

# Peptide synthesis utilizing micro-flow technology

Literature Seminar #1

2020.11.19

B4 Kazuki Oikawa

# Contents

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## 1. Introduction

## 2. Micro-flow technology for peptide synthesis

- Solid-phase  $\alpha$ -peptide synthesis
- Solution-phase  $\alpha$ -peptide synthesis
- Cyclic peptide synthesis

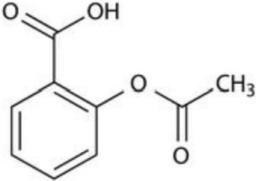
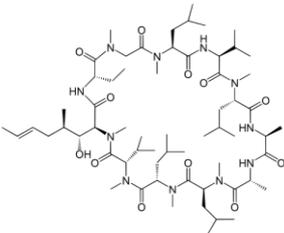
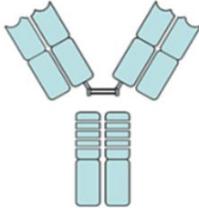
## 3. Application and latest development

- Total synthesis of Feglymycin
- Rapid total synthesis of protein
- Optimization by Deep learning

## 4. Summary

# Introduction

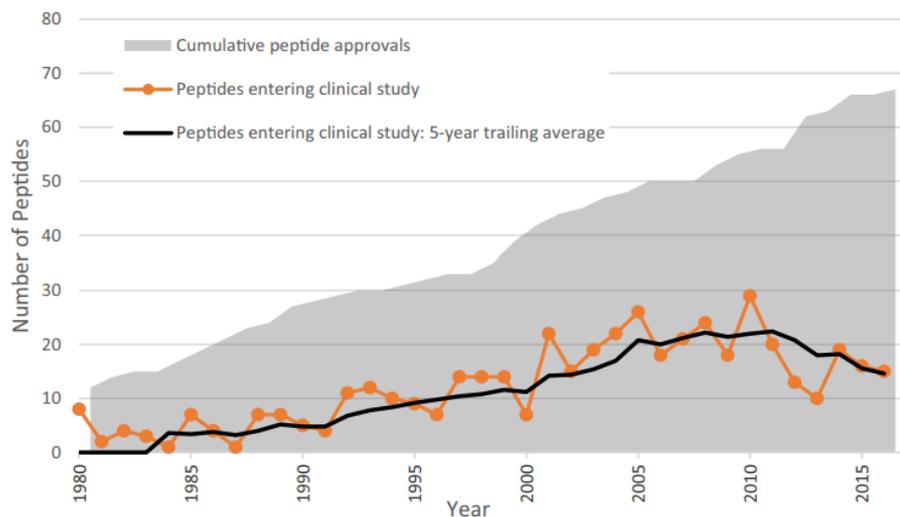
## ○ Peptide drugs

	Small molecule drugs	<b>Peptide drugs</b>	Macromolecular drugs
			
Molecular weight	300-500	<b>1000-10000</b>	50000-150000
Oral administration	○	○ or x	x
Cell penetration	◎	○	x
Target selectivity	x	○	◎
Synthetic approach	Chemical	<b>Chemical or Biological</b>	Biological
Manufacturing cost	Low	<b>Relatively Low</b>	High
Possibility to launch	Low	<b>Relatively High</b>	Relatively High

**Peptide drugs have the merits of both small molecular drugs and macromolecular drugs!!**

# Introduction

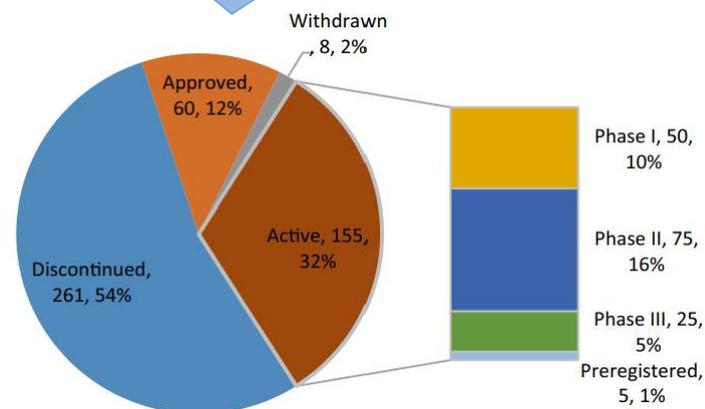
## ○ Peptide drugs



Since 1980, the number of approved peptide drugs gradually increased and over **60** drugs are approved.

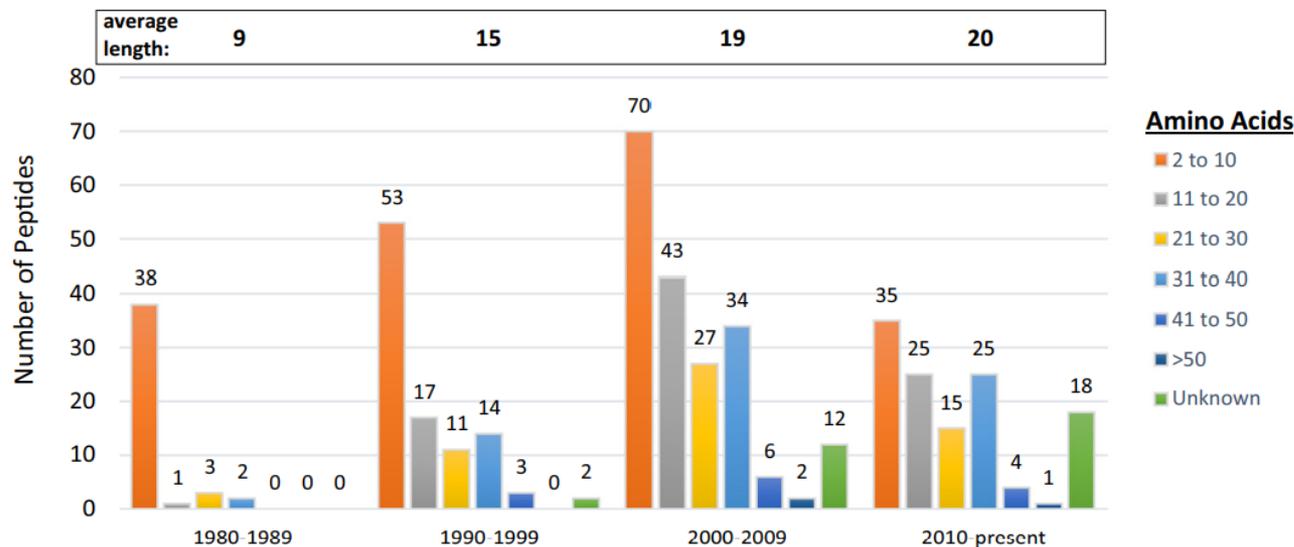
**155** peptide drugs are in active clinical development(2017)

Total sales of peptide drugs is **\$50 billion** and **5%** of total pharmaceutical sales. This percentage is estimated to grow **9-10%** annually!



# Introduction

## ○ Peptide drugs



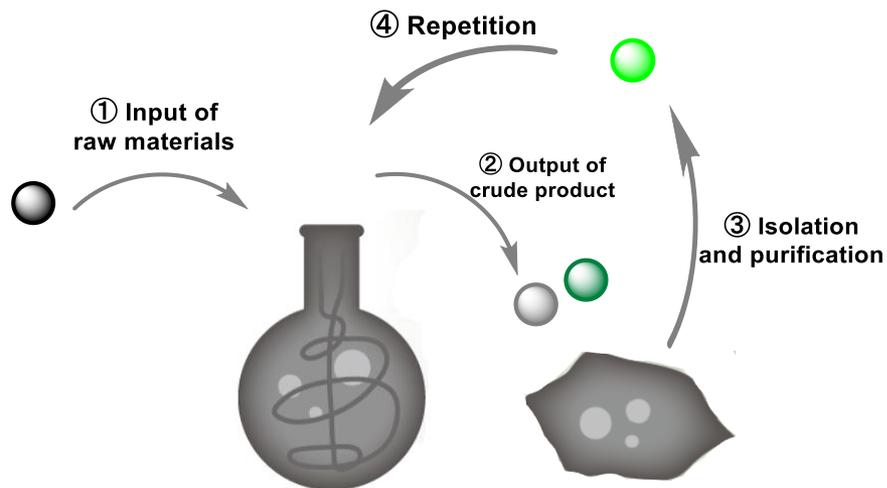
The number of long-chain, complex peptide drugs are increasing due to improvements in peptide synthesis and manufacture technology

**More scaled-up, high-yielding, cost-effective, and less-wasteful method of peptide synthesis are needed...**

# Introduction

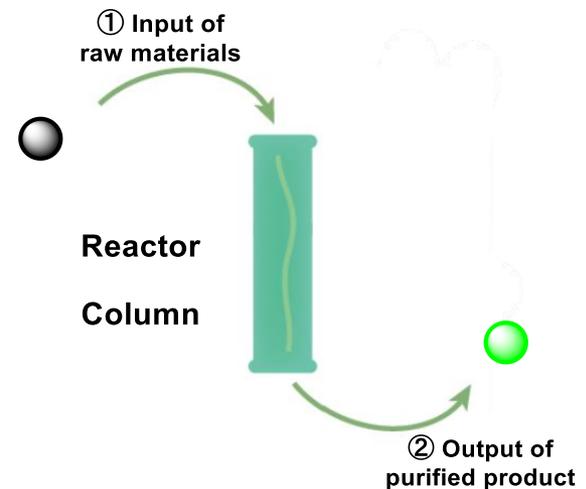
## ○ What is Flow reaction?

### Batch reaction



- Large reactor
- Charging, reaction, recovery is conducted one by one

### Flow reaction



- Small channel
- Continuous reaction

# Introduction

## ○ Equipment for micro-flow reaction

### Micro reactor



### Components

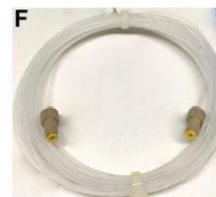
Pump



Mixer



Reactor coil



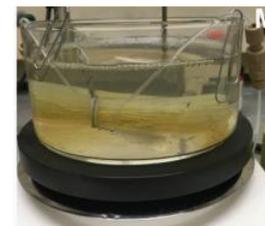
Packed bed reactor



Thermal jacket



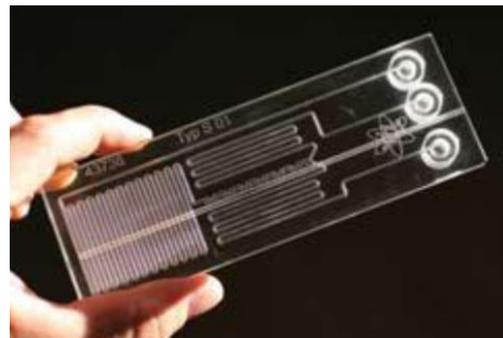
Oil bath



# Introduction

## ○ Features of micro-flow reaction

- ✓ Short diffusion length
  - ⇒ **Precise control of short reaction time**
- ✓ Large surface-to-volume
  - ⇒ **Rapid and precise temperature control**
- ✓ Thin nature of microreactor
  - ⇒ **Light penetration efficiency**
- ✓ Small reaction space
  - ⇒ **Minimized risk in handling dangerous compounds**
- ✓ Continuous operation or increasing the number of reactors
  - ⇒ **Easy scale-up**



**Micro-flow technology enables the use of highly reactive, unstable, explosive or toxic compounds**

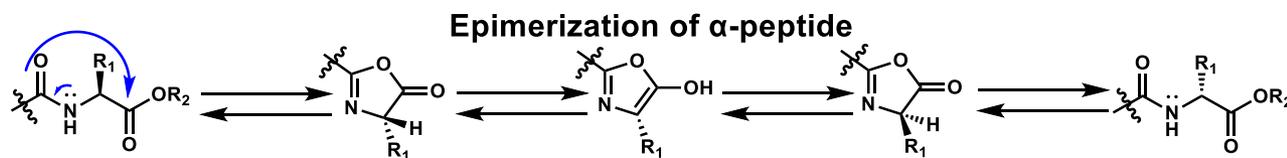
# Introduction

## ○ Features especially in peptide syntheses

### Problems of conventional syntheses

- Liquid-phase approach
  - ⇒ ○ Large scale
  - × Complicated purification Long reaction time
- Solid-phase approach
  - ⇒ ○ Rapid reaction Easy purification
  - × Low atom economy Small scale
- Both approaches
  - ⇒ × Epimerization Aggregation

Micro-flow peptide synthesis focuses on lined (○○○) points



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- Cyclic peptide synthesis

## 3. Application and latest development

- Total synthesis of Feglymycin
- Rapid total synthesis of protein
- Optimization by Deep learning

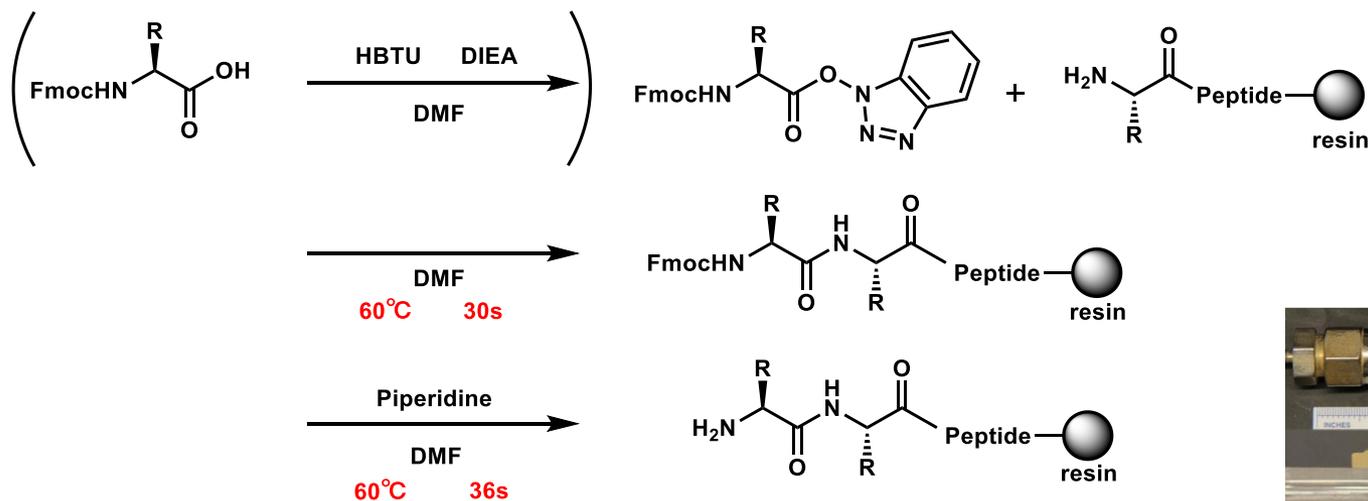
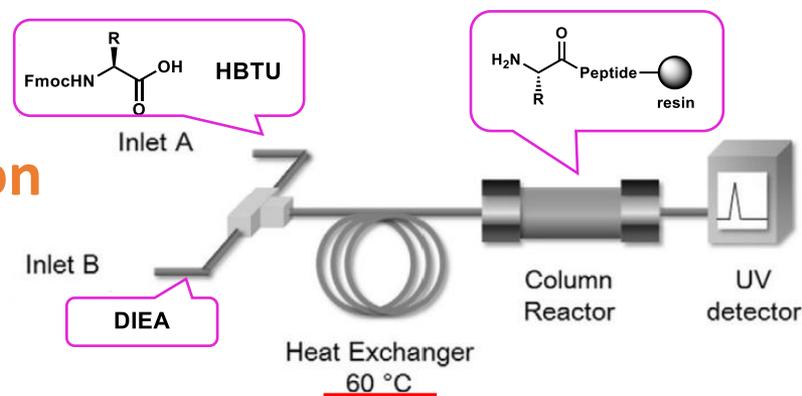
## 4. Summary

# Solid-phase $\alpha$ -Peptide synthesis

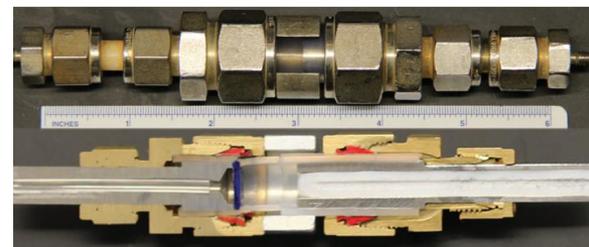
## ○ Synthesis on a solid-phase column reactor

Rapid preheat

⇒ Rapid reaction without degradation



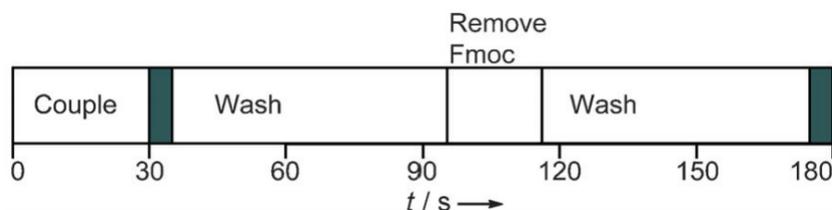
Reaction vessel



# Solid-phase $\alpha$ -Peptide synthesis

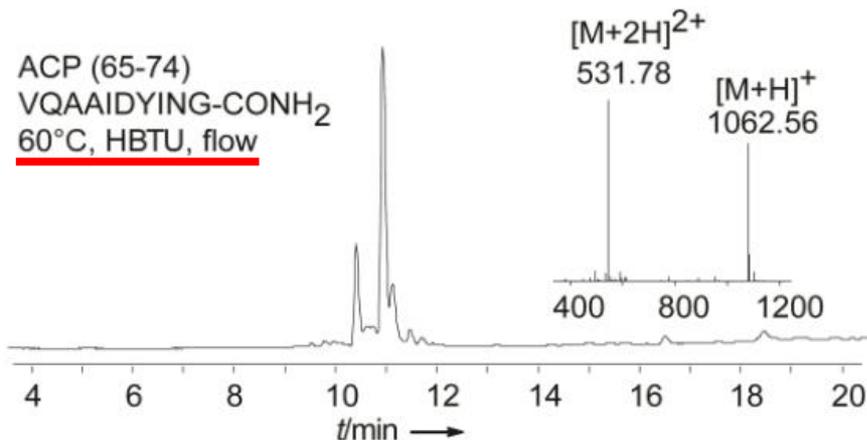
## ○ Synthesis on a solid-phase column reactor

### 1 cycle of peptide chain elongation

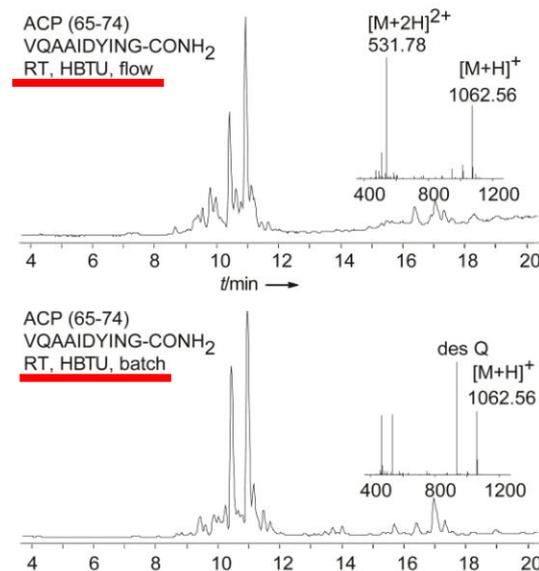


Achieved rapid peptide synthesis that took only **3 minutes** to extend 1 residue!

### Synthesis of part of protein

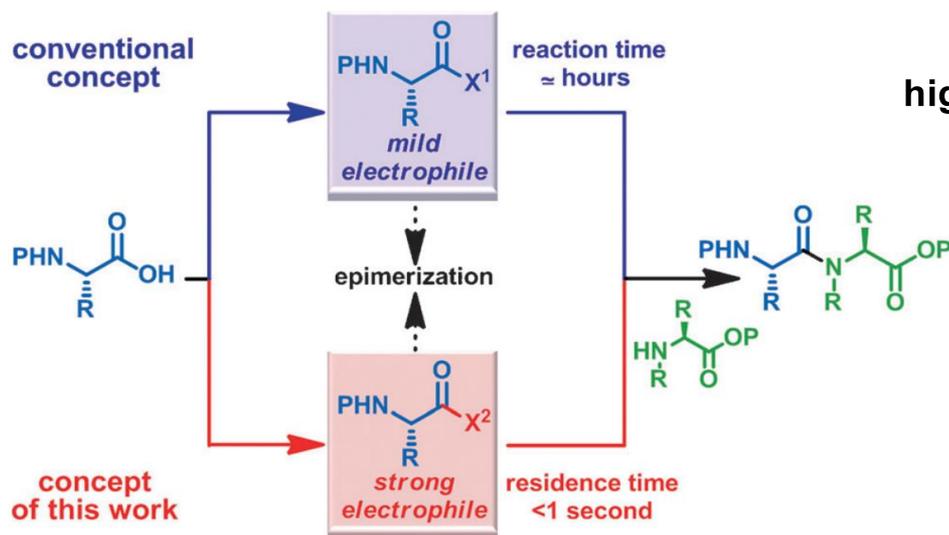


### Data for comparison



# Solution-phase $\alpha$ -Peptide synthesis

## ○ Synthesis via rapid and strong activation using Triphosgene

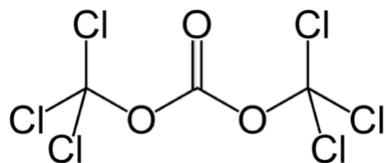


Strong electrophile wasn't used because of high risk of side reactions such as epimerization



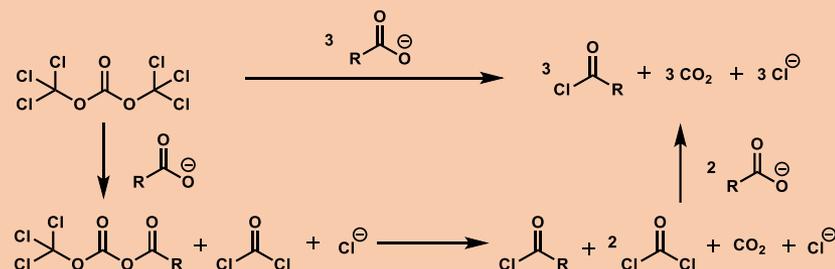
Rapid activation and short residence time enabled suppression of epimerization

Strong activation by triphosgene enabled rapid reaction with less nucleophilic amines, including **N-methyl amino acids**



Triphosgene

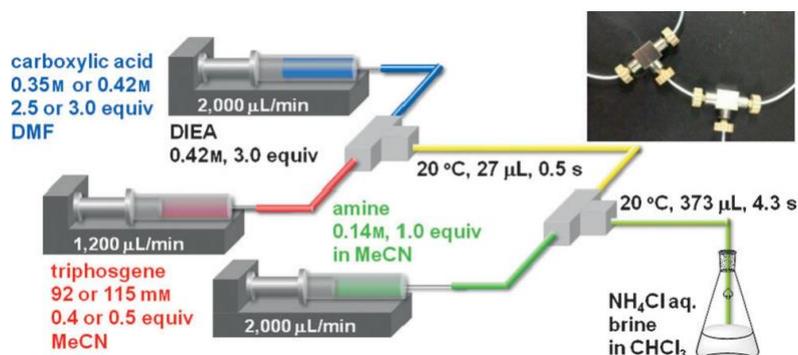
### Mechanism of acid chloride formation



# Solution-phase $\alpha$ -Peptide synthesis

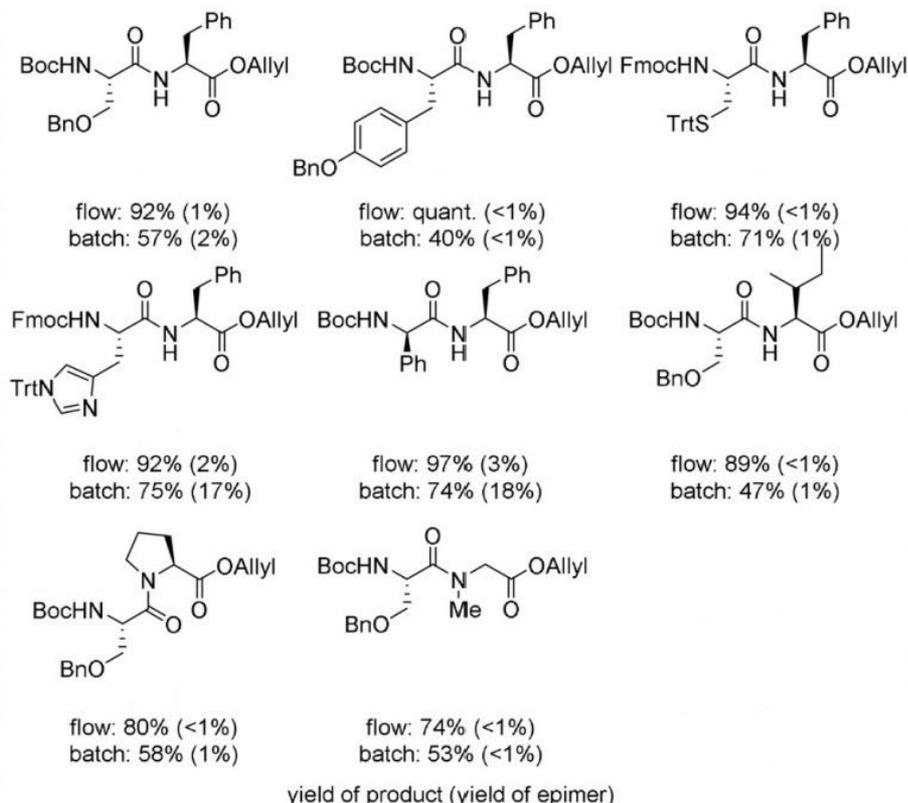
## Synthesis via rapid and strong activation using Triphosgene

### Optimized conditions



- ✓ Short reaction time
- ✓ High yield and Low epimerization level (compared to batch reaction)

### Substrate scope



(Reaction time of batch synthesis was 30s)

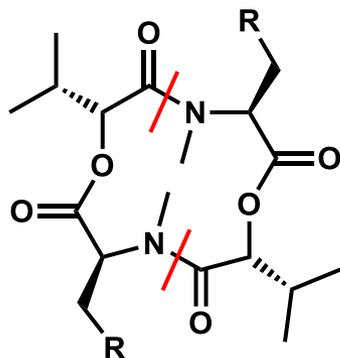
# Cyclic peptide synthesis

## Synthesis of CODs

### Cyclooligomeric depsipeptides (CODs)

- Structures derived from repeated oligopeptidol monomer units
- Exhibit a wide variety of biological activities

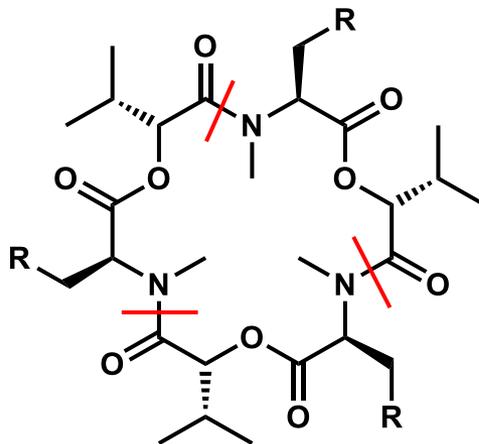
12-membered CODs



R= Ph

R= <sup>i</sup>Pr

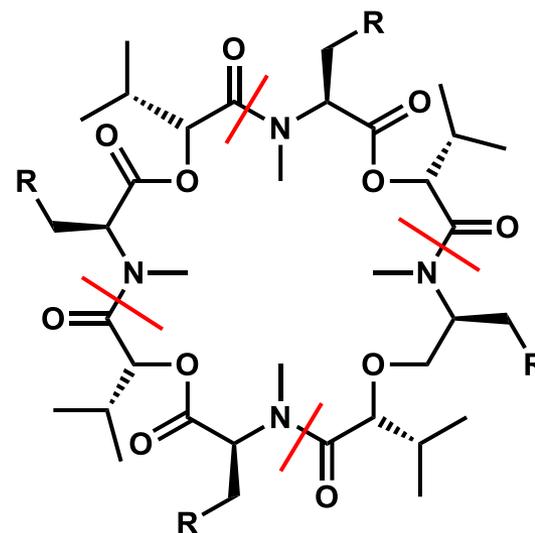
18-membered CODs



R= Ph beauvericin

R= <sup>i</sup>Pr enniatin C

24 membered CODs



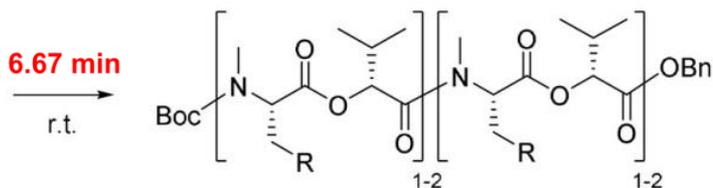
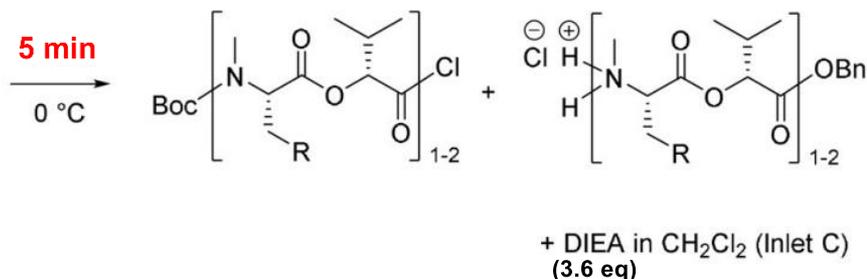
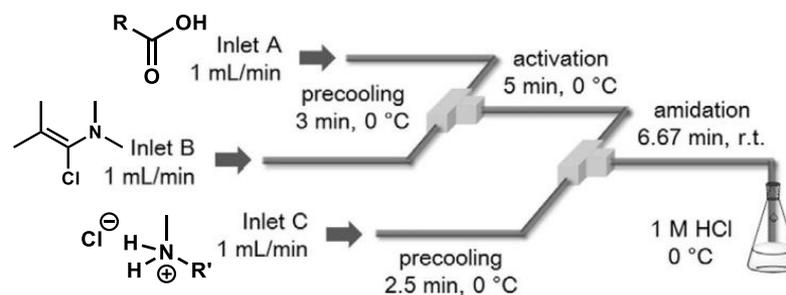
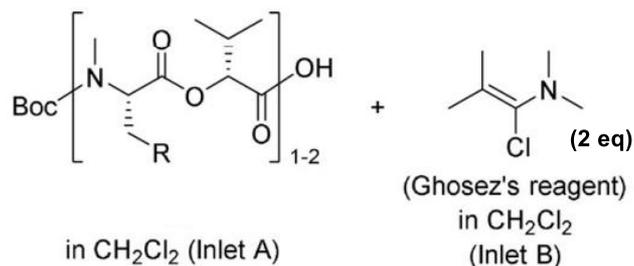
R= Ph

R= <sup>i</sup>Pr bassianolide

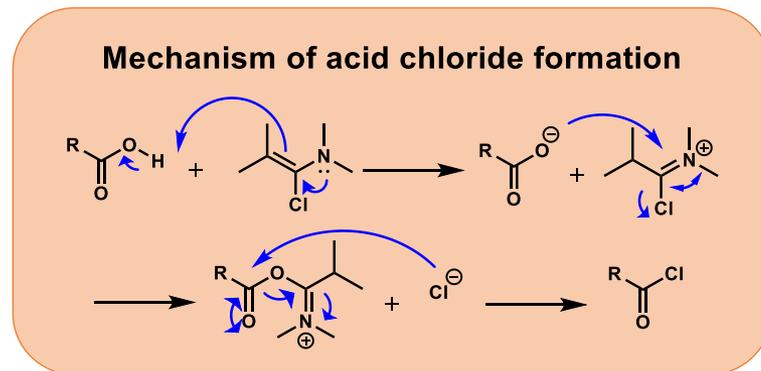
# Cyclic peptide synthesis

## Synthesis of CODs

### Synthesis of linear depsipeptides



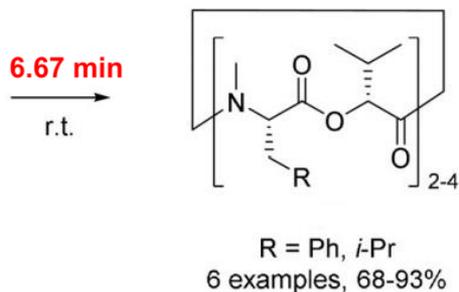
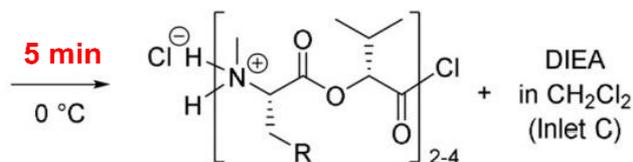
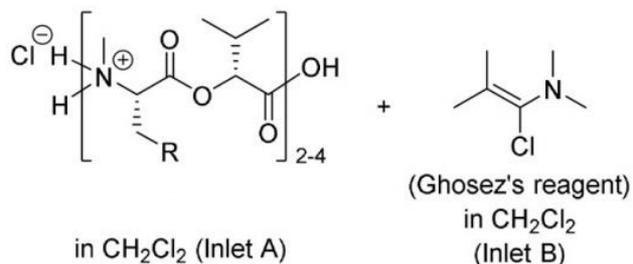
R = Ph, *i*-Pr  
6 examples, 67-90%



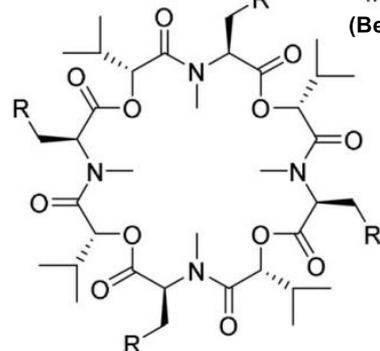
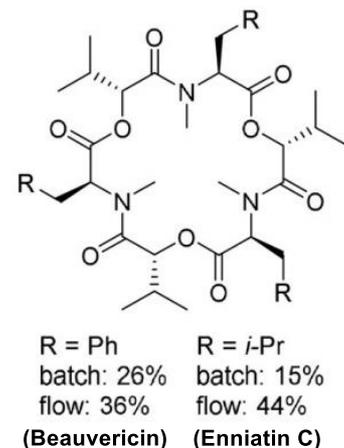
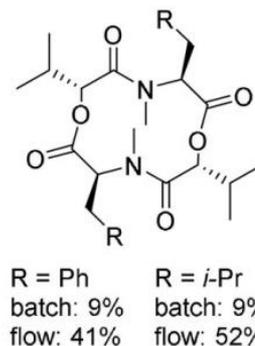
# Cyclic peptide synthesis

## Synthesis of CODs

### Macrocyclization



### Overall yields



Optimized flow conditions allowed inter- and intramolecular couplings in **high yields** and with **reduction in effort** compared to batch method.

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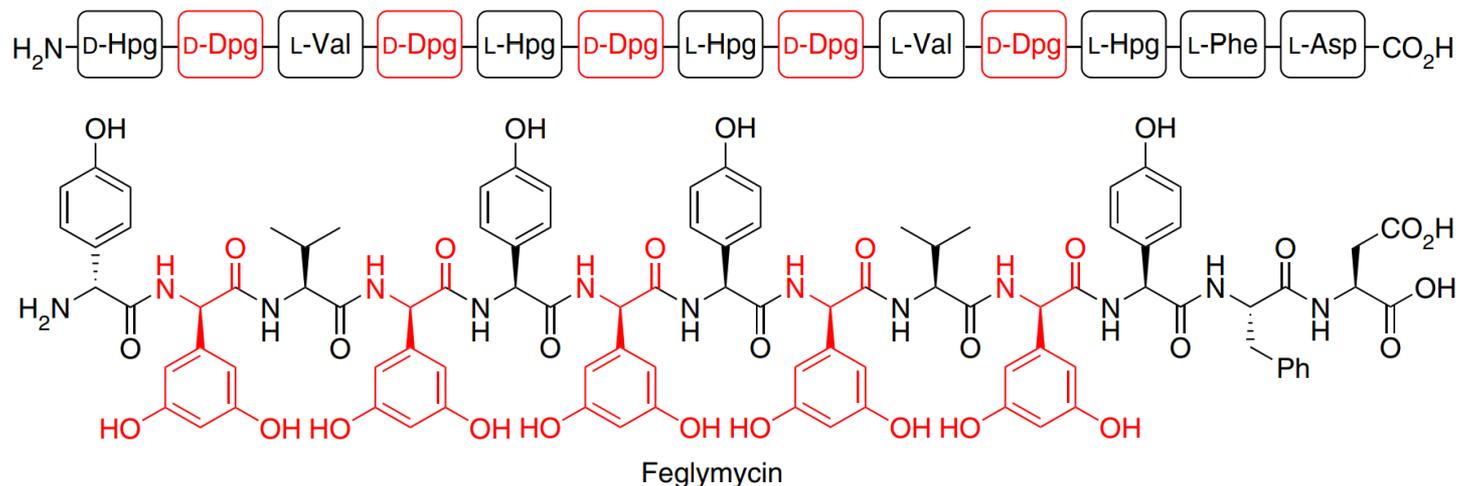
## 4. Summary

# Total synthesis of Feglymycin

## ○ Feglymycin

- Oligopeptide composed of 13 amino acids
- Strong anti-HIV activity and moderate antimicrobial activity
- A lot of **highly epimerization-prone** residues (Hpg, **D-Dpg**)

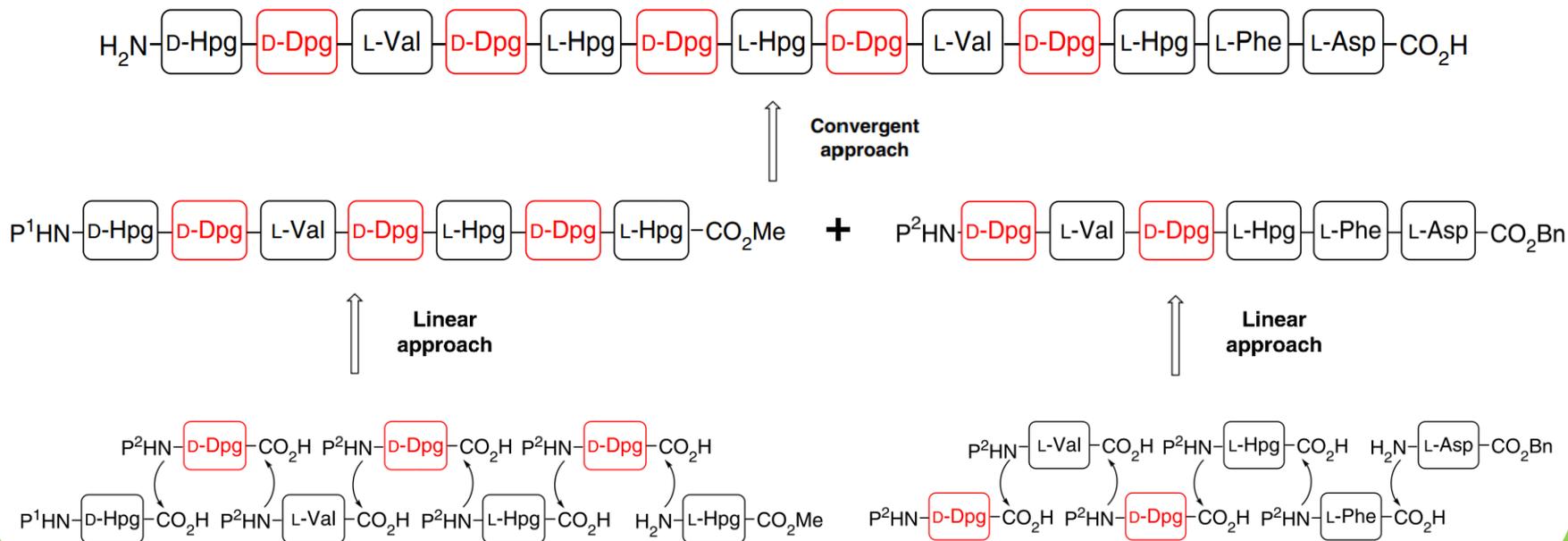
⇒ Despite the importance, synthetic methodology is very limited...



# Total synthesis of Feglymycin

## Synthetic protocol

### Linear/Convergent hybrid approach



### Linear strategy

- Easy installation of various amino acids
- Minimum protecting group manipulation

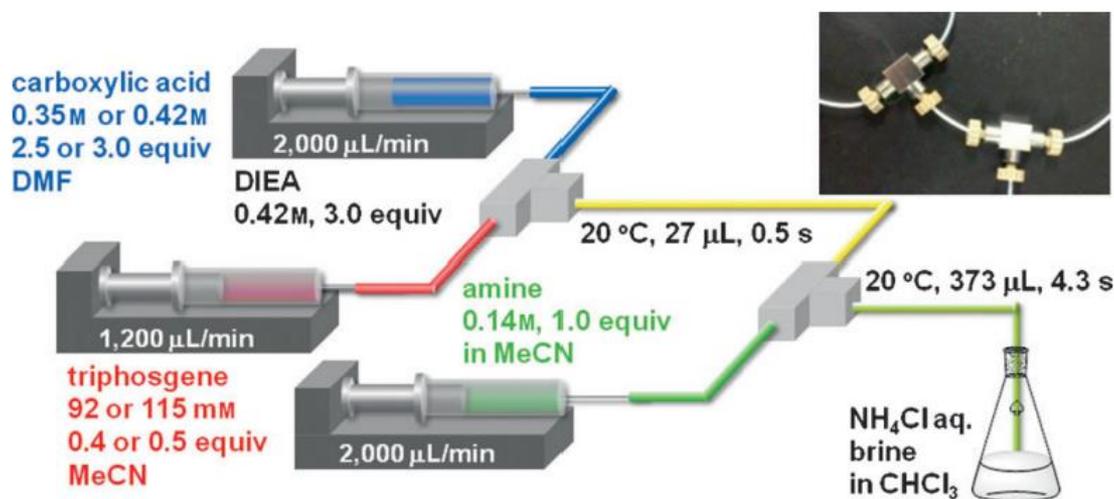
### Convergent strategy

- Avoid poor solubility of longer peptides
  - Easy purification

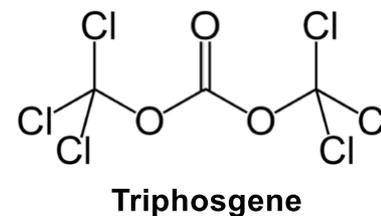
# $\alpha$ -Peptide synthesis (redisplay)

## ○ Synthesis via rapid and strong activation using Triphosgene

### Optimized conditions

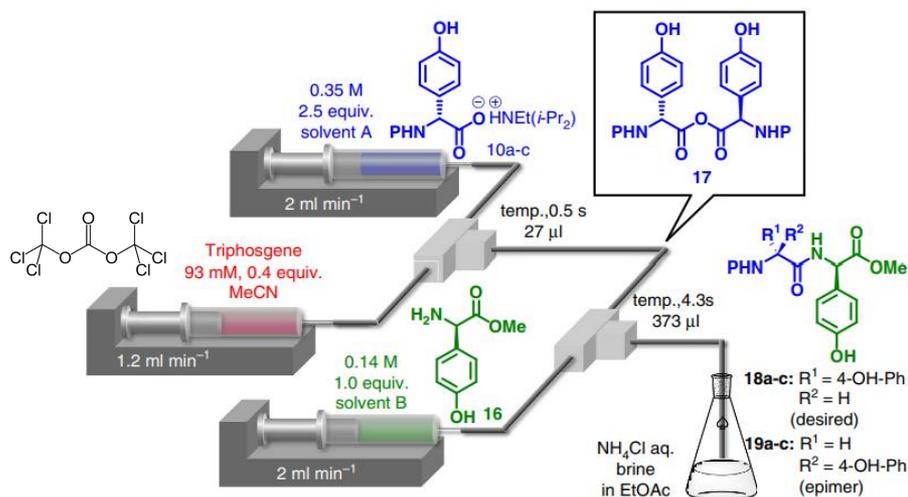


- ✓ Short reaction time
- ✓ High yield and Low epimerization level (compared to batch reaction)



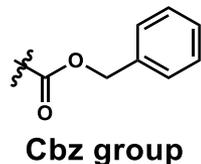
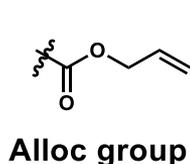
# Total synthesis of Feglymycin

## Optimization



Solvents, reaction temperature, and protection groups were optimized in order to increase yields and decrease epimerization level

Succeeded in suppressing epimerization level to **1%** for highly racemizable substrate!!



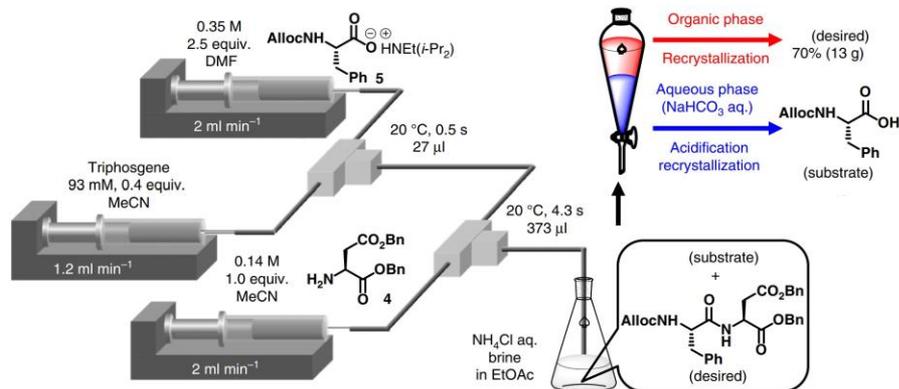
Entry	10 (P)	solv. A	solv. B	temp. (°C)	18 a-c	19 a-c
1	10a (Boc)	DMA	DMA	20	19%	1%
2	10b (Cbz)	DMA	DMA	20	84%	4%
3	10c (Alloc)	DMA	DMA	20	82%	4%
4	10b (Cbz)	DMA	DMA	10	76%	1%
5	10c (Alloc)	DMA	DMA	10	83%	1%
6	10b (Cbz)	DMA	H <sub>2</sub> O/MeCN = 1/2	10	81%	1%
7	10c (Alloc)	DMA	H <sub>2</sub> O/MeCN = 1/2	10	82%	1%
8 <sup>†</sup> (batch)	10c (Alloc)	DMA	H <sub>2</sub> O/MeCN = 1/2	10	82%	3%
9	10c (Alloc)	NMP	H <sub>2</sub> O/MeCN = 1/2	10	68% <sup>‡</sup>	
10	10c (Alloc)	DMPU	H <sub>2</sub> O/MeCN = 1/2	10	45% <sup>‡</sup>	

\*Yield was determined by HPLC-UV analysis.

<sup>†</sup>Reaction time for the activation of carboxylic acid and the amidation: 30 s.

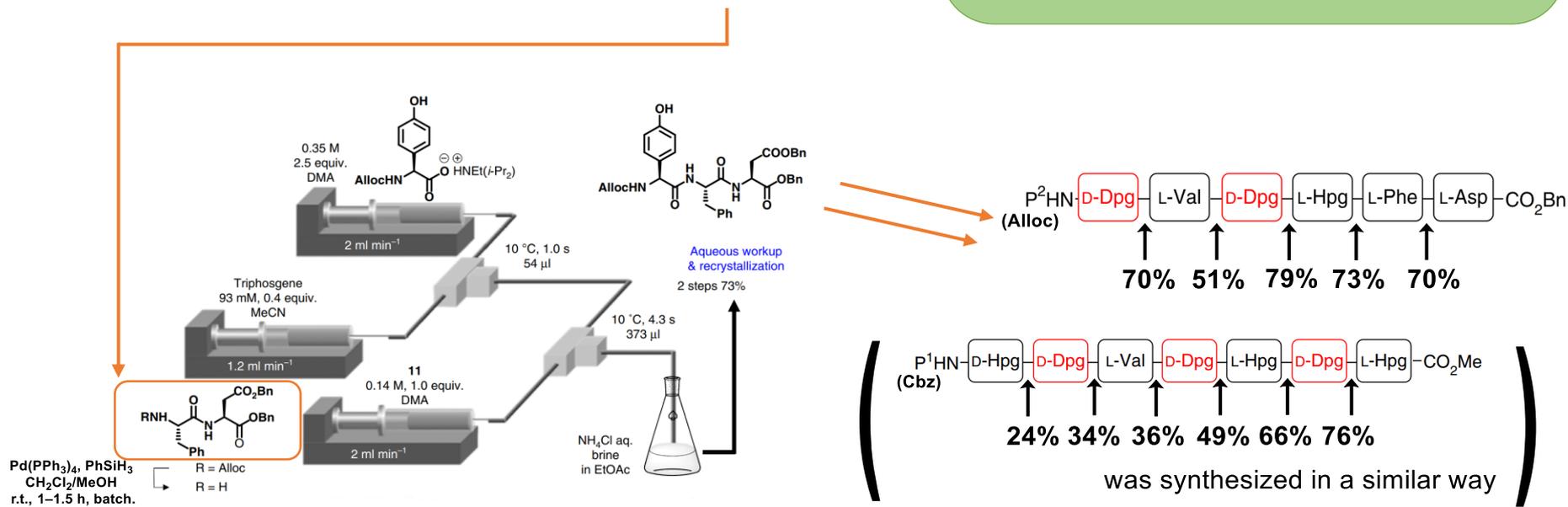
# Total synthesis of Feglymycin

## Linear synthesis



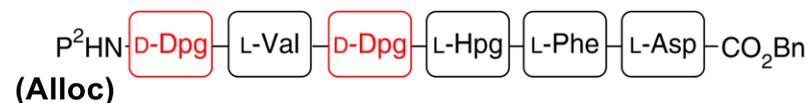
### Linear synthesis protocol

- ① Coupling using micro-flow reaction
- ② Purification
- ③ Deprotection of N-terminus
- ④ Repetition of ①~③ and elongation to 6-7 residues



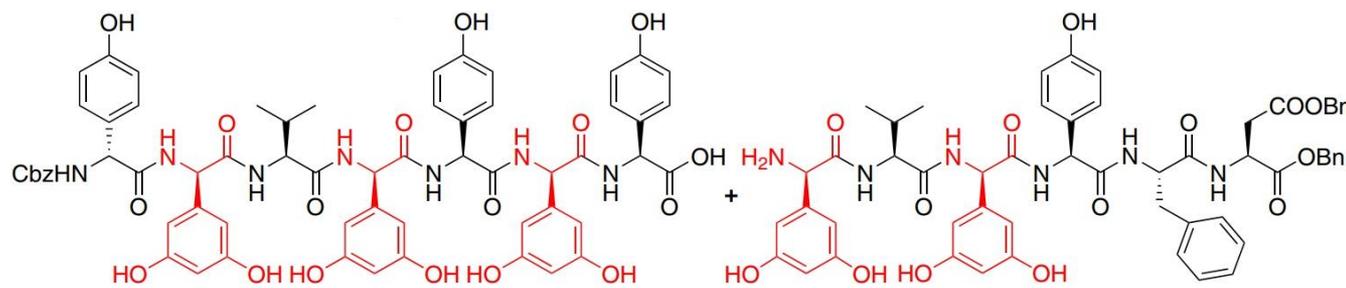
# Total synthesis of Feglymycin

## ○ Convergent synthesis



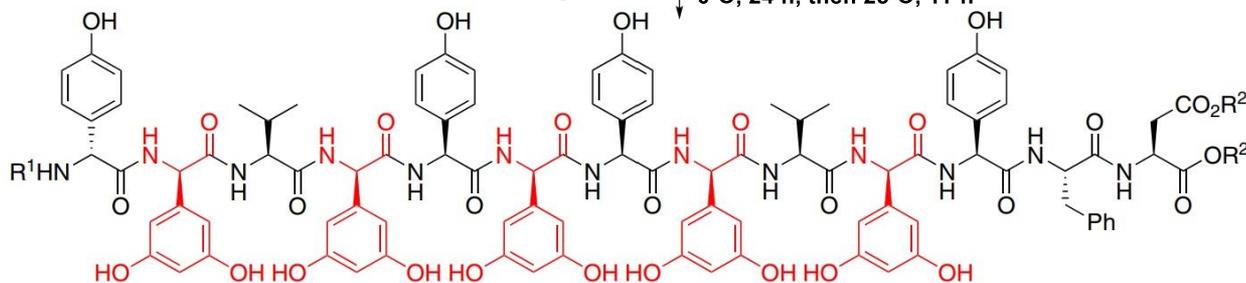
Me<sub>3</sub>SnOH, 1,2-dichloroethane  
85°C, 3.5 h

PS-Ph<sub>3</sub>P-Pd, PhSiH<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>/MeOH/H<sub>2</sub>O  
r.t., 50 min



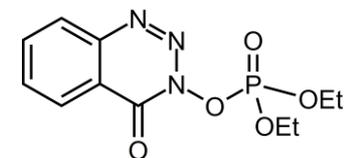
2 step 44%

DEPBT, NaHCO<sub>3</sub>, DMF  
0°C, 24 h, then 25°C, 11 h



H<sub>2</sub>, Pd/C, MeOH,  
r.t., 3 h  
quant.

R<sup>1</sup> = Cbz, R<sup>2</sup> = Bn  
R<sup>1</sup> = R<sup>2</sup> = H



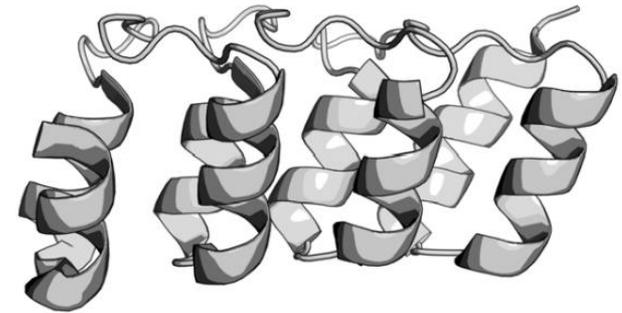
DEPBT

# Rapid total synthesis of protein

## ○ DARPin pE59 & Barnase

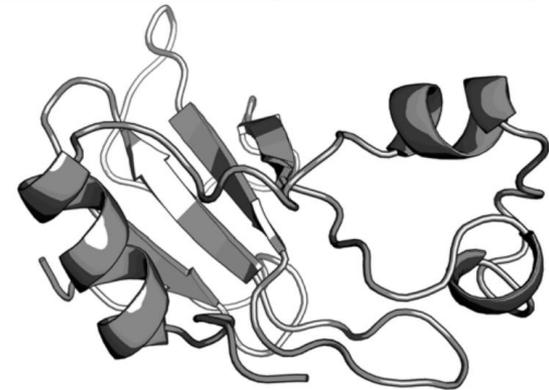
### DARPin (designed ankyrin repeat protein) pE59

- **130-residue protein**
- **Established versatility as a protein-binding scaffold (DARPin)**
- **Nanomolar affinity for pERK 2 (pE59)**



### Barnase

- **113-residue protein**
- **Potent RNase with endonuclease activity**
- **Not require cofactors or metal ions for folding or catalytic activity.**



# Rapid total synthesis of protein

## ○ Synthetic protocol

### Combination of micro-flow technology and NCL

Four fragments: Micro-flow synthesis on a solid-phase column reactor (D[1]~D[4], B[1]~B[4] respectively)

Authentic protein: Fragment coupling using NCL (Native chemical ligation)

#### DARPin pE59

D[1] <sup>1</sup>Gly-Gly-Gly-Gly-Gly-Ser-Asp-Leu-Gly-<sup>10</sup>Lys-  
<sup>11</sup>Lys-Leu-Leu-Glu-Ala-Ala-Arg-Ala-Gly-<sup>20</sup>Gln-  
D[2] <sup>21</sup>Asp-Asp-Glu-Val-Arg-Ile-Leu-Nle-Ala-<sup>30</sup>Asn-  
<sup>31</sup>Gly-Ala-Asp-Val-Asn-Ala-Leu-Asp-Glu-<sup>40</sup>Asp-  
<sup>41</sup>Gly-Leu-Thr-Pro-Leu-His-Leu-Ala-Ala-<sup>50</sup>Gln-  
D[3] <sup>51</sup>Leu-Gly-His-Leu-Glu-Ile-Val-Glu-Val-<sup>60</sup>Leu-  
<sup>61</sup>Leu-Lys-Tyr-Gly-Ala-Asp-Val-Asn-Ala-<sup>70</sup>Glu-  
<sup>71</sup>Asp-Asn-Phe-Gly-Ile-Thr-Pro-Leu-His-<sup>80</sup>Leu-  
D[4] <sup>81</sup>Ala-Ala-Ile-Arg-Gly-His-Leu-Glu-Ile-<sup>90</sup>Val-  
<sup>91</sup>Glu-Val-Leu-Leu-Lys-His-Gly-Ala-Asp-<sup>100</sup>Val-  
<sup>101</sup>Asn-Ala-Gln-Asp-Lys-Phe-Gly-Lys-Thr-<sup>110</sup>Ala-  
D[4] <sup>111</sup>Phe-Asp-Ile-Ser-Ile-Asp-Asn-Gly-Asn-<sup>120</sup>Glu-  
<sup>121</sup>Asp-Leu-Ala-Glu-Ile-Leu-Gln-Lys-Leu-<sup>130</sup>Asn

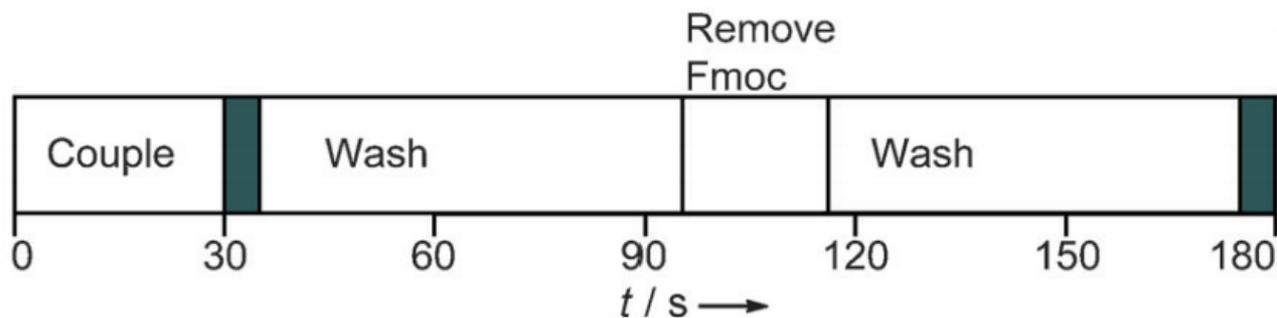
#### Barnase

B[1] <sup>1</sup>Gly-Gly-Gly-Ala-Gln-Val-Ile-Asn-Thr-<sup>10</sup>Phe-  
<sup>11</sup>Asp-Gly-Val-Ala-Asp-Tyr-Leu-Gln-Thr-<sup>20</sup>Tyr-  
B[2] <sup>21</sup>His-Lys-Leu-Pro-Asp-Asn-Tyr-Ile-Thr-<sup>30</sup>Lys-  
<sup>31</sup>Ser-Glu-Ala-Gln-Ala-Leu-Gly-Trp-Val-<sup>40</sup>Ala-  
B[3] <sup>41</sup>Ser-Lys-Gly-Asn-Leu-Ala-Asp-Val-Ala-<sup>50</sup>Pro-  
<sup>51</sup>Gly-Lys-Ser-Ile-Gly-Gly-Asp-Ile-Phe-<sup>60</sup>Ser-  
<sup>61</sup>Asn-Arg-Glu-Gly-Lys-Leu-Pro-Gly-Lys-<sup>70</sup>Ser-  
<sup>71</sup>Gly-Arg-Thr-Trp-Arg-Glu-Ala-Asp-Ile-<sup>80</sup>Asn-  
B[4] <sup>81</sup>Tyr-Thr-Ser-Gly-Phe-Arg-Asn-Ser-Asp-<sup>90</sup>Arg-  
<sup>91</sup>Ile-Leu-Tyr-Ser-Ser-Asp-Trp-Leu-Ile-<sup>100</sup>Tyr-  
<sup>101</sup>Lys-Thr-Thr-Asp-His-Tyr-Gln-Thr-Phe-<sup>110</sup>Thr-  
<sup>111</sup>Lys-Ile-<sup>113</sup>Arg

# $\alpha$ -Peptide synthesis (redisplay)

## ○ Synthesis on a solid-phase column reactor

### 1 cycle of peptide chain elongation



Achieved rapid peptide synthesis  
that took only **3 minutes** to  
extend 1 residue!

# Rapid total synthesis of protein

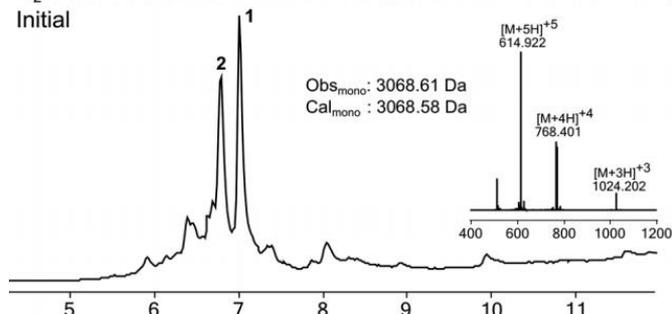
## ○ Typical synthetic problems

### Fragment D[1]

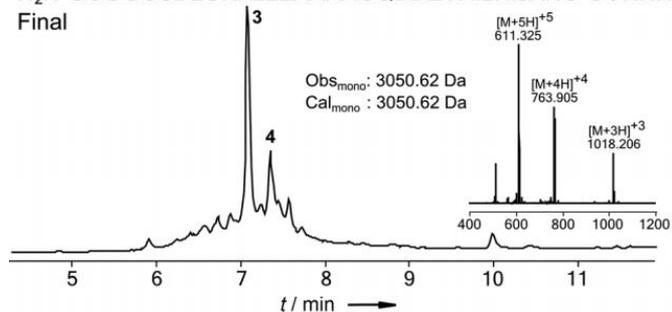
Oxidation of Met<sub>28</sub> occurred  
 ⇒ Substituted Met<sub>28</sub> with norleucine(Nle)

DARPin fragment 1, D[1]

H<sub>2</sub>N-GGGGGSDLGKKLLEAARAGQDDEVRLMANG-CONHNH<sub>2</sub>  
 Initial



H<sub>2</sub>N-GGGGGSDLGKKLLEAARAGQDDEVRLNleANG-CONHNH<sub>2</sub>  
 Final



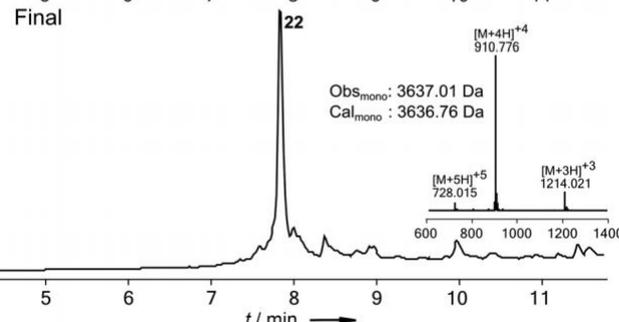
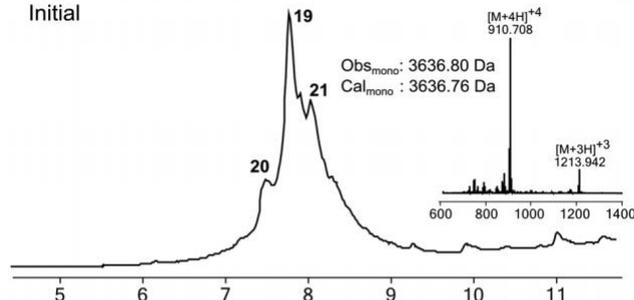
Peak #	Obs <sub>mono</sub>	Identified As
1	3068.61	Expected D[1]
2	3084.59	ox- <sup>28</sup> Met
3	3050.62	Expected D[1] (M28Nle)
4	2922.60	des-Lys/Gln

### Fragment D[4]

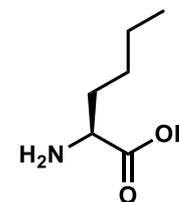
Aspartimide formation occurred  
 ⇒ Protection of Asp<sub>116</sub> by methylpentyl group

DARPin fragment 4, D[4]

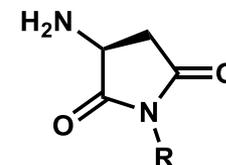
H<sub>2</sub>N-CDVNAQDKFGKTAFDISIDNGNEDLAEILQKLN-CONH<sub>2</sub>  
 Initial



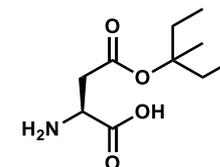
Peak #	Obs <sub>mono</sub>	Identified As
19 & 22	3636.9	Expected D[4]
20	3636.79	Isomer of D[4]
21	3703.88	Piperidine adduct



norleucine



Aspartimide



Asp(OMpe)

# Rapid total synthesis of protein

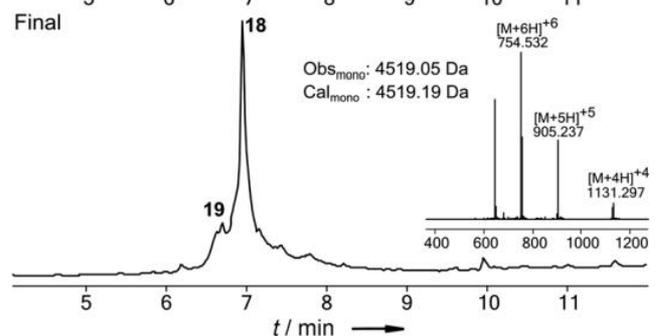
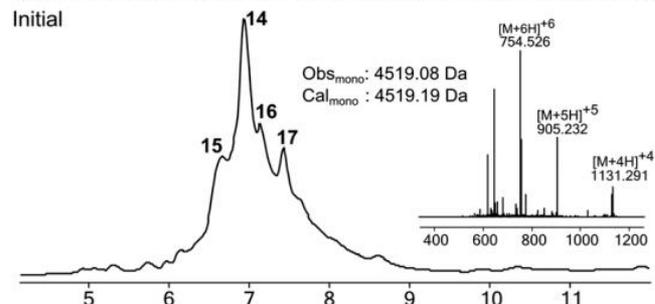
## ○ Typical synthetic problems

### Fragment B[4]

Numerous deletion products  
 ⇒ Used different resin (PEG resin)

#### Barnase fragment 4, B[4]

H<sub>2</sub>N-CDINYTSGFRNSDRILYSSDWLIYKTTDHYQTFTKIR-CONH<sub>2</sub>



Peak #	Obs <sub>mono</sub>	Identified As
14 & 18	4519.1	Expected B[4]
15 & 19	4519.2	Isomer of B[4]
16	4363.09	des-Arg
17	2932.49	Truncated sequence

### Fragment B[1+2]

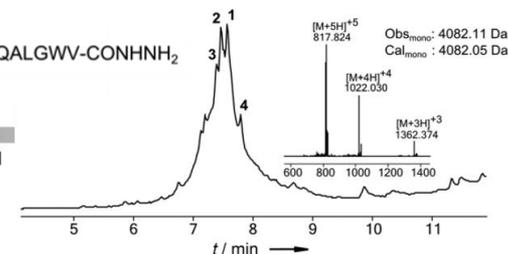
Purification was impossible  
 ⇒ Divided B[1+2] into two fragments

#### Barnase fragment 1+2, B[1+2]

H<sub>2</sub>N-AQVINTFDGVADYLQTYHKLPDNYITKSEAQALGWV-CONH<sub>2</sub>

Initial

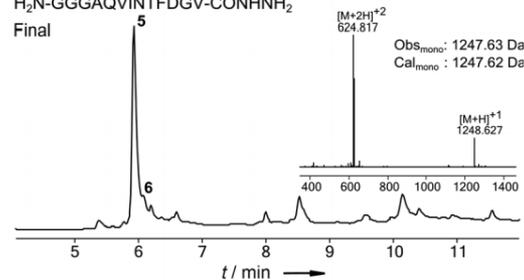
Peak #	Obs <sub>mono</sub>	Identified As
1	4082.11	Expected B[1+2]
2	3983.09	des-Val
3	4064.20	-H <sub>2</sub> O
4	3968.07	des-Asn



#### Barnase fragment 1, B[1]

H<sub>2</sub>N-GGGAQVINTFDGV-CONH<sub>2</sub>

Final

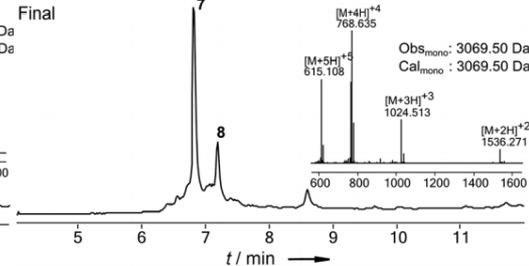


Peak #	Obs <sub>mono</sub>	Identified As
5	1247.63	Expected B[1]
6	1133.59	des-Asn

#### Barnase fragment 2, B[2]

H<sub>2</sub>N-CDYLQTYHKLPDNYITKSEAQALGWV-CONH<sub>2</sub>

Final

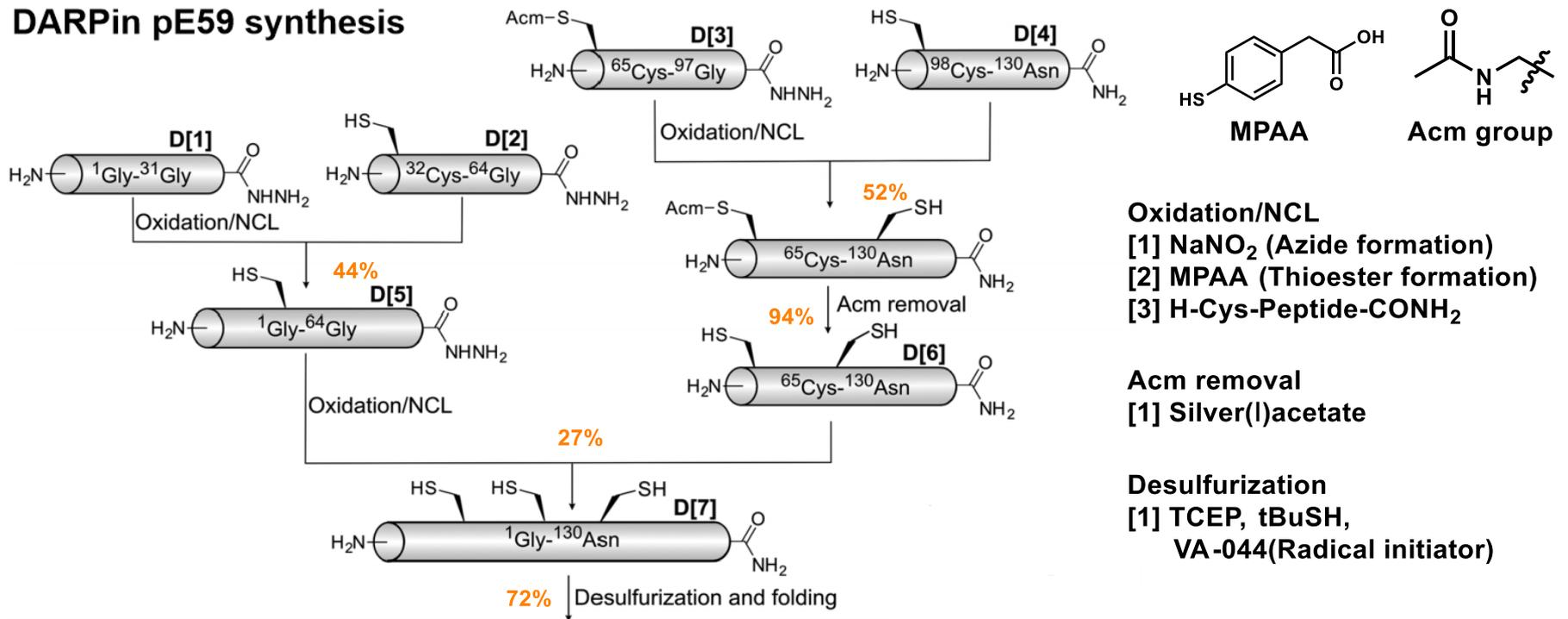


Peak #	Obs <sub>mono</sub>	Identified As
7	3069.50	Expected B[2]
8	2932.49	des-His

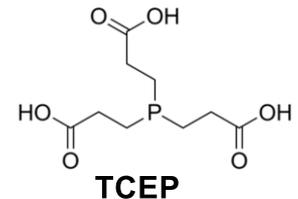
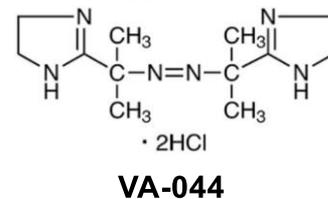
# Rapid total synthesis of protein

## Native chemical ligation (NCL)

### DARPin pE59 synthesis



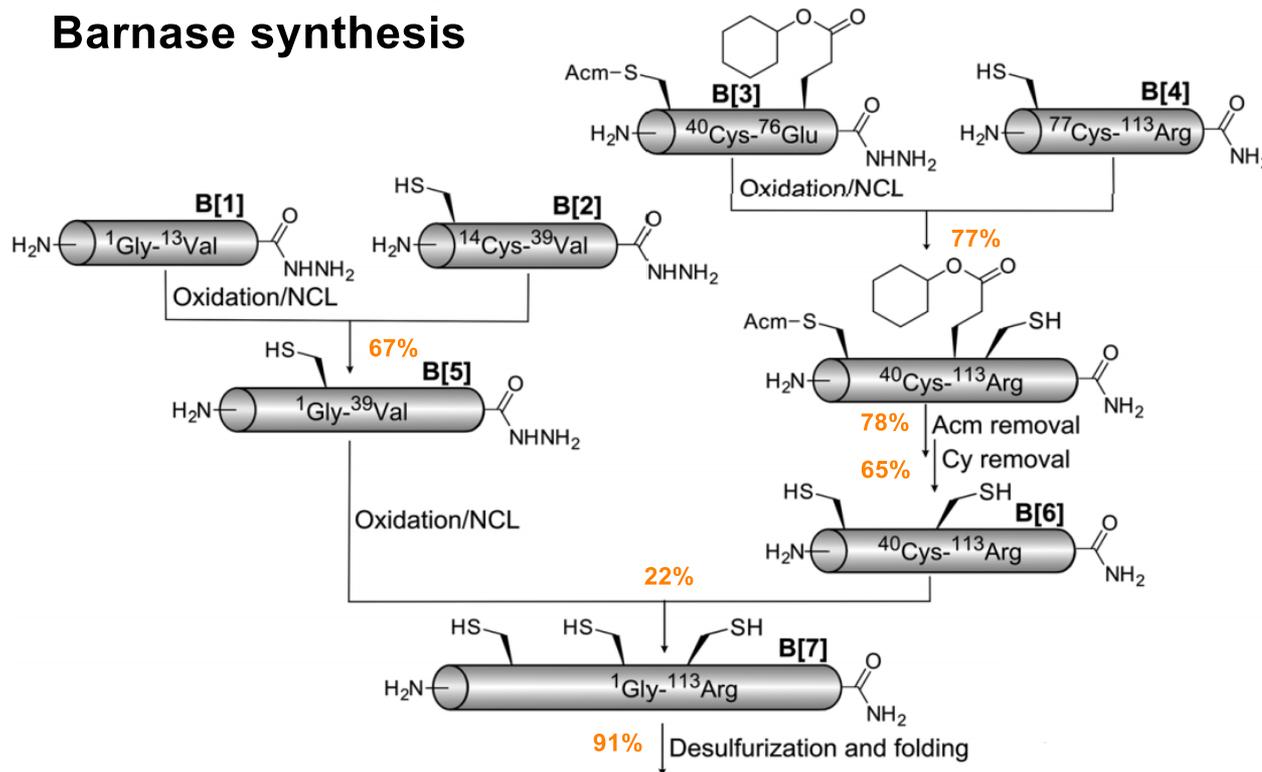
**DARPin pE59**



# Rapid total synthesis of protein

## ○ Native chemical ligation (NCL)

### Barnase synthesis



### Barnase

#### Oxidation/NCL

- [1] NaNO<sub>2</sub> (Azide formation)
- [2] MPAA (Thioester formation)
- [3] H-Cys-Peptide-CONH<sub>2</sub>

#### Acm removal

- [1] Silver(I)acetate

#### Cy removal

- [1] HF

#### Desulfurization

- [1] TCEP, tBuSH,  
VA-044(Radical initiator)

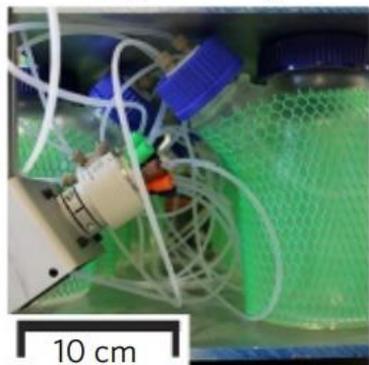
# Optimization by Deep learning

## ○ Fully automated synthesizer

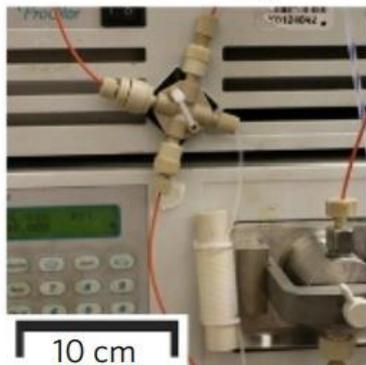
- ✓ High fidelity
- ✓ Rapid
- ✓ Minimum amount of biproducts
- ✓ Incorporation of non-canonical amino acids



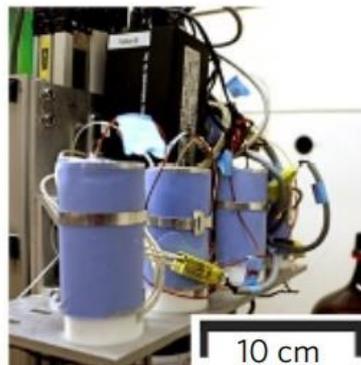
Reagent storage



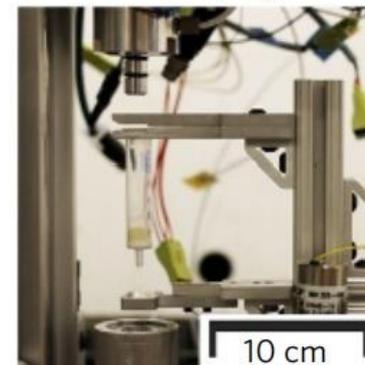
Fluid mixing



Activation

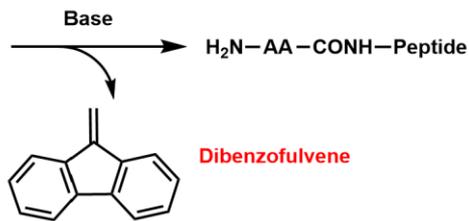
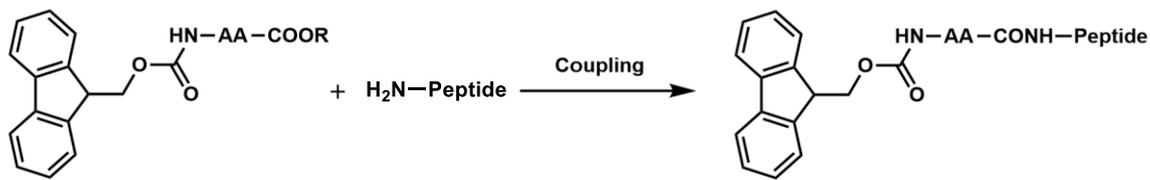


Coupling

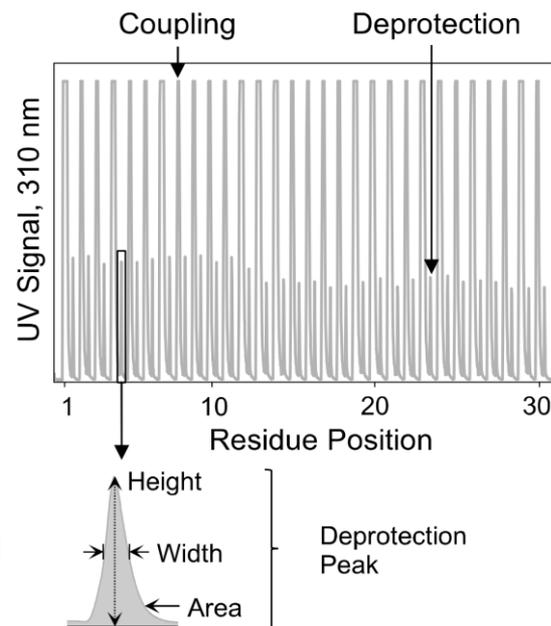
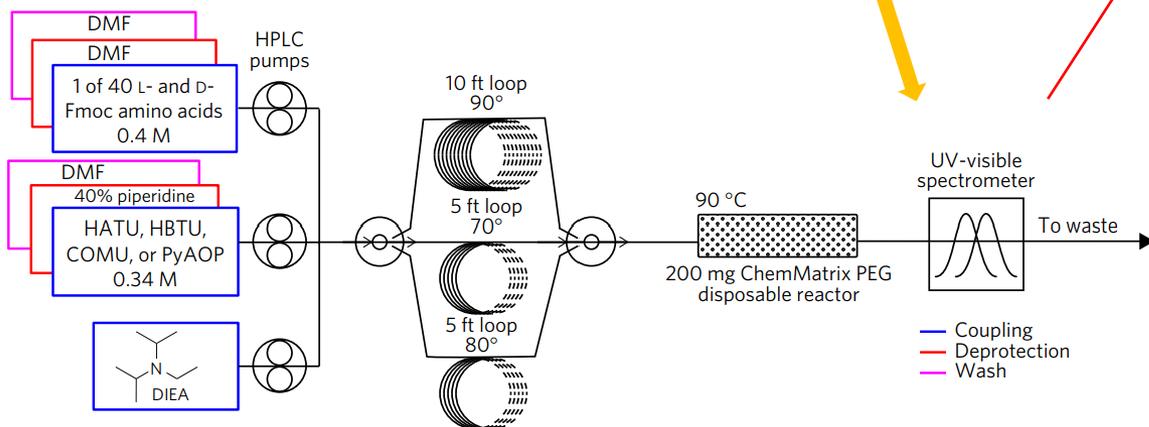


# Optimization by Deep learning

## ○ Access to highly reproducible data



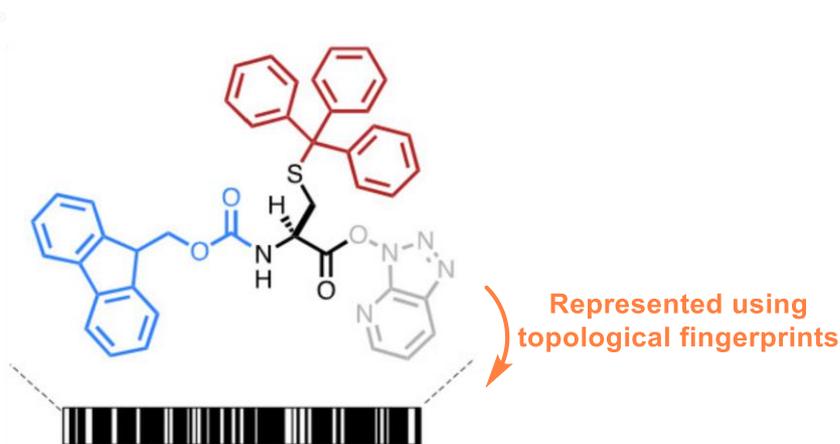
This data can be used to indirectly obtain information on stepwise coupling and overall synthesis performance



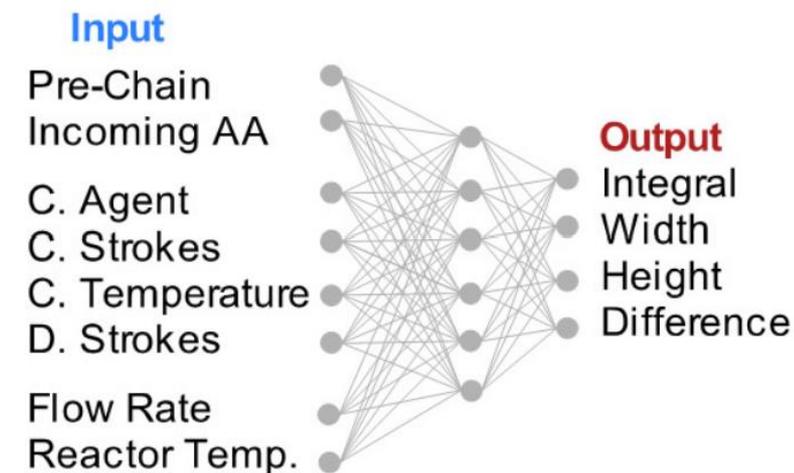
# Optimization by Deep learning

## Prediction of Fmoc deprotection traces

### Representation of amino acids for learning model

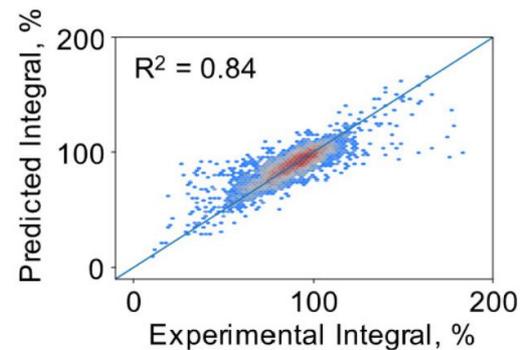


### Schematic of the machine learning model



Prediction errors were under **4%** (RMSE/range)

$$RMSE = \sqrt{\frac{1}{N} \sum_{i=1}^N (y_i - \hat{y}_i)^2}$$



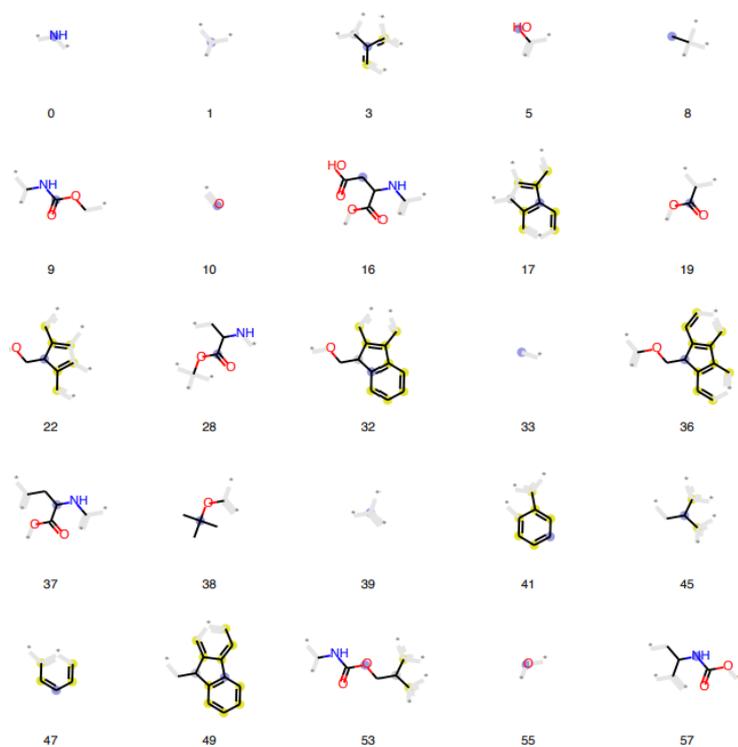
# Optimization by Deep learning

## ○ Supplement about fingerprint

### Incoming amino acid

Fmoc- and side-chain protected representations are used

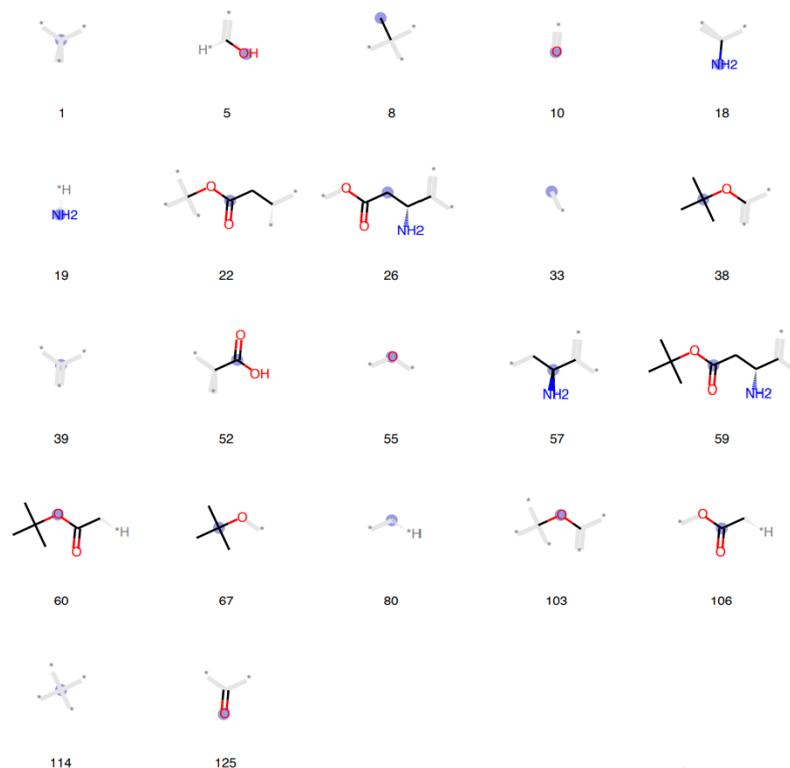
#### Fmoc-Asp(O<sup>t</sup>Bu)-OH



### Pre-chain

Side-chain protected representations are used

#### H-Asp(O<sup>t</sup>Bu)-OH

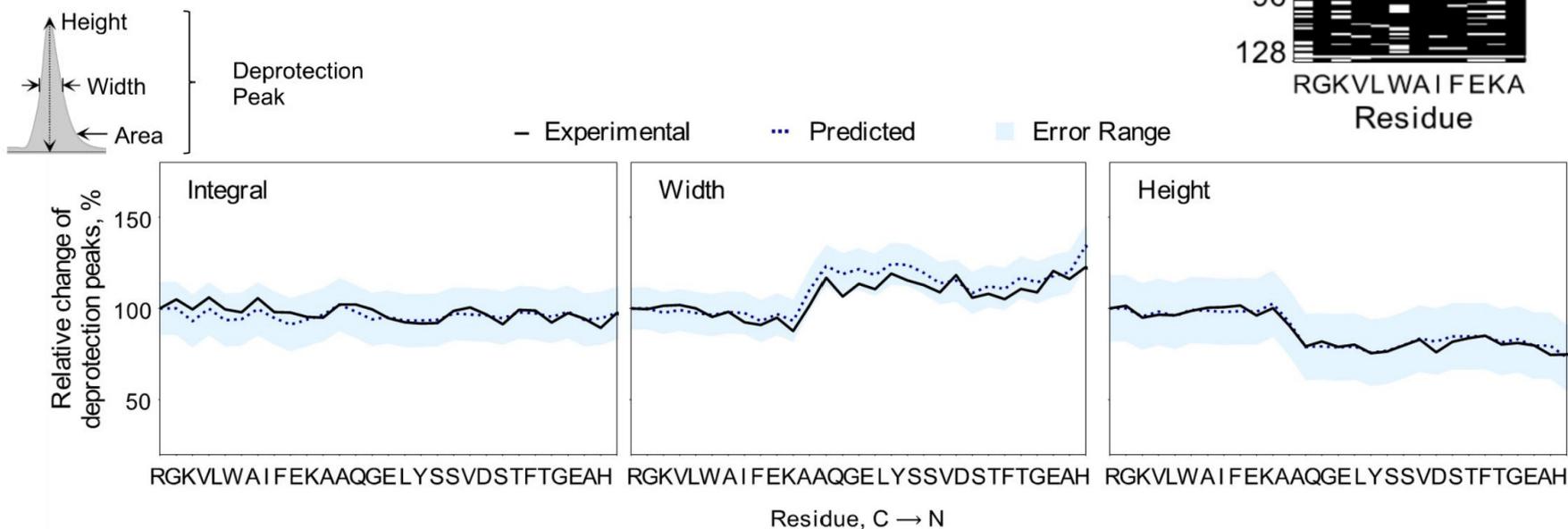
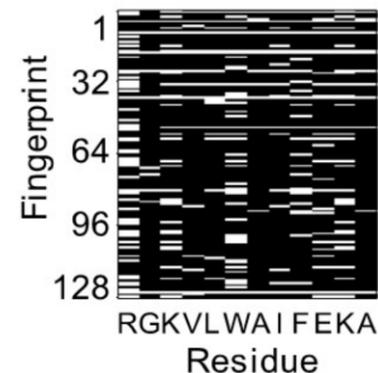


# Optimization by Deep learning

## Prediction for GLP-1 synthesis

Sequence of Human GLP-1

His|Ala|Glu|Gly|Thr|Phe|Thr|Ser|Asp|Val|Ser|Ser|Tyr|Leu|Glu|Gly|Gln|Ala|Ala|Lys|Glu|Phe|Ile|Ala|Trp|Leu|Val|Lys|Gly|Arg



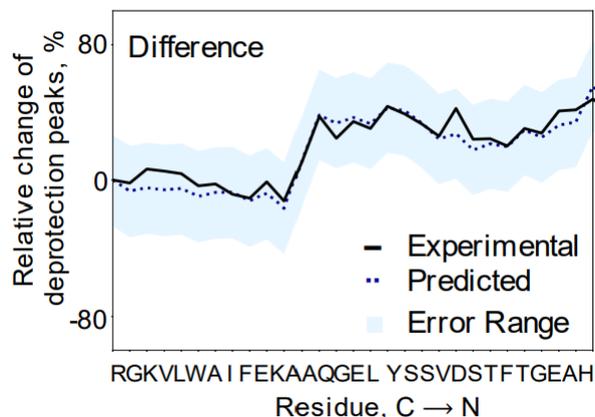
**Predictions from the model match the experimental values**

**⇒ However, what is width and height useful for...?**

# Optimization by Deep learning

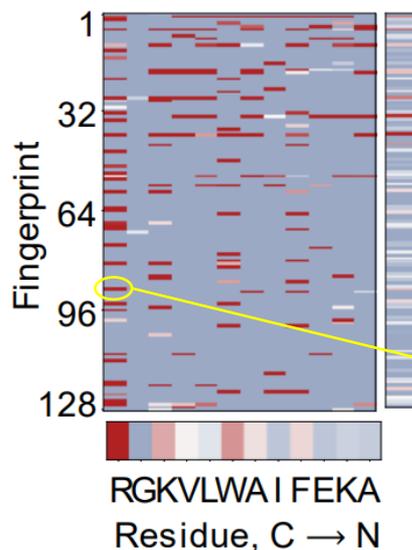
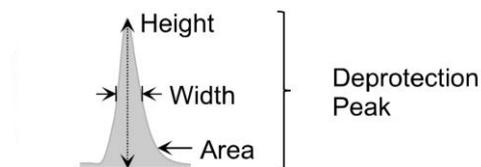
## ○ Interpretation of aggregation

Aggregation is a sequence-dependent event that results in poor synthetic outcome

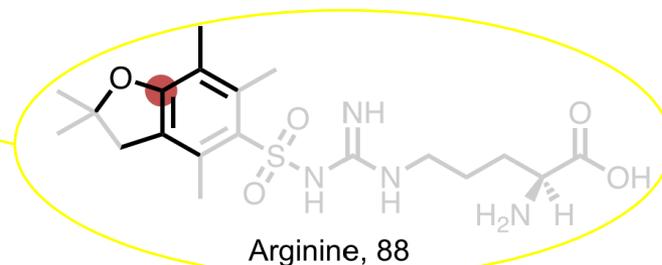


Aggregation causes mass transfer issues and slow reaction kinetics, and they are reflected in **flattened, wider** UV-Vis deprotection peaks

⇒ **Difference (Width - Height)** was used as index of aggregation



Furthermore, identification of the residues which are responsible for aggregation was achieved using minimal model

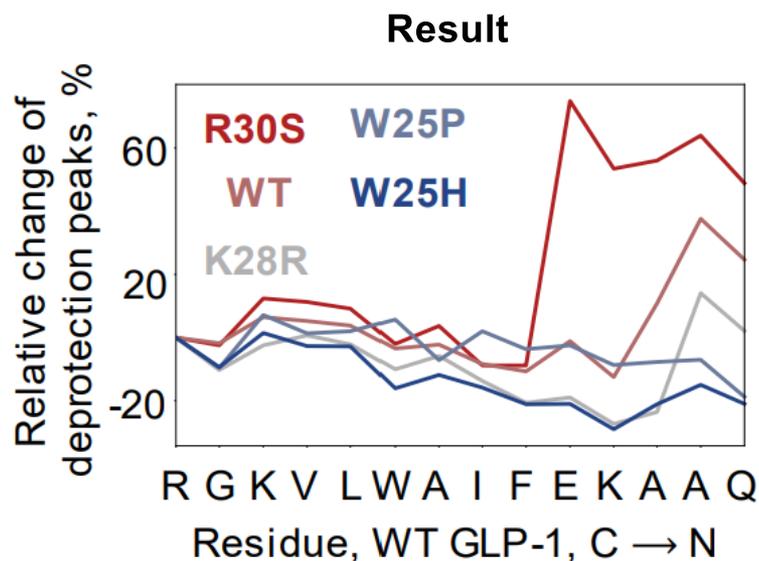
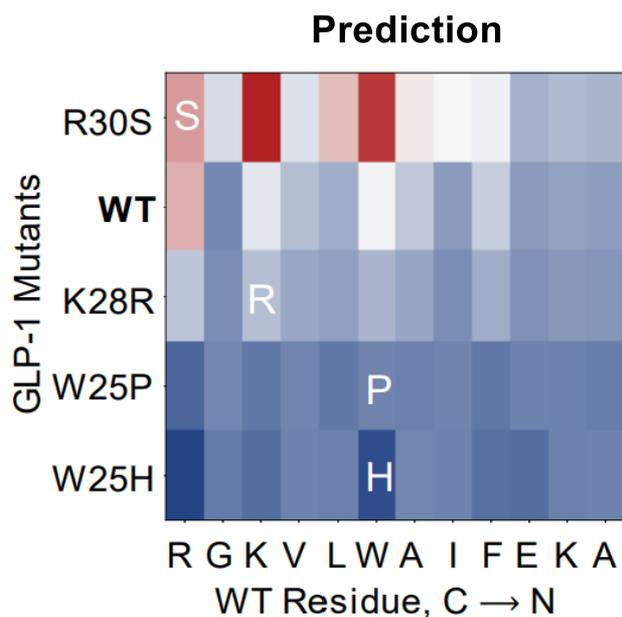


# Optimization by Deep learning

## ○ Sequence optimization using single-point mutations

Mutation of amino acids which were activated for aggregation led to a **decrease of aggregation** in most cases

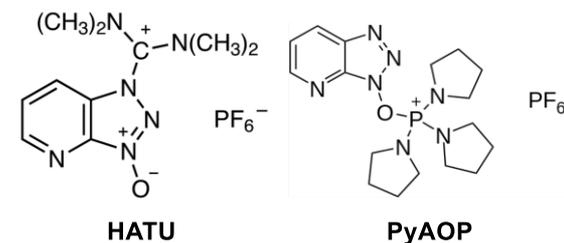
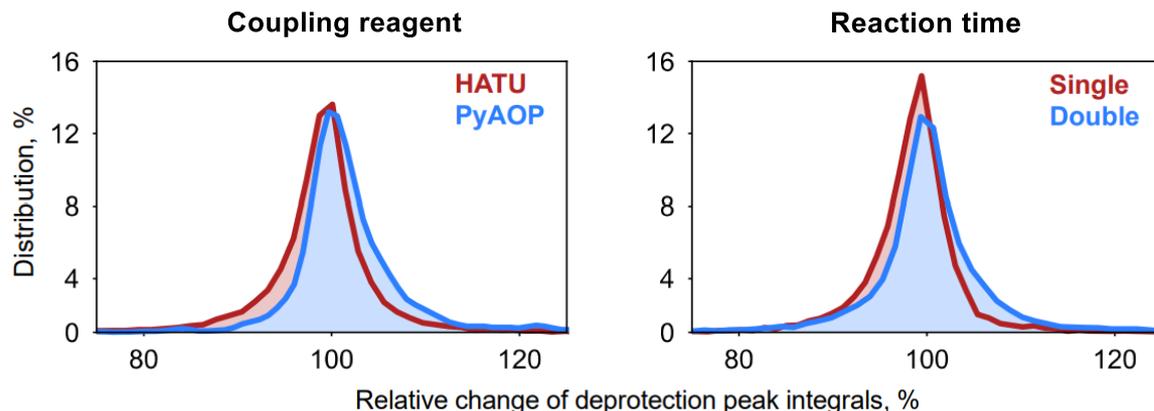
#	GLP-1 Mutants
<b>R30S</b>	-QAAK EFI AW LVKGS
<b>WT</b>	-QAAK EFI AW LVKGR
<b>K28R</b>	-QAAK EFI AW LVRGR
<b>W25P</b>	-QAAK EFI AP LVKGR
<b>W25H</b>	-QAAK EFI AH LVKGR



# Optimization by Deep learning

## ○ Future optimization

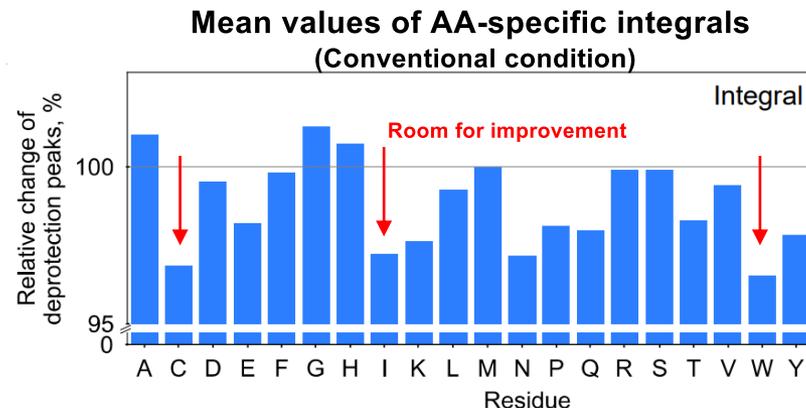
Reaction conditions also have room for improvement



In prediction, use of **PyAOP** and **extended coupling time** will improve the yields

The difference is small, but these minor effects add up to have a **detrimental** impact in long peptide synthesis

>99% coupling efficiency per incorporated amino acid is crucial



# Contents

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## 1. Introduction

## 2. Micro-flow technology for peptide synthesis

- Solid-phase  $\alpha$ -peptide synthesis
- Solution-phase  $\alpha$ -peptide synthesis
- Cyclic peptide synthesis

## 3. Application and latest development

- Total synthesis of Feglymycin
- Rapid total synthesis of protein
- Optimization by Deep learning

## 4. Summary

# Summary

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- Peptide drugs have been attracting attention in recent years and more efficient synthesis method is needed
- Micro-flow technology has great possibility to develop peptide synthesis in terms of precise reaction control and ease of scale-up
- In solution-phase, rapid and low-epimerization level synthesis was achieved.
- In solid-phase, rapid and automated continuous synthesis was achieved.

# Appendix

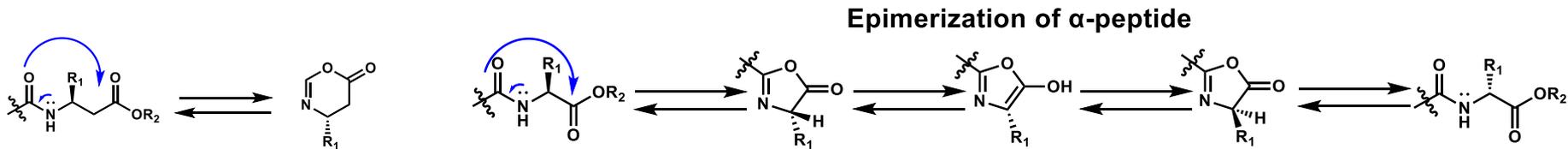
# $\beta$ -Peptide synthesis

## ○ $\beta$ -amino acids

Amino acids which have amino group bonded to the  $\beta$  carbon

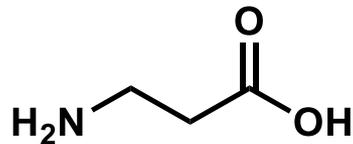
- Many of  $\beta$ -amino acids lack chiral center at  $\alpha$ -position

→ Epimerization doesn't occur

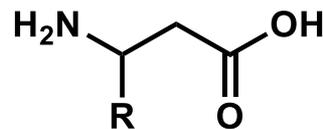


- Interesting properties from structural and biological viewpoint

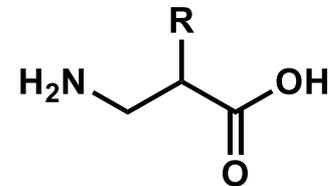
(ex: stable against proteolytic degradation)



$\beta$ -Alanine



$\beta^3$ -amino acid



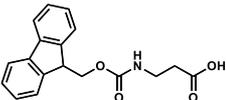
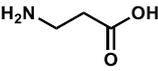
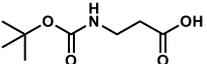
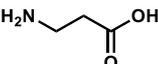
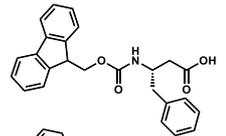
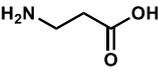
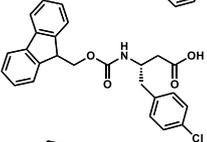
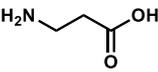
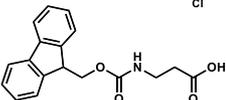
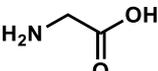
$\beta^2$ -amino acid



# $\beta$ -Peptide synthesis

## ○ First report of micro-flow synthesis

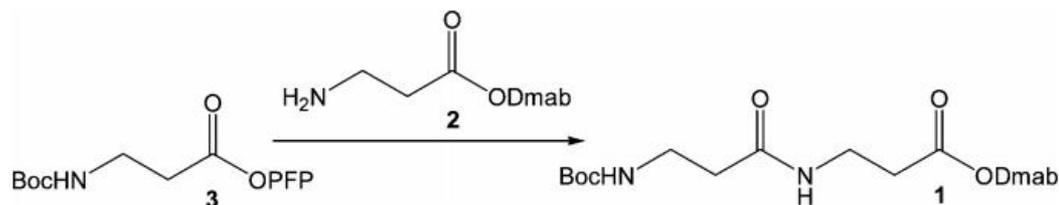


Entry	PG-AA1	AA2	Yield(Flow)	Yield(Batch)
1			quant.	46%
2			quant.	57%
3			quant.	35%
4			quant.	36%
5			quant.	35%

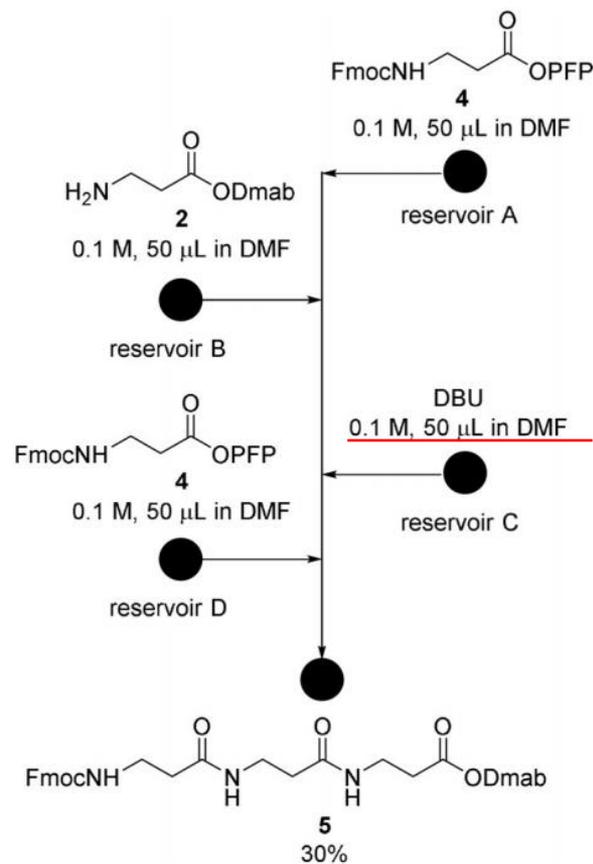
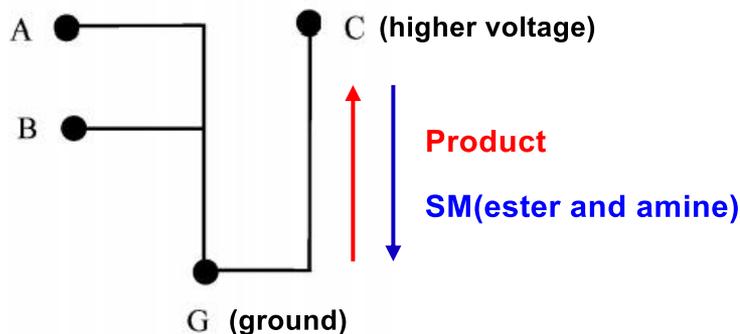
# $\beta$ -Peptide synthesis

## ○ First report of micro-flow synthesis

- ✓ Deprotection using only 1 equiv. of base
- ✓ Achieved multi-step synthesis
- ✓ Separation using electrophoresis



Scheme 1 Synthesis of dipeptide 1.



# $\beta$ -Peptide synthesis

## ○ EOF (Electroosmotic flow)

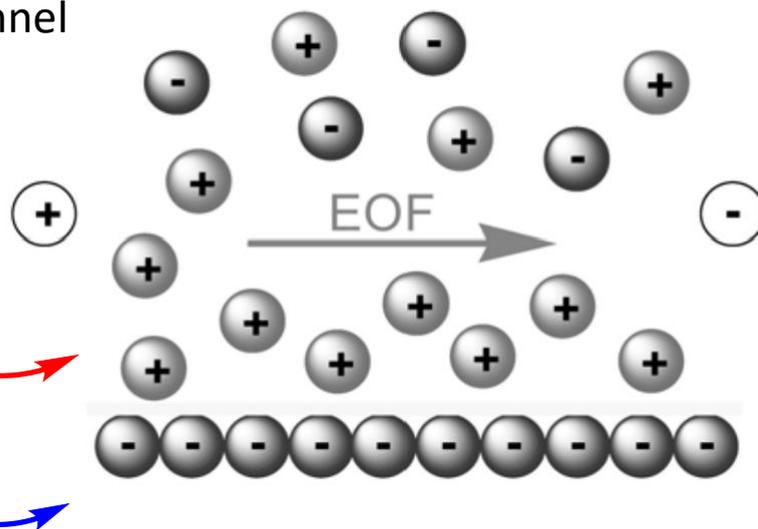
The motion of liquid induced by an applied potential across microchannel

An electric field causes layer to move towards the negative electrode

→ Causes bulk liquid to move within the channel

Positive counter ions form a double layer

Negatively charged material on the surface



# $\beta$ -Peptide synthesis

## ○ Dmab

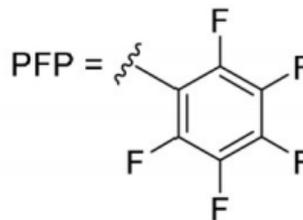
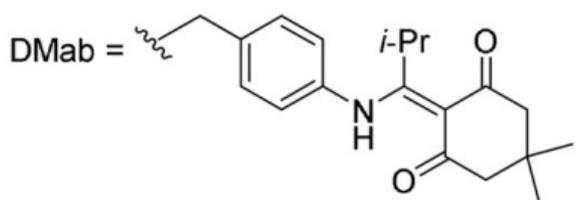
A protecting group which is orthogonal to both acid(TFA) and piperidine-labile protecting groups(PG).

This PG is removed under mild conditions( $\text{NH}_2\text{NH}_2$ ). It is crucial because EOF is retarded if the pH is less than 3.

## ○ PFP (pentafluorophenyl)

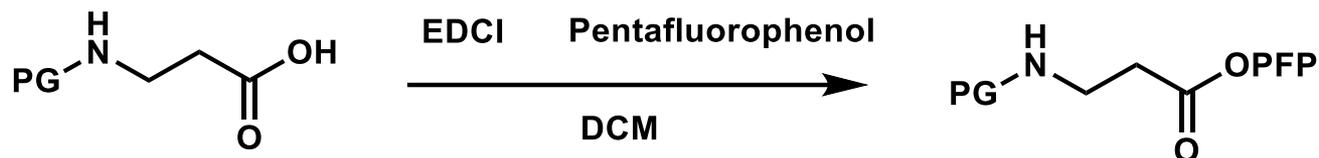
PFP esters are active esters which are useful for laboratory peptide synthesis.

They are less susceptible to spontaneous hydrolysis during conjugation reaction.

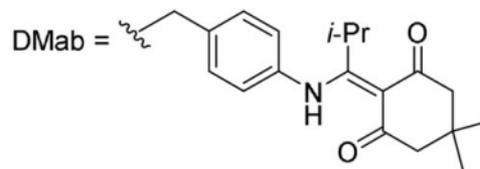
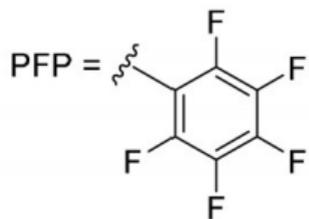
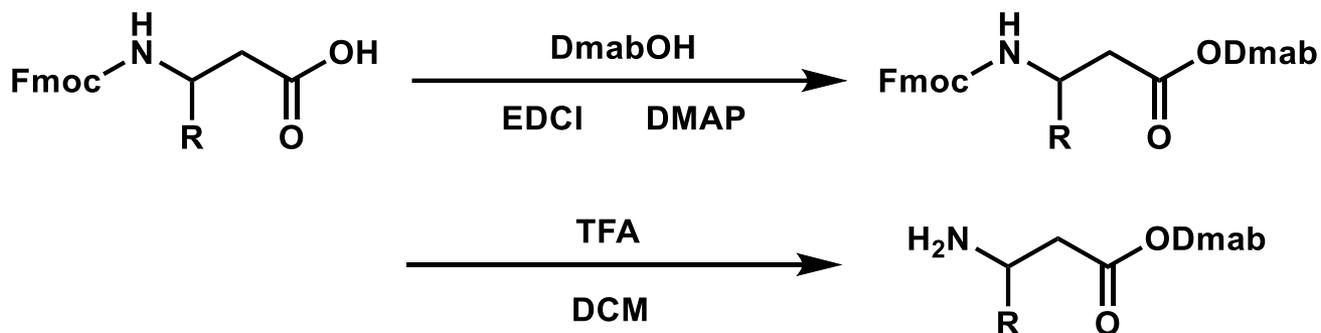


# $\beta$ -Peptide synthesis

## ○ Preparation of PFP ester

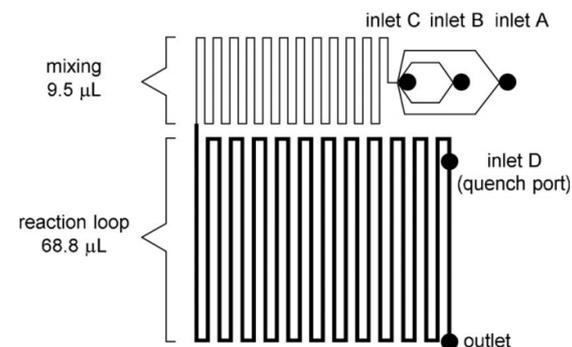
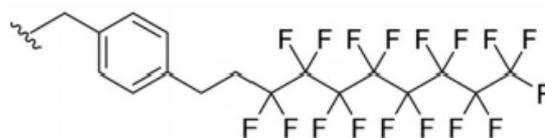
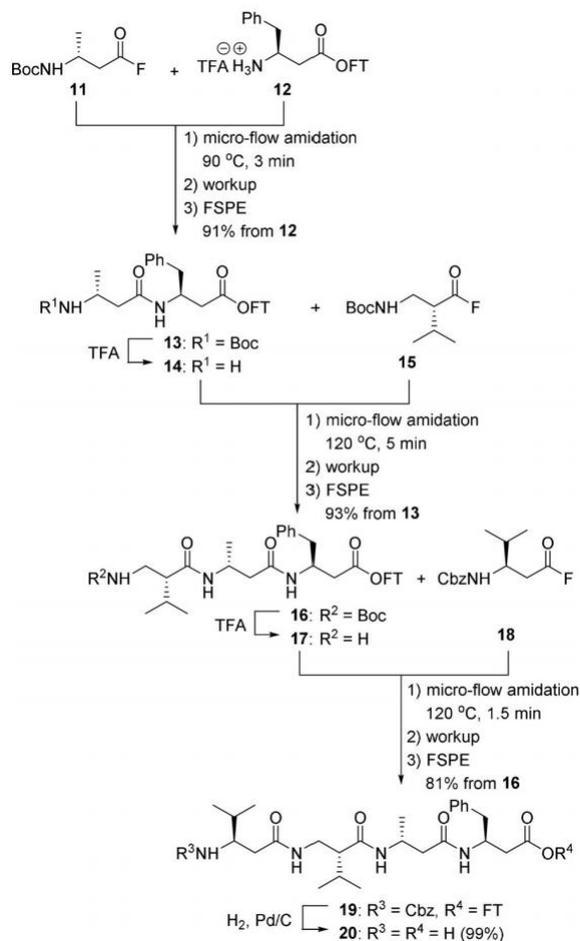


## ○ Preparation of Dmab-protected amino acids



# $\beta$ -Peptide synthesis

## ○ Synthesis using fluororous tag



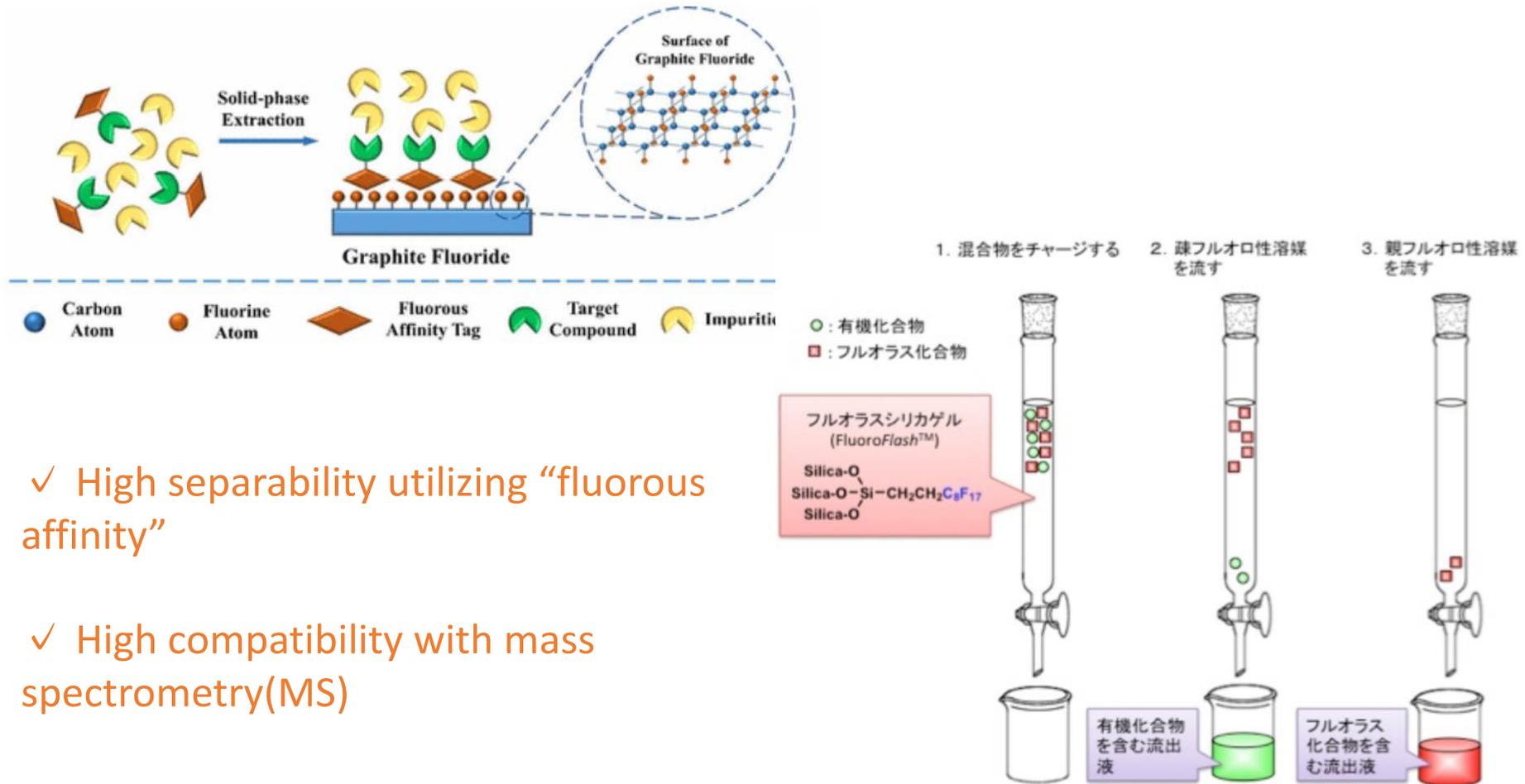
**Silicon microreactor**

Fluororous compounds show high affinity for fluororous solvents

- $\Rightarrow$
- Easy purification (using FSPE)
  - Overcome poor solubility

# $\beta$ -Peptide synthesis

## ○ Fluorous solid-phase extraction (FSPE)



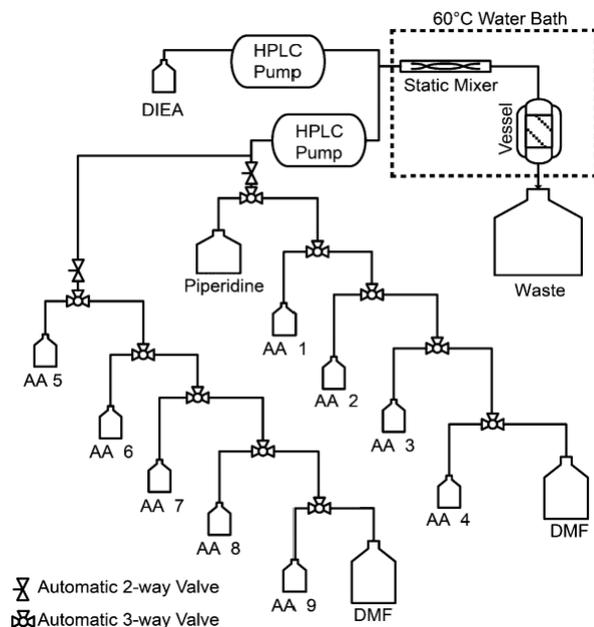
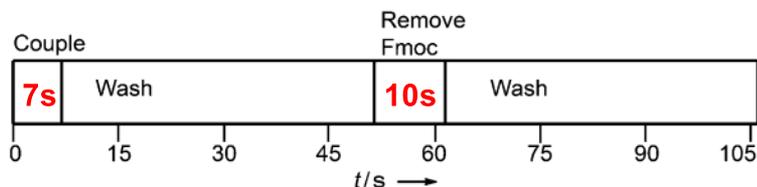
✓ High separability utilizing “fluorous affinity”

✓ High compatibility with mass spectrometry (MS)

# Solid-phase $\alpha$ -Peptide synthesis

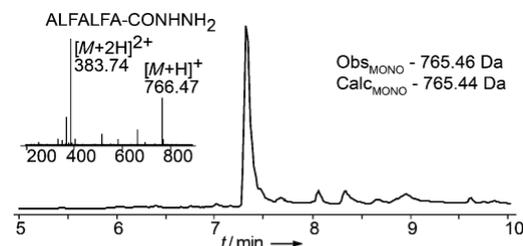
## ○ Synthesis on a solid-phase column reactor

Automated peptide synthesis in a short time was achieved



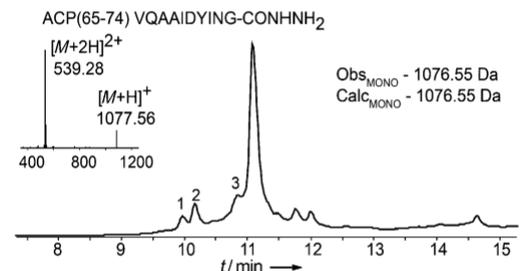
① 7 residue model peptide

12.5 min



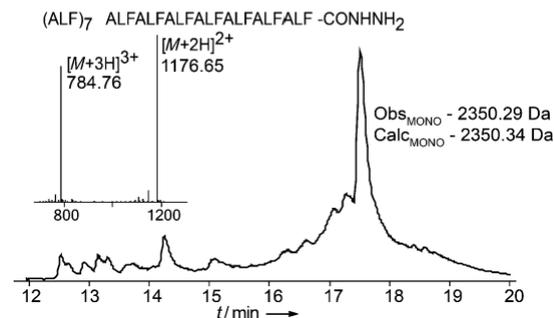
② ACP(65-74)

17.8 min



③ 21 residue model peptide

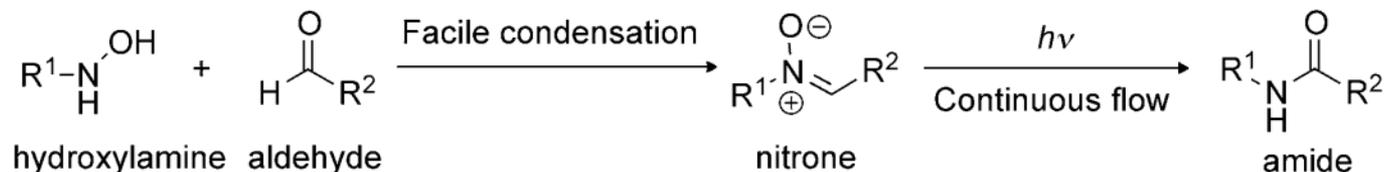
37.5 min



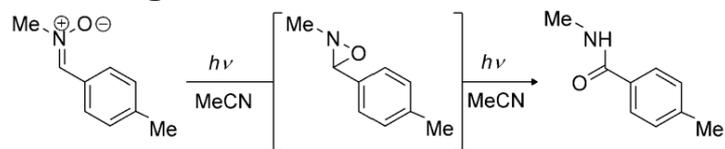


# Solution-phase $\alpha$ -Peptide synthesis

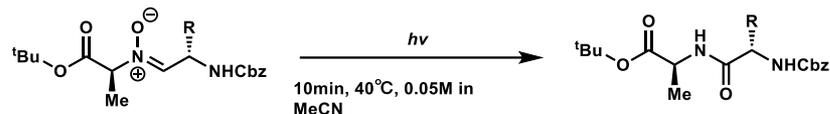
## ○ $\alpha$ -Peptide synthesis via photochemical reaction



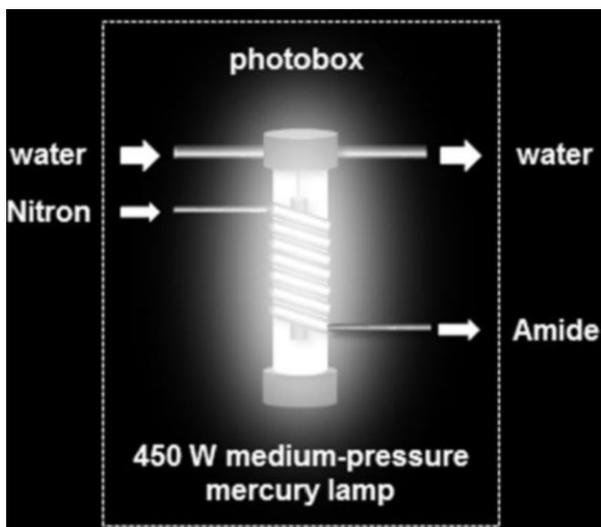
### Rearrangement of nitron to amide



### Substrate scope



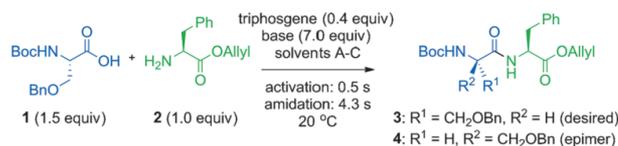
Entry	Substrate	Product
1		 (54%)
2		 (60%)
3		 (56%)
4		 (50%)
5		 (50%) (5min)
6		 (37%) (20min)



# Solution-phase $\alpha$ -Peptide synthesis

## ○ Synthesis via rapid and strong activation using Triphosgene

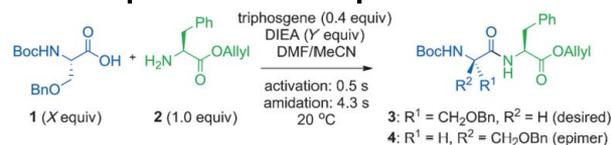
### Optimization of solvents and bases



Entry	Solvent			Base	Yield [%]	
	A	B	C		3	4
1	CH <sub>2</sub> Cl <sub>2</sub>	CH <sub>2</sub> Cl <sub>2</sub>	CH <sub>2</sub> Cl <sub>2</sub>	DIEA	31	–
2	1,4-dioxane	1,4-dioxane	1,4-dioxane	DIEA	– <sup>[b]</sup>	–
3	MeCN	MeCN	MeCN	DIEA	48	2
4	<i>i</i> PrOH	MeCN	MeCN	DIEA	– <sup>[c]</sup>	–
5	MeCN	MeCN	MeCN/H <sub>2</sub> O (1:1)	DIEA	52	3
6	NMP	MeCN	MeCN	DIEA	< 55	< 10
7	DMF	MeCN	MeCN	DIEA	62	9
8	MeCN/H <sub>2</sub> O (9:1)	MeCN	MeCN	DIEA	56	15
9	MeCN/DMF (9:1)	MeCN	MeCN	DIEA	55	2
10	DMF/H <sub>2</sub> O (9:1)	MeCN	MeCN	DIEA	58	9
11	DMF	MeCN	MeCN	Et <sub>3</sub> N	– <sup>[b]</sup>	–
12	DMF	MeCN	MeCN	Me <sub>2</sub> NEt	52	10
13	DMF	MeCN	MeCN	Cy <sub>2</sub> NMe	63	15
14	DMF	MeCN	MeCN	lutidine	48	1
15	DMF	MeCN	MeCN	collidine	– <sup>[b]</sup>	–
16	DMF	MeCN	MeCN	DBU	24	9
17	DMF	MeCN	MeCN	DABCO	– <sup>[b]</sup>	–
18	H <sub>2</sub> O	MeCN	MeCN	LiOH	– <sup>[b]</sup>	–

[a] Flow rate A: 2000  $\mu\text{L min}^{-1}$ , flow rate B: 1200  $\mu\text{L min}^{-1}$ , flow rate C: 2000  $\mu\text{L min}^{-1}$ . [b] Insoluble salts were generated. [c] A complex mixture was obtained. Boc = *tert*-butoxycarbonyl, DABCO = 1,4-diazabicyclo-[2,2,2]octane, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DIEA = *N,N*-diisopropylethylamine, DMF = *N,N*-dimethylformamide, NMP = *N*-methylpyrrolidone.

### Optimization of quantities



Entry	X	Y	Yield [%]	
			3	4
1	1.5	7.0	62	9
2	2.0	7.0	73	7
3	2.5	7.0	77	4
4	2.5	5.0	72	2
5	2.5	3.0	92	1

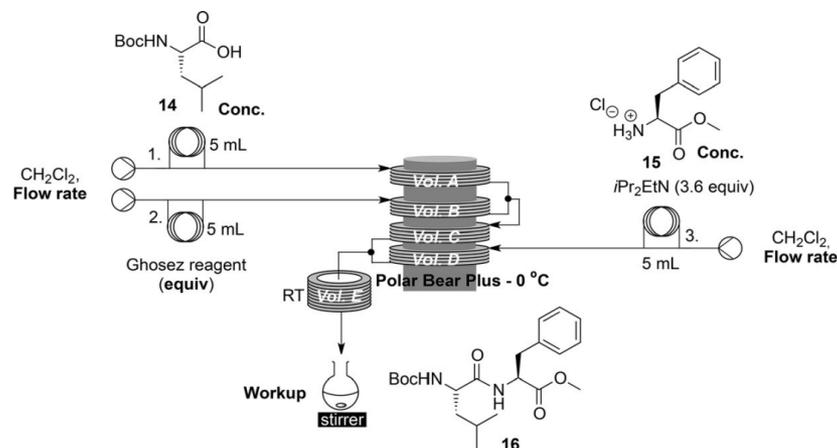
[a] Flow rate A: 2000  $\mu\text{L min}^{-1}$ , flow rate B: 1200  $\mu\text{L min}^{-1}$ , flow rate C: 2000  $\mu\text{L min}^{-1}$ .

### Optimization of residence time

entry	reaction tube 1	temp. (°C)	yield 3 (%)	yield 4 (%)	yield S1 (%)
1	inner diameter: 0.5 mm, length: 41 mm, volume: 8 $\mu\text{L}$ , reaction time: 0.15 s	20	66	0	4
2		30	77	1	0
3		40	78	1	0
4		50	83	2	0
5	inner diameter: 0.8 mm, length: 54 mm, volume: 27 $\mu\text{L}$ , reaction time: 0.5 s	20	92	1	0
6		30	86	1	0
7		40	84	1	0
8		50	80	2	0
9	inner diameter: 0.8 mm, length: 159 mm, volume: 80 $\mu\text{L}$ , reaction time: 1.5 s	20	93	1	0
10		30	78	1	0
11		40	77	1	0
12		50	69	2	0

# Cyclic peptide synthesis

## ○ Optimization

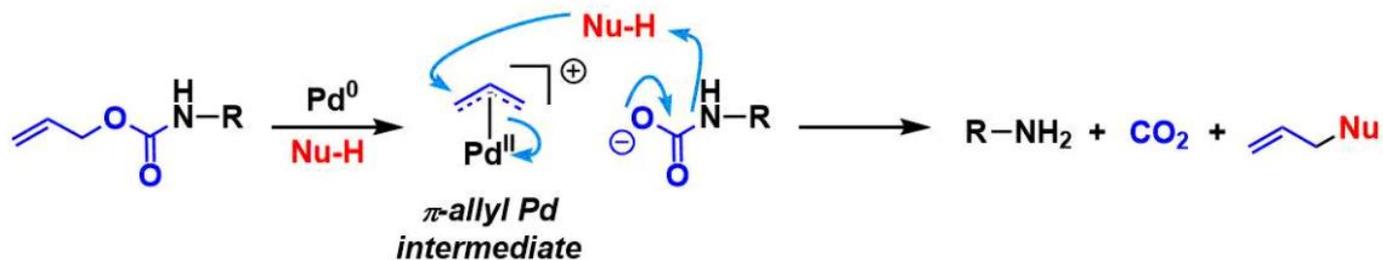


Entry	Flow rate per pump [mL·min <sup>-1</sup> ]	Scale [mmol]	Conc. [M]	T solvent	Activation time [min]	Vol. [mL]					Equiv. Ghosez reagent	Reaction time [min]	Workup	Yield [%]
						A	B	C	D	E				
1	batch	0.50	0.10	–	20	–	–	–	–	–	1	930	RT/1 M HCl	14
2	0.50	0.50	0.10	RT	3	2.5	2.5	3	3	10	1	6.67	RT/1 M HCl	3
3	0.50	0.50	0.10	RT	3	2.5	2.5	3	3	10	2	6.67	RT/1 M HCl	19
4	0.50	0.50	0.10	0 °C	3	2.5	2.5	3	3	10	2	6.67	RT/1 M HCl	18
5	0.25	0.50	0.10	RT	6	2.5	2.5	3	3	10	2	13.3	RT/1 M HCl	11
6	1.00	0.50	0.10	RT	1.5	2.5	2.5	3	3	10	2	3.33	RT/1 M HCl	12
7	1.00	0.50	0.10	RT	1.5	2.5	2.5	3	3	20	2	6.67	RT/1 M HCl	11
8	0.50	0.50	0.10	RT	3	2.5	2.5	3	3	20	2	13.3	RT/1 M HCl	18
9	1.00	0.50	0.10	RT	5	3	3	10	2.5	20	2	6.67	RT/1 M HCl	19
10	1.00	0.50	0.10	RT	5	3	3	10	2.5	20	2	6.67	0 °C/1 M HCl	33
11	1.00	0.50	0.10	RT	5	3	3	10	2.5	20	2	6.67	0 °C/sat. NH <sub>4</sub> Cl	31
12	1.00	0.50	0.10	RT	5	3	3	10	2.5	20	2	6.67	0 °C/H <sub>2</sub> O	28
13	1.00	0.10	0.02	RT	5	3	3	10	2.5	20	2	6.67	0 °C/1 M HCl	44
14	1.00	0.05	0.01	RT	5	3	3	10	2.5	20	2	6.67	0 °C/1 M HCl	46
15	batch	0.05	0.01	–	5	–	–	–	–	–	2	6.67	0 °C/1 M HCl	46
16	batch	0.60	0.01	–	5	–	–	–	–	–	2	6.67	0 °C/1 M HCl	23
17	1.00	0.60 <sup>[a]</sup>	0.01	RT	5	3	3	10	2.5	20	2	6.67	0 °C/1 M HCl	47

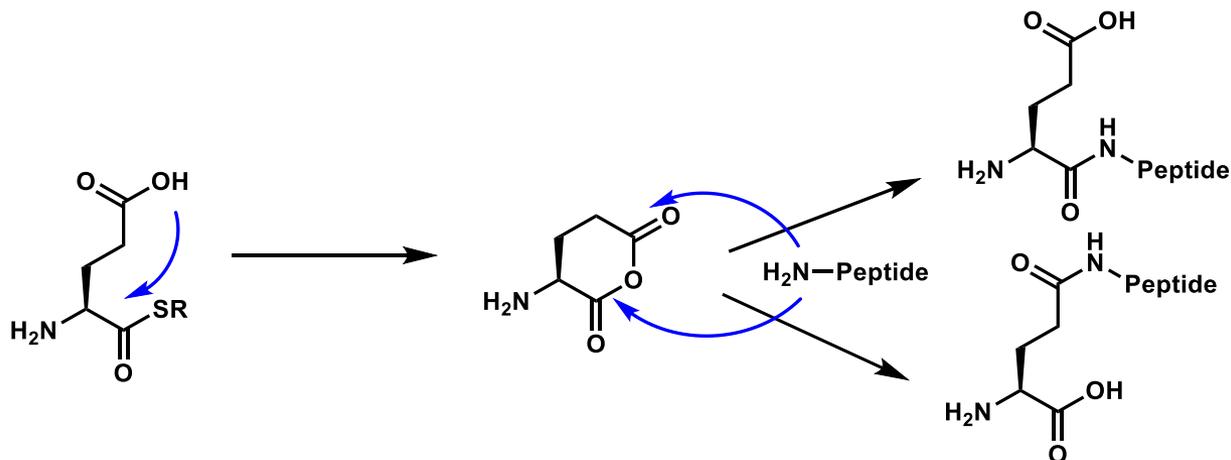
[a] Reaction was run continuously for 1 h after reaching steady state

# Total synthesis of Feglymycin

## ○ Deprotection of Alloc group



## ○ Isomerization of C-terminal Glu

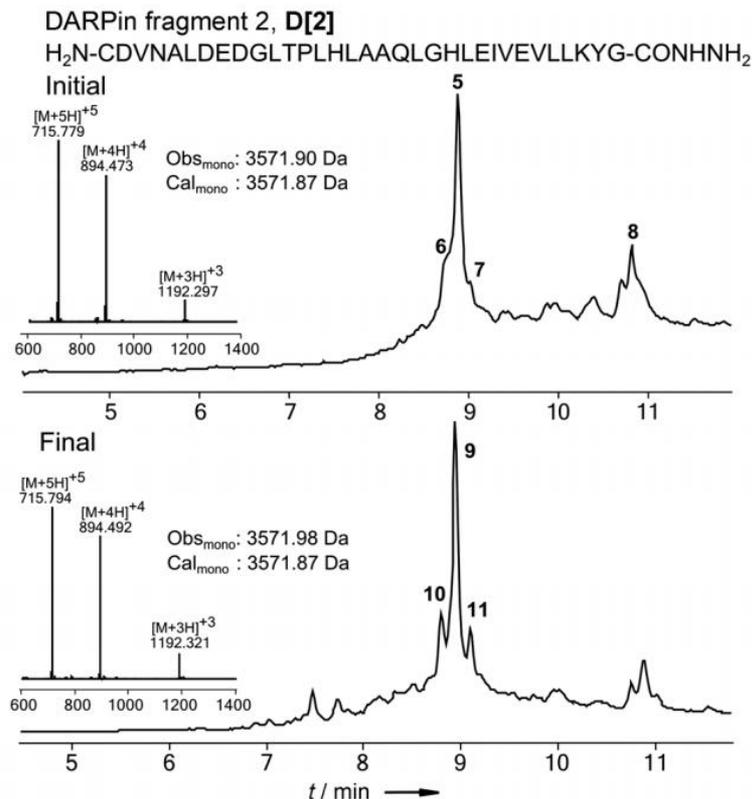




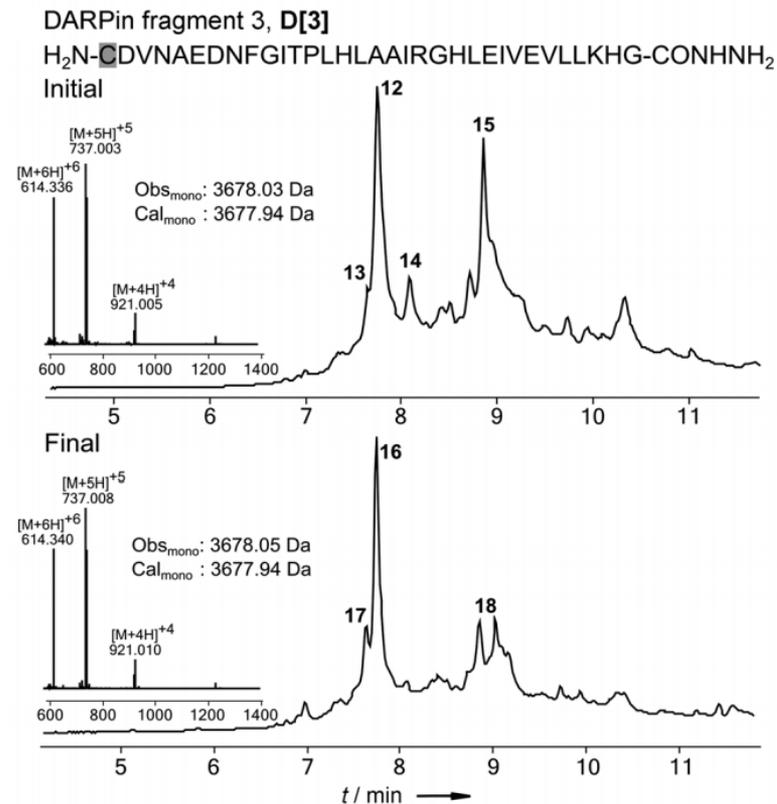


# Total synthesis of protein

## ○ LC-MS data of DARPin pE59 fragments



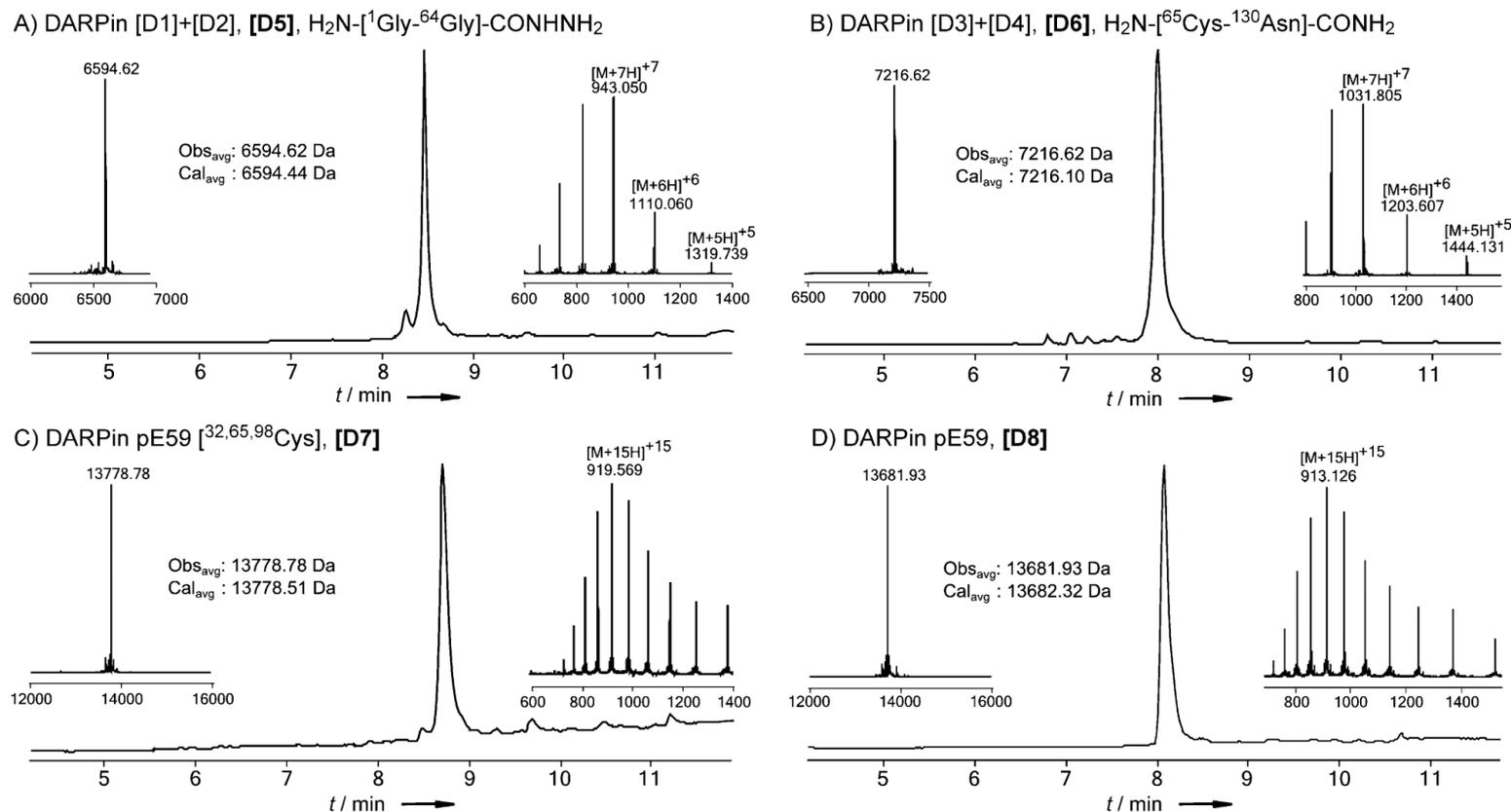
Peak #	Obs <sub>mono</sub>	Identified As
5 & 9	3572.0	Expected D[2]
6 & 10	3571.9	Isomer of D[2]
7, 8 & 11	3434.8	des-His



Peak #	Obs <sub>mono</sub>	Identified As
12 & 16	3678.0	Expected D[3]
13 & 17	3678.0	Isomer of D[3]
14	3427.84	des-His + des-Leu/Ile
15 & 18	3540.9	des-His

# Total synthesis of protein

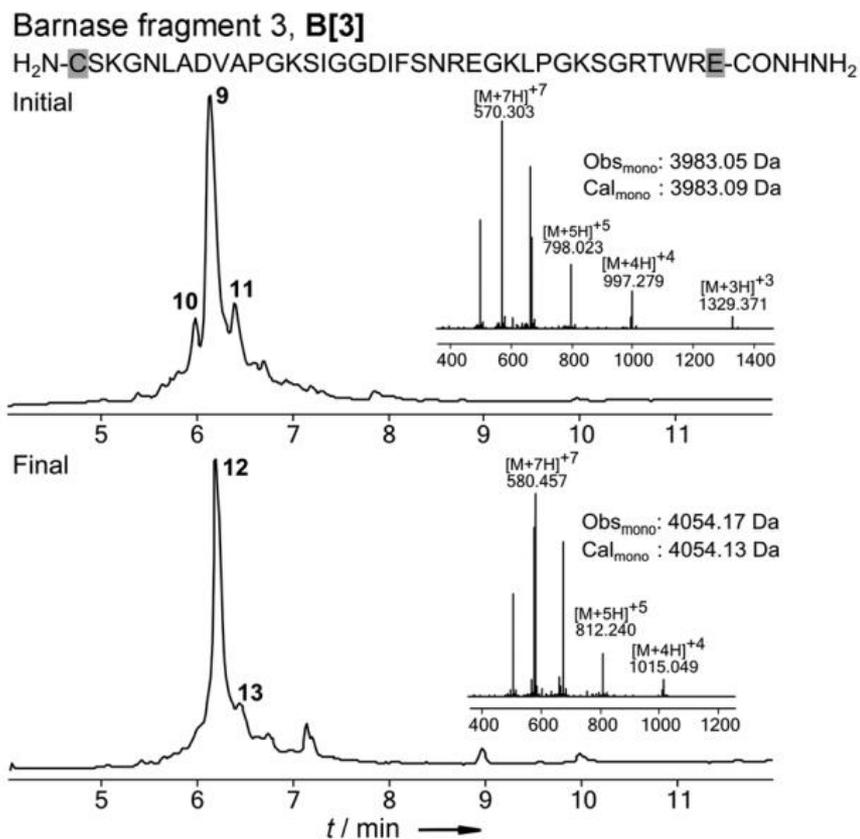
## ○ LC-MS data of DARPin pE59 fragments



**Figure 4.** LC-MS data (total ion current vs. time) of purified DARPin ligation products for purified A) N-terminal polypeptide H<sub>2</sub>N-[Gly1-Gly64]-CONH<sub>2</sub>, B) C-terminal polypeptide H<sub>2</sub>N-[Cys65-Asn130]-CONH<sub>2</sub>, C) full-length DARPin [Cys32,65,98], and D) full-length, native, desulfurized DARPin pE59. Each panel also displays MS of the major peak (inset), comparison of average calculated and observed molecular masses for the expected product, and deconvolution result. The charge state series (inset spectra) indicate the most abundant ions; observed and calculated masses are averages.

# Total synthesis of protein

## ○ LC-MS data of Barnase fragments

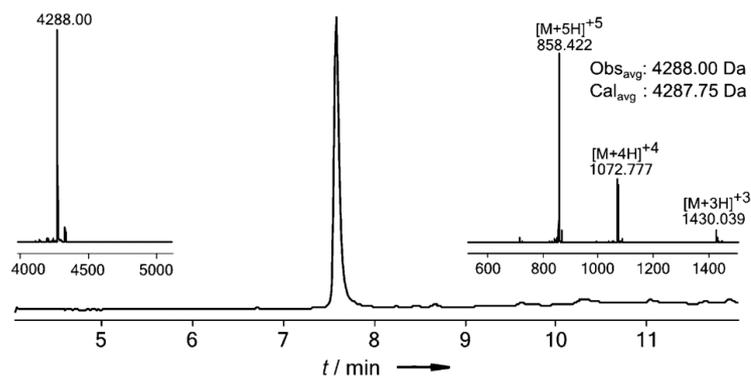


Peak #	Obs <sub>mono</sub>	Identified As
9	3983.05	Expected B[3]
10	3983.14	Isomer of B[3]
11	4079.11	Trifluoacetylated B[3]
12	4054.17	Expected B[3]
13	4150.18	Trifluoacetylated B[3]

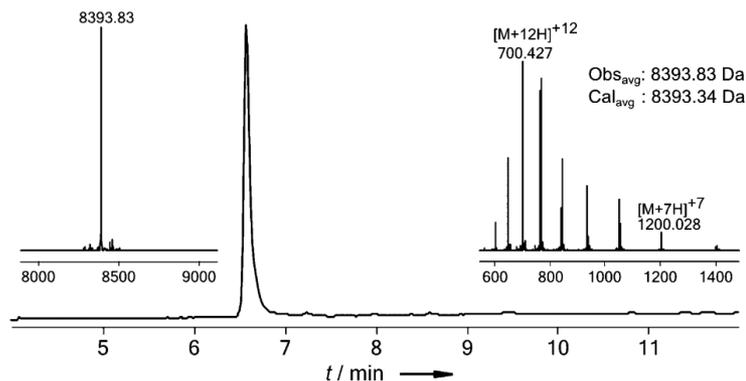
# Total synthesis of protein

## ○ LC-MS data of Barnase fragments

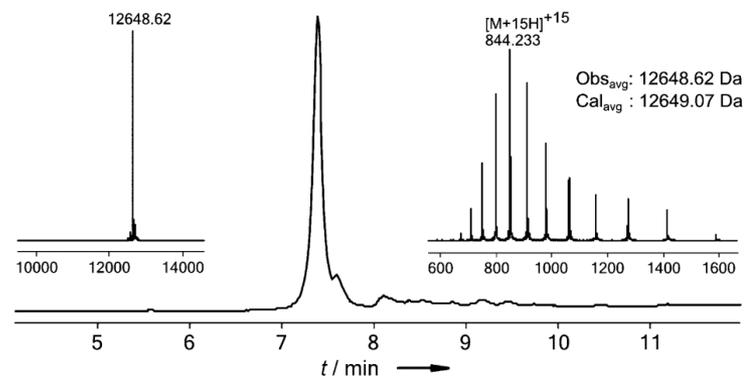
A) Barnase [B1]+[B2], **B[5]**, H<sub>2</sub>N-[<sup>1</sup>Gly-<sup>39</sup>Val]-CONHNH<sub>2</sub>



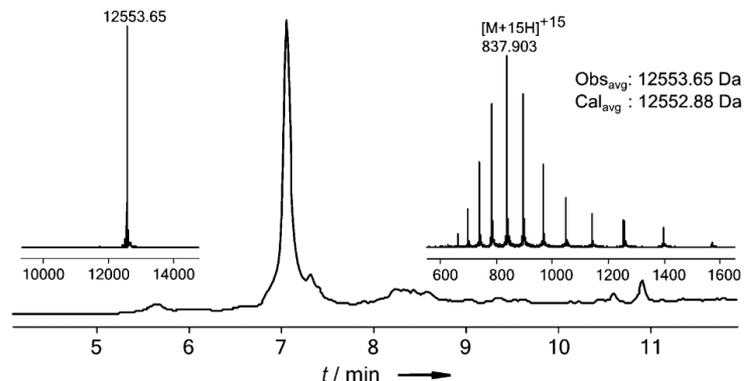
B) Barnase [B3]+[B4], **B[6]**, H<sub>2</sub>N-[<sup>40</sup>Cys-<sup>113</sup>Arg]-CONH<sub>2</sub>



C) Barnase [<sup>14,40,74</sup>Cys], **B[7]**



D) Barnase, **B[8]**

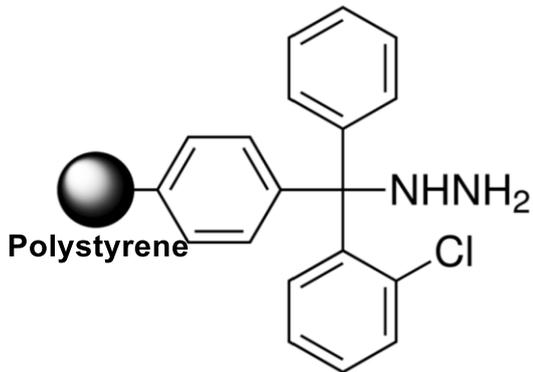


**Figure 5.** LC-MS data (total ion current vs. time) of purified Barnase ligation products: A) N-terminal polypeptide H<sub>2</sub>N-[Gly1-Val39]-CONHNH<sub>2</sub>, B) C-terminal polypeptide H<sub>2</sub>N-[Cys40-Arg113]-CONH<sub>2</sub>, C) full-length Barnase [Cys14,40,77], and D) full-length, native, desulfurized Barnase. Each panel also displays MS of the major peak (inset), comparison of average calculated and observed molecular masses for the expected product, and deconvolution result. The charge state series (inset mass spectra) display the most abundant ions; observed and calculated masses are averages.

# Total synthesis of protein

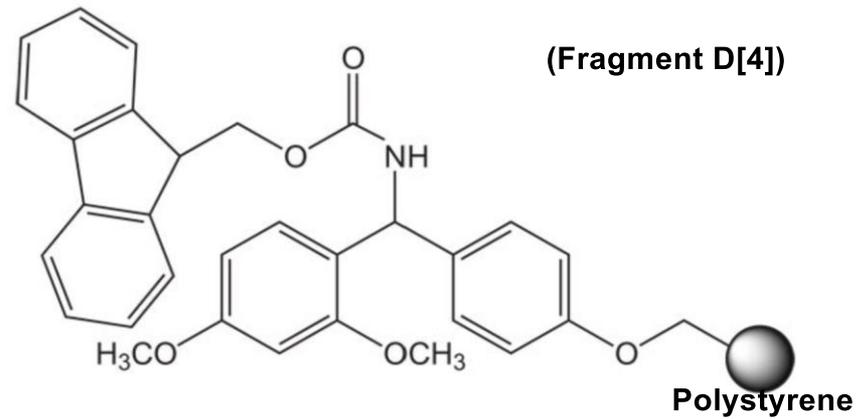
## ○ Resin

### 2-chlorotrityl hydrazine resin



(Fragment D[1]~D[3], B[1]~B[3])

### Rink-AM resin



### Rink-PEG resin

