Peptide chemistry in different environment (industrial and continuous flow synthezes)

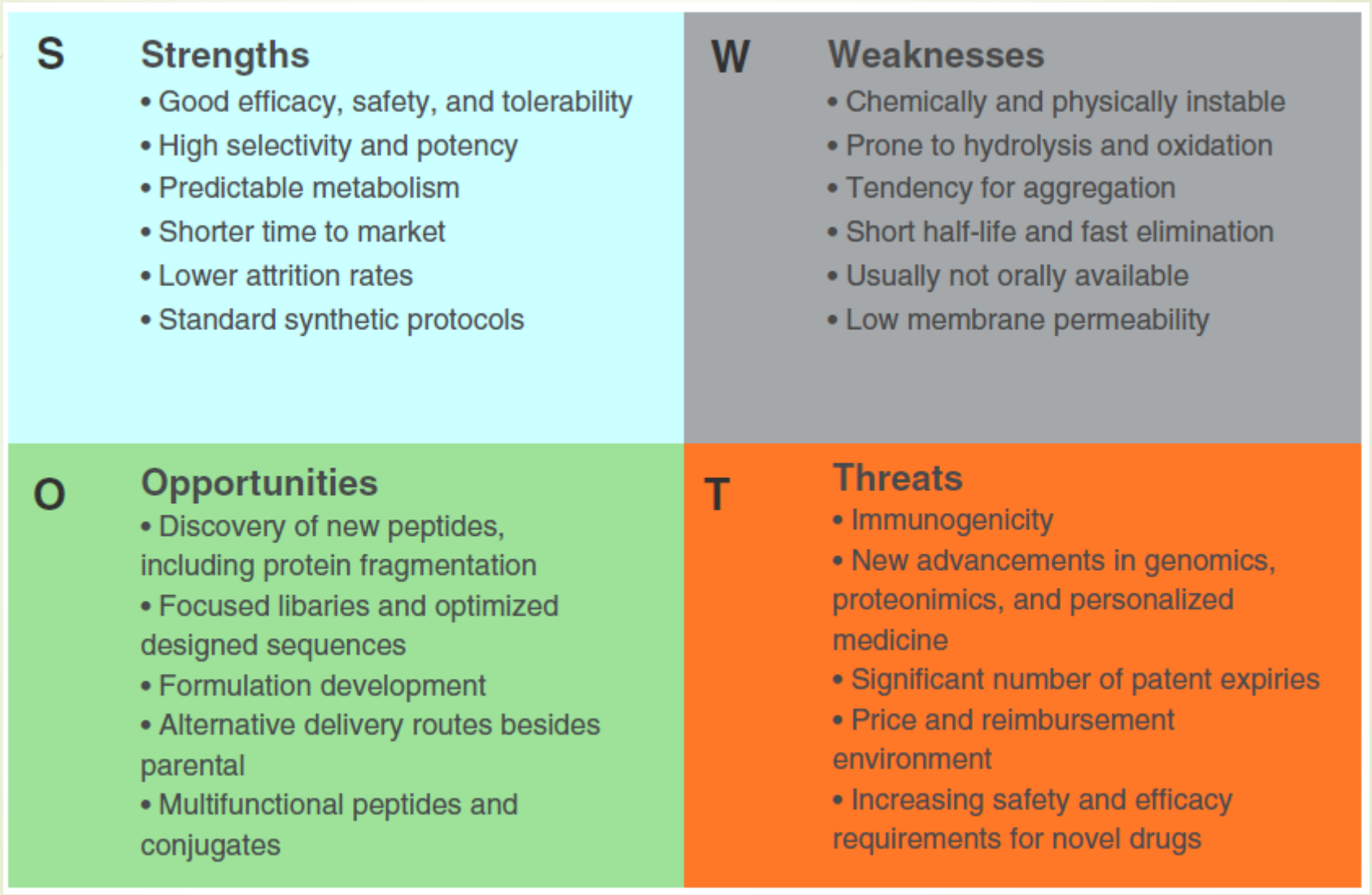
László Kocsis

Robert Bosch Kft.

Outline

- Peptides in the Pharmaceutical industry
- Examples of industrial peptide synthesis
- Quality assurance
- Flow chemistry introduction
- Continuous flow peptide synthesis – the beginning
- Continuous flow peptide synthesis – the current trends

Peptides in the pharma (SWOT)



S — Strengths
- Good efficacy, safety, and tolerability
- High selectivity and potency
- Predictable metabolism
- Shorter time to market
- Lower attrition rates
- Standard synthetic protocols

W — Weaknesses
- Chemically and physically instable
- Prone to hydrolysis and oxidation
- Tendency for aggregation
- Short half-life and fast elimination
- Usually not orally available
- Low membrane permeability

O — Opportunities
- Discovery of new peptides, including protein fragmentation
- Focused libaries and optimized designed sequences
- Formulation development
- Alternative delivery routes besides parental
- Multifunctional peptides and conjugates

T — Threats
- Immunogenicity
- New advancements in genomics, proteonimics, and personalized medicine
- Significant number of patent expiries
- Price and reimbursement environment
- Increasing safety and efficacy requirements for novel drugs

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Peptides in the pharma

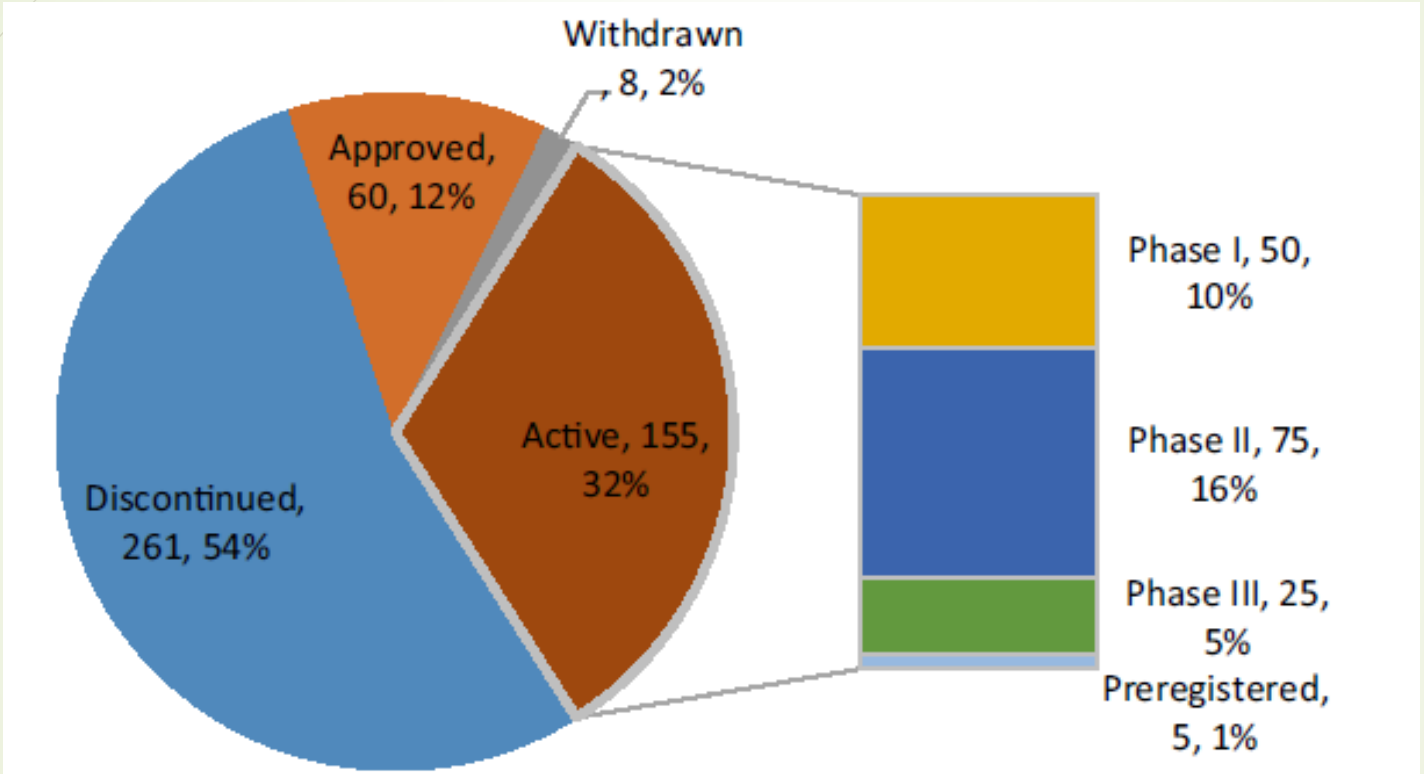


Fig. 1. Current development status of therapeutic peptides. Numbers refer to the number and percentage of all peptides in the given category. "Withdrawn" refers to previously approved products no longer on the market; "Discontinued" refers to programs terminated prior to approval, and the "Active" category encompasses all peptides in active clinical development.

Lau J.L., Dunn M.K. Bioorg. Med. Chem. (2017), http://dx.doi.org/10.1016/j.bmc.2017.06.052

Peptides in the pharma (probability of approval)

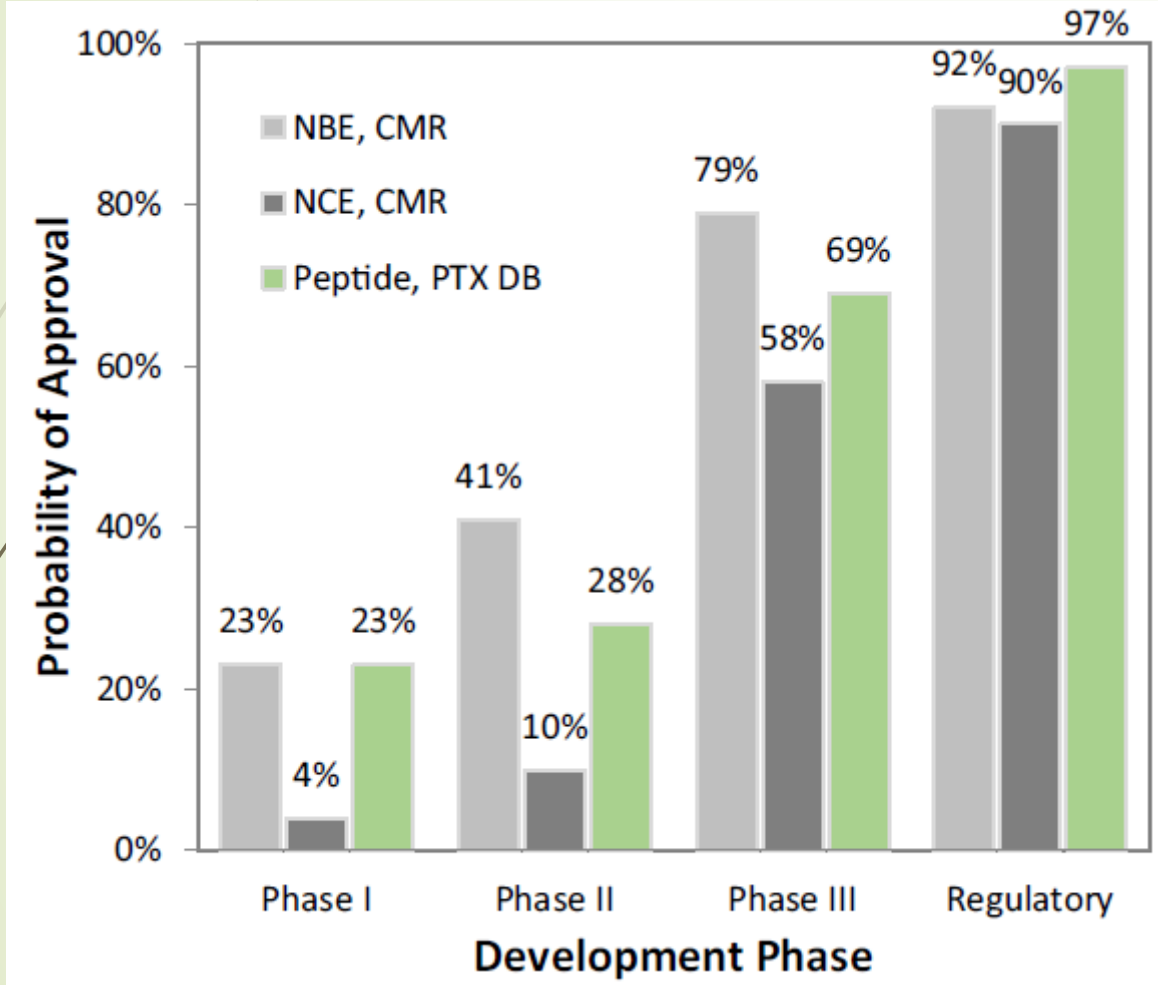


Fig. 9. Probability of success for agents at various stages of clinical development. NBE: new biological entity; NCE: new chemical entity; "Regulatory" refers to the approval rate of marketing applications by regulatory bodies (i.e. FDA, EMA). Success rates for NBE and NCE drugs are taken from the 2015 CMR International Pharmaceutical R&D Factbook, an annual report on industry trends. Peptide success rates are taken from the authors' peptide therapeutics database (PTX DB). See the Supplementary Information for a comparison of these methodologies, which differ slightly in their classifications of clinical development phases and their timeframes for data inclusion.

Lau J.L., Dunn M.K. Bioorg. Med. Chem. (2017), http://dx.doi.org/10.1016/j.bmc.2017.06.052

Peptides in the pharma (Length)

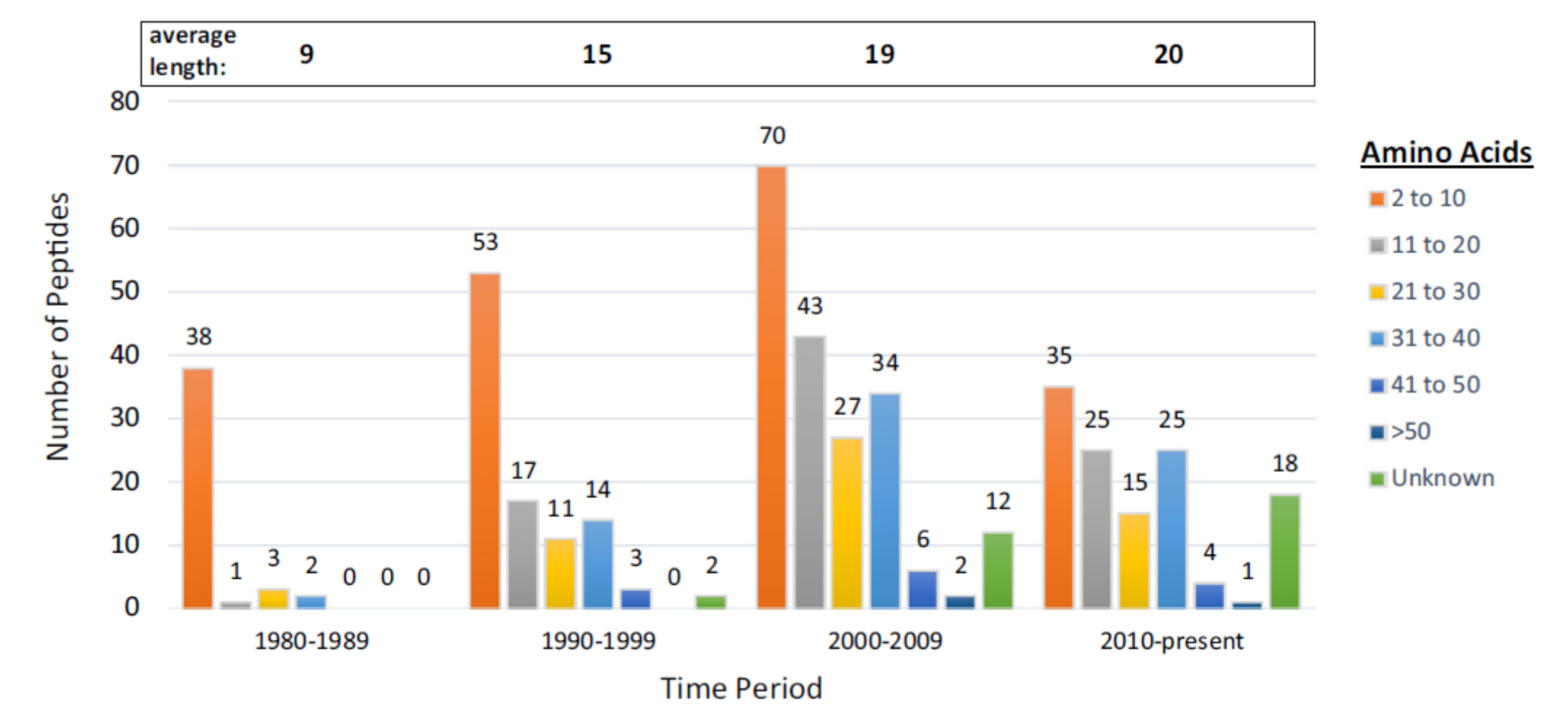


Fig. 3. Length of peptides entering clinical development, by decade. Peptides with unknown length were not included in the average length calculation.

Lau J.L., Dunn M.K. Bioorg. Med. Chem. (2017), http://dx.doi.org/10.1016/j.bmc.2017.06.052

Peptides in the pharma (chemical basis)

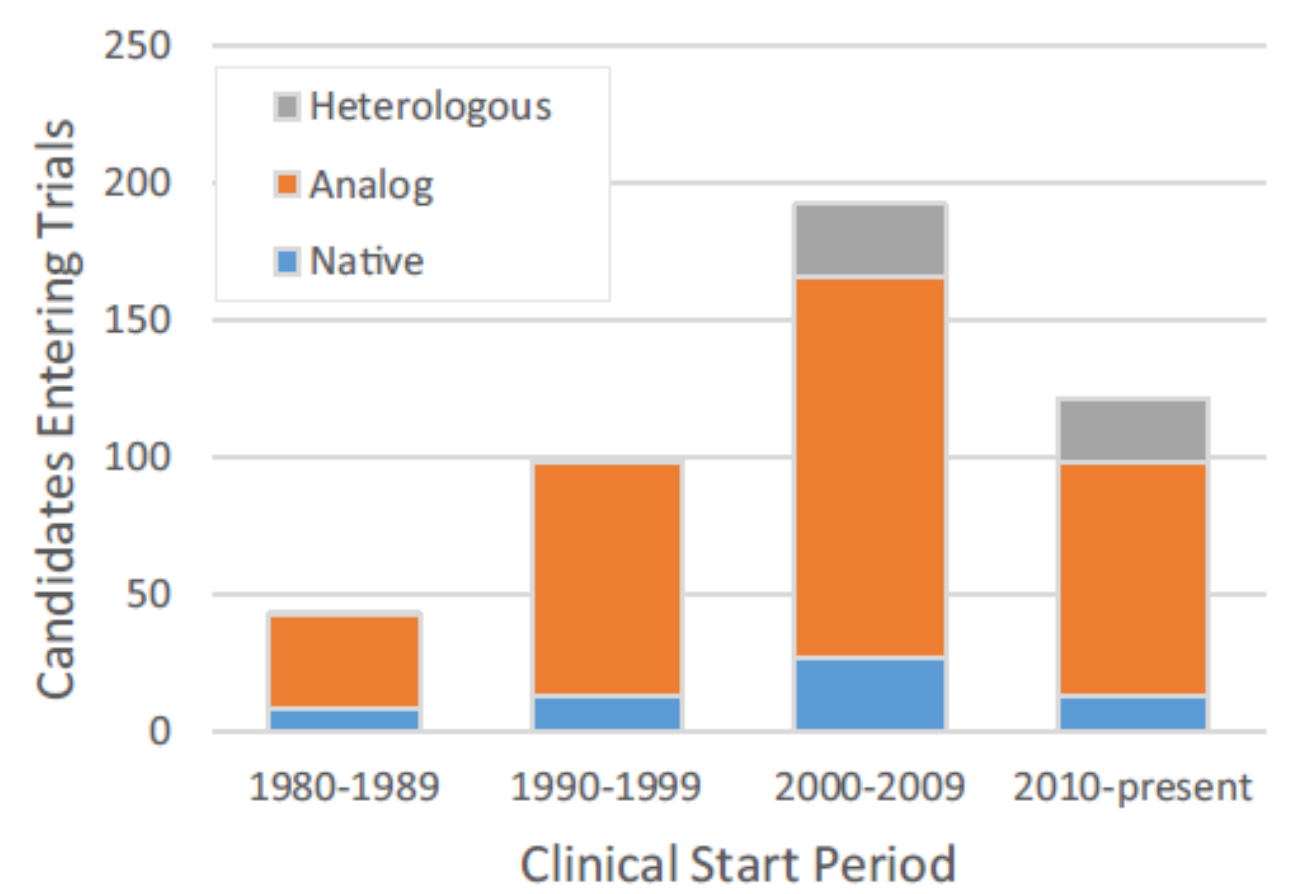
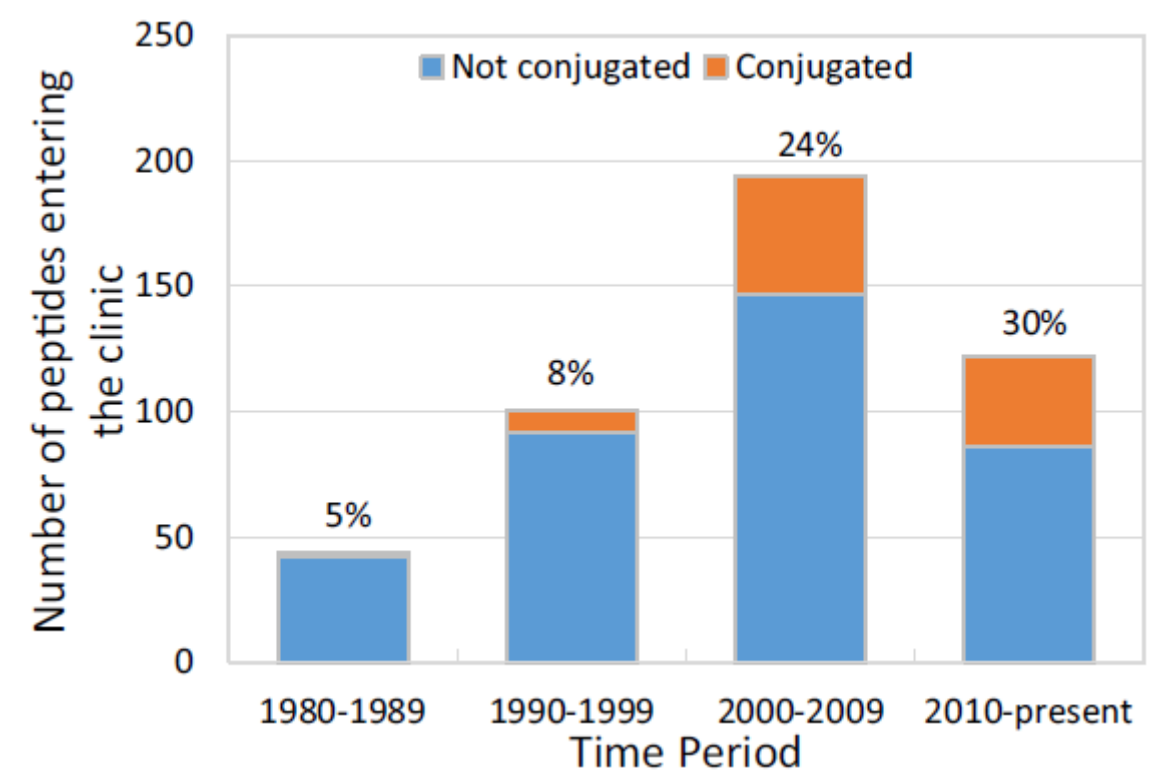


Fig. 4. Chemical basis of peptides entering clinical development.

Lau J.L., Dunn M.K. Bioorg. Med. Chem. (2017), http://dx.doi.org/10.1016/j.bmc.2017.06.052

Peptides in the pharma (conjugates)



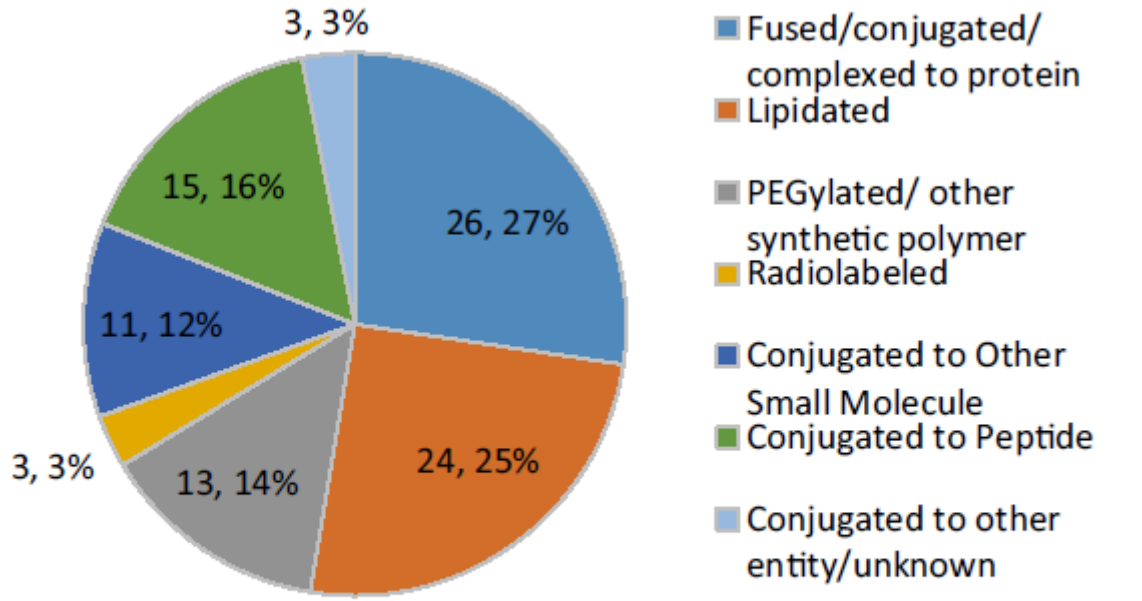


Fig. 5. Number and percentage of conjugated peptides entering clinical development (top) and distribution of all conjugated moieties in the peptide database (bottom).

Lau J.L., Dunn M.K. Bioorg. Med. Chem. (2017), http://dx.doi.org/10.1016/j.bmc.2017.06.052

Peptides in the pharma (therapeutic areas)

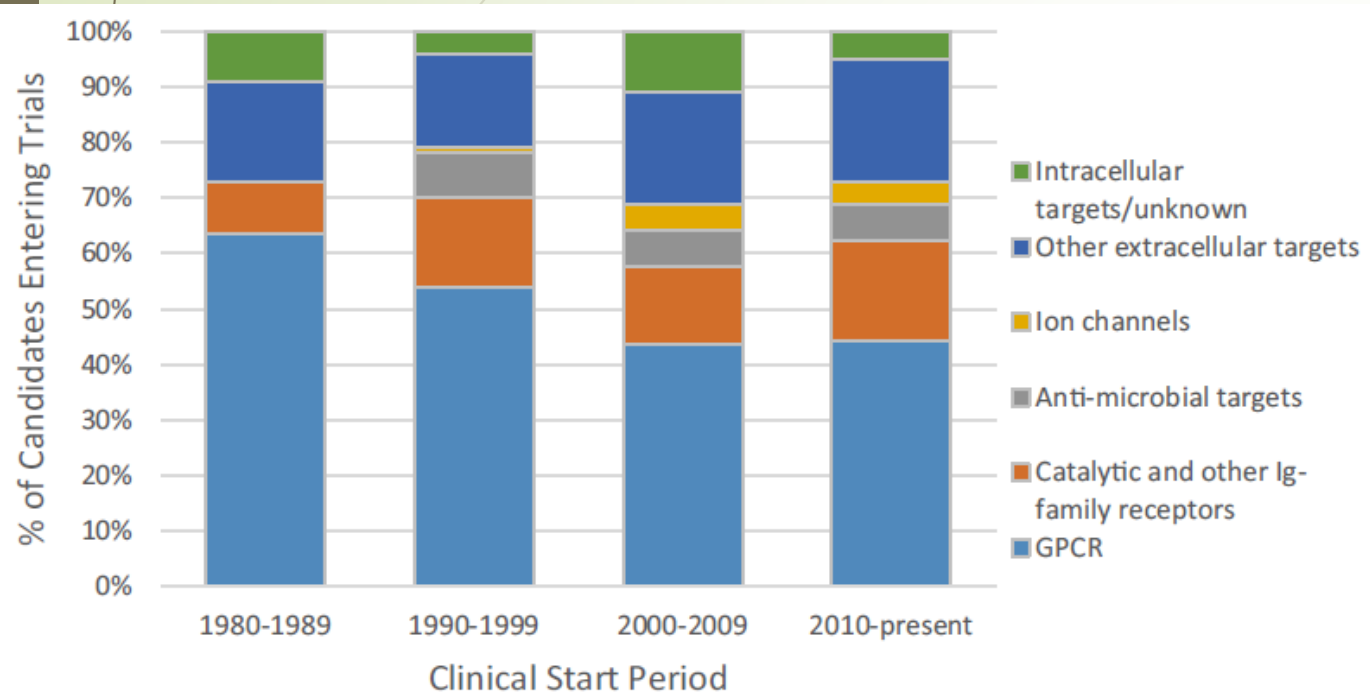


Fig. 6. Molecular targets of peptides entering clinical development.

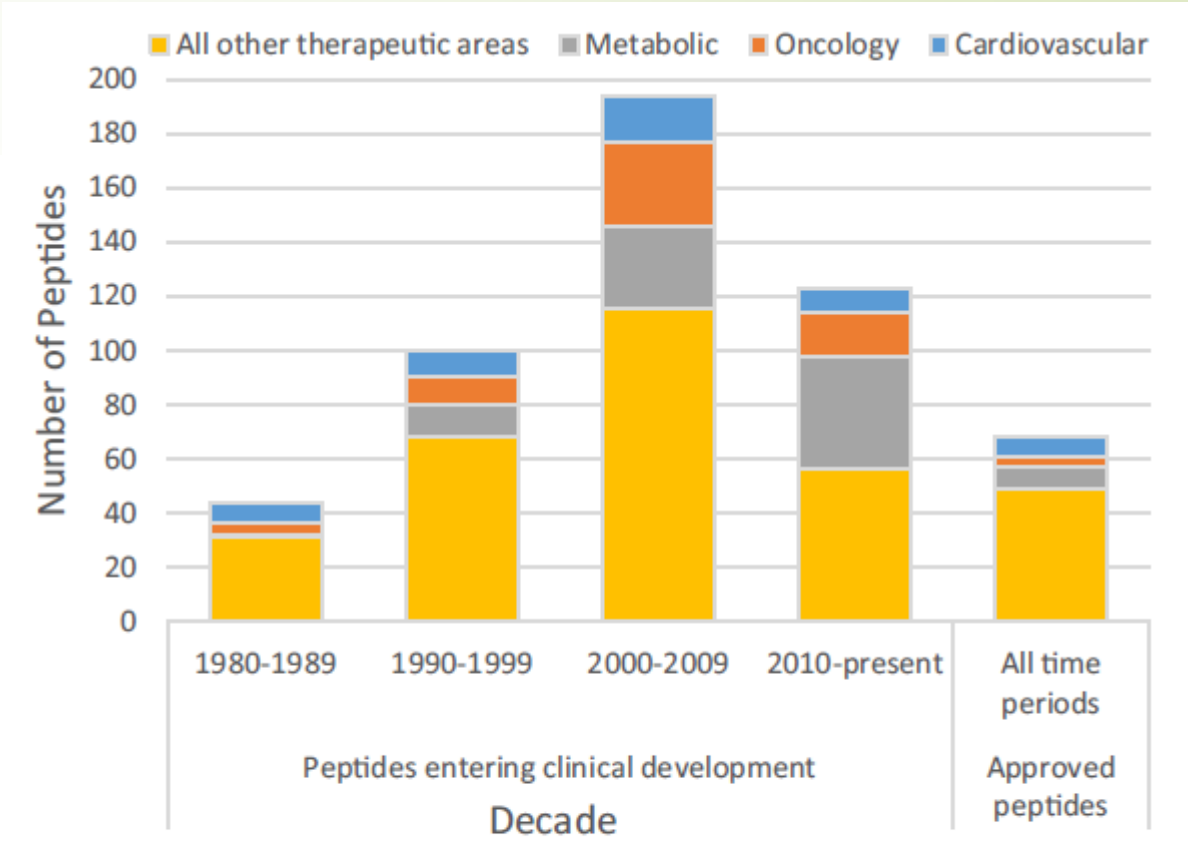


Fig. 7. Primary therapeutic area for peptides by time period of clinical initiation, compared with the primary therapeutic area for approved peptides across all time periods (including pre-1980). This figure highlights peptides in the three most popular therapeutic areas (at present). See the Supplementary Information for therapeutic area definitions.

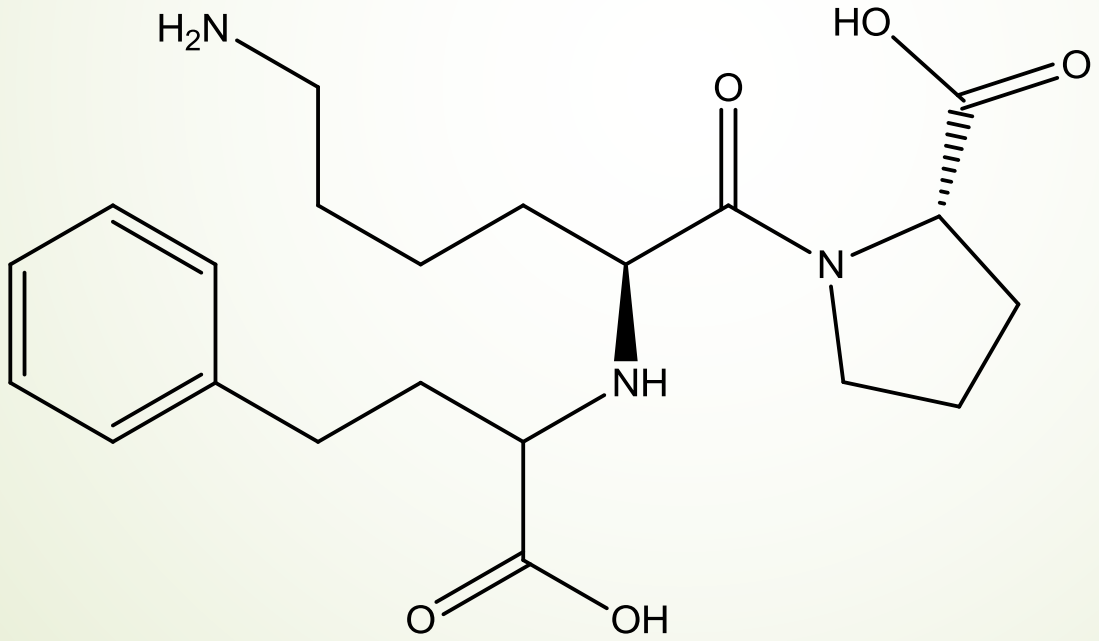
Lau J.L., Dunn M.K. Bioorg. Med. Chem. (2017), http://dx.doi.org/10.1016/j.bmc.2017.06.052

Synthetic possibilities

- Solution phase synthesis
- Solution phase polymerization
- Solution phase synthesis + fragment condensation
- Solid phase synthesis
- Solid phase synthesis + fragment condensation
- Solid phase synthesis + ligation
- Biotechnological synthesis

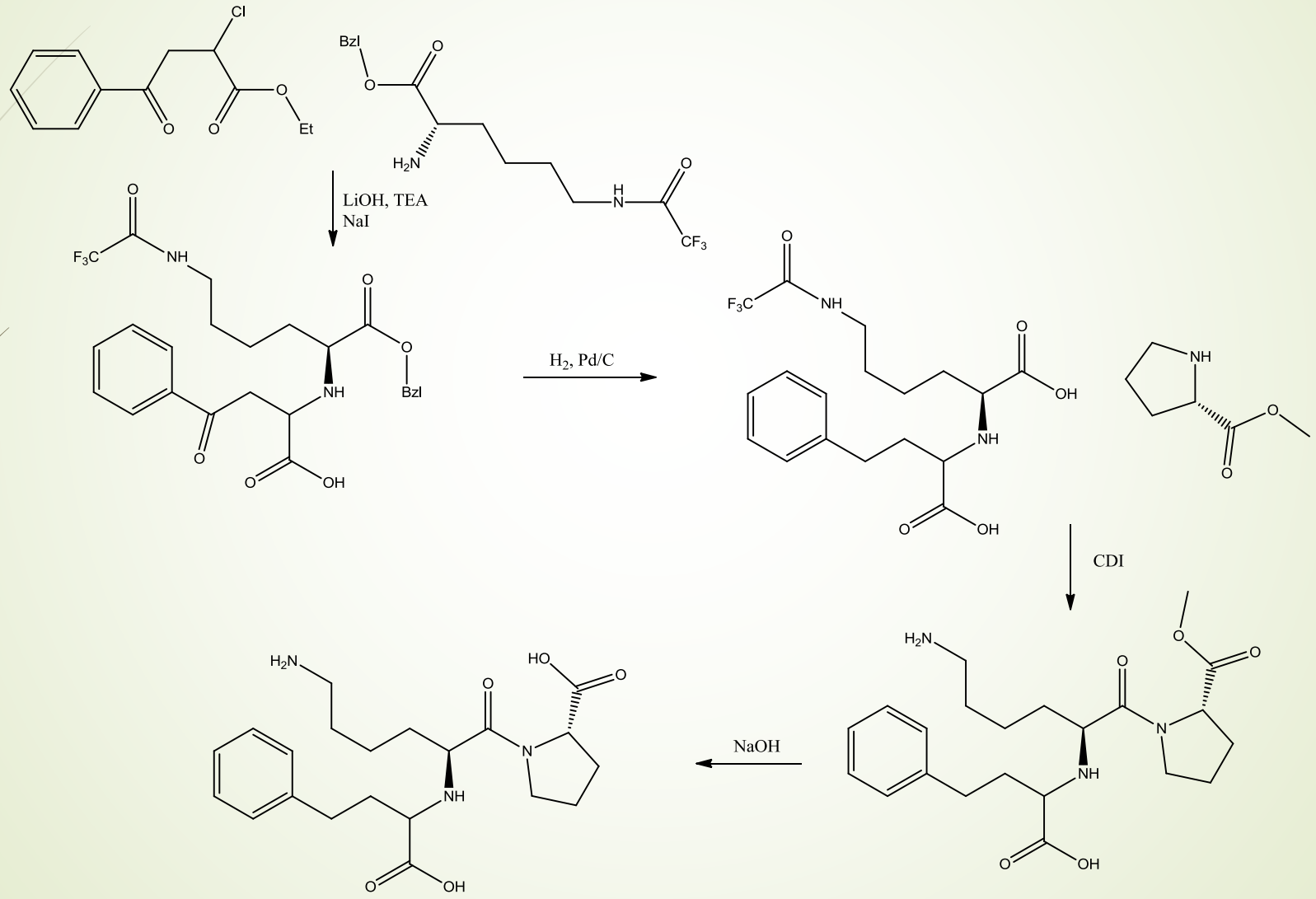
Solution phase synthesis

- Lisinopril (Lisopress) – angiotensin-converting enzyme (ACE) inhibitor class used primarily in treatment of high blood pressure, heart failure, and after heart attacks. It is also used for preventing kidney and eye complications in people with diabetes.

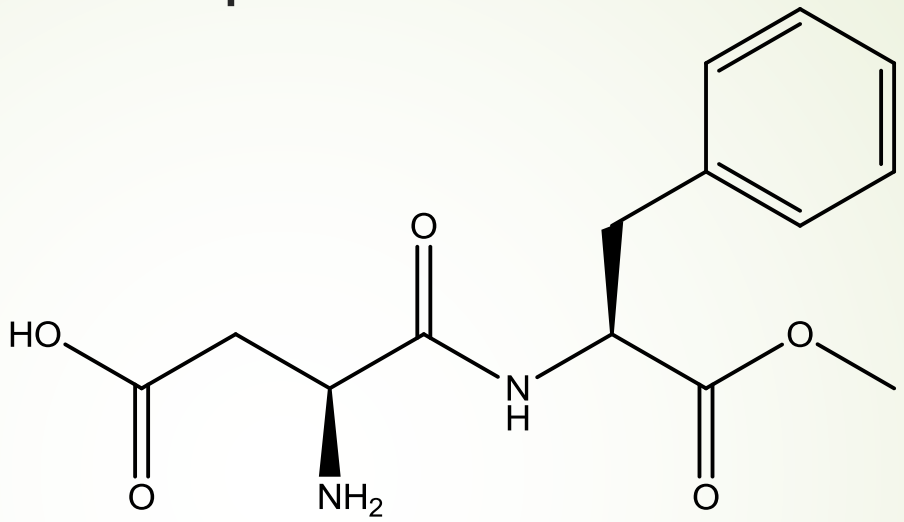


N^2-[(1S)-1-karboxi-3-fenilpropil-L-lizil-L-prolin

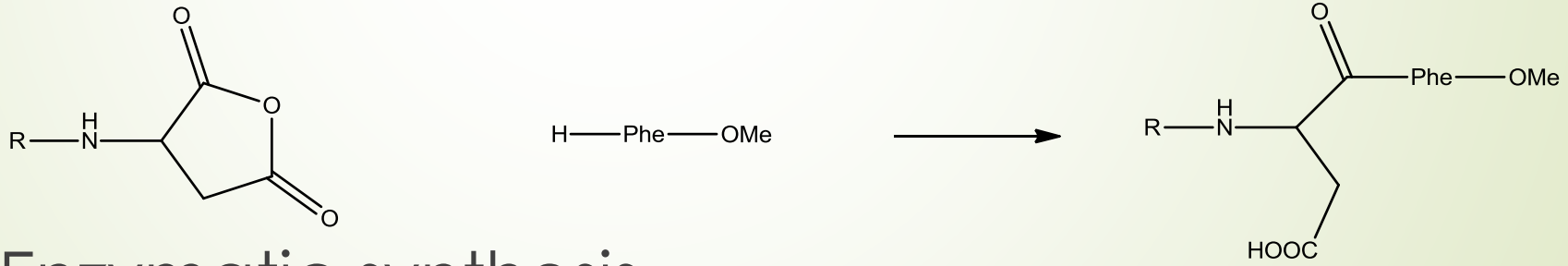
Synthesis of Lisinopril



Synthesis of aspartame



- Chemical synthesis



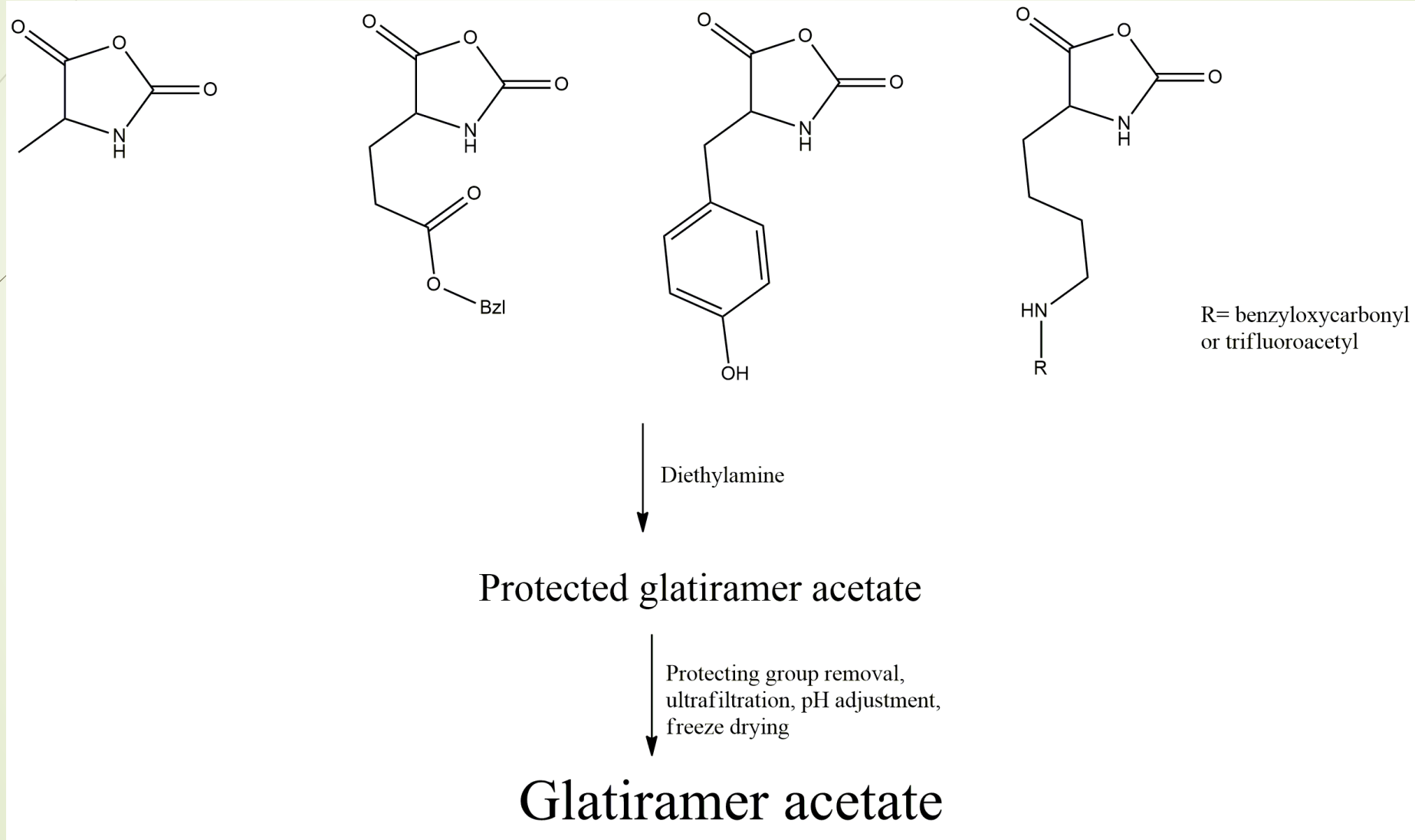
- Enzymatic synthesis
  - Bacillus thermoproteolyticus

Solution phase polymerization

- Glatiramer acetate (Copolymer-1; Copaxone)

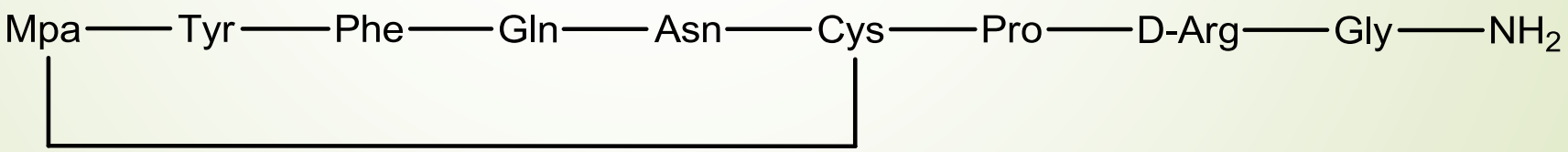
- It is an immunomodulator medication currently used to treat multiple sclerosis (reducing the number of the sudden deriorating sections)
- Subcutaneous injection
- Linear polymer of L-glutamic acid, L-alanine, L-lysine and L-tyrozine – the amino acids of myelin
  - Myelin basic protein is the antigen in the myelin sheaths of the neurons that stimulates an autoimmune reaction in people with MS, so the peptide may work as a decoy for the attacking immune cells.
- Average molecular weight: 6.4 kDa (4.7 – 11 kDa)

Synthesis of Glatiramer acetate

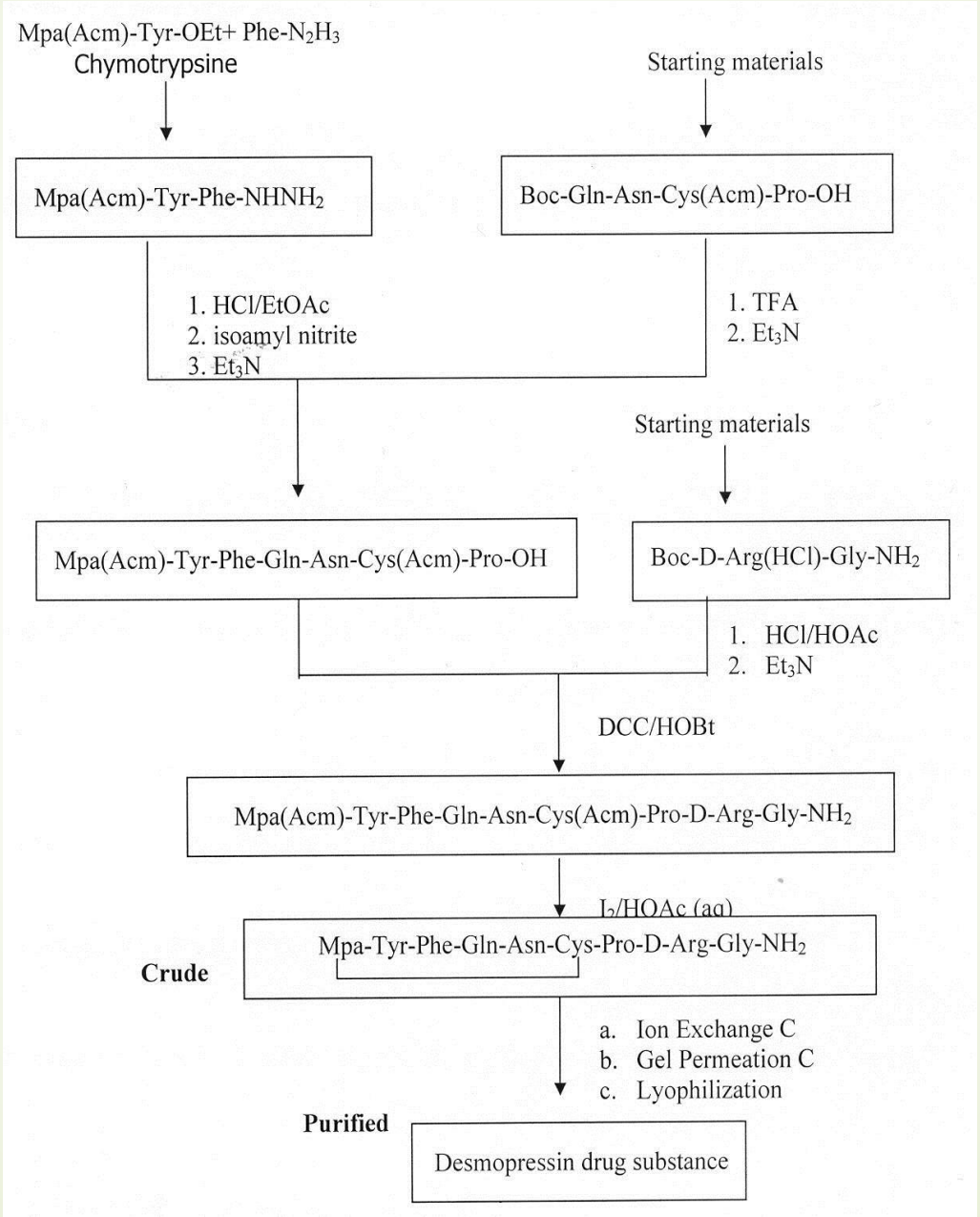


Solution phase synthesis + fragment condensation

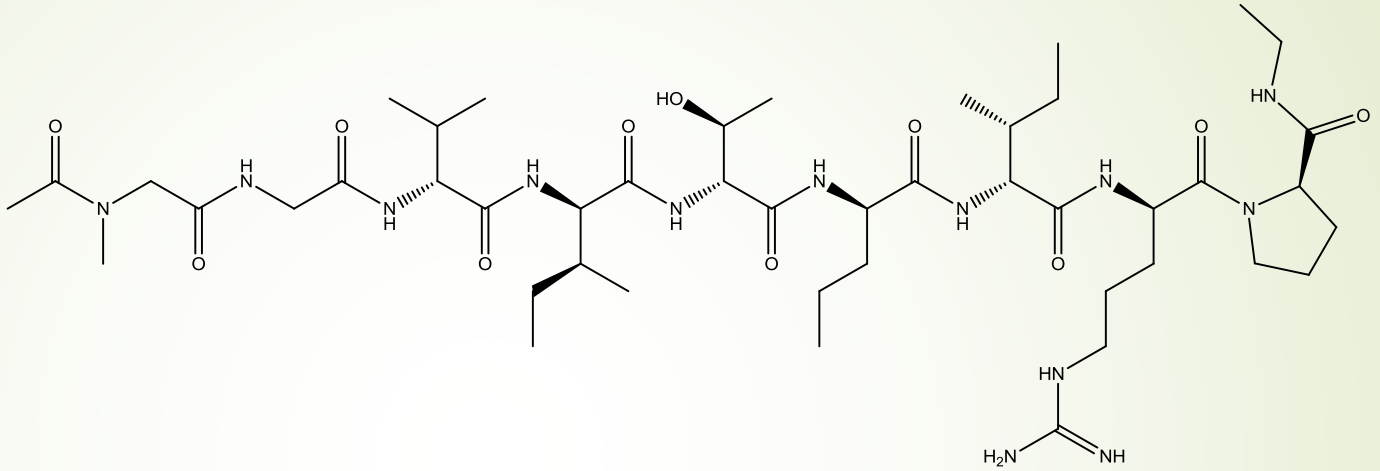
- Desmopressin (1-deamino-8-D-Arginyl vasopressin)
- Used in: diabetes insipidus, bedwetting, hemophilia A, von Willebrand disease, and high blood urea levels
- Was approved for medical use in the United States in 1978
- Typical monthly supply costs 100 – 200 USD
- Yearly production volume 10 – 100 kg



Synthesis of Desmopressin



Optimization of the synthesis of ABT-510



Ac—Sar–Gly–Val—D-allo-Ile–Thr—Nva—Ile—Arg—Pro—NHEt

- Synthetic possibilities
  - Stepwise solid phase synthesis
  - Stepwise solution phase synthesis
  - Solution phase fragment condenzation – 5+4
  - Solution phase fragment condenzation – (2+4)+3

SPPS of ABT-510

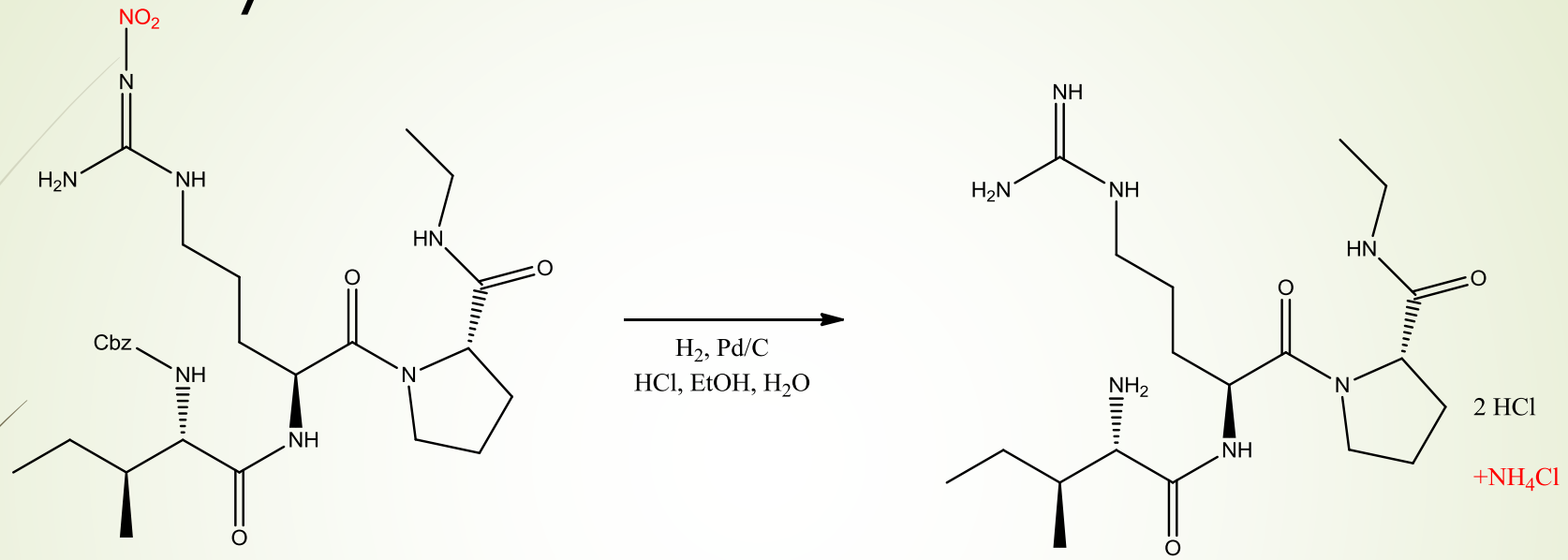
- Fmoc/tBu-strategy
- Purification by RP-HPLC
- Ion exchange chromatography
- Freeze-drying (Lyophilization)

It is not economical in a yearly ~500 kg production volume

Solution phase synthesis
Synthesis of the building blocks

- Ac-Sar-Gly-OH
  - Raw materials: H-Sar-OH, H-Gly-OBzl*HCl
  - 3 synthetic steps
  - Very stable, well crystallized, pure substance (> 99,5%; MeOH-MTBE)
- Cbz-Val-D-allo-Ile-Thr-Nva-OMe
  - Raw materials: Cbz-Val-OSu, Cbz-D-allo-Ile-OH*DCHA, Cbz-Thr-OH, H-Nva-OMe*HCl
  - 5 synthetic steps (3 couplings, 2 Hydrogenation)
  - Very stable, well crystallized, pure substance(> 99,5%, DMF-THF-MTBE)
- H-Ile-Arg-Pro-NHEt * 2HCl
  - Raw materials: Cbz-Ile-OSu, Boc-Arg(NO_2)-OH, Cbz-Pro-OH
  - 6 synthetic steps (3 couplings, 1 Boc deprotection, 2 Hydrogenation)
  - Needed to be adjusted to reach an appropriate quality

Optimization of H-Ile-Arg-Pro-NHEt * 2HCl synthesis



Original process:

NH_4Cl removal by reverse osmosis

Distillation

Precipitation EtOH/EtOAC

Purity: ~97%

Optimized process:

Alkalizing with NaOH (NH_3 + NaCl

Removal of NH_3 by distillation

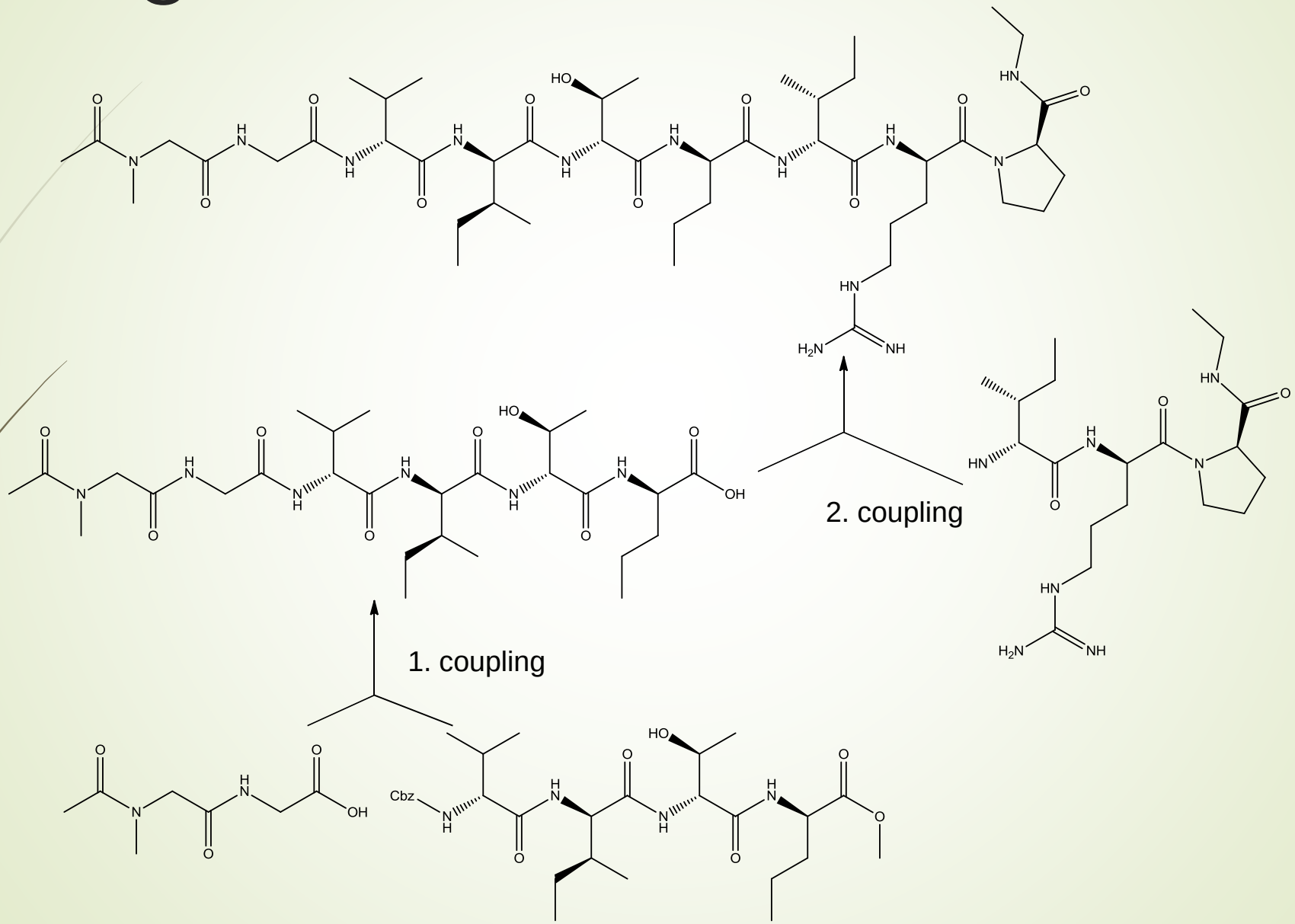
pH adjustment (HCl)

Filtration of NaCl

Crystallization EtOH/EtOAc

Purity: >99%

Fragment condenzations I.

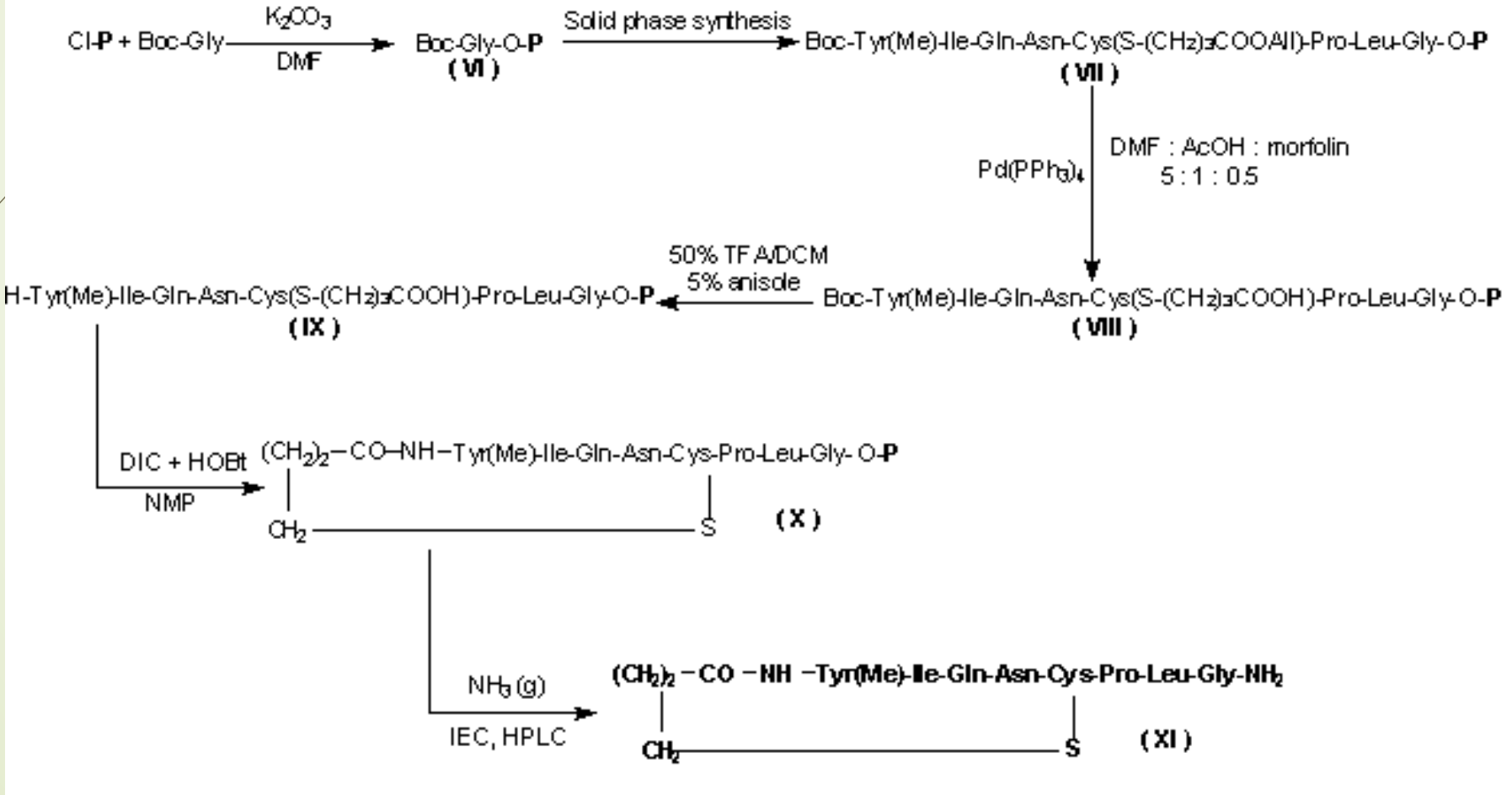


Fragment condensations II.

- 1. coupling (batch size: 30 kg)
  - Hydrogenation in N-methyl-pyrrolidone
  - Coupling in NMP with EDC + HOBt
  - Hydrolysis of the methyl ester without isolation
  - Isolation after setting the isoelectric point
  - Production time: ~ 2 days, Purity > 99%
- 2. coupling(batch size: 10 kg)
  - Coupling in DMF with EDC+HOBt+2,4,6-collidine
  - Crystallization in 2-propanol (removal of DMF, EDU, HOBt, collidine, D-Nva-epimer)
  - Ion exchange to form acetate
  - Recrystallization(Water: IPA: IpOAc = 1.2: 2.5: 20)
  - Production time ~1 day
  - Yield ~ 90%, Purity > 99,7%

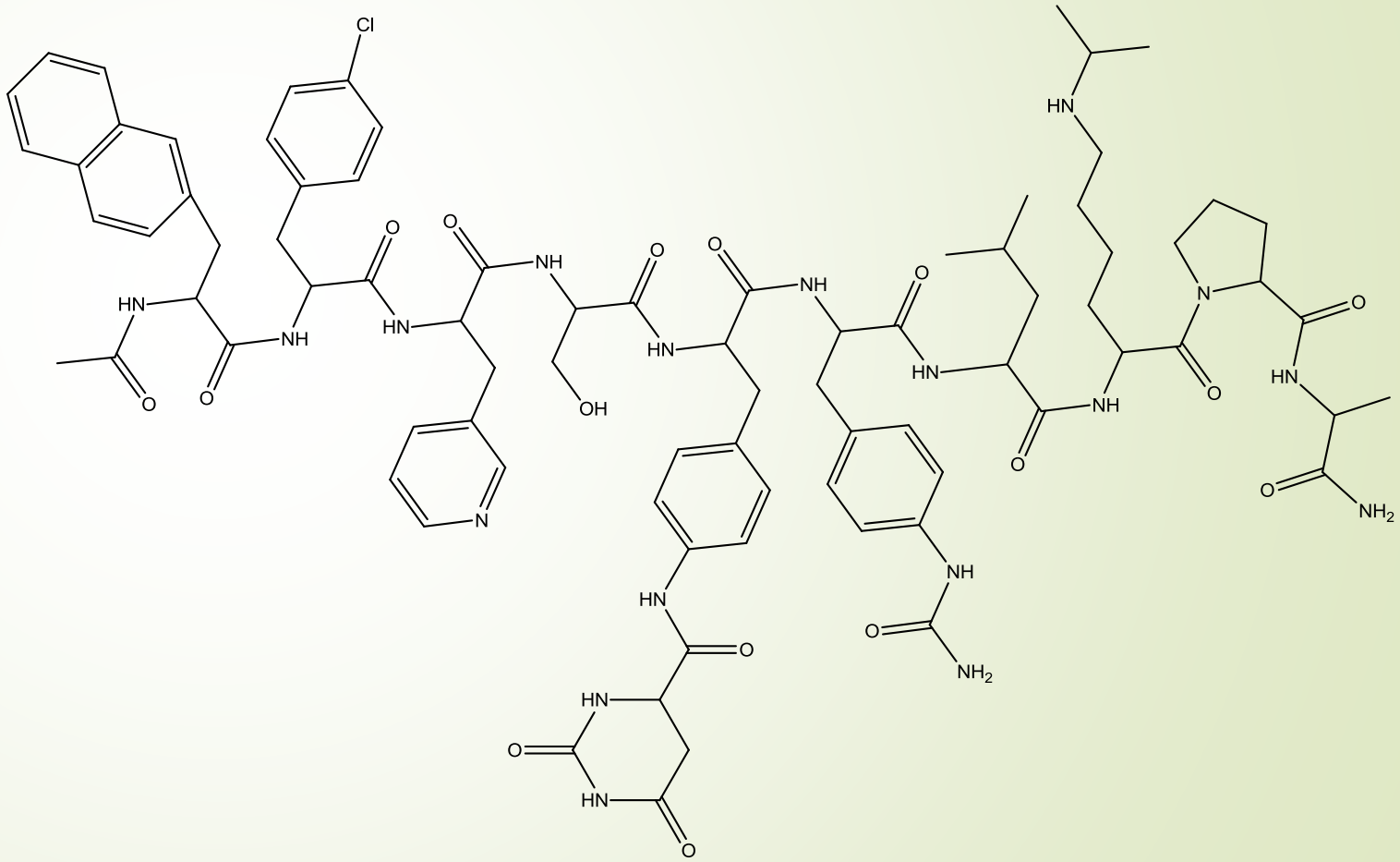
Solid phase synthesis 1. Boc/Bzl strategy

- Carbetocin – an oxytocin analogue



Solid phase synthesis 2. Fmoc/tBu strategy

- Degarelix (Firmagon)
- hormonal therapy used in the treatment of prostate cancer
- GnRH antagonist
- Suppress the testosteron production



Ac—D-2-Nal—D-Phe(4-Cl)·D-3-Pal—Ser—Aph(Hor)–D-Aph(Cbm)·Leu—Ilys—Pro—D-Ala—NH_2

Synthesis of Degarelix

- Solid phase synthesis
- Fmoc/tBu-strategy
- Raw materials: Fmoc-D-2-Nal-OH, Fmoc-D-Phe(4-Cl)-OH, Fmoc-D-3-Pal-OH, Fmoc-Ser(tBu)-OH, Fmoc-Aph(Hor), Fmoc-D-Aph(Cbm)-OH, Fmoc-Leu-OH, Fmoc-Ilys(Boc)-OH, Fmoc-Pro-OH, Fmoc-D-Ala-OH
- Fmoc-Knorr(Rink) linker
- Coupling agent: DIC-HOBt
- Cleavage/deprotection: TFA, water, ammonium-acetate
- Purification: RP-HPLC, ultra filtration

SPPS + Fragment condensation

- Enfuvirtide (T-20, Fuseon)

  Ac-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Tyr-Trp-Ala-Ser-Leu-Trp-Asn-Trp-Phe-NH_2

HIV fusion inhibitor, the first of a novel class of antiretroviral drugs used in combination therapy for the treatment of HIV-1 infection

History:

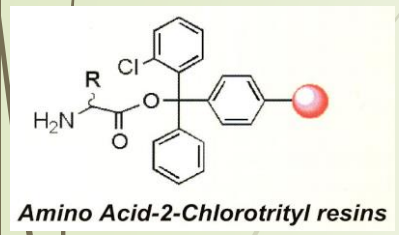
Duke University – Timeris (1996) – Hoffmann-La Roche (1999) – FDA(2003)

Stepwise synthesis of Enfuvirtide

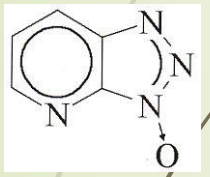
- Route 1: Linear Solid Phase Peptide Synthesis
- Fmoc SPPS conducted for the 36 residue sequence
- Greater than 2 equivalents of Fmoc-AA were used per coupling
- Furthermore, upon cleavage from the resin, the peptide was only ~30-40% pure
- This required difficult, low throughput chromatographic separation
- Overall yield was 6-8%
- This was an expensive and inefficient initial synthesis, but allowed access to enough material for clinical trials

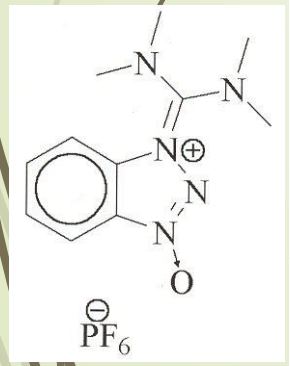
Nat. Rev. Drug Disc., **2003,** 2, 587-593

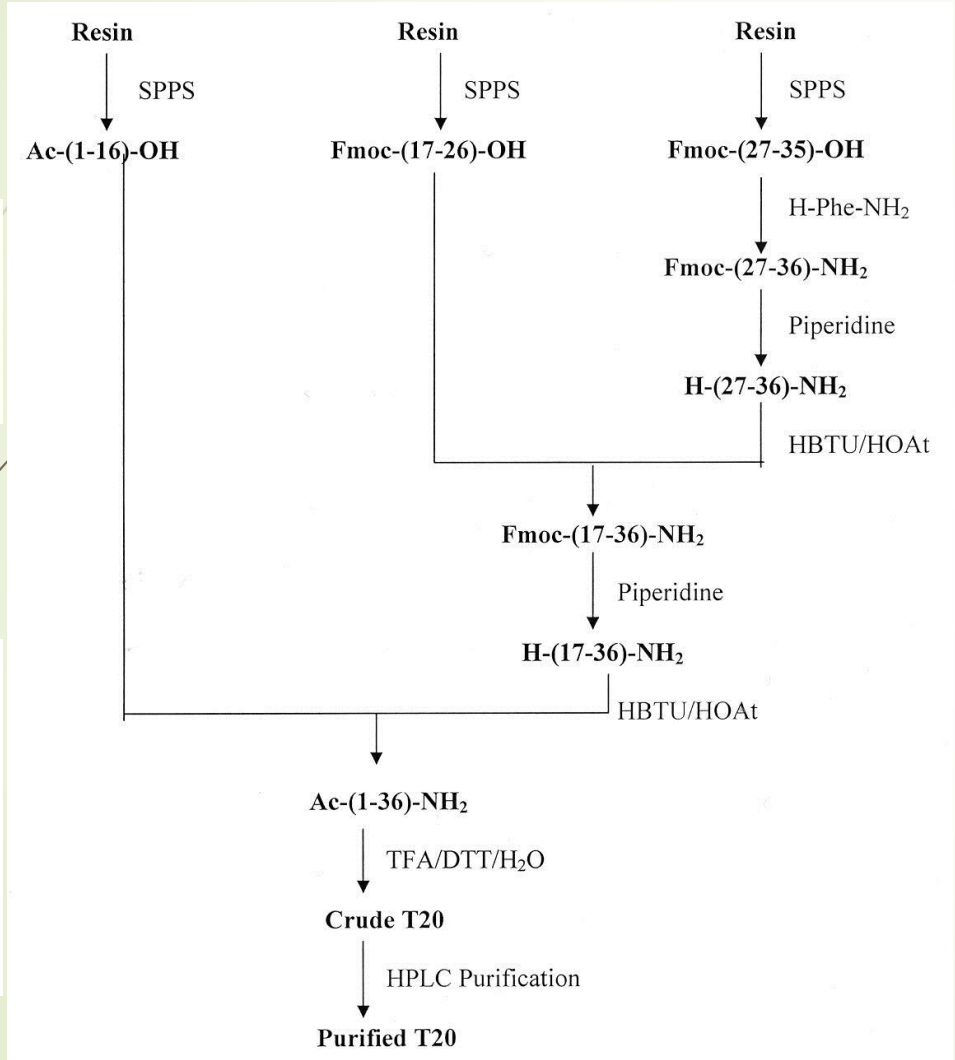
Fragment based synthesis of Enfuvirtide



Amino Acid-2-Chlorotrityl resins







- Three side chain protected fragments are constructed using a super acid sensitive resin, 2-chlorotrityl resin
- Resin is not patent protected and can be easily recycled, also attachment is racemization-free
- The three fragments were synthesized using HBTU/HOBt and 1.5 eq of Fmoc protected amino acids, no re-couple cycles were necessary
- Each fragment is isolated in >85% yield and >90% purity
- Each fragment can be synthesized in one week and in 300-500 kg scales
- To make the process efficient solvent recycling must occur, while yields are >99% per coupling, the cost is 75L of solvent per kilogram resin
- Five solution phase reactions complete the peptide which is then isolated in 30% overall yield
- The segment condensations were optimized to show less than 1% racemization

Ac-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-Trp-Ala-Ser-Leu-Trp-Asn-Trp-Phe-NH_2

Nat. Rev. Drug Disc., **2003,** 2, 587-593

Enfuvirtide production in numbers

- Yearly production volume: 3,700 kg
- 106 synthetic steps
- The time demand of one batch is more than 6 months
- 45,000 kg raw material to produce 1,000 kg product
- Yearly cost of 1 treatment 25.000 USD

Industrial scale peptide synthesis

Peptide synthesizer in the lab

SPPS reactor

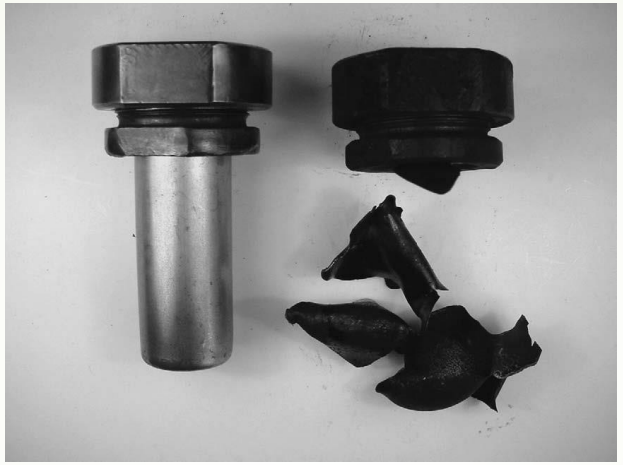
HPLC column

Problems with SPPS in industrial scale

- High amount of raw material is needed
  - Process optimization, raw material price reduction
- Huge solvent demand
  - Recycling
- Coupling agents
  - Risk of explosion

Explosive risk of coupling agents

Dry HOBt
Koenen-test 10mm



50% wet HOBt
Koenen-test 2mm

TBTU
Koenen-test 2mm

Quality assurance (GMP)

- Good manufacturing practice guidelines provide guidance for manufacturing, testing, and quality assurance in order to ensure that a manufactured product is safe for human consumption or use
- Basic principles
  - Manufacturing facilities must maintain a clean and hygienic manufacturing area.
  - Manufacturing facilities must maintain controlled environmental conditions in order to prevent cross-contamination from adulterants and allergens that may render the product unsafe for human consumption or use.
  - Manufacturing processes must be clearly defined and controlled. All critical processes are validated to ensure consistency and compliance with specifications.
  - Manufacturing processes must be controlled, and any changes to the process must be evaluated. Changes that affect the quality of the drug are validated as necessary.
  - Instructions and procedures must be written in clear and unambiguous language using good documentation practices.
  - Operators must be trained to carry out and document procedures.
  - Records must be made, manually or electronically, during manufacture that demonstrate that all the steps required by the defined procedures and instructions were in fact taken and that the quantity and quality of the food or drug was as expected. Deviations must be investigated and documented.
  - Records of manufacture (including distribution) that enable the complete history of a batch to be traced must be retained in a comprehensible and accessible form.
  - Any distribution of products must minimize any risk to their quality.
  - A system must be in place for recalling any batch from sale or supply.
  - Complaints about marketed products must be examined, the causes of quality defects must be investigated, and appropriate measures must be taken with respect to the defective products and to prevent recurrence

Source: wikipedia

Quality of the raw materials

Fmoc-Lys(Boc)-OH specification:

Test	Result
Appearance	White almost white powder
Identity (IR)	Identical with standard
Assay (titration)	98.0 – 102.0 %
Solubility (0.5 mmol/ ml DMF)	clear, max 150 Hazen
Optical rotation (c=1, DMF)	-11.0^{o} ~ -13.0^{o}
Melting range	126.0 – 132.0 ^{o}C
Ash (SO4)	max 0.2%
Water content (KF)	max 1.0%
Chromatographic purity(TLC)	
$CHCl_3$:MeOH:AcOH=90:8:2 (V/V)	min 99%
H-Lys(Boc)-OH (TLC)	max 0.2%
Chromatographic purity (HPLC)	min 98.5%
Fmoc-β-Ala-OH (HPLC)	max 0.1%
Fmoc-β-Ala-Lys(Boc)-OH (HPLC)	max 0.1%
D-enantiomer	max 0.2%
Other amino acids	max 0.2%
Residual solvents	To be reported

Production documentation 1.

- What should be documented
  - Performed production process steps, actions
  - Equipment cleaning processes and their inspection
  - In process control investigations and their check
  - Operation of the supporting facilities
  - Planned/sudden deviations and their evaluation (change controll, deviation)

Production documentation 2.

PolyPeptide Laboratories

MBPR číslo: MBPR-4110
Vydání: 02
Strana 29 z 29

Jméno produktu: Oxytocin surový	**Materiálové číslo:** 4110 **Číslo šarže:**

		Provedl	Kontrola	Datum
IV.6	*Označení zařízení* Aparatura pro rozpuštění Bis-Acm-Oxytocinu se označí štítkem obsahujícím jméno a materiálové číslo produktu a číslo vyráběné šarže	_____		_____
IV.7	*Vyloučení kontaminace obsahu aparatury* Nikde v místnosti se nezachází s žádnými suchými chemikáliemi	_____	_____	_____
IV.8	*Rozpuštění Bis-Acm-Oxytocinu* V den reakce, maximálně jeden den předem, se do kotle napustí _____l vody, materiálové číslo 1663 a za míchání se přidá _____g Bis-Acm-Oxytocinu, materiálové číslo 4105 číslo šarže_____ Roztok se míchá do úplného rozpuštění. Začátek rozpouštění_____ Konec rozpouštění_____ Celková doba rozpouštění_____min.	_____ _____ _____ _____ _____ _____	_____ _____ _____	_____ _____ _____
IV.9	**Příprava roztoku pro cyklizaci** *Příprava roztoku jodu v DMF* V den reakce se do Erlenmayerovy baňky vhodného objemu naváží _____g jodu, materiálové číslo 1659 číslo šarže_____	_____ _____	_____ _____	_____

Review of analytical evaluation of 3 regularly manufactured batches

Parameters	Specification	120801298A	080800598A	070800598A
Characteristics	White freeze dried fluffy lyophilizate	comply	comply	comply
Identification /AAA,HPLC/	Retention time identifical to reference sample	conforms	conforms	conforms
Optical rotation /calc.with reference to anhydrous, acetic acid-free subst./	-71* to -81*	-78,5*	-79,7*	-78,7*
Aminoacid analysis	Asp Gly Pro Glu 0,95-1,05 Ile Leu Tyr Cys C3-present	Asp 1,00 Gly 0,97 Pro 1,00 Glu 1,02 Ile 0,99 Leu 1,01 Tyr 1,00 Cys C3-present	Asp 0,97 Gly 1,01 Pro 1,00 Glu 1,00 Ile 0,99 Leu 1,01 Tyr 0,97 Cys C3-present	Asp 0,98 Gly 1,01 Pro 1,02 Glu 0,97 Ile 0,98 Leu 1,01 Tyr 0,97 Cys C3-present
Water content /Karl Fischer, coulometric detection/	max.6%	3,1%	3,8%	3,0%
Solvent residues / Acetic acid /	max.5%	5,4%	3,4%	3,6%
Peptide related impurities / Sum of impurities /	max.5%	1,9%	2,2%	3,0%
Individual impurity	1,0%	0,6%	0,6%	0,9%
Peptide content:				
content of Carbetocin /free base/ in % of the total mass of substance	min. 85,0%	89,7%	89,9%	91,0%

Drug Master File

Table of contents – Open Part

Requirements about the quality of the final APIs

ICH - The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (1990 to present)

To achieve greater harmonization in the interpretation and application of technical guidelines and requirements for product registration.

Q1: Stability(1992)
Q2: Analytical validation (1993)
Q3: Impurities (1994)
Q4: Pharmacopoeias
Q5: Quality of biotechnological products (1995)
Q6A: Specifications: test procedures and acceptance criteria for new drug substances and new drug products: chemical substances (1999)
Q6B: Specifications: test procedures and acceptance criteria for biotechnological/biological products (1998)
Q7A: Good Manufacturing Practice API (2000)
Q8: Pharmaceutical development (2004)
Q9: Quality risk management (2005)
Q10: Pharmaceutical quality system(Step 2 2007)
Q11: Development and manufacture of drug substances
Q12: Lifecycle management

Required data to register a peptide drug

- Manufacturer (Name, address, responsibility status in each and every production site, analysis location and the owner of the DMF)
- Documentation of the production process' development
- Documentation of the used materials (amino acids, amino acid derivatives, reagents, solvents, resins)
- Product identification (Amino acid analysis, MS, Amino acid sequencing, disulfide bridges, spectroscopy, electrophoresis, NMR, X-ray diffraction)
- Physicochemical identification (isoelectric point, solubility, coefficient of extinction, spectral characterization, pH of the solution)
- Biological characterization (biological effect, strength, immunogenity, in vitro, in vivo effects)

Purity requirements

- Detection limit of impurities: 0.03%
- Identification limit of impurities: 0.05%
- Quantification limit of impurities: 0.05%

Potential impurities:

- Peptide related impurities (racemic, deficient, truncated, side produc, isomers, oxidized derivatives, mixed-up disulfide bridges, aggregates
- Non-peptide related impurities (residual solvents, coupling agent residuals, protecting group residuals, inorganic asalts)

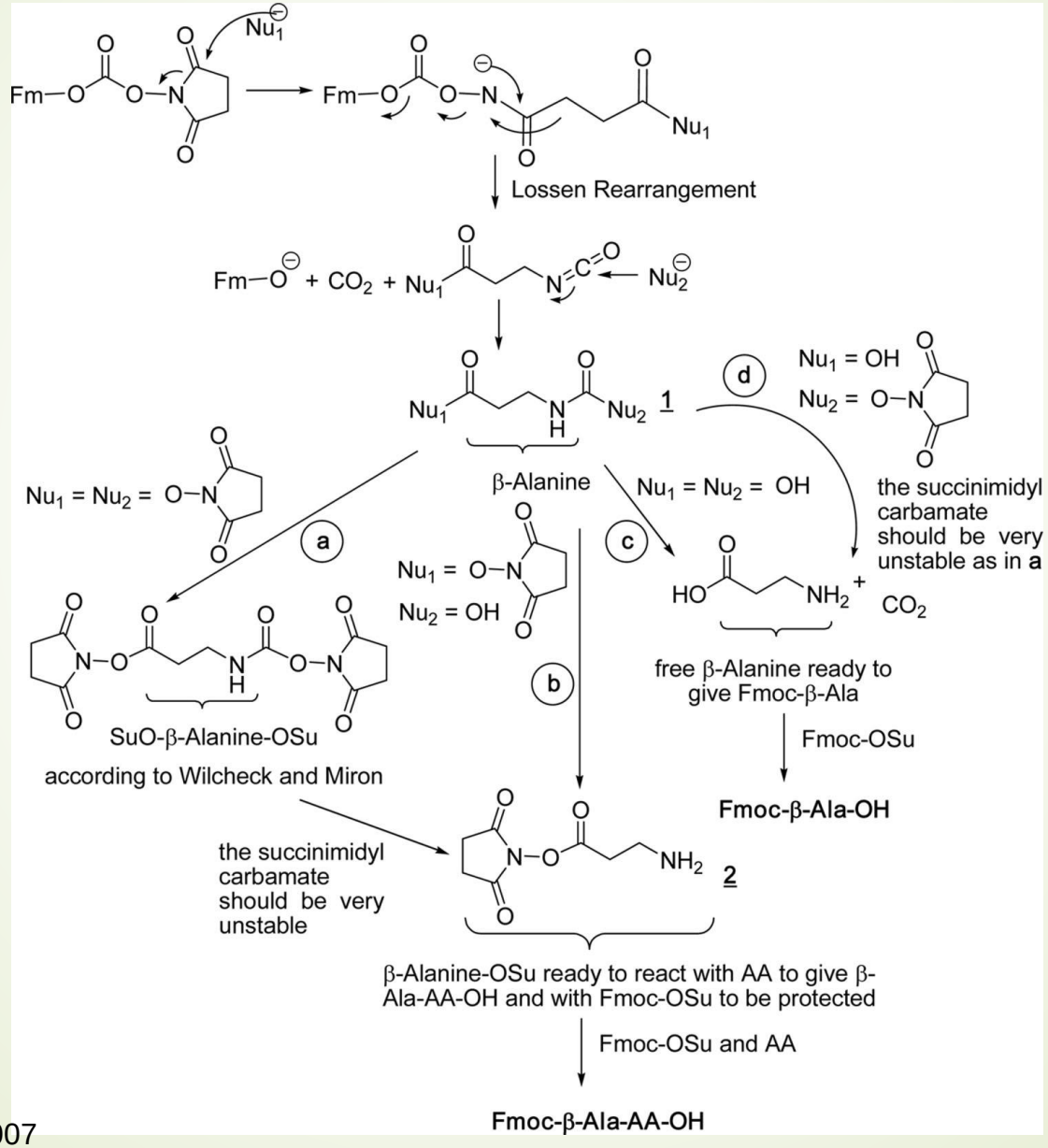
β-Alanine impurity

- > 20 AA containing peptide was prepared for clinical study. A new impurity was appeared in one of the batches wiht a 0.84 RRT compared to the main product (0.49%)
- It can be purified, but with extreme losses (~50%)
- The impurity was identified: Chromatography, MS/MS, amino acid analysis, sequencing and structure proving by synthesis
- The impurity was β-alanine and it was originated by the used protected amino acids

Where did it come from?

Hlebowicz et al, J.Pept.Res, 2005

„Formation" of β-Alanine impurity

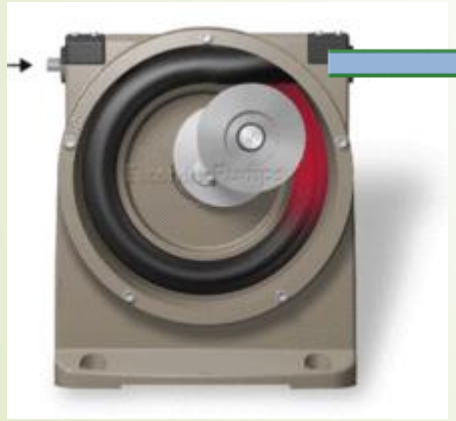


Izidoro et al Biopolymers, 2007

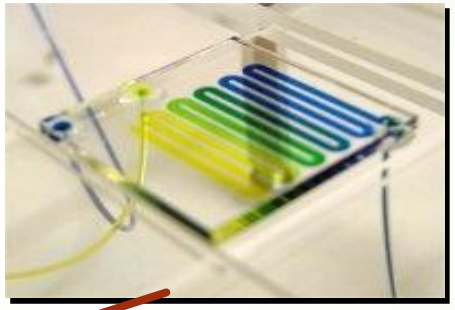
Continuos Flow synthesis

What is flow chemistry?

- Performing a reaction continuously, typically on small scale,
- through either a coil or fixed bed reactor.



Pump

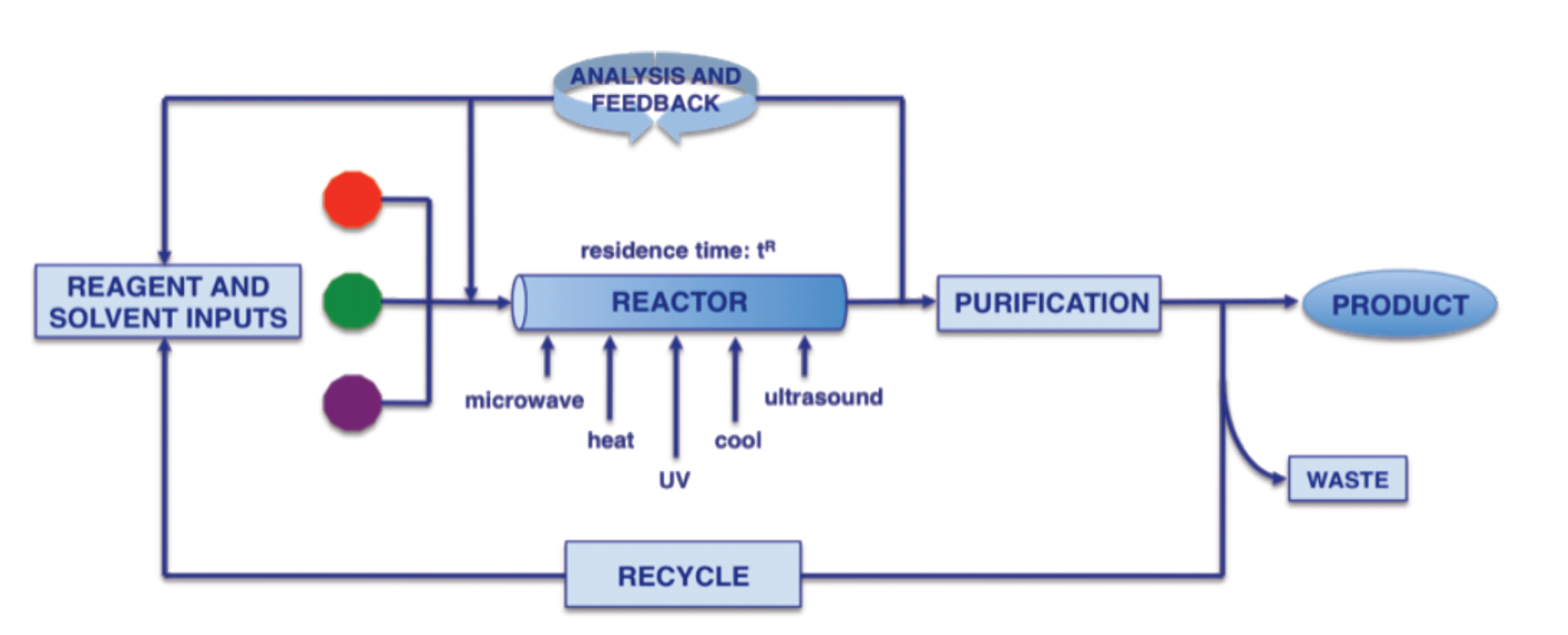


OR

Reactor

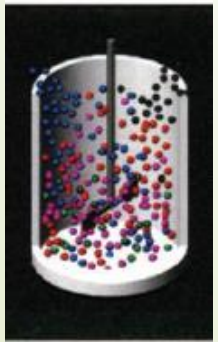
Collection

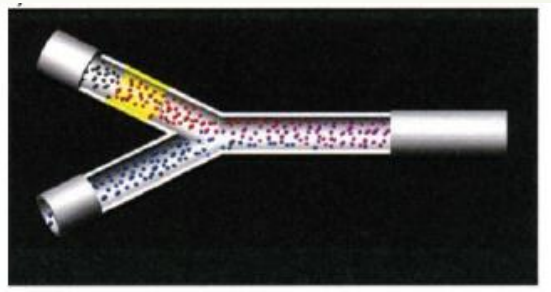
Continuous flow „environment”



Batch and flow

- Fundamental differences between batch and flow reactions:
- stoichiometry vs. flow rates,
- reaction time vs. residence time,
- reaction completion - „reaction window" depends on time *in batch*, while time and space (distance) *in flow*,
- Temperature control is different for batch and flow (particularly for fast reactions, mixing etc.)





Where is flow chemistry applied best?

Exothermic Reactions

- Very good temperature control
- Accurate residence time control
- Efficient mixing
- Less chance for thermal run-away
- Higher productivity per volume
- High selectivity

Endothermic Reactions

- Control over T, p and residence time
- High selectivity
- Accessing new chemistry
- Higher productivity per volume
- High atom efficiency

Reactions with gases

- Accurate gas flow regulation
- Increased safety
- Easy catalyst recycling
- High selectivity
- Higher productivity per volume

Scale up

- Increased safety
- Higher productivity per volume
- Selectivity
- Reproducibility

Miniaturization: Enhanced temperature control

Large surface/volume rate

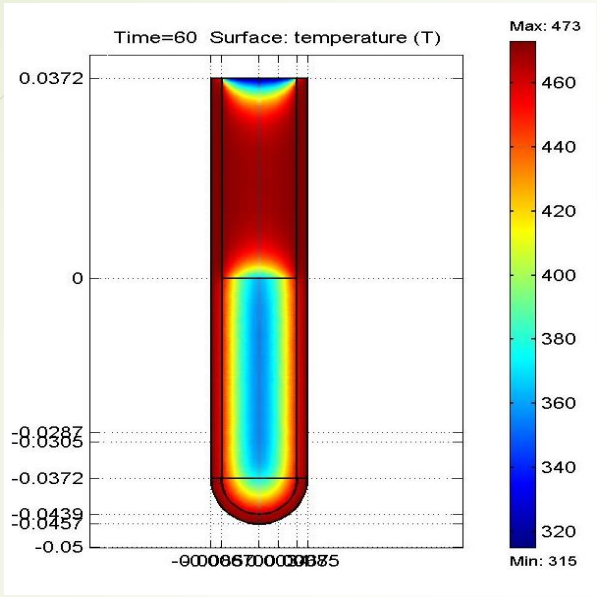
- Microreactors have higher surface-to-volume ratio than macroreactors, heat transfer occurs rapidly in a flow microreactor, enabling precise temperature control.

Size	1/100
Surface area	1/10000
Volume	1/1000000
Surface / Volume	100

Yoshida, Green and Sustainable Chemical Synthesis Using Flow Microreactors, ChemSusChem, 2010

Heating Control

Batch



- Larger solvent volume.
- Lower temperature control.

Outcome:

-More difficult reaction control.
- Higher possibility of exotherm.

Flow

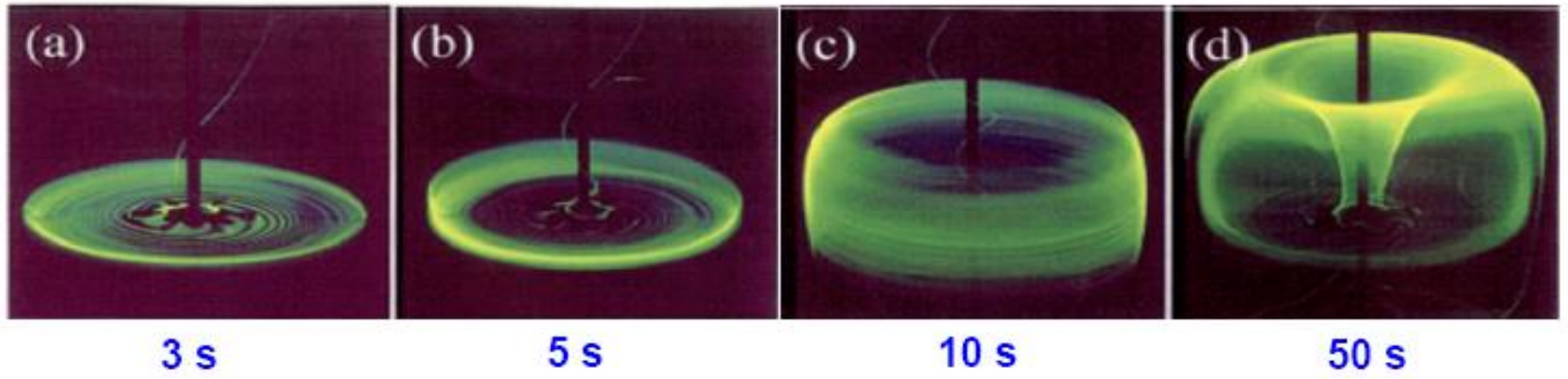
- Lower reaction volume.
- Closer and uniform temperature control

Outcome:

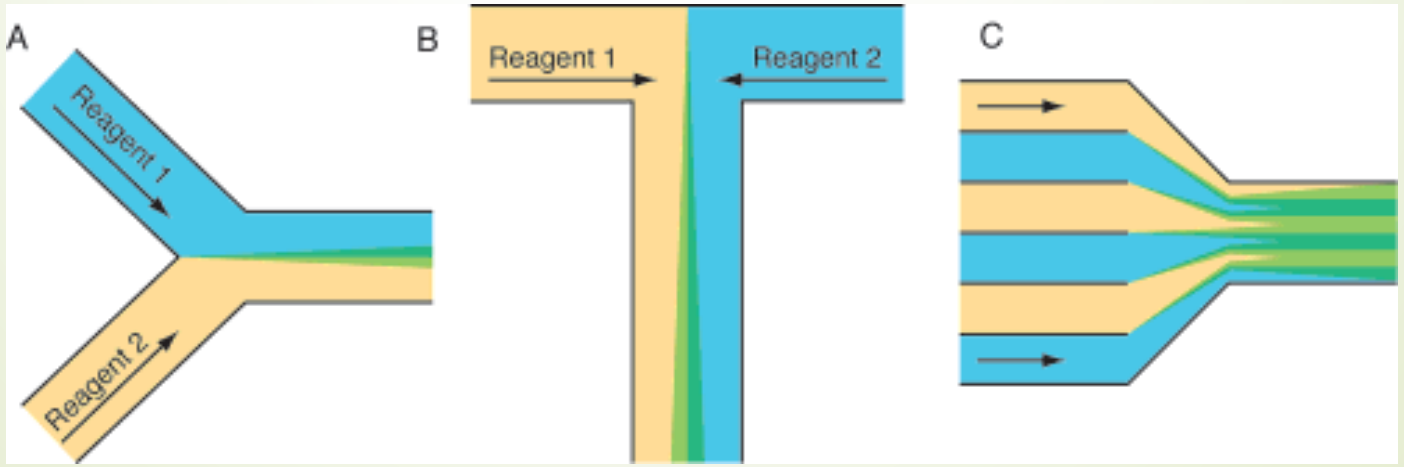
- Safer chemistry.
- Lower possibility of exotherm.

Improved Mixing Compared to Batch

- Fluorescent dye in glycerin (20 L reactor, impeller mixing)



Flow reactors can achieve homogeneous mixing and uniform heating in microseconds (suitable for fast reactions)



First trials

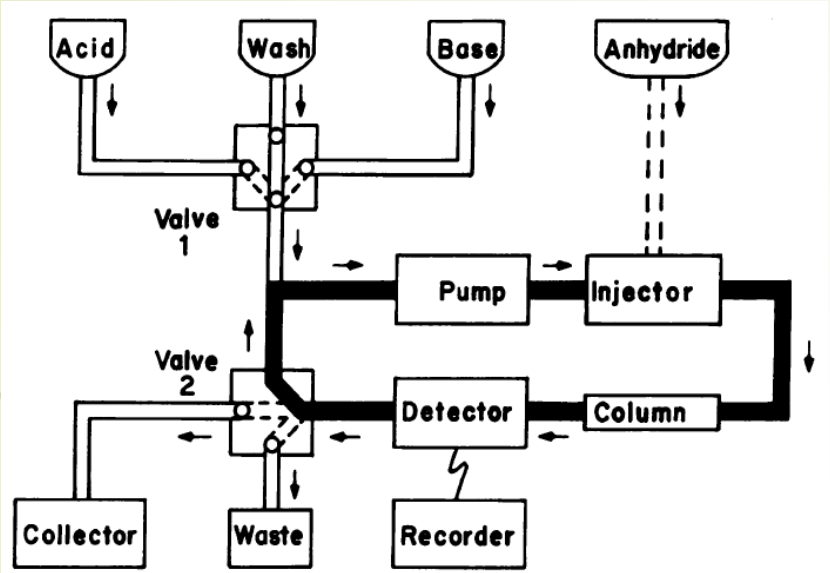


Table 4. Synthetic cycles for assembly of ovalbumin-(238–254)

Cycle step	Discontinuous: Reagent	Discontinuous: Shaking time, min	Continuous flow: Reagent	Continuous flow: Pumping time, min
Deprotection	6.5 M CF_3COOH	1 + 20	0.01 M CH_3SO_2OH 0.10 M CF_3COOH	6
Wash	None	6 × 1	None	6
Neutralization	0.30 M DIEA	2 × 2	0.30 M DIEA	3
Wash	None	6 × 1	None	6
Coupling	0.08 M Boc-aa 0.08 M DCC (12 equiv. each)	120	0.10 M Boc-aa anhydride (16 mol equiv.)	45
Wash	None	8 × 1	None	6
Repeat last four steps				

Table 3. Distribution of Leu-Ala-Gly-Val and deletion peptides after continuous-flow synthesis

Synthesis code	Amino protection	Solid support*	Valine loading, mmol/g	30-min couplings per cycle	Peptide distribution† relative mol %: LAGV	AGV	LAV	LGV	LV
S-7	Boc	A	0.13	1	89.1	4.2	5.2	1.1	0.2
S-8	Boc	A	0.13	2	98.3	1.3	0.4	<0.1	0.1
S-9	Boc	A	0.13	2	99.3	0.3	0.2	0.2	<0.1
S-10	Boc	A	0.64	2	99.1	0.5	0.4	<0.1	<0.1
S-11	Boc	B	0.27	1	86.6	9.7	0.6	2.9	0.2
S-12	Boc	B	0.27	2	83.3	16.1	0.5	0.3	0.4
S-13	Fmoc‡	C	0.80	2	96.1	2.3	1.6	<0.1	<0.1
S-14	Fmoc‡	C	0.44	2	96.3	0.2	3.5	<0.1	<0.1
S-15	Fmoc§	C	0.44	2	98.6	<0.1	1.4	<0.1	<0.1

* Support A, microporous hydroxymethyl-PAM-polystyrene prepared from copoly(styrene-1% divinylbenzene) (Bio-Beads SX-1, Bio-Rad); support B, hydroxymethyl-PAM-polystyrene prepared from macroporous polystyrene (Dionex, Sunnyvale, CA); support C, microporous 4-(hydroxymethyl)phenoxymethyl-copoly(styrene-1% divinylbenzene) (*p*-alkoxybenzylalcohol resin, Chemical Dynamics, South Plainfield, NJ). Each synthesis used 0.08–0.10 g of Boc-Val-resin in a 0.39 × 6.0 cm column, except S-9 (0.76 g, 0.10 mmol) and S-10 (0.70 g, 0.45 mmol) in an 0.78 × 12 cm column pumped at 6 ml/min.

† L, leucine; A, alanine; G, glycine; V, valine.

‡ Soluble Fmoc-peptides were deprotected with 1 M piperidine in dioxane/water, 1:1 (vol/vol).

§ Soluble Fmoc-peptides were deprotected with 2 M piperidine in DMF, and the resulting free peptides were precipitated by addition of ether.

Lukas et al.
PNAS 1981
78(5) 2791

Current trends (low amino acid excess)

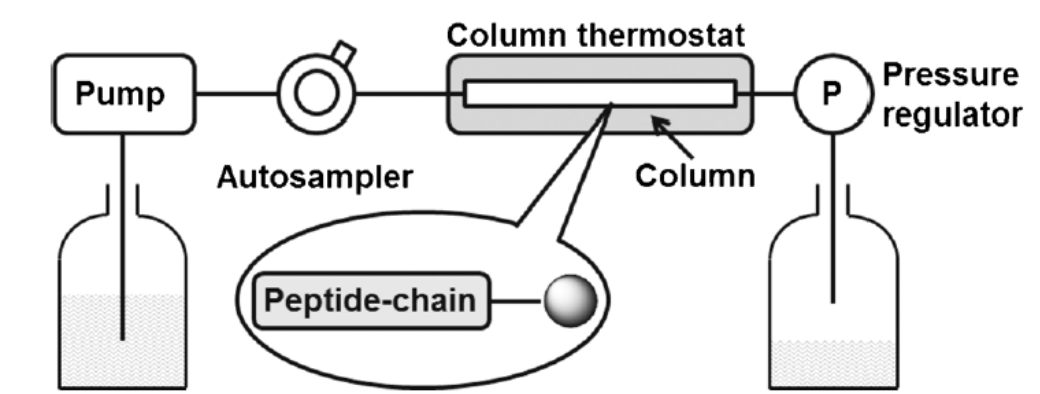
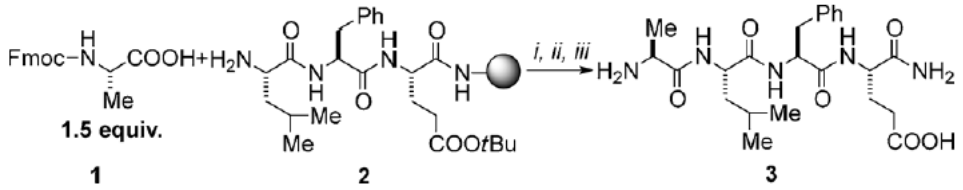


Figure 1. Schematic representation of the constructed CF reactor.



Scheme 1. Reaction used for the fine-tuning of CF-SPPS coupling conditions. (*i*) 1.5 equiv of HATU, 3 equiv of DIPEA in DMF; *ii*) 2% DBU and 2% piperidine in DMF; *iii*) 95% TFA+5% H_2O).

TentaGel resin
50 – 70°C, 60bar, 6.7min cycletime

Mándity et al. ChemSusChem 2014, 7(11) 3172

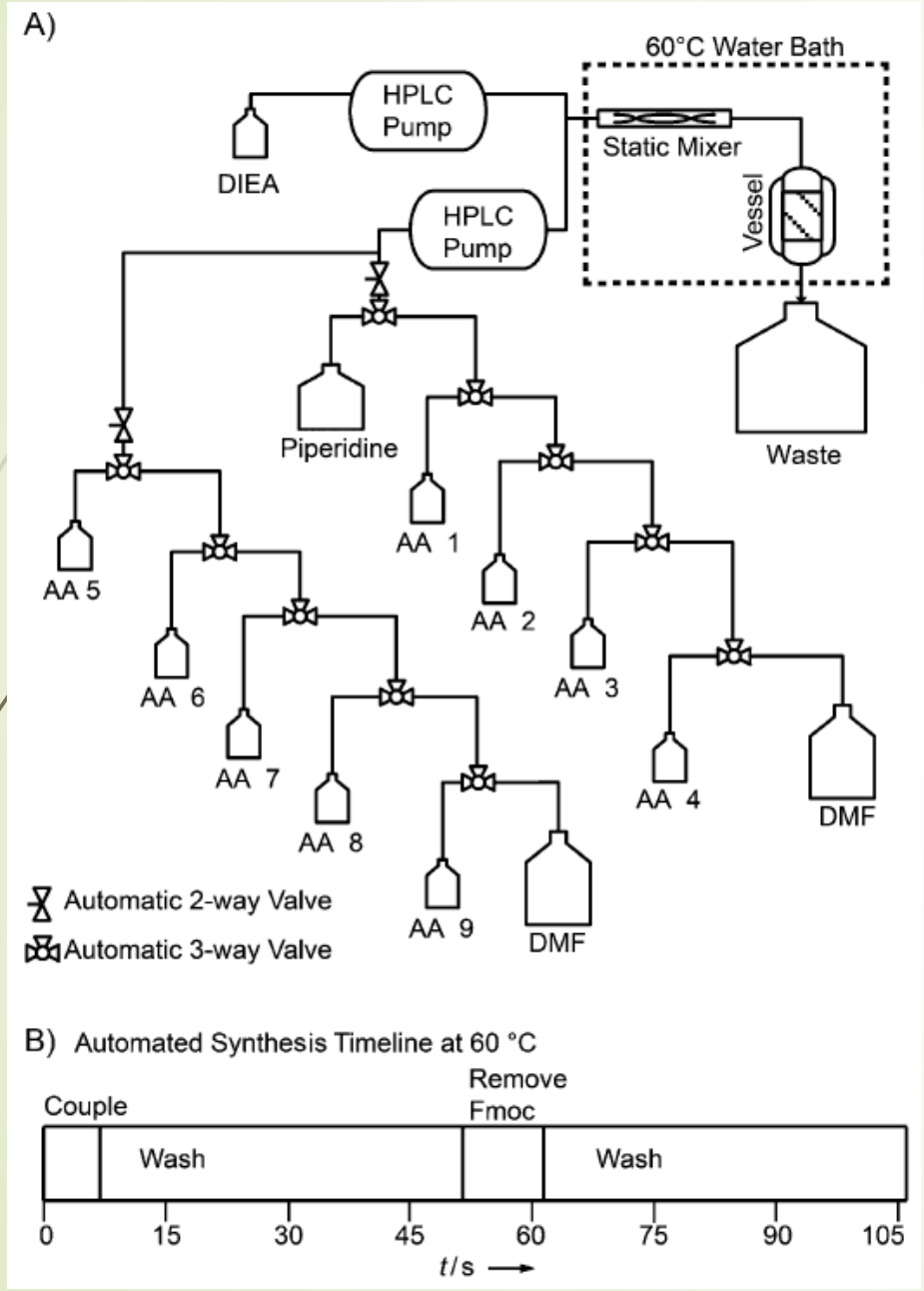
Sequence 23: ACP 65-74
(H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2)
Sequence 24: CD8 fragment
(H-Gly-Leu-Ile-Thr-Val-Ser-Val-Ala-Val-NH_2)

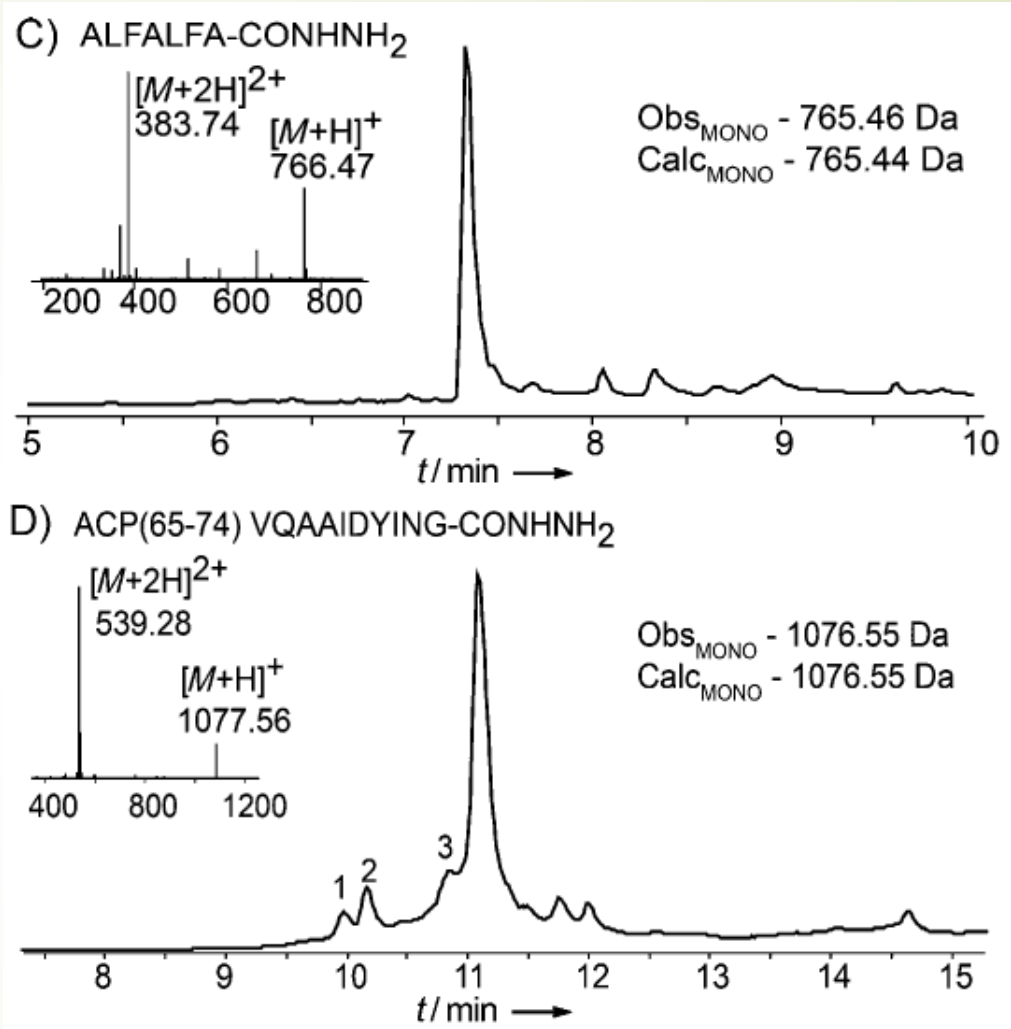
Table 2. Comparison of the results of the syntheses of difficult sequences with literature data.[a]

Property	Sequence **23**		Sequence **24**	
	CF-SPPS	Literature[4g]	CF-SPPS	Literature[17a]
Crude purity	86%[a]	94%	84%[a]	89%
Amino acid equivalents	1.5	5	1.5	5
Coupling time	6.7 min	60 min	6.7 min	10 min
Solvent used[a]	35 mL	46.2 mL	31.5 mL	183.6 mL

[a] Given for the same scale of synthesis.

Current trends (low cycle time)





Fmoc-Rink-MBHA resin
6 eqv Fmoc-AA-OH, HBTU

Simon et al. ChemBioChem 2014, 15(5) 713

Automated flow SPPS

Figure 1 – The CF-SPPS setup, consisting of two R2 pump modules, one R4 reactor module, autosampler and in-line UV detector.

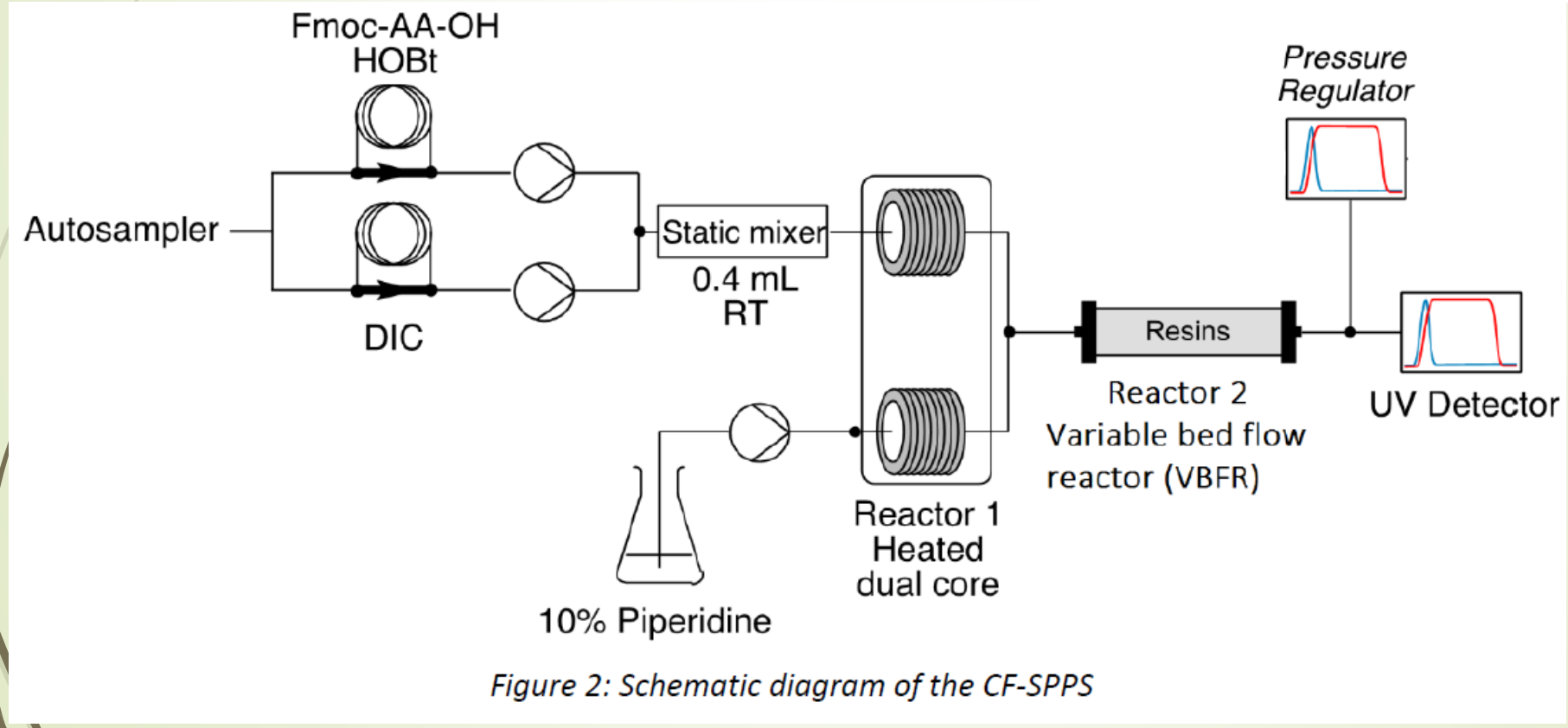


Figure 2: Schematic diagram of the CF-SPPS

Source: Vapourtec

Results of the synthesis

- ACP 65-74 was synthesized (H_2N-VQAAIDYING-$CONH_2$) on Rink-amide resin

Table 1 – Optimisation process for ACP synthesis

Entry	Scale (mmol)	Double couple V	Solvent for V coupling	des-V by-product	Overall ACP purity
1 (TK147)	0.06–0.16	N	DMF	4.7	85.3
2 (TK144R)	0.06–0.16	Y	DMF	2.7	86.1
3 (TK148R)	0.06–0.16	N	50% DMSO in DMF	1.7	88.9
4 (TK149)	0.12–0.32	N	50% DMSO in DMF	2.3	88.1
5 (TK150)	0.30–0.80	N	50% DMSO in DMF	0.9	93.7

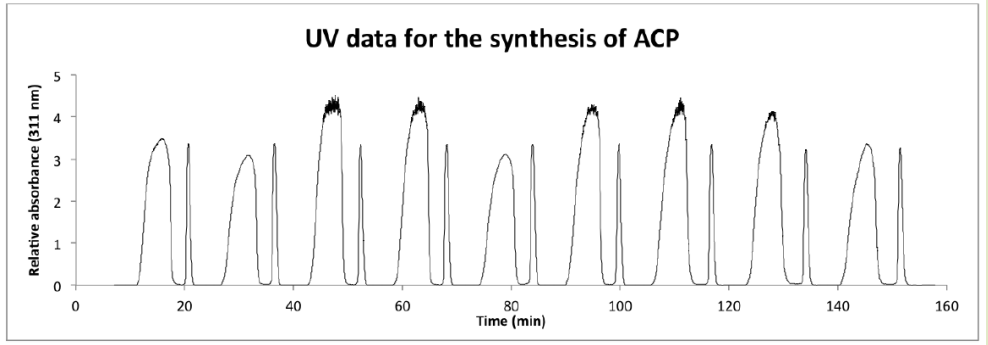


Figure 5 – UV data for synthesis of ACP.

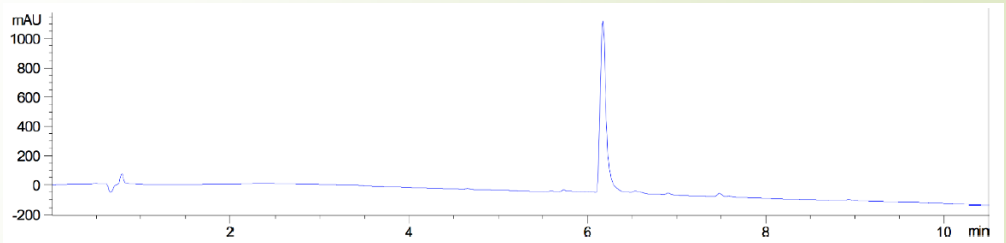


Figure 8 - HPLC trace of crude ACP (Entry 5, Table 1). Gradient = 5–40% B over 10 min. Purity = 93.7%.

Source: Vapourtec

Results of the synthesis

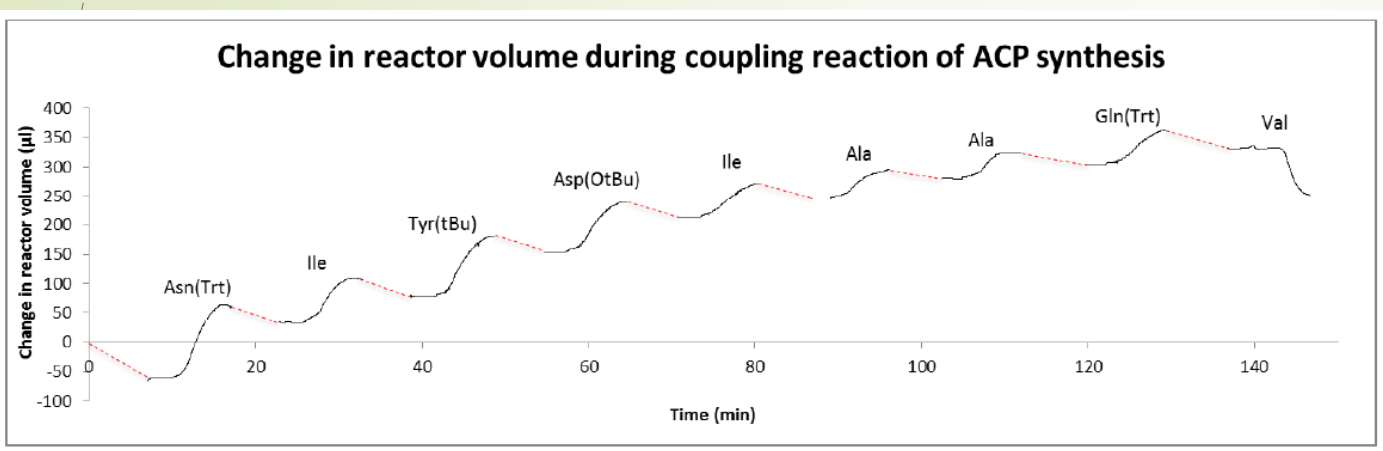


Figure 4 – Variable bed reactor (VBFR) data (black solid line) showing resin swelling during coupling reactions of ACP. The VBFR data during Fmoc-deprotection and washing steps are omitted (shown as red dotted lines). The reactor volume reduced significantly during Val coupling, suggesting potential peptide chain aggregation and lowering coupling efficiency.

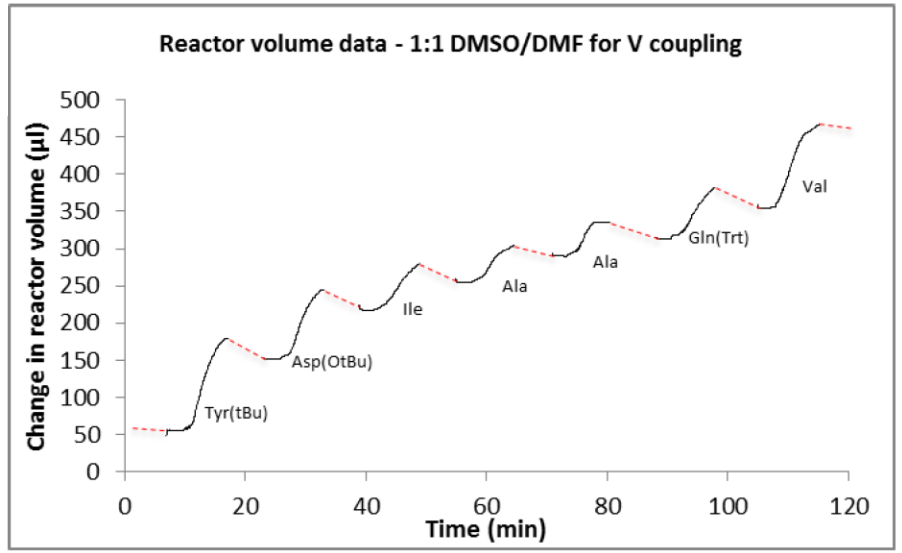


Figure 7 – Variable bed flow reactor (VBFR) data (black solid line) showing resin swelling for the last 7 coupling reactions of ACP. The VBFR data for the first two couplings, Fmoc-deprotection and washing steps are omitted (shown as red dotted lines). Using 50% DMSO/DMF (v/v) for Val coupling prevented the drop in column height and restored the characteristic swelling of the resins during coupling reaction

Source: Vapourtec

Thank you for your attention!