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In situ Fmoc removal – a sustainable solid-phase peptide synthesis approach†

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Solid-phase peptide synthesis (SPPS) is the strategy of choice for the synthesis of peptides for research and production purposes. From a green chemistry perspective, SPPS has several positive features. However, it is hampered by high solvent consumption for washings after each of the two main steps, namely deprotection and coupling. Here we propose to combine the two steps into one. In this regard, once the coupling is completed, piperidine or 4-methylpiperidine is added up to a concentration of 20% to the coupling cocktail, which contains an excess of Fmoc-aa-OxymaPure (active ester) and Fmoc-peptide resin. We further demonstrate that the deactivation of the OxymaPure ester is faster than Fmoc removal and therefore this *in situ* Fmoc removal strategy avoids the double incorporation of the amino acid into the peptide chain. Furthermore, we also show that this single wash is more efficient at removing traces of piperidine when 1% of OxymaPure, which is a weak acid, is added to the washing solvent. This strategy brings about a saving of 75% of the solvent. We envisage that the modifications to this new protocol will be added to the green toolbox for SPPS and will make this strategy more sustainable.

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Introduction

Peptides are a family of chemical compounds formed by the consecutive linking of amino acids found in living organisms. As regulators of many biochemical pathways, these molecules have always been viewed as potential drugs or templates for new ones.[1] Although the structure of peptides is simple, it was not until the end of the last century that they were unquestionably considered to be drugs. This identity was due to the large number of steps required for the synthesis of a medium-/large-sized peptide. Without taking into account the starting building blocks, the synthesis of a peptide of 15 amino acids, for instance, requires at least 31 synthetic steps with the corresponding workups and the isolation and often purification of the intermediates. With the development and later implementation of the solid-phase peptide synthesis (SPPS) methodology carried out by the Nobel laureate R. Bruce Merrifield, a new scenario was envisaged.[2] Thanks to automatic synthesizers, it is now possible to synthesize a few milligrams of a medium-/large-sized peptide for research purposes in only a few hours and multiple kilograms of a peptide-based active pharmaceutical ingredient (API) in a few weeks.[3,4] Thanks to SPPS, two important steps of the drug discovery process, namely discovery and API production, have advanced enormously. As an example, in 2021, the US Food and Drug Administration (FDA) approved ten drugs containing peptides, a number that accounts for 25% of the 2021 drug harvest.[5] In the context of green chemistry, SPPS has large "green brushstrokes". In this regard, this strategy has the following characteristics:[6–8] (i) all reactions are carried out in the same reactor, with no mass transfer between reactors, and no major cleaning procedures; (ii) intermediates are not isolated, thereby lowering solvent consumption; (iii) excellent yields are obtained, which facilitates purification and reduces the use of solvents; (iv) high boiling point solvents are used; (v) solvents can be recycled and the resin can be reused [when a chlorotrityl chloride (CTC) resin is used]; (vi) excess reactants and soluble side products are removed by simple filtration and washing with the corresponding solvents; and, finally, (vii) high efficiency and reduced labor hours are achieved.

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However, from the green chemistry perspective, these advantages are weighed down by the following disadvantages, which require mitigation: (i) the presence of high molecular weight protecting groups in the predominant fluorenylmethoxycarbonyl (Fmoc)-*tert*-butyl (*t*Bu) strategy translates into poor atom economy and (ii) good yields are favored by the use of large excesses of reagents, a practice that is untenable for research purposes. This drawback is moderated in large-scale production, where excesses are very close to those used in solution synthetic organic chemistry.

Given the definition of green chemistry by Paul Anastas in 1991, "Green Chemistry is the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances",[9] many academic and industrial groups have studied the replacement of *N*,*N*′-dimethylformamide (DMF) and *N*-methylpyrrolidone (NMP), solvents of choice in SPPS, by green solvents.[10–23] However, few efforts have been devoted to solvent reduction by this strategy.

A "classical" Fmoc/*t*Bu coupling cycle comprises the following four steps: (i) coupling of Fmoc-aa-OH, very often carried out with *N*,*N*′-diisopropylcarbodiimide (DIC) and OxymaPure;[24] (ii) washings with DMF to remove the excess of reagents (Fmoc-aa-OH, DIC, OxymaPure) and side-products [*N*,*N*′-diisopropylurea (DIU)]; (iii) Fmoc removal, in most cases using 20% piperidine (PIP) in DMF; and (iv) washings with DMF to remove the excess PIP (which could remove the Fmoc group of the next incoming Fmoc-aa-OH) and *N*-fluorenylmethylpiperidine formed as the side product. In this context, as washings (iv) to remove the excess PIP cannot be omitted, efforts focused on the removal of the washings (ii) after the coupling. Thus, once the coupling is finished, the PIP solution is added and the original protocol is followed with minor adjustments. In this novel *in situ* Fmoc removal protocol, the deprotection step is carried out immediately after the coupling in the same step. Ajiphase, a liquid phase peptide synthesis methodology, proposes a kind of similar approach for avoiding liquid-liquid extractions, which are demanding manipulations. Furthermore, in some of the accelerated microwave CEM programs the piperidine solution is added at the end of the coupling step.[25] Research efforts have been devoted not only to removing the washing steps (ii) but also reducing the amount of solvent used in steps (ii) and (iii).

Results and discussion

In situ Fmoc removal involves Fmoc removal directly after coupling (without filtration) using 20% PIP in DMF. Several experiments were designed and performed as described below. In all cases, at the end of the sequence elongation, global deprotection using trifluoroacetic acid (TFA)–triisopropylsilane (TIS)–H_2O (95 : 2.5 : 2.5) cleaved the peptide from the resin. The residue was washed with cold ether to afford the peptide, which was analyzed by HPLC.

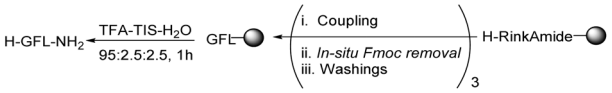
SPPS of H-Gly-Phe-Leu-NH_2 (H-GFL-NH_2) using *in situ* Fmoc removal

To test the validity of the *in situ* Fmoc removal approach, the model tripeptide (H-GFL-NH_2) was synthesized following two distinct protocols. In the first case, a standard SPPS protocol was used (coupling → washing → Fmoc removal → washing), whereas in the second case, *in situ* Fmoc removal was performed after coupling and the washing step was eliminated (coupling → Fmoc removal → washing). The tripeptide was assembled on the Fmoc-RinkAmide-AM-PS resin (0.1 g, 0.63 mmol g^{-1}) using four synthetic protocols (A–D) as shown in Table 1. In all cases, Fmoc-aa-OH, OxymaPure, and DIC were dissolved in 0.5 mL of DMF. In experiments A–C, 3 eq. of reagents were used, while 1.5 eq. were used in D. After 1 h of coupling, 0.12 mL of neat PIP (approximately 6 eq.) was added and left to react. Finally, all peptidyl resins were washed three times with 3 mL of DMF. The rationale of the four experiments was to first determine their efficacy and also the impact of deprotection time (# C and D), high temperature (# B), and a greater amount of coupling reagents (# A–C) on the purity of the final product.

A priori, double or even triple incorporation of the incoming amino acid would be expected once PIP has removed Fmoc from the peptidyl resin. In this side reaction, PIP could have two purposes, first removing the Fmoc group and second scavenging the active species (OxymaPure ester), thereby deactivating it. If Fmoc removal occurs more quickly than the deactivation of the OxymaPure ester, the latter can over-acylate itself or acylate the free amino peptidyl resin, causing the formation of peptides with double/triple hits. In contrast, faster deactivation of the OxymaPure ester *vs.* Fmoc removal will support the *in situ* Fmoc removal strategy. Temperature may play an important role in these two reactions, favoring the former. The second side reaction could be the incomplete removal of the Fmoc group, which may lead to either a Fmoc-protected peptide or a deletion peptide. *A priori*, experiments A and B are the most conflictive because they contain more coupling cocktail than D, which may have a greater acylation capacity and at the same time a higher consumption of PIP, and/or less deprotection time than C and D. Both factors are detrimental to the quality of the final product because they may favor the formation of deletion peptides.

The HPLC analysis (Fig. 1) of experiments A and B indicated that the temperature did not affect the purity of the final product. Although the four experiments yielded excellent purity, A and B, which as discussed above could be the most demanding, performed slightly better than C and D. With

Table 1 Synthesis of H-GFL-NH_2 using different protocols



Protocol	A	B	C	D
Coupling[a]	0.6 M	0.6 M	0.6 M	0.3 M
Fmoc Removal[b]	7 min, rt	7 min, 45 °C	20 min, rt	20 min, rt
Washing	3 × 3 mL DMF			

[a] Fmoc-AA-OH–DIC–OxymaPure [1 : 1 : 1], 1 min of pre-activation, 1 h, rt; for experiments A–C, 3 eq. of each reagent were used whereas for D 1.5 eq. were used. [b] *In situ* Fmoc removal using 20% PIP in DMF.

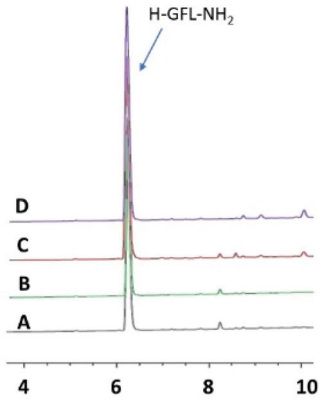


Fig. 1 HPLC of H-GFL-NH_2 synthesized using *in situ* Fmoc removal.

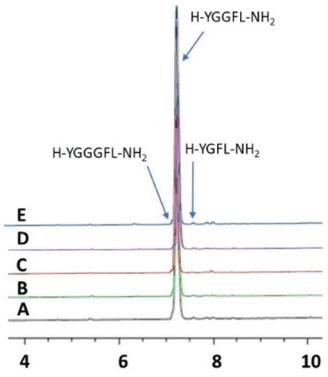


Fig. 2 HPLC of H-YGGFL-NH_2 prepared using *in situ* Fmoc removal (experiments A–E).

these results in hand, we tested the synthesis of Leu-Enkephalin (YGGFL) as a model peptide.

SPPS of H-Tyr-Gly-Gly-Phe-Leu-NH_2 (H-YGGFL-NH_2) using *in situ* Fmoc-removal

After the proof of concept, *in situ* Fmoc removal was tested under a broad range of conditions for the coupling of Fmoc-G-OH and Fmoc-Y(*t*Bu)-OH on the H-GFL-NH-RinkAmide-resin, which was prepared on a large scale for the experiments. First, similar conditions to those given in Table 1 were studied as a reference (Table 2).

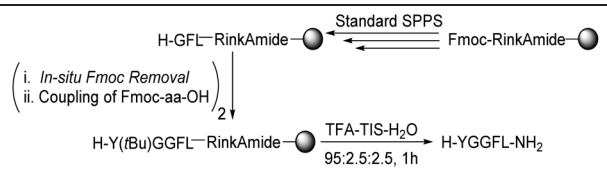
The purity of the pentapeptide in all cases was excellent and again temperature was not a factor to be taken into consideration (# A *vs.* B and D *vs.* E). Furthermore, longer deprotection times (# C) did not appear to have a true impact on the use of 3 or 1.5 eq. of the coupling reagent (# A and B *vs.* D and E) (Fig. 2).

SPPS of H-YGGFL-NH_2 for understanding the effect of PIP during the coupling

To understand the effect of traces of PIP during the next coupling, three experiments were performed with the H-GFL-RinkAmide resin (0.1 mmol). In each experiment, 0.1 mL of PIP in DMF was added after the washings and before addition of the coupling reagents (Table 3). In the first set of experiment, 0.1 mL of 20% PIP in DMF (0.20 mmol) was used in protocols A–C, representing approximately a 2-fold excess with respect to the amino groups anchored on the resin and therefore a 66% and 133% excess with respect to the coupling reagents when 3 eq. and 1.5 eq. were used, respectively. In the second set of experiments, 0.1 mL of 5% PIP in DMF (0.05 mmol) was used in protocols A–C, representing a 50% excess with respect to the amino groups anchored on the resin and therefore a 16% and 33% excess with respect to the coupling reagents when 3 eq. and 1.5 eq. were used, respectively. In the last set of experiments, 0.1 mL of 1% PIP in DMF (0.01 mmol) was used, representing a 10% excess with respect to the amino groups anchored on the resin and therefore a 3% and 6% excess with respect to coupling reagents when 3 eq. and 1.5 eq. were used, respectively.

As mentioned earlier, PIP during the coupling can have two functions: (i) removal of the Fmoc group of the incoming amino acid either activated in solution or already coupled on the resin and (ii) consumption of the activated amino acid still containing Fmoc or after Fmoc has been removed. If the first reaction is predominant and/or the fastest one, the formation

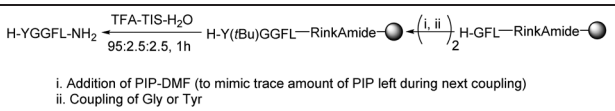
Table 2 Synthesis of H-YGGFL-NH_2 using *in situ* Fmoc removal



Protocol	A	B	C	D	E
Coupling[a]	0.6 M	0.6 M	0.6 M	0.3 M	0.3 M
Fmoc Removal[b]	7 min, rt	7 min, 45 °C	20 min, rt	7 min, rt	7 min, 45 °C
Washing	3 × 3 mL DMF				

[a] Fmoc-AA-OH : DIC : OxymaPure [1 : 1 : 1], 1 min of pre-activation, 1 h, rt; for experiments A–C, 3 eq. of each reagent were used whereas for D and E 1.5 eq. were used. [b] *In situ* Fmoc removal using 20% PIP in DMF.

Table 3 Synthesis of H-YGGFL-NH_2 in the presence of PIP during the coupling



	PIP in DMF[a] (% purity of H-YGGFL-NH_2)		
Protocol	20%	5%	1%
A	3 eq. rt (51.5%)	3 eq. rt (96.7%)	3 eq. rt (96.3%)
B	1.5 eq. rt (7.2%)	1.5 eq. rt (82.8%)	1.5 eq. rt (92.5%)
C	1.5 eq. 45 °C (4.8%)	1.5 eq. 45 °C (93.5%)	1.5 eq. 45 °C (94.0%)

[a] PIP in DMF was added to the peptidyl resin followed by coupling cocktail (Fmoc-AA-OH : DIC : OxymaPure [1 : 1 : 1], 1 min of pre-activation) for 1 h. Fmoc removal for 7 min at rt.

of peptides containing extra residues (Gly and Tyr in this case) should be observed. The second reaction should translate into the formation of deletion peptides due to the lack of the activating reagent. After addition of PIP/DMF, the coupling cocktail was added (as shown in Table 3). After global deprotection, the purity of H-YGGFL-NH_2 was analyzed by HPLC and confirmed by LCMS. The analysis showed that (i) only deletion peptides are obtained as major impurities (des-Gly, des-Tyr and des-Tyr-Gly), with no significant appearance of a double hit (a double hit of Gly is already very common in normal SPPS); (ii) the lowest purity is obtained when 20% PIP in DMF is added; (iii) the use of 1.5 eq. of PIP/DMF gives poorer results than the use of 3 eq.; (iv) the use of 45 °C to carry out the experiment does not appear to be detrimental; (v) when 1% PIP in DMF is added, the purity of the final peptide is similar to that obtained when 5% is added. From these results, the following main conclusions can be drawn: (i) deactivation of the active ester is faster than the removal of Fmoc in the absence of double hit peptides; (ii) the use of 45 °C favors coupling over deactivation of the active ester, based on the best results of the experiment #C *vs.* B with 5% and 1% of PIP in DMF; (iii) the final purity of the peptide is encouraging, except in the case of adding 20% PIP in DMF (Fig. 3).

SPPS of H-YGGFL-NH_2, and PIP neutralization by adding 1% OxymaPure during washings

To achieve an efficient removal of traces of PIP before adding the coupling mixture, washings were carried out with 1% OxymaPure. Again, the presence of PIP was mimicked by adding it before the washings. In this series of experiments, H-GFL-resin (0.1 mmol) was treated with 0.1 mL of 20% PIP in DMF (as explained in the previous experiment), followed by washing with 1% OxymaPure-DMF before addition of the coupling cocktail. Table 4 and Fig. 4 summarize the experimental protocol and results obtained.

Comparison of these results with those outlined in Table 3 demonstrates the effect of the washing with 1% OxymaPure-DMF. Just a single wash brings about an increase in purity from 51.5%, 7.2%, and 4.8% to 98.2%, 91.7%, and 97.2% for A (3 eq.), B (1.5 eq.), and C (1.5 eq., 45 °C), respectively. Additionally, these results confirm the previous observation that deactivation of the active ester is faster than the removal of the Fmoc group and that either an excess of the coupling mixture or an additional washing step allows safe coupling.

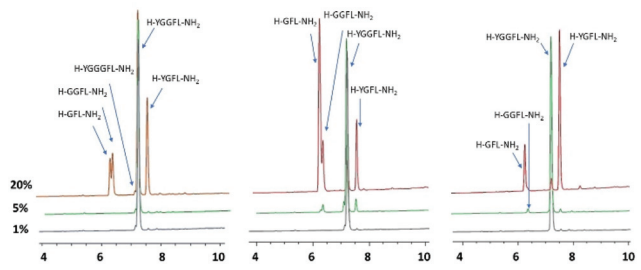
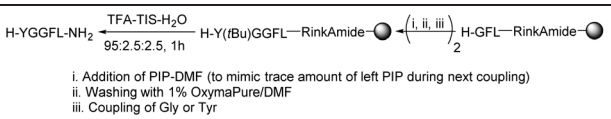


Fig. 3 HPLC analysis of H-YGGFL-NH_2 when PIP in DMF is added before the coupling (Table 3).

Table 4 Synthesis of H-YGGFL-NH_2 by washing with 1% OxymaPure



	1% OxymaPure in DMF[a] (% purity of H-YGGFL-NH_2)	
Protocol	1 × 1 mL	2 × 1 mL
A	3 eq. rt (98.2%)	NA[b]
B	1.5 eq. rt (91.7%)	1.5 eq. rt (98.3%)
C	1.5 eq. 45 °C (97.2%)	1.5 eq. 45 °C (98.4%)

[a] 0.1 mL of 20% PIP in DMF was added to the peptidyl resin followed by washing using 1% OxymaPure in DMF. Coupling cocktail (Fmoc-aa-OH : DIC : OxymaPure [1 : 1 : 1], 1 min of pre-activation) was added for 1 h. A: 3 eq.; B: 1.5 eq.; C: 1.5 eq. at 45 °C. [b] Since the results obtained in B and C were satisfactory, an attempt with a higher concentration was made.

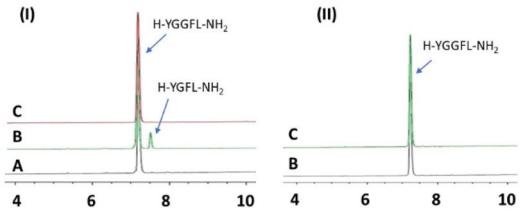


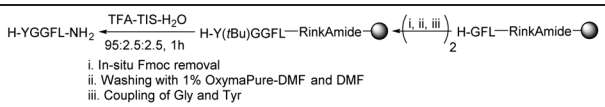
Fig. 4 HPLC analysis of H-YGGFL-NH_2 to determine the effect of the washing resin (containing 20% PIP in DMF) with 1% OxymaPure-DMF before coupling.

SPPS of H-YGGFL-NH_2 using 1% OxymaPure-DMF for washing after *in situ* Fmoc removal

As described above, the trace amount of PIP was quenched by washing with 1% OxymaPure-DMF. In this series of experiments, Fmoc was removed *in situ* after coupling, as explained in the previous experiments. The resin was then washed as follows: twice with 1% OxymaPure-DMF (protocols A and B); once with 1% OxymaPure-DMF (protocol C); once with DMF and a second wash with 1% OxymaPure-DMF (protocol D). After washing, coupling was performed. The cleaved peptide was then analyzed by HPLC. Table 5 and Fig. 5 summarize the experimental protocols and the results obtained.

In all the protocols, the peptide with >97% purity was obtained except in protocol C. The low yields could be attributed to the fact that one wash was not sufficient to eliminate trace PIP from the reaction mixture. This trace PIP might have led to the formation of des-Tyr as a major impurity. In the rest of the cases, the formation of a trace amount of double hit Gly was observed (Fig. 5). The double hit may not be related to the presence of PIP because in protocol C, in which the washing was not so efficient (as demonstrated by the presence of the

Table 5 Synthesis of H-YGGFL-NH_2 involving washing the resin with 1% OxymaPure-DMF after *in situ* Fmoc removal before coupling



Protocol[a]	Washing after *in situ* Fmoc removal (% purity of H-YGGFL-NH_2)
A	2 × 1 mL 1% OxymaPure-DMF (97.2%)
B[b]	2 × 1 mL 1% OxymaPure-DMF (97.7%)
C	1 × 1 mL 1% OxymaPure-DMF (80.2%)
D	1 × 1 mL DMF + 1 × 1 mL 1% OxymaPure/DMF (97.2%)

[a] Coupling was then performed using Fmoc-AA-OH : DIC : OxymaPure [1 : 1 : 1]; 1.5 eq., 1 min of pre-activation for 1 h at rt. [b] Coupling and deprotection at 45 °C.

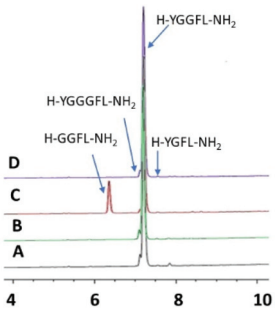


Fig. 5 HPLC analysis of H-YGGFL-NH_2 to study the effect of the washing resin with 1% OxymaPure-DMF prior to coupling.

deletion peptide), the double hit was not superior to that achieved in the rest of the cases.

4-Methyl piperidine (4-MP) as a substitute of PIP for *in situ* Fmoc removal

Although Fmoc removal using PIP is widely accepted, 4-MP emerges as a promising greener substitute.[26–28] Prompted by the encouraging results obtained so far, an attempt was made to replace PIP with 4-MP for *in situ* Fmoc removal. Coupling was performed using Fmoc-AA-OH–DIC–OxymaPure [1 : 1 : 1] with 1.5 eq. after 1 min of pre-activation for 1 h at rt. Fmoc was removed by adding 4-MP up to a concentration of 20% for 7 min at rt, followed by washing with 1 × 1 mL of DMF + 1 × 1 mL of 1% of OxymaPure-DMF. After the cleavage from the resin, the peptide was analyzed by HPLC. The peptide was obtained with >98.0% purity with some impurities like a double hit of Gly (1.24%) and des Gly (0.23%). Comparison of the results revealed that PIP and 4-MP gave similar peptide purities (4-MP, slightly higher purity than PIP). Therefore, 4-MP emerges as a substitute for PIP for Fmoc removal, thereby paving the way for a greener SPPS strategy (Fig. 6). In this regard, for all the following experiments, 4-MP was used for *in situ* Fmoc removal.

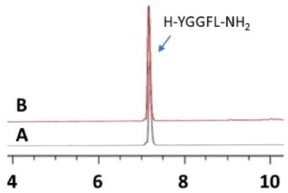


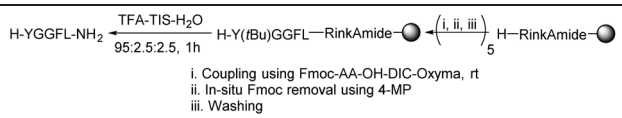
Fig. 6 Comparative study of peptide synthesis using PIP [A] and 4-MP [B] for *in situ* Fmoc removal.

Complete synthesis of H-YGGFL-NH_2 using *in situ* Fmoc removal (using 4-MP)

After the success of *in situ* Fmoc removal with 4-MP and washing with 1% OxymaPure for the coupling of the Gly and Tyr units on the tripeptidyl resin (H-GFL-Rink-amide-resin), this protocol was applied to the total synthesis of Leu-Enkephalin pentapeptide. Table 6 shows the list of experiments performed for H-YGGFL-NH_2 synthesis. All the syntheses of peptides, except M, were performed on a 0.1 mmol scale using 1.5 eq. of Fmoc-AA-OH–DIC–OxymaPure.

Protocol A involved the synthesis of the peptide following the standard protocol (coupling → washing → deprotection → washing) at rt for 1 h using 0.3 M coupling cocktail (1.5 eq.) with 1 min of pre-activation. Fmoc was removed using 20% 4-MP in DMF (1 × 1 min: 1 × 7 min) at rt. Each coupling and deprotection step were followed by washing with DMF (after

Table 6 Synthesis of H-YGGFL-NH_2 using *in situ* Fmoc removal



Protocol	Coupling (1.5 eq.) Fmoc-AA-OH–DIC–OxymaPure	Fmoc removal 4-MP-DMF (20%) *in situ* Fmoc removal	Washing	Yield, %
A[a,b,d]	1 : 1 : 1; 1 h	7 min	3 × 1 mL DMF	93.2
B[b,d]	1 : 1 : 1; 1 h	7 min	1 × 1 mL DMF	83.5
C[b,d]	1 : 1 : 2 1 h	7 min	+ 1 × 1 mL 1%	84.2
D	1 : 1 : 1; 1 h	7 min	OxymaPure-	95.4
E[c]	1 : 1 : 1 1 h	7 min	DMF	94.9
F	1 : 1 : 2; 1 h	7 min		94.2
G[e]	1 : 1 : 1; 1 h	7 min		86.8
H[f]	1 : 1 : 1; 1 h	10 min		96.3
I	1 : 1 : 1; 1 h	10 min		90.2
J	1 : 1 : 1; 2 h	20 min		95.2
K	1 : 1 : 1; 1 h	10 min	2 × 1 mL 1%	96.3
L[g]	1 : 1 : 1; 1.5 h	20 min	OxymaPure-	96.6
M[g,h]	1 : 1 : 1; 1.5 h	20 min	DMF	95.4

[a] Standard cycle (coupling → washing → deprotection → washing) for peptide synthesis. [b] 1 min of pre-activation for coupling cocktail. [c] Half of the DIC added along with coupling cocktail; the remaining added after 15 min. [d] Pre-activation [for the rest of the protocols (D–M) *in situ* activation was performed]. [e] Fmoc removal for two Gly residues was achieved after filtration. [f] Filtration after each coupling before Fmoc removal. [g] Additional 0.3 eq. of DIC added after 30 min of coupling. [h] 1 g scale.

coupling 2 × 1 mL and after deprotection 3 × 1 mL). After global deprotection and washing with diethyl ether, the purity of the peptide (93.3%) was determined by HPLC, which revealed impurities of desLeu (3.3%, 5.4 min), desGly (3.0%, 7.5 min), and a double hit of Gly (0.4%, 7.2 min). The next experiment was performed (protocol B), where washing after coupling was eliminated and the *in situ* Fmoc removal protocol was adopted (4-MP was added up to a concentration of 20%). Coupling then proceeded as previously described after washing the resin (1 × 1 mL DMF; 1 × 1 mL 1% OxymaPure-DMF). The synthetic cycle was therefore coupling → deprotection → washing. The purity of the peptide was found to be 83.5%, with impurities of desLeu (2.5%), desGly (5.6%), a double hit of Gly (5.0%), a double hit of Leu (1.5%, 8.8 min) and a double hit of Phe (0.9%, 9.1 min). Therefore, the synthesis was attempted again, this time using 2 eq. of OxymaPure (protocol C) with 1 min of pre-activation of the coupling cocktail. However, while the purity of the peptide was 84.2%, which is slightly better than that achieved with protocol B, it was still not acceptable.

Another attempt like protocol B was made. In this regard, instead of pre-activation for 1 min, the coupling cocktail was activated *in situ* (protocol D). Using this strategy, the purity of the peptide increased from 83.5% to 95.4%. The absence of a double hit of Leu and Phe indicates that this reaction is associated with the pre-activation step, where the active species can react with itself, rather than with the *in situ* Fmoc removal step. Upon using only 1.5 eq. of reagents, the double hit is favored more than that achieved when a larger excess of reagents is used because the Fmoc-dipeptide is less reactive than Fmoc-aa-OH and therefore more Fmoc-aa-OH, which couples faster, is present when using a larger excess of them. Prompted by this improvement in purity, in protocol E, a slight modification from protocol D was attempted wherein half of the DIC was added along with the coupling cocktail at 0 min whereas the other half was added after 15 min of coupling time (1 eq.). The purity of the peptide obtained was 94.9% (comparable to that of protocol D). To further enhance the synthetic protocol, 3 eq. of OxymaPure were used to neutralize the trace 4-MP to avoid side reactions (protocol F). However, the purity of the peptide was still only 94.2%, with no significant improvement. In all the above protocols, des-Gly appeared as a major impurity. In protocol G, to confirm that des-Gly was not related to the traces of 4-MP, Fmoc removal of two Gly residues was attempted after filtration, while the rest of the synthetic protocol remained the same (in the case of protocol D). However, the purity of the peptide was lower (86.8% *vs.* 95.4%). Protocol H involved filtration after each coupling and Fmoc removal was extended to 10 min (unlike 7 min in all earlier protocols). In this case, the peptide was achieved with 96.3% purity. With this improvement, in the next protocol, I, *in situ* Fmoc removal, was applied for 10 min. Protocol H differed from I; the former involved Fmoc removal after filtration whereas the latter involved *in situ* Fmoc removal. However, this change did not enhance the purity of the peptide (90.2% in protocol I compared to 96.3% in protocol H). To improve protocol I, the coupling time (2 h instead of 1 h) and deprotection time (20 min instead of 10 min) were extended (protocol J), which enhanced the purity of the peptide from 90.2% to 95.2%. Protocol J showed almost similar purity of the peptide to that achieved with protocol H but had the advantage of being greener (as filtration after each coupling was omitted in the case of protocol J) (Fig. 7).

In all the earlier attempts, washing after *in situ* Fmoc removal was attempted once with DMF and then with 1% OxymaPure/DMF. In protocol K (similar to protocol I), the peptide was synthesized but the washing after Fmoc removal was performed twice using 1 mL of 1% OxymaPure/DMF. This approach improved the purity of the peptide from 90.2% to 96.3%. Thus, washing twice with 1% OxymaPure/DMF made a significant contribution to increasing the purity. In protocol L, the coupling and Fmoc removal time were enhanced from 1 h to 1.5 h and 10 min to 20 min, respectively. This modification, wherein the coupling time was 1.5 h (0.3 eq. of DIC added after 30 min of coupling) and the deprotection time was 20 min, afforded the peptide with 96.6% purity. It is important to highlight that the peptide purity obtained using protocols K and L was superior to that achieved with protocol A, which is the standard procedure with a differentiated Fmoc removal step and washings after Fmoc removal and coupling steps.

Finally, protocol M, which is similar to protocol L, was performed but on a 0.64 mmol (1.0 g) scale. The purity on a large scale was found to be 95.4% with impurities of des-Gly (1.6%) and others (<1%). In this series of experiments, we successfully synthesized H-YGGFL-NH_2 on a large scale under *in situ* Fmoc removal conditions. Again, the purity of protocol M was superior to that achieved with protocol A, which is the standard procedure. In protocol M, the solvent consumption was also calculated. The total manual synthesis of H-YGGFL-NH_2 generated only 200 mL of waste compared to the standard procedure (800 mL (approx.)). This total waste was from coupling,

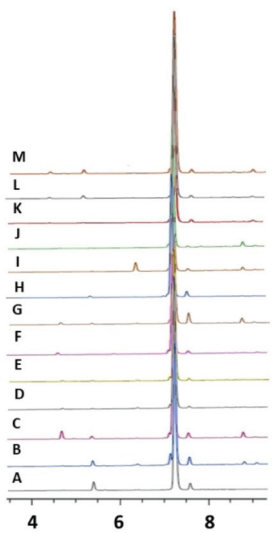


Fig. 7 Comparative study of H-YGGFL-NH_2 synthesis as explained in Table 6.

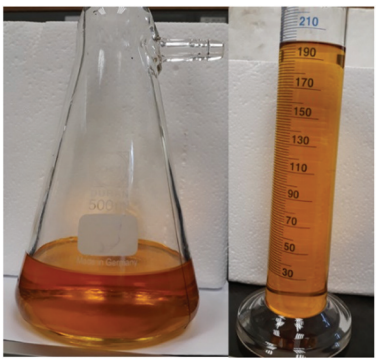


Fig. 8 Solvent waste during the synthesis of H-YGGFL-NH_2 on a 1 g scale (0.64 mmol): protocol M from Table 6.

in situ Fmoc removal and washing. This reduction of waste using the *in situ* Fmoc removal approach (washing with 1% OxymaPure) proves to be a greener SPPS strategy (Fig. 8).

The process mass intensity (PMI) and complete environmental factor (E-factor) are key mass-based metrices to evaluate the green credentials of an individual step or sequence of reactions during the synthetic process and chemical development.[29,30]

$$\text{PMI} = \frac{\text{mass of reactants}}{\text{mass of product}}$$

$$E\text{-Factor} = \frac{\text{mass of waste(solvent + spent resin)}}{\text{mass of product}}$$

These two metrics were calculated for 0.64 mmol scale Leu-Enkephalin synthesis using the "*in situ* Fmoc removal protocol" and compared with that of standard SPPS (using an excess of the solvent and 3 eq. of the coupling cocktail). The PMI and E-factor for the former were calculated as 571.0 and 558.0, respectively, whereas those of standard SPPS were found to be 2242.6 and 2224.0, respectively. This fair reduction of the PMI and E-factor (around 4 times) indicates the feasibility of the "*in situ* Fmoc removal" protocol for the efficient synthesis of the peptide without compromising the yield and purity of the final peptide.

Experimental

General information

All reagents and solvents were purchased from commercial suppliers and used without further purification. Fmoc amino acids and Fmoc-Rink amide AM resin (loading 0.64 mmol g^{-1}) were purchased from Iris Biotech. OxymaPure and DIC were received as gifts from Luxembourg Biotech. Piperidine (PIP)/4-methylpiperidine (4-MP) was supplied by Sigma-Aldrich. Organic solvents dimethylformamide (DMF) and HPLC quality acetonitrile (CH_3CN) were purchased from Merck. Milli-Q water was used for RP-HPLC analyses. Analytical HPLC was performed on an Agilent 1100 system using a Phenomenex Aeris™ C18 (3.6 μm, 4.6 × 150 mm) column, with a flow rate of 1.0 mL min^{-1} and UV detection at 220 nm. The Chemstation software was used for data processing; buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in CH_3CN. LC-MS was performed on a Thermo Fisher Scientific UltiMate 3000 UHPLC-ISQTM EC single quadrupole mass spectrometer in the 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 CH_3CN. **HPLC method:** 5–60% B into A in 15 min.

SPPS

All peptides were assembled manually in plastic syringes fitted with a porous polyethylene disc employing the Fmoc/*t*Bu methodology using DIC/OxymaPure as the coupling agent. Fmoc removal was carried out *in situ i.e.*, PIP or 4-MP was added to the peptidyl resin without filtering the coupling cocktail after coupling. In some experiments, Fmoc was removed by treatment with a solution of 20% PIP or 4-MP in DMF (v/v).

SPPS of tripeptide H-Gly-Phe-Leu-NH_2 (H-GFL-NH_2) using *in situ* Fmoc removal

The tripeptide H-Gly-Phe-Leu-NH_2 was synthesized using the Fmoc-Rink-amide AMPS resin (loading 0.64 mmol g^{-1}). The coupling was performed using Fmoc-AA-OH/DIC/Oxyma [at a ratio of 1 : 1 : 1, 3.0 or 1.5 eq.] in DMF at rt for 1 h after 1 min of pre-activation. Fmoc was removed *in situ* with PIP at different times and temperatures (7 min, rt/45 °C; 20 min, rt). The tripeptide was cleaved from the resin by treatment with TFA/TIS/H_2O (95 : 2.5 : 2.5) for 1 h at rt and precipitated with chilled diethyl ether. The HPLC analysis method (5–60% B into A) was used for coupling quantification.

SPPS of the tripeptidyl resin (H-GFL-Rink amide AMPS) for coupling of Fmoc-Gly-OH and Fmoc-Tyr(*t*Bu)-OH units

The tripeptidyl resin (H-GFL-resin) for the coupling of the Gly and Tyr units was synthesized on Rink-amide-AMPS (loading 0.64 mmol g^{-1}) following the standard protocol (coupling → washing → deprotection → washing). The coupling of each residue was performed using Fmoc-AA-OH/DIC/Oxyma [at a ratio of 1 : 1 : 1, 3.0 eq.] in DMF at rt for 1 h after 1 min of pre-activation. Fmoc was removed using 20% PIP in DMF (v/v) for 1 × 1 min + 1 × 7 min at rt.

Coupling of Fmoc-Gly-OH and Fmoc-Tyr(*t*Bu)-OH, on the tripeptidyl resin (H-GFL-Rink amide AMPS) and *in situ* Fmoc removal

Couplings for 60 min were performed by *in situ* activation or pre-activating 0.6 M solutions of the coupling cocktail [Fmoc-AA-OH : OxymaPure : DIC (1 : 1 : 1, 3.0 eq.) in 0.5 mL of DMF] for 1 min, before addition to the tripeptidyl resin. In the case of 1.5 eq. of the reagent, the concentration of the coupling cocktail was 0.3 M at rt/45 °C. Fmoc was removed by treatment with neat PIP or 4-MP, *i.e.*, 0.12 mL of PIP or 4-MP was added without filtering the coupling reaction mixture and washing the peptidyl resin (*in situ* Fmoc removal) for 1 × 7 min or 10 or 20 min at rt/45 °C. After Fmoc removal, the resin was washed with DMF, 1% OxymaPure, or both.

SPPS of Leu-Enkephalin pentapeptide (H-Tyr-Gly-Gly-Phe-Leu-NH_2)

Previous standard SPPS protocol for comparison. SPPS using the Fmoc/*t*Bu methodology was carried out manually with plastic syringes fitted with polyethylene porous discs. The Fmoc-Rink-amide AM resin (0.64 mmol g^{-1}) was washed with DMF (2 × 5 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% PIP in DMF (1 × 1 min and 1 × 7 min) followed by washing with DMF. The protected Fmoc-amino acids (1.5 eq.) and OxymaPure (1.5 eq.) in 0.5 mL of DMF (0.6 M) were preactivated for 1 min with DIC (1.5 eq.) before being added to the resin. The reaction was continued for 1 h at rt. Deprotection and coupling were repeated until the pentapeptides were achieved. Washings after couplings with DMF (2 × 3 mL) and after deprotection (3 × 3 mL) were performed. The peptide was cleaved from the resin by treatment with TFA/TIS/H_2O (95 : 2.5 : 2.5) for 1 h at rt. The residue was precipitated with chilled diethyl ether and centrifuged to afford the desired peptide which was analyzed by HPLC and LCMS.

Newly adopted SPPS protocol (*in situ* Fmoc removal). SPPS was carried out manually with plastic syringes fitted with polyethylene porous discs using the Fmoc/*t*Bu methodology. The Fmoc-Rink-amide AM resin (0.64 mmol g^{-1}) was washed with DMF (2 × 1 mL for 5 min). The Fmoc group was deprotected by treatment of the resin with 20% 4-MP/DMF (1 × 7 min) followed by washing with 1% OxymaPure/DMF (2 × 1 mL). The protected Fmoc-amino acids (1.5 eq.) and OxymaPure (1.5 eq.) in 0.5 mL DMF (0.3 M) were preactivated for 1 min with DIC (1.5 eq.) as a coupling system before addition to the resin, for 1 h at rt. After 1 h of coupling, 0.12 mL of neat 4-MP was added for 1 × 7 min at rt without removing the coupling reaction mixture and washing. After 7 min, the reaction mixture was filtered and the peptidyl resin was washed with 1% OxymaPure/DMF (2 × 1 mL). Coupling and deprotection were repeated until the peptide was assembled onto the resin. All the peptides were dried and cleaved from the resin by treatment with TFA/TIS/H_2O (95 : 2.5 : 2.5) for 1 h at rt. The cleavage mixture was precipitated with chilled diethyl ether, centrifuged to afford the desired peptide, and confirmed by HPLC and LCMS analyses.

Conclusions

By this *in situ* Fmoc removal SPPS strategy, the typical sequence of coupling → washing → deprotection → washing can be efficiently converted to coupling → deprotection → washing. Briefly, this new approach involves adding PIP or 4-MP up to a concentration of 20% to the coupling cocktail. After 20 min, the resin is filtered off and washed twice with 1% OxymaPure-DMF. PIP or 4-MP removes efficiently the Fmoc group and scavenges the active species of the Fmoc-amino acid avoiding two potential side-reactions: deletion peptides (incomplete Fmoc removal) and peptides with double hits (over reaction). Several experiments were carried out by adding 3-methyl-1-butane thiol or benzyl amine as the additional quencher of the OxymaPure ester, but the results did not show any apparent improvements (results not shown).[25]

This *in situ* Fmoc removal strategy implies a solvent saving of around 75% (two PIP/4-MP solutions, four washing steps after the coupling and two after the deprotection), which implies a 4 times reduction of the PMI and E-factor. If required, a third washing using 1% OxymaPure-DMF can be introduced. However, even in this case, there is a considerable saving of the solvent. The addition of OxymaPure to the PIP solution has also been used to prevent the formation of aspartimide and δ-lactam and other side reactions.[31,32] It is important to highlight that washing with 1% OxymaPure does not pose any risk in the formation of HCN, as it has been previously described when in contact with DIC.[22f,33] Provided the given washing conditions, at no time will OxymaPure (1%) be in contact with DIC, because this would have already been consumed by reaction with the incoming Fmoc-amino acid and/or with the OxymaPure present during coupling. Nevertheless, as OxymaPure is used just as an acid rectifier, other similar compounds such as HOBt, Cl-HOBt, and HOPO can be used for the same purpose.

A preliminary study was conducted in the green solvent *N*-butylpyrrolidinone (NBP) at 45 °C (due to the high viscosity of NBP).[17] Similar results to those of DMF (Fig. 6) were obtained (HPLC in the ESI† HPLC-S47).

It is envisaged that modifications of this new protocol will find their way into the green toolbox for SPPS, thereby making this strategy more sustainable.

Author contributions

A. K. performed the experiments. A. K. and A. S. analysed the results of the experiments, and prepared the first draft. B. G. T. and F. A. conceived and designed the manuscript. The four authors contributed to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

1 M. Muttenthaler, G. F. King, D. J. Adams and P. F. Alewood, *Nat. Rev. Drug Discovery*, 2021, **20**, 309.
2 R. B. Merrifield, *J. Am. Chem. Soc.*, 1963, **85**, 2149.

3 T. Bruckdorfer, O. Marder and F. Albericio, *Curr. Pharm. Biotechnol.*, 2004, **5**, 29.

4 A. A. Zompra, A. S. Galanis, O. Werbitzky and F. Albericio, *Future Med. Chem.*, 2009, **1**, 361.

5 D. Al Shaer, O. Al Musaimi, F. Albericio and B. G. de la Torre, *Pharmaceuticals*, 2022, **15**, 222.

6 Y. E. Jad, A. Kumar, A. El-Faham, B. G. de la Torre and F. Albericio, *ACS Sustainable Chem. Eng.*, 2019, **7**, 3671.

7 O. Al Musaimi, B. G. de la Torre and F. Albericio, *Green Chem.*, 2020, **22**, 996.

8 L. Ferrazzano, M. Catani, A. Cavazzini, G. Martelli, D. Corbisiero, P. Cantelmi, T. Fantoni, A. Mattellone, C. De Luca, S. Felletti, W. Cabri and A. Tolomelli, *Green Chem.*, 2022, **24**, 975.

9 P. Anastas and N. Eghbali, *Chem. Soc. Rev.*, 2010, **39**, 301.

10 (*a*) A. Isidro-Llobet, M. N. Kenworthy, S. Mukherjee, M. E. Kopach, K. Wegner, F. Gallou, A. G. Smith and F. Roschangar, *J. Org. Chem.*, 2019, **84**, 4615; (*b*) M. C. Bryan, P. J. Dunn, D. Entwistle, F. Gallou, S. G. Koenig, J. D. Hayler, M. R. Hickey, S. Hughes, M. E. Kopach, G. Moine, P. Richardson, F. Roschangar, A. Steven and F. J. Weiberth, *Green Chem.*, 2018, **20**, 5082.

11 (*a*) F. P. Byrne, S. Jin, G. Paggiola, T. H. M. Petchey, J. H. Clark, T. J. Farmer, A. J. Hunt, C. R. McElroy and J. Sherwood, *Sustainable Chem. Processes*, 2016, **4**, 7; (*b*) J. Sherwood, H. L. Parker, K. Moonen, T. J. Farmer and A. J. Hunt, *Green Chem.*, 2016, **18**, 3990.

12 (*a*) R. K. Henderson, C. Jimenez-Gonzalez, D. J. C. Constable, S. R. Alston, G. G. A. Inglis, G. Fisher, J. Sherwood, S. P. Binksa and A. D. Curzons, *Green Chem.*, 2011, **13**, 854; (*b*) C. M. Alder, D. Hayler, R. K. Henderson, A. M. Redman, L. Shukla, L. E. Shuster and H. F. Sneddon, *Green Chem.*, 2016, **18**, 3879.

13 (*a*) K. Hojo, M. Maeda and K. Kawasaki, *J. Pept. Sci.*, 2001, **7**, 615; (*b*) K. Hojo, M. Maeda and K. Kawasaki, *Tetrahedron*, 2001, **60**, 1875; (*c*) K. Hojo, M. Maeda and K. Kawasaki, *Tetrahedron Lett.*, 2004, **45**, 9293; (*d*) K. Hojo, H. Ichikawa, M. Maeda, S. Kida, Y. Fukumori and K. Kawasaki, *J. Pept. Sci.*, 2007, **13**, 493; (*e*) K. Hojo, H. Ichikawa, Y. Fukumori and K. Kawasaki, *Int. J. Pept. Res. Ther.*, 2008, **14**, 373; (*f*) K. Hojo, H. Hara, H. Kitai, M. Onishi, H. Ichikawa, Y. Fukumori and K. Kawasaki, *Chem. Cent. J.*, 2011, **4**, 49; (*g*) K. Hojo, M. Onishi, H. Ichikawa, Y. Fukumori and K. Kawasaki, *J. Pept. Sci.*, 2011, **17**, 487; (*h*) K. Hojo, H. Ichikawa, H. Hara, M. Onishi, K. Kawasaki and Y. Fukumori, *Protein Pept. Lett.*, 2012, **19**, 1231; (*i*) K. Hojo, N. Shinozai, Y. Nozawa, Y. Fukumori and H. Ichikawa, *Appl. Sci.*, 2013, **3**, 614; (*j*) K. Hojo, S. Fujiwara, H. Inai, Y. Manabe and Y. Tsuda, *Int. J. Pept. Res. Ther.*, 2022, **28**, 51.

14 (*a*) M. Cortes-Clerget, J. Y. Berthon, I. Krolikiewicz-Renimel, L. Chaisemartin and B. H. Lipshutz, *Green Chem.*, 2017, **19**, 4263; (*b*) M. Cortes-Clerget, N. R. Lee and B. H. Lipshutz, *Nat. Protoc.*, 2019, **14**, 1108.

15 A. S. Galanis, F. Albericio and M. Grøtli, *Org. Lett.*, 2009, **11**, 4488.

16 K. Wegner, K. Manzor, D. Barnes, A. Jardine and D. Moran, *Green Chem. Lett. Rev.*, 2021, **14**, 153.

17 (*a*) J. Lopez, S. Pletscher, A. Aemissegger, C. Bucher and F. Gallou, *Org. Process Res. Dev.*, 2018, **22**, 494; (*b*) B. G. de la Torre, A. Kumar, M. Alhassan, C. Bucher, F. Albericio and J. Lopez, *Green Chem.*, 2020, **22**, 3162.

18 (*a*) V. Martin, S. Jadhav, P. H. G. Egelund, R. Liffert, H. Johansson Castro, T. Krüger, K. F. Haselmann, S. Thordal Le Quement, F. Albericio, F. Dettner, C. Lechner, R. Schönleber and D. S. Pedersen, *Green Chem.*, 2021, **23**, 3295; (*b*) S. Jadhav, V. Martin, P. H. G. Egelund, H. Johansson Castro, T. Krüger, F. Richner, S. Thordal Le Quement, F. Albericio, F. Dettner, C. Lechner, R. Schönleber and D. S. Pedersen, *Green Chem.*, 2021, **23**, 3312.

19 S. Knauer, N. Koch, C. Uth, R. Meusinger, O. Avrutina and H. Kolmar, *Angew. Chem., Int. Ed.*, 2020, **59**, 12984.

20 (*a*) L. Ferrazzano, D. Corbisiero, G. Martelli, A. Tolomelli, A. Viola, A. Ricci and W. Cabri, *ACS Sustainable Chem. Eng.*, 2019, **7**, 12867; (*b*) L. Ferrazzano, G. Martelli, T. Fantoni, A. Daka, D. Corbisiero, A. Viola, A. Ricci, W. Cabri and A. Tolomelli, *Org. Lett.*, 2020, **22**, 3969; (*c*) G. Martelli, P. Cantelmi, A. Tolomelli, D. Corbisiero, A. Mattellone, A. Ricci, T. Fantoni, W. Cabri, F. Vacondio, F. Ferlenghi, M. Mor and L. Ferrazzano, *Green Chem.*, 2021, **23**, 4095; (*d*) G. Martelli, P. Cantelmi, C. Palladino, A. Mattellone, D. Corbisiero, T. Fantoni, A. Tolomelli, M. Macis, A. Ricci, W. Cabri and L. Ferrazzano, *Green Chem.*, 2021, **23**, 8096.

21 (*a*) S. Lawrnson, M. North, F. Peigneguy and A. Routledge, *Green Chem.*, 2017, **19**, 952; (*b*) S. B. Lawrnson, R. Arav and M. North, *Green Chem.*, 2017, **19**, 1685; (*c*) Y. Ran, F. Byrne, I. D. V. Ingram and M. North, *Chem. – Eur. J.*, 2019, **25**, 4951; (*d*) C. Amadi-Kamalu, H. Clarke, M. McRobie, J. Mortimer, M. North, Y. Ran, A. Routledge, D. Sibbald, M. Tickias, K. Tse and H. Willway, *ChemistryOpen*, 2020, **9**, 431.

22 (*a*) J. Pawlas, T. Nuijens, J. Persson, T. Svensson, M. Schmidt, A. Toplak, M. Nilsson and J. H. Rasmussen, *Green Chem.*, 2019, **21**, 6451; (*b*) J. Pawlas, B. Antonic, M. Lundqvist, T. Svensson, J. Finnman and J. H. Rasmussen, *Green Chem.*, 2019, **21**, 2594; (*c*) J. Pawlas, T. Svensson and J. H. Rasmussen, *RSC Adv.*, 2019, **9**, 38928; (*d*) J. Pawlas and J. H. Rasmussen, *Green Chem.*, 2019, **21**, 5990; (*e*) J. Pawlas and J. H. Rasmussen, *ChemSusChem*, 2021, **14**, 3231; (*f*) M. Erny, M. Lundqvist, J. H. Rasmussen, O. Ludemann-Hombourger, F. Bihel and J. Pawlas, *Org. Process Res. Dev.*, 2020, **24**, 1341.

23 (*a*) Y. E. Jad, G. A. Acosta, S. N. Khattab, B. G. de la Torre, T. Govender, H. G. Kruger, A. El-Faham and F. Albericio, *Org. Biomol. Chem.*, 2015, **13**, 2393; (*b*) Y. E. Jad, G. A. Acosta, S. N. Khattab, B. G. de la Torre, T. Govender, H. G. Kruger, A. El-Faham and F. Albericio, *Amino Acids*, 2016, **48**, 419; (*c*) Y. E. Jad, G. A. Acosta, T. Govender, H. G. Kruger, A. El-Faham, B. G. de la Torre and F. Albericio, *ACS Sustainable Chem. Eng.*, 2016, **4**, 6809; (*d*) A. Kumar, Y. E. Jad, A. El-Faham, B. G. de la Torre and

F. Albericio, *Tetrahedron Lett.*, 2017, **58**, 2986; (*e*) A. Kumar, Y. E. Jad, J. M. Collins, F. Albericio and B. G. de la Torre, *ACS Sustainable. Chem. Eng.*, 2018, **6**, 8034; (*f*) A. Kumar, A. Thompson-Adewumi, K. P. Nandhini, J. M. Collins, F. Albericio and B. G. de la Torre, *Org. Process Res. Dev.*, 2019, **23**, 1096; (*g*) A. Kumar, M. Alhassan, J. Lopez, F. Albericio and B. G. de la Torre, *ChemSusChem*, 2020, **13**, 5288; (*h*) A. Kumar, A. Sharma, B. G. de la Torre and F. Albericio, *Green Chem. Lett. Rev.*, 2021, **14**, 545.

24 R. Subirós-Funosas, R. Prohens, R. Barbas, A. El-Faham and F. Albericio, *Chem. – Eur. J.*, 2009, **15**, 9394.

25 (*a*) T. Daisuke, I. Tatsuji and F. Tatsuya, *Angew. Chem.*, 2017, **56**, 7803; (*b*) S. K. Singh and J. M. Collins, *Methods Mol. Bio.*, 2020, **2103**, 95.

26 A. Kumar, A. Sharma, B. G. de la Torre and F. Albericio, *Molecules*, 2019, **24**, 4004.

27 O. F. Luna, J. Gomez, C. Cárdenas, F. Albericio, S. H. Marshall and F. Guzmán, *Molecules*, 2016, **21**, 1542.

28 A. Přibylka, M. Pastorek, M. Grepl and E. P. Schütznerová, *Tetrahedron*, 2021, **99**, 132452.

29 R. A. Sheldon, *ACS Sustainable Chem. Eng.*, 2018, **6**, 32–48.

30 E. R. Monteith, P. Mampuys, L. Summerton, J. H. Clark, B. U. W. Maes and C. R. McElroy, *Green Chem.*, 2020, **22**, 123–135.

31 A. Pryyma, S. Gunasekera, J. Lewin and D. M. Perrin, *Bioconjugate Chem.*, 2020, **31**, 2685.

32 R. Subirós-Funosas, A. El-Faham and F. Albericio, *Tetrahedron*, 2011, **67**, 8595.

33 (*a*) A. D. McFarland, J. Y. Buser, M. C. Embry, C. B. Held and S. P. Kolis, *Org. Process Res. Dev.*, 2019, **23**, 2099; (*b*) S. R. Manne, O. Luna, G. A. Acosta, M. Royo, A. El-Faham, G. Orosz, B. G. de la Torre and F. Albericio, *Org. Lett.*, 2021, **23**, 6900; (*c*) S. R. Manne, A. El-Faham, B. G. de la Torre and F. Albericio, *Tetrahedron Lett.*, 2021, **85**, 153462.

