Peptide Modification

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DNA-Catalyzed Introduction of Azide at Tyrosine for Peptide Modification

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Abstract: *We show that DNA enzymes (deoxyribozymes) can introduce azide functional groups at tyrosine residues in peptide substrates. Using in vitro selection, we identified deoxyribozymes that transfer the 2′-azido-2′-deoxyadenosine 5′-monophosphoryl group (2′-Az-dAMP) from the analogous 5′-triphosphate (2′-Az-dATP) onto the tyrosine hydroxyl group of a peptide, which is either tethered to a DNA anchor or free. Some of the new deoxyribozymes are general with regard to the amino acid residues surrounding the tyrosine, while other DNA enzymes are sequence-selective. We use one of the new deoxyribozymes to modify free peptide substrates by attaching PEG moieties and fluorescent labels.*

Synthesis of modified peptides and proteins is essential for understanding the functions of natural post-translational modifications, monitoring protein distributions in vivo, modulating therapeutic properties of peptides, proteins, antibodies, and antibody-drug conjugates, and other applications.[1] In nature, tyrosine is modified via a wide array of chemical changes.[2] Tyrosine has often been targeted for synthetic modification on the basis of its electron-rich nature and typically low abundance on protein surfaces. Approaches for tyrosine modification include reaction with electron-deficient diazonium salts,[3] three-component Mannich-type reaction with aldehydes and anilines,[4] alkylation with π-allylpalladium complexes,[5] reaction with preformed imines,[6] and aqueous ene-type reaction with PTAD derivatives.[7] Oxidative couplings have also been explored,[8] as have enzyme cascades.[9] However, these approaches each rely solely on differential physical accessibilities to discriminate among several tyrosines in the substrate. Some tyrosine modification reactions suffer from off-target reactivity of other electron-rich residues such as tryptophan.[4c,7,8] Therefore, alternative approaches are needed for sequence-selective tyrosine modification.

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We pursued a two-step approach to tyrosine modification using deoxyribozymes (DNA enzymes; Figure 1 a), which are

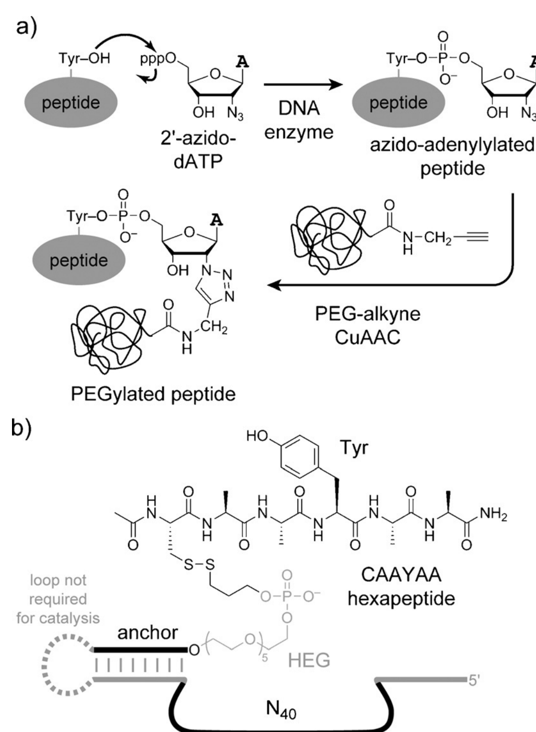

Figure 1. Overview of DNA-catalyzed introduction of azide at tyrosine residues. a) Two-step peptide modification by DNA-catalyzed azido-adenylylation and CuAAC, with PEGylation as the specific example. b) Arrangement of initially random DNA pool (N_{40}) and peptide substrate for in vitro selection of deoxyribozymes for azido-adenylylation. In each selection round, PAGE-shift capture of active DNA sequences is achieved by CuAAC with PEG_{5k}-alkyne. See the Supporting Information, Figure S1 for nucleotide details of the selection process. The dashed loop enables selection but is dispensable for catalysis.

particular DNA sequences that have catalytic activity.[10] In our approach, a deoxyribozyme first catalyzes the reaction between the tyrosine side chain and 2′-azido-2′-deoxyadenosine 5′-triphosphate, or 2′-Az-dATP, to form a phosphodiester bond (azido-adenylylation). Second, a particular modification of interest is attached to the azido group by copper-catalyzed azide–alkyne cycloaddition (CuAAC)[1h,11] using an alkyne-functionalized reagent.

We initially performed in vitro selection[12] to establish viability of this approach, using the hexapeptide CAAYAA as the substrate (Figure 1 b). This hexapeptide was covalently attached via a disulfide linkage and a hexa(ethylene glycol) [HEG] tether to a DNA anchor oligonucleotide, which was

bound by Watson–Crick base pairs to one of the fixed-sequence binding arms of the random N_{40} DNA pool. After exposure of the substrate-conjugated N_{40} pool to 2′-Az-dATP, the catalytically active DNA sequences, which were now attached to an azido group, were increased in mass by CuAAC with alkyne-modified poly(ethylene glycol) that has an average molecular weight of 5000 (PEG_{5k}-alkyne). PEGylation is an important protein modification that increases renal clearance time, suppresses aggregation, increases solubility, reduces immunogenicity and toxicity, and improves in vivo stability.[13] For catalytically active DNA sequences, this "capture step"[10i] attachment of PEG induces a sufficiently large polyacrylamide gel electrophoresis (PAGE) shift that enables the selection process. After PAGE separation, treatment with dithiothreitol (DTT) cleaved the disulfide bond, and another PAGE separation was performed. If the PEG-modified 2′-Az-dAMP group is attached not at the tyrosine residue but instead at some undesired position within the DNA itself (for example, the 5′-hydroxyl group or a nucleobase functional group), then disulfide cleavage will lead to only a small PAGE shift, in comparison with the much larger PAGE shift upon removal of the entire PEG-modified hexapeptide. This additional DTT/PAGE operation proved necessary to avoid emergence of DNA sequences that catalyze undesired reactions (data not shown).

In each selection round, the incubation conditions included 100 μM 2′-Az-dATP in 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C for 14 h (the divalent metal ions were included because each has proven useful in past DNA enzyme selection experiments). After 8 rounds, the azido-adenylylation activity of the pool reached 16 % (Supporting Information, Figure S2 a), and individual deoxyribozymes were cloned. A single sequence, named DzAz1 (Supporting Information, Figure S3), was identified. Partial randomization and reselection of DzAz1 (Supporting Information, Figure S2 b) revealed that the 5′-half sequence of the N_{40} region could be varied extensively, whereas the 3′-half sequence was highly conserved (Supporting Information, Figure S3). With the DNA-anchored CAAYAA substrate, the reselected variant DzAz1b (11 mutations in the variable region) has 58 % single-turnover azido-adenylylation yield with k_{obs} of $0.38 \pm 0.03\ h^{-1}$, and variant DzAz1c (10 mutations) has 56 % yield with k_{obs} of $0.19 \pm 0.03\ h^{-1}$; parent DzAz1 has 40 % yield with k_{obs} of $0.79 \pm 0.07\ h^{-1}$ (each $n = 3$, ± sd; Figure 2). Product identity was validated by MALDI mass spectrometry (Supporting Information, Table S2). Both Zn^{2+} and one of Mn^{2+} or Mg^{2+} are required for catalysis; Mn^{2+} is much more effective (Supporting Information, Figure S4). DzAz1 retains substantial activity when natural ATP is used in place of 2′-Az-dATP (Figure 2, k_{obs} $0.3\ h^{-1}$ for DzAz1 and both reselected variants), suggesting utility of this general approach for studying natural tyrosine adenylylation (AMPylation).[2,14]

With this proof-of-principle validation in hand, we sought to modify mixed-sequence peptides that have a variety of amino acids near the tyrosine. In two recent efforts with tyrosine-modifying deoxyribozymes, we found that peptide sequence-selective catalysis by DNA can be found from selection experiments in which mixed-sequence peptides are the substrates.[15] Here, four new in vitro selection experiments were performed, each using one of four specific DNA-anchored octapeptides CLQTYPRT, CQQPYITN, CERSYLMK, or CFQPYMQE. The first sequence comprises amino acids 19–25 from the 32-mer salmon calcitonin (sCT),[16] and the remaining three sequences correspond, respectively, to amino acids 15–21, 54–60 (with C56S), and 78–84 of human interleukin-22 (146-mer hIL-22, amino acids 34–179 of the genomic sequence);[17] an artificial N-terminal cysteine was appended onto each peptide to allow disulfide linkage to the DNA anchor oligonucleotide. sCT is a hormone prescribed for bone-related disorders such as osteoporosis, Paget's disease, and hypercalcemia; hIL-22 is a cytokine involved in proliferation, host defense, and the inflammatory response. Each selection experiment was performed using the same incubation conditions as for the DNA-anchored CAAYAA. After 8–12 rounds, each selection experiment showed 16–44 % pool azido-adenylylation activity (Supporting Information, Figure S2 c–f), and individual deoxyribozymes were cloned.

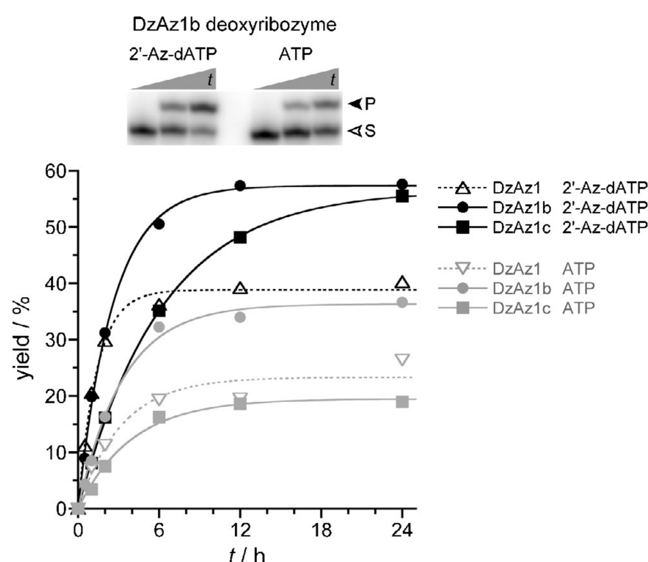

Figure 2. Single-turnover kinetic assays of the DzAz1 deoxyribozyme, which was identified by in vitro selection using the DNA-anchored hexapeptide CAAYAA, and reselected variants DzAz1b and DzAz1c. Incubation conditions: 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C with 100 μM 2′-Az-dATP or natural ATP. In the PAGE image, representative time points are shown for DzAz1b at $t = 0$, 2, and 24 h. S = substrate, P = product. See the Supporting Information, Figure S3 for deoxyribozyme sequences, Table S2 for MALDI mass spectrometry, Figure S4 for assays of divalent metal ion dependence, and Figure S5 for assays with mixed-sequence peptide substrates.

Five unique deoxyribozyme sequences designated DzAz2 through DzAz6 were identified from the selection experiment with the sCT-derived octapeptide CLQTYPRT, while the other three selections with the hIL-22 peptides each led to a single DNA sequence, respectively named DzAz7, DzAz8, and DzAz9 (Supporting Information, Figure S3). The eight new DNA enzymes share no obvious sequence conservation among themselves or with DzAz1. DzAz2, DzAz7, and DzAz8 catalyze azido-adenylylation of their corresponding

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peptides in 61–87% yield in 24 h with k_{obs} 0.1–0.5 h^{-1} (Figure 3a); the other deoxyribozymes have lower 9–27% yields (Supporting Information, Figure S6a). Natural ATP is generally tolerated well in place of 2′-Az-dATP (Supporting Information, Figure S6b).

All nine of DzAz1 through DzAz9 were evaluated for azido-adenylylation activity with each of the four mixed-sequence DNA-anchored peptides (Figure 3b; Supporting Information, Figure S5). DzAz8 did not discriminate among any of the peptide sequences. In sharp contrast, DzAz2 functioned well only with the CLQTYPRT substrate that was used during its identification. The other seven deoxyribozymes each exhibited partial discrimination among the four peptide sequences, as exemplified by DzAz7.

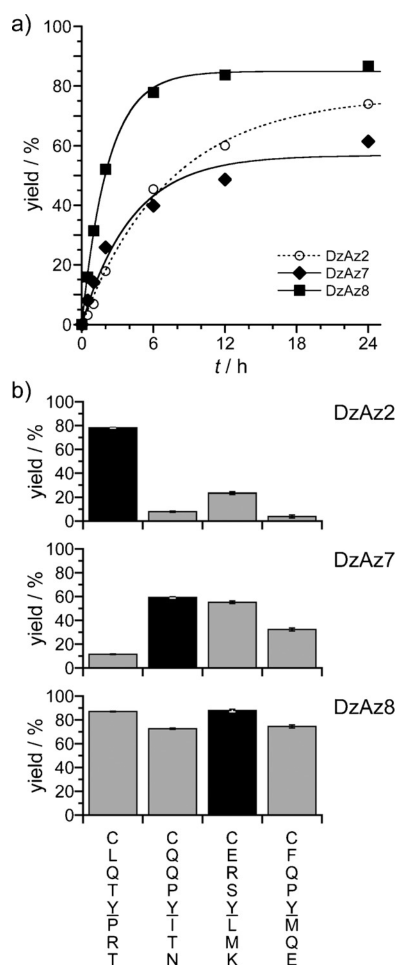

Figure 3. Assays of the DzAz2 through DzAz9 deoxyribozymes, which were identified by in vitro selection using DNA-anchored octapeptides of mixed amino acid sequences. Incubation conditions as in Figure 2. a) Single-turnover kinetic assays with the particular peptide substrate used during identification of each deoxyribozyme. Data are shown for DzAz2, 7, and 8 (k_{obs} 0.14, 0.24, and 0.46 h^{-1}). See the Supporting Information, Figure S6a for data with the other deoxyribozymes. b) Assays with all four peptide sequences (yield at 24 h; each $n=3$, ± sd); see Figure S5 for data with the other deoxyribozymes. Black bars denote the particular peptide substrate used during identification of that deoxyribozyme; gray bars are for the other peptide substrates. See the Supporting Information, Figure S3 for deoxyribozyme sequences and Figure S6b for assays with natural ATP in place of 2′-Az-dATP.

The peptide sequence dependence of DzAz2 was evaluated in greater detail by testing its azido-adenylylation activity with systematic mutants of the DNA-anchored CLQTYPRT substrate. As expected, mutation of the Tyr to either Phe or Ser abolished activity (Supporting Information, Figure S7). The six amino acids surrounding the Tyr were then individually replaced with Ala, revealing that the required motif is YPR (Figure 4). Introduction of this YPR motif into

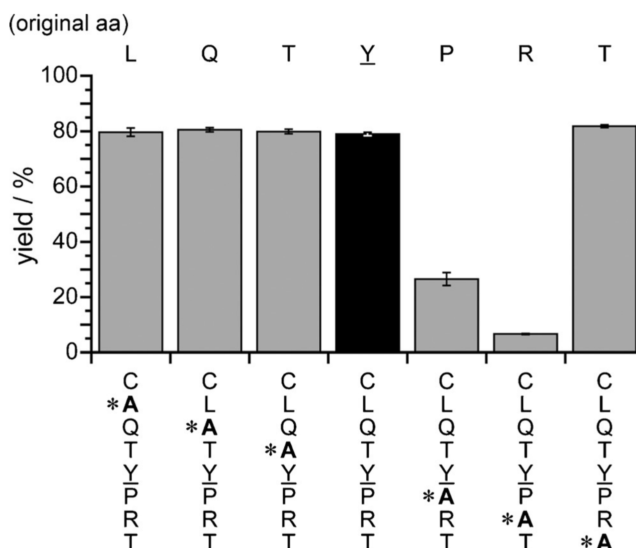

Figure 4. Peptide sequence dependence of DzAz2. Azido-adenylylation yields at 24 h are shown, each with a series of DNA-anchored peptide substrates in which a single amino acid was mutated to Ala ($n=3$, ± sd). See Figure S7 for mutation of Tyr to Phe or Ser, which abolished activity.

the other three peptide substrates enabled their azido-adenylylation by DzAz2 (Supporting Information, Figure S8), establishing YPR as both necessary and sufficient for DzAz2 reactivity. DzAz2 was also able to discriminate between two Tyr within one longer peptide substrate on the basis of their sequence contexts (Figure 5). In contrast, although the activity of the non-selective DzAz8 deoxyribozyme was also abolished upon mutating Tyr to Ser (as expected), all Ala mutations of CLQTYPRT were tolerated well (60–90% yield; Supporting Information, Figure S9).

Deoxyribozymes such as DzAz8 will be most useful if they can function with free peptide substrates that are not tethered to a DNA anchor oligonucleotide. Azido-adenylylation of untethered sCT was achieved by DzAz8 in 14% yield in 24 h (Figure 6a). In contrast, the sCT-specific DzAz2 did not function with untethered sCT ($<1\%$; data not shown). DzAz8 was also assayed with the 28-mer N-terminal fragment (C-terminal Tyr) of atrial natriuretic peptide (atriopeptin, ANP), which can induce natriuresis (sodium excretion in urine) and vasodilation and is commercialized in Japan for treatment of heart failure.[18] Azido-adenylylation of ANP by DzAz8 in 59% yield in 24 h was observed (Figure 6a). Both sCT and ANP were also adenylylated with natural ATP lacking the 2′-azido group, in respective yields of 6.7% and 16% (Supporting Information, Figure S10). The HPLC-

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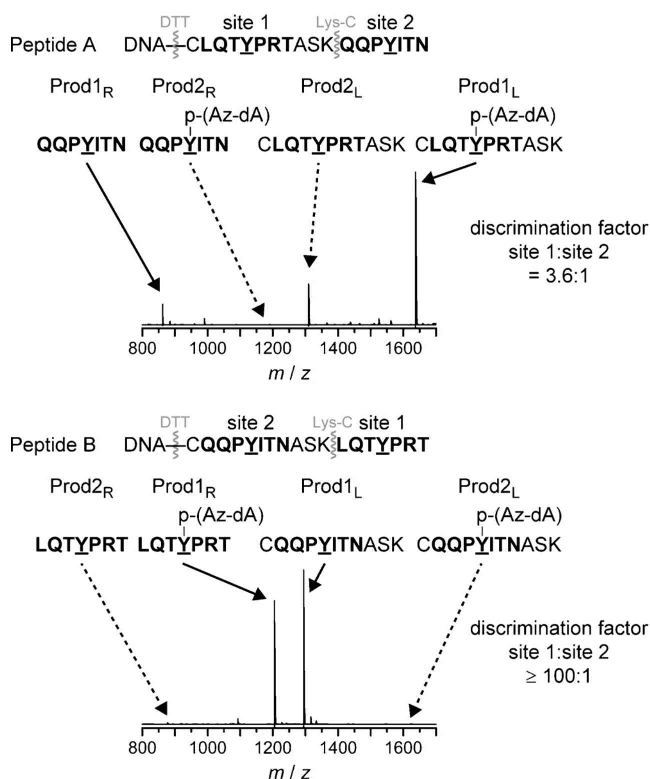

Figure 5. DzAz2 differentiates between two Tyr in one peptide substrate. The LQTYPRT (site 1) and QQPYITN (site 2) sequence motifs (where DzAz2 is selective for the first motif; see Figure 3) were concatenated in either order within a longer peptide substrate via an arbitrary linker (ASK). The DNA-anchored peptide substrate was azido-adenylylated by DzAz2, and the product (as a mixture of site 1 and site 2 modifications) was PAGE-separated. In each case, DzAz2 preferentially modified LQTYPRT (site 1), as revealed by MALDI mass spectrometry after Lys-C cleavage to separate the two peptide segments and DTT treatment to remove the DNA anchor oligonucleotide. Each peptide product fragment is labeled Prod1 or Prod2 corresponding to site 1 or site 2 modification, and with a subscripted L or R corresponding to the fragment location on the left or right side of the longer peptide before Lys-C cleavage. See the Supporting Information, Table S2 for MS data and Supporting Information text for procedure and calculation of discrimination factors from the mass spectrometry data.

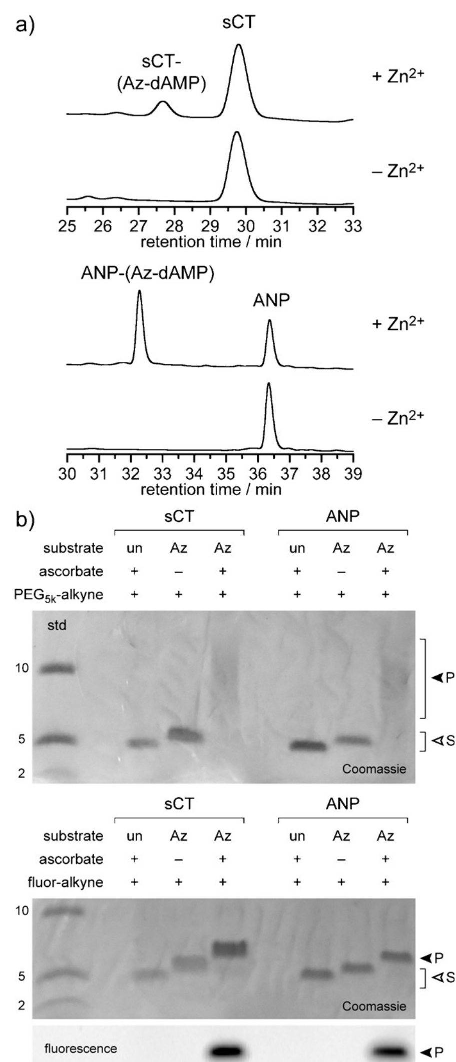

Figure 6. Azido-adenylylation and subsequent PEG or fluorescein modification of untethered (free) peptide substrates by DzAz8. a) Azido-adenylylation by DzAz8 of sCT and ANP, assayed by HPLC ($t=24$ h). b) Modification via CuAAC of azido-adenylylated sCT and ANP with PEG_{5k}-alkyne and fluorescein-alkyne, assayed by SDS-PAGE and imaging by Coomassie stain or fluorescence. un = unmodified substrate, Az = azido-adenylylated substrate, S = substrate, P = product, std = 2, 5, 10 kDa. Ascorbate is the reducing agent required for CuAAC. sCT (32 aa) CSNLSTCVLGKLSQELHKLQTYPRTNTGSGTP; ANP (28 aa) SLRRSSCFGGRMDRIGAQSGLGCNSFRY (disulfide bridge between the two C within both peptides).

purified azido-adenylylated sCT and ANP were further derivatized quantitatively via CuAAC using either PEG-alkyne or fluorescein-alkyne (Figure 6b), with product validation by MALDI mass spectrometry (Supporting Information, Table S2).

In summary, we have identified DNA enzymes for azido-adenylylation of tyrosine residues in peptide substrates. One such deoxyribozyme, DzAz2, is selective for the YPR sequence motif and is able to discriminate between tyrosine residues within a single peptide on the basis of sequence context. Another DNA enzyme, DzAz8, is peptide sequence-general, functions with free peptides, and allows their subsequent CuAAC labeling with moieties such as PEG and fluorescein. Finding peptide sequence selectivity by deoxyribozymes such as DzAz2 adds to the growing list of such observations that have now been made for three different DNA-catalyzed reactions,[15] establishing that DNA enzymes have the broader ability to interact with side chains of peptide substrates. In ongoing work, we are seeking to combine the key features of peptide sequence selectivity (for example, DzAz2) and reactivity with free peptide substrates (for example, DzAz8). We are also seeking to extend DNA-catalyzed reactivity from peptides to larger protein substrates.[19]

Keywords: deoxyribozymes · DNA · in vitro selection · peptide modification · peptides

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[1] a) J. Kalia, R. T. Raines, *Curr. Org. Chem.* **2010**, *14*, 138–147; b) N. Stephanopoulos, M. B. Francis, *Nat. Chem. Biol.* **2011**, *7*, 876–884; c) L. Davis, J. W. Chin, *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 168–182; d) M. Rashidian, J. K. Dozier, M. D. Distefano, *Bioconjugate Chem.* **2013**, *24*, 1277–1294; e) M. F. Debets, J. C. M. van Hest, F. P. J. T. Rutjes, *Org. Biomol. Chem.* **2013**, *11*, 6439–6455; f) Y. Takaoka, A. Ojida, I. Hamachi, *Angew. Chem. Int. Ed.* **2013**, *52*, 4088–4106; *Angew. Chem.* **2013**, *125*, 4182–4200; g) C. D. Spicer, B. G. Davis, *Nat. Commun.* **2014**, *5*, 4740; h) C. S. McKay, M. G. Finn, *Chem. Biol.* **2014**, *21*, 1075–1101; i) E. V. Vinogradova, C. Zhang, A. M. Spokoyny, B. L. Pentelute, S. L. Buchwald, *Nature* **2015**, *526*, 687–691; j) O. Boutureira, G. J. L. Bernardes, *Chem. Rev.* **2015**, *115*, 2174–2195; k) N. Krall, F. P. da Cruz, O. Boutureira, G. J. L. Bernardes, *Nat. Chem.* **2016**, *8*, 103–113; l) V. Chudasama, A. Maruani, S. Caddick, *Nat. Chem.* **2016**, *8*, 114–119; m) C. Zhang, M. Welborn, T. Zhu, N. J. Yang, M. S. Santos, T. Van Voorhis, B. L. Pentelute, *Nat. Chem.* **2016**, *8*, 120–128; n) S. Bondalapati, M. Jbara, A. Brik, *Nat. Chem.* **2016**, *8*, 407–418; o) P. Akkapeddi, S.-A. Azizi, A. M. Freedy, P. M. S. D. Cal, P. M. P. Gois, G. J. L. Bernardes, *Chem. Sci.* **2016**, *7*, 2954–2963.

[2] L. H. Jones, A. Narayanan, E. C. Hett, *Mol. BioSyst.* **2014**, *10*, 952–969.

[3] J. M. Hooker, E. W. Kovacs, M. B. Francis, *J. Am. Chem. Soc.* **2004**, *126*, 3718–3719.

[4] a) N. S. Joshi, L. R. Whitaker, M. B. Francis, *J. Am. Chem. Soc.* **2004**, *126*, 15942–15943; b) D. W. Romanini, M. B. Francis, *Bioconjugate Chem.* **2008**, *19*, 153–157; c) J. M. McFarland, N. S. Joshi, M. B. Francis, *J. Am. Chem. Soc.* **2008**, *130*, 7639–7644.

[5] S. D. Tilley, M. B. Francis, *J. Am. Chem. Soc.* **2006**, *128*, 1080–1081.

[6] H.-M. Guo, M. Minakawa, L. Ueno, F. Tanaka, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1210–1213.

[7] H. Ban, J. Gavrilyuk, C. F. Barbas III, *J. Am. Chem. Soc.* **2010**, *132*, 1523–1525.

[8] a) K. L. Seim, A. C. Obermeyer, M. B. Francis, *J. Am. Chem. Soc.* **2011**, *133*, 16970–16976; b) A. M. ElSohly, M. B. Francis, *Acc. Chem. Res.* **2015**, *48*, 1971–1978.

[9] A.-W. Struck, M. R. Bennett, S. A. Shepherd, B. J. C. Law, Y. Zhuo, L. S. Wong, J. Micklefield, *J. Am. Chem. Soc.* **2016**, *138*, 3038–3045.

[10] a) R. R. Breaker, G. F. Joyce, *Chem. Biol.* **1994**, *1*, 223–229; b) A. Peracchi, *ChemBioChem* **2005**, *6*, 1316–1322; c) S. K. Silverman, *Chem. Commun.* **2008**, 3467–3485; d) K. Schlosser, Y. Li, *Chem. Biol.* **2009**, *16*, 311–322; e) S. K. Silverman, *Acc. Chem. Res.* **2009**, *42*, 1521–1531; f) S. K. Silverman, *Angew. Chem. Int. Ed.* **2010**, *49*, 7180–7201; *Angew. Chem.* **2010**, *122*, 7336–7359; g) J. Liu, Z. Cao, Y. Lu, *Chem. Rev.* **2009**, *109*, 1948–1998; h) X. B. Zhang, R. M. Kong, Y. Lu, *Annu. Rev. Anal. Chem.* **2011**, *4*, 105–128; i) S. K. Silverman, *Acc. Chem. Res.* **2015**, *48*, 1369–1379; j) M. Hollenstein, *Molecules* **2015**, *20*, 20777–20804.

[11] a) Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* **2003**, *125*, 3192–3193; b) V. Hong, S. I. Presolski, C. Ma, M. G. Finn, *Angew. Chem. Int. Ed.* **2009**, *48*, 9879–9883; *Angew. Chem.* **2009**, *121*, 10063–10067; c) A. A. Ahmad Fuaad, F. Azmi, M. Skwarczynski, I. Toth, *Molecules* **2013**, *18*, 13148–13174.

[12] a) C. Tuerk, L. Gold, *Science* **1990**, *249*, 505–510; b) A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818–822; c) D. L. Robertson, G. F. Joyce, *Nature* **1990**, *344*, 467–468; d) G. F. Joyce, *Annu. Rev. Biochem.* **2004**, *73*, 791–836; e) G. F. Joyce, *Angew. Chem. Int. Ed.* **2007**, *46*, 6420–6436; *Angew. Chem.* **2007**, *119*, 6540–6557.

[13] a) M. J. Roberts, M. D. Bentley, J. M. Harris, *Adv. Drug Delivery Rev.* **2002**, *54*, 459–476; b) A. P. Chapman, *Adv. Drug Delivery Rev.* **2002**, *54*, 531–545; c) M. Werle, A. Bernkop-Schnürch, *Amino Acids* **2006**, *30*, 351–367; d) P. Bailon, C. Y. Won, *Expert Opin. Drug Delivery* **2009**, *6*, 1–16; e) G. Pasut, F. M. Veronese, *J. Controlled Release* **2012**, *161*, 461–472; f) N. Nischan, C. P. Hackenberger, *J. Org. Chem.* **2014**, *79*, 10727–10733.

[14] a) C. A. Worby, S. Mattoo, R. P. Kruger, L. B. Corbeil, A. Koller, J. C. Mendez, B. Zekarias, C. Lazar, J. E. Dixon, *Mol. Cell* **2009**, *34*, 93–103; b) A. Itzen, W. Blankenfeldt, R. S. Goody, *Trends Biochem. Sci.* **2011**, *36*, 221–228; c) M. Grammel, P. Luong, K. Orth, H. C. Hang, *J. Am. Chem. Soc.* **2011**, *133*, 17103–17105; d) C. Smit, J. Blumer, M. F. Eerland, M. F. Albers, M. P. Muller, R. S. Goody, A. Itzen, C. Hedberg, *Angew. Chem. Int. Ed.* **2011**, *50*, 9200–9204; *Angew. Chem.* **2011**, *123*, 9367–9371; e) M. Broncel, R. A. Serwa, E. W. Tate, *ChemBioChem* **2012**, *13*, 183–185; f) M. P. Müller, M. F. Albers, A. Itzen, C. Hedberg, *ChemBioChem* **2014**, *15*, 19–26; g) D. M. Lewallen, A. Sreelatha, V. Dharmarajan, F. Madoux, P. Chase, P. R. Griffin, K. Orth, P. Hodder, P. R. Thompson, *ACS Chem. Biol.* **2014**, *9*, 433–442; h) C. Hedberg, A. Itzen, *ACS Chem. Biol.* **2015**, *10*, 12–21.

[15] a) C. Chu, O. Wong, S. K. Silverman, *ChemBioChem* **2014**, *15*, 1905–1910; b) S. M. Walsh, S. N. Konecki, S. K. Silverman, *J. Mol. Evol.* **2015**, *81*, 218–224.

[16] a) R. M. Epand, R. F. Epand, A. R. Stafford, R. C. Orlowski, *J. Med. Chem.* **1988**, *31*, 1595–1598; b) C. H. Chesnut 3rd, M. Azria, S. Silverman, M. Engelhardt, M. Olson, L. Mindeholm, *Osteoporosis Int.* **2008**, *19*, 479–491; c) M. W. Jones, G. Mantovani, C. A. Blindauer, S. M. Ryan, X. Wang, D. J. Brayden, D. M. Haddleton, *J. Am. Chem. Soc.* **2012**, *134*, 7406–7413.

[17] a) R. A. P. Nagem, D. Colau, L. Dumoutier, J.-C. Renauld, C. Ogata, I. Polikarpov, *Structure* **2002**, *10*, 1051–1062; b) C. Eidenschenk, S. Rutz, O. Liesenfeld, W. Ouyang, *Curr. Top. Microbiol. Immunol.* **2014**, *380*, 213–236; c) J. A. Dudakov, A. M. Hanash, M. R. M. van den Brink, *Annu. Rev. Immunol.* **2015**, *33*, 747–785.

[18] D. L. Vesely, *Clin. Exp. Pharmacol. Physiol.* **2006**, *33*, 169–176.

[19] C. Chu, S. K. Silverman, *Org. Biomol. Chem.* **2016**, *14*, 4697–4703.

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