

Supplementary Data

Chemically synthesized circular RNAs with phosphoramidate linkages enable rolling circle translation

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Table S1 Chemically synthesized RNA fragments for circular RNA synthesis.

Name	Sequence (5' to 3') ^a
L48	AUU AUU GAC UAC AAG GAC GAC GAU GAC AAA AUU AUU AUU AUU AAG GAG
L78	AUA UAU CCG AUG AUU AUU GAC UAC AAG GAC GAC GAU GAC AAA AUU AUU GAC UAC AAG GAC GAC GAU GAC AAA CUG CUG
L48N	AUU AUU GAC UAC AAG GAC GAC GAU GAC AAA AUU AUU AUU AUU AAG GAG-NH₂
L78N	AUA UAU CCG AUG AUU AUU GAC UAC AAG GAC GAC GAU GAC AAA AUU AUU GAC UAC AAG GAC GAC GAU GAC AAA CUG CUG-NH ₂
L59N	AC UAC AAG GAC GAC GAU GAC AAA AUU AUU GAC UAC AAG GAC GAC GAU GAC AAA CUG CUG-NH ₂
L67N	AUU AUU GAC UAC AAG GAC GAC GAU GAC AAA AUU AUU AUU AUU AAG GAG AUA UAU CCG AUG AUU AUU G-NH₂
L48N*	AUU AUU GAC UCA GAA GAC ACG GAU GAC AAA AUU AUU AUU AUU AAG GAG-NH₂
L78N*	AUA UAU CCG AUG AUU AUU GAC UCA GAA GAC ACG GAU GAC AAA AUU AUU GAC UCA GAA GAC ACG GAU GAC AAA CUG CUG-NH ₂

^a5'-end of the RNAs were phosphorylated. G-NH₂ denotes 3'-amino-3'-deoxy G.

Highlighted in blue denotes FLAG-coding sequence. Highlighted in gray denotes SD sequence. Highlighted in yellow denotes randomized FLAG-coding sequence. Squared denotes a start codon (Met).

Table S2 Chemically synthesized splint DNAs for circular RNA synthesis.

Name	Sequence (5' to 3')
T1	TCG GAT ATA TCT CCT TAA TA
T2	CTT GTA GTC AAT AAT CAG CAG TTT GTC ATC
T3	TCC TTG TAG TCA ATA ATC AT
T5	TTC TGA GTC AAT AAT CAG CAG TTT GTC ATC

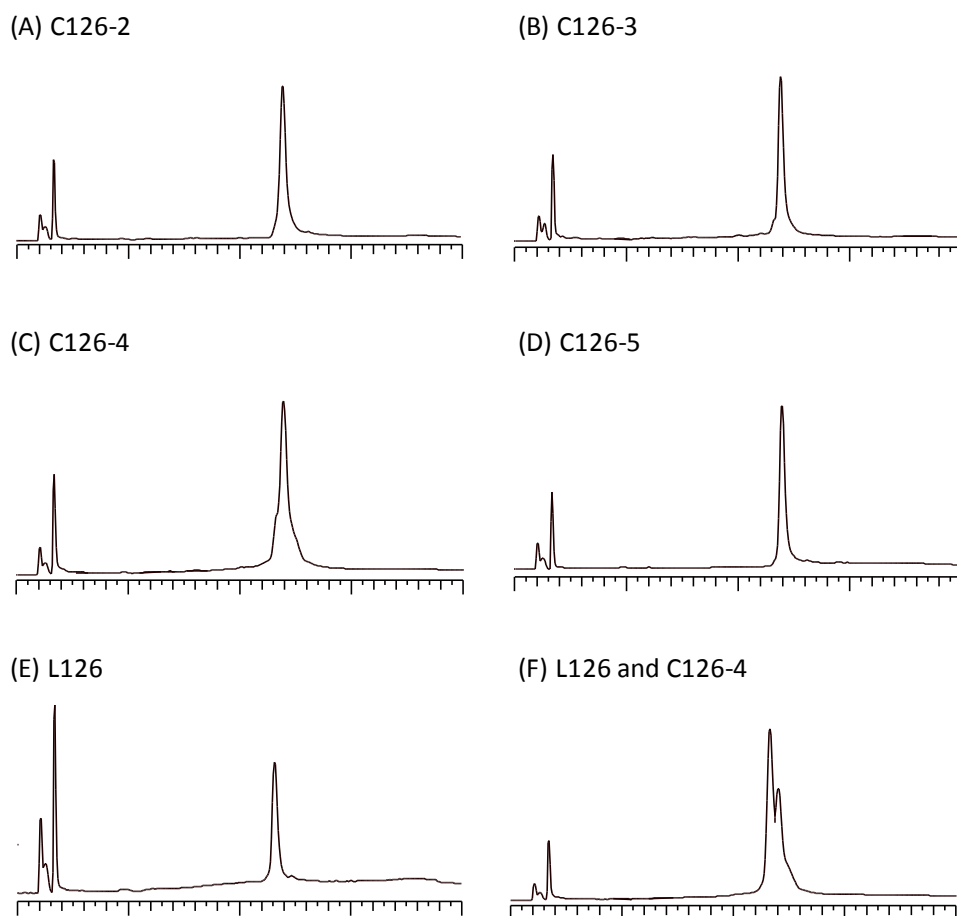


Figure S1. RNA purities for synthesized circular RNAs by RP-HPLC. (A) C126-2. (B) C126-3. (C) C126-4. (D) C126-5. (E) Linear 126 mer RNA (L126). (F) Co-injection result of a linear 126 mer RNA and circular 126 mer RNA. Linear 126 mer RNA and circular 126 mer RNA show the different retention time (11.67 min. for linear and 12.05 min. for circular RNA). HPLC analysis was performed with DNAPac RP column (Thermo Scientific, 100×2.1 mm, particle size: 4 μ m). Eluent A was 5% acetonitrile (MeCN) in 50 mM triethylammonium acetate (TEAA) buffer (pH 7.0), and eluent B was 100% MeCN. The gradient program was as follows: 0–20% B (0–20 min), 75% B (20.1–25 min) and 5% B (25.1–30.0 min) at 60 °C.

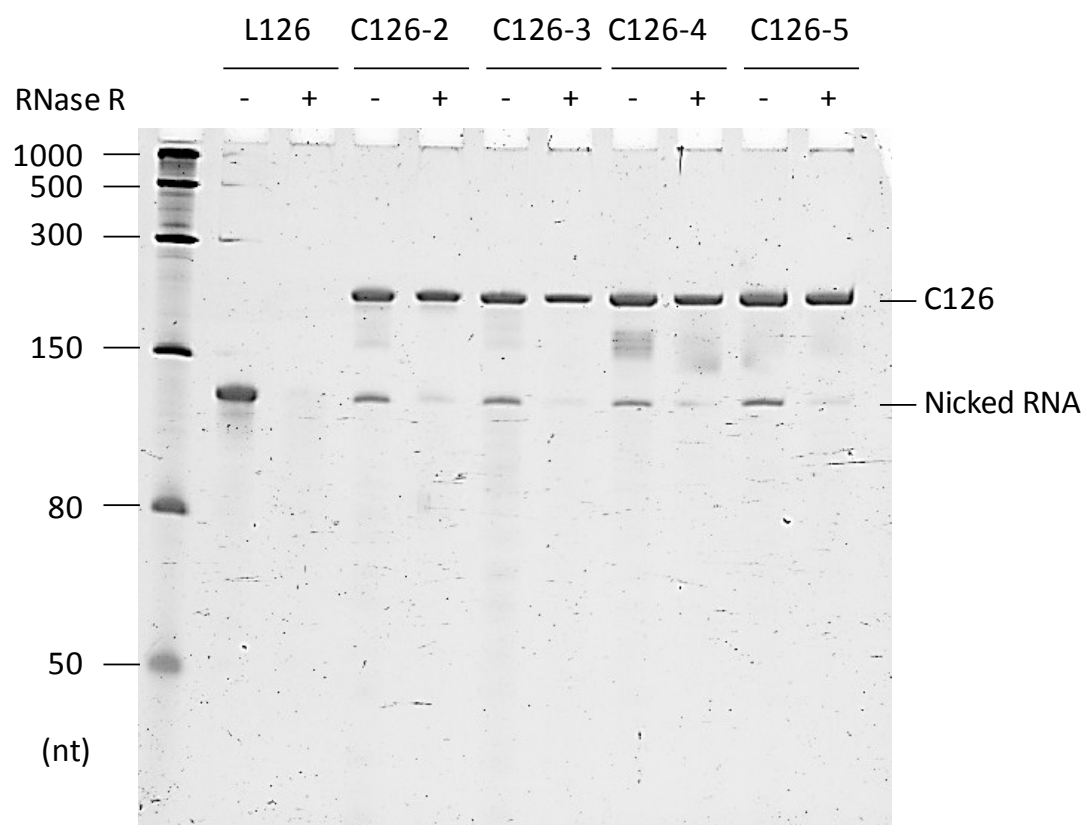
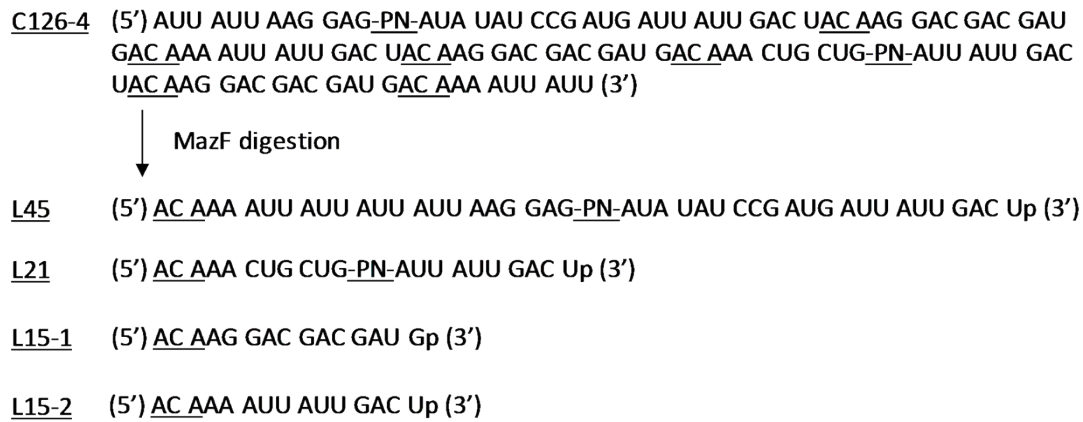
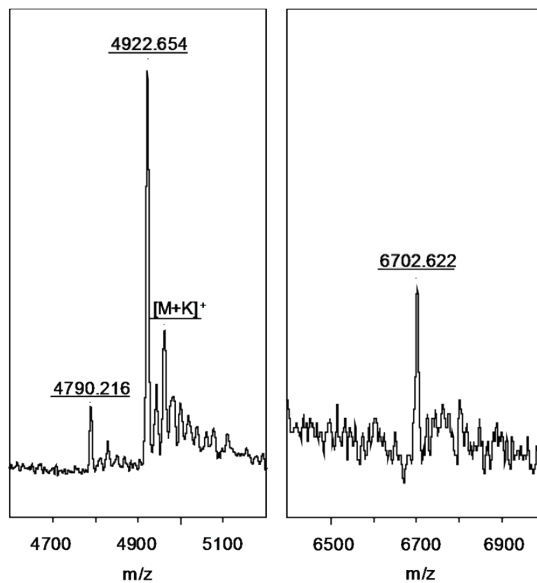


Figure S2. Circularization test by RNase R treatment. After the reaction, crude samples were analyzed by 8% denaturing PAGE containing 8 M urea (30 min, 25 mA constant). The gel was visualized by SYBR Green II staining.

(A)



(B)



(C)

Linear RNAs	Calcd. MS	Observed MS
L45	14421.59	-
L21	6703.00	6702.62
L15-1	4923.01	4922.65
L15-2	4790.87	4790.22

Figure S3. MALDI-TOF/MS analysis of C126-4 after MazF digestion reaction. (A) Sequences of original C126-4 and expected RNA fragments after MazF digestion. (B) MALDI-TOF/MS spectra of the RNA fragments. (C) Calculated MS [M] and observed MS [M+H]⁺ of the RNA fragments.

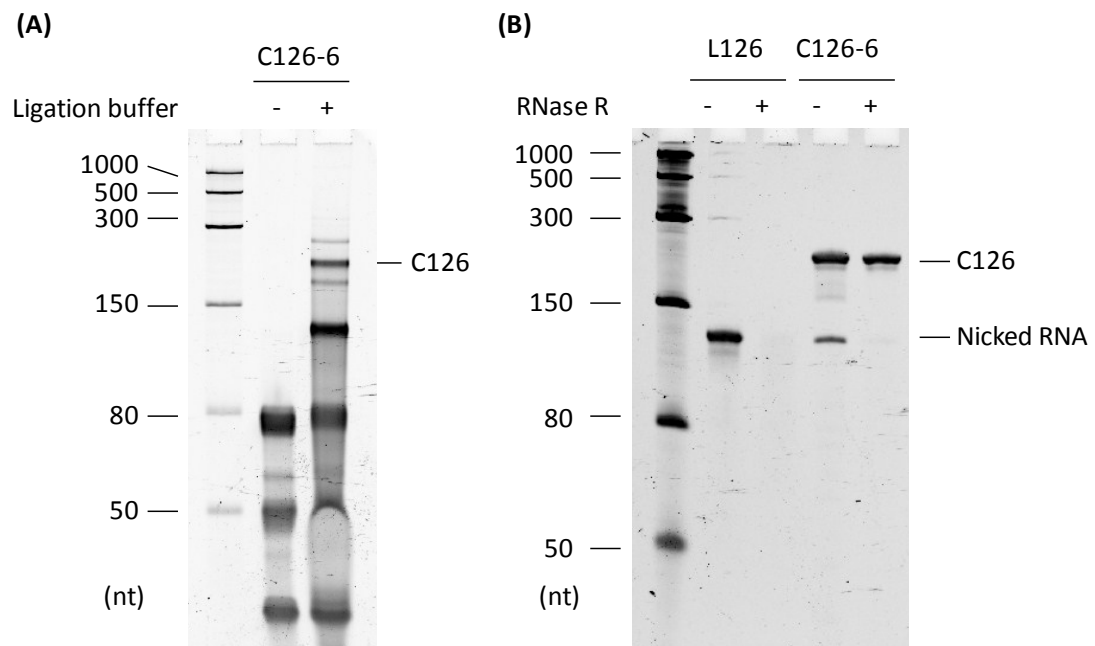


Figure S4. (A) One-pot chemical synthesis of C126-6. (B) RNase R treatment of C126-6. Both reaction crude was analyzed by eight percent denaturing PAGE (8 M urea, 30 min, 25 mA constant). The gel was visualized by SYBR Green II staining.

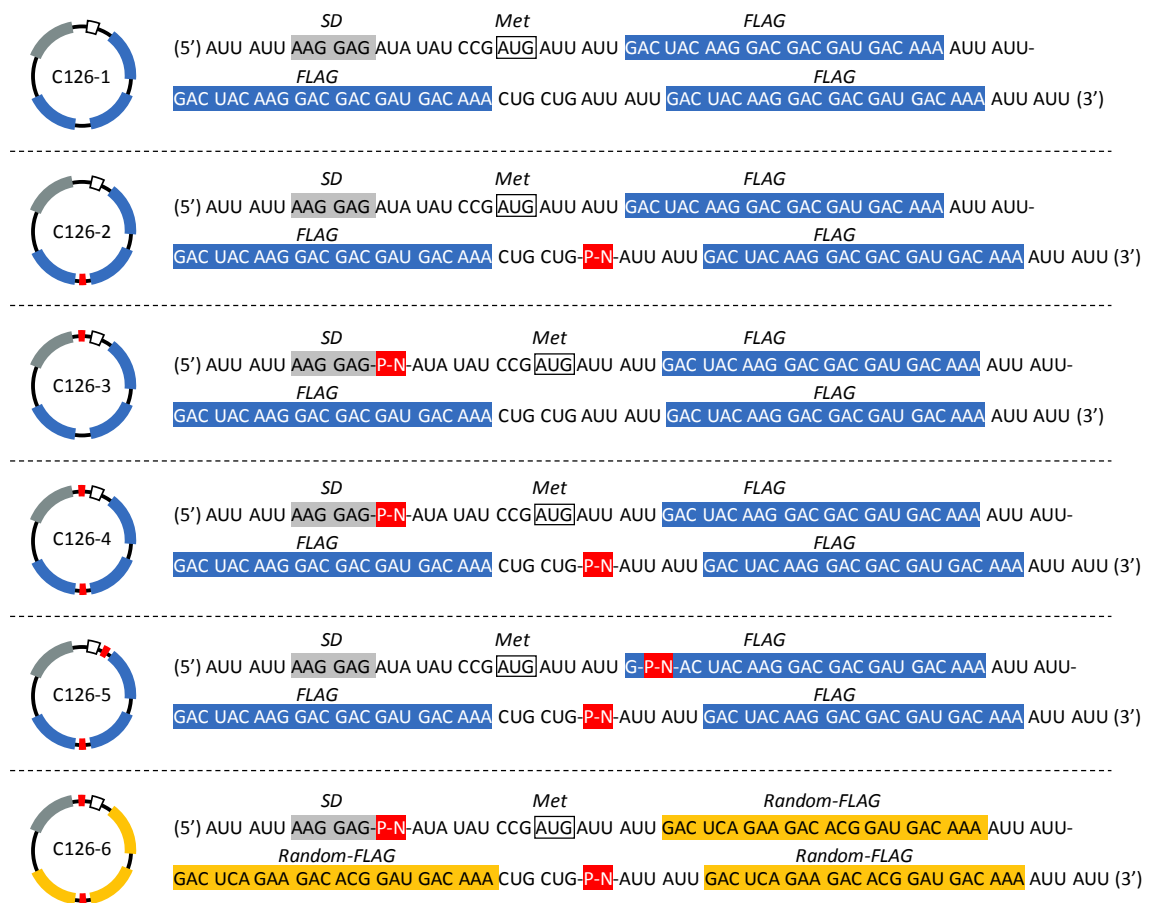


Figure S5. The sequences of circular RNA used in this study. Position of P-N bonds was indicated as P-N.

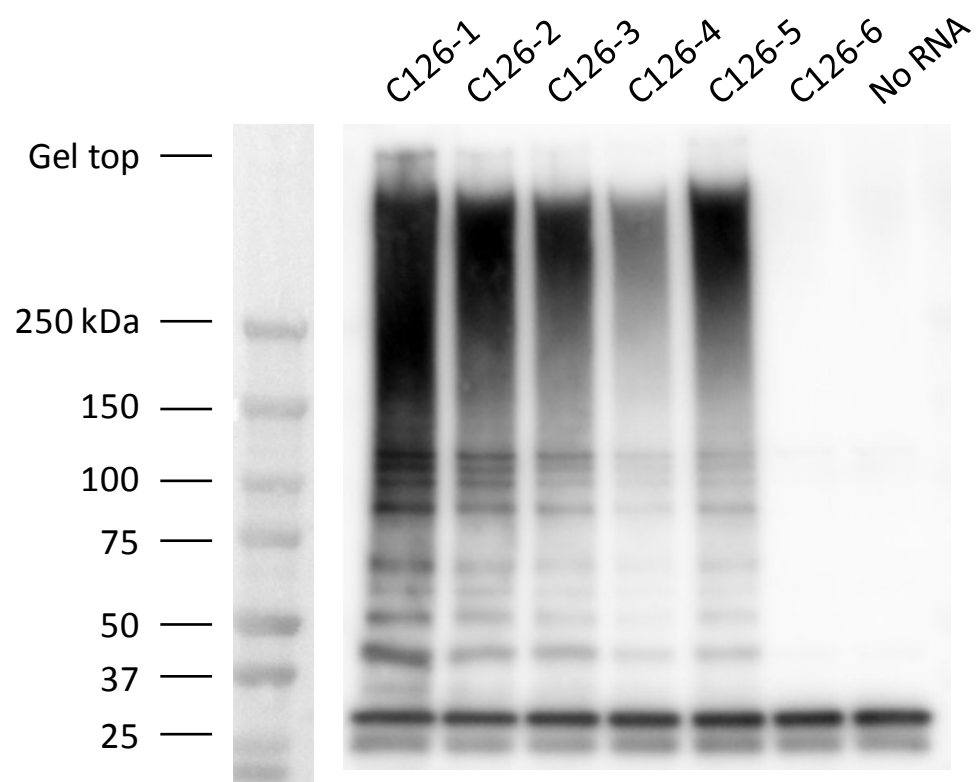


Figure S6. Row data of Figure 6 with protein size marker (Precision Plus Protein Dual Color Standards, Bio-Rad) and no RNA control.

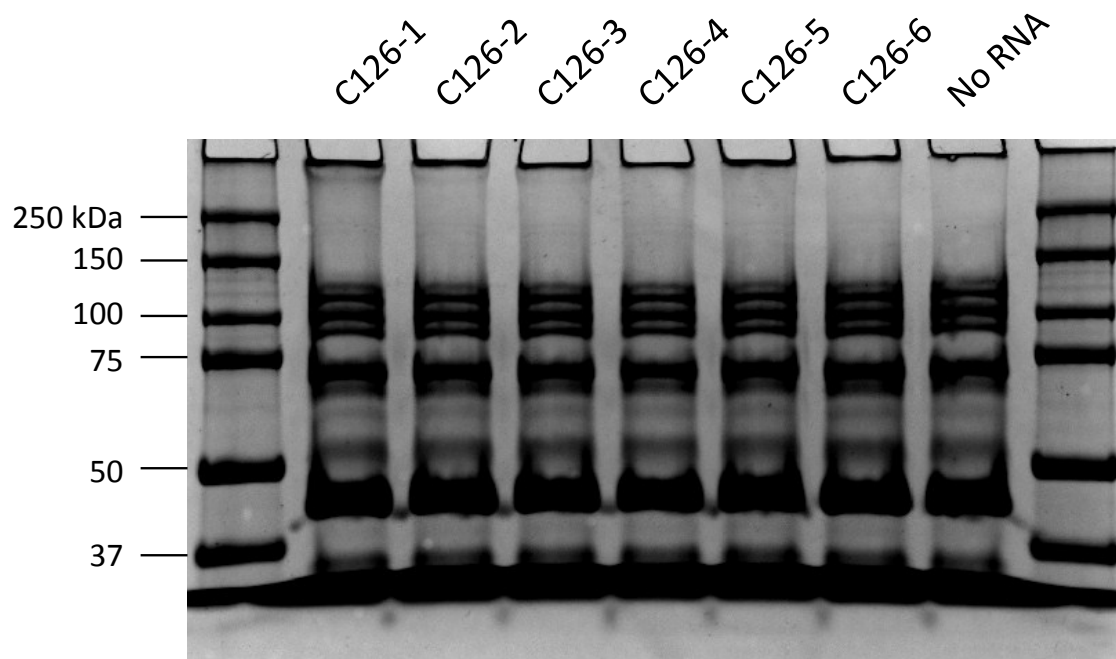


Figure S7. Translation products were analyzed by coomassie blue staining. 7.5% SDS-PAGE was used for the analysis.

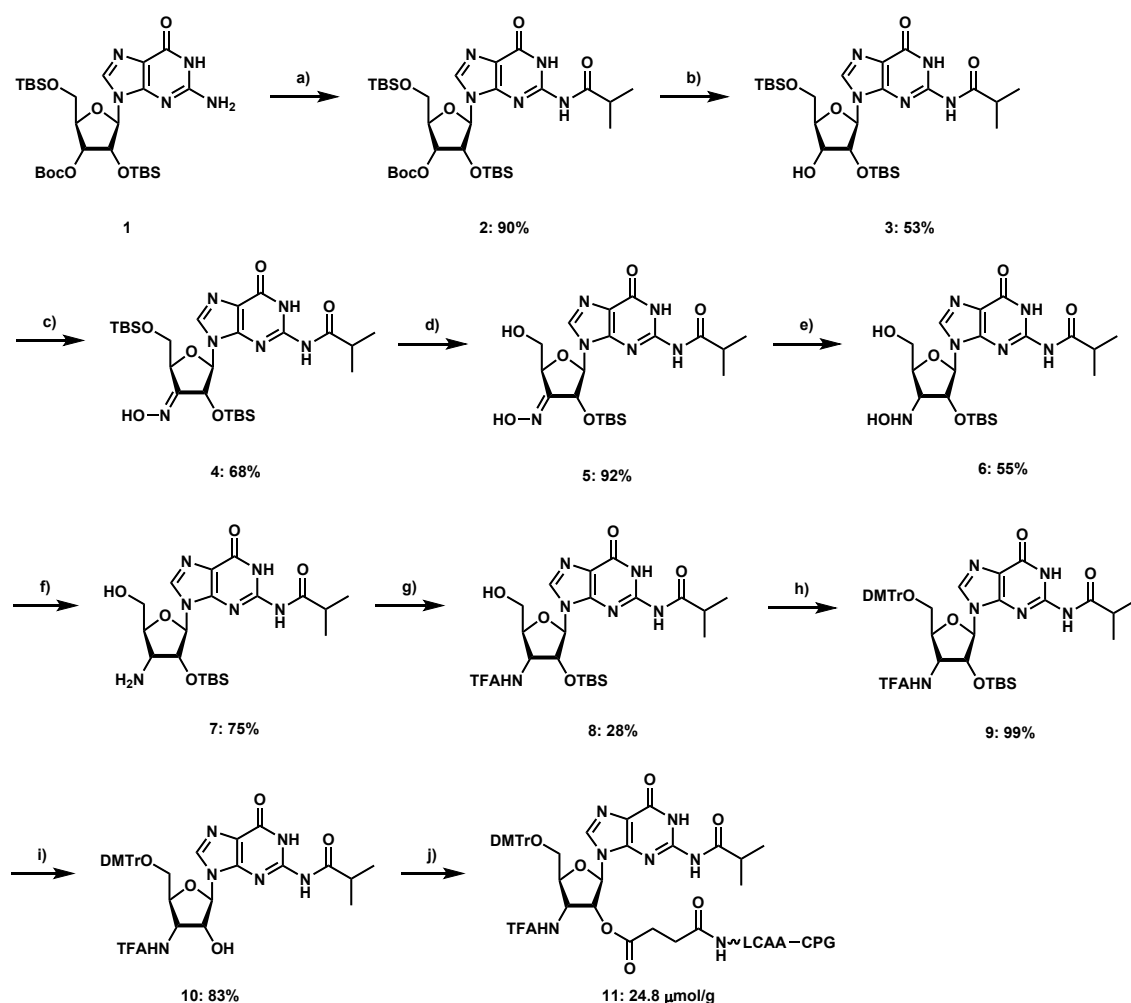
Experimental procedure

General Remarks

High-resolution mass spectra (HRMS) data were acquired in positive ion mode using micrOTOF-QII (Bruker) with an electrospray ionization (ESI) source. Mass data of oligonucleotides were obtained using a microflex MALDI-TOF mass spectrometer (Bruker Daltonics) by positive mode using a mixture of 3-hydroxypicolinic acid (HPA) and ammonium citrate as a matrix. Nuclear magnetic resonance (NMR) spectra were acquired on a JMM ECS-400 (JEOL) spectrometer with 400 MHz for proton (^1H NMR) and 100 MHz for carbon (^{13}C NMR); chemical shifts are reported in ppm (δ).

Synthesis of 3'-Amino G CPG

3'-amino modified G CPG (**11**) was synthesized as shown in Scheme S1. Compound **1** was synthesized according to reported method¹. Compound **2-11** were newly synthesized in this report.



Scheme S1. Synthesis of 3'-amino modified G CPG. a) Isobutyryl chloride, pyridine, CH_2Cl_2 , 0 °C. b) *o*-dichlorobenzene, reflux. c) 1) CrO_3 , Ac_2O , pyridine, room temperature. 2) $\text{NH}_2\text{OH}\cdot\text{HCl}$, pyridine, room temperature. d) 90% aq. TFA, 0 °C. e) NaBH_4 , AcOH , 0 °C. f) H_2 , 10% Pd/C, 90% aq. AcOH , room temperature. g) $\text{CF}_3\text{CO}_2\text{Et}$, Et_3N , MeOH , room temperature. h) DMTrCl , pyridine, room temperature. i) TBAF, THF, room temperature. j) 1) Succinic anhydride, Et_3N , DMAP, ACN, room temperature. 2) Native Amino lcaa CPG (1000 Å), EDC·HCl, HOAt, DMF, room temperature.

*N*²-Isobutyryl-3'-(*tert*-butoxycarbonyl)-2',5'-bis-*O*-(*tert*-butyldimethylsilyl) guanosine (2).

To a solution of **1** (1.60 g, 2.61 mmol) in pyridine (8 mL) was slowly added dropwise a solution of isobutyryl chloride (0.61 mL, 5.75 mmol) in CH₂Cl₂ (2 mL) at 0 °C under stirring. After completion of addition, the reaction mixture was stirred at room temperature for 1.5 hours and concentrated under vacuum to dryness. The residue was partitioned between EtOAc and H₂O and the organic layer was washed with sat. NaHCO₃ aq., brine, dried (Na₂SO₄), filtered, and concentrated. The residue was purified by column chromatography (SiO₂, 2% MeOH in CH₂Cl₂) to give **2** (1.61 g, 2.36 mmol, 90%).

¹H NMR (400 MHz, CDCl₃) δ 11.96 (1H, s), 8.38 (1H, s), 8.0d (1H, d *J* = 3.6), 5.87 (1H, q, *J* = 3.6 Hz), 5.24 (d, 1H, *J* = 4.8), 4.61 (dd, 1H, *J* = 8.8, 5.6 Hz), 4.28 (1H, s), 3.93 (d, *J* = 12.0 Hz), 3.84 (d, *J* = 12.4 Hz), 2.68 – 2.61 (1H, m), 1.50 (9H, d, *J* = 3.6), 1.29 – 1.25 (6H, m), 0.92 (9H, d, *J* = 3.6), 0.77 (9H, d, *J* = 3.2), 0.12 (6H, d, *J* = 3.6), -0.03 (3H, d, *J* = 3.6), -0.19 (3H, d, *J* = 3.2)

¹³C NMR (100 MHz, CDCl₃) δ 178.5, 155.7, 152.8, 148.4, 147.6, 136.9, 121.4, 87.7, 83.2, 83.1, 76.0, 75.0, 62.9, 36.5, 27.9, 36.1, 25.5, 19.2, 18.9, 18.5, 18.0, -5.1, -5.3, -5.4

ESI-HRMS: calcd. for C₃₁H₅₆N₅O₈Si₂ 682.3667 [M+H]⁺, found : 682.3675 [M+H]⁺

*N*²-Isobutyryl-2',5'-bis-*O*-(*tert*-butyldimethylsilyl)guanosine (**3**).

2 was dissolved in *o*-dichlorobenzene and refluxed for 3.5 hours. The reaction mixture was directly purified by column chromatography (SiO₂, 2% MeOH in CH₂Cl₂) to give **3** (0.31 g, 0.53 mmol, 53%).

¹H NMR (400 MHz, CDCl₃) δ 12.01 (1H, s), 8.50 (1H, s), 8.07 (1H, s), 5.86 (1H, d, *J* = 6.0 Hz), 4.47 (1H, s), 4.24 – 4.23 (1H, m), 4.22 – 4.21 (1H, m), 3.93 (1H, dd, *J* = 11.6, 2.0 Hz), 3.82 (1H, dd, *J* = 11.6, 2.0 Hz), 2.66 (1H, d, *J* = 6.8 Hz), 1.27 (3H, d, *J* = 6.8 Hz), 1.25 (3H, d, *J* = 6.8 Hz), 0.93 (9H, s), 0.82 (9H, s), 0.13 (3H, s), 0.12 (3H, s), -0.07

(3H, s), -0.20 (3H, s)

^{13}C NMR (100 MHz, CDCl_3) δ 179.0, 155.7, 148.5, 148.7, 147.8, 136.8, 121.0, 87.4, 85.4, 77.6, 71.8, 63.6, 36.2, 25.9, 25.4, 19.1, 18.7, 18.3, 17.8, -5.3, -5.4, -5.5, -5.6

ESI-HRMS: calcd. for $\text{C}_{26}\text{H}_{48}\text{N}_5\text{O}_6\text{Si}_2$ 582.3143 $[\text{M}+\text{H}]^+$, found : 582.3141 $[\text{M}+\text{H}]^+$

*N*²-Isobutyryl-2',5'-bis-*O*-(*tert*-butyldimethylsilyl)-3'-deoxy-3'-(hydroxyimino)guanosine (**4**).

To a suspension of CrO_3 (0.13 g, 1.29 mmol) and 4 Å MS (0.26 g) in CH_2Cl_2 (2.6 mL) was slowly added dropwise pyridine (207 μL , 2.58 mmol) at 0 °C under stirring. Ac_2O (122 μL , 1.29 mmol) was then added to the mixture and stirred at 0 °C for 30 min. This mixture was added a solution of **3** (0.25 g, 0.43 mmol) in CH_2Cl_2 (1.3 mL) and stirred at room temperature for 3 hours. The reaction mixture was diluted with EtOAc and filtered through Silica pad. A filter cake was washed with EtOAc and the filtrate was concentrated to give light brownish solid. This crude was used in next reaction without further purification.

To a solution of crude compound in pyridine (4.0 mL) was added $\text{NH}_2\text{OH}\cdot\text{HCl}$ (0.30 g, 4.30 mmol) and the mixture was stirred at room temperature for 24 hours. The reaction mixture was concentrated under vacuum and the residue was partitioned between EtOAc and H_2O . The organic layer was washed with brine, dried (Na_2SO_4), filtered, and concentrated. The residue was purified by column chromatography (SiO_2 , 2% MeOH in CH_2Cl_2) to give **4** (0.26 g, 0.44 mmol, 68% for 2 steps).

^1H NMR (400 MHz, CDCl_3) δ 12.14 (1H, s), 9.27 (1H, s), 8.78 (1H, s), 8.11 (1H, s), 5.78 (1H, d, $J = 7.6$ Hz), 5.09 (1H, s), 4.92 (1H, d, $J = 7.2$ Hz), 4.14 (1H, d, $J = 11.4$ Hz), 3.92 (1H, d, $J = 11.4$ Hz), 2.79 – 2.74 (1H, m), 1.27 – 1.21 (6H, m), 0.91 (9H, s), 0.71 (9H, s),

0.10 (3H, s), 0.07 (3H, s), -0.10 (3H, s), -0.23 (3H, s)

^{13}C NMR (100 MHz, CDCl_3) δ 178.9, 157.8, 155.6, 148.7, 147.8, 136.8, 120.8, 87.5, 86.5, 62.2, 36.3, 25.9, 25.5, 25.2, 19.1, 18.8, 18.3, 18.0, -5.0, -5.5, -5.6, -5.7

ESI-HRMS: calcd. for $\text{C}_{26}\text{H}_{47}\text{N}_6\text{O}_6\text{Si}_2$ 595.3096 $[\text{M}+\text{H}]^+$, found : 595.3064 $[\text{M}+\text{H}]^+$

*N*²-Isobutyryl-2'-*O*-(*tert*-butyldimethylsilyl)- 3'-deoxy-3'-(hydroxyimino)guanosine (**5**).

4 (129 mg, 0.22 mmol) was treated with 90% aq. TFA (1 mL) at 0 °C for 1 hour. The mixture was concentrated under vacuum. The residue was co-evaporated three times with H_2O and toluene (1:1, v/v) under vacuum to give colorless solid. The residual solid was purified by column chromatography (SiO_2 , 2 to 3% MeOH in CH_2Cl_2) to give **5** (96 mg, 0.20 mmol, 92%)

^1H NMR (400 MHz, CD_3OD) δ 8.36 (1H, s), 5.87 (1H, d, $J = 7.6$ Hz), 5.18 (1H, dd, $J = 7.6, 2.0$ Hz), 5.02 (1H, d, $J = 2.0$ Hz), 4.11 (1H, dd, $J = 12.0, 2.0$ Hz), 3.92 (1H, d, $J = 12.0, 2.0$ Hz), 2.71 (1H, sept., $J = 7.2$ Hz), 1.21 (6H, d, $J = 7.2$ Hz), 0.72 (9H, s), 0.00 (3H, s), -0.16 (3H, s)

^{13}C NMR (100 MHz, CD_3OD) δ 181.8, 157.4, 156.8, 151.0, 150.0, 139.8, 121.3, 88.4, 79.7, 76.5, 61.6, 36.9, 25.9, 19.4, 19.2, -4.5, -5.5

ESI-HRMS: calcd. for $\text{C}_{20}\text{H}_{32}\text{N}_6\text{NaO}_6\text{Si}$ 503.2050 $[\text{M}+\text{Na}]^+$, found : 503.2024 $[\text{M}+\text{Na}]^+$

*N*²-Isobutyryl-2'-*O*-(*tert*-butyldimethylsilyl)- 3'-deoxy-3'-(hydroxyamino)guanosine (**6**).

To a solution of **5** (93 mg, 0.19 mmol) in AcOH (1.9 mL) was slowly added NaBH_4 (15 mg, 0.38 mmol) at 0 °C. The resulting mixture was stirred at room temperature for an hour. The reaction mixture was concentrated, and the residue was partitioned between CH_2Cl_2 and H_2O . The aqueous layer was extracted with CH_2Cl_2 . The combined organic

layers were washed with brine, dried (Na₂SO₄), filtered, and concentrated. The residue was purified by column chromatography (SiO₂, 5% MeOH in CH₂Cl₂) to give **6** (51 mg, 0.11 mmol, 55%).

¹H NMR (400 MHz, CD₃OD) δ 8.34 (1H, s), 6.06 (1H, d, *J* = 6.0 Hz), 4.75 (1H, t, *J* = 6.4 Hz), 4.27 (1H, d, *J* = 2.8 Hz), 3.86 (1H, dd, *J* = 12.4, 2.0 Hz), 3.73 (1H, d, *J* = 12.4, 2.0 Hz), 3.62 – 3.60 (1H, m), 2.71 (1H, sept., *J* = 6.8 Hz), 1.21 (6H, d, *J* = 6.8 Hz), 0.82 (9H, s), -0.02 (3H, s), -0.23 (3H, s)

¹³C NMR (100 MHz, CD₃OD) δ 181.8, 157.4, 150.8, 149.8, 139.6, 139.4, 121.1, 89.7, 84.5, 77.7, 65.4, 65.2, 36.9, 26.0, -5.2, -5.3

ESI-HRMS: calcd. for C₂₀H₃₅N₆O₆Si 483.2387 [M+H]⁺, found : 483.23264 [M+H]⁺

*N*²-Isobutyryl-2'-*O*-(*tert*-butyldimethylsilyl)-3'-amino-3'-deoxy-guanosine, acetic acid (**7**).

To a solution of **6** (50 mg, 0.10 mmol) in 90% aq. AcOH (1.5 mL) was added 10% Pd/C (20 mg). The resulting mixture was stirred under H₂ gas atmosphere at room temperature for 18 hours. The mixture was filtered through celite pad and filtrate was concentrated. The residual white solid was purified by column chromatography (SiO₂, 6 to 10% MeOH in CH₂Cl₂) to give **7** (41 mg, 0.08 mmol, 78%).

¹H NMR (400 MHz, CD₃OD) δ 8.32 (1H, s), 5.99 (1H, s), 4.60 (1H, s), 3.95 – 3.68 (4H, m), 2.73 (1H, br. s), 1.22 (6H, br. s), 0.05 (3H, s), -0.06 (3H, s)

ESI-HRMS: calcd. for C₂₀H₃₄N₆NaO₅Si 489.2258 [M+Na]⁺, found : 489.2231 [M+Na]⁺

*N*²-Isobutyryl-2'-*O*-(*tert*-butyldimethylsilyl)-3'-(trifluoroacetyl-amino)-3'-deoxy-guanosine (**8**).

To a solution of **7** (40 mg, 0.078 mmol) and Et₃N (106 μL, 0.76 mmol) in MeOH (760 μL) was added CF₃CO₂Et (45 μL, 0.38 mmol). The resulting mixture was stirred at room temperature for 24 hours. The reaction mixture was concentrated, and the residue was purified by column chromatography (SiO₂, 5 to 11% MeOH in CH₂Cl₂) to give **8** (12 mg, 0.024 mmol, 28%).

¹H NMR (400 MHz, CDCl₃) δ 12.26 (1H, s), 10.11 (1H, s), 7.76 (1H, s), 7.26 (1H, d, *J* = 3.6 Hz), 5.71 (1H, d, *J* = 3.6 Hz), 4.98 (1H, dd, *J* = 6.8 Hz), 4.78 (1H, dd, *J* = 6.8, 3.6 Hz), 4.21 (1H, d, *J* = 6.8 Hz), 4.03 (1H, dd, *J* = 11.2 Hz), 3.82 (1H, dd, *J* = 11.2 Hz), 2.79 (1H, sept., *J* = 6.8 Hz), 1.26 (3H, d, *J* = 6.8 Hz), 1.24 (3H, d, *J* = 6.8 Hz), 0.85 (9H, s), -0.01 (3H, s), -0.11 (3H, s)

¹³C NMR (100 MHz, CDCl₃) δ 179.8, 158.0, 157.7, 157.3, 156.9, 155.2, 148.3, 147.3, 138.6, 122.0, 120.0, 117.1, 114.2, 111.3, 91.6, 83.7, 74.5, 61.3, 51.0, 36.1, 25.2, 18.9, 17.7, -5.0, -5.4

ESI-HRMS: calcd. for C₂₂H₃₃F₃N₆NaO₆Si 585.2081 [M+Na]⁺, found : 585.2085 [M+Na]⁺

*N*²-Isobutyryl-2'-*O*-(*tert*-butyldimethylsilyl)-3'-(trifluoroacetyl amino)-3'-deoxy-5'-*O*-[bis(4-methoxy)trityl]guanosine (**9**).

To a solution of **8** (10 mg, 0.017 mmol) in pyridine (1 mL) was added DMTrCl (36 mg, 0.106 mmol). The resulting mixture was stirred at room temperature for 1.5 hours. The reaction mixture was quenched by addition of MeOH (1 mL), and then concentrated. The residue was dissolved in EtOAc and washed with H₂O, brine, dried (Na₂SO₄), filtered, and concentrated. The residual yellow oil was purified by column chromatography (SiO₂, 17 to 33% EtOAc in hexane) to give **9** (15 mg, 0.017 mmol, 99%).

^1H NMR (400 MHz, CDCl_3) δ 11.99 (1H, s), 10.11 (1H, s), 8.07 (1H, s), 7.81 (1H, s), 7.45 (2H, dd, $J = 8.2, 2.0$ Hz), 7.32 (4H, dd, $J = 9.2, 3.6$ Hz), 7.24 – 7.29 (3H, m), 7.01 (1H, d, $J = 7.2$ Hz), 6.76 (4H, $J = 9.2, 3.6$ Hz), 5.71 (1H, d, $J = 4.2$ Hz), 5.16 (1H, dd, $J = 6.4, 4.2$ Hz), 4.20 – 4.17 (1H, m), 3.76 (3H, s), 3.75 (3H, s), 3.56 (1H, dd, $J = 11.2, 2.8$ Hz), 3.22 (1H, dd, $J = 11.2, 2.8$ Hz), 1.82 (1H, d, $J = 6.8$ Hz), 0.97 (3H, d, $J = 6.8$ Hz), 0.68 (9H, s), 0.79 (3H, d, $J = 6.8$ Hz), 0.04 (3H, s), -0.06 (3H, s)

^{13}C NMR (100 MHz, CDCl_3) δ 171.2, 158.7, 158.0, 157.6, 157.2, 156.8, 155.4, 147.6, 147.2, 144.8, 139.2, 135.9, 135.4, 130.0, 127.9, 127.1, 122.6, 120.0, 117.0, 114.1, 111.2, 90.1, 86.3, 81.7, 73.4, 62.4, 60.4, 55.2, 51.4, 36.1, 25.4, 18.4, 17.8, -5.0, -5.3

ESI-HRMS: calcd. for $\text{C}_{43}\text{H}_{52}\text{F}_3\text{N}_6\text{O}_8\text{Si}$ 865.3568 $[\text{M}+\text{H}]^+$, found : 865.3533 $[\text{M}+\text{H}]^+$

*N*²-Isobutyryl-3'-(trifluoroactylamino)-3'-deoxy-5'-*O*-[bis(4-methoxy)trityl]guanosine (**10**).

To a solution of **9** (14 mg, 0.016 mmol) in THF (1 mL) was added TBAF (1 M in THF, 19 μL), and the mixture was stirred at room temperature for 1 hours. The reaction mixture was concentrated, and resulting residue was purified by column chromatography (SiO_2 , 3 to 6 % MeOH in CH_2Cl_2) to give **10** (11 mg 0.014 mmol, 83%).

^1H NMR (400 MHz, CDCl_3) δ 12.12 (1H, br. s), 8.76 (1H, br. s), 7.74 (1H, s), 7.81 (1H, s), 7.68 (1H, d, $J = 5.4$ Hz), 7.48 (2H, d, $J = 7.6$ Hz), 7.37 (2H, d, $J = 9.2$ Hz), 7.34 (2H, d, $J = 9.2$ Hz), 7.25 – 7.21 (2H, m), 7.17 (1H, t, $J = 7.2$ Hz), 6.81 (2H, d, $J = 9.2$ Hz), 6.78 (2H, d, $J = 9.2$ Hz), 5.80 (1H, d, $J = 4.0$ Hz), 5.35 (1H, br. s), 5.08 (1H, dd, $J = 12.4, 6.4$ Hz), 4.30 – 4.29 (1H, m), 3.76 (3H, s), 3.74 (3H, s), 3.57 – 3.53 (1H, m), 3.29 – 3.26 (1H, m), 1.84 – 1.57 (1H, m), 0.94 (3H, d, $J = 6.8$ Hz), 0.68 (3H, d, $J = 6.8$ Hz)

^{13}C NMR (100 MHz, CDCl_3) δ 179.4, 158.6, 158.4, 158.0, 157.6, 157.3, 147.8, 147.2,

144.8, 139.4, 136.3, 135.7, 130.1, 129.9, 128.1, 128.0, 127.0, 121.0, 120.0, 117.1, 114.2, 111.3, 91.2, 86.1, 82.5, 71.5, 62.7, 55.1, 51.2, 35.9, 18.5, 18.2

ESI-HRMS: calcd. for $C_{37}H_{38}F_3N_6O_8$ 751.2703[M+H]⁺, found : 751.2722[M+H]

3'-amino modified G CPG (11)

A mixture of **10** (0.90 g, 1.20 mmol), succinic anhydride (0.24 g, 2.40 mmol), Et₃N (0.42 mL, 3.0 mmol), and DMAP (29 mg, 0.24 mmol) in CH₃CN (12 mL) was stirred at room temperature for 1.5 hours. The mixture was partitioned between EtOAc and aqueous NaHCO₃ (saturated). The organic layer was washed with brine, dried (Na₂SO₄), and concentrated to give the corresponding succinate. A mixture of the succinate, Native amino lcaa CPG (1000 Å, 84 µmol/g, 1.20 g, 0.10 mmol), HOBt (136 mg, 1.01 mmol), and EDC·HCl (193 mg, 1.01 mmol) in DMF (2.5 mL) was shaken at room temperature for 12 hours. After the resin was washed with DMF and CH₂Cl₂, 10% Ac₂O in pyridine (0.5 mL) was added and the whole mixture was shaken at room temperature for 12 hours. The resin was washed with pyridine, CH₂Cl₂, and dried in vacuo. The amount of the compound **9** loaded on the solid support was 24.8 µmol/g from the calculation based on absorbance of dimethoxytrityl cation released by treating with 3% TCA in CH₂Cl₂ (10 mL).

Chemical DNA/RNA synthesis and purification.

The linear RNAs and splint DNAs were synthesized on a Gene World H-8-SE DNA synthesizer (Gene World, Tokyo, Japan) or NR-2A 7MX synthesizer (Nihon Techno Service, Ushiku, Ibaraki, Japan). The RNA oligonucleotides were synthesized using 2'-O-tri-iso-propylsilyloxymethyl protected β-cyanoethyl phosphoramidites (ChemGenes,

Wilmington, USA). The 5'-phosphorylation reactions were performed on the synthesizer using 5'-Phosphate-ON Reagent (ChemGenes). The 3'-amino modification were performed using 3'-amino modified G CPG (**11**). The synthesized oligonucleotides were deprotected according to the procedures provided by the supplier. The deprotected RNAs were purified using 15% denaturing polyacrylamide gel electrophoresis (PAGE), and isolated using the crush and soak method. The ratio of acrylamide to bis-acrylamide used throughout this study to separate the RNAs was 19:1 (wt/wt). Low Range ssRNA Ladder (New England Biolabs) was used as the size marker of single-stranded RNA.

Two-step chemoenzymatic synthesis of circular RNA

C126-2) After being annealed with T2 (44 μ M), L78 (44 μ M) and L48N (44 μ M) was incubated in a mixture of 250 mM HEPES-NaOH (pH 8.7), 500 mM EDC, 500 mM HOBt, 100 mM NaCl, and 100 mM MgCl₂ at room temperature overnight. After iPrOH precipitation, the ligated product was purified by 8% denaturing PAGE, and isolated using the crush and soak method to give linear 126 mer product. Linear RNA product (2 μ M) was then annealed with T1 (4 μ M), and incubated with T4 RNA ligase 2 (0.08 U/ μ L, New England Biolabs, MA, USA) in a mixture of 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 1 mM DTT, 400 μ M ATP at room temperature for 2.5 hours. After iPrOH precipitation, the ligated product was purified by 8% denaturing PAGE, and isolated using the crush and soak method to give **C126-2** (3.7%, 2 steps).

C126-3) After being annealed with T1 (44 μ M), L78N (44 μ M) and L48 (44 μ M) was incubated in a mixture of 250 mM HEPES-NaOH (pH 8.7), 500 mM EDC, 500 mM HOBt, 100 mM NaCl, and 100 mM MgCl₂ at room temperature overnight. After iPrOH

precipitation, the ligated product was purified by 8% denaturing PAGE, and isolated using the crush and soak method to give linear 126 mer product. Linear RNA product (2 μ M) was then annealed with T2 (4 μ M), and incubated with T4 RNA ligase 2 (0.08 U/ μ L) in a mixture of 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 400 μ M ATP at room temperature for 2.5 hours. After iPrOH precipitation, the ligated product was purified by 8% denaturing PAGE, and isolated using the crush and soak method to give **C126-2** (3.2%, 2 steps).

One-pot chemical synthesis of circular RNA

C126-4) After being annealed with T1 (20 μ M) and T2 (20 μ M), L78N (20 μ M) and L48N (20 μ M) was incubated in a mixture of 250 mM HEPES-NaOH (pH 8.7), 500 mM EDC, 500 mM HOBt, 100 mM NaCl, and 100 mM $MgCl_2$ at room temperature overnight. After iPrOH precipitation, the ligated product was purified by 8% denaturing PAGE, and isolated using the crush and soak method to give **C126-4** (12.5%).

C126-5) After being annealed with T1 (20 μ M) and T3 (20 μ M), L69N (20 μ M) and L59N (20 μ M) was incubated in a mixture of 250 mM HEPES-NaOH (pH 8.7), 500 mM EDC, 500 mM HOBt, 100 mM NaCl, and 100 mM $MgCl_2$ at room temperature overnight. After iPrOH precipitation, the ligated product was purified by 8% denaturing PAGE, and isolated using the crush and soak method to give **C126-5** (7.6%).

C126-6) After being annealed with T1 (20 μ M) and T5 (20 μ M), L48N* (20 μ M) and L78N* (20 μ M) was incubated in a mixture of 250 mM HEPES-NaOH (pH 8.7), 500 mM EDC, 500 mM HOBt, 100 mM NaCl, and 100 mM $MgCl_2$ at room temperature overnight.

After iPrOH precipitation, the ligated product was purified by 8% denaturing PAGE, and isolated using the crush and soak method to give **C126-5** (2.8%).

RNase R treatment of synthesized RNAs

Synthesized linear or circular RNA (2 μ M) was incubated with RNase R (0.8 U/ μ L, Lucigen) in 20 mM Tris-HCl (pH 8.0), 100 mM KCl and 0.1 mM MgCl₂ at room temperature for 20 min. After being added an equal volume of 2 $\times$ formamide loading solution [5 μ L; 80% formamide, 10 mM EDTA (pH 8.0), 0.01% xylene cyanol, 0.01% bromophenol blue], the reaction mixture was analyzed by 8% denaturing PAGE. The gels were stained by SYBR Green II (Lonza, Rockland, ME, USA), and visualized by scanning on a ChemiDoc XRS plus system (BioRad, Hercules, CA, USA).

MazF digestion of circular RNA

C126-4 (6 μ M) was incubated with MazF enzyme (2 U/ μ L, Takara, Japan) in the reaction buffer at 37 °C for 20 min. After desalting using Zip Tip C₁₈ (Merck), the eluent was analyzed by MALDI-TOF/MS.

Translation reaction of circular RNA in *PURE* system and the western blotting analysis.

RNAs were translated using the PURExpress in vitro protein expression kit that includes releasing factor (New England Biolabs) in a minimum volume of 10 μ L. The reaction mixture was incubated at 37 °C for 2 hours before an aliquot (1 μ L) was taken and mixed with 9 μ L of 2 $\times$ SDS sample loading solution [125 mM Tris-HCl (pH 6.8), 2% SDS, 30% glycerol, 5% 2-mercaptoethanol, 0.03% bromophenol blue] and 4 μ L of water. After

being heated at 95°C for 5 min, the mixture was separated using a 5-20% gradient gel (e-PAGEL, ATTO, JAPAN) using 1× running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Precision Plus Protein Dual Color Standards (Bio-Rad, Hercules, CA, USA) were used as the size marker. Peptides were electrotransferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) in a mixture of 25 mM Tris, 192 mM glycine, and 20% methanol using the semi-dry method. The blot was blocked in 3% BSA in TBS-T (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, and 0.05% Tween-20) at room temperature for 1 h. The blot was incubated with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:2,000 (0.5 µg/mL) in the blocking buffer for 1 h at room temperature. After being washed in TBS-T, the blot was mixed with anti-mouse IgG peroxidase conjugate (Sigma-Aldrich) at a dilution of 1:10,000 for 1 h at room temperature and then washed in TBS-T. The FLAG-containing peptide bands were visualized on a ChemiDoc XRS plus system (BioRad) using the SuperSignal West Femto maximum sensitivity substrate kit (Thermo Fisher Scientific).

Coomassie blue staining analysis of the translation products

The translation products in Fig.6 were also separated by 7.5% SDS-PAGE using 1× running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). After the running, gel was stained by Simply Blue Safe Stain (Thermo Fisher Scientific) according to the manufacturer protocol.

Reference

1. J. McCormick, Y. Li, K. McCormick, H. I. Duynstee, A. K. Van Engen, G. A. Van Der Marel, B. Ganem, J. H. Van Boom and J. Meinwald, *J. Am. Chem. Soc.*, 1999, **121**, 5661–

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