Solid-Phase Synthesis and Screening of Macrocyclic Nucleotide-Hybrid Compounds Targeted to Hepatitis C NS5B

Michael Smietana,[a] Robert B. Johnson,[b] Q. May Wang,[b] and Eric T. Kool*[a]

Abstract: A convergent strategy for the synthesis of cyclic nucleotide-hybrid molecules on controlled pore glass is reported. A major advantage of the approach is the lack of restrictions on the sequence and structural variation, allowing the incorporation of modified ribonucleosides (such as 2′-OMe-ribonucleotides), as well as threoninol derivatives. This methodology allows a fully automated assembly by means of standard phosphoramidite chemistry and is based on a recently published procedure for the preparation of cyclic oligodinucleotides in the DNA series (M. Smietana, E. T. Kool, *Angew. Chem.* **2002**, *114*, 3856–3859; *Angew. Chem. Int. Ed. Engl.* **2002**, *41*, 3704–3707). A library of potential cyclic hybrid inhibitor compounds targeting hepatitis C virus NS5B enzyme (the replicating polymerase of HCV) was generated by means of the parallel-pool strategy. Screening of the library revealed that cyclic hybrid *c*(C_{OME}-EthenodA) was a significant inhibitor of NS5B, with an IC_{50} of 40 μM. Preliminary structure–activity studies of this lead compound are described.

Keywords: antiviral agents · DNA structures · solid-phase synthesis · structure–activity relationships

Introduction

Cyclic molecules are frequently found in nature. The discovery of numerous naturally occurring cyclic metabolites has encouraged scientists to systematically study the chemical, physical, and biological properties of these compounds. In this context, cyclic DNAs and RNAs play a key role reflected by their unusual chemical and biological activities.[1] Such molecules are attractive targets for study as they combine two favorable properties: i) reduced conformational flexibility of the backbone, which can enhance receptor selectivity and binding affinity, and ii) lowered susceptibility to degradation in biological systems.[2,3] Both aspects make such cyclic molecules potentially good leads for drug discovery.[1] However, macrocyclic molecules, such as cyclic oligonucleotides, are more difficult to synthesize than linear congeners because of the entropic cost of ring closure. Typically, the preparation of large cyclic DNAs ($>\approx 30$ nucleotides) involves a template-directed ligation of a linear precursor oligonucleotide. The template can be provided as a separate sequence[4] or it can involve an internal secondary structure.[5] Template-free methodologies on a modified solid support have been described for the synthesis of small- to medium-sized cyclic DNAs[6] and RNAs.[7,8]

We recently described the first solid-phase synthesis of cyclic oligonucleotides that utilizes the standard β-cyanoethylphosphoramidite method,[9] which is based on a rapid and inexpensive automated 5′-iodination method developed in our laboratory.[10] The main advantage of this method is that cyclization occurs spontaneously during deprotection of the oligonucleotide, leading to cyclic compounds bearing a 5′-bridging phosphorothioate linkage with good-to-excellent yields (Scheme 1, R = H).[9] Since the chemical diversity of natural nucleotides is limited, we sought to increase the chemical and structural variation by replacing natural monomers with modified nucleotides and non-nucleotide moieties. We now report on the extension of this methodology to the preparation of cyclic 2′-(OMe) RNA, and cyclic DNA-hybrid threoninol derivatives. The preparation of a library of such compounds targeted to viral polymerases is also described. Screening of this library against the replicative polymerase of the hepatitis C virus revealed a member that is inhibitory at low micromolar concentrations.

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Results and Discussion

Our goals in this work were to investigate whether our previously developed chemistry could be adapted to the prepa-

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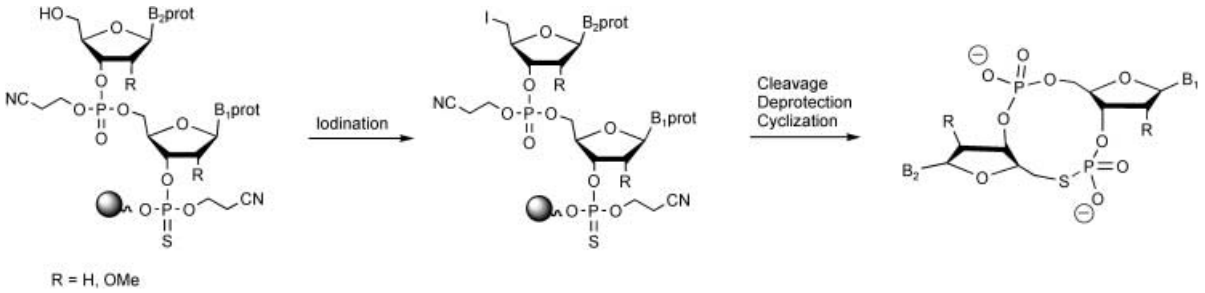


Scheme 1. Formation of cyclic oligonucleotides.

ration of more structurally and chemically diverse molecules, and whether such molecules might be active as inhibitors of viral polymerases. Although the conditions previously described work well for the preparation of cyclic DNAs, as exemplified by the synthesis of *c*(QQ)[11] (Table 1, entry 1), cyclic oligoribonucleotides, because of their greater reactivity, are much more difficult to prepare than the corresponding 2′-deoxy analogues. To circumvent the difficulties generally encountered with the classic 2′-*tert*-butyldimethylsilyl-protected amidites, the use of the 2′-*O*-triisopropylsilyloxymethyl (TOM) protecting group has been described.[8] A strict application of the conditions developed in our laboratory for cyclic DNAs showed us that 2′-*O*-methoxyribonucleotides are well-iodinated on the 5′-end, furnishing high yields of cyclic, mixed ribo- and deoxy-oligonucleotides (Table 1, entry 2). However, the synthesis of cyclic oligo-di-(2′-OMe)-ribonucleotides appeared more difficult to achieve. A brief investigation revealed sulfurization to be the step that failed. Our initial conditions were based on a protocol that required a single delivery of Beaucage reagent (0.05 M, 15 equiv), followed by a waiting period of 30 s.[12] A series of experiments were executed in which the number of equivalents of Beaucage reagent and the reaction times were varied. Complete sulfurization efficiency was achieved with two deliveries of 20 equiv of reagents, each one followed by a waiting period of 500 s. Clearly, the requirements for sulfurization of 2′-substituted linkages are more stringent than for their DNA counterparts. Application of these conditions allowed cyclization of mixed cyclic oligonucleotides (entry 3) as well as cyclic oligodi-(2′-OMe)-ribonucleotides without any restrictions on the nucleobases (entries 4–7) (Scheme 1, R = OMe). These conditions also allow the preparation of fully modified trimers (entry 8).

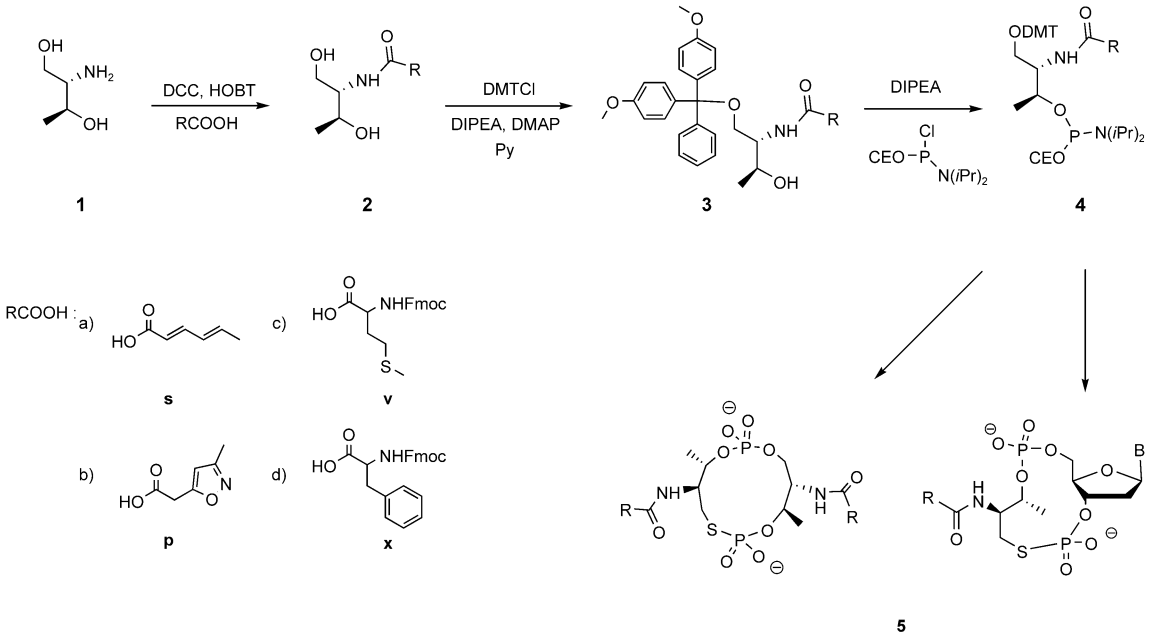
We then turned our attention toward the formation of cyclic nucleotide-hybrid compounds. Cyclic peptide-DNA hybrids with ring sizes of 30–40 atoms have already been described in the literature with respect to their potential protein-binding properties.[13] In order to preserve the more rigid and smaller 12-membered ring structure of cyclic oligodinucleotides, we decided to use D-threoninol derivatives as the hybrid backbone. Both L- and D-threoninol enantiomers have already been synthesized and introduced into ODNs for several purposes;[14] however, they have never been part of cyclic structures. The D-threoninol backbone was chosen for three reasons: i) it mimics the spacing of the traditional ribose moiety, which has three carbons between the primary and secondary alcohols, ii) it can be modified to be suitable for standard phosphoramidite chemistry, and iii) the secondary amino group can be condensed with a series of carboxylic sidechains to provide the diversity necessary for the synthesis of a library.

The synthesis of cyclic hybrids **5** is shown is Scheme 2. The secondary amino group of D-threoninol was modified by means of an amide linkage with a variety of carboxylic acids, by treatment with DCC (1 equiv), HOBt (1 equiv), and diisopropylethylamine (1.2 equiv) in DMF. For the purpose of selective protection of the primary alcohol, either 4,4′-dimethoxytrityl (DMT) or 4-monomethoxytrityl (MMT) groups were used. The latter could be completely removed by following the standard protocol on a DNA synthesizer. Finally, the secondary hydroxyl group was activated with bis(diisopropylamino)-2-cyanoethylphosphine. In the purification of such products by silica-gel column chromatography, 2% triethylamine was typically used to prevent decomposition. Compounds **4** were used along with standard nucleobases on an automated DNA synthesizer following the conditions used for 2′-*O*-methoxyribonucleo-

Table 1. Synthesis and characterization of cyclic compounds.

Entry	Product	^{31}P NMR		Yield[b] [%]	Mass[c]	
		P(O)[a]	P(S)[a]		calcd	found
1	*c*(QQ)	−0.12	20.32	45	636.1	637.1
2	*c*(U_{OMe}T)	−0.81	20.45	85	638.0	638.9
3	*c*(TU_{OMe})	−0.36	19.33	90	638.0	638.9
4	*c*($C_{OMe}C_{OMe}$)	−0.98	19.47	70	652.1	653.1
5	*c*($C_{OMe}U_{OMe}$)	−0.42	19.27	93	653.1	654.1
6	*c*($C_{OMe}G_{OMe}$)	−1.04	19.40	77	692.1	693.1
7	*c*($C_{OMe}A_{OMe}$)	−0.77	19.46	75	676.1	677.1
8	*c*(QQC_{OMe})	−0.31, −0.21	17.98	38	952.1	953.1
9	*c*(T**s**)	−0.55	19.18	96	579.1	580.0
10	*c*(**vs**)	−0.34	19.08	37	565.1	588.0
11	*c*(T**p**)	−0.53	18.92	75	616.1	616.9
12	*c*(**x**T)[d]	−0.51, −0.35	19.65, 19.71	80	618.1	619.1

[a] ^{31}P chemical shift measured with respect to 85% H_3PO_4 as an external standard. [b] Absolute HPLC yield determined as a percentage of all areas displayed. [c] Electrospray MS. [d] Serinol derivative.



Scheme 2. Synthesis of cyclic DNA hybrids.

tides. Deprotection of bases, release from the solid support, and cyclization with aqueous ammonia at room temperature overnight gave cyclic DNA-hybrids **5**. Structural characterization of **5** included ^{31}P NMR spectroscopy and ES-MS (Table 1). Exploratory experiments showed that these derivatives can be incorporated as the primary as well as the secondary substrate on the column (entries 9–11), even though complete hybrid structures led to more complex mixtures of products in some cases.

An exception was noted with amino acid derivatives **4v** and **4x**; if incorporated as the secondary substrate, alkaline deprotection liberates the free amino group which is ideally placed for an intramolecular attack on the 5′-iodo intermediate, leading to a six-membered cycle. To circumvent this side reaction, these derivatives—which were synthesized for potentially enhanced membrane permeability on account of their ionizable N-termini—had to be placed in the primary position (entries 10 and 12), or in the middle of a trimer. In case of compound **x**, prochiral serinol condensed with phenylalanine was used in place of D-threoninol leading to two diastereomeric dimers (entry 12). Although no diastereomer was clearly apparent in ^{31}P NMR (Table 1, entry 10), or by HPLC, we cannot rule out the possibility that some racemization might occur at the amino acid moiety during alkaline deprotection.

With these optimized conditions, we envisioned the feasibility of a library. For this purpose, the synthesis of 11 threoninol-like core structures containing heteroatomic and amino acid sidechains was achieved. Associated with standard or modified nucleobases, a set of 24 monomers was obtained (Scheme 3). A parallel-pool library strategy that incorporates the well-known techniques of split-mix and parallel combinatorial synthesis was chosen.[15] In this way, small pools of dimers (4 members each) were synthesized through a unique mixing and splitting step. Each pool can be assessed in a particular biological assay, and if positive, deconvoluted by synthesis of subpools and unique members. In this strategy, the CPG support was functionalized with 5′end-ODMT derivatives in the four separate columns of an ABI394 DNA synthesizer yielding intermediates **6**. Each pool was then mixed in equal amounts, and split into four batches. Each refilled column **7** was then reacted with a different 5′end-ODMT derivative to produce sets of supported dimers. Treatment with ammonium hydroxide liberated a library of 256 cyclic dimers as pools of 4 members (Figure 1).

All pools were purified as a mixture by preparative HPLC, although in most cases four well-separated chromatographic peaks were visible. For more than 80% of the

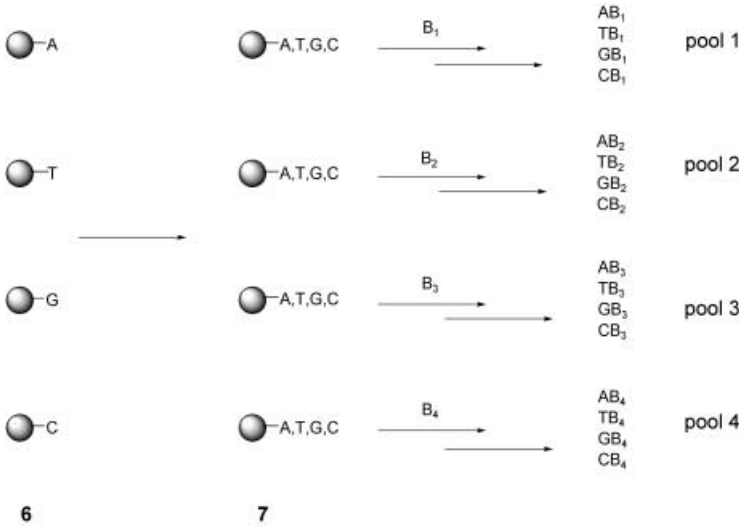
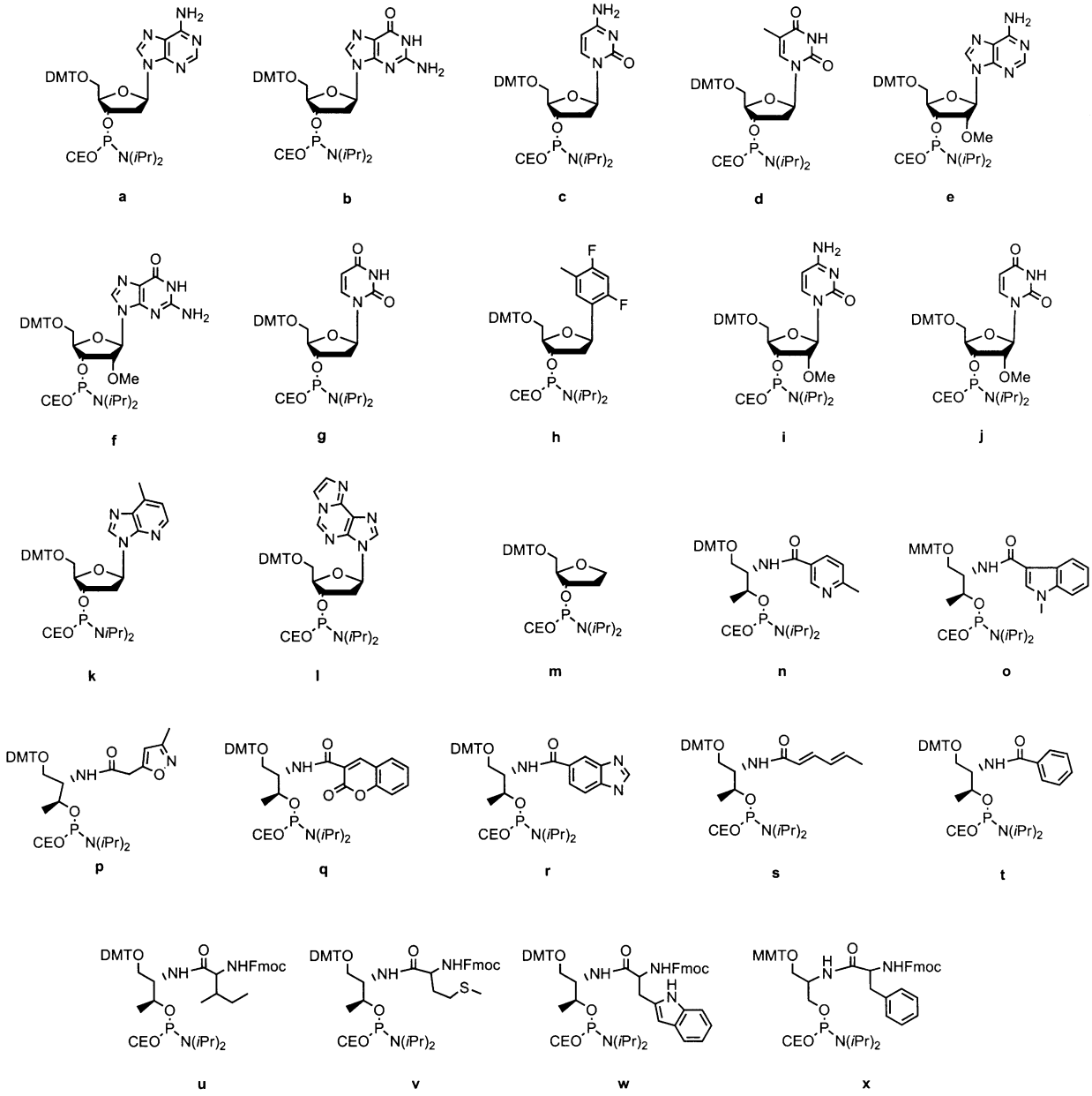


Figure 1. Parallel-pool synthesis of a library of cyclic dinucleotides and nucleotide-hybrid molecules (B_{1-4} = natural and modified bases).



Scheme 3. Monomers present in the library.

pools only minor amounts of other compounds were evident, as exemplified by pool 8 (Figure 2).

Finally, we investigated the potency of the library pools toward the RNA-dependent RNA polymerase NS5B encoded by the hepatitis C virus; this was carried out following an elongation assay already described.[16] Each pool was individually examined for antiviral activity. Although significant inhibition was exhibited by some pools (Figure 3), unique members obtained after deconvolution displayed lower activities than the initial pool.[17] We surmise that a small unidentified synthesis side product was active, or that multiple compounds were active only in combination. Nevertheless, despite this complication, we were able to identify cyclic hybrid *c*(C_{OMe}EthenodA) (*c*(il) in Scheme 3) (Table 2) as an active compound in our library with an IC_{50} value in the micromolar range (IC_{50} 40 μM).

Preliminary structure–activity relationship studies were carried out on second-generation analogues of this compound, again by the use of our solid-supported synthesis procedure. The results show that both the etheno and the 2′-OMe parts of the molecule are necessary for activity (Table 2, entries 1–3). As expected, the position of the sulfur bridge does not influence the IC_{50} value (entry 4). Interestingly, the IC_{50} value obtained with *c*(U_{OMe}EthenodA) (entry 5) suggests that hydrogen bonding with the cytosine enhances the potency of the inhibitor. Further modification at the 2′-*O*-alkyl position may be the most promising strategy to provide a further increase in the biological activity of this lead compound.

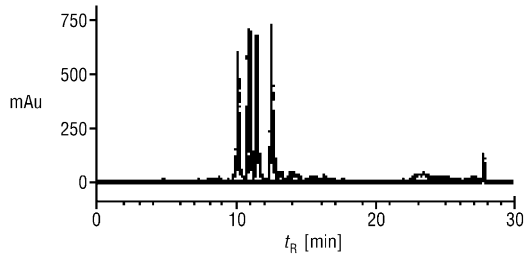


Figure 2. Analytical HPLC chromatogram of crude pool 8, containing four discrete library members.

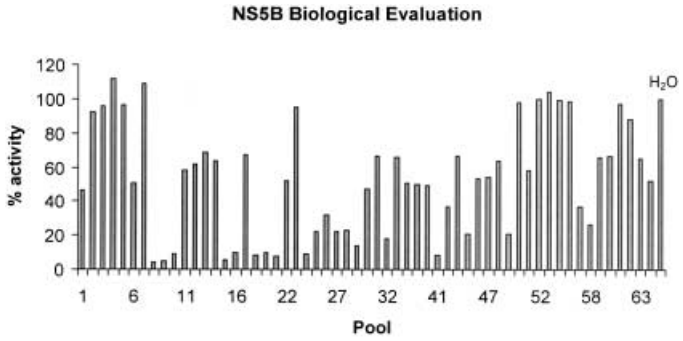
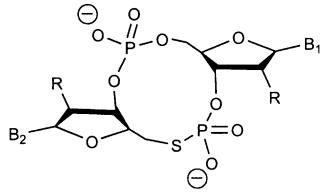


Figure 3. Inhibition of NS5B by pools 1–64. Each pool contains four cyclic compounds. Each assay was performed in duplicate at a concentration of 100 μM of each compound by pool.

Table 2. Inhibition of hepatitis C virus NS5B by cyclic dinucleotides.



Entry	B_1[a]	B_2[a]	IC_{50} [μM][b]
1	$C_{(OMe)}$	EthenodA	40
2	$C_{(OMe)}$	A	>1000
3	C	EthenodA	>1000
4	EthenodA	$C_{(OMe)}$	40
5	$U_{(OMe)}$	EthenodA	800
6	5-Me-$C_{(OMe)}$	EthenodA	>1000
7	2′-F-C	EthenodA	1000

[a] Corresponding to Scheme 3: $C_{(OMe)}$: **i**; EthenodA: **L**; C: **c**; $U_{(OMe)}$: **j**. [b] The IC_{50} values are the mean of three separate experiments.

Conclusion

In conclusion, we have developed a solid-phase synthesis of cyclic DNA/RNA and D-threoninol-DNA- or -RNA hybrids. This approach allows fully automated assembly of commercial and modified phosphoramidite derivatives. More diverse libraries are created by condensing the threoninol amino group with a larger number of carboxylic acids. The advantages in synthetic efficiency offered by the parallel-pool synthesis, as well as all mixture-based synthetic combinatorial libraries, are tempered by the false positives or additive effects that these mixtures may induce. Nevertheless, this methodology enables rapid preparation of hundreds of compounds that may be separated and screened individually. In doing so, we have demonstrated that members of this library have biological activities that can serve as leads for the possible development of new therapeutic agents in the treatment of chronic HCV disease.

Experimental Section

General: All commercially available chemical reagents were used without purification. ^1H and ^{13}C NMR spectra were recorded on a INOVA 500 spectrometer at 500 and 125 MHz, respectively. The signals of the residual protonated solvent ($CDCl_3$ or CD_3OD) were used as reference signal. ^{31}P NMR spectra were recorded on a INOVA 300 spectrometer at 121.5 MHz. ^{31}P chemical shifts were measured with respect to 85% H_3PO_4 as an external standard. Mass spectra were measured on a Finnigan LCQ for ESI-MS (analysis performed in the negative mode for cyclic oligonucleotides and cyclic hybrids). HRMS analyses were performed on a VG ZAB2SE high resolution mass spectrometer (UC Riverside). Cyclic structures were synthesized on a 1 μmol scale with a ABI394 DNA synthesizer (Applied Biosystems) according to standard protocol in "trityl-off" mode. HPLC purifications were performed on a Shimadzu system. A Hypersil BDS C18 5 μm column (250×4.6 mm, flow rate 1 mL min^{-1}, Alltech) was used for analytical purification and a HS Hyper prep 100 BDS C18 8 μ-column (250×10 mm, flow rate 3 mL min^{-1} Alltech) for preparative purification, with a linear gradient of acetonitrile (0–20%) in 50 mM triethylammonium acetate (TEAA, pH 7.0). For determination of oligonucleotide concentrations, the following extinction coefficients (λ = 260 nm) [L mol^{-1} cm^{-1}] were used: adenosine 15300, guanosine 11700, cytosine 7400, uridine 9900, thymine 8400, ethenodA 4800, 2′-OMe-cytosine 7000, 2′-OMe-uridine 9750. [α-^{32}P]-Uridine triphosphate was purchased from Amersham. Autoradiography was performed with a Molecular Dynamics Storm860 Phosphorimager.

Preparation of active HCV NS5B enzyme was performed by RBJ and QMW at Lilly Research Laboratories following a procedure described previously.[16] The library synthesis, assay of activity[16] and screening of compounds was performed by MS and ETK at Stanford University.

Monomers synthesis

General procedure for the coupling step: DCC (1 equiv) and HOBT (1 equiv) were dissolved in a 0.4 M solution of the acid (1 equiv) in DMF. After 1 h at room temperature, D-threoninol in DMF was added, and the resulting mixture was stirred overnight. The mixture was then filtered, and the DMF was removed by distillation. The crude compound was purified by silica-gel chromatography (usually AcOEt/MeOH 1:1).

General procedure for the protection of the primary alcohol: 0.3 equiv DMAP and 1.5 equiv DIPEA were added to a solution of the threoninol derivative in dry pyridine. A solution of 4,4′-dimethoxytrityl (DMT) chloride (or 4-momethoxytrityl chloride (MMT)) (1.2 equiv) in pyridine was added slowly over a period of 45 min. The reaction was followed by TLC analysis, and quenched with methanol after completion. The pyridine was evaporated, and the compound was purified by silica-gel chromatography.

General procedure for the protection of the secondary alcohol: The DMT ether (or MMT ether) was dissolved in dry dichloromethane (0.4 M solution), and DIPEA (1.2 equiv) and *N*,*N*′-diisopropylchlorophosphoramidite (1.5 equiv) were added. The reaction mixture was stirred for 90 min at room temperature and then quenched by the addition of a saturated solution of NaCl. The organic layer was extracted, washed with water, dried, and purified by silica-gel chromatography. The compound was obtained as a mixture of two diastereoisomers.

***N*-(2-Hydroxy-1-hydroxymethylpropyl)-6-methyl-nicotinamide (2n)**: R_f = 0.2 (AcOEt/MeOH 8.5:1.5); yield: 92%; ^1H NMR (500 MHz, CD_3OD): δ = 1.20 (d, J = 6.5 Hz, 3H), 2.56 (s, 3H), 3.71 (dd, J = 6.5 Hz, 1H), 3.77 (dd, J = 5.5 Hz, 1H), 4.07 (m, 2H), 4.96 (s, 2H), 7.36 (d, J = 8.0 Hz, 1H), 8.15 (d, J = 8.0 Hz, 1H), 8.88 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 20.55, 23.89, 58.34, 62.65, 67.27, 124.56, 129.31, 137.46, 148.65, 162.43, 168.35; MS (ESI): m/z: 225.1 (100) $[M+H]^+$; HRMS (EI): m/z: calcd for $C_{11}H_{17}N_2O_3$: 225.1233; found: 225.1239).

***N*-(2-Hydroxy-1-hydroxymethylpropyl)-1-methyl-1*H*-indole-3-carboxamide (2o)**: R_f = 0.5 (AcOEt/MeOH 8:2); yield: 66%; ^{1}H NMR (500 MHz, CD_3OD): δ = 1.23 (d, J = 6.5 Hz, 3H), 3.68 (s, 3H), 3.77 (m, 2H), 4.07 (m, 1H), 4.14 (m, 1H), 7.20 (m, 2H), 7.31 (d, J = 8.0 Hz, 1H), 7.79 (s, 1H), 8.04 (d, J = 8.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 20.73, 33.31, 56.99, 63.04, 67.15, 110.63, 111.00, 121.53, 122.24, 123.49, 127.28, 133.40, 138.56, 168.13; MS (ESI): m/z: 263.0 (100) $[M+H]^+$; HRMS (EI): m/z: calcd for $[C_{14}H_{19}N_2O_5]^+$: 263.1396; found: 263.1401.

***N*-(2-Hydroxy-1-hydroxymethylpropyl)-2-(3-methylisoxazol-5-yl)acetamide (2p)**: R_f = 0.3 (AcOEt/MeOH 8:2); yield: 60%; ^{1}H NMR (500 MHz, CD_3OD): δ = 1.14 (d, J = 6.0 Hz, 3H), 2.24 (s, 3H), 3.60 (dd, J = 6.5 Hz, 1H), 3.66 (dd, J = 6.0 Hz, 1H), 3.77 (d, 2H), 3.85 (m, 1H), 3.99 (m, 1H), 4.87 (s, 2H), 6.20 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 11.22, 20.36, 34.88, 57.68, 62.67, 66.89, 104.95, 161.57, 168.50, 169.95; MS (ESI): m/z: 229.0 (100) $[M+H]^+$; HRMS (EI): m/z: calcd for $C_{10}H_{17}N_2O_4$: 229.1188; found: 229.1196.

***N*-(2-Hydroxy-1-hydroxymethylpropyl)-2-oxo-2*H*-chromene-3-carboxamide (2q)**: R_f = 0.15 (AcOEt) Yield: 70%; ^{1}H NMR (500 MHz, CD_3OD): δ = 1.04 (d, J = 6.5 Hz, 3H), 3.58 (d, J = 5.5 Hz, 2H), 3.83 (m, 1H), 4.01 (m, 1H), 4.31 (brs, 2H), 7.24 (m, 2H), 7.53 (m, 2H), 8.68 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 19.49, 55.61, 62.14, 65.94, 116.13, 118.11, 125.06, 129.58, 134.06, 148.28, 154.03, 160.97, 162.20; MS (ESI): m/z: 278.2 (100) $[M+H]^+$; HRMS (EI): m/z: calcd for $C_{14}H_{16}NO_5$: 278.1028; found: 278.1031.

***N*-(2-Hydroxy-1-hydroxymethylpropyl)-1*H*-benzimidazole-5-carboxamide (2r)**: The synthesis of this product was realized in a mixture of NMP/DMF (1:1). R_f = 0.4 (AcOEt/MeOH 8:2); yield: 93%; ^{1}H NMR (500 MHz, CD_3OD): δ = 1.22 (d, J = 6.5 Hz, 3H), 3.72 (dd, J = 6.0 Hz, 1H), 3.77 (dd, J = 5.5 Hz, 1H), 4.12 (m, 2H), 5.07 (s, 3H), 7.62 (d, J = 8.5 Hz, 1H), 7.80 (d, J = 8.5 Hz, 1H), 8.17 (s, 1H), 8.28 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 20.60, 58.23, 62.79, 67.33, 115.75, 116.39, 123.17, 130.47, 138.83, 140.69, 144.68, 171.01; MS (ESI): m/z: 250.1 (100) $[M+H]^+$; HRMS (EI): m/z: calcd for $C_{12}H_{16}N_3O_3$: 250.1192; found: 250.1192.

***N*-(2-Hydroxy-1-hydroxymethylpropyl)-hexa-2,4-dienamide (2s)**: R_f = 0.4 (AcOEt/MeOH 9.5:0.5); yield: 70%; ^{1}H NMR (500 MHz, CD_3OD): δ = 1.18 (d, J = 6.5 Hz, 3H), 1.87 (d, J = 6.5 Hz, 3H), 3.65 (dd, J = 6.0 Hz, 1H), 3.70 (dd, J = 6.0 Hz, 1H), 3.93 (m, 1H), 4.05 (m, 1H), 4.96 (brs, 3H), 6.01 (m, 1H), 6.11 (m, 1H), 6.22 (m, 1H), 7.14 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 18.65, 20.36, 57.42, 62.77, 67.08, 122.81, 131.12, 138.74, 142.33, 169.48; MS (ESI): m/z: 200.0 (100) $[M+H]^+$; HRMS (EI): m/z: calcd for $C_{10}H_{18}NO_3$: 200.1287; found: 200.1278.

***N*-(2-Hydroxy-1-hydroxymethylpropyl)-benzamide (2t)**: R_f = 0.6 (AcOEt/MeOH 8:2); yield: 60%; ^{1}H NMR (500 MHz, CD_3OD): δ = 1.14 (d, J = 6.5 Hz, 3H), 3.76 (d, J = 4.6 Hz, 2H), 3.94 (m, 1H), 4.14 (m, 1H), 7.32 (m, 3H), 7.74 (d, J = 8.0 Hz, 2H), 8.12 (brs, 2H); ^{13}C NMR (125 MHz, CD_3OD): δ = 20.04, 55.48, 63.34, 67.23, 126.98, 128.36, 131.53, 133.96, 168.67; MS (ESI): m/z: 210.0 (100) $[M+H]^+$; HRMS (EI): m/z: calcd for $C_{11}H_{16}NO_5$: 210.1130; found: 210.1134.

9*H*-Fluoren-9-ylmethyl ester of [1-(2-hydroxy-1-hydroxymethylpropylcarbamoyl)-2-methyl-butyl]-carbamic acid (2u): R_f = 0.34 (AcOEt); yield: 62%; ^{1}H NMR (500 MHz, CD_3OD): δ = 0.84 (m, 6H), 0.97 (d, J = 6.5 Hz, 3H), 1.12 (m, 1H), 1.41 (m, 1H), 1.74 (m, 1H), 2.50 (s, 1H), 3.28–3.46 (m, 2H), 3.63 (m, 1H), 3.92 (m, 2H), 4.21 (m, 2H), 4.61 (m, 2H), 7.30–7.45 (m, 2H), 7.74 (m, 2H), 7.89 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD): δ = 11.73, 16.18, 20.71, 24.92, 37.20, 47.37, 56.30, 60.13, 60.88, 64.45, 66.32, 120.80, 126.03, 127.76, 128.34, 141.39, 144.44, 144.61, 156.71, 172.03; MS (ESI): m/z: 441.0 (100) $[M+H]^+$; HRMS (FAB): m/z: calcd for $C_{25}H_{33}N_2O_5$: 441.2389; found: 441.2377.

9*H*-Fluoren-9-ylmethyl ester of [1-(2-hydroxy-1-hydroxymethylpropylcarbamoyl)-3-methylsulfanylpropyl]-carbamic acid (2v): R_f = 0.55 (AcOEt/MeOH 9.5:0.5); yield: 80%; ^{1}H NMR (500 MHz, CD_3OD): δ = 1.14 (d, J = 6.5 Hz, 3H), 1.93 (m, 2H), 2.11 (s, 3H), 2.55 (m, 2H), 3.64 (m, 2H), 3.81 (m, 1H), 4.03 (m, 1H), 4.26 (m, 2H), 4.42 (m, 2H), 4.92 (brs, 4H), 7.35 (m, 2H), 7.41 (m, 2H), 7.68 (m, 2H), 7.81 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD): δ = 14.10, 19.40, 30.06, 31.79, 54.66, 56.23, 61.57, 65.49, 66.78, 119.77, 125.03, 127.02, 127.63, 141.44, 143.92, 144.22, 157.34, 173.63; MS (ESI): m/z: 458.9 (100) $[M+H]^+$; HRMS (FAB): m/z: calcd for $C_{24}H_{31}N_2O_5S$: 459.1954; found: 459.1949.

9*H*-Fluoren-9-ylmethyl ester of [1-(2-hydroxy-1-hydroxymethylpropylcarbamoyl)-2-(1H-indol-2-yl)-ethyl]-carbamic acid (2w): R_f = 0.62 (AcOEt/MeOH 9.5:0.5); yield: 70%; ^{1}H NMR (500 MHz, CD_3OD): δ = 0.91 (d, J = 6.5 Hz, 3H), 3.11 (m, 1H), 3.60 (m, 2H), 3.78 (m, 1H), 3.92 (m, 1H), 4.13 (m, 1H), 4.21 (m, 1H), 4.30 (m, 1H), 4.49 (m, 1H), 7.08 (m, 3H), 7.28 (m, 2H), 7.37 (m, 3H), 7.55 (m, 2H), 7.66 (d, J = 7.5 Hz, 1H), 7.76 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD): δ = 18.84, 28.09, 47.14, 56.13, 56.64, 61.29, 65.42, 66.91, 109.90, 111.16, 118.26, 118.71, 119.71, 121.28, 123.46, 125.05, 127.01, 127.58, 136.94, 141.35, 144.06, 157.17, 173.82; MS (ESI): m/z: 514.1 (100) $[M+H]^+$, 536.2 (30) $[M+Na]^+$; HRMS (FAB): m/z: calcd for $C_{30}H_{32}N_2O_5$: 514.2342; found: 514.2319.

9*H*-Fluoren-9-ylmethyl ester of [1-(2-hydroxy-1-hydroxymethylpropylcarbamoyl)-2-phenylethyl]-carbamic acid (2x): R_f = 0.5 (AcOEt); yield: 75%; ^{1}H NMR (500 MHz, CD_3OD): δ = 2.90 (m, 1H), 3.15 (m, 1H), 3.51 (m, 1H), 3.63 (d, J = 5.5 Hz, 2H), 3.91 (m, 1H), 4.15 (m, 1H), 4.36 (m, 2H), 7.31 (m, 8H), 7.59 (m, 2H), 7.80 (d, 2H); ^{13}C NMR (125 MHz, CD_3OD): δ = 12.21, 26.07, 26.75, 34.76, 54.31, 58.01, 61.59, 68.00, 120.91, 126.17, 127.75, 128.77, 129.45, 130.44, 142.54, 145.19, 174.08; MS (ESI): m/z: 483.1 (100) $[M+Na]^+$; HRMS (FAB): m/z: calcd for $C_{27}H_{28}N_2O_5Na$: 483.1896; found: 483.2319.

***N*-(1-*O*-[4,4′-Dimethoxytrityl]-2-hydroxypropyl)-6-methyl-nicotinamide (3n)**: R_f = 0.40 (AcOEt/Et_3N 98:2); yield: 67%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.21 (d, J = 6.5 Hz, 3H), 2.55 (s, 3H), 3.35 (dd, J = 3.5 Hz, 1H), 3.56 (dd, J = 4.5 Hz, 1H), 3.75 (s, 6H), 4.12 (m, 1H), 4.22 (m, 1H), 6.79 (m, 5H), 7.19 (m, 6H), 7.52 (d, J = 7.0 Hz, 1H), 7.80 (d, J = 7.5 Hz, 2H), 8.31 (d, J = 7.0 Hz, 1H), 9.15 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 17.9, 20.9, 56.0, 57.2, 64.2, 68.5, 88.8, 114.6, 124.3, 126.6, 128.4, 129.0, 129.4, 135.3, 137.2, 143.0, 152.1, 157.1, 159.5, 167.6; MS (ESI): m/z: 549.2 (100) $[M+H]^+$; HRMS (EI): m/z: calcd for $C_{32}H_{34}N_2O_5Na$: 549.2365; found: 549.2337.

***N*-(2-Hydroxy-1-*O*-[4-monothoxytrityl]-propyl)-1-methyl-1*H*-indole-3-carboxamide (3o)**: R_f = 0.33 (AcOEt/hexane/Et_3N 60:38:2); yield: 78%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.27 (d, J = 6.5 Hz, 3H), 3.4 (m, 1H), 3.64 (m, 1H), 3.75 (s, 6H), 3.77 (s, 3H), 4.26 (m, 2H), 6.82 (m, 3H), 7.21–7.36 (m, 10H), 7.48 (d, J = 7.0 Hz, 4H), 7.71 (s, 1H), 8.08 (d, J = 8.0 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.04, 33.07, 53.42, 55.01, 65.32, 68.78, 86.84, 109.97, 110.40, 113.11, 120.02, 121.44, 122.37, 125.13, 126.93, 126.96, 127.87, 128.06, 130.16, 132.51, 134.77, 137.09, 143.76, 143.91, 158.50, 165.35; MS (ESI): m/z: 557.1 (100) $[M+Na]^+$; HRMS (FAB): m/z: calcd for $C_{34}H_{34}N_2O_4Na$: 557.2444; found: 557.2416.

***N*-(1-*O*-[4,4′-Dimethoxytrityl]-2-hydroxypropyl)-2-(3-methylisoxazol-5-yl)-acetamide (3p)**: R_f = 0.5 (AcOEt/Et_3N 98:2); yield: 77%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.11 (d, J = 6.5 Hz, 3H), 2.26 (s, 3H), 3.26 (dd, J = 3.5 Hz, 1H), 3.41 (dd, J = 4.5 Hz, 1H), 3.74 (s, 2H), 3.94 (s, 6H), 3.95 (m, 1H), 4.08 (m, 1H), 6.84 (d, J = 7.0 Hz, 4H), 7.23–7.37 (m, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 11.38, 19.90, 34.88, 53.72, 55.18, 64.85, 68.42, 86.73, 104.08, 113.26, 126.99, 127.78, 127.99, 129.82, 135.09, 135.30, 144.19, 158.56, 160.15, 165.74, 166.75; MS (ESI): m/z: 553.1 (100) $[M+Na]^+$; HRMS (FAB): m/z: calcd for $C_{31}H_{34}N_2O_6Na$: 553.2315; found: 553.2340.

***N*-(1-*O*-[4,4′-Dimethoxytrityl]-2-hydroxypropyl)-2-oxo-2*H*-chromene-3-carboxamide (3q)**: R_f = 0.44 (AcOEt/hexane/Et_3N 49:49:2); yield: 57%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.18 (d, J = 6.5 Hz, 3H), 3.20 (brs, 1H), 3.37 (dd, J = 3.5 Hz, 1H), 3.52 (dd, J = 4.5 Hz, 1H), 3.77 (s, 6H), 4.18 (m, 2H), 6.84 (m, 4H), 7.20–7.41 (m, 10H), 7.69 (m, 2H), 8.89 (s, 1H), 9.51 (d, J = 8.5 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.04, 54.38, 55.11, 64.64, 68.77, 86.67, 113.16, 116.61, 118.28, 118.51, 125.20, 126.83, 127.92, 129.73, 129.93, 134.06, 135.29, 135.45, 144.24, 148.35, 154.45, 158.43, 161.09, 161.98; MS (ESI): m/z: 602.1 (100) $[M+Na]^+$; HRMS (FAB): m/z: calcd for $C_{35}H_{33}NO_7Na$: 602.2155; found: 602.2131.

***N*-(1-*O*-[4,4′-Dimethoxytrityl]-2-hydroxypropyl)-1*H*-benzimidazole-5-carboxamide (3r)**: R_f = 0.5 (AcOEt/hexane/Et_3N 90:8:2); yield: 90%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.02 (d, J = 6.5 Hz, 3H), 3.74 (s, 6H), 3.86 (m, 2H), 4.06 (m, 1H), 4.22 (m, 1H), 6.44 (d, J = 7.0 Hz, 1H), 6.62 (d, J = 7.0 Hz, 1H), 6.79 (d, J = 8.0 Hz, 4H), 6.98–7.12 (m, 4H), 7.28 (m, 3H), 7.42 (m, 1H), 7.54 (d, J = 7.0 Hz, 1H), 7.91 (s, 1H), 7.98 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.33, 54.97, 55.12, 64.03, 67.96, 113.33, 127.93, 128.06, 128.63, 128.71, 129.48, 130.93, 133.00, 134.35,

158.91, 168.11; MS (ESI): *m/z*: 574.1 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{33}H_{34}N_3O_5$: 552.2498; found: 552.2496.

***N*-(1-*O*-[4,4′-Dimethoxytrityl]-2-hydroxypropyl)-hexa-2,4-dienamide (3s)**: R_f = 0.25 (AcOEt/hexane/Et_3N 40:58:2); yield: 80%; ^1H NMR (500 MHz, $CDCl_3$): δ = 1.13 (d, J = 6.5 Hz, 3H), 1.82 (d, J = 6.0 Hz, 3H), 3.28 (m, 1H), 3.42 (m, 1H), 3.73 (s, 6H), 4.03 (m, 1H), 4.12 (m, 1H), 5.82 (m, 1H), 6.06 (m, 1H), 6.14 (m, 1H), 6.38 (m, 1H), 6.81 (d, J = 8.5 Hz, 4H), 7.18–7.31 (m, 7H), 7.40 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 19.36, 19.78, 54.88, 60.15, 64.56, 67.84, 86.34, 112.98, 121.21, 126.66, 127.68, 127.70, 129.46, 129.69, 135.26, 135.39, 137.70, 141.25, 144.32, 158.28, 166.55; MS (ESI): *m/z*: 524.2 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{31}H_{35}NO_5Na$: 524.2413; found: 524.2434.

***N*-(1-*O*-[4,4′-Dimethoxytrityl]-2-hydroxypropyl)-benzamide (3t)**: R_f = 0.45 (AcOEt/hexane/Et_3N 49:49:2); yield: 67%; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.21 (d, J = 6.5 Hz, 3H), 3.35 (dd, J = 3.5 Hz, 1H), 3.56 (dd, J = 4.5 Hz, 1H), 3.75 (s, 6H), 4.12 (m, 1H), 4.22 (m, 1H), 6.79 (m, 5H), 7.25 (m, 6H), 7.44 (m, 5H), 7.80 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 17.2, 56.4, 57.6, 64.3, 68.5, 89.8, 126.2, 127.3, 128.4, 129.2, 131.9, 133.5, 135.3, 143.0, 159.5, 171.1; MS (ESI): *m/z*: 512.1 (100) $[M+H]^+$; HRMS (EI): *m/z*: calcd for $C_{32}H_{34}NO_5$: 512.2498; found: 512.2437.

9*H*-Fluoren-9-ylmethyl ester of (1-{1-*O*-[4,4′-dimethoxytrityl]-2-hydroxypropylcarbamoyl}-2-methylbutyl)-carbamic acid (3u): R_f = 0.5 (AcOEt/hexane/Et_3N 40:58:2); yield: 60%; ^1H NMR (500 MHz, $CDCl_3$): δ = 0.81–0.90 (m, 6H), 1.03 (d, J = 6.5 Hz, 3H), 1.42 (m, 2H), 1.81 (m, 1H), 3.15 (m, 1H), 3.34 (m, 1H), 3.64 (s, 6H), 3.82–4.31 (m, 6H), 6.72 (d, J = 8.5 Hz, 4H), 7.14–7.29 (m, 13H), 7.43 (m, 2H), 7.66 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 11.34, 14.09, 15.48, 19.93, 24.63, 37.56, 47.06, 53.63, 55.04, 59.87, 64.77, 66.95, 68.07, 86.63, 113.20, 119.85, 125.00, 126.89, 126.98, 127.59, 127.85, 127.91, 129.80, 129.83, 135.21, 135.43, 141.17, 143.63, 144.23, 156.15, 158.53, 171.38; MS (ESI): *m/z*: 765.2 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{46}H_{50}N_2O_7Na$: 765.3516; found: 765.3552.

9*H*-Fluoren-9-ylmethyl ester of (1-{1-*O*-[4,4′-dimethoxytrityl]-2-hydroxypropylcarbamoyl}-3-methylsulfanylpropyl)-carbamic acid (3v): R_f = 0.3 (AcOEt/hexane/Et_3N 40:58:2); yield: 50%; ^1H NMR (500 MHz, $CDCl_3$): δ = 1.06 (d, J = 6.5 Hz, 3H), 1.99 (m, 5H), 2.51 (t, J = 7.0 Hz, 2H), 3.00 (brs, 1H), 3.20 (m, 1H), 3.35 (m, 1H), 3.66 (s, 6H), 4.05 (m, 3H), 4.40 (m, 3H), 6.75 (d, 4H), 7.11–7.32 (m, 15H), 7.68 (d, J = 8.0 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 15.03, 19.87, 29.90, 53.77, 54.97, 60.23, 64.36, 66.88, 67.92, 86.50, 113.11, 119.77, 124.92, 126.81, 126.92, 127.54, 127.82, 129.78, 135.19, 135.39, 141.07, 143.46, 143.76, 144.23, 155.87, 158.42, 171.33; MS (ESI): *m/z*: 783.2 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{45}H_{48}N_2O_7SNa$: 783.3080; found: 783.3046.

9*H*-Fluoren-9-ylmethyl ester of [1-{1-*O*-[4,4′-dimethoxytrityl]-2-hydroxypropylcarbamoyl}-2-(1*H*-indol-2-yl)-ethyl]-carbamic acid (3w): R_f = 0.28 (AcOEt/hexane/Et_3N 40:58:2); yield: 50%; ^1H NMR (500 MHz, $CDCl_3$): δ = 0.87 (d, J = 6.5 Hz, 3H), 2.84 (brs, 1H), 3.15–3.30 (m, 4H), 3.88 (s, 6H), 4.01 (m, 2H), 4.06 (m, 3H), 4.32 (m, 2H), 4.58 (m, 1H), 6.70 (d, J = 8.5 Hz, 4H), 7.15–7.45 (m, 19H), 7.70 (d, J = 7.5 Hz, 2H), 8.24 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 19.47, 20.81, 28.67, 46.86, 54.07, 54.92, 60.21, 63.93, 66.95, 86.34, 110.06, 111.29, 113.02, 118.48, 119.55, 119.74, 122.03, 123.28, 124.95, 126.75, 126.91, 127.13, 127.49, 127.74, 129.79, 135.16, 135.28, 136.11, 141.03, 143.47, 143.67, 144.37, 155.88, 158.35, 171.65; MS (ESI): *m/z*: 838.3 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{51}H_{49}N_3O_7Na$: 838.3468; found: 838.3504.

9*H*-Fluoren-9-ylmethyl ester of (1-{1-hydroxymethyl-2-*O*-[(-[4-monomethoxytrityl]-ethylcarbamoyl}-2-phenylethyl)-carbamic acid (3x): R_f = 0.45 (AcOEt/hexane/Et_3N 80:18:2); yield: 70%; ^1H NMR (500 MHz, $CDCl_3$): δ = 3.14 (m, 2H), 3.33 (m, 1H), 3.69 (m, 1H), 3.50 (m, 1H), 3.76 (m, 4H), 4.12 (m, 1H), 4.29 (m, 1H), 4.37 (m, 1H), 4.46 (m, 1H), 4.59 (m, 1H), 6.85 (d, J = 8.5 Hz, 4H), 7.19–7.47 (m, 18H), 7.53 (d, J = 7.5 Hz, 2H), 7.58 (d, J = 7.5 Hz, 1H), 7.79 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 38.72, 46.76, 51.12, 54.89, 56.10, 60.24, 62.08, 62.37, 66.80, 83.35, 112.99, 119.72, 124.85, 126.69, 126.84, 127.53, 127.72, 128.03, 128.09, 128.32, 128.44, 129.09, 130.06, 134.86, 136.27, 141.00, 143.50, 143.89, 155.75, 158.39, 171.04; MS (ESI): *m/z*: 755.1 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{47}H_{44}N_2O_6Na$: 755.3113; found: 755.3097.

3-*O*-[4,4′-Dimethoxytrityl]-1-methyl-2-[(6-methylpyridine-3-carbonyl)-amino]-propyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4n): R_f = 0.30 (AcOEt/hexane/Et_3N 40:58:2); yield: 60%; ^1HNMR (500 MHz, $CDCl_3$): δ = 1.01 (d, J = 7.0 Hz, 3H), 1.13–1.31 (m, 12H), 2.38 (m, 2H), 2.61 (s, 3H), 3.32 (d, J = 5.5 Hz, 2H), 3.55 (m ; 2H), 3.77 (s, 6H), 4.38 (m, 1H), 4.43 (m, 1H), 6.81 (m, 4H), 7.28 (m, 8H), 7.43 (d, J = 7.0 Hz, 2H), 8.01 (d, J = 8.0 Hz, 1H), 8.88 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 19.62, 20.10, 24.38, 42.97, 54.63, 55.02, 57.97, 62.49, 68.80, 85.94, 112.92, 117.66, 122.88, 126.62, 126.66, 127.25, 127.66, 127.95, 128.03, 129.86, 129.91, 135.18, 135.73, 144.60, 147.39, 158.28, 161.46, 165.31; MS (ESI): *m/z*: 727.1 (100) $[M+H]^+$, 749.3 (45) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{41}H_{52}N_4O_6P$: 727.3625; found: 727.3595.

3-*O*-[(4-Monomethoxytrityl]-1-methyl-2-[(1-methyl-1*H*-indole-3-carbonyl)-amino]-propyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4o): R_f = 0.33 (AcOEt/hexane/Et_3N 40:58:2); yield: 65%; ^1HNMR (500 MHz, $CDCl_3$): δ = 11.18 (d, J = 7.0 Hz, 6H), 1.20 (d, J = 7.0 Hz, 6H), 1.37 (d, J = 6.5 Hz, 3H), 2.36 (dt, J = 6.25 Hz, 2H), 3.28 (dd, J = 7.0 Hz, 1H), 3.43 (dd, J = 7.0 Hz, 1H), 3.52–3.62 (m, 4H), 3.79 (s, 3H), 3.82 (s, 3H), 4.59 (m, 2H), 6.41 (d, J = 9.0 Hz, 1H), 6.83 (d, J = 9.0 Hz, 2H), 7.24 (m, 3H), 7.29–7.39 (m, 5H), 7.37 (m, 3H), 7.50 (d, J = 8.5 Hz, 4H), 7.75 (s, 1H), 7.99 (d, J = 8.0 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 19.61, 20.03, 24.27, 25.52, 53.72, 55.06, 58.10, 68.93, 69.05, 86.21, 110.02, 110.71, 112.92, 117.66, 119.89, 121.18, 122.28, 125.02, 126.76, 127.67, 127.70, 128.37, 128.41, 130.32, 132.68, 135.42, 137.12, 144.26, 144.31, 158.35, 164.72; MS (ESI): *m/z*: 757.4 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{43}H_{51}N_4O_5PNa$: 757.3495; found: 757.3513.

3-[4,4′-Dimethoxytrityl]-1-methyl-2-[2-(3-methylisoxazol-5-yl)-acetylamino]-propyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4p): R_f = 0.35 (AcOEt/hexane/Et_3N 60:38:2); yield: 70%; ^1H NMR (500 MHz, $CDCl_3$): δ = 11.11 (d, J = 6.5 Hz, 6H), 1.14 (d, J = 7.0 Hz, 6H), 1.18 (d, J = 6.5 Hz, 3H), 2.19 (s, 3H), 2.38 (m, 2H), 3.18 (d, J = 6.0 Hz, 2H), 3.48 (m, 4H), 3.68 (s, 2H), 3.75 (s, 6H), 4.20 (m, 1H), 4.31 (m, 1H), 6.06 (s, 1H), 6.82 (d, J = 8.5 Hz, 4H), 7.19–7.30 (m, 7H), 7.41 (d, J = 8.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 11.03, 19.20, 19.92, 19.97, 23.94, 24.00, 24.30, 24.36, 34.56, 42.68, 42.78, 54.28, 54.32, 54.84, 57.80, 57.95, 62.24, 68.16, 68.28, 85.71, 103.72, 112.74, 117.79, 126.46, 127.48, 127.83, 129.75, 129.77, 135.50, 135.55, 144.44, 158.12, 159.68, 165.81, 166.18; MS (ESI): *m/z*: 753.3 (100) $[M+Na]^+$; HRMS (EI): *m/z*: calcd for $C_{40}H_{51}N_4O_7PNa$: 753.3393; found: 753.3395.

3-*O*-(4,4′-Dimethoxytrityl)-1-methyl-2-[(2-oxo-3,8a-dihydro-2*H*-chromene-3-carbonyl)-amino]-propyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4q): R_f = 0.4 (AcOEt/hexane/Et_3N 30:68:2); yield: 80%; ^1H NMR (500 MHz, $CDCl_3$): δ = 1.00–1.16 (m, 15H), 2.43–2.58 (m, 4H), 3.22 (m, 1H), 3.37 (m, 1H), 3.53 (m, 2H), 3.75 (s, 6H), 4.40 (m, 2H), 6.82 (m, 4H), 7.18–7.36 (m, 8H), 7.47 (m, 2H), 7.61 (m, 2H), 8.92 (d, J = 8.5 Hz, 1H), 9.07 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 19.30, 19.92, 24.10, 24.27, 42.79, 45.93, 54.30, 54.50, 54.87, 57.70, 62.54, 68.56, 85.82, 122.81, 116.23, 117.54, 118.22, 124.95, 126.41, 127.52, 127.95, 129.48, 129.82, 133.76, 135.68, 135.82, 144.61, 148.07, 154.14, 158.11, 160.82, 161.26; MS (ESI): *m/z*: 802.2 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{44}H_{52}N_3O_8PNa$: 802.3233; found: 802.3251.

2-[(1*H*-Benzimidazole-5-carbonyl)-amino]-3-*O*-[4,4′-dimethoxytrityl]-1-methylpropyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4r): R_f = 0.75 (AcOEt/hexane/Et_3N 60:38:2); yield: 60%; ^1H NMR (500 MHz, $CDCl_3$): δ = 1.07–1.19 (m, 15H), 2.57 (m, 4H), 3.55 (m, 4H), 3.68 (s, 6H), 4.22 (m, 2H), 6.77 (m, 4H), 7.01–7.25 (m, 10H), 7.42–7.95 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 19.10, 20.02, 24.27, 42.75, 54.49, 54.90, 58.07, 74.59, 113.06, 113.12, 115.23, 117.43, 117.57, 119.63, 122.29, 127.66, 127.80, 127.87, 129.30, 130.73, 132.93, 133.01, 134.38, 136.66, 141.06, 143.97, 145.22, 145.84, 146.64, 158.71, 166.69, 167.37; MS (ESI): *m/z*: 774.3 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{42}H_{50}N_5O_6PNa$: 774.3396; found: 774.3577.

3-*O*-[4,4′-Dimethoxytrityl]-2-hexa-2,4-dienoylamino-1-methylpropyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4s): R_f = 0.42 (AcOEt/hexane/Et_3N 30:68:2); yield: 70%; ^1H NMR (500 MHz, $CDCl_3$): δ = 1.01 (d, J = 7.0 Hz, 3H), 1.13–1.28 (m, 12H), 1.83 (d, J = 6.5 Hz, 3H), 2.40 (m, 1H), 2.57 (m, 1H), 3.53 (m, 4H), 3.78 (s, 6H), 4.28–4.42 (m, 2H), 5.72–5.95 (m, 2H), 6.09 (m, 1H), 6.16 (m, 1H), 6.83 (m, 4H), 7.18–7.33 (m, 7H), 7.43 (d, J = 7.0 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 19.87, 20.58, 20.66, 24.67, 24.80, 43.27, 43.37, 54.20, 55.44, 58.14, 68.90,

69.95, 86.33, 118.11, 118.28, 121.94, 126.99, 128.06, 128.43, 128.52, 129.99, 130.32, 130.42, 136.27, 137.92, 141.48, 145.12, 158.66, 166.50; MS (ESI): *m/z*: 724.4 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{40}H_{52}N_3O_6PNa$: 724.3491; found: 724.3497.

2-Benzoylamino-3-*O*-[4,4′-dimethoxytrityl]-1-methylpropyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4t): R_f = 0.45 (AcOEt/hexane/Et_3N 30:68:2); yield: 92%; ^1H NMR (500 MHz, $CDCl_3$): δ = 0.90–1.21 (m, 15H), 2.36 (t, J = 6.5 Hz, 2H), 2.47 (m, 2H), 3.29 (m, 1H), 3.47 (m, 3H), 4.19 (m, 2H), 7.76 (d, J = 9.0 Hz, 4H), 7.18–7.45 (m, 12 H), 7.76 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 13.18, 19.42, 23.32, 23.54, 42.07, 42.31, 53.89, 54.18, 57.14, 59.35, 61.86, 85.18, 112.20, 125.91, 125.99, 126.06, 126.93, 127.29, 127.35, 127.67, 129.24, 130.53, 133.86, 135.13, 135.25, 144.032, 157.66, 166.29; MS (ESI): *m/z*: 734.3 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{41}H_{50}N_3O_6PNa$: 734.3335; found: 734.3356.

3-*O*-(4,4′-Dimethoxytrityl)-1-methyl-2-((*N*-Fmoc-isoleucine)-amino)-propyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4u): R_f = 0.55 (AcOEt/hexane/Et_3N 30:68:2); yield: 70%; ^1H NMR (500 MHz, $CDCl_3$): δ = 0.84–0.99 (m, 8H), 1.03–1.22 (m, 15H), 2.32 (t, J = 6.0 Hz, 2H), 2.49 (m, 1H), 3.11 (m, 2H), 3.46 (m, 4H), 3.68 (s, 6H), 4.01–4.31 (m, 4H), 6.75 (d, J = 8.5 Hz, 4H), 7.10–7.48 (m, 15H), 7.69 (d, J = 7.5 Hz, 2H) ; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 11.51, 15.38, 19.79, 20.19, 24.25, 24.62, 37.74, 42.92, 43.16, 47.07, 55.07, 57.96, 59.84, 62.46, 66.91, 86.03, 112.99, 119.83, 125.07, 126.68, 127.59, 127.71, 128.15, 130.04, 135.84, 141.15, 143.96, 144.67, 156.41, 158.36, 171.15; MS (ESI): *m/z*: 948.2 (100) $[M+H]^+$, 965.3 (30) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{55}H_{67}N_4O_8PNa$: 965.4594; found: 965.4598.

3-*O*-(4,4′-Dimethoxytrityl)-1-methyl-2-((*N*-Fmoc-methionine)-amino)-propyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4v): R_f = 0.50 (AcOEt/hexane/Et_3N 40:58:2); yield: 75%; ^1H NMR (500 MHz, $CDCl_3$): δ = 0.90–1.21 (m, 15H), 2.02 (m, 5H), 2.32 (t, J = 7.0 Hz, 2H), 2.50 (m, 2H), 3.13 (m, 2H), 3.44 (m, 4H), 3.69 (s, 6H), 4.10–4.33 (m, 4H), 6.77 (m, 4H), 7.12–7.50 (m, 15H), 7.70 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 15.15, 19.66, 20.25, 24.16, 24.48, 29.97, 32.32, 42.93, 43.18, 47.06, 54.18, 55.10, 62.41, 66.98, 86.04, 113.00, 119.88, 125.05, 126.72, 127.01, 127.63, 127.74, 128.16, 130.04, 135.86, 141.17, 144.67, 158.39, 170.85; MS (ESI): *m/z*: 983.4 (100) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{54}H_{65}N_4O_8PSNa$: 983.4158; found: 983.4174.

3-*O*-[4,4′-Dimethoxytrityl]-1-methyl-2-((*N*-Fmoc-trypthophane)-amino)-propyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4w): R_f = 0.38 (AcOEt/hexane/Et_3N 40:58:2); yield: 75%; ^1H NMR (500 MHz, $CDCl_3$): δ = 0.89–1.31 (m, 15H), 2.27 (t, J = 6.0 Hz, 3.05–3.24 (m, 4H), 3.72–3.76 (s, 6H), 4.13–4.25 (m, 4H), 6.79 (m, 4H), 7.24–7.83 (m, 22H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.09, 19.07, 20.00, 24.11, 24.46, 27.92, 42.86, 45.78, 47.02, 54.00, 55.08, 57.82, 57.97, 60.29, 62.67, 67.00, 68.64, 68.77, 86.01, 111.42, 112.88, 117.94, 118.33, 119.79, 120.91, 121.97, 122.04, 123.66, 125.02, 126.71, 126.95, 127.00, 127.49, 128.22, 130.09, 135.72, 135.76, 141.11, 143.55, 143.78, 144.94, 155.89, 158.34, 171.05; MS (ESI): *m/z*: 1038.4 (100) $[M+Na]^+$, HRMS (FAB): *m/z*: calcd for $C_{60}H_{66}N_5O_8PNa$: 1038.4547; found: 1038.4576.

3-*O*-(4-Monomethoxytrityl)-2-(2-((*N*-Fmoc-phenylalanine)-amino)-propyl ester of 1-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4x): R_f = 0.28 (AcOEt/hexane/Et_3N 30:68:2); yield: 70%; ^1H NMR (500 MHz, $CDCl_3$): δ = 1.09–1.20 (m, 12H), 2.46 (m, 2H), 3.09–3.28 (m, 4H), 3.55–3.77 (m, 12H), 4.21–4.44 (m, 4H), 6.83 (m, 2H), 7.17–7.56 (m, 23H), 7.78 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.08, 20.76, 24.28, 34.46, 42.67, 42.79, 46.79, 49.57, 54.85, 55.97, 57.81, 58.01, 58.19, 60.10, 60.92, 66.68, 86.10, 112.84, 117.66, 119.68, 124.89, 126.69, 126.80, 127.44, 127.58, 128.09, 128.28, 128.38, 128.44, 129.05, 130.10, 134.94, 134.99, 136.22, 140.96, 143.44, 143.56, 143.85, 155.52, 158.29, 170.84; MS (ESI): *m/z*: 933.1 (100) $[M+H]^+$, 955.4 (95) $[M+Na]^+$; HRMS (FAB): *m/z*: calcd for $C_{56}H_{61}N_4O_7PNa$: 955.4176; found: 955.4167.

Cyclic oligonucleotide synthesis: All cyclic oligonucleotides and cyclic hybrids were prepared on a DNA synthesizer by means of standard phosphoramidite chemistry and standard synthesis cycles. After coupling the second nucleotide (DMT-off), the columns were removed and 1 mL syringes were attached to both ends of the columns. $(PhO)_3PCH_3I$ (1 mL, 0.5 M in anhydrous DMF) was then passed from one syringe, through the column, to the other syringe for 5 min before the sample was put on a shaker at room temperature for 25 min. The iodination reagent was then removed and the column washed with CH_2Cl_2 (10 mL), CH_3CN (10 mL), and CH_2Cl_2 (10 mL). Cleavage and cyclization of the nucleotides was achieved by soaking the beads in concentrated ammonia at room temperature for 24 h. Cyclic compounds were purified by preparative reverse-phase HPLC with a gradient of 0–20% of acetonitrile over 20 min at a flow rate of 3 mL min^{-1}.

HCV assay: Poly(A) was used as the template (10 μg mL^{-1}), oligu(U)$_{12}$ as the primer (1 μg mL^{-1}), 10 μM α-UTP and 0.025 μCi of [α-^{32}P]UTP as the substrate, in a total volume of 5 μL reaction mix containing 20 mM tris-HCl pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, and purified NS5B sample (0.04 mg mL^{-1}). The reaction was started by adding the template, primer, enzyme, and buffer at 0 °C for 1 h. The inhibitor and the substrate were then added and the reaction mixture was incubated at 30 °C for 60 min. Reactions were terminated by adding 1 volume of stop buffer (95% formamide, 20 mM EDTA (pH 8.0), 0.05% xylene cyanol α,δ-bromophenol blue), and reactions mixtures were kept 1 h at −20 °C.

Aliquots of each reaction mixture (2 μL) were then spotted uniformly onto a Hybond N$^+$ nylon membrane filter, kept at ambient temperature for 15 min, and washed with phosphate buffer (pH 7). Finally, the filter was thoroughly dried and the amount of ^{32}P that remained bound to the Hybond paper was quantified by PhosphoImager analysis.

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