

Electronic Supplementary Information**DNA-Catalyzed Reactivity and Selectivity for Serine Side Chains**

Amit Sachdeva and Scott K. Silverman*

Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, IL 61801 (USA)

Table of Contents

Substrates, in vitro selection, PAGE standards, and assay procedures	page S2
Assays for initial Ala, Ser, and Tyr selections with 3'-phosphate and 3'-OH substrates.....	page S3
Assays for Ser and Tyr selections redirected with 3'-ddC substrates.....	page S3
Sequences of SerB1–SerB4 and TyrB1 deoxyribozymes	page S4
Kinetic assays for SerB1–SerB4 and TyrB1 deoxyribozymes.....	page S4
Mn ²⁺ and Mg ²⁺ requirements of SerB1–SerB4 and TyrB1 deoxyribozymes	page S5
K _{d,app} (Mn ²⁺) determinations for SerB1–SerB4 and TyrB1 deoxyribozymes	page S6
Product characterization assays for SerB1–SerB4 and TyrB1 deoxyribozymes.....	page S7
Assays for positional selectivities of SerB1–SerB4 deoxyribozymes.....	page S9
References for Supplementary Information	page S9

Substrates, in vitro selection, PAGE standards, and assay procedures

DNA-tripeptide conjugates were synthesized using the protocols described previously,¹ with a minor modification to introduce a capping step during the solid-phase synthesis. After each coupling step, the unreacted amino group was capped using a mixture of acetic anhydride, pyridine, and 1-methylimidazole (5% each) in THF that was prepared by mixing 500 μ L of Glen Research Cap Mix A (10% each of acetic anhydride and pyridine in THF) with 500 μ L of Cap Mix B (10% 1-methylimidazole in THF). The glass beads were incubated with 1 mL of this solution for 30 min, washed with 10 mL of acetonitrile, and washed with 10 mL of CH_2Cl_2 .

The in vitro selection experiments and deoxyribozyme cloning procedures were performed essentially as described previously.^{2,3} DNA and RNA sequences used during selection are shown in Figure S1.

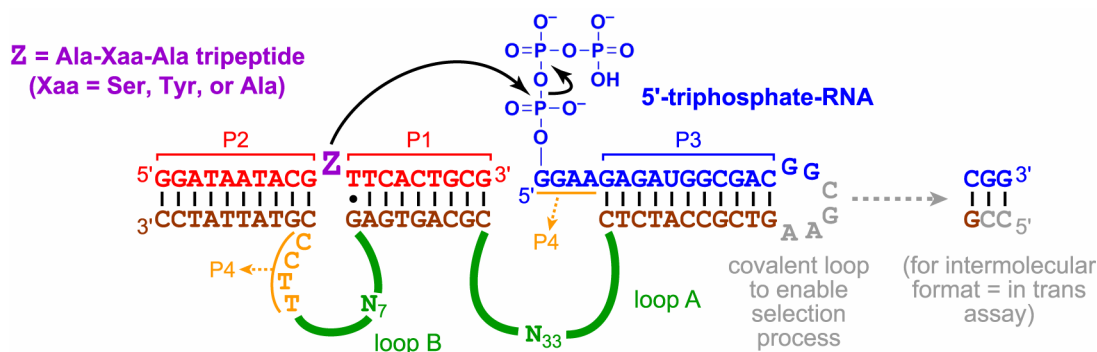


Figure S1. DNA and RNA sequences used in the selection experiments. The 3'-terminus of the tripeptide-containing substrate (red) was either 3'-OH, 3'-phosphate, or 3'-ddC, as described in the text. To assay individual deoxyribozymes in the intermolecular (in trans) format using the tripeptide-containing substrate and the free 5'-triphosphate-RNA (blue), the grey CGAA nucleotides were removed and replaced with 5'-CC on the deoxyribozyme strand, thereby fully complementing the 5'-triphosphate-RNA. The G•T wobble pair at the end of P1 is present because the 5'-NH₂-T nucleotide (the only commercially available 5'-NH₂ nucleotide phosphoramidite) was required for synthesis of the tripeptide-containing substrate, and the deoxyribozyme pool strand with the corresponding G nucleotide was available from our previous work.

A branched PAGE standard was prepared using the previously described 9HR17 deoxyribozyme, which forms a 2',5'-branched nucleic acid product from DNA-rA-DNA and 5'-triphosphate-RNA substrates.¹ The 9HR17 product has similar molecular weight and architecture as the expected nucleopeptide ligation products from the Figure S1 selections. This product was used directly as the branched standard for all intermolecular (in trans) assays, and it was ligated by T4 RNA ligase to the deoxyribozyme pool to provide the size standard used during each selection round.

For activity assays using deoxyribozymes prepared by solid-phase synthesis, the general approach was described previously.² The DNA-peptide-DNA substrate is designated as the L (left-hand) substrate, and the 5'-triphosphate-RNA substrate is designated as the R (right-hand) substrate. In most assays, the ³²P-radiolabeled L substrate was the limiting reagent relative to the deoxyribozyme (E) and the R substrate. The L:E:R ratio was <1:5:10, with the concentration of E equal to 0.5 μ M in 20 μ L assays. Values of k_{obs} were obtained by fitting the yield versus time data directly to first-order kinetics; i.e., $\text{yield} = Y(1 - e^{-kt})$, where $k = k_{\text{obs}}$ and Y is the final yield.

Assays for initial Ala, Ser, and Tyr selections with 3'-phosphate and 3'-OH substrates

As shown in Figure S2a, the round 4 products from all four selections with 3'-phosphate substrates were assayed by PAGE, in the intermolecular format (Figure S1) alongside the branched standard described on page S2. In each case, substrate with either 3'-phosphate or 3'-OH was tested with 20 h incubation. For Xaa = Ser, the ligation yields were 0.8% and 1.3%, respectively; for Tyr, the ligation yields were 5.8% and 7.0%. For both Ser and Tyr, the migration rate clearly indicated that the great majority of the product is branched rather than linear; i.e., that reaction is taking place at or near the tripeptide portion of the substrate, rather than at the 3'-terminus. For Ala, the ligation yields were 1.0% and <0.5%. In contrast to Ser and Tyr, the migration rate for the product with Ala suggested a linear rather than branched product, consistent with the considerable yield difference in favor of the 3'-phosphate substrate.

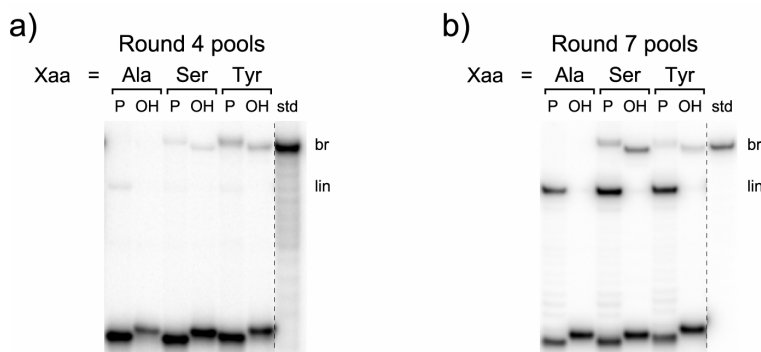


Figure S2. Assays of round 4 and round 7 selection products formed using either 3'-phosphate (P) or 3'-OH tripeptide-containing substrate (Xaa of substrate matches Xaa of selection pool). a) Round 4. b) Round 7. Std = branched standard, as described on page S2. Incubation conditions: 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl and 20 mM Mn^{2+} , and 40 mM Mg^{2+} at 37 °C for 21 h.

After three additional rounds, the round 7 products were assayed in the same way (Figure S2b). For Xaa = Ala, the result was similar to the round 4 observations. However, for both Ser and Tyr, the predominant product was now linear, with only 3–7% of the total product clearly branched (using the 3'-phosphate substrate). When the 3'-OH substrate was used, only the branched product was observable, consistent with nucleophilic reaction of the 3'-phosphate group during formation of the undesired linear product.

Assays for Ser and Tyr selections redirected with 3'-ddC substrates

The uncloned round 10 products from the 3'-ddC-redirected Ser and Tyr selections were assayed in the intermolecular format with the 3'-ddC substrates (Figure S3). The ligation yields were 32% (Ser) and only 3.1% (Tyr), versus 32% and 9% observed in the corresponding selection rounds. For both selections, no activity was observed with a substrate containing Ala (a substrate containing Lys was also tested and found not to support any activity). However, the round 10 Ser selection pool did show 7.5% yield with Tyr substrate, and the round 10 Tyr selection pool showed 3.5% yield with the Ser substrate. Cloning from round 10 of the Ser selection led to deoxyribozymes with varying selectivities for Ser over Tyr (Figure 2b). In contrast, cloning from round 10 of the Tyr selection led only to the TyrB1 deoxyribozyme, which is highly selective for Tyr over Ser (Figure 2a). The deoxyribozymes in the uncloned round 10 pool that were responsible for the observed activity with the Ser substrate were not identified upon cloning of round 10, which led only to TyrB1.

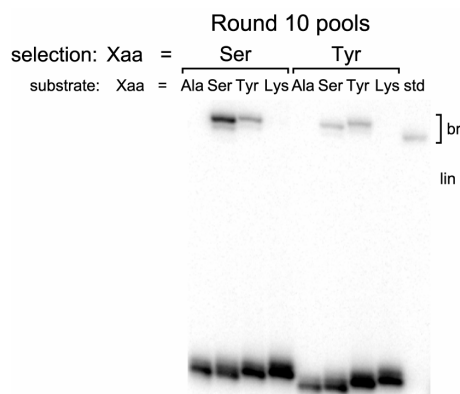


Figure S3. Assays of round 10 selection products (Xaa = Ser or Tyr) formed using 3'-ddC tripeptide-containing substrates (Xaa = Ala, Ser, Tyr, or Lys). Incubation conditions as in Figure S2. Note that the branched standard lacks the 3'-ddC that is present on the substrate, and therefore the branched product migrates slightly slower than the branched standard.

Sequences of SerB1–SerB4 and TyrB1 deoxyribozymes

a)

deoxyribozyme	sequence
SerB1	5'-P3-ACGCCGTGTGTCTTACCGCGTACAGCGAATAGTT-P1-TGCAGCG-P4-P2-3'
SerB2	5'-P3-CTACAGTCGAGTAATGTAGTCAGCAAGATCGTCA-P1-TGTAGCG-P4-P2-3'
SerB3	5'-P3-CAGACGTACCGGACTGACAGCAAGATTGTTAGTA-P1-TGTAGCG-P4-P2-3'
SerB4	5'-P3-CACAGTATTGTGTCTAGCAAGAAGTGGGATGTTTTP1-TGTAGCG-P4-P2-3'
TyrB1	5'-P3-CGCCATTGTGGGTACAAAATGCGCCCGAGAAGTT-P1-CGCGGAA-P4-P2-3'

b)

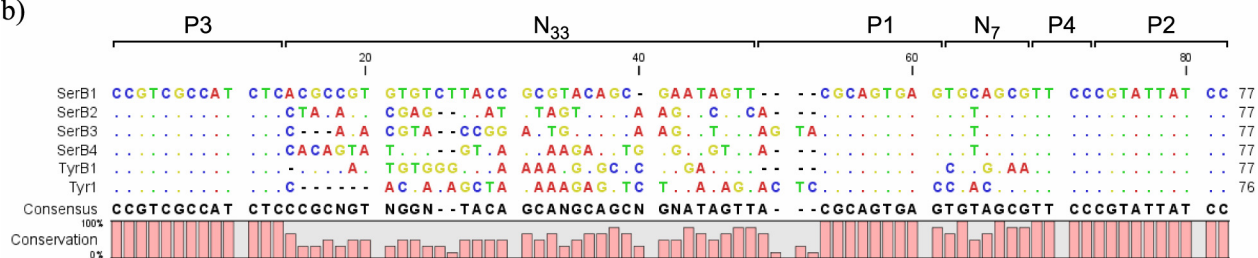


Table S1. Sequences and alignment of the SerB1–SerB4 and TyrB1 deoxyribozymes. a) The sequences are shown for the intermolecular (in trans) assay format as described in Figure S1, with the nucleotides of the P3, P1, P4, and P2 regions as shown in that figure. b) Alignment of the SerB1–SerB4, TyrB1, and TyrB deoxyribozymes, as computed using the CLC Sequence Viewer program.

Kinetic assays for SerB1–SerB4 and TyrB1 deoxyribozymes

Kinetic assays for the SerB1–SerB4 and TyrB1 deoxyribozymes were performed as described on page S2. Representative full PAGE kinetic data for SerB2 are shown in Figure S4a. Kinetic plots for SerB3 and SerB4 are shown in Figure S4b (see Figure 2 for TyrB1, SerB1, and SerB2 data). Representative data comparing reactivities of SerB1–SerB4 with four Ala-Xaa-Ala (Xaa = Ala, Ser, Tyr, or Lys) substrates, along with the three single-amino-acid substrates (Ser, Tyr, or Lys), are shown in Figure S4c.

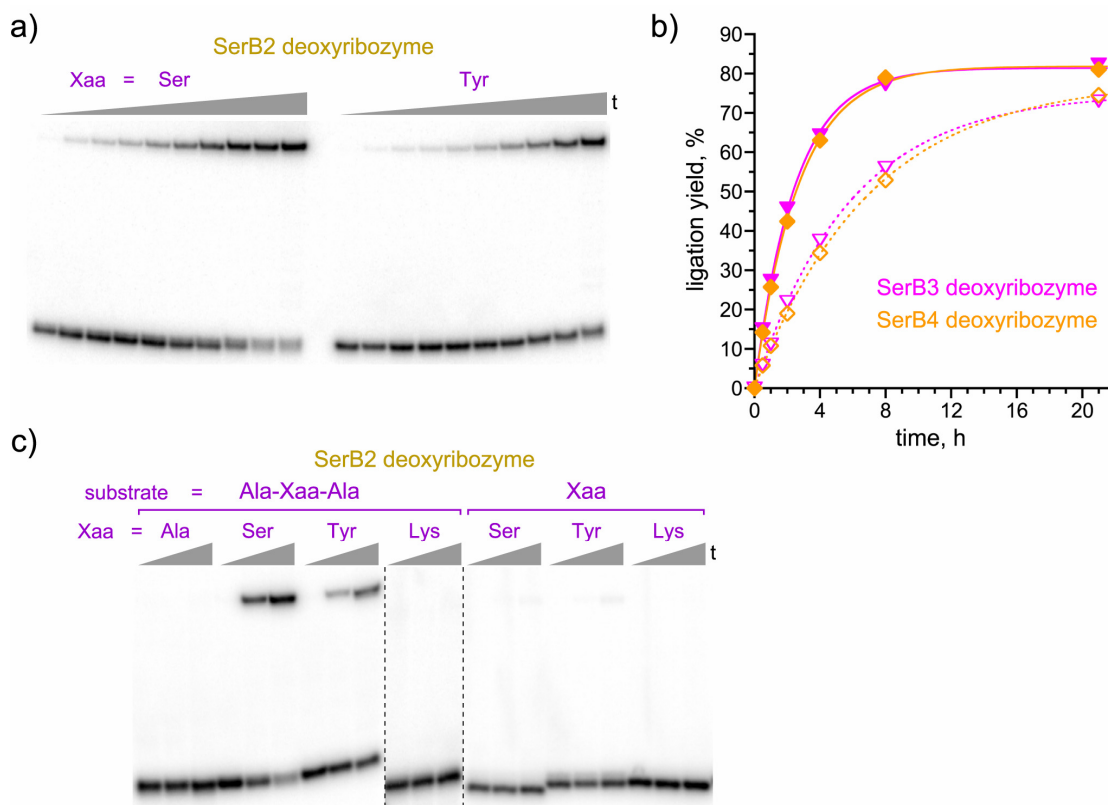


Figure S4. Ligation assays in 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, 20 mM Mn^{2+} , and 40 mM Mg^{2+} at 37 °C. a) Representative full PAGE kinetic data for SerB2. t = 0, 4, 8, 15, 30 min; 1, 2, 4, 8, and 21 h. b) Kinetic plots for SerB3 and SerB4 (see Figure 2 for TyrB1, SerB1, and SerB2 data). Xaa = Ser (\blacktriangledown , SerB3; \blacklozenge , SerB4) or Tyr (∇ , SerB3; \diamond , SerB4). k_{obs} values: SerB3, 0.40 h^{-1} with Ser and 0.17 h^{-1} with Tyr; SerB4, 0.37 h^{-1} with Ser and 0.14 h^{-1} with Tyr. c) Representative assays for SerB2 with substrates containing a tripeptide or a single amino acid. t = 0, 1, and 5 h. Similarly, neither Ala nor Lys led to reactivity with the other four deoxyribozymes.

Mn^{2+} and Mg^{2+} requirements of SerB1–SerB4 and TyrB1 deoxyribozymes

For the SerB1–SerB4 and TyrB1 deoxyribozymes, the ligation activities were comparable when the assays were performed with 20 mM Mn^{2+} alone, rather than 20 mM Mn^{2+} + 40 mM Mg^{2+} as in Figure 2 (Figure S5). No activities were observed with 40 mM Mg^{2+} alone.

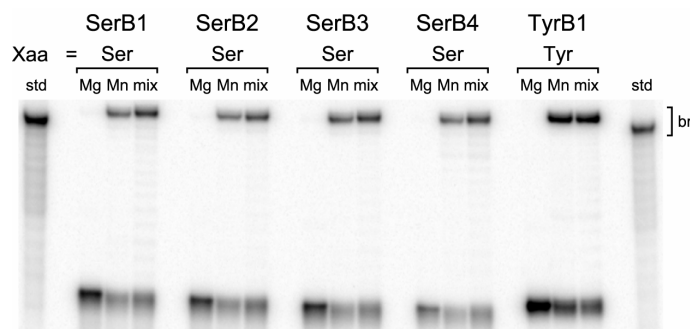


Figure S5. Ligation assays for the SerB1–SerB4 and TyrB1 deoxyribozymes in 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl and either 40 mM Mg^{2+} , 20 mM Mn^{2+} , or 20 mM Mn^{2+} + 40 mM Mg^{2+} (i.e., "mix") at 37 °C. The 21 h timepoints are shown using the 3'-ddC tripeptide-containing substrates with Xaa corresponding to the selection experiment. The k_{obs} and yield for each deoxyribozyme with 20 mM Mn^{2+} alone or additionally with 40 mM Mg^{2+} were comparable (data not shown).

$K_{d,app}(Mn^{2+})$ determinations for SerB1–SerB4 and TyrB1 deoxyribozymes

The Mn^{2+} dependence of each of the SerB1–SerB4 and TyrB1 deoxyribozymes was determined using the corresponding Ser or Tyr tripeptide-containing substrate (Figure S6). Above 20–60 mM Mn^{2+} for all five deoxyribozymes, activity suppression was observed, via a decrease in ligation yield typically accompanied by a decrease in k_{obs} . The data points for which such suppression was evident were excluded from the plots. The illustrated data allow the conclusion that $K_{d,app}$ is >10 mM for each of SerB1–SerB3; 13 ± 2 mM for SerB4; and 17 ± 3 mM for TyrB1. No Mg^{2+} was included in these experiments. The k_{obs} values at 20 mM Mn^{2+} without Mg^{2+} (as shown in Figure S6) were slightly different from the k_{obs} values at 20 mM Mn^{2+} and 40 mM Mg^{2+} (as shown in Figures 2 and S5).

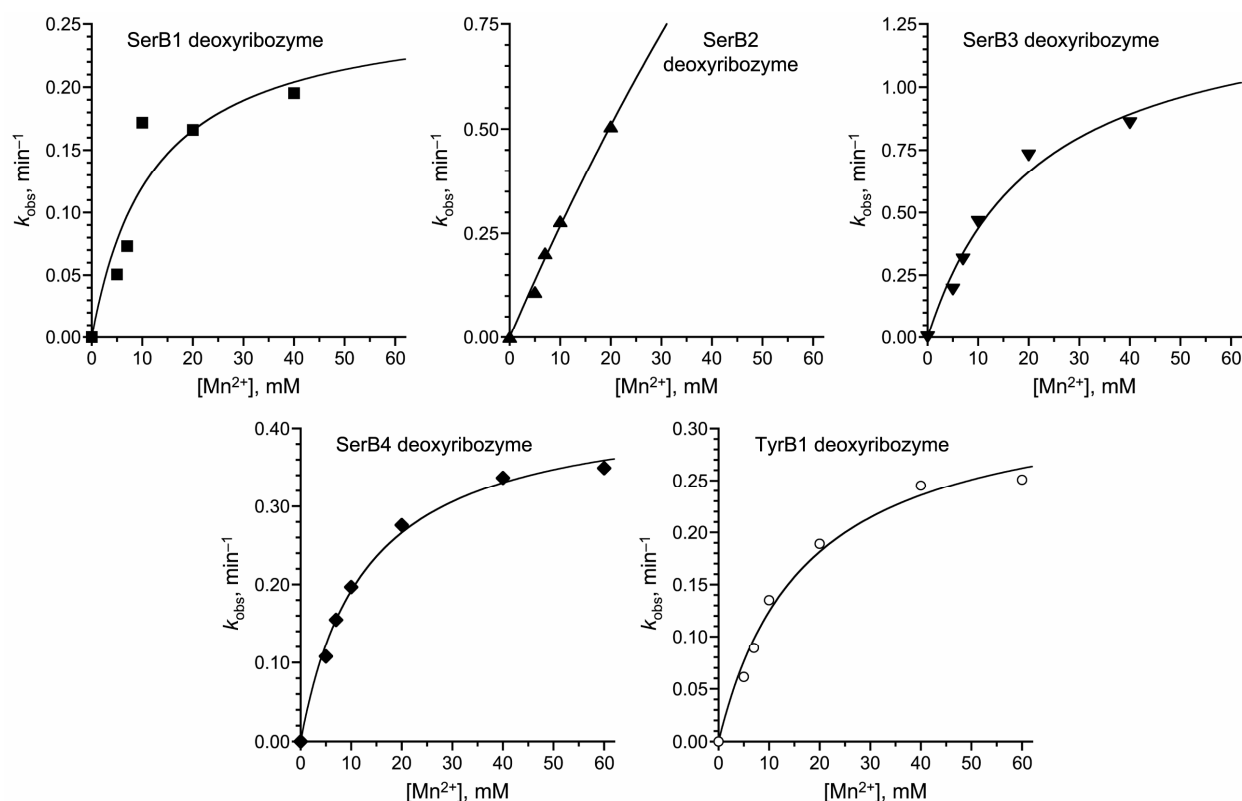


Figure S6. Determinations of $K_{d,app}(Mn^{2+})$ for the SerB1–SerB4 and TyrB1 deoxyribozymes. Assays were performed in 50 mM HEPES, pH 7.5, 150 mM NaCl, and 2 mM KCl additionally containing 0–60 mM Mn^{2+} at 37 °C. Data were fit to the conventional curve $k_{obs} = k_{max} \times [Mn^{2+}] / (K_d + [Mn^{2+}])$.

Product characterization assays for SerB1–SerB4 and TyrB1 deoxyribozymes

MALDI-TOF mass spectrometry was used to corroborate the product assignments for the TyrB1, SerB1, and SerB2 deoxyribozymes (Figure S7). In all three cases, the observed mass was within experimental error of the expected value for the assigned product.

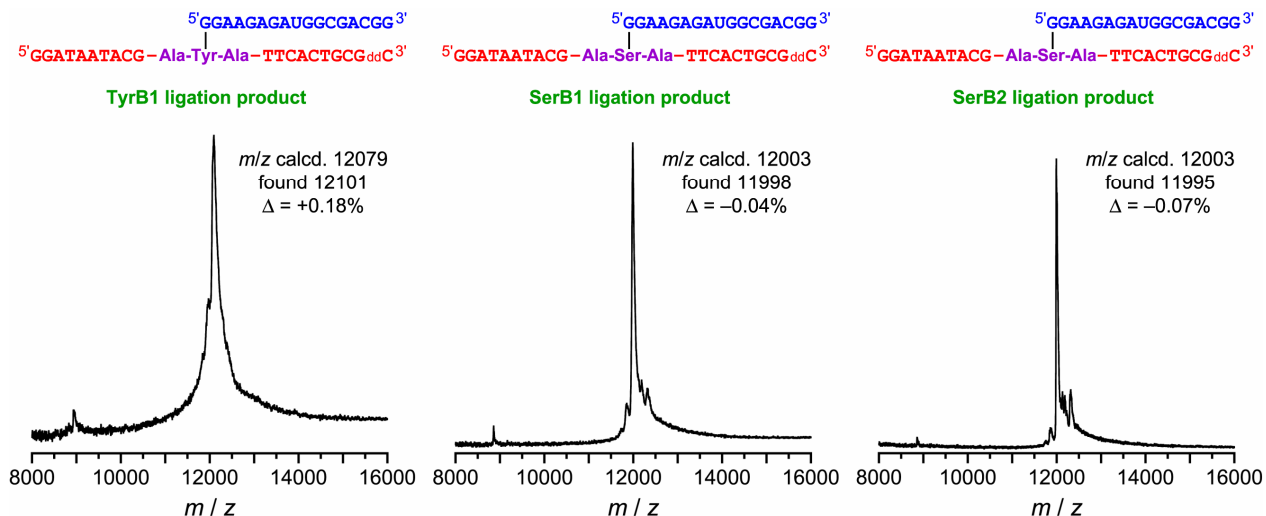


Figure S7. MALDI-TOF mass spectrometry of the TyrB1, SerB1, and SerB2 ligation products.

Biochemical assays were performed to provide additional evidence regarding the substrate reaction site for the SerB1 ligation product (Figure S8; data for the TyrB1 ligation product was essentially equivalent). These assays involved treatment of each ligation product with 80% aqueous acetic acid, which is known to hydrolyze phosphoramidate (P–N) bonds.⁴ The tripeptide-containing substrate has a phosphoramidate linkage directly to the 5'-side of the tripeptide (Figure 1b; also see Figure S8a). Therefore, phosphoramidate hydrolysis should lead to a readily predicted PAGE pattern if the product has the Tyr/Ser residue of the tripeptide-containing substrate attached directly to the RNA substrate. Furthermore, the observed pattern should depend on whether the tripeptide-containing substrate was initially 5'-³²P-radiolabeled or 3'-³²P-radiolabeled. For 5'-radiolabeling the phosphoramidate linkage is between the ³²P-radiolabel and the tripeptide, whereas for 3'-radiolabeling the tripeptide is between the phosphoramidate linkage and the ³²P-radiolabel. In addition, ligation products were synthesized using a substrate that has a second phosphoramidate linkage between the two T nucleotides immediately to the 3'-side of the tripeptide (Figure S8b; prepared by incorporating two 5'-NH₂-T phosphoramidite monomers instead of only one such monomer during solid-phase synthesis of the tripeptide-containing substrate). For DNA-catalyzed ligation products prepared using this alternative substrate, phosphoramidate hydrolysis should cleave the tripeptide region (now connected to the RNA substrate) from the ³²P radiolabel regardless of whether the label is at the 5'- or 3'-end of the substrate. For both the TyrB1 and SerB1 ligation products, the assay outcomes were entirely in accord with expectations (Figure S8c and S8d), supporting the nucleopeptide linkage assignments made on the basis of our other data.

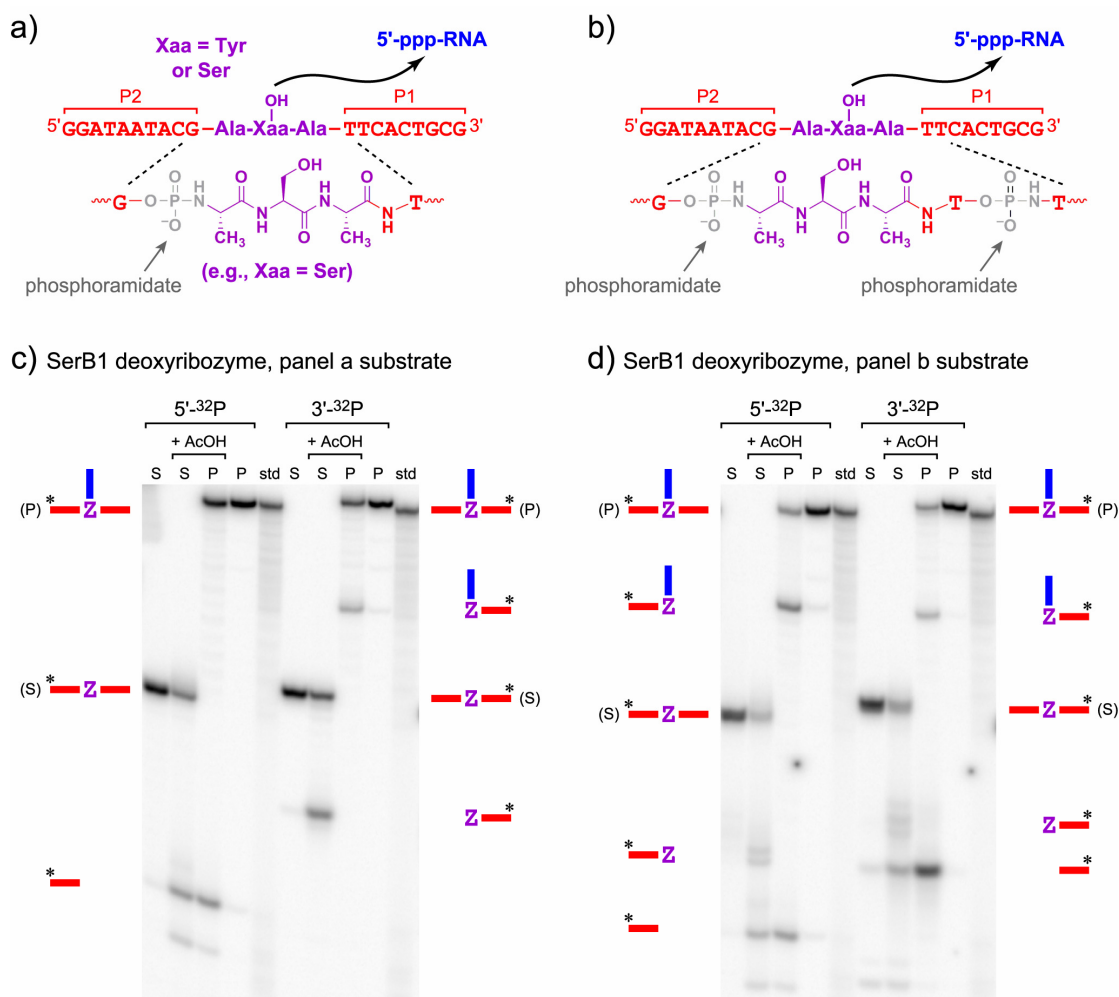


Figure S8. Biochemical assays to support the assignment of substrate reaction site for the SerB1 ligation products. a) Tripeptide-containing substrate with a single phosphoramidate (P–N) linkage on the 5' side of the tripeptide, as used during selection and all other assays in this report (same substrate as depicted in Figure 1b). b) Tripeptide-containing substrate with a second phosphoramidate linkage incorporated on the other (3') side of the tripeptide. For both panels a and b, the substrate had 3'-OH when 5'-³²P-radiolabeled (γ -³²P-ATP, T4 PNK) and 3'-dC-OH when 3'-³²P-radiolabeled (α -³²P-dCTP, terminal deoxytransferase). c) and d) Treatment of the SerB1 ligation products, made using the substrates of panels a and b, respectively, with 80% aqueous AcOH to cleave the phosphoramidate linkage(s). S = substrate; P = product. Z denotes the Ala-Ser-Ala tripeptide portion of the substrate. The incubation time of 5 h was chosen to cleave only a fraction of the potentially cleavable P–N bonds, which allows observation of both unhydrolyzed and hydrolyzed ligation product.

Assays for positional selectivities of SerB1–SerB4 deoxyribozymes

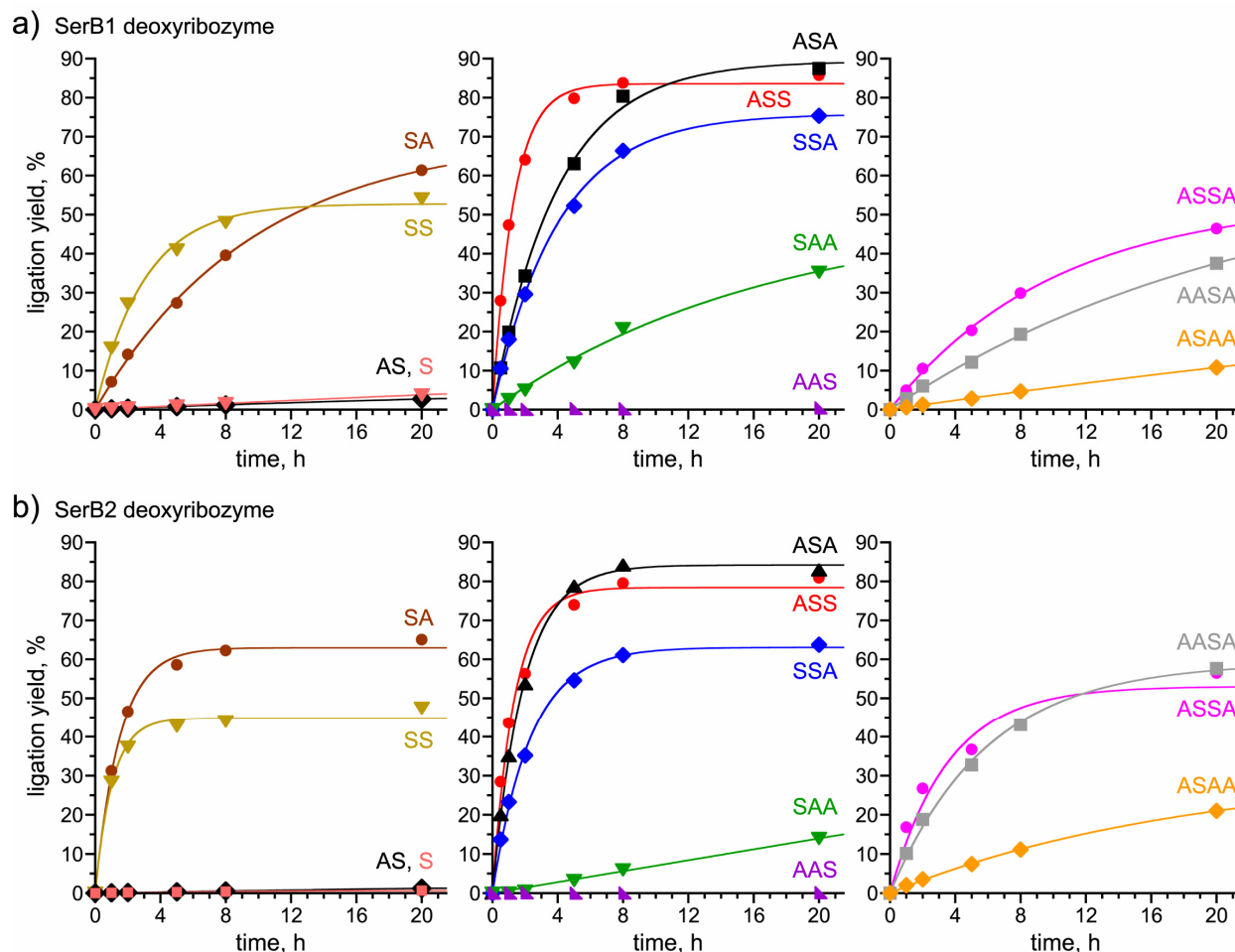


Figure S9. Side chain positional selectivity for serine hydroxyl groups by the SerB1 and SerB2 deoxyribozymes. Data for SerB2 in Figure 3 are the same as shown in this figure in color to facilitate comparisons.

References for Supplementary Information

1. P. I. Pradeepkumar, C. Höbartner, D. A. Baum and S. K. Silverman, *Angew. Chem. Int. Ed.*, 2008, **47**, 1753-1757.
2. A. Flynn-Charlebois, Y. Wang, T. K. Prior, I. Rashid, K. A. Hoadley, R. L. Coppins, A. C. Wolf and S. K. Silverman, *J. Am. Chem. Soc.*, 2003, **125**, 2444-2454.
3. D. M. Kost, J. P. Gerdt, P. I. Pradeepkumar and S. K. Silverman, *Org. Biomol. Chem.*, 2008, **6**, 4391-4398.
4. T. Wada, T. Moriguchi and M. Sekine, *J. Am. Chem. Soc.*, 1994, **116**, 9901-9911.
5. J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, 1961, **83**, 649-658.