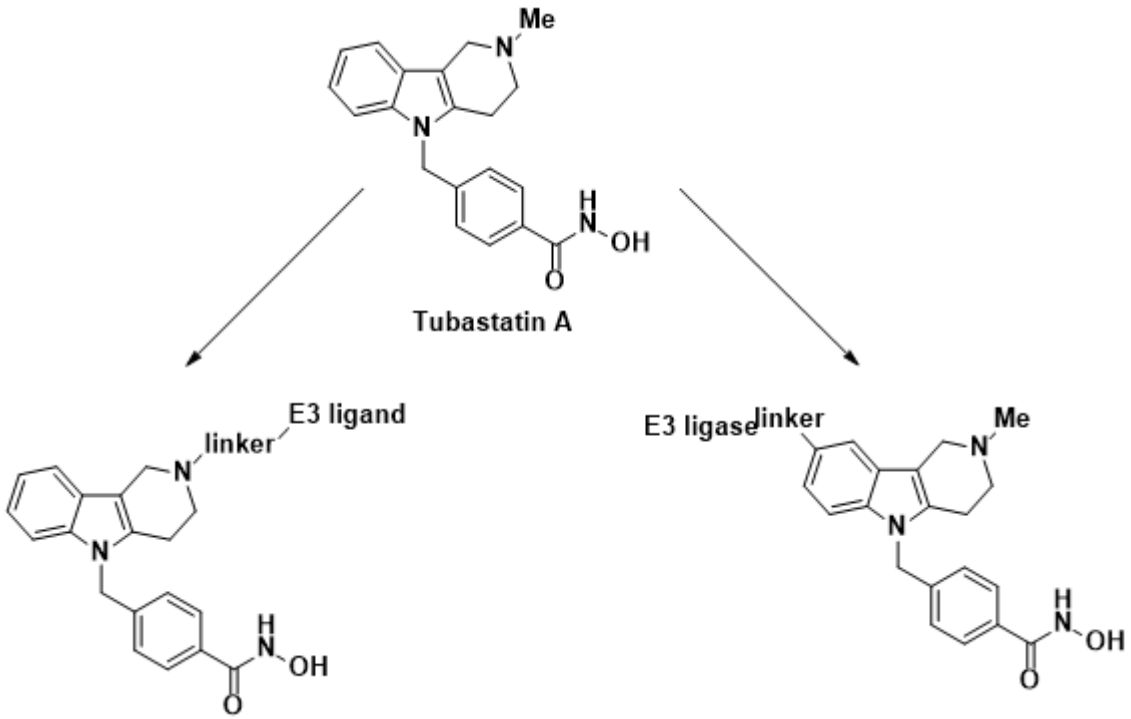


Figure 4.2. Design of TubastatinA based PROTAC

Furthermore, to perform the protein degradation promoted by PROTACs, a linker with the right length is necessary, so that different linkers have been introduced. For what concern the E3 portion, VHL and CEREBLON scaffolds have been modified to promote the coupling with the different linkers (Figure 4.3).

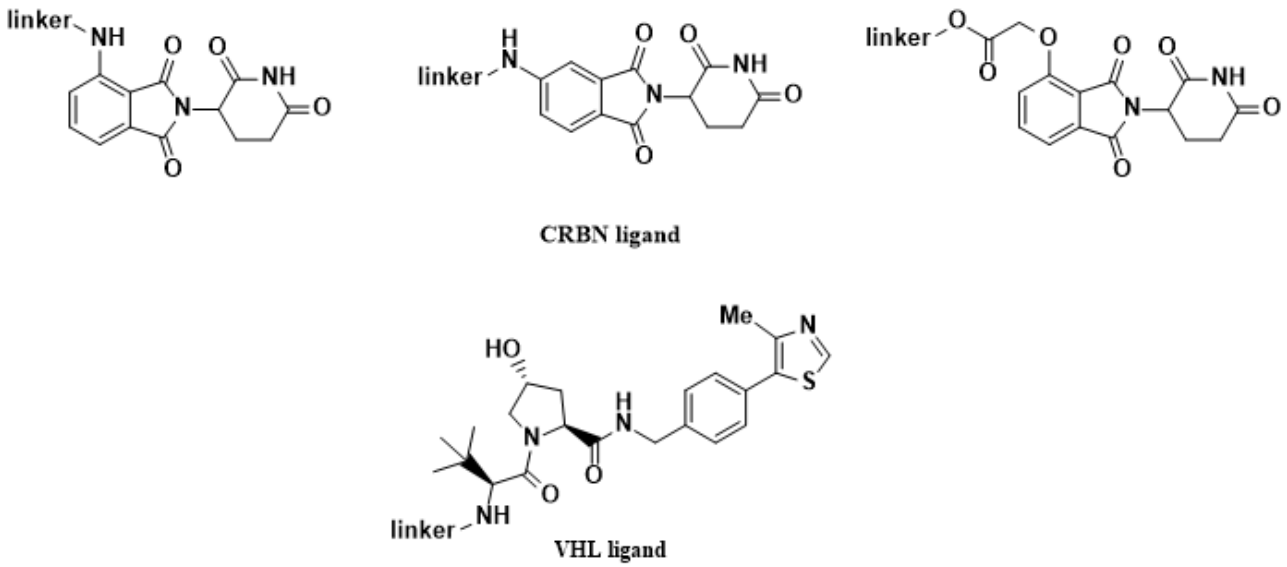
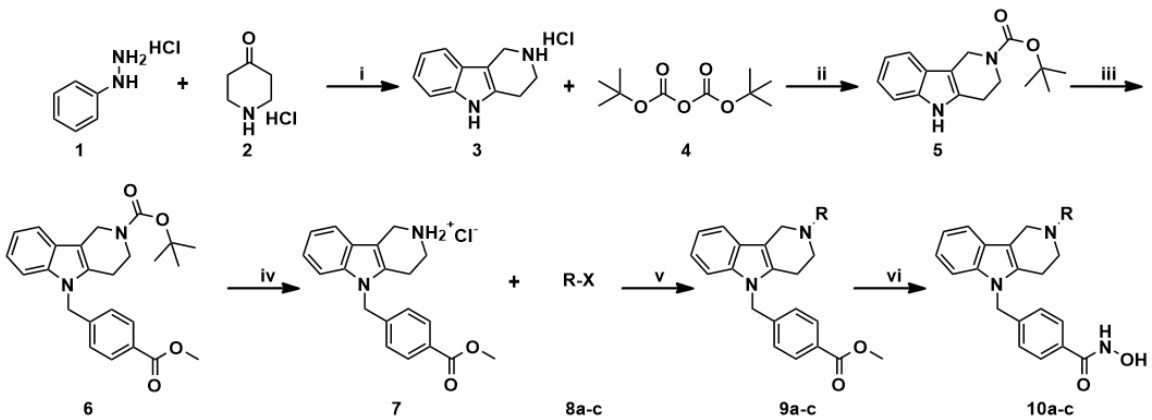


Figure 4.3. Modifications at CEREBLON and VHL scaffolds.

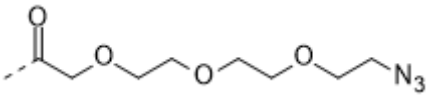
At first, the Tubastatin A scaffold was synthetized and modified with different substituent at the nitrogen of carboline ring to find the right linker strategy. The synthetic pathway to obtain Tubastatin-A scaffold started with the Fischer-indole reaction, using phenylhydrazine hydrochloride

1 and piperidone mono hydrate mono hydrochloride **2**, in absolute EtOH (Scheme 4.1). The nitrogen of the carboline ring of **3** was protected using di-tert-butyl dicarbonate ($BocO_2$) **4**, DIPEA as a base in dry DCM to obtain intermediate **5**. Then, through alkylation of the aromatic nitrogen of the indole **5** with methyl 4-(bromomethyl)benzoate, using NaH and dry DMF under argon, intermediate **6** was obtained. The Boc protecting group was removed operating with a 2M solution of HCl in Et_2O, using DCM as solvent. Different linkers **8a-c** were then introduced to afford intermediates **9a-c**. The hydroxamates **10a-c** were prepared using 60% aqueous hydroxylamine (NH_2OH) solution, KCN and dioxane. Compounds **9a-c** were tested using a cell-based target occupancy (BRET) assay.[23]



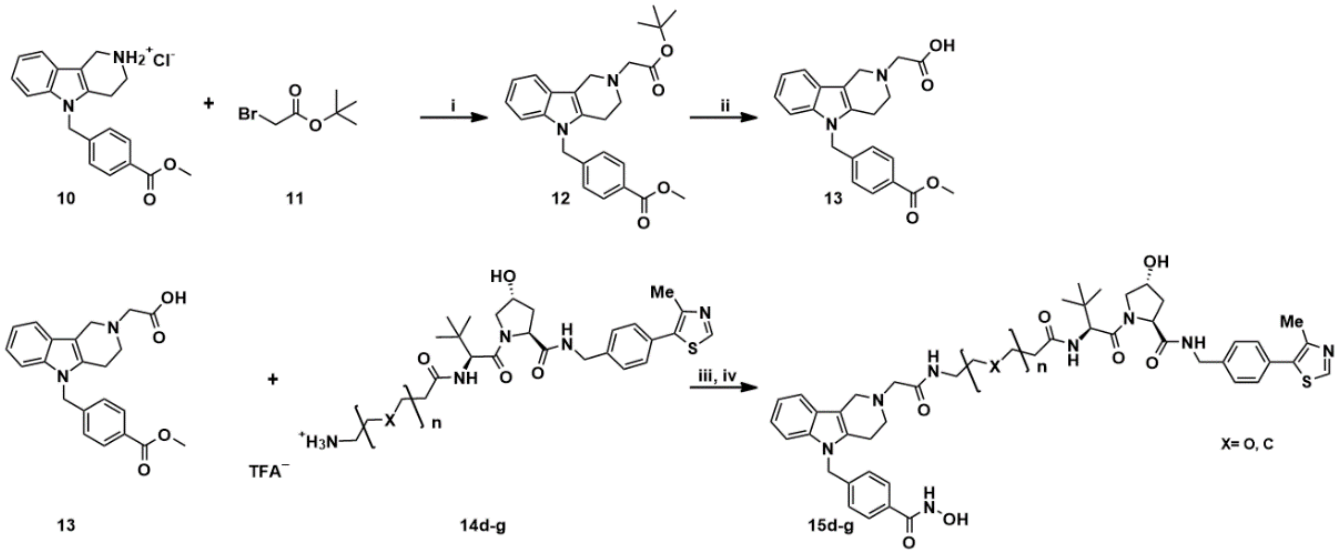
Scheme 4.1. General synthetic procedure for **10a-c**. i) Absolute EtOH, 90°C, 4h; ii) DIPEA, CH_2Cl_2, r.t. 6h; iii) methyl-4-(bromomethyl)benzoate, NaH, DMF, r.t., 6h; iv) HCl 2M in Et_2O, DCM, r.t. 12h; v) Et_3N, DMF, 60°C, 5h; vi) NH_2OH 60% in H_2O, KCN, Dioxane.

Table 4.1. Results of BRET assay for **10a-c**.

Compound	R	IC_{50} HDAC6 (nM)	IC_{50} HDAC10 (nM)
10°		464.0	695.0
10b		623.0	633.0
10c		887.0	12.0
Tubastatin A	-	53.0	8.1

The preliminary test on Tubastatin A derivatives **9a-c** showed the importance of the bond between the linker and the nitrogen atom of the carboline ring (Table 4.1). Comparing the activity between Tubastatin A and the acetylated analog **9a** it is possible to observe a significant decrease of activity on both HDAC6 and HDAC10, as well as in the case of **9b**, bearing a long linker. The compound **9c** showed weak activity against HDAC6 but good activity against HDAC10, likewise to

TubastatinA. From these results it is possible to speculate that a basic nitrogen in the carboline ring might be crucial for a potent HDAC10 binding,[23] so that in the case of HDAC10-PROTACs it is important to introduce a linker which satisfy this condition. For this reason, the short liker **11** was introduced at the TubastatinA scaffold by alkylation, using DIPEA in boiling DMF (Scheme 4.2). Then, the tert-butyl group from intermediate **12** was removed using TFA in DCM solution to obtain the compound **13**. On this intermediate, different linker already bounded with the E3 ligase VHL **14d-g** were introduced using EDCI as coupling agent. The reaction was performed in dry DMF solution, in the presence of HOBt. Four different linkers has been introduced, two of them are alkyl chains (compounds **15d** and **15e**) and the other two (compounds **15f** and **15g**) are poly(ethylene glycol) chains.

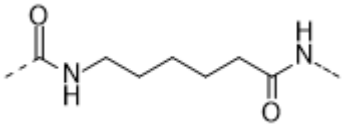
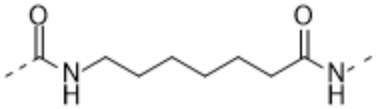
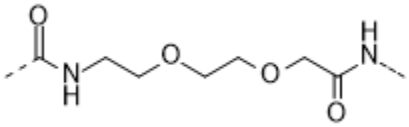
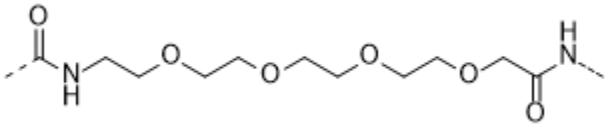


Scheme 4.2. Synthetic procedure for **15d-g** i) Et_3N, DMF, 60°C, 5h; ii) 20% TFA in DCM, r.t. 24h; iii) EDCI, HOBt, DIPEA, DMF, r.t. 6h; iv) NH_2OH 60% in H_2O, KCN, Dioxane.

Four different linkers has been introduced, two of them are alkyl chains (compounds **15d** and **15e**) and the other two (compounds **15f** and **15g**) are poly(ethylene glycol) chains.

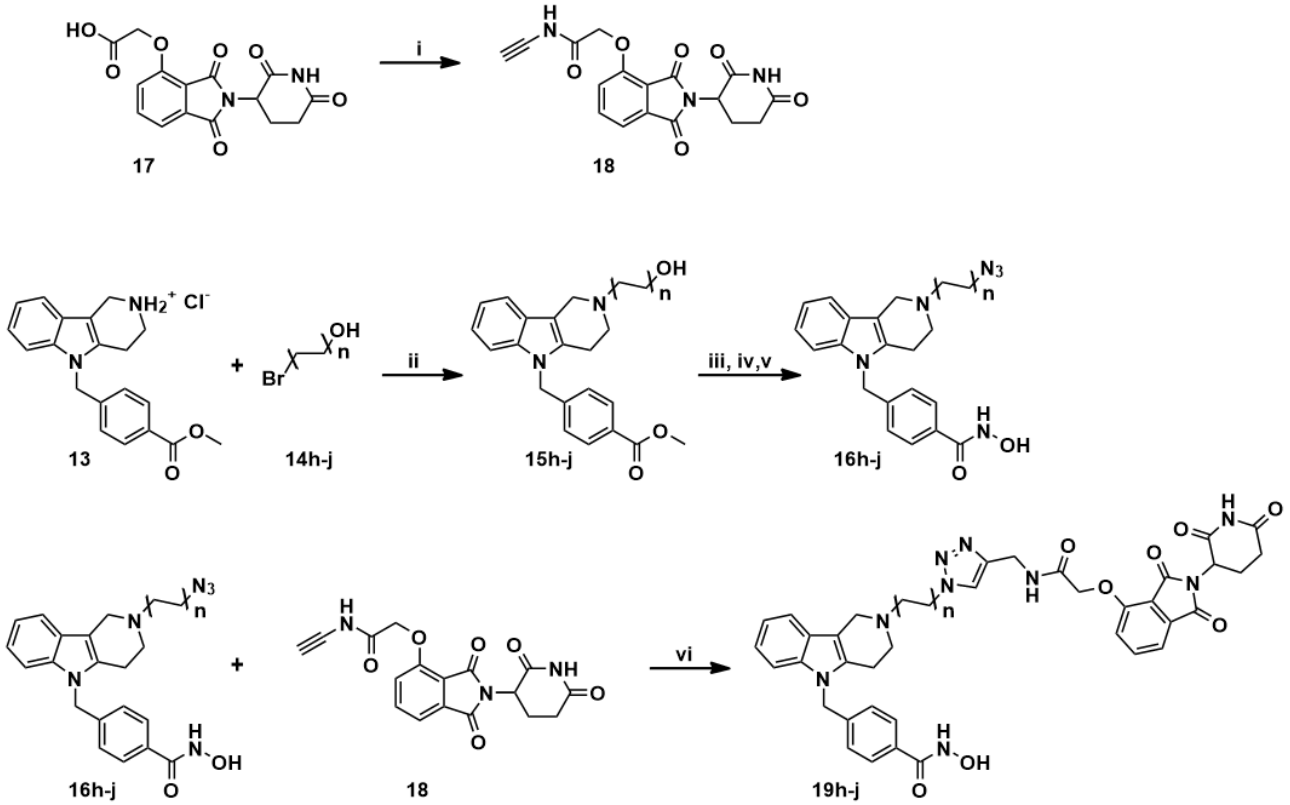
In the BRET test (Table 4.2) compound **15d** resulted about 45-fold more selective against HDAC10 if compared with the HDAC6 inhibitory activity, while compound **15e** equipped with a longer alkyl chain showed comparable IC_{50} values against HDAC6 and HDAC10. The substitution of the alkyl chain with a short poly(ethylene glycol) one, as in compound **15f**, partially restored the selectivity against HDAC10. Compound **15g** endowed with a long poly(ethylene glycol) linker resulted the best compound of the series with an IC_{50} of 0.32 μM and it is about 120-fold more selective against HDAC10 than HDAC6.

Table 4.2. Results of BRET assay for **15d-g**

Compound	Linker	IC_{50} HDAC6 (μM)	IC_{50} HDAC10 (μM)
15d		56.22	1.25
15e		2.54	1.03
15f		32.97	11.4
15g		38.51	0.32
Tubastatin	-	0.0053	0.00081

A second series of PROTACs based on Tubastatin scaffold bearing CEREBLON as E3 ligase has been synthetized (Scheme 4.3). In this series an important modification has been introduced in the linker strategy: the connection of the two-active part of the molecule using the "click chemistry" tool. The cycloaddiction catalysed by Copper(I) (CuAAC) between an azide group and an alkyne is the most representative example of click chemistry[24] and is largely used in drug development due to the high yields and absence of by-products. In this context, click chemistry may be a straightforward solution to connect the two-active part of the PROTAC.[25]

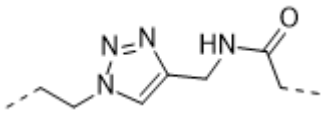
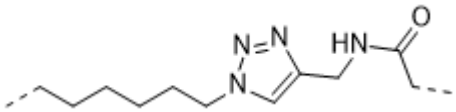
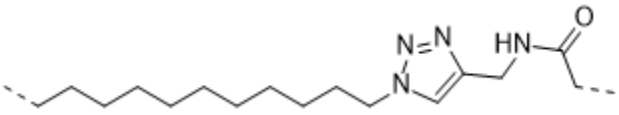
The second series of PROTAC **19h-j** was obtained through a CuAAC, in the presence of tris((1-(tert-butyl)-1*H*-1,2,3-triazol-4-yl)methyl)amine (TTA) in dry THF solution. The synthetic pathway to obtain the two intermediates **16h-j** and **18** for the final click reaction started with the reaction between the thalidomide analog **17** and propargylamine, using EDCI as coupling reagent in dry DMF under argon to obtain the intermediate **18**. Different alkyl alcohols **14h-j** were introduced on intermediate **13** via alkylation in boiling DMF solution in the presence of triethylamine (TEA). Then, the hydroxy group of derivatives **15h-j** were first converted in the corresponding mesylates by methanesulfonyl chloride in the presence of TEA in dry DCM solution. Azide group was then introduced using NaN_3 in boiling DMF under argon to obtain the desired intermediates **16h-j**.



Scheme 4.3. Synthesis of compounds **29h-j**. Reagents and conditions; i) propargylamine, EDCI, HOBt, DMF, r.t. 6h; ii) Et_3N, DMF, 60°C, 5h; iii) Methanesulfonyl chloride, Et_3N, dry $MeCN_2$, r.t. 4h; iv) NaN_3, DMF, 120°C, 5h; v) NH_2OH 60% in H_2O, KCN, Dioxane; vi) CuI, tris((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amine (TTA), dry tetrahydrofuran (THF), r.t. 8h.

The modifications in the linker and the E3 ligase produced an unexpected loss of selectivity against HDAC10 (Table 4.3), indeed all the compounds **19h-j** showed about the same IC_{50} values for both HDAC6 and HDAC10. Compound **19h**, endowed with a short linker, showed good potency against both isoforms (IC_{50} HDAC6 4.46 µM, IC_{50} HDAC10 4.07 µM).

Table 4.3. Results of BRET assay for compounds **19h-j**

Compound	Linker	IC_{50} HDAC6 (µM)	IC_{50} HDAC10 (µM)
19h		4.46	4.07
19i		3.59	6.28
19j		4.22	3.53
TubastatinA	-	0.0053	0.00081

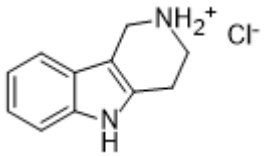
Increasing the number of carbon atoms of the linker chain to give compound **19i** resulted in a decrease of activity against HDAC10 (IC_{50} 6.28 µM) but on the other hand, the activity against HDAC6 was improved (IC_{50} 3.59 µM). A further increase of the chain length to give compound **19j** restored the activity against HDAC10 (IC_{50} 3.53 µM). Taken together BRET results showed that linkers with different chain may influence the selectivity of the inhibitor.

These preliminary assays indicated compounds **15d**, **15g**, **19h** and **19j** as good candidates for the *in-vitro* tests to evaluate their ability to promote the degradation of HDAC10, and ultimately their ability to promote HDAC10-associated phenotypes, e.g. chemotherapy sensitization in neuroblastoma cell lines.

4.3 Experimental

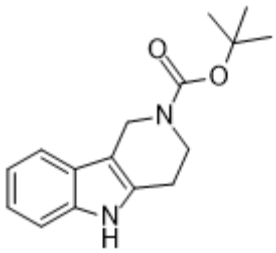
Chemicals and solvents were purchased from commercial sources at the highest level of purity and used without purification. Anhydrous dichloromethane, toluene, acetonitrile, and tetrahydrofuran were prepared with an MBraun SPS800 solvent purification system. Purification was performed by flash column chromatography using SiliCycle SiliaFlash P60 (40− 63 μm, 60 Å particle size) or via RP-HPLC (Agilent 1260 Infinity and an ES quadrupole Agilent 6120, column: Kinetex 5 μm C18 100 Å, AXIA packed LC column 250 × 21.2 mm; temperature = 40 °C; solvent A = H2O, 0.05% TFA; solvent B = MeCN, 0.05% TFA; flow-rate = 15.0 mL/min; method: gradient: 5% B to 30% B [over 4 min], then 30% B to 60% B [over 8 min], then 0% B to 95% B [over 4 min]). For certain substances, optimization of the HPLC gradient was performed. Analytical LC/MS was performed on an Agilent 1260 infinity system using reverse phase. Column: Kinetex 2.6 μm C18 100 Å, LC column 50 × 2.1 mm; temperature = 40 °C; solvent A = H_2O, 0.01% HCO_2H; solvent B = MeCN, 0.01% HCO_2H; flow rate = 0.60 mL/ min; method: gradient: 1% B to 90% B [over 6 min] then 90% B to 99% B [over 2 min]. High-resolution mass spectrometry was recorded on a Bruker ApexQe FT-ICR instrument, (Department of Organic Chemistry, University of Heidelberg). NMR spectra were recorded on Bruker 400 or 600 MHz instruments at 298.1 K.

2,3,4,5-Tetrahydro-1*H*-pyrido[4,3-*b*]indol-2-ium chloride (3)



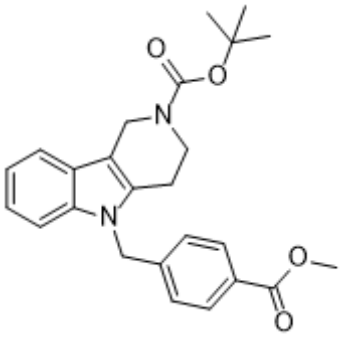
To a mixture of 4-piperidone monohydrate hydrochloride (7.23g, 50 mmol) in absolute EtOH, phenylhydrazine hydrochloride (7.68g, 50 mmol) was added. The mixture was refluxed for 5 hours and the formed solid was filtered off, washed with cold EtOH and used without further purification. Yield 70%. ^{1}H NMR (DMSO-d6) δ 2.78 (m, 2H, CH_2), 3.71 (s, 2H, CH_2), 4.54 (s, 2H, CH_2), 7.30 (d, *J* = 8.0 Hz, 2H, Ar), 7.37 (d, *J* = 7.5 Hz, 2H, Ar), 10.89 (s, 1H, NH), 11.25 (s, 2H, NH_2). Elemental analysis: calculated for $C_{11}H_{12}N_2$ (172.10) %C, 76.27, %H 7.56, %N 16.17, found %C 76.24, %H 7.58, %N 16.19. M/z 173.

Tert-butyl 3,4-dihydro-1*H*-pyrido[4,3-*b*]indole-2(5*H*)-carboxylate (5)



To a suspension of 2,3,4,5-Tetrahydro-1*H*-pyrido[4,3-*b*]indole (6.26 g, 30 mmol) in DCM (40 mL), DIPEA (12 mL, 45 mmol) was added and the resulting solution was stirred rt for 10 minutes. Then, $BocO_2$ (6.547g, 30 mmol) was added and the reaction mixture was stirred rt for 2h. The reaction was quenched with saturated NH_4Cl aqueous solution and extracted with DCM (3 x 10 mL). The organic layer was washed with water (2 x 10 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. The crude material was purified with Flash Chromatography (20% AcOEt - 80% Hexane) to obtain the pure material. Yield 72%. ^{1}H NMR (DMSO-d6) δ 1.44 (s, 9H, $3CH_3$), 2.78 (m, 2H, CH_2), 3.71 (s, 2H, CH_2), 4.54 (s, 2H, CH_2), 6.96 (d, *J* = 7.5 Hz, 1H, Ar), 7.03 (d, *J* = 9.5 Hz, 2H, Ar), 7.30 (d, *J* = 8.0 Hz, 1H, Ar), 7.36 (d, *J* = 9.0 Hz, 1H, Ar) 10.89 (s, 1H, NH). Elemental analysis: calculated for $C_{16}H_{20}N_2O_2$ (272.34) %C, 70.56, %H 7.40, %N 10.29, found %C 70.52, %H 7.38, %N 10.31. M/z 273.

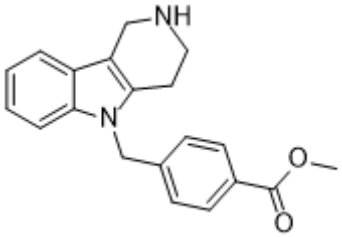
Tert-butyl 5-(4-(methoxycarbonyl)benzyl)-3,4-dihydro-1*H*-pyrido[4,3-b]indole-2(5*H*)-carboxylate (6)



To a mixture of tert-butyl 3,4-dihydro-1*H*-pyrido[4,3-*b*]indole-2(5*H*)-carboxylate (2.723 g, 10 mmol) and NaH (0.44 g, 10 mmol) under argon dry DMF (10 mL) was added. The mixture was stirred at rt for 30 minutes, then methyl 4-(bromomethyl)benzoate (2.291 g, 10 mmol) was added and stirring continued for further 5h. The mixture was poured in water and extracted with AcOEt (3 x15 mL). The organic layer was washed with water (3 x 20 mL) and brine. The organic layer was dried over anhydrous $MgSO_4$ and evaporated under vacuum. The crude material was purified with Flash Chromatography (30% AcOEt - 70% Hexane) to obtain the pure title compound. Yield 57%. ^{1}H NMR (DMSO-d_6): δ 1.42 (s, 9H, 3 CH_3), 2.70 (m, 2H, CH_2), 3.71 (s, 2H, CH_2), 3.81 (s, 3H, CH_3), 4.57 (s, 2H, CH_2), 5.45 (s, 2H, CH_2), 7.05 (m, 1H, Ar), 7.08 (t, *J* = 5.5, 2.5 Hz, 1H, Ar), 7.15

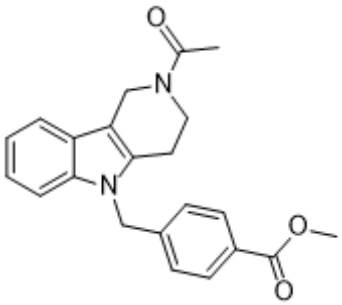
(d, *J* = 8.5 Hz, 2H, Ar), 7.39 (d, *J* = 8.0 Hz, 1H, Ar), 7.46 (d, *J* = 7.5 Hz, 1H, Ar), 7.91 (m, 2H, Ar). Elemental analysis: calculated for $C_{25}H_{28}N_2O_4$ (420.50) %C, 71.41, %H 6.71, %N 6.66, found %C 71.47, %H 6.73, %N 6.62. M/z 421.

Methyl 4-((3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (7)



To a stirred solution of tert-butyl 5-(4-(methoxycarbonyl)benzyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indole-2(5*H*)-carboxylate (2.101 g, 5 mmol) in DCM (5 mL), an aqueous HCl 2M solution (5 mL, 10 mmol) in Et_2O was added dropwise. The resulting mixture was stirred at rt overnight. The formed solid was filtered off and washed with Et_2O (4 x 5 mL), dried, and used without further purification. Yield 52%. ^{1}H NMR (DMSO-d_6) δ 3.02 (m, 2H, CH_2), 3.48 (s, 2H, CH_2), 3.83 (s, 3H, CH_3), 4.34 (s, 2H, CH_2), 5.52 (s, 2H, CH_2), 7.08 (d, *J* = 7.5 Hz, 1H, Ar), 7.14 (m, 1H, Ar), 7.21 (d, *J* = 8.5 Hz, 2H, Ar), 7.46 (d, *J* = 8.0 Hz, 1H, Ar), 7.55 (d, *J* = 7.5 Hz, 1H, Ar), 7.89 (m, 2H, Ar), 9.79 (s, 1H, NH). Elemental analysis: calculated for $C_{20}H_{20}N_2O_2$ (320.38) %C, 74.98, %H 6.29, %N 8.74, found %C 75.01, %H 6.33, %N 8.76. M/z 321.

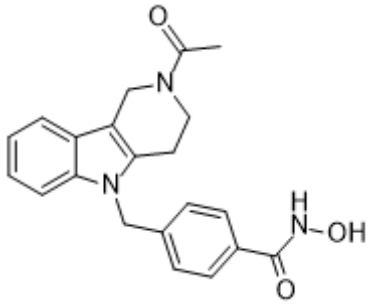
Methyl 4-((2-acetyl-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (9a)



To a suspension of methyl 4-((3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (0.178 g, 0.5 mmol) in DCM (2mL) under argon, TEA (0.2 mL, 1.2 mmol) was added and the resulting solution was cooled to 0 °C. At this solution, acetyl chloride (0.48 mL, 0.6 mmol) was added dropwise and the solution was heated to rt. The reaction mixture was stirred rt for 1.5h. The solution was poured in water, extract with DCM (3 x 10 mL), washed with NH_4Cl (2 x 10 mL), water (2 x 10 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. The crude material was purified with Flash Chromatography (1% MeOH - 99% DCM) to obtain the pure material. Yield 66%. ^{1}H NMR (DMSO-d_6) δ 2.13 (m, 3H, CH_3), 2.15 (m, 1H, CH), 2.67 (m, 2H, CH_2) 3.76 (m, 1H, CH), 3.81 (s, 3H, CH_3), 3.93 (m, 1H, CH), 4.66 (s, 1H, CH), 4.81

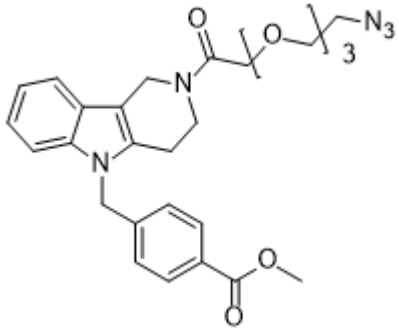
(s, 1H, CH), 5.23 (s, 2H, CH_2), 7.00 (m, 2H, Ar), 7.10 (m, 3H, Ar), 7.44 (d, *J* = 7.0 Hz, 1H, Ar), 7.87 (d, *J* = 8.5 Hz, 2H, Ar). Elemental analysis: calculated for $C_{22}H_{22}N_2O_3$ (362.42) %C, 72.91, %H 6.12, %N 7.73, found %C 72.95, %H 6.13, %N 7.70. M/z 363.

4-((2-Acetyl-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)-N-hydroxybenzamide (10a)



To a solution of methyl 4-((2-acetyl-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (0.363 g, 1 mmol) in 1,4-dioxane (5 mL), KCN (0.13 g, 2 mmol) and 50% aqueous NH_2OH (5 mL) were added. The reaction mixture was stirred at rt overnight and monitored by TLC. The solution was quenched with saturated $NaHCO_3$ solution, extract with AcOEt (3 x 5 mL) and washed with $NaHCO_3$ (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. Yield 58%. ^{1}H NMR (DMSO-d_6) δ 2.12 (m, 3H, CH_3), 2.19 (m, 1H, CH), 2.72 (m, 2H, CH_2) 3.17 (m, 1H, CH), 3.79 (m, 1H, CH), 3.85 (s, 1H, CH), 4.68 (s, 1H, CH), 5.41 (s, 2H, CH_2), 7.05 (m, 4H, Ar), 7.40 (d, *J* = 7.5 Hz, 1H, Ar), 7.49 (d, *J* = 7.0 Hz, 1H, Ar), 7.65 (m, 2H, Ar), 8.95 (s, 1H, NH), 11.12 (s, 1H, OH). Elemental analysis: calculated for $C_{21}H_{21}N_3O_3$ (363.55) %C, 69.41, %H 5.82, %N 11.56, found %C 69.49, %H 5.79, %N 11.60. M/z 364.

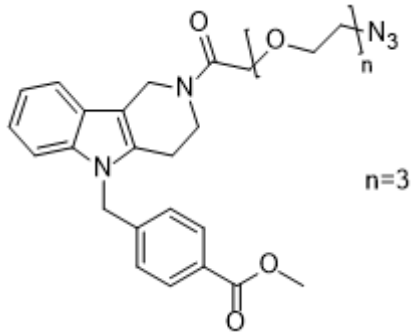
Methyl 4-((2-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (9b)



To a solution of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetic acid (0.156 g 0.5 mmol) in DMF (2mL), EDCI (0.095 g, 0.5 mmol) and HOBt (0.075 g, 0.5 mmol) were added and the resulting mixture was stirred for 15 minutes, then methyl 4-((3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (0.178 g, 0.5 mmol) in DMF (2 mL) was added. The reaction mixture was stirred rt overnight and monitored by TLC. The reaction was quenched with saturated $NaHCO_3$ aqueous solution, extracted with AcOEt (3 x 5 mL) and washed with saturated $NaHCO_3$ aqueous

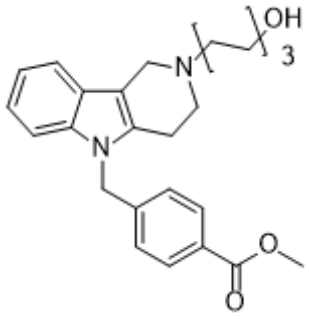
solution (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. Yield 64%. ^{1}H NMR (DMSO-d_6) δ 2.88 (m, 3H, OCH_3), 3.38 (m, 2H, CH_2) 3.63 (m, 12H, CH_2), 4.31 (s, 2H, CH_2), 4.39 (s, 2H, CH_2), 4.71 (s, 2H, CH_2), 5.45, (s, 2H, CH_2) 7.09 (m, 3H, Ar), 7.13 (d, *J* = 8.0 Hz, 2H, Ar), 7.44 (m, 2H, Ar), 7.67 (d, *J* = 8.5 Hz, 2H, Ar). Elemental analysis: calculated for $C_{28}H_{33}N_5O_6$ (535.59) %C, 62.79, %H 6.21, %N 13.08, found %C 62.87, %H 6.24, %N 13.03. M/z 536.

4-((2-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)acetyl)-3,4-dihydro-1*H*-pyrido[4,3-b]indol-5(2*H*)-yl)methyl)-N-hydroxybenzamide (10b)



Following the procedure described for **10a** the title compound was obtained in 33% yield. ^{1}H NMR (DMSO-d_6): δ 2.74 (m, 3H, OCH_3), 3.40 (m, 2H, CH_2), 3.69 (m, 12H, CH_2), 4.33 (s, 2H, CH_2), 4.37 (s, 2H, CH_2), 4.78 (s, 2H, CH_2), 5.45, (s, 2H, CH_2) 7.11 (m, 3H, Ar), 7.21 (d, *J* = 7.0 Hz, 2H, Ar), 7.49 (m, 2H, Ar), 7.63 (d, *J* = 7.5 Hz, 2H, Ar), 8.98 (s, 1H, NH), 11.09 (s, 1H, OH). Elemental analysis: calculated for $C_{27}H_{32}N_6O_6$ (536.58) %C, 60.44, %H 6.01, %N 15.66, found %C 60.50, %H 6.02, %N 15.62. M/z 537

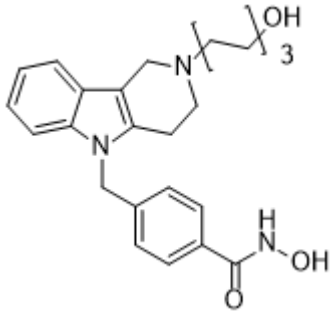
Methyl 4-((2-(6-hydroxyhexyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (9c)



To a suspension of methyl 4-((3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2H)-yl)methyl)benzoate (0.178 g, 0.5 mmol) in DMF (2mL), 6-bromo-1-hexanol (0.091 g, 0.5 mmol) was added. The resulting mixture was stirred at 60 °C for 5h, after then the mixture was poured in water, extract with AcOEt (3 x 5 mL) and washed with saturated $NaHCO_3$ aqueous solution (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. Yield 68%. ^{1}H NMR (DMSO-d_6) δ 1.43 (m, 6H, CH_2), 1.67 (m, 4H, CH_2) 1.72 (m, 2H, CH_2), 2.67 (s, 2H, CH_2), 2.80 (s,

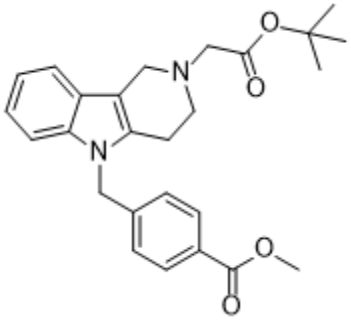
2H, CH_2), 2.97 (s, 2H, CH_2), 3.87, (s, 3H, OCH_3), 5.29, (s, 2H, CH_2) 7.02 (d, *J* = 8.0 Hz, 2H, Ar), 7.11 (m, 3H, Ar), 7.41 (m, 1H, Ar), 7.88 (d, *J* = 7.5 Hz, 2H, Ar), 12.13 (s, 1H, OH). Elemental analysis: calculated for $C_{26}H_{32}N_2O_3$ (420.54) %C, 74.26, %H 7.67, %N 6.66, found %C 74.18, %H 7.64, %N 6.68. M/z 421.

N-Hydroxy-4-((2-(6-hydroxyhexyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzamide (10c)



Following the procedure described for **10a** the title compound was obtained in 47% yield. ^{1}H NMR (DMSO-d_6) 1.46 (m, 6H, CH_2), 1.65 (m, 4H, CH_2) 1.70 (m, 2H, CH_2), 2.66 (s, 2H, CH_2), 2.84 (s, 2H, CH_2), 3.01 (s, 2H, CH_2), 5.33, (s, 2H, CH_2) 7.10 (d, *J* = 8.0 Hz, 2H, Ar), 7.16 (m, 3H, Ar), 7.38 (m, 1H, Ar), 7.91 (d, *J* = 7.5 Hz, 2H, Ar), 9.21 (s, 1H, NH), 11.68, (s, 1H, OH) 12.13 (s, 1H, OH). Elemental analysis: calculated for $C_{25}H_{31}N_3O_3$ (421.53) %C, 71.23, %H 7.41, %N 9.97, found %C 71.30, %H 7.40, %N 10.00. M/z 422.

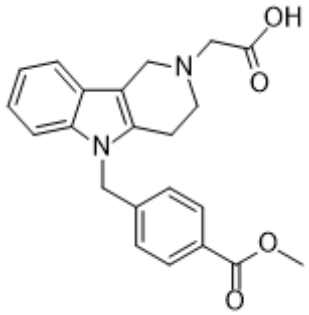
Methyl 4-((2-(2-(tert-butoxy)-2-oxoethyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (12)



To a suspension of methyl 4-((3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (17.84 g, 50 mmol) in DMF (20mL) under argon, TEA (20 mL, 120 mmol) was added, followed by tert-butyl 2-bromoacetate (9.75 g, 50 mmol). The resulting mixture was heated to 60°C for 8h, after that the mixture was poured in water, extract with AcOEt (3 x 5 mL) and washed with saturated $NaHCO_3$ aqueous solution (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. Yield 68%. ^{1}H NMR (DMSO-d_6) 1.55 (s, 9H, $3CH_3$), 2.81 (s, 2H, CH_2) 3.09 (s, 2H, CH_2), 3.48 (s, 2H, CH_2), 3.90 (s, 3H, OCH_3), 3.95 (s, 2H, CH_2), 5.32, (s, 2H, CH_2) 7.07 (d, *J* = 8.5 Hz, 2H, Ar), 7.18 (m, 3H, Ar), 7.48 (m, 1H, Ar), 7.95 (d, *J* = 8.0 Hz, 2H,

Ar). Elemental analysis: calculated for $C_{26}H_{30}N_2O_4$ (434.53) %C, 71.87, %H 6.96, %N 6.45, found %C 71.95, %H 6.93, %N 6.43. M/z 435.

2-(5-(4-(Methoxycarbonyl)benzyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-2(5*H*)-yl)acetic acid (13)

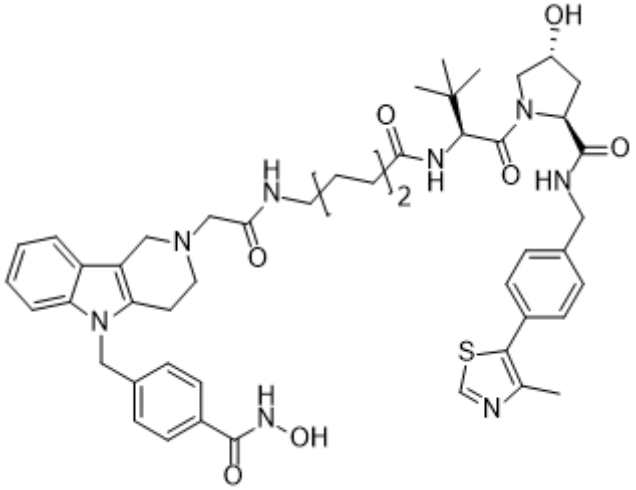


To a solution of methyl 4-((2-(2-(tert-butoxy)-2-oxoethyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (1.08 g, 25 mmol) in DCM (20 mL), then TFA (15 mL) was added dropwise and the solution was stirred at r.t. overnight. The solvent was removed under reduced pressure and the resulting solid was filtered off and washed with Et_2O to obtain the pure compound. Yield 88%. ^{1}H NMR (DMSO-d_6) 3.15 (s, 2H, CH_2), 3.79 (s, 2H, CH_2) 3.87 (s, 3H, OCH_3), 4.36 (s, 2H, CH_2), 4.68 (s, 2H, CH_2), 5.57 (s, 2H, CH_2), 7.11 (d, *J* = 8.0 Hz, 2H, Ar), 7.22 (m, 3H, Ar), 7.49 (m, 1H, Ar), 7.94 (d, *J* = 8.0 Hz, 2H, Ar). Elemental analysis: calculated for $C_{22}H_{22}N_2O_4$ (378.42) %C, 69.83, %H 5.86, %N 7.40, found %C 69.74, %H 5.84, %N 7.44. M/z 379.

General procedure for the synthesis of compounds 15d-g

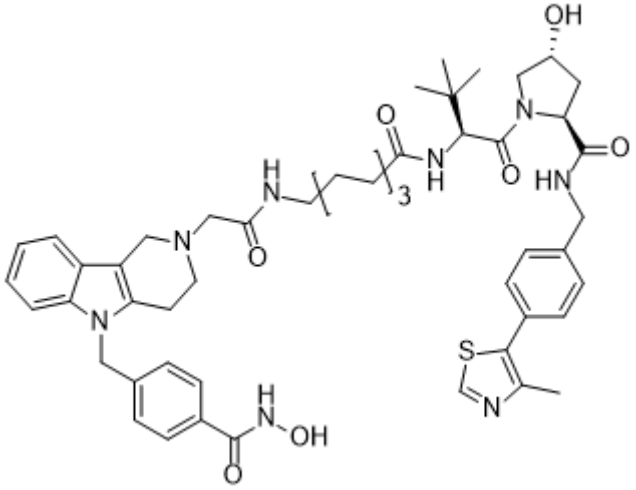
To a solution of 2-(5-(4-(methoxycarbonyl)benzyl)-3,4-dihydro-1*H*-pyrido[4,3-b]indol-2(5*H*)-yl)acetic acid (0.156 g, 0.5 mmol) in DMF (2mL), EDCI (0.095 g, 0.5 mmol) and HOBt (0.075 g, 0.5 mmol) were added and the resulting mixture was stirring for 15 minutes, then a solution of **14d-g** (0.5 mmol,) in DMF (2 mL) was added. The reaction mixture was stirred at rt overnight and monitored by TLC. The reaction was quenched with saturated $NaHCO_3$ aqueous solution, extract with AcOEt (3 x 5 mL) and washed with saturated $NaHCO_3$ aqueous solution (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. The resulting intermediate was dissolved in 1,4-dioxane (5 mL) then KCN (0.035 g, 0.5 mmol) and 50% aqueous NH_2OH (1 mL) were added. The reaction mixture was stirred at rt overnight and monitored by TLC. The solution was quenched with saturated $NaHCO_3$ solution, extract with AcOEt (3 x 5 mL) and washed with $NaHCO_3$ (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum to give the title compounds.

(2*S*,4*R*)-4-Hydroxy-1-((*S*)-2-(6-(2-(5-(4-(hydroxycarbamoyl)benzyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-2(5*H*)-yl)acetamido)hexanamido)-3,3-dimethylbutanoyl)-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (15d)



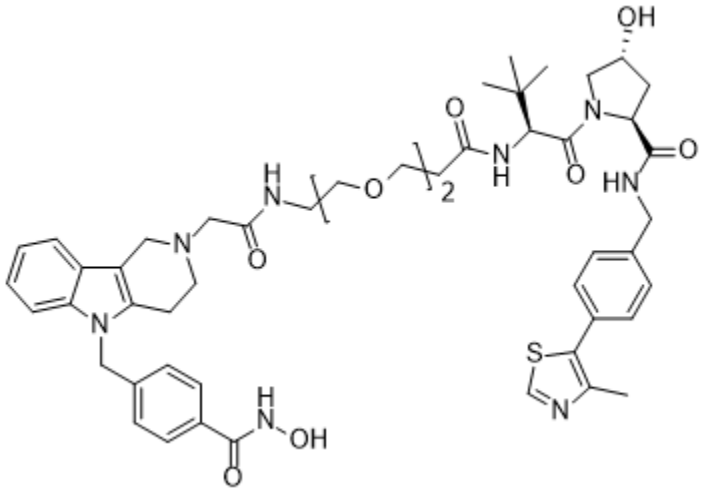
Following the general procedure, the title compound was obtained in yield 56%. ^{1}H NMR (DMSO-d_6) δ 0.79 (s, 9H, 3CH_3), 0.84 (s, 3H, CH_3), 1.79 (m, 10H, CH_2), 1.95 (s, 2H, CH_2), 2.22 (m, 4H, CH_2), 2.69 (s, 2H, CH_2), 2.88 (m, 1H, CH), 2.91 (m, 2H, CH_2), 3.03 (m, 1H, CH), 3.23 (m, 4H, CH_2), 3.78 (s, 2H, CH_2), 5.23, (s, 2H, CH_2) 6.12 (m, 1H, Ar), 7.00 (d, *J* = 8.5 Hz, 2H, Ar), 7.16 (m, 2H, Ar), 7.37 (m, 4H, Ar), 7.44 (m, 1H, Ar), 7.94 (d, *J* = 8.5 Hz, 2H, Ar), 8.11 (s, 1H, NH), 8.27 (s, 1H, NH) 8.69 (s, 2H, NH), 9.81 (s, 1H, OH) 10.12 (s, 1H, OH). Elemental analysis: calculated for $C_{50}H_{61}N_7O_7S$ (905.12) %C, 65.02, %H 6.68, %N 12.38, found %C 65.09, %H 6.66, %N 12.35. M/z 906.

(2*R*,4*S*)-4-Hydroxy-1-((*R*)-2-(7-(2-(5-(4-(hydroxycarbamoyl)benzyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-2(5*H*)-yl)acetamido)heptanamido)-3,3-dimethylbutanoyl)-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (15e)



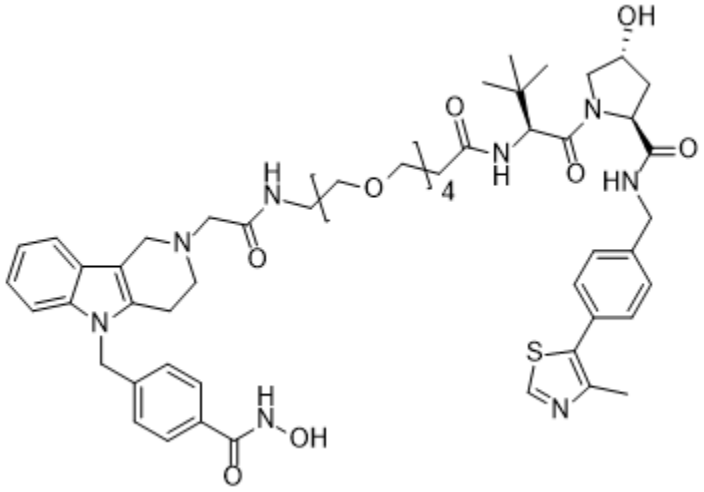
Following the general procedure, the title compound was obtained in yield 48%. ^{1}H NMR (DMSO-d_6): δ 0.76 (s, 9H, 3CH_3), 0.88 (s, 3H, CH_3), 1.82 (m, 12H, CH_2), 1.93 (s, 2H, CH_2), 2.22 (m, 4H, CH_2), 2.66 (s, 2H, CH_2), 2.89 (m, 1H, CH), 2.92 (m, 2H, CH_2), 3.07 (m, 1H, CH), 3.28 (m, 4H, CH_2), 3.75 (s, 2H, CH_2), 5.27, (s, 2H, CH_2) 6.08 (m, 1H, Ar), 7.05 (d, *J* = 8.5 Hz, 2H, Ar), 7.19 (m, 2H, Ar), 7.34 (m, 4H, Ar), 7.42 (m, 1H, Ar), 7.96 (d, *J* = 8.0 Hz, 2H, Ar), 8.16 (s, 1H, NH), 8.29 (s, 1H, NH) 8.72 (s, 2H, NH), 9.93 (s, 1H, OH) 10.17 (s, 1H, OH). Elemental analysis: calculated for $C_{50}H_{61}N_7O_7S$ (919.14) %C, 65.34, %H 6.80, %N 12.19, found %C 65.27, %H 6.83, %N 12.16. M/z 920.

(2*S*,4*R*)-1-((*S*)-2-(Tert-butyl)-14-(5-(4-(hydroxycarbamoyl)benzyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-2(5*H*)-yl)-4,13-dioxo-6,9-dioxa-3,12-diazatetradecan-1-oyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (15f)



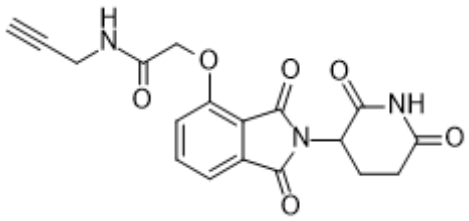
Following the general procedure, the title compound was obtained in yield 66%. ^{1}H NMR (DMSO-d_6): δ 0.88 (s, 9H, 3CH_3), 0.95 (s, 3H, CH_3), 1.92 (s, 2H, CH_2), 2.12 (m, 4H, CH_2), 2.39 (m, 8H, CH_2) 2.77 (s, 2H, CH_2), 2.84 (m, 1H, CH), 2.85 (m, 2H, CH_2), 2.92 (m, 1H, CH), 3.26 (m, 4H, CH_2), 3.55 (s, 2H, CH_2) 3.74 (s, 2H, CH_2), 5.26, (s, 2H, CH_2) 6.19 (m, 1H, Ar), 7.08 (d, *J* = 8.5 Hz, 2H, Ar), 7.21 (m, 2H, Ar), 7.30 (m, 4H, Ar), 7.38 (m, 1H, Ar), 7.87 (d, *J* = 8.0 Hz, 2H, Ar), 8.19 (s, 1H, NH), 8.24 (s, 1H, NH) 8.79 (s, 2H, NH), 9.85 (s, 1H, OH) 10.22 (s, 1H, OH). Elemental analysis: calculated for $C_{49}H_{60}N_8O_9S$ (937.11) %C, 62.80, %H 6.45, %N 11.96, found %C 62.89, %H 6.47, %N 11.93. M/z 938.

(2*S*,4*R*)-1-((*S*)-2-(tert-butyl)-20-(5-(4-(hydroxycarbamoyl)benzyl)-3,4-dihydro-1*H*-pyrido[4,3-b]indol-2(5*H*)-yl)-4,19-dioxo-6,9,12,15-tetraoxa-3,18-diazaicosan-1-oyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (15g)



Following the general procedure, the title compound was obtained in yield 73%. ^{1}H NMR (DMSO-d_6): δ 0.88 (s, 9H, $3CH_3$), 0.92 (s, 3H, CH_3), 1.99 (s, 2H, CH_2), 2.17 (m, 4H, CH_2), 2.38 (m, 16H, CH_2) 2.75 (s, 2H, CH_2), 2.85 (m, 1H, CH), 2.91 (m, 2H, CH_2), 2.99 (m, 1H, CH), 3.21 (m, 4H, CH_2), 3.54 (s, 2H, CH_2) 3.77 (s, 2H, CH_2), 3.91 (s, 3H, OCH_3), 5.24, (s, 2H, CH_2) 6.09 (m, 1H, Ar), 7.11 (d, J = 7.5 Hz, 2H, Ar), 7.19 (m, 2H, Ar), 7.37 (m, 4H, Ar), 7.50 (m, 1H, Ar), 7.98 (d, J = 7.0 Hz, 2H, Ar), 7.99 (s, 1H, NH), 8.66 (s, 2H, NH), 9.82 (s, 1H, OH). Elemental analysis: calculated for $C_{54}H_{69}N_7O_{11}S$ (1025.22) %C, 63.32, %H 6.79, %N 9.57, found %C 63.40, %H 6.77, %N 9.54. M/z 1026.

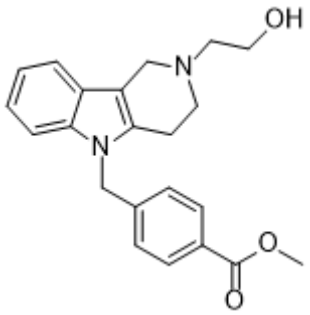
2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)-N-(prop-2-yn-1-yl)acetamide (18)



To a solution of 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetic acid (16) (3.22 g, 10 mmol) in DMF (10 mL), EDCI (1.91g, 10 mmol) and HOBt (1.35 g, 10 mmol) were added and the resulting mixture was stirred for 15 minutes, then propargylamine (17) (0.550 g, 10 mmol) in DMF (5mL) was added. The reaction mixture was stirred at rt overnight and monitored by TLC. The reaction was quenched with saturated $NaHCO_3$ aqueous solution, extract with AcOEt (3 x 5 mL) and washed with saturated $NaHCO_3$ aqueous solution (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. Yield 87%. ^{1}H NMR (DMSO-d_6) δ 2.75 (s, 1H, CH), 2.91 (s, 2H, CH_2), 3.99 (m, 2H, CH_2), 4.86 (m, 2H, CH_2), 5.16 (m, 1H, CH), 7.41 (d, *J* = 8.0 Hz, 1H, Ar), 7.52 (d, *J* = 7.5 Hz, 1H, Ar), 7.84 (m, 1H, Ar), 7.98 (s, 1H,

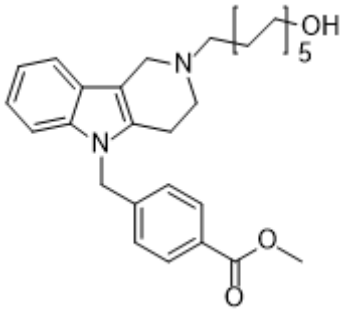
NH), 8.44 (s, 1H, NH). Elemental analysis: calculated for $C_{18}H_{15}N_3O_6$ (369.33) %C 58.54, %H 4.09, %N 11.38, found %C 58.48, %H 4.07, %N 11.35. M/z 370.

Methyl 4-((2-(2-hydroxyethyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (15h)



To a suspension of methyl 4-((3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (0.35 g, 1 mmol) in DMF (4 mL), 3-bromo-1-ethanol (0.124 g, 1 mmol) was added. The resulting mixture was stirred at 60°C for 5h, after that the mixture was poured in water, extract with AcOEt (3 x 5 mL) and saturated $NaHCO_3$ aqueous solution (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. Yield 87%. ^{1}H NMR (DMSO-d6) δ 1.49 (m, 4H, CH_2), 1.77 (m, 2H, CH_2), 2.85 (s, 2H, CH_2), 2.92 (s, 2H, CH_2), 3.84, (s, 3H, OCH_3), 5.27, (s, 2H, CH_2) 7.08 (d, *J* = 8.0 Hz, 2H, Ar), 7.18 (m, 3H, Ar), 7.49 (m, 1H, Ar), 7.92 (d, *J* = 8.5 Hz, 2H, Ar), 12.9 (s, 1H, OH). Elemental analysis: calculated for $C_{22}H_{24}N_2O_3$ (364.44) %C, 72.50, %H 6.64, %N 7.69, found %C 72.59, %H 6.62, %N 7.66. M/z 365.

Methyl 4-((2-(11-hydroxyundecyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (15j)



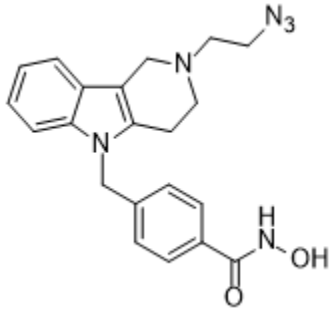
To a suspension of methyl 4-((3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)benzoate (0.35 g, 1 mmol) in DMF (4 mL), 11-bromo-1-undecanol (0.49 g, 1 mmol) was added. The resulting mixture was stirred at 60°C for 5h, after that the mixture was poured in water, extract with AcOEt (3 x 5 mL) and washed with saturated $NaHCO_3$ aqueous solution (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum. Yield 87%. ^{1}H NMR (DMSO-d_6): δ 1.63 (m, 22H, CH_2), 1.77 (m, 2H, CH_2), 2.89 (s, 2H, CH_2), 3.06 (s, 2H, CH_2), 3.88, (s, 3H, OCH_3), 5.22, (s, 2H, CH_2) 7.05 (d, *J* = 8.5 Hz, 2H, Ar), 7.21 (m, 3H, Ar), 7.44 (m, 1H, Ar),

7.90 (d, *J* = 8.5 Hz, 2H, Ar), 12.98 (s, 1H, OH). Elemental analysis: calculated for $C_{31}H_{42}N_2O_3$ (490.68) %C, 75.88, %H 8.63, %N 5.71, found %C 75.95, %H 8.61, %N 5.69. M/z 491.

General procedure for the synthesis of compounds (16h-j)

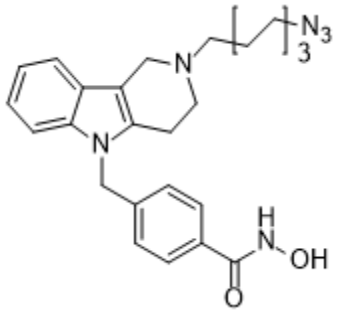
To a stirred solution of 15h-j (0.5 mmol) in dry DCM (3 mL), Et_3N (0.14 mL, 1 mmol) was added and the mixture was cooled to 0 °C. Methanesulfonyl chloride (0.11 g, 1 mmol) was added dropwise and the solution was stirred for 5 h at 24 °C. The solvent was removed under vacuum and the crude material was dissolved in DMF (2 mL) and treated with NaN_3 (0.73g, 1.2 mmol). The mixture was stirred at 90 °C for 6 h then KCN (0.035 g, 0.5mmol) and 50% aqueous NH_2OH (1 mL) were added. The reaction mixture was stirred rt overnight and monitored by TLC. The solution was quenched with saturated $NaHCO_3$ solution, extract with AcOEt (3 x 5 mL) and washed with $NaHCO_3$ (3 x 5 mL) and brine. The organic layer was dried over with anhydrous $MgSO_4$ and evaporated under vacuum to give the titled compounds.

4-((2-(2-Azidoethyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)-N-hydroxybenzamide (16h)



Following the general procedure, the title compound was obtained in yield 48%. ^{1}H NMR (DMSO-d_6): δ 1.88 (m, 2H, CH_2), 2.39 (m, 4H, CH_2), 2.88 (s, 2H, CH_2), 2.97 (s, 2H, CH_2), 5.29, (s, 2H, CH_2) 7.11 (d, *J* = 7.0 Hz, 2H, Ar), 7.22 (m, 3H, Ar), 7.56 (m, 1H, Ar), 7.83 (d, *J* = 6.5 Hz, 2H, Ar), 8.75 (s, 1H, NH), 11.23 (s, 1H, OH). Elemental analysis: calculated for $C_{21}H_{22}N_6O_2$ (390.44) %C, 64.68, %H 5.68, %N 21.25, found %C 64.62, %H 5.66, %N 21.19. M/z 391.

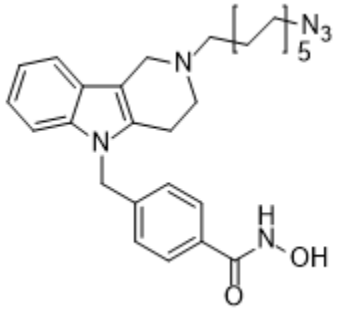
4-((2-(6-Azidohexyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)-N-hydroxybenzamide (16i)



Following the general procedure, the title compound was obtained in yield 52%. ^{1}H NMR (DMSO-d_6): δ 1.96 (m, 2H, CH_2), 2.44 (m, 12H, CH_2), 2.82 (s, 2H, CH_2), 3.05 (s, 2H, CH_2), 5.62, (s, 2H,

CH_2) 7.09 (d, *J* = 7.5 Hz, 2H, Ar), 7.24 (m, 3H, Ar), 7.55 (m, 1H, Ar), 7.88 (d, *J* = 7.0 Hz, 2H, Ar), 8.72 (s, 1H, NH), 11.28 (s, 1H, OH). Elemental analysis: calculated for $C_{25}H_{30}N_6O_2$ (446.54) %C, 67.24, %H 6.77, %N 18.82, found %C 67.31, %H 6.79, %N 18.86. M/z 447.

4-((2-(11-Azidoundecyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)-N-hydroxybenzamide (16j)

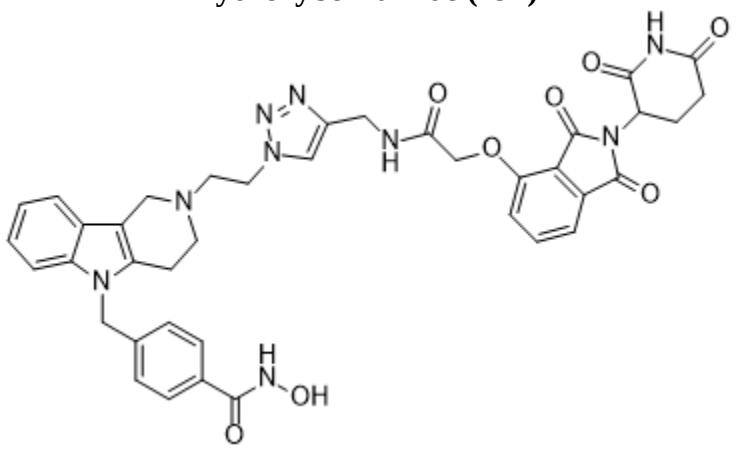


Following the general procedure, the title compound was obtained in yield 34%. ^{1}H NMR (DMSO-d_6) δ 1.96 (m, 2H, CH_2), 2.44 (m, 22H, CH_2), 2.63 (s, 2H, CH_2), 3.08 (s, 2H, CH_2), 5.70, (s, 2H, CH_2) 7.12 (d, *J* = 7.5, 2H, Ar), 7.28 (m, 3H, Ar), 7.60 (m, 1H, Ar), 7.84 (d, *J* = 7.0 2H, Ar), 8.68 (s, 1H, NH), 11.33 (s, 1H, OH). Elemental analysis: calculated for $C_{30}H_{40}N_6O_2$ (516.68) %C, 69.74, %H 7.80, %N 16.27, found %C 69.68, %H 7.83, %N 16.23. M/z 517.

General procedure for compounds (19h-j)

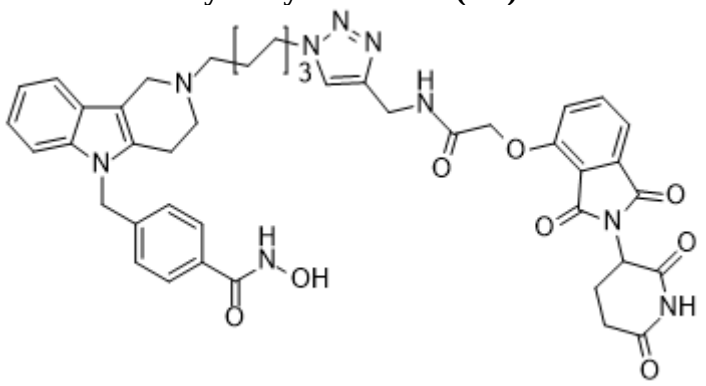
To a stirred solution of CuI (0.01 g, 0.05 mmol) in dry THF (2 mL), TTA (0.014 g 0.05 mmol) was added and the corresponding mixture was stirred at rt for 40 minutes. After that, 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)-N-(prop-2-yn-1-yl)acetamide 18 (0.185 g, 0.5 mmol) and the corresponding azide 16h-j (0.5 mmol) were added and the mixture was stirred at rt for 6 h. The THF was removed under vacuum and the crude material was dissolved in DMSO and purified via RP-HPLC (solvent A = H_2O, 0.05% TFA; solvent B = MeCN, 0.05% TFA; flow rate = 15.0 mL/min; method: gradient: 5% B to 30% B [over 4 min], then 30% B to 60% B [over 8 min], then 0% B to 95% B [over 4 min]).

4-((2-(2-(4-((2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamido)methyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)-*N*-hydroxybenzamide (19h)



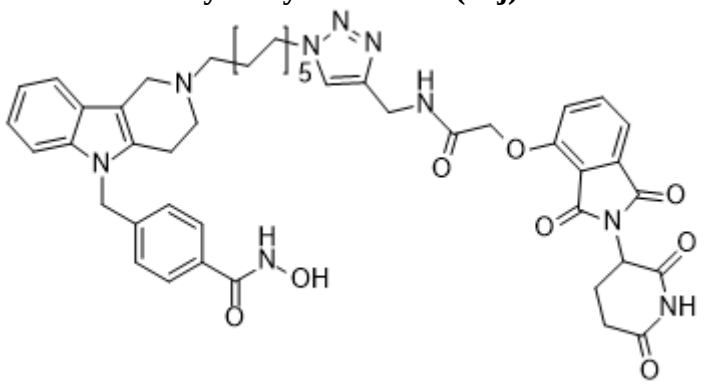
Following the general procedure, the title compound was obtained in yield 28%. ^{1}H NMR (DMSO-d_6): δ 1.91 (m, 2H, CH_2), 2.44 (m, 4H, CH_2), 2.94 (m, 4H, CH_2), 3.08 (s, 2H, CH_2), 3.84 (m, 4H, CH_2), 4.68 (s, 1H, CH), 4.84 (m, 2H, CH_2), 5.17 (m, 2H, CH_2), 5.33 (s, 1H, CH), 7.09 (d, *J* = 7.5 Hz, 2H, Ar), 7.24 (m, 3H, Ar), 7.44 (d, *J* = 8.0 Hz, 1H, Ar), 7.55 (m, 2H, Ar), 7.82 (m, 3H, Ar), 7.90 (s, 1H, NH), 8.48 (s, 1H, NH), 8.79 (s, 1H, NH), 11.28 (s, 1H, OH). Elemental analysis: calculated for $C_{39}H_{37}N_9O_8$ (759.77) %C, 61.65, %H 4.91, %N 16.59, found %C 61.59, %H 4.89, %N 16.63. M/z 760.

4-((2-(6-(4-((2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamido)methyl)-1*H*-1,2,3-triazol-1-yl)hexyl)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indol-5(2*H*)-yl)methyl)-*N*-hydroxybenzamide (19i)



Following the general procedure, the title compound was obtained in yield 33%. ^{1}H NMR (DMSO-d_6): δ 1.88 (m, 2H, CH_2), 2.40 (m, 12H, CH_2), 2.87 (m, 4H, CH_2), 3.04 (s, 2H, CH_2), 3.82 (m, 4H, CH_2), 4.66 (s, 1H, CH), 4.80 (m, 2H, CH_2), 5.19 (m, 2H, CH_2), 5.32 (s, 1H, CH), 7.12 (d, *J* = 7.5 Hz, 2H, Ar), 7.26 (m, 3H, Ar), 7.42 (d, *J* = 8.0 Hz, 1H, Ar), 7.50 (m, 2H, Ar), 7.84 (m, 3H, Ar), 7.96 (s, 1H, NH), 8.49 (s, 1H, NH), 8.77 (s, 1H, NH), 11.30 (s, 1H, OH). Elemental analysis: calculated for $C_{43}H_{45}N_9O_8$ (815.87) %C, 63.30, %H 5.56, %N 15.45, found %C 63.25, %H 5.58, %N 15.41. M/z 816.

4-((2-(11-(4-((2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamido)methyl)-1*H*-1,2,3-triazol-1-yl)undecyl)-3,4-dihydro-1*H*-pyrido[4,3-b]indol-5(2*H*)-yl)methyl)-N-hydroxybenzamide (19j)



Following the general procedure, the title compound was obtained in yield 24%. ^{1}H NMR (DMSO-d_6) δ 1.94 (m, 2H, CH_2), 2.46 (m, 22H, CH_2), 2.85 (m, 4H, CH_2), 3.08 (s, 2H, CH_2), 3.84 (m, 4H, CH_2), 4.70 (s, 1H, CH), 4.82 (m, 2H, CH_2), 5.11 (m, 2H, CH_2), 5.31 (s, 1H, CH), 7.15 (d, *J* = 8.0 Hz, 2H, Ar), 7.29 (m, 3H, Ar), 7.45 (d, *J* = 8.5 Hz, 1H, Ar), 7.58 (m, 2H, Ar), 7.80 (m, 3H, Ar), 8.02 (s, 1H, NH), 8.51 (s, 1H, NH), 8.69 (s, 1H, NH), 11.26 (s, 1H, OH). Elemental analysis: calculated for $C_{48}H_{55}N_9O_8$ (886.01) %C, 65.07, %H 6.26, %N 14.23, found %C 65.13, %H 6.24, %N 14.28. M/z 887.

4.4 References

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5.0 Conclusion

In this thesis I studied different series of compounds using three important approaches in drug discovery: the single target approach, the multitarget approach and the PROTAC approach. Using the single target approach, Carbonic Anhydrase inhibitors were synthetized, and several compounds selectively inhibited the cancer related isoforms hCAIX and hCAXII in low nanomolar range. In the first series, arylthiazolin-4-one derivatives was synthetized, showing that the 3,4,5-trimethoxyphenyl and the naphtyl derivatives are the best hCAIX inhibitors, with Ki 17.6 nM and 20.9 nM respectively. A small library of N^1-acetylpyrazoline sulfamates were synthetized, showing that the position of sulfamate moiety on 5 or 3-aryl ring is strictly correlated with the inhibitory activity: the sulfamic group on the 3- or 4- position of the 5-aryl is necessary to selectively inhibit hCAIX and hCAXII, with an electron-withdrawing group on the 4-postion of the 3-aryl ring. Furthermore, two series of benzensulfonamide derivatives bearing carbonyl ureido and thioureido moieties were designed and the SAR analysis showed that the presence at 4-position of fluorine atom or methyl group gave the most potent hCAIX inhibitors. The 4-methoxybenzyl derivative displayed the best inhibitory activity on hCAXII (6.4 nM), resulting about 7-fold more selective as compared to both hCAII and hCAIX inhibitory activity. The 2,6-substituted compounds showed interesting inhibitory profile and selectivity against both the cancer related isoform as compared to hCAII inhibitory activity. These studies were further combined with the development of Steroid Sulfatase inhibitors to obtain two series of dual CA/STS inhibitors. In the first series the 3-methylphenyl derivative showed inhibitory activity against hCA IX at sub nanomolar levels, with Ki 0.91 nM, and a good STS residual activity (10.4% ± 1.9) while the 2,3-dimethylphneyl derivative displayed STS residual activity better than **7** (5.9% ± 0.8) and it is also endowed with inhibitory activity against hCAXII at low nanomolar levels (Ki 1.0 nM). In the second series the 3,4-trimethoxyphenyl derivative displayed Ki 24.2 nM against hCAIX and STS residual activity of 19.0% ± 3.1, while the benzofuran-2-yl derivative showed inhibitory activity at low nanomolar range against both hCA IX (6.7 nM) and hCA XII (1.0 nM) and STS residual activity of 4.7% ± 0.7. These compounds that showed interesting dualistic properties may be attractive for potential *in vivo* evaluation. Furthermore, polyphenols-based hydrazones bearing benzofuran, indole and benzimidazole scaffolds were studied, displaying antioxidant and photoprotective activity. The SAR data obtained from DPPH, FRAP and ORAC assays, showed an interesting correlation between the number and the position of hydroxyl groups on arylidene moiety and the antioxidant activity, as well as the presence of 2-hydroxy-4-diethylamino group. Furthermore, mono-hydroxylated compounds, as well as the activity of the 2-hydroxynaphtyl and 2-hydroxy-4-diethylamino compounds, comparable with the photoprotective capabilities of commercial PBSA sunscreen filter.

These multitarget compounds also showed promising antiproliferative activity due to *in vitro* results on human melanoma Colo38 and erythroleukemic K562 cell lines. Among them, the 2-hydroxy-4-diethylamino derivatives showed the best multitarget activity, with good antioxidant, photoprotective and antiproliferative properties. Starting from the good results of arylhydrazones compounds, a new series of 2-arylbenzimidazole derivatives were synthesized in the context of multitarget study. Results from antioxidant assays showed that the presence of a sulfonic acid at 5-position of benzimidazole scaffold, is the least favourable whereas benzimidazole bearing carboxyl or cyano groups in the same position showed various antioxidant activity. The best antioxidant compounds were investigated for their photoprotective activity, showing that the presence of two hydroxyl groups and 2-hydroxy-4-diethylamino groups is correlated with good photoprotective activity. Selected compounds were also tested against Colo38 cell line showing interesting antiproliferative activity. To conclude, a new approach in drug discovery was performed, the PROTAC approach. New Tubastatin-based PROTACs bearing VHL and CEREBLON as E3 ligases were prepared with the aim of promote degradation of Histone Deacetilase 10 a new important target in Neuroblastoma treatment. Results from BRET test showed that activity of the inhibitors is correlated with the length of the linkers and that selected compounds may be good candidates for the *in-vitro* tests to evaluate their ability to promote the degradation of HDAC10

6.0 Acknowledgment

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