Green Chemistry

PAPER

View Article Online
View Journal | View Issue

Cite this: *Green Chem.*, 2025, **27**, 14911

Advancing sustainable peptide synthesis: methanesulfonic acid–formic acid as a greener substitute for TFA in final global deprotection

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Received 11th August 2025,
Accepted 16th October 2025

DOI: 10.1039/d5gc04192a

rsc.li/greenchem

Solid-phase peptide synthesis is the preferred technique for producing peptides in both research and industrial applications. At the end of the synthetic process, the peptides are retrieved from the solid support by treatment with trifluoroacetic acid (TFA) along with scavengers. TFA, however, is a polyfluoroalkyl substance (PFAS) and poses a serious risk to human health and has a considerable ecological impact. Herein, the use of 2% methanesulfonic acid (MSA) with formic acid (FA) as a solvent in the presence of TIS as a scavenger has been employed for the final global deprotection. 2% MSA with FA is able to successfully cleave all protecting groups in 2–3 h. Peptides containing Ser, Thr, Trp, and Tyr showed formylation, which was successfully eliminated by treatment with 0.5 M NH_4OH. After cleavage with MSA–TIS–FA (2 : 2.5 : 95.5), further tests revealed no traces of peptides remaining anchored to the RinkAmide resin, indicating in most cases quantitative cleavage of the peptide from the resin. This cleavage yield using MSA–FA is superior to that obtained with the classical TFA method. Furthermore, the use of MSA–FA reduces strong acid consumption by approximately 98% (from 95% when TFA is used to just 2% with MSA) and replaces the most harmful PFAS (TFA) with a far more environmentally friendly acid (MSA). This protocol has been successfully applied to the final global deprotection of several biologically important peptides, including tirzepatide, one of the most important active pharmaceutical ingredient (API) blockbusters currently on the market.

Green foundation

1. We are tackling the most important unmet requirement in green peptide synthesis, the replacement of trifluoroacetic acid (TFA), which is an important member of PFAS.
2. Methanesulfonic acid (MSA), which is probably the greener organic acid, is successfully proposed to replace TFA.
3. MSA–formic acid will replace TFA in both research and industrial modes on a ton scale.

Introduction

Peptides have gained immense interest over the last few decades owing to their exponential growth in U.S. Food and Drug Administration (FDA) drug approvals.[1–4] The most used approach for production in industry is *via* Solid Phase Peptide Synthesis (SPPS), thanks to the concept of solid support introduced by R. Bruce Merrifield in 1964.[5] In this strategy, the synthesis is performed such that each amino acid is incorporated sequentially to obtain the final peptide of interest assembled onto the solid support (or resin).[6] The peptide is then cleaved off the solid support using a strong acid like trifluoroacetic acid (TFA). The American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable (ACS GCIPR) is promoting a shift toward more sustainable peptide synthesis methods, with an emphasis on minimizing environmental harm.[7] This includes replacing hazardous solvents and reagents such as *N*,*N*-dimethylformamide (DMF), piperidine and trifluoroacetic acid (TFA), which pose risks to human health and have a considerable ecological impact.[7]

Among these, TFA is classified as a polyfluoroalkyl substance (PFAS).[8] PFAS are a group of chemicals that are increasingly scrutinized by regulatory authorities and are likely to face

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future usage restrictions.[9] These compounds degrade very slowly in the environment and pose significant risks to both human health and ecosystems.[10] Additionally, TFA is volatile and irritates the eyes.[11,12] It is a strong acid and highly corrosive to the skin and respiratory system.[12] Its rapid absorption into body tissues renders its use in routine small-scale syntheses consistently hazardous. In large-scale synthesis, where substantially larger quantities are handled, TFA presents an even greater and extremely serious risk. Subsequently, identifying suitable alternatives to TFA is of considerable importance.[13,14] To the best of our knowledge, only two studies to date have demonstrated a PFAS-free approach for cleaving unprotected peptides from resin.

Pawlas *et al.* recently suggested combining Brønsted–Lowry acids (BAs) and Lewis acids (LAs)—for example, HCl/$FeCl_3$ or acetic acid (AcOH)/$FeCl_3$, alongside polar solvents like dimethyl carbonate (DMC) and acetonitrile (ACN) as a cleavage cocktail for an effective PFAS-free alternative to TFA.[13]

Methanesulfonic acid (MSA) is well documented in the literature as a green solvent, as it is a natural product and part of the natural sulfur cycle.[15] It exhibits a very low vapor pressure and a high boiling point, and under normal operating conditions, it does not release any hazardous volatile compounds. MSA has a pK_a of −1.86, comparable to strong inorganic acids like nitric acid and sulfuric acid.[16] Its stronger acidity allows it to be used at lower concentrations than TFA, offering advantages in both handling and final disposal. Furthermore, it is easily biodegradable and breaks down into sulfate and carbon dioxide as end products (Fig. 1).[16,17]

Owing to its high acidity, our group used MSA for global deprotection in the presence of solvents like acetic acid (AcOH) or DMC.[14] The protocol reported was solvent-dependent. MSA seems to work more efficiently with dichloromethane (DCM) compared to polar solvents.[14] However, DCM is a very hazardous solvent and problematic for the work-up. In our previous work, the best conditions to cleave the peptide from the RinkAmide resin were found to be 8–12% MSA in AcOH or DMC with triisopropylsilane (TIS) as a scavenger in each case.[14] However, complete detritylation of the side chains of Gln and Asn was not achieved even with an increased amount of MSA (16%). In the present work, a novel combination of MSA and formic acid (FA) is proposed. The inclusion of FA fundamentally changes the process, enabling the removal of DMC while substantially reducing MSA usage from 16% to just 2%, with the well-recognized environmental benefits this entails. Application of this method to the final global deprotection of several model and biologically important peptides, including tirzepatide, one of the most important blockbuster active pharmaceutical ingredients (APIs) currently on the market, is presented.

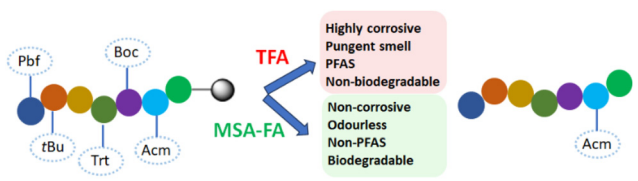

Fig. 1 General comparison for global deprotection using TFA and MSA.

Results and discussion

Leu enkephalin (H-YGGFL-NH_2) was synthesised as a model peptide using RinkAmide-polystyrene (PS) resin. The peptidyl resin was then treated with the standard TFA-based cleavage cocktail, TFA–TIS–H_2O (95 : 2.5 : 2.5), for 2 h. The peptide was then precipitated using ether to afford the desired peptide. The peptide was evaluated for its purity using HPLC (Fig. 2A, #1). Owing to MSA's high acidity, 2% MSA was used in 2.5% TIS with the remaining 95.5% being FA. In our earlier report, DMC or AcOH was used. However, upon comparison of the dielectric constant, FA has a much higher dielectric constant and is more acidic compared to AcOH. Therefore, in the current protocol, MSA–TIS–FA (2 : 2.5 : 95.5) was first used to cleave the Leu-enkephalin peptide (Fig. 2A, #2). After 2 h of stirring in the above cocktail, the peptide was precipitated using ether to afford the peptide. Slight formylation was observed, as depicted by the peak at 7.6 min. This formylation and deformylation exist in solution, and the reaction is reversible. However, upon treatment with 0.5 M NH_4OH overnight (on), deformylation was achieved at a faster rate, eliminating

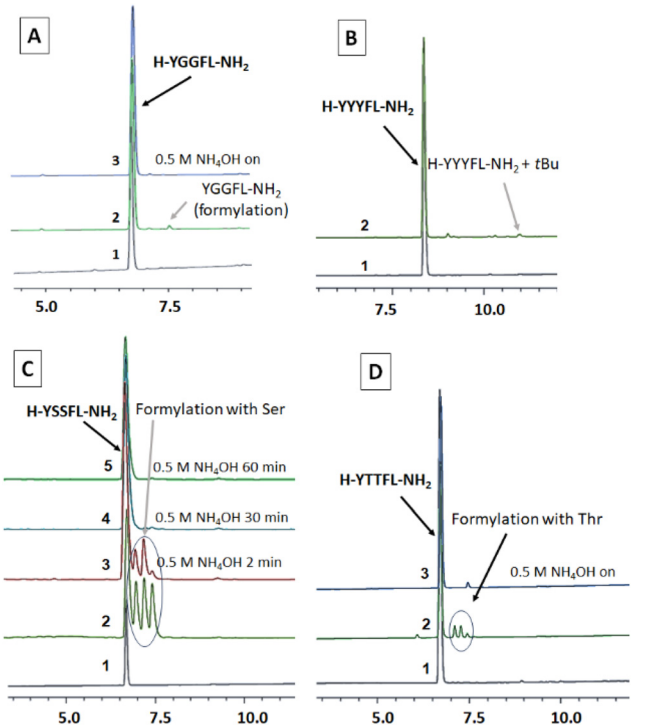

Fig. 2 Comparison of different cleavage conditions in the case of (A) H-YGGFL-NH_2; (B) H-YYYFL-NH_2; (C) H-YSSFL-NH_2; and (D) H-YTTFL-NH_2 from RinkAmide resin. #1, TFA cleavage; #2, MSA cleavage; #3–5, 0.5 M NH_4OH treatment after MSA cleavage.

the formylated product (Fig. 2A, #3). The purity of the peptide was found to be comparable to that of TFA cocktail cleavage (in both cases, 98% purity). In addition, 99% cleavage yield was obtained in the case of MSA cleavage. After the MSA cleavage, the residual resin was treated with TFA–TIS–H_2O (95.0 : 2.5 : 2.5) for 2 h to calculate cleavage yields. The residual solution was precipitated using chilled ether. The solution either did not show any precipitation or showed trace amounts (<0.1%), thereby confirming that MSA cleavage was almost quantitative and represents an optimal condition for the complete peptide cleavage from the resin.

With these promising results in hand, modifications in Leu enkephalin (H-YGGFL-NH_2) have been made further, wherein the "GG" residue has been replaced with other side chain-protected amino acids. The use of adjacent double amino acids was proposed to increase the likelihood of partial removal and/or the occurrence of side reactions. In the first set of experiments, the modified Leu enkephalin was synthesized using standard SPPS with replacement of "GG" with either a phenolic or alcoholic group to afford H-YYYFL-NH_2, H-YSSFL-NH_2, and H-YTTFL-NH_2, respectively.

In the case of Tyr, the peptide purity was comparable under both cleavage conditions, as shown in Fig. 2B (#1 for TFA cleavage and #2 for MSA cleavage). The major drawback witnessed in the MSA cleavage was the formylation of the side chain in the case of Ser (Fig. 2C) and Thr (Fig. 2D). This was not witnessed in the case of Tyr (Fig. 2B, #2), maybe due to its weak nucleophilicity, which makes the formylation more difficult. The purity of the peptide was found to be 99% and 98% in the case of TFA and MSA cleavage, respectively. The cleavage yield was calculated to be >99% for MSA cleavage. However, in the case of Ser and Thr, due to their high nucleophilicity, formylation takes place to produce the peptide of interest along with the formylated product at the reactive sites. Herein, 0.5 M NH_4OH has been used. In the case of Ser, the deformylation is achieved in 60 min (Fig. 2C, #3–5). However, in the case of Thr, the reaction was performed overnight for completion of deformylation (Fig. 2D, #3). In each case, the peptide was obtained with high purity compared to that of the TFA cocktail. In the case of Ser, the purity of the peptide was found to be 98% and 99% for TFA and MSA cleavage, respectively. In the case of Thr, the purity was found to be 99% and 98% for TFA and MSA cleavage, respectively. In addition, the cleavage yield for H-YTTFL-NH_2 was found to be 95% in the case of MSA cleavage.

Furthermore, the peptide containing Asp (D) and Glu (E) were synthesized, *viz.*, H-YDDFL-NH_2 and H-YEEFL-NH_2. It can be seen from Fig. 3 that the formation of aspartimide in the case of MSA cleavage (Fig. 3A, #2) was more important compared to that of TFA (Fig. 3A, #1). This could be attributed to the stronger acidity of MSA compared to TFA. Furthermore, the greater polarity of FA compared to TFA may also play a role. In addition, adjacent peaks also appear, which correspond to the same mass as that of the parent peptide (H-YDDFL-NH_2). This afforded the overall peptide in 92.4% purity. The peptide purity was quite comparable in the case of

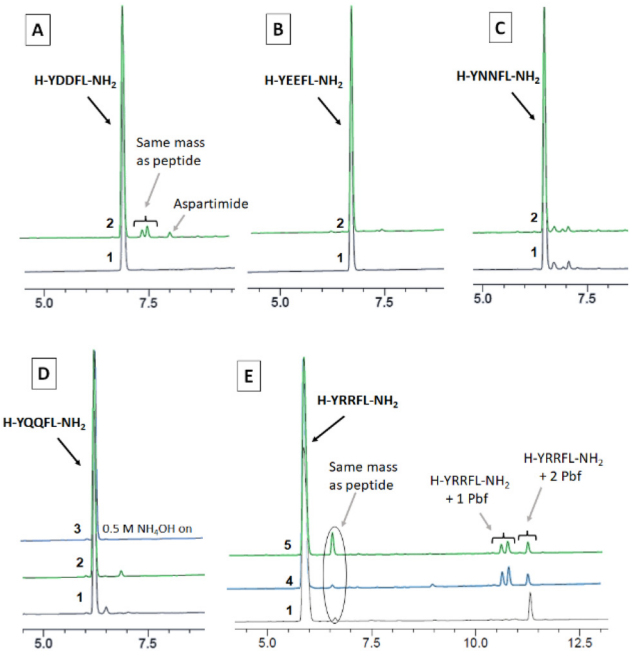

Fig. 3 Cleavage study in the case of (A) H-YDDFL-NH_2, (B) H-YEEFL-NH_2, (C) H-YNNFL-NH_2, (D) H-YQQFL-NH_2 and (E) H-YRRFL-NH_2 from RinkAmide resin. #1, TFA cleavage, #2, MSA cleavage, #3, 0.5 M NH_4OH treatment after MSA cleavage, #4, MSA cleavage for 2 h, #5, MSA cleavage for 3 h.

H-YEEFL-NH_2, as depicted in Fig. 3B (#1 for TFA cleavage and #2 for MSA cleavage). In the case of H-YEEFL-NH_2, 99% and 95% purity was obtained for TFA and MSA cleavage, respectively. The cleavage yield from the resin in both cases of H-YDDFL-NH_2 and H-YEEFL-NH_2 was found to be >98%.

In the case of H-YNNFL-NH_2 (Fig. 3C), the peptide was found to be 92% pure for TFA cleavage (Fig. 3C, #1) and 93% for MSA cleavage (Fig. 3C, #2). The cleavage yield was calculated to be 93% for MSA cleavage. However, in the case of H-YQQFL-NH_2 upon treatment with the MSA cocktail (Fig. 3D, #2), slight formylation was obtained, maybe on Tyr, which was eliminated by treating the reaction mixture with 0.5 M NH_4OH overnight (Fig. 3D, #3). The peptide purity was found to be 98%, which is slightly better than that for TFA cleavage, which was 97% (Fig. 3D, #1). The cleavage yield was calculated to be 93% and 99% for MSA cleavage in the case of H-YNNFL-NH_2 and H-YQQFL-NH_2, respectively. Removal of the Pbf group in the case of Arg was also studied. H-YRRFL-NH_2 was synthesised and cleaved using the TFA cleavage cocktail (Fig. 3E, #1) and also using MSA (Fig. 3E, #4) for 2 h in both cases. It was found that, in both cases, HPLC showed the presence of the parent peptide along with the Pbf-protected peptide. The cleavage was repeated for 3 h (Fig. 3E, #5) and it was observed that the Pbf group was not removed completely, also leading to further side impurities. Upon comparison of both cases, it was found that in the case of 3 h (Fig. 3E, #5), an extra peak appeared with the same mass as that of the parent peptide. The complete removal of the Pbf group was not achieved even

after a longer reaction time. The peptide purity was found to be 92%, 85% and 80% in the case of TFA, 2 h MSA and 3 h MSA cleavage, respectively. The cleavage yield in the case of MSA was found to be 96% and 99% for 2 h and 3 h, respectively.

In the case of H-YKKFL-NH_2, TFA cleavage (Fig. 4A, #1) afforded a pure peptide, whereas for MSA cleavage (Fig. 4A, #2), in addition to the product peak, a small amount of formylation was observed. Upon treatment with 0.5 M NH_4OH overnight, the formylation was eliminated, and the product was obtained in high purity (Fig. 4A, #3). The peptide purity was found to be >99% in the case of TFA. In the case of MSA, 98% purity was obtained with 95% cleavage yield.

In the case of H-YWWFL-NH_2, a small amount of formylation was observed for the MSA cleavage cocktail compared to that of TFA cleavage, as shown in Fig. 4B (#1 for TFA cleavage and #2 for MSA cleavage). Formylation was eliminated by treating with 0.5 M NH_4OH overnight (Fig. 4B, #3). No trace of the formylated product was found and the purity of the peptide in MSA cleavage (97%) was comparable to that of TFA cleavage (98%). The cleavage yield was found to be 99%.

In the case of His, two peptides were studied. In the first case, His(Boc) was used (Fig. 4C) whereas in the second case, His(Trt) (Fig. 4D) was used for peptide elongation. In the case of His(Boc), the peptide purity was found to be 97% and 96% for TFA (Fig. 4C, #1) and MSA (Fig. 4C, #2), respectively. However, in the case of His(Trt), TFA cleavage afforded the peptide (Fig. 4D, #1) in good purity. However, the MSA cocktail for 2 h at rt (Fig. 4D, #2) did not completely remove the Trt protecting group from the His side chain. The cleavage was then studied at 40 °C for 3 h (Fig. 4D, #3). Under these conditions, the complete removal of Trt was observed, thereby improving the peptide purity to 97%, which was quite comparable to that of TFA cleavage (99%). Cleavage yields were calculated to be >99% and 96% in the case of His(Boc) and His(Trt), respectively.

In the case of H-YMMFL-NH_2, under the TFA cleavage conditions, the peptide was found to be 92% pure (Fig. 5A, #1). The major impurity was found to be the *tert*-butylated peptide. However, in the case of MSA cleavage, the percentage of this side product was quite high (Fig. 5A, #2). This side reaction was reverted by treatment of a lyophilised peptide with 5% AcOH in H_2O at 40 °C for 36 h (Fig. 5A, #3), wherein S*t*Bu was completely eliminated. The purity of the peptide in MSA cleavage was found to be 96% with a cleavage yield of 93%.

In the case of Cys, two syntheses were attempted, one using the Acm-protecting group and the other using Trt. In the case of Acm, H-YC(Acm)C(Acm)FL-NH_2, the purity was found to be 98% for TFA cleavage (Fig. 5B, #1). Even in the case of MSA cleavage, the peptide was obtained in 96% purity with 93% cleavage yield (Fig. 5B, #2). Removal of Trt from Cys is challenging and requires optimisation with time and different scavengers. The results for the Trt protecting group are provided in the SI.

After attempts of cleavage using the MSA cocktail in the model peptides containing the standard side chain protecting groups of Trt, Boc, *t*Bu, Pbf, *etc.*, an attempt was made to employ the strategy on real peptides. In this context, several peptides like ACP(65–74) (H-VQAAIDYING-NH_2), ABRF1992 (H-GVRGDKGNPGWPGAPY-NH_2), bradykinin (H-RPPGFSPFR-NH_2), the semaglutide fragment (SGT 8-mer) (H-AWLVRGRG-OH), the tirzepatide fragment (TZP 18-mer) (H-FVQWLIAGGPSSGAPPPS-NH_2), and the full tirzepatide (TZP 1–39) peptide (H-Y-Aib-EGTFTSDYSI-Aib-LDKIAQK(AEEA-AEEA-γ-Glu-eicosanedioic acid) AFVQWLIA-GPSSGAPPPS-NH_2) were used as shown in Fig. 6.

ACP(65–74) contains two Trt, thereby making Trt removal a challenge, alongside the presence of Asp(O*t*Bu)-Tyr(*t*Bu), which are prone to aspartimide formation. This makes the ACP decapeptide an ideal model to be studied under these cleavage conditions. During the cleavage of the ACP decapeptide, TFA cleavage revealed a Des-Val peak at 6.0 min,

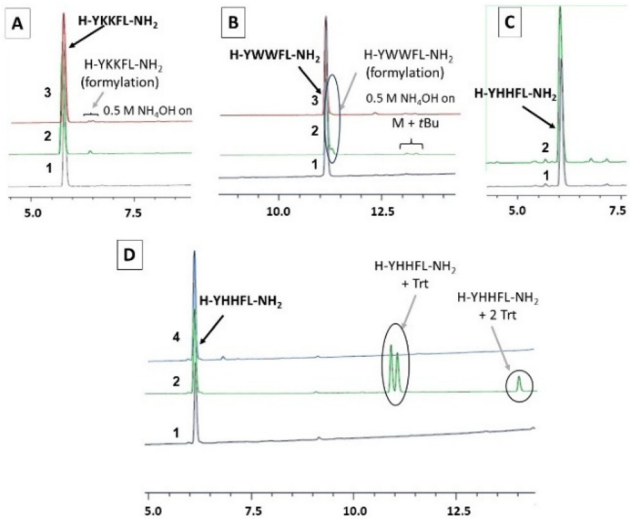

Fig. 4 Cleavage study in the case of (A) H-YKKFL-NH_2, (B) H-YWWFL-NH_2, (C and D) H-YHHFL-NH_2 from RinkAmide-resin at different time intervals. #1, TFA cleavage, #2, MSA cleavage, #3, 0.5 M NH_4OH treatment after MSA cleavage, #4, MSA cleavage at 40 °C for 3 h.

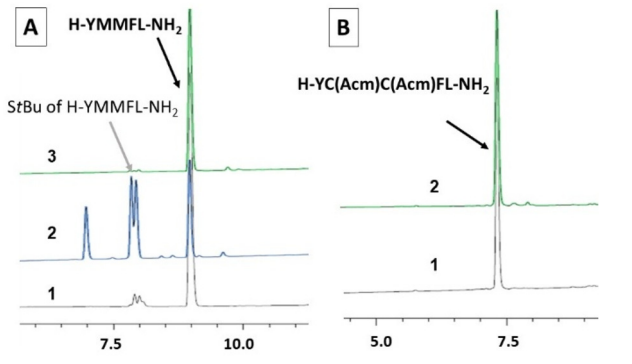

Fig. 5 Cleavage study in the case of (A) H-YMMFL-NH_2 and (B) H-YCCFL-NH_2 from RinkAmide-resin at different time intervals. #1, TFA cleavage, #2, MSA cleavage, and #3, 5% AcOH in H_2O at 40 °C for 36 h.

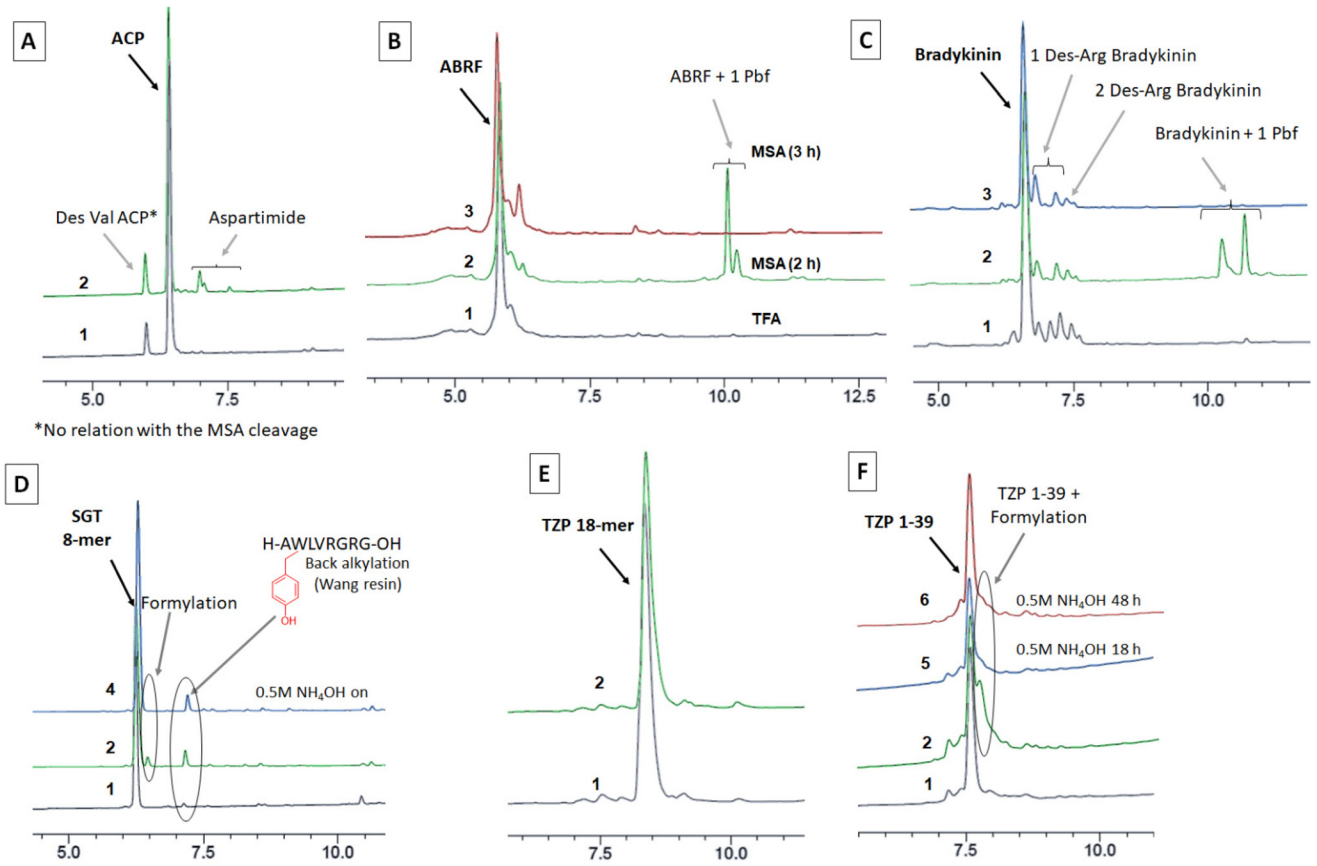

Fig. 6 Cleavage study in the case of (A) ACP(65–74), (B) ABRF1992, (C) bradykinin, (D) the semaglutide fragment (SGT 8-mer), (E) the tirzepatide fragment (TZP 18-mer), and (F) full tirzepatide (TZP 1–39). #1, TFA cleavage; #2, MSA cleavage in 2 h; #3, MSA cleavage in 3 h; #4, 0.5 M NH_4OH treatment after MSA cleavage; and #5 and 6, 0.5 M NH_4OH treatment after MSA cleavage.

affording the parent peptide with 91% purity (Fig. 6A, #1), which is a deletion peptide formed during peptide elongation and has nothing to do with the cleavage. The abovementioned MSA cocktail was also in parallel used for cleavage of the ACP peptide (Fig. 6A, #2). HPLC revealed the presence of aspartimide formation (confirmed by LCMS, see the SI). The peptide purity was found to be 81% in the case of MSA cleavage with 8% aspartimide formation. In our earlier work, MSA–TIS–DMC (16 : 20 : 64) was used, wherein 8% aspartimide formation was observed.[14] Herein, in order to tackle aspartimide formation, a lower amount of MSA, *i.e.*, 1.5% (instead of 2%), was further explored. The percentage of aspartimide was reduced from 8.0% to 5.0% in a lower amount of MSA. Further optimization is needed for complete elimination of aspartimide formation to afford the peptide with better purity. The cleavage yield was calculated to be 98%.

MSA cleavage conditions for the ABRF1992 peptide were next evaluated. The ABRF1992 peptide contains Arg(Pbf), Asp(O*t*Bu), Lys(Boc), Asn(Trt), and Trp(Boc), which eventually can also lead to potential side reactions like aspartimide formation. Removal of Pbf could also be challenging. The cleavage performed using TFA afforded crude ABRF1992 (Fig. 6B, #1) with a purity of 77%. In the case of MSA cleavage for 2 h, peptides with partial cleavage of the Pbf group (Fig. 6B, #2) were obtained. As mentioned earlier, the cleavage was repeated for 3 h for ABRF1992 (Fig. 6B, #3) and the peptide was obtained with 66% purity and a cleavage yield of 96%.

In addition, bradykinin, which contains two Arg(Pbf) and one Ser(*t*Bu), was also studied. The peptide was found to be 72% pure during TFA cleavage (Fig. 6C, #1). Similar to the case of ABRF1992, MSA cleavage for bradykinin was performed similarly for 2 h, rendering the partial cleavage of the Pbf group (Fig. 6C, #2). Then, the reaction time was increased to 3 h (Fig. 6C, #3), thereby eliminating the Pbf group. The peptide purity obtained in both cases of TFA and MSA was found to be the same, 72%. The cleavage yield was found to be 98%.

In the case of semaglutide (SGT 8-mer), containing two Arg(Pbf), one Trp(Boc), and one Ser(tBu), the peptide was synthesized on Wang resin, affording an acid peptide. The cleavage in the case of TFA afforded a peptide of 95% purity with traces of alkylation due to the Wang linker (Fig. 6D, #1). During MSA cleavage, formylation was also observed along with alkylation. The use of methylbenzhydryl bromide (MBH Br) resin should avoid this side product. This is confirmed by the presence of a peak at 7.2 min (Fig. 6D, #2). Formylation

was eliminated by treating with 0.5 M NH_4OH overnight (Fig. 6D, #4). The peptide purity in the case of MSA cleavage was found to be 94% with a cleavage yield of 96%.

As the last set of experiments, the TZP fragment (TZP 18-mer) and TZP 1–39 were also subjected to TFA cleavage (Fig. 6E, #1) for comparison with MSA cleavage results (Fig. 6E, #2). In the case of TZP 18-mer, the purity obtained was similar to that of the TFA cocktail (91% in both cases). However, in the TZP 1–39 peptide, as obtained earlier, formylation was witnessed. This was eliminated by treating with 0.5 M NH_4OH overnight (Fig. 6F). The purity after the treatment was found to be 72% compared to that of TFA cleavage (74%). The cleavage yield in both cases was found to be 99%.

Experimental

Materials and methods

All reagents and solvents were purchased from commercial suppliers and used as received without further purification. Fmoc amino acids were purchased from Chempure (Bangalore, India). Fmoc-RinkAmide resin (loading 0.67 mmol g^{-1}), PS-PEG-RinkAmide AM resin (loading 0.37 mmol g^{-1}) and Wang resin (loading 0.57 mmol g^{-1}) were gifted from Sunresin (Xi'an, China). DIC and OxymaPure were generous gifts from Luxembourg Bio Technologies (Nes Ziona, Israel). Organic solvents, dimethylformamide (DMF) and HPLC-grade acetonitrile (ACN) were purchased from Merck. Methanesulfonic acid (MSA) and triisopropylsilane (TIS) were purchased from Sigma, and formic acid (FA) was purchased from Merck. Milli-Q water was used for RP-HPLC. Analytical HPLC was carried out on a Shimadzu system using a Phenomenex Aeris™ C18 column (3.6 μm, 4.6 × 150 mm), with a flow rate of 1.0 mL min^{-1} and UV detection at 220 nm. LabSolution software was used for data processing. Buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in ACN. LCMS was performed on a Thermo Fisher Scientific UltiMate 3000 UHPLC-ISQTM EC single quadrupole mass spectrometer in positive ion mode using a Phenomenex Aeris™ C18 (3.6 μm, 4.6 × 150 mm) column. Buffer A: 0.1% formic acid in H_2O; buffer B: 0.1% formic acid in ACN.

General procedure for SPPS

All peptides were synthesized using a standard Fmoc/*t*Bu-based solid-phase peptide synthesis (SPPS) protocol. Fmoc-RinkAmide-aminomethyl (AM) resin (0.67 mmol g^{-1}), PS-PEG-RinkAmide AM resin (loading 0.37 mmol g^{-1}) and Wang resin (0.57 mmol g^{-1}) served as the solid support for the peptide assembly. The resin was swelled in DMF for 30 min and then washed with DMF. The Fmoc group was removed by treating the resin with 20% piperidine in DMF (1 × 3 min and 1 × 10 min) followed by thorough washing with DMF (×4). Protected Fmoc-amino acids (3.0 eq.) were coupled using DIC (3.0 eq.) and OxymaPure (3.0 eq.) in DMF as the coupling agents for 1 h at rt. This process was repeated sequentially until the full peptide was assembled.

SPPS of Leu-enkephalin pentapeptides (H-YXXFL-NH_2)

The Leu-enkephalin pentapeptides (H-YXXFL-NH_2) were synthesized using the Fmoc-RinkAmide AM-PS resin (loading 0.67 mmol g^{-1}) (where XX: GG, YY, SS, TT, EE, DD, WW, KK, RR, NN, QQ, HH, CC, and MM). The coupling was performed using Fmoc-AA-OH/DIC/OxymaPure [1.0 : 1.0 : 1.0, 3.0 eq.] in DMF at rt for 1 h after 1 min of pre-activation. Fmoc was removed using 20% piperidine in DMF (v/v) for 1 × 3 min + 1 × 10 min at rt. The pentapeptides were cleaved from the resin by treatment with TFA–TIS–H_2O (95 : 2.5 : 2.5) for 2 h at rt and precipitated with chilled ether. The HPLC analysis method (5–60% B into A) was used for coupling quantification.

Solid-phase peptide synthesis (SPPS) of semaglutide (SGT) 8-mer using Wang resin

The first Fmoc-amino acid was incorporated with Fmoc–Gly–OH–DIC–DMAP (1.0 : 1.0 : 0.1, 3 eq.) in DMF for 2 h. To cap the unreacted sites, acetic anhydride (Ac_2O)–DMAP (1.0 : 0.1, 10 eq.) was used in DMF for 30 min. Fmoc removal and coupling of the remaining amino acids were performed as mentioned above for Leu-enkephalin pentapeptide synthesis.

Solid-phase peptide synthesis (SPPS) of tirzepatide 18-mer (H22-39-NH_2), ABRF, bradykinin, and ACP decapeptide using Fmoc-RinkAmide-aminomethyl (AM) resin

The procedure for the synthesis of these peptides was similar to that of Leu enkephalin mentioned above.

Solid-phase peptide synthesis (SPPS) of tirzepatide (TZP, H1-39-NH_2) using PS-PEG-RinkAmide AM resin

Tirzepatide (TZP, H1-39-NH_2) was synthesized using PS-PEG-RinkAmide AM resin using the standard protocol mentioned for the Leu-enkephalin pentapeptide.

Standard global deprotection of peptides

Global deprotection was performed using TFA–H_2O–TIS (95 : 2.5 : 2.5) as the cleavage cocktail for 2 h at rt, and the respective peptides were precipitated with cold ether. The crude peptides were then dissolved in ACN/H_2O and analysed using HPLC and LCMS.

Global deprotection of peptides with MSA

The cleavage cocktail MSA–TIS–FA (2.0 : 2.5 : 95.5) was used for the cleavage study. Peptidyl resins (10 mg) were placed into an Eppendorf tube and a cleavage solution (0.2 mL per 10 mg of resin) was added. The Eppendorf tube was then kept for shaking for 2 h (or 3 h) at rt (or 40 °C in the case of His). After completion, the filtrate was collected, and cold diethyl ether was added to precipitate the peptide. The crude peptide was obtained after centrifugation and removal of the supernatant, followed by drying the residue. This process was repeated twice, followed by dissolving the crude peptides in the combination of ACN and H_2O and then injected into HPLC and LCMS for analysis.

For the calculation of the cleavage yields, the same resin after MSA cleavage was treated with TFA–TIS–H_2O (95 : 2.5 : 2.5) for 2 h at rt. After 2 h, the filtrate was collected, and cold ether was added to obtain a precipitate, which was then centrifuged and injected into HPLC. Comparison of the HPLC of the products obtained in each cleavage allowed for the calculation of the cleavage yield.

Conclusions

Herein, we have demonstrated that MSA in combination with FA is excellent for the final global deprotection step in SPPS in a green context. In comparison with the previous method reported by our group,[14] the use of FA represents an important change in the rules of the game. FA presents several advantages over DMC and/or AcOH as a solvent. The first reason could be the facilitation of the precipitation of the peptide in the last manipulation. FA is more polar than DMC and even AcOH, and the precipitation of the peptide is better when chilled ether is added. Very importantly, only 2% of MSA is required to cleave the peptide completely from the RinkAmide-resin and remove the most demanding protecting groups such as Pbf from Arg and Trt from Asn/Gln. When AcOH or DMC was used,[14] 8–16% of MSA was needed for a similar performance. Thus, the final global deprotection of ACP(65–74) anchored to the RinkAmide-resin could be carried out with 1.5–2% of MSA in FA, with quantitative cleavage from and no traces of Asn/Gln(Trt). In the case of our previous work with AcOH/DMC,[14] 16% of MSA was needed to accomplish almost quantitative cleavage, but some traces of Trt remained in the peptide. Regarding aspartimide formation, 5–8% was formed with 1.5–2% MSA in FA and 8% with 16% MSA in DMC. This represents a reduction of the consumption of MSA by approximately 95–90%.

We believe that in the case of peptides giving aspartimides, their formation could be further optimized with even less MSA and/or lower temperatures. The current protocol provides similar or superior cleavage results compared to TFA cocktail conditions. An important additional advantage is that 2% of MSA is much more manageable than 16%. This also applies when compared with TFA, which is used in more than 90% concentration. Furthermore, disposal of 2% of the green and biodegradable acid, such as MSA, will be much more convenient and affordable than 95% of a hazardous and stable acid, such as TFA, which is a PFAS. Compared with the TFA used in the classical method, this represents a 98% reduction in the use of a strong acid, fully aligned with the 12 principles of Green Chemistry, as proposed by Paul Anastas.[18]

It is important to highlight that all the present work has been carried out using TIS as the only scavenger. Further optimization will be performed by testing different scavengers. For instance, in the previous work, *m*-cresol exhibited superior performance to TIS in several cases.[14]

As it has been realized in this work, MSA and FA can lead to formylation of nucleophiles and the side chains of Ser, Thr, Tyr, Trp, and even traces of formyl Lys have been detected. This does not represent a problem at all, because all these formylations are reversible reactions that can be easily reverted by treatment with 0.5 M NH_4OH (1 h for Ser and overnight for Thr, Trp and Lys). Met, as it also occurs during the TFA cleavage, can be *tert*-butylated, but again this *tert*-butylation is reversible and can be reverted by treatment overnight with 5% AcOH.[19] In some sequences, the removal of Pbf from Arg required 3 h of treatment. Regarding the removal of the Trt group, whose reaction is also reversible, 2 h was sufficient to remove it from Asn/Gln. However, the removal of Trt from His required 3 h at 40 °C. On the other hand, when imidazole of His is protected with Boc, this is removed nicely in 2 h at room temperature. Removal of Trt from Cys has been demonstrated to be a challenge and will require an optimization using different scavengers and reaction times. However, Acm from Cys is stable to MSA in FA and can be removed later with *N*-chlorosuccinimide (NCS) or iodine, which are standard treatment in Cys-containing peptides.

This cleavage cocktail has been positively tested first with model peptides (H-Y(*t*Bu)XXFL-NH-RinkAmide-resin, where XX are all the trifunctional amino acids), and then with other peptide such as ACP(65–74), bradykinin, ABRF1992, the C-terminal part of semaglutide, the C-terminal fragment of tirzepatide, and even the full linear sequence of tirzepatide, one of the most important active pharmaceutical ingredient (API) blockbusters currently on the market. Its global production is estimated at several tons per year.[20] If MSA is used instead of TFA, the savings from using a strong acid (TFA/MSA) will be very significant, with the added benefit that the acid used, MSA, is green and biodegradable, with significant environmental and economic repercussions. In other industries such as the Zn industry, MSA is recovered by vacuum distillation at a pressure of 0.04 mbar and a vapor temperature of 110 °C, with an efficiency of distillation recovery of 88 ± 2 vol%.[16] Alternatively, MSA can also be recovered *via* an electrochemical approach. This reaffirms the sustainability of the use of MSA.[17] Table 1 summarizes the key features of conventional peptide cleavage using TFA compared with the current global deprotection approach using MSA.

Table 1 Salient features of global deprotection using TFA and MSA

Property	Conventional peptide cleavage using TFA	Current peptide cleavage using MSA
Percentage used	95%	2%
Solvent	None	Formic acid
PFAS	✓	✗
Deprotection efficiency	Fast	Moderate to fast
Formylation	✗	✓
Removing formylation	Not applicable	0.5 N NH_4OH
Smell/odor	✓	✗
Volatility	✓	✗
Environmental impact	Non-biodegradable	Biodegradable
Greener process	✗	✓
Potential cost saving	✗	✓
Waste handling	Hazardous	Non-hazardous

Although further optimization involving different scavengers, reaction times, and temperatures should be carried for minimizing side reactions, we envisage that MSA in formic acid will replace TFA cleavage in peptide synthesis.

Author contributions

FF and AK performed the reactions and carried out the cleavage study; ML, OM, SB, FA, and BGT supervised the research work; AS and AK contributed to the preparation of the first draft. The final version of the manuscript was prepared with consent of all the authors.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information: HPLC and LC-MS. See DOI: https://doi.org/10.1039/d5gc04192a.

Acknowledgements

We thank Yoav Luxembourg for encouraging this work.

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