2-(2-Thienyl)-5,6-dihydroxy-4-carboxypyrimidines as Inhibitors of the Hepatitis C Virus NS5B Polymerase: Discovery, SAR, Modeling, and Mutagenesis

Uwe Koch,*,† Barbara Attenni,† Savina Malancona,† Stefania Colarusso,† Immacolata Conte,† Marcello Di Filippo,† Steven Harper,† Barbara Pacini,† Claudia Giomini,† Steven Thomas,‡ Ilario Incitti,† Licia Tomei,† Raffaele De Francesco,† Sergio Altamura,† Victor G. Matassa,§ and Frank Narjes*,†

Istituto Di Ricerche Di Biologia Molecolare, P. Angeletti S.p.A. (Merck Research Laboratories, Rome), Via Pontina Km 30,600, 00040 Pomezia, Italy, and Merck Sharp & Dohme Research Laboratories, The Neuroscience Research Centre, Terling's Park, Harlow, Eastwick Road, Essex, CM20 2QR, United Kingdom

Received October 21, 2005

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 dihydroxypyrimidine carboxylates **2** as novel, reversible inhibitors of the HCV NS5B polymerase. This series was further developed into 5,6-dihydroxy-2-(2-thienyl)pyrimidine-4-carboxylic acids such as **34** (EC_{50} 9.3 μM), which now show activity in the cell-based HCV replication assay. The structure−activity relationship of these inhibitors is discussed in the context of their physicochemical properties and of the polymerase crystal structure. We also report the results of mutagenesis experiments which support the proposed binding model, which involves pyrophosphate-like chelation of the active site Mg ions.

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] In contrast to hepatitis B virus infections, no vaccine or general therapy is available. Current therapy is based upon a combination of pegylated α-interferon (IFN) and ribavirin.[4−6] Although more efficacious than previous treatments, which were based on IFN monotherapy,[4] 50% of patients infected with the genotype 1 form of the virus fail to show a sustained virological response.[7,8] Another drawback of this treatment is its poor tolerability, which is associated with severe flu-like symptoms, depression and anemia, causing about 20% of patients to discontinue treatment.[9] 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.[10−16]

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.[17] This polyprotein undergoes maturational processing in the cytoplasm or in the endoplasmic reticulum of infected cells to generate structural and nonstructural (NS) viral proteins. Among the NS proteins are two viral enzymes, which have been shown to be 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. This protein synthesizes both a complementary (−)-strand of HCV RNA and, using this as a template, the (+)-stranded HCV RNA genome itself.[18,19]

NS5B 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.[20−24]

Until very recently, it was not possible to propagate the virus in cell culture,[25,26] and as a consequence a subgenomic replicon system had been adapted for efficient RNA replication in the human hepatoma Huh-7 cell-line, allowing the study of the activity of key enzymes such as the NS3 protease-helicase and the NS5B polymerase.[27,28] This assay has been validated by the observation that an inhibitor of the NS3 protease, which was also a potent inhibitor of the replicon system, elicited a strong reduction in viral load in a phase 1b clinical trial.[29−31]

NS5B is normally not expressed in uninfected cells and presents an attractive target for the development of safe antiviral drugs.[32−34] Several laboratories, including ours, have disclosed allosteric inhibitors, which inhibited HCV replication in the replicon system. Several of these allosteric inhibitors have been reported to block the initiation step of viral replication either by preventing binding of RNA to the polymerase or by blocking a necessary conformational change.[35−41]

Apart from allosteric inhibitors, two classes of active-site inhibitors have been described. The first are modified chain-terminating nucleoside (substrate) analogues, which were shown to block RNA replication in the replicon system.[42−44] The second class are pyrophosphate (product) analogues, namely diketo acids **1** (Figure 1). We have recently shown that **1** inhibits selectively the HCV RdRP elongation step.[45] Kinetic experiments indicated that **1** and Foscarnet, a known pyrophosphate mimic and an approved antiviral drug[46] interact at a common binding site.[47] Due to the reactivity of the diketo moiety, **1** was further elaborated into 5,6-dihydroxypyrimidine-4-carboxylic acids such as **2**.[48,49] Intensive SAR studies directed at improving

* Corresponding authors. U.K., Tel.: +39 0691093644, e-mail: uwe_koch@merck.com; F.N., Tel. +39 0691093625, Fax: +39 0691093654, e-mail: frank_narjes@merck.com.

† Merck Research Laboratories, Rome.

‡ Merck Sharp & Dohme Research Laboratories, United Kingdom.

§ Current address: Almirall SA, Calle Treball 2-4, Sant Just Desvern, Barcelona 08960, Spain.

10.1021/jm051064t CCC: $33.50
Published on Web 01/25/2006

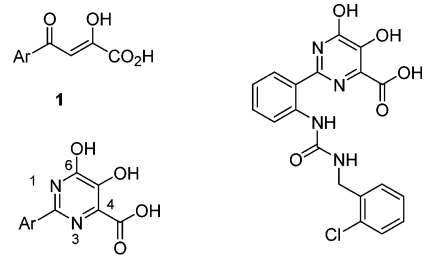


Figure 1.

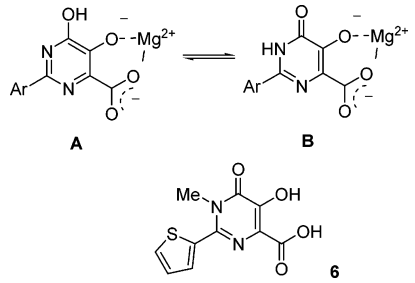


Figure 2.

the potency of **2**, led to substitution in the ortho or meta position of the phenyl group on the 2-position of the pyrimidine with amides, ureas and sulfonamides, preferentially terminating in an aryl residue.[50,51] Compound **5** (IC_{50} 0.48 μM), with a 60-fold improvement in potency over **2**, is an example of this effort. Exchanging the phenyl group for a 2-thienyl residue also gave an order of magnitude increase in potency (**3**, IC_{50} 2.6 μM), while the pyridine **4** (IC_{50} > 50 μM) was inactive.[52] None of the compounds **1**−**5** showed any activity in the replicon system.

Diketo acids have been proposed to inhibit HIV-integrase by a mechanism involving chelation of active site magnesium ions.[53] By analogy it was hypothesized that the dianion formed by deprotonation of the acid and the 5-hydroxy group in **2** could be chelating one or both of the magnesium ions in the active site (**A** in Figure 2) and thus act as a pyrophosphate mimic, thereby blocking the elongation step of RNA synthesis. This was proven by biochemical studies which established that arylpyrimidines are competitive with diketo acids and other classical pyrophosphate analogues.[47] A more indirect proof came from SAR studies, where it was shown that the free carboxylic acid and the 5-hydroxy group on the pyrimidine are essential for activity.[49,52]

Dihydroxypyrimidine **A** can exist in the tautomeric pyrimidone form **B** (Figure 2), which in principle is also capable of magnesium ion chelation. Presently, it is not known if **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 a possible inhibitor was shown with *N*-methyl pyrimidone **6** (IC_{50} 4.7 μM), an equipotent analogue of dihydroxypyrimidine **3**.[52]

We have continued to develop our dihydroxypyrimidine series of NS5B inhibitors for several reasons. The genetic variability of the virus is high. It can be classified in six major genotypes, each of which is subdivided into several subtypes.[54] Allosteric inhibitors have been shown to be highly specific for one genotype.[55] In contrast, due to the structural constraints necessary to maintain the catalytic function, the active site is more conserved and active site inhibitors are potent against a majority of viral genotypes.[55] For the same reason mechanism-based inhibitors are expected to develop resistance mutations less fast than allosteric inhibitors. Finally, we also expect that combination therapy will be required for an effective treatment, and an active site inhibitor together with an allosteric inhibitor would present a good combination, as is the case in the treatment of HIV infection.

In this paper we describe our efforts to further improve the potency of the dihydroxypyrimidine series, with the aim to arrive at compounds which would show activity in the subgenomic replicon assay. We also provide further evidence which supports the binding model of dihydroxypyrimidines as pyrophosphate isosteres.

Biology. The compounds described were routinely assessed for activity against the purified ΔC55 NS5B enzyme[56] in the presence of a homopolymeric template RNA. Selected compounds were also tested on the full-length and ΔC21 enzyme forms confirming potency (data not shown, except for **34**). Expression in *E. coli* BL21(DE3) and purification of the HCV 1b-BK strain NS5BΔC55 (residues 1−536), NS5BΔC21 (residues 1−570), mutants ΔC55-R158M and ΔC55-R158K were carried out as already described.[56,57] The full-length HCV NS5B protein was purified from Sf9 cells infected with a recombinant baculovirus as described.[56]

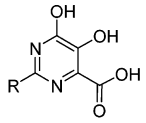
Inhibition of replication of subgenomic HCV RNA was measured in Huh-7 cells using a modification of the procedure of Bartenschlager[27] in the presence of 10% fetal calf serum.[36]

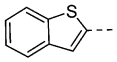
Results and Discussion

SAR in the 2-Position of the Pyrimidine. The lack of activity of dihydroxypyrimidines in the replicon assay was thought to be due to two factors, lack of intrinsic potency, and the physiochemical properties of the compounds. At physiological pH the dihydroxypyrimidines can exist as a mono- or dianion, depending on the pK_a of the hydroxy group, which reduces their ability to cross cell membranes. Since previous studies had shown that the 5,6-dihydroxy-4-carboxylic acid moiety is crucial for activity, our initial efforts concentrated on improving the potency of these inhibitors. The exchange for a phenyl group to a thiophene had produced a 10-fold gain in potency, and consequently we expanded the SAR in the 2-position. As the results in Table 1 demonstrate, an aryl substituent directly attached to the pyrimidine is required for activity, since substituents such as cyclohexyl (**7**, IC_{50} > 50 μM), *n*-butyl (**8**, IC_{50} > 50 μM) or benzyl (**9**, IC_{50} > 50 μM) lost significant in activity. Preferred are five-membered aromatic heterocycles, with furan **10** being equipotent to thiophene **3**, and the electron-poor thiazole **11** gaining about 4-fold (IC_{50} 0.76 μM). Relatively basic heterocycles such as imidazole **13**, as well as its precursor, *N*-benzyl imidazole **12** are at least 10-fold less active, as was already seen with pyridine **4**. Conversion of the pyridine into the less polar pyrazine, which has a much reduced dipole moment compared to pyridine, results in compound **14** (IC_{50} 5.3 μM), which is nearly as potent as **3**. The benzothiophene derivative **15** is 4-fold less potent than **3**, indicating steric constraints in the binding pocket.

This set of results seemed to indicate that an electron-withdrawing effect, going from thiophene to the more electron-withdrawing thiazole is beneficial for potency. To test this hypothesis, we prepared the sulfide **16**, which shows 55% inhibition at 50 μM. Oxidation to the more electron-withdrawing sulfone gives compound **17**, which now has an IC_{50} of 2.4 μM. The Hammett constant, which measures the effect of substituents in the para-position of benzoic acids on the pK_a, changes from 0 for the sulfide to 0.72 for the sulfone. Similarly, the thienyl-

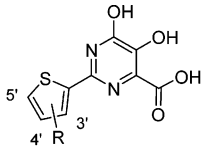
Table 1. Inhibition of NS5B RdRP by 2-Substituted Dihydroxypyrimidines **3**−**17**

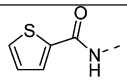


No.	R	IC_{50} (μM)[a]	No.	R	IC_{50} (μM)[a]
3		2.6 ± 1.1	**12**		30% @ 50
7		31% @ 200	**13**		50% @ 50
8	n-butyl	0% @ 50	**14**		5.3 ± 2.3
9		24% @ 50	**15**		11.6 ± 0.2
10		2.9 ± 1.4	**16**		55% @ 50
11		0.76 ± 0.23	**17**		2.4

[a] IC_{50} values are the average of at least three independent determinations.

Table 2. Inhibition of NS5B RdRP by 2-(2-Thienyl)dihydroxypyrimidines



No.	R	IC_{50} (μM)[a]	No.	R	IC_{50} (μM)[a]
3	H	2.6	**23**	4' 	13.8
18	5'-Me	48.6	**24**	3'-NH_2	1.9
19	3'-Me	5.6			
			25	3'-NHC(O)NHBn	0.23
20	5'-NO_2	16.0	**26**	3'-NHC(O)OBn	0.29
21	4'-NO_2	9.4	**27**	3'-$NHSO_2$-2-naphthyl	1.42
22	3'-NO_2	0.68	**28**	3'-NHC(O)Bn	0.38

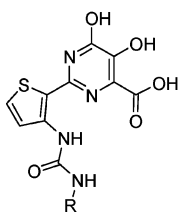
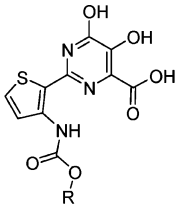
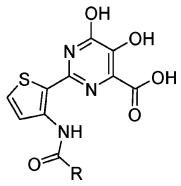
[a] IC_{50} values are the average of at least two independent determinations. The standard error is within ±20% of the average.

substituted pyrimidine **3** has a larger Hammett constant (0.05) than the cyclohexyl-substituted **7** (−0.22).[58] These results suggest that the importance of the 2-substituent being electron-withdrawing is due to their effect on the pK_a of the pyrimidine. In addition, the polarity of the substituent, steric limitations and its conformational preference with respect to the pyrimidine seem to be important. However, none of these compounds showed any inhibition of the replicon assay at a fixed concentration of 100 μM.

We then proceeded to investigate the possibility of incorporating the potency enhancing side-chains found in the phenyl series. Although thiazole **11** was the most potent compound in this preliminary round of SAR, we decided to conduct the studies of the side-chain SAR with thiophene **3**, since it allows investigation of SAR in three different positions.

It became immediately apparent that substitution in the 5′- and the 4′-position of the thiophene ring of **3** were detrimental, since residues such as methyl or nitro in these positions led to a significant loss in potency (**18**, IC_{50} 48.6 μM; **20**, IC_{50} 16 μM; **21**, IC_{50} 9.4 μM, Table 2). Previously a thienyl acetamide residue in the meta position of the phenyl ring in **2** had given a 10-fold improvement in activity,[50] but in the 4′-position of the thiophene more than 4-fold loss (**23**, IC_{50} 13.8 μM) was observed. This left only the 3′-position, where a methyl group leads to a 2-fold loss (**19**, IC_{50} 5.6 μM), but the electron-withdrawing nitro group gave a 4-fold gain in potency (**22**, IC_{50}

Table 3. Inhibition of NS5B RdRP by 2-(2-Thienyl)dihydroxypyrimidines

R	compd	IC_{50} (μM)[a]	EC_{50} (μM)[a,b]	compd	IC_{50} (μM)[a]	EC_{50} (μM)[a,b]	compd	IC_{50} (μM)[a]	EC_{50} (μM)[a,b]
Ph	**29**	0.58	>50	-			**30**	0.80	>50
$(CH_2)_2$Ph	**31**	0.62	>50	**32**	0.46	>50	**33**	0.58	>50
CH_2(2-Cl-Ph)	**34**	0.15	9.3 ± 2.8	**35**	0.14	31.5 ± 6.4	**36**	0.21	53.3 ± 6.3
CH_2(2-Cl-6-Me-Ph)	**37**	0.09	13.6 ± 3.2	-			-		
CH_2(2,6-Cl_2-Ph)	-			**38**	0.19	18.6 ± 2.3	-		
CH_2-3-indolyl	-			-			**39**	0.20	>50
CH_2-3-benzothienyl	**40**	0.22	30.0 ± 4.1	-			**41**	0.23	>50
CH_2-1-naphthyl	-			**42**	0.14	13.6 ± 2.3	-		
CH_2-(2-Ph-3-indolyl)	-			-			**43**	0.28	>50

[a] IC_{50} and EC_{50} values are the average of at least three independent determinations. Standard deviations for the IC_{50} are within 30% of the average. [b] Compounds were initially tested in the replicon assay at a fixed concentration of 100 μM. EC_{50} values were determined only for compounds showing >50% inhibition.

0.68 μM) with respect to **3**, making this compound equipotent to thiazole **11**. Converting the nitro group to the electron-donating amine **24** (IC_{50} 1.9 μM) reduces activity to the levels of **3**, again underscoring the importance of the electronic properties of the 2-substituent on the activity of the pyrimidines.

Acylation of amine **24** with a selection of side-chains previously described in the phenyl series[50,51] gave another order of magnitude in potency for the benzyl urea **25** (IC_{50} 0.23 μM) and the corresponding carbamate **26** (IC_{50} 0.29 μM), confirming the SAR already observed in the phenyl series. The SAR was different for the sulfonamide and the amide linking groups. Naphthylsulfonamide **27** (IC_{50} 1.42 μM) gave only a 1.5-fold gain in activity instead of the 10-fold observed in the phenyl series (IC_{50} 2.6 μM),[51] while for amide **28** (IC_{50} 0.38 μM) the opposite was observed. The amide linkage had no impact on activity in the phenyl series, where the corresponding compound was equipotent with **2** (IC_{50} 33 μM).[50] None of these compounds inhibited the replicon assay to any significant extent at a concentration of 100 μM.

Further SAR then concentrated on ureas, carbamates and amides. Reducing or elongating the chain gave a 2-fold loss in potency across all linking groups (Table 3, see compounds **29**−**33**). Chlorination of the aromatic residue led to a small improvement in intrinsic potency and, more important, to activity in the replicon assay. Cell-based activity was best in the urea series, where compound **34** had an EC_{50} of 9.3 μM and showed no cytotoxicity up to 100 μM. Carbamate **35** and amide **36** were 3- and 5-fold less active, despite similar IC_{50} values.

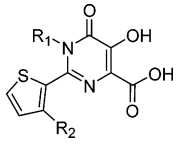
In an attempt to further improve potency we used a rapid analogue approach to prepare a diverse set of ureas, carbamates and amides. Residues R (Table 3) were initially chosen to maximize diversity among them and between the three linking groups. Despite a large number of compounds made, no major improvement in potency could be achieved, and the SAR remained rather flat. Aromatic residues were always preferred over aliphatic ones (results not shown), and a simple methylene group was the preferred spacer between the aromatic group and the linker. Moving the chlorine on the aromatic ring of **34** from the ortho- to the meta- or the para-position gave a 2−3-fold loss in potency (results not shown). Further substitution on the aromatic group with methyl groups or halogens led to a slight increase in intrinsic potency, but not to an improvement in cell-based activity (see urea **37**, IC_{50} 0.09 μM, EC_{50} 13.6 μM). The same was observed for substitution in the benzylic position, where a variety of groups were accepted, but activity in the replicon system remained poor (results not shown). In summary, a large number of aromatic groups and substitution patterns are accepted and, irrespective of the linking group, gave potent enzyme inhibitors. Cell-based activity in the low micromolar range was best in the urea series, followed by carbamates, whereas amides were significantly less active.

From this investigation the urea **34** emerged as one of the more active compounds. It loses 60-fold in potency in the cell-based assay with respect to the enzyme assay. This is not due to the enzyme form used in our in vitro assay, the ΔC55 polymerase, since **34** inhibits the ΔC21 (IC_{50} 0.045 μM) and the full-length version of the NS5B polymerase (IC_{50} 0.13 μM) with similar IC_{50}s. It was also active on the ΔC21 NS5B polymerase of genotype 2a (IC_{50} 0.044 μM) and 3a (IC_{50} 0.048 μM), thus showing that this class of inhibitors has the potential to be active against a broad range of viral isolates.

The side-chain on the thiophene does not only increase the potency of the inhibitors, but renders them also more lipophilic, as evidenced by the distribution coefficient (log *D*) at pH 7.4 of unsubstituted **3** (−1.95) versus urea **34** (+0.37). This certainly aids cell-penetration. More polar compounds, such as thiazole **11** (log *D* −2.12) or carbamate **32** (−0.43), are only 3−5-fold less active in the enzyme assay, but show less than 20% inhibition of the replicon at 100 μM. A further increase in lipophilicity can be achieved by substitution on the aryl ring (**37**, log *D* +0.6), or by its conversion into a naphththalene or other bicyclic heteroaramatic groups (**39**, calculated log *D* 1.93; **42** calculated log *D* 1.09). This led to good or even improved intrinsic potency, but not improved replicon potency with respect to **34**. We believe that the loss of potency in the replicon assay is due to a large extent to the polar anionic character of the compound and to some smaller extent to protein binding. This is reflected by running the replicon assay in the absence of serum, where an EC_{50} of 3.1 μM resulted, still 20-fold above its IC_{50}. In line with this, **34** showed poor permeability in Caco-2 cell line, both in the apical-basal and the basal-apical direction, and is highly bound to plasma proteins (0.2% free in human plasma).

In an attempt to reduce the polar character of our compounds, we investigated substitutions on the N-1 nitrogen of the

Table 4. Inhibition of NS5B RdRP by *N*-Alkylpyrimidinones



no.	R_1	R_2	IC_{50} (μM)[a]
22	H	NO_2	0.68
44	Me	NO_2	8.7
45	allyl	NO_2	69.3
46	Bn	NO_2	79.3
47	Me	NH(CO)NH-(2-ClPh)	1.2

[a] Values are the average of at least two independent determinations.

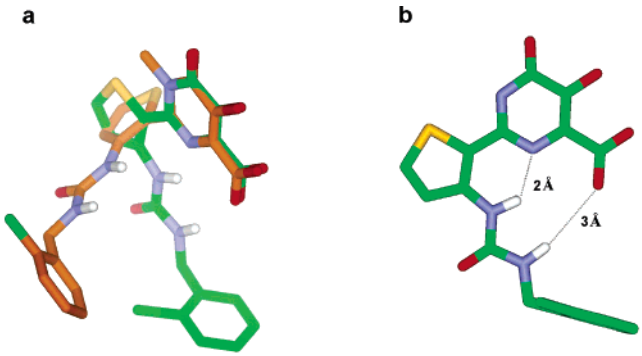


Figure 3. (a) Superposition of models of **34** (green) and the *N*-methylpyrimidone **47**. (b) Possible hydrogen bonding network in the syn orientation of urea and acid.

pyrimidine, also based on the precedence that the *N*-methyl pyrimidone **6** is as active as the dihydroxypyrimidine **3**. As Table 4 shows, alkylation of nitrothienyl pyrimidine **22** leads to a loss of potency, which increases with the size of the substituent. The O-alkylated compounds did not show activity. The *N*-methyl group is tolerated, and so we converted compound **44** to the urea analogue **47**. Compared to the dihydroxypyrimidine **34**, the pyrimidone **47** is about 1 order of magnitude less potent in the enzyme assay and does not inhibit the replicon system at 100 μM. Modeling suggests that the loss of potency is due to the position of the side-chain, which as a consequence of the twist induced by the methyl group at N-1, now occupies a different region of space. The minimum energy conformer, obtained in water using an MMFF force field, shows a twist with an angle between thiophene and pyrimidone of 70° (±20°) (Figure 3a).

Modeling for the dihydroxypyrimidine **34** on the other hand, showed a planar compound, despite the presence of a substituent in the 3-position of the thiophene. The syn and anti orientation of the urea and the carboxylate, obtained by a 180° rotation around the pyrimidine thiophene axis, are almost isoenergetic. For both orientations the planar arrangement is only possible with the inner urea NH forming a close intramolecular contact to either to the N-3 or N-1 nitrogen atoms of the pyrimidine. Amides and carbamates can have similar interactions. In the syn conformation it is possible for the urea to form a second close contact between the outer urea NH and the carboxylic acid (Figure 3b), an arrangement which we term intramolecular hydrophilic collapse, which would enforce the planar arrangement. We speculate that this effect could explain the enhanced activity of ureas in the replicon assay with respect to the equipotent carbamates and amides (see compound **34** versus **35** and **36**, Table 3).

The SAR suggests that the substituent in the C-2 position has an influence on the pK_a of the 5-hydroxy group. In analogy

Table 5. IC_{50} and pK_a of the 5-hydroxy Group of Selected Pyrimidines

compd	IC_{50} (μM) Mg^{2+} (Mn^{2+})	pK_a[a]
2	30 (2.3)	8.6 ± 0.2
3	2.6 (0.24)	7.7 ± 0.2
6	4.2 (0.46)	9.1 ± 0.5
7	>50 (>25)	8.7
10	2.9 (0.24)	7.4 ± 0.1
11	0.76 (0.21)	6.9 ± 0.3
22	0.68 (0.065)	6.9 ± 0.2
34	0.15 (0.036)	6.6 ± 0.1
47	1.2 (0.08)	9.2 ± 0.4

[a] Average of three independent determinations. Data aquired using a Sirius Profiler SGA (see Experimental Section).

to the related diketo acids, which have been shown to inhibit NS5B by the same mechanism involving Mg-chelation,[59] we assume the change of the pK_a to influence the Mg-chelating capability. To test this assumption we determined the apparent pK_a value of the 5-hydroxy group of some selected compounds.

Importantly 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 5 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 **11** and nitrothiophene **22** 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 observed for example a 2-fold gain in potency for thiazole **11** 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). Taken together these results provide evidence for the hypothesis that the doubly deprotonated pyrimidine is the bioactive species. Possibly this is due to its better capability to chelate Mg ions.

Introduction of the urea substituent **34** leads to the most potent compound in this series. The urea also causes a significant increase in acidity compared to the compound with an unsubstituted thiophene **3**. This strong effect is somewhat unexepected since the urea is not directly attached to the pyrimidine and its electron-withdrawing effect has to be transmitted through the thiophene. A possible explanation comes from the modeling which suggests for **34** a planar conformation (Figures 3a and 3b). This type of arrangement with a contact between a hydrogen bond acceptor and donor within a six-membered ring is similar to that observed in the enolic form of 1,3-diketones and could help to increase acidity by stabilizing the bis-anion. In fact the urea **34** has the lowest pK_a in this series. **34** is also the most potent compound in this series. Affinity seems to be further increased by hydrophobic interaction of the terminal aryl group in a hydrophobic pocket.

Introduction of an *N*-methyl group to generate the 2-thieno-substituted *N*-methyl pyrimidone **6** increases the pK_a by more than one unit without having a significant effect on potency. According to the pK_a of **6** only a small quantity of this compound should be present in its bis-anionic form at pH 7.4. Thus, at least for the thieno-substituted compound, the monoanionic form of the *N*-methyl pyrimidone has the same potency

as the corresponding, doubly deprotonated pyrimidine. We speculate that in case of the pyrimidines it is the higher affinity of the doubly charged form for the Mg ion which is responsible for the pK_a dependence of the SAR. After *N*-methylation only a single tautomer is possible which by itself in its monoanionic form may have a high affinity for the Mg ion. This implies the importance of the charge to be due to its effect on chelation not on a direct interaction with the protein. That chelation is of central importance for the affinity of these compounds is in agreement with the strong effect on affinity observed when Mg is exchanged for Mn in the NS5B assay (see Table 5).[49]

The *N*-methyl group of the pyrimidone **47** sterically prevents a planar arrangement of the 2-thienyl group and the pyrimidine (Figure 3a) and as a consequence the 2-substituent occupies an area different from the same substituent of **34** after superposition of the pyrimidine moiety. Interestingly, we do not observe any decrease in the pK_a compared to the unsubstituted pyrimidone **6**, confirming the hypothesis that the formation of the close intramolecular contact between the urea NH and the pyrimidine helps to increase acidity.

Molecular Modeling and Mutagenesis. To gain further insight into the mechanism of inhibition we have generated a binding model for the pyrimidines on the basis of the existing crystal structures of the NS5B protein. The active site of NS5b binds two Mg ions which have been shown crystallographically to mediate the binding of the triphosphate of a cocrystallized nucleotide.[23] During elongation, we assume, in analogy to other polymerases,[60−62] one Mg ion to chelate the primer ribose hydroxy group and the α-phosphate of the incoming nucleoside. The second Mg ion is bound between the β- and γ-phosphate of the incoming nucleotidetriphosphate (NTP), which after addition of this nucleotide to the primer is cleaved off as the pyrophosphate.

SAR and biochemical data suggest that pyrimidines have a similar binding mode as the pyrophosphate. Accordingly we propose the pyrimidine to interact with the Mg ion chelating the β- and γ-phosphate groups of the triphosphate. We have used crystallographic information about the standard Mg−oxygen interaction geometry to generate a binding model of the pyrimidine bound to the Mg ion in the crystal structure of NS5B. The position and orientation of the pyrimidine was optimized to reduce steric interactions with the protein while maintaining the chelation geometry.

The model suggests the 2-thiophene of the pyrimidine to be stacked against Arg158 which in the crystal structure with GTP forms hydrogen bonds to the α-phosphate.[23] By analogy to the T7 DNA polymerase and HIV reverse transcriptase,[60,62] which both contain a conserved basic residue in contact with the α-phosphate, we expect Arg158 to play a critical role in activating the α-phosphate for nucleophilic attack by the 2′-OH of the primer and at the same time interacting lipophilically with the base of the incoming nucleosidetriphosphate. To test the importance of this residue for inhibition by the pyrimidines we suggested two mutations of Arg158.

Since the interactions between Arg158 and the thiophene seem to be mainly of lipophilic nature a mutation into Met, a more lipophilic amino acid with similar size but without a charge, should maintain the affinity of the pyrimidine. Mutation into the more polar Lys however should decrease the capability to interact lipophilically with the thiophene while maintaining a positive charge. We observe a decrease of inhibition for pyrimidines **3**, **11**, and **35** for both mutations (Table 6). However, in the case of thiophene **3** the decrease in potency is significantly larger for the Arg158Lys mutation (35-fold) than for the Arg158Met (3-fold) suggesting that the lipophilic component of the interaction between Arg158 and the 2-thiophene is particularly important. The same kind of behavior is observed for thiazole **11**, and also for compound **35**, with a potency enhancing side-chain. Here the loss in potency on the Arg158Lys mutant is less severe (6-fold), but still larger than on the Arg158Met mutant (2-fold). The K_m for the substrate UTP has been measured and does not show significant differences between wt and mutant enzymes (Table 6). These results confirm a mode of binding of pyrimidines which involves chelation of Mg ions and lipophilic interactions of the substituent in position 2 of the pyrimidine with Arg158.

Table 6. Activity of **3**, **11**, and **35** in HCV NS5B Containing Site-Directed Mutations

	IC$_{50}$ (μM), K_m for UTP		
compd	wt	R158M[a]	R158K[a]
3	2.6	9.3	92.0
11	0.76	2.77	16.0
35	0.14	0.34	0.90
UTP	6.0	15.0	20.0

[a] Measured in duplicate.

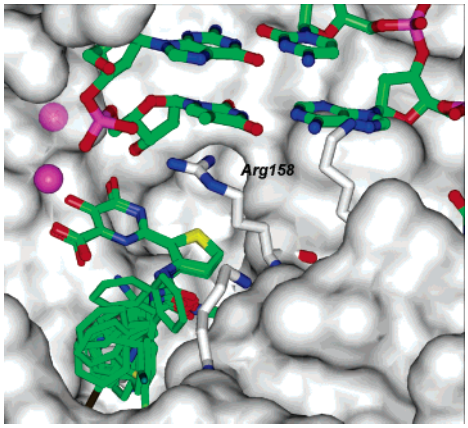


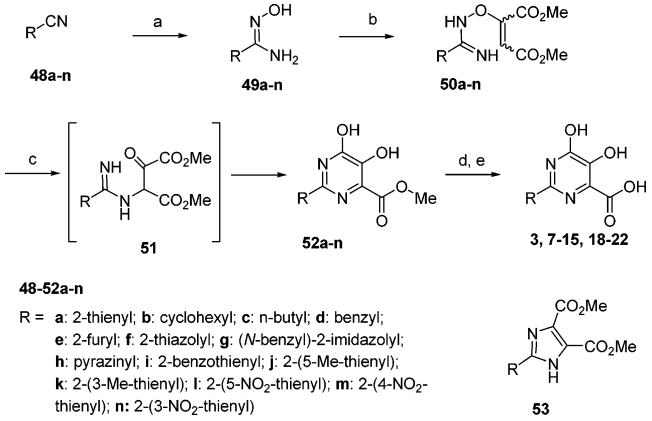
Figure 4. Model of active 2′-substituted 2-thienyl-pyrimidines in the active site together with a primer and template. The pyrimidines correspond to those in Table 3. The two Mg ions are indicated as violet spheres, carbon atoms are shown in green, oxygen in red, nitrogen in blue and sulfur in yellow.

In agreement with SAR this model shows that the space available to substituents in positions 4 and 5 of the thiophene is limited. The substituents in position 3 are bound in a cavity characterized by the presence of a number of basic and lipophilic residues (Figure 4). Interaction with these residues is responsible for the increase in potency of some compounds containing lipophilic substituents in this position. The binding site is further characterized by the presence of a number of basic amino acids explaining the lack of activity of the 2-imidazolo substituted pyrimidines **12** and **13**. The conformational change introduced by the *N*-methyl group of pyrimidone **47** is not compatible with the binding mode described for the dihydroxypyrimidine. In the twisted conformation the *o*-chlorobenzyl moiety cannot be accommodated in the binding pocket requiring either a conformational change or a less optimal Mg-chelation geometry, both of which accompanied by a loss of binding energy and thus affinity.

Conclusion

We have described the further development of the dihydroxypyrimidine class of NS5B inhibitors, which were inactive in the cell-based HCV replicon assay. We have identified different

Scheme 1. Synthesis of Pyrimidine Carboxylates **3**, **7**−**15**, **18**−**22**[a]



[a] Reagents and conditions: (a) $H_2NOH{\cdot}HCl$, Na_2CO_3, EtOH, H_2O, rt; (b) DMAD, $CHCl_3$, rt to reflux; (c) xylenes, 140−150 °C; (d) NaOH, 80 °C; (e) for **52g**: PvCl, DMAP, pyridine, rt; HCO_2NH_4, Pd/C, MeOH, rt; then step d.

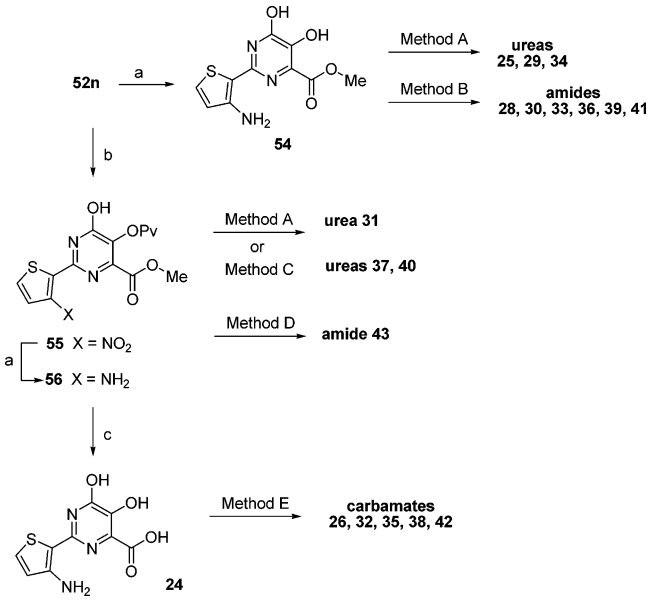
properties which contribute to binding. Modulation of the pK_a by substituents in position 2 of the pyrimidine influence affinity by their effect on Mg chelation. We have described the physicochemical and conformational properties of the substituents attached to the pyrimidine which favor affinity. Molecular modeling studies were used to suggest a mode of binding of pyrimidines, on the basis of which site directed mutations have been designed which confirm the proposed model. This model involves pyrophosphate like chelation of the active site Mg ions and identifies a lipophilic pocket which can be used as the basis for the development of more potent inhibitors. SAR data confirm the binding mode of the dihydroxypyrimidine. Urea **34** emerged from these studies as one of the most potent compounds, which showed the same potency on a variety of polymerases from different viral isolates and also inhibited the cell-based replicon assay. This makes the dihydroxypyrimidine class of inhibitors a promising starting point for future development of more active compounds.

Chemistry. For the synthesis of 2-substituted dihydroxypyrimidine carboxylates **3**−**15** and **18**−**22** the method of Culbertson was used.[63] Briefly, amidoximes **49a**−**n**, obtained from nitriles **48a**−**n**, either commercially available or prepared as described in the Experimental Section, were reacted with dimethylacetylene dicarboxylate (DMAD) to yield adducts **50a**−**n** as a mixture of geometrical isomers (Scheme 1). Usually these adducts were not isolated, but cyclized directly in refluxing xylene to yield the desired pyrimidine methyl esters **52**. In most cases the esters precipitated from the reaction mixture and were isolated by filtration generally in good purity and yields ranging from 3 to 60%. Polymerization of more sensitive substrates such as **48d** (9%) and **48e** (3%) under the reaction conditions was the major side-reaction. As a side-product imidazole **53** was observed in several cases in the LC-MS trace of the crude mixtures and isolated and characterized in the case of **53n**. It is assumed that the reaction from **50** to **52** follows a Claisen-type rearrangement via intermediate **51** shown in Scheme 1,[63] which after tautomerization of the amine could cyclize onto the ketone to give the imidazole or onto one ester group to yield **52**.

Hydrolysis of the ester gave the final products in about 70−90% purity. Imidazole **52g** was first acylated with pivaloyl chloride, which made the compound soluble enough for the subsequent hydrogenation to give **13**.

Scheme 2 illustrates the chemistry used for the investigation of the side-chain SAR in the thiophene series. Ester **52n** was

Scheme 2[a]



[a] Reagents and conditions: (a) Pd/C, H_2, MeOH, rt; (b) PvCl, 4-DMAP, py, rt; (c) NaOH, 80 °C; Method A: RCNO, py, rt;, temp.; then (c); Method B: RCO_2H, CDI, DMF, add **54**, rt; then (c); Method C: PhOC(O)Cl, DCM, rt, add RNH_2, rt; then (c); Method D: RCO_2H, BOP-Cl, DCM, NEt_3, 0 °C, 1 h; add **56**, NEt_3; then (c); Method E: ROH, DSC, CH_3CN, Et_3N, rt, overnight; add **24**, CH_3CN, H_2O, rt.

hydrogenated to amine **54**. Reaction with isocyanates then led to the desired ureas. Addition of **54** to a solution of carboxylic acids, previously activated with CDI and triethylamine, led to the amides. Reactions with amine **54** were sometimes capricious, due to acylation of the 5-hydroxy group and due to the low solubility of some of the products, which made a workup from organic solvents difficult. Protection of the 5-hydroxy group as its pivaloate ester solved both of these problems and amine **56** was used for the rapid analogue synthesis. Ureas were prepared either by reaction with isocyanates or via the intermediate phenylcarbamate.[64] Amides were accessed by the BOP-Cl mediated coupling of acids.[65,66] After workup and deprotection of the ester groups products usually precipitated upon acidification of the hydrolysis mixture and were isolated by filtration. They were then purified to >95% purity by reversed phase HPLC.

Carbamates were also accessible from **56**, but in some cases hydrolysis of the carbamate occurred under the harsh conditions employed for methyl ester hydrolysis. To avoid this amino acid **24** was reacted directly with the appropriate alcohol in the presence of *N*,*N*′-disuccinidyl carbonate.[67] For the synthesis of sulfonamide **27** a cyclic methylene acetal was used to block the reactive diol functionality.[51]

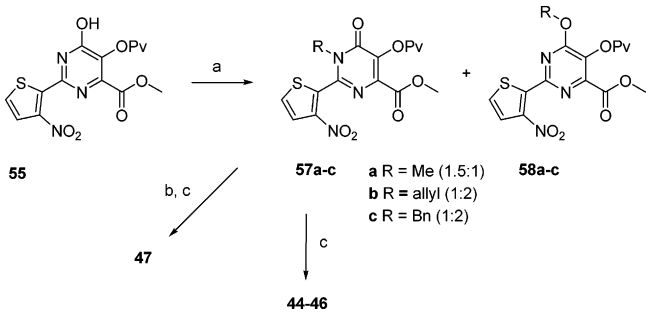
Alkylation of **55** with alkylhalides produced a mixture of the products of N- and O-alkylation, which were easily separated by column chromatography. The synthesis then proceeded as described above for the dihydroxypyrimidines (Scheme 3). Sulfide **16** and sulfone **17** were prepared from dichloropyrimidine **59** as shown in Scheme 4.

Experimental Section

Synthesis and Characterization of Inhibitors. General Procedures. Solvents and reagents were used as received from commercial sources.

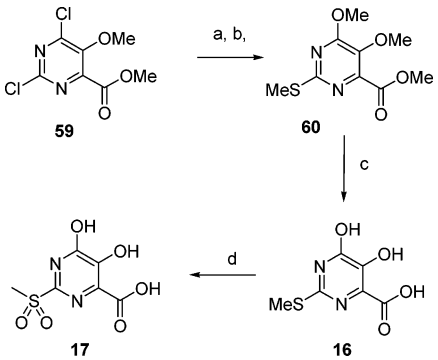
Melting points were determined in open capillaries using a Buechi 535 apparatus and are reported uncorrected. Nuclear magnetic resonance spectra (^{1}H NMR recorded at 400 or 300 MHz,

Scheme 3. Synthesis of *N*-Alkylpyrimidones **44**−**47**[a]



[a] Reagents and conditions: (a) Cs_2CO_3, THF, 50 °C; add electrophile; **57a** Me_2SO_4; **57b** allyl bromide; **57c** benzyl bromide; (b) Pd/C, H_2, MeOH, rt; DCM, py, 2-chlorobenzyl isocyanate; (c) NaOH, 80 °C.

Scheme 4. Synthesis of Inhibitors **16** and **17**



[a] Reagents and conditions: (a) NaOMe, MeOH, 90%; (b) NaSMe, NMP, 50 °C, 70%; (c) BBr_3, DCM, then RP-HPLC, 50% (d) H_2O_2, AcOH, then RP-HPLC, 24%.

^{13}C NMR recorded at 100 or 75 MHz) were obtained on Bruker AMX spectrometers and are referenced in ppm relative to TMS or the solvent signal. Low-resolution mass spectra (*m*/*z*) were recorded on a Perkin-Elmer API 100 (electrospray ionization) mass spectrometer. High-resolution mass measurements were performed on a Finnigan TSQ Quantum Ultra AM in selected ion monitoring (SIM) mode. Elemental analyses were performed by the analytical chemistry group at IRBM.

Thin-layer chromatography was performed on aluminum plates precoated with Merck silica gel F_{254}, which were visualized by the quenching of the UV fluorescence (λ_{max} 254 nm) or by staining with 5% phosphomolybdic acid in 95% ethanol. Flash chromatography was accomplished on Merck silica gel (200−400 mesh). Reversed phase analytical HPLC was performed on a Waters Alliance separation module 2695, using H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) as eluents. Peaks were detected with a Waters 996 PDA detector using the Maxplot option (210−400 nM) contained in the PDA program. Inhibitor purity was determined using the following methods: Method A: Waters Symmetry C18 column (150 × 3.9 mm, 5 μm), flow rate 1 mL/min, gradient: 90% A (or 100% for polar compounds), 0.5 min isocratic, linear to 0% A in 10 min, then isocratic; Method B: Phase Separations Spherisorb S5 ODS2 column (25 × 4.6 mm, 5 μm), flow 1.5 mL/min, gradient: 90%A, 3 min isocratic, linear to 50%A in 15 min; Method C: Waters X-terra C18 column (50 × 2.5 mm, 3.5 μm), flow rate 0.5 mL/min, gradient: 100% A, 2.0 min isocratic, linear to 0% A in 7 min. Preparative HPLC was conducted on a Waters Symmetry C18 column (150 × 19 mm, 7 μm) or on Waters Prep-NovaPak column (HR C18, 40 × 100 mm, 6 micron) using H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) as eluents; detection at 220 nm with a Waters 486 absorbance detector. All reactions were run under inert atmosphere (nitrogen or argon) in oven or flame dried glassware.

The following compounds were prepared according to described procedures: 1,3-thiazole-2-carbonitrile[68] (**48f**), 1-benzyl-1*H*-imidazole-2-carbonitrile[69] (**48g**).

General Procedure for the Synthesis of 2-Substituted 5,6-Dihydroxypyrimidine-4-carboxylic Acids (3, 11, 15, 19, 22). The procedure from Culbertson was followed.[63] Usually amidoximes **49** and Michael adducts **50** were used without further purification. The preparation of 5,6-dihydroxy-2-(3-nitro-2-thienyl)pyrimidine-4-carboxylic acid (**22**) is a representative example.

3-Nitro-2-thiophenamidoximate (49n). Nitrile **48n** (86.93 mmol, 13.4 g) was suspended in water (360 mL) and ethanol (48 mL). Sodium carbonate (15.7 g, 147.8 mmol) and hydroxylamine (18.7 g, 287 mmol) were added, and the mixture was left at room temperature for 24 h. The orange solid was isolated by filtration, washed with a small portion of diethyl ether and dried. The amidoxime (14.0 g, 86%) was obtained as an orange solid and existed as a mixture *E*/*Z* of about 4:1* as determined by ^{13}C-spectroscopy. ^{1}H NMR (400 MHz, DMSO-d_6) δ, 9.95 (bs, 1 H), 7.68 (d, J = 5.3 Hz, 1H), 7.60 (d, J = 5.3 Hz, 1H) 6.08 (bs, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.5, 145.5, 144.6*, 139.5*, 136.6, 127.5*, 127.0, 124.2, 123.5*; *m*/*z* 188 (M + H); recrystallization from dichloromethane/*n*-pentane, mp 201−202 °C; Anal. ($C_5H_5N_3O_3S$) C, H, N, S.

Dimethyl 2-({[1-Amino(3-nitro-2-thienyl)methylene]amino}-oxy)but-2-enedioate (50n). Amidoxime **49n** (11.87 g, 63.90 mmol) was suspended in dichloromethane (250 mL). Triethylamine (0.5 mL) and dimethyl acetylenedicarboxylate (9.53 g, 67.1 mmol) were added. The mixture was refluxed for 3 h and became homogeneous during this time. Evaporation of the dichloromethane gave 20.88 g of a red oil, which was dissolved in ethyl acetate (400 mL). After washing with water and brine, the organic phase was dried over sodium sulfate and evaporated. The reddish oil (20.5 g, 97%) was used without further purification. ^{1}H NMR ($CDCl_3$, two diastereomers 2.5:1*) δ 7.61*, 7.58 (d, J = 5.6 Hz, 1H), 7.38*, 7.33 (d, J = 5.6 Hz, 1H) 6.10, 5.84* (bs, 2H), 5.94, 5.88* (s, 1H), 3.90*, 3.83 (s, 3H), 3.71, 3.68* (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.4*, 163.9, 161.6, 158.3*, 153.1, 149.1, 148.8*, 144.7*, 131.1, 130.9*, 126.2*, 125.9, 124.3*, 124.2, 104.3, 95.4*, 52.0*, 51.8, 50.7, 50.5*; *m*/*z* 330 (M + H).

Methyl 5,6-dihydroxy-2-(3-nitro-2-thienyl)pyrimidine-4-carboxylate (52n). To a flask containing adduct **50n** (30.7 g, 93.28 mmol) was added xylene (212 mL), and the reaction was left at 140 °C until the disappearance of the starting material. The reaction mixture was stored in a refrigerator at 4 °C overnight and the precipitate isolated by filtration. The brown solid was washed with ethyl acetate and petrol ether and dried under vacuum to give the product as a beige powder (13.3 g, 48%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.2 (bs, 1H), 7.88 (d, J = 5.5 Hz, 1H), 7.72 (d, J = 5.5 Hz, 1H), 3.82 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.6, 158.9, 147.2, 145.2, 139.3, 135.4, 129.6, 128.5, 124.2, 52.7; *m*/*z* 296 (M − H); Anal. ($C_{10}H_7N_3O_6S$) C, H, N.

Evaporation of the mother liquor under reduced pressure and purification of the residue by flash chromatography on silica gel (PE/EtOAc 1:1) gave a brown solid which was recrystallized from methanol and water to give **dimethyl 2-(3-nitro-2-thienyl)-1*H*-imidazole-4,5-dicarboxylate (53n)** as a yellow powder in 4% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 14.0 (bs, 1H), 7.90 (d, J = 5.5 Hz, 1H), 7.75 (d, J = 5.5 Hz, 1H) 3.85 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.2, 144.0, 138.2, 130.7, 130.3, 128.9, 124.2, 51.9; *m*/*z* 312 (M + H); HRMS calc. for $C_{11}H_{10}N_3O_6S$ (M + H) 312.0285, fd. 312.0269; HPLC: A: 4.25 min, 97.6%; B: 6.81 min, 98.1%.

5,6-Dihydroxy-2-(3-nitro-2-thienyl)pyrimidine-4-carboxylic Acid (22). To a suspension of **52n** (72 mg, 0.24 mmol) in methanol (1 mL) and water (1 mL) was added 0.5 N NaOH (2 equiv), and the mixture was heated to 80 °C. After 15 min hydrolysis was complete, as indicated by HPLC. The solution was cooled to room temperature, and then 1 N HCl was added dropwise until a pH of 2 was reached. The resulting solid was isolated by filtration and washed with water, MeOH and EtOAc and then dried under vacuum to give **22** as a yellow powder (29 mg, 43%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.3 (bs, 1H), 11.8 (bs, 1H), 7.89 (d, J = 5.4 Hz, 1H), 7.71 (d, J = 5.4 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ

169.5, 159.1, 149.6, 145.6, 139.7, 135.9, 129.9, 129.2, 124.5; *m/z* 282 (M − H); Anal. ($C_9H_5N_3O_6S$) C, H, N.

5,6-Dihydroxy-2-(2-thienyl)pyrimidine-4-carboxylic Acid (3). Adduct formation and cyclization gave **52a** as a brown powder (30%); hydrolysis and purification by RP-HPLC gave **3** as a beige powder (65%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.4 (bs, 1H), 7.84 (d, J = 3.5 Hz, 1H), 7.61 (d, J = 4.9 Hz, 1H) 7.09 (dd, J = 3.5, 4.9, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.8, 158.5, 146.4, 142.0, 136.7, 130.9, 128.7, 128.2, 127.9; *m/z* 239 (M + H); Anal. ($C_9H_6N_2O_4S$·0.5 H_2O·0.5 TFA) C, H, N.

5,6-Dihydroxy-2-(1,3-thiazol-2-yl)pyrimidine-4-carboxylic Acid (11). Adduct formation and cyclization gave **52f** as a brown powder (35%); hydrolysis and purification by RP-HPLC yielded **11** as a beige solid (71%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.51 (bs, 1H), 7.96 (d, J = 2.95, 1H), 7.91 (d, J = 2.95, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.3, 161.5, 159.2, 154.3, 143.5, 137.1, 127.0, 123.8; *m/z* 238 (M − H); *m/z* 240 (M + H); HRMS calc. for $C_8H_6N_3O_4S$ (M + H) 240.0074, fd. 240.0072; HPLC: A (100%): 3.22 min, 95.0%; B: 4.39 min; 96.2%.

2-(1-Benzothien-2-yl)-5,6-dihydroxypyrimidine-4-carboxylic Acid (15). Adduct formation and cyclization gave **52i** as a pale yellow solid (92%); hydrolysis and precipitation gave **15** as a beige solid after extensive washing with water, MeOH and EtOAc (46%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.2 (bs, 1H), 8.27 (s, 1H), 7.97 (d, J = 2.1 Hz, 1H), 7.86 (d, J = 2.1 Hz, 1H) 7.37−7.46 (m, 2H); *m/z* 289 (M + H); Anal. ($C_{13}H_8N_2O_4S$·0.5 H2O) C, H, N.

5,6-Dihydroxy-2-(3-methyl-2-thienyl)pyrimidine-4-carboxylic Acid (19). Adduct formation and cyclization gave 24% of **52k**; hydrolysis and purification by RP-HPLC gave **19** (24%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.2 (bs, 1H), 7.53 (d, J = 4.0 Hz, 1H), 7.93 (d, J = 4.0 Hz, 1H) 2.36 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.0, 161.3, 157.4, 136.9, 131.0, 128.4, 126.3, 14.9; *m/z* 251 (M − H); Anal. ($C_{10}H_8N_2O_4S$·0.5 H_2O) C, H, N.

Synthesis of 5,6-Dihydroxy-2-(methylthio)pyrimidine-4-carboxylic Acid (16). 2,6-Dichloro-5-methoxypyrimidine-4-carboxylate (59). *N*,*N*-dimethylaniline (0.856 mL, 6.76 mmol) was added to a stirred solution of methyl 2,6-dihydroxy-5-methoxypyrimidine-4-carboxylate[70] (1.0 g, 5 mmol) in $POCl_3$ (34.5 mL). After refluxing overnight $POCl_3$ was evaporated and the residue was poured on ice water and extracted into diethyl ether. The combined ethereal layers were washed with brine, dried over sodium sulfate and evaporated in vacuo. Purification by flash chromatography (SiO_2; petroleum ether/EtOAc 4:1) gave methyl 2,6-dichloro-5-methoxypyrimidine-4-carboxylate (**59**) (1.07 g, 90%) as a beige solid. ^{1}H NMR (300 MHz, DSMO) δ 3.95 (s, 3H), 3.93 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 161.97, 157.79, 151.25, 151.14, 148.47, 62.40, 53.45; *m/z* 238.0 (M + H).

M**ethyl 5,6-Dimethoxy-2-(methylthio)pyrimidine-4-carboxylate (60).** Powdered sodium methoxide (0.14 g, 2.5 mmol) was added to a stirred solution of **59** (0.5 g, 2.1 mmol) in anhydrous methanol (5 mL) at room temperature and stirred for 30 min. The reaction mixture was then diluted with ethyl acetate and washed with water and with brine. The organic layer was dried over sodium sulfate and evaporated to afford pure methyl 2-chloro-5,6-dimethoxypyrimidine-4-carboxylate (0.44 g, 90%) as a white solid. ^{1}H NMR (400 MHz, DMSO) δ 4.04 (s, 3H), 3.89 (s, 3H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 165.13, 162.81, 150.74, 148.45, 139.71, 61.26, 55.55, 52.94; *m/z* 232 (M + H). A solution of this compound (0.100 g, 0.43 mmol) in anhydrous NMP (1 mL) was treated with sodium methanethiolate (0.033 g, 0.47 mmol) in one portion and stirred at 50 °C for 16 h. The reaction mixture was diluted with ethyl acetate, washed with water and brine, dried over sodium sulfate and evaporated in vacuo, leaving methyl 5,6-dimethoxy-2-(methylthio)pyrimidine-4-carboxylate (**60**) (0.073 g, 70%) as a white solid. ^{1}H NMR (400 MHz, DMSO) δ 4.01 (s, 3H), 3.88 (s, 3H), 3.76 (s, 3H), 2.51 (s, 3H); *m/z* 244.8 (M + H).

5,6-Dihydroxy-2-(methylthio)pyrimidine-4-carboxylic Acid (16). A solution of **60** (0.080 g, 0.33 mmol) in anhydrous DCM (1 mL) was treated with BBr_3 (1 M solution in DCM, 1.98 mL, 0.198 mmol). The resulting solution was allowed to warm to room temperature and stirred for 4 h. The volatiles were evaporated, and the residue was treated with HCl (1 N, 8 mL) and stirred for 10 min. The solution was diluted with AcCN and purifed by Prep HPLC using a Waters Prep-NovaPak column. Lyophilization gave **16** (33 mg, 50%) as a colorless powder. ^{1}H NMR (300 MHz, DMSO) δ 2.47 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 169.37, 158.91, 148.94, 145.98, 128.68, 13.22; *m/z* 201 (M − H); HRMS calc. for $C_6H_7N_2O_4S$ (M + H) 203.0121, fd. 203.0125; HPLC: A (100%): 2.13 min, 98.1%; B: 3.38, 97.8%.

5,6-Dihydroxy-2-(methylsulfonyl)pyrimidine-4-carboxylic Acid (17). A solution of **16** (33 mg, 50%) (0.022 g, 0.11 mmol) in glacial acetic acid (1 mL) was treated with a 35% aqueous solution of hydrogen peroxide (0.5 mL). The resulting solution was stirred at room temperature for 16h then diluted with water/AcCN (1:1) and lyophilized. The crude material was purifed by Prep HPLC using a Waters Prep-NovaPak column. Lyophilization gave **17** (0.006 g, 24%) as acolorless powder. ^{1}H NMR (300 MHz, DMSO) δ 3.27 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 39.46 (SO_2*Me*); *m/z* 233 (M − H); HRMS calc. for $C_6H_5N_2O_6S$ (M − H) 233.0098, fd. 233.0069; HPLC (from 100% A): A: 1.1 min, 99%; B: 2.3 min, 98.7%.

Methyl 2-(3-Amino-2-thienyl)-5,6-dihydroxypyrimidine-4-carboxylate (54). To a solution of **52n** (1.5 g, 5.05 mmol) in ethyl acetate/methanol (2:1, 200 mL) was added Pd/C (10% Pd, 1.5 g) and the reaction stirred under an atmosphere of hydrogen for 5 h at ambient temperature. The catalyst was removed by filtration and washed with hot ethyl acetate and methanol. After evaporation **54** (1.08 g, 80%) was obtained as a yellow solid, which was used without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.80 (bs, 3H), 7.48 (d, J = 5.2 Hz, 1H), 6.67 (d, J = 5.2 Hz, 1H) 3.85 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.04, 160.32, 145.82, 144.34, 142.52, 130.11, 128.94, 122.82, 110.07, 52.85; *m/z* 268 (M + H).

(3-Amino-2-thienyl)-5,6-dihydroxypyrimidine-4-carboxylic Acid (24). A solution of methyl 2-(3-amino-2-thienyl)-5-[(2,2-dimethylpropanoyl)oxy]-6-hydroxypyrimidine-4-carboxylate (**56**) (0.080 g, 0.22 mmol) in 0.5 N NaOH (1.2 mL) was heated at 90 °C for 30 min. The cooled reaction mixture was acidified with 1 N HCl to pH 1 allowing the formation of a precipitate. The solid was washed with water, acetone and diethyl ether and then dried under vacuum. **24** (30 mg, 30%) was obtained as the hydrochloride salt. ^{1}H NMR (400 MHz, D_2O−NaOH, pH = 7) δ 7.65 (d, J = 4.9 Hz, 1H), 6.62 (d, J = 4.9 Hz, 1H); ^{13}C NMR (100 MHz, 330K, DMSO-d_6) δ 169.5, 159.5, 150.5, 146.7, 144.0, 128.9, 128.4, 121.8, 102.9; *m/z* 252 (M − H).

Methyl 5-[(2,2-Dimethylpropanoyl)oxy]-6-hydroxy-2-(3-nitro-2-thienyl)pyrimidine-4-carboxylate (55). 52n (3.0 g, 10 mmol) was dissolved in anhyd pyridine (30 mL) at room temperature. A catalytic amount of 4-DMAP (0.12 g, 1 mmol) was added, followed by the dropwise addition of neat pivaloyl chloride (1.29 mL, 10.5 mmol). After 3 h at room temperature, the pyridine was removed in vacuo, the residue dissolved in ethyl acetate and washed with 1 N HCl (2×) and brine. Drying over Na_2SO_4 and removal of the solvents gave a brown solid, which was crystallized from EtOAc to give **55** (2.5 g, 66%) as a beige solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.9 (bs, 1H), 7.96 (d, J = 5.5 Hz, 1H), 7.73 (d, J = 5.5 Hz, 1H), 3.83 (s, 3H), 1.30 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.9, 162.2, 157.2, 147.6, 145.2, 142.2, 137.7, 133.5, 130.0, 123.7, 52.6, 38.2, 26.3; *m/z* (ES^+) 382 (M + H); Anal. ($C_{15}H_{15}N_3O_7S$) calc. C 47.24, H 3.96, N 11.02; fd. C 46.61, H 3.71, N 10.75.

Methyl 5-[(2,2-Dimethylpropanoyl)oxy]-6-hydroxy-2-(3-amino-2-thienyl)pyrimidine-4-carboxylate (56). 55 (1.47 g, 3.8 mmol) was dissolved in MeOH/AcOEt (1:1 v/v, 45 mL) and hydrogenated at atmospheric pressure in the presence of 10% Pd/C (0.7 g). After 2 h, the catalyst was removed by filtration and extensively washed with hot MeOH and EtOAc. Evaporation of the filtrate gave the amine (1.19 g, 89%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO) δ 9.12 (bs, 2H), 7.55 (d, J = 5.2 Hz, 1H), 6.65 (d, J = 5.2 Hz, 1H), 3.85 (s, 3H), 1.29 (s, 9H); ^{13}C NMR (100 MHz, DMSO-

d_6) δ 175.0, 163.3, 159.9, 154.3, 151.6, 143.2, 141.5, 131.0, 121.5, 103.3, 52.9, 30.7, 26.8; *m/z* 352 (M + H).

Method A. Synthesis of Ureas from Amine 54. The preparation of **2-[3-({[(2-chlorobenzyl)amino]carbonyl}amino)-2-thienyl]-5,6-dihydroxypyrimidine-4-carboxylic acid** (**34**) is a representative example. Amine **54** (300 mg, 1.12 mmol) was dissolved in pyridine (10 mL). 2-Chloro-benzylisocyanate was added dropwise at room temperature and the reaction monitored by analytical HPLC. When all the amine was consumed, the solvent was evaporated and the residue was taken into a large volume of ethyl actetate, washed with hydrochloric acid (1 N), brine, and dried over sodium sulfate. The methyl ester obtained after evaporation was hydrolyzed using sodium hydroxide (4.5 mL, 1 N) and methanol (3 mL) at 80 °C for 30 min. The solution was then cooled in an ice-bath and acidified with hydrochloric acid (1 N) until a pH of 2 was obtained. The precipitated solid was isolated by filtration, washed with water and diethyl ether and dried. It was boiled once in ethyl acetate, filtered and washed again with ethyl acetate and diethyl ether, and dried under vacuum to give 270 mg of crude product. This was purified by preparative reversed phase HPLC using a Waters PrepNovaPak column to give 131 mg of **34** (28%) as a colorless powder. ^{1}H NMR (DMSO-d_6) δ 12.7 (bs, 1H), 11.05 (bs, 1H), 7.83 (d, J = 5.4 Hz, 1H), 7.61 (d, J = 5.4 Hz, 1H), 7.42−7.50 (m, 2H), 7.27−7.38 (m, 2H), 7.00 (bs, 1H), 4.42 (bd, J = 5.9 Hz, 2H); ^{13}C NMR (75 MHz, DMSO/HCl, 330K) δ 167.8, 159.3, 153.9, 142.1, 142.0, 140.6, 136.6, 131.6, 128.6, 128.6, 128.1, 127.2, 126.8, 122.1, 112.3, 40.6; *m/z* 419, 421 (M − H); HRMS calc. for $C_{17}H_{14}ClN_4O_5S$ 421.0373, fd. 421.0228; HPLC: A: 6.77 min, 100%, B: 10.34, 99%; Anal. ($C_{17}H_{13}N_4O_5S\cdot H_2O$) C, H, N, S.

2-(3-{[(Benzylamino)carbonyl]amino}-2-thienyl)-5,6-dihydroxypyrimidine-4-carboxylic Acid (25). ^{1}H NMR (DMSO-d_6) δ, 12.8 (bs, 1H), 11.05 (bs, 1 H), 7.85 (d, J = 5.4 Hz, 1H), 7.61 (d, J = 5.4 Hz, 1H), 7.30−7.41 (m, 4H), 7.18−7.27 (m, 1H), 6.93 (bs, 1H), 4.36 (bd, J = 5.7 Hz, 2H); *m/z* 387 (M + H), 385 (M − H); HRMS calc. for $C_{17}H_{15}N_4O_5S$ (M + H) 387.0763, fd. 387.0728; HPLC: A: 6.1 min, 96.2%; B: 9.1 min, 98.7%.

Method B: Synthesis of Amides from Amine 54. A solution of the carboxylic acid (0.27 mmol) and 1,1-carbonyldiimidazole (43 mg, 0.27 mmol) in anhydrous DMF (0.5 mL) was stirred at room temperature under nitrogen overnight. Amine **54** (60 mg, 0.22 mmol) was added as a solid and stirring continued for 24 h. HCl (1 N, 2 mL) was then added to the reaction mixture and the precipitate formed was isolated by centrifugation, washed with H_2O (2 × 2 mL) and dried. The solid was dissolved in THF/H_2O (1:1 v/v, 2 mL) and treated with 1 N NaOH (0.66 mmol) for 1 h at 80 °C. The reaction mixture was acidified with 1 N HCl and the precipitate isolated by filtration and purified by RP-HPLC.

5,6-Dihydroxy-2-{3-[(phenylacetyl)amino]-2-thienyl}pyrimidine-4-carboxylic Acid (28). ^{1}H NMR (400 MHz, DMSO) δ 12.90 (bs, 1H), 12.52 (bs, 1H), 7.96 (d, J = 7.0 Hz, 1H), 7.63 (d, J = 5.1 Hz, 1H), 7.38−7.31 (m, 2H), 7.30 (t, J = 7.5 Hz, 2H), 7.20 (m, 1H), 3.71 (s, 2H); *m/z* 370 (M − H); HRMS calc. for $C_{17}H_{14}N_3O_5S$ (M + H) 372.0654, fd. 372.0660; HPLC: A: 6.47 min, 96.0%; B: 9.39 min, 96.2%.

2-(3-{[(2-Chlorophenyl)acetyl]amino}-2-thienyl)-5,6-dihydroxypyrimidine-4-carboxylic Acid (36). 33 mg (89%) of a yellow powder obtained; 96% pure by HPLC. ^{1}H NMR (DMSO-d_6) δ, 13.0 (bs, 1H), 12.5 (s, 1 H), 11.05 (bs, 1H), 7.96 (d, J = 5.4 Hz, 1H), 7.71 (d, J = 5.4 Hz, 1H), 7.42−7.47 (m, 2H), 7.30−7.35 (m, 2H), 3.98 (s, 2H); *m/z* 404, 406 (M − H); HRMS calc. for $C_{17}H_{13}ClN_3O_5S$ (M + H) 406.0264, fd. 406.0277; HPLC: A: 6.74 min, 97.0%; B: 9.95 min, 96.6%.

5,6-Dihydroxy-2-{3-[(1*H*-indol-3-ylacetyl)amino]-2-thienyl}-pyrimidine-4-carboxylic Acid (39). ^{1}H NMR (300 MHz, DMSO) δ 12.42 (bs, 1H), 10.92 (bs, 1H), 8.01 (d, J = 5.2 Hz, 1H), 7.68 (d, J = 5.2 Hz, 1H), 7.63 (d, J = 7.9 Hz, 1H), 7.33 (m, 2H), 7.06 (t, J = 7.6 Hz, 1H), 6.96 (t, J = 7.6 Hz, 1H) 3.86 (s, 2H); ^{13}C NMR (75 MHz, DMSO/HCl) δ 169.09, 168.76, 160.06, 145.78, 145.27, 140.66, 136.46, 128.80, 128.32, 127.39, 124.41, 122.52, 121.38, 119.07, 118.84, 113.79, 111.755, 108.37, 34.60; *m/z* 409 (M − H); Anal. ($C_{19}H_{14}N_4O_5S\cdot H_2O$) C, H, N, S.

Method C: Synthesis of Ureas from Amine 56. The preparation of 2-[3-({[(2-chloro-6-methylbenzyl)amino]carbonyl}amino)-2-thienyl]-5,6-dihydroxypyrimidine-4-carboxylic acid (**37**) is a representative example. Amine **56** (1.6 g, 4.55 mmol) was dissolved in dry THF under nitrogen. After cooling at 0 °C, pyridine (0.5 mL, 5.69 mmol) and phenyl chloroformate (0.6 mL, 4.78 mmol) were added. The ice bath was removed and the reaction was stirred 2 h at RT. The mixture was diluted with EtOAc and washed with HCl (1 N), water, saturated $NaHCO_3$ and brine. Evaporation of the solvent gave methyl 5-[(2,2-dimethylpropanoyl)oxy]-6-hydroxy-2-{3-[(phenoxycarbonyl)amino]-2-thienyl}-pyrimidine-4-carboxylate in 97% yield. ^{1}H NMR (DMSO-d_6) δ 11.68 (bs, 1H), 7.85 (d, J = 5.4 Hz, 1H), 7.80 (d, J = 5.4 Hz, 1H), 7.45 (m, 2H), 7.29 (m, 3H), 3.83 (s, 3H), 1.31 (s, 9H); *m/z* 470 (M − H). This compound (100 mg, 0.21 mmol) was suspended in 0.5 mL of DMSO. 2-Chloro-6-methylbenzylamine (34 mg, 0.22 mmol) was added and the reaction mixture heated at 60 °C for 3 h. After cooling to room temperature, the reaction was diluted with EtOAc and washed with HCl (1 N), water, NaOH (1 M), brine, dried over sodium sulfate and evaporated. The crude compound was dissolved in methanol (2 mL) and treated with NaOH (1M, 0.5 mL). The reaction was stirred 1 h at 80 °C. The solution was then cooled in an ice-bath and acidified with hydrochloric acid (1 N) until a pH of 2 was obtained. The precipitated solid was isolated by filtration, washed with water and diethyl ether and dried affording **37** (19 mg, 15%). ^{1}H NMR (DMSO-d_6) δ 12.72 (bs, 1H), 11.01 (bs, 1H), 7.86 (d, J = 5.7 Hz, 1H), 7.61 (bs, 1H), 7.2−7.3 (m, 3H), 6.40 (s, 1H), 4.48 (d, J = 4.43 Hz, 2H), 2.45 (s, 3H); *m/z* 435 (M + H); HRMS calc. for $C_{18}H_{16}ClN_4O_5S$ (M + H) 435.0530, fd. 435.0506; HPLC: A: 7.09 min, 96.1%; B: 11.4 min, 95.2%.

Method D: Synthesis of Amides from Amine 56. A solution of the carboxylic acid (0.2 mmol) and triethylamine (0.034 mL, 0.22 mmol) in DCM (0.7 mL) was cooled to 0 °C and treated with BOP-Cl (56 mg, 0.22 mmol) in one portion. After 30 min amine **56** (70 mg, 0.20 mmol) and triethylamine (0.034 mL, 0.22 mmol) were added sequentially and the reaction mixture was allowed to warm to RT and stirred overnight. The reaction mixture was then diluted with EtOAc, washed with 1 N HCl, saturated $NaHCO_3$ and brine, and dried over Na_2SO_4. The crude residue obtained after evaporation of the volatiles was dissolved in THF and water and treated with triturated with 1 N NaOH (0.5 mL). The reaction mixture was heated at 80 °C for 1 h and then cooled to RT and acidified with 1 N HCl. The precipitate formed was filtered off, washed with H_2O and with diethyl ether and further purified by RP-HPLC.

5,6-Dihydroxy-2-(3-{[(2-phenyl-1*H*-indol-3-yl)acetyl]amino}-2-thienyl)pyrimidine-4-carboxylic Acid (43). 48 mg (50%) of a yellow powder obtained; 96% pure by HPLC. ^{1}H NMR (DMSO-d_6) δ, 13.1 (bs, 1H), 12.65 (s, 1H), 11.3 (bs, 1H), 8.01 (d, J = 5.4 Hz, 1H), 7.82 (d, J = 7.5, 2H); 7.71 (d, J = 5.4 Hz, 1H), 7.59 (d, J = 7.9 Hz, 1H), 7.49 (t, J = 7.6 Hz, 2H), 7.33−7.42 (m, 2H), 7.10 (t, J = 7.7 Hz, 1H), 6.95 (t, J = 7.4 Hz, 1H), 4.04 (s, 2H); *m/z* 485 (M − H); HRMS calc. for $C_{25}H_{19}N_4O_5S$ (M + H) 487.1183, fd. 487.1189; HPLC: A: 7.46 min, 95.2%; B: 11.5 min, 95.6%.

Method E: Synthesis of Carbamates from 24. The alcohol (0.38 mmol) was dissolved in CH_3CN (1.0 mL) and treated with DSC (97.4 mg, 0.38 mmol) and Et_3N (0.13 mL, 0.95 mmol) at room temperature,[67] and the resulting solution was stirred overnight. Amine **24** (55 mg, 0.19 mmol) dissolved in 1:1 mixture of CH_3CN and H_2O (1 mL) and Et_3N (0.080 mL, 0.57 mmol) was added, and the resulting mixture was stirred at room temperature overnight. The volatiles were evaporated, and the residue was treated with HCl (1 N, 2 mL × 3) and washed respectively with H_2O and diethyl ether and further purified by RP-HPLC.

2-(3-{[(Benzyloxy)carbonyl]amino}-2-thienyl)-5,6-dihydroxypyrimidine-4-carboxylic Acid (26). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.29 (s, 1H), 7.78 (d, J = 4.7 Hz, 1H), 7.70 (d, J = 4.7 Hz, 1H) 7.48−7.27 (m, 5H), 5.20 (s, 2H); ^{13}C NMR (300 MHz, DMSO-d_6) δ 168.8, 159.7, 153.0, 146.0, 144.8, 139.7, 136.7, 128.6, 128.3, 128.0, 127.6, 122.0, 113.9, 66.3; *m/z* 386 (M − H); *m/z* 389 (M +

H); HRMS calc. for $C_{17}H_{14}N_3O_6S$ (M + H) 388.0603, fd. 388.0581; HPLC A: 7.18 min, 98.0%; B: 10.93 min, 99.1%.

2-[3-({[(2-Chlorobenzyl)oxy]carbonyl}amino)-2-thienyl]-5,6-dihydroxypyrimidine-4-carboxylic Acid (35). ^{1}H NMR (300 MHz, 300K, DMSO-d_6) δ 11.46 (bs, 1H), 7.79 (d, J = 5.52 Hz, 1H), 7.69 (d, J = 5.31 Hz, 2H), 7.61−7.58 (m, 1H), 7.52−7.48 (m, 1H), 7.44−7.37 (m, 2H), 5.28 (s, 2H); *m*/*z* 420 (M − H). HRMS calc. for $C_{17}H_{13}ClN_3O_6S$ (M + H) 422.0214, fd. 422.0228; HPLC: A: 7.80 min, 96.6%; B: 12.5 min, 95.2%.

2-[3-({[(2,6-Dichlorobenzyl)oxy]carbonyl}amino)-2-thienyl]-5,6-dihydroxy-pyrimidine-4-carboxylic Acid (38). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.13 (s, 1H), 7.85−7.69 (m, 2 H), 7.57 (d, J = 7.6 Hz, 2 H) 7.52−7.45 (m, 1H), 5.44 (s, 2H); ^{13}C NMR (300 MHz, DMSO-d_6) δ 168.6, 159.6, 152.4, 139.0, 136.0, 131.4, 130.9, 128.7, 128.1, 121.7, 61.6; *m*/*z* 455 (M − H); *m*/*z* 457 (M + H); HRMS calc. for $C_{17}H_{12}Cl_2N_3O_6S$ (M + H) 455.9824, fd. 455.9799; HPLC: A: 8.51 min, 97.2%; B: 12.6 min, 96.3%.

5,6-Dihydroxy-2-(3-{[(1-naphthylmethoxy)carbonyl]amino}-2-thienyl)pyrimidine-4-carboxylic Acid (42). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.35 (s, 1H), 8.17−8.07 (m, 1 H), 8.01−7.89 (m, 2H) 7.83−7.74 (m, 1H), 7.73−7.47 (m, 5H), 5.69 (s, 2H); *m*/*z* 436 (M − H); HRMS calc. for $C_{21}H_{16}N_3O_6S$ 438.0760 (M + H), fd. 438.0766; HPLC: A: 7.98 min, 95.0%; B: 13.1 min, 94.2%.

Methyl 5-[(2,2-Dimethylpropanoyl)oxy]-1-methyl-2-(3-nitrothien-2-yl)-6-oxo-1,6-dihydro-pyrimidine-4-carboxylate (57a). Pyrimidine **55** (0.8 g, 2.10 mmol) was dissolved in THF (10 mL). Cesium carbonate (1.03 g, 1.5 equiv) was added, and the mixture was heated to 50 °C. After 5 min neat dimethyl sulfate (0.53 g, 2 equiv) was added dropwise and the reaction was then stirred for 1 h at this temperature. After cooling to room temperature, the reaction mixture was diluted with ethyl acetate and the organic phase washed with NH_4Cl (2 M) and brine. Flash chromatography (PE/EA 3:1) gave the *O*-methylated compound **58a** (0.33 g, 39.8%) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, J = 5.4 Hz, 1H), 7.41 (d, J = 5.4 Hz, 1H), 4.01 (s, 3H), 3.96 (s, 3H), 1.40 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0, 163.5, 162.6, 154.1, 147.4, 146.5, 136.5, 132.6, 127.8, 124.2, 55.5, 53.1, 39.2, 26.9; *m*/*z* 396 (M + H); recrystallization from dichloromethane/*n*-pentane, mp 112−114 °C; Anal. ($C_{16}H_{17}N_3O_7S$) C, H, N.

The eluent was then changed to PE/EA 1:1 to yield **57a** (0.45 g, 54.7%) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, J = 5.5 Hz, 1H), 7.57 (d, J = 5.5 Hz, 1H), 3.92 (s, 3H), 3.40 (s, 3H), 1.43 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0, 162.5, 157.8, 147.8, 146.0, 140.3, 139.4, 134.3, 127.9, 123.7, 53.1, 39.3, 33.5, 27.0; *m*/*z* 396 (M + H); recrystallization from dichloromethane/*n*-pentane, mp 159−162 °C; Anal. ($C_{16}H_{17}N_3O_7S$) C, H, N.

5-Hydroxy-1-methyl-2-(3-nitrothien-2-yl)-6-oxo-1,6-dihydro-pyrimidine-4-carboxylic Acid (44). Pyrimidone **57a** (90 mg) was hydrolyzed using sodium hydroxide as described for compound **22**. Purification by RP-HPLC gave **44** (30 mg, 44%) as a colorless powder. ^{1}H NMR (400 MHz; DMSO-d_6) δ 7.97 (d, J = 5.5 Hz, 1H), 7.78 (d, J = 5.5 Hz, 1H), 3.25 (s, 3H); ^{13}C NMR (100 MHz; DMSO-d_6) δ 168.1, 158.2, 147.7, 145.0, 139.6, 135.5, 129.5, 126.7, 122.8, 32.8; *m*/*z* 296 (M − H); HRMS calc. for $C_{10}H_8N_3O_6S$ (M + H) 298.0128, fd. 298.0123; HPLC: 4.32 min, 100%; C: 4.62, 99.2%.

2-[3-({[(2-Chlorobenzyl)amino]carbonyl}amino)-2-thienyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxylic Acid (47). Pyrimidone **57a** was dissolved in methanol (0.02 M). Pd/C (10% Pd, 20% in weight) was added, and the reaction stirred for 4 h under an atmosphere of hydrogen. Removal of the catalyst by filtration, exhaustive washing of the catalyst and evaporation of the solvents gave methyl 5-[(2,2-dimethylpropanoyl)oxy]-1-methyl-2-(3-aminothien-2-yl)-6-oxo-1,6-dihydropyrimidine-4-carboxylate (93%) as a yellow powder, which was used without further purification. ^{1}H NMR (400 MHz; DMSO-d_6) δ 7.68 (d, J = 5.5 Hz, 1H), 7.05 (bs, 2H), 6.72 (d, J = 5.5 Hz, 1H), 3.83 (s, 3H), 3.67 (s, 3H), 1.29 (s, 9H); *m*/*z* 366 $(M + H)^+$.

The amine (80 mg, 0.22 mmol) was dissolved in dichloromethane (2 mL) and pyridine (26 mg, 1.5 equiv) and neat 2-chlorobenzyl isocyanate (40 mg, 1.1 equiv) were added at 0 °C. The resulting solution was stirred overnight at room temperature, and then another 1.1 equiv of isocyanate was added. The reaction was brought to reflux for 5 h and cooled to room temperature. After dilution with ethyl acetate, the organic phase was washed with hydrochloric acid (1 N), water, sat. aq sodium hydrogen carbonate, and brine. The crude product, obtained after evaporation of the solvents, was directly hydrolyzed using sodium hydroxide (0.5 M, 3 equiv) in methanol (2 mL) and THF (1.5 mL) at 80 °C for 1 h. The solution was cooled to room temperature and acidified to pH 2 with hydrochloric acid (1 N). The crude product was purified by RP-HPLC. After lyophilization **47** (36 mg, 38%) was obtained as a colorless powder. ^{1}H NMR (400 MHz; DMSO-d_6) δ 8.88 (bs, 1H), 7.66 (d, J = 5.4 Hz, 1H), 7.53 (d, J = 5.4 Hz, 1H), 7.42 (d, J = 6.7 Hz, 1H), 7.22−7.35 (m, 3H), 6.73 (t, J = 5.8 Hz, 1H), 4.31 (d, J = 5.8 Hz, 2H), 3.32 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.1, 159.4, 154.8150.1, 147.2, 143.3, 138.7, 137.4, 132.4, 129.6, 129.3, 129.1, 127.7, 127.0, 122.7, 113.6, 41.2, 33.7; *m*/*z* 433, 435 (M − H); HRMS calc. for $C_{18}H_{16}ClN_4O_5S$ (M + H) 435.0524, fd. 435.0521; A: HPLC: 6.61 min, 98.8%; B: 12.7 min, 97.5%.

Determination of the pK_a. Data were acquired using the Sirius Profiler SGA (Sirius Analytical, Forest Row, East Sussex, UK) and the built-in software. A solution of the compound in DMSO 10 mmol, 20 μL) was diluted to 2 mL using deionized water and infused at 0.25 mL/min into a 1 mL/min pH gradient. Changes in the UV spectrum were recorded with the diode array detector during the 2 min run with the pH changing from 2 to 12 and used to calculate the apparent pK_a of the compound.

Molecular Modeling. Conformational analysis was performed with MacroModel[71] generating 1000 initial conformers for each compound using the Monte Carlo algorithm. The conformers were subsequently energy-minimized with the MMFF method.[72,73] Information about the geometric details of magnesium chelation were obtained from small molecule crystal structures as deposited in the CSD.[74] This information was used to place the pyrimidine oxygen atoms into positions corresponding to a typical chelation geometry. Finally the compound was optimized in the active site of the protein. The binding of the primer-template duplex and the nucleoside triphosphate was inferred from the HIV RT ternary complex as described by Bressanelli.[62,75]

Acknowledgment. We thank Silvia Pesci and Renzo Bazzo for NMR experiments. We are grateful to Francesca Naimo and Anna Alfieri for analytical chemistry, to Fabio Bonelli and Vincenzo Pucci for HRMS determination, and to Nadia Gennari, Monica Bisbocci, Sergio Serafini and Mauro Cerretani for the inhibition data on the NS5B enzyme and the replicon. We are indebted to Marco Poma for the resynthesis of compound **3**. We thank Michael Rowley and Joerg Habermann for proofreading of the manuscript and for helpful discussions. This work was supported in part by a grant from the MIUR.

Supporting Information Available: Synthetic procedures for the preparation of nitriles **48i**, **48n**, and inhibitors **21**, **23**, **27**, **45** and **46**. NMR and MS data for compounds **7**−**10**, **12**−**14**, **18**, **20**, **29**−**33**, **40** and **41**. Protocols for biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Wasley, A.; Alter, M. J. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin. Liver Dis.* **2000**, *20*, 1−16.
(2) Lauer, G. M.; Walker, B. D. Hepatitis C virus infection. *N. Engl. J. Med.* **2001**, *345*, 41−52.
(3) McHutchison, J. G. Understanding hepatitis C. *Am. J. Manag. Care* **2004**, *10*, S21−29.
(4) Tan, S. L.; He, Y.; Huang, Y.; Gale, M., Jr. Strategies for hepatitis C therapeutic intervention: now and next. *Curr. Opin. Pharmacol.* **2004**, *4*, 465−470.
(5) Wong, J. B.; Davis, G. L.; McHutchison, J. G.; Manns, M. P.; Albrecht, J. K. Economic and clinical effects of evaluating rapid viral response to peginterferon alfa-2b plus ribavirin for the initial treatment of chronic hepatitis C. *Am. J. Gastroenterol.* **2003**, *98*, 2354−2362.

(6) McHutchison, J. G.; Manns, M.; Patel, K.; Poynard, T.; Lindsay, K. L.; Trepo, C.; Dienstag, J.; Lee, W. M.; Mak, C.; Garaud, J. J.; Albrecht, J. K. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* **2002**, *123*, 1061−1069.

(7) Dymock, B. W. Emerging therapies for hepatitis C virus infection. *Expert Opin. Emerg. Drugs* **2001**, *6*, 13−42.

(8) Fried, M. W.; Shiffman, M. L.; Reddy, K. R.; Smith, C.; Marinos, G.; Goncales, F. L., Jr.; Haussinger, D.; Diago, M.; Carosi, G.; Dhumeaux, D.; Craxi, A.; Lin, A.; Hoffman, J.; Yu, J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* **2002**, *347*, 975−982.

(9) Fried, M. W. Side effects of therapy of hepatitis C and their management. *Hepatology* **2002**, *36*, S237−244.

(10) Tan, S. L.; Pause, A.; Shi, Y.; Sonenberg, N. Hepatitis C therapeutics: current status and emerging strategies. *Nat. Rev. Drug Discovery* **2002**, *1*, 867−881.

(11) De Francesco, R.; Rice, C. M. New therapies on the horizon for hepatitis C: are we close? *Clin. Liver Dis.* **2003**, *7*, 211−242, xi.

(12) Ni, Z. J.; Wagman, A. S. Progress and development of small molecule HCV antivirals. *Curr. Opin. Drug Discovery Devel.* **2004**, *7*, 446−459.

(13) Sarisky, R. T. Nonnucleoside inhibitors of the HCV polymerase. *J. Antimicrob. Chemother.* **2004**, *54*, 14−16.

(14) Gordon, C. P.; Keller, P. A. Control of hepatitis C: a medicinal chemistry perspective. *J. Med. Chem.* **2005**, *48*, 1−20.

(15) Brass, V.; Blum, H. E.; Moradpour, D. Recent developments in target identification against hepatitis C virus. *Expert Opin. Ther. Targets* **2004**, *8*, 295−307.

(16) De Francesco, R.; Migliaccio, G. Challenges and successes in developing new therapies for hepatitis C. *Nature* **2005**, *436*, 953−960.

(17) Lahm, A.; Yagnik, A.; Tramontano, A.; Koch, U. Hepatitis C virus proteins as targets for drug development: the role of bioinformatics and modelling. *Curr. Drug Targets* **2002**, *3*, 281−296.

(18) Behrens, S. E.; Tomei, L.; De Francesco, R. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J.* **1996**, *15*, 12−22.

(19) Wang, Q. M.; Hockman, M. A.; Staschke, K.; Johnson, R. B.; Case, K. A.; Lu, J.; Parsons, S.; Zhang, F.; Rathnachalam, R.; Kirkegaard, K.; Colacino, J. M. Oligomerization and cooperative RNA synthesis activity of hepatitis C virus RNA-dependent RNA polymerase. *J. Virol.* **2002**, *76*, 3865−3872.

(20) Biswal, B. K.; Cherney, M. M.; Wang, M.; Chan, L.; Yannopoulos, C. G.; Bilimoria, D.; Nicolas, O.; Bedard, J.; James, M. N. Crystal structures of the RNA-dependent RNA polymerase genotype 2a of hepatitis C virus reveal two conformations and suggest mechanisms of inhibition by non-nucleoside inhibitors. *J. Biol. Chem.* **2005**, *280*, 18202−18210.

(21) Love, R. A.; Parge, H. E.; Yu, X.; Hickey, M. J.; Diehl, W.; Gao, J.; Wriggers, H.; Ekker, A.; Wang, L.; Thomson, J. A.; Dragovich, P. S.; Fuhrman, S. A. Crystallographic identification of a noncompetitive inhibitor binding site on the hepatitis C virus NS5B RNA polymerase enzyme. *J. Virol.* **2003**, *77*, 7575−7581.

(22) Wang, M.; Ng, K. K.; Cherney, M. M.; Chan, L.; Yannopoulos, C. G.; Bedard, J.; Morin, N.; Nguyen-Ba, N.; Alaoui-Ismaili, M. H.; Bethell, R. C.; James, M. N. Nonnucleoside analogue inhibitors bind to an allosteric site on HCV NS5B polymerase. Crystal structures and mechanism of inhibition. *J. Biol. Chem.* **2003**, *278*, 9489−9495.

(23) Bressanelli, S.; Tomei, L.; Rey, F. A.; De Francesco, R. Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides. *J. Virol.* **2002**, *76*, 3482−3492.

(24) Butcher, S. J.; Grimes, J. M.; Makeyev, E. V.; Bamford, D. H.; Stuart, D. I. A mechanism for initiating RNA-dependent RNA polymerization. *Nature* **2001**, *410*, 235−240.

(25) Lindenbach, B. D.; Evans, M. J.; Syder, A. J.; Wolk, B.; Tellinghuisen, T. L.; Liu, C. C.; Maruyama, T.; Hynes, R. O.; Burton, D. R.; McKeating, J. A.; Rice, C. M. Complete replication of hepatitis C virus in cell culture. *Science* **2005**, *309*, 623−626.

(26) Zhong, J.; Gastaminza, P.; Cheng, G.; Kapadia, S.; Kato, T.; Burton, D. R.; Wieland, S. F.; Uprichard, S. L.; Wakita, T.; Chisari, F. V. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9294−9299.

(27) Lohmann, V.; Korner, F.; Koch, J.; Herian, U.; Theilmann, L.; Bartenschlager, R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **1999**, *285*, 110−113.

(28) Bartenschlager, R. Hepatitis C virus replicons: potential role for drug development. *Nat. Rev. Drug Discovery* **2002**, *1*, 911−916.

(29) Lamarre, D.; Anderson, P. C.; Bailey, M.; Beaulieu, P.; Bolger, G.; Bonneau, P.; Bos, M.; Cameron, D. R.; Cartier, M.; Cordingley, M. G.; Faucher, A. M.; Goudreau, N.; Kawai, S. H.; Kukolj, G.; Lagace, L.; LaPlante, S. R.; Narjes, H.; Poupart, M. A.; Rancourt, J.; Sentjens, R. E.; St George, R.; Simoneau, B.; Steinmann, G.; Thibeault, D.; Tsantrizos, Y. S.; Weldon, S. M.; Yong, C. L.; Llinas-Brunet, M. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* **2003**, *426*, 186−189.

(30) Bartenschlager, R. The hepatitis C virus replicon system: from basic research to clinical application. *J. Hepatol.* **2005**, *43*, 210−216.

(31) Goudreau, N.; Llinas-Brunet, M. The therapeutic potential of NS3 protease inhibitors in HCV infection. *Expert Opin. Investig. Drugs* **2005**, *14*, 1129−1144.

(32) Wu, J. Z.; Hong, Z. Targeting NS5B RNA-dependent RNA polymerase for anti-HCV chemotherapy. *Curr. Drug Targets: Infectious Disorders* **2003**, *3*, 207−219.

(33) Beaulieu, P. L.; Tsantrizos, Y. S. Inhibitors of the HCV NS5B polymerase: new hope for the treatment of hepatitis C infections. *Curr. Opin. Investig. Drugs* **2004**, *5*, 838−850.

(34) Condon, S. M.; LaPorte, M. G.; Herbertz, T. Allosteric inhibitors of hepatitis C NS5B RNA-dependent RNA polymerase. *Curr. Med. Chem.: Anti-Infective Agents* **2005**, *4*, 99−110.

(35) Harper, S.; Avolio, S.; Pacini, B.; Di Filippo, M.; Altamura, S.; Tomei, L.; Paonessa, G.; Di Marco, S.; Carfi, A.; Giuliano, C.; Padron, J.; Bonelli, F.; Migliaccio, G.; De Francesco, R.; Laufer, R.; Rowley, M.; Narjes, F. Potent Inhibitors of Subgenomic Hepatitis C Virus RNA Replication through Optimization of Indole-N−Acetamide Allosteric Inhibitors of the Viral NS5B Polymerase. *J. Med. Chem.* **2005**, *48*, 4547−4557.

(36) Harper, S.; Pacini, B.; Avolio, S.; Di Filippo, M.; Migliaccio, G.; Laufer, R.; De Francesco, R.; Rowley, M.; Narjes, F. Development and preliminary optimization of indole-N-acetamide inhibitors of hepatitis C virus NS5B polymerase. *J. Med. Chem.* **2005**, *48*, 1314−1317.

(37) Di Marco, S.; Volpari, C.; Tomei, L.; Altamura, S.; Harper, S.; Narjes, F.; Koch, U.; Rowley, M.; De Francesco, R.; Migliaccio, G.; Carfi, A. Interdomain Communication in Hepatitis C Virus Polymerase Abolished by Small Molecule Inhibitors Bound to a Novel Allosteric Site. *J. Biol. Chem.* **2005**, *280*, 29765−29770.

(38) Ma, H.; Leveque, V.; De Witte, A.; Li, W.; Hendricks, T.; Clausen, S. M.; Cammack, N.; Klumpp, K. Inhibition of native hepatitis C virus replicase by nucleotide and non-nucleoside inhibitors. *Virology* **2005**, *332*, 8−15.

(39) Gopalsamy, A.; Lim, K.; Ciszewski, G.; Park, K.; Ellingboe, J. W.; Bloom, J.; Insaf, S.; Upeslacis, J.; Mansour, T. S.; Krishnamurthy, G.; Damarla, M.; Pyatski, Y.; Ho, D.; Howe, A. Y.; Orlowski, M.; Feld, B.; O'Connell, J. Discovery of pyrano[3,4-b]indoles as potent and selective HCV NS5B polymerase inhibitors. *J. Med. Chem.* **2004**, *47*, 6603−6608.

(40) Beaulieu, P. L.; Bousquet, Y.; Gauthier, J.; Gillard, J.; Marquis, M.; McKercher, G.; Pellerin, C.; Valois, S.; Kukolj, G. Non-nucleoside benzimidazole-based allosteric inhibitors of the hepatitis C virus NS5B polymerase: inhibition of subgenomic hepatitis C virus RNA replicons in Huh-7 cells. *J. Med. Chem.* **2004**, *47*, 6884−6892.

(41) Gu, B.; Johnston, V. K.; Gutshall, L. L.; Nguyen, T. T.; Gontarek, R. R.; Darcy, M. G.; Tedesco, R.; Dhanak, D.; Duffy, K. J.; Kao, C. C.; Sarisky, R. T. Arresting initiation of hepatitis C virus RNA synthesis using heterocyclic derivatives. *J. Biol. Chem.* **2003**, *278*, 16602−16607.

(42) Tomassini, J. E.; Getty, K.; Stahlhut, M. W.; Shim, S.; Bhat, B.; Eldrup, A. B.; Prakash, T. P.; Carroll, S. S.; Flores, O.; MacCoss, M.; McMasters, D. R.; Migliaccio, G.; Olsen, D. B. Inhibitory effect of 2′-substituted nucleosides on hepatitis C virus replication correlates with metabolic properties in replicon cells. *Antimicrob. Agents Chemother.* **2005**, *49*, 2050−2058.

(43) Eldrup, A. B.; Allerson, C. R.; Bennett, C. F.; Bera, S.; Bhat, B.; Bhat, N.; Bosserman, M. R.; Brooks, J.; Burlein, C.; Carroll, S. S.; Cook, P. D.; Getty, K. L.; MacCoss, M.; McMasters, D. R.; Olsen, D. B.; Prakash, T. P.; Prhavc, M.; Song, Q.; Tomassini, J. E.; Xia, J. Structure−activity relationship of purine ribonucleosides for inhibition of hepatitis C virus RNA-dependent RNA polymerase. *J. Med. Chem.* **2004**, *47*, 2283−2295.

(44) Carroll, S. S.; Tomassini, J. E.; Bosserman, M.; Getty, K.; Stahlhut, M. W.; Eldrup, A. B.; Bhat, B.; Hall, D.; Simcoe, A. L.; LaFemina, R.; Rutkowski, C. A.; Wolanski, B.; Yang, Z.; Migliaccio, G.; De Francesco, R.; Kuo, L. C.; MacCoss, M.; Olsen, D. B. Inhibition of hepatitis C virus RNA replication by 2′-modified nucleoside analogs. *J. Biol. Chem.* **2003**, *278*, 11979−11984.

(45) Summa, V.; Petrocchi, A.; Pace, P.; Matassa, V. G.; De Francesco, R.; Altamura, S.; Tomei, L.; Koch, U.; Neuner, P. Discovery of alpha,gamma-diketo acids as potent selective and reversible inhibitors of hepatitis C virus NS5b RNA-dependent RNA polymerase. *J. Med. Chem.* **2004**, *47*, 14−17.

(46) De Clercq, E. Antiviral drugs in current clinical use. *J. Clin. Virol.* **2004**, *30*, 115−133.

(47) De Francesco, R.; Tomei, L.; Altamura, S.; Summa, V.; Migliaccio, G. Approaching a new era for hepatitis C virus therapy: inhibitors of the NS3−4A serine protease and the NS5B RNA-dependent RNA polymerase. *Antiviral Res.* **2003**, *58*, 1−16.
(48) Pace, P.; Nizi, E.; Pacini, B.; Pesci, S.; Matassa, V.; De Francesco, R.; Altamura, S.; Summa, V. The monoethyl ester of meconic acid is an active site inhibitor of HCV NS5B RNA-dependent RNA polymerase. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3257−3261.
(49) Summa, V.; Petrocchi, A.; Matassa, V. G.; Taliani, M.; Laufer, R.; De Francesco, R.; Altamura, S.; Pace, P. HCV NS5b RNA-dependent RNA polymerase inhibitors: from alpha, gamma-diketoacids to 4,5-dihydroxypyrimidine- or 3-methyl-5-hydroxypyrimidinonecarboxylic acids. Design and synthesis. *J. Med. Chem.* **2004**, *47*, 5336−5339.
(50) Crescenzi, B.; Poma, M.; Ontoria, J. M.; Marchetti, A.; Nizi, E.; Matassa, V.; Gardelli, C. Phenyldihydroxypyrimidines as HCV NS5B RNA Dependent RNA Polymerase Inhibitors. Part I: Amides and Ureas. *Lett. Drug Des. Discovery* **2005**, *2*, 451−455.
(51) Ponzi, S.; Giuliano, C.; Donghi, M.; Poma, M.; Matassa, V. G.; Stansfield, I. Phenyldihydroxypyrimidines as HCV NS5B RNA Dependent RNA Polymerase Inhibitors. Part II: Sulfonamides. *Lett. Drug Des. Discovery* **2005**, *2*, 456−461.
(52) Stansfield, I.; Avolio, S.; Colarusso, S.; Gennari, N.; Narjes, F.; Pacini, B.; Ponzi, S.; Harper, S. Active site inhibitors of HCV NS5B polymerase. The development and pharmacophore of 2-thienyl-5,6-dihydroxypyrimidine-4-carboxylic acid. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5085−5088.
(53) Hazuda, D. J.; Anthony, N. J.; Gomez, R. P.; Jolly, S. M.; Wai, J. S.; Zhuang, L.; Fisher, T. E.; Embrey, M.; Guare, J. P., Jr.; Egbertson, M. S.; Vacca, J. P.; Huff, J. R.; Felock, P. J.; Witmer, M. V.; Stillmock, K. A.; Danovich, R.; Grobler, J.; Miller, M. D.; Espeseth, A. S.; Jin, L.; Chen, I. W.; Lin, J. H.; Kassahun, K.; Ellis, J. D.; Wong, B. K.; Xu, W.; Pearson, P. G.; Schleif, W. A.; Cortese, R.; Emini, E.; Summa, V.; Holloway, M. K.; Young, S. D. A naphthyridine carboxamide provides evidence for discordant resistance between mechanistically identical inhibitors of HIV-1 integrase. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11233−11238.
(54) Simmonds, P.; Holmes, E. C.; Cha, T. A.; Chan, S. W.; McOmish, F.; Irvine, B.; Beall, E.; Yap, P. L.; Kolberg, J.; Urdea, M. S. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J. Gen. Virol.* **1993**, *74 (Pt 11)*, 2391−2399.
(55) Ludmerer, S. W.; Graham, D. J.; Boots, E.; Murray, E. M.; Simcoe, A.; Markel, E. J.; Grobler, J. A.; Flores, O. A.; Olsen, D. B.; Hazuda, D. J.; LaFemina, R. L. Replication fitness and NS5B drug sensitivity of diverse hepatitis C virus isolates characterized by using a transient replication assay. *Antimicrob. Agents Chemother.* **2005**, *49*, 2059−2069.
(56) Tomei, L.; Vitale, R. L.; Incitti, I.; Serafini, S.; Altamura, S.; Vitelli, A.; De Francesco, R. Biochemical characterization of a hepatitis C virus RNA-dependent RNA polymerase mutant lacking the C-terminal hydrophobic sequence. *J. Gen. Virol.* **2000**, *81*, 759−767.
(57) Biroccio, A.; Hamm, J.; Incitti, I.; De Francesco, R.; Tomei, L. Selection of RNA aptamers that are specific and high-affinity ligands of the hepatitis C virus RNA-dependent RNA polymerase. *J. Virol.* **2002**, *76*, 3688−3696.
(58) Kubinyi, H. *QSAR. Hansch Analysis and Related Approaches (Methods and Principles in Medicinal Chemstry*; VCH: Weinheim, 1993.
(59) Grobler, J. A.; Stillmock, K.; Hu, B.; Witmer, M.; Felock, P.; Espeseth, A. S.; Wolfe, A.; Egbertson, M.; Bourgeois, M.; Melamed, J.; Wai, J. S.; Young, S.; Vacca, J.; Hazuda, D. J. Diketo acid inhibitor mechanism and HIV-1 integrase: implications for metal binding in the active site of phosphotransferase enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 6661−6666.
(60) Doublie, S.; Ellenberger, T. The mechanism of action of T7 DNA polymerase. *Curr. Opin. Struct. Biol.* **1998**, *8*, 704−712.
(61) Jeruzalmi, D.; Steitz, T. A. Structure of T7 RNA polymerase complexed to the transcriptional inhibitor T7 lysozyme. *EMBO J.* **1998**, *17*, 4101−4113.
(62) Huang, H.; Chopra, R.; Verdine, G. L.; Harrison, S. C. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **1998**, *282*, 1669−1675.
(63) Culbertson, T. P. Synthesis of methyl 5,6-dihydroxy-2-phenyl-4-pyrimidinecarboxylate: a corrected structure. *J. Heterocycl. Chem.* **1979**, *16*, 1423−1424.
(64) Thavonekham, B. A practical synthesis of ureas from phenyl carbamates. *Synthesis* **1997**, 1189−1194.
(65) Cabre, J.; Palomo, A. L. New experimental strategies in amide synthesis using N,N-bis[2-oxo-3-oxazolidinyl]phosphorodiamidic chloride. *Synthesis* **1984**, 413−417.
(66) Diago-Meseguer, J.; Palomo-Coll, A. L.; Fernandez-Lizarbe, J. R.; Zugaza-Bilbao, A. A new reagent for activating carboxyl groups: preparation and reactions of N,N-bis[2-oxo-3-oxazolidinyl]phosphorodiamidic chloride. *Synthesis* **1980**, 547−551.
(67) Ghosh, A. K.; Duong, T. T.; McKee, S. P.; Thompson, W. J. N,N′-Disuccinimidyl carbonate: a useful reagent for alkoxycarbonylation of amines. *Tetrahedron Lett.* **1992**, *33*, 2781−2784.
(68) Henry, D. W. Chemotherapeutic nitroheterocycles. Derivatives of 5-nitrothiazole-2-carboxaldehyde and 5-nitrothiazole-2-carboxylic acid. *J. Med. Chem.* **1969**, *12*, 303−306.
(69) Whitten, J. P.; McCarthy, J. R.; Matthews, D. P. Cyanogen bromide-dimethylaminopyridine (CAP). A convenient source of positive cyanide for the synthesis of 2-cyanoimidazoles. *Synthesis* **1988**, 470−472.
(70) Ivin, B. A.; Rutkovskii, G. V.; Rusavskaya, T. N.; Smorygo, N. A.; Sochilin, E. G. Synthesis and rearrangement of some ethyl esters of alpha-substituted hydantoin- Δ5, alpha-acetic acids. *Chem. Heterocycl. Compd.* **1979**, *15*, 336−341; (translation from *Khimiya Geterotsiklicheskikh Soedinenii* **1974**, *11*, 1527−1535).
(71) *MacroModel Version 7.0*: Schroedinger Inc., Portland OR 97201. (http://www.schrodinger.com/Products/macromodel.html.
(72) Halgren, T. A. Potential energy functions. *Curr. Opin. Struct. Biol.* **1995**, *5*, 205−210.
(73) Halgren, T. A. *J. Comput. Chem.* **1996**, *17*, 490−586.
(74) *ConQuest 1.6 User Guide, Cambridge Crsytallographic Data Centre*: Cambridge, CB2 1EZ, UK.
(75) Bressanelli, S.; Tomei, L.; Roussel, A.; Incitti, I.; Vitale, R. L.; Mathieu, M.; De Francesco, R.; Rey, F. A. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 13034−13039.

JM051064T