JOC The Journal of Organic Chemistry

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J. Org. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.joc.0c00599 • Publication Date (Web): 10 Jun 2020

Environmentally friendly SPPS II: Scope of green Fmoc removal protocol using NaOH and its application for synthesis of commercial drug triptorelin

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KEYWORDS: green chemistry, solid-phase peptide synthesis, protecting groups, Fmoc, 2-methyltetrahydrofuran, sodium hydroxide, triptorelin, aspartimide

ABSTRACT: With the growing necessity to consider environmental impacts when synthesizing peptide-based drugs and to expand upon the recently published short communication report, we herein present a thorough evaluation of a green Fmoc removal protocol. Our protocol avoids the use of hazardous components (using piperidine as a base and DCM and DMF as solvents) and relies on the utilization of the green mineral base NaOH in combination with the greener solvent 2-MeTHF mixed with MeOH. For the original Fmoc removal cocktail (solvents ratio of 1:1), we evaluated the impact of quality/purity of the used 2-MeTHF, scale-up, ratio of 2-MeTHF/MeOH, utilized hydroxide, temperature, and reaction time. An alternative 3:1 protocol was examined using various amino acids, and only Gly required the optimization of the Fmoc removal cocktail composition. The optimized protocol used to remove Fmoc from Gly-residue was proved by the synthesis of Leu-enkephalin. We also investigated the stability of the conventional amino acid side-chain protecting groups, *t*-Bu, Boc, Trt, and Pbf, and the formation of aspartimide as an undesirable side reaction that occurs during Fmoc solid-phase peptide synthesis (SPPS). The applicability of this synthetic strategy was documented by evaluating the SPPS of a commercial drug used for prostate and breast cancer treatments - decapeptide triptorelin.

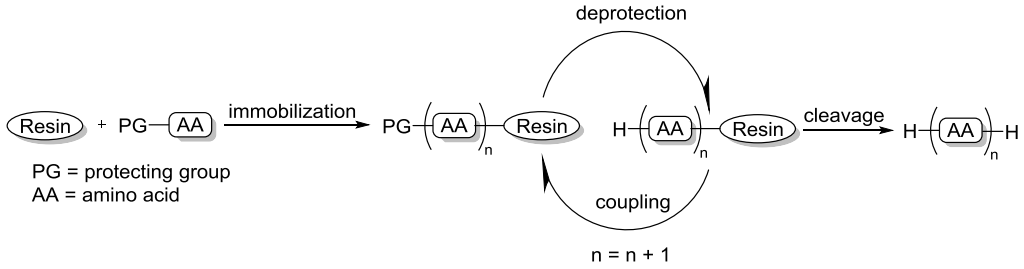
INTRODUCTION

While "small-molecule" drugs may suffer from a reduced target selectivity, peptide-based pharmaceuticals exhibit high biological activity and high specificity.[1] However, the interest of the pharmaceutical industry in peptide chemistry has been reduced due to the low oral bioavailability and low stability of these "Nature's pharmaceuticals" under physiological conditions. Recent advances in a field of chemical modification and drug-delivery technology led pharmaceutical companies to refocus their attention to peptide-based drugs, which is documented by the increasing number of therapeutic peptides on the market and drug candidates in several clinical and preclinical trials.[2-6] The commercial production of peptides used as active pharmaceutical ingredients (APIs) is scaling up to multi-ton quantities.[7-9] Moreover, peptides are expected to play an important role as future therapeutics, and therefore, their manufacturing processes represent an environmental issue.

The primary environmental concerns of the processes used to manufacture every API involve solvents since solvents are the major component of the reaction mixture.[10,11] Regarding peptide synthesis, among others, there are two key chemical strategies - solution-phase and solid-phase peptide synthesis (SPPS) as well as their combinations, which are referred to as hybrid synthesis methods.[12-14] These approaches include the consumption of large amounts of solvents that are used during the preparation and purification steps, and in SPPS methods, solvents are also used to wash the resin. The most often employed solvents used in SPPS are undoubtedly DMF and DCM. Both of these solvents are considered hazardous according to several green selection guides,[15] and DMF is likely to be restricted by REACh.[16] SPPS is used not only in research laboratories but also in industry. For this reason, environmentally friendly approaches are highly desirable. Notable, greener SPPS belongs to key green chemistry research areas identified by ACS Green Chemistry Institute Pharmaceutical Roundtable (GCIPR).[17]

SPPS was introduced in 1963 by B. R. Merrifield,[18] who was awarded the Nobel Prize for this finding. The methodology starts with the immobilization of the first protected amino acid (AA) via the carboxylic terminus onto insoluble support (resin), which then plays the role of

Scheme 1. Repetitive nature of SPPS – deprotection/coupling cycle



the C-terminal protecting group (PG). The peptide chain assembly is performed by a repetitive stepwise process based on repeating the coupling and deprotecting cycles of *N*-protected AAs, which is alternated with washing steps (Scheme 1 was adapted from reference[19]). When the peptide elongation process is complete, the whole procedure is accomplished by the final cleavage of the synthesized peptide from the resin concurrently with the global deprotection of the side-chain PGs.[13,20] In combination with the appropriate PGs,[21] SPPS became a widespread practical tool used in peptide synthesis. Although both coupling reagents[22-24] and resins have evolved since then, SPPS has not changed much until now. There is a collection of polyethylene glycol (PEG)-based solid supports, with advantages in the swelling of solvents from medium to high polarities, which range from toluene to water.[25] Despite that, the most popular solid support is still a polystyrene (PS) resin cross-linked with divinylbenzene (1-2% DVB), and the main reason is the financial aspect.

There have been attempts to make SPPS more environmentally friendly, which follows the current trend of green chemistry principles.[26] Greening SPPS is an extensively studied process, and the term green SPPS (GSPPS) should be used only after each aspect of the performance is addressed under green conditions. The number of publications dedicated to this topic per year has increased since 2011,[27] and the relevance of this field is proven by the fact that since 2018 till now, four reviews summarizing the results of developing GSPPS were published.[27-30] Specifically, performing SPPS in water, supercritical carbon dioxide and alternative organic solvents was clearly summarized in the review by Lawrenson.[28] Varnava and Sarojini extended their summary of the literature by also considering solvent-free techniques as well as evolving the use of greener and safer reagents.[29] And perhaps because Albericio's group published most of the original papers, they offered an insider perspective by comprehensively examining all the aspects of SPPS in the context of green chemistry studied so far, including steps such as the final cleavage from the resin and precipitation of peptides.[27] And very recently, they reviewed greening of each individual step of the Fmoc/*t*-Bu-SPPS process.[30]

The utilization of water as the greenest solvent requires using unusual hydrophilic PGs, or the traditional Fmoc- and Boc-protected AAs have to be transformed by nanosizing into water-dispersible nanoparticles, etc. Meanwhile, the simple substitution of the harmful solvents DMF and DCM by a green solvent with a better EHS (environmental, health, and safety) profile[15] enables the process to keep the advantages of traditional solid-phase synthesis, including the utilization of traditional commercially available reagents.

Alternative solvents were most intensively studied by Albericio and co-workers.[31-40] They investigated less problematic alternatives, such as MeCN and THF, only for the coupling step in combination with a PEG-based resin.[31] However, also studied greener alternative 2-MeTHF exhibited a good solubility for all the Fmoc-AAs and acylation agents as well as good swelling abilities for both the PS- and PEG-based resins.[32] 2-MeTHF was used as a universal solvent for the SPPS steps, including the attachment of the first AA onto both the Wang[41] and chlorotrityl chloride (CTC)[42] resins (PS-based resins), the assembly of the peptide chain,[32] and the precipitation step[36]. However, the Fmoc removal step requiring an elevated temperature appeared to be critical to the final crude purity of the synthesized peptide.[35] To look for suitable green solvents for Fmoc removal, Albericio's group evaluated γ-valerolactone (GVL) and *N*-formylmorpholine (NFM).[33] However, both solvents exhibited some limitations. While NFM performed well only with the PEG-based resin, GVL exhibited good results when it was combined with both PS-[34] and PEG-based resins in amide bond formation,[33] and was compatible with microwaves.[39] GVL was also a suitable solvent for the immobilization of the first AA onto the Wang resin (but not suitable for the CTC resin).[37] However, the solvent reacts with the less hindered Gly residues because the ring opens under the Fmoc removal conditions, resulting in acylation as a side reaction. The authors solved this issue by the incorporation of the corresponding Fmoc-AA-Gly-OH dipeptides.[38] Another green solvent, cyclopentyl methyl ether (CPME), was shown to be a good precipitating ether for use in SPPS from both a chemical point of view[43] and also from a morphological perspective.[40] However, CPME exhibits poor solubility of Fmoc-AAs.[32] In addition to Alberico's group, Lawrenson at al.[44] studied the utilization of propylene carbonate (PC) on a PEG support in SPPS. More recently, Lopez et al.[45] from Novartis Pharma AG published a systematic evaluation of more than thirty green solvents in combination with a PS-based resin, and their best green candidate for SPPS was *N*-butylpyrrolidinone (NBP). The concept of green solvent mixtures for SPPS (GM-SPPS) introduced by Ferrazzano and Cabri et al.[46] relying on combinations of cyrene, sul-

folane, or anisol with dimethyl carbonate or diethyl carbonate enabled to tailor a media of optimal properties.

Since Fmoc removal is one of the two-repeating-steps of SPPS (coupling/deprotection), the green performance would have huge environmental impacts. Fmoc[47-49] is an α-amino PG utilized by the Fmoc/*t*-Bu SPPS strategy,[50,51] which is the most commonly used SPPS approach, where the α-amino group is protected with the base-labile Fmoc and AA side chains with acid-labile groups.[52] This methodology superseded the original one introduced by Merrifield, relying on Boc/Bzl protection.

The removal of Fmoc is typically carried out under mildly basic conditions, and piperidine is almost exclusively used as a reagent for Fmoc removal in SPPS. Piperidine is toxic and scored 6.9 points (yellow/usable) on a scale 0 – 10 in GSK's acid and base selection guide.[53] Moreover, piperidine is classified as a controlled substance serving as an intermediate for the synthesis of illegal narcotic drugs.[54-56] The main reported obstacle of using piperidine is the mediation of aspartimide formation, a notorious sequence, and conformation-sensitive side reaction that occurs in SPPS.[57,58] Last but not least, piperidine is also an expensive reagent.

Attempts to make the Fmoc removal step greener were dedicated to the utilization of lower concentrations of piperidine[59] or piperidine alternatives, such as methyl piperidines,[60-62] DBU,[63,64] piperazine,[58,62] a mixture of piperazine and DBU,[65] 4-methylpiperazine,[61] a series of amines,[66] or morpholine.[67] All these piperidine alternatives were studied only in DMF. Notable, Lopez et al. were the first researchers to combine a green solvent and an alternative reagent for Fmoc cleavage (4-methylpiperidine in NBP).[45] More recently, Pawlas et al. also used 4-methylpiperidine in a mixture of green solvents NBP/EtOAc[68] and DMSO/EtOAc.[69] On the other hand, most of the papers dedicated to greening SPPS still used 20% piperidine in the evaluated solvent.

By focusing on greening the SPPS process, step-by-step, and considering that the Fmoc removal step is also crucial for SPPS to be successful, we were interested in possible means of improving this aspect. At the beginning of 2019, we published a short communication describing a new green protocol for removing the Fmoc group. The cleavage cocktail consisted of 0.2 M NaOH in a mixture of 2-MeTHF and MeOH (1:1).[19] The green inorganic base NaOH scored an 8 according to GSK's guide[53] and was also generally recognized as safe (GRAS) by the FDA.[70] The utilization of NaOH in solid-phase synthesis is not new; it has a long history since R. B. Merrifield used saponification by aq. NaOH in EtOH in his revolutionary paper published in 1963.[18] Our research group have used NaOH in a mixture of solvents (THF/MeOH) for the ester hydrolysis of heterocycles immobilized via amides[71] on a Rink resin,[72] and as a cleavage cocktail of ester-linked compounds on a Wang resin.[73] However, the lability of the Fmoc group toward NaOH has been reported already in 1980,[49,74] and to best of our knowledge, we have not found any evidence of the utilization of this inorganic base in SPPS. The only exception is the work by Hojo et al., which explored water-dispersible Fmoc-AAs in combination with PEG-based resins.[75,76]

In our green Fmoc removal protocol, we substituted the typically utilized THF[71,73] for the greener alternative 2-MeTHF.[77] 2-MeTHF is a bio-based solvent that can be produced from renewable resources derived from lignocellulosic biomass, such as furfural or levulinic acid, and moreover, 2-MeTHF is biodegradable.[77,78] Furthermore, 2-MeTHF has an acceptable toxicological profile for pharmaceutical chemical process development.[79] However, the assessment of 2-MeTHF by selection guides is not ambiguous (classified as recommended/green by Sanofi,[80] usable/yellow by Pfizer,[81] yellow by GCIPR and GSK, hazardous/red by Astra Zeneca[82]). To conclude, it is a preferable solvent substitute in comparison to DMF and DCM. 2-MeTHF was shown to be superior to DMF in SPPS when it was used only in the coupling step, but Fmoc removal was still performed with piperidine/DMF.[35] In the case of the fully green solvent performance using only 2-MeTHF in combination with the widely used PS resin, the Fmoc removal step was not optimal.[33]

In our previously published short communication,[19] the usability of the Fmoc removal protocol was documented on selected single Fmoc-AAs attached to a Rink resin and also on the synthesis of pentapeptide Leu-enkephalin amide. Moreover, the protocol is compatible with PS-based resin, the AA racemization was excluded, and also the comparison of NaOH with other organic bases, such as piperidine, morpholine, and DBU, was accomplished. However, other features are required to be addressed in order to make this protocol applicable for the sustainable manufacturing of therapeutic peptides. Additionally, this protocol has the potential to become commonly used not only in GSPPS, but also in green solid-phase organic synthesis (GSPOS) of peptidomimetics and nature-inspired heterocycles utilizing Fmoc-AAs as building blocks. Here, we report our green Fmoc removal protocol in terms of the optimization of the cleavage conditions, the stability of the acid-labile PGs, and the formation of aspartimide as an undesired side-product. The applicability of this protocol was documented by synthesizing the commercial peptide drug triptorelin. Triptorelin (pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2) is a synthetic decapeptide and belongs to a family of gonadotrophin-releasing hormone (GnRH) analogs. Triptorelin is an agonist to the luteinizing hormone-releasing hormone (LH-RH) receptor, possesses greater potency than the native hormone, and is resistant to proteolysis. Triptorelin is a commercial peptide-based drug with the following clinical applications: prostate cancer, breast cancer, endometriosis, uterine fibroids, assisted reproduction, endometrial thinning, and central precocious puberty.[83,84]

RESULTS AND DISCUSSION

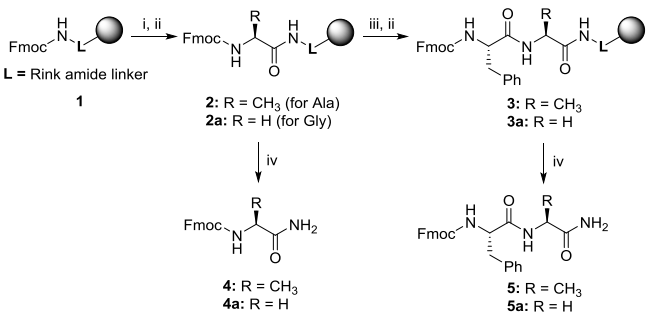
Optimization of the Fmoc removal conditions. To investigate the scope and limitation of the presented protocol, numerous Fmoc removal experiments (Tables 2 – 8) were tested on the model compound (resin **2**) prepared according to Scheme 2. Briefly, Fmoc-Ala-OH (or

Table 1. Specification overview of the evaluated 2-MeTHF with different qualities

Label	Vendor	Grade	Declared purity	Stabilizer BHT[a]	% Water[b]	% Water by K. F.[c]
A	Sigma-Aldrich	ReagentPlus®	≥99.5%	150 – 400 ppm	≤1.0	0.0124 ± 0.0005
B	VWR	GPR RECTAPUR® for synthesis	≥99.0%	150 – 400 ppm	≤0.03	0.0132 ± 0.0009
C	Merck	EMPLURA®	~98%	>100 ppm	≤0.1	0.0059 ± 0.0009

[a]BHT is the abbreviation for butylhydroxytoluene (2,6-di-tert-butyl-4-methylphenol). [b]Water content declared by the supplier. [c]Water content determined by coulometric Karl Fisher titration from 5 measurements.

Scheme 2. Evaluation of the Fmoc removal conditions using a Fmoc-Ala (or Fmoc-Gly) attached to a Rink resin[a]



[a]Reagents and conditions: (i) 50% piperidine in DMF, rt, 15 min; (ii) Fmoc-AA-OH, DIC, HOBt, DCM/DMF (1:1), rt, 1 h; (iii) NaOH, 2-MeTHF/MeOH, -2 – +40 °C, 1 – 15 min, or DBU in MeTHF, rt, 15 min (for details, see Tables 2 – 8); (iv) 50% TFA/DCM, rt, 30 min.

Fmoc-Gly-OH for Table 8) as the first AA was attached to Rink amide resin (**1**) in the traditional manner (using hazardous components DMF, DCM, piperidine). Then the resin **2** was exposed to a NaOH solution under green conditions and subsequently reacted with a second Fmoc-AA to form Fmoc-dipeptide (**3**). After the TFA-mediated cleavage of the product from the resin, the ratio of **4:5** was estimated by HPLC at 300 nm. To obtain better chromatographic separation of Fmoc-Ala-NH_2 (**4**) and the dipeptide (**5**), Fmoc-Phe was used as the second AA. The HPLC ratio of **4** and **5** after TFA cleavage revealed the amount of Fmoc removed, i. e., a >99% presence of **5** indicated the quantitative NaOH-mediated removal of the Fmoc group. Typically, the experiments were performed on an analytical scale using the volume of the Fmoc removal cocktail 1 mL, the amount of the treated resin in each experiment was 20 mg, and exposure to NaOH cocktail was 15 min if not stated otherwise.

By exploring the possibilities of optimizing the Fmoc removal cocktail, several aspects needed to be addressed. In the beginning, we turned our attention to the composition of the cocktail. We were interested in the impact of the quality of the used 2-MeTHF since vendors offer various purities for different prices (Table 1). We purchased high-quality 2-MeTHFs from Sigma-Aldrich (purity ≥99.5%, for a simplification we use label A) and VWR (≥99.0%, label B). Additionally, we also evaluated low-cost EMPLURA from Merck (reportedly, its purity exceeds 98%, label C).[85] First, ^{1}H NMR of each 2-MeTHF type did not reveal any significant difference (the spectra are shown in the Supporting Information).

In the next step, the efficiencies of NaOH-based cleavage cocktail of various concentrations consisting of a mixture of MeOH with 2-MeTHFs mentioned above of different qualities were evaluated. Quantitative Fmoc removal was achieved using 0.3 M NaOH (entries 1 – 3, Table 2) as well as 0.2 M NaOH (entries 4 – 6), irrespectively of the purity of 2-MeTHF. At a very low concentration of NaOH, 0.05 M (entries 10 – 12), all 2-MeTHFs provided incomplete Fmoc removal (81 – 91%). To conclude, the differences between evaluated 2-MeTHFs were negligible. From a practical point of view, all three 2-MeTHFs performed similarly and are suitable for the presented protocol.

Table 2. Effect of the NaOH concentration and 2-MeTHF source on the Fmoc removal from Fmoc-Ala-Rink 2[a]

Entry	c_{NaOH}	2-MeTHF	**4:5**
1	0.3 M	A	<1:>99
2	0.3 M	B	<1:>99
3	0.3 M	C	<1:>99
4	0.2 M	A	<1:>99
5	0.2 M	B	<1:>99
6	0.2 M	C	<1:>99
7	0.1 M	A	2:98
8	0.1 M	B	<1:>99
9	0.1 M	C	<1:>99
10	0.05 M	A	19:81
11	0.05 M	B	9:91
12	0.05 M	C	12:88

[a]All the reactions were performed using 2-MeTHF/MeOH 1:1 at 25 – 26 °C.

To prove our protocol to be applicable also for the synthesis of peptides at a larger (preparative) scale regardless of the quality of used 2-MeTHF, the efficiency of Fmoc removal was evaluated using the cocktail of 0.2 M NaOH in a mixture of 2-MeTHF (A – C) and MeOH (1:1) on 250 mg and 1 g of Fmoc-Ala-Rink resin **2**. All three studied 2-MeTHFs achieved quantitative Fmoc removal for both evaluated scales.

Other experiments were focused on changing the ratio of 2-MeTHF and MeOH in the Fmoc removal cocktail. The utilized solvents play different roles. MeOH is important for dissolving NaOH, but at the same time, it causes the resin to shrink. On the other hand, 2-MeTHF ensures the swelling of the resin. To achieve better resin

swelling, the solutions of 2-MeTHF with MeOH in the ratio of 1:1, 3:1, and 4:1 and all without NaOH addition were studied (an evaluation of the resin swelling[86] is included in the Supporting Information). Next, the efficiency of Fmoc removal by using cocktails of NaOH with a 2-MeTHF/MeOH ratio of 3:1, and 4:1, was studied on the model Fmoc-Ala-Rink resin **2** (Table 3). 0.2 M NaOH in both studied solvent ratios provided a quantitative Fmoc cleavage (entries 1 and 2), but the solubility of NaOH in the 4:1 solution was slightly reduced. Decreasing the concentration to 0.1 M NaOH showed incomplete Fmoc cleavage (entry 6), indicating a worsening trend (compare to entry 9 in Table 2). Therefore, to find out if more concentrated (0.2 M) NaOH cocktail with the ratio of solvents 4:1 tolerates the presence of higher water content, which could increase the solubility of NaOH in cleavage cocktail, three additions of water up to 20% (v/v) were evaluated (entries 3 – 5). In all three cases, Fmoc was quantitatively removed; however the use of 20% addition of water into cleavage cocktail led to the formation of an emulsion (entry 5), which is not suitable for synthesis. The Fmoc removal cocktail containing 5% addition of water (entry 3) was also successfully applied for the Fmoc removal step at a preparative scale (up to 1 g of resin).

Another notable point is the reaction temperature. To easily observe a potential improvement or deterioration of Fmoc removal efficiency, a low concentrated (0.05 M) NaOH-based cleavage cocktail providing only 88% efficiency at room temperature (entry 2 in Table 4) was selected for this study. The lower temperature of -2 °C slowed the reaction to achieve only 21% (entry 1), whereas the elevated temperature of 40 °C (entry 3) removed Fmoc almost quantitatively.

In an attempt to accelerate the Fmoc removal step, the reaction time was evaluated as another parameter of the protocol. The experiments were performed by applying 0.2 M NaOH in 2-MeTHF/MeOH 1:1 (Table 5). While 1 and 5 min reactions resulted in partial Fmoc cleavage, only 32% and 73%, respectively (entries 1 and 2), 10 min treatment already led to quantitative Fmoc removal (entry 3), and 15 min Fmoc removal is a guarantee (entry 4).

In the next step, the effect of the utilized hydroxide was evaluated. Potassium hydroxide and sodium hydroxide scored an 8 according to GSK's guide[53] and are both GRAS by the FDA. On the other hand, lithium hydroxide receives only a 5.7 on the scale of greenness and is not GRAS.[70] For this study, 2-MeTHF B was mixed with MeOH to achieve ratios of 1:1 and 3:1. Under these conditions, NaOH provided quantitative Fmoc removal, and KOH reached very similar results (entries 3 and 4 in Table 6). In contrast, LiOH was significantly less soluble than the other studied hydroxides, and also, its efficiency of Fmoc removal was only 53% and 65% (entries 5 and 6). For all these reasons, LiOH is not a suitable alternative.

To make our protocol greener, the possibility of exchanging MeOH for EtOH, alcohol with a better EHS profile,[85,87] was tested. Probably due to the significantly lower solubility of NaOH in EtOH than in MeOH, even 0.3 M (the highest concentration studied) or lower concentrations of NaOH provided, at most, only 41% Fmoc removal (entry 1, Table 7). To increase the solubility of NaOH, water was added into the studied mixture. The Fmoc cleavage cocktail containing 3% or 10% addition of water improved the outcome to 65% and 79%, respectively (entries 2 and 3). However, the higher addition of water (20%) into the mixture led to the formation of emulsion, providing a low efficiency of Fmoc removal (entry 4). Therefore, MeOH cannot be replaced by EtOH as greener alcohol.

Table 3. Effect of the ratio of 2-MeTHF/MeOH on the Fmoc removal from Fmoc-Ala-Rink 2[a]

Entry	c_{NaOH}	2-MeTHF/MeOH	Water addition	**4:5**
1	0.2 M	3:1	0%	<1:>99
2	0.2 M	4:1	0%	<1:>99
3	0.2 M	4:1	5%	<1:>99
4	0.2 M	4:1	10%	<1:>99
5	0.2 M	4:1	20%	<1:>99
6	0.1 M	4:1	0%	3:97

[a]All the reactions were performed at 24 – 26 °C. For all these experiments, 2-MeTHF C was used.

Table 4. Effect of the temperature on the Fmoc removal from Fmoc-Ala-Rink 2[a]

Entry	Temperature	**4:5**
1	-2 °C	79:21
2	25 °C	12:88
3	40 °C	2:98

[a]All the reactions were performed using 0.05 M NaOH in 2-MeTHF C with MeOH 1:1.

Table 5. Effect of the reaction time on the Fmoc removal from Fmoc-Ala-Rink 2[a]

Entry	Time	**4:5**
1	1 min	68:32
2	5 min	27:73
3	10 min	<1:>99
4	15 min	<1:>99

[a]All the reactions were performed using 0.2 M NaOH in 2-MeTHF B with MeOH 1:1 at 25 °C.

Table 6. Effect of the utilized hydroxide on the Fmoc removal from Fmoc-Ala-Rink 2[a]

Entry	Hydroxide	2-MeTHF/MeOH	**4:5**
1	NaOH	1:1	<1:>99
2	NaOH	3:1	<1:>99
3	KOH	1:1	3:97
4	KOH	3:1	2:98
5	LiOH	1:1	47:53
6	LiOH	3:1	35:65

[a]All the reactions were performed using 0.2 M hydroxide (prepared from a 4.7 M resp. 12.5% aq. stock solution; resulting water content in the Fmoc cleavage cocktail was 4%) in 2-MeTHF B with MeOH at 26 °C.

Table 7. Effect of exchanging MeOH for EtOH on the Fmoc removal from Fmoc-Ala-Rink 2[a]

Entry	2-MeTHF/EtOH	Water addition	**4:5**
1	1:1	0%	59:41
2	1:1	3%	35:65
3	1:1	10%	21:79
4	1:1	20%	53:47

[a]All reactions were performed at 23 – 24 °C using 0.3 M NaOH in 2-MeTHF C with EtOH 1:1, and with or without an appropriate amount of water addition.

To conclude this section, the optimal and also recommended concentration of NaOH for achieving a quantitative Fmoc removal is 0.2 M. The ratio of 2-MeTHF and MeOH in cleavage cocktail may range from 1:1 to 4:1. The recommended reaction time for quantitative Fmoc removal is 15 min; however, 10 min should also be suitable. NaOH can be eventually substituted by KOH, but not by LiOH. The presence of MeOH in the Fmoc removal cocktail is necessary, mainly to serve as a solvent for dissolving NaOH. However, MeOH cannot be replaced with greener alcohol, such as EtOH.

According to green solvent selection guides, MeOH is a less desirable solvent in the mixture of 2-MeTHF/MeOH, but its role is inherent. Since MeOH could not be exchanged with EtOH, we tried to use the minimum MeOH amount necessary. Therefore, we evaluated 3:1 (2-MeTHF/MeOH) an optimized (0.2 M NaOH) protocol for all the previously studied[19] Fmoc-AAs (Gly, Phe, Lys(Boc), Pro, Ser(O*t*-Bu), Thr(O*t*-Bu), Tyr(O*t*-Bu), Asp(O*t*-Bu), Glu(O*t*-Bu), Val, Leu, and Ile), and we newly extended the protocol used for Trp(Boc) and His(Boc) and compared it to the original 1:1 (2-MeTHF/MeOH) protocol. The complete Fmoc removal was achieved using both protocols for all the tested Fmoc-AAs with only one exception – Gly, which was not in accordance with our previous results.[19] Although Gly is, from a structural point of view, the simplest AA, it cannot be considered a typical AA, and therefore, an in-depth study was required. The results are summarized in Table 8. While 0.2 M NaOH ensured quantitative Fmoc removal for other AAs, in the case of Fmoc-Gly-Rink resin **2a** (Scheme 2), the efficiency of Fmoc removal was only 81% (entry 2). Increasing or decreasing the hydroxide concentration provided very similar results (entries 1 and 4). The expected trend was observed at elevated temperature. The ratio of the cleaved Fmoc group increased to 95% when the reaction was performed at 40 °C (entry 3). Considering the solvent content of the Fmoc cleavage cocktail, the utilization of the 3:1 protocol to achieve better swelling than the 1:1 protocol did not have a significant influence on the outcome (entry 5). Surprisingly, the 5% addition of water into the 3:1 mixture (entry 6) resulted in quantitative Fmoc removal. This composition of the Fmoc cleavage cocktail was also successfully applied at a semi-preparative scale (up to 1 g of resin), and therefore, it was afterward included in the utilized methods for the synthesis of triptorelin. Further increasing the ratio of 2-MeTHF up to 4:1 did not form a clear solution (entry 7).

Table 8. The Fmoc removal from Fmoc-Gly-Rink 2a using NaOH or DBU as base[a]

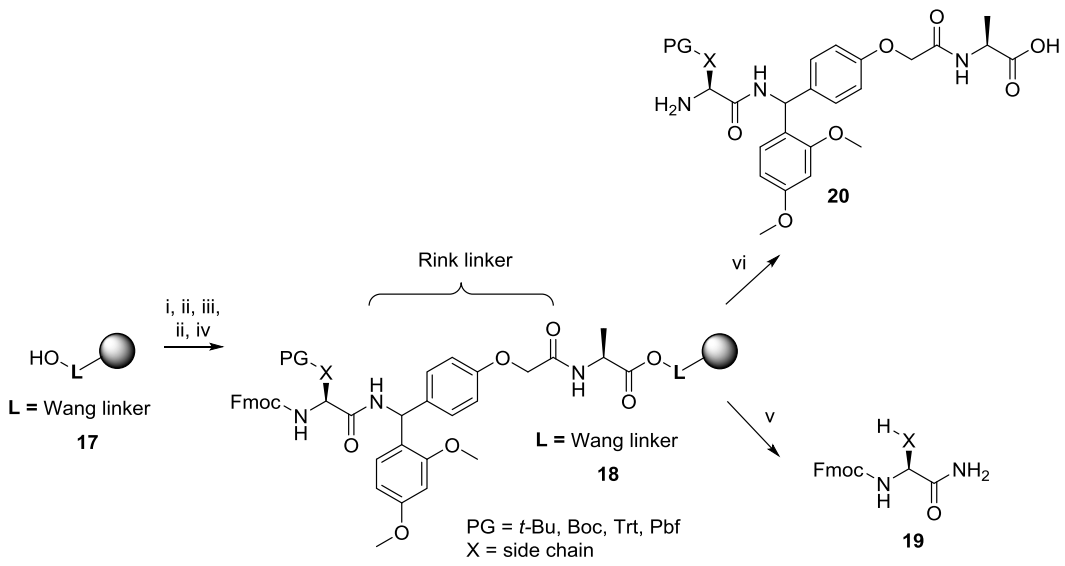
Entry	Base	2-MeTHF/ MeOH	Water addition	**4a:5a**
1	0.3 M NaOH	1:1	0%	17:83
2	0.2 M NaOH	1:1	0%	19:81
3	0.2 M NaOH	1:1	0%	5:95[b]
4	0.1 M NaOH	1:1	0%	19:81
5	0.2 M NaOH	3:1	0%	14:86
6	0.2 M NaOH	3:1	5%	<1:>99
7	0.2 M NaOH	4:1	5%	3:97
8	1% DBU	100:0	0%	<1:>99
9	0.5% DBU	100:0	0%	<1:>99

[a]For all these experiments, 2-MeTHF C was used, and the reaction temperature was 25 °C except for entry 3. [b]The reaction was performed at 40 °C.

To determine if this unexpected behavior of Gly will also manifest with bases other than NaOH, we investigated DBU for removing Fmoc from the Fmoc-Gly residue. In contrast to NaOH, 1%, and 0.5% DBU (entries 8 and 9) were the concentrations evaluated to remove Fmoc completely.

To verify the optimized protocol for the Fmoc-Gly residue, we synthesized the pentapeptide Leu-enkephalin amide with a structure of H-Tyr-Gly-Gly-Phe-Leu-NH_2, which contains two troublesome Gly residues in the sequence. For the synthesis, a protocol used in a previously published short communication report was adopted.[19] Acylation was carried out in 2-MeTHF B, which was also used for the Fmoc removal step. The ratio of the solvents was 3:1 to achieve better swelling of the resin and also to provide an alternative to the previous 1:1 protocol. When the Fmoc group from Gly was being removed, the optimized protocol with the 5% addition of water was applied. For the Fmoc removal of both Fmoc-Gly residues, we evaluated at an analytical scale (with ca. 20 mg of resin) all three protocols: 1:1, 3:1, and 3:1 + 5% addition of water (see the chromatograms in the Supporting Information). It should be noted that removing Fmoc from the Fmoc-Gly residue with Gly in the position of the third AA at solvent ratios of 1:1 and 3:1 + 5% addition of water was quantitative, whereas, in the case of the 3:1 protocol, a significant amount of the Fmoc-derivative was detected. Removing Fmoc from the following Gly residue in the position of the fourth AA was even more difficult. Both protocols without water addition partly failed, and only the 3:1 protocol with 5% addition of water removed Fmoc quantitatively at an analytical scale (20 mg of resin). However, the preparative performance of using 0.5 g of Fmoc-Gly-Gly-Phe-Leu-NH-Rink resin was carried out, and the Fmoc removal process required one repetition. It is worth to mention that we have not noticed such difficulties previously when synthesizing Leu-enkephalin amide.[19] The reason might be that the used 2-MeTHF solvent with an EMPLURA grade obtained from Sigma-Aldrich (not from Merck) contained a higher amount of water. In any case, the optimized SPPS protocol for

Scheme 3. Design of the first PG stability experiment[a]



[a]Reagents and conditions: (i) Fmoc-Ala-OH, HOBt, DIC, DMAP, DCM/DMF (1:1), rt, on; (ii) 50% piperidine in DMF, rt, 15 min; (iii) Fmoc-Rink linker, HOBt, DIC, DCM/DMF (1:1) rt, 1 h; (iv) Fmoc-AA(X-PG)-OH, HOBt, DIC, DCM/DMF (1:1), rt, 1 h; (v) 50% TFA/DCM, rt, 30 min; (vi) 0.2 M NaOH, 2-MeTHF/MeOH (1:1), rt, 30 min.

removing Fmoc from Gly residues and final isolation process, as described in the previous short communication report,[19] produced the target pentapeptide in a crude purity of 99% and an overall yield of 72% (refer to the chromatogram and ^{1}H NMR spectrum in the Supporting Information). These values proved the good applicability of this novel Fmoc removal protocol comparable to the traditional protocol that uses piperidine and DMF with DCM as solvents.

Stability of AA side-chain protecting groups. In Fmoc/*t*-Bu SPPS, the AA side-chain functionalities are protected with orthogonal acid-labile PGs.[21] To evaluate the stability of these PGs toward our Fmoc removal cocktail, we designed two sets of experiments involving side-chain PGs *t*-Bu, Boc, Trt, and Pbf by utilizing Fmoc-Ser(O*t*-Bu)-OH (**6**, Figure 1), Fmoc-Tyr(O*t*-Bu)-OH (**7**), Fmoc-Asp(O*t*-Bu)-OH (**8**), Fmoc-Glu(O*t*-Bu)-OH (**9**), Fmoc-Lys(Boc)-OH (**10**), Fmoc-His(Boc)-OH (**11**), Fmoc-Trp(Boc)-OH (**12**), Fmoc-Asn(Trt)-OH (**13**), Fmoc-Gln(Trt)-OH (**14**), Fmoc-His(Trt)-OH (**15**), and Fmoc-Arg(Pbf)-OH (**16**).

In the first set of experiments, the Fmoc-AA(X-PG)-Rink-linker-Ala-Wang resin models (**18**, Scheme 3) were synthesized. The Rink linker ensured reliable chromatographic separation, and in addition, it is also a good chromophore as well as the Fmoc group. Treatment with the TFA cleavage cocktail (conditions (v)) resulted in the liberation of Fmoc-AA(XH)-NH_2 (**19**) and PG removal from the side chain. On the other hand, the NaOH cocktail (conditions (vi)) cleaved only the ester-based attachment of Ala to the Wang resin and also removed the Fmoc group, while the PGs of all the studied AAs stayed intact (**20**) except for Boc on Fmoc-His (**11**). On the contrary, Trt, as another studied acid-labile PG of His (**15**) remained intact. Interestingly, Boc was stable to NaOH treatment when serving as protection of aliphatic amino group of Lys (**10**) or aromatic nitrogen of Trp (**12**). The combination of the racemic Rink linker and two optically pure AAs (**20**) resulted in a mixture of two diastereomers (see the chromatograms in the Supporting Information).

In the second approach, the Fmoc-AAs (Figure 1) were immobilized on the Wang linker (**21**, Scheme 4). Then, the Fmoc group was removed using piperidine/DMF, and the liberated amino group was reacted with 4-nitrobenzensulfonyl chloride to ensure the good absorbance and

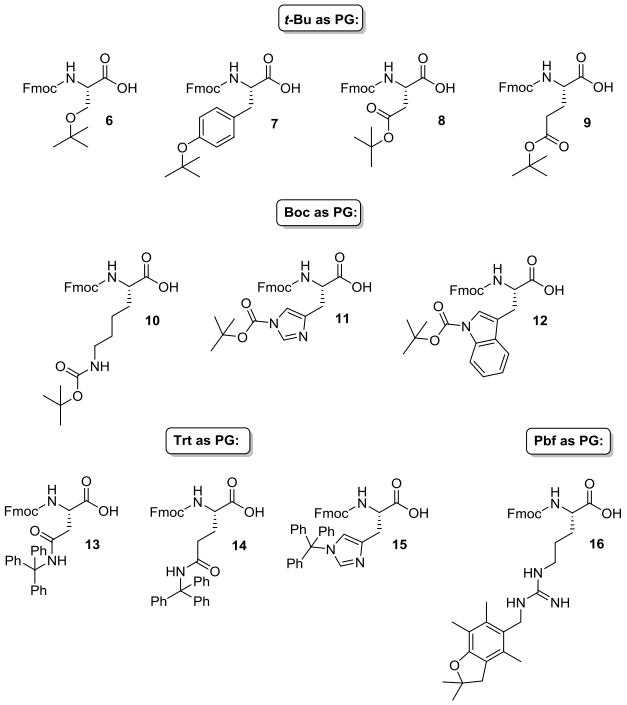
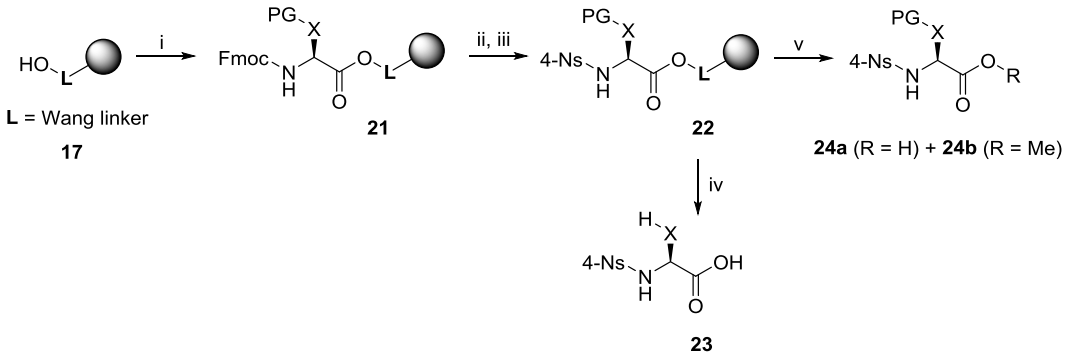


Figure 1. Structures of selected Fmoc-AAs with side-chain protection

Scheme 4. Design of the second PG stability experiment[a]

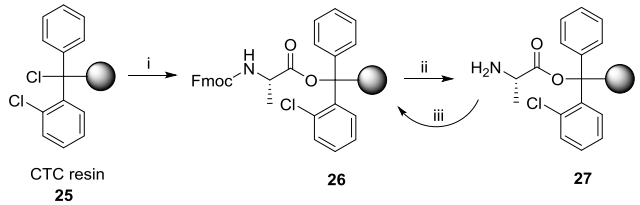


[a]Reagents and conditions: (i) Fmoc-AA(X-PG)-OH, HOBt, DMAP, DIC, DCM/DMF (1:1), rt, on; (ii) 50% piperidine in DMF, rt, 15 min; (iii) 4-Ns-Cl, lutidine, DCM, rt, 2.5 h; (iv) 50% TFA/DCM, rt, 30 min; (v) 0.2 M NaOH, 2-MeTHF/MeOH (1:1), rt, 30 min.

also better chromatographic retention of the cleaved compounds (**23**, **24**). An analytical amount of resin **22** was treated using acidic (conditions (iv)) and basic (conditions (v)) cleavage cocktails, and the presence/absence of the PGs was evaluated using HPLC-MS. Obtained results were in agreement with previous experiments. The TFA cocktail caused the cleavage of material from the resin and, also, the removal of the PGs (**23**). On the other hand, NaOH cleaved the compounds from the resin, and the PGs remained intact (**24**). Only in the case of Fmoc-His(Boc) the material without PG was detected, which can be avoided using Trt for the side chain protection. Notably, the methanolic NaOH cleavage cocktail caused re-esterification into methyl ester derivatives **24b**, and saponification led to the formation of carboxylic acids **24a** (see the chromatograms in the Supporting Information).

Compatibility with amide- and ester-based types of attachments. The NaOH Fmoc removal cocktail is compatible only with the amide-based attachment, which is achieved, for example, via the Rink amide resin, resulting in peptide amides such as GnRH analogs. However, the stability of *tert*-butyl esters (Asp(O*t*-Bu) and Glu(O*t*-Bu)) led us to assume that the ester linkage formed via the trityl-based CTC resin might be more stable than the Wang linkage due to steric hindrances, allowing for the preparation of terminal carboxylic acid peptides. This hindered resin has already been shown to be convenient for avoiding diketopiperazine formation as a side reaction.[88] Therefore, we performed the following sequence of experiments (Scheme 5). Fmoc-Ala-OH was attached to the CTC resin (**25**), and the loading was determined by HPLC to be 0.44 mmol/g. The exposure to the NaOH cocktail was followed by the reaction with Fmoc-OSu, and the loading decreased to only 0.002 mmol/g, indicating that almost no material remained attached to the resin. To conclude, the ester-type of attachment formed via the CTC resin is not compatible with the presented green protocol.

Scheme 5. Evaluation of the stability of the trityl-based CTC resin toward the NaOH cocktail[a]



[a]Reagents and conditions: (i) Fmoc-Ala-OH, DIEA, dry DCM, rt, on, first determination of loading; (ii) 0.2 M NaOH, 2-MeTHF/MeOH (1:1), rt, 30 min; (iii) Fmoc-OSu, DCM, rt, 1 h, second determination of loading.

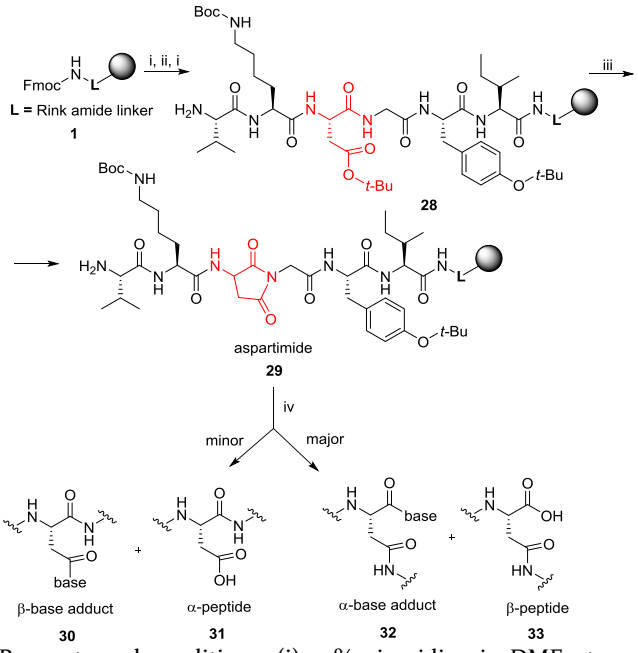
Aspartimide formation. To evaluate the effect of the NaOH treatment on this serious side reaction in SPPS, a susceptible peptide sequence was synthesized in a traditional manner (Scheme 6) and subsequently exposed to an extended treatment of the base. The evaluated hexapeptide was a fragment of the scorpion toxin II H-Val-Lys-Asp-Gly-Tyr-Ile-NH_2, which is particularly prone to aspartimide formation.[58,89] Our green Fmoc removal protocol was examined and compared to a reagent typically used for Fmoc removal – piperidine, and green base DBU. Both piperidine and DBU[58,63,64] are known to induce aspartimide formation. Piperidine, as a nucleophilic base, also causes subsequent adduct formation. Scheme 6, which shows the pathway of aspartimide (**29**) and the by-products (**30** – **33**) formation, was adapted according to a reference.[58] First, the Fmoc-hexapeptide using standard Fmoc-SPPS protocols was prepared, and then, the Fmoc group was cleaved using 50% piperidine in DMF to obtain peptide **28**. This polymer-supported peptide was subsequently exposed to 50% piperidine in DMF, 3% DBU in DMF or 2-MeTHF, and 0.2 M NaOH in 2-MeTHF/MeOH 1:1 and 3:1 at room temperature for 24 h. The products were cleaved by 50% TFA/DCM for 1 h to secure the cleavage of the Boc and *t*-Bu PGs. The cleaved compounds were analyzed by HPLC-MS (see the chromatograms in the Supporting Information). The results showed that piperidine induced substantial degrees of both aspartimide and base adduct formation, and only 13% of the target peptide remained intact. DBU caused 37%, resp. 16% aspartimide formation. The chromatogram obtained for the extended treatment with NaOH, the 1:1 protocol, was almost identical to this obtained for the untreated one, indicating no aspartimide formation. In

Table 9. Methods used for the preparation of the model decapeptide triptorelin[a]

Method	(A)	(B)	(C)	(D)
Swelling	3x DCM 3x DMF	3x 2-MeTHF	3x 2-MeTHF	3x 2-MeTHF
Deprotection	50% PIP/DMF rt, 15 min	0.2 M NaOH 2-MeTHF/MeOH (1:1)[b] rt, 15 min	0.2 M NaOH 2-MeTHF/MeOH (1:1)[b] rt, 15 min	0.2 M NaOH 2-MeTHF/MeOH (3:1)[b] rt, 15 min
Washing		3x 2-MeTHF 3x DCM	5x 2-MeTHF	5x 2-MeTHF
Coupling	Fmoc-AA-OH, HOBt, DIC			
	DMF/DCM (1:1) rt, 1 h	DMF/DCM (1:1) rt, 1 h	2-MeTHF rt, 1 h	2-MeTHF rt, 1 h
Washing	3x DMF 3x DCM	3x DMF 3x DCM	3x 2-MeTHF/MeOH (1:1) 3x 2-MeTHF	3x 2-MeTHF/MeOH (1:1) 3x 2-MeTHF

[a]Reaction steps performed using hazardous components are in red color, and environmentally friendly approaches are in green. [b]0.2 M NaOH in 2-MeTHF/MeOH 3:1 + 5% addition of water was used to remove Fmoc from the Gly residue.

Scheme 6. Synthesis of model peptide 28 and base-mediated formation of aspartimide and by-products[a]



[a]Reagents and conditions: (i) 50% piperidine in DMF, rt, 15 min; (ii) Fmoc-AA-OH, DIC, HOBt, DCM/DMF (1:1), rt, 1 h; repeating steps (i), (ii) until the completed elongation of the desired hexapeptide; (iii) base; (iv) nucleophilic base.

the case of 3:1 protocol, an additional peak (12%, with a higher retention time) with the same mass as that of linear hexapeptide **28** (88%) without a PG, presumably the β-peptide (**33**), was detected.

Synthesis of commercial peptide triptorelin. To document the applicability of the presented green Fmoc removal protocol, we used the SPPS method to synthesize the commercial drug decapeptide triptorelin (pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2). This commercial peptide has a challenging sequence since it includes AAs with heterocyclic side-chain substitutions, namely, two Trp (one as D-isomer) and His. Furthermore, there is another difficult structural moiety in Arg with the guanidine unit. The synthesis was carried out using Fmoc-AAs on a Rink amide PS/DVB-based resin.[72] The swelling, deprotecting, coupling, and washing steps are given in Table 9. The traditional protocol (method A) relied on the utilization of hazardous reagents DMF, DCM, and piperidine and served as a standard for comparison. The combination of traditional acylation and green deprotection (method B) was included to simplify the evaluation of the green Fmoc removal impact on the crude purity of the final decapeptide. As well two fully green protocols (methods C and D) using 2-MeTHF for coupling and NaOH in 2-MeTHF/MeOH, 1:1, and 3:1, for deprotection were evaluated.

The decapeptides synthesized according to methods A – D were cleaved from the resin by a mixture of TFA/H_2O (95:5). The cleavage cocktail was then evaporated, and the peptides were precipitated by diethyl ether. Isolated decapeptides were characterized by HRMS and ^{1}H NMR. The crude purities were determined from the HPLC traces and are summarized in Table 10. One example of chromatogram illustrating the presence of side-product is shown in Figure 2. Yields were calculated from NMR spectra with respect to the resin loading. The identity of prepared decapeptides was proven using a commercially available triptorelin standard purchased by AAPPTec (see the chromatograms and ^{1}H NMR spectra in the Supporting Information). Note that the SPPS process proceeded well for all four methods until the penultimate step – the Fmoc removal from His residue – before the last acylation step with pGlu. The treatment with NaOH resulted in the formation of earlier eluted impurity/side-product with the same mass spectrum. The ratios of side-product to product are shown in Table 10. In the case of method D, when the 3:1 protocol was used, 30% of the side-product was formed. Therefore, this side-product was isolated from this mixture and characterized. The HRMS spectra were comparable to those of triptorelin, but ^{1}H NMR showed slight differences in the aromatic part. The comparison suggested the correct peptide sequence with

Table 10. Purities and yields of the decapeptide triptorelin prepared using different methods

Method	Crude purity[a]	Purity including side-product	SP:P[b]	Yield[c]
A	96%	96%	0:100	83%
B	72%	77%	6:94	71%
C	72%	87%	17:83	53%
D	57%	82%	30:70	48%
E[d]	83%	83%	0:100	80%

[a]Purities were estimated from the HPLC traces obtained at 220–400 nm. [b]SP:P means the ratio of side-product to product. [c]Yields were calculated from the NMR spectra of the decapeptides following cleavage with TFA/H_2O (95:5) at rt for 4 h, evaporation with N2, and precipitation with diethyl ether. [d]Modified Method A, where Fmoc removal from His residue was performed using 3% DBU in 2-MeTHF at rt for 15 min.

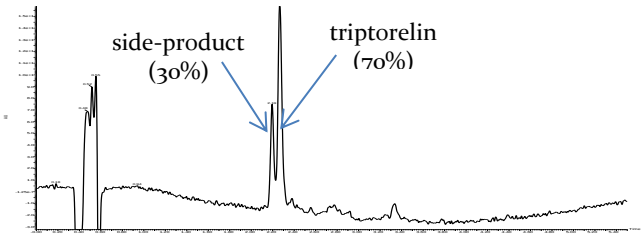


Figure 2. HPLC chromatogram of triptorelin prepared using Method D.

differences in chemical shifts of His and Trp signals (see ^{1}H NMR spectra in the Supporting Information). To eliminate potential racemization of His, we prepared triptorelin by incorporating D-His instead of L-His using a traditional protocol to obtain a peptide containing a D-isomer. The chromatographic co-elution of the isolated side-product with the triptorelin sequences having L- or D-His residues clearly eliminated the racemization of His. Also, the racemization studied on a simpler dipeptide model (according to Scheme 2, when first AA = His instead of Ala) did not indicate any epimerization. Keeping on mind the lability of Boc PG on His under NaOH treatment, triptorelin with incorporated Trt protected His was prepared using traditional protocol up to the critical step and Fmoc from His(Trt) residue was cleaved using 3:1 green protocol. Regardless of used His side chain PG, the identical side-product was formed, indicating no effect of the PG. To eliminate a potential *cis*/*trans* isomerism, solutions of isolated triptorelin and side-product in DMSO were exposed to 80 °C for 2.5 h. However, no transformation occurred, and both compounds were stable under these conditions. To prove that the formation of side-product is connected to the presence of His in triptorelin sequence, modified decapeptide replacing His for Phe was prepared in a traditional protocol up to the step of Fmoc removal from Phe residue, and subsequently exposed to green Fmoc removal cocktail, the 3:1 protocol, which was more prone to the formation of the side-product. The synthesis was accomplished by the coupling with pGlu, and decapeptide was isolated. HPLC trace of the crude peptide showed only one peak corresponding to the main product with no side-product occurrence (see the Supporting Information). These results indicated that the side-product formation is caused by the presence of His. Therefore His was incorporated into entirely unrelated peptide Leu-enkephalin to evaluate its impact on the side-product formation. Polymer-supported Leu-enkephalin was acylated with Fmoc-His(Boc)-OH in a traditional manner, and the Fmoc removal was carried out using the green 3:1 protocol. Interestingly, no side-product was detected (see Supporting Information). Based on this experiment, it was confirmed that our green Fmoc removal protocol can be applied for the preparation of His-containing peptides. Furthermore, we have proved that the formation of the side-product in the synthesis of triptorelin is sequence-dependent. It has to be noted that to entirely eliminate the formation of the undesired triptorelin side-product, 3% DBU in 2-MeTHF should be applied as a green alternative for removing Fmoc from the His residue (protocol E, Table 10) resulting in decapeptide of 83% purity. Obtained yields ranged from 48 to 83% depending on used protocol (see Table 10). The combination of green Fmoc removal and traditional acylation (Method B) provided comparable yield to traditional approach (Method A) indicating that green Fmoc removal step did not cause substantial decrease of peptide yield. Both protocols employing fully green solvent performance (Methods C and D) led to lower yields (53% and 48%, respectively) suggesting that green acylation in 2-MeTHF might be responsible for this decrease. Using DBU in 2-MeTHF only for Fmoc removal from His residue did not significantly affect isolated yield (compare Methods A and E).

To conclude, the presented method for green Fmoc removal was successfully applied in SPPS of commercial peptide triptorelin and proved to be amenable for the production of peptides.

CONCLUSIONS

Following the short communication published in January 2019, in this full paper, we address further aspects of a new green Fmoc removal protocol employing mineral base NaOH. All features of the presented protocol were evaluated in detail on selected model compound containing Fmoc-Ala, and optimized conditions were subsequently screened also for other Fmoc-AAs. Notably, the polymer-supported Fmoc-Gly residue required a specific protocol utilizing a 5% addition of water in the Fmoc removal cocktail. Further, the AA side-chain PGs, such as *t*-Bu, Boc, Trt, and Pbf, proved their stability by remaining intact after exposure to NaOH-based Fmoc removal cocktail with only one exception of Boc group protecting His. Using a hexapeptide fragment of scorpion toxin II, it was confirmed that our evaluated green protocol does not induce aspartimide formation, which is another advantage over using hazardous piperidine. Furthermore, the protocol is compatible with the most commonly used PS-based resin when the amide-type of attachment uses the Rink amide resin. Wang and CTC resins are not com-

patible since their attachment via an ester is unstable under the NaOH treatment. Despite this limitation, there are numerous peptide amides produced on a large scale, such as analogs of oxytocin and LH-RH. To document the applicability, the commercially produced decapeptide triptorelin, a synthetic agonist analog of LH-RH, was synthesized using several green protocols. The crude purities of the prepared decapeptide ranged from 57 to 83% were acceptable regarding the one achieved with the traditional protocol using hazardous piperidine in combination with hazardous solvents DMF and DCM.

EXPERIMENTAL SECTION

HRMS of triptorelin prepared using protocol A:

HRMS (ESI-TOF) *m/z* calculated for $C_{64}H_{82}N_{18}O_{13}$ $[M+H]^+$ 1311.6382, found 1311.6387; $[M-H]^-$ 1309.6225, found 1309.6221.

HRMS of triptorelin prepared using protocol B:

HRMS (ESI-TOF) *m/z* calculated for $C_{64}H_{82}N_{18}O_{13}$ $[M+H]^+$ 1311.6382, found 1311.6384; $[M-H]^-$ 1309.6225, found 1309.6226.

HRMS of triptorelin prepared using protocol C:

HRMS (ESI-TOF) *m/z* calculated for $C_{64}H_{82}N_{18}O_{13}$ $[M+H]^+$ 1311.6382, found 1311.6387; $[M-H]^-$ 1309.6225, found 1309.6225.

HRMS of triptorelin prepared using protocol D:

HRMS (ESI-TOF) *m/z* calculated for $C_{64}H_{82}N_{18}O_{13}$ $[M+H]^+$ 1311.6382, found 1311.6387; $[M-H]^-$ 1309.6225, found 1309.6230.

HPLC and NMR spectra are included in the Supporting Information.

Material and Methods

Solvents were used without further purification. The Rink amide resin (100-200 mesh, 1% DVB, 0.57 mmol/g), Wang linker (100-200 mesh, 1% DVB, 0.9 mmol/g) and 2-chlorotrityl chloride resin (100-200 mesh, 1% DVB, 0.85 mmol/g) were used. The synthesis was carried out on Domino Blocks (www.torviq.com) in disposable polypropylene reaction vessels.

All reactions were carried out at ambient temperature (~24 °C) unless stated otherwise. The volume of wash solvent was 10 mL per 1 g of resin unless stated otherwise. For washing, resin slurry was shaken with the fresh solvent for at least 1 min before changing the solvent. After adding a reagent solution, the resin slurry was manually vigorously shaken to break any potential resin clumps. Resin-bound intermediates were dried by a stream of nitrogen for prolonged storage and/or quantitative analysis.

For the LC-MS analysis, a sample of resin (~5 mg) was treated with 50% TFA in DCM for 30 min or with H2O/TFA (5:95) for 1 h, the cleavage cocktail was evaporated by a stream of nitrogen, and cleaved compounds extracted into 1 mL of MeOH.

The LC-MS analyses were carried out using UPLC Waters Acquity equipped with PDA and QDa detectors. The system comprised XSelect® HSS T3 (Waters) 3 x 50 mm C18 reverse phase column XP, 2.5 µm particles. Mobile phases: 10 mM ammonium acetate in HPLC grade water (A) and gradient grade acetonitrile for HPLC (B). A gradient was mainly formed from 20% to 80% of B in 4.5 min, kept for 1 min, with a flow rate of 0.6 mL/min. The MS ESI operated at cone voltage 25 V, and at probe temperature 600 °C and source temperature 120 °C.

Purification was carried out using semipreparative HPLC Agilent on YMC-Actus Pro 20 x 100 mm C18 reverse phase column, 5 µm particles. Mobile phases: 0.1% TFA in HPLC grade water (A) and gradient grade acetonitrile for HPLC (B). A gradient was formed from 10% to 50% in 6 min, with a flow rate of 15 mL/min.

All ^{1}H NMR experiments were performed at magnetic field strengths of 9.39 T (with operating frequencies 399.78 MHz) at ambient temperature (20 °C). All the spectra were referenced relative to the signal of DMSO (δ = 2.49 ppm).

HRMS analyses were performed using UPLC Dionex Ultimate 3000 equipped with Orbitrap Elite high-resolution mass spectrometer Thermo Exactive plus operating at full scan mode (120,000 FWMH) in the range of 100 – 1800 m/z. The settings for electrospray ionization were as follows: oven temperature of 150 °C and the source voltage of 3.6 kV. The acquired data were internally calibrated with diisooctyl phthalate as a contaminant in MeOH (m/z 391.2843). Prior UPLC separation (column Phenomenex Gemini C18, 2 x 50 mm, 3 µm particles), the samples diluted in MeOH were injected by direct infusion into the MS using an autosampler. Isocratic elution was performed using the mobile phase formed of acetonitrile/isopropyl alcohol/10 mM ammonium acetate (40:5:55), and a flow rate was 0.3 mL/min.

Determination of water content in evaluated 2-MeTHFs was performed using coulometric Karl Fischer titration on 831 KF Coulometer (Metrohm) with CombiCoulomat frit Karl Fischer reagent (Merck).

Experimental Procedures

Fmoc removal of the Fmoc Rink amide resin and subsequent acylation with Fmoc-AA-OH (Resin 2):

The Fmoc Rink amide AM resin, resin **1** (1 g, 0.61 mmol/g, 100 – 150 mesh), was washed with DCM (3× 10 mL) and DMF (3× 10 mL) and treated with a solution of 50% piperidine in DMF (10 mL) at rt for 15 min. The resin was thoroughly washed with DMF (5× 10 mL) and DCM (3× 10 mL). Then, a solution of Fmoc-AA (2 mmol), HOBt•H_2O (2 mmol, 306 mg) and DIC (2 mmol, 313 µL) in 10 mL of DCM/DMF (1:1) was added, and the reaction slurry was shaken at rt for 1 h. The resin was washed with DMF (3× 10 mL) and DCM (3× 10 mL).

Example of the treatment with NaOH in 2-MeTHF/MeOH:

Resin **2** (ca. 20 mg) was washed 3× with 2-MeTHF and treated with 0.2 M NaOH (10.6 µL of 50% (w/v) NaOH ~ 18.94 M NaOH aq. solution) in 2-MeTHF/MeOH (1:1, v/v; 1 mL) for 15 min at rt. The resin was then washed 5× with 2-MeTHF and cleaved by 50% TFA in DCM for 30 min at rt, and the cleaved compound was extracted into MeOH for the HPLC-MS measurement.

Evaluation of NaOH, KOH and LiOH as hydroxides:

A 4.735 M, resp. 12.5% (w/v) aqueous solution of NaOH was prepared by dissolving the stock solution (50% (w/v) NaOH ~ 18.94 M NaOH aq. solution) in water. Appropriate amounts of solids were dissolved in water to prepare the 4.735 M, resp. 12.5% (w/v) aqueous stock solutions of KOH and LiOH. For each hydroxide, 42.2 µL of a 4.735 M (12.5%) aqueous solution was added to 960 µL of a solvent to achieve an appropriate ratio of 2-MeTHF and MeOH. The percentage water content in the Fmoc removal cocktail was approximately 4% in all the cases.

Example of the treatment with DBU in 2-MeTHF:

Resin **2** (ca. 20 mg) was washed 3× with 2-MeTHF and treated with 1% DBU (10 µL) in 2-MeTHF (990 µL) for 15 min at rt. The resin was then washed 5× with 2-MeTHF and treated by 50% TFA in DCM for 30 min at rt. The cleaved compound was extracted into MeOH for the HPLC-MS measurement.

Immobilization of Fmoc-AA-OH onto the Wang resin (resin 21):

Wang resin **17** (250 mg, 0.9 mmol/g, 100 – 200 mesh) was washed with DCM (3× 3 mL). A solution of Fmoc-AA (0.5 mmol), HOBt•H_2O (0.5 mmol, 77 mg), DMAP (0.12 mmol, 15 mg) and DIC (0.5 mmol, 78 µL) in 2.5 mL of DCM/DMF (1:1) was added and the reaction slurry was shaken overnight at rt. The resin was then washed with DMF (3× 3 mL) and DCM (3× 3 mL).

Acylation of amino group with Fmoc-Rink amide linker (after Fmoc removal and subsequent acylation with Fmoc-AA = resin 18) or with Fmoc-AA (resin 3):

Resin (250 mg) acylated with Fmoc-AA-OH was after Fmoc removal washed with DCM (3× 3 mL) and treated with a solution of Fmoc-Rink amide linker or Fmoc-AA- OH (0.5 mmol) with HOBt•H_2O (0.5 mmol, 77 mg) and DIC (0.5 mmol, 78 µL) in 2.5 mL of DCM/DCM (1:1) and the reaction slurry was shaken at rt for 1 h. The resin was washed with DMF (3× 3 mL) and DCM (3×3 mL).

Cleavage of the product from the resin by 50% TFA/DCM and preparation of the sample for HPLC-MS (compounds 19 and 23):

An analytical amount of resin **18** or resin **22** (ca. 5 mg) was transferred into an Eppendorf tube and treated with 1 mL of a solution of 50% TFA in DCM for 30 min at rt. The TFA solution was evaporated by a stream of nitrogen. The cleaved compound was extracted into 1 mL of MeOH.

Cleavage from the resin by 0.2 M NaOH and preparation of the sample for HPLC-MS (compounds 20 and 24):

An analytical amount of resin **18** or resin **22** (ca. 10 mg) placed in a plastic syringe with a frit was treated with 1 mL of a solution of 0.2 M NaOH (10.6 µL of 50% (w/v) NaOH ~ 18.94 M NaOH aq. solution) in 2-MeTHF/MeOH (1:1, v/v; 1 mL) for 30 min at rt. The resin was filtered off, and the obtained solution was neutralized with AcOH and diluted with MeOH to achieve a ratio of 1:1.

Reaction with 4-Ns-Cl (resin 22):

After Fmoc removal, resin **21** (250 mg) was washed with DCM (3× 3 mL), and a solution of 4-Ns-Cl (0.75 mmol, 165 mg) and 2,6-lutidine (0.83 mmol, 95 µL) in 2.5 mL of DCM was added to the resin. The resulting slurry was shaken at rt for 2.5 h and subsequently washed with DCM (3× 3 mL).

Immobilization of Fmoc-Ala-OH onto the CTC resin (resin 26):

CTC resin **25** (125 mg, 0.85 mmol/g, 100 – 200 mesh) was washed with anhydrous DCM (3× 3 mL). A solution of Fmoc-Ala-OH (0.2 mmol, 63 mg) and DIEA (0.5 mmol, 88 µL) in 3 mL of anhydrous DCM was added, and the reaction slurry was shaken overnight at rt. The resin was washed with DMF (3× 3 mL) and DCM (3× 3 mL), and the resin loading was quantified.

Quantification of the resin loading:

A sample of resin **26** was washed with DCM (5× 3 mL) and MeOH (3× 3 mL) and dried with nitrogen. Then, 5 mg of the resin was cleaved with 50% TFA in DCM for 30 min. The cleavage cocktail was evaporated by a stream of nitrogen, and the cleaved compound was extracted into 1 mL of MeOH. This sample of the Fmoc derivative was analyzed by HPLC, and the quantity was compared with the analysis of the standard (Fmoc-Ala-OH; concentration of 0.25 mg/mL). The loading of the resin was determined by an external standard method by the integration of the UV response at 300 nm.

Treatment with NaOH in 2-MeTHF/MeOH (resin 27):

Resin **21** (ca. 100 mg) was washed 3× with 2-MeTHF and treated with 0.2 M NaOH (21.1 µL of 50% (w/v) NaOH ~ 18.94 M NaOH aq. solution) in 2-MeTHF/MeOH (1:1, v/v; 2 mL) for 30 min at rt. The resin was then washed 5× with 2-MeTHF.

Reaction with Fmoc-OSu (resin 26):

Resin **22** (ca. 100 mg) was washed 3× with DCM, and a solution of Fmoc-OSu (0.5 mmol, 169 mg) in 1 mL of DCM was added to the resin. The resulting slurry was shaken at rt for 1 h. The resin was subsequently washed 5× with DCM, and the resin loading was quantified.

SPPS of Leu-enkephalin amide by starting with the Fmoc-Rink amide resin:

Green Fmoc removal by 0.2 M NaOH in 2-MeTHF/MeOH:

The resin (0.5 g) was washed 3× with 2-MeTHF (≥99.0% purity purchased from VWR) and treated with 0.2 M NaOH (52.8 µL of 50% (w/v) NaOH ~ 18.94 M NaOH aq. solution) in 2-MeTHF/MeOH (3:1, v/v; 5 mL) or (3:1, v/v; 4.75 mL) + 5% H_2O (250 µL) at rt for 15 min. The resin was then washed 5× with 2-MeTHF.

Acylation with Fmoc-AA:

The resin (0.5 g) was washed 3× with 2-MeTHF. A solution of Fmoc-AA (1 mmol), HOBt•H_2O (1 mmol, 154 mg) and DIC (1 mmol, 156 µL) in 5 mL of 2-MeTHF was added to the resin, and the reaction slurry was shaken at rt for 1 h. Then, the resin was washed 3× with 2-MeTHF, 3× with 2-MeTHF/MeOH (1:1), and 3× with 2-MeTHF.

SPPS of triptorelin by starting with the Fmoc-Rink amide resin:

The synthesis method was carried out according to methods A – D by starting with 0.5 g of the Fmoc-Rink amide resin. For methods B – D, 2-MeTHF (GPR RECTAPUR® for synthesis, ≥99.0%) purchased from VWR was used in both the acylation and Fmoc removal steps.

Acylation with Fmoc-Arg(Pbf)-OH in methods C and D:

Due to the low reactivity of Fmoc-Arg(Pbf)-OH with the H-Pro-Gly-NHRink resin, the acylation in 2-MeTHF had to be repeated 2× – 3× at rt for 1 h to obtain quantitative conversion. In the traditional protocol using DMF/DCM (1:1) at rt for 1 h, the acylation step did not require repetition.

Acylation with pyroglutamic acid in methods C and D:

Due to the low solubility of pyroglutamic acid in 2-MeTHF, the acylation step was performed by adding 10% DMF into 2-MeTHF at rt for 1 h.

Cleavage and isolation of triptorelin:

250 mg of resin-supported pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH-Rink was washed 3× with MeOH and 3× with DCM. The resin was treated with 3 mL of a solution of TFA/H_2O (95:5) at rt for 4 h. The TFA solution was collected, and the resin was washed with DCM. The combined extracts were concentrated under a stream of nitrogen, and the crude peptide was precipitated with diethyl ether (3 mL), filtered and subsequently lyophilized.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org. ^{1}H NMR spectra of different used 2-MeTHFs and prepared peptides (Leu-enkephalin amide and triptorelin), swelling properties of PS-Rink resin in chosen solvents, HPLC chromatograms (selected steps in SPPS of Leu-enkephalin amide, stability evaluation of side-chain PGs, aspartimide formation, prepared peptides), HRMS data of prepared peptides (PDF).

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The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This research was supported by the Department of Chemistry and Biochemistry, University of Notre Dame, and research grant 18-17978Y from the Czech Grant Agency (GACR). We thank Jana Skopalová, Ph. D., for determination of water content by coulometric Karl Fisher titration.

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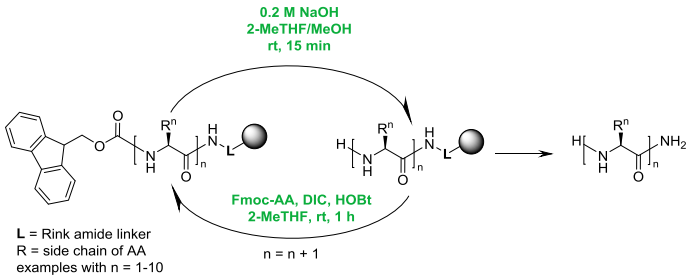
0.2 M NaOH
2-MeTHF/MeOH
rt, 15 min
Fmoc-AA, DIC, HOBt
2-MeTHF, rt, 1 h
n = n + 1
L = Rink amide linker
R = side chain of AA
examples with n = 1-10