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# Sustainable Peptide Synthesis Enabled by a Transient Protecting Group

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**Abstract:** The growing interest in synthetic peptides has prompted the development of viable methods for their sustainable production. Currently, large amounts of toxic solvents are required for peptide assembly from protected building blocks and switching to water as reaction medium remains a major hurdle in peptide chemistry. We report an aqueous solid-phase peptide synthesis strategy that is based on a water-compatible 2,7-disulfo-9-fluorenylmethoxycarbonyl (Smoc) protecting group. This approach enables peptide assembly under aqueous conditions, real-time monitoring of building block coupling and efficient post-synthetic purification. The procedure for the synthesis of all natural and several non-natural Smoc-protected amino acids is described, as well as assembly of 22 peptide sequences and the fundamental issues of SPPS, among them protecting group strategy, coupling and cleavage efficiency and stability under aqueous conditions, and crucial side reactions.

## Introduction

Peptides, natural biopolymers that comprise a chain of up to 100 covalently linked amino acids, are elementary components in all living systems and regulate many biological processes. Currently, synthetic peptides are industrially produced for the treatment of cancer, diabetes, cardiovascular and neurodegenerative diseases,[1-3] used in cosmetic,[4-5] diagnostic, and medical technology products, as well as in veterinary medicine, agrochemistry or as dietary supplements.[1, 6]

The history of peptide synthesis dates back to the turn of the twentieth century, when Theodor Curtius[7] and then Emil Fisher[8-9] presented the early protocols for the chemical assembly of oligopeptides, thus having started "a systematic attack on a field of natural substances that have previously been avoided by chemists."[10] Following the introduction of readily cleavable protecting groups that enabled condensation of multifunctional amino acids,[11-12] the solution-phase synthetic methods allowed to produce impressively long peptidic molecules.[13] However, the real breakthrough in the field of synthetic peptides was achieved when Robert Bruce Merrifield presented his concept of the solid-phase peptide synthesis (SPPS) in 1963.[14] Over the following more than five decades peptide chemistry has been significantly developed towards more and more efficient synthetic procedures, having taken advantage of the automation,[15-16] novel protection strategies,[17] microwave assistance,[18] improved isolation and purification methods, as well as advanced analytics. As a result, the field of synthetic peptides keeps rapidly growing, and the market of peptide pharmaceutics amounts ca. 15 billion USD.[2] Over the last decade, more than 100 peptides have been approved as drugs or for diagnostic applications.[2-3]

To date, peptide synthesis has gained almost optimal efficiency in terms of coupling yield and duration. However, solvent consumption that has been recognized as an economic and environmental issue since a decade, makes SPPS one of the most inefficient approaches known.[19-22] In view of sustainability, an ideal procedure could be outlined as a fully automated process with no waste of organic solvent, reduced consumption of all chemicals and shortened reaction times, with online monitoring of each reaction step if required, and with successive automated purification.[23] Recent advantages in microwave-assisted SPPS successfully coped with the task of reduced chemical expenditure.[24-25] However, even when scaled-down, consumption of dimethylformamide (DMF) and *N*-methyl-2-pyrrolidone (NMP), which are currently the solvents of choice in SPPS, is undesirable as they are classified by the European Reach Regulation as substances of very high concern due to their carcinogenic, mutagenic, or toxic for reproduction (CMR) properties. Whereas a sustainable, green alternative to existing SPPS scheme is highly desirable,[26] switching to water – the natural medium for peptide biosynthesis – remains one of the most fascinating unsolved problems in organic chemistry.[27]

To take this hurdle, a number of alternative approaches has been proposed, among them peptide synthesis in less harmful organic solvents or those referred to as the "green" ones (cyrene, diethyl carbonate, anisole, etc.)[28], solvent-free methods based on ball-milling, and application of some water-compatible systems in view of protecting groups and solvents.[21-22, 27, 29-37] However, the scope of these procedures is often limited in frames of peptide length, choice of amino acid derivatives, orthogonality, and compatibility with the desired synthetic step. Indeed, the longest sequence accessed by these methods comprised ten amino acids.[21-22, 27, 29-37]

Effective online monitoring at each synthetic step is another challenge in SPPS as the final yield strongly depends on coupling efficiency. Among two main approaches for solid-phase assembly of peptides, which are named according to the respective transient protection the Boc- and the Fmoc-SPPS, the latter allows one to follow an *N*-terminal deprotection, but only when the coupling step has been already accomplished and therefore cannot be repeated in case of insufficient coupling.[38] To date, no reliable method for inline SPPS monitoring has been reported. In

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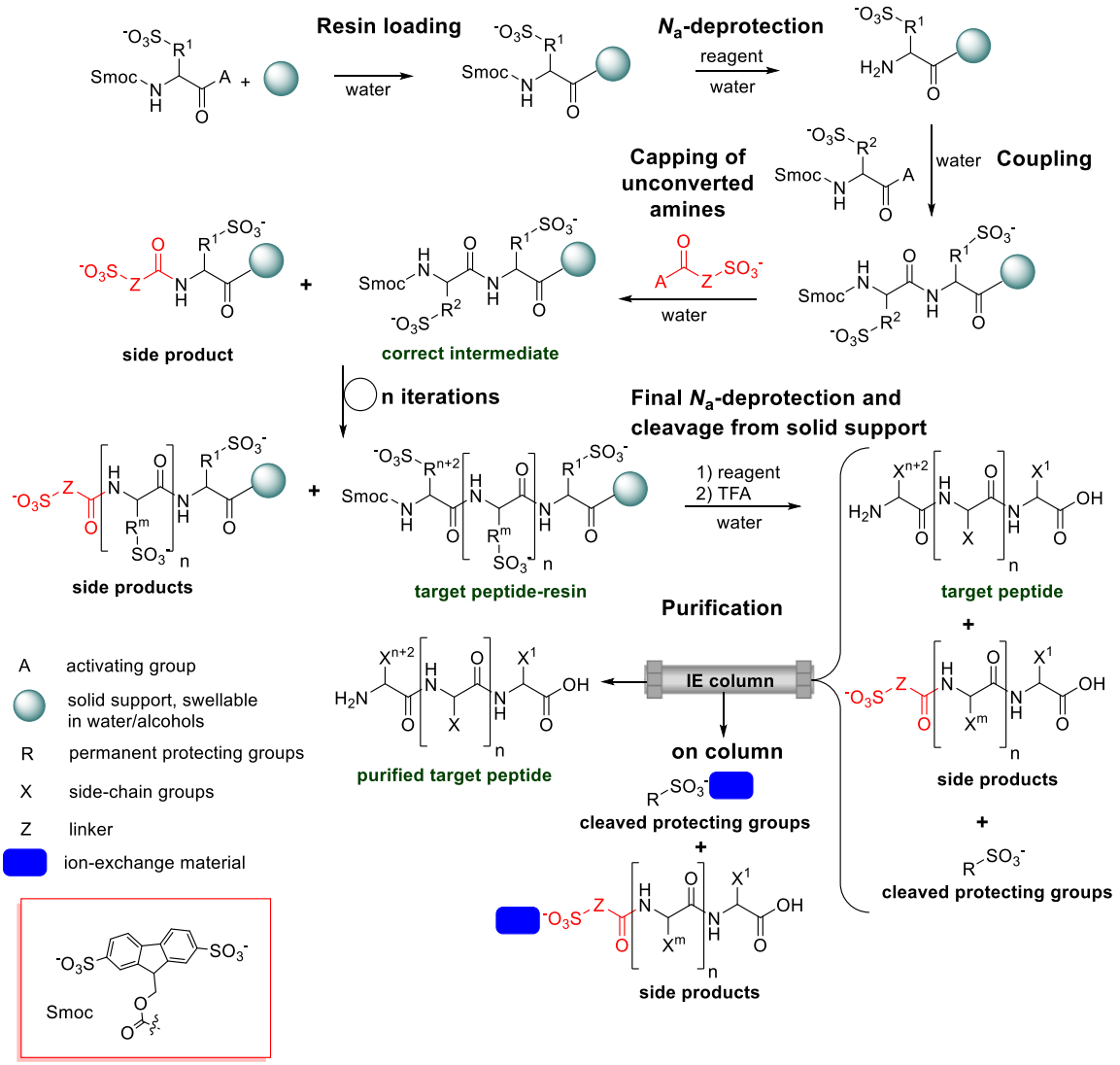


**Scheme 1.** General scheme of ASPPS

addition, efficient purification is one of the bottlenecks of peptide production, which mainly relies on reversed-phase high-performance liquid chromatography (HPLC) and requires large amounts of organic solvents and tremendous time efforts.

Herein, we present a novel approach to peptide assembly in water as a sustainable alternative to the state-of-the-art process (**Scheme 1**). The cornerstone of our aqueous solid-phase peptide synthesis (ASPPS) is a water-compatible 2,7-disulfo-9-fluorenylmethoxycarbonyl (Smoc) protecting group that combines a fluorenyl system with charged sulfonic pendants (**Figure 1**). Amino acids bearing this group at the *N*-terminus possess both high water solubility and pronounced fluorescence, which makes them suitable for the real-time monitoring (**Figure 1**).

## Results and Discussion

Our study started from the conversion of Fmoc-chloride **1** to Smoc-chloride **2** upon treatment with oleum (**Figure 1**, Section Methods, ESI Section 1.1). A further access to Smoc-protected building blocks (3-32, **Table 2**) was achieved in two ways.

**Table 1.** Comparison of protecting schemes for Boc-, Fmoc-SPPS and ASPPS

| Boc-SPPS[a] | Fmoc-SPPS | ASPPS |
|---|---|---|
| Boc-Arg(Tos)-OH | Fmoc-Arg(Pbf)-OH | Smoc-Arg-OH |
| Boc-Asn(Trt)-OH | Fmoc-Asn(Trt)-OH | Smoc-Asn-OH |
| Boc-Asp(OBzl)-OH | Fmoc-Asp(OtBu)-OH | Smoc-Asp(OtBu)-OH |
| Boc-Cys(Acm)-OH | Fmoc-Cys(Trt)-OH | Smoc-Cys(Trt)-OH |
| Boc-Gln(Trt)-OH | Fmoc-Gln(Trt)-OH | Smoc-Gln-OH |
| Boc-Glu(OBzl)-OH | Fmoc-Glu(OtBu)-OH | Smoc-Glu(OtBu)-OH |
| Boc-His(Dnp)-OH | Fmoc-His(Trt)-OH | Smoc-His(Trt)-OH[a] |
| Boc-Lys(Cbz)-OH | Fmoc-Lys(Boc)-OH | Smoc-Lys(Boc)-OH |
| Boc-Ser(Bzl)-OH | Fmoc-Ser(tBu)-OH | Smoc-Ser(OtBu)-OH |
| Boc-Thr(Bzl)-OH | Fmoc-Thr(tBu)-OH | Smoc-Thr(OtBu)-OH |
| Boc-Trp(For)-OH | Fmoc-Trp(Boc)-OH | Smoc-Trp(Boc)-OH[b] |
| Boc-Tyr(Bzl)-OH | Fmoc-Tyr(tBu)-OH | Smoc-Tyr(OtBu)-OH |

[a] Smoc-His was synthesized without side-chain protection as well. [b] Smoc-Trp was synthesized without side-chain protection as well.

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A direct conversion of $N_\alpha$-Fmoc amino acids lacking functional side-chains towards the respective Smoc derivatives (**3**, **4**, **7**, **12**, **16**, **21**, **30**, **31, Figure 1**) was performed applying sulfuric acid with subsequent neutralisation of its excess by calcium carbonate (ESI Section 1.2). The second route relying on the usage of Smoc-Cl **2** (**Figure 1**) to address the free *N*-terminus was successfully applied to all canonical amino acids as well as to a number of non-natural building blocks, giving desired $N_\alpha$-Smoc counterparts in high yields (**Table 2**, ESI Section 1.2). Moderate conversion obtained for 2-aminoisobutyric acid (Aib, **32**) was most likely due to obvious steric hindrance. NMR studies performed with $N_\alpha$-Smoc amino acids **3-32** confirmed their structure (ESI, Section 1.3).

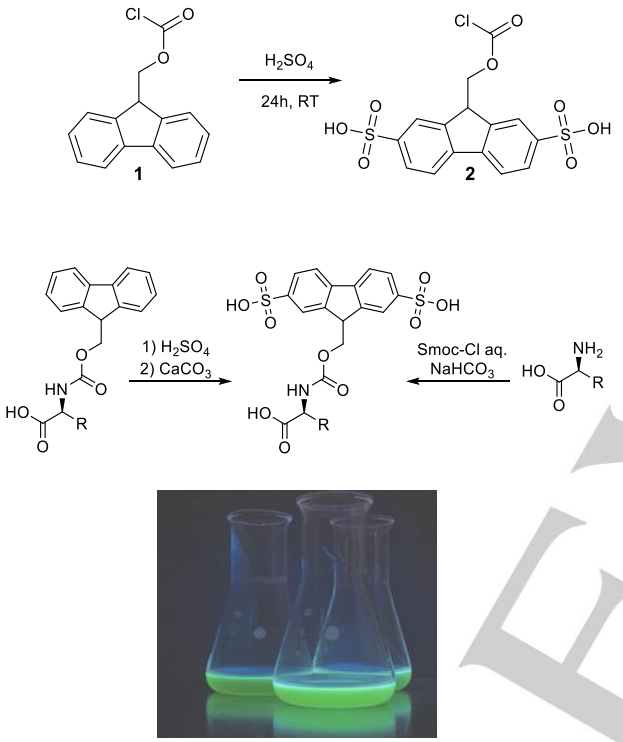


**Figure 1.** Top: Synthesis of Smoc chloride **2** from Fmoc chloride **1**. Middle: synthetic access to N-Smoc amino acids. Bottom: Solution of **2** under UV irradiation.

Amino acids with functional side chains either combined $N_\alpha$-Smoc with the standard side-chain protecting groups or, with the exception of Lys, Glu, Asp, Ser, Thr and Cys, were used unprotected in aqueous SPPS (**Figure 1**, **Table 1**, **Table 2**, ESI, Section 1.2). Hence, compared to the state-of-the-art peptide-synthetic methods (**Table 1**), ASPPS offers significant advantages in terms of atom economy and reduced time required to remove side-chain protecting groups.[39-40] Moreover, its application could be advantageous in view of aggregation via side-chain protecting groups. Indeed, avoiding bulky and hydrophobic moieties as i.e. trityl or Pbf could be desirable upon assembly of "difficult" peptides often bearing arginine, asparagine, or glutamine residues.

In course of the solid-phase peptide assembly, the $N_\alpha$-protecting group is cleaved at the beginning of each cycle (**Scheme 1**) under mild conditions to retain the orthogonality with side chains.[40] Therefore, we examined various aqueous bases or their solutions in polar solvents for the ability to cleave an Smoc group (ESI, Section 1.4). We found that aqueous piperidine, piperazine, sodium hydroxide, ethanolamine and ammonia readily liberated the *N*-terminus within 5 minutes at ambient temperature along with the formation of the respective disulfonated dibenzofulvene and the products of water or base addition (ESI Section 1.4, Figure S33).

**Table 2.** Synthesized N-Smoc amino acids

| Nr | Abbreviation | Yield [%] |
|---|---|---|
| **3** | Smoc-L-Ala-OH | 87.2 |
| **4** | Smoc-D-Ala-OH | 86.9 |
| **5** | Smoc-L-Arg-OH | 85.7 |
| **6** | Smoc-L-Arg(Pbf)-OH | 85.1 |
| **7** | Smoc-L-Asn-OH | 90.4 |
| **8** | Smoc-L-Asp(OtBu)-OH | 86.7 |
| **9** | Smoc-L-Cys(Trt)-OH | 85.1 |
| **10** | Smoc-L-Gln-OH | 90.8 |
| **11** | Smoc-L-Glu(OtBu)-OH | 88.2 |
| **12** | Smoc-Gly-OH | 93.7 |
| **13** | Smoc-L-His-OH | 92.4 |
| **14** | Smoc-L-His(Trt)-OH | 86.6 |
| **15** | Smoc-L-Ile-OH | 88.9 |
| **16** | Smoc-L-Leu-OH | 90.6 |
| **17** | Smoc-D-Leu-OH | 88.7 |
| **18** | Smoc-L-Lys(Boc)-OH | 87.4 |
| **19** | Smoc-L-Met-OH | 95.1 |
| **20** | Smoc-L-Phe-OH | 93.7 |
| **21** | Smoc-L-Pro-OH | 85.8 |
| **22** | Smoc-L-Ser-OH | 90.0 |
| **23** | Smoc-L-Ser(OtBu)-OH | 87.9 |
| **24** | Smoc-L-Thr-OH | 92.2 |
| **25** | Smoc-L-Thr(OtBu)-OH | 89.2 |
| **26** | Smoc-L-Trp-OH | 90.7 |
| **27** | Smoc-L-Trp(Boc)-OH | 86.9 |
| **28** | Smoc-L-Tyr-OH | 89.7 |
| **29** | Smoc-L-Tyr(tBu)-OH | 91.4 |
| **30** | Smoc-L-Val-OH | 87.2 |
| **31** | Smoc-β-Ala-OH | 92.5 |
| **32** | Smoc-Aib-OH | 57.8 |

After the conditions for the Smoc group deprotection have been thoroughly examined, the stability of $N_\alpha$-Smoc amino acids during aqueous peptide synthesis was studied upon incubation of Smoc-Arg-OH **5**, Smoc-Ile-OH **15**, Smoc-Phe-OH **20**, Smoc-Pro-OH **21** and Smoc-Ser-OH **22** with 3 eq. aqueous $NaHCO_3$. HPLC analysis after 7, 14 and 21 days showed that the examined constructs were sufficiently stable (ESI Section 1.5). The fact that all Smoc-protected amino acids were soluble and stable at concentrations typically used in peptide synthesizers, makes them suitable building blocks for automated SPPS.

Since the early years of peptide synthesis, it has been generally accepted that formation of a peptide bond implies anhydrous coupling conditions as otherwise the required active species are

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**Table 3.** Overview of synthesized peptides

| No | Peptide sequence | Solvent | Yield, mg (%)* |
|---|---|---|---|
| **48** | H-AGELS-$NH_2$ (Pentapeptide-31) | water | 13.7 (57.7) |
| **49** | H-GPQGPQ-OH (Hexapeptide-9) | water | 9.2 (38.7) |
| **50** | H-EEMQRR-$HN_2$ (Hexapeptide-3) | water | 21.7 (51.3) |
| **51** | Ac-EEMQRR-$HN_2$ (Acetylhexapeptide-3) | water | 18.7 (42.1) |
| **52** | Leu-Enkephalin amide H-YGGFL-$NH_2$ | water | 19.6 (70.8) |
| **53** | Met- Enkephalin H-YGGFM-OH | 30% $MeCN_{aq}$ | 9 (63) |
| **54** | Leu-Enkephalin H-YGGFL-OH | 30% $MeCN_{aq}$ | 10.3 (71.8) |
| **55** | (ACP) 65-74 H-VQAAIDYING-OH | 50% $MeCN_{aq}$ | 18 (36) |
| **56** | (ACP) 65-74 H-VQAAIDYING-$NH_2$ | 50% $MeCN_{aq}$ | 23 (46) |
| **57** | H-GPRP-OH | water | 8 (38) |
| **58** | Smoc-VVIA-$NH_2$ | water | 26 (67) |
| **59** | Smoc-DIIW-OH | water | 22 (50) |
| **60** | Smoc-E(OtBu)K(Boc)R(Pbf)S(tBu)C(Trt)-OH | 50% $MeCN_{aq}$ | 18 (44) |
| **61** | H-CYEIS-$NH_2$ | 30% $MeCN_{aq}$ | 15 (48.9) |
| **62** | H-ANKPG-$NH_2$ | 30% $MeCN_{aq}$ | 13 (35.6) |
| **63** | Pal-GHK-OH** | 20% $EtOH_{aq}$ | 14.1 (54.2) |
| **64** | Pal-GQPR-OH** | 20% $EtOH_{aq}$ | 18.7 (59.7) |
| **65** | H-GPRPA-$NH_2$ Vialox | 30% $MeCN_{aq}$ | 12 (48.4) |
| **66** | Oxytocin | 30% $MeCN_{aq}$ | 18 (35.7) |
| **67** | Vasopressin | 30% $MeCN_{aq}$ | 20 (39.7) |
| **68** | Heptaarginine | 30% $MeCN_{aq}$ | 23 (41.4) |
| **69** | Leuphasyl | 20% $MeCN_{aq}$ | 17.3 (57.8) |

*calculated from average loading; ** Pal: Palmitic acid

hydrolysed and the equilibrium is shifted towards initial reagents. However, in water the acidity of carboxylic acids is increased compared to that in polar aprotic solvents commonly applied in peptide synthesis.[41] Therefore, the acidic proton of the carboxylic group is transferred to a water molecule, thus increasing reactivity of the carboxylate ion towards carbodiimides.[42] In the present study, we evaluated different coupling reagents or active ester-forming compounds in the synthesis of a dipeptide Smoc-l-Pro-l-Tyr-OMe (ESI Section 1.6) at ambient temperature. The results are summarized in ESI Section 1.6 and Table S11. In our hands, the combination of EDC-HCl/Oxyma[43] in the presence of sodium bicarbonate as a general base gave 90.7% conversion after 25 min in water at ambient temperature. Interestingly, in 30% aq. acetonitrile, ethanol or isopropanol reaction yield increased to almost quantitative (discussed in detail in ESI Section 1.6).

With all natural and some non-canonical amino acids as $N_\alpha$-protected Smoc building blocks possessing significant stability in basic aqueous solutions, but easily losing their aminoterminal protection upon mild treatment with particular water-soluble bases, we expanded the classic Merrifield protocol[14, 40] towards an aqueous solid-phase peptide synthesis (ASPPS, Scheme 1). As a solid support we used a water-swellable resin from the repertoire of commercially available SPPS polymers (e.g. Tentagel, ChemMatrix, PEGA, etc.).[44] Following loading of the first amino acid (**Scheme 1**) in the case when a commercial preloaded resin was not available, the Smoc deprotection was conducted with either aqueous NaOH, $NH_3$, piperazine or ethanolamine. Then, coupling of the next activated amino acid was performed followed if required by capping of unconverted amines with special sulfo-tags based on sulfoacetic acid or similar compounds (**Scheme 1**). This step ensured labelling of all remaining free amines thus allowing for an easy purification after global cleavage. This deprotection-coupling-capping cycle was repeated until the desired length of a peptide had been reached.

In the present study, we assembled 22 peptides of different lengths and complexity, most of them being bioactive molecules used in cosmetic or pharmaceutical applications (**Table 3**).

For the detailed experimental procedure, refer to ESI Section 1.7. Briefly, all peptides were assembled manually on either a commercially available ChemMatrix H-Rink amide or on a preloaded HMPB-ChemMatrix resin. The ChemMatrix H-Rink amide resin was loaded in a double coupling using a solution of $N_\alpha$-Smoc amino acid (3 eq.), EDC-HCl **37** (5.5 eq.), and Oxyma **39** (3 eq.) in aqueous $NaHCO_3$. Smoc was deprotected with either 1M NaOH, 25% aq. ethanolamine or 5-10% aq. piperazine. Coupling of the amino acids was performed similar to the loading of the first amino acid. For the details see ESI Section 1.7. As expected, the lack of side-chain protection significantly reduced the global cleavage duration. Indeed, even in the case of peptides with multiple arginines[39] (**50**, **51**, **68**) cleavage time did not exceed 1 h, and purity of the crude peptide was reasonable (**Figure 2**).

To verify our "sulfo-tag" purification approach we synthesized two model peptides, **49** and **77**, having taken all amino acids for coupling in deficiencies (0.95 eq. to the loading of prior amino

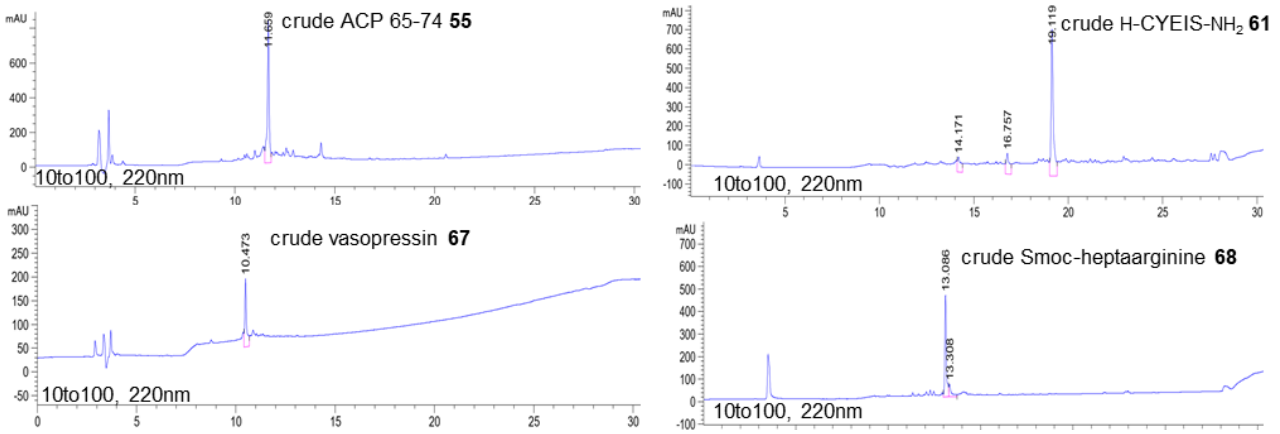


**Figure 2.** Selected RP-HPLC traces of crude **55** (a standard validation sequence), **61** (a model peptide for racemization studies), **67** (natural peptide vasopressin), **68** (an arginine-rich peptide). HPLC traces show peptides prior to any purification step.

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acid) to facilitate formation of wrong, shortened sequences. (**Figure 3** and ESI Section 1.8) After global acidolytic cleavage, all labelled side products, the "correct" peptide of desired length during the ASPPS corresponded to that observed upon an Fmoc-based SPPS in DMF. The formation of aspartimide, a well-documented side reaction occurring frequently at Asn-R or Asp-R

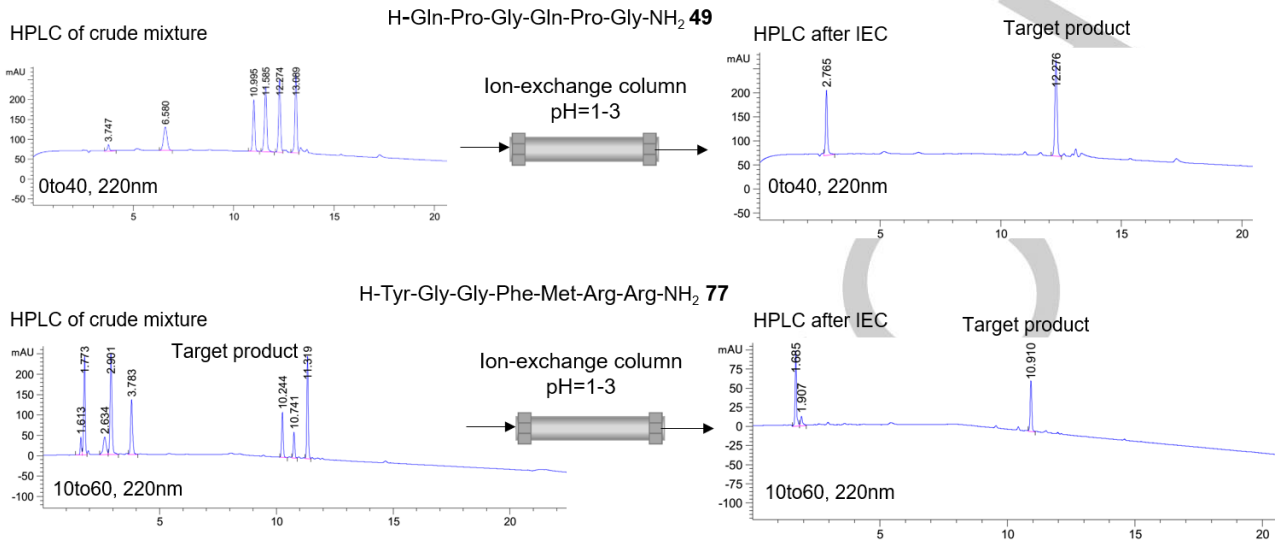


**Figure 3.** Sulfo-tag purification concept. Two model peptides, **49** and **77**, were assembled with all amino acids taken in deficiencies to facilitate formation of by-products. Peptide 77 was synthesized in a classic DMF based synthesis to show applicability in Fmoc-SPPS.

and the free side-chain protecting groups resided in solution. The mixture was loaded onto an ion-exchange column, and only the desired peptide lacking a sulfo-tag at the *N*-terminus was able to run through; all negatively charged sulfonated impurities were retained by the column (as shown in **Figure 3**). Since this is a fast, easy-to-handle, and sustainable purification option, ion-exchange chromatography could serve as a pre-purification step to remove most of the side products, thus facilitating subsequent HPLC separation.

In addition, two important side reactions commonly occurring upon SPPS were studied. Racemization upon ASPPS was assessed by assembly of two model peptides *H*-CYEIS-$NH_2$ **61**, *H*-ANKPG-$NH_2$ **62.** The results of this study are summarized in ESI Section 1.9. Briefly, the racemization of the amino acids where R is an amino acid such as Gly, Ala or Ser[45-46], was studied on four model peptides derived from peptide scorpion toxin II (*H*-VKDGYI-$NH_2$ **70**, *H*-VK(d-D)GYI-$NH_2$ **71**, *H*-VKNGYI-$NH_2$ **72** and *H*-VK(β-D)GYI-$NH_2$ **73**). Our data summarized in ESI Section 1.10 clearly indicate that the aspartimide formation in water is temperature dependent. Thus, at 4 °C the ratio of a *β*- to an *α*-product (Section 1.10 Fig. S78) is 1:3 and at 40 °C – 2.5:1. Therefore, deprotection of the sequences prone to aspartimide formation in water should be performed at reduced temperature. Interestingly, the β-branching is the only aspartimide side reaction detected in ASPPS.

An astonishing feature of the Smoc group is its fluorescence. Indeed, due to its particular electronic structure it possesses a distinct greenish glow (**Figure 1**) both being attached to an $N_{\alpha}$-

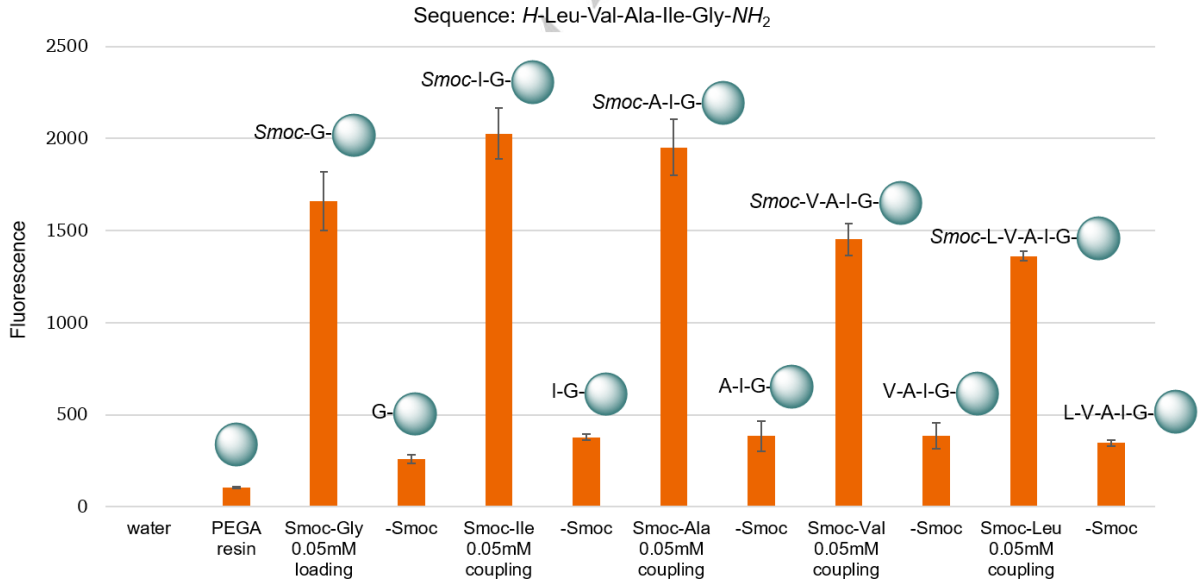


**Figure 4.** Fluorescence monitoring of coupling and deprotection steps during ASPPS of a peptide *H*-Leu-Val-Ala-Ile-Gly-$NH_2$.

atom and cleaved (see ESI Section 1.11, 1.12 for details). These properties allow the detection in solution or even when bound to the solid support, thus enabling for the first time a real-time monitoring of both the coupling and the deprotection step. Assessment of fluorescence at each coupling and deprotection step during the synthesis of a pentapeptide *H*-LVAIG-$NH_2$ was performed by excitation at 280 nm and emission at 340 nm (**Figure 4,** ESI section 1.12). The fluorescence allows distinguishing between the reaction steps. After Smoc deprotection, the fluorescence adjusts itself on a baseline value with a specific auto-fluorescence. After coupling of an $N_\alpha$-Smoc amino acid, the fluorescence increases (**Figure 4**). The amino acids show intrinsically different fluorescence; this could be compensated by a normalisation that takes the quantum yield of each amino acid into account.

## Conclusion

To summarize, we developed the working concept of efficient aqueous solid-phase peptide synthesis and demonstrated its applicability to the synthesis of 22 biologically active peptides. To make access to aqueous peptide synthesis, coupling efficiency was assessed in water-based systems applying respective $N_\alpha$-Smoc amino acids and using different activation approaches. In our hands, several water-compatible activating additives were found appropriate, with EDC-HCl **37**, Oxyma **39** and HOPO **40** being the most efficient ones. Our experiments showed that although coupling of amino acids in pure water gave reasonable yields and purity of peptides, the addition of organic co-solvents enhanced coupling performance significantly. Additional studies on enantiomeric composition showed no increased racemization levels during the ASPPS process. Ionic properties of the Smoc protecting group gave rise to an elegant approach towards a reliable purification of synthetic peptides. Already Merrifield showed that a mono-sulfonated Fmoc derivative could be applied to peptide isolation with IEC.[47] This method was further optimized and integrated as capping strategy into the ASPPS-based peptide assembly. To that end, all the by-products originating from incomplete couplings are labelled with charged sulfo-tags and can be easily removed upon successive IEC after cleavage from solid support. Our studies showed that sulfo-tag capping could also be applied to Fmoc-SPPS. This method allows tailoring of purification strategies depending on required peptide purity grade. Moreover, the same method could be used to refine the waste water (Section ESI 1.8, Figure S71).

It is important to mention that development of specific, water-optimized resins and respective linker systems still remains the challenge that must be addressed. However, this issue definitely does not belong to the field of the present work. The next goal in the frame of this study is development of automated protocols for small- and large-scale sustainable peptide synthesis.

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## Author Contributions

SK initiated, designed and coordinated the project; SK, CU, NK: designed the experiments, performed all experiments and analysis; OA, SK: wrote the manuscript; RM: performed NMR studies and acquisition. OA and HK advised on all aspects. All authors discussed the results and commented on the manuscript.

## Conflict of Interests

The authors declare competing financial interests. S.K., H.K, and C.U. are the founders of Sulfotools GmbH, a small chemical company interested in aqueous peptide synthesis. N.K. is an employee of Sulfotools GmbH. O.A., S.K., H.K, and C.U. are named inventors on a patent application (WO 2016 050764) filed by the Technische Universität Darmstadt and Sulfotools GmbH on the aqueous peptide synthesis methodology described in this work. R.M. declares no competing financial interest.

**Keywords:** peptide synthesis • solid phase peptide synthesis SPPS • sustainable chemistry • Smoc protecting group • water based synthesis

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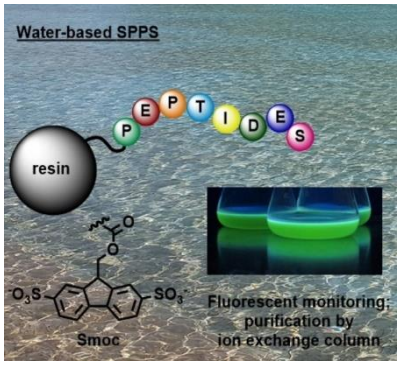


Peptide synthesis goes green. A novel Smoc protecting group enables efficient peptide assembly under aqueous conditions, advanced with fluorescence assessment of synthesis progress and a viable post-synthetic purification.