Synthesis and Biological Characterization of B-Ring Amino Analogues of Potent Benzothiadiazine Hepatitis C Virus Polymerase Inhibitors

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Benzothiadiazine inhibitors of the HCV NS5B RNA-dependent RNA polymerase are an important class of non-nucleoside inhibitors that have received considerable attention in the search for novel HCV therapeutics. Research in our laboratories has identified a novel series of tetracyclic benzothiadiazine inhibitors of HCV polymerase bearing a benzylamino substituent on the B-ring. Compounds in this series exhibit low-nanomolar activities in both genotypes 1a and 1b polymerase inhibition assays and subgenomic replicon assays. Optimization of pharmacokinetic properties in rat led to compound **30**, which has good oral bioavailability ($F = 56\%$) and a favorable tissue distribution drug profile, with high liver to plasma ratios. Compound **30** is a potent inhibitor in replicon assays, with EC_{50} values of 10 and 6 nM against genotypes 1a and 1b, respectively.

Introduction

HCV infection is a major concern for healthcare throughout the world. The most recent estimate of the current size of the problem is chronic HCV infection in some 2−3% of the world population (over 170 million infected people).[1] Because these individuals typically remain asymptomatic for 10−20 years after the initial infection, a large percentage of those currently infected will likely advance to end-stage liver disease in the coming 1−2 decades.[2] This expected increase in the number of HCV infected individuals experiencing liver complications will result in a dramatic increase in the number of cases of cirrhosis and hepatocellular carcinoma throughout the world. In the U.S. alone, complications due to HCV infection are estimated to cause over 8000 deaths per year,[3] a number that is expected to increase in the coming decade because of increased mortality within the population of currently infected individuals.[4]

The grim statistics of the global HCV infection problem are in no way relieved by the current line of agents available for treatment and/or prevention. A clinically proven vaccine for HCV is not currently available to help control the spread of HCV infection.[5] Furthermore, the task of treating HCV infection is complicated by the fact that there are six major genotypes, with numerous subtypes for each genotype, making it difficult to discover a vaccine and effective therapeutics to cover all of these variant forms of the virus. The present standard of care is combination therapy using pegylated interferon and ribavirin.[6] While this drug combination is effective for treating patients infected with genotype 2 or 3 virus (>80% sustained virologic response, or SVR), it is far less effective for treating other genotypes, including genotype 1 (<50% SVR) which accounts for the majority of infections in the U.S. (75% genotype 1a or 1b), Europe, Asia, and elsewhere across the globe. Other concerns include the length of therapy (48 weeks to treat genotype 1 infection) and a number of moderate to severe side effects experienced by a majority of patients receiving therapy. Thus, there exists a great need for new agents to combat HCV infection, in particular those effective for treating patients infected with genotype 1 virus.

Research to discover novel HCV therapeutics has focused in large part on the identification of compounds that inhibit the NS5B RNA-dependent RNA polymerase.[7,8] A number of small molecule inhibitors have been identified that bind to several sites of the enzyme. These research efforts have been aided by X-ray crystallography and molecular modeling studies of enzyme−inhibitor complexes that have revealed details of binding of these molecules to polymerase. One class of non-nucleoside inhibitors that has received considerable attention are benzothiadiazine analogues, which bind to a unique site at the base of the so-called "palm domain", in closer proximity to the enzyme catalytic site than other known non-nucleoside inhibitors.[9,10] These compounds inhibit an initial step in viral RNA synthesis and do not compete with the incorporation of NTP.

We recently described a series of thiadiazine compounds that exhibit low nanomolar potency as inhibitors of the NS5B polymerase of genotype 1 HCV.[11] The most active compounds in this series bear a geminal dialkyl-substitution pattern on the B-ring (Figure 1). Isoamyl analogue **1** was found to be inferior to neohexyl analogue **2** both in in vitro activity (compound **2** is 1.5-fold more active than **1** against genotypes 1a and 1b polymerase) and in pharmacokinetic properties. The advantage of the neohexyl analogue **2** in vivo is the result of improved metabolic stability, as it was found that tertiary alcohol **3** was the major metabolite of **1** in in vitro metabolism studies using hepatocytes. Thus, the neohexyl substitution pattern in **2** effectively blocks the primary site for oxidative metabolism in **1**, resulting in improved exposures on oral administration in

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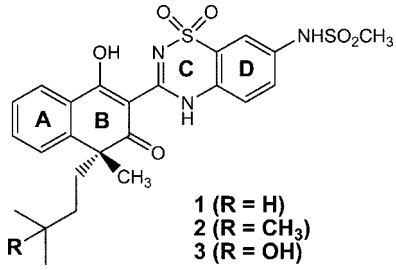


Figure 1. B-Ring dialkylthiadiazine HCV polymerase inhibitors.

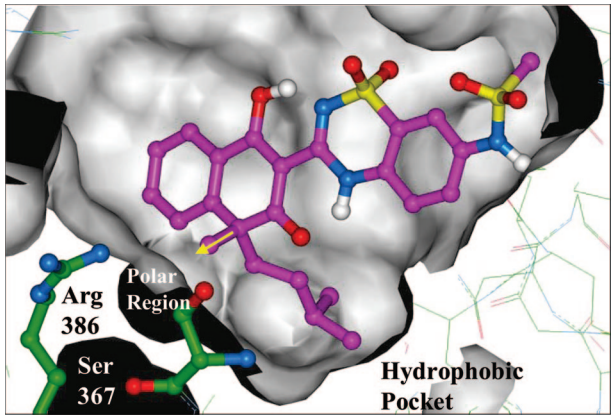


Figure 2. Molecular model showing **1** bound to HCV polymerase, which revealed that the B-ring methyl group projected toward a polar region of the enzyme occupied by residues Arg 386 and Ser 367.

rat (3.5-fold improvement in AUC for **2** over **1** following a 5 mg/kg oral dose).

We used molecular modeling to generate a model of inhibitor **1** occupying the thiadiazine binding site of HCV polymerase (Figure 2). The planar ABCD ring structure was oriented in like manner to that reported for crystallographic studies of enzyme−inhibitor complexes of other thiadiazine inhibitors.[12] This model places the isoamyl side chain on the B-ring into a hydrophobic pocket defined by residues Leu 384, Pro 197, and Tyr 415. This orientation revealed that the methyl side chain on the B-ring was projected toward a polar region of the enzyme occupied by residues Arg 386 and Ser 367. There have been no reports, to date, of inhibitors that occupy this region of the enzyme. We envisioned a strategy for exploring this region of the enzyme by replacing the B-ring methyl group with an amino function that would allow for facile late-stage substitution to rapidly prepare analogues for testing. It was our hope that such modifications might result in inhibitors with improved properties, such as better in vitro potency, improved pharmacokinetics, and/or improved physicochemical properties.

Chemistry

Synthesis of an amino derivative of isoamyl analogue **1** is shown in Scheme 1. 2-Hydroxy-1,4-naphthoquinone (**4**) was converted to *E*-oxime analogue **5** by reaction with methoxyamine in methanol, followed by allylation using an allylindium organometallic reagent generated by the addition of allyl bromide to a suspension of indium metal in DMF.[13] The resulting allyl intermediate **6** was reacted with isobutyraldehyde to give oxazolidine intermediate **7**.[14] The allyl chain of **7** was extended by olefin metathesis using 2-methyl-2-butene and Hoveyda−Grubbs second generation catalyst[15] to give prenyl analogue **8**. Hydrogenation of **8** using Adam's catalyst provided an intermediate with the desired isoamyl chain, which was further elaborated by acid hydrolysis to give methoxyamine **9**.

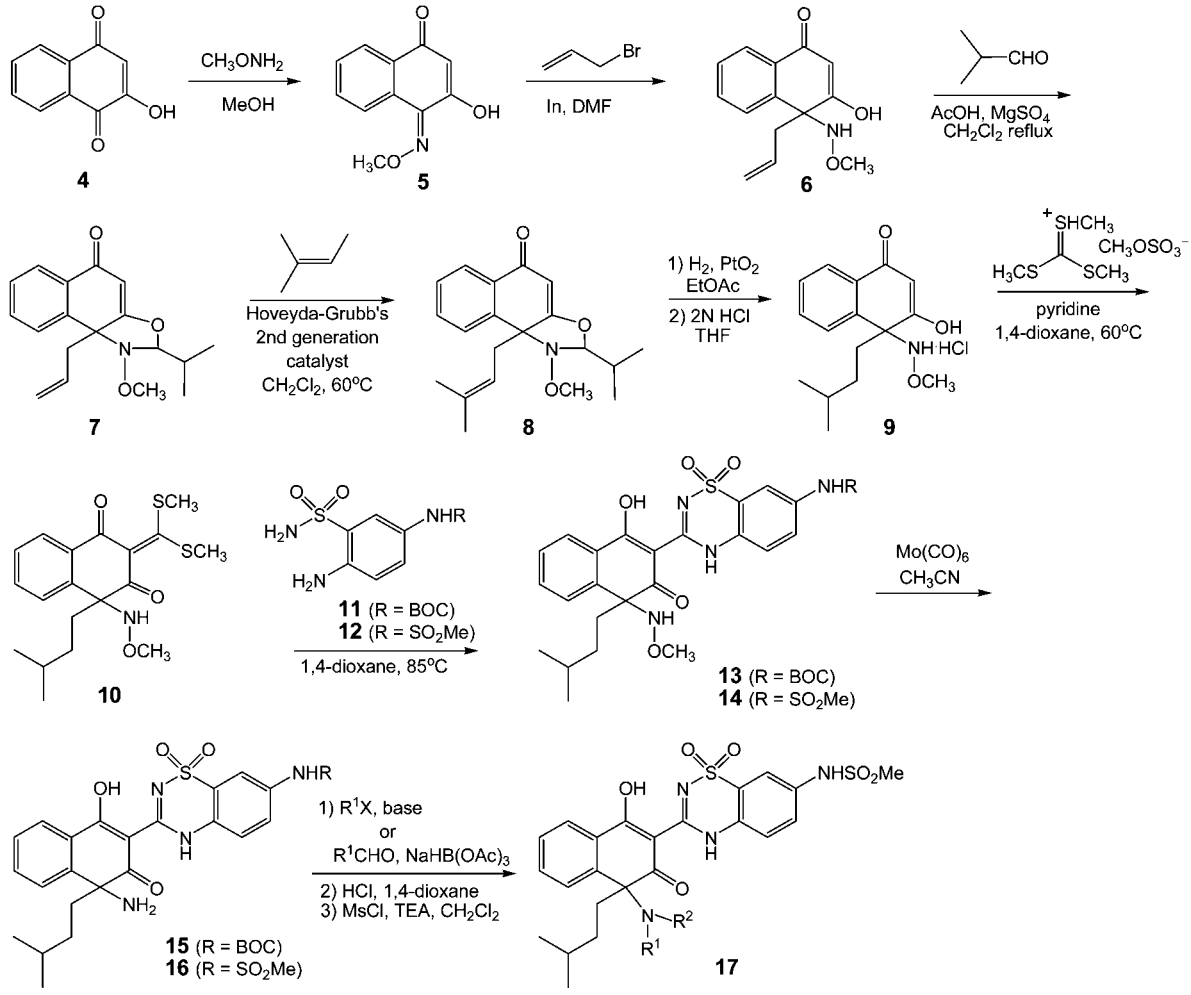
Bicyclic intermediate **9** was reacted with tris(methylthio)-methyl methylsulfate[16] to give dithioketeneacetal intermediate **10**, properly functionalized for conversion to the desired thiadiazine tetracycles. This was accomplished by reaction with *o*-aminobenzenesulfonamide **11** or **12** to provide thiadiazine analogues substituted in the 7-position with either a BOC-protected amino group (**13**) or a methanesulfonamide group (**14**), respectively. The N−O bond of the methoxyamine was cleaved by reduction using molybdenumhexacarbonyl to provide B-ring amine compounds **15** and **16**.[17] Substitution of the amino group was accomplished using standard conditions of acylation or reductive alkylation. Compounds derived from BOC-protected intermediate **15** were further converted to the desired 7-methanesulfonylbenzothiadiazine analogues by BOC removal with acid hydrolysis, followed by reaction with methanesulfonyl chloride. In this way, a series of inhibitors represented by structure **17** having a diverse set of functionality on the amino group (R^1 = acyl, alkyl, sulfonyl, carbamoyl, etc.) was prepared.

Asymmetric synthesis of chiral AB ring portions of these compounds is shown in Scheme 2. Reaction of allyl methoxyamine intermediate **6** with D-glyceraldehyde acetonide gave a mixture of two stereoisomeric oxazolidine analogues **18** and **19**, which were readily separated by chromatography on silica gel. Crystallization gave each of these compounds in high configurational purity as determined by ^{1}H NMR analysis. Chiral intermediates **18** and **19** were converted to chiral isoamyl-substituted AB ring fragments **20** and **21** using the methods previously described for the synthesis of **9**. Further elaboration of each of these enantiomeric methoxyamines to give tetracyclic benzothiadiazine derivatives was accomplished using the methods shown in Scheme 1. The resulting chiral inhibitors were obtained in high optical purity as determined by chiral HPLC analysis.[18]

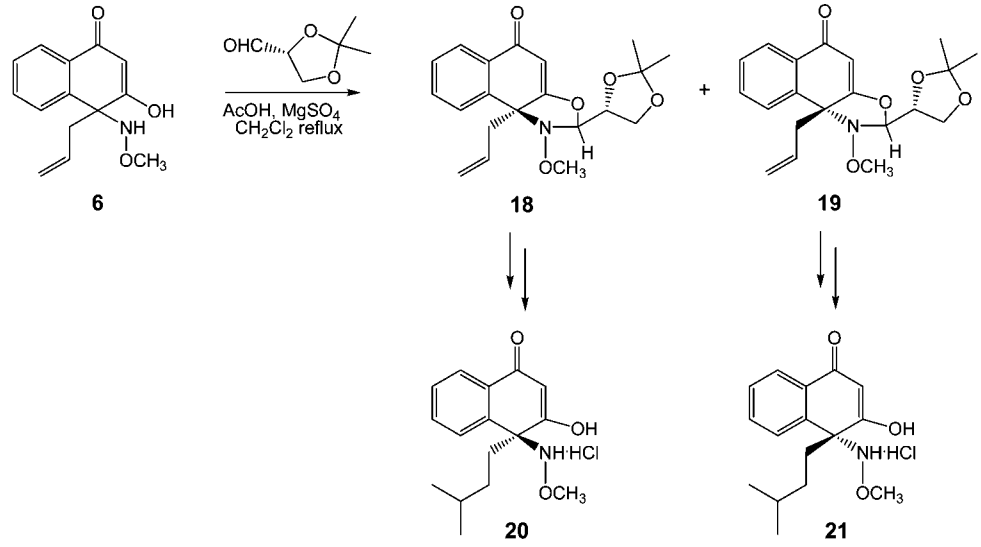
Neohexyl B-ring amino analogues were synthesized as shown in Scheme 3. Alkylation of **4** using an organotitanium reagent generated by the addition of neohexyl Grignard to titanium(IV) isopropoxide at −78 °C afforded tertiary alcohol intermediate **22**.[19] Compound **22** was converted to thiadiazine **23** by the two-step sequence of reactions involving initial formation of the dithioketeneacetal intermediate using tris(methylthio)methyl methylsulfate,[16] followed by reaction with aminosulfonamide **12**. Acetamide **24** was prepared from **23** by a Ritter reaction in acetonitrile in essentially quantitative yield.[20] Acid hydrolysis removed the acetyl group to give primary amine **25**, which was further converted to secondary amine analogues **26** by reductive alkylation with a variety of aldehydes. This method provided a series of compounds for testing having a variety of functionality extending from the B-ring amino group.

Chiral resolution was used to separate racemic amine **25** into the individual enantiomers as shown in Scheme 4. Acylation of **25** with chloroformate **27**, prepared from (*R*)-4-phenyloxazolidin-2-one by reaction with diphosgene, gave a 1:1 mixture of diastereomers that was separated by column chromatography on silica gel.[21] This method allowed the desired diastereomer **28**, having the *S*-configuration at the tertiary carbon of the B-ring, to be isolated in excellent optical purity (>98% de).[22] The oxazolidinone ring was displaced using lithium trimethylsilylethoxide to give the desired amine protected as the trimethylsilylethylcarbamate, which was removed with TFA to give chiral amine **29**, isolated as the TFA salt.[23] Reductive alkylation of **29** using 2,6-dimethylbenzaldehyde afforded **30**.

Scheme 1. Synthesis of Isoamyl-Substituted Analogues of B-Ring Amino Tetracyclic Thiadiazine Inhibitors of HCV Polymerase



Scheme 2. Synthesis of Chiral Isoamyl AB Ring Fragments



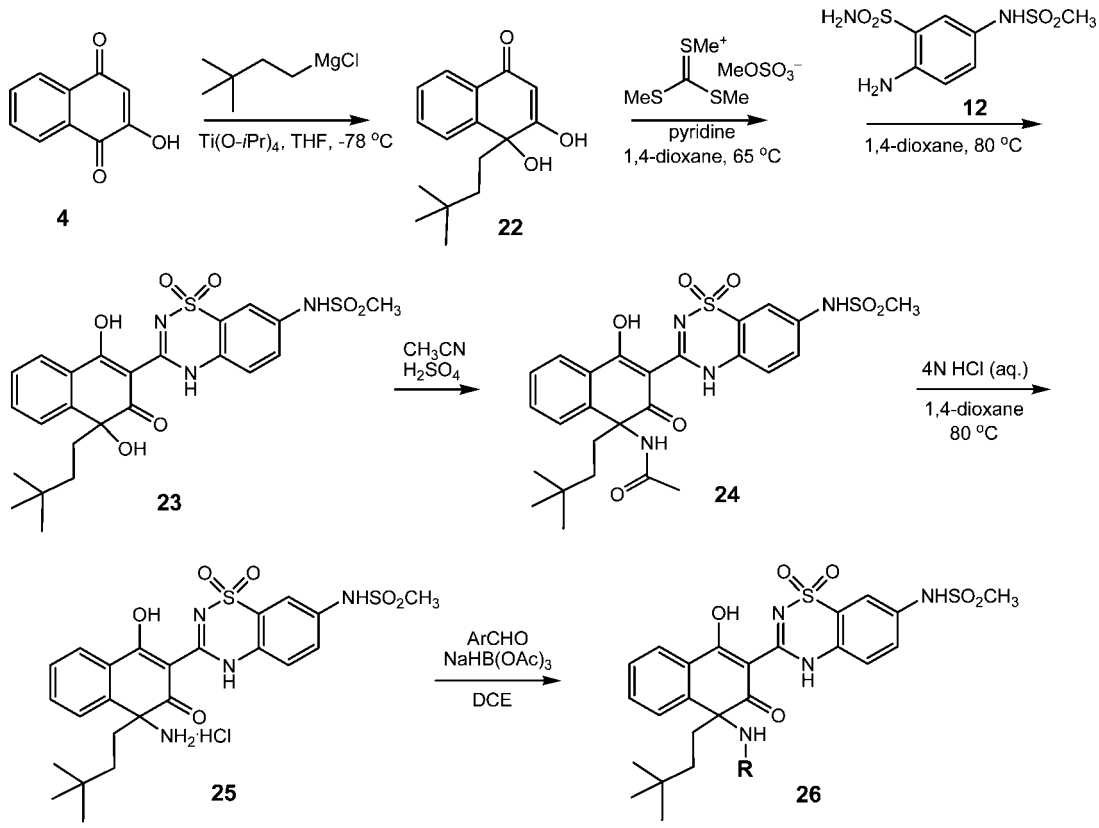
Chiral HPLC analysis of **30** confirmed that this compound contained only trace amounts of the *R*-enantiomer (<0.5%).

Biology

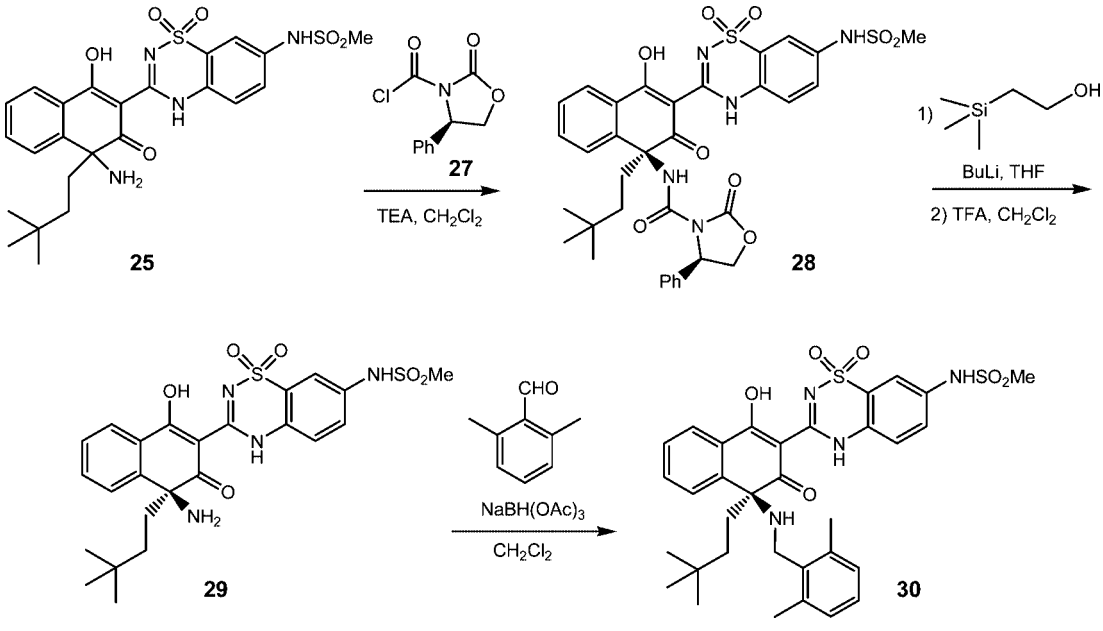
Biochemical activity of compounds was determined by measuring their ability to inhibit the formation of RNA products in a standard polymerase inhibition assay. Inhibition of genotypes 1a (H77) and 1b (BK) HCV NS5B was determined by measuring the amount of ^{3}H-UTP incorporated into RNA by scintillation counting. IC_{50} values for inhibitors were calculated using a standard IC_{50} equation.

Cell-culture activity of compounds was determined using subgenomic replicons transfected into Huh-7 cells. The ability of compounds to inhibit replication of genotype 1a (H77)

Scheme 3. Synthesis of Neohexyl-Subtituted Analogues of B-Ring Amino Thiadiazine Inhibitors of HCV Polymerase



Scheme 4. Chiral Resolution of Neohexyl B-Ring Aminothiadiazine Inhibitors



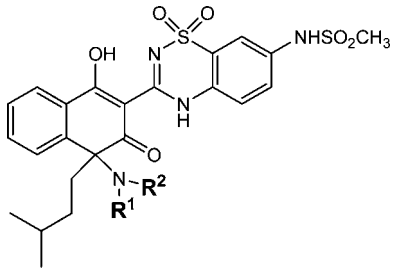
and 1b (Con1) replicons expressing the firefly luciferase gene was determined by measuring the level of luciferase in the cell lysate using a luminometer. Replicon inhibition activity, reported as EC_{50}, was measured both in the absence of human serum (HS) and in the presence of 40% HS in order to determine the extent to which inhibitor−protein interactions affect compound potency.

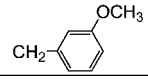
Pharmacokinetic properties of compounds were determined using Sprague−Dawley rats ($n = 3$ for each experiment) via both intravenous and oral administration of a 5 mg/kg dose. Compounds were formulated using DMSO/PEG400 (1:9,v/v).

Results and Discussion

A series of isoamyl-substituted B-ring amino analogues were prepared and evaluated for their ability to inhibit RNA synthesis catalyzed by genotype 1a NS5B.[24] Compounds with $IC_{50} < 30$ nM were further evaluated for their ability to inhibit the 1a replicon assay. A summary of SAR for this series is shown in

Table 1. Activity Data for Analogues of Isoamyl-Substituted B-Ring Aminothiadiazine Inhibitors of HCV Polymerase



Compd	R^1	R^2	IC_{50} (nM)[a]	EC_{50} (nM)[b]
16	H	H	12	29
14	OCH_3	H	10	16
17a	OCH_3	$COCH_3$	83	ND
17b	COPh	H	108	ND
17c	CO_2CH_3	H	25	210
17d	$CONH_2$	H	27	576
17e	SO_2CH_3	H	24	69
17f	CH_2Ph	H	19	34
17g		H	14	38

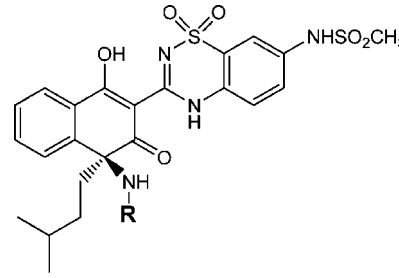
[a] 1a enzyme activity. [b] 1a replicon activity; ND = not determined.

Table 1. Primary amine **16** is a potent inhibitor in both the enzyme (IC_{50} = 12 nM) and replicon (EC_{50} = 29 nM) assays. Methoxyamine derivative **14** is equally active to **16** in the biochemical assay, and 2 times more active in replicon (EC_{50} = 16 nM). Compound **17a**, the *N*-acetyl derivative of **14**, was several times less active than **14**. In general, amide analogues were found to be somewhat less potent inhibitors of polymerase, as demonstrated by benzamide **17b** (IC_{50} = 108 nM). Carbamates are more active than amides in the biochemical assay (see methylcarbamate **17c**, IC_{50} = 25 nM). However, **17c** is significantly less active as an inhibitor of the replicon assay (EC_{50} = 210 nM). The same is true for urea analogues, such as **17d**, which has good enzyme activity (IC_{50} = 27 nM) but relatively poor replicon activity (EC_{50} = 576 nM). Sulfonamide analogues, such as methanesulfonamide **17e**, were somewhat more potent inhibitors of replicon (EC_{50} = 69 nM for **17e**) than acylated derivatives such as **17c** and **17d**.

Alkylation of the B-ring amine resulted in compounds with improved activity in the replicon assay relative to other substituted analogues investigated. It was found that a wide range of alkyl groups provided potent inhibition of polymerase function. Of the secondary amine analogues investigated, benzyl analogues such as **17f** and **17g** gave the best overall performance, since they were found to be potent inhibitors in both the enzyme and replicon assays.

Single enantiomers of a select group of the more potent compounds from this series were prepared to determine both in vitro activity and pharmacokinetic properties in rat.[18] The data are summarized in Table 2. The *S*-enantiomer methoxyamine analogue **31** (IC_{50} = 6 nM) was found to be 10 times more potent as an inhibitor of genotype 1a polymerase than the *R*-enantiomer (IC_{50} = 64 nM), confirming our expectation that the *S*-isomer of these compounds would be the more active component.[22] Amine derivatives **32** and **33** were both found to be potent inhibitors of 1a polymerase, with the primary amine **32** (IC_{50} = 4 nM) showing somewhat better potency than benzylamine derivative **33** (IC_{50} = 10 nM). However, unsubstituted amine **32** showed a nearly 10-fold decline in activity

Table 2. Comparison of Activities against Genotype 1a and Pharmacokinetic Properties in Rat for Chiral Isoamyl B-Ring Amino Analogues



compd	R	IC_{50} (nM)/[a] EC_{50} (nM)[b]	CL_p[c]	$t_{1/2}$[d]	AUC[e]	*F*, %[f]
31	OMe	6/12	2.7	0.6	0.35	34.1
32	H	4/37	2.12	0.3	0	0
33	Bn	10/16	1.16	1.7	0.6	13.3
34	Ms	7/56	4.3	0.2	0	0

[a] 1a enzyme activity. [b] 1a replicon activity. [c] Clearance (L/(h·kg)) following 5 mg/kg iv dose in rat. [d] Half-life in hours following 5 mg/kg iv dose in rat. [e] $AUC_{0-\infty}$ following 5 mg/kg oral dose (μg·h/mL). [f] Percent bioavailable following 5 mg/kg oral dose in rat.

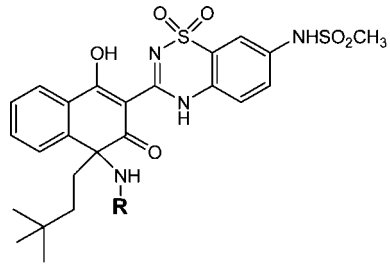
in replicon, whereas benzylamine **33** lost less than 2-fold activity in replicon (EC_{50} = 16 nM) relative to activity in the enzyme inhibition assay. Methanesulfonamide **34** gave a similar profile to unsubstituted amine **32**, with potent inhibition in the biochemical assay (IC_{50} = 7 nM) but substantially less activity in replicon (EC_{50} = 56 nM).

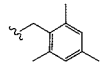
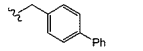
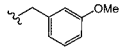
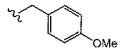
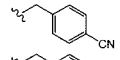
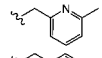
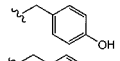
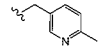
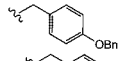
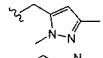
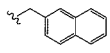
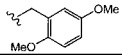
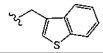
Of the compounds tested, only methoxyamine analogue **31** and benzylamine analogue **33** were found to be bioavailable following oral administration in rat. Compound **33** gave the best overall pharmacokinetic profile, with better overall exposure, a longer half-life (1.7 h), and a more moderate rate of clearance following iv dosing. Primary amine **32** and methanesulfonamide **34** gave no measurable plasma concentrations following oral dosing.

In an effort to improve the plasma concentration of these compounds in rat following oral administration, the isoamyl side chain on the B-ring was replaced with a neohexyl group. This strategy was based on a discovery made during our investigation of B-ring dialkyl tetracyclic thiadiazine inhibitors,[11] where it was determined that the neohexyl side chain blocked a site for oxidative metabolism in the isoamyl side chain (see Figure 1 and the accompanying discussion). Given the success of benzylamino analogues in the isoamyl series (i.e., compound **33**), our attention in the neohexyl series focused on arylmethylamino derivatives. Thus, a series of compounds was synthesized in parallel from neohexylamine intermediate **25** by reductive alkylation with a variety of carboxaldehydes to give arylmethyl derivatives **26** (see Scheme 3).

The SAR for compounds in the neohexyl series of inhibitors is summarized with the examples shown in Table 3. A comparison of benzamide **26a** and benzylamine **26b** confirms that amide analogues are substantially less active in both the neohexyl and isoamyl series of inhibitors. In general, it was found that a wide range of functionality for the arylmethyl group provided potent inhibition of 1a polymerase. All of the compounds in this series were found to have IC_{50} values against 1a polymerase of less than 50 nM. A particularly attractive property of these compounds was the excellent activity in the 1a replicon assay, which tended to be in good agreement with activity in the biochemical assay. Replicon EC_{50} values tended to be no greater than 2- to 3-fold higher than the enzyme IC_{50} values, with many compounds giving equal or greater inhibition

Table 3. Activity Data for Analogues of Neohexyl-Substituted B-Ring Aminothiadiazine Inhibitors of HCV Polymerase



Compd	R	IC_{50} (nM)[a] / EC_{50} (nM)[b]	compd	R	IC_{50} (nM)[a] / EC_{50} (nM)[b]
26a		151 / ND	**26l**		24 / 21
26b		17 / 10	**26m**		33 / 60
26c		15 / 6	**26n**		27 / ND
26d		13 / 20	**26o**		12 / 10
26e		13 / 13	**26p**		10 / 15
26f		13 / 17	**26q**		15 / 13
26g		20 / 13	**26r**		14 / 11
26h		27 / 118	**26s**		5 / 15
26i		33 / 35	**26t**		9 / 29
26j		17 / 8	**26u**		23 / 16
26k		10 / 15	**26v**		19 / 13

[a] 1a enzyme activity. [b] 1a replicon activity; ND = not determined.

Table 4. Pharmacokinetic Properties in Rat for Neohexyl-Substituted B-Ring Aminothiadiazines

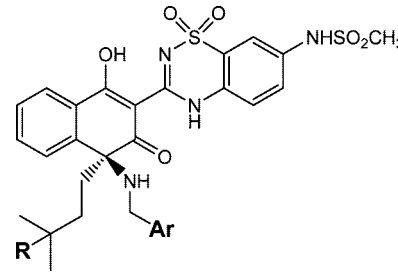
compd	$t_{1/2}$[a]	CL_p[b]	C_{max}[c]	AUC[d]	*F*, %[e]
26c	3.2	1.5	0.40	1.6	48.0
26j	2.2	1.2	0.08	0.41	8.9
26l	5.0	0.78	0.46	3.5	53.7
26q	1.0	1.7	0.15	1.34	33.8
26r	0.5	5.9	0.0	0.0	0.0
26u	2.2	1.4	0.18	1.02	28.1

[a] Half-life in hours following 5 mg/kg iv dose in rat. [b] Clearance (L/(h·kg)) following 5 mg/kg iv dose in rat. [c] C_{max} following 5 mg/kg oral dose in rat (μg/mL). [d] $AUC_{0-\infty}$ following 5 mg/kg oral dose in rat (μg·h/mL). [e] Percent bioavailable following 5 mg/kg oral dose in rat.

in replicon. Benzylamine analogues having a wide range of one or more substituents all resulted in similar activity in both biochemical and replicon assays. Potent assay inhibition was maintained regardless of the position of the substituent or the nature of the substituting group (electron withdrawing or donating, charged groups, etc.). Larger substituents, such as the benzyl ether group in **26h**, did result in a significant reduction in replicon activity. A variety of heteroaryl analogues, as well as bicyclic aryl and heteroaryl analogues, all gave similar activities in both assays.

Pharmacokinetic properties in rat for a subset of compounds from the neohexyl-substituted series of inhibitors are shown in Table 4. Dimethylbenzyl analogue **26l** had properties that were significantly better than other compounds tested, including a longer half-life, lower rate of clearance, and greater plasma exposure and bioavailability following oral dosing. On the basis

Table 5. Comparison of in Vitro and in Vivo Properties of Compounds **30** and **33**



compd	R	Ar
33	H	Ph
30	Me	2,6-di-Me-Ph

	polymerase IC_{50} (nM)[a]		replicon EC_{50} (nM)[b]		
compd	1a	1b	1a	1b	1b (HS)[c]
33	10 ± 4	16 ± 8	68 ± 31	15 ± 1	273
30	16 ± 7	19 ± 7	10 ± 6	6 ± 0	270

	$t_{1/2}$[d]	CL_p[e]	C_{max}[f]	AUC[g]	*F*, %[h]
33	1.7 ± 0.2	1.16 ± 0.09	0.08 ± 0.03	0.6 ± 0.4	13.3 ± 8.5
30	3.1 ± 0.6	0.70 ± 0.19	0.62 ± 0.28	4.21 ± 1.30	55.9 ± 17.2

	1 h[i]	6 h[i]	12 h[i]
33	3.59 ± 0.75 (12)	2.35 ± 0.22 (6)	0.88 ± 0.16 (3)
30	5.86 ± 0.41 (12)	4.36 ± 1.09 (15)	2.11 ± 0.24 (11)

[a] Inhibition of isolated enzyme activity. [b] Inhibition of subgenomic replication in Huh-7 cells. [c] Inhibition measured in the presence of 40% human serum. [d] Half-life in hours following 5 mg/kg iv dose in rat. [e] Clearance (L/(h·kg)) following 5 mg/kg iv dose in rat. [f] C_{max} following 5 mg/kg oral dose in rat (μg/mL). [g] $AUC_{0-\infty}$ following 5 mg/kg oral dose in rat (μg·h/mL). [h] Percent bioavailable following 5 mg/kg oral dose in rat. [i] Compound concentration in liver (μg/g) at time indicated after dosing; liver/plasma ratio in parentheses.

of the superior in vivo performance of this compound, dimethylbenzyl analogue **26l** was selected for chiral synthesis to evaluate the more active *S*-enantiomer.

Activity data against genotypes 1a and 1b polymerase in both enzyme inhibition and replicon assays for chiral neohexyl analogue **30** are compared with data for chiral isoamyl analogue **33** in Table 5. While both compounds had similar activity as inhibitors of genotypes 1a and 1b polymerase, neohexyl analogue **30** was found to be significantly more active than isoamyl analogue **33** in 1a and 1b replicon assays. However, when replicon activity was measured in the presence of 40% human serum, both compounds gave a similar EC_{50} value. This large serum-attenuating effect on replicon activity (17-fold and 47-fold for **33** and **30**, respectively) was typical for inhibitors in this series.

Pharmacokinetic studies in rat revealed that the in vivo performance of neohexyl analogue **30** was significantly improved relative to isoamyl analogue **33**. A comparison of iv and po data is shown in Table 5 and Figure 3. Following a 5 mg/kg iv dose, the plasma half-life increased to 3.1 h for **30**, versus 1.7 h for **33**. Furthermore, **30** was cleared from the plasma more slowly (0.7 L·h/kg) than **33** (1.16 L·h/kg). Plasma exposure of **30** following a 5 mg/kg oral dose was greatly enhanced relative to **33**, with a 7-fold increase in both maximum concentration (C_{max}) and overall exposure (AUC) for **30**. The oral bioavailability for **30** was 55.9%, a 4-fold increase relative to **33**.

Because hepatitis infection affects the liver, it is generally considered to be a desirable property that HCV drugs have organ

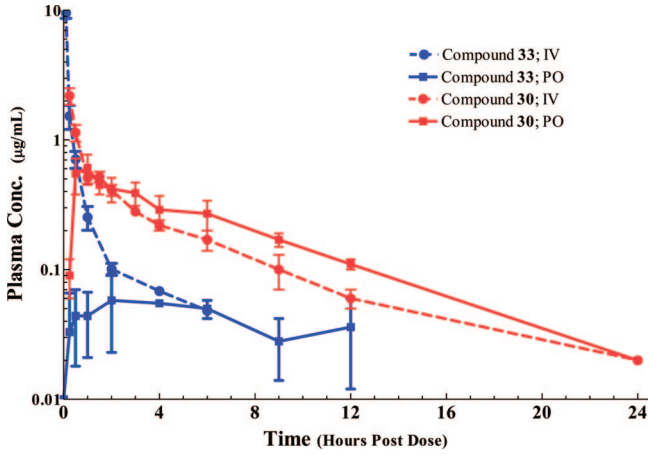


Figure 3. Plasma concentrations following administration of a 5 mg/kg iv or oral dose of **30** or **33** in rat.

distribution properties that favor exposure in the liver. In order to determine the extent to which these inhibitors are present in the target organ, we measured the concentration of **30** and **33** in liver following a 5 mg/kg oral dose in rat. Both compounds gave good liver concentrations at 1, 6, and 12 h after dosing, with neohexyl analogue **30** giving significantly enhanced levels relative to isoamyl analogue **33** at each time point. In the case of neohexyl analogue **30**, a liver/plasma ratio of >10 was maintained through 12 h after dosing. Furthermore, the liver concentration at 12 h (3.25 μM) for **30** exceeds the measured 1b replicon serum-adjusted EC_{50} (0.27 μM) by 12-fold, suggesting this compound may be suitable for convenient dosing regimens.

Conclusions

Our continued investigations into novel inhibitors of HCV NS5B polymerase of the benzothiadiazine class have provided B-ring amino analogues with promising characteristics as potential drug candidates for the treatment of HCV infection. As predicted on the basis of molecular modeling studies of B-ring dialkylthiadiazine inhibitors, substitution of this site was well tolerated, with groups ranging in size from amine to substituted benzylamine giving low-nanomolar inhibitors of genotypes 1a and 1b polymerase. Benzylamine analogues were found to be potent inhibitors of replication in subgenomic replicon assays such that the cell-culture activity for these compounds was within a few-fold of enzyme inhibition results. Neohexyl analogue **30** had good pharmacokinetic properties in rat following oral dosing, with an oral bioavailability of 56%. Given results previously described, in which the neohexyl side chain was found to block a primary site for oxidative metabolism in isoamyl analogues of B-ring dialkylthiadiazine HCV polymerase inhibitors, it is likely that the improvement in PK performance for **30** over isoamyl analogue **33** is the result of the neohexyl side chain affording a similar advantage for B-ring aminothiadiazine inhibitors. The tissue distribution properties of both **30** and **33** make them attractive as potential HCV therapeutics for patients infected with genotype 1 virus.

Experimental Section

General Methods. Reagents and solvents, including anhydrous solvents, were obtained from commercial sources and used as supplied. Column chromatography was carried out on silica gel. Chiral chromatography was carried out using a Chiralpak AD-H column (0.46 cm × 25 cm), Daicel Chemical Ind., Ltd. ^{1}H NMR spectra were measured using a Bruker AMX 300 (300 MHz) NMR spectrometer. Chemical shifts are reported in ppm (δ) and referenced to an internal standard of tetramethylsilane (δ 0.00 ppm). ^{1}H−^{1}H couplings are assumed to be first-order, and peak multiplicities are reported in the usual manner. MS analysis was conducted using a Finnigan SSQ7000 (ESI) mass spectrometer.

4-(3,3-Dimethylbutyl)-3,4-dihydroxynaphthalen-1(4*H*)-one (22). To a stirred mixture of Mg turnings (2.9 g, 119 mmol) in anhydrous THF (150 mL) at 0 °C was added neohexyl chloride (14.2 g, 118 mmol), followed by dropwise addition of 1,2-dibromoethane (2.5 mL, 29 mmol). The resulting mixture was stirred at room temperature under N_2 for 3 days, during which time a nearly homogeneous solution was obtained. The mixture was cooled to −78 °C, and titanium(IV) isopropoxide (33.9 mL, 115 mmol) was added dropwise. The resulting opaque yellow solution was stirred at −78 °C for 1 h, and then a solution of 2-hydroxy-1,4-naphthoquinone (5.0 g, 28.7 mmol) in anhydrous THF (80 mL), precooled to 0 °C, was added dropwise via canula over 20 min. Following the addition, the resulting mixture was allowed to slowly warm to room temperature and was stirred under N_2 overnight. The mixture was poured into 1 N aqueous HCl (500 mL) and extracted with ethyl acetate (2 × 300 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was subjected to column chromatography on Florisil, eluting with a gradient of 1−5% MeOH in CH_2Cl_2. The still impure product was purified by column chromatography on silica gel using 1:1 ethyl acetate/hexanes as eluent to give **22** as an orange-yellow solid (2.06 g, 28%): ^{1}H NMR (DMSO-d_6) δ 7.82 (d, J = 6.6 Hz, 1H), 7.61−7.69 (m, 2H), 7.57 (t, J = 7.0 Hz, 1H), 7.35−7.45 (m, 1H), 5.47 (s, 1H), 4.08 (s, 1H), 1.60−2.07 (m, 2H), 0.74−0.98 (m, 1H), 0.68 (s, 9H), 0.47 (m, 1H).

***N*-[3-[4-(3,3-Dimethylbutyl)-1,4-dihydroxy-3-oxo-3,4-dihydronaphthalen-2-yl]-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-7-yl}methanesulfonamide (23).** To a solution of **22** (5.75 g, 22.1 mmol) in anhydrous 1,4-dioxane (100 mL) was added tris(methylthio)methyl methylsulfate[16] (29.0 g, 110 mmol) and pyridine (8.7 mL, 110 mmol). The resulting mixture was stirred at 65 °C for 2 h and then concentrated in vacuo. The crude product was purified by column chromatography on silica gel using 1:1 ethyl acetate/hexanes to give a yellow oil (7.28 g, 90%). The oil was dissolved in anhydrous 1,4-dioxane (100 mL), and to the solution was added **12** (5.78 g, 21.8 mmol). The resulting mixture was stirred at 80 °C for 16 h and then cooled to room temperature to give **23** as a crystalline solid that was collected by filtration and dried (8.5 g, 77%): ^{1}H NMR (DMSO-d_6) δ 13.33 (br s, 1H), 10.16 (s, 1H), 8.03 (d, J = 7.7 Hz, 1H), 7.65−7.75 (m, 2H), 7.47−7.62 (m, 4H), 3.57 (s, 1H), 3.06 (s, 3H), 1.89−2.04 (m, 1H), 1.74−1.89 (m, 1H), 0.87−1.04 (m, 1H), 0.62−0.80 (m, 10H); MS (ESI) m/z 534 (M + H)$^+$.

***N*-(1-(3,3-Dimethylbutyl)- 4-hydroxy-3-{7-[(methylsulfonyl)-amino]-1,2-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl}-2-oxo-1,2-dihydronaphthalen-1-yl}acetamide (24).** To a vigorously stirred solution of **23** (5.7 g, 10.7 mmol) in CH_3CN (180 mL) at 0 °C was added concentrated H_2SO_4 (60 mL) dropwise over 20 min. The resulting mixture was allowed to warm to room temperature, stirred for 4 h, and then poured onto crushed ice (~400 mL). The mixture was allowed to warm to room temperature, and the product was collected by filtration to give **24** as an off-white solid (6.1 g, 99%): ^{1}H NMR (DMSO-d_6) δ 13.65 (br s, 1H), 10.26 (s, 1H), 9.15 (s, 1H), 8.12 (d, J = 7.0 Hz, 1H), 7.69−7.79 (m, 2H), 7.47−7.65 (m, 4H), 3.06 (s, 3H), 2.50 (s, 3H), 1.74−2.06 (m, 2H), 0.84−1.00 (m, 1H), 0.66 (s, 9H), 0.39−0.58 (m, 1H); MS (ESI) m/z 575 (M + H)$^+$.

***N*-{3-[4-Amino-1-hydroxy-4-(3,3-dimethylbutyl)-3-oxo-3,4-dihydronaphthalen-2-yl]-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-7-yl}methanesulfonamide Hydrochloride (25).** To a solution of **24** (6.1 g, 10.6 mmol) in 1,4-dioxane (60 mL) was added 4 N aqueous HCl (30 mL), and the resulting mixture was heated at 80 °C for 5 days. The cooled mixture was concentrated in vacuo to ~20 mL and was extracted with 3:1 CH_2Cl_2/2-PrOH (3 × 50 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo to give an oil that was suspended in ethyl

acetate (~100 mL) to give a solid that was filtered and dried to afford **25** as a light-yellow solid (6.0 g, 99%): ^{1}H NMR (DMSO-d_6) δ 14.44 (br s, 1H), 9.95 (s, 1H), 8.62 (br s, 2H), 8.13 (d, J = 7.7 Hz, 1H), 7.59−7.74 (m, 2H), 7.48−7.59 (m, 2H), 7.42−7.48 (m, 1H), 7.33−7.39 (m, 1H), 3.01 (s, 3H), 1.94−2.12 (m, 1H), 1.76−1.94 (m, 1H), 1.01−1.18 (m, 1H), 0.70−0.80 (m, 1H), 0.69 (s, 9H); MS (ESI) m/z 533 $(M + H)^+$.

***N*-(1-(3,3-Dimethylbutyl)-4-hydroxy-3-{7-[(methylsulfonyl)-amino]-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl}-2-oxo-1,2-dihydronaphthalen-1-yl)benzamide (26a).** To a vigorously stirred suspension of **23** (20 mg, 38 μmol) in PhCN (0.2 mL) at 0 °C was added concentrated H_2SO_4 (0.1 mL) dropwise. The resulting mixture was allowed to warm to room temperature and stirred for 3 h. The mixture was added to ice (~2 mL) and was allowed to warm to room temperature. The resulting mixture was extracted with Et_2O (3 × 2 mL), and the combined organic layers were concentrated to give a solid. The crude product was purified by crystallization from Et_2O/hexanes to give **26a** as a colorless solid (22 mg, 92%): ^{1}H NMR (DMSO-d_6) δ 13.67 (br s, 1H), 10.26 (s, 1H), 9.54 (s, 1H), 8.16 (d, J = 7.7 Hz, 1H), 7.40−7.94 (m, 11H), 3.08 (s, 3H), 2.02−2.28 (m, 2H), 0.91−1.07 (m, 1H), 0.68 (s, 9H), 0.42−0.55 (m, 1H); MS (ESI) m/z 637 $(M + H)^+$.

General Method for Reductive Alkylation of 25 To Give 26b−v.[25] ***N*-{3-[4-(Benzylamino)-4-(3,3-dimethylbutyl)-1-hydroxy-3-oxo-3,4-dihydronaphthalen-2-yl]-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-7-yl}methanesulfonamide (26b).** To a suspension of **25** (0.12 g, 0.21 mmol) in dichloroethane (2 mL) was added *N*,*N*-diisopropylethylamine (73 μL, 0.42 mmol). The mixture was stirred at room temperature until it was homogeneous (approximately 5 min), and then benzaldehyde (38 μL, 3.2 mmol) and $MgSO_4$ (25 mg) were added. The reaction mixture was heated at 50 °C for 18 h. The mixture was cooled to room temperature and filtered, and the filtrate was treated with sodium triacetoxyborohydride (72 mg, 0.34 mmol) and AcOH (36 μL, 0.63 mmol). The mixture was stirred at room temperature for 18 h, diluted with CH_2Cl_2 (2 mL), washed with H_2O (2 × 2 mL), and dried over Na_2SO_4. The drying agent was filtered off, and the filtrate was concentrated in vacuo to give a crude product that was purified by column chromatography on silica gel, eluting with 5% MeOH in CH_2Cl_2 to give **26b** as a light-yellow solid (84 mg, 60%): ^{1}H NMR (DMSO-d_6) δ 13.99 (s, 1H), 9.98 (s, 1H), 9.61 (s, 1H), 8.18−8.24 (m, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.68−7.77 (m, 1H), 7.62 (t, J = 7.5 Hz, 1H), 7.45−7.56 (m, 2H), 7.28−7.43 (m, 6H), 3.43 (d, J = 12.9 Hz, 2H), 3.02 (s, 3H), 2.20−2.36 (m, 1H), 1.90−2.05 (m, 1H), 0.98−1.14 (m, 1H), 0.67 (s, 9H), 0.39−0.55 (m, 1H); MS (ESI) m/z 623 $(M + H)^+$.

2-Oxo-4-(*R*)-phenyloxazolidin-3-carbonyl Chloride (27). To a solution of (*R*)-(−)-4-phenyloxazolidin-2-one (5.0 g, 30.6 mmol) in anhydrous THF (200 mL) at 0 °C under N_2 was slowly added a 1.6 M solution of *n*-butyllithium in hexane (19.0 mL, 30.4 mmol). The mixture was stirred at 0 °C for 30 min and then cooled to −78 °C. Trichloromethyl chloroformate (7.8 mL, 45.9 mmol) was added, and the resulting mixture was stirred at −78 °C for 30 min and then allowed to warm to room temperature. The mixture was concentrated in vacuo, and the resulting residue was taken up in CH_2Cl_2 (200 mL) and filtered. The filtrate was concentrated in vacuo to afford **27** as an off-white solid (4.5 g, 65%): ^{1}H NMR (DMSO-d_6) δ 7.29−7.45 (m, 5H), 4.89−4.99 (m, 1H), 4.68 (t, J = 8.5 Hz, 1H), 4.00 (dd, J = 8.5, 6.6 Hz, 1H).

2-Oxo-4-(*R*)-phenyloxazolidine-3-carboxylic Acid [1-(*S*)-(3,3-Dimethylbutyl)-4-hydroxy-3-(7-methanesulfonylamino-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)-2-oxo-1,2-dihydronaphthalen-1-yl]amide (28). To a solution of **25** (3.0 g, 5.3 mmol) in anhydrous CH_2Cl_2 (50 mL) was added triethylamine (2.2 mL, 15.8 mmol) followed by **27** (1.25 g, 5.5 mmol). The mixture was stirred at room temperature for 3 h and then diluted with CH_2Cl_2 (100 mL) and washed with 0.1 N aqueous HCl (3 × 50 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel using a gradient of 1−7% MeOH in CH_2Cl_2 as eluent. Compound **28** eluted as the least polar diastereomeric component and was obtained as a light-yellow solid (1.35 g): ^{1}H NMR (DMSO-d_6) δ 13.60 (br s, 1H), 10.20 (s, 1H), 8.77 (s, 1H), 8.09 (d, J = 7.0 Hz, 1H), 7.46−7.74 (m, 6H), 7.32−7.41 (m, 2H), 7.18−7.31 (m, 3H), 5.30 (dd, J = 8.3, 3.1 Hz, 1H), 4.78 (t, J = 8.5 Hz, 1H), 4.17 (dd, J = 8.6, 3.1 Hz, 1H), 3.07 (s, 3H), 1.90−2.01 (m, 2H), 0.98−1.13 (m, 1H), 0.81−0.95 (m, 1H), 0.71 (s, 9H); MS (ESI) m/z 722 $(M + H)^+$.

***N*-{3-[4-(*S*)-Amino-4-(3,3-dimethylbutyl)-1-hydroxy-3-oxo-3,4-dihydronaphthalen-2-yl]-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-7-yl}methanesulfonamide Trifluoroacetic Acid Salt (29).** To a solution of 2-(trimethylsilyl)ethanol (0.71 mL, 8.6 mmol) in anhydrous THF (5 mL) under N_2 at 0 °C was added a 1.6 M solution of *n*-butyllithium in hexane (5.4 mL, 8.6 mmol), dropwise over 10 min. Following the addition, the reaction mixture was stirred for an additional 30 min at 0 °C before a solution of **28** (0.62 g, 0.86 mmol) in anhydrous THF (5 mL) was added dropwise. Following the addition, the mixture was allowed to warm to room temperature and was stirred at room temperature for 16 h. The mixture was poured into ethyl acetate (50 mL) and washed with 0.1 N aqueous HCl (50 mL) and H_2O (50 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography on silica gel using 1:4 ethyl acetate/hexanes as the eluent to give the trimethylsilylethylcarbamate of **29** as a yellow solid (0.40 g, 69%). This solid was dissolved in CH_2Cl_2 (4 mL), and the solution was cooled to 0 °C before trifluoroacetic acid (2 mL) was added dropwise. The reaction mixture was stirred at 0 °C for 1 h and was then allowed to warm to room temperature and was stirred for 1 h. The solution was concentrated in vacuo to afford **29** as a yellow solid (0.38 g, quantitative): ^{1}H NMR (DMSO-d_6) δ 14.45 (s, 1H), 9.95 (s, 1H), 8.60 (br s, 3H), 8.13 (d, J = 7.4 Hz, 1H), 7.28−7.72 (m, 6H), 3.01 (s, 3H), 1.95−2.10 (m, 1H), 1.78−1.94 (m, 1H), 0.99−1.17 (m, 1H), 0.65−0.92 (m, 10H); MS (ESI) m/z 533 $(M + H)^+$; $[\alpha]_D$ −78° (c 0.5, MeOH).

***N*-{3-[4-(*S*)-[(2,6-Dimethylbenzyl)amino]-4-(3,3-dimethylbutyl)-1-hydroxy-3-oxo-3,4-dihydronaphthalen-2-yl]-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-7-yl}methanesulfonamide (30).** To a suspension of **29** (0.38 g, 0.59 mmol) in anhydrous dichloroethane (6 mL) was added *N*,*N*-diisopropylethylamine (0.22 mL, 1.23 mmol). The mixture was stirred at room temperature until it was homogeneous before 2,6-dimethylbenzaldehyde (0.17 g, 1.23 mmol) and $MgSO_4$ (0.15 g) were added. The reaction mixture was heated at 50 °C for 18 h, cooled to room temperature, and filtered. To the filtrate was added sodium triacetoxyborohydride (0.21 g, 0.99 mmol) and glacial acetic acid (0.11 mL, 1.86 mmol), and the resulting mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with CH_2Cl_2 (25 mL), washed with H_2O (2 × 10 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel using 3% MeOH in CH_2Cl_2 as the eluent to give **30** as a light-yellow solid (0.22 g, 58%): ^{1}H NMR (DMSO-d_6) δ 13.96 (s, 1H), 9.98 (s, 1H), 9.25 (br s, 1H), 8.22 (dd, J = 7.7, 1.5 Hz, 1H), 8.03 (d, J = 7.7 Hz, 1H), 7.70−7.79 (m, 1H), 7.64 (t, J = 7.4 Hz, 1H), 7.45−7.55 (m, 2H), 7.32−7.42 (m, 1H), 7.14−7.22 (m, 1H), 7.04−7.10 (d, J = 7.7 Hz, 2H), 3.64−3.79 (m, 1H), 3.38−3.43 (m, 1H), 3.02 (s, 3H), 2.31−2.43 (m, 1H), 2.27 (s, 6H), 1.99−2.12 (m, 1H), 0.94−1.12 (m, 1H), 0.68 (s, 9H), 0.32−0.48 (m, 1H); MS (ESI) m/z 651 $(M + H)^+$; $[\alpha]_D$ −8° (c 0.4, MeOH).

Assay Conditions for Determining HCV NS5B Polymerase Inhibition. Two-fold serial dilutions of the inhibitors were incubated with 20 mM Tris-Cl (pH 7.4), 2 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA), 60−125 μM GTP, and 20−50 nM NS5B [HCV genotype 1b (BK, Genbank accession number AF054247) or HCV genotype 1a (H77, Genbank accession number AF011751)] for 15 min at room temperature. The reaction was initiated by the addition of 20 μM CTP, 20 μM ATP, 1 μM ^{3}H-UTP (10 mCi/umol), 5 nM template RNA, and 0.1 U/μL RNase inhibitor (RNasin, Promega) and allowed to proceed for 2 to 4 h at room temperature. Reaction volume was 50 μL. The reaction was terminated by the addition of 1 volume of 4 mM spermine in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA. After incubation for at least

15 min at room temperature, the precipitated RNA was captured by filtering through a GF/B filter (Millipore) in a 96-well format. The filter plate was washed three times with 200 μL each of 2 mM spermine, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, and 2 times with EtOH. After air-drying, 30 μL of Microscint 20 scintillation cocktail (Packard) was added to each well, and the retained cpm values were determined by scintillation counting. IC_{50} values were calculated by a two-variable nonlinear regression equation using an uninhibited control and a fully inhibited control sample to determine the minimum and maximum for the curve.

Assay for Determining Inhibition of Replication of Subgenomic HCV Replicons. The inhibitory effects of compounds on HCV replicon replication were determined by measuring activity of the luciferase reporter gene. Stable replicon cell lines based on genotype 1a (H77) and genotype 1b (Con1) sequences were used. Briefly, replicon-containing cells were seeded into 96-well plates at a density of 5000 cells per well in 100 μL of DMEM containing 5% FBS. The following day compounds were diluted in DMSO to generate a 200× stock in a series of eight half-log dilutions. The dilution series was then further diluted 100-fold in the medium containing 5% FBS. Medium with the inhibitor was added to the overnight cell culture plates already containing 100 μL of DMEM with 5% FBS. In assays measuring inhibitory activity in the presence of human plasma, the medium from the overnight cell culture plates was replaced with DMEM containing 40% human plasma and 5% FBS. The cells were incubated for 3 days in the tissue culture incubators. For the luciferase assay, 30 μL of Passive Lysis buffer (Promega) was added to each well, and then the plates were incubated for 15 min with rocking to lyse the cells. Luciferin solution (100 μL, Promega) was added to each well, and luciferase activity was measured with a Victor II luminometer (Perkin-Elmer). The percent inhibition of HCV RNA replication was calculated for each compound concentration, and the EC_{50} value was calculated using GraphPad Prism 4 software.

Methods for Pharmacokinetic Evaluation of Compounds in Rat. Compounds were formulated in a DMSO/PEG400 (1:9, v/v) for single intravenous or oral dosing of 5 mg/mL in overnight fasted Sprague−Dawley rats ($n = 3$). Heparinized plasma samples were withdrawn at 0.1 (iv only), 0.25, 0.5, 1, 2, 4, 6, 9, 12, and 24 h after dosing. Liver and plasma samples from separate animals were collected at 1, 6, and 12 h after oral dose. Plasma and liver drug concentrations were determined by a liquid chromatography−mass spectrometry (LC−MS) assay. Mean plasma concentration data were submitted to multiexponential curve fitting using WinNonlin. The area under the mean concentration−time curve from 0 to t hours (time of the last measurable concentration) after dosing (AUC_{0-t}) was calculated using the linear trapezoidal rule for the concentration−time profile. The residual area was extrapolated to infinity, determined as the final measured mean concentration (C_t) divided by the terminal elimination rate constant (β), and was added to AUC_{0-t} to produce the total area under the curve ($AUC_{0-\infty}$).

Supporting Information Available: Experimental details for the synthesis of isoamyl analogues **14**, **16**, and **17a−g**, chiral isoamyl analogues **31−35**, and neohexyl analogues **26b−v**; HPLC data for all compounds presented in Tables 1−5, including chiral chromatography conditions and results for compounds **30** and **33**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(23) Optical rotation measurement confirmed that **29** was the (−)-enantiomer {$[\alpha]_D$ −72° (*c* 0.5, MeOH)}, which we expected to be the desired *S*-stereoisomer based on polarimetry studies of chiral isoamyl analogues. Details are available in the Supporting Information.
(24) Our initial investigations of B-ring amino thiadiazine inhibitors of HCV polymerase focused on a series of compounds bearing an isoamyl side chain. This initial series of analogues was made prior to the discovery that a neohexyl side chain on the B-ring offered advantages over an isoamyl B-ring side chain as the result of improved metabolic stability.
(25) Experimental details for the synthesis of **26b**−**v** can be found in the Supporting Information.

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