UNIVERSITÀ DEGLI STUDI DI CATANIA

International PhD in Basic and applied Biomedical Sciences XXX Cycle

---

Dott.ssa GIULIA MONCIINO

Synthesis and biological evaluation of pirrolidine, oxazolidin-2-imine and mercaptoketones derivates as HCV, HIV and HDAC inhibitors

Coordinatore:
Chiar.mo Prof. FERDINANDO NICOLETTI

Relatore:
Chiar.mo Prof. UGO CHIACCHIO

2014-2017

INDEX

PREFACE

During the period of my PhD I developed a project that is part of a more extensive program carried out over the years at the Faculty of Pharmacy of the University of Catania by Prof. Ugo Chiacchio's team, with the collaboration of several researchers from the same University and Prof. Pedro Merino of the University of Zaragoza.

The main objective of this thesis is to investigate the value of various aromatic systems such as antiviral agents, specifically RNA viruses, and those that belong to the families of *Flaviviridae* and *Retroviridiidae*.

Among them there are pathogens responsible for diseases with a high epidemiological impact, such as HCV (cause of type C disease), that is also diffused in Europe. But above all, agents like the HIV virus which are responsible for diseases characterized by a high degree of mortality and related illnesses very common in the countries of the so-called Third World, for which there is no adequate and effective therapeutic treatment, so far. Over the years, some vaccines have been created, but these obviously may be used only for prior prevention and are ineffective in the case of already infected individuals.

My thesis deals with the development and synthesis of new inhibitors of viral replication, which interfere with non-structural proteins of HCV virus ((NS) NS3/4A, NS4B, NS5A and NS5B) and the design, synthesis and biological evaluation of a new series of compounds, where structural elaboration has been performed towards inhibiting different targets of HIV-1.

However, during my PhD research I also focused my interest on the study of novel HDAC Inhibitors. Histone deacetylases (HDACs) have a crucial role in the remodeling of chromatin, and are involved in the epigenetic regulation of gene expression. Recently, inhibition of HDACs came out as a target for correcting epigenetic changes associated with cancer and other diseases.

HDAC enzyme are overexpressed and associated with oncogenes transcription factors, mutated in cancer and it has been observed that high levels of HDACs are involved in carcinogenesis. HDACi already investigated and tested, showed a potent inhibitory activity in the growth of cell lines of different histological nature and in animal tumors. They have shown low toxicity, both in vitro and in vivo, against healthy cells; despite they induce hyper acetylation both in healthy cells and in tumor cells.

Moreover, the selective inhibition of some classes of HDAC is involved in the process of eradication of HIV infection as some HDACi showed effects on the latent pools of HIV in infected persons; because they are expressed in the nucleus of the CD4 + T cells and are associated with the promoter of HIV in cellular cell lines of latency.

RNA- VIRUS

Among the molecules able to fight infection caused by RNA virus belong more than 350 human pathogen agents and most of the aetiological agents of emerging or re-emerging disease.

Recently the genomic and structural characterization of RNA virus has been taken into account.

RNA viruses of clinical interest can be divided into three main groups according to the nature of the genome.

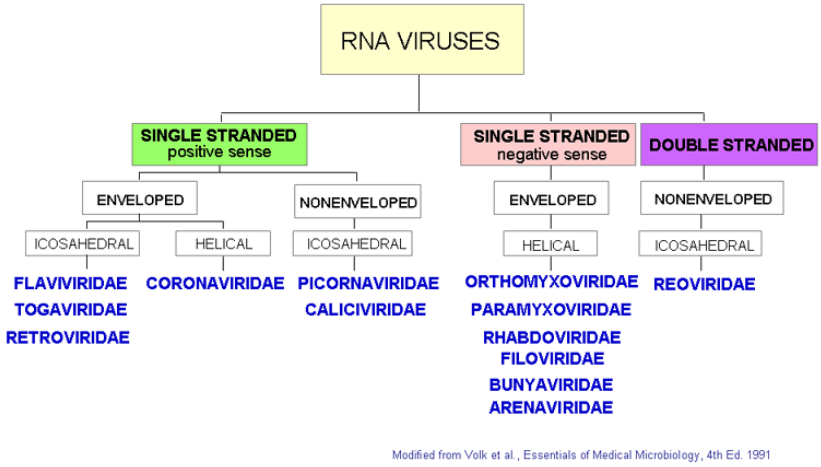


Figure 1

Positive-sense single-stranded virus: A positive-sense single-stranded RNA virus (or (+)ssRNA virus) is a virus that uses positive sense, single-stranded RNA as its genetic material. Single stranded RNA viruses are classified as positive or negative

depending on the sense or polarity of the RNA. The positive-sense viral RNA genome can also serve as messenger RNA and can be translated into protein in the host cell. They are included in Group IV of the Baltimore classification. Among them, the family of *Flaviviridae* made up of about 80 pathogenic virus including some emerging agents such as Yellow fever, Encephalitis virus, Hepatitis C etc.

To the same group belong the Retroviridae family, a retrovirus class with isometric capside and genome consisting of two positive polarities monocatenary RNA molecules. The most known being the human immunodeficiency virus (HIV), indicated as the aetiologic agent of AIDS.

Negative-sense single-stranded virus: A negative-sense single-stranded RNA virus (or (-)ssRNA virus) uses negative sense single-stranded RNA as its genetic material. The negative viral RNA is complementary to the mRNA and need to be converted to a positive RNA by RNA polymerase before translation. Consequently, the purified RNA of a negative sense virus is not infectious by itself but it needs to be converted to a positive sense RNA for replication. These viruses are comprised in Group V of the Baltimore classification. The *Filoviridae* and the *Paramyxoviridae* makes part of this group, the former being responsible mainly for Ebola virus and the latter being responsible for some important human disease such as mumps, measles, and respiratory syncytial virus (RSV), which is the major cause of bronchiolitis and pneumonia in infants and children. Furthermore the *Orthomyxoviridae* **is** an important negative-sense single-stranded virus family, that

includes that includes seven genera: Influenza virus A (that causes all flu pandemics), Influenza virus B, Influenza virus C, Influenza virus D, Isavirus, Thogotovirus and Quaranjavirus.

Double-stranded RNA viruses: are a diverse group of viruses that vary widely in host range (humans, animals, plants, fungi, and bacteria), genome segment number (one to twelve) and virion organization (T-number, capsid layers or turrets). Rotaviruses are includes in this group, are globally known as a common cause of gastroenteritis in young children, and bluetongue virus, an economically important pathogen of cattle and sheep.

The *Reoviridae* family is the largest and most diverse in terms of host range.

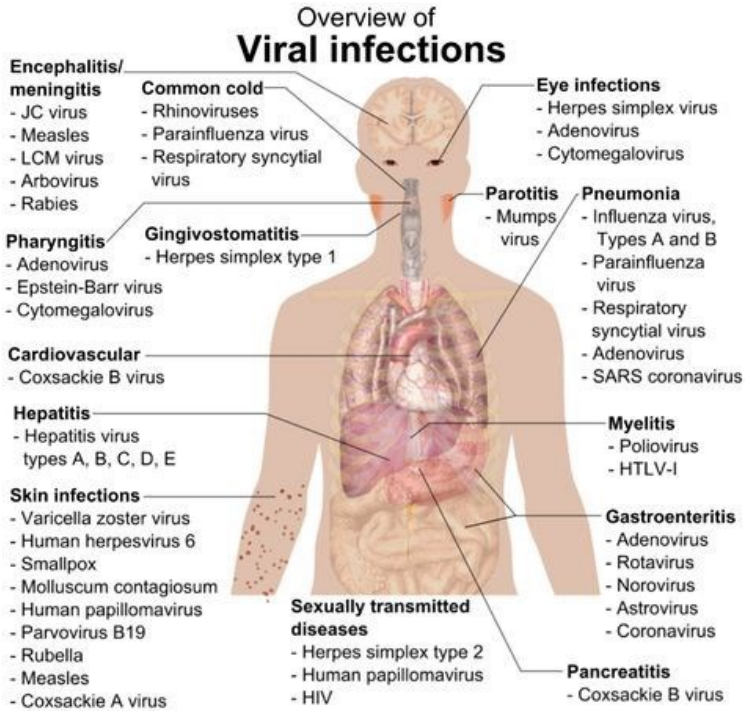


Figure 2

ANTIVIRAL DRUGS

Antiviral drugs already on the market or in the trial phase act with different mechanisms and have been designed and synthesized to interact with specific phases of viral replication. These can be divided, according to their chemical structure, into three major classes: nucleoside analogues, nucleotide analogues and non-nucleoside derivatives.

Also, according to their mechanism of action, they can be classified in reverse transcriptase inhibitors, protease inhibitors, polymerase inhibitors, viral coating blockers, viral mRNA capping blockers etc.

Unfortunately, currently for most of them the therapy only helps to reduce the severity of viral infection, but with new therapies it is must be highlighted that, for some patients, antiviral treatments are a cure.

The key to developing a new antiviral is the selectivity of its action. A successful drug must interfere with a specific viral function or with a cellular function implicate in the replication. At last researchers face with pharmacokinetic problems cause ideally a drug should be water-soluble, chemically and metabolically stable, easily absorbable and at the same time non-toxic, carcinogenic, mutagenic or teratogenic.[1]

2. HEPATITIS C

Hepatitis C is a virus that can infect the liver. The responsible agent of the affection is a small (55–65 nm in size), enveloped, positive-sense single-stranded RNA virus belonging to the genus *Hepacivirus* and relating to the family *Flaviviridiae*[2].

If left untreated, it cause serious and potentially life-threatening damage to the liver over many years.

However, with modern treatments the infection can be cure and most people will have a normal life expectancy.

The infection can be spread in various ways include: post transfusional factors, with needles and syringes, vertical transmission[2].

Hepatitis C often does not have any noticeable symptoms until the liver has been significantly damaged, many people have the infection without realising it, and symptoms can be mistaken for another condition. Symptoms can include: flu-like symptoms, such as muscle aches and a high temperature (fever) feeling tired all the time loss of appetite abdominal (tummy) pain feeling and being sick. The only way to know if these symptoms are caused by hepatitis C is to get tested.

HCV

The hepatitis C virus (Figure 3) is made up of a small spherical particle and consists of a *core* containing genetic information surrounded by an icosahedral protective shell of protein, and further encased in a lipid envelope of cellular origin. Two viral envelope glycoproteins, E1 and E2, are embedded in the lipid envelope.

Core and envelope ,together, form the nucleocapsid containing the viral genome capable of encoding for a polyprotein which due to processes performed by viral and host proteases, gives rise to structural proteins of the envelope and nucleocapside (encoded by genes E1 and E2), and non-structural or functional proteins that play an important role in viral replication. Functional proteins include RNA polymerase RNA dependent, helicase and viral protease. (Figure 4)

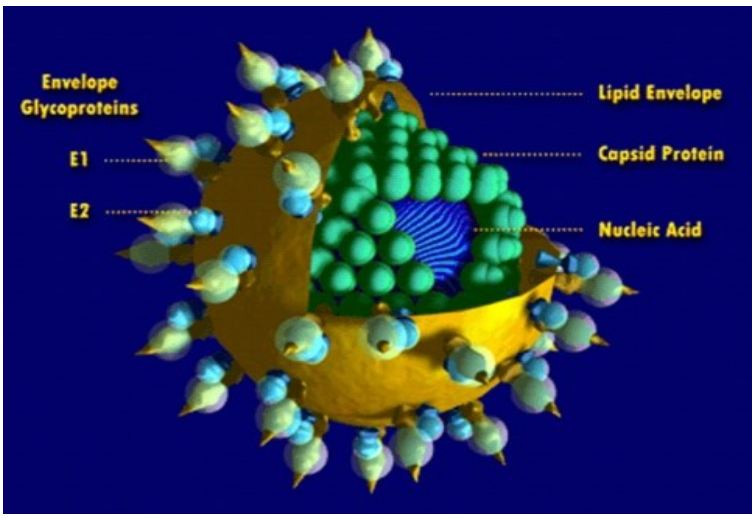


Figure 3

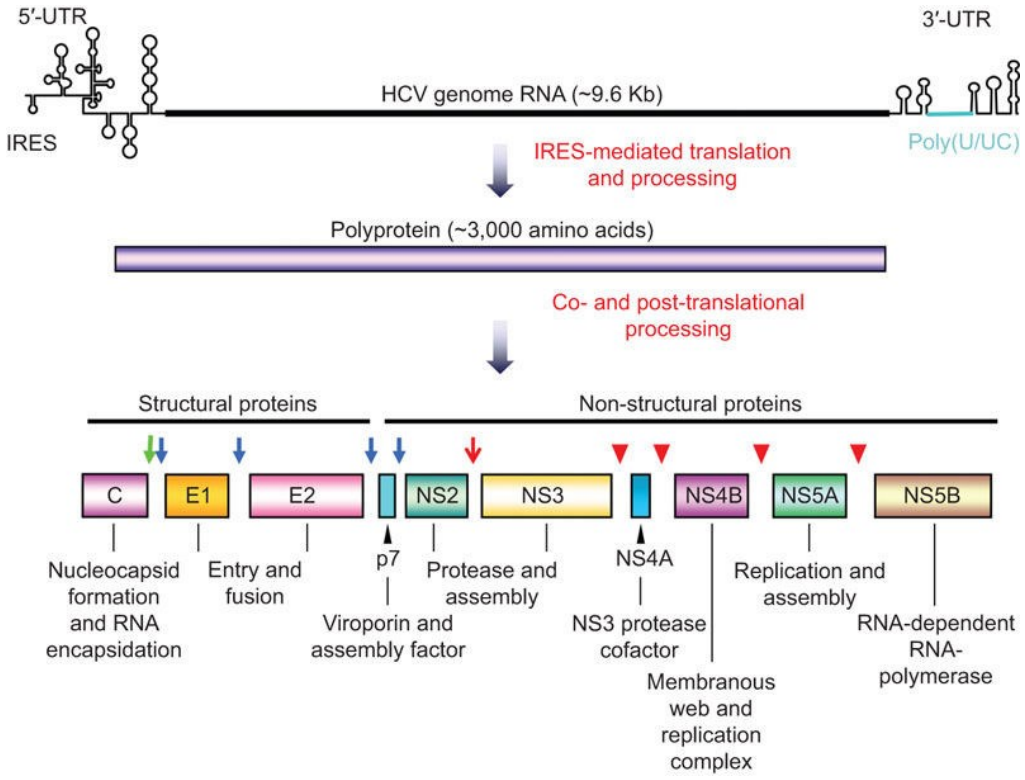


Figure 4

HCV is an RNA virus, subject to cytosolic replication that undergoes to frequent and spontaneous mutations, which involve different segments of the genome. These mutations make the viral population so polymorphic and genetically wide to give the virus the connotation of "*almost species"*, that is, the ability of the infecting agent's to generate, to evade immunological pressure of the infected person, a diverse set of genomic forms that share a dominant sequence that is conserved and a myriad of mutations[4-.6]

These features of HCV involve a series of biological effects as the resistance that it is able to develop against the drugs used in the treatment of the pathology and the enormous difficulty in setting the appropriate vaccine.

The most important property of the HCV is the great variability of the genomic sequence. Because of this genetic heterogeneity, the viral isolates that greatly differ in their genomic sequence have been divided into *types* or *genotypes*. Within each genotype isolated viruses, as they were not able to suggest a classification, were grouped into various "*subtypes*". According to the classification proposed by Simmonds in 1993, the different HCV isolates should be included in one of the six distinct genotypes (indicated with Arabic numbers), comprising 18 subtypes (indicated by letters, tiny, of the alphabet).

Therefore, HCV is indicated as 1a, 1b, 2a, 2b, 3, 4, 5 and 6. The geographical distribution of the different genotypes of HCV appear to be extremely variable. In Italy and Europe, as well as in the United States, there is a clear prevalence of genotype 1, and in particular genotype 1b. Genotype 3 is predominantly spread in India, while genotype 4 appears to be mainly spread across the African continent and in particular, in Zaire and Egypt; genotype 5 is the most common in South Africa where it is responsible for over 50% of cases of infection; genotype 6 and its subtypes have, however, more spreading in Asia (Table 1).[11]

Table 1. Geographical distribution of HCV clades, genotypes and prominent subtypes

HCV clade	Genotype	Subtype	Geographical distribution
1	1	a	United States, Northern Europe
		b	Global distribution
2	2	a	North America, Europe and Japan
		b	North America, Europe and Japan
		c	Northern Italy
3	3	a	Indian Subcontinent, Europe and United States
	10		Indonesia
4			North Africa and Middle East
5			South Africa
6	6		Hong Kong and Southeast Asia
	7		Vietnam, Thailand, Indonesia and Burma
	8		Vietnam, Indonesia and Burma
	9		Vietnam, Thailand, Indonesia and Burma
	11		Indonesia

FIGURE 1: Data quality scores (by country) and HCV genotype distribution by Global Burden of Disease Regions

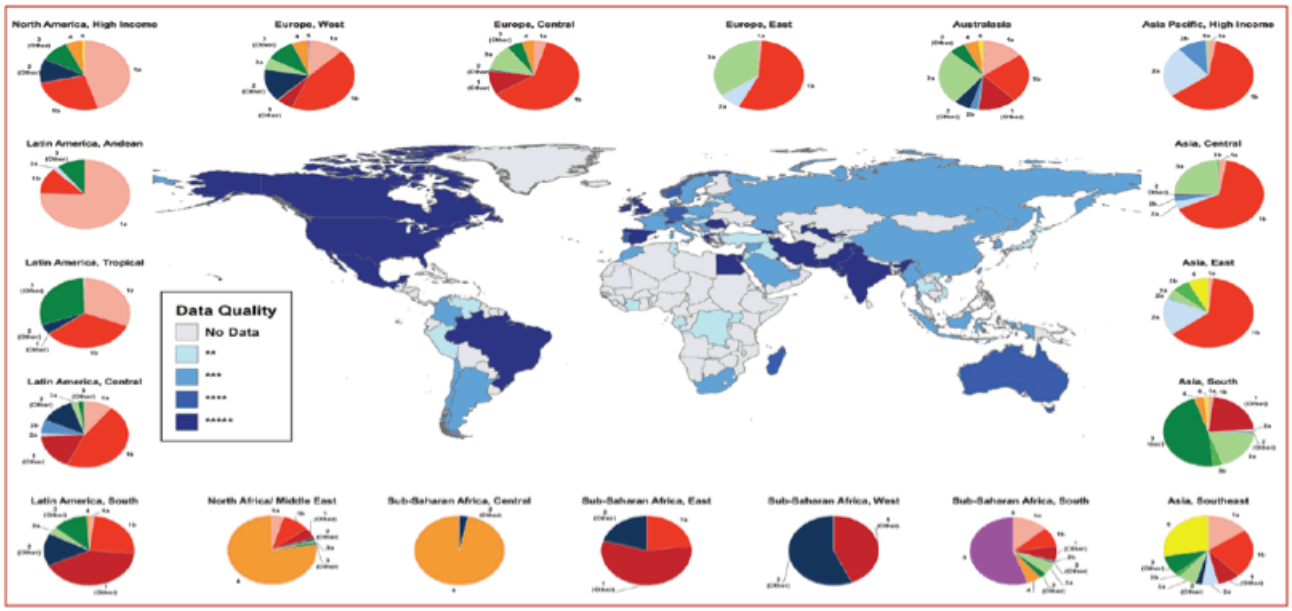


Figure 5

2.1 Mechanism of cell infection

HCV once bound to the specific hepatocellular receptor by its antigen, penetrates into the hepatocyte and begins to replicate causing flogosis and liver death.

The infectivous agent of HCV is transmitted parenterally, more frequently via horizontally than via vertically and the main contagion vehicles are: infected needles and syringes, inadequately sterilized surgical instruments, sexual transmission, and in the past through transfusion[4].

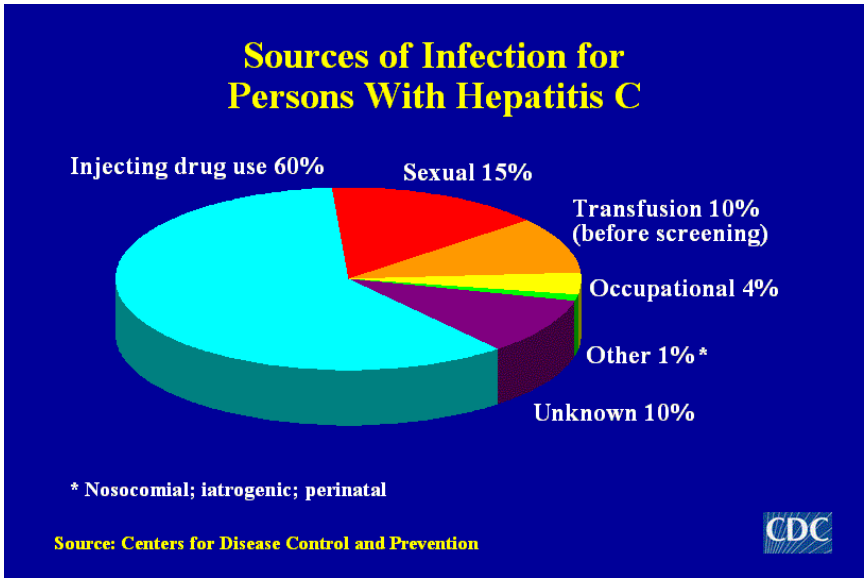


Figure 6

2.2 Therapy

There are lots of studies regarding new therapies to treat hepatitis C. After more than a decade in which the only available therapy was based on the dual therapy with IFN, PEG-IFN and Ribavirin in recent years a revolution is taking place in the treatment of

this disease with new molecules that will ensure the elimination of interferon first and then the Ribavirin

New antiviral drugs have been approved from FDA in the last two years. The molecules in dual or in a 3D therapy, initially with interferon or ribavirin, are Sofosbuvir, Simeprevir, Daclatasvir, that inhibit the enzyme RNA-dependent RNA polymerase (NS5B), the NS3 protease and NS5A respectively.

In the second half of the nineties, the only available therapy was IFN-α in monotherapy, with limited efficacy and a cost-benefit ratio not beneficial. The first therapy used was IFN-α pegylated (PEG-IFN) and Ribavirin (RBV) for 6 to 12 months depending on the genotype and patient's response to the treatment and represented the Standard of Care (SoC) for about fifteen years.

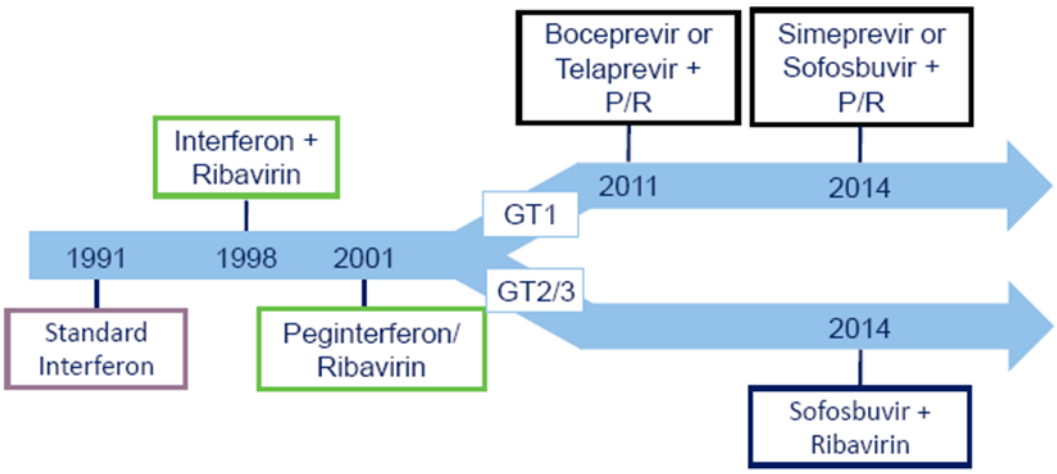


Figure 7

In 2011, new Direct Acting Antiviral (DAA) anti-HCV drugs were introduced on the market to be associated with PEG-IFN and RBV therapy. These molecules were introduced with the names of Boceprevir (Victrelis®, MSD Italy) and Telaprevir (Incivo®, Janssen Cilag SpA) and represent the first Generation of DAA anti-HCV.

Currently, DAA are generally divided into four main classes of drugs:

• Viral protease inhibitors (NS3) inhibitors acting on the NS5B protein of the viral polymerase protein complex distinguished by:

• nucleoside and

• non-nucleoside

• inhibitors acting on NS5A protein of the viral polymerase protein complex.

These drugs have the same mechanism of action: they are inhibitors of HCV protease NS3; bounding in a covalent but reversible way, to the active site of the NS3 protease; they stop viral replication in host cells and thus act differently from IFN and RBV. There are several ways to administer: Boceprevir (BOC) is introduced after 4 weeks of "induction" with PEG-IFN and RBV, and it is continued in combination with double therapy for 24, 32 or 44 weeks, as appropriate. Telaprevir (TEL), on the other hand, is administered in association with SoC from the start, for a total duration of 12 weeks followed by another 12 or 36 weeks of dual therapy.

Sofosbuvir is an antiviral drug that inhibits the enzyme "RNA-dependent RNA polymerase (NS5B), essential for hepatitis C virus replication. The commercial name

is SOVALDI® and is available as tablets. It can only be used for the treatment of chronic hepatitis C in adult patients.

The substantial difference between sofosbuvir and other drugs used to eliminate the hepatitis C virus (such as Peg-IFN and ribavirin) is that this new drug acts directly against the virus, blocking the replication process. Interferon, on the other hand, stimulates the immune system's response to the virus and ribavirin indirectly interferes with viral replication. Combined intake of these drugs increases the possibility of definitive virus eradication. Sofosbuvir cannot be taken in monotherapy but should be taken with ribavirin with or without PEG-interferon in double or triple therapy. It can an also be administered in combination with other DAA (daclatasvir and simeprevir) with or without ribavirin. The dose of sofosbuvir should not be reduced.

Simeprevir is a new antiviral drug that inhibits serine protease (NS3 / 4A). The trade name is OLYSIO® and is available in the form of capsules. Like the Sofusbuvir the combined therapy with other drugs, can increase the possibility of definitive virus eradication.

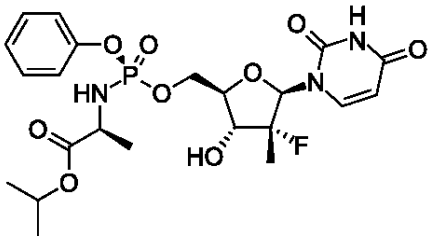
It can be taken exclusively with pegylated interferon and / or ribavirin, or in addition to another antiviral since the first day of treatment, forming a dual or triple therapy.

When considering the treatment with simeprevir in combination with peginterferon alfa and ribavirin in HCV genotype 1a patients, before starting treatment is strongly recommended a test to evaluate the presence of the virus with NS3 Q80K polymorphism.

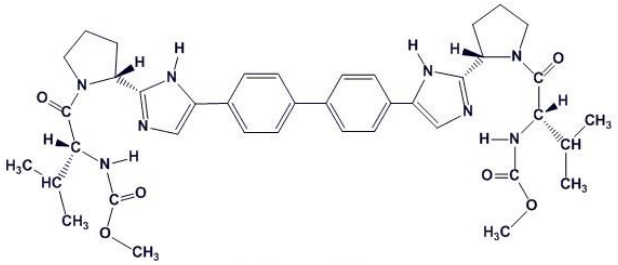
Daclatasvir is a potent inhibitor of NS5A complex for hepatitis C virus replication. The commercial name is DAKLINZA®; its use is recommended in combination with other antivirals and in particular sofosbuvir, with or without ribavirin, for the treatment of patients with genotype 1, 3, 4 and in particular cases in genotype 2 patients.

It is similar to other new generation antivirals and acts directly against the virus, blocking the replication process. In some cases, the combination of daclatasvir with ribavirin is recommended because that increase the possibility of a definitive virus eradication. When it is used in combination with sofosbuvir, it is a wholly orally interferon-free regime that in clinical trials provided healing rates of up to 100%, including patients with advanced liver disease, in genotype 3 and in those who had previously failed treatment with a protease inhibitor.

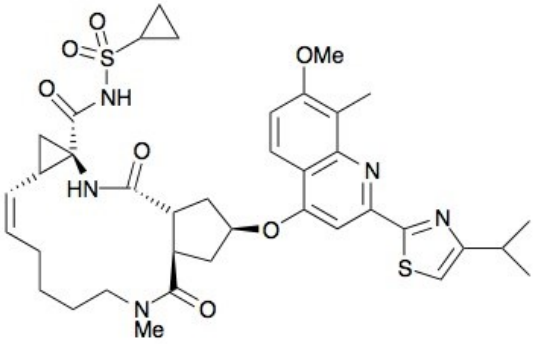
Daclatasvir is the first NS5A-approved European Union (EU) inhibitor and is used in combination with other medicines, ensuring shorter treatment duration (12 or 24 weeks) than 48 weeks of interferon-ribavirin-based regimens.



Sofusbovir



DACLATASVIR



Simeprevir

2.2.1 Drugs and their molecular targets

In order to design compounds with specific targeting, it is necessary to identify the main homologies between the replication steps of the virus and those of the host cell. As it happens with a large number of other antiviral agents nucleosides analogs, with direct action to the *Flaviviridiae,* converted into their triphosphate form by the action of kinases cell or viral induced should be potentially able to affect selectively certain enzymes of the replicative cycle of virus.

An interesting target is the non-structural protein NS5B, which is the most important enzyme in the replication of HCV, identified as RNA polymerase-RNA dependent

(RpRd)[14], as it plays a key role in catalyzing the synthesis of the negative and positive filament of the RNA[15].

Viral genome replication occurs at cytoplasmic level by a negative polarity RNA (said replication intermediate) produced by NS5B, hat acting as a mold to produce new positive polarity RNA molecules. These new positive RNA molecules can and may be used for the synthesis of new negative filaments, for the polypeptide expression, or for packing in the viral progeny.

In recent years, scientific literature has reported three different classes of compounds that interfere, by different mechanisms, with the activity of this enzyme.

1) The inhibitors that bind to the allosteric site of polymerase giving making it conformationally inactive. In this category there are different compounds with different chemical structure. However, due to frequent viral mutations, often occur resistance phenomena.

2) The inhibitors that bind to the pyrophosphate site. That compounds act on the active site of the polymerase chelating magnesium and manganese metal ions necessary in the process of elongation of the RNA chain.

3) Nucleoside inhibitors acting as competitive inhibitors and chain terminators. A modified nucleoside capable of interacting with the NS5B polymerase must be characterized by the presence of a ribose-like spacer group and in particular the presence on the C-2 'of a hydroxyl group or isosteric group with which it is essential for molecular recognition by the NS5B.

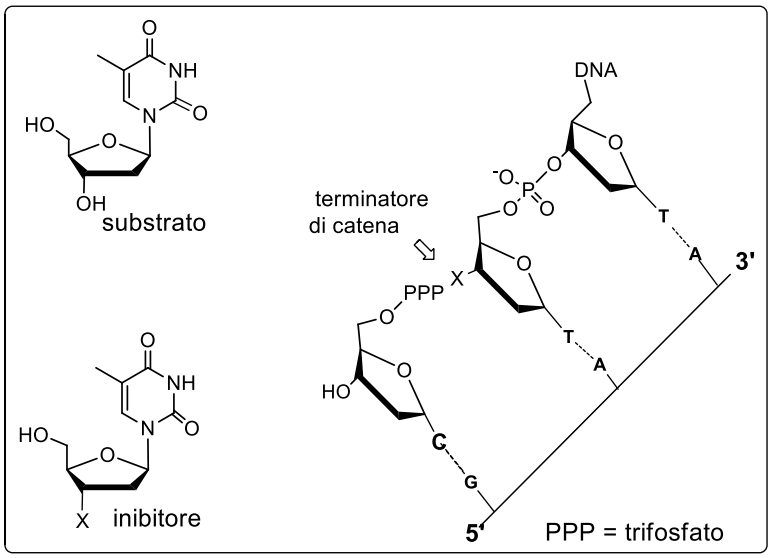

substrato
inibitore
terminatore
di catena
DNA
PPP
PPP = trifosfato
3'
5'

Figure 8

2.3 AZANUCLEOSIDES

Particular interest, during the PhD, has been addressed towards the synthesis and the biological activity of nucleoside analogues, in which structural modifications of the heterocyclic bases and/or the sugar moiety of natural nucleosides have been performed.[2-3]

In general, nucleosides, consisting of both a base moiety and a sugar moiety, are commonly defined as carbocyclic nucleosides, thionucleosides, phosphanucleosides and azanucleosides. Moreover, they are classified into two mayor divisions that are *N*-nucleosides and *C*-nucleosides. *N*-nucleosides consist of a bond between the anomeric carbon of the sugar moiety and the nitrogen of the nucleobase, while *C*-nucleosides are compounds having a bond between the anomeric carbon of the sugar and the carbon of the base portion[10].

The biological activity of nucleoside analogues showing antiviral properties is strictly linked to their conversion, through cellular enzymes, to the corresponding biologically active triphosphate form. It interacts with viral transcriptase or interferes with cell growth, slowing the cell cycle progression (Figure 9).

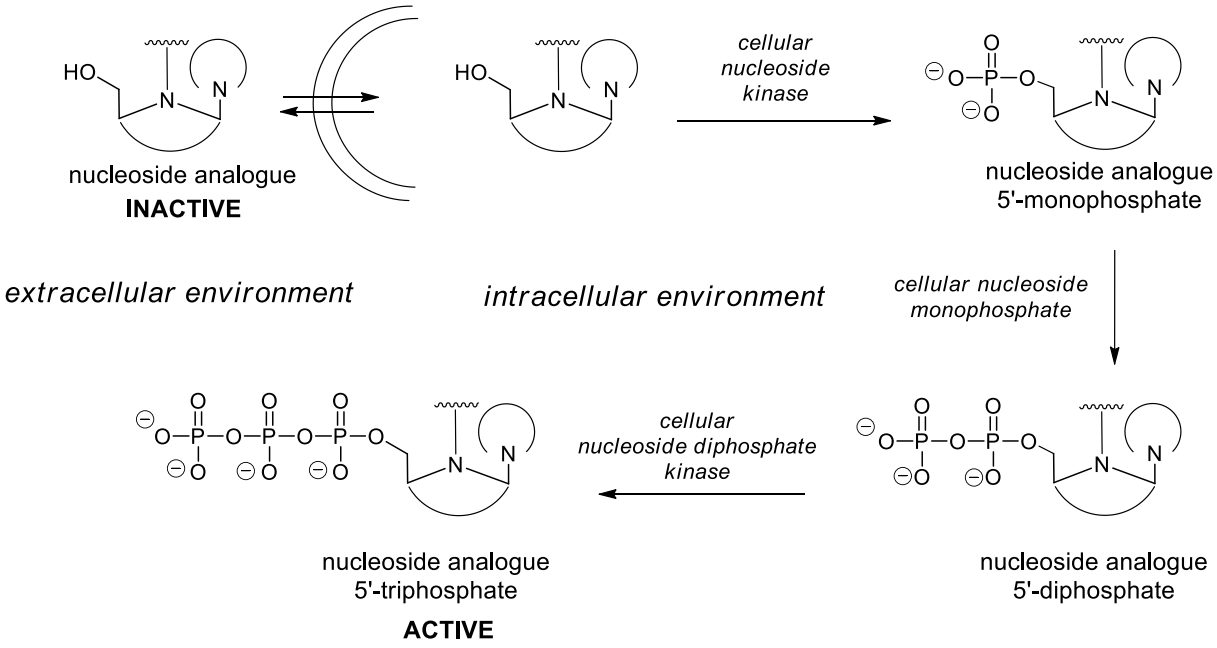


Figure 9

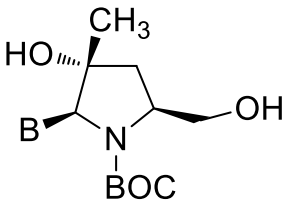
One of the metabolic drawbacks of nucleoside analogues is the retention of their stability following the triphosphorylation inside the host cell. Several strategies to overcome the initial selective phosphorylation step have been designed; in particular, phosphonate analogues, by miming the nucleoside monophosphates, overcome the instability of nucleotides towards phosphodiesterase and enhance the cellular uptake by bypassing the initial phosphorylation step.

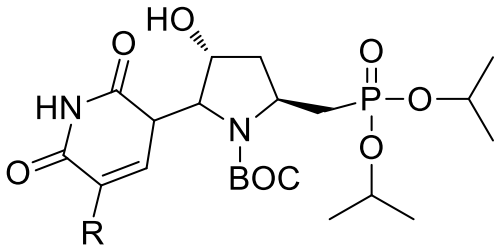
In this context, our research group has recently reported the synthesis of a new class of aza-nucleosides possessing low toxicity and interesting anti-HCV properties.

According to these studies , we have extended our interest to the synthesis of 3-alkyl aza-nucleosides such as *(3R,5S)-tert-butyl 3-hydroxy-5-(hydroxymethyl)-3-methyl-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)pyrrolidine-1-carboxylate* and to the synthesis of 5-phosphonated azanucleosides such as *(3R,5S)-tert-butyl 5-*

((diethylphosphoryl) methyl)-3-hydroxy-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)pyrrolidine-1-carboxylate , in order to evaluate their anticancer and antiviral activities. The rational of our choice is based on the consideration that the presence of a methyl group at C3 of the pyrrolidine moiety can enhance the lipophilicity of the molecule and the related permeability in the cell.

On the contrary, the presence of the phosphonic unit at C5 of the pyrrolidine ring can produce new compounds that can bypass the limiting monophosphorylation step, promoting the cellular uptake and leading to biologically active compounds.



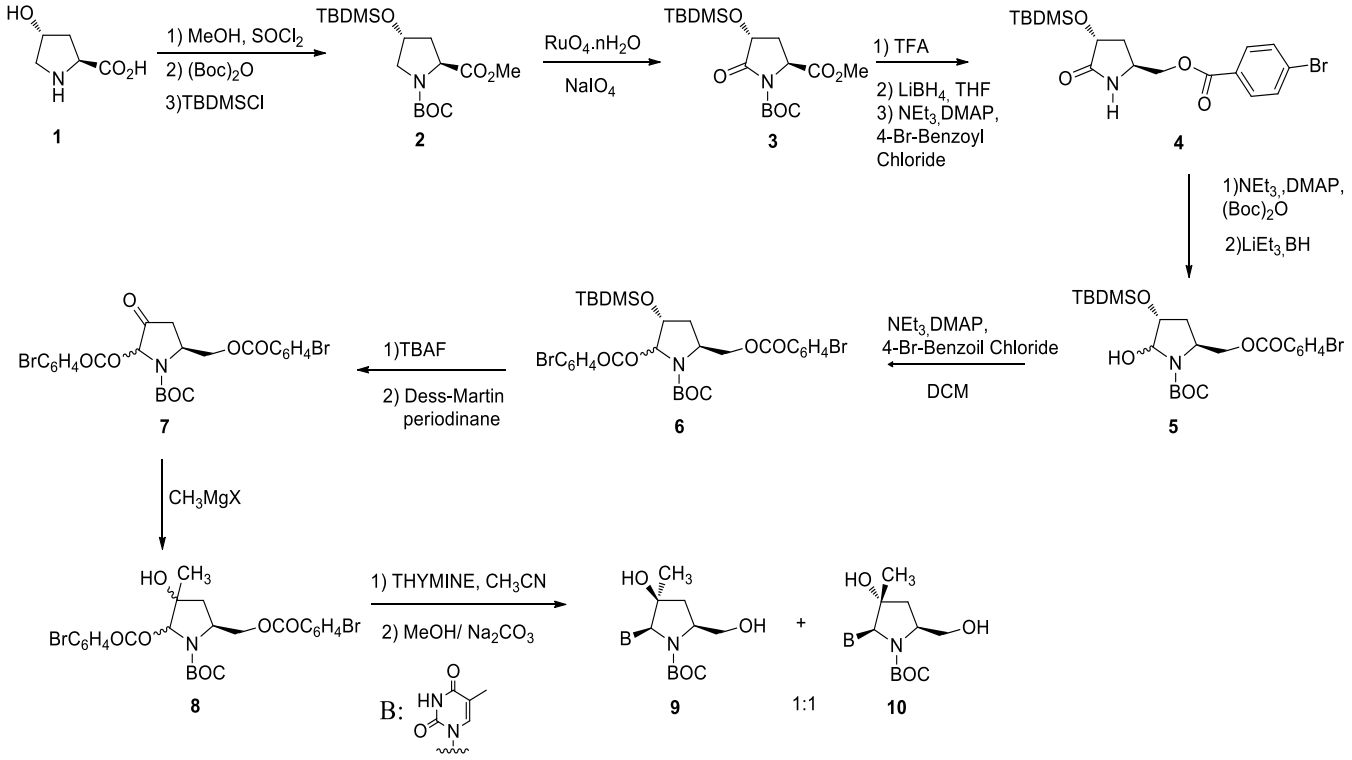


2.3.1 Chemistry

The synthetic approach toward *(3R,5S)-tert-butyl 3-hydroxy-5-(hydroxymethyl)-3-methyl-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)pyrrolidine-1-carboxylate* (**9**,**10**) reported in Scheme 1 is a multistep sequence which starts from *trans* 4-hydroxy- *L*-proline **1**. This compound was converted into **2** and oxidized with $RuO_4.nH_2O$ to **3**, further reaction with TFA, reduction with $LiBH_4$ and subsequently reaction with 4-Br-Benzoyl Chloride afforded the derivative **4.** Protection of amide group with BOC followed by reduction of carbonyl bond with Super Hydride gave a

mixture of **5**. Further reaction with 4-Br-Benzoyl Chloride afforded the O-Bz derivative **6**. TBAF treatment and Dess-Martin oxidation give rise to the formation of compound **7** which was converted to a diasteromeric mixture of C3 methylated derivative **8** by reaction with CH_3MgX. Nucleosidation with silylated Thymine followed by transesterification with MeOH/ NO_2CO_3 gave a mixture of compound **9** and **10** in a 1:1 ratio.

Both compounds obtained are under biological screening.

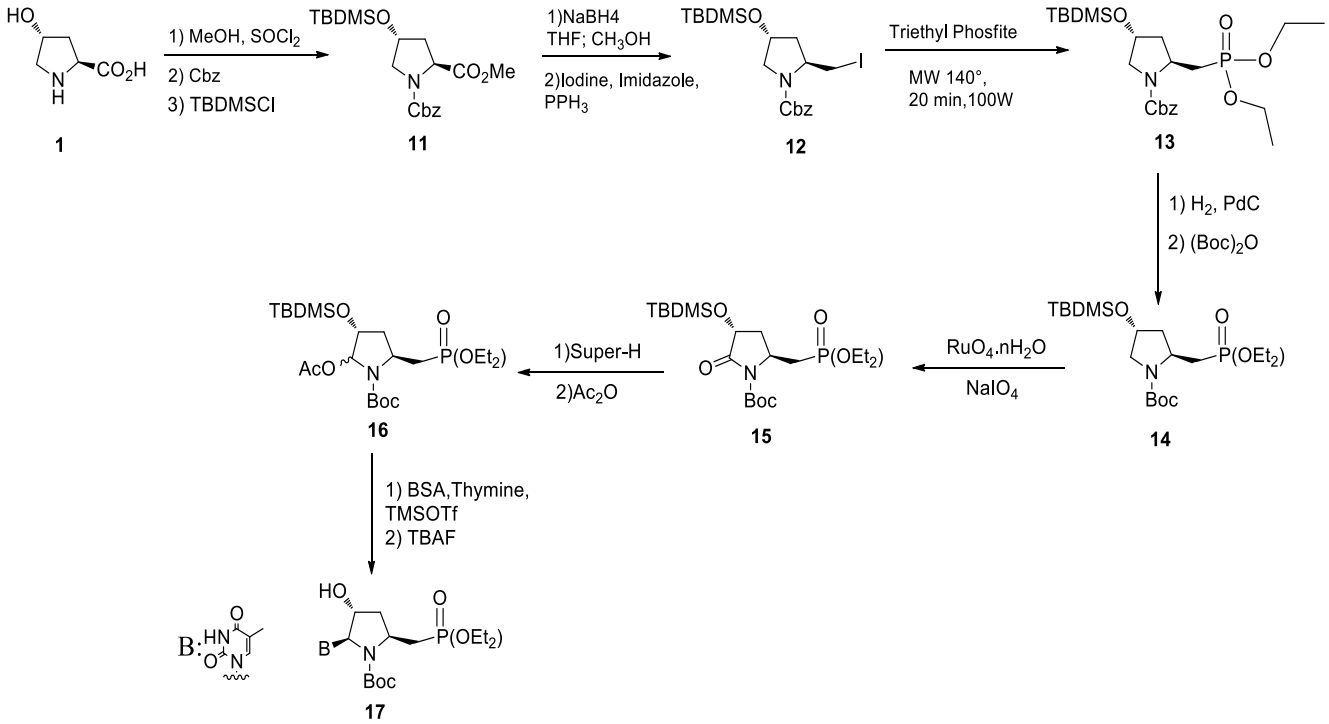


Scheme 1

The synthetic approach toward *(3R,5S)-tert-butyl 5-((diethylphosphoryl) methyl)-3-hydroxy-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)pyrrolidine-1-*

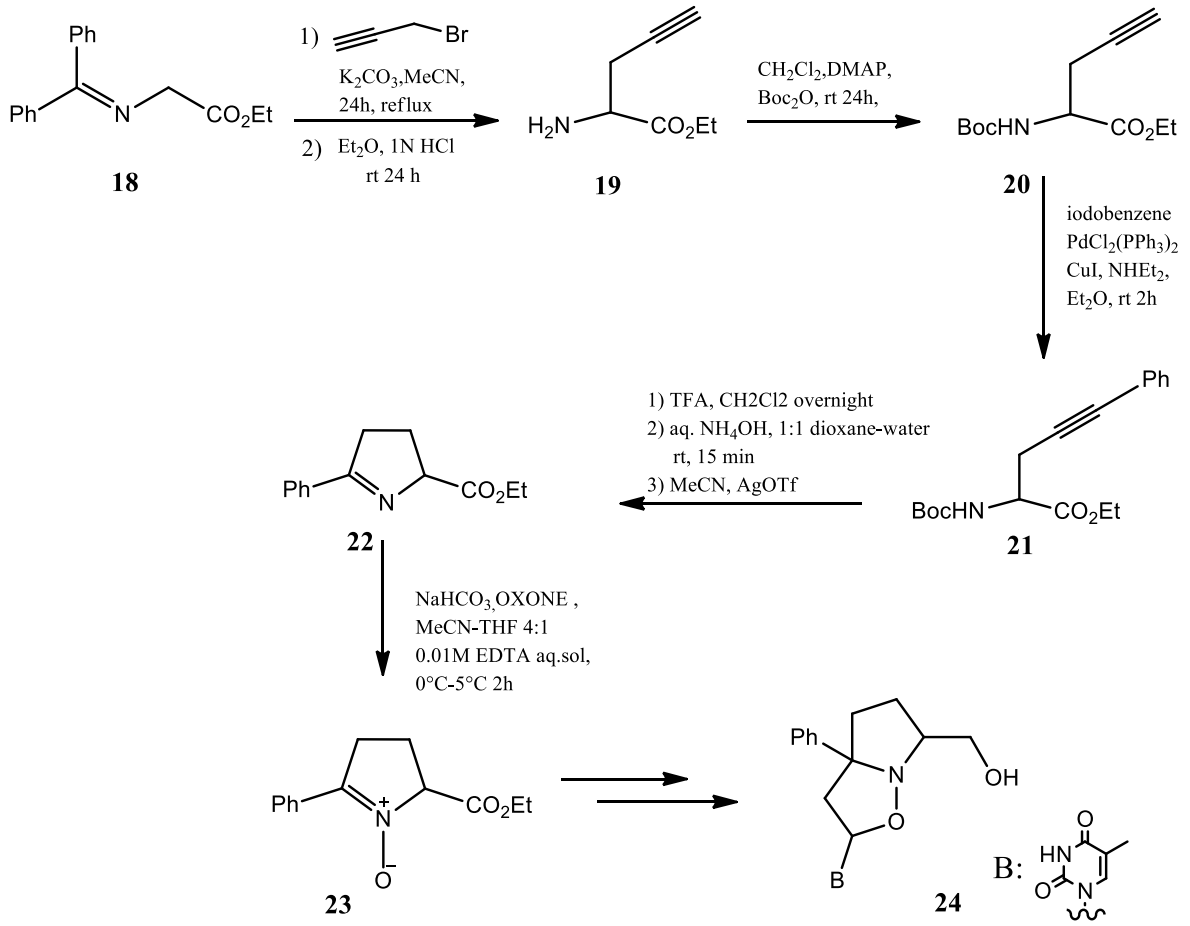
carboxylate reported in Scheme 2 is a multistep sequence which starts from *trans* 4-hydroxy- *L*-proline **1**. This compound was converted into **11**, thus was reduced with $NaBH_4$ in THF/MeOH to give the alcohol, and subsequently Michaelis-Arbuzov reaction with Iodine, imidazole and PPH_3 afforded the iodide derivative **12**. Therefore, the reaction of **12** with triethylphosphite was carried out without use of solvent under microwave irradiation (MW) at 140°C, 100 W for 20 minute to give the corresponding phosphonate **13**. The hydrogenolysis and then the subsequently protection of amide group with BOC gave **14**. This compound was oxidized to **15** with $RuO_4.nH_2O$ then reduced with Super Hydride and acetylated to diasteromeric mixture of acetylated aminals **16**. Finally, compound **16** was nucleosidated with silylated Thymine and converted to target compound **17** after treatment with TBAF.

This phosphonate derivative is under biological screening.



Scheme 2

With aim to obtain pyrrolidine skeleton, useful precursor of bicyclic of oxazanucleosides such us **7** , applying the methodology of 1,3 dipolar cycloaddition , part of my PhD thesis was spent at the Faculty of Sciences of the University of Zaragoza, to synthetize the nitrone **6** (Scheme 3).



Scheme 3

Thus starting from *N-(Diphenylmethylene)glicine ethylester* **18** nitrone **23** was obtained according to scheme 3. In particular compound **18** was reacted with propargyl bromide to give **19** by nucleophilic addition and by hydrolysis in Et_2O/HCl. BOC reaction followed by Sonogashira coupling reaction lead to **21** which was converted by intramolecular cyclization to pyrroline **22** and finally oxidized with oxone to give the expected nitrone **23**. Nitrone 23 was characterized according to its NMR spectral.

Materials and methods

All chemicals were purchased from Sigma–Aldrich Chemical Co. The solvent was removed at aspirator pressure using a rotary evaporator. TLC was performed with Merck precoated TLC plates, and the compounds were made visible using a fluorescent inspection lamp and iodine vapor. Gravity chromatography was done with Merck silica gel 60 (mesh size 63–200 μm). Nuclear magnetic resonance spectra were recorded on a Varian Inova instrument, operating at 500 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR. Chemical shifts (δ) for ^{1}H NMR spectra are reported in ppm downfield relative to the center line of $CDCl_3$ triplet at 7.26 ppm. Chemical shifts for ^{13}C NMR spectra are reported in ppm downfield relative to the center line of $CDCl_3$ triplet at 77.23 ppm. The abbreviations s, d, t, and m stand for the resonance multiplicities singlet, doublet, triplet, and multiplet, respectively. ^{13}C spectra, are ^{1}H decoupled, and multiciplities were determined by APT pulse sequence. The melting points were recorded on a Boëtius hot plate microscope. FT-IR spectra were recorded on FT-IR Shimadzu spectrometer (4000–400 $cm^{-}1$. EI-MS and HRMS were performed with Finnigan MAT 95, EI: 70 eV, R:10000.

(2S,4R)-4-hydroxy-2-(metoxycarbonyl)pyrrolidin chloride

In a 3-necked flask containing anhydrous methanol (33 mL), placed in an ice bath, acetyl chloride (4.14 g, 52.8 mmol) was added dropwise under nitrogen atmosphere.

Then, acid [(2S, 4R) -4-hydroxypyrrolidine-2-carboxylic acid (5.0 g, 37.7 mmol) was added and the reaction mixture was first brought to room temperature and then refluxed for 8 hours. After bringing the reaction mixture to room temperature, ethyl ether was added until complete precipitation of (2S, 4R) -4-hydroxy-2- (methoxycarbonyl) pyrrolidine chloride. 99% yield; white solid; M. P. 149-152 ° C

^{1}H NMR (DMSO, 500 MHz): δ 2.07 (ddd, 1H, J = 4.5, 11.0, 13.0 Hz), 2.18 (m, 1H), 3.05 (d, 1H, J =12.0 Hz), 3.35 (m, 1H), 3.74 (s, 3H, OMe), 4.40 (t, 1H, J = 4,5 Hz), 4.44 (dd, 1H, J = 6.5, 11.0 Hz), 4.68 (bs, OH), 9.32 (1H, NH), 10.44 (1H, NH). ^{13}C NMR (DMSO, 50 MHz): δ 37.0, 53.0, 57.4, 57.8, 68.4, 169.0.

Anal. Calc for $C_6H_{12}ClNO_3$: C, 39.68; H, 6.66; Cl, 19.52; N, 7.71%. Found: C, 39.66; H, 6.64; Cl, 19.50; N, 7.71%.

1-*ter*-butyl-2-metil-(2S,4R)-4-hydroxypyrrolidin-1,2-dicarboxylate

To a suspension of compound 1 (6.32 g, 34.8 mmol) in 45 mL of anhydrous CH_2Cl_2 at 0 ° C, was added triethylamine (10.1 g, 100 mmol) and then di-tert-butyl dicarbonate (8.3 g, 38.0 mmol). The reaction was maintained between 0 and 5 ° C for 12 hours under N_2 flow. The mixture was then treated with a 1 M solution of H_3PO_4 and then with a saturated solution of $NaHCO_3$ (3x45 mL). The organic phase was dried over $MgSO_4$, filtered and evaporated. A yellow oil was obtained, yield 91%.

^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.37 (s, 6H), 1.43 (s, 3H), 2.03 (m, 1H), 2.26 (m, 1H), 2.97 (m, 1H), 3.46 (m, 1H), 3.59 (m, 1H), 3.69 (s, 3H), 4.36 (m, 1H), 4.44 (m, 1H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 27.9, 37.1, 54.0, 57.9, 61.3, 72.0, 83.4, 160.1, 168.4.

Anal. Calc per $C_{11}H_{19}NO_5$: C, 53.87; H, 7.81; N, 5.71%. Trovato: C, 53.84; H, 7.77; N, 5.69%.

1-tert-butyl-2-methyl (2S, 4R) -4 - {[tert-butyl (dimethyl) silyl] oxy} -pyrrolidine-1,2-dicarboxylate (2)

The imidazole (7.15 g, 105 mmol) was added to the solution of compound (11.7 g, 47.7 mmol) in 60 mL of anhydrous DMF. Then the tert-butyldimethylsilylchloride (7.91 g, 52.5 mmoles) was added dropwise at 0 ° C and allow the reaction to proceed at room temperature overnight. The organic phase was washed with H_2O (3x100 mL), dried over sodium sulfate and filtered; the organic solvent was removed under reduced pressure. A light yellow oil was obtained, 92% yield.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.01 (s, 6H), 0.82 (s, 9H), 1.35 (s, 6H), 1.39 (s, 3H), 1.96 (m, 1H), 2.09 (m, 1H), 3.29 (m, 1H), 3.53 (m, 1H), 3.65 (s, 3H), 4.32 (m, 2H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ -4.6, 17.9, 25.6, 28.3, 38.9, 51.9, 54.5, 54.8, 69.7, 78.0, 159.8, 173.9.

Anal. Calc per $C_{17}H_{33}NO_5Si$: C, 56.79; H, 9.25; N, 3.90; Si, 7.81%. Found: C, 56.76; H, 9.23; N, 3.88; Si, 7.84%.

(2S,4R)-1-tert-butyl 2-methyl 4-((tert-butyldimethylsilyl)oxy)-5-oxopyrrolidine-1,2-dicarboxylate (3)

In a 3-necked flask containing NaIO4 (7.5 g, 35.0 mmol) in H2O (127 mL), $Ru_2O_2H_2O$ (0.35 g, 2.66 mmol) was added under N2 flow and left under stirring for about 30 minutes. Then a solution of compound **2** (5.0 g, 14.0 mmol) is added, employing about

60 mL of ethyl acetate. The mixture becomes first yellow and then black after 4 hours. Then (9.0 g, 42 mmol) of NaIO4 are added and the mixture is yellow, ensuring that the color is maintained over time. The organic phase separated from the aqueous phase is treated with a saturated solution of NaHSO3 (3 x 60 mL), filtered on celite, dried over sodium sulfate and evaporated to dryness to give a yellow oil, yield 60%

((2S,4R)-4-((tert-butyldimethylsilyl)oxy)-5-oxopyrrolidin-2-yl)methyl 4-bromobenzoate (4)

A solution compound **3** (6 g) in 20 mL of CH_2Cl_2 was treated with TFA (10 eq.) for 2 h. The solvent was removed *in vacuo* and the crude was dissolved in $CHCl_3$, washed with saturated $NaHCO_3$, and brine. Dried over MgSO4, filtered, concentrated to provide the product as a beige solid, 90% yield.

The solid (4,5 g 11,86 mmol) was dissolved in a solution of 40 mL of THF and 8 mL of CH_3OH, refluxed at 55°C, $NaBH_4$ (0.09 g, 2,37 mmol) was added. The reaction proceded for 1 hour, after bringing the reaction mixture to room temperature was quenched with water, the solvent was removed *in vacuo* and the crude was extracted in CH_3COOEt, dried over MgSO4, filtered, concentrated to provide the product as a pink oil, 91% yield.

Then solution of the crude product (5 g, 19,3 mmol) in 20 mL of anhydrous DMF was added NaH (1,1eq) at 0 ° C until it was totally dissolved; then was added dropwise 4-br-benzoylchloride (1,1 eq.) and allow the reaction to proceed at room temperature overnight. The reaction was quenqued with H_2O (30 mL), The organic phase was

extracted with CH_2Cl_2 (3x50 mL), dried over sodium sulfate and filtered; the organic solvent was removed under reduced pressure.The result product was purified by Flash chromatography (THF/nEXANE 5%) rf. 0.2 to afford a white solid, yield 85% yield.

((2S,4R)-1-(1-(tert-butoxy)vinyl)-4-((tert-butyldimethylsilyl)oxy)-5-hydroxypyrrolidin-2-yl)methyl 7-bromohepta-2,4,6-triynoate (5)

To a suspension of compound **4** (4 g, 9,35 mmol) in 45 mL of anhydrous CH_2Cl_2 at 0 ° C, was added triethylamine (5eq) and then di-tert-butyl dicarbonate (1 eq). The reaction was maintained between 0 and 5 ° C for 12 hours under N_2 flow. The mixture was then treated with a 1 M solution of H_3PO_4 and then with a saturated solution of $NaHCO_3$ (3x45 mL). The organic phase was dried over $MgSO_4$, filtered and evaporated. A yellow oil was obtained, yield 90%.

To a solution of the oil (3,9 g, 2.68 mmol) in 80 ml of anhydrous THF at -78 ° C under N2 flow, lithium triethylborohydride (0.5 eq,) was added and stirred under agitation for 1 h. After a subsequent addition of a reducing agent (0.69 eq), the reaction is conducted at -78 ° C for one hour more, water is added to the same temperature. The solvent was removed, the organic layers extracted with ethyl acetate the organic phase was dried over $MgSO_4$, filtered and evaporated. A yellow oil was obtained, 71% yield.

(3R,5S)-tert-butyl 2-((7-bromohepta-2,4,6-triynoyl)oxy)-5-(((7-bromohepta-2,4,6-triynoyl)oxy)methyl)-3-((tert-butyldimethylsilyl)oxy)pyrrolidine-1-carboxylate (6)

To a solution of the **5** (4 g, 9,8 mmol) in 20 mL of anhydrous DMF was added NaH (1,1eq) at 0 ° C until it was totally dissolved; then was added dropwise 4-br-benzoylchloride (1,1 eq.) and allow the reaction to proceed at room temperature overnight. The reaction was quenqued with H_2O (30 mL), The organic phase was extracted with CH_2Cl_2 (3x50 mL), dried over sodium sulfate and filtered; the organic solvent was removed under reduced pressure.The result product was purified by Flash chromatography (THF/nEXANE 5%) rf. 0.25 to afford a white solid, yield 85% yield.

(5S)-tert-butyl 2-((7-bromohepta-2,4,6-triynoyl)oxy)-5-(((7-bromohepta-2,4,6-triynoyl)oxy)methyl)-3-oxopyrrolidine-1-carboxylate (7)

To a solution of compound **6** (3.1 g, 4.34mmol) in 60 mL of anhydrous THF, TBAF (4.34 mmol) was added at 0 ° C. After 2 hours the reaction was treated with the addition of 120 mL of H_2O. The solvent THF was removed, extracted with ethyl acetate, dried over $MgSO_4$, filtered and evaporated and a yellow oil was obtained. To a solution of the oil in 50 mL of anhydrous CH_2Cl_2, 8 mL of Dess-Martin was added, the reaction is conducted under N_2 at room temperature for 24 hours, washed with sodium thiosulphate and brine, filtered and the solvent evaporated under reduced pressure. White solid was obtained by chromatography purification using as eluent acetate / cyclohexane (50:50).

(5S)-tert-butyl 2-((7-bromohepta-2,4,6-triynoyl)oxy)-5-(((7-bromohepta-2,4,6-triynoyl)oxy)methyl)-3-hydroxy-3-methylpyrrolidine-1-carboxylate (8)

To a solution of compound **7** (2g, 3,34mmol) in THF dry at -78°C was added dropwise CH_3Li 1,6M in diethyl ether (5,26 mL). The reaction mixture was stirred at -78°C for 2 h and then quenqued with a NH4Cl solution; the solvent was removed and the aqueous layers extracted with CH_2Cl_2.the organic phase was dried over $MgSO_4$, filtered and the solvent evaporated under reduced pressure.

(2R,3S,5S)-tert-butyl 3-hydroxy-5-(hydroxymethyl)-3-methyl-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)pyrrolidine-1-carboxylate (9)

(2R,3R,5S)-tert-butyl 3-hydroxy-5-(hydroxymethyl)-3-methyl-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)pyrrolidine-1-carboxylate (10)

To a suspension of thymine (0.06 g of 0.55 mmol) in 4 mL of anhydrous CH_3CN was added dropwise N, O-bis-trimethylsilylacetamide (0.45 g, 2.23 mmol) and allowed to reflux under stirring for 15 minutes. A solution of compound **8** (0.16 g, 0.46 mmol) was added using 4 mL of CH3CN and then trimethylsilyltriflate (0.15 g, 0.69 mmol). After 1 hour, the reaction was quenched with a saturated solution of $NaHCO_3$ at 0 ° C. The organic solvent was removed, the sample dissolved in CH_2Cl_2, washed with (2x20 mL), dried, filtered and the solvent removed. The product was purified by flash chromatography employing an eluent mixture consisting of methanol / chloroform (10 x 90), rf = 0.13; Yield 76%; White solid m.p. = 119.3-121.8 ° C.

To a solution of the afforded product (0.12 g, 2.91 mmol) was added a saturated solution of sodium carbonate in CH_3OH, the reaction was carried out at room temperature for one hour, the reaction mixture was quenqued with saturated solution

of sodium chloride, the organic solvent was removed under reduced pressure, extracted with ether and a white solid was obtained. The product was purified by flash chromatography using as eluent ethyl acetate / cyclohexane (50:50).

(2S,4R)-1-benzyl 2-methyl 4-((tert-butyldimethylsilyl)oxy)pyrrolidine-1,2-dicarboxylate (11)

To a suspension of compound **1** previously treated with Thionyl Chloride (8,47 g, 46,2 mmol) in 45 mL of dioxane/H_2O, was added triethylamine (9,62 mL) and then benzylchloroformiate (15,76 g, 92,4 mmol). The reaction was maintainedat r.t for 4h. The mixture was then treated with a 0.5 M solution of HCl and then with a saturated solution of $NaHCO_3$ (3x45 mL). The organic phase was dried over $MgSO_4$, filtered and evaporated. A oil was obtained, yield 91%.

(2S,4R)-benzyl 4-((tert-butyldimethylsilyl)oxy)-2-(iodomethyl)pyrrolidine-1-carboxylate (12)

Compound **11** (5 g 12,7 mmol) was dissolved in a solution of 40 mL of THF and 8 mL of CH_3OH, refluxed at 55°C, $NaBH_4$ (0.09 g, 25,3 mmol) was added. The reaction proceeded for 1 hour, after bringing the reaction mixture to room temperature was quenched with water, the solvent was removed *in vacuo* and the crude was extracted in CH_3COOEt, dried over MgSO4, filtered, concentrated to provide the product as a transparent oil, 90% yield.

To a suspension of the afforded compound (5.8 g, 16 mmol), imidazole (2.74g, 40,2 mmol) and triphenylphospine (10,5 g, 40.18 mmol) in 20 mL of anhydrous THF under

N_2 flow, was added in three times during ten minutes the iodine (7.66g, 30.18 mmol). After 1 hours at room temperature the reaction was quenched with water, the solvent was removed *in vacuo* and the crude product was extracted in CH_2Cl_2 (3x25 mL) dried over MgSO4, filtered, concentrated *in vacuo*. The result product was purified by Flash chromatography (THF/nEXANE 5%) rf. 0.2 to afford a transparent oil, yield 70%.
^{1}H-NMR (400 MHz, CDCI3): 6 0.03-0.07 (6H, m, Si(CH3)2), 0.85 (9H, s, SiC(CH3)3), 1.84-1.94 (1H, m, C3-H), 1.97-2.14 (1H, m, C3-H), 3.36.3.74 (4H, m, C5-H2, CH2I), 3.88-4.00 (1H, m, C2-H), 4.34-4.40 (1H, m, C4-H), 5.06-5.20 (2H, m, CH2Ph), 7.30 (5H, m, Ph). ^{13}C-NMR (90 MHz, CDC13): 6 -4.82 and -4.76 (Si(CH3)2), 12.92 and 13.00 (CH2I, rotamers), 17.95 (SiC__(CH3)3), 25.70 (SiC(CH3)3), 41.39 and 42.17 (C3, rotamers), 55.98 and 56.02 (C5, rotamers), 56.35 and 56.41 (C2, rotamers), 66.79 and 67.14 (CH2Ph, rotamers), 69.52 and 69.90 (C4, rotamers),127.68, 127.82, 127.93, 128.04, 128.48, and 127.57 (Ph, rotamers), 155.29 (CO2).

(2S,4R)-benzyl 4-((tert-butyldimethylsilyl)oxy)-2 ((diethylphosphoryl)methyl)pyrrolidine-1-carboxylate (13)

A suspension of compound **12** (0.5g, 1.08 mmol), in 1 mL of triethyl phosphite , was poured into microwave for 40 minutes at 100 W and 140°. The solvent was removed *in vacuo* and the crude product was was purified by Flash chromatography (THF/nEXANE 5%) rf. 0.2 to afford a colourless oil, yield 30%.
^{1}H-NMR (400 MHz, CDCI3): b 0.04-0.07 (6H, m, Si(CH3)2), 0.86 (9H, s, SiC(CH3)3), 1.08-1.30 (12H, m, CH(CI-h)2 x 2), 1.65-1.78 (1H, m, C3-H), 1.97-2.15 (2H, m, CH2P), 2.40-2.50 (1H, m, C3-H), 3.42-3.52 (2H, m, C5- H2), 4.08-4.18 (1H, m, C2-H), 4.32-4.40 (1H, m, C4-H), 4.50-4.60 (2H, m, CIt(CH3)2 x 2), 5.10-5.22 (2H, m, CH2Ph), 7.29 (5H, m, Ph). ^{13}C-NMR (90 MHz, CDCI3): 8 -4.86 and -4.77 (Si(CH3)2), 17.94 (SiC_C_(CH3)3), 23.55, 23.71, 23.82, and 23.98 (each d, 3jc,p=4.4, OCH(CH3)2), 24.04 and 25.70 (Si(CH3)3, rotamers), 31.41 (d, 1Jc,p=135, CH2P), 40.75 and 41.49 (Ca, rotamers), 51.96 and 52.51 (C2, rotamers), 54.82 and 55.04 (C5, rotarners), 66.54 and 66.93 (CH2Ph, rotamers), 70.10 and 70.16 (each d, 2jc,p=7.4,

OC_H(CH3)2), 71.91 and 71.97 (C4, rotamers), 127.68, 127.93, 128.32, 128.45, 129.85, 129.93, 136.65, and 136.89 (Ph, rotamers), 155.10 (CO2).

(2S,4R)-tert-butyl 4-((tert-butyldimethylsilyl)oxy)-2-((diethylphosphoryl)methyl)pyrrolidine-1-carboxylate (14)

A mixture of **13** (3.0 g, 5.8 mmol),p-toluenesulfonic acid (2.4 g, 12 mmol), and 10% paradium on carbon (0.4 g) in methanol (50 ml) was stirred under H2 atmosphere at room temperature for 3 h. The catalyst was tilted off and the filtrate was concentrated *in vacuo* to give a residue. Di-tert-butyl dicarbonate (1.1 g, 8.6 mmol) and triethylamine (2.3 g, 22 mmol) was added to the mixture of the resulting residue in dichloromethane (100 mL) and the mixture was refluxed for 12 h. 1M aqueous NaOH solution was added to the mixture, the mixture was extracted with dichloromethane (60 ml). The extract was washed with brine and dried over Na_2SO_4.

Concentration of the solvent in vacuo gave a residue, which was purified by column chromatography (ethylacetate/methanol: 10/1)to give 10 (1.3 g, 63%)as a pale yellow oil.

(3R,5S)-tert-butyl 3-((tert-butyldimethylsilyl)oxy)-5-((diethylphosphoryl)methyl)-2-oxopyrrolidine-1-carboxylate (15)

In a 3-necked flask containing NaIO4 (7.5 g,) in H2O (127 mL), $Ru_2O_2H_2O$ (1,9 mmol) was added under N2 flow and left under stirring for about 30 minutes. Then a solution of compound **14** (5.0 g, 10.4 mmol) is added, employing about 60 mL of ethyl acetate. The mixture becomes first yellow and then black after 4 hours. Then (9.0 g,) of NaIO4 are added and the mixture is yellow, ensuring that the color is maintained over time. The organic phase separated from the aqueous phase is treated with a saturated solution

of NaHSO3 (3 x 60 mL), filtered on celite, dried over sodium sulfate and evaporated to dryness to give a yellow oil, yield 65%.

(3R,5S)-1-tert-butyl 2-methyl 3-((tert-butyldimethylsilyl)oxy)-5-((diethylphosphoryl)methyl)pyrrolidine-1,2-dicarboxylate (16)

To compound **15** (3,9 g, 7.25 mmol) in 80 ml of anhydrous THF at -78 ° C under N2 flow, lithium triethylborohydride (0.5 eq,) was added and stirred under agitation for 1 h. After a subsequent addition of a reducing agent (0.69 eq), the reaction was conducted at -78 ° C for one hour more, water was added to the same temperature. The solvent was removed, the organic layers extracted with ethyl acetate the organic phase was dried over $MgSO_4$, filtered and evaporated. A yellow oil was obtained, 60% yield.

To a solution of the oil (2.5 g,) in 60 mL of anhydrous CH2Cl2 was added dropwise, acetic anhydride (1 eq) and then triethylamine (2eq) and the reaction stirred at room temperature overnight. The organic phase was washed with H2O (3x60 mL), dried over sodium sulfate and the solvent evaporated *in vacuo*. The reaction product was purified by flash chromatography employing the ethyl acetate / cyclohexane (25:75); a yellow oil was obtained; yield 75%.

(3R,5S)-tert-butyl 3-hydroxy-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-5-(phosphorylmethyl)pyrrolidine-1-carboxylate compound with 1-propoxypropane (1:1) (17)

To a suspension of thymine (5 g of 3.9 mmol) in 20 mL of anhydrous CH_3CN was added dropwise N, O-bis-trimethylsilylacetamide (0.45 g, 2.23 mmol) and allowed to

reflux under stirring for 15 minutes. A solution of compound **16** (1,5 g, 3.6 mmol) was added using 15 mL of CH3CN and then trimethylsilyltriflate (1.5eq). After 1 hour, the reaction was quenched with a saturated solution of $NaHCO_3$ at 0 ° C. The organic solvent was removed, the sample dissolved in CH_2Cl_2, washed with (2x30 mL), dried, filtered and the solvent removed. The product was purified by flash chromatography methanol / chloroform (10:90), rf = 0.2. a pure white solid was obtained.

To a solution of the solid (1,2 g, 2,61mmol) in 60 mL of anhydrous THF, TBAF 1M in THF (2,6 mmol) was added at 0 ° C. After 2 hours the reaction was treated with the addition of 120 mL of H_2O. The solvent THF was removed, extracted with ethyl acetate, dried over $MgSO_4$, filtered and evaporated and a white solid was obtained.

Ethyl 2-aminopent-4-ynoate (19)

N-(Diphenylmethylene)glicine ethylester **18** (1.7g, 6.359 mmol), propargyl bromide (1.5 eq.) and potassium carbonate (5 eq.) in dry CH_3CN (25 mL) was refluxed for 24h. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure, than the crude product was extracted with Et_2O (100 mL), washed with water, brine and dried over $MgSO_4$. Evaporation of solvent gave the crude alkylated Schiff-base product.

The crude alkylated product was hydrolyzed in Et_2O (25 mL) and 1N HCl (10 mL) stirred at rt. for 24 h. Than the aqueous layer was separed and washed with excess of Et_2O to remove unwanted organic residues. The aqueous layer was basified with ammonia solution to pH ~ 10 and than extraxted with Ch3COOEt.

The combined ethylacetate layer was washed with water, brine dried over $MgSO_4$. Evaporation of solvent gave the amino ester.A yellow oil was obtained, yield 95%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.26 – 4.05 (m, 2H), 3.62 (d, *J* = 6.1, 5.3 Hz, 1H), 2.71 – 2.52 (m, 2H), 2.15 (d, *J* = 0.5 Hz, 1H), 2.09 – 1.92 (m, 2H), 1.27 (t, *J* = 7.1, 0.5 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.59, 79.50, 71.24 (s), 61.30, 53.11, 24.77, 14.16.

Ethyl 2-((tert-butoxycarbonyl)amino)pent-4-ynoate (20)

Compound **19** was dissolved in dry CH_2Cl_2, Boc_2O (1.2 eq.), DMAP (1eq.) and NEt_3 (1 eq.) was subsequently added at rt. and stirred for 24h. The solvent was removed and ET_2O added, washed with HCL 0.1 M, $NaHCO_3$ brine, dried over $MgSO_4$, filtered and the solvent removed. The result product was purified by Flash chromatography (CH_3COOEt/EXANE 5%) rf. 0.19 to afford a transparent oil, yield 60%.

^1H NMR (400 MHz, $CDCl_3$) δ 5.35 (d, J = 7.6 Hz, 1H), 4.47 – 4.37 (m, 1H), 4.30 – 4.15 (m, 2H), 2.71 (d, J = 7.5, 6.1, 3.7 Hz, 1H), 2.03 (t, J = 2.6 Hz, 1H), 1.45 (s, 9H), 1.32 – 1.25 (t, 3H).

Ethyl 2-((tert-butoxycarbonyl)amino)-5-phenylpent-4-ynoate (21)

To a solution of substrate **20** (0.364g, 1.50mmol), dissolved in 20mL of ET_2O, iodobenzene (0.203 ml, 1.82mmol), NEt_3 (1.04 ml, 7.53), CuI (0.0287 g, 0.15mmol) and $PdCl_2(PPh3)_2$ (0.075mmol) were added and the resulting mixture was stirred for 2h at rt. The reaction mixture was poored into a satured acqueous solution of NH_4Cl (20 ml) and after separation of the organic layer the acq. one was extracted with ET_2O (2x25 ml).The organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated in *vacuo*. The crude product was purified by Flash chromatography (CH_3COOEt/EPTANE 1:6) rf. 0.3 to afford a yellow oil, 80% yield.

^1H NMR (400 MHz, $CDCl_3$) δ 7.68 – 7.58 (m, 1H), 7.32 – 7.18 (m, 2H), 7.08 – 6.98 (m, 2H), 5.35 – 5.23 (m, 1H), 4.41 – 4.32 (m, 1H), 4.25 – 4.09 (m, 1H), 2.87 m, 2H), 2.76 – 2.70 (m, 1H), 2.70 – 2.60 (m, 1H), 1.96 (t, J = 2.6 Hz, 1H), 1.45 (s, 9H), 1.26 – 1.16 (m, 3H).

Ethyl 5-phenyl-3,4-dihydro-2H-pyrrole-2-carboxylate (22)

A solution of the precursor **21** (0.2g 0.63mmol) was dissolved in CH_2Cl_2 and at 0°C TFA (0.092 ml, 1.26mmol) was added and stirred at rt. overnight. After remove of the solvent a solution of the HCl-salt in dioxane/H_2O 1:1 (15 mL) was treated dropwise at rt. with 25% aq. NH_4OH until the mixture reached pH 10. The aqueous solution was

extracted with CH_2Cl_2 (3x 20 ml), the combined organic layer were dried over $MgSO_4$, filtered and concentrated in *vacuo* to afford a brown oil. The crude amino ester was purified by Flash chromatography (CH_3COOEt/EXANE 15%) rf. 0.25 to afford a light yellow oil, 75 % yield.

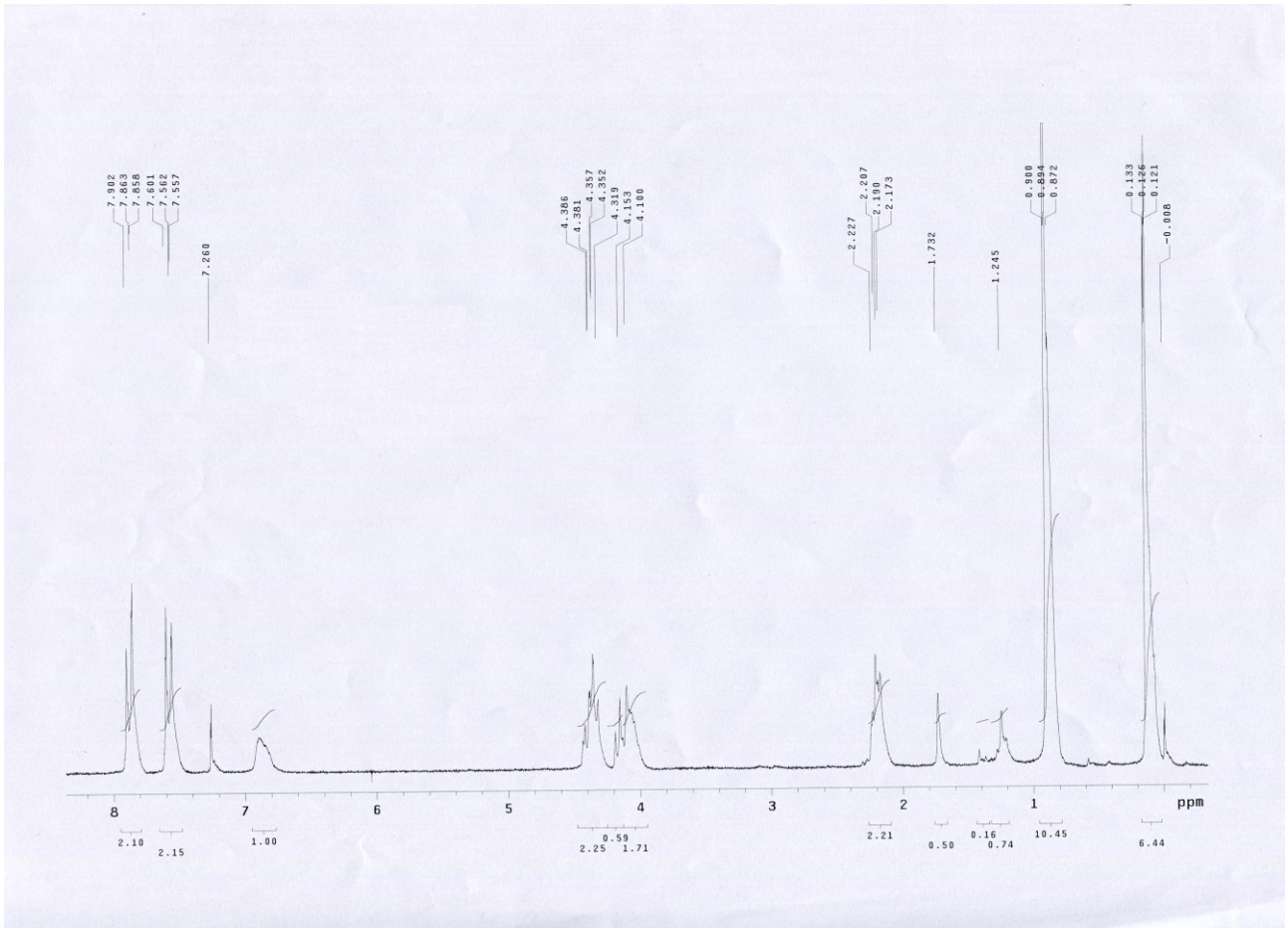
^1H NMR (400 MHz, $CDCl_3$) δ 7.86 – 7.76 (m, 1H), 7.65 – 7.55 (m, 1H), 7.51 – 7.26 (m, 3H), 4.82 (q, *J* = 14.3, 7.2 Hz, 1H), 4.05 (q, *J* = 7.1 Hz, 2H), 3.15 – 3.01 (m, 1H), 3.01 – 2.84 (m, 1H), 2.40 – 2.24 (m, 1H), 2.18 (m, 1H), 1.43 – 1.31 (m, 3H).

2-(ethoxycarbonyl)-5-phenyl-3,4-dihydro-2H-pyrrole 1-oxide (23)

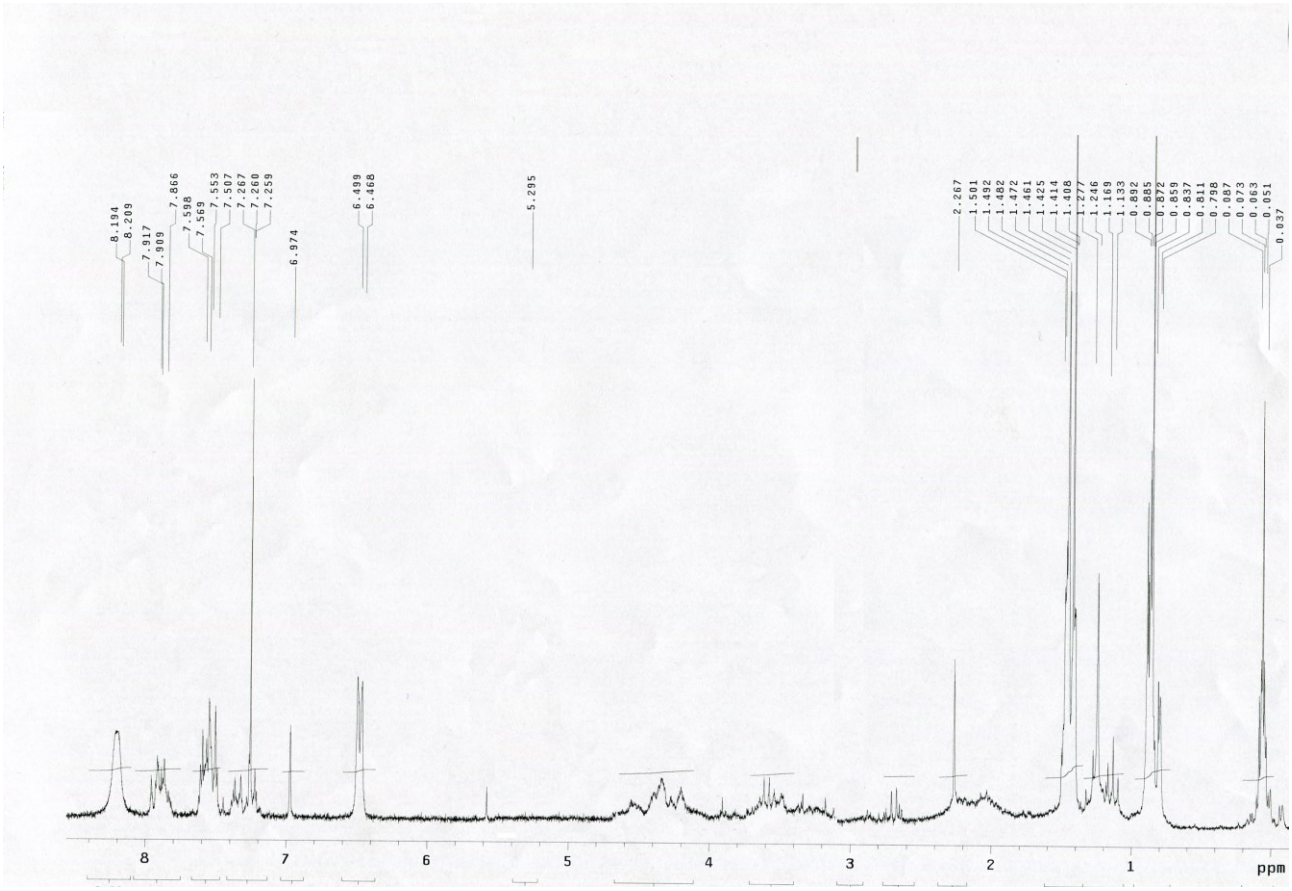
To a solution of the imine (**22**, 0.167 g,0 .77mmol) in MeCN /THF 4:1 and 0.01 –m EDTA aq.solution, at 0°C-5°C $NaHCO_3$ (0.323g, 3.84mmol) was added, sequentially over 2 h under vigorous stirring oxone (0.497g, 0.80mmol) was added in portions. Stirring the reaction all the time at 0°C-5°C. After 2h CH_3COOEt 10 mL and H_2O 2ml was added and the phases separated, the aqueous layer was extracted twice with CH_3COOEt, dried over $MgSO_4$, filtered and concentrated in *vacuo*. The crude product was purified by Flash chromatography (CH_3COOEt/EXANE 10%) rf. 0.32 to afford a yellow oil, 63 % yield.

^1H NMR (300 MHz, $CDCl_3$) δ 7.94 – 7.81 (m, 1H), 7.53 – 7.21 (m, 4H), 5.15 (d, J = 8.1 Hz, 1H), 4.34 – 4.00 (m, 2H), 3.17 – 2.88 (m, 1H), 2.75 – 2.35 (m, 1H), 2.35 – 2.14 (m, 1H), 2.09 – 1.83 (m, 2H), 1.23 – 1.11 (m, 3H).

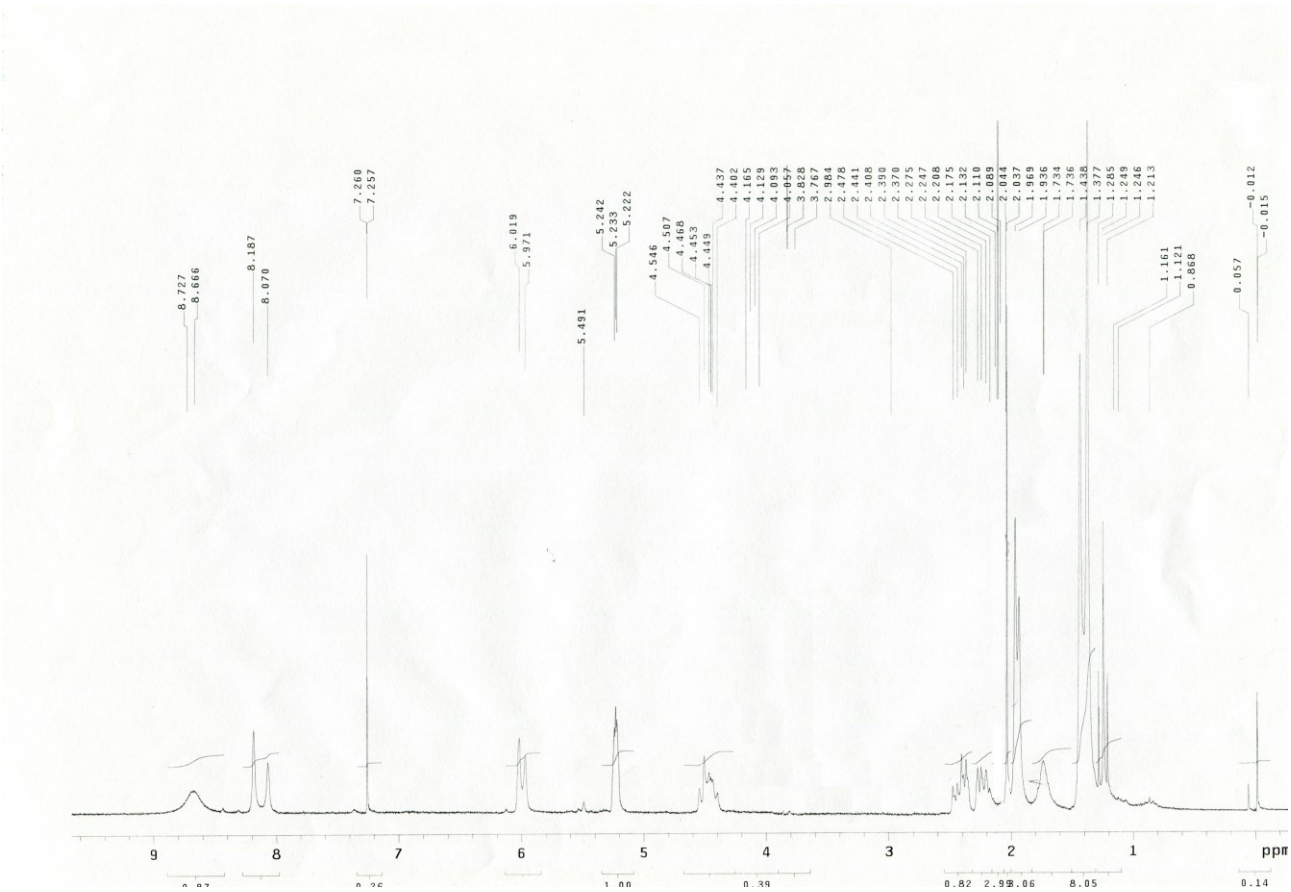
^{1}H NMR compound 4



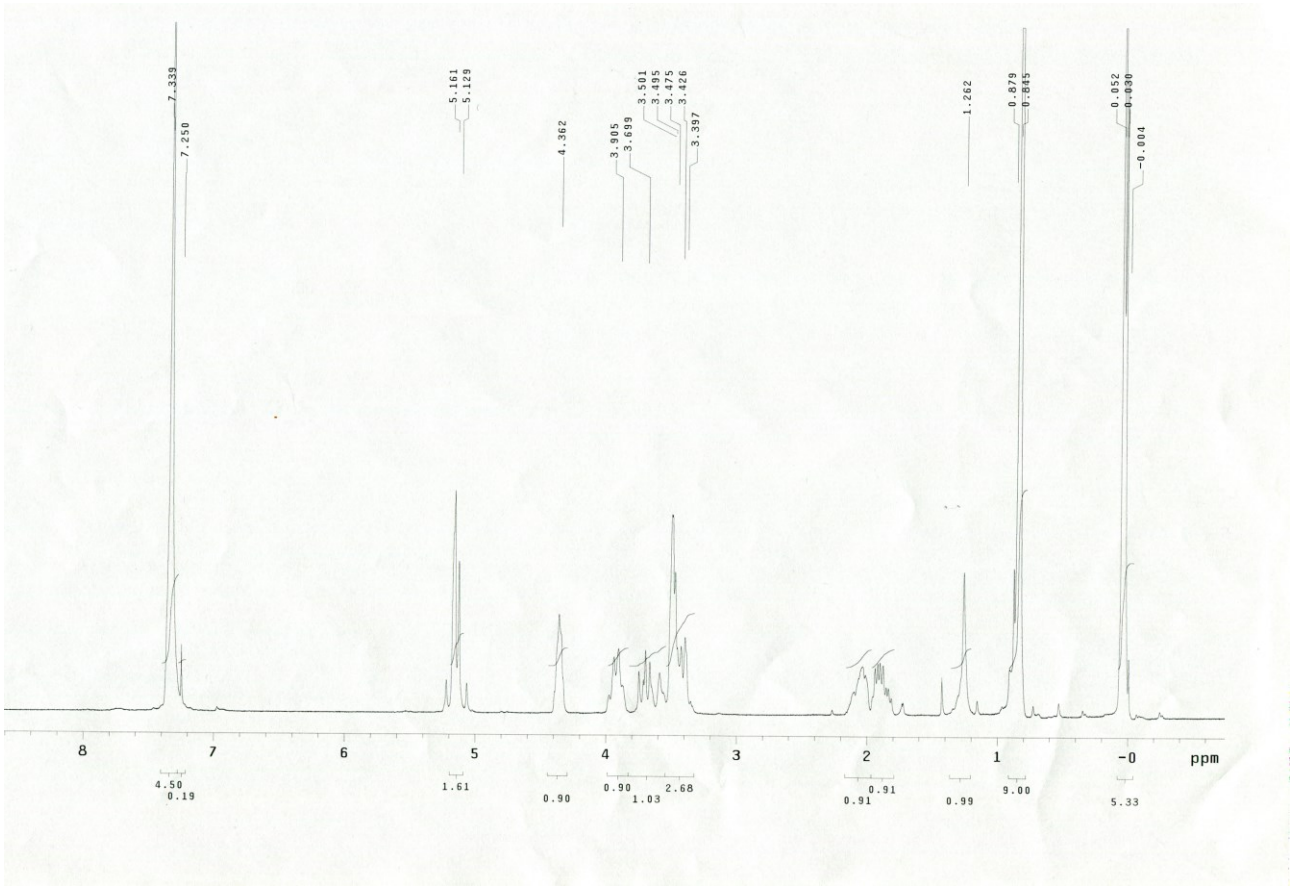
^{1}H NMR compound 6



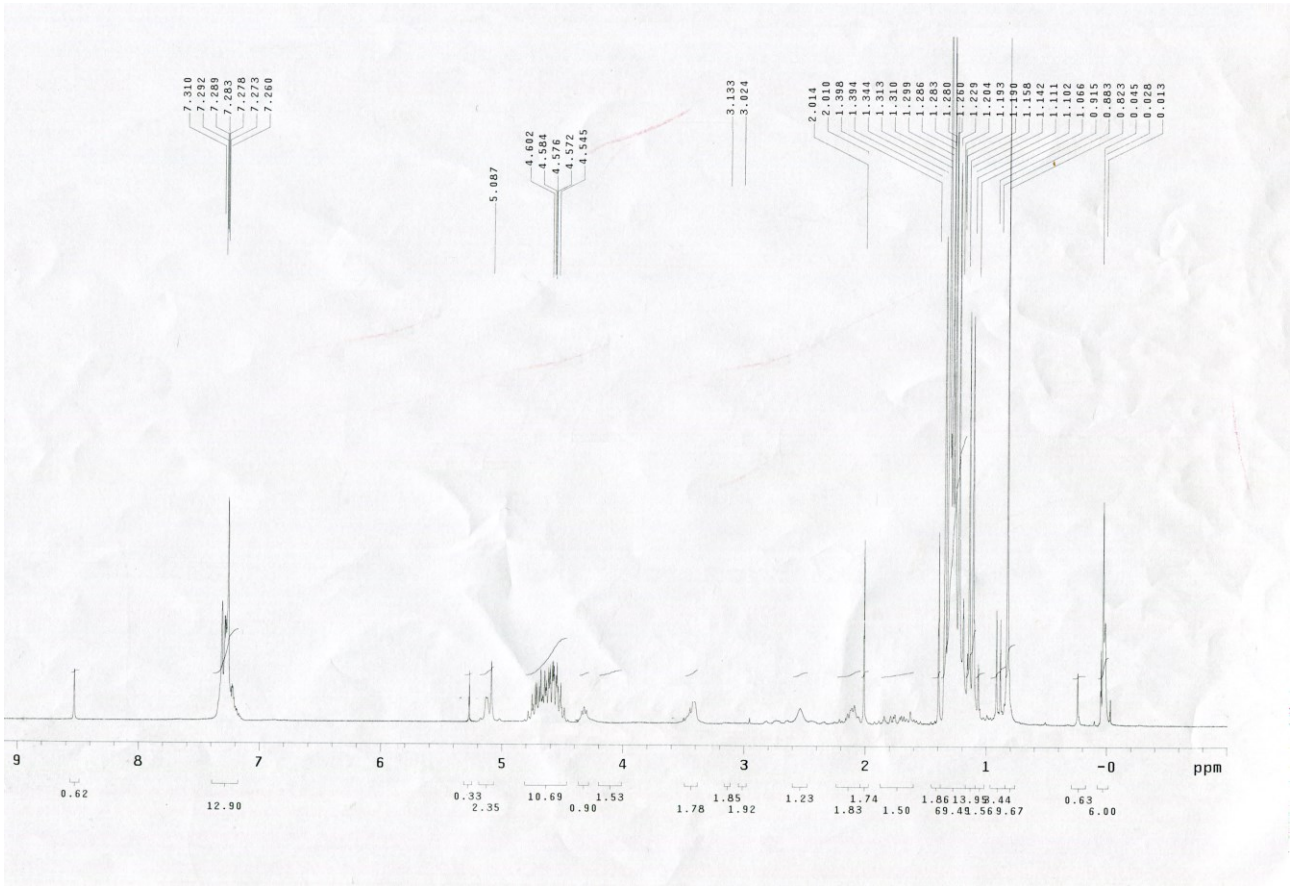
^{1}H NMR compound 9-10



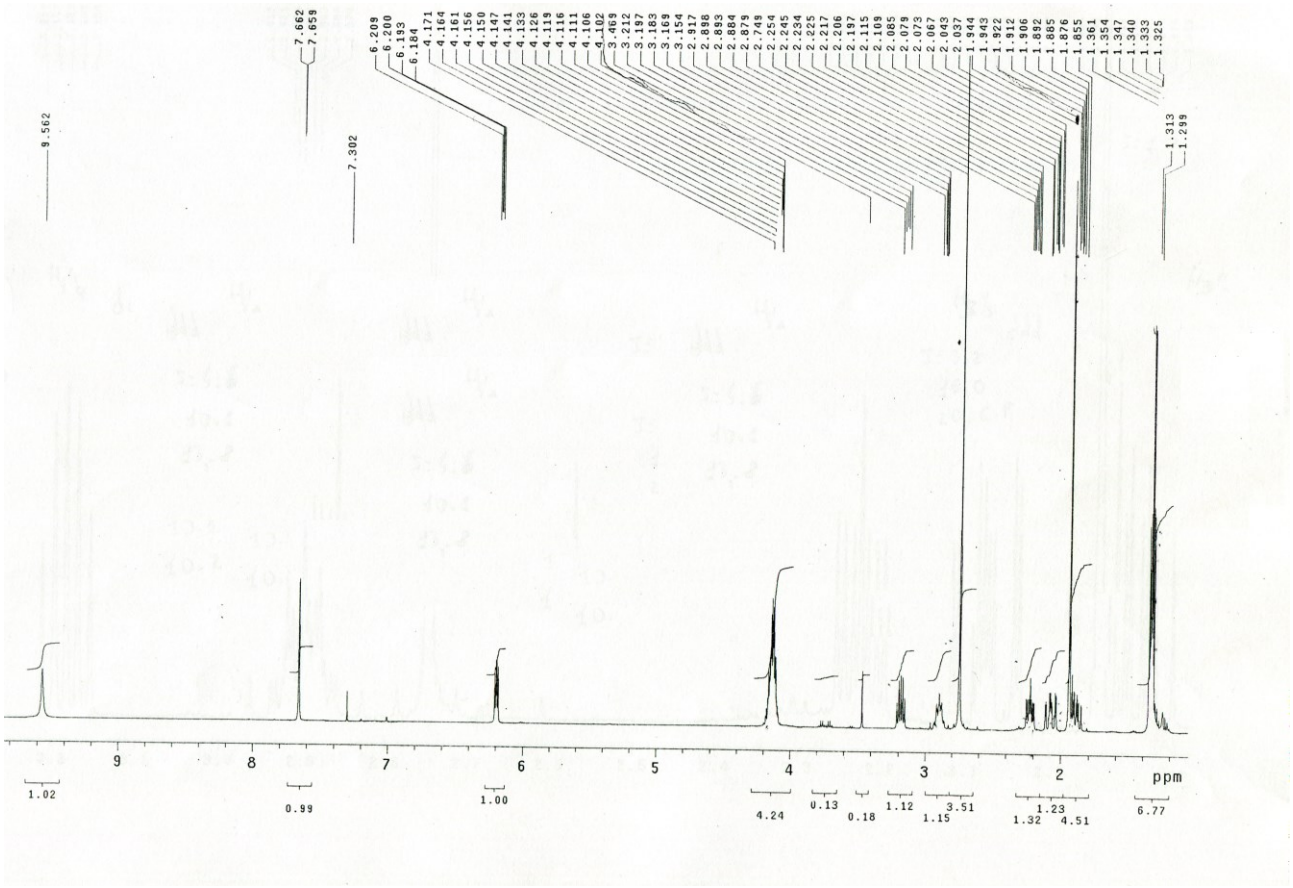
^{1}H NMR compound 12



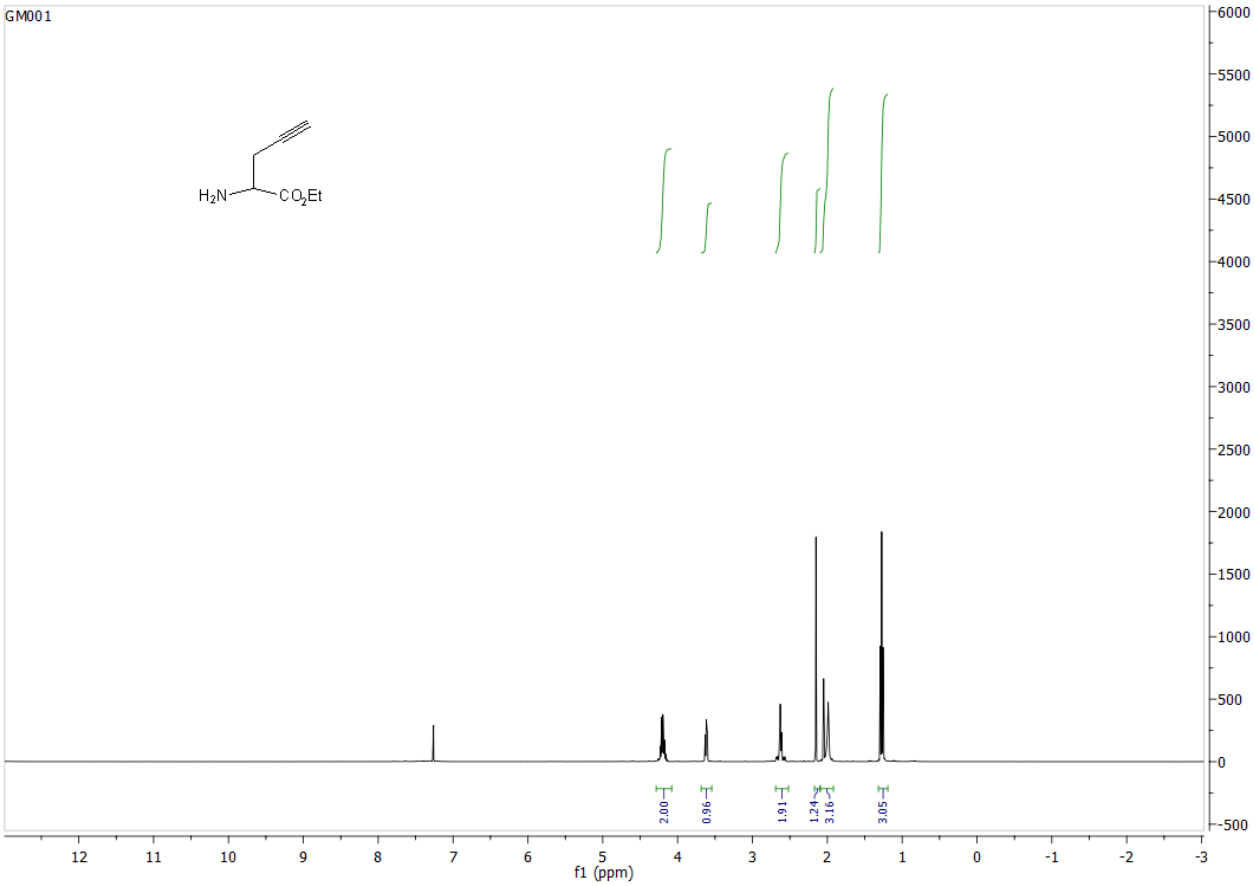
^{1}H NMR compound 13



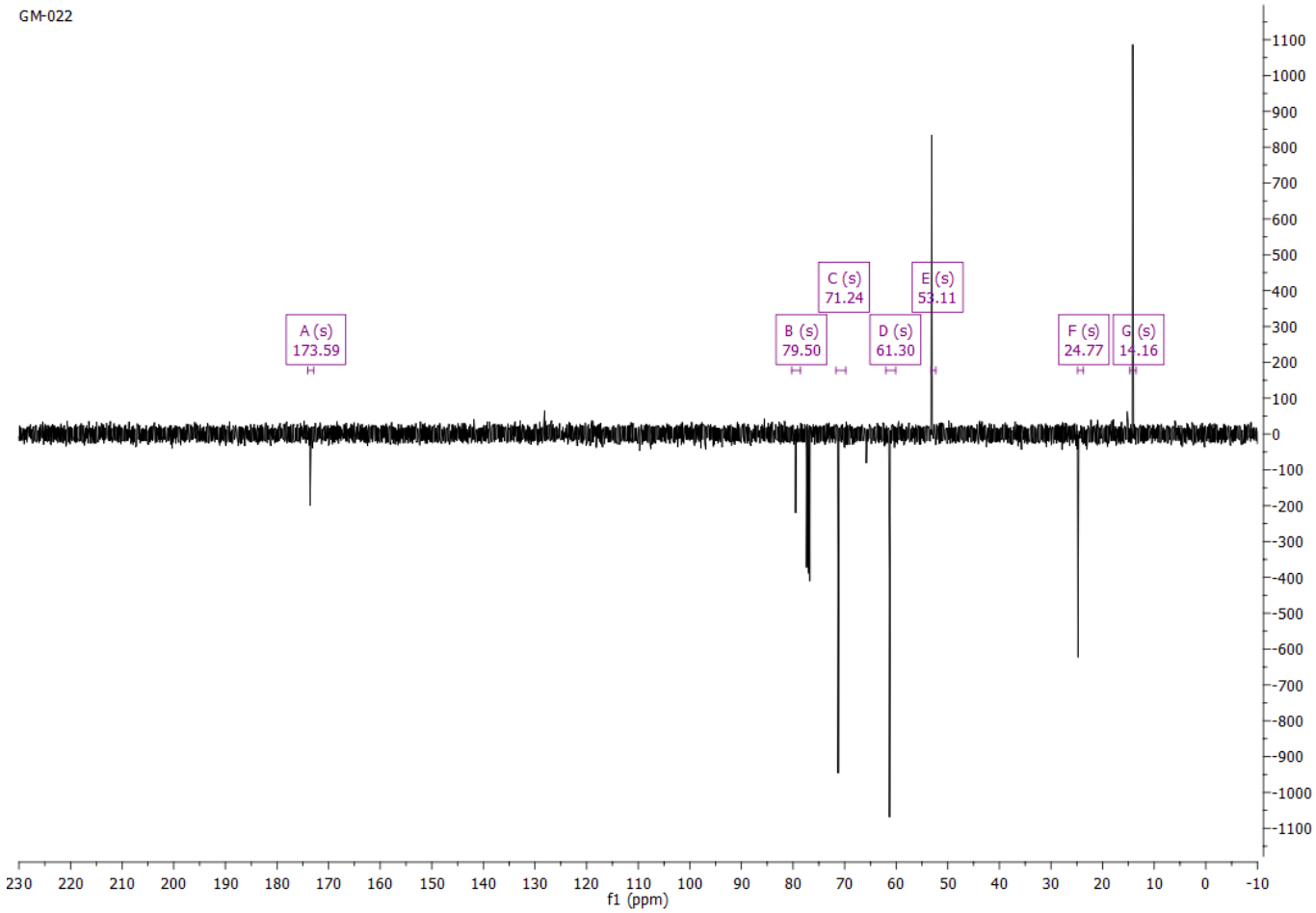
^{1}H NMR compound 17



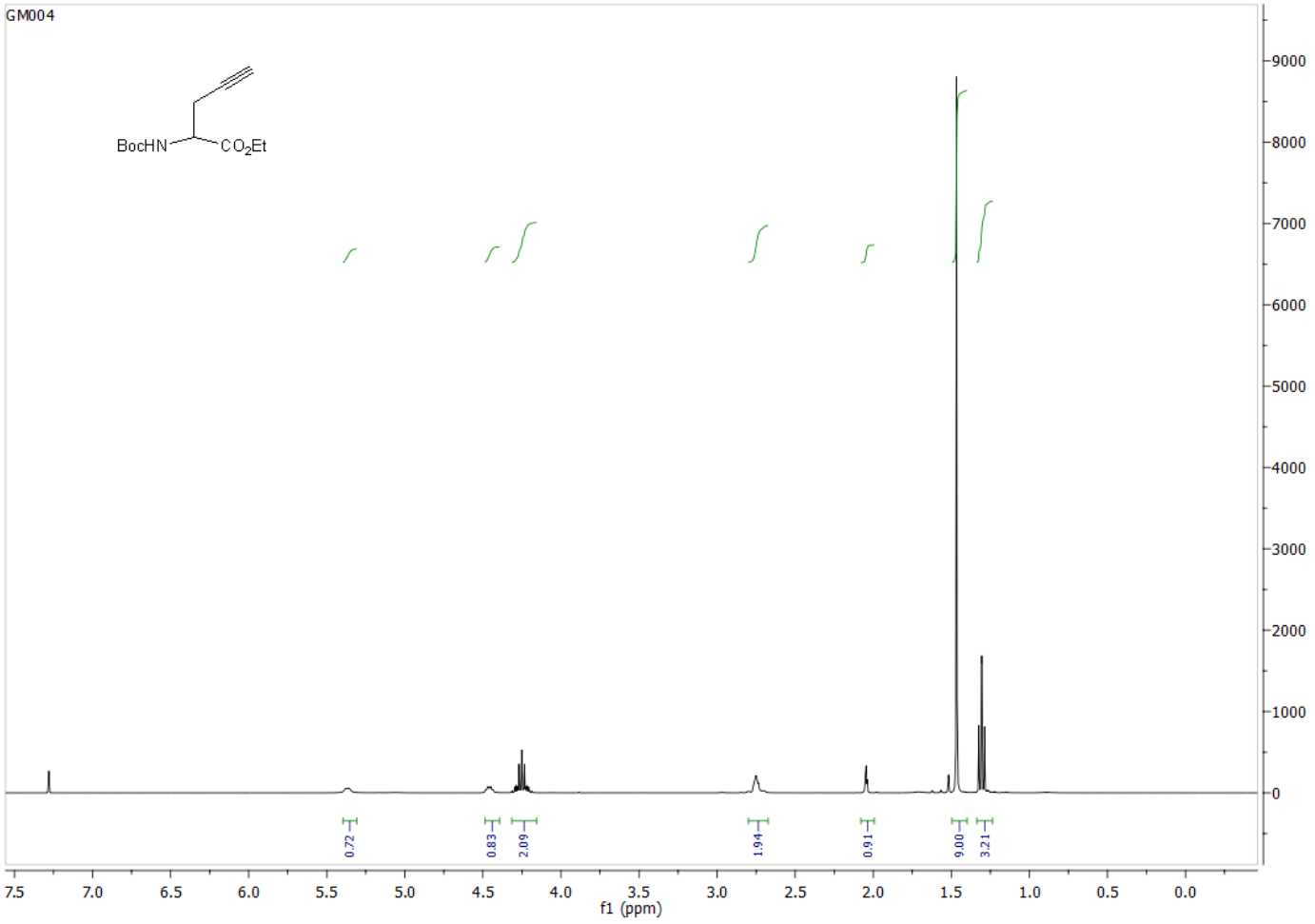
^{1}H NMR compound 19



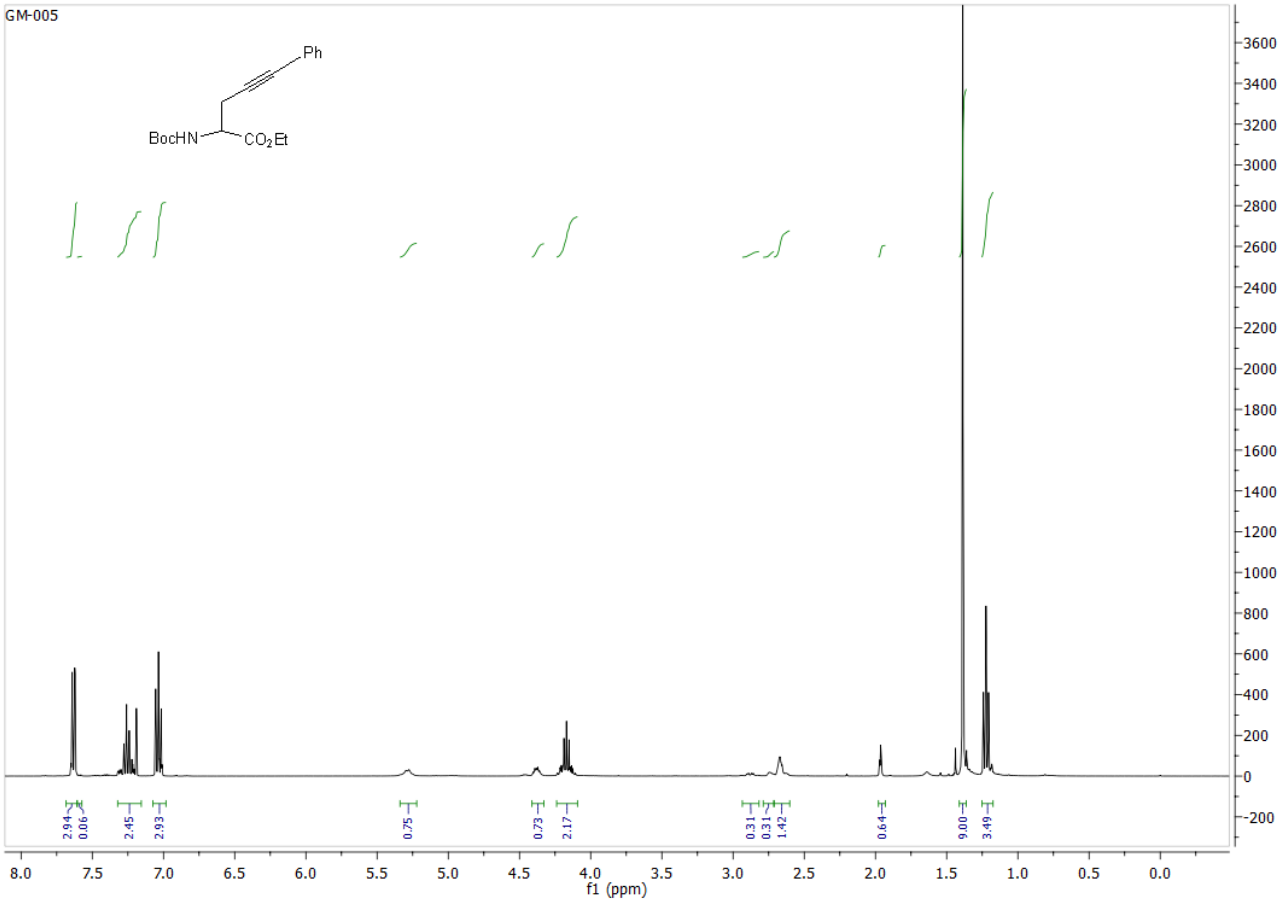
^{13}C NMR compound 19



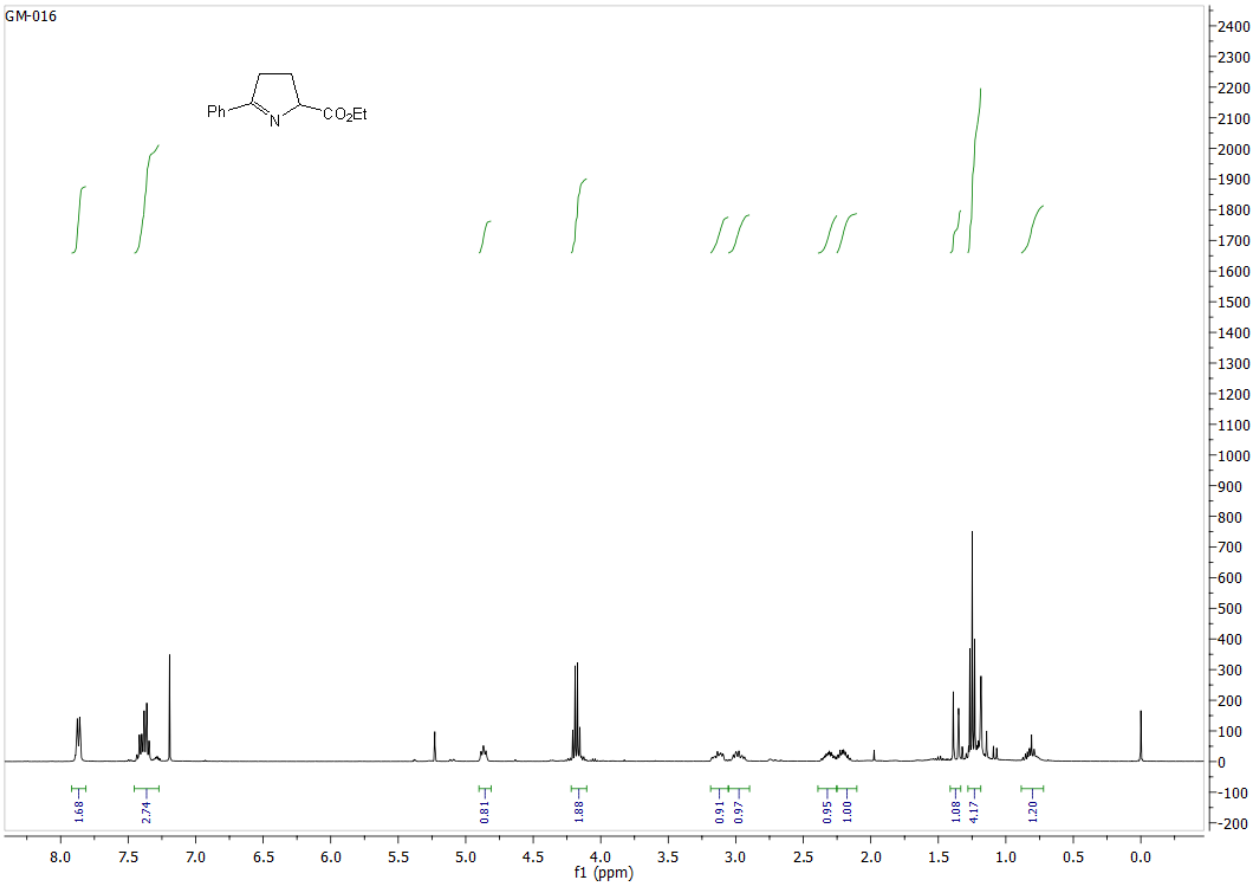
^{1}H NMR compound 20



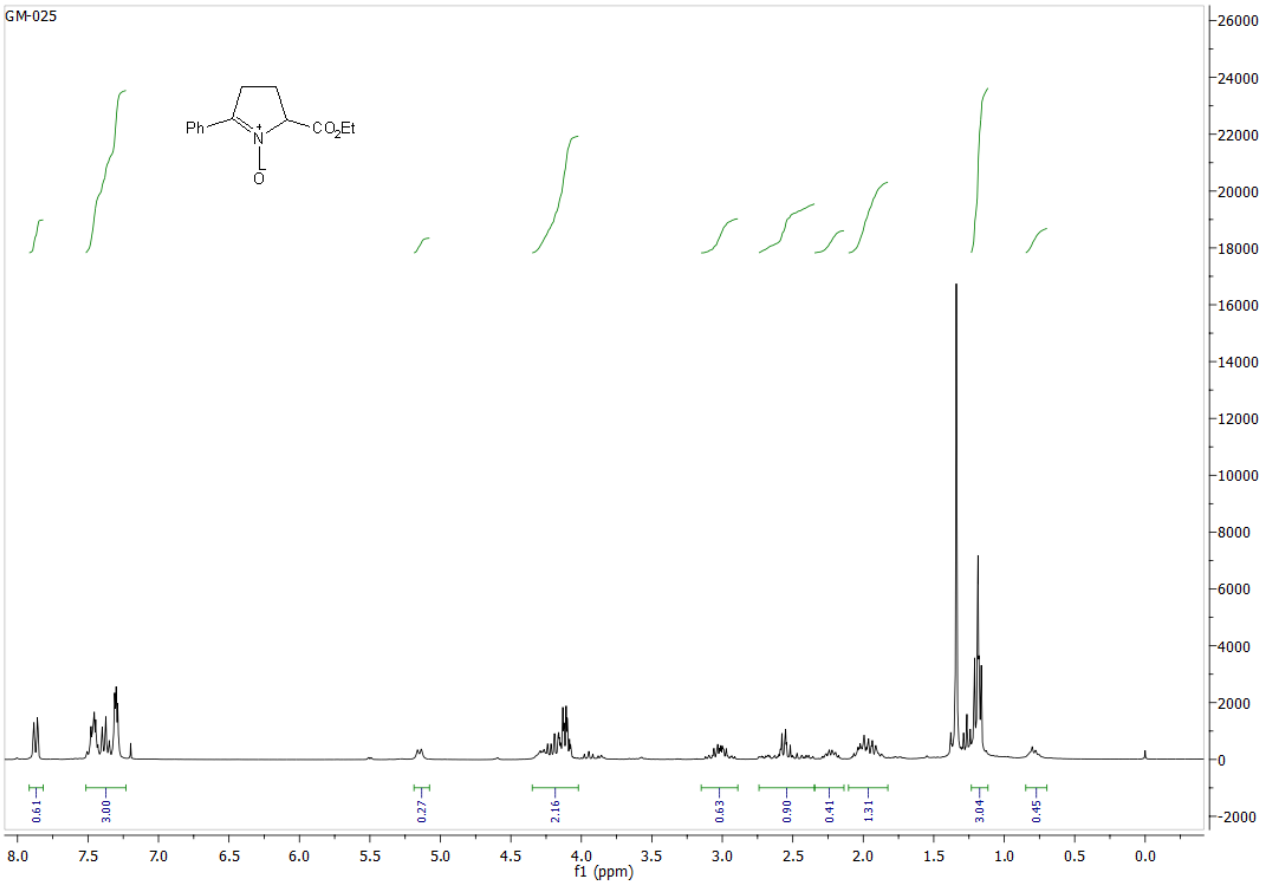
^{1}H NMR compound 21



^{1}H NMR compound 22



^{1}H NMR compound 23



3. HIV

The Human Immunodeficiency Virus (HIV) belongs to the *Retroviridae* family. Three subfamilies can be distinguished: Oncovirinae, Lentivirinae, Spumavirinae.

HIV takes part to the Lentivirus family. These are viruses that cause persistent infections and cause immunosuppression and chronic degenerative diseases due to the fact that the target of the virus are the CD4 + lymphocytes, cells that are involved in the immune response.

HIV is an RNA virus with a spherical shape, with a diameter of 80-120 nm. The viral envelope , is made up of a double-layer lipid membrane that is acquired by the cytoplasmic membrane of the host cell during the phase of viral healing. The vial contains viral glycoproteins, gp120 and gp41, formed following the proteolytic cleavage of the gp160 polyprotein. Gp120 is a glycosylated protein, has a molecular weight of 120 kD, protruding from the surface of the viral particle. This glycoprotein is responsible for the tissue tropism of HIV. It is recognized by neutralizing antibodies and its antigenicity and receptor specificity may vary during a chronic HIV infection. Gp41 is the transmembrane glycoprotein (41 kD), responsible for the fusion of viruses and target cells.

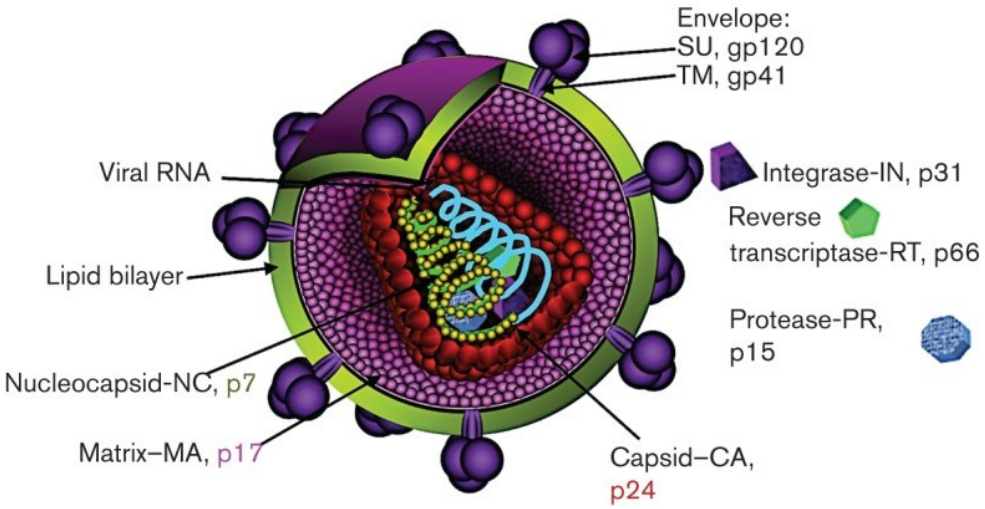


Figure 10

The envelope surround the capsid and is constituted by an external portion called the matrix, and by a central portion, the core or nucleocapside.

The matrix is made up of the p17 protein, which extends between the inner portion of the viral envelope and the nucleocapside. Protein p17 plays an important role in transporting structural precursors and viral glycoproteins to the cytoplasmic membrane of the host cell and promotes incorporation into the nascent virion.

The core of HIV virion, structured like a cone-shaped, is mainly composed of p24 protein.

Within the nucleus, two single-stranded genomic RNA molecules having positive polarity, these are covalently bound to reverse transcriptase and associated with two basic proteins, p7 and p9, to form a nucleoprotein complex.

The nucleocapside is constituted by protease, reverse transcriptase, integrase and RNase H, all viral enzymes necessary for the replication of virions. The protease acts

the clivage of viral polyproteins and process the replication enzymes.

Whereas , integrase are the enzyme needed for the integration of the viral genome in the host cell DNA[22]. Finally, RNase H, deriving from the clivage of the RT, is an endonuclease capable of producing molds for reverse transcriptase[23].

Reverse transcriptase (RT) is indispensable for the synthesis of linear double strand DNA from genomic RNA.

Reverse transcriptase has two activities:

- DNA polymerase activity: it is able to synthetize the corresponding DNA molecule by using either a RNA molecule or a single filament DNA as a mold. It requires a fragment of DNA or RNA to use as a starter to initiate the polymerization reaction;
- RNase H activity: it can deteriorate the RNA from the RNA-DNA hybrids that are formed during the polymerization reaction, in order to form a double filament of DNA from a single RNA filament.[24]
- Figure 17 shows that inverse HIV transcriptase is a heterodimer whose structure has the shape of a grabbing hand and consists of two subunits which have respectively molecular weights of 66 and 51 kDa respectively named as p66, 560 aa, and p51, of 440 aa. The p51 subunit derives from the proteolytic cleavage of the p66 C-

terminus which involves the loss of the Rnase H domain and is operated by a viral protease.

DNA-Polymerasic and Ribonucleasic catalyst activities are carried out by the p66 enzymatic subunit, while the p51 subunit is essential for stabilizing the tRNA primer binding at the beginning of the retrotrascription, for chain shifting and the processing step during DNA synthesis.

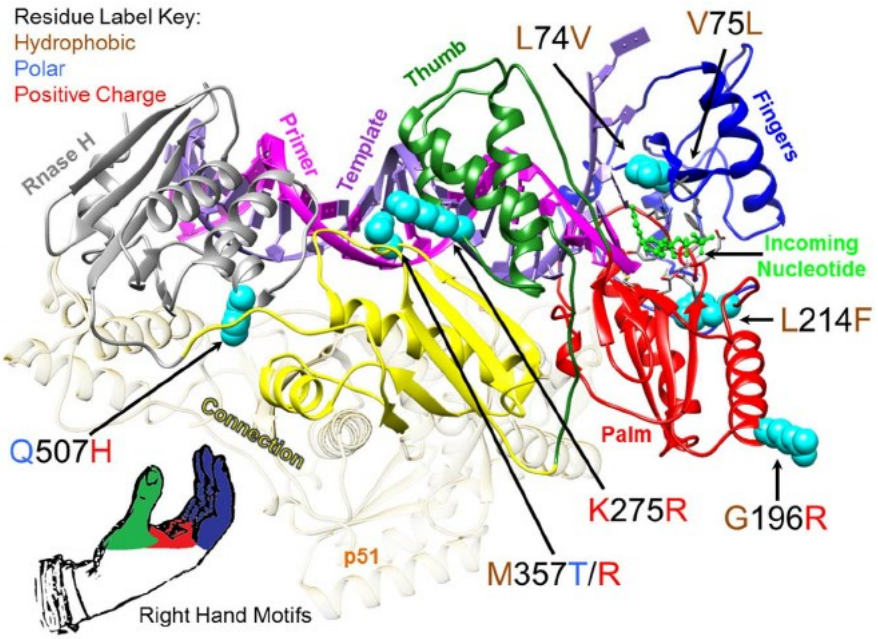


Figure 11

Being Reverse Transcriptase an essential viral enzyme for replication, it is one of the major drug targets used in current antiretroviral therapies.

Figure 12

There are two important categories of drugs that inhibit the enzyme activity:

• **Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTIs and NtRTIs):** They are structural analogues of the natural substrate (dNTP) and compete with the binding site (dTTP). They act as chain terminators as they have a free oxydril group in 3'. Unfortunately, they are harmful to the cells.

• **Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs):**

These molecules are chemically different from the natural substrate. They block the polymerase enzyme activity by interacting with it in a non-competitive way. Non-nucleoside reverse transcriptase inhibitors bind to an allosteric site far from the catalytic site. The allosteric site is made up of two tryptophan residues surrounding the NNRTI molecule and incorporating it[25].

These molecules are thought to prevent the conformational changes essential to stretching the DNA strand. Moreover, they do not cause cytotoxicity at therapeutic doses.

Early drug design studies show that the structures of some classes of NNRTIs seem to diverge, but from more in-depth studies it has been noted that most have common features:

• a hydrophilic "body" represented by a carboxylic or (thio) acetamide or ureid group, surrounded by

• two "hydrophobic" wings.

Hence, the structure evokes the shape of a butterfly, with a body (hydrophilic) and two wings (hydrophobic).

This "Butterfly-like" model has been confirmed by crystallographic analysis.

3.1 NNRTIs

NNRTIs have received lot of attentions because of their favourable/positive potency and low cytotoxicity. Five NNRTIs have been approved for AIDS treatment, so far and about 50 classes of structurally diverse NNRTIs are being widely investigated[26].

In this context, the pyrimidine nucleus represents a versatile chemical core in the design of many antiretroviral agents acting as NNRTIs. Modified pyrimidines constitute the backbone of many non nucleoside reverse transcriptase inhibitors [27].

1-[(Hydroxyethoxy)methyl]-6-(phenyl-sulfanyl)thymine (HEPT **1**) and its analogue (TNK-651 **2**) 2-alkoxy-6-benzyl-3,4-dihydro-4-oxopyrimidine (DABO **3**), diaryl-pyrimidines (DAPYs **4**) and truncated reverse isoxazolidinyl nucleosides (TRINs **5**) (Figure 19) are all families of potent NNRTIs that, through binding at the allosteric, non-nucleoside binding pocket (NNIBP) of RT, prevent the conformational transition needed for the formation of a productive polymerase–RNA complex.

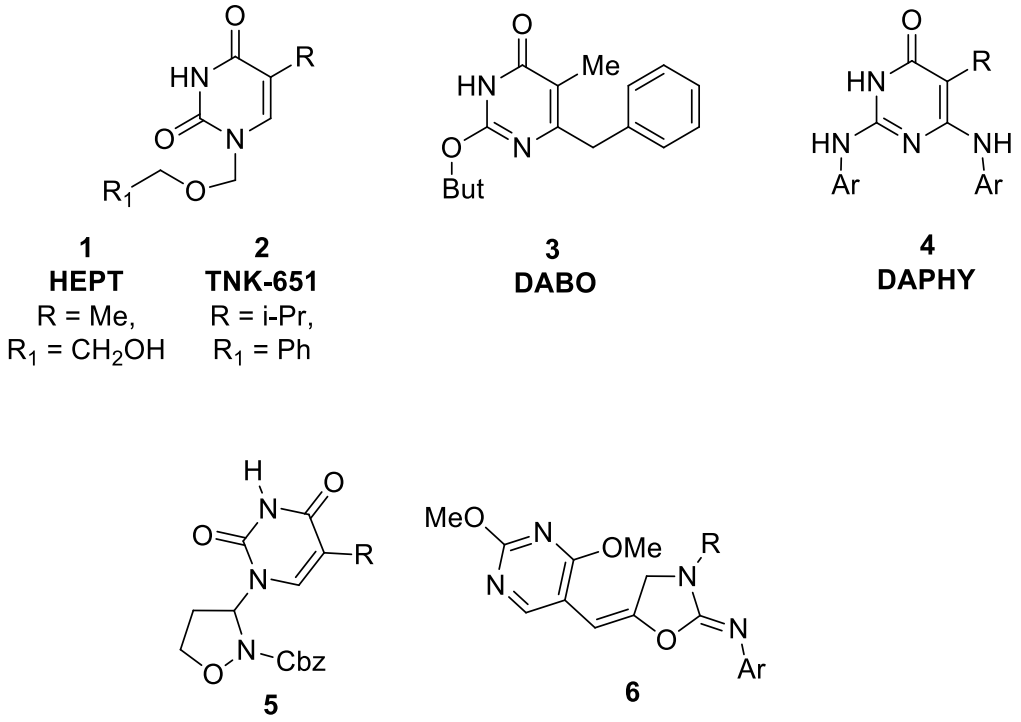


Figure 13

In the exploitation of chemotypes amenable to the construction of conformationally controlled new compounds endowed with significantly improved drug resistance profiles, compared with the first generation of NNRTIs[28], I report the design, the synthesis and the biological evaluation of a new series of compounds, **6**, amenable to derivatives **3**, where structural elaboration has been performed towards inhibiting different targets of HIV-1. We made core refinements and several modifications on the substituents of the DABO series trying to obtain improved anti-HIV activity. In particular, the synthesized derivatives are characterized by the replacement of the methyl substituent at C-5 with a methyleneisoxazolidine 2'-imino unit: this modification should affect the biological properties by controlling the conformational behavior. Moreover the introduction of various substituents on the imino group should

improve the lipocifility of the compounds allowing a better interaction with the enzyme.

3.2 DESIGN AND SINTHESIS

Recently our research group has developed a new synthesis of the piperazinone ring in only three steps through a copper-intramolecular reaction catalyzed by stoichiometric amount of NBS, followed by a subsequent *cross- coupling* reaction performed with Pd (0).

An extension of this study has led to hypothesize the synthesis of oxazolidinone derivatives applying the same methodology.

These heterocycles, for their chemical nature, are biologically relevant; they present an α,α'-disubstituted amino ketone. The masked form of 2-amino ketone function increase the lipophilicity and, consequently, the easiest "*drug delivery*".

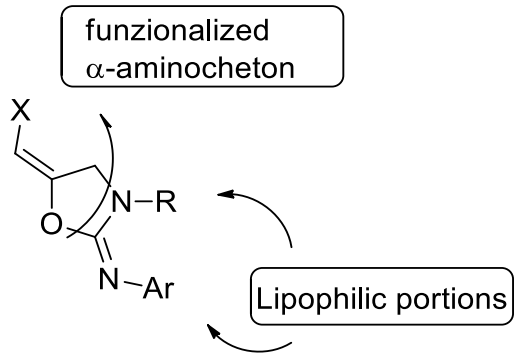


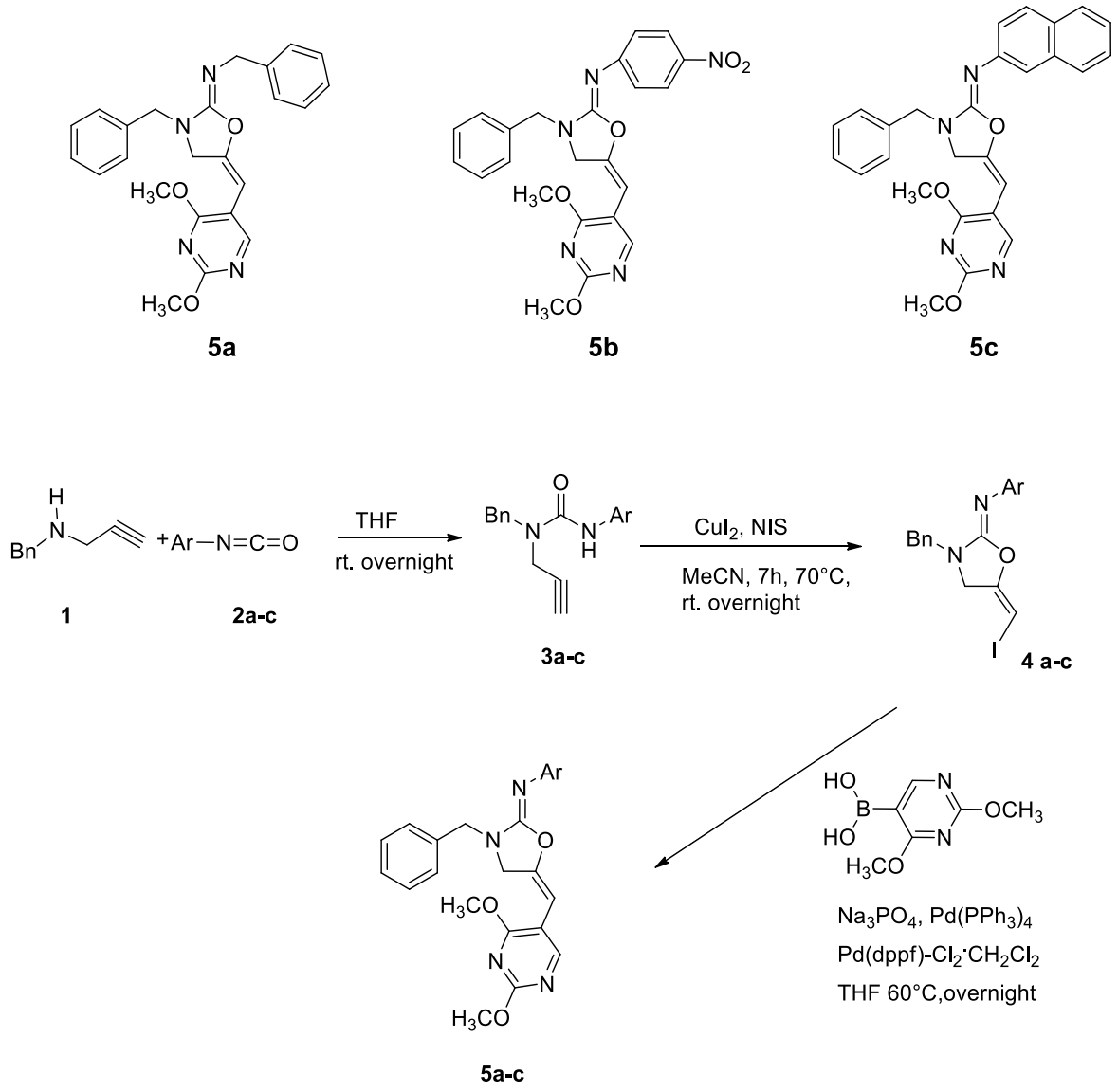
Figure 14

The modification of this structure with the introduction of pyrimidine ring at the methylidene junction provides a new series of structurally related pyrimidine-1,3-oxazolidin-2-arylimino hybrids which contain two heterocycles that are widely used in

medicinal chemistry. With this in mind we have designed the synthesis of *(2,4-dimethoxypyrimidin-5-yl) methylene) oxazolidin-2-imines (**5a-c**)*, structurally related to DABO as potential non-nucleoside reverse transcriptase inhibitors.

3.3 Chemistry

The synthetic approach of the designed compounds was achieved through three steps and is shown in the general scheme 6.



Scheme 4

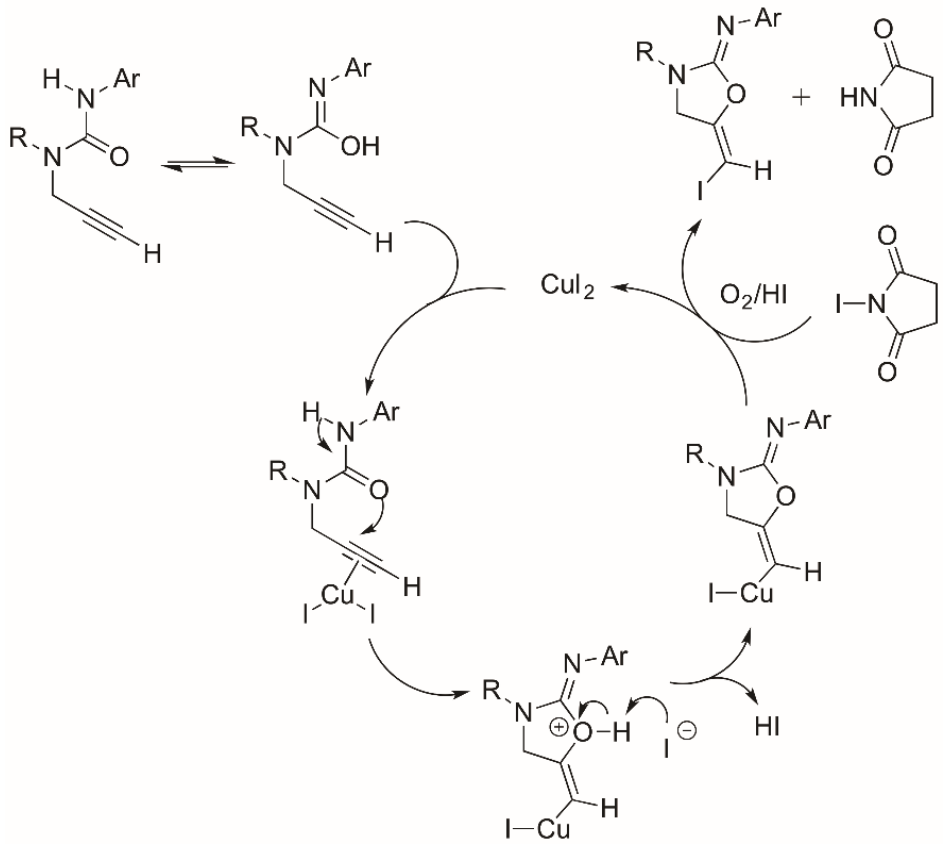
The alchinylureas **3a-c** were prepared by reacting 1.2 equivalents of N-benzyl-propargylamine **1** in anhydrous THF with an equivalent of the respective isocyanates **2a-c**: benzylisocianate, 4-nitrophenyl isocyanate and 1-naphthyl isocyanate. The reaction was conducted under inert atmosphere and stirring at room temperature overnight.

Oxazolidine derivatives **4a-c** were prepared by reacting 1 equivalent of **3a-c**, solubilized in anhydrous CH_3CN with 1 equivalent of N-iodosuccinimide and 1 equivalent of CuI_2. The reaction was conducted under N_2 and reflux at a temperature of 70 ° C for 7 h and then at room temperature for 14 h. Subsequently, the reaction mixture was filtered over silica gel, washing with ethyl acetate. The solution was then concentrated in *vacuo*. The compounds **4a-c** were purified by flash chromatography and subsequently characterized for ^{1}H and ^{13}C NMR.

The ^{1}H NMR spectrum, in $CDCl_3$of compound **4b**, selected as a model compound, shows three singlets at 5.77, 4.65, and 4.04 ppm, which can be referred to the allyl, methylene and benzylic protons respectively. The signals in the aromatic range from 7.35 to 7.31 ppm is attributed to the benzene aromatic protons and the double doublets of protons in ortho and para of the nitro group centered at 8.1 and 7.097 with a J of 9 Hz.

On the basis of the experimental evidence a plausible mechanism to explain the alkoxyalogenation reaction leading to formation of the variously substituted **4a-c** N-

aryl-1,3-oxazolidin-2-imines with CuI_2 and N-iodosuccinimide is shown in the following general scheme:



The catalytic cycle would proceed through the vinyl copper intermediate **A**, generated from an 5-exo-dig cyclization by nucleophilic attack of the oxygen atom on the activated carbon-carbon triple –bond. The subsequent deprotonation of **A** forms the intermediate **B**, which is able to interact with NIS, in presence of HI and oxigen , providing the final oxazolidine-imine and regenerating the copper catalyst. The evolution of intermediate B could also proceed by Copper (III) species that undergoes reductive elimination.[29]

The final step which lead to the pyrimidine-1,3-oxazolidin-2-arylimino hybrids **5a-c** was performed according to Suzuki-Miyaura reaction[30] involving as reagents 4a-c and 2-4-dimethoxypyrimidine boronic acid in the presence of catalytic amount of [1,1′-Bis(diphenylphosphino)ferrocene]dichloropalladium(II), complex with dichloromethane. **5a-c** compounds were purified through flash chromatography and then characterized by ^{1}H NMR and ^{13}C NMR.

The ^{1}H NMR spectrum, in deuterated chloroform of the compound **5b** chosen as lead compound , shows the two signals of pyrimidine methoxyl at 3.98 and 3.96 ppm as singlet; the signal of the methylene protons of the oxazolidine ring as singlet at 4.22 ppm and a peak at 6.20 ppm that refers to the allyl proton. The signals of the aromatic protons belonging to the imino-linked phenyl group are easily attributed. However, hydrogen in ortho position to the nitro group can be observed at 8.16 ppm and 8.20 ppm, and the meta ones at 7.21 and 7.22 ppm. The aromatic zone of the spectrum is thick with multipletes overlapping, making their single attribution difficult. The multiplets are identified between 7.34 and 7.40ppm. Hydrogen belonging to the pyrimidine ring is at 7.88ppm.

^{13}C spectrum shows the signal of methylenic carbon of oxazolidine core at 47.7 ppm, CH2 benzyl at 48.7 ppm and methoxyl carbons at 54.9 ppm and 54.1 ppm.

In addition, we can distinguish the signals of the carbon of oxazolidine nucleus, the one that we found at 158.6 ppm is the signal of the imminic carbon, and the one at153.6 ppm refers to the alkenyl carbon.

The C-H bond of the alkenyl group shows a chemical shift at 109.0 ppm. The aromatic carbon signals dropped to 146.6 ppm are relative to the carbon of the phenyl group directly linked to imminic nitrogen, at 135.1 ppm correspond the carbon of the pyrimidine ring linked to the double bond. In the area of aromatic signals, we can see two deschermed signals of the C-H belonging to the aryl bound of imminic nitrogen. They are at 123.8 ppm (carbons in orto) and 108.1 ppm (carbons in meta).

We might note the peak at 150.4 ppm which is directly linked to the nitro group. Peaks at 128.2 ppm, 129.0 ppm, 135.1 ppm and finally 146.6 ppm represent benzyl carbons. Allylic carbon drops to 95.6 ppm. The aromatic carbon of the pyrimidine ring linked to the double bond, more deschermed, is at 109.0 ppm. The carbons bound to the two methoxy groups fall to 165.0 ppm and 168.5 ppm. The peak at 164.1 ppm shows the C-H of the pyrimidine ring.

The series of pyrimidine-1,3-oxazolidin-2-arylimino hybrids **5a-c** thus synthetized are now under biological screening to verify their anti-HIV activity as predicted by docking studies.

3.4 MOLECULAR DOCKING

In order to study the interaction modalities of synthesized compounds with the HIV enzyme, silica studies were performed using the compound (**5b**) as a model. The 3D structure of the complex that includes the non-nucleous inhibitor (TNN-RNA), TNK-651, with the HIV enzyme was recovered from the Protein Data Bank (code 1RT2). Subsequently, the TNK-651 inhibitor was removed from the model and the missing atoms were rebuilt and optimized locally. Hence, hydrogen atoms were added using the MolProbity Web server, and assigned the charges according to the Gasteiger-Marsili scheme. The protein, with its ligand repositioned in the binding site, has been optimized using MMFF94s as a force field. Computational docking studies were conducted using AutoDock Vina with a grid spacing equal to 0.375 Å and a grid size of 30 × 30 × 30, capable of covering the entire active site. I have been generating 9 poses for the selected ligand. The results of docking studies have shown that compound **5b** is a good candidate for NNRTI since it shows good ability to fit within the enzyme allosteric site with a ΔG bind of -8.1 kcal / mol.

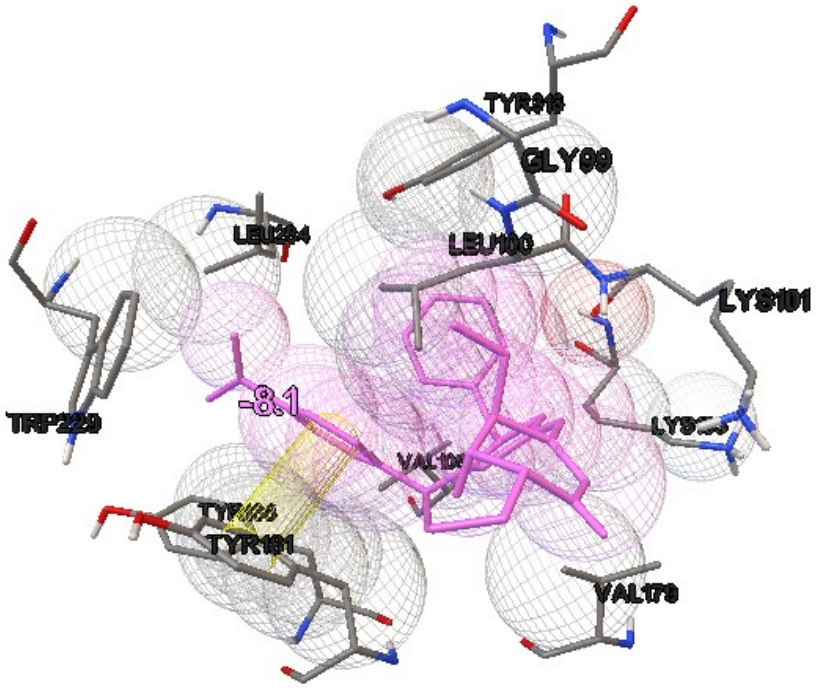


Figure 15

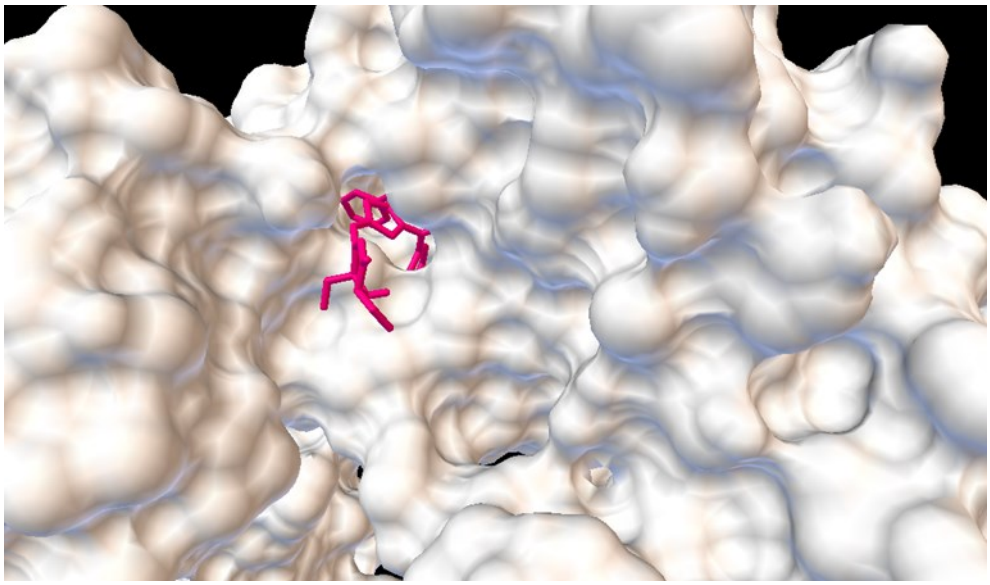


Figure 16

3.5 Materials and methods

All chemicals were purchased from Sigma–Aldrich Chemical Co. The solvent was removed at aspirator pressure using a rotary evaporator. TLC was performed with Merck precoated TLC plates, and the compounds were made visible using a fluorescent inspection lamp and iodine vapor. Gravity chromatography was done with Merck silica gel 60 (mesh size 63–200 μm). Nuclear magnetic resonance spectra were recorded on a Varian Inova instrument, operating at 500 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR. Chemical shifts (δ) for ^{1}H NMR spectra are reported in ppm downfield relative to the center line of $CDCl_3$ triplet at 7.26 ppm. Chemical shifts for ^{13}C NMR spectra are reported in ppm downfield relative to the center line of $CDCl_3$ triplet at 77.23 ppm. The abbreviations s, d, t, and m stand for the resonance multiplicities singlet, doublet, triplet, and multiplet, respectively. ^{13}C spectra, are ^{1}H decoupled, and multiciplities were determined by APT pulse sequence. The melting points were recorded on a Boëtius hot plate microscope. FT-IR spectra were recorded on FT-IR Shimadzu spectrometer (4000–400 $cm^{-}1$. EI-MS and HRMS were performed with Finnigan MAT 95, EI: 70 eV, R:10000.

1,3-dibenyl-1-etynilurea (3a)

To a solution of N-benzyl-propargylamine **1** (0.305 mL, 2.06 mmol) in 5 mL of anhydrous THF was added benzyl isocyanate (0.383 mL, 3.09 mmol). The reaction was conducted under inert atmosphere and proceeded at room temperature for 24 h. The solution has a yellow appearance. The solvent is then removed under reduced pressure and the product obtained is a yellow solid. Urea obtained was characterized by ^{1}H NMR. Yield 84%. ^{1}H NMR (CDCl3, 500 MHz):^{1}H NMR: 2.78 ppm (1H, s), 4.50 ppm (2H, s), 4.79 (2H, s), 7.19-7.38 ppm (10H,m).

1-benzyl-3-(4-nitrophenyl)-1etinylurea (3b)

To a solution of N-benzyl-propargylamine **1** (0.300 mL, 2.7 mmol) in 5 mL of anhydrous THF was added 4-nitrophenyl isocyanate (0.366 g, 2.23 mmol). The reaction was stirred under inert atmosphere and proceeded at room temperature for 24 h. The solution was yellow. The solvent is removed under reduced pressure. An intense yellow solid is obtained. Urea has been characterized by ^{1}H NMR.

Yield 78%. Yellow solid. ^{1}H NMR (CDCl3, 500 MHz): 2.78 ppm (1H, s), 4.50 (2H, s), 4.79 (2H, s), 7.19-7.38 (10H).

1-benzyl-1-etynil-3-naphtalen-2-il-urea (3c)

To a solution of N-benzyl-propargylamine **1** (0.305 mL, 2.07 mmol) in 5 mL of anhydrous THF was added 1-naphthyl isocyanate (0.44 mL, 3.102 mmol). The reaction was conducted under N_2 and proceeded at room temperature for 20 h. The solution was yellow. Then the solvent was removed at low pressure.

(2*Z*,5*E*)-*N*,3-dibenzyl-5-(iodomethylene)oxazolidin-2-imine (4a)

To a solution of allylurea (0.3 g, 1.07 mmol) in CH3CN anhydrous (14 mL) was added N-iodosuccinimide (0.3 g, 1.07 mmol) and CuCl2 (0.0183 g, 0.107 mmol). The reaction proceeds under N_2. It was refluxed for 7 h at 70 ° C and then left for at room temperature 14 h. The reaction mixture assumes a red-brown appearance. Subsequently, the reaction mixture formed a precipitate, it was filtered on a silica gel

septum, washing with ethyl acetate. After evaporating the whole solvent, an orange oil was obtained. The residue was purified by flash chromatography (ethyl acetate / cyclohexane 10:90). Oil ,Yield 63%. Rf: 0,5.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 – 7.29 (m, 8H), 7.23 –7.18 (m, 2H), 5.62 (t, *J* = 2.6 Hz, 1H), 4.52 (d, *J* = 2.6 Hz, 2H), 3.81 (s, 2H), 3.79 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.4 (s), 154.5 (s), 142.0 (s), 137.4 (s), 129.5 (d), 129.2 (d), 129.0 (d), 128.3 (d), 127.7 (d), 52.3 (d),51.5 (t), 51.4 (t), 49.5 (t). HRMS-EI (m/z) [M^+] calcd for $C_{18}H_{17}IN_2O$ 404.0433 found 404.0431

(2*Z*,5*E*)-3-benzyl-5-(iodomethylene)-*N*-(4-nitrophenyl) oxazolidin-2-imine (4b)

To a solution of allylurea (0.3 g, 1.02 mmol) in CH3CN anhydrous (14 mL) was added N-iodosuccinimide (0.3 g, 1.02 mmol) and CuCl2 (0.0173 g, 0.101 mmol). The reaction proceeds under N_2. It was refluxed for 7 h at 70 ° C and then left for at room temperature 14 h. The reaction mixture assumes a red-brown appearance. Subsequently, the reaction mixture formed a precipitate,it was filtered on a silica gel septum, washing with ethyl acetate. After evaporating the whole solvent, an orange oil is obtained. The residue was purified by flash chromatography (ethylene / cyclohexane acetate 5:95). Oil,Yield 76%

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15 (d, *J* = 9.0 Hz, 2H), 7.43 – 7.33 (m, 5H), 7.16 (d, J = 9.0 Hz, 2H), 5.77 (t, *J* = 2.7 Hz, 1H), 4.64 (s 2H), 4.04 (t, *J* = 2.7 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.4 (s), 154.9 (s), 154.4 (s), 141.6 (s), 137.3 (s), 129.3 (d), 129.0

(d), 128.9 (d), 126.2, (d), 123.5 (d), 52.7 (d), 51.8 (t), 49.4 (t). HRMS-EI (m/z) [M^+] calcd for $C_{17}H_{14}IN_3O_3$ 435.0127 found 435.0124.

(2Z,5E)-3-benzyl-5-(iodomethylene)-*N*-(naphthalen-1-yl)oxazolidin-2-imine (4c). To a solution of alkylurea (0.3 g, 0.954 mmol) in CH3CN anhydrous (14 mL) was added N-iodosuccinimide (0.214 g, 0.954 mmol) and CuCl2 (0.0162 g, 0.0954 mmol). The reaction proceeds under an inert, reflux atmosphere. It was left under stirring for 7 h at 70 ° C and then for 14 h at room temperature. The reaction mixture assumes a red-brown appearance. During the reaction a precipitate is formed which has to be eliminated. The solution was then filtered over silica gel septum, washing with ethyl acetate. The mixture is brought to dry under reduced pressure. After evaporating the whole solvent, a dark red oil is obtained. The residue was purified by flash chromatography using as eluent ethylene / cyclohexane acetate 5:95 mixture. Yield 79%. Oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.90 (d, *J* = 8.4 Hz, 1H), 7.52 (d, J = 8.5 Hz 1H), 7.44 –7.32 (m, 8H), 7.16 (d, 1H), 6.91 (d, *J* = 8.4 Hz, 1H), 5.56 (t, J = 2.6 Hz, 1H), 4.72 (s, 2H), 4.00 (t, J = 2.6 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.5 (s), 154.3 (s), 147.2 (s), 137.4 (s), 136.5 (s), 129.3 (d), 129.1 (d), 128.9 (d), 127.9, (d), 127.5 (d), 127.0 (d), 126.5 (s), 125.8 (d), 124.2 (d), 122.4 (d), 121.2 (d), 52.4 (d), 51.5 (t), 49.6 (t). RMS-EI (m/z) [M^+] calcd for $C_{21}H_{17}IN_2O$ 440.0433 found 440.0435.

(2*E*,5*E*)-*N*,3-dibenzyl-5-((2,4-dimethoxypyrimidin-5-yl) methylene) oxazolidin-2-

imine (5a)

To a solution of 3M of THF water in the substrate (4a) (0.300 g, 0.7139 mmol) was added 2-4-dimethoxypyrimidine boronic acid (0.522 g, 2.85 mmol) and Na3PO4 (0.702 g, 4.28 mmol) and finally the Pd (0.0427). The reaction was refluxed at 60 ° C for one night. The solvent was removed and the residue washed with water and saturated NaCl solution, dried over $MgSO_4$ filtered and concentrated in *vacuo*. The product was purified by flash chromatography (ethyl acetate / cyclohexane 20:80). Pale yellow-yellow oil with gummy consistency, 45% yield.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.86 (s, 1H), 7.41-7.39 (m, 2H), 7.37 – 7.28 (m, 7H), 7.24 7.21 (m, 1H), 6.13 (t, *J* = 2.4 Hz, 1H), 4.59 (s, 2H), 4.55 (s, 2H), 4.08 (d, *J* = 2.4 Hz, 2H), 3.98 (s, 3H), 3.96 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.2 (s), 163.5 (s), 157.8 (d),155.2 (s), 146.4 (s), 142.3 (s), 137.5 (s), 129.7 (s), 129.2 (d), 128.2 (d), 127.3 (d), 107.9 (d), 95.4 (d), 54.8 (q), 54.1 (q), 51.6 (t), 51.3 (t), 46.7 (t). HRMS-EI (m/z) [M^+] calcd for $C_{24}H_{24}N_4O_3$ 416.1896 found 416.1893.

(2*Z*,5*E*)-3-benzyl-5-((2,4-dimethoxypyrimidin-5-yl)methylene)-*N*-(4-nitrophenyl)oxazolidin-2-imine (5b)

To a solution of 3M of THF water of the substrate (4b) (0.300 g, 0.689 mmol) was added 2-4-dimethoxypyrimidine boronic acid (0.504 g, 2.75 mmol) and Na3PO4 (0.678 g, 4.13 mmol) and finally Pd (0.0505 g). The reaction was refluxed at 60 ° C for one night. The solvent under reduced and the residue washed with water and saturated NaCl solution, dried over $MgSO_4$ filtered and concentrated in *vacuo*. The crude product

was purified by flash chromatography,(ethyl acetate / cyclohexane 25:75). In TLC the product wass visible as a yellow spot. The product obtained is a orange.solid ,87% yield Rf:0,26.

^{1}H NMR (500 MHz, cdcl3) δ 8.20-8.16 (t, J = 8.1 Hz, 2H), 7.88 (s, 1H), 7.41 – 7.33 (m, 5H), 7.24 – 7.20 (m, 1H), 6.20 (t, 1H), 4.67 (s, 2H), 4.22 (d, J = 2.5 Hz, 2H), 3.98 (s, 3H), 3.97 (s, 3H).^{13}C NMR (125 MHz, $CDCl_3$) δ 168.6 (s), 164.5 (s), 158.6 (d), 153.5 (s), 150.4 (s), 146.6 (s), 142.5 (s), 135.0, (s), 128.9 (d), 128.2 (d), 124.6 (d), 123.8 (d), 108.5 (s), 95.6 (d), 54.8 (q), 54.1 (q), 48.6 (t), 47.6 (t). HRMS-EI (m/z) [M^+] calcd for $C_{23}H_{21}N_5O_5$ 447.1590 found 447.1591.

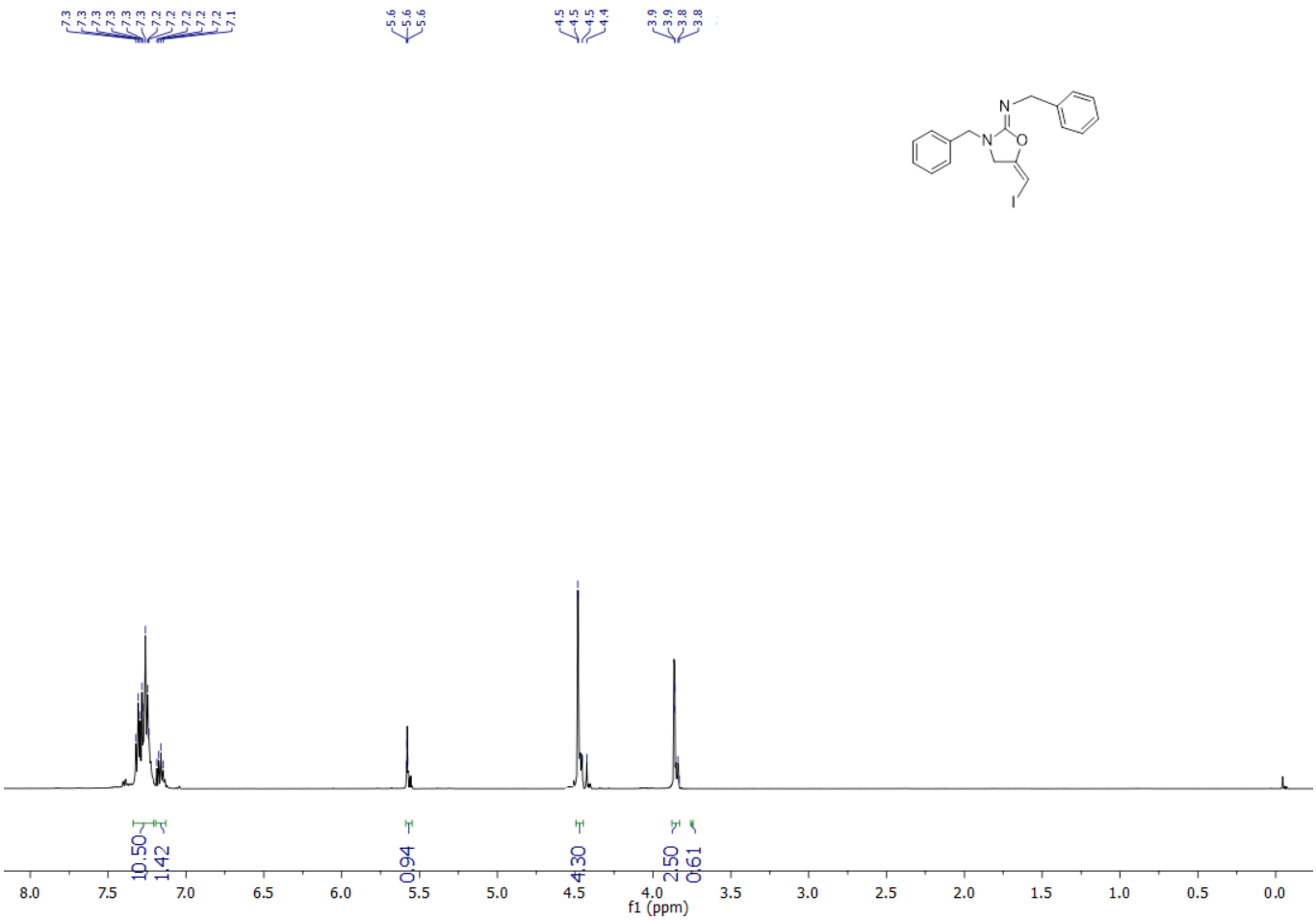
(2*Z*,5*E*)-3-benzyl-5-((2,4-dimethoxypyrimidin-5-yl)methylene)-*N*-(naphthalen-1-yl)oxazolidin-2-imine (5c)

To a solution of 3M of THF water of the substrate (4c) (0.300 g, 0.683 mmol) was added 2-4-dimethoxypyrimidine boronic acid (0.499 g, 2.73 mmol) and Na3PO4 (0.671 g, 4.09 mmol) and finally Pd (0.0500 g). The reaction was refluxed at 60 ° C overnight. The solvent removed and the residue washed with water and saturated NaCl solution, then dried over $MgSO_4$ filtered and concentrated in *vacuo*. The product was purified by flash chromatography(ethyl acetate / cyclohexane 18:82). In TLC the product was visible as a dark spot. The product obtained was a reddish-brown oil and sticky consistency.

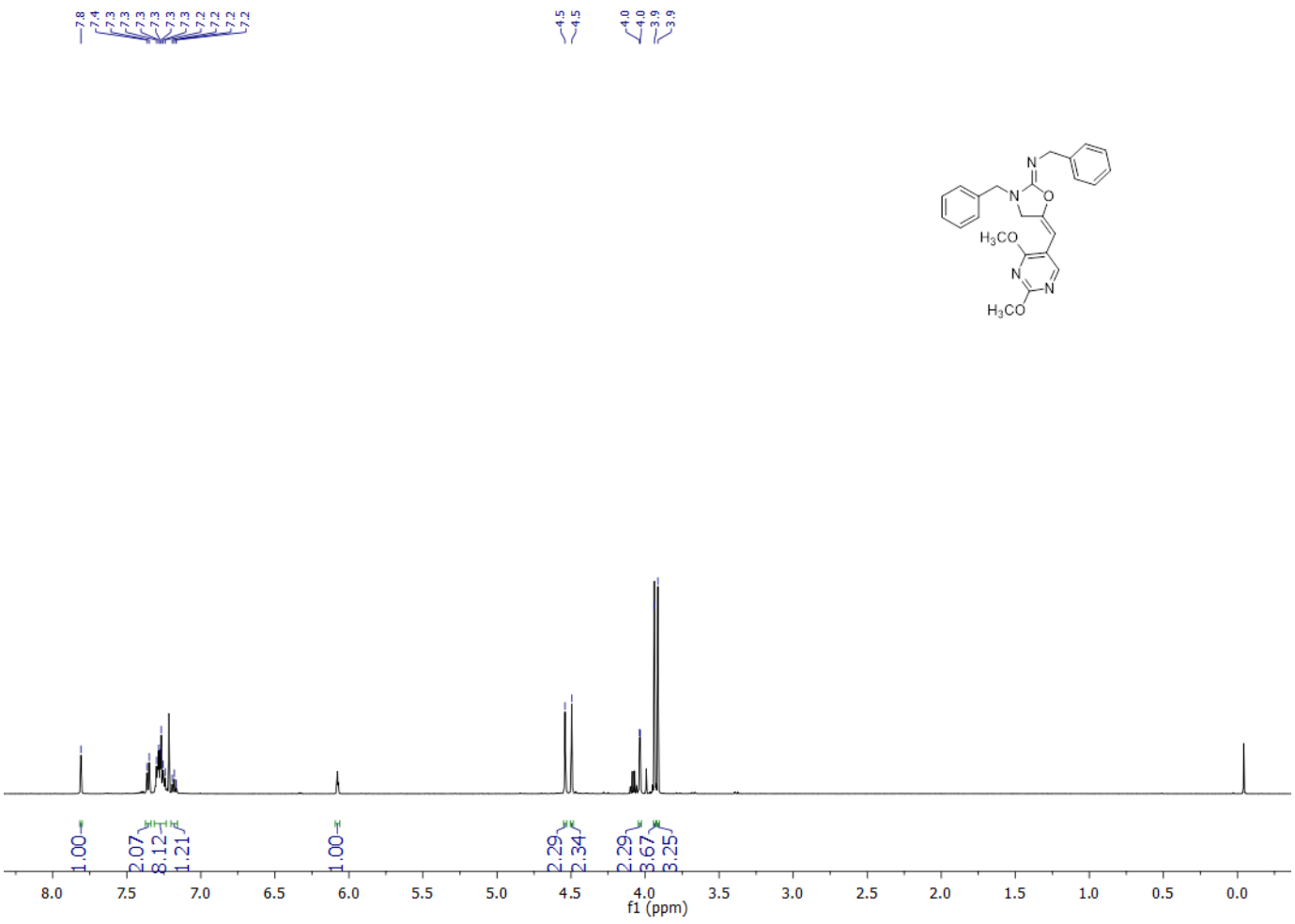
74% yield Rf:0,5.

^{1}H NMR (500 MHz, cdcl3) δ 7.76 (d, J = 6.6 Hz, 1H), 7.37 (s, 1H), 7.32 (d, J = 6.2, Hz, 1H), 7.06 (d, J = 8.2 Hz, 1H), 6.99 – 6.83 (m, 7H), 6.75 (d, J = 0.7 Hz, 2H), 5.58 (t, 1H), 4.30 (s, 2H), 3.74 (d, J = 2.5 Hz, 2H), 3.46 (s, 3H), 3.45 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.9 (s), 163.3 (s), 154.6 (d),149.2 (s), 149.9 (s), 142.9 (s), 135.8 (s), 134.9 (s), 129.4 (d), 128.9 (d), 128.3 (d), 128.0 (d), 127.7 (d), 127.5 (d), 126.5 (s), 125.9 (d), 125.6(d), 124.9 (d), 124.1 (d), 117.7 (s), 94.6 (d), 54.8 (q), 54.1 (q), 48.9 (t), 47.9 (t). HRMS-EI (*m/z*) [M^+] calcd for $C_{27}H_{24}N4O_3$ 452.1896 found 452.1894.

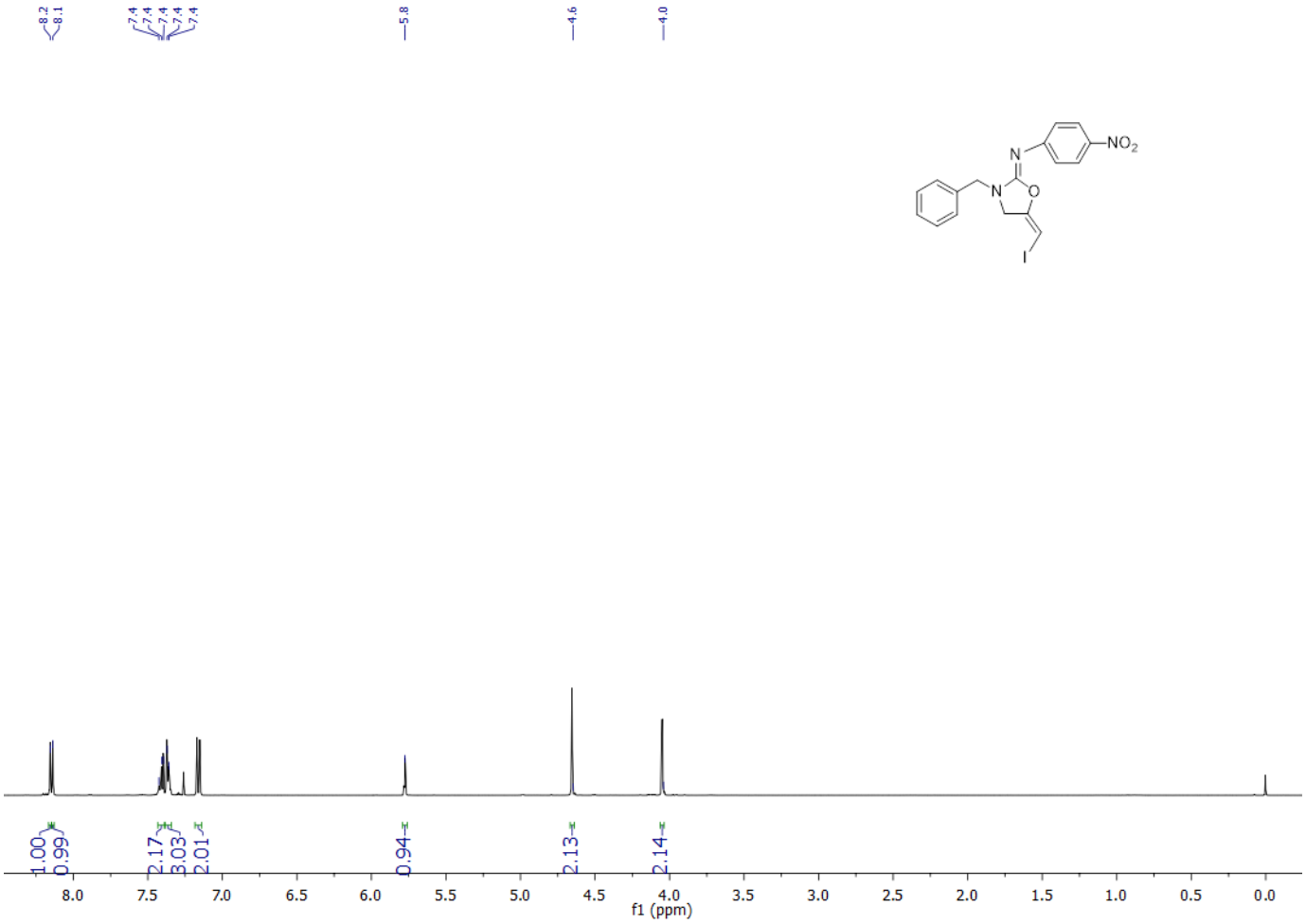
^{1}H NMR compound 4a



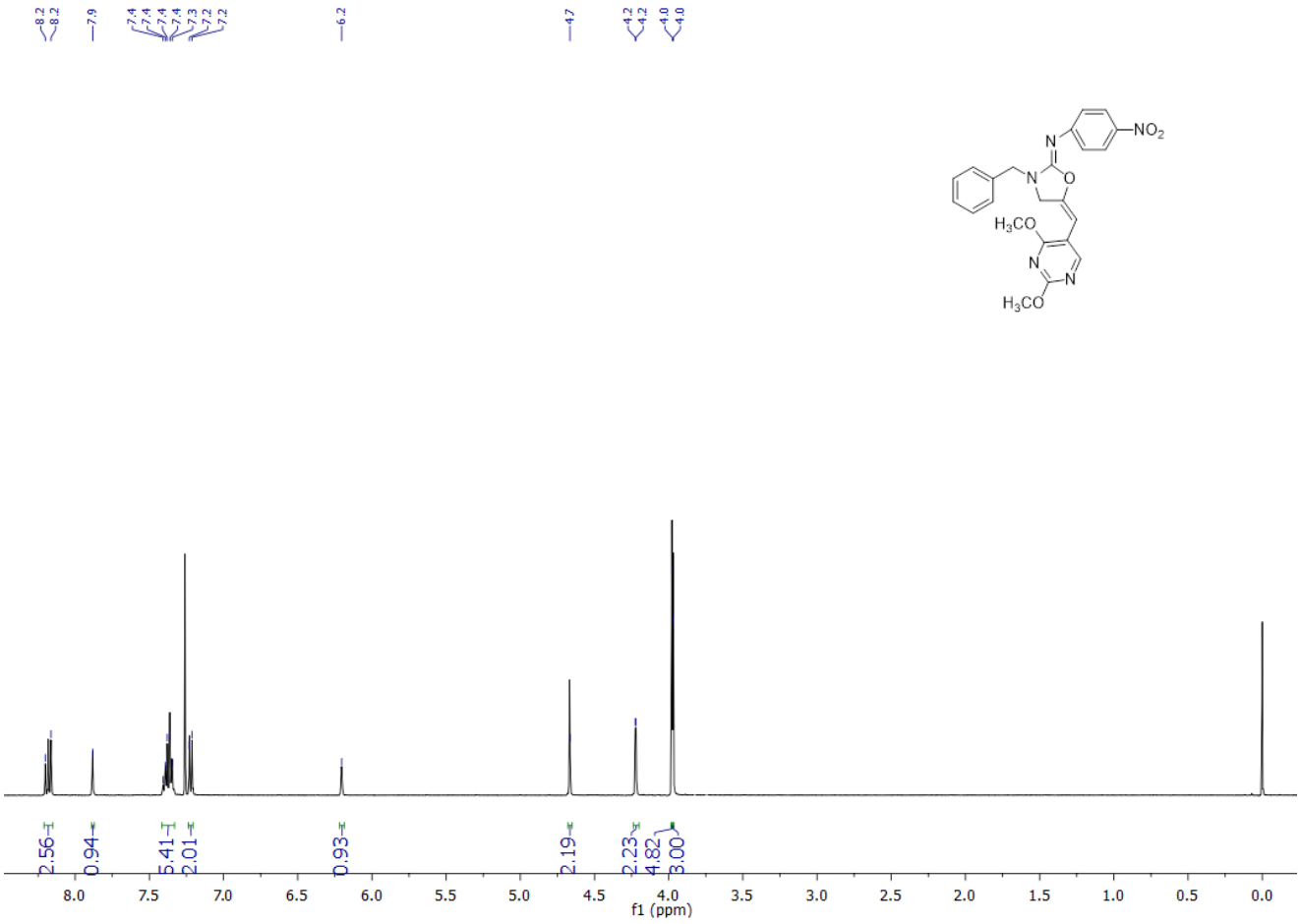
^{1}H NMR compound 5a



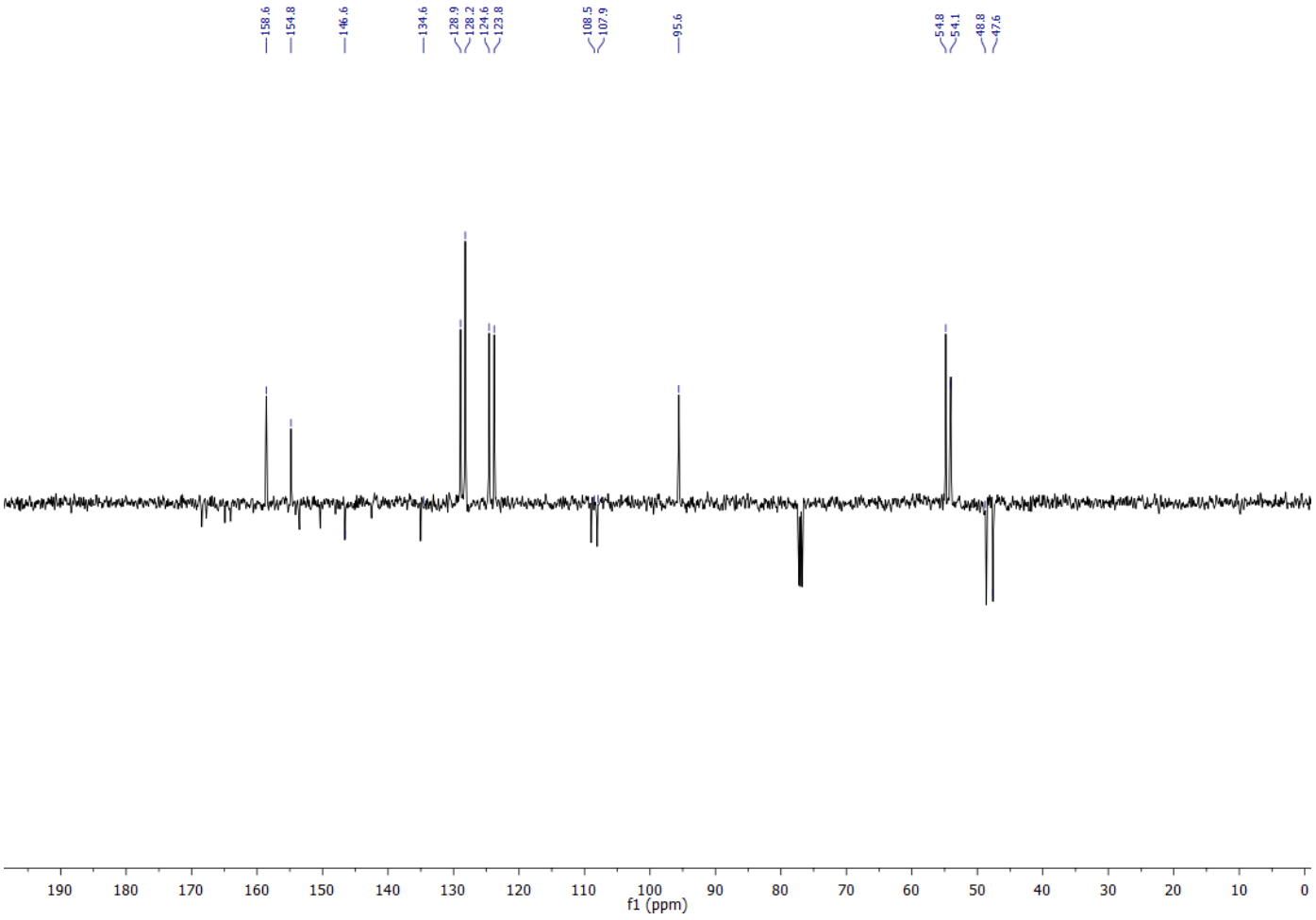
^{1}H NMR compound 4b



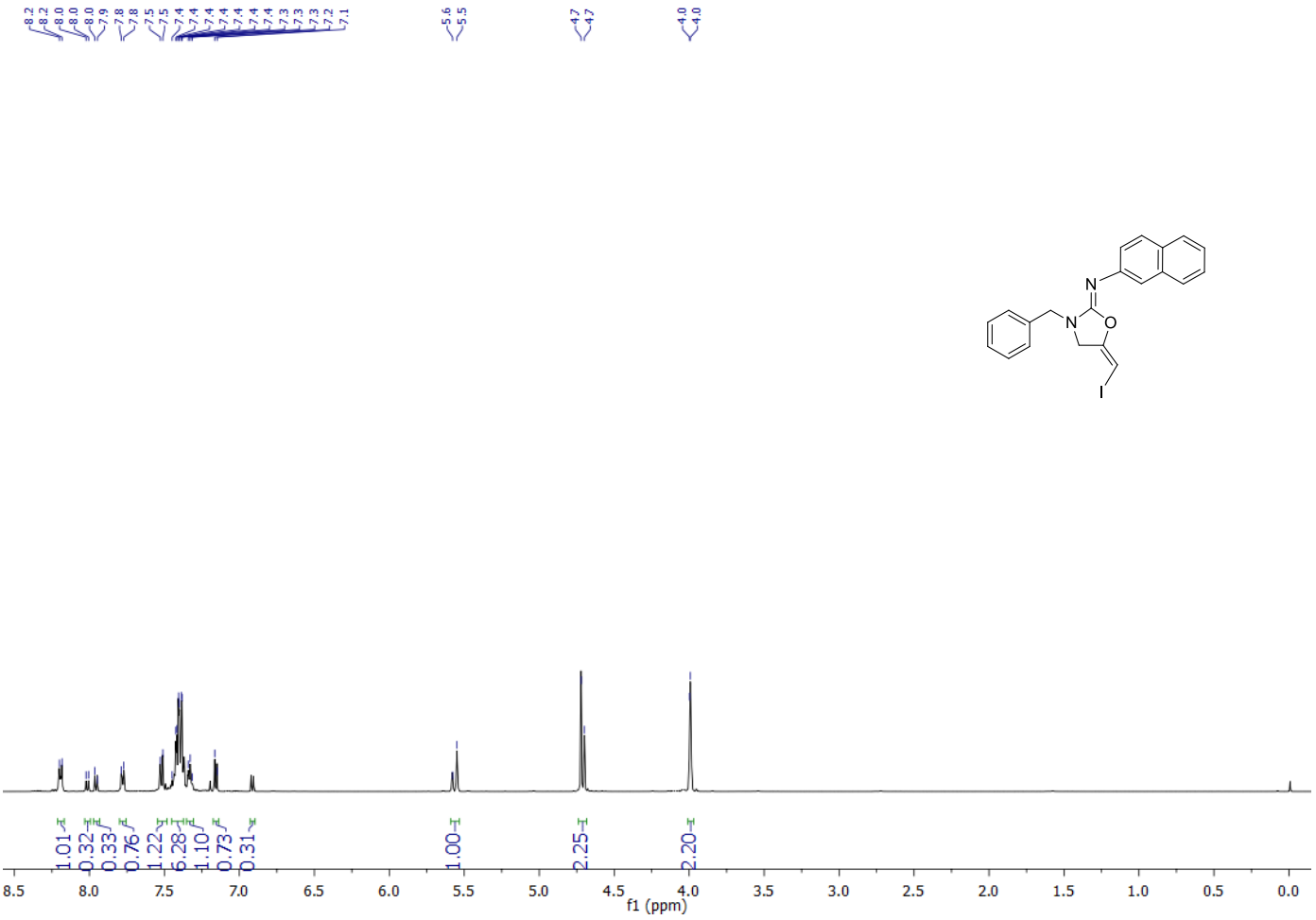
^{1}H NMR compound 5b



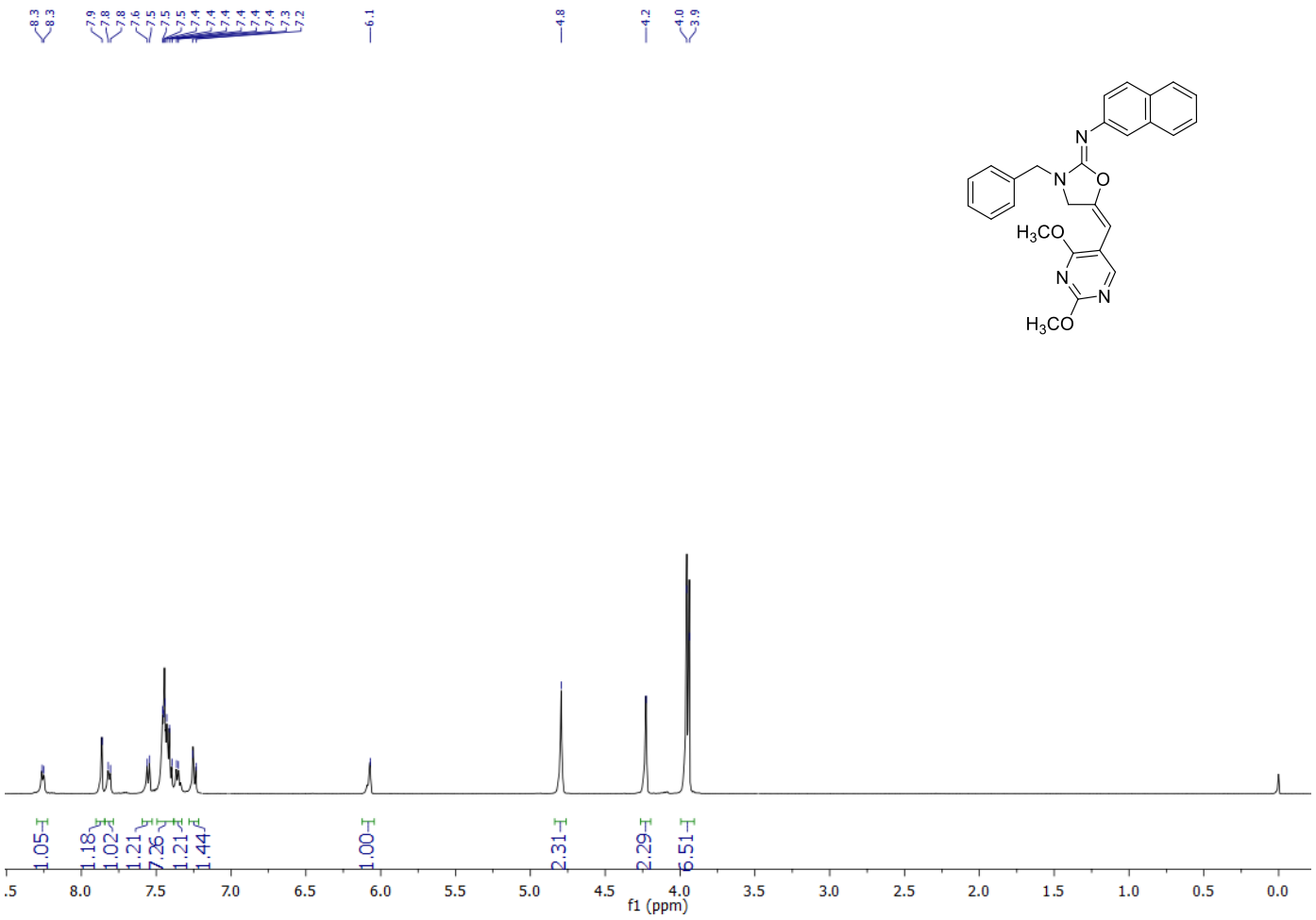
^{13}C NMR compound 5b



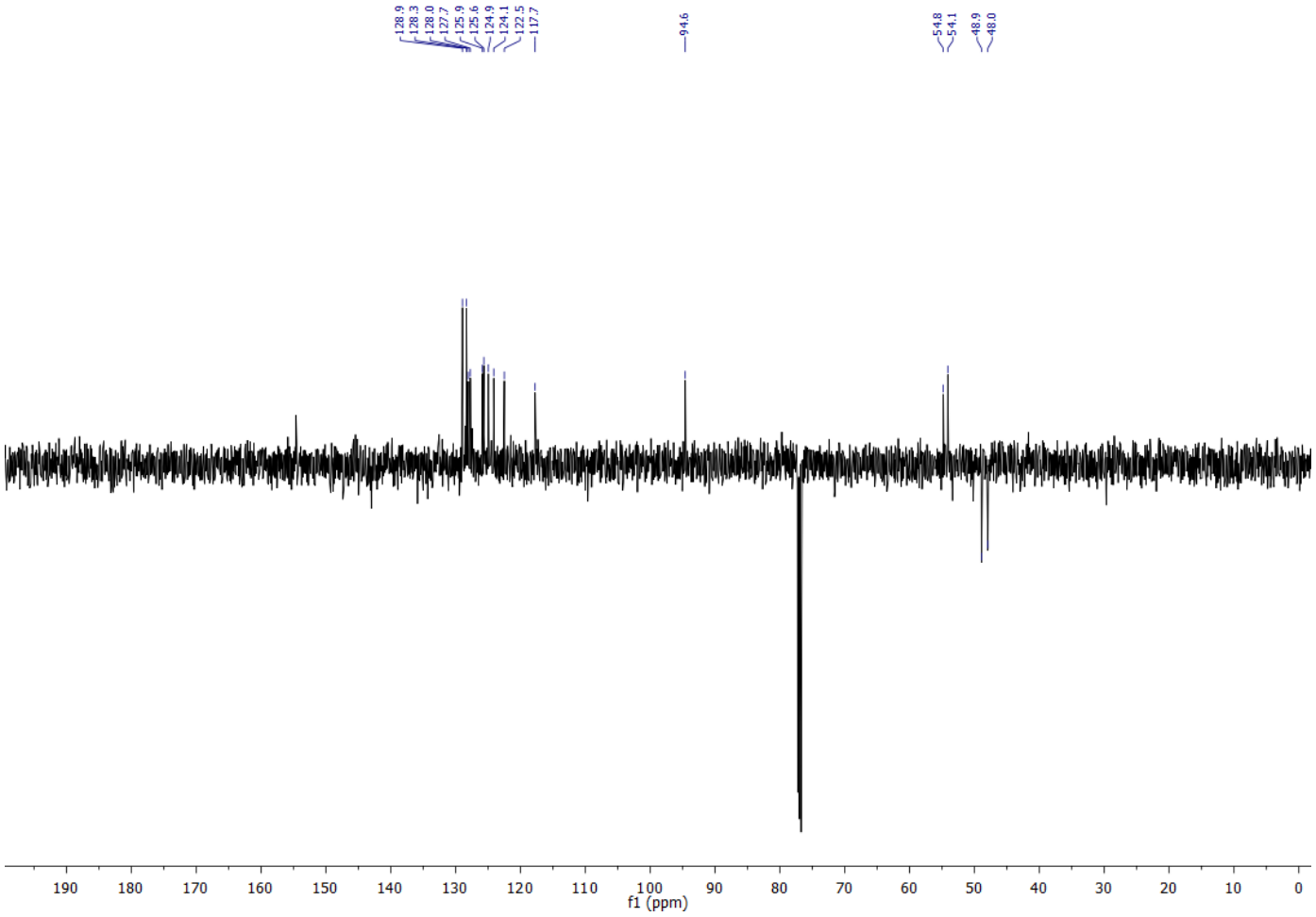
^{1}H NMR compound 4c



^{1}H NMR compound 5c



^{13}C NMR compound 5c



4. HISTONE DEACETYLASE

Histone deacetylases (HDACs) play a crucial role in the remodeling of chromatin, and are involved in the epigenetic regulation of gene expression. In the last decade, inhibition of HDACs came out as a target to correct epigenetic changes associated with cancer and other diseases. Over twenty HDAC inhibitors have entered in clinical studies so far, and some of them (e.g. vorinostat, romidepsin) have been approved for the treatment of cutaneous T-cell lymphoma[17,18]

Whereas, eighteen HDACs have been identified and classified into four classes according to their homology with yeast proteins[19,20]:

- Class I: HDAC1, HDAC2, HDAC3, HDAC8
- Class II: HDAC4, HDAC6, HDAC7, HDAC9, HDAC10
- Class III: Sir2
- Class IV: HDAC11

Table 2. Classification of histone deacetylases (HDAC) enzymes.

HDAC family member (Homology to yeast)		Localization	Size (amino acids)
Class I (Rdp3)	HDAC1	Nucleus	483
	HDAC2	Nucleus	488
	HDAC3	Nucleus	428
	HDAC8	Nucleus	377
Class IIa (Hda1)	HDAC4	Nucleus/cytoplasm	1084
	HDAC5	Nucleus/cytoplasm	1122
	HDAC7	Nucleus/cytoplasm	855

	HDAC9	Nucleus/cytoplasm	1011
Class IIb (Hda1)	HDAC6	Mainly cytoplasm	1215
	HDAC10	Mainly cytoplasm	669
Class IV (Rdp3/Hda1)	HDAC11	Nucleus/cytoplasm	347

Class I, which is homologous to Rpd3 in yeast, includes HDACs 1, 2, 3, and 8, has a nuclear localization. It is ubiquitously expressed in human cell lines and tissues. Class II is homologous to yeast Hda1 and can be subdivided into two subclasses: IIa (HDAC 4, 7, and 9) and IIb (HDAC 6 and 10). Class II exhibits tissue-specific expression and can shuttle between the nucleus and cytoplasm, which suggests that this class of HDACs may be involved in the acetylation of non-histone proteins. The recently discovered HDAC11 is the only member of the class 4 HDACs and is homologous with both class I and class II. The class III HDACs, also called sirtuins (SIRT1-7), includes a group of proteins that are homologous with the yeast Sir2 family of proteins. The subcellular distribution and the specific tissue expression of this class are unknown. Class I and class II HDACs are sensitive to the classical HDAC inhibitor trichostatin A (TSA), whereas those of class III are insensitive to this inhibitor and require the coenzyme NADþ as a cofactor. They are also overexpressed and associated with oncogenes transcription factors, mutated in cancer, and are potential targets for small inhibitors molecules. Recently it has been observed that high levels of HDACs are involved in carcinogenesis, so HDACi can be considered as potential anticancer agents.

All the studied and tested HDACi, showed a potent inhibitory activity in the growth of cell lines of different histological nature and in animal's tumors. They have also shown low toxicity, both in vitro and in vivo, against healthy cells; despite they induce hyper acetylation both in healthy cells and in tumor cells.

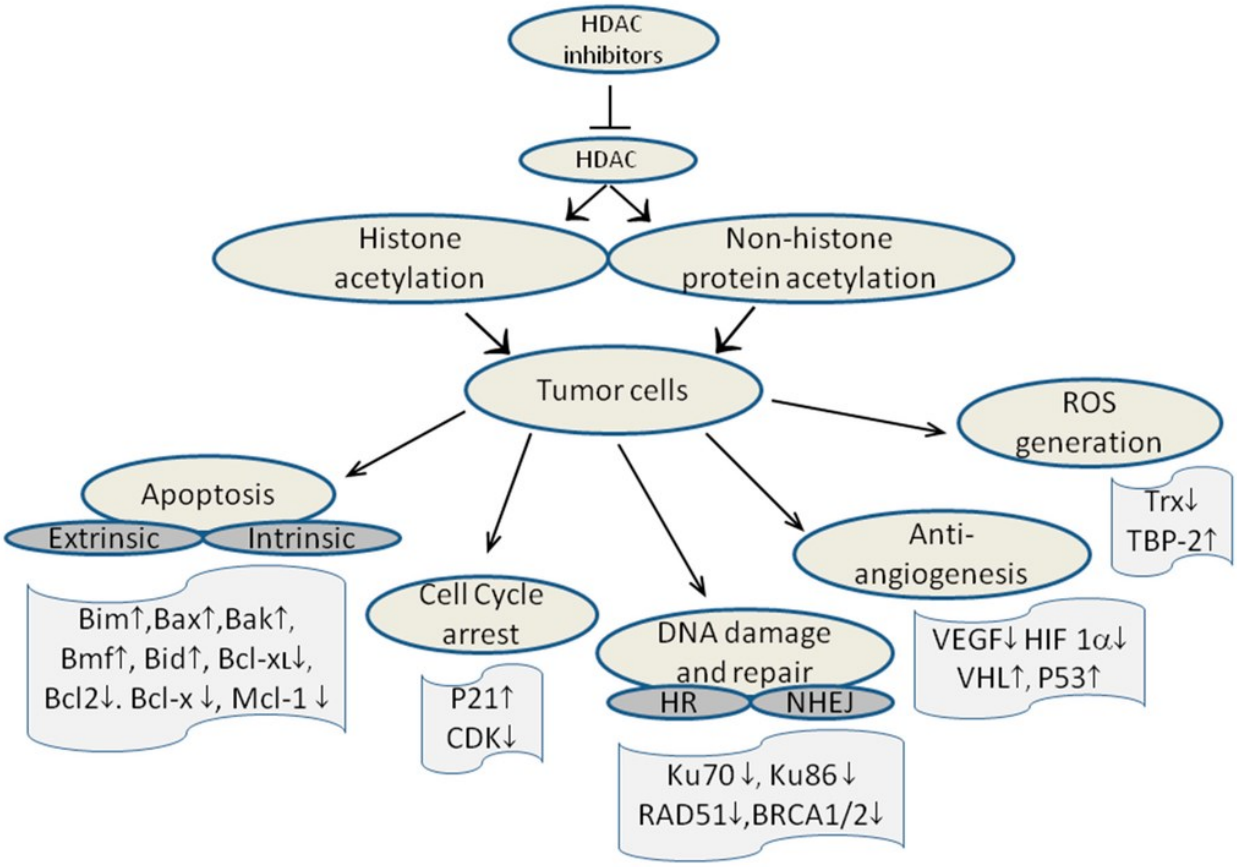


FIGURE 17

Effects of activation of hdac

The effects of the activation of HDAC give rise to (Figure 12):

1- Stopping cell cycle
2- Angiogenesis
3- Immunomodulation

4- Apoptosis

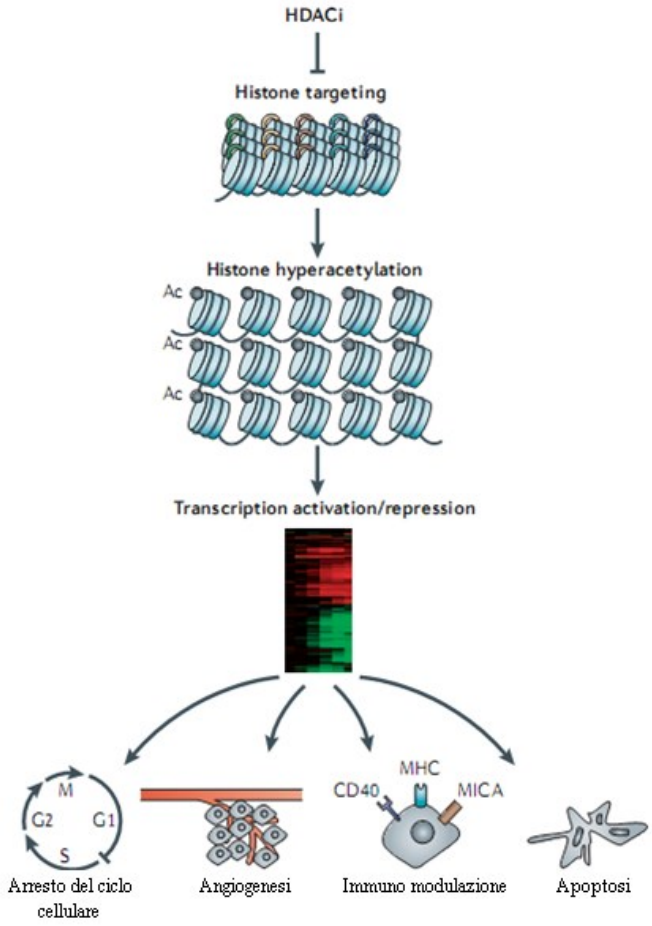


Figure 18

4.1 HISTONE DEACETYLASE INHIBITORS

The histone deacetylase inhibitors (HDACi), are generally constituted by a zinc chelating portion, by a linker and a "cap" that interacts with the enzyme through of hydrophobic interactions.

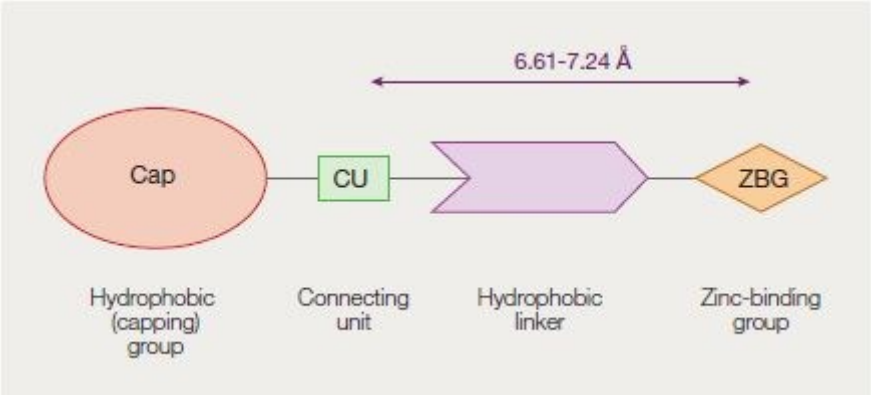


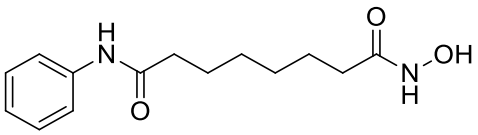
Figure 19

HDACis are structurally divided into four main classes:

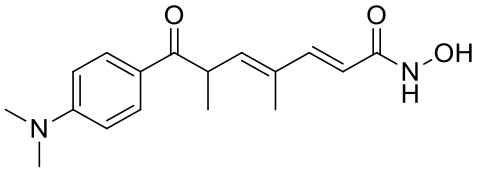
- hydroxamic acids,
- benzamides,
- aliphatic acids,
- cyclic peptides
- mercaptoketones

The most studied are hydroxamic acid derivatives and benzamides. Below are listed some examples of HDACis that show good activity.

HYDROXAMIC ACIDS



(SAHA)

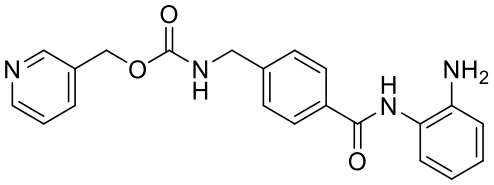


(TSA)

IC_{50} HDAC2 = 0,05 μM

IC_{50} HDAC1-B = 0,05 μM

BENZAMIDES

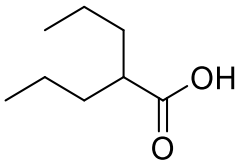


ENTINOSTAT (MS275)

IC_{50} HDAC1 = 0,51 μM

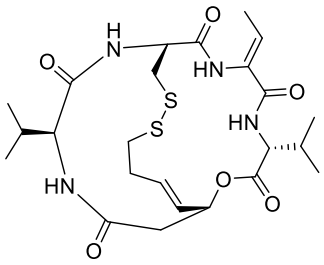
IC_{50} HDAC3 = 0,17 μM

ALIPHATIC ACIDS



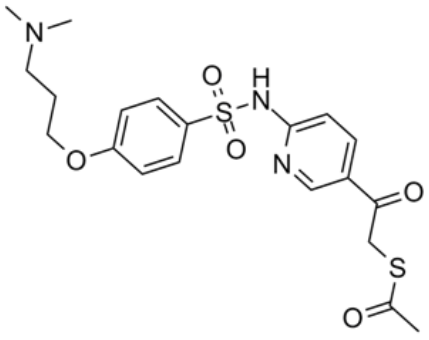
VALPROIC ACID

CYCLIC PEPTIDE



ROMIDEPSIN
(DEPSIPEPTIDE;FK-228)

MERCAPTOKETONES



KD5170

IC_{50} HDAC1 = 20 nM

IC_{50} HDAC2 = 2 μM

IC_{50} HDAC4 = 26 nM

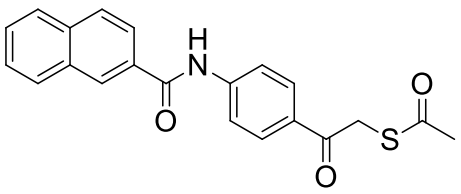
IC_{50} HDAC6 = 14 nM

The majority of HDAC inhibitors (HDACi), including the recently approved SAHA (ZolinzaTM), use a hydroxamic acid motif to chelate zinc in the active site of the enzyme.

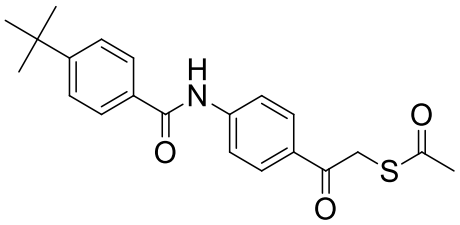
However, this motif typically confers undesired properties such as poor pharmacokinetics, off-target cross-reactivity and poor solubility. Disulfide prodrug strategy to modulate largazole-based compounds resulted in enzymatic activities comparable to the natural product largazole. KD5170, a mercaptoketone-based Class I and II HDAC inhibitor which is another thioester prodrug demonstrated broad spectrum cytotoxicity against a range of human tumor-derived cell lines. The thioester prodrug undergoes hydrolysis to generate mercaptoketone that coordinates Zn^{2+} in a bidentate or monodentate fashion in the active site of HDACs. In the proposed mechanism of action, the thioester prodrug undergoes hydrolysis to generate mercaptoketone that coordinates Zn^{2+} in a bidentate or monodentate fashion in the active site of HDACs[14].

4.2 Sinthesis and biological evaluation

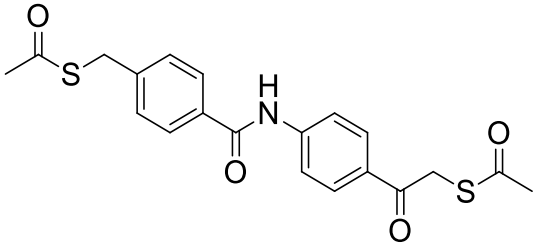
With the research group, I have synthesized three molecules bearing the mercaptoketone group.



NAFT-SCO

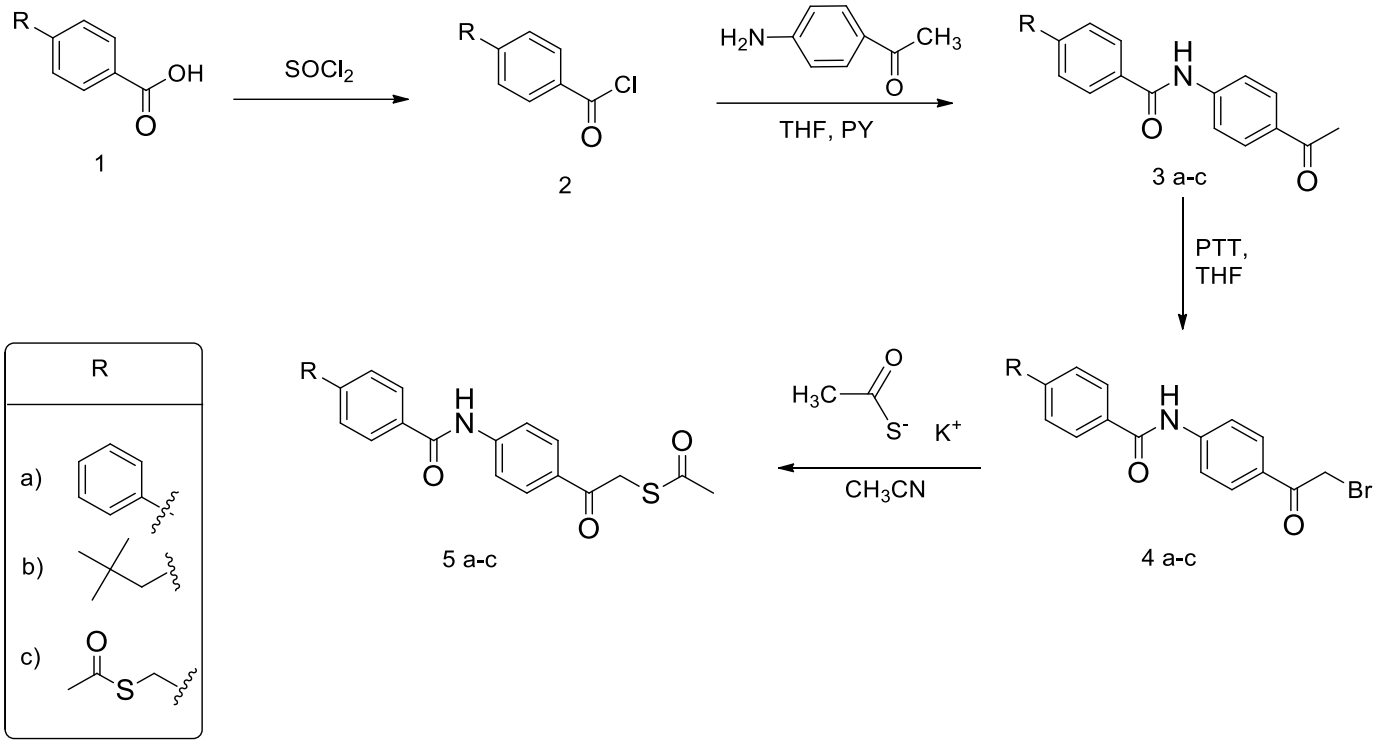


t-BUT-SCO



DISCO

The synthesis starts from substituted benzoic acids that are converted into the corresponding acyl-chloride **2** by $SOCl_2$ reaction. Compounds **2a-c** react with *p*-aminoacetophenone to form the amides **3a-c** that undergo an α-bromination to lead compound **4a-c**. Finally, nucleophilic substitution using potassium thioacetate gives rise the target compounds **5a-c**. (Scheme 5)



Scheme 5

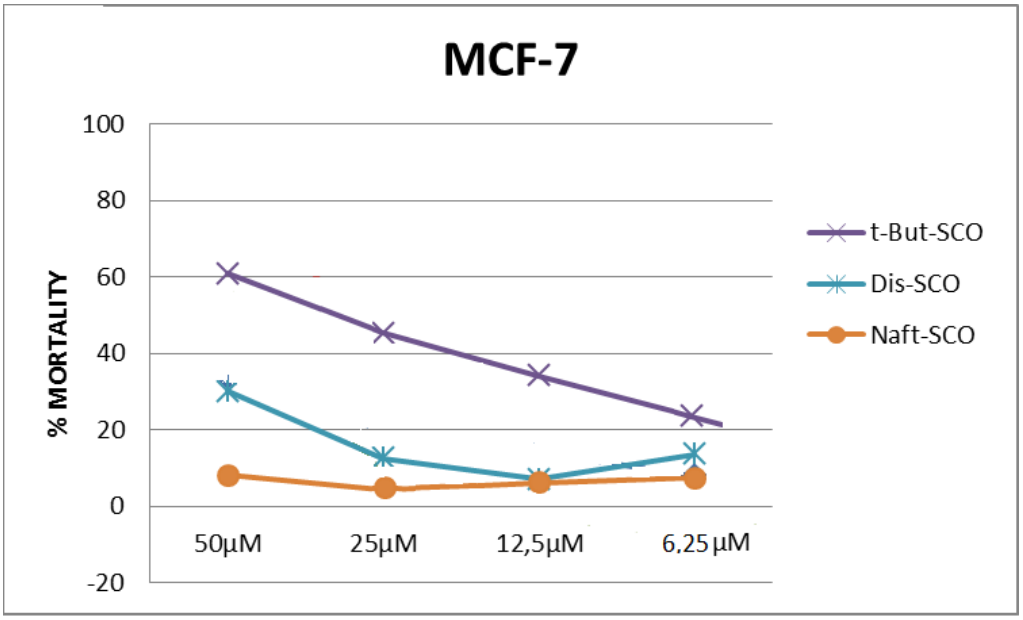
These compounds have been assayed as antitumor agents by the research group of Prof. F. Nicoletti and in particular by Prof. K. Mangano.

The compounds t-But-SCO, Dis-SCO and Naft –SCO were screened in different cancer cell lines and a significant cytotoxic effect was observed upon the treatment with t-But-SCO in the breast cancer line MCF-7 (60% of cell mortality at the dose of 50μM) and colon adenocarcinoma cell line SW620 (75% of cell mortality at the doses of 50 and 25 μM). (Figure 20)

In addition this compound was added to peripheral mononuclear cells (PBMCs) isolated by the blood of healthy donors to ascertain the absence of toxic effects in primary normal cells.

These data represent a preliminary evidence for the further development of this drug in breast and colon cancer. A deeper investigation of the mechanism of action should be undertaken to discriminate the reason for its efficacy as antitumor drug in these particular cancer cell lines compared to other tumour cells.

Based on these results additional studies are warranted in xenograft animal models of breast and colon cancer.



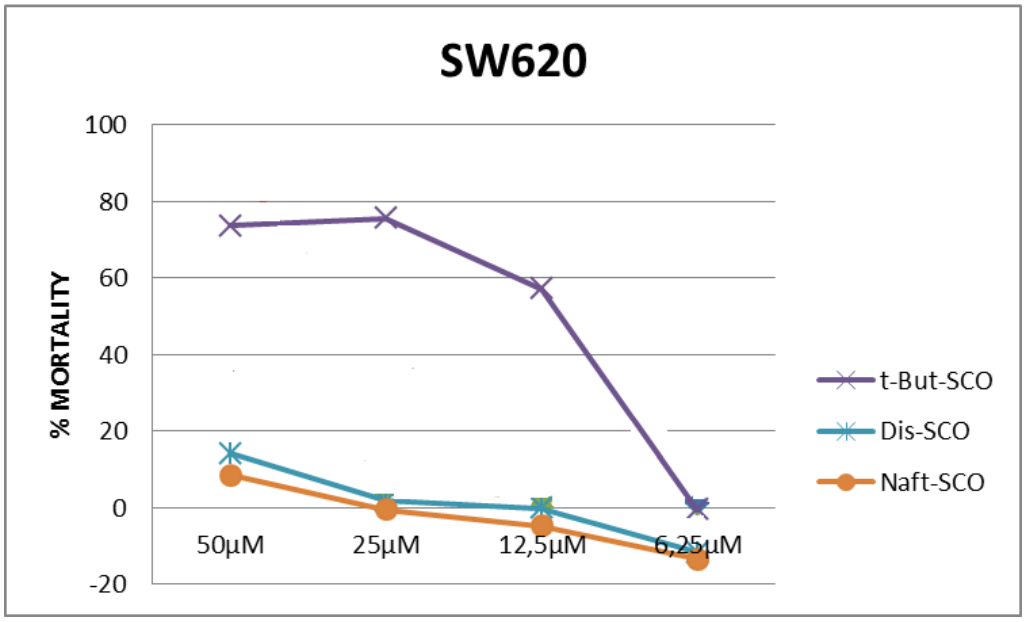
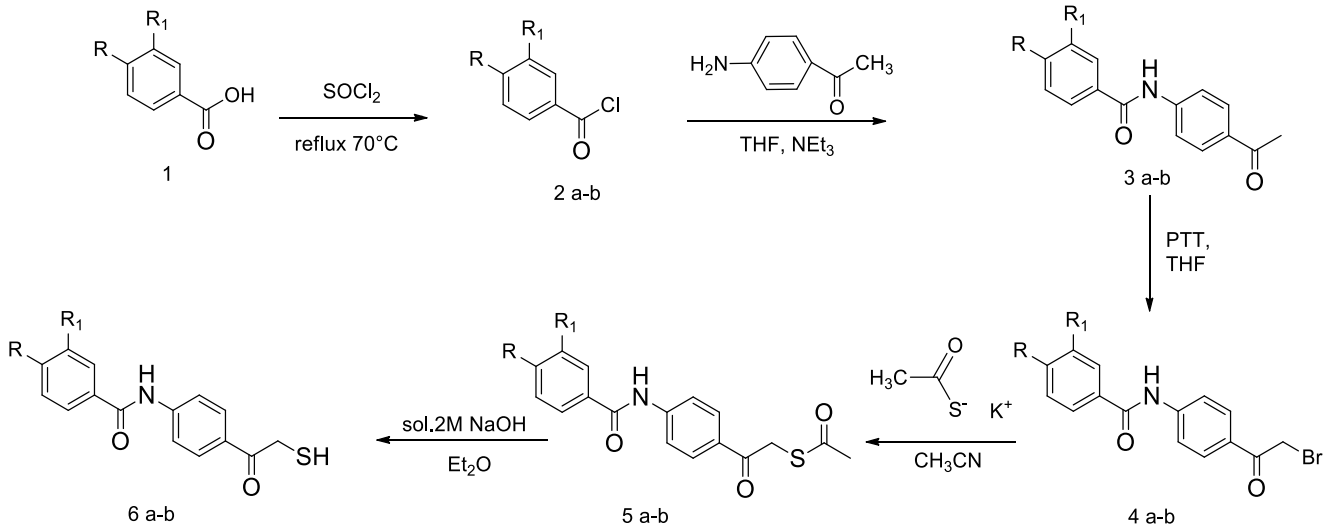


Figure 20

Subsequently to the promising results of the first synthesized molecules I synthetized similar molecules with different substituents. Eventually I tried the reaction of hydrolysis in order to make the molecule more bioavailable with the free SH portion (Scheme 6).

Pharmacological tests, together with the enzymatic assay of synthesized compounds are currently in progress, in order to verify their possible biological activity as HDAC inhibitors.



R	R_1
a) CHO	H
b) NO_2	CF_3

Scheme 6

4.3 Materials and methods

All chemicals were purchased from Sigma–Aldrich Chemical Co. The solvent was removed at aspirator pressure using a rotary evaporator. TLC was performed with Merck precoated TLC plates, and the compounds were made visible using a fluorescent inspection lamp and iodine vapor. Gravity chromatography was done with Merck silica gel 60 (mesh size 63–200 μm). Nuclear magnetic resonance spectra were recorded on a Varian Inova instrument, operating at 500 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR. Chemical shifts (δ) for ^{1}H NMR spectra are reported in ppm downfield relative to the center line of $CDCl_3$ triplet at 7.26 ppm. Chemical shifts for ^{13}C NMR spectra are reported in ppm downfield relative to the center line of $CDCl_3$ triplet at 77.23 ppm. The abbreviations s, d, t, and m stand for the resonance multiplicities singlet, doublet, triplet, and multiplet, respectively. ^{13}C spectra, are ^{1}H decoupled, and multiciplities were determined by APT pulse sequence. The melting points were recorded on a Boëtius hot plate microscope. FT-IR spectra were recorded on FT-IR Shimadzu spectrometer (4000–400 $cm^{-}1$. EI-MS and HRMS were performed with Finnigan MAT 95, EI: 70 eV, R:10000.

4-formylbenzoyl chloride (2a)

A solution of 4-formil-benzoic acid (1 g,6.6 mmol) in 15 mL of thionyl chloride was refluxed at 80°C over night. The solvent was removed under reduced pressure. Yield 90%. Yellow solid.

N-(4-acetylphenyl)-4-formylbenzamide (3a)

To a solution of **2a** (0.885 g, 5.24 mmol) in 15 mL of THF dry at 0° C was added 4-amino-acetophenone (0,717 g, 5.3 mmol) and NEt_3 (1.074 ml, 10.6 mmol), the reaction mixture was stirred overnight at r.t. The mixture was neutralized with $NaHCO_3$

solution, the solvent removed and the crude product extracted in CH_3COOEt (3x50mL) dried over MgSO4, filtered, concentrated *in vacuo*. The result product was purified by Flash chromatography (CH_3COOEt /nEXANE 25%) rf. 0.2 to afford a brown solid, yield 70%.

N-(4-(2-bromoacetyl)phenyl)-4-formylbenzamide (4a)

To a solution of **3a** (0.5 g, 1,9 mmol) in 15 mL of THF at r.t. was added 4-amino-PTT (0,417 g, 1,4 mmol), the reaction mixture became orange coloured and proceed until it became colorless. The mixture was quenqued with H_2O, the solvent removed and the crude product extracted in CH_3COOEt (3x50mL) dried over MgSO4, filtered, concentrated *in vacuo* to afford a brown solid.

S-(2-(4-(4-formylbenzamido)phenyl)-2-oxoethyl) ethanethioate (5a)

To a solution of α-alogenoketone (1eq) in CH_3CN was added potassium thioacetate (1eq), the reaction mixture was stirred for 3h at r.t.. The solvent was removed and the crude product filtered on silica gel with ethyl acetate as eluent then concentrated *in vacuo* to afford a yellow solid.

4-formyl-N-(4-(2-mercaptoacetyl)phenyl)benzamide (6a)

To a solution of **5a** (0.1 g) in dry EtO_2 a 2M solution of NaOH (0,1 mL)was added and the reaction stirred for 2hours at r.t. The organic layers separed and the aqueous one cooled to 0°C and acidified, the extracted with CH_2Cl_2, dried over MgSO4, filtered, concentrated *in vacuo* to afford a white solid.

4-nitro-3-(trifluoromethyl)benzoyl chloride (2b)

A solution of 3-Methyl-4-nitrobenzoic acid (1 g, 5,5 mmol) in 15 mL of thionyl chloride was refluxed at 80°C over night. The solvent was removed under reduced pressure. Yield 90%. Yellow solid.

N-(4-acetylphenyl)-4-nitro-3-(trifluoromethyl)benzamide (3b)

To a solution of **2b** (0.780 g, 3 mmol) in 15 mL of THF dry at 0° C was added 4-amino-acetophenone (0,417 g, 3 mmol) and NEt_3 (0,85 ml, 6 mmol), the reaction mixture was stirred overnight at r.t. The mixture was neutralized with $NaHCO_3$ solution, the solvent removed and the crude product extracted in CH_3COOEt (3x50mL) dried over MgSO4, filtered, concentrated *in vacuo*. The result product was purified by Flash chromatography (CH_3COOEt /nEXANE 25%) rf. 0.2 to afford a brown solid, yield 70%.

4-(4-nitro-3-(trifluoromethyl)benzamido)benzoyl bromide (4b)

To a solution of **3b** (0.5 g, 1,4 mmol) in 15 mL of THF at r.t. was added 4-amino-PTT (0,417 g, 1,4 mmol), the reaction mixture became orange coloured and proceed until it became colorless. The mixture was quenqued with H_2O, the solvent removed and the crude product extracted in CH_3COOEt (3x50mL) dried over MgSO4, filtered, concentrated *in vacuo* to afford a brown solid.

S-(2-(4-(4-nitro-3-(trifluoromethyl)benzamido)phenyl)-2-oxoethyl) ethanethioate

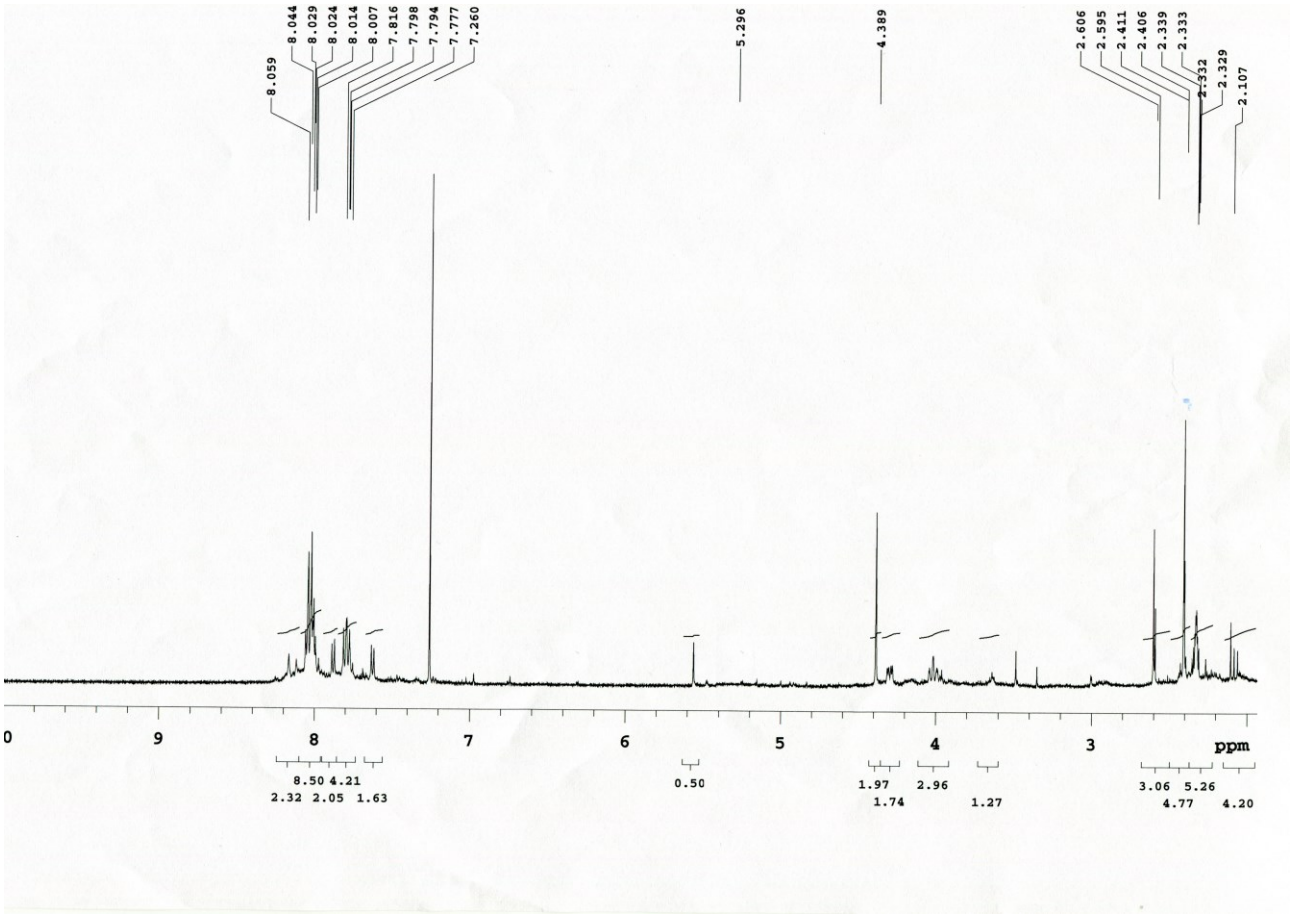
(5b)

To a solution of α-alogenoketone (1eq) in CH_3CN was added potassium thioacetate (1eq), the reaction mixture was stirred for 3h at r.t. The solvent was removed and the crude product filtered on silica gel with ethyl acetate as eluent then concentrated *in vacuo* to afford a orange solid.

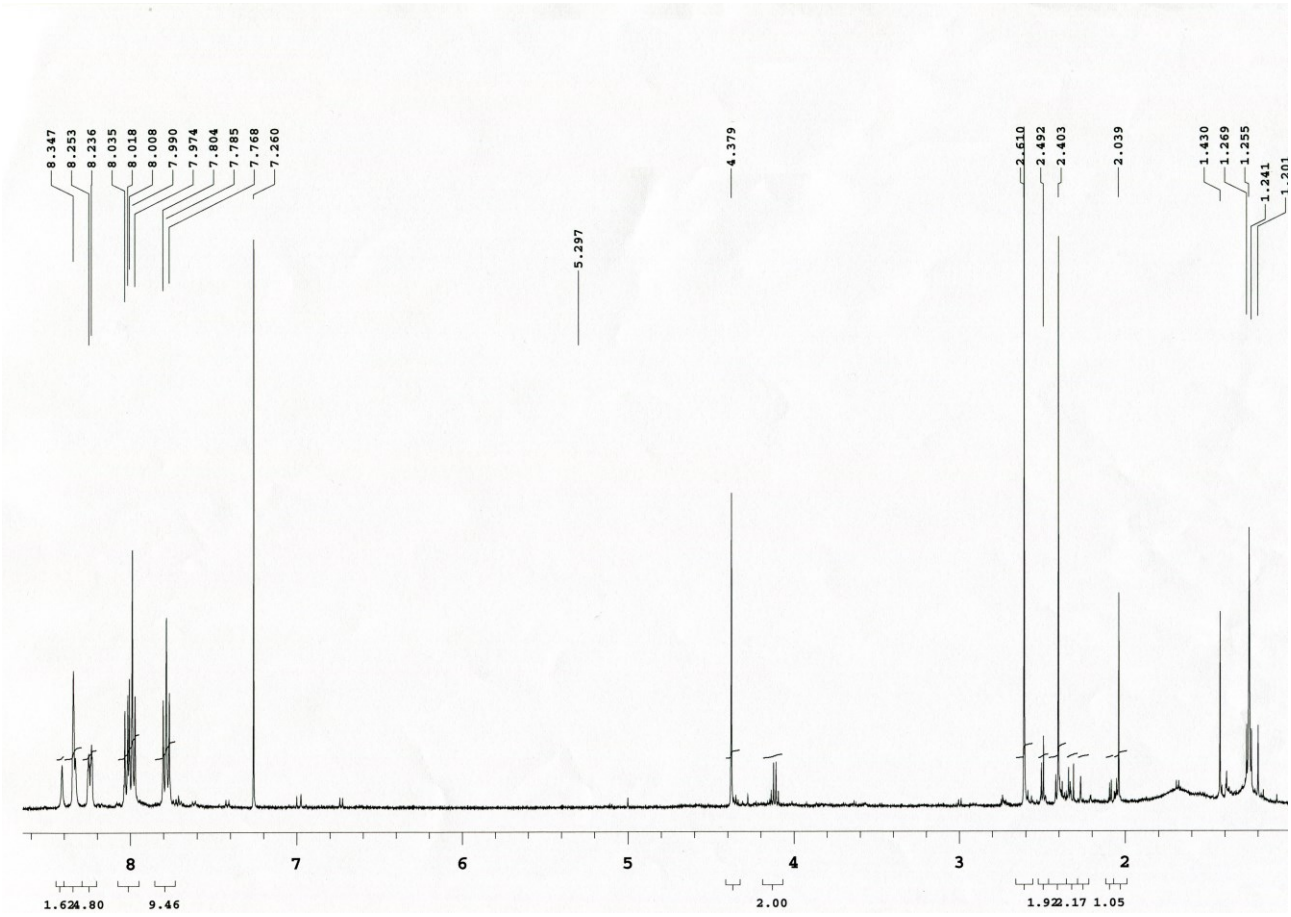
N-(4-(2-mercaptoacetyl)phenyl)-4-nitro-3-(trifluoromethyl)benzamide (6b)

To a solution of **5a** (0.1 g) in dry EtO_2 a 2M solution of NaOH (0,1 mL)was added and the reaction stirred for 2hours at r.t. The organic layers separed and the aqueous one cooled to 0°C and acidified, the extracted with CH_2Cl_2, dried over MgSO4, filtered, concentrated *in vacuo* to afford a white solid.

^{1}H NMR compound 5a



^{1}H NMR compound 5b



Bibliography

[1] Drews. J. Science. 2000, 287, 1960-4.

[2] Choo QL, Kuo G, Winer AJ et Al. “Isolation of a cDNA clone derived from a blood-borne

non-A, non-B hepatitis genome". *Science* **1989**, *244*, 359−362.

[3]Fattovich G et al. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. *Gastroenterology* **1997**, *112*, 463−472.

[4]D'amico G et al. Survival and prognostic indicators in compensated and decompensated cirrhosis. *Dig Dis Sci* **1986**, *31*, 468−475.

[5]Perrillo R. P., Thuluvath P. J., Rothstein K., et al. Improved work productivity, safety and quality of life with pegylated (40KDA) interferon alfa-2a (PegasysTM) therapy in the treatment of chronic hepatitis C [abstract]. *Hepatology* **2000**, *32* (Pt 2) 362.

[6]Perrillo R. P., Rothstein K. D., Imperial J., et al. Therapy with Pegasys® demonstrates similar efficacy and significantly improved tolerability, quality of life and work productivity compared with RebetronTM in patients with chronic hepatitis C [abstract]. *J. Hepatol.* **2001**, *34* Suppl. 1, 146−7.

[7]. Li, X.; Zhan, P.; de Clercq, E.; Liu, X. The HIV-1 non-nucleoside reverse transcriptase inhibitors (Part 5): Capravirine and its analogues. *Curr. Med. Chem*. **2012**, 19, 6138–6149.

[8].. Sari, O.; Roy, V.; Agrofoglio, L.A. Nucleosides modified at the base moiety. In Chemical Synthesis of Nucleoside Analogues, 1st ed.; Merino, P., Ed.; Wiley-VCH: Hoboken, NJ, USA, **2013**; p. 49.

[9]Chiacchio, U.; Corsaro, A.; Giofrè, S.V.; Romeo, G. Isoxazolidinyl nucleosides. In Chemical Synthesis of Nucleoside Analogues, 1st ed.; Merino, P., Ed.; Wiley-VCH: Hoboken, NJ, USA, **2013**; p. 781.

[10]. Romeo, R.; Carnovale, C.; Rescifina, A.; Chiacchio, M.A. Phosphonated nucleoside analogues. In Chemical Synthesis of Nucleoside Analogues, 1st ed.; Merino, P., Ed.; Wiley-VCH: Hoboken, NJ, USA, **2013**; p. 163.

[11]. Chronic hepatitis C and genotyping: the clinical significance of determining HCV genotypes, *H James Hnatyszyn*, Bayer Institute for Clinical Investigation (BICI), Bayer HealthCare – Diagnostics Division, Berkeley, CA, USA

[12]. Roberto Romeo , Caterina Carnovale, Salvatore V. Giofrè, Giulia Monciino, Maria A. Chiacchio, Claudia Sanfilippo, and Beatrice Macchi. Enantiomerically Pure Phosphonated Carbocyclic 2'-Oxa-3'-Azanucleosides: Synthesis and Biological Evaluation. *Molecules* **2014**, *19*, 14406-14416.

[13]. Xiao-Long Qui and Feng-Ling Qing, Sinthesis of 3'-Deoxy-3'-difluoromethyl azanucleosides from *trans*-4-Hydroxy-*L*-proline. *J.Org:Chem,* **2005**,*70*,3826-3837.

[14]. Behrens S.E., Tomei L., De Francesco R. *Embo J.* **1996**, 15, 12-22.

Lohmann V., Overton H., Bartenschlager R. *J. Biol. Chem.* **1999**, 274, 10807-15.,

[15]. Ferrari E., Wright-Minogue J., Fang J.W., Baroudy B.M., Lau J.Y., Hong Z. *J. Virol.* **1999**, 73, 1649-54.

[16]. Santiago Ropero, Manel Esteller, The role of histone deacetylases (HDACs) in human cancer. *Molecular oncology,* **2007**,19-25.

[17]Reed, J. C.; Jurgensmeier J. M.; Matsuyama S. *Biochim. Biophys. Acta*, **1998**, *1366*, 127–137. b) Bolden J. E.; Peart M. J.; Johnstone, R. W. *Nature*, **2006**, *5*, 769–784.

[18] Chiara Zagni, Giuseppe Floresta, Giulia Monciino, and Antonio Rescifina The Search for Potent, Small-Molecule HDACIs in Cancer Treatment: A Decade After Vorinostat,

[19]. Madhusoodanan Mottamal, Shilong Zheng, Tien L. Huang and Guangdi Wang. Histone Deacetylase Inhibitors in Clinical Studies as Templates for New Anticancer Agents. Molecules **2015**, *20*, 3898-3941.

[20]. Carmeliet, P.; Jain, R. K. *Nature*, **2000**, *407*, 249-257. b) Buys, C. H. C. M.; *N. Engl. J. Med.*, **2000**, *342*, 1282-1283. c) Chambers, A. F.; Groom, A. C.; MacDonald, 123 I. C. *Nat. Rev. Cancer*, **2002**, *2*, 563-557. d) Greider, C. W.; Blackburn, E. H. *Sci. Am. Feb.*, **1996**, 80-85.

[21]. Joseph E. Payne, Céline Bonnefous, Christian A. Hassig , Kent T. Symons , Xin Guo , Phan-Manh Nguyen , Tami Annable, Paul L. Wash, Timothy Z. Hoffman a , Tadimeti S. Rao, Andrew K. Shiau, James W. Malecha, Stewart A. Noble , Jeffrey H. Hager, Nicholas D. Smith. Identification of KD5170: A novel mercaptoketone-based histone deacetylase inhibitor. *Bioorganic & Medicinal Chemistry Letters,* **2008**,*18* , 6093–6096.

[22.] Masuda et al., **1997**; Brown, **1997**.

[23.] Davies et al., **1991**; Hostomsky et al., **1991**.

[24.] Baltimore, D. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature 226 (**1970**) 1209–1211).

[25].Mao, C.; Sudbeck, E. A.; Venkatachalam, T. K.; Uckun, F. M. Biochemical Pharmacology. **2000**, 60, 1251-1265.

[26]. Chen, Y.L., Lin, S.Z., Chang, J.Y., Cheng, Y.L., Tsai, N.M., Chen, S.P., Chang, W.L., Harn, H.J., 2006. In vitro and in vivo studies of a novel potential anticancer agent of isochaihulactone on human lung cancer A549 cells. Biochem. Pharmacol. 72, 308–319.

[27]. Ali, M.A., Shaharyar, M., Siddiqui, A.A., 2007. Synthesis, structural activity relationship and anti-tubercular activity of novel pyrazoline derivatives. Eur. J. Med. Chem. 42, 268–275. Bashir, R., Ovais, S., Yaseen, S., Hamid, H., Alam, M.S., Samim, M., Singh, S., Javed, K.,

2011. Synthesis of some new 1,3,5-trisubstituted pyrazolines bearing benzene sulfonamide as anticancer and anti-inflammatory agents. Bioorg. Med. Chem. Lett. 21, 4301–4305.

[28] .Hassan, S.Y., 2013. Synthesis, antibacterial and antifungal activity of some new pyrazoline and pyrazole derivatives. Molecules 18, 2683–2711

[29] *J.Org. Chem*. **2015**, 80,7226-7234

[30]N. Miyaura, A. Suzuki, J.Chem.Soc.Comm., **1979**, 866-867.

List of original papers

1) R. Romeo, C. Carnovale, S. V. Giofrè, G. Monciino, M. A. Chiacchio, C. Sanfilippo, B. Macchi. **Enantiomerically Pure Phosphonated Carbocyclic 2'-Oxa-3'-Azanucleosides : Syntesis and Biological Evaluation,** Molecules,2014, 19,14406-14416 ; ISSN 1420-3049

2) C. Zagni, G.Floresta, G. Monciino, and A. Rescifina. **The Search for Potent, Small-Molecule HDACIs in Cancer Treatment: A Decade After Vorinostat,** Published online in Wiley Online Library (wileyonlinelibrary.com), DOI 10.1002/med.21437, 2017

3) Maria G. Varrica, Chiara Zagni, Placido G. Mineo, Giuseppe Floresta, Giulia Monciino, Venerando Pistarà, Antonio Abbadessa, Angelo Nicosia, Rogerio M. Castilho, Emanuele Amata. **DNA intercalators based on (1,10-phenanthrolin-2-yl)isoxazolidin-5-yl core with better growth inhibition and selectivity than cisplatin upon head and neck squamous cells carcinoma** https://doi.org/10.1016/j.ejmech.2017.11.067