Knud J. Jensen

KVL A UNIVERSITY OF LIFE SCIENCES

Solid-Phase Peptide Synthesis with Microwave Heating

The Royal Veterinary and Agricultural University DENMARK

As of January 1 2007: University of Copenhagen

Acknowledgement

- Pernille Tofteng Shelton
- Dr. Malene Brandt
- Prof. Steen Gammeltoft, Glostrup Hospital
- Andreas Hoel, Biotage
- Dr. Andrew Coffey, Polymer Laboratories
- Funding: Lundbeck foundation

Overview: presentation

- Why peptides?
  - Peptides as drugs!
  - Peptides as biomedical probes
- Solid-phase peptide synthesis
  - Much more than formation of amide bonds
- Phosphopeptide proteomics
- General study of manual SPPS using precise microwave heating
- Conclusion

Peptides as drugs

- All "peptide" products (synthetic peptides; peptidomimetics, proteins including antibodies but excluding vaccines) are worth $28 billion (2000).
- 3 sub-groups of products (2000):
  - Recombinant proteins : $14 billion
  - Monoclonal antibodies : $ 4 billion
  - Synthetic products : $10 billion
- Synthetic erythropoietin protein (2003)
  - Gryphon (100 mill. USD from Roche)

A. Loffet, *J. Peptide Sci.*, 2002, 8, 1-7

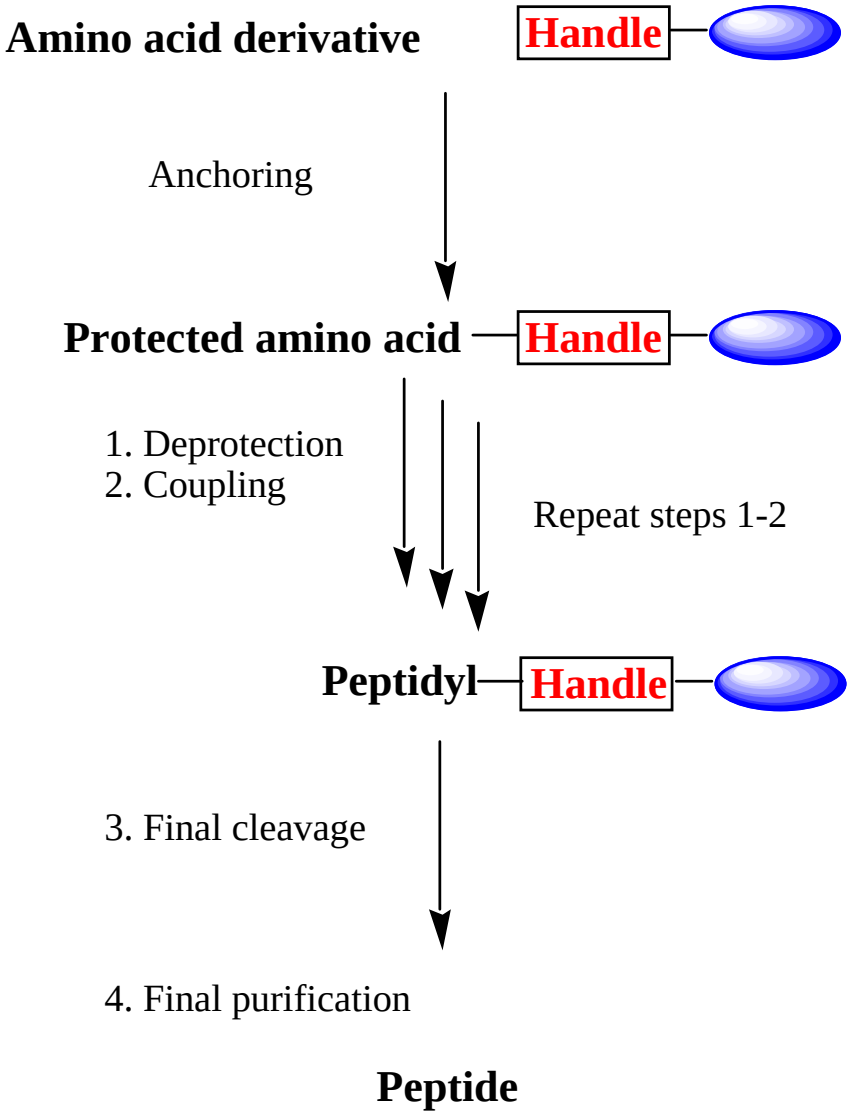
Peptides as drugs: Fuzeon (T-20)

- First member of the third generation of drugs for the treatment of HIV/AIDS: Trimeris and Roche
- Inhibits fusion between viral particles and cells
- Synthesized by condensation of three segments, which are prepared by SPPS
- A synthetic peptide produced on a ton-scale

Synthetic peptides *for* drugs

- Biomedical research
- Starting point for the development of small-molecule drugs
- Peptides for drug target validation
- Understanding peptide binding and processing proteins
- Proteomics etc.
- Vaccinations
- ... and a lot more

Solid-phase peptide synthesis



R. B. Merrifield,
Nobel Lecture,
Angew. Chem. Int. Ed. Engl.
1985, 24, 799-810.

Essentials of peptide synthesis

- Defined by the N^{α}-protecting group, which will be removed after each coupling
- The two most important are Fmoc and Boc
- The choice of semi-permanent side-chain protecting group and handle (linker) depends on the chosen N^{α}-protecting group
- The linkage to the solid support defines the chemistry possible in the following steps

Essentials of peptide synthesis

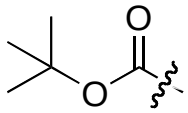
- Merrifield *et al.* develop the use of the Boc group, which requires TFA for removal
- Fmoc, developed by Carpino, utilized by Sheppard *et al.*, requires typically secondary amines like piperidine
- Fmoc chemistry provides generally milder conditions

Essentials of peptide synthesis: Fmoc

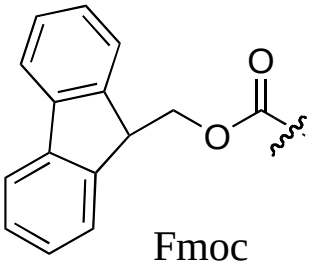
- N^{α}-Fmoc-AA-OH
- Handles: acid-labile, base-labile, photo-labile, fluoride-labile
- Acid-labile handles: TFA cocktails
- Side-chain protecting groups: most are TFA-labile
  - Combined release and deprotection of peptide
  - Tert-butyl, Trityl, Pbf
  - Dde, Alloc
- Orthogonality

Protecting groups and coupling reagents

N^{α}-amino protecting groups

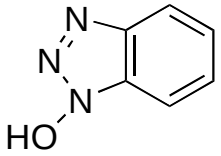


Boc



Fmoc

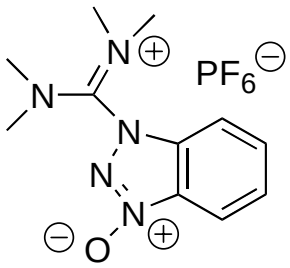
Auxiliary nucleophiles



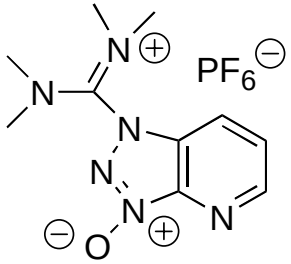
HOBt

HOAt

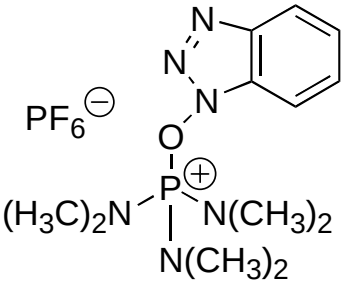
Coupling reagents: Electrophiles (nucleophiles released)



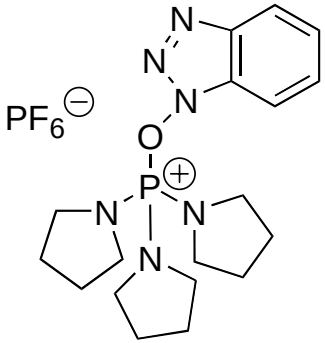
HBTU



HATU

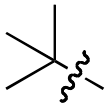


BOP



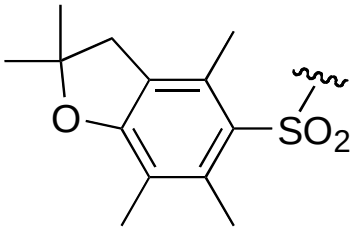
PyBOP

Common side-chain protecting groups for general Fmoc SPPS



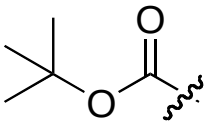
Bu^t

Ser, Thr, Tyr, Asp, Glu: TFA



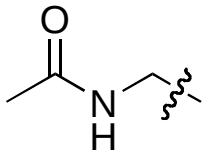
Pbf

Arg: TFA with scavenger



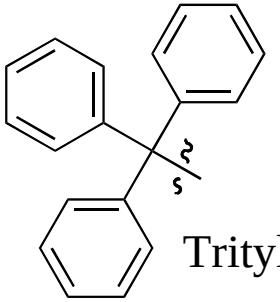
Boc

Lys, Trp (His): TFA



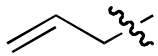
Acm

Cys: I_2, Tl(tfa)3, Hg(II)

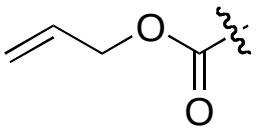


Trityl

His, Cys, Asn, Gln: TFA with silane



Asp, Glu Pd(0):



Lys: Pd(0)

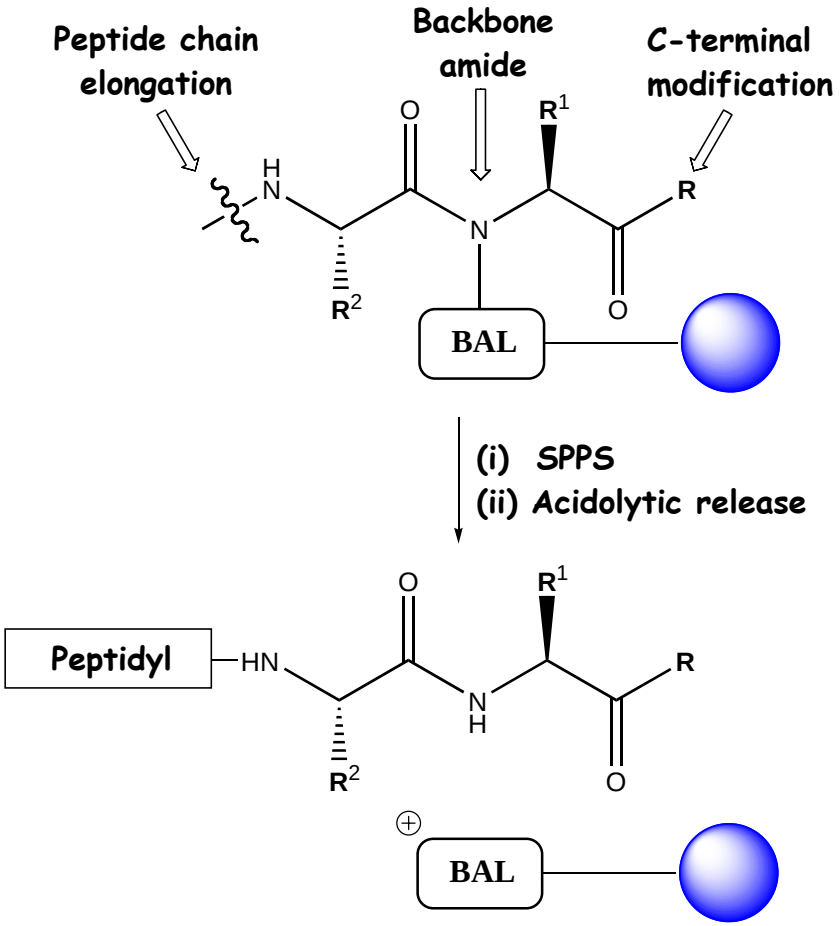
Essentials of peptide synthesis
The solid support (resin; matrix)

- **Polystyrene (PS)**
  - **Many variations**
- **PEG attached to PS**
  - **Tentagel, PEG-PS, Argonaut, etc.**
- **PEGA**

Handles (linkers)

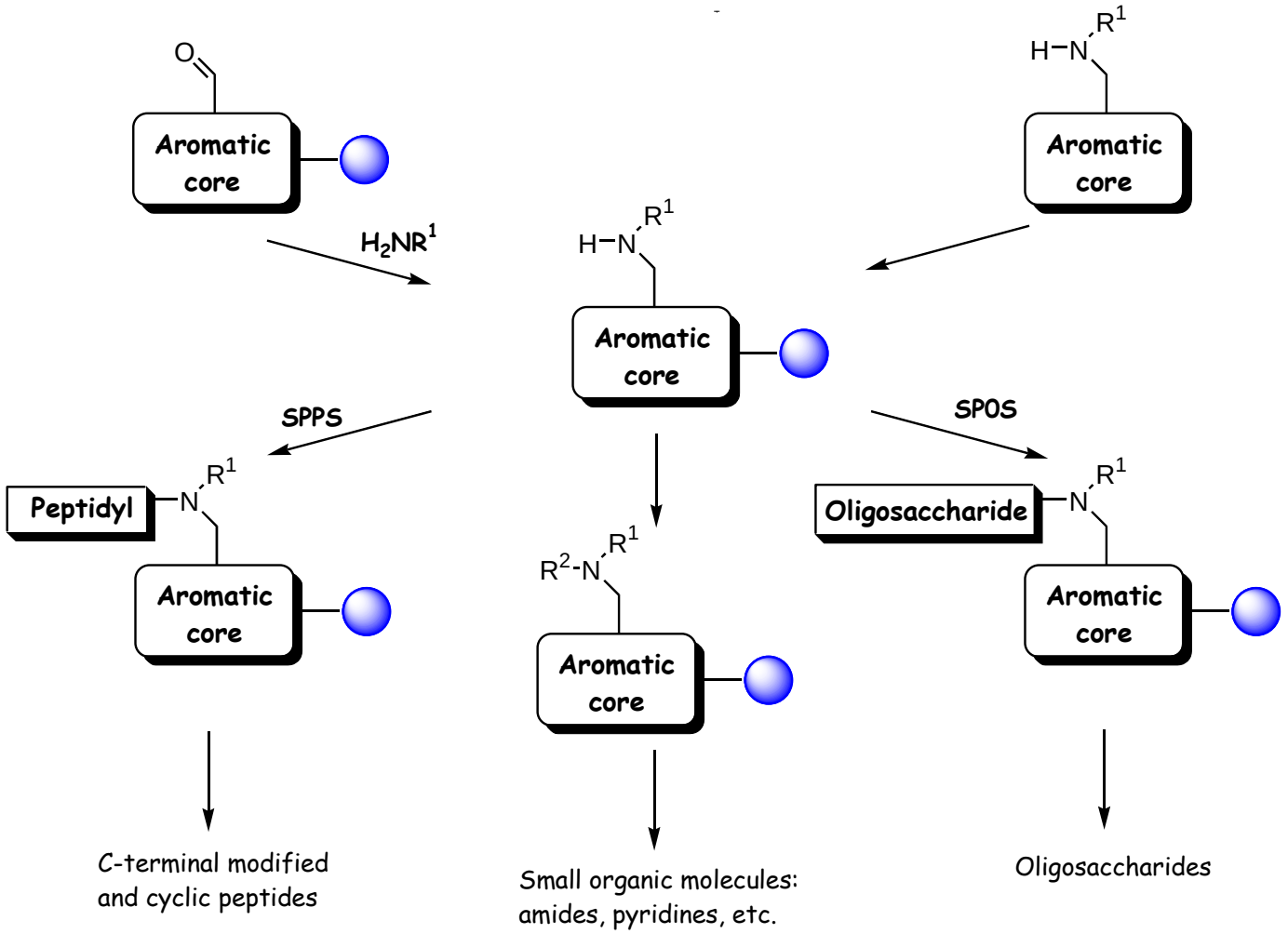
- Hydroxybenzyl: Wang
- Trityl based: ClTrt,
- Rink amide, PAL (for peptide amides)
- BAL
- HMBA (release by nucleophiles)

Backbone Amide Linker (BAL): Theme



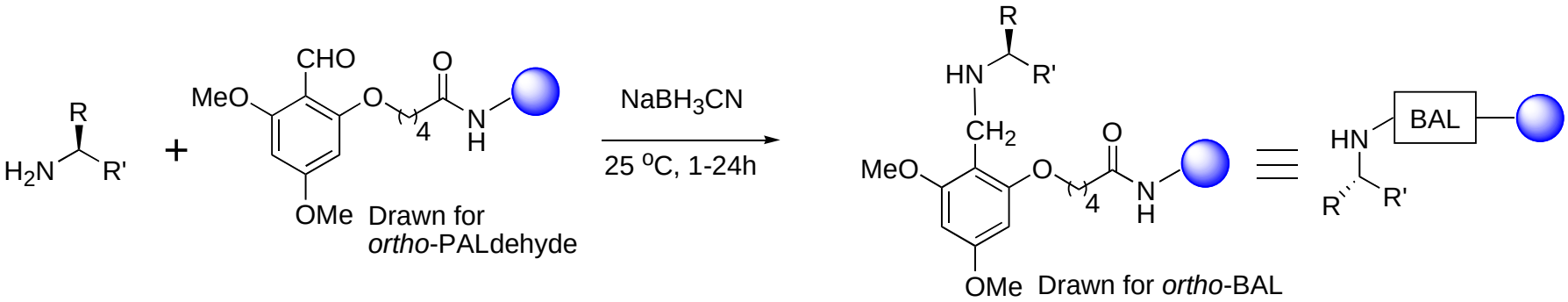
K. J. Jensen, J. Alsina, M. F. Songster, J. Vagner, F. Albericio, G. Barany, *J. Am. Chem. Soc.*, 120, 1998, 5441-5452.

Backbone Amide Linker (BAL): Theme



K. J. Jensen, J. Alsina, M. F. Songster, J. Vagner, F. Albericio, G. Barany, *J. Am. Chem. Soc.*, 120, 1998, 5441-5452.

Backbone Amide Linker (BAL)



U. Boas, J. Brask, J. B. Christensen, K. J. Jensen,
J. Comb. Chem., 2002, 4, 223-228.

Instrumentation for solid-phase Peptide synthesis

- First automated synthesizers developed in the 60's
- Semi-automated synthesizers
- Automated synthesizers
- Parallel synthesizers
- Synthesizer with microwave heating

Essentials of peptide synthesis
The problems

- **Incorporation of post-translation modifications**
  - **Phosphorylation, glycosylation, lipidation, etc**

- **Current problems in peptide synthesis:**
- **Longer peptides**
- **Aggregation during synthesis**
- **Difficult couplings/sequences**
- **Asn/Asp**
- **Dehydration**
- **Peptide thioesters etc.**
- **Arrays etc.**

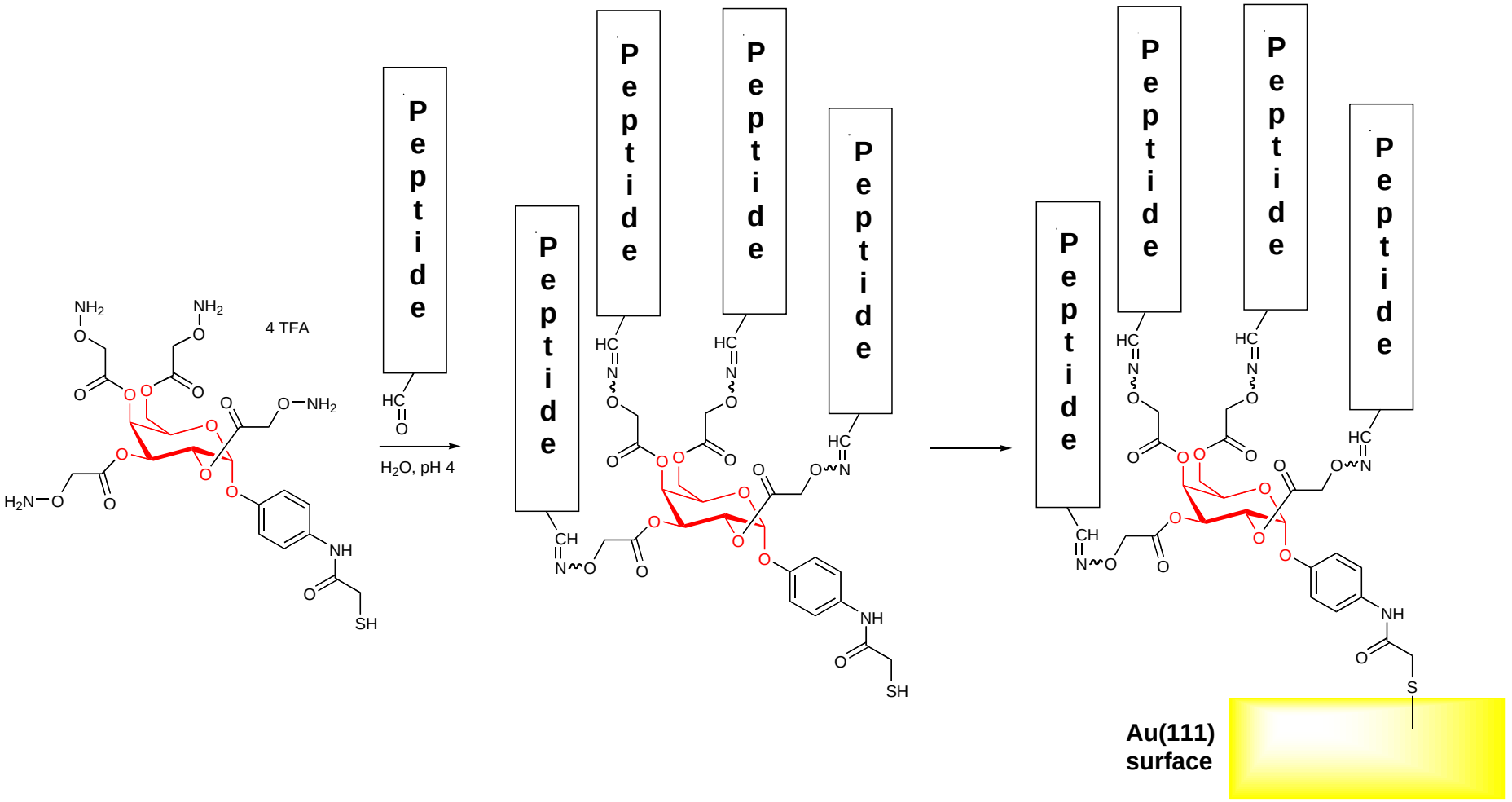
Why do we need heating in SPPS?

- In a globalized world, follow a NASA motto:
- Faster, better, cheaper

- Aggregation during synthesis: low yields if any; complex product mixtures

- Non-amide bond formation: other types of chemistry

Synthetic proteins by oxime ligation: Requires peptide aldehydes



J. Brask, H. Wackerbarth, K. J. Jensen, J. Zhang, I. Chorkendorff, J. Ulstrup
J. Am. Chem. Soc., 125, 2003, 94-104.

Why do we need heating in SPPS?

- Cool nanobioscience
- Enabled by synthesis of cool peptides used for assembly of *de novo* designed proteins
- The next slide shows how peptides can be used to study electron transport at the single molecule level
- Cool peptides synthesized using microwave heating

In situ STM image of synthetic dimeric Protein: single-molecule detection

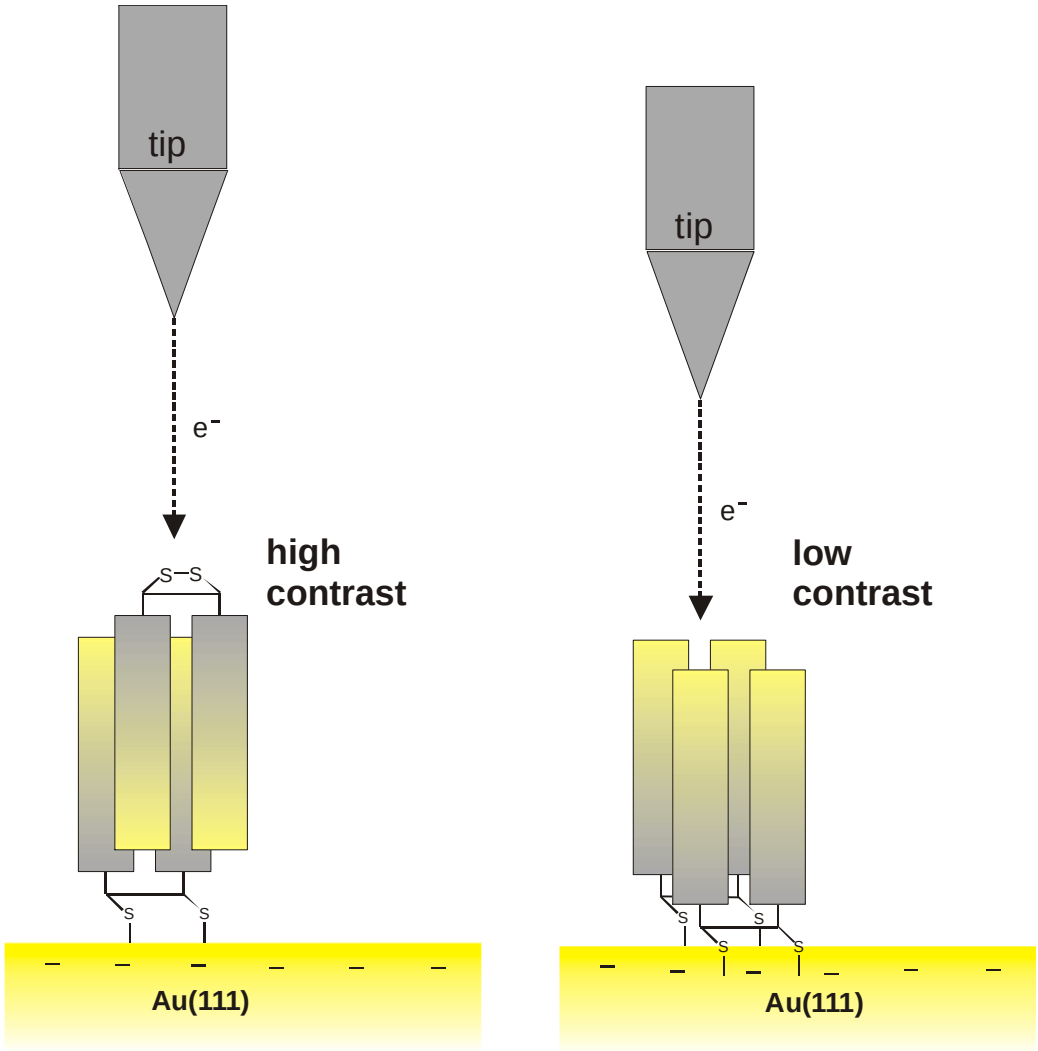


Illustration of 2x2 helix bundle protein adlayer adsorbed on the Au(111) surface. The parallel and antiparallel conformation of the 2x2 helix bundle protein and the electron flow from the tip to the Au(111) substrate are indicated

H. Wackerbarth, A. P. Tofteng, I. Chorkendorff, J. Ulstrup, K. J. Jensen, *Langmuir*, 2006, in press

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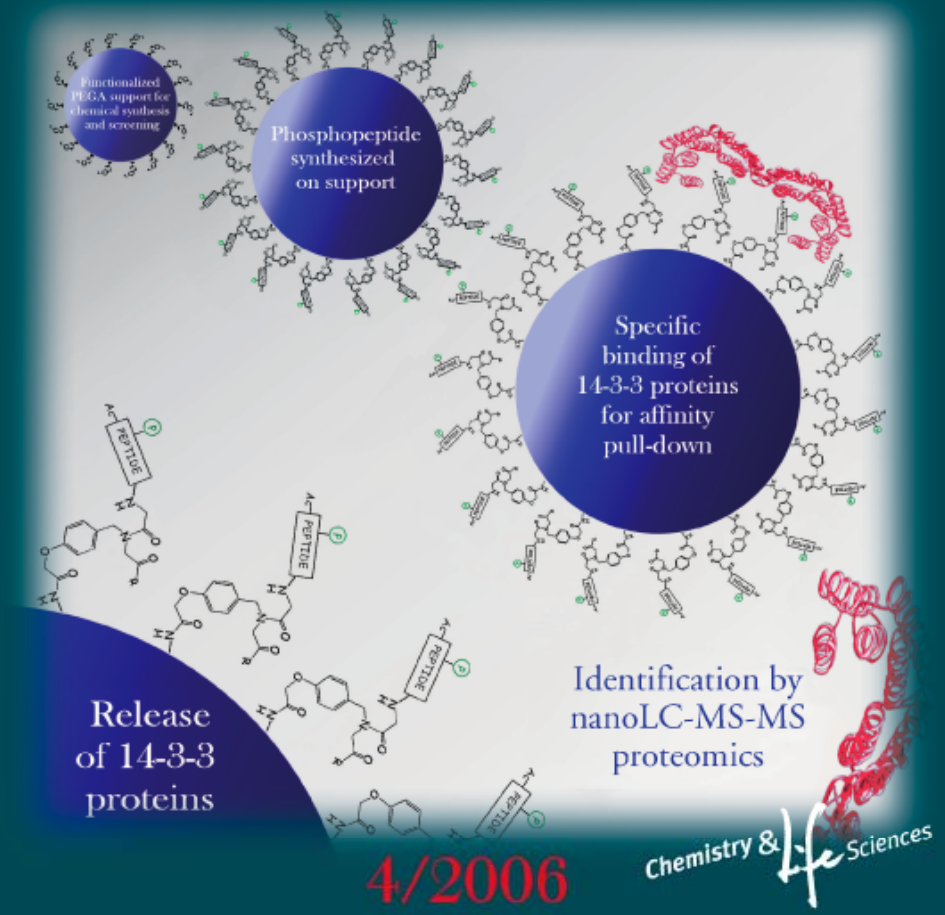


4/2006

EUChemSoc

Review: Dynamic Structural Investigations on the *Torpedo* Nicotinic Acetylcholine Receptor by Time-Resolved Photoaffinity Labeling
Plus Original Contributions

WILEY-VCH

Phosphopeptide proteomics

- Affinity pull-down of phosphopeptide binding proteins
- Solid-phase synthesis of peptides on PEGA suitable for pull-down
- Cell lysates
- Transfected and endogenous 14-3-3ζ
- SDS-PAGE gel
- Digested with trypsin
- Identification by nanoLC-MS-MS

Microwave heating for SPPS

- Precise heating during solid-phase synthesis
- Biotage Initiator
- Dedicated solid-phase vial from Biotage
- Manual SPPS with heating to 60 °C.

A. Pernille Tofteng Shelton et al., in preparation
M. Brandt, S. Gammeltoft, K. J. Jensen, Int. J. Pept. Res. Ther, 2006, *in press*

Microwave heating for SPPS: Reactions

- Coupling of handle (linker)
- Reductive amination
- Coupling with symm. anhydride
- Amide bond formation
- Fmoc deprotection

M. Brandt, S. Gammeltoft, K. J. Jensen, Int. J. Pept. Res. Ther, 2006, *in press*

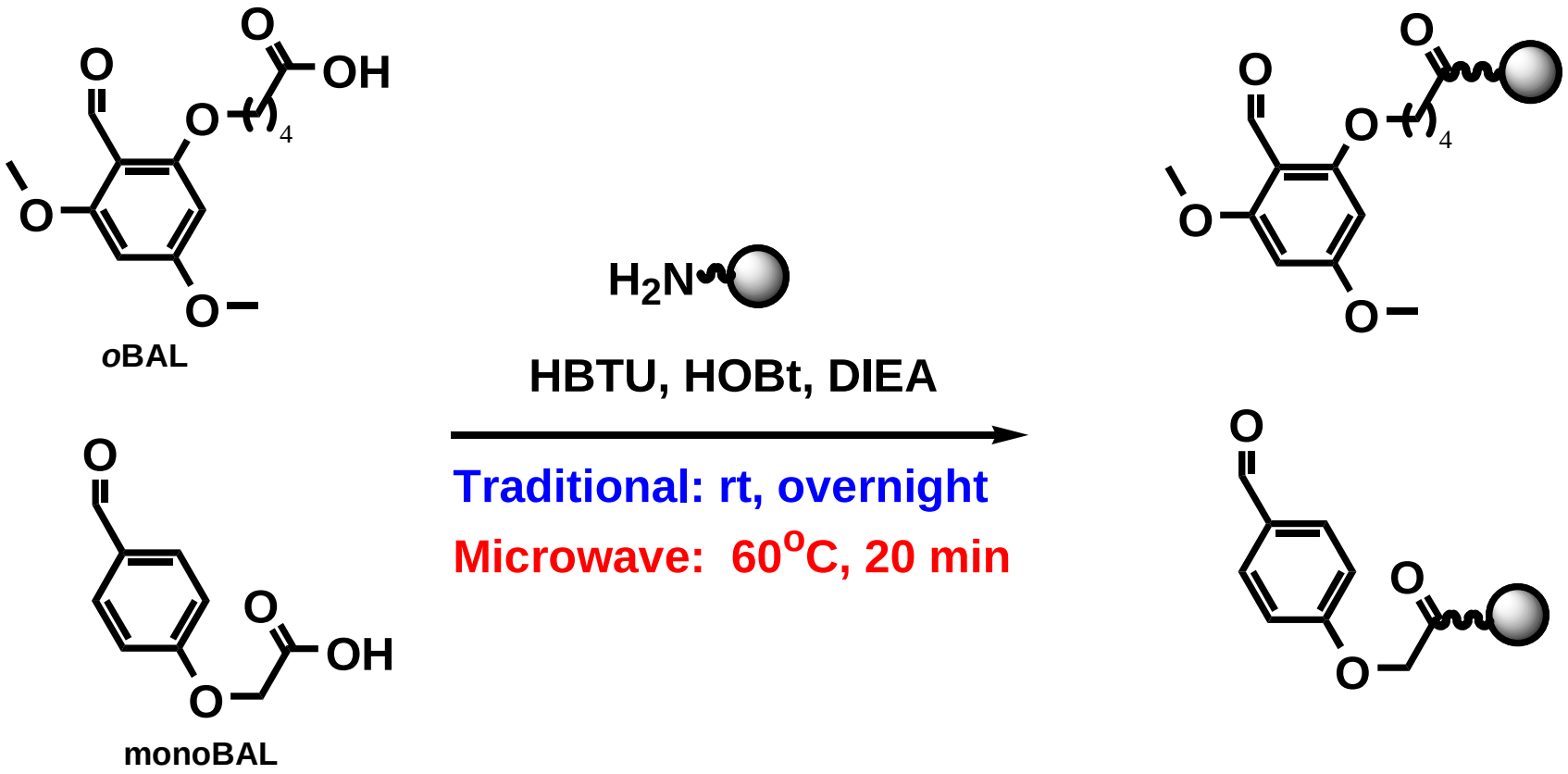
Microwave heating for SPPS: Results

No.	Sequence	Calc. MS	Found	Assigned
1	**Fmoc-YGGFL-NH_2**	776.35	777.36 - 777.40	$[M + H]^{+}$
2	**Ac-PFRGRSR-pS-APPNLGG-NH_2**	1688.83	845.37 - 845.45	$[M + 2H]^{2+}$
			563.91 - 563.93	$[M + 3H]^{3+}$
3	**Ac-PFRGRSRSAPPNLGG-NH_2**	1609.85	805.40	$[M + 2H]^{2+}$
			537.26	$[M + 3H]^{3+}$

Three test sequences were synthesized, one of which was a phospopeptide for a proteomics project

M. Brandt, S. Gammeltoft, K. J. Jensen, Int. J. Pept. Res. Ther, 2006, *in press*

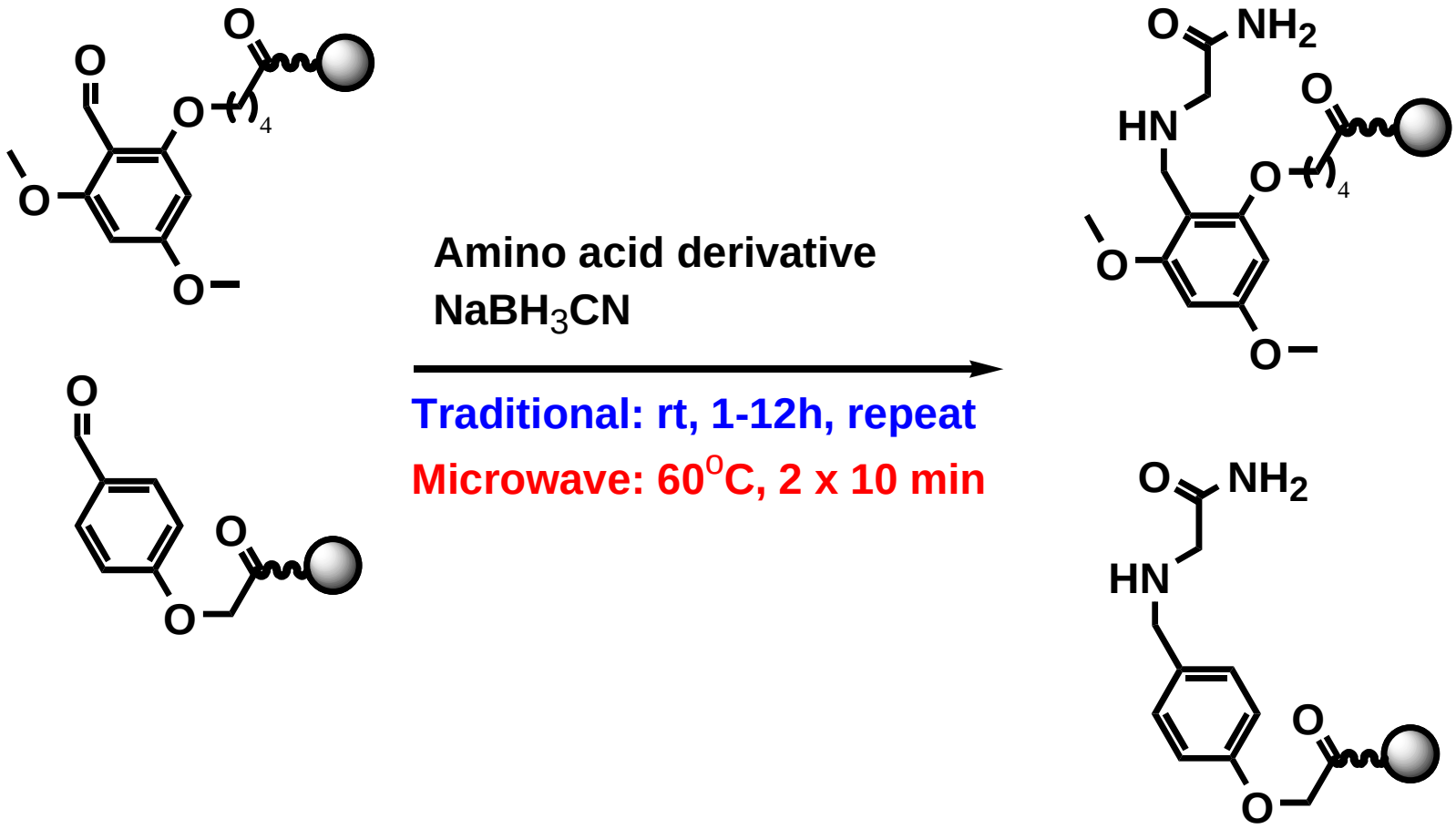
Anchoring of handle



A. Pernille Tofteng Shelton et al., in preparation

M. Brandt, S. Gammeltoft, K. J. Jensen, Int. J. Pept. Res. Ther, 2006, *in press*

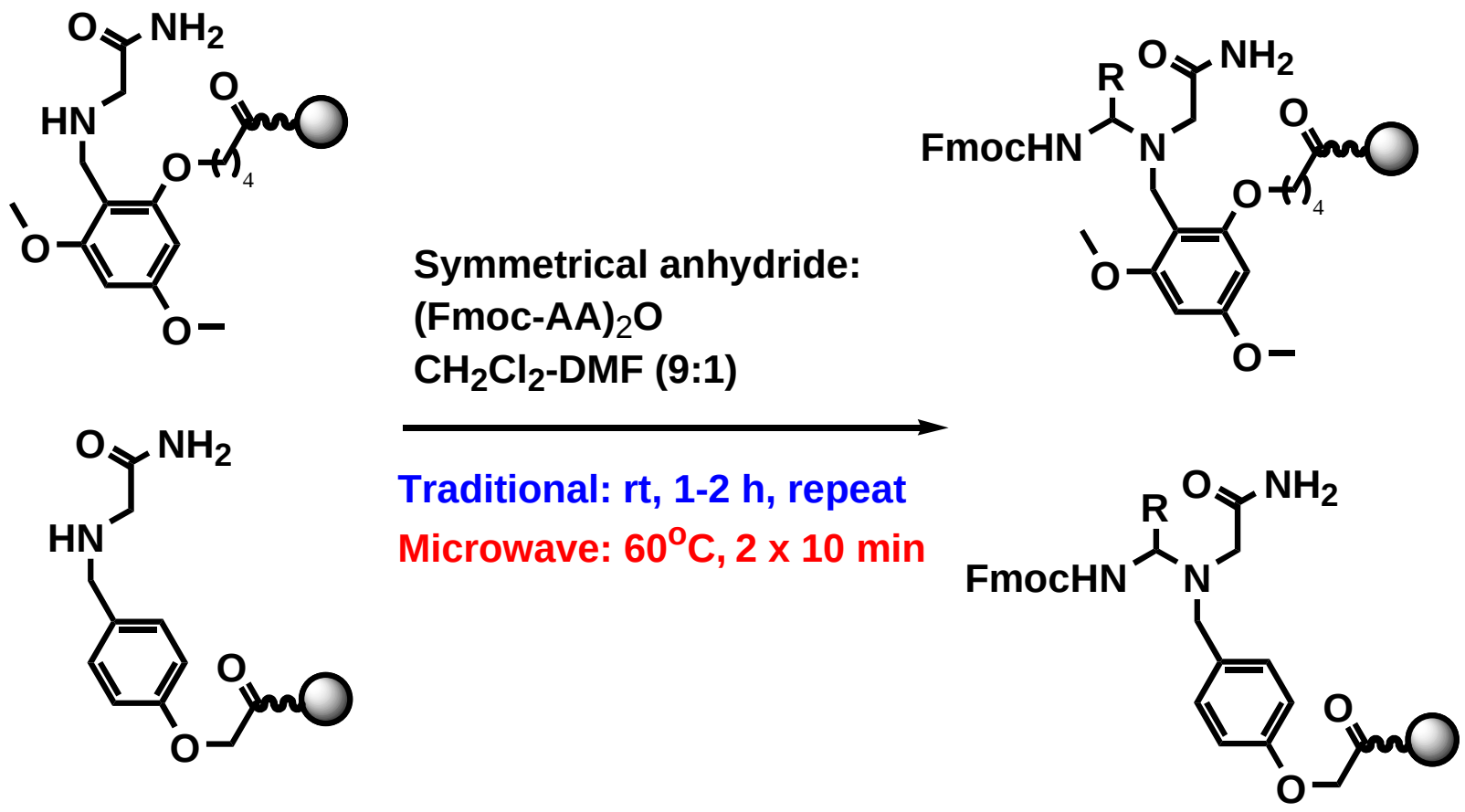
Reductive amination of handle



A. Pernille Tofteng Shelton et al., in preparation

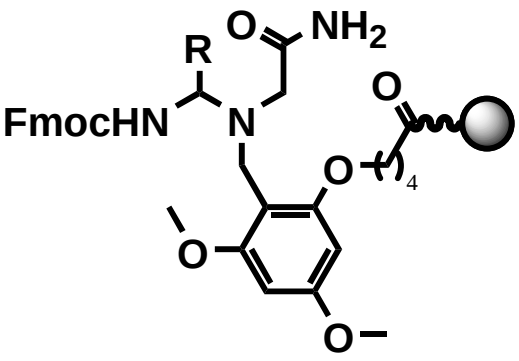
M. Brandt, S. Gammeltoft, K. J. Jensen, Int. J. Pept. Res. Ther, 2006, *in press*

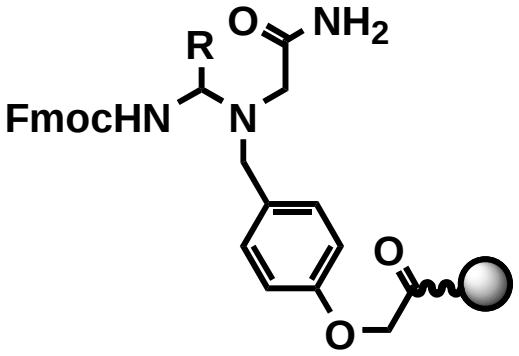
Acylation of BAL secondary amine



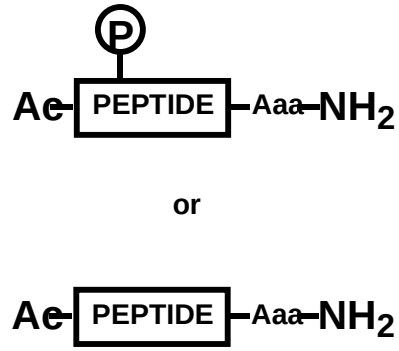
M. Brandt, S. Gammeltoft, K. J. Jensen, Int. J. Pept. Res. Ther, 2006, *in press*

Peptide chain elongation: oBAL, monoBAL



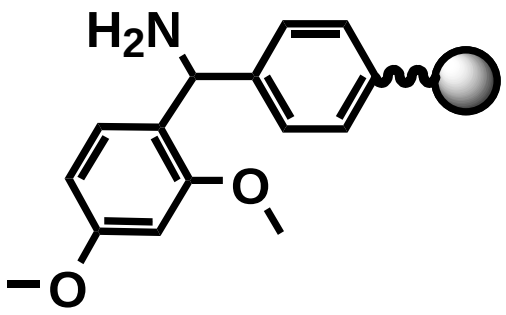


(1) Peptide synthesis
(mw, 60^{O}C, 2-10 min)
(2) N-terminal capping
(3) Removal of protecting groups and release from support (Rink)
(4) Removal of protecting groups (monoBAL)
(5) Release from support (monoBAL)



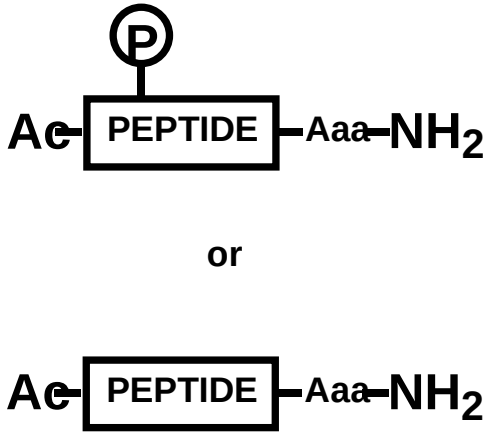
M. Brandt, S. Gammeltoft, K. J. Jensen, Int. J. Pept. Res. Ther, 2006, *in press*

Peptide chain elongation: Rink amide linker



Rink Amide Linker

(1) Peptide synthesis
(mw, 60°C, 2-10 min)
(2) N-terminal capping
(3) Removal of protecting groups and release from support (Rink)



M. Brandt, S. Gammeltoft, K. J. Jensen, Int. J. Pept. Res. Ther, 2006, *in press*

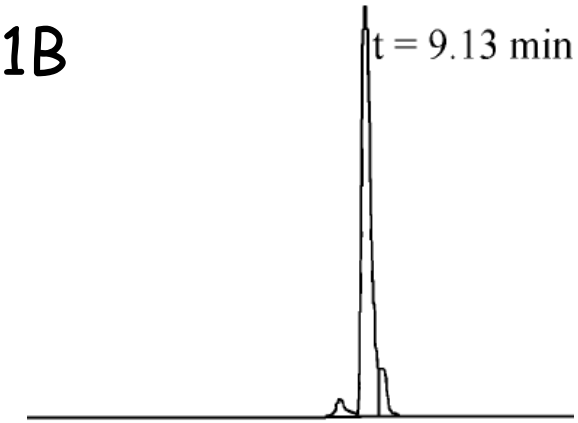
Synthesis of peptide 1: Fmoc-YGGFL-NH_2

Peptides	Handle (linker)	Resin	Fmoc-Deprotection	Coupling	Cleavage/ Release (TFA-H_2O)	Purity[a] (%)
Fmoc-YGGFL-NH_2 **1A**	Rink	TG	1 + 2x5 min rt	2 min	2 h, rt*	75
1B	Rink	TG	1 + 2x5 min rt	4 min	2 h, rt*	85
1C	Rink	TG	1 + 2x5 min rt	10 min	rt (2 h)* or 60°C or 80°C (5 min)	97 95 94
1D	Rink	TG	1 + 5 min mw	10 min	2 h, rt*	89
1E	Rink	PS	1 + 2x5 min rt	10 min	rt (2 h) or 60°C* or 80°C (5 min)	71 82 76
1F	*o*BAL	PS	1 + 2x5 min rt	10 min[b]	rt (2 h) or 60°C* or 80°C (5 min)	49 76 52

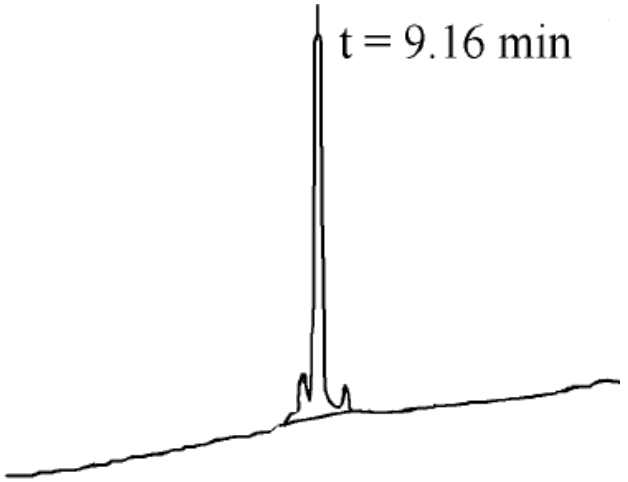
[a] HPLC at 265 nm

Peptides 1B and 1E

1B



1E



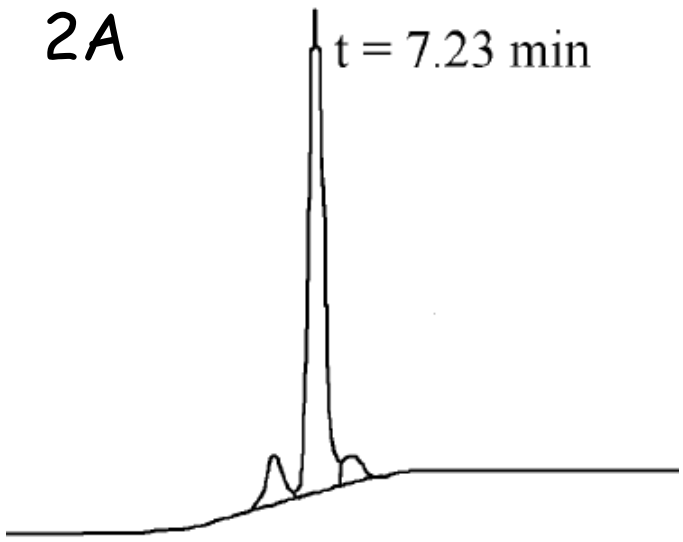
HPLC chromatograms at 265 nm of peptides released from peptidyl-resins **1B** and 1E with TFA-H_2O at rt for 2h.

Peptide 2:
Ac-PFRGRSR-pS-APPNLGG-NH_2

Peptide	Handle (linker)	Resin	Fmoc-deprotection	Coupling	Cleavage/ Release[a]	Purity[b] (%)
2A	monoBAL	PEGA	1 + 2x5 min rt	2 min[c]	1) B* 2) TFA-TFMSA	42
2B	monoBAL	PEGA	1 + 2x5 min rt	10 min[d]	1) B* 2) TFA-TFMSA	45

[a] B*: TFA-TES-H_2O (95:2.5:2.5). Cleavages at rt: 2h, for TFA-TFMSA cleavage: mw: 5 min; unless otherwise specified; HPLC at 215 nm.

Peptide 2:
Ac-PFRGRSR-pS-APPNLGG-NH_2



HPLC chromatograms at 215 nm of peptide released from peptidyl-resin **2A**.

Peptide 2:
Ac-PFRGRSR-pS-APPNLGG-NH_2

Peptide	Handle (linker)	Resin	Fmoc-deprotection	Coupling	Cleavage/ Release[a]	Purity[b] (%)
2C	Rink	PS	1 + 2x5 min rt	10 min	TFA-H_2O	39
2D	Rink	PS	1 + 2x5 min rt	10 min	1) B*[e] 2) TFA-TFMSA	47
2E	Rink	PS	1 + 2x5 min rt	10 min	B*	38
2F	Rink	PS	1 + 2x5 min rt	10 min	B*[e]	33

[a] B*: TFA-TES-H_2O (95:2.5:2.5). Cleavages at rt: 2h, for TFA-TFMSA cleavage: mw: 5 min; unless otherwise specified; [e] 20h; HPLC at 215 nm

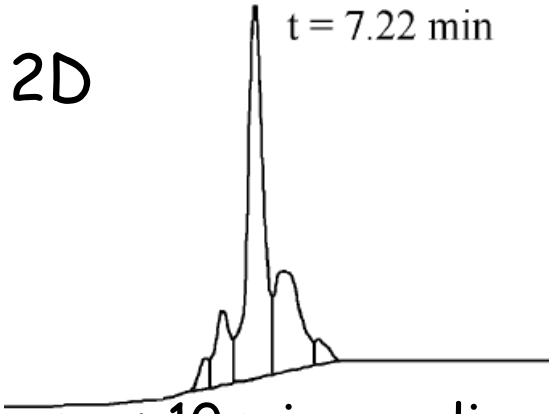
Peptide 2:
Ac-PFRGRSR-pS-APPNLGG-NH_2

Peptide	Handle (linker)	Resin	Fmoc-deprotection	Coupling	Cleavage/ Release[a]	Purity[b] (%)
2G	*o*BAL	TG	1 + 2x5 min rt	10 min[d]	B*	31
2H	*o*BAL	TG	1 + 2x5 min rt	10 min[d]	B*[f]	22
2I	Rink	TG	1 + 3 min mw	2 min mw	TFA-H_2O rt	41
2J	Rink	TG	1 + 3 min mw	2 min mw	B* rt	49
2K	Rink	TG	1 + 2x5 min rt	2 min rt	TFA-H_2O rt	23
2L	Rink	TG	1 + 2x5 min rt	2 min rt	B* rt	25

[a] B*: TFA-TES-H_2O (95:2.5:2.5). Cleavages at rt: 2h, for TFA-TFMSA cleavage: mw: 5 min; unless otherwise specified.
[e] 20h; [f] 23h; HPLC at 215 nm.

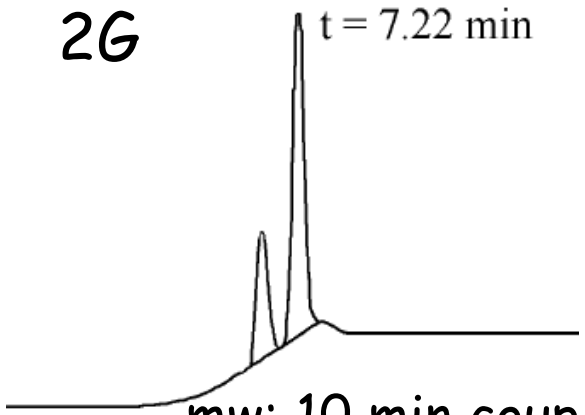
Peptide 2:
Ac-PFRGRSR-pS-APPNLGG-NH_2

2D



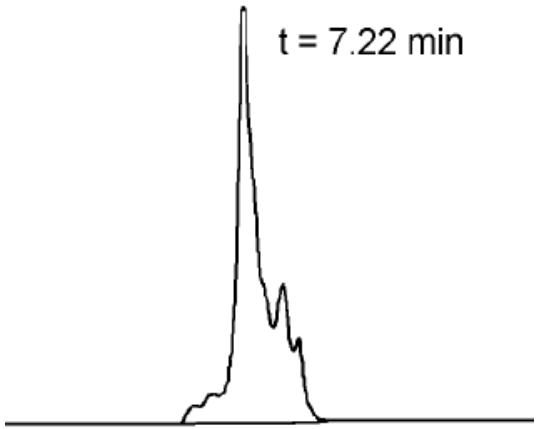
mw: 10 min coupling
(20 h B*)

2G



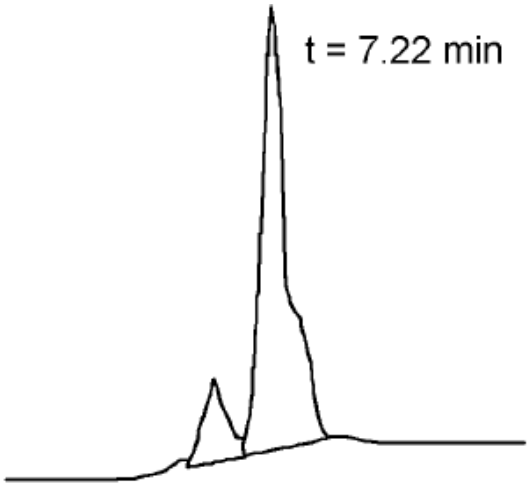
mw: 10 min coupling

2J



mw: 2 min coupling

2L



rt: 2 min coupling

Peptide 3:
Ac-PFRGRSRSAPPNLGG-NH_2

Peptide	Handle (linker)	Resin	Fmoc-deprotection	Coupling	Cleavage/ Release[a]	Purity[b] (%)
3A	Rink	TG	1 + 3 min mw	2 min mw	TFA/H_2O rt	59
3B	Rink	TG	1 + 3 min mw	2 min mw	B* rt	77
3C	Rink	TG	1 + 3 min mw	10 min	TFA/H_2O	70
3D	Rink	TG	2 + 4 min rt	3-4 min rt	TFA/H_2O rt	55
3E	Rink	TG	2 + 4 min rt	3-4 min rt	B* rt	43

[a] B*: TFA-TES-H_2O (95:2.5:2.5). Cleavages at rt: 2h, for TFA-TFMSA cleavage: mw: 5 min; unless otherwise specified.
[c] Loading of handle: 20 min; reductive amination: 2 min; symmetrical anhydride: 2 min; [e] 20h; [f] 23h

Microwave heating for SPPS: Summary

- Coupling of handle (linker)
  - RT: overnight MW: 20 min
- Reductive amination
  - RT: 2× 1-12 h MW: 2×10 min
- Coupling with symm. anhydride
  - RT: 2× 1-2h MW: 2×10 min
- Amide bond formation
  - RT: 45 min MW: 2-10 min
- Release from support: MW 5 min

M. Brandt, S. Gammeltoft, K. J. Jensen, Int. J. Pept. Res. Ther, 2006, *in press*