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Inhibitors of HCV NS5B polymerase. Part 1: Evaluation of the southern region of (2Z)-2-(benzoylamino)-3-(5-phenyl-2-furyl)acrylic acid

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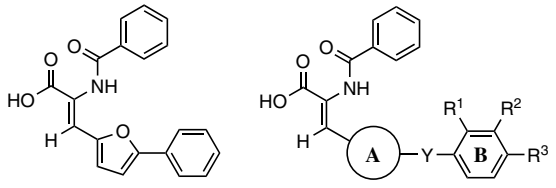
Abstract—A novel series of nonnucleoside HCV NS5B polymerase inhibitors were prepared from (2*Z*)-2-(benzoylamino)-3-(5-phenyl-2-furyl)acrylic acid, a high throughput screening lead. SAR studies combined with structure based drug design focusing on the southern heterobiaryl region of the template led to the synthesis of several potent and orally bioavailable lead compounds. X-ray crystallography studies were also performed to understand the interaction of these inhibitors with HCV NS5B polymerase.

1. Introduction

Hepatitis C virus (HCV) is the major causative agent of blood-borne non-A, non-B hepatitis, and it is estimated to infect over 170 million people worldwide. Chronic HCV infections have been associated with liver fibrosis, liver cirrhosis, and heptacellular carcinoma.[1,2] Current HCV therapy consists of injectable α-interferon used alone or in combination with ribavirin.[2] Although effective in certain patient populations, interferon therapy has several limitations including high cost and significant side effects that often require dose adjustment or discontinuation of therapy. Moreover, while not well understood, interferon therapy has been shown to be most effective against genotype 2 and 3 HCV infections and least effective against genotype 1 infections. Unfortunately, since 60% of patients in North America are infected with the genotype 1 strains, a significant portion of HCV positive patients will not benefit from interferon therapy.[1] As a consequence of these limitations, a medical need exists for new, orally administered, anti-HCV agents with good efficacy, spectrum, and tolerability.

From a therapeutic perspective, hepatitis C virus offers a number of potential targets for small molecule intervention.[3,4] The viral genome encodes for several nonstructural (NS) proteins including a protease (NS3/NS4a), a helicase (NS3) and an RNA-dependent polymerase (NS5B).[4] In this report, we describe our efforts to develop nonnucleoside inhibitors of the NS5B RNA-dependent RNA polymerase.[5] Given the essential role of this enzyme in viral replication, it is anticipated that agents capable of disrupting its function will prove efficacious in the treatment of HCV infections.



PNU-248809 (**1**) (**2**)

Figure 1. Lead compound (**1**) and SAR strategy (**2**).

Keywords: HCV Polymerase; Inhibitors.
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High throughput screening of our internal compound collection against HCV polymerase led to the identification of PNU-248809 (**1**, Fig. 1) as a modest inhibitor of CΔ21 NS5B polymerase[6] (IC_{50} = 6.7 μM). Herein we describe our SAR strategy (**2**, Fig. 1) to investigate the structural requirements of the A- and B-ring regions of this template. In the following report, we describe modifications to the northern region of the template.

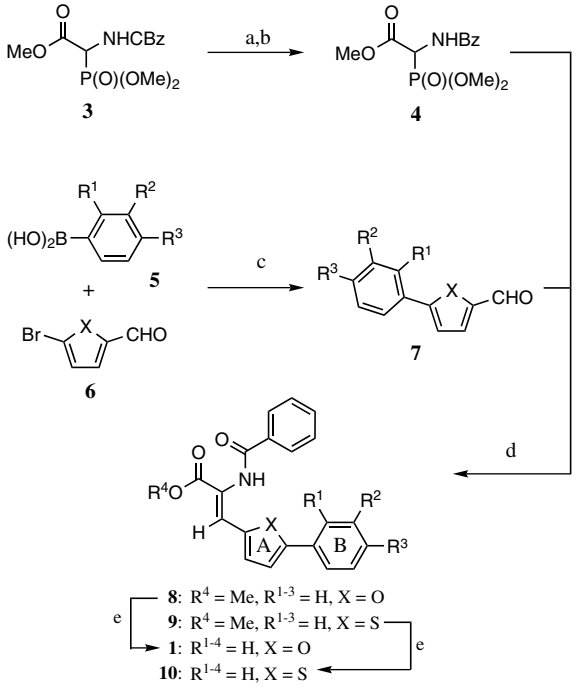
2. Chemical synthesis

Initial synthesis of PNU-248809 (**1**) established a convenient route for the parallel synthesis of analogs as outlined in Scheme 1. Commercially available *N*-(Cbz)-α-phosphonoglycine trimethyl ester (**3**, Scheme 1) was first deprotected by hydrogenolysis[7] to afford an amine that was acylated with benzoyl chloride in the presence of triethylamine to provide, after recrystallization, *N*-(Bz)-α-phosphonoglycine trimethyl ester **4** in 50% yield over two steps. Intermediate **4** was then condensed with various substituted 5-phenyl-2-furaldehydes (**7**, X = O) or 5-phenyl-2-thiophenecarboxaldehydes (**7**, X = S) to afford an enamide product [**8** (X = O) or **9** (X = S)] as a single *Z*-stereoisomer.[8] The aldehydes (i.e., **7**) required for this condensation were either purchased commercially or prepared via palladium-mediated coupling of a substituted phenyl boronic acids **5** with 5-bromo-2-furanaldehyde (**6**, X = O) or 5-bromo-2-thiophenecarboxaldehyde (**6**, X = S).[9] The synthesis was completed by saponification of the methyl ester of intermediate [**8** (X = O) or **9** (X = S)] with aqueous LiOH in dioxane to afford the final products. This sequence was used to prepare a series of substituted B-ring analogs.

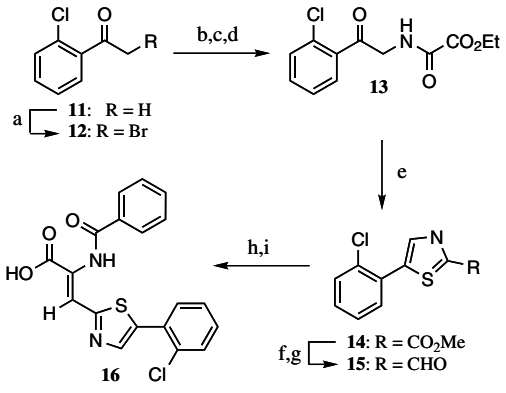
In addition to the furan and thiophene A-rings described in Scheme 1, several other heterocyclic and aryl A-rings were also prepared. Since preliminary SAR data (vide infra) suggested that a 2-chloro substituent on the B-ring of the template afforded optimal inhibitory activity against HCV NS5B polymerase, all subsequent A-ring analogs were synthesized with this optimal substitution pattern. As illustrated in Scheme 2, a thiazole A-ring analog was synthesized via initial α-bromination of 2-chloroacetophenone **11** to provide bromide **12**. Bromoketone **12** was then reacted with the modified Gabriel reagent $NaN(CHO)_2$[10] to afford, after acid catalyzed deformylation, an amine intermediate that was acylated with ethyl chlorooxacetate to provide amide **13**. Treatment of intermediate **13** with P_2S_5 at elevated temperature provided thiazole **14** in modest yield.[11] The ester of thiazole **14** was then converted to the corresponding aldehyde **15** using a two-step reduction–oxidation sequence. The resulting aldehyde **15** was condensed with *N*-(Bz)-α-phosphonoglycine trimethyl ester **4** in the presence of DBU to give, after hydrolysis of the intermediate methyl ester, thiazole **16**.

As illustrated in Scheme 3, several analogs containing six-membered aryl and heteroaryl A-rings were also prepared. In this general sequence, various aryl bromides (**17**–**19**) were coupled with 2-chlorophenyl boronic acid in the presence of a $Pd(PPh_3)_4$ to afford biaryls (**20**, **21**, **23**). Subsequently, the esters of heterobiaryls (**21** and **23**) were converted to the corresponding aldehydes (**22** and **24**) via DIBAL reduction. Finally aldehydes (**20**, **22**, **24**) were subjected to condensation with *N*-(Bz)-α-phosphonoglycine trimethyl ester (**4**) and hydrolysis generating compounds **25**–**27**.

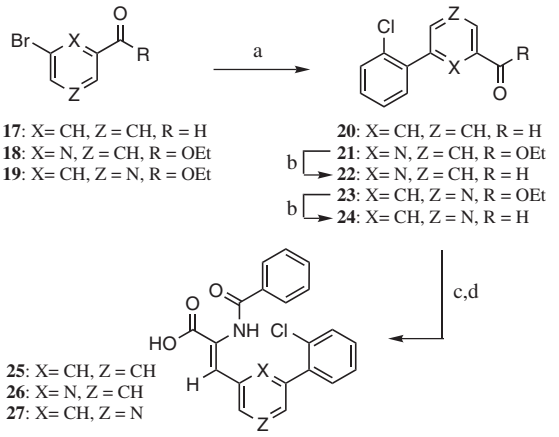
Finally an analog possessing a phenyl ether A-ring was prepared as outlined in Scheme 4. Initial aromatic nucleo-



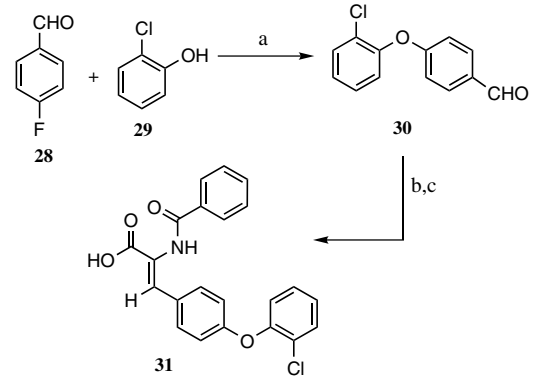
Scheme 1. Reagents and conditions: (a) 10% Pd–C, H_2, MeOH, 25 °C, 8 h; (b) Bz–Cl, Et_3N, CH_2Cl_2, 0 → 25 °C, 50% over two steps; (c) TBAB, K_2CO_3, $Pd(OAc)_2$, H_2O, 25 °C, 16 h; (d) DBU, CH_2Cl_2, 25 °C, 2 h; (e) LiOH (aq), 1,4-dioxane, 25 °C, 2 h.



Scheme 2. Reagents and conditions: (a) Br_2, $AlCl_3$, Et_2O, 0 °C, 8 h, 40%; (b) $NaN(CHO)_2$, MeCN, 25 → 70 °C, 2 h, 65%; (c) 5% HCl/EtOH, 25 °C, 24 h, 85%; (d) ethyl chlorooxacetate, Et_3N, CH_2Cl_2, 25 °C, 2 h, 34%; (e) P_2S_5, $CHCl_3$, 60 °C, 16 h, 95%; (f) DIBAL, CH_2Cl_2, −78 °C, 3 h, 79%; (g) Dess Martin reagent, $NaHCO_3$, CH_2Cl_2, 0 → 25 °C, 4 h, 82%; (h) *N*-(Bz)-α-phosphonoglycine trimethyl ester (**4**), DBU, CH_2Cl_2, 2 h, 65%; (i) LiOH (aq), 1,4-dioxane, 25 °C, 2 h, 91%.



Scheme 3. Reagents and conditions: (a) 2-$ClC_6H_4B(OH)_2$, $Pd(Ph_3P)_4$, Na_2CO_3, 80 °C, 12 h; (b) DIBAL, CH_2Cl_2, −78 °C, 3 h; (c) *N*-(Bz)-α-phosphonoglycine trimethyl ester (**4**), DBU, CH_2Cl_2, 25 °C, 2 h; (d) LiOH (aq), 1,4-dioxane, 25 °C, 2 h.



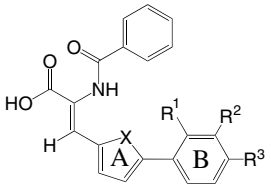
Scheme 4. Reagents and conditions: (a) K_2CO_3, DMA, 150 °C, 12 h, 79%; (b) *N*-(Bz)-α-phosphonoglycine trimethyl ester (**4**), DBU, CH_2Cl_2, 25 °C, 2 h, 71%; (c) LiOH (aq), 1,4-dioxane, 25 °C, 2 h, 89%.

philic displacement reaction between 4-fluorobenzaldehyde (**28**) and 2-chlorophenol (**29**) in the presence of K_2CO_3 at 150 °C to afforded 4-(2-chlorophenoxy)benzaldehyde (**30**). This aldehyde was condensed with *N*-(Bz)-α-phosphonoglycine trimethyl ester (**4**) in the presence of DBU and the resulting intermediate saponified to provide compound **31**.

3. Results and discussion

Our SAR strategy provided for initial screening of new analogs against HCV polymerase in a continuous read Picogreen® assay using a genotype 1b CΔ21 NS5B polymerase enzyme construct.[12] Evaluation of the compounds from Table 1 (B-ring substitution with A-rings selected from either furan or thiophene) revealed several interesting trends. First, A-ring thiophenes had preferred inhibitory activity compared with A-ring furans as illustrated by several compound pairs (**10** > **1**), (**32** > **33**), and (**34** > **35**). Second, small hydrophobic substituents were preferred at the R^1-position of the B-ring as highlighted by compound **32** (IC_{50} = 0.66 μM). In general, increasing size and/or polarity of a substituent at the R^1 position beyond Cl or Me led to decreased activity as did substitution at R^2 and R^3. These observations suggested that the B-ring binding pocket was shallow and hydrophobic.

Table 1. Activity of compounds **1**, **10**, and **32**–**57** against HCV NS5B polymerase



Compound	X	R^1	R^2	R^3	NS5b IC_{50} (μM)
1	O	H	H	H	6.7
10	S	H	H	H	1.5
32	S	Cl	H	H	0.66
33	O	Cl	H	H	1.3
34	S	Me	H	H	1.4
35	O	Me	H	H	3.0
36	S	F	H	H	2.6
37	O	F	H	H	3.1
38	O	H	F	H	3.9
39	O	OCF_3	H	H	4.0
40	O	H	H	Cl	4.6
41	O	CF_3	H	H	5.6
42	O	H	Cl	H	6.0
43	O	NO_2	H	H	6.9
44	O	H	CF_3	H	8.7
45	O	H	H	F	12
46	O	H	H	CF_3	13
47	O	OMe	H	H	20
48	S	CH_2OH	H	H	20
49	O	H	H	OCF_3	22
50	O	H	NO_2	H	25
51	O	H	H	SMe	27
52	O	H	OCF_3	H	66
53	O	H	H	NO_2	>100
54	O	H	CH_2OH	H	>100
55	O	H	H	CH_2OH	>100
56	O	H	SO_2NH_2	H	>100
57	O	H	H	SO_2NH_2	>100

Evaluation of several alternative A-rings (Table 2) revealed that the majority were less potent than the thiophene ring (e.g., compound **10**). Surprisingly however, phenyl ether **31** proved to be more potent (IC_{50} = 0.20 μM). As a follow-up, the 2- and 3-isomers (not shown) of compound **31** (4-isomer) were prepared.

Table 2. Activity of A-ring analogs against HCV NS5B polymerase

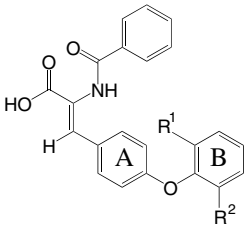
Compound	A-ring	NS5b IC_{50} (μM)
33 (Table 1)	Furan	1.3
31 (Scheme 4)	Phenyl ether	0.20
10 (Scheme 1)	Thiophene	0.66
25 (Scheme 3)	Phenyl	1.3
16 (Scheme 2)	Thiazole	5.2
26 (Scheme 3)	Pyridine	11
27 (Scheme 3)	Pyridine	>100

The 2-isomer was inactive while the 3-isomer was two-fold less potent than **31**.

In order to further improve the activity of compound **31**, additional analogs were prepared (according to the method of Scheme 4) and evaluated. Consistent with the previous results of Table 1, preliminary data in this phenyl ether series suggested that substitution at the B-ring R^1 position was necessary to achieve optimal activity while substitution at B-ring *meta*- or *para*-positions was generally deleterious to activity (data not shown). Table 3 highlights that for mono-substituted analogs (R^2 = H) halogens were preferred in the R^1 position with activity positively correlated with increasing halogen size (I > Br > Cl > F).

To better understand the interaction of these inhibitors with HCV polymerase, a representative compound, **59**, was soaked into crystalline CΔ21 NS5B enzyme and the structure was solved (Fig. 2) revealing that the inhibitor interacts at a unique binding site on the polymerase.[13–15] The site is comprised of a deep hydrophobic pocket located adjacent to the sequence motif (residues 363–369) that forms a β hairpin linking the palm domain to the thumb domain of the polymerase. In models of template-primer binding to RNA-dependent DNA polymerases, the backbone of this hairpin interacts with the phosphate backbone of the substrate primer strand, so we have denoted this binding site as the *primer grip* site.[16]

Table 3. Activity of compounds **58**–**77** against HCV NS5B polymerase



Compound	R^1	R^2	NS5b IC_{50} (μM)
58	I	H	0.04
59	Br	H	0.10
31	Cl	H	0.20
60	Me	H	0.58
61	CF_3	H	1.3
62	SMe	H	1.8
63	F	H	6.9
64	Et	H	9.4
65	OMe	H	15
66	H	H	17
67	*i*-Pr	H	28
68	Ph	H	48
69	O-*i*-Pr	H	44
70	CN	H	47
71	NO_2	H	73
72	*t*-Bu	H	>100
73	Br	F	0.03
74	Cl	F	0.07
75	Cl	Me	0.20
76	Cl	NH_2	0.31
77	Cl	NO_2	5.5

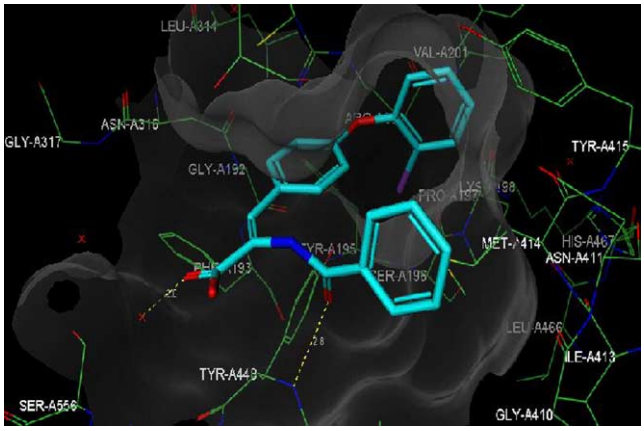


Figure 2. X-ray crystal structure (2.5 Å resolution) of compound **59** (Table 3) bound to the primer grip site of CΔ21 NS5B polymerase.

Interaction of a small molecule at this primer grip site of could be expected to inhibit with the activity of NS5B by interfering with initiation and/or elongation. Examination of the inhibition kinetics of compound **59** in fact revealed both effects. The NS5B polymerase assay used to evaluate these analogs was run under conditions for de novo initiation. The ssRNA template consisted of 32 mer heterpolymer RNA with no secondary structure and the sequence CCC at the 3′ terminus. Ribonucleoside triphosphates, rATP, rCTP, and rUTP were present at 2 μM; rGTP was present at 50 μM for de novo initiation opposite CC at the 3′ terminus.[17] Under these conditions, **59** and related analogs bound at the *primer grip* site decreased both the rate of de novo initiation and the processive polymerase reaction (elongation of nascent RNA strand).

Bresannelli et al. have modeled the de novo initiation complex from X-ray crystallography of HCV NS5B with ribonucleoside triphosphates (rNTP) and divalent cations bound to the enzyme.[18] The structural data indicates three rNTP are bound at the C (catalytic) site, P (primer) site, and I (interrogation) site of NS5B. Ser367, Arg386, and Arg394, which hydrogen bond with α and β phosphates of rNTP at the P site,[18] contribute to the formation of the *primer grip* site described in this report. Distortion of the P site by inhibitor bound at the *primer grip* site appears to decrease the rate of de novo initiation. Inhibition by compound **59** was dependent on rGTP concentration suggesting partial competition between compound **59** and rGTP for binding to the enzyme. Binding of inhibitors at the *primer grip* site could affect elongation of the nascent RNA strand by (i) lowering the affinity of the P site for primer strand or (ii) distorting the adjacent C site where incoming rNTP are added to the primer strand.

From a structure-based design perspective, the crystal structure of bound **59** revealed several key inhibitor–enzyme interactions including a water-mediated hydrogen bond between the carboxylic acid and Ser556. Additionally, the carbonyl of the benzamide functionality accepts a hydrogen bond from the backbone amide of the Tyr448 residue. Interestingly, the A- and B-rings of the

inhibitor extend into a deep, lipophilic pocket within the enzyme (formed by residues Pro197, Leu384, Met414, Tyr415, and Tyr448) affording substantial hydrophobic interactions. Moreover, there appears to be an electronically favorable interaction between the B-ring 2-Br substituent and Tyr448, which is positioned at the bottom of this pocket. Further examination of the crystal structure revealed that a second tyrosine residue (Tyr415) as well as a serine residue (Ser368) were in fact in close proximity to the B-ring opposite the 2-Br substituent. The location of these residues suggested that a suitable substituent occupying the 6-position might favorable engage one (or both) of these residues. The preferred activity of selected 2,6-disubstituted compounds (**73** and **74**, Table 3) suggests that such a binding mode might in fact be operative.

In order to further evaluate the potential antiviral efficacy of this series of NS5B polymerase inhibitors, several leading analogs were evaluated in a cellular sub-genomic replicon assay.[19] As illustrated in Table 4, analogs **31**, **58**, **59**, and **73** had superior cellular activity relative to the original screening lead (**1**), and all of the compounds proved to be nontoxic at the doses evaluated. It is notable however, that while compounds **31**, **58**, **59**, and **73** proved to be very potent against isolated NS5B polymerase, their activities in the cellular replicon system were rather modest. We have rationalized that this differential in enzyme versus cellular activity might be attributable to a number of potential factors,[20] including: (a) low membrane permeability due to the presence of a carboxylic acid; (b) high levels of serum protein binding; and/or (c) the inability of compounds bound at this site of NS5B to effectively block replication in a functional replication system. In the following manuscript, we attempt to address several of these potential issues through modification of the northern region of this template.

Finally, several of the leading analogs from this series were selected for pharamacokinetic profiling to evaluate the overall potential of this series of inhibitors to deliver an orally administered anti-HCV agent. As shown in Table 5, compounds **31** and **59** were dosed to rats (Sprague-Dawley) at 5 mpk and had oral bioavailabilities of 42% and 21%, respectively. At this dose, the compounds achieved C_{max} levels of 22 μM (**31**) and 9 μM (**59**) with half-lives under 2 h and relatively low levels of clearance.

In conclusion, we have reported the preparation of a novel series of nonnucleoside inhibitors of HCV NS5B polymerase. Structure–activity studies have afforded a 200-fold improvement in activity for selected compounds relative to the original screening lead. Moreover, crystallography studies have suggested that these compounds bind to the polymerase at a unique site, which we have denoted as the primer grip site. Leading analogs demonstrated only modest cellular activity, but exhibited reasonable PK properties. The results of additional SAR studies on this template are reported in the following article.

Table 4. Activity of selected compounds in sub-genomic relicon assay[19]

Compound	IC_{50} (μM)	ED_{50} (μM)	CC_{50} (μM)
1	6.7	>100	>100
31	0.20	60 ± 9	>100
59	0.10	28 ± 7	>100
58	0.04	29 ± 4	>100
73	0.03	50 ± 7	>100

Table 5. Pharmacokinetic properties of compounds **31** and **59**

Compound	%F	C_{max} (μM)	$t_{1/2}$ (h)	CL (L/h/kg)
31	42	22	1.9	0.31
59	21	9	1.5	0.20

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truncated version of the enzyme, denoted CΔ21, is employed in crystallography and preliminary biochemistry work. All enzyme inhibition data presented herein is from a CΔ21 genotype 1b.

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