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2-(3-Thienyl)-5,6-dihydroxypyrimidine-4-carboxylic acids as inhibitors of HCV NS5B RdRp

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ABSTRACT

A series of 2-(3-thienyl)-5,6-dihydroxypyrimidine-4-carboxylic acid inhibitors of the hepatitis C virus (HCV) NS5B polymerase enzyme are reported. Sulfonyl urea substituted analogs in this series proved to be the most potent active site non-nucleoside inhibitors of NS5B reported to date. These compounds had low nanomolar enzyme inhibition across HCV genotypes 1–3 and showed single digit micromolar inhibition in the HCV replicon assay. This improved cell-based activity allowed the binding mode of these compounds to be probed by selection of resistant mutations against compound 21. The results generated are in broad agreement with the previously proposed binding model for this compound class.

Around 2% of the world's population is infected with hepatitis C virus (HCV) and at risk of developing serious liver diseases such as cirrhosis and hepatocellular carcinoma.[1] HCV is a leading indication for liver transplantation and is thought to lead to around 10,000 deaths annually in the United States alone.[2] The current standard of care for HCV, which is based on a combination of interferon-α and ribavarin, can cure HCV infections, but is often inadequate. In addition to serious side effects such as fatigue, flu-like symptoms, and hemolytic anemia[3] interferon-based therapy also has a low success rate (<50%) in the main viral genotype.[4] The need for improved therapies is pressing because although the incidence of new HCV infections is declining, mortality is expected to increase into the middle the next decade.[5]

The HCV genome is highly variable in sequence with six main genotypes that are encoded by (+)-stranded RNA's containing around 9.6 kilobases.[6] Translation of the viral genome in hepatocytes and post-translational processing events liberate six non-structural (NS) proteins that comprise the viral replication machinery. The NS5B enzyme is one of these proteins, and has been identified as an RNA dependent RNA polymerase. NS5B is essential for replication since it both effects the synthesis of a (−)-stranded HCV RNA template and regenerates the (+)-stranded genomic RNA.[7] The NS5B active site is located in the palm domain of the enzyme where two conserved aspartic acid residues chelate a pair of Mg^{2+} ions that catalyze the nucleotidyl transfer reaction. The Mg^{2+} ions have the dual role of activating the 3′-OH of the elongating RNA chain for nucleophilic attack, and stabilizing the pyrophosphate leaving group on the incoming nucleoside triphosphate.

The HCV polymerase enzyme is seen as an attractive target for drug intervention because of its fundamental role in viral replication. Clinical proof-of-concept for antivirals operating through an allosteric mechanism of action has now been demonstrated for several classes of NS5B inhibitors.[8] However, allosteric compounds typically have genotype sensitivities that can result in drastically reduced effectiveness against HCV from genotypes 2 or 3.[9] To date, clinical reductions in HCV viral load from allosteric compounds has been limited to patients infected with genotype 1 HCV.[10] In contrast, the highly conserved active site of NS5B favors the development of inhibitors that are active across genotypes. Active site nucleoside inhibitors such as R7128 have shown comparably strong clinical effects in patients infected with all three of the main HCV genotypes (genotypes 1–3).[11] Despite the attraction of the active site with a view to identifying pan-genotype inhibitors, very few series of non-nucleoside inhibitors have been reported. 5,6-Dihydroxypyrimidine-4-carboxylic acids (e.g., **1**) that evolved from a diketoacid lead[12] are the most promising active site non-nucleosides reported to date. Our earlier work in this area (Fig. 1) demonstrated that replacement of the C2 phenyl group in **1** with five-membered ring heterocycles (such as 2-thiophene, **2**) improved biochemical potency by an order of magnitude.[13] Optimization of **2** led to the 3′-substituted thiophene **3** that shows good NS5B enzyme inhibition (IC_{50} 158 nM) but has poor activity in the cell-based HCV replicon assay (EC_{50} 10.9 μM).[14] In a continuation of our pursuit for active-site inhibitors with improved potency we describe here our work in a related series of 3-thiophene substituted 5,6-dihydroxypyrimidine-4-carboxylic acids. In addi-

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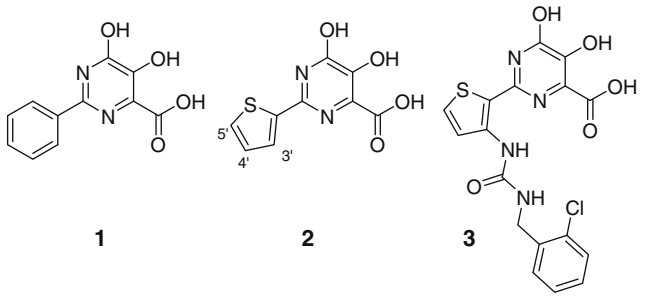


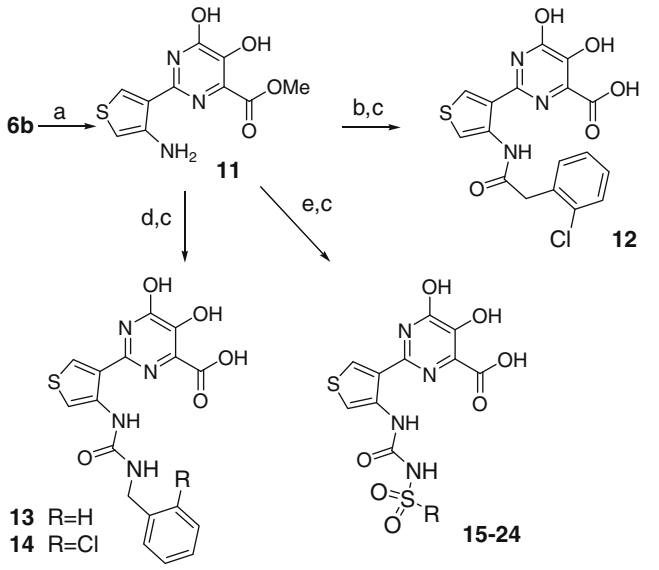
Figure 1. Previously reported 2-aryl-5,6-dihydroxypyrimidine-4-carboxylic acid inhibitors of the HCV NS5B polymerase.

tion to more than 10-fold improved biochemical potency, this series provided for the first time active-site non-nucleoside inhibitors that are sufficiently active in the replicon system to allow the study of resistance to this compound class.

2-(3-Thienyl)-5,6-dihydroxypyrimidine-4-carboxylic acids were prepared following literature precedent as outlined in Scheme 1.[14] Thus amidoximes **5a–d** (available from the corresponding carbonitriles **4a–d**) were condensed with dimethyl acetylene dicarboxylate and the resulting adducts were cyclized under thermal conditions. Routine hydrolysis of the pyrimidine methyl esters **6a–d** furnished the target inhibitors **7–10**.

Based on previous work[14] structure–activity investigations at the 2′- and 4′-position of 3-thiophene **7** were of interest. However, the free amine from *N*-Cbz deprotection of **6d** proved to be unstable, and compound **10** was the only 2′-substituted 3-thiophene evaluated. In contrast, removal of the *N*-Boc protecting group from **6b** (Scheme 2) afforded the free 4′-aminothiophene **11** that reacted smoothly with electrophiles. Thus treatment of **11** with 2-chlorobenzoic acid and *N*,*N*′-carbonyldiimidazole followed by saponification gave the amide **12**. Ureas **13–14** were prepared in the same fashion through reaction of **11** with the appropriate benzyl isocyanates. Sulfonyl ureas **15–24** were obtained from commercial sulfonyl isocyanates or with ethyl sulfonyl carbamates (that were prepared by treatment of the corresponding aryl sulfonamides with ethyl chloroformate).

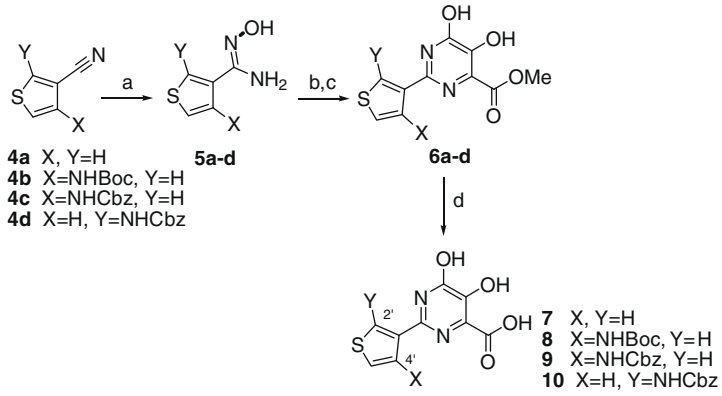
Table 1 summarises our initial exploration of 3-thiophene substituted 5,6-dihydroxypyrimidine-4-carboxylic acids. In line with results for the isomeric 2-thiophene analog **2**, compound **7** (IC_{50} 6.1 μM) had improved potency with respect to our original phenyl substituted lead **1** (IC_{50} 30 μM).[14] This is thought to stem from acidification of the pyrimidine 5-OH group when five-membered ring heterocycles are present at the pyrimidone C-2 position, an effect believed to favorably influence Mg^{2+} chelation in the



Scheme 2. Reagents and conditions: (a) TFA/CH_2Cl_2, 25 °C, 99%; (b) 2-Cl–C_6H_4–CH_2CO_2H, NEt_3, CDI, DMF, 25 °C; (c) LiOH·H_2O, THF/H_2O, 50 °C, 40–95%; (d) RC_6H_4-CH_2NCO, Py, 25 °C; (e) $ArSO_2NHCO_2Et$, NEt_3, toluene, reflux or $ArSO_2NCO$, Py, 25 °C.

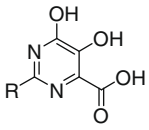
NS5B active site. The greater improvement for the 2-thiophene compound is in line with the somewhat stronger electron withdrawing effect for this isomer.[15] Structure–activity relationships at the 4′-position of the thiophene ring of **7** proved to be somewhat changed with respect to those at the 3′-position of 2-thiophene **2**. An example was the 2-chlorobenzyl amide **12** which brought only a modest improvement in potency over **7** (rather than the order of magnitude gain seen in the isomeric 2-thiophene series). The benzyl carbamoyl substituted **9**, as well as its 2′-isomer **10**, each improved potency by around 20-fold with respect to the unsubstituted analog **7**. In contrast, no gain in potency came from the simple alkyl carbamate in **8**. Benzyl ureas, which had given the most active inhibitors in the 2-thiophene series (i.e., **3**) were extensively explored in the 3-thiophene area. Although gains in potency were achieved with respect to the simple benzyl urea **13** (IC_{50} 873 nM) they were consistently modest; the 2-chlorobenzyl urea **14** again was optimal (IC_{50} 258 nM).

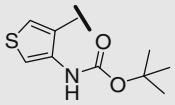
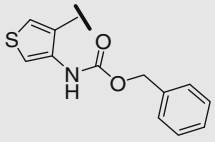
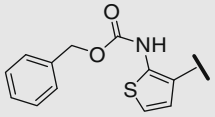
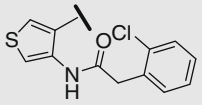
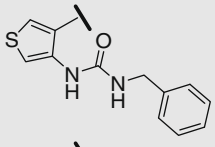
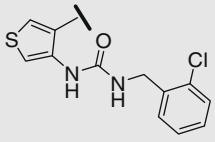
More notable improvements in potency came from introduction of a sulfonyl urea group at the 4′-position of thiophene **7** (Table 2). The phenyl sulfonyl urea **15** was around 100-fold more active than **7**, and was also the first compound in this regioisomeric thiophene series to show cell-based activity (EC_{50} 32 ± 12 μM). Efforts to



Scheme 1. Reagents and conditions: (a) NH_2OH·HCl, TEA, MeOH, 50 °C, 95%; (b) DMAD, $CHCl_3$, 75 °C, 85%; (c) *p*-xylene, 160 °C, 40%; (d) LiOH·H_2O, THF/H_2O, 50 °C, 80%.

Table 1
Enzyme inhibition for 3-thiophene substituted 5,6-dihydroxypyrimidine-4-carboxylic acid; initial SAR at the 2′- and 4′-positions

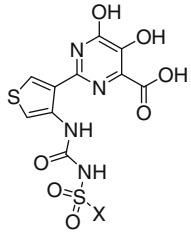


Compd	R	IC_{50}[a] (nM)
1		30,000 ± 707[b]
2		2600 ± 2163
7		6151 ± 1803
8		8280[b]
9		326 ± 5
10		343 ± 11
12		2610[b]
13		873 ± 61
14		258 ± 98

[a] IC_{50} measured against ΔC_{55} NS5B enzyme from genotype 1b HCV.
[b] $n = 1$; Unless otherwise stated results in this and subsequent tables are expressed as mean ± standard error (or half range for $n = 2$) for 2–18 independent determinations.

further improve the sulfonyl urea compounds focused on changes to the phenyl ring in the thiophene side-chain of **15**. The introduction of substituents at the *ortho*-position gave analogs **16**–**18** that, despite similar biochemical potency to **15,** had improved cell-based activities. In particular, the halogenated compounds **16** and **18** had efficacy in the replicon assay that was improved by around an order of magnitude. In contrast, compounds with a halogen substituent at the 3- or 4-positions of the phenyl ring (**19**–**20**) did not bring any improvement over **15**. 2,3-Disubstituted aromatic rings such as the 1-naphthyl derivative **21** or the 2,3-dichloro compound **23** were the most active inhibitors in this study. Both these compounds are low nanomolar inhibitors of

Table 2
Intrinsic potency and cell-based assay results for sulfonyl urea compounds **15**–**24**



Compd	X	IC_{50}[a] (nM)	EC_{50}[b] (1b, μM)	EC_{50}[c] (2a, μM)
15	Ph	62 ± 13	32 ± 12	
16	2-Cl–Ph	78 ± 24	2.6 ± 1.1	6.6[c]
17	2-CH_3–Ph	47 ± 1	16[d]	
18	2-Br–Ph	74 ± 12	3.4 ± 1.9	
19	3-Br–Ph	38 ± 9	37[d]	
20	4-Cl–Ph	85 ± 2	26[d]	
21	1-Naphthyl	36 ± 5	3.6 ± 0.7[e]	3.7[c]
22	2-Naphthyl	48 ± 2	32[d]	
23	2,3-Cl–Ph	12 ± 1	2.4 ± 0.3	4.9[c]
24	Me	426 ± 72	—	

[a] IC_{50} measured against ΔC_{55} NS5B enzyme from genotype 1b HCV.
[b] Replicon assay run over 96 h in HUH-7 cells harboring genotype 1b HCV.
[c] Full length genotype 2a infectivity assay.
[d] $n = 1$.
[e] $n = 18$.

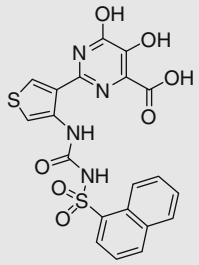
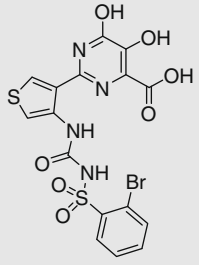
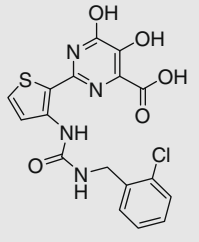
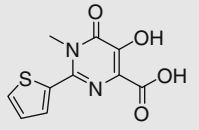
the NS5B enzyme and show low micromolar inhibition in the cell-based assay.[16] The importance of the position of the substituents on the phenyl ring was again apparent from the 2-naphthyl compound **22** that had much weaker cell based activity than its isomer **21**.[17] The improvement of inhibition observed for compounds with a sulfonyl urea side chain was thought likely to be related to the acidity of this group. This was confirmed by preparation of the methyl sulfonyl urea **24** that was 14-fold more active than the unsubstituted thiophene **7**. Potency gains of this magnitude are not achieved when alkyl groups are attached to a thiophene through non-acidic linkers; the *tert*-butylcarbamate **8** is one such example.

In line with our expectation that inhibitors targeting the highly conserved active site should block HCV replication for all viral genotypes, compound **21** displayed potent inhibition against NS5B isolates from all three of the major HCV genotypes. Thus **21** showed potent activity against the ΔC_{21} NS5B enzymes from genotype 1b (IC_{50} 5.6 nM), 2a (IC_{50} 2.1 nM), 2b (IC_{50} 5.8 nM) and 3a (IC_{50} 3.9 nM). Blockade of HCV in HUH-7 cells stably transfected with HCV RNA from the 2b genotype was also observed (EC_{50} 9.6 μM) and cell-based activity was confirmed (for **21**, **16** and **23**) in a full-length genotype 2a infectivity assay (Table 2). Following intravenous administration of compound **21** (3 mg/kg) to rat, an excellent plasma half life ($t_{1/2}$ = 15 h) was measured; this reflects the expected low volume of distribution (0.1 L/kg) and turnover (0.2 mL/min/kg) for this acidic compound that is highly bound to rat serum proteins (>99%). Oral exposure (3 mg/kg) was 3.3 μM h and though oral bioavailability was low (%$F_{p.o.}$ = 2), high levels of systemic exposure could be achieved through intramuscular administration of the compound (%$F_{i.m.}$ = 89).

Under a modification to the replicon assay protocol, cell survival in response to antibiotic neomycin sulfate can be tied to HCV replication by incorporation of a neomycin phosphotransferase gene into the HCV RNA.[18] Efficient suppression of HCV replication, as expected by inhibition of NS5B, sensitizes cells to antibiotic and links cell survival to the development of escape mutations that restore replication competency. These characteristics have been exploited for the selection of cell colonies resistant to compound **21** from the current series. Sequencing of the HCV genomes in the surviving

colonies revealed two specific mutations in the NS5B region, P156S and G152E, each of which was demonstrated by reverse genetics sufficient to confer resistance to **21**. Thus when compound **21** was tested in HUH-7 cells transfected with sub-genomic HCV RNA harboring either mutation G152E or P156S, it showed at least a six-fold loss in activity. Compound **21**, as well as the related sulfonyl urea **18,** the urea **3** and the simple pyrimidone **25** were also tested against an isolated HCV NS5B enzyme containing either the P156S or G152E mutations. The results in Table 3 illustrate that sulfonyl ureas **21** and **18** as well as **3** all showed significant impairment of activity against the G152E mutant with IC_{50} values between five and eightfold reduced with respect to wild-type. In contrast, compound **25** (which has an unsubstituted thiophene ring) showed no decrease in response suggesting a role for the thiophene side-chain in conferring resistance. The loss of cell-based activity against P156S that had been seen in a cell-based setting was not recapitulated when this mutation was incorporated into a genotype 1b ΔC_{55} NS5B enzyme. Apparently the interaction between **21** and NS5B in the context of a cell-based replication complex is not fully recaptured in the in-vitro assay.[19]

Table 3
IC_{50} values for compounds **21**, **18**, **3** and **25** in ΔC_{55}-con1 enzyme either wild type or single point NS5B mutated

Compd	Structure	Wild-type IC_{50} (μM)	G152E IC_{50} (μM)	P156S IC_{50} (μM)
21		0.04	0.3	0.04
18		0.06	0.4	0.07
3		0.18	0.8	0.17
25[a]		4.6	3.2	5.6

[a] Assay run in the presence of $MnCl_2$.

In large part the resistance data observed are consistent with the binding model that we previously proposed for these active site inhibitors. In the C6/N1 amide tautomer (that has been found to have low ground state energy, and which preliminary SAR suggested may be the active species) the conformation of the sulfonylurea side chain would be dictated by an intramolecular hydrogen bond between the 3-NH group on the thiophene ring and the N3 atom of the pyrimidone. Modeling of **18** into the pyrophosphate binding region of the NS5B active site in this planar orientation (Fig. 2) highlights contacts between the sulfonylurea side chain and both the P156 and particularly the G152 residues.

In light of the above considerations it became clear that compound **26** (Fig. 3) was an attractive target since it constrains both the side-chain orientation and pyrimidone tautomerism in our proposed binding model. Unlike the *N*-methylpyrimidone **27,** the ring constrained analog **26** ought to have a conformation in which the thiophene and pyrimidone rings are approximately coplanar. The change of side chain orientation for *N*-methyl compounds such as **27** is thought to explain their reduced inhibition; compound **27** (IC_{50} 600 nM) is an 8-fold weaker inhibitor than **16**. Compound **27** was prepared as outlined in Scheme 3. Regioselective deprotonation of the thiophene **4b** (LDA, −78 °C) followed by alkylation with TBS protected 1,2-iodoethanol furnished **29**, albeit in modest yield. This compound was elaborated as previously described to the pyrimidine **31**. Protection of the pyrimidine 5-OH as a pivaloate ester followed by TBS deprotection of the primary alcohol set up the cyclization step that was smoothly effected (50% yield) under Mitsunobu-conditions. Compound **33** was elaborated to the target

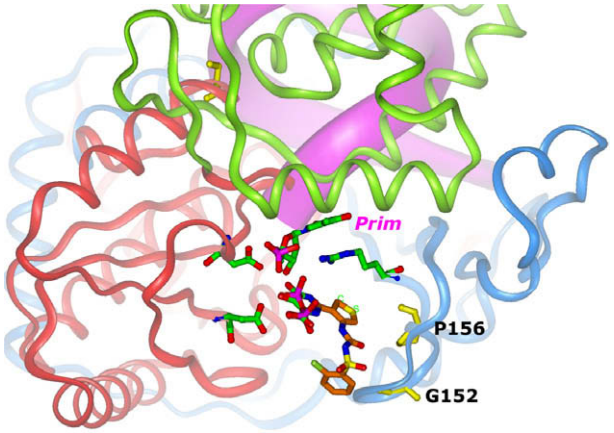


Figure 2. Binding model of **18** superimposed to the pyrophosphate in the active site together with a primer. The thumb, palm and fingers domains are colored in green, red and blue, respectively. Carbon atoms are shown in green (for **18** carbon atoms are shown in green and orange), oxygen in red, nitrogen in blue, sulfur in yellow, phosphorous in violet and bromine in pale green. The two catalytic aspartates and Arg 158 are shown. P156 and G152 are located in the Λ2 fingertips loop, in close contact with the specificity portion of **18** but not with its metal chelating portion.

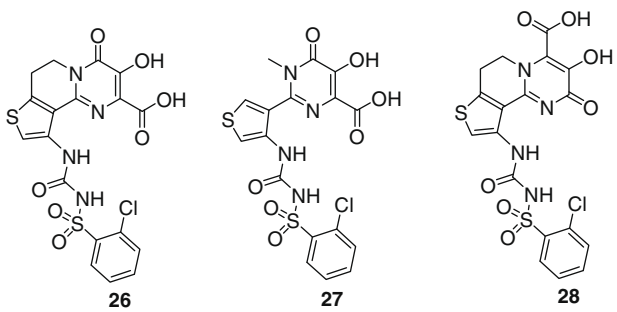
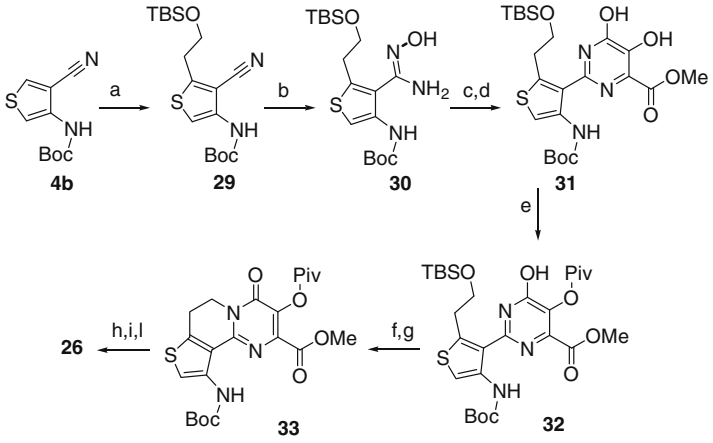


Figure 3.



Scheme 3. Reagents and conditions: (a) LDA, ICH_2CH_2OTBS, −78 °C, 24%; (b) NH_2OH, TEA, EtOH, 0.3 M, 80 °C, 30%; (c) DMAD, $CHCl_3$, 90 °C; (d) *p*-xylene, reflux; (e) Piv-Cl, Py, 25 °C, 32%; (f) THF:Py:HF.Py, −78 °C; (g) DEAD, PPh_3, 25 °C, 50%; (h) CH_2Cl_2:TFA; (i) 2-Cl–C_6H_4–SO_2NCO, Py, 25 °C; (l) LiOH·H_2O, THF/H_2O, 50 °C, 16%.

inhibitor by removal of the pivaloate and *N*-Boc protecting groups followed by installation of the side chain using 2-chlorophenylsulfonyl isocyanate; the synthesis was completed by methyl ester hydrolysis to give **26**.

Compound **26** was tested against the genotype 1b NS5B enzyme which it proved to have extremely weak potency (IC_{50} 10 μM). This result was somewhat contrary to our expectation based on the structure activity relationships and modeling hypotheses generated across several series of inhibitor. A simple explanation may be that there is insufficient space to accommodate the newly formed six-membered ring that has been installed into the inhibitor in **26**. However, this result may alternatively point to the importance of an alternative binding orientation, in which a 180° rotation occurs about the thiophene–pyrimidine bond (such as is constrained in compound **28**).

In summary, we have described a series of active site inhibitors of NS5B that achieved around a 15-fold improvement in biochemical potency over our previous work. Strong enzyme inhibition (*c.* 5 nM) was achieved against ΔC_{21} NS5B from HCV genotypes 1–3 and cell-based efficacy was improved to a level that for the first time allowed resistance to this compound class to be assessed. Mutation at either of residues P156 and G152 in NS5B was found sufficient to confer resistance to sulfonylurea **21**, and compounds **3** and **18** showed cross-resistance. The binding model previously proposed for 5,6-dihydroxypyrimidine-4-carboxylic acids was supported by resistance data, but the weak inhibitor **26** (which was designed based upon this model) highlights that further studies are required to fully understand the interaction of this compound class at the active site.

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