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A peptidyl transferase ribozyme capable of combinatorial peptide synthesis

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Abstract—The formation of peptide bonds is a key step in both the chemical and biological synthesis of peptides. The ribozyme can use a wide range of amino acids as its substrate for the dipeptide synthesis. A library containing 29 peptides whose synthesis was catalyzed by this unique ribozyme was analyzed by mass spectrometry. These results implicate that ribozyme may have potential application in the peptide synthesis.

1. Introduction

There is considerable interest in the peptide synthesis. Polypeptide synthesis using solid-phase methods pioneered by Merrifield[1] is capable of producing the peptides up to 100 amino acid residues. Although solid-phase peptide synthesis has made great progresses in recent years, such syntheses still contain the number of side products that accumulate over the many coupling steps that often makes purification of the final product laborious. Chemical synthesis of proteins is one of the most powerful approaches for constructing proteins of novel design and structure.[2] However, an ideal biocatalyst like the ribosomal peptidyl transferase is not available, and multi-enzyme complexes in bacterial peptide synthesis are limited to specific purposes, only proteases can be used for formation of the peptide bond.[3] The protein-synthesizing enzyme in principle would display high catalytic efficiency for all theoretically possible combinations of amino acids. But an ideal applicable method for the formation of peptide bond has still not been found. The catalytic RNA (ribozyme) might have the potential application as peptidyl transferase for peptide synthesis.

In recent years, in vitro selection and in vitro evolution methods[4] have provided powerful tools for isolating ribozymes that can catalyze a wide range of chemical and biochemical reactions. Molecules have been isolated from pools of random RNA sequences that confer polynucleotide kinase activity,[5] catalyze a polymerase-like reaction,[6] and promote alkylation[7] and carbon-sulfur bond formation.[8] A RNA molecule isolated from a large library of RNAs catalyzed the isomerization of a bridged biphenyl,[9] the Diels–Alder reaction,[10] the insertion of copper into porphyrin,[11] aminoacyl transfer reactions to form 3′-terminal[12] or 2′-internal aminoacyl esters,[13] and 5′-terminal esters or amide bonds.[14] RNA can also catalyze nucleotide synthesis,[15] peptide bond formation,[16] Michael-addition reaction[17] and RNA polymerization.[18] These findings have greatly expanded the catalytic diversity and versatility of RNA.

Through in vitro selection we have isolated one ribozyme family that catalyzed the peptide bond formation using *N*-biotinylated aminoacyl-adenylates as its substrates.[19] One ribozyme (R180) has been characterized by kinetic and structural studies, which can efficiently catalyze the peptide bond formation using almost any amino acid. In the ribozyme reaction, the amino group of phenylalanine of the peptidyl acceptor as a nucleophile attacks the carbonyl carbon of methionine of *N*-biotinylamidocaproyl-methionyl-adenylate to form a peptide bond (Fig. 1). The ribozyme demonstrated little amino acid specificity for the peptide bond-forming reactions and can use almost all possible combinations of amino acids to make the peptide bond. Here, we report the combinatorial dipeptide synthesis catalyzed by the R180 ribozyme.

Keywords: Combinatorial synthesis; Ribozyme; Peptide bond formation; Peptidyl transferase.
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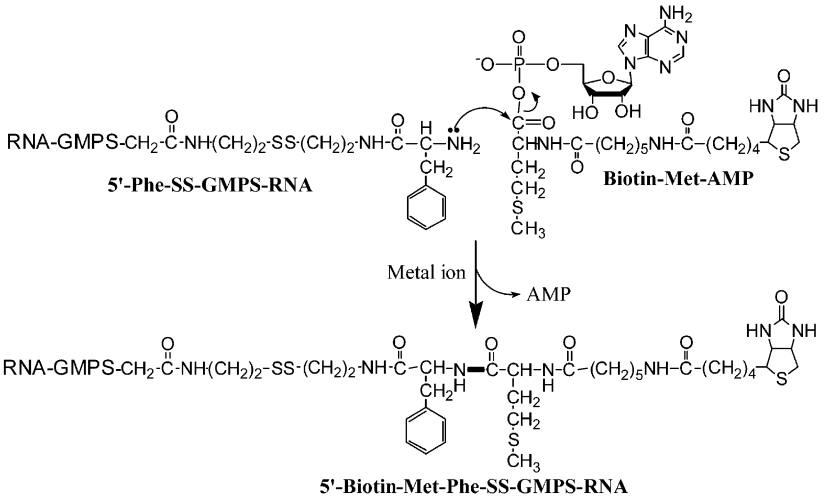

Figure 1. The peptide bond-forming reaction of biotin-Met-adenylate with 5′-Phe-SS-GMPS-RNA.

2. Results and discussion

2.1. Preparation of the substrates and ribozyme

N-Biotinylamidocaproyl-methionyl-adenylate anhydride (biotin-Met-AMP) was synthesized by the modified report method.[20] The biotinylamidocaproic acid was coupled with *N*-hydroxysuccinimide (NHS) catalyzed by 1,3-dicyclohexyl carbodiimide (DCC) and then reacted with L-amino acids to give *N*-biotinylamidocaproyl-L-amino acids. The *N*-biotinylamidocaproyl-L-amino acid was coupled with adenosine-5′-monophosphate in the presence of DCC in pyridine-water and 0.8 mM HCl to produce the desired biotin-aminoacyl-adenylate anhydrides in 20–50% yield. The final product was purified by the reverse-phase chromatography eluted with a gradient of water/methanol (0–50%) and characterized by proton and phosphorus-NMR spectroscopy, HPLC, and mass spectroscopy. The *N,N*′-bis(bromoacetyl)-cystamine was synthesized by the coupling reaction of cystamine with the three times excess of bromoacetyl-*N*-hydroxysuccinimide in anhydrous DMF. The synthesis of L-aminoacyl-pyridyldithioethylamide (aa-PDA) was accomplished in three steps.[21] First, cysteamine was reacted with a 2-fold excess of 2,2′-dithiodipyridine to produce the pyridyldithioethylamine (PDA) that was then coupled with *N*-Boc-L-aminoacyl-*N*′-hydroxysuccinimide esters to produce the Boc-L-aminoacyl-PDA that was treated with TFA to remove the Boc group to afford the desired products L-aminoacyl-PDA. The final products were purified by silica-gel column eluted with a gradient of methanol/chloroform (0–30%). The ribozyme containing phenylalanine attached via a disulfide linker to the 5′-end of the RNA molecules was prepared by a two-step method to incorporate different amino acids into the 5′-end of RNA molecules. The in vitro transcribed 5′-GMPS-RNA was first coupled with *N,N*′-bis(bromoacetyl) cystamine and then treated with dithiothreitol (DTT) to generate 5′-HS-$CH_2CH_2NHCOCH_2$-GMPS-RNA, and finally reacted with L-aminoacyl-pyridyldithioethylamide to produce the 5′-aminoacyl-$NHCH_2CH_2SSCH_2CH_2NHCOCH_2$-GMPS-RNA (5′-aminoacyl-SS-GMPS-RNA).

2.2. Dipeptide library synthesis

Figure 2 depicts the synthesis of a 5′-aminoacyl-SS-GMPS-RNA pool and the set up for investigating combinatorial dipeptide synthesis. The R180 ribozyme-linked amino acid pool was prepared by coupling 5′-cysteamine-GMPS-RNA with an equimolar mixture of five aminoacyl-PDA (Phe-PDA, Leu-PDA, Gln-PDA, Lys-PDA, and Trp-PDA). Because the disulfide exchanging reaction of 5′-$HSCH_2CH_2NHC(O)CH_2$-GMPS-RNA with aminoacyl-PDA was fast and displayed no preference for amino acid side chains, the aminoacyl moieties were linked equally to the 5′-end of the R180 ribozyme. The aminoacyl-SS-GMPS-RNA mixture was reacted with an equimolar mixture of six biotin-aminoacyl-AMP anhydrides (Met, Leu, Phe, Gln, and Arg) to generate a peptide library containing thirty dipeptides.

Five authentic biotin-aa′-aa-cysteamines, Ala-Gln, Gln-Gln, Met-Phe, Leu-Trp, and Phe-Phe, were chemically synthesized to identify the peptide library. We used these five authentic compounds to optimize the HPLC conditions and to refine the methods for mass spectrometry. The peptide library was identified by LC-MS with a positive ion mode. The results are presented in Table 1. We observed the mass peaks (M + 1) for all expected biotin-aa-aa′-cysteamine peptides except biotin-Met-Trp-cysteamine that might not be detected by MS by experimental error and we believed that the library should contain this peptide product. In Table 1, the third column lists the calculated exact mass and fourth column lists the observed mass peaks for each peptide compound. All observed mass peaks of the peptide products agreed with the calculated exact masses. The mass spectra of five authentic compounds are shown in Figure 3A. Figure 3B shows twenty-four mass spectra of the R180 ribozyme-mediated peptide products. The mass peaks of biotin-Ala-Gln-cysteamine, biotin-Gln-Gln-cysteamine, biotin-Met-Phe-cysteamine, biotin-Leu-Trp-cysteamine, and biotin-Phe-Phe-cysteamine were almost identical with the mass peaks of the five authentic compounds. Although some peptide

products could not be determined by MS because they have share the same or a close mass, they had a different retention times by HPLC. For example, the calculated masses were 615.29 and 615.32 for biotin-Ala-Gln-cysteamine and biotin-Ala-Lys-cysteamine, and 691.32, 691.32, and 691.35 for biotin-Gln-Phe-cysteamine, biotin-Phe-Gln-cysteamine, and biotin-Phe-Lys-cysteamine, respectively. We observed two components on the total ion chromatogram (TIC) with a mass of 616.5 and three components on the TIC for the masses of 692.9/692.8. The combination of HPLC and ESI-MS constituted a rapid and effective method for characterizing the peptide products generated by the R180 ribozyme. Overall, the LC-MS analyses identified 29 peptide products generated by the R180 ribozyme. These results demonstrate that the R180 ribozyme is able to synthesize

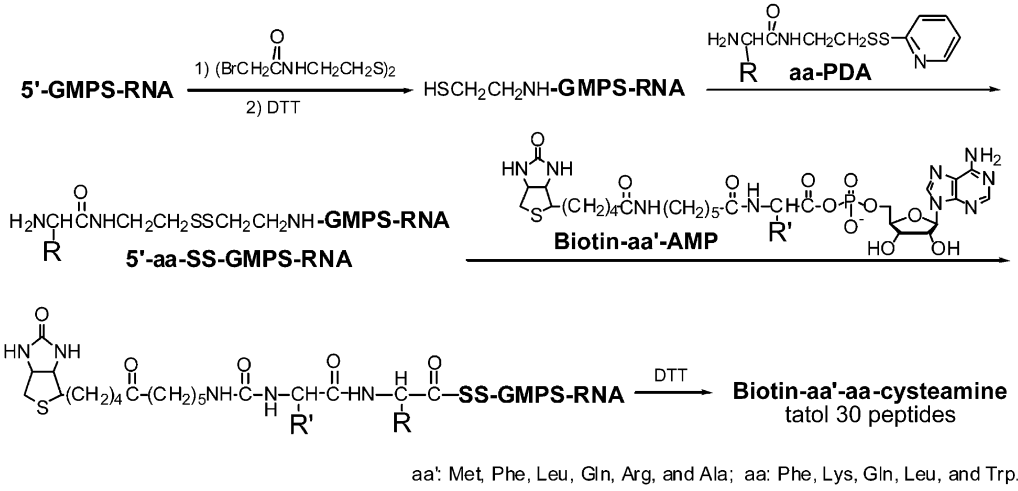

Figure 2. Random dipeptide synthesis by the R180 ribozyme. The in vitro transcribed 5′-GMPS-RNA was reacted with *N,N′*-bromoacetylcystamine and then treated with DTT to yield 5′-cysteamine-GMPS-RNA. 5′-Cysteamine-GMPS-RNA was coupled with an equimolar mixture of aminoacyl-pyridyldithioethylamide (aa′-PDA) to produce 5′-aa-SS-GMPS-RNAs (aa = Phe, Lys, Gln, Leu, and Trp) that were then incubated with six biotin-aa′-AMP substrates (Met, Leu, Phe, Gln, and Arg).

Table 1. LC-MS analysis of random-synthesized peptide products by the R180 ribozyme[a]

Entry	Product	Calculated exact mass	Observed mass peaks $(M+1)^+$	Authentic comp. $(M+1)^+$ and $(M+Na)^+$
1	Biotin-Ala-Leu-cysteamine	600.31	601.1	—
2	Biotin-Ala-Gln-cysteamine	615.29	616.5, 616.5	616.3, 638.7
3	Biotin-Ala-Lys-cysteamine	615.32		—
4	Biotin-Ala-Phe-cysteamine	634.30	635.1	—
5	Biotin-Leu-Leu-cysteamine	642.36	643.7	—
6	Biotin-Leu-Gln-cysteamine	657.33		—
7	Biotin-Gln-Leu-cysteamine	657.33	658.5	—
8	Biotin-Leu-Lys-cysteamine	657.37		—
9	Biotin-Met-Leu-cysteamine	660.32	661.1	—
10	Biotin-Gln-Gln-cysteamine	672.31	673.0, 673.1	673.4, 695.5
11	Biotin-Gln-Lys-cysteamine	672.35		—
12	Biotin-Ala-Trp-cysteamine	673.31	674.1	—
13	Biotin-Met-Gln-cysteamine	675.29	676.5, 676.6	—
14	Biotin-Met-Lys-cysteamine	675.33		—
15	Biotin-Leu-Phe-cysteamine	676.34	677.8	—
16	Biotin-Phe-Leu-cysteamine	676.34		—
17	Biotin-Arg-Leu-cysteamine	685.38	686.4	—
18	Biotin-Gln-Phe-cysteamine	691.32	692.9, 692.8, 692.9	—
19	Biotin-Phe-Gln-cysteamine	691.32		—
20	Biotin-Phe-Lys-cysteamine	691.35		—
21	Biotin-Met-Phe-cysteamine	694.30	695.1, 717.0 (M + Na)	695.4, 717.5
22	Biotin-Arg-Gln-cysteamine	700.35	701.7	—
23	Biotin-Arg-Lys-cysteamine	700.39		—
24	Biotin-Phe-Phe-cysteamine	710.33	711.1	711.4, 733.6
25	Biotin-Leu-Trp-cysteamine	715.35	716.8	716.4, 738.6
26	Biotin-Arg-Phe-cysteamine	719.36	720.0	—
27	Biotin-Gln-Trp-cysteamine	730.33	731.7	—
28	Biotin-Met-Trp-cysteamine	733.31	None	—
29	Biotin-Phe-Trp-cysteamine	749.34	750.2	—
30	Biotin-Arg-Trp-cysteamine	758.37	759.6	—

[a] Analytic HPLC conditions: C18 reverse-phase column; 500 μL/min flow rate; 10 mM acetic acid, pH 3.45; gradient 10–40% acetonitrile/30 min, 40–100% acetonitrile/5 min, and 100% acetonitrile/5 min. ESI-MS conditions: positive ion electrospray mode, 250 °C capillary temperature, 17.0 V capillary voltage, and −4.5 kV spray voltage.

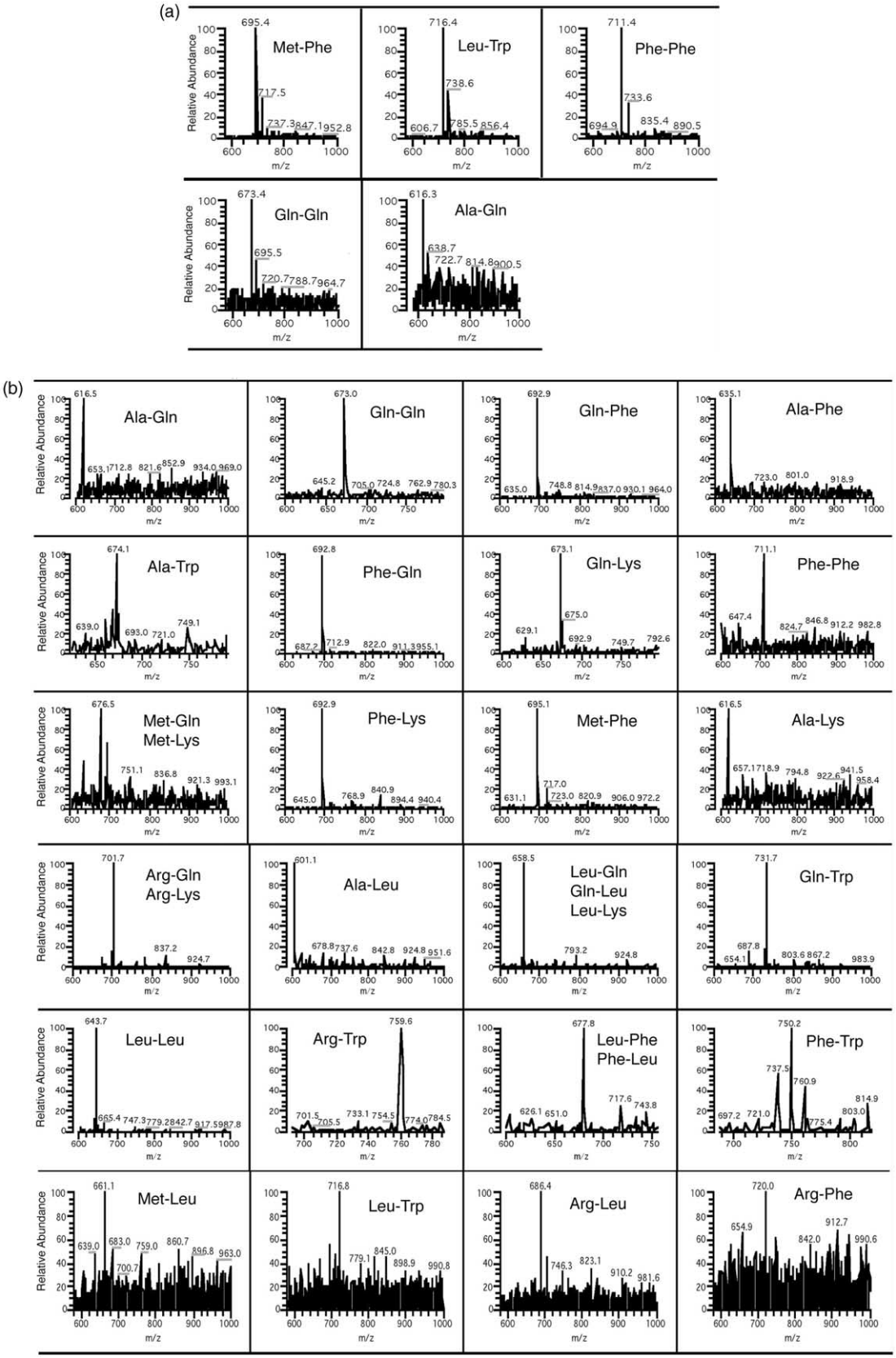

Figure 3. ESI-MS spectra of Biotin-aa′-aa-cysteamine. Y axis is relative abundance and X axis is m/z. (**A**) ESI-MS positive ion spectra of five authentic biotin-aminoacyl-aminoacyl-cysteamine compounds: Gln-Gln, Phe-Phe, Met-Phe, Leu-Trp, and Arg-Leu. Authentic compounds were used for optimizing the HPLC conditions and the LC-MS analysis. (**B**) ESI-MS spectra of the random dipeptide products synthesized by the R180 ribozyme.

a highly diverse combinatorial dipeptide library whose complexity approaches the theoretical prediction.

2.3. Leaving group activity

The aminoacyl-adenylate is an active intermediate for the protein biosynthesis in all living systems. The aminoacyl-adenylate is not very stable and its synthesis often gives a low yield. In order to approach the practical application of the peptidyl transferase ribozyme for peptide synthesis, we have investigated the peptide bond formation catalyzed by the R180 ribozyme using a various substrates with a different leaving group. The biotin-methionyl-*p*-nitrophenyl ester (biotin-Met-PNP) and biotin-methionyl-*N*-hydroxy-succinimide ester (biotin-Met-NHS) were synthesized by the DCC coupling reaction of *N*-biotin-methionine with *p*-nitrophenol and *N*-hydroxy-succinimide, respectively. The biotin-methionyl-cyanomethyl ester (biotin-Met-OCH_2CN) and biotin-methionyl-imidazolide (biotin-Met-Im) were also prepared by the standard manners. Figure 4A displays the streptavidin gel-shift results of the peptide bond formation of biotin-Met-PNP (lanes 16–20, Fig. 4A), biotin-Met-NHS (lanes 11–15, Fig. 4A), biotin-Met-Im (lane 6–10, Fig. 4A), and biotin-Met-OCH_2CN (lanes 21–25, Fig. 4A) with 5′-Phe-SS-GMPS-RNA under the same conditions as described above. Surprisingly, all substrates are active for the peptide bond formation by R180 ribozyme. The biotin-Met-NHS and biotin-Met-Im are almost 10-fold more active than biotin-Met-AMP for the peptide bond formation (Fig. 4B). Interestingly, the biotin-Met-PNP ester is also about 2.5 times more active than biotin-Met-AMP for the R180 ribozyme. The biotin-Met-PNP ester might be an interesting substrate for the peptide synthesis catalyzed by the ribozyme because aminoacyl-PNP esters are easy to

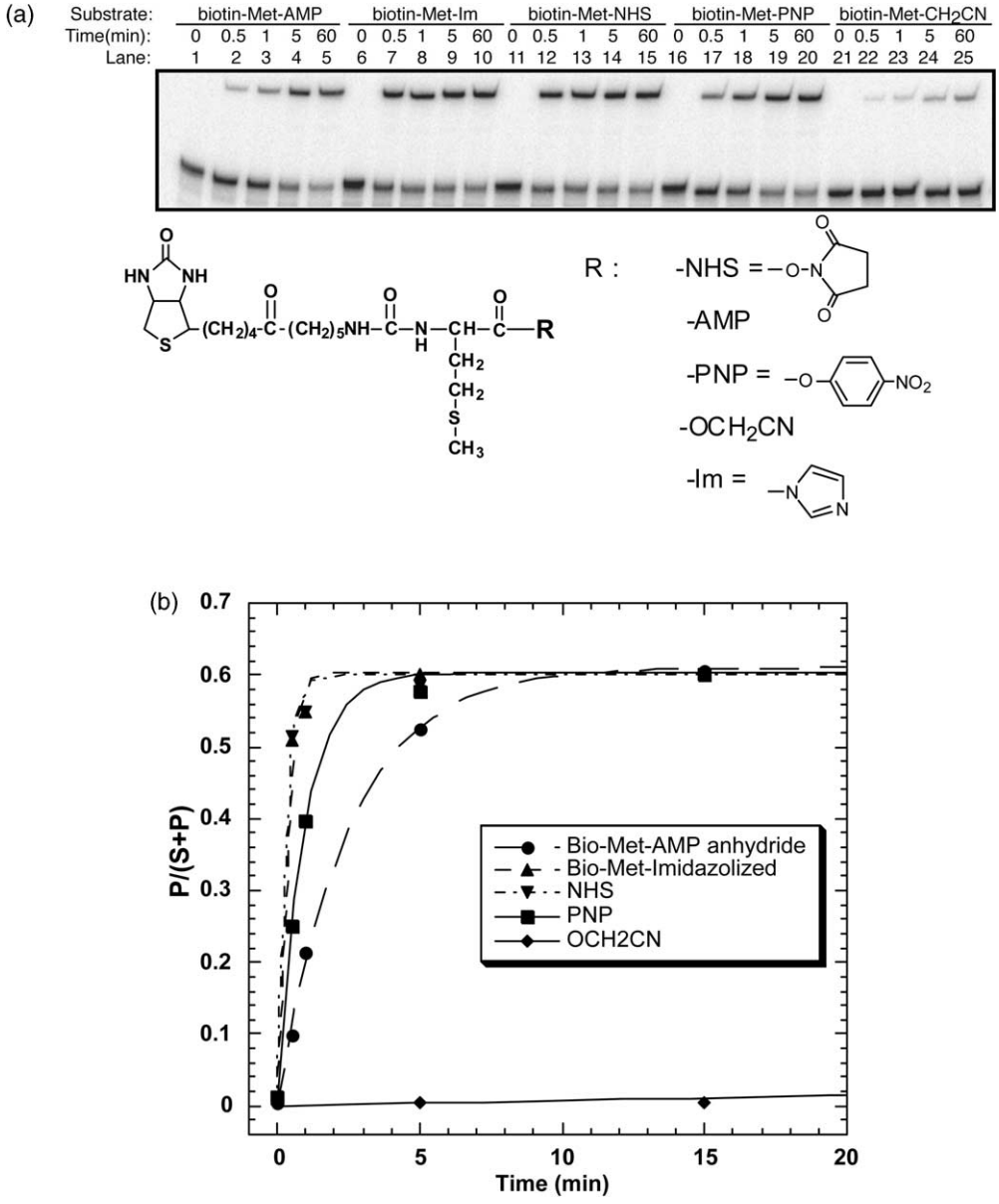

Figure 4. The peptide bond-forming activity of the substrates with a different leaving group. (**A**) Autoradiogram of peptide bond-forming reactions of 5′-Phe-SS-GMPS-RNAs with five biotin-Met derivatives. The conditions were the same as described in Section 4.4 of the Experimental. (**B**) The plots of the time courses of the peptide bond formation by R180 ribozyme.

be synthesized. These results suggested that the leaving group of the substrate is not the binding site for R180 ribozyme and the biotin of the substrate is the binding site (data not shown).

3. Conclusion

This study implicates that the catalytic RNA may have the potential application in the peptide synthesis. The ribozyme can function as the peptidyl transferase for the protein synthesis. Our selected ribozyme is capable of catalyzing random dipeptide synthesis, thus further supporting the powerful RNA catalysis. As proposed that the polypeptide may be synthesized by multiple ribozymes mimicking the modular polyketide and non-ribosomal polypeptide synthesis.[19] Such, the study implied that the ribozyme might be able to make randomized polypeptide library. The ribozyme may function as a general peptide-synthesizing enzyme to make the peptide.

4. Experimental

4.1. Ribozyme preparation

The double stranded DNA as the template for in vitro transcription was made by PCR amplification in 10 mM Tris–HCl; pH 8.3; 50 mM KCl; 0.01% (W/V) gelatin; 0.05% Tween 20; 0.2 mM dNTPs; 1.0 μM primers and 3.0 mM $MgCl_2$, in 30 cycles (94 °C, 1 min; 52 °C, 1 min; 72 °C, 2 min). In vitro transcription was performed with 10 μg of PCR-amplified 196-mer DNA in a 250 μL reaction mixture (40 mM Tris–HCl, pH 7.5; 10 mM DTT; 4 mM spermidine; 0.05% Triton X-100; 12 mM $MgCl_2$; 1.0 mM each ATP, CTP, UTP; 8 mM GMPS; 1.0 mM GTP; and T7 RNA polymerase) for 3 h at 37 °C. For ^{32}P-labaled RNA was transcribed in the presence of 10 μCi [α-^{32}P]-ATP. 5′-GSMP-RNA was purified by electrophoresis on a denaturing 8% polyacrylamide gel. 5′-GMPS-RNA was reacted with *N*-bromoacetyl-*N*′-phenylalanyl-cystamine (10 mM) in the chemical linkage buffer (40 mM HEPES, pH 8.0; 150 mM NaCl, and 1 mM EDTA) for 2 h at room temperature. The reaction mixture was extracted with chloroform/phenol once, chloroform once, and precipitated with ethanol to give 5′-Phe-SS-GMPS-RNA.

4.2. Biotin-L-aminoacyl-adenylate synthesis

The described[20] synthesis of biotin-Met-AMP anhydride was modified as follows. 0.8 M HCl (138 μL) was added to a mixture of *N*-biotinylamidocaproyl-L-amino acid (0.1 mmol) and nucleoside-5′-monophophate (0.11 mmol) in water (51 μL) and pyridine (572 μL) at 0 °C. A solution of dicyclohexylcarbodiimide (DCC) (2.6 mmol) in pyridine (600 μL) was added. The mixture was stirred in an ice-water bath for 3.5 h. After removing the precipitation, the solution was precipitated with acetone at −70 °C and collected by centrifugation. The pellet was washed with ethyl ether (30 mL), dissolved in water at 0 °C, and immediately applied to a C18 reverse-phase column and eluted with water/methanol (0–50%). The desired fractions were evaporated quickly within 10 min by vacuum pump and the aqueous solution was lyophilized to yield a white solid. ^{1}H NMR (D_2O): δ 8.45 (m, 1H), 8.26 (m, 1H), 6.12 (d, $J = 6.87$ Hz, 1H), 4.74 (m, 1H), 4.57–4.46 (m, 3H), 4.36 (m, 2H), 4.21 (m, 2H), 3.25 (m, 1H), 3.09 (m, 2H), 2.96 (m, 1H), 2.72 (m, 1H), 2.52-2.39 (m, 2H), 2.25–1.89 (m, 9H), 1.68–1.22 (m, 12H); ^{31}P NMR (D_2O): δ-7.52; ESI-MS (M-H): calculated 816.3, found 816.3. Other biotin-aminoacyl-AMP anhydrides were prepared as above. The compounds were characterized by NMR, HPLC, and mass spectrometry.

4.3. Synthesis of L-aminoacyl-pyridyldithioethylamide

Pyridyldithioethylamine (PDA) was prepared by the procedures described in the literature.[21] The L-aminoacyl-PDA derivatives (Phe-PDA, Leu-PDA, Gln-PDA, Lys-PDA, and Trp-PDA) were synthesized as follows. A solution of PDA (0.74 g, 3.3 mmol) and 1.0 mL of diisopropylethylamine (DIEA) in 10 mL of anhydrous dimethylformamide (DMF) at room temperature was added into a solution of *N*-Boc-aminoacyl-*N*-hydroxysuccinimidyl (NHS) ester (3.0 mmol) in 10 mL of anhydrous DMF. The mixture was stirred for two days at room temperature. After removing the DMF solvent, the solid was dissolved in 100 mL of chloroform and then washed with water three times. The organic layer was dried over anhydrous Na_2SO_4. The crude product was purified by a silica gel column eluted with methanol/chloroform (0–5%). The pure Boc-aminoacyl-PDA compounds were dissolved in 20 mL of methylene chloride and 2.0 mL of trifluoroacetic acid (TFA) was dropwise added at 0 °C. The mixture was stirred for one h at 0–5 °C and 2 h at room temperature. The reactions were monitored by thin layer chromatography (TLC) of silica gel plate run in chloroform/methanol, 8:2 (vol/vol). After removing TFA and solvent, the material was applied to flash column chromatography (silica gel) and eluted with chloroform/methanol (0–20%). The final products were confirmed by NMR and mass spectrometry.

4.4. Activity assay

The reactions were performed with 0.5 μM 5′-Phe-SS-GMS-RNA (R180) and 50 μM biotin-Met-AMP in the presence of 300 mM KCl, 100 mM $MgCl_2$, and 50 mM HEPES buffer (pH 7.4) at 25 °C. The RNA was pre-incubated for 10 min at 50 °C and then slowly cooled to room temperature. Reactions were initiated after addition of biotin-aminoacyl-AMP substrate. Aliquots of 2 μL were removed from a 20 μL reaction mixture at specific time points, quenched with equal volumes of stop buffer [100 mM HEPES (pH 7.4), 100 mM EDTA, 90% formamide, 0.01% bromophenol blue, and 0.025% xylene cyanol] and frozen on dry ice. Thawed samples were incubated with 7.5 μg of streptavidin for 20 min. The biotinylated RNA products were resolved by electrophoresis on 7.5 M urea/8% polyacrylamide gels with 1×TBE buffer at 800 V at 4 °C. The fraction of product formation relative to total substrate and product at each time point was quantitated using a Molecular Dynamics PhosphorImager.

4.5. Dipeptide library synthesis

The in vitro transcribed 5′-GMPS-RNA was reacted with 2 mM *N, N*′-di(bromoacetyl)-cystamine in the chemical linkage buffer at room temperature for 3 h and then treated with 10 mM DTT to yield 5′-cysteamine-GMPS-RNA. After chloroform/phenol extraction, RNA was precipitated with ethanol and dissolved in degassed H_2O. 5′-Cysteamine-GMPS-RNA was coupled with five 20 mM aminoacyl-pyridyldithioethyl amides (Phe, Leu, Lys, Gln, and Trp) mixture in chemical linkage buffer for 3 h to yield 5′-aminoacyl-SS-GMPS-RNA pool. A 4 μM aminoacyl-SS-GMPS-RNA was preincubated in the presence of 100 mM $MgCl_2$, 300 KCl, and 50 mM HEPES buffer (pH 7.4) for 10 min at 50 °C. The reaction was initially incubated with an equimolar mixture of 100 μM biotin-aminoacyl-AMP substrates (Met, Leu, Ala, Phe, Gln, and Arg) in a total 2.0 mL volume. The biotin-aminoacyl-AMP was added to the reaction mixture as 20 μL portions of a 5.0 mM stock in 5 intervals every 1 h. The final reaction mixture was extracted with phenol/chloroform twice and chloroform once, and then precipitated with 3 volumes of ethanol. The RNA product obtained by centrifugation was dissolved in deionized water. After treatment with DTT, the mixture was divided into two parts. One part was used directly for LC-MS analysis. The other was extracted twice with phenol/chloroform and once with chloroform. The combined organic layers were evaporated to dryness and then dissolved in DMF for LC-MS analysis.

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