EMMI Intensive Programme "Design, Synthesis and Validation of Imaging Probes"
Torino, 19-30 September 2011

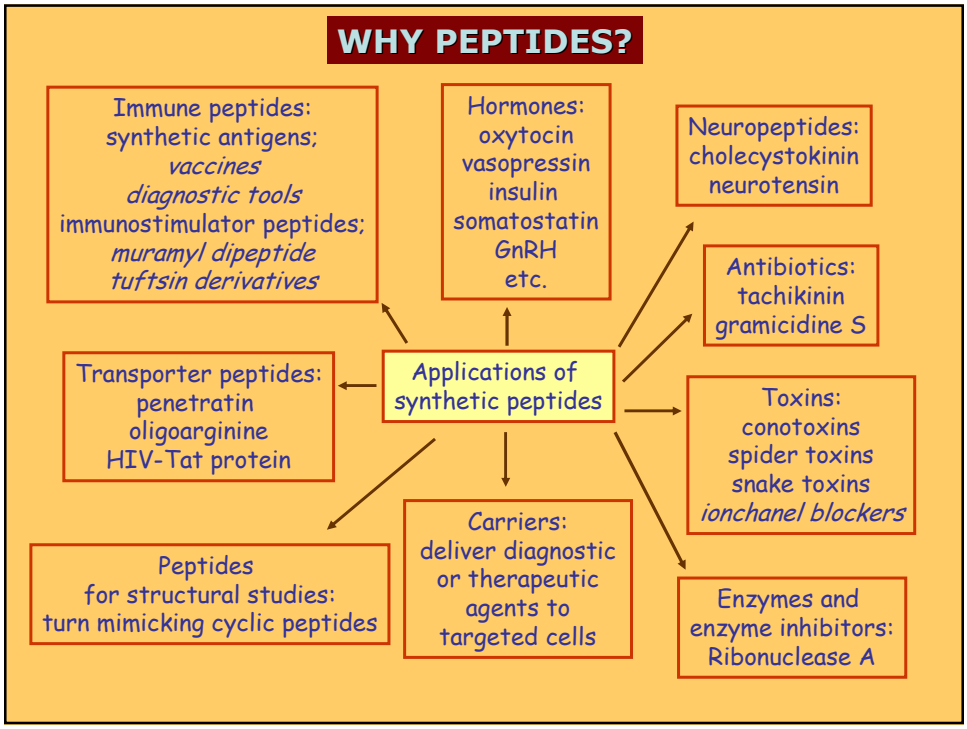
Basic principles and procedures of solid phase peptide synthesis

Lorenzo Tei, PhD

Dipartimento di Scienze dell'Ambiente e della Vita
Università del Piemonte Orientale "Amedeo Avogadro"
Viale T. Michel 11, 15121, Alessandria

Contents:

- Introduction
- Historical background
- Different strategies for SPPS
- Possible problems
- Aminoacids and protections
- Amide bond formation
- Solid supports and linkers
- Analytical tests
- Peptide isolation and purification



Advantages of utilizing peptides as therapeutic molecules:

- High activity
- High specificity
- Unique 3D characteristics
- No accumulation in organs
- Low toxicity
- Less immunogenic than antibodies

- They can be made synthetically, by recombinant methods or by chemical modification of an isolated natural product.

Peptides developed for therapeutic applications:

- Allergy/Asthma
- Arthritis
- Cancer
- Diabetes
- Cardiovascular diseases
- Inflammation
- Vaccines
- Ophtalmology
- Central Nervous System diseases
- Antiviral
- Antibacterial

Peptide synthesis:

Solution synthesis:

- Optimisation of reaction conditions and yields.
- Purification of every step.
- Time consuming.
- Synthesis of small peptides.
- Useful for large-scale manufacturing.

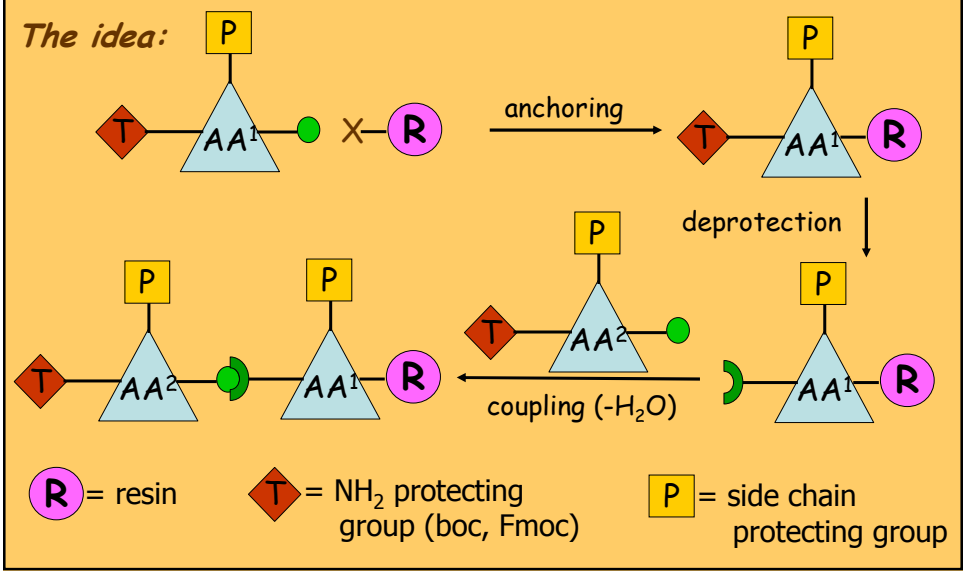
Solid phase synthesis:

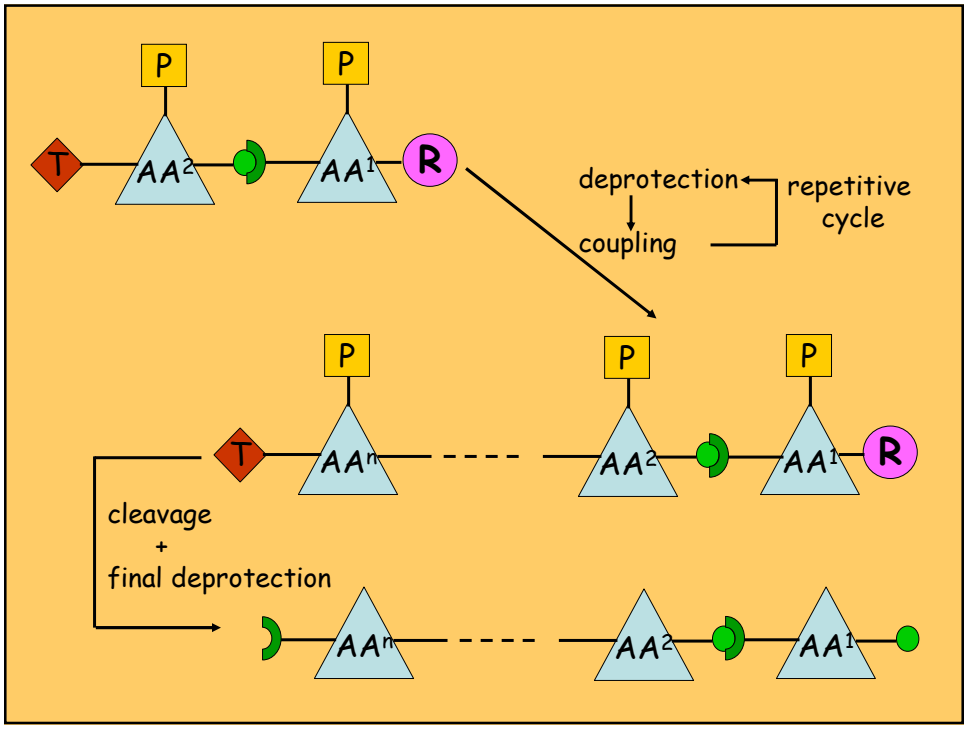
- Anchoring of the peptide chain on a resin.
- Use of excess reagents removed by filtration and washing procedures.
- Simple, rapid and repetitive steps.
- High yields.
- Possible automation
- Synthesis of long peptides.

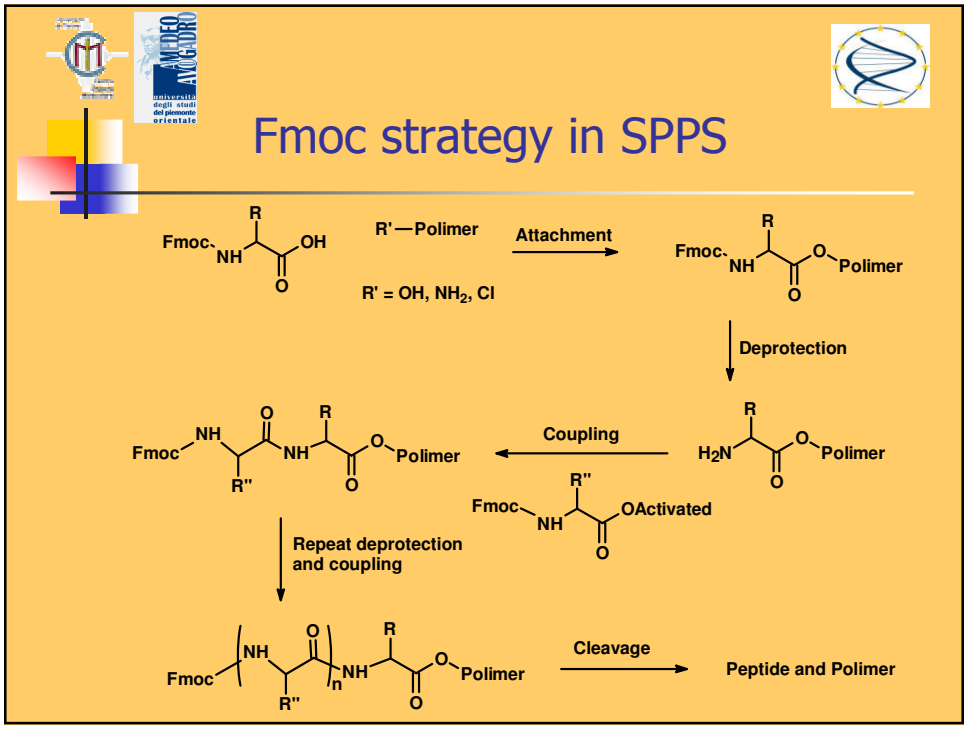
Beginnings of the SPPS

- Merrifield in 1963 introduced the use of a polystyrenic support for the synthesis of peptides.
- Development of the Boc strategy (use of quite strong acidic conditions)
- In the '70 different polymeric supports and more labile protecting groups were developed.
- In 1978 the Fmoc-based SPPS was introduced.
- In the last 15 years the Fmoc approach has prevailed over the Merrifield SPPS.

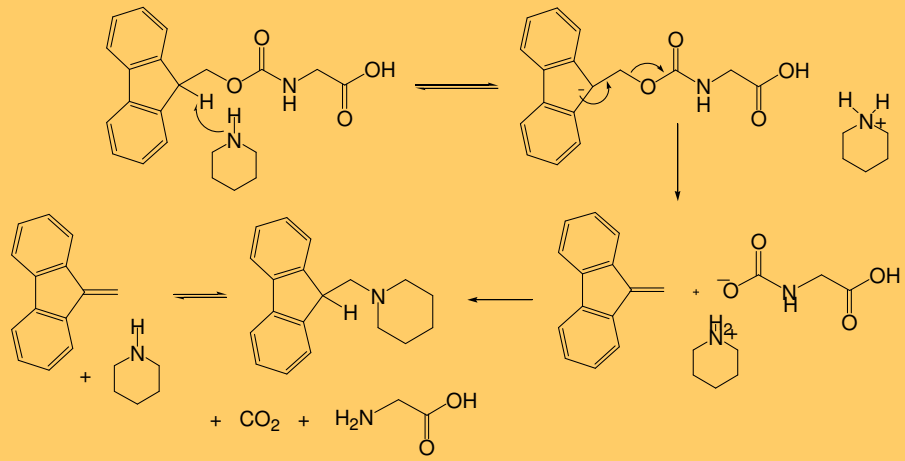
Solid phase peptide synthesis



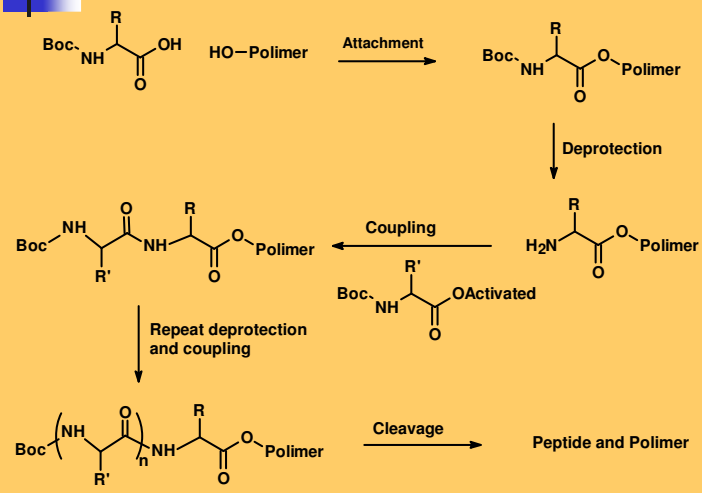

P
P
T
AA2
AA1
R
deprotection
coupling
repetitive
cycle
P
P
P
T
AAn
AA2
AA1
R
cleavage
+
final deprotection
AAn
AA2
AA1


Fmoc strategy in SPPS
Fmoc
NH
R
OH
O
R'—Polimer
R' = OH, NH2, Cl
Attachment
Fmoc
NH
R
O
Polimer
O
Deprotection
H2N
R
O
Polimer
O
Coupling
Fmoc
NH
R"
OActivated
O
Fmoc
NH
O
R
NH
O
Polimer
R"
O
Repeat deprotection
and coupling
Fmoc
NH
O
R
NH
n
O
Polimer
R"
O
Cleavage
Peptide and Polimer

Fmoc deprotection

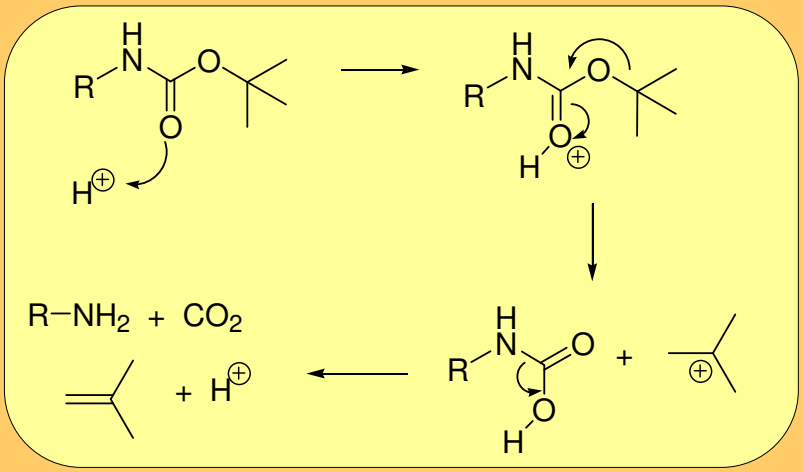


Boc Strategy in SPPS



The Boc approach needs to use highly toxic liquid HF for cleavage from the resin.

Boc deprotection

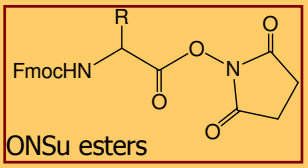


Common AA side-chain protecting groups used in Fmoc SPPS

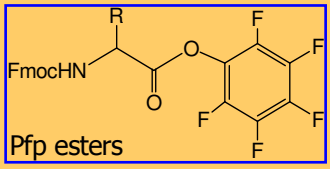
Side-chain fuctionality of amino acids	Commonly used protecting group	Abbreviation
Arg		Pbf
Asp, Glu, Ser, Thr, Tyr		tBu
Lys, Trp		Boc
Asn, Gln, Cys, His		trt

Coupling methods in Fmoc SPPS
Pre-activation method:

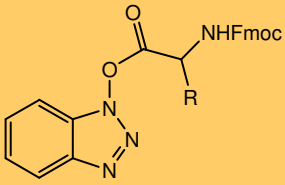
- Pre-activation of the acid via activated esters.
- N-hydroxysuccinimide (ONSu); slow formation of the amide bond.
- Pentafluorophenyl (OPfp) ester; in the presence of HOBt the rate of reaction is very rapid.



ONSu esters



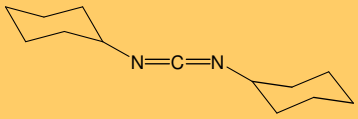
Pfp esters



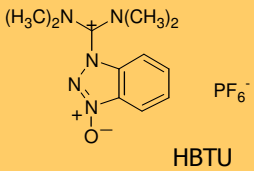
HOBt esters

Coupling methods in Fmoc SPPS
In situ - activation method:

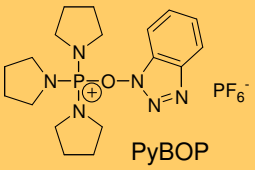
- Carbodiimides (DCC and DIC)
- Benzotriazole PF_6 salts (HBTU, PyBOP, HATU)



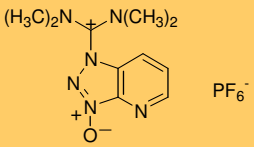
Dicyclohexylcarbodimide (DCC)



HBTU

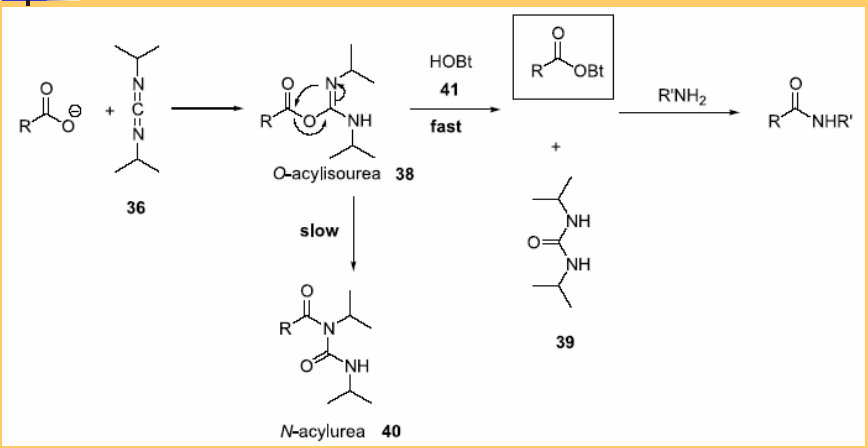


PyBOP



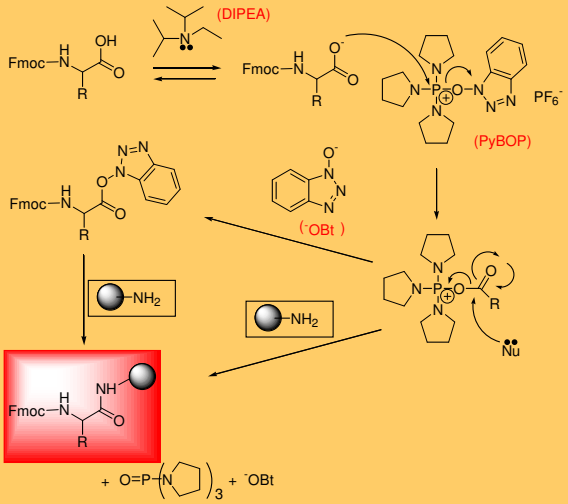
HATU

Coupling using Diisopropylcarbodiimide



The use of HOBt minimises the formation of unreactive *N*-acylurea

Mechanism of amide bond formation using PyBOP/HOBt/DIPEA

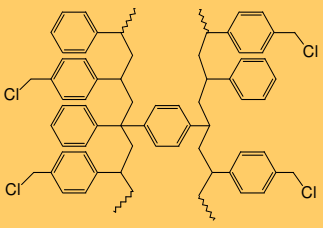


Solid supports:

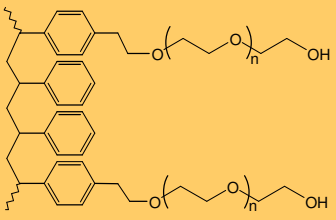
- Dry polystyrene beads have an average diameter of 50 μm, but in DMF or DCM they swell 2.5 to 6.2 fold in volume.
- SPPS takes place within a well solvated gel containing mobile and reagent accessible peptide chains.
- Small particle sized resins with low cross linking allows rapid diffusion of reagents inside the beads.
- Solid support and peptide backbone may be of comparable polarities (polyamides, PEG).

Resins for SPPS

Resin	Composition	Functional group
Chloro/Aminomethyl polystyrene	1% divinylbenzene cross-linked polystyrene	CH_2Cl, CH_2NH_2, benzhydrylamino
Tentagel	Polystyrene-polyethylene glycol graft polymer	CH_2OH, CH_2NH_2



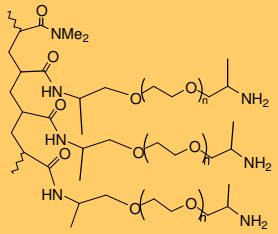
Merrifield



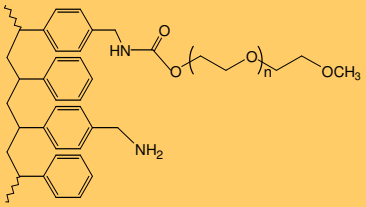
Tentagel

Resins for SPPS

PEGA	Bis 2-acrylamidoprop-1-ylPEG, 2-acrylamidoprop-1-yl[2-aminoprop-1-yl]PEG dimethylacrylamide co-polymer	CH_2NH_2
Novagel™	(4'-O-methylpolyethylene glycoxycarbonylamino-methyl)polystyrene	CH_2NH_2



PEGA



Novagel™

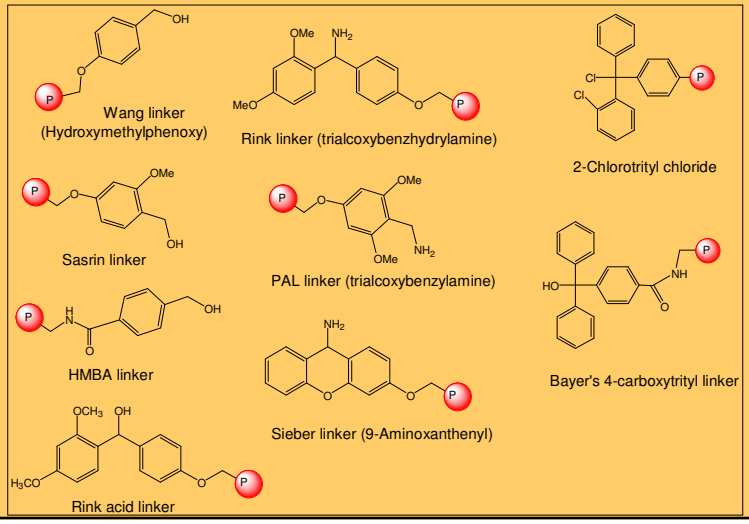
Linkers:

The purpose of the linker are:

- provide a reversible linkage between the synthetic peptide chain and the solid support.
- Protect the C-terminal α-carboxyl group during the process of chain extension.
- Most linkers release peptide acids or amides upon treatment with TFA.
- Some linkers permit the cleavage with nucleophiles, used for the preparation of C-terminal modified peptides such as esters secondary amides, aldehydes, alcohol.

Linkers:

Hydroxymethyl-based linkers	Aminomethyl-based linkers	Trityl chloride-based linkers



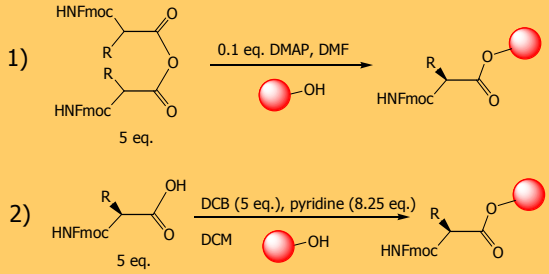
Resin handling procedures

- Apart from PEGA resin, all resins have to be swollen before use.
- Underivatized polystyrene resins swell only in dichloromethane.
- All the other are swollen in DMF.
- Resins (except PEGA) can be dried under vacuum without damage.
- Resins are fragile and has to be handled carefully (do not use magnetic stirrers!)

Attachment of 1st amino acid

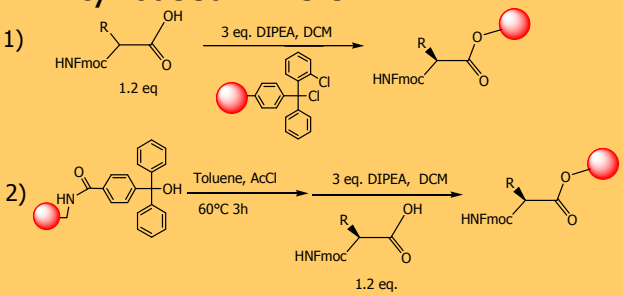
For hydroxymethyl based linkers quite harsh conditions has to be employed:

1. use of the symmetrical anhydride of the Fmoc amino acid and DMAP in DMF.
2. Use of the Fmoc amino acid and dichlorobenzoyl chloride in DMF/pyridine



Attachment of 1st amino acid

Trityl based linkers:



Aminomethyl-based linkers:

- Generally the amino function is Fmoc protected
- Standard piperidine deprotection and subsequent coupling of the 1st amino acid

Synthesis protocol:

- Swelling of the resin
- 1st Fmoc amino acid attachment
- Deprotection Fmoc group with piperidine in DMF
- Washings with DMF (between 5 to 10 times)
- Fmoc amino acid coupling (Activators/DIPEA/DMF)
- Washings with DMF
- Deprotection Fmoc and so on…
- After last coupling the resin has to be dried *in vacuo* before cleavage

Analytical tests

Qualitative amine tests:

- Kaiser test (ninhydrin test)
  http://www.natureprotocols.com/2007/10/24/monitoring_solid_phase_peptide.php
- TNBS test
- Chloranil test
  http://www.chempep.com/ChemPep-Fmoc-Solid-Phase-Peptide-Synthesis.htm

Fmoc determination for estimation of first residue attachment:

- The loading of the 1st AA can be calculated determining the absorbance at 290 nm (dibenzofulvene formed in the Fmoc deprotection)

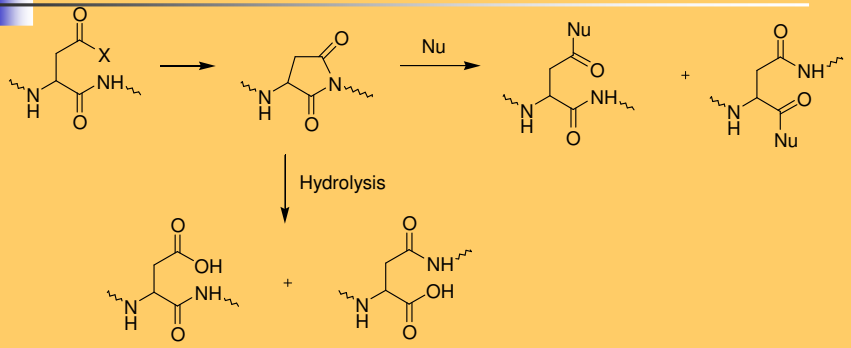
Possible problems

Aggregation
Peptide chains on resin can form secondary structures or aggregates either with other peptide chains or with the polymer support. Mainly caused by H-bonding and hydrophobic interactions (high proportion of Ala, Ile, Asn or Gln)

Enantiomerization
During coupling the acidity of the H atom on the α-carbon is enhanced by the carboxy group activation.
May occur on attachment of Cys, His, Pro, Met, or Trp on hydroxymethyl-based resins

Side reactions
Rare if the AA are protected and the correct procedures are followed. Aspartimide formation can occur if Asp is coupled to Gly, Ser, Asn, Thr or Arg.

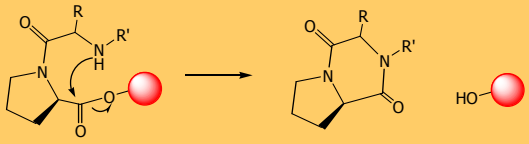
Aspartimide formation



Base catalyzed aspartimide formation involves attack by the nitrogen atom attached to the α-carboxy group of either aspartic acid or asparagine on the side chain ester or amide group, respectively.
Nucleophilic attack then causes subsequent ring opening, which gives rise to a mixture of α-aspartyl and β-aspartyl peptides.

Diketopiperazine formation

- Peptides containing proline or N-alkylated amino acids attached via benzyl ester to the resin may have the problem of diketopiperazine formation
- This not only causes a yield reduction but may also lead to the generation of truncated sequences.
- In these cases is better to use a more hindered trityl resin



Cleavage from the resin

- 95% TFA for 1-3h cleaves the peptide from the resin and deprotect t-butyl groups, Pbf group from Arg, Trt group from Cys, Gln, Asn and His.
- Highly reactive cationic species are generated that can react with amino acids containing electron rich functional groups: Tyr, Trp, Met and Cys.
- Nucleophilic reagents (scavengers) are added to the TFA to quench these ions: water, 1,2-Ethandithiol (EDT), triisopropylsilane (TIS), thioanisole.

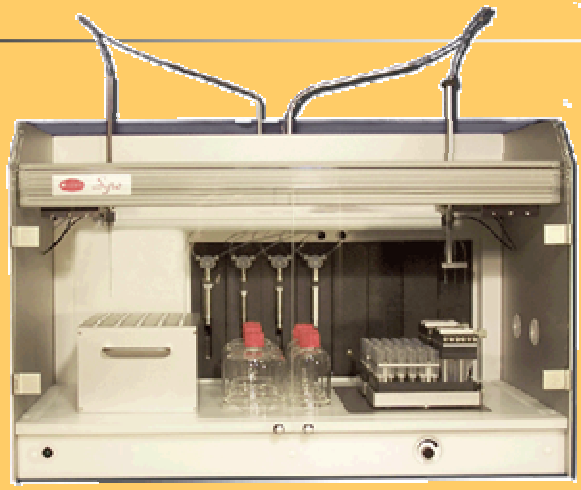
Cleavage cocktails

- If Cys is absent we normally use TFA, TIS 95:5 *v/v.*
- With Cys we add EDT (TFA, TIS, EDT; 95:3:2 *v/v*).
- With Met we add thioanisole (TFA, TIS, Thioanisole; 95:3:2 *v/v*)
- Reagent K (TFA, thioanisole, H_2O, phenol, EDT; 82.5:5:5:5:2.5 *v/v*).
- Reagent R (TFA, thioanisole, anisole, EDT; 90:5:3:2).
- With Chlorotrityl chloride resins protections on the AA side-chains can be mantained using DCM, AcOH, trifluoroethanol 80:10:10 *v/v.*
- With Trp a further step with a 5% water solution of AcOH is required to obtain the fully deprotected peptide.

Peptide isolation and purification

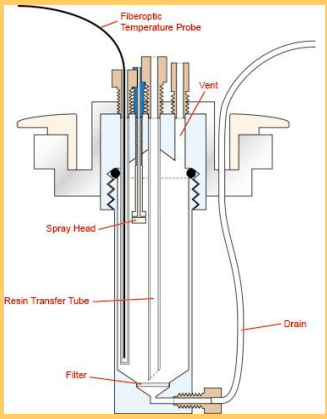
- Precipitated by addition of cold diethyl ether
- Directly from TFA solution or following evaporation of TFA and volatile scavengers.
- Centrifuge and wash with Et_2O 3 times
- Dry the solid in vacuo
- Purification by HPLC
- Characterisation by MALDI-TOF mass spectrum, NMR spectrometry.

Multisyntech SYRO II



Syro II automated peptide synthesizer

CEM Liberty



Liberty automated peptide synthesizer shown with single-mode microwave reaction vessel