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Regiospecific Solid-Phase Synthesis of Branched Oligoribonucleotides That Mimic Intronic Lariat RNA Intermediates

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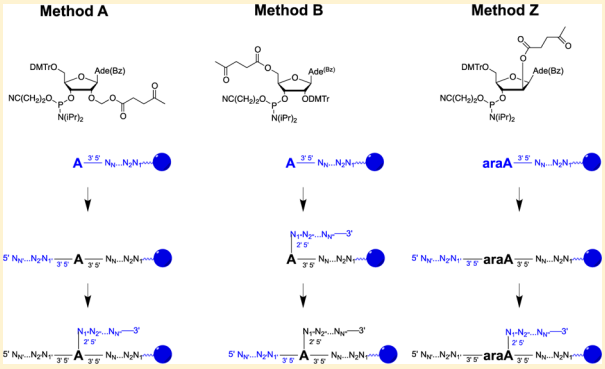
Supporting Information

ABSTRACT: We have developed new solid phase methods for the synthesis of branched RNAs that mimic intronic lariat RNA intermediates. These methods produce branched oligoribonucleotide sequences of arbitrary length, base composition, and regiochemistry at the branchpoint junction. The methods utilize branching monomers that allow for the growth of each branch regioselectively from any of the hydroxyl positions (5′, 3′, or 2′) at the branch-point junction. The integrity and branchpoint connectivity of the synthetic products have been confirmed by HPLC and MS analysis, and cleavage of the 2′,5′ linkage by recombinant debranching enzyme. Nonhydrolyzable branched RNA analogues containing arabinose instead of ribose at the branchpoint junction were shown to inhibit debranching activity and, hence, represent "decoys" for sequestering RNA binding proteins thought to drive amyotrophic lateral sclerosis (ALS).



INTRODUCTION

The spliceosome removes introns from eukaryotic precursor mRNA (pre-mRNA) in the form of RNA lariats that possess unusual 2′,5′-phosphodiester linkages (Figure 1).[1,2] This linkage endows enormous stability to the RNA lariat[3] and must be hydrolyzed by the RNA intron debranching enzyme (Dbr1)[4] before the lariat can be efficiently metabolized or converted into other critical cellular factors such as snoRNA[5] or miRNA.[6] Dbr1 has also been implicated in the propagation of retrotransposons[7] and in the replication of HIV-1 in human cells, ostensibly through a 2′,5′-phosphodiester intermediate that is transiently formed during reverse transcription.[8] However, this claim has since been challenged by other groups.[9,10] Several other branched structures such as multicopy single-stranded DNAs (msDNA) have been detected in living cells,[11,12] all of which contain the same common structural features of lariat RNA, namely, vicinal 2′,5′- and 3′,5′-phosphodiester bonds at a branchpoint nucleotide (Figure 1).

Synthetic branched RNAs (bRNAs) are important model systems for studying bRNA structure,[13] pre-mRNA splicing[14] and intron debranching.[15,16] They also represent lead compounds in the development novel nucleic acid-based therapeutics.[17] For example, inhibition of Dbr1 causes RNA lariats to accumulate in the cytoplasm and act as "decoys" in sequestering toxic RNA binding proteins thought to drive amyotrophic lateral sclerosis (ALS).[17] Structural studies have also recently yielded high-resolution crystal structures of bRNA bound to the Dbr1 active site (Montemayor et al., manuscript in preparation), establishing a platform for rationally designing new and more optimal inhibitors of the enzyme. Toward this end, the synthetic protocols described here allow production of milligram quantities of branched substrates needed for further structural and pharmacological investigation.

Ogilvie and co-workers have prepared branched tri- and tetranucleotides containing identical nucleotides at the branchpoint 2′ and 3′ positions, and their methods have subsequently been adapted to the synthesis of bRNA isomers with different nucleotides at the 2′ and 3′ positions.[18] Regiospecific synthesis of tetranucleotide branchpoints have been performed by Kierzek et al. using solution-phase phosphoramidite chemistry.[19] Chattopadhyaya and co-workers have synthesized and structurally characterized heptameric bRNAs and RNA minilariats with self-cleavage properties.[20,21] Although all of these approaches have their own advantages, they are generally labor intensive, particularly in the synthesis of medium size bRNAs (>5 nucleotides). As a result, much current effort has focused on ways to synthesize larger bRNAs via solid-phase methods. Our first such approach utilized 2′,3′-bis-*O*-phosphoramidite to couple two support-bound RNA

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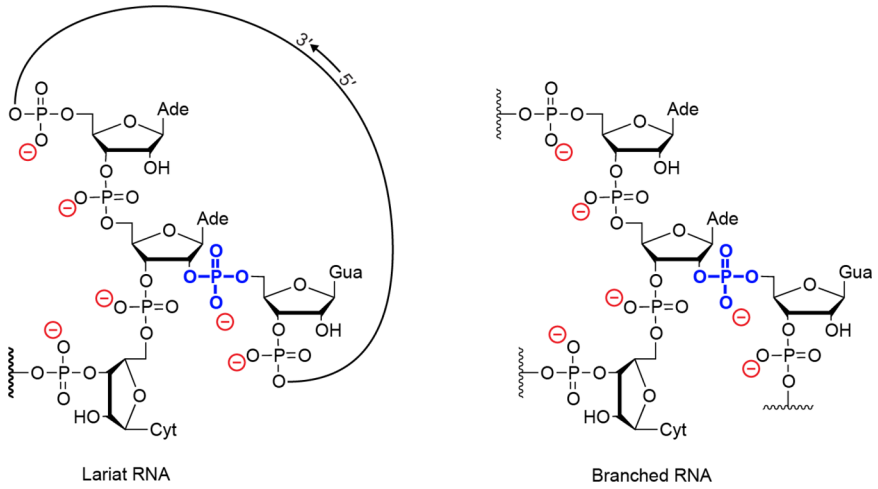

Figure 1. Structures of lariat and branched RNA (bRNA). The 2′,5′-phosphodiester bond found in these RNAs (blue) is hydrolyzed by the RNA intron debranching enzyme Dbr1. After hydrolysis, the phosphate moiety remains attached to the 5′ position of guanosine, and the branchpoint adenosine has a 2′ hydroxyl group.

chains, thus forming a branch juncture with the desired vicinal 2′,5′- and 3′,5′-phosphodiester bonds.[22] With this method, dendritic (multibranched), Y-shaped (branched), and V-shaped (branch lacking the 5′ branch segment) molecules having identical 2′ and 3′ chains were readily assembled.[22,23] The method was later adapted to the synthesis of bRNA mixtures with 2′ and 3′ chains of different base composition.[24]

Sproat and colleagues have reported novel methods toward the regiospecific solid-phase synthesis of small to medium-sized bRNA molecules[25,26] and branched DNA/RNA chimeras.[27] Although elegant, their methodologies necessitated numerous orthogonal protecting groups as well as in-house synthesis of complex sets of phosphoramidite building blocks and branchpoint introduction synthons, making this approach also highly labor intensive. Similarly, a divergent approach has been adapted to the regioselective synthesis of branched DNA[28−30] and branched DNA/RNA chimeras similar to the msDNA molecule of the prokaryote *Myxococcus xanthus*.[31] The latter work relied on the chemoselective removal of the 2′-TBDMS group (embedded within a 2′-fpmp protected RNA) to allow chain extension from the 2′-hydroxyl of the branch with inverted DNA 5′-phosphoramidites. As reverse RNA phosphoramidite monomers were not available at the time, the strategy was initially incapable of generating all-RNA branched structures. Furthermore, extra care was necessary to prevent cleavage of the oligomer from the CPG solid support during fluoride-mediated cleavage of the TBDMS group. Silverman and colleagues have introduced an elegant, deoxyribozyme-based system that catalyzes the formation of 2′,5′-branched RNA linkages with very high selectivity.[32] In contrast to synthetic methods, this approach is limited by synthesis scale (pico/nanomol), a requirement of a deoxyribozyme (>70 nucleotides), limited sequence tolerance at the branchpoint nucleotide, and variable binding domain sequence of the excess DNA "catalyst" used.

Here, we describe reliable procedures for large-scale, regiospecific solid-phase synthesis of bRNAs with mixed base compositions. Our method utilizes branch-site monomers that allow for the growth of each branch regioselectively from any of the hydroxyl positions (5′, 3′, or 2′), providing an end product of TBDMS-protected bRNA molecules that can be released from the support and deprotected following well established RNA synthesis methods.[33] Ready availability of the forward and reverse RNA phosphoramidite monomers and trivial synthesis of the branchpoint monomers allows this approach to be used in synthesizing milligram (micromol) quantities of bRNA of arbitrary length and base composition around the branch-point junction, limited only by the total length of the oligonucleotides. Additionally, we describe a third regioselective approach to synthesize branched compounds whose branchpoint sugar moiety has been modified to an arabinose.

■ RESULTS AND DISCUSSION

The present work is an extension of our previously described[31] "divergent" method for the synthesis of chimeric branched DNA-RNA and focuses on the synthesis of branched nucleic acids consisting entirely of ribonucleotide units (see Scheme 2A,B). A key development of the present work is the use of branch-site 2′-acetal levulinic ester (ALE) (**A**) and 2′-dimethoxytrityl (DMTr) (**B**) protected monomers that are used at the branchpoint and their use in conjunction with the reverse (**R**) and standard (**S**) 2′-TBDMS monomers. A suitable protecting group for the 2′-hydroxyl of the branchpoint must be orthogonal to protecting groups on the sugar (5′ and 3′ hydroxyls) and the nucleobases (exocyclic amines). It must additionally be orthogonal to the TBDMS protecting group at the 2′ hydroxyls of all remaining nucleotides, so that only the 2′ hydroxyl of the designated branchpoint can be deprotected selectively. The ALE and DMTr groups meet this requirement as they can be removed by hydrazinolysis or acidolysis, respectively, and these conditions do not affect the 2′-TBDMS or amide-based *N*-protecting groups generally used for RNA synthesis.

Synthesis of Branchpoint Monomers. 3′,5′-Bis-silylated N-Bz adenosine with a thiomethyl ether in position C2′ (**1**)[34] was activated with SO_2Cl_2 and reacted with cesium bicarbonate activated levulinic acid to yield the acetal levulinic ester (ALE) protected compound **2** in 59% yield.[35] The ALE group[35] is used instead of levulinic ester to avoid migration of the ester from 2′ to 3′. Subsequent cleavage of the silyl protection yielded compound **3** in 73% that was tritylated and phosphitylated to give compound **A** (Scheme 1A). Our lab has previously developed 2′-DMTr protected guanosine phosphoramidites for the synthesis of oligoribonucleotides

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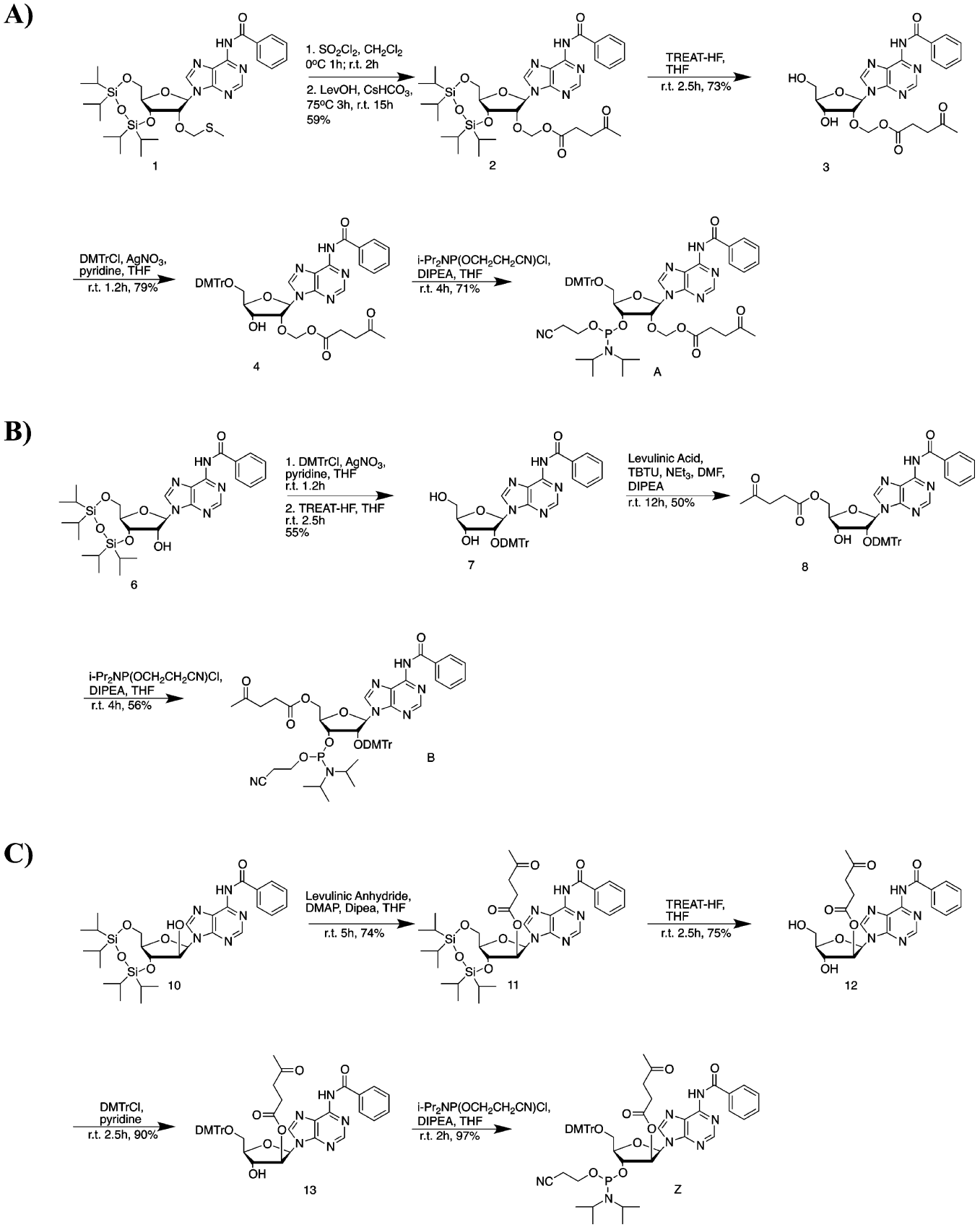
Scheme 1. Synthesis of Branchpoint Synthons



with a 2′ phosphate monoester moiety.[36] Compound **6** was protected with DMTr in position C2′ followed by silyl deprotection with triethylamine trihydrofluoride (TREAT-HF) to give compound **7** in 55% yield. Compound **7** was

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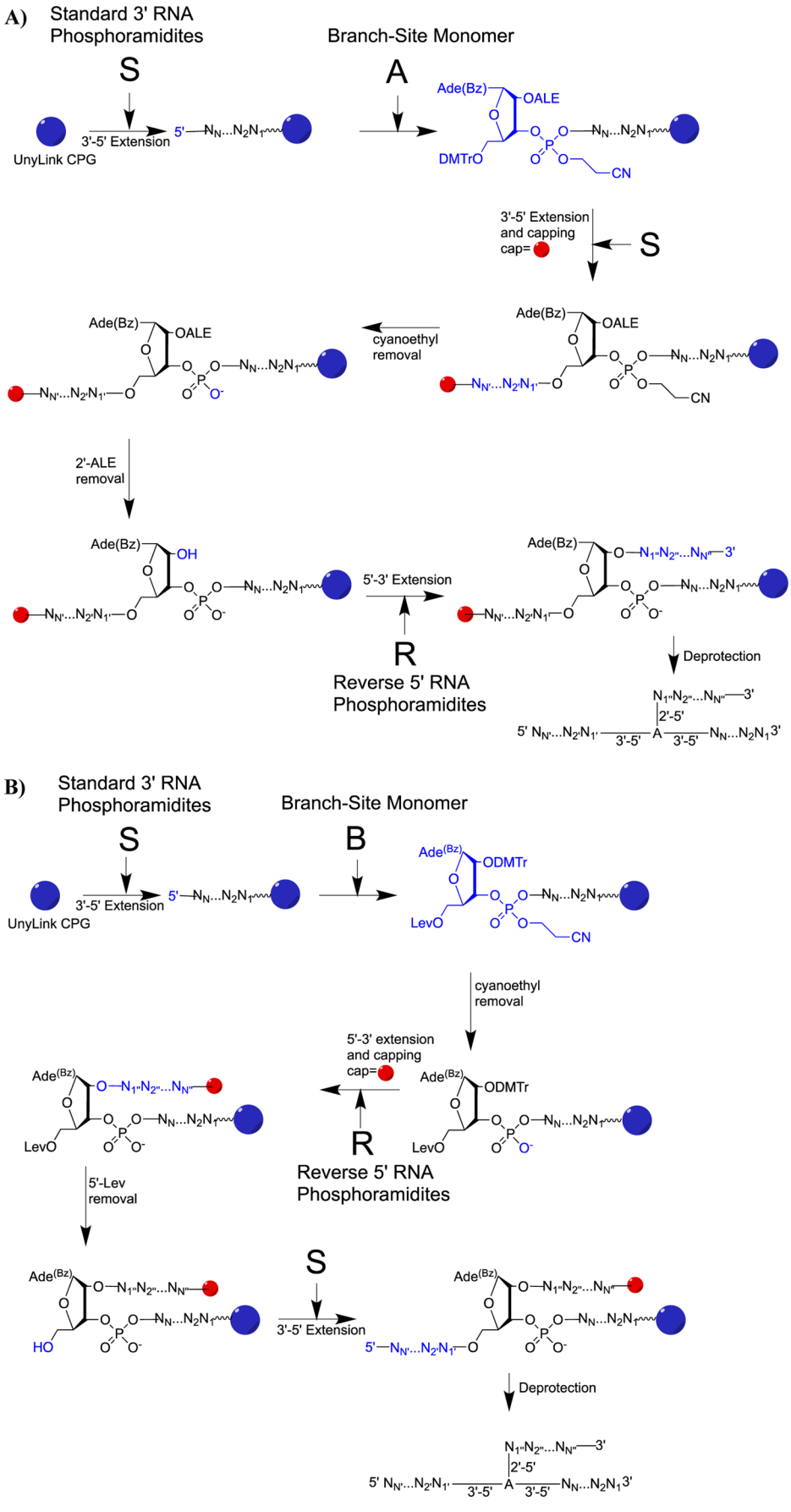
Scheme 2. Solid-Phase Synthesis of Branched RNAs



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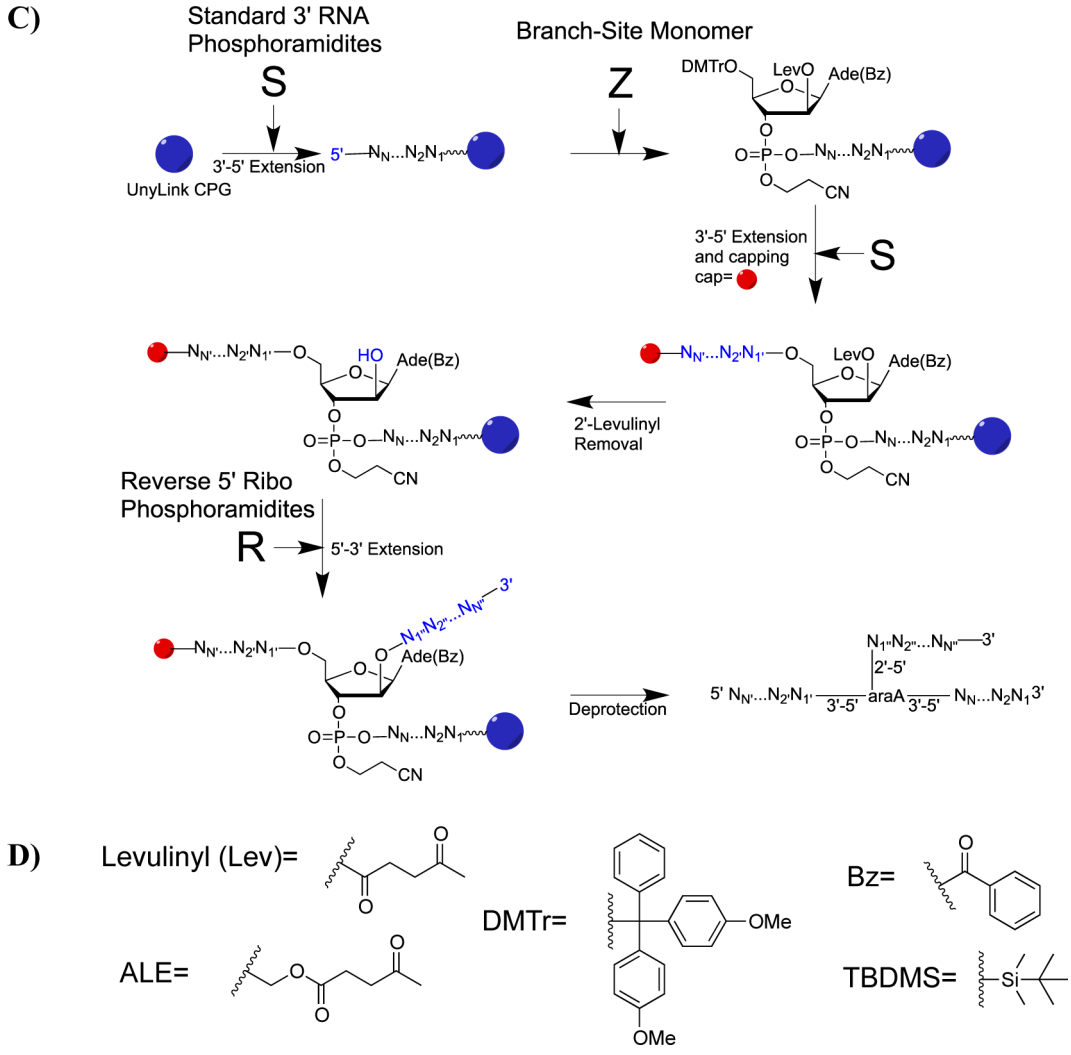
Scheme 2. continued



then treated with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and levulinic acid to selectively install the levulinyl group on the 5′-hydroxyl in 50%. Finally, phosphitylation of the 3′-hydroxyl group afforded branching monomer **B** in 56% yield (Scheme 1B). The 5′,3′ silyl protected *N*-Bz arabinoadenosine (**10**) was reacted with levulinic anhydride in the presence of *N*,*N*-dimethylaminopyridine (DMAP) to yield compound **11** in 74% yield. Subsequent cleavage of the silyl protection yielded compound **12** in 75% that was tritylated and phosphitylated to give compound **Z** (Scheme 1C). NMR characterization data (spectra) are provided in the Supporting Information.

Synthesis of Native Branched RNAs by Method A. This method synthesizes the 2′ branched segment from an adenosine unit located in the middle of the RNA chain. The key features of this method are (a) solid-supported synthesis of an RNA strand (via the incorporation of standard TBDMS RNA monomers (**S**)) bearing a single 2′-ALE unit at the branch-site monomer (**A**); (b) selective sequential removal of cyanoethyl phosphodiester groups followed by the 2′-ALE group; and (c) 5′ to 3′ growth of the 2′-branch segment using reverse RNA monomers (**R**) (Scheme 2A). Specifically, the first two branch segments are synthesized in the conventional 3′ to 5′ direction using commercially available 2′-TBDMS 3′-phosphoramidite monomers followed by branching synthon **A**, thus yielding a linear RNA chain (5′-$N_{N'}$...$N_{2'}N_{1'}$-$A_{2'\text{-ALE}}$-N_N...N_2N_1-3′-CPG). After capping of the 5′ end, the cyanoethyl phosphodiester protecting groups are removed by treatment with NEt_3/MeCN at room temperature for 90 min. This is a necessary step to perform before removal of the 2′-ALE group to prevent isomerization or cleavage at the branchpoint 3′,5′-internucleotide linkage. The 2′-ALE groups are then removed by treatment with buffered 0.5 M hydrazine hydrate at room temperature for 15 min. The elongation of the last branch from the 2′ position of nucleotide (**A**) is then achieved using commercially available reverse 5′ phosphoramidites, thereby generating the branched RNA. To force branching at the sterically hindered 2′-hydroxyl group, both the concentration and the coupling time of the first 5′-amidite were increased to 0.3 M and 30 min, respectively.[30]

Synthesis of Native Branched RNAs by Method B. In contrast to Method A, this method introduces the 2′ branch segment earlier in the synthesis, immediately after coupling of the branch-site monomer (**B**) (Scheme 2B). The first branch is synthesized in the conventional 3′ to 5′ direction and completed by the incorporation of synthon (**B**) at the 5′ end, producing $^{5'\text{-Levulinyl}}A_{2'\text{-DMTr}}$-$N_N$...$N_2N_1$-3′-CPG. Next, the cyanoethyls are cleaved by triethylamine in acetonitrile (2:3 v/v) and the 2′-DMTr group removed, allowing the growth of a 2′ chain using reverse 5′-phosphoramidites (**R**). As for method A,

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Table 1. Solid-Phase Synthesis of Branched RNAs

molecule	sequence	method	HPLC yield (%)	isolated yield (%)	calculated molecular weight (Da)	calculated exact mass (Da)	observed mass (m/z)
I	UAA(2′GU)CA	A	56	25	2188.4	2187.3	2187.3
I	UAA(2′GU)CA	B	54	24	2188.4	2187.3	2187.4
IZ	UAaraA(2′GU)CA	Z	64	21	2188.4	2187.3	2187.3
II	UAA(2′GUAUGU)GU	A	44	26	3491.1	3490.5	3491.5
II	UAA(2′GUAUGU)GU	B	46	20	3491.1	3490.5	3491.4
IIZ	UAaraA(2′GUAUGU)GU	Z	67	32	3491.1	3490.5	3491.5
III	UAA(2′GU)GUAUGU	A	40	11	3491.1	3490.5	3491.7
III	UAA(2′GU)GUAUGU	B	45	26	3491.1	3490.5	3491.7
IIIZ	UAaraA(2′GU)GUAUGU	Z	65	33	3491.1	3490.5	3491.5
IV	UACUAA(2′GUAUG) CAAGU	A	19	6	5090.1	5087.7	5089.2
IV	UACUAA(2′GUAUG) CAAGU	B	27	10	5090.1	5087.7	5089.2
IVZ	UACUAaraA(2′GUGUG) CAAGU	Z	55	26	5106.1	5103.7	5106.7
IVZ2	UACUAaraA(2′GUAUG) CAAGU	Z	62	18	5090.1	5087.7	5087.7

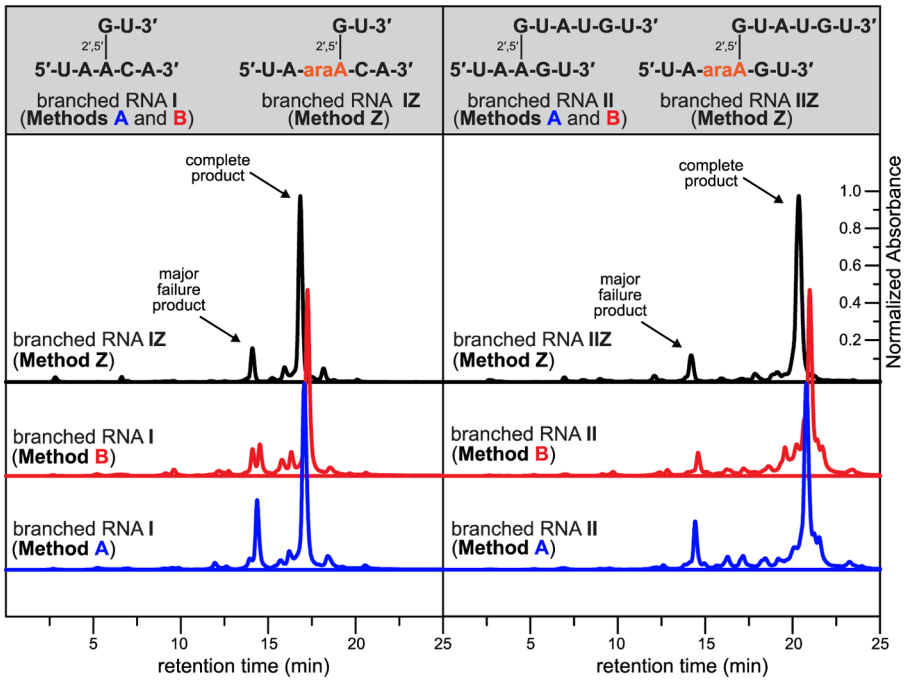

Figure 2. Anion-exchange HPLC chromatograms of crude synthetic products for branched oligonucleotides **I** and **II**, generated by Methods A and B, and equivalent length syntheses of molecules **IZ** and **IIZ**, generated by Method Z.

the concentration and the coupling time of the first 5′-phosphoramidite is increased to 0.3 M and 30 min, respectively. Capping and removal of the 5′-levulinyl group from the branchpoint are carried out in buffered 0.5 M hydrazine hydrate at room temperature for 15 min, which permits completion of the Y-shaped branched RNA molecule through the coupling of standard 3′ phosphoramidite monomers (**S**). Analogous to our Method B, branch and hyperbranch RNAs constructed from a 2′-MMTr uridine, 5′-levulinyl, 3′-phosphoramidite branchpoint synthon[37] were effectively prepared by Sabatino and co-workers for down-regulating GRP78 expression and inducing cell death in HepG2 liver cancer cells.[38]

Synthesis of Arabinose Modified Branched RNAs. In addition, we have refined the synthesis of substrates with an altered configuration at the C2′ of the branchpoint sugar. Specifically, we have prepared arabinoadenosine derivatives, where the 2′ hydroxyl group is *cis*-oriented with respect to the nucleobase, and incorporated it at the branching point. Branched compounds with this modification are not hydrolyzed by Dbr1 and have been previously shown to inhibit the debranching enzyme toward native lariat RNAs in pre-mRNA splicing.[39,40] However, until now, the synthetic methodology employed to generate these compounds required a 2′,3′-bis-*O*-phosphoramidite of the arabinose nucleoside that was coupled simultaneously to two growing RNA chains.[39,40] To achieve 2′ and 3′ and regioselectivity at the branchpoint, as well as increase the overall yield of branched RNA synthesis, our approach utilizes a novel 2′-*O*-levulinylated arabinose monomer (**Z**) (Scheme 2C). The synthetic strategy for preparing the arabinose modified bRNA is almost identical to Method A, with the exception that the decyanoethylation step becomes

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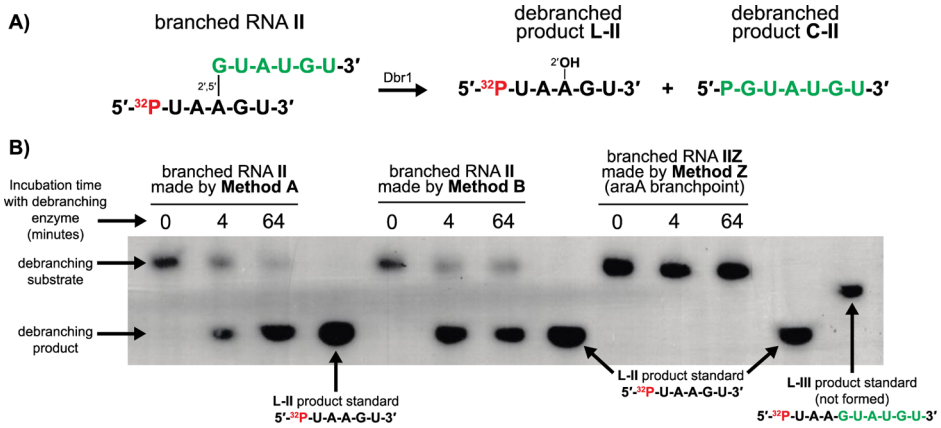

Figure 3. Polyacrylamide gel electrophoresis of ^{32}P-labeled branched oligoribonucleoitdes before and after treatment with Dbr1. (A) Anticipated debranching reaction of radiolabeled compound **II**, showing expected products **L-II** and **C-II**. (B) Radiolabeled compounds generated by Methods A and B are indeed substrates of the debranching enzyme. Inhibitor **IIZ** (generated by Method Z) is not hydrolyzed by the debranching enzyme. The **L-III** standard confirms the absence of unintended 2′,3′ isomerization during synthesis, which would not be detectable via mass spectrometry of isolated bRNA products (Table 1). In addition, this figure can be used to assess the quality of the purified branched products.

unnecessary as the 2′ hydroxyl of the arabinose branchpoint is no longer *cis* to the adjacent 3′,5′ phosphotriester linkage.

Optimization of bRNA Synthesis. Four different branched compounds were prepared using each of the above-mentioned methods (Table 1). The first was a short heptamer with two nucleotides branching off from the central adenosine unit (molecules **I** and **IZ**; Table 1). Molecules **II** and **III** (as well as **IIZ** and **IIIZ**) were 11-mers, each having 2 nucleotide units on two branch segments, while the remaining segment was extended to 6 nucleotides. Finally molecule **IV** was a 16-mer with 5 nucleotides on each branch segment.

Preliminary syntheses of these compounds produced the desired products; however, the HPLC traces of the crude materials contained significant amounts of failure sequences (HPLC analysis; Supporting Information Figure S1A). Thus to augment the yields we sought to synthesize bRNA **II** via Method A while modifying several essential synthetic parameters. Once optimized, these parameters were applied to Method B as well. Among the parameters tested were coupling agents (5-ethylthio-1*H*-tetrazole versus 4,5-dicyanoimidazole), deprotection time (delevulination and decyanoethylation), and nucleoside loading on the solid support (CPG). The products from these trials were assessed by HPLC (Supporting Information Figure S2). It was found that the yields were most affected by reduction of the decyanoethylation time from 3 to 1.5 h and reduction of the delevulination time from 30 to 15 min. The latter change, which actually increased yields, might be significant considering the potential risk of loss of certain base protecting groups upon prolonged treatment with hydrazine. We were unable to obtain any branched product with a CPG support having a loading of 78 μmol/g; however, supports with 30−40 μmol/g loadings afforded branched products in good yields (Supporting Information Figure S2). The conditions that were best yielding (Supporting Information Figure S2) were chosen to resynthesize the bRNAs listed in Table 1.

After synthesis and deprotection, the crude products were analyzed by anion exchange HPLC (Figure 2, Supporting Information Figure S1B) and found to contain the expected full-length products. Isolated in yields ranged from 6 to 26% (Table 1). The high purity of the isolated bRNAs (77−99%; average purity: 92%) was confirmed by PAGE and HPLC (Figure 3; Supporting Information Figures S3 and S4). In terms of yields, Methods A and B afforded comparable results (Table 1). Longer sequences generally provided less material (6−10%) than shorter sequences (11−26%), regardless of the method used (Table 1). Incorporation of arabinose (via Method Z) afforded slightly cleaner crude products (up to 66% by HPLC) and higher isolated yields (up to 33%). In Methods A and Z, and to a lesser extent B, there is one major failure product, which, on the basis of retention time and mass spectrometry (Figure 2; see also Supporting Information Figure S5), corresponds to a linear product that has not undergone branching at C2′. This unbranched species likely arises from inefficient coupling of the first 5′-phosphoramidite despite the higher concentration (0.3 M) used. This results from the sterically hindered 2′-hydroxyl that is vicinal to a phosphodiester bond (in the case of branchpoint ribose) or adenine base (in the case of the arabinose branchpoint), which reduces overall coupling efficiency.[31] This coupling defect was observed to a lesser extent in Method B, possibly as coupling at the 2′ branch segment takes place before the 5′ segment is introduced, thereby reducing steric strain at that step.

HPLC analysis of products generated from Methods A and B reveals the presence of failure products that elute before the intact bRNA product but after the unbranched side product, suggesting a significant reduction in the stepwise coupling yields after initial branchpoint formation. One potential source of this problem is the decyanoethylation step preceding the deblocking of a 2′ vicinal hydroxyl. In this step, the cyanoethyl groups are removed in a triethylamine solution, thus converting all existing phosphotriesters to phosphodiesters, a mandatory action that prevents 2′,3′ isomerization and/or strand cleavage due to nucleophilic attack from the deblocked 2′ vicinal hydroxyl (Supporting Information Figure S6B).[41−43] This has been observed and solved by decyanoethylation during the regiospecific solution phase synthesis of branched ribonucleotides.[19] However, this process also deblocks cyanoethyl groups from the phosphotriesters of all previously incorporated nucleotides. It has been reported that this conversion inhibits subsequent couplings and reduces the yield of each step from 99 to 93%.[44] It was assumed that the main cause of the coupling inhibition is the presence of several phosphodiester linkages, which are competing with the 2′ hydroxyl for activated

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amidite. However, subsequent couplings can be restored to 96–97% efficiency by washing the growing oligonucleotide with 0.1 M DBU and 0.1 M 1*H*-tetrazole after each detritylation step to form a tetrazolium salt with the phosphate.[44] Unfortunately, in our hands, this protocol failed to improve postbranching coupling yields (Supporting Information Figure S2). Evidently, this limitation does not affect synthesis of the arabinose bRNAs as it lacks the decyanoethylation step, since the arabinose 2′ hydroxyl is *trans* to the 3′ phosphotriester and prevents 2′-3′ isomerization or strand cleavage.

Characterization of Branched Compounds by Exposure to Debranching Enzyme (Dbr1). Samples of branched RNA **II** (synthesized by Methods A and B) were exposed to a recombinant preparation of the intron debranching enzyme from *Entamoeba histolytica* (Dbr1) in order to determine the efficacy of this compound as a mimic of cellular lariat and branched RNAs. The samples were radiolabeled at the 5′ position in order to assay the debranching reaction in vitro using denaturing polyacrylamide gel electrophoresis. In this assay, only one of the two products is visible after debranching and has a significantly enhanced migration through the gel relative to the unhydrolyzed substrate (Figure 3 and Supporting Information Figure S3). The resulting data confirm bRNA **II** is bona fide substrate of the enzyme. As expected from previous studies, the arabinoadenosine branched derivative **IIZ** is not hydrolyzed by the enzyme. These findings are consistent with other preparations of bRNA made previously in our lab and elsewhere.[39,40]

In addition, some of the products of hydrolysis were purified by HPLC (Figure 4 and Supporting Information Figure S7) and their composition analyzed by MS (Supporting Information Figure S7). The data confirm these products match the anticipated masses for RNA chains independent of the synthetic methods used, confirming Methods A and B are regioselective around the branchpoint.

Inhibition of Dbr1 by Arabinose Branched Analogues. As the product of Method Z is not hydrolyzed by Dbr1, and similar compounds have also been shown to inhibit the enzyme,[39,40] we evaluated the effect of increasing concentrations of molecules **IZ** and **IVZ** on debranching activity toward a native branchpoint. In this approach, molecule **II** (generated by Method A) was ^{32}P-labeled and exposed to Dbr1, and the debranching products were monitored by denaturing PAGE electrophoresis. Concentrations of **IZ** or **IVZ** and linear control UAACA (**L-I**) were increased from 0 to 32 μM (Figure 5 and Supporting Information Figure S8). The results were subsequently subjected to densitometry calculations, from which the maximal reaction progression was determined. The resulting data show that both arabinose molecules **IZ** and **IVZ** inhibit Dbr1 and that **IVZ** is apparently more potent.

CONCLUSIONS

In summary, we have synthesized various bRNAs of distinct sequences using two different approaches. An additional approach was developed to generate branched compounds containing an arabinose branchpoint. The purity of the crude products varied between the methods and the compounds: Method Z, with an arabinose branchpoint, generated the highest yield of branched compound, likely because it avoids a decyanoethylation step that reduces the yields of all subsequent coupling steps. Exposure to Dbr1 resulted in hydrolysis

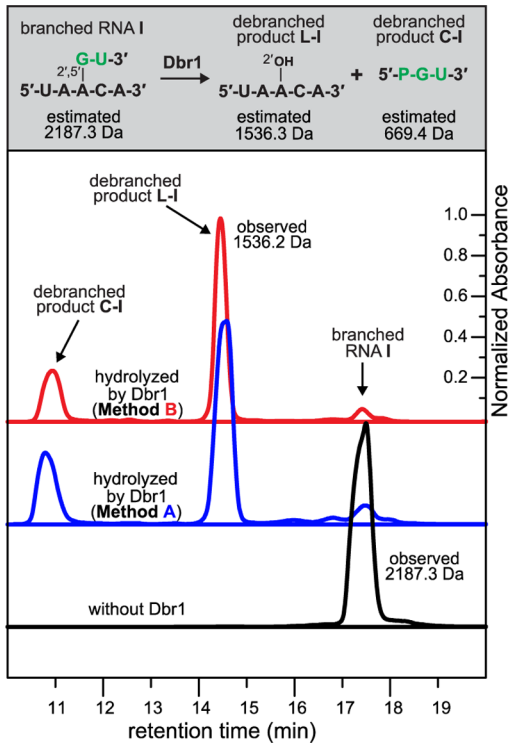

Figure 4. Anion-exchange chromatograms of substrate **I** before and after treatment with debranching enzyme Dbr1. Near-complete debranching is observed for substrates made by Methods A and B. It was not feasible to measure an experimental mass for purified product **C-I**.

products of the correct length and revealed that the arabinoadenosine compound made by this approach can inhibit debranching activity. These findings will aid in the future development and large-scale production of therapeutic bRNA compounds.

EXPERIMENTAL SECTION

Solid Phase Synthesis of Branched RNAs. *Materials and Reagents.* Branched oligonucleotide syntheses were carried out using a DNA/RNA synthesizer and a universal (UnyLink) solid support on a 1 μmol scale (loading 29.9 μmol/g). Conventional and reverse 2′ TBDMS and bis-cyanoethyl-*N*,*N*-diisopropyl phosphoramidite were used (0.15 M in MeCN). Phosphoramidites were dissolved in MeCN and activated with 5-ethylthio-1*H*-tetrazole (0.25 M in MeCN). Capping was carried out by the simultaneous delivery of acetic anhydride in pyridine/THF and *N*-methylimidazole (16% in THF) and contacting the solid support for 6 s. Oxidation of phosphite triester intermediates was effected with 0.1 M iodine in pyridine/H_2O/THF (20 s). A solution of 3% trichloroacetic acid in THF, delivered over 1.8 min, was used to deblock DMTr groups. Anhydrous triethylamine/MeCN (2:3 v/v) and 0.5 M hydrazine hydrate in pyridine/acetic acid (3:2 v/v) were used to remove, respectively, cyanoethyl (CNEt) phosphate and Levulinyl/ALE protecting groups. Oligonucleotides were deblocked and released from the solid support using a concentrated ammonium hydroxide solution. TBDMS groups were cleaved with TREAT-HF.

Method A (Scheme 2A). 5′-$N_{N'}$...$N_{2'}N_{1'}$-$A_{2'\text{-ALE}}$-N_N...N_2N_1-3′ was grown on the solid support (1 μmol columns) in the conventional 3′ to 5′ direction using standard 3′ RNA phosphoramidites (denoted as **S** in Scheme 2). The $A_{2'\text{-ALE}}$ unit in this sequence was introduced by coupling monomer (**A**) for 10 min (0.13 M in MeCN, activator: 5-ethylthio-1*H*-tetrazole). Subsequently the 5′-DMTr was removed, and the deblocked hydroxyl was capped affording a 5′-*O*-acetylated linear oligomer. Anhydrous triethylamine/MeCN (2:3 v/v) solution was

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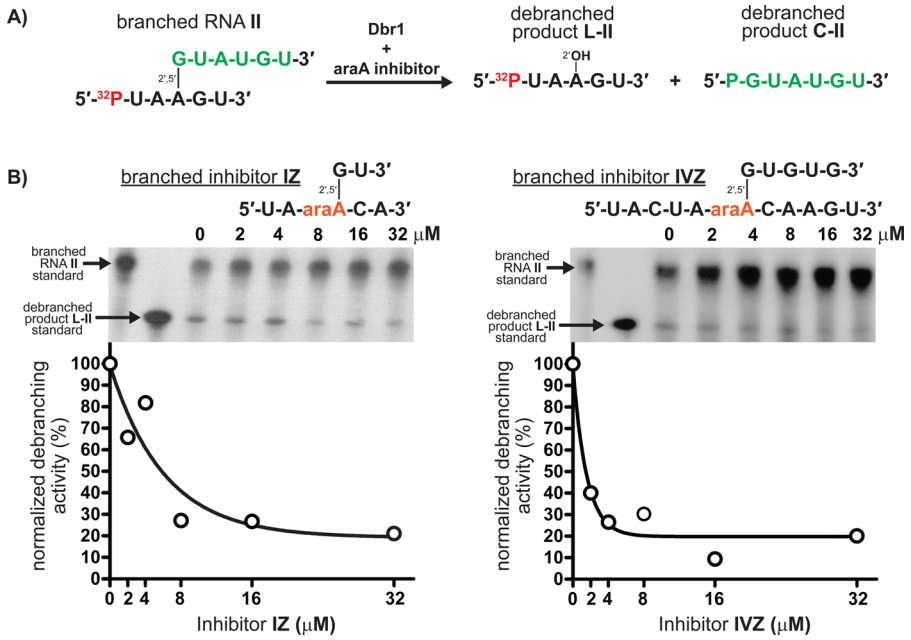

Figure 5. bRNAs with arabinoadenosine branchpoints are effective inhibitors of Dbr1. (A) Anticipated debranching reaction for substrate **II**, which contains a natural adenosine branchpoint. (B) The arabinoadenosine compounds inhibit debranching of substrate **II** in a dose-dependent fashion. The larger arabinoadenosine compound **IVZ** is apparently a more effective inhibitor than the smaller compound **IZ**. A similar titration series with product analogue 5′-UAACA-3′ (**L-I**) was used to ensure the observed inhibition is specific to the arabinoadenosine branchpoint (Supporting Information Figure S8).

passed through the solid support and allowed to react for 20 min. This step was repeated four times to ensure complete removal of the cyanoethyl phosphodiester protecting groups. After washing with MeCN (5 min) and drying the support over Ar stream (10 min), the synthesis columns were temporarily removed from the synthesizer and placed in a round-bottom flask under a high vacuum for one hour. The columns were placed back on the synthesizer, and a freshly prepared solution of 0.5 M hydrazine hydrate in pyridine/acetic acid (3:2 v/v) was flowed through the columns for 20 s and allowed to react for 3.75 additional minutes. This step was repeated four times to ensure complete removal of the 2′-ALE groups. After washing (MeCN, 10 min) and drying (Ar gas, 10 min), the solid supports were dried under a high vacuum (1 h). The synthesis columns containing the solid supports were returned to the synthesizer, and a reverse phosphoramidite corresponding to $N_{1''}$ position (0.3 M in MeCN) was coupled for 30 min. The remaining units were coupled for 15 min at the same concentration, and completion of this branch segment afforded the fully protected branched RNAs.

Method B (Scheme 2B). $^{5\prime\text{-Levulinyl}}A_{2'\text{-DMTr}}\text{-}N_N...N_2N_1\text{-}3'$ was grown on the solid support (1 μmol columns) in the conventional 3′ to 5′ direction using standard 3′ phosphoramidites (S). The $^{5\prime\text{-Levuliny-}l}A_{2'\text{-DMTr}}$ unit in this sequence was introduced by coupling monomer (**B**) for 15 min (0.15 M in MeCN, activator: 5-ethylthio-1*H*-tetrazole). Anhydrous triethylamine/MeCN (2:3 v/v) solution was passed through the solid support and allowed to react for 20 min. This step was repeated four times to ensure complete removal of the cyanoethyl phosphotriester protecting groups. After washing with MeCN (5 min) and drying the support over Ar stream (10 min), the synthesis columns were temporarily removed from the synthesizer and placed in a round-bottom flask under a high vacuum for one hour. The columns were placed back on the synthesizer, and the growing oligonucleotide was then detritylated and extended by units $N_{1''}N_{2''}...N_{N''}$ in the 5′ to 3′ direction. Subsequently the 3′-DMTr was removed, and the deblocked hydroxyl was capped affording a 3′-*O*-acetylated V-shaped oligomer. Subsequently, a freshly prepared solution of 0.5 M hydrazine hydrate in pyridine/acetic acid (3:2 v/v) was flowed through the columns for 20 s and allowed to react for 3.75 additional minutes. This step was repeated four times to ensure complete removal of the 2′-ALE groups. After washing (MeCN, 10 min) and drying (Ar gas, 10 min), the solid supports were dried under a high vacuum (1 h). The synthesis columns containing the solid supports were returned to the synthesizer, and 3′-5′ synthesis of units $N_{1'}N_{2'...}N_{N'}$ resumed. For the final branch segment the phosphoramidites were dissolved in 0.3 M MeCN, and coupling time was set to 30 min for the first unit and 15 min for the remaining ones.

Method Z (Scheme 2C). $5'\text{-}N_{N'}...N_{2'}N_{1'\text{-Ara}}A^{2'\text{-Levulinyl}}\text{–}N_N...N_2N_1\text{-}3'$ was grown on the solid support (1 μmol columns) in the conventional 3′ to 5′ direction using standard 3′ RNA phosphoramidites (denoted as **S** in Scheme 2). The $_{Ara}A^{2'\text{-Levulinyl}}$ unit in this sequence was introduced by coupling monomer (**Z**) for 10 min (0.13 M in MeCN, activator: 5-ethylthio-1*H*-tetrazole). Subsequently the 5′-DMTr was removed, and the deblocked hydroxyl was capped affording a 5′-*O*-acetylated linear oligomer. The sample was then washed with MeCN (5 min), and the support was dried over Ar stream (10 min). Subsequently, a freshly prepared solution of 0.5 M hydrazine hydrate in pyridine/acetic acid (3:2 v/v) was flowed through the columns for 20 s and allowed to react for 3.75 additional minutes. This step was repeated four times to ensure complete removal of the 2′-Levulinyl groups. After washing (MeCN, 10 min) and drying (Ar gas, 10 min), the solid supports were dried under a high vacuum (1 h). The synthesis columns containing the solid supports were returned to the synthesizer, and a reverse phosphoramidite corresponding to $N_{1''}$ position (0.3 M in MeCN) was coupled for 30 min. The remaining units were coupled at 0.15 M for 15 min, and completion of this branch segment afforded the fully protected branched RNA analogues.

Deprotection. Solid supports were transferred into a screw cap centrifuge tube into which 250 μL of anhydrous ethanol and 750 μL cold (−20 °C) concentrated ammonia were added. The samples were shaken for 48 h at room temperature to simultaneously remove the remaining cyanoethyl groups, deblock base protecting groups, and release the oligonucleotides from the support. Samples were centrifuged for 10 min at 11000*g*. The supernatant was cooled over dry ice for 5 min and evaporated using a speedvac evaporation system. TREAT-HF (150 μL/μmol of branched RNA) was added, and the solution was shaken for 48 h at room temperature. The crude branched compounds were isolated by adding cold *n*-butanol (1 mL, −20 °C), followed by 3 M NaOAc (30 μL; pH 5.5), and centrifuging the resulting mixture at 11000*g*. After removing the supernatant, the

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remaining pellet (which contained the bRNA) was dried, taken up in DEPC-treated autoclaved water (1 mL), and filtered.

Purification. Branched compounds were purified by HPLC (chromatograms not shown) using an anion exchange column (Protein PAK DEAE 5PW 21.5 mm × 15 cm). The buffer system consisted of water (solution A) and 1 M aq lithium perchlorate (solution B), at a flow rate of 4 mL/min. The gradient was 0−40% B over 50 min at 60 °C. Under these conditions the desired peaks eluted at 25−35 min. The collected samples were lyophilized and redissolved in 1 mL of autoclaved Milli-Q H_2O and loaded into a pre-eluted size exclusion column filled with NAP-25 matrix. The column was eluted with more Milli-Q H_2O and 7 × 1 mL fractions were collected. Each of these fractions was quantified by absorbance (λ = 260 nm) and assayed for salt content (λ < 230 nm). To obtain the number of nanomoles, a conversion factor was used, (12.99 nmol/OD for bRNA **I**, 8.42 nmol/OD for bRNAs **II**, **IIZ**, **III**, **IIIZ** and 6.04 for bRNA **IV** and **IVZ**) as calculated for a linear RNA of the same sequence by the IDT DNA oligoanalyzer program.[45] The masses of the molecules were confirmed by LC−MS ESI-Q-TOF or ESI-TOF (Table 1, Supporting Information Figure S9). The quality of the purified products was also assessed by reinjection into the HPLC (Supporting Information Figure S4).

Oligonucleotide Handling. Before and after purification, the oligonucleotides were handled with sterilized (16 psi, 125 °C 30 min) pipet tips, microcentrifuge tubes, Sephadex (size-exclusion NAP-25 columns), and diethylpyrocarbonate (DEPC) treated water (less than 1 month old). Oligonucleotides were then typically stored at −20 °C.

Radiolabeling the bRNA Samples. 100 pmol of sample was reacted with γ-^{32}P-ATP in presence of T4 polynucleotide kinase (2000 units) and 1× PNK buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, pH 7.6 at 25 °C). The reaction was carried out in a total volume of 20 μL at 37 °C for 1 h. After completion of the reaction, the mixture was loaded into a size-exclusion column (NAP-10), eluted with sterilized water, and collected as 500 μL fractions. The early most radioactive fractions were combined, evaporated, and redissolved to 20 000 CPM/μL.

Analytical HPLC. The crude samples as well as the products of the debranching assays were assayed on HPLC using an anion exchange column (Protein DEAE 5PW 7.5 × 75 mm). The buffer system consisted of water (solution A) and 1 M aq lithium perchlorate (solution B), at a flow rate of 1 mL/min. The gradient was 0−24% solution B over 30 min at 65 °C Under these conditions the desired peaks eluted at roughly 18 min for compound **I** and 21.5 min for compounds **II**, **IIZ**, **III**, and **IIIZ** (Figure 2 and Supporting Information Figures S1, S2, and S5).

Expression and Purification of Debranching Enzyme. A codon-optimized version of the *DBR1* gene from *Entamoeba histolytica* was cloned into a modified pET15b plasmid for expression of a hexahistidine-tagged protein in *Escherichia coli*. The protein was purified using immobilized metal ion chromatography and cation exchange chromatography. SDS-PAGE was used to confirm the purity of the protein used in activity assays. A more detailed purification protocol is to be reported elsewhere (Montemayor et al., manuscript in preparation). The purified protein was found to be very sensitive to repeated cycles of freezing and thawing, with an approximate 2-fold decrease in observed debranching activity for each freeze/thaw cycle.

Debranching Assays and Analysis by HPLC. Purified branched RNA (10 nmol) was digested by 20 pmol of debranching enzyme in 100 μL debranching buffer (50 mM NaCl, 10 mM HEPES, 10 mM $MnCl_2$, 10% glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP), pH 7.0) for 2 h. The reaction mixture was directly subjected to anion exchange HPLC purification and peaks collected, dried down, and subsequently analyzed by mass spectrometry.

Debranching Assays and Analysis by Autoradiography. Reactions were carried out at 2 μM substrate concentrations (including 800 CPM/μL radioactive substrate), 10 nM enzyme in debranching buffer (100 mM NaCl, 20 mM HEPES, 10% glycerol, 1 mM TCEP, 0.1 mM $MnCl_2$, pH 7.0). Aliquots were removed and added to an equal volume of formamide gel loading buffer to quench the reaction. The products were heated to 95 °C and loaded onto a 16% polyacrylamide gel with 7 M Urea and Tris-Borate-EDTA buffer in an electrophoresis apparatus. After 2−3 h at 1000 V, the gel was frozen in a cassette with an X-ray film overnight, and developed.

Inhibition Assays and Analysis by Autoradiography. Inhibition reactions were performed at 28 nM substrate, 10 nM enzyme and an identical buffer to the above debranching assays. Reactions were carried out in the presence of increasing concentrations of inhibitors (0, 2, 4, 8, 16, and 32 μM) and stopped 2 min into the reaction by addition of an equal volume of formamide loading buffer. The samples were loaded on the gel in ascending order of inhibitor concentration (Figure 5 and Supporting Information Figure S8).

Autoradiography was performed as above. The films were scanned, and densitometry was performed using an image processing program (ImageJ). The inhibition data were fit by nonlinear regression to a simple exponential decay function of form $y = e^{-kx} + C$ using the program GraphPad Prism.

Synthesis of the Branchpoint Monomer (A) for Method A (Scheme 1A). The synthesis of N^6-benzoyl-2′-*O*-(acetyl levulinic ester)-3′-*O*-[(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite]-5′-*O*-(4,4′-dimethoxytrityl)-adenosine (**A**) was performed according to a previous published method[35] with slight modifications.

*N^6-Benzoyl-2′-O-[(methylthio)methyl]-3′,5′-O-[1,1,3,3-tetrakis(1-methylethyl)-1,3-disiloxanediyl]-adenosine (**1**).* Title compound was synthesized according to previously published protocols.[34]

*N^6-Benzoyl-2′-O-[acetal levulinic ester]-3′,5′-O-[1,1,3,3-tetrakis-(1-methylethyl)-1,3-disiloxanediyl]-adenosine (**2**).* $CsHCO_3$ (582 mg, 3 mmol) was suspended in DMF (2.5 mL), levulinic acid (0.625 mL, 6.1 mmol) was added, and the mixture was heated to 75 °C for 3 h. Compound **1** (1.33 g, 1.97 mmol) was dissolved in CH_2Cl_2 (20 mL) and cooled to 0 °C under Ar. Freshly made SO_2Cl_2 (10 mL, 1 M in CH_2Cl_2, 10 mmol) was added dropwise over 20 min, and the mixture was stirred at 0 °C for 1 h and then 2 h at room temperature. The mixture was concentrated under N_2, redissolved in CH_2Cl_2 (15 mL), and cannulated to the Cs-activated levulinic acid. The flask was washed with CH_2Cl_2 (5 mL), and the reaction mixture was stirred at room temperature. After 15 h, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ and subsequently once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2; the combined organic extracts were washed once with brine and subsequently combined with the original organic layer. The combined organic layers were dried with $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (SiO_2, 1:1→1:2 Hexanes/EtOAc) to give compound **2** (871 mg, 59%) as an amorphous white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.23 (s, 1H), 8.63 (d, *J* 2.3 Hz, 1H), 8.52 (s, 1H), 8.05−7.99 (m, 2H), 7.63 (t, *J* 7.4 Hz, 1H), 7.53 (t, *J* 7.6 Hz, 2H), 6.10 (s, 1H), 5.47 (d, *J* 6.5 Hz, 1H), 5.37 (d, *J* 6.5 Hz, 1H), 5.07 (dd, *J* 9.0 Hz, 5.2, 1H), 4.93 (d, *J* 5.3 Hz, 1H), 4.07−4.01 (m, 1H), 3.98−3.89 (m, 2H), 2.63 (t, *J* 6.4 Hz, 2H), 2.47−2.35 (m, 2H), 1.97 (s, 2H), 1.12−0.96 (m, 28H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 209.9, 206.9, 175.7, 171.9, 166.1, 152.5, 152.1, 151.0, 143.5, 133.7, 132.9, 128.95, 128.91, 126.2, 98.9, 88.6, 86.9, 86.1, 81.3, 71.1, 61.3, 37.4, 29.8, 27.6, 17.74, 17.72, 17.69, 17.66, 17.54, 17.49, 13.58, 13.57, 13.4, 13.3, 13.21, 13.16, 12.8; HRMS (ESI Q-TOF) calcd for $C_{35}H_{52}N_5O_9Si_2$ $(M + H)^+$ 742.3304, found 742.3298.

*N^6-Benzoyl-2′-O-[acetal levulinic ester]-adenosine (**3**).* Compound **2** (2.61 g, 3.46 mmol) was dissolved in THF (8.7 mL) and stirred at room temperature under Ar. TREAT-HF (1.0 mL, 1.84 mmol) was added. After 2.5 h the mixture was diluted with 100 mL of CH_2Cl_2 and concentrated. The residue was purified by column chromatography (SiO_2, 1:200 → 1:25 CH_3OH/CH_2Cl_2) to yield compound **3** (1.20 g, 73%) as an amorphous white solid: ^{1}H NMR (300 MHz, acetone-d_6) δ 10.20 (s, 1H), 8.63−8.69 (m, 1H), 8.56−8.63 (m, 1H), 8.12 (dd, *J* 9.8, 8.6 Hz, 2H), 7.48−7.69 (m, 3H), 6.21 (t, *J* 6.4 Hz, 1H), 5.18−5.38 (m, 3H), 5.01 (dd, *J* 6.2, 4.9 Hz, 1H), 4.45−4.69 (m, 2H), 4.19 (q, *J* 2.5 Hz, 1H), 3.66−3.96 (m, 2H), 3.06 (s, 1H), 2.55−2.69 (m, 2H), 2.21−2.34 (m, 2H), 2.01−2.10 (m, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.5, 183.1, 172.0, 164.9, 152.1, 150.6, 150.3, 143.5, 133.3, 133.0, 128.8, 128.0, 124.3, 89.0, 88.9, 87.9, 82.6,

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71.8, 63.0, 37.8, 29.7, 27.7; HRMS (ESI Q-TOF) calcd for $C_{23}H_{25}N_5O_8Na$ $(M + Na)^+$ 522.1601, found 522.1585.

*N^6-Benzoyl-2′-O-[acetal levulinic ester]-5′-O-[4,4′-dimethoxytrityl]-adenosine (**4**).* Compound **3** (508 mg, 1.02 mmol) and $AgNO_3$ (206 mg, 1.21 mmol) were dissolved in THF (4.95 mL) and pyridine (0.55 mL) and stirred under Ar. 4,4′-Dimethoxytrityl chloride (411 mg, 1.21 mmol) was added, and the reaction was stirred at room temperature. After 1.2 h, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ and once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2, and the combined organic extracts were washed once with brine. They were subsequently combined with the original organic layer, dried with $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (SiO_2, 1:200 → 3:50 CH_3OH/CH_2Cl_2) to yield compound **4** (653 mg, 79%) as an amorphous white solid: ^{1}H NMR (300 MHz, acetone-d_6) δ 8.61 (s, 1H), 8.49 (d, J 6.4 Hz, 1H), 8.12 (d, J 7.3 Hz, 2H), 7.43−7.71 (m, 5H), 7.13−7.41 (m, 7H), 6.84 (dd, J 8.9 Hz, 3.1, 4H), 6.29 (d, J 4.7 Hz, 1H), 5.40 (dd, J 26.9, 6.5 Hz, 2H), 5.21 (t, J 4.9 Hz, 1H), 4.79 (d, J 4.2 Hz, 1H), 4.27 (q, J 4.1 Hz, 1H), 3.75 (s, 6H), 3.47 (dd, J 13.1, 6.4 Hz, 2H), 2.66 (t, J 6.4 Hz, 2H), 2.37 (t, J 6.4 Hz, 2H), 2.07 (d, J 9.0 Hz, 3H); ^{13}C NMR (75 MHz, acetone-d_6) δ 206.0, 171.9, 158.7, 152.1, 151.8, 150.4, 145.1, 143.0, 135.9, 135.8, 134.2, 132.3, 130.1, 128.5, 128.3, 128.1, 127.7, 126.7, 125.1, 113.0, 88.4, 87.2, 86.2, 84.0, 81.2, 70.2, 63.5, 54.6, 37.2, 28.4, 27.6; HRMS (ESI Q-TOF) calcd for $C_{44}H_{43}N_5O_{10}Na$ $(M + Na)^+$ 824.2908, found 824.2911.

*N^6-Benzoyl-2′-O-(acetyl levulinic ester)-3′-O-[(2-cyanoethyl-N,N-diisopropyl)phosphoramidite]-5′-O-(4,4′-dimethoxytrityl)-adenosine (**A**).* Compound **4** (567 mg. 0.71 mmol) was dissolved in 4.24 mL THF, and the mixture was stirred at room temperature under Ar. *N,N*-Diisopropylethylamine (0.9 mL, 5.58 mmol) was added, and subsequently 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.195 mL, 0.78 mmol) was added. After 4 h, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ and once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2, and the combined extracts were washed once with brine. Subsequently the extracts were combined with the original organic layer, dried with $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (SiO_2, 1:2 hexanes/EtOAc + 1% NEt_3) to yield compound **5** (508 mg, 71%) as an amorphous white solid: ^{1}H NMR (300 MHz, acetone-d_6) δ 8.62 (d, J 2.5, 1H), 8.53 (d, J 1.2 Hz, 1H), 8.07−8.20 (m, 2H), 7.04−7.75 (m, 12H), 6.80−6.92 (m, 4H), 6.31 (t, J 5.0 Hz, 1H), 5.29−5.53 (m, 2H), 4.96−5.09 (m, 1H), 3.79−4.46 (m, 1H), 3.76 (d, J 2.2 Hz, 7H), 3.34−3.74 (m, 6H), 2.59−2.96 (m, 4H), 2.38 (dt, J 9.9, 6.4 Hz, 2H), 1.18−1.34 (m, 12H), 1.13 (t, J 8.2 Hz, 3H); ^{13}C NMR (75 MHz, acetone-d_6) δ 206.0, 205.9, 172.0, 171.8, 158.7, 152.1, 152.0, 151.8, 150.5, 145.10, 145.06, 143.4, 143.2, 135.83, 135.81, 135.75, 134.2, 132.4, 130.2, 130.10, 130.07, 128.5, 128.32, 128.26, 128.2, 128.1, 127.73, 127.71, 126.72, 126.69 125.3, 125.2, 118.3, 118.0, 113.0, 88.5, 88.3, 87.9, 87.4, 86.29, 86.26 83.6, 83.2, 83.1, 80.3, 79.8, 79.7, 72.5, 71.7, 71.5, 71.4, 71.1, 63.1, 62.9, 61.1, 59.4, 59.1, 58.7, 58.5, 58.3, 58.2, 54.68, 54.67 45.0, 44.9, 43.22, 43.16 43.1, 43.0, 37.17, 37.15 27.7, 27.6, 24.24, 24.21, 24.1, 24.0, 22.32, 22.29, 22.26, 22.2, 20.0, 19.9, 19.8, 19.5, 19.4; ^{31}P NMR (81 MHz, DMSO-d_6) δ 149.5, 149.0, 13.9, −2.0; HRMS (ESI Q-TOF) calcd for $C_{53}H_{61}N_7O_{11}P$ $(M + H)^+$ 1002.4167, found 1002.4160.

Synthesis of the Branchpoint (B) for Method B (Scheme 1B). The synthesis of N^6-benzoyl-2′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-[(2-cyanoethyl-*N,N*-diisopropyl) phosphoramidite]-5′-*O*-levulinyl-adenosine (**B**) was done following a published synthesis of the analogous guanosine[36] (N^6-benzoyl-2′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-[(2-cyanoethyl-*N,N*-diisopropyl) phosphoramidite]-5′-*O*-levulinyl-guanosine).

*N^6-Benzoyl-3′,5′-O-[1,1,3,3-tetrakis(1-methylethyl)-1,3-disiloxanediyl]-adenosine (**6**).* Title compound was made according to previously established protocols.[46]

*N^6-Benzoyl-2′-O-(4,4′-dimethoxytrityl)-adenosine (**7**).* Compound **6** (7.14 g, 11.6 mmol) and $AgNO_3$ (2.37 g, 13.9 mmol) were dissolved in THF (52 mL) and pyridine (5.8 mL) and stirred under Ar. 4,4′-Dimethoxytrityl chloride (4.73 g, 13.9 mmol) was added, and the reaction was stirred at room temperature. After 1.2 h, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ and once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2, and the combined organic extracts were washed once with brine. They were subsequently combined with the original organic layer, dried with $MgSO_4$, filtered, and concentrated. The crude syrup was coevaporated with toluene to yield N^6-benzoyl-2′-*O*-(4,4′-dimethoxytrityl)-3′,5′-*O*-[1,1,3,3-tetrakis(1-methylethyl)-1,3-disiloxanediyl]-adenosine.

The crude N^6-benzoyl-2′-*O*-(4,4′-dimethoxytrityl)-3′,5′-*O*-[1,1,3,3-tetrakis(1-methylethyl)-1,3-disiloxanediyl]-adenosine was dissolved in THF (29 mL), and TREAT-HF (3.3 mL, 20 mmol) was added. After 2.5 h, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ and once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2, and the combined organic extracts were washed once with brine and subsequently combined with the original organic layer. The combined organic phase was dried with $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (SiO_2, 1:200 → 3:50 CH_3OH/CH_2Cl_2) to yield compound **7** (4.34 g, 55% over 2 steps) as an amorphous white solid: ^{1}H NMR (300 MHz, acetone-d_6) δ 10.45 (s, 1H), 8.48 (s, 1H), 8.46 (s, 1H), 8.16 (d, J 7.3 Hz, 2H), 7.47−7.64 (m, 3H), 7.41 (dd, J 8.9, 7.5 Hz, 2H), 7.28 (d, J 8.9 Hz, 2H), 7.07−7.20 (m, 5H), 6.73 (d, J 8.9 Hz, 2H), 6.61 (d, J 8.9 Hz, 2H), 6.33 (d, J 7.6 Hz, 1H), 5.55 (d, J 8.6 Hz, 1H), 5.12 (dd, J 7.6 Hz, 4.3, 1H), 4.08 (s, 1H), 3.81 (d, J 1.9 Hz, 1H), 3.69 (d, J 13.9 Hz, 7H), 3.45 (t, J 10.5 Hz, 1H), 3.32 (s, 1H), 3.21 (s, 1H); ^{13}C NMR (75 MHz, acetone-d_6) δ 165.4, 158.9, 158.6, 151.3, 151.1, 150.8, 145.3, 143.9, 135.5, 135.3, 134.1, 132.5, 130.3, 130.1, 129.6, 128.6, 128.4, 127.8, 127.7, 126.8, 125.8, 113.0, 112.9, 88.8, 87.8, 87.3, 75.7, 71.6, 62.8, 54.7, 54.7; HRMS (ESI Q-TOF) calcd for $C_{38}H_{36}N_5O_7$ $(M + H)^+$ 674.2615, found 674.2599.

*N^6-Benzoyl-2′-O-(4,4′-dimethoxytrityl)-5′-O-levulinyl-adenosine (**8**).* *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethylammonium tetrafluoroborate (TBTU, 3.02 g, 9.45 mmol) was dissolved in DMF (22.7 mL) and *N,N*-diisopropylethylamine (3.3 mL) and stirred at room temperature under Ar. Freshly distilled levulinic acid (0.96 mL, 9.45 mmol) was added, and the reaction was stirred at room temperature for 30 min. Compound **7** (2.12 g, 3.15 mmol) was dried under a vacuum in a round-bottom flask with a magnetic stirrer. Ten milliliters of the TBTU/levulinic acid reaction was added to the nucleoside and stirred at room temperature for 2 h. Subsequently the remainder of the TBTU/levulinic acid reaction mixture was cannulated to the nucleoside, and the reaction was stirred at room temperature. After 10 h, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ and once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2. Next, the combined organic extracts were washed once with brine and subsequently combined with the original organic layer, dried with $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (SiO_2, 1:200 → 1:50 CH_3OH/CH_2Cl_2) to yield compound **8** (1.44 g, 84%) as an amorphous white solid. There was a difficulty fully purifying the compound from the starting material; therefore, the NMR analysis is that of the purest fraction, but the entire material was carried through to the next step: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.20 (s, 1H), 8.56 (s, 1H), 8.35 (s, 1H), 8.10−7.99 (m, 2H), 7.63 (d, J 7.3 Hz, 1H), 7.54 (t, J 7.5 Hz, 2H), 7.38 (dd, J 6.0, 2.3 Hz, 2H), 7.25 (d, J 8.9 Hz, 2H), 7.13 (ddd, J 14.7, 11.5, 4.2 Hz, 5H), 6.70 (d, J 9.0 Hz, 2H), 6.60 (d, J 9.0 Hz, 2H), 5.86 (d, J 5.4 Hz, 1H), 5.40 (d, J 5.9 Hz, 1H), 4.98 (t, J 5.3 Hz, 1H), 4.07−4.22 (m, 2H), 3.92−4.04 (m, 1H), 3.64 (d, J 14.5 Hz, 6H), 3.43 (d, J 4.6 Hz, 1H), 2.62 (t, J 6.5 Hz, 2H), 2.28−2.41 (m, 2H), 2.04 (s, 3H); ^{13}C NMR (75 MHz, acetone-d_6) δ 205.8, 205.5, 171.9, 165.3, 159.0, 158.8, 151.6, 150.4, 145.3, 143.3, 136.1, 135.6, 135.3, 134.1, 132.4, 130.4, 130.3, 130.0, 129.8, 129.6, 129.2, 128.5, 128.3, 128.1, 127.8, 127.7, 127.6, 126.9, 126.5, 125.4, 113.1, 113.0, 112.9, 112.7, 87.3, 82.7, 75.4, 70.7, 63.7, 54.7, 37.9, 37.3, 28.8, 27.5; HRMS (ESI Q-TOF) calcd for $C_{43}H_{41}N_5O_9Na$ $(M + Na)^+$ 794.2802, found 794.2782.

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N^6-Benzoyl-2′-O-(4,4′-dimethoxytrityl)-3′-O-[(2-cyanoethyl-N,N-diisopropyl)phosphoramidite]-5′-O-levulinyl-adenosine (**B**). Compound **8** (1.44 g. 1.86 mmol) was dissolved in 11.1 mL THF, and the mixture was stirred at room temperature under Ar. *N*,*N*-Diisopropylethylamine (2.35 mL, 14.57 mmol) was added, followed by 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.46 mL, 2.1 mmol). After 16 h, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ and once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2. Next the combined organic extracts were washed once with brine and subsequently combined with the original organic layer, dried with $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (SiO_2, CH_2Cl_2 + 1% NEt_3) to yield compound **9** (1.00 g, 56%) as an amorphous white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.55 (dd, *J* 21.4, 19.9 Hz, 1H), 8.05 (d, *J* 7.2 Hz, 2H), 7.61−7.68 (m, 1H), 7.52−7.59 (m, 2H), 6.96−7.30 (m, 10H), 6.73 (dd, *J* 13.0, 9.0 Hz, 2H), 6.59 (dd, *J* 9.0, 5.8 Hz, 2H), 6.22 (dd, *J* 31.1, 7.5 Hz, 1H), 5.44 (dd, *J* 7.5, 4.2 Hz, 1H), 5.23−5.29 (m, 1H), 3.72−4.41 (m, 1H), 3.61−3.69 (m, 9H), 3.35−3.52 (m, 1H), 3.09−3.25 (m, 1H), 2.60−2.86 (m, 10H), 2.48 (s, 4H), 2.07 (t, *J* 2.9 Hz, 3H), 1.03−1.32 (m, 15H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 207.1, 207.0, 182.2, 172.3, 172.2, 166.2, 160.7, 158.84, 158.76, 158.5, 145.3, 145.2, 135.8, 135.5, 135.2, 133.8, 132.9, 130.6, 130.0, 129.9, 129.0, 128.9, 127.94, 127.92, 119.5, 119.3, 113.4, 113.3, 86.8, 82.6, 73.4, 72.8, 63.9, 60.9, 59.6, 58.7, 55.4, 48.9, 47.3, 43.1, 38.7, 37.7, 30.0, 29.8, 27.7, 24.9, 24.6; ^{31}P NMR (81 MHz, DMSO-d_6) δ 151.5, 149.0; HRMS (ESI Q-TOF) calcd for $C_{52}H_{59}N_7O_{10}P$ $(M + H)^+$ 972.4061, found 972.4029.

Synthesis of the Branchpoint Monomer (Z) for Method Z (Scheme 1C). *N*^{6}-Benzoyl-3′,5′-O-[1,1,3,3-tetrakis(1-methylethyl)-1,3-disiloxanediyl]-adenosine-9-β-D-arabinofuranoside (**10**). Title compound was synthesized according to previously published protocols.[33]

N^6-Benzoyl-2′-O-levulinyl-3′,5′-O-[1,1,3,3-tetrakis(1-methylethyl)-1,3-disiloxanediyl]-adenosine-9-β-D-arabinofuranoside (**11**). *N*,*N*′-Dicyclohexylcarbodiimide (3.35 g, 16.2 mmol) was dissolved in 40 mL of anhydrous THF. Freshly distilled levulinic acid (3.3 mL, 33 mmol) was added to the mixture yielding almost immediate precipitation. After 2 h, the mixture was filtered under nitrogen through an oven-dried filtration apparatus designated for anhydrous filtration. This yielded a 0.41 M solution of levulinic anhydride in THF. Compound **10** (7.21 g, 11.7 mmol) and 4-dimethylaminopyridine (845 mg, 6.91 mmol) were dissolved in THF (55.4 mL), *N*,*N*-diisopropylethylamine (17 mL, 20 mmol) was added, and the mixture was stirred at room temperature under Ar. The filtered levulinic anhydride solution (31.4 mL, 12.9 mmol) was added. After 3 h, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ (sat aq) and once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2. Next the combined organic extracts were washed once with brine and subsequently combined with the original organic layer, dried with $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (SiO_2, 1:200 → 1:20 CH_3OH/CH_2Cl_2) to yield compound **11** (6.21 g, 74%) as an amorphous white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.23 (s, 1H), 8.63−8.70 (m, 1H), 8.35−8.42 (m, 1H), 8.02 (dd, *J* 5.2, 3.3 Hz, 2H), 7.56−7.68 (m, 1H), 7.47−7.56 (m, 2H), 6.56 (d, *J* 7.0 Hz, 1H), 5.60 (dd, *J* 8.0, 7.0 Hz, 1H), 5.11 (t, *J* 8.3 Hz, 1H), 4.14−4.24 (m, 1H), 3.97 (ddd, *J* 13.7, 8.0, 3.0 Hz, 2H), 2.20−2.36 (m, 2H), 2.05−2.18 (m, 1H), 1.89 (d, *J* 3.1 Hz, 3H), 1.83 (dt, *J* 17.0, 6.3 Hz, 1H), 1.20−1.30 (m, 1H), 1.16−1.09 (m, 6H), 0.99−1.06 (m, 20H), 0.94 (ddd, *J* 9.6, 8.7, 4.6 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 206.1, 171.6, 166.1, 152.3, 151.0, 143.8, 133.8, 132.9, 131.6, 128.94, 128.89, 128.6, 127.9, 125.9, 80.8, 79.6, 77.5, 73.7, 62.0, 37.2, 29.6, 27.4, 17.8, 17.7, 17.63, 17.61, 17.3, 17.25, 17.23, 17.1, 13.1, 12.9, 12.7, 12.4; HRMS (ESI Q-TOF) *m*/*z* calcd for $C_{34}H_{50}N_5O_8Si_2$ $(M + H)^+$ 712.3198, found 712.3184.

N^6-Benzoyl-2′-O-levulinyl-adenosine-9-β-D-arabinofuranoside (**12**). Compound **11** (4.79 mg, 6.72 mmol) was dissolved in THF (16.5 mL) and stirred at room temperature under Ar. Subsequently TREAT-HF (2.0 mL, 12 mmol) was added, and the reaction was stirred at room temperature. After 2.5 h the mixture was concentrated. The residue was purified by column chromatography (SiO_2, 1:100 → 1:20 CH_3OH/CH_2Cl_2) to yield compound **12** (2.37 g, 75%) as an amorphous white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.20 (s, 1H), 8.73 (s, 1H), 8.57−8.64 (m, 1H), 7.98−8.08 (m, 2H), 7.44−7.69 (m, 3H), 6.58 (d, *J* 5.8 Hz, 1H), 5.88 (s, 1H), 5.35 (t, *J* 5.9 Hz, 1H), 5.11 (s, 1H), 4.46 (s, 1H), 3.90 (ddd, *J* 6.6, 4.9, 3.5 Hz, 1H), 3.71 (ddd, *J* 17.2, 12.2, 4.2 Hz, 2H), 2.36 (t, *J* 6.7 Hz, 2H), 2.18−2.29 (m, 1H), 1.98 (dd, *J* 11.7, 5.2 Hz, 1H), 1.94 (d, *J* 3.0 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 206.5, 171.5, 166.0, 152.4, 152.2, 150.7, 143.7, 133.8, 132.9, 128.9, 125.8, 83.6, 81.8, 77.9, 72.0, 60.7, 37.4, 29.7, 27.6; HRMS (ESI Q-TOF) calcd for $C_{22}H_{23}N_5O_7Na$ $(M + Na)^+$ 492.1495, found 492.1487.

N^6-Benzoyl-2′-O-levulinyl-5′-O-[4,4′-dimethoxytrityl]-adenosine-9-β-D-arabinofuranoside (**13**). Compound **12** (2.24 g, 4.76 mmol) was dissolved in pyridine (34 mL) and stirred under Ar. 4,4′-Dimethoxytrityl chloride (1.78 g, 5.24 mmol) was added, and the reaction was stirred at room temperature. After 10 h, $NaHCO_3$ (sat aq, 30 mL) was added, and the reaction mixture placed on a rotary evaporator to remove most of the solvent. Subsequently, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ and once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2, and the combined organic extracts were washed once with brine. Subsequently, the extracts were combined with the original organic layer, dried with $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (SiO_2, 1:100 → 1:20 CH_3OH/CH_2Cl_2) to yield compound **13** (3.30 g, 90%) as an amorphous white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.22 (s, 1H), 8.67 (s, 1H), 8.35 (s, 1H), 8.03 (d, *J* 7.3 Hz, 2H), 7.63 (t, *J* 7.4 Hz, 1H), 7.53 (t, *J* 7.6 Hz, 2H), 7.35−7.42 (m, 2H), 7.15−7.27 (m, 7H), 6.76−6.89 (m, 4H), 6.63 (d, *J* 5.9 Hz, 1H), 5.93 (d, *J* 5.6 Hz, 1H), 5.31 (t, *J* 5.8 Hz, 1H), 4.52 (dd, *J* 12.0, 5.9 Hz, 1H), 4.11 (td, *J* 6.9, 3.1 Hz, 1H), 3.70 (t, *J* 4.4 Hz, 6H), 3.42 (dd, *J* 10.3, 7.3 Hz, 1H), 3.31−3.35 (m, 1H), 3.27 (dd, *J* 10.3, 2.9, 1H), 2.31 (t, *J* 6.9 Hz, 2H), 2.17 (dt, *J* 16.9, 6.8 Hz, 1H), 1.87−1.98 (m, 4H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 206.5, 171.6, 166.0, 158.5, 152.3, 152.2, 150.8, 145.2, 143.7, 136.0, 135.8, 133.8, 132.9, 130.2, 128.9, 128.2, 128.1, 127.1, 125.6, 113.6, 85.9, 82.0, 81.7, 78.0, 73.2, 63.9, 55.4, 37.4, 29.7, 27.6; HRMS (ESI Q-TOF) calcd for $C_{43}H_{41}N_5O_9Na$ $(M + Na)^+$ 794.2802, found 794.2832.

N^6-Benzoyl-2′-O-(4,4′-dimethoxytrityl)-3′-O-[(2-cyanoethyl-N,N-diisopropyl)phosphoramidite]-5′-O-levulinyl-adenosine-9-β-D-arabinofuranoside (**14**). Compound **13** (3.19 g. 4.13 mmol) was dissolved in 25 mL THF, and the mixture was stirred at room temperature under Ar. *N*,*N*-Diisopropylethylamine (5.2 mL, 32 mmol) was added, followed by 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.97 mL, 4.3 mmol). After 2 h, CH_2Cl_2 and $NaHCO_3$ (sat aq) were added, and the mixture was separated. The organic phase was washed twice with $NaHCO_3$ and once with brine. The combined aqueous layers were extracted 3 times with CH_2Cl_2, and the combined organic extracts were washed once with brine and subsequently combined with the original organic layer, dried with $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (SiO_2, CH_2Cl_2 + 1% NEt_3) to yield compound **9** (3.78 g, 97%) as an amorphous white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.22 (s, 1H), 8.62 (s, 1H), 8.40 (d, *J* 13.7 Hz, 1H), 8.03 (d, *J* 7.9 Hz, 2H), 7.63 (t, *J* 7.3 Hz, 1H), 7.54 (t, *J* 7.6 Hz, 2H), 7.32−7.42 (m, 3H), 7.12−7.29 (m, 6H), 6.74−6.86 (m, 4H), 6.68 (dd, *J* 8.1, 6.1 Hz, 1H), 5.52 (dt, *J* 30.6, 5.8 Hz, 1H), 4.83−4.97 (m, 1H), 4.16−4.35 (m, 1H), 3.66−3.80 (m, 7H), 3.35−3.64 (m, 4H), 3.31 (d, *J* 7.1 Hz, 2H), 2.73 (t, *J* 5.9 Hz, 1H), 2.62 (t, *J* 5.9 Hz, 1H), 2.44−2.54 (m, 2H), 2.24−2.37 (m, 2H), 2.07−2.23 (m, 1H), 1.83−2.00 (m, 3H), 1.01−1.35 (m, 10H), 0.91 (dd, *J* 24.7, 7.0 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 207.1, 206.9, 172.4, 172.2, 166.1, 158.8, 158.6, 158.5, 152.4, 152.3, 151.5, 150.9, 150.9, 145.3, 145.2, 144.9, 144.5, 135.8, 135.6, 135.3, 135.2, 133.8, 132.9, 130.6, 130.0, 129.9, 129.0, 128.9, 128.7, 128.1, 127.9, 127.2, 126.9, 126.7, 119.4, 119.3, 113.5, 113.3, 87.2, 86.8, 86.7, 82.3, 81.3, 73.4, 72.7, 63.8, 63.4, 60.7, 59.7, 59.5, 58.2, 58.0, 55.5, 55.4, 55.3, 52.4, 43.5, 43.3, 43.1, 42.9, 38.7, 37.7, 37.7, 29.9, 27.8, 27.7, 25.1, 25.0, 24.8, 24.5, 24.4, 20.5, 20.4, 20.2, 20.1, 7.6; ^{31}P NMR (81 MHz,

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DMSO-d_6) δ 149.55, 149.30, 13.86; HRMS (ESI Q-TOF) calcd for $C_{52}H_{59}N_7O_{10}P$ $(M + H)^+$ 972.4061, found 972.4088.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional data for molecules listed in Table 1. These include HPLC chromatograms and PAGE gels on crude bRNA products, debranching assays, inhibition studies. Additionally, optimization parameters are listed in Figure S2. Finally, characterization raw data are provided, including mass spectrometry of oligonucleotides, ^{1}H, ^{13}C, ^{31}P NMR and HRMS of branchpoint amidites and intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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