Identification of Efficient and Sequence Specific Bimolecular Artificial Ribonucleases by a Combinatorial Approach

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Synthesis of monomers

Scheme S1. Synthesis of phosphoramidites for incorporation of monomers X, Y, Z and P.

General
All reagents and solvents were of analytical grade and obtained from commercial suppliers and used without further purification except for CH₂Cl₂, which was distilled prior to use. Anhydrous CH₂Cl₂ and anhydrous toluene were dried through storage over activated 4Å molecular sieves. Reactions were monitored by thin layer chromatography (TLC) using silica gel (Merck, Silica gel 60) or neutral aluminum oxide (Merck, Aluminum oxide 60) coated plates with indicator. Column chromatography was performed on silica gel (Merck, Silica gel 60, 0.063-0.200 mm) or neutral aluminum oxide (Merck, Aluminum oxide 90 active neutral, 0.063-0.200 mm). After chromatography fractions containing product were pooled, evaporated to dryness and dried under vacuum for 12 hours. NMR spectra were recorded on a Varian Gemini 2000
300MHz instrument. Chemical shifts are reported in ppm relative to TMS (1H; internal), solvents peaks (13C) and H3PO4 (31P; external). Assignments of the NMR signals are based on 2D correlation experiments. Signals in the NMR spectra originating from terpyridine, N,N-bis(2-pyridylmethyl)glycyl and dimetoxytrityl moieties are indicated with Ter, Pyr and DMT subscripts, respectively. High resolution mass spectra (HRMS) were recorded in positive mode on an IonSpec Fourier Transform MALDI mass spectrometer. Starting compounds 1, 2, 3 and 4 were prepared as described in the literature.

2'-Amino-2'-deoxy-5'-O-[(4,4'-dimethoxytrityl)-2'-N-(6-(2,2':6',2''-terpyridin-4'-yloxy)hexanoyl)uridine (2). A mixture of 6-(2,2':6',2''-terpyridin-4'-yloxy)hexanolic acid, and N,N-bis(2-pyridylmethyl)glycine were co-evaporated with anhydrous toluene (2 × 5 mL) and dried in vacuo. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (108 mg, 0.56 mmol) and anhydrous CH2Cl2 (10 mL) were added, and the mixture was stirred for 24 hours at rt under an atmosphere of nitrogen. The reaction mixture was diluted with CH2Cl2 (15 mL) and washed with brine (2 × 10 mL). The aqueous phases were combined and extracted with CHCl3 (3 × 10 mL). The combined organic phase was dried over Na2SO4, filtered and evaporated to dryness. The resulting residue was purified by column chromatography (neutral Al2O3, 0-10% MeOH in CH2Cl2, v/v) to give amide derivative 2 (289 mg, 72%) as a white solid material. TLC: Rf 0.8 (neutral Al2O3, 10% MeOH in CH2Cl2, v/v). 1H NMR (300 MHz, CDCl3): δ 8.53 (2H, d, H3 Ter, H3"Ter, J3-4 = J3"-4" = 7.8 Hz), 8.51 (2H, dd, H6Ter, H6"Ter, J6-5 = J6"-5" = 4.7 Hz, J6-4 = J6"-4" = 1.8 Hz), 7.87 (2H, s, H3"Ter, H5"Ter), 7.76 (2H, td, H4Ter, H4"Ter, J4-3 = J4"-3" = 8.3 Hz, J4-5 = J4"-5" = 8.3 Hz, J4-6 = J4"-6" = 1.8 Hz), 7.56 (1H, d, H6, J6-5 = 8.2 Hz), 7.32-6.70 (16H, m, DMT, H5 Ter, H5"Ter, NH-2'), 6.07 (1H, d, H1', J1'-2' = 8.3 Hz), 5.41 (1H, d, H5, J5-6 = 8.2 Hz), 4.61 (IH, app. q, H2', J2'-1' = J2"-1" = J2'-NH = 8.3 Hz), 4.35 (1H, d, H3', J3'-2' = 8.3 Hz), 4.20-4.10 (3H, m, H4', H6), 3.67 (6H, s, 2×OC2H5), 3.30-3.24 (2H, m, H5'a, H5'b), 2.19 (2H, t, Hα, Jα-β = 6.5 Hz), 1.77 (2H, quintet, Hδ, Jδ-γ = Jδ-ε = 6.2 Hz), 1.67 (2H, quintet, Hβ, Jβ-γ = Jβ-α = 6.5 Hz), 1.45 (2H, quintet, Hγ, Jγ-β ~ Jγ-δ = 6.5 Hz). 13C NMR (75 MHz, CDCl3): δ 173.80 (C2'), 167.13 (C1), 158.57 (C4DMT, C4'DMT), 156.66 (C2'Ter, C6'Ter), 155.96 (C1Ter,
C1\(^{"Ter}\), 151.13 (C2), 148.62 (C6\(^{"Ter}\), C6\(^{""Ter}\)), 144.10 (C1\(^{"DMT}\)), 140.11 (C6), 137.11 (C4\(^{"Ter}\), C4\(^{""Ter}\)), 135.26 and 135.11 (C1\(^{"DMT}\), C1\(^{"DMT}\), C6\(^{"DMT}\)), 128.06 and 127.96 (C2\(^{"DMT}\), C6\(^{"DMT}\) and C3\(^{"DMT}\), C5\(^{"DMT}\)), 127.00 (C4\(^{"DMT}\)), 123.96 (C5\(^{"Ter}\), C5\(^{""Ter}\)), 121.71 (C3\(^{"Ter}\), C3\(^{""Ter}\)), 113.23 (C3\(^{"DMT}\), C3\(^{"DMT}\), C5\(^{"DMT}\), C5\(^{"DMT}\), C5\(^{"DMT}\)), 107.47 (C3\(^{"Ter}\), C5\(^{"Ter}\)), 102.90 (C5), 86.97 (Ar 3\(^{C}\)ODMT), 86.68 (C1'), 85.82 (C4'), 71.22 (C3'), 68.49 (C\(\varepsilon\)), 63.67 (C5'), 55.45 (C2'), 55.20 and 55.16 (2×O\(\varepsilon\)C\(\varepsilon\)H\(\varepsilon\)), 36.05 (C\(\alpha\)), 27.92 (C\(\delta\)), 25.93 (C\(\gamma\)), 25.10 (C\(\beta\)).

HRMS: \(m/z\) 913.3547 ([M+Na]\(^+\), C\(_{51}\)H\(_{50}\)N\(_{6}\)O\(_{9}\)Na\(^+\) calcd. 913.3532).

### 2'-Amino-3'-(2-cyanoethoxy(diisopropylamino)phosphino)-2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-2'-N-(6-(2,2':6',2"-terpyridin-4'-yloxy)hexanoyl)uridine (3).

Amide derivative 2 (225 mg, 0.25 mmol) was co-evaporated with anhydrous toluene (2 × 5 mL) and dried \textit{in vacuo}. Anhydrous CH\(_2\)Cl\(_2\) (5 mL) and diisopropylethylamine (437 µL, 323 mg, 2.50 mmol) were added. The resulting solution was stirred at rt under an atmosphere of nitrogen, and 2-cyanoethyl \(N,\ N\)-diisopropylphosphoramidochloridite (85 µL, 90 mg, 0.38 mmol) was added dropwise. After 1 hour, the reaction mixture was diluted with CH\(_2\)Cl\(_2\) (10 mL), washed with sat. aq. NaHCO\(_3\) (2 × 5 mL), with brine (5 mL) and dried by filtration through a layer of Na\(_2\)SO\(_4\). After evaporation to dryness, the residue was purified by column chromatography (neutral Al\(_2\)O\(_3\), 0-3% MeOH in CH\(_2\)Cl\(_2\), v/v containing 2 % pyridine) to give phosphoramidite 3 (261 mg, 95%) as a white solid material. TLC: \(R_f\) 0.7 (neutral Al\(_2\)O\(_3\), 5% MeOH in CH\(_2\)Cl\(_2\)). \(^{31}\)P NMR (121.5 MHz, CDCl\(_3\)): \(\delta\) 151.25 and 150.00 HRMS: \(m/z\) 1113.4556 ([M+Na]\(^+\), C\(_{60}\)H\(_{67}\)N\(_{8}\)O\(_{10}\)PNa\(^+\) calcd. 1113.4610).

### 2'-Amino-2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-2'-N-(6-(2,2':6',2"-terpyridin-4'-yloxy)hexanoyl)uridine (4).

Amide derivative 4 (245 mg, 49%) was obtained as a white solid material starting form nucleoside 1\(^1\) (486 mg, 0.86 mmol), 5-(2,2':6',2"'-terpyridin-4'-yloxy)pentanoic acid\(^3\) (200 mg, 0.57 mmol) and \(N\)-(3-dimethylaminopropyl)-\(N\)'-ethylcarbodiimide hydrochloride (137 mg, 0.71 mmol), following the same procedure as described for the synthesis of amide 2. TLC: \(R_f\) 0.71 (neutral Al\(_2\)O\(_3\), 10% MeOH in CH\(_2\)Cl\(_2\), v/v). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 8.57 (2H, d, H3\(^{"Ter}\), H3\(^{""Ter}\), \(J_{3-4} = J_{3"-4"} = 7.7\) Hz), 8.56 (2H, m, H6\(^{"Ter}\), H6\(^{""Ter}\)), 7.90 (2H, s, H3\(^{"Ter}\), H5\(^{"Ter}\)), 7.83 (2H, td, H4\(^{"Ter}\), H4\(^{""Ter}\), \(J_{4-3} = J_{4"-3"} = J_{4-5} = J_{4"-5"} = 7.7\) Hz, \(J_{4-6} = J_{4"-6"} = 1.5\) Hz), 7.56 (1H, d, H6, \(J_{6-5} = 8.3\) Hz),
7.36-6.73 (16H, m, DMT, H5′, H5′′, NH-2′), 6.07 (1H, d, H1′, J1'-2' = 8.1 Hz), 5.49 (1H, d, H5, J5,6 = 8.3 Hz), 4.55 (1H, app. q, H2′, J2'-1' = J2'-3' = J5'-NH = 8.1 Hz), 4.40 (1H, dd, H3′, J3'-2' = 8.1 Hz, J3'-4' = 1.8 Hz), 3.75 (6H, s, 2×OCH3), 3.37-3.30 (2H, m, H5′, H5′′), 2.39 (2H, t, Hα, Jα-β = 6.2 Hz), 2.08-1.72 (4H, m, Hβ, Hγ). 13C NMR (75 MHz, CDCl3): δ 173.41 (CO(2′)), 166.86 (C4′Ter), 162.99 (C4), 158.54 (C4DMT, C4′DMT), 156.69 (C2′Ter, C6′Ter), 155.93 (C2Ter, C2′Ter), 150.92 (C2), 148.62 (C6Ter, C6′Ter), 144.17 (C1′DMT). 140.11 (C6), 137.20 (C4Ter, C4′Ter), 135.36 and 135.21 (C1DMT, C1′DMT), 129.99 (C2DMT, C2′DMT, C6DMT, C6′DMT), 128.06 and 127.92 (C2′DMT, C6′DMT and C3′DMT, C5′DMT), 126.99 (C4′DMT), 124.03 (C5Ter, C5′Ter), 121.77 (C3Ter, C3′Ter), 113.20 (C3DMT, C3′DMT, C5DMT, C5′DMT), 107.50 (C3′Ter, C5′Ter), 102.83 (C5), 87.21 (C1′), 86.81 (Ar3COdMT), 85.69 (C4′), 70.40 (C3′), 68.40 (Cδ), 63.63 (C5′), 55.45 (C2′), 55.17 (2×OCH3), 34.94 (Cu), 27.49 (Cγ), 22.40 (Cβ). HRMS: m/z 899.3398 ([M+Na]+, C51H50N6O9Na+ calcd. 899.3375).

2'-Amino-3'-O-(2-cyanoethoxy(diisopropylamino)phosphino)-2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-2'-N-(5-(2,2':6',2''-Terpyridin-4'-yloxy)pentanoyl)uridine (5). Phosphoramidite 5 (206 mg, 61%) was obtained as a white solid starting material from amide derivative 4 (195 mg, 0.22 mmol) and 2-cyanoethyl N,N-diisopropylphosphoramidochloridite (98 µL, 104 mg, 0.44 mmol) following the same procedure as described for 3. TLC: Rf 0.63 (neutral Al2O3, 5% MeOH in CH2Cl2, v/v). 31P NMR (121.5 MHz, CDC13): δ 149.17 and 148.17. HRMS: m/z 1099.4415 ([M+Na]+, C62H69N8O10PNa+ calcd. 1099.4454).

2'-Amino-5'-O-(4,4'-dimethoxytrityl)-2'-N-(6-(2,2':6',2''-terpyridin-4'-yloxy)hexanoyl)thymidine (7). Amide derivative 7 (231 mg, 72%) was obtained as a white solid material starting from nucleoside 62 (200 mg, 0.35 mmol), 6-(2,2':6',2''-terpyridin-4'-yloxy)hexanoic acid3 (191 mg, 0.53 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (88 mg, 0.46 mmol), following the same procedure as described for the synthesis of amide 2. TLC: Rf 0.67 (neutral Al2O3, 10% MeOH in CH2Cl2, v/v). 1H NMR (300 MHz, d6-DMSO, major rotamer): δ 8.59-8.54 (4H, m, H3Ter, H3′Ter, H6Ter, H6′Ter), 7.92 (2H, s, H3', Ter, H5′Ter), 7.83 (2H, td, H4Ter, H4′Ter, Jα-β = J4'-3' = J4'-5' = 7.5 Hz, J4,4'-6,6' = 1.7 Hz), 7.56 (1H, s, H6), 7.40-6.77 (16H, m, DMT, H5Ter, H5′Ter, NH-2′), 6.29 (1H, b, 3′OH), 5.43
(1H, s, H1’), 4.47 (1H, m, H2’), 4.32-4.20 (3H, m, H3’, Hε), 3.78 and 3.77 (6H, s, 2×OC3H3), 3.62 (1H, d, H5’a, J5’a-5’b = 10.9 Hz), 3.55 (1H, d, H5”a, J5”a-5”b = 11.1 Hz), 3.45 (1H, d, H5’b, J5’b-5’a = 10.9 Hz), 3.43 (1H, d, H5”b, J5”b-5”a = 11.1 Hz), 2.55 (2H, t, Hα, Jα-β = 7.5 Hz), 2.00-1.85 (4H, m, Hβ, Hδ), 1.65 (2H, quintet, Hγ, Jγ-β = Jγ-δ = 6.8 Hz), 1.38 (3H, s, CH3). 13C NMR (75 MHz, CDCl3): δ 173.08 (C O(2’)), 167.12 (C4’Ter), 163.53 (C4), 158.68 (C4 DMT, C4’DMT), 156.68 (C2’Ter, C6’Ter), 155.94 (C2 Ter, C2”Ter), 150.00 (C2), 148.86 (C6Ter, C6”Ter), 144.25 (C1’DMT), 137.21 (C4Ter, C4”Ter), 135.25 and 135.24 (C1’DMT, C1’’DMT), 134.23 (C6) 130.05 and 130.0 (C2DMT, C6DMT, and C2’DMT, C6’’DMT), 128.06 and 127.99 (C2’’DMT, C6’’DMT and C3’’DMT, C5’’DMT), 127.11 (C4’’DMT), 123.98 (C5Ter, C5’’Ter), 121.77 (C3Ter, C3’’Ter), 113.28 (C3DMT, C3’’DMT, C5DMT, C5’’DMT), 110.24 (C5), 107.72 (C3’Ter, C5’’Ter), 87.93 (Ar3CODMT), 87.41 (C1’), 86.66 (C4’), 69.39 (C3’), 68.62 (C6), 63.89 (C2’), 59.08 (C5’), 55.23 (2×OCH3), 51.21 (C5”), 34.72 (Cα), 27.63 (Cδ), 26.35 (Cγ), 24.35 (Cβ), 12.35 (CH3). HRMS: m/z 939.3657 ([M+Na]+, C53H52N6O9Na+ calcd. 939.3688). 2’-Amino-3’-O-(2-cyanoethoxy(diisopropylamino)phosphino)-5’-O-(4,4’-dimethoxytrityl)-2’-N,4’-methylene-2’-N-(6-(2,2’:6’,2”-terpyridin-4’yloxy)hexanoyl)thymidine (8). Phosphoramidite 8 (156 mg, 61%) was obtained as a white solid starting material from amide derivative 7 (213 mg, 0.23 mmol) and 2-cyanoethyl N,N-diisopropylphosphoramidochloridite (77 µL, 81 mg, 0.34 mmol) following the same procedure as described for 3. TLC: Rf 0.56 and 0.81 (neutral Al2O3, 5% MeOH in CH2Cl2, v/v). 31P NMR (121.5 MHz, CDCl3): δ 149.46 and 147.71. HRMS: m/z 1139.4823 ([M+Na]+, C62H69N8O10PNa+ calcd. 1139.4767). 2’-Amino-2’-N-[N,N-bis(2-pyridylmethyl)glycyl]-2’-deoxy-5’-O-(4,4’-dimethoxytrityl)uridine (9). Amide derivative 9 (900 mg, 76%) was obtained as a white solid material starting from nucleoside 1 (819 mg, 1.50 mmol), N,N-bis(2-pyridylmethyl)glycine4 (579 mg, 2.25 mmol) and N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (361 mg, 1.88 mmol), following the same procedure as described for the synthesis of amide 2, except that purification was performed by silica gel column chromatography (0-6% MeOH in CH2Cl2). TLC: Rf 0.59 (silica, 15% MeOH in CH2Cl2, v/v). 1H NMR (300 MHz, CDCl3): δ 8.82 (1H, d, NH-2’, JNH-2’-2’ = 10.0 Hz), 8.55 (2H, dd, H6Pyr, H6’Pyr, J6,5 = J6’,5’ = 4.8 Hz, J6-4 = J6’-4’ = 1.1 Hz), 7.79 (1H, d, H6,
$J_{6.5} = 8.2\, \text{Hz}$, 7.62 (2H, t, H4$_{\text{Pyr}}$, H4’$_{\text{Pyr}}$, $J_{4.3} = J_{4.5} = J_{4'.3'} = J_{4'.5'} = 7.6\, \text{Hz}$), 7.43 (2H, d, H3$_{\text{Pyr}}$, H3’$_{\text{Pyr}}$, $J_{3.4} = J_{3'.4'} = 7.6\, \text{Hz}$), 7.34-8.85 (15H, m, DMT, H5$_{\text{Pyr}}$, H5’$_{\text{Pyr}}$), 6.30 (1H, d, H1’, $J_{1'.2'} = 8.7\, \text{Hz}$), 5.37 (1H, d, H5, $J_{5.6} = 8.2\, \text{Hz}$), 5.00 (1H, m, H2’), 4.44 (1H, d, H3’, $J_{3'.2'} = 4.7\, \text{Hz}$), 4.32 (1H, s, H4’), 3.85 (2H, d, 2×NCH$_a$H$_b$Ar, $J_{a-b} = 13.5\, \text{Hz}$), 3.78-3.69 (6H, m, 2×OCH$_3$, 2×NCH$_a$H$_b$Ar), 3.46 (2H, s, H5’), 3.40 (1H, d, C$_{\alpha}$H$_a$H$_b$, $J_{b-a} = 16.4\, \text{Hz}$), 3.20 (1H, d, C$_{\alpha}$H$_a$H$_b$, $J_{b-a} = 16.4\, \text{Hz}$).

$^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 171.39 (C$_{\text{O(2')}}$), 163.14 (C4), 158.62 (C4DMT, C4’DMT), 157.02 (C2Pyr, C2’Pyr), 150.87 (C2), 149.42 (C6Pyr, C6’Pyr), 144.11 (C1”DMT), 140.32 (C6), 137.15 (C4$_{\text{Pyr}}$, C4’$_{\text{Pyr}}$), 135.29 and 135.08 (C1DMT, C1’DMT), 130.13 (C2DMT, C2’DMT, C6DMT, C6’DMT), 128.18 and 127.98 (C2’’DMT, C6’’DMT and C3’’DMT, C5’’DMT), 127.05 (C4’’DMT), 124.00 (C5$_{\text{Pyr}}$, C5’$_{\text{Pyr}}$), 122.79 (C3$_{\text{Pyr}}$, C3’$_{\text{Pyr}}$), 113.25 (C3DMT, C3’DMT, C5DMT, C5’DMT), 102.67 (C5), 87.12 (C1’), 85.45 (Ar$_3$CO$_{\text{DMT}}$), 84.84 (C4’), 72.51 (C3’), 63.99 (C5’), 59.74 and 58.41 (2×NCH$_2$Ar), 55.45 (C2’), 55.18 (2×OCH$_3$), 46.07 (C$\alpha$).

2’Amino-2’-N-(N,N-bis(2-pyridylmethyl)glycyl)-3’-O-(2-cyanoethoxy(diisopropylamino)phosphino)-2’-deoxy-5’-O-(4,4’-dimethoxytrityl)uridine (10). Phosphoramidite 10 (858 mg, 81%) was obtained as a white solid starting material from amide derivative 9 (851 mg, 1.08 mmol) and 2-cyanoethyl N,N-diisopropylphosphoramidochloridite (306 µL, 324 mg, 1.37 mmol) following the same procedure as described for 3 except that purification was performed by silica gel column chromatography (0-5% MeOH in CH$_2$Cl$_2$, containing 2% pyridine). TLC: $R_f$ 0.62 (silica gel, 15% MeOH in CH$_2$Cl$_2$, v/v). $^{31}$P NMR (121.5 MHz, CDCl$_3$): $\delta$ 150.7 and 151.3.

**Synthesis and purification of oligonucleotides**

Synthesis of oligonucleotides was performed in 0.2 µmol scale using an automated DNA synthesizer (PerSpective Biosystems Expedite 8909). Oligonucleotides bearing a modified monomer at the 3’-end were synthesized using universal support (Glen Research, Universal Support II). Standard cycle procedures were applied for unmodified phosphoramidites using 0.25 M solution of 4,5-dicyanoimidazole as activator. In case of phosphoramidite 8 (for incorporation of monomer Z) and LNA phosphoramidites (monomers A$^L$ and T$^L$) both coupling time and oxidation time were extended to 30 min.
and 1 min, respectively. To accomplish incorporation of monomers X and Y (phosphoramidites 3 and 5, respectively), a double coupling procedure with extended coupling time (2 × 30 min) was performed. Incorporation of monomer P (phosphoramidite 10) required the use of 0.5 M pyridinium hydrochloride solution in acetonitrile as activator, together with double coupling procedure and extended coupling time (2 × 30 min). Stepwise coupling yields, as determined by a spectrophotometric DMT+ assay, were >99% for standard and LNA phosphoramidites, >98% for phosphoramidite 8, >85% for phosphoramidites 3 and 5, and >40% for phosphoramidite 10.

Removal of the nucleobase protecting groups and cleavage from solid support was effected using standard conditions (32% aqueous ammonia for 12 h at 55 °C). When universal support was used the cleavage was performed by 12 h treatment with saturated methanolic ammonia solution at rt, followed by addition of 32% aqueous ammonia and heating for 12 h at 55 °C.

Purification of all oligonucleotides was performed by DMT-ON RP-HPLC using a Waters Prep LC 4000 HPLC machine equipped with an Xterra MS C18-column (10 µm, 300 mm × 7.8 mm). The following eluent system was used: A-buffer: 95% 0.1 M Et₃NH·HCO₃, 5% CH₃CN; B-buffer: 25% 0.1 M Et₃NH·HCO₃, 75% CH₃CN. Gradient: 0-5 min isocratic hold of 100% A-buffer, followed by a linear gradient to 55% B-buffer over 75 min at a flow rate of 1.0 mL/min. Fractions containing pure oligonucleotides were collected and evaporated on a speed-vac, followed by detritylation (80% aq. AcOH, 20 min), precipitation (abs. EtOH, −18 °C, 12 h) and washing with abs. EtOH.

Unmodified RNA was obtained from a commercial supplier.

**MALDI-MS of synthesized oligonucleotides**

The composition of oligonucleotides was verified by MALDI-MS analysis (Table S1) whereas the purity (>80%) was verified by ion-exchange HPLC using a LaChrom L-7000 system (VWR International) equipped with a Gen-Pak Fax column (100 mm × 4.6 mm). The following eluent system was used: A-buffer: 25 mM Tris-Cl, 1 mM EDTA, pH 8.0; B-buffer: 1 M NaCl. Gradient: 0-5 min isocratic hold of 95% A-buffer, followed by a linear gradient to 70% B-buffer over 41 min at a flow rate of 0.75 mL/min.
Table S1. MALDI-MS of synthesized ONs.

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Experimental details of the RNA cleavage reactions

5'-End-labeling of the RNA substrate. The RNA substrate (1 pmol), [γ-^32P] ATP (1 μCi) and T4 polynucleotide kinase were incubated for 60 min at 37 °C in a buffer containing 70 mM Tris HCl pH 7.6, 10 mM MgCl₂ and 5 mM DTT. After denaturating the enzyme by heating to 80 °C for 15 min., the labeled RNA was mixed with non-labeled RNA (499 pmol) and the volume was adjusted with water to 100 μL (obtained stock solution with final RNA concentration of 5 μM was used in the cleavage experiments).^5^ RNA cleavage. All RNA cleavage reactions were performed at 37°C in 100 mM NaCl, 20 mM Tris HCl pH 7.4, with an RNA concentration of 1 μM. The reactions were carried out with concentrations of oligonucleotide cleaving agents and Cu²⁺ (added as CuCl₂ solution) as stated in the communication with total reaction volumes of 5-15 μL. Prior to starting the reaction by addition of the Cu²⁺ solution, denaturation was performed by heating to 80 °C for 2 min. Aliquots (1 μL) were taken at specific times and the cleavage reactions were stopped by addition of EDTA and formamide followed by heating to 80 °C for 2 min. Samples were analyzed on 20% urea polyacrylamide gel.\(^5\)}
RNA cleavage by oligonucleotides DNA1-N and DNA2-N

The ability of oligonucleotides DNA1-N and DNA2-N to individually cleave the RNA target was studied. As seen from the results depicted in Figure S1, this resulted in much lower cleavage efficiencies relative to combined use of DNA1-N and DNA2-N, providing an evidence for RNA cleavage cooperativity for the bimolecular artificial ribonuclease system.

**Figure S1.** Denaturating gel electrophoresis of cleavage reactions catalyzed by either DNA1-N or DNA2-N (16 h; $c_{\text{DNAx-N}} = 4 \mu\text{M}; c_{\text{Cu}^{2+}} = 8 \mu\text{M}; c_{\text{RNA}} = 1 \mu\text{M}; 37^\circ\text{C}$).

**Thermal denaturation studies**

Concentrations of oligonucleotides were calculated using the following extinction coefficients (OD$_{260}/\mu$mol): G, 10.5; A, 13.9; T/U, 7.9; C, 6.6; terpyridine, 8.0;$^6$ $N$,$N$-bis(2-pyridylmethyl), 6.0 ($2 \times$ OD$_{260}/\mu$mol for pyridine$^7$). Oligonucleotides (1.0 $\mu$M of each strand) and Cu$^{2+}$ (added as CuCl$_2$ solution) were thoroughly mixed in $T_m$-buffer (100 mM NaCl, pH 7.0 adjusted with 10 mM NaH$_2$PO$_4$/5 mM Na$_2$HPO$_4$) and denaturation was performed by heating at 80 for 2 min which was followed by cooling to the starting temperature of the experiment. Quartz optical cells with a path-length of 1.0 cm were used. Thermal denaturation temperatures ($T_m$ values$^\circ\text{C}$) were measured on a Perkin Elmer Lambda 35 UV/VIS spectrometer equipped with a PTP-6 Peltier temperature programmer and determined as the maximum of the first derivative of the thermal denaturation curve ($A_{260}$ vs. temperature). A temperature ramp of 1.0 $^\circ\text{C}$/min was used in
all experiments. Reported thermal denaturation temperatures are an average of two measurements within ±1.0 °C.

**Table S2.** Thermal denaturation temperatures ($T_m$ values (ºC)) of duplexes formed between DNA1-Y, DNA2-Y and complementary RNA at different concentration of Cu$^{2+}$.

<table>
<thead>
<tr>
<th>Code</th>
<th>Sequence</th>
<th>Cu$^{2+}$ per duplex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 eq.</td>
</tr>
<tr>
<td>DNA1-Y</td>
<td>5’-d(CACACCAA CY)</td>
<td>RNA 3’-r(GUG GUU GAA GAA GGU GU)</td>
</tr>
<tr>
<td>DNA2-Y</td>
<td>5’-d(YCTTCCA CA)</td>
<td>RNA 3’-r(GUG GUU GAA GGU GU)</td>
</tr>
<tr>
<td>DNA1-Y</td>
<td>5’-d(CACACCAA CY)</td>
<td>DNA2-Y 5’-d(YCTTCCA CA)</td>
</tr>
</tbody>
</table>

$^a$ not determined; $^b$ single transition observed

**References**