Inhibitors of the hepatitis C virus RNA-dependent RNA polymerase

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Abstract
Infections caused by Hepatitis C Virus (HCV) are a significant world health problem for which novel therapies are in urgent demand. The polymerase of HCV is responsible for the replication of viral RNA. We recently disclosed diketoacids **1** and dihydroxypyrimidine carboxylates **3** as novel, reversible inhibitors of the HCV NS5B polymerase. We report here the further development of **3** into the more potent 5,6-dihydroxy-2-(2-thienyl)pyrimidine-4-carboxylic acids. The structure activity relationship of these inhibitors is discussed in the context of their physicochemical properties, supporting the proposed binding model, which involves pyrophosphate like chelation of the active site Mg-ions. We also report on the discovery and synthesis of two related scaffolds, the *N*1-substituted pyrimidones and pyridine-*N*-oxides.

Keywords: Hepatitis C virus, NS5B polymerase, pyrimidines, *N*1-substituted pyrimidones, pyridine-*N*-oxides

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Introduction

Hepatitis C Virus (HCV) infection constitutes a global health problem, which affects more than 170 million individuals.[1,2] If untreated, more than 60% of these individuals will develop chronic liver disease, which in 15-20% of the cases can lead to chronic hepatitis, liver cirrhosis and even hepatocellular carcinoma.[3] The current therapy, a combination of pegylated α-interferon (IFN) and ribavirin, is of limited efficacy and poorly tolerated.[4] This has stimulated intense research programs in the pharmaceutical industry to find a broadly effective antiviral therapy. By analogy to HIV research, most efforts to develop antiviral agents for HCV have focused on inhibition of the essential viral enzymes.[5-7]

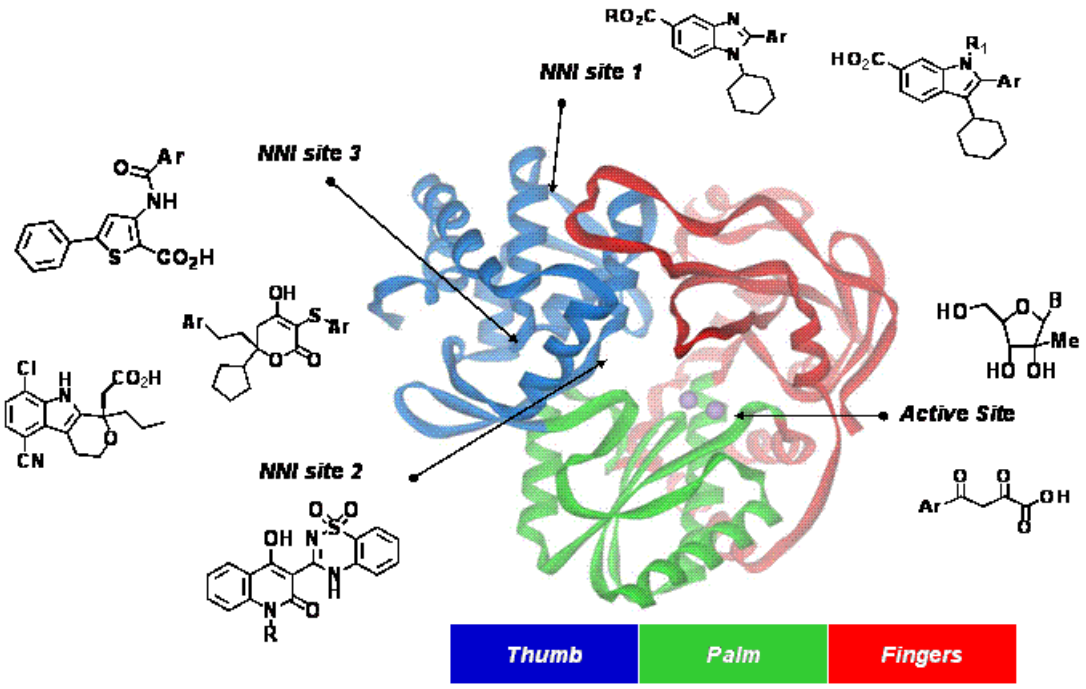


Figure 1. Structure of the HCV NS5B RNA polymerase with structures of selected inhibitors and their respective binding sites

HCV is a small, enveloped virus, belonging to the Flaviviridae, a family of positive strand RNA viruses. Its genome is approximately 9600 nucleotides in length and encodes a single polyprotein of about 3000 amino acids. This polyprotein undergoes maturational processing in the cytoplasm or in the endoplasmic reticulum of infected cells to generate structural and non-structural (NS) viral proteins.[8] Among the NS proteins are two viral enzymes, which are crucial for viral replication and consequently were selected as potential targets for antiviral therapy. The NS3 protease-helicase processes the viral polyprotein downstream of it and liberates the NS5B RNA dependent RNA polymerase (NS5B RdRp), the catalytic core of the HCV replication machinery.[9]

The NS5B RdRP has been characterized by biochemical methods and by X-ray crystallography. It adopts a tertiary structure which resembles a right hand, a motif common to most nucleotide polymerizing enzymes. The catalytic activity of the enzyme is mediated by two magnesium ions in the active site, which serve to activate the 3'-OH of the elongating RNA and position the incoming nucleotide-triphosphate for nucleophilic attack.[10-12]

NS5B is not expressed in uninfected cells, and due to its unique features presents an attractive target for the development of safe antiviral drugs. Several laboratories, including ours,[13] have disclosed non-nucleoside (NNI) inhibitors. These include a variety of heterocyclic systems, which have been shown to bind to three distinct sites on the polymerase (Figure 1).[14,15]

Apart from allosteric inhibitors, classical chain-terminating nucleoside (substrate) analogs,[16] and pyrophosphate (product) analogs like diketoacids **1** have been described (Figure 1, 2).[17] The latter were found by screening of the Merck compound collection. Diketoacids have been proposed to inhibit HIV-integrase by a mechanism involving chelation of active site magnesium ions.[18] Recently we reported that 1 inhibits selectively the NS5B RdRP elongation step.[17] Kinetic experiments indicated that **1** and Foscarnet,[19] a known pyrophosphate mimic and an approved antiviral drug, interact at a common binding site.

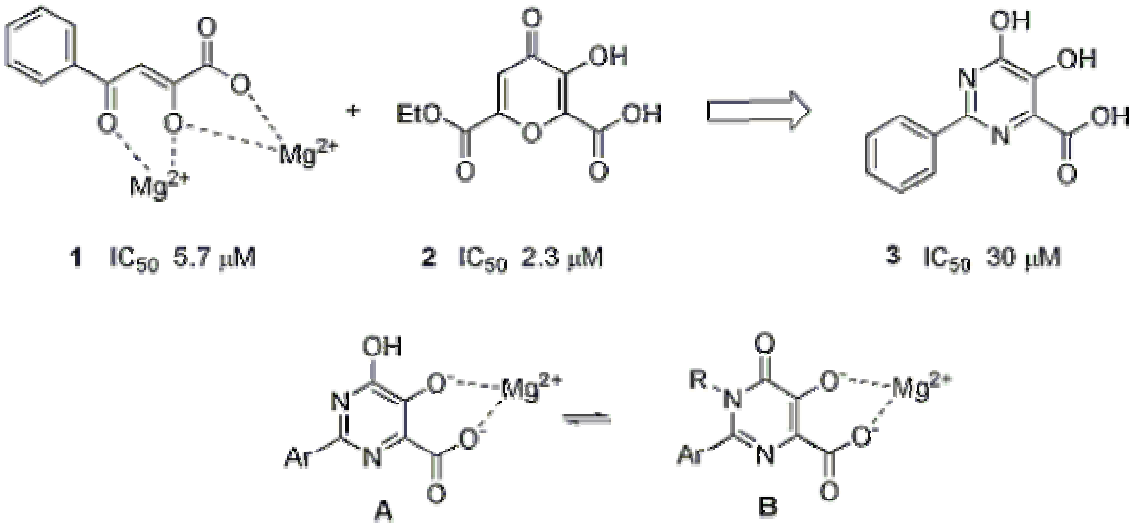


Figure 2

While structures like **1** could be converted to potent inhibitors of the NS5B polymerase by substitution on the phenyl ring,[17] the inherent reactivity of the diketoacid moiety presented a problem for the further development of these compounds as drug candidates.

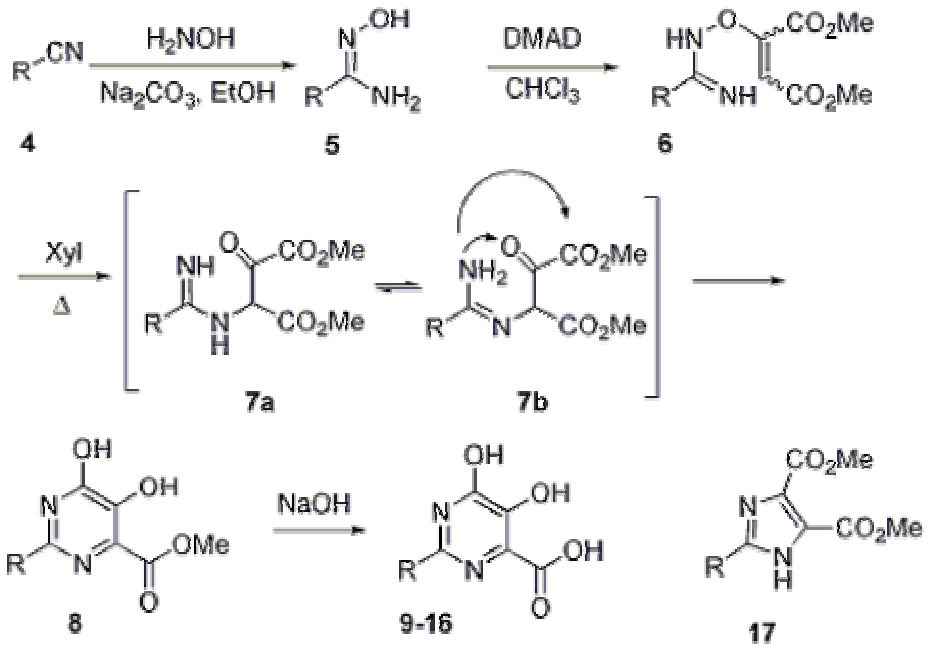
As second hit from random screening was the meconic acid derivative **2** (Figure 2). This compound was more potent than **1**, but the chemical instability prevented further exploration of this scaffold.[20] In the search for chemically and biologically stable diketoacid replacements 2-aryl-5,6-dihydroxy-pyrimidine-4-carboxylic acid **3** was identified.[21] Similar to diketoacids,

binding of **3** is mediated by divalent cations such as Mg^{2+} or Mn^{2+}. Furthermore, **3** was shown to be competitive with diketoacids, indicating that both interact at a common binding site, and suggesting that the dihydroxy acid moiety of **3** chelates the active site metal, as shown for structure **A** in its bis-anionic form. Dihydroxypyrimidine **A** can also exist in the tautomeric pyrimidone form **B** (Figure 2), which in principle is also capable of magnesium ion chelation. Presently, it is not known whether **A** or **B** or a mixture of them is responsible for the observed inhibition of NS5B RdRP, or if a bis- or monoanion is the active species. That pyrimidone **B** is an inhibitor was shown by *N*-methylation (R = Me) which gave an equally active pyrimidone.[22]

1. 5,6-Dihydroxypyrimidine-4-carboxylic acids

1.1 SAR in the 2-position

With respect to the diketoacid **1** the pyrimidine **3** is about 5-fold less active. In an attempt to improve the potency we successfully introduced side-chains onto the phenyl group of **3**,[23,24] and also investigated more broadly the SAR of the substituent in the 2-position of **3.** These derivatives were prepared using the published method by Culbertson (Scheme 1).[25]



Scheme 1. Synthesis of 2-substituted 4,5-dihydroxy-pyrimidine-4-carboxylic acids

Amidoximes **5**, obtained from nitriles **4**, were reacted with dimethylacetylene dicarboxylate (DMAD) to yield the Michael-adducts **6** as a mixture of geometrical isomers. Heating these in refluxing xylene gave the desired pyrimidine methyl esters **8** in yields ranging

from 3-65%, which after hydrolysis with sodium hydroxide gave the final products. Imidazoles **17** were observed in several cases in the LC-MS trace of the crude mixtures. Mechanistically, it is assumed that conversion of **6** to **8** follows a Claisen-type rearrangement via intermediate **7a**, which after tautomerization to **7b** could cyclize onto the ketone to give imidazoles or onto one ester group to yield pyrimidine esters.[25]

A wide variety of compounds were prepared and some of the more interesting analogs are presented in Table 1.[26] As the results show, an aryl group directly attached to the 2-position is clearly required for activity, since substituents such as cyclohexyl (**9**, IC_{50} > 50 μM), benzyl (**10**, IC_{50} > 50 μM) or 3-piperidinyl (**11**, IC_{50} > 50 μM) were essentially inactive with respect to **3**. Preferred are 5-membered aromatic heterocycles such as a thiophene (**13**, IC_{50} 2.6 μM), a furan (**14**, IC_{50} 2.9 μM), or a thiazole (**15**, IC_{50} 0.76 μM), the latter being one of the most active compounds.

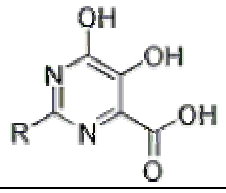
The electron-withdrawing nitro group in the 3-position of the thiophene led to another gain in potency, making **16** equipotent to thiazole **15**. For these pyrimidines we observed the dependence of the IC_{50} from the metal ion used in the assay (Table 1), as has been observed in the ketoacid series. This set of results indicated that an electron-withdrawing effect, going from thiophene to the more electro-withdrawing thiazole or nitrothiophene seems to be beneficial for potency, possibly by influencing the pK_a of the 5-hydroxy group. We assume that this group, together with the acid is involved in chelation of an active site Magnesium ion (see Figure 2), and changes in its pK_a will influence its chelating capabilities. Determination of the apparent pK_a -value of the hydroxyl group supports this hypothesis (Table 1).[26]

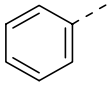
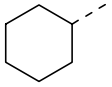
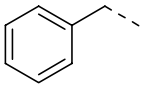
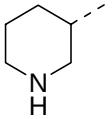
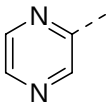
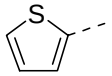
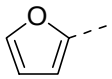
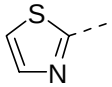
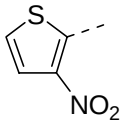
The pK_a of the phenolic hydroxyl group of all compounds with low micromolar activity is close to seven with inhibition being measured at pH 7.4. Thus the pH is close to the inflection point of the pH-dependent dissociation curve and consequently small variations of the pK_a can significantly change the ratio of the mono- and bis-anionic form under the assay conditions. Depending on which protonation state corresponds to the bio-active form this influences the measured inhibition. From the results presented in Table 1 it becomes clear that generally a more electron-withdrawing substituent in position 2 of the pyrimidine lowers the pK_a of the 5-hydroxy group.

The most active compounds, thiazole **15** and nitrothiophene **16** also have the lowest pK_a value. This means that decreasing pK_a increases the amount of the bis-anionic form under our assay condition (pH 7.4).

To test whether an increase in the amount of the doubly deprotonated species leads to an increase in potency the IC_{50} of the same compound was measured at different pH values. We observe for example a 2-fold gain in potency for **15** when the pH is raised to 8.0 (IC_{50} 0.34 μM instead of 0.76 μM), and a drop of 2-3-fold at pH 7.0 (IC_{50} 1.9 μM). Taking together these results provide evidence for the hypothesis that the doubly deprotonated pyrimidine **A** (Figure 2) is the bioactive species. Possibly this is due to its better capability to chelate Mg-ions.

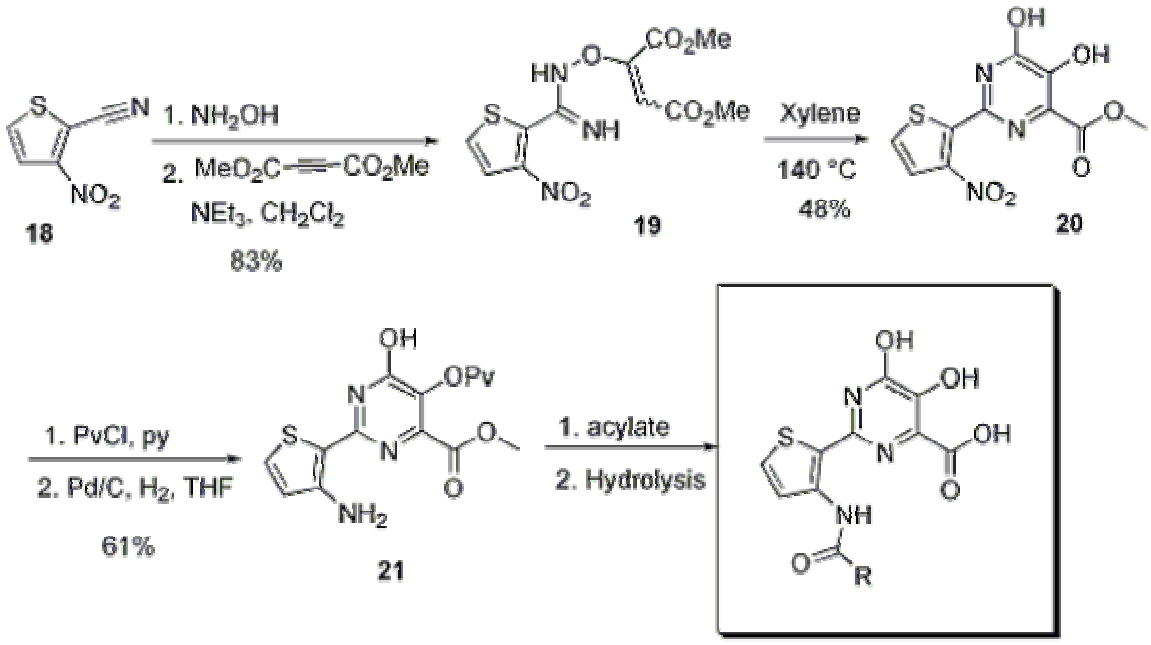
Table 1. Inhibition of NS5B RdRP by 2-substituted dihydroxypyrimidines **3, 9-16**



No.	R	IC_{50} (μM) Mg^{2+}	IC_{50} (μM) Mn^{2+}	pK_a
3		30	2.3	8.6
9		> 50		8.7
10		> 50		-
11		> 50		-
12		5.3		7.3
13		2.6	0.24	7.7
14		2.9	0.24	7.4
15		0.76	0.21	6.9
16		0.68	0.07	6.9

1.2. 2-(2-Thienyl)-5,6-dihydroxy-4-carboxy-pyrimidines

Further SAR around the thiophene **13** established that only substitution of the 3-position was tolerated. A library of around 200 compounds was prepared using the readily available amine **21**, which was reacted with a wide variety of acylating agents (Scheme 2) to give ureas, amides and carbamates.[26]



Scheme 2

From this work the benzyl urea **22** emerged as one of the most potent compounds (Figure 3). An aryl group at some distance from the thiophene further increased activity with respect to **13**. It was also found that the two hydroxyl groups as well as the acid are absolutely required for activity. The urea substituent causes a significant increase in the acidity of the 5-hydroxy group (pK_a 6.6) compared to unsubstituted **13**, which is somewhat unexpected since the urea is not directly attached to the pyrimidine. A possible explanation comes from modeling, which suggests a planar conformation for **22**, despite the presence of a substituent in the 3-position of the thiophene. In the conformation shown in Figure 3, it is possible for the inner urea NH to form a close intramolecular contact with the N3 of the pyrimidine and for the outer urea NH a second interaction with the carboxylate is possible. These interactions would enforce a planar conformation and could help to increase the acidity of **22**. An indirect proof comes from the *N*-methyl pyrimidone **23**, which is one order of magnitude less potent than **22**. As a consequence of the twist induced by the *N*-methyl group, the side-chain now occupies a different region of space, which explains the loss of potency. Interestingly, the pK_a of **23** is higher compared to **22** and does not change with respect the unsubstituted *N*-methyl 2-thienyl pyrimidone **27** (pK_a 9.1, see

Figure 4), confirming the hypothesis that the formation of close intramolecular contacts between the urea NH and the pyrimidine helps to increase acidity.

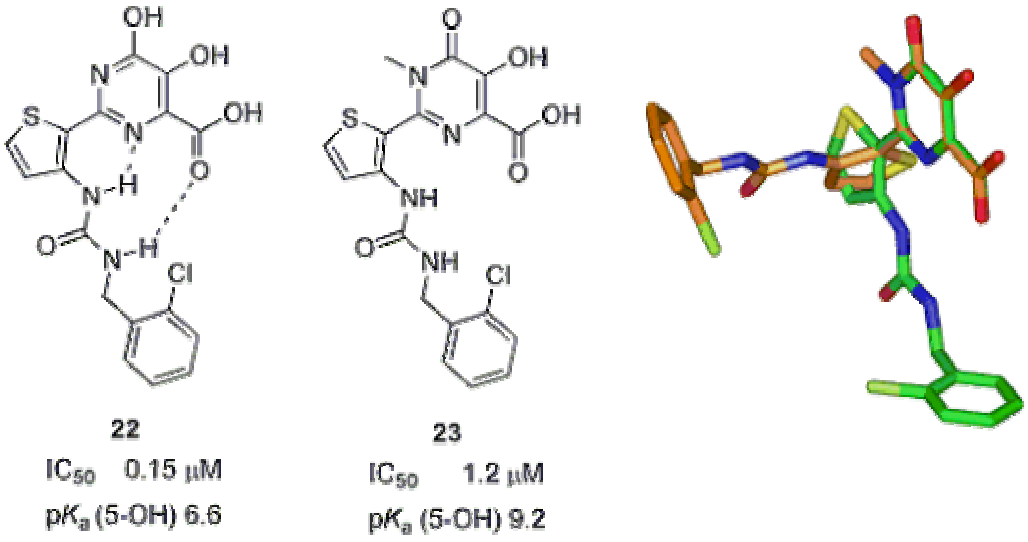


Figure 3. Structures and superposition of inhibitors **22** (green) and **23** (orange).

2. Investigation of the dihydroxypyrimidine core

During the development of the SAR around **13** we also investigated replacements of the dihydroxypyrimidine core, with the aim to replace some of the polar functionality on the pyrimidine. As the structures in Figure 4 show, this was not successful, except in the case of the *N*-methylpyrimidone **27** and the pyridine-*N*-oxide **28**, which showed similar potency with respect to the parent dihydroxypyrimidines **13** and **3**.[22,27]

The initial synthesis for **27** and **28** did not allow the introduction of a wide variety of substituents, and thus limited a full exploration of the SAR. To overcome these limitations we investigated several possible routes towards these novel ketoacid replacements.

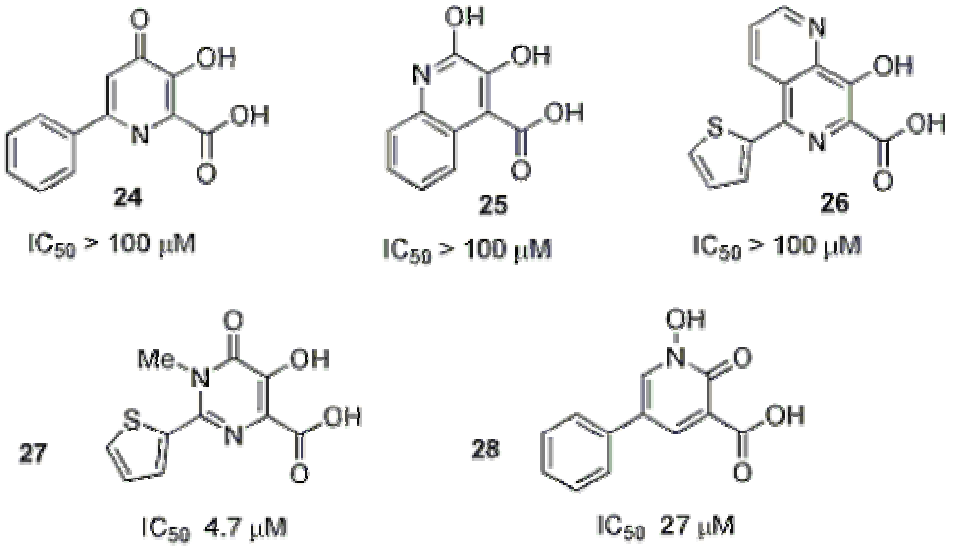
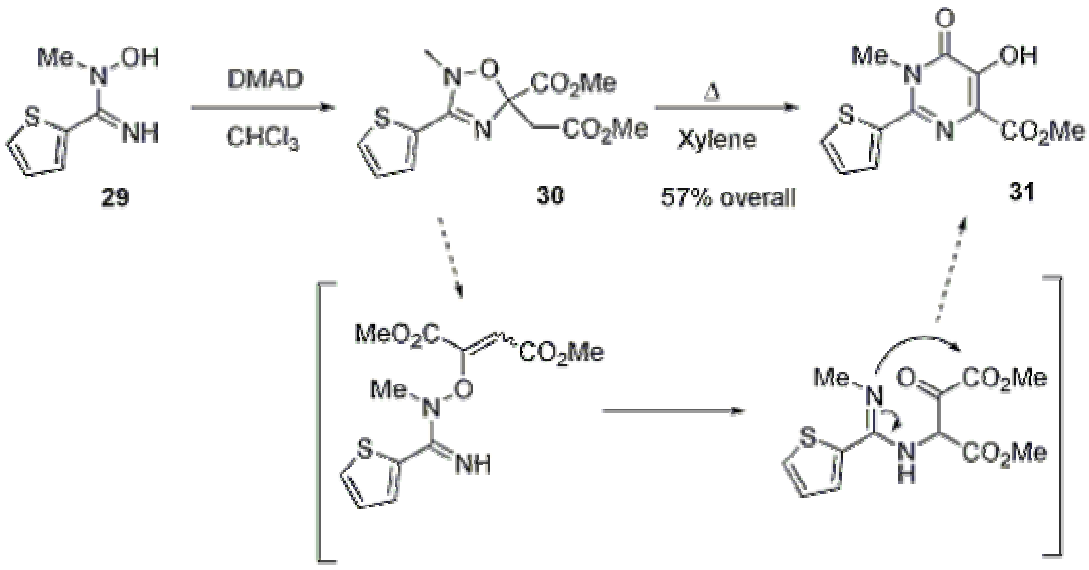


Figure 4

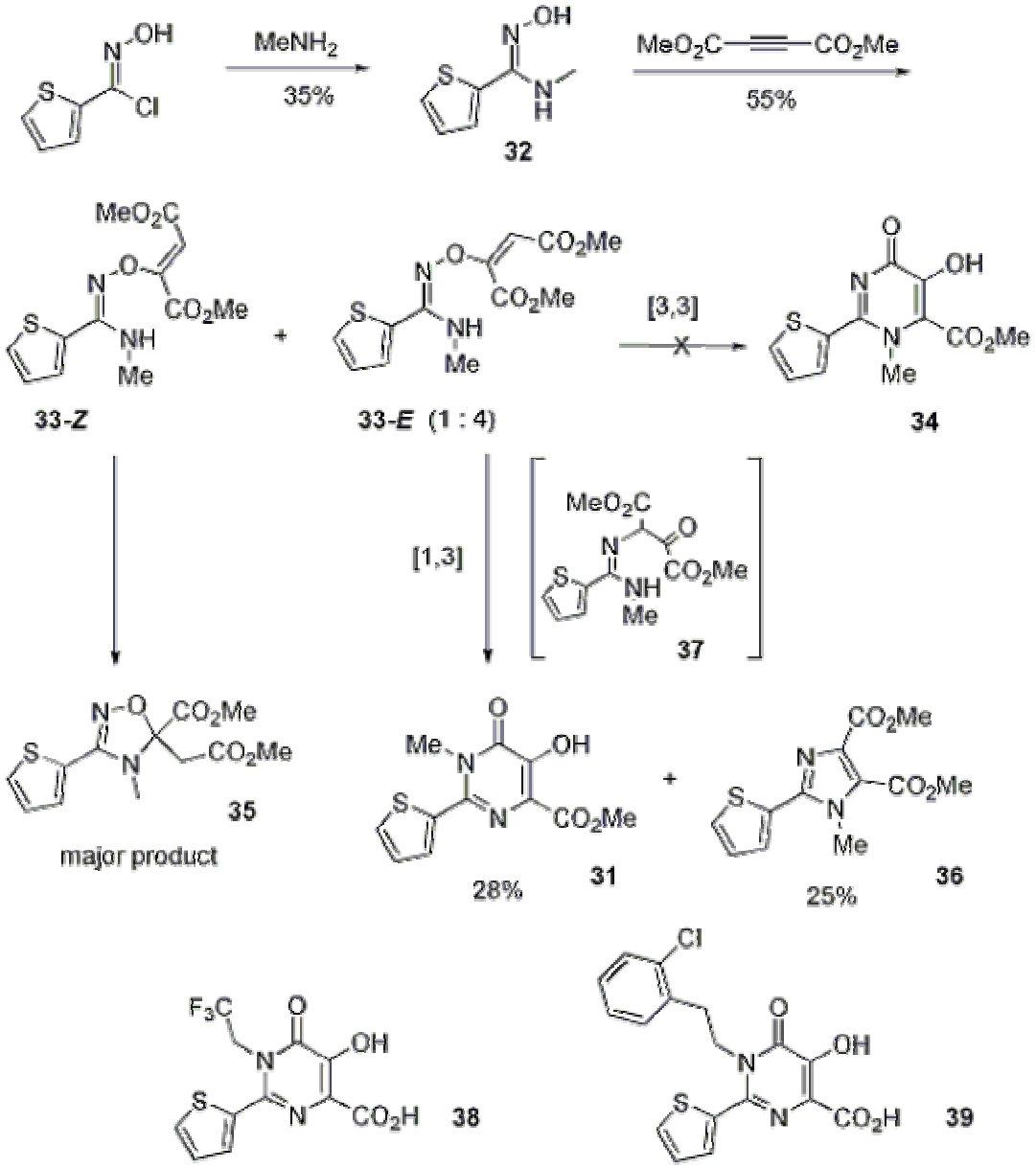
2.1 *N*1-substituted pyrimidones

The first approach to *N*1-alkylated pyrimidones relied on the alkylation of a suitably protected dihydroxypyrimidine and gave mixtures of the *O*- and *N*-alkylated product. Another drawback was that the reaction worked only with reactive electrophiles, which severely limited the range of substituents we could introduce. Use of the *N*-methyl amidoxime **29** as a starting material solved the problem of the non-selective alkylation, as Scheme 3 shows. Reaction of **29** with DMAD gave the 2-methyl-1,2,4-oxadiazole **30** in a double Michael addition as the sole product in good yield. Heating **30** in xylene then produced the desired *N*-methyl pyrimidone ester **31** in good isolated yield, probably via opening of the oxadiazole, followed by a Claisen-type rearrangement and subsequent ring closure, as was described for the dihydroxypyrimidines (Scheme 1).[28]



Scheme 3

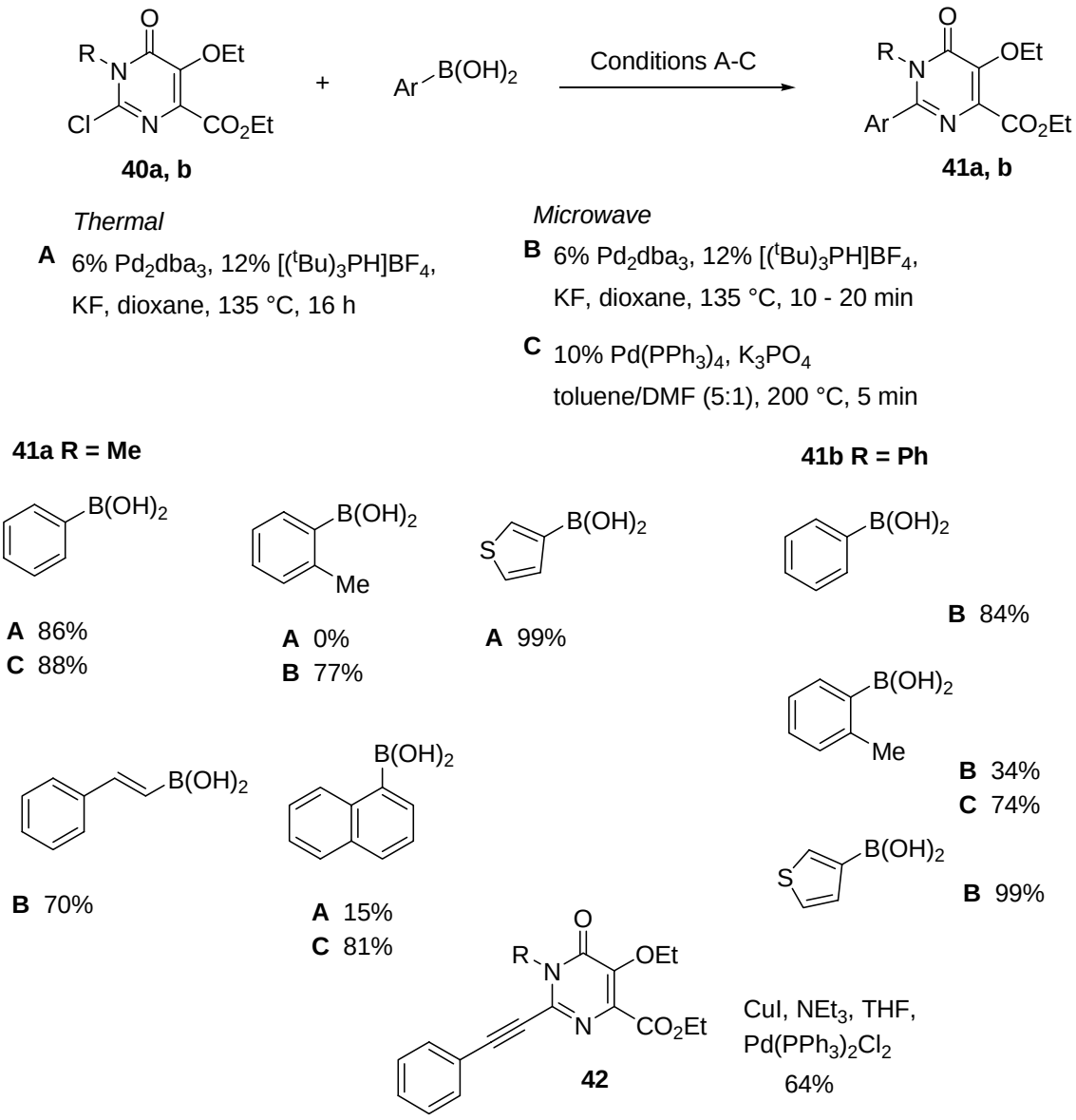
Interestingly, the isomeric thiophenecarboximidamide **32** did not give the *N*3-methyl pyrimidone **34**, but the *N*1-methyl pyrimidone **31** (Scheme 4). Our initial investigation of this reaction revealed that **32** on reaction with DMAD gave a mixture of the regioisomeric mono-Michael adducts **33** (Scheme 4). The Z-adduct **33-*Z*** cyclized to the 4-methyl-1,2,4-oxadiazole **35** on heating, but did not rearrange to a pyrimidone, even after prolonged heating, unlike its regioisomer **30**. The E-adduct **33-*E*** on the other hand gave rise to the *N*1-methyl pyrimidone **31** together with imidazole **36**. None of the *N*3-methylpyrimidone **34** was formed¸ suggesting that the reaction does not proceed via a Claisen-type [3,3]-rearrangement, as it is hypothesized for the formation of dihydroxypyrimidines (Scheme 1) and *N*-alkylpyrimidones (Scheme 3). We currently think that here a [1,3]-shift is operating, which via intermediate **37** leads to the observed products **31** and **36**.[28]



Scheme 4

Although the overall yield for this process was low, we were able to obtain products such as **38** and **39**, which were not accessible by the alkylation methodology. A drawback of the approaches described in Schemes 3 and 4 is that both the *N*1- and the 2-substituent have to be introduced in the first steps of the synthesis.

Recently we developed a novel approach, relying on palladium catalyzed cross-coupling for the carbon-carbon-bond formation in the 2-position and avoiding the non-selective *N*1-functionalization.[29] This method is virtually unprecedented in the literature and judging from the first results seems to be quite versatile (Scheme 5).



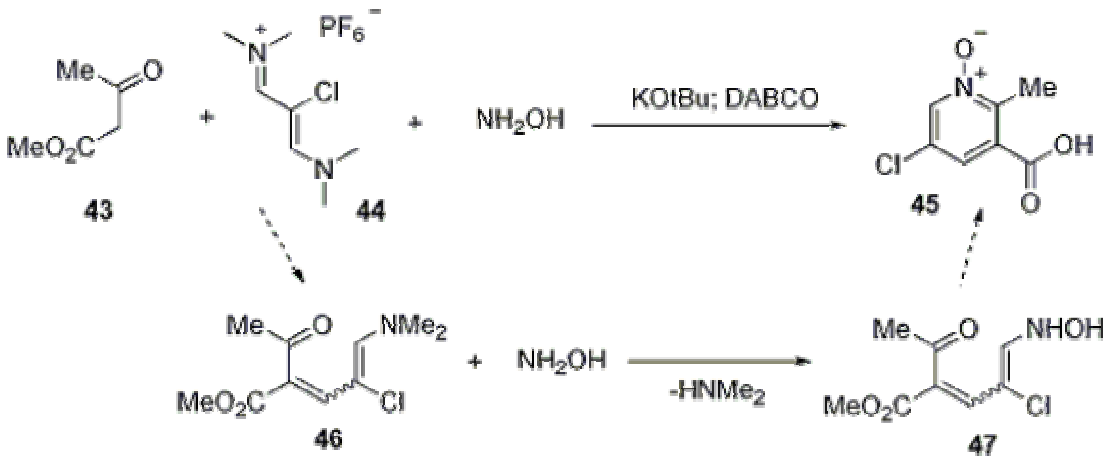
Scheme 5. Carbon-carbon bond formation in the 2-position of *N*1-substituted pyrimidones

2-Chloro-1-substituted pyrimidones **40**, which are available in multi-gram quantities undergo Suzuki or Sonogashira reactions using standard coupling protocols. The *N*-Methyl derivative **40a** for example gives good yields in the cross-coupling with most boronic acids under thermal conditions. Increased steric demand on the boronic acid partner led to low yields or no coupling product, as is shown for the case of the *o*-tolyl derivative. In this case microwave irradiation promoted coupling in good yield. The *N*-phenyl pyrimidone **40b** reacted only under microwave irradiation. Proper choice of catalyst and irradiation time allowed also the coupling of the sterically hindered *o*-tolyl boronic acid.

The acetylene **42** was obtained under standard Sonogashira coupling conditions.

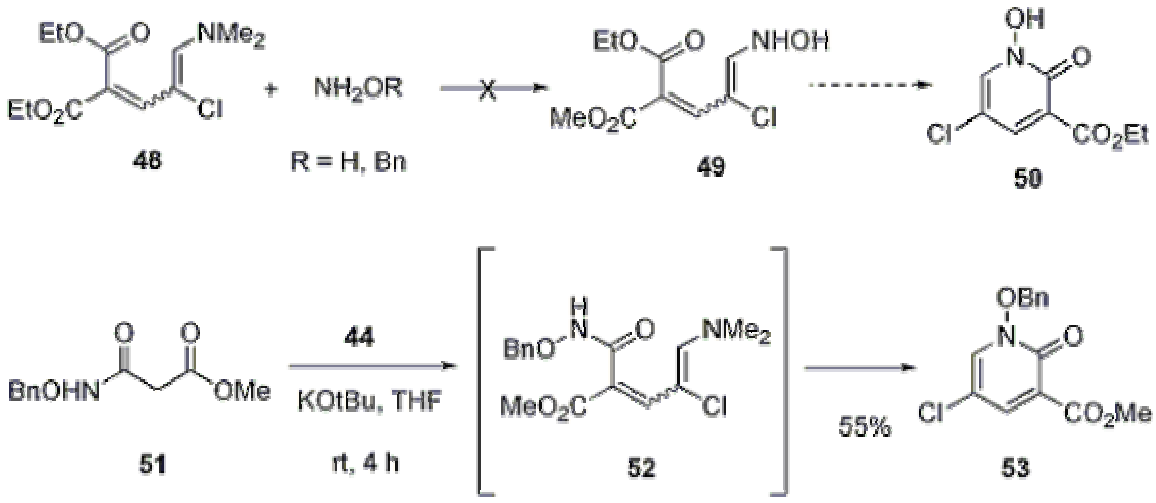
2.2. Pyridine-*N*-oxides

The initial synthesis of pyridine-*N*-oxide **28** relied on the oxidation of the pyridine to its *N*-oxide in the late stage of the synthesis. This step was low yielding, and moreover the product was difficult to separate from the starting pyridine. The harsh reaction conditions would also not compatible with sensitive functionality. We envisioned a milder way, relying on a [3+2+1] annulation strategy recently published by the Merck process group.[30,31] Here a vinamidinium salt **44** is reacted with the β-ketoester **43** and hydroxylamine in the presence of two bases to give directly pyridine-*N*-oxide **45**. Mechanistic investigations confirmed that **46** and **47** are intermediates in this process.[31]



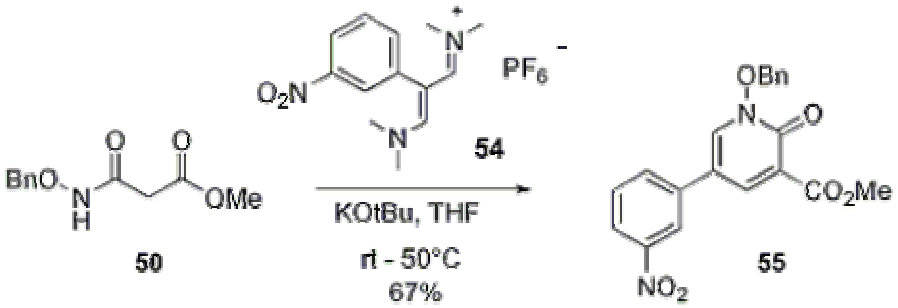
Scheme 6

Using the published conditions and several variations thereof, we were unable to bring about the reaction of malonate-derived **48** to *N*-oxide **50**. A possible explanation could be the lower reactivity of the ester group in **49** compared to the ketone in **47** in the final cyclization step. It was not really clear however, if **49** was formed at all from **48** and hydroxylamine, since the reaction mixtures became difficult to analyze after prolonged reaction time. We hypothesized that by bringing in the hydroxylamine from the beginning, thus using a [3+3] annulation instead of the [3+2+1], might circumvent this problem. As it turned out, when the 3-[(benzyloxy)amino]-3-oxopropanoate **51** was prepared and reacted with **44** at room temperature in THF, the protected *N*-oxide **53** was obtained in good isolated yield.[32]



Scheme 7

Unfortunately, chloride in **53** did not undergo Suzuki or Stille-type cross-coupling conditions under a variety of conditions, so we resorted to the preparation of aryl-vinamidinium salts such as **54**. Also these reacted smoothly with **51** to give the desired product **55**. Further investigations in this area are on-going.



Scheme 8

Conclusions

By investigating the SAR of dihydroxypyrimidine **3** in the 2-position, we identified several groups that led to a 10-40-fold gain in potency. Further SAR around the 2-thienyl analog **13** then led to inhibitors like **22**, which had sub-micromolar affinity on the NS5B polymerase. During this work we were able to identify the factors which contribute to binding of the pyrimidines to the HCV NS5B polymerase. Modulation of the pK_a by substituents in the 2-position of the

pyrimidine influences affinity by their effect on Mg-chelation. A substituent in the 3-position of the thiophene terminating with a lipophilic aryl residue leads to a further increase in potency. The urea group enforces a planar arrangement of the pyrimidine, as suggested by modeling, and due to a hydrogen-bonding network further increases the acidity of the 5-hydroxy group.
SAR around the pyrimidine scaffold led to the discovery of *N*1-substituted pyrimidones and pyridine-*N*-oxides. The work on the former offered some insight into the rearrangement mechanism of the Michael adducts, which are intermediates in the formation of dihydroxypyrimidines and pyrimidones. Different mechanisms might be operating for different series, but a thorough investigation of the reaction mechanism is needed.

A robust methodology relying on palladium mediated coupling was subsequently developed for the carbon-carbon-bond formation in *N*1-substituted pyrimidones. This methodology has the potential to give access to a broad variety of substitution patterns in this position. For the pyridine-*N*-oxides a novel and mild cyclization strategy was found, which allowed access to the desired target molecules.

Acknowledgements

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