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Discovery of benzimidazole-diamide finger loop (Thumb Pocket I) allosteric inhibitors of HCV NS5B polymerase: Implementing parallel synthesis for rapid linker optimization

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ABSTRACT

Previously described SAR of benzimidazole-based non-nucleoside finger loop (Thumb Pocket I) inhibitors of HCV NS5B polymerase was expanded. Prospecting studies using parallel synthesis techniques allowed the rapid identification of novel cinnamic acid right-hand sides that provide renewed opportunities for further optimization of these inhibitors. Novel diamide derivatives such as **44** exhibited comparable potency (enzymatic and cell-based HCV replicon) as previously described tryptophan-based inhibitors but physicochemical properties (e.g., aqueous solubility and lipophilicity) have been improved, resulting in molecules with reduced off-target liabilities (CYP inhibition) and increased metabolic stability.

The hepatitis C virus (HCV) has infected an estimated 170–200 million people world-wide.[1,2] The infection becomes chronic in a majority of cases and may lead to severe or fatal liver damage (cirrhosis and hepatocellular carcinomas). Currently available therapies are based on the combined use of pegylated interferons (e.g., PEG-IFN-α) and ribavirin which have limited efficacy (~50% for genotype 1 infected patients) and tolerability.[3] The last years have witnessed extensive efforts from the pharmaceutical industry, devoted to the development of specific antiviral agents for the treatment of HCV infection that would provide improved efficacy, tolerability, and compliance in addressing the unmet medical need.[4,5]

Since its inception, combinatorial chemistry has become an integrated part of the drug discovery process. On the one hand, the synthesis of large screening libraries has contributed to compound collections in pharmaceutical companies, occupying a significant proportion of screening pools. On the other hand, the synthesis of small focused libraries through parallel synthesis techniques has played an equally important, yet complementary role in lead optimization. Recent developments in new synthetic methodologies as well as new instrumentation for synthesis and purification of compounds have contributed to significantly increasing the utilization of parallel synthesis in lead optimization and the generalization of these techniques to increasingly complex chemistries.

Traditionally, the use of parallel synthesis in lead optimization has focused mostly on expanding the diversity of substituents around a core scaffold. While certainly worthwhile and useful in rapidly providing structure–activity relationships (SAR), complementary uses of parallel chemistry techniques and principles can be envisaged that concretely advance lead optimization projects by providing more profound structural alterations and novel opportunities for further optimization. Herein, we report a novel strategy for identifying an alternative lead series through the synthesis of small libraries based on established SAR and acquired knowledge of preferred pharmacophores. This approach was successfully applied to our HCV NS5B polymerase inhibitor project and provided new opportunities for expansion and diversification of a previously established lead compound.

We previously reported the discovery of specific benzimidazole-based non-nucleoside inhibitors (NNI) that bind to an allosteric site in the thumb domain of HCV NS5B polymerase (Thumb Pocket I).[6,7] This class of compounds is thought to interfere with a protein conformational change that is required to initiate viral RNA synthesis by the enzyme. The inhibitors bind in a hydrophobic pocket at the top of the thumb domain, and compete with intra-molecular binding of a loop that extends from the finger domain of the enzyme, to prevent the formation of a completely enclosed active site required for productive template binding and RNA synthesis.[7]

The initial benzimidazole screening hit (**1**, NS5B IC_{50} = 14 μM) was optimized to provide 5-hydroxytryptophan derivatives such

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as **2** with nanomolar potency in the enzymatic assay (Fig. 1).[8,9] Unfortunately the polarity and ionization of these structures was incompatible will cell permeation and the compounds were found inactive in a cell-based HCV replicon assay.

Removal of the two ionizable carboxyl groups led to neutral inhibitors (**3**) with low micromolar potency in the cell-based assay and additional biological validation of the mechanism of polymerase inhibition.[10] While replacement of the benzimidazole scaffold of these inhibitors by a more lipophilic indole isostere provided significant improvements in cell culture activity with EC_{50} values in the low nanomolar range (e.g., **4**),[11,12] these molecules exhibited undesirable physicochemical characteristics which we believed would eventually compromise their further development. In particular, aqueous solubility was extremely low and the high lipophilicity of the molecules (*c*Log *P* = 6.1) increased potential for off-target activity.[13] In addition, electron-rich indole derivatives are potential substrates for bioactivation that may lead to toxicity.[14] As a result, we initiated a search for alternative right-hand sides with potential to confer superior drug-like properties to the molecules and that would be more readily amenable to rapid analog synthesis. Our objective was the identification of novel right-hand sides that would provide inhibitors with enzymatic potency in the nanomolar range, at least low micromolar cellular potency and structural diversity from preceding series. Initial SAR in the benzimidazole series had identified the cyclohexyl ring and the carbonyl group on the benzimidazole scaffold as key contributors to potency, whereas the C-2 position was more tolerant of structural changes.[7,8] Our search for replacements of the right-hand side portion of inhibitors was carried out using the synthetically accessible benzimidazole scaffold, retaining these two anchor points. The 3-furyl group was chosen for the C-2 substituent as it had been shown to confer good potency. SAR and NMR studies on the protein-bound conformation of inhibitors suggested that the central substituent extending from the asymmetric α-carbon of the tryptamine portion of inhibitors such as **2**–**4**, served mainly as an orienting group that positions the benzimidazole scaffold and the right-hand side indole scaffold in an optimal arrangement for interacting with the protein binding site.[10,15] Attachment of a carboxylic acid function at the far right-hand side of the inhibitor was previously shown to provide solubility and improve potency (cf. compound **2**).[9] Thus, this function was maintained in the design of our prospecting libraries. A hypothetical pharmacophore model for benzimidazole inhibitors and the library designs are depicted in Figure 2.

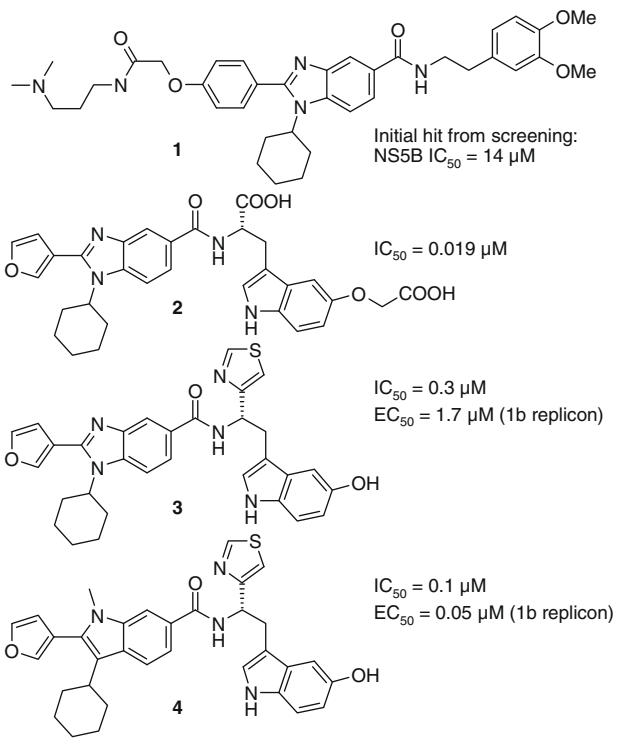


Figure 1. Allosteric 'finger loop' inhibitors of HCV polymerase from early optimization studies.

The library based on generic structure **6** suggested initial coupling of benzimidazole carboxylic acid **9** to various diamino spacers that were selected based on their ability to ultimately project the terminal carboxylic acid pharmacophore at variable distances and regions in space relative to the left-hand side of the molecule. As shown in Scheme 1, coupling of benzimidazole **9**[8] with Boc mono-protected diamines **10 a**–**f** under standard conditions provided amides **11a**–**f** (ca. 75% yield) that were subsequently deprotected under acidic conditions to provide amines **12a**–**f**. Each of these amine fragments were then reacted with a set of aldehydes (**13**–**18**) under reductive amination conditions to provide benzylamine library I or coupled with isocyanate esters (**19**–**23**) to provide urea library II as shown in Scheme 1. Following deprotection with NaOH (library II) and purification by reversed-phase HPLC, inhibitors of general formula **6** were obtained in yields ranging from 29% to 60% (library I, 34/36 compounds) and 12% to 80% (library II, 20/30 compounds). The 54 compounds thus obtained were tested in our standard enzymatic NS5B assay,[16] but unfortunately, all were found to be less active ($IC_{50} \sim 3$–50 μM) than the free carboxylic acid **9** (IC_{50} = 1.6 μM).[8]

Consequently, our strategy focused on the general motif represented by diamide structure **7** (Fig. 2) in which an aniline carboxylic acid derivative is attached to benzimidazole scaffold **9** either directly or through an amino acid linker (*d,l*-alanine or *d,l*-β-alanine), thus providing various spacer lengths between the left-hand side benzimidazole scaffold and the right-hand side carboxylic acid pharmacophore. The libraries were synthesized on a solid support as described in Scheme 2. Seventeen nitroarenes carrying a carboxylic acid function (**24**–**40**) were attached to bromo-Wang resin under standard conditions and the nitro functionality reduced to provide anilines **41**. Coupling of the aniline to fragment **9** provided library A. Coupling with Fmoc-*d,l*-Ala or Fmoc-*d,l*-β-Ala followed by deprotection provided intermediates **42** that were subsequently

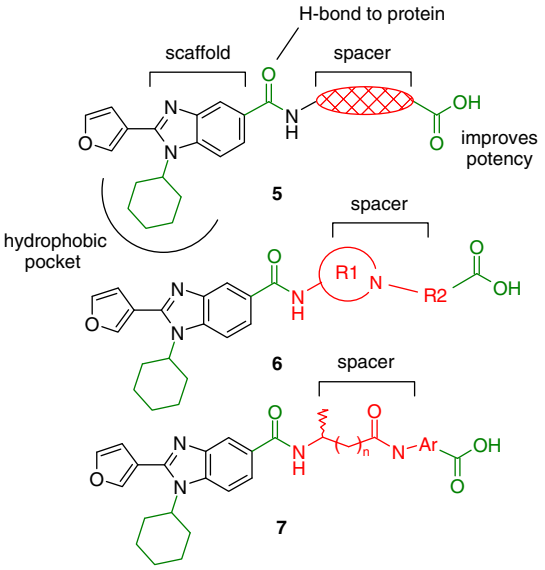
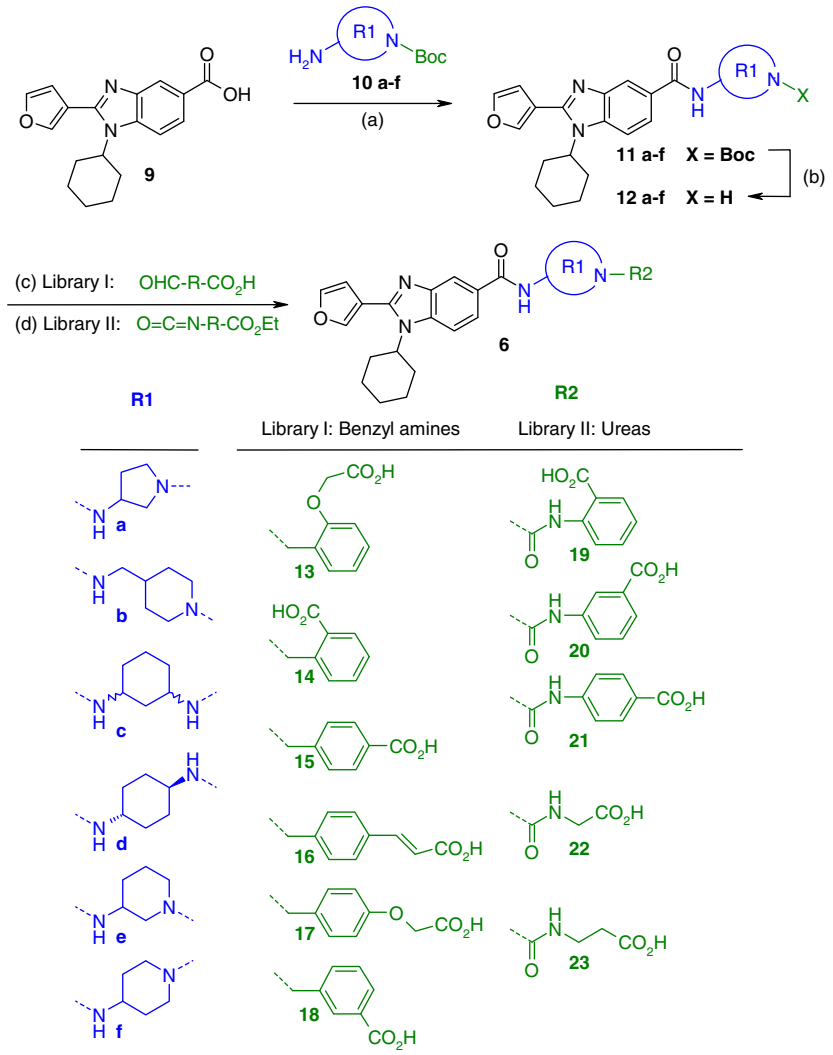


Figure 2. Pharmacophore model and library design for right-hand side exploration.



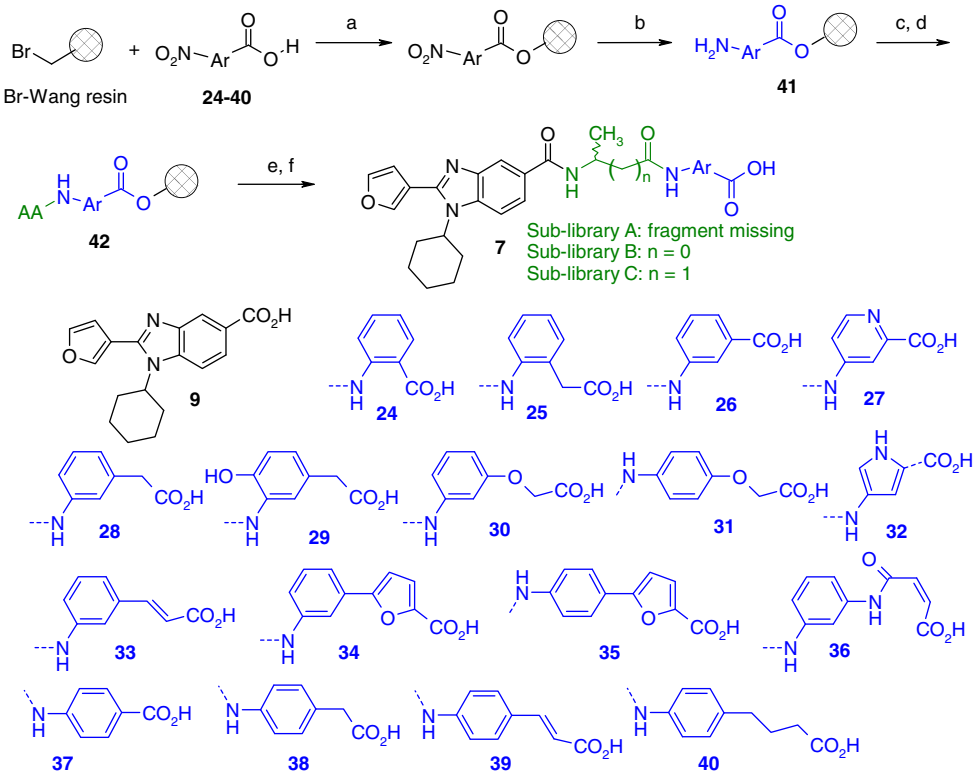
Scheme 1. Libraries of benzylamine and urea derivatives synthesized from a diamine spacer. Reagents and conditions: (a) amine 1.1 equiv, HATU (1.1 equiv, DIPEA (1.5 equiv)/DMF; (b) 25% TFA/CH_2Cl_2, 30 min; (c) library I: OHC-R (10 equiv), $NaCNBH_3$ (5 equiv), AcOH/trimethylorthoformate/DMF 1:1, 3 h then 0.1 M aqueous HCl; (c) library II: (*Note:* the carboxylic acid was introduced as ester derivative) (i) O=N=C–R (1.5 equiv)/DMF, 1.5 h (ii) 1 M aqueous NaOH (3.3 equiv), 14 h; (iii) AcOH (6.6 equiv).

coupled to benzimidazole **9** to provide libraries B and C (Ala and β-Ala spacers, respectively). Libraries A (no spacer) and C (*d*,l-β-Ala spacer) did not provide compounds with significantly improved potency compared to benzimidazole carboxylic acid derivative **9**, suggesting that no additional interactions with the protein could be engaged with these structures. IC_{50} values in library A ranged from 9 to 34 μM. The most potent analog in library C was the 4-aminocinnamic acid derivative **C-39** (IC_{50} = 0.6 μM) which provided a modest 2.5-fold improvement in potency over **9**.

Library B (*d*,*l*-Ala spacer) in contrast, provided more encouraging results. The most interesting analogs displayed IC_{50} values <500 nM in our enzymatic assay (Table 1) along with HCV genotype 1b replicon cell-based potency in the 10–30 μM range.

For example, 4-benzoic amide analog **B-37** provided a fourfold improvement in intrinsic potency (IC_{50} = 0.39 μM) compared to carboxylic acid derivative **9** and unlike the latter, inhibited the 1b cell-based replicon with EC_{50} = 30 μM.[17] Extending the carboxylic acid function by a methylene atom was detrimental to potency (**B-38**) as was modifying the substitution pattern from a *para*- to a *meta*-arrangement (**B-26**). Phenoxyacetic acid derivative **B-31** which extends the carboxylic acid function one atom further from the aromatic ring was tolerated but offered no advantage, presumably as a consequence of the high degree of rotational freedom. In support of this argument, conformationally-restricted cinnamic acid derivative **B-39** was identified as the most potent inhibitor in this series (IC_{50} = 0.34 μM). Furthermore, the decrease in ionization associated with the cinnamic acid moiety compared to benzoic or aliphatic carboxylic acid derivatives resulted in improved cellular permeation and a beneficial effect on inhibition of the replicon (EC_{50} = 13 μM). Moving the cinnamic acid moiety from the *para*- to the *meta*-position of the aromatic ring (**B-33**) resulted in a modest threefold reduction in intrinsic potency, suggesting that the acid function is unlikely to be involved in a strong ionic interaction with protein residues. Conformationally restricted furoic acid derivative **B-35** had comparable intrinsic potency as cinnamic derivative **B-39** but the increased ionization of the carboxylic acid function was detrimental to cell culture activity.

During this initial prospecting study, we identified novel diamide right-hand side functionalities (e.g., **B-39**) for our benzimidazole-based NS5B inhibitors that provided comparable intrinsic potency to inhibitors such as **3** and offered new opportunities for further optimization. All compounds in library B had been synthesized in racemic form, and one of our objectives was to probe conformational preferences at the central amino acid position by



Scheme 2. Libraries A–C prepared using no spacer or *d*,*l*-alanine and *d*,*l*-β-alanine spacers. Reagents and conditions: (a) nitro acid (4 equiv), DIPEA (6.8 equiv)/DMF, 15 h; (b) $SnCl_2$ dihydrate (13 equiv)/DMF, 24 h; (c) Fmoc-*d*,*l*-Ala or Fmoc-*d*,*l*-β-aminobutyric acid (1.5 equiv), TBTU (1.5 equiv), DIPEA (3 equiv)/DMF, 3 h at 60 °C and 5 h at rt; second coupling: Fmoc-*d*,*l*-Ala or Fmoc-*d*,*l*-β-amino-butyric acid (4.5 equiv), HATU (4.5 equiv), DIPEA (6.5 equiv)/DMF, 15 h rt; (d) 20% v/v/DMF 20 min; (e) **9** (1.5 equiv), TBTU (1.5 equiv), DIPEA (3 equiv)/DMF, 18 h; (f) 50% TFA/DCE, 1h.

Table 1
Most potent compounds from library B (*d*,*l*-Ala spacer)

Compds	NS5B IC_{50}, μM[16]	1b Replicon EC_{50}, μM[17]
B-37	0.39 (±0.02)	30 (*n* = 1)
B-38	1.52 (*n* = 1)	
B-26	1.61 (±0.04)	
B-31	0.9 (±0.2)	
B-39	0.34 (±0.07)	13 (*n* = 1)
B-33	0.9 (±0.2)	
B-35	0.45 (*n* = 1)	28 (*n* = 1)

synthesizing configurationally-defined analogs. Previous studies in the tryptophan series had identified a conformational bias for this position, resulting from rigidification of the inhibitor backbone toward the bioactive conformation.[10,15] Compound **B-39** was thus resynthesized using configurationally-defined (*R*)- and (*S*)-alanine spacers and the results are shown in Table 2.

The (*R*)-configuration at the asymmetric center of the alanine spacer provided a twofold improvement in potency over the racemic mixture. This result is consistent with previous findings in the tryptophan series where a similar orientation of a carboxyl, alkyl or heterocyclic substituent conferred preferential binding to the protein (cf. compounds **2** and **3**).[15] In support of this observation, replacement of the (*R*)-alanine linker by the more flexible glycine residue (compound **45**) resulted in a twofold loss in intrinsic potency. Diversifying SAR was performed at the linker position with the aim of identifying superior linkers. A variety of amino acids were introduced in place of the alanine residue of **44** such as *dl*-Ile, *dl*-Phe, *dl*-cyclohexylalanine, *dl*-norleucine, *dl*-phenylglycine, *dl*-cyclohexylglycine, *dl*-2-thienylglycine, (*R*)-homoPhe, (*R*)-homoSer, (*R*)-4-phenylPhe, and (*R*)-ethylglycine. None proved superior to compound **44** (IC_{50} = 0.4 μM for the homologated ethylglycine analog **46** to 8 μM for the large lipophilic 4-phenylPhe analog; results not shown). These observations are again consistent with the amino acid side chain pointing to solvent and providing conformational rigidification toward a bioactive conformation. Modest improvements in intrinsic potency could be achieved through conformational rigidification of the right-hand side cinnamic acid moiety in compound **44** (Fig. 3).

Table 2
Inhibitors with configurationally-defined Ala spacers

Compds	Spacer	NS5B IC_{50}, μM[16]	1b Replicon EC_{50}, μM[17]
B-39	*d*,*l*-Ala	0.34 (±0.07)	13 (*n* = 1)
43	(*S*)-Ala	0.36 (±0.08)	25 (*n* = 1)
44	(*R*)-Ala	0.13 (±0.02)	6.9 (±0.8)
45	Gly	0.28 (±0.08)	41 (*n* = 1)
46	(*R*)-Ethylglycine	0.40 (±0.07)	6.5 (*n* = 1)
47	(*R*)-Ala	0.09 (±0.02)	>42
48	(*R*)-Ala	0.06 (±0.01)	>40

Both benzofuran (**47**) and indole (**48**) carboxylic acid derivatives provided up to an additional twofold improvement in enzymatic potency with IC_{50} values in the 60–90 nM range (Table 2), but the increased ionization of the carboxylic acid of these analogs was detrimental to cell permeation and replicon inhibition (EC_{50} >40 μM). Compound **44** was also tested against the RNA-dependent RNA polymerase of the poliovirus and a mammalian DNA-dependent RNA polymerase isolated from calf thymus.[6,17] No inhibition was detected against these enzymes at concentrations up to 200 μM, indicating that specificity against HCV was maintained in this series (TI >1500).

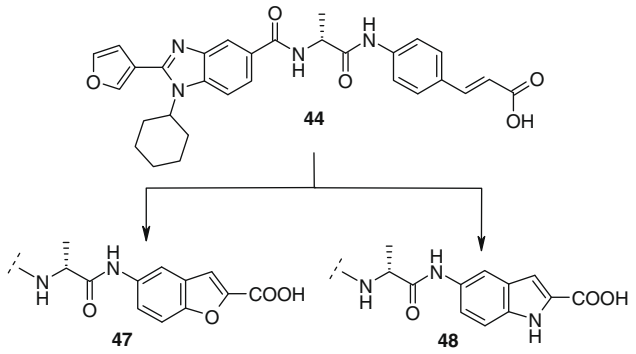


Figure 3. Conformationally-restricted cinnamic acid isosteres.

Table 3
Comparison of ADMET parameters for tryptophan and diamide-based benzimidazole NS5B inhibitors

Compds	Log *D* (pH 7.4)	Sol[a] (μg/mL)	HLM[b] (min)	CYP450 IC_{50} (μM)				
				1A2	2C9	2C19	2D6	3A4
3	3.8	<0.07	26	>30	2.3	2.4	4.0	0.4
44	2.2	171	44	>30	>30	>30	>30	12.3

[a] Twenty-four hours solubility of amorphous material at pH 7.2.
[b] $T_{1/2}$ at 10 μM in human liver microsomes (min).

In addition to identifying novel structural features that would provide opportunities for diversification of the SAR in the benzimidazole series, we also aimed to improve the physicochemical properties of our inhibitors. 5-Hydroxytryptophan derivatives such as **3** suffered from high lipophilicity (Log *D* = 3.8 at pH 7.4) and poor solubility (typically <0.1 μg/mL in pH 7.2 buffer). These undesirable properties often lead to liabilities in ADMET (e.g., CYP450 inhibition, poor metabolic stability) and toxicity in vivo.[13] In contrast, compound **44**, with comparable potency to **3** against NS5B and the cell-based replicon, provided for improved physicochemical properties (Log *D* = 2.2 at pH 7.4 and solubility = 171 μg/mL at pH 7.2), reduced potential for metabolism by human liver microsomes and CYP450 inhibition as depicted in Table 3.

In conclusion, a prospecting study using parallel synthesis techniques allowed the rapid identification of novel right-hand sides that offer renewed opportunities for further optimization of our benzimidazole-based HCV NS5B polymerase inhibitors. The novel diamide derivatives exhibit comparable potency (enzymatic and cell-based replicon) as previously described tryptophan-based inhibitors and improved physicochemical properties. Further optimization of these inhibitors will be reported in the near future.

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16. All inhibitors in this study were purified to >95% homogeneity by reversed-phase HPLC and isolated as TFA salts. All compounds were characterized by mass spectrometry and gave ^{1}H NMR spectra consistent with expected structures. IC_{50} values were determined as described in Ref. 6 and are the average of at least two independent determinations unless indicated otherwise.
17. EC_{50} determinations in the cell-based replicon assay were performed in duplicates using RT-PCR for RNA quantification as described in: Beaulieu, P. L.; Fazal, G.; Goulet, S.; Kukolj, G.; Poirier, M.; Tsantrizos, Y.; Jolicoeur, E.; Gillard, J.; Poupart, M.-A.; Rancourt, J. WO Patent WO 03/010141, 2003. Total replicon RNA levels were normalized to control cellular GAPDH mRNA. Compounds were devoid of cytotoxicity at inhibitory concentrations. For an overview and basic protocols on the use of HCV replicons, see Lohmann, V. *Meth. Mol. Biol.* **2009**, *510*, 145: *Hepatitis C: Methods and Protocols*, 2nd ed.; Tang, H., Ed.; Humana Press.