How was an artificial ribozyme developed into bio-venture.

~ Case Study of Peptidream ~

April 26th (Sat.), 2014

Takuya Matsumoto (D2)

2013年06月11日東大発ＶＢのペプチドリーム、東証マザーズ上場－自社創薬に本腰

http://www.peptidream.com/index.html

Why Peptide?

Small Molecule

O High permeability of intestinal or cell membrane (oral dose)

X Too small for inhibiting protein-protein interaction

Antibody

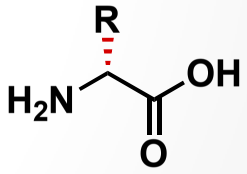
O Inhibitive capacity of protein-protein interaction
High selectivity (low side-effects)

X Potential immunogen
Low permeability of membrane

'Non-Standard' Peptide

High permeability of membrane

High selectivity and high binding affinity



D-configurations

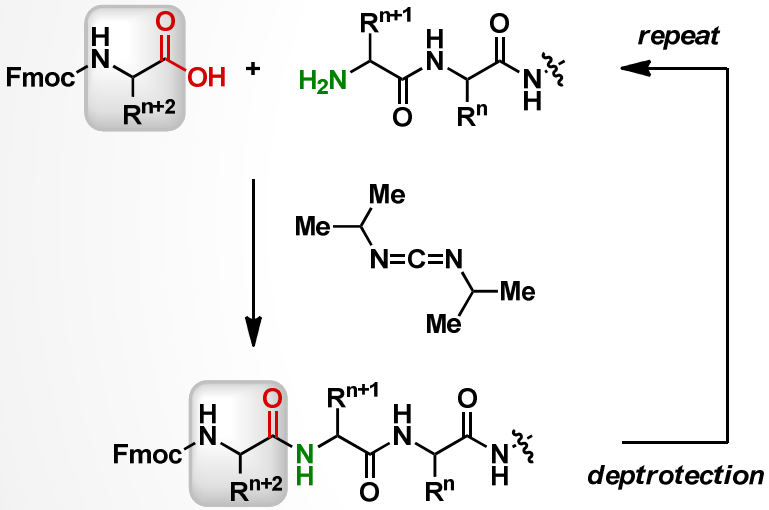
N-methyl

What is Peptidream doing?

A major obstacle to the development of peptide drugs

- **the lack of a suitable synthetic methodology for producing the diverse compound libraries required for drug discovery**

Chemical synthesis



Insufficient library size
(adaptability to any amino acids)

Ribosomal synthesis

1st	2nd U	2nd C	2nd A	2nd G	3rd
U	Phe / Leu	Ser	Tyr / Stop	Cys / Stop / Trp	U C A G
C	Leu	Pro	His / Gln	Arg	U C A G
A	Ile / fMet/Met	Thr	Asn / Lys	Ser / Arg	U C A G
G	Val	Ala	Asp / Glu	Gly	U C A G

Limited to canonical amino acids
(> 10 peptide bond formation / sec)

- **the lack of a suitable screening methodology from the huge peptide libraries**

Prof. Hiroaki Suga

Education

1986: B.Sc., Okayama University (Prof. Sigeru Torii)
1987: University of Lausanne (Prof. Manfred Schlossor)
1989: M.Sc., Okayama University (Prof. Sigeru Torii)
1994: Ph.D., Massachusetts Institute of Technology (Prof. Satoru Masamune)

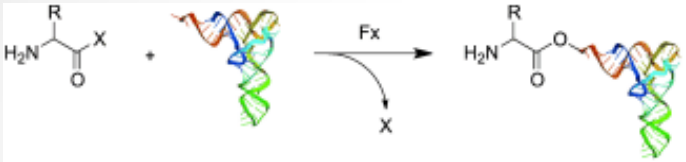
Professional career

1994-1997:
Postdoctoral fellow, Massachusetts General Hospital,
Harvard Medical School (***Prof. Jack W. Szostak***)
1997-2002:
Assistant professor, University at Buffalo,
The State University of New York
2002-2003:
Associate professor (tenured), University at Buffalo,
The State University of New York
2003-2005:
Associate professor, Research Center for Advanced Science and Technology,
The University of Tokyo
2005-2010:
Professor, Research Center for Advanced Science and Technology,
The University of Tokyo
2010-present:
Professor, Graduate School of Science, The University of Tokyo

Peptidream's Technology (Today's Contents)

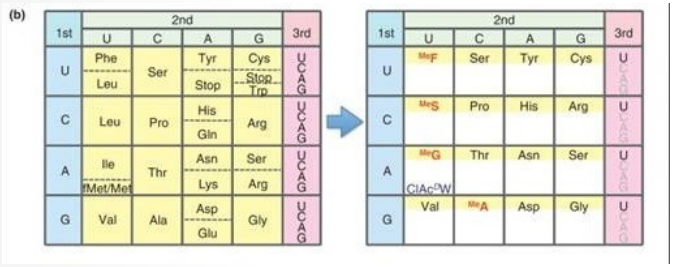
Peptide Discovery Platform System

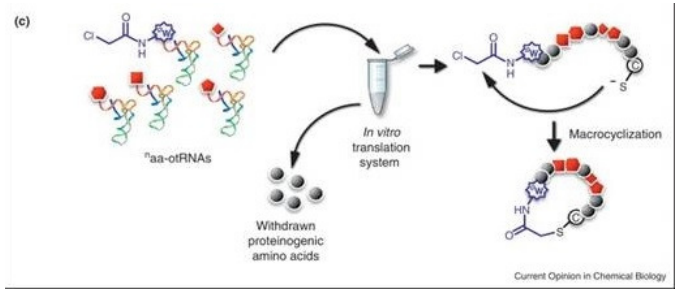
1. **Flexizyme** (an RNA-based artificial aminoacyl-tRNA synthetase)



Catalyst

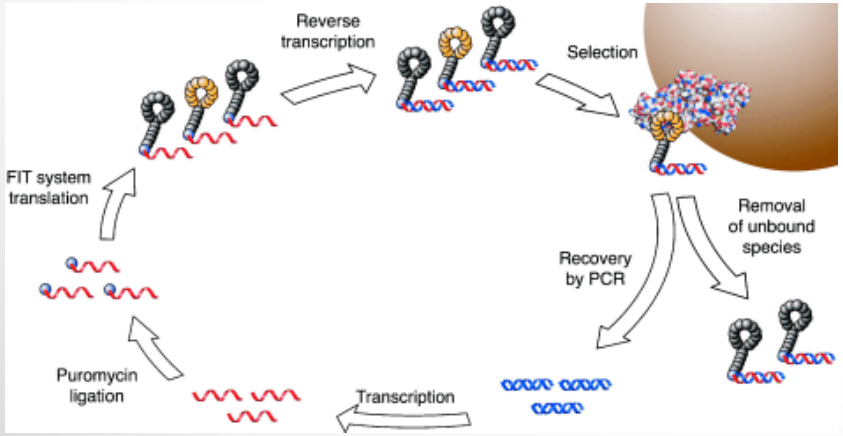
2. **Flexible In-vitro Translation (FIT) system**





Library (Synthesis)

3. **RAndom Peptide Integrated Discovery (RAPID) system**



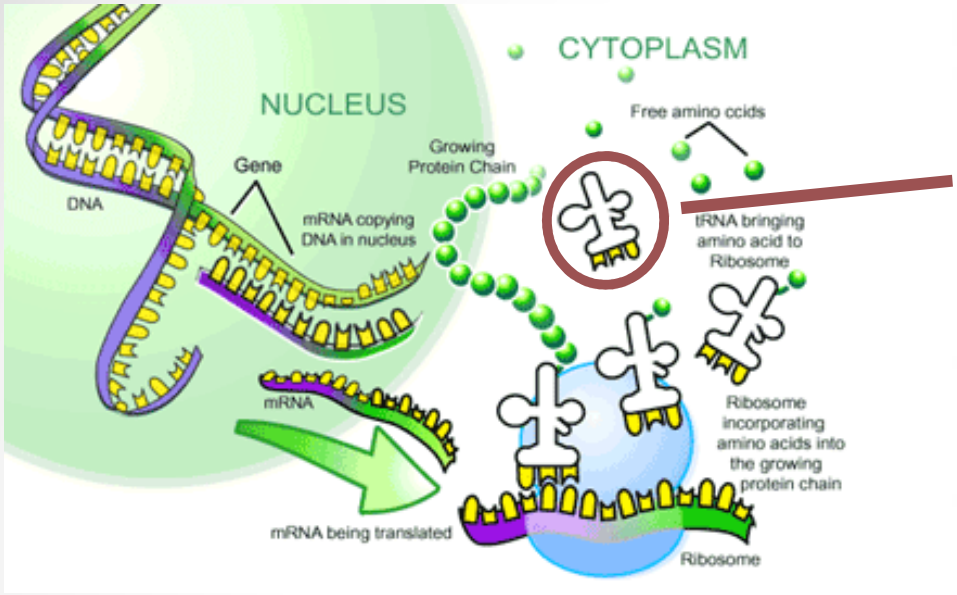
Screening

1. Flexizyme

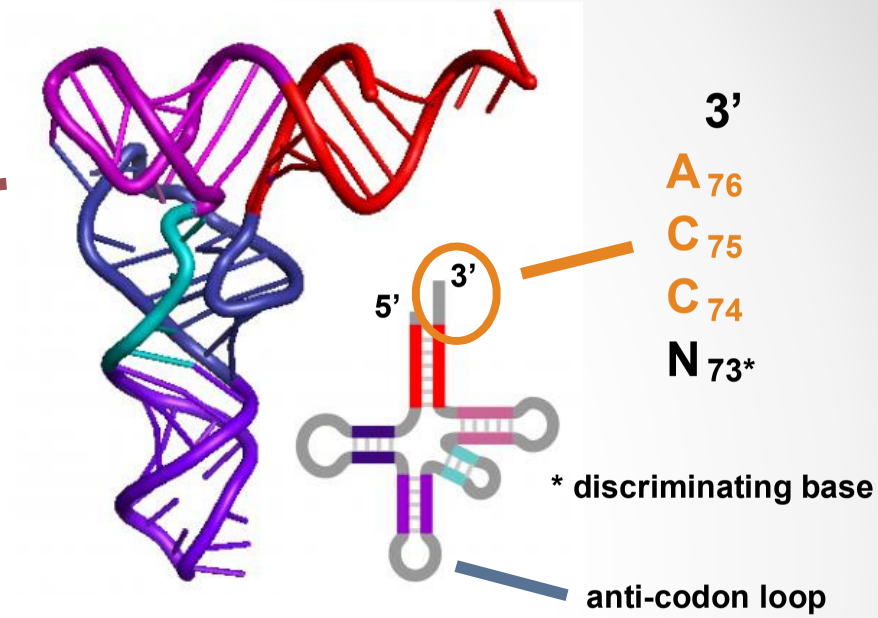
~ *Acc. Chem. Res.*, **2011**, *44* (12), pp 1359–1368. ~

Overview

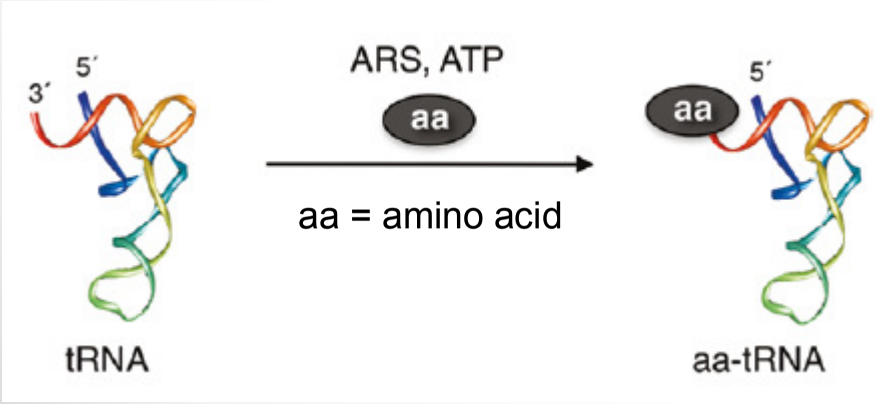
Transcription & Translation



tRNA



Charging amino acids onto tRNAs



Aminoacyl - tRNA synthetases (ARSs):
a family of protein enzymes which charge amino acids onto the 3’-terminus of tRNA

VS

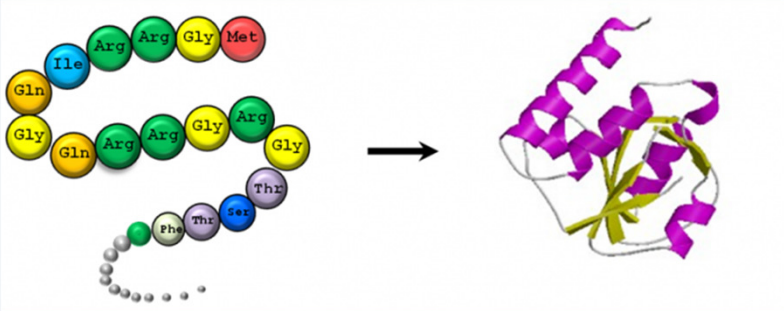
Flexizyme:
an RNA-based artificial ARSs (rybozyme), which has broad substrate scope of both amino acids and tRNAs.

Historical Background (RNA World Hypothesis)

The RNA world hypothesis:
hypothesis that self-replicating RNA molecules were precursors to current life, which is based on DNA, RNA and proteins.

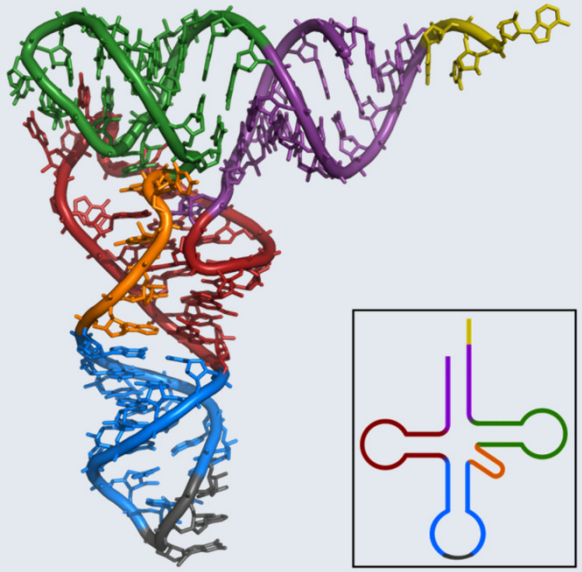
Current life

Catalyst for chemical reactions = **Protein**



Storage of genetic information = **DNA**

Early life on earth



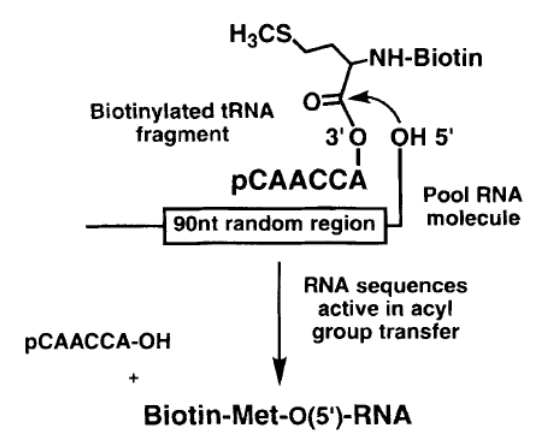
RNA

In order to prove the hypothesis, many artificial ribozymes that resemble various functions of current protein enzymes have been developed.

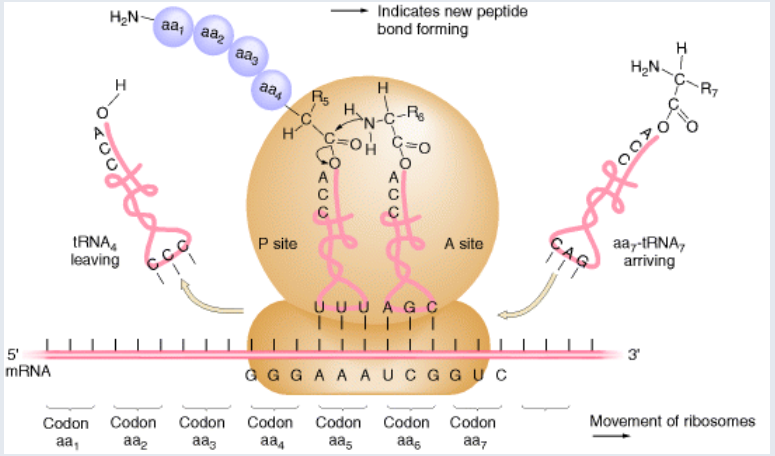
Peptidyl Transferase

The first artificial ribozyme scoping of tRNA-like molecule

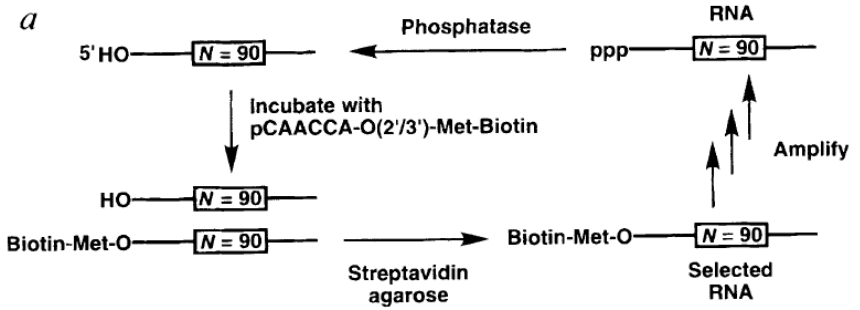
Target reaction

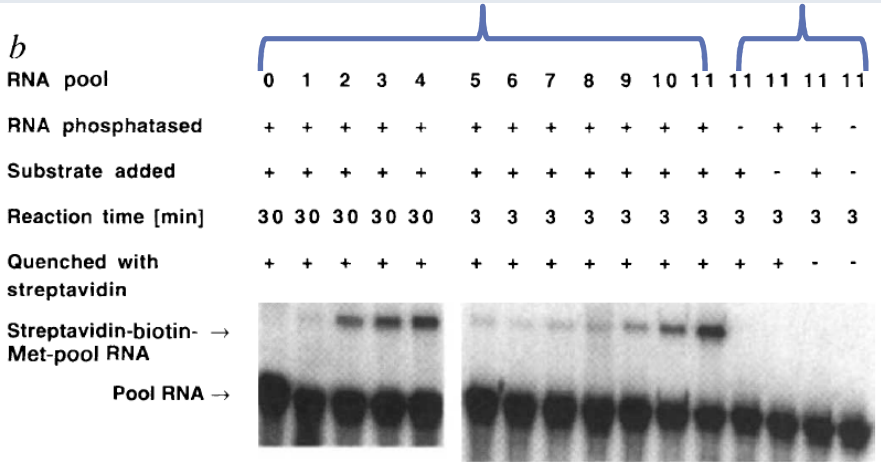


Original reaction (Peptidyl Transfer)



Methods (In vitro selection) & Results

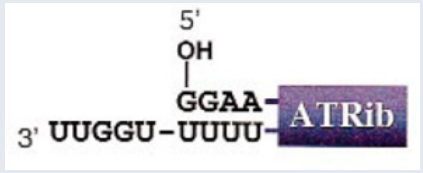




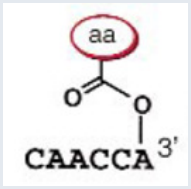
The pool RNA was constructed from completely random-sequence RNA, without bias toward any known sequence or structure.

Lohse, P. A.; Szostak, J. *Nature* **1996**, *381*, 442.

The First Artificial Aminoacyl-tRNA Synthetase (step1)

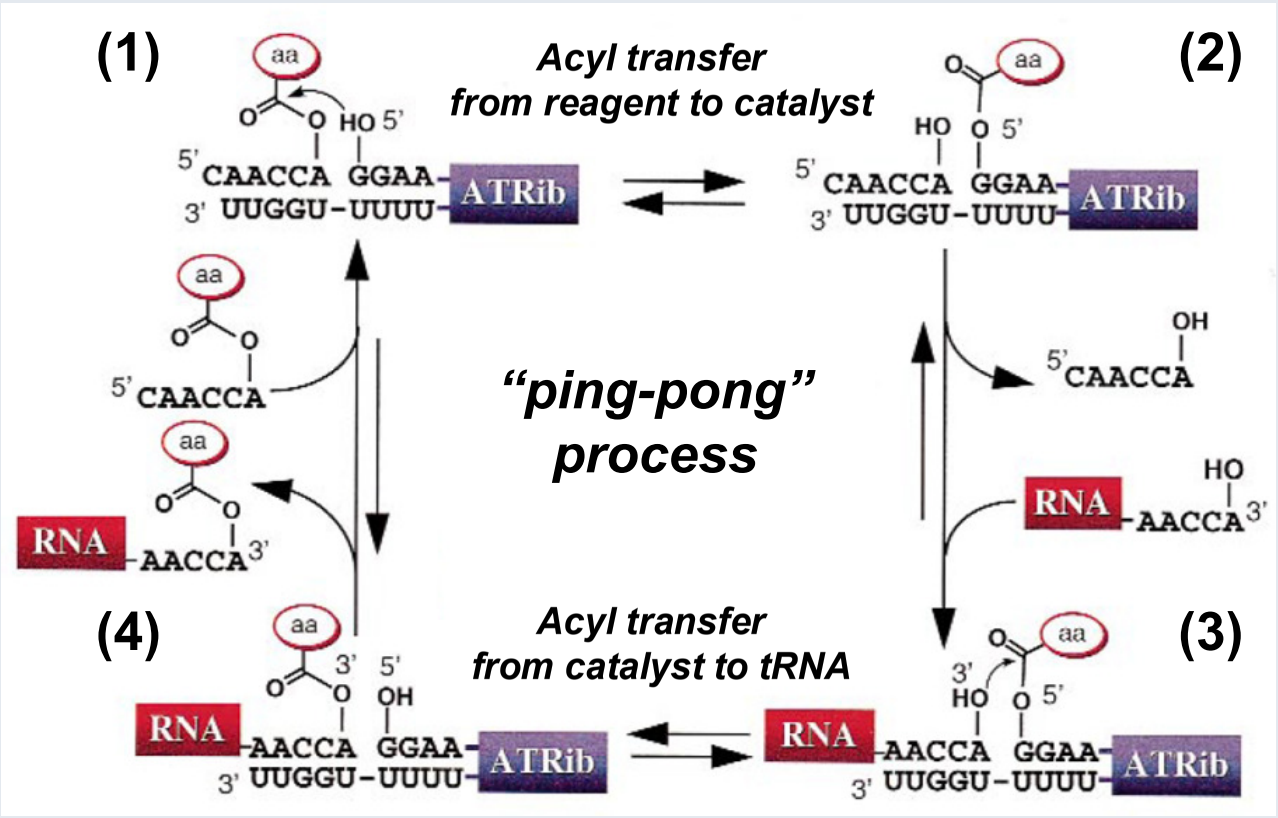


catalyst

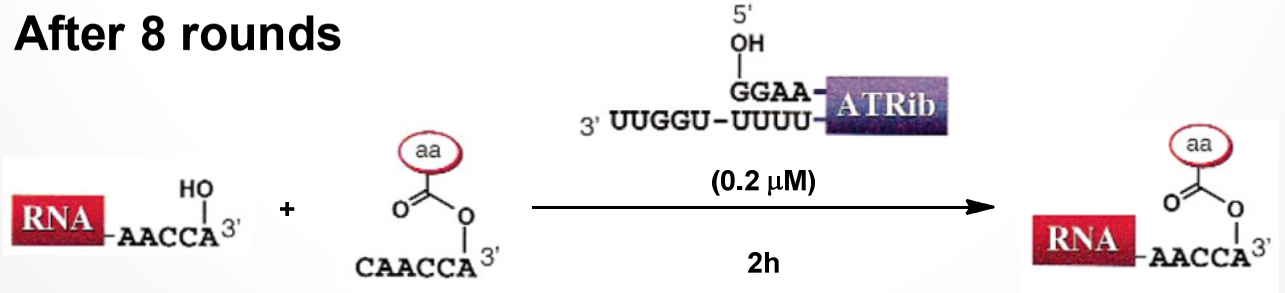


acylating reagent

tRNA



After 8 rounds

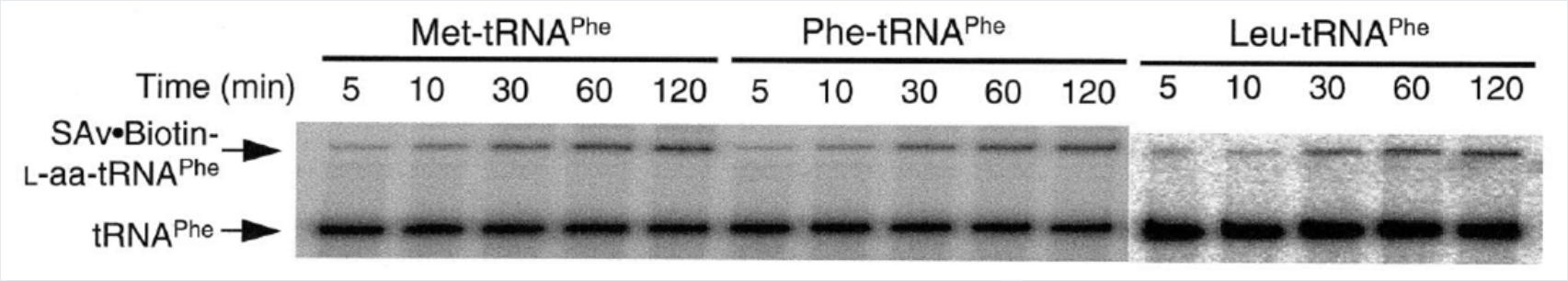


(1 μM) (10 μM) 30 ~ 40 %

Lee, N.; Bessho, Y.; Wei, K.; Szostak, J. W.; Suga, H. *Nat. Struct. Biol*. **2000**, *7*, 28.

The First Artificial Aminoacyl-tRNA Synthetase (step2) 1

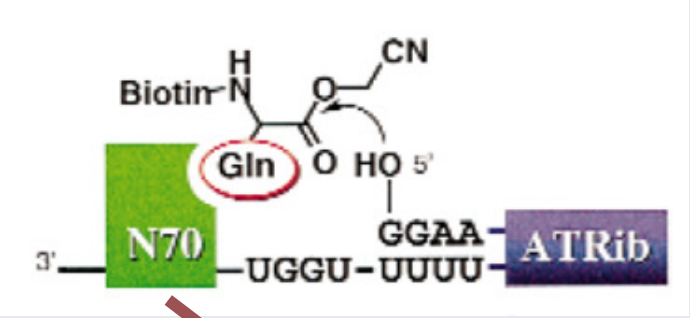
Previous artificial ribozyme (ATRib) → no selectivity toward amino acid



VS

Ribozyme in RNA world (?) → selectivity toward specific amino acid

New design



Amino acid recognizing region

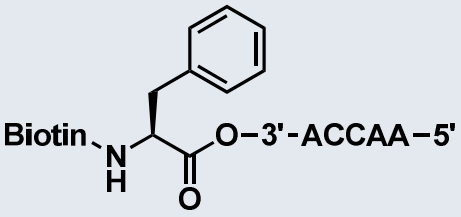
Evolution pressure

1.

Biotin-(L)-Gln-CME

Selectivity toward side chain of Gln

2.



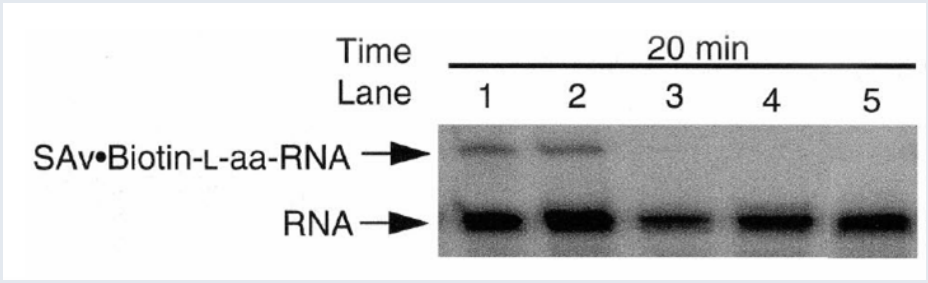
Biotin-L-Phe-3'-ACCAAC-5'

Retention of the original oligonucleotide-ribozyme acyl-transfer reaction

Lee, N.; Bessho, Y.; Wei, K.; Szostak, J. W.; Suga, H. *Nat. Struct. Biol*. **2000**, *7*, 28.

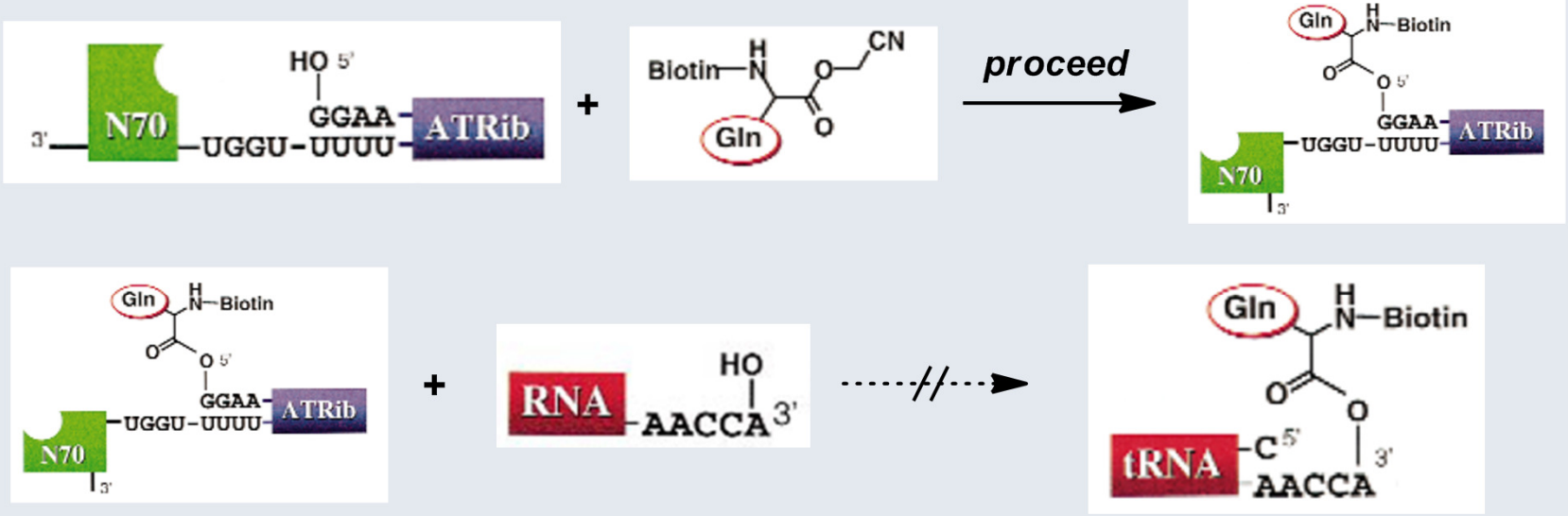
The First Artificial Aminoacyl-tRNA Synthetase (step2) 2

Amino acids selectivity of evolved ribozyme



1. Biotin-(L)-**Gln**-CME
2. Biotin-(L)-**Phe-3'-ACCAAC-5'**
3. Biotin-(L)-**Phe**-CME
4. Biotin-(L)-**Leu**-CME
5. Biotin-(L)-**Val**-CME

Ribozyme-catalyzed aminoacylation of tRNA



The yield of aminoacylated tRNA was barely above background

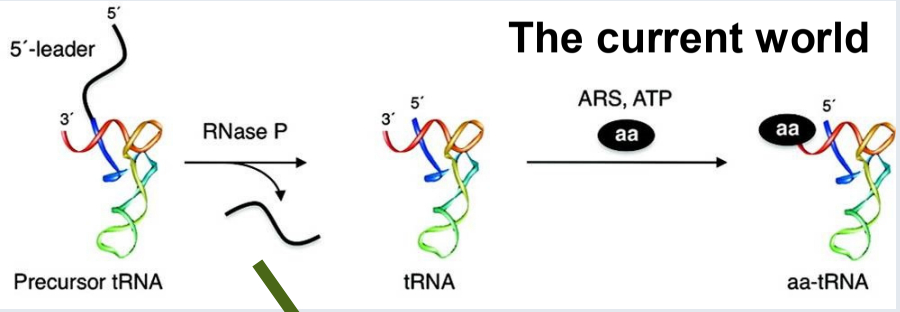
the complex mechanisms involving the equilibrium shift of acyl-transfer chemistry → **suspended**

Lee, N.; Bessho, Y.; Wei, K.; Szostak, J. W.; Suga, H. *Nat. Struct. Biol.* **2000**, *7*, 28.

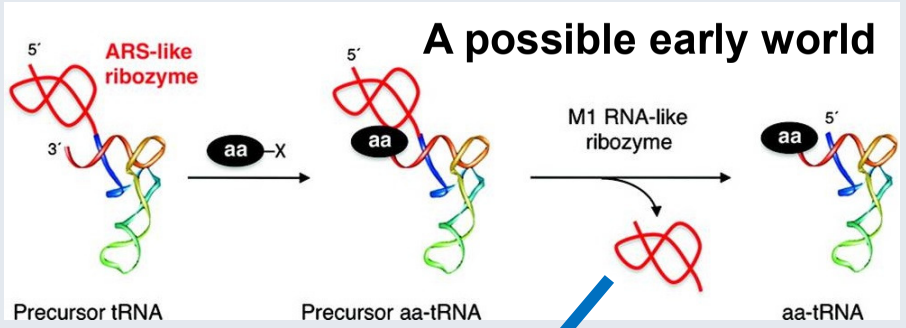
Next Design (Catalysis by 5'-Leader)

How to charge specific amino acid onto tRNA ?

aa-tRNA maturation pathway

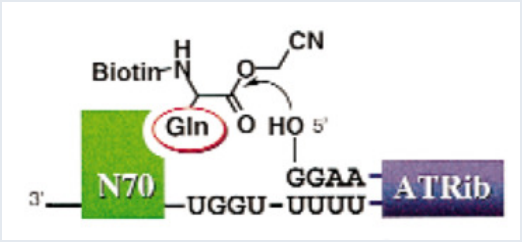


5' leader domain has no function
In the "modern world".



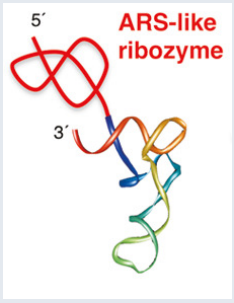
Catalytic activity ?

Previous Strategy



- *trans*-acting rybozyme
- catalyst domain and recognition domain were evolved *separately.*

This time Strategy

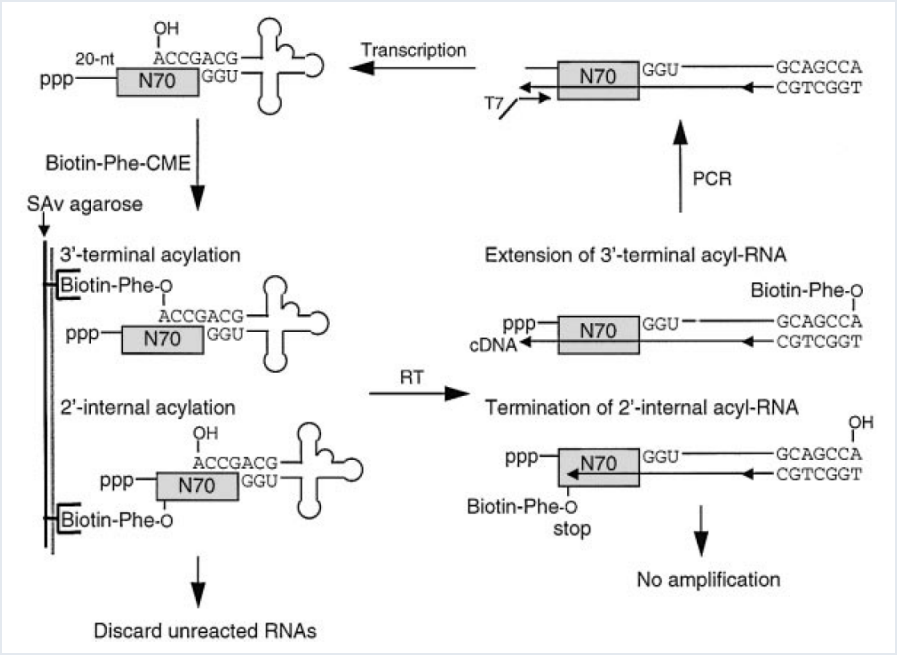


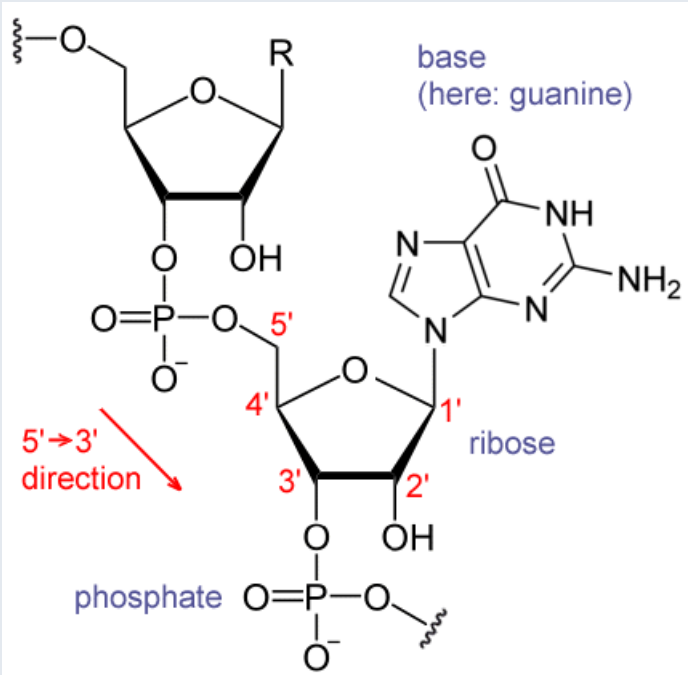
- *cis*-acting rybozyme
- catalyst domain and recognition domain were evolved *at the same time*.

5' leader catalytic domain can be removed by M1-like ribozyme to yield mature aminoacyl-tRNAs.

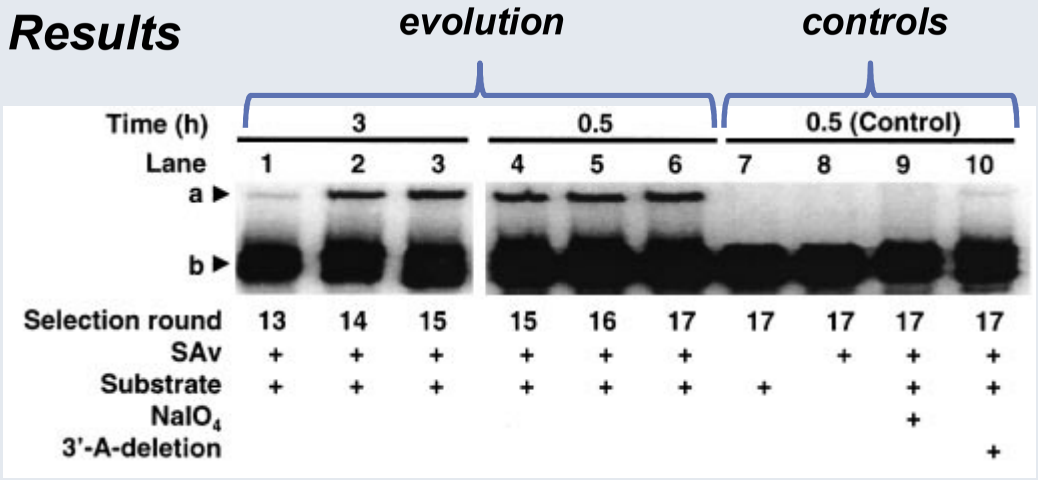
Aminoacylation by 5'-Leader Sequence Domain 1

Methods





Results



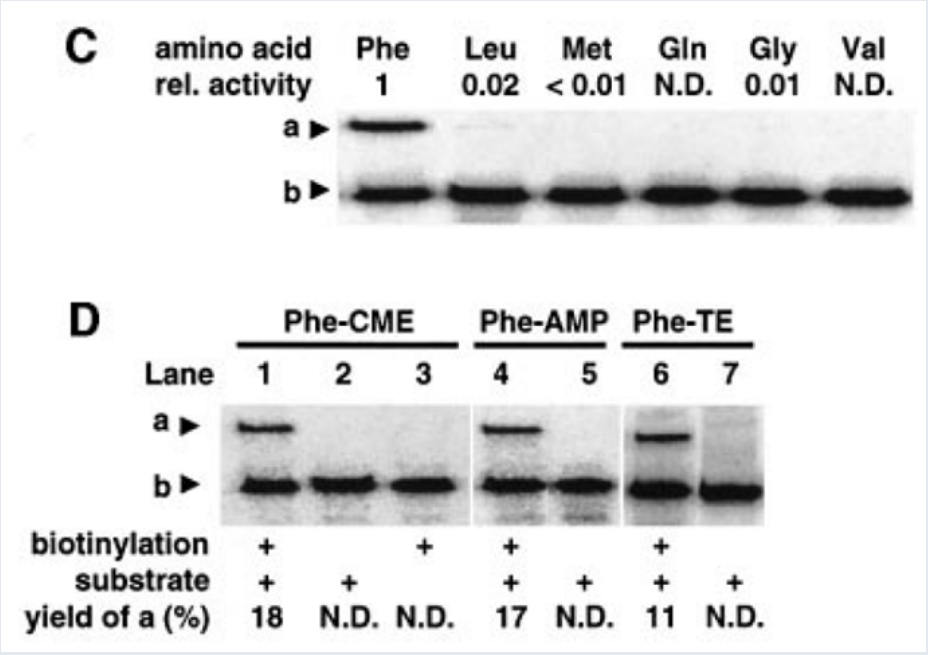
3'-OH of 3'-terminal adenosine was selectively aminoacylated !

for detail discussion,
J. Am. Chem. Soc. **2001**, *123*, 7178.

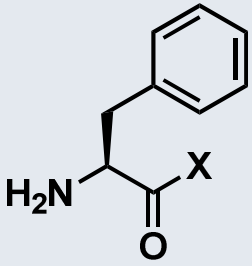
Saito, H.; Kourouklis, D.; Suga, H. *EMBO J.* **2001**, *20*, 1797.

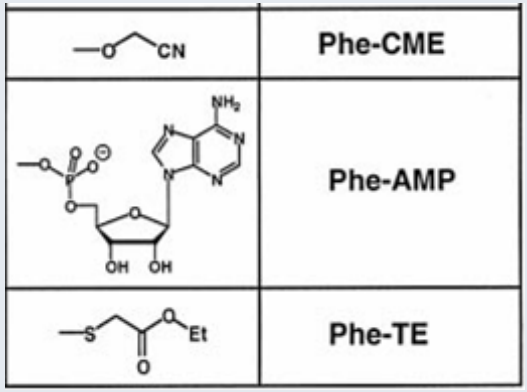
Aminoacylation by 5'-Leader Sequence Domain 2

Amino acids selectivity



(L)-Tyr 0.55; (D)-Phe 0.18

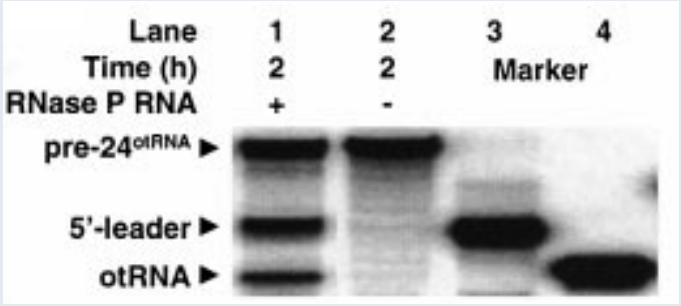




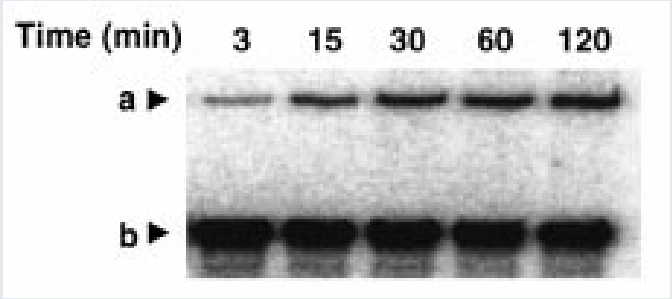
The critical recognition element is the phenylalanyl (benzyl) side chain.

After aminoacylation, the biotinylation was performed.

RNase P RNA cleavage of pre-tRNA



Trans-aminoacylation !



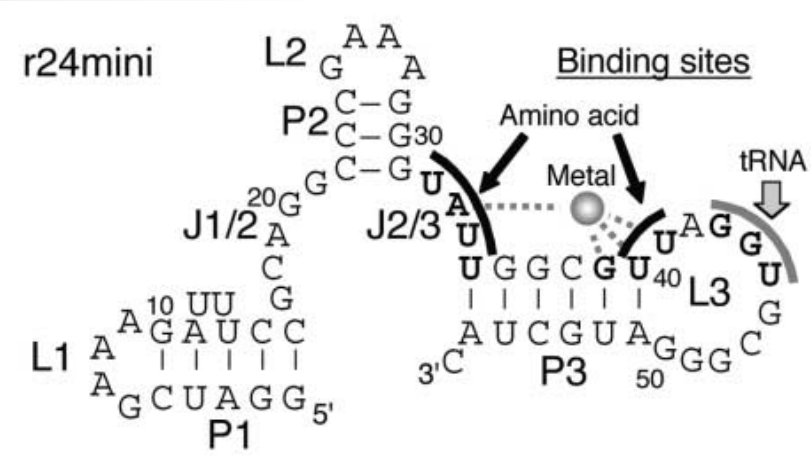
Saito, H.; Kourouklis, D.; Suga, H. *EMBO J.* **2001**, *20*, 1797.

Basic Science → **Specificity**

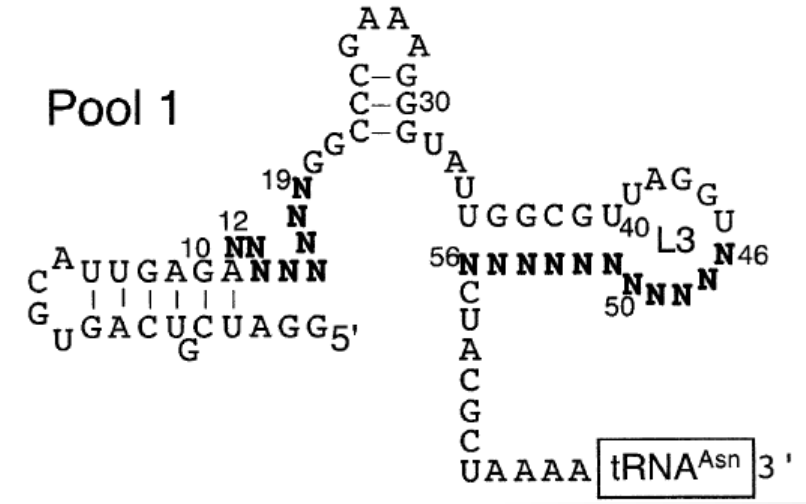
Applied Science → **Versatility**

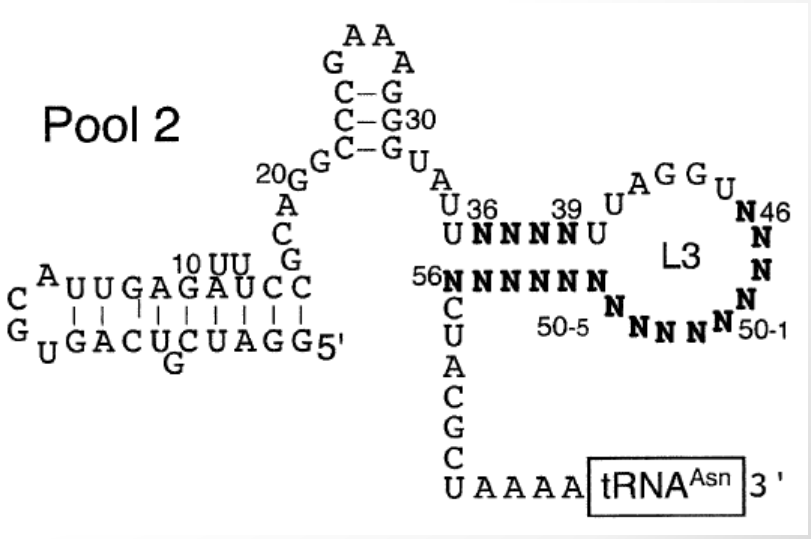
Expanding the Scope of tRNA 1

Original Ribozyme



Modification Design





for biochemical structural studyies,

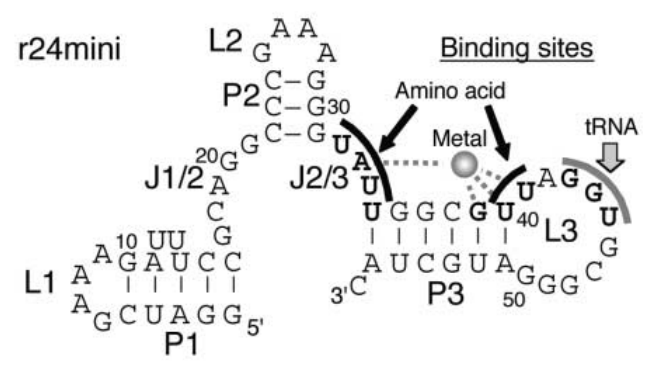
RNA **2001**, *7*, 1867.

Nucleic Acids Res. **2002**, *30,* 5151.

Murakami, H.; Saito, H.; Suga, H. *Chem. Biol.* **2003**, *10*, 655.

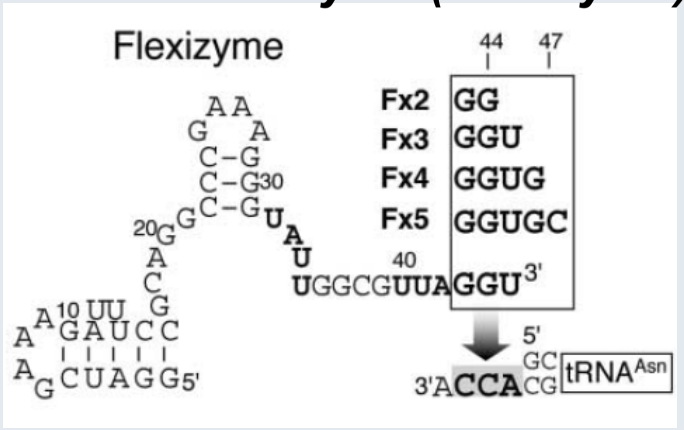
Expanding the Scope of tRNA 2

Original ribozyme (r24mini)

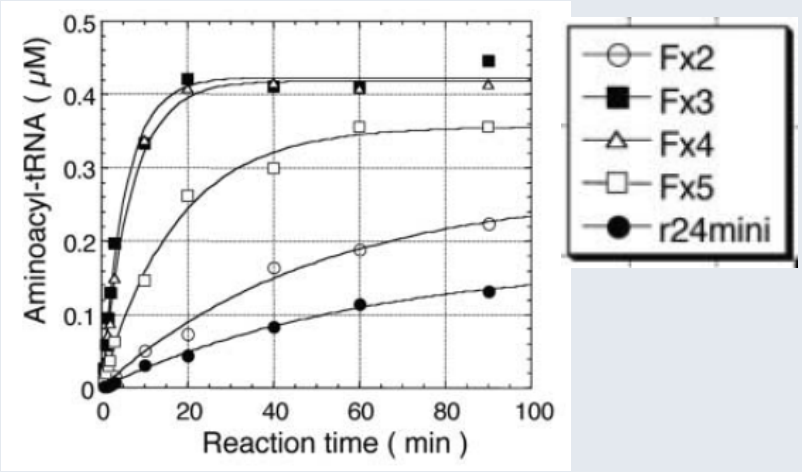


Just Deleting
N^{46} ~ N^{56}

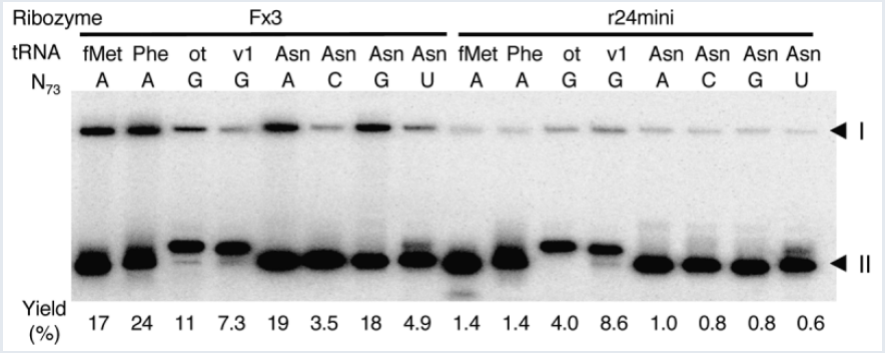
Evolved ribozyme (Flexizyme)



Comparison of the the ribozyme activities between Flexizymes (Fx2–5) and r24mini.



Aminoacylation activities of Fx3 and r24mini toward various tRNAs.

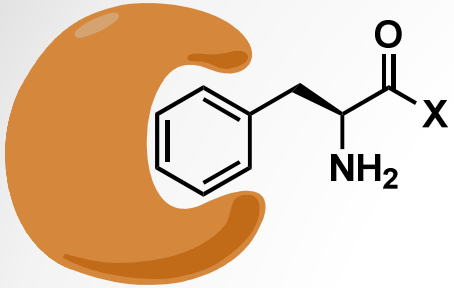


Flexizyme (Fx3) shows greater activity toward various tRNAs !

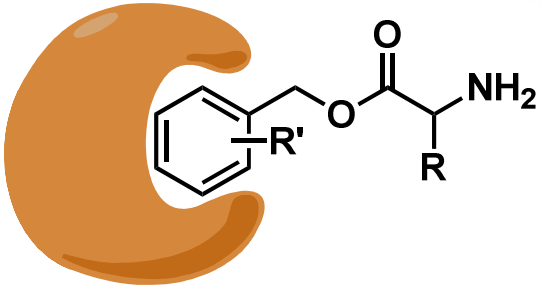
Murakami, H.; Saito, H.; Suga, H. *Chem. Biol.* **2003**, *10*, 655.

Expanding the Scope of Amino Acids

Hypothesis



Fx3 only recognize the benzyl group.

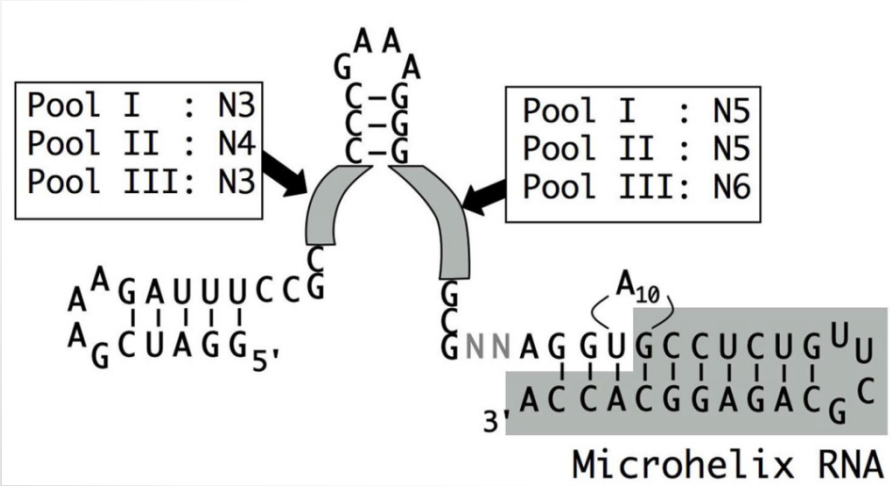


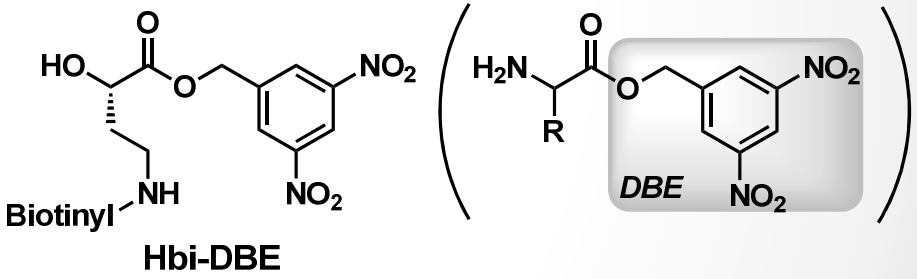
Benzyl group is embedded in leaving groups.

DBE → **dFx**

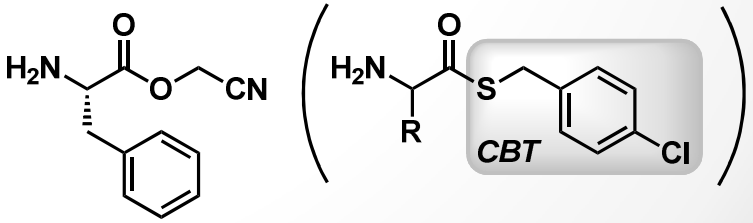
CBT → **eFx**

Modification Design





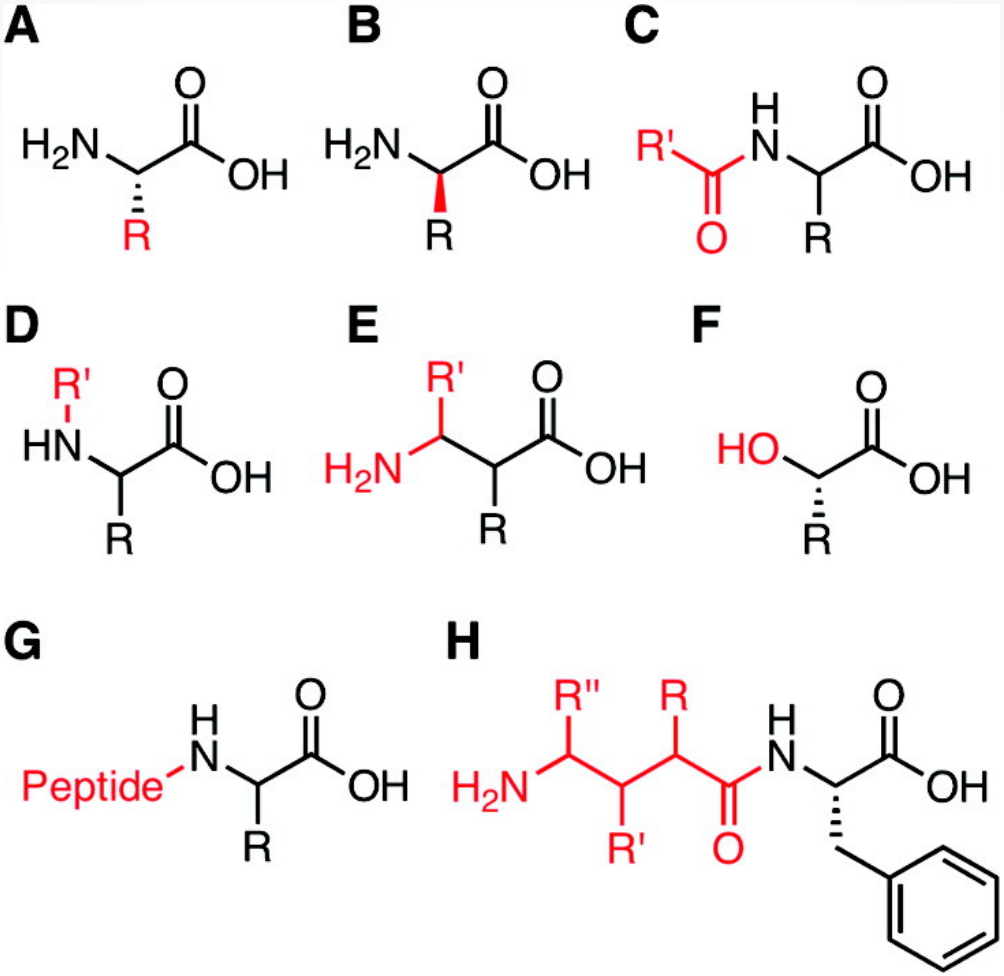
Hbi-DBE



Phe-CME

Murakami, H.; Ohta, A.; Ashigai, H.; Suga, H. *Nat. Methods* **2006**, *3*, 357.

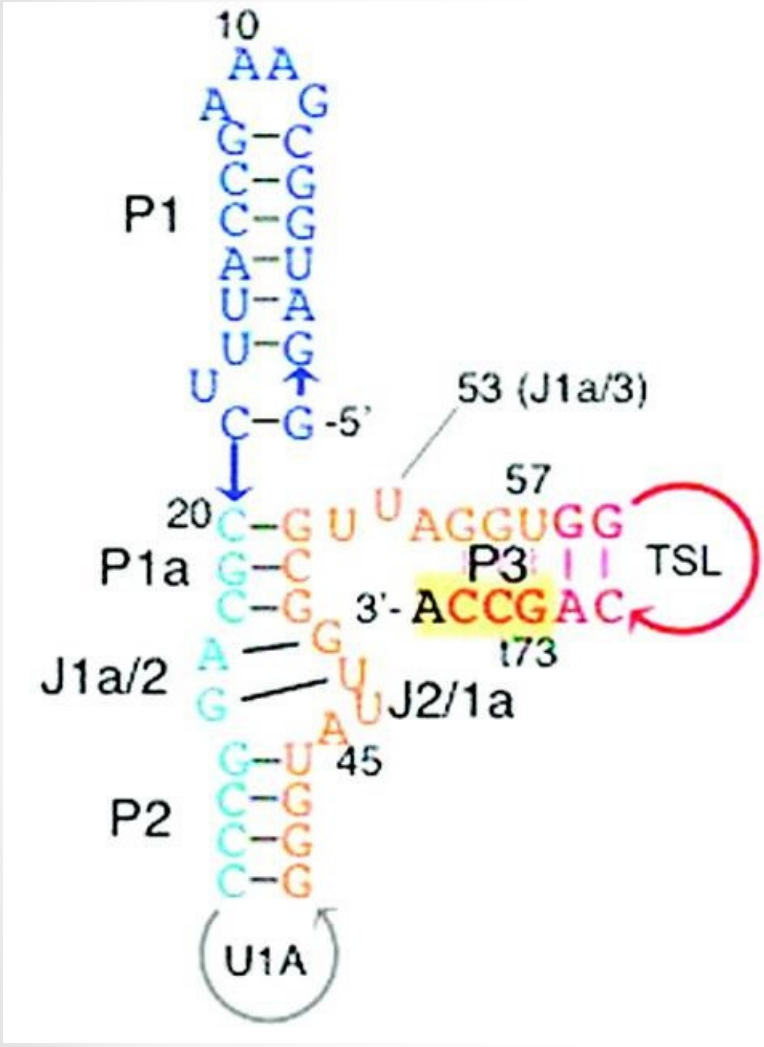
Scope of side chains



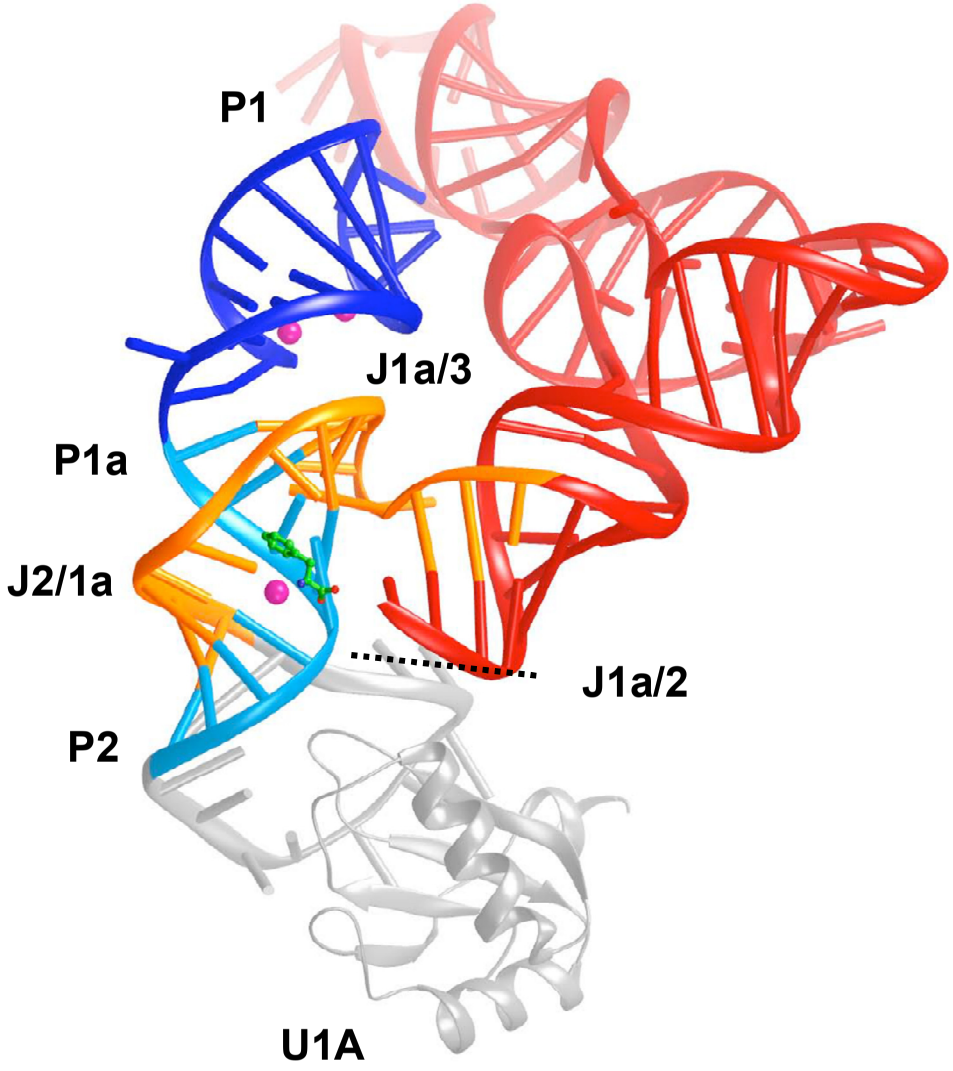
(A) L-Amino acid with nonproteinogenic side chain. (B) D-Amino acid. (C) R-*N*-acyl-amino acid.
(D) R-*N*-Alkyl-amino acid. (E) β-Amino acid. (F) R-Hydroxy acid.
(G) R-*N*-Peptidyl-amino acid. (H) γ-Aminoacyl-amino acid.

Flexizyme Structure (Crystal Structural Studies) 1

The secondary structure

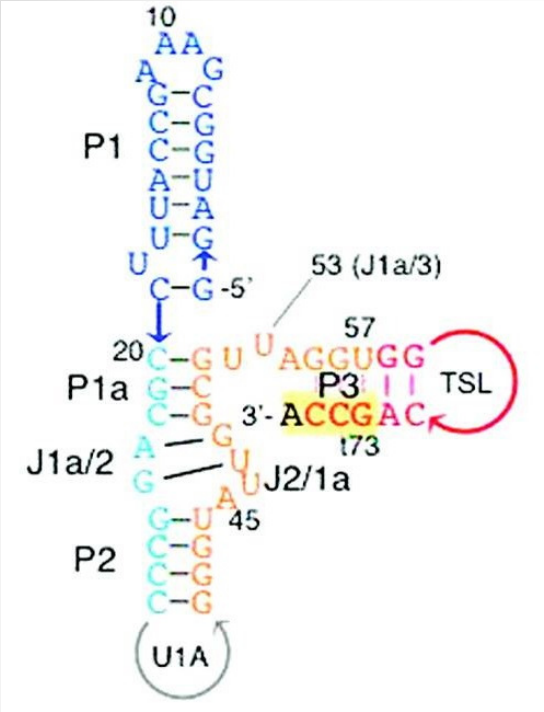


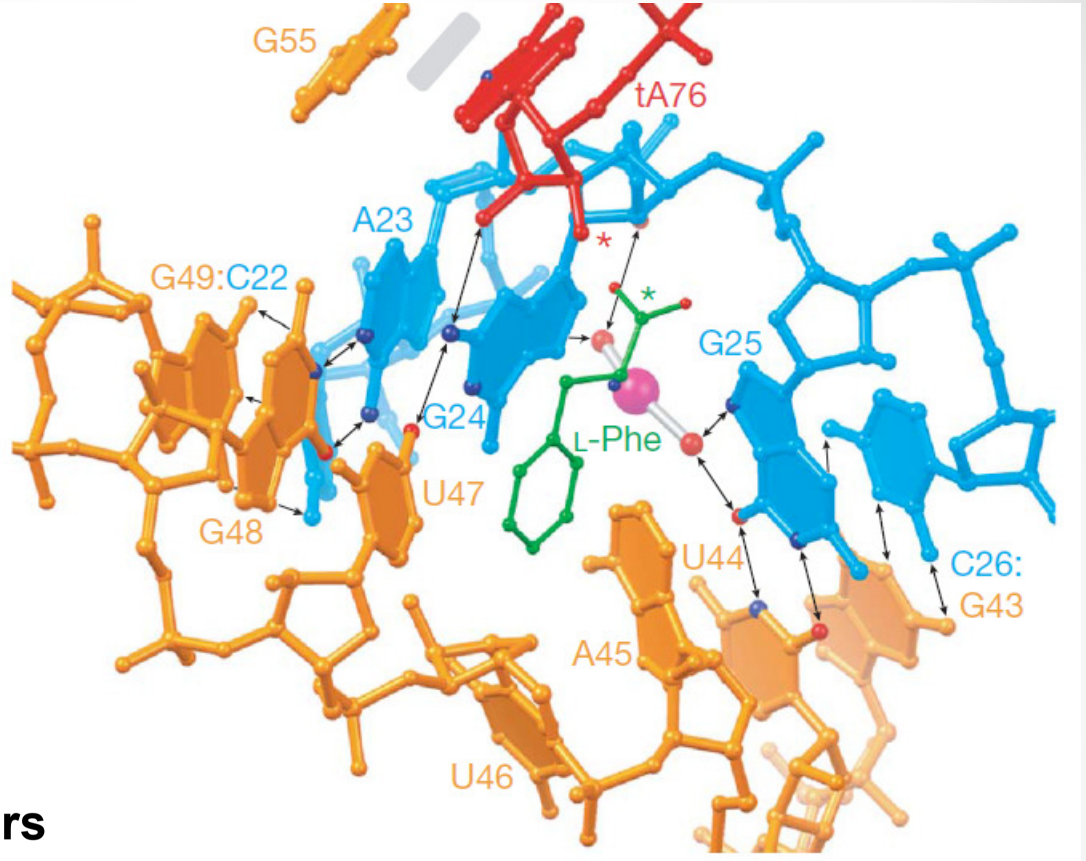
Interaction model with full-length tRNA



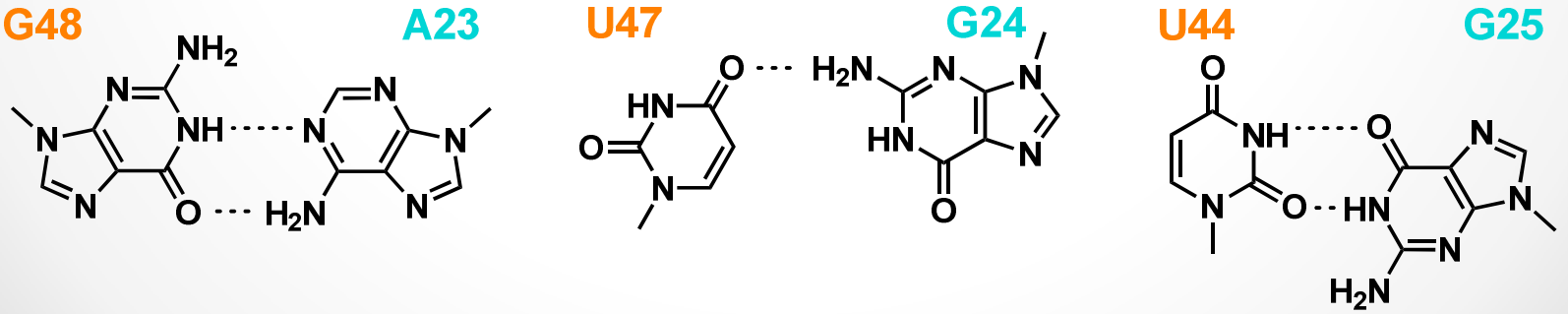
Xiao, H.; Murakami, H.; Suga, H.; Ferre-D'Amare, A. R. *Nature* **2008**, *454*, 358.

Flexizyme Structure (Crystal Structural Studies) 2



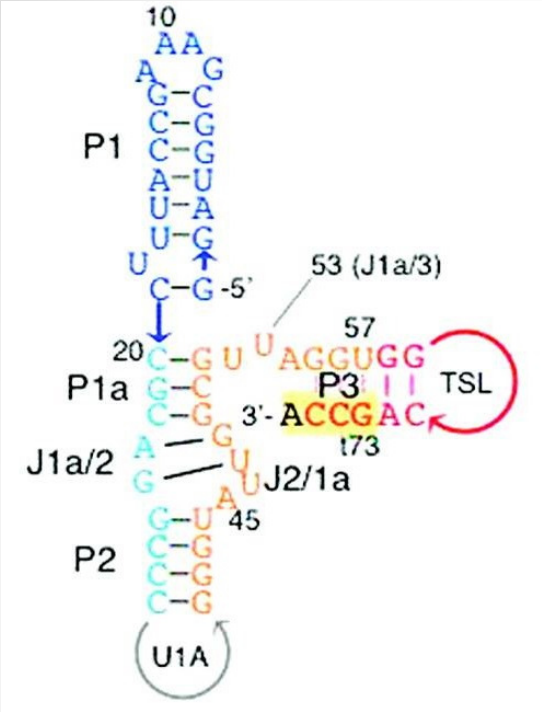


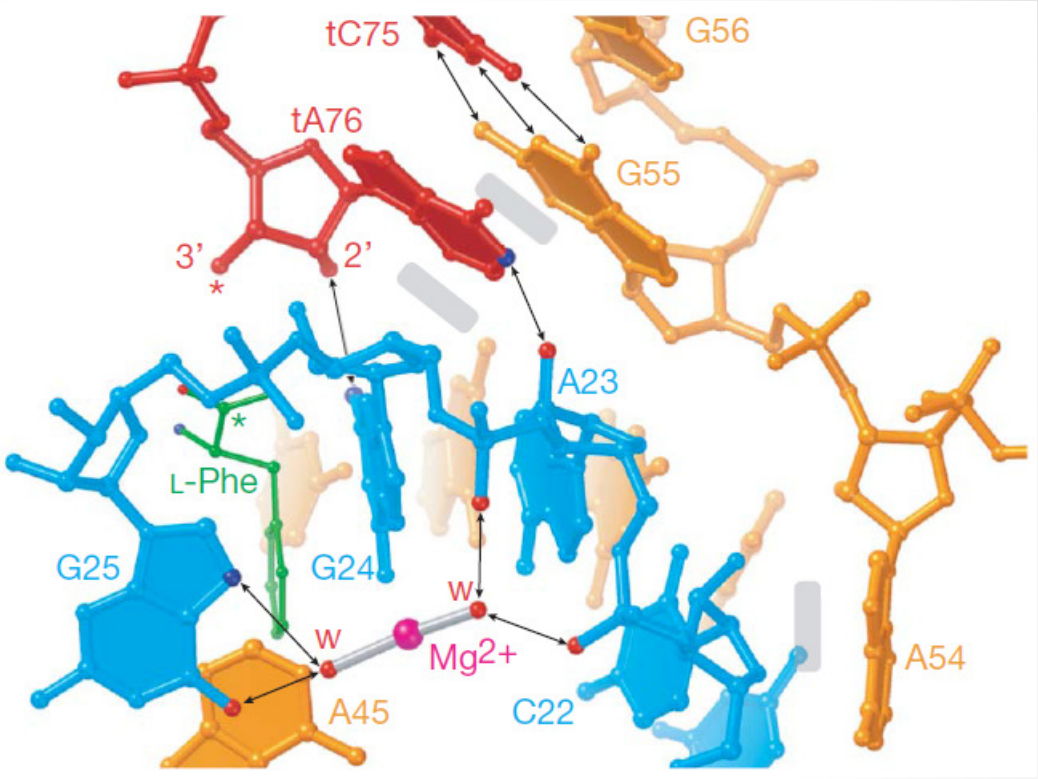
critically import base-pairs



Xiao, H.; Murakami, H.; Suga, H.; Ferre-D'Amare, A. R. *Nature* **2008**, *454*, 358.

Flexizyme Structure (Crystal Structural Studies) 3





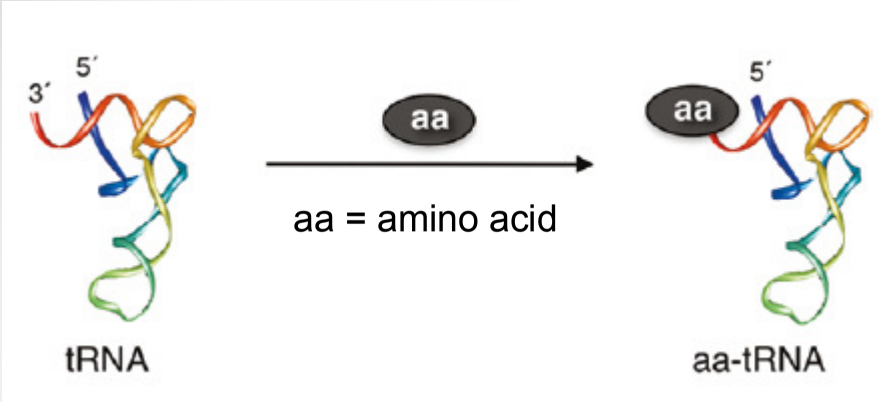
Amino Acid Recognition:
Interaction with O6 of G24 by its partial positive charge at the center of the phenyl ring

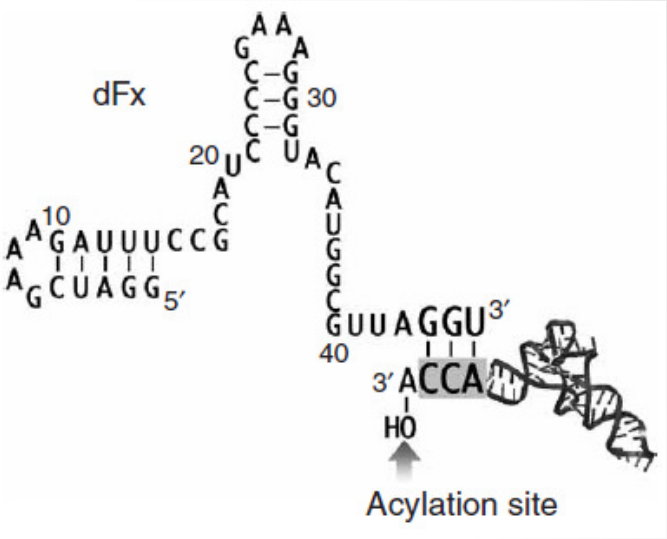
tRNA Acceptor End Recognition:
(1) a partial cross-strand stack of the base of tA76 with tC75-G55 pair
(2) the base of tA76 makes van der Waals contact with the ribose of G24
(3) a hydrogen bond between N1 of tA76 and the 2'-OH of A23
(4) a hydrogen bond between the 2'-OH of tA76 and N2 of G24

Xiao, H.; Murakami, H.; Suga, H.; Ferre-D'Amare, A. R. *Nature* **2008**, *454*, 358.

Small summary

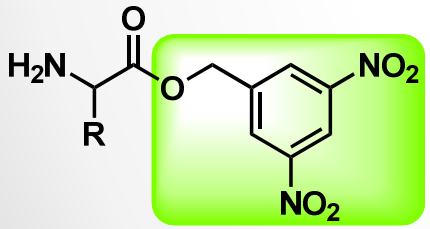
Charging amino acids onto tRNAs



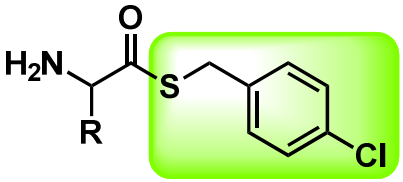


Amino Acid Recognition: Bn of LG

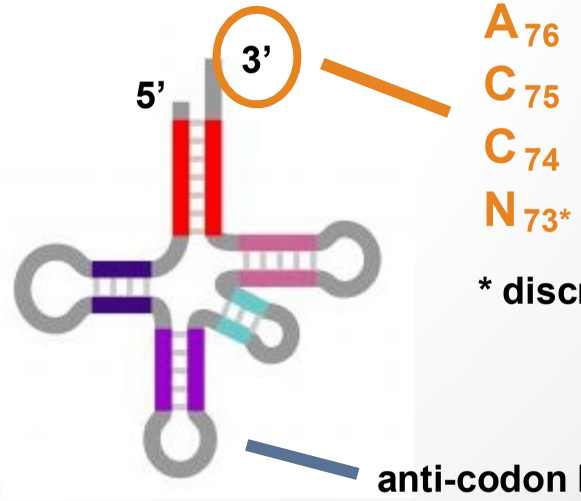
DBE (for dFx)



CBT (for eFx)



tRNA Recognition: N73-C75 (+A76)

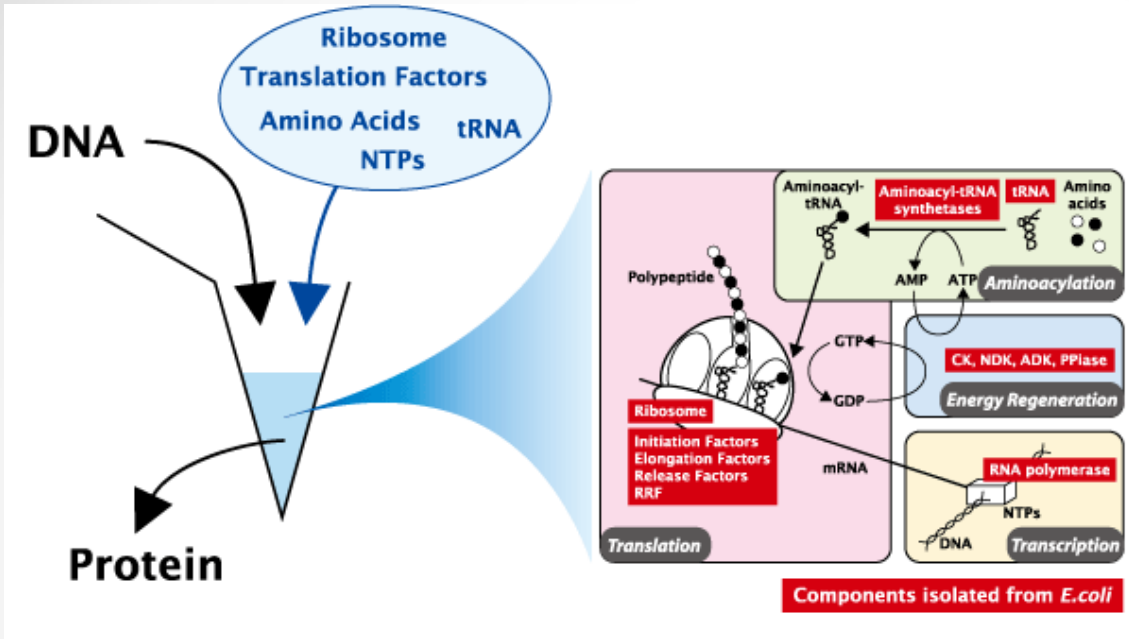


2. FIT system

~ *Nat. Protoc.* **2011**, *6, pp779–790.* ~

Overview

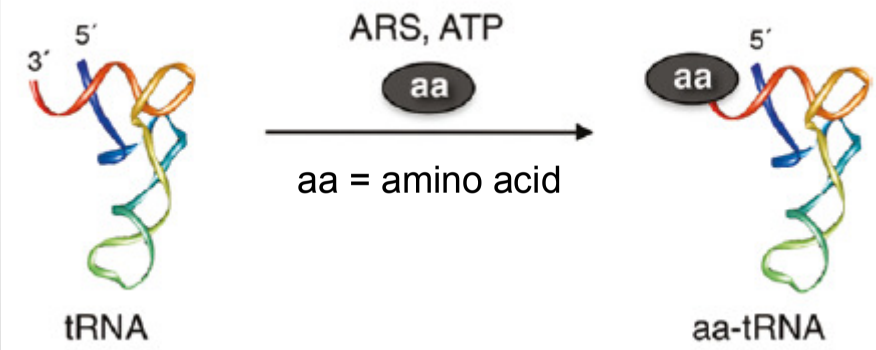
Reconstituted cell-free translation system



(from GeneFrontier Corporation)

+

Flexizyme

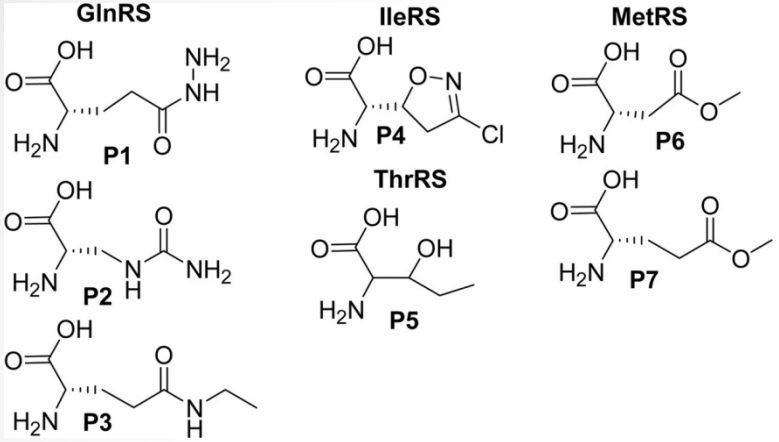


= FIT system

Non-Canonical AAs onto tRNA (the Other Ways)

1. Aminoacyl - tRNA Synthetases (ARSs)

Miss-acylation with natural ARSs



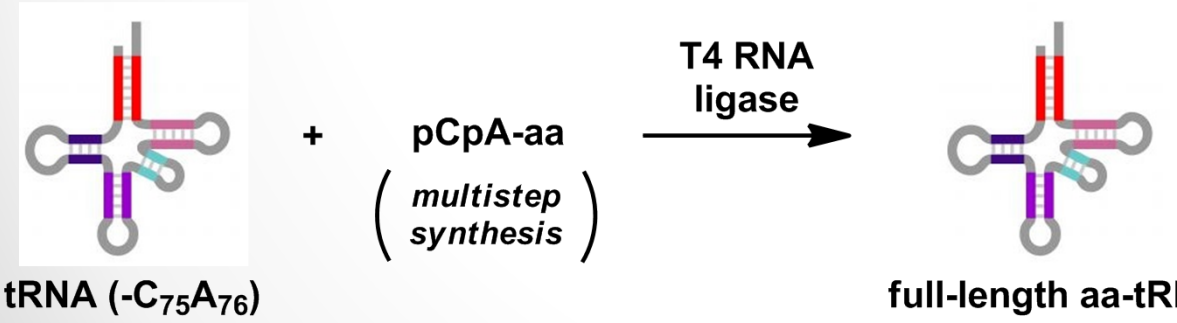
Engineered ARSs

Tirrell, D. A. *et al. Methods*, **2005**, *36*, 291.

Szostak J. W. *et al. Proc. Natl. Acad. Sci. USA* **2006**, *103*, 4356.

Szostak J. W. *et al. PLoS One 2* **2007**, e972.

2. Chemical Synthesis

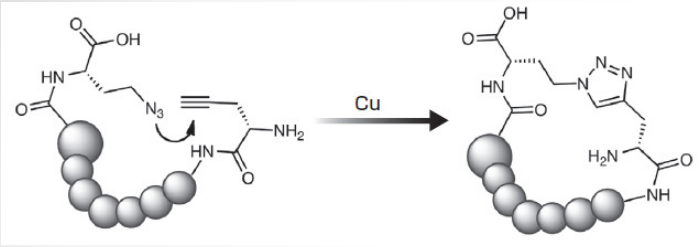


Hecht, S.M.; Alford B.L.; Kuroda Y.; Kitano S. *J. Biol. Chem.* **1978**, *253*, 4517.

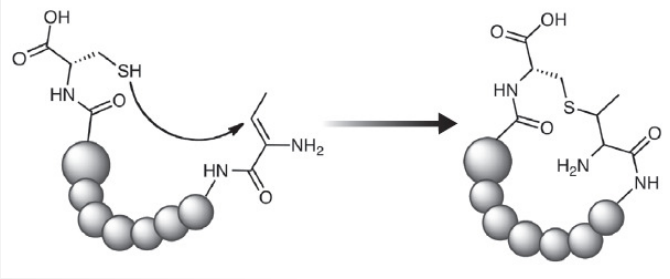
Schultz, P.G. *et al. J. Am. Chem. Soc.* **1991**, *113*, 2722.

Application

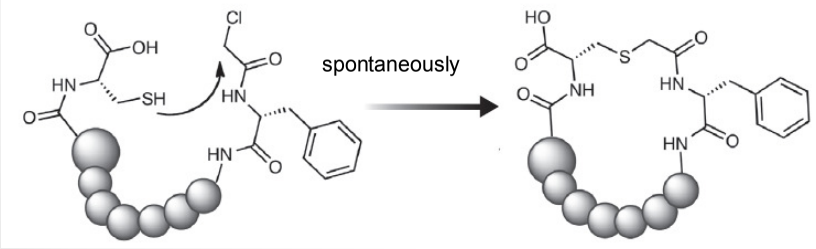
Huisgen Cycloaddition



Michael Addition



S_N2 Reaction



a

1st	2nd U	2nd C	2nd A	2nd G	3rd
U		^{Me}Ala	Tyr stop	Cys	U C A G
C		Pro	^{Me}Tyr	^{Me}Gly	U C A G
A	^{ClAc}Phe		Lys		U C A G
G			Asp		U C A G

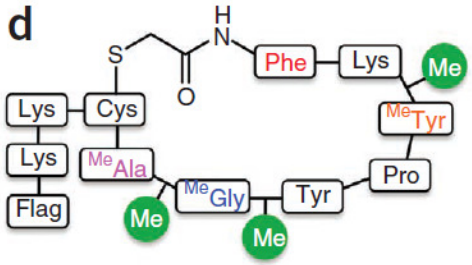
b

1st	2nd U	2nd C	2nd A	2nd G	3rd
U		^{Bu}Gly	Tyr stop	Cys	U C A G
C		Pro	^{Pr}Gly	^{Me}Gly	U C A G
A	^{ClAc}D-Phe		Lys		U C A G
G			Asp		U C A G

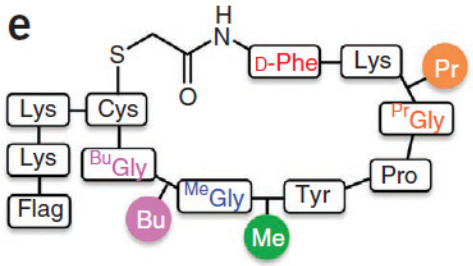
c

mRNA	AUG	AAG	CAC	CCA	UAC	CGC	UCC	UGC	(KK-Flag)	UAA
N-methyl peptide	^{ClAc}Phe	Lys	^{Me}Tyr	Pro	Tyr	^{Me}Gly	^{Me}Ala	Cys	KK-Flag	stop
Peptoid	^{ClAc}D-Phe	Lys	^{Pr}Gly	Pro	Tyr	^{Me}Gly	^{Bu}Gly	Cys	KK-Flag	stop

d



e



Peptoid:
artificially designed peptides composed of N-substituted glycine building blocks

Goto, Y.; Katoh, T.; Suga, H. *Nat. Protoc.* **2011**, *6*, 779.

3. RAPID system

~ *Chemistry & Biology* **2011**, *18*, 1562. ~

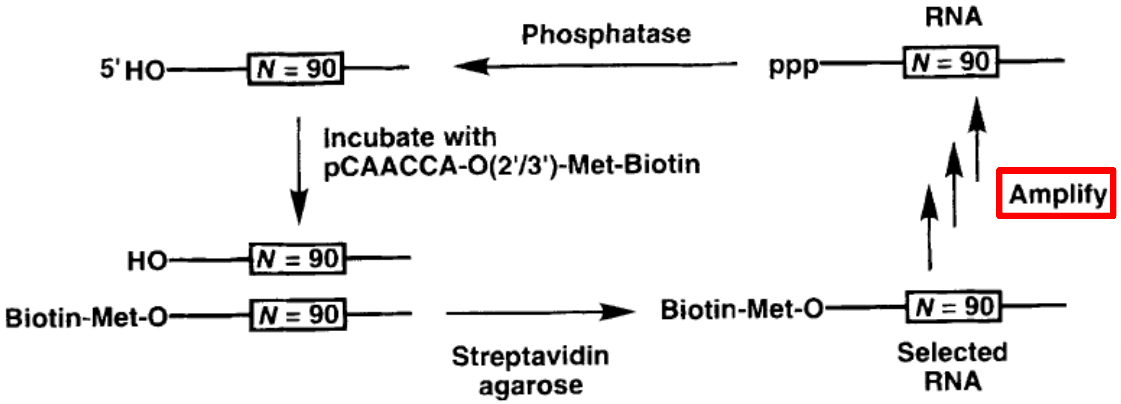
How to Select Bioactive-Peptide ?

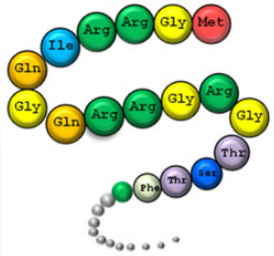
Information Flow

mRNA	AUG	AAG	CAC	CCA	UAC	CGC	UCC	UGC	(KK-Flag)	UAA
N-methyl peptide	ClAc-Phe	Lys	Me-Tyr	Pro	Tyr	Me-Gly	Me-Ala	Cys	KK-Flag	stop

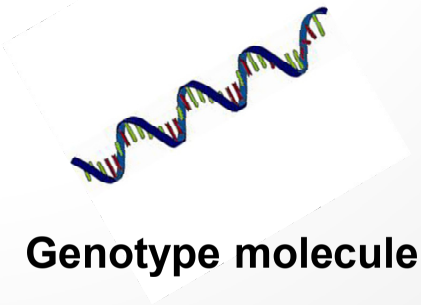
Screening peptides one by one ???

In vitro selection of RNA and DNA



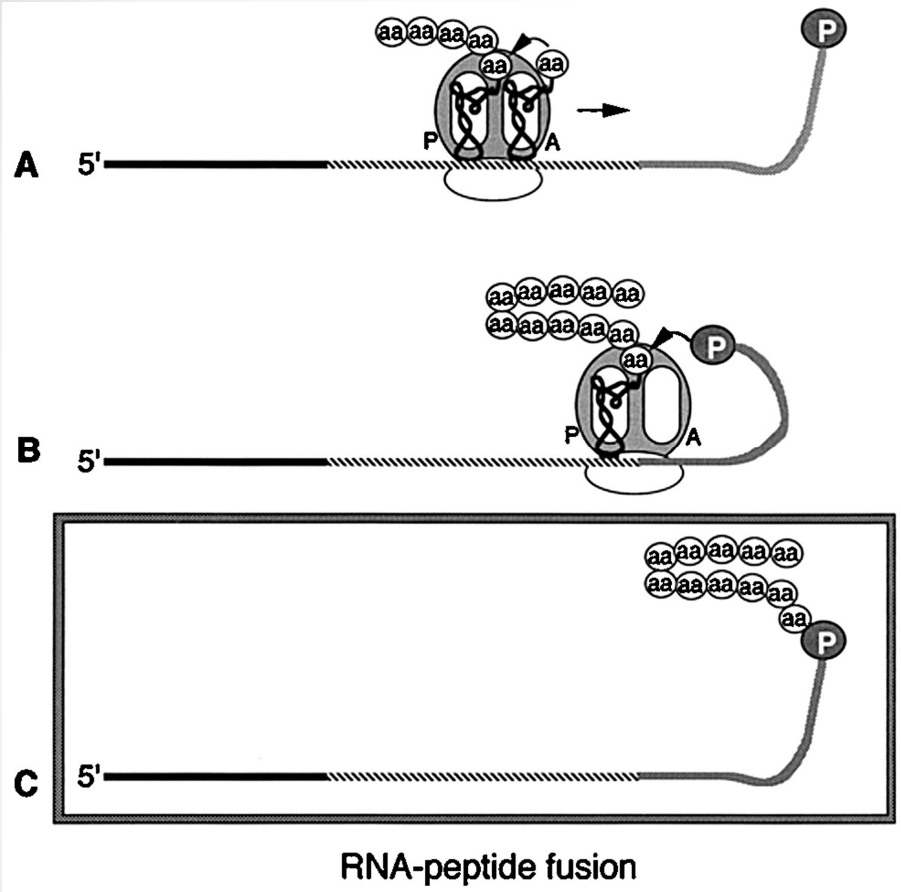


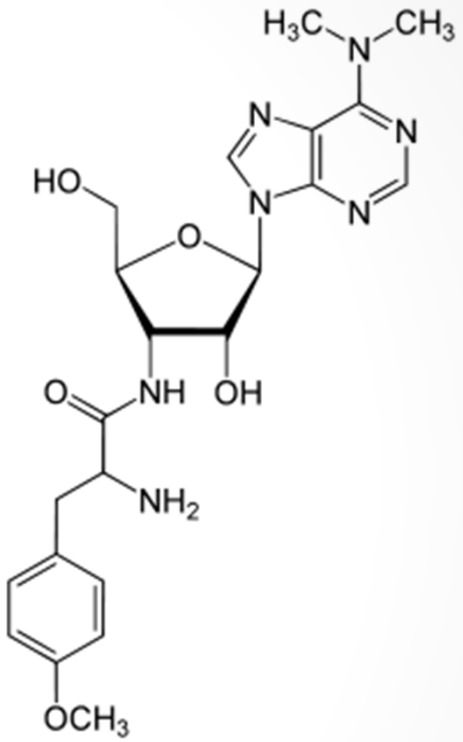
Phenotype molecule



Genotype molecule

mRNA Display (In Vitro Virus)



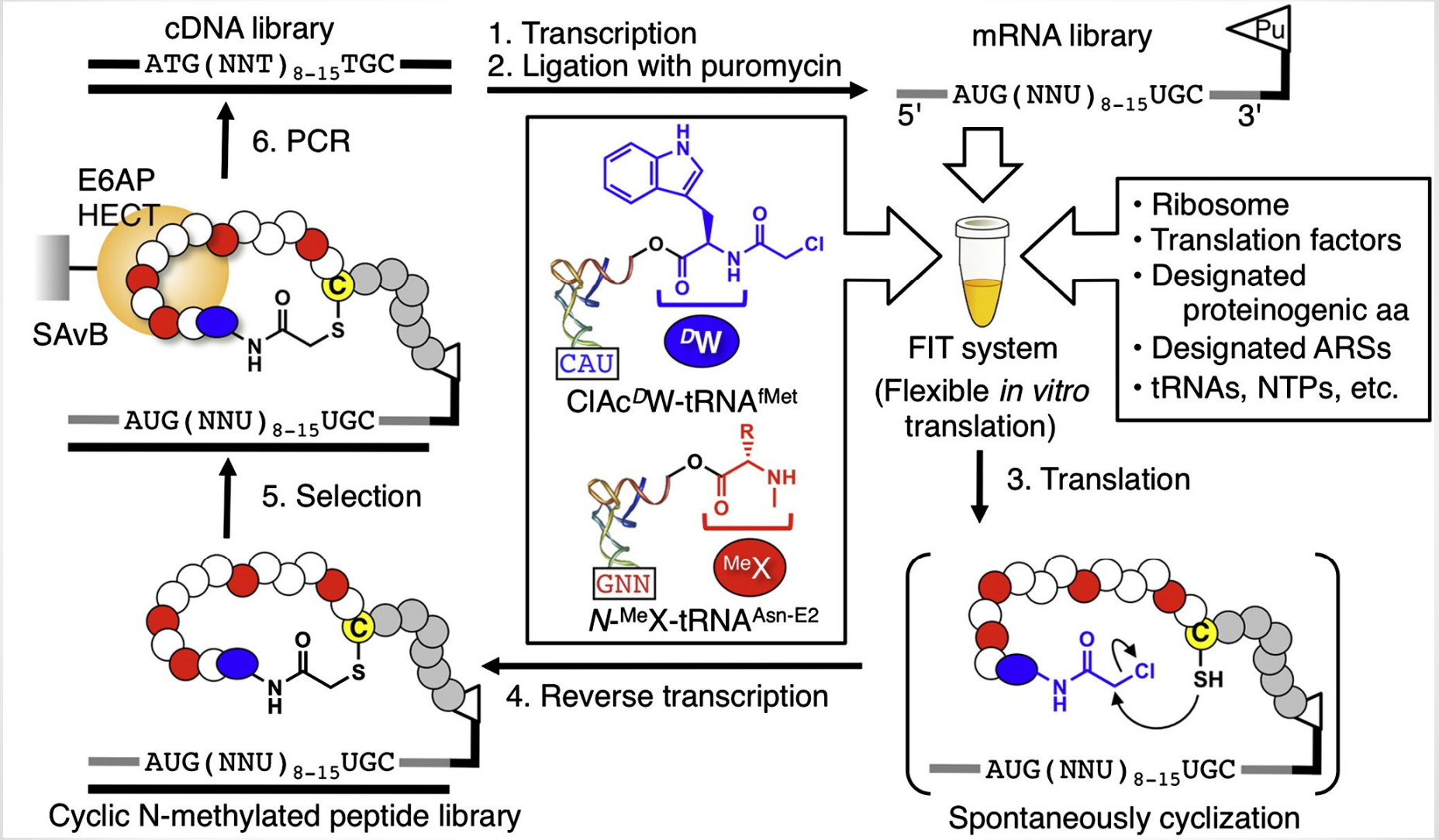


Nemoto, N.; Miyamoto-Sato, E.; Husimi, Y.; Yanagawa, H. *FEBS Lett*. **1997**, *414*, 405.

Roberts, R. W.; Szostak, J. W. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12297.

Keefe, A. D.; Szostak, J. W. *Nature* **2001**, *410*, 715.

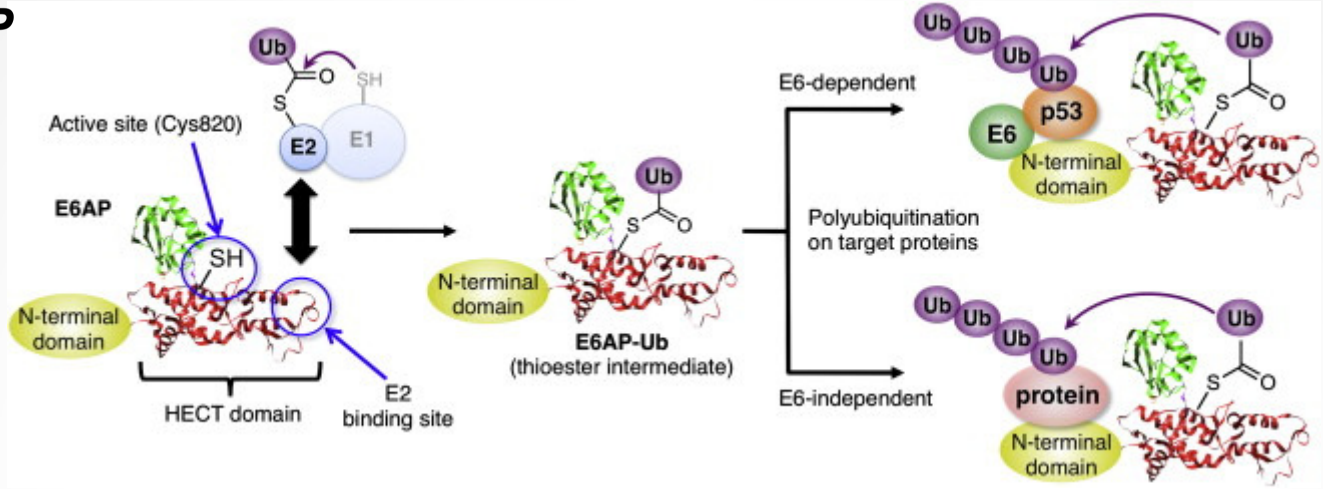
FIT System + mRNA Display = RAPID System



Yamagishi, Y.; Shoji, I.; Miyagawa, S.; Kawakami, T.; Katoh, T.; Goto, Y.; Suga, Y. *Chemistry & Biology* **2011**, *18*, 1562.

Application to the selective inhibitor against E6AP

E6AP

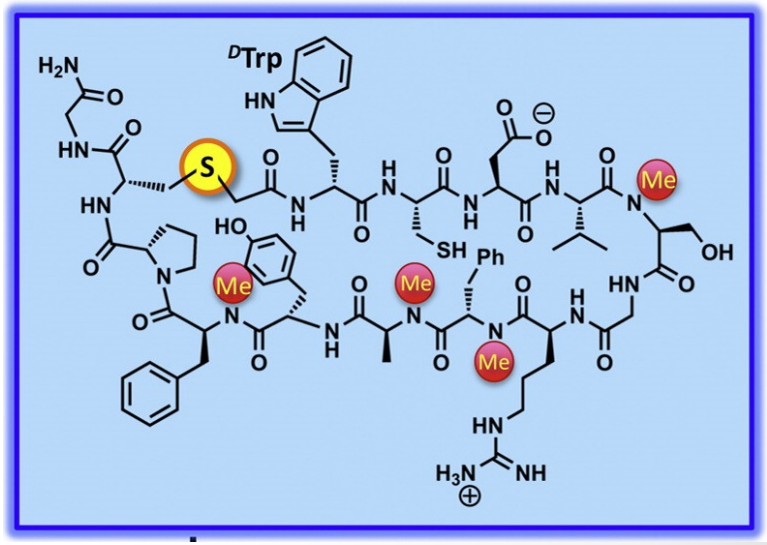


E6 protein originates from the high-risk types 16 and 18 human papillomavirus (HPV).

Reprogrammed genetic code

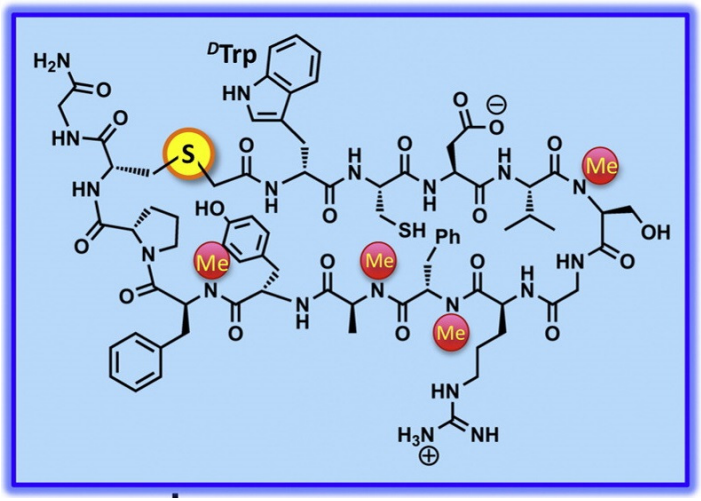
1st	2nd U	2nd C	2nd A	2nd G	3rd
U	^{Me}F	Ser	Tyr	Cys	U C A G
C	^{Me}S	Pro	His	Arg	U C A G
A	^{Me}G ClAc^{D}W	Thr	Asn	Ser	U C A G
G	Val	^{Me}A	Asp	Gly	U C A G

Selected Macrocyclic N-methyl-peptide



Yamagishi, Y.; Shoji, I.; Miyagawa, S.; Kawakami, T.; Katoh, T.; Goto, Y.; Suga, Y. *Chemistry & Biology* **2011**, *18*, 1562.

Application to the selective inhibitor against E6AP



S

$Ac^{D}WCDV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG\text{-}NH_2$

Kd = 0.60 nM

$Ac^{D}WCDV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG\text{-}NH_2$

Linear (no thioeter linkage)
Kd = 180 nM

S

$Ac^{D}WCDVSGRFGYFPCG\text{-}NH_2$

***N*-H amino acids (no *N*-Me)**
Kd > 1000 nM

Both thioester linkage & *N*-Me modification are essential !

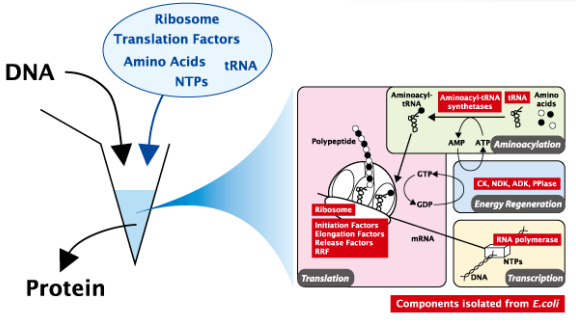
Yamagishi, Y.; Shoji, I.; Miyagawa, S.; Kawakami, T.; Katoh, T.; Goto, Y.; Suga, Y.
Chemistry & Biology **2011**, *18*, 1562.

4. Summary

The RNA world hypothesis

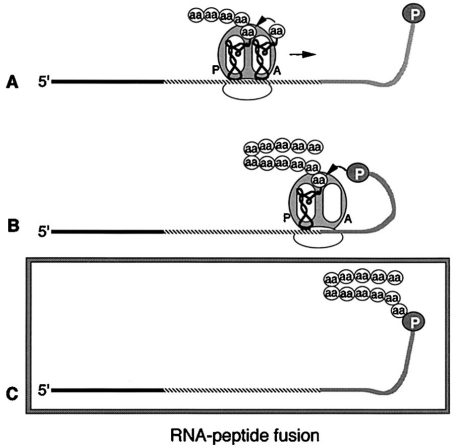
Flexizyme

Reconstituted cell-free translation system



FIT system

mRNA Display



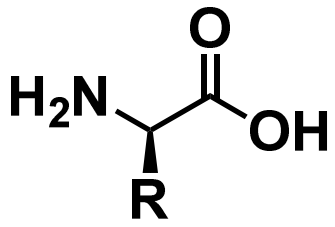
RAPID system

Peptidream Inc.

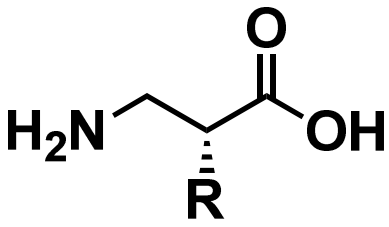
Ribosomal Synthesis of Unnatural Peptides
Josephson, K.; Hartman, M. C. T.; Szostak, J. W.
J. Am. Chem. Soc., **2005**, *127*, 11727.

Limitation

Even though flexizymes facilitate the preparation of tRNAs charged with D-amino acids or β-amino acids,
some of these amino acids could not be consecutively elongated because of poor compatibility of naturally occurring ribosomes.
(It should be noted that the initiation event turned out to be more amenable to a wide variety of amino acids.)



D-amino acid



β-amino acid