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2′-Deoxy-2′-spirocyclopropylcytidine Revisited: A New and Selective Inhibitor of the Hepatitis C Virus NS5B Polymerase

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The current therapy for hepatitis C virus (HCV) infection has limited efficacy, in particular against the genotype 1 virus, and a range of side effects. In this context of high unmet medical need, more efficacious drugs targeting HCV nonstructural proteins are of interest. Here we describe 2′-deoxy-2′-spirocyclopropylcytidine (**5**) as a new inhibitor of the HCV NS5B RNA-dependent RNA polymerase, displaying an EC_{50} of 7.3 μM measured in the Huh7-Rep cell line and no associated cytotoxicity (CC_{50} > 98.4 μM). Computational results indicated high similarity between **5** and related HCV inhibiting nucleosides. A convenient synthesis was devised, facilitating synthesis of multigram quantities of **5**. As the exposure measured after oral administration of **5** was found to be limited, the 3′-mono- and 3′,5′-diisobutyryl ester prodrugs **20** and **23**, respectively, were evaluated. The oral dosing of **23** led to substantially increased exposure to **5** in both rats and dogs.

Introduction

Over the past decade, the number of hepatitis C patients has steadily increased, and current estimates are that approximately 175 million people are infected with hepatitis C virus (HCV[a]) worldwide. The infection is thought to be the major cause of liver diseases including fibrosis, cirrhosis, and hepatocellular carcinoma.[1] There are at least six major HCV genotypes and multiple subtypes.[2]

The current standard of care (SOC) for the treatment of HCV infection consists of a combination of pegylated interferon-α and the broad-spectrum antiviral nucleoside analogue ribavirin. In patients infected with genotype 2 or 3, a sustained virological response (SVR) is observed in 80% of treated patients, while for genotype 1 patients only around 50% SVR is achieved.[3] Moreover, the side effects associated with current therapy are responsible for the relatively high percentage of treatment discontinuations observed during HCV treatment.[4] The anticipated introduction of HCV NS3/4A protease inhibitors (PIs) (with telepravir (VX-950)[5] and boceprevir (SCH 503034)[6] being the most advanced) is expected to result in a more successful SOC in the near future. However, several patient populations, e.g., partial or null responders, will still remain untreatable and the emergence of resistance mutations as a result of treatment with PIs remains a risk. Consequently, there is still a need for new and more effective treatment options for HCV.

The HCV genome consists of a 9.6 kb positive-sense, single-stranded RNA. It encodes at least 10 proteins, including both structural and nonstructural (NS) proteins, the latter being the replication enzymes.[7] The HCV NS5B polymerase is an RNA-dependent RNA polymerase that catalyzes the synthesis of positive (genomic) and negative (template) strand HCV RNA.[8,9] Like other polymerases, NS5B has the typical "right-hand" three-dimensional motif with a thumb and fingers domain surrounding the catalytic site in the base of the palm domain.[10] Six classes of NS5B inhibitors have been identified. Non-nucleoside inhibitors (NNI) are chemically diverse and bind at one of five allosteric binding sites (NNI-1, NNI-2, NNI-3, NNI-4, and NNI-5).[11] Following metabolism to their 5′-triphosphate derivatives by cellular kinases, nucleoside inhibitors (NI) compete with the binding of natural nucleotide 5′-triphosphates at the NS5B active site, which, after incorporation in the nascent nucleic acid, leads to a chain termination. Because of relatively strict conservation of the nucleoside binding site across HCV genotypes, NIs are generally expected to show high genotypic coverage.[12] In in vitro experiments NIs were found to have a higher genetic barrier to

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[a] Abbreviations: HCV, hepatitis C virus; SVR, sustained virological response; SOC, standard of care; NNI, non-nucleoside inhibitor; NI, nucleoside inhibitor; PI, protease inhibitor; rmsd, root-mean-square deviation; RDPR, ribonucleotide diphosphate reductase; NTP, 2′-α-hydroxy moiety of ribonucleoside 5′-triphosphate; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; TBAF, tetra-*n*-butylammonium fluoride; MMTr, monomethoxy tritylchloride; EC_{50}, 50% effective concentration in Huh-7-Luc replicon cells; CC_{50}, 50% cytotoxic concentration; C_{max}, mean maximum concentration; t_{max}, time to C_{max}; LC−MS, liquid chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; ESI, electrospray ionization; LRMS, low-resolution mass spectrometry; TIPDSCl, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane; THF, tetrahydrofuran; DIPEA, diisopropylethylamine; DIAZALD, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide; DMAP, 4-dimethylaminopyridine; CMV, cytomegalovirus.

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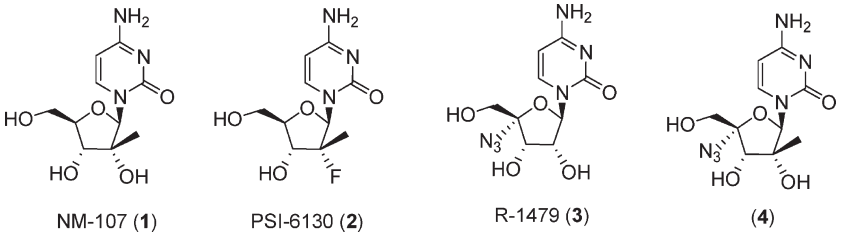
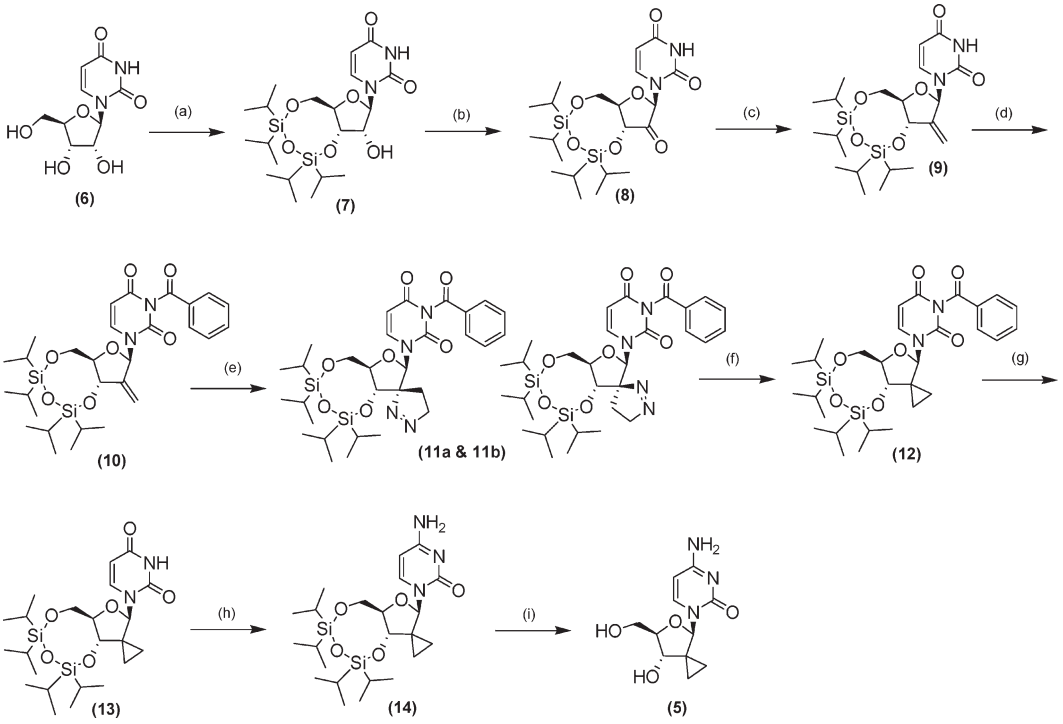


Figure 1. 2′- and 4′-modified nucleosides.

Scheme 1. Synthesis of 2′-Deoxy-2′-spirocyclopropylcytidine (**5**)[a]



[a] (a) TIPDSCl, pyridine, room temp, 12 h, 70%; (b) Dess−Martin periodinane, DCM, room temp, 12 h, 87%; (c) methyltriphenylphosphonium bromide, *s*-BuLi, THF, room temp, 12 h, 60%; (d) BzCl, Hünig's base, DCM, room temp, 12 h, 86%; (e) CH_2N_2, diethyl ether, room temp, 48 h, 84%; (f) hν, toluene/CH_3CN, benzophenone, room temp, 3 h, 84%; (g) NH_3, MeOH, room temp, 2 h, 40%; (h) (1) 2,4,6-triisopropylbenzenesulfonyl chloride, DMAP, Et_3N, CH_3CN, room temp, 12 h; (2) NH_3/H_2O, CH_3CN, room temp, 3 h, 93%; (i) TBAF, THF, then Dowex WX40 and recrystallization (MeOH), 62%.

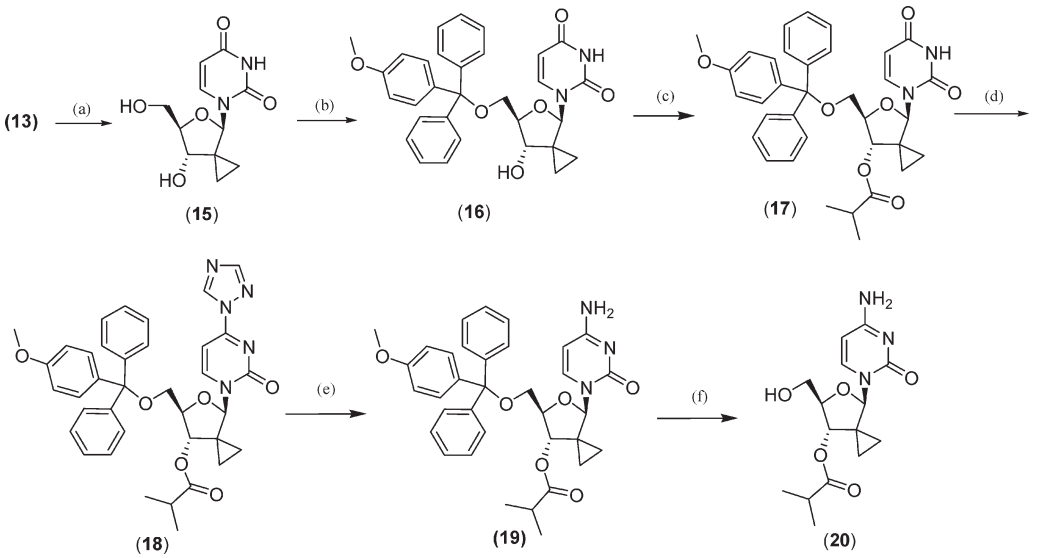
the development of resistance than NNIs and protease inhibitors.[13]

The structure of NS5B polymerase has been studied extensively,[14] although detailed understanding of the structural basis for nucleotide recognition has not yet been attained. Although RNA polymerases are assumed to interact with the 2′-α-hydroxy moiety of ribonucleoside 5′-triphosphates (NTPs), thereby discriminating among 2′-deoxyribonucleoside 5′-triphosphates (dNTPs),[15,16] the HCV NS5B polymerase promiscuously accommodates diversity in both the base and pentose moieties of the nucleotide, and chain-terminating 2′-deoxynucleotides that are incorporated into nascent RNA have been described.[17] As a result, numerous 2′-substituted nucleoside derivatives have been studied as potential HCV drugs.

To date, despite numerous approaches and the exploration of many novel scaffolds[18−21] including carbocyclic derivatives,[22] only two different classes of HCV-active nucleosides have progressed through preclinical evaluation to establish proof of concept in HCV-infected patients.[23−27] The first such class of HCV NIs encompasses 2′-modified ribonucleoside derivatives, with 2′-*β*-*C*-methyl analogues being particularly important. Among these derivatives, the combination of a 2′-*β*-methyl substituent with an α-fluorine or α-hydroxyl led to the most promising HCV NIs. Although larger groups have been reported (e.g., 2′-*β*-ethyl, 2′-α-OCH_3), these modifications resulted in less effective inhibitors.[28,20] The 4′-modified ribonucleoside derivatives are the second distinct structural class of HCV NIs. Various substituents at the 4′-position of the sugar are accommodated by NS5B polymerase. In the cytidine series, however, it has been shown that only the 4′-azido group yields an acceptable activity vs toxicity index.[29] From a viral resistance perspective, the 2′-modified compounds (NM-107 (**1**)[30] and PSI-6130 (**2**),[31] Figure 1) are susceptible to the S282T mutation of the enzyme while the 4′-modified analogue R-1479 (**3**)[32] is impacted by the S96T and M142T mutations. It is noteworthy that the combination of the 2′-methyl and 4′-azido substituents leads to the inactive hybrid **4**.[33]

As stated, several nucleoside derivatives that are inhibitors of the HCV NS5B RNA-dependent RNA polymerase have progressed into the clinical phase. However, the occurrence of

Scheme 2. Synthesis of the Monoester (**20**)[a]



[a] (a) TBAF, THF, room temp, 1 h, then Dowex WX40, 84%; (b) MMTrCl, pyridine, 0 °C to room temp, 12 h; (c) isobutyric anhydride, pyridine, 10 °C to room temp, 12 h; (d) $POCl_3$, 1*H*-1,2,4-triazole, triethylamine, DCM, room temp, 4 h, 74%; (e) NH_4OH, THF, room temp, 12 h; (f) AcOH (80%), room temp, 9 h, 62%.

severe unwanted side effects has resulted in the discontinuation of their development. NM-283, the 3′-valine ester prodrug of **1**, was halted because of severe GI tract toxicity and hematological disorders,[34,35] while R-1626, the 2,3,5-triisobutyryl ester prodrug of **3**, was stopped mainly because of excessive neutropenia observed in some patients.[36] Consequently, there is still a need for safe and well tolerated derivatives of this class of compounds.

Our laboratories are actively involved in several research programs aimed at identifying new HCV inhibitors. These efforts have led to the discovery of TMC435, a macrocyclic NS3/4A serine protease inhibitor currently in phase 2b clinical evaluation,[37,38] as well as two new classes of benzodiazepines found to be allosteric NS5B NNI-3 site inhibitors.[39,40] We now report our findings on 2′-deoxy-2′-spirocyclopropylcytidine (**5**), a new inhibitor of the HCV NS5B RNA-dependent RNA polymerase with a previously unreported 2′-substitution pattern.

Chemistry

While the 2′-deoxy-2′-spirocyclopropylcytidine **5** is novel to the HCV field, it was previously proposed as a potential inhibitor of ribonucleotide diphosphate reductase (RDPR), a key enzyme in the de novo synthesis of deoxynucleoside precursors for DNA synthesis.[41] No biological data on its RDPR inhibitory activity have been reported previously. We postulated that **5** could provide a useful analogue in the class of HCV-inhibiting nucleosides and considered it to be particularly attractive because the 2′-carbon of the 2′-spirocyclopropyl derivative is not stereogenic, unlike that of **1** and **2**. On the basis of earlier work reported by Samano and Robins,[42] a new and convenient procedure, enabling multigram-scale synthesis of **5**, was elaborated. As depicted in Scheme 1, the 3-benzoyl-1-{(6a′*R*,8′*R*,9a′*S*)-2′,2′,4′,4′-tetraisopropyltetrahydrospiro[cyclopropane-1,9′-furo[3,2-*f*][1,3,5,2,4]trioxadisilocine]-8′-yl}pyrimidine-2,4(1*H*,3*H*)-dione (**12**) is a key intermediate for the synthesis of **5**.

The reaction sequence starts with the simultaneous protection of the 3′- and 5′-OH groups of uridine **6**, capturing them as a cyclic disilylether. Dess−Martin oxidation of the 2′-OH group of **7** yielded ketone **8**. Introduction of the exocyclic double bond was accomplished through Wittig olefination using methyltriphenylphosphonium bromide to give **9** in 60% yield. To prevent methylation of the N^3-atom of the pyrimidine, **9** was benzoylated to yield intermediate **10**. The creation of the spirocyclopropyl fragment was subsequently achieved through a 1,3-dipolar cycloaddition reaction of diazomethane on the vinylic precursor **10**. The mixture of spiropyrazolines **11a** and **11b** thus obtained was subjected to a photochemically induced nitrogen extrusion reaction to provide **12** in 84% isolated yield. Notably, attempts to synthesize the cyclopropyl moiety via Simmons−Smith carbene-mediated approaches were either unsuccessful or inferior to the diazomethane-photochemistry sequence. Similar failure for structurally related 2-deoxy-2-methylenearabinopyranose has been reported, attributing the ineffectiveness of this approach to the highly electron deficient nature of the double bond.[43] Additionally, the exocyclic double bond in **10** might be sterically hindered, thus hampering the reaction with a bulky carbene reagent. With the fully protected spirocyclopropyl intermediate **12** in hand, the synthesis of **5** was completed by debenzoylation, yielding **13**, followed by conversion of the uracil moiety to the corresponding cytidine derivative. For the latter reaction, a two-step procedure (conversion of **13** into its 1-(4-dimethylamino)-pyridinium derivative, followed by ammonolysis)[44,45] was used, yielding intermediate **14**, which was desilylated by tetra-*n*-butylammonium fluoride (TBAF) to provide the target nucleoside (**5**), obtained in an analytically pure form after treatment with a Dowex cation-exchange resin[46] followed by recrystallization from methanol.

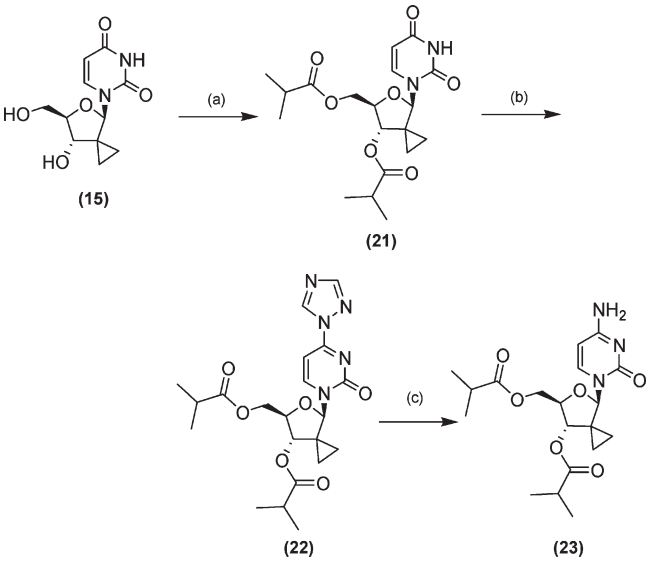
It is well-documented that the oral administration of nucleosides in preclinical studies often results in low overall exposure.[47] Nucleosides are highly polar, resulting in low intestinal permeability, and unless their uptake is mediated through nucleoside-specific active transport systems, low oral bioavailability is often encountered.[48] In anticipation of such difficulties, the 3′-mono and 3′-5′-diisobutyryl derivatives of **5** were synthesized and their pharmacokinetic properties were compared to those of the parent nucleoside. Approaches using

mono- and diisobutyryl derivatives to achieve higher exposure following oral administration have been reported for related nucleosides.[23,24,49,50] Schemes 2 and 3 illustrate the synthesis of these ester prodrugs.

The synthesis of the monoisobutyryl ester of **5** is shown in Scheme 2. Intermediate **13** was desilylated using TBAF, giving 2′-deoxy-2′-spirocyclopropyluridine **15**. Selective protection of the primary 5′-OH group was achieved by reaction with monomethoxy tritylchloride (MMTr), giving **16** in 84% yield. Subsequent esterification with isobutyric anhydride led to **17**, which was converted into the cytidine analogue **19** by successive treatment with phosphorus oxychloride and 1*H*-1,2,4-triazole, followed by ammonolysis of intermediate **18**. Finally, acidic removal of the MMTr protecting group afforded monoester **20**.

As shown in Scheme 3, the synthesis of the diisobutyryl ester of **5** started from compound **15**, which was first esterified with isobutyric anhydride followed by uracil-to-cytidine conversion, similar to the sequence described for intermediate **19**.

Scheme 3. Synthesis of the Diester (**23**)[a]



[a] (a) Isobutyric anhydride, pyridine, room temp, 12 h, 96%; (b) $POCl_3$, 1*H*-1,2,4-triazole, triethylamine DCM, room temp, 2.5 h, 86%; (c) NH_4-OH, THF, 3.5 h, 84%.

Table 1. Inhibition of HCV Replication in Huh-7-Rep Cells (EC_{50}, Luciferase Assay) for Reference Nucleosides **1**−**3** and Cyclopropyl Analogues **5**, **20**, and **23**, and Cytotoxicity (CC_{50}) Measured in Huh7-CMV-Luc and MT4-LTR-Luc Cells

		CC_{50} (μM)[a]	
compd	EC_{50} (μM)[a]	Huh7-CMV-Luc	MT4-LTR-Luc
1	4.6	>98.4	>98.4
2	1.9	>98.4	>98.4
3	4.3	>98.4	>98.4
5	7.3	>98.4	>98.4
20	42.8	>98.4	>98.4
23	20.3	>98.4	>98.4

[a] Average values of at least four repeats.

Biological and Pharmacokinetic Results

Antiviral Activity. The parent nucleoside **5** was tested on a Huh7 replicon cell line containing the subgenomic bicistronic replicon clone ET with a luciferase readout[51] to evaluate its HCV-inhibitory properties. Gratifyingly, **5** showed unambiguous inhibition of HCV replication in a range comparable to that of **1**, **2**, and **3**, with no apparent cellular toxicity (Table 1). In addition, ester prodrugs **20** and **23** showed clear, albeit lesser, inhibitory properties. These results indicate that both prodrugs were hydrolyzed in the cellular medium during the course of the assay, ultimately releasing the parent nucleoside **5**.

Pharmacokinetic Profile. Plasma kinetics and liver-plasma distribution after a single oral administration of **5**, **20**, and **23** using 20% hydroxypropyl-β-cyclodextrin as a vehicle were determined in male Sprague−Dawley rats (Figure 2, Table 2). Although only low plasma concentrations of **5** were achieved after administration of the parent compound **5** or its monoester **20**, they were quantifiable up to 7 h postadministration. For the diester **23**, high mean maximum concentrations (C_{max}) of **5** in the plasma were achieved at 0.7−0.8 h postdose (t_{max}), indicating rapid absorption and hydrolysis of **23** to **5**. At lower dose, the oral exposure of **5** increased approximately 10-fold after administration of **20** and more than 24-fold after administration of **23**. A dose-proportional increase in AUC of **5** was observed upon dose escalation from 15 to 90 mg/kg of **23** (AUC of 7.22 and 40 μg·h/mL after administration of 15 and 90 mg/kg, respectively). Compound **20** was undetectable in plasma after administration.

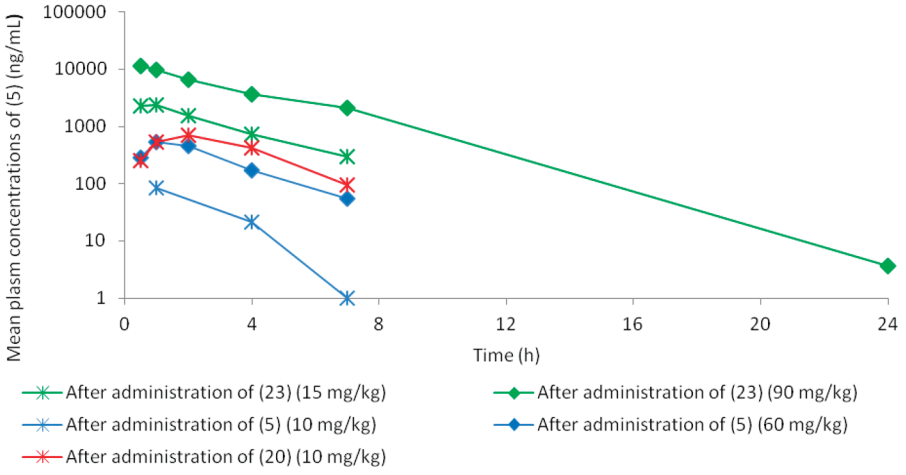


Figure 2. Mean plasma concentrations of **5** after single oral administration of parent **5**, its monoisopropyl prodrug **20**, or its diisopropyl prodrug **23** to Sprague−Dawley rats.

Following administration of **23**, low concentrations of **20** were detected in plasma (around 0.2% of the plasma concentrations of **5**) and **23** was not detected. The liver-to-plasma AUC_{0-7h} ratio of **5** was found to be approximately 1 for all of the tested compounds.

The diisopropyl ester **23** was evaluated in dogs (Figure 3, Table 3). Plasma concentrations were detectable up to 48 h postadministration. Exposure increased proportionally for the two doses tested (15 and 75 mg/kg). The diester was undetectable in plasma at the low dose, while at high dose diester plasma concentrations below 13 ng/mL were measured at the earlier time points. The corresponding monoester **20** exposure represented less than 0.4% of the parent **5** exposure after administration of either dose.

Computational Modeling

After demonstrating that **5** selectively inhibited HCV NS5B RNA-dependent RNA polymerase, we decided to study its properties computationally. The 2′-modified ribose derivatives **1** and **2** are sterically similar, but the 2′-hydroxyl group of **1** is hydrophilic and can act as a hydrogen bond donor and/or acceptor while the 2′-fluorine of **2** is hydrophobic and can only act as a weak hydrogen bond acceptor. Compound **5** differs from **1** and **2** at the 2′-position, with its bulkier and completely hydrophobic cyclopropyl substituent. The lowest energy conformation for each of the nucleosides **1**, **2**, and **5** was calculated with the modeling package MOE (version 2009.10, Chemical Computing Group, Montreal, Canada) using MMFF94x (unpublished but see Halgren[52] and references therein) and a Born solvation model.[53] Compounds **1** and **5** share a slight energetic preference for the 3′-endo (Northern) conformation (less than 1 kcal mol^{-1}), consistent with both conformational states being significantly populated in solution. Under these conditions **2** shows a 4 kcal mol^{-1} preference for the 3′-endo conformation. Figure 4 shows the superposition of the calculated low energy conformers of **1**, **2**, and **5**, underscoring the overall similarity of the three analogues and also highlighting the difference at the 2′-position.

Superposition of the non-hydrogen atoms common to the three nucleosides (i.e., omitting the 2′-substituents) yields differences less than 0.1 Å rmsd for both the **1**−**5** and **2**−**5** pairs. To further quantify the conformational match discussed above and shown in Figure 4, a three-dimensional shape comparison between the low energy conformations of **5** and those of **1** and **2** was performed with the Tanimoto shape algorithm ROCS.[54,55] ROCS compares molecules on the basis of shape Tanimoto score, a quantitative measure of three-dimensional overlap where a value of 1 means complete overlap (i.e., identical shape) and a fraction indicates partial overlap.[54−56] The shape similarity between the cyclopropyl analogue **5** and the reference compounds **1**

Table 2. Mean Noncompartmental PK Parameters of **5** after Single Oral Administration of the Parent **5**, its Monoisobutyryl Prodrug **20**, and its Diisobutyryl Prodrug **23** to Sprague−Dawley Rats

	compound administered				
	5	**20**	**23**	**5**	**23**
dose (μmol/kg)	36	28.8	36	216	216
doses (mg/kg)	10	10	15	60	90
C_{max} (μg/mL)	0.085	0.184	2.39	0.527	11.4
t_{max} (h)	1	0.7	0.8	1.3	0.7
AUC_{0-7h} (μg·h/mL)	0.248	2.62	7.22	1.64	40.0[a]

[a] AUC_{0-24h}.

Table 3. Mean Noncompartmental PK Parameters after Single Oral Administration of the Diisopropyl Prodrug **23** to Beagle Dogs

parameter		
dose (μmol/kg)	36	180
dose (mg/kg)	15	75
C_{max} (μg/mL)	6.3	42.8
t_{max} (h)	1.5	1.3
AUC_{0-24h} (μg·h/mL)	37	204

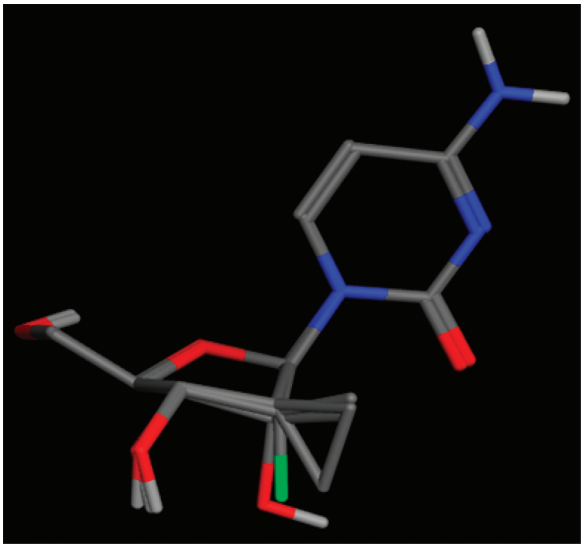


Figure 4. Superimposed low energy conformations of nucleosides **1**, **2**, and **5**. For clarity, only polar hydrogens are shown. The 3′-hydroxyl of **5** was manually adjusted to optimize the overlay of this group over the three nucleosides.

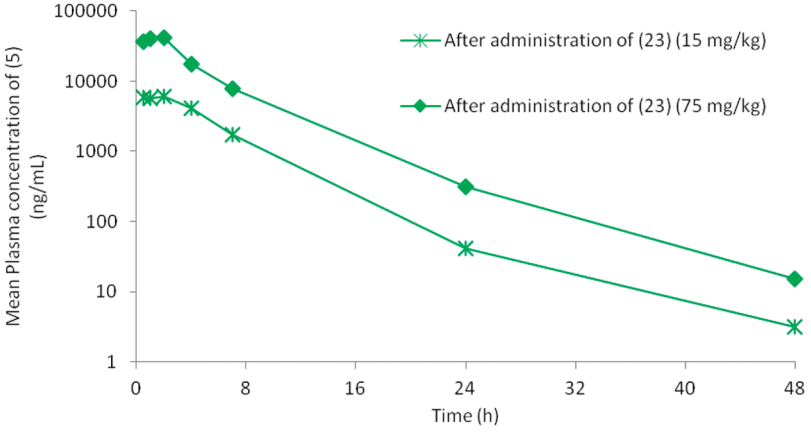


Figure 3. Mean plasma concentrations of parent **5** after single oral administration of its diisopropyl prodrug **23** to Beagle dogs.

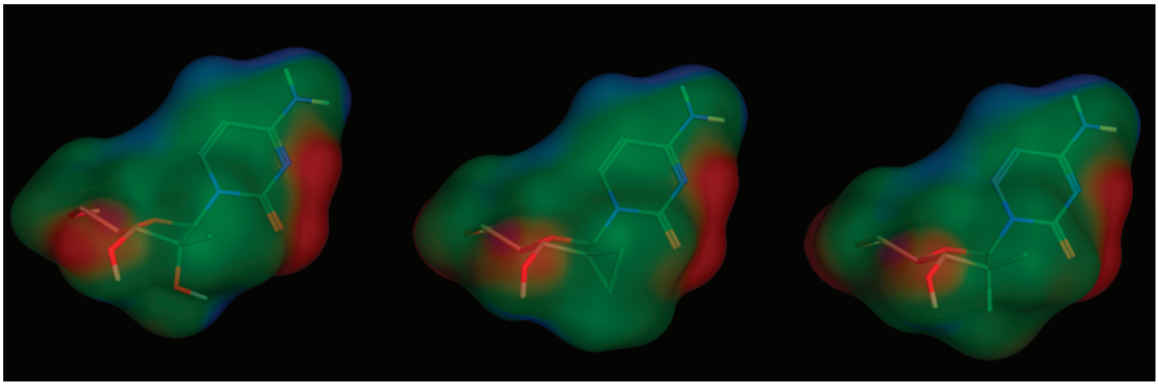


Figure 5. Electron density of 2′-substituted ribonucleosides **1** (left), **5** (middle), and **2** (right). Electrostatic potentials are mapped onto Connolly surfaces. Red represents negative values, while blue represents positive values. Values close to zero are green.

and **2** was extremely high; for both comparisons a Tanimoto similarity score of 0.98, indicating 98% overlap, was calculated.

Having established the structural similarity of **1**, **2**, and **5**, electronic properties of the three nucleosides were then compared. Electrostatic potentials calculated for the aligned lowest energy conformations were mapped onto Connolly surfaces[57] (calculations with MOE; Figure 5). The overall electrostatic potential surfaces of the three scaffolds were found to be strikingly similar (Figure 5). To quantify electrostatic similarity, the aligned conformation of **5** was compared to those of **1** and **2** with the Openeye package EON. Generally, Coulombic electrostatic Tanimoto indexes range from 1 (identical) to negative values resulting from the overlap of positive and negative charges.[56] In this case, the Coulombic electrostatic Tanimoto indices were 0.91 between **5** and **1** and 0.92 between **5** and **2**, indicating high overall electrostatic similarity. The only region of the surfaces showing visible difference is the 2′-position, consistent with the observation that the 2′-position can accommodate some variation.[28]

Our computational analyses confirmed the high overall similarity of **1**, **2**, and **5**, consistent with the 2′-spirocyclopropyl substituent being a useful surrogate in this series of anti-HCV nucleosides. The significance of the slight-to-moderate energetic preference for the 3′-endo (Northern) conformation in these molecules is unclear. Since both the Northern and Southern conformers likely exist in solution, both may be available in the therapeutically relevant biological setting. Furthermore, additional factors including nucleoside uptake, polymerase affinity, efficiency of phosphorylation (to the therapeutically relevant nucleotide; manuscript in preparation), and susceptibility to deamination may contribute to the observed antiviral activities of nucleosides. A cogent discussion of the interplay between various factors that contribute to nucleoside antiviral activity has been presented by Eldrup.[58]

Conclusions

2′-Deoxy-2′-spirocyclopropylcytidine **5** was discovered as a new member of the class of 2′-modified nucleoside derivatives that have HCV inhibiting properties. Compound **5** is a novel, potent (EC_{50} = 7.3 μM), and selective (CC_{50} > 98.4 μM) inhibitor of the HCV NS5B RNA-dependent RNA polymerase, a clinically validated target for HCV treatment. A convenient synthesis of **5** was developed in which the 2′-spirocyclopropyl fragment was constructed via a dipolar cycloaddition/photochemistry sequence. Pharmacokinetic evaluation of **5** as well as its prodrugs **20** and **23** revealed an increased exposure to **5** after administration of the prodrug derivatives, especially so for the diester prodrug **23**. A dose-proportional increase in exposure to **5** was seen in rats and dogs after administration of the diisobutyryl ester derivative **23**. Molecular modeling studies indicated close structural similarity between **1**, **2**, and **5** and supported the potential of the 2′-spirocyclopropyl moiety as a surrogate for the 2′-methyl group. The further biological, pharmacological, and pharmacokinetic evaluation of 2′-deoxy-2′-spirocyclopropylcytidine **5** and its related diester prodrug **23** is ongoing, results of which will be reported elsewhere.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz for ^{1}H. Chemical shifts are given in ppm and J values in Hz. Multiplicity is indicated using the following abbreviations: d for doublet, t for a triplet, m for a multiplet, etc. Low-resolution mass spectra were measured on a Waters Alliance 2695 high-performance liquid chromatography (HPLC) coupled to a single quadrupole (Waters ZQ) or a time-of-flight (Waters LCT) mass spectrometer using electrospray ionization (ESI) in positive or negative mode.

The purity of all tested compounds was confirmed to be ≥95% using two of four independent chromatographic systems. In method 1, an Atlantis T3 column (3.0 μm, 4.6 mm × 50 mm) and mobile phases A (10 mM NH_4OOCH_3 + 0.1% HCOOH in H_2O) and B (CH_3OH) were used. Analyses were run at 50 °C using a flow rate of 2 mL/min applying the following gradient: 0 min, 0% B; 4.2 min, 95% B; 5.4 min, 0% B. Method 2 was performed on a Vision HT C18P column (1.5 μm, 2.1 mm × 20 mm) using mobile phases A (0.1% HCOOH in H_2O/CH_3CN 95:5) and B (CH_3CN). Analyses were run at 50 °C using a flow rate of 0.6 mL/min applying the following gradient: 0 min, 0% B; 1.5 min, 100% B; 2.1 min, 100%; 2.7 min, 0% B. For Method 3, a SunFire C18 column (3.5 μm, 4.6 mm × 100 mm) and mobile phases A (10 mM NH_4OOCH_3 + 0.1% HCOOH in H_2O) and B (CH_3OH) were employed. Analyses were run at 50 °C using a flow rate of 1.5 mL/min applying the following gradient: 0 min, 35% B; 7.0 min, 95% B; 9.6 min, 95% B; 9.8 min, 35% B; 12 min, 35% B. The fourth system (method 4) used an XS Strategy column (1.7 μm, 2.1 mm × 20 mm) and mobile phases A (0.1% HCOOH in H_2O/CH_3CN 95/5) and B (CH_3CN). Analyses were run at 50 °C using a flow rate of 0.6 mL/min applying the following gradient: 0 min, 0% B; 1.5 min, 100% B; 2.1 min, 100%; 2.7 min, 0% B. Eluted peaks were detected at a single wavelength (273 nm).

Thin-layer chromatography (TLC) was performed on 5 cm × 10 cm aluminum sheets coated with silica gel 60 F_{254} (Merck KGaA). Preparative HPLC was done on a Synergi 250 mm × 77 mm × 10 μm column, using 95% MeCN in water (0.2% FA) as the mobile phase. The flow rate was 150 mL/min. Detection was done using a Gillson 215 detector operating at 220 nm. All reagents were purchased from commercial sources and were used as received.

Preparation of 1-((6a*R*,8*R*,9*R*,9a*S*)-9-Hydroxy-2,2,4,4-tetraisopropyl-6a,8,9,9a-tetrahydro-6*H*-furo[3,2-*f*][1,3,5,2,4]trioxadisilocin-8-yl)pyrimidine-2,4(1*H*,3*H*)-dione (7). To a solution of uridine **6**

(1.0 equiv, 100 g) in pyridine (1000 mL) was added dropwise 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPDSCl; 1.02 equiv, 131.6 g) over 20 min. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated by distillation, and the residue was dissolved in CH_2Cl_2 (500 mL). The CH_2Cl_2 solution was washed with water (3 × 400 mL), dried over $MgSO_4$, and concentrated by rotavapor. The crude product was purified by column chromatography on silica gel using petroleum ether/ethyl acetate 4:1 to give intermediate **7** as a white foam (139 g, 70% yield). Liquid chromatography−mass spectrometry (LC−MS): t_R = 3.23 min, m/z = 487 $(M + H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.78−1.23 (m, 28 H), 3.30 (br s, 1 H), 4.01 (dd, J = 13.20, 2.05 Hz, 1 H), 4.12 (d, J = 8.61 Hz, 1 H), 4.15−4.25 (m, 2 H), 4.36 (dd, J = 8.61, 4.89 Hz, 1 H), 5.70 (d, J = 8.22 Hz, 1 H), 5.74 (s, 1 H), 7.71 (d, J = 7.82 Hz, 1 H), 9.09 (br s, 1 H).

Preparation of 1-((6a*R*,8*R*,9a*R*)-2,2,4,4-Tetraisopropyl-9-oxotetrahydro-6*H*-furo[3,2-*f*][1,3,5,2,4]trioxadisilocin-8-yl)pyrimidine-2,4(1*H*,3*H*)-dione (8). To a solution of **7** (1.0 equiv, 159 g) in dry CH_2Cl_2 (1600 mL) at 0 °C was added Dess−Martin periodinane (1.2 equiv, 162 g). The reaction mixture was stirred at 25−30 °C overnight. Then 2000 mL of diethyl ether was added to the reaction mixture and the precipitates were filtered off over diatomite. The resulting filtrate was washed with 3000 mL of saturated $NaHCO_3$ solution containing 185 g of $Na_2S_2O_3$. The organic layer was collected and washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give intermediate **8** as a white foam (138 g, 87% yield) which was used as such in the next step. LC−MS: t_R = 3.24−3.45 min, m/z = 485 $(M + H)^+$ (hydrate of the ketone is observed under the LC−MS conditions). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.70−1.30 (m, 28 H), 3.90−4.02 (m, 1 H), 4.05 (br s, 2 H), 4.99 (d, J = 9.04 Hz, 1H), 5.49 (s, 1H), 5.70 (d, J = 7.90 Hz, 1 H), 7.77 (d, J = 7.96 Hz, 1 H), 11.60 (br s, 1 H).

Preparation of 1-((6a*R*,8*R*,9a*S*)-2,2,4,4-Tetraisopropyl-9-methylenetetrahydro-6*H*-furo[3,2-*f*][1,3,5,2,4]trioxadisilocin-8-yl)pyrimidine-2,4(1*H*,3*H*)-dione (9). Under N_2 atmosphere, *sec*-butyllithium in hexane (293 mL, 1.4 N, 2.0 equiv, 0.41 mol) was added dropwise to a solution of triphenylmethylphosphonium bromide (2.5 equiv, 183 g) in dry THF (1000 mL) cooled at −78 °C. Then a solution of **8** (1.0 equiv, 100 g, 0.2 mol) in dry THF (300 mL) was added to the reaction mixture under N_2. The cooling bath was removed, and the reaction mixture was stirred for 16 h at room temperature. THF was evaporated by rotavapor, and CH_2Cl_2 (500 mL) was added to dissolve the residue. The CH_2Cl_2 solution was washed with water (3 × 300 mL), dried over $MgSO_4$, and concentrated by rotavapor. The crude product was purified by column chromatography on silica gel using petroleum ether/ethyl acetate 8:1 to 5:1 to give intermediate **9** as a white foam (60 g, 60% yield). LC−MS: t_R = 3.55 min, m/z = 483 $(M + H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.02−1.13 (m, 28 H), 3.69 (ddd, J = 8.8, 2.6, 2.0 Hz, 1 H), 4.05 (dd, J = 13.3, 2.6 Hz, 1 H), 4.14 (dd, J = 13.3, 2.0 Hz, 1 H), 4.82 (dd, J = 8.8, 1.2 Hz, 1 H), 5.46 (t, J = 1.7 Hz, 1 H), 5.53−5.55 (m, 1 H), 5.70 (dd, J = 8.1, 2.1 Hz, 1 H), 6.51 (d, J = 0.8 Hz, 1 H), 7.44 (d, J = 8.1 Hz, 1 H), 8.69 (br s, 1 H).

Preparation of 3-Benzoyl-1-((6a*R*,8*R*,9a*S*)-2,2,4,4-tetraisopropyl-9-methylenetetrahydro-6*H*-furo[3,2-*f*][1,3,5,2,4]trioxadisilocin-8-yl)pyrimidine-2,4(1*H*,3*H*)-dione (10). To a solution of **9** (1.0 equiv, 190 g) in CH_2Cl_2 (2000 mL) was added *N*,*N*-diisopropylethylamine (DIPEA; 1.02 equiv, 61 g). Then benzoyl chloride (1.1 equiv, 60 g) was added dropwise and the reaction mixture was stirred at room temperature overnight. The reaction mixture was washed with a saturated $NaHCO_3$ solution (3 × 500 mL), dried with $MgSO_4$, and concentrated by rotavapor. The crude product was purified by column chromatography on silica gel using petroleum ether/ethyl acetate 8:1 to give intermediate **10** as a white foam (200 g, 86% yield). LC−MS: t_R = 3.83 min, m/z = 587 $(M + H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.04−1.13 (m, 28 H), 3.71 (ddd, J = 8.9, 2.7, 1.8 Hz, 1 H), 4.07 (dd, J = 13.3, 2.7 Hz, 1 H), 4.18 (dd, J = 13.3, 1.8 Hz, 1 H), 4.85 (dd, J = 8.9, 1.3 Hz, 1 H), 5.47 (t, J = 2.0 Hz, 1 H), 5.57−5.58 (m, 1 H), 5.82 (d, J = 8.2 Hz, 1 H), 6.51 (d, J = 1.0 Hz, 1 H), 7.51 (t, J = 7.6 Hz, 2 H), 7.60 (d, J = 8.2 Hz, 1 H), 7.66 (t, J = 7.6 Hz, 1 H), 7.94 (d, J = 7.6 Hz, 2 H).

Preparation of 3-Benzoyl-1-((3′*R*,6a*R*,8*R*,9a*S*)-2,2,4,4-tetraisopropyl-4′,5′,6,6a,8,9a-hexahydrospiro[furo[3,2-*f*][1,3,5,2,4]trioxadisilocine-9,3′-pyrazole]-8-yl)pyrimidine-2,4(1*H*,3*H*)-dione (11a) and Its Epimer 3-Benzoyl-1-((3′*S*,6a*R*,8*R*,9a*S*)-2,2,4,4-tetraisopropyl-4′,5′,6,6a,8,9a-hexahydrospiro[furo[3,2-*f*][1,3,5,2,4]trioxadisilocine-9,3′-pyrazole]-8-yl)pyrimidine-2,4(1*H*,3*H*)-dione (11b). 2-(2-Ethoxyethoxy)ethanol (200 mL) and diethyl ether (80 mL) were added to a flask containing a solution of KOH (90 g, 30.6 equiv) in water (100 mL). The flask was heated to 60 °C, and a solution of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (DIAZALD; 152 g 13.7 equiv) in diethyl ether (500 mL) was added dropwise in about 2 h. During this period, the generated diazomethane was condensed with diethyl ether and dropped into another flask which was cooled by dry ice. The diazomethane/diethyl ether solution was then added to a solution of **10** (30 g) in diethyl ether (50 mL) cooled on an ice bath. The reaction mixture was stirred at room temperature for 48 h, and the reaction was checked by LC−MS and TLC until completion. Acetic acid was added dropwise until the yellow color disappeared. The reaction mixture was evaporated on a rotavapor. The residue was dissolved in CH_2Cl_2 and evaporated to dryness. The crude product was purified by column chromatography on silica gel using petroleum ether/ethyl acetate 5:1 to 2:1 to give a mixture of intermediates **11a** and **11b** as a white foam (27 g, 85% yield). LC−MS: t_R = 3.67 min, m/z = 629 $(M - H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.82−1.19 (m, 28 H), 1.57−1.64 (m, 2 H), 3.82−5.06 (m, 6 H), 5.84−5.94 (m, 2 H), 7.47−7.53 (m, 2 H), 7.60−7.69 (m, 1 H), 7.88−7.94 (m, 2 H), 8.05−8.15 (m, 1 H).

Preparation of 3-Benzoyl-1-((6a′*R*,8′*R*,9a′*S*)-2′,2′,4′,4′-tetraisopropylhexahydrospiro[cyclopropane-1,9′-furo[3,2-*f*][1,3,5,2,4]trioxadisilocine]-8′-yl)pyrimidine-2,4(1*H*,3*H*)-dione (12). The mixture of intermediates **11a** and **11b** (8 g, 1.0 equiv) was dissolved in dry toluene (400 mL) and acetonitrile (400 mL), and benzophenone (0.23 g, 0.1 equiv) was added. The mixture was stirred and illuminated by a medium pressure mercury lamp for 3 h until **11a** and **11b** were consumed as monitored by TLC and LC−MS. The solvent was removed by rotavapor, and the residue (intermediate **12**) was used in the next step without further purification. LC−MS: t_R = 3.90 min, m/z = 601 $(M + H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.57−0.64 (m, 1 H), 0.66−0.73 (m, 1 H), 0.86−0.94 (m, 1 H), 0.98−1.14 (m, 28 H), 1.16−1.22 (m, 1 H), 3.86 (dd, J = 8.7, 2.3 Hz, 1 H), 4.07 (dd, J = 13.5, 2.3 Hz, 1 H), 4.21 (d, J = 13.5 Hz, 1 H), 4.51 (d, J = 8.7 Hz, 1 H), 5.83 (d, J = 8.2 Hz, 1 H), 5.86 (s, 1 H), 7.50 (t, J = 7.5 Hz, 2 H), 7.65 (t, J = 7.5 Hz, 1 H), 7.89−7.95 (m, 3 H).

Preparation of 1-((6a′*R*,8′*R*,9a′*S*)-2′,2′,4′,4′-Tetraisopropylhexahydrospiro[cyclopropane-1,9′-furo[3,2-*f*][1,3,5,2,4]trioxadisilocine]-8′-yl)pyrimidine-2,4(1*H*,3*H*)-dione (13). Crude intermediate **12** (110 g) was dissolved in CH_2Cl_2 (200 mL), and NH_3/methanol (6 N, 1100 mL) was added to the mixture. The reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated to dryness by rotavapor, and the residue was purified by column chromatography on silica gel using petroleum ether/ethyl acetate 5:1 to 2:1 to give intermediate **13** as a white foam (48 g, 91% purity on LC−MS and NMR). Intermediate **13** was then repurified by preparative HPLC (water/CH_3CN, with formic acid as modifier). Fractions containing **13** were combined and neutralized to pH 8 with NH_3. CH_3CN was evaporated by rotavapor, and the water phase was extracted with ethyl acetate. The organic phase was dried over $MgSO_4$ and evaporated to give pure intermediate **13** as a white foam (35 g, 40% yield). LC−MS: t_R = 3.66 min, m/z = 497 $(M + H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.54−0.62 (m, 1 H), 0.70−0.76 (m, 1 H), 0.82−0.90 (m, 1 H), 0.95−1.12 (m, 28 H), 1.16−1.24 (m, 1 H), 3.83 (d, J = 8.7 Hz, 1 H), 4.04 (d, J = 13.5 Hz, 1 H), 4.17

(d, J = 13.5 Hz, 1 H), 4.47 (d, J = 8.7 Hz, 1 H), 5.71 (d, J = 8.2 Hz, 1 H), 5.87 (s, 1 H), 7.76 (d, J = 8.2 Hz, 1 H), 8.19 (br s, 1 H).

Preparation of 4-Amino-1-((6a′*R*,8′*R*,9a′*S*)-2′,2′,4′,4′-Tetraisopropylhexahydrospiro[cyclopropane-1,9′-furo[3,2-*f*][1,3,5,2,4]trioxadisilocine]-8′-yl)pyrimidin-2(1*H*)-one (14). Triethylamine (0.41 g, 2.0 equiv) was added to a stirring solution of intermediate **13** (10 g, 1.0 equiv) in CH_3CN (300 mL). 2,4,6-Triisopropylbenzenesulfonyl chloride (10.2 g, 2.0 equiv) and 4-dimethylaminopyridine (DMAP; 5.1 g, 2.0 equiv) were added. The reaction mixture was stirred at room temperature for 16 h. The mixture was evaporated to dryness on a rotavapor. The residue was taken into CH_3CN (100 mL), and NH_3 (28% aqueous solution, 200 mL) was added. This reaction mixture was stirred at room temperature for 3 h. Then the solvent was removed by rotavapor and the residue was dissolved in CH_2Cl_2 and washed with brine. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by column chromatography on silica gel using ethyl acetate/methanol 9:1 to give intermediate **14** as a white foam (9.28 g, 93% yield) that was used directly in the next step. LC−MS: t_R = 3.44 min, m/z = 496 $(M + H)^+$.

Preparation of (4*R*,6*R*,7*S*)-4-Amino-1-(7-hydroxy-6-hydroxymethyl-5-oxaspiro[2.4]hept-4-yl)-1*H*-pyrimidin-2-one (5). Intermediate **14** (20 g, 1.0 equiv) was dissolved in THF (600 mL), and TBAF (21.62 g, 2.05 equiv) was added to the mixture. The reaction mixture was stirred at room temperature for 1 h. Then Dowex resin (Dowex 50WX4, 202 g) and $CaCO_3$ (67 g) were added, and the mixture was stirred at room temperature for 1 h. The reaction mixture was filtered, and the Dowex resin was washed twice with a mixture of pyridine, methanol, and water (1600 mL, 3:1:1). The combined filtrates were concentrated to dryness by rotavapor. The residue was purified twice by column chromatography (10−25% methanol in $CHCl_3$) to give compound **5** as a white solid (7.5 g, 92% purity).

In order to increase the purity, recrystallization was carried out. Compound **5** (7.5 g, 92% purity) was dissolved in HPLC-grade methanol (6 mL), and the solvent was allowed to evaporate slowly at room temperature until less than 1 mL remained. White crystals were formed and collected by filtration and the crystals were washed with cold methanol to give compound **5** as white crystals (6.13 g, >98% purity, 62% yield). MS: m/z = 254 $(M + H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.31−0.61 (m, 3 H), 0.86−1.09 (m, 1 H), 3.49−3.63 (m, 1 H), 3.63−3.78 (m, 2 H), 3.85−4.02 (m, 1 H), 4.98 (br s, 1 H), 5.12 (d, J = 5.28 Hz, 1 H), 5.72 (d, J = 7.24 Hz, 1 H), 6.00 (s,1 H), 6.96−7.32 (m, 2 H), 7.77 (d, J = 7.24 Hz, 4 H). HPLC purity determination: method 1, t_R = 0.22, purity 99.4%; method 2, t_R = 0.68, purity 98.7%.

Preparation of (4*R*,6*R*,7*S*)-1-(7-Hydroxy-6-hydroxymethyl-5-oxaspiro[2.4]hept-4-yl)-1*H*-pyrimidin-2,4(1*H*,3*H*)-dione (15). Intermediate **13** (11.00 g, 22.14 mmol) was dissolved in THF (280 mL), and TBAF (59.8 mL, 59.8 mmol) was added. The mixture was stirred at room temperature for 1 h. A mixture of pyridine, methanol, and water (80 mL, 3:1:1) was added, followed by a strongly acidic cation exchanger, Dowex 50WX4 (128 g), in a mixture of pyridine, methanol, and water (320 mL, 3:1:1). The reaction mixture was stirred for 45 min and filtered. The Dowex residue was washed twice with a mixture of pyridine, methanol, and water (320 mL, 3:1:1), and the combined filtrates were concentrated under reduced pressure. The mixture was purified by silica gel chromatography with gradient elution of 0−10% methanol in ethyl acetate, resulting in intermediate **15** (5.597 g, 84%) as a white foam. LC−MS: t_R = 2.05 min, m/z = 253 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.50−0.60 (m, 3 H), 1.01−1.09 (m, 1 H), 3.55−3.62 (m, 1 H), 3.68−3.76 (m, 2 H), 4.03 (t, J = 4.5 Hz, 1 H), 5.02 (t, J = 4.5 Hz, 1 H), 5.20 (d, J = 5.1 Hz, 1 H), 5.64 (d, J = 8.0 Hz, 1 H), 5.92 (s, 1 H), 7.82 (d, J = 8.0 Hz, 1 H), 11.28 (br s, 1 H).

Preparation of (4*R*,6*R*,7*S*)-1-(7-Hydroxy-6-((4-methoxyphenyldiphenyl)methoxymethyl)-5-oxaspiro[2.4]hept-4-yl)-1*H*-pyrimidin-2,4(1*H*,3*H*)-dione (16). A solution of **15** (350 mg, 1.377 mmol) in dry pyridine (15 mL) was cooled on an ice−water bath, and monomethoxytrityl chloride (MMTrCl; 900 mg, 2.91 mmol) was added. The reaction mixture was left on a melting ice−water bath and was then stirred at room temperature overnight. Excess methanol was added, and after 30 min, the reaction mixture was concentrated, dried, and used as such in the next reaction. LC−MS: t_R = 2.48 min, m/z = 525 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.54−0.61 (m, 3 H), 1.04−1.09 (m, 1 H), 3.25−3.32 (m, 2 H), 3.74 (s, 3 H), 3.85−3.91 (m, 1 H), 4.21 (dd, J = 7.5, 5.9 Hz, 1 H), 5.25 (d, J = 5.9 Hz, 1 H), 5.38 (d, J = 8.0 Hz, 1 H), 5.90 (s, 1 H), 6.90 (d, J = 8.4 Hz, 2 H), 7.23−7.28 (m, 4 H), 7.29−7.35 (m, 4 H), 7.37−7.42 (m, 4 H), 7.74 (d, J = 8.0 Hz, 1 H), 11.32 (br s, 1 H).

Preparation of (4*R*,6*R*,7*S*)-(4-(2,4-Dioxopyrimidin-1(2*H*)-yl)-6-[(4-methoxyphenyldiphenyl)methoxymethyl]-5-oxaspiro[2.4]hept-7-yl) Isobutyrate (17). Intermediate **16** (250 mg, 0.475 mmol) was dissolved in dry pyridine (10 mL), and the solution was cooled in a with cold water bath. To the solution was added via syringe isobutyric anhydride (236 μL, 1.424 mmol), and the mixture was stirred at room temperature for 2 h. More isobutyric anhydride (236 μL, 1.424 mmol) was added, and the mixture was stirred further for 2 h. Then additional isobutyric anhydride (236 μL, 1.424 mmol) was added and the mixture was stirred overnight. After this time, the excess isobutyric anhydride was quenched by addition of methanol. The solution was stirred for 20 min at room temperature and then concentrated to dryness. The residue was taken into ethyl acetate (30 mL) and the solution washed with saturated aqueous $NaHCO_3$ (2 × 20 mL). The organic phase was dried over Na_2SO_4, the solid was filtered off, and the solvent removed by evaporation, resulting in **18** as a colorless oil which was used as such in the next reaction. LC−MS: t_R = 3.07 min, m/z = 273 $(M + H)^+$.

Preparation of (4*R*,6*R*,7*S*)-(4-(2-Oxo-4-([1,2,4]triazol-1-yl)-pyrimidin-1(2*H*)-yl)-6-[(4-methoxyphenyldiphenyl)methoxymethyl]-5-oxaspiro[2.4]hept-7-yl) Isobutyrate (18). $POCl_3$ (102 μL, 1.089 mmol) was added to a cooled mixture of intermediate **17** (250 mg, 0.419 mmol), 1*H*-1,2,4-triazole (327 mg, 4.73 mmol), triethylamine (661 μL, 4.73 mmol), and CH_2Cl_2 (6.0 mL) while the reaction temperature was maintained below 25 °C (resulting in a white precipitate). The reaction mixture was stirred at room temperature for 4 h. When the reaction was completed, the excess $POCl_3$ was quenched by careful addition of cold H_2O. The organic layer was separated and concentrated by evaporation under vacuum. The mixture was purified by silica gel chromatography using gradient elution CH_2Cl_2/ethyl acetate 90:10 to 85:15, resulting in intermediate **18** as an oil (200 mg, 74%). LC−MS: t_R = 3.15 min, m/z = 648 $(M + H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.67−0.73 (m, 1 H), 0.78−0.85 (m, 1 H), 0.97−1.05 (m, 1 H), 1.08−1.13 (m, 1 H), 1.15 (d, J = 7.0 Hz, 3 H), 1.18 (d, J = 7.0 Hz, 3 H), 2.57 (spt, J = 7.0 Hz, 1 H), 3.48 (dd, J = 10.7, 3.3 Hz, 1 H), 3.55 (dd, J = 10.7, 2.5 Hz, 1 H), 3.81 (s, 3 H), 4.26 (ddd, J = 4.7, 3.3, 2.5 Hz, 1 H), 5.42 (d, J = 4.7 Hz, 1 H), 6.48 (s, 1 H), 6.66 (d, J = 7.2 Hz, 1 H), 6.87 (d, J = 8.6 Hz, 2 H), 7.27−7.36 (m, 8 H), 7.40−7.48 (m, 4 H), 8.10 (s, 1 H), 8.59 (d, J = 7.2 Hz, 1 H), 9.23 (s, 1 H).

Preparation of (4*R*,6*R*,7*S*)-(4-(4-Amino-2-oxopyrimidin-1(2*H*)-yl)-6-[(4-methoxyphenyldiphenyl)methoxymethyl]-5-oxaspiro[2.4]hept-7-yl) Isobutyrate (19). Intermediate **18** (200 mg, 0.309 mmol) was dissolved in THF (5 mL) and treated with concentrated aqueous NH_4OH (0.6 mL). After 4 h, more concentrated aqueous NH_4OH (0.3 mL) was added and the mixture was stirred overnight. The solvent was removed in vacuo, and the oil was taken up in ethyl acetate and washed with water and brine. After drying with Na_2SO_4, filtration, and evaporation of the volatiles, the residue **19** was used as such in the next reaction. LC−MS: t_R = 2.86 min, m/z = 594 $(M - H)^-$.

Preparation of (4*R*,6*R*,7*S*)-(4-(4-Amino-2-oxopyrimidin-1(2*H*)-yl)-6-hydroxymethyl-5-oxaspiro[2.4]hept-7-yl) Isobutyrate (20). Intermediate **19** (180 mg, 0.302 mmol) was dissolved in 80% aqueous acetic acid (5 mL), and the reaction mixture was stirred

at room temperature. After 9 h, the volatiles were removed and the mixture was purified by silica gel chromatography by gradient elution with 5−15% methanol in CH_2Cl_2. The obtained residue was triturated with $^iPr_2O$ and dried in vacuo, resulting in compound **20** (60.8 mg, 62%). MS: m/z = 324 $(M + H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.33−0.41 (m, 1 H), 0.62−0.71 (m, 1 H), 0.74−0.82 (m, 2 H), 1.08−1.14 (m, 6 H), 2.55−2.64 (1H, m), 3.62−3.68 (m, 2H), 3.99−4.04 (m, 1 H), 4.98−5.03 (m, 1 H), 5.12 (t, J = 5.2 Hz, 1 H), 5.76 (d, J = 7.4 Hz, 1 H), 6.27 (s, 1 H), 7.14−7.33 (m, 2 H), 7.80 (d, J = 7.4 Hz, 1 H). HPLC purity determination: method 3, t_R = 2.40, purity 97.2%; method 4, t_R = 0.51, purity 95.5%.

Preparation of (4*R*,6*R*,7*S*)-(4-(2,4-Dioxopyrimidin-1(2*H*)-yl)-6-(isobutyryloxymethyl)-5-oxaspiro[2.4]hept-7-yl) Isobutyrate (21). Intermediate **15** (5.16 g, 20.30 mmol) was dissolved in dry pyridine (100 mL), and the solution was externally cooled with cold water. Isobutyric anhydride (16.85 mL, 101 mmol) was added, and the mixture was allowed to proceed at room temperature overnight. The mixture was externally cooled with cold water, and the excess isobutyric anhydride was quenched by addition of methanol. After the mixture was stirred for 20 min at room temperature and after evaporation of the volatiles, ethyl acetate was added and the mixture was washed with saturated aqueous $NaHCO_3$ (2×). The organic phase was dried with $MgSO_4$ and concentrated in vacuo to give intermediate **21** (7.68 g, 96%) as a white solid. LC−MS: t_R = 2.26 min, m/z = 393 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.57−0.63 (m, 1 H), 0.66−0.72 (m, 1 H), 0.87−0.96 (m, 2 H), 1.10 (d, J = 6.8 Hz, 12 H), 2.59 (m, 2 H), 4.21−4.32 (m, 3 H), 5.10 (d, J = 4.3 Hz, 1 H), 5.69 (d, J = 8.1 Hz, 1 H), 6.13 (s, 1 H), 7.61 (d, J = 8.1 Hz, 1 H), 11.37 (br s, 1 H).

Preparation of (4*R*,6*R*,7*S*)-(4-(4-([1,2,4]Triazol-1-yl)-2-oxopyrimidin-1(2*H*)-yl)-6-(isobutyryloxymethyl)-5-oxaspiro[2.4]hept-7-yl) Isobutyrate (22). $POCl_3$ (4.72 mL, 50.6 mmol) was added to a cooled mixture of **21** (7.68 g, 19.47 mmol), 1*H*-1,2,4-triazole (15.20 g, 220 mmol), and triethylamine (30.7 mL, 220 mmol) in dry CH_2Cl_2 (50 mL). The mixture was stirred at room temperature for 2.5 h. The excess $POCl_3$ was quenched by addition of cold water, and the organic layer was separated and concentrated in vacuo. The mixture was purified by silica gel chromatography by gradient elution with CH_2Cl_2/ethyl acetate 90:10 to 85:15, resulting in intermediate **22** (7.5 g, 86%). LC−MS: t_R = 2.38 min, m/z = 446 $(M + H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.56−0.64 (m, 1 H), 0.75−0.83 (m, 1 H), 1.00−1.06 (m, 1 H), 1.13−1.19 (m, 1 H), 1.19−1.26 (m, 12 H), 2.63 (spt, J = 6.8 Hz, 2 H), 4.35−4.41 (m, 2 H), 4.51 (dd, J = 12.7, 5.7 Hz, 1 H), 4.97 (d, J = 3.1 Hz, 1 H), 6.60 (s, 1 H), 7.09 (d, J = 7.2 Hz, 1 H), 8.14 (s, 1 H), 8.27 (d, J = 7.2 Hz, 1 H), 9.26 (s, 1 H).

Preparation of (4*R*,6*R*,7*S*)-(4-(4-Amino-2-oxopyrimidin-1(2*H*)-yl)-6-(isobutyryloxymethyl)-5-oxaspiro[2.4]hept-7-yl) Isobutyrate (23). Intermediate **22** (7.49 g, 16.81 mmol) was dissolved in THF (200 mL) and treated with concentrated aqueous NH_4OH (15 mL). After 3.5 h, the volatiles were removed under reduced pressure. The mixture was purified by silica gel chromatography with gradient elution of 0−5% methanol in CH_2Cl_2. The product was dissolved in ethyl acetate, and the mixture was washed with water (2×) and brine (2×). The organic phase was dried with $MgSO_4$ and after filtration concentrated in vacuo, resulting in compound **23** (5.597 g, 84%) as a white foam. MS: m/z = 394 $(M + H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.39−0.44 (m, 1 H), 0.67−0.73 (m, 1 H), 0.80−0.89 (m, 2 H), 1.09−1.13 (m, 12 H), 2.54−2.65 (m, 2 H), 4.21−4.35 (m, 3 H), 5.01 (d, J = 3.1 Hz, 1 H), 5.77 (d, J = 7.4 Hz, 1 H), 6.25 (s, 1 H), 7.22−7.31 (m, 2 H), 7.55 (d, J = 7.4 Hz, 1 H). HPLC purity determination: method 3, t_R = 4.58, purity 99.1%; method 4, t_R = 0.71, purity 97.5%.

Replicon Activity. The HCV 1b subgenomic luciferase reporter replicon (replicon clone ET obtained from R. Bartenschlager, adapted from Lohmann[59] with adaptive mutations E1202G, T1280I, K1846T) was used to measure anti-HCV activity as described before.[37] Briefly, 2500 cells were incubated with compounds plated in a 384-well nine-point dilution ($^1/_4$ dilutions) format for 3 days. Cellular activity was then detected by the measurement of luciferase activity, and 50% effective concentrations (EC_{50}) of compounds were determined.

Cellular Toxicity. Cellular toxicity was determined in Huh7-CMV-Luc and MT4-LTR-Luc assays. In brief, Huh7 cells, stably transformed with a luciferase reporter gene under control of the cytomegalovirus (CMV) constitutive promoter, or MT4 cells expressing a luciferase reporter controlled by the HIV long terminal repeat (LTR) promoter, were cultured in the presence or absence of test compound concentrations. After 3 days of incubation at 37 °C in a humidified 5% CO_2 atmosphere, cell proliferation was quantified by measuring the luciferase activity and expressed as CC_{50} values (cytotoxicity, 50% inhibitory concentration of cell growth). Tests were performed in 384-well plates.

Pharmacokinetic Studies. Pharmacokinetic studies were performed in Sprague−Dawley rats and Beagle dogs. Three animals per dose regimen were administered a single oral solution of test compound using 20% hydroxylpropyl-β-cyclodextrin. Rats were dosed at around 36 and 216 μmol/kg with compound **5** and its prodrug **23**, while prodrug **20** was only dosed at around 36 μmol/kg. In dogs, only **20** was given at 36 and 180 μmol/kg. Blood samples were collected on EDTA and put on ice protected from light, at 0.5, 1, 2, 4, 7, and 24 h in rats and at 0.5, 1, 2, 4, 6, 8, 24, and 48 h in dogs. Blood samples were centrifuged at 4 °C within 1 h after collection of blood to prepare plasma. Plasma samples were stored at −20 °C until further analysis.

Analytical Method for Parent 5. After solubilization and protein precipitation (with acetonitrile), plasma samples were quantified on an Atlantis HILIC LC column (3 μm, 50 mm × 4.6 mm; Waters). Mobile phases consisted of 0.01 M NH_4OOCH_3 (solvent A) and CH_3CN (solvent B). Chromatographic separation was obtained by gradient elution (from 5% solvent A/95% solvent B as starting conditions to 50% solvent A/50% solvent B in 2.5 min) at a flow rate of 1.2 mL/min.

Analytical Method for Prodrugs 20 and 23. After solubilization and protein precipitation (with CH_3CN), plasma samples were quantified on X-bridge C18 LC-column (3.5 μm, 50 mm × 4.6 mm; Waters). Mobile phases consisted of 0.01 M CH_3COONH_4 (solvent A) and CH_3CN (solvent B). Chromatographic separation was obtained by gradient elution (from 45% solvent A/55% solvent B as starting conditions to 5% solvent A/95% solvent B in 3 min) at a flow rate of 1.2 mL/min.

For all the different compounds, LC−MS/MS analysis was carried out on an API-4000 MS/MS instrument (Applied Biosystems), which was coupled to a HPLC system (Agilent). The API-4000 MS/MS instrument, operating in the positive ion mode using the TurboIonSpray interface (electrospray ionization), was optimized for the quantification of the compounds. The limit of quantification was at least 5.00 ng/mL for plasma samples. The accuracy (intrabatch accuracy from independent quality control samples) was between 85% and 115% of the nominal value over the entire range for plasma samples. The typical range of the standard curve was 5−2000 ng/mL.

Pharmacokinetic Analysis. Noncompartmental PK analysis was performed on the individual plasma concentration-versus-time data (WinNonlin, version 5.2.1; Pharsight Corp.).

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