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Synthesis and Incorporation of the Phosphoramidite Derivative of 2′-*O*-Photocaged 3′-*S*-Thioguanosine into Oligoribonucleotides: Substrate for Probing the Mechanism of RNA Catalysis

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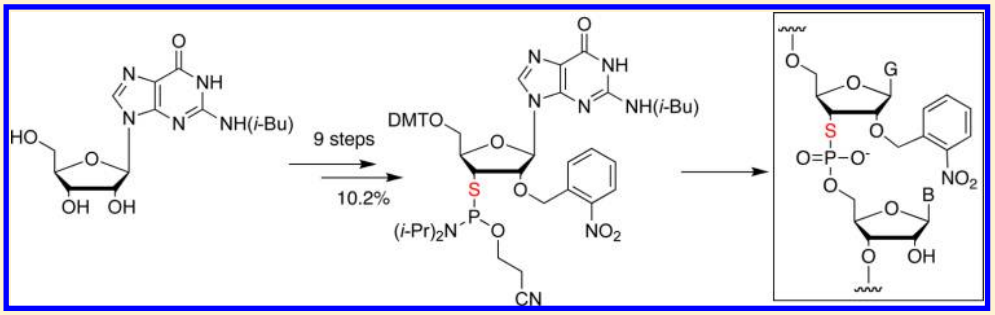
Supporting Information



ABSTRACT: Oligoribonucleotides containing 3′-*S*-phosphorothiolate linkages possess properties that can reveal deep mechanistic insights into ribozyme-catalyzed reactions. "Photocaged" 3′-*S*- RNAs could provide a strategy to stall reactions at the chemical stage and release them after assembly steps have occurred. Toward this end, we describe here an approach for the synthesis of 2′-*O*-(*o*-nitrobenzyl)-3′-thioguanosine phosphoramidite starting from N^2-isobutyrylguanosine in nine steps with 10.2% overall yield. Oligonucleotides containing the 2′-*O*-(*o*-nitrobenzyl)-3′-*S*-guanosine nucleotide were then constructed, characterized, and used in a nuclear pre-mRNA splicing reaction.

Oligonucleotides containing phosphorothiolate linkages, in which sulfur replaces the 3′- or 5′-bridging oxygen connected to the ribofuranose ring (Figure 1, **II** and **III**), serve as powerful biochemical probes to investigate fundamental features of enzyme catalysis.[1−4] RNAs containing a 3′-*S*-phosphorothiolate linkage have been used in metal ion rescue experiments to elucidate the mechanism of metalloribozymes such as the group I intron, the group II intron, and the spliceosome.[5−8] These studies rely on careful comparison of the 3′-*O* and 3′-*S* substrates and must include assays that monitor the chemical step. Despite broad utility of these substrates in the investigation of phosphoryl transfer reactions, complex assembly processes and conformational changes that accompany biological catalysis frequently mask analysis of the chemical step. Photocaging provides a well-established strategy to protect functional groups from chemical reactions until released by UV irradiation.[9−11] In this respect, having access to "photocaged" 3′-*S*-modified RNAs could provide a strategy to stall reactions at the chemical stage and release them only after assembly steps have occurred. Moreover, photocaging provides the added advantage of increasing the stability of these phosphorothiolate-modified oligonucleotides by eliminating side reactions involving 2′-*O*-transphosphorylation.[1]

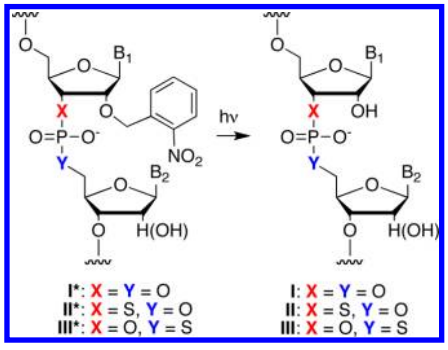


Figure 1. Structures of wild type, 3′-*S*-modified, and 5′-*S*-modified oligonucleotides.

The 2′-*O*-(*o*-nitrobenzyl) derivatives of uridine,[12] adenosine,[13] cytidine,[13] and guanosine[14] have been synthesized and applied to the synthesis of oligoribonucleotides containing one or multiple 2′-*O*-photoliable groups (Figure 1, **I***).[12,13,15−18] Dinucleotides and trinucleotides containing a 2′-*O*-(*o*-nitrobenzyl) group were usually synthesized via a solution method involving condensation between the 3′-phosphate or 5′-phosphate of one nucleoside and the 5′-OH or 3′-OH group of another nucleoside.[12,13,17] Longer oligoribonucleotides (>3-mer) containing 2′-*O*-(*o*-nitrobenzyl) groups could be

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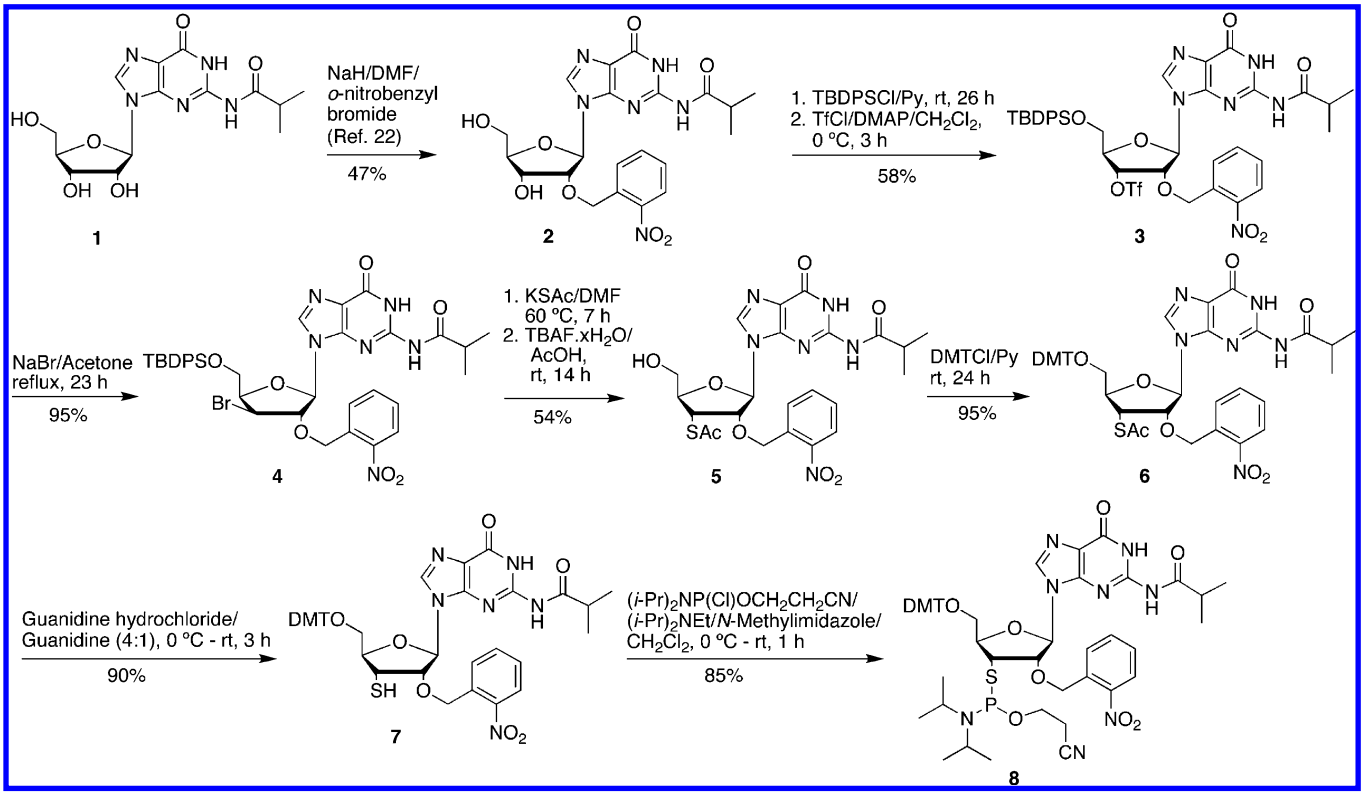
Scheme 1



efficiently synthesized via solid-phase synthesis using 3′-phosphoramidites[15,16] or 3′-*H*-phosphonates of 2′-*O*-(*o*-nitrobenzyl) nucleosides.[18] After removal of the 2′-*O*-(*o*-nitrobenzyl) group by photolysis, these RNAs could initiate efficient and accurate ribozyme-catalyzed reactions.[15,16] This RNA-caging approach has also been used to investigate spliceosome assembly during pre-mRNA splicing through introducing a 2′-*O*-(*o*-nitrobenzyl) group into the branch adenosine nucleotide, which blocks the first step of splicing, thereby allowing the study of the precatalytic stages during assembly.[19]

The synthesis of oligonucleotides containing a 2′-*O*-photoliable group and 3′-*S*-phosphorothiolate linkage (Figure 1, **II***) has not yet been reported. Here we report the synthesis of the phosphoramidite derivative of the 2′-*O*-(*o*-nitrobenzyl)-3′-thioguanosine and its application for the construction of oligonucleotides containing a 2′-*O*-photocaged 3′-*S*-phosphorothiolate linkage via solid-phase synthesis.

For the synthesis of 2′-*O*-(*o*-nitrobenzyl)-3′-thioguanosine phosphoramidite **8** (Scheme 1), we adapted previous approaches for the synthesis of 2′-*O*-TBS-3′-thioguanosine phosphoramidite[20] and 2′-*O*-methyl-3′-thioguanosine phosphoramidite.[21] We chose the *o*-nitrobenzyl group in this study because the starting material *o*-nitrobenzyl bromide is commercially available and relatively inexpensive. Additionally, use of the *o*-nitrobenzyl group is well-established for the synthesis of 2′-*O*-photocaged RNAs. N^2-Isobutyryl-2′-*O*-(*o*-nitrobenzyl)guanosine (**2**) was prepared from N^2-isobutyryl-guanosine (**1**) in 47% yield as described previously, except that we quenched the reaction with dilute aqueous HCl.[22] Reaction of **2** with (*tert*-butyl)diphenylsilyl chloride gave the corresponding 5′-silyl derivative in 94% yield. In the presence of 1.4 molar equiv of DMAP, the 5′-silyl derivative reacted with 1.05 molar equiv of trifluoromethanesulfonyl chloride at 0 °C to give the corresponding 3′-triflate derivative **3** in 62% yield. When 2.0 molar equiv of DMAP and 1.5 molar equiv of trifluoromethanesulfonyl chloride were used, the reaction afforded 3′-triflate derivative **3** in 50% yield along with the guanosine 3′,O^6-ditriflate derivative (∼13% yield). Subsequent S_N2 substitution with 4 molar equiv of NaBr in refluxing acetone afforded 3′-*β*-bromo derivative **4** in 95% yield. Reaction of **4** with KSAc in DMF at 60 °C gave a mixture of the 3′-*S*-acetyl and 3′,4′-unsaturated derivatives in a 1.3/1 ratio. Desilylation of the mixture with TBAF·xH$_2$O/AcOH in THF and purification by silica gel chromatography gave the desired pure 5′-deprotected derivative **5** in 54% yield over the two steps. Although the 3′-*β*-iodo derivative could be prepared by S_N2 substitution of **3** with NaI in 96% yield, the subsequent reaction with KSAc, followed by desilylation of the reaction mixture, produced predominantly the 3′,4′-unsaturated derivative and gave the desired 5′-deprotected derivative **5** only in 19% yield. Protection of **5** with DMTCl in pyridine for 24 h afforded the 5′-*O*-DMT ether **6** in 95% yield. The 3′-*S*-acetyl group of **6** was selectively removed by treatment with a 5:1 mixture of guanidine hydrochloride and guanidine[23] to give 3′-SH derivative **7** in 90% yield. Phosphitylation of compound **7** with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite yielded the corresponding 2′-*O*-(*o*-nitrobenzyl)-3′-thioguanosine phosphoramidite **8** in 85% yield.

Incorporation of phosphoramidite **8** into an 18-mer oligonucleotide was then accomplished by manual coupling using our previously described protocol.[24] The modified oligonucleotide, 5′-UUU AG$_{3'S,2'\text{-}O\text{-}(o\text{-NBn})}$ A GGU UGC UGC UUU-3′ (2′-*O*-photocaged-3′-*S*-RNA), was obtained in 1.5% yield after standard deprotection and reverse-phase HPLC purification. The presence of the modification was confirmed by MALDI-TOF MS (Figure S1 in Supporting Information). Additionally, the oligonucleotide was 5′-radiolabeled with γ^{32}-ATP and further characterized by UV deprotection, alkaline

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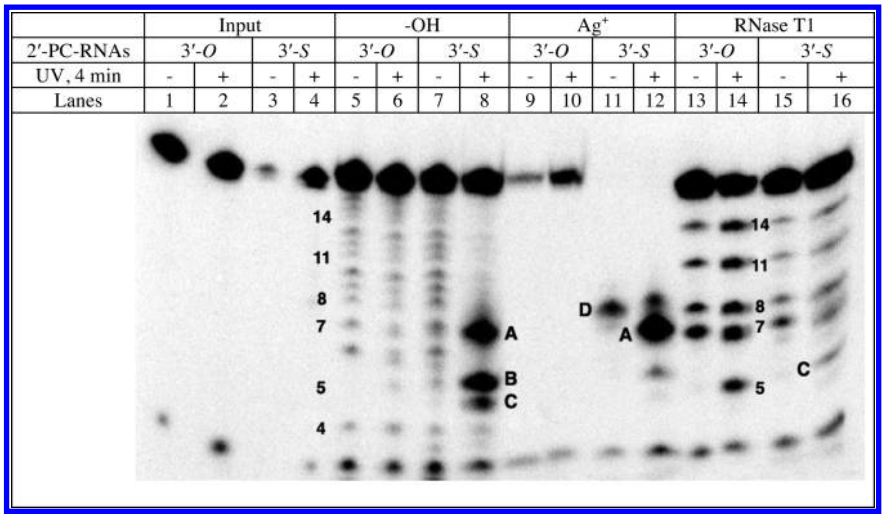

Figure 2. Alkaline hydrolysis, silver ion cleavage, and RNase T1 cleavage of 2′-photocaged 3′-*O*-substrate (5′-UUU $AG_{3'O,2'\text{-}O\text{-}(o\text{-NBn})}$ A GGU UGC UGC UUU-3′) and 2′-photocaged 3′-*S*-substrate (5′-UUU $AG_{3'S,2'\text{-}O\text{-}(o\text{-NBn})}$ A GGU UGC UGC UUU-3′): (A) 5′-pUUUA$G_{2'\text{-}OH,3'\text{-}SH}$-3′, (B) 5′-pUUUA$G_{2'\text{-}O,3'\text{-}SP}$-3′ (2′-*O*,3′-*S*-cyclic phosphoryl 5-mer), (C) 5′-pUUUA$G_{2'\text{-}OH,3'\text{-}SP}$-3′ (3′-*S*-phosphoryl 5-mer), and (D) 5′-pUUUA-$G_{2'\text{-}O\text{-}(o\text{-NBn}),3'\text{-}SH}$-3′. The numbers in the picture are assigned for the ladders of alkaline hydrolysis and T1 treatment of these two 18-mer RNAs.

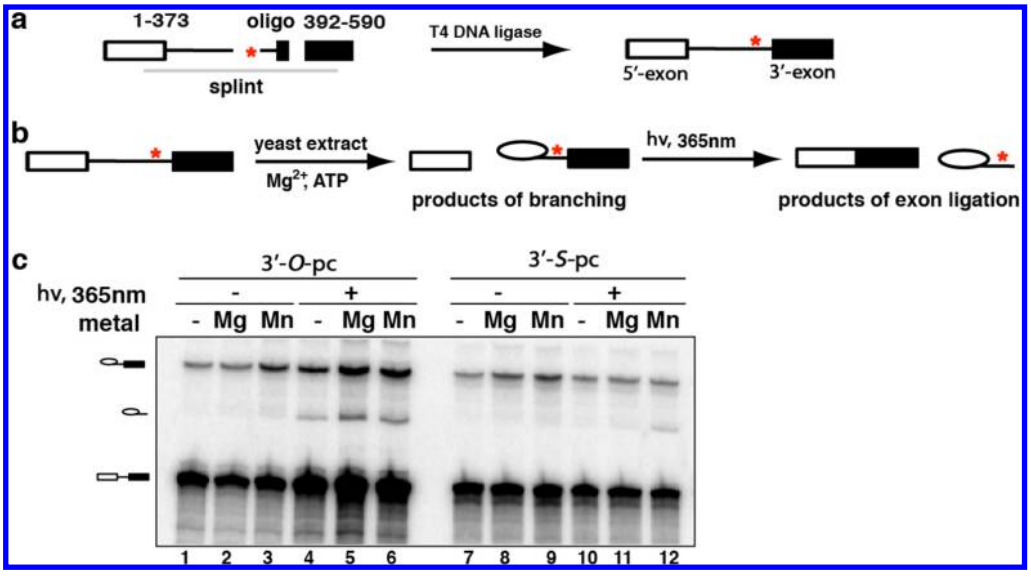

Figure 3. (a) Schematic for synthesis of *ACT1* yeast splicing substrates. RNA is shown as black lines and boxes; the DNA splint is shown in gray. The boxes represent the exons, and the black line represents the intron. The red star indicates the position of the radiolabel. The modified 2′-*O*-photocaged residue is the last nucleotide of the intron. (b) Schematic of the two steps of splicing. (c) Exon ligation of the 3′-*O*-pc and 3′-*S*-pc substrates in an in vitro splicing assay (60 mM K_2PO_4 (pH 7), 3% PEG 8000, 2.5 mM $MgCl_2$, 2 mM ATP, 40% yeast extract in the initial incubation); "metal" indicates the addition of 0.5 mM $MgCl_2$, $MnCl_2$, or nothing as indicated in the main text.

hydrolysis, silver ion cleavage, and RNase T1 cleavage (Figure 2). For comparison, we prepared the corresponding 2′-photocaged wild-type RNA, 5′-UUU $AG_{3'O,2'\text{-}O\text{-}(o\text{-NBn})}$ A GGU UGC UGC UUU-3′ (2′-*O*-photocaged-3′-*O*-RNA), by solid-phase synthesis using 2′-*O*-(*o*-nitrobenzyl)guanosine phosphoramidite. The UV-deprotected 3′-*S*-RNA was readily cleaved under alkaline conditions (pH 9) to form predominantly three 5-nucleotide products. Three cleavage products from top to bottom on line 8 of Figure 2 were assigned as (A) 5′-pUUUA$G_{2'\text{-}OH,3'\text{-}SH}$-3′, (B) 5′-pUUUA$G_{2'\text{-}O,3'\text{-}SP}$-3′ (2′-*O*,3′-*S*-cyclic phosphosphate 5-mer), and (C) 5′-pUUUA$G_{2'\text{-}OH,3'\text{-}SP}$-3′ (3′-*S*-phosphate 5-mer).[25,26] These product assignments correspond to those identified for alkaline cleavage of the dinucleotide pIspU.[26] As anticipated, the UV-deprotected 3′-*S*-oligonucleotide was more susceptible toward hydroxide-catalyzed strand scission than the 2′-photocaged 3′-*S*-oligo (Figure 2, compare lane 7 to 8). Exposure to silver ion of both 2′-*O*-photocaged 3′-*S*-RNA and UV-deprotected 3′-*S*-RNA resulted in the cleavage products assigned as 5′-pUUUA-$G_{2'\text{-}O\text{-}(o\text{-NBn}),3'\text{-}SH}$-3′ and 5′-pUUUA$G_{2'\text{-}OH,3'\text{-}SH}$-3′, respectively (Figure 2, lanes 11 and 12).[26] These results confirm that the RNA contains a 3′-*S*-phosphorothiolate linkage, and the ratio of the D and A product band indicates that UV irradiation converted ~90% of the 2′-*O*-photocaged 3′-*S*-RNA to 3′-*S*-RNA (lane 12). Moreover, neither 2′-*O*-photocaged guanosine 3′-*O*-RNA nor 2′-*O*-photocaged guanosine 3′-*S*-RNA was cleaved by RNase T1 at the photocaged residue until after UV deprotection (Figure 2, compare lanes 13 to 14 and 15 to 16). These results confirm that the RNA contains a 2′-photocaged 3′-phosphorothiolate linkage at the designated

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position and shows that the photocage can be removed efficiently.

We next tested whether the 2′-*O*-photocaged 3′-*S*-oligonucleotide could be used to stage nuclear pre-mRNA splicing reactions at the chemical step. Nuclear pre-mRNA splicing is catalyzed by the spliceosome, a complex ribonucleoprotein particle that undergoes assembly steps and rearrangements en route to the chemical steps.[27] We incorporated the photocaged 3′-*S*-oligonucleotide into a widely used *ACT1* yeast splicing substrate and used it in metal rescue experiments with the spliceosome (Figure 3a).[28] The oligonucleotide was designed to contain the photocaged residue at the intron terminus, such that the phosphorothiolate was in the leaving group position for the second step of splicing. To assemble the full-length substrate, we ^{32}P-radiolabeled the O- and S-containing oligonucleotides and then ligated them to two RNA oligonucleotides generated by in vitro transcription (Figure 3a). Next we incubated these RNA substrates in splicing-competent *Saccharomyces cerevisiae* extract so that spliceosomes could assemble on them and catalyze branching (Figure 3b). The lariat intermediate product of the branching step runs aberrantly slowly and can be seen as the top band of the splicing gel (Figure 3c).

With the photocage in place, the spliceosomes stalled prior to exon ligation regardless of the identity of the atom (O or S) in the leaving group position (Figure 3c, lanes 1 and 7). To remove the photocage and permit exon ligation (Figure 3b), we irradiated spliceosomes for 5 min with 365 nm UV light (lanes 4−6 and 10−12). To initiate exon ligation, we then added additional $MgCl_2$, $MnCl_2$, or nothing and assayed for exon ligation. As expected, appearance of the product of exon ligation of the 3′-*O*-pc substrate proceeded efficiently in the UV-treated spliceosomes (lanes 4−6) but not without irradiation (lanes 1−3). Exon ligation of the 3′-*S*-pc substrate only proceeded efficiently with UV treatment and in the presence of $MnCl_2$ (compare lane 12 with lanes 7−11). These results demonstrate that the photocage comprises an effective block to splicing chemistry and that, upon removal, splicing occurs in the presence of an added metal ion. The dependence of the 3′-*S*-pc substrate reaction on the "thiophilic" manganese(II) ion reflects the role of the metal ion in leaving group stabilization.[8]

In summary, we have developed an efficient synthesis of the phosphoramidite derivative of 2′-*O*-(*o*-nitrobenzyl)-3′-thioguanosine starting from N^2-isobutyrylguanosine in nine steps. From this phosphoramidite, an 18-nucleotide RNA containing a 2′-*O*-photoliable group adjacent to a 3′-*S*-phosphorothiolate linkage was successfully synthesized by the solid-phase synthesis. Irradiation with UV light released the photocaging group without affecting the integrity of the 3′-*S* modification. This capability can enable effective use of the 3′-sulfur modification to perform metal rescue experiments even in systems that undergo complex assembly and conformational changes en route to the chemical step. As proof-of-principle, we have incorporated this modified RNA into a model yeast pre-mRNA splicing substrate and used it to probe for catalytic metal ion interactions in the spliceosome.[8]

EXPERIMENTAL SECTION

N^2-Isobutyryl-2′-*O*-(*o*-nitrobenzyl)guanosine (2). Under argon, N^2-isobutyrylguanosine **1** (1.72 g, 4.86 mmol) was treated with NaH (307 mg, 95%, 12.15 mmol) in DMF (40 mL) at 0 °C. After hydrogen gas generation ceased (45 min), *o*-nitrobenzyl bromide (1.58 g, 7.30 mmol) was added, and the mixture was stirred at rt for 5 h. The reaction was neutralized with 1 N HCl. The mixture was evaporated, and the residue was purified by silica gel chromatography, eluting with 4−6% methanol in dichloromethane to give the product **2a**[14,22] as a yellow foam (1.12 g, 47% yield).

5′-*O*-(*tert*-Butyldiphenylsilyl)-N^2-isobutyryl-2′-*O*-(*o*-nitrobenzyl)-3′-*O*-trifluoromethylsulfonylguanosine (3). To a stirred solution of N^2-isobutyryl-2′-*O*-(*o*-nitrobenzyl)guanosine (**2**) (2.312 g, 4.73 mmol) in dry pyridine (20 mL) was added *tert*-butyldiphenylsilyl chloride (1.82 mL, 7.10 mmol) under argon. The mixture was stirred at rt for 24 h, then quenched with CH_3OH (4.0 mL), and evaporated to a syrup. The residue was dissolved in CH_2Cl_2 and washed with H_2O. The organic layer was dried over anhydrous $MgSO_4$. After filtration and removal of solvent, the residue was isolated by silica gel chromatography, eluting with 2% CH_3OH in CH_2Cl_2 to give 5′-*O*-(*tert*-butyldiphenylsilyl)-N^2-isobutyryl-2′-*O*-(*o*-nitrobenzyl)guanosine as a white foam: 3.24 g (94% yield); ^{1}H NMR ($CDCl_3$/TMS) δ 12.20 (brs, 1H), 9.95 (brs, 1H), 7.97 (s, 1H), 7.96 (d, 1H, J = 8.8 Hz), 7.70−7.60 (m, 5H), 7.53 (m, 1H), 7.45−7.25 (m, 7H), 6.03 (d, 1H, J = 2.8 Hz), 5.28 (d, 1H, J = 15.0 Hz), 5.14 (d, 1H, J = 15.0 Hz), 4.61 (m, 1H), 4.28 (m, 1H), 4.22 (m, 1H), 4.05 (m, 1H), 3.88 (m, 1H), 2.84 (m, 1H), 1.26 (d, 3H, J = 4.8 Hz), 1.24 (d, 3H, J = 4.8 Hz), 1.02 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 179.6, 155.6, 147.9, 147.7, 146.9, 136.8, 135.5, 135.4, 134.0, 133.8, 132.7, 132.4, 129.84, 129.80, 128.7, 128.4, 127.8, 127.7, 124.6, 121.2, 86.9, 84.6, 83.3, 69.1, 68.8, 63.0, 36.1, 26.8, 19.0, 18.9, 18.8; HRMS calcd for $C_{37}H_{43}N_6O_8Si$ [MH$^+$] 727.2906, found 727.2903. Under argon, to a solution of 5′-*O*-(*tert*-butyldiphenylsilyl)-N^2-isobutyryl-2′-*O*-(*o*-nitrobenzyl)guanosine (3.24 g, 4.46 mmol) and DMAP (763 mg, 6.25 mmol) in dry CH_2Cl_2 (30 mL) at 0 °C was added CF_3SO_2Cl (0.50 mL, 4.7 mmol). After the mixture was stirred at 0 °C for 3 h, the reaction was quenched with ice water (20 mL) and the mixture stirred for 15 min. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 30 mL). The organic layers were combined, washed with brine, and subsequently dried over anhydrous $MgSO_4$. After filtration and removal of solvent, the residue was purified by silica gel chromatography, eluting with 1.5−2% methanol in CH_2Cl_2 to give **3** as a white foam: 2.37 g (62% yield); ^{1}H NMR ($CDCl_3$/TMS + a few drops of D_2O) δ 8.00 (d, 1H, J = 8.4 Hz), 7.85 (s, 1H), 7.70−7.50 (m, 6H), 7.50−7.30 (m, 7H), 6.03 (d, 1H, J = 4.8 Hz), 5.71 (m, 1H), 5.30 (d, 1H, J = 14.8 Hz), 5.09 (d, 1H, J = 14.8 Hz), 4.89 (t, 1H, J = 4.8 Hz), 4.46 (m, 1H), 4.08 (dd, 1H, J = 3.4, 12.0 Hz), 3.86 (dd, 1H, J = 3.2, 12.0 Hz), 2.63 (m, 1H), 1.27 (d, 3H, J = 6.0 Hz), 1.26 (d, 3H, J = 6.0 Hz), 1.04 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 178.8, 155.4, 147.8, 147.7, 147.0, 137.1, 135.6, 135.5, 134.3, 133.3, 132.2, 132.0, 130.5, 130.4, 128.8, 128.6, 128.18, 128.15, 124.8, 122.1, 118.6, 86.4, 82.2, 81.9, 80.1, 69.9, 61.9, 36.6, 26.9, 19.2, 19.02, 18.98; HRMS calcd for $C_{38}H_{42}N_6O_{10}F_3SiS$ [MH$^+$] 859.2399, found 859.2399.

3′-β-Bromo-5′-*O*-(*tert*-butyldiphenylsilyl)-N^2-isobutyryl-2′-*O*-(*o*-nitrobenzyl)guanosine (4). A mixture of **3** (1.641 g, 1.91 mmol) and NaBr (786 mg, 7.64 mmol) in acetone (20 mL) was heated under reflux for 23 h under argon. The solvent was removed, and the residue was isolated by silica gel chromatography, eluting with 2% methanol in CH_2Cl_2 to give **4** as pale yellow foam: 1.43 g (95% yield); ^{1}H NMR ($CDCl_3$/TMS) δ 12.15 (brs, 1H), 9.76 (brs, 1H), 8.07 (d, 1H, J = 7.2 Hz), 7.84 (s, 1H), 7.80−7.60 (m, 6H), 7.55−7.30 (m, 7H), 6.00 (s, 1H), 5.56 (d, 1H, J = 14.8 Hz), 5.11 (d, 1H, J = 14.8 Hz), 4.60−4.47 (m, 3H), 4.11 (dd, 1H, J = 5.8, 10.6 Hz), 4.03 (dd, 1H, J = 5.8, 10.6 Hz), 2.83 (m, 1H), 1.27 (d, 6H, J = 7.2 Hz), 1.09 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 179.5, 155.6, 148.0, 147.4, 146.9, 136.7, 135.59, 135.57, 134.3, 133.6, 132.8, 130.0, 128.8, 128.7, 127.89, 127.87, 124.9, 121.4, 90.6, 89.5, 82.4, 69.3, 64.8, 50.3, 36.3, 26.8, 19.2, 19.1, 18.9 ppm; HRMS calcd for $C_{37}H_{42}N_6O_7SiBr$ [MH$^+$] 789.2062, found 789.2059.

3′-*S*-Acetyl-N^2-isobutyryl-2′-*O*-(*o*-nitrobenzyl)-3′-thioguanosine (5). Under argon to a solution of **4** (2.99 g, 3.78 mmol) in dry DMF (25 mL) was added potassium thioacetate (1.30 g, 11.4 mmol), and the mixture was stirred at 60 °C for 7 h. After the solvent was removed under reduced pressure, the residue was partitioned between a saturated aqueous $NaHCO_3$ solution/brine (v/v, 1:1) and CH_2Cl_2.

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The organic layer was dried over $MgSO_4$. After filtration and removal of the solvent, the residue was dissolved in THF (45 mL), and AcOH (1.23 mL, 21.7 mmol) and *n*-$Bu_4NF{\cdot}3H_2O$ (2.74 g, 8.69 mmol) were added. The mixture was stirred at rt for 14 h, then diluted with CH_2Cl_2, washed with saturated aqueous $NaHCO_3$ and brine, and dried over $MgSO_4$. After filtration and removal of solvent, the residue was purified by silica gel chromatography, eluting with 3% MeOH in CH_2Cl_2 to give **5** as a white foam: 1.12 g (54% yield); ^{1}H NMR (CD_3OD/TMS) δ 8.37 (s, 1H), 8.00 (dd, 1H, J = 1.2, 8.4 Hz), 7.71 (d, 1H, J = 6.8 Hz), 7.66 (dt, 1H, J = 1.2, 7.6 Hz), 7.51 (dt, 1H, J = 1.2, 7.8 Hz), 6.19 (d, 1H, J = 2.0 Hz), 5.25 (d, 1H, J = 13.6 Hz), 5.03 (d, 1H, J = 13.6 Hz), 4.55−4.44 (m, 2H), 4.25−4.18 (m, 1H), 3.92 (dd, 1H, J = 2.4, 12.8 Hz), 3.72 (dd, 1H, J = 2.8, 12.8 Hz), 2.75 (m, 1H), 2.36 (s, 3H), 1.25 (d, 3H, J = 4.4 Hz), 1.23 (d, 3H, J = 4.4 Hz); ^{13}C NMR ($CDCl_3$) δ 196.0, 181.6, 157.4, 149.7, 149.1, 139.2, 134.7, 134.4, 130.8, 130.0, 125.8, 121.5, 89.2, 86.4, 85.2, 70.8, 61.4, 44.6, 37.0, 30.4, 19.4 19.3 ppm; HRMS calcd for $C_{23}H_{27}N_6O_8S$ [MH^+] 547.1606, found 547.1606.

3′-*S*-Acetyl-5′-*O*-(dimethoxytrityl)-*N*^{2}-isobutyryl-2′-*O*-(*o*-nitrobenzyl)-3′-thioguanosine (6). To a solution of **5** (861 mg, 1.58 mmol) in dry pyridine (15 mL) under argon was added DMTCl (1.07 g, 3.15 mmol). The mixture was stirred at rt for 24 h and quenched with MeOH. The solvent was removed, the residue was partitioned between 5% aqueous $NaHCO_3$ and CH_2Cl_2. The organic layer was washed with brine and dried over $MgSO_4$. After filtration and removal of solvent, the residue was isolated by silica gel chromatography, eluting with 0−1% MeOH in CH_2Cl_2 containing 0.5% Et_3N to give **6** as a pale yellow foam: 1.28 g (95% yield); ^{1}H NMR ($CDCl_3$/TMS) δ 12.04 (brs, 1H), 9.18 (brs, 1H), 8.08 (d, 1H, J = 8.0 Hz), 7.98 (s, 1H), 7.82 (d, 1H, J = 7.6 Hz), 7.69 (m, 1H), 7.50−7.15 (m, 10H), 6.80 (d, 4H, J = 8.8 Hz), 6.09 (s, 1H), 5.51 (d, 1H, J = 15.6 Hz), 5.15 (d, 1H, J = 15.2 Hz), 4.84 (dd, 1H, J = 5.2, 11.2 Hz), 4.42 (d, 1H, J = 4.8 Hz), 4.35 (m, 1H), 3.77 (s, 6H), 3.51 (dd, 1H, J = 2.8, 11.2 Hz), 3.32 (m, 1H), 2.71 (m, 1H), 2.30 (s, 3H), 1.26 (d, 3H, J = 7.2 Hz), 1.24 (d, 3H, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$) δ 193.9, 179.0, 158.6, 155.5, 147.7, 147.1, 146.8, 144.3, 137.0, 135.6, 135.5, 134.34, 134.25, 130.1, 128.6, 128.5, 128.2, 127.9, 127.0, 124.8, 122.0, 113.1, 88.7, 86.5, 84.8, 82.9, 69.5, 61.4, 55.3, 43.7, 36.5, 30.5, 19.0 18.8 ppm; HRMS calcd for $C_{44}H_{45}N_6O_{10}S$ [MH^+] 849.2912, found 849.2922.

5′-*O*-(Dimethoxytrityl)-*N*^{2}-isobutyryl-2′-*O*-(*o*-nitrobenzyl)-3′-thioguanosine (7). To a solution of guanidine hydrochloride (108 mg, 1.13 mmol) in a mixed solvent of MeOH/CH_2Cl_2 (9:1, v/v, 10 mL) was added NaOMe (0.5 M in MeOH, 0.40 mL, 0.20 mmol) at 0 °C. After the mixture was stirred at 0 °C for 10 min, the solution was transferred to the flask containing **6** (160 mg, 0.188 mmol) in dry CH_2Cl_2 (10 mL). The mixture was warmed to rt and stirred for 3 h, and then partitioned between H_2O and CH_2Cl_2. The organic layer was separated, washed with brine, and dried over anhydrous $MgSO_4$. The solvent was removed, and the residue was isolated by silica gel chromatography, eluting with 1−2% MeOH in CH_2Cl_2 containing 0.5% Et_3N to give the product as a white foam: 137 mg (90% yield); ^{1}H NMR ($CDCl_3$/TMS) δ 12.08 (brs, 1H), 9.20 (brs, 1H), 8.07 (d, 1H, J = 7.2 Hz), 8.06 (s, 1H), 7.89 (d, 1H, J = 7.6 Hz), 7.68 (m, 1H), 7.53−7.18 (m, 10H), 6.83 (d, 4H, J = 7.6 Hz), 6.07 (s, 1H), 5.67 (d, 1H, J = 15.6 Hz), 5.21 (d, 1H, J = 15.6 Hz), 4.20 (m, 1H), 4.15 (d, 1H, J = 5.2 Hz), 3.77 (s, 6H), 3.70 (m, 1H), 3.62 (m, 1H), 3.49 (dd, 1H, J = 2.8, 11.2 Hz), 2.75 (m, 1H), 1.26 (d, 6H, J = 7.0 Hz); ^{13}C NMR ($CDCl_3$) δ 179.2, 158.7, 155.5, 147.9, 147.1, 146.7, 144.3, 136.1, 135.5, 134.6, 130.15, 130.06, 128.5, 128.4, 128.14, 128.08, 127.1, 124.8, 121.9, 113.4, 88.1, 86.7, 86.5, 85.6, 69.1, 60.6, 55.3, 38.6, 36.4, 19.1, 18.9 ppm; HRMS calcd for $C_{42}H_{43}N_6O_9S$ [MH^+] 807.2807, found 807.2819.

5′-*O*-(Dimethoxytrityl)-*N*^{2}-isobutyryl-2′-*O*-(*o*-nitrobenzyl)-3′-thioguanosine-3′-*S*-(cyanoethyl *N*,*N*-diisopropylphosphoramidite) (8). Under argon to a solution of **7** (137 mg, 0.17 mmol) in dry dichloromethane (10 mL) were added *N*,*N*-diisopropylethylamine (0.15 mL, 0.86 mmol), 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (96 μL, 0.43 mmol), and 1-methylimidazole (6.2 μL, 0.078 mmol). The mixture was stirred at rt for 1 h, quenched with MeOH (1 mL), and stirred for an additional 5 min. The solvent was removed, and the residue was purified by silica gel chromatography, eluting with 0−5% acetone in CH_2Cl_2 containing 0.5% Et_3N to give product as a white foam: 0.146 g (85% yield); ^{31}P NMR (CD_3CN) δ 164.6, 159.2 ppm; HRMS calcd for $C_{51}H_{60}N_8O_{10}PS$ [MH^+] 1007.3885, found 1007.3884.

5′-*O*-(Dimethoxytrityl)-*N*^{2}-isobutyryl-2′-*O*-(*o*-nitrobenzyl)-guanosine 3′-*N*,*N*-Diisopropyl(cyanoethyl)phosphoramidite (9). To a solution of 5′-*O*-(dimethoxytrityl)-*N*^{2}-isobutyryl-2′-*O*-(*o*-nitrobenzyl)guanosine[22] (47 mg, 0.059 mmol) in dry CH_2Cl_2 (5.0 mL) under Ar were added *N*,*N*-diisopropylethylamine (52 μL, 0.30 mmol), 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (42 mg, 0.18 mmol), and 1-methylimidazole (5.0 μL, 0.059 mmol). The mixture was stirred at rt until all starting material was consumed (1 h). The reaction was quenched with MeOH (1 mL) and stirred for 5 min. After the solvent was removed, the crude product was purified by silica gel chromatography, eluting with 1% MeOH in CH_2Cl_2 containing 0.5% Et_3N, to give the corresponding phosphoramidite as a yellow foam: 56 mg (95% yield); ^{31}P NMR (CD_3CN) δ 152.8, 152.6; HRMS calcd for $C_{51}H_{60}N_8O_{11}P$ [MH^+] 991.4119, found 991.4110.

Oligonucleotide Synthesis. The oligonucletide 5′-UUU $AG_{3'S,2'\text{-}O\text{-}o\text{NBn}}$A GGU UGC UGC UUU-3′ (ACT1-3′-*S*) was synthesized on an Expedite 8900 DNA synthesizer by manual coupling of phosphoramidite **8** through a modified protocol as previously described.[24] Following the standard oligonucleotide deprotections[29] (a, concentrated NH_4OH/EtOH, 3:1 (v/v), 55 °C, 17 h; b, TEA-3HF/TEA/NMP, 65 °C, 1.5 h), the oligonucleotide (1.5% yield) was obtained by reverse-phase HPLC purification (C18 column, 0−30% aceonitrile/100−70% 0.1 M TEAA pH 7.0 over 30 min) and confirmed by the MALDI-TOF MS: calcd for ($M + NH_4$) 5854.8, found 5853.1.

Oligonucleotide Characterization. Alkaline hydrolysis: 4k cpm of the 5′-radiolabeled oligonucleotide (1 μL) with or without UV deprotection (UVP-B1000, 365 nm, 4 min) was treated with $NaHCO_3$ (pH 9, 50 mM, 2 μL) in a total volume of 10 μL solution at 90 °C for 10 min. Formamide loading dye (2×, 10 μL) was added, and the mixture was run on a 20% dPAGE gel.

Silver ion cleavage: 4k cpm of the 5′-radiolabeled oligonucleotides (2 μL) with or without UV deprotection (UVP-B1000, 365 nm, 4 min) was treated with $AgNO_3$ (100 mM, 0.4 μL) in a total volume of 20 μL solution in the dark at rt for 60 min. DTT (100 mM, 0.6 μL) was then added, and the mixture was spun at full speed for 5 min. A 15 μL aliquot of solution was withdrawn, added to 2× formamide loading dye (15 μL), and run on a 20% dPAGE gel.

Rnase T1 treatment: 4k cpm of the 5′-radiolabeled oligonucleotides (1 μL) with or without UV deprotection (UVP-B1000, 365 nm, 4 min) was combined with 8 M urea (pH 9, 6 μL), 200 mM sodium citrate (pH 5, 1 μL), and 1 unit of RNase T1 in a final reaction volume of 9 μL. After a 10 min incubation at 37 °C, 2× formamide loading dye (8 μL) was added and the sample was run on a 20% dPAGE gel.

Synthesis of *ACT1* Yeast Splicing Substrates.[8] The ACT1-1-373 (nucleotides 1−373) was synthesized by in vitro transcription from a plasmid template linearized with *Hin*dIII restriction and containing ACT1-1-373 followed by an HDV ribozyme sequence. In cases where HDV cleavage was inefficient during transcription, the RNA was resuspended in Tris (10 mM, pH 7.5) and $MgCl_2$ (20 mM). Ribozyme cleavage was induced via 2−4 cycles of 90 °C for 1 min, rt for 15 min, and 37 °C for 15 min. The buffer conditions were then adjusted for T4 PNK treatment of the transcript to remove the 2′,3′-cyclic phosphate left by the ribozyme. The ACT1-392-590 (nucleotides 392−590) was synthesized by in vitro transcription using a PCR-derived template generated using plasmid bJPS149. As the subsequent ligation requires a 5′-monophosphate group, a 4-fold excess of GMP over GTP was included in the transcription reaction.

ACT1 ligation reactions consisted of 500 pmol of ACT1-1-373, 50 pmol of oligonucleotide ACT1-3′-*O* or ACT1-3′-*S*, and 500 pmol of ACT1-392-590. The RNA was hybridized to 50 pmol of ACT1 splint in buffer TEN50 (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 50 mM NaCl) on a thermal cycle by heating to 90 °C for 2 min followed by reduction of the temperature by 1 °C for 1 min to 24 °C, then cooling to 4 °C for 5 min. T4 DNA ligase (∼100 pmol, synthesized in-house)

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or T4 RNA ligase 2 (2 units, New England Biolabs) was then added, and reactions were incubated at 37 °C for 4 h. The ligation reactions were DNase-treated (RNase-free DNase) for 15 min to remove splint, phenol−chloroform extracted, and ethanol precipitated before purification on 6% denaturing polyacrylamide gel. Bands containing full-length ACT1 pre-mRNA were excised and recovered by passive elution in TEN250 buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 250 mM NaCl) overnight at 4 °C. Yields typically ranged from 200 to 600 fmol, enough for 50−150 splicing reactions.

Splicing Reactions. Yeast splicing-competent extracts were prepared using the liquid nitrogen method, as described in the literature.[30] In vitro splicing reactions consisted of ^{32}P-labeled substrates (0.2−0.4 nM), 40% yeast extract that was pretreated with 1 mM EDTA, 3% PEG 8000, 60 mM K_2PO_4 (pH 7), 3.5 mM $MgCl_2$, and 2 mM ATP. Reactions were incubated at 20 °C for 20 min, then on ice for 5 min for 365 nm UV light treatment. Metal ion concentrations were adjusted to 4 mM with either $MgCl_2$ or $MnCl_2$, and the reactions were incubated at 20 °C for another 20 min. The reactions were then quenched and analyzed by 6% dPAGE gel as described previously.[8]

■ ASSOCIATED CONTENT

Supporting Information

MALDI-TOF MS of 2′-photocaged 3′-*S*-RNA, ^{1}H NMR and ^{13}C NMR spectra of compounds **3**−**7**, ^{1}H NMR and ^{31}P NMR of phosphoramidites **8** and **9**. 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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