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Inhibitors of HCV NS5B polymerase. Part 2: Evaluation of the northern region of (2*Z*)-2-benzoylamino-3-(4-phenoxy-phenyl)-acrylic acid

Jeffrey A. Pfefferkorn,[a,*] Richard Nugent,[b] Rebecca J. Gross,[c] Meredith Greene,[c] Mark A. Mitchell,[c] Matthew T. Reding,[d] Lee A. Funk,[e] Rebecca Anderson,[c] Peter A. Wells,[e] John A. Shelly,[a] Robert Anstadt,[c] Barry C. Finzel,[a] Melissa S. Harris,[a] Robert E. Kilkuskie,[b] Laurice A. Kopta[b] and Francis J. Schwende[a]

[a]*Pfizer Global Research & Development, Michigan Laboratories, 2800 Plymouth Road, Ann Arbor, MI 48105, USA*
[b]*Pfizer Global Research & Development, Research Technology Center, 620 Memorial Drive, Cambridge, MA 02139, USA*
[c]*Pfizer Global Research & Development, Michigan Laboratories, 301 Henrietta Street, Kalamazoo, MI 49007, USA*
[d]*Pfizer Global Research & Development, 800 North Lindbergh Blvd., Creve Coeur, MO 63167, USA*
[e]*Pfizer Global Research & Development, La Jolla Laboratories, 10628 Science Center Dr., San Diego, CA 92121, USA*

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Abstract—A novel series of non-nucleoside HCV NS5B polymerase inhibitors was prepared from a (2*Z*)-2-benzoylamino-3-(4-phenoxy-phenyl)-acrylic acid template. Solution and solid phase analog synthesis focused on the northern region of the template combined with structure based design led to the discovery of several potent and orally bioavailable lead compounds.

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, heptacellular carcinoma, and other forms of liver dysfunction.[1,2] Given the wide spread impact of this disease, there is a substantial medical need for new anti-HCV agents to compliment current therapies. In this and the preceding article, we report on our efforts to develop orally bioavailable, non-nucleoside inhibitors of HCV NS5B RNA-dependent RNA polymerase.[3]

In the preceding report, we described the identification of (2*Z*)-2-(benzoylamino)-3-(5-phenyl-2-furyl)acrylic acid (**1**, Fig. 1) as a novel non-nucleoside inhibitor of HCV NS5B polymerase.[3] Optimization of the southern region of this compound led to analogs **2** and **3** as potent inhibitors. However, despite the potent activity of these

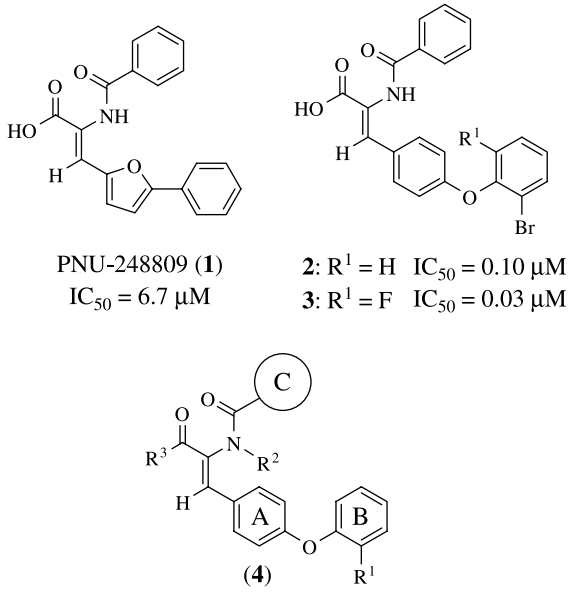

Figure 1. Previous lead compounds (**1**–**3**) and SAR strategy (**4**).

* Corresponding author. Tel.: +1 734 622 1471; fax: +1 734 622 3171; e-mail: jeffrey.a.pfefferkorn@pfizer.com

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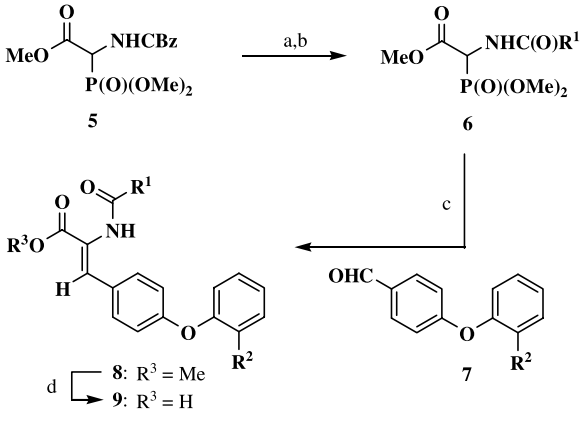
compounds against isolated NS5B polymerase, the compounds exhibited only moderate activity in a cellular subgenomic replicon assay (**2**: $ED_{50} = 28 \pm 7$ μM and **3**: $ED_{50} = 60 \pm 7$ μM).[3]

Herein, we describe the optimization of the northern region of this template including modification of the benzamide (i.e., C-ring) and carboxylic acid motifs (**4**, Fig. 1). In undertaking these studies, we hoped to improve the potency of these inhibitors against NS5B polymerase and also establish an improved correlation between enzyme and cellular replicon activity relative to that observed for previous lead compounds **2** and **3**.

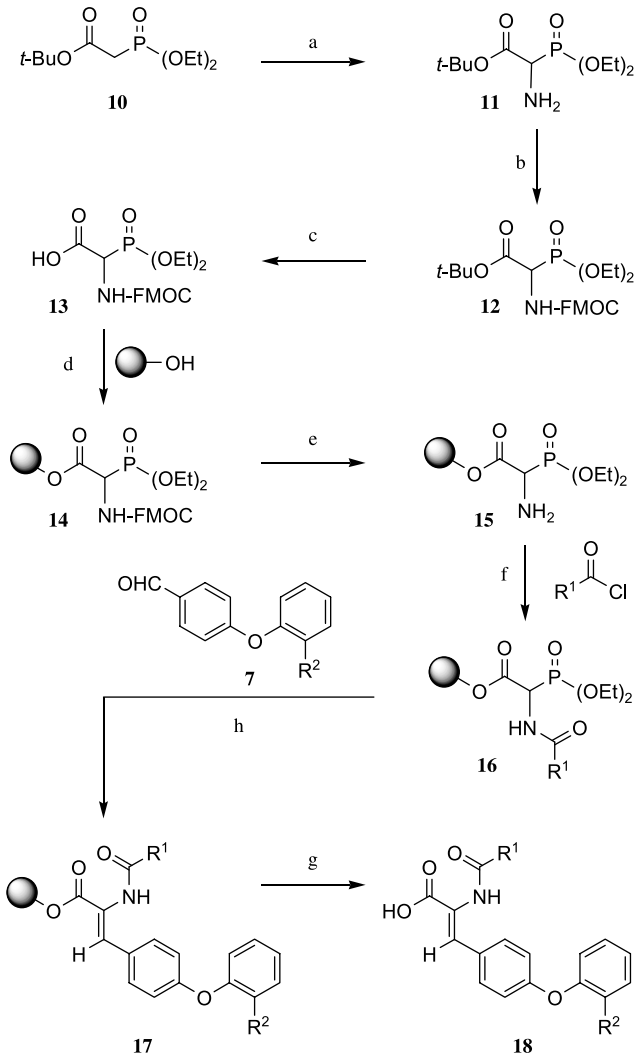
2. Chemical synthesis

Analogs for the current studies were prepared using a combination of solution and solid phase synthetic routes. Solution phase synthesis of C-ring amide analogs (**4**, Fig. 1) was accomplished as outlined in Scheme 1. As described in the preceding report, *N*-(Cbz)-α-phosphonoglycine trimethyl ester **5** was first deprotected by hydrogenolysis[4] to afford an amine that was acylated with various acid chlorides [$R^1C(O)Cl$] in the presence of triethylamine to provide, after recrystallization, compounds **6** where R_1 was aryl, heteroaryl, or aliphatic. Intermediate **6** was then condensed with optimally substituted 4-phenoxybenzaldehydes (**7**, R^2 = Br or I) to afford an enamide product **8** as a single *Z*-stereoisomer.[5] The synthesis was completed by saponification of the methyl ester **8** with aqueous LiOH in dioxane to afford the carboxylic acid **9**.

As a compliment to this solution phase method, an analogous solid phase synthesis was developed to facilitate rapid synthesis of additional analogs. Strategically, a route was envisioned that would enable the installation of multiple elements of diversity on solid support. As shown in Scheme 2, the loading precursor was prepared from *tert*-butyl *P*,*P*-dimethylphosphonoacetate **10** by treatment with *O*-(diphenyl phosphinyl)hydroxylamine[6] in the presence of NaH to affect α-amination.[7] The



Scheme 1. Reagents and conditions: (a) 10% Pd–C, H_2, MeOH, 25 °C, 8 h; (b) $R^1C(O)Cl$, Et_3N, CH_2Cl_2; (c) DBU, CH_2Cl_2, 25 °C, 2 h; (d) LiOH (aq), 1,4-dioxane, 25 °C, 2 h.

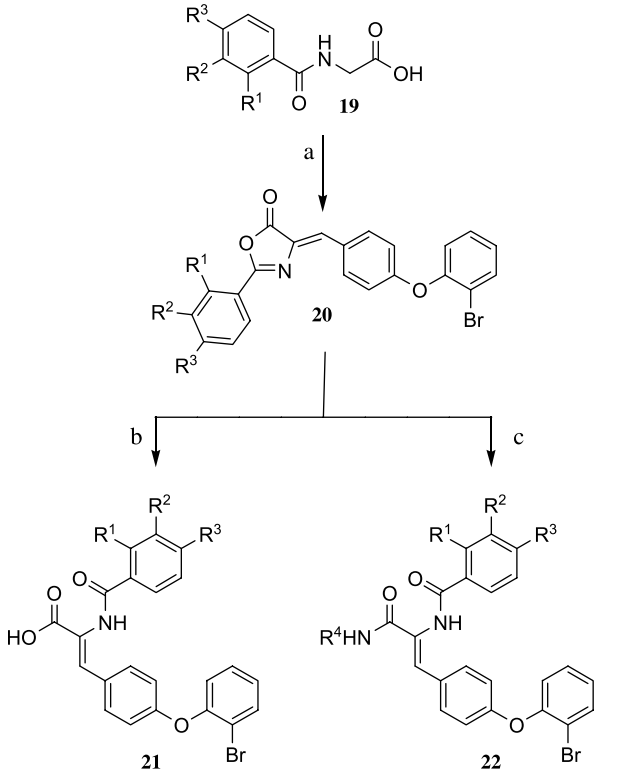


Scheme 2. Reagents and conditions: (a) NaH, $Ph_2P(O)ONH_2$,[6] THF, −78 → 25 °C, 12 h, 74%; (b) FMOC-Cl, $NaHCO_3$, dioxane:water, 25 °C, 12 h, 91%; (c) TFA, CH_2Cl_2, 25 °C, 5 h, 70%; (d) i. Wang resin (1.0 mmol/g), DIC, DMAP, CH_2Cl_2, 25 °C, 12 h (92% loading determined via FMOC analysis); ii. Ac_2O, pyridine, CH_2Cl_2, 25 °C, 0.5 h; (e) piperidine, DMF, 25 °C, 2 h; (f) $R^1C(O)Cl$, diisopropylethylamine, CH_2Cl_2, 25 °C, 12 h; (h) DBU, **7**, CH_2Cl_2, 25 °C, 12 h; (g) TFA, CH_2Cl_2, 25 °C, 0.5 h.

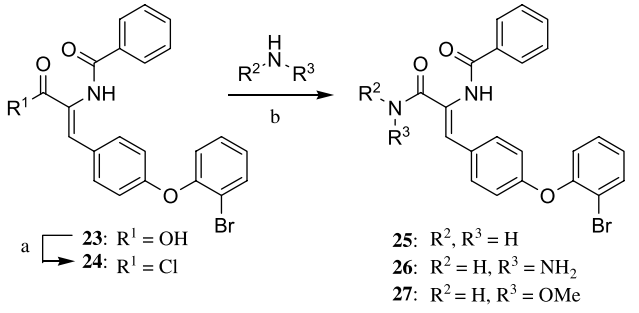
resulting amine **11** was protected as its FMOC derivative **12** and the *tert*-butyl ester hydrolyzed with TFA to provide carboxylic acid **13** suitable for loading onto solid support. In that event, a mixture of carboxylic acid **13** and Wang resin was treated with DIC and catalytic DMAP for 12 h. After the coupling reaction, any unreacted loading sites were derivatized with acetic anhydride. The FMOC protecting group was subsequently removed by treatment with piperidine. The liberated resin-bound amine **14** was derivatized with various acid chlorides [$R^1C(O)Cl$] in the presence of *N*,*N*-diisopropylethylamine to provide resin-bound amide **16**.

Intermediate **16** was then condensed with an optimally substituted 4-phenoxybenzaldehyde (**7**, R_2 = Br or I) to afford olefin **17**. Finally, the product was cleaved from solid support by treatment with TFA to afford carbox-

ylic acid **18**. After cleavage, the final compounds were purified by automated LC/MS. A third method for the solution phase preparation of (2*Z*)-2-benzoylamino-3-(4-phenoxy-phenyl)-acrylic acid analogs employed an Erlenmeyer azalactone strategy as outlined in Scheme 3.[8] Hence, a substituted hippuric acid **19** was treated with 4-(2-bromophenoxy)benzaldehyde, acetic anhydride, and sodium acetate to afford azalactone **20**.[8] In a subsequent step, azalactone **20** was hydrolyzed to carboxylic acid analogs **21** or treated with an amine (R^4NH_2) to afford amide **22**. This method was utilized to prepare selected analogs for which the requisite substituted hippuric acids were commercially available or readily prepared.



Scheme 3. Reagents and conditions: (a) 4-(2-bromophenoxy)benzaldehyde, NaOAc, Ac_2O, 60 °C, 1 h; (b) NaOH, acetone, 70 °C, 1 h; (c) R^4NH_2, EtOH, 70 °C, 2 h.



Scheme 4. Reagents and conditions: (a) $(COCl)_2$, DMF (cat), CH_2Cl_2, 45 °C, 2 h; (b) R^2R^3NH, triethylamine, CH_2Cl_2, 25 °C, 2 h.

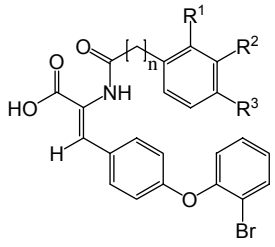
Several carboxylic acid derivatives were prepared as outlined in Scheme 4 where carboxylic acid **23** was converted to the corresponding acid chloride **24** via treatment with oxayl chloride and catalytic DMF. Acid chloride **24** was then separately treated with ammonia, hydrazine, and *O*-methylhydroxylamine to afford primary amide **25**, hydrazide **26**, and *N*-hydroxyl amide **27**, respectively.

3. Results and discussion

To assess the effects of our structural modifications, all analogs were evaluated against HCV polymerase in a continuous read picogreen® assay using a genotype 1b CΔ21 NS5B polymerase enzyme construct.[9] Additionally, compounds with IC_{50} <10 μM were also evaluated in a cellular subgenomic replicon assay for which ED_{50} and CC_{50} values are reported.[10] Our structure–activity discussion will begin by examining the effects of C-ring (**4**, Fig. 1) changes and then evaluate the effects of modifications to the carboxylate.

Analogs containing substituted aryl ring C-rings are presented in Table 1. The most potent of these analogs had a benzamide C-ring (i.e., $n = 0$) with an oxygen substituent at the R^1 position as illustrated by compounds **28** (R^1 = OEt, IC_{50} = 0.05 μM) and **29** (R^1 = OH, IC_{50} =

Table 1. Activity of substituted aryl amide analogs **28**–**45** against HCV NS5B polymerase



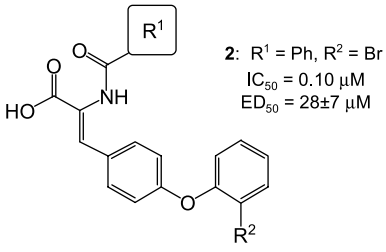
Compd	R^1	R^2	R^3	*n*	NS5B IC_{50} (μM)	ED_{50} (μM)	CC_{50} (μM)
2	H	H	H	0	0.10	28 ± 7	>100
28	OEt	H	H	0	0.05	70 ± 7	>100
29	OH	H	H	0	0.08	>100	>100
30	H	F	H	0	0.09	53 ± 11	>100
31	H	H	Cl	0	0.11	>100	>100
32	H	H	Br	0	0.12	>100	>100
33	F	H	H	0	0.18	>100	>100
34	F	H	F	0	0.23	>100	>100
35	H	F	F	0	0.23	>100	>100
36	H	H	Me	0	0.25	>100	>100
37	H	H	Br	1	0.82	>100	>100
38	H	H	Cl	1	0.94	>100	>100
39	H	H	I	1	1.00	>100	>100
40	H	H	CF_3	1	1.00	>100	18 ± 0
41	H	H	F	1	2.2	>100	>100
42	H	H	CF_3	2	0.37	>100	>100
43	H	H	Cl	2	0.50	>100	>100
44	H	H	F	2	0.60	>100	>100
45	H	H	Me	2	2.6	>100	>100

0.08 μM). The addition of small, non-polar substituents to the benzamide ring was tolerated, but did not afford increased potency relative to the unsubstituted case (i.e. **2**) as illustrated by compounds **30**–**36**. Most other mono- or poly-substituted benzamide derivatives (not shown) were less active.

Additional C-ring analogs were prepared containing an aliphatic linker [$-CH_2-$ or $-(CH_2)_2-$] between a substituted aromatic ring and the carbonyl of the amide as highlighted in Table 1. Overall, analogs of this type proved less potent than their benzamide counterparts. Examining the phenyl acetyl compounds ($n = 1$, **37**–**41**) revealed that substitution at the R^3 position with halogens provided optimal relative activity (i.e., compounds **37**–**39**); however, these compounds were approximately 10-fold less active than the simple unsubstituted benzamide (**2**). Similarly, for phenyl ethyl amide compounds ($n = 2$, **42**–**45**) substitution of the R^3 position with CF_3, Cl, and F provided optimal relative activity, but overall analogs of this type also had lower potency than benzamide **2**. Finally, as highlighted in Table 1, despite reasonable enzyme potency none of these C-ring aryl amides (i.e., **28**–**45**) demonstrated improved cellular activity relative to lead compound **2**.

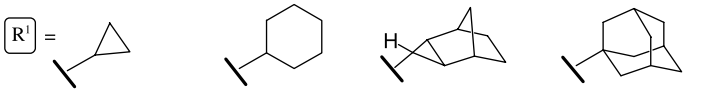
Beyond the aromatic C-ring analogs described above, various substituted alkyl and cycloalkyl derivatives were also prepared with representative examples presented in Table 2 (**46**–**58**). In the case of cycloalkyl substituents, inhibition potency increased with increasing size such that compound **46** (R^1 = cyclopropyl) was least potent and compound **49** (R^1 = adamantyl) was most potent ($IC_{50} = 0.07$ μM). Extended cycloalkyl analogs proved less potent than their directly bonded counterparts (e.g., **47** > **52** > **53**). The introduction of polar functionality including alcohols (**54**–**56**) and carboxylic acids (**57** and **58**) at the C-ring position did not afford improved activity. As was the case with the aryl amides described above, most of the alkyl and cycloalkyl amides analogs

Table 2. Activity of alkyl and cycloalkyl C-ring amide analogs **46**–**58**



2: R^1 = Ph, R^2 = Br
IC_{50} = 0.10 μM
ED_{50} = 28±7 μM

Cycloalkyl Groups[a]

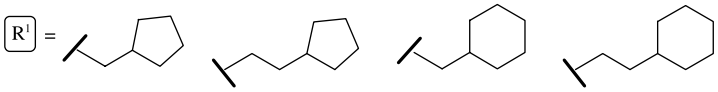


46: R^2 = I
IC_{50} = 2.8 μM
ED_{50} >100 μM

47: R^2 = Br
IC_{50} = 0.2 μM
ED_{50} >100 μM

48[b]: R^2 = Br
IC_{50} = 0.1 μM
ED_{50} >100 μM

49: R^2 = Br
IC_{50} = 0.07 μM
ED_{50} >100 μM



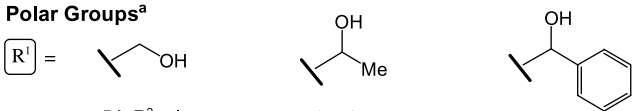
50: R^2 = Br
IC_{50} = 0.85 μM
ED_{50} >100 μM

51: R^2 = I
IC_{50} = 7.0 μM
ED_{50} = 15±4 μM

52: R^2 = Br
IC_{50} = 2.2 μM
ED_{50} >100 μM

53: R^2 = Br
IC_{50} = 13 μM
ED_{50} >100 μM

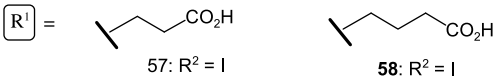
Polar Groups[a]



54: R^2 = I
IC_{50} = 2.0 μM
ED_{50} >100 μM

55[b]: R^2 = I
IC_{50} = 4.8 μM
ED_{50} >100 μM

56[b]: R^2 = I
IC_{50} = 3.1 μM
ED_{50} >100 μM

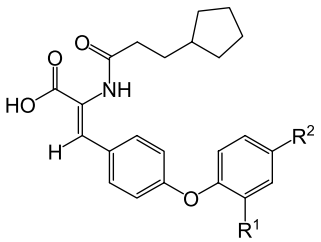


57: R^2 = I
IC_{50} = 0.79 μM
ED_{50} >100 μM

58: R^2 = I
IC_{50} = 0.83 μM
ED_{50} >100 μM

[a]All compounds demonstrated CC_{50} >100 mM; [b]Compounds were evaluated as racemic mixtures.

Table 3. Activity of selected cyclopentylethyl C-ring analogs



Compd	R^1	R^2	NS5B IC_{50} (μM)	ED_{50} (μM)	CC_{50} (μM)	ED_{50}/IC_{50}
2	—	—	0.10	28 ± 7	>50	280
51	I	H	7.0	15 ± 4	>50	2
59	Br	H	6.0[a]	12 ± 5	>100	2
60	CF_3	H	6.3[a]	5 ± 2	>100	0.7
61	Et	H	7.1	15 ± 0	>100	2
62	Br	F	15[a]	12 ± 9	>100	0.8
63	CF_3	NH_2	14[a]	5 ± 2	>50	0.4

[a] IC_{50} values determined on the sodium salt of the carboxylic acid due to solubility considerations.

of Table 2 did not have improved cellular activity (relative to compound **2**) with the notable exception of compound **51**.

Interestingly, analog **51** (Table 2) exhibited consistent replicon activity (ED_{50} = 15 ± 4 μM). A structure–activity comparison of **51** versus **50** or **52** suggested that this replicon activity was rather anomalous; nevertheless, additional analogs of compound **51** were prepared as highlighted in Table 3. Examination of these analogs revealed that while only moderately potent against NS5B, these inhibitors had consistent activity in the replicon assay. Compounds **60** and **63** exhibited the best activities (ED_{50} = 5 ± 2 μM for both). Moreover, the ratios of cellular to enzyme activity (i.e., ED_{50}/IC_{50}) were improved approaching unity for compounds **51** and **59**–**63**, whereas the same ratio for compound **2** was several hundreds reflecting its substantial differential in enzyme versus cellular activity.

In order to better understand the activity of the cycloalkyl amides of Table 2, several compounds were selected to be soaked into crystalline CΔ21 NS5B such that inhibitor bound crystal structures could be obtained. The highly potent adamantyl derivative (compound **49**) was initially selected and its bound structure is presented in Figure 2.[11] As was observed in the preceding report,[3] this compound binds in the primer grip site and engages in a hydrogen bond between its carboxylic acid and the hydroxyl of Ser 556. There is a second hydrogen bond between the amide carbonyl to backbone amide of Tyr 448 (N–O distance is 2.7 Å). The phenyl ether motif again occupies a deep hydrophobic pocket formed by residues Pro 197, Leu 384, Met 414, Tyr 415, and Tyr 448. Examination of the binding pocket region surrounding the C-ring reveals a relatively large cavity explaining the diversity of functionality that is tolerated at that position. Additionally, the residues surrounding the C-ring pocket are generally hydrophobic thereby explaining the preference for lipophilic substituents at this position.

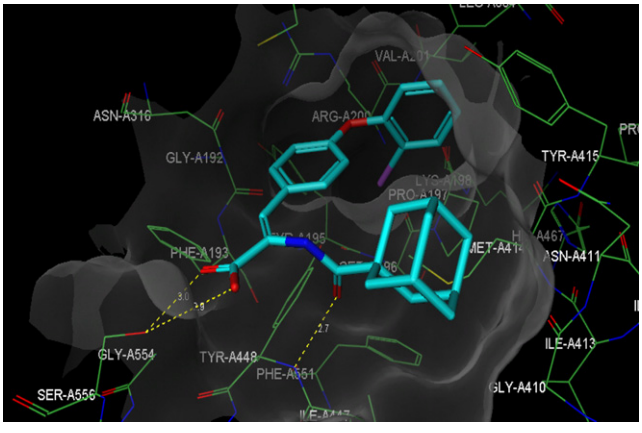


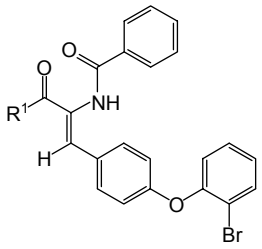
Figure 2. X-ray crystal structure (2.8 Å resolution) of compound **49** (Table 2) bound to the Primer Grip site of CΔ21 NS5B polymerase.

We next attempted to obtain a crystal structure of compound **51** (Table 3) bound to NS5B polymerase. The reader will recall that this compound, containing a cyclopentylethyl C-ring, exhibited consistent replicon activity. Despite substantial efforts, we were unable to obtain a crystal structure of **51** (or several related compounds from Table 3) bound to NS5B polymerase. Intrigued by these results and seeking to better understand the interaction of this analog with the polymerase, resistance selection studies were undertaken in the replicon system with compound **51**. These studies revealed a mutation (M423 → T423) that rendered inhibitor **51** inactive in the cellular assay. By comparison, lead compound **2** retained its activity in this resistant (M423T) replicon line.[12] Interestingly, M423 is not a part of the primer grip binding site previously described (Fig. 2). Based on the literature reports, M423 is located in the allosteric binding site of the polymerase.[13,14] Based on these data and the fact that the SAR data surrounding compound **51** are inconsistent with those of the other analogs from this template, we are proposing that introduction of the cyclopentylethyl group into this analog resulted in its ability to interact with NS5B polymerase at the allosteric site. Follow up studies on this potentially new series of inhibitors will be reported separately.[15]

The final component of our SAR program on the (2*Z*)-2-benzoylamino-3-(4-phenoxy-phenyl)-acrylic acid template involved modification of the carboxylic acid. Given that the carboxylate motif might be contributing to poor membrane permeability and/or protein binding, it was anticipated that modification as an amide might lead to improved cellular activity for compounds bound to the primer grip site. As highlighted in Table 4, various amide derivatives were prepared.

Examination of Table 4 illustrates that primary amide **25**, hydrazide **26**, and *N*-methoxyamide **27** were less active than the parent carboxylic acid **2**. Encouragingly, ethanolamide **64** while having modest enzyme potency (IC_{50} = 2.7 μM) demonstrated reproducible replicon activity (ED_{50} = 8 ± 2 μM). X-ray studies on a related

Table 4. Activity of analogs containing modifications to the carboxylate



Compound	R^1	NS5B IC_{50} (μM)	ED_{50} (μM)	CC_{50} (μM)	ED_{50}/IC_{50}
2	OH	0.10	28 ± 7	>50	280
25	NH_2	0.5	20 ± 7	45 ± 7	—
26	$NHNH_2$	1.0	>50	>50	—
27	NHOMe	0.8	>50	22	—
64	$NH(CH_2)_2OH$	2.7	8 ± 2	>50	3
65	$NHCH_2CO_2H$	0.06	>50	>50	—
66	$NHCH_2Ph$	>100	>50	>50	—
67	NHPh	>100	>50	>50	—
68	NH[(4-OH)–Ph]	12	>50	22 ± 0	—
69	NH[(3-OH)–Ph]	1.9	15 ± 0	25 ± 0	—
70	NH[(2-OH)–Ph]	15	>50	>50	—
71	NH[(2-CO_2H)–Ph]	0.4	>50	>50	—

ethanolamide analog (not shown) confirmed that compounds of this type do in fact bind at the primer grip site of NS5B in a manner identical to that previously described for compound **49** (Fig. 2). In the case of the ethanolamide containing analogs, the position of Ser 556 is perturbed slightly to accommodate a hydrogen bond to the terminal alcohol of the amide. Preparation of the carboxylic acid equivalent of compound **64** (i.e., glycimide **65**) afforded a dramatic increase in enzyme potency (IC_{50} = 0.06 μM) with a concomitant reduction in cellular activity (ED_{50} >50 μM). Simple amides such as anilide **66** or benzyl amide **67** were less active; however, installation of hydroxyl or carboxylic acid functional group onto the anilide (i.e., compounds **68**–**71**) restored activity against NS5B polymerase.

Taken together, these results (along with the previously described X-ray structure, Fig. 2) indicate that analogs of this template require a functional group capable hydrogen bonding at the carboxylate position. While carboxylic acids at this position afford high inhibitory potency (as evidenced by **2** and **65**), their potentially poor membrane permeability and/or serum protein binding properties prevent them from achieving substantial efficacy in a cellular assay. Encouragingly, the activity of **64** suggests that it may be possible to satisfy the hydrogen bonding requirements of this position using functional groups that offer improved permeability and protein binding properties.[16]

Finally, in order to evaluate the potential of compounds such as **64** as orally administered anti-HCV agents, a pharmacokinetic profile of the compound was obtained (Table 5). Compound **64** was dosed to rats (Sprague-Dawley) at 5 mpk. As illustrated, amide **64** demonstrated improved oral bioavailabilities relative to the parent carboxylic acid **2**, and it also had higher rates of clearance and a slightly lower half-life.

Table 5. Pharmacokinetic properties of selected compounds

Compound	%*F*	C_{max} (μM)	$t_{1/2}$ (h)	CL (Lh/kg)
2	21	9	1.5	0.20
64	76	1.5	1.0	3.2

In conclusion, we have reported on the structure–activity evaluation of a novel series of non-nucleoside inhibitors of HCV NS5B polymerase. In the preceding report, we described as to how the modification of the southern region of (2*Z*)-2-(benzoylamino)-3-(5-phenyl-2-furyl)-acrylic acid afforded potent inhibitors such as compound **2** that interact with NS5B polymerase at the primer grip site as determined by X-ray crystallography. The modest cellular efficacy of such compounds prompted us to undertake modifications to the northern region of the template. These efforts resulted in the identification of **64** as a lead compound with reasonable activity and PK properties; additional optimization studies on this molecule will be reported in due course. Finally, this work led to the serendipitous identification of **51** and related analogs as novel polymerase inhibitors that may interact with NS5B at the allosteric site.

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tors at Chiron Corporation for providing us with CΔ21 HCV NS5B.

References and notes

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12. Efforts to similarly generate a replicon line resistant to compound **2** were not successful.
13. For a description of the HCV allosteric site: (a) Love, R. A.; Parge, H. E.; Yu, H.; Hickey, M. J.; Diehl, W.; Gao, W.; Wriggers, H.; Ekker, A.; Wang, L.; Thomson, J. A.; Dragovich, P. S.; Fuhrman, S. A. *J. Virol.* **2003**, *77*, 7575; (b) Wang, M.; Ng, K. K.-S.; 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. G. *J. Biol. Chem.* **2003**, *278*, 9489.
14. At the present time, we do not have an explanation for our inability to soak compound **51** into the allosteric site of NS5B and solve the structure. A possible explanation is that our crystal packing form (Ref. 11) constricts the allosteric site.
15. Competitive binding studies between a labeled variant of compound **51** and a published allosteric inhibitor (Ref. 13b) supported the conclusion that **51** interacts at the allosteric site. As a control, a labeled variant of compound **2** did not compete for the allosteric site. Details of these studies along with additional SAR around compound **51** will be reported separately.
16. Preliminary studies confirmed that analogs bearing carboxylic acids were highly protein bound and had low membrane permeability. For example, compound **2** had $K_d = 0.35\ \mu M$ for human serum albumin (HSA, fraction V) and low Caco-2 permeability. In contrast, compound **64** had $K_d = 10\ \mu M$ for HSA and high Caco-2 permeability.