Peptido

~ The Road To Becoming A Peptide Master ~

4th Edition

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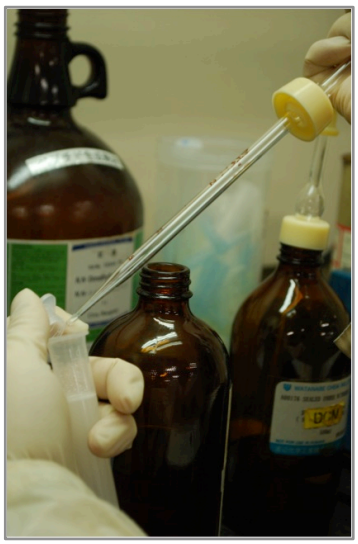
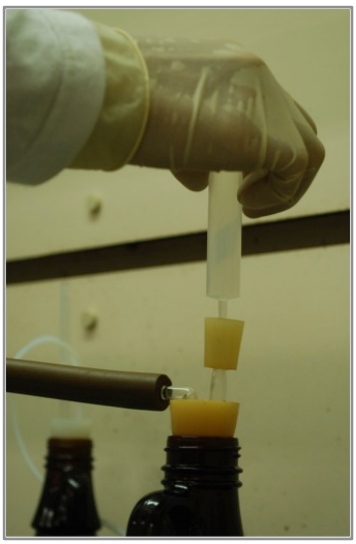
Basic action in Peptido



Addition of solvent

Mixing

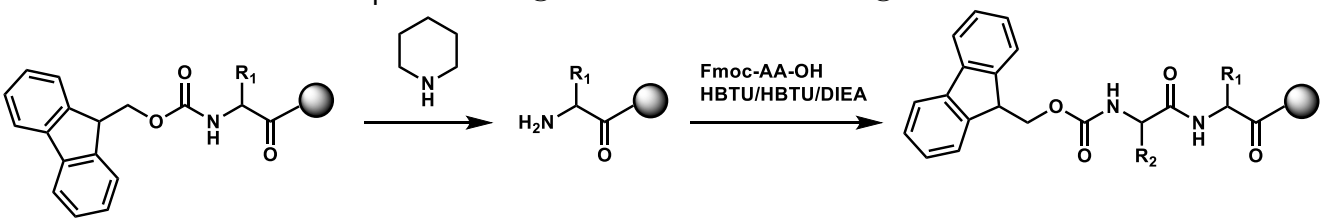


Removal of solvent

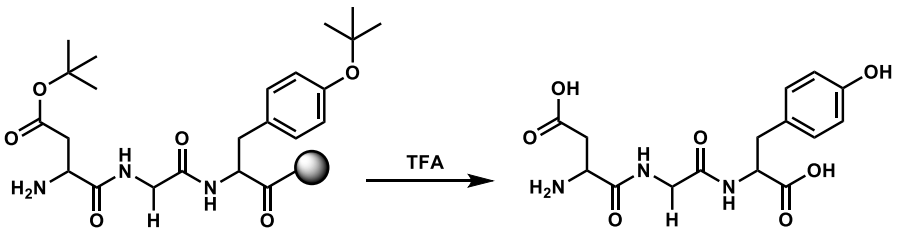
1-1. Reaction

① Fmoc deprotection by secondary amine (piperidine)
② Extension reaction of peptide sequence by coupling reagent

Scheme. Fmoc deprotection ① & extension reaction ②



Scheme. Deprotection & cleavage from resin by strong acid（TFA）

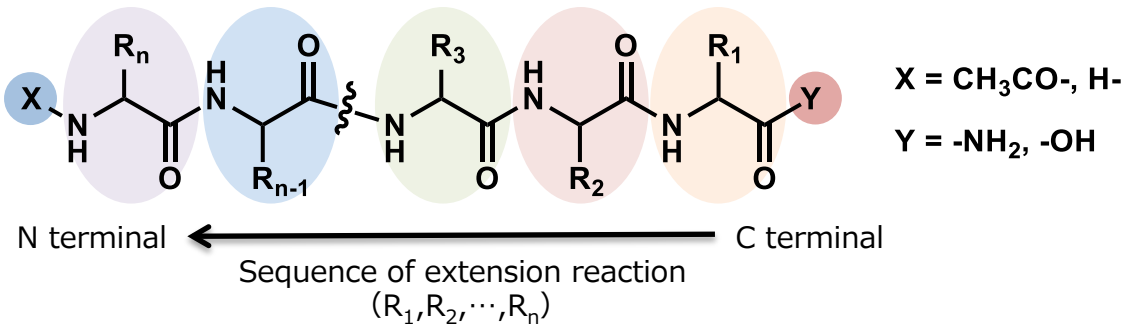


※ Fmoc is not deprotected by TFA.

1-2. Molecular design

The functional group in C terminal（Y）depends on the type of resin. After cleavage from resin by TFA, Y becomes -NH_2 and -OH in the case of carboxamide and peptide acid, respectively.
On the other hand, the functional group in N terminal（X）becomes amine group（H-）after Fmoc deprotection. Therefore, it is possible to acetylate（X = CH_3CO-）or modify carboxyl group（X = HOOC-$(CH_2)_2$-CO-）to N terminal.

Fig. Peptide structure



1-3. Solid phase synthesis

Unlike liquid phase synthesis, we can synthesize a large amount of peptide with easy reaction steps and high reaction efficiency in solid phase synthesis. We can also get the peptide with high purity if we optimize the reaction steps（handling, coupling reagents, etc）.
In addition, we can synthesize not only linear peptide, but also circular peptide and peptide which side chain is modified. As for selective deprotection of amino acid (AA) side chain, please refer to chapter 7 and solid phase synthesis handbook[1].

2-1. General reagents

Table. Reagents used in general synthesis

Reagent name	Abb	Application
dichloromethane	DCM	Swelling resin
N,N-dimethylformamide	DMF	Reaction solvent
N-methylpyrrolidone	NMP	Reaction solvent, resin storage solvent
methanol	MeOH	Shrinking resin
piperidine	PPD	Fmoc deprotection
2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate	HBTU	Coupling agent (condensing agent)
1-Hydroxy-1H-benzotriazole, monohydrate	HOBt	Coupling agent (activating agent)
(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate	COMU	Coupling agent (condensing agent)
diisopropylethylamine	DIEA	Organic base
phenol		Kaiser test
ninhydrin		Kaiser test
ethanol	EtOH	Kaiser test
0.0013% KCN/pyridine		Kaiser test (domestic chemical)
acetic anhydride	Ac_2O	Acetylation of unreacted points
trifluoroacetic acid	TFA	Deprotection of protecting group, Cleavage from resin
triisopropylsilane	TIS	Scavenger for deprotection of protecting group
ethanedithiol	EDT	Scavenger for deprotection of protecting group
diethylether	Et_2O	Ether precipitation

Table. Reagents used in special cases

Reagent name	Abb	Application
dicyclohexylcarbodiimide	DCC	Introduction of amino acid to hydroxy resin
N,N-dimethyl-4-aminopyridine	DMAP	Organic base
benzoic anhydride		Benzoylation of unreacted points
pyridine		Cleavage from Sieber, 2-Chlorotrityl resin
hydrazine monohydrate		Deprotection of nvDde
2-mercaptoethanol		Deprotection of tButhio
iodine	I_2	Deprotection of Acm & Trt、-SS- formation

2-2. Instruments

Table. Instruments and consumable items

Instrument name	Application
Komagome pipette/pipette	Addition of solvent (2 mL-Komagome is useful.)
vortex mixer	Mixing of column (Should have resistance for organic solvent)
diaphragm pump/suction bottle	Removal of solvent (Suction bottle: made by gallon bottle & rubber stopper)
glass tube/hot plate	Kaizer test IWAKI 9830-1007
desiccator/vacuum pump	Drying sample
UV absorptiometer	Fmoc quantification
centrifuge	Collection of deprotected peptide
evaporator	Distilling away solvent

3-1. Selection of resin

Generally speaking, it is better to use「Rink Amide AM resin HL」thanks to good cost-effectiveness and high yield. In the case of long and bulky peptide or α-helix peptide synthesis, it may be difficult to extend peptide. In that case, you should use the resin which has low reaction point density and PEG chain.
There are some types of resin which we don't need 4-1 <Fmoc deprotection> step. Before using, please check resin structure.

Table. Type of resin and method for utilization[1]

Resin name	Reaction point density*	Base material	Price /g	1st loading	Cleavage
Peptide acid					
Fmoc-AA-Wang resin	1.0 mmol/g	PS		pre-loaded	6-1.
NovaSyn TGA resin	0.2 mmol/g	PEG-PS	¥9,500	4-2.	6-1.
Peptide carboxamide					
Rink amide PEGA resin	0.2 mmol/g	PEG-AAm	¥21,000	4-1.	6-1.
NovaSyn TGR resin	0.3 mmol/g	PEG-PS	¥15,000	4-1.	6-1.
Rink Amide AM resin LL	0.4 mmol/g	PS	¥8,000	4-1.	6-1.
Rink Amide AM resin HL	0.6 mmol/g	PS	¥8,000	4-1.	6-1.
Rink Amide NovaGel	0.7 mmol/g	PEG-PS	¥13,600	4-1.	6-1.
Protected peptide acid					
2-Chlorotrityl chloride resin	1.3 mmol/g	PS	¥6,400	4-3.	6-2.
NovaSyn TGT alcohol resin	0.2 mmol/g	PEG-PS	¥14,800	4-2.	6-2.
Protected peptide carboxamide					
Sieber Amide resin	0.6 mmol/g	PEG-PS	¥18,100	4-1.	6-2.

* It depends on Lot. Check the value on the bottle.

3-2. Selection of column

Table. Synthesis column

Column name	Optimum scale	Price	Other info.
PD-10 Empty Columns	~0.1 mmol	¥19,800/50	(A) GE, 17-0435-01
Econo-Pac Chromatography Columns	~0.3 mmol	¥27,000/50	(B) BIO RAD, 732-1010

Select column according to reaction scale (the amount of solvent). We should choose the optimal column which is enough for resin to soak in reaction solvent. Although we can reuse the column, should be careful about degradation of filters and caps. Especially, the bottom cap tends to crack due to repeated vortex mixing. Before addition of strong acid for deprotection or cleavage, you should check carefully (You can also use the cap for PetiSyzer (p.16)). If the filter floats, reset it.

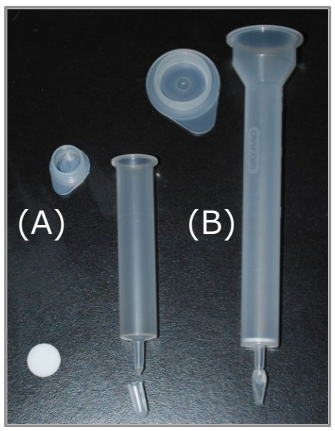


Fig. synthesis column

3-3. Swelling of resin

Selected resin is weighed, transfer into the column, and soaked in DMF overnight. You can also use DCM which has higher swelling coefficient (2-3 hours). Please note that DCM is easy to evaporate and the resin which has low reaction point density tends to float on solution level (Here, swelling time is longer than usual. It is acceptable to soak resin in DCM for 30 min).

3-4. Fmoc amino acid

3 eq. mol (relative to reaction point on the resin) of a AA is taken in a tube.
The separated AA is recommended to keep in dry and without light.

Table. Main Fmoc AA we use (AAs shown in red are kept at -20℃.)

AA	Abb	MW	AA	Abb	MW	AA	Abb	MW
Ala	A	311.3	Leu	L	353.4	Lys(biotin)	Kb	594.7
Arg(Pbf)	R	648.8	Lys(Boc)	K	468.6	Lys(ivDde)	Ki	574.7
Asn(Trt)	N	596.7	Met	M	371.5	Lys(Mtt)	Km	624.8
Asp(OtBu)	D	411.5	Phe	F	387.4	Asp(Odmab)	Do	666.8
Cys(Trt)	C	585.7	Pro	P	337.4	Ser(PO(Obzl)OH)	pS	497.4
Gln(Trt)	Q	610.7	Ser(tBu)	S	383.5	Thr(PO(Obzl)OH)	pT	511.5
Glu(OtBu)	E	425.5	Thr(tBu)	T	397.5	Tyr(PO(Obzl)OH)	pY	573.5
Gly	G	297.3	Trp(Boc)	W	526.6	Cys(Acm)	Ca	414.4
His(Trt)	H	619.7	Tyr(tBu)	Y	459.6	Cys(tButhio)	Ct	431.6
Ile	I	353.4	Val	V	339.4	Cys(Mmt)	Cm	615.7

3-5. Preparation of coupling reagents

Coupling reagents are prepared at the time of use according to reaction scale of synthesis. When you use sonication to dissolve these reagents in solvents, prevent water being mixed into prepared solutions. If you want to store them temporarily, keep them in refrigerator away from the light, after treating them with nitrogen gas. You can use them for about 1 month. However, you should use up them in one synthesis because these reaction activities are gradually decreasing. In order to avoid decrease of reagents activities due to repeated opening and closing of a cap, you should separate these reagents into several bottles at first.

Table. Recipe for coupling reagents

0.45M coupling cocktail		
HBTU	6.1 g	3.05 g
HOBt·H_2O	2.5 g	1.25 g
DMF	32 ml	16 ml
Available number of reaction times with 0.1 mmol scale	~ 45 times	~ 20 times

0.9M DIEA		
DIEA	5.5 ml	2.75 ml
NMP	29.5 ml	14.25 ml
Available number of reaction times with 0.1 mmol scale	~ 45 times	~ 20 times

(Additive amount of each solution for one reaction in 0.1 mmol scale : 700 μL)

3-6. Preparation of Kaiser test reagents

We can purchase reagents 1~3 from domestic chemical company (product number: 2590077, Reagents for Kaiser Test, ¥9,000/set). We can also prepare reagents 1 and 2 by ourselves because these are relatively safe. If the color of reagent 1 turns to dark, prepare it again. Since it takes time to prepare for reagent 2, you should check whether it is enough or not before your synthesis. After preparation of them, confirm whether these reagents make resin which has amine groups blue.

Table. Recipe for Kaiser test reagents

Kaiser test reagent	Solute	Solvent
1. ninhydrin/EtOH	0.5 g ninhydrin	10 ml EtOH
2. phenol/EtOH	8 g phenol	2 ml EtOH
3. KCN/pyridine	0.13 mg KCN	10 ml pyridine

4-1. Introduction to Fmoc protected resin

We can introduce AA to the resin according to the same protocol as 4-4. Peptide extension.

4-2. Introduction to hydroxy resin based on DCC/DMAP method (0.1 mmol scale)

Synthesis column

〈Coupling〉

- 0.1 mmol Hydroxy resin in DCM or DMF
- Swell overnight

25 ml Eggplant flask

- 0.5 mmol Fmoc-AA-OH
- 0.5 mmol DCC
- in DMF (amount of dissolution, less than 5 ml)
- Stir for 30 min at 0℃

- 0.05 mmol DMAP
- Stir for 1 h at RT

※ If DCC is not dissolved in DMF, you can use DIC instead of DCC.
※ If the amount of solvent is too small to stir, add DMF more as you think proper.

〈Removal of impurities & Masking of unreacted points〉

- 2 ml DMF wash ×3
- 2 ml DCM/EtOH (50/50) wash ×3
- 2 ml DCM wash ×3
- 2 ml DMF wash ×3
- 0.5 mmol benzoic anhydride in 2mL 20% pyridine/DMF
- Stir for 1 h at RT
- DMF wash ×3
- DCM wash ×3

Move on to 4-4. Peptide extension

※ Calculate introduction efficiency according to 5-1. Fmoc quantification

4-3. Introduction to active trityl resin (0.1 mmol scale)

25 ml Eggplant flask (or synthesis column)

〈Coupling〉

- 0.1 mmol 2-Chlorotrityl chloride resin in DCM (amount of soaking)
- Stir for 10 min at RT
- 0.15 mmol Fmoc-AA-OH
- 0.5 mmol DIEA (80 µl)
- in DCM (amount of dissolution)
- Stir for 2 hs at RT

※ If AA is not dissolved in DCM, you can add the small amount of DMF as you think proper.

Synthesis column

〈Inactivation of unreacted points〉

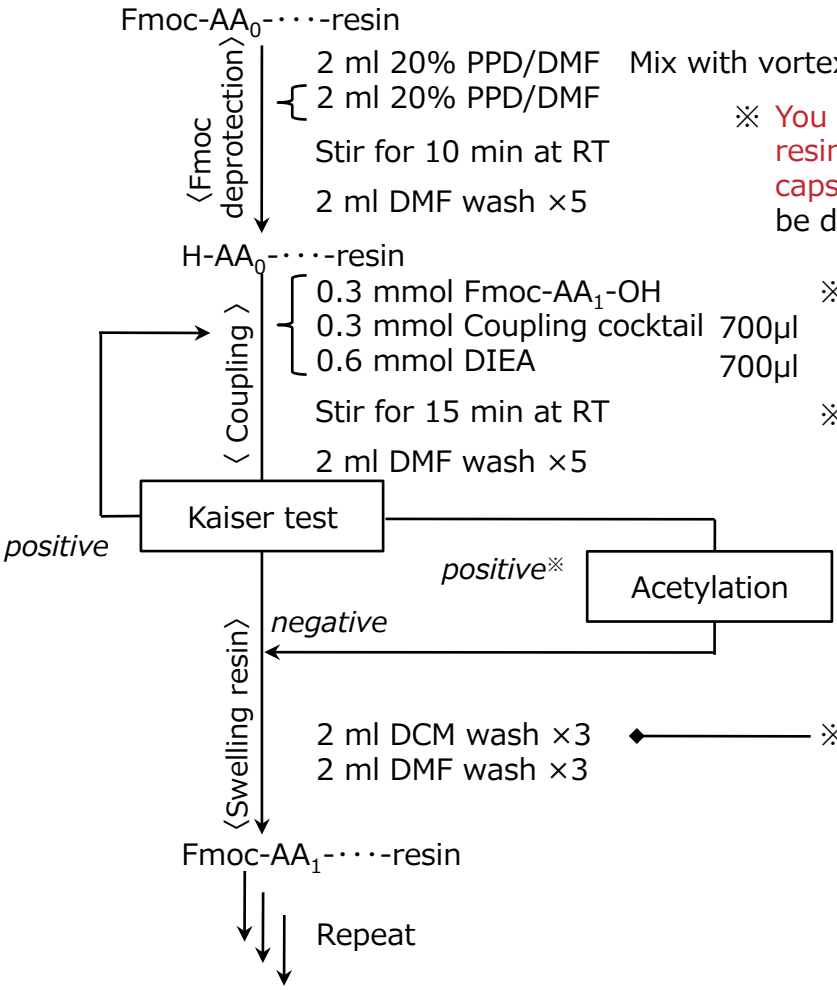
- 2 ml DCM/MeOH/DIEA=17/2/1 wash ×3
- 2 ml DCM wash ×3
- 2 ml DMF wash ×3
- 2 ml DCM wash ×3

Move on to 4-4. Peptide extension

※ Calculate introduction efficiency according to 5-1. Fmoc quantification

※ In 4-2・4-3, you should select the kind of solvent which can make AA conc. from 0.3 to 0.5M.

4-4. Peptide extension (0.1 mmol scale)



※ You should wash the solvent and resin on the upper and the bottom caps carefully, otherwise Fmoc may be deprotected during storing.

※ After mixing and dissolving all of them completely, add the mixture to resin.

※ You can change the reaction time, additive amount of reagents and the number of reaction times. Optimize them based on analysis.

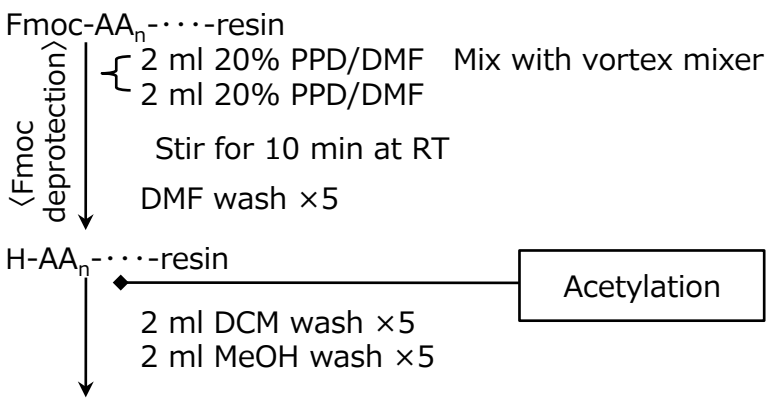
If Kaiser test shows positive after 2nd – 3rd reaction, acetylate unreacted points.

※ When you keep your peptide in the middle of your synthesis overnight, you should stop your synthesis in this step, soak the resin in DCM or NMP, and keep it in refrigerator.

※ When you change your reaction scale, the mol ratio in 〈Coupling〉 step should be Fmoc-AA-OH : Coupling cocktail : DIEA : H_2N-(Resin) = 3: 3 : 6 : 1.
And also, the amount of solvent in 〈Fmoc deprotection〉 or each washing step should be about 1.5 eq. (relative to the resin volume) .

※ You need to remember that coupling reagents such as HBTU or COMU, are for activating the carboxyl groups.
In the common peptide extension, AA after being activated is added to the free amine groups on the resin. In an opposite case, in which an amine compound is added to carboxyl groups on the resin, coupling reagents should be directly added to the resin. Then in the second step amine compound will be added to the activated resin.

4-5. Ending of peptide extension



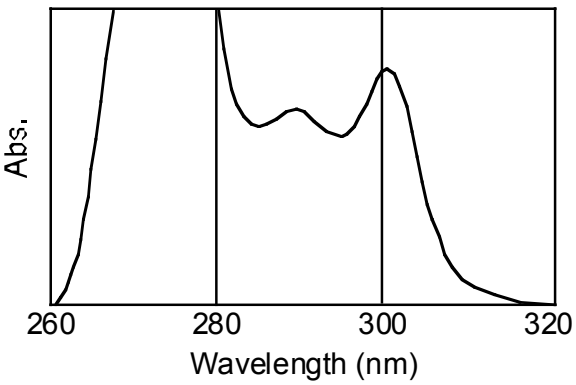
When you synthesize the peptide whose N terminal is acetylated, you should acetylate it in this step.

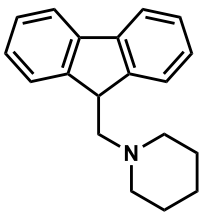
5-1. Quantification of introduction ratio of amino acid

If we assume that 100 % Fmoc group is deprotected, the introduction ratio of the AA can be estimated by the amount of Fmoc group which is eluted in 〈Fmoc deprotection〉 step. You should quantify the amount of Fmoc group at least for 1st, middle and final AAs during the waiting time in <Coupling> step.

Protocol

- Collect all filtrate in 4-4. 〈Fmoc deprotection〉 step and measure that volume with measuring cylinder (The volume will be about 14 mL in 0.1 mmol scale, so you can use a 15 ml-centrifuge tube for measuring the volume).
- Dilute collected filtrate 10~100 times with DMF.
- Measure absorbance of the prepared sample at 301 nm.
  Blank absorbance should be obtained with DMF.
- Calculate the amount of Fmoc group by using ε_{301nm} = 7,800 $M^{-1}cm^{-1}$.





Three Abs peaks can be observed from 260~320 nm.

5-2. Kaiser test

It can be checked whether there are unreacted amine groups or not based on ninhydrine reaction.

Protocol

- Mix each of 20 µl Kaiser test reagents in a glass tube
- Add very tiny amount of resin to the glass tube
- Heat the glass tube with boiling water for 1 min
- Check the resin or solution color

dark red~blue : positive / yellow : negative

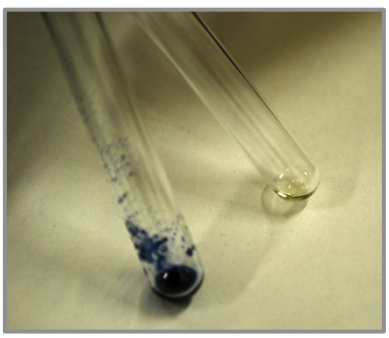


Fig. Kaiser test result
Left : positive / Right : negative

5-3. Acetylation

Acetylation enables us to mask unreacted amine groups or amine groups in N terminal. If Ac_2O is old, its pH may be lower than usual due to hydrolysis. Thus, be careful if you use the resin which cleaves peptides with dilute acid.

Protocol (0.1 mmol scale)

2 ml DCM wash ×3
2 ml 25% Ac_2O/DCM Mix with vortex mixer for 30 sec
(Stir for 5 min at RT) ← In the case of acetylation for N terminal
2 ml DCM wash ×3
2 ml DMF wash ×5

6-1. Cleavage and deprotection by strong acid

Protection groups on the side chains of Fmoc AAs can be deprotected by TFA with scavenger to avoid side reactions. Since deprotection is exothermic reaction, deprotection cocktail and TFA should be cooled in advance.

Table. Selection of deprotection cocktail

Deprotection cocktail	Volume ratio	Volume in 0.1 mmol scale (μl)
If peptide has Cys (Trt) or Met		
TFA/water/TIS/EDT	94/2.5/1.0/2.5	2350/62.5/25/62.5
If peptide does not have Cys (Trt) or Met		
TFA/water/TIS	95/2.5/2.5	2375/62.5/62.5

Protocol 1 Resin in synthesis column

〈Cleavage・Deprotection〉
- Deprotection cocktail (Cooled in advance)
  Keep for 90 min at RT (Stir every 30 min)
  Collect filtrate into a 50 ml centrifuging tube
- 1.5 ml cooled TFA ×3
  → Collect each filtrate into a 50 ml centrifuging tube

Wash cooled Et_2O as you think proper

Protocol 2 Crude peptide solution in a 50 ml centrifuging tube

〈Ether precipitation〉
- 40 ml cooled Et_2O
  Stir well
  Centrifuge (4℃, 3,500×*g*, 5 min)
  Discard supernatant
  } Repeat 3 times

Keep it in hood for about 10 min to evaporate Et_2O
Dry in desiccator over 2 hrs

Crude peptide

〈Reference 1〉 Peptide purification

Check crude yield by weighing crude peptide. If crude yield is too low, there are possibilities such as

- Introduction efficiency of each AA is low.
- There are still protected peptides on the resin due to the lack of the deprotection.

We can solve these problems by Fmoc quantification and redoing deprotection, respectively.

Prepare crude peptide solution with about 10 mg/ml with 0.1% TFA solution. If there is precipitate, try the following methods to dissolve it.

- Add more TFA till its conc. becomes 1% in the case of basic peptide
- Dissolve into mixture of acetonitrile/water

After filtration, check whether target peptide has been synthesized by MALDI-TOF-MS.

Sort target peptide by using RP-HPLC of water/acetonitrile. It can be purified by Sephadex G-10~25 in advance. Then, you should care about changing of solubility with pH.

Lyophilize and obtain target peptide powder after removal of acetonitrile by an evaporator (Obtained peptide is TFA salt. If it contains acetonitrile, it may be difficult to lyophilize).

According to peptide sequence, there is optimal peptide storage solution. You should check it out in advance.

6-2. Cleavage of protected peptide by dilute acid (0.1 mmol scale)[2]

Protocol 1

Protected peptide on resin in synthesis column

〈Cleavage〉

〈Swell with DCM〉

2 ml 1% TFA/DCM
Mix with vortex mixer for 2 min
Collect filtrate into test tube (5 ml) with 2 ml 10% pyridine/MeOH

Repeat 10 times.
After that, transfer all filtrate from 10 test tubes to one 200 ml eggplant flask.

2 ml DCM ×3
2 ml MeOH ×3
2 ml DCM ×3
2 ml MeOH×3

Collect each filtrate into eggplant flask

※ You can check the end of the elution by TLC.

Protocol 1

Protected peptide solution

〈Reprecipitation〉

Condense by an evaporator
40 ml cooled H_2O
Filter precipitate and wash it with cooled H_2O
Dry in a desiccator over 2 hrs

Protected peptide

Basically, protected peptides cannot be purified by HPLC. Thus, peptide extension reaction should be done efficiently. For example,

- To make molar ratio and reaction time in 〈Coupling〉 step
  - Fmoc-AA-OH : Coupling cocktail: DIEA : H_2N-(Resin) = 4: 4 : 8 : 1
  - 10 min

  in order to avoid side reactions.
- Whichever Kaiser test result shows, acetylate N terminal.

The protocol for deprotection of side chain is the same as 6-1.

〈Reference 2〉 Head-to-tail cyclic peptide synthesis

According to 6-2, you can obtain protected peptide acid whose N terminal is not protected. When low-concentrated this peptide is exposed to amide coupling reaction, you can get intramolecularly-cyclized peptide.

Protocol　Cyclization of RGD peptide (RGDfX)

H-Arg(Pbf)-Gly-Asp(OtBu)-Dphe-Xxx-OH

〈Coupling〉

DMF (peptide final conc. ≦ 5mM)
5 eq. $NaHCO_3$
3 eq. DPPA

〈Crude purification〉

Stir for 24 hrs
Filter solid $NaHCO_3$
Remove DMF by oil pump
Precipitate with cold water
Suction filtrate & wash precipitate with water and ether
Dry in a desiccator over 2 hrs

-Arg(Pbf)-Gly-Asp(OtBu)-Dphe-Xxx-

7-1. Fmoc-Lys(ivDde)-OH[3]

ivDde group is stable against 20% PPD/DMF and TFA, and deprotected by hydrazine selectively. Therefore, you can synthesize branched peptides by using ε amine group of Lys as bifurcation. Please note that hydrazine deprotects not only ivDde but also Fmoc. IvDde deprotection step can be monitored by measurement of Abs at 290 nm as well as Fmoc quantification.

Protocol

Ac-AA-‥‥-Lys(ivDde)-‥‥-resin in synthesis column

〈ivDde deprotection〉
- 2 ml 2% hydrazine monohydrate/DMF
- Mix with vortex mixer for 1 min
- 2 ml 2% hydrazine monohydrate/DMF
- Stir for 30 min at RT
- 2 ml DMF wash ×5

Ac-AA-‥‥-Lys-‥‥-resin in synthesis column

Extension reaction from ε amine group

※ Check that Kaiser test shows positive. The color should be weak blue.

7-2. Fmoc-Cys(Acm)-OH

Acm group is stable against 20% PPD/DMF and TFA, and deprotected by I_2 selectively. At that time, it makes disulfide bond. Moreover, in the case of Fmoc-Cys(Trt)-OH which is used in general Fmoc solid phase synthesis, Trt is also deprotected by I_2 and Cys makes disulfide bond. One of the main differences between these AAs is stability for acid.

Protocol Iodine oxidation on resin[4]

Fmoc-AA-‥‥-Cys(Acm)-‥‥-Cys(Trt)-‥‥-resin in synthesis column

〈Iodine oxidation〉
- 1 mmol I_2 (250 mg) in 2 ml DMF
- Keep for 4 hrs at RT (Mix every 1hr)
- 2 ml DMF wash ×10
- 2 ml DCM wash ×3
- 2 ml DMF wash ×3

〈Fmoc deprotection Removal of remained HI〉
- 2 ml 20% PPD/DMF Mix with vortex mixer for 1 min
- 2 ml 20% PPD/DMF
- Stir for 15 min at RT
- 2 ml DMF wash ×5

H-AA-‥‥-Cys-‥‥-Cys-‥‥-resin in synthesis column
SS

Don't use EDT, which cleave disulfide bond, as deprotection scavenger.
If reaction point density is high, there may be disulfide bond between molecules.
You should adjust introduction efficiency of 1st AA or choose an optimal resin.

〈Reference 3〉 Iodine oxidation in liquid phase[5]

Cys(Acm) containing crude peptide is dissolved in water/acetonitrile =1/5, and delivered I_2 solution by drops with stirring till solution shows color. After keeping for 30 min, ascorbic acid sodium salt is added to quench I_2. After removal of solvent, it can be purified by RP-HPLC.
The selectivity of disulfide bond formation within or between molecules should be controlled by peptide concentration.

7-3. Fmoc-Cys(tButhio)-OH[6]

tButhio group is deprotected selectively by reduction with thiol group.

Protocol

····-Cys(tButhio)-····-resin in synthesis column

〈tButhio deprotection〉
- 2 ml 20% 2-mercaptoethanol/DMF
- Stir for 4 hrs at RT
- 2 ml DMF wash ×5

-Cys-····-resin in synthesis column

7-4. Fmoc-Cys(Mmt)-OH[6] It can also be used for Fmoc-Lys(Mtt)-OH.

Mmt group is deprotected by dilute acid reversibly. It can be deprotected without side reactions by using TIS as scavenger. Mmt deprotection step can be monitored by measurement of Abs at 460 nm as well as Fmoc quantification. If it is difficult to deprotect Mmt、Mtt, repeat deprotection step.

Protocol

····-Cys(Mmt)-····-resin in synthesis column

〈Mmt deprotection〉
- 2 ml TFA/TIS/DCM=1/5/94
- Stir for 5 min at RT
- 2 ml DCM wash ×3

Repeat 3 times

····-Cys-····-resin in synthesis column

〈Reference 4〉 Disulfide bond formation on resin by *in situ* activation with Npys[6]

Disulfide bond formation on resin can be monitored by using activated thiol formation by 2,2'-dithiobis(5-nitropyridine) (DTNP) (Npys group) and 7-3,4.

····-Cys(Mmt)-····-Cys(tButhio)-····-resin in synthesis column

↓ Protocol 7-3.

····-Cys(Mmt)-····-Cys-····-resin in synthesis column

〈Npys group〉
- 2 ml DCM wash ×3
- 1.0 mmol DTNP (310 mg) in 2ml DCM
- Stir for 1 hr at RT
- 2 ml DCM wash ×5

····-Cys(Mmt)-····-Cys(Npys)-····-resin in synthesis column

〈-SS- formation〉
- 2 ml TFA/TIS/DCM=1/5/94
- Stir for 5 min at RT
- 2 ml DCM wash ×3

Measure Abs of filtrate.
Repeat till Abs of DTNP at 386 nm disappears.

····-Cys-····-Cys-····-resin in synthesis column
SS

Don't use EDT, which cleave disulfide bond, as deprotection scavenger.
If reaction point density is high, there may be disulfide bond between molecules.
You should adjust introduction efficiency of 1st AA or choose an optimal resin.

Is that reagent fresh?

Almost all reagents used in peptide synthesis are prone to be deteriorated by moisture or O_2. Reagents should be purchased with consideration for frequency of synthesis and also separated into some bottles at first as necessary.
It depends on the kinds of reagents or frequency of opening and closing of their cap, but reagents should be used within 1 year after purchasing.
Don't buy too much amount of reagent with a large bottle even if it is cheaper!

Method of storage and using is important. For example, reagents which is kept in refrigerator or freezer such as protected AAs, should be kept at RT about 30 min before using in order to avoid moisture. DIEA which tends to be deteriorated by oxidation or CO_2 should be stored under N_2 gas.

Method of reagent storage is directly related with peptide yield or purity! Manage reagents properly!

When should I prepare for amino acids?

If necessary AAs are weighed the day before using, you can use well the waiting time for reaction on the day for synthesis. Needless to say, the bottles of AAs should be put at RT in preparation for recoupling.
On the other hand, in the case of peptide which has only 1~3 AAs in the sequence, it is not difficult to weigh AAs and dissolve them during the waiting time for 〈Fmoc deprotection〉 step. It depends on your experimental style to use the 10 minute waiting time efficiently.

Synthesis by hand vs PetiSyzer

It is much easier to synthesize by PetiSyzer than by hand. One of the merits of PetiSyzer is ability to control temperature. However, a usable amount of resin in PetiSyzer is lower than that in synthesis by hand because of thin synthesis column for PetiSyzer.

By hand : ~ 0.3 mmol PetiSyzer : ~100 mg

Except for quantity synthesis, it is recommended to use PetiSyzer.

Trick in the synthesis by PetiSyzer

If there are a lot of synthesis columns (over 4?), it is faster to add washing solvents by washing bottles. Even if molecular sieve is in the bottle, it is not hamper unlike Komagome pipette which is inserted into a solvent bottle.

When Kaiser test & Fmoc quantification are not enough for you to have confidence …

You can cleave small amount of intermediate peptide from resin and analyze it.
The same amount of resin for Kaiser test is taken from synthesis column to an Eppendorf tube. Then, small amount of cleavage cocktail is added to the tube. After about 30 min, ether precipitation is repeated a few times by using a desk centrifuge. After drying and dissolving in water, it can be analyzed by MALDI-TOF-MS or RP-HPLC.
This step is not troublesome. Therefore, it may relieve you when your resin has color due to introduction of dye or peptide chain is long.

Is it really easy to synthesize by an automatic peptide synthesizer?

It is really easy to synthesize peptides by a peptide synthesizer. If you want to synthesize a lot of kinds of peptides with small scale, peptide synthesizer is useful. However, peptide purity with peptide synthesizer tends to be low because the synthesizer doesn't do Kaiser test and recoupling. Especially, if you use a peptide synthesizer whose operation rate is low with little maintenance, it may be difficult to purify the target compound by RP-HPLC, which results in a huge amount of time in the purification step.
Even though it is a little troublesome to synthesize peptides by hand, it is better to do so with good purity, which results in saving time in purification step and acetonitrile.

Troubleshooting

When the yield of your target compound is low …

There may be problems in the steps of Fmoc deprotection, peptide extension reaction or cleavage.

Predictable reasons could be ① mixing steps are not enough, ② activity of coupling reagents decreases, ③ it is difficult for an AA to react due to its steric hindrance. Troubleshooting methods may lead to ① change the size of reactors, the amount of the solvent, mixing time or strength, ② remake coupling reagents, ③ change the kind of the coupling reagent (such as HATU or COMU which has higher reaction efficiency than HBTU[7]). Whichever step you have a trouble in, you can analyze and improve it based on Fmoc quantification. In terms of troubleshooting in the cleavage step, please refer to 「Cleavage」as below.

When you use COMU as a coupling reagent, COMU 3.1 g/DMF 18 ml (0.4M)とDIEA 2.1 ml /NMP 18 ml (0.6M)

Kaiser test

If the washing is not enough, the color of the solvent may turn to blue, red or purple. However, it should be negative if the color of the resin doesn't change. In addition, it often tends to be negative when the color of the resin is red (especially in the case of using active trityl resin).

Storage of the resin

The resin should be kept in ① the solvent or ② the dry state. In the case of ①, it is better to use DCM or NMP. Please note that DCM is easy to evaporate. DMF is not recommended, because DMF is degraded during long storage and it produces amine groups, which may result in undesired Fmoc deprotection. ② is suitable for long storage of the resin. Please note that you need to swell the dried resin with DCM for about 30 min before you restart your experiment.

Cleavage

This step is one of the reasons of decrease in yield. Because the cleavage reaction is exothermic reaction, it is better to cool cleavage cocktail and TFA in advance. Depending on your peptide sequence, you need to select the optimal kind of the deprotection cocktail or adjust the reaction time. Since some peptide sequences show difficulty for deprotection of protection groups and need some scavengers in peptide cleavage step to avoid side reactions. Moreover, if the amount of TFA or cooled Et_2O is too low or too high in the reprecipitation step, the yield may be low. Thus you should check the yield every time. Rough standard is TFA/Et_2O=1/20 v/v.

Fluorophore labeling

Generally speaking, fluorophores are more expensive than Fmoc AAs. Therefore you should care about your reaction scale.

If it is a reaction with 1.2 eq. mol (relative to reaction point) of a fluorophore, it is possible that almost 100% reaction is succeeded by increase of the fluorophore conc. and reaction time and temperature. However, if the fluorophore conc. is too high, sometimes it is difficult to dissolve it or mix it.

Know-how of synthesis

① Optimize reaction conditions! (conc., pH & the kind of catalysts, the amount of solvents, mixing time, reaction temperature)

② Think about the purpose of each step! (reaction, washing, cleavage etc…each handling has each optimal way.)

③ Don't neglect to check the progress of each reaction! (Fmoc quantification, MS analysis, kaizer test, weighing the yield etc)

For example, you can check the progress of each reaction and activities of your reagents by Fmoc quantification!

HPLC

Peptide samples are dissolved in the A/B mixture. The ratio of solvent B should be lower than the initial value of the solvent gradient. Dusts or impurities should be removed by filtration or centrifuge. If the sample cannot be dissolved in the A/B mixture, the other solvents can be used such as DMSO. If you use the other solvents, please check that it is not insolubilized in the column.

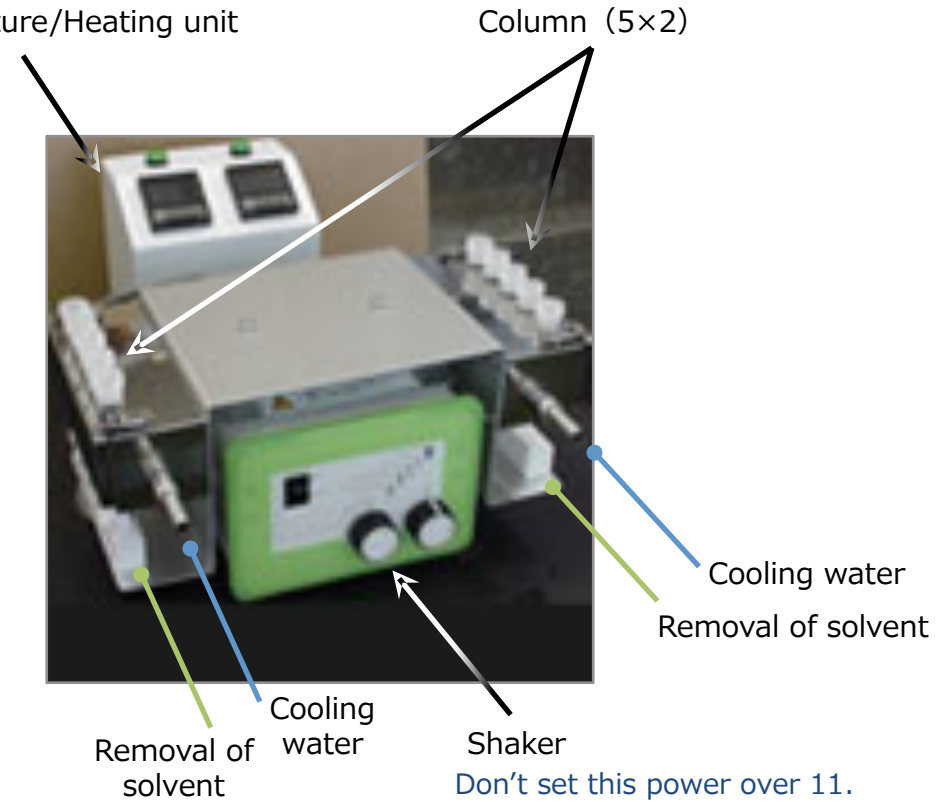
MS

Peptide solution and matrix solution are mixed with the ratio of 1:1, 1:10, 1:100. When it is crystalized after drying, it is easy to be ionized. If it cannot be dried, you should change the kind of the matrix or solvent (CHCA, DHBA, HPA etc).

Explanation of an equipment

Power button is in the back. You can control 2 independent temperature between left and right sides by using the green buttons. Please connect cords properly!

You should order special columns for HiPep directly. You can order columns, upper caps and bottom caps separately. Please refer to the catalog from HiPep when you want to know these information such as price and product number.



Don't set this power over 11.
It is dangerous!

It is connected to suction bottle/diaphragm pump.
When you turn a side finger grip vertically, you can suck only your target column.

Protocol

The maximum amount of the resin is up to about 150 mg. Ideal amount is 100 mg.
During the coupling reaction, the temperature should be 50℃. From the end of the reaction to the treatment with PPD, the temperature should be decreased to RT with cooling water (If the amount of the solvent is small, it may be meaningless to control the temperature).
In the cleavage step, column is stirred slowly with upper and bottom caps at 50℃. When the deprotection cocktail is added to column, it should not be cooled.

Maintenance

It is exposed to organic solvents.
After using, please wipe away the organic solvents and keep clean!
Especially, the shaker and the monitor on heating unit don't have resistance for organic solvents. Therefore please wipe away organic solvents as soon as possible if these parts are exposed to the solvents.
The plastic part, where column is inserted, for removal of the solvent is removable. If it gets to be dirty, please wash it in order to avoid choking.

After using, make it sure to turn off the vacuum pump and water flow.

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Peptido ~ The Road To Becoming A Peptide Master ~
4th edition

Released in December 2015

The purpose of this booklet is to hand down knowledge and technique about solid phase peptide synthesis to the next generation. If you synthesize peptides according to this book, you can obtain a lot of skills which people on the Peptido should have. However, still the road to becoming a peptide master should be hard and steep. We wish somebody with strong pioneering heart will find new secrets about Peptido and edit a greater book than this one.