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CRITICAL REVIEW

Microwave heating in solid-phase peptide synthesis

Søren L. Pedersen, A. Pernille Tofteng, Leila Malik and Knud J. Jensen*

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The highly refined organic chemistry in solid-phase synthesis has made it the method of choice not only to assemble peptides but also small proteins – mainly on a laboratory scale but increasingly also on an industrial scale. While conductive heating occasionally has been applied to peptide synthesis, precise microwave irradiation to heat the reaction mixture during coupling and N^{α}-deprotection has become increasingly popular. It has often provided dramatic reductions in synthesis times, accompanied by an increase in the crude peptide purity. Microwave heating has been proven especially relevant for sequences which might form β-sheet type structures and for sterically difficult couplings. The beneficial effect of microwave heating appears so far to be due to the precise nature of this type of heating, rather than a peptide-specific microwave effect. However, microwave heating as such is not a panacea for all difficulties in peptide syntheses and the conditions may need to be adjusted for the incorporation of Cys, His and Asp in peptides, and for the synthesis of, for example, phosphopeptides, glycopeptides, and *N*-methylated peptides. Here we provide a comprehensive overview of the advances in microwave heating for peptide synthesis, with a focus on systematic studies and general protocols, as well as important applications. The assembly of β-peptides, peptoids and pseudopeptides are also evaluated in this *critical review* (254 references).

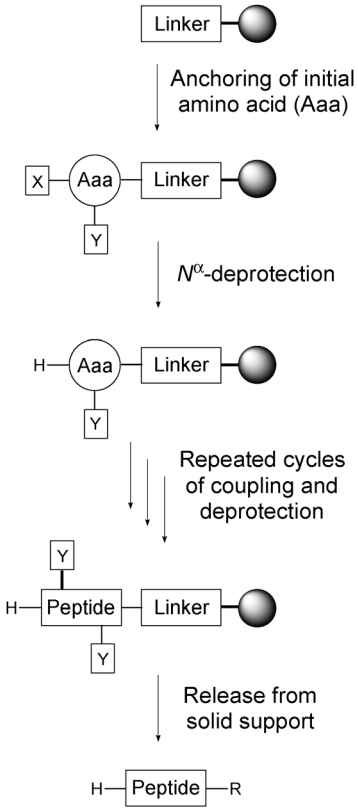
Introduction

Solid-phase peptide synthesis (SPPS) has become the primary source for synthetic peptides which are essential for research in biology, biomedicine, drug discovery and many other fields. Merrifield's introduction of functionalized solid supports, which allows for anchoring of an amino acid, revolutionized the field of peptide science and inaugurated the SPPS methodology.[1] Since then, all aspects of SPPS have been further developed and refined, thus extending the reach of synthetic peptide chemistry tremendously. SPPS is defined by the set of N^{α}-protecting groups, side-chain protecting groups, coupling reagents, linkers (handles), as well as resins and other solid supports. Suitably N^{α}- and side-chain protected amino acids are coupled sequentially to a growing peptide chain attached to a solid support (resin) in the $C \rightarrow N$ direction. Typically, the *C*-terminal amino acid is first anchored at the carboxy terminus to the solid support *via* a cleavable handle. Then, the N^{α}-protecting group can be removed without affecting the side-chain protecting groups, thus the polypeptide chain is prepared for the next coupling cycle. SPPS reactions are driven to completion by the use of soluble reagents in excess, which can be removed by filtration and washing. Following the completion of the desired sequence of amino acids, the peptide is released from the solid support, and simultaneously the semi-permanent side-chain protecting groups are typically removed concomitantly. The principle is illustrated in Scheme 1. However, the peptides that are synthetically accessible, *e.g.* their maximal length, specific post-translational modifications, and unnatural modifications, are defined by the effectiveness and limitations of this underlying organic chemistry, thus calling for further developments.

The two most widely used N^{α}-protecting groups in SPPS are the fluoren-9-ylmethyloxycarbonyl (Fmoc)[2–4] and the *tert*-butoxycarbonyl (Boc),[1,5] each defining an overall strategy for SPPS. The chemical conditions for removal of these transient protecting groups, *i.e.* base *vs.* acid, each define a 'chemical window' of opportunities for the other chemical steps in the overall SPPS strategy. The Boc strategy, initially introduced by Merrifield, requires trifluoroacetic acid (TFA) or a similar acid for repetitive removal of the Boc groups, while often relying on hydrofluoric acid (HF) for release of the assembled peptide from the support. Thus, the Boc strategy also relies on differences in acidlability of the N^{α}-and side-chain protecting groups.[1,5] The Fmoc strategy is often preferred over the Boc strategy for routine synthesis, as the latter normally requires the use of corrosive and toxic HF and the necessity for a HF apparatus. The Fmoc group can be removed under mild conditions with secondary amines, typically 1 : 4 piperidine–DMF (Scheme 2).[6,7]

The semi-permanent protecting groups (side-chain protecting groups) for the Fmoc strategy have been extensively refined during the past decades. For trifunctional amino acid residues, *e.g.* Cys, Asp, Glu, Lys, and Arg side-chain protection is essential

University of Copenhagen, Faculty of Life Sciences, IGM, Section for Nanobioscience, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark. E-mail: kjj@life.ku.dk



Scheme 1 The principle of SPPS. X: Temporary N^{α}-protecting group. Y: Semi-permanent side-chain amino acid (Aaa) protecting groups. R: *C*-terminal functionality, typically OH or NH_2.

for successful peptide synthesis. The generally used protecting groups are: *tert*-butyl (*t*-Bu) for Glu, Asp, Ser, Thr, and Tyr; 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for Arg; and trityl (Trt) for Cys, Asn, Gln, and His.

It is a prerequisite that the activated carboxylic acid moiety formed from the amino acid and the coupling reagent is able to react with the N^{α}-amino group of the growing peptide chain (Scheme 3). Carbodiimide-based coupling reagents, such as DCC (*N*,*N*′-dicyclohexylcarbodiimide)[8–10] or DIC (*N*,*N*′-diisopropylcarbodiimide),[11] were amongst the first coupling reagent introduced and have been used for decades. Potential side-reactions with carbodiimide-based reagents is the $O \rightarrow N$ rearrangement of the *O*-acylisourea intermediate and 'over-activation' by formation of the symmetrical anhydride,[12] thus carbodiimides are used in combination with auxiliary nucleophiles such as HOBt or HOAt. Among carbodiimides, DIC is preferred over DCC in automated syntheses.

Auxiliary nucleophiles, such as HOBt (1-hydroxybenzotriazole),[13] ensure that the optical integrity of the stereogenic center at the *C*-terminal of the activated amino acid residue is maintained throughout the coupling step (Scheme 3). Numerous coupling reagents have been developed to reduce coupling time and minimize epimerization, since the carbodiimide-based coupling reagents were introduced – the most important are HBTU (*N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide),[14,15] HATU (*N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-*b*]pyridine-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide),[15] PyBOP (1-benzotriazolyloxy-tris-pyrrolidinophosphonium hexafluorophosphate)[16] and the novel COMU

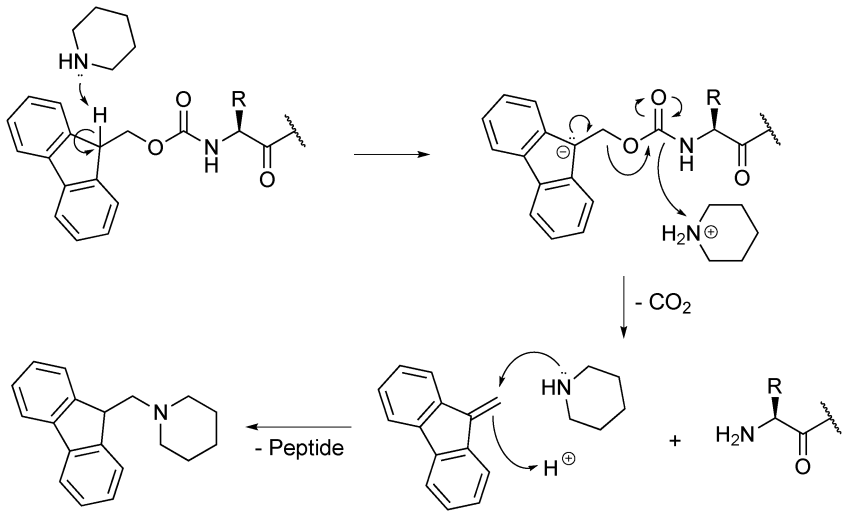
A. Pernille Tofteng, Knud J. Jensen, Leila Malik, Søren L. Pedersen

Dr Søren Ljungberg Pedersen (right) earned his MSc degree in Pharmaceutical Chemistry in 2005 from the University of Southern Denmark, Faculty of Natural Sciences and PhD in Bioorganic Chemistry in 2010 from University of Copenhagen, Faculty of Life Sciences in collaboration with Rheoscience. Subsequently he undertook post-doctoral studies with Prof. Knud J. Jensen in the Lundbeck Foundation Center for Biomembranes in Nanomedicine (CBN) at University of Copenhagen. His research interests are biopharmaceutical drug development, from peptide drug design and synthesis to chemical protein modification, as well as new applications for solid-phase peptide chemistry.

Dr Anne Pernille Tofteng (left) obtained her MSc degree in Chemical Engineering from the Technical University of Denmark in 2004. After this, she earned her PhD from the University of Copenhagen for her work on the total chemical synthesis and biomimetic folding of insulin and its analogues in collaboration with Novo Nordisk. Since completing her PhD in 2008 she has worked as a post-doctoral scientist with Prof. Knud J. Jensen at the University of Copenhagen developing new strategies for solid-phase peptide synthesis. Her research interests include the design and synthesis of pharmaceutical peptides and proteins, microwave-assisted peptide synthesis and novel chemical strategies for peptide modification.

Leila Malik (second from right) obtained her MSc degree in Organic Chemistry from the University of Copenhagen. She subsequently worked within the pharmaceutical industry and has several years of experience in medicinal chemistry from companies such as Novo Nordisk, Denmark, Isis Pharmaceuticals, California (2001) and Acadia Pharmaceuticals, Sweden (2008). Currently, she is finishing her PhD with Prof. Knud J. Jensen at University of Copenhagen. Her research interests range from drug discovery using synthetic organic chemistry to peptide based drug design and chemical protein modification.

Prof. Knud J. Jensen (second from left) holds degrees in Organic Chemistry and Philosophy from the University of Copenhagen. He obtained a PhD degree in Bioorganic Chemistry with Prof. Morten Meldal in 1992. He did postdoctoral research with Prof. George Barany, University of Minnesota. Afterwards, he became Assistant Prof. at the Technical University of Denmark in '97 and Associate Prof. at the Royal Veterinary and Agricultural University in Copenhagen, which in 2007 became part of the University of Copenhagen. The same year he was promoted to full Prof. in Nanobioscience. His research covers a broad range of topics in synthetic bioorganic chemistry and nanobioscience.

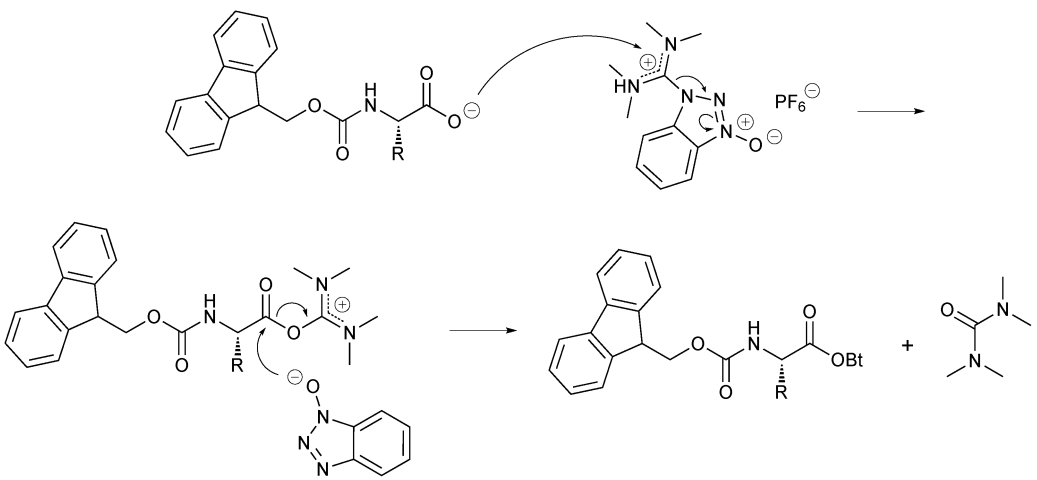


Scheme 2 N^{α}-deprotection by piperidine as base and nucleophilic scavenger.

(1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholino-methylene)]methanaminium hexafluorophosphate)[17] reagents (Fig. 1).

The amino acid protecting groups, coupling reagents, and resins have been refined over the last three decades and they are now very efficient which allows their use in routine syntheses. Moreover, the development of commercially available automated peptide synthesizers has come a long way enabling a high degree of predictability and reproducibility. There is a growing expectation that SPPS in the near future will be able to reliably provide small proteins by direct synthesis. However, low purities and sometimes even failure in achieving the desired peptide sequence is still a frequently occurring problem – especially as the peptide becomes longer. The main reasons are believed to be steric hindrance and intra- and intermolecular aggregation. Amino acids which are prone to form β-sheets often lead to aggregation during peptide strand elongation most likely due to their hydrogen bonding and hydrophobic properties (especially peptides containing a high proportion of Ala, Val, Ile, Asn or Gln).[18–23] These problems often lead to premature terminations or deletions of the elongating peptide sequence that tend to be very tedious to purify on a preparative scale. Intermolecular aggregation often leads to poor solvation of the peptidyl-polymer, but it is less pronounced when resins with a low-loading are being used.

Several strategies to suppress or reduce on-resin aggregations have been described and include pseudoprolines,[24] solvent composition,[25] and chaotropic salts,[26,27] however, the utility is limited and the efficiency is variable. Heating has emerged[28–33] as an additional parameter in SPPS and is likely to reduce both inter- and intramolecular aggregation, consequently leading to reduced coupling times and improved coupling efficiency of bulky and β-branched amino acids. Is heating above room temperature during the chemical synthesis of peptides a good idea? After all, peptides are complex molecules with many functional groups and a propensity for degradation. However, in a biological context, thermophiles are active at temperatures well above 40 °C and their proteins are compatible with these elevated temperatures. Until recently, with only a few exceptions, synthesis of peptides was performed at room temperature or below ambient, *e.g.* for epimerization-prone reaction. It is tempting to say that peptide chemistry had been developed for room temperature. However, as described in the following,



Scheme 3 The amino acid activation mechanism using HBTU and HOBt.

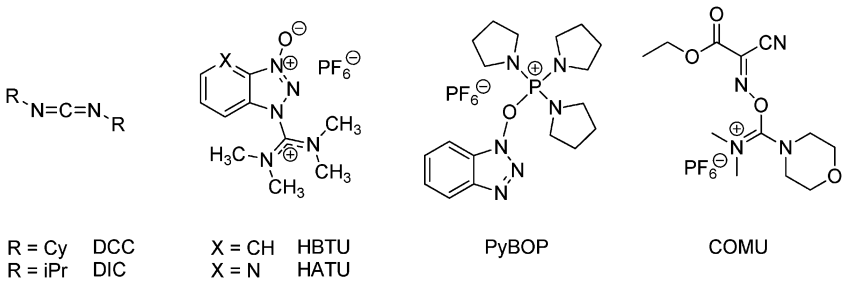


Fig. 1 Commonly used coupling reagents.

SPPS is generally very compatible with elevated temperature. Nevertheless caution should be taken with, for example, epimerization-prone reactions.

Conductive heating had sporadically been applied to SPPS over the last decades, especially in the coupling step. For example, Varanda and Miranda synthesized the acyl carrier protein fragment 65–74 (ACP(65–74)) and the un-sulfated cholecystokinin-8 as model peptides at different temperatures, and found 60 °C to be the most appropriate for the coupling steps.[32] Kaplan and co-workers synthesized long peptides (84–107 residues) using elevated temperatures both in the N^{α}-deprotection (40 °C) and the coupling (55 °C) steps.[29] Following this initial work, several reports described the use of elevated temperature for peptide couplings (30–80 °C).[28–33] Wang and co-workers described the SPPS of ACP(65–74) as well as two other peptides using a slightly modified domestic microwave oven and showed improved coupling yields at elevated temperature.[34] During the 1990s the use of microwave heating in organic synthesis evolved as a new parameter for improving both the reaction speed and yield, and several special microwave instruments for organic synthesis became commercially available. Erdélyi and Gogoll showed that special microwave reactors indeed could be used to improve speed and purity in SPPS. Moreover, the data showed that the solid support did not degrade under the microwave-assisted synthesis.[35] Publications describing microwave-assisted SPPS have within the last decade confirmed the initial findings of Erdélyi and Gogoll. Impressive improvements both in the speed of the coupling and N^{α}-deprotection reactions as well as in terms of crude peptide purity have been reported.[35–89] Applying microwave heating to direct and linear SPPS methodology has resulted in the synthesis of a considerable number of peptides and proteins up to at least 109 amino acid residues.[35–129]

Microwave synthesizers

As described above, the initial studies describing microwave-assisted SPPS utilized modified domestic microwave ovens, which did not allow for precise control of the microwave irradiation, leading to in-homogenous heating.[34] Two different microwave reactor designs are currently available for organic synthesis: multimode and monomode (single-mode) reactors. The microwaves in a multimode instrument (in principle like in a domestic microwave oven) are reflected by the walls of the relatively large cavity, which can generate pockets of high and low energy as the moving waves either reinforce or cancel out each other leading to a non-homogenous microwave field in the cavity. Thus, to ensure that the field is as homogeneous as possible most multimode instruments are equipped with a mechanical mode stirrer that continuously changes the instantaneous field pattern inside the cavity. In the monomode cavities a standing wave is created when the electromagnetic irradiation is passed by a waveguide that directs the microwaves directly through the reaction vessel that is positioned in a fixed distance from the radiation source. The key differences between the multimode and monomode reactors are that the multimode can perform parallel synthesis, but suffers from inhomogeneous heating in the cavity, in contrast, the monomode reactor provides a high degree of reproducibility due to a very precise heating.

The first report, by Erdélyi and Gogoll, describing SPPS in a special microwave reactor for organic synthesis was performed using a synthesizer from Personal Chemistry.[35] Also the Green Motif I microwave synthesis reactor (IDX corp.),[66,67,70] the Milestone CombiChem microwave synthesizer[130] and the Prolabo Synthwave 402 monomode microwave reactor[131] have been utilized for microwave-assisted SPPS. In 2003, the first dedicated microwave-assisted peptide synthesizer was introduced (Liberty, CEM), which is a completely automated valve-based system.[132] A manual system, from the same company, is also available (Discover SPS), in which the reactor is manually moved back and forth between the microwave reactor and a washing station.[132] In 2006 Brandt *et al*. reported the use of a Biotage Initiator for manual peptide synthesis,[88] which was later developed further to perform semi-automated synthesis with manual addition of amino acids and automated washing and N^{α}-deprotection.[44] In 2010 the first automated X-Y robot for peptide synthesis with microwave heating (Syro *Wave*™, Biotage) was introduced commercially. This system is a valve-free instrument which is very flexible due to its ability to perform either single-mode microwave-assisted SPPS or conventional parallel SPPS at room temperature.[45,133] Moreover, the mode of stirring in the two systems is different, as the Liberty uses nitrogen bubbling, while the Syro *Wave*™ vortexes within the cavity. Finally, the two instruments have different reactor sizes (CEM Liberty 10–125 mL, Biotage Syro *Wave*™ 2–10 mL).

Parallel microwave-assisted peptide synthesis has been reported by Murray and Gellman for library synthesis of β-peptides (*vide infra*) using 96-well polypropylene filter plates inside the multimode microwave cavity (MARS, CEM).[134,135] Multi-well polypropylene filter plates are heat stable under the conditions used for microwave-assisted SPPS. Even though the temperature is controlled by an internal fiber-optic sensor,

the setup suffers from in-homogeneity throughout the plate, as a temperature variation of ±5 °C at different positions in the 96-well plate has been observed.[135] The plate is manually moved to a draining station in between microwave irradiated reactions and the reagents as well as wash solutions are manually added.[134,135]

Microwave theory and effect

In conductive heating, energy is transferred to the reaction mixture *via* convective currents or thermal conductivity, however, microwave heating (dielectric heating at 2.45 GHz) occurs by disposing the energy directly to the solvent (and some reagents), due to interactions of the material with the alternating electric field. Materials interact with the electromagnetic field differently, *i.e.* materials store and convert the energy to heat to different extents, which have huge impact on their ability to be heated by microwaves. *N*,*N*-Dimethylformamide (DMF) and *N*-methyl-2-pyrrolidinone (NMP), the most common solvents for both coupling and N^{α}-deprotection in SPPS, have a very good ability to be heated by microwaves (loss factor: tan δ 0.161 and tan δ 0.275, DMF[136] and NMP[137] respectively), and the addition of amino acids and coupling reagents to NMP has an additional positive influence on the microwave absorption.[138] Besides the solvent, several other factors influence the dielectric properties, such as sample volume, vessel material, and the mode of stirring, *i.e.* vortexing or N_2 bubbling. Microwave-assisted SPPS without mixing leads to inhomogeneous temperature distributions in the reactor vessel, as the common solvents, DMF and NMP, strongly absorb microwave energy which may give short microwave penetration, and the solvents are furthermore viscous and the addition of a resin will increase the viscosity. As the most common commercially available monomode microwave synthesizers measure the temperature by an IR sensor (the Discover SPS and the Liberty from the bottom and the Syro *Wave*™ from the side at a defined height) the mixing is of high importance for precise temperature measurement. Kappe and co-workers have shown huge differences between the temperature measured by IR and internal fiber-optic probes in the absence of adequate mixing.[138–140] Moreover, the group of Martinez has demonstrated the importance of mixing, by synthesizing a model nonapeptide with and without the use of magnetic stirring during microwave-assisted SPPS – major differences in crude peptide purity was shown, 76% *vs.* 42%, respectively.[77] It has to be noted that standard magnetic stirring is not recommended during SPPS due to the risk of grinding of the solid support, however, it might be preferred over un-mixed reactions.

Several reports speculate that microwaves to a significant extent interact directly with amide dipoles of the dipole moments in peptides and that this effect causes direct heating of peptides, as opposed to indirect, thermal heating by contact with the solvent molecules. It is then speculated that this could explain why microwave heating in SPPS often is not only faster but also provides higher purities compared to conventional room temperature SPPS.[85,141,142] This hypothesis is difficult to address in an experimentally precise manner. However, Bacsa *et al.* addressed this question by comparing microwave heating with conventional heating during SPPS.[138] Three peptides, varying in length from 9 to 24 AA, were tested by microwave and oil-bath heating at 86 °C on two different resins and resulted in similar crude purities. Moreover, the degree of epimerization and the impurity profiles were identical for the two peptides (microwave *vs.* conventional heating). Finally, the 60 °C increase in temperature (ambient to 86 °C) leads to an estimated 50-fold increase in reaction rate from both processes, in agreement with the Arrhenius equation, thus this kinetic effect is probably responsible for providing peptides in high speed and purity.[138]

Peptide couplings

Fmoc and Boc strategies have successfully been applied together with microwave-irradiation to increase reaction rates and crude peptide purity. In by far the most papers on microwave-assisted SPPS, variants of the Fmoc strategy have been used. The performances of all the most common coupling reagents has been investigated together with microwave heating and only the thermal stability of the auxiliary nucleophile Oxyma (ethyl 2-cyano-2-(hydroxyimino)acetate) has been reported to be relatively low when subjected to heating[143] In early work by Erdélyi and Gogoll, brief heating to 110 °C during coupling was reported.[35] However, the majority of controlled microwave-assisted peptide couplings were performed at temperatures in the range 50–80 °C, except for amino acids with an increased risk of epimerization which often were coupled at lower temperatures (<50 °C).[85]

Systematic studies where coupling reagents, temperature and time were varied have been published.[35,39,44,45,47,51,61,74,76,85,87,88,138,144,145] Coupling reagents show almost identical ranking in performance at elevated temperature as at room temperature, however, the 'uronium' type coupling reagent COMU has been reported to considerably outperform the classical HBTU and HATU in the synthesis of an Aib containing pentapeptide,[61] and PyBOP, DIC, TSTU, HCTU, HBTU as well as HATU in the synthesis of the *C*-terminus of the MuLV CTL epitope.[45,133] Our literature survey showed that the most common coupling reagents and additives used in conventional SPPS were also those which were applied in microwave-assisted SPPS. Typically the amino acids and coupling reagents were used in 3–5 molar excess, however, for difficult sequences up to 10 molar excess has been reported (Table 1).[35–40,42,44–55,57–71,73–88]

In a few cases, a protocol where intermediate cooling was applied has been used and mainly performed on a manual microwave instrument, the Discover SPS (CEM). The reaction mixture was cooled to −10 °C in an ice–ethanol solution, followed by irradiation with 100 watts for 5 s which gave a temperature of ~30 °C, and the cycle was repeated 5 times.[146–149] This alternative protocol was expected to suppress different side-reactions and epimerization, however, to our knowledge no thorough study has been reported describing the effects of intermediate cooling.

Only relatively few publications describe the synthesis of long peptides or proteins, although in such cases microwave irradiation should be especially valuable. First, β-amyloid(1–42) was successfully synthesized using microwave heating (Table 1, entry 13 and 14).[45,46] This peptide is a current example of a long

Table 1 Overview of peptides assembled by microwave-assisted solid-phase synthesis and the coupling conditions used

		Coupling					
Entry	Peptide	Coupling reagents	Equiv.	Time/min	Temp./°C	Crude purity (%)	Ref.
1	Cyclotides	HBTU	4	5	75	—	36
2	Granzyme C substrates	HCTU	5	5	75	—	37
3	*N*-methyl tri-peptide	DIC/HOAt	3	2×10	75	83	38
4	PTHrP1-34	TBTU	5	5	75	77	39
5	Human islet amyloid polypeptide	HBTU	5	5	75	75	40[a]
6	Dicarba hGH fragment	HBTU/HOBt	—	10	75	—	41[a]
7	Exon 1 huntingtin peptide	HBTU	4	2×5	75	—	42[b]
8	Affibody	HBTU	10	4	—		43
9	MuLV CTL epitope, *C*-terminus	HBTU/HOBt	4	10	80	44	44
10	MuLV CTL epitope, *C*-terminus	COMU/HOBt/HOAt	5.2	5	75	70	45
11	LysM, *C*-terminus	HBTU/HOBt	4	10	80	48	44
12	LysM, *C*-terminus	HBTU/HOBt/HOAt	5.2	2 × 10	75	75	45
13	β-amyloid 1–42	HBTU/HOBt/HOAt	5.2	2 × 5	75	72	45
14	β-amyloid 1–42	DIC/HOBt	5	10	86	78	46[c]
15	Amylin	HBTU	5 or 10	15 + 15 + 6	25 + 73 + 90	—	47
16	PKB/Akt inhibitors	HBTU	3	5	75	—	48[d]
17	CLN025	HATU	5	10	75	—	49
18	Cyclic pentapeptide	DIC/HOBt	3	20	60	90	50
19	GLP-1	HBTU/HOBt	4	6	50	93	51
20	Antifreeze glycopeptides	TBTU	5	10	40	—	52[e]
21	Tn glycopeptides	HBTU	5	5	80	—	53, 54[f]
22	Collagen-mimetic lipopeptide	HBTU/HOBt	5	5	50	50	55, 59[g]
23	NY-ESO-1 fragment 140–180	HBTU	5	5	75	46	58[h]
24	Human Insulin Glargine	HBTU	4 or 5	5	75	—	57, 64
25	Zwitterionic peptide	HCTU	—	5	75	—	60
26	H-Tyr-Aib-Aib-Phe-Leu-NH_2	COMU	10	6	80	89	61[i]
27	Lys-like polypeptoid	DIC/HOBt	3	20	60	—	62
28	Conotoxin Rg1A analogues	HBTU/HOBt	—	10	75	—	63
29	RNA-stabilizing peptides	TBTU	5	5	80	—	65
30	Muc-1 glycopeptides	HBTU/HOBt	3	10	50	67	66–71[j]
31	Human Insulin peptide 3	HBTU	5	5	75	—	73
32	Cyclotides	HBTU/HOBt	5	5	87	—	74[k]
33	GLP-1	HBTU/HOBt	4	10	60	—	75
34	[^{8}Gly]-GLP-1	HBTU/HOBt	3	10	50	60	76
35	Exendin-4	HBTU/HOBt	3	10	50	42	76
36	Model nonapeptide	DIC/HOBt	4	5	75	76	77
37	Phosphopeptides	HBTU	5	5	75	30–93	78[h]
38	DesB30 Insulin	DIC/HOAt	6	5	70	—	79
39	GLP-1 analogues	HBTU/HOBt	4	10	75	—	80
40	PNA-peptide hybride	HBTU/HOBt	5.5	20	60	—	81
41	Lipopeptides	HBTU/HOBt	5	5	55	—	82[g]
42	CSF114(Glc)	TBTU/HOAt	2.5	5	70–75	—	83, 86
43	Calmodulin-binding peptide	DIC/HOBt	3	3	60	95	84
44	20-mer model peptide	HBTU/HOBt	5	5	80	84	85[l]
45	Gramacidin A	TBTU/HOAt	2.5	5	70–75	72	86[m]
46	Calmodulin-binding peptide	DIC/HOBt	3	2	0 to 96	86	87[n]
47	Phospho-Ser peptide	HBTU/HOBt	4	2	60	49	88
48	Fmoc-TVI-NH_2	PyBOP	3	20	110	60	35

[a] Arg was double coupled. His and Cys were coupled at max 50 °C. [b] The first 12 couplings were performed using standard CEM conditions and the poly-Q stretch was coupled at room temperature plus an additional coupling step at 75 °C (5 min at RT + 10 min at 75 °C). [c] His was coupled at room temperature for 1 h. [d] Two of the analogues were synthesized at 75 °C for 20 min using HATU as coupling reagent. [e] Glyco-amino acids were coupled at 40 °C for 15 min. [f] Glyco-amino acids were coupled using HATU, HOBt, 80 °C for 20 min. [g] Arg was double coupled at room temperature. [h] Arg was coupled for 25 min at room temperature followed by 5 min at 75 °C. Cys or His were coupled for 10 min at room temperature followed by 5 min at 47 °C. Phosphorylated amino acids were coupled for 15 min at 72 °C. [i] 6 equiv. for Aib couplings. [j] Glycoamino acids were coupled using only 1.5 equiv. coupling reagents and 20 min coupling times. [k] Prepared by Boc chemistry. [l] Cys and His were coupled at room temperature. [m] Coupled by double couplings – single couplings only give 44% purity. [n] Pulsed microwave irradiation with intermittent cooling to sub-ambient temperature (4 times 30 s).

and so-called difficult 'sequence'. Secondly, the complex peptide hormone insulin, which contains two peptide chains and three disulfide bridges, has been synthesized by conventional SPPS using complex strategies.[150–152] Microwave heating allowed the synthesis of desB30 insulin analogues in reasonable yields through the assembly of a 60-mer linear precursor, which after folding was processed to two-chain insulin (Table 1, entry 38).[79] Thirdly, a 58-mer peptide composed of three α-helices, a so-called affibody, was successfully synthesized using CEM standard conditions, however, microwave-assisted synthesis under these particular conditions led to an increase in deletion sequences as well as an increase in aspartimide formation compared to room temperature (Table 1, entry 8).[43] Fourthly, another affibody, a 66-mer peptide, was synthesized in 41%

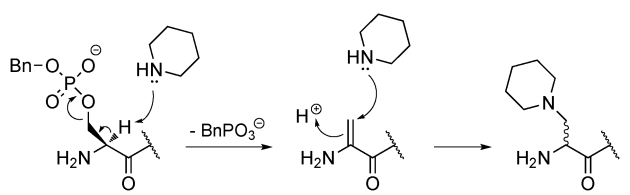
crude purity using microwave-assisted SPPS.[126] Finally, the synthesis of human exon 1 huntingtin was published recently, which consist of a 42 residue poly-Gln stretch and two poly-Pro stretches of 10 and 11 residues, respectively, connected by spacer regions giving a total of 109 amino acid residues (Table 1, entry 7).[42] This is to our knowledge the currently longest peptide sequence reported to have been assembled by microwave-assisted SPPS.

Phosphopeptides

Phosphopeptides are often used to study the role of protein phosphorylation/dephosphorylation which governs a myriad of cellular processes, thus the development of efficient solid-phase synthesis methods are an important process. The synthesis is generally performed by incorporation of phosphorylated amino acid derivatives into the growing peptide chain.[153] Fmoc-Xxx(PO_3Bn,H)–OH (Xxx = Ser, Thr or Tyr) have proven particularly useful in Fmoc based SPPS due to their high chemical stability upon storage over long periods and because they can be directly used in standard SPPS protocols. Furthermore, the phospho-protecting group is removed during the final TFA treatment. At room temperature the introduction of phosphorylated derivatives often requires double coupling,[154] however, microwave heating can increase the yield of the incorporation of the phosphorylated amino acid as well as shorten the coupling time.[78,88] Piperidine induced phosphoryl β-elimination of Ser(PO_3Bn,H)-containing peptides has been reported to be increased at elevated temperature.[155] However, the use of 50% cyclohexylamine in DCM, 5% DBU in DMF or 5% piperazine in DMF instead of 20% piperidine in DMF for the N^{α}-deprotection of Fmoc-Ser(PO_3Bn,H)-peptidyls circumvented some of the β-elimination problems.[155] Piperidine-induced β-elimination only occurs for phosphoseryl residues (Scheme 4), not for Tyr, and most likely only during the N^{α}-deprotection of the Ser(PO_3Bn,H) residue.[155] Using monobenzylated phosphorylated amino acid building blocks Brandt *et al.* showed that microwave irradiation both during coupling and N^{α}-deprotection provided phosphopeptides in moderate purity, however, the authors did not comment on β-elimination during the N^{α}-deprotection steps.[88]

O- and *N*-glycopeptides

O- and *N*-glycosylation are ubiquitous posttranslational modifications and they play important roles in a wide range of biological processes including cell adhesion, inflammation, immune response, and cell growth.[156] It has been estimated



Scheme 4 Mechanism of piperidine induced β-elimination of *N*-terminal phosphoseryl residues followed by piperidine addition.[254]

that at least 50% of all human proteins are likely to be glycosylated.[157] The majority of oligosaccharides in naturally occurring glycoproteins can be classified as either *N*-glycosides where the acetylglucosamine is linked to the side-chain of Asn or as *O*-glycosides where the oligosaccharide is linked to the hydroxyl side-chains of Ser, Thr, Tyr, and others.[158] In addition, less common glycosylations where the oligosaccharide is linked to a, for example, hydroxyproline or hydroxylysine are also well documented.[159] The synthesis of glyco-peptides by conventional synthesis at room temperature has been extensively reviewed,[157,160–163] thus herein the focus is merely on microwave-assisted SPPS of glycopeptides.

The most common methodology for the synthesis of glycopeptides relies on pre-synthesized glycosylated amino acid building blocks[164–168] incorporated into stepwise SPPS, which allows for complete control over the attachment of the carbohydrate moiety in the peptide chain. When employing the Fmoc strategy, the protecting group scheme is suitable for glycopeptide synthesis and standard coupling reagents such as DIC, HBTU and HATU in combination with an auxiliary nucleophile are generally utilized. However, the complexity and size of the glycosylated amino acid building blocks can make the coupling reaction a time consuming process and even with powerful coupling reagents, completion of the coupling can be problematic. Furthermore, the cost of the glycosylated building blocks encourages the use of a minimum excess to be used in SPPS and therefore faster and more efficient protocols are preferable. The short and efficient couplings in standard microwave-assisted SPPS the microwave methodology have also been applied in solid-phase glycopeptide synthesis (SPGS). The stability of the glycosylated building block during the coupling step and in particular throughout the N^{α}-deprotection is the main concern when applying microwave irradiation to the SPGS. The piperidine treatment during N^{α}-deprotection could potentially lead to β-elimination of the glycan moiety which might be more pronounced when heating is applied. Nevertheless, despite all the challenges and the complexity, several groups have reported promising results on the use of microwave heating during SPGS.[44,52,66–71,86,117–119,124,169,170]

The mucin-type glycopeptide is the most studied glycopeptide in respect to microwave-assisted SPGS.[169] In particular the MUC1 type *O*-glycans have been studied and their synthesis by conventional SPPS is established.[170] Nishimura and co-workers have synthesized a variety of MUC1-related glycopeptides where the glycosylated amino acids are incorporated using microwave-assisted SPGS (Table 2, Fig. 2, peptides **1–9**).[66–71] A core-2 trisaccharide on Ser or Thr residues are one of the most common building blocks (Table 2, Fig. 2, peptides **1–4** and **6**), but other saccharides have also been incorporated into the MUC1 type glycopeptides (Table 2, Fig. 2, peptides **2**, **3**, **5**, and **7–9**).[68–71] The most complex glycosylated building block incorporated by microwave-assisted SPGS to date carried an *O*-linked pentasaccharide (Table 2, Fig. 2, peptide **3**) and was incorporated at 50 °C for 20 min.[69] The most complex glycopeptide synthesized contained five core-2 trisaccharide on Ser and Thr residues and the overall synthesis time required for the synthesis of this 20-mer was reduced from 4 days to 7 hours with comparable overall yields (Table 2, Fig. 2, peptide **1**).[66,67] The incorporation of

Reaction conditions for the synthesis of different glycopeptides by SPGS

		Coupling of the glyco-amino acids				N^{α}-Deprotection		
Peptide[a]	Name	Coupling reagents	Equiv.	Time/min	Temp./°C	Time/min	Temp./°C	Ref.
1	MUC1	HBTU/HOBt/DIEA	1.5	20	50	3	50	66, 67
2	MUC1	HBTU/HOBt/DIEA	1.5	20	50	3	50	68
3	MUC1	HBTU/HOBt/DIEA	1.5	20	50	3	50	69
4	MUC1	HBTU/HOBt/DIEA	1.5	20	50	3	50	70
5	MUC1	HBTU/HOBt/DIEA	1.5	15	50	3	50	70
6	MUC1	HBTU/HOBt/DIEA	1.5	20	50	3	50	71
7	MUC1	HBTU/HOBt/DIEA	1.5	20	50	3	50	71
8	MUC1	HBTU/HOBt/DIEA	1.5	20	50	3	50	71
9	MUC1	HBTU/HOBt/DIEA	1.5	20	50	3	50	71
10	CSF114(Glc)	TBTU/NMM	2.5	5	70–75	0.5 + 3	70–75	86, 119
11	Peptide aldehyde	HBTU/HOBt/DIEA	4.0	10	80	1 + 2	RT + 60	44
12	Tn glycopeptides	HATU/HOAt/DIEA	1.5	20	80	0.5 + 3	80	53
13	Tn glycopeptides	HATU/HOAt/DMAP/collidine	1.5	20	80	0.5 + 3	80	54
14	Tn glycopeptides	HATU/HOAt/DMAP/collidine	1.5	20	80	0.5 + 3	80	54
15	Antifreeze peptide	HATU/HOAt/DIEA	2.5	15	40	7	40	52
16	Antifreeze peptide	HBTU/DIEA	1.5	15	80	0.5 + 3	80	118
17	FAN(635-655)	DIC/HOBt/DIEA	3	15	55	0.5 + 3	75	117

[a] The peptide sequences are presented in Fig. 2 (except peptide **17**).

glycosylated amino acid building blocks and the following N^{α}-deprotection was, in all five examples, performed at 50 °C and no additional by-product caused by the heating was reported.[66,67] This solid-phase strategy was used to synthesize a variety of similar glycopeptides, which were elongated enzymatically after SPGS by glycosyltransferases.[68–71] Nishimura and co-workers also used the strategy to incorporate a *N*-linked Asn(Ac_3-βGlcNAc) residue into a 10-mer glycopeptide which was then further elongated enzymatically (Table 2, Fig. 2, peptide **5**).[70]

Papini, Rovero, and co-workers reported that in the synthesis of CSF114,[83,86,171] a *N*-glycosylated peptide acting as an immunological probe, the incorporation of the glycosylated building block benefitted from the increased temperature and shorter coupling time. The *N*-linked Asn(Ac_4-βGlc) moiety was incorporated at 70–75 °C for 5 min and the N^{α}-deprotection was performed at 70–75 °C for 3–3.5 min (Table 2, Fig. 2, peptide **10**).[83,86] The microwave irradiation led to a reduction in cycle time (from 2 h to 30 min) and an increase in crude peptide purity as well as overall yield (from <20% to >70% and 10% to 46%, respectively). The developed microwave-assisted strategy was also used in later studies where a variety of glycopeptides with a *N*-linked Asn(βGlc) residue were synthesized.[117,119]

Recently, Brimble and co-workers synthesized fluorescein-labelled, *O*-linked Ser/Thr (αGalNAc) glycopeptides as immunological probes (Table 2, Fig. 2, peptides **12–14**), by incorporating 1–3 monosaccharide units and investigating the limitations of microwave-assisted SPGS.[53,54] The most complicated contained three sequential Thr(αGalNAc) units as well as a fluorescein label in a 7-mer peptide (Table 2, Fig. 2, peptide **13**). This was accomplished by applying high temperatures (80 °C) during the coupling (20 min) of the glycosylated building blocks as well as during N^{α}-deprotection (0.5 + 3 min).

Antifreeze glycopeptides and analogues thereof are another class of peptides which has been synthesized by microwave-assisted SPGS.[52,118,172] The antifreeze glycopeptides enable fish to live at temperatures below the freezing point of physiological solutions. They consist of the tripeptide units $(Ala–Ala–Thr)_n$ with minor sequence variation and bear an *O*-linked β-Gal-(1–3)-αGalNAc at every Thr residue (Table 2, Fig. 2, peptide **15**).[173] In a study by Sewald and co-workers a Thr residue with an *O*-linked α-GalNAc was incorporated using microwave irradiated SPGS with a maximum temperature of 40 °C leading to a reduced cycle time (3 h to 45 min).[52] Higher temperatures were investigated, however instead of a further improvement in the crude peptide purity, the elevation in temperature resulted in a decomposition and de-glycosylation of the peptides.[52] This particular result is somewhat in contradiction with the data for the microwave-assisted SPGS of an antifreeze peptide analogue containing four neoglycosylation sites (Table 2, Fig. 2, peptide **16**) where the coupling step was performed at high temperature (80 °C) over 15 min. Decomposition or de-glycosylation of the target peptide was not reported and an overall peptide yield of 72% was achieved.[118] However, a glycopeptide aldehyde, synthesized by microwave-assisted SPGS using elevated temperature during the coupling step (80 °C for 10 min) resulted in partial *O*-deacetylation which may originate from the DIEA in the coupling step. However, following complete deprotection of the glycan hydroxyl groups the fully linked glycopeptides aldehyde was obtained in good yields.[44]

Unverzagt and co-workers have recently published fragment condensation of an analog of RNase 1–39. Three fragments were coupled onto a resin-bound glycopeptide using microwave irradiation for 2 × 30 min at 55 °C. The glycopeptides, having an unprotected GlcNAc moiety, was not reported to decompose or de-glycosylate, *i.e.* after 3 hours of microwave heating at 55 °C.[174]

The area of microwave heating during SPGS is still relatively unexplored, but the current data suggest that heating can improve the yields of synthesized peptides and minimize the reaction times. Nevertheless, only limited data are currently available on what effect the additional heating has on stability of the glycosylated amino acids and only a small selection of glycosylated amino acids have been incorporated using this technique. Some studies suggest that in some cases temperatures >40 °C gave decomposition and de-glycosylation, although

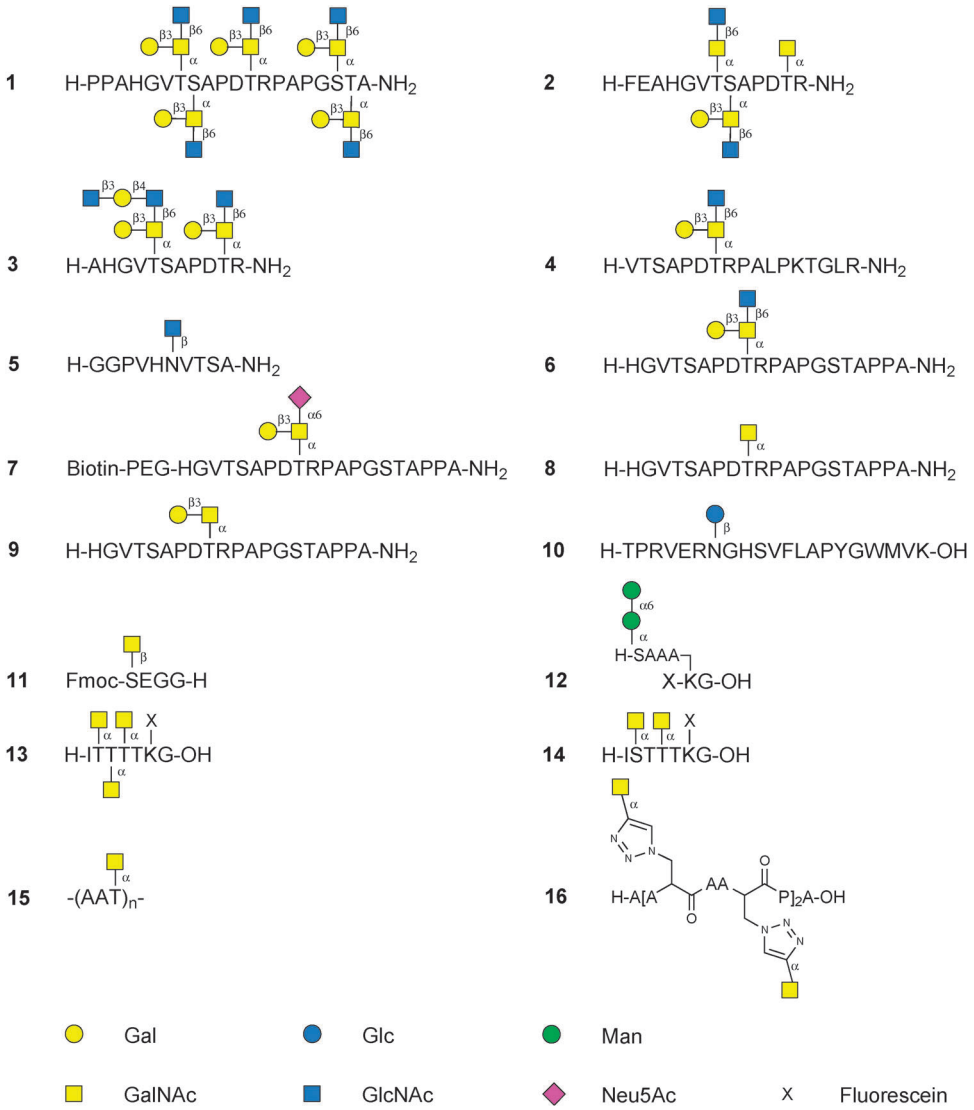


Fig. 2 Glycosylated peptides synthesized by microwave-assisted SPGS.

several other glycopeptides were synthesized at 80 °C without any reported side-reactions.

Interestingly, a solution study on de-*O*-glycosylation of glycopeptides using the mild base dimethylamine (DMA) and microwave heating was recently published.[175] The aim was to obtain quantitative release of the *O*-glycans for identification purposes. Microwave heating for 70 min at 70 °C gave complete *O*-glycan release in 40% aqueous DMA. These findings suggest that there is limitation in the stability of the glycan moieties during microwave irradiation. The stability of the glycan moieties are most likely sequence dependent, thus it is recommended that caution must be taken when applying heat during the peptide couplings and, more importantly, during N^{α}-deprotection using piperidine.

N^{α}-methylated peptides

Introducing *N*-methyl amino acids in bio-active peptides has resulted in analogues with improved stability,[176,177] activity,[178,179] and bioavailability.[180,181] Conventional synthesis of peptides containing *N*-methyl amino acid residues has been reviewed recently.[182,183] Generally, *N*-methylated peptides are synthesized either by the method of Miller and Scanlan, which was further developed by Kessler *et al.*,[184] where the amine is *N*-terminally methylated in a three-step procedure using dimethylsulfate as the methylating agent,[183–185] or by incorporating Fmoc *N*-methyl amino acid building blocks into the sequence by standard SPPS conditions.[186–190] The subsequent coupling onto *N*-methyl amino acids generally occurs in low yields and often requires coupling reagents such as triphosgene, HATU or PyBOP, and double coupling.[186–190] Only a few reports describe the use of the microwave heating for the assembly of *N*-methylated peptide. Albericio and co-workers have published a protocol for the synthesis of short peptides containing several *N*-methyl amino acids successively to each other using microwave irradiation (35 °C, 20 min) and DIC/HOAt as coupling reagents, in DCM.[142] The short *N*-methylated peptides were synthesized in reasonable purity. The authors report that two of the peptides could not be obtained by conventional methods at room temperature even when using a double or triple coupling protocol.

These two peptides both contained *N*-methylated β-branched amino acid residues, which are highly sterically demanding. Due to the low solubility of the protected amino acids in DCM, a different protocol was recently reported where DIC/HOAt in DMF (2 × 10 min at 75 °C) was used.[38] Coupling onto these highly sterically demanding residues is very troublesome and microwave heating appears to increase the yield of these couplings tremendously. An alternative method, the use of intermediate cooling, for incorporating single *N*-methylated amino acid residues, has recently been applied to synthesize several neurotensin fragment sequences (NT).[146] Even analogues having *N*-methylated Ile in the sequences were successfully synthesized using the intermediate cooling methodology. The effect of intermediate cooling compared to typical microwave heating would need to be investigated further to conclude whether it has a general effect. Nevertheless, microwave heating as such has a significant impact on the degree of acylation onto a resin-bound *N*-methylated amino acid residue.

β-Peptides

β-Peptides (oligomers of β-amino acids) are a class of foldamers that can adopt a variety of secondary structures and they have been proven to be proteolytically and metabolically stable as well as very useful for biomedical applications.[191–198] The major obstacles when applying conventional SPPS conditions for the assembly of β-peptides have been to obtain high yielding amide bond formation and *N*-deprotection. The difficulties are mainly attributed to aggregation and folding of the peptidyl intermediates, however, as for α-peptides, some of the aggregation problems can be alleviated by use of a chaotropic salt additive.[27] While microwave irradiation has been explored to overcome some of these challenges in the synthesis of α-peptides as well as for increasing the reaction rate, only a few protocols using microwave heating have been reported for the solid-phase synthesis of β-peptides.

Murray and Gellman reported the first microwave-assisted application for the assembly of a β-peptide, the extensively studied 14-helical β-peptides, by using β^3-amino acids and *trans*-2-aminocyclohexane carboxylic acid (ACHC, Fig. 3), which has a constrained six-membered ring.[134,199] A hexa- and a deca-β-peptide were synthesized at room temperature using double couplings and double *N*-deprotections of the ACHC residues (Fig. 3). The hexa-β-peptide was reported to have a moderate crude purity of 55% in contrast to the penta-β-peptide precursor that had a high crude purity of 95%.[199] However, applying microwave irradiation to the synthesis of the hexa-β-peptide, during the coupling (60 °C, 2 min) and *N*-deprotection (50 °C, 4 min) steps, afforded an improved purity of 80%. The deca-β-peptide sequence was a greater synthetic challenge, which under conventional room temperature SPPS condition only gave a crude purity of 21%, however, applying microwave heating resulted in a crude peptide purity of 57%. The low yields of the two β-peptides originated from an incomplete coupling of the ACHC residues, which was addressed by performing the microwave-assisted couplings in a 0.8 M LiCl in NMP affording an improvement in purity to 88% and 94% for the deca-β-peptide and hexa-β-peptide, respectively.[199]

The latter methodology was applied to a parallel synthesis of a hexa-β-peptide library using a 96-well polypropylene filter plate in a MARS multimode microwave reactor from CEM (*vide supra*).[134] The designed hexa-β-peptide library resulted in a variation in average product purity between 50% and 69%. The variation in crude purity was most likely due to inhomogeneous microwave heating over the plate area.[134] In an effort to locate an acceptable platform to synthesize β-peptides, Murray and Gellman also published the use of microwave irradiation to synthesize combinatorial libraries on PS macrobeads in a monomode microwave reactor (CEM Liberty).[200] First, the previously described MARS parallel system methodology was applied, which afforded very low yields. However, using an alternative protocol with 6 cycles of heating and cooling while not changing reagents was reported to provide acceptable results.[200] Moreover, the alternative microwave protocol was compared to conventional heating in an oil bath, which led to the conclusion that the increase in reaction rate and peptide purity was most likely of thermal nature.[200] The microwave protocol was then utilized to make a 100-member library based on an octa-β-peptide, which was reported to block the interaction between the MDM2 protein and a 17-residue peptide from the *N*-terminal region of p53. The average purity of this library was reported to be 65%.[201]

The multimode synthesis protocol was also exploited by Pomerantz *et al.* for the synthesis of β-peptides with a diversity of applications, such as the formation of lyotropic crystals and

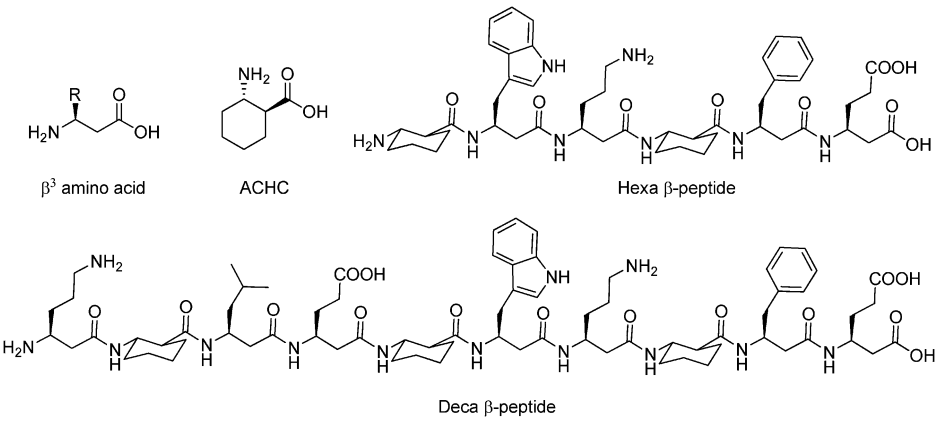


Fig. 3 β-amino acids and the hexa and deca β-peptide.[199]

the synthesis of β-peptides on gold surfaces.[202–205] In a recent paper by Petersson and Schepartz an optimized protocol was reported for the synthesis of the 28-mer β-peptide Z28, which gave an increase in isolated peptide yield, from <1% up to 19%, when using PyAOP/HOAt as coupling reagents instead of PyBOP/HOBt (60 °C for 6 min at 60 °C followed by cooling for 5 min at room temperature), as well as 20% piperidine in DMF followed by two times 2% DBU in DMF for *N*-deprotection (70 °C for 4 min followed by cooling for 5 min at room temperature).[206] The latter results demonstrates that microwave irradiation can improve the synthesis of especially long sequences of β-peptides, which otherwise would be difficult to obtain in reasonable purities and yields using conventionally methods.

Peptoids

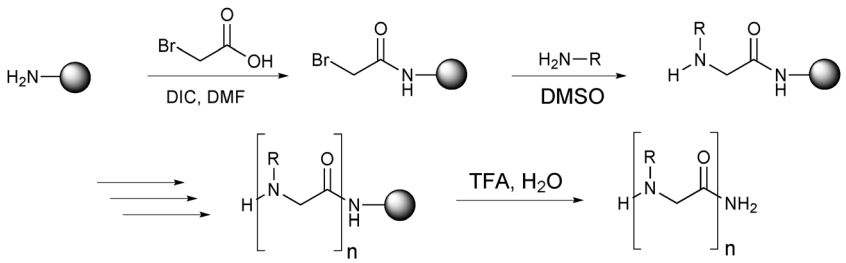
Peptoids are poly-*N*-substituted glycines where the side-chain is connected to the amide instead of the α-carbon. They were originally developed as a new motif for chemically diverse combinatorial libraries.[207] In contrast to the α-peptide backbone, peptoid backbones lack both chiral centers and hydrogen bond donors, and this, as well as the conformational heterogeneity arising from tertiary amide isomerism, complicates the design of well-folded peptoid motifs. Therefore the understanding of the intramolecular interactions that direct folding is still at an early state, in comparison to other foldamer systems such as β-peptides. The stable three-dimensional structures that peptoid can adopt is beyond the scope of this review and will therefore not be discussed herein.[208–211]

Peptoids exhibit enhanced stability towards proteolyses and bioavailability relative to natural peptides,[212] and are often synthesized *via* the solid-phase sub-monomer method developed by Zuckermann *et al*.[213] This method consists of iterative acylation steps performed by addition of bromoacetic acid and DIC followed by iterative amination steps by nucleophilic displacement of bromide with a primary amine (Scheme 5). The methodology makes it ideal for automated and combinatorial synthesis. The drawbacks are the long reaction times per residue at room temperature, that can be up to 3 h,[214] which are exacerbated in the synthesis of longer peptoids and with the incorporation of amines with low reactivity.

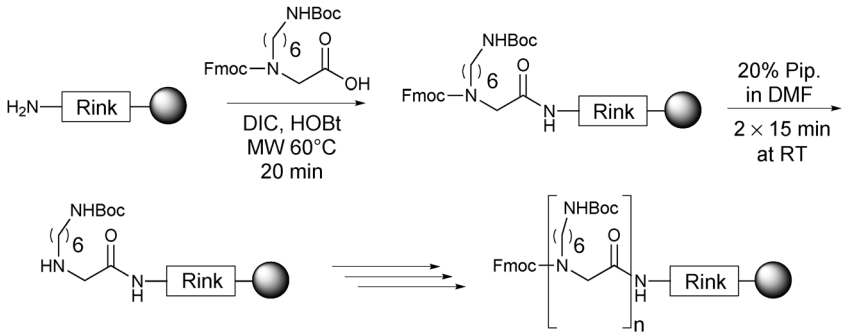
Using the Zuckermann methodology,[213] Kodadek and co-workers reported the use of microwave-assisted solid-phase synthesis of peptoids and that microwave heating reduced the total synthesis time for a 9-residue peptoid up to 10-fold.[215,216] However, the microwave experiments were performed in a domestic microwave oven without adequate control of the temperature, thus the microwave irradiation in both the acylation and amination steps were limited to 2 × 15 s (~35 °C). Yield and purity was generally higher for the microwave method than for those made at room temperature. Recently the same group used this methodology to synthesize large libraries of peptoids.[217,218] Furthermore, Blackwell and co-workers have used a laboratory microwave reactor with some temperature and pressure control (Milestone Ethos Microsynth microwave), and demonstrated that incorporation of electronically deactivated benzyl amide side-chains into peptoids was significantly improved by microwave irradiation.[219,220] A pentamer of 1-(pentafluorophenyl)ethylamine (fpe) units was synthesized at room temperature which afforded a crude purity of 22%, however, when applying microwave-assisted reaction conditions the crude purity was improved to 56%. The acylations were performed for 25 s at 35 °C and the amination reactions for 90 s at 95 °C, which resulted in an overall reaction time of 2 min per monomer unit. Microwave irradiation is not always required for high yields, *i.e*. peptoids consisting of only unhindered primary amines are easily made even at room temperature.[219,220] The molecular scaffold of peptoids makes them amenable for combinatorial strategies, which was recently utilized in a pentamer positional scanning library. Here the above microwave-assisted protocol by Blackwell was utilized using the so called 'tea bag' approach, which enabled them to identify new trypsin inhibitors.[221]

Recently, Blackwell and co-workers extensively studied the folding mechanism in peptoids, in which more than 38 peptoids were presented,[222] of which six were assembled by microwave-assisted solid-phase method on Rink amide linker PS resin using the previously used application protocol.[219] In another study by Blackwell and co-workers peptoid nonamers containing nitroaromatic monomer units were synthesized using microwave irradiation and studied by circular dichroism spectroscopy.[223] However, here Blackwell and co-workers report that their previously reported microwave methodology resulted in low yield and poor crude purity. The low yields could be due to the decreased nucleophilicities of these α-chiral nitro aromatic amines, which led to the development of an optimized protocol where the amination reaction time was increased from 90 s to 1 h and the temperature was lowered from 95 °C to 60 °C, which increased the yield from 13% to 20%.[223]

The above mentioned peptoids were synthesized using the classical Zuckermann method for peptoid synthesis (Scheme 5),[213] however, Peretto *et al*. later developed an alternative solid-phase route using Fmoc peptoid building blocks for the synthesis of peptoid oligomers (Scheme 6).[224] The monomer unit *N*-[6-(*tert*-butyloxycarbonyl)amino-hexyl]-*N*-Fmoc-glycine, a secondary



Scheme 5 The Zuckermann method for the synthesis of peptoids.[213]



Scheme 6 Synthesis of peptoids using the Bradley protocol.[62]

amine, necessitates multiple couplings using PyBrOP as coupling reagent for full incorporation. Later, Bradley and co-workers presented a microwave-assisted coupling protocol with DIC/HOBt: the secondary amine was coupled for 20 min at 60 °C using three equiv. of monomer and the *N*-deprotections were performed using 20% piperidine in DMF (2 × 15 min) at room temperature.[225] The Bradley coupling protocol has some similarities to coupling onto N^{α}-methyled amino acid residues and the optimal coupling conditions are therefore very similar.[38] The protocol resulted in a quickly synthesized hepta-peptoid with high purity (98%), which was superior to the conventional methods at room temperature.[225] Several groups have applied microwave irradiation to synthesize labeled peptoids with a variety of fluorophores and fluorescence quenchers to study the protease activity as well as to make fluorescein-tagged peptoids with cell penetrable and intracellular ability and for the synthesis of peptoid dendrimers.[62,226,227] The microwave irradiation protocols outperformed conventional synthesis at room temperature by providing the peptoids in less time and in much higher crude purities.

Pseudopeptides

Besides backbone modifications such as *N*-methylated peptides, peptoids, and β-peptides other pseudopeptides have been synthesized using microwave irradiation. They include amide bond modifications such as a reduced amide, Ψ(CH_2NH),[228] that are used for studying the importance of particular peptide bonds.[229–232] Pseudopeptides containing reduced amide bonds are generally obtained by reductive amination of the growing peptide with Boc- or Fmoc-protected aminoaldehydes.[233] In 2004 microwave irradiation was first reported for the synthesis of pseudopeptides in solution phase to improve the synthesis of Ψ(CH_2NH), *i.e.* both in the conversion of amino acids to aldehydes and in the reductive amination.[234] Later, Lee and co-workers reported the use of microwave heating in a solid-phase approach for the synthesis of reduced amide bond surrogates. Dipeptides were prepared by reacting an aldehyde in the presence of 1% acetic acid to give an imine, which was reduced to an amine bond using mild reducing agent ($NaBH_3CN$).[235] To prevent the elimination of the Fmoc group the reaction was performed at a temperature below 80 °C. The microwave protocol led to an increase in coupling yields compared to those achieved at room temperature. Furthermore, the synthesis time was significantly reduced from 5 h to 8.5 min per cycle, room temperature and microwave heating, respectively, without an increase in the degree of epimerization. Lee and co-workers synthesized a 5-mer and a 12-mer pseudopeptide having reduced amide bonds and especially the latter resulted in a significantly enhancement in yields compared to the unheated method (from 10 to 80%) as well as major decrease in reaction time.[235] The microwave protocol is indeed an efficient route for the synthesis of reduced amide pseudopeptides.

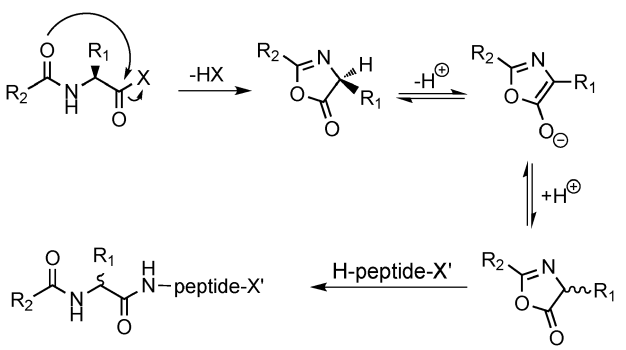
Another type of pseudopeptides, containing ester bonds, were recently prepared by Lee and co-workers utilizing microwave irradiation.[236] Like reduced amide bonds, ester bonds can be used to investigate the role of hydrogen bonding of amides in proteins and peptides.[237–239] Several different pseudodipeptides were synthesized by microwave-assisted solid-phase chemistry using (*S*)-2-hydroxy-4-methylpentanoic acid (α-hydroxy-Leu), DIC, DMAP (0.1 equiv.) and *N*-ethylmorpholine (NEM) (1.2 equiv.) for 12 min at 90 °C, and compared to the synthesis at room temperature. The experiment resulted in almost identical yields, but the overall coupling time was reduced from 7 h to 12 min.[236] However, the synthesis of two pseudopeptides, a 6- and a 12-mer, containing an ester bond in the *N*-terminal segment, was also considerably improved after elevating the temperature by microwave heating to 90 °C (yield raised from <45% to >80%).[236] Thus, the microwave-assisted procedure is indeed a fast and efficient method for the synthesis of pseudopeptides containing ester bonds.

N^{α}-deprotection and potential side-reactions

Microwave heating cannot only be applied during the coupling but also during the N^{α}-deprotection, *i.e.* removal of the N^{α}-protecting group, typically Fmoc. Several groups have reported the use of microwave heating during the deprotection step, but it is less widely used.[45,48,49,54,63,66,72,79,85,138,144,195,240,241] In most applications for Fmoc chemistry, 20% piperidine in DMF is the standard N^{α}-deprotection reagent although variations, such as using 20–50% piperidine in DMF or NMP, have also been exploited.[36,45,48,138] The N^{α}-deprotection often follows a two-step procedure which commences with a 2–3 min treatment to remove the initially formed high concentrations of dibenzofulvene. This is followed by filtration and a second 10–20 min reaction to complete the N^{α}-deprotection. The same approach is used when employing microwave heating although the reaction times can be decreased to approximately 0.5 + 3 min

at 37–80 °C.[36,54,63,85,138] One of the major concerns when applying heating during the N^{α}-deprotection step is the possibility for an increased level of side-reactions, such as aspartimide formation or epimerization. In order to suppress side-reactions, different reagents have been investigated for Fmoc removal, for example, 5% piperazine or 20% piperidine solutions with 0.5 M HOBt in DMF, which was recommended for Fmoc removal of 'difficult peptide sequences'.[45,49,72,80,85,241] The use of piperazine has successfully suppressed β-elimination in microwave-assisted solid-phase phosphopeptide synthesis.[155] Another approach for microwave assisted N^{α}-deprotection is 5 × 5 s at 100 W with intermediate cooling after each 5 s reaction.[146–149] However, the cooling time and thus the total deprotection time (contact time with piperidine) is in most cases not reported. The intermediate cooling approach has been invoked to suppress side-reactions but, to our knowledge, no comparative data have been reported supporting this hypothesis.

Microwave heating can accelerate amide bond formation and N^{α}-deprotection but an important question is whether microwave irradiation also accelerates competing side-reactions such as epimerization and aspartimide formation. Epimerization during standard Fmoc SPPS is well-studied, however it occurs infrequently during coupling and to an even lesser extent during N^{α}-deprotection. The 19 common chiral proteogenic amino acids have a chiral center at the α-carbon and Thr as well as Ile have an additional chiral center at the β-carbon in their side-chain. It is crucial to maintain the correct configuration of these chiral centers. Chiral amino acids have a potentially acidic hydrogen atom at the α-carbon which, by subsequent removal and reattachment, represents a potential site for epimerization through enolization. The main mechanism by which epimerization occurs is *via* an oxazolone intermediate which is formed by attack on the activated carboxyl group on the adjacent amide bond (Scheme 7).[242] Epimerization during peptide chain assembly is a risk when incorporating Cys or His residues.[243,244] Often, a peptide with an epimerized chiral center can be separated from the all-L sequence by reverse-phase HPLC. The presence of D-enantiomers in a crude peptide mixture can explicitly be analyzed by several chiral techniques and normally the content of D-enantiomer is <0.1%. It has been speculated that the reason His residues are more prone to epimerization is that the imidazole group may aid in removing the α-H from the His residue.



Scheme 7 Epimerization *via* oxazolone formation.[242] R_1 and R_2 represent different amino acid side-chains. X′ represents the modification on the *C*-terminal.

In Cys residues, the β-sulfur could be stabilizing the anion at the α-carbon of an activated Cys derivative. Epimerization during peptide assembly can be reduced by avoiding pre-activation time, base-free activation,[245] change of base from *N*,*N*-diisopropylethylamine (DIEA) to 2,4,6-trimethylpyridine (TMP)[243,246] or coupling with pre-formed 2,3,4,5,6-pentafluorophenyl (Pfp) esters.[247] Epimerization of the chiral α-carbon is a recurring problem in esterifications, for example, when the first (*C*-terminal) amino acid is coupled to a hydroxyl. This is often required in the synthesis of *C*-terminal peptide acids.

Heating during coupling could conceivably increase the degree of epimerization and it is thus important to consider when employing microwave irradiation in SPPS. However, explicit analysis of the degree of epimerization requires additional effort and it is not always performed. One commonly used method for measuring the degree of epimerization in peptides is through chiral amino acid analysis using GC-MS with a chiral column.[144] Collins and co-workers reported in 2007 epimerization levels for microwave-assisted SPPS in the synthesis of a 20-mer peptide containing all the proteogenic amino acids.[85] Prior to this study other groups had evaluated the epimerization problem but only when applying conventional heating such as oil baths.[248] Collins and co-workers set the temperature to 80 °C for both coupling and N^{α}-deprotection and reported increased epimerization, particularly for the Cys, His and Asp residues, with increased temperatures.[85] Regarding His and Cys the problem could be suppressed by lowering the temperature from 80 °C to 50 °C. This finding was confirmed in an additional study by Kappe and co-workers in 2008 who investigated a magainin-II analogue containing a His residue as well as a Cys residue.[138] It was demonstrated that SPPS assisted by conventional heating or microwave irradiation afforded comparable epimerization levels.[138] Recently, Kappe and co-workers suggested that incorporating His and Cys should be conducted at room temperature to prevent epimerization. To investigate this protocol it has been applied in the synthesis of β-amyloid which contains three His residues.[46] Chiral GC-MS analysis showed a very low epimerization level at 0.3% D-His for room temperature coupling *versus* 7% for coupling at 86 °C.[46] Loffredo *et al.* suggest that a change in solvent from DMF to the binary, aprotic mixture DMSO–toluene (1 : 3) also suppresses epimerization of most natural amino acids during microwave-assisted SPPS at 60 °C using either Fmoc or Boc chemistry. However, the problem with epimerization of the Cys residue was not solved and yet again it was recommended that heating was completely avoided or lowered to 50 °C.[144,145]

Epimerization of Asp and aspartimide formation increases drastically when the temperature is raised above room temperature during piperidine-induced N^{α}-deprotection (20% piperidine in DMF).[43,85] However, a change to piperazine lowers the level of D-Asp formation from 9.6% to 1.2% and the degree of aspartimide formation from 31.5% to 3.15%.[85] However, circumventing heating during the N^{α}-deprotection may also solve this problem, however, it was not suggested in this study. Furthermore, other groups have synthesized shorter peptides containing Asp where the N^{α}-deprotection was carried out using standard condition (20% piperidine in DMF at 60 °C using microwave irradiation) resulting in only minor

amounts of D-Asp present (0.7%).[144] This indicates that there are still some unsolved issues concerning heating during the N^{α}-deprotection step and it appears that the aspartimide formation is highly sequence dependent.

Solid supports

Several solid supports have been used in the microwave-assisted assembly of peptides. The traditional polystyrene (PS) resins, however, have often been outperformed by the poly(ethylene glycol) (PEG) modified PS supports (TentaGel, TG)[249] and the fully PEG-based ChemMatrix (CM) resin.[250] This is likely due to a lowering of the intra- and intermolecular aggregation in the latter. Both the TG and the CM resins swell well in the common solvents used in peptide synthesis (DMF, NMP, DCM as well as TFA).[249,250] Kappe and co-workers synthesized the nonapeptide, H-GILTVSVAV-NH_2, with DIC/HOBt-mediated peptide couplings. The coupling time was 20 min at 60 °C using 10 equiv. reagents on different resins. The Rink Amide MBHA PS resin (loading: 0.64 mmol g^{-1}) and the Rink Amide TG resin (loading: 0.24 mmol g^{-1}) both gave a crude yield of 85%, but in contrast to the MBHA PS resin the amount of coupling reagents could be reduced to 5 equiv. without major changes in the crude peptide purity (83%). However, using Rink Amide CM resin (loading: 0.50 mmol g^{-1}) and 10 equiv. or 5 equiv. of coupling reagents gave a crude purity of 90% and 91%, respectively, of the nonapeptide (Table 3, entry 1–10).[138] Kappe and co-workers also reported that at 75 °C using only 3 equiv. of coupling reagents, the CM resin outperformed the TG resin, *i.e.* 71% *vs.* 91% crude purity (Table 3). Finally, at 86 °C the CM resin (3 equiv. of coupling reagents, 95% crude purity) also resulted in a higher crude purity of the nonapeptide sequence compared to the TG resin (5 equiv. of coupling reagents, 92% crude purity).[138] The superior performance of the PEG-based resins were also reported by Galanis *et al.*, who showed an increase in crude purity when synthesizing α-Conotoxin analogues on TG resin (loading: 0.24 mmol g^{-1}) compared to PS resin (loading: 0.43 mmol/g^{-1}) (Table 3, entry 11–18). Moreover, the differences in resin performance, PS *vs.* TG, were more or less identical at room temperature and microwave heating.[251]

Besides the common PS, TG and CM resins there are also some reports of microwave-assisted SPPS using PEGA, CLEAR and Wang resins. Nishimura *et al.* have shown that PEGA (loading: 0.055 mmol g^{-1}) outperformed TG (loading: 0.26 mmol g^{-1}) in the synthesis of a MUC1-related glycopeptides of 20 amino acid residues (Table 2, entry 1). TG resin resulted in a crude peptide purity of 44% which was considerably lower than for the PEGA (poly(ethylene glycol)-poly(*N*,*N*-dimethylacrylamide) copolymer) resin, which gave a crude purity of 67%.[66] Another PEG-based resin, the CLEAR resin, was used for the synthesis of triple helical collagen-mimetic lipopeptides in reasonable purity.[55]

Papini and co-workers have previously described the synthesis of Gramicidin A and CSF114(Glc) using Glycinol 2-chlorotrityl PS (loading: 1.1 mmol g^{-1} and 0.51 mmol g^{-1}) and Fmoc-Lys(Boc)-Wang PS resin (loading: 0.67 mmol g^{-1}), respectively. An acceptable HPLC purity of Gramicidin A was only achieved using the lower loading (0.51 mmol g^{-1}) of the resin and double couplings, however the CSF114(Glc) peptide was synthesized in 98% crude purity using the pre-loaded Wang resin.[86]

Generally, our literature studies revealed that PEG-based resins outperformed the PS-based resins, however, the degree of PEG, the amount of cross-linking and diversities in batches have significant influence on the probability of accessing the amino terminal of the growing peptidyl polymer. Thus, when synthesizing medium to long peptide sequences choosing the optimal resin is likely to be a very important factor.

Microwave-assisted release of peptides

A number of groups have reported the use of microwave irradiation to increase the speed of peptide release from the solid support and the concurrent side-chain deprotection.[55,78,88]

Table 3 Resin performance when microwave irradiation was applied

			Coupling				
Entry	Sequence	Resin and loading/mmol g^{-1}	Equiv.	Time/min	Temp./°C	Purity (%)	Ref.
1	H-GILTVSVAV-NH_2	PS (0.64)	10	20	60	85	138
2	H-GILTVSVAV-NH_2	PS (0.64)	10	10	75	81	138
3	H-GILTVSVAV-NH_2	TG (0.24)	10	20	60	85	138
4	H-GILTVSVAV-NH_2	TG (0.24)	5	20	60	83	138
5	H-GILTVSVAV-NH_2	TG (0.24)	3	10	75	71	138
6	H-GILTVSVAV-NH_2	TG (0.24)	5	10	86	92	138
7	H-GILTVSVAV-NH_2	CM (0.50)	10	20	60	90	138
8	H-GILTVSVAV-NH_2	CM (0.50)	5	20	60	91	138
9	H-GILTVSVAV-NH_2	CM (0.50)	3	10	75	95	138
10	H-GILTVSVAV-NH_2	CM (0.50)	3	10	86	95	138
11[a]	H-GX_1X_2SNPVX_1HLEHSNLX_2-NH_2	PS (0.43)	3	5–7	60	85	251
12[a]	H-GX_1X_2SNPVX_1HLEHSNLX_2-NH_2	TG (0.24)	3	5–7	60	91	251
13[a]	H-GX_3X_2SNPVX_1HLEHSNLX_2-NH_2	PS (0.43)	3	5–7	60	87	251
14[a]	H-GX_3X_2SNPVX_1HLEHSNLX_2-NH_2	TG (0.24)	3	5–7	60	93	251
15[a]	H-GX_2X_1SNPVX_2HLEHSNLX_1-NH_2	PS (0.43)	3	5–7	60	86	251
16[a]	H-GX_2X_1SNPVX_2HLEHSNLX_1-NH_2	TG (0.24)	3	5–7	60	92	251
17[a]	H-GX_2X_3SNPVX_2HLEHSNLX_1-NH_2	PS (0.43)	3	5–7	60	86	251
18[a]	H-GX_2X_3SNPVX_2HLEHSNLX_1-NH_2	TG (0.24)	3	5–7	60	88	251

[a] X_1 = Cys(Mmt), X_2 = Cys(Acm), X_3 = Cys(S^{t}Bu).

The studies were conducted using standard resins and linker types (Rink Amide TG, Wang ChemMatrix, CLEAR Acid resin, Rink Amide PS, *o*-BAL PS).[55,78,88] The cleavage time was decreased from 2–5 hours down to minutes. Moreover, two papers by Clearhout *et al.*[50] and Kluczyk *et al.*[252] describe how TFA under microwave irradiation can substitute the use for HF in the cleavage of peptides from the Merrifield resin and *meta*-dialkoxy-BAL PS, respectively, however, the methods suffer from lower yield. Recently, a fully side-chain protected linear version of a cyclotide was cleaved from a NovaSyn TGT resin (PEG-PS-copolymer functionalised with 4-carboxy-tritylchloride) using AcOH/TFE/DCM (1/1/8) for 45 min at ~40 °C.[36] Heating during TFA treatment may be of limited value for PEG-containing supports, as they may degrade at high temperatures.

Some safety-catch type linkers release the peptide by nucleophilic displacement, *i.e.* under non-acidic conditions. In these cases, microwave heating may indeed be very valuable. Park and Lee have taken advantage of microwave irradiation in the release of peptides from activated safety-catch linker by amines and showed that microwave heating increased the yield from 59% (25 °C, 100 min) to 92% (130 °C, 10 min) in the release of a small dipeptide.[240] Tofteng *et al.* used microwave and conventional heating for the thiolytic release of protected peptide thioesters from the pyro-Glu linker.[253]

Conclusion

Numerous peptides and a few small proteins have successfully been synthesized using microwave-assisted SPPS, which suggests that most peptide sequences can probably be synthesized using microwave heating. However, this does not necessarily imply that dramatic rate- and yield-enhancements can be reached in all cases. Many peptide sequences are readily synthesized and microwave irradiation will not necessarily lead to significant increases in rate and yield. On the other hand, acylation of sterically demanding residues and syntheses of peptides that are prone to aggregation can benefit hugely from microwave heating. Conductive heating is also able to increase reaction rates and crude peptide purities. However, microwave instruments provide fast and precise heating and allow fast cooling by pressurized air, they provide homogeneous and reproducible heating, and they are easily controllable.

Currently, there are two manufacturers of microwave-assisted peptide synthesizers, CEM and Biotage, and both provide fully automated synthesizers. The major differences between the two systems are the liquid handling and the mixing of the reaction mixture, as the valve-based CEM instrument relies on nitrogen bubbling, while the Biotage instrument is a valve-free robot that uses vortexing. Most, if not all, microwave-assisted peptide syntheses reported in literature were on a scale below 0.2 mmol.

Generally the coupling of the proteogenic amino acids by Fmoc microwave-assisted SPPS can be performed using acylation times of 5 min at 75 °C, however, sterically hindered amino acids, such as peptoids, *N*-methylated, as well as glycosylated and phosphorylated amino acid residues, may need prolonged coupling times, *e.g.* 20 min at 75 °C. The activation of the amino acids should be performed as for conventional room temperature synthesis, except for the auxiliary nucleophile Oxyma, which is not stable at elevated temperature. More caution should be taken when performing N^{α}-deprotection using microwave heating, as epimerization, aspartimide formation, and β-elimination are potential side-reactions. Thus, it seems prudent to minimize microwave heating during N^{α}-deprotections of especially Asp, Cys, His, phosphorseryl and glycosylated amino acid residues.

Microwave-assisted peptide synthesis is a general technique that enables the assembly of highly pure peptides, however, often after optimization of each particular synthesis. Microwave irradiation in SPPS could lead to the beginning of a new era for peptide and protein applications due to the accessibility of 'difficult' and long sequences as well as sequences with unnatural amino acids.

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