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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Ribosomal Synthesis of Peptides with Multiple β-Amino Acids

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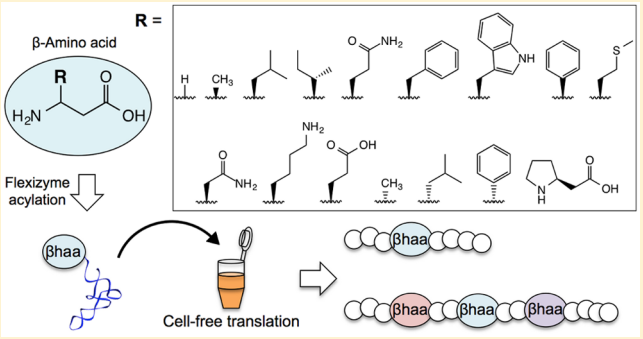
Supporting Information

ABSTRACT: The compatibility of β-amino acids with ribosomal translation was studied for decades, but it has been still unclear whether the ribosome can accept various β-amino acids, and whether the ribosome can introduce multiple β-amino acids in a peptide. In the present study, by using the *Escherichia coli* reconstituted cell-free translation system with a reprogramed genetic code, we screened β-amino acids that give high single incorporation efficiency and used them to synthesize peptides containing multiple β-amino acids. The experiments of single β-amino acid incorporation into a peptide revealed that 13 β-amino acids are compatible with ribosomal translation. Six of the tested β-amino acids (βhGly, L-βhAla, L-βhGln, L-βhPhg, L-βhMet, and D-βhPhg) showed high incorporation efficiencies, and seven (L-βhLeu, L-βhIle, L-βhAsn, L-βhPhe, L-βhLys, D-βhAla, and D-βhLeu) showed moderate incorporation efficiencies; whereas no full-length peptide was produced using other β-amino acids (L-βhPro, L-βhTrp, and L-βhGlu). Subsequent double-incorporation experiments using β-amino acids with high single incorporation efficiency revealed that elongation of peptides with successive β-amino acids is prohibited. Efficiency of the double-incorporation of the β-amino acids was restored by the insertion of Tyr or Ile between the two β-amino acids. On the basis of these experiments, we also designed mRNA sequences of peptides, and demonstrated the ribosomal synthesis of peptides containing different types of β-amino acids at multiple positions.



INTRODUCTION

It has been found that the ribosome has a remarkable tolerance for various nonproteinogenic amino acids, although natural translation system commonly employs 20 proteinogenic α-amino acids as substrates. The ribosomal tolerance toward backbone-modified amino acids,[1] such as *N*-methyl amino acids and D-amino acids, has been studied,[2−16] but the results of these studies were partially inconsistent presumably because of the insufficient optimization of the cell-free translation systems to study the tolerance of ribosome. In the previous report,[17] we successfully applied our optimized translation system to reveal D-amino acids compatibility with ribosomal translation, and found various D-amino acids that could be used for the ribosomal peptide synthesis. In this study, we used our cell-free translation system to bare the intrinsic ability of ribosome toward β-amino acids.

β-Amino acids are nonproteinogenic amino acids with elongated backbones of α-amino acids. Incorporation of the β-homoglycine (βhGly) into a peptide or protein was initially studied via amber suppression method using βhGly-tRNA$_{CUA}$ prepared by chemoenzymatic acylation.[18] The incorporation efficiency of βhGly into a peptide (MGLYLGLF-βhGly-GLYLGLF) in rabbit reticulocyte extract was determined as 11% on the basis of the ratio of [^{35}S]Met and [^{3}H]Leu in the product peptide, although there was no direct evidence of the incorporation of βhGly rather than the misincorporation of a proteinogenic amino acid.[16] Incorporation of βhGly into T4 lysozyme in an *Escherichia coli* (*E. coli*) extract was also tested, but the incorporation efficiency was less than 5%.[3] Other β-amino acids with different side chains (α-methyl-βhGly, β,β-dimethyl-βhGly, D-βhPhg, and D-*p*-bromo-βhPhg) as well as βhGly were further tested for incorporation into dihydrofolate reductase.[19,20] However, the incorporation efficiencies of these β-amino acids were only just above the background when an *E. coli* extract with wild type ribosome was used, and certain mutations of the ribosome were required to improve the incorporation efficiency.

In the above studies, the undesired competition between the β-amino acid incorporation and the termination event triggered by release factor 1 could be a major problem. To avoid this problem, a reconstituted *E. coli* cell-free translation system[21,22] without release factor 1[23] was used for studying the compatibility of βhGly. Even though there was no undesired termination, no peptide containing βhGly was detected and only Gln misincorporation was observed. A strategy of codon reassignment[24−26] has also been used to study β-amino acid compatibility. L-βhAla and L-βhPhe were tested for incorpo-

Received: November 30, 2015
Published: January 25, 2016

DOI: 10.1021/jacs.5b12482
J. Am. Chem. Soc. 2016, 138, 1962−1969

ration into a tripeptide at the GUU sense codon in an mRNA using a reconstituted cell-free translation system in which all aminoacyl-tRNA synthetases and amino acids were omitted, but neither a peptide containing L-βhAla nor that containing L-βhPhe were detected.[9] Various β-aminoacyl-tRNAs synthesized by aminoacyl-tRNA synthetases have also been used to study the compatibility of β-amino acids, but no peptide containing a β-amino acid has been observed.[11] These studies using a reconstituted *E. coli* cell-free translation system have indicated that no β-amino acid was compatible with ribosomal translation. However, in these reconstituted systems,[9,23] the concentrations of the elongation factor Tu (EF-Tu, 1 and 3.6 μM) and aminoacyl-tRNA (1 and 18 μM) were significantly lower than those (10 μM EF-Tu and 50 μM aminoacyl-tRNA) in the optimized translation system.[27] We have previously used our optimized translation system in a study that re-evaluated D-amino acid compatibility with ribosomal translation.[17] We found that various D-amino acids, including the ones reported as incompatible by other researchers,[3,9,16,28] were compatible with translation. This result encouraged us to use the optimized reconstituted translation system for studying β-amino acid compatibility.

In the present study, we comprehensively evaluated the β-amino acid compatibility with ribosomal translation (Figure 1). We prepared 16 β-aminoacyl-tRNAs using the flexizyme (dFx),[26,29] and used these β-aminoacyl-tRNAs to determine the intrinsic efficiency of single incorporation of a β-amino acid into a peptide. Furthermore, using β-amino acids with high compatibility, we successfully synthesized peptides containing these β-amino acids at multiple positions. Our study elucidated β-amino acid compatibility with ribosomal translation, which would make it possible to create nonstandard peptide libraries containing β-amino acids. In the future, the combination of a ribosomal translation system with β-amino acids and an *in vitro* selection method,[30,31] such as our high-speed selection method (TRAP display, transcription-translation coupled with the association of a puromycin linker),[32] will allow the rapid development of therapeutic peptidomimetics from these highly diverse nonstandard peptide libraries.[1,33]

EXPERIMENTAL SECTION

Materials. Flexizyme, $tRNA^{EnAsn}_{XXX}$ (an *E. coli* $tRNA^{Asn}$ derivative for carrying amino acids; XXX represents an anticodon), and $microhelix^{EnAsn}$ (the acceptor stem of $tRNA^{EnAsn}$ for analyzing flexizyme-aminoacylation efficiency) were prepared according to previous reports (see also Supporting Information).[26,34] The general procedure for the synthesis of amino acid substrates is presented in the Supporting Information.

Preparation of Aminoacyl-$tRNA^{EnAsn}_{XXX}$. Aminoacylation of $tRNA^{EnAsn}_{XXX}$ (50 μL) was performed under the following conditions: 50 mM bicine buffer (pH 9.0), 20 mM $MgCl_2$, 40% DMSO (or 20% DMSO for L-βhLys), 25 μM $tRNA^{EnAsn}_{XXX}$, 25 μM dFx, and 5 mM amino acid substrate (2.5 mM amino acid substrate for L-βhLys). Each reaction mixture was incubated on ice for 22 h. The reaction was stopped by the addition of 200 μL of 0.3 M sodium acetate (pH 5). The RNA was recovered by ethanol precipitation, and the pellet was rinsed twice with 70% ethanol containing 0.1 M sodium acetate (pH 5) and once with 70% ethanol. The pellet was dried and was stored at −80 °C. The pellet was dissolved in 5 μL of 1 mM sodium acetate, and the solution was used for the translation reaction.

Preparation of DNA Templates for Peptide Synthesis. The template DNAs for R1−R15 mRNAs were prepared as follows. Extension (20 μL) was performed under the following conditions: 1× PCR buffer [10 mM Tris-HCl (pH 8.4); 50 mM KCl; 0.1% (v/v) Triton X-100], 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 μM each of a forward primer and a reverse primer (the primers are shown in Tables S1 and S2), and Taq DNA polymerase. Extension was performed by heating the mixture at 95 °C for 1 min, and five cycles of 50 °C for 1 min and 72 °C for 1 min.

PCR (1 mL) was performed in the following conditions: 1× PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 10 μL of the extended DNA solution, 0.5 μM T7ex5F22 (5′-GGCGT AATAC GACTC ACTAT AG-3′), 0.5 μM FlaguaaR33 (5′-CGAAG CTTAC TTGTC GTCGT CGTCC TTGTA GTC-3′), and Taq DNA polymerase. Amplification was performed by 12 cycles of 95 °C for 40 s, 50 °C for 40 s, and 72 °C for 40 s. After phenol/chloroform extraction, the

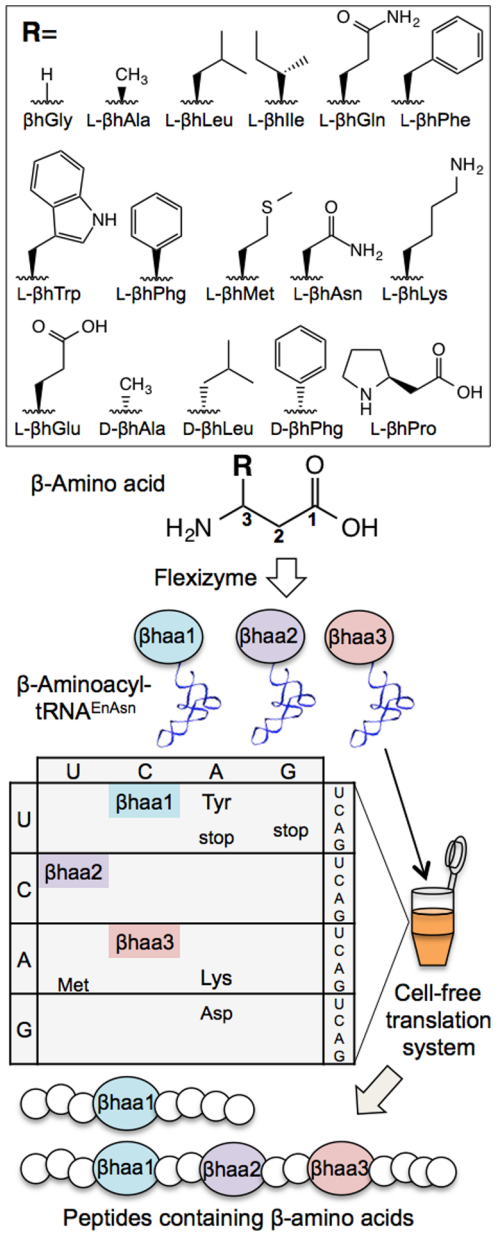


Figure 1. Scheme of the evaluation of β-amino acid compatibility with the elongation event in ribosomal translation. Sixteen β-amino acids were charged onto $tRNA^{EnAsn}$ using the flexizyme acylation system. Resulted β-aminoacyl-$tRNA^{EnAsn}$(s) were added to a custom-made *E. coli* reconstituted cell-free translation system containing four amino acids and four corresponding aminoacyl tRNA synthetases. In this translation system, β-amino acid(s) were assigned to blank codon(s). Analysis of peptide products were performed by tricine-SDS-PAGE to quantify the peptide yields and by MALDI-TOF-MS to confirm the production of full-length peptides. Abbreviation: βhaa, β-amino acid.

DOI: 10.1021/jacs.5b12482
J. Am. Chem. Soc. 2016, 138, 1962−1969

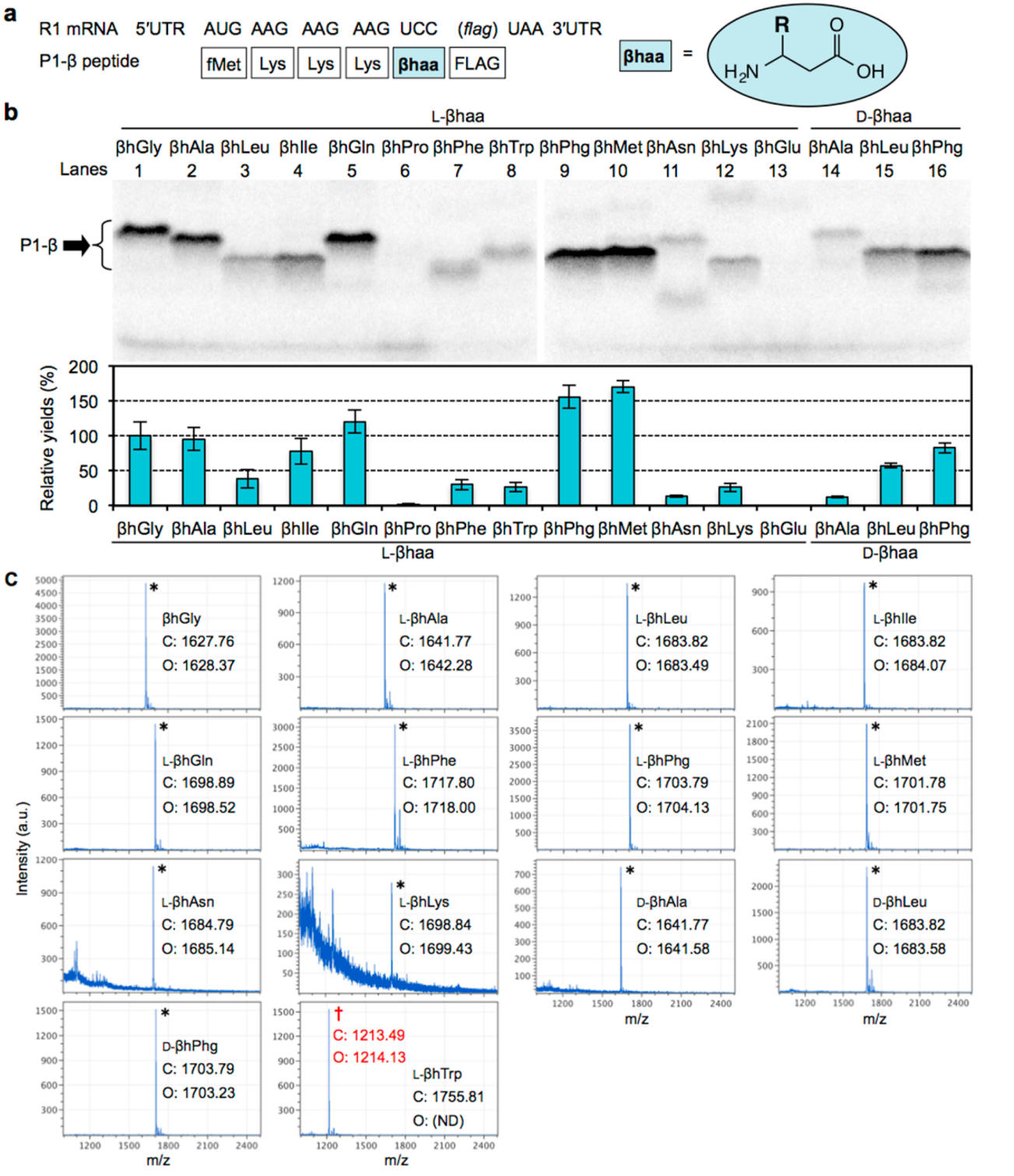


Figure 2. Single incorporation of 16 *β*-amino acids into a peptide chain. (a) Sequence of the R1 mRNA and the corresponding P1-*β* peptide. Each *β*-amino acid was assigned to the blank codon UCC. FLAG represents the FLAG-tag peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). (b) The peptide productions quantified by tricine-SDS-PAGE. The resulted peptides were labeled with [^{14}C]Asp included in FLAG-tag sequence. Each lane shows the production of the P1-*β* peptide under the condition containing 50 μM *β*-aminoacyl-tRNA$^{EnAsn}_{GGA}$. The band intensity of each peptide was normalized against the intensity of the peptide produced with 50 μM *β*hGly-tRNA$^{EnAsn}_{GGA}$. Error bar shows the standard deviation of each experiment in triplicate. (c) MALDI-TOF-MS spectra of P1-*β* peptides. Unlabeled Asp was used instead of [^{14}C]Asp for the MS analysis. The calculated mass (C) and the observed mass (O) of the singly charged P1-*β* peptides $[M + H]^+$ are shown in each spectrum. The peaks are labeled as follows: the P1-*β* peptide containing single *β*-amino acid (*) and truncated peptide (*β*haa-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) (†). Abbreviations: UTR, untranslated region; *β*haa, *β*-amino acid; ND, not detected.

product DNA was collected by ethanol precipitation and was dissolved in 100 μL of ultrapure water.

Translation Reaction. Translation systems without the 20 proteinogenic amino acids and the 20 aminoacyl-tRNA synthetases

DOI: 10.1021/jacs.5b12482
J. Am. Chem. Soc. 2016, 138, 1962−1969

(Tables S3 and S4) were prepared by a similar procedure in the previous reports.[22,27,35,36] EF-Tu was purified as a complex of EF-Tu and EF-Ts (elongation factor Ts); thus, the concentration of EF-Tu described in this study represented that of the EF-Tu and EF-Ts complex. A translation reaction mixture (2.5 μL) containing 0.04 μM of DNA template; 0.5 mM each of Tyr, Met, Ile (only for double incorporation with Ile insertion), and Lys; 50 μM [^{14}C]Asp; 0.02 μM TyrRS; 0.03 μM MetRS; 0.11 μM LysRS; 0.13 μM AspRS; 0.4 μM IleRS (only for double incorporation with Ile insertion); and aminoacyl-tRNA$^{EnAsn}_{XXX}$ (see figure legends for the concentration) was incubated at 37 °C for 60 min. The concentration of aminoacyl-tRNA described in the figure legend was the concentration of the transcribed tRNA$^{EnAsn}_{XXX}$; thus, it included the nonacylated tRNA (Figure S1 for the aminoacylation efficiency with each amino acid). The products were analyzed by tricine-SDS-PAGE and autoradiography (Pharos FX imager, Bio-Rad). Alternatively, the reaction was performed with Asp instead of [^{14}C]Asp, and the resulting product was analyzed using Autoflex II (Bruker Daltonics). For optimization of the EF-Tu concentration, 20 aminoacyl-tRNA synthetases (Table S5), 0.5 mM of each proteinogenic amino acid and 10 ng/μL green fluorescent protein template[37] were added to the translation mixture (2.5 μL), in which 0, 3.6, or 10 μM EF-Tu was present. The reaction mixtures were incubated at 37 °C. The reaction was quenched by the addition of 80 μL of 50 mM Tris-HCl (pH 8.0) buffer. Fluorescence of the resulting solutions was measured using SpectraMax M5 (Molecular Devices) at λ_{ex} 486 nm and λ_{em} 530 nm.

RESULTS AND DISCUSSION

Flexizyme Acylation System for β-Amino Acids. We used the flexizyme acylation system for the preparation of β-aminoacyl-tRNAs.[26,29] Because the flexizyme (dFx) recognizes only the dinitrobenzyl moiety in the amino acid substrates, we synthesized dinitrobenzyl esters of 16 commercially available β-amino acids (L-βhGly, L-βhAla, L-βhLeu, L-βhIle, L-βhGln, L-βhPro, L-βhPhe, L-βhTrp, L-βhPhg, L-βhMet, L-βhAsn, L-βhLys, L-βhGlu, D-βhAla, D-βhLeu, and D-βhPhg; L and D represent the configuration at the C^3 carbon by considering $-CH_2COOH$ of the β-amino acids as $-COOH$ of an α-amino acid; Figure 1), and tested them for acylation of microhelix^{EnAsn} using the flexizyme (dFx). Acid-PAGE analysis showed that all of the β-amino acids were successfully charged on the microhelix (Figure S1). The aminoacylation efficiency varied from 25% to 55%, which were sufficiently high for further β-amino acid incorporation studies.

Incorporation of βhGly into Peptide. For the evaluation of β-amino acid compatibility with ribosomal translation, we first used βhGly, the β-amino acid most commonly studied for incorporation into peptides. We prepared βhGly-tRNA$^{EnAsn}_{GGA}$, and added it to a custom-made *E. coli* reconstituted cell-free translation system in which the UCC (Ser) codon was made vacant by omitting all amino acids and aminoacyl-tRNA synthetases except those (Met, Tyr, Asp, Lys) used in the P1-β peptide fMKKK-βhGly-FLAG (Figure S2a; FLAG represents the FLAG-tag peptide). We varied the concentrations of aminoacyl-tRNA (1, 18, or 50 μM) and EF-Tu (1, 3.6, or 10 μM) to reveal the differences of translation systems used in previous studies[9,23] and our optimized translation system (EF-Tu concentration was optimized by green fluorescent protein expression; Figure S3). The P1-β peptide was expressed in the presence of [^{14}C]Asp, and the resulting peptide was analyzed by tricine-SDS-PAGE (Figure S2b). As we expected, the yield of the P1-β peptide was enhanced with higher EF-Tu and tRNA concentrations, whereas these higher concentrations of EF-Tu and tRNA also improved the yield of P1-α peptide fMKKK-αGly-FLAG. We then investigated whether the observed molecular weight of the P1-β peptide corresponded to its calculated molecular weight by MALDI-TOF-MS (Figure 2c, Figure S4). The major peak of the P1-β peptide was consistent with the calculated molecular weight. These results clearly showed that the peptide synthesized in the optimized cell-free translation system contained βhGly; therefore, we concluded that βhGly was compatible with ribosomal translation.

Incorporation of Various L-β-Amino Acids into Peptide. Next, we extended our study to the 12 L-β-amino acids (L-βhAla, L-βhLeu, L-βhIle, L-βhGln, L-βhPhe, L-βhPhg, L-βhMet, L-βhAsn, L-βhLys, L-βhTrp, L-βhPro, and L-βhGlu). We prepared each L-βhaa-tRNA$^{EnAsn}_{GGA}$ (where βhaa represents one of the tested β-amino acids) using the flexizyme (dFx), and added it to the reconstituted cell-free translation system with the blank codon UCC. The P1-β peptides fMKKK-βhaa-FLAG (Figure 2a) were expressed and were analyzed by tricine-SDS-PAGE followed by autoradiography. The yields of the peptides containing one of the L-β-amino acids were normalized to those of fMKKK-βhGly-FLAG. The result of PAGE analysis showed that P1-β peptides containing one of L-βhAla, L-βhLeu, L-βhIle, L-βhGln, L-βhPhe, L-βhPhg, L-βhMet, L-βhAsn, L-βhLys, and L-βhTrp were synthesized, whereas no peptide was produced for L-βhPro and L-βhGlu (Figure 2b, Figure S5).

To determine whether the observed molecular weights of the synthesized peptides corresponded to the calculated molecular weights of the P1-β peptides, each product was analyzed by MALDI-TOF-MS (Figure 2c, Figure S4). The major peak of each product, except L-βhTrp, was consistent with the calculated molecular weight of the P1-β peptide containing one of the nine β-amino acids (L-βhAla, L-βhLeu, L-βhIle, L-βhGln, L-βhPhe, L-βhPhg, L-βhMet, L-βhAsn, and L-βhLys). For L-βhTrp, only a truncated peptide consistent with L-βhTrp-FLAG was detected. This truncated peptide could be produced by the following mechanism. After the EF-Tu-mediated delivery of L-βhTrp-tRNA to the aminoacyl-tRNA binding site (A site) in the ribosome, fMKKK-tRNA dropped off from the peptidyl-tRNA binding site (P site),[38,39] presumably because of its poor peptidyl-transfer efficiency. However, the translocation of L-βhTrp-tRNA to the P site occurred, so that L-βhTrp-FLAG was produced.

To summarize these results, β-amino acid incorporation efficiencies were clearly affected by the properties of β-amino acid side chains (Figure S6). β-Amino acids with small side chains (βhGly and L-βhAla) showed high incorporation efficiencies (Figure 2b, lanes 1 and 2). β-Amino acids with aliphatic side chains (L-βhLeu and L-βhIle) showed moderate incorporation efficiencies (Figure 2b, lanes 3 and 4). The incorporation efficiencies of β-amino acids with amide side chains (L-βhGln and L-βhAsn) were different; L-βhGln showed high and L-βhAsn showed low efficiency (Figure 2b, lanes 5 and 11). No peptide was observed for L-βhPro (Figure 2b, lane 6), presumably because of lower reactivity of its secondary amino group. The incorporation efficiencies of β-amino acids with aromatic side chains (L-βhPhg, L-βhPhe, and L-βhTrp) also varied (Figure 2b, lanes 7–9). L-βhPhg showed high and L-βhPhe showed low efficiency; L-βhTrp yielded only a truncated peptide in MS analysis. β-Amino acids with charged side chain (L-βhLys and L-βhGlu) showed low or no incorporation (Figure 2b, lanes 12 and 13).

These correlations between side chain structure and incorporation efficiency observed for β-amino acids are similar to those previously reported for *N*-methylamino acids, D-amino

DOI: 10.1021/jacs.5b12482
J. Am. Chem. Soc. 2016, 138, 1962–1969

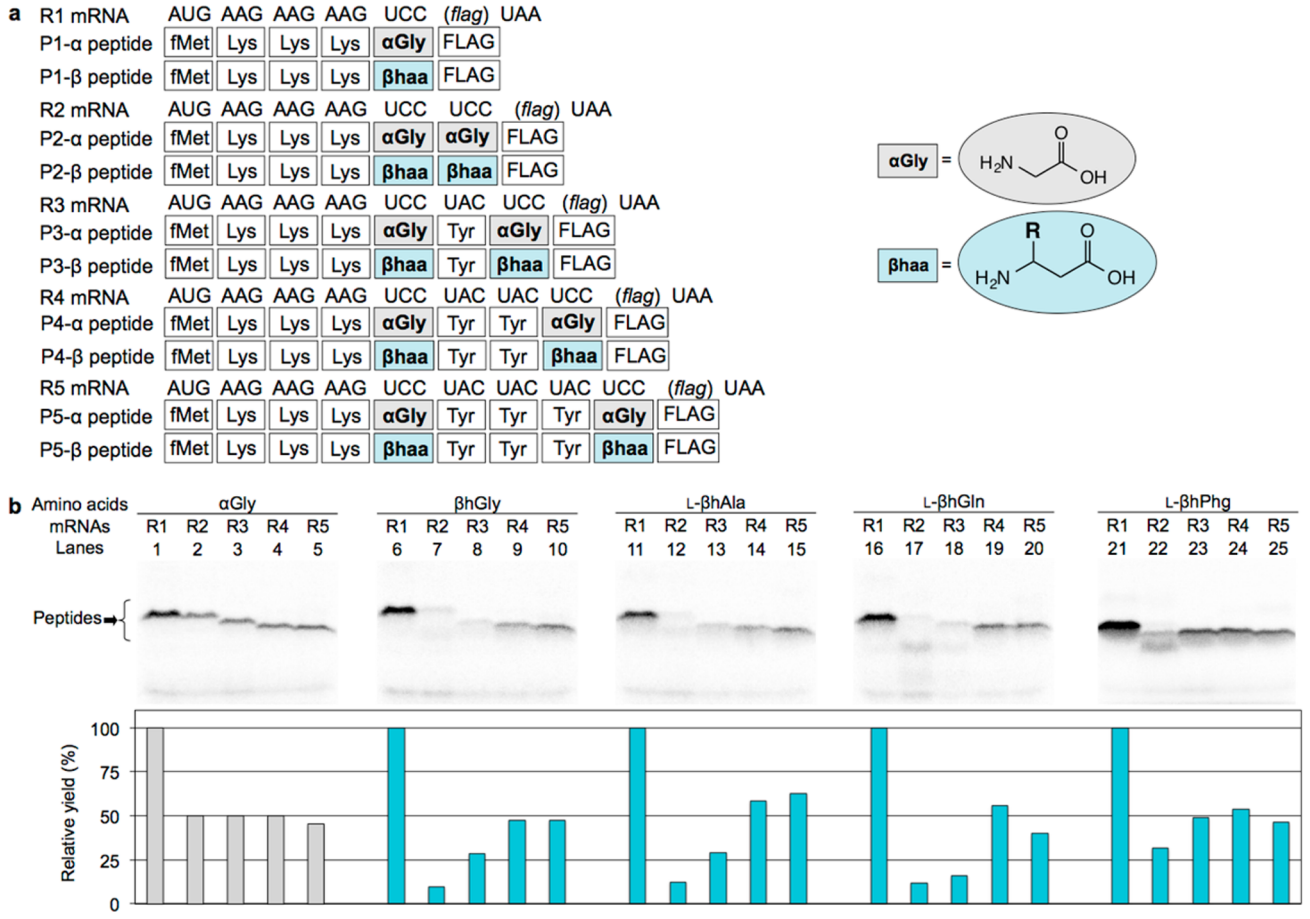


Figure 3. Double incorporation of *β*-amino acids into a peptide. (a) Sequences of the R1, R2, R3, R4, and R5 mRNAs and the corresponding P1, P2, P3, P4, and P5 peptides. *α*Gly, *β*hGly, L-*β*hAla, L-*β*hGln, and L-*β*hPhg were assigned to the blank codon UCC. 5′- and 3′-UTR are not shown. (b) Tricine-SDS-PAGE analysis of peptides. The peptides production was analyzed as described in Figure 2b. The lanes 1−5, 6−10, 11−15, 16−20, and 21−25 show the production of the peptides under the condition containing 50 μM of *α*Gly-tRNA$^{EnAsn}_{GGA}$, *β*hGly-tRNA$^{EnAsn}_{GGA}$, L-*β*hAla-tRNA$^{EnAsn}_{GGA}$, L-*β*hGln-tRNA$^{EnAsn}_{GGA}$, and L-*β*hPhg-tRNA$^{EnAsn}_{GGA}$, respectively. The band intensity of each peptide was normalized against the corresponding P1 peptide. Abbreviation: *β*haa, *β*-amino acid.

acids, and *N*-substituted glycines.[13,17,40] This similarity may be caused by the different affinities of the EF-Tu toward *β*-amino acids on tRNA (Figure S6I) because the affinity of EF-Tu varies among proteinogenic L-amino acids on tRNA.[41,42] It could also be explained by the difference in the affinity to the A site of the ribosome (Figure S6II) or the rate of peptide bond formation in the peptidyl-transferase center (Figure S6III) since the puromycin derivatives of L-(4-Me)-*β*hPhe and L-*β*hAla showed distinct IC_{50} values for the translation of globin.[43] The peptidyl-transfer reaction may be particularly slow for L-*β*hPro since the reaction observed for L-Pro using quench-flow translation kinetics was slower than that of other L-amino acids.[44] After *β*-amino acid incorporation, the *β*-amino acid at the nascent peptide on the peptidyl-tRNA might inhibit formation of the next peptide-bond (Figure S6IV).

We also elucidated the effect of the codon coding a *β*-amino acid on the efficiency of single incorporation. We assigned *α*Gly, *β*hGly, and L-*β*hGlu to ACC and CAC codons by adding aminoacyl-tRNA$^{EnAsn}_{GGU}$ and aminoacyl-tRNA$^{EnAsn}_{GUG}$ to the translation system with templates carrying corresponding codons (Figure S7). Tricine-SDS-PAGE analysis of the produced peptides showed that *β*hGly was incorporated into the peptide with similar efficiency among all three tested codons, while L-*β*hGlu was not incorporated into the peptide. This result indicated that the single incorporation of *β*-amino acids was less influenced by the type of codon involved. We also expressed fMYKY-Xaa-FLAG and fMKYY-Xaa-FLAG (Xaa = *α*Gly, *β*hGly, and L-*β*hGlu) to study the effect of the peptide sequence on the efficiency of single incorporation (Figure S8). *β*hGly was incorporated into fMYKY-Xaa-FLAG with similar efficiency as into fMKKK-Xaa-FLAG, but not into fMKYY-Xaa-FLAG, while L-*β*hGlu was hardly incorporated into all three tested peptides. This suggested that the peptide sequences might affect the efficiency of single incorporation.

Effect of Chirality at the C^3 Carbon of *β*-Amino Acids on Incorporation Efficiency. It is well-known that the translation system has a preference for L- over D-*α*-amino acids,[2,3,6,9,16] although we have previously shown that some D-*α*-amino acids are compatible with translation.[17] We speculated whether the chirality at the C^3 carbon would affect the incorporation efficiency of the *β*-amino acids like that of the C^2 carbon of *α*-amino acids.

To investigate the effect of the chirality at the C^3 carbon of *β*-amino acids on incorporation efficiency, the P1-*β* peptides, fMKKK-*β*haa-FLAG with each of D-*β*hAla, D-*β*hLeu, and D-*β*hPhg, were expressed and were analyzed by the same method used for the L-*β*-amino acids. The MALDI-TOF-MS analysis showed that the peptides with the corresponding molecular weight were synthesized by the translation reaction for all the three tested D-*β*-amino acids (Figure 2c). The stereoselectivity

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J. Am. Chem. Soc. 2016, 138, 1962−1969

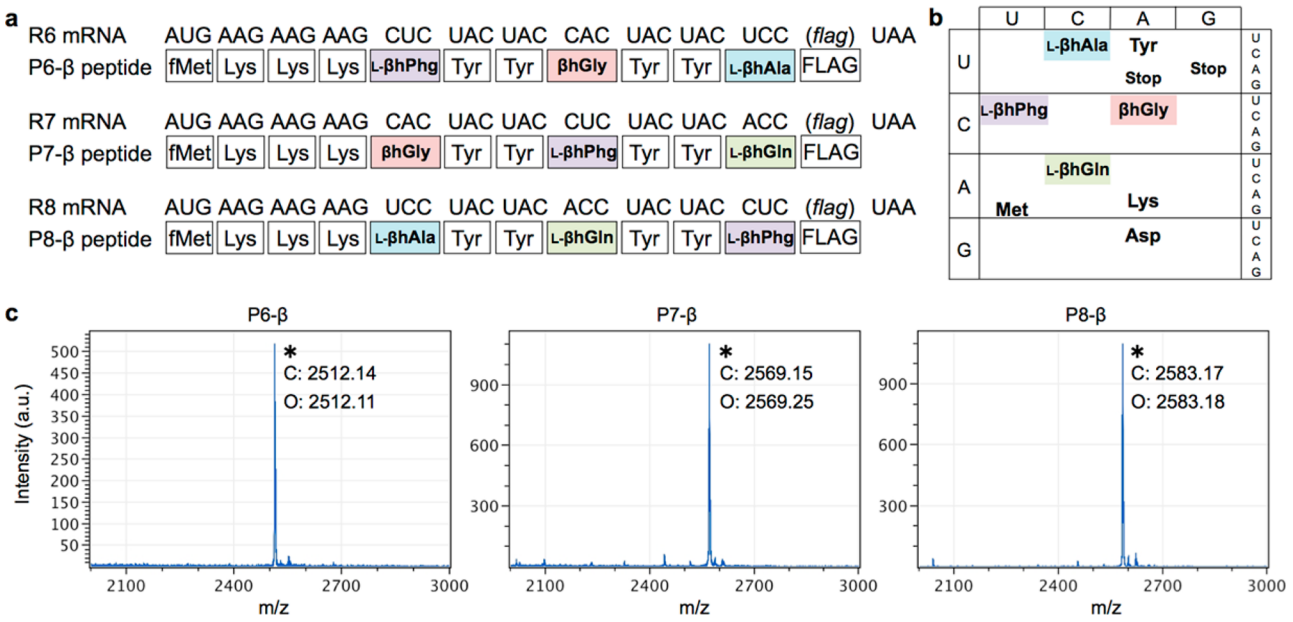

Figure 4. Incorporation of three types of *β*-amino acids into a peptide. (a) Sequences of the R6, R7, and R8 mRNA and the corresponding P6-*β*, P7-*β*, and P8-*β* peptide. (b) The codon table showing the correspondence of four *β*-amino acids and four blank codons. *β*hGly, L-*β*hAla, L-*β*hGln, and L-*β*hPhg were assigned to the blank codons CAC, UCC, ACC, and CUC, respectively. (c) MALDI-TOF-MS spectra of P6-*β*, P7-*β*, and P8-*β* peptides containing three *β*-amino acids produced under the condition containing 50 μM each of *β*-aminoacyl-tRNAs (*β*hGly-tRNA$^{EnAsn}_{GUG}$, L-*β*hAla-tRNA$^{EnAsn}_{GGA}$, L-*β*hGln-tRNA$^{EnAsn}_{GGU}$, and L-*β*hPhg-tRNA$^{EnAsn}_{GAG}$). The calculated mass (C) and the observed mass (O) of the singly charged P1 peptide $[M + H]^+$ are shown in each spectrum. The peaks labeled with asterisk (*) are the desired peptides with triple *β*-amino acids.

of the translation reaction for *β*-amino acids was not necessarily the same as that for *α*-amino acids. The incorporation efficiencies of D-*β*hAla and D-*β*hPhg were lower than those of the corresponding L-isomers (Figure 2b, lane 14 vs lane 2, lane 16 vs lane 9), whereas that of D-*β*hLeu was higher than that of L-*β*hLeu (Figure 2b, lane 15 vs lane 3). Because these *β*-amino acids have one methylene insertion between the carbonic acid and the chiral center of their original substrates, the extra methylene may account for the violation of the rule governing ribosome stereoselectivity.

Double Incorporation of *β*-Amino Acids into Peptide. We further investigated whether *β*-amino acids could be successively incorporated into a peptide in the ribosomal translation system. On the basis of the results of the single incorporation of *β*-amino acids, we selected *β*hGly, L-*β*hAla, L-*β*hGln, and L-*β*hPhg as building blocks for this experiment. The successive incorporation of *β*-amino acids was tested by expressing the P2-*α* peptide (fMKKK-*α*Gly-*α*Gly-FLAG) and P2-*β* peptides (fMKKK-*β*haa-*β*haa-FLAG) (Figure 3a). An intense band was observed for the synthesis of the P2-*α* peptide (Figure 3b, lane 2). In contrast, only faint bands were observed for the synthesis of the P2-*β* peptides containing one of *β*hGly, L-*β*hAla, or L-*β*hGln (Figure 3b, lanes 7, 12, and 17). Moreover, MALDI-TOF-MS analysis showed that the synthesized peptides mostly contained a single corresponding *β*-amino acid (Figure S9, first column). These results indicated that the precise successive incorporation of *β*-amino acid into a peptide was prohibited, even if the *β*-amino acids could be incorporated efficiently at a single position (Figure 2b, lanes 1, 2, and 5). The peptide containing a single *β*-amino acid was probably produced by "hopping" of the ribosome[45] from codon 5 to 6 on the R2 mRNA. For the synthesis of the P2-*β* peptide containing L-*β*hPhg, two faint bands were observed by tricine-SDS-PAGE (Figure 3b, lane 22). MS analysis revealed that the synthesized peptide was a mixture of a peptide containing a single L-*β*hPhg and the full-length peptide containing two L-*β*hPhg.

We hypothesized that the slight movements of both a carbonyl group of a *β*-amino acid on a peptidyl-tRNA and an amino group of *β*-amino acid on an aminoacyl-tRNA away from their original positions cooperatively decrease the efficiency of peptide bond formation. Thus, we investigated whether the insertion of one, two, or three Tyr residues between the two *β*-amino acids allowed the corresponding peptides to be expressed. Although the detected bands of P3-*β* peptides, fMKKK-*β*haa-Y-*β*haa-FLAG, were faint for tested *β*-amino acids (*β*hGly, L-*β*hAla, and L-*β*hGln; Figure 3b, lanes 8, 13, and 18) except for L-*β*hPhg (Figure 3b, lane 23), MS analysis confirmed the production of the P3-*β* peptides containing two *β*-amino acids (Figure S9, second column). Furthermore, intense bands of P4-*β* and P5-*β* peptides were observed for all tested *β*-amino acids by tricine-SDS-PAGE analysis (Figure 3b, lanes 9, 10, 14, 15, 19, 20, 24, and 25). MS analysis of these peptides confirmed that the molecular weights were consistent with those of the desired P4-*β* or P5-*β* peptides containing two *β*-amino acids (Figure S9, third and fourth columns). These results indicated that the insertion of Tyr between the *β*-amino acids restored the double-incorporation efficiency. We also performed similar experiments using Ile instead of Tyr as the inserted amino acids. We found that Ile insertion could also recover the yields of peptides containing two *β*-amino acids in most cases (Figure S10).

Incorporation of Multiple *β*-Amino Acids into Peptide. On the basis of this knowledge, we designed the P6-*β*, P7-*β*, and P8-*β* peptides, fMKKK-*β*haa^1-YY-*β*haa^2-YY-*β*haa^3-FLAG, to test the incorporation of multiple *β*-amino acids into a peptide (Figure 4a). Four tRNAs with these *β*-amino acids (*β*hGly-tRNA$^{EnAsn}_{GUG}$, L-*β*hAla-tRNA$^{EnAsn}_{GGA}$, L-*β*hGln-tRNA$^{EnAsn}_{GGU}$, and L-*β*hPhg-tRNA$^{EnAsn}_{GAG}$) were prepared using the flexizyme acylation system, and added to a custom-

DOI: 10.1021/jacs.5b12482
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made reconstituted cell-free translation system in which CAC (His), UCC (Ser), ACC (Thr), and CUC (Leu) codons were vacant. As a result, βhGly, L-βhAla, L-βhGln, and L-βhPhg were reassigned to the CAC, UCC, ACC, and CUC codons, respectively (Figure 4b). Although the yields of these peptides containing multiple β-amino acids were 10%−20% of those of corresponding peptides with αGly (Figure S11), MALDI-TOF-MS analysis of each synthesized peptide showed a peak corresponding to the one containing three β-amino acids (Figure 4c, Figure S12). These results clearly demonstrated that peptides containing multiple types of β-amino acids could be synthesized in the ribosomal translation system.

Interestingly, we identified the fMKKK-L-βhPhg-YY and L-βhGln-YY-L-βhPhg-FLAG fragments as byproducts in the MALDI-TOF-MS analysis. Moreover, in all of the above single and double incorporation experiments, we observed fragments ending just before the β-amino acid or starting from the β-amino acid; however, no fragments ending or starting just after the β-amino acid were observed (Figure 2c, L-βhTrp; Figure S9, P4-β and P5-β). This might indicate that the addition of a β-amino acid to a nascent peptide is a more difficult step than the elongation of a nascent peptide chain after a β-amino acid.

CONCLUSION

We have evaluated the compatibility of 16 β-amino acids with elongation during translation by using an *E. coli* reconstituted cell-free translation system in which the Ser codon was reassigned to one of the β-amino acids. Single-incorporation experiments revealed that 13 β-amino acids could be incorporated into the peptide in the cell-free translation system. The incorporation efficiencies of β-amino acids were affected by the properties of the β-amino acid side chain; those with small side chains showed high incorporation efficiencies, those with aliphatic side chains showed moderate incorporation efficiencies, and those with charged side chains showed very low or no incorporation efficiencies. Moreover, we found that the successive incorporation of two β-amino acids was prohibited, but the double-incorporation efficiency was restored by the insertion of Tyr or Ile between two β-amino acids. On the basis of the double-incorporation study, we designed mRNAs encoding peptides containing multiple types of β-amino acids, and demonstrated the synthesis of these peptides in the translation system.

Additionally, β-amino acids receive attention as the building blocks of peptide analogues for pharmaceutical uses, because β-amino acids improve the stability of the peptide analogues containing them against proteolysis.[20,46] Our study greatly increased the knowledge for the use of β-amino acids in a ribosomal translation system, so it makes β-amino acids available for the construction of the peptide library. Such highly diverse peptide libraries containing β-amino acids would become a useful source for therapeutic peptidomimetics in the future.[1,33]

Abbreviations. Tris, Tris(hydroxymethyl)aminomethane; SDS-PAGE, sodium dodecyl sulfate−polyaclylamid egel electrophoresis; DMSO, dimethyl sulfoxide; TyrRS, tyrosyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase; MALDI-TOF-MS, matrix assisted laser desorption ionization time of flight mass spectrometry.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b12482.

Detailed experimental procedures and additional tables and figures (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Funding Program for Next Generation World-Leading Researchers (LR011 to H.M.) and Grants-in-Aid for JSPS Fellows (26-9520 to T.F.) from Japan Society for the Promotion of Science (JSPS).

REFERENCES

(1) Fujino, T.; Murakami, H. *Chem. Rec.* **2016**, DOI: 10.1002/tcr.201500239.
(2) Calendar, R.; Berg, P. *J. Mol. Biol.* **1967**, *26*, 39.
(3) Ellman, J. A.; Mendel, D.; Schultz, P. G. *Science* **1992**, *255*, 197.
(4) Chung, H. H.; Benson, D. R.; Schultz, P. G. *Science* **1993**, *259*, 806.
(5) Mendel, D.; Cornish, V. W.; Schultz, P. G. *Annu. Rev. Biophys. Biomol. Struct.* **1995**, *24*, 435.
(6) Dedkova, L. M.; Fahmi, N. E.; Golovine, S. Y.; Hecht, S. M. *J. Am. Chem. Soc.* **2003**, *125*, 6616.
(7) Frankel, A.; Millward, S. W.; Roberts, R. W. *Chem. Biol.* **2003**, *10*, 1043.
(8) Merryman, C.; Green, R. *Chem. Biol.* **2004**, *11*, 575.
(9) Tan, Z.; Forster, A. C.; Blacklow, S. C.; Cornish, V. W. *J. Am. Chem. Soc.* **2004**, *126*, 12752.
(10) Dedkova, L. M.; Fahmi, N. E.; Golovine, S. Y.; Hecht, S. M. *Biochemistry* **2006**, *45*, 15541.
(11) Hartman, M. C.; Josephson, K.; Lin, C. W.; Szostak, J. W. *PLoS One* **2007**, *2*, e972.
(12) Zhang, B.; Tan, Z.; Dickson, L. G.; Nalam, M. N.; Cornish, V. W.; Forster, A. C. *J. Am. Chem. Soc.* **2007**, *129*, 11316.
(13) Kawakami, T.; Murakami, H.; Suga, H. *Chem. Biol.* **2008**, *15*, 32.
(14) Sando, S.; Masu, H.; Furutani, C.; Aoyama, Y. *Org. Biomol. Chem.* **2008**, *6*, 2666.
(15) Subtelny, A. O.; Hartman, M. C.; Szostak, J. W. *J. Am. Chem. Soc.* **2008**, *130*, 6131.
(16) Bain, J. D.; Wacker, D. A.; Kuo, E. E.; Chamberlin, A. R. *Tetrahedron* **1991**, *47*, 2389.
(17) Fujino, T.; Goto, Y.; Suga, H.; Murakami, H. *J. Am. Chem. Soc.* **2013**, *135*, 1830.
(18) Heckler, T. G.; Zama, Y.; Naka, T.; Hecht, S. M. *J. Biol. Chem.* **1983**, *258*, 4492.
(19) Dedkova, L. M.; Fahmi, N. E.; Paul, R.; del Rosario, M.; Zhang, L.; Chen, S.; Feder, G.; Hecht, S. M. *Biochemistry* **2012**, *51*, 401.
(20) Maini, R.; Nguyen, D. T.; Chen, S.; Dedkova, L. M.; Chowdhury, S. R.; Alcala-Torano, R.; Hecht, S. M. *Bioorg. Med. Chem.* **2013**, *21*, 1088.
(21) Forster, A. C.; Weissbach, H.; Blacklow, S. C. *Anal. Biochem.* **2001**, *297*, 60.
(22) Shimizu, Y.; Inoue, A.; Tomari, Y.; Suzuki, T.; Yokogawa, T.; Nishikawa, K.; Ueda, T. *Nat. Biotechnol.* **2001**, *19*, 751.
(23) Sando, S.; Abe, K.; Sato, N.; Shibata, T.; Mizusawa, K.; Aoyama, Y. *J. Am. Chem. Soc.* **2007**, *129*, 6180.
(24) Forster, A. C.; Tan, Z.; Nalam, M. N.; Lin, H.; Qu, H.; Cornish, V. W.; Blacklow, S. C. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 6353.

DOI: 10.1021/jacs.5b12482
J. Am. Chem. Soc. 2016, 138, 1962−1969

(25) Josephson, K.; Hartman, M. C.; Szostak, J. W. *J. Am. Chem. Soc.* **2005**, *127*, 11727.
(26) Murakami, H.; Ohta, A.; Ashigai, H.; Suga, H. *Nat. Methods* **2006**, *3*, 357.
(27) Reid, P. C.; Goto, Y.; Katoh, T.; Suga, H. *Methods Mol. Biol.* **2012**, *805*, 335.
(28) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182.
(29) Ohuchi, M.; Murakami, H.; Suga, H. *Curr. Opin. Chem. Biol.* **2007**, *11*, 537.
(30) Nemoto, N.; Miyamoto-Sato, E.; Husimi, Y.; Yanagawa, H. *FEBS Lett.* **1997**, *414*, 405.
(31) Roberts, R. W.; Szostak, J. W. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 12297.
(32) Ishizawa, T.; Kawakami, T.; Reid, P. C.; Murakami, H. *J. Am. Chem. Soc.* **2013**, *135*, 5433.
(33) Kawakami, T.; Murakami, H. *J. Nucleic Acids* **2012**, *2012*, 713510.
(34) Ohta, A.; Murakami, H.; Higashimura, E.; Suga, H. *Chem. Biol.* **2007**, *14*, 1315.
(35) Goto, Y.; Katoh, T.; Suga, H. *Nat. Protoc.* **2011**, *6*, 779.
(36) Ohashi, H.; Shimizu, Y.; Ying, B. W.; Ueda, T. *Biochem. Biophys. Res. Commun.* **2007**, *352*, 270.
(37) Murakami, H.; Kourouklis, D.; Suga, H. *Chem. Biol.* **2003**, *10*, 1077.
(38) Herr, A. J.; Wills, N. M.; Nelson, C. C.; Gesteland, R. F.; Atkins, J. F. *J. Mol. Biol.* **2001**, *311*, 445.
(39) Cruz-Vera, L. R.; Magos-Castro, M. A.; Zamora-Romo, E.; Guarneros, G. *Nucleic Acids Res.* **2004**, *32*, 4462.
(40) Kawakami, T.; Murakami, H.; Suga, H. *J. Am. Chem. Soc.* **2008**, *130*, 16861.
(41) LaRiviere, F. J.; Wolfson, A. D.; Uhlenbeck, O. C. *Science* **2001**, *294*, 165.
(42) Asahara, H.; Uhlenbeck, O. C. *Biochemistry* **2005**, *44*, 11254.
(43) Starck, S. R.; Qi, X.; Olsen, B. N.; Roberts, R. W. *J. Am. Chem. Soc.* **2003**, *125*, 8090.
(44) Pavlov, M. Y.; Watts, R. E.; Tan, Z.; Cornish, V. W.; Ehrenberg, M.; Forster, A. C. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 50.
(45) Kane, J. F.; Violand, B. N.; Curran, D. F.; Staten, N. R.; Duffin, K. L.; Bogosian, G. *Nucleic Acids Res.* **1992**, *20*, 6707.
(46) Frackenpohl, J.; Arvidsson, P. I.; Schreiber, J. V.; Seebach, D. *ChemBioChem* **2001**, *2*, 445.

DOI: 10.1021/jacs.5b12482
J. Am. Chem. Soc. 2016, 138, 1962−1969