International Journal of Peptide Research and Therapeutics (2025) 31:43
https://doi.org/10.1007/s10989-025-10703-4

Scalable and Sustainable DMF-Free Solid-Phase Synthesis of Liraglutide by 1-Tert-Butyl-3-Ethylcarbodiimide-Mediated Couplings and Catch-and-Release Acylation and Purification Strategies

Lorenzo Pacini[1,2] · Manoj Kumar Muthyala[3] · Robert Zitterbart[3] · Oleg Marder[4] · Paolo Rovero[2,5] · Anna Maria Papini[1,2]

Accepted: 10 February 2025

Abstract

Introduction The growing need for sustainable practices in pharmaceutical manufacturing has stimulated advancements in peptide synthesis. This study focuses on applying green chemistry principles to the synthesis of the Glucagon-Like Peptide-1 analog liraglutide.

Material and Methods The safer coupling reagent 1-tert-butyl-3-ethylcarbodiimide (TBEC) was tested in combination with eco-friendly binary solvents such as dimethyl sulfoxide and butyl acetate to propose novel and sustainable solid-phase synthetic and purification strategies of liraglutide.

Results Two synthetic strategies were developed for liraglutide production. The first strategy was based on a "direct synthesis", incorporating a lipidated lysine building block into the peptide sequence, achieving 86% HPLC purity after catch-and-release purification. The second strategy based on "catch-lipidation-and-release" approach, allowed to obtain the peptide precursor without the lipid moiety, which was later linked during a controlled lipidation step. This latter strategy yielded purities exceeding 90% and reduced reliance on preparative HPLC. TBEC minimizes hazardous byproducts, such as hydrogen cyanide, and enhances solvent compatibility, achieving crude purities and yields comparable to conventional syntheses.

Conclusion This work underscores the potential of green chemistry to align pharmaceutical innovation with environmental responsibility. In particular our findings highlight the effectiveness of TBEC and green solvent systems optimizing scalable and sustainable SPPS processes and improving resource efficiency. Thus, we propose a viable pathway to produce the therapeutic peptide ingredient liraglutide significantly reducing the environmental impact while maintaining high efficiency and quality of the synthesis.

Keywords Solid-phase peptide synthesis · GLP-1 agonist · Catch-and-release purification · Green binary solvent mixtures · TBEC coupling reagent

✉ Anna Maria Papini
annamaria.papini@unifi.it

1 Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino, Italy

2 Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, Peptlab, MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy

3 Gyros Protein Technologies Inc., 4675 South Coach Drive, Tucson, AZ 85714, USA

4 Luxembourg Bio Technologies, 8 Sapir Pinhas St., 7403631 Nes Ziona, Israel

5 Department of Neurosciences, Psychology, Drug Research and Child Health, Section of Pharmaceutical Sciences and Nutraceutics, University of Florence, 50019 Sesto Fiorentino, Italy

Introduction

The rising focus on sustainable practices in chemical synthesis has led to significant advancements in green chemistry, especially within pharmaceutical production, where reducing hazardous waste and minimizing environmental impact are critical objectives (Kar et al. 2022). Peptide-based therapeutics, increasingly vital in treating a wide array of conditions, from metabolic disorders to autoimmune diseases (Selmi et al. 2011; Sharma et al. 2023), are one area where environmentally friendly approaches are especially valuable (Ferrazzano et al. 2022). However, solid-phase peptide synthesis (SPPS), a key method for producing peptides, has traditionally relied on toxic solvents, high reagent

Published online: 22 February 2025

consumption, and other practices that contribute to environmental and safety concerns. Integrating green chemistry principles into SPPS is therefore essential to develop more sustainable, less hazardous methodologies for peptide production (Martin et al. 2020).

One promising strategy for green SPPS involves optimized coupling reagents for amide bond formation (Yang et al. 2023). Among these, *N*,*N*′-diisopropylcarbodiimide (DIC) is commonly employed but is known for its associated safety risks, including toxicity, side reactions, and undesired by-products (Pawlas and Rasmussen 2023). 1-tert-Butyl-3-ethylcarbodiimide (TBEC), a novel and safer coupling reagent, offers a compelling alternative (Pawlas et al. 2023). TBEC reduces the health hazards and environmental impact typically associated with traditional reagents, while maintaining high efficiency and low epimerization rates in peptide synthesis (Fantoni et al. 2024). Importantly, compatibility of TBEC with green binary solvent mixtures, such as combinations of dimethyl sulfoxide (DMSO) and tert-butyl acetate (BuOAc), further supports its use in sustainable SPPS protocols (Ferrazzano et al. 2019; Al Musaimi et al. 2020; Wegner et al. 2021; Pacini et al. 2024). These green solvents provide a more environmentally friendly medium for synthesis compared to traditional toxic solvents such as dimethylformamide (DMF) or dichloromethane (DCM), making TBEC and green solvent systems an ideal pairing for advancing green peptide chemistry. Last but not least, while DIC reacts with OxymaPure® at a 1:1 ratio, yielding the byproduct oxadiazole and releasing HCN, TBEC reacts with OxymaPure® under the same conditions, forming oxadiazine only, what makes TBEC a more health and safety friendly reagent (Manne et al. 2021, 2022).

The development and application of greener SPPS methodologies are especially relevant in production of Glucagon-Like Peptide-1 (GLP-1) agonists, which are prominent in treating diabetes and obesity (Staby et al. 2020; Liu et al. 2020; Hach et al. 2024). GLP-1-based drugs have revolutionized the therapeutic landscape in metabolic health, with agonists like liraglutide achieving blockbuster status due to their efficacy, long lasting action, and favorable safety profiles. However, as demand for these peptides rises, the need for scalable, sustainable synthetic routes that align with environmental responsibility goals is dramatically increasing. Designing green synthetic strategies for GLP-1 agonists would meet this market demand while reducing the ecological footprint of their production (Barredo-Vacchelli et al. 2024). Herein, two synthetic strategies, involving the use of PurePep EasyClean (PEC) catch & release technology, are described (Zitterbart et al. 2021). In both cases, we successfully achieved levels of liraglutide purity that makes further purification through chromatography much easier.

In summary, this work highlights the importance of TBEC as a safer, more sustainable coupling reagent, its compatibility with green solvent systems within the production of liraglutide, and the broader role of green SPPS approaches in producing key therapeutic peptide ingredients. Additionally, it has been demonstrated that the use of heating together with TBEC/Oxyma Pure accelerates SPPS and further enhances reaction rates, contributing to the efficiency of green peptide synthesis. By promoting eco-friendly methods for the synthesis of GLP-1 agonists, decreasing the need of time-and-solvent-consuming preparative HPLC, we can meet the growing demand for these drugs while setting a new standard for environmental responsibility in pharmaceutical manufacturing.

Materials and Methods

Fmoc-amino acids, Oxyma Pure and Fmoc-Gly-2-Chlorotrityl resin were provided directly by Gyros Protein Technologies (Tucson, AZ, USA). *N*,*N*′-Diisopropylcarbodiimide was purchased from Carbosolution Chemicals GmbH (St. Ingbert, Germany). Dimethylformamide (peptide grade: amine content < 0.0003% and water content < 0.01%), ethyl acetate, propyl acetate, butyl acetate (synthesis grade acetates: water content < 0.1%), dimethyl sulfoxide (synthesis grade: water content < 0.5%), acetic anhydride, pyridine, anisole (synthesis grade: water content < 0.1%), and triisopropylsilane were obtained from Carl Roth GmbH (Karlsruhe, Germany). Piperidine, trifluoroacetic acid, and Rink amide AM resin (0.64 mmol/g), Fmoc-Lys(Palm-Glu-OtBu)-OH, Palm-L-Glu(OSu)-OtBu were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Fmoc-Gly-Tentagel S resins (0.17 mmol/g) was acquired from Rapp Polymere (Tuebingen, Germany). 1-tert-Butyl-3-ethylcarbodiimide (TBEC) was provided by Luxembourg Bio Technologies (Ness Ziona, Israel).

Solubility Tests

After calibrating a 15 mL plastic tube, 1 mmol of each coupling reagent was weighed, and 1 mL of the tested solvent was added. The mixture was stirred at room temperature for 30 min. If the reagent remained insoluble, the solvent volume was gradually increased to check solubility at concentrations of 0.5, 0.25, and 0.1 M.

Solid-Phase Peptide Synthesis

All syntheses were performed using the PurePep® Chorus (Tucson, AZ, USA) peptide synthesizer in parallel synthesis mode, with induction heating and at room temperature. For 100 µmol scale syntheses, 303 mg of Fmoc-Gly-2-Chlorotrityl resin (0.33 mmol/g loading), 417 mg of Fmoc-Gly-Tentagel S resin (0.24 mmol/g loading), and 588 mg of

Tentagel S RAM resin (0.17 mmol/g loading) were weighed. Fmoc-amino acids were dissolved in the tested mixture or in DMF using an ultrasonic bath at 0.3 M concentration. DIC or TBEC and Oxyma Pure were dissolved in the tested mixture or in DMF at 1 M concentration. The final Fmoc-amino acids concentration, after the additions of coupling reagents, is 0.19 M. The solvents were freshly prepared and transferred to the appropriate bottles corresponding to separate lines on the machine. After synthesis, the loaded resins were washed with isopropyl alcohol and dried under vacuum. Before starting each synthesis with different solvents, the instrument was fully calibrated to ensure that the correct volume of solvent was delivered.

Test Cleavage

A small amount (2–4 mg) of dry loaded resin was transferred into a 500 µL plastic tube, and 100 µL TFA, 5 µL TIS, and 5 µL H_2O were added. After 1.5 h of mechanical mixing, the suspension was filtered and cold diethyl ether (− 20 °C) was added to the peptide TFA solution. The precipitated peptide was isolated by centrifugation and dried under vacuum. The crude peptides were dissolved in water/ACN 1:1 and analyzed by RP-UPLC coupled with ESI–MS.

PurePep EasyClean (PEC) Lipidation

The first step of this process is the coupling of the reductively, traceless cleavable linker PEC-RC+, TFA cleavage resistant, that allows further attachment on activated agarose beads, on the N-terminal amino function of the to-be-purified peptide still on resin that was conducted at room temperature for 18 h under mechanical shaking using 4 equivalents of the linker, 6 equivalents of Oxyma pure, and 6 equivalents of DiPEA in DMSO/EtOAc 1:9 (v/v) mixture. After monitoring with LC–MS, the linker-precursor peptide was cleaved from the resin, and all protecting groups on the amino acid side chains were removed. The linker-peptide was solubilized in TFA/H_2O 9:1 (v/v) and cooled at 0 °C. Pyridine was added dropwise, the solution was diluted with ACN and added to polymethacrylate (PMA) activated beads. In the case of activated Agarose beads, the linker-peptide was solubilized with DMSO and added directly on the beads. The mixture was shaken for 5 h at room temperature, and, after immobilization, the beads were washed with 3 × H_2O. Unreacted aldehyde functions were blocked on beads using a solution of 1% (w/v) NH_2OMe in 0.1 M aqueous citric acid buffer (pH 4.5) for 5 min. The loaded beads (agarose or PMA) were washed with DMSO/butyl acetate 3:7 (v/v). A solution of 4 equivalents of Palm-L-Glu(OSu)-OtBu, 4 equivalents of coupling reagent (HOAt or Oxyma Pure) and 6 equivalents of base (DiPEA or Pyridine) were added to the loaded beads after 5 min of pre-activation. The formation of the new amide bond occurred in 5 h.

PurePep EasyClean (PEC) Purification

After immobilization (and acylation, in the case of the second strategy), the beads were washed with 3 × 0.9 M guanidinium chloride (GdmCl) in DMSO and with 3 × 0.1 M NaCI in H_2O/EtOH 3:7 (v/v). The linker on the peptide was reduced using a solution of 0.3 M dithiothreitol (DTT) in 0.6 M aqueous $NaHCO_3$ (pH 8) for 15 min at room temperature. Then, the beads were washed with H_2O and ACN. The final release consisted in treatment with TFA/H_2O 95:5 (v/v) for 45 min at room temperature for PMA beads and TFA/H_2O 40:60 (v/v) for 1 h at room temperature followed by precipitation in cold diethyl ether and lyophilization. (Scheme S1).

Results and Discussion

Green Solvents Binary Mixtures and TBEC

The first part of our study involved the test of a safer coupling reagent such as TBEC in combination with binary mixtures previously studied. TBEC is a non-symmetric carbodiimide as showed in Fig. 1 (Manne et al. 2022). In the very enlightening work from Pawlas and Rasmussen (2023), it was demonstrated that the coupling system TBEC/Oxyma Pure, during the formation of a new amide bond, releases a marked lower quantity of HCN deriving from Oxyma Pure in comparison with the standard coupling system DIC/Oxyma Pure even if the performances, in terms of coupling reaction rate are essentially the same. Another important feature is that the TBEC derived urea, 1-tert-butyl-3-ethyl urea (TBEU), is more soluble in DMF and in many other solvent systems. Despite the higher cost, TBEC can be considered superior for its safety profile.

We investigated the coupling system TBEC/Oxyma Pure to synthesize, at 100 µmol scale and at 90 °C, three model peptides using the green binary mixture butyl acetate/DMSO, propyl acetate/DMSO or anisole/DMSO for coupling and Fmoc removal reactions, and ethyl acetate/DMSO for washings.

The three model peptides are ACP(65–74)-NH_2 (H-VQAAIDYING-NH_2), a peptide commonly employed to assess the efficiency of synthetic protocols, polyALA (H-AAAAAAAAAK-NH_2) a well-known self-assembling peptide even on resin, and AlaSTD (H-AKADEVSLHKWYG-NH_2) a

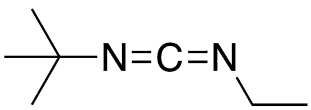


Fig. 1 Structure of TBEC

standard peptide used as a reference non-challenging sequence (Pacini et al. 2024).

The sequences were produced with the PurePep® Chorus automated synthesizer (Gyros Protein Technologies, Tucson, AZ, USA), employing mechanical shaking and nitrogen bubbling for mixing. The exact protocol, outlining volumes and durations, is provided in Tables S1 and S2.

The data reported in Table 1 show that the crude purities of the three peptide syntheses performed in green binary solvent mixtures and DMF are fairly comparable at 90 °C. On the other hand, as previously demonstrated, at r.t. DMF performances are better than BuOAc/DMSO (Figs. S1–S3) (Pacini et al. 2024). There is no single solvent mixture that consistently outperforms the others at 90 °C. For instance, the crude purity of ACP-NH_2 is higher when synthesized with DMF, but this trend does not hold for all sequences. The purity of PolyALA is higher when anisole/DMSO mixture was used, while GTPstd achieves better purity with a BuOAc/DMSO mixture. On average, the purities of the three sequences are comparable when using DMF and BuOAc/DMSO (Figs. S4–S15).

At this stage, we decided to investigate the use of TBEC in association with BuOAc/DMSO mixture also for the synthesis of the linear precursor of liraglutide at high temperature (90 °C) without double couplings and use of pseudo prolines. The synthesis yielded an over 70 A/A% purity and 93% crude yield (isolated mass/calculated mass %) demonstrating the full comparability with DIC/Oxyma Pure synthesis in BuOAc/DMSO mixture used for the second strategy (Fig. 2).

Green Synthesis Approaches for Liraglutide Production

In the second part of this work, we focused on the synthetic strategy of liraglutide, a blockbuster drug included in the class of GLP-1 agonists. This study presents two synthetic strategies that utilize green binary solvent

Table 1 Crude HPLC Purities of peptides synthesized using TBEC/Oxyma Pure in green solvents binary mixtures

	DMF Oxyma Pure TBEC 90 °C, 2 min	BuOAc DMSO Oxyma Pure TBEC 90 °C, 2 min	Anisole DMSO Oxyma Pure TBEC 90 °C, 2 min	PrOAc DMSO Oxyma Pure TBEC 90 °C, 2 min
Peptide	HPLC Purity (% A/A)	HPLC Purity (% A/A)	HPLC Purity (% A/A)	HPLC Purity (% A/A)
ACP-NH_2 VQAAIDYING-NH_2	89.1	85.4	78.6	76.8
PolyALA-NH_2 AAAAAAAK-NH_2	77.4	76.4	79.9	64.6
GTPstd-NH_2 * AKADEVSLHKWYG-NH_2	89.6	93.3	83.9	77.4

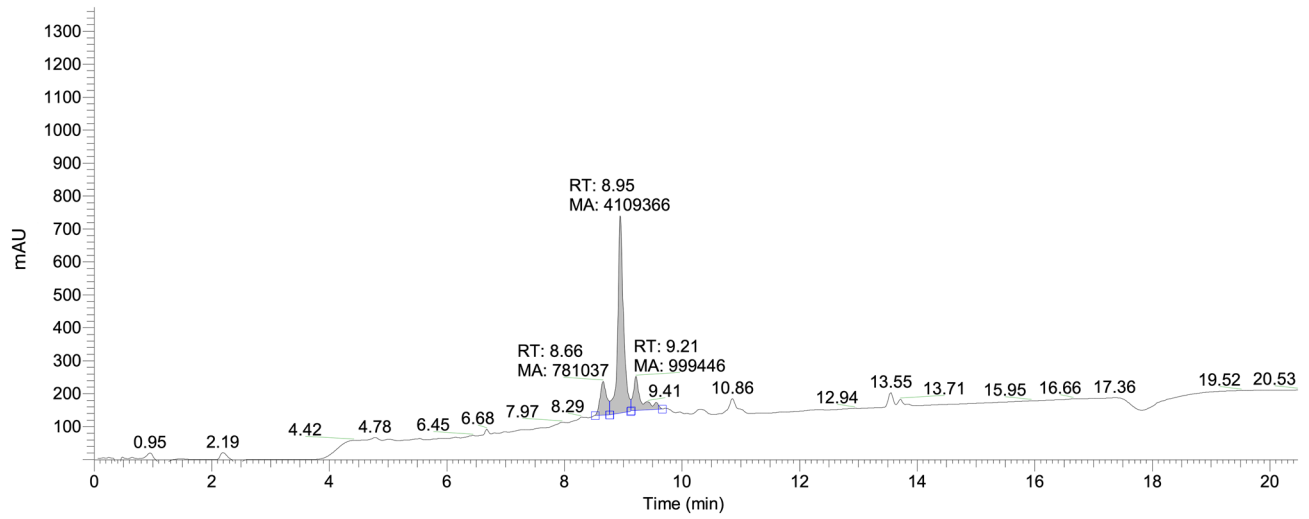
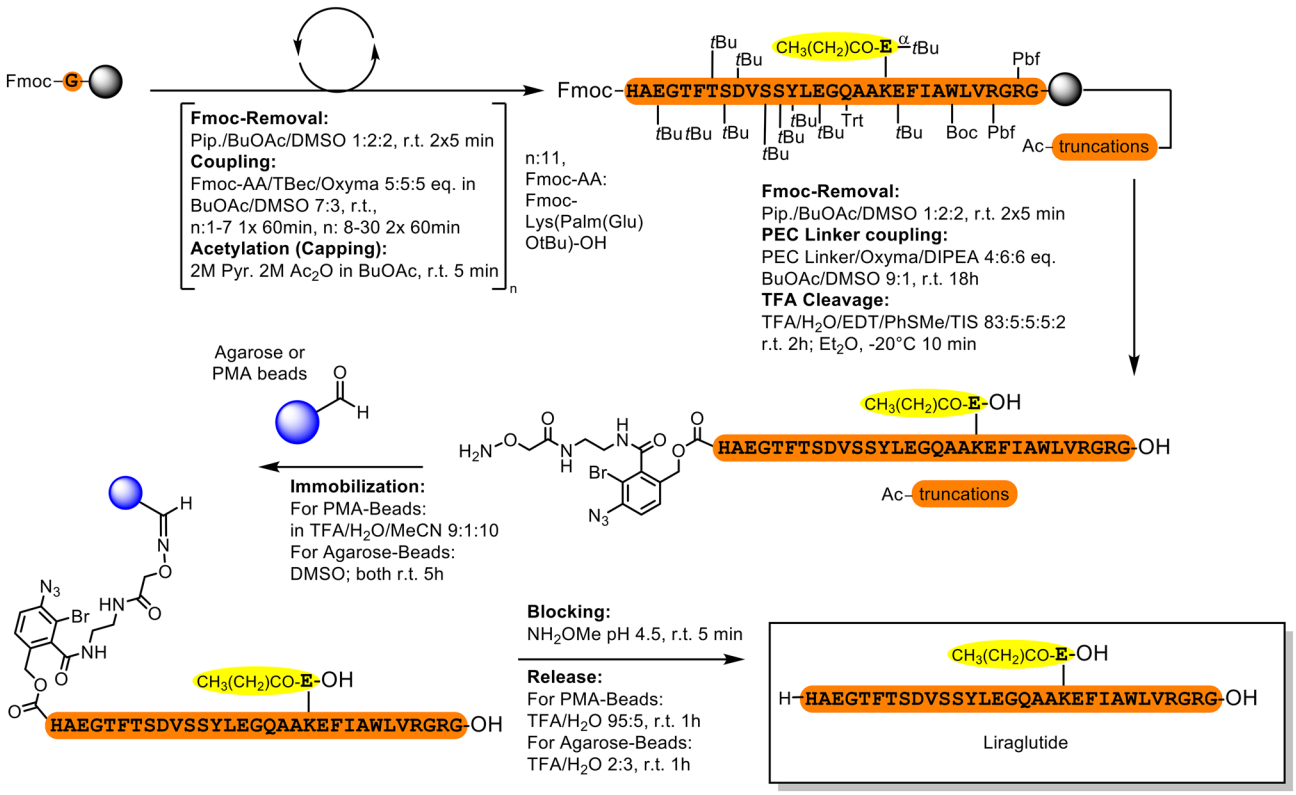


Fig. 2 RP-UHPLC traces of the precursor of liraglutide synthesized using BuOAc/DMSO and TBEC/Oxyma Pure as coupling system. C4 column Kromasil 300-5-C4 (300 Å, 5 µm, 4.6×150 mm); temperature 35 °C; flow, 1 mL/min; eluent, 0.1% (v/v) TFA in H_2O (**A**) and 0.1% (v/v) TFA in CH_3CN (**B**); λ, 215 nm, gradient, 5–95% B in 20 min. Rt = 8.95 min: precursor liraglutide 70.1% purity



Scheme 1. "Direct synthesis" of liraglutide

mixtures. The first strategy involves the synthesis of the complete liraglutide sequence, incorporating the building block Lys(Palm-Glu-OtBu) directly in the sequence via green SPPS followed by catch-and-release PEC purification as reported in Scheme 1. The catch-and-release technique represents a significant advancement toward greener peptide synthesis, as it reduces both time and solvent consumption in the most critical bottleneck of the process, i.e. purification (Fig. 3; Table S3).

In contrast, the second strategy involves the synthesis of the linear sequence of liraglutide without the lipid moiety attached to the lysine residue (Scheme 2). This approach also employs green binary solvent mixture for a green solid-phase peptide synthesis. Following the completion of the SPPS, cleavage from the resin and complete removal of side-chain protecting groups in the resulting peptide undergoes catch-and-release purification (PEC).

After the "catch" step, lipidation occurs directly on the agarose solid phase. This process involves the free primary amine on the side chain of the lysine residue, which is the only reactive amine present in the peptide. This strategy allows for precise control over the lipidation process, thereby optimizing the final yield and purity.

The "Direct Synthesis" Strategy

In this pathway, the entire peptide was synthesized using solid-phase peptide synthesis (SPPS) on a 100 µmol scale, utilizing the PurePep Chorus instrument by Gyros Protein Technology (Tucson, AZ, USA). In line with green chemistry principles applied to peptide synthesis, and based on our previous work (Pacini et al. 2024), we selected environmentally friendly binary solvent mixtures for the synthesis of liraglutide. Specifically, the EtOAc/DMSO mixture (8:2, v/v) was used as the primary solvent for washings. For amide bond formation and Fmoc deprotection, BuOAc/DMSO mixtures (ratios of 1:1 and 7:3, respectively) were employed due to their varying polarity.

Additionally, these mixtures were designed for use at elevated temperatures, as their boiling points exceed 100 °C, potentially enhancing yield, purity, and reducing synthesis time. However, for this study, all reactions were conducted at room temperature to facilitate scale-up on the PurePep Sonata + instrument by Gyros Protein Technology (Tucson, AZ, USA), which lacks a heating system.

For coupling, we used a system consisting of diisopropylcarbodiimide (DIC) and Oxyma Pure (5 equivalents

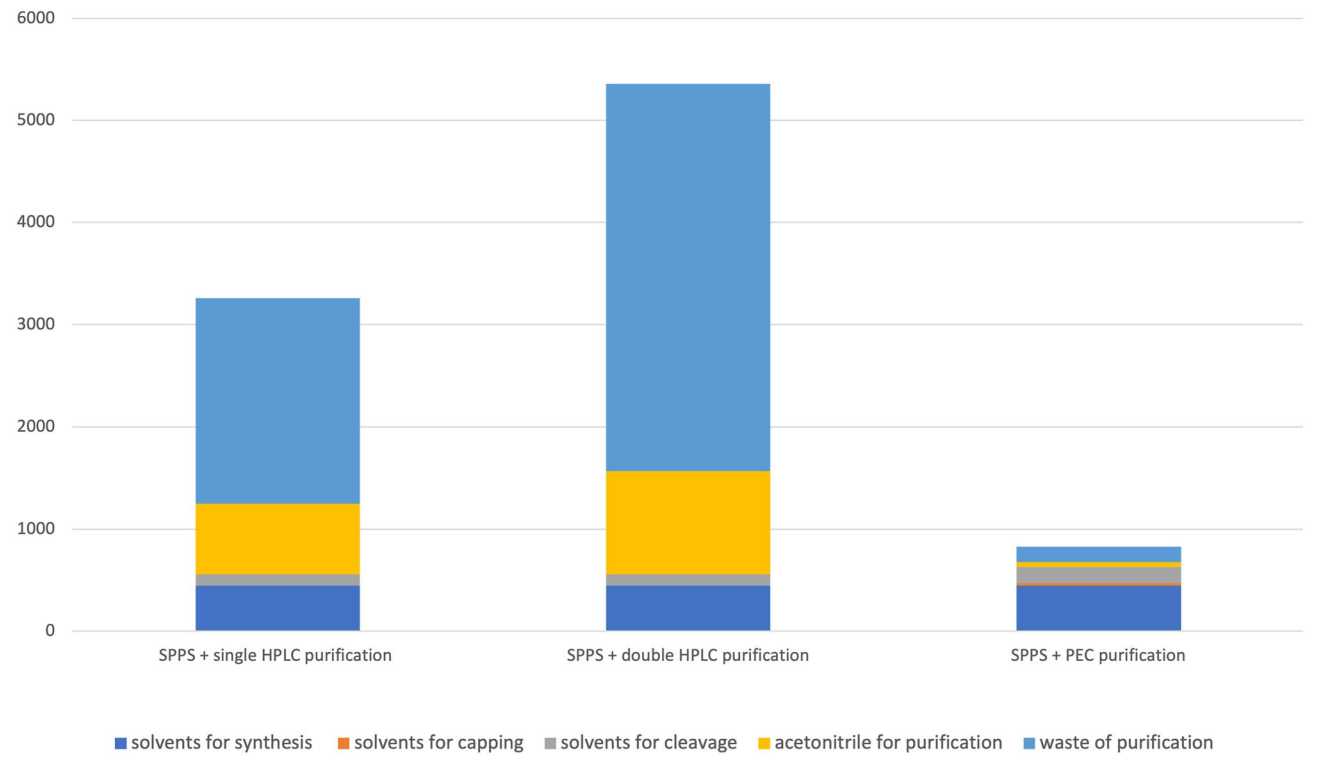


Fig. 3 Comparison of solvent consumption (mL)

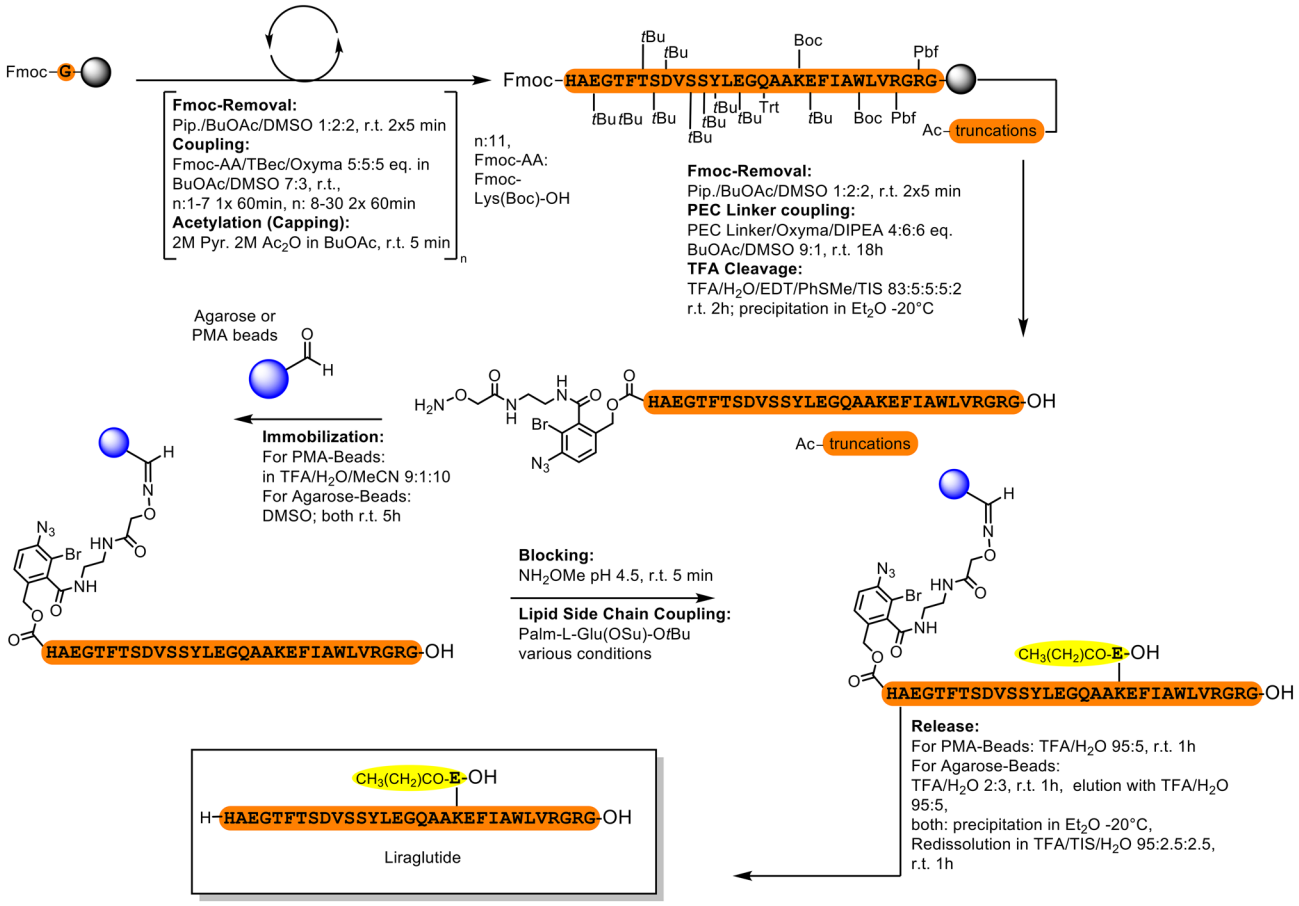
each), along with 5 equivalents of Fmoc-protected amino acids. The reaction was performed for one hour at room temperature, mixing by both nitrogen bubbling and mechanical shaking. Instead, the coupling reaction with the building block Fmoc-Lys(Palm-Glu-OtBu)-OH required 2 equivalents of the building block, along with 2 equivalents each of DIC and Oxyma Pure. From the 8th cycle onwards, the reaction was repeated twice to achieve a conversion rate as close as possible to 100%. Fmoc removal was performed in two 5-min treatments using the appropriate solution, except for the second amino acid in the sequence, where a shorter treatment (5 + 3 min) was applied to prevent the formation of diketopiperazines, which could significantly reduce the overall yield, as previously demonstrated for another GLP-1 agonist product (Wang et al. 2022). In order to monitor and prevent the formation of diketopiperazines (DKP), we simultaneously synthesized the sequences using two different types of resin functionalization, i.e., Wang and 2-chlorotrityl resin, expecting differences between the two resins due to the steric hindrance of the trityl group on trityl resin that should prevent the formation of diketopiperazine better than Wang resin. We were unable to detect any DKP during the Fmoc removal of the second amino acid on both types of resin. The rapid treatment (5 + 3 min) effectively deprotected the N-terminal of the second amino acid without resulting in the formation of DKPs (data not shown).

Another important reaction is acetylation after each coupling step, which ensures that only the N-terminal of the desired sequence remains free at the end of the synthesis.

After the synthesis was completed on the instrument, a test cleavage was performed, and the HPLC analysis showed that the purity of the peptide was 45.1% A/A (Fig. S16).

At this stage, the loaded resins were divided into two aliquots: one half (made of halves of 2-chlorotrityl resin and Wang resins combined in one batch) was cleaved and purified by preparative HPLC, while the others separately underwent PEC catch-and-release purification. For the latter, with only the N-terminal of the desired sequence accessible on the resin, the PEC linker was coupled using 4 equivalents of linker, 6 equivalents of Oxyma Pure, and 6 equivalents of DiPEA. This reaction proceeded in 16 h at room temperature in a green binary mixture of BuOAc and DMSO (7:3, v/v), with reaction progress monitored by the chloranil test. After the cleavage step, we evaluated the yield based on the isolated crude product mass. Both syntheses on the two types of resin yielded approximately 75% (isolated mass/calculated mass).

The two crudes deriving from the two resin types were combined in one batch and PEC purified obtaining a final product with an HPLC purity of 86.1 A/A% and a final yield of 24% (Fig. 4).



Scheme 2. "Catch-lipidation-and-release" strategy for the synthesis of liraglutide

The Catch-Lipidation-And-Release Strategy

The peptide was assembled on Fmoc-Gly- 2-Chlorotrityl resin using standard Fmoc-protected amino acids, following the same green mixtures described in the previous strategy. However, this time Fmoc-Lys(Boc)-OH was used instead of the more expensive building block Fmoc-Lys(Palm-Glu-OtBu)-OH. As a result, at the end of the SPPS, we obtained a liraglutide precursor without the fatty acid plus Glu as linker moiety on the peptide (HPLC crude purity 65.1 A/A% and yield 78%) (Fig. S17).

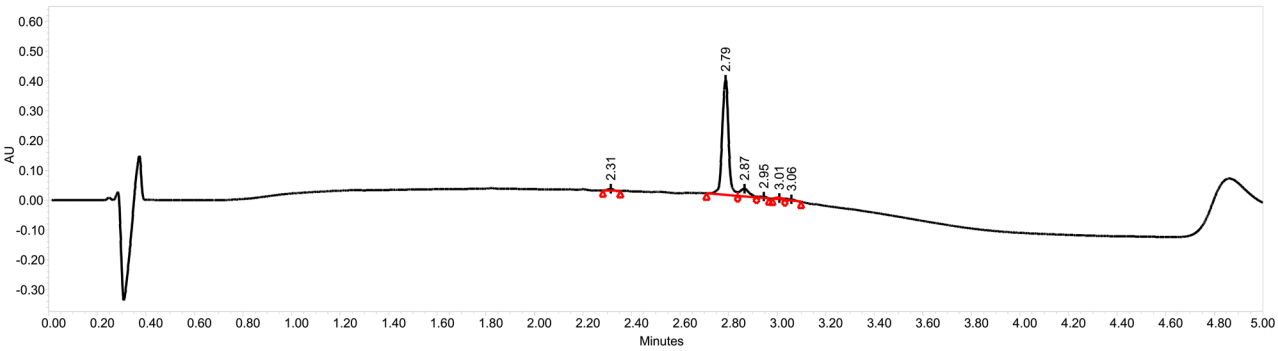


Fig. 4 RP-UHPLC traces of PEC purified liraglutide synthesized using BuOAc/DMSO, direct synthesis strategy. C18 column Waters Acquity BEH (130 Å, 1.7 µm, 2.1×50 mm); temperature 65 °C; flow, 0.5 mL/min; eluent, 0.1% (v/v) TFA in H_2O (**A**) and 0.1% (v/v) TFA in CH_3CN (**B**); λ, 215 nm, gradient, 10–90% B in 5 min. Rt = 2.79 min: liraglutide 86.05% purity

After PEC linker coupling and cleavage, we obtained a 53.8 A/A% HPLC pure crude (yield 71%, Fig. S18). At this stage, we immobilized 5 µmol of PEC-linker-liraglutide precursor on both agarose and PMA aldehyde-functionalized beads for acylation as a proof of concept. Following immobilization, the first acylation attempt used 4 equivalents of the building block Palm-L-Glu(OSu)-OtBu along with 4 equivalents of Oxyma Pure, dissolved in DMF. After 5 min of preactivation at room temperature, the solution was added to the peptide-loaded beads (agarose and PMA). We hypothesized that the primary amine on lysine would be more reactive than histidine and more prone to nucleophilic attack on the OSu-activated carboxylic group of the building block.

The reaction was monitored after 2 and 4 h drawing a small sample of beads. The product was cleaved after 4 h from the beads when it yielded a conversion rates of 95% for agarose beads (HPLC purity 75.7 A/A%) and 91% for PMA beads (HPLC purity 63.3 A/A%) (Table 2).

We observed that the cocktail acidity (40% v/v TFA in water) used to release liraglutide from agarose beads was not sufficient to completely remove the tBu group from the carboxylic function on the α-carbon of Palm-Glu (Fig. 5).

The remaining tBu ester (~65%) was removed by treating the crude peptide with TFA/TIS/H_2O (95:2.5:2.5 v/v/v) at room temperature for 1 h, which resulted in the completely deprotected liraglutide (5 mg, yield 23%, final purity 72.5 A/A%) (Fig. S19).

This first encouraging result prompted us to explore different acylation conditions for the active ester Palm-Glu(OSu)-OtBu by adding a base and the well-known acylation catalyst azahydroxybenzotriazole (HOAt) (Carpino 1993; Subirós-Funosas et al. 2009) in the binary solvent mixture BuOAc/DMSO 7:3 v/v, also used for SPPS.

We investigated 3 different coupling systems (c.s.) still at 5 µmol, reported in Table 3, both on agarose and PMA beads.

In all these coupling systems, we added 6 equivalents of base to compensate for the minimal basicity of DMF, substituted with BuOAc/DMSO, that provides the optimal environment for the coupling reaction. The best result (79.7 A/A%, conversion 99%), was achieved after 5 h with the coupling system 2 (HOAt and DIPEA) on agarose beads

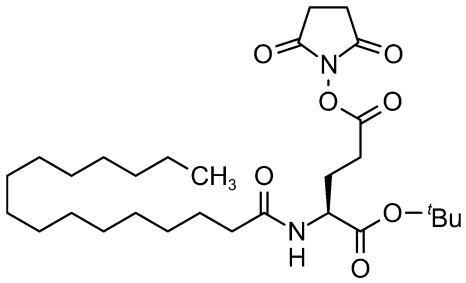


Fig. 5 Structure of Palm-L-Glu(OSu)-OtBu

(Table 4). All the attempts on PMA beads gave lower purity, conversion, and yield.

Based on this result, we decided to scale up the reaction by a factor of 10 using agarose beads.

50 µmol were treated with coupling system 2 in BuOAc/DMSO for 5 h at room temperature obtaining, after the release from agarose beads and a further treatment with TFA/TIS/H_2O, 90.5 A/A%, HPLC crude purity and a conversion over 99% (Fig. 6). Moreover, we recovered 45 mg of the final product (25% yield).

Conclusions

This study highlights the successful integration of green chemistry principles into solid-phase peptide synthesis (SPPS), offering a practical and environmentally friendly alternative for producing therapeutic peptide ingredients. By using 1-tert-butyl-3-ethylcarbodiimide (TBEC) as a safer coupling reagent in combination with eco-friendly solvent systems like dimethyl sulfoxide (DMSO) and butyl acetate (BuOAc), we reduced the environmental footprint and safety risks commonly associated with traditional synthesis methods. These approaches maintained high efficiency and delivered high-quality peptide products, demonstrating that sustainability and performance can go hand in hand.

We explored two innovative strategies for synthesizing liraglutide, a key GLP-1 agonist used to treat diabetes and obesity. The "direct synthesis" approach incorporated a lipidated lysine building block directly into the peptide

Table 2 In-Process-Control of Acylation study on liraglutide precursor

	Bead type	Conversion (%)	Purity (A/A%)
Check 2 h	Agarose	87	69.5
	PMA	55	43.8
Final release 4 h	Agarose	95	75.7
	PMA	91	63.3

Table 3 Three Coupling Systems evaluated for the optimization of acylation

c.s. 1	c.s. 2	c.s. 3		
BB-Osu			4 equiv	20 µmol
OxymaPure	HOAt	OxymaPure	4 equiv	20 µmol
DiPEA	DiPEA	Pyridine	6 equiv	30 µmol
			In 150 µL BuOAc/DMSO	

Table 4 Results of coupling attempts on agarose and PMA beads

	Coupling systems	Conversion	Purity after 5 h (A/A %)	Purity after 1 h TFA treatment (A/A %)	Yield (%)
Agarose beads	1	97	22.3	75.3	23
	2	99	20.6	79.7	24
	3	86	19.2	65.4	19
PMA beads	1	92	57.6		18
	2	95	65.2		20
	3	64	43.8		15

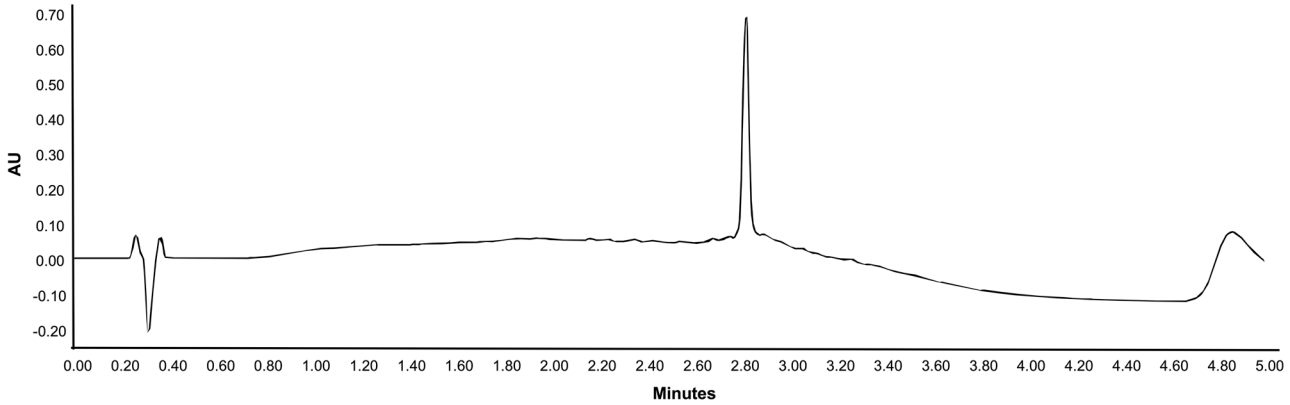


Fig. 6 RP-UHPLC traces of PEC purified liraglutide synthesized using BuOAc/DMSO, Acylation strategy. C18 column Waters Acquity BEH (130 Å, 1.7 µm, 2.1×50 mm); temperature 65 °C; flow, 0.5 mL/min; eluent, 0.1% (v/v) TFA in H_2O (**A**) and 0.1% (v/v) TFA in CH_3CN (**B**); λ, 215 nm, gradient, 10–90% B in 5 min. Rt = 2.81 min: liraglutide 90.5% purity

sequence, achieving excellent purity after catch-and-release purification. The "catch-lipidation-and-release" strategy took a different route, attaching the lipid moiety in a controlled, post-synthesis step, which allowed for better precision and higher final yields. Both methods proved to be scalable and significantly reduced the need for extensive preparative HPLC, saving time and resources.

This work demonstrates that adopting greener methods in peptide synthesis is not only possible but practical for meeting the pharmaceutical industry growing demand for sustainability. Our results show that using TBEC and green solvents can deliver the same high-quality outcomes as conventional methods while being more friendly to the environment. Additionally, the catch-and-release purification technique enhanced efficiency, making these methods suitable for large-scale applications.

Looking ahead, this research sets the stage for further innovation in sustainable pharmaceutical manufacturing. As the need for peptide-based therapeutics like liraglutide continues to grow, these methods offer a reliable, eco-conscious way to meet that demand. By aligning cutting-edge science with environmental responsibility, this study underscores the potential to redefine how we produce life-saving medicines, benefiting both people and the planet.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10989-025-10703-4.

Acknowledgements Lorenzo Pacini acknowledges MUR and EU-FSE for the financial support of the PhD fellowship PON Research and Innovation 2014–2020 (D.M. 1061/2021) XXXVII Cycle in Chemical Sciences: "Greening peptide chemistry, a necessary step to the future". The project was funded in part by EUniWell: 5th seed funding call for the TTPep project and 3rd Well-Being Research Incubator Call for the project "Sustainable and green upscaling processes for peptide(-based) in vitro diagnostics and cosmeceuticals".

Author Contribution L.P. performed the experiments, revised data, and wrote the original draft of the manuscript. L.P., M.K.M., R.Z., and A.M.P. conceived the work on the instrumentations. L.P., O.M., and A.M.P. conceived the work on the reagents. A.M.P. and P.R. supervised the work. All authors reviewed the manuscript.

Funding Open access funding provided by Università degli Studi di Firenze within the CRUI-CARE Agreement.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Conflict of Interest Manoj Kumar Muthyala and Robert Zitterbart are employers of Gyros Protein Technologies Inc. and declare competing financial interests: the PurePep® Chorus synthesizer and the PurePep

EasyClean technology described in the manuscript can be purchased from Gyros Protein Technologies. Oleg Marder is an employer of Luxembourg Bio Technologies and declare competing financial interests: T-Bec® described in the manuscript can be purchased from Luxembourg Bio Technologies.

References

Al Musaimi O, De La Torre BG, Albericio F (2020) Greening Fmoc/*t*Bu solid-phase peptide synthesis. Green Chem 22:996–1018. https://doi.org/10.1039/C9GC03982A

Barredo-Vacchelli GR, Rodríguez JA, Eloy JA, Camperi SA (2024) A novel method for liraglutide synthesis and purification. Pept Sci 116:e24351. https://doi.org/10.1002/pep2.24351

Carpino LA (1993) 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive. J Am Chem Soc 115:4397–4398. https://doi.org/10.1021/ja00063a082

Fantoni T, Orlandin A, Di Stefano I et al (2024) Solid phase peptide synthesis using side-chain unprotected arginine and histidine with Oxyma Pure/TBEC in green solvents. Green Chem 26:10929–10939. https://doi.org/10.1039/D4GC03209H

Ferrazzano L, Catani M, Cavazzini A et al (2022) Sustainability in peptide chemistry: current synthesis and purification technologies and future challenges. Green Chem 24:975–1020. https://doi.org/10.1039/D1GC04387K

Ferrazzano L, Corbisiero D, Martelli G et al (2019) Green solvent mixtures for solid-phase peptide synthesis: a dimethylformamide-free highly efficient synthesis of pharmaceutical-grade peptides. ACS Sustain Chem Eng 7:12867–12877. https://doi.org/10.1021/acssuschemeng.9b01766

Hach M, Engelund DK, Mysling S et al (2024) Impact of manufacturing process and compounding on properties and quality of follow-On GLP-1 polypeptide drugs. Pharm Res. https://doi.org/10.1007/s11095-024-03771-6

Kar S, Sanderson H, Roy K et al (2022) Green chemistry in the synthesis of pharmaceuticals. Chem Rev 122:3637–3710. https://doi.org/10.1021/acs.chemrev.1c00631

Liu X, Zhang N, Gu X et al (2020) Total synthesis of semaglutide based on a soluble hydrophobic-support-assisted liquid-phase synthetic method. ACS Comb Sci 22:821–825. https://doi.org/10.1021/acscombsci.0c00134

Manne SR, Akintayo DC, Luna O et al (2022) *tert* -Butylethylcarbodiimide as an efficient substitute for diisopropylcarbodiimide in solid-phase peptide synthesis: understanding the side reaction of carbodiimides with OxymaPure. Org Process Res Dev 26:2894–2899. https://doi.org/10.1021/acs.oprd.2c00220

Manne SR, Luna O, Acosta GA et al (2021) Amide formation: choosing the safer carbodiimide in combination with OxymaPure to avoid HCN release. Org Lett 23:6900–6904. https://doi.org/10.1021/acs.orglett.1c02466

Martin V, Egelund PHG, Johansson H et al (2020) Greening the synthesis of peptide therapeutics: an industrial perspective. RSC Adv 10:42457–42492. https://doi.org/10.1039/D0RA07204D

Pacini L, Muthyala M, Aguiar L et al (2024) 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 30:e3605. https://doi.org/10.1002/psc.3605

Pawlas J, Choi J-H, Von Bargen C et al (2023) Elevating 1- *tert* -Butyl-3-ethylcarbodiimide (TBEC) as a reagent for sustainable peptide synthesis: quality assessment and minimizing racemization, precipitation, and radical-induced side reactions by TBEC/Oxyma couplings in an environmentally sensible solvent. Org Process Res Dev 27:1348–1364. https://doi.org/10.1021/acs.oprd.3c00120

Pawlas J, Rasmussen JH (2023) Assessment of sulfur impurities in GMP-grade diisopropylcarbodiimide and their impact on coupling reagent-induced side reactions in peptide synthesis. Org Process Res Dev 27:982–992. https://doi.org/10.1021/acs.oprd.3c00099

Selmi C, Papini AM, Pugliese P, Alcaro MC, Gershwin ME (2011) Environmental pathways to autoimmune diseases: the cases of primary biliary cirrhosis and multiple sclerosis. Arch Med Sci 7(3):368–380. https://doi.org/10.5114/aoms.2011.23398

Sharma K, Sharma KK, Sharma A, Jain R (2023) Peptide-based drug discovery: current status and recent advances. Drug Discov Today 28:103464. https://doi.org/10.1016/j.drudis.2022.103464

Staby A, Steensgaard DB, Haselmann KF et al (2020) Influence of production process and scale on quality of polypeptide drugs: a case study on GLP-1 analogs. Pharm Res 37:120. https://doi.org/10.1007/s11095-020-02817-9

Subirós-Funosas R, Prohens R, Barbas R et al (2009) Oxyma: an efficient additive for peptide synthesis to replace the benzotriazole-based HOBt and HOAt with a lower risk of explosion[1]. Chem A Eur J 15:9394–9403. https://doi.org/10.1002/chem.200900614

Wang J, Berglund MR, Braden T et al (2022) Mechanistic study of diketopiperazine formation during solid-phase peptide synthesis of tirzepatide. ACS Omega 7:46809–46824. https://doi.org/10.1021/acsomega.2c05915

Wegner K, Barnes D, Manzor K et al (2021) Evaluation of greener solvents for solid-phase peptide synthesis. Green Chem Lett Rev 14:153–164. https://doi.org/10.1080/17518253.2021.1877363

Yang J, Huang H, Zhao J (2023) Active ester-based peptide bond formation and its application in peptide synthesis. Org Chem Front 10:1817–1846. https://doi.org/10.1039/D2QO01686A

Zitterbart R, Berger N, Reimann O et al (2021) Traceless parallel peptide purification by a first-in-class reductively cleavable linker system featuring a safety-release. Chem Sci 12:2389–2396. https://doi.org/10.1039/D0SC06285E