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Combinatorial Nucleoside-Deletion-Scanning Mutagenesis of Functional DNA**

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Table of Contents

| Statement on use of butantriol-derived spacer Δ | page | S2 |
|--|------|---------|
| General Information | page | S2 |
| Chemical synthesis of phosphoramidite 1 | page | S2-S7 |
| Oligonucleotide sequences | page | S8 |
| General methods for synthesis and purification of oligonucleotides | page | S8-S9 |
| Method development for NDS | page | S9 |
| General workflow and experimental details of combinatorial NDS mutagenesis | page | S11-S18 |
| References | page | S18 |

| Index of Supporting Figures | | |
|---|--------|-------|
| Figures S1-S8. ¹ H, ¹³ C, ³¹ P NMR spectra | page S | S3-S7 |
| Figures S9-S10. Incorporation efficiency of 1 | page S | S10 |
| Figures S11. Optimization of hydrolysis conditions | page S | S11 |
| Figures S12. Workflow of NDS mutagenesis | page S | S11 |
| Figures S13. HPLC traces of NDS libraries for 8LV13 and 9HR17 | page S | S12 |
| Figures S14. Hydrolysis gels for NDS of 8LV13 and 9HR17 | page S | S14 |
| Figures S15. Interference values for 8LV13 | page S | S15 |
| Figures S16. Interference values for 9HR17 | page S | S15 |
| Figures S17. Kinetic gels for 8LV13 mutants | page S | S16 |
| Figures S18. Mutation cycle for 8LV13 | page S | S17 |
| Figures S19. Kinetic gels for 9HR17 mutants | page S | S18 |

Index of Supporting Tables

| Table S1. Ligation yields for NDS library separation | page S13 |
|---|----------|
| Table S2. 8LV13 mutants | page S16 |
| Table S3. 9HR17 mutants | page S17 |
| Table S4. Comparison of ligation kinetics for 9HR17 mutants | page S17 |
| | |

Statement on use of butantriol-derived spacer Δ (see footnote [5] in manuscript)

The butantriol-derived spacer Δ is advantageous over an alternative glycerol-based derivative; here, Δ exclusively leads to cleavage at the 3'-phosphate (due to the formation of a 5-membered instead of 7membered heterocycle in the trigonal-bipyramidal transition state), and therefore results in a unique hydrolysis pattern; in contrast, a glycerol-derived spacer would have equal probability to cleave at the 3' and 5' phosphate, and therefore hydrolysis ladders could be ambiguous. As an other alternative to the flexible spacer Δ , a cyclic tetrahydrofurane derivative was considered, which resembles an abasic site ribonucleotide. We decided to use the flexible structure Δ to avoid interference from the 2'-OH group in the rigid construct, which could mask the effect of nucleobase deletion (similar to 2'-OH interference in CoMA and dNAIM). The flexible spacer Δ thus gives stronger results by firmly indicating the dispensability of nucleobase and ribose at the same time.

General Information

All chemicals and solvents were obtained from commercial sources. Solvents were dried over activated molecular sieves. Silica gel plates precoated with fluorescent indicator were used for thin layer chromatography (TLC) and the plates were visualised either with UV light or conc. H_2SO_4 and heating. Silica gel 60, 0.032-0.063 mm (230-450 mesh), pretreated with NEt₃ was used for column chromatography. ¹H NMR and ¹³C NMR were recorded on a 400 MHz instrument and ³¹P NMR was recorded in 300 MHz instrument. The chemical shifts (in ppm) are reported downfield from TMS (0 ppm) or CDCl₃ (7.26 ppm for ¹H and 77.16 ppm for ¹³C). Spin multiplicities in ¹H NMR are reported as singlet (s), broad singlet (bs), doublet (d), doublet of doublet (dd), triplet (t) or multiplet (m).

Standard 5'-*O*-DMT-protected 3'- β -cyanoethyl phosphoramidites of *N*⁶-benzoyl-2'-deoxyadenosine, *N*⁴acetyl-2'-deoxycytidine, *N*²-(*N*,*N*-dimethylamino)methylene-2'-deoxyguanosine, and thymidine were from SAFC/Sigma-Aldrich. Benzylthiotetrazole (BTT) was from Carbosynth (UK). Ribonucleotide triphosphates (NTPs) for in vitro transcription reactions were purchased from Jena Bioscience, and α -³²PdATP (3000 Ci/mmol) and γ -³²P-ATP (3000 Ci/mmol) was from Perkin Elmer. T4 RNA ligase, T4 DNA ligase and T4 polynucleotide kinase (PNK) were from Fermentas, and Klenow fragment was from Jena Bioscience.

Chemical synthesis of phosphoramidite 1



Scheme S1. Synthesis of phosphoramidite 1. Reagents and conditions: a) DMT-Cl, pyridine, RT, 6 h, 99%; b) AD-mix- β , tBuOH, H₂O, 0°C, 24 h, 73%; c) tBDMS-Cl, imidazole, CH₂Cl₂, RT, 16 h, 74%; d) CEP-Cl, Me₂NEt, CH₂Cl₂, RT, 45 min, 75%.

4-(4,4'-Dimethoxytrityl)oxy-1-butene (3). To the solution of 3-buten-1-ol (**2**) (100 mg, 1.38 mmol) in dry pyridine (6.2 mL), dimethoxytrityl chloride (DMT-Cl, 700 mg, 2.1 mmol) was added under argon and the mixture was stirred for 6 h at RT. The reaction mixture was quenched with methanol (3 mL) and diluted with dichloromethane (DCM, 50 mL). The organic layer was washed with 5% aq. NaHCO₃ (20 mL × 3). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The crude compound was purified by column chromatography (2% NEt₃, 20% ethyl acetate in hexane) to give a colorless jelly product **3** (536 mg, 1.43 mmol, 99%). $R_f = 0.54$ (15% ethyl acetate / hexane; TLC plate pretreated with NEt₃); ¹H NMR (400 MHz, CDCl₃) δ 7.51-7.22 (m, 9 H), 6.87 (d, J = 8.4 Hz, 4 H), 5.97-5.84 (m, 1 H), 5.09 (2 dd, J = 17.6 Hz, 10.9 Hz, 2 H), 3.84 (s, 6 H), 3.17 (m, 2 H), 2.42 (m, 2 H); ¹³C NMR (100 MHz, CDCl₃) δ 158.6, 145.6, 136.9, 136.1, 130.4, 128.5, 128.1, 126.9, 116.6, 115.6, 113.4, 86.2, 63.5, 55.7, 35.2; ESI-MS calcd. for C₂₅H₂₆O₃ [M+Na]⁺ 397.19, found [M+Na]⁺ 397.1.



Figure S2. ¹³C-NMR (100 MHz, CDCl₃) of compound 3

4-(4,4'-Dimethoxytrityl)oxy-1,2(*R***)-butandiol (4).** A mixture of tBuOH (4.3 mL), water (4.3 mL) and AD-mix-β (1.2 g) was stirred at RT until a clear biphasic solution with the lower aqueous layer being bright yellow, was produced. The clear solution was cooled to 0°C whereupon some of the dissolved salts precipitated. The solution was added to compound **3** (322 mg, 0.86 mmol). The heterogeneous slurry was stirred vigorously for 24 h at 0°C. Solid Na₂SO₃ (1.2 g) was added at 0°C to the bright yellow or orange mixture. Upon stirring for 45 min at RT, the solution turned almost colorless. Ethyl acetate (12 mL) was added, and the layers were separated. The aqueous phase was further extracted with ethyl acetate (15 mL × 3); the combined organic phases were dried over Na₂SO₄, the solvent was evaporated and the crude product was purified by column chromatography (2% NEt₃, 50-80% ethyl acetate in hexane) to afford compound **4** as a colorless, viscous oil (257 mg, 0.63 mmol, 73%). *R_f* = 0.58 (7% MeOH/DCM; TLC plate pretreated with NEt₃); ¹H NMR (400 MHz, CDCl₃) δ 7.45-7.35 (m, 9 H), 6.83 (d, J = 8.8 Hz, 4 H), 3.93-3.88 (m, 1 H), 3.78 (s, 6 H), 3.59 (dd, J = 10.9 Hz, 3.3 Hz, 1 H), 3.46 (dd, J = 11.4 Hz, 6.6 Hz, 1 H), 3.40-3.34 (m, 1 H), 3.30-3.25 (m, 1H), 1.83-1.71 (m, 2 H); ¹³C NMR (100 MHz, CDCl₃) δ 158.2, 144.5, 135.8, 135.7, 129.73, 127.8, 127.7, 126.6, 113.0, 86.6, 71.1, 66.5, 61.4, 55.1, 33.0; ESI-MS calcd. for C₂₅H₂₈O₅ [M+Na]⁺ 431.5, found [M+Na]⁺ 431.2.





Figure S4. ¹³C-NMR (100 MHz, CDCl₃) of compound 4

1-(*tert.*-Butyldimethylsily)oxy-4-(4,4'-dimethoxytrityl)oxy-2(*R*)-butanol (5). To a solution of compound 4 (232 mg, 0.57 mmol) and imidazole (69.7 mg, 1.02 mmol) in dry DCM (3 mL), tBDMS-Cl (128.5 mg, 0.85 mmol) was added at 0°C in three portions (first 2/3 amount, then 1/6 amount twice at 1 h interval) under argon. The mixture was stirred overnight at RT, quenched with water (0.5 mL) and diluted with DCM (20 mL). The DCM layer was washed with brine (10 mL × 3). The organic phase was dried over Na₂SO₄, the solvent was evaporated and the crude product was purified by column chromatography (2% NEt₃, 8% ethyl acetate in hexane) to afford **5** as a colorless, sticky compound (233 mg, 0.44 mmol, 74%). $R_f = 0.51$ (20% ethyl acetate / hexane, TLC plate pretreated with NEt₃); ¹H NMR (400 MHz, CDCl₃) δ 7.46-7.21 (m, 9 H), 6.84 (d, J = 8.4 Hz, 4 H), 3.89-3.85 (m, 1 H), 3.80 (s, 6 H), 3.62 (dd, J = 9.9 Hz, 4.4 Hz, 1 H), 3.48 (dd, J = 9.9 Hz, 6.6 Hz, 1 H), 3.36-3.20 (m, 2 H), 2.85 (bs, 1 H), 1.78 (m, 2 H), 0.91 (s, 9 H), 0.07 (s, 6H); ¹³C NMR (100 MHz, CDCl₃) δ 158.4, 145.1, 136.4, 136.3, 130.1, 128.2, 127.7, 126.8, 113.2, 86.4, 70.7, 67.3, 61.3, 55.4, 33.5, 26.2, 18.6, - 5.0; ESI-MS calcd. for C₃₁H₄₂O₅Si [M+Na]⁺ 545.75, found [M+Na]⁺ 545.2.



Figure S5. ¹H-NMR (400 MHz, CDCl₃) of compound 5



Figure S6. ¹³C-NMR (100 MHz, CDCl₃) of compound 5

1-(*tert.*-Butyldimethylsilyl)oxy-4-(4,4'-dimethoxytrityl)oxy-butane, 2(*R*)-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (1). To a mixture of compound 5 (233 mg, 0.44 mmol) and dimethylethylamine (0.5 mL, 4.5 mmol) in DCM (6.2 mL), CEP-Cl (126.6 mg, 0.54 mmol) was added under argon and the mixture was stirred for 45 min at RT. The reaction was quenched with 0.5 mL methanol, evaporated to dryness and purified by column chromatography (2% NEt₃, 2% ethyl acetate in hexane) to give compound 1 as a colorless sticky oil (251 mg, 0.35 mmol, 75%). $R_f = 0.53$ (15% ethyl acetate / hexane, TLC plate pretreated with NEt₃); ¹H NMR (400 MHz, CDCl₃) δ 7.45-7.20 (m, 9 H), 6.82 (m, 4 H), 3.99-3.49 (m, 15 H), 3.17 (m, 2 H), 2.59 (m, 1 H), 2.41 (m, 1 H), 2.02–1.83 (m, 1 H), 1.16-1.04 (m, 12 H), 0.90 (s, 9 H), 0.05 (s, 6 H); ³¹P NMR (121 MHz, CDCl₃) δ 148.18, 147.95; ESI-MS calcd. for C₄₀H₅₉N₂O₆PSi [M+H]⁺ 724.0 and [M+Na]⁺ 746.0, found [M+H]⁺ 723.0 and [M+Na]⁺ 745.4.



Figure S8. ³¹P-NMR (121 MHz, CDCl₃) of phosphoramidite 1

Oligonucleotide sequences

| RNA / DNA substrates | | | |
|-------------------------------------|-----------------------------|----------------|---------------------------|
| | 5'-sequence-3' | mol. wt. calc. | mol.wt. found (ESI-MS) |
| scaffold strand S1 (8LV13) | GGATAATACG (rA) CTCACTGCG | 6142.0 | 6141.7 |
| scaffold strand S2 (9HR17) | GGATAATACG (rC) TTCACTGCG | 6133.0 | 6131.4 |
| adaptor strand A1 (8LV13) | (rA)ppGGAAGAGATGGCGACr(GGA) | 6119.9 | 6119.1 |
| Precursor to adaptor strand A1, pA1 | pGGAAGAGATGGCGACr(GGA) | 5790.7 | 5789.8 |
| adaptor strand RNA A2 (9HR17) | r(pppggaagagauggcgacgg) | 5847.4 | 5846.7 |

Deoxyribozymes

DNA oligonucleotides for adenylation of adaptor strand pA1^[3] template T1 5'-GCCATCTCTTCCTATAGTGAGTCGTAT-3' mismatch acceptor M1 5'-CTGTAATACGACTCACTATC-3'

Template and promoter strand for in vitro transcription of adaptor strand A2template T25'-CCGTCGCCATCTCTTCCTATAGTGAGTCGTATTACAG-3'T7 promoter5'-CTGTAATACGACTCACTATA-3'

Template DNA for 3'-³²P-labeling of deoxyribozyme libraries T3 5' -GTGGATAATACGGGAAp-3'

DNA oligonucleotide for optimization of hydrolysis conditions 5' -ACGTXTGCAT-3'

The unmodified DNA oligonucleotides, including mutants of 8LV13 and 9HR17 deoxyribozymes were purchased from Sigma Aldrich and purified by PAGE (10% polyacrylamide). The ribonucleotide-containing oligonucleotides for scaffold strands and adaptor A1 were prepared by solid phase synthesis. The adaptor strand A1 for the 8LV13-catalyzed reaction was prepared by 5'-adenylation of its precursor DNA pA1 using T4-DNA ligase, ATP, DNA template T1 and mismatch acceptor M1.^[3] The RNA adaptor substrate A2 was prepared by in vitro transcription using T7 RNA polymerase and a synthetic DNA template T2.^[4] The template T3 for 3'-³²P-labeling via Klenow-catalyzed single-nt ligation^[5] was prepared by solid-phase synthesis using a 3'-phosphate support.

General methods for solid-phase synthesis, deprotection and purification of oligonucleotides

Solid-phase synthesis was performed on 0.7 µmol polystyrene custom primer supports from GE Healthcare, using a Pharmacia Gene Assembler Plus. Phosphoramidite solutions, tetrazole solution, and acetonitrile were dried over activated molecular sieves overnight. Synthesis conditions were as follows:

page S8

Detritylation: 3% (v/v) dichloroacetic acid in 1,2-dichloroethane for two minutes. Activation and coupling: 75 µL of 0.1 M phosphoramidites in acetonitrile; 250 µL of 0.25 M benzyl thiotetrazole in acetonitrile. Coupling time: 4 min. Capping: 1:1 (v/v) A/B for one minute (A, Ac₂O/sym-collidine/acetonitrile, 2:3:5 (v/v); B, 0.5 M 4-(dimethylamino)pyridine in acetonitrile). Oxidation: 10 mM I₂ in acetonitrile/sym-collidine/water (10:1:5, v/v) for one minute.

Deprotection of rN- or Δ -containing oligonucleotides was achieved with 33% ammonium hydroxide:ethanol 3:1 for 18 hours at 55°C, followed by cleavage of the 2'-O-silyl groups with tetrabutylammonium fluoride trihydrate (TBAF·3H₂O) in THF (1 M, 1000 µL) for 15 hours at 40°C. The reaction was quenched with Tris.HCl (1 M, pH 7.4, 1000 µL), and the crude oligonucleotides were isolated by desalting on a Sephadex G 10 column (3x5 mL HiTrap column, GE Healthcare) monitored by UV-detection at 280 nm.

The quality of the synthesized oligonucleotides was analyzed by anion exchange HPLC (Dionex DNAPac PA200 column: eluant A, 25 mM Tris.HCl, pH 8.0, 6 M urea; eluant B, 25 mM Tris.HCl, pH 8.0, 0.5 M NaClO₄, 6 M urea) and then purified by denaturing PAGE [10-20% polyacrylamide, 1x TBE (89 mM each Tris and boric acid, pH 8.3, 2 mM EDTA), 7 M urea, 0.7 mm, 20x30 cm, 35W]. Oligonucleotides were visualized by UV shadowing and extracted by crush and soak into TEN (10 mM Tris.HCl, pH 8.0, 1 mM EDTA, 300 mM NaCl) at 37°C for 2x 3-5 h, followed by precipitation with ethanol at -80°C. The quality was confirmed by denaturing anion-exchange HPLC and ESI-MS.

5'-Adenylation of adaptor strand A1

The 5'-adenylation was performed using a C-T acceptor-template mismatch and T4 DNA ligase.^[3] A sample in a total volume of 280 μ L containing 2 nmol 5'-phosphorylated DNA pA1, 2.2 nmol template and 2.4 nmol acceptor M1 was annealed in 5 mM Tris.HCl (pH 7.5), 15 mM NaCl and 0.1 mM EDTA by heating at 95°C for 3 min and cooling at 25°C for 20 min. The solution was brought to a final volume of 400 μ l, containing 1x T4 DNA ligase buffer [40 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP], 6 mM ATP and 2 μ L T4 DNA ligase. The solution was incubated at 25°C for 2 h, followed by ethanol precipitation using 40 μ L 3M NaCl. The residue was dissolved in water (20 μ L) and purified by gel electrophoresis (20% polyacrylamide, 35 W, 0.4 mm, 2 h). The product was confirmed by ESI-MS. The yield was 25-40% in repeated experiments.

In vitro transcription of 5'-triphosphorylated RNA adaptor strand A2

The in vitro transcription reaction with T7 RNA polymerase was done in 100 μ L using 1 μ M reverse strand T2 and 1 μ M promoter strand in 40 mM Tris-HCl, pH 8.0, 30 mM MgCl₂, 10 mM DTT, 4 mM each NTP and 2 mM spermidine at 37°C for 5 h. The RNA transcript was purified by gel electrophoresis (20% polyacrylamide, 35 W, 0.7 mm, 2 h) yielding 3.5 nmol A2, which was characterized by ESI-MS.

Method development for combinatorial NDS mutagenesis

Coupling efficiency of phosphoramidite 1 in competition with DNA phosphoramidites

The incorporation efficiency of **1** in competition with deoxyribonucleotide phosphoramidites was determined by HPLC analysis of 5-mer model oligonucleotides (ACGTT). During the solid phase synthesis, one coupling step was performed with mixtures of **1** and one of the four dN amidites, containing 15-30% of **1** at a final phosphoramidite concentration of 100 mM. The mixtures were prepared from 100 mM stock solutions. Coupling conditions were as stated above for four minutes with BTT as activator. Cleavage of the oligonucleotide and deprotection of the phosphate backbone and nucleobases were achieved by 33% ammonium hydroxide:ethanol 3:1 for 18 hours at 55°C. The crude product was isolated and analyzed by denaturing anion exchange HPLC. The ratio of unmodified DNA pentamer and

the pentamer containing one tBDMS-protected Δ was calculated from the area integration (peak detection at 260 nm). For comparison, the modified pentamer AC Δ TT was synthesized using a 100 mM solution of 1 for position Δ .



Figure S9. HPLC analysis of tBDMS- Δ -containing DNA oligonucleotides using dN/1 mixtures of 70/30 (except for the control oligo (AC Δ TT on the left, which was synthesized with 100% 1). Dionex DNAPac PA200, 0 – 32% B in 24 min. Eluant A. 25 mM Tris.HCl, pH 8.0, 6 M urea; Eluant B. 25 mM Tris.HCl, pH 8.0, 0.5 M NaClO₄, 6 M urea; 80°C, detection at 260 nm.



Figure S10. Exemplary analysis of incorporation efficiency for $1 (\Delta)$ in competition with dG.

Optimization of alkaline hydrolysis

A 10-mer model oligonucleotide (E) containing Δ at a single position was synthesized and used for analysis of the hydrolysis behavior of the nucleoside deletion resembling an incomplete ribose. Aliquots of the oligonucleotide were incubated at 95°C with various concentrations of NaOH, and the hydrolysis products were analyzed by anion exchange HPLC (Figure S11). The expected hydrolysis products P1 and P2 were detected. The initially formed cyclic phosphate P1° was further hydrolyzed to the isomeric 2' and 3' open phosphates P1°. Even with 100 mM NaOH, only 15% of the oligonucleotide was hydrolyzed after incubation at 95°C for 30 min, while 400 mM NaOH resulted in 87% hydrolysis. The high flexibility of the non-nucleotidic spacer demanded for higher base concentrations. Based on these results, we decided to use a final concentration of 400 mM NaOH at 95°C for 15 min for hydrolysis of NDS libraries, which resulted in optimal hydrolysis at Δ , with negligible background hydrolysis of other phosphodiester bonds. Supporting Information for Samanta and Höbartner, Angewandte Chemie



Workflow of combinatorial NDS mutagenesis



Figure S12. General workflow of combinatorial NDS mutagenesis. Experimental details for the individual steps are given in the following sections, for the application on nucleic-acid ligating deoxyribozymes.

page S11

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A) Solid-phase synthesis of combinatorial deletion libraries of deoxyribozymes

Following the analysis of the incorporation efficiency of phosphoramidite 1, the deoxyribozyme libraries for 8LV13 and 9HR17 were synthesized using dN/1 mixtures of 70/30. This resulted in 4-7% incorporation at each nucleotide position in the two loop regions containing 40 nucleotides in total. The binding arms were synthesized using standard DNA phosphoramidites (this required manual exchange of the phosphoramidite solutions, since the Pharmacia Gene assembler plus has only six phosphoramidite ports, but eight different solutions are needed). Figure S13 shows the anion exchange HPLC analysis of crude and purified DNA libraries.



Figure S13. HPLC profiles of crude and purified deoxyribozyme libraries containing statisticially distributed Δ substitutions in loops A and B. Dionex DNAPac PA200, 0 – 100% B in 75 min. Eluant A. 25 mM Tris.HCl, pH 8.0, 6 M urea; Eluant B. 25 mM Tris.HCl, pH 8.0, 0.5 M NaClO₄, 6 M urea; 80°C, detection at 260 nm.

B) Separation of active deoxyribozyme mutants

B1) Enzymatic 5'-phosphorylation of NDS deoxyribozyme libraries

The Δ -containing NDS deoxyribozyme libraries (2 nmol) were incubated with 5 units of T4 PNK in presence of 1x PNK buffer (50 mM Tris.HCl, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA), 1 mM ATP in a final volume of 40 μ L for 4 h at 37°C. The enzyme was denatured by heating at 70°C for 5 min. The 5'-phosphorylated products were isolated by precipitation with ethanol at -80°C and then used for the ligation reactions without further purification.

B2) Ligation of deoxyribozymes libraries to the corresponding adaptor strand substrates

The 5'-phosphorylated deoxyribozyme library (1 nmol) and the adaptor strand (1.13 nmol) were annealed in a final volume of 23 μ L containing 1x annealing buffer (5 mM Tris.HCl, pH 7.6, 15 mM NaCl, 0.1 mM EDTA) by heating at 95°C for 2 min and slow cooling at RT for 30 min. The ligation reaction was initiated by adding 3 μ L 10x T4 RNA ligase buffer (500 mM Tris.HCl, pH 7.5, 100 mM DTT, 50 mM

page S12

MgCl₂), 0.6 μ L 50 mM ATP and 10 unit of T4 RNA ligase (10 u/ μ L) adjusting the final volume to 30 μ L. The reaction mixture was incubated at 25 °C for 10 h and then at 4°C for 12 h, quenched by 30 μ L stop solution (80% formamide, 1x TBE, 50 mM EDTA, 0.0025% each of bromophenol blue and xylene cyanol). The ligation product was separated by denaturing PAGE (10% polyacrylamide, 0.7 mm, 35 W, 1 h 26 min). The products were visualised by UV shadowing and isolated as described above. The isolated yield was 10-15%.

B3) 3'-³²P-labeling of DNA

The deoxyribozyme libraries and the deoxyribozyme libraries ligated to their corresponding adaptor substrates were 3'-³²P-radiolabelled by α -³²P-dATP using a DNA template and Klenow DNA polymerase. The DNA (50 pmol) and the template T3 (250 pmol) were annealed in a 10 µL volume by heating at 95 °C for 2 min and subsequent cooling at RT for 30 min. The reaction mixture was incubated at 37°C for 1.5 h after adding 1.5 µL of 10x Klenow reaction buffer (100 mM Tris.HCl, pH 7.5, 50 mM MgCl₂, 75 mM DTT), α -³²P-dATP (10 µCi) and 5 u Klenow DNA polymerase while the final volume was adjusted to 15 µL. The 3'-³²P-labeled products were isolated by denaturing PAGE (10%, 0.7 mm, 35 W, 45 min).

B4) Separation of active and inactive deoxyribozyme mutants by DNA-catalyzed synthesis of 2',5'branched nucleic acids

The adaptor-ligated, 3° -³²P-labeled DNA library (~25 pmol) and the corresponding scaffold substrate (166 pmol) were annealed in the presence of 5 mM HEPES, pH 7.5, 15 mM NaCl, 0.1 mM EDTA by heating at 95°C for 3 min and cooling on ice for 5 min or at RT for 45 min in a total volume of 24 µL. The ligation reaction was initiated by adding 8 µL 5x HEPES ligation buffer (250 mM HEPES, pH 7.5, 750 mM NaCl, 10 mM KCl) and 8 µL 100 mM MnCl₂ to a final volume of 40 µL. The final Mn²⁺ concentration was 20 mM. For each enzyme pool, the ligation reaction was performed at 37 °C for 30 min, 1.5 h and 3 h. The ligation products were separated by denaturing PAGE and the active (ligated) and inactive (unligated) fractions were isolated (10% PAGE, 0.7 mm, 35 W, 1 h).

The separation of the 9HR17 pool was also performed in the presence of Mg^{2+} instead of Mn^{2+} , at pH 9.0 instead of pH 7.5. The adaptor-ligated, 3'-³²P-labelled 9HR17-library (~25 pmol) was annealed to the scaffold strand S2 (166 pmole) in the presence of 5 mM HEPES, pH 7.5, 15 mM NaCl, 0.1 mM in a total volume of 30 µL. The reaction was initiated by adding 8 µL 5x CHES ligation buffer (250 mM CHES, pH 9.0, 750 mM NaCl, 10 mM KCl) and 2 µL 800 mM MgCl₂ to a final volume of 40 µL. The final Mg²⁺ concentration was 40 mM. The ligation reaction was performed at 37°C for 1.5 h and 3 h. The ligation products were again separated by denaturing PAGE and active (ligated) and inactive (unligated) fractions were isolated (10% PAGE, 0.7 mm, 35 W, 1 h).

The ligation yields of the DNA-catalyzed ligation for 8LV13 and 9HR17 libraries are summarized in Table S1.

| JIINT / NDS HUTaries. | | | | |
|-----------------------|------------------------|-------------------------|-------------------------|--|
| ligation time (h) | yield % | yie | ld % | |
| | 8LV13 library | 9HR17 | 7 library | |
| | 20 mM Mn ²⁺ | 20 mM Mn^{2+} | 40 mM Mg^{2+} | |
| 0.5 | 26.8% | 19.1% | 7.6% | |
| 1.5 | 31.1% | 23.6% | 12.2% | |
| 3.0 | 31.0% | 25.5% | 15.9% | |

Tabel S1. Ligation yields for DNA-catalyzed formation of 2',5'-branched nucleic acids for 8LV13 and 9HR17 NDS libraries.

C) Alkaline hydrolysis

Aliquots (3 μ L) of the active and inactive fraction as well as the unseparated NDS library were mixed with an equal volume of 800 mM aq. NaOH solution (final NaOH concentration 400 mM) and incubated

Supporting Information for Samanta and Höbartner, Angewandte Chemie page S14

at 95°C for 15 min in a PCR thermocycler with a heated lid. To the reaction mixture, loading dye (80% formamide, 1x TBE, 50 mM EDTA, 0.025% each bromophenol blue and xylene cyanol, 10 μ L) was added. From this sample, 5 - 6 μ L were loaded on a denaturing sequencing gel [20% polyacrylamide, 0.4 mm, 20 × 40 cm 35 W, 3 h 15 min (for loop B) or 5 h / 5 h 45 min (for loop A)]. Gels were dried at 80°C under vacuum for 30 min and exposed to a Phosphor storage screen for 18 - 38 h before scanning by a Storm PhosphorImager. The full hydrolysis gels for the 1.5 h reaction are depicted in Figure S14.

D) Analysis and quantification of interference pattern

The analysis was similar to the procedure described for combinatorial mutation interference analysis (CoMA).^[4a] Individual band intensities were quantified using the area analysis tool of ImageQuant software (Molecular Dynamics). The sum of all band intensities was normalized for minor loading differences. The interference values were calculated for every nucleotide position as the ratio of the band intensity in the unselected library by the band intensity in the active fraction. Deletion interference values larger than ten were set to 10.0. Values <2 (i.e., less than 50% change in intensity) were considered insignificant. The interference values were determined for the separated fraction after various ligation periods for both deoxyribozyme NDS libraries. The results are summarized in Figure S15 for 8LV13 and Figure S16 for 9HR17.

The results for loop A are discussed in the main manuscript. For loop B, nucleotide deletion was detrimental at most positions (exceptions: nt 34-35 in loop B of 8LV13, and nt 35 in 9HR17). The observation that Δ was not tolerated at nt 41-44 (i.e., the Watson-Crick base-paired region of loop B with the adaptor substrate) is in agreement with the importance of this stem for formation of the three helix junction structure in 2',5'-branched nucleic acid-forming deoxyribozymes.



Figure S14. Full hydrolysis gel images of a) 8LV13 and b),c) 9HR17 NDS libraries using active fraction after ligation reaction for 1.5 hours. In each panel, the left image shows the full hydrolysis gel; for better resolution of loop A, a second gel (right image) was run for longer time (in this case, fragments of loop B ran off the gel).



Figure S15. Interference values for 8LV13 NDS library; Separation of the active fraction was performed after ligation in the presence of 20 mM Mn^{2+} for 0.5, 1.5, and 3 hours. The nucleotide positions are numbered on the xaxis from 5' to 3' direction (loop A: nt 1-33, loop B: nt 34-40, paired region with adaptor substrate: nt 41-44)

1



Figure S16. Interference values for 9HR17 NDS libraries. a) Selection in the presence of 40 mM Mg²⁺ for 1.5 h and 3 h. b) Selection in the presence of 20 mM Mn²⁺ for 0.5, 1.5, and 3 h. The nucleotide positions are numbered on the x-axis from 5' to 3' direction (loop A: nt 1-33, loop B: nt 34-40, paired region with adaptor substrate: nt 41-44).

E-F) Kinetic assay for DNA-catalyzed synthesis of 2',5'-branched nucleic acids

Kinetic assays were performed in a trimolecular format under single-turnover conditions. The 5'-³²Plabeled scaffold strand was the limiting reagent relative to the deoxyribozymes and adaptor strand. The ratio of scaffold:deoxyribozyme:adaptor was 1:3:15. The scaffold strand (1 pmol), adaptor strand (15 pmol) and deoxyribozyme (3 pmol) were annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl and 0.1 mM EDTA in a 6 µL volume by heating at 95°C for 3 min and cooling for 5 min in ice or 45 min at RT. The ligation reactions were performed in 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl and 20 mM MnCl₂ (for both 8LV13 and 9HR17) or 50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl and 40 mM

36 37 38 39 40 41

MgCl₂ (only for 9HR17). The reaction mixture was incubated at 37°C. Aliquots of 0.8 μ L were removed at desired timepoints and quenched into 4 μ L stop solution (80% formamide, 1x TBE, 50 mM EDTA, 0.025% each bromophenol blue and xylene cyanol). Samples were analysed by PAGE (20% polyacrylamide, 0.4 mm, 35 W, 1 h 45 min). Gels were dried and exposed to a phosphor storage screen. Band intensities were analyzed by ImageQuant. The yield versus time data were fit to the equation of a first order kinetics i.e. yield = Y*(1-e^{-kt}), where $k=k_{obs}$ and Y= final yield.

The sequences of individual DNA mutants and the kinetic parameters are summarized in Tables S2 and S3. The corresponding gel images are depicted in Figures S17 and S19. Figure S18 shows the relationship of mutant 8LV13 sequences, and visualizes the effects on k_{obs} of classified sequence changes (see Figure caption for details).

| Table | S2. | 8LV13 | mutants |
|-------|-----|-------|---------|
|-------|-----|-------|---------|

| number | seq | uence | : | | | | | | | | | | <i>k</i> _{obs} | <i>k</i> _{rel} |
|-------------------------|-----|-----------------------|-------|-------|-------|-------|------|------|-------|-------------------|------|-----|-------------------------|-------------------------|
| | 1 | 5 | 10 | 15 | 20 | 25 | 30 | | P1 | | 35 | 40 | min ⁻¹ | mut/wt |
| 8LV13-wt ^[1] | CGA | GAGCO | TGAAI | GTTGG | TTGAG | GCACA | GTGT | ACGC | AGTGA | AG <mark>A</mark> | GGGA | AAC | 0.17 | 1.0 |
| M1 | CGA | GAGCG | TGAAI | GTTGG | TTGAG | GCACA | GTGT | ACGC | AGTGA | AG <mark>A</mark> | GGA | AAC | 0.09 | 0.5 |
| M2 | CGA | GAGCG | TGAAI | GTTGG | TTGAG | | i | ACGC | AGTGA | AG <mark>A</mark> | GGGA | AAC | 0.16 | 1.0 |
| M8 | CGT | T GCG | TGAAI | GTTGG | TTGAG | | i | ACGC | AGTGA | AG <mark>A</mark> | GGGA | AAC | 0.02 | 0.1 |
| M3 | CGA | GAGCG | TGAAI | GTGT | GAG | | i | ACGC | AGTGA | AG <mark>A</mark> | GGGA | AAC | 0.80 | 4.7 |
| M4 | CGT | T GCG | TGAAI | GTGT | GAG | | i | ACGC | AGTGA | AG <mark>A</mark> | GGGA | AAC | 0.25 | 1.5 |
| M5 | CGT | T GCG | TGAAI | GTGT | GAG | | i | ATTT | TGTGA | AG <mark>A</mark> | GGGA | AAC | 0.73 | 4.3 |
| M7 | CGA | GA <mark>G</mark> CG | TGAAI | GTGT | GAG | | i | ACGC | AGTGA | AG <mark>A</mark> | GGA | AAC | 0.01 | 0.06 |
| M6 | CGA | GA <mark>G</mark> CG | TGAAI | GTGT | GAG | | i | A | GTGA | AG <mark>A</mark> | GGGA | AAC | 0.29 | 1.7 |
| M9 | CGT | T GCG | TGAAI | GTGT | GAG | | i | A | GTGA | AG <mark>A</mark> | GGGA | AAC | 0.04 | 0.25 |
| M10 | CGA | .GA <mark>G</mark> CC | TGAAI | GATA | GAG | | i | ACGC | AGTGA | AG <mark>A</mark> | GGGA | AAC | 0.001 | 0.006 |
| M11 | CGA | GAGCG | TGAAI | GACAC | GAG | | i | ACGC | AGTGA | AG <mark>A</mark> | GGGA | AAC | <10 ⁻⁴ | |
| M12 | CGA | GAGCG | TGAAI | GTAAA | TGAG | | i | ACGC | AGTGA | AG <mark>A</mark> | GGGA | AAC | <10 ⁻⁴ | |
| M13 | CGT | T GCG | TGAAI | GTTTT | TTGAG | | i | ACGC | AGTGA | AG <mark>A</mark> | GGGA | AAC | <10 ⁻⁴ | |

^a Only the sequence of the loopA-P1-loopB region is shown. Each sequence has additional constant binding arms at the 5' and 3' end. The given sequences are preceded by 5'-CCGTCGCCATCTC- and followed by -TCCCGTATTATCC-3'. Ligation kinetics were measured at pH 7.5, in the presence of 20 mM Mn²⁺, at 37°C.



Figure S17. Gel images for kinetic assays of 8LV13 mutants (k_{obs} values see Table S2). The timepoints are 0, 2, 5, 10, 15, 30, 60, 90, 180 min. Selected kinetic graphs are depicted in Figure 3 of the manuscript.



Figure S18. Mutation cycle depicting the relationship of 8LV13 mutants. The deletion and replacement mutations are designated as A (deletion of nt 35), B (deletion of nt 24-32), C (replacement of nt 15-20 by TGT), D (shortening of P1 to 5 base-pairs), D' (introducing four mismatches in P1 by changing CGCA to TTTT), and E (replacing nt 3-5 by TT). The numbers below the arrows indicate the relative change in k_{obs} . Acceleration results mostly from mutation C (replacement of nt 15-20 TTGGTT by TGT).

| Table S3. 9HR17 mutants. | | | | | | | | | | | | | | |
|--------------------------|-----------------------|--------|---------------------|-------|------|-------|---------------|--------|------|------|-------------------------|-------|-------------------------|-------|
| | | | | | | | | | | | <i>k</i> _{obs} | yield | <i>k</i> _{obs} | yield |
| number | sequence ^a | L | | | | | | | | | Mn ²⁺ | at 3h | Mg ²⁺ | at 3h |
| | 1 5 | 10 | 15 | 20 | 25 | 30 | I | 21 | - 35 | 40 | [min⁻¹] | % | [min ⁻¹] | % |
| 9HR17-wt ^[2] | CAGCTATAI | IGTGCT | GGAC | TGAGA | GGGG | TAGTT | rcgca | AGTGAG | GTGT | 'AGG | 0.40 | 90 | 0.025 | 90 |
| M10 | CAGCTATAI | IGTGCT | GGAC | TGAGA | G | | CGCZ | AGTGAG | GTGT | 'AGG | 0.13 | 88 | n.d. | |
| M1 | CAGCTATAI | IGTGCT | GGAC | TGAGA | G | TAAAT | CGCZ | AGTGAG | GTGT | 'AGG | 0.15 | 87 | 0.002 | 36 |
| M11 | CAGCTATAI | IGTGCT | GGAC | TGAGA | G | T | CGCZ | AGTGAG | GTGT | 'AGG | 0.03 | 87 | 0.001 | 12 |
| M12 | CAGTT | CT | 'GGA <mark>C</mark> | TGAGA | G | T | CGC | AGTGAG | GTGT | 'AGG | 0.003 | 44 | n.d. | |
| M13 | CAGTT | CT | 'GGA <mark>C</mark> | TGAGA | GGGG | TAGTT | rcgc <i>i</i> | AGTGAG | GTGT | 'AGG | 0.06 | 85 | n.d. | |
| M2 | CAGTTCG | CT | 'GGA <mark>C</mark> | TGAGA | GGGG | TAGTT | FCGCA | AGTGAG | GTGT | 'AGG | 0.20 | 90 | 0.004 | 42 |
| M3 | CAG | CT | GGAC | TGAGA | GGGG | TAGTT | rcgca | AGTGAG | GTGT | 'AGG | 0.07 | 89 | n.d. | |
| M4 | С | | GGAC | TGAGA | GGGG | TAGTT | rcgca | AGTGAG | GTGT | 'AGG | 0.01 | 72 | n.d. | |
| M14 | CTAT | ΤG | AC | TGAGA | GGGG | TAGTT | rcgca | AGTGAG | GTGT | 'AGG | 0.03 | 86 | n.d. | |
| M5 | CAG | CT | GGAC | TGAGA | G | | CGCZ | AGTGAG | GTGT | 'AGG | 0.01 | 66 | n.d. | |
| M6 | CAGCTAT | TGCT | GGAC | TGAGA | G | | CGCZ | AGTGAG | GTGT | 'AGG | 0.15 | 90 | n.d. | |
| M7 | CAGCTAT | TGCT | GGAC | TGAGA | G | | | GTGAG | GTGT | 'AGG | 0.36 | 87 | n.d. | |
| M8 | CAGCTAT | TGCT | GGAC | TGAGA | G | | TTTT | GTGAG | GTGT | 'AGG | 0.31 | 89 | n.d. | |
| M9 | CAGCTAT | TGCT | GGAC | TGAGA | GGGG | TAGTT | rcgcz | AGTGAG | GTGT | 'AGG | 0.32 | 92 | 0.022 | 89 |

^a Only the sequence of the loopA-P1-loopB region is shown. Each sequence has additional constant sequences (binding arms) at the 5' and 3' end. The given sequences are preceded by 5'-CCGTCGCCATCTC- and followed by -TCCCGTATTATCC-3'. Ligation kinetics were measured at pH 7.5, in the presence of 20 mM Mn²⁺ and at pH 9.0 in the presence of 40 mM Mg²⁺ at 37°C.

| Tal | ble S4. | Comparison | of ligation | kinetics | for 9HR17 | mutants. |
|-----|---------|------------|-------------|----------|-----------|----------|
|-----|---------|------------|-------------|----------|-----------|----------|

| number | <i>k</i> _{obs} | yield | k _{rel} | <i>k</i> _{obs} | yield | k _{rel} | k _{obs} ratio |
|----------|-------------------------|-------|------------------|-------------------------|-------|------------------|------------------------|
| | Mn ²⁺ | at 3h | | Mg ²⁺ | at 3h | | Mn^{2+} |
| | [min⁻¹] | % | | [min ⁻¹] | % | | Mg ²⁺ |
| 9HR17-wt | 0.40 | 90 | 1.00 | 0.025 | 90 | 1.00 | 16 |
| M1 | 0.15 | 87 | 0.38 | 0.002 | 36 | 0.08 | 75 |
| M11 | 0.03 | 87 | 0.08 | 0.001 | 12 | 0.04 | 30 |
| M2 | 0.20 | 90 | 0.50 | 0.004 | 42 | 0.16 | 50 |
| M9 | 0.32 | 92 | 0.80 | 0.022 | 89 | 0.88 | 15 |

| 9HR17-wt | 9HR17-M1 | 9HR17-M2 | 9HR17-M3 | 9HR17-M4 | 9HR17-M5 |
|--|-----------|-----------|----------|------------------------------------|-----------|
| | | | | | |
| Number of Street | - | | | | |
| 9HR17-M6 | 9HR17-M7 | 9HR17-M8 | 9HR17-M9 | 9HR17-M10 | 9HR17-M11 |
| - | | | * | | * |
| | - | · | | - | - |
| 9HR17-M12 | 9HR17-M13 | 9HR17-M14 | 9HR17-wt | 40 mM Mg ²⁺ 9HR17-M1 | 9HR17-M9 |
| | * | | | * | * |
| - | _ | | _ | - | _ |

Figure S19. Gel images for kinetic assays of 9HR17 mutants (k_{obs} values see Table S3). The timepoints are 0, 2, 5, 10, 15, 30, 60, 90, 180 min (for the mutants marked with *, an additional timepoint at 240 min was analyzed). Selected kinetic graphs are depicted in Figure 4 of the manuscript.

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