Green Pharmacy | Aspects of Peptide Synthesis and Peptide Therapeutics | A Review

Insights into the green pharmacy aspects of Semaglutide, Liraglutide, BMS-986189 and cyclosporine and introducing Green by Design for therapeutic peptides

Abstract

The field of peptide therapeutics is rapidly growing. Unfortunately, the production of these peptides generates large amounts of waste. The production mass intensity (PMI) of a peptide therapeutic using solid-phase peptide synthesis is around 13000 while the PMI for small drug molecules is around 300. Furthermore, the main waste in this process consists of toxic and reprotoxic solvents such as DMF, DCM and DMAc. The use of different solvents with lower toxicity and a better environmental footprint has been investigated. Furthermore, improved synthetic methods allowed for a significant reduction in the overall PMI of various peptide therapeutics and more benign coupling agents have been implemented. Lastly, the metabolic stability versus the environmental stability of active pharmaceutical ingredients is discussed. It is expected that the use of machine learning and quantitative structure–activity relationship can help to reduce this excessive stability.

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1. Layman’s summary

Peptide medicijnen zijn opgebouwd uit aminozuren en worden steeds meer gebruikt tegen verschillende ziektes. Een bekend voorbeeld hiervan is Ozempic dat Semaglutide bevat als actieve stof en gebruikt wordt in patiënten met diabetes type 2. De productie van deze medicijnen genereert ontzettend grote hoeveelheden afval. Gemiddeld komt er bij de synthetische productie van 1 kg van deze medicatie 13000 kg afval vrij. Bij de productie van meer gangbare medicijnen, zoals paracetamol of ibuprofen, komt er ongeveer 170 kg tot 300 kg afval vrij per kilogram van de actieve stof. Dit laat zien dat het proces voor de productie van de peptide medicijnen erg milieu belastend is en dat hier dus verbeteringen gemaakt moeten worden zodat de hoeveelheid afval afneemt. Daarnaast is het soort afval wat vrijkomt bij deze productie erg schadelijk voor mens en milieu.

In het huidige proces worden deze peptide medicijnen gemaakt door aminozuren één voor één aan elkaar te koppelen in een schadelijk oplosmiddel tot het product is gemaakt. Ondanks dat deze koppeling erg efficiënt gedaan kan worden is het uiteindelijke totaalplaatje erg inefficiënt. Dit komt doordat deze koppeling soms wel 30 keer gedaan moet worden voordat het peptide medicijn geproduceerd is.

Om dit proces te verbeteren zijn er onderzoeken gedaan om het schadelijke oplosmiddel te vervangen voor minder schadelijke alternatieven waardoor er nu verschillende betere opties mogelijk zijn. Ook zijn er goede alternatieven gevonden voor het type synthese dat gebruikt wordt. Deze hebben allemaal zo hun voor-, en nadelen. Het grootste probleem is dat er momenteel geen goed alternatief is dat breed toepasbaar is op productieschaal. Wel is er recent een onderzoek gepubliceerd waarin de gebruikte techniek breed toepasbaar was, en zeer waarschijnlijk goed op grote schaal uitvoerbaar is. De onderzoekers lieten zien dat hun nieuwe techniek de hoeveelheid afval met een factor 20 kon verminderen.

Een andere zorg onder wetenschappers is het ophopen van medicijnen, of deelstructuren van de medicijnen in de natuur. Onderzoek liet zien dat in bijna elke rivier in de wereld medicijnresten te vinden zijn en dat dit dus een algemeen probleem is. Het is belangrijk dat medicatie stabiel genoeg is om zijn functie in de patiënt uit te voeren maar dit kan er dus ook voor zorgen dat deze medicatie ophoopt in de natuur waarvoor de gevolgen niet altijd even goed voorspeld kunnen worden. Om deze reden is in kaart gebracht welke structuren makkelijk worden afgebroken en welke moeilijk afbreekbaar zijn in de natuur. Deze informatie is belangrijk om te gebruiken in de ontwikkeling van nieuwe medicijnen omdat naast patiëntveiligheid ook rekening gehouden moet worden met mogelijke milieuschade door deze nieuwe medicijnen.

2. List of abbreviations

ACN:	Acetonitrile
AE:	Atom economy
Aib:	Aminoisobutyric acid
Ala:	Alanine
API:	Active pharmaceutical ingredient
CEPS:	Chemo-enzymatic peptide synthesis
CF-SPPS:	Continuous flow solid-phase peptide synthesis
COMU:	(1-(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylaminomorpholino-methylene) ethaminiumhexafluorophosphate)
CY:	Chemical yield
CyA:	Cyclosporine A
DCM:	Dichloromethane
DEE:	Diethyl ether
DIC:	*N,N*-Diisopropylcarbodiimide
DIPEA:	Diisopropyl ethyl amine
DMAc:	*N,N*-Dimethylacetamide
DMC:	Dimethyl carbonate
DMF:	*N,N*-Dimethylformamide
DMSO:	Dimethyl sulfoxide
DOL:	1,3-Dioxolane
DPDTC:	Dipyridyl dithiocarbonate
DPP-IV:	Dipeptidyl peptidase IV
EtOAc:	Ethyl acetate
ETT:	5-(Ethylthio)-1H-tetrazole
Fmoc:	Fluorenylmethyloxycarbonyl
GLP-1:	Glucagon-like peptide 1
GSolPPS:	Green solution phase-peptide synthesis
HATU:	*N,N,N',N'*-Tetramethyl-O-(1H-7-azabenzotriazol-1-yl uranium hexafluorophosphate
HBTU:	*N,N,N',N'*-Tetramethyl-O-(1H-benzotriazol-1-yl uranium hexafluorophosphate
HOAt:	1-Hydroxy-7-azabenzotriazole
HOBt:	1-Hydroxybenzotriazole
HPLC:	High pressure liquid chromatography
IEC:	Ion-exchange chromatography
LMICs:	Low- to middle-income countries
Lys:	Lysine
MCSGP:	Multicolumn countercurrent solvent gradient purification
ML:	Machine learning
MP-RPLC:	Medium-pressure reversed-phase liquid chromatography
MTBE:	Methyl-tert-butyl ether
PD-1:	Programmed cell death protein-1
PD-L1:	Programmed death ligand-1
NMF:	*N*-Formylmorpholine
NMP:	*N*-Methylpyrrolidone
PMI:	Process mass intensity
NOP:	*N*-Octyl-2-pyrrolidone

QSAR:	Quantitative structure-activity relationship
RP-HPLC:	Reversed-phase high pressure liquid chromatography
SAR:	Structure-activity relationship
SFC:	Supercritical fluid chromatography
SI:	Solvents intensity
SMoc:	2,7-Disulfo-9-fluorenylmethoxycarbonyl
SPPS:	Solid-phase peptide synthesis
TBEC:	1-(tert-Butyl)-3-ethylcarbodiimide
TEP:	Triethyl phosphate
TFA:	Trifluoroacetic acid
TFFH:	N,N,N',N'-Tetramethylformamidinium hexafluorophosphate
THF:	Tetrahydrofuran

3. Introduction

The field of peptide therapeutics in the pharmaceutical industry is growing and is currently responsible for roughly 5% of global revenue in the pharmaceutical market. Production of these peptides started in 1922 with the medicinal use of insulin, isolated from animal pancreases. However, interest from the pharmaceutical industry in peptide therapeutics increased around 1954 with the successful synthetic production of oxytocin followed by vasopressin and somatostatin [1]. Furthermore, improvements in drug design, formulation and delivery as well as the introduction of solid-phase peptide synthesis (SPPS) has made the production of peptide therapeutics commercially more viable [2]. The number of approved therapeutic peptides is growing rapidly (Figure 1). Currently, over 100 peptide therapeutics are approved for commercial production while another 150 peptide compounds are in clinical developments as well as over 400 peptides which are investigated in preclinical studies [1, 2, 3]. These peptide therapeutics are of utmost importance to be able to bind new targets which currently seems undruggable using small drug molecules. However, SPPS is the main method used for the synthesis of these peptide therapeutics, which requires a large excess of reagents and uses a large quantity of toxic solvents, which has a negative environmental impact [2].

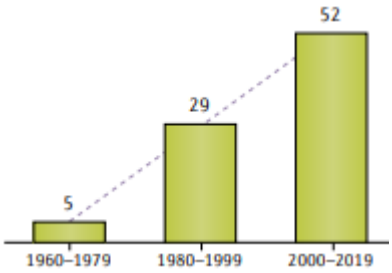


Figure 1: Taken from Nat. Rev. Drug. Discov. (2021) Muttenthaler, M. et al. Number of therapeutic peptides approved *[1].*

Multiple efficiency metrics are described in green chemistry which can be used to compare different processes and consider their respective environmental effect and give insight into the sustainability of a production process. For example, atom economy (AE), chemical yield (CY), process mass intensity (PMI), solvents intensity (SI) among others can be used to compare synthetic processes. AE describes how efficiently the reagents used are incorporated into the final product while chemical yield looks at the efficiency of the reaction themself. However, these efficiency metrics do not take reaction solvents, washing solvents nor purification materials into account. PMI and SI do incorporate solvent waste in which SI only looks to the amount of solvent used while PMI is described by the total mass of materials used in the process divided by the mass of the product [2, 4]. The PMI of a small drug molecule is usually between 168 and 308 while the PMI for peptides therapeutics on average is around 13000. The PMI of peptide therapeutics is currently not improved for the peptides in phase 1 and phase 2 clinical studies [2]. This information, combined with the knowledge that peptide therapeutics is a growing field, should show the necessity to improve peptide synthesis to significantly reduce solvent waste and thus improve the PMI.

Early peptide therapeutics suffered from short half-lives for which multiple solutions were thought of, such as unnatural amino acids, *N*-capping and disulfide bond mimetics among others, to improve their metabolic stability [1]. However, this increased stability has also resulted in increased stability of metabolites and active pharmaceutical ingredients (APIs) in the aqueous environment. Here, they can form a risk to aquatic life, wildlife in general, as well as human health [5]. A global study towards API pollution in rivers showed that it is a global issue in which low- to middle-income countries (LMICs) are disproportionately affected, likely caused by poor sewage treatment plants [6]. This pollution is expected to grow as a result of increased access to drugs in LMICs without being able to efficiently remove these in sewage treatment plants. Therefore, the GREENER concept, as described by Moermond *et al.*, should be used in the development of new drugs to reduce the environmental impact without reducing the efficacy, safety or patient wellbeing [5].

In this review a focus will be given on four different peptide therapeutics, namely BMS-986189, cyclosporine A (CyA), Semaglutide and Liraglutide in which their current production procedures will be discussed followed by the attempts to incorporate green chemistry and reducing the environmental burden of SPPS. After this, the build-up of APIs and their metabolic remnants in nature will be discussed, highlighting the concept: “Green by design” which will focus on how the stability of metabolic remnants can be influenced to reduce long lasting chemicals in the environment.

4. Discovery and production of peptide therapeutics

BMS-986189 is a therapeutic peptide which is able to act as an antagonist in the interaction between programmed cell death protein-1 (PD-1) and programmed death ligand-1 (PD-L1) and is therefore of interest in immuno-oncology [3, 7]. This compound was discovered via a structure-activity relationship (SAR) study in which many different peptide macrocycles were synthesized and tested for their binding affinity against different targets including the PD-1/PD-L1 protein-protein interaction [8]. Currently, BMS-986189 is mainly synthesized using the SPPS-method in which the amino acid chain is constructed in multiple cycles to obtain the protected amino acids in the correct order. These are then deprotected followed by a macrocyclization reaction between amino acid 1 and 14 to obtain BMS-986189. The compound consist out of multiple nonproteinogenic amino acids (depicted by red numbers) and natural amino acids (depicted by blue numbers) (Figure 2). Because of the incorporation of the nonproteinogenic amino acids it is currently not feasible to produce this via recombinant or semi-recombinant techniques. Therefore, SPPS is the preferred method for its production. The PMI of the synthetic steps is around 910 which is not that extreme compared to the PMI of small drug molecules. However, the purification and isolation steps increase the PMI by over 13000 and the cyclization also adds another 1160 resulting in a total PMI of more then 15000 [3].

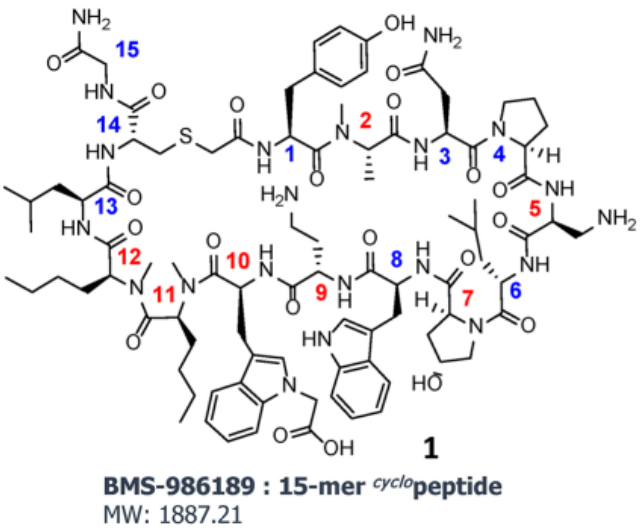


Figure 2: Picture taken from J. Org. Chem. (2024) Mukherjee, S. et al. Structure of BMS-986189 with the amino acid numbering starting at the N*-terminus. Red numbers indicate nonproteinogenic amino acids while the blue indicate natural amino acids [3].*

Cyclosporine A (CyA) is an important therapeutic peptide which is used as an immunosuppressant to improve one-year graft survival rates after transplant operations. CyA is a natural cyclic peptide which consists of eleven amino acids with multiple nonproteinogenic amino acids (Figure 3). It was first isolated from the culture broth of the fungus *T. inflatum Gams* in 1970 which is still the preferred method of production. This fermentative production process has been improved over the years by optimizing the culture medium and conditions and by introducing different mutations in the fungi [9, 10]. The first synthetic route, reported in 1984, used liquid-phase peptide coupling to connect multiple fragments to obtain the protected linear peptide chain. Deprotection followed by a cyclization step gave CyA in 27.5% overall yield [11]. Total synthesis of CyA using SPPS was performed in 1997 in which an overall yield around 30% from starting material was achieved [12]. Although the exact PMI for these processes is not reported, estimation using average numbers can be performed. The average PMI for the bioproduction of therapeutic peptides is reported to be on average 8300 while synthetically produced peptides via SPPS have an average of 875 per natural amino acid [2]. Thus, for cyclosporine the calculated PMI would be around 9625 using SPPS, not considering the synthesis of the unnatural amino acids, while its production using the fungi is slightly better at an estimate of 8300.

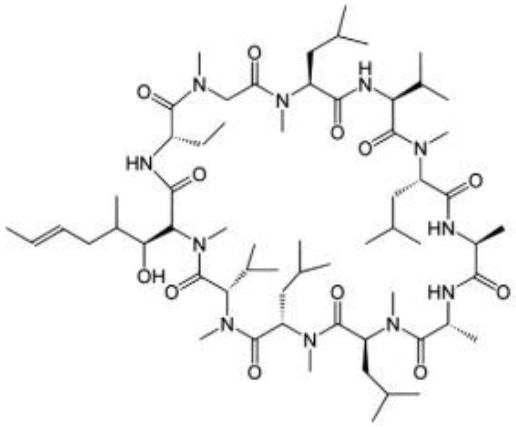


Figure 3: Picture taken from Biotech. Adv. (2011) Survase, S.A. et al. Structure of cyclosporine A [10]

Two other therapeutic peptides are Liraglutide and Semaglutide which are both used in the treatment of diabetes and obesity. The structure of these peptides are based on the glucagon-like peptide 1 (GLP-1) hormone. The discovery that this GLP-1 hormone is able to control the blood glucose levels led to the efforts to produce a drug for type 2 diabetes. GLP-1 itself cannot be used as a treatment option because of its short half-life of roughly 90 seconds in blood. An alanine scan of GLP-1 and exendin-4, a similar peptide from an animal, was performed in order to investigate which amino acids are important in binding. With this method it was determined that dipeptidyl peptidase IV (DPP-IV) degradation happens on the *N*-terminus of GLP-1, causing its short half-life. This degradation does not occur for exendin-4 which contains a glycine (Gly) instead of alanine (Ala) in the second position of the *N*-terminus. However, substitution of Ala for Gly in GLP-1 resulted in a significantly reduced binding affinity. Changing Ala for aminoisobutyric acid (Aib) resulted in a GLP-1 peptide mimetic with high affinity to the GLP-1 receptor with a good stability against DPP-IV degradation. Furthermore, albumin binding seemed to be an interesting method to further prolong the half-life because of reduced renal clearance for drugs bound to albumin [13]. Therefore, a screening was performed in which multiple fatty-acids were introduced on different acylation sites of the core structure as well as changes in the core structure itself by varying a few amino acids [14]. This resulted in the discovery of Liraglutide which was selected for its great binding affinity and therapeutic suitable half-life of 8-15 h [15]. Liraglutide does not contain Aib on the second position but is unchanged at the *N*-terminus. The increased half-life is likely caused by the reversible albumin binding and protection against DPP-IV degradation by steric hinderance of the fatty-acid [13]. The large-scale production of Liraglutide starts by the expression of the peptide sequence in genetically transformed yeast. The obtained GLP-1 peptide mimetic can then be acylated at the lysine (Lys) position with *N*-palmitoyl glutamic acid to obtain Liraglutide [14, 16].

Semaglutide was discovered in the pursuit to improve patient care since Liraglutide needs to be injected once daily. Semaglutide improves on this by only needing a once-weekly injection to be efficacious with an increased half-life of 165 h. The main difference between Liraglutide and Semaglutide is the fatty-acid linker which has an improved binding to albumin and the introduction of Aib in the second position of the *N*-terminus domain as seen in Figure 4 [15]. Semaglutide is produced via a semi-recombinant method in which a large part of the peptide fragment is produced via a yeast expression system in which a part of the GLP-1 peptide mimetic, namely, (R34)GLP-1(8-37) or (R34)GLP-1(9-37), backbone is encoded. After isolation the peptide mimetic undergoes standard acylation with the fatty-acid linker followed by propagation of the peptide chain to obtain Semaglutide [14].

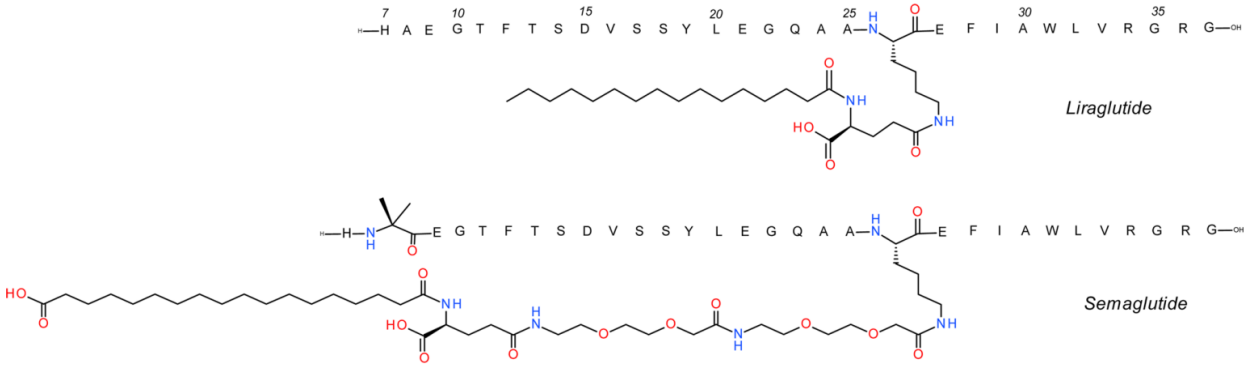


Figure 4: Picture taken from J. Med. Chem. (2015) Lau, J. et al. Structures of Liraglutide and Semaglutide [15].

Using the same average PMI per amino acids as previously mentioned this would result in a PMI of roughly 26000 for the production Liraglutide using SPPS. The production of Semaglutide is performed via semi-recombinant techniques in which the main part is produced recombinantly followed by the addition of 2 amino acids. This would result in a PMI of roughly 10000 for the semi-recombinant production and 26000 for the fully synthetic production.

This data shows that the production of therapeutic peptides, using recombinant, semi-recombinant or other bioproduction techniques, is more environmentally friendly compared to the production using SPPS. However, the discovery process of therapeutic peptides using recombinant techniques is long and expensive while a large number of potential nonnatural therapeutic peptides can be synthesized using the SAR method [14]. Furthermore, as observed for BMS-986189, it is not always feasible to produce these therapeutic peptides using these techniques due to the incorporation of nonproteinogenic peptides. Therefore, if we want to significantly reduce the environmental pollution in the production of therapeutic peptides, we should aim to incorporate as few nonproteinogenic amino acids as possible and, if they are required, place them in strategic locations to allow for semi-recombinant production. However, the number-one goal in drug discovery should still be the discovery of the best working API. Therefore, the SPPS process should also be a target itself to reduce the amount of waste generated by this synthetic process as it allows for the production of more therapeutic peptides.

5. Green approaches in the synthesis of peptide therapeutics

The environmental impact of SPPS is not only caused by the large amount of waste generated but also due to the type of waste. Main solvent waste in the synthetic and washing steps consists of *N,N*-dimethylformamide (DMF), *N,N*-dimethylacetamide (DMAc) and *N*-methylpyrrolidone (NMP), which are known to be reprotoxic, as well as the toxic solvents dichloromethane (DCM), diethyl ether (DEE) and methyl-tert-butyl ether (MTBE). Furthermore, the amino acids are usually protected with fluorenylmethyloxycarbonyl (Fmoc) which require deprotection using piperidine after each coupling step resulting in a poor atom economy [2, 17]. Lastly, as observed when dissecting the PMI of BMS-986189, the PMI for purification steps accounts for roughly 85% of all waste generated in the entire process and thus needs to be reduced [3]. Therefore, clear steps need to be taken to reduce the volume of toxic solvents needed in the synthesis, improvements in atom economy need to be made by using alternative coupling agents, improved protection groups which should be cleavable using less harmful chemicals and lastly, improvements in purification is required to be able to significantly reduce the PMI.

5.1 Using different solvents

The use of DMF and NMP is generally preferred in SPPS due to their great technical profile for which the requirements are listed in Ferrazzano *et al*. [18]. However, as mentioned before, there is a clear need to substitute these solvents for less toxic ones. Furthermore, recovery of these solvents would be preferable as this would greatly reduce the PMI in the synthetic part. A ranking of different solvents is shown in Table 1 and shows that DMF and NMP belong to the hazardous solvents. Many different solvent combinations have been reported, such as acetonitrile (ACN)/tetrahydrofuran (THF), dimethyl sulfoxide (DMSO)/ethyl acetate (EtOAc) and *N*-formylmorpholine (NFM)/1,3-dioxolane (DOL) among others. Even though these solvents have shown promising results in some research papers, they lack industrial application due to their low flash points and increased costs compared to DMF [19, 20]. Furthermore, the performances of most green mono-solvent systems seem to be case dependent with mostly a reduction in yield and purity when comparing them to DMF [21]. A potential green solvent for SPPS is triethyl phosphate (TEP) which is able to dissolve almost all Fmoc protected amino acids and most coupling reagents except for the, on production scale, less frequently used *N,N,N',N'*-tetramethyl-O-(1H-7-azabenzotriazol-1-yl uranium hexafluorophosphate (HATU) and *N,N,N',N'*-tetramethyl-O-(1H-benzotriazol-1-yl uranium hexafluorophosphate (HBTU). The solvent itself shows low toxicity and degrades into the fairly harmless ethanol and phosphoric acid. However, recovery from the process waste material was not reported. Thus, although it could reduce toxic waste in the SPPS process, it would not reduce the PMI [22]. Another green solvent mixture consisting of 20% dimethyl carbonate (DMC) in *N*-octyl-2-pyrrolidone (NOP) also conforms to the solvent requirements for SPPS and can be easily recovered from the process waste mixture by distillation. Hereby, the use of this solvent combination does not only reduce the amount of toxic solvents used in the synthetic part of SPPS but the recovery of these solvents also leads to a PMI reduction of roughly 65%. The production of NOP produces more kg CO_2 per kg NOP produced compared to the production of DMF and NMP. However, because of NOP's easy recovery by a single distillation, the overall carbon footprint is reduced compared to the use of DMF and NMP [20]. Furthermore, its recovery drives down the production costs which is of great importance if we want the pharmaceutical industry to use these green alternatives.

Table 1: Table adapted from Green Chem. (2022) Ferrazzano, L. et al. Overview of different solvent categorized by their safety and environmental effect [18].

Highly hazardous	Hazardous	Problematic or hazardous
1,2-dichloroethane	1,4-Dioxane	Cyclohexane
Benzene	Diethyl ether	Dichlormethane
Carbon tetrachloride	Dimethylacetamide	Formic acid
Chloroform	Methoxy ethanol	Methyl *tert*-butyl ether
Nitromethane	*N,N*-Dimethylformamide	Pyridine
	n-Hexane	Tetrahydrofuran
	N-Methylpyrrolidone	
	n-Pentane	
	Triethylamine	
Problematic	**Recommended or problematic**	**Recommended**
Acetonitrile	Acetic acid	Anisole
Chlorobenzene	Acetic anhydride	Ethanol
Dimethylsulfoxide	Acetone	Ethyl acetate
Methyl cyclohexane	Benzyl alcohol	*i*-Propanol
Methyl tetrahydrofuran	Cyclohexanol	*i*-Propyl acetate
n-Heptane	Ethylene glycol	*n*-Butanol
Toluene	Methanol	*n*-Butyl acetate
Xylenes	Methyl acetate	Water
	Methyl ethyl ketone	
	Methyl *i*-butyl ketone	
	tert-Butanol	

5.2 Convergent peptide synthesis

The peptide chain in SPPS is normally build in a linear fashion starting from the C-terminus. This method is known to form impurities which are difficult to remove. These impurities contain deletion peptides, which are peptide chains in which one or more amino acid residues are lacking. The deletion peptides are difficult to remove since they are closely structurally related to the desired peptide. Furthermore, quantitative yield of each propagation cycle is required for high yielding products because a 98% yield for each coupling step would result in a overall yield of 55% after 30 cycles [23]. This issue can be reduced by convergent peptide synthesis in which multiple smaller building blocks are prepared with SPPS followed by the coupling of these building blocks into a single peptide chain (Figure 5). The difficulty of convergent peptide synthesis lies in choosing the correct condensation site to be able to couple the building blocks. Carboxylic acid activation of peptides can lead to fast isomerization and hereby form a difficult to remove diastereoisomer. The advantage of this method is that it allows for easier scale-up and for purification using normal-phase

chromatography of the synthesized fragments. Furthermore, it is thought to significantly improve overall yield and purity of the final peptide [7, 16].

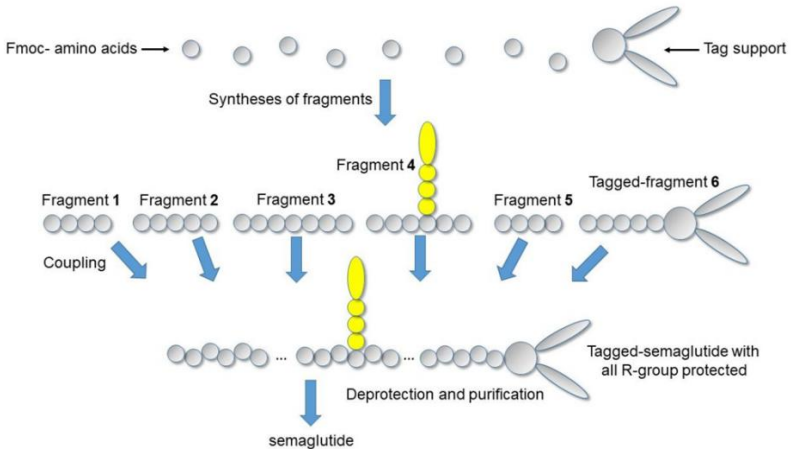


Figure 5: Picture taken from ACS Comb. Sci. (2020) Liu, X. et al. Convergent synthetic strategy for the synthesis of Semaglutide [24].

The discovery of a soluble hydrophobic-support compound led to the ability to use this material in liquid-phase peptide coupling. This support allows for peptide coupling and deprotection in the liquid-phase after which an easy isolation is possible by precipitation of the hydrophobic-support coupled peptide using a polar antisolvent. After multiple cycles the smaller peptide fragments can be obtained in great yield and purity. After coupling of the smaller fragments the compound can be cleaved from the hydrophobic-support resin and deprotected to obtain the final product. This method was applied for the synthesis of Semaglutide in which six smaller fragments were synthesized and coupled to improve yield and purity [24]. Although the authors were pleased with the results, their method still require a significant amount of solvent. They reported the use of 500 ml ACN and 500 ml THF after each coupling or deprotection reaction while only using 1.34 g starting material. Deprotection of the tagged peptide required 60 ml THF as solvents and coupling reactions were performed in 60 ml DCM. In total 52 cycles were required to obtain the 6 different fragments which gave a total of roughly 58 liter of solvent to obtain 609 mg of Semaglutide. However, not all material was used after each step thus calculating the exact PMI of their process is difficult. The possible yield of Semaglutide would be around 6 g if all material was used after each step which would result in a PMI of roughly 20000-30000 which is still very high. Also, the method still required the reprotoxic DMF and the problematic THF. The advantage of this method is the ability to scale the process and the similarity to the current production process. This allows easier incorporation from an industrial perspective. Furthermore, other support material can be used to improve isolation after coupling or deprotection. For example, newer hydrophobic-support materials do not require precipitation to isolate the peptide but can be isolated using liquid-liquid extraction. This could significantly improve the overall PMI of this method and is reported to roughly reduce the overall waste by a factor 10 [25].

The use of a convergent method for the synthesis of BMS-986189 did not work as hoped. Two fragments were synthesized using SPPS of which one was purified using normal-phase chromatography while the other fragment did not need purification after resin cleavage. After obtaining the two fragments, multiple liquid-phase couplings were tried. Unfortunately, the coupling of these fragments gave unsatisfactory results. The obtained yield was below <70% and the impurity profile of the obtained mixture was worse compared to linear SPPS because of diastereoisomer formation [7].

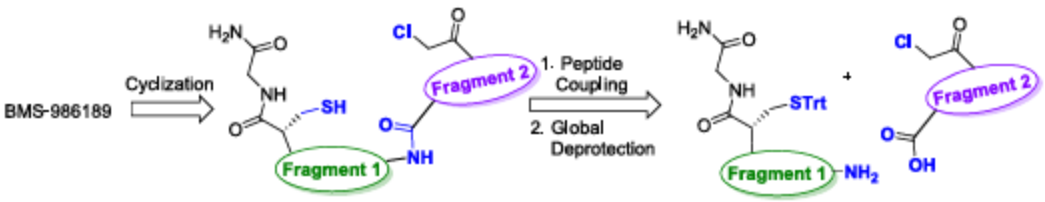


Figure 6: Taken from J. Org. Chem. (2024) Kempson, J. et al. Convergent strategy for the synthesis of BMS-986189 [7].

Introducing pseudoproline (Figure 7) instead of threonine, serine or cysteine can prevent this racemization upon carboxylic acid activation and is shown in an example for the synthesis of Liraglutide and Semaglutide. Furthermore, pseudoproline can reduce aggregation of peptides during SPPS. It allowed the researchers to build three segments of Liraglutide and Semaglutide and condensate these in two steps on a solid support to obtain the final product in increased yield and purity. Furthermore, they describe that the impurities which are formed are more easily separated from the product. However, cleaving of pseudoproline from the resin was slightly more difficult as the standard cleaving method also resulted in a cyclization reaction of pseudoproline forming diketopiperazine. Therefore, an extra optimization was required in the development process to obtain a proper protocol for peptide cleavage. [16].

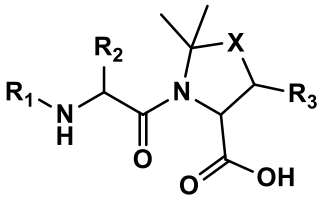


Figure 7: The possible structures of pseudoproline in which R_1 is the remainder of the peptide chain, R_2 is the remainder of any amino acid, R_3 = H or CH_3 (H for cysteine and serine, CH_3 for threonine) and X = O or S (O for serine and threonine and S for cysteine).

Another convergent ligation method uses the spontaneous rearrangement of a thioester with a *N*-terminus cysteine (Figure 8). This is formed by the reversible coupling between a C-terminal thioester and the *N*-terminal cysteine and is also referred to as native chemical ligation. The rearrangement is irreversible and driven by a favorable geometric arrangement in which the formed thioester rearranges into the wanted peptide bond. This method is usually combined with SPPS, which is used to synthesize the different fragments, followed by the coupling of these fragments with the chemical ligation method [26, 27, 28, 29]. This method has similar advantages as the other convergent methods. Another advantage of this method is the ability to perform the ligation reaction in aqueous solution, thus eliminating the need for toxic solvents in this part of the synthesis. Furthermore, great yields can be obtained with this technique, no racemization is observed and no protection groups on the peptide building blocks are required [27]. This method has been applied for the one-pot coupling of three peptide building blocks in the total synthesis of crambin. Using this method the researchers were able to improve the overall yield of crambin from roughly

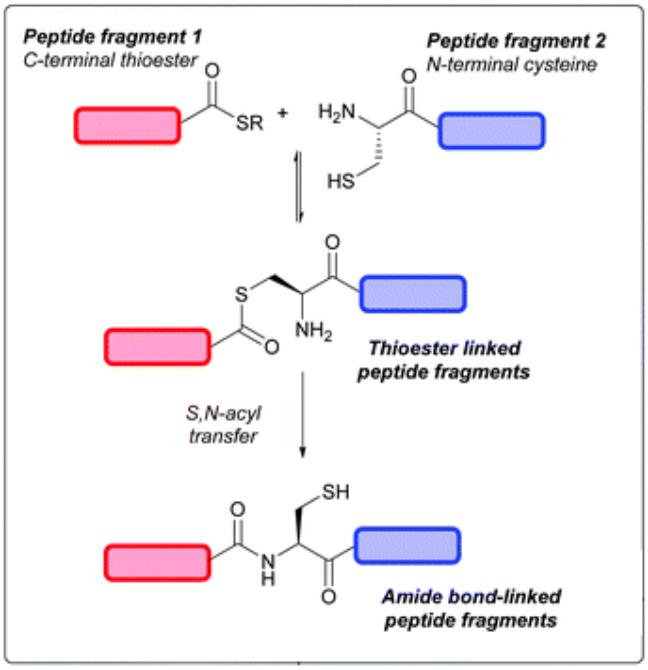


Figure 8: Picture taken from Chem. Soc. Rev. (2021) Spears, R.J. et al. The native chemical ligation method by the formation of a thioester which rearranges into an amide bond [29].

25% to roughly 40% and reduce the time required for its synthesis from ten days to two days [26]. However, some researchers mentioned that this native chemical ligation method cannot be used on large scale because of poor stability of the peptide thioester [30].

Improving the yield and purity of a production process indirectly influences the PMI of a process as less material is required to obtain the same quantity of product when yield is improved. Furthermore, increased purity might lead to a reduction in purification efforts which could reduce the amount of waste produced in the purification and isolation steps. Especially, the isolation seems to be an important factor in the total PMI as observed for the data from BMS-986189. Thus, the improved purity with the easier removed impurities might lead to a significant reduction in PMI. However, the pseudoproline method will require a better understanding of possible condensation site of peptide fragments. Maybe, we should focus on the discovery of other pseudo-amino acids which are able to prevent epimerization during condensation and form into natural amino acids during cleaving or deprotection. This would allow more condensation sites in a peptide and thus allow more fragmentation sites to find improvements in the convergent peptide synthesis. Furthermore, the convergent ligation method using the thioester rearrangement is quite limited as it requires a cysteine moiety in a logical coupling site.

5.3 Chemo-enzymatic peptide synthesis

Another methodology to reduce the environmental waste generated in the production of therapeutic peptides is the use of chemo-enzymatic peptide synthesis (CEPS). In CEPS the peptide ligation is performed using various ligases which have multiple advantages over the previously mentioned convergent peptide ligation methods. For example, enzymatic ligation usually have improved selectivity, require mild reaction conditions and can be performed on deprotected peptides [17, 18, 30]. The use of these deprotected peptides also reduced solvating issues seen with the protected peptides in other convergent ligation methods [23]. However, the technique is currently rarely applied because of the specificity of the enzymes which limit their broad application. Also, the enzymes are able two perform two different reactions, namely, the coupling reaction and hydrolysis of the peptide. The ratio between these is reported as synthesis over hydrolysis (S/H) ratio. Some enzymes from nature, like butelase and sortase, are able to perform peptide ligations in aqueous environments. However, these enzymes require specific sequences to recognize the peptide to be able to perform the ligation.

Peptiligase is a mutated enzyme which is able to perform chemo-enzymatic ligations of various peptide fragments as well as head-to-tail macrocyclizations of linear peptides in great yield in water (Figure 9) [30]. A recent study used this enzyme in the synthesis of aviptadil, a therapeutic peptide consisting of 28 amino acids, which can be used after COVID-19 infection. Two segments were synthesized using SPPS and these were coupled in a 76% yield which significantly improved on the existing methods in which the best overall yield obtained was 54% [23]. The utility of CEPS has also been shown in the synthesis of longer peptides using a combination of SPPS with CEPS. The synthesis of a heterodimer named PEP40233 required this method which allowed the coupling of a 61 amino acid fragment with a 47 amino acid fragment to obtain the product with 108 amino acids which would be very difficult to isolate using the standard SPPS method [31].

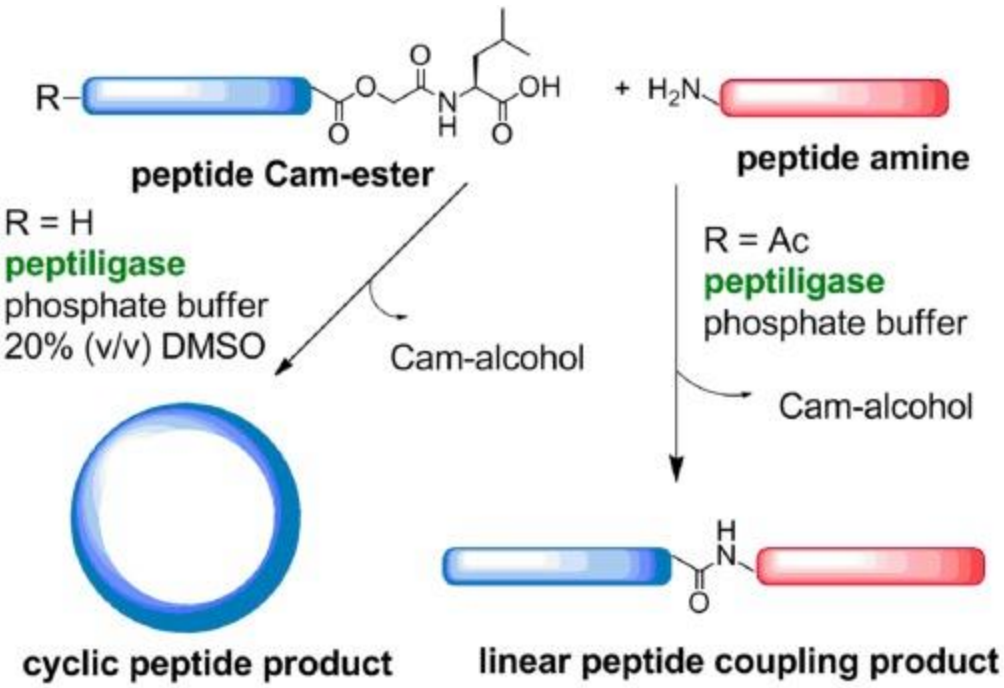


Figure 9: Adapted from Adv. Synth. Catal. (2016) Toplak, A. et al. Use of peptiligase for either a cyclization reaction or a linear peptide ligation coupling [30].

It is reported that the combination of SPPS with CEPS could reduce the environmental pollution and is competitive in the production costs compared to SPPS [19]. It mainly reduces the environmental pollution by using water as reaction solvent, removing the need to use the Fmoc protecting group and lastly by reducing the equivalents of reagents required compared to standard coupling methods [18]. However, it should be noted that this technique requires the development of a suitable biocatalyst for each coupling. Also, the pharmaceutical industry would require the complete removal of the biocatalyst from the API which needs to be identified using approved analytical methods [19]. Furthermore, conventional SPPS with all its negative impact on the environment is still a requirement in this method. Thus, even though CEPS could significantly reduce the PMI by improving yield and reduce toxic waste, we should aim to improve the environmental impact of the SPPS method itself as well. Another option might be to fully assemble therapeutic peptides via CEPS methods. However, significant steps would be required in this field to investigate this possibility since therapeutic peptide synthesis using CEPS is currently only performed in combination with other synthetic strategies, such as SPPS [18].

5.4 Continuous flow

Another method to reduce waste when using SPPS is the use of continuous flow (CF)-SPPS (Figure 10). In this method the solid resin is packed in a similar manner as silica and the amino acids are passed through this column for coupling. It starts with mixing of an amino acid with a coupling agent which are preactivated at an elevated temperature before being pumped through the column. Completion of the coupling can be determined by measuring the UV-Vis spectrum of the exiting mixture after which a piperidine solution can be pumped through the column to deprotect the peptide bound to the solid resin. The important factors to be controlled using this set-up were linear velocity, acid activation temperature, bed temperature and amino acid activation time. The optimum

conditions were determined on small scale using a column with a diameter of 10 mm for the synthesis of a peptide containing ten amino acids. These optimum conditions could also be used for a column with a diameter of 35 mm to be able to synthesize the peptide on a 20 g scale. The crude peptide was obtained in 90% purity and after isolation a yield of 89% was obtained in a total of 4 hours from starting material. The PMI of this process was reported to be 47.6 per amino acid which significantly improves on the current PMI of SPPS with its average of 875 per amino acid [32]. This method is still in the development stage and the current most up to date method allows for the synthesis of 10 to 20 g of API. Some reported advantages of this method include reduction in solvent waste, in-line process monitoring, reducing excess reagents and reducing overall synthesis time. Therefore, it should be investigated how this method can be scaled properly to obtain the API in the kilogram or ton scale to be of commercial interest.

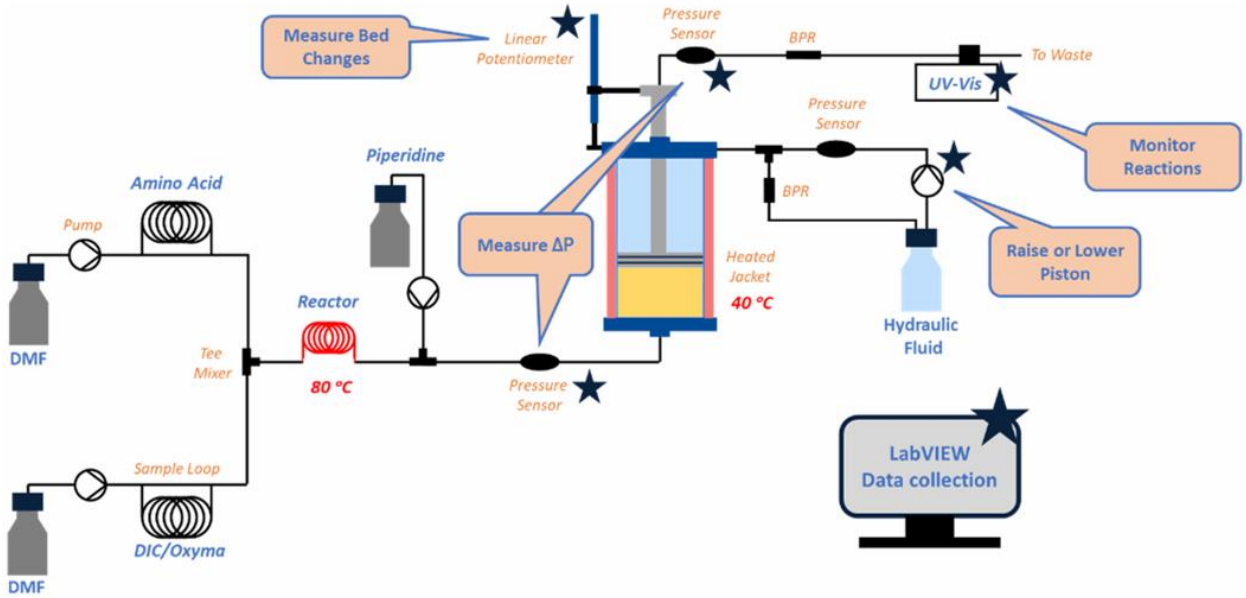


Figure 10: Picture taken from Org. Process Res. Dev. (2024) Ruhl, K. E. et al. Schematic overview of the CF-SPPS set-up [32].

For drug discovery it might also be interesting to use the so-called automated fast-flow peptide synthesis method. In this method high pressure liquid chromatography (HPLC) pumps are used to pump the required solutions to a disposable ChemMatrix PEG reactor which contains the resin required for SPPS. The solutions which are pumped towards the reactor are first mixed together followed by activation in a heating module. Using this method each coupling could be performed in 7 seconds instead of the usual hours seen in SPPS. Furthermore, the process was automated by measuring the UV/VIS-spectra of the waste stream to determine when the coupling or deprotection step was finished. This allowed for quick succession with another amino acid after each cycle is finished. Furthermore, this method is highly reproducible since it is fully automated and can be precisely controlled by the HPLC pumps [33]. Using this method, multiple long peptides have been synthesized containing 86 to 164 amino acids in only 3.5h to 6.5h. This significantly improves the speed of synthesis of these long peptides. However, the quantities obtain are in the low milligram scale which only allows for some binding assays and structure evaluation [34]. Although this method might not be usable for large-scale application, it could reduce the amount of time it takes to discover new therapeutic peptides.

5.5 Improvements in the coupling chemistry

Currently, the main amide bond formation method in the pharmaceutical industry requires carboxylic acid activation with 1-hydroxybenzotriazole (HOBt), *N,N*-diisopropylcarbodiimide (DIC) and ethyl 2-hydroxyimino-2-cyanoacetate (OxymaPure), called coupling agents. These coupling agents are needed in excess to obtain good yield and reduce racemization and epimerization of the amino acids. Furthermore, the amino acids used in the synthesis are almost always Fmoc-protected which require deprotection with excess piperidine after each coupling [17, 35]. The fact that these coupling agents are not catalytically used and that each equivalent of amino acid used also adds one equivalent of Fmoc to the generated waste results in a very poor overall atom economy. Therefore, researchers are trying to find other protecting groups which are less bulky to improve atom economy as well as safer coupling agents which would preferably be used catalytically.

Some other commonly used coupling agents are HATU, HBTU and 1-hydroxy-7-azabenzotriazole (HOAt). All these coupling agents contain some disadvantage which makes them not green or unsafe. The simultaneous use of OxymaPure with DIC is known to form the toxic gas HCN, although some methods have already been developed to make the use of these coupling agents safe. Furthermore, OxymaPure is classified as a green coupling agent [36, 37]. HATU and HBTU are also often used as coupling agents. However, they are thought to be sensitizers and, after prolonged use, can cause allergic reactions and even anaphylaxis [38]. DIC is also known to be an allergen and its use should therefore be limited [37]. Furthermore, HOAt and HOBt are categorized as a class 1 explosive and are known to be environmentally unfriendly [17]. Some known green alternatives are propylphosphonic anhydride (T3P®) [37, 39]. 5-(ethylthio)-1H-tetrazole (ETT) in combination with 1-(tert-butyl)-3-ethylcarbodiimide (TBEC) [40], ynamide derivatives [41, 42], (1-(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylaminomorpholino-methylene) ethaminiumhexafluorophosphate) (COMU), N,N,N',N'-tetramethylformamidinium hexafluorophosphate (TFFH) [37] and dipyridyl dithiocarbonate (DPDTC) (Figure 11) [43].

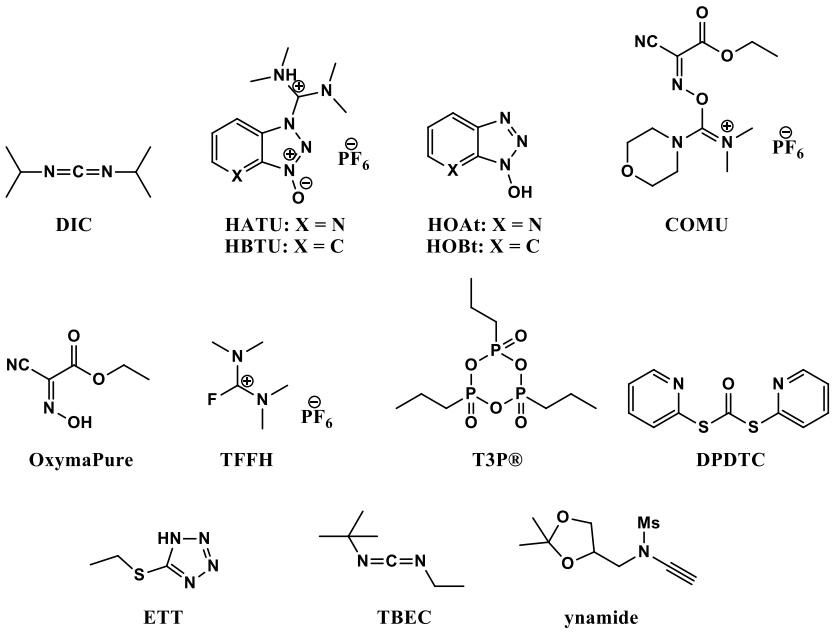


Figure 11: Figure adapted from Chem. Select (2021) Musaimi, O. A. et al. Overview of the different coupling agents used in peptide chemistry [37].

T3P® has been known for over a 40 years and is currently already used for large-scale amidation reaction in industry. However, research towards its usability for peptide couplings in SPPS is currently still very limited. The first article describing SPPS with T3P® was published in 2021 by Musaimi *et al*. Here, they showed that T3P® is compatible with SPPS and that it can be used in combination with green solvents. Furthermore, a decapeptide could be synthesized with satisfactory purity and limited side product formation [37]. T3P® has also been applied in green solution phase-peptide synthesis (GSolPPS) of a pentapeptide. Mattellone *et al*. reported the use of T3P® in GSolPPS while also removing the need for piperidine as deprotection agent by using *N*-benzyloxycarbonyl as protection group instead of Fmoc. The *N*-benzyloxycarbonyl group could be easily removed via hydrogenation. This led to the possibility to perform multiple coupling and deprotection cycles in a single pot without purifications. First, the starting amine was dissolved in EtOAc followed by the addition of T3P®, Diisopropyl ethyl amine (DIPEA) and a *N*-benzyloxycarbonyl protected amino acid under nitrogen atmosphere to obtain a protected dipeptide. $Pd/C_{10\%}$ was added after complete coupling and the flask was placed under a hydrogen atmosphere to deprotect the dipeptide. After deprotection the next amino acid can be coupled under nitrogen atmosphere by adding more T3P® and DIPEA and the coupling and deprotection cycle was repeated until the wanted peptide was obtained. Using this method the hazardous piperidine and DMF were not needed, PMI per amino acid was reduced to 30 and a less harmful coupling agent was used [39]. Although this method already significantly improved on the current SPPS methods it could still use significant improvements to be a true green protocol. T3P® and DIPEA are required in excess for the addition of each amino acid. Furthermore, a large protection group is still used and removed after each coupling which negatively effects the overall atom economy of this protocol.

The use of DPDTC might also be interesting in future for peptide synthesis and has currently been applied for the synthesis of amides bonds. The coupling of an amine and carboxylic acid using DPDTC has been tried without solvent, with a concentration of 2M in EtOAc and in aqueous conditions using micelle techniques. All gave satisfactory results and can be used depending on the nature of the starting materials used. The reaction mechanism is similar to that of other coupling agents. DPDTC is able to react with the acid to form a thioester intermediate which activates the acid to allow the attack of an amine to form the amide bond. The reaction has been tried in the presence of many other functionalities and contains a single example in which one amino acid substrate was used. Furthermore, only a minor excess of the amine and coupling agent were required to obtain good yield [43]. This method might be interesting to further expand if it can be used in the synthesis of longer peptides using a wider variety of amino acids. Since the reaction can be performed in EtOAc it could be applied to SPPS or even in solution-phase peptide synthesis.

The use of ETT also requires more research to expand its scope and determine its suitability in peptide synthesis. A first investigation into its use showed that the formed intermediate was slightly more prone to racemization because of a higher acidity of the formed leaving group. This was not an issue for serine and cysteine, however, it did give higher levels of racemization when trying to couple histidine [35]. Its combined use with TBEC has also been investigated in which the sterically hindered coupling between two Aib residues could be performed. The combination has also been used in the synthesis of a decapeptide in which a purity of 99% could be obtained. However, the overall yield was not reported nor the exact percentage of racemization [40].

Another interesting coupling agent is an ynamide derivative. This coupling agent has been reported to show no racemization or epimerization. Furthermore, its use in peptide chemistry has been proven by the synthesis of a tetrapeptide named carfilzomib. The overall yield improved from 26%-

36% to 70% when using ynamide as coupling agent compared to other coupling agents. Another advantage of ynamide is that it can be removed from the reaction mixture by a simple acidic aqueous work-up. This removes the need to perform column chromatography to remove the formed byproducts which is normally needed when using other coupling agents [42].

To summarize, great improvements have been made in the last few years to implement green coupling agents in peptide synthesis. Furthermore, these green coupling agents have been applied in combination with greener solvents which is also important for the overall improvement to obtain a green SPPS. Multiple examples have been given in which great successes were obtained. Now, it is important to optimize these existing protocol for a wider scope of peptides on production scale and verify their utility for the synthesis of longer peptides. Furthermore, it is still important to discover a catalytic coupling agent to get to the next step in green sustainable chemistry. Some catalytic coupling agents have been discovered, however, they are currently to be applied in SPPS.

Replacing the Fmoc protecting group is currently understudied and could also lead to a drop in PMI and improve the AE [2]. Most replacement studies performed are focused on easier removal of the protection group or focus on other limitations of Fmoc. For example, 2,7-Disulfo-9-fluorenylmethoxycarbonyl (Smoc) has been developed to allow peptide synthesis in aqueous environments while also removing the need for piperidine as deprotecting agent (Figure 12). Its easy removal with sodium hydroxide, ammonia or other aqueous bases are a first step to a greener protecting and deprotecting cycle in peptide chemistry [44]. However, significantly smaller in Mw protecting agents should be investigated in the future to obtain improvements in the AE.

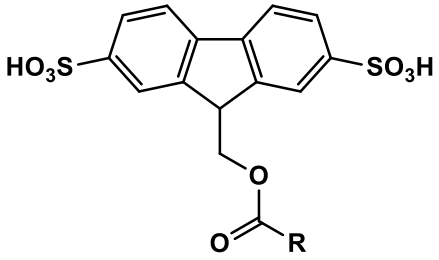


Figure 12: Structure of SMoc in which R can be any amino acid.

5.6 Improvements in purification

In contrast to small drug molecules, final peptide products are usually purified using reversed-phase (RP)-HPLC or ion-exchange chromatography (IEC), while small drug molecules are purified using recrystallization. RP-HPLC is not preferred on large-scale because it requires the use of high volumes of solvents to obtain satisfactory purity and, in most cases, multiple columns are needed to remove the impurities. This inefficient purification system accounts for roughly 50% of the total PMI, although the waste mainly consist of water and acetonitrile which are considered green and problematic respectively. Unfortunately, recrystallization of middle to large linear peptides is usually not possible since a high crude purity is required. Also, the increase in purity using recrystallization is only minimal for larger peptides [2]. Therefore, improved HPLC methods and other techniques have been investigated to purify therapeutic peptides and reduce the environmental pollution.

One of the improvements made for RP-HPLC was changing ACN for the more environmentally friendly DMC. A case study, using this solvent in the purification process of Icatibant, reported an improved separation in which faster run-times, lower solvent usage and similar peptide recovery rates were obtained [45]. This small change can be easily implemented in existing protocols and might be combined with other improved purification methods to further reduce their environmental footprint.

A study towards improving the purification of BMS-986189 used a combination of medium-pressure reversed-phase LC (MP-RPLC) and supercritical fluid chromatography (SFC) [46]. SFC is thought to be less polluting because it uses less harmful solvents compared to RP-HPLC and implements the use of CO_2 as mobile phase which allows for easier recovery. Also, SFC can use shorter protocols because of better separation of compounds which reduces solvent consumption [2]. First, the crude peptide, with a purity of roughly 50%, was purified using MP-RPLC to remove residual trifluoroacetic acid (TFA) from the peptide cleaving process as well as other polar impurities. This resulted in roughly 80% purity and could be scaled in the lab to a maximum loading of 20 g crude peptide per injection. Isocratic SFC was performed next with the idea to increase the throughput of the purification method by allowing stacking of injections (Figure 13). The injections were separated by 2.5 min and fractions containing the desired compound were combined to obtain a >95% purity with a decent recovery of >70%. Around 30 g of the therapeutic peptide was isolated by this method with a throughput of 0.96 g/h and was described as the first large-scale peptide purification with SFC [46]. The PMI of this purification method has not been calculated by the authors, however, they do mention a lower solvent usage. Also, stacking of the injections allowed for the purification of multiple injections at the same time which likely contributes to the lower solvent usage and allows for a continuous process. Furthermore, the combination of MP-RPLC and SFC are both scalable to allow the isolation of larger quantities.

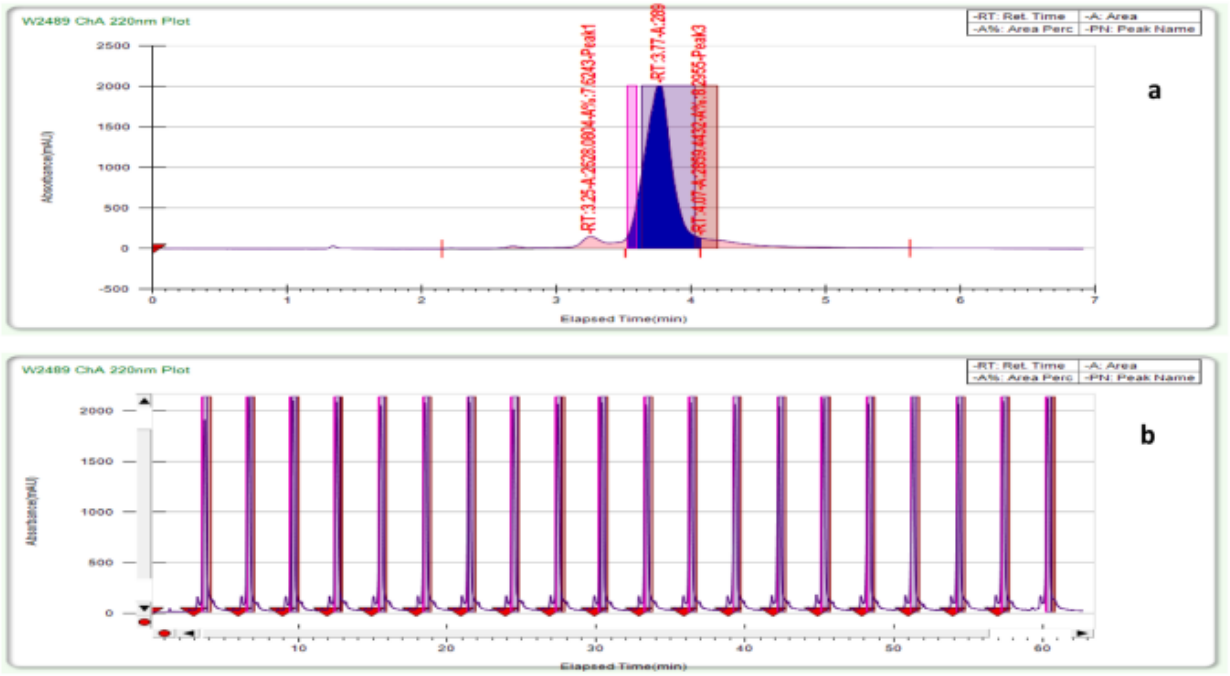


Figure 13: Picture taken from J. Chroma. A (2024) Li, P et al. Chromatogram of a stacked injection method when using isocratic SFC in which a) shown the spectra of a single injection and b) the spectra of a stacked injection with an interval of 2.5 min *[46]*.

Another method to reduce environmental pollution is multicolumn countercurrent solvent gradient purification (MCSGP). This method focusses on improving the recovery during purification instead of reducing waste in the purification step by using multiple columns. Any loss of product during purification results in the use of a larger amount of starting material to make up for this loss of product. Thus, this increases the produced waste throughout the entire production process. Therefore, maximizing the recovery during purification without reducing purity can significantly reduce the overall PMI even though the purification method itself does not reduce waste [2, 18]. This

method has been applied for the purification of glucagon in which a two-column system was used. Both columns used an identical stationary and mobile phase but are shifted by a half cycle (Figure 14). In the first cycle the first column is loaded with fresh feed (zone 2) and starts eluting in zone 4. This zone only contains weaker absorbed material (W) and can be discarded. Zone 5 contains a mixture of W and the product (P) which is transferred to the second column (zone 5 to zone 1) which starts its loading phase (zone 1, 2 and 3). After this mixture pure P elutes which is collected followed by a mixture of P and stronger absorbed material (S) in zone 7. This stream is also transferred to the second column which will be in zone 3. Lastly, in zone 8 only S is eluted which is discarded. After this, the second column becomes the first and the first column becomes the second to allow the cycle to continue. Note that the uptake of different streams by the second column require a mobile phase in which none of the compounds starts eluting. Furthermore, before loading of the different streams from the first column onto the second column, a dilution step is required to prevent elution of compounds on the second column. An improved recovery of glucagon from 71% using the standard batch mode RP-HPLC to 88% using this MCSGP set-up was obtained [47]. Unfortunately, the authors did not mention how this affected the overall PMI of the production and isolation. However, this significant improvement in isolation should reduce the overall PMI even though this method required a slightly higher solvent usage. Furthermore, this method can be further improved on by implementing the green solvent DMC instead of the used ACN.

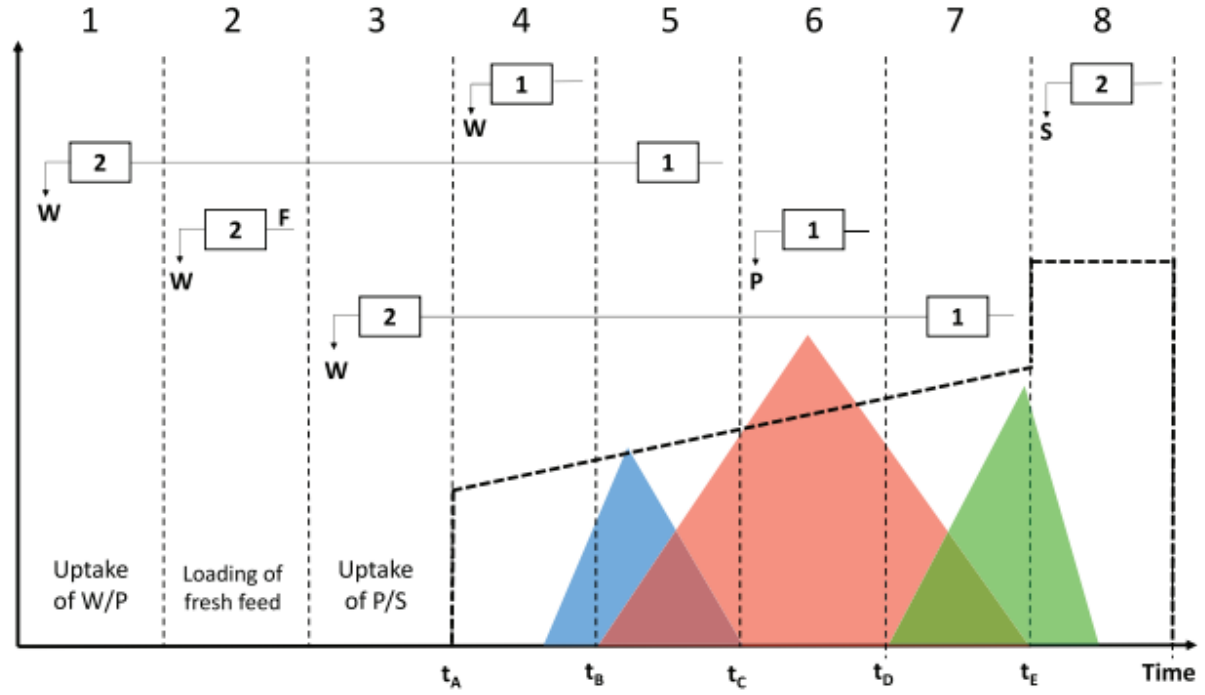


Figure 14: Picture taken from J. Cromat. A (2020) De Luca, C. et al. Schematic overview of the principle of a two-column system for the purification of a therapeutic peptide using MCSGP [47].

6. Green by design

Access to drugs, including peptide pharmaceuticals, is essential for human health. However, their use can have significant downstream effects on the environment after use [5, 48]. As mentioned in the introduction, a global study towards API pollution in the world rivers shows the significance of this pollution. However, it should be noted that this study did not contain any peptide therapeutic but consisted of over 60 small drug compounds [6]. As the environmental health also impacts human health we must look for methods to reduce the environmental pollution of APIs. An acronym to address this issue is GREENER which stands for: "***G****ood practice for patients,* ***R****educed off-target effects, high specificity,* ***E****xposure reduction via less emissions,* ***E****nvironmental (bio)degradability,* ***N****o PBT (persistent, bioaccumulative as well as toxic) substances,* ***E****ffects reduction: avoid undesirable moieties and,* ***R****isk and hazard mitigation*". The goal of this concept is the reduction of the environmental impact of APIs after patients use [5].

A reduction in environmental pollution after patient use in developed countries could be to improve the current sewage treatment plants by implementing membrane filtration [49]. However, this method does not target the underlaying issue, namely the excessive stability of pharmaceutics after use, and will likely not help in LMICs. Therefore, the focus should be towards a reduced environmental stability of future APIs without decreasing their efficacy or stability when in humans. It seems impossible to need good stability when a compound is in use and a poor stability after use. However, some examples already exist to prove its feasibility such as replacing a C-F bond for a C-OH bond (Figure 15). This minor change already significantly improved the biodegradability without reducing the pharmaceutical properties [50]. Furthermore, APIs might be made light sensitive, prone to hydrolysis or oxidation and/or easily degraded by environmental bacteria, which does not have to be an issue when correctly using the compound, and helps environmental degradation [51]. Some chemical structures are known to improve biodegradation while others reduce this leading to significant longer degradation times (Table 2) [52].

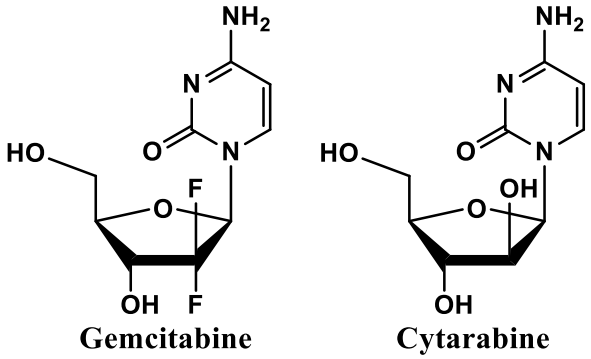
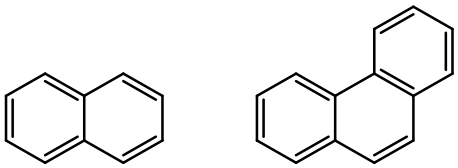
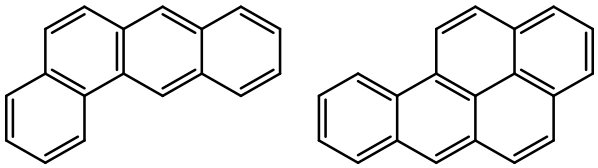
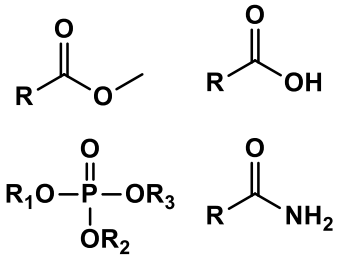
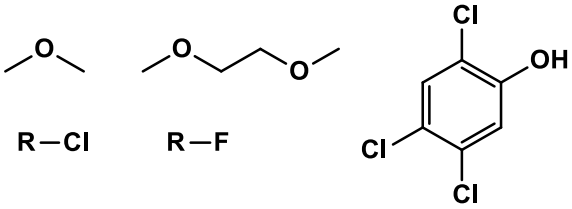
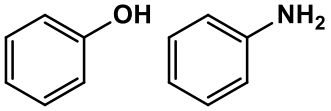
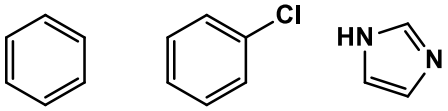
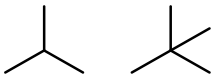
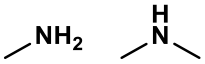
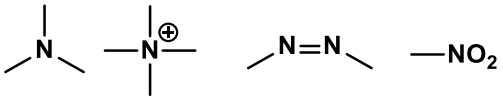
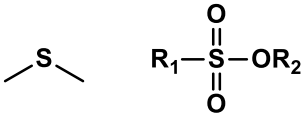
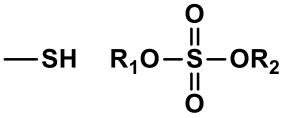


Figure 15: Figure adapted from Green Chem. (2007), Kummerer, K. et al. Structures of Gemcitabine and Cytarabine [50].

Based on this table the structures of the four different therapeutic peptides should be decently biodegradable. The amide bonds and esters, which are the main functionalities, should improve the biodegradability of these compounds. However, BMS-986189 and CyA contain quite a few methylated amides which might hinder biodegradability. This table also shows that the linker used in Liraglutide is likely easier to degrade compared to the linker used in Semaglutide, which contains multiple ether functionalities.

Table 2: Table adapted from Green Chem. (2023), Castiello, C. et al. Overview of different functional gropus which either improve or hinder biodegradability of APIs [52].

Enhancing biodegradability	**Hindering biodegradability**
Polycyclic aromatics < 3 rings 	Polycyclic aromatics > 3 rings 
Carboxylic acid, (phosphate-)ester, amide 	Aliphatic esters and halogens (Especially polyhalogenated, >3) 
Phenol and aniline (Especially with electron-donating substitution) 	Benzol and heterocycles (Especially electron-withdrawing substitution) 
Unbranched alkanes or alkenes (Especially C_4-C_5) 	Branched alkanes (Especially electron-withdrawing substitution) 
Primary and secondary amines 	Tertiary/quaternary amines, azo-, nitro-groups 
Thioesters and sulfonates 	Thiols and sulphates 

The incorporation of quantitative structure–activity relationship (QSAR) tests and machine learning (ML) into the development process of new APIs might also be used for the development of greener APIs. These tools could help in obtaining expected toxicologic data and biodegradability of new compounds before synthesizing and testing them. Furthermore, these techniques might be used to determine which functionalities might be interchangeable to improve the expected biodegradability without reducing the theoretical binding affinity. Although this could significantly increase the speed of discovery of potential new compounds, it could also lead to missing data by rejecting poor results of the ML-data without experimentally confirming this. However, it expected that these modeling techniques will significantly improve the efficiency in drug discovery which could lead to environmentally safer products [48].

7. Conclusion

Many great attempts have been made to improve the production of peptide therapeutics. Currently SPPS, recombinant or semi-recombinant techniques are used for the production of these peptides which are highly pollutive production methods. The production of peptides using recombinant or semi-recombinant techniques produce less waste compared to the present large scale SPPS production process. The issues addressed for SPPS are, the use of large amount of toxic solvents, the use of environmentally harmful coupling agents, its poor AE, and the waste produced in purification. Multiple reports have shown that the toxic solvent DMF can be replaced for more environmentally safe solvents such as TEP or the combination of NOP and DIC. The amount of solvent used, as well of other waste products, can be significantly reduced by the use of convergent, chemo-enzymatic or continuous flow synthetic methods. Of these methods the continuous flow production method seems to be most promising as it was able to reduce the PMI required per amino acid addition from the average of 875 to 47.6. Furthermore, this method has been shown to be effective on multigram scale and is further scalable according to the authors. Other reports have shown that the harmful coupling agents, which are currently in use, can be changed for other less harmful ones. One of these is T3P® which has been applied in a GSolPPS which allowed the researchers to produce a pentapeptide with a PMI of 30 per amino acid. An understudied part in SPPS is the improvement in AE by replacing the bulky Fmoc protection group. The protection strategy of the α-amine should be rethought in the near future to be able to significantly improve the AE. Purification of therapeutic peptides is another part which has been addressed in literature. Improvements have been made by using either a combination of MP-RPLC and SFC or by using MCSGP in which the latter required less adaption from the current chromatographic separation methods. Lastly, the concept: “Greener” shows which steps are required to prevent build-up of long lasting chemicals in nature. Also, the use of QSAR and ML should help to predict biodegradability of new compounds and thus help researchers in the drug discovery process.

8. Future prospects

Some improvements in the AE of SPPS have been described by Fantoni *et al.* and Yang *et al*. These articles focused on the use of side-chain unprotected amino acids, such as Arg/His/Tyr. Thus, reducing the need to protect and deprotect these side-chains in SPPS and improve the AE when coupling these amino acids.

- Fantoni T, Orlandin A, Di Stefano I, et al. Solid phase peptide synthesis using side-chain unprotected arginine and histidine with Oxyma Pure/TBEC in green solvents. *Green Chem*. 2024;26(1):10929-10939. doi:10.1039/D4GC03209H
- Yang Y, Hansen L, Per R. Side-Chain Unprotected Fmoc-Arg/His/Tyr-OH Couplings and Their Application in Solid-Phase Peptide Synthesis through a Minimal-Protection/Green Chemistry Strategy. *Org. Process Res. Dev.* 2022;26(5):1520-1530. doi:10.1021/acs.oprd.2c00083

Other improvements to reduce the PMI of SPPS are described by the authors below. Collins *et al.* describes the use of microwave irradiation to remove the Fmoc-deprotection agent, pyrrolidine, to allow for a wash-free protocol. This reduced the produced waste by roughly 80% for the production of Liraglutide without effecting the purity. Jaradat *et al.* gives an in depth review of new aqueous methods in SPPS which eliminates the use of toxic solvents in the synthetic part of the production process.

- Collins JM, Singh SK, White TA, et al. Total wash elimination for solid phase peptide synthesis. *Nat Commun*. 2023;14(1):8168. doi:10.1038/s41467-023-44074-5
- Jaradat DMM, Al Musaimi O, Albericio F. Advances in solid-phase peptide synthesis in aqueous media (ASPPS). *Green Chem.* 2022;24(1):6360-6372. doi:10.1039/D2GC02319A

Another improvement in the purification process has been described by Barredo-Vacchelli, *et al*. In this article a final two-step deprotection method has been developed. In this method the peptide is first deprotected followed by cleaving the unprotected peptide from the resin. This significantly improved the purity profile, reduced the amount of washing solvent required and allowed for an easier downstream purification method.

- Barredo-Vacchelli GR, Rodríguez JA, Eloy JA, Camperi SA. A Novel Method for Liraglutide Synthesis and Purification. 2024;116(5):e24351. doi:10.1002/pep2.24351

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