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Grau de Química

Treball Final de Grau

Literature search about the impact of green chemistry on solid-phase peptide synthesis.

Recerca bibliogràfica sobre l'impacte de la química verda en la síntesi de pèptids en fase sòlida.

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Si he fet descobriments inavaluables ha estat més per tenir paciència més que qualsevol altre talent.

Isaac Newton

M'agradaria agrair-li al meu tutor Dr. Ernesto Nicolás Galindo tot el temps que ha dedicat a ajudar-me i guiar-me al llarg d'aquest projecte. Gràcies als seus ànims i la seva amabilitat ha estat un camí molt més fàcil.

REPORT

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1. SUMMARY

Solid-phase peptide synthesis is a widely used strategy today, to produce peptides that can later be used in the pharmaceutical industry as they have therapeutic properties, among many others. In order to produce a peptide, throughout the synthesis solvents, reagents, additives, and bases are used that are considered toxic, dangerous, and even some have restrictions on their use. In addition, the strategy generates a large amount of waste because it is usual to work with an excess of reagents and a number of washes have to be performed. As it is a widely used synthesis, both at an industrial and research level, and thanks to the awareness of the ecological footprint that has been growing in recent years, the synthesis of peptides is being influenced by the use of green chemistry. That is, ecological alternatives to traditional products have been studied over the last few years, with the aim of producing peptides with similar yields and purities to those obtained with the classical approach but reducing toxicity and waste. In this work, a bibliographic study has been carried out on the green alternatives that have been investigated over the last years from the information given by recent reviews (2019 and 2020). Since the synthesis published by Merrifield for the first time dated in 1963, it was not until the decade of the present century that green chemistry has begun to be involved in the seek of synthetic alternatives more healthy and amenable to the environment. Several protocols have been proposed so far that replace the traditional products with less harmful and more compatible with the green chemistry postulates, and that have been considered promising by researchers according to the results achieved in different aspects of the synthesis (resin swelling, the solubility of reagents, yields and purities). This work tries to give the state-of-the-art in this field.

Keywords: Solid Phase Peptide Synthesis, Green Chemistry, SPPS in Green Chemistry.

2. RESUM

La síntesi de pèptids en fase sòlida és una estratègia molt utilitzada en l'actualitat, per a produir pèptids que poden ser utilitzats en la indústria farmacèutica per tenir propietats terapèutiques, entre moltes altres. Per produir un pèptid, al llarg de la síntesi s'utilitzen dissolvents, reactius, additius i bases que es consideren tòxiques, perilloses i fins i tot algunes tenen restriccions en el seu ús. A més, l'estratègia genera una gran quantitat de residus perquè és habitual treballar amb un excés de reactius i cal realitzar una sèrie de rentats. Al tractar-se d'una síntesi molt utilitzada, tant a nivell industrial com de recerca, i gràcies a la consciència de l'empremta ecològica que ha anat creixent en els últims anys, la síntesi de pèptids està sent influenciada per l'ús de la química verda. És a dir, en els últims anys s'han estudiat alternatives ecològiques als productes tradicionals, amb l'objectiu de produir pèptids amb rendiments i pureses similars als obtinguts amb l'enfocament clàssic, però reduint la toxicitat i els residus. En aquest treball s'ha realitzat un estudi bibliogràfic sobre les alternatives verdes que s'han investigat en els últims anys a partir de la informació aportada per les revisions recents (2019 i 2020). Des de la síntesi publicada per Merrifield per primera vegada el 1963, no és fins a la dècada d'aquest segle que la química verda ha començat a involucrar-se en la recerca d'alternatives sintètiques més saludables i amigables amb el medi ambient. Fins al moment s'han proposat diversos protocols que reemplacen els productes tradicionals per altres menys nocius i més compatibles amb els postulats de la química verda, i que han estat considerats prometedors pels investigadors segons els resultats obtinguts en diferents aspectes de la síntesi (inflament de la resina, solubilitat de reactius, rendiments i pureses). Aquest treball intenta mostrar les últimes investigacions d'aquest camp.

Paraules clau: Síntesi de pèptids en fase sòlida, química verda, SPPS en química verda.

3. INTRODUCTION

Throughout the years, different fields of application of peptides have been discovered, from hormonal functionality to antibiotics. Furthermore, studies have shown the potential of peptides in drug synthesis.[1,2] The importance that peptides have acquired in recent years is due to their therapeutic role on different diseases such as diabetes, cardiovascular problems, and cancer, among others. Their use in other fields such as cosmetics, supplements, immunology, or diagnostics has also increased. All the interest they are generating is due to their high specificity, low toxicity and fewer side effects compared to other small molecules that used to be used for drug production in the past.[3,4] For these reasons, researchers and the pharmaceutical industry have been involved in the development and production of peptides in recent years, where clinical trials have been carried out where it has been possible to commercialize some of the peptides. All this is leading to an increase in the production of pharmaceutical peptides.

Peptides are molecules formed by the union of amino acids; these are linked together by covalent bonds known as peptide bonds. The formation of the peptide bond is a dehydration reaction, which involves the loss of a water molecule for each peptide bond generated. All peptide structures are made up of a terminal amino and a terminal carboxyl, as seen in *Figure 1*. Furthermore, the side chains may or may not be functionalized. The most common way to name amino acids is by using the 3-letter code. In *Figure 1* there are three amino acids, glycine, serine, and valine linked together by peptide bonds, whose 3-letter code is Gly, Ser and Val, respectively. The peptide that these amino acids make up through the 3-letter code would be H-Gly-Ser-Val-OH.

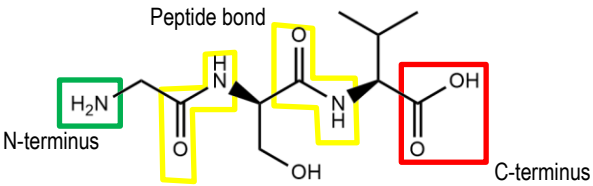

Figure 1. An example of a peptide (tripeptide), H-Gly-Ser-Val-OH. These amino acids in particular are naturally proteinogenic, of which there are 20. The configuration of these amino acids is S. tBu

3.1. SOLID-PHASE PEPTIDE SYNTHESIS

The most used strategy to synthesize peptides is the solid-phase peptide synthesis (SPPS), of which Merrifield first published in 1963.[5] Due to the wide applicability of peptides there has been an increasing interest in this synthesis. Consequently, the improvements carried out have allowed many advances in peptide chemistry, such as starting by synthesizing simple peptide chains and today it is possible to do it with longer and more complex chains.

In the SPPS, here are two strategies: The fluorenylmethoxycarbonyl/*tert*-butoxycarbonyl (Fmoc/tBu) strategy and the *tert*-butoxycarbonyl/benzyl (Boc/Bzl) strategy (*See annex 1*).[6] These protecting groups can be divided into temporary and permanent. Fmoc and Boc belong to the group of temporary protecting groups since they are removed each time an amino acid is added to the peptide sequence. On the other hand, bzl and *t*Bu are permanent protecting groups since they are not eliminated until the end of the synthesis because they protect side chains.[7]

In the Fmoc strategy, resin and side-chain groups eliminations are orthogonal (*Figure 2*). Thus, Fmoc group elimination is performed using piperidine (PP) (basic conditions), whereas peptide-resin bond cleavage and side-chain groups removal are performed by an acid like trifluoroacetic acid (TFA). However, in the Boc strategy, this elimination takes place by strong and hazardous acids like hydrofluoric acid (HF) or trifluoromethanesulfonic acid (TFMSA) (*Figure 3*).

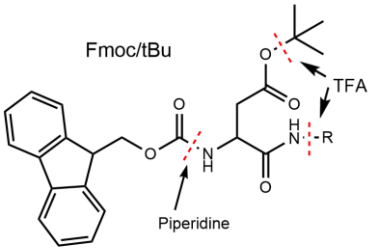

Figure 2. Fmoc Deprotection and cleavage points.

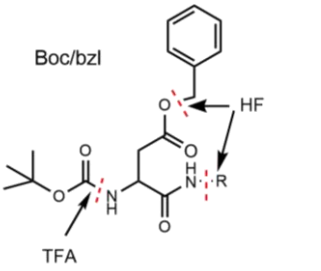

Figure 3. Boc Deprotection and cleavage points.

The reagents used in each strategy for deprotection are the main reason to use Fmoc/*t*Bu as usual and Boc/benzyl on specific occasions, due to HF is a strong acid and it is highly toxic and dangerous.

Although the Fmoc strategy is less dangerous than the Boc strategy, it is necessary to increase its ecological footprint and to combine it with green chemistry because of the toxic solvents used during the process like *N*,*N*-dimethylformamide (DMF) and dichloromethane (DCM), among others, and the amount of them that is utilized because of the number of washes and steps of synthesis.

Scheme 1 shows a generalized scheme of SPPS since both the Fmoc and Boc protocols can be applied. A crucial issue of the synthesis falls on the swelling of the resin because it will influence the process since the same solvent can perform differently depending on the resin. The base composition of the resins differentiates 3 groups: polystyrene (PS), polyethylene glycol (PEG) and those grafted that are a mixture of PS and PEG. The swelling of the resin is a critical step in the synthesis, since with good swelling the reactive functional groups of the resin are available on the surface of the resin to allow the coupling of amino acids and thus carry out a successful synthesis. DCM, DMF and *N*-Methylpyrrolidone (NMP) are the most widely used solvents in the traditional protocols.

The coupling of the first amino acid to the resin uses to be through an ester bon and the experimental conditions depend on the type of functionalization of the resin. The right functionalization for the synthesis is achieved by utilizing a suitable linker.

Amino acid couplings take place by prior activation of the carboxyl group of the amino acid. Carbodiimides can be used for activation (*See annex 2*) but they can provoke racemisation. To remedy this, additives such as 1-Hydroxy-7-azabenzotriazole (HOAt) and (1-hydroxybenzotriazole (HOBt) (*See annex 1*), or other sophisticated coupling agents with *N*,*N*-diisopropylethylamine (DIPEA) as a base (*See annex 1*) are used. The capping step consists of an additional reaction in which acetic anhydride is used to block amino acids that have not reacted due to ineffective coupling.

In the Fmoc strategy, Fmoc removal is performed by using 20% of PP in DMF (*See annex 2*) and a mixture of TFA with a carbocation scavenger is utilized to remove the side chain protecting groups from the peptide and to cleave peptide from resin.

In spite of being the SPPS so useful, the synthetic process requires the use of a large number of toxic and dangerous solvents such as DMF and NMP which are classified in Registration, Evaluation, Authorisation, and restriction of Chemicals (REACH) by European chemicals Agency (ECHA) as very high concern.[8,9] Moreover, a number of washes are needed to remove the by-products and excess of reagents. These problems can cause serious damage to people and it also makes a negative environmental impact.

For this reason, there have been several studies over time to reduce environmental impact and contribute to more efficient and sustainable production implementing the principles of green chemistry on the process.

Scheme 1. SPPS of the tripeptide H-Gly-Ser-Val-OH

3.2. GREEN CHEMESTRY

The goal of Green Chemistry is not only to reduce, or even avoid, the use of dangerous solvents during a chemical process, it also improves and changes other factors that can influence on chemical procedures and reactions such as the designing of safer procedures and products. This kind of chemistry focus on find greener alternatives to those procedures and solvent which are hazardous and harm the environment. Besides that, chemical industrialization has been suffering an increase over the last years, and consequently, more waste and pollution has been generated. In 1998, Anastas et al presented what has been called the principles in which the green chemistry is based[10,11]:

1. Waste prevention: It is preferable to reduce waste products rather than clean the waste that has produced.
2. Atom economy: Optimize processes so that the use of reagents is the maximum over the final products. In other words, the number of reactive atoms should be the maximum possible on the products.
3. Less dangerous chemical synthesis: Reorganize chemical procedures in a way that has reduced toxicity and hazard using less harmful products and reactions.
4. Develop safer chemicals and products: Design products using efficient chemical processes and reduce their damaging effects in the meantime.
5. Safer solvents and auxiliaries: Promote the use of safe and green solvents and avoid the use of auxiliaries which produce more waste, it is also essential that when we use them use the innocuous ones.
6. Energy efficiency: Minimize the economic and environmental effects of the chemical processes in energy requirements. For this reason, use environmental pressure and temperature are preferable.
7. Renewable feedstocks: Avoid the use of consumable materials and promote the use of renewable raw materials.
8. Avoid or reduce derivates: Use derivates generates extra waste because of extra steps reagents needed.
9. Catalysis in favor: Catalysis has shown more efficiency than stoichiometric reactions, and it also allows us to carry out unfavorable reactions.
10. Biodegradation: Chemical products produced should reduce themselves after use. Chemical products should have an innocuous degradation.

11. Real-time analysis to prevent pollution: It helps to monitored procedures and control the prejudicial waste formation
12. Accident prevention: Planning solvents and chemical substances before starting a procedure to reduce of accidents such as explosions and releases, among others.

4. OBJECTIVES

Due to the great relevance that the SPPS has achieved, the main goal of this project has been to carry out a bibliographic study of the way in which green chemistry is influencing the classical synthetic approach.

5. METHODS

Based on the objective of the project, a bibliographic review was proposed based on two reviews,[12,13] for which an intersection of references from the two reviews was carried out, in addition to seeking complementary information on some concepts. Because green chemistry in SPPS is a concept that has started to be implemented relatively recently, starting in '00s, the search has focused on the last two decades. The databases that have been used are Scifinder and Reaxys, updating the search weekly and in this way being up-to-date with all the articles published about studies on the use of green chemistry protocols in SPPS.

At the time of writing, an order has been followed, starting with solvents, and swelling, followed by coupling reagents and additives, and ending with the elimination of the Fmoc group. The writing has been developed temporarily, starting with the first years, and ending with the most recent about each specific solvent, reagent, or base.

6. RESULTS AND DISCUSSION

Among the different aspects of SPPS, the studies have been focussed mainly in the search for alternatives to the traditional solvents that are used, such as DMF, DCM and NMP, since they are harmful to both health and the environment, and do not contribute to improving the environmental impact. *Table 1* shows the classification of the three main solvents and different guidelines such as Pfizer, Sanofi and GSK among others, which focus on analysing them by their characteristics.

Solvent	AZ	ACS GCI-PR	GSK	Pfizer[a]	Sanofi[b]	Overall[c]	Hazardous Attributes
DMF				Und.	S.R		Reproductive toxicity, harmful, irritant
DCM				Und.	S.A	CC	Cancerogenic, irritation.
NMP				Und.	S.R		Reproductive toxicity Irritant, harmful.

Table 1. Classification of the traditional solvents used in SPPS and different guidelines. Green: recommended, yellow: Problematic, orange: problematic or hazardous, red: Hazardous. [a]Und.: Undesirable. [b]S.A: substitution advisable, S.R: substitution requested. [c]CC: Candidate to be confirmed by EACH, according to the REACH regulation for substances of very high concern (SVHC).[14,15,16,17,18,19]

The substitution of PP by other solvents has also been studied over the years. This base is used to remove the temporary protecting group (Fmoc/Boc), which is flammable, corrosive and irritating. Besides, PP is a controlled and regulated substance, according to the 91/109/EC recommendation by the Drug Enforcement Agency (DEA), since it is used in the synthesis of narcotic drugs and psychotropic substances and generates large amounts of toxic waste, consequently increasing the manufacturing costs of peptides.

The substitution of coupling reagents and additives, derived from benzotriazol, has also been considered due to the explosive properties that they can have in some situations.[20] Therefore, they are not considered very stable products.

6.1. SOLVENTS

Two types of solvents are distinguished in spps. These are the aprotic and protic solvents, where different types of solvents make up both groups (see *table 2*).

Solvent↓	Swelling	Coupling 1st aa	Fmoc removal	Coupling step	Cleavage step	Washings
2-MeTHF	✓	✓	✓	✓		✓
Anisole	✓			✓	✓	✓
CPME	✓		✓ *	✓		
Isosorbide	✓		✓ *			
GVL	✓	✓	✓	✓		✓
EtOAc	✓		✓			✓
NBP	✓				✓	
NFM	✓		✓	✓		
PC	✓			✓		
MEK	✓					
TFT	✓					
H_2O		✓	✓	✓		
EtOH	✓			✓		
IPA	✓		✓			
EtOAc/TMO	✓					
EtOAC/PC	✓					
2-MeTHF/MeOH	✓		✓			
An/DCM	✓			✓		
NBP/EtOAc	✓			✓		
MeTHF/MeCN	✓					

Table 2. Summary of the solvents studied as alternatives to the traditional ones and their application in the different steps of the synthesis. *: moderate performance

6.1.1 Aprotic solvents

These are the solvents that do not form hydrogen bonds since they do not have O-H or N-H bonds. Ethers, esters, ketones, or carbonates are some of the kind of solvents that conformed aprotic ones.

6.1.1.1 Ether solvents

Ether solvents are the most widely used as alternatives in SPPS, being 2-Methyltetrahydrofuran (2-MeTHF) (*See annex 1*) the most relevant. It is considered a green solvent because it comes from biomass and is also biodegradable, it is known as not mutagenic or irritant.[2,1,3,5,6]

In order to achieve the best synthetic results, it is important that the solvents used in the synthesis have a good swelling capacity since in this way the reagents can penetrate the resin and reach the reactive points, and therefore, be able to carry out the synthesis correctly. Otherwise, accessibility to the functional group would not be facilitated. As a general rule, PEG resins have better swelling capacity than PS resins, but this is not the case for 2-MeTHF.[21] *Table 3* shows the swelling capacity of 2-MeTHF, which is considered moderate in grafted and PEG resins with TentaGel S as an exception, and good capacity in PS resins with the exceptions of 2-Chlorotrityl chloride (CTC) resin that present moderate swelling.

In 2015, Jad Y. et al.,[22] evaluated the solubility of Fmoc-amino acids in 2-MeTHF, using Fmoc-Gly-OH as a representative sample among others, the results were successful. Once these results were obtained, the authors proceeded to examine the solubility of coupling reagents, which showed good solubility in 2-MeTHF.

Next year, in 2016, the same research group,[23] synthesized the pentapeptide α-amino isobutyric acid-enkephalin (Aib-enkephalin) (*See annex 1*) and the best results were obtained on ChemMatrix resin using 2-MeTHF as solvent in the coupling, using *N*,*N'*-Diisopropylcarbodiimide/Ethyl 2-cyano-2-(hydroxyimino)acetate (DIC/Oxyma) as coupling reagent, and Fmoc removal steps, as well as in the washings. Furthermore, the use of 2-MTHF minimized the formation of the des-Aib by-product (5%), compared to traditional solvents DCM and DMF. The authors also synthesized the decapeptide Aib- acyl Carrier Protein (ACP) (*See annex 1*) and observed that if they modified the deprotection conditions by increasing the temperature, better yields were obtained and the des-Aib secondary product was minimized (8.9%) compared to DMF protocol.

In 2018, Al Musaimi O. et al.,[24] studied the ability of 2-MeTHF to incorporate the first amino acid into the resin on CTC and Wang resins. To carry out this study, were chosen as model amino acids those that had lower solubility, hindered side-chains, and did not have side chain protecting groups. The results obtained were similar in 2-MeTHF and DCM. A similar study was carried out on Wang resin, where the amino acid models incorporated into the resin were six, among them Fmoc-Phe-OH, Fmoc-Leu-OH, Fmoc-Ser(*t*Bu)-OH. 2-MeTHF showed a first amino acid incorporation yield similar to DMF. In both resins, the degree of racemization when the first Fmoc amino acid was incorporated, was lower when using 2-MeTHF than when using DCM. The degree of racemization ranged from 0.1 to 1.2% when 2-MeTHF was used, but it ranged from 0.2 to 5.5%

when DCM was used. With these results, the authors concluded that 2-MeTHF is an alternative to replace DMF and DCM in all the steps of SPPS (incorporation of the first amino acid, coupling and Fmoc removal). Moreover, using a single solvent minimizes the degradation of the resin and allows its reuse.

Anisole (An) (*See annex 1*) is another solvent used by a few research groups due to its green attributes. It is biodegradable and low-cost.[13,15,17] *Table 3* shows that anisole has a good swelling in almost all the resins except for PS, CTC and SpheriTide, a PEG resin.

Lopez J. et al.,[25] studied the coupling reaction, using DIC/Oxyma as coupling reagents, of Fmoc-Leu-OH to H-Phe-OMe in anisole and a fast reaction was observed. In addition, the authors studied the Fmoc removal step, where it was found that anisole is not efficient since the reaction is very slow (uncomplete after 30 min).

In 2020, Alhassan M. et al.,[26] proposed a protocol for the TFA cleavage of a pentapeptide from a CTC resin using different solvents and comparing them with the traditional one (DCM). The results showed that anisole provided the best cleavage yields.

Cyclopentyl methyl ether (CPME) (*See annex 1*) is described as non-mutagenic and non-irritant solvent.[13,15,17] *Table 3* shows that it has moderate swelling in both PS and ChemMatrix (CM) resins and gives moderate-poor results in the rest of the PEG resins. As happened with the swelling of resins with 2-MeTHF, in the case of CPME the same happens because swelling is higher in PS resins than in PEGs.

In 2015, Jad Y. et al.,[22] conducted a study to measure the solubility of coupling reagents in CPME, the results of which showed the traditional reagent derived from benzotriazole (HBTU, HATU, *See annex 1*) and an ecological alternative to it derivate from Oxyma (COMU) (*See annex 2*), as the only soluble. The authors also carried out the synthesis of the Aib-enkephalin in a PS resin with different coupling reagents, where it was found that the yields obtained when CPME was used were low since they ranged from 0.8 to 29, 2%, and amounts of the des-Aib by-product ranged from 5.6 to 82.5%. Specifically, when CPME was used with HOBt and Oxyma as coupling additives the results were better than the obtained with uronium salts.

In 2017, Jad et al.,[27] published a study related to the synthesis of a heptapeptide on a Rink Amide (RA)-PS resin and in a CM resin. They focused the attention on the Fmoc elimination step using CPME together with 20% PP. Poor yields were obtained with the PS resin, and moderate with the CM resin (26.3%).

Isosorbide dimethyl ether (*See annex 1*) is another green solvent because this product is derived from biomass and renewable resources whose origin occurs in the catalytic hydrogenation of glucose.

Table 3 shows that isosorbide has good swelling on some grafted, PS and CM resins.

In 2017, Jad Y. E., et al.,[27] reported on the ability of isosorbide to remove the Fmoc group during the synthesis of an heptapeptide, on RA-PS and CM resins. This solvent showed a lower removal capacity compared with DMF and *N*-formylmorpholine (NFM).

	Solvent → Resin ↓	DMF	DCM	NMP	2-MeTHF	Anisole	CPME	Isosorbide
PS	PS			UN				
	Merrifield		G. S					
	Sieber Amide						UN	UN
	Wang-PS						UN	UN
	2-CTC						UN	UN
	JandaJel				G. S	G. S		
	ParaMax							
	RA-MBHA-PS						UN	UN
	AM-PS					G. S		
PEG	CM		G. S					
	SpheriTide							
	RA-CM		G. S				UN	UN
	AM-CM		G. S					UN
	Wang-CM		G. S				UN	UN
Grafted	HypoGel 200							
	ArgoGel							
	TentaGel S							
	NovaGel							

Table 3. Swelling capacity comparative between traditional solvents and most researched ether solvents. G.P: Great swelling (8< mL/g), Green: Good swelling (4< mL/g), yellow: Moderate swelling (2-4 mL/g), red: Poor swelling (2>mL/g), UN: Unknown results (Not tested)[13,24,25,28,29]

6.1.1.2 Ester solvents

Another group of solvents of interest that have been tested in the field of SPPS are esters.

The most well-known and used ester solvent to date is γ-Valerolactone (GVL) (*See annex 1*), which is known as green solvent because it is biodegradable, comes from renewable resources (as biomass) and it is non-toxic.[13,14,17] *Table 4* shows the swelling capacities of the GVL, which are good in both PS and PEG resins, while in grafted resins it has a moderate capacity. It can be

common that both CTC and AM-PS resins present the worst swelling capacity in this solvent. In addition, some studies report that the swelling of PEG resins is more effective than those based on PS.[13,27]

In 2017, Jad Y. E. et al.,[27] studied the use of GVL in the Fmoc removal step during the synthesis of an heptapeptide on RA-PS and CM resins. The Fmoc protecting group was removed using 20% PP in GVL obtaining successful yields that were better in CM resin than in PS resin. The results obtained with GVL were similar to those obtained with DMF and a study was carried increasing the reaction time from 45 s to 7 min for both resins. Under these conditions, the performance of the Fmoc group elimination reaction was excellent (95,5% on PS resin and 100% on CM resin).

In the same year, Kumar A. et al.,[30] proved that GVL had excellent coupling capacity, using DIC/Oxyma as coupling reagents, by synthetising the pentapeptide Aib-enkephalin on RA-PS resin (99,2% yield). Moreover, they also synthetized the difficult decapeptide Aib-ACP in the same conditions also achieving very good yields (89,7%). Previously, the authors proved the good solubility of the amino acids in GVL. Another interesting result was that the formation of des-Aib side-product was reduced in GVL if compared to DMF. As the results were so positive, the authors synthesized the decapeptide Aib-ACP on a RA-PS resin using GVL as the sole solvent for the entire process. The final yield was good (52%) but lower compared to the previous syntheses.

In 2018, Kumar A. et al.,[31] reported better efficiencies that derived to a reduction in waste when using microwave (MW) compared to the traditional protocol. The authors synthetized a tripeptide, H-Leu-Phe-Gly-NH_2, with a purity obtained was higher (around 95%) than the reported before. Moreover, the synthesis of the ACP peptide was performed under MW on a PS resin and changing the time conditions in different ways (traditional protocol, increasing deprotection time, increasing coupling time, and increasing both deprotection and coupling times). All the modifications showed a quality improvement of the peptide, resulting the best result when increasing of both times. In addition to that, a synthesis of the difficult decapeptide called JungRedemann (*See annex 1*) was carried out on a RA-CM resin. GVL gave the best purity results (68%) and no side reactions were observed in comparison with DMF (57% purity). These successful results moved to the synthesis of the 20-mer (ABC) and the 28-mer Thymosin (*See annex 1*) peptides on PS and CM resins using the latter modified protocol (extended times for coupling and deprotection steps). The high-performance liquid chromatography (HPLC) analysis

showed a good quality peptide synthesis although in the case of the ABC peptide an impurity was found corresponding to a secondary reaction of Met oxidation on the cleavage step. In the case of thymosin, the PS resin gave purity yields poorer than the CM resin.

In 2019, Jad Y.E. et al.,[32] compared the results obtained by using GVL with those when utilizing 2-MeTHF, in the synthesis of long peptides of up to 28 amino acids. The latter showed poorer yields than the former with a PS resin. The same authors,[33] proposed an improvement of the protocol using GVL due to the secondary reaction formed by the Gly residue during the elimination of Fmoc due to Gly acylation with GVL. For this, the synthesis of the ABRF-1992 (*See annex 1)* peptide was carried out on CTC, focused on the formation of dipeptides of Fmoc-Aaa-Gly-OH, where Aaa was Pro, Lys, Arg, the results of this study showed a minimization of side reactions. In another study, the same authors,[34] decided to test GVL on a Wang resin when incorporating the first amino acid. Eighteen amino acids were coupled successfully at room temperature (rt).

Other ester that was studied in SPPS is ethyl acetate (EtOAc) (*See annex 1*), whose greenness falls in its attributes as non-irritant neither phototoxic nor photo allergenic.[13] *Table 4* shows its swelling capacity in some resins. As shown in the table, It has a good capacity of selling in CM, PS, JandaJel and ArgoGel resin.

	Solvent → Resin ↓	DMF	DCM	NMP	GVL	EtOAc
PS	PS			UN		
	Merrifield		G. S			
	Sieber Amide					
	Wang-PS					
	2-CTC					
	JandaJel					
	ParaMax					
	RA-MBHA-PS					
	AM-PS					
PEG	CM		G. S			
	SpheriTide					
	RA-CM		G. S			
	AM-CM		G. S			
	Wang-CM		G. S			
Grafted	HypoGel 200					
	ArgoGel					
	TentaGel S					
	NovaGel					

Table 4. Swelling capacity comparative between traditional solvents and most researched ester solvents. G.P: Great swelling (8< mL/g), Green: Good swelling (4< mL/g), yellow: Moderate swelling (2-4 mL/g), red: Poor swelling (2>mL/g), UN: Unknown results (Not tested)[25,28,29]

In 2016, Jad Y. E. et al.,[23] presented some protocols to carry out the synthesis of the pentapeptide Aib-enkephalin and the decapeptide Aib-ACP on RA-PS and RA-CM resins. One of these protocols was about using EtOAc for the deprotection and washing steps, together with the use of 2-MeTHF for the washing and coupling steps. The results were compared to those obtained with DMF, using 2-MeTHF as the sole solvent. Des-Aib was the most formed by-product in the synthesis of Aib-enkephalin, on CM resin. Because of this, when studying a solvent, the amount of secondary product formed was considered in addition to the yield of the peptide. The peptide yields were higher when the CM resin was used than in the case of the PS resin. For both resins, the yield of the side reaction was lower than the one obtained with DMF and similar to the obtained when using 2-MeTHF.

6.1.1.3 Amide solvents

Table 5 shows the amide solvents most studied over the years. Although there is not much information about them, some studies of resin swelling have been carried out. *N*-butylpyrrolidinone (NBP) (*See annex 1*) shows a good swelling in PS resins, whereas NFM gives better results with the CM resin. NBP is non-mutagenic neither toxic nor biodegradable, but its price is higher than DMF.[13]

In 2018, Lopez J. et al.,[25] successfully proved the solubility in NBP of the amino acids Fmoc-Gln-OH and Fmoc-Gly-OH, and the coupling reagents DIC and additive Oxyma. Moreover, the synthesis of a linear Octreotide, an octapeptide (*See annex 1*) was performed, showing that some couplings were slower in comparison with DMF, and the results obtained for the Fmoc removal were similar to those obtained with DMF and quicker enough to consider this solvent as an alternative. It was suggested that the coupling reaction is successful when low polar aprotic solvents like 2-MeTHF are used, but in Fmoc removal more polar solvents like NBP are required. There is a problem here, and it is that when using two different solvents the structure of the resin is decomposing and its reuse is more complicated. In addition, it makes recycling and waste reduction difficult. Moreover, using NBP the peptides produced have a lower quality but similar impurities in comparison with DMF. Finally, NBP is compatible with PS resins, because it could be used as alternative to DMF.

In 2019, Jad. Y. E. et al.,[32] also reported that NBP had a successful performance in Fmoc removal and a good capacity to dissolve Fmoc amino acids and coupling reagents. The swelling capacity on both RA-PS and RA-CM resins is higher in DMF than in NBP. However, serine, cysteine and histidine tend to racemize in the former more than in the latter. Also, another side-reaction of SPPS, aspartimide formation (*See annex 2*), was considered in this study and the synthesis of an hexapeptide was carry out for this purpose. The results showed that the synthesis made in NBP gave better quality peptides than DMF, that is, the lower apolarity of NBP reduced the formation of aspartimide. The following year, Kumar A. et al.,[35] studied reactions under stress conditions using NBP which results showed a fewer production of waste and impurities than using DMF.

In 2017, NFM (*See annex 1*) was investigated by Jad Y. E. et al.,[27] as green solvent because it is not carcinogenic. Although its high price and unknown stability, it has been considered as an alternative in this field. It was used in the Fmoc removal step by synthetising an heptapeptide in

both PS and CM resins. Despite of having poor swelling in the PS resin, it showed a good performance in the deprotection step. However, the yield was lower than in the CM resin. As this first essay was successful, another one was tried by synthetising the same peptide extending the reaction time from 45 s to 7 min. Results were successful with the CM resin, similar to those obtained with DMF, but were moderate with the PS resin.

	Solvent → Resin ↓	DMF	DCM	NMP	NBP	NFM
PS	PS			UN		
	Merrifield		G. S		UN	UN
	Sieber Amide				UN	UN
	Wang				UN	UN
	2-CTC				UN	UN
	JandaJel				UN	UN
	ParaMax				UN	UN
	RA-MBHA-PS				UN	UN
	AM-PS					UN
PEG	CM		G. S		UN	
	Spheritide				UN	UN
	RA-CM		G. S		UN	UN
	AM-CM		G. S		UN	UN
	Wang-CM		G. S		UN	UN
Grafted	HypoGel 200				UN	UN
	ArgoGel				UN	UN
	TentaGel				UN	UN
	NovaGel				UN	UN

Table 5. Swelling capacity comparative between traditional solvents and amide solvents used in SPPS. G.P: Great swelling (8< mL/g), Green: Good swelling (4< mL/g), yellow: Moderate swelling (2-4 mL/g), red: Poor swelling (2>mL/g), UN: Unknown results (Not tested).[6]

In 2017, Kumar A. et al.,[30] demonstrated that the use of NFM for washing and during couplings provided successful yields (93,1%) using DIC/Oxyma as coupling reagent, by synthesizing the pentapeptide Aib-enkephalin on a RA-PS resin, but a performance related to the des-Aib side-product (3,6%) poorer than using DMF with GVL in coupling and washing (0,8%). The synthesis of the decapeptide Aib-ACP in the same resin was also performed in comparison with the DMF protocol, using NFM, GVL and 2-MeTFH as sole solvents in each protocol, obtaining as yields 54%, 52% and 25%, respectively.

6.1.1.4 Carbonate Solvents

Propylene carbonate (PC) (*See annex 1*) is a cyclic carbonate considered non-carcinogenic, neither mutagenic, nor irritant, its price is lower than DMF and it has low toxicity.[13]

In 2017, Lawrenson S. B et al.,[36] described PC as an alternative to DMF for the coupling step. The yield was successful (93%) for the synthesis of a dipeptide using EDC/HOBt as coupling agents, on a CM resin. Also, the synthesis of a nonapeptide, Bradykinin (*See annex 1*), on the same resin gave a lower yield in comparison with the dipeptide yield, but the authors considered PC an alternative to DMF. No significant impurities and racemisation were found when PC was used in the synthesis of the nonapeptide whereas in DMF appeared impurities.

	Solvent → Resin ↓	DMF	DCM	NMP	PC
PS	PS			UN	
	Merrifield		G. S		UN
	Sieber Amide				UN
	Wang				UN
	2-CTC				UN
	JandaJel				UN
	ParaMax				UN
	RA-MBHA-PS				UN
	AM-PS				UN
PEG	CM		G. S		
	SpheriTide				UN
	RA-CM		G. S		UN
	AM-CM		G. S		UN
	Wang-CM		G. S		UN
Grafted	HypoGel 200				UN
	ArgoGel				UN
	TentaGel				UN
	NovaGel				UN

Table 6. Swelling capacity comparative between traditional solvents and carbonate solvents used in SPPS. G.P: Great swelling (8< mL/g), Green: Good swelling (4< mL/g), yellow: Moderate swelling (2-4 mL/g), red: Poor swelling (2>mL/g), UN: Unknown results (Not tested).[13]

6.1.1.5 Ketone solvents

The green attributes of buta-2-one (MEK) (*See annex 1*) lie in the fact that it is a non-mutagenic nor carcinogenic compound.[13] Protocols where the solvent plays an important role in

peptide synthesis have not been developed and only swelling capacities have been tested in some resins.[29] *Table 7* shows that it is a solvent that has a good swelling capacity in few resins, such as Merrifield, some PEG (like CM) and ArgoGel (grafted), while in the rest of the resins it has a moderate swelling, as PS, Wang, CTC, SpheriTide, TentaGel, among others.

	Solvent → Resin ↓	DMF	DCM	NMP	MEK
PS	PS			UN	
	Merrifield		G. S		
	Sieber Amide				
	Wang				
	2-CTC				
	JandaJel				
	ParaMax				
	RA-MBHA-PS				
	AM-PS				
PEG	CM		G. S		
	SpheriTide				
	RA-CM		G. S		
	AM-CM		G. S		
	Wang-CM		G. S		
Grafted	HypoGel 200				
	ArgoGel				
	TentaGel S				
	NovaGel				

Table 7. Swelling capacity comparative between traditional solvents and ketone solvents in SPPS. G.P: Great swelling (8< mL/g), Green: Good swelling (4< mL/g), yellow: Moderate swelling (2-4 mL/g), red: Poor swelling (2>mL/g), UN: Unknown results (Not tested).[13,27]

6.1.1.6 Fluorocarbon solvents

ααα-trifluorotoluene (*See annex 1*) is classified as non-carcinogenic nor irritant. *Table 8* shows its compatibility with CM resins due its swelling capacity. It provided better Fmoc removal results and final yields with CM resin(31,1%) than PS resin (12,1%). Extending the time for Fmoc removal from 45 s to 7 min an increase in performance was observed and it provided moderate results in both resins, giving CM better Fmoc removal yields (58,6%) in comparison with RA-PS (47,7%).[27]

	Solvent → / Resin ↓	DMF	DCM	NMP	ααα-Trifluorotoluene
PS	PS			UN	
	Merrifield		G. S		UN
	Sieber Amide				UN
	Wang				UN
	2-CTC				UN
	JandaJel				UN
	ParaMax				UN
	RA-MBHA-PS				UN
	AM-PS				UN
PEG	CM		G. S		
	SpheriTide				UN
	RA-CM		G. S		UN
	AM-CM		G. S		UN
	Wang-CM		G. S		UN
Grafted	HypoGel 200				UN
	ArgoGel				UN
	TentaGel S				UN
	NovaGel				UN

Table 8. Swelling capacity comparative between traditional solvents and fluorocarbon solvents in SPPS.[13,27]

6.1.2 Protic solvents

They are the solvents that have O-H or N-H bonds. Alcohols and water are the most tried in SPPS.

6.1.2.1 Water

Water is the most ecologic solvent due it is a natural product which is not inflammable nor toxic and it has lower price than the other solvents. For these reasons, and because H_2O is not an organic solvent, it has been testing in SPPS. The main problem is the poor solubility of Fmoc amino acids in water, which prevents the synthesis from being carried out.

To solve this problem, in 2007, Hojo K. et al.,[37] carried out the first experiment, where water was used as a solvent together with Fmoc amino acids that had previously been processed and converted into dispersible nanoparticles. The advantage of nano amino acids is that these can be removed by filtration and washed with water. In a RA-PEG resin was carry out the synthesis of the pentapeptide Leu-enkephalin (*See annex 1*) under these conditions with a yield of 67%. Another experiment was performed using water as the solvent in a PEG resin, but the peptide could not be synthesized.

In 2011, Hojo K. et al.,[38] carried out an experiment using the Boc strategy in PEG as dispersant to enhance water solvent protocol. A tripeptide was prepared using water dispersible Boc nanoamino acids with a yield of 82% and a purity of 90%. The synthesis of a tetrapeptide and Leu-enkephalin was also carried out with yields higher than the obtained in the former experiment.

The same authors[39]

published in 2011 an article about catalytic reactions with water as solvent in SPPS. Water-dispersible Fmoc-Phe-OH nanoparticles were coupled in a RA-TentaGel resin, obtaining a quantitative yield after 30 min. Moreover, Leu-enkephalin was synthesized in a 67% yield using processed Fmoc nanoamino acid particles in the same resin and coupling reagents that are soluble in water. The same experiment was carried out with unprocessed Fmoc nanoamino acids in PEG, but the results were not satisfactory. In another experiment, the synthesis of Leu-enkephalin was performed with the protocol using water as the solvent and nanoparticles in the presence of Triton X-100 as a dispersing agent since it inhibits peptide aggregation. The results were better than those obtained previously, and the purity was high (93%) and similar when using the traditional Fmoc strategy.

In 2012, Hojo k. et al.,[40] developed a water synthetic strategy using Fmoc nanoamino acids with MW to increase reaction rates. The peptides were synthesized in a RA-TentaGel resin using Triton X-100 as dispersant. Leu-enkephalin was prepared under these conditions using soluble coupling reagents. Low racemization was observed, and the yield was high (76%). Moreover, ACP was synthesized with a high purity and a 38% yield. All together indicated that this strategy could be applied in simple peptide syntheses.

To improve the MW-water strategy, in 2013 the same authors[41] focused their research on racemization of cysteine because it is one of the amino acids most prone to this process. A nonapeptide was synthesized in a RA-PEG resin at pH around 7 to minimize racemization during the coupling reaction. The results were satisfactory, showing a reduction of the side-reaction (<3%). Furthermore, in 2014, Hojo K. et al.[42] studied the histidine racemization, another amino acid sensitive to this side reaction. A low racemisation was obtained in the synthesis of the hexapeptide NPW30 in RA-PEG resin.

6.1.2.2 Alcohols

From the point of view of swelling, ethanol (EtOH) has been studied more than isopropanol (IPA), although there isn't much information performance about both of them. EtOH and IPA have similar swelling capacity, as shown in *Table 9*, but EtOH seems to have a better performance than IPA for all resins.[28] IPA has one of the worst sweeling capacities on a CM resin, whereas EtOH has the best swelling capacity in a SpheriTide resin, whose swelling is poor in all green solvents. In the case of PEG resins, both solvents induce poor swelling.

In 2011, Hojo K. et al.[39] developed a protocol based on the use of aqueous EtOH (50%). Leu-enkephalin was synthesized using Triton X-100 as dispersant with high yield and purity.

In 2017, the use of IPA in the Fmoc removal step was investigated as green solvent by Jad Y. E. et al.[27] An heptapeptide was synthetised on PS an CM resins with unsuccessful results in both cases, in comparison with the other green solvents and DMF.

	Solvent → **Resin ↓**	DMF	DCM	NMP	EtOH	IPA
PS	PS					
	Merrifield		G. S			
	Sieber Amide					
	Wang					
	2-CTC					
	JandaJel					
	ParaMax					
	RA-MBHA-PS					
	AM-PS					
PEG	CM		G. S			
	SpheriTide					
	RA-CM		G. S			
	AM-CM		G. S			
	Wang-CM		G. S			
Grafted	HypoGel 200					
	ArgoGel					
	TentaGel S					
	NovaGel					

Table 9. Swelling capacity comparative between traditional solvents and alcohols solvents. G.P: Great swelling (8< mL/g), Green: Good swelling (4< mL/g), yellow: Moderate swelling (2-4 mL/g), red: Poor swelling (2>mL/g), UN: Unknown results (Not tested)[27,28,29]

6.1.3 Mixed solvents

Another alternative to traditional solvents is binary solvent mixtures, which are made up of previously studied green solvents. Several investigations have been carried out to find out the effectiveness of these mixtures.

In 2019 Ran Y. et al.[43] performed some experiments to check the swelling on a Merrifield resin of different binary mixtures. The mixtures that gave the best results were used in the synthesis of a tripeptide. The solvents chosen were PC, EtOAc and 2,2,5,5-tetramethyltetrahydrofuran (TMO) (*See annex 1*). TMO originates from renewable raw materials, does not lead to the formation of toxic peroxides and is more ecological than toluene. These solvents were compared with the mixtures EtOAc:PC (90:10) and TMO:PC (different ratios). The former gave the best swelling capacity (4,6mL/g) and the best yields (307mg/g) in the synthesis of the tripeptide H-Leu-Ala-Phe-OH, although the mixtures TMO:PC (90:10) and (40:60) provided good swelling capacity (3,8 mL/g in both mixtures).

In that same year, Přibylka A. et al.[44] used a mixture of 2-MeTHF and MeOH in the study for the elimination of the Fmoc group in the presence of NaOH as a base as an alternative to piperidine. Lue-enkephalin pentapeptide was synthesized in a RA-PS resin with results comparable to those obtained with DMF, DCM and piperidine. The same authors in 2020[45] studied the formation of aspartimide (*See annex 2*) and the stability of amino acid side chain protecting groups. The results obtained for the stability of the amino acids residues protecting groups *t*-Bu, Boc, Trt, and Pbf (*See annex 1*) were in general successful since it was found that they continued intact when carrying out the Fmoc elimination, except in the case of the Boc protection for histidine. It was also confirmed that this protocol avoids the formation of aspartimide. The purities obtained by synthesizing a variety of peptide sequences using this protocol were successful.

In 2019, Ferrazzano L. et al.[46] studied various solvent mixtures on Wang-PS, RA-PS, Wang-Tentagel, RA-CM and RA-Tentagel resins. The selected mixtures were Cyrene (Cyr)/diethyl carbonate (DEC) (30:70), Sulfolane (Sul)/DEC (30:70) and (An)/DEC (70:30). Aib-enkephalin was synthesized on Wang-PS and RA-CM resins using different mixtures for the coupling step using DIC/Oxyma as coupling reagents), obtaining peptide better yields (from 43,6% to 60,6%) than those achieved by DMF (13,5% on Wang-PS resin and 53% on RA-CM resin), together with lower levels of des-Aib by-products (from 5% to 38%) in comparison with DMF (13,5% and 53%).

The authors concluded that the mixtures were compatible in both PS and PEG resins regardless of the functionalization used. Moreover, the most relevant results were obtained when the mixture An/DCM(70:30) was used to synthesize Aib-ACP, Aib-enkephalin and Octreotide.

In 2020, trials with binary mixtures continued with the study of Erny M. et al.[47] Fmoc-Ser(t-Bu)−OH peptide was synthesized on a Ramage aminomethylstyrene (H-RMG AMS) resin using DIC/Oxyma as the coupling system and the formation of the by-product hydrocyanic acid (HCN) was evaluated (*See annex 2*). NBP and PC, two previously investigated green solvents, were used together with EtOAc, which inhibits the formation of secondary chains. In comparison with DMF, NBP and NBP/EtOAc (1:4) gave the lowest formation of HCN. From here they investigate the most efficient ratio of NBP/EtOAc to use, it led to better NBP/EtOAc results (1:4).

In that same year, another study carried out by Magtaan J. K. et al.,[29] compared the swelling of traditional solvents with green solvents. Specifically, the mixture 2-MeTHF/acetonitrile (MeCN) (1:1) had a moderate swelling capacity in PS resins and a little bit better in PEG resins. Other green solvents such as anisole, GVL or 2-MeTHF were found to have better swelling capacity.

6.2 REAGENTS AND ADDITIVES

Not only is necessary find alternatives for solvents but also reagents and additives used in some steps can contribute to the greening of chemistry, from the coupling steps to elimination of the Fmoc group. For this purpose, experiments have been carried on reducing toxicity and hazardousness.

6.2.1 Coupling reagents and additives

6.2.1.1 Coupling reagents in organic solvents

The classical coupling agents such as DCC or DIC (*See annex 1*), among others, are necessary for the activation of the carboxyl group, which allows a rapid and quantitative amide bond formation. The addition of these reagents is usually accompanied by racemization, and this poses a purity problem in the synthesis of the peptide. To avoid or reduce this racemization additives are used, the usual ones are benzotriazoles such as HOBt and HOAt, which in some circumstances they are explosive.[20] In order to minimize these risks, searches were carried out to find safer alternatives.[12,13] In this sense, Oxyma and COMU (*See annex 1 and 2*) have been

described as the best environmentally friendly alternative to benzotriazoles and derivative coupling reagents.[32,51]

In 2009,[48] some experiments were made for first time using Oxyma as an additive with DIC, which is considered less hazardous than benzotriazole derivates such as HOBt and HOAt. It was found with the synthesis of a tripeptide on a Fmoc-RA-PS resin that elongation yields were better with Oxyma than with HOAt and HOBt, and that the racemization levels were similar to those obtained with the classical additives. Previously, simpler peptides were synthesized to find the best conditions resulting Oxyma the best yield (90,8%) in the case of the H-Gly-Cys-Phe-NH_2 synthesis. More complicated peptides were tested on the same resin, such as ACP or Leu-enkephalin analogues. In the synthesis of Leu-enkephalin, with 5 min coupling time, HOAt showed the best yield (94,9%), whereas in the synthesis of Leu-enkephalin analogue, with 30 min coupling time, Oxyma showed the best yield (79%). In all the syntheses, an improvement on inhibition of the racemization and deletion by-products was observed when Oxyma was used.

In 2014, Jad Y. E., et al.[49] studied an alternative to Oxyma in the synthesis of, H-Gly-Ser-Phe-NH_2, H-Gly-Cys-Phe-NH_2, among others, on a RA-PS resin .In this study the authors found that 5-(hydroxyimino)-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione (B-Oxyma) (*See annex 1*) suppressed racemization (0,3-1%) better than Oxyma (0,4-3%). Aib was also synthetised using Oxyma with a 42,8% yield and the lowest percentage of by-product des-Aib (50,4%). B-oxyma presented lower yields than Oxyma and more by-product formation.

In 2018, experiments carried out by Albericio F. and El-aham A.[22] revealed a leak from the synthesized peptide sequence when using Oxyma. To solve this problem the potassium salt of Oxyma was introduced as an alternative (K-Oxyma) (*See annex 1*), which provided better solubilities and thermal stability than Oxyma.

In 2019, Pawlas J. and Rasmussen J. H.,[50] demonstrated that DIC/Oxyma had the best solubility capacity to dissolve an Fmoc amino acids in comparison with other coupling systems such as COMU/DIPEA, DIC/HOBt, HBTU and HATU (*See acronyms*). This study was carried out with a PS resin for the synthesis of a Aib-ACP analogue, where the mixture dimethylsulfoxide (DMSO)/EtOAc (1:9) was used as solvent. Oxyma gave a yield of 92% and a purity of 70% with and low level of secondary reactions (0,39%). Moreover, similar studies carried out by other authors showed that the Oxyma suppresses racemisation under the different conditions that were used.[48,49,51,52]

As an alternative to benzotriazole derivatives has been developed the reagent COMU (*see annex 2*), an Oxyma derivate based in an uronium salt with a morpholino carbon skeleton.[53] Hydrolytic stabilities showed to be better in COMU than benzotriazole derivates such as HATU or HBTU (*See annex 2*).[54] It was stablished that Oxyma derivates such as COMU and HDMODC (*See annex 1*) were the most soluble in comparison with other coupling reagents. These excellent solubilities will improve coupling yields and removal of waste (excess of reagents and by-products). COMU can be used with two different bases, *N,N*-diisopropylethylamine (DIPEA) or 2,4,6-trimethylpyridine (TMP) (*See annex 1*),the latter reducing racemization. COMU provided the best coupling yields in the syntheses of a pentapeptide and a decapeptide, with racemization levels lower than those obtained with HATU. Moreover, a stability experiment showed that COMU turned out to be the best because it was the most stable and with the most controlled decomposition, thus being safer than benzotriazoles. Moreover, COMU requires a lower amount of base (1 equiv).[55] Experts recommend the use of DIC/Oxyma in MW automatized synthesis, whereas in a manual synthesis they suggest using COMU with DIPEA or TMP, which are soluble in 2-MeTHF, DMC or EtOAc.

In 2020, Kumar A. et al.,[35] tested the solubility of different coupling reagents in NBP at rt. They found that both the traditional coupling reagents and Oxyma derivatives were soluble, being COMU the best (0,33M).

6.2.1.2 Coupling reagents and additives soluble in water.

To carry out the SPPS in the greenest solvent which is water, it is necessary to use solvents and reagents soluble in water for the different reactions of the synthesis to take place. For this reason, coupling reagents used during the syntheses of peptides in water must be soluble in water. Reagents and additives such as HOBt, HOAt, Oxyma or COMU are not soluble, therefore several investigations have been carried out to reach a successful result.

In 2007, Hojo K. et al.,[16] described a protocol where water was used as green solvent. The amino acid derivative Fmoc-Phe-OH was coupled on a Leu-RA-grafted resin, which coupling yield became quantitative in a 30-min reaction. The water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) *(See annex 1)* and the water-soluble additive *N*-hydroxy-5-norbornene-endo-2,3-dicarboximide (HONB) (*See annex 1*) were used in the presence of DIPEA to perform these coupling reactions.

In 2011, the same authors[39] carried out a synthesis of Leu-enkephalin, among other peptides, on a RA-Tentagel resin using the Boc strategy to assay a new coupling reagent efficiency, 4-(4,6-dimethoxy-1,3,5- triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (*See annex 1*). This additive was used without any additive and with NMM as base. Water was used as sole solvent and triton X-100 as dispersant, resulting a yield of 86% and a purity of 93%. Comparing both water-soluble coupling reagents, DMTMM provided better yields in coupling reactions than EDC with HONB.

In 2012, Hojo K. et al.[40] synthetized Leu-enkephalin and an heptapeptide to study the coupling reaction time compared to EDC with DMTMM giving the latter better results than the former. In addition, it was observed that DMTMM was compatible with the use of MW in the synthesis of ACP, from which good yields and purities were obtained.

In the following years, the same authors studied the reduction of the racemization levels in peptides containing cysteine[41] and histidine[42] amino acids. The authors synthetised peptides containing these amino acids, such as NPW30, by using MW in water and following a similar protocol and then they observed that the racemization of these amino acids was inhibited. For this reason, DMTMM and EDC were used to reach a quantitative coupling reaction. In the case of NPW30 the peptide yield was over 28% and level of His racemization was over 20%, whereas in the synthesis of the L,L-Cys-oxytocin the yield was over 24% while racemization was not significant.

6.3 Fmoc REMOVAL

PP (in solution of DMF) are the traditional conditions used in the Fmoc removal step. Traditional Fmoc removal strategy is performed in a highly basic medium due to PP (pKa 11.2). Moreover, this amine has strong nucleophilic character. Finally, due its restrictions, it has been necessary to find an alternative to this base. For this reason, some investigations have been carried out.

In 2006, Hachmann J. and Lebl M.[56] introduced for the first time 4-methylpiperidine (4MP) (*See annex 1*), which is not restricted by DEA, as a possible alternative to PP in the elimination of the Fmoc protecting group. The study was carried out with ACP, enkephalin and LHRH (*See annex 1*) peptides, resulting a similar behaviour to PP.

In 2013, Ralhan K. et al[57] published for the first time a kinetic study about piperazine (PZ) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (*See annex 1*). The former is considered as an alternative to PP because it is less hazardous, and its use is not restricted. Both bases showed an efficient Fmoc removal in the syntheses of the Poly-Ala (*See annex 1*). One of the problems that affect the use of strong bases for Fmoc removal is the generation of side reactions as the formation of aspartimide and epimerization of cysteine or histidine. To prove these bases as successful ones, the authors synthetized Scorpion toxin (*See annex 1*) on a CTC resin. The results showed that the best yield was obtained using 5% PZ and 1% formic acid (FA) in DMF (92,8%). Moreover, it was possible to reduce the formation of aspartimide o 7,2%. A similar yield was obtained with 5% PZ and 1% DBU + 1% FA in DMF, minimizing racemisation to 1,41%.

In 2014, Vergel Galeano et al.[58] synthesized some peptides, including the difficult Lactoferricin and L1-HPV. Fmoc removal was carried out using 40% 4MP in DMF and the results were excellent to the point of considering 4MP as a potential alternative to PP.

In 2016 a study was carried out by Luna O. F. et al.,[59] synthesizing peptides such as NBC155, NBC759 and NBC1951 (*See annex 1*) on a RA-resin to compare the efficiency of PP, 4MP and PZ under MW conditions. The yields were similar using the three bases but deletions of Ala, Lys, Glu or His resulted when using these solvents.

In 2020, Guzmán F. et al.[60] studied PP, 4MP and PZ using the protocol developed in 1985 called the 'Teabag Protocol'. This protocol is more practical because allows to prepare different peptides in parallel. In addition, the number of reagents, solvents and waste is reduced. This protocol was carried out on RA-polypropylene resin and several peptides, such as NBC759, NBC1951and NBC155, were used as models 4MP provided the most satisfactory results, 40% peptide yield in NBC759, NBC1951 provided 36% yield, NBC155 resulted in 60% peptide yield, which purities in all syntheses were above 70-75% .

7. CONCLUSIONS

This work, based on the bibliographic search of the impact of green chemistry in SPPS, has shown that there is great interest in finding green alternatives to the traditional strategy, due to the numerous investigations that have been carried out in the last years. These have ranged from the swelling capacity of solvents in a resin to the substitution of the PP base in the Fmoc elimination step, through the selection of solvents and coupling reagents and additives.

In general, it has been demonstrated that the swelling capacity is greater in PEG resins, but 2-MeTHF and CPME have been the exception solvents since their swelling has been greater in PS resins. Furthermore, the solvents that have had the highest solubility with both the Fmoc amino acids and coupling agents have been 2-MeTHF, anisole and GVL.

Despite the wide selection of alternative solvents that have been studied, the number of them is reduced when it comes to checking their compatibility (swelling or solubility), and the final synthetic results (yields, reduction of the formation of by-products and side-reactions). These parameters have been crucial to allowing the use of a single solvent in the entire synthetic process. 2-MeTHF and GVL have proved to be the most efficient aprotic solvents throughout the process, although some solvents gave quantitative yields only in specific parts of the synthesis, such as CMPE in the Fmoc elimination step or anisole in the cleavage step. The problem with these particular cases is the need to use more than one solvent when synthesizing a peptide, which could have a negative effect on the resin since it could be damaged, making its reuse difficult. In the case of protic solvents, water proved to be the effective alternative when used with Trition X-100 as a dispersant, producing peptides in quantitative yields and with high purities. Moreover, Water and GVL proved to be compatible with the use of MW, which allowed to improve the synthetic results. NFM is another successfully studied alternative that provided better PS resin yields than GVL and 2-MeTHF, but its purities were lower and by-product formation was higher than the latter.

In the case of additives for coupling, Oxyma showed optimal results in the performance of the coupling reaction and reduction of racemization when compared to HOAt and HOBt. However, B-

Oxyma afforded peptides with lower yields and more by-product formation than Oxyma when synthesizing more difficult peptides. Regarding the coupling agents, it was found that the Oxyma derivative COMU gave in general better yields compared to benzotriazole derivatives HATU or HBTU. It was also found to be soluble in various ecological solvents such as 2-MeTHFand NBP. It is worth mentioning that several authors recommend the use of Oxyma as additive of the coupling agent DIC, and COMU with DIPEA or TMP, both bases giving similar results.

The solubility of the coupling reagents and additives is a key point when using water as the solvent for SPPS and several studies have been carried out in this respect. In this sense, EDC with HONB as additive and DMTMM have been found as effective soluble coupling reagents. The former has been used with DIPEA and the latter together with NMM as bases. Both coupling reagents have achieved quantitative results in the synthesis of difficult peptides. In addition, they have proved to be effective in the reduction of racemisation of Cysteine and histidine, amino acids prone to give this side reaction.

Another interesting point is the need to find an alternative to PP, the base used for removing the Fmoc group. This product is controlled by DEA for being used in drug synthesis and, therefore, difficult to purchase, apart from being corrosive and irritating. Two potential alternatives have been found so far, 4MP and PZ. 4MP has provided yields similar to PP and higher than PZ. Moreover, 4MP is cheaper, for which it has been considered the best alternative to PP.

As has been proven, nowadays it is affordable both economically and synthetically to use more ecological protocols for the synthesis of peptides in moderate to high yields, reducing at the same time toxicity, danger, and waste. Despite these advances, scientific community claim for continuing the research in this field in order to improve the synthetic yields and the purity of the product by using a protocol as green as possible.

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9. ABBREVIATIONS/ACRONYMS

ACP	Acyl Carrier Protein
ACS GCI PR	American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable
Aib	α- aminoisobutyric acid
Ala	Alanine
AMS	Aminomethylstyrene
An	Anisole
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
AZ	AstraZeneca
Boc	*Tert*-butoxycarbonyl
B-Oxyma	5-(hydroxyimino)-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione
Bzl	Benzyl
CM	ChemMatrix
COMU	1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate
CPME	Cyclopentyl methyl ether
CTC	2-Chlorotrityl chloride
Cyr	Cyrene
Cys	Cysteine
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene

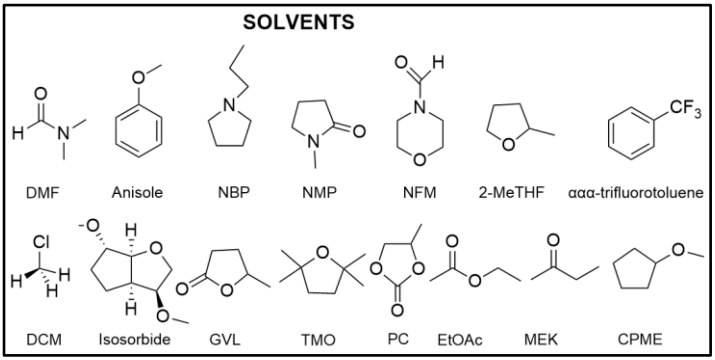
DCC	*N*,*N'*-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DEC	Diethyl carbonate
Des-aa	Peptide deletion in which the amino acid aa has not been incorporated
DIC	*N*,*N'*-Diisopropylcarbodiimide
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
DMTMM	4-(4,6-dimethoxy-1,3,5- triazin-2-yl)-4-methylmorpholinium chloride
DVB	Divinylbenzene
ECHA	European Chemicals Agency
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EtOAc	Ethyl acetate
EtOH	Ethanol
Fmoc	Fluorenylmethoxycarbonyl
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GSK	GlaxoSmithKline
GVL	γ-Valerolactone
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCN	Hydrocyanic acid
HDMODC	1-[(1-(dicyanomethyleneaminooxy)dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate
HF	Hydrofluoric acid

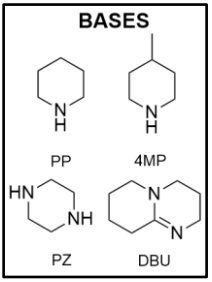
His	Histidine
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HONB	*N*-hydroxy-5-norbornene-endo-2,3-dicarboximide
HPLC	High-performance liquid chromatography
K-Oxyma	Ethyl (Z)-2-cyano-3-hydroxyacrylate, potassium salt
Ile	Isoleucine
IPA	Isopropanol
Leu	Leucine
Lys	Lysine
MeCN	Acetonitrile
MEK	2-Butanone
Met	Methionine
4MP	4-methylpiperidine
2-MeTHF	2-Methyltetrahydrofuran
NBP	*N*-butylpyrrolidinone
NFM	*N*-formylmorpholine
NMP	*N*-Methylpyrrolidone
Oxyma	Ethyl 2-cyano-2-(hydroxyimino)acetate
PC	Propylene carbonate
PEG	Polyethylene glycol
Phe	Phenylalanine
PP	Piperidine
Pro	Proline
PS	Polystyrene
PZ	Piperazine
RA	Rink Amide

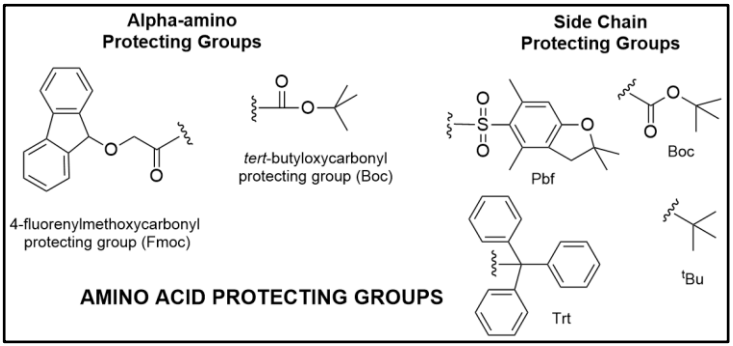
REACH	Registration, Evaluation, Authorisation, and restriction of Chemicals
RMG	Ramage
Ser	Serine
SPPS	Solid-phase peptide synthesis
Sul	Sulfolane
*t*Bu	*Tert*-butyl
TFA	Trifluoroacetic acid
TFMSA	trifluoromethanesulfonic acid
TFT	ααα-trifluorotoluene
Thr	Threonine
TIS	Triisopropylsilane
TMO	2,2,5,5-tetramethyltetrahydrofuran
TMP	2,4,6-trimethylpyridine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valin

APPENDICES

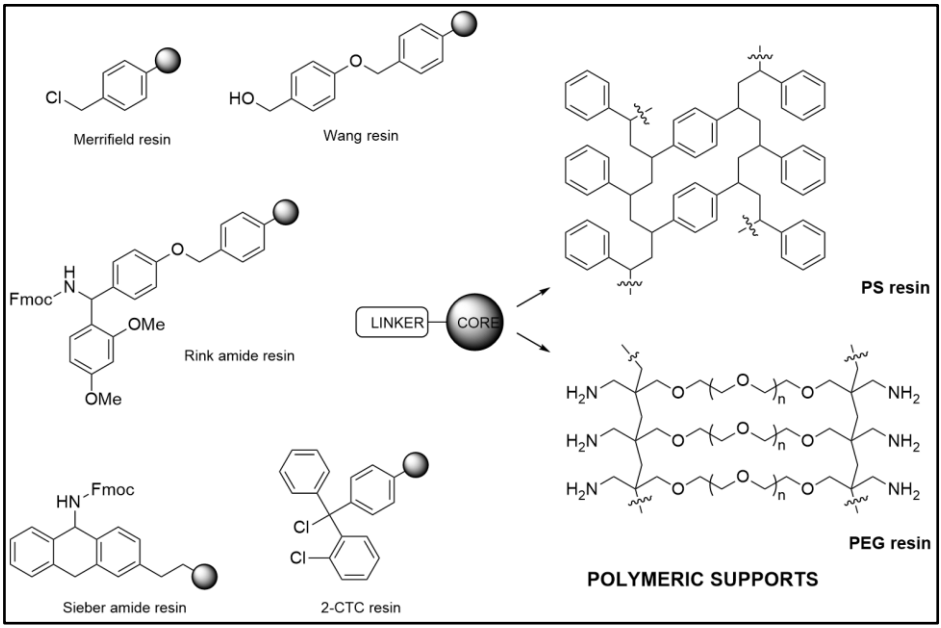
APPENDIX 1: STRUCTURES

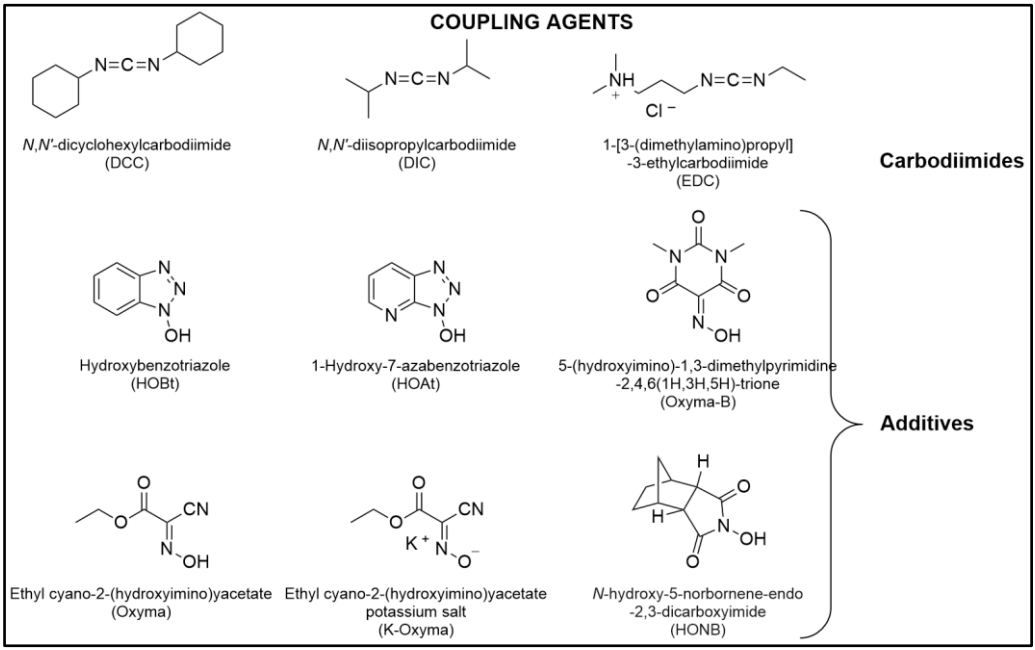




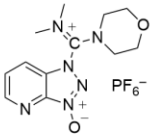


ABC	H-Val-Tyr-Trp-Thr-Ser-Pro-Phe-Met-Lys-Leu-Ile-His-Glu-Gln-Cys-Asn-Arg-Ala-Asp-Gly-NH_2
ABRF-19992	H-Gly-Val-Arg-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-Pro-Tyr-NH_2
ACP	H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2
Aib-ACP	H-Val-Gln-Aib-Aib-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2
Aib-enkephalin	H-Tyr-Aib-Aib-Phe-Leu-NH_2
Bradykinin	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
JungRedemann	H-Trp-Phe-Thr-Thr-Leu-Ile-Ser-Thr-IleMet-NH_2
Lue-enkephalin	H-Tyr-Gly-Gly-Phe-Leu-NH_2
LHRH	H-Glu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2
Linear Octreotide	H-$_D$-Phe-Cys-Phe-$_D$-Trp-Lys-Thr-Cys-Thr-ol
NBC155	H-Thr-Leu-Glu-Glu-Phe-Ser-Ala-Lys-Leu-NH_2
NBC759	H-Lys-Lys-Trp-Arg-Trp-Trp-Leu-Lys-Ala-Leu-Ala-Lys-Lys-NH_2
NBC1915	H-Val-Ala-Pro-Ile-Ala-Lys-Tyr-Leu-Ala-Thr-Ala-Leu-Ala-Lys-Trp-Ala-Leu-Lys-Gln-Gly-Phe-Ala-Lys-Leu-Lys-Ser-NH_2
Poly-Ala	H-Tyr-Ala_{10}-Lys-OH
Scorpion toxin II	H-Val-Lys-Asp-Gly-Tyr-Ile-NH_2
Thymosin	H-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-NH_2

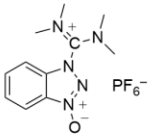

Merrifield resin
Wang resin
Rink amide resin
Sieber amide resin
2-CTC resin
LINKER
CORE
PS resin
PEG resin
POLYMERIC SUPPORTS


COUPLING AGENTS
N,N'-dicyclohexylcarbodiimide (DCC)
N,N'-diisopropylcarbodiimide (DIC)
1-[3-(dimethylamino)propyl] -3-ethylcarbodiimide (EDC)
Carbodiimides
Hydroxybenzotriazole (HOBt)
1-Hydroxy-7-azabenzotriazole (HOAt)
5-(hydroxyimino)-1,3-dimethylpyrimidine -2,4,6(1H,3H,5H)-trione (Oxyma-B)
Additives
Ethyl cyano-2-(hydroxyimino)yacetate (Oxyma)
Ethyl cyano-2-(hydroxyimino)yacetate potassium salt (K-Oxyma)
N-hydroxy-5-norbornene-endo -2,3-dicarboxyimide (HONB)

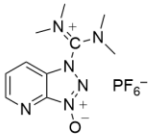
COUPLING AGENTS



1-((dimethylamino)(morpholino)methylene) -1H[1,2,3]triazolo[4,5-b]pyridinium hexafluorophosphate-3-oxide (HDMA)

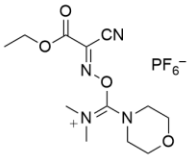


hexafluorofosfat de 3-òxid-*N*-[(1H-benzotriazol-1-il)-(dimetilamino)metilen]-*N*-metilmetanamini (HBTU)

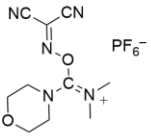


(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)

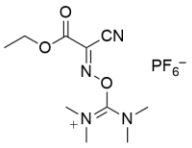
Benzotriazople derivatives



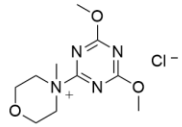
1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy) -dimethylaminomorpholinomethylene)] methanaminium hexafluorophosphate (COMU)



1-[(1-(dicyanomethyleneaminooxy)dimethylamino -morpholinomethylene)] methanaminium hexafluorophosphate (HDMODC)



O-[cyano(ethoxycarbonyl)methyleneamino] -N,N,N′,N′-tetramethyluronium hexafluorophosphate (HOTU)

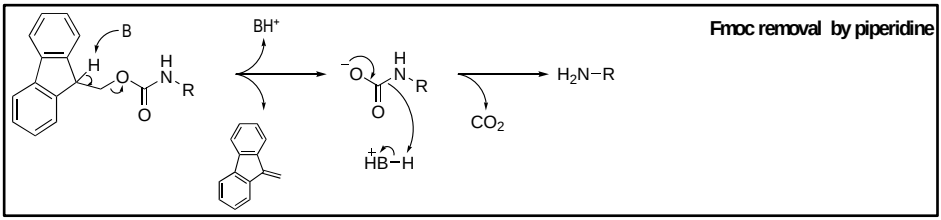


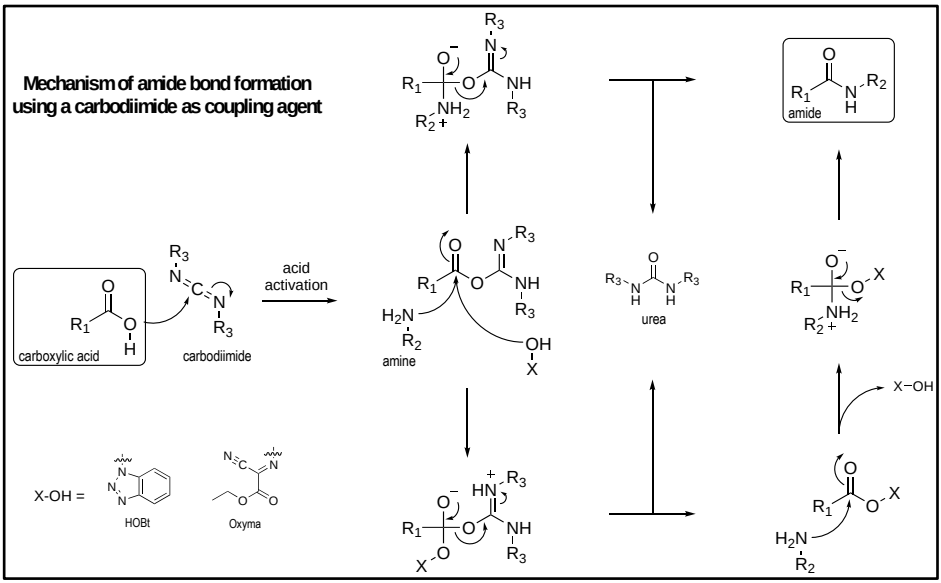
4-(4,6-dimethoxy-1,3,5- triazin-2-yl) -4-methylmorpholinium chloride (DMTMM)

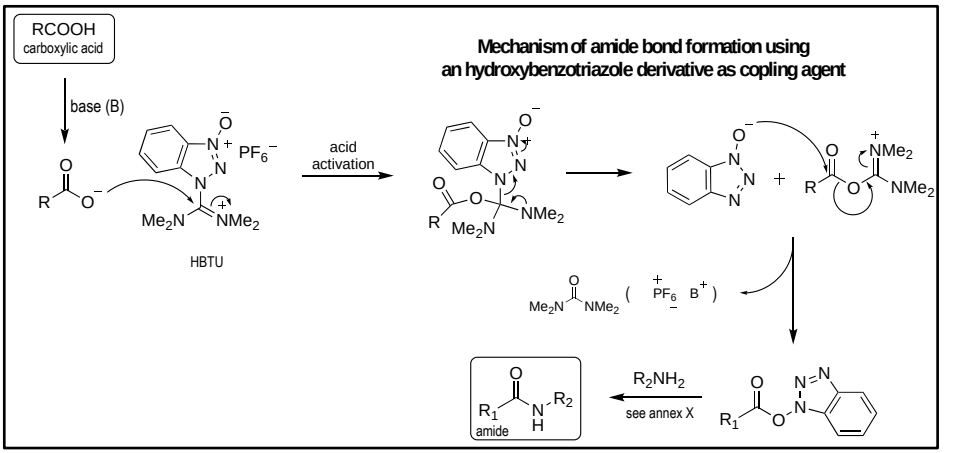
Oxyma derivatives

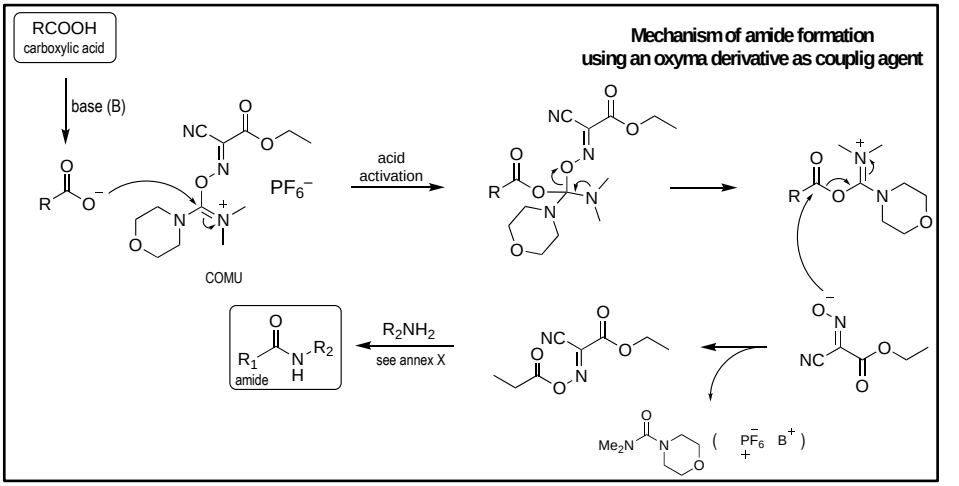
Water soluble reagents

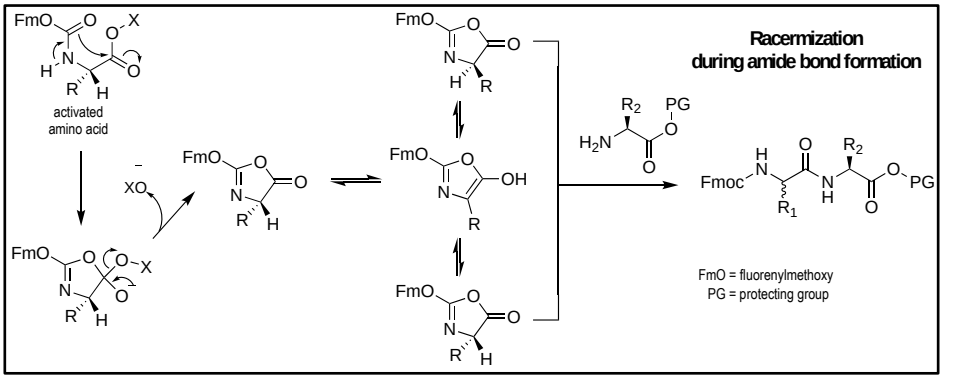
APPENDIX 2: MECHANISMS

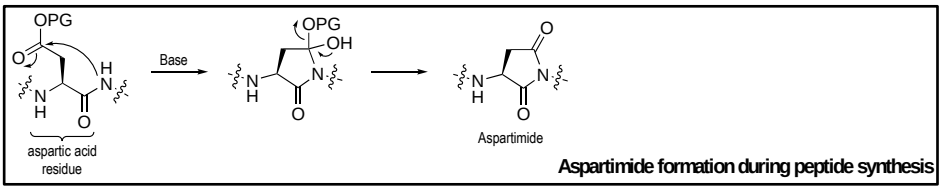


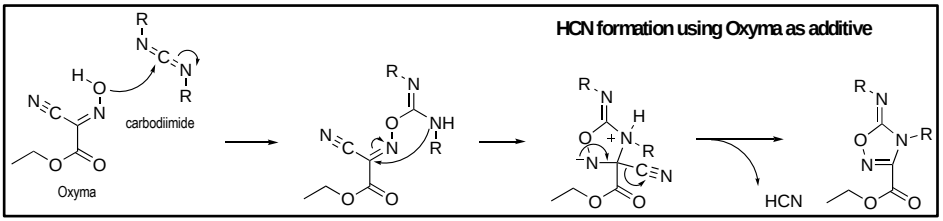



RCOOH
carboxylic acid
base (B)
HBTU
acid activation
Mechanism of amide bond formation using an hydroxybenzotriazole derivative as copling agent
amide
R_2NH_2
see annex X


RCOOH
carboxylic acid
base (B)
COMU
acid activation
Mechanism of amide formation using an oxyma derivative as couplig agent
amide
R_2NH_2
see annex X


activated amino acid
Racemization during amide bond formation
FmO = fluorenylmethoxy
PG = protecting group


OPG
O
H
N
N
H
O
aspartic acid
residue
Base
OPG
OH
N
H
N
O
O
N
H
N
O
Aspartimide
Aspartimide formation during peptide synthesis


HCN formation using Oxyma as additive
R
N
C
N
R
H
O
N
C
O
O
Oxyma
carbodiimide
HCN