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Construction of a library of structurally diverse ribonucleopeptides with catalytic groups

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ABSTRACT

Functional screening of structurally diverse libraries consisting of proteins or nucleic acids is an effective method to obtain receptors or aptamers with unique molecular recognition characteristics. However, further modification of these selected receptors to exert a newly desired function is still a challenging task. We have constructed a library of structurally diverse ribonucleopeptides (RNPs) that are modified with a catalytic group, in which the catalytic group aligns with various orientations against the ATP binding pocket of RNA subunit. As a proof-of-principle, the screening of the constructed RNP library for the catalytic reaction of ester hydrolysis was successfully carried out. The size of both the substrate-binding RNA library and the catalytic group modified peptide library are independently expandable, and thus, the size of RNPs library could be enlarged by a combination of these two subunits. We anticipate that the library of functionalized and structurally diverse RNPs would be expanded for various other catalytic reactions.

1. Introduction

Biomacromolecular receptors provide useful frameworks for constructing sensors with high specificity for a particular ligand. Efforts have been taken to construct biosensors through modification of proteins and nucleic acids receptors with appropriate fluorophores[1–5] or artificial enzymes with a catalytic group to achieve proximity effect between the substrate and the catalytic group.[6,7] However, naturally occurring receptors do not always provide the recognition characteristics for the molecule of interest, and rational design of a receptor with desired specificity is still a challenging task. A library of RNA molecules that differ in their three-dimensional structures by means of randomized nucleotide sequences has been applied for the selection of receptors for the target ligands.[8–10] Although the library-based selection offers one of the promising methods to obtain aptamers with the specific recognition characteristics for the molecule of interest, further modification of the selected aptamers to exert a newly desired function is often difficult due to the lack of their structural information. In addition, the original substrate binding characteristics of the aptamer could be impaired by the introduction of a new functional group. Synthetic functional groups were incorporated to biomacromolecules in the library by modification of proteins or nucleic acids via chemical modification or genetic mutation.[11,12] These chemical or genetic methods require laborious tasks to generate a library with limited size. Therefore, an alternative method is required to construct a large size library to increase the possibility of selecting a "hit" biomacromolecule with the function of interest.

Ribonucleopeptide (RNP) is one of the appropriate scaffolds to construct a library with structurally diverse RNA-peptide complexes. We have reported a method to obtain ATP-binding RNP receptors by in vitro selection of a library of RNP with randomized RNA sequences.[13] Because the RNA subunit works as a receptor for the substrate, the selected ATP-binding RNP receptor was further converted into a new RNP library by complexation of the RNA subunit and a library of Rev peptides, in which a peptide loop with randomized amino acid residues was incorporated at the N-terminus of Rev peptide. An ATP-binding RNP receptor selected from this peptide-based RNP library exerted higher ATP-binding specificity than the original RNP (Fig. 1a).[14] A fluorescent RNP library was also constructed by combining the RNA subunit library and a library containing fluorophore-modified Rev peptides (Fig. 1a).[15–17] The original substrate-binding ability of the RNP receptor was maintained in the selected RNP sensor even upon modification of the Rev peptide by a fluorophore. By taking advantage of the noncovalent complex formation of RNP, combination of the RNA library and the peptide library would dramatically increase the size of the RNP library. Therefore, RNP is a good candidate of scaffolds to construct a library of structurally diverse biomolecular assemblies containing a substrate-binding pocket and a synthetic functional group.

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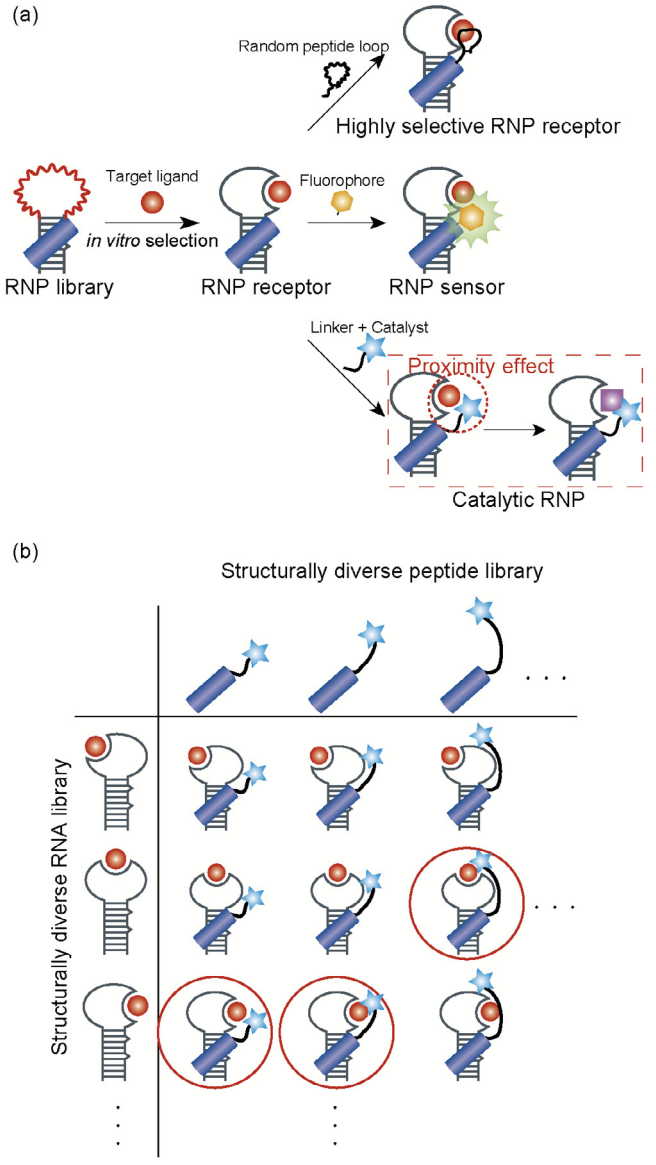


Fig. 1. (a) Schematic illustration of the stepwise molding of RNP receptors. The RNP receptor was first selected by in vitro selection of the RNP library. The resulting RNP receptor was converted to more selective RNP receptors by a suitable peptide loop containing randomized amino acid residues[14] or to fluorescent RNP sensors by fluorophore modification.[15–17] Also, an RNP library containing catalytic groups in the peptide subunit could provide catalytic RNPs. (b) Combination of a structurally diverse RNA library and a structurally diverse peptide library affords the construction of a library of RNPs, in which the catalytic group aligns with various orientations to the substrate-binding pocket.

In this study, we have constructed a library of structurally diverse RNP receptors equipped with a synthetic functional group. A series of RNA subunits obtained by the in vitro selection of ATP-binding RNP receptors[15,17] afforded a library of structurally diverse RNAs consisting of the ATP-binding region and the RRE (*R*ev *R*esponsive *E*lement) nucleotide sequence. A library of peptides was constructed by modification of the Rev peptide with catalytic groups through various peptide linkers (Fig. 1b). Combination of these two types of libraries by the complex formation between the RRE RNA and the Rev peptide generated a library of functionalized and structurally diverse RNPs, in which the catalytic group would align with various orientations against the ATP binding pocket of RNA subunits.

The functionalized and structurally diverse RNPs were expected to exhibit the ATP-binding ability. An adenosine derivative with an ester group was utilized to select the RNPs that could accelerate the ester hydrolysis reaction by the proximity effect between the ATP binding domain and the catalytic group on the peptide (Fig. 1a). Screening of the RNP library was successfully performed for the hydrolysis reaction of the ester derivative of adenosine to obtain catalytic RNPs.

2. Results and discussion

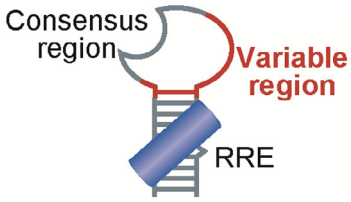
2.1. Construction of a functionalized structurally diverse RNP library

A library of structurally diverse RNPs with various geometries between the substrate binding pocket and the catalytic group was constructed by combination of the RNA subunits of ATP-binding RNP and a library of Rev peptide modified with catalytic groups. From the RNA-oriented RNP library, a series of ATP-binding RNP receptors was obtained through in vitro selection[15,17] with a variation in the RNA sequences (Fig. 1a). The nucleotide sequence of ATP-binding RNP consisted of three segments, the RRE, consensus, and variable regions (Fig. 2). The consensus region of the ATP-binding RNP receptors forms the binding pocket for the substrate adenosine.[15,17] The consensus and the RRE regions were connected through the variable region that differ in various length and sequences of the nucleotides. The structural diversity in the variable region affords diverse structures of RNPs, which in turn exhibits a variety of geometries between the ATP-binding pocket and the Rev peptide that is bound to the RRE sequence.

The N-terminus of Rev peptide in RNP can be chemically modified without diminishing the binding affinity of Rev peptide to the RRE sequence.[14–17] Judging from the structure of Rev-RRE complex,[18] the N-terminus of Rev peptide in RNP should locate closer to the binding-pocket than its C-terminus. Therefore, the N-terminus of Rev peptide is a suitable position for the modification by a catalytic group. N,N-Dimethyl-4-aminopyridine (dmap), 4,4′-dimethyl-2,2′-bipyridine (bpy) and histidine (H) were chosen as possible catalytic groups for the ester hydrolysis reaction. In addition, peptide linkers with various amino acid sequences were introduced between the catalytic groups and the N-terminus of Rev peptides. Four types of short peptide motifs were selected from known RNP structures[19–22] (Fig. 3) to achieve a structural diversity (Fig. 4). The motif pa (Fig. 3b) traverse the phosphate backbone as found in the eukaryotic ribosome structure (Fig. 3a), the motif pb (Fig. 3d) snugly fits in the major groove of RNA duplex in the Tav2b/siRNA complex (Fig. 3c), the motif pc (Fig. 3f) binds along the phosphate backbone in the complex of Mss16p DEAD-box helicase domain 2/RNA duplex (Fig. 3e), and the motif pd (Fig. 3h) passes over the phosphate backbone from the major groove to the minor groove as found in the small RNA methyltransferase HEN1 complex (Fig. 3g). Synthetic flexible linkers containing multiple repeating sequences (GGS) were also incorporated between the N-terminus of Rev peptide and the catalytic group (pe and pf). Combination of the RNA subunit library and the peptide subunit library afforded a structurally diverse RNP library with catalytic group, in which the substrate ATP resided in various orientation and distances to the catalytic group (Fig. 1b).

2.2. Design and synthesis of an ester derivative of adenosine for facile screening of catalytic RNPs

Spectroscopic monitoring of the *p*-nitrophenyl ester hydrolysis is a rapid, sensitive and quantitative way to evaluate the catalytic activity of biomacromolecules.[23,24] The *p*-nitrophenyl ester has been used as the reporter motif of substrate because it shows little or no absorbance of around 400 nm in water but provide characteristic absorbance of *p*-nitrophenolate upon hydrolysis of the



No.	RRE	Variable region	Consensus region		Consensus region	Variable region	RRE
A02	GGUCUGGGCGCA	UUGCAU	GUUGUGG	GUA	UGUGUGUG	AUGUAU	UGACGGUACAGGCC
A08	----------	UUUUCAUGGCC	CUUGUGG	GAAGGAU	UGUG	A	----------
A09	----------	CUGGUGU	GUAGUGG	GC	UGUGUGUG	AUGCCG	----------
A14	----------	UACUGC	GUAGUGG	UU	GGUGUGUG	GCGGUUU	----------
A15	----------	GCAGU	GUAGUGG	UU	UGCGUGUG	AUUGCUUA	----------
A17	----------	UUAGAU	GUAGUGG	GU	AGUGUGUG	UUUUCUG	----------
A24	----------	UGAUUGC	GUAGUGG	UU	UGUGUGUG	GUUGUC	----------
A25	----------	UGCUG	GUAGUGG	GUA	UGUGUGUG	CGGCAUU	----------
A26	----------	UUCCG	GUAGUGG	UUG	UGUGUGUG	CGGUUUU	----------
A30	----------	UAUACC	GUAGUGG	UU	UCUGUCGG	GGUUGG	----------
A33	----------	GUUUGCUGUUGCCGU	GUAGUGG	UU	UGUGUG		----------
A34	----------	U	GUAGUGG	UGCCUGUGAUGGC	UGUGAUGG	A	----------
A36	----------		GUGGUGG	UUCG	UCUGGGUG	CGCUGGUUUGU	----------
An02	----------	CAAUAUC	GUAGUGG	UGA	GGUGUGUG	GAUAU	----------
An03	----------	UU	GUAGUGG	UUUC	UCUGUCGG	AA	----------
An08	----------	AU	GUAGUGG	UUU	UGUGUGUG	AUU	----------
An09	----------	AC	GAAGUGG	UUU	UGAGUGUG	GU	----------
An10	----------		UUAGUGG	UUU	UGUGUGUG	U	----------
An15	----------	AU	GUAGUGG	UU	UGUGUGUG	AU	----------
An16	----------	C	GUAGUGG	UG	UGUGUGUG	G	----------
An17	----------		UUAGUGG	UU	UGUGUGUG		----------
An18	----------	AC	GUAGUGG	GU	UGAGUGUG	GU	----------
An19	----------		UUAGUGG	UUU	UGUGUGUG		----------
An22	----------	U	GCAGUGG	GUA	UGUGUGUG	A	----------
An29	----------	GCU	GUAGUGG	UU	UGAGUGUG	AGU	----------
An30	----------	AGUCAAUC	GUAGUGG	AC	UGUGUGUG	GAU	----------
An33	----------	C	GUAGUGG	AA	UGUGUGUG	G	----------
An34	----------	AC	UUAGUGG	UU	UGUGUGUG	GU	----------
An35	----------	AUCAC	GUAGUGG	AU	UGUGUGUG	GUGGU	----------

Fig. 2. Nucleotide sequences of the RNA subunit of ATP-binding RNP receptor.[15,17] Nucleotides in the consensus region forms the binding pocket to the substrate ATP. The red squared regions indicate the variable region that determines the relative orientation of the ATP-binding pocket to the peptide subunit.

ester.[23–25] For such measurements, it is important to optimize the conditions for the detection with high S/N ratio to accurately identify the "hit" molecules. The signals resulting from the reaction in the presence of RNPs should be distinguishable from that in the absence of RNP. Generally, autohydrolysis of the *p*-nitrophenyl ester is accelerated in the basic condition, which causes the increase of the background noise. Though the reaction in acidic condition is desirable to avoid the autohydrolysis of *p*-nitrophenyl ester, the reaction in acidic condition results little or no significant absorbance of *p*-nitrophenolate due to the high pK_a value of the *p*-nitrophenol (7.15 at 25 °C).[26] The extinction coefficient at 400 nm (ε_{400}) of *p*-nitrophenol at pH 6.2 is only 2800 (Table S1). Thus, the accurate determination of the hydrolysis reaction is difficult by the high-through-put UV measurement in plate reader. In contrast, the fluorescent emission of umbelliferone is detectable by plate reader even in slightly acidic conditions because of its high quantum yield ($\Phi_f = 0.79$).[27]

In addition, the fluorescence intensity of an umbelliferyl ester is quite low ($\Phi_f = 0.0011$ for 7-acetoxycoumarin).[27] The fluorescence detection has an advantage in reducing the background noise caused by aggregation or bubble formation in the wells of the plate when compared to the UV absorbance detection. These characteristics of umbelliferyl ester prompted us to utilize umbelliferone as the reporter motif of the ester derivative of adenosine for screening the catalytic activity of structurally diverse RNPs library (see Scheme 1).

The ATP-binding RNP receptor binds to the adenine base of ATP by forming the Hoogsteen U:A:U triple (Fig. S1) as has been determined by NMR measurements.[17] Due to this recognition mechanism, modification of the ribose moiety of an adenosine derivative would not significantly reduce its affinity of RNP receptors. We designed an umbelliferyl ester derivative of adenosine (Umb-Ado) **7** as the substrate for hydrolysis reaction by structurally diverse RNPs. For the synthesis of Umb-Ado **7**, 2′,3′-isopropylideneadenosine[28] **2** was converted to an azide compound **3**, then to 5′-deoxy-5′-amino-2′,3′-isopropylideneadenosine **4** upon hydrogenation.[29] Condensation of **4** with succinic acid anhydride gave 5′-calboxylic acid **5**, which was further converted to the umbelliferyl ester **6** by WSC. Deprotection of the ester **6** gave the designed substrate Umb-Ado **7**.

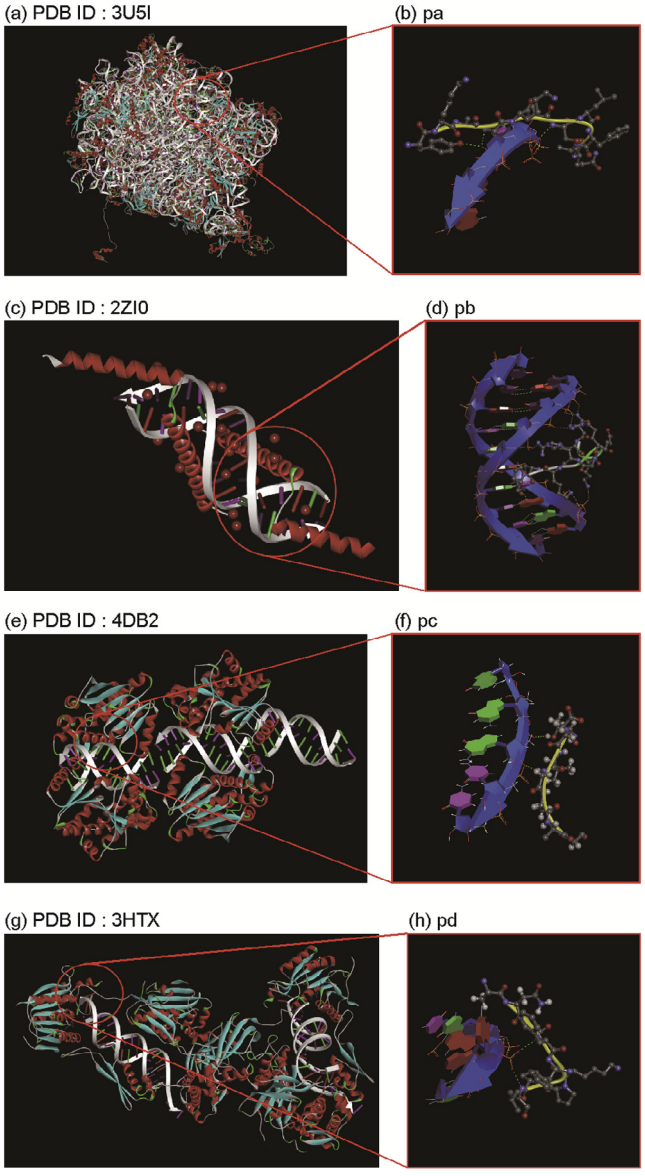
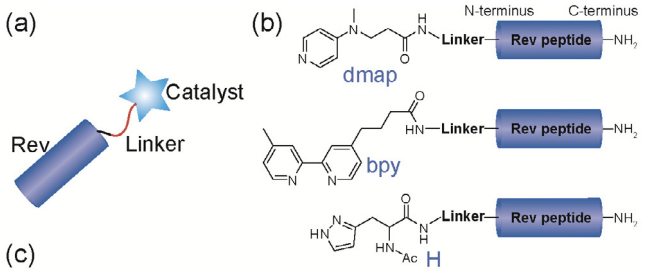


Fig. 3. Structural models of short peptide motifs derived from known RNP structures. RNP structures of (a) eukaryotic ribosome (PDB ID: 3U5I),[19] (c) Tav2b/siRNA complex (PDB ID: 2ZI0),[20] (e) Mss116p DEAD-box helicase domain 2/RNA duplex (PDB ID: 4DB2),[21] and (g) small RNA methyltransferase HEN1 (PDB ID: 3HTX)[22] were used for the short peptide motifs (b) pa, (d) pb, (f) pc, and (h) pd, respectively.



Peptide Name	Catalyst-linker	Rev
dmap-Rev	dmap-	TRQARRNRRRRWRERQRAAAAR
dmap-pa1-Rev	dmap-YKAGKASLFAQGG	TRQARRNRRRRWRERQRAAAAR
dmap-pa2-Rev	dmap-YKAGKASLFAQGSGG	TRQARRNRRRRWRERQRAAAAR
dmap-pb1-Rev	dmap-NRKERGHKSPGSGG	TRQARRNRRRRWRERQRAAAAR
dmap-pb2-Rev	dmap-NRKERGHKSPGGSGG	TRQARRNRRRRWRERQRAAAAR
dmap-pc1-Rev	dmap-TDVGAGSG	TRQARRNRRRRWRERQRAAAAR
dmap-pc2-Rev	dmap-TDVGAGSGG	TRQARRNRRRRWRERQRAAAAR
dmap-pd1-Rev	dmap-ANAFFKPPLGGSGG	TRQARRNRRRRWRERQRAAAAR
dmap-pf1-Rev	dmap-SGG	TRQARRNRRRRWRERQRAAAAR
dmap-pf2-Rev	dmap-SGGSGG	TRQARRNRRRRWRERQRAAAAR
dmap-pf3-Rev	dmap-SGGSGGSGG	TRQARRNRRRRWRERQRAAAAR
bpy-Rev	bpy-	TRQARRNRRRRWRERQRAAAAR
bpy-pa1-Rev	bpy-YKAGKASLFAQGG	TRQARRNRRRRWRERQRAAAAR
bpy-pb2-Rev	bpy-NRKERGHKSPGGSGG	TRQARRNRRRRWRERQRAAAAR
bpy-pc2-Rev	bpy-TDVGAGSGG	TRQARRNRRRRWRERQRAAAAR
bpy-pd1-Rev	bpy-ANAFFKPPLGGSGG	TRQARRNRRRRWRERQRAAAAR
H-Rev	H	TRQARRNRRRRWRERQRAAAAR
H-pe1-Rev	HG	TRQARRNRRRRWRERQRAAAAR
H-pe3-Rev	HGSG	TRQARRNRRRRWRERQRAAAAR
H-pe5-Rev	HGGSGG	TRQARRNRRRRWRERQRAAAAR
H-pe8-Rev	HGGSGGSGG	TRQARRNRRRRWRERQRAAAAR

Fig. 4. (a) Schematic illustrations of catalyst modified Rev peptide through linker peptide. (b) Structures of catalytic groups modified to N-terminus of the peptide subunit. (c) Amino acid sequences of Rev peptide derivatives. Short peptide motifs (in orange, Fig. 3) are introduced through flexible linkers composed by G and S to the N-terminus of Rev peptide (TRAQRRNRRRRWRERQRAAAAR). As the catalytic groups for hydrolysis, dmap, bpy and histidine were modified at the N-terminus of peptides.

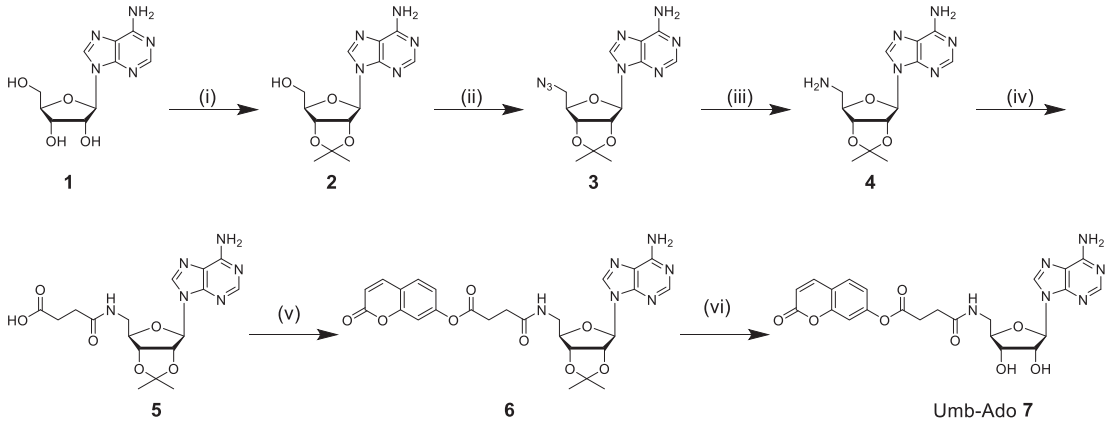
2.3. Conditions for the screening of catalytic RNPs

Time-dependent spectral change of Umb-Ado alone was evaluated at pH 6 (Fig. S2). Autohydrolysis of Umb-Ado was monitored by the time-dependent fluorescence intensity change (λ_{Ex} = 355 nm/λ_{Em} = 455 nm). Time course profiles of the background hydrolysis reaction at 20 °C were monitored at different pH conditions to determine the optimum conditions for screening the ester hydrolysis activity of RNPs in buffers containing 100 mM NaCl, 5 mM $MgCl_2$, 0.005% Tween 20 as described previously for the ligand-binding studies of ATP-binding RNP.[15,17] Autohydrolysis of Umb-Ado in the buffer at pH 7 resulted in a conversion yield of 80% after three hours at 20 °C (Fig. 5). At pH 6, the conversion yield was 10% after three hours. Almost no hydrolysis of Umb-Ado was observed at pH 5 as judged from the emission intensity, but the quantum yield for the fluorescence emission of umbelliferone was too low to determine the accurate concentration at pH 5. These results taken together, the condition in a buffer containing 30 mM Bis-Tris-HCl (pH 6.0), 100 mM NaCl, 5 mM $MgCl_2$ and 0.005% Tween 20 was used for screening the hydrolytic activity of RNPs library at 20 °C.

Formation of the 1:1 complex of An15 RNA (see Fig. 2) and the dmap-pa1-Rev peptide (see Fig. 4) at 20 °C in this buffer was confirmed by gel shit assay using 8% native polyacrylamide gel electrophoresis (PAGE) for several ratios of RNA to peptide (Fig. S3). This RNP showed almost quantitative complex formation with one equivalent of peptide to RNA at the concentration of 5 μM. Formation of the ATP-binding RNP and Umb-Ado complex was next analyzed by titration of a 5FAM-labeled Rev peptide (5FAM-Rev)[15] and An15 RNA complex with Umb-Ado (Fig. 6) and the estimated dissociation constant (K_D) was 2.6 ± 0.2 μM in the above mentioned conditions. This result indicated that the designed substrate for the hydrolytic reaction, Umb-Ado, maintained the binding ability to the ATP-binding RNP receptor.[15,17]

2.4. Screening of the structurally diverse RNP library for the identification of catalytic RNPs

Screening of the library of structurally diverse RNPs with the catalytic group was first performed by using roughly purified RNAs and peptides in a high-through-put manner. RNA was purified with



Scheme 1. Synthetic scheme of umbelliferyl ester of adenosine (Umb-Ado **7**). Reaction conditions: (i) TsOH, acetone; (ii) dppa, DBU, 15-crown 5-ether, NaN_3, 1,4-dioxane; (iii) H_2, Pd/C, MeOH; (iv) succinic anhydride, pyridine; (v) WSCI·HCl, umbelliferone, DMAP, CH_2Cl_2; (vi) 50% TFA/H_2O.

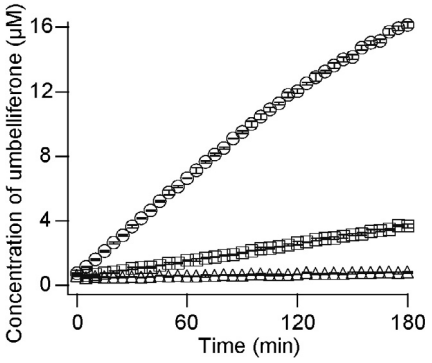


Fig. 5. Time course plots of the autohydrolysis reaction of Umb-Ado in sodium phosphate buffer, pH 7 (circle), Bis-Tris-HCl buffer, pH 6 (square), and acetate buffer, pH 5 (triangle) at 20 °C.

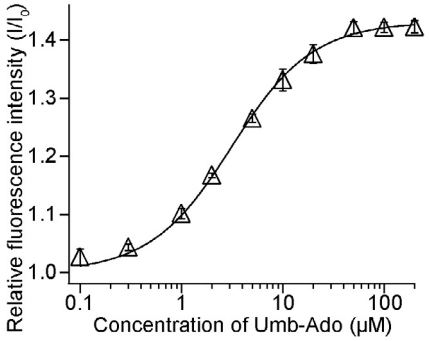


Fig. 6. A titration curve for the relative fluorescence intensity changes of An15/5FAM-Rev with increasing concentrations of Umb-Ado in the buffer containing 30 mM Bis-Tris-HCl (pH 6.0) with 100 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20 at 20 °C.

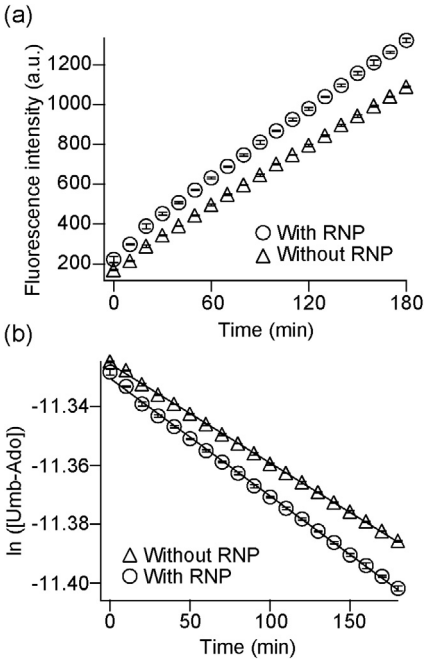


Fig. 7. (a) Time course plots of the intensity of the sample containing Umb-Ado with (circle) or without (triangle) An15/dmap-pa1-Rev. (b) Time course plots of ln ([Umb-Ado]) of the sample containing Umb-Ado with (circle) or without (triangle) An15/dmap-pa1-Rev. All samples were measured in the buffer containing 30 mM Bis-Tris-HCl (pH 6.0), 100 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20 at 20 °C.

size-exclusion chromatography after the transcription reaction, and each peptide was purified only by the diethyl ether extraction after deprotection and cleavage from the resin. In total, 609 RNPs were obtained by the combination of 29 RNAs and 21 peptides (Table S2). Each RNP of the library was mixed with Umb-Ado in the screening buffer, then time course changes of the fluorescence emission intensity at 455 nm was monitored. Progress of the ester hydrolysis was estimated by using a standard curve of the umbelliferon emission (Fig. S4). 17 RNPs showed acceleration of the reaction rate over 1.2-fold higher than that in the condition without RNP in the first screening (Table S2). These RNPs were selected for the second screening, in which the RNA and the peptide components were purified by denaturing PAGE and HPLC, respectively. In the second screening, RNP with An15 RNA and dmap-pa1-Rev showed the highest acceleration of the hydrolysis (Fig. 7a). An15 RNA or dmap-pa1-Rev by itself showed no acceleration of the ester hydrolysis reaction (Fig. S5), indicating that the RNP complex formation by An15 and dmap-pa1-Rev was necessary to accelerate the reaction. The proximity effect between Umb-Ado in the binding pocket of An15 and the dmap group of dmap-pa1-Rev upon the RNP complex formation likely accounts for the observed acceleration of the ester hydrolysis. To support this, hydrolysis of 7-acetoxycoumarin, an umbelliferyl ester without the adenosine moiety, did not show any acceleration in the presence of An15/dmap-pa1-Rev RNP (Fig. S6). The pseudo-first order kinetic constants (k_{obs}) for the hydrolysis of Umb-Ado in the presence and absence of An15/dmap-pa1-Rev were 4.0 and 3.4 × 10^{-4} min^{-1}, respectively.

The k_{obs} in the presence of RNP was 1.2-fold higher than that in the absence of RNP (Fig. 7b).

3. Conclusion

A library of structurally diverse RNPs with catalytic group was successfully prepared by a combination of an RNA library that has a binding pocket for adenosine derivatives with a variety of overall structures and a Rev peptide library that contained catalytic group through various peptide linkers. The noncovalent complex formation of RNA and the Rev peptide derivative enables the facile expansion of the RNPs library size. An umbelliferone ester of adenosine derivative Umb-Ado was useful for the fluorescence detection of ester hydrolysis reaction even in the acidic condition. The umbelliferone ester is a sensitive alternative for the *p*-nitrophenol esters widely used as the substrate for hydrolytic reactions especially in acidic conditions. The functionalized and structurally diverse RNPs library was screened for the ester hydrolysis of Umb-Ado. An RNP of An15 RNA and dmap-pa1-Rev, one of the RNPs selected by the screening, clearly showed significant acceleration of the hydrolysis of Umb-Ado. It is noteworthy that catalytic RNPs albeit with moderate activity were selected from the small size library, less than 1000, in this study. Because the numbers of ATP-binding RNA and/or the Rev peptide with catalytic group are readily increased, the size of RNP library would be expanded conveniently by the combination of these two subunits. Alternatively, introduction of random mutation to RNA of the selected RNP would afford a focused library for further selection of catalytic RNP. The functionalized and structurally diverse RNPs library would be quite useful for exploring catalytic RNPs not only for the ester hydrolysis but also for various other chemical reactions.

4. Materials and methods

PrimeSTAR HS DNA polymerase for PCR reactions was obtained from TaKaRa Bio Inc. (Shiga, Japan). T7-Scribe™ Standard RNA IVT Kit for RNA preparation was obtained from CELLSCRIPT™ (Madison, USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydro-chloride (WSCI·HCl), N-α-Fmoc-protected amino acids, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophos-phate (HBTU), 1-hydroxybenzotriazole (HOBt), SP grade N,N-dimethylformamide (DMF), trifluoroacetic acid (TFA) and 4-[(2,4-Dimethoxyphenyl)-N-(9-fluorenylmethoxy-carbonyl) aminomethyl]phenoxy-acetamido polyethyleneglycol resin (Fmoc-NH-SAL-PEG resin) were obtained from Watanabe Chemical Industries (Hiroshima, Japan). Dichloromethane (DCM), 1,8-diazabicyclo [5,4,0]-7-undecene (DBU) and umbelliferon were purchased from Nacalai Tesque (Kyoto, Japan). N,N-Diisopropylethylamine (DIEA), HPLC-grade acetonitrile and N,N-dimethyl-4-aminopyridine (dmap), and *p*-toluenesulfonic acid monohydrate were obtained from Sigma-Aldrich (St. Louis, USA). Adenosine, 15-crown 5-ether and diphenylphosphoryl azide (dppa) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Reversed-phase C18 columns (4.6 × 150 mm, Ultron VX-ODS; Shinwa Chemical Industries, Kyoto, Japan and 10 × 150 mm, 5C18-ARII; Nacalai Tesque, Kyoto, Japan) were used for purification of peptides for preparative purposes. Sodium azide, succinic anhydride, gel electrophoresis grade acrylamide, bisacrylamide, phenol, thioanisol, 1,2-ethanditiol, 10% palladium on carbon and distilled organic solvents were purchased from Wako Chemicals (Tokyo, Japan).

4.1. Preparation of the RNA subunit library

The plasmid DNA templates were prepared as described previously.[13–17] The double-stranded DNA templates for RNA transcription were constructed by PCR amplification to add the promoter for T7 RNA polymerase using PrimeSTAR DNA polymerase (TaKaRa) with 5′-DNA primer (5′-TCTAATACGACTCACTATAGGTCTGGGC GCA-3′: T7 RNA promoter is underlined) and 3′-DNA (5′-GGCCTGTACCGTC-3′). RNA transcription was performed using an T7-Scribe™ Standard RNA IVT Kit (CELLSCRIPT™) for 3 h at 37 °C, according to the supplier's recommended protocols. The resulting RNA was extracted with phenol/chloroform, then precipitated with ethanol, and pelleted by centrifugation. The size-exclusion with Micro Bio-Spin 6 column (Bio-Rad) was performed after dissolving the RNA pellet in TE buffer. For the first screening, the RNA was used without further purification. For the second screening, the RNA purified by denaturing polyacrylamide gel electrophoresis and eluted. Concentrations of RNAs were determined by UV spectroscopy.

4.2. Synthesis of the catalyst-modified peptide library

The peptides were synthesized as follows. Fmoc-NH-SAL-PEG resin was placed in a dry flask, and a sufficient amount of DMF was added to soak the resin; this mixture was allowed to swell for 30 min. The resin was loaded onto an automated peptide synthesizer (Liberty; CEM/PSSM-8; Shimadzu), and the subsequent synthesis was performed according to Fmoc chemistry protocols using protected Fmoc-amino acids and HBTU. Unnatural catalytic groups (3-(Methyl-4-pyridylamino)propionic acid, 4′-Methyl[2,2′-bipyridine]-4-butanoic acid) were directly coupled to N-terminus deprotected Rev peptides on the resin in DMF containing WSC·HCl. Acetylated histidine was introduced by usual Fmoc chemistry protocol and acetylation. The synthesized peptides were used as crude for the first screening and purified by HPLC before carrying out the second screening.

4.3. Synthesis of 5′-deoxy-5′-(umbelliferyl butylate)amido-adenosine (Umb-Ado **7**)

4.3.1. Synthesis of 2′,3′-O-isopropylideneadenosine **2**[28]

A mixture of adenosine (4.96 g, 18.56 mmol), *p*-toluenesulfonic acid monohydrate (3.53 g, 18.56 mmol) and acetone (150 mL) was stirred at room temperature in an open vessel. After 46 h from starting reaction, 5.10 g of *p*-toluenesulfonic acid monohydrate was added. After 2 h, 5.21 g of *p*-toluenesulfonic acid monohydrate was added (total 13.94 g, 73.3 mmol). After 2 h from adding the acid, the spot of compound **1** disappeared. The mixture turned into a yellow solution. The reaction mixture was quenched by adding 12.3 mL of triethylamine (88.4 mmol, 4.73 eq.). The mixture was evaporated and purified by column chromatography (chloroform/methanol 20:1) to give compound **2** (4.50 g, 14.6 mmol, 79%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.33 and 1.55 (2s, 6H, CMe_2), 3.52–3.56 (m, 2H, H5′), 4.19–4.23 (m, 1H, H4′), 4.95–4.98 (m, 1H, H 3′), 5.25 (vbs, 1H, OH), 5.33–5.36 (m, 1H, H 2′), 6.11–6.12 (m,1H, H 1′), 7.34 (bs, 2H, NH_2), 8.15 (s, 1H, H2), 8.34 (s, 1H, H8); ESI-TOF-MS for $C_{13}H_{17}N_5O_4$: (MNa^+) calcd: 330.12, found: 330.03.

4.3.2. Synthesis of 5′-deoxy-5′-azido-2′,3′-O-isopropylideneadenosine **3**[29]

Compound **2** (1.0 g, 3.25 mmol, 1.0 eq.), dppa (1.4 mL, 6.51 mmol, 2.0 eq) and DBU (1.47 mL, 9.77 mmol, 3.0 eq.) were dissolved in 1,4-dioxane 10 mL under nitrogen atmosphere. The mixture was stirred at room temperature for 2 h followed by addition of sodium azide (1.0 g, 16.3 mmol, 5.0 eq.) and 15-crown 5-ether (6.5 μL, 33 μmol, 0.01 eq.). The mixture was refluxed for 1 h and filtered. The filtrate was evaporated and purified by column chromatography (chloroform/methanol 29:1) to give the compound **3** (0.87 g, 2.6 mmol, 80%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.39 and

1.62 (2 s, 6H, CMe_2), 3.52–3.64 (m, 2H, H5′), 4.36–4.41 (m, 1H, H4′), 5.06 (dd, 1H, J = 3.3, 6.3 Hz, H3′), 5.46 (dd, 1H, J = 2.5, 6.5 Hz, 2H′), 5.89 (br, 2H, NH_2), 6.11 (d, 1H, J = 2.5 Hz, H1′), 7.93 (s, 1H, H8), 8.36 (s, 1H, H2). ESI-TOF-MS for $C_{13}H_{16}N_8O_3$: (MNa^+) calcd: 355.12, found: 355.08.

4.3.3. Synthesis of 5′-deoxy-5′-amino-2′,3′-O-isopropylideneadenosine 4[29]

Compound **3** (1.0 g, 3.3 mmol) and 10% Pd/C (200 mg) in methanol (50 mL) were stirred in room temperature under hydrogen atmosphere. After 4 h, the reaction mixture was filtered. This resulting mixture containing compound **4** was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 and 1.64 (2 s, 6H, CMe_2), 3.01–3.07 (m, 2H, H5′), 4.30–4.31 (m, 1H, H4′), 5.05 (dd, J = 6.3, 3.3 Hz, 1H, H3′), 5.48 (dd, J = 6.3, 3.0 Hz, 1H, H2′), 6.12 (d, J = 3.0 Hz, 1H, H1′), 8.18 (s, 1H, H2), 8.82 (s, 1H, H8); ESI-TOF-MS for $C_{13}H_{16}N_8O_3$: (MNa^+) calcd: 307.14, found: 307.11.

4.3.4. Synthesis of 5′-deoxy-5′-(3″-carboxypropyl) amido-2′,3′-O-isopropylideneadenosine 5

Compound **4** (313 mg, 1.02 mmol) and succinic anhydride (795 mg, 7.5 mmol) were stirred in distilled pyridine (20 mL) under the nitrogen atmosphere at 0 °C. The temperature was increased to room temperature over 30 min. After 12 h, the organic layer was washed with hydrochloric acid, conc. sodium bicarbonate, brine and then dried over anhydrous sodium sulfate to give compound **5** (230 mg, 0.57 mmol, 56%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35 and 1.69 (2 s, 6H, CMe_2), 2.78–2.84 (m, 4H, $(CH_2)_2$), 3.23 (d, J = 14.7 Hz, 1H, H5′), 3.64–3.76 (m, 2H, NH_2), 4.20–4.28 (m, 1H, H5′), 4.52 (d, J = 2.1 Hz, 1H, H4′), 4.78 (dd, J = 6.3, 1.8 Hz, 1H, H3′), 5.30 (m, 1H, H2′), 5.78 (d, J = 5.4 Hz, 1H, H1′), 7.87 (s, 1H, H2), 8.31 (d, J = 9.6 Hz, 1H, NH), 8.38 (s, 1H, H8); ESI-TOF-MS for $C_{17}H_{22}N_6O_6$: (MH^+) calcd: 407.17, found: 407.09.

4.3.5. Synthesis of 5′-deoxy-5′-(umbelliferyl butylate)amido-2′,3′-O-isopropylideneadenosine 6

Compound **5** (28 mg, 0.07 mmol), and umbelliferone (57 mg, 0.35 mmol) were stirred in dichloromethane (15 mL) under nitrogen atmosphere at 0 °C. DMAP (0.86 mg, 0.007 mmol) and WSCI·HCl (16 mg, 0.084 mmol) were added and the reaction temperature was increased to room temperature over 30 min. After 24 h, the resulting solution was evaporated and purified by HPLC (column: 5C18-AR-II 20 mm × 150 mm/mobile phase 5% acetic acid with 24–48% acetonitrile) to give compound **6** (0.011 mmol, 16%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.18 and 1.58 (2 s, 6H, CMe_2), 2.69–2.75 (m, 1H, H5′), 2.86–2.95 (m, 2H, H8′), 3.16–3.25 (m, 2H, H9′), 4.24–4.32 (m, 1H, H5′), 4.51 (d, J = 1.9 Hz, 1H, H4′), 4.78 (dd, J = 6.2, 1.9 Hz, 1H, H3′), 5.31 (t, J = 5.7 Hz, 1H, H2′), 5.74–5.78 (b, 3H, H1′ and NH_2), 6.34 (d, J = 9.4 Hz, 1H, Ar-H), 7.06 (dd, J = 8.5, 2.2 Hz, 1H, Ar-H), 7.13 (d, J = 2.2 Hz, 1H, Ar-H), 7.44 (d, J = 8.5 Hz, 1H, Ar-H), 7.67 (d, J = 9.6 Hz, 1H, Ar-H), 7.84 (s, 1H, H8), 8.36 (s, 1H, H2), 8.79 (d, J = 9.3 Hz, 1H, NH); ESI-TOF-MS for $C_{26}H_{26}N_6O_8$: (MH^+) calcd: 551.18, found: 551.20.

4.3.6. Synthesis of 5′-deoxy-5′-(umbelliferyl butylate)amidoadenosine (Umb-Ado) 7

A solution containing compound **6** (20 mg, 0.036 mmol) was stirred in 50% TFA/H_2O and after 48 h it was purified by HPLC (column: 5C18-AR-II 20 mm × 150 mm/mobile phase 5% acetic acid with 24–48% acetonitrile) to give compound **7** (0.008 mmol, 24%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.75–2.81 (m, 2H, H9′), 2.95–3.06 (m, 2H, H8′), 3.34–3.38 (m, 3H, H4′ and H5′), 4.29 (d, J = 2.57, 1H, H3′), 4.75 (t, J = 6.2 Hz, 1H, H2′), 5.81 (d, J = 6.6 Hz, 1H, H1′), 6.42 (d, J = 9.4 Hz, 1H, Ar-H), 7.10 (dd, J = 8.4, 2.2 Hz, 1H, Ar-H), 7.17 (d, J = 1.8 Hz, 1H, Ar-H), 7.55 (d, J = 8.4 Hz, 1H, Ar-H), 7.79 (d, J = 9.5 Hz, 1H, Ar-H), 8.01 (s, 1H, H8), 8.30 (s, 1H, H2), 8.82 (d, J = 7.0 Hz, 1H, NH); ESI-TOF-MS for $C_{23}H_{22}N_6O_8$: (MH^+) calcd: 511.16, found: 511.09.

4.4. Determination of the binding affinity of Umb-Ado to RNP by fluorescence titrations

The fluorescence measurements were performed using 96-well plates on a Wallac ARVOsx 1420 multilabel counter or Tecan infinity pro for the determination of the binding affinity of Umb-Ado to RNP. A binding solution (100 μL) containing 1 μM of fluorescent RNP (An15/5FAM-Rev) in 30 mM Bis-Tris-HCl (pH 6.0), 100 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20 with indicated concentration of substrate was gently swirled for few minutes and allowed to settle for 30 min at 20 °C. Emission spectra were measured with an appropriate filter set for each fluorophore. Excitation and emission wavelengths for 5FAM-Rev were 485 and 535 nm, respectively. The binding affinity of fluorescent RNP to Umb-Ado was obtained by fitting the substrate titration data using the equation:

$$F_{obs} = A\{([FRNP]_T + [substrate]_T + K_D) - (([FRNP]_T + [substrate]_T + K_D)^2 - 4[FRNP]_T[substrate]_T)^{1/2}\}/2\,[FRNP]_T$$

where A is the increase in fluorescence at saturating substrate concentrations ($F_{max} - F_{min}$), K_D is the equilibrium dissociation constant, and $[FRNP]_T$ and $[substrate]_T$ are the total concentrations of fluorescent RNP and the substrate, respectively.

4.5. Screening of RNP library

Fluorescence measurements in 96-well plates were performed on a Wallac ARVOsx 1420 multilabel counter. A solution (100 μL) containing 5 μM of each combination of RNPs and 20 μM Umb-Ado in reaction buffer containing 30 mM Bis-Tris-HCl (pH 6.0), 100 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20 was gently swirled for 30 s and the time-dependent emission intensity of the umbelliferone, which is a product of the hydrolysis, was measured every 20 min for 3 h. Excitation and emission wavelengths for umbelliferone were 355 and 455 nm, respectively. Concentration of the umbelliferone was estimated by using a standard curve.

4.6. Calculation of the pseudo-first order kinetics constant (k_{obs})

The pseudo-first order kinetics constant (k_{obs}) was obtained from the slope of the log plot of the time-dependent concentration of umbelliferone.

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A. Supplementary material

Supplementary data (pH dependence of extinction coefficient of *p*-nitrophenol (Table S1), summary of the acceleration ratio for the reaction rate (k_{obs}) in the first screening (Table S2), binding mode for the ATP-binding RNP receptor and adenosine (Fig. S1), time dependent spectra of Umb-Ado in the buffer (Fig. S2), autoradiograms of the gel shift assay to confirm the complex formation of RNP (Fig. S3), standard curve for the fluorescent intensity of

umbelliferone (Fig. S4), time course plots for the intensity of samples containing Umb-Ado (Fig. S5), time course plots for the intensity of samples containing 7-acetoxycoumarin (Fig. S6), ^{1}H NMR spectra of compound **6** and **7** (Figs. S7 and S8) and HPLC chromatogram of dmap-pa1-Rev (Fig. S9)) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2017.02.007.

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