

Aqueous Solid-Phase Peptide Synthesis (ASPPS) using Standard Fmoc/tBu-Protected Amino Acids

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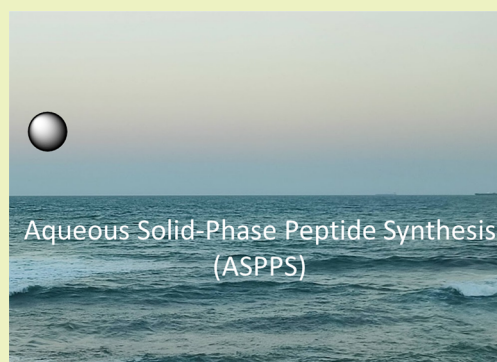
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ABSTRACT: In the context of growing environmental concerns and regulatory pressures, the peptide synthesis community is increasingly turning its attention to green chemistry. Traditional solid-phase peptide synthesis (SPPS), although widely used in research and industry, heavily relies on hazardous solvents, particularly *N,N*-dimethylformamide (DMF), which poses sustainability and safety issues. Despite various efforts to replace DMF with greener solvents or solvent mixtures, limitations remain, especially for synthesizing medium to large peptides. Aqueous solid-phase peptide synthesis (ASPPS) has emerged as a promising alternative, yet progress has been hampered by the poor solubility of standard Fmoc-amino acids and the instability of newly designed water-compatible protecting groups. Recent developments, such as more soluble protected amino acids and Fmoc-amino acid nanoparticles, have shown potential to overcome these obstacles in ASPPS but have the disadvantage of requiring distinct protected amino acids and/or nonstandard manipulations. Here we present a novel ASPPS protocol that closely mirrors conventional SPPS strategies while being performed entirely in aqueous media. This method utilizes standard Fmoc-protected amino acids and hydrophilic resins. Our findings offer a potential scalable, environmentally friendly approach to peptide synthesis that addresses key limitations of current green methodologies, opening new avenues for sustainable pharmaceutical manufacturing.

KEYWORDS: *aspartimide, green solvents, isopropanol, sustainability, water*



INTRODUCTION

Although the term “green chemistry” was coined by Anastas in the 1990s,¹ the field of synthetic peptide chemistry paid little attention to this concept until recent years, when several factors converged to promote the development of green approaches to peptide synthesis. In this regard, the first factor has undoubtedly been greater societal awareness of the urgent need for more sustainability, reflected, for example, in the cosmetics industry, which was the first to require the sustainable production of active ingredients.² The second factor regards the increasing tendency of governments to restrict the use of certain hazardous reagents or solvents. In the European Union, as of December 12, 2023, the manufacture, use, or sale of *N,N*-dimethylformamide (DMF) is prohibited unless exposure levels are restricted to 6 mg/m³ (2 ppm) for inhalation and 1.1 mg kg⁻¹ day⁻¹ for skin contact.³ Finally, the surge in peptide-based pharmaceuticals driven in large part by the launch of GLP-1 and GIP agonists, specifically liraglutide, semaglutide, and tirzepatide has greatly contributed to growing interest in green chemistry within the peptide synthesis community. We have moved from producing peptides at the gram scale to manufacturing them at the ton scale, with all the associated implications for raw material consumption, as well

as the management and disposal of excess reagents and side-products.

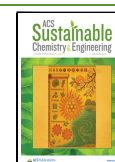
Solid-phase peptide synthesis (SPPS), first proposed by the Nobel Laureate R. Bruce Merrifield,⁴ continues to be the strategy of choice for synthesizing peptides in both research and industrial modes. In terms of sustainability, the main problem associated with this method is the use of excess reagents and their removal along with soluble side-products by extensive washing steps. Despite EU restrictions on the use of DMF, it continues to be the solvent of choice for SPPS. Attempts to replace DMF with a single solvent have been unsuccessful. *N*-Butylpyrrolidinone (NBP) has come closest,⁵ but its high viscosity makes its performance somewhat inferior to that of DMF. However, with slight heating to temperatures of 40 °C, satisfactory results have been achieved with NBP.^{6,7} However, the EU is expected to eventually restrict the use of all amidated solvents.³ Other solvents that are greener than DMF

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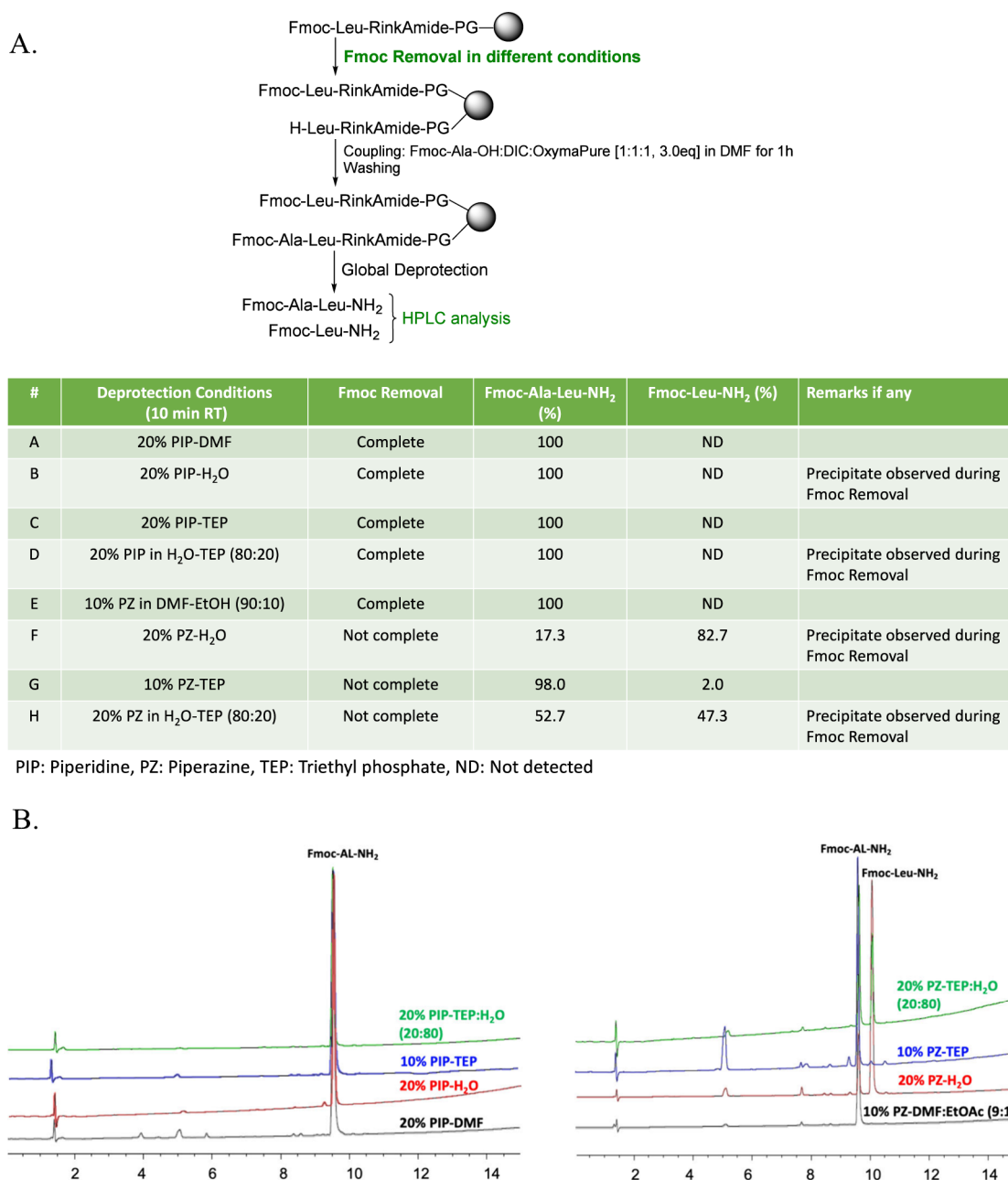


Figure 1. (A) Strategy and results of the performance of PZ vs PIP for Fmoc removal. (B) HPLC of Fmoc deprotection with both piperidine (PIP) and piperazine (PZ) in the different solvents. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–95% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

have been tested, including acetonitrile (ACN),^{8,9} tetrahydrofuran (THF),⁹ 2-methyltetrahydrofuran (Me-THF),^{10,11} cyclopentylmethyl ether (CPME),¹¹ γ -valerolactone (GVL),^{12–14} N-formylmorpholine,¹⁴ propylene carbonate (PC),^{15,16} N-octyl pyrrolidinone (NOP),¹⁷ anisole,¹⁸ PolarClean,^{19,20} dipropylene glycoldimethyl ether (DMM),²¹ and triethylphosphate (TEP).²² In all cases, these solvents allowed the synthesis of small peptides. For the preparation of medium-large peptides, the use of microwave techniques and/or ChemMatrix resin,²³ which is an excellent polyethylene glycol (PEG) resin but is no longer commercially available, was required.

Given the difficulty identifying a single solvent capable of replacing DMF, several groups have proposed solvent mixtures designed to mimic its physical properties. In this regard, some

have suggested mixtures of NBP-ethyl acetate (EtOAc),²⁴ cyrene, sulfolane, or anisole,²⁵ dimethyl sulfoxide (DMSO) with either EtOAc or 1,3-dioxolane (DOL),²⁶ DMSO-*n*-butyl acetate (AcOBu),²⁷ NOP-anisole,²⁸ DMSO-isopropyl alcohol (IPA),²⁹ or DMSO-TEP.^{29,30} These mixtures outperform single solvents and, in most cases, allow the synthesis of large peptides with results comparable to those obtained using DMF.

Although water is often regarded as the greenest solvent par excellence, its use in SPPS has received little attention compared to organic solvents.³¹ In solution peptide chemistry, despite the significant input from, primarily, Lipshutz³² and also other colleagues, no significant progress has been seen for the synthesis of medium and large-sized peptides. See Bordignon et al's recent review on this topic.³³

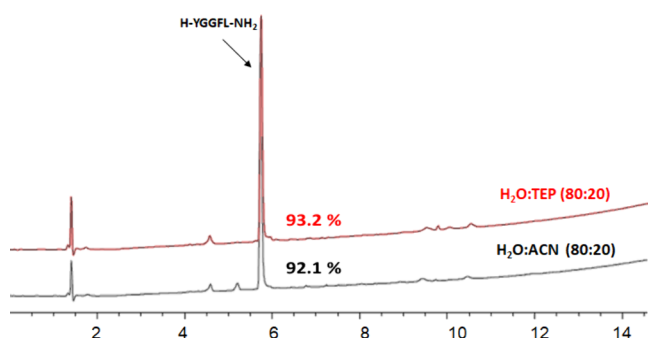
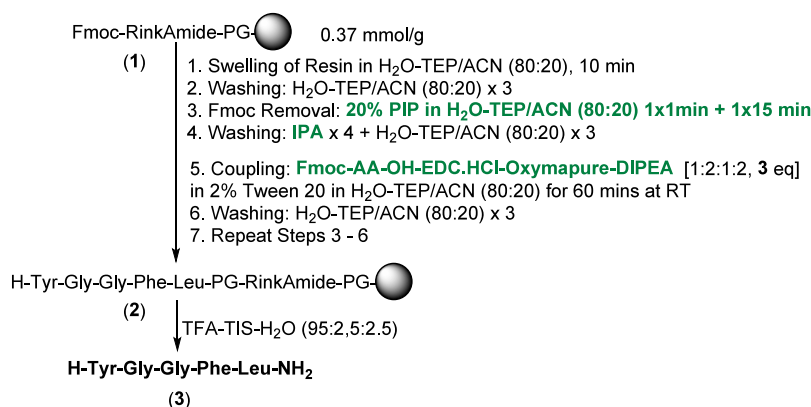
Scheme 1. Protocol Used for the Synthesis of H-Tyr-Gly-Gly-Phe-Leu-NH₂ (3)

Figure 2. HPLC of H-YGGFL-NH₂ NH₂ (3) with PG-resin and Fmoc-AA-OHs with H₂O-TEP or H₂O-ACN (80:20) as the solvent at RT. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–95% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

Early efforts in aqueous solid-phase peptide synthesis (ASPPS) focused on the development of new protecting groups for the α -amino group of the amino acid with ionizable moieties to favor the solubility of the protected amino acid in aqueous media. In this regard, the pioneering efforts of the Hojo and Kawasaki group in developing the first water-compatible protecting groups are highly commendable.³⁴ They

proposed a series of derivatives where β -substituted ethyl-oxycarbonyl protecting groups contained either sulfonio or sulfonic salts.^{35–38} These groups were removed through a β -elimination similar to Fmoc removal. Although the design of these protecting groups was innovative, their lability toward secondary amines during deprotection and their stability throughout stepwise synthesis were suboptimal, compromising the purity of the final peptide.^{37,39} More recently, Kolmar and co-workers, inspired by the pioneering work of Merrifield, who devised mono-sulfoFmoc as a purification tool,⁴⁰ developed di-sulfoFmoc [2,7-disulfo-9-fluorenylmethoxycarbonyl (Smoc)] for solubilizing the Fmoc-protected amino acids.^{41,42} Using Smoc-amino acids, this group synthesized peptides with acceptable purity by ASPPS.

Although Smoc-amino acids for proteinogenic amino acids are commercially available, complete replacement of the well-established and standard Fmoc amino acids remains a distant goal. Not only all proteinogenic protected amino acids are needed, but also D-amino acids, as well as a large number of natural and unnatural amino acids commonly found in therapeutic peptides or their development.

In this context, and despite the outstanding contributions of Hojo and Kawasaki as well as Kolmar to the development of new protecting groups, more work is focusing on the use of standard Fmoc-amino acids. To address the limited solubility

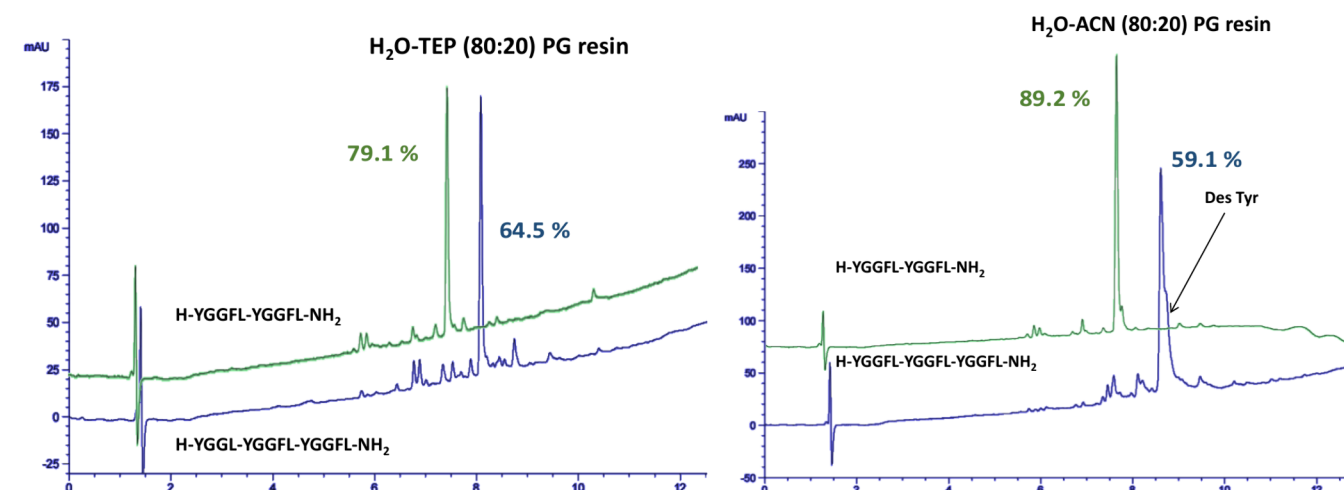


Figure 3. HPLC of H-(YGGFL)_{2,3}-NH₂ with PG-resin and Fmoc-AA-OHs with H₂O-ACN or H₂O-TEP (80:20) as the solvent at RT. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–95% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

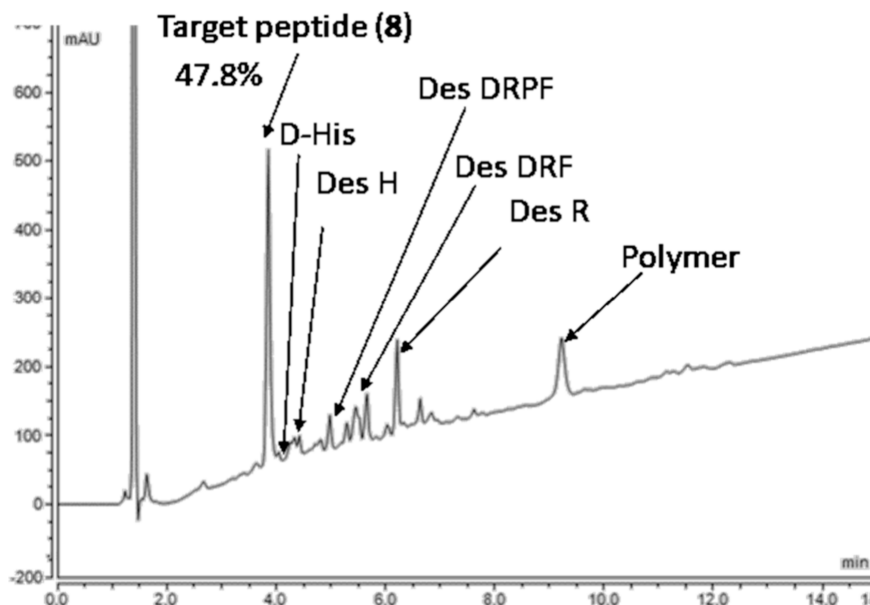
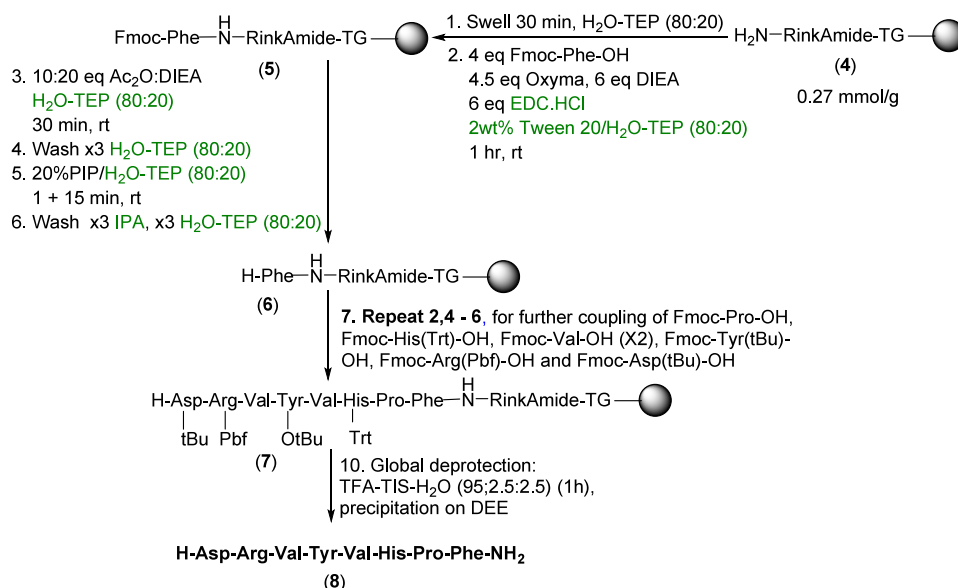
Scheme 2. Protocol Used for the Synthesis of the Angiotensin II Analog [H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-NH₂ (8)]

Figure 4. HPLC profile of the angiotensin II analog [H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-NH₂ (8)] synthesized as described in Scheme 2. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–95% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

of the latter in aqueous media, Hojo and Kawasaki's group proposed performing ASPPS with water-dispersible Fmoc-amino acid nanoparticles, which can be produced by ball mill pulverization with ZrO beads in the presence of PEG as a dispersant.^{43–45} Several model peptides were synthesized via this innovative strategy. In some of their studies, the performance of the synthesis was enhanced by microwave heating. Our group also used this microwave-based technique for ASPPS using standard Boc-amino acids to prepare a small peptide.⁴⁶ In addition, Collins used a microwave-assisted automatic synthesizer in combination with 1,1-dioxobenzothio-2-ylmethyloxycarbonyl (Bsmoc)-amino acids for ASPPS.⁴⁷ Bsmoc, which is an α,β -unsaturated sulfone-based protecting group, was first developed by Carpino as a Fmoc substitute.⁴⁸ The presence of the sulfone makes Bsmoc more

compatible than Fmoc for aqueous chemistry. Bsmoc-amino acids came close to commercial availability on two occasions, highlighting the challenges involved in launching a new protecting group.

Pawlas and Rasmussen were the first to propose the use of standard Fmoc-amino acids for ASPPS.²⁰ The synthesis of Leu-enkephalineamide was carried out in H₂O–PolarClean (4:1) using *N,N,N',N'*-tetramethylchloroformamidinium hexafluorophosphate (TCFH)/collidine (1:3) as coupling method. All these research groups used hydrophilic resins, such as PEG-based resins [Tentagel (TG),⁴⁹ poly(ethylene glycol) (PG), or ChemMatrix²³].

Here we present a novel ASPPS strategy designed to closely mimic the steps and chemical principles of conventional SPPS

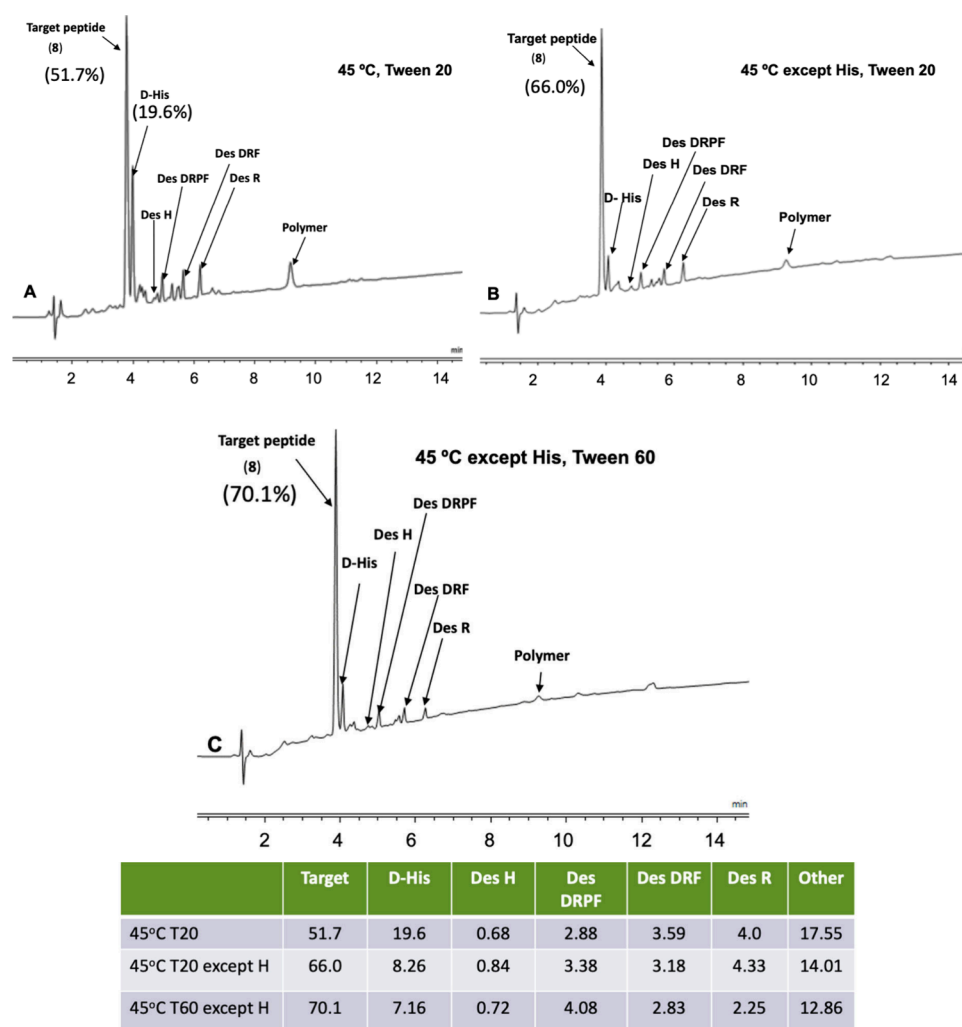


Figure 5. HPLC profile of the angiotensin II analog [H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-NH₂ (8)] synthesized as described in Scheme 2, but at 45 °C for the coupling of all Fmoc-amino acids (A), except Fmoc-His(Trt)-OH, and extended reaction times (30 min deprotection, 120 min coupling) (B); and similar to (B) but with Tween 60 (C). HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–95% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

protocols based on organic solvents but adapted to perform effectively in aqueous media.

RESULTS AND DISCUSSION

First, α -amino protecting groups, resins, coupling reagents, and cosolvents were screened. Only commercially available reagents and solvents with affordable prices and large-scale availability were tested. From this preliminary study, we concluded that Fmoc standard amino acids, PEG–PS resins, water-soluble carbodiimides, piperidine (PIP), or 4-methylpiperidine (MePIP) to remove the α -amino protecting group, and ACN, TEA, and IPA were the most appropriate as cosolvents for the new ASPPS strategy. The presence of surfactants (Tween 20 and 60 and TPGS-750-M, Figure S1) for the coupling reaction was tested with inconclusive results in this preliminary step.

The validation of protecting group removal in solid-phase synthesis requires two key steps, namely assessment of efficiency to deprotect, and confirmation of an effective removal of side products (the dibenzofulvene, in the case of the Fmoc group) through washing.

The first set of experiments was carried out using H₂O–ACN or H₂O–TEA (80:20) and PG resin for all steps. In the preliminary study, PIP or MePIP outperformed 1 M NaOH. The latter removed the protecting group, but the purity of the final peptide was poorer than that achieved when PIP/MePIP were used.

Piperazine (PZ) was also proposed to substitute PIP for Fmoc removal.⁵⁰ The main drawback of PZ in DMF-based SPPS is its poor solubility in DMF, so a 10% solution of PZ in DMF–EtOH (9:1) is recommended. In ASPPS, PZ solubility should not pose a problem, and it is envisaged that the dibenzofulvene (DBF)–PZ adduct would be more soluble in the aqueous medium than the PIP counterparts. To study the capacity of PZ to remove the Fmoc group in ASPPS, the protocol shown in Figure 1 was followed, with the results summarized below.

Figure 1 shows that while PIP was efficient in all conditions, even in H₂O alone, PZ was only able to remove the Fmoc group in DMF–EtOH. The experiments with PZ (#E–H) confirmed that the reaction kinetics in aqueous mixtures were slower compared to organic solvents, and therefore, the reaction times were extended.

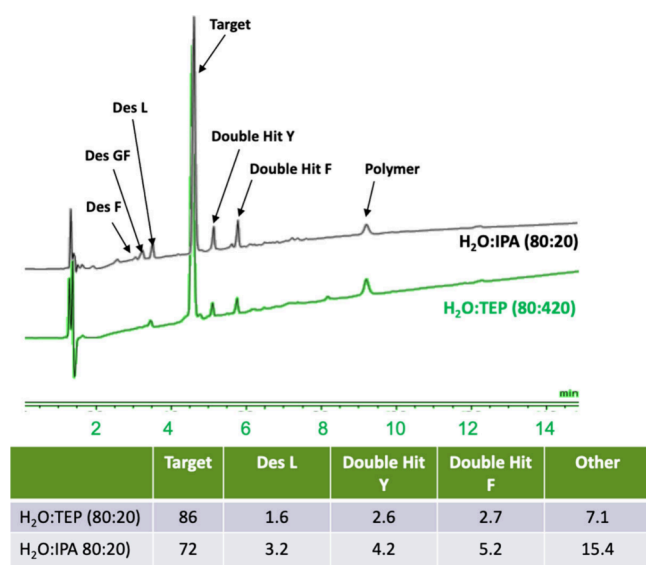


Figure 6. Comparison of the syntheses of H-YGGFL-NH₂ (3) carried out with H₂O-IPA or H₂O-TEP (80:20) at 45 °C and extended reaction times (30 min deprotection, 120 min coupling). HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–95% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

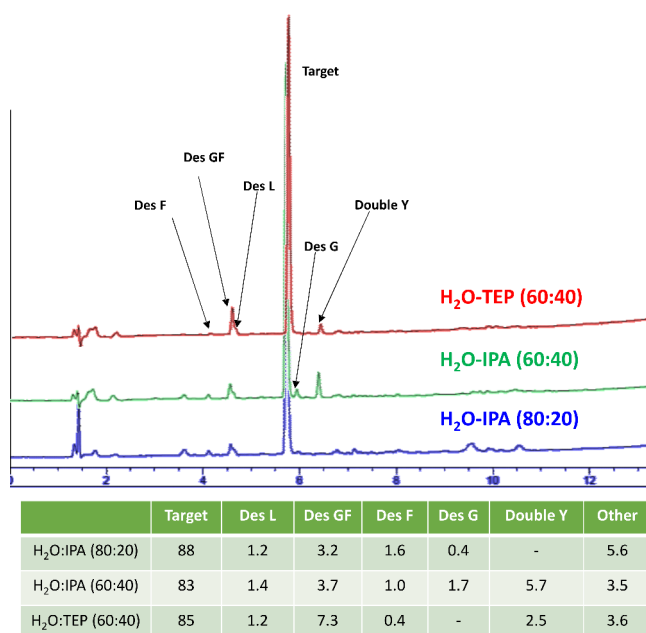


Figure 7. Comparison of the syntheses of H-YGGFL-NH₂ (3) carried out with IPA and TEP in H₂O at RT and extended reaction times (30 min deprotection, 120 min coupling). HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–95% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

On the other hand, and as Figure 1 indicates, in all cases in which the reaction was carried out in aqueous media (#B, D, F, H), a precipitate formed, presumably caused by the DBF-adduct. However, 0.2 M HCl in H₂O-TEP (taking advantage of the basic character of the side-product) (Figure S2) or IPA (Figure S3), three and two washings, respectively, effectively removed it. Thereafter, IPA washings were adopted after Fmoc removal in aqueous media.

Next, two syntheses of the model peptide Leu-enkephalin amide [H-Tyr-Gly-Gly-Phe-Leu-NH₂ (3)] in H₂O-TEP or H₂O-ACN (80:20) were carried out using the protocol shown in Scheme 1, which is virtually identical to a standard Fmoc/tBu protocol but using aqueous media instead of DMF.

For coupling, the Fmoc-AA-OH was suspended in 10 vol. of the aqueous mixture, with respect to the resin (1 mL/100 mg), and *N,N*-diisopropylethylamine (DIPEA) was then added. In all cases, the Fmoc-AA-OH was completely dissolved. Oxyma was introduced, and the clear yellow solution was added to the resin. Finally, EDC-HCl was added, and the mixture was left to react for 1 h at room temperature (RT). For Fmoc removal, the reaction times were extended slightly (1 + 15 min) with respect to the standard protocols, as discussed above. The final peptides in both syntheses presented satisfactory purity (Figure 2).

Next, H-(YGGFL)_{2,3}-NH₂ was synthesized following the same protocol described in Scheme 1. For the larger peptides, TEP outperformed ACN (Figure 3).

An angiotensin II analog (H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-NH₂) was prepared in H₂O-TEP (80:20) following the synthetic scheme shown in Scheme 2, which involves the use of TG resin instead of PG, a larger excess of Fmoc-AA-OH and coupling reagents, and acetylation after the incorporation of Fmoc-Phe-OH. TG resin contains more PEG and therefore have less loading capacity compared to PG resin (0.27 mmol/g for TG resin and 0.37 mmol/g for PG-resin).

Figure 4 shows that the target peptide was the major peak, accompanied by several more nonpolar impurities.

The synthesis was repeated at 45 °C. The impurities were approximately the same with a reduction of Des R, but with a notable increase of D-His as expected (Figure 5A). The synthesis was then repeated at 45 °C, except for the incorporation of His and with extended reaction times: 30 min for Fmoc removal instead of 1 + 15 min and 2 h for coupling instead of 1 h. This approach led to a reduction in epimerization and impurities (Figure 5B). Finally, the synthesis was repeated at 45 °C, except for the coupling of His at room temperature, and with extended reaction times, but using Tween 60 instead of 20 (Figure 5C). The purity of the target peptide was similar, although slightly purer (70% vs 66%).

Next, we examined IPA as a cosolvent. IPA has several advantages over TEP in terms of availability and, more importantly, cost. First, H-YGGFL-NH₂ (3) was synthesized using the same conditions described in Scheme 2, but at 45 °C, with extended reaction times (30 min deprotection, 120 min coupling), and without surfactant. The results obtained with IPA were slightly inferior to those achieved with TEP (Figure 6). The main differences are attributable to the presence of the double incorporation of Phe and Tyr, which is enhanced by the heating.

The synthesis of H-YGGFL-NH₂ was then carried out with a higher proportion of IPA-TEP at RT and again using extended reaction times (30 min deprotection, 120 min coupling). Similar results were obtained using 20% and 40% IPA-TEP (Figure 7).

Next, the angiotensin II analog was prepared using a higher proportion of IPA-TEP (40%) at RT and using extended reaction times (30 min deprotection, 120 min coupling), similar to the previous synthesis of H-YGGFL-NH₂. Based on the results shown in Figure 8, it can be concluded that H₂O-IPA (60:40) renders good results (87% purity), evidenced not

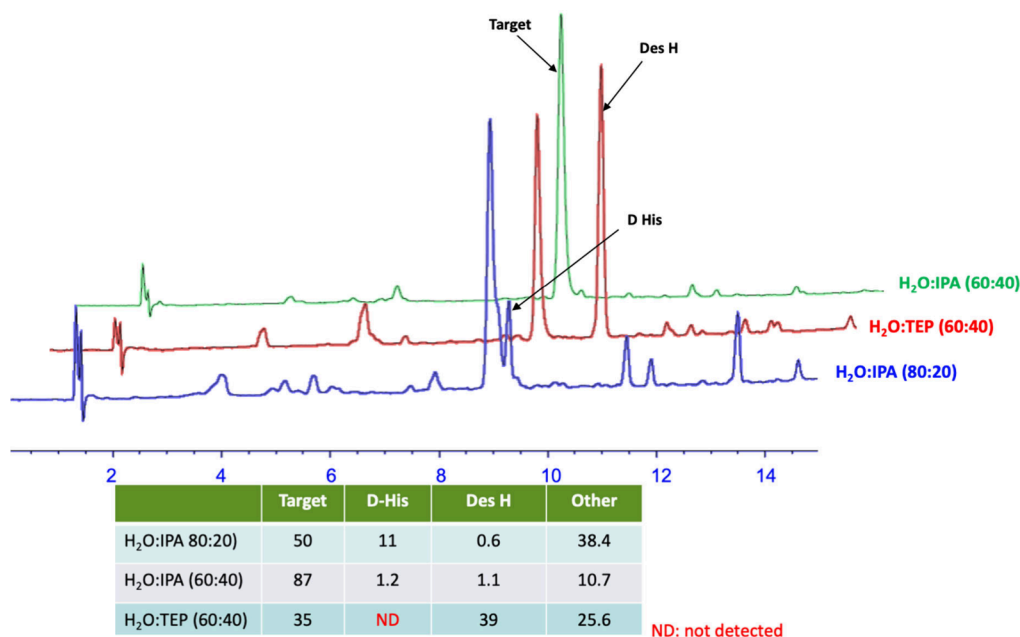


Figure 8. Comparison of the syntheses of angiotensin II analog (8) carried out with IPA and TEP in H₂O at RT and extended reaction times (30 min deprotection, 120 min coupling). HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–40% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

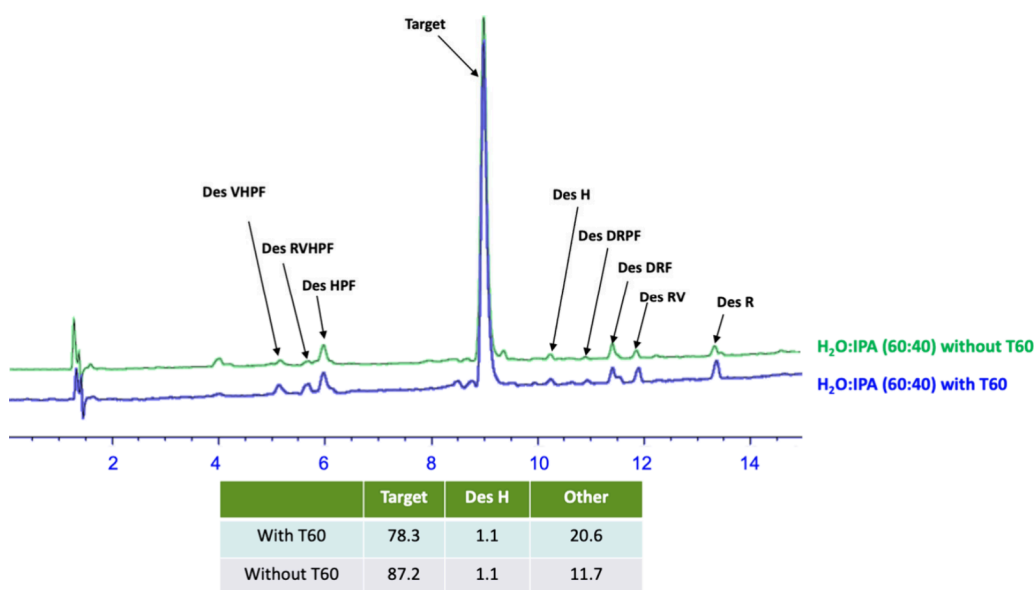


Figure 9. Comparison of the syntheses of angiotensin II analog (8) carried out with H₂O–IPA (60:40) with and without Tween T60 at RT and extended reaction times (30 min deprotection, 120 min coupling). HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–40% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

only by the absence of the D-His peptide but also by the small number of other impurities.

Next, the need for the surfactant was examined by preparing the angiotensin II analog at RT, using H₂O–IPA (60:40) and extended reaction times (30 min deprotection, 120 min coupling). The results shown in Figure 9 indicated that purity without Tween 60 was superior.

The same peptide was prepared under identical conditions but using H₂O–TEP (60:40). Similar results were achieved, but the final peptides showed slightly less purity. This observation confirms that IPA is not inferior to TEP (Figure S4).

A similar outcome regarding the importance of the surfactant—showing no significant benefit from its use—was observed in the synthesis of H-Tyr-Gly-Gly-Phe-Leu-NH₂ using H₂O–TEP (60:40) (Figure S5).

Analysis of Figures 9 and S4 shows that the vast majority of impurities were not deletion peptides (incomplete Fmoc elimination or coupling), but rather deletions of peptide fragments.

For example, the series formed by Des HPF and Des VHPF implies that, in these two side-products, elongation did not begin until the fourth amino acid (Val in Des HPF) or until the fifth one (Tyr in Des VHPF). This could suggest that the

Scheme 3. Protocol Used for the Syntheses of Tir-14 (11) and Tir-20 (12)

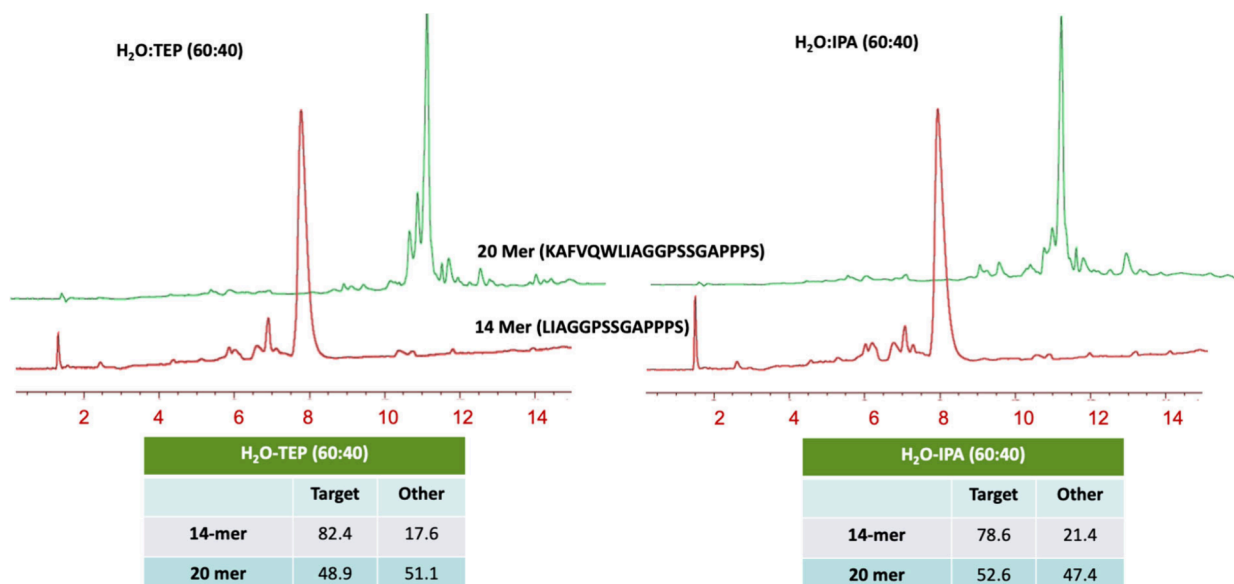
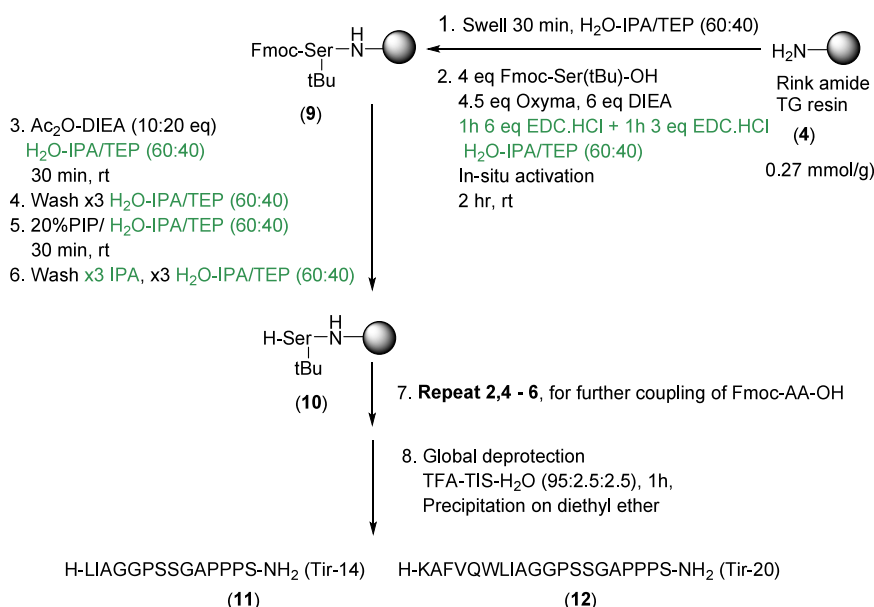
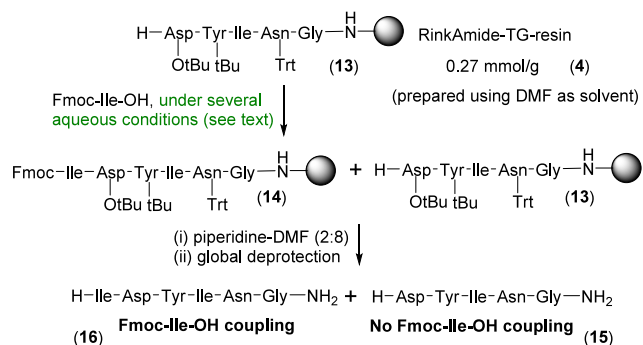


Figure 10. Comparison of the syntheses of Tir-14 (11) and Tir-20 (12) carried out with H₂O-IPA or H₂O-TEP (60:40) following the protocol shown in Scheme 3. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μm, 4.6 mm × 150 mm), 0–40% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

Scheme 4. Synthetic Scheme Used to Identify Optimal Conditions for the Coupling of Fmoc-Ile-OH on the Resin



peptide chain collapses, due to aggregation or even precipitation, during synthesis but subsequently becomes soluble again, rendering a peptide lacking several amino acids. Such collapse may be enhanced by the aqueous media.

Next, two peptides corresponding to the C-terminal part of Tirzepatide containing 14 and 20 residues (Tir-14 and Tir-20) were prepared following the protocol shown in Scheme 3 at RT, and with extended reaction times (30 min deprotection, 120 min coupling), and H₂O-IPA or H₂O-TEP (60:40) as the solvent.

All of the peptides showed acceptable purities, with no significant difference between those prepared with IPA or TEP (78.6%, 52.6% for IPA and 82.4%, 47.9% for TEP) (Figure 10). Both IPA and TEP showed good performance, but IPA

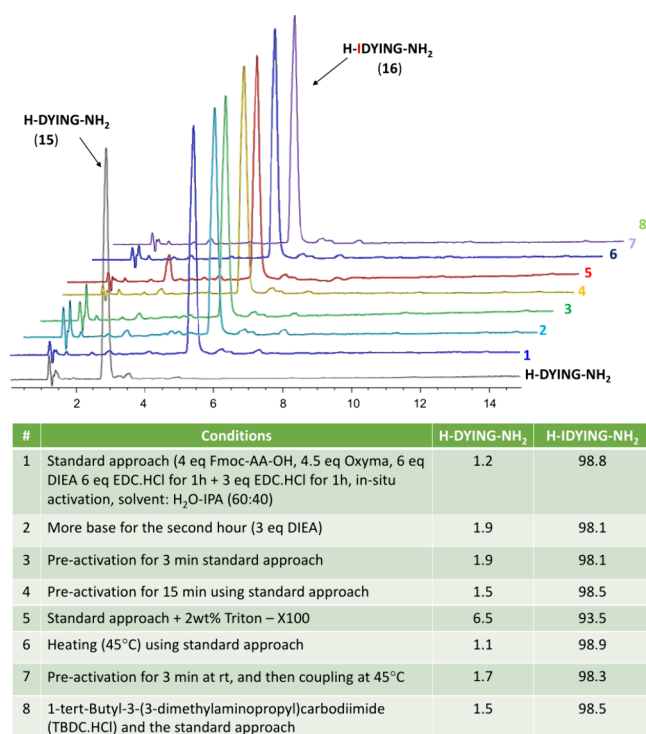


Figure 11. HPLC and table summarizing the coupling experiments of Scheme 4. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 10–30% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

has the added advantages of greater accessibility and a lower price.

Having demonstrated that this ASPPS strategy, based on standard Fmoc-AA-OH, water-soluble carbodiimides (EDC·HCl, TBDC·HCl) for coupling, piperidine for Fmoc-removal, in H₂O–IPA (60:40) as a solvent, enabled the synthesis of a substantial number of peptides, an optimization phase was performed using ACP(65–74) (H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH₂) as a model. ACP(65–74) is a medium-sized peptide whose synthesis is known to be challenging. In this regard, the removal of the Fmoc of Ile(69) is difficult and has been used as a model for studying

this reaction using green solvents and reagents⁵¹ and couplings of Gln and Val are also problematic. In addition, the peptide contains Gln and Asn residues with side chains protected by a trityl (Trt) group, which can cause solubility issues. Furthermore, the peptide is prone to aspartimide formation.

First, the coupling was studied following Scheme 4. The following conditions were tested using H₂O–IPA (60:40) at RT in all cases. Conditions 2–8 are based in the modification of conditions 1 (standard approach):

1. Standard approach: 4 equiv of Fmoc-AA-OH, 4.5 equiv of Oxyma, 6 equiv of DIEA, 6 equiv of EDC·HCl for 1 h + 3 equiv of EDC·HCl for 1 h, in situ activation
2. Use of an additional base for the second hour (3 equiv of DIEA)
3. Preactivation for 3 min using the standard approach
4. Preactivation for 15 min using the standard approach
5. Addition of 2 wt % Triton-X100 using the standard approach
6. Heating (45 °C) using the standard approach
7. Preactivation for 3 min, and then coupling at 45 °C
8. Use of 1-tert-butyl-3-(3-(dimethylamino)propyl)-carbodiimide (TBDC·HCl) instead of EDC·HCl

As shown in Figure 11, all couplings except the one performed in the presence of Triton (#5, Figure 11) gave essentially comparable results, exceeding 98% completion but not reaching full conversion (it is possible that the removal of the Fmoc of Asp was not quantitative, and therefore around 1% the deletion peptide is present by default).

A new set of experiments was carried out to corroborate these results. In this regard, the excess was reduced from 4 equiv to 2 equiv, and heating was at 35 °C. Finally, two controls were conducted, one using DMF instead of H₂O–IPA (60:40) but with EDC·HCl, and the second using DIC and Oxyma in DMF. Samples were taken after 2 h of coupling for HPLC analysis. For all conditions, see Table 1. Similar results were obtained with DIC and water-soluble carbodiimides (EDC·HCl and TBDC·HCl), reaffirming that the deletion peptide is likely caused by incomplete removal of Fmoc from the previous Fmoc-peptide-resin (Figure 12).

Fmoc removal was evaluated using the protocol described in Scheme 5. This removal is considered difficult and is used as a

Table 1. Conditions Used for Corroborating the Results of Figure 11

expt	solvent	coupling conditions (in situ activation)	t (h)	T (°C)	remarks
Control 1	DMF	Fmoc-AA/Oxyma/DIC (2:2.5:3 equiv)	6	25	standard DMF coupling samples for HPLC every 2 h
Control 2	DMF	Fmoc-AA/Oxyma/EDC·HCl/DIEA (2:2.5:3:3) + EDC·HCl/DIEA (2:2) + EDC·HCl/DIEA (2:2)	2 2 2	25	samples for HPLC every 2 h
A	H ₂ O–IPA (60:40)	Fmoc-AA/Oxyma/EDC·HCl/DIEA (4:4.5:6:6) + EDC·HCl (3)	2 2	25	As #1 in the table in Figure 11 samples for HPLC every 2 h
B	H ₂ O–IPA (60:40)	Fmoc-AA/Oxyma/TBDC·HCl/DIEA (4:4.5:6:6) + EDC·HCl/DIEA (2:2)	2 2	25	similar to A, but TBDC·HCl instead of EDC·HCl samples for HPLC every 2 h
C	H ₂ O–IPA (60:40)	Fmoc-AA/Oxyma/EDC·HCl/DIEA (2:2.5:3:3) + EDC·HCl/DIEA (2:2) + EDC·HCl/DIEA (2:2)	2 2 2	25	similar to Control 2, but in H ₂ O–IPA (60:40) samples for HPLC every 2 h
D	H ₂ O–IPA (60:40)	Fmoc-AA/Oxyma/EDC·HCl/DIEA (2:2.5:3:3) + EDC·HCl/DIEA (2:2) + EDC·HCl/DIEA (2:2)	2 2 2	35	similar to C, but at 35 °C, samples for HPLC every 2 h

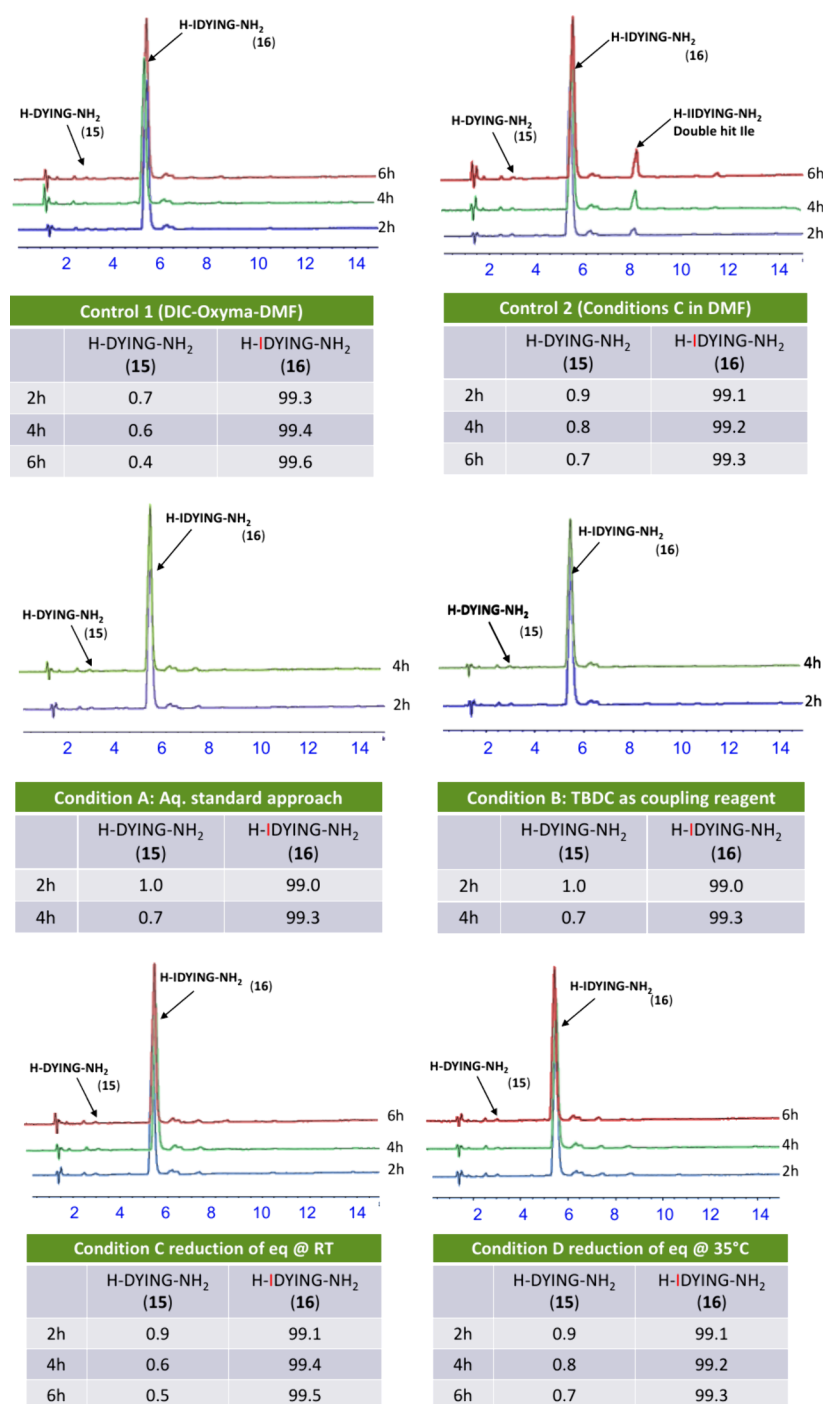


Figure 12. Results of the comparison between DMF and H₂O–IPA (60:40) and between DIC and EDC·HCl/TBDC·HCl. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 10–30% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

model for studying Fmoc removal reaction.⁵¹ The following removal conditions were studied:

- 5 + 5 min 20% PIP/H₂O–IPA (60:40)
- 15 + 15 min 20% PIP/H₂O–IPA (60:40)
- 1 + 5 min 2% DBU + 5% MOR/H₂O–IPA (60:40)
- 1 + 15 min 2% DBU + 5% MOR/H₂O–IPA (60:40)
- 1 + 10 min NaOH/H₂O–IPA (60:40)
- 1 + 20 min NaOH/H₂O–IPA (60:40)
- 5 + 5 min 20% PIP/H₂O–IPA (60:40) + 2 wt % T60
- 5 + 5 min 20% PIP/H₂O–IPA (60:40) + 2 wt % Triton-X100

I. 15 + 15 min 20% PIP/H₂O–IPA (60:40) at 45 °C

As expected, this Fmoc removal approach was demanding and required longer times with PIP (B vs A) and DBU (D), or some heating (I) (Figure 13). Furthermore, the presence of surfactants was beneficial, although longer times were needed (G, H vs A). The use of NaOH was detrimental for the synthesis, although it performed slightly better than PIP over short times. NaOH produced a large number of side products (Figure S6).

These results demonstrated that the Fmoc removal conditions in aqueous media required further optimization.

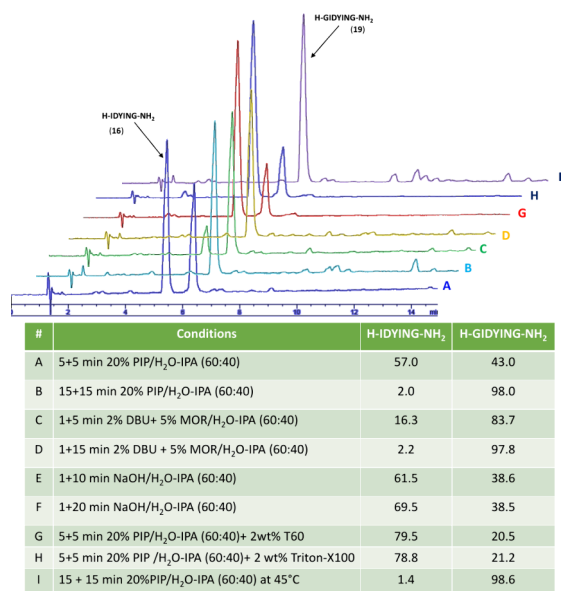


Figure 13. HPLC and table summarizing the Fmoc removal experiments using Scheme 5. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 10–30% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm. For HPLCs of #E and F, see Figure S6.

In this second synthetic round, Fmoc-Ile-Asp-Tyr-Ile-Asn-Gly-NH₂ versus H-Ile-Asp-Tyr-Ile-Asn-Gly-NH₂ was analyzed by HPLC. The following conditions were studied:

- Control DMF. 2 $\times$ 15 min 20%PIP-DMF (sample), + 15 min (sample), + 15 min (sample), total 1 h
 A. 2 $\times$ 15 min 20% PIP/H₂O-IPA (60:40) (sample), + 15 min (sample) + 15 min (sample), total 1 h

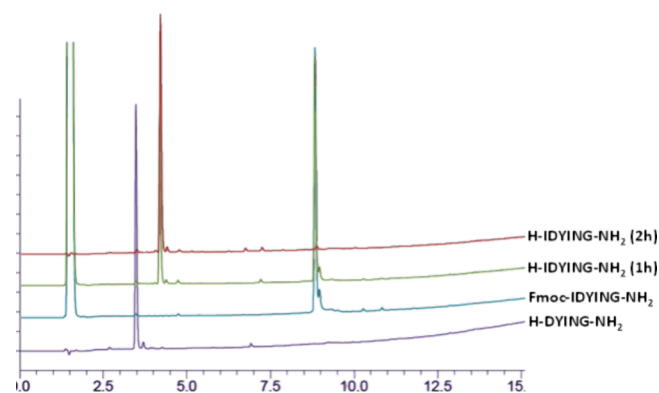
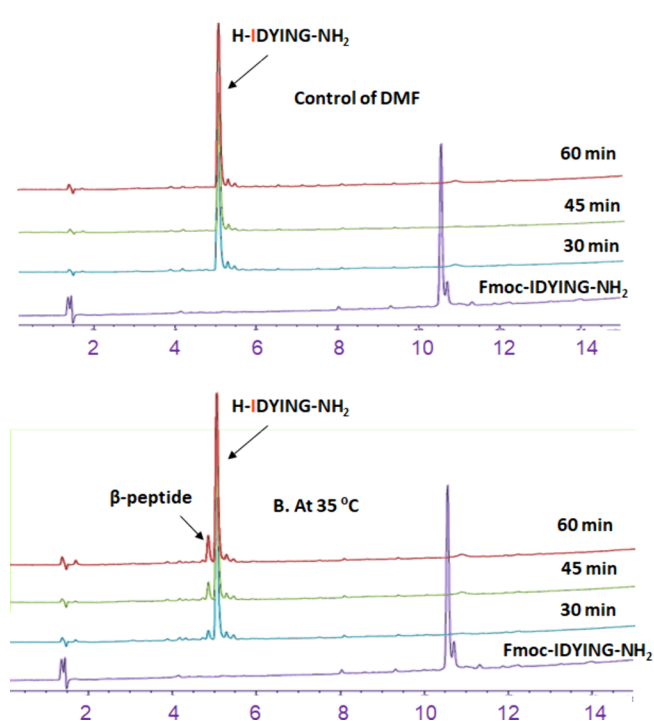


Figure 15. Fmoc removal from Fmoc-IDYING-NH-RinkAmide-TG-resin using 50% MOR in H₂O-IPA (60:40) at 45 $^{\circ}$ C. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–80% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

B. Repeat A at 35 $^{\circ}$ C

C. 2 $\times$ 10 min 2% DBU+ 5% MOR/H₂O-IPA (60:40), + 10 min (sample), total 30 min

Figure 14 shows that 30 min of 20% PIP in H₂O-IPA (60:40) effectively removed Fmoc from this difficult Ile-peptide-resin. However, of note, the peak just before that corresponding to the target, which only appeared in the aqueous treatments (A–C), corresponded to the β -peptide. This peak was more pronounced with longer times, and even greater when heating or DBU were used. The β -peptide arose from the formation of aspartimide, which may be favored by the aqueous media, and further hydrolysis.

In conclusion, the optimal synthetic cycle involves a treatment of 30 min with 20% PIP in H₂O-IPA (60:40) to remove completely the Fmoc group and a coupling for 2 h also

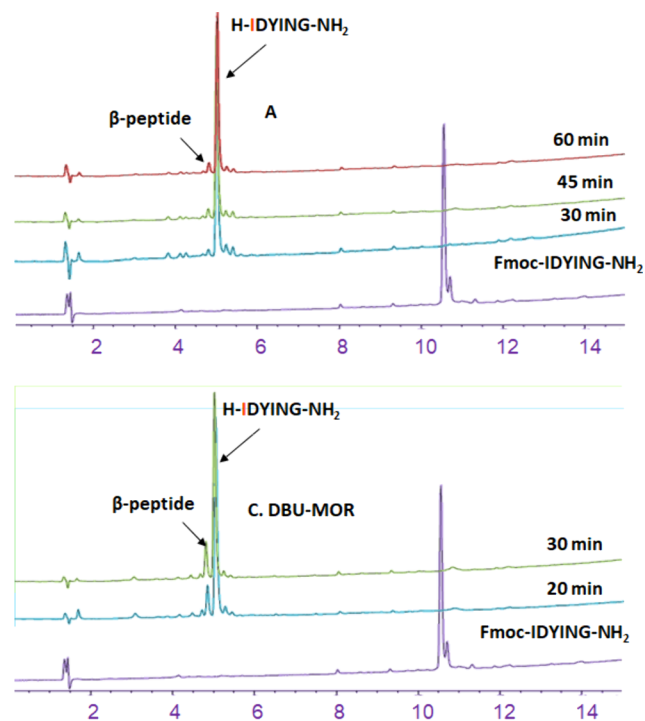
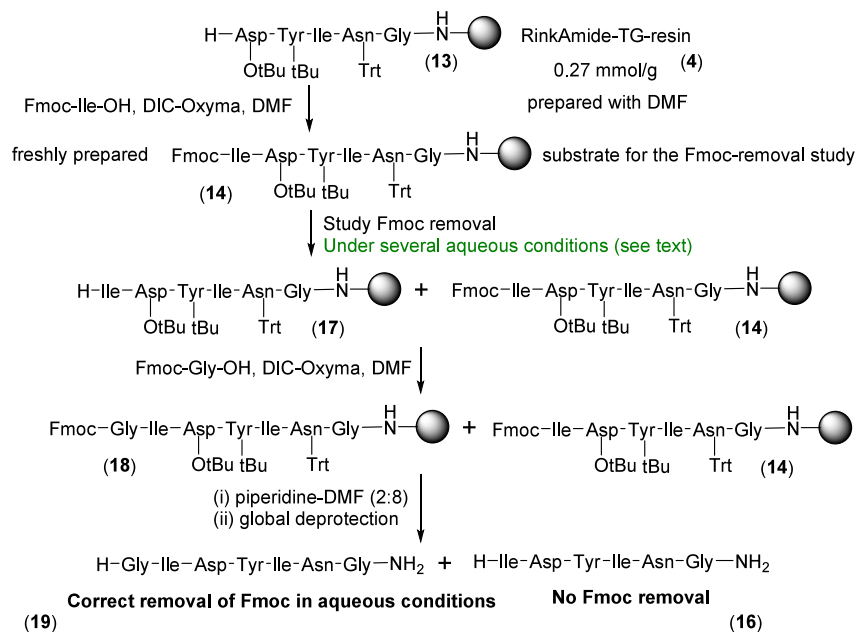
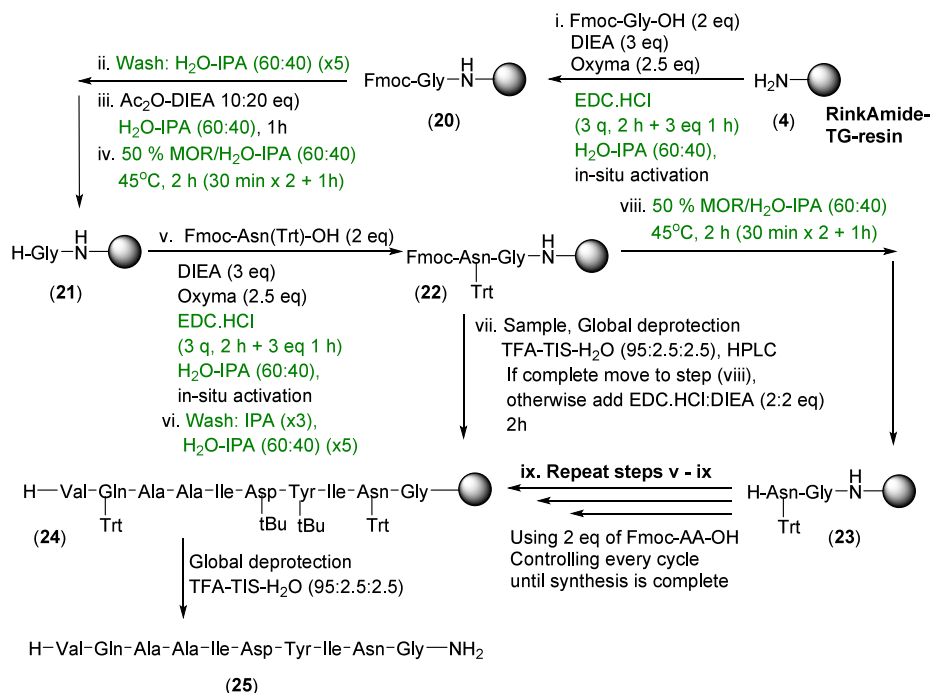


Figure 14. Optimization of Fmoc removal. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 5–80% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

Scheme 5. Synthetic Scheme Used to Identify Optimal Conditions for Fmoc Removal from the Ile-Peptide-Resin



Scheme 6. Scheme Followed for the Synthesis of ACP(65–74) (25) in Aqueous Media



in H₂O-IPA (60:40). However, in Asp-containing peptides, the formation of the β -peptide was studied and subsequently either removed or at least mitigated. First, 20% PIP in IPA was tested, but after 2 h, Fmoc removal was very poor (Figure S7). Finally, 50% morpholine (MOR) in H₂O-IPA (60:40) at 45 °C was tested. Our group has demonstrated that MOR is less efficient than PIP at removing the Fmoc group, but even at high temperatures, it does not form aspartimides or the β -peptide.⁵² Figure 15 shows that after 2 h at 45 °C, Fmoc was removed without β -peptide formation, thereby corroborating previous work.⁵² Interestingly, the adduct of MOR and DBF did not precipitate.

Finally, ACP(65–74) was prepared using the synthetic protocol shown in Scheme 6. In this case, as aspartimide formation takes place after the incorporation of the second amino acid [Asn(Trt)], all the Fmoc removal steps were carried out with 50% MOR in H₂O-IPA (60:40) at 45 °C for 2 h. In the synthesis of other peptides without the risk of aspartimide formation, 20% PIP in H₂O-IPA (60:40) at RT for 30–45 min can be used. Coupling reactions were carried out for 3 h, and additional EDC·HCl (3 equiv) was added after the second hour. All washings were carried out with H₂O-IPA (60:40). A similar synthesis was also carried out in parallel using DMF instead of H₂O-IPA (60:40), with exactly the

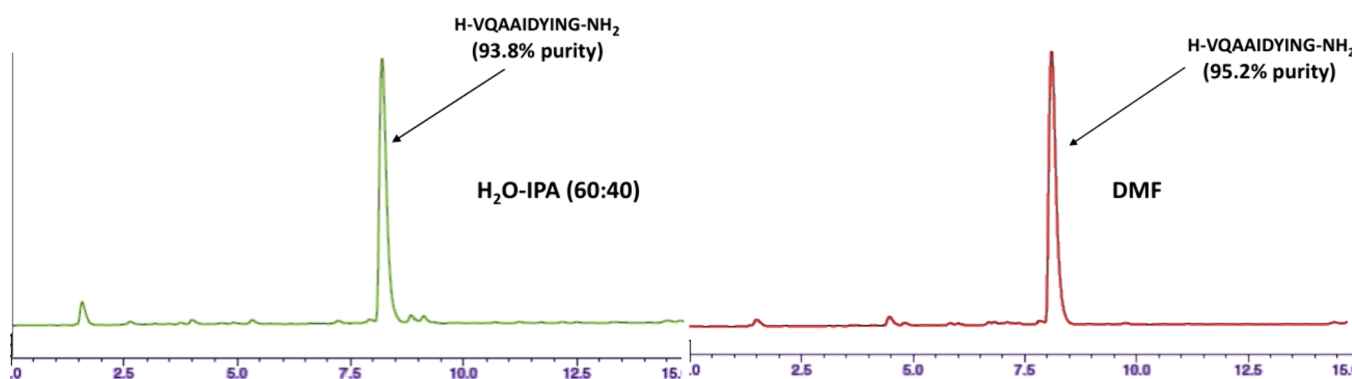


Figure 16. HPLC of ACP(65–74) (25) synthesized on RinkAmide-TG-resin using H₂O–IPA(60:40) and DMF following the protocol detailed in Scheme 6. HPLC conditions 10–30% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm. HPLC conditions: Phenomenex AerisTMC₁₈ (3.6 μ m, 4.6 mm $\times$ 150 mm), 0–30% B (ACN with 0.1% TFA) into A (H₂O with 0.1% TFA), 15 min, 220 nm.

same excess of reagents and reaction times at 45 °C, for Fmoc removal. Some intermediates of the syntheses were also analyzed (Figure S8). The HPLC of both syntheses indicated a very similar purity of the synthetically difficult peptide when only H₂O–IPA (60:40) was used, compared to when DMF was used (Figure 16).

CONCLUSIONS

Medium-sized peptides of up to 20 amino acids can be synthesized by ASPPS strategy employing the following: PEG–PS resins; standard Fmoc/tBu amino acids; EDC·HCl/Oxyma as coupling reagent; and H₂O–IPA (60:40) as the solvent for coupling, Fmoc removal, and washings. All Fmoc derivatives of the 20 proteinogenic amino acids having their side-chain protected were soluble in H₂O–IPA (60:40) after adding DIEA [0.2 mmol of Fmoc-AA-OH in 1 mL of H₂O–IPA (60:40) and 0.3 mmol of DIEA] and sonication for 1 min.

The kinetics of coupling and Fmoc removal are slower than when DMF is used, and therefore, extended reaction times are needed. The coupling is carried out for 3 h with just 2 equiv of the Fmoc-AA-OH, 3 equiv of DIEA, 2.5 equiv of Oxyma, and 6 equiv of EDC·HCl, the latter added in two portions (3 equiv at the beginning and 3 equiv after 2 h of coupling). Washings with IPA and H₂O–IPA (60:40) are used throughout the synthetic procedure. These solvents can remove the PIP-dibenzofulvene adduct formed during the Fmoc removal step. The presence of surfactants has not been shown to be necessary.

In the synthesis of Asp/Asn-containing peptides in aqueous media, β -peptide formation through the corresponding aspartimide is favored compared to when DMF is used. Therefore, for these peptides, Fmoc should be removed with 50% MOR in H₂O–IPA (60:40) at 45 °C for 2 h instead of 20% PIP in H₂O–IPA (60:40) at RT for 30 min.

During the synthesis of the angiotensin II analog, peptides lacking not only one amino acid (deletion peptide) but also dipeptides or tripeptides were observed. This suggests that the growing peptide may precipitate in the aqueous medium and subsequently redissolve after two or more synthetic cycles, allowing peptide elongation to continue. This phenomenon warrants further attention, and appropriate strategies should be developed to prevent such precipitation. Notably, peptide precipitation or chain collapse was found to coincide with overnight interruptions of the synthesis.

Finally, we would like to emphasize the importance of addressing the aqueous waste generated during ASPPS, as well as in other aqueous-based synthetic chemistry, as highlighted by colleagues in the field.^{53,54} The adoption of water as a green solvent depends not only on its effectiveness as a solvent but also on the responsible management of its waste. It is also important to recognize that in certain regions of the world, such as Africa, water is extremely scarce and must be regarded as a valuable resource. That said, a deeper discussion of this issue lies beyond the scope of the present work.

EXPERIMENTAL SECTION

Materials and Reagents. All chemicals, reagents, and solvents were obtained from commercial sources and used without additional purification unless otherwise specified. Fmoc-protected amino acids were sourced from Chempure (Bangalore, India). The solid support Fmoc-RinkAmide-PG-resin (loading: 0.37 mmol/g) was kindly provided by Sunresin (Xian, China), and Fmoc-RinkAmide-TG-resin (loading: 0.27 mmol/g) by IRIS Biotech (Marktredwitz, Germany). DIC, Oxyma, and triethylphosphate (TEP) were generous gifts from Luxembourg Bio Technologies (Nes Ziona, Israel). Organic solvents, including dimethylformamide (DMF), isopropyl alcohol (IPA), and HPLC-grade acetonitrile (ACN), were purchased from Merck, as was triisopropylsilane (TIS). Milli-Q ultrapure water was used in all reverse-phase HPLC (RP-HPLC) procedures.

Instrumentation and Analytical Conditions. Analytical HPLC was performed using a Shimadzu system equipped with a Phenomenex AerisTMC C₁₈ column (3.6 μ m, 4.6 mm $\times$ 150 mm), operating at a flow rate of 1.0 mL/min with UV detection at 220 nm. Data acquisition and processing were carried out using LabSolutions software. Mobile phases consisted of buffer A [0.1% trifluoroacetic acid (TFA) in water] and buffer B (0.1% TFA in ACN). LC-MS analyses were conducted on a Thermo Fisher Scientific UltiMate 3000 UHPLC system coupled to an ISQTM EC single quadrupole mass spectrometer in positive ion mode, using the same Phenomenex AerisTMC C₁₈ column. The mobile phases were buffer A (0.1% FA in water) and buffer B (0.1% FA in ACN).

Optimized Procedure for Aqueous Solid-Phase Peptide Synthesis. Peptides were synthesized manually in polypropylene syringes fitted polyethylene filter disks. Solid supports, including Rink Amide-TentaGel resin (0.27 mmol/g) and Rink Amide-PG resin (0.37 mmol/g), were first swollen in H₂O–IPA (60:40) for 30 min and washed with H₂O–IPA (60:40). A scale of 0.05 to 0.1 mmol was used depending on the study that was conducted. Fmoc deprotection was achieved by treating the resin with 20% piperidine in H₂O–IPA (60:40) for (15 + 15 min), followed by five H₂O–IPA (60:40) washes. Amino acid couplings were performed using 2 equiv of each Fmoc-protected amino acid, 3 equiv of EDC·HCl, 2.5 equiv of OxymaPure, and 3 equiv of DIEA in H₂O–IPA (60:40), with agitation for 2 h at room temperature (RT), followed by more

equivalents of EDC·HCl and DIEA (2:2) for an additional hour. The coupling cycle was repeated sequentially until the desired peptide sequence was completed.

Peptide Cleavage and Global Deprotection. Global deprotection and peptide cleavage were performed with a cocktail of TFA–H₂O–TIS (95:2.5:2.5 v/v/v) for 1 h at RT. The released peptides were precipitated in chilled diethyl ether, centrifuged, collected, and subsequently dissolved in ACN–H₂O mixtures for characterization by HPLC and LC-MS. The final cleavage yields for all the syntheses were between 80–96% as calculated by a double cleavage as reported earlier.⁵⁵

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssuschemeng.5c09191>.

HPLC and LC-MS of the model peptides synthesized herein (DOCX)

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Notes

The authors declare no competing financial interest.

■ ADDITIONAL NOTE

“DMSO–IPA was first presented by H. Saneii at the 37th European Peptide Symposium (Firenze, Italy, August 2024).

■ REFERENCES

(1) Anastas, P.; Eghbali, N. Green chemistry: principles and practice. *Chem. Soc. Rev.* **2010**, *39* (1), 301–312.

(2) Thompson, E. C.; Anastas, P.; Bialk, H.; D'Alessandro, D.; Hoven, V. P.; Kedwards, T. J.; Liu, Z.; Mudring, A.-V.; Saito, K.; Zeidler, V. Z.; et al. Advancing green chemistry performance assessment: the Estée Lauder Companies' continuing journey towards meaningful transparency. *Green Chem.* **2025**, *27* (18), S015–S026.

(3) Sherwood, J.; Albericio, F.; de la Torre, B. G. *N,N*-Dimethyl Formamide European Restriction Demands Solvent Substitution in Research and Development. *ChemSusChem* **2024**, *17* (8), No. e202301639.

(4) Merrifield, R. B. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85* (14), 2149–2154.

(5) Lopez, J.; Pletscher, S.; Aemissegger, A.; Bucher, C.; Gallou, F. *N*-Butylpyrrolidinone as Alternative Solvent for Solid-Phase Peptide Synthesis. *Org. Process Res. Dev.* **2018**, *22* (4), 494–503.

(6) Kumar, A.; Alhassan, M.; Lopez, J.; Albericio, F.; de la Torre, B. G. *N*-Butylpyrrolidinone for Solid-Phase Peptide Synthesis is Environmentally Friendlier and Synthetically Better than DMF. *ChemSusChem* **2020**, *13*, S288–S294.

(7) de la Torre, B. G.; Kumar, A.; Alhassan, M.; Bucher, C.; Albericio, F.; Lopez, J. Successful development of a method for the incorporation of Fmoc-Arg(Pbf)-OH in solid-phase peptide synthesis using *N*-butylpyrrolidinone (NBP) as solvent. *Green Chem.* **2020**, *22* (10), 3162–3169.

(8) Acosta, G. A.; del Fresno, M.; Paradis-Bas, M.; Rigau-De Llobet, M.; Cote, S.; Royo, M.; Albericio, F. Solid-phase peptide synthesis using acetonitrile as a solvent in combination with PEG-based resins. *J. Pept. Sci.* **2009**, *15* (10), 629–633.

(9) Jad, Y. E.; Acosta, G. A.; Khattab, S. N.; de la Torre, B. G.; Govender, T.; Kruger, H. G.; El-Faham, A.; Albericio, F. Peptide synthesis beyond DMF: THF and ACN as excellent and friendlier alternatives. *Org. Biomol. Chem.* **2015**, *13* (8), 2393–2398.

(10) Jad, Y. E.; Acosta, G. A.; Govender, T.; Kruger, H. G.; El-Faham, A.; de la Torre, B. G.; Albericio, F. Green Solid-Phase Peptide Synthesis 2. 2-Methyltetrahydrofuran and Ethyl Acetate for Solid-Phase Peptide Synthesis under Green Conditions. *ACS Sustainable Chem. Eng.* **2016**, *4* (12), 6809–6814.

(11) Jad, Y. E.; Acosta, G. A.; Khattab, S. N.; de la Torre, B. G.; Govender, T.; Kruger, H. G.; El-Faham, A.; Albericio, F. 2-Methyltetrahydrofuran and cyclopentyl methyl ether for green solid-phase peptide synthesis. *Amino Acids* **2016**, *48* (2), 419–426.

(12) Kumar, A.; Jad, Y. E.; Collins, J. M.; Albericio, F.; de la Torre, B. G. Microwave-assisted green solid-phase peptide synthesis using γ -valerolactone (GVL) as solvent. *ACS Sustainable Chem. Eng.* **2018**, *6* (6), 8034–8039.

(13) Kumar, A.; Sharma, A.; de la Torre, B. G.; Albericio, F. Scope and limitations of γ -valerolactone (GVL) as a green solvent to be used with base for Fmoc removal in solid phase peptide synthesis. *Molecules* **2019**, *24* (21), No. 4004.

(14) Kumar, A.; Jad, Y. E.; El-Faham, A.; de la Torre, B. G.; Albericio, F. Green solid-phase peptide synthesis 4. γ -Valerolactone and *N*-formylmorpholine as green solvents for solid phase peptide synthesis. *Tetrahedron Lett.* **2017**, *58* (30), 2986–2988.

(15) Lawrenson, S. B.; Arav, R.; North, M. The greening of peptide synthesis. *Green Chem.* **2017**, *19* (7), 1685–1691.

(16) Varró, N.; MánditynéHuszka, B.; Erdei, E.; Mándoki, A.; Mándity, I. M. Upscaled Synthesis of α - and β -Peptides in a Continuous-Flow Reactor Using Propylene Carbonate as an Eco-Friendly Solvent. *Chem. Methods* **2025**, *5*, No. 2500010.

(17) Martelli, G.; Cantelmi, P.; Tolomelli, A.; Corbisiero, D.; Mattellone, A.; Ricci, A.; Fantoni, T.; Cabri, W.; Vaccondio, F.; Ferlenghi, F.; Mor, M.; Ferrazzano, L. Steps towards sustainable solid phase peptide synthesis: use and recovery of *N*-octyl pyrrolidone. *Green Chem.* **2021**, *23* (11), 4095–4106.

(18) Příbylka, A.; Pastorek, M.; Grepl, M.; Schütznerová, E. P. The application of anisole in greener solid-phase peptide synthesis protocols - Compatibility with green bases in Fmoc removal and new green binary mixture for coupling. *Tetrahedron* **2021**, *99*, No. 132452.

- (19) Kumar, A.; Sharma, A.; de la Torre, B. G.; Albericio, F. Rhodiasolv PolarClean - a greener alternative in solid-phase peptide synthesis. *Green Chem. Lett. Rev.* **2021**, *14* (3), 545–550.
- (20) Pawlas, J.; Rasmussen, J. H. Circular Aqueous Fmoc/t-Bu Solid-Phase Peptide Synthesis. *ChemSusChem* **2021**, *14* (16), 3231–3236.
- (21) Vivenzio, G.; Scala, M. C.; Marino, P.; Manfra, M.; Campiglia, P.; Sala, M. Dipropylene glycol Dimethylether, New Green Solvent for Solid-Phase Peptide Synthesis: Further Challenges to Improve Sustainability in the Development of Therapeutic Peptides. *Pharmaceutics* **2023**, *15* (6), No. 1773.
- (22) Nandhini, K. P.; Cele, N.; de la Torre, B. G.; Albericio, F. Triethyl phosphate (TEP) as a green solvent for solid-phase peptide synthesis (SPPS). *Green Chem. Lett. Rev.* **2024**, *17* (1), No. 2330639.
- (23) Garcia-Martin, F.; Quintanar-Audelo, M.; Garcia-Ramos, Y.; Cruz, L. J.; Gravel, C.; Furic, R.; Cote, S.; Tulla-Puche, J.; Albericio, F. ChemMatrix, a Poly(ethylene glycol)-Based Support for the Solid-Phase Synthesis of Complex Peptides. *J. Comb. Chem.* **2006**, *8* (2), 213–220.
- (24) Pawlas, J.; Antonic, B.; Lundqvist, M.; Svensson, T.; Finnman, J.; Rasmussen, J. H. 2D green SPPS: green solvents for on-resin removal of acid sensitive protecting groups and lactamization. *Green Chem.* **2019**, *21* (10), 2594–2600.
- (25) Ferrazzano, L.; Corbisiero, D.; Martelli, G.; Tolomelli, A.; Viola, A.; Ricci, A.; Cabri, W. Green Solvent Mixtures for Solid-Phase Peptide Synthesis: A Dimethylformamide-Free Highly Efficient Synthesis of Pharmaceutical-Grade Peptides. *ACS Sustainable Chem. Eng.* **2019**, *7* (15), 12867–12877.
- (26) Martin, V.; Jadhav, S.; Egelund, P. H. G.; Liffert, R.; Johansson Castro, H.; Krüger, T.; Haselmann, K. F.; Thordal Le Quement, S.; Albericio, F.; Dettner, F.; Lechner, C.; Schönleber, R.; Pedersen, D. S. Harnessing polarity and viscosity to identify green binary solvent mixtures as viable alternatives to DMF in solid-phase peptide synthesis. *Green Chem.* **2021**, *23* (9), 3295–3311.
- (27) Pacini, L.; Muthyala, M.; Aguiar, L.; Zitterbart, R.; Rovero, P.; Papini, A. M. Optimization of peptide synthesis time and sustainability using novel eco-friendly binary solvent systems with induction heating on an automated peptide synthesizer. *J. Pept. Sci.* **2024**, *30* (9), No. e3605.
- (28) Vivenzio, G.; Scala, M. C.; Auriemma, G.; Sardo, C.; Campiglia, P.; Sala, M. Application of a new green protocol in solid-phase peptide synthesis: identification of a new green solvent mixture compatible with TBEC/ETT. *Green Chem. Lett. Rev.* **2024**, *17* (1), No. 2404234.
- (29) Lastella, L.; Zecca, M.; Centomo, P.; Jerábek, K.; Formaggio, F.; Guryanov, I.; Ricci, A.; Biondi, B. Toward a Green SPPS: The Use of an Innovative Mesoporous pDVB Support for Environmentally Friendly Solvents. *J. Pept. Sci.* **2025**, *31* (8), No. e70038.
- (30) Noki, S.; Lastella, L.; Serban, S.; Basso, A.; Orosz, G.; G de la Torre, B.; Albericio, F. Triethyl phosphate (TEP) - dimethylsulfoxide (DMSO), as a green solvent mixture for solid-phase peptide synthesis (SPPS). *Green Chem. Lett. Rev.* **2025**, *18* (1), No. 2438068.
- (31) Jaradat, D. s. M. M.; Al Musaimi, O.; Albericio, F. Advances in solid-phase peptide synthesis in aqueous media (ASPPS). *Green Chem.* **2022**, *24* (17), 6360–6372.
- (32) Cortes-Clerget, M.; Lee, N. R.; Lipshutz, B. H. Synthetic chemistry in water: applications to peptide synthesis and nitro-group reductions. *Nat. Protoc.* **2019**, *14* (4), 1108–1129.
- (33) Bordignon, F.; Scarso, A.; Angelini, A. Challenges and Achievements of Peptide Synthesis in Aqueous and Micellar Media. *ChemBioChem* **2025**, *26* (11), No. e202500099.
- (34) Hojo, K.; Maeda, M.; Kawasaki, K. A new water-soluble N-protecting group, 2-[phenyl(methyl)sulfonio]ethoxycarbonyl tetrafluoroborate, and its application to solid phase peptide synthesis in water. *J. Pept. Sci.* **2001**, *7* (12), 615–618.
- (35) Hojo, K.; Maeda, M.; Takahara, Y.; Yamamoto, S.; Kawasaki, K. A new reagent, 2-[phenyl (methyl) sulfonio] ethyl-4-nitro-phenyl-carbonate tetrafluoroborate (Pms-ONp), for preparing water-soluble N-protected amino acids. *Tetrahedron Lett.* **2003**, *44* (14), 2849–2851.
- (36) Hojo, K.; Maeda, M.; Kawasaki, K. Solid-phase peptide synthesis in water. Part 3: A water-soluble N-protecting group, 2-[phenyl (methyl) sulfonio] ethoxycarbonyl tetrafluoroborate, and its application to solid phase peptide synthesis in water. *Tetrahedron* **2004**, *60* (8), 1875–1886.
- (37) Hojo, K.; Maeda, M.; Kawasaki, K. 2-(4-Sulfophenylsulfonyl) ethoxycarbonyl group: a new water-soluble N-protecting group and its application to solid phase peptide synthesis in water. *Tetrahedron Lett.* **2004**, *45* (50), 9293–9295.
- (38) Hojo, K.; Maeda, M.; Tanakamaru, N.; Mochida, K.; Kawasaki, K. Solid phase peptide synthesis in water VI: evaluation of water-soluble coupling reagents for solid phase peptide synthesis in aqueous media. *Protein Pept. Lett.* **2006**, *13* (2), 189–192.
- (39) Hojo, K.; Maeda, M.; Smith, T. J.; Kita, E.; Yamaguchi, F.; Yamamoto, S.; Kawasaki, K. Peptide synthesis in water IV. Preparation of N-ethanesulfonylethoxycarbonyl (Esc) amino Acids and their application to solid phase peptide synthesis. *Chem. Pharm. Bull.* **2004**, *52* (4), 422–427.
- (40) Merrifield, R. B.; Bach, A. E. 9-(2-Sulfo)-fluorenylmethoxycarbonyl chloride, a new reagent for the purification of synthetic peptides. *J. Org. Chem.* **1978**, *43* (25), 4808–4816.
- (41) Knauer, S.; Koch, N.; Uth, C.; Meusinger, R.; Avrutina, O.; Kolmar, H. Sustainable peptide synthesis enabled by a transient protecting group. *Angew. Chem., Int. Ed.* **2020**, *59* (31), 12984–12990.
- (42) Uth, C.; Englert, S.; Avrutina, O.; Kolmar, H.; Knauer, S. Novel amino-Li resin for water-based solid-phase peptide synthesis. *J. Pept. Sci.* **2023**, *29* (12), No. e3527.
- (43) Hojo, K.; Ichikawa, H.; Hara, A.; Onishi, M.; Kawasaki, K.; Fukumori, Y. Aqueous Microwave-Assisted Solid-Phase Peptide Synthesis Using Fmoc Strategy: In-Water Synthesis of “Difficult Sequences”. *Protein Pept. Lett.* **2012**, *19*, 1231–1236.
- (44) Hojo, K.; Ichikawa, H.; Maeda, M.; Kida, S.; Fukumori, Y.; Kawasaki, K. Solid-phase peptide synthesis using nanoparticulate amino acids in water. *J. Pept. Sci.* **2007**, *13* (7), 493–497.
- (45) Hojo, K.; Shinozaki, N.; Hara, A.; Onishi, M.; Fukumori, Y.; Ichikawa, H. Aqueous Microwave-Assisted Solid-Phase Peptide Synthesis Using Fmoc Strategy. II. Racemization, Studies and Water Based Synthesis of Cysteine-Containing Peptides. *Protein Pept. Lett.* **2013**, *20*, 1122–1128.
- (46) Galanis, A. S.; Albericio, F.; Grotli, M. Solid-phase peptide synthesis in water using microwave-assisted heating. *Org. Lett.* **2009**, *11* (20), 4488–4491.
- (47) Collins, J. Water Soluble Solid Phase Peptide Synthesis. WO 2013/115813 A1, 2013.
- (48) Carpino, L. A.; Ismail, M.; Truran, G. A.; Mansour, E. M. E.; Iguchi, S.; Ionescu, D.; El-Faham, A.; Riemer, C.; Warrass, R. The 1,1-Dioxobenzo[b]thiophene-2-ylmethoxycarbonyl (Bsmoc) Amino-Protecting Group. *J. Org. Chem.* **1999**, *64* (12), 4324–4338.
- (49) Bayer, E.; Rapp, W. Polystyrene-Immobilized PEG Chains. In *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Application*; Plenum Press: New York, 1992; p 325.
- (50) Luna, O. F.; Gomez, J.; Cardenas, C.; Albericio, F.; Marshall, S. H.; Guzman, F. Deprotection reagents in Fmoc solid phase peptide synthesis: Moving away from piperidine? *Molecules* **2016**, *21* (11), No. 1542.
- (51) Jad, Y. E.; Govender, T.; Kruger, H. G.; El-Faham, A.; de la Torre, B. G.; Albericio, F. Green Solid-Phase Peptide Synthesis (GSPPS) 3. Green Solvents for Fmoc Removal in Peptide Chemistry. *Org. Process Res. Dev.* **2017**, *21* (3), 365–369.
- (52) Mthembu, S. N.; Chakraborty, A.; Schönleber, R.; Albericio, F.; de la Torre, B. G. Morpholine, a strong contender for Fmoc removal in solid-phase peptide synthesis. *J. Pept. Sci.* **2024**, *30* (2), No. e3538.
- (53) Krell, C.; Schreiber, R.; Hueber, L.; Sciascera, L.; Zheng, X.; Clarke, A.; Haenggi, R.; Parmentier, M.; Bagaia, H.; Rodde, S.; Gallou, F. Strategies to Tackle the Waste Water from α -Tocopherol-Derived Surfactant Chemistry. *Org. Process Res. Dev.* **2021**, *25* (4), 900–915.

(54) Fleck, N.; Roschangar, F.; Haydl, A. M. API Syntheses in Aqueous Media: Assessing the Environmental Footprint en route from Academic Discovery to Industrial Applications as “Green Opportunity” for Process Chemistry. *Org. Process Res. Dev.* **2023**, 27 (5), 822–830.

(55) Gamal, H.; Kaushal, M.; Kumar, A.; Noki, S.; Zhang, C.; Wang, C.; Kou, X.; Basso, A.; Serban, S.; de la Torre, B. G.; Albericio, F. 4-Methylbenzhydryl Bromine Resin: Bridging the Gap between 2-Chlorotrityl Chloride and Wang-Type Resins for the Solid-Phase Synthesis of C-Terminal Acid Peptides. *Chem. Methods* **2025**, 5, No. 202500092.