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SCUOLA DI DOTTORATO IN
SCIENZE FARMACEUTICHE
XXIII CICLO

HETEROCYCLIC COMPOUNDS ENDOWED WITH ANTIVIRAL ACTIVITY

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INTRODUCTION

Integrase (IN) of HIV-1, ribonuclease H (RNase H) of HIV-1 and NS5B RNA polymerase RNA dependent (NS5b) of HCV are three enzymes that belong to two different classes,[1-3] but have in common the presence of divalent metal ions, such as Mg^{2+}, in their catalytic core domine. Each of these enzymes utilized the Mg^{2+} ions for catalyse the reactions in which are involves. These metal ions are chelated by a highly conserved motive that consisted in three acidic amino acid residues namely D-X4-D motive. In IN this motive is represented by D64, D116 and E152 aminoacids; the Mg^{2+} ions are coordinated by D64 and D116 along with water molecole, whereas E152 that lies closed to D64 dose not partecipate in metal binding. [4] In RNase H this motive is represented by D443, E478, D498 and D549 amino acids, [5] whereas in NS5B is represented by D220, D318 and D319 amino acids. [6] The architetture of the amino acids within the catalytic site and the geometry of the catalytic metals are highly conserved among a superfamily of nuclease and polynucleotidyl trasferase.

2,4-Diketo acids (DKAs) are the most important class of IN, RNase H and NS5B inhibitors and provided the first proof-of-concept of antiviral agents in the cell based assay. The mechanism of action of these inhibitors is likely a consequence of the interaction with metals in the active site, resulting in a functional impairment by chelating of critical metal cofactors. [7] The discovery of DKA was based on the hypothesis of metal cation/DKA motif coordination that provides a solution to the two-metal-ions active site binding pharmacophore. Selective DKA could be designed, incrising bulk and complexity of DKA substituents that could relax the requirement for either single or double metal ion interaction, but also confer selectivity by steric exclusion. Among the polynucleotidyl trasferase, RNase H shows high similarities with the structure of HIV-1 IN, which has been study for long times as a target for drug development and for which, a new drug was recently introduced in clinical practice. [8] Given the structural similarities between the domines of these three viral enzimes, several DKAs initially developed as IN inhibitors were also screened against RNase H or NS5b and viceversa.

Here I report the design, synthesis and comparative evaluation of series of molecules that contain the DKA moiety like pharmacophore, and that are able to inhibit each of this three enzimes selectively.

First chapter

INTEGRASE OF HIV-1

Human immunodeficiency virus type-1 (HIV-1) and acquired immunedeficiency disease (AIDS) have cost the lives of millions of people world wide since the epidemic began. Currently the World Health Organization estimates that 33,3 million peoples are infected with HIV-1, constituting a global pandemic illness with significant social and economic impact (Figure 1). [9]

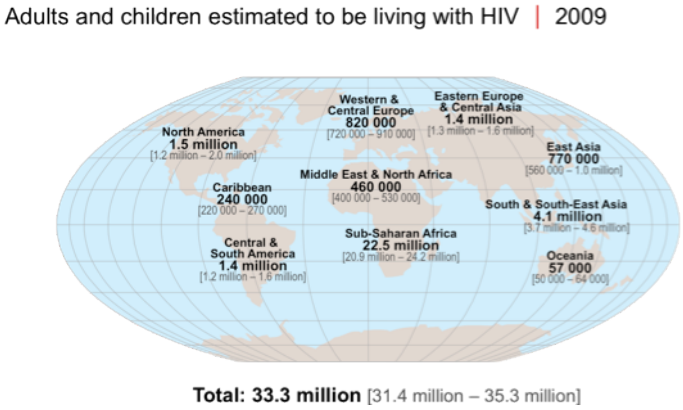


Figure 1. Adults and children estimated to be living with HIV (2009)

In response to this medical crisis, the scientific and medical communities have discovered and developed antiretroviral (ARV) drugs that directly target viral enzymes or processes essential for viral replication (Figure 2). These ARV drugs, most of which are small molecule, include nucleoside/tide reverse transcriptase inhibitors (NRTIs, 8 approved drugs), non-nucleoside reverse transcriptase inhibitors (NNRTIs, 4 approved drugs), protease inhibitors (PIs, 9 approved drugs), and most recently the entry inhibitor maraviroc, and the integrase inhibitor (INSTIs), raltegravir. Moreover, a peptidic inhibitor, enfuvirtide, targets viral fusion. Combinations of ARV drugs used as highly active antiretroviral therapy (HAART) have become the standard of care for HIV therapy and have produced significant declines in the morbidity and mortality associated with HIV-1 infection, especially in those countries where access to ARV medications is widely available.

Among the three enzymes encoded by HIV-1, protease (PR), reverse transcriptase (RT), and integrase (IN), the latter was until recently an “orphan” in terms of approved ARV drugs. Only in the 2007, the FDA approved raltegravir (RAL, MK-0518, Isentress®, Merck Inc.), a strand transfer inhibitor of IN (INSTI), for the treatment of HIV-1 infection for just ARV treated subjects. [10] RAL was also

approved by the FDA for use in firstline ARV therapy in June 2009 and is undergoing further phase 3 studies in ARV treatment-naïve subjects. A second INSTI, elvitegravir (EVG, GS-9137, JTK-303, discovered by Japan Tobacco and developed by Gilead Sciences, Inc.) is undergoing phase 3 studies in ARV treatment-experienced subjects. [11] S/GSK-1349572 is another INSTI, which recently entered phase 2 testing, suggesting that additional drugs may become available in this new class. [12]

Viral integration is a particularly desirable target because, like other retroviruses, HIV-1 requires integration into the host genome for its replication and propagation. Moreover, HIV-1 integrase has no human equivalent and offers the possibility of a high drug specificity with limited cellular toxicity. [13]

1.1 Biology of integrase of HIV-1 enzyme.

1.1.1 Structure and function of HIV-1 integrase enzyme.

Despite its acute importance for antiretroviral drug discovery and decades of rigorous research, the complete structure of IN, either as a separete protein or in the context of the functional intasome, is laking. The structural organization of the IN active site is unknown. Because clinically useful INSTIs preferentially bind to and inhibit the intasome complex compared to free IN, the mechanism of drug action is poorly undestood. [14]

IN recognized and acts on the termini of the linear double-strand DNA molecule, produced during the reverse trascription, and mediate its integration into the host DNA of the infected cell to produced functional provirus. Integration is the final step before irreversibile and productive HIV-1 infection of a target cell is achieved. [15] IN, encoded by HIV-1 *pol* gene, is a 32 kDa enzyme costituited by 288 amino acids. IN is divided into three structural and functional domine: the N-terminus domine (NTD), the catalytic core domine (CCD), and the C-terminus domine (CTD). [16]

The N-terminal domain of the integrase (amino acids 1-50) is characterized by two pairs of highly conserved histidine and cysteine residues (histidine residues 12 and 16; cysteine residues 40 and 43) that form a “HH-CC” or zinc finger motif that involves the chelation of one zinc atom per integrase monomer. The N-terminal domain is required for high-order multimerization that is stimulated by zinc. [17] A zinc atom is required to stabilize the folded structure of the N-terminal domain of

HIV-1 integrase and is necessary for optimal enzymatic activity.

The catalytic core domain includes amino acids 51- 212 of HIV-1 integrase. The catalytic core domain forms a dimer in solution and in the functional enzyme; the monomer structure of the catalytic core is similar to the folds of RNaseH. The catalytic core domain is required for the cleavage and formation of phosphodiester bonds. The active site of the catalytic core domain contains three highly conserved acidic amino acids forming a catalytic triad (residues D64, D116, and E152, also known as the D, D E motif), which coordinate two divalent metal ions and are absolutely essential for efficient integrase enzymatic activity. [18] Each of these amino acids is required for the catalysis of viral integration into host DNA; substitution of one of these residues significantly reduces the enzymatic activity of HIV-1 integrase. Interestingly, although the catalytic core domain can also catalyze the disintegration reaction (the reverse of integration) by itself, the N-terminal and C-terminal domains of integrase are also required for the 3′ processing and strand-transfer reactions. The C-terminal domain (amino acids 213-288) is the least conserved of the three domains. The HIV-1 C-terminal domain shares some structural homology with the SH3 class of DNA-binding domain, can bind DNA nonspecifically, and is required for both the 3′ processing and strand-transfer activities of HIV-1 integrase. [19]

1.1.2 Integration of HIV-1 into the human genome.

The role of IN during HIV-1 replication begins in the cytoplasm after completation of reverse trascription. The resulting double strand intermediate DNA undergoes two reaction catalyzed by IN. IN selectively recruits an unperfected reverse-trascripted DNA in the cytoplasm, and binds its LTR sequence. [15] After the binding, IN catalyzed the removal of the highly conserved GT dinucleotides at 3'-end and, in consequence, the formation of reactive 3'-OH ends in both strands of the viral c-DNA, by a nucleophilic attack of the phosphodiester bond between the deoxyguanosine and deoxyadenosine, in a reaction called 3'-processing (3'-P). With the 3'-P, IN formed together with viral DNA and a series of host (barrier to autointegration factor-1 (BAF), interactor-1, heat shock protein 60 (HSP60), high mobility group protein A-1 (HMG-A1), and lens epithelium derived growht factor (LEDGF/p75) [20] and viral (matrix, Vpr, p7/nucleocapsid and RT) [21] protein a preintegration complex (PIC), which is activelly trasported to the nucleous where the second reaction called strand transfer (ST) occurs.

ST reaction consists in a nucleophilic attack by the two reactive 3'-OH recessed ends of the viral DNA (DNA donor), formed during 3'-P, to the host DNA (DNA acceptor); the 3'-OH ends of viral DNA are taken closely by coordination, for making possible the reaction with the mayor groove of the genomic host DNA. The gap formed, after the nucleophilic attack, between the 5'-end of DNA donor and 3'-end of DNA acceptor is filled probably by the cellular DNA repair enzymes, finishing the integration process. [22]

The completion of the ST reaction produces a functional integrated proviral DNA which begin the template for transcription of new viral RNAs, needed for translation of viral proteins and enzymes, and transcription of new full length viral RNAs, required for packaging into new virions. Each integrated provirus allows for the production of thousands of new virions, amplifying the initial infection and leads the infected CD4+ T-cell to the death. Without successful integration, viral replication would leading to a rapid decline due to the lack of amplification of the infection. The development of INSTIs nevertheless required over a decade of basic research before representatives of this drug class began to emerge in the clinic.

1.2 Pharmacophore analysis and mechanisms of ST integrase inhibitors.

IN inhibitors include compounds that exhibit a broad structural diversity. However, different compounds seem to follow a common pattern: almost all the compounds identified contain metal-chelating motifs. Based on pharmacophore analysis of several integrase inhibitors, their structural features can be divided into two domains: (i) the enzyme-binding domain and (ii) the catalytic triad domain.

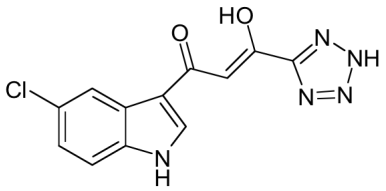
The enzyme-binding domain facilitates and potentially stabilizes inhibitor binding to the integrase enzyme, thereby allowing the catalytic triad domain to be presented to the catalytic triad. The enzyme-binding domain is highly flexible, composes by aromatic hydrophobic group(s), and can tolerate aromatic moieties of various sizes. The enzyme-binding domain of the inhibitors can be viewed as "anchoring residues" that binds to the complementary region in the integrase enzyme (corresponding to the "inhibitor binding" region); furthermore it also facilitates the transport of the integrase inhibitor across the cell membrane. The structural flexibility of the enzyme-binding domain allows the attachment of additional hydrophobic residues that further facilitate cell membrane permeability as well as enhance the binding of the molecule

to the hydrophobic regions of IN. The catalytic triad domain of integrase inhibitor is highly conserved and shares a common motif. A general feature of this domain is that they all contain divalent metal ion chelating motifs such as catechol, 1,2-diols, β-dicarbonyls, α-hydroxy acids, or quinolinols. Another important feature concern the planar configuration of the catalytic triad domain with the enzyme-binding domain. [13] Among all known IN inhibitors, only the α,γ-diketo acids (DKA) are valid bioligical inhibitors. The INSTs are thought to engage metal ion cofactors in the IN active site by interactions with uniquely positioned oxygen atoms of the pharmacophore. Recently its publicated the first crystal structures of the full-length IN from the prototipe foamy virus in complex with its cognate viral DNA; this was refine using diffraction data collected on crystals soaked in presence of Mg^{2+} and the clinical INST RAL or EVG. On the basis of the structures, the two INSTs seem to have similar mode of binding and action. Their metal chelating oxygen atoms orient towards the metal cofactor of the active site, whereas the halobenzyl groups fit within a tight poket created by displacement of the 3' adenosine. The two drugs are implicated in different interaction with the enzyme: RAL makes Van der Waals interactions with the bases of invariant CA dinucleotide in addiction with hydrophobic and stacking interaction with the side chains of Pro 214 and Tyr 212 with its isopropyl and methyl-oxadiazole groups; EVG through its quinolone base and isopropyl group, interact with Pro 214. The intasome become inactive after the displacement of reactive 3' viral DNA end from the active site. The extensive contacts with the viral DNA end observed in these structures determine why the INSTs preferentially interact and inhibit the DNA-bound form of HIV-1 IN. [14]

1.3 Development of ST integrase inhibitors: DKA.

The discovery of small molecole was greatly enhanced after the publication by the Merck researcher of the *in vitro* assay for the analysis of IN activity and IN inhibitors. [23] The following years were characterized by an intensive research of natural product and synthetic molecule that are able to inhibit IN. The first natural products were identify among the fungal compounds, [24] like equisitina, and were characterized by the presence of pharmacophoric mojety like β-hydroxyketo group, diketo group (DKA) or a carboxylate group. These compounds were not selective inhibitors of ST, but can inhibit also the 3'-P reaction. The first selective ST

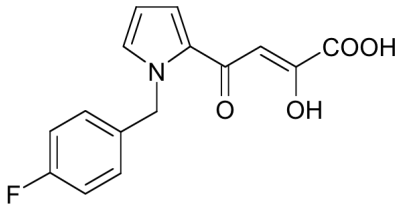
inhibitors was 5-CITEP (**1**), discovered by Shionogi in 1999. [25]



5 - CITEP

1

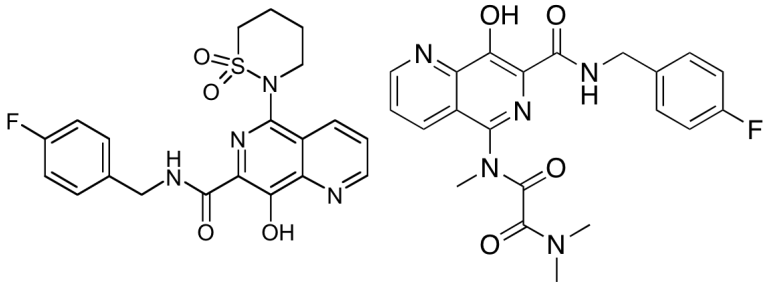
This compound presents the triazole cycle as bioisoster of carboxylic acid group; furthermore 5-CITEP was the first compound for which the crystal structure in complex with CCD subunit was available. Thanks to this knowledge, was possible to define the mechanism of inhibition of DKA: the DKA mojety are able to chelate the Mg^{2+} ions presentes in the catalytic core domain. The indole base was demostrated to enhance the metal dependency, infact its substitution with benzyl base induced the decrease of inhibitory activity. To 5-CITEP follows a series of compound that were characterized by the presence of DKA mojety and were designed on the bases of crystal structure of 5-CITEP with CCD. Another important inhibitor was the Merck inhibitor L-731,988 (**2**), a pyrrolydiketo acid with a p-fluorobenzyl group bounded to the pyrrolic nitrogen. [26] This compound along with L-708,906, demonstrated an increase of ST inhibition on cell based assay and was utilized for selected resistance mutation.



L-731,988

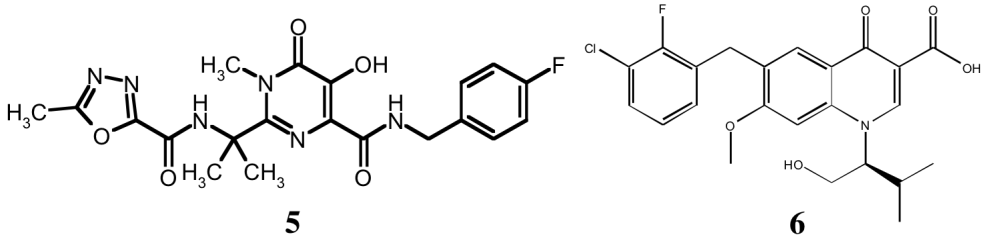
2

Noteworthy are also Merck naphthyiridine componds like L-870,810 [27] (**3**) and L-870-812 [28] (**4**) that represents the evolution of the first compounds because contains a p-fluorobenzyl amide instead of carboxylic acid and the α,γ-diketo acid mojety was fused in the naphthyridine ring. The first was brought up in clinical trials until phase II. Its study was discontinous for its hepatotoxicity observed in long-term safety study. The second was the most extensively characterized compound of this series but for its capability to develop primary resistance after only one month of treatment, was abandoned.

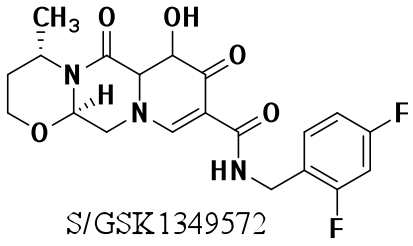


3 **4**

Successful development and approval of an HIV-1 INSTI for the treatment of HIV-1 infection was ultimately achieved with the discovery of RAL (**5**) by the Merck research laboratories, a pyrimidine compound, that present a new scaffold compared with the previous inhibitors. RAL was approved for clinical used by FDA in 2007. Another important compound introduced in phase III of clinical trials is EVG (**6**) discovered by Japan Tobacco; its structure derived from quinolinone atibiotic. Recentlt another compound is introduced in phase II of clinical trials, the naphthyridinone S/GSK 1349572 (**7**) [29] discovered by Shonogi and Glaxo Smith Kline.



5 **6**



S/GSK1349572

1.3.1 Raltegravir. [12]

RAL is the first INSTs have been introduced in clinical practice in the 2007 after the approval by FDA. From the chemical point of view, RAL is a 5-hydroxydihydropyrimidin-6-one-4-carboxamide belonging to the DKA class. The pharmacophore, or the catalityc triade domain, is not represented by the

characteristic sequence of the α,γ-diketo acid mojety, but in this case the metal chelating group is conceal in the pyrimidinone cycle; for guarantee the chelation of the metal ions in the pharmacophoric group of RAL are respected the sequence and the kind of atoms that characterized the α,γ-diketo acid mojety. Infact the carbonyl group on 6 position and the hydroxyl group in 5 position of the pyrimidine ring, and the carboxyl group of the 4-fluorobenzyl amide imitate the two carbonyl group and the carboxyl acid respectively, of the α,γ-diketo acid mojety (see structure 5). Merck had discovered a series of DKA scaffolds active against HIV-1 IN and the HCV NS5b RNA-dependent RNA polymerase and mechanistically these compounds were expected to act similarly against these enzymes, namely via the chelation of active site metals. An interesting and key observation of this work was that a dihydroxypyrimidine caboxamide derived from DKAs in the HCV program that had no activity against HCV NS5B, acted as a potent, reversible and selective HIV INSTI with an IC_{50} of 85 nM in an enzymatic IN ST assay. Optimization of the SAR on the carboxamide led to the identification of p-flurobenzyl as the optimal amide residue and a gem-dimethyl as the optimal 2-substituent for the dihydroxypyrimidine core. Further optimization of capping groups of the amine portion of the scaffold demonstrated that heteroaromatic groups were the most potent. Five membered ring heterocycles were also investigated and compounds bearing 2 or 3 heteroatoms were the most potent in cell based antiviral assays, particularly those with a heteroatom in the 2 position. The compound that ultimately became known as RAL was an oxadiazole with three heteroatoms and was one of the most potent compounds investigated in cell based assays with an IC_{95} of 31 nM in the presence of 50% human serum. RAL was highly selective against other enzymes working through Mg^{2+} based mechanisms being inactive against HCV polymerase, HIV RT and RNaseH, and human, α, β and γ polymerases, and showed no activity up to 10 μM concentration on a panel of 150 enzymes, channels and receptors. RAL also did not have activity against the major cytochrome P450 isoforms including 1A2, 2C9, 2D6, 3A4 and 2C9 nor did its how time dependent inhibition of 3A4. Binding affinity on cardiac HERG channels was >50 μM, suggestive of a good cardiac safety profile. Pharmacokinetic studies in animals showed good bioavailability with a multiphasic elimination, including a relatively short phase and a prolonged phase. Studies of metabolism demonstrated that RAL was primarily eliminated as a glucuronidated

metabolite through bile and urine with the glucoronidation occurring on the 5-hydroxyl group of the pyrimidinone ring. Based on the need to keep the plasma concentration above the cell culture 95% inhibitory concentration (CIC_{95}) and considering a variety of key factors including the biphasic elimination, the metabolic stability, protein binding and plasma clearance, the human dosing regimen of RAL was predicted to be twice daily (BID). Metabolism of RAL occurs primarily through glucoronidation and not via Cyp3A4 therefore RAL cannot be boosted with ritonavir as is the case for EVG. The most common side effects are upset stomach, headache, tiredness, itching, diarrhea, constipation, flatulence, and sweating.

Based on the clinical trials studies, RAL has been shown to be a potent, well tolerate drug that can be combined with existing ARVs to provide highly efficacious treatment to both ARV experienced and naïve subjects. Further clinical studies will define the strengths and weaknesses of RAL and of the INSTI class ingeneral.

1.3.2 Elvitegravr.

Elvitegravir is a second INSTI in phase 3 clinical development in ARV treatment-experienced subjects and is also undergoing phase 2 development in ARV naïve subjects as part of a fixed dose combination regimen. EVG was discovered by researchers at Japan Tobacco who described a new pharmacophore, specifically 4-quinolone-3-glycoxylicacid, which maintained the coplanarity observed in DKA INSTIs. Compounds containing the 4-quinolone-3-carboxylic acid motif, but not the 4-quinolone-3-glycoxylic acid, were inhibitors of IN, with the coplanar monoketo acid motif in 4-quinolone-3-carboxylic acids providing an alternative to the DKA motif (see structure 6). Elvitegravir showed potent anti-HIV activity *in vitro* against HIV-1 of multiple subtypes (EC_{50} ranging from 0.1 to 1.26 nM) as well as against HIV-2 strains (EC_{50} 1.4–2.8 nM), and both SIV and murine leukemia viruses. Elvitegravir, like raltegravir has also shown potent antiviral activity *in vivo* against HIV-1 carrying resi stance mutations to multiple antiretroviral drug classes. Development of EVG, has focused on boosting it to QD dosing via inhibition of CyP3A4 metabolism. The clinical study demonstrated that EVG is metabolized by CYP3A4 and that the plasma concentration of elvitegravir can be boosted by the addition of ritonavir. EVG pharmacokinetics was significantly improved when the drug was boosted with ritonavir; infact its plasma half-time was increased from three

to nine hours in the presence of ritonavir, thereby permitting once-daily dosing of this new integrase inhibitor [13]. Tha most common side effect is loss of appetite.

1.3.3 Resistance to Raltegravir and Elvitegravir.

Drug resistance in HIV-1 evolves due to the lack of proof reading activity of HIV reverse transcriptase. Consequently, nearly every viral genome contains at least one mutation, some of which can impart drug resistance. During the development of INSTIs, a significant amount of information has accumulated on the IN mutations leading to drug resistance (Table 1). As currently understood, resistance to INSTIs can be mediated by a number of IN mutations that can be considered "primary" resi stance mutations which are all located in the CCD of IN, close to the INSTI binding site. Within this set of mutations, which occur at codons T66, E92, Y143, Q148 and N155, varying effects on susceptibility to EVG and RAL are observed. Numerous additional IN mutations, some of which are natural poly- morphisms and most of which are also found in the CCD, comprise secondary resistance mutations which increase phenotypic resistance when added to primary mutations. Many of these secondary mutations have only limited effects on susceptibility to EVG and RAL when present on their own. Some secondary resistance mutations have been noted in the CTD of IN including mutations at codons S230 and R263; however, no resistance mutations have been described in the NTD of IN. In many respects, resistance to INSTIs has features in common with that observed for other ARV drug classes, notably the accumulation of mutations leads to higher levels of resistance.

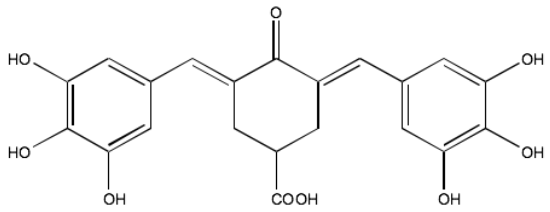
Table 1. HIV-1 integrase mutations selected by INSTIs *in vitro*.

Compound	Group	Selected mutations in IN of HIV-1
RAL	Merck	E138A, G140A, Q148K
RAL	Merck	T66A, Q95K, Y143C or T66A, Y143R or N155H
RAL	Tibotec	E138K, G140A, Q148R
EVG	Kyoto University	H51Y, E92Q, S147G, E157Q or T66I, Q95K, E138K Q146P, S147G
EVG	Gilead	T66I, F121Y or T66I, S153Y or T66I, R263K
EVG	GSK	T66I/A/K, V72A, E92Q/V, P145S, Q146S/L, Q148K/R

EVG	Merck	T66A, V72I, N155S or Q148R
EVG	Tibotec	T66I alone or R20K, T66I, L74M, S230R or T66I, A128T, E138K, Q148R, S230R or E92Q alone or T66A, E92Q or E92Q, E138K, Q148R

1.4 Aim

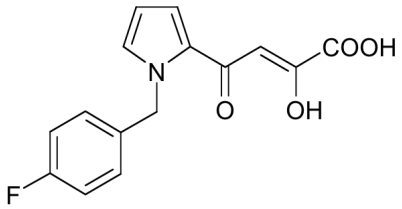
The research group where I spent my PhD period by several years is involved in design and synthesis of organic compounds inhibitors of IN of HIV-1. This approch is started at the end of the nineties, with the synthesis of curcumina analogues polyoxydryl compounds that exhibited high activity in enzyme and cell-based assaies. [30]



8

IC_{50} = 0.2 μM (3'P)
IC_{50} = 0.3 μM (ST)
EC_{50} = 4 μM

Afterwards, the Merck Inc. had finished a random screening on about 250000 compounds, from which was emerged a new hit compound (**2, L-731,988**) characterized by a diketoacid mojety linked on a phenylmethyl pyrrolic group, proven to be a potent inhibitor on ST, in cell-based assay too.



L-731,988

2

These interesting results lead us to design a series of 6-(1-arylmethyl-1H-pyrrol-2-yl)-2,4-dioxo-5-hexenoic acids that risen from a superposition of cinnamoyl and dioxobutanoic mojeties (Fig. 8).

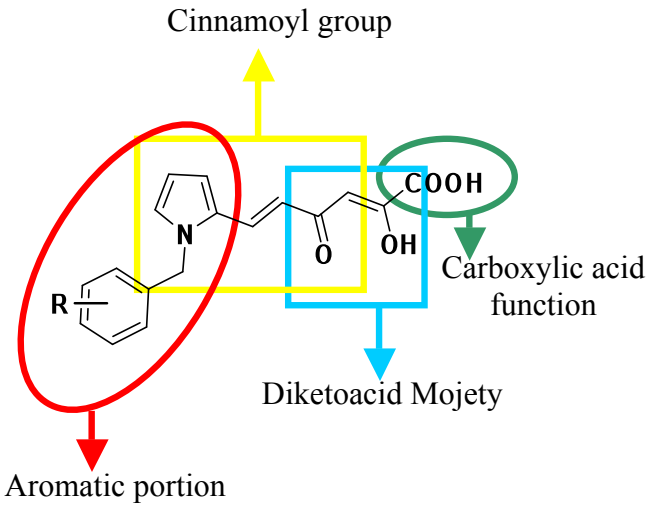


Figure 8. Different features of 6-(1-arylmethyl-1H-pyrrol-2-yl)-2,4-dioxo-5-hexenoic acids.

All new synthesized compounds was tested by research group of Prof. Y. Pommier at National Institute of Healt in Bethesda, USA.

From an initial analysis of obtained biologycal data, some mojety was resulted essential to have actived compounds against IN of HIV-1. In particular is emerged that:

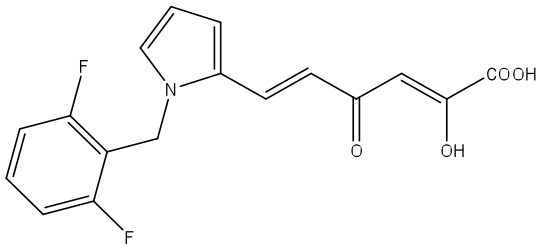
1 a cinnamoyl group is presented both in natural and synthetic compound;

2 the α,β-diketo group characterized the aryldiketoacids series;

3 The carboxylic acid function is found in natural (L-cicoric acid), diketoacid compound (L-731,988) and cinnamoyl derivative (**8**);

4 an aromatic portion is presented in all compounds.

Among the serie of pyrrolyldiketoacid derivatives synthesized by us, the best resultes was obtained with the 2,6-difluorosubstitutes derivative **9**.



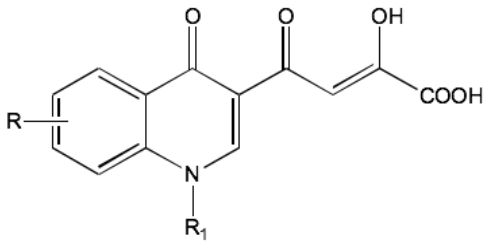
9

9 has demonstrated an IC_{50} value of 0.15 μM, EC_{50} value of 0.022 μM, CC_{50} value of >50 μM and a selective index > 2273 that lead it to be three times more active of Merck derivative **L-731,988**.

On the bases of this structural analysis, my research group has decided to preserve

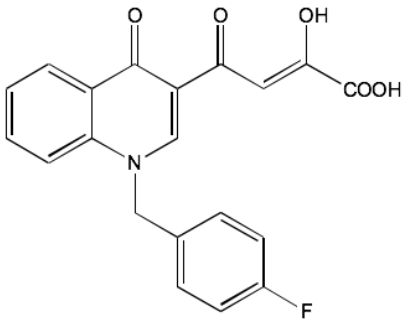
unchanged the essential structural features for the antiviral activity, or rather the cinnamoyl group, the α,β-diketo group, the carboxylic acid function and the aromatic portion. In particolar, the cinnamoyl group was built in a rigid ring as the oxoquinolonyl system, to generate conformational blocked analogue of **9**, to increase the interaction with the enzymatic active site and the biologycal activities.

In this way was designed and synthesized the quinolonyldioxobutanoic acids **10**.



10

Initially was led studies on the substitution on benzene ring of quinolonyl system, to obtain selective compound active on ST; after that was studied the substitution on nitrogen atom of quinolonyl system, in particular was found that the best substituent was the benzyl group and specifically the 4-fluorobenzyl derivative **11**.



11

The results obteined with derivative **11** was excellent: infact, it presents an IC_{50} value of 0.013 μM for ST inhibition and an EC_{50} value of 1.17 μM.

Noteworthy, passed between **9** to **10** series it possible to note an increase of activity on ST inhibition, assessable of 2-3 order of magnitude.

Analizing the activities data, compound **11** shows an interesting inhibitory activity in enzymatic assay, but a more restricted activity in cell-based assay on infected cell by HIV-1.

On the bases of this beckground, my research work was to develop the IN inhibitors with three different approaches:

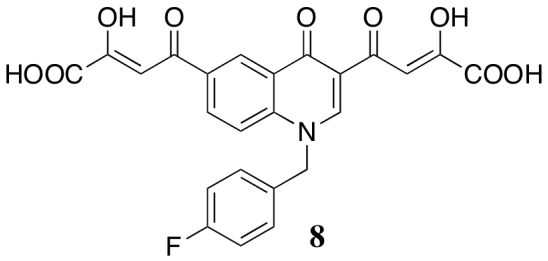
1. carry on the design and synthesis of quinolonyldioxobutanoic acids analogue of **11**, to increase the selectivity for ST reaction and the activity in cell-based assay on infected cell by HIV-1;
2. designed and synthesized a new scaffolds ables to chelate the two metal ions present in the active site of the enzyme and given an inhibitory activity in cell-based assay on infected cell by HIV-1;
3. develop INSTIs that can be tested for its microbicide activity.

In the following paragraphs, I'm going to describe how these three different approaches was developed.

1.5 Quinolonyldiketo acids

1.5.1 Introduction

Recent studies on quinolinonyl diketo acid derivatives led us to discover the bifunctional compound **12** as a potent IN inhibitor for both 3′-P and ST.[32] Moreover, **12** inhibits HIV-1 replication in acutely infected cells.[32]



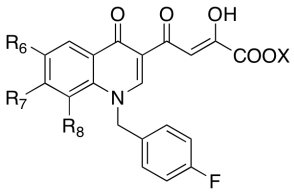
8

Docking studies on the binding mode of **12** to the IN catalytic site also suggested a peculiar interaction of the drug involving both the acceptor and the donor DNA binding sites of the enzyme. This hypothesis was confirmed by our recent cross-linking experiment studies that pointed out a specific interaction of **12** with K156 and K159 amino acid residues of the IN catalytic core domain.15 This binding mode could account for the high potency of **12** against both 3′-P and ST. The aim of the present project was the design of new quinolinone derivatives endowed with a selective activity against ST. This project could provide new information regarding the interactions of quinolinonyl diketo acids with the IN active site and consequently increase our knowledge about the catalytic mechanisms of IN, which in the absence of structural information on IN-DNA and drug molecular structures are still far from being totally elucidated. In the present paragraph, I describe novel quinolinonyl diketo acid derivatives **13a-i**, **14a-i**, **15a**,**b** and **16a**,**b** designed by replacement of the 6-diketo acid chain of **12**, responsible for binding to the donor DNA binding site, with smaller substituents in the 6-, 7-, or 8-position of the quinolinone ring.

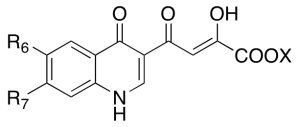
These substituents should exhibit reduced binding to the donor viral cDNA site due to their small size/length or to their limited ability to form hydrogen bonds. Thus, these structural modifications should provide novel quinolinonyl diketo acid derivatives with increased ST selectivity. Furthermore, I have delved into the substitution in 7-position of the quinolinone ring with different cyclic and linear bases, designed and synthesized novel quinolinonyl diketo acid derivatives **13j-q, 14j-q** analogues of **13i** and **14i** and **17a,b** and **18a,b** analogues of **12**, in which is presented a lipophilic and basic portion that may counterbalance the polarity of the

diketoacid mojety and enhanced the activity in cell-based assay on infected cell by HIV-1.

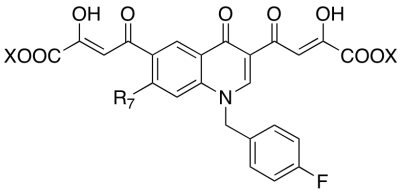
Table 2. Compound **13a-q**, **14a-q, 15a,b, 16a,b, 17a,b** and **18a,b.**



13a-q 14a-q



15a,b 16a,b



17a,b 18a,b

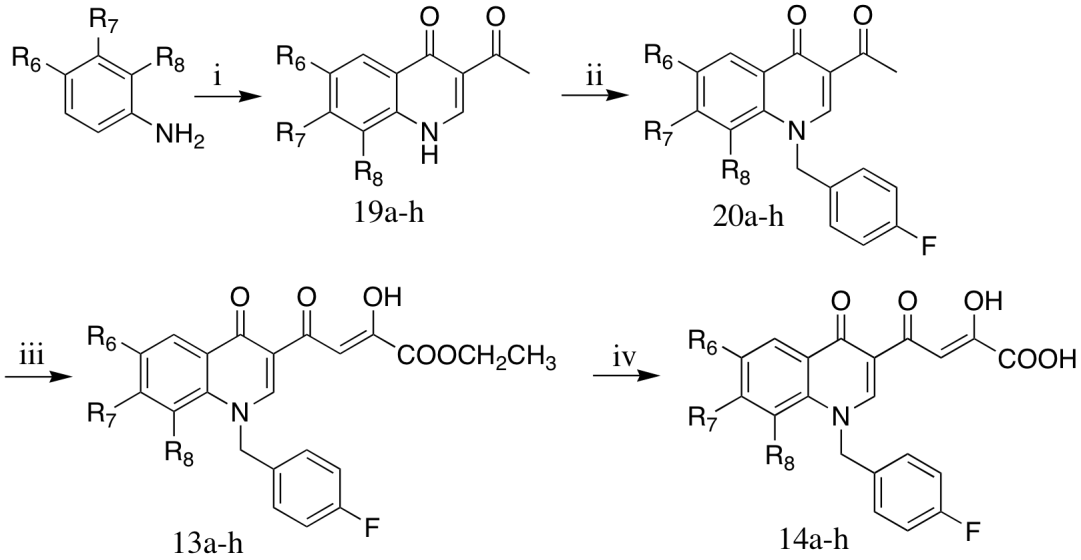
Cpd	R6	R7	R8	X
13a	H	H	H	Et
13b	F	H	H	Et
13c	H	F	H	Et
13d	F	H	F	Et
13e	Cl	H	H	Et
13f	H	Cl	H	Et
13g	H	H	Cl	Et
13h	Cl	Cl	H	Et
13i	H	1-Pyrrolydinyl	H	Et
13j	H	1-Pyperazinyl	H	Et
13k	H	*N*-Methyl-1- Pyperazinyl	H	Et
13l	H	*N*-Ethyl-1- Pyperazinyl	H	Et
13m	H	*N*-Acethyl-1- Pyperazinyl	H	Et
13n	H	1-Thiomorpholinyl	H	Et
13o	H	1-Morpholinyl	H	Et
13p	H	*N*-(3-chloroprop-1-yl)-1-pyperazinlyl	H	Et
13q	H	*N,N*-dimethylamine	H	Et
15a	Cl	H	-	Et
15b	H	Cl	-	Et
17a	-	*N,N*-dimethylamine	-	Et
17b	-	*N*-Methyl-1- Pyperazinyl	-	Et

Cpd	R6	R7	R8	X
14a	H	H	H	H
14b	F	H	H	H
14c	H	F	H	H
14d	F	H	F	H
14e	Cl	H	H	H
14f	H	Cl	H	H
14g	H	H	Cl	H
14h	Cl	Cl	H	H
14i	H	1-Pyrrolydinyl	H	H
14j	H	1-Pyperazinyl	H	H
14k	H	*N*-Methyl-1- Pyperazinyl	H	H
14l	H	*N*-Ethyl-1- Pyperazinyl	H	H
14m	H	*N*-Acethyl-1- Pyperazinyl	H	H
14n	H	1-Thiomorpholinyl	H	H
14o	H	1-Morpholinyl	H	H
14p	H	*N*-(3-chloroprop-1-yl)-1-pyperazinlyl	H	H
14q	H	*N,N*-dimethylamine	H	H
16a	Cl	H	-	H
16b	H	Cl	-	H
18a	-	*N,N*-dimethylamine	-	H
18b	-	*N*-Methyl-1- Pyperazinyl	-	H

1.5.2 Results and discussion

Synthesis of derivatives **13a-q**, **14a-q, 15a,b, 16a,b, 17a,b** and **18a,b** is outlined in Schemes 1–4. 3-Acetyl-4(1*H*)-quinolinones **19a-h** were prepared by reaction of the proper aniline with ethyl orthoformate and ethyl acetoacetate, which were thermally condensed in the presence of an inert heating medium (Dowtherm A) under argon atmosphere, according to the Yoshizawa procedure. [33] Then, **17a-h** were alkylated with 4-fluorobenzylbromide in alkaline medium (K_2CO_3) to give the N-1 substituted quinolones **18a-h**. These compounds were condensed with diethyl oxalate in the presence of sodium ethoxide to provide ethyl esters **13a-s,** which were in turn hydrolyzed with 6 N NaOH to afford the corresponding acids **14a-s** (Scheme 1).

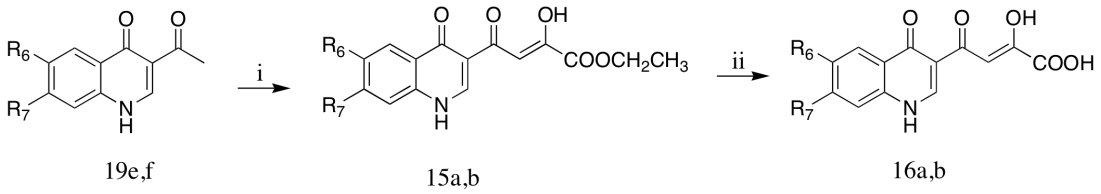
Scheme 1. [a]



[a] Reagents and conditions: (i) ethyl orthoformate, ethyl acetoacetate, Dowtherm A, 95-254 °C, 8 h; (ii) 4-fluorobenzyl bromide, K_2CO_3, DMF, 100 °C, 1 h; (iii) diethyl oxalate, C_2H_5ONa, THF, room temp, 2 h; (iv) 1 N NaOH, THF/CH_3OH, room temp, 40 min.

Synthesis of 1-unsubstituted derivatives **15a**,**b** and **16a**,**b** was achieved in a similar fashion by coupling of 4-quinolinones **19e**,**f** with diethyl oxalate in the presence of the sodium ethoxide, followed by alkaline hydrolysis of the resulting ethyl esters **15a**,**b** to provide acids **16a**,**b** (Scheme 2).

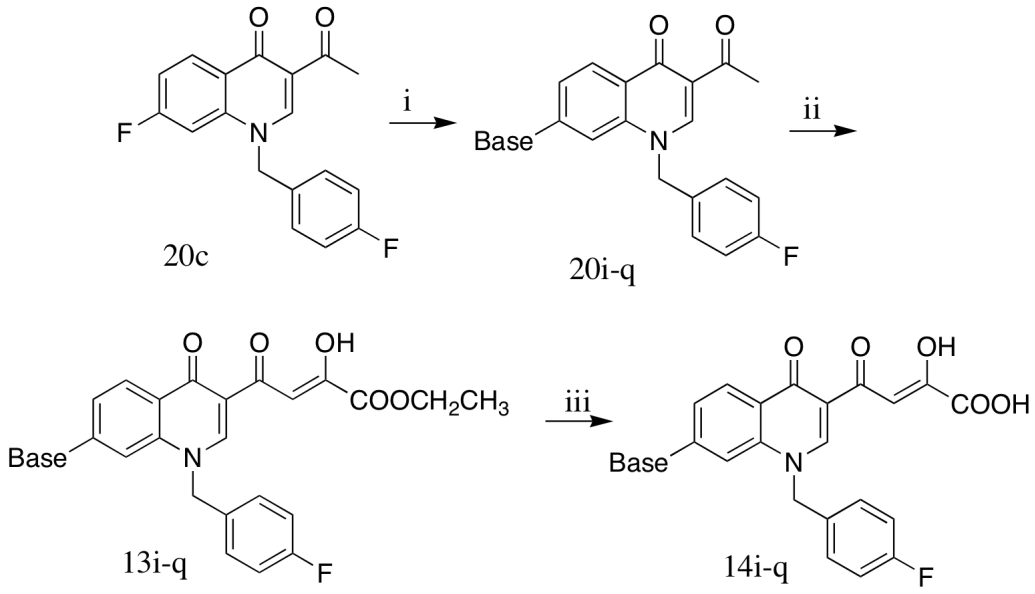
Scheme 2. [a]



[a] Reagents and conditions: (i) diethyl oxalate, C_2H_5ONa, THF, room temp, 2 h; (ii) 1 N NaOH, THF/CH_3OH, room temp, 40 min.

Derivatives **13i-q** and **14i-q** were synthesized as described in Scheme 3. Derivatives **17i-q** were obtained in few minutes with good yields by substitution of fluorine atom of **17c** with the appropriate base in the presence of NEt_3 or in aqueous solution under microwave irradiation. **17i-s** was then condensed with ethyl oxalate, and the ester **13i-s** that formed was hydrolyzed to afford the required acid **14i-s**.

Scheme 3. [a]

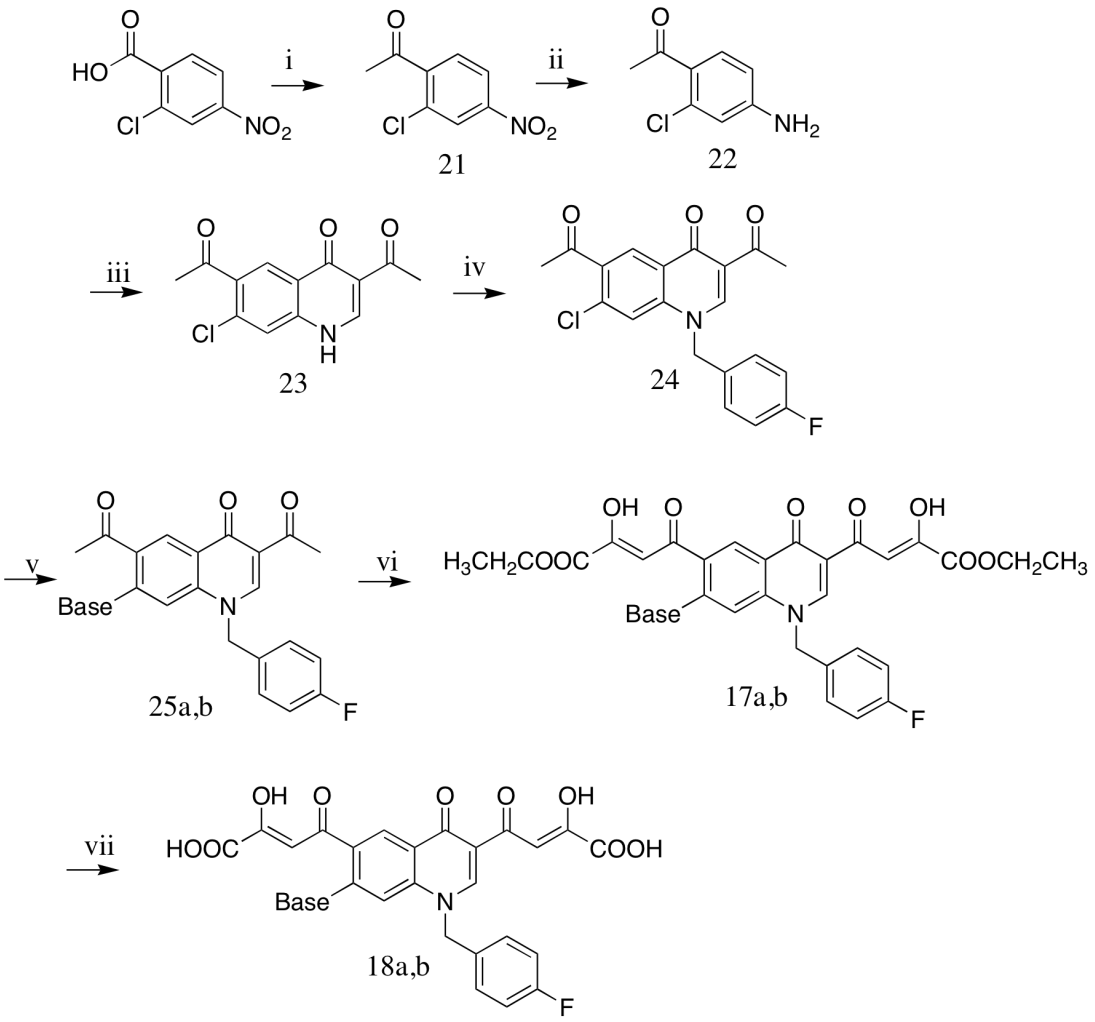


[a] Reagents and conditions: (i) base, Et_3N, DMF, microwave, 100 W, 153 °C, 10 min, or *N,N*-dimethylamine acq. sol. 40%, 100 W, 175 °C, 10 min; (ii) diethyl oxalate, C_2H_5ONa, THF, room temp, 2 h; (iii) 1 N NaOH, THF/CH3OH, room temp, 40 min.

The derivatives **17a,b** and **18a,b** were synthesized as described in Scheme 4, starting from the commercially available 4-nitro-2-chlorobenzoic acid that reacted with carbonyldiimidazole (CDI) to form the activated amide that reacted with Meldrum's acid (2,2–dimethyl-1,3-dioxano-4,6-dione) to form the product of substitution that was hydrolized in presence of HCl 10 % to obtain the acethyl derivative **21.** Subsequently, It was sujected to a reduction reaction, which the nitro group was

reduced to amino group in presence of Pd/C and H_2 at atmospheric pressure and room temperature obtaining the derivative **22,** that was the substratum for the cyclization reaction with ethyl orthoformate and ethyl acetoacetate, which were thermally condensed in the presence of an inert heating medium (Dowtherm A) under argon atmosphere, according to the Yoshizawa procedure. [33] The 3,6-Diacetyl-4(1*H*)-quinolinone **23** was alkylated in presence of 4-fluorobenzylbromide in alkaline medium (K_2CO_3) to give the N-1 substituted quinolones **24.** Derivatives **25a,b** were obtained in few minutes with good yields by substitution of fluorine atom of **17c** with *N*-methylpyperazine in the presence of NEt_3 or *N,N*-dimethylamine in aqueous solution under microwave irradiation. **25a,b** was then condensed with ethyl oxalate, and the ester **17a,b** that formed was hydrolyzed to afford the required acid **18a,b**.

Scheme 4. [a]

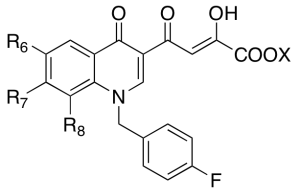


[a] Reagents, conditions and yields: (i) 1) CDI,CH_2Cl_2, room temp, 2 h; 2) Meldrum's acid, room

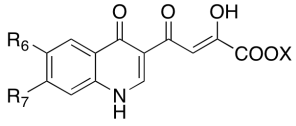
temp,72 h, 3) HCl 10% , room temp, 2 gg, 10 %; (ii) Pd/C, H_2, ethyl acetate, 1 Atm, room temp, 2.5 h, 98 %; (iii) ethyl orthoformate, ethyl acetoacetate, Dowtherm A, 95-254 °C, 8 h; 50 %; (iv) 4-fluorobenzylbromide, K_2CO_3, DMF, 100 °C, 1 h, 17 %; (v) *N*-Methylpyperazine, Et_3N, DMF, microwave, 100 W, 153 °C, 10 min, 50 %, or *N,N*-dimethylamine acq. sol. 40%, 100 W, 175 °C, 10 min, 51%; (vi) diethyl oxalate, C_2H_5ONa, THF, room temp, 2 h, 76%; (vii) NaOH 1N, THF_a, CH_3OH, room temp, 5 min, 77 %.

Derivatives **13a-q**, **14a-q, 15a,b, 16a,b, 17a,b** and **18a,b** were tested in vitro for ST inhibition in the presence of magnesium (Mg^{2+}) using a recently described high throughput electrochemiluminescent (HTECL) assay. [30] IC_{50} values were generated from duplicate experiments (Table 3). Compounds **13a-q**, **14a-q, 15a,b, 16a,b, 17a,b** and **18a,b** were also tested for ST and 3′-P using gel-based assays carried out in the presence of Mg^{2+}. IC_{50} values were calculated using dose response curves and are summarized in Table 3.

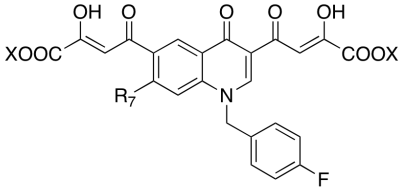
Table 3. Activities data for compound **13a-q**, **14a-q**, **15a**,**b**, **16a**,**b, 17a,b** and **18a,b.**



13a-q 14a-q



15a,b 16a,b



17a,b 18a,b

					ANTI-IN activity IC_{50}[a]			ANTIVIRAL activity		
Cpd	**R6**	**R7**	**R8**	**X**	**Mg^{2+b}**	**Mg^{2+c}**	**Mg^{2+}_c**	**CC_{50}[d]**	**EC_{50}[e]**	**SI[f]**
13a	H	H	H	Et	0.67	0.79, 0.44	135	nt[g]	nt[g]	
13b	F	H	H	Et	0.58	0.23, 0.44	>333	>200	>50	
13c	H	F	H	Et	1.3	1.2, 2.9	>333	>200	>50	
13d	F	H	F	Et	26	5.2	>333	>200	>50	
13e	Cl	H	H	Et	21	3.0	>333	nt[g]	nt[g]	
13f	H	Cl	H	Et	14	3.6	>333	nt[g]	nt[g]	
13g	H	H	Cl	Et	32	5.2	>333	>200	>50	
13h	Cl	Cl	H	Et	3.9	1.8	135	72	>50	
13i	H	1-Py[h]	H	Et	2.3	2.7	110	>200	4.1	48.8
13j	H	1-Pyperazinyl	H	Et	24.4	nt[g]	>333	nt[g]	nt[g]	
13k	H	*N*-Methyl-1-Pyperazinyl	H	Et	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
13l	H	*N*-Ethyl-1-Pyperazinyl	H	Et	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
13m	H	*N*-Acethyl-1-Pyperazinyl	H	Et	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
13n	H	1-Thiomorpholinyl	H	Et	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
13o	H	1-Morpholinyl	H	Et	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
13p	H	*N*-(3-chloroprop-1-yl)-1-pyperazinlyl	H	Et	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
13q	H	*N,N*-dimethylamine	H	Et	0.37	nt[g]	39	nt[g]	nt[g]	
15a	Cl	H	H	Et	3.3	2.1	120	nt[g]	nt[g]	
15b	H	Cl	H	Et	>111	224	>333	nt[g]	nt[g]	
17a	-	*N,N*-dimethylamine	H	Et	2.9	nt[g]	41	nt[g]	nt[g]	
17b	-	*N*-Methyl-1-Pyperazinyl	H	Et	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
14a	H	H	H	H	0.040	0.06,	14	>200	1.17	>171

						0.16				
14b	F	H	H	H	0.030	0.14	2.3	>200	>50	
14c	H	F	H	H	0.020	0.050	4.4	>200	>50	
14d	F	H	F	H	0.018	0.16	16	>200	>50	
14e	Cl	H	H	H	0.028	0.40	6.4	>200	46.1	4.3
14f	H	Cl	H	H	0.019	0.11	4.0	>200	>50	
14g	H	H	Cl	H	0.023	0.51	44	>200	>50	
14h	Cl	Cl	H	H	0.033	0.075	11, 34	156	3.2	62.5
14i	H	1-Pyrrolidinyl	H	H	0.028	0.18	14, 9.0	>200	0.17	>1176
14j	H	1-Pyperazinyl	H	H	2.01	nt[g]	65	nt[g]	nt[g]	
14k	H	*N*-Methyl-1-Pyperazinyl	H	H	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
14l	H	*N*-Ethyl-1-Pyperazinyl	H	H	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
14m	H	*N*-Acethyl-1-Pyperazinyl	H	H	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
14n	H	1-Thiomorpholinyl	H	H	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
14o	H	1-Morpholinyl	H	H	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
14p	H	*N*-(3-chloroprop-1-yl)-1-pyperazinlyl	H	H	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
14q	H	*N,N*-dimethylamine	H	H	0.054	nt[g]	2.7	nt[g]	nt[g]	
16a	Cl	H	H	H	>111	56	166	nt[g]	nt[g]	
16b	H	Cl	H	H	37	17	>333	nt[g]	nt[g]	
18a	H	*N,N*-dimethylamine	H	H	0.15	nt[g]	5.1	nt[g]	nt[g]	
18b	H	*N*-Methyl-1-Pyperazinyl	H	H	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	

[a] Inhibitory concentration 50% (μM) determined from dose response curves. [b] Experiments performed in duplicate using the high throughput electrochemiluminescent (HTECL) assay (ST assay in the presence of Mg^{2+}). [c] Experiments performed in a gel-based assay in the presence of Mg^{2+} (see Figure 8). [d] Cytotoxic concentration 50% (μM). [e] Effective concentration 50% (μM). [f] Selectivity index (CC_{50}/EC_{50}). [g] nt: not tested.

The newly synthesized DKAs **13a-i**, **14a-i**, **15a**,**b**, **16a**,**b** exhibited high potency against IN with high selectivity against ST, thus confirming the hypothesis that the removal of the diketo acid branch in the 6-position of the quinolinone ring of **12** can lead to ST-selective inhibitors (**14a-i** showed 3′-P/ST ratios ranging from 45 to 667). The acid derivatives **14a-i** were more potent than the corresponding esters **13a-i** (Figure 8A and Figure 8B, compare compounds **14h** and **13h**), and the 1-*p*-F-benzyl substituted quinolinones (**13e**,**f** and 1**4e**,**f**) were markedly more active than their unsubstituted counterparts (**15a**,**b** and **16a**,**b**). This trend is consistent with the results obtained previously on the bifunctional DKA (BDKA) series.[30] In particular, the relevant role played by the *p*-F-benzyl moiety at the 1-position of the quinolinone ring was previously noted for the DKA derivatives. The newly synthesized acid derivatives **14a-i** were potent inhibitors, showing IC_{50} values in the range of 18-40 nM for the ST step (Table 3). The most active compound of this series is **14d** with a ST IC_{50} value of 18 nM, which is comparable to reference drugs **2** and **3**. Removal of the diketo acid chain from the 6-position of our BDKA lead molecule (compound **12**) [30] led to compound **14a** that retains the potent anti-IN activity of the parent derivative with an IC_{50} value of 40 nM for ST (Table 3). However, **14a** is a more ST

selective inhibitor than **12**.14 Introduction of a halogen (F or Cl) in the 6-, 7-, or 8-position of **14a** led to compounds **14b-g**, which show similar anti-IN activities **(**IC_{50}) 18-30 nM, (Table 3) as their unsubstituted counterpart **14a**. Interestingly, the anti-IN activities appear to be influenced by the position of the halogen. Indeed, inhibitory activities decrease in the following order:

8-F(Cl) ~ 7-F(Cl) > 6-F(Cl) and 7-Cl > 8-Cl > 6-Cl.

The introduction of a second chlorine atom in the structure of the potent inhibitor **14f** led to **14h**, which is approximately 1.5 times less potent than the parent counterpart. Similarly, replacement of the chlorine atom of **14f** with a pyrrolidine ring led to **14i**, which is approximately 1.5 times less potent than the parent compound. In conclusion, the activities of compounds with a substituent in the 7-position decrease in the following order: Cl > F > 1-pyrrolidinyl > H (Table 3). Compounds **13a-i** and **13a-i** also inhibited integrase with similar potency in the presence of Mg^{2+} or Mn^{2+} (data not shown). The activity in the presence of Mg^{2+} in addition to the selectivity for ST represents a trademark of the most potent DKAs active against HIV replication in cells.

Activities data relatively the newly synthesized DKAs **13j-q**, **14j-q**, **17a**,**b**, **18a**,**b** are uncomplete, thus it cannot possible to formulate valid structure-activity relationships. Now are availabled enzymatic activity data only for compounds **13-14j,q** and **17-18a.** On the strength of the available data is possible to affirm that the acid derivatives **14j-q** and **18a** were more potent than the corresponding esters **13j**,**q** and **17a** and exhibited high potency against IN with high selectivity against ST. The substitution with the dimethylamine on quinolinone ring led to compound **14q** that mantein the inhibitory activity on enzymatic assay if compare with the lead **14i**; this trend is not followed by derivative **14j** in which the enzymatic activity is decreased of hundred times. Compound **18a** is a derivative of **12**, compared with the BDKA lead molecule **12** its enzymatic activity is diminished of ten times, but with the insertion of dimethylamine substituent is increased the selectivity for ST respect to 3'-P reaction.

Cytotoxicity and antiviral activities of compounds **13a-i**, **14a-i**, **15a**,**b**, and **16a**,**b** are presented in Table 3. Among the tested compounds, derivatives **13a**,**i**, and **14a**,**e**,**h**,**i** show EC_{50} < 50 μM when tested against HIV-1-infected H9/ HTLVIIIB cells. In particular, **13i** and **14a**,**h**,**i** are active in the micromolar range, with SI ranging from 48.8 to >1176. The most potent derivative of this series is **14i** (EC_{50}) 0.17 μM and SI

> 1176). Compound **14i** is in fact more potent than reference derivatives **2** and **12**. In general, all the active compounds were characterized by low cytotoxicity against human histiocytic lymphoma (U937) cell line, showing CC_{50} values in the range of 99 to >200 μM.

1.5.3 Conclusions

I designed, synthesized, and tested a series of novel quinolinonyl diketo acid derivatives in both enzyme- and cellbased assays as anti-HIV-1 agents to selectively target the ST step of integration. We were able to generate such new compounds, which are potent ST-selective IN inhibitors and active against HIV-1 replication in acutely infected cells. These compounds could be useful tools to study the mechanism of action of HIV-1 IN.

1.5.4 Experimental section

Chemistry

General. Melting points were determined with a Büchi 530 capillary apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Spectrum-one spectrophotometer. ^{1}H NMR spectra were recorded on a bruker AC 400 specrtometer. Merk silica gel 60 F_{254} plates were used for analytical TLC. Column chromatogrephies were performed on silica gel (Merck; 70-230 mesh) or alumina (Merck; 70-230 mesh). Concentration of solution after reactions and extractions involved the use of a rotary evaporator operating at reduced pressure of approximately 20 Torr. Analytical results agreed to within ±0.40% of the theoretical values.

Microwave irradiation experiments. Microwave reactions were conduced using a CEM Discover system unit (CEM. Corp., Matthews, NC). The machine consists of a continuous focused microwave-power delivery system with operator selectable power output from 0 to 300 W. The temperature of the contents of the vessel was monitored using a calibrated infrared temperature control mounted under the reaction vessel. All experiments were performed using a stirring option whereby the contents of the vessel are stirred by means of a rotating magnetic plate located below the floor of the microwave cavity and a Teflon-coated magnetic stir bar in the vessel.

Synthesis of 1-(2-chloro-4-nitrophenyl)ethanone (21). To a suspention of 2-chloro-4-nitrobenzoic acid commercially available (10 g, 0.0496 mol) in dichloromethane (46.6 mL) was added CDI (8.3 g, 0.051 mol) and stirred at room temp for 2 h (until begin a solution). To the obtained solution was added Meldrum's acid (7.15 g, 0.0496 mol) and stirred for 72 h at room temp. Afterwards, was added 59.5 mL of HCl 10%, and the suspention was stirred and heated at reflux for 8 h. After cooling, the organic layer was collected, washed with acq. sol $NaHCO_3$ 5%, brine and dried on Na_2SO_4 anydrus, obtaining 2.23 g of crude product as orange oil. Purification of crude products was performed by chromatography on silica gel column (chloroform/ethyl acetate 1:2 as eluent) to give 310 mg of pure **21** (8% yield). Chemical and physical of derivative **21** are reported in Table 4.

Synthesis of 1-(4-amino-2-chlorophenyl)ethanone (22). A solution of starting

material **21** (4.31 g, 0.0216 mol) in 282 mL of ethyl acetate was stirred in presence of 720 mg of 10% Pd/C under H_2 atmosphere at atmospheric pressure and room temperature for 2.5 h. The mixture was then filtered and the Pd residue washed with ethyl acetate. The combined organic layer were concentrated at reduced pressure to yield 3.36 g of pure product as yellow oil (98% yield). Chemical and physical of derivative **22** are reported in Table 4.

General Procedure for the Synthesis of 3-Acetyl-4(1*H*)-quinolinones 19a-h, 23. Ethyl orthoformate (4.0 g, 27 mmol), ethyl acetoacetate (3.5 g, 27 mmol), the appropriate substituted aniline (27 mmol), and Dowtherm A (5.6 mL) were charged in a three-necked flask equipped with a water separator. This mixture was stirred under argon atmosphere, while the temperature was increased to 95 °C in 1 h, then gradually up to 162 °C in a further hour. Then the mixture was stirred at this temperature for 6 h. After this time, the resulting solution was added in portions during 3 h into 42 mL of Dowtherm A stirred in a three-necked flask equipped with thermometer and water separator and heated at 253-254 °C. After addition the mixture was heated at the same temperature for 2 h. Then the mixture was cooled at 90 °C, treated with isopropanol (10 mL), cooled at 30 °C, filtered, and washed with isopropanol and light petroleum ether in turn to give pure derivatives **19a-h, 23**. Chemical and physical data of derivatives **19a-h** and **23** are reported in Table 4.

General Procedure for the Synthesis of 1-(4-fluorophenyl)methyl-4(1*H*)-quinolinones 20a-h, 24. A mixture of the proper **19a-h** or **23** derivative (1.1 mmol), 4-fluorophenylmethyl bromide (610 mg, 3.3 mmol), and anhydrous K_2CO_3 (210 mg, 1.5 mmol) in dry DMF (10 mL) was stirred at 100 °C for 1 h. After the mixture was cooled, water was added (40 mL) and the precipitate that formed was filtered, washed with water and light petroleum ether in turn, and then dried under IR lamp to provide pure derivatives **20a-h, 24**. Chemical and physical data of derivatives **20a-h** and **24** are reported in Table 4.

General Procedure for the Synthesis of 7-(base)-4(1*H*)-quinolinones (20i-p, 25b). A mixture of **20c** or **24** (8.5 mmol), base (25.6 mmol), and NEt_3 (7.7 mmol) in dry DMF (40 mL) was irradiated with microwave at 153 °C for 10 min (applied potency 100 W), in an open vessel equipped with a condenser. After cooling, the reaction

mixture was diluted with water (100 mL) and treated with 1 N HCl until pH 7. The solid that formed was collected by filtration. Purification of crude product was performed by chromatography on silica gel column (chloroform/ethanol 10:1 as eluent) to give of pure **20i-p, 25a**. Chemical and physical data of derivatives **20i-p** and **25a** are reported in Table 4.

General procedure for the synthesis of 7-(dimethylamino)-4(1*H*)-quinolinones (20q, 25a). A mixture of dimethylamine in H_2O (40%, 0.0013 mol), and quinolinone **20c** or **24** (0.00045 mol) was irradiated with microwave at 175 °C, 100 Watt, for 10 minutes. After cooling the reaction mixture was diluited with water. The formed precipitate was collected by filtration on gouch, washed with water, and diethylic ether. Purification of crude products was performed by chromatography on silica gel column (chloroform/ethanol 5:1 as eluent) to give pure products**.** Chemical and physical data of derivatives **20q** and **25a** are reported in Table 4.

General Procedure for the Synthesis of Diketo Esters 13a-q, **15a,b and 17a,b.** Sodium ethoxide (390 mg, 5.5 mmol) was added into a well stirred mixture of the appropriate acetyl derivative **19e,f, 20a-q** or **25a-b** (2.7 mmol) and diethyl oxalate (790 mg, 5.4 mmol) in anhydrous THF (2.7 mL) under nitrogen atmosphere. The mixture was stirred at room temperature for 2 h, then was poured into *n*-hexane (50 mL). The collected precipitate was vigorously stirred for 30 min in 1 N HCl (50 mL). The yellow solid that formed was filtered, washed with water, and dried under IR lamp to afford the pure diketo esters **13a-q**, **15a,b** and **17a,b**. Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR are reported for each of the following compounds.

4-[1-(4-Fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13a). 82%; 175-177 °C; ethanol; IR *v* 3400 (OH), 1721 (C=O ester), 1661, 1635, and 1607 (C=O ketone) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.44 (t, 3H, CH_3), 4.42 (q, 2H, C*H*$_2$$CH_3$), 5.48 (s, 2H, CH_2), 7.06-7.26 (m, 4H, benzene H), 7.37-7.65 (m, 3H, quinolinone C6-H, C7-H, and C8-H), 8.16 (s, 1H, butenoate C3-H), 8.56 (m, 1H, quinolinone C5-H), 8.77 (s, 1H, quinolinone C2-H), 15.50 (bs, 1H, OH).

4-[6-Fluoro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2- hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13b). 92%; 152-153 °C; toluene/cyclohexane; IR *v* 1740 (C=O ester), 1650 and 1624 (C=O ketone) cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.29 (t, 3H, CH_3), 4.30 (q, 2H, CH_2CH_3), 5.78 (s, 2H, CH_2), 7.16-7.20 (m, 2H, benzene H), 7.29-7.37 (m, 2H, benzene H), 7.66 (m, 1H, quinolinone C7-H), 7.81 (m, 1H, quinolinone C8-H), 7.97 (m, 1H, quinolinone C5-H), 8.00 (s, 1H, butenoate C3-H), 9.13 (s, 1H, quinolinone C2-H), 15.33 (bs, 1H, OH).

4-[7-Fluoro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13c). 84%; 151-152 °C; benzene/cyclohexane; IR *v* 1726 (C=O ester), 1644 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-*d*6) δ 1.30 (t, 3H, CH_3), 4.30 (q, 2H, CH_2CH_3), 5.72 (s, 2H, CH_2), 7.17-7.21 (m, 2H, benzene H), 7.31-7.40 (m, 3H, benzene H and quinolinone C6-H), 7.62 (m, 1H, quinolinone C8-H), 7.97 (s, 1H, butenoate C3-H), 8.36 (m, 1H, quinolinone C5-H), 9.10 (s, 1H, quinolinone C2-H), 14.00 (bs, 1H, OH).

4-[8-Fluoro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13d). 83%; 179-180 °C; benzene/cyclohexane; IR *v* 3300 (OH), 1744 (C=O ester), 1648 and 1604 (CdO ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.27 (t, 3H, CH_3), 4.30 (q, 2H, CH_2CH_3), 5.78 (s, 2H, CH_2), 7.14-7.24 (m, 4H, benzene H), 7.48-7.53 (m, 1H, quinolinone C6-H), 7.59-7.69 (m, 1H, quinolinone C7-H), 7.97 (s, 1H, butenoate C3-H), 8.17-8.19 (m, 1H, quinolinone C5-H), 9.01 (s, 1H, quinolinone C2-H), 15.50 (bs, 1H, OH).

4-[6-Chloro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13e). 34%; 131-133 °C; washed with cyclohexane; IR *v* 3400 (OH), 1722 (C=O ester), 1700 and 1628 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.33 (t, 3H, CH_3), 4.33 (q, 2H, CH_2CH_3), 5.71 (s, 2H, CH_2), 7.14-7.34 (m, 4H, benzene H), 7.71-7.77 (m, 2H, quinolinone C7-H and C8-H), 7.91 (s, 1H, butenoate C3-H), 8.25 (m, 1H, quinolinone C5-H), 9.09 (s, 1H, quinolinone C2-H), 12.70 (bs, 1H, OH).

4-[7-Chloro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13f). 76%; 173-175 °C; washed with isopropanol; IR

v 3300 (OH), 1727 (C=O ester), 1647 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.30 (t, 3H, CH_3), 4.32 (q, 2H, CH_2CH_3), 5.76 (s, 2H, CH_2), 7.16-7.25 (m, 2H, benzene H), 7.36-7.38 (m, 2H, benzene H), 7.54 (m, 1H, quinolinone C6-H), 7.88 (m, 1H, quinolinone C8-H), 7.96 (s, 1H, butenoate C3-H), 8.29 (m, 1H, quinolinone C5-H), 9.08 (s, 1H, quinolinone C2-H), 12.70 (bs, 1H, OH).

4-[8-Chloro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13g). 98%; 164-165 °C; toluene; IR *v* 3400 (OH), 1749 (C=O ester), 1644 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.29 (t, 3H, CH_3), 4.30 (q, 2H, CH_2CH_3), 6.09 (s, 2H, CH_2), 7.14-7.16 (m, 4H, benzene H), 7.15 (m, 1H, quinolinone C6-H), 7.82 (m, 1H, quinolinone C7-H), 7.91 (s, 1H, butenoate C3-H), 8.34 (m, 1H, quinolinone C5-H), 8.81 (s, 1H, quinolinone C2-H), 15.25 (bs, 1H, OH).

4-[6,7-Dichloro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13h). 98%; 144-145 °C; toluene/cyclohexane; IR *v* 1727 (C=O ester), 1638 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.35 (t, 3H, CH_3), 4.38 (q, 2H, CH_2CH_3), 5.82 (s, 2H, CH_2), 7.18-7.30 (m, 2H, benzene H), 7.43-7.46 (m, 2H, benzene H), 7.98 (s, 1H, butenoate C3-H), 8.14 (s, 1H, quinolinone C8-H), 8.41 (s, 1H, quinolinone C5-H), 9.14 (s, 1H, quinolinone C2-H), 16.00 (bs, 1H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(pyrrolidin-1-yl)-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13i). 82%; 170 °C (dec); methanol; IR *v* 3300 (OH), 1735 (C=O ester), 1621 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.31 (t, 3H, CH_3), 1.98-2.10 (m, 4H, pyrrolidine H), 3.32-3.36 (m, 1H, pyrrolidine H), 4.30 (q, 2H, CH_2CH_3), 5.64 (s, 2H, CH_2), 6.37-6.39 (m, 1H, quinolinone C8-H), 6.77-6.79 (m, 1H, quinolinone C6-H), 7.29-7.32 (m, 2H, benzene H), 7.45-7.48 (m, 2H, benzene H), 8.12-8.16 (m, 2H, quinolinone C5-H and butenoate C3-H), 8.79 (s, 1H, quinolinone C2-H), 13.50 (bs, 1H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(pyperazin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13j): 98%; 150-152 °C; washed with isopropanole; IR: cm^{-1} 3347 (OH enole), 1711 (C=O ester), 1666 (C=O ketone). ^1H

NMR: (DMSO-d_6) δ 1.32-1.29 (t, 3H, CH_3CH_2), 3.33 (m, 4H, pyperazine H), 3.38 (m, 4H, pyperazine H), 4.33-4.28 (q, 4H, CH_2CH_3), 5.71 (s, 2H, CH_2 benzyl), 6.86 (s, 1H, C8-H quinolinone), 7.23-7.17 (m, 3H, benzene H and C6-H quinolinone), 7.44-7.40 (m, 2H, benzene H), 8.00 (s, 1H, CH enole), 8.11-8.09 (d, 1H, C5-H quinolinone), 9.00 (s, 1H, C2-H quinolinone).

4-[1-(4-Fluorophenyl)methyl-7-(*N*-methylpyperazin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13k): 99%; 96-99 °C; washed with isopropanole; IR: cm^{-1} 3381 (OH enole), 1713 (C=O ester), 1605 (ketone); ^1H NMR: (DMSO-d_6) δ 1.02 (s, 3H, CH_3 pyperazine); 1.23-1.22 (t, 3H, CH_3CH_2); 2.33 (m, 4H, pyperazine H); 3.24 (m, 4H, pyperazine H); 4.11-4.10 (q, 4H, CH_2CH_3); 5.54 (s, 2H, CH_2 benzyl); 6.72 (s, 1H, C8-H quinolinone); 7.07-7.05 (d, 1H, C6-H quinolinone); 7.19 (m, 2H, benzene H); 7.29 (m, 2H, benzene H); 8.08-8.06 (d, 1H, C5-H quinolinone); 8.55 (s, 1H, CH enole); 8.61 (s, 1H, C2-H quinolinone).

4-[1-(4-Fluorophenyl)methyl-7-(*N*-ethylpyperazin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13l): 99%; 200 °C (dec); washed with isopropanole; IR: cm^{-1} 3388 (OH enole), 1711 (C=O ester), 1606 (ketone). ^1H NMR: (DMSO-d_6) δ 1.15-1.18 (t, 3H, CH_3CH_2 pyperazine), 1.24-1.18 (t, 3H, CH_3CH_2), 2.19 (q, 3H, CH_2CH_3 pyperazine), 2.39 (m, 4H, pyperazine H), 3.24 (m, 4H, pyperazine H), 4.12-4.10 (q, 4H, CH_2CH_3), 5.55 (s, 2H, CH_2 benzyl), 6.72 (s, 1H, C8-H quinolinone), 7.07-7.01 (m, 2H, CH enole and C6-H quinolinone), 7.20-7.18 (m, 2H, benzene H), 7.29 (m, 2H, benzene H), 8.08-8.05 (d, 1H, C5-H quinolinone), 8.54 (s, 1H, C2-H quinolinone).

4-[1-(4-Fluorophenyl)methyl-7-(*N*-acethylpyperazin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13m): 91%; 178-180 °C; washed with isopropanole; IR: cm^{-1} 3409 (OH enole), 1716 (C=O ester), 1604 (ketone). ^1H NMR: (DMSO-d_6) δ 1.22-1.26 (t, 3H, CH_3CH_2), 2.03 (s, 3H, CH_3 acethylpyperazine), 3.26 (m, 4H, pyperazine H), 3.54 (m, 4H, pyperazine H), 4.12-4.10-4.14 (q, 4H, CH_2CH_3), 5.56 (s, 2H, CH_2 benzyl), 6.75 (s, 1H, C8-H quinolinone), 7.01 (s, 1H, CH enole), 7.04-7.06 (d, 1H, C6-H quinolinone), 7.18-7.22 (m, 2H, benzene H), 7.28-7.32 (m, 2H, benzene H), 8.08-8.10 (d, 1H, C5-H quinolinone), 8.53 (s, 1H, C2-H quinolinone).

4-[1-(4-Fluorophenyl)methyl-7-(tiomorpholin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13n): 99%; 170 °C; washed with isopropanole; IR: cm^{-1} 3388 (OH enole), 1711 (C=O ester), 1606 (ketone). ^{1}H NMR: (DMSO-d_6) δ 1.24-1.18 (t, 3H, CH_3CH_2), 3.24 (m, 4H, tiomorpholine H), 3.72 (m, 4H, tiomorpholine H), 4.12-4.10 (q, 4H, CH_2CH_3), 5.56 (s, 2H, CH_2 benzyl), 6.77 (s, 1H, C8-H quinolinone), 7.37-7.08 (m, 6H, CH enole, C6-H quinolinone and benzene H), 8.10 (d, 1H, C5-H quinolinone), 8.60 (s, 1H, C2-H quinolinone).

4-[1-(4-Fluorophenyl)methyl-7-(morpholin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13o): 76%; 175-177 °C; trituration on diethyl ether; IR: cm^{-1} 3388 (OH enole), 1711 (C=O ester), 1606 (ketone). ^{1}H NMR: (DMSO-d_6) δ 1.24-1.18 (t, 3H, CH_3CH_2), 3.24 (m, 4H, morpholine H), 3.72 (m, 4H, morpholine H), 4.12-4.10 (q, 4H, CH_2CH_3), 5.56 (s, 2H, CH_2 benzyl), 6.77 (s, 1H, C8-H quinolinone), 7.37-7.08 (m, 6H, CH enole, C6-H quinolinone and benzene H), 8.10 (d, 1H, C5-H quinolinone), 8.60 (s, 1H, C2-H quinolinone).

4-[1-(4-Fluorophenyl)methyl-7-(*N*-(3-chloroprop-1-yl)-1-pyperazinlyl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13p): 77%; 175-177 °C; washed with acetone; IR: cm^{-1} 3488 (OH enole), 1723 (C=O ester), 1617 (ketone). ^{1}H NMR: (DMF-d_7) δ 1.35-1.39 (t, 3H, CH_3CH_2), 1.96-1.99 (q, 2H, $ClCH_2CH_2CH_2N$ pyperazine), 2.48-2.54 (m, 6H, pyperazine H and $ClCH_2CH_2CH_2N$ pyperazine), 3.38-3.39 (m, 4H, pyperazine H), 3.73-3.76 (q, 4H, CH_2CH_3), 4.35-4.39 (q, 2H, $ClCH_2CH_2CH_2N$ pyperazine), 5.87 (s, 2H, CH_2 benzyl), 6.98 (s, 1H, C8-H quinolinone), 7.22-7.29 (m, 3H, C6-H quinolinone and benzene H), 7.58-7.62 (m, 2H, benzene H), 8.10 (m, 2H, CH enole and C5-H quinolinone), 9.14 (s, 1H, C2-H quinolinone).

4-[1-(4-Fluorophenyl)methyl-7-(dimethylamino)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (13q): 98%; 150 °C (dec); washed with isopropanole; ^{1}H NMR: (DMF-d_7) δ 1.32-1.28 (t, 3H, CH_3CH_2), 3.00 (s, 6H, CH_3 dimethylamine), 4.22-4.16 (q, 4H, CH_2CH_3), 5.65 (s, 2H, CH_2 benzyl), 6.52 (s, 1H, C8-H quinolinone), 6.89-6.87 (s, 1H, C6-H quinolinone), 7.18 (s, 1H, CH enole), 7.25-7.21 (m, 2H, benzene H), 7.48-7.45 (m, 2H, benzene H), 8.20-8.18 (d, 1H, C5-H quinolinone), 8.70 (s, 1H, C2-H quinolinone).

4-(6-Chloro-4(1*H*)-quinolinon-3-yl)-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (15a). 57%; 209-211 °C; ethanol; IR *v* 3300 (NH and OH), 1734 (C=O ester), 1684 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.31 (t, 3H, CH_3), 4.25 (q, 2H, CH_2), 7.40-8.00 (m, 3H, quinolinone C7-H, C8-H and butenoate C3-H), 8.20 (m, 1H, quinolinone C5-H), 8.50 (s, 1H, quinolinone C2-H), 12.75 (bs, 2H, NH and OH).

4-(7-Chloro-4(1*H*)-quinolinon-3-yl)-2-hydroxy-4-oxo-2-butenoic Acid Ethyl Ester (15b). 77%; 187-189 °C; washed with isopropanol; IR *v* 3200, 3064 (NH and OH), 1726 (C=O ester), 1700 and 1624 (C=O ketone) cm-1. 1H NMR (DMSO-d_6) δ 1.30 (t, 3H, CH_3), 4.30 (q, 2H, CH_2), 7.47 (m, 1H, quinolinone C6-H), 7.68 (s, 1H, quinolinone C8-H), 7.94 (s 1H, butenoate C3-H), 8.18 (m, 1H, quinolinone C5-H), 8.77 (s, 1H, quinolinone C2-H), 13.00 (bs, 1H, NH and OH).

4,4'-[7-(Dimethylamino)-1-(4-fluorobenzyl)-4-oxo-(1H)-quinolinon-3,6-diyl] bis 2-hydroxy-4-oxobut-2-enoic Acid Ethyl Ester (17a): 77%; 185 (dec) °C; washed with isopropanole; IR: cm^{-1} 3300 (OH enole), 1720 (C=O ester); ^1H NMR: (DMSO-d_6) δ 1.31-1.29 (t, 3H, CH_3CH_2), 2.81 (s, 3H, CH_3 dimethylamine), 4.30-4.30 (q, 2H, CH_3CH_2), 5.70 (s, 2H, CH_2 benzyl), 6.69 (s, 1H, C8-H quinolinone), 7.24-7.20 (t, 2H, benzene H), 7.42-7.41 (m, 2H, benzene H), 7.99 (bs, 1H, CH enole), 8.20 (s, 1H, C5-H quinolinone), 9.05 (s, 1H, C2-H quinolinone).

4,4'-[7-(*N*-methylpyperazin-1-yl)-1-(4-fluorobenzyl)-4-oxo-(1H)-quinolinon-3,6-diyl] bis 2-hydroxy-4-oxobut-2-enoic Acid Ethyl Ester (17b): 82%; >240 °C; washed with isopropanole; IR: cm^{-1} 3407 (OH enole), 1717 (C=O ester), 1634 (C=O ketone); ^1H NMR: (DMSO-d_6) δ 1.22-1.25 (t, 3H, CH_3CH_2), 2.17 (s, 3H, CH_3 pyperazine), 2.40 (s, 4H, pyperazine), 2.99 (s, 4H, pyperazine), 4.10-4.11 (q, 2H, CH_3CH_2), 5.58 (s, 2H, CH_2 benzyl), 6.90 (s, 1H, C8-H quinolinone), 7.24-7.20 (m, 3H, benzene H and CH enole), 7.32-7.38 (m, 2H, benzene H), 8.29 (s, 1H, C5-H quinolinone), 8.56 (s, 1H, C2-H quinolinone).

General Procedure for the Synthesis of Diketo Acids 14a-i, 16a,b and 18a,b. A mixture of 1 N NaOH (6.5 mL) and the appropriate ester **13a-q**, **15a,b** or **17a,b** (1.3 mmol) in 1:1 THF/methanol (12 mL) was stirred at room temperature for 40 min and then poured onto crushed ice. The aqueous layer was separated and treated with 1 N

HCl until pH 3 was reached, and the yellow solid that formed was collected by filtration, then washed with water, hot dry ethanol, and light petroleum ether to afford pure acids **14a-i, 16a,b** and **18a,b**. Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR are reported for each of the following compounds.

4-[1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14a). 50%; 207-209 °C; washed with anhydrous ethanol; IR *v* 3400 (OH), 1732 (C=O acid), 1619 (C=O ketone) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 5.79 (s, 2H, CH_2), 7.22-7.38 (m, 4H, benzene H), 7.53-8.34 (m, 5H, quinolinone C6-H, C7-H, C8-H and butenoate C3-H), 9.16 (s, 1H, quinolinone C2-H), 15.50 (bs, 2H, OH).

4-[6-Fluoro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14b). 95%; 183-184 °C; washed with anhydrous ethanol; IR *v* 3100 (OH), 1730 (C=O acid), 1613 (C=O ketone) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 5.76 (s, 2H, CH_2), 7.16-7.20 (m, 2H, benzene H), 7.33-7.36 (m, 2H, benzene H), 7.65 (m, 1H, quinolinone C7-H), 7.80 (m, 1H, quinolinone C8-H), 7.94-7.97 (m, 2H, quinolinone C5-H and butenoate C3-H), 9.10 (s, 1H, quinolinone C2-H), 14.67 (bs, 2H, OH).

4-[7-Fluoro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14c). 95%; 162-163 °C; washed with anhydrous ethanol; IR *v* 3300 (OH), 1719 (C=O acid), 1635 (C=O ketone) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 5.70 (s, 2H, CH_2), 7.14-7.21 (m, 2H, benzene H), 7.31-7.37 (m, 3H, benzene H andquinolinone C6-H), 7.60 (m, 1H, quinolinone C8-H), 7.93 (s,1H, butenoate C3-H), 8.34 (m, 1H, quinolinone C5-H), 9.05 (s,1H, quinolinone C2-H), 14.60 (bs, 2H, OH).

4-[8-Fluoro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14d). 78%; 180-181 °C; washed with hot anhydrous ethanol; IR *v* 3300 (OH), 1732 (C=O acid), 1634 and 1602 (C=O ketone) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 5.86 (s, 2H, CH_2), 7.12-7.23 (m, 4H, benzene H), 7.45-7.50 (m, 1H, quinolinone C6-H), 7.57-7.62 (m, 1H, quinolinone C7-H), 7.70 (s, 1H, butenoate C3-H), 8.16-8.18 (m, 1H, quinolinone C5-H), 8.96 (s, 1H, quinolinone C2-H), 14.30 (bs,

2H, OH).

4-[6-Chloro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14e). 71%; 194-197 °C; DMF/water; IR *v* 3385 (OH), 1710 (C=O acid), 1663 and 1622 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 5.72 (s, 2H, CH_2), 7.18-7.32 (m, 4H, benzene H), 7.75-7.85 (m, 2H, quinolinone C7-H and C8-H), 8.22 (m, 1H, C5-H quinolinone), 7.90 (s, 1H, butenoate C3-H), 9.08 (s, 1H, quinolinone C2-H), 13.00 (bs, 2H, OH).

4-[7-Chloro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14f). 64%; 188-190 °C; washed with isopropanol; IR *v* 3400 (OH), 1734 (C=O acid), 1646 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 5.75 (s, 2H, CH_2), 7.21-7.36 (m, 4H, benzene H), 7.55 (m, 1H, quinolinone C6-H), 7.83-7.85 (m, 2H, quinolinone C8-H and butenoate C3-H), 8.30 (m, 1H, quinolinone C5-H), 9.07 (s, 1H, quinolinone C2-H), 13.00 (bs, 2H, OH).

4-[8-Chloro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14g). 82%; 175-177 °C; washed with anhydrous ethanol; IR *v* 3300 (OH), 1742 (C=O acid), 1637 and 1603 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 6.08 (s, 2H, CH_2), 7.00-7.30 (m, 4H, benzene H), 7.44-7.46 (m, 1H, quinolinone C6-H), 7.81-7.83 (m, 2H, butenoate C3-H and quinolinone C7-H), 8.33-8.35 (m, 1H, quinolinone C5-H), 8.89 (s, 1H, quinolinone C2-H), 14.70 (bs, 2H, OH).

4-[6,7-Dichloro-1-(4-fluorophenyl)methyl-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14h). 86%; 175-177 °C; washed with isopropanol; IR *v* 3300 (OH), 1722 (C=O acid), 1630 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 5.75 (s, 2H, CH_2), 7.17-7.22 (m, 2H, benzene H), 7.32-7.39 (m, 2H, benzene H), 7.88 (s, 1H, quinolinone C8-H), 8.06 (s, 1H, butenoate C3-H), 8.32 (s, 1H, quinolinone C5-H), 9.05 (s, 1H, quinolinone C2-H), 16.00 (bs, 2H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(pyrrolidin-1-yl)-4(1*H*)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14i). 87%; 197-199 °C; washed with isopropanol; IR *v* 3300 (OH), 1718 (C=O acid), 1614 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ

2.06-2.13 (m, 4H, pyrrolidine H), 3.26-3.30 (m, 4H, pyrrolidine H), 5.77 (s, 2H, CH_2), 6.42-6.45 (m, 1H, quinolinone C8-H), 6.81-6.85 (m, 1H, quinolinone C6-H), 7.18-7.25 (m, 2H, benzene H), 7.49-7.52 (m, 2H, benzene H), 8.02 (s, 1H, butenoate C3-H), 8.15-8.17 (m, 1H, quinolinone C5-H), 9.09 (s, 1H, quinolinone C2-H), 14.00 (bs, 2H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(pyperazin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14j): 98%; 224 °C; washed with isopropanole; IR: cm^{-1} 3500-2000 (OH acid), 1700 (C=O acid), 1649 (C=O keton). ^{1}H NMR: (DMSO-d_6) δ 3.35 (m, 4H, pyperazine H), 3.46 (m, 4H, pyperazine H), 5.64 (s, 2H, CH_2 benzyl), 6.82 (s, 1H, C8-H quinolinone), 7.20-7.12 (m, 3H, benzene H, C6-H quinolinone), 7.41-7.37 (m, 2H, benzene H), 8.08 (d, 1H, C5-H quinolinone), 8.42 (s, 1H, CH enole), 8.75 (s, 1H, C2-H quinolinone), 16.00-14.00 (bs, 2H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(*N*-methylpyperazin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14k): 99%; 224 °C; washed with isopropanole; IR: cm^{-1} 3500-2000 (OH acid), 1700 (C=O acid), 1599 (C=O ketone). ^{1}H NMR: (DMSO-d_6) δ 2.38 (s, 3H, CH_3 pyperazine), 2.66 (m, 4H, pyperazine H), 3.35 (m, 4H, pyperazine H), 5.67 (s, 2H, CH_2 benzyl), 6.81 (s, 1H, C8-H quinolinone), 7.29-7.08 (m, 3H, benzene H, C6-H quinolinone), 7.31-7.48 (m, 2H, benzene H); 7.69 (bs, 1H, CH enole), 8.08-8.06 (d, 1H, C5-H quinolinone), 8.93 (s, 1H, C2-H quinolinone), 16.00-14.00 (bs, 2H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(*N*-ethylpyperazin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14l): 99%; >240 °C; washed with isopropanole; IR: cm^{-1} 3500-2000 (OH acid), 1700 (C=O acid), 1578 (C=O ketone). ^{1}H NMR: (DMSO-d_6) δ 1.06 (t, 3H, CH_3CH_2 pyperazine), 2.22 (q, 3H, CH_2CH_3 pyperazine), 2.39 (m, 4H, pyperazine H), 3.35 (m, 4H, pyperazine H), 5.65 (s, 2H, CH_2 benzyl), 6.78 (s, 1H, C8-H quinolinone), 7.12 (s, 1H, C6-H quinolinone), 7.20 (m, 2H, benzene H), 7.37 (m, 2H, benzene H), 7.69 (bs, 1H, CH enole), 8.07-8.05 (d, 1H, C5-H quinolinone), 8.90 (s, 1H, C2-H quinolinone), 16.00-14.00 (bs, 2H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(*N*-acethylpyperazin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14m): 90%; 195-197 °C; washed with acetonitrile; IR: cm^{-1} 3392-2000 (OH enole and acid), 1716 (C=O acid), 1599 (C=O ketone). ^{1}H NMR: (DMSO-d_6) δ 2.05 (s, 3H, CH_3 acethylpyperazine), 3.32 (m, 4H, pyperazine H), 3.55 (m, 4H, pyperazine H), 5.71 (s, 2H, CH_2 benzyl), 6.81 (s, 1H, C8-H quinolinone), 7.22 (m, 3H, benzene H and C6-H quinolinone), 7.40 (m, 2H, benzene H), 7.97 (bs, 1H, CH enole), 8.10 (d, 1H, C5-H quinolinone), 9.01 (s, 1H, C2-H quinolinone), 16.00-14.00 (bs, 2H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(tiomorpholin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14n): 30%; 190 °C; washed with isopropanole; IR: cm^{-1} 3500-2000 (OH acid), 1711 (C=O acid), 1606 (C=O ketone). ^{1}H NMR: (DMSO-d_6) δ 2.53 (m, 4H, tiomorpholine H), 3.79 (m, 4H, tiomorpholine H), 5.58 (s, 2H, CH_2 benzyl), 6.66 (s, 1H, C8-H quinolinone), 7.16-7.10 (m, 4H, CH enole, C6-H quinolinone and benzene H), 7.35-7.32 (m, 2H, benzene H), 7.23-7.21 (d, 1H, C5-H quinolinone), 8.78 (s, 1H, C2-H quinolinone), 16.00-14.00 (bs, 2H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(morpholin-1-yl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14o): 99%; 187-189 °C; washed with isopropanole; IR: cm^{-1} 3500-2000 (OH acid), 1726 (C=O acid), 1600 (C=O ketone). ^{1}H NMR: (DMSO-d_6) δ 3.27 (m, 4H, morpholine H), 3.71 (m, 4H, morpholine H), 5.70 (s, 2H, CH_2 benzyl), 6.83 (s, 1H, C8-H quinolinone), 7.29-7.10 (m, 3H, C6-H quinolinone and benzene H), 7.32-7.48 (m, 2H, benzene H), 7.99 (bs, 1H, CH enole), 8.11-8.09 (d, 1H, C5-H quinolinone), 9.02 (s, 1H, C2-H quinolinone), 16.00-14.00 (bs, 2H, OH).

4-[1-(4-Fluorophenyl)methyl-7-(*N*-(3-chloroprop-1-yl)-1-pyperazinlyl)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14p): 100%; 175-177 °C; washed with THF; IR: cm^{-1} 3410-2000 (OH), 1717 (C=O ester), 1655 (ketone). ^{1}H NMR: (DMF-d_7) δ 2.18 (q, 2H, $ClCH_2CH_2CH_2N$ pyperazine), 2.73-2.79 (m, 6H, pyperazine H and $ClCH_2CH_2CH_2N$ pyperazine), 3.59 (m, 4H, pyperazine H), 3.91-

3.92 (q, 2H, Cl*CH*$_2$CH$_2$CH$_2$N pyperazine), 6.03 (s, 2H, CH$_2$ benzyl), 7.16 (s, 1H, C8-H quinolinone), 7.38-7.45 (m, 3H, C6-H quinolinone and benzene H), 7.74-7.78 (m, 2H, benzene H), 8.37-8.39 (m, 2H, CH enole and C5-H quinolinone), 9.27 (s, 1H, C2-H quinolinone), 16.00-14.00 (bs, 2H, OH).

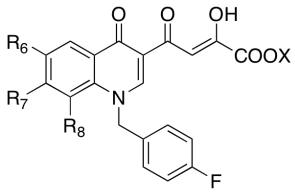
4-[1-(4-Fluorophenyl)methyl-7-(dimethylamino)-4-(1H)-quinolinon-3-yl]-2-hydroxy-4-oxo-2-butenoic Acid (14q): 98%; 203-205 °C; washed with isopropanole; ^{1}H NMR: (DMF-d_7) δ 3.22 (s, 6H, CH$_3$ dimethylamine), 6.00 (s, 2H, CH$_2$ benzyl), 6.82 (s, 1H, C8-H quinolinone), 7.15-7.12 (s, 1H, C6-H quinolinone), 7.46-7.42 (m, 2H, benzene H), 7.76 (m, 2H, benzene H), 8.37-8.35 (d, 1H, C5-H quinolinone), 8.41 (s, 1H, CH enole), 9.27 (s, 1H, C2-H quinolinone), 16.00-14.00 (bs, 2H, OH).

4-(6-Chloro-4(1*H*)-quinolinon-3-yl)-2-hydroxy-4-oxo-2-butenoic Acid (16a). 50%; 220 °C (dec); toluene/cyclohexane; IR *v* 3400, 3200 (NH and OH), 1718 (C=O acid), 1640 and 1611 (C=O ketone) cm^{-1}. ^{1}H NMR (DMSO-d_6) *δ* 7.72-7.75 (m, 2H, quinolinone C7-H and C8-H), 8.13 (s, 1H, butenoate C3-H), 8.53 (m, 1H, quinolinone C5-H), 8.80 (s, 1H, quinolinone C2-H), 13.00 (bs, 3H, NH and OH).

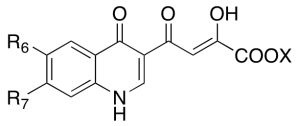
4,4'-[7-(Dimethylamino)-1-(4-fluorobenzyl)-4-oxo-(1H)-quinolinon-3,6-diyl] bis 2-hydroxy-4-oxobut-2-enoic Acid (18a): 77%; >240 °C; washed with isopropanole; IR: cm^{-1} 3300-2000 (OH acid), 1578 (C=O acid); ^{1}H NMR: (DMSO-d_6) δ 2.82 (s, 3H, CH$_3$ dimethylamine), 5.70 (s, 2H, CH$_2$ benzyl), 6.71 (s, 1H, C8-H quinolinone), 7.24-7.20 (t, 2H, benzene H), 7.42-7.41 (m, 2H, benzene H), 7.90 (bs, 1H, CH enole), 8.22 (s, 1H, C5-H quinolinone), 9.02 (s, 1H, C2-H quinolinone), 16.00-14.00 (bs, 2H, OH).

4,4'-[7-(*N*-methylpyperazin-1-yl)-1-(4-fluorobenzyl)-4-oxo-(1H)-quinolinon-3,6-diyl] bis 2-hydroxy-4-oxobut-2-enoic Acid (18b): 32%; >240 °C; washed with isopropanole; IR: cm^{-1} 3338-2000 (OH enole and acid), 1700 (C=O acid), 1595 (C=O ketone).

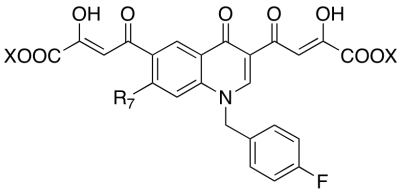
Table 4. Chemical and physical data of derivatives **19a-h**, **20a-q, 21, 22, 23, 24** and **25a,b.**



13a-q 14a-q



15a,b 16a,b



17a,b 18a,b

Cpd	R6	R7	R8	mp (°C)	Recryst solvent[a]	Yield (%)
19a	H	H	H	242-244	a	56
19b	F	H	H	>300	b	29
19c	H	F	H	270-271	c	41
19d	F	H	F	268-270	b	40
19e	Cl	H	H	>300	b	29
19f	H	Cl	H	>300	b	96
19g	H	H	Cl	>300	b	55
19h	Cl	Cl	H	114-115	d	19
20a	H	H	H	213-214	d	65
20b	F	H	H	234-235	e	94
20c	H	F	H	186-187	e	100
20d	F	H	F	193-194	f	78
20e	Cl	H	H	214-216	e	92
20f	H	Cl	H	185-187	e	73
20g	H	H	Cl	215-217	g	92
20h	Cl	Cl	H	235-237	d	49
20i	H	1-Pyrrolydinyl	H	175-177	e	56
20j	H	1-Pyperazinyl	H	230-232	d	32
20k	H	*N*-Methyl-1-Pyperazinyl	H	223-224	d	14
20l	H	*N*-Ethyl-1-Pyperazinyl	H	220-223	d	21
20m	H	*N*-Acethyl-1-Pyperazinyl	H	>240	d	52
20n	H	1-Thiomorpholinyl	H	220-223	g	52
20o	H	1-Morpholinyl	H	227	g	35
20p	H	*N*-(3-chloroprop-1-yl)-1-pyperazinlyl	H	230	d	54
20q	H	*N,N*-dimethylamine	H	225	e	100
21	-	-	-	oil	oil	8
22	-	-	-	-	-	98
23	acethyl	Cl	H	>240	h	50
24	acethyl	Cl	H	128-130	g	17
25a	acethyl	*N,N*-dimethylamine	H	180	e	51
25b	acethyl	*N*-Methyl-1-Pyperazinyl	H	>240	h	82

[a] Recrystallization solvents: (a) aceton, (b) ethanol, (c) isopropanol, (d) toluene, (e) toluene/cyclohexane, (f) benzene/cyclohexane, (g) benzene, (h) methanol.

Biology

Compounds **13a-q**, **14a-q**, **15a**,**b**, **16a**,**b, 17a,b** and **18a,b** were tested for their ability to inhibit HIV-1 integrase in vitro using a gel-based assay in addition to a 96-well plate-based high throughput electrochemiluminescent assay.[30] In the gel assay, a 5′-end-labeled 21-mer double-stranded DNA oligonucleotide corresponding to the last 21 bases of the U5 viral LTR is used to follow both 3′-P and ST. Briefly, a DNA-enzyme complex is preformed by mixing the drug at the destre concentration with 400 nM recombinant HIV-1 integrase in a buffer containing 50 mM MOPS, pH 7.2, 7.5 mM $MnCl_2$, and 14.3 mM β-mercaptoethanol. The integration reaction is then initiated by the addition of 20 nM 5′-labeled double-stranded DNA template and continued in a total volume of 10 μL for 60 min at 37 °C. The reaction samples are stopped by adding the same volume of electrophoresis denaturing dye and loaded on 20% 19/1 acrylamide denaturing gel. Gels were exposed overnight and analyzed using a Molecular Dynamics hosphorimager (Sunnyvale, CA). The 96-well plate-based high throughput electrochemiluminescent assay was performed using a BioVeris M series analyzer (Gaithersburg, MD) as described previously.[30] Briefly, DNA substrates were obtained from BioVeris and used according to the manufacturer's recommendations. Donor DNA is incubated for 30 min at 37 °C in the presence of 250 nM recombinant HIV-1 integrase. After addition of the drug, the integration reaction is initiated by addition of target DNA. Reaction is carried out for 60 min at 37 °C and then read on the BioVeris M series analyzer.

The anti-HIV drug testing was performed in 96-well plates with a defined, previously titered inoculum of a laboratory strain (HTLV-IIIB) to minimize the inoculum effect. In brief, all compounds were dissolved in dimethyl sulfoxide and diluted in cell culture medium at concentrations ranging from 0.1 to 50 μM. Exponentially growing human T lymphocytes (H9 cell line) were added at 5000 cells/well. After the infectivity of a virus stock (HTLV-IIIB) is quantified, an aliquot containing 1 x 10^5 50% tissue culture infectious dose (TCID50) per 5000 H9 cells per well is used as inoculum in each set of in vitro infections of H9 cells. Uninfected cells with the compound served as a toxicity control, and infected and uninfected cells without the compound served as basic controls. Culture were incubated at 37 °C in a 5% CO_2 atmosphere for 4 days. Supernatant fluid of infected wells (in the absence of drug and at each of a number of drug concentrations) is harvested. HIV p24 antigen is quantified, and the 50% inhibitory concentration (IC_{50}) of drug is

determined using the median effect equation.

Cytotoxicity of test compounds has been evaluated on human histiocytic lymphoma (U937) cell line obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were plated in a 96 well-plate at a concentration of 5 x 10^3/mL in RPMI-1640 without phenol red, supplemented with 20% fetal calf serum and antibiotics. Two hours after plating, compounds were added at different concentrations ranging from 1 to 200 μM and cells were incubated at 37 °C for 24 h. Then cells were incubated at 37 °C for 3 h with 1 mg/mL of MTT (Sigma-Aldrich, Milan, Italy). After incubation, the remaining water insoluble formazan was solubilized in absolute isopropanol containing 0.1 N HCl. Absorbance of converted dye was measured in an ELISA plate reader at a wavelength of 570 nm. The cytotoxicity of the compounds was calculated as the percentage reduction of the viable cells compared with the drug-free control culture. The drug concentration required to reduce the cell viability by 50% has been called IC_{50}.

1.6 New scaffolds

1.6.1 Introduction

Studies led on the diketoacid compounds have highlighted their limited capacity to cross cell membrane and lower activities in cell-based assay on infected cell by HIV-1. Infact, compounds sintesized by us [31, 32] have a marked inhibitory activity in enzymatic assay, but a more limited activity in cell-based assay on infected cell by HIV-1. The molecular portion that negatively influenced the EC_{50} value is the diketo acid mojety, which carry out two opposing functions: the first and most important one, of farmacofore; the second one of group that block the crossing of cell membrane. Contemporaneously, the scientific research is oriented to developp scaffolds that can include the diketo acid portion into an etherocyclic ring like dihydroxypyrimidinone, [10] naphthyridine ring, [27, 28, 29] pyrrolopyridine, [34] dihydroisoindolone ring [35]; furthermore, in these compounds the carboxylic acid function is replaced by a 4-fluorobenzylamide or a hydroxamic acid functions.

On the bases of these knowledge, I have designed and synthesized newly inhibitors that do not present the spitting α,γ-diketoacid motif, but molecules in which this motif is disguised in the oxoquinolonyl ring, so that are kept to the fondamental distances between the different atoms or groups (O, N, etc) responsable of the chelation of Mg^{2+} ions and the inhibitor activity on IN of HIV-1.

The designe of newly compounds were realized on structural knowledge of utilized in clinical practice inhibitor, Raltegravir, so I have made a superposition of farmacophoric group of RAL with that of the most active compound synthesyzed by us **11** (figure 9) to focused the attention on the essential structural features for the activity.

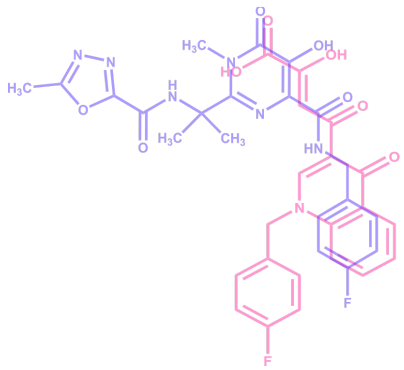


Figure 9. Superposition of Raltegravir (blu) and **11** (red).

From this preliminary analysis, is emerged that the following groups are essential for activities: a first carbonyl group, an enolic OH, and a second carbonyl group.

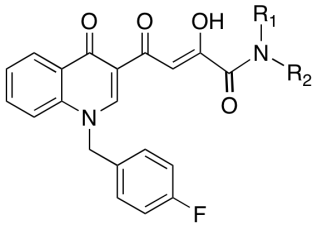
In RAL the first carbonyl group is represented by the amidic carbonyl bloked in the pyrimidinonic ring, so the first modification on the structure **11** is the replaced of carboxylic function of the diketoacid mojety with a amide function (**26** and **27**).

Another derivatives are designed by the schift of two positions of the 1,3-diketobutyric acid chain in the oxoquinolonil ring, obtaining derivatives **28a-d** and **29a-e**, in which the phenolic OH or anilinic NH_2 and the quinolonic carbonyl are superpositioned with the enolic OH and carbonyl of 4-fluorobenzylamide of RAL respectively. The third chelating group are represented by the carbonyl of 4-fluorobenzylamide (**29a-e**) or of carboxylic acid (**28a-d**).

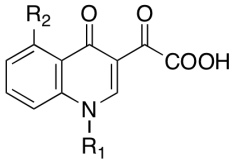
A third series of derivatives are obtained by a insertion of a second phenolic OH in position 6 of benzene ring leading to a designe of 5,6-dihidroxyquinolonil derivative **30** and 5,6-dihydroxybenzopyranone derivative **31**. The two OH in position 5 and 6 are superpositioned with the pyrimidinonic amidic carbonyl and enolic OH of RAL.

Finally I have designed derivatives **32** and **33** that are structural analogues of RAL in which the dihydropyrimidinone ring are substituted with a 4-oxoquinolonil (**32**) or with a 2-oxoquinolonil (**33**) ring.

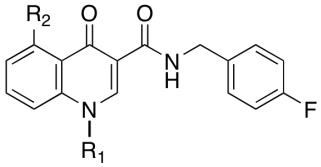
Table 1. compound **26**, **27, 28a-d** and **29a-e.**



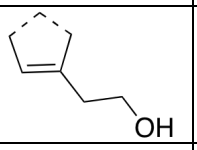
26, 27

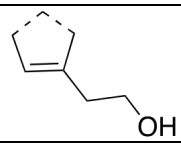


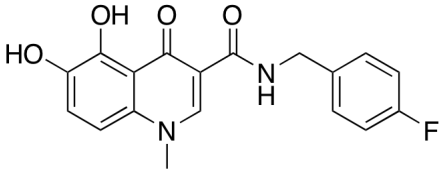
28a-d



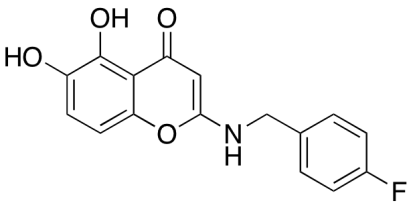
29a-e

Cpd	R1	R2
26	CH_3	CH_3
		
28a	H	OH
28b	Et	OH
28c	Et	OEt
28d		OH
29a	H	OH
29b	Et	OH
29c	Et	OEt

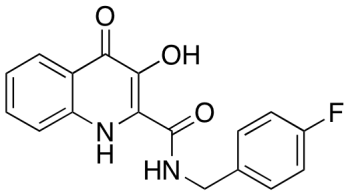
29d		OH
29e	Et	NH_2



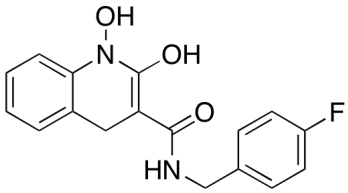
30



31



32



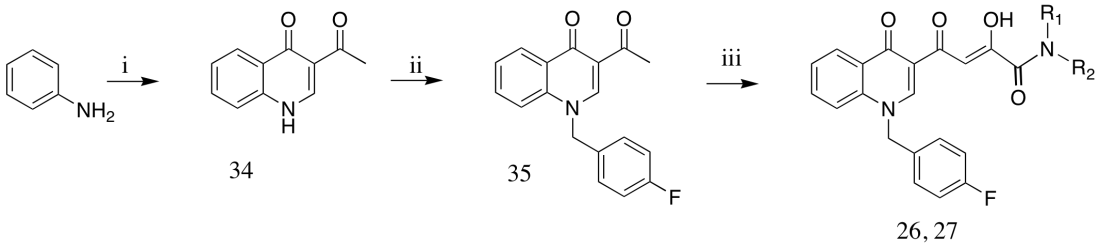
33

1.6.2 Results and discussion

Synthesis of derivatives **26, 27, 28a-d, 29a-e, 30, 31, 32,** and **33** is outlined in Schemes 1–8.

Compounds **26** and **27** (Scheme 1) were synthesized utilizing the same procedure emploied for the quinolonyldiketo acids [31, 32] until the formation of alkilation product; infact the 3-acethylquinolone **34** were prepared by reaction of aniline with ethyl orthoformate and ethyl acetoacetate, which were thermally condensed in the presence of an inert heating medium (Dowtherm A) under argon atmosphere, according to the Yoshizawa procedure. [33] Then, **34** was alkylated with 4-fluorobenzylbromide in alkaline medium (K_2CO_3) to give the N-1 substituted quinolone **35**. For the insertion of amidic portion, quinolinone **35** were reacted with the appropriate oxamate. At the first time the reaction was led in according with the literature, [36] in presence of LDA (lithium diisopropylamide) in THF at –72 °C, but the low temperature had decreased the solubility of quinolinone **35** and was impossible to isolate the final product. Therefore, the reaction was performed at room temperature in presence of another kind of alkaline medium (potassium *tert* butoxide) obtaining the desire products **26** and **27**.

Scheme 1. [a]

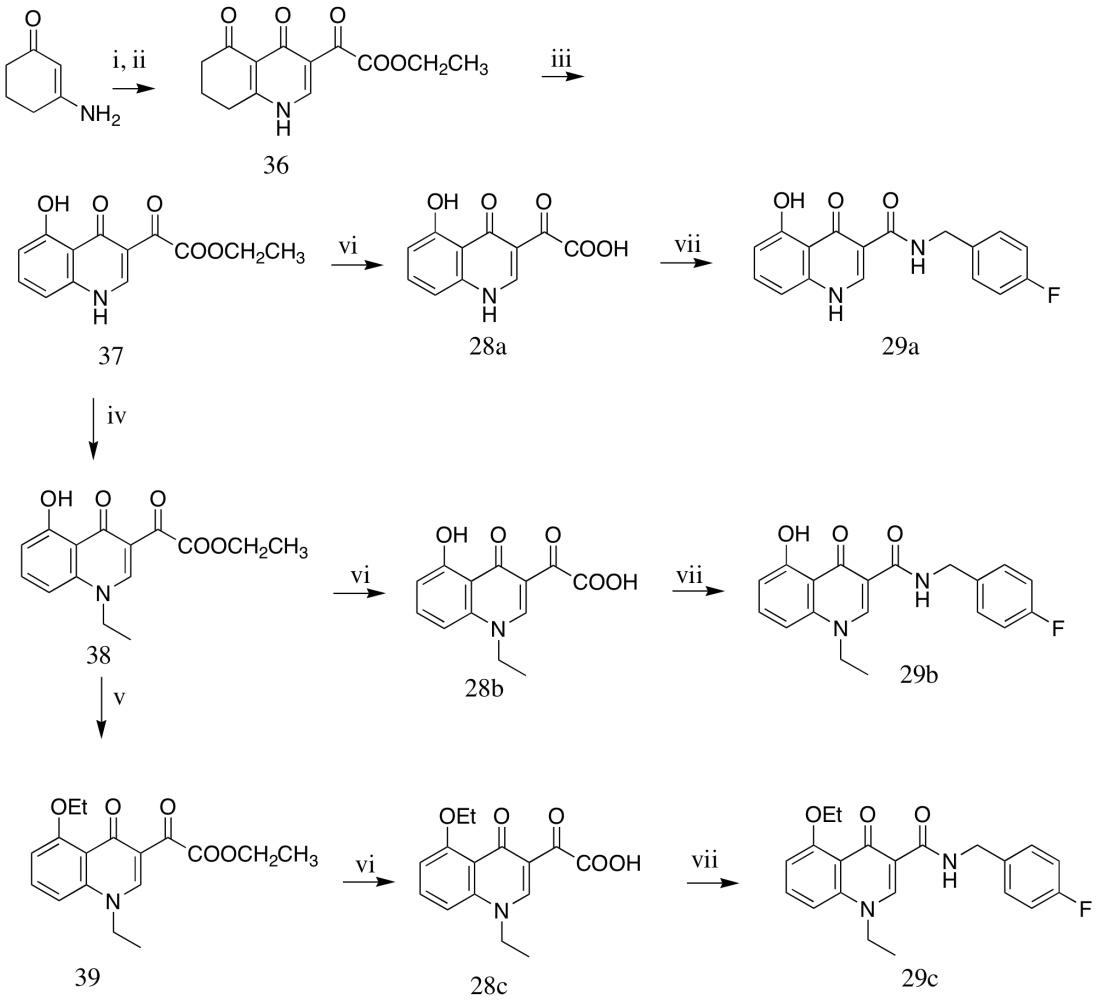


[a] Reagents, conditions and yields: (i) ethyl orthoformate, ethyl acetoacetate, Dowtherm A, 95-254 °C, 8 h, 56% (ii) 4-fluorobenzyl bromide, K_2CO_3, DMF, 100 °C, 1 h, 65% (iii) oxamate, THF_a, putassium *tert*-butoxide, room temp, 20 min, 80%.

Compound **28a-c** and **29a-c** (Scheme 2) was synthesized in according with literature procedure. [37] The commercially available 3-aminocyclohexanone was reacted with diethyl ethoxymethylenemalonate at 130 °C obatining an intermediate that was thermally condensed in presence of an inert heating medium (diphenyl eter) under argon atmosphere yielding product **36**. The next oxidation reaction, in presence of molecular iodine in ethanol at reflux temperature, leading to the key intermediate **37** that was alkylated in alkaline medium (K_2CO_3) to the corresponding *N*-

ethylquinolinone **38**, that in turn was alkylated to the *N*-ethyl-5-ethoxyquinolinone **39**. Compounds **37**, **38** and **39** were hydrolyzed in alkaline medium (sodium hydroxide) to the corresponding acid **28a-c**, that was then converted to the corresponding amide **29a-c** under microwave irradiation (100 °C, 150 Watt, 10 min) in presence of PS-carbodiimide, HOBT (hydroxybenzotriazole), *N*-methylpirrolidone and 4-fluorobenzylamine. [38]

Scheme 2. [a]

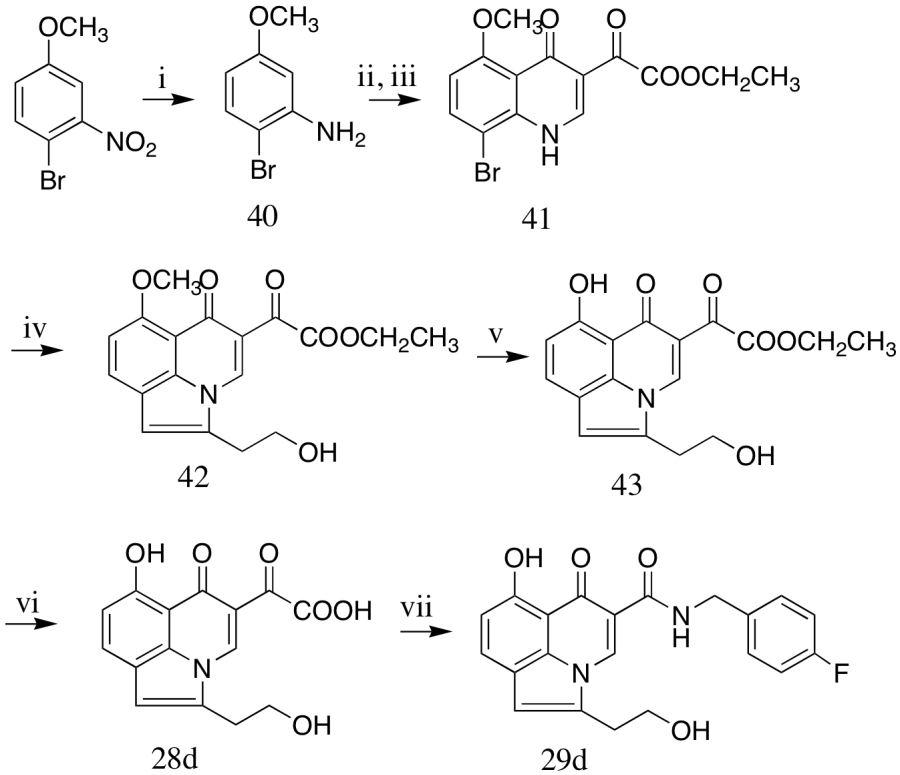


[a] Reagents, conditions and yields: (i): EMME, 130 °C, 3 h; 74%; (ii): diphenyl eter, reflux,1 h, 58%; (iii): iodine, EtOH, reflux, 17 h, 76%; (iv): K_2CO_3, CH_3CH_2I, DMF, 80 °C, 12 h, 43%; (v): K_2CO_3, CH_3CH_2I, DMF, 80 °C, 12 h, 83%; (vi): NaOH 10%, 100°C, 1h; (vii): HOBT, PS- carbodimmide, p-fluorobenzylamine, NMP, MW (100°C, 150 W, 200 PSI, 10 min).

Compound **28d** and **29d** was synthesized in according with the Scheme 3. The commercially available 3-nitro-4-bromoanisole was reducted to the corresponding 1-bromo-4-methoxyaniline **40** for a redox reaction in presence of $SnCl_2$, H_2O, HCl_{conc} and ethanol 96° at room temperature. The amine was reacted with diethyl

ethoxymethylenemalonate at 130 °C obatining an intermediate that was condensed in presence of an acidic medium (Eaton's reagent; [39] $CH_4O_3SO_5P_2$, 7,7% w/w P_2O_5 in metansulfonic acid) under argon atmosphere yielding product **41.** To obtaining the compound **42** was performed a Sonogashira coupling in presence of CuI, $Cl_2Pd(PPh_3)_2$, NEt_3 and 3-butin-1-ole at reflux temperature for 3 days. [39] The compound **28d** was obtained converted **42** to the demethylated compound **43** for deprotection by BBr_3 at –45 °C, that was successively hydrolyzed to the desire acid. Amide **29d** was yielded with the same procedure of amides **29a-c.**

Scheme 3. [a]

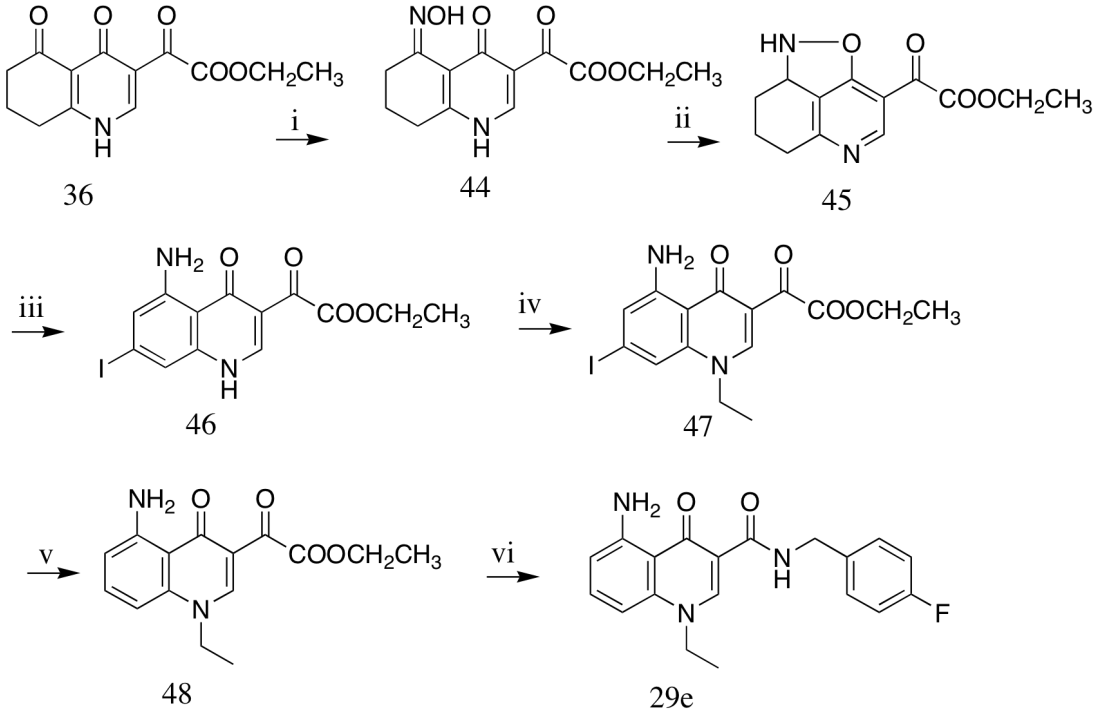


[a] Reagents, conditions and yields: (i): $SnCl_2$, H_2O, HCl_{conc}, ethanol 96°, room temp, 1 night, 98%; (ii): EMME, 130 °C, 3 h; 74%; (iii): Eaton's reagent, 90 °C, 5 h, 60%; (iv): CuI, $Cl_2Pd(PPh_3)_2$, NEt_3, 3-butin-1-ole, reflux, 3 days, 40%; (v): BBr_3 at –45 °C, 12 h, 30%; (vi): NaOH 10%, 100°C, 1h, 98%; (vii): HOBT, PS- carbodimmide, p-fluorobenzylamine, NMP, MW (100°C, 150 W, 200 PSI, 10 min), 35%.

Compound **29d** (Scheme 4) was synthesized in according with literature procedure. [37] Compound **36** was obtained with the same procedure of Scheme 1. The insertion of 5-amine group was performed in three steps: an addiction reaction to the carbonyl group in presence of hydroxilamine hydrochloride at reflux temperature (**44**), followed by a reaction of pretection (ethylchloroformate, pyridine, room temp) to

fournish a condensed isoxazole ring **45**, which was oxidized with KI, I_2, ethanole absolute at reflux temperature to yield derivative **46**. The nitrogen atom of **46** was alkylated in alkaline medium (K_2CO_3) to **47**, that was subjected to a reductive dehalogenation catalized by Pd/C 10% in presence of H_2 (3 Atm) and triethylamine at 40 °C obtaining the ethyl ester **48** that was directly converted to the corresponding amide **29e** under microwave irradiation (120 °C, 100 Watt, 15 min) in presence of 4-fluorobenzylamine. [40]

Scheme 4. [a]

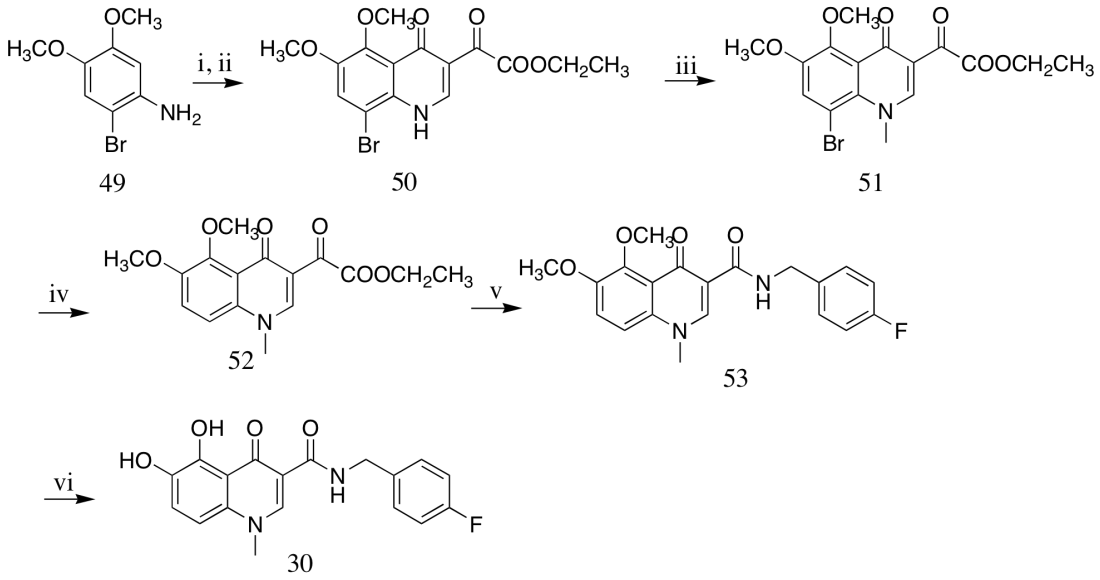


[a] Reagents, conditions and yields: (i): hydroxilamine hydrochloride, reflux, 3 h, 78%; (ii): ethylchloroformate, pyridine, room temp, 30 min, 76%; (iii): KI, I_2, EtOH, reflux, 1 h, 43%; (iv): K_2CO_3, CH_3CH_2I, DMF, 80 °C, 12 h, 43%; (v): Pd/C, H_2 3 Atm, NEt_3, 40 °C, 7 h, 30%; (vi): p-fluorobenzylamine, MW (120°C, 100 W, 200 PSI, 15 min).

Compound **30** was synthesized in according with the Scheme 5. Compound **49**, synthesized according with the literature, [41] was converted to the quinolinone **50** with the same procedure of Scheme 3. Quinolinone **50** was alkylated in alkaline medium (K_2CO_3) to fournish *N*-methylquinolinone **51**, that was subjected to a reductive dehalogenation catalized by Pd/C 10% in presence of H_2 (3 Atm) and triethylamine at 40 °C obtaining the ethyl ester **52** that was directly converted to the corresponding amide **53** under microwave irradiation (120 °C, 100 Watt, 15 min) in presence of 4-fluorobenzylamine. [40] Compound **30** was obtained for deprotection with BBr_3 at –45

°C.

Scheme 5. [a]

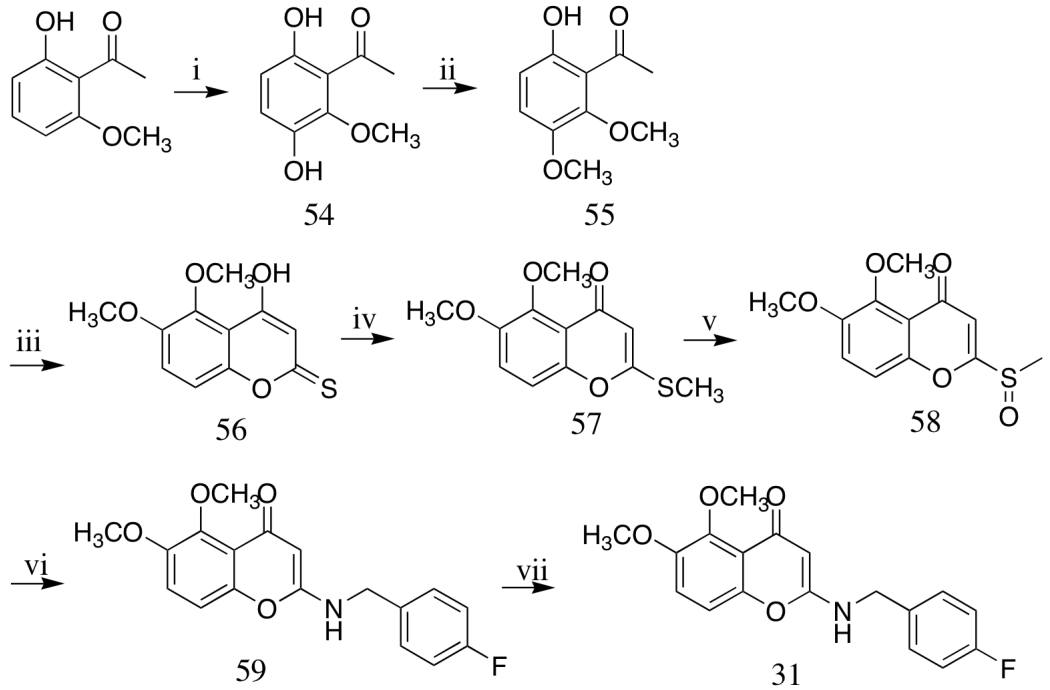


49 50 51 52 53 30

[a] Reagents, conditions and yields: (i): EMME, 130 °C, 3 h; 74%; (ii): Eaton's reagent, 90 °C, 5 h, 55%; (iii): K_2CO_3, CH_3CH_2I, DMF, 80 °C, 12 h, 45%; (iv): Pd/C, H_2 3 Atm, NEt_3, 40 °C, 7 h, 87%; (vii): p-fluorobenzylamine, MW (120°C, 150 W, 200 PSI, 15 min), 35%; (iv): BBr_3 at –45 °C, 12 h, 30%.

Compound **31** was synthesized in according with the Scheme 6. The commercially available 2-hydroxy-6-methoxyacetophenone was oxydized to the corresponding quinone **54** in presence of hydrogen peroxyde. The new formed hydroxyl group was alkylated in alkaline medium (K_2CO_3) to fournish *O*-methyl derivative **55** that was cyclized in presence of CS_2 (carbonium disulfide) in alkaline medium (*t*-BuOK) to yield the 4-hydroxybenzo-2-tiopyranone **56**. The insertion of the 4-fluorobenzylamine group was obtained firstly converting the 2-tiopyranone in the corresponding 2-methyltiopyranone **57**, and than oxydizing it to the better leaving group methylsulfoxyde in presence of metachloroperbenzoic acid, obtaining compound **58,** which was reacted with 4-fluorobenzyl amine yielding compound **59**. The desire compound **31** was obtained for deprotection of methoxide group in 5 and 6 positions of benzopyranone ring of **59** in presence of BBr_3 under inert atmosphere.

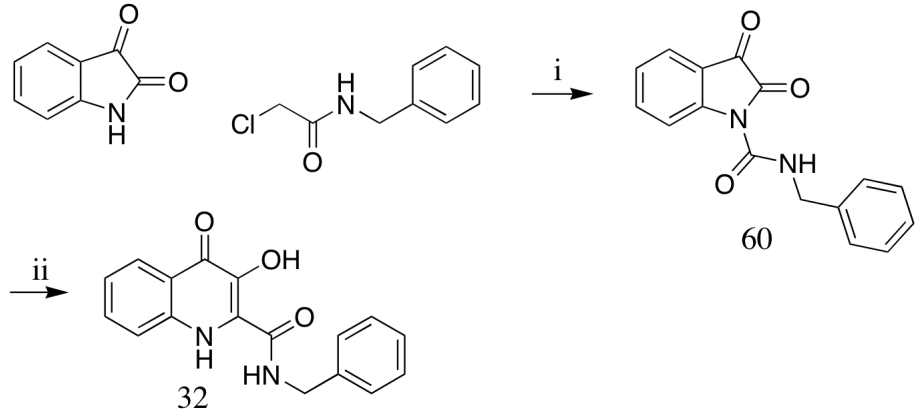
Scheme 6. [a]



[a] Reagents, conditions and yields: (i): H_2O_2 35%, PTSA, acetic acid, 50 °C, 24 h, 27%; (ii): dimethyl sulfate, K_2CO_3, acetone, room temp, 2 h, 48%; (iii): CS_2, putassium *tert*-butoxyde, THF_a, 45 °C, 3 h, 45%; (iv): CH_3I, K_2CO_3, acetone, reflux, 30 min, 100%; (v): metachloroperbenzoic acid, dichloromethane, room temp, 63%; (vi): 4-fluorobenzyl amine, aceto nitrile, room temp, 1,5 h, 28%; (vii): BBr_3 at –45 °C, 12 h, 16%.

Compound **32** was obtained in two steps from isatine and chloroacethylbenzyl amide (Scheme 7) in according with the leterature. [42] Compound **60** was than converted to compound **32** with a trasposition reaction involving the isatine ring in alkaline medium (CH_3ONa).

Scheme 7. [a]

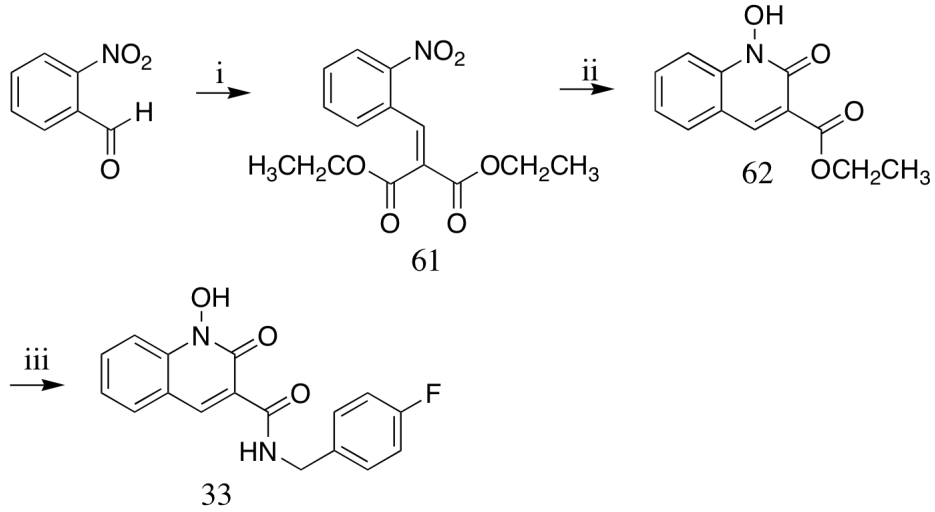


[a] Reagents, conditions and yields: (i): K_2CO_3, DMF_a, 1,5 h, 87%; (ii): CH_3ONa, CH_3OH_a, 2 h, 120 °C; 10%.

Compound **33** was obtained in three steps (Scheme 8) in according with the

literature. [43] First, *o*-nitro-benzaldehyde and diethylmalonate react in a classical Knoevenagel reaction. Then compound **62** is obtained via a one pot reduction and cyclization. Finally, aminolysis of the remaining ester function, with the 4-fluorobenzyl amine, yielding compound **33**.

Scheme 8. [a]



[a] Reagents, conditions and yields: (i): Diethylmalonate, $NaHCO_3$, acetic anidrhyde, 100 °C, 6 h, 45%; (ii): PtO_2, H_2 (1 Atm), acetic acid, room temp, 22 h, 27%; (iii): 4-fluorobenzyl amine, 100 °C, 4 h, 83%.

Derivatives **26, 27, 28a-d, 29a-e, 30, 31, 32** and **33** were tested in vitro for ST inhibition in the presence of magnesium (Mg^{2+}) using a recently described high throughput electrochemiluminescent (HTECL) assay. [30] IC_{50} values were generated from duplicate experiments (Table 5). Compounds **26, 27, 28a-d, 29a-e, 30, 31, 32** and **33** were also tested for ST and 3′-P using gel-based assays carried out in the presence of Mg^{2+}. IC_{50} values were calculated using dose response curves and are summarized in Table 5.

Table 5. Activities data for compounds **26, 27, 28a-d, 29a-e, 30, 31, 32** and **33.**

	ANTI-IN activity IC_{50}[a]			**ANTIVIRAL activity**		
Cpd	**Mg^{2+} [b]**	**Mg^{2+} [c]**	**Mg^{2+} [c]**	**CC_{50}[d]**	**EC_{50}[e]**	**SI[f]**
26	0.92	nt[g]	34.2	nt[g]	3.9	
27	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
28a	55	nt[g]	>333	nt[g]	>50	
28b	>333	nt[g]	>333	nt[g]	>50	
28c	>333	nt[g]	>333	nt[g]	>50	
28d	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
29a	>333	nt[g]	>333	nt[g]	>50	
29b	>333	nt[g]	>333	nt[g]	>50	

29c	>333	nt[g]	>333	nt[g]	>50	
29d	>333	nt[g]	>333	nt[g]	>50	
29e	>333	nt[g]	>333	nt[g]	>50	
30	>333	nt[g]	>333	nt[g]	>50	
31	106	nt[g]	>111	nt[g]	>50	
32	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	
33	nt[g]	nt[g]	nt[g]	nt[g]	nt[g]	

[a] Inhibitory concentration 50% (μM) determined from dose response curves. [b] Experiments performed in duplicate using the high throughput electrochemiluminescent (HTECL) assay (ST assay in the presence of Mg^{2+}). [c] Experiments performed in a gel-based assay in the presence of Mg^{2+}. [d] Cytotoxic concentration 50% (μM). [e] Effective concentration 50% (μM). [f] Selectivity index (CC_{50}/EC_{50}). [g] nt: not tested.

Activities data relatively the newly synthesized compounds **26, 27, 28a-d, 29a-e, 30, 31, 32** and **33** are uncompleted, thus it can not possible to formulate valid structure-activity relationships.

Compouds that exhibite better activities data are **26, 28a** and **31.**

Compound **26** is closely related to the quinolonildiketoacids, [31] of which the inhibitory activity in enzymatic assay and cell-based assay are well known. However, is interesting that the amide function conveies activity as well as carboxylic acid function. This trend reflected those of the dihydroxypyrimidine like RAL.

In the series of **28a-d** and **29a-e** the enzymatic activity is dependent on the presence of carboxylic acid function, fenolic OH and unsubstituted quinolonic NH. Infact the only compound that shows an interesting activity data on enzymatic assay is **28a,** while other compounds that are substituted on fenolic OH and/or quinolonic NH (**28b-d)** and presented the 4-fluorobenzylamide function (**29a-e)** instead of carboxylic acid function are completely inactive.

Compound **31** is not structurally related to other compound and its activity in enzymatic assay is an interesting starting point for the development of new INSTIs.

1.6.3 Conclusions

I designed, synthesized, and tested a series of novel derivatives in both enzyme- and cellbased assays as anti-HIV-1 agents to selectively target the ST step of integration. We were able to generate such new compounds, some of which (**26, 28a** and **31)** are ST-selective IN inhibitors and active (**26**) against HIV-1 replication in acutely infected cells. These compounds could be useful tools to develop new INSTIs and to study the mechanism of action of HIV-1 IN.

1.6.4 Experimental section

Chemistry

General. Melting points were determined with a Büchi 530 capillary apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Spectrum-one spectrophotometer. ^{1}H NMR spectra were recorded on a bruker AC 400 specrtometer. Merk silica gel 60 F_{254} plates were used for analytical TLC. Column chromatogrephies were performed on silica gel (Merck; 70-230 mesh) or alumina (Merck; 70-230 mesh). Concentration of solution after reactions and extractions involved the use of a rotary evaporator operating at reduced pressure of approximately 20 Torr. Analytical results agreed to within ±0.40% of the theoretical values.

Microwave irradiation experiments. Microwave reactions were conduced using a CEM Discover system unit (CEM. Corp., Matthews, NC). The machine consists of a continuous focused microwave-power delivery system with operator selectable power output from 0 to 300 W. The temperature of the contents of the vessel was monitored using a calibrated infrared temperature control mounted under the reaction vessel. All experiments were performed using a stirring option whereby the contents of the vessel are stirred by means of a rotating magnetic plate located below the floor of the microwave cavity and a Teflon-coated magnetic stir bar in the vessel.

3-Acetyl-4(1*H*)-quinolinone 34. Ethyl orthoformate (4.0 g, 27 mmol), ethyl acetoacetate (3.5 g, 27 mmol), aniline (27 mmol), and Dowtherm A (5.6 mL) were charged in a three-necked flask equipped with a water separator. This mixture was stirred under argon atmosphere, while the temperature was increased to 95 °C in 1 h, then gradually up to 162 °C in a further hour. Then the mixture was stirred at this temperature for 6 h. After this time, the resulting solution was added in portions during 3 h into 42 mL of Dowtherm A stirred in a three-necked flask equipped with thermometer and water separator and heated at 253-254 °C. After addition the mixture was heated at the same temperature for 2 h. Then the mixture was cooled at 90 °C, treated with isopropanol (10 mL), cooled at 30 °C, filtered, and washed with isopropanol and light petroleum ether in turn to give pure derivative **34**. Chemical and physical data of derivative **34** are reported in Table 6.

1-(4-Fluorophenyl)methyl-4(1*H*)-quinolinone 35. A mixture of **34** (1.1 mmol), 4-

fluorophenylmethyl bromide (610 mg, 3.3 mmol), and anhydrous K_2CO_3 (210 mg, 1.5 mmol) in dry DMF (10 mL) was stirred at 100 °C for 1 h. After the mixture was cooled, water was added (40 mL) and the precipitate that formed was filtered, washed with water and light petroleum ether in turn, and then dried under IR lamp to provide pure derivatives **35**. Chemical and physical data of derivatives **35** are reported in Table 6.

General procedure for synthesis of 4-[1-((4-flurophenyl)methyl)-4-oxo-4(1H)-quinolon-3-yl]-2-hydroxy-4-oxobut-2-enamide 26, 27. Potassium tertbuthoxide (380 mg, 3.4 mmol) was added into a well stirred mixture of **35** (1.7 mmol) and the appropriate oxamate (3.4 mmol) in anhydrous THF (2.7 mL) under nitrogen atmosphere. The mixture was stirred at room temperature for 2 h, then was poured into *n*-hexane (50 mL). The collected precipitate was vigorously stirred for 30 min in 1 N HCl (50 mL). The yellow solid that formed was filtered, washed with water, and dried under IR lamp to afford the pures **26** and **27**. Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR are reported for each of the following compounds.

4-[1-((4-Flurophenyl)methyl)-4-oxo-4(1H)-quinolon-3-yl]-2-hydroxy-*N*,*N*-dimethyl-4-oxobut-2-enamide (26). 80%; 190-195 °C; ethanol; IR *v* 1630 (C=O amide), 1611 (C=O ketone) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 2.92-2.94 (m, 3H, CH_3 amide), 4.39 (s, CH_2 ketone), 5.76-5.78 (s, 2H, CH_2 benzyl), 7.17-7.22 (m, 2H, benzene H), 7.35-7.38 (m, 2H, benzene H), 7.46 (bs, CH enole), 13.00 (bs, 3H, NH and OH).

4-[1-((4-Flurophenyl)methyl)-4-oxo-4(1H)-quinolon-3-yl]-2-hydroxy-pyperidin-4-oxobut-2-enamide (27). 81%; 153 °C; benzene/cyclohexane; IR *v* 1630 (C=O amide), 1601 (C=O ketone) cm^{-1}. ^{1}H NMR (Acetone-d_6) δ 1.43-1.74 (m, 6H, pyperidine H), 3.50-3.60 (m, 4H, pyperidine H), 5.83 (d, 2H, CH_2 benzyl), 7.15-7.19 (m, 2H, benzene H), 7.43-7.51 (m, 3H, benzene H and quinolinone C8-H), 7.64 (bs, 1H, CH enole), 7.69-7.76 (m, 2H, quinolinone C6-H and C7-H), 8.41-8.43 (d, 1H, quinolinone C5-H), 9.02 (s, 1H, quinolinone C2-H), 13.00 (bs, 1H, OH).

Ethyl 4,5-dioxo-1,4,5,6,7,8-exahydroquinolin-3-carboxylate (36). A suspention of

commercially available 3-aminocyclohexanone (5 g, 45 mmol) in ethyl ethoxymethylenemalonate (10.7 g, 45 mmol) was heated for 3 h at 130 °C. The reaction mixture was concentrated at reduced pressure obtained 15 g of crude product as a brown oil that was utilized for the following reaction without further purification. The crude product was dissolved in diphenyl ether (93 mL) and refluxed for 1 h. The reaction mixture was poured into *n*-hexane (60 mL) and the precipitate collected by filtration, washed with *n*-hexane obtaining 2.4 g of pure **36** as a brown solid. Chemical and physical data of derivatives **36** are reported in Table 7.

Ethyl 5-hydroxy-4-oxo-4(1H)-dihydroquinolin-3-carboxylate (37). 36 (9.3 mmol) was dissolved into 97 mL of ethanol, I_2 (25.8 mmol) was added and the mixture refluxed for 17 h. After cooling the reaction mixture was concentrated at reduced pressure and the residue washed with ethyl acetate, H_2O, acetone and petroleum ether obtaining 1.03 g of crude product **37** as a brown solid. Chemical and physical data of derivatives **37** are reported in Table 7.

Greneral procedure for alkylation (38, 39, 47 and 51). A mixture of appropriate substrate **37, 38, 46** [37] or **50** (1.1 mmol), alkyl iodide (3.3 mmol), and anhydrous K_2CO_3 (1.5 mmol) in dry DMF (10 mL) was stirred at 100 °C. After the mixture was cooled, water was added (40 mL) and the precipitate that formed was filtered, washed with water and light petroleum ether in turn, and then dried under IR lamp to provide pure derivatives **38, 39, 47** and **51**. Chemical and physical data of derivatives **38, 39, 47** and **51** are reported in Table 7 and 8.

General procedure for hydrolysis (28a-d). A mixture of the appropriate ethyl ester **37, 38, 39** or **43** (1.4 mmol) in 42 mL of NaOH acquous solution 10% was heated for 1h at 100 °C. After colling the mixture was treated with 1 N HCl until pH 1-2 was reached, the acquous extract with $CHCl_3$, the organic layer washed with brine, dried over Na_2SO_3 and evaporated at reduced pressure yielding pure **28a-d.** Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR are reported for each of the following compounds.

5-Hydroxy-4-oxo-4(1H)-dihydroquinolin-3-carboxylic acid (28a). IR *v* 2833 (OH and NH), 1702 (C=O acid), 1640 (C=O ketone) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 6.84-

6.86 (d, 1H, quinolinone C8-H), 7.20-7.22 (d, 1H, quinolinone C6-H), 7.69-7.73 (t, 1H, quinolinone C7-H), 8.85 (s, 1H, quinolinone C2-H), 12.60 (s, 1H, OH), 13.60 (bs, 1H, NH).

5-Hydroxy-1-ethyl-4-oxo-4(1H)-dihydroquinolin-3-carboxylic Acid (28b). 42%; 245 °C; ethanol; IR *v* 3345 (OH), 1705 (C=O acid), 1635 (C=O ketone) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.52 (t, 3H, $CH_2\mathit{CH_3}$), 4.38 (m, 4H, $\mathit{CH_2}CH_3$), 6.79 (m, 2H, quinolinone C6-H and quinolinone C8-H), 7.51 (t, 1H, quinolinone C7-H), 8.46 (m, 1H, quinolinone C2-H), 14.66 (bs, 1H, COOH).

5-Ethoxy-1-ethyl-4-oxo-4(1H)-dihydroquinolin-3-carboxylic Acid (28c). 55%; 213 °C; Ethanol; IR *v* 2969 (OH), 1712 (C=O acid), 1620 (C=O ketone) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.60 (m, 6H, $CH_2\mathit{CH_3}$), 4.30 (m, 4H, $\mathit{CH_2}CH_3$), 6.97 (m, 1H, quinolinone C6-H), 7.14 (m, 1H, quinolinone C8-H), 7.71 (m, 1H, quinolinone C7-H), 8.72 (m, 1H, quinolinone C2-H), 15.62 (bs, 1H, COOH).

General procedure for amide formation (29a-d). A suspention of appropriate acid **28a-d** (0.57 mmol), NMP (2 mL), HOBT (0.57 mmol), PS-carbodiimide (1.36 g) and 4-fluorobenzylamine was reacted under microwave irradiation (100 °C, 150 Watt) for 10 min. The mixture was filtered on Si-carbonate, washed with methanol alternated with $CHCl_3$, obtaining a crude producte that was purified by silica gel column chromatography using ethyl acetate/ethanol 3:1 as eluent obtaining pure **29a-d.** Yield (%), melting point (°C), recrystallization solvent, IR, ^1H NMR are reported for each of the following compounds.

5-Hydroxy-*N*-((4-fluorophenyl)methyl)-4-oxo-4(1H)-dihydroquinolin-3-carboxamide (29a). IR *v* 3366-2161 (OH and NH), 1629 (C=O amide) cm^{-1}. ^1H NMR (DMSO-d_6) δ 4.53-4.55 (d, 2H, CH_2 benzylamide), 6.72-6.74 (d, 1H, quinolinone C6-H), 7.09-7.11 (d, 1H, quinolinone C8-H), 7.14, 7.19 (m, 2H, benzene H), 7.37-7.41 (m, 2H, benzene H), 7.59-7.63 (t, 2H, quinolinone C7-H), 8.76 (s, 1H, quinolinone C2-H), 9.70 (m, 1H, NH), 13.74 (s, 1H, OH).

5-Hydroxy-1-ethyl-*N*-((4-fluorophenyl)methyl)-4-oxo-4(1H)-dihydroquinolin-3-carboxamide (29b). 28%; 210 °C; benzene/cyclohexane; IR *v* 3263 (NH), 3038

(OH), 1654 (C=O ketone), 1601 (C=O amide) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.57 (t, 3H, CH_2CH_3), 4.30 (q, 2H, CH_2CH_3), 4.66 (s, 2H, CH_2-benzyl), 6.87 (m, 1H, quinolinone C6-H), 6.92 (m, 1H, quinolinone C8-H), 7.04 (m, 2H, benzene H), 7.37 (m, 2H, benzene H), 7.61 (m, 1H, quinolinone C7-H), 8.81 (m, 1H, quinolinone C2-H), 9.89 (bs, 1H, NH).

5-Ethoxy-1-ethyl-*N*-((4-fluorophenyl)methyl)-4-oxo-4(1H)-dihydroquinolin-3-carboxamide (29c). 32%; 165 °C; benzene/cyclohexane; IR *v* 2925 (NH), 1649 (C=O ketone), 1601 (C=O amide) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.51-1.67 (m, 6H, CH_2CH_3), 4.23-4.29 (m, 4H, CH_2CH_3), 4.64 (s, 2H, CH_2-benzyl), 6.88 (m, 1H, quinolinone C6-H), 6.99 (m, 2H, benzene H), 7.06 (m, 1H, quinolinone C8-H), 7.54 (m, 2H, benzene H), 7.59 (m, 1H, quinolinone C7-H), 8.73 (m, 1H, quinolinone C2-H), 12.2 (bs, 1H, NH).

***N*-((4-fluorophenyl)methyl)-7-hydroxy-2-(2-hydroxyethyl)-6-oxo-6H-pyrrol[3,2,1-ij]quinolin-5-carboxamide (29d).** 30%; 200-205 °C; methanol; IR *v* 3389 (OH), 3247 (NH), 1675 (C=O ketone), 1634 (C=O amide) cm^{-1}. ^1H NMR (DMSO-d_6) δ 3.17-3.20 (t, 2H, CH_2CH_2OH), 3.77 (t, 2H, CH_2CH_2OH), 4.60 (s, 2H, CH_2-benzyl), 4.50 (bs, 1H, OH), 6.93 (s, 1H, Pyrrole-β-protone), 7.00 (m, 1H, quinolinone C6-H), 7.16-7.20 (m, 2H, benzene H), 7.41-7.45 (m, 3H, quinolinone C8-H and benzene H), 7.96-7.98 (m, 1H, quinolinone C7-H), 9.35 (m, 1H, quinolinone C2-H), 9.80 (bs, 1H, NH), 11.5 (s, 1H, OH).

2-Bromo-5-methoxyaniline (40). To a solution of $SnCl_2 \cdot 2H_2O$, (3.0 mmol) in 72.26 mL of HCl_{conc} was added 62 mL of ethanol 96° and 3-nitro-4-bromoanisole (8.6 mmol) slowly. The reaction mixture was stirred at room temperature for 1 night, diluted with a little of water, treated with NaOH 6 N until pH 8 was reached. The mixture was filtered on boukner, the solid washed with THF and the filtrate concentrated at reduced pressure. The residue was extract with ethyl acetate, the organic layer washed with brine, dried over Na_2SO_3 and concentrated under reduced pressure to obtain 5 g of crude product **40** that was utilized for the following reaction without further purification (98% yield). Chemical and physical data of derivatives **40** are reported in Table 7.

General procedure for cyclization with Eaton's Reagent (41, 50). A suspention of the appropriate amine **40** or **49** [41] (45 mmol) in ethyl ethoxymethylenemalonate (10.7 g, 45 mmol) was heated for 3 h at 130 °C. The reaction mixture was concentrated at reduced pressure obtained crude product that was utilized for the following reaction without further purification. The crude product (8.06 mmol) was dissolved in 40 mL of Eaton reagent's ($CH_4O_3SO_5P_2$, 7.7% w/w P_2O_5 in methanesulfonic acid, 4 eq) at room temperature under inert atmosphere. After dissolution the mixture was stirred at 90 °C for 5 h under inert atmosphere. After cooling, was added crushed ice and the mixture was treated with NaOH 6 N until pH 7-8 was reached. The residue was extract with ethyl acetate, the organic layer washed with brine, dried over Na_2SO_3 and concentrated under reduced pressure to obtain crude product **41** and **50** that was purified by silica gel column chromatography using ethyl acetate/ethanol 10:1 as eluent obtaining pure **41** and **50.** Chemical and physical data of derivatives **41** and **50** are reported in Table 7 and 8.

Ethyl 2-(2-hydroxyethyl)-7-methoxy-6-oxo-6H-pyrrol[3,2,1-i,j]quinolin-5-carboxylate (42). Starting material **41** (3.07 mmol) was dissolved into 13.7 mL of absolute ethanol. CuI (0.307 mmol), $Cl_2Pd(PPh_3)_2$ (0.0307 mmol), NEt_3 (6.14 mmol) and 3-buthyn-2-ole (4.6 mmol) were added. The reaction mixture was stirred at reflux temperature for 3 days making every day repeated addiction in 8h of the reactants. After cooling to room temperature, the reaction mixture was poured into H_2O, treated with HCl 1 N until pH 1-2 was reached. The residue was extract with ethyl acetate, the organic layer washed with brine, dried over Na_2SO_3 anhydrous and concentrated under reduced pressure to obtain crude product **42** that was purified by silica gel column chromatography using ethyl acetate/ethanol 10:1 as eluent obtaining 450 mg of pure **42** (40% yield)**.** Chemical and physical data of derivative **42** are reported in Table 7 and 8.

2,5-dihydroxy-6-methoxyacetophenone (54). To a solution of 2-hydroxy-6-methoxyacetophenone (3 mmol) in 7 mL of acetic acid was aded H_2O_2 (6 mmol) and PTSA (0.3 mmol). The solution was stirred for 24 h at 50 °C. After cooling, acetic acid was removed by evaporation at reduced pressure and the resulting was extract with ethyl acetate. The organic layer was washed with brine and dried over Na_2SO_3 anhydrous and concentrated under reduced pressure to obtain crude product **54** as

yellow oil, that was purified by silica gel column chromatography using ethyl acetate/chloroform 1:3 as eluent obtaining 150 mg of pure **54** (27% yield)**.** Chemical and physical data of derivative **54** are reported in Table 9.

2-Hydroxy-5,6-dimethoxyacetophenone (55). A mix of **54** (5 mmol), K_2CO_3 (5.3 mmol), in 3 mL of acetone anhydrus was stirred for 10 min at room temperature. Dimethyl sulfate (5 mmol) was added and the reaction mixture was stirred at room temperature for 2 h. Acetone was removed by evaporation at reduced pressure and the resulting was extract with ethyl acetate. The organic layer was washed with brine and dried over Na_2SO_3 anhydrous and concentrated under reduced pressure to obtain crude product **54** as dark oil, that was purified by silica gel column chromatography using ethyl acetate/chloroform 1:10 as eluent obtaining 550 mg of pure **55** (48% yield)**.** Chemical and physical data of derivative **55** are reported in Table 9.

5,6-Dimethoxy-4-hydroxybenzo-2-tiopyranone (56). A solution of **55** (18.5 mmol), CS_2 (18.5 mmol) in 13 mL of THF_a was added to a suspention of *t*-BuOK (55 mmol) under inert atmosphere. The reaction was stirred at 45 °C for a night. After cooling, THF was removed by evaporation at reduced pressure and the resulting was extract with chloroform. The organic layer was washed with brine and dried over Na_2SO_3 anhydrous and concentrated under reduced pressure to obtain crude product **56** as dark red solid (45% yield), that was utilized for the next reaction without further purification**.** Chemical and physical data of derivative **56** are reported in Table 9.

5,6-Dimethoxy-2-methyltiobenzopyran-4-one (57). To a solution of **56** (4.5 mmol) in 15 mL of acetone anhydrus was added K_2CO_3 (5 mmol) and CH_3I (20 mmol). The reaction mixture was stirred at reflux temperature for 30 min. After cooling H_2O was added, acetone evaporated at reduced pressure and the resulting material was extract with ethyl acetate, the organic layer was washed with brine and dried over Na_2SO_3 anhydrous and concentrated under reduced pressure to obtain crude product **57** as dark red solid (100% yield), that was utilized for the next reaction without further purification**.** Chemical and physical data of derivative **57** are reported in Table 9.

5,6-Dimethoxy-2-(methylsulfo)benzopyran-4-one (58). To a solution of **57** (1.19

mmol) in 10 mL of dichloromethane at 0 °C was added m-chloroperbenzoic acid (1.48 mmol). The ice bath was remuved and the reaction was stirred at room temperature for 1 h. The formed solid was remuved by filtration and the filtrate was washed with aq. $NaCO_3$ 5%, H_2O, and $NaHSO_{4ss}$. The organic layer was dried over Na_2SO_4 anhydrus and evaporated at reduced pressure obtaining 200 mg of crude product **58** (62% yield) that was utilized for the next reaction without further purification**.** Chemical and physical data of derivative **58** are reported in Table 9.

5,6-Dimethoxy-2-(4-fluorobenzylamine)benzopyran-4-one (59). To a solution of **58** (0.7 mmol) in 5 mL of acetonitrile was added 4-fluorobenzylamine (0.9 mmol). The reaction mixture was stirred at room temperature for 1.5 h. The solvent was evaporated at reduced pressure and the residue solved with ethyl acetate. The organic layer washed with HCl 1 N, brine and dried over Na_2SO_4 anhydrus and evaporated at reduced pressure obtaining crude product **59** as brown solid, that was purified by silica gel column chromatography using ethyl acetate as eluent obtaining 70 mg of pure **59** (28% yield)**.** Chemical and physical data of derivative **59** are reported in Table 9.

General procedure for dealkylation (30, 31, 43). To a solution of appropriate alkyloxy derivative **42, 53** or **59** (0.25 mmol) in 4.5 mL of CH_2Cl_2 at -45 °C was added dropwise a solution of BBr_3 1M in the same solvent (1.2 mmol). The reaction mixture was stirred at the same temperature for 1 night. After room temperature was reached, the reaction mixture was poured into water and the fourmed precipitate collected by filtration, washed with water and diethyl ether obtaining crude product that was purified by silica gel column chromatography using ethyl acetate as eluent obtaining pure **30, 31** and **43.** Chemical and physical data of derivative **43** are reported in Table 7. Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR are reported for the following compounds.

***N*-((4-fluorophenyl)methyl)-5,6-dihydroxy-1-methyl-4-oxo-4(1H)-dihydroquinolin-3-carboxamide (30).** 30%; >270 °C; Methanol; IR *v* 3408 (OH), 3082 (NH), 1648 (C=O ketone), 1603 (C=O amide) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 3.96 (s, 3H, CH_3), 4.54-4.55 (s, 2H, CH_2-benzyl), 7.08-7.19 (m, 3H, benzene H and quinolinone C8-H), 7.33-7.39 (m, 3H, benzene H and quinolinone C7-H), 8.83 (s,

1H, quinolinone C2-H), 9.40 (bs, 1H, OH), 9.89 (bs, 1H, NH), 14.16 (bs, 1H, OH).

5,6-Dihydroxy-2-(4-fluorobenzylamine)benzopyran-4-one (31). 15.6%; oil; ^{1}H NMR ($CDCl_3$) δ 4.42-4.43 (s, 2H, CH_2 benzylamide), 5.31 (s, 1H, benzopyranone C3-H), 5.48 (bs, 1H, NH), 6.64-6.68 (d, 1H, benzopyranone C8-H), 7.07-7.10 (m, 3H, benzopyranone C7-H and benzene H), 7.30-7.35 (m, 2H, benzene H), 13.34 and 13.47 (bs, 1H, OH).

General procedure for the reductive dehalogenation (48, 52). A solution of starting material **47** or **51** (1.6 mmol) in 120 mL of ethanol was stirred in presence of 300 mg of 10% Pd/C under H_2 atmosphere at 3 Atm and 40 °C for 7/1.5 h respectivelly. The mixture was then filtered and the Pd residue washed with ethyl acetate. The combined organic layer were concentrated at reduced pressure to yield crude product that was purified. **48**: alluminium oxide column chromatography using ethyl acetate/ethanol 50:1 as eluent; **52**: silica gel column chromatography using ethyl acetate as eluent. Chemical and physical of derivatives **48** and **52** are reported in Tables 7 and 8.

General procedure for amide formation (29e, 53). Appropriate starting material **48** or **52** (3.02 mmol) an the 4-fluorobenzylamine (30.2 mmol) was reacted under microwave irradiation (120 °C, 100 Watt) for 10 min. The reaction mixture was poured into diethyl ether, the formed solid collected by filtration, washed with ether yielding pure **29e** and **53.** Chemical and physical of derivative **53** are reported in Table 8. Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR are reported for the following compound.

5-Amino-1-ethyl-*N*-((4-fluorophenyl)methyl)-4-oxo-4(1H)-dihydroquinolin-3-carboxamide (29e). 30%; 250 °C; Methanol; IR *v* 3263 (NH), 3008 (NH_2), 1634 (C=O ketone), 1600 (C=O amide) cm^{-1}. ^{1}H NMR (Acetone-d_6) δ 1.48-1.52 (t, 3H, CH_2*CH_3*), 4.37-4.42 (q, 2H, *CH_2*CH_3), 4.62 (s, 2H, CH_2-benzyl), 6.59-6.61 (m, 1H, quinolinone C6-H), 6.75-6.77 (m, 1H, quinolinone C8-H), 7.08-7.13 (m, 2H, benzene H), 7.37-7.47 (m, 3H, quinolinone C7-H and benzene H), 8.68 (m, 1H, quinolinone C2-H), 9.06 (bs, 1H, NH).

60. A mixture of isatine (23mmol) K_2CO_3 (27 mmol) and haloderivative (27 mmol)

in 25 mL of DMF_a was stirred at 80 °C for 1.5 h. After cooling, the reaction mixture was purified in crushed ice and the formed precipitate was collected by filtration, washed with H_2O and diethyl ether obtaining 5.89 g of crude product **60** (87% yield), that was utilized for the next reaction without further purification. Chemical and physical of derivative **60** are reported in Table 10.

***N*-Benzyl-3-hydroxy-4-oxo-4(1H)-dihydroquinolin-2-carboxamide (32).** To a solution of CH_3ONa (13 mmol) in methanol anhydrus (3.3 mL) heated at 120 °C was added **60** (1.7 mmol). The reaction mixture was stirred at the same temperature for 2 h. After cooling, the reaction mixture was purified in crushed ice and the formed precipitate was filtered off, water was acidified with HCl 1 N until pH 2 was reached. The formed precipitate was collected by filtration, washed with H_2O and diethyl ether obtaining crude product **32**, that was purified by silica gel column chromatography using ethyl acetate as eluent obtaining pure **32.** Yield (%), melting point (°C), recrystallization solvent, IR, ^1H NMR are reported for the following compound.

***N*-Benzyl-3-hydroxy-4-oxo-4(1H)-dihydroquinolin-2-carboxamide (32).** 10%; 230-232 °C; trituration in diethyl eter; IR *v* 3223 (OH), 3058 (NH), 1637 (C=O amide), 1585 (C=O ketone) cm^{-1}. ^1H NMR (DMSO-d_6) δ 4.63-4.65 (d, 2H, CH_2 benzylamine), 7.21-7.41 (m, 8H, benzene H, quinolinone C6-H, C7-H and C8-H), 7.57-7.59 (d, 1H quinolinone C5-H), 8.12-8.14 (d, 1H, NH), 11.36 (bs, 1H, OH), 12.53 (s, 1H, NH).

2-(2-Nitro-benzylidene)-malonic Acid Diethyl Ester (61). To a solution of diethyl malonic ester (27 mmol), acetic acid (9 mmol), and piperidine (2.7 mmol) in benzene (60 mL) was added 2-nitrobenzaldehyde (22.5 mmol). The reaction mixture was refluxed for 72 h and then evaporated. The crude product was dissolved in ethyl acetate, washed 4 times with aq. $NaHCO_3$ 5% and once with brine, dried over Na_2SO_4 anhydrus, filtered and evaporated. The product was purified over silica gel with cyclohexane/ethyl acetate 95:5 as eluent to give **61** as a colorless oil (12% yield). Chemical and physical of derivative **61** are reported in Table 10.

1-Hydroxy-2-oxo-1,2-dihydro-qoinoline-3-carboxylic Acid Ethyl Ester (62). Compound **61** (1.05 mmol) was dissolved in acetic acid (5 mL), and PtO_2 (5 mg) was added. The reaction mixture was stirred 24 h at room temperature under hydrogen, then filtered over celite, and washed with methanol. The solvents were evaporated under reduce pressure. The product was purified on *n*-hexane for 1 night obtaining pure **62** as a beige powder (55% yield). Chemical and physical of derivative **62** are reported in Table 10.

1-Hydroxy-2-oxo-1,2-dihydro-quinoline-3-carboxylic Acid 4-fluoro-benzylamide (33). Compound **62** (0.16 mmol) was dissolved in 4-fluorobenzylamine (2 mL), and the reactionmixture was beate at 60 °C for 4 h, and then cooled at room temperature. Ethyl acetate was added, and the organic layerwas washed 4 times with HCl 1 N, once with water, once with brine, dried over Na_2SO_4 anhydrus, filtered and evaporated. The residue was precipitated over dichloromethane and filtered to give **33** as a beige powder. Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR are reported. 87%; 230-232 °C; precipitated over dichloromethane; IR *v* 3255 (OH), 3050 (NH), 1655 (C=O amide), 1609 (C=O ketone) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 4.55-4.59 (d, 2H, CH_2 benzylamine), 7.15-7.20 (m, 2H, benzene H), 7.39-7.42 (m, 3H, benzene H and quinolinone C6-H), 7.78-7.80 (m, 2H, quinolinone C7-H and C5-H), 8.04-8.06 (d, 1H quinolinone C8-H), 8.85 (s, 1H, quinolinone C4-H), 10.07 (s, 1H, NH), 11.87 (bs, 1H, OH).

Table 6. Chemical and physical data of synthesis intermediate of derivatives **26** and **27.**

Cpd	mp (°C)	Recryst solvent[a]	Yield (%)
34	242-244	a	56
35	213-214	b	65

[a] Recrystallization solvents: (a) aceton, (b) toluene.

Table 7. Chemical and physical data of synthesis intermediate of derivatives **28a-d** and **29a-e.**

Cpd	mp (°C)	Recryst solvent[a]	Yield (%)
36	170-171	a	58
37	294-296	d	60
38	155	b	78
39	155	b	89
40	oil	oil	98
41	184-185	a	60
42	oil	oil	40
43	-	-	98

44	245-248	c	78
45	102	b	77
46	238 (dec)	d	44
47	179-180	e	34
48	200-201	f	30

[a] Recrystallization solvents: (a) methanol, (b) benzene/cyclohexane, (c) ethanol, (d) DMF, (e) ethyl acetate, (f) ethyl acetate/ethanol

Table 8. Chemical and physical data of synthesis intermediate of derivative **30.**

Cpd	**mp (°C)**	Recryst solvent[a]	**Yield (%)**
49	oil	oil	100
50	178-180	a	55
51	75-80	a	46
52	105	b	100
53	244	a	58

[a] Recrystallization solvents: (a) toluene, (b) *n*-hexane.

Table 9. Chemical and physical data of synthesis intermediate of derivatives **31.**

Cpd	**mp (°C)**	Recryst solvent	**Yield (%)**
54	oil	oil	27.5
55	oil	oil	48
56	oil	oil	45
57	oil	oil	100
58	oil	oil	62
59	oil	oil	28

Table 10. Chemical and physical data of synthesis intermediate of derivatives **32** and **33.**

Cpd	**mp (°C)**	Recryst solvent	**Yield (%)**
60	190-193	-	87
61	oil	oil	12
62	-	-	55

Biology

Compounds **13a-i**, **14a-i**, **15a**,**b**, **16a**,**b, 17a,b** and **18a,b** were tested for their ability to inhibit HIV-1 integrase in vitro using a gel-based assay in addition to a 96-well plate-based high throughput electrochemiluminescent assay.[30] In the gel assay, a 5′-end-labeled 21-mer double-stranded DNA oligonucleotide corresponding to the last 21 bases of the U5 viral LTR is used to follow both 3′-P and ST. Briefly, a DNA-enzyme complex is preformed by mixing the drug at the destre concentration with 400 nM recombinant HIV-1 integrase in a buffer containing 50 mM MOPS, pH 7.2, 7.5 mM $MnCl_2$, and 14.3 mM β-mercaptoethanol. The integration reaction is then initiated by the addition of 20 nM 5′-labeled double-stranded DNA template and continued in a total volume of 10 μL for 60 min at 37 °C. The reaction samples are stopped by adding the same volume of electrophoresis denaturing dye and loaded on 20% 19/1 acrylamide denaturing gel. Gels were exposed overnight and analyzed using a Molecular Dynamics hosphorimager (Sunnyvale, CA). The 96-well plate-based high throughput electrochemiluminescent assay was performed using a BioVeris M series analyzer (Gaithersburg, MD) as described previously.[30] Briefly, DNA substrates were obtained from BioVeris and used according to the manufacturer's recommendations. Donor DNA is incubated for 30 min at 37 °C in the presence of 250 nM recombinant HIV-1 integrase. After addition of the drug, the integration reaction is initiated by addition of target DNA. Reaction is carried out for 60 min at 37 °C and then read on the BioVeris M series analyzer.

The anti-HIV drug testing was performed in 96-well plates with a defined, previously titered inoculum of a laboratory strain (HTLV-IIIB) to minimize the inoculum effect. In brief, all compounds were dissolved in dimethyl sulfoxide and diluted in cell culture medium at concentrations ranging from 0.1 to 50 μM. Exponentially growing human T lymphocytes (H9 cell line) were added at 5000 cells/well. After the infectivity of a virus stock (HTLV-IIIB) is quantified, an aliquot containing 1 x 10^5 50% tissue culture infectious dose (TCID50) per 5000 H9 cells per well is used as inoculum in each set of in vitro infections of H9 cells. Uninfected cells with the compound served as a toxicity control, and infected and uninfected cells without the compound served as basic controls. Culture were incubated at 37 °C in a 5% CO_2 atmosphere for 4 days. Supernatant fluid of infected wells (in the absence of drug and at each of a number of drug concentrations) is harvested. HIV p24 antigen is quantified, and the 50% inhibitory concentration (IC_{50}) of drug is

determined using the median effect equation.

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Second chapter

RIBONUCLEASE H OF HIV-1

Among the enzymes that are codified by *pol* gene of HIV-1, reverse trascriptase (RT) is the most and longer studied. [1] RT is a multifunctional enzyme that are implicated in several associated activities, all of these essentials for HIV-1 infection. Infact RT parteciped to the life cycle of the virus as RNA- and DNA-dependent DNA polymerase (RDDP and DDDP, respectively), ribonuclease H (RNase H), strand transfer, strand dispacement synthesis and nucleotide excission. [2,3,4] To catalyse these reactions, RT utilized two different catalytic sites which, albeit the HIV-1 RT is composed by two polypetide subunits (p66 and p51), reside in p66 subunit at the distance of 18 base pairs from each other and are strictly inter-dependent: RT-associated polymerase function and RNase H functon.

As RT-associated polymerase function, RT carried out a conversion of the viral single strand RNA genome into a double strand DNA, which is subsequently traslocated into the cell nucleus and integrated into the cell host DNA. [2,3] As RNase H function, RT catalyzed the degradation of RNA strand of the RNA:DNA hybrid intermediate to permited the synthesis of double strand viral DNA. This passage is essential for the HIV-1 infection. [5]

Although RT is a multifunctional enzyme, all approved RT inhibitors (NRTIs and NNRTs) target only the RT-associated polymerase function. Therefore, the identification of new RT inhibitors that target other essential RT-associated function such as RNase H activity, could be an attractive approach to enhanced the anti-HIV-1 drug armamentarium effectiveness. [6]

2.1 Biology of RNase H function

2.1.1 Structure of reverse trascriptase

RT is codify by *pol* gene as p66 polypeptide (66 kDa) that is partially cleaved by the viral protease into two p51 and p15 polypeptides (51 and 15 kDa, respectively). The active RT is an heterodimeric enzyme composed by two subunits: p66, contenents 560 amino acidic residues, and p51, contenents 440 amino acidic residues.

The p66 subunit contains two differents catalytic domains: the first located in the N-

terminus, possessing RNA- and DNA-plymerase activity, and catalyzing the reverse trascription of viral RNA in double strand proviral DNA; the second located in the C-terminus, possessing ribonuclease H activity, and catalyzing the degradation of RNA strand of the RNA:DNA hybrid intermediate. The tridimentional structure of the p66 is similar to other polymerase and are compared to a closed hand consisting of four different domains namely palm domain (residues 86-117 and 156-237), fingers domain (residues 1-85 and 118-155) and thumb domain (residues 319-426) and by a connection domain (residues 427-560), [7] ables to envelop the viral genome and accomodate it in the way which can cross the active site of the enzyme.

The p51 subunit shows a different spatial arrangiament, it has no cleft for the template binding and the residues requied for catalysis are buried into the subunit and, as a result, does not contains functional site. Infact the catalytic site of RT-associated polymerase function is costituted by Asp110, Asp185, and Asp186 amino acids residues, which are highly conserved and are located in the palm domain of p66. On the contrary, the corresponding Asp residues in p51 are concealed and have lost the catalytic functions of the enzyme. [5]

Thogeter with IN and other enzyme like transposase and RuvC resolvase, RNase H belong to the polynucleotidyl transferase family. RNase H of HIV-1 catalyzed phosphoryl transfer through nucleophilic substitution reactions on phosphate ester, requiring the concerted action of a general base activating the nucleophile and a general acid protonating the leaving group. In particolar catalysis occurs by deprotonation of a water molecule to form a nucleophilic hydroxide group that attacks the scissile phosphate goup on RNA. To accomplish this reaction, RNase H function requred the presence of metal ions such as Mg^{2+} or Mn^{2+}. Differently to other transposase, the structure of RNase H domain has been proposed to need two divalent cations that, consistetly with the phosphoryl transfer geometry, seem to be coordinated by the active site carboxylates D443, E478, D498 and D549. [8]

These residues composed a three amino acids DDE motive, highly conserved in many HIV-1 strains in the core domain of the RNase H active site: mutations in any of the D443, E478, D498 or D549 abolish enzyme activities. [9]

2.1.2 Role of RNase H function in the reverse trascriptase

The reverse transcription of HIV-1 genome needs a primer of double strand RNA. This primer is furnish by the cellular apparatus and is constituted by a specific

sequence tRNALys3 which is complementary to the primer binding site (PBS) localized at the 5' end of the viral RNA. Therefore, the RT-associated RDDP function starts the transcription of the first molecule of DNA with negative polarity. The product of this first step is the hybrid RNA:DNA intermediate that will move from RDDP function active site to RNase H function active site. In the RNase H function active site the RNA strand of the hybrid is hydrlolyzed to obtain a minor-single-strand DNA, that is then transfered to the 3' end of the same molecule of RNA in correspondence to LTR sequence (intramoleular transfer) or to other copy of viral genome within virion (itermolecular transfer) to allow further elongation. This double-strand RNA:DNA segment become the primer for the elongation of the DNA strand catalyzed by the RDDP function of RT. The product of this second polymerization is newly a RNA:DNA hybrid intermediate which is partially hydrolized by RNase H to furnish a plus-singol-strand of DNA with a polypurine tract (PPT) of RNA which is not degradated. The PPT is the primer for the synthesis of the second DNA positive strand catalyzed by the DDDP function of RT. Now the removals of the PPT and tRNA primers by the RNase H function makes possible a second strand transfer which occurs with the interaction of the PBS sequences of the two DNA strands; or rather the 3' end of DNA (+) is transfered on the PBS in the 3' end of the DNA (-) to allow the DDDP function of RT to synthesize a complete double strand DNA. [5] Finally, it is assumed that the replication intermediate is circularized and that bidirectional DNA synthesis continues, during which an RT-associated strand displacement function is required.

2.1.3 Mechanism of action of RNase H function

The reaction catalyzed by RNase H function consist in the hydrolysis of phosphodiester bond of the viral RNA backbone which form an hybrid with new synthesized DNA strand. The reaction involved is a nucleophilic substitution carried out by a water molecule on the phosphodiester bond, that needs the concerted action of a general base activating the water molecule and a general acid protonating the negative charge of leaving group. The transition state has a bipyramid geometry which presents the five oxygen atoms in the vertices bounded to the pentavalent phosphorus atom. The oxygen atoms of the nocleophile and the leaving group occupy the axial positions while the residue oxygen atoms are in equatorial position.

The generals base and acid need for the hydrolysis of phosphodiester bond are

represent by the two bivalent metal ions which interact with carboxyl groups of the amino acids Asp443, Glu478, Asp498 and Asp549 in the active site of RNase H function. One of each metal ion acts like a general base, lowering the pka value of the water molecule which rise up the catalytic site. The water molecule is converted to hydroxid ion that represents the nucleophile needed for the reaction. The other one, acts like a general acid activating a second water molecule, which protoning the negative oxygen atom in the 3' end of the leaving group. Both metal ions contibute to orientate and stabilize the pentavalent transition state and to neutralize the emerged negative charge on the leaving group. [10]

The properties of the two metal ions are differents for each of the two differents positions. Infact the metal ion in the A position must have a lower value of pka for increasing the catalytic activity and the local concentration of the hydroxid ions; on the contrary, the presence of metal ions with lower pka value decrease the capacity to neutralize the negative charge on the leaving group by a indissociate water molecule. [11] On the basis of this consideration, is predictable that the activity of RNase H is enhanced in presence of metal ions such as Mn^{2+} (pka= 10,6) or Co^{2+} (pka= 8,9) then with Mg^{+2} (pka= 11,4). But crystallographic study, cofirmed by calorimetric method and NMR spectoscopy, reveal that the presence of two metal ions contraddistinc by two ions of Mg^{2+} are indispensable for the activity of the RNase H of HIV-1. [12]

2.2 Inhibitors of ribonuclease H function

During the last decades, significant progress has been made in the field of RT polymerase function inhibitors as antiretroviral agents and some of these compounds are widely used in clinical practice as drugs in the highly active anti retroviral therapy (HAART). [13] Conversely, specific inhibitors for RNase H function were not identified until recently. However, the RT-associated RNase H function was proven as an essential step in the virus life cycle. Thus, efforts in the development of new compounds targeting the RNase H activity are relevant to enhance the antiretroviral armamentarium. However, few specific RNase H inhibitors are known and little studies have been performed to assess the efficacy of these inhibitors in the treatment of HIV. Knowing the active site of the RNase H function and the mechanism of the catalysis, the inhibitors can:

- Modify the interaction between RNase H and the substrate;
- Modify the alignment between the substrate in the active site;
- Induce modification in the removal of the primer after the extension of DNA strand and alter the hydrolysis of the hybrid RNA:DNA.

On the bases of different studies, are known different kind of RNase H inhibitors which can divided into two classes:

A-Inhibitor with unknown binding site;

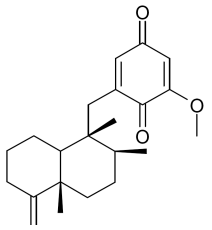
B-Inhibitors with known binding site.

To the firs group belong compounds with unclear mechanism of action.

2.2.1 A-Inhibitor with unknown binding site

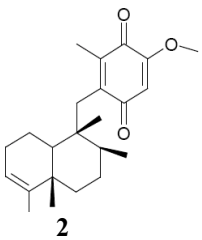
QUINONES AND NAPHTHOQUINONES

One of the first compound that was descrive to inhibite RNase H of HIV-1 is the Illimaquinone (**1**), a secondary metabolite producted by the Read sea sponge *Smenospongia sp.* This compound presents a inhibitory activity against the RNase H of HIV-1 about 15 μM, but is proven to inhibit also RT of HIV-2 and RNase h of *Escherichia coli* with an IC_{50} value ranging from 30 to 63 μM.

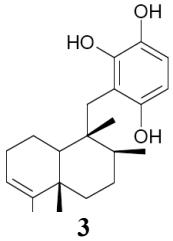


1

The SAR of Illimaquinone was studied synthesizing two analogue that present the oxydated or riduced pharmacophore, like the compound **2** and **3**. The modifications apported to this compound leaded to a decreasing of the activity against RNase H of HIV-1 and RT of HIV-2 and.



2

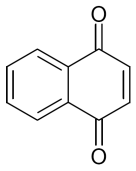


3

The binding site of illimaquinone was proven to be in close proximity to cysteine 280 in both HIV-1 and HIV-2 RTs. [14] However, no data were reported on its efficacy

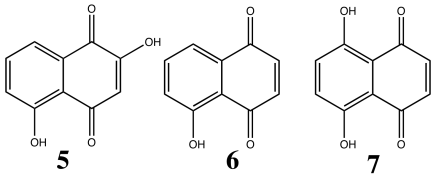
in the inhibition of the HIV replication cycle in cell-based assays.

The 1,4-naphthoquinone **4** are the compound demonstrating the best inhibitory activity and selectivity in enzymatic assay between naphthoquinone compounds. Infact its IC_{50} against RNase H is 9.5 μM without significant activity against RDDP function.



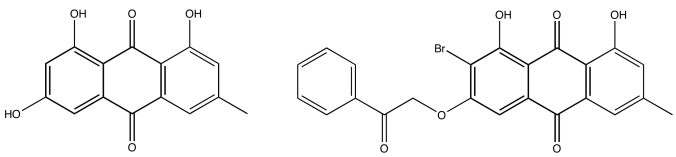
4

The relationship between structure and activity for this compound was studuied and a serie of derivatives were synthesized. The new derivatives presented an hydroxyl group in C2, C5 or C8 positions (**5**, **6**, **7**); all the modification to **4** structure lead to a decreasing of RNase H inhibition. [15]



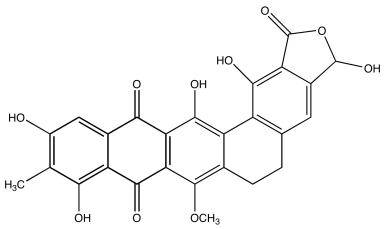
5 **6** **7**

Among naphthoquinones, another important compound is a derivative of frangula-emodinie, an ingredient of chinese herbes. The **8** compound was tested in *in vitro* test against RNase H of HIV-1 showing an IC_{50} value of 75 μM. With some modifications of the base structure of **8**, like an introduction of bromine atom and a alkylation with a benzoylmethyl substituent, was synthesized compound **9** which has demonstrated to be more potent of **9** in test against RNase H function, infact its IC_{50} value is about 14 μM. [16]



8 **9**

More structurally complicated quinones were studied by Marchand *at al.* Madurahydroxylactone (MHL, **10**) is a secondary metabolite from the soil bacterium *Nonomuraea rubra*, that belongs to the family of benzo[a]naphthacenequinone antibiotics. [17]



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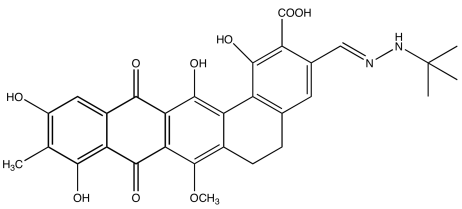
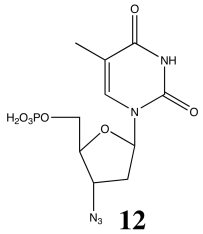


11

Despite **10** has a broad spectrum of action, a series of semi-synthetic derivatives were evaluated for dual inhibition of HIV-1 IN and RNase H. While most of the compounds exhibited similar potencies for both enzymes (IC_{50} ranging from 0.3 to 22 μM), some derivatives showed 10- to 100-fold higher selectivity for each enzyme. The most potent and selective MHL derivative identified within this series was the hydrazone **11**, which showed IC50 values of 0.69 and 122 μM against RNase H and IN, respectively.

NUCLEOTIDES AND OLIGONUCLEOTIDES

Some nucleotides were proven to be weak inhibitors of HIV RNase H activity in enzyme assays. In particular, AZT-MP (**12**), inhibited the HIV RNase H activity more potently than other nucleotide monophosphates. [18] Its inhibitory potency was dependent on the divalent metal ion used, changing in the assay. In fact, the measured IC_{50} values were 5 μM and 0.05 μM when Mn^{2+} or Mg^{2+} were used as divalent cation activator, respectively. 2',3'-Dideoxyadenosine 5'-monophosphate (ddAMP) and 2',3'-dideoxyguanosine 5'-monophosphate (ddGMP), also inhibited the RNase H activity with IC_{50} values of 0.2 and 0.5 μM, respectively, in the presence of Mn^{2+}, while they were inactive in the presence of Mg^{2+}. [19]



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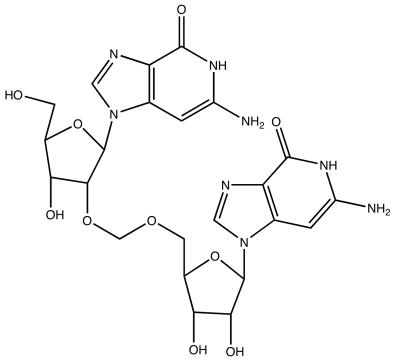


13

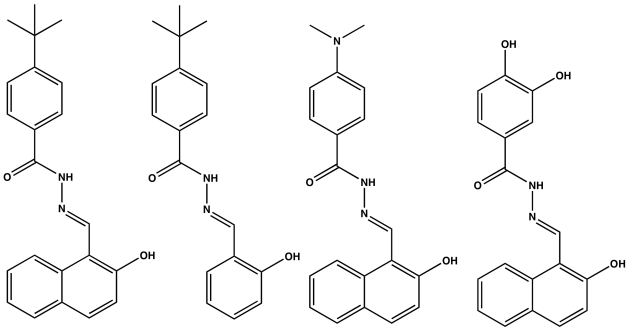
A selective inhibitor of RNase H and influenza endonuclease was the dinucleotide dimer diguanosine (rGrG). Infact it is not an inhibitor of either RNase H of *Escherichia coli* and a nuclear extract of Hela cell; despite it showed an IC_{50} value of

15 μM against RNase H of HIV-1. Also its derivative **13** evidence a similar activity but is more potent then parent compound showing an IC_{50} value of 5 μM against RNase H of HIV-1. This compound is obtained replacing the 3'-5'-phosphodiester linker with a 2'-5'-formacetal of the parent compound. [20]

HYDRAZONES

The *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH, **14**) was discovered after a random screening of a large number of molecules which has in common the capacity to chelate metal ions, resulting active against RNase H and RT of HIV-1 with IC_{50} value between 0.8 and 15 μM, respectivly. To the contrary, RNase H and RT of HIV-2 are resistant to the inhibition induced by BBNH (IC_{50} >50 μM). Interestingly, **14** was also active in cell-based assays, showing EC_{50} value of 1.5 μM for the inhibition of HIV-1 replication in cord blood mononuclear cells. BBNH inhibited both RNase H and RDDP activities of HIV-1 RTs mutated in residues K103N, Y181I, Y188H, Y188L with potency similar to the one obtained for wild type RT. Differently, when assayed on the mutated RT Y181C, **14** was not active on the RNase H activity while it inhibited the RDDP function with an IC_{50} value of 1.6 μM. Therefore, BBNH (**14**) was hypothesized to bind to two different sites on HIV-1 RT. The first one in the polymerase domain possibly nearby the NNRTI-binding site and the second one possibly located in the RNase H domain. After a molecular modeling study was suggested that the compound **14** makes two main interactions with the enzyme in the RNase H function active site: the first chelating the divalent metal ions presents in the active site of the enzyme and the second making a π-stacking interaction with the aromating ring of Tyr 501 and its second ring of naphthyl group. For validate this theory was sintetized the analogue of **14** changing the naphthyl moiety with a phenole group obtained the compound **15** which loss the inhibitory potency against RNase H function (IC_{50}> 50 μM), while retain the ability to inhibit RT function (IC_{50}= 2.5 μM). [21] Further studies on the mechanism of action of BBNH (**14**) led to hypothesize that its binding to RT could impact with the protein-protein interactions of the heterodimeric enzyme. To evaluate whether this observed phenomenon was mediated by **14** binding to one or more sites in RT, a random screening was conduced over 100 molecules structurally analogue of **14**, and the compound **16** was resulted the most selective against RNase

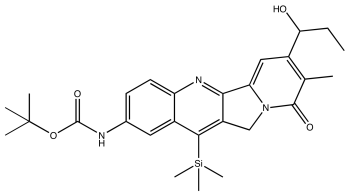
H function then RT function (IC_{50} RNase H = 4 μM, IC_{50} RT >40 μM). The identification of these specific inhibitors, in combination with other biochemical data, suggested a model in which two molecules of BBNH bind the RT heterodimer. On this regard, only the binding of hydrazone molecules in the DNA polymerase active domain elicits the observed destabilization of the HIV-1 RT heterodimer. Finally, the strain to increased the selectivity of this molecules against RNase H domine lead to the synthesis of dihydroxy-benzoyl-naphthyl-hydrazone **17** (DHBNH), which is revealed a potent and selective inhibitor of RNase H function (IC_{50}= 0.5 μM, IC_{50} RT= 50 μM). To better elucidate the mechanism of action of this compound, the crystal structure of HIV-1 RT in complex with DHBNH was solved, showing that it binds near the polymerase active site in a pocket different from the NNRTI-binding site, and also >50 Å away from the RNase H active site. It was hypothesized that **17** perturbs the trajectory of the template–primer so that RNase H cannot operate on its substrate. Alternatively, **17** may also bind to a second site in or near the RNase H domain that was not seen in the crystal. [21]



14 **15** **16** **17**

MAPPICINE ANALOGUES

Mappicine is a minor alkaloid from *Mappia foetida Miers* that is related to camptothecin. A few analogs (**18-20**) of this natural product have been described as selective HIV RNase H inhibitors. [22] In fact, these compounds inhibited the HIV RNase H activity in enzyme assay with IC_{50} values between 2.1 and 10 μM. The mappicine analogs may bind to a binding site on the HIV-1 RT not overlapping with the NNRTI binding site.



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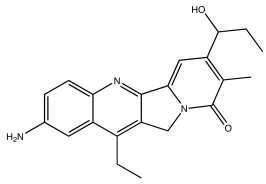

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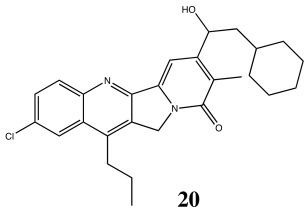
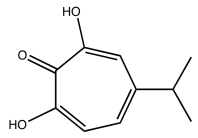


20

2.2.2 B-Inhibitors with known binding site

TROPOLONES

Tropolone and derivatives are natural compounds isolated from the heartwood of cupressaceous plants (*Thuja plicata, Thuja occidentalis* and *Chamaecyparis obtusal)* that are endowed of many biological activities including antifungal, antitumor, insecticide and antimicrobial effects. During a high-throughput screening of the American National Cancer Institute was identified the hydroxylated tropolone derivatives β-thujaplicinol (2,7-dihydroxy-4-1(methylethyl)-2,4,6-cycloheptatrien-1-one) (**21**) and manicol (1,2,3,4-tetrahydro-5-7-dihydroxy-9-methyl-2-(1-methylethenyl)-6Hbenzocyclohepten-6-one) (**22**), as potent and selective inhibitors of the HIV-1 RNase H. [23]



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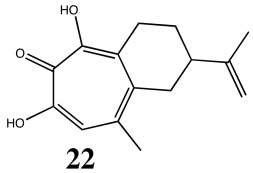


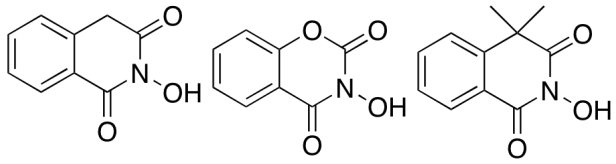
22

21 inhibited *in vitro* the HIV-1, HIV-2, *E. coli* and human RNase Hs with IC_{50} values of 0.2 μM, 0.7 μM, 50 μM and 5.7 μM, respectively. Similarly, **22** inhibited the HIV-1 and *E. coli* RNases H with IC_{50} of 1.5 μM and 40 μM, respectively. Both **21** and **22** failed to inhibit RDDP activity of HIV-1 RT at a concentration of 50 μM, suggesting that they are specific for the C-terminal RNase H domain. SAR studies within this class of compounds, led to conclude that the 2,7-dihydroxy function of these tropolone analogs is very relevant for the anti-RNase H activity, through a role in metal chelation at the RNase H active site. The vital role the 2,7-dihydroxy function of these tropolone analogues was definitively proven by the means of RX diffraction studies performed on a crystal structure of **21** bound to the RNase H active site of

both HIV-1 RT and an isolated RNase H domain. In fact, **21** was found to chelate the two divalent metal ions at the RNase H active site. [24]

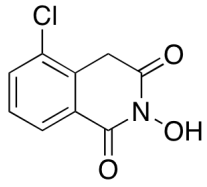
N-HYDROXYIMIDES

A series of *N*-hydroxyimides was previously synthesized as inhibitor of influenza endonuclease enzyme, but after the identification of the analogies between endonuclease and RNase H active sites, these compound was tested also aganst the RNase H function of HIV-1. The compound **23** was identified as the most active of this series showing an inhibitory activity against RNase H function with an IC_{50} value of 0.6-1 μM in enzymatic assay, while the inhibition of RDDP function was more mild with an IC_{50} value of 40 μM; this compound was unaffected against RNase H of *E. coli*. [25]



23 **24** **25**

Interesting SAR were described within this series. First, all three *N*-hydroxyimide oxygens were essential for inhibitory activity of the compounds and substitutions on the hydroxyl group were not tolerated, as well as its replacement with an amino group. The possibility to obtain an enolizable form was vital for the anti-RNase H activity, too. In fact, derivatives in which the structure blocked the tautomerization, were totally inactive (see compound **24** and **25**). After these coniderations, a large number of derivatives was synthesized aimed for enhance the inhibitory activity. All the synthesized compounds were weaker inhibitors of the RNase H activity (IC_{50} ranged from 5.7 to 58 μM among the active compounds) if compared to the prototype **23** and no clear structure-activity relationship emerged from the IC_{50} values. Thus the pharmaco-modulation of the isoquinoline ring in position 7 did not yield better candidates than **23**, and did not give compounds active in the submicromolar range against RNase H. [26] Only the compond **26,** the 5-chloro derivative, has demonstrated better activity then **23 (**IC_{50}= 0.3 μM). [27]



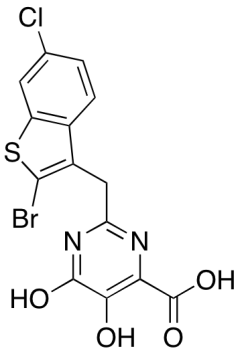
26

The enzymatic inhibitory properties of these *N*-hydroxyimides may be related to the metal binding abilities of the compounds. Infact, physicochemical studies performed by the means of NMR and UV-vis experiments, were consistent with a 1/1 stoichiometry of the magnesium complexes in solution, and the metal complexation was strictly dependent on the enolization abilities of the compounds. Unfortunately, all tested compounds exhibited high cellular cytotoxicity in cell culture which limits their applications as antiviral agents.

HYDROXYPYRIMIDINE CARBOXYLATE

Based on an electronical analysis of three different classes of RNase H inhibitors (DKA, *N*-hydroxyimides and hydroxytropolones) was designed new inhibitors characterized by the pyrimidinolcarboxylate moiety. [28]

SAR studies within this series as RNase H inhibitors in enzyme assays were focused on variations of the 2-substituent and led to the following conclusions: i) the best linker between the pyrimidinol ring and the 2-substituent as a second aromatic ring was a methylene group; ii) disubstituted compounds on this aryl portion were more potent than unsubsituted or monosubstituted analogues; iii) the best activity was obtained with a substituted benzothiophene ring as the aryl moiety. The most potent compound was the thiophene derivative **27**, that showed an IC_{50} value of 0.17 μM against HIV-1 RNase H and 70 μM against human RNase H. [29]



27

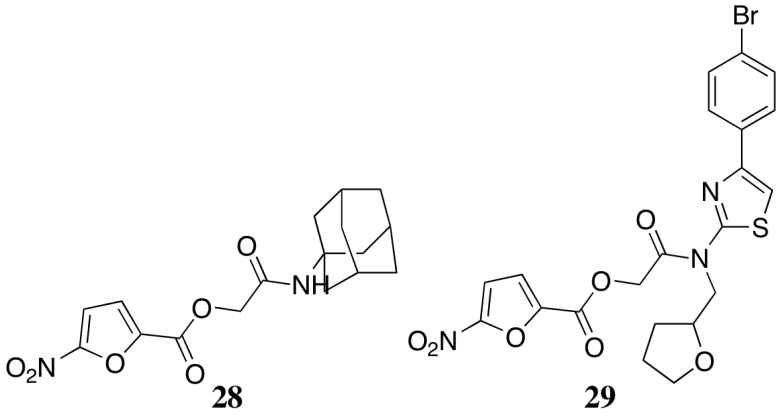
It is worth to note that the compounds reported in this work were inactive in a multi-

cycle MT-4 HIV infectivity assays at concentrations up to 100 μM. This could be ascribed to: insufficient intrinsic potency, low cell permeability, or high protein binding.

NITROFURAN-2-CARBOXYLIC ACID CARBAMOYLMETHYL ESTER (NACME) DERIVATIVES

During a screening for RNase H inhibitors, 20000 small molecules were tested and a novel RNase H inhibiting group of compounds was identified that was characterized by a 5-nitro-furan-2-carboxylic acid carbamoylmethyl ester (NACME) moiety. [30]

5-Nitro-furan-2-carboxylic acid adamantan-1-carbamoylmethyl ester (**28**) and 5-nitro-furan-2-carboxylic acid [[4-(4-bromo-phenyl)-thiazol-2-yl]-(tetrahydro-furan-2-ylmethyl)-carbamoyl]-methyl ester (**29**) were the most interesting NACMEs. They effectively blocked HIV-1 and MLV RT-associated RNase H activities with IC_{50} values of 3-30 μM but had little effect on bacterial RNase H activity in biochemical assays.



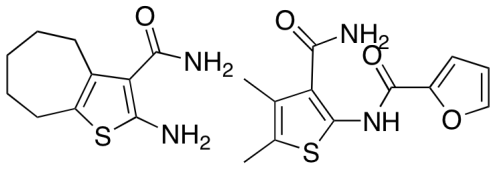
Both HIV-1 IN and RT RDDP enzymatic activities were not detectably affected by these compounds, therefore, they could be considered specific RNase H inhibitors. Docking studies indicated that the nitrofuran group of **28** chelates the two Mg^{2+} ions and orients toward the conserved His539 residue to form a possible hydrogen bond. For compound **29**, in addition to the above interactions, the nitrogen atom of the thiazole ring binds the Asp549 by hydrogen bond.

The SAR within this series led to conclude that: i) derivatives carrying various substituents at the end of the carbamoylmethyl ester group retained RNase H inhibitory activity; ii) substitution of the 5-nitrofuran moiety with various heterocycles resulted in a loss of RNase H inhibition, indicating a critical role for the 5-nitrofuran group within this series of RNase H inhibitiors; iii) derivatives with

relatively long and bulky groups at the end of the carbamoylmethyl ester group, displayed RNase H inhibitory activity; iv) compounds with relatively small volume groups in the same position showed no inhibitory activity.

VINYLOGOUS UREA

A high-throughput screening of National Cancer Institute libraries of synthetic and natural compounds was carried out and were identified the vinylogous ureas 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (NSC727447, **30**) and *N*-[3-(aminocarbonyl)-4,5-dimethyl-2-thienyl]-2-furancarboxamide (NSC727448, **31**), as novel HIV-1 and HIV-2 RNase H inhibitors.



30 **31**

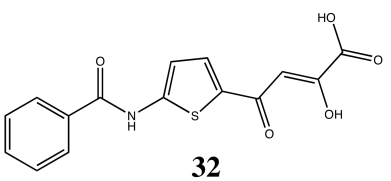
NSC727447 (**30**) did not inhibit DNA polymerase and pyrophosphorolysis activities at a concentration of 50 μM, indicating preferential inhibition of RNase H function. [31] A mass spectrometric protein footprinting based on biotin modification of exposed lysine residues in the free protein and the protein-inhibitor complex, revealed that Cys280 and Lys281, located in helix I of the thumb subdomain, were protected from modification by inhibitor binding. Proximity between the p51 (thumb) and the p66 domains implies that the urea vinylog binds near the catalytic center affecting either positioning of the nucleic acid substrate in the active site or divalent metal coordination. Site-directed mutagenesis using reconstituted p66/p51 heterodimers substituted with various amino acids shows that the alteration of the p66 RNase H primer grip, significantly affects inhibitor sensitivity. The above data suggested that regions within the RNase H domain distinct from the catalytic center can be targeted by small-molecule antagonists. The vinylogous ureas occupy a binding pocket outside the RNase H catalytic center and represent a novel class of allosteric RNase H antagonists to complement active-site directed, metal-chelating inhibitor.

DIKETOACID (DKA)

The DKA represents a significant class of RNase H inhibitors of HIV-1. Infact these compound, like *N*-hydroxyimides and tropolones, are able to chelate divalent metal ions which are presented in the active site of RNase H function. The idea of the

possible activity of DKA on RNase H function born from the known activity of these compounds on IN enzyme of HIV-1, which belonges to the same enzymatic class of RNase H enzyme; infact either enzyme are classified as polynucleotidyl transferases within high similarity in the catalytic site. In the active site of both enzyme are presented two metal ions, such as Mg^{2+} and the chelation of these bring to an inhibition of the catalytic activity. In *in vitro* test the α,γ-diketoacid mojety has dimonstred the ability to compete for the bond to the active site with native substrate because are able to form a ligand-metal-enzyme complex. Given the structural similarities between the domains of the two viral enzymes (IN and RNase H), several DKAs initially developed as IN inhibitors were also screened against the HIV-1 RNase H activity. Among them, the 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid (BTDBA, **32**) was the first HIV RNase H inhibitor reported. [32] It inhibited HIV-1 RNase H as part of full-length HIV RT showing an IC_{50} value of 3.2 μM, and also inhibited the isolated HIV-1 RNase H domain chimera carrying the *E. coli* RNase H basic helix loop motif with a similar IC_{50} value of 4.7 μM, while the IC_{50} values for HIV RT and *E. coli* RNase H inhibition were above 50 μM. However, BTDBA (**32**) also inhibited the HIV-1 IN with an IC_{50} value of 1.9 μM, showing to be not able to discriminate between the two HIV-1 targets, and most importantly, it did not block the viral replication in cell-based assays. [32] Further characterizations with isothermal titration calorimetry demonstrated that i) BTDBA requires the presence of a divalent cation in order to bind to the RNase H domain; ii) its metal-dependent K_d value is comparable to its IC_{50} value; iii) the nucleic acid substrate is not needed for its binding to RNase H.

Interestingly, **32** inhibited the strand transfer activity of HIV-RT synergistically with either a NNRTI, a nucleoside inhibitor (AZTTP), or a polymerase active site binding inhibitor (phosphonoformic acid, PFA, Foscarnet). These results are consistent with the requirement of both polymerase and RNase H activity for strand transfer and suggest that regimens combining polymerase and RNase H inhibitors may be therapeutically beneficial. [33]



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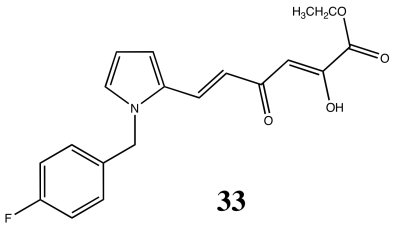


33

More recently another DKA (**33**) was identify as RNase H inhibitors. [34] This compound is structurally correlated with BTDBA for the presence of α,γ-diketoacid mojety and the aromatic portion, a pyrrole ring, responsable for the bound with the enzyme. RDS 1643 (**33**), inhibited the HIV-1 RNase H activity in enzyme assays with an IC_{50} value of 13 μM, showing lower potency if compared to BTDBA (**32**). It did not affect neither the HIV-1 RDDP function nor the AMV and *E. coli* RNase H activity, while it weakly inhibited the HIV-1 IN reaction (IC_{50} = 90 μM). Differently from BTDBA (**32**), RDS 1643 (**33**) inhibited the replication of wild type HIV-1 in cell-based assays showing an EC_{50} value of 13 μM and a CC_{50} value of 63 μM. Furthermore, **33** blocked the replication of three HIV-1 NNRTI resistant viral mutants (RT mutations were Y181C; K103N/Y181C; K103R/V179D/P225H) with EC_{50} values ranging from 7 to 19 μM. Mode of action studies demonstrated that **33** maximum adsorbance shifted in the presence of the Mg^{2+} ions, suggesting a mechanism of action similar to that shown by BTDBA (**32**). The effective concentration of **33**, although higher then some NNRT, is promised for outline an hypothetic association of DKAs with traditional therapy; moreover, the mutations of the amino acids in the active site of the enzyme less influence its activity then the drugs of HAART.

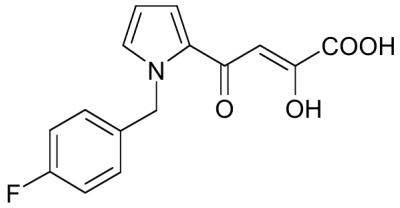
In conclusion the results suggested that: i) the DKAs have a favourable orientation in the interaction with the two metal ions in the active site of RNase H function; ii) the base structure have a lipofilic aromatic portion no much hindered for the bound with the amino acids residues and the stabilization of the compound-enzyme complex.

2.3 Aim

With the introduction of the HAART, the infection had partially lost the caracteristic of irreverible patology. The pharmacological association of several drugs has brought to the extention of the extimated life-time of the patients with infection of HIV, determined a decline of the incidence of desease correlated with the AIDS infection. However, the resistant viral strains to the chemotherapy and the toxicity of the utilized drugs have higlighted the necessity of a new associative scheme, with the introduction of new drugs able to target vital enzymes of the replicative viral cycle.

RNase H of HIV-1 belonge to the superfamily of polinucleotidyl trasferase, that also include the core domine of IN of HIV-1, another essential retroviral enzyme. From a structural analysis is emerged that these two proteines share most similar catalytic core domines, even tough carried out different byological activities. By this observation, was proposed the possibility to test the active molecules on the IN of HIV-1 also on the RNase H function.

The research group where I spent my PhD, by several years is involved in studies on integrase of HIV-1 inhibitors. Moreover, the decennal interest on pyrrole chemistry, has brought to development of pyrrolyldiketo acid derivative **33** analogue of compound **32**, first reported in literature inhibitor of HIV-1 RNase H function. The α,γ-diketo acid mojety of **32** is the pharmacophoric group that form the ligand-metal-enzyme tertiary complex with the two metal ions of catalytic active site, blocking the bound of naturale substrate. In a first time, we carried out a study of selectivity for Merck derivative L-731,988 on both RNase H function and polymerase function. Data furnished has higlighted that L-731,988 (**34**) does not selectivelly inhibits two catalytic domines of RT (also at concentration > 100 μM). Therefore in the structure of derivative **33** a vinilic mojety was been introduced between the diketo acid portion and the pyrrole ring, and was conserved DKA pharmacophoric mojety and aromatic portion allowing to value the importance of the distance between these two group in the molecule.

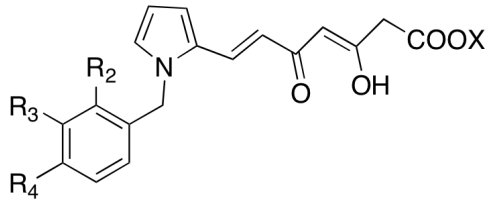


L-731,988

34

Our compound was tested on both RNase H function and RDDP function of RT, resulting a potent and selective inhibitor of RNase H function of HIV-1 (IC_{50} = 13 μM, IC_{50} > 100 μM, respectively), representing a new lead compound. Starting from the structure of **33** we have firstly investigeted the benzyl portion introducing mono- or di-substituted benzyl groups, for designed *N*-benzylpyrroles **35a-r, 36a-q, 37a-k** and **38a-k** (Tables 1 and 2)**.**

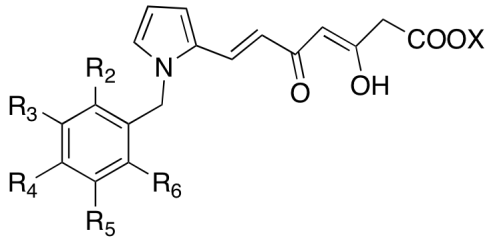
Table 1. Compounds **35a-r** and **36a-q**



Cpd	R2	R3	R4	X
35a	H	H	H	Et
35b	Cl	H	H	Et
35c	CN	H	H	Et
35d	F	H	H	Et
35e	CH_3	H	H	Et
35f	OC_2H_5	H	H	Et
35g	OCH_3	H	H	Et
35h	H	Cl	H	Et
35i	H	CN	H	Et
35j	H	F	H	Et
35k	H	Me	H	Et
35l	H	OCH_3	H	Et
35m	H	H	Cl	Et
35n	H	H	CN	Et
35o	H	H	F	Et
35p	H	H	CH_3	Et
35q	H	H	OCH_3	Et
35r	H	H	NO_2	Et

Cpd	R2	R3	R4	X
36a	H	H	H	H
36b	Cl	H	H	H
36c	CN	H	H	H
36d	F	H	H	H
36e	CH_3	H	H	H
36f	OC_2H_5	H	H	H
36g	OCH_3	H	H	H
36h	H	Cl	H	H
36i	H	CN	H	H
36j	H	F	H	H
36k	H	Me	H	H
36l	H	OCH_3	H	H
36m	H	H	Cl	H
36n	H	H	CN	H
36o	H	H	F	H
36p	H	H	CH_3	H
36q	H	H	OCH_3	H

Table 2. Compounds **37a-k** and **38a-k**



Cpd	R2	R3	R4	R5	R6	X
37a	F	F	H	H	H	Et
37b	Cl	H	Cl	H	H	Et
37c	F	H	F	H	H	Et
37d	CH_3	H	CH_3	H	H	Et
37e	F	H	H	F	H	Et
37f	Cl	H	H	H	Cl	Et
37g	F	H	H	H	F	Et
37h	H	F	F	H	H	Et
37i	H	Cl	H	Cl	H	Et
37j	H	F	H	F	H	Et
37k	H	CH_3	H	CH_3	H	Et

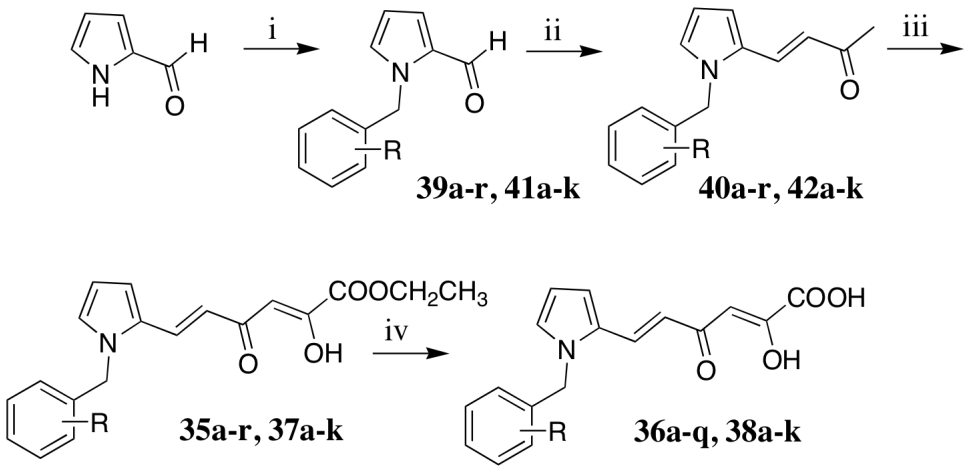
Cpd	R2	R3	R4	R5	R6	X
37a	F	F	H	H	H	Et
37b	Cl	H	Cl	H	H	Et
37c	F	H	F	H	H	Et
37d	CH_3	H	CH_3	H	H	Et
37e	F	H	H	F	H	Et
37f	Cl	H	H	H	Cl	Et
37g	F	H	H	H	F	Et
37h	H	F	F	H	H	Et
37i	H	Cl	H	Cl	H	Et
37j	H	F	H	F	H	Et
37k	H	CH_3	H	CH_3	H	Et

2.3.1 Results and discussion

Scheme 1, reported above, describes the synthesis of derivatives **35a-r, 36a-q 37a-k** and **38a-k.**

The synthesis of compound **35a-r, 36a-q, 37a-k** and **38a-k** (Scheme 1) started from the commercially available pyrrole-2-carboxaldehyde, that was alkylated in alkaline medium (K_2CO_3) with the appropriate benzylbromide to obtain **39a-r** and **41a-k** N-substuted-pyrrole-2-carboxaldehyde. The aldehydes **39a-r** and **41a-k** were converted to the corresponding 3-buten-2-ones **40a-p** and **42a-k** in a condensation reaction with acetone in presence of sodium hydroxyde (NaOH). To afford ethyl esters **35a-r** and **37a-k,** 3-buten-2-ones **40a-p** and **42a-k** was treated with diethyl oxalate utilyzing a weak base like sodium ethoxyde (NaOEt). The hydrolysis of esters function led to the corresonding acids **36a-q** and **38a-k**.

Scheme 1. [a] Synthesis of derivatives **35a-r, 36a-q 37a-k** and **38a-k.**



[a] Reagents and conditions: i: benzyl bromide, K_2CO_3, 110 °C; ii: acetone, NaOH 5 N, 50 °C; iii: diethyl oxalate, sodium ethoxyde, THF_a, rt; iv: NaOH 1 N, 1:1 THF-Methanol, rt.

All synthesized compounds are tested in *in vitro* assay on ricombinant RT and in cell-base assay on MT-4 cells by Prof. Enzo Tramontano of Cagliari University. Table 3 and 4 report the enzyme inhibitory activities (IC_{50}), cell inhibitory activities (EC_{50}), citotoxic concentration (CC_{50}) and selective index (SI). For the evaluation of inhibitory selectivity on RNase H function, compounds **35a-r, 36a-q 37a-k** and **38a-k** are also tested on IN of HIV-1 (Table 5 and 6) by Prof. Yves Pommier of National Institute of Healt in Bethesda, and on RDDP function of RT by Prof. Enzo

Tramontano (data not shown).

Analizing synthesized compounds IC_{50} values on RNase H function, it stands to reason that acids compounds are not always more active then the corresponding esters (Tables 3 and 4). This kind of reactivity is different confronting with the inhibition of IN of HIV-1 and NS5B of HCV by DKAs. Infact, in the series of DKAs inhibitors of IN of HIV-1 (see chapter 1) and NS5B of HCV (see chapter 3) the acids compounds are always more actives then the corresponding esters; furthemore, the IC_{50} values of acids are greater 10- 100-folds then the corresponding values of esters. Infact, when compounds **35a-r, 36a-q 37a-k** and **38a-k** was tested on IN (Tables 5 and 6), its IC_{50} values mantaining this trend.

The most active compounds of these two series (monosubstituted- and disubstituted-*N*-benzyl-pyrrolyldiketohexenoic acids and esters) are compounds **35e, 36o** and **37h**, more active then the lead (**35o).** The first and the last one compound are esters, instead of **36o** that is an acid: its IC_{50} values on RNase H in *in vitro* enzyme assay are comparables (3 μM, 2.5 μM and 3 μM, respectively), while its IC_{50} values on IN in *in vitro* enzyme assay are completely different (32 μM, 0.026 μM, 1.6 μM, respectively) because in this second case the acid compound, **36o,** is more active then the other two. Compound **35e** is more selective for RNase H function, because its IC_{50} value in *in vitro* enzyme assay is >10-folds lower then the IC_{50} value in *in vitro* enzyme assay on IN of HIV-1. Compouns **36o** and **37h** are not selective, because are able to inhibit both RNase H and IN enzymes: compound **36o** is ≈100-folds while compound **37h** is twice-folds more active against IN enzyme.Therefore, in comparison with the lead, the new synthesized compound are more active but less selective. However, each of these compounds shows an EC_{50} value comparable with own IC_{50} value, therefore are able to across the cell membrane and all the synthesized compound does not inhibit the RDDP function of RT (data not shown).

Among orto-benzylsubstituted pyrrolyldiketohexenoic esters and meta-benzylsubstituted pyrrolyldiketohexenoic acids, the activity is correlated with the nature of substituent in the same manner. Infact in both case the activity decrease in the following order:

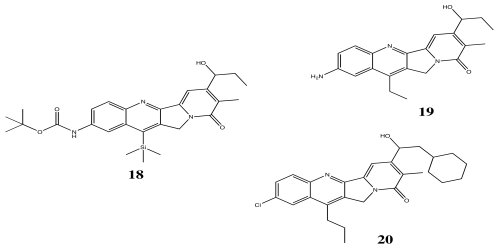
$$CH_3 > Cl, CN, F > OCH_3, OEt$$

Among orto-benzylsubstituted pyrrolyldiketohexenoic acids, the electron-withdrawings substituents (**36b,c,d,** chloro, ciano and fluoro respectively) are the most tolerated then the electron-donors (**36e,f,g,** methyl, ethoxy and methoxy

respectively). This trend is overturn among the para-benzylsubstituted pyrrolyldiketohexenoic esters (Table 3). Among meta-benzylsubstituted pyrrolyldiketohexenoic esters and para-benzylsubstituted pyrrolyldiketohexenoic acids, the inhibitory activity are not well correlated with the nature of substituent.

The modifications brought to disubstituted-N-benzyl-pyrrolyldiketohexenoic esters and acids do not give the whole picture for the structure-activity relationships (SAR), because substituents with differents electron effects and/or polarity was not bound in every position (Table 4). At this time are take into consideration chloro, fluoro and methyl groups in 2,4- and 3,5-disubstitution and chloro and fluoro groups in 2,6-disubstitution. Only fluorine atom was also bound in 2,3- and 3,4-positions.

Table 3. Inhibition of RNase H, enzymatic activities, cell activity, citotoxicity and selective index of compound **35a-r** and **36a-q.**



Cpd	R2	R3	R4	X	IC_{50} (μM) RH[a]	EC_{50} (μM)[b]	Cititoxicity[c]	SI[d]
35a	H	H	H	Et	-	0.25	>50	>200
36a	H	H	H	H	15	0.35	>50	>142
35b	Cl	H	H	Et	9.8	3.00	>50	>16
36b	Cl	H	H	H	5.0	1.3	>50	>38
35c	CN	H	H	Et	32	4.50	32	>7
36c	CN	H	H	H	6	21.00	>50	>2
35d	F	H	H	Et	6.3	0.33	>50	>152
36d	F	H	H	H	6.4	0.35	>50	>142
35e	CH_3	H	H	Et	3.0	1.4	>50	>35
36e	CH_3	H	H	H	26	3.7	>50	>13
35f	OC_2H_5	H	H	Et	> 100 (56%)	0.6	40	66.6
36f	OC_2H_5	H	H	H	64	0.055	>50	>909
35g	OCH_3	H	H	Et	> 100 (55%)	0.2	46	124
36g	OCH_3	H	H	H	16	0.057	>50	>877
35h	H	Cl	H	Et	19	0.79	>50	>63
36h	H	Cl	H	H	9	11.00	>50	>4
35i	H	CN	H	Et	5	7.1	>50	>7
36i	H	CN	H	H	5	>50	>50	-
35j	H	F	H	Et	9.0	2.2	>50	>21
36j	H	F	H	H	14	3.80	>50	>13
35k	H	Me	H	Et	9.6	2.8	>50	>18
36k	H	Me	H	H	4.6	1.9	>50	>26
35l	H	OCH_3	H	Et	20	0.35	>50	>142
36l	H	OCH_3	H	H	> 100 (69%)	0.61	>50	>82
35m	H	H	Cl	Et	8.0	26.3	>50	>2
36m	H	H	Cl	H	5.0	>50	>50	-

35n	H	H	CN	Et	8.0	>50	>50	-
36n	H	H	CN	H	6.0	8.00	>50	>6
35o	H	H	F	Et	13.0	<0.2	>50	>250
36o	H	H	F	H	2.5	0.63	>50	>80
35p	H	H	CH_3	Et	7.3	17.4	>50	>2
36p	H	H	CH_3	H	17.0	>50	>50	-
35q	H	H	OCH_3	Et	6.7	>50	>50	-
36q	H	H	OCH_3	H	3.0	17.4	>50	>2
35r	H	H	NO_2	Et	12.0			

[a] = compound concentrations neaded for reduce to 50% the activity of RnaseH;

[b] = effective concentration 50% on HIV-1 infected cell;

[c] = citotoxic concentration 50%;

[d] = selective index = CC_{50}/EC_{50};

Table 4. Inhibition of RNase H, enzymatic activities, cell activity, citotoxicity and selective index of compound **37a-k** and **38a-k.**

Cpd	R2	R3	R4	R5	R6	X	IC_{50} (μM) RH[a]	EC_{50} (μM)[b]	Cititoxicity[c]	SI[d]
37a	F	F	H	H	H	Et	8	<0.2	>50	>250
38a	F	F	H	H	H	H	6	0.39	>50	>128
37b	Cl	H	Cl	H	H	Et	9	>50	38	-
38b	Cl	H	Cl	H	H	H	7	16.2	>50	>2
37c	F	H	F	H	H	Et	19	<0.2	50	>250
38c	F	H	F	H	H	H	10	0.5	>50	>100
37d	CH_3	H	CH_3	H	H	Et	13	2.5	>50	>20
38d	CH_3	H	CH_3	H	H	H	100	17	30	-
37e	F	H	H	F	H	Et	9	0.81	>50	>60
38e	F	H	H	F	H	H	5	0.93	>50	>54
37f	Cl	H	H	H	Cl	Et	22	2.5	27	-
38f	Cl	H	H	H	Cl	H	28	1.4	42	16
37g	F	H	H	H	F	Et	19	0.095	>50	>526
38g	F	H	H	H	F	H	50	0.022	>50	>2273
37h	H	F	F	H	H	Et	3	2	>50	>25
38h	H	F	F	H	H	H	86	0.046	>50	>1087
37i	H	Cl	H	Cl	H	Et	-	3	>50	>16.6
38i	H	Cl	H	Cl	H	H	30	30.00	>50	>1.7
37j	H	F	H	F	H	Et	5	3.9	>50	>7
38j	H	F	H	F	H	H	14	1.7	22	>10
37k	H	CH_3	H	CH_3	H	Et	6	11.00	>50	>4
38k	H	CH_3	H	CH_3	H	H	16	5.6	>50	>8

[a] = compound concentrations neaded for reduce to 50% the activity of RnaseH;

[b] = effective concentration 50% on HIV-1 infected cell;

[c] = citotoxic concentration 50%;

[d] = selective index = CC_{50}/EC_{50};

Table 5. Inhibition of IN (ST) of compound **35a-r** and **36a-q.**

Cpd	R2	R3	R4	X	IC_{50} (μM) IN[a]
35a	H	H	H	Et	15
36a	H	H	H	H	0.09
35b	Cl	H	H	Et	8.0

36b	Cl	H	H	H	4.0
35c	CN	H	H	Et	9.00
36c	CN	H	H	H	6
35d	F	H	H	Et	0.98
36d	F	H	H	H	0.98
35e	CH_3	H	H	Et	32
36e	CH_3	H	H	H	0.17
35f	OC_2H_5	H	H	Et	12
36f	OC_2H_5	H	H	H	0.31
35g	OCH_3	H	H	Et	23
36g	OCH_3	H	H	H	0.53
35h	H	Cl	H	Et	6
36h	H	Cl	H	H	0.31
35i	H	CN	H	Et	-
36i	H	CN	H	H	0.75
35j	H	F	H	Et	11
36j	H	F	H	H	0.92
35k	H	Me	H	Et	8.0
36k	H	Me	H	H	1.3
35l	H	OCH_3	H	Et	60
36l	H	OCH_3	H	H	0.49
35m	H	H	Cl	Et	42
36m	H	H	Cl	H	>4.1
35n	H	H	CN	Et	>111
36n	H	H	CN	H	1.7
35o	H	H	F	Et	98
36o	H	H	F	H	0.026
35p	H	H	CH_3	Et	>111
36p	H	H	CH_3	H	1.2
35q	H	H	NO_2	Et	-
35r	H	H	OCH_3	Et	110
36r	H	H	OCH_3	H	4.1

[a] = inhibitory concentrations 50% on ST determined by a dose-response curve.

Table 6. Inhibition of IN of compound **37a-k** and **38a-k.**

Cpd	R2	R3	R4	R5	R6	X	IC_{50} (μM) IN[a]
37a	F	F	H	H	H	Et	0.53
38a	F	F	H	H	H	H	0.059
37b	Cl	H	Cl	H	H	Et	>111
38b	Cl	H	Cl	H	H	H	4.9
37c	F	H	F	H	H	Et	1.00
38c	F	H	F	H	H	H	0.042
37d	CH_3	H	CH_3	H	H	Et	>111
38d	CH_3	H	CH_3	H	H	H	4.40
37e	F	H	H	F	H	Et	0.45
38e	F	H	H	F	H	H	0.052
37f	Cl	H	H	H	Cl	Et	1.70
38f	Cl	H	H	H	Cl	H	0.17
37g	F	H	H	H	F	Et	4.00
38g	F	H	H	H	F	H	0.15
37h	H	F	F	H	H	Et	0.60
38h	H	F	F	H	H	H	1.60
37i	H	Cl	H	Cl	H	Et	8.0
38i	H	Cl	H	Cl	H	H	0.97
37j	H	F	H	F	H	Et	0.49
38j	H	F	H	F	H	H	1.2
37k	H	CH_3	H	CH_3	H	Et	19
38k	H	CH_3	H	CH_3	H	H	1.6

[a] = inhibitory concentrations 50% on ST determined by a dose-response curve.

2.3.2 Conclusion

The high incidence of resistance in therapy-experienced and newly infected patients and the adverse effects of the existing treatments underscore the need for new HIV-1 inhibitors. The RT polymerase and RNase H activities as well as the IN 3'-end processing and strand transfer activities all require divalent cations and are essential for viral replication. While several inhibitors of RT polymerase activity are in the clinic and inhibitors of IN strand transfer activity have been tested in clinical trials, RNase H inhibitors are only at the earlier steps of the drug discovery pipeline.

The DKA represents a significant class of RNase H inhibitors of HIV-1. Infact these compound are able to chelate divalent metal ions which are presented in the active site of RNase H function. The idea of the possible activity of DKA on RNase H function born from the known activity of these compounds on IN enzyme of HIV-1, which belonges to the same enzymatic class of RNase H enzyme, the superfamily of polynucleotidyl transferases within high similarity in the catalytic site. Infact, in the active site of both enzyme are presented two metal ions, such as Mg^{2+} and the chelation of these bring to an inhibition of the catalytic activity. In *in vitro* test the α,γ-diketoacid mojety has dimonstred the ability to compete for the bond to the active site with native substrate because are able to form a ligand-metal-enzyme complex.

The work presented in this second chapter reguards the designed, synthesis and biological evaluation of new pyrrolyldiketohexenoic esters and acids **35a-r, 36a-q 37a-k** and **38a-k,** in own inhibitory activity on RT and IN of HIV-1, starting from compound **34** that represent our lead compoumd. Compounds **35e, 36o** and **37h** are the most active synthesized, more then lead compound (**32** or **35o)**, but only compound **35e** shows a selectivity on RNase H.

From these initial data, the optimization of the pyrrolyl-derivatives structure is the base for future studies to develop more potent and selective inhibitors of RNase H function.

2.3.3 Experimental section

Chemistry

Melting points were determined with a Büchi 530 capillary apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Spectrum-one spectrophotometer. ^{1}H NMR spectra were recorded on a bruker AC 400 specrtometer. Merk silica gel 60 F_{254} plates were used for analytical TLC. Column chromatogrephies were performed on silica gel (Merck; 70-230 mesh) or alumina (Merck; 70-230 mesh). Concentration of solution after reactions and extractions involved the use of a rotary evaporator operating at reduced pressure of approximately 20 Torr. Analytical results agreed to within ±0.40% of the theoretical values.

General procedure for the synthesis of N-**[(phenyl)metyl]pyrrole-2-carboxaldehyde 39a-r and 41a-k**. A solution of pyrrole-2-carboxaldehyde (0.0092 mol) in 20 mL of dry DMF was treated with K_2CO_3 (0.0147 mol). Than was added the appropriate benzyl alogenure (0.011 mol) and was stirred with bascular stirring in Büchi Syncore for 1 night at 110 °C. The solution was diluted with water and the solid that formed was filtered on flash vac. The solid was washed with water and light petroleum ether to give derivatives **39a-r** and **41a-k**. Chemical, physical, and analytical data of derivatives **39a-r** and **41a-k** are reported in Table 4.

General procedure for the synthesis of 4-(pyrrol-3-yl)but-3-en-2-one 40a-r and 42a-k. A solution of appropriate aldehyde **39a-r** or **40a-k** (0.0311 mol) in 105.7 mL of acetone was treated with 45.2 mL of NaOH 5 N and stirred at 50 °C for 2 night and then was treated with water. The mixture was extracted with ethyl acetate. The collected organic extract was washed with brine (three times), and dried, and the solvent was evaporated under reduced pressure to obtain pure derivatives **40a-r** and **42a-k**. Chemical, physical, and analytical data of derivatives **40a-r** and **42a-k** are reported in Table 4.

General procedure for the synthesis of Diketo Esters 35a-r and 37a-k. The appropriated acetyl derivative **40a-r** or **42a-k** (0.031 mol) and diethyl oxalate (0.062 mol) were dissolved in 31 mL of dry THF and treated, under argon stream, with

NaOEt obtained by the dissolution of Na (0.063 mol) in 56.36 mL of absolute ethanol. The mixture was stirred at room temperature for 1 h 30 min, then was pured into *n*-hexane (704 mL). The collected precipitate was vigorously stirred for 30 min in 1 N HCl (704 mL). The solid that formed was filtered, washed with water and light petroleum ether and dried under IR lamp to efford the pure diketo ester **35a-r** and **37a-k.** Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR, and analytical data are reported for each of the following compounds.

6-[1-(Phenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35a). 68%; 79-81 °C; ligroina; IR: ν 3400 (OH), 1732 ($COOC_2H_5$), 1621 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.38-1.45 (t, 3H, CH_3), 4.33-4.43 (q, 2H, CH_2), 5.27 (s, 2H, CH_2), 6.33-6.43 (m, 3H, pyrrole β, hexanoate C3-H and hexanoate C5-H), 6.90-6.97 (m, 1H, pyrrole β), 7.11 (m, 1H, pyrrole α), 7.30-7.38 (m, 5H, benzene H), 7.64-7.72 (d, 1H, J_t = 15.0, hexanoate C6-H).

6-[1-(2-Chlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35b). 100%; 98-99 °C; ligroina; IR: ν 3400 (OH), 1720 ($COOC_2H_5$), 1600 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.35-1.38 (t, 3H, CH_3), 4.34-4.40 (q, 2H, CH_2), 5.33 (s, 2H, CH_2), 6.33-6.40 (m, 3H, pyrrole β, hexanoate C3-H and hexanoate C5-H), 6.64 (m, 1H, pyrrole β), 6.85-6.92 (m, 2H, benzene H and pyrrole α), 7.13-7.27 (m, 2H, benzene 2H), 7.44-7.48 (m, 1H, benzene H), 7.56-7.60 (d, 1H, J_t = 15.0, hexanoate C6-H).

6-[1-(2-Cianophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35c). 80%; 179-180 °C; benzene/cyclohexane; IR: ν 3400 (OH), 2227 (CN), 1746 ($COOC_2H_5$), 1578 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.42-1.45 (t, 3H, CH_3), 4.38-4.43 (q, 2H, CH_2), 5.52 (s, 2H, CH_2), 6.41-6.46 (m, 3H, pyrrole β, hexanoate C3-H and hexanoate C5-H), 6.82-6.84 (d, 1H, benzene H), 6.94-6.95 (m, 1H, pyrrole β) 7.01-7.02 (m, 1H, pyrrole α), 7.44-7.48 (m, 1H, benzene H), 7.56-7.60 (m, 2H, benzene H and hexanoate C6-H), 7.77-7.79 (m, 1H, benzene H).

6-[1-(2-Fluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35d). 87%; 103-105 °C; benzene; IR: ν 3400 (OH), 1731 ($COOC_2H_5$), 1680

(CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.41-1.46 (t, 3H, CH_3), 4.38-4.44 (q, 2H, CH_2), 5.34 (s, 2H, CH_2), 6.36-6.38 (t, 1H, pyrrole β), 6.41-6.46 (m, 2H, J_t = 15.2, hexanoate C5-H and hexanoate C3-H), 6.86-6.92 (m, 2H, benzene 1H, pyrrole β), 7.00 (s, 1H, pyrrole α), 7.12-7.18 (m, 2H, benzene 2H), 7.32-7.37 (m, 1H benzene H), 7.71-7.75 (d, 1H, J_t = 15.2, hexanoate C6-H).

6-[1-(2-Methylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35e). 89%; 104-105 °C; benzene/cyclohexane; IR: ν 3400 (OH), 1720 ($COOC_2H_5$), 1610 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.41-1.46 (t, 3H, CH_3), 2.36 (s, 1H, CH_3), 4.38-4.44 (q, 2H, CH_2), 5.22 (s, 2H, CH_2), 6.27-6.45 (m, 3H, J_t = 15.3, hexanoate C5-H, hexanoate C3-H and pyrrole β), 6.69-6.72 (m, 3H, benzene 1H, pyrrole α, β), 7.16-7.31 (m, 3H, benzene 3H), 7.61-7.69 (d, 1H, J_t = 15.3, hexanoate C6-H).

6-[1-(2-Ethoxyphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35f). 85%; 73-75 °C; benzene/cyclohexane; IR: ν 3400 (OH), 1731 ($COOC_2H_5$), 1680 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.38-1.37 (t, 3H, CH_3), 1.45-1.46 (t, 3H, CH_3), 4.11-4.13 (q, 2H, CH_2), 4.33-4.37 (q, 2H, CH_2), 5.25 (s, 2H, CH_2), 6.26-6.28 (t, 1H, pyrrole β), 6.33 (s, 1H, hexanoate C3-H), 6.33-6.37 (d, 1H, J_t = 15.3, hexanoate C5-H), 6.83-6.89 (m, 3H, benzene 2H, pyrrole β), 6.97 (s, 1H, pyrrole α), 7.21-7.23 (d, 1H, benzene H), 7.71-7.74 (d, 1H, J_t = 15.4, hexanoate C6-H).

6-[1-(2-Methoxyphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35g). 100%; 116-118 °C; benzene/cyclohexane; IR: ν 3400 (OH), 1730 ($COOC_2H_5$), 1620 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.38-1.37 (t, 3H, CH_3), 3.90 (s, 3H, CH_3), 4.31-4.37 (q, 2H, CH_2), 5.21 (s, 2H, CH_2), 6.26-6.28 (t, 1H, pyrrole β), 6.32-6.36 (d, 1H, J_t = 15.3, hexanoate C5-H), 6.37 (s, 1H, hexanoate C3-H), 6.72-6.74 (m, 1H, pyrrole β), 6.83-6.94 (m, 4H, benzene 2H, pyrrole β and pyrrole α), 7.24-7.28 (m, 1H, benzene H), 7.72-7.76 (d, 1H, J_t = 15.4, hexanoate C6-H).

6-[1-(3-Chlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35h). 75%; 93-95 °C; ligroina; IR: ν 3400 (OH), 1721 ($COOC_2H_5$), 1574

(CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.34-1.41 (t, 3H, CH_3), 4.29-4.40 (q, 2H, CH_2), 5.21 (s, 2H, CH_2), 6.32-6.40 (m, 3H, pyrrole β, hexanoate C3-H and hexanoate C5-H), 6.86-7.01 (m, 4H, benzene 2H and pyrrole α, β), 7.25-7.28 (m, 2H, benzene 2H), 7.54-7.62 (d, 1H, J_t = 15.3 hexanoate C6-H).

6-[1-(3-Cianophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35i). 83%; 105-106 °C; acetone; IR: ν 3400 (OH), 2227 (CN), 1746 ($COOC_2H_5$), 1578 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.80-1.84 (t, 3H, CH_3), 4.77-4.82 (q, 2H, CH_2), 6.11 (s, 2H, CH_2), 6.87 (m, 1H, pyrrole β), 6.92 (s, 1H, hexanoate C3-H), 7.13-7.17 (d, 1H, J_t = 15.5, hexanoate C5-H), 7.55 (m, 1H, pyrrole α), 7.18 (s, 1H, pyrrole β) 7.95-8.12 (m, 3H, benzene 3H), 8.17-8.21 (m, 2H, benzene H, J_t = 15.4 hexanoate C6-H).

6-[1-(3-Fluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35j). 76%; 84-86 °C; ligroina; IR: ν 3400 (OH), 1700 ($COOC_2H_5$), 1600 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.35-1.38 (t, 3H, CH_3), 4.30-4.36 (q, 2H, CH_2), 5.25 (s, 2H, CH_2), 6.33-6.41 (m, 3H, pyrrole β, hexanoate C3-H and hexanoate C5-H), 6.71 (s, 1H, pyrrole β), 6.82-7.06 (m, 4H, benzene 3H and pyrrole α), 7.27-7.38 (m, 1H, benzene H), 7.53-7.61 (d, 1H, J_t = 15.4 hexanoate C6-H).

6-[1-(3-Methylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35k). 71%; 76-78 °C; cyclohexane; IR: ν 3400 (OH), 1700 ($COOC_2H_5$), 1590 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.35-1.38 (t, 3H, CH_3), 2.32 (s, 3H, CH_3), 4.30-4.36 (q, 2H, CH_2), 5.19 (s, 2H, CH_2), 6.32-6.40 (m, 3H, pyrrole β, hexanoate C3-H and hexanoate C5-H), 6.38-7.26 (m, 6H, benzene 4 H, pyrrole α, β), 7.61-7.65 (d, 1H, J_t = 15.3 hexanoate C6-H).

6-[1-(3-Methoxyphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35l). 100%; 80-82 °C; ligroina; IR: ν 3400 (OH), 1729 ($COOC_2H_5$), 1585 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.35-1.38 (t, 3H, CH_3), 3.76 (s, 3H, CH_3), 4.30-4.36 (q, 2H, CH_2), 5.20 (s, 2H, CH_2), 6.30 (s, 1H, pyrrole β), 6.34-6.37 (m, 2H, hexanoate C3-H and hexanoate C5-H), 6.56-6.68 (m, 2H, benzene H), 6.80-6.85 (m, 2H, benzene H and pyrrole β), 6.92 (s, 1H, pyrrole α), 7.21-7.28 (m, 1H, benzene H),

7.61-7.65 (d, 1H, J_t = 15.3 hexanoate C6-H).

6-[1-(4-Chlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35m). 60%; 134-136 °C; ligroina; IR: ν 3400 (OH), 1700 ($COOC_2H_5$), 1600 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.37-1.46 (t, 3H, CH_3), 4.33-4.40 (q, 2H, CH_2), 5.24 (s, 2H, CH_2), 6.35-6.43 (m, 3H, hexanoate C3-H, hexanoate C5-H and pyrrole β), 6.88-7.04 (m, 4H, benzene 2H and pyrrole α, β), 7.30-7.36 (m, 2H, benzene 2H), 7.58-7.66 (m, 1H, J_t = 15.4, hexanoate C6-H).

6-[1-(4-Cianophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35n). 82%; 128-130 °C; ligroina; IR: ν 3400 (OH), 2227 (CN), 1746 ($COOC_2H_5$), 1578 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.80-1.84 (t, 3H, CH_3), 4.77-4.82 (q, 2H, CH_2), 6.11 (s, 2H, CH_2), 6.87 (m, 1H, pyrrole β), 6.92 (s, 1H, hexanoate C3-H), 7.13-7.17 (d, 1H, J_t = 15.5, hexanoate C5-H), 7.55 (m, 1H, pyrrole α), 7.18 (s, 1H, pyrrole β), 7.95-8.12 (m, 3H, benzene H), 8.17-8.21 (m, 2H, benzene H, J_t = 15.4, hexanoate C6-H).

6-[1-(4-Fluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35o). 78%; 96-97 °C; ligroina; IR: ν 3400 (OH), 1698 ($COOC_2H_5$), 1605 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.38-1.46 (t, 3H, CH_3), 4.37-4.41 (q, 2H, CH_2), 5.25 (s, 2H, CH_2), 6.36-6.44 (m, 3H, J_t = 15.5, pyrrole β, hexanoate C3-H, hexanoate C5-H), 6.80 (m, 1H, pyrrole β), 6.96 (m, 1H, pyrrole α), 7.05-7.09 (m, 4H, benzene H), 7.65-7.73 (d, 1H, J_t = 15.5, hexanoate C6-H).

6-[1-(4-Methylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35p). 60%; 85-86 °C; *n*-hexane; IR: ν 3400 (OH), 1721 ($COOC_2H_5$), 1605 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.38-1.45 (t, 3H, CH_3), 2.36 (s, 3H, CH_3), 4.33-4.44 (q, 2H, CH_2), 5.23 (s, 2H, CH_2), 6.32-6.43 (m, 3H, J_t = 15.4, pyrrole β, hexanoate C3-H, hexanoate C5-H), 6.90-6.96 (m, 2H, pyrrole α, β), 6.98-7.02 (d, 2H, benzene H), 7.16-7.20 (m, 2H, benzene H), 7.65-7.73 (d, 1H, J_t = 15.4, hexanoate C6-H).

6-[1-(4-Methoxyphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35q). 64%; 84-86 °C; ethanol; IR: ν 3400 (OH), 1701 ($COOC_2H_5$), 1598 (CO)

cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.38-1.45 (t, 3H, CH_3), 3.82 (s, 3H, CH_3), 4.33-4.44 (q, 2H, CH_2), 5.20 (s, 2H, CH_2), 6.32-6.43 (m, 3H, J_t = 15.4, pyrrole β, hexanoate C3-H, hexanoate C5-H), 6.86-7.08 (m, 6H, benzene 4H and pyrrole α, β), 7.67-7.75 (d, 1H, J_t = 15.4, hexanoate C6-H).

6-[1-(4-Nitrophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (35r). 61%; 61-63 °C; ligroina; IR: ν 3400 (OH), 1700 ($COOC_2H_5$), 1580 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.37-1.44 (t, 3H, CH_3), 4.33-4.43 (q, 2H, CH_2), 5.39 (s, 2H, CH_2), 6.37-6.45 (m, 3H, pyrrole β, hexanoate C3-H, hexanoate C5-H), 6.90-7.10 (m, 2H, pyrrole α, β), 7.18-8.30 (m, 2H, benzene H), 7.50 (d, 1H, hexanoate C6-H), 8.21-8.25 (m, 2H, benzene H).

6-[1-(2,3-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37a). 79%; 67-69 °C; acetone; IR: ν 3400 (OH), 1727 ($COOC_2H_5$), 1611 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.79-1.81 (t, 3H, CH_3), 4.76-4.78 (q, 2H, CH_2), 6.05 (s, 2H, CH_2), 6.81-6.87 (m, 2H, pyrrole β and hexanoate C3-H), 7.11-7.26 (m, 2H, pyrrole α and J_t = 14.9 hexanoate C5-H), 7.48 (s, 1H, pyrrole β), 7.61-7.84 (m, 3H, benzene 3H), 8.19-8.27 (d, 1H, J_t = 14.9 hexanoate C6-H).

6-[1-(2,4-Dichlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37b). 100%; 110-113 °C; benzene/cyclohexane; IR: ν 3400 (OH), 1773 ($COOC_2H_5$), 1610 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.28-1.31 (t, 3H, CH_3), 4.22-4.28 (q, 2H, CH_2), 5.48 (s, 2H, CH_2), 6.35-6.36 (m, 2H, pyrrole β and hexanoate C3-H), 6.51-6.53 (d, 1H, benzene H), 6.76-6.78 (m, 1H, hexanoate C5-H), 7.02 (s, 1H, pyrrole β), 7.26 (s, 1H, pyrrole α) 7.41-7.47 (m, 2H, benzene H and hexanoate C6-H), 7.74-7.75 d, 1H, benzene H).

6-[1-(2,4-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37c). 82%; 119-121 °C; ligroina; IR: ν 3400 (OH), 1737 ($COOC_2H_5$), 1601 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.42-1.48 (t, 3H, CH_3), 4.38-4.40 (q, 2H, CH_2), 5.29 (s, 2H, CH_2), 6.36-6.38 (m, 1H, pyrrole β), 6.41-6.46 (m, 2H, hexanoate C5-H and hexanoate C3-H), 6.85-6.99 (m, 5H, pyrrole α, pyrrole β and benzene 3H) 7.68-7.62 (d, 1H, J_t = 15.27 hexanoate C6-H).

6-[1-(2,4-Dimethylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37d). 98%; 102-104 °C; benzene/cyclohexane; IR: ν 3400 (OH), 1747 (COO C_2H_5), 1711 (CO) cm^{-1}; ^1H NMR (DMF): δ 1.42-1.46 (t, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 4.38-4.43 (q, 2H, CH_2), 5.21 (s, 2H, CH_2), 6.33-6.34 (m, 1H, pyrrole β), 6.40-6.44 (m, 2H, J_t = 15.3 hexanoate C5-H and hexanoate C3-H), 6.65-6.67 (d, 1H, benzene H), 6.81-6.82 (m, 1H, pyrrole β), 6.92-6.93 (m, 1H, pyrrole α), 7.00-7.02 (d, 1H, benzene H), 7.09 (s, 1H, benzene H), 7.68-7.72 (d, 1H, J_t = 15.3 hexanoate C6-H,), 12-18 (sb, 1H, COOH).

6-[1-(2,5-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37e). 82%; 84-85 °C; acetone; IR: ν 3400 (OH), 1724 ($COOC_2H_5$), 1618 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.81-1.85 (t, 3H, CH_3), 4.77-4.83 (q, 2H, CH_2), 6.04 (s, 2H, CH_2), 6.86 (s, 1H, pyrrole β) 6.96 (s, 1H, hexanoate C3-H), 7.01-7.19 (m, 2H, pyrrole α and J_t = 15.3 hexanoate C5-H), 7.55 (s, 1H, pyrrole β), 7.64-7.78 (m, 3H, benzene 3H), 8.24-8.28 (d, 1H, J_t = 15.3 hexanoate C6-H).

6-[1-(2,6-Dichlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37f). 69%; 146-148 °C; ligroina; IR: ν 3400 (OH), 1730 ($COOC_2H_5$), 1578 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.42-1.47 (t, 3H, CH_3), 4.40-4.45 (q, 2H, CH_2), 5.51 (s, 2H, CH_2), 6.26-6.28 (m, 1H, pyrrole β), 6.49-6.53 (m, 2H, J_t = 15.24 hexanoate C5-H and hexanoate C3-H), 6.64 (s, 1H, pyrrole β), 6.89-6.90 (m, 1H, pyrrole α), 7.35-7.37 (t, 1H, benzene H), 7.45-7.47 (d, 2H, benzene 2H), 7.96-8.00 (d, 1H, J_t = 15.24 hexanoate C6-H).

6-[1-(2,6-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37g). 50%; 125-127 °C; ligroina; IR: ν 3400 (OH), 1710 ($COOC_2H_5$), 1610 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.40-1.44 (t, 3H, CH_3), 4.32-4.42 (q, 2H, CH_2), 5.27 (s, 2H, CH_2), 6.25-6.26 (m, 1H, pyrrole β), 6.37-6-45 (d, 1H, J_t = 15.2 hexanoate C5-H), 6.48 (s, 1H, hexanoate C3-H), 6.80 (m, 1H, pyrrole β), 6.91-7.01 (m, 3H, benzene 2H and pyrrole α), 7.27-7.40 (m, 1H, benzene H), 7.91-7.99 (d, 1H, J_t = 15.2 hexanoate C6-H).

6-[1-(3,4-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37h). 60%; 74-75 °C; acetone; IR: ν 3400 (OH), 1737 ($COOC_2H_5$), 1633 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.81-1.84 (t, 3H, CH_3), 4.77-4.82 (q, 2H, CH_2), 6.01 (s, 2H, CH_2), 6.84-6.85 (m, 1H, pyrrole β) 6.93 (s, 1H, hexanoate C3-H), 7.12-7.16 (d, 1H, J_t = 15.4 hexanoate C5-H), 7.51-7.63 (m, 3H, benzene 2H and pyrrole β), 7.78-7.86 (m, 2H, benzene H and pyrrole α) 8.18-8.22 (d, 1H, J_t = 15.4 hexanoate C6-H).

6-[1-(3,5-Dichlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37i). 62%; 112-113 °C; ligroina; IR: ν 3400 (OH), 1723 ($COOC_2H_5$), 1646 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.35-1.42 (t, 3H, CH_3), 4.33-4.37 (q, 2H, CH_2), 5.20 (s, 2H, CH_2), 6.35-6.42 (m, 3H, pyrrole β, hexanoate C3-H and hexanoate C5-H), 6.88-6.91 (m, 4H, benzene 3H and pyrrole β), 7.27-7.29 (m, 1H, pyrrole α), 7.49-7.57 (d, 1H, J_t = 15.3 hexanoate C6-H).

6-[1-(3,5-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37j). 58%; 75-78 °C; ligroina; IR: ν 3400 (OH), 1735 ($COOC_2H_5$), 1630 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.42-1.48 (t, 3H, CH_3), 4.37-4.43 (q, 2H, CH_2), 5.28 (s, 2H, CH_2), 6.40-6.45 (m, 3H, pyrrole β, hexanoate C3-H and hexanoate C5-H), 6.57-6.59 (m, 2H, pyrrole α and pyrrole β), 6.76-6.80 (m, 1H, benzene 1H), 6.92-6.93 (m, 1H, benzene 1H), 6.97-6.98 (m, 1H, benzene H) 7.55-7.59 (d, 1H, J_t = 15.29 hexanoate C6-H).

6-[1-(3,5-Dimethylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (37k). 46%; 99-101 °C; ligroina; IR: ν 3400 (OH), 1729 ($COOC_2H_5$), 1616 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.35-1.42 (t, 3H, CH_3), 2.27 (s, 6H, CH_3), 4.31-4.37 (q, 2H, CH_2), 5.14 (s, 2H, CH_2), 6.29-6.32 (m, 1H, pyrrole β), 6.33-6.37 (m, 2H, J_t = 15.1 hexanoate C5-H and hexanoate C3-H), 6.67 (s, 1H, benzene H), 6.84-6.85 (m, 1H, pyrrole β), 6.90-6.91 (m, 2H, pyrrole α and benzene H), 7.64-7.67 (d, 1H, J_t = 15.1 hexanoate C6-H), 12-18 (sb, 1H, COOH).

General procedure for the synthesis of Diketo Acids 36a-q and 38a-k. A mixture of 1 N NaOH (9.48 mL) and the appropriated ester **35a-q** or **37a-k** (0.0019 mol) in

1:1 THF-Methanol (9.32 mL) was stirred at room temperature for 30 min and then pured into crushed ice. The acquous mixture was treated with 1 N HCl until Ph 3 was reached, and extracted with ethyl acetate (three times). The collected organic extract was washed with brine (three times), and dried, and the solvent was evaporated under reduced pressure to give the pure diketo acids **36a-r** and **38a-k.** Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR, and analytical data are reported for each of the following compounds.

6-[1-(Phenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36a). 68%; 146-148 °C; toluene; IR: ν 3400 (OH), 1710 (COOH), 1598 (CO) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 5.43 (s, 2H, CH_2), 6.30-6.33 (m, 1H, pyrrole β), 6.45 (s, 1H, hexanoate C3-H), 6.58-6.67 (d, 1H, J_t = 15.4, hexanoate C5-H), 7.00-7.02 (m, 1H, pyrrole β), 7.08-7.12 (m, 1H, pyrrole α), 7.27-7.40 (m, 5H, benzene H), 7.61-7.79 (d, 1H, J_t = 15.4, hexanoate C6-H).

6-[1-(2-Chlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36b). 61%; 157-159 °C; toluene; IR: ν 3400 (OH), 1688 (COOH), 1588 (CO) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 5.47 (s, 2H, CH_2), 6.30-6.34 (m, 1H, pyrrole β), 6.43 (s, 1H, hexanoate C3-H), 6.52-6.57 (m, 1H, pyrrole β), 6.65-6.70 (d, 1H, J_t = 15.5, hexanoate C5-H), 7.02-7.03 (s, 1H, pyrrole α), 7.25-7.32 (m, 3H, benzene 3H), 7.48-7.58 (m, 2H, J_t = 15.5, benzene H and hexanoate C6-H).

6-[1-(2-Cianophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36c). 58%; 179-180 °C; isopropanole; IR: ν 3400 (OH), 2227 (CN), 1746 (COOH), 1578 (CO) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 5.71 (s, 2H, CH_2), 6.39-6.46 (m, 1H, pyrrole β), 6.53 (s, 1H, hexanoate C3-H), 6.72-6.76 (m, 2H, hexanoate C5-H and benzene H), 7.09 (s, 1H, pyrrole β), 7.31 (s, 1H, pyrrole α), 7.50-7.55 (m, 1H, benzene H), 7.63-7.71 (m, 2H, benzene H and hexanoate C6-H), 7.96-7.94 (m, 1H, benzene H).

6-[1-(2-Fluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36d). 85%; 132-133 °C; benzene/toluene; IR: ν 3400 (OH), 1710 (COOH), 1590 (CO) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 5.46 (s, 2H, CH_2), 6.30-6.34 (m, 1H, pyrrole β), 6.66-6.70 (d, 1H, J_t = 16.02 hexanoate C5-H), 6.87-6.95 (m, 2H, hexanoate C3-H and pyrrole

β), 7.14-7.38 (m, 5H, benzene 4H and pyrrole α), 7.62-7.68 (m, 1H, J_t = 16.02, hexanoate C6-H).

6-[1-(2-Methylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36e). 95%; 146-147 °C; benzene/toluene; IR: ν 2923 (OH), 1700 (COOH), 1580 (CO) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 2.35 (s, 3H, CH_3), 5.39 (s, 2H, CH_2), 6.30-6.43 (m, 3H, pyrrole β and hexanoate C3-H), 6.62-6.69 (d, 1H, J_t = 15.2, hexanoate C5-H), 6.98 (s, 1H, pyrrole β), 7.06-7.25 (m, 5H, benzene 4H and pyrrole α), 7.45-7.52 (d, 1H, J_t = 15.2, hexanoate C6-H), 12-18 (sb, 1H, COOH).

6-[1-(2-Ethoxyphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36f). 80%; 159-160 °C; toluene; IR: ν 2923 (OH), 1731 (COOH), 1601 (CO) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 1.32-1.38 (t, 3H, CH_3), 4.05-4.10 (q, 2H, CH_2), 5.33 (s, 2H, CH_2), 6.23-6.25 (t, 1H, pyrrole β), 6.42 (s, 1H, hexanoate C3-H), 6.60-6.64 (d, 1H, J_t = 15.4, hexanoate C5-H), 6.73-6.75 (d, 1H, benzene H), 6.80-6.85 (t, 1H, bezene H), 6.95 (s, 1H, pyrrole β), 6.98-7.00 (d, 1H, benzene H), 7.20-7.23 (m, 2H, benzene H, pyrrole α), 7.64-7.68 (d, 1H, J_t = 15.4, hexanoate C6-H), 12-18 (sb, 1H, COOH).

6-[1-(2-Methoxyphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36g). 100%; 142-143 °C; toluene; IR: ν 3400 (OH), 1732 (COOH), 1601 (CO) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 3.85 (s, 3H, CH_3), 5.21 (s, 2H, CH_2), 6.25 (m, 1H, pyrrole β), 6.41 (s, 1H, hexanoate C3-H), 6.60-6.64 (d, 1H, J_t = 15.3, hexanoate C5-H), 6.69-7.03 (m, 4H, benzene 3H and pyrrole β), 7.20-7.23 (m, 2H, benzene H, pyrrole α), 7.64-7.68 (d,1H, J_t = 15.3, hexanoate C6-H), 12-18 (sb, 1H, COOH).

6-[1-(3-Chlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36h). 79%; 144-145 °C; toluene/cycloexane; IR: ν 3400 (OH), 1750 (COOH), 1610 (CO) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 5.43 (s, 2H, CH_2), 6.31 (m, 1H, pyrrole β), 6.38 (s, 1H, hexanoate C3-H), 6.62-6.70 (d, 1H, J_t = 15.2, hexanoate C5-H), 6.97-7.40 (m, 6H, benzene 4H and pyrrole β, α), 7.54-7.62 (d, 1H, J_t = 15.3, hexanoate C6-H), 12-18 (sb, 1H, COOH).

6-[1-(3-Cianophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36i). 44%; 126-128 °C; isopropanol/isopropylic ether; IR: ν 3400 (OH), 2231 (CN), 1717 (COOH), 1582 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.52 (s, 2H, CH_2), 6.34 (m, 1H, pyrrole β), 6.46 (s, 1H, hexanoate C3-H), 6.66-6.71 (d, 1H, J_t = 15.3, hexanoate C5-H), 7.01 (s, 1H, pyrrole β), 7.34-7.37 (m, 2H, benzene H), 7.52-7.66 (m, 3H, benzene H, J_t = 15.3 hexanoate C6-H, pyrrole α), 7.76-7.82 (d, 1H, benzene H), 12-18 (sb, 1H, COOH).

6-[1-(3-Fluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36j). 76%; 132-135 °C; toluene; IR: ν 3400 (OH), 1700 (COOH), 1600 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.44 (s, 2H, CH_2), 6.31 (m, 1H, pyrrole β), 6.40 (s, 1H, hexanoate C3-H), 6.60-6.68 (d, 1H, $J_t \approx 15$, hexanoate C5-H), 7.79-7.19 (m, 4H, benzene 4H and pyrrole β), 7.26-7.47 (m, 2H, benzene 2H), 7.58-7.66 (d, 1H, $J_t \approx 15$, hexanoate C6-H), 12-18 (sb, 1H, COOH).

6-[1-(3-Methylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36k). 95%; 121-123 °C; benzene/cyclohexane; IR: ν 3400 (OH), 1700 (COOH), 1590 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.49 (s, 3H, CH_3), 5.33 (s, 2H, CH_2), 6.28 (m, 2H, hexanoate C3-H and pyrrole β), 6.63 (d, 1H, J_t = 15.7, hexanoate C5-H), 6.79-7.34 (m, 6H, benzene 4H, pyrrole α, β), 7.58 (d, 1H, J_t = 15.7, hexanoate C6-H), 12-18 (sb, 1H, COOH).

6-[1-(3-Methoxyphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36l). 100%; 145-147 °C; toluene; IR: ν 3400 (OH), 1713 (COOH), 1614 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 3.70 (s, 3H, CH_3), 5.36 (s, 2H, CH_2), 6.27 (m, 1H, pyrrole β), 6.43 (s, 1H, hexanoate C3-H), 6.60-6.64 (m, 3H, benzene 2H and hexanoate C5-H), 6.82 (m, 1H, benzene H), 6.97 (m, 1H, pyrrole β), 7.22 (m, 1H, benzene 1H), 7.29 (m, 1H, pyrrole α), 7.60-7.66 (d, 1H, J_t = 15.4, hexanoate C6-H), 12-18 (sb, 1H, COOH).

6-[1-(4-Chlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36m). 76%; 170-172 °C; benzene/toluene; IR: ν 3400 (OH), 1685 (COOH), 1548 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.44 (s, 2H, CH_2), 6.36-6.38 (m, 1H, pyrrole β), 6.48 (s,

1H, hexanoate C3-H), 6.69-6.73 (d, 1H, J_t = 15.5, hexanoate C5-H), 7.05 (s, 1H, pyrrole β), 7.08-7.12 (m, 2H, benzene H), 7.32 (m, 1H, pyrrole α), 7.40-7.44 (m, 2H, benzene H), 7.55-7.65 (d, 1H, J_t = 15.5 hexanoate C6-H).

6-[1-(4-Cianophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36n). 93%; >250 °C; washed with isopropylic ether; IR: ν 3400 (OH), 2227 (CN), 1746 (COOH), 1578 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.59 (s, 2H, CH_2), 6.36-6.38 (m, 1H, pyrrole β), 6.48 (s, 1H, hexanoate C3-H), 6.69-6.73 (d, 1H, J_t = 15.4, hexanoate C5-H), 7.05 (s, 1H, pyrrole β), 7.22-7.24 (m, 2H, benzene H), 7.36 (m, 1H, pyrrole α), 7.58-7.62 (d, 1H, J_t = 15.4 hexanoate C6-H), 7.85-7.87 (m, 2H, benzene H,).

6-[1-(4-Fluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36o). 54%; 98-99 °C; benzene/toluene; IR: ν 3400 (OH), 1689 (COOH), 1598 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.38 (s, 2H, CH_2), 6.25 (m, 2H, pyrrole β and hexanoate C3-H), 6.58-6.66 (d, 1H, J_t = 15.6, hexanoate C5-H), 6.93-7.01 (m, 3H, benzene 2H and pyrrole β), 7.13-7.17 (d, 2H, benzene H), 7.26 (s, 1H, pyrrole α), 7.42-7.50 (d, 1H, J_t = 15.6, hexanoate C6-H,), 12-18 (sb, 1H, COOH).

6-[1-(4-Methylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36p). 72%; 149-151 °C; benzene/toluene; IR: ν 3400 (OH), 1705 (COOH), 1575 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.27 (s, 3H, CH_3), 5.34 (s, 2H, CH_2), 6.27 (m, H, pyrrole β) 6.37 (d, 1H, hexanoate C5-H), 6.55 (s, 1H, hexanoate C3-H), 6.90 (m, 1H, pyrrole β), 7.13-7.24 (m, 5H, benzene 4H and pyrrole α), 7.42-7.50 (d, 1H, J_t = 15.6, hexanoate C6-H,), 12-18 (sb, 1H, COOH).

6-[1-(4-Methoxyphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (36q). 64%; >300 °C; washed with isopropylic ether; IR: ν 3400 (OH), 1698 (COOH), 1600 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 3.73 (s, 3H, CH_3), 5.34 (s, 2H, CH_2), 6.27-6.40 (m, 2H, pyrrole β and hexanoate C3-H) 6.62-6.68 (d, 1H, hexanoate C5-H), 6.89-6.93 (m, 3H, benzene 2H and pyrrole β), 7.05-7.09 (d, 2H, benzene H), 7.28 (s, 1H, pyrrole α), 7.62-7.68 (d, 1H, J_t = 15.6, hexanoate C6-H,), 12-18 (sb, 1H, COOH).

6-[1-(2,3-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38a). 47%; 147-148 °C; isopropanole/*n*-hexane; IR: ν 3400 (OH), 1758 (COOH), 1630 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.54 (s, 2H, CH_2), 6.35 (m, 1H, pyrrole β), 6.50 (s, 1H, hexanoate C3-H), 6.69-6.75 (m, 2H, pyrrole β and hexanoate C5-H), 7.03 (s, 1H, pyrrole α), 7.19-7.45 (m, 3H, benzene 3H), 7.70-7.73 (d, 1H, J_t = 15.3, hexanoate C6-H,), 12-18 (sb, 1H, COOH).

6-[1-(2,4-Dichlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38b). 68%; 145-146 °C; toluen; IR: ν 3400 (OH), 1758 (COOH), 1630 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.51 (s, 2H, CH_2), 6.36-6.38 (m, 1H, pyrrole β), 6.47 (s, 1H, hexanoate C3-H), 6.55-6.57 (m, 1H, benzene H), 6.61-6.65 (d, 1H, J_t = 15.32 hexanoate C5-H), 7.07 (s, 1H, pyrrole β), 7.29 (s, 1H, pyrrole α), 7.41-7.44 (m, 1H, benzene 1H), 7.55-7.59 (d, 1H, J_t = 15.32 hexanoate C6-H and), 7.73-7.74 (d, 1H, benzene 1H), 12-18 (sb, 1H, COOH).

6-[1-(2,4-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38c). 67%; 130-132 °C; ethyl acetate; IR: ν 3400 (OH), 1758 (COOH), 1630 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.46 (s, 2H, CH_2), 6.31-6.32 (m, 1H, pyrrole β), 6.50 (s, 1H, hexanoate C3-H), 6.70-6.74 (d, 1H, J_t = 13.68 and hexanoate C5-H), 6.97-7.03 (m, 2H, pyrrole β and benzene H), 7.08-7.13 (m, 1H, benzene H) 7.28-7.37 (m, 2H, pyrrole α and benzene H), 7.67-7.72 (d, 1H, J_t = 13.68 hexanoate C6-H,), 12-18 (sb, 1H, COOH).

6-[1-(2,4-Dimethylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38d). 59%; 151-153°C; toluene; IR: ν 3400 (OH), 1747 (COOH), 1711 (CO) cm^{-1}; ^1H NMR (DMF): δ 2.47 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 5.68 (s, 2H, CH_2), 6.55-6.57 (m, 1H, pyrrole β), 6.69-6.73 (m, 2H, benzene H and hexanoate C3-H), 6.93-6.97 (d, 1H, J_t = 15.7 hexanoate C5-H), 7.16-7.18 (d, 1H, benzene H), 7.28 (s, 1H, benzene H), 7.33 (s, 1H, pyrrole β), 7.42 (s, 1H pyrrole α), 7.92-7.96 (d, 1H, J_t = 15.7 hexanoate C6-H,), 12-18 (sb, 1H, COOH).

6-[1-(2,5-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38e). 51%; 141-143 °C; toluene/*n*-hexane; IR: ν 3400 (OH), 1747 (COOH), 1711

(CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.50 (s, 2H, CH_2), 6.34-6.37 (m, 1H, pyrrole β), 6.51 (s, 1H, hexanoate C3-H), 6.68-6.77 (m, 2H, pyrrole β and hexanoate C5-H), 7.04 (s, 1H, pyrrole α), 7.21-7.39 (m, 3H, benzene 3H), 7.70-7.74 (d, 1H, J_t = 15.2 hexanoate C6-H,), 12-18 (sb, 1H, COOH).

6-[1-(2,6-Dichlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38f). 84%; 132-134 °C; toluene; IR: ν 3400 (OH), 1758 (COOH), 1630 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.55 (s, 2H, CH_2), 6.21-6.25 (m, 1H, pyrrole β), 6.61 (s, 1H, hexanoate C3-H), 6.67 (s, 1H, pyrrole β), 6.79-6.83 (d, 1H, J_t = 15.7 hexanoate C5-H), 7.01 (s, 1H, pyrrole α), 7.50-7.54 (m, 1H, benzene H), 7.63-7.65 (m, 2H, benzene 2H), 7.93-7.96 (d, 1H, J_t = 15.7 hexanoate C6-H), 12-18 (sb, 1H, COOH).

6-[1-(2,6-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38g). 64%; 182-183 °C; toluene; IR: ν 3400 (OH), 1720 (COOH), 1610 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.40 (s, 2H, CH_2), 6.20-6.24 (m, 1H, pyrrole β), 6.50 (s, 1H, hexanoate C3-H), 6.68-72 (d, 1H, J_t = 15.4 hexanoate C5-H), 6.91-6.92 (m, 1H, pyrrole β), 7.10-7.20 (m, 2H, pyrrole α and benzene H), 7.41-7.49 (m, 1H, benzene H), 7.83-7.91 (d, 1H, J_t = 15.4 hexanoate C6-H and), 12-18 (sb, 1H, COOH).

6-[1-(3,4-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38h). 100%; >270 °C; DMF/H_2O; IR: ν 3400 (OH), 1758 (COOH), 1630 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.38 (s, 2H, CH_2), 5.80 (s, 1H, pyrrole β), 6.30 (s, 1H, hexanoate C3-H), 6.53 (m, 1H, hexanoate C5-H), 6.80-6.90 (m, 2H, pyrrole β and benzene H), 7.14-7.60 (m, 4H, benzene 2H, hexanoate C6-H and pyrrole α), 12-18 (sb, 1H, COOH).

6-[1-(3,5-Dichlorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38i). 95%; >300 °C; toluene/cyclohexane; IR: ν 3400 (OH), 1743 (COOH), 1521 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.38 (s, 2H, CH_2), 6.24 (s, 1H, pyrrole β), 6.50-6.58 (m, 1H, hexanoate C5-H), 6.33 (s, 1H, hexanoate C3-H), 7.02-7.35 (m, 5H, benzene 3H, pyrrole β and hexanoate C6-H), 7.50 (s, 1H, pyrrole α), 12-18 (sb, 1H, COOH).

6-[1-(3,5-Difluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38j). 60%; 146-149 °C; washed with ethanol; IR: ν 3400 (OH), 1712 (COOH), 1595 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.29 (s, 2H, CH_2), 6.36 (s, 1H, pyrrole β), 6.47 (s, 1H, hexanoate C3-H), 6.70-6.78 (m, 3H, benzene 2H and hexanoate C5-H), 7.03 (s, 1H, pyrrole β), 7.18-7.22 (m, 1H, benzene 1H), 7.35 (s, 1H, pyrrole α), 7.59-7.62 (d, 1H, hexanoate C6-H and), 12-18 (sb, 1H, COOH).

6-[1-(3,5-Dimethylphenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid (38k). 78%; 145-147 °C; benzene; IR: ν 3400 (OH), 1713 (COOH), 1608 (CO) cm^{-1}; ^1H NMR (DMF): δ 2.18 (s, 6H, CH_3), 5.29 (s, 2H, CH_2), 6.25-6.27 (m, 1H, pyrrole β), 6.44 (s, 1H, hexanoate C3-H), 6.59-6.63 (d, 1H, J_t = 15.4 hexanoate C5-H), 6.68 (s, 1H, benzene H),6.86 (s, 1H, pyrrole β), 6.95-6.96 (d, 1H, benzene H), 7.26 (s, 1H, pyrrole α), 7.59-7.63 (d, 1H, J_t = 15.4 hexanoate C6-H,), 12-18 (sb, 1H, COOH).

In the table 4 are reported the chemical-physical characteristic of the syntetic intermediete **39a-r, 40a-r, 41a-k,** and **42a-k.**

Table 4. Chemical, Physical, and Analytical Data of Derivatives **39a-r, 40a-r, 41a-k,** and **42a-k**

Cpd	R_2	R_3	R_4	R_5	R_6	mp (°C)	recryst solvent[a]	Yeld (%)
39a	H	H	H	H	H	oil	oil	76
39b	Cl	H	H	H	H	81-82	b	100
39c	CN	H	H	H	H	138-140		85
39d	F	H	H	H	H	34-36	b	100
39e	CH_3	H	H	H	H	38-40	b	100
39f	OC_2H_5	H	H	H	H	oil	oil	71
39g	OCH_3	H	H	H	H	56-58	b	100
39h	H	Cl	H	H	H	oil	oil	100
39i	H	CN	H	H	H	98-100	c	70
39j	H	F	H	H	H	oil	oil	100
39k	H	CH_3	H	H	H	oil	oil	82
39l	H	OCH_3	H	H	H	oil	oil	69
39m	H	H	Cl	H	H	78-80	b	96
39n	H	H	CN	H	H	-	-	100
39o	H	H	F	H	H	oil	oil	65
39p	H	H	CH_3	H	H	49-51	b	86
39q	H	H	OCH_3	H	H	oil	oil	77
39r	H	H	NO_2	H	H	110-112	-	52
40a	H	H	H	H	H	oil	oil	100
40b	Cl	H	H	H	H	54-56	a	85

40c	CN	H	H	H	H	87-89	c	75
40d	F	H	H	H	H	46-47	a	92
40e	CH_3	H	H	H	H	76-77	a	58
40f	OC_2H_5	H	H	H	H	81-82	a	47
40g	OCH_3	H	H	H	H	120-122	a	37
40h	H	Cl	H	H	H	78-80	a	63
40i	H	CN	H	H	H	oil	oil	68
40j	H	F	H	H	H	oil	oil	69
40k	H	CH_3	H	H	H	65-67	a	81
40l	H	OCH_3	H	H	H	50-52	b	66
40m	H	H	Cl	H	H	oil	oil	100
40n	H	H	CN	H	H	95-98	c	32
40o	H	H	F	H	H	oil	oil	83
40p	H	H	CH_3	H	H	oil	oil	87
40q	H	H	OCH_3	H	H	oil	oil	100
40r	H	H	NO_2	H	H	90-92	-	32
41a	F	F	H	H	H	oil	oil	71
41b	Cl	H	Cl	H	H	75-77	c	100
41c	F	H	F	H	H	40-41	b	100
41d	CH_3	H	CH_3	H	H	oil	oil	98
41e	F	H	H	F	H	50-52	b	87
41f	Cl	H	H	H	Cl	70-72	b	72
41g	F	H	H	H	F	oil	oil	87
41h	H	F	F	H	H	47-49	b	69
41i	H	Cl	H	Cl	H	61-63	b	100
41j	H	F	H	F	H	56-58	b	100
41k	H	CH_3	H	CH_3	H	oil	oil	52
42a	F	F	H	H	H	oil	oil	81
42b	Cl	H	Cl	H	H	88-90	c	58
42c	F	H	F	H	H	63-65	c	28
42d	CH_3	H	CH_3	H	H	79-80	c	84
42e	F	H	H	F	H	oil	oil	72
42f	Cl	H	H	H	Cl	89-90	c	47
42g	F	H	H	H	F	86-88	a	100
42h	H	F	F	H	H	oil	oil	87
42i	H	Cl	H	Cl	H	75-77	a	85
42j	H	F	H	F	H	62-64	c	35
42k	H	CH_3	H	CH_3	H	76-78	b	72

[a] Recrystallization solvent: (a) ligroina; (b) *n*-hexane; (c) cyclohexan

Biology

HIV-1 rRT purification

Heterodimeric rRT was expressed essentially as described. Briefly, *E. coli* strain M15 containing the P6HRT-prot vector were grown up to an OD600 of 0.8 and induced with IPTG 1.7 mM for 5 h. Protein purification was carried out with an AKTA-prime FPLC (Amersham Biosciences) with a Ni^{2+}-sepharose chromatography. Briefly, cell pellets were resuspended in Lyses buffer (20mM Hepes pH 7.5, 0.5 M NaCl, 5mM β-mercaptoethanol, 5mM imidazole, 0.4 mg/mL lysozime), incubated on ice for 20 min, sonicated and centrifuged at 30,000 X *g* for 1 h. The supernatant was applied to a His-binding resin column and washed thoroughly withWash buffer (20 mM Hepes pH 7.5, 0.3 M NaCl, 5 mM β-mercaptoethanol, 60 mM imidazole, 10% glycerol). rRT was gradient-eluted with Elute buffer (Wash buffer with 0.5 M imidazole), fractions were collected and enzyme activity was assessed; protein purity was checked by SDS-PAGE and found to be higher than 90%. Enzyme containing fractions were pooled and loaded onto a Hi-Trap desalting column equilibrated with Storage buffer (50 mM Tris–Hcl pH 7.0, 25 mM NaCl, 1 mM EDTA, 10% glycerol). Fractions were collected, catalytic activities and protein concentration were determined. Enzyme-containing fractions were pooled and aliquots were stored at −80 ◦C.

RNase H polymerase-independent cleavage assay

When the tC5U/p12 hybrid was used as reaction substrate the RNase H activity was measured as described. Briefly, the tC5U RNA oligonucleotide was labeled at its 5'-end with [γ^{32}P]-ATP and T4 polynucleotide kinase. The unincorporated [γ^{32}P]-ATP was separated from the labeled RNA by running the sample through a G-25 Sephadex quick spin column. The labeled tC5U oligonucleotide was annealed to the p12 DNA oligonucleotide in 0.1 M NaCl by heating at 80 °C and slowly cooling to room temperature overnight. RNase H activity was measured in 15 μL reaction volume containing 50 mM Tris–HCl pH 7.8, 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 80 mM KCl, 50 nM tC5U/p12 labeled hybrid, 2 nM rRT and different drug concentrations. The reaction mixture was incubated for 30 min at 37 °C and stopped by the addition of 7.5 μL of sample buffer (96% formamide, 20 mM EDTA, 0.08 % Bromophenol blue and 0.08 % Xylene cyanol). Samples were heated at 100 °C for 3

min, and 10 μL of them were layered onto a denaturing 20 % polyacrylamide gel (7 M urea, 0.09 M Tris–borate (pH 8.3), 2 m MEDTA, 20 % acrylamide) and run for 1.5 h at 80 W. The gel was dried and reaction products were visualized and quantified by a Bio-Rad FX Phosphoimager. When the poly(dC)-[3H]poly(rG) hybridwas used as reaction substrate the RNaseH activitywas measured as described (Starnes and Cheng, 1989). Briefly, RNaseH activity was measured in 50 μL reaction volume containing the same reaction mixture as above with the exception of 4 nM poly(dC)-[3H]poly(rG) and 1 nM rRT. The reaction mixture was incubated for 30 min at 37 °C and 40 μL aliquots were spotted on glass fiber filters (Whatman GF/A) and processed for determination of trichloroacetic acid-insoluble radioactivity. When AMV and *E. coli* RNase H activities were measured, the poly(dC)-[3H]poly(rG) hybrid was used as reaction substrate with the buffer conditions indicated by the manufacturer, and the trichloroacetic acidinsoluble radioactivity was determined as above.

RNA-dependent DNA polymerase assay

The RDDP activity of HIV-1 rRT was measured as described in 50 μL volume containing 50 mM Tris–HCl pH 7.8, 80 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL bovine serum albumine, 0.3 O.D./mL poly(rC)-oligo$(dG)_{12–18}$, 10 μM [3H]-dGTP (1 Ci $mmol^{-1}$). The reaction mixture was incubated for 30 min at 37 °C, 40 μL aliquots were spotted on glass fiber filters (Whatman GF/A) and processed for determination of trichloroacetic acid-insoluble radioactivity.

HIV-1 replication assay

Wt and resistant HIV-1 strains stock solutions had titers of (2–4.5) X 10^6 50% cell culture infectious dose (CCID50)/mL. Cytotoxicity evaluation was based on the viability of mock-infected MT-4 cells, as monitored by the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Activity of the compounds against the HIV-1 multiplication in acutely infected cells was based on inhibition of virus-induced cytopathicity in MT-4 cells and was determined by the MTT method.

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Third Chapter

NS5B RNA-DEPENDENT RNA POLYMERASE OF HCV

Due to the similarity of its genomic organization to that of pesti- and flaviviruses, HCV has been classified as one of three genera constituting the family Flaviviridae. [1] Since the identification and molecular cloning of hepatitis C virus (HCV) in the late 1980s, it has been estimated that more than 170 million people are infected with the virus. In approximately 80% of infections the virus is able to elude the body's immune response and succeeds in establishing a chronic infection. [2] The number of individuals infected with HCV continues to increase and persistently infected persons are at risk of developing cirrhosis and hepatocellular carcinoma.

HCV has a single-stranded positive sense, 9.6 kb RNA genome that is flanked at each terminus by a 5' and 3' non-translated region (NTR) and contains one long open reading frame that encodes a precursor polyprotein of about 3000 amino acids. [3] Translation of the polyprotein is directed by the internal ribosome entry site (IRES) located within the 5' end.

The polyprotein is subsequently processed into both structural (core, envelope 1, envelope 2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins by cellular and viral proteases. [4] Core protein is a highly basic protein that forms the nucleocapsid. The envelope proteins E1 and E2 are highly glycosylated transmembrane proteins that are non-covalently associated to form the viral envelope. Protein p7 is a highly hydrophobic polypeptide that forms hexamers and has been reported to have ion channel activity. NS2 is a cis-acting autoprotease that is essential for viral replication. NS2 catalyzes the cleavage of the polyprotein precursor at the NS2/NS3 junction, and also plays an essential role in virus assembly. NS3 is a bifunctional protein with serine protease activity in the N-terminus, which is responsible for cleavage at the NS3/NS4A, NS4A/NS4B, NS4b/NS5A and NS5A/NS5B sites, and NTPase/helicase activities in the C-terminus of the protein. The NS4A polypeptide functions as a cofactor for NS3 protease activity. NS4B was demonstrated to induce specific cellular membrane changes, creating a membranous web that serves as a scaffold for the formation of the viral replication complex. NS5A is a phosphoprotein with multiple functions and is essential for viral replication and assembly. NS5B catalyzes the synthesis of both minus-strand and

plus-strand RNA. [5]

The most attractive targets for future anti-HCV agents are those that specifically target the viral replication cycle (Figure 2): internal ribosome entry site (IRES) inhibitors, viral protease inhibitors and transcription (polymerase) inhibitors and virus assembly inhibitors.

It is known that translation of HCV RNA is initiated by internal entry of the 50 non-coding region or IRES into ribosomes *in vitro*. [6] IRES inhibitors are RNA structures that lead to inhibition of HCV polyprotein translation and includes anti-sense oligonucleotides (e.g., ISIS 14803; Isis Pharmaceuticals, Carlsbad, CA), small interfering RNAs (siRNA) and ribozymes. To date, the clinical experience with oligonucleotides has been disappointing, due to evidence of toxicity [7] and poor antiviral activity. The most promising recent advances have come in molecules that target the NS3/4A serine protease and the NS5B RNA-dependent RNA polymerase.

The structural solution of the NS3/4A protease and the HCV NS5B polymerase and the development of a (sub)genomic replicon system have enabled the development and testing of HCV specific compounds. As other viral infections, it is widely recognized that combinations of drugs that act via different mechanisms are required to suppress the emergence of resistance effectively, and agents that inhibit the RNA polymerase NS5B are, therefore, of great research interest.

3.1 Biology of NS5B polymerase enzyme

3.1.1 Structure and function of NS5B polymerase enzyme

HCV, like other (+)-strand RNA viruses, uses its viral genomic RNA as a template for both translation and generation of a complementary (-)-stranded RNA intermediate. The (-)-stranded RNA is then used as the template for the synthesis of molar excess of (+)-stranded progeny RNA molecules.

A membrane-associated replicase enzyme complex consisting of virally encoded and host proteins is responsible for the replication of viral RNA. The catalytic subunit of the replicase complex is the HCV encoded non structural 5B protein (NS5B), which contains all the sequence motifs highly conserved among all the known RNA-dependent RNA polymerases (RdRps). [8] By extension of studies from the HIV-1 and -2, where the reverse transcriptase is a primary target for effective antivirals, the HCV RdRp is considered an important target for drug development. [9]

The NS5B is an enzyme of 65 kDa and contains six conserved motifs designated A-

F. The HCV RdRp has discernable fingers, palm and thumb subdomains. Anusual feature of this polymerase is that, due to the extensive interactions between the finger and thumb subdomains, the HCV RdRp has an encircled active site. [10] These contacts restrict the flexibility of each subdomains and between them. Structural studies with RdRp from the HCV genotype 2a indicate the presence of two conformations of the protein even in the absence of template RNA, where the key difference between the two forms is the relative orientation of the thumb domain in relation to fingers and palm domains. [11] Since both conformations lacked RNA, remains to be determined whether the template RNA will induce the same structural change. Another unusual feature of NS5B is a β-hairpin loop that protrudes into the active site located at the base of the palm subdomain. This 12 amino acid loop was suggested to interfere with binding to double stranded RNA due to steric hindrance. [12] The poliovirus RdRp lacks a similar β-loop, a feature that may be related to the poliovirus RdRp normally directing genome replication with a protein-nucleotide primer. [13] It was suggested that the β-loop could be involved in positioning the 3' terminus of the viral RNA for correct initiation. [12]

The C-terminal region of NS5B also lines the RNA binding cleft in the active site. This region, which immediately precedes the C-terminal membrane anchorage domain, forms a hydrophobic pocket and interacts extensively with several important structural elements including the β-loop. [14] Deletions of up to 55 residues of the C-terminal tail resulted in increased RdRp activity, suggesting a direct role for the C-terminal tail in RNA synthesis activity and providing evidence for regulation at the active site. [15]

The active sites of HCV NS5B and HIV-1 RT are very similar and can be superimposed without significant steric clashes. The residues involved in nucleotidyl transfer are found in palm motifs A and C. Motif A harbors the metal binding residue D220 which is a part of conserved D-X4-D motif, while motif C has the conserved metal binding and nucleotidyl transfer residues D318 and D319.

D225 within motif A, forms a H-bond with the ribose 2'-hydroxyl group of the nucleotidyl triphosphate natural substrate (NTP) and is thought to discriminate against the use of dNTPs. Crystal soaking experiments of HCV NS5B with ribonucleoside triphosphates (rNTPs) revealed several residues in the catalytic pocket that contact the triphosphates of rNTPs. These include R158, S367, R386, T390 and R394.

Unfortunately, it was not possible to identify the base-interacting residues. Soaked NS5B crystals with GTP also revealed a low affinity GTP binding site on the surface of the protein. [16] This second site, which is apparently specific for GTP, lies between the fingers and thumb subdomains, approximately 30 Å away from the catalytic pocket. By virtue of its position and the requirement of higher GTP concentrations to saturate the pocket, it was suggested that it might play a regulatory role in RNA synthesis. [16] To date, any allosteric properties of this low affinity site in response to GTP has not been determined.

3.1.2 Activity of NS5B polymerase

The activity of NS5B polymerase can be synthesized as followed:

- coupled of RNA template molucule and of NTD;
- initiation;
- elongation;
- ending and release of template.

The NS5B polymerase can begin the synthesis of a new molecule of RNA depending on three mechanisms:

- primer-dipendent;
- copy back;
- *de novo.*

The initiation primer-dependent is a method utilized by several viruses for a faithful and effective replication. In this case is a viral protein to act as a primer, to which the polymerase addes the NTD depending on the complementarity with the template. The copy-back mechanism is highlight in *in vitro* experiment for the lack of the RNA primer. The 3'-OH teminus of the template molecule is used as a primer for a replication process that lead to synthesis of a new double strand molecule. The *de novo* synthesis is highlight in *in vitro* experiment too.

The synthesis of a new molecule of RNA started from the 3' end of the genome by the utilization of NTP. The terminal cytidyne is responsible for the specificity in the entry of RNA template in the catalytic active site of the polymerase. Infact the steric hindrance in the binding pocket block the entrance of more voluminouse base like adenine and guanosine, and the electrostatic interaction do not favour the binding of uracil. Furthermore, the β-hairpin is able to correctly direct the RNA template, which

is caught in a vice like a grip into the catalytic active site. The hypitesis of the *de novo* mechanism is confemed by the tridimentional structure of NS5B in a complex with NTD. In the catalytic active site was been identified three different binding site for the NTD, called initiation site, elongation site, and interrogation site. The initiation phase of the replication of the viral genome is the more difficult phase, because the ending sequence responsable of the entry of the RNA template must be highly conserved in every replicative cycle. [10]

To the initiation follows the elongation, that is a rapid process which is repeated thousend times in the replication of the viral genome. This long time intervall allows the selective inhibition by the traditional HIV antiviral drugs, like AZT and 3TC. At the end of the elongation phase the new RNA molecule goes out from the catalytic active site of the polymerase. [17]

Polymerase require divalent metal ions for activity. RNA synthesis by NS5B is increased by 4-20 fold when Mn^{2+} is present in the reaction in comparison to a reaction with only Mg^{2+}. However, other divalent metal ions such as Co^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} did not support RdRp activity. [15] Recently it was shown that iron binds specifically to the Mg^{2+} binding site of NS5B and can inhibit RNA synthesis. [18] Mn^{2+} appears to more specifically contribute to *de novo* initiation by lowering the KM for GTP by about 30-fold. While the concentration of Mn^{2+} used *in vitro* is far higher than concentrations present in the cell, physiologically relevant Mn^{2+} levels can increase *de novo* initiation with GTP.

Analysis of proteins with C-terminal deletions revealed that the C-terminus of the HCV RdRp plays a role in Mn^{2+} induced *de novo* initiation and can contribute to the suppression of primer extension. Spectroscopy examining the intrinsic tryptophan and tyrosine fluorescence of the HCV RdRp produced results consistent with the protein undergoing a conformational change in the presence of divalent metals. [15]

3.2 Inhibitors of NS5B polymerase

In the past decade, intensive efforts have focused on the discovery of both nucleos(t)ide and non-nucleoside inhibitors of the HCV NS5B polymerase. These efforts have resulted in several promising agents advancing in clinical development.

The crystal structure of the catalytic domain of N5SB (lacking the C-terminal membrane-anchoring region) has revealed several significant features. A helix at the tip of the fingers domain binds to a lipophilic pocket in the back of the thumb

domain; it is this interaction that is disrupted by inhibitors that bind at the thumb pocket I site. Two other notable features of the apo structure are the presence of a β-hairpin from the thumb domain that reaches down into the palm domain, and the residual C-terminal strand that folds over the palm domain. Both of these features have to move to enable processive synthesis of RNA. Thumb pocket II inhibitors bind to a remote region of the thumb domain and are presumed to affect the flexibility of this region. Palm pocket I inhibitors bind to elements of the β-hairpin and the palm domain simultaneously. Palm pocket II inhibitors, the binding site of which overlaps partially with the palm pocket I site, do not interact directly with the β-hairpin, but occlude the region that would be occupied by the incoming nucleotide prior to ligation. Thus, the selectivity that is evident in non-nucleoside inhibitors (NNIs) for NS5B compared with other polymerases is understandable in the context of the unique structural features of NS5B. [19]

3.2.1 Nucleos(t)ide inhibitors

The key residues in the HCV NS5B polymerase active site are conserved across genotypes, and replicon systems containing resistance mutations for nucleos(t)ide analogs demonstrate greatly reduced replication capacity, indicating a high barrier to resistance generation. [20] Nucleos(t)ide analogs served as the cornerstone of successful antiviral therapy for herpes viruses, HBV and HIV. Reflecting the success of these compounds, more then 20 distinct nucleos(t)ide agents have been approved by regulatory agencies globally. Nucleos(t)ide analogs may therefore play a critical role in virally targeted HCV combination therapy in the future. The major approved nucleos(t)ide analog antiviral compounds are endogenous 2'-deoxynucleos(t)ides, whereas the inhibition of the RNA-dependent RNA polymerase activity of HCV NS5B requires ribonucleos(t)ide analogs, which typically have a greater potential toxicity than 2'-deoxynucleos(t)ides and poorly defined mechanism(s) of toxicity. The explanation of the high preclinical and clinical problems, observed for the nucleos(t)ide class of compounds in the treatment of HCV infections, is the inadequate tolerability.

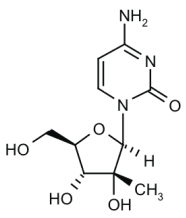
Nucleos(t)ide analogs that directly target the viral polymerase have a unique pharmacology, and require phosphorylation to generate the triphosphate (TP) forms to demonstrate antiviral activity; therefore the activity of these compounds depend on successive interactions with the cellular enzymes involved in the regulation of

endogenous nucleotide pools for activation to their respective TP forms. The first phosphorylation step, catalyzed by nucleoside kinases and phosphotransferases, can be bypassed effectively by administration of a monophosphate (MP) prodrug. [21] The levels of the phosphorylated forms are regulated by nucleotidases and phosphatases. Although nucleos(t)ides can be catabolized by diverse pathways, cytochrome P450 (CYP) enzymes have not been demonstrated to play a role in the degradation of these molecules, and many nucleos(t)ide analogs are unchanged when eliminated in the urine.

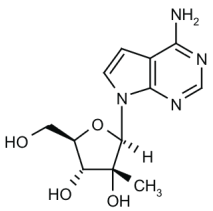
In the late 1960s, approximately 20 years before the identification of HCV, a research group at Merck described several ribonucleoside analogs, including branched-chain sugar nucleosides with 2'-alkyl substitutions, as antiviral agents. [22] 2'-*C*-Methyl ribonucleos(t)ides were the first to be reported to have potent anti-HCV activity; [23] the EC_{50} value of 2'-*C*-(CH3)-adenosine in a replicon system was 0.3 μM, and 2'-*C*-(CH3)-ATP inhibited purified NS5B by non-obligate chain termination with an IC_{50} value of 1.9 μM. Idenix Pharmaceuticals Inc (formerly Novirio Pharmaceuticals Ltd) was the first company to advance a member of this class of compounds into clinical trials: a prodrug of **NM 107** (2'-*C*-(CH3)-cytidine), valopicitabine dihydrochloride (**NM 283**) was investigated in phase II clinical trials, but this compound is no longer being developed.

An investigation into various base modifications led to the identification of **MK 0608** (2'-*C*-(CH3)-7-deaza-adenosine), which is stable against adenosine deaminase (the enzyme that limits the oral bioavailability of 2'-*C*-(CH3)-A), maintains replicon activity, and was demonstrated to result in a 20-fold improvement in the IC_{50} of the TP form against NS5B, compared with 2'-*C*-(CH3)-A. [24] Administration of **MK 0608** (1 mg/kg qd, po) for 37 days to two chimpanzees infected with HCV caused a 4.6-log reduction in viral load in one animal and reduced viral load to undetectable levels in the other. Although clinical development of **MK 0608** did not progress beyond a phase I clinical trial, these preclinical results established the potential of nucleos(t)ide analogs as potent HCV inhibitors.

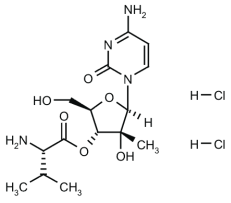
R 1479 (4'-



NM 107

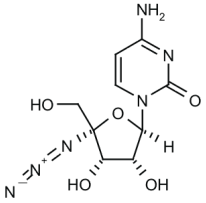


MK 0608

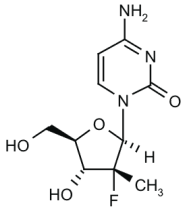


NM 283

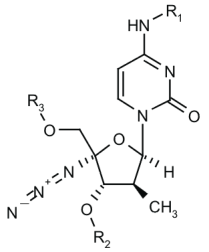
azido-cytidine), a nucleos(t)ide analogue lacking the 2'-*C*-(CH3) group, demonstrated selective replicon potency that was caused by non-obligate chain termination of NS5B activity. [25] In addition, analogues with a 2'-deoxy-2'-fluoro-2'-*C*-(CH3) ribose sugar moiety have been demonstrated to have anti-HCV activity: the cytidine analog **PSI 6130** exhibited the greatest potency, with an EC_{90} value of 5.4 μM. [26] Medivir has described 4'-azido-2'-deoxy nucleoside analogs (eg, 2'-*C*-(CH3)-2'-deoxy-4'-azido-cytidine analogs) [27] as potentially useful in the treatment of HCV infection.



R 1479

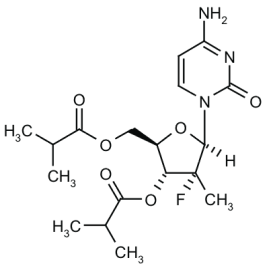


PSI 6130

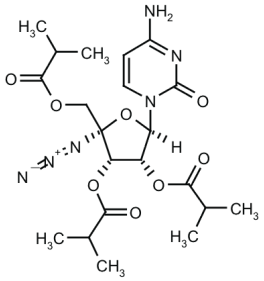


2'-*C*-(CH3)-2'-deoxy-4'-azido-cytidine analogs

Adopting a tactic used successfully for acyclovir, a 3'-valyl ester prodrug of **NM 107**, valopicitabine dihydrochloride (**NM 283**), was produced. It has demonstrated a markedly improved oral bioavailability compared with the original drug. This result was presumably attribute to the uptake by peptide transporters in the intestine. [28] Capping the free hydroxyl groups of **R 1479** and **PSI 6130** as isobutyryl esters yielded the prodrugs Balapiravir (**R 1626**) and **RG 7128**, [29] respectively. Both compounds were advanced to clinical trials; Balapiravir is no longer being developed, while **RG 7128** (EC_{50} = 1340 nm) is in phase II trials. [30]



RG 7128



Balapiravir (R 1626)

The initial phosphorylation step for most nucleoside analogs is limiting; thus, high doses of the prodrug agents were required to observe clinical efficacy.

3.2.2 Non nucleoside inhibitors

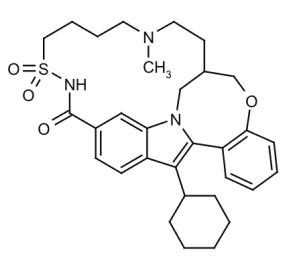
Several non nucleosedi inhibitors of the NS5B recently has been described and some of them are the object of preclinic and clinic study. Great interest is focused to compounds caracterized by a benzimidazole or benzothiadiazine based.

The non nucleoside inhibitors can be distinguished in according to the binding site with the enzyme. Therefore, the non nucleoside inhibitors are classify into:

- NS5B thumb pocket I inhibitors;
- NS5B thumb pocket II inhibitors;
- NS5B palm pocket I inhibitors;
- NS5B palm pocket II inhibitors;
- NS5B inhibitors binding the metal ions chelating site.

NS5B thumb pocket I inhibitors:

Ligands binding to the thumb pocket I site form a salt bridge with a guanidinium residue (Arg503) and several lipophilic interactions with neighboring regions of the protein (**1, 2**).



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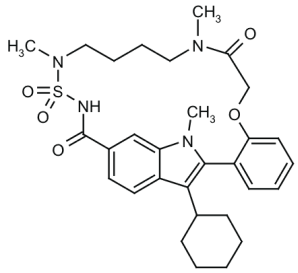
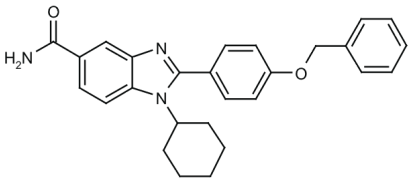
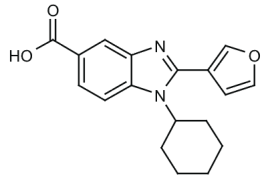


2

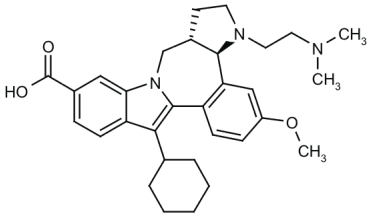
The best characterized resistance mutations are variants of Pro495, which is exposed on the protein surface and forms van der Waals interactions with the inhibitors. This pocket has been widely studied by several pharmaceutical companies that carried out lead optimization researches, that led to a substitution of the early benzimidazole based (**3, 4**) with a more lipophilic indole core, and a introduction of a ring connecting the indole with the 2-aryl substituent; this changes led to improve cellular activity (**5, 6**). [31]



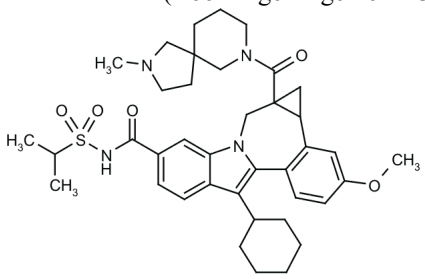
3 (Japan Tobacco)



4 (Boehringer Ingelheim Corp)



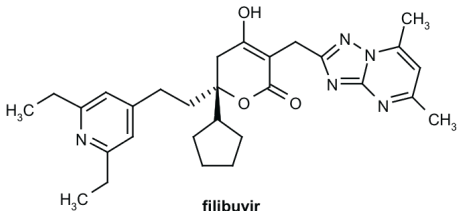
5 (IRBM)



6 (Bristol-Myers Squibb Co)

NS5B thumb pocket II inhibitors:

The thumb pocket II binding site is formed by changes in the conformation of residues Met423 and Leu497. Inhibitors occupy the resultant spaces with lipophilic substituents and form polar interactions with the backbone amides of Ser476 and Tyr477, either directly or via a bridging water molecule. Mutations at Met423 have been observed clinically. Initial research on inhibitors binding at this site was first reported at approximately the same time as research on thumb pocket I compounds and early leads also contained an acidic group with an adjacent lipophilic substituent, with stringent demands on geometry and shape. However, over time, several chemotypes have emerged that have reached clinical trials. The most advanced of these compounds is filibuvir (**7,** EC_{50} = 52nM), which is characterized by a weakly acidic dihydropyranone core. [33]



filibuvir

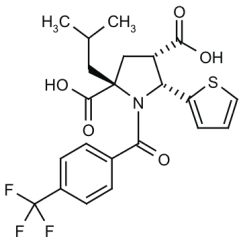
7 (Pfizer Inc.)
IC_{50} = 52 nM

NS5B palm pocket I inhibitors:

Ligands for the palm pocket I binding site contain lipophilic substituents suitably disposed relative to a group that forms a polar interaction with the backbone amide of Tyr448. While many resistance mutations have been reported in *in vitro* studies, Met414, Tyr448, Gly554 and Cys316 are associated with clinical isolates from

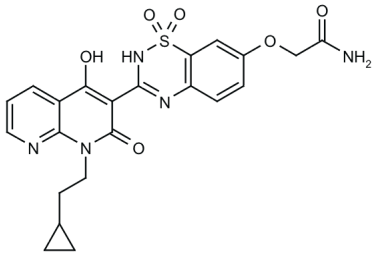
patients treated with drugs binding at this site.

An early series of palm pocket I inhibitors are the acyl pyrrolidines. Research on this series culminated in the identification of GSK-625433 (**8**), which reached phase I clinical trials.



8

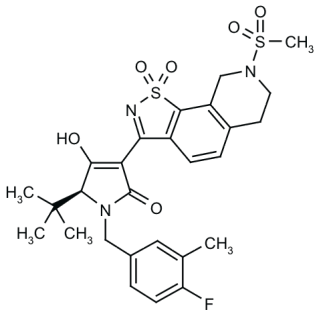
Around the same period, a series of quinolone-benzothiadiazines (**9**) was discovered at GSK, and further details of the optimization program, which explored heterocyclic alternatives to phenyl rings in the original lead, have been described. [34]



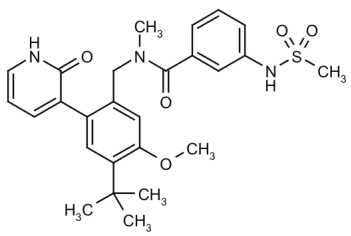
9

A common feature that greatly improves cellular potency and that has been broadly adopted is the incorporation of a hydrogen bond donor group (typically sulfonamide) at the 7-position of the benzothiadiazine or a related heterocycle.

Further studies led to identification of tetramic acids (**10**) [35] and pyridinones (**11**) [36] as core for NS5B palm pocket I inhibitors.



10 (La Roche)

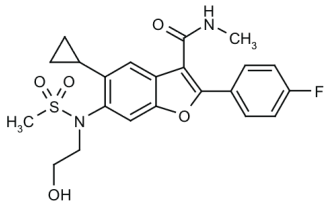


11 (La Roche)

NS5B palm pocket II inhibitors:

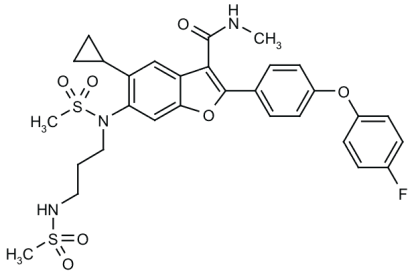
The palm pocket II site is formed upon movement of the side chain of Arg200,

creating a space that can be filled by an aromatic substituent on a molecule also bearing a polar group to interact with the side chain hydroxyl of Ser365. The most notable historical example of NS5B palm pocket II inhibitors is HCV-796 (**12**), which demonstrated the potential for clinical efficacy via this mechanism of inhibition, but was subsequently withdrawn from development because of liver toxicity. Mutations at Cys316 have been observed clinically for resistance to this compound.



12

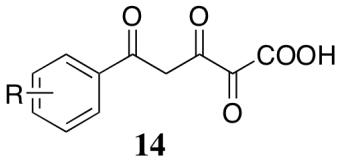
A research group at Roche has filed a patent application claiming more complicated 2-substituents on the benzofuran core (**13**). [37]



13

NS5B inhibitors binding the metal ions chelating site:

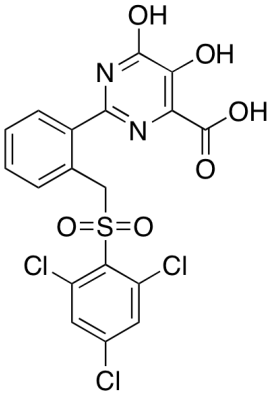
During a screening of more then 200000 compounds, the Merck & co. has been identify the α,γ-diketoacids (DKA) (**14**) as reversible inhibitor, with submicromolar and selective activity against NS5B polymerase. [38] The bond of the DKAs to the active site of the RdRp is mediated by divalent cations, like Mg^{2+} or Mn^{2+}, and occurred with a non competitive mechanism toward the RNA template molecule. Probably its activity is dued to a direct interaction of the diketo acid mojety with the divalent metal ions, which are essential for the activity of the polymerase.



14

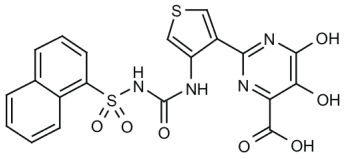
In the 2003 SAR studies begun by Summa *et al.* [39] on this new class of inhibitors,

has been demonstrated the importance of the arilic ring; infact the introduction of alifatic mojety like methyl or *t*-buthyl group determined a decrease of the activity. The substitution of phenil with a thiophene ring brought to a compound with comparable activity, while the introduction of more polar etherocycles, like 3-pyridine and 2-furane, was not well tolerated; the diketo acid mojety was largely investigated too: simple modifications lead to a compounds completly inactives. [39]
Recently the Merck & co. has descibed a new class of NS5B inhibitors 2-aril-4,5-dihydroxypyrimidin-6-carboxilyc acids (**15**) highly selective for the polymerase.



15

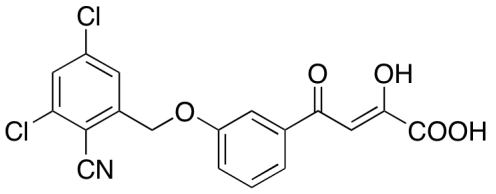
A study by Pacini *et al* has expanded the SAR of dihydroxypyrimidine carboxylic acids that inhibit NS5B by chelating to the associated Mg^{2+} ions; this study demonstrated, using the weak cellular activity of analogs such as compound **16** that mutations adjacent to this site (Pro156Ser and Gly152Glu) conferred resistance against the series. [40]



16

3.3 Aim

After a screening on 200000 α,γ-diketo acid compounds, the Merck reserchers have concluded that molecules with this mojety are selective inhibitors of NS5B polymerase of HCV. [38] Among them, three compounds (**17:** IC_{50} = 0.056 μM; **18:** IC_{50} = 7.9 μM; **19:** IC_{50} = 12.8 μM) have demonstrated micromolar IC_{50} values against NS5B.



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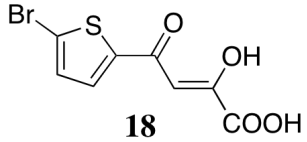
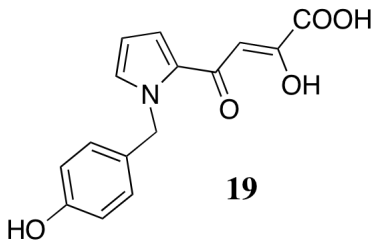


18

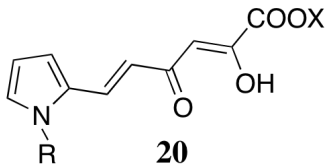


19

Therefore **17, 18** and **19** are not selective because are able to inhibite also the polymerase of hepatitis B and integrase of HIV-1. The inhibitory activity are dued to their ability to chelate the Mg^{2+} ions, and in conseguence to interfere with the bond of the phosphoric group of the nucleotidic substrate with the catalytic active site of the polymerase, preventing the formation of phosphodiester bond catalyzed by the enzyme.

The research group where I spent my Ph.D., by several years is involved in studies on integrase of HIV-1 inhibitors. Moreover, the decennal interest on the pyrrole chemistry, has brought to the development of pyrrolyldiketo acids derivatives analogues of **19** compound, for testing their inhibitory activity against NS5B.

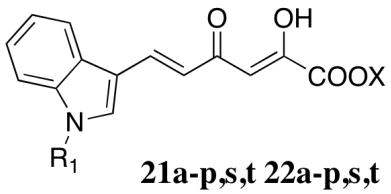
Starting from **19** compound, a vinilic mojety was been introduced between the diketo acid portion and the pyrrole ring, obtaining compounds with general structure **20**.



20

The IC_{50} values obtained in *in vitro* assay against the NS5B polymerase are included between 72.2 and 272 μM. To elaborate SAR study, the pyrrole ring was substituted with other etherocycle rings like indole. For that derivatives was studied the importance of the substitution on the nitrogen atom of the eterocycle ring: the indolyldiketoexenoic derivatives it was been substituted with aliphatic (alkylic and alkenylic) and aromatic (benzyl and phenil) groups, obtaining compounds **21a-p,s-t** and **22a-p,s-t** (Table 1).

Table 1. Compound **21a-p,s-t** and **22a-p,s-t**



21a-p,s,t 22a-p,s,t

Cpd	R	R_1	X
21a	-	$(CH_2)_2CH_3$	Et
21b	-	$(CH_2)_3CH_3$	Et
21c	-	$(CH_2)_2CH(CH_3)_2$	Et
21d	-	$CH=CHCH_3$	Et
21e	-	$CH_2CH=CHCH_3$	Et
21f	-	$CH=C(CH_3)_2$	Et
21g	-	$CH_2C(CH=CH)CH_3$	Et
21h	-	Bn[a]	Et
21i	-	4-Cl-Bn[a]	Et
21j	-	4-F-Bn[a]	Et
21k	-	4-CN-Bn[a]	Et
21l	-	4-OCH_3-Bn[a]	Et
21m	-	4-OH-Bn[a]	Et
21n	-	Ph[b]	Et
21o	-	4-OH-Ph[b]	Et
21p	-	4-Cl-Ph[b]	Et

Cpd	R	R_1	X
22a	-	$(CH_2)_2CH_3$	H
22b	-	$(CH_2)_3CH_3$	H
22c	-	$(CH_2)_2CH(CH_3)_2$	H
22d	-	$CH=CHCH_3$	H
22e	-	$CH_2CH=CHCH_3$	H
22f	-	$CH=C(CH_3)_2$	H
22g	-	$CH_2C(CH=CH)CH_3$	H
22h	-	Bn[a]	H
22i	-	4-Cl-Bn[a]	H
22j	-	4-F-Bn[a]	H
22k	-	4-CN-Bn[a]	H
22l	-	4-OCH_3-Bn[a]	H
22m	-	4-OH-Bn[a]	H
22n	-	Ph[b]	H
22o	-	4-OH-Ph[b]	H
22p	-	4-Cl-Ph[b]	H

21s	-	$CH_2(C=O)N(CH_3)_2$	Et	**22s**	-	$CH_2(C=O)N(CH_3)_2$	H
21t	-	$CH_2(C=O)Morph^c$	Et	**22t**	-	$CH_2(C=O)Morph^c$	H

[a] Bn: benzyl; [b] Ph: Phenyl; [c] Morph: morpholine.

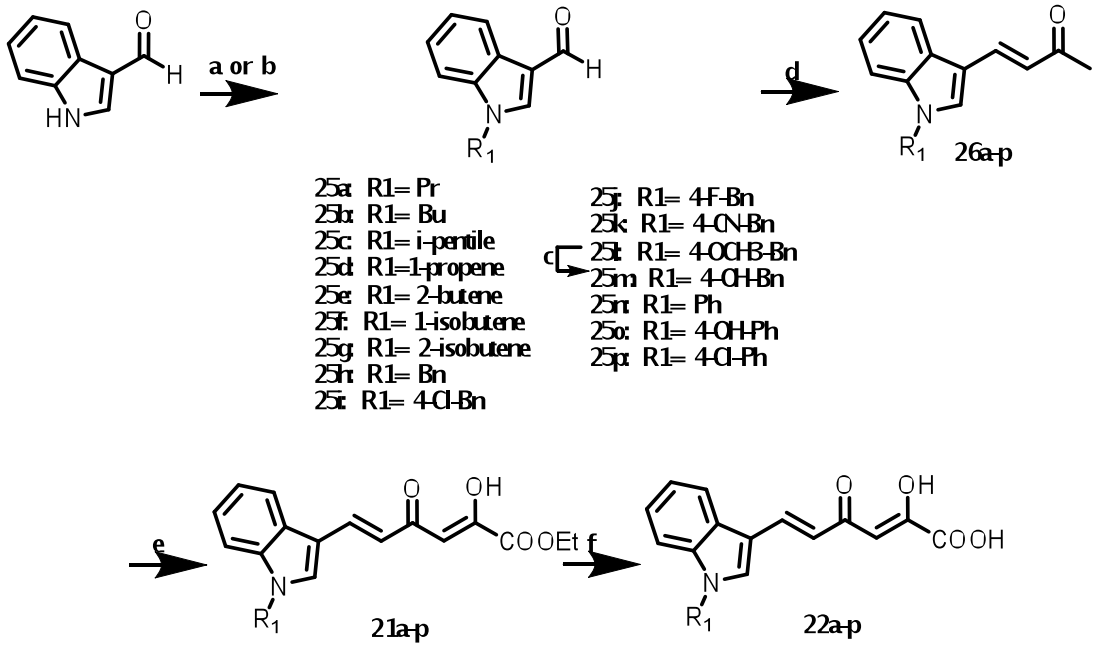
All these compounds are tested in *in vitro* assay on ricombinant RdRp.

3.3.1 Results and discussion

Scheme 1, 2 and 3 reported above, describes the synthesis of derivatives **21a-p,s-t**, **22a-p,s-t.**

The synthesis of compound **21a-p** and **22a-p** (Scheme 1) started from the commercially available indole-3-carboxaldehyde, that was alkylated in alkaline medium (NaH) on the nitrogen atom with the appropriate alkyl, alkenyl or benzyl bromide to obtain **25a-l** N-substuted-indole-3-carboxaldehyde. To obtain the **25n-p** derivatives the indole-3-carboxaldehyde was allowed to react with the appropriate phenylboronic acid in a coupling reaction in presence of cupric acetate as catalyst, that was carried out utilyzing microwaves irradiation in an open-vessel experiment. In comparison with the betch reaction, in the microwave reaction the yield was increased. The 4-methoxybenzyl derivative **5l** afforded 4-hydroxybenzyl derivative **5m** after treatment with boron tribromide (BBr_3). The aldehydes **25a-p** were converted to the corresponding 3-buten-2-ones **26a-p** in a condensation reaction with acetone in presence of sodium hydroxyde (NaOH). To afford ethyl esters **21a-p,** 3-buten-2-ones **26a-p** was treated with diethyl oxalate utilyzing a weak base like sodium ethoxyde (NaOEt). The esters was converted to the corresonding acids **22a-p** for hydrolysis.

Scheme 1. [a]

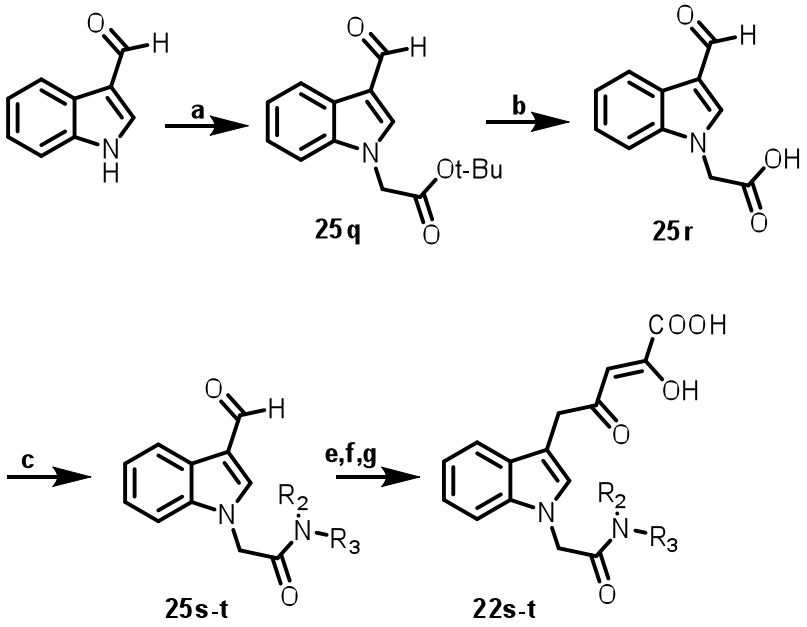


[a]Reagents and conditions: (a) DMF, NaH, alkylic or benzylic alogenure, 1 h, r.t; (b) phenylboronic acid, cupric acetate, NMP-pyridine, MW (60W, 120 °C, 50s, open vessel); (c) BBr_3, CH_2Cl_2, -45 °C,

1 h; (d) NaOH 5 N, acetone, 2 night, 50 °C; (e) diethyl oxalate, NaOEt, THF, 1 h 30 min, r.t.; (f) NaOH 1 N, THF-Methanol, 30 min, r.t.

The amides **25s-t** (Scheme 2) was obtained allowed a reaction between the indole-3-carboxaledehyde with tert-butylbromo acetate; the ester function now formed (**25q**) was hydrolysed in acidic ambient afforded the corresponding acid **25r.** The amides formation was performer on acid **25r** in presence of HATU and DIEA as catalysts. The successive passages are the same described for the Scheme 1.

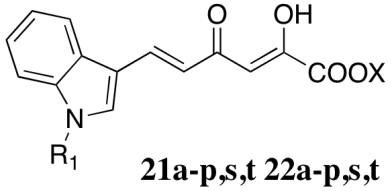
Scheme 2. [a]



[a]Reagents and conditions: (a) DMF, NaH, tert-butylbromo acetate, 1 h, r.t.; (b) dichloromethane-trifluoroacetic acid mixture 1:1, 16 h, r.t.; (c) base, HATU, DIEA, 2 days, r.t.; (e) NaOH 5 N, acetone, 2 night, 50 °C; (f) diethyl oxalate, NaOEt, THF, 1 h 30 min, r.t.; (g) NaOH 1 N, THF-Methanol, 30 min, r.t.

All these compounds are tested in *in vitro* assay on ricombinant RdRp. Table 3 and 4 reports the enzyme inhibitory activities in *in vitro* assay on ricombinant NS5B of HCV.

Table 3. Activities data of compound **21a-p,s-t** and **22a-p,s-t**



21a-p,s,t 22a-p,s,t

Cpd	R	R_1	X	NS5B (μM)

Cpd	R	R_1	X	NS5B (μM)

				IC_{50}
21a	-	$(CH_2)_2CH_3$	Et	45
21b	-	$(CH_2)_3CH_3$	Et	25
21c	-	$(CH_2)_2CH(CH_3)_2$	Et	52
21d	-	$CH=CHCH_3$	Et	12
21e	-	$CH_2CH=CHCH_3$	Et	33
21f	-	$CH=C(CH_3)_2$	Et	62
21g	-	$CH_2C(CH=CH)CH_3$	Et	73.3
21h	-	Bn[a]	Et	97.3
21i	-	4-Cl-Bn[a]	Et	36.5
21j	-	4-F-Bn[a]	Et	180
21k	-	4-CN-Bn[a]	Et	32.3
21l	-	4-OCH_3-Bn[a]	Et	72
21m	-	4-OH-Bn[a]	Et	29.6
21n	-	Ph[b]	Et	99.4
21o	-	4-OH-Ph[b]	Et	>200
21p	-	4-Cl-Ph[b]	Et	83
21s	-	$CH_2(C=O)N(CH_3)_2$	Et	NA
21t	-	$CH_2(C=O)$Morph[c]	Et	NA

				IC_{50}
22a	-	$(CH_2)_2CH_3$	H	25
22b	-	$(CH_2)_3CH_3$	H	15
22c	-	$(CH_2)_2CH(CH_3)_2$	H	48
22d	-	$CH=CHCH_3$	H	12.5
22e	-	$CH_2CH=CHCH_3$	H	21
22f	-	$CH=C(CH_3)_2$	H	32
22g	-	$CH_2C(CH=CH)CH_3$	H	46.6
22h	-	Bn[a]	H	27.5
22i	-	4-Cl-Bn[a]	H	27.3
22j	-	4-F-Bn[a]	H	76.4
22k	-	4-CN-Bn[a]	H	23.6
22l	-	4-OCH_3-Bn[a]	H	10.7
22m	-	4-OH-Bn[a]	H	30
22n	-	Ph[b]	H	30
22o	-	4-OH-Ph[b]	H	21
22p	-	4-Cl-Ph[b]	H	34.6
22s	-	$CH_2(C=O)N(CH_3)_2$	H	42
22t	-	$CH_2(C=O)$Morph[d]	H	NA

[a] Bn: benzyl; [b] Ph: Phenyl; [c] Morph: morpholine.

In the enzymatic assay are tested both esters and acids derivatives. By a first analysis are evident that the diketo acid mojety are essential for the biological activity, infact the acid derivatives are always more active then the corresponding esters. The discrepance between these two result may be attribuite to the capacity of the acids function to chelate the metal ions presents in the active site. The IC_{50} values for the indolyldiketohexenoic esters **21a-p,s,t** are ranging from 12 to >200 μM, while, the indolyldiketohexenoic acids **22a-p,s-t** have IC_{50} values ranging from 10.7 to 46.6 μM.

In the serie of indolyldiketohexenoic acids the best substituent of the nitrogen atom of the indole ring is the 4-methoxybenzoyl group; infact the derivative **22l** presenting this substituent, has a IC_{50} value of 10.7 and is the compound that inhibit the NS5B polymerase with lower IC_{50}. Among the *N*-benzyl substituted indolyldiketohexenoic acids, all the substituent in position 4 of the benzyl group are well tolerated, with the exception of the fluorine atom that influence negativelly the inhibitory activity (IC_{50} = 76.4 μM). The IC_{50} value for the *N*-benzyl substituted indolyldiketohexenoic acids **22h,i,k-m** are ranging from 10.7 to 30 μM.

The *N*-alkyl and -alkenyl substituted indolyldiketohexenoic acids **22a-g** shows a good NS5B inhibitory activities; in particular the *N*-allyl indolyldiketohexenoic acids

22d exhibit IC_{50} value of 12.5 μM, comparable with the IC_{50} value of the more active compound **22l.**

The substitution on nitrogen atom of indole ring with phenyl **(22n-p)** and acetamide (**22s,t)** groups does not lead to any improvement in the NS5B inhibitory activity. **22o** has a similar IC_{50} value of the *N*-alkyl and -alkenyl substituted derivatives **22a,b,e** (21 μM)**,** whereas **22n,p,s,t** are slightly less inhibitory activity (30-42 μM) (Table 3)**.**

3.3.2 Conclusion

The hepatitis C virus (HCV) is an RNA virus, belonging to the flaviviride family, responsable of the insorgence of non-A and non-B hepatitis. About 3% of the world population are infected by HCV virus and developed a cronic epatitis, that lead to cirrhosis and hepatocellular cancer. Actually does not exist a really effective care for the eradication of the cronic hepatitis associated with the infection of the HCV virus. This disease are treated mainly with an association of interferon and ribavirine, that mostly bringh to a failure of the cure. Is evident the necessity of new drugs that are able to block the viral replication.

The NS5B polymerase of HCV represent a interesting target for the development of new potent and selective drugs.

The work presented in this third chapter reguards the development, synthesis and evaluation of biological activity of new indolyldiketohexenoic acids. Some of the new derivatives synthesized (**22l** and **22d**) have demonstated a IC_{50} values (10.7 and 12.5 μM respectively) comparable with the IC_{50} values of more promising compound known in letterature.

On the bases of these data, it is possible to claim that the derivative **22l** and **22d** can be considered new lead compound for future study.

3.3.3 Experimental section

Chemistry

General. Melting points were determined with a Büchi 530 capillary apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Spectrum-one spectrophotometer. ^{1}H NMR spectra were recorded on a bruker AC 400 specrtometer. Merk silica gel 60 F_{254} plates were used for analytical TLC. Column chromatogrephies were performed on silica gel (Merck; 70-230 mesh) or alumina (Merck; 70-230 mesh). Concentration of solution after reactions and extractions involved the use of a rotary evaporator operating at reduced pressure of approximately 20 Torr. Analytical results agreed to within ±0.40% of the theoretical values.

Microwave irradiation experiments. Microwave reactions were conduced using a CEM Discover system unit (CEM. Corp., Matthews, NC). The machine consists of a continuous focused microwave-power delivery system with operator selectable power output from 0 to 300 W. The temperature of the contents of the vessel was monitored using a calibrated infrared temperature control mounted under the reaction vessel. All experiments were performed using a stirring option whereby the contents of the vessel are stirred by means of a rotating magnetic plate located below the floor of the microwave cavity and a Teflon-coated magnetic stir bar in the vessel.

General procedure for the synthesis of N**-substituted indole-3-carboxaldehyde (25a-p)**. A solution of indole-3-carboxaldehyde (1.34 g – 0.0092 mol) in 20 mL of dry DMF was treated with NaH (580 mg – 0.0147 mol). After development of H_2, the solution was treated with the appropriate alogenure (0.011 mol) and was stirred with bascular stirring in Büchi Syncore for 1 h at room temperature. The solution was diluted with water and the solid that formed was filtered on flash vac. The solid was washed with water and light petroleum ether to give derivatives **25a-p**. Chemical, physical, and analytical data of derivatives **25a-p** are reported in Table 3.

1-(4-hydroxybenzyl)-1H-indole-3-carboxaldehyde (25m). A solution of **25l** (6 g-0.0226 mol) in 246 mL of CH_2Cl_2 was added dropwise to a solution of BBr_3 1 M in CH_2Cl_2 (0.0063 mol, 119.25 mL) cooled at -45 °C under argon stream. The mixture was stirred at -45 °C for 1 h and then was treated with water. After placing the reaction at room temperature, the formed precipitate was collected by filtration. The

solid was washed with water and light petroleum ether to give 4.96 g (76%) of pure **25m** as a red solid. Chemical, physical, and analytical data of derivative **25m** are reported in Table 3.

General procedure for the synthesis of 1-(4-phenyl)-1H-indole-3-carboxaldehyde (25n-p). In a 100 mL round bottom flask were placed appropriate phenylboronic acid (0.0084 mol), cupric acetate (0.012 mol), indole-3-carboxaldehyde (0.0048 mol) and 1:1 NMP:Pyridine mixture (5 mL). The bottom was placed into the microwave cavity (60 W, 120 °C, 50 s, open vessel). The reaction was repeat for six times and after each cycle, the reaction bottom was cooled and cupric acetate (0.012 mol), phenylboronic acid (0.0084 mol) and NMP:Pyridine mixture (5 ml) was added. After cooling the mixture was diluted with tetrahydrofuran and filtered off, and the solvent was evaporated. The residue was dissolved in ethyl acetate, washed with 1N HCl (20 times) and brine (three times), dried, and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (ethyl acetate-chloroform in differents ratio) to furnish the derivatives **25n-p**. Chemical, physical, and analytical data of derivatives **25n-p** are reported in Table 3.

***tert*-Butyl 2-(3-formyl-1*H*-indol-1-yl)acetate (25q).** A solution of indole-3-carboxaldehyde (4 g, 0.0276 mol) in 90 mL of dry DMF, was treated with NaH (1.55 g, 0.039 mol). After development of H_2, the solution was treated with tert-butylbromo acetate (10.75 g, 0.055 mol) and was stirred with bascular stirring in Büchi Syncore for 1 h at room temperature. The residue was dissolved in ethyl acetate, washed with 1 N HCl (3 times) and brine (three times), dried, and the solvent was evaporated under reduced pressure. The crude product was chromatographed with flash chromatography on silica gel (ethyl acetate-n-exane mixture 1:5 as eluent) to furnish 4.05 g (56.6%) of pure **25q** as a white solid. Chemical, physical, and analytical data of derivative **25q** are reported in Table 3.

2-(3-formyl-1*H*-indol-1-yl)acetic acid (25r). 25q (3.95 g, 0.0152 mol) was treated with dichloromethane-trifluoroacetic acid mixture 1:1 (305 mL) to obtain a solution 0.05 M. The mixture was stirred for 16 h at room temperature. After removal of the solvent, the residue was triturated with diethyl ether for 1 h. The solid was filtered

and washed with diethyl ether to obtain 2.83 g (92%) of pure **25r** as a pink solid. Chemical, physical, and analytical data of derivative **25r** are reported in Table 3.

General procedure for the synthesis of 3-formyl-1H-indole 25s-t. 25r (0.0026 mol) is solubilized in 13 mL of dry DMF to obtain a 0.2 M solution. The solution was trited with base (0.0026 mol) and HATU (0.0026 mol). The solution was cooled at 0 °C and treated with DIEA (0.0078 mol). After the addiction, the mixture was stirred for 2 days at room temperature. Ethyl acetate was added to the reacton and the obtained mixture was washed with HCl 1 N (three times), $NaHCO_{3ss}$ (three times) and brine (three times), dred and the solvent was evaporated under reduced pressure to obtain derivatives **25s-t**. Chemical, physical, and analytical data of derivatives **25s-t** are reported in Table 3.

General procedure for the synthesis of 4-(indol-3-yl)but-3-en-2-one 26a-p,s-t. A solution of appropriate aldehyde **25a-p** or **25s-t** (0.0311 mol) in 105.7 mL of acetone was treated with 45.2 mL of NaOH 5 N and stirred at 50 °C for 2 night and then was treated with water. The mixture was extracted with ethyl acetate. The collected organic extract was washed with brine (three times), and dried, and the solvent was evaporated under reduced pressure to obtain pure derivatives **26a-p,s-t**. Chemical, physical, and analytical data of derivatives **6a-p,s-t** are reported in Table 3.

General procedure for the synthesis of Diketo Esters 21a-p,s-t. The appropriated acetyl derivative **26a-p,s-t** (0.031 mol) and diethyl oxalate (0.062 mol) were dissolved in 31 mL of dry THF and treated, under argon stream, with NaOEt obtained by the dissolution of Na (0.063 mol) in 56.36 mL of absolute ethanol. The mixture was stirred at room temperature for 1 h 30 min, then was pured into *n*-hexane (704 mL). The collected precipitate was vigorously stirred for 30 min in 1 N HCl (704 mL). The solid that formed was filtered, washed with water and light petroleum ether and dried under IR lamp to efford the pure diketo ester **21a-p**,**s-t.** Yield (%), melting point (°C), recrystallization solvent, IR, ^{1}H NMR, and analytical data are reported for each of the following compounds.

6-(1-propyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21a). 76%; 105-107°C; toluene/cyclohexane; IR: ν 3400 (OH), 1750 ($COOC_2H_5$),

1725 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.88-0.92 (t, 3H, CH_3), 1.27-1.36 (t, 3H, CH_3), 1.83-1.88 (q, 2H, CH_2), 4.23-4.35 (m, 4H, CH_2), 6.72 (s, 1H, hexanoate C3-H), 6.95-6.99 (d, 1H, J_t = 14.8 Hz, hexanoate C5-H), 7.28-7.37 (m, 2H, indole C5-H, C6-H), 7.66-7.68 (d, 1H, indole C7-H), 8.05-8.09 (d, 1H, J_t = 14.8 Hz, hexanoate C6-H), 8.18 (m, 2H, indole C2-H, C4-H), 12-18 (sb, 1H, OH).

6-(1-butyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21b). 14%; 90-92°C; washed with isopropylic ether; IR: ν 3400 (OH), 1745 ($COOC_2H_5$), 1715 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.92-0.95 (t, 3H, CH_3), 1.28-1.36 (m, 5H, CH_2 and CH_3), 1.80-1.83 (q, 2H, CH_2), 4.26-4.34 (m, 4H, CH_2), 6.72 (s, 1H, hexanoate C3-H), 6.95-6.99 (d, 1H, J_t = 15.7 Hz, hexanoate C5-H), 7.28-7.37 (m, 2H, indole C5-H, C6-H), 7.66-7.68 (d, 1H, indole C7-H), 8.05-8.09 (d, 1H, J_t = 15.7 Hz, hexanoate C6-H), 8.18 (m, 2H, indole C2-H, C4-H), 12-18 (sb, 1H, OH).

6-(1-isopentyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21c). 58%; 107-109°C; washed with isopropylic ether; IR: ν 3400 (OH), 1725 ($COOC_2H_5$), 1617 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.92-0.98 (m, 6H, CH_3), 1.32-1.38 (t, 3H, CH_3), 1.55-1.58 (m, 1H, CH), 1.72-1.74 (q, 2H, CH_2), 4.28-4.29 (m, 4H, CH_2), 6.57 (s, 1H, hexanoate C3-H), 7.06 (sb, 1H, hexanoate C5-H), 7.27-7.36 (m, 2H, indole C5-H, C6-H), 7.62-7.64 (d, 1H, indole C7-H), 7.97-8.01 (d, 1H, hexanoate C6-H), 8.13 (s, 2H, indole C2-H, C4-H), 12-18 (sb, 1H, OH).

6-(1-(prop-1-enyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21d). 62%; 81-82°C; washed with isopropylic ether; IR: ν 3400 (OH), 1725 ($COOC_2H_5$), 1617 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.33-1.37 (t, 3H, CH_3), 1.83-1.86 (m, 3H, CH_3), 4.31-4.36 (m, 4H, CH_2), 5.78-5.82 (m, 1H, CH), 6.76 (s, 1H, hexanoate C3-H), 7.07-7.11 (m, 2H, hexanoate C5-H and CH), 7.33-7.41 (m, 2H, indole C5-H, C6-H), 7.55-7.57 (d, 1H, indole C7-H), 8.11-8.15 (d, 1H, J_t = 15.9 Hz, hexanoate C6-H), 8.21-8.23 (s, 1H, indole C4-H), 8.29 (s, 1H, indole C2-H), 12-18 (sb, 1H, OH).

6-(1-(but-2-enyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21e). 66%; 124-126°C; washed with isopropylic ether; IR: ν 3400 (OH), 1729

($COOC_2H_5$), 1611 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.33-1.36 (t, 3H, CH_3), 1.69-1.70 (d, 3H, CH_3), 4.30-4.36 (q, 2H, CH_2), 4.84-4.85 (d, 2H, CH_2), 5.72-5.76 (m, 2H, CH), 6.73 (s, 1H, hexanoate C3-H), 6.69-7.00 (d, 1H, J_t = 15.8 Hz, hexanoate C5-H), 7.28-7.36 (m, 2H, indole C5-H, C6-H), 7.62-7.64 (d, 1H, indole C7-H), 8.06-8.10 (d, 1H, J_t = 15.8 Hz, hexanoate C6-H), 8.13 (s, 1H, indole C2-H), 8.16-8.18 (d, 1H, indole C4-H), 12-18 (sb, 1H, OH).

6-(1-(2-methylprop-1-enyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21f). 56%; 130-132°C; washed with isopropanol; IR: ν 3400 (OH), 1729 ($COOC_2H_5$), 1611 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.32-1.36 (t, 3H, CH_3), 1.73 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 4.31-4.32 (q, 2H, CH_2), 6.73 (sb, 1H, hexanoate C3-H), 6.83 (s, 1H, CH), 7.08 (sb, 1H, hexanoate C5-H), 7.32-7.39 (m, 2H, indole C5-H, C6-H), 7.45-7.47 (d, 1H, indole C7-H), 8.05 (sb, 1H, hexanoate C6-H), 8.13 (s, 1H, indole C2-H), 8.23-8.24 (d, 1H, indole C4-H), 12-18 (sb, 1H, OH).

6-(1-(2-methylallyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (1g). 74%; 81-84°C; washed with isopropylic ether; IR: ν 3400 (OH), 1729 ($COOC_2H_5$), 1611 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.32-1.36 (t, 3H, CH_3), 1.73 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 4.31-4.32 (q, 2H, CH_2), 6.73 (sb, 1H, hexanoate C3-H), 6.83 (s, 1H, CH), 7.08 (sb, 1H, hexanoate C5-H), 7.32-7.39 (m, 2H, indole C5-H, C6-H), 7.45-7.47 (d, 1H, indole C7-H), 8.05 (sb, 1H, hexanoate C6-H), 8.13 (s, 1H, indole C2-H), 8.23-8.24 (d, 1H, indole C4-H), 12-18 (sb, 1H, OH).

6-(1-benzyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21h). 100%; 118-120°C; Toluene; IR: ν 3400 (OH), 1727 ($COOC_2H_5$), 1630 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.24-1.28 (t, 3H, CH_3), 4.21-4.22 (q, 2H, CH_2), 5.46 (s, 2H, CH_2), 6.53 (sb, 1H, hexanoate C3-H), 7.08 (sb, 1H, hexanoate C5-H), 7.18-7.33 (m, 7H, benzene H, indole C5-H, C6-H), 7.55-7.57 (d, 1H, indole C7-H), 7.87 (sb, 1H, hexanoate C6-H), 8.08 (s, 1H, indole C2-H), 8.14-8.16 (d, 1H, indole C4-H), 12-18 (sb, 1H, OH).

6-(1-(4-chlorobenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21i). 100%; 120-122°C; washed with isopropanol; IR: ν 3400 (OH), 1724 ($COOC_2H_5$), 1608 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.31 (t, 3H, CH_3), 4.28 (q, 2H, CH_2), 5.53 (s, 2H, CH_2), 6.71 (sb, 1H, hexanoate C3-H), 7.00 (sb, 1H, hexanoate C5-H), 7.25-7.34 (m, 4H, benzene H, indole C5-H, C6-H), 7.43-7.45 (m, 2H, benzene H), 7.60-7.62 (d, 1H, indole C7-H), 8.05 (s, 1H, J_t = 13.95 Hz, hexanoate C6-H), 8.16-8.23 (m, 2H, indole C2-H and C4-H), 12-18 (sb, 1H, OH).

6-(1-(4-fluorobenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21j). 91%; 116-119°C; washed with isopropanol; IR: ν 3400 (OH), 1741 ($COOC_2H_5$), 1619 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.35 (t, 3H, CH_3), 4.34 (q, 2H, CH_2), 5.53 (s, 2H, CH_2), 6.75 (s, 1H, hexanoate C3-H), 7.00-7.02 (s, 1H, J_t = 15.7 Hz, hexanoate C5-H), 7.19-7.22 (m, 4H, benzene H, indole C5-H, C6-H), 7.38-7.42 (m, 2H, benzene H), 7.65-7.67 (d, 1H, indole C7-H), 8.05-8.08 (s, 1H, J_t = 15.7 Hz, hexanoate C6-H), 8.18-8.20 (d, 1H, indole C4-H), 8.30 (s, 1H, indole C4-H), 12-18 (sb, 1H, OH).

6-(1-(4-cyanobenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21k). 100%; 108-110°C; washed with isopropanol; IR: ν 3400 (OH), 2226 (CN), 1720 ($COOC_2H_5$), 1600 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.33 (t, 3H, CH_3), 4.34 (q, 2H, CH_2), 5.67 (s, 2H, CH_2), 6.74 (s, 1H, hexanoate C3-H), 7.02-7.05 (s, 1H, J_t = 16.8 Hz, hexanoate C5-H), 7.30-7.32 (m, 2H, indole C5-H, C6-H), 7.44-7.45 (d, 2H, benzene H), 7.58-7.60 (d, 1H, indole C7-H), 7.85-7.86 (d, 2H, benzene H), 8.05-8.08 (s, 1H, J_t = 16.8 Hz, hexanoate C6-H), 8.18-8.20 (d, 1H, indole C4-H), 8.32 (s, 1H, indole C4-H), 12-18 (sb, 1H, OH).

6-(1-(4-methoxybenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21l). 68%; 128-130°C; washed with isopropanol; IR: ν 3400 (OH), 1718 ($COOC_2H_5$), 1608 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.30 (t, 3H, CH_3), 3.74 (s, 3H, CH_3), 4.33 (q, 2H, CH_2), 5.39 (s, 2H, CH_2), 6.70-6.76 (m, 3H, benzene H and hexanoate C3-H), 6.98-7.01 (bs, 1H, hexanoate C5-H), 7.19-7.21 (d, 2H, benzene H), 7.26-7.35 (m, 2H, indole C5-H, C6-H), 7.65-7.66 (d, 1H, indole C7-H), 8.06-8.08 (bs, 1H, hexanoate C6-H), 8.15-8.16 (d, 1H, indole C4-H), 8.28 (s, 1H, indole C4-

H), 12-18 (sb, 1H, OH).

6-(1-(4-hydroxybenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21m). 80%; 148-150°C; washed with isopropanol; IR: ν 3400 (OH), 1724 ($COOC_2H_5$), 1614 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.30 (t, 3H, CH_3), 4.33 (q, 2H, CH_2), 5.39 (s, 2H, CH_2), 6.70-6.76 (m, 3H, benzene H and hexanoate C3-H), 6.98-7.01 (s, 1H, J_t = 15.9 Hz, hexanoate C5-H), 7.19-7.21 (d, 2H, benzene H), 7.26-7.35 (m, 2H, indole C5-H, C6-H), 7.65-7.66 (d, 1H, indole C7-H), 8.06-8.08 (s, 1H, J_t = 15.9 Hz, hexanoate C6-H), 8.15-8.16 (d, 1H, indole C4-H), 8.28 (s, 1H, indole C4-H), 10.00 (sb, 1H OH fenole), 12-18 (sb, 1H, OH).

6-(1-phenyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21n). 64%; °C; washed with isopropanol; IR: ν 3400 (OH), 1725 ($COOC_2H_5$), 1610 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.29 (t, 3H, CH_3), 4.28 (q, 2H, CH_2), 6.1 (s, 1H, hexanoate C3-H), 7.06-7.10 (s, 1H, J_t = 15.9 Hz, hexanoate C5-H), 7.32-7.34 (m, 2H, indole C5-H, C6-H), 7.47-7.63 (m, 6H, benzene H and indole C7-H), 8.05-8.11 (s, 1H, J_t = 15.9 Hz, hexanoate C6-H), 8.22 (s, 1H, indole C4-H), 8.37 (s, 1H, indole C4-H), 12-18 (sb, 1H, OH).

6-(1-(4-hydroxyphenyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21o). 73%; 151-153°C; toluene; IR: ν 3400 (OH), 1726 ($COOC_2H_5$), 1618 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.35 (t, 3H, CH_3), 4.33 (q, 2H, CH_2), 6.76 (s, 1H, hexanoate C3-H), 7.00-7.03 (d, 2H, benzene H), 7.08-7.12 (d, 1H, J_t = 15.9 Hz, hexanoate C5-H), 7.36-7.37 (m, 2H, indole C5-H, C6-H), 7.45-7.49 (m, 4H, benzene H and indole C7-H), 8.11-8.15 (s, 1H, J_t = 15.9 Hz, hexanoate C6-H), 8.26 (m, 1H, indole C4-H), 8.32 (s, 1H, indole C4-H), 10.00 (bs, 1H, OH fenole), 12-18 (sb, 1H, OH).

6-(1-(4-chlorophenyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21p). 74%; 142-144°C; washed with isopropanol; IR: ν 3400 (OH), 1726 ($COOC_2H_5$), 1618 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.35 (t, 3H, CH_3), 4.33 (q, 2H, CH_2), 6.76 (s, 1H, hexanoate C3-H), 7.00-7.03 (d, 2H, benzene H), 7.08-7.11 (d, 1H, J_t = 15.6 Hz, hexanoate C5-H), 7.36-7.37 (m, 2H, indole C5-H, C6-H), 7.45-

7.49 (m, 4H, benzene H and indole C7-H), 8.11-8.14 (s, 1H, J_t = 15.6 Hz, hexanoate C6-H), 8.26 (m, 1H, indole C4-H), 8.32 (s, 1H, indole C4-H), 12-18 (sb, 1H, OH).

6-(1-((dimethylcarbamoyl)methyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21s). 100%; 188-190°C; washed with isopropylic ether; IR: ν 3400 (OH), 1734 (COOH), 1618 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 4.78-4.81 (m, 6H, CH_3), 6.69 (sb, 1H, hexanoate C3-H), 6.80 (s, 1H, CH_2), 7.00 (sb, 1H, hexanoate C5-H), 7.31-7.38 (m, 2H, indole C5-H, C6-H), 7.45-7.47 (d, 1H, indole C7-H), 8.06-8.10 (d, 1H, hexanoate C6-H), 8.12 (s, 1H, indole C2-H), 8.18-8.20 (d, 1H, indole C4-H), 12-18 (sb, 2H, OH).

6-(1-(2-morpholino-2-oxoethyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21t). 74%; 183°C; washed with isopropylic ether; IR: ν 3400 (OH), 1730 (COOH), 1620 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.24 (m, 4H, morpholine H), 3.72 (m, 4H, morpholine H), 6.69 (sb, 1H, hexanoate C3-H), 6.85 (s, 1H, CH_2), 7.00 (sb, 1H, hexanoate C5-H), 7.31-7.38 (m, 2H, indole C5-H, C6-H), 7.45-7.47 (d, 1H, indole C7-H), 8.06-8.10 (d, 1H, hexanoate C6-H), 8.12 (s, 1H, indole C2-H), 8.18-8.20 (d, 1H, indole C4-H), 12-18 (sb, 2H, OH).

General procedure for the synthesis of Diketo Acids 22a-p,s-t. A mixture of 1 N NaOH (9.48 mL) and the appropriated ester **21a-p,s-t** (0.0019 mol) in 1:1 THF-Methanol (9.32 mL) was stirred at room temperature for 30 min and then pured into crushed ice. The acquous mixture was treated with 1 N HCl until Ph 3 was reached, and extracted with ethyl acetate (three times). The collected organic extract was washed with brine (three times), and dried, and the solvent was evaporated under reduced pressure to give the pure diketo acids **22a-p,s-t.** Yield (%), melting point (°C), recrystallization solvent, IR, ^1H NMR, and analytical data are reported for each of the following compounds.

6-(1-(2-morpholino-2-oxoethyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid Ethyl Ester (21t). 74%; 183°C; washed with isopropylic ether; IR: ν 3400 (OH), 1730 (COOH), 1620 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.24 (m, 4H, morpholine H), 3.72 (m, 4H, morpholine H), 6.69 (sb, 1H, hexanoate C3-H), 6.85 (s,

1H, CH_2), 7.00 (sb, 1H, hexanoate C5-H), 7.31-7.38 (m, 2H, indole C5-H, C6-H), 7.45-7.47 (d, 1H, indole C7-H), 8.06-8.10 (d, 1H, hexanoate C6-H), 8.12 (s, 1H, indole C2-H), 8.18-8.20 (d, 1H, indole C4-H), 12-18 (sb, 2H, OH).

6-(1-propyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22a). 93%; 153-155°C; washed with isopropanol; IR: ν 3400 (OH), 1720 (COOH), 1591 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.88-0.92 (t, 3H, CH_3), 1.27-1.36 (t, 3H, CH_3), 1.83-1.88 (q, 2H, CH_2), 4.23-4.35 (m, 4H, CH_2), 6.69 (s, 1H, hexanoate C3-H), 6.92-6.95 (d, 1H, J_t = 15.5 Hz, hexanoate C5-H), 7.27-7.36 (m, 2H, indole C5-H, C6-H), 7.65-7.67 (d, 1H, indole C7-H), 8.03-8.07 (d, 1H, J_t = 15.5 Hz, hexanoate C6-H), 8.16 (m, 2H, indole C2-H, C4-H), 12-18 (sb, 2H, OH)

6-(1-butyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22b). 87%; 144-146°C; washed with hot absolute ethanol; IR: ν 3400 (OH), 1754 (COOH), 1586 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.92-0.95 (t, 3H, CH_3), 1.28-1.32 (q, 2H, CH_2), 1.80-1.83 (q, 2H, CH_2), 4.26-4.29 (q, 2H, CH_2), 6.68 (s, 1H, hexanoate C3-H), 6.96 (d, 1H, hexanoate C5-H), 7.27-7.36 (m, 2H, indole C5-H, C6-H), 7.65-7.67 (d, 1H, indole C7-H), 8.02-8.06 (d, 1H, J_t = 15.7 Hz, hexanoate C6-H), 8.18 (m, 2H, indole C2-H, C4-H), 12-18 (sb, 2H, OH).

6-(1-isopentyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22c). 30%; 134-136°C; washed with hot absolute ethanol; IR: ν 3400 (OH), 1707 (COOH), 1570 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.96-0.98 (m, 6H, CH_3), 1.57-1.58 (m, 1H, CH), 1.72-1.76 (q, 2H, CH_2), 4.27-4.31 (q, 2H, CH_2), 6.74 (s, 1H, hexanoate C3-H), 6.94 (sb, 1H, hexanoate C5-H), 7.27-7.37 (m, 2H, indole C5-H, C6-H), 7.64-7.66 (d, 1H, indole C7-H), 8.03-8.05 (d, 1H, hexanoate C6-H), 8.18 (s, 2H, indole C2-H, C4-H), 12-18 (sb, 2H, OH).

6-(1-(prop-1-enyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22d). 64%; 126-128°C; washed with isopranol; IR: ν 3400 (OH), 1728 (COOH), 1663 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.84-1.86 (m, 3H, CH_3), 5.76-5.80 (m, 1H, CH), 6.76 (s, 1H, hexanoate C3-H), 7.04-7.09 (m, 2H, hexanoate C5-H and CH), 7.32-7.40 (m, 2H, indole C5-H, C6-H), 7.54-7.56 (d, 1H, indole C7-H), 8.05 (sb, 1H,

hexanoate C6-H), 8.18-8.24 (m, 2H, indole C2-H and C4-H), 12-18 (sb, 2H, OH).

6-(1-(but-2-enyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22e). 88%; 163-165°C; washed with hot absolute ethanol; IR: ν 3400 (OH), 1754 (COOH), 1588 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.69-1.70 (d, 3H, CH_3), 4.84-4.85 (d, 2H, CH_2), 5.68-5.79 (m, 2H, CH), 6.71 (s, 1H, hexanoate C3-H), 6.93-6.97 (d, 1H, J_t = 15.6 Hz, hexanoate C5-H), 7.27-7.36 (m, 2H, indole C5-H, C6-H), 7.61-7.63 (d, 1H, indole C7-H), 8.05-8.09 (d, 1H, J_t = 15.6 Hz, hexanoate C6-H), 8.12 (s, 1H, indole C2-H), 8.14-8.16 (d, 1H, indole C4-H), 12-18 (sb, 2H, OH).

6-(1-(2-methylprop-1-enyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22f). 78%; 165°C; washed with hot absolute ethanol; IR: ν 3400 (OH), 1719 (COOH), 1588 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.73 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 6.71 (sb, 1H, hexanoate C3-H), 6.83 (s, 1H, CH), 7.04 (sb, 1H, hexanoate C5-H), 7.31-7.38 (m, 2H, indole C5-H, C6-H), 7.45-7.47 (d, 1H, indole C7-H), 8.06-8.10 (d, 1H, J_t = 15.8 Hz, hexanoate C6-H), 8.12 (s, 1H, indole C2-H), 8.18-8.20 (d, 1H, indole C4-H), 12-18 (sb, 2H, OH).

6-(1-(2-methylallyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22g). 86%; 149-151°C; washed with hot absolute ethanol; IR: ν 3400 (OH), 1719 (COOH), 1588 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.73 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 6.71 (sb, 1H, hexanoate C3-H), 6.83 (s, 1H, CH), 7.04 (sb, 1H, hexanoate C5-H), 7.31-7.38 (m, 2H, indole C5-H, C6-H), 7.45-7.47 (d, 1H, indole C7-H), 8.06-8.10 (d, 1H, J_t = 15.8 Hz, hexanoate C6-H), 8.12 (s, 1H, indole C2-H), 8.18-8.20 (d, 1H, indole C4-H), 12-18 (sb, 2H, OH).

6-(1-benzyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22h). 68%; 178-180°C; DMF/H_2O; IR: ν 3400 (OH), 1754 (COOH), 1619 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 5.48 (s, 2H, CH_2), 6.64 (sb, 1H, hexanoate C3-H), 6.93 (sb, 1H, hexanoate C5-H), 7.20-7.33 (m, 7H, benzene H, indole C5-H, C6-H), 7.55-7.57 (d, 1H, indole C7-H), 7.99 (sb, 1H, hexanoate C6-H), 8.09 (d, 1H, indole C4-H), 8.21 (s, 1H, indole C2-H), 12-18 (sb, 2H, OH).

6-(1-(4-chlorobenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22i). 64%; 192-194°C; DMF/H_2O; IR: ν 3400 (OH), 1727 (COOH), 1607 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 5.59 (s, 2H, CH_2), 6.71 (sb, 1H, hexanoate C3-H), 6.96-6.98 (s, 1H, J_t = 13.95 Hz, hexanoate C5-H), 7.23-7.25 (m, 4H, benzene H, indole C5-H, C6-H), 7.41-7.46 (m, 2H, benzene H), 7.60-7.62 (d, 1H, indole C7-H), 8.05 (s, 1H, J_t = 13.95 Hz, hexanoate C6-H), 8.16 (d, 1H, indole C4-H), 8.27 (s, 1H, indole C2-H), 12-18 (sb, 2H, OH).

6-(1-(4-fluorobenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22j). 77%; 168-170°C; DMF/H_2O; IR: ν 3400 (OH), 1725 (COOH), 1603 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 5.60 (s, 2H, CH_2), 6.71 (sb, 1H, hexanoate C3-H), 6.96-6.99 (s, 1H, J_t = 15.5 Hz, hexanoate C5-H), 7.19-7.24 (m, 2H, benzene H), 7.27-7.34 (m, 2H, indole C5-H, C6-H), 7.39-7.41 (m, 2H, benzene H), 7.60-7.62 (d, 1H, indole C7-H), 8.05 (s, 1H, J_t = 15.5 Hz, hexanoate C6-H), 8.16 (d, 1H, indole C4-H), 8.28 (s, 1H, indole C2-H), 12-18 (sb, 2H, OH).

6-(1-(4-cyanobenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22k). 91%; 168-170°C; ethanol; IR: ν 3400 (OH), 2227 (CN), 1708 (COOH), 1600 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 5.60 (s, 2H, CH_2), 6.73 (sb, 1H, hexanoate C3-H), 6.96-7.00 (s, 1H, J_t = 15.7 Hz, hexanoate C5-H), 7.30-7.33 (m, 2H, indole C5-H, C6-H), 7.43-7.44 (d, 2H, benzene H), 7.59-7.61 (d, 1H, indole C7-H), 7.86 (d, 2H, benzene H), 8.05-8.08 (s, 1H, J_t = 15.7 Hz, hexanoate C6-H), 8.19 (d, 1H, indole C4-H), 8.29 (s, 1H, indole C2-H), 12-18 (sb, 2H, OH).

6-(1-(4-methoxybenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22l). 60%; 182-184°C; DMF/H_2O; IR: ν 3400 (OH), 1727 (COOH), 1604 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.75 (s, 3H, CH_3), 5.39 (s, 2H, CH_2), 6.71-6.76 (m, 3H, benzene H and hexanoate C3-H), 6.96-7.00 (s, 1H, J_t = 15.8 Hz, hexanoate C5-H), 7.20 (d, 2H, benzene H), 7.26-7.33 (m, 2H, indole C5-H, C6-H), 7.65-7.66 (d, 1H, indole C7-H), 8.05-8.09 (s, 1H, J_t = 15.8 Hz, hexanoate C6-H), 8.25 (d, 1H, indole C4-H), 8.29 (s, 1H, indole C2-H), 12-18 (sb, 2H, OH).

6-(1-(4-hydroxybenzyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22m). 100%; 158-160°C; DMF/H_2O; IR: ν 3400 (OH), 1723 (COOH), 1606 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 5.39 (s, 2H, CH_2), 6.71-6.76 (m, 3H, benzene H and hexanoate C3-H), 6.96-7.00 (s, 1H, J_t = 15.8 Hz, hexanoate C5-H), 7.20 (d, 2H, benzene H), 7.26-7.33 (m, 2H, indole C5-H, C6-H), 7.65-7.66 (d, 1H, indole C7-H), 8.05-8.09 (s, 1H, J_t = 15.8 Hz, hexanoate C6-H), 8.25 (d, 1H, indole C4-H), 8.29 (s, 1H, indole C2-H), 9.5 (sb, 1H, OH fenole), 12-18 (sb, 2H, OH).

6-(1-phenyl-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22n). 98%; °C; washed with isopropanol; IR: ν 3400 (OH), 1754 (COOH), 1616 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 6.57 (bs, 1H, hexanoate C3-H), 7.05 (bs, 1H, hexanoate C5-H), 7.30-7.32 (m, 2H, indole C5-H, C6-H), 7.46-7.64 (m, 6H, benzene H and indole C7-H), 8.01 (s, 1H, hexanoate C6-H), 8.20 (d, 1H, indole C4-H), 8.33 (s, 1H, indole C2-H), 12-18 (sb, 2H, OH).

6-(1-(4-hydroxyphenyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22o). 64&; 167-169°C; DMF/H_2O; IR: ν 3400 (OH), 1764 (COOH), 1606 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 6.75 (bs, 1H, hexanoate C3-H), 7.00-7.08 (m, 3H, benzene H and hexanoate C5-H), 7.34-7.36 (m, 2H, indole C5-H, C6-H), 7.45-7.50 (m, 3H, benzene H and indole C7-H), 8.09 (bs, 1H, hexanoate C6-H), 8.24 (d, 1H, indole C4-H), 8.30 (s, 1H, indole C2-H), 9.93 (s, 1H, OH fenole), 12-18 (sb, 2H, OH).

6-(1-(4-chlorophenyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22p). 97%; 168-170°C; washed with hot absolute ethanol; IR: ν 3400 (OH), 1764 (COOH), 1606 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 6.75 (bs, 1H, hexanoate C3-H), 7.00-7.08 (m, 3H, benzene H and hexanoate C5-H), 7.34-7.36 (m, 2H, indole C5-H, C6-H), 7.45-7.50 (m, 3H, benzene H and indole C7-H), 8.09 (bs, 1H, hexanoate C6-H), 8.24 (d, 1H, indole C4-H), 8.30 (s, 1H, indole C2-H), 12-18 (sb, 2H, OH).

6-(1-((dimethylcarbamoyl)methyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22s). 72%; 185-187°C; washed with isopropylic ether; IR: ν 3400 (OH), 1720 (COOH), 1598 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 4.80-4.84 (m, 6H, CH_3), 6.71 (sb, 1H, hexanoate C3-H), 6.83 (s, 1H, CH_2), 7.04 (sb, 1H, hexanoate

C5-H), 7.31-7.38 (m, 2H, indole C5-H, C6-H), 7.45-7.47 (d, 1H, indole C7-H), 8.06-8.10 (d, 1H, hexanoate C6-H), 8.12 (s, 1H, indole C2-H), 8.18-8.20 (d, 1H, indole C4-H), 12-18 (sb, 2H, OH).

6-(1-(2-morpholino-2-oxoethyl)-1*H*-indol-3-yl)-2-hydroxy-4-oxo-2,5-hexadienoic Acid (22t). 70%; 185°C; washed with hot absolute ethanol; IR: ν 3400 (OH), 1719 (COOH), 1578 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.24 (m, 4H, morpholine H), 3.72 (m, 4H, morpholine H), 6.71 (sb, 1H, hexanoate C3-H), 6.78 (s, 1H, CH_2), 7.00 (sb, 1H, hexanoate C5-H), 7.31-7.38 (m, 2H, indole C5-H, C6-H), 7.45-7.47 (d, 1H, indole C7-H), 8.06-8.10 (d, 1H, hexanoate C6-H), 8.12 (s, 1H, indole C2-H), 8.18-8.20 (d, 1H, indole C4-H), 12-18 (sb, 2H, OH).

In Table 5 are reported the chemical-physical characteristic of the syntetic intermediete **25a-t, 26a-p,s,t.**

Table 5. Chemical, Physical, and Analytical Data of Derivatives **25a-t, 26a-p,s,t.**

Cpd	R	R_1	mp (°C)	recryst solvent[a]	Yeld (%)
25a	-	$(CH_2)_2CH_3$	104-106	a	76
25b	-	$(CH_2)_3CH_3$	oil	oil	50
25c	-	$(CH_2)_2CH(CH_3)_2$	oil	oil	88
25d	-	$CH=CHCH_3$	oil	oil	38
25e	-	$CH_2CH=CHCH_3$	-	-	18
25f	-	$CH=C(CH_3)_2$	oil	oil	32
25g	-	$CH_2C(CH=CH)CH_3$	oil	oil	88
25h	-	Bn[b]	102-104	a	93
25i	-	4-Cl-Bn[b]	115-117	e	66
25j	-	4-F-Bn[b]	112-114	e	62
25k	-	4-CN-Bn[b]	146-148	b	81
25l	-	4-OCH_3-Bn[b]	123-126	f	68
25m	-	4-OH-Bn[b]	180-182	b	76
25n	-	Ph[c]	-	-	47
25o	-	4-OH-Ph[c]	233-234	c	12
25p	-	4-Cl-Ph[c]	161-163	c	27
25q	-	$CH_2COOtBu$	125-127	h	56
25r	-	CH_2COOH	205-207	d	92
25s	-	$CH_2(C=O)N(CH_3)_2$	127-128	b	88
25t	-	$CH_2(C=O)$Morph[d]	105-106	b	100
26a	-	$(CH_2)_2CH_3$	oil	oil	97
26b	-	$(CH_2)_3CH_3$	oil	oil	100
26c	-	$(CH_2)_2CH(CH_3)_2$	oil	oil	87

26d	-	$CH=CHCH_3$	oil	oil	100
26e	-	$CH_2CH=CHCH_3$	oil	oil	100
26f	-	$CH=C(CH_3)_2$	oil	oil	99
26g	-	$CH_2C(CH=CH)CH_3$	93-95	e	80
26h	-	Bn[b]	127-129	b	92
26i	-	4-Cl-Bn[b]	147-149	g	98
26j	-	4-F-Bn[b]	126-128	g	83
26k	-	4-CN-Bn[b]	158-160	c	68
26l	-	$4\text{-}OCH_3$-Bn[b]	126-128	g	60
26m	-	4-OH-Bn[b]	154-156	c	100
26n	-	Ph[c]	oil	oil	79
26o	-	4-OH-Ph[c]	162-164	b	62
26p	-	4-Cl-Ph[c]	153-155	g	92
26s	-	$CH_2(C=O)N(CH_3)_2$	oil	oil	100
26t	-	$CH_2(C=O)$Morph[d]	179-181	g	32

[a] Recrystallization solvent: (a) toluene/cyclohexane; (b) toluene; (c) ethanol; (d) DMF/H_2O; (e) n-exane; (f) cyclohexane; (g) benzene; (h) toluene/benzene; (i) isopropanol/isopropylic ether [b] Bn: benzyl; [c] Ph: Phenyl; [d] Morph: morpholine.

Biology

The RNA-dependent RNA polymerase assay was performed in a total volume of 40 μL containing 1 nM enzyme, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20 units of RNase inhibitor, 50 μg/mL of actinomycin D, 5 μCi of [α-^{32}P]UTP, 0.5 mM each of remaining NTPs (ATP, CTP, GTP) with 10 μg/mL of RNA template. The concentration of the limiting nucleotide was adjusted to 10 μM. The reaction mixture were incubated at 30 °C for 2 h. After incubation, the reaction was stopped by digestion with 50 μg of protease K in a protease K buffer (150 mM NaCl, 50mM Tris-HCL (pH 7.5), and 0.5% SDS) for 30 min.The RNA products were extracted phenol/chloroform (1:1) before ethanol precipitation. After heat denaturation (94 °C, 2 min), these products were analized by 8M urea, 8% PAGE. The gels were dried and analyzed using a BAS 1000 Bioimage analyzer system (Fuji).

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