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# Sequence-Dependent Acylation of Peptide Lysine Residues by DNAzymes

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Methods for modifying intact peptides are useful but can be unselective with regard to amino acid position and sequence context. In this work, we used in vitro selection to identify DNAzymes that acylate a Lys residue of a short peptide in sequence-dependent fashion. The DNAzymes do not acylate Lys when placed at other residues in the peptide, and the acylation activity depends on the Lys sequence context. A high acylation yield is observed on the preparative nanomole scale. These findings are promising for further development of DNAzymes for broader application to top-down Lys acylation of peptide and protein substrates.

## Introduction

Lysine (Lys) acylation is an important post-translational modification of peptides and proteins.[1] Acyl modifications of Lys can be introduced into peptides during their linear preparation by bottom-up solid-phase synthesis[2] or into proteins by nonsense codon suppression.[3] Alternatively, after unmodified peptide preparation is complete, modifications like Lys acyl groups can be appended in top-down fashion by chemical or enzymatic approaches. Chemical methods for peptide and protein acylation typically lack selectivity among multiple Lys positions and sequence contexts, although these methods are improving.[4] Enzymatic approaches are in principle capable of high selectivity, but they typically require creating a non-native protein with an inserted target sequence that is recognized by the enzyme.[5] In this study, we seek a new enzymatic approach to site-selective Lys acyl modification of peptides using DNAzymes.

DNAzymes are artificial DNA sequences with catalytic activity,[6] identified by in vitro selection from random sequence populations.[7] The repertoire of DNAzyme catalysis notably includes RNA cleavage[8] and peroxidase activity[9] as well as many other reactions.[6] Achieving site-selective Lys acylation of peptides by DNAzymes will broaden DNAzyme catalysis and provide a practically useful method for sequence-dependent peptide modification.

We reported DNAzymes that acylate Lys of a peptide that was covalently tethered to a DNA anchor oligonucleotide (Figure 1A, *top*).[10] In that effort, the acyl donor was a 5′-aryl ester oligonucleotide, such that both substrates, the Lys aliphatic amine nucleophile and the aryl ester electrophile, were noncovalently bound to the DNAzyme via remote Watson-Crick base pairing. We found that in this arrangement, a 2,3,5,6-tetrafluorophenyl ester (TFPE) acyl donor was too reactive; i.e., the DNA-splinted but uncatalyzed background reaction rate was too high to allow DNAzyme catalysis. In contrast, the less reactive 4-fluorophenyl and phenyl esters (4FPE, PE) were suitably reactive to enable identification of DNAzymes, with highest rate enhancement above background ($k_{obs}/k_{bkgd}$) of 1100. Separately, we reported DNAzymes that acylate DNA nucleobase aromatic amines, now using the TFPE acyl donor, with highest rate enhancement of 300 (Figure 1A, *bottom*).[11]

In this current work, the Lys aliphatic amine is presented on a peptide that is not covalently tethered to a DNA anchor oligonucleotide (Figure 1B). For practical application of DNAzymes to peptide modification, there cannot be a requirement that the peptide is covalently tethered. This untethered arrangement substantially increases the challenge for DNAzyme catalysis, because in addition to catalyzing the Lys acylation, the DNAzyme must bind the untethered peptide substrate without the benefit of Watson-Crick base pairing to the anchor oligonucleotide. Our previous DNAzymes that were identified by selection with a tethered peptide had no activity with an untethered peptide.[10] Consistent with the above-mentioned chemical considerations as well as our findings on DNAzyme-catalyzed tethered peptide Lys reactivity with phosphorus electrophiles,[12] here in our new experiments we used the more reactive TFPE acyl donor. We report the successful identification of DNAzymes for site-selective and amino acid context-dependent untethered peptide Lys acylation.

## Results and Discussion

To identify DNAzymes by in vitro selection, one of the substrates must be covalently attached to the initially random DNA population (pool), so that catalytically active DNA

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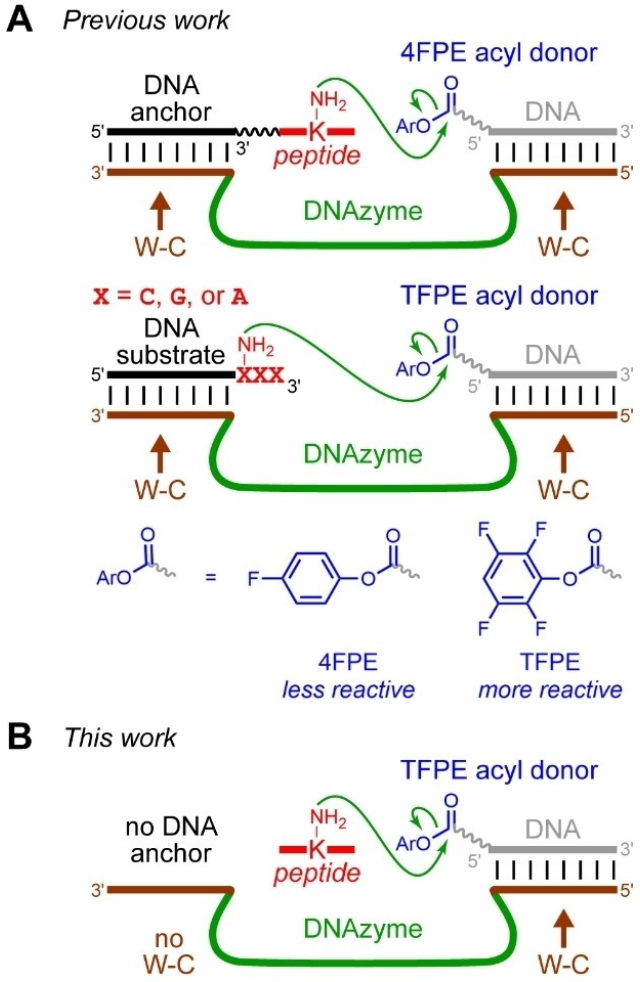


**Figure 1.** DNAzymes for amine acylation in several contexts. (A) Previous work. *Top*, acylation of Lys aliphatic amine in a peptide that is covalently tethered to a DNA anchor oligonucleotide.[10] The acyl donor is a 5′-aryl ester oligonucleotide, here as the 4-fluorophenyl ester (4FPE, illustrated) or phenyl ester (PE). The DNAzymes that emerged from this selection effort had no catalytic activity with an untethered peptide. *Bottom*, DNAzymes for acylation of DNA nucleobase amines.[11] Here the acyl donor is the more reactive 2,3,5,6-tetrafluorophenyl ester (TFPE). In both reactions, each substrate is anchored or bound to the DNAzyme binding arms by Watson-Crick base pairs (brown arrows). (B) This work. We seek DNAzymes for Lys acylation of a peptide that is not covalently tethered to a DNA anchor oligonucleotide. Because of the absence of Watson-Crick base pairs to bind the untethered peptide substrate, we anticipated the need for the more reactive TFPE as the acyl donor.

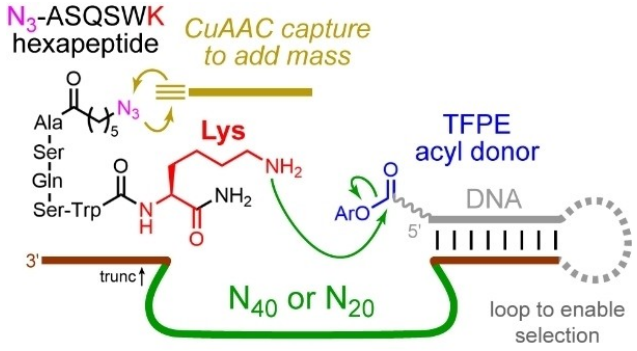


**Figure 2.** In vitro selection design for DNAzyme-catalyzed peptide Lys acylation. See Figure S2 for nucleotide details. Each DNAzyme that emerges from the selection process is assayed without the dashed loop at far right, such that the TFPE acyl donor substrate is bound intermolecularly by the DNAzyme (in trans).The loop is required to enable selection, because acylation of the peptide and subsequent CuAAC (DNA splint not shown) results in a substantial upward PAGE shift only for catalytically active DNA sequences. The black arrow marks the 3′-truncation site for each new DNAzyme. The 5′-TFPE acyl donor oligonucleotide was prepared by activation of the 5′-$CO_2H$ oligonucleotide with EDC and TFP.

sequences can be separated by polyacrylamide gel electrophoresis (PAGE) in each iterated selection round. By choosing to use an untethered peptide substrate, we must covalently attach the acyl donor substrate to the DNA pool (Figure 2). In this format we performed six in vitro selection experiments, at three pH values of 6.5, 7.4, and 8.5 and 37 °C, each with an $N_{40}$ or $N_{20}$ random region and using a 5′-TFPE oligonucleotide as the acyl donor. With $N_{40}$, we sample only $10^{-10}$ of sequence space ($4^{40} \approx 10^{24}$ possible sequences; initial pool size of 200 pmol = $10^{14}$ actual sequences). With $N_{20}$, we survey all of sequence space ($4^{20} \approx 10^{12}$ possible sequences; ~$10^2$ copies of each sequence in the initial 200 pmol pool sample), but the smaller DNA length may be less able to support both substrate binding and catalysis. Ultimately the successful random region lengths for any given catalytic activity must be determined empirically.[13] At pH 6.5 and 7.4, we included 40 mM $MgCl_2$, 20 mM $MnCl_2$, and 1 mM $ZnCl_2$, because all three metal ions at these concentrations have been useful for various DNAzyme activities. At pH 8.5, only $Mg^{2+}$ can be included because $Mn^{2+}$ oxidizes and $Zn^{2+}$ precipitates.

For each selection, the model hexapeptide substrate was ASQSWK, thereby placing tryptophan (Trp) directly adjacent to the Lys residue that is intended to be acylated. This was done based on the precedent that aromatic groups can foster binding to nucleic acids, notably with slow off-rate modified aptamers (SOMAmers)[14] and tRNA aminoacylation ribozymes (flexizymes).[15] The remainder of the peptide sequence was chosen for solubility, as well as to include two serine (Ser) as potentially competing nucleophiles for acylation. The intended Lys acylation site was arbitrarily placed at the hexapeptide C-terminus, flanked by two amide groups that include the C-terminal amide.

Unlike in our selection experiments with a covalently tethered peptide,[10] here a successful DNAzyme-catalyzed amine acylation reaction does not, by itself, result in a sufficient mass addition and large PAGE shift of the corresponding DNA pool sequence. Therefore, based on our prior work with DNAzymes for modifying peptide tyrosine (Tyr) residues by Tyr-RNA and Tyr-DNA conjugation,[16] at the peptide N-terminus we included an azido group from 6-azidohexanoic acid in place of the standard *N*-acetyl cap. This azido group enables CuAAC attachment of a 3′-alkyne oligonucleotide in each selection round. Performing this capture step after the Lys acylation step induces a substantial PAGE shift for catalytically active DNA sequences, thus enabling their enrichment in each round.

At each of the three pH values, we first performed background assays with the $N_{40}$ pool to establish a suitable peptide concentration and incubation time to use during

selection (Figure S1). Our general guideline is to seek $<5\%$ background reaction, where 5% background limits the enrichment factor per selection round to no higher than 20×. We found that at pH 6.5 and 7.4, 100 μM peptide and 16 h incubation time led to 3.2% and 1.3% background. In contrast, at pH 8.5 and 16 h, a lower peptide concentration of 10 μM was necessary to have 4.0% background (vs. 26% at 100 μM). All of these background yields were obtained in trans, i.e., without the 5′-TFPE acyl donor oligonucleotide covalently attached to the pool, thus obviating the need for the CuAAC step in order to observe the PAGE shift. Therefore, these background yields are about two-fold higher than would be observed if CuAAC were also performed, because the CuAAC yield in our arrangement was ~50%.

The progressions of the six selection experiments are in Figure 3A. At pH 6.5 and 8.5 for both $N_{40}$ and $N_{20}$, the pool yield was sufficiently high at round 11–13 to move forward to cloning and identifying individual DNAzymes. In all four cases, imposing additional selection pressure by decreasing the peptide concentration from 100 μM–10 μM was unproductive; the yield dropped and did not recover after three rounds. In contrast to the outcomes at pH 6.5 and 8.5, for the selection at pH 7.4 little pool yield was observed above background, likely related to the observed relative instabilty of TFPE at this intermediate pH value,[11] and thus neither pH 7.4 selection was cloned. For the $N_{40}$ pool, each of the pH 6.5 and pH 8.5 selections led to a set of DNAzymes (five 12LK2 and two 11LM2 DNAzymes, respectively; Figure 3B). For the $N_{20}$ pool, the selections at pH 6.5 and 8.5 gave an overlapping set of four 13LN2 DNAzymes, which was possible because of the sequence space oversampling. Thus, from the four selection experiments combined, we found a total of 11 new DNAzymes for peptide Lys acylation.

The 11 new individual peptide acylation DNAzymes were assayed in various ways. An initial survey (Table S1) showed yields as high as 80% in 48 h, in some cases approaching those high yields at much earlier times. We varied the $Mg^{2+}$, $Mn^{2+}$, and $Zn^{2+}$ concentrations, to assess whether the 40 mM $Mg^{2+}$, 20 mM $Mn^{2+}$, and 1 mM $Zn^{2+}$ used during selection are optimal for each DNAzyme (only $Mg^{2+}$ for the pH 8.5 DNAzymes; all three metal ions for the pH 6.5 DNAzymes). For all 11 DNAzymes, no improvement was found with 120 or 240 mM $Mg^{2+}$, 10 or 30 mM $Mn^{2+}$, and 0.5 or 1.5 mM $Zn^{2+}$. The subsequent assays were thus performed using the appropriate initial combinations of 40 mM $Mg^{2+}$, 20 mM $Mn^{2+}$, and 1 mM $Zn^{2+}$. We evaluated truncation of the fixed 3′-segment of each DNAzyme. When all but three nucleotides were removed from the fixed 3′-segment (see truncation site marked with a small black arrow in Figure 2), full yield was retained, so our later assays used these 3′-truncated DNAzyme variants.

The rate constant $k_{obs}$ was obtained for each DNAzyme, where this rate constant is single turnover with respect to the 5′-TFPE acyl donor oligonucleotide (Figure 4A). The highest $k_{obs}$ of 0.46 $h^{-1}$ was found for the 12LK10 DNAzyme, with 82% yield at pH 6.5 and 100 μM peptide. Background rate constants ($k_{bkgd}$ values) for all DNAzymes were determined using initial-rate kinetics, with the random $N_{40}$ pool in place of the DNAzyme

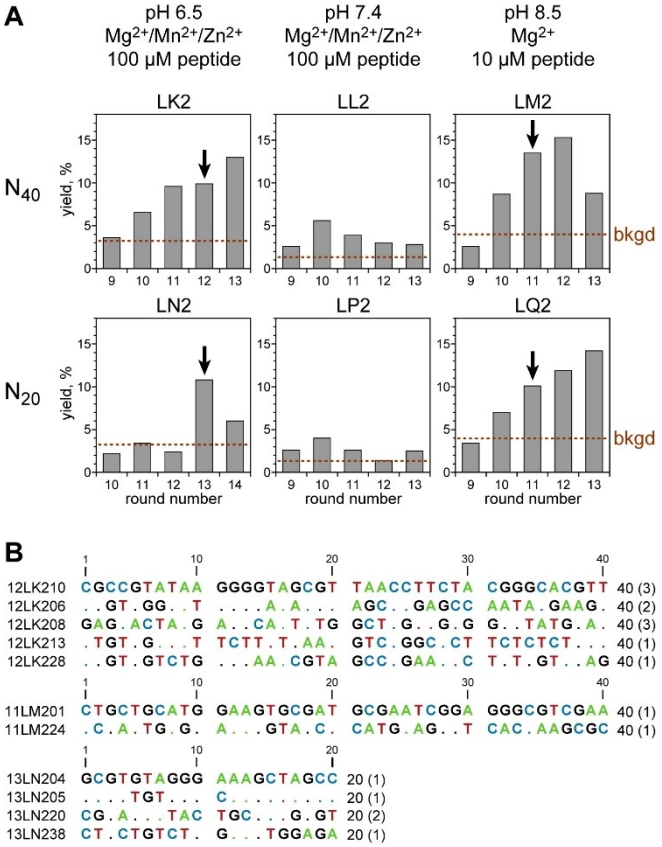


**Figure 3.** Selection progressions and DNAzyme sequences. (A) Progressions of the six in vitro selection experiments. Each selection has an arbitrary alphanumeric designation, LK2 through LQ2, depending on the combination of random region length ($N_{40}$ or $N_{20}$) and incubation conditions during the enrichment step of selection (37 °C, 16 h; pH, metal ions, and peptide concentration as shown; 40 mM $Mg^{2+}$, 20 mM $Mn^{2+}$, 1 mM $Zn^{2+}$). The dashed line labeled "bkgd" marks the uncatalyzed background yield (2–4% in all cases) under the same incubation conditions. Each background yield was obtained in trans, without CuAAC reaction, and is thus ~2× higher than the background yield if CuAAC were performed. The cloned selection rounds are marked with black arrows. Specific cloned rounds were chosen in part in an effort to obtain a greater diversity of DNAzyme sequences (e.g., cloning LK2 at round 12 rather than round 13). (B) Individual DNAzyme sequences. Only the initially random ($N_{40}$ or $N_{20}$) sequences are shown. All DNAzymes were initially assayed as 5′-TCCGTCGCCATCTCTTC-$N_{40}$/$N_{20}$-ATAGTGAGTCGTATTA-3′, with the 3′-truncation site marked in Figure 2 located between the two underlined nucleotides. In each alignment, a dot denotes conservation, i.e., the same nucleotide as in the uppermost sequence within the family. On the far right is shown the sequence length and (in parentheses) the number of times that the particular sequence was found during cloning. No common or conserved motifs were apparent, except 13LN204 and 13LN205 are similar. See Table S3 and Figure S6 for predicted secondary structures.

(Figure S1; there is no difference between $N_{40}$ and $N_{20}$ background). The highest rate enhancement $k_{obs}/k_{bkgd}$ of 72 was found for 12LK210. For the nine DNAzymes identified at pH 6.5, assays using all combinations of the three metal ions revealed that $Mn^{2+}$ alone is sufficient for full catalytic activity, with varying metal ion dependence in other ways (Figure S3). Various other DNAzymes and ribozymes are also active with $Mn^{2+}$ alone.[12,17]

Functional peptide binding was assessed for all 11 new DNAzymes by determining their yields at 16 h for a range of peptide concentrations (Figures 4B and S4A). The midpoint

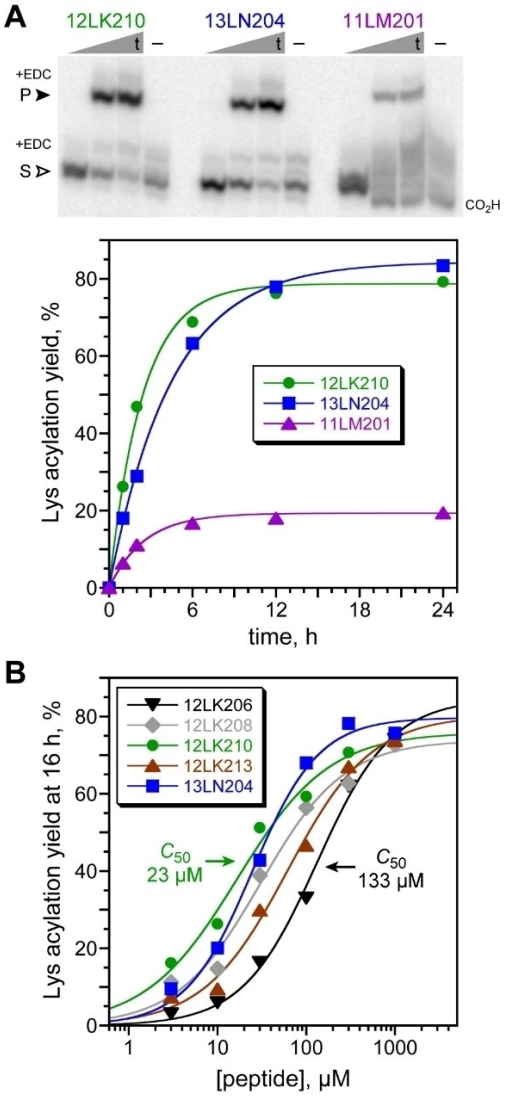


**Figure 4.** Kinetics and peptide $C_{50}$ values for peptide Lys acylation DNAzymes. (A) Representative PAGE data ($t = 30$ s, 6 h, 24 h; 37 °C) and single-turnover kinetic plots for three of the DNAzymes (12LK210 and 13LN204 at pH 6.5 with 100 μM peptide; 11LM201 at pH 8.5 with 10 μM peptide). – = peptide omitted. S = substrate; P = product. For 12LK10 and 13LN204 (pH 6.5), nonspecific EDC adducts of the substrate and product are evident, as marked on the left side of the gel image and confirmed by MALDI-TOF mass spectrometry (Table S2). For 11LM201 (pH 8.5), substantial hydrolysis of the 5′-TFPE to the 5′-$CO_2H$ is evident, as marked on the right side, followed by EDC adduct formation. $k_{obs}$ values ($h^{-1}$, ± half of range, each $n = 2$) and 48 h yields: 12LK206 0.078 ± 0.002, 73%; 12LK208 0.31 ± 0.01, 78%; 12LK210 0.46 ± 0.01, 82%; 12LK213 0.13 ± 0.01, 61%; 12LK228 0.11 ± 0.01, 78%; 11LM201 0.46 ± 0.01, 20%; 11LM224 0.33 ± 0.01, 19%; 13LN204 0.23 ± 0.01, 84%; 13LN205 0.11 ± 0.01, 75%; 13LN220 0.12 ± 0.02, 78%; 13LN238 0.12 ± 0.02, 49%. $k_{bkgd}$ values (Figure S1) were $6.4\times10^{-3}$ $h^{-1}$ and $7.6\times10^{-3}$ $h^{-1}$, respectively, at pH 6.5 and 8.5. Rate enhancements ($k_{obs}/k_{bkgd}$): 12LK206 12; 12LK208 48; 12LK210 72; 12LK213 20; 12LK228 17; 11LM201 61; 11LM224 43; 13LN204 36; 13LN205 17; 13LN220 19; 13LN238 19. For all DNAzymes, high yield was retained when the 6-azidohexanoate group on the hexapeptide was replaced with acetyl. (B) Peptide concentrations at which 50% of maximal yield was observed (the $C_{50}$ values, determined at 16 h). 12LK206 133; 12LK208 31; 12LK210 23 (one individual data plot is shown; 23 ± 5, mean ± sd, $n = 3$) ; 12LK213 62; 12LK228 103; 11LM201 23; 11LM224 37 13LN204 25; 13LN205 84; 13LN220 72; 13LN238 92.

value $C_{50}$, which represents the peptide concentration at which 50% of the maximal yield was observed, ranged from 23–133 μM. Separately, for three DNAzymes (12LK10, 12LK213, and 13LN204), a more formal $K_m$ determination using initial-rate kinetics revealed $K_m > 1$ mM (Figure S4B). Although $K_m \gg C_{50}$, the practical utility of such DNAzymes will depend largely on the $C_{50}$ value. For instance, note in Figure 4A that > 75% yield is observed in 12 h for both 12LK10 and 13LN204 at 100 μM peptide, where this concentration is well above $C_{50}$ for both of these DNAzymes, but far below $K_m$.

We evaluated all 11 new DNAzymes with a tethered version of the hexapeptide substrate, in which this peptide is covalently attached to a DNA oligonucleotide anchor by CuAAC (Figure S5). For two of the DNAzymes, 12LK208 and 12LK210, at most several-fold rate enhancement was evident relative to the DNA-splinted but uncatalyzed background reaction, whereas the other nine DNAzymes did not have any rate enhancement. This outcome reinforces our finding that these new DNAzymes all achieve substantial rate enhancement for catalysis of untethered peptide Lys acylation. For comparison, in the previous study, we found DNAzymes that catalyze tethered peptide Lys acylation relative to the splinted background reaction,[10] but those earlier DNAzymes used the 4FPE and PE acyl donors (Figure 1A) because the splinted background reaction was too fast with TFPE, and those DNAzymes could not detectably modify an untethered peptide.

For four DNAzymes (12LK210, 12LK213, 13LN204, and 11LM201), MALDI-TOF mass spectrometry of the PAGE-isolated product confirmed peptide acylation (Table S2). For all 11 DNAzymes, product cleavage assay by base treatment established > 99% (pH 6.5) and > 97% (pH 8.5) Lys N-acylation rather than Ser O-acylation (Figure 5; see caption for explanation). Mfold analysis[18] led to many predicted secondary structures (Table S3 and Figure S6), although these predictions are of limited utility at the present stage. Three-dimensional structural information for any DNAzymes is currently quite limited,[19] and we have not pursued such analysis here.

Some of our DNAzymes that create Tyr-RNA/DNA conjugates,[16] as well as other DNAzymes from our efforts for Tyr phosphorylation[20] and Tyr adenylylation,[21] have catalytic activity that depends on the peptide sequence. Here we investigated the peptide sequence requirements of the 11 new DNAzymes, in two ways. First, we determined the DNAzyme activities when Lys was located other than at its original K6 position in the parent hexapeptide sequence. We assayed each DNAzyme at 24 h with the peptide that lacks any Lys at all (i.e., the K6S mutant peptide, where S replaces K to retain solubility), along with double mutant peptides in which K6S was accompanied by one of the two original S changed to K (S4K or S2K; Figures 6A and S7A). For all 11 DNAzymes, no acylation yield above background was observed with K6S or K6S/S2K. Ten of the 11 DNAzymes also had no yield with K6S/S4K, although 13LN220 was able to modify K4 with lower yield than for the parent sequence. From these data, we conclude that these DNAzymes have high site selectivity for K6 over the other tested Lys positions within the peptide.

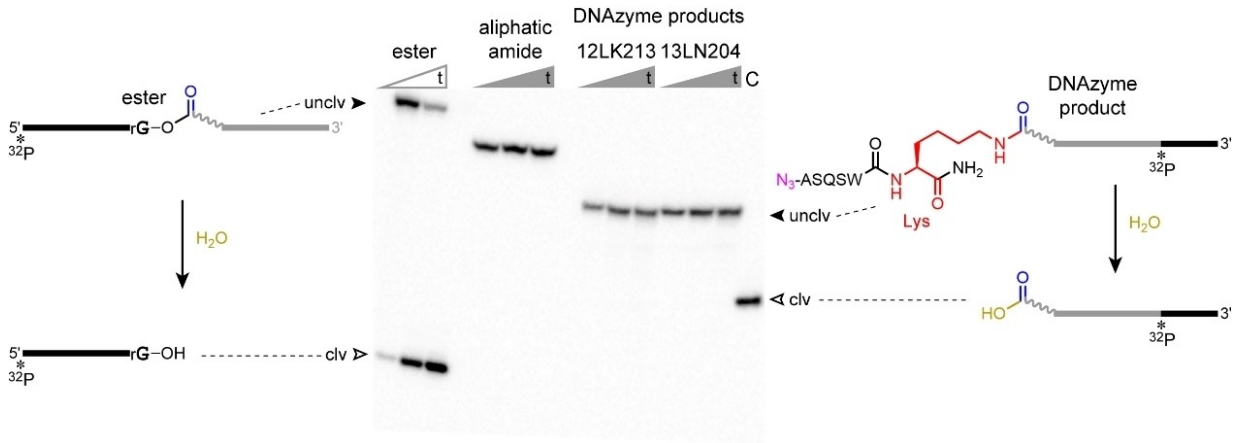


**Figure 5.** Product cleavage assays to establish peptide Lys N-acylation rather than Ser O-acylation by the new DNAzymes. Representative data are shown for the cleavage products (clv) of 12LK213 and 13LN204; the products of all nine other DNAzymes similarly remained uncleaved (unclv). C = 5′-$CO_2H$ oligonucleotide standard. Ester: $t$ = 30 s, 5 min, 2 h. All other samples: $t$ = 30 s, 2 h, 6 h. As a positive control, the ester-linked standard is readily cleaved by base treatment (*left*), whereas an aliphatic amide-linked standard is uncleaved (*center*). The DNAzyme products are also uncleaved (*right*), thus establishing that they are not O-acylation products.

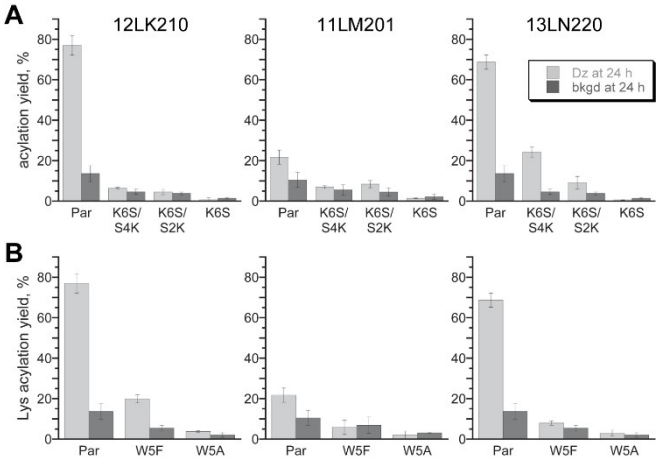


**Figure 6.** Peptide sequence requirements of the DNAzymes. (A) Evaluating site selectivity for Lys within the peptide substrate. (B) Evaluating sequence context requirements by mutating the Trp adjacent to Lys. In both panels, each data bar represents the peptide Lys acylation yield at 24 h, for either the DNAzyme (Dz, light) or the background (dark; DNAzyme replaced with $N_{40}$ pool). Values are mean ± sd ($n$ = 3). Par = parent peptide substrate (ASQSWK). Data for three DNAzymes is shown here; data for the other eight DNAzymes is in Figure S7.

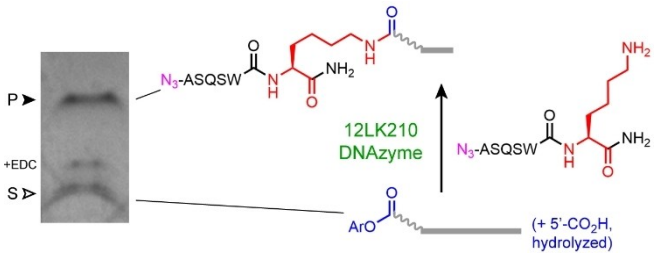


**Figure 7.** Preparative-scale DNAzyme-catalyzed peptide Lys acylation reaction. The 40 μL sample contained 800 pmol of peptide (20 μM), 960 pmol of 12LK10 DNAzyme, and 1120 pmol of 5′-TFPE acyl donor oligonucleotide (ratio of 1.0 : 1.2 : 1.4). Reaction conditions: 105 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $ZnCl_2$ and 150 mM NaCl, with final pH of 6.5, at 37 °C for 44 h. The 20% PAGE image was acquired by UV shadowing, where only oligonucleotides are visible, with the DNAzyme evident higher in the lane (not shown). See Figure 4A for description of EDC adduct (+EDC). After gel extraction, we obtained 238 pmol of acylated peptide (P) and 327 pmol of unreacted acyl donor (S and its EDC adduct), for 30% isolated yield of acylated peptide, 50% gel extraction efficiency, and 60% calculated chemical yield of acylated peptide relative to the limiting amount of peptide starting material.

Second, we determined the activities of the DNAzymes when the Trp residue adjacent to the original Lys was mutated to either Phe or Ala (W5F or W5A peptide; Figures 6B and S7B). Ten of the 11 DNAzymes had no acylation yield with either W5F or W5A, whereas 12LK210 had low yield only with W5F. We conclude that these DNAzymes require Trp adjacent to the Lys, as was present during the in vitro selection process. This requirement suggests a key DNAzyme–substrate interaction involving this Trp, consistent with our initial design consideration.[14,15]

As a final experiment, we performed preparative-scale peptide Lys acylation reactions, now with the untethered peptide as limiting reagent (Figure 7). We used the 12LK210 DNAzyme, which had low $C_{50}$ value of 23 μM in the functional peptide binding assay of Figure 4B, at 20 μM. This DNAzyme was able to provide a high yield of Lys-acylated peptide on the nanomole scale.

## Conclusions

In this study, we achieved our goal of identifying DNAzymes that catalyze sequence-dependent peptide lysine acylation at a practically useful peptide concentration. Only the Lys residue that was present in the peptide during in vitro selection is modified by each new DNAzyme, whereas nearby amino acids are unreactive when mutated to Lys. Also, the catalytic activity of the DNAzymes strongly depends on the amino acids near the Lys. Thus we have

established the ability of DNAzymes both to acylate a peptide substrate that is not covalently tethered to a DNA anchor oligonucleotide and to discriminate site-selectively among Lys residues with amino acid context dependence. This outcome is an important step in our continued development of DNAzymes that modify peptide and proteins.

## Experimental Section

### Oligonucleotide and Peptide Preparative Procedures

DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) or prepared by solid-phase synthesis on an ABI 394 instrument using reagents from Glen Research, including the 5′-$CO_2H$ modifier (5′-carboxy-modifier C10, 10–1935) and the 3′-alkyne-serinol linker (20-2992). Oligonucleotides were purified by 7 M urea denaturing 20% or 8% PAGE with running buffer 1× TBE (89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3), extracted from the polyacrylamide with TEN buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 300 mM NaCl), and precipitated with ethanol. The 17 nt 5′-$CO_2H$ oligonucleotide was purified by HPLC, using a Shimadzu Prominence instrument with a Phenomenex Gemini-NX $C_{18}$ column (5 μm, 10×250 mm), solvent A (20 mM triethylammonium acetate in 50% acetonitrile/50% water, pH 7.0), solvent B (20 mM triethylammonium acetate in water, pH 7.0), gradient of 15% A/85% B at 0 min to 30% A/70% B at 45 min, and flow rate of 3.5 mL/min, with retention time of 23.7 min. Each hexapeptide was prepared by solid-phase synthesis using Fmoc Rink amide MBHA resin as described.[22] 6-Azidohexanoic acid was coupled at the N-terminus as described.[16]

### In Vitro Selection and Cloning Procedures

Full procedures are in the supporting information.

### Single-Turnover DNAzyme Assay Procedure

The general single-turnover assay procedure for each DNAzyme using a 5′-aryl ester oligonucleotide substrate was as follows. Each DNAzyme was assayed as 5′-TCCGTCGCCATCTCTTC-$N_{40/20}$-ATA-3′, where the particular 40 or 20 nt are in Figure 3B. The 5′-$CO_2H$ oligonucleotide was internally radiolabled by splint ligation to a 9 mer that was 5′-$^{32}$P-radiolabeled using γ-$^{32}$P-ATP and T4 polynucleotide kinase (see depiction in Figure 5; see nucleotide details in Figure S2). An 8 μL sample containing 0.5 pmol of internally $^{32}$P-radiolabeled 5′-$CO_2H$ substrate and 5 pmol of DNAzyme was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The 5′-$CO_2H$ oligo was activated at room temperature for 1.5 h in a total volume of 12 μL containing 50 mM TFP (added from a 600 mM stock solution in DMF) and 50 mM EDC (added from a freshly prepared 600 mM stock solution in water). The DNAzyme-catalyzed reaction was initiated by bringing the sample to 20 μL total volume with one of two sets of contents: (a) 100 μM peptide $N_3$-ASQSWK (added from a 1 mM stock in water), 105 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $ZnCl_2$ and 150 mM NaCl, with 30 mM each EDC/TFP and final pH of 6.5; or (b) 10 μM peptide $N_3$-ASQSWK (added from a 100 μM stock in water), 240 mM AMPSO, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl, with 30 mM each EDC/TFP and final pH of 8.5. The $Mg^{2+}$ and $Mn^{2+}$ were added from 10× stock solutions containing 400 mM $MgCl_2$ and 200 mM $MnCl_2$. The $Zn^{2+}$ was added from a 10× stock solution containing 10 mM $ZnCl_2$, 20 mM $HNO_3$, and 200 mM HEPES at pH 7.5; this stock solution was freshly prepared from a 100× stock of 100 mM $ZnCl_2$ in 200 mM $HNO_3$. For samples at pH 6.5, 20 mM of the HEPES was from the $ZnCl_2$ stock solution. The metal ion stocks and then the peptide were added last to the final sample, which was incubated at 37 °C. At each time point, a 2 μL aliquot was quenched with 7 μL of stop solution (80% formamide, 1× TBE [89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3], 50 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol). Quenched samples were separated by 20% PAGE and quantified using a Phosphorimager. Values of $k_{obs}$ were obtained by fitting the yield versus time data directly to first-order kinetics; i.e., yield $= Y^{\bullet}(1-e^{-kt})$, where $k = k_{obs}$ and $Y$ = final yield. Each $k_{obs}$ value is reported with error calculated as the half of the range from two independent determinations. When $k_{obs}$ was sufficiently low such that an exponential fit was not meaningful (background reactions and peptide $K_m$ determinations), the initial points were fit to a straight line, and $k_{obs}$ was taken as the slope of the line. For the plots in Figures 4B and S4, the data were fit to yield $= Y^{\bullet}$[peptide]$^n$/([peptide]$^n + C^n$)), where $n$ = Hill coefficient, $C = C_{50}$ or $K_m$ as appropriate, and $Y$ = final yield. All data were plotted and all curves fit in KaleidaGraph.

### Preparative-Scale Peptide Lys Acylation Procedure

Following the single-turnover DNAzyme assay procedure, the reaction sample of Figure 7 included 800 pmol (20 μM) of $N_3$-ASQSWK peptide, 960 pmol of 12LK210 DNAzyme, and 1120 pmol of 5′-TFPE acyl donor oligonucleotide (ratio of 1.0 : 1.2 : 1.4), with annealing volume of 16 μL, activation volume of 24 μL, and final volume of 40 μL. The sample was incubated at 37 °C for 44 h, separated by 20% PAGE, and visualized by UV shadowing.

### Mass Spectrometry of DNAzyme Products

The products from representative DNAzymes were analyzed by MALDI-TOF mass spectrometry (see Table S2 for data). Following the single-turnover DNAzyme assay procedure, each reaction sample included 1 nmol of DNA substrate, 1.5 nmol of DNAzyme, and 100 μM or 10 μM peptide $N_3$-ASQSWK, with annealing volume of 16 μL, activation volume of 24 μL, and final incubation volume of 40 μL. The sample was incubated at 37 °C for 44 h, separated by 20% PAGE, and desalted by Millipore $C_{18}$ ZipTip. Data were acquired on a Bruker UltrafleXtreme MALDI-TOF mass spectrometer with matrix 3-hydroxypicolinic acid in positive ion mode at the UIUC School of Chemical Sciences Mass Spectrometry Laboratory. All $m/z$ values are for $[M+H]^+$.

### Product Cleavage Assay by Base Treatment

Each DNAzyme product was formed by the single-turnover DNAzyme assay procedure, using 3 pmol of internally $^{32}$P-radiolabeled 5′-$CO_2H$ oligonucleotide and 10 pmol of DNAzyme, with annealing volume of 8 μL, activation volume of 12 μL, and final incubation volume of 20 μL for 48 h, then separated by 20% PAGE. The ester standard was formed by DNA-splinted reaction using 5 pmol of 5′-$^{32}$P-radiolabeled 3′-rG DNA substrate, 10 pmol of DNA splint, and 20 pmol of 5′-$CO_2H$ oligonucleotide, using the procedure for DNAzyme-catalyzed and splinted background reactions as described.[11] The aliphatic amide standard was formed by DNA-splinted reaction using 5 pmol of 5′-$^{32}$P-radiolabeled 3′-$C_3$-$NH_2$ DNA substrate, 10 pmol

of DNA splint, and 20 pmol of 5′-$CO_2H$ oligonucleotide, using the amide coupling procedure as described.[23] For the product cleavage assay, 0.5 pmol of the DNAzyme product or standard and 10 pmol of 60 nt $(AAC)_{20}$ as carrier oligonucleotide were brought to 40 μL total volume containing 10 mM NaOH (added from a 100 mM stock solution), for final pH of 12.0, and incubated at 55 °C. At each time point, a 2 μL aliquot was quenched with 7 μL of stop solution containing 300 mM sodium acetate, pH 5.2. Samples were separated by 20% PAGE.

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## Conflict of Interests

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

**Keywords:** Deoxyribozymes · In vitro selection · Oligonucleotides · Peptides · Ribozymes

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