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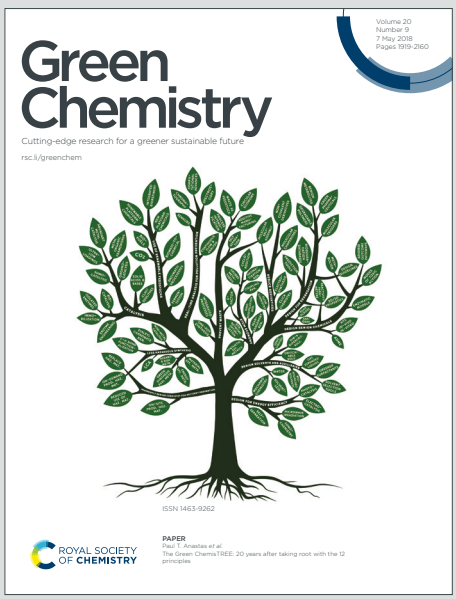

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Greening Fmoc/*t*Bu Solid-Phase Peptide Synthesis

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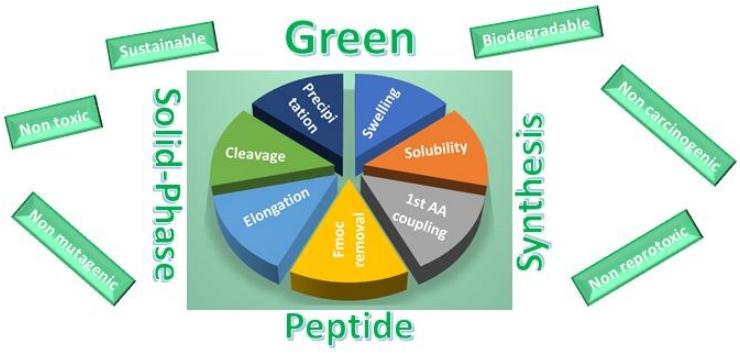
Abstract



Peptides are gaining considerable attention as potential drugs. The so-called Fmoc/*t*Bu Solid-Phase Synthesis is the method of choice for the synthesis of these molecules in both research and industrial settings. This synthetic strategy involves a solid polymeric protecting group and allows the use of an excess of reagents to achieve quantitative yields. Intermediates are not isolated. However, extensive washing with solvents is carried out between the synthetic steps. Hazardous solvents, mainly DMF, NMP, and CH_2Cl_2, are currently being used for the chemical reactions and also for the washings. In recent years, several studies have proposed the use of greener solvents in Solid-Phase Peptide Synthesis (SPPS). Here we compile all the greening efforts done in this regard and analyze each synthetic step separately. In summary, we can conclude that in many cases green solvents do not impair the synthetic process and that their adoption in current synthetic schemes will be translated into less impact on the environment and on human health.

1. Introduction

In 1990, the United States Environmental Protection Agency (US EPA) passed a Pollution Prevention Act [1]. This measure could be considered the starting point of the sustainable chemistry era. A year

later, Paul Anastas defined "Green Chemistry", saying "Green chemistry is the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances." [2]. Thereafter, research into green chemistry took off [3]. Under the auspices of the Division of Environmental Chemistry, the first green symposium, entitled "Benign by Design: Alternative Synthetic Design for Pollution Prevention", was held in 1993 during the 206th National Meeting of the American Chemical Society, in Chicago, Illinois [4]. The first Gordon Research Conference on green chemistry, called "Environmentally Benign Organic Synthesis", then followed in 1996. In 1998, the 12 principles of green chemistry were first published in a book named "Green Chemistry: Theory and Practice" [5]. From the very outset of research into green chemistry, the goal of this endeavor was to involve diverse sectors, from academia and research centers, right through to the industrial sector [6,7]. In this regard, in 2005, the ACS Green Chemistry Institute® Pharmaceutical Roundtable (GCIPR) was established to foster the adoption of green chemistry by the pharmaceutical industry. The key research areas were established in 2006 [6] and then updated in 2016 [7]. The GCIPR started with a focus on small molecules and biomolecules. More recently, medium-sized molecules (1000–5000 Da), peptides and oligonucleotides have also been included due to their potential as active pharmaceutical ingredients (APIs) [6,7].

Peptides play an important role in the fields of drug discovery and delivery [8-10], immunology [11], diagnostics [12], and biomaterials [13]. This relevance is attributed to their potential as drugs with high efficacy and minimal side effects. Indeed, hundreds of peptides are currently being tested in preclinical trials. In addition, about 150 peptides have yielded promising results in clinical phases, and approximately 70 others are already on the market [8,9,14,15].

Since the pioneering work of Bruce Merrifield in 1963 [16], solid-phase peptide synthesis (SPPS) has received increasing attention by the industrial sector and has finally been adopted as the strategy of choice for the production of peptides [17-19]. SPPS is based on the use of a polymeric support as the protecting group of the first amino acid [16]. This technique outperforms the solution phase strategy in various aspects, in particular with respect to the easiness and effectiveness of its protocol. SPPS consists of the following three main steps: addition of the reagents and reactants to perform the reaction; filtration; and washing. This straightforward protocol, which can even be automated, is capable of producing satisfactory yields for peptides comprising up to 50 amino acid residues. The introduction of automatic synthesizers has facilitated the synthetic strategy and allowed the introduction of techniques such as conventional or microwave heating [20], flow chemistry [21], and "on line" real-time monitoring techniques [22], thereby increasing access to peptides that are difficult to prepare using more conventional methods. An important advantage of the SPPS strategy is that the scaling-up process can be carried out very smoothly in most cases. In the period 2017-2019, the Food

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and Drug Administration (FDA) approved 14 TIDES-based drugs, 13 of which were prepared using the solid-phase approach [8,9].

Although several strategies based on a variety of protecting groups have been published, the so-called 9-fluorenylmethoxycarbonyl (Fmoc) [23]/*tert*-butyl (*t*Bu) approach is, without doubt, the predominant strategy in both research and industrial settings. However, the Fmoc group cannot be considered green because it is produced from charcoal-derived products. Consequently, there is a call for the development of other protecting groups (see Concluding Remarks and Future Perspectives). In this context, this review is centered on the efforts that have been made to green the current Fmoc/*t*Bu methodology with regard to solvents.

Solvents are the major component of the total mass in any manufacturing process [24]. Ashcroft and co-workers reviewed trends in the use of solvents in SPPS between 1997 and 2002. They concluded that the use of *N,N*-dimethylformamide (DMF) and CH_2Cl_2 remained constant. In contrast, the use of *N*-methyl-2-pyrrolidone (NMP) increased three-fold over the same period [25]. All of these solvents raise both serious environmental [26] and health concerns because they are all reprotoxic [27]. Furthermore, CH_2Cl_2 is carcinogenic and is therefore being called to be banned [28,29]. Accordingly, it is of paramount priority to introduce green alternatives for these substances.

The greenness of a solvent is determined by means of the environmental health and safety (EHS) assessment method [30] or life-cycle assessment (LCA) method [31]. These approaches focus on various solvent characteristics, namely sustainability, biodegradability, ease of incineration, recyclability, ozone depletion impact, volatile organic compounds (VOC) and their emission potential, impact on water and on air, risk to human health, and safety hazard [32-34]. Thus, new alternative solvents must be non-toxic, sustainable and have properties that resemble those of the solvents being replaced [35].

Next, this review will discuss each individual step of the Fmoc/*t*Bu SPPS process. At the end of the paper, the use of water, which could be considered the greenest solvent of all, will also be addressed.

2. Conventional Fmoc/*t*Bu SPPS

The introduction of the Fmoc protecting group in 1970 by Carpino [23] facilitated SPPS. The so-called Fmoc/*t*Bu strategy is based on an orthogonal protecting group scheme (the two groups are removed by a different mechanism and in any order [36]). The Fmoc group is removed from the α-amino group through a β-elimination reaction with a base, mainly secondary amines such as piperidine or 4-methylpiperidine, while the side-chain protecting groups [*t*Bu, trityl (Trt), 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf)] and the peptide resin bond are cleavable by acid,

mainly trifluoroacetic acid (TFA). These two orthogonal cleavage mechanisms can be fine-tuned, thereby enhancing performance. Furthermore, the Fmoc/*t*Bu strategy does not involve the use of hazardous reagents like anhydrous HF and trifluoromethanesulfonic acid (TFMSA), and it minimizes the use of TFA to one step per synthesis. The *tert*-butyloxycarbonyl (Boc)/benzyl (Bzl) strategy requires the use of anhydrous HF or TFMSA and the concourse of TFA in each cycle for the removal of the Boc group [37]. The implementation of the Fmoc/*t*Bu strategy has democratized SPPS in that it is now possible for any decently equipped lab to synthesize peptides. However, the Fmoc approach has some disadvantages with respect to Boc. The Fmoc group is more hydrophobic than Boc, thereby hindering its use in an aqueous context. In addition, the Fmoc group is less soluble in many solvents, including green ones. The aromatic character of the Fmoc group favors π-π interactions, which very often lead to the collapse of the peptide resin and generate deletion peptides as a result of poor removal of the Fmoc group. In fact, only a few research laboratories use the Boc/Bzl strategy for the preparation of peptide thioesters required for the chemical ligation of proteins. At the industrial level, Boc chemistry is used only for the preparation of some small peptides whose Drug Master File was filed in the last century.

Several key points are required to assure successful SPPS. These include, but are not limited to, the correct choice of polymeric support, solvents, linkers, coupling reagents, protected amino acids, cleavage conditions, and precipitating ether [38]. Figure 1 shows a schematic representation of various SPPS steps and the reagents currently in use. Those reagents that are considered green are highlighted in this color, while red indicates those that should be replaced by greener ones.

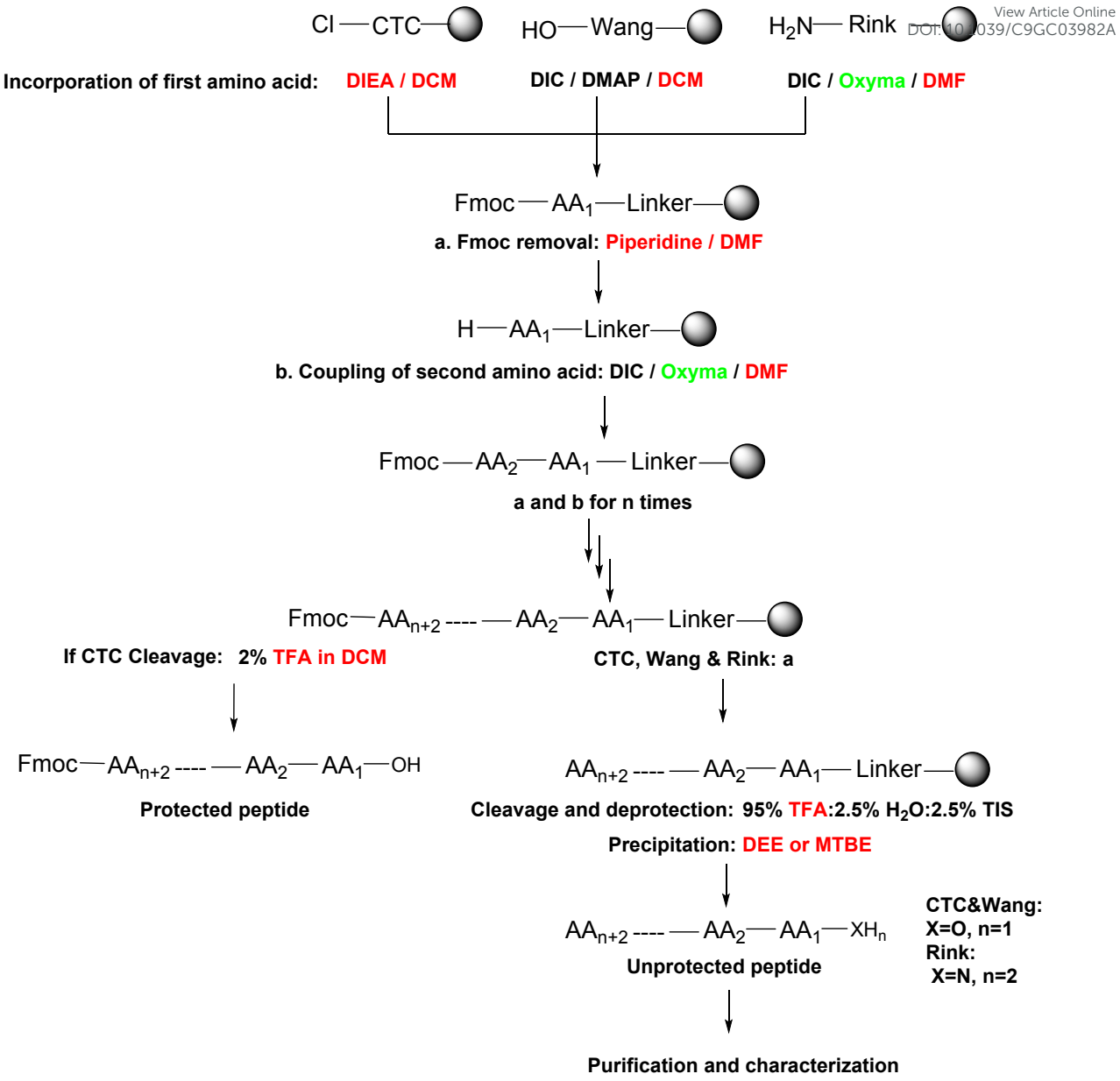

Figure 1. Schematic representation of SPPS and the reagents used.

3. Solvents

Solvents are the predominant component in chemical reactions. They are essential for the completion of reactions, as well as for purifying purposes [39]. Solvents account for 80–90% of the mass used in chemical synthesis, both in the pharmaceutical and fine chemical industries [24]. Furthermore, less than 50% of the solvents are currently recycled and reused [24]. The consumption of solvents is even greater in the SPPS mode as these substances are used in repeated washings that are intercalated between the different steps. In addition, various environmental and health concerns are associated with most solvents [26-29]. Given this context, there is a social mandate to find greener alternatives.

Several guidelines on solvents have been published by big pharmaceutical companies like Pfizer [40], Sanofi [41], GlaxoSmithKline (GSK) [26], and AstraZeneca [42], in addition to the GCIPR [43] and the Innovative Medicines Initiative (IMI)-CHEM21 [44]. These guidelines classify solvents on the basis of the parameters previously discussed and seek to facilitate the selection of the most adequate and greener solvents for each synthetic step. Figure 2 shows the green solvents proposed by the abovementioned guidelines. The solvents are grouped by chemical functionality (ethers, ketones, amides and derivatives, esters, aromatics, sulf-oxide/-ones, liquid ionics, alcohols, other and mixtures of the same). The green attributes associated with each solvent are detailed in Table 1 [44-96].

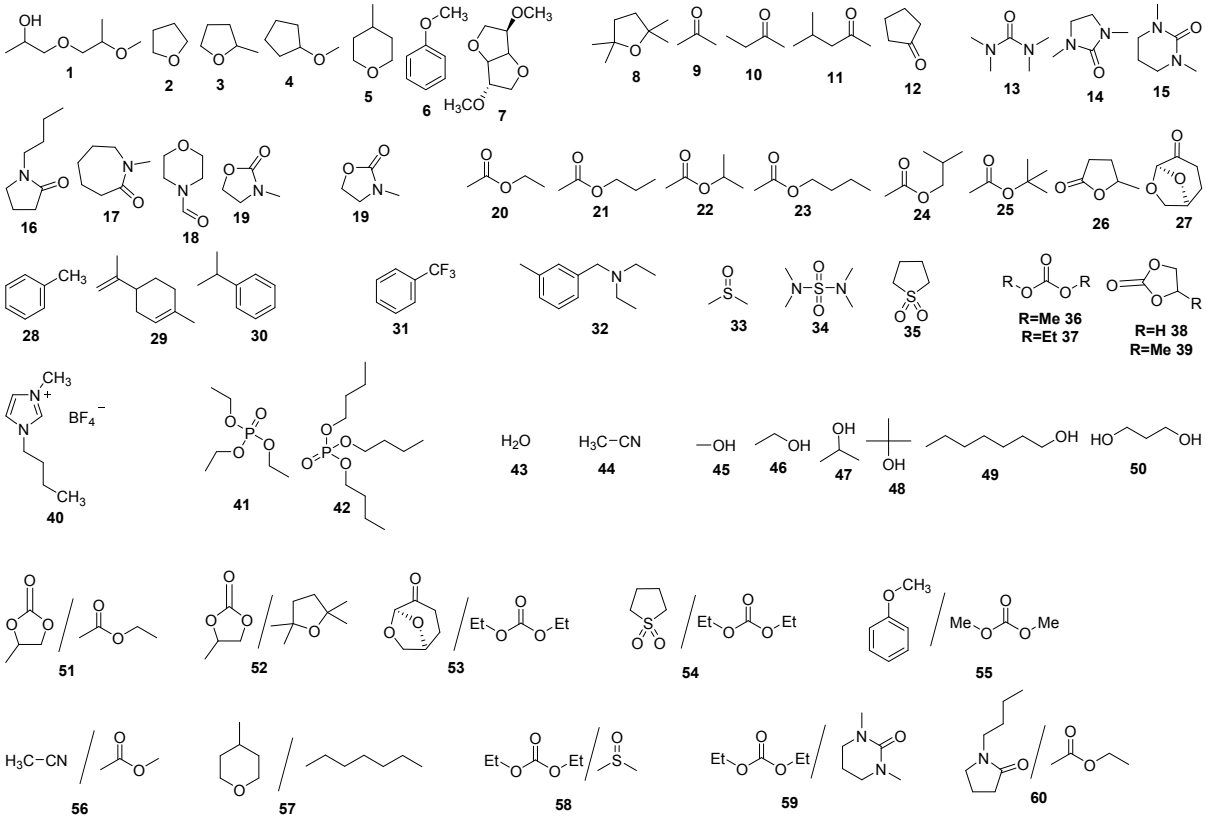

Figure 2. Chemical structures of common green solvents.

Table 1. Green solvents and their green attributes. The information is recompiled from [44-96].

#	Solvent	Green attributes						Reference
		Biodegradable	Non-carcinogenic	Non-mutagenic	Non-skin irritant, or sensitizer	Derived from biomass/ renewable resources	Others	
1	Dipropylene glycol dimethyl ether (DMM)	■					Non-toxic to aquatic organisms	[45]
2	Tetrahydrofuran (THF)	■		■	■			[46]
3	2-Methyl tetrahydrofuran (2-MeTHF)			■		■		[44,47-49,92]
4	Cyclopentyl methyl ether (CPME)			■	■		A high boiling point (106 °C). Low formation of peroxides. Low acute or subchronic toxicity.	[50,93]
5	4-Methyl tetrahydropyran		■	■			Not listed as toxic substance.	[51]
6	Anisole	■					Low-cost. Non-toxic.	[52]
7	Isosorbide dimethyl ether					■		[53]
8	2,2,5,5-Tetramethyloxolane (TMO)					■	Does not form hazardous peroxides.	[90,91]
9	Acetone		■	■			Exempt from restrictions placed on most volatile organic compounds (VOCs).	[54,55]
10	Butan-2-one (MEK)		■	■				[56]
11	4-methylpentan-2-one (Methyl isobutyl ketone)	■		■			Low mammalian and aquatic toxicity.	[57]
12	Cyclopentanone					■		[58]

13	Tetramethylurea (TMU)		■					[71]
14	1,3-Dimethyl-2-imidazolidinone (DMI)		■	■				[59]
15	1,3-Dimethyl-3,4,5,6-tetrahydro-2- pyrimidinone (DMPU)		■	■				[59]
16	*N*-Butylpyrrolidone (NBP)	■		■			Non-reproductively toxic.	[60]
17	N-Methylcaprolactam (MCL)		■					[73]
18	N-Formylmorpholine (NFM)		■					[62]
19	3-Methyl-2-oxazolidinone (MOL)		■					[61]
20	Ethyl acetate (EtOAc)				■		Non-phototoxic or photo allergenic.	[63]
21	Propyl acetate	■						[64]
22	Isopropyl acetate	■						[65]
23	Butyl acetate	■			■		Non-phototoxic or photo allergenic.	[63,66]
24	Isobutyl acetate	■						[66]
25	*t*-Butyl acetate	■					Exempt from restrictions placed on most volatile organic compounds (VOCs).	[54,66]
26	γ-Valerolactone (GVL)	■				■	Non-toxic.	[67]
27	Cyrene					■		[68]
28	Toluene		■	■				[69]
29	Limonene	■						[45]

30	p-Cymene								[70]
31	α,α,α-Trifluorotoluene							Hazardous Air Pollutant (HAPs)-free Not ozone depleter.	[45,94,96]
32	N,N-Diethyl-meta-toluamide (DEET)								[72]
33	Dimethyl sulfoxide (DMSO)							Non-toxic.	[74]
34	N,N,N',N'-Tetraethyl sulfamide (TES)							Not listed as toxic substance.	[75]
35	Sulfolane								[76,77]
36	Dimethyl carbonate (DMC)							Exempt from restrictions placed on most VOCs. Non-toxic.	[54,78]
37	Diethyl carbonate (DEC)							Low toxicity.	[79]
38	Ethylene carbonate (EC)								[45]
39	Propylene carbonate (PC)							Low toxicity. Economical manufacturing. Non-phototoxic. Non-photosensitizing.	[80,81,95]
40	1-Butyl-3-methylimidazolium tetrafluoroborate [Bmim][BF_4]							Not a reproductive toxicant. Non-measurable vapor pressure.	[82]
41	Triethyl phosphate							Low toxicity. Non-mutagenic. Non-neurotoxic.	[83]
42	Tributyl phosphate							Non-neurotoxic. Low toxicity.	[84]
43	Water								
44	Acetonitrile (ACN)							Non-toxic	[85]
45	Methanol (MeOH)							Not a human reproductive toxicant.	[86]

46	Ethanol (EtOH)						[45]
47	Isopropyl alcohol (IPA)					Does not cause teratogenic effects.	[87]
48	*t*-Butanol						[45]
49	Heptanol					No fertility effects. Not harmful if swallowed.	[88]
50	Ethylene glycol						[89]

Table 1 shows an extensive list of green or at least less hazardous solvents to those currently in use whose application in SPPS has been envisaged. However, for a determined solvent to be considered useful for this synthetic technique, it should have various key properties. In this regard, it should be able to dissolve the protected amino acids and the other reagents used; it should have the capacity to swell the polymeric support; and, given the presence of a heterogeneous phase in SPPS, it should not show high viscosity. In regard to the latter, a high viscosity will hinder the diffusion of the reagents containing solution inside the polymeric support, thus impeding them from reaching the reactive sites and producing concomitant poor yields. A slightly high viscosity could be compensated by increasing the temperature. The use of solvent mixtures could also improve those requirements more flexible.

4. Swelling

SPPS is a heterogeneous reaction in which the polymeric support is the solid matrix on which the growing molecule is anchored, and the other reactants and reagents are in solution. Consequently, the presence of the solid component requires the solvents to meet further criteria. Thus, in addition to having solvation capacity, the solvents must also swell the resin in order to allow the reactants to reach the active sites. In this regard, solvents with poor swelling capacity inhibit the reaction rate and can also fuel aggregation pathways during the synthetic process [97,98]. Importantly, in a green context, swelling must be maintained at a moderate level. Although superior swelling capacity favors reagent access to the active sites, it requires extra amounts of solvents and, on an industrial scale, it would require the use of larger capacity reactors.

Overall, three main types of polymeric support are used in SPPS, namely polystyrene (PS) [16], PS functionalized with polyethylene glycol (PEG) (PEG-PS; TentaGel, ArgoGel) [99,100], and PEG alone (PEGA, ChemMatrix, CM) [101-104]. PS swells nicely in non-polar solvents and to a lower but satisfactory extent in polar aprotic ones, depending on the Hansen solubility parameters of the polymeric support, loaded amount, type of linker and the solvent in contact [105,106]. PEG-PS/TentaGel swells better than PS in both types of solvent and shows increased compatibility with polar protic solvents [99,100]. CM shows greater swelling capacity in almost all solvents than the previous supports and better compatibility with water and aqueous buffers [102].

With respect to peptide production, PS is the polymeric support of choice due to considerations of price and commercial availability. In contrast, in the research context, the choice is based on the synthetic difficulty of the peptide in question (size, hydrophobic peptide regions, fatty acids, cyclic structures, multi copies of the same peptide…) and PEG-PS or CM are often chosen.

In the search for alternatives to DMF, NMP, and CH_2Cl_2, the first parameter to be addressed is swelling capacity, which is determined mainly following Griffith's method [107]. Thus, solvents can be classified on the basis of their swelling capacity as follows: ≥4 mL/g (good), 2–4 mL/g (moderate) and < 2 mL/g (poor).

Several groups have independently examined the swelling capacity of a number of solvents for various types of polymeric supports. Table 2 compiles results from our group [102,108-110], Lopez *et al.* at Novartis group [111], Ferrazzano and Cabri *et al.* [112], Pawlas and co-workers [113,114], and North *et al.* [115]. Some of these groups have also studied green binary mixtures and found that the swelling capacity of certain mixtures outperforms that of the individual components [112,113,115,116]. Thus, using the Hansen solubility parameter in practice (HSPiP) software, North *et al.* concluded that a green binary mixture of PC with EtOAc or with TMO shows a satisfactory capacity to swell PS and that it outperforms each individual solvent [116].

Table 2. Swelling capacity of green solvent alternatives on various types of polymeric support. The information is recompiled [102,108-117].

		Polymeric Support Type		
#*	**Solvent**	**PS**	**PS-PEG**	**CM**
1	DMM	**Moderate**	**NP**	**NP**
2	THF	**Good**	**NP**	**Good**
3	2-MeTHF	**Good**	**Moderate**	**Good**
4	CPME	**Good**	**Moderate**	**Moderate**
5	4-Methyltetrahydropyran	**Good**	**NP**	**NP**
6	Anisole	**Moderate**	**Moderate**	**Good**
7	Isosorbide dimethyl ether	**Good**	**Good**	**Good**
8	TMO	**Poor**	**NP**	**NP**
9	Acetone	**Poor**	**Moderate**	**Good**
10	MEK	**Moderate**	**Moderate**	**Good**
11	4-Methylpentan-2-one	**Moderate**	**Moderate**	**Moderate**
12	Cyclopentanone	**Good**	**Good**	**Good**
13	TMU	**Good**	**NP**	**NP**
14	DMI	**Good**	**NP**	**NP**
15	DMPU	**Good**	**NP**	**NP**
16	NBP	**Good**	**NP**	**NP**
17	MCL	**Poor**	**NP**	**NP**
18	NFM	**Poor**	**NP**	**Good**
19	MOL	**Poor**	**NP**	**NP**
20	EtOAc	**Moderate**	**Moderate**	**Good**
21	Propyl acetate	**Moderate**	**NP**	**NP**
22	Isopropyl acetate	**Moderate**	**Moderate**	**Moderate**
23	Butyl acetate	**Moderate**	**NP**	**NP**
24	Isobutyl acetate	**Moderate**	**Moderate**	**Moderate**
25	*t*-Butyl acetate	**Poor**	**NP**	**NP**
26	GVL	**Moderate**	**Moderate**	**Good**
27	Cyrene	**Poor**	**Moderate**	**Good**
28	Toluene	**Good**	**NP**	**NP**

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29	Limonene	Poor	Moderate	Good
30	p-Cymene	Poor	Moderate	Poor
31	α,α,α-Trifluorotoluene	Poor	NP	Good
32	DEET	Poor	NP	NP
33	DMSO	Poor	NP	Good
34	TES	Poor	NP	NP
35	Sulfolane	Poor	NP	NP
36	DMC	Moderate	Moderate	Good
37	DEC	Moderate	Moderate	Good
38	EC	Poor	Moderate	Good
39	PC	Poor	Moderate	Good
40	[Bmim][BF_4]	Poor	Moderate	Good
41	Triethyl phosphate	Poor	NP	NP
42	Tributyl phosphate	Poor	NP	NP
43	Water	Poor	Moderate	Good
44	ACN	Poor	NP	Good
45	MeOH	Poor	Moderate	Good
46	EtOH	Poor	Moderate	Moderate
47	IPA	Poor	Poor	Poor
48	*t*-Butanol	Poor	NP	NP
49	Heptanol	Poor	Moderate	Good
50	Ethylene glycol	Poor	NP	NP
	Green binary mixtures			
51	PC-EtOAc(1:9)	Good	NP	NP
52	PC-TMO (1:9), (3:7), (4:6)*	Moderate	NP	NP
53	Cyrene-DEC (3:7)**	Moderate	Moderate	Poor
54	Sulfolane-DEC (3:7)**	Good	Good	Good
55	Anisole-DMC (7:3)**	Good	Good	Good
56	CAN-EtOAc (1:1)	Good	NP	NP
58	DMSO-EtOAc (0.2:9.8), (1:9), (5:5)*	Good	NP	NP
59	DMPU-EtOAc (0.2:9.8), (1:9), (5:5)*	Good	NP	NP

* # refers to those indicated in Figure 2

**Several solvent ratios were investigated. The results in the table reflect the optimal ratio.

Good (green)-good swelling >4mL/ g and more; Moderate (Yellow)-tolerable swelling 2-4mL/g; Poor (Red)-poor swelling < 2mL/g; and NP (Gray)-not performed

The data shown in Table 2 reveal that several potential green solvents, namely isosorbide dimethyl ether and cyclopentanone (Table 2, # 7, 12), and to a lower extent THF and 2-MeTHF (Table 2, # 2, 3), swell the various types of polymeric supports in a satisfactory manner. CPME can swell PS only (Table 2, # 4). The following solvents were tested only with PS and showed good swelling capacity: 4-methyltetrahydropyran; DMI; TMU; DMPU; and NBP (Table 2, # 5, 13, 14, 15, 16). In contrast anisole, acetone, MEK, NFM, EtOAc, GVL, cyrene, limonene, α,α,α-trifluorotoluene, DMSO, DMC, DEC, EC, PC, [Bmim][BF_4], water, ACN, MeOH, and heptanol revealed good swelling capacity only for CM (Table 2, # 6, 9, 10, 18, 20, 26, 27, 29, 31, 33, 36, 37, 38, 39, 40, 43, 44, 45, 49).

Although not all the solvents have been tested in all types of polymeric supports, we can conclude that, as expected, PEG-based resins swell better than PS-based resins in a broad range of solvents.

The binary mixtures described in the literature generally show better swelling properties in PS than single solvents. Thus, sulfolane-DEC (3:7) and anisole-DMC (7:3) performed well for the three types of polymeric support (Table 2, # 54, 55). On the other hand, cyrene-DEC (3:7) showed a moderate capacity to swell PS and PS-PEG only (Table 2, # 53). In addition, PC-EtOAc (1:9), ACN-EtOAc (1:1), DMSO-EtOAc (0.2:9.8), (1:9), (5:5), and DMPU-EtOAc (0.2:9.8), (1:9), (5:5) were tested only on PS and also showed good performance (Table 2, # 51, 56, 58, 59), and to a lesser extent PC-TMO (1:9), (3:7), (4:6) (Table 2, # 52).

5. Fmoc-amino acids and reagent solubility

The solubility of Fmoc-amino acids, as well as the coupling reagents, must be assured prior to considering any green alternative. The data outlined in Tables 2 and 3 are compiled from results described by our group [109,118], Lopez *et al.* at Novartis [111], Pawlas and Rasmussen [114], and Ferrazzano and Cabri *et al.* [112]. The latter have also proposed that binary solvent mixtures, such as those mentioned in the previous sections, can optimize solvent properties.

Interestingly, Lopez *et al*. also checked the efficiency of the solvents to solubilize by-products [111]. Ferrazzano and Cabri *et al.* considered the addition of the coupling reagents (due to better solubility of the activated ester in comparison to the free amino acid) [112]. The capacity of a solvent to dissolve Fmoc-amino acids and coupling reagents was examined at concentrations of between 0.1 and 0.2 M, a range usually considered for automatic synthesizers and also at production scale.

Table 3. Green solvents and their solubilization efficiency. The information is recompiled from [109,111,116-118]

#*	Solvent	Fmoc-AA(PG)-OH, PG: sidechain protecting group													Oxyma		DIC	DIU	HOBT	HOAt	HATU	HBTU	COMU	EDC.HCl	DCC
		Gln(Trt)	Gly	Asn(Trt)	Phe	Val	Tyr(*t*Bu)	Arg(Pbf)	Trp(Boc)	Ala	Leu	Aib	Pure	B	K										
2	THF	S	S	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	NP	NP	S	S	NP	NP	NP	NP	NP	NP	NP	NP
3	2-MeTHF	S	S	SS	S	S	S	S	S	NP	NP	NP	S	S	SS	S	SS	S	SS	SS	SS	SS	NP	NP	NP
4	CPME	I	I	I	I	S	I	I	S	NP	NP	NP	S	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
6	Anisole	I	I	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	NP	NP	S	I	NP	NP	NP	NP	NP	NP	NP	NP
8	TMO	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	NP	NP	NP	NP	I	NP	NP	I	NP	S	NP	NP
13	TMU	S	S	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	NP	NP	S	S	NP	NP	NP	NP	NP	NP	NP	NP
14	DMI	S	S	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	NP	NP	S	S	NP	NP	NP	NP	NP	NP	NP	NP
15	DMPU	S	S	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	NP	NP	S	S	NP	NP	NP	NP	NP	NP	NP	NP
16	NBP	S	S	NP	NP	NP	NP	S	NP	NP	S	NP	S	NP	NP	S	S	NP	NP	NP	NP	NP	NP	NP	NP
18	NFM	NP	NP	NP	S	NP	S	NP	NP	NP	S	S	S	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
20	EtOAc	S	I	NP	NP	NP	NP	NP	NP	S	S	NP	S	NP	NP	S	I	NP	NP	NP	NP	NP	NP	NP	NP
21	Propyl acetate	S	I	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	NP	NP	S	I	NP	NP	NP	NP	NP	NP	NP	NP
26	GVL	S	S	S	S	S	S	S	S	S	S	S	S	NP	NP	S	NP	NP	NP	NP	NP	NP	NP	NP	NP
28	Toluene	I	I	NP	NP	NP	NP	NP	NP	NP	NP	NP	I	NP	NP	S	I	NP	NP	NP	NP	NP	NP	NP	NP
33	DMSO	S	S	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	NP	NP	S	S	NP	NP	NP	NP	NP	NP	NP	NP
37	DEC	I	I	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	NP	NP	S	I	NP	NP	NP	NP	NP	NP	NP	NP
38	EC	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	S
39	PC	NP	NP	NP	NP	NP	NP	NP	NP	S	S	NP	NP	NP	NP	NP	NP	S	NP	NP	S	NP	S	S	S

* # refers to those indicated in Figure 2

S (Green)-soluble; SS (Yellow)-sparingly soluble; I (Red)-insoluble; and NP (Gray)-not performed. OxymaPure: ethyl-2-cyano-2-hydroxyimino acetate; DIC: *N,N'*-diisopropylcarbodiimide; ; DIU: *N,N'*-diisopropylurea; HOBt: 1-hydroxybenzotriazole; HOAt: 1-hydroxy-7-azabenzotriazole; HATU: N- [(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene)-Nmethylmethanaminium hexafluorophosphate N-oxide; HBTU: N- [(1H-benzotriazol-1-yl)-(dimethylamino)methylene]N-methylmethanaminium hexafluorophosphate N-oxide; COMU: 1-(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylaminomorpholino-methylene) methaminium hexafluorophosphate; EDC.HCl: [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride]; DCC: N,N'-dicyclohexylcarbodiimide

Table 4. Green binary mixtures and their solubilization efficiency. The information is recompiled from [112,114,116].

#*	Solvent	Fmoc-AA(PG)-OH																						HOBT	HBTU
		Gly	Ala	Leu	Ile	Aib	Phe	D-Phe	Tyr (*t*Bu)	D-Trp (Boc)	Lys (Boc)	Glu (*t*Bu)	Arg (Pbf)	Cys (Trt)	Asn (Trt)	Gln (Trt)	Asp (*t*Bu)	Pro	Thr (*t*Bu)	Val	Trp (Boc)	Ser (*t*Bu)	Met	His (Trt)		
51	PC-EtOAc (1:9)	NP	S	S	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	S
52	PC-TMO (1:9)	NP	S	S	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	I	I
	(3:7)		S	S																					SS	SS
	(4:6)		S	S																					S	S
53	Cyrene-DEC (3:7)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	NP	NP	NP	NP	NP	NP
54	Sulfolane-DEC (3:7)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	NP	NP	NP	NP	NP	NP
55	Anisole-DMC (7:3)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	NP	NP	NP	NP	NP	NP
58	DMSO-EtOAc (1:9)	S	S	S	S	NP	S	NP	S	NP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	NP	NP

* # refers to those indicated in Figure 2

S (Green)-soluble; SS (Yellow)-sparingly soluble; I (Red)-insoluble; and NP (Gray)-not performed; for solvents 53, 54 and 55 coupling reagents were added (DIC/OxymaPure) to enhance the solubility.

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The data compiled in Tables 3 and 4 reveal several potential green solvents with excellent capacity to dissolve Fmoc-amino acids, coupling reagents, additives, and side products. The following solvents dissolved selected reactants during their corresponding screening study: THF; 2-MeTHF; TMU; DMI; DMPU; NBP; NFM; GVL; DMSO; and PC (Table 3, # 2, 3, 13, 14, 15, 16, 18, 26, 33, 39).

Inspection of the solvents that dissolved Fmoc-amino acids and other reagents and that showed a good capacity to swell PS (Table 2) reveals that only TMU and NBP fall into this category (Table 3, # 13, 16). On the other hand, CPME, which showed a good capacity to swell PS, did not dissolve most protected amino acids and other reagents (Table 3, # 4) and is therefore unsuitable for the purpose of SPPS.

Furthermore, of the green binary mixtures, the following solvent mixtures showed a good capacity to dissolve all reagents: PC-EtOAc (1:9); PC-TMO (4:6); cyrene-DEC (3:7); sulfolane-DEC (3:7); anisole-DMC (7:3); and DMSO:EtOAc (1:9) (Table 4, # 51, 52, 53, 54, 55, 58). However, # 53, 54, and 55 pairs showed this capacity only in the presence of the coupling reagents DIC and OxymaPure. Although this observation is positive, it limits the use of these mixtures. Considering these properties and swelling capacities (Table 4), binary mixtures show good performance regarding both aspects.

Overall, further research into the use of more binary and even tertiary mixtures will be helpful to identify other optimal mixtures. However, in the context of the green arena, the recovery or recycling of mixtures is tedious and, in some cases, impossible.

6. Fmoc removal

The two key reactions in the SPPS approach are the removal of the Fmoc group of the α-amino function, and amide/peptide bond formation. Although it is widely, but erroneously, believed that the latter reaction is the most difficult to achieve, Fmoc removal is also a very demanding reaction and it can often lead to poor synthetic results. Intra- and inter-chain interactions during both Fmoc removal and coupling are probably the main factors leading to poor SPPS. As mentioned earlier, the aromatic character and hydrophobicity of the Fmoc moiety play a fundamental role in favoring such interactions.

The removal of the Fmoc group has to be tackled from two perspectives, namely base and solvent. Traditionally, piperidine in DMF has been broadly used. However, piperidine is considered a hazardous substance as per the green guidelines [119]. Furthermore, it is also a controlled substance as it is the intermediate for the synthesis of narcotic drugs, thus jeopardizing its use in several countries [120,121]. Several groups have therefore introduced alternatives to piperidine. Wade *et al.* introduced piperazine [122], while Hachmann and Lebl tested several piperidine derivatives, proposing 4-

methylpiperidine as the optimal alternative [123]. Our group has recently reported a similar performance of piperidine, 4-methylpiperidine, and piperazine for removal of the Fmoc-group [124].

The results shown in Table 5 were obtained either by performing a complete SPPS and evaluating the purity and yield of the final product or by assessing the efficiency of the Fmoc removal step (on its own) by quantifying the unprotected fragment using HPLC or LC-MS.

Table 5. Green alternates for the Fmoc cleavage step. The information is recompiled from [108,111-114,116,117,122,123,125,126]

#	Solvent	Polymeric Support PS	CM
	Alternatives to DMF		
A	Piperidine/NFM	**Moderate**	**Good**
B	Piperidine/2-MeTHF	**Good**	**Good**
C	Piperidine/GVL	**Good**	**Good**
D	Piperidine/α,α,α-Trifluorotoluene	**Moderate**	**Moderate**
E	Piperidine/PC	**Poor**	**Good**
	Green binary mixtures		
F	Piperidine/Cyrene:DEC (3:7)	**Poor**	**NP**
G	Piperidine/Sulfolane:DEC (3:7)	**Moderate**	**Moderate**
H	Piperidine/Anisole:DMC (7:3)	**Good**	**Good**
	Alternatives to piperidine		
I	4-Methylpiperidine/EtOAc	**Poor**	**NP**
J	4-Methylpiperidine/Anisole	**Poor**	**NP**
K	4-Methylpiperidine/2-MeTHF	**Poor**	**NP**
L	4-Methylpiperidine/THF	**Poor**	**NP**
M	4-Methylpiperidine/Toluene	**Poor**	**NP**
N	4-Methylpiperidine/DMSO	**Good**	**NP**
O	4-Methylpiperidine/DMPU	**Good**	**NP**
P	4-Methylpiperidine/DMI	**Good**	**NP**
Q	4-Methylpiperidine/NBP	**Good**	**NP**
R	4-Methylpiperidine/DMSO-EtOAc (0.2:9.8), (1:9), (5:5)	**Good**	**NP**
S	4-Methylpiperidine/DMPU-EtOAc (0.2:9.8), (1:9), (5:5)	**Good**	**NP**
T	4-Methylpiperidine/NBP-EtOAc (1:1)	**Good**	**NP**
U	50% Morpholine/2-MeTHF	**Good**	**NP**
V	0.5% DBU*/2-MeTHF	**Good**	**NP**
W	0.5M NaOH/THF-MeOH (1:1)	**Good**	**NP**
X	0.1M NaOH*/2-MeTHF-MeOH (1:1)	**Good**	**NP**

*Several concentrations were tested. The results in the table reflect the optimal concentration.
Good (Green)-complete Fmoc removal; Poor (Red)-incomplete Fmoc removal; and NP (Gray)-not performed.

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Table 5 shows that piperidine prepared in either 2-MeTHF or GVL [108], as a single solvent (Table 5, # B, C), or in binary mixture of anisole-DMC (7:3) (Table 5, # H) [112], and to a lesser extent in α,α,α-trifluorotoluene (Table 5, D) or in sulfolane-DEC (3:7) (Table 5, # G) are the best choices to replace DMF in PS and CM. On the other hand, for NFM, the results were satisfactory only for CM and to a lesser extent for PS (Table 5, # A) [108]. This finding can be attributed to the poor capacity of this solvent to swell PS (refer to Table 4).

Lopez *et al.* at Novartis tested 4-methylpiperidine in several green solvents during their screening study [111]. Furthermore, Pawlas and Rasmussen tested the 4-methylpiperidine in three green binary mixtures [DMSO-EtOAc, DMPU-EtOAc and NBP-EtOAc] with distinct compositions [114]. From that work, DMSO, DMPU, DMI, NBP, DMSO-EtOAc, DMPU-EtOAc, and NBP-EtOAc emerged as potential alternatives to DMF and piperidine (Table 5, # N, O, P, Q, R, S, T). However, it should be noted that those authors' work was based only on PS [111,113,114].

Interestingly, Přibylka and co-workers introduced morpholine in 2-MeTHF, DBU in 2-MeTHF, and NaOH in either THF-MeOH or 2-MeTHF-MeOH for Fmoc removal in syntheses carried out on Rink-amide PS resin (Table 5, # U, V, W, X). All these Fmoc removal solutions emerged as potential greener alternatives to piperidine and DMF [125].

In the case of GVL, which showed good results in removing the Fmoc group from PS and CM, its ability to react with amines by ring opening and amide formation was demonstrated. For instance, the piperidine or 4-methylpiperidine solution rendered 4-hydroxypentanoic piperidide and 4-hydroxypentanoic 4-methylpiperidide, respectively (Figure 3) [127].

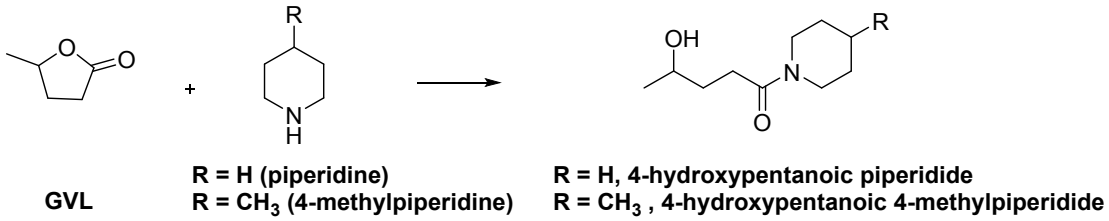


Figure 3. Ring opening pathways of GVL in the presence of piperidine or 4-methylpiperidine.

From a practical point of view, this is translated in the fact that the piperidine solutions are not fully stable in GVL (Figure 3). Overall, after 24 h, 66% of the piperidines remained in the solution and this percentage decreased to 36% for 4-methylpiperidine and 20% for piperidine after 96 h. The slightly greater stability of GVL in the presence of 4-methylpiperidine supports its preferred used [127]. Nevertheless, it is recommended that a fresh solution of 25% 4-methylpiperidine in GVL be prepared every 24 h. In this regard, the slightly higher concentration of a freshly prepared piperidine solution ensures excellent performance for Fmoc removal.

As GVL was found to react with amines, its capacity to react with the α-amino function of the growing peptide chain catalyzed with piperidines was studied [128] (Figure 4). This reaction must be considered only during Fmoc removal of the *N*-terminal Gly peptide resin due to the poor hindrance of Gly using microwave-assisted heating. Only in this case is it recommended that the dipeptide formed by the Gly and the subsequent Fmoc-protected amino acid be incorporated. This Fmoc-AA-Gly-OH can be prepared in a straightforward manner using CTC resin and green solvents. After cleavage, the CTC resin can be easily recycled and used again.

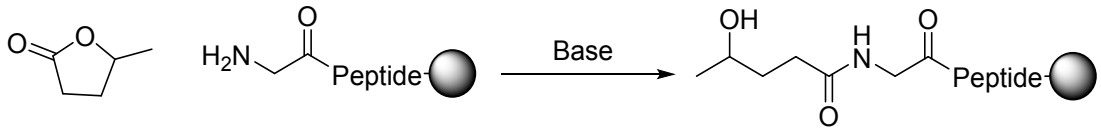
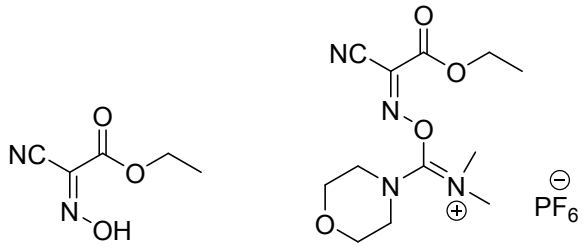


Figure 4. Potential side reaction between GVL and amine in the presence of base.

7. Amide (peptide) bond formation

Regarding amide (peptide) bond formation, the carboxylic acid moiety of one of the amino acids needs to be activated in an appropriate manner. The coupling concept was introduced by Sheehan and Hess in 1955 [129]. Various coupling reagents are currently available for this purpose [130,131]. Carbodiimide is the most widely used coupling reagent, including DCC, for Boc/Bzl chemistry, and DIC for Fmoc/*t*Bu chemistry. The reaction of the amino acid with these coupling reagents generates a highly reactive *O*-acylisoaurea, which can give rise to several undesired side reactions. To prevent this, the activation is carried out in the presence of N-OH-containing reagents, which render more suitable active esters. In the past, HOBt [132] and the more reactive HOAt [133] were used. However, the explosive property of these two reagents [134] encouraged the development of alternative safer reagents. In turn, OxymaPure (Figure 5) is considered safer and of even higher efficiency for the previous purpose, in terms of yield and the minimization of racemization [135]. In this regard, the GCIPR [136] has classified OxymaPure and its uronium salt COMU (Figure 5) [137,138] as two of the greenest coupling reagents. In this green context, COMU is preferred to its benzotriazole counterparts HBTU and HATU.



OxymaPure **COMU**

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Figure 5. Chemical structure of OxymaPure and COMU.

Recently, Kolis and co-workers at Eli Lilly have described that, in the absence of a nucleophile (amino component), the reaction of Fmoc-aa-OH with DIC and OxymaPure leads to the formation of hydrogen cyanide [139]. Although this reaction is much more exacerbated when large excesses of DIC and OxymaPure are used (3 fold more of each one with respect to the protected amino acid), it can be also observed when OxymaPure and DIC are used in substoichiometric amounts relative to the protected amino acid (0.5:0.5:1). However, this side reaction has not been reported in the presence of the amino component, which corresponds to the real case in peptide synthesis.

Peptide bond formation is currently carried out using DMF or NMP. In an effort to find a substitute for these solvents, a number of groups have introduced several green alternatives and exemplified their efficiency by synthesizing peptides on PS and CM. Table 6 recompiles these results.

Table 6. Performance of green solvents for peptide bond formation. The information is recompiled from [109-114,116-118,125,126,128,140,141].

#*	Solvent	Polymeric Support				Reference
		PS	Comments**	CM	Comments**	
2	THF	**Good**	Aib-Leu-enkephalin (5 mer)	**Good**	Aib-Leu-enkephalin (5 mer) Aib-ACP (10 mer)	[109,140]
3	2-MeTHF	**Good**	Leu-enkephalin (5 mer) Aib-Leu-enkephalin (5 mer)/ 40 °C	**Good**	Aib-Leu-enkephalin (5 mer) Aib-ACP (10 AAs)/40 °C	[125,126]
4	CPME	**Poor**	Aib-Leu-enkephalin (5 mer)	**Moderate**	Aib-Leu-enkephalin (5 mer)	[109]
8	TMO	**Very Poor**	Leu-Ala-Phe	**NP**		[116]
13	TMU	**Good**	Octreotide (8 mer)/ automatic synthesizer	**NP**		[111]
14	DMI	**Good**	Octreotide (8 mer)/ automatic synthesizer	**NP**		[111]
15	DMPU	**Poor**	Octreotide (8 mer)/ automatic synthesizer	**NP**		[111]
16	NBP	**Good**	Octreotide (8 mer)/ automatic synthesizer/	**NP**		[111]
18	NFM	**Moderate**	Aib-Leu-enkephalin (5 mer) Aib-ACP (10 mer)	**NP**		[118]

20	EtOAc	**Good**	Leu-Ala-Phe	**NP**		[116]
26	GVL	**Good**	Aib-Leu-enkephalin (5 mer) Aib-ACP (10 mer) ---------- Microwave-assisted automatic synthesizer/90 °C ACP (10 mer) ABC (20 mer) Thymosin (28 mer)	**Good**	Microwave-assisted automatic synthesizer/90 °C JR (10 mer) ABRF 1992 (16 mer) ABC (20 mer) Thymosin (28 mer)	[118,128,141]
33	DMSO	**Poor**	Octreotide (8 mer)/ automatic synthesizer	**NP**		[111]
39	PC	**Poor**	Leu-Ala-Phe	**Good**	Leu-Ala-Phe Bradykinin (9 mer)	[116] [117]
44	ACN	**NP**		**Good**	Leu-enkephalin (5 mer) ACP (10 mer) Aib-Leu-enkephalin (5 mer) Aib-ACP (10 mer)	[110,140]
			Green binary mixtures			
51	PC-EtOAc (1:9)	**Good**	Leu-Ala-Phe	**NP**		[116]
52	PC-TMO (1:9), (6:4) (7:3)	**Moderate**	Leu-Ala-Phe	**NP**		[116]
53	Cyrene-DEC (3:7)	**Poor**	Aib-Leu-enkephalin (5 mer)	**NP**		[112]
54	Sulfolane-DEC (3:7)	**Moderate**	Aib-Leu-enkephalin (5 mer)	**Moderate**	Aib-Leu-enkephalin (5 mer) Aib-ACP (10 mer)	[112]
55	Anisole-DMC (7:3)	**Good**	Aib-Leu-enkephalin (5 mer) Octreotide (8 mer), some steps at 40°C	**Good**	Aib-ACP (10 mer)	[112]
58	DMSO-EtOAc (1:9)	**Good**	Aib-ACP (10 mer)/50 °C	**NP**		[114]
60	NBP-EtOAc (1:1)	**Good**	Ac-Nle-*c*(Asp-His-Phe-Arg-Trp-Lys)	**NP**		[113]

* # refers to those indicated in Figure 2

**Syntheses are carried out manually at RT if other conditions are not indicated.

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Good (Green)-efficient synthesis; Moderate (Yellow)-tolerable synthesis; Poor (Red)-inefficient synthesis; and NP (Gray)-not performed.

From the color code in Table 6, it can be clearly appreciated that CM-based resins outperform PS-based ones. In this regard, all but one synthesis carried out on CM rendered good purities. However, this same analysis allows us to conclude that PS-based resins are also compatible with a large number of green solvents.

Although the synthesis of small peptides is easier to handle than that of larger and/or more complex peptides, the potential compatibility of the solvent in the former can be envisaged. Thus, TMO and PC were unable to accomplish the synthesis of even a simple tripeptide on PS (Table 6, # 8, 39). Of note, the mixture of TMO and PC in different proportions [PC-TMO (1:9), (6:4), (7:3)] (Table 6, # 52) allowed the synthesis of the same tripeptide on PS. Interestingly, PC and TMO in the proportion 6:4 rendered an immiscible solvent mixture, which adds an additional degree of heterogeneity to the system but allows the preparation of the small peptide. The authors claimed that in terms of green chemistry, this immiscibility could be favorable because it may facilitate the separation of the two solvents and even the isolation of some of the reagents for further recycling if they are soluble mainly in one of the two solvents.

The octapeptide octreotide has been synthesized by the Novartis group using several solvents (TMU, DMI, DMPU, and NBP) (Table 6, # 13, 14, 15, 16). The best results were obtained with NBP. This is particularly interesting because NBP is structurally similar to NMP, one of the solvents most widely used in SPPS at production scale, but has fewer and less hazardous properties. In this regard, NBP was successfully applied for the semi-industrial synthesis of the linear precursor of the octapeptide octreotide using PS at 25 °C. Although the purity of the crude peptide synthesized with NBP was only slightly inferior to that prepared with DMF (80% vs. 86%), the impurity profile was similar in both cases. This slight difference in purity could be due to the high viscosity of NBP (4.0 cP vs. 0.8 cP for DMF) [111].

As mentioned earlier, solvent viscosity is key aspect in the SPPS mode as it facilitates the diffusion of the reagents inside the polymeric support. In this regard, our group has used cyrene to synthesize Leu-Enkephalin (5 mer) using on PS. However, several deletion peptides were observed due, presumably, to the high viscosity of cyrene (14.4 cP) (unpublished data).

For most solvents, the synthesis of larger or more complex peptides requires the use of relatively high temperatures or even microwave-assisted heating. Thus, 2-MeTHF allows the synthesis of Aib (the hindered amino acid α,α-dimetylglycine) containing peptides with slight heating (Table 6, # 3).

Our group has studied the use of GVL in depth. Using a microwave-assisted automatic synthesizer at 90 °C, GVL allowed the synthesis of ACP (65-74) (10 mer), JR (10 mer), ABRF1992 (16 mer), ABC (20 mer) and thymosin (28 mer) using PS- and CM-type resins (Table 6, # 26). As mentioned earlier, the incorporation of the Gly was carried out through the dipeptide to avoid the previously discussed side reaction [128]. To the best of our knowledge, Thymosin (28 mer) is the largest peptide synthesized so far using a green solvent [141].

In addition to North's group (Table 6, # 51-52), Ferrazzano and Cabri *et al.* have also tested several binary mixtures, namely cyrene-DEC, sulfolane-DEC, and anisole-DMC (Table 6, # 53-55). First, they screened several mixtures for each pair of solvents in terms of suitability for the swelling of the polymeric support and the capacity to dissolve the protected amino acids and other reagents (see previous sections). The coupling was then studied with the best mixtures, which were cyrene-DEC (3:7), sulfolane-DEC (3:7), and anisole-DMC (7:3). The best results were achieved with the latter, as shown for the synthesis of Aib-Leu-enkephalin (5 mer), Aib-ACP (10 mer), and octeotride (8 mer) (Table 6, # 55).

Pawlas and Rasmussen from Polypeptide, one of the most important research contract organizations, achieved good results with two binary mixtures based on EtOAc and using PS-based resins. In this regard, they used NBP-EtOAc (1:1) for the synthesis of the cyclic peptide Ac-Nle-c(Asp-His-Phe-Arg-Trp-Lys)-NH2 for both the elongation of the peptide and cyclization in solid phase (Table 6, # 60); and DMSO-EtOAc (1:9) for the synthesis of Aib-ACP (Table 6, # 58). Regarding the DMSO-EtOAc mixture, it is important to highlight that it is less expensive than other rare green solvents and even DMF and/or NMP. Finally, this group was the first to demonstrate the possibility of recycling up to 86% of solvents and OxymaPure from the SPPS stream. The work was exemplified by indistinguishable syntheses using the recycled reagents versus the virgin ones.

Although the work carried out using binary and even ternary mixtures could open up a new arena for SPPS endeavors in green terms, additional research efforts and optimization are still required.

8. First amino acid incorporation through ester formation

The SPPS of *C*-terminal carboxylic acid peptides requires the concourse of two supports, namely CTC resin and Wang resin. CTC resin allows peptide release with a low concentration of TFA (1–3 %), rendering protected peptides. The treatment of both resins with a high concentration of TFA in the presence of scavengers leads to the unprotected peptide. On CTC resin, the first Fmoc protected amino acid is incorporated through a nucleophilic substitution reaction in the presence of a base, usually *N,N*-diisopropylethylamine (DIEA) in CH_2Cl_2. In contrast, activation with DIC in the presence of

4-dimethylaminopyridine (DMAP) in CH_2Cl_2-DMF is required for Wang resin. In both cases, CH_2Cl_2 is the solvent to be substituted as DMF (for the Wang resin) is added only to solubilize the Fmoc-AA-OH.

Our group has demonstrated that 2-MeTHF and GVL are the best green solvents in terms of swelling capacity, solubilization capability, and reactivity [108,109,118]. In this context, we have studied the use of these two solvents for the incorporation of the first amino acid onto CTC and Wang resins, in terms of incorporation yield, and of absence of racemization and formation of dipeptides in the case of Wang resin. While 2-MeTHF rendered excellent results for both supports [142], GVL was effective only for Wang resin [143]. When this solvent was tested on CTC resin, very low incorporation values were obtained (e.g., Fmoc-Gly-OH, 0.10 mmol/g; compared with 0.82 mmol/g in case of 2-MeTHF) [143].

For Wang resin, 2-MeTHF rendered comparable loading values and lower racemization than the conventional solvent (CH_2Cl_2) using Fmoc-AA-OH-DIC-DMAP at a ratio of 5:2.5:0.25 (symmetrical anhydride) [142]. On the other hand, the use of GVL was not as straightforward [143]. Thus, for some Fmoc protected amino acids, the use of the symmetrical anhydride of Fmoc-AA-OH rendered good results. However, for other Fmoc-AA-OH, the use of *N*-methylimidazole (NMI) as a catalyst and double incorporation reaction [Fmoc-AA-OH-DIC-NMI (2.5:2.5:0.25)] gave superior results to those obtained with DMAP. Again, for Wang resin, the formation of Fmoc-Gly-Gly-OH during the incorporation of less hindered and more reactive Fmoc-Gly-OH was very low for both solvents [142,143].

It is important to highlight that CTC resin can be recycled several times by means of treatment with neat TFA to remove the remaining peptide anchored and then further reactivated with $SOCl_2$ [128,144].

9. Peptide cleavage

After assembly of the desired peptide sequence, the final peptide has to be cleaved from the solid support. Using conventional Fmoc/*t*Bu SPPS mode, two main cleavage strategies are performed: (i) global deprotection with a high content of TFA in the presence of scavengers, which cleave the peptide from the resin with concomitant removal of the protecting groups; and (ii) treatment of the most acid-labile resins, such as CTC and Sieber resins, with organic solutions that contain a low proportion of TFA (1–4%), thereby rendering the protected peptides for further use for the preparation of larger peptides in hybrid solid-phase/solution schemes [145]. In both cases, TFA is the source of protons. Although it would be desirable to substitute TFA for a mineral acid, work carried out in this regard, such as that performed by Stetsenko and co-workers, has not been successful to date [146]. While the presence of organic solvents is almost residual in the global deprotection, these substances are clearly

the major component in the cleavage of protected peptides. So far, only the hazardous CH_2Cl_2 is being used, and therefore a greener substitute is called for.

In this arena, only a couple of examples using a simple dipeptide have been published to date. Pawlas and co-workers examined 2-MeTHF and a green binary mixture of EtOAc-ACN (1:1) to replace CH_2Cl_2 using the dipeptide Fmoc-Gly-Gly anchored to CTC resin as a model [113]. While the use of 2-MeTHF cleaved the dipeptide with very low yield (8%), the binary solvent allowed a satisfactory yield (96%) [113]. However, results obtained with the cleavage of a dipeptide cannot be used for the generalization of the strategy. In fact, preliminary and unpublished results obtained by our laboratory using 2-MeTHF and other green solvents confirm the difficulty encountered by Pawlas and co-workers and allow us to affirm that the size and composition of the protected peptide play a key role in this manipulation and that further efforts are required in order to address this issue.

10. Peptide precipitation

After the peptide is released from the polymeric support, it must be precipitated and separated from the resin. Ethers, including diethyl ether (DEE) and *tert*-butyl methyl ether (MTBE), are used for this purpose after the final global deprotection. [147]. However, these ethers raise concerns regarding low boiling and flash points, among others. In addition, MTBE is not stable under cleavage conditions and tends to *tert*-butylate some residues, such as Met and Trp, of the final peptide [147].

To make this process eco-friendlier, our laboratory has introduced green ethers to replace the hazardous ones previously used. CPME and 2-MeTHF have shown excellent performance in the precipitation step [142,148]. In this regard, comparable HPLC purities and similar recovery percentages have been obtained using these two solvents for the synthesis of a broad range of peptides with distinct amino acid contents and sizes (up to 28 AAs) [142,148] (Table 7). Only the small Leu-Enkephalin (5 AAs) peptide rendered poor recovery yields when CPME was used [148]. However, our laboratory has also commonly experienced similar behavior with the same peptide for the common reagent DEE.

Pawlas and co-workers introduced 4-methyltetrahydropyran ether either on its own or as a mixture in n-heptane as a green alternative to precipitate the peptide after the final global deprotection step [113]. They reported good recovery of the peptide after the precipitation step [113] (Table 7).

Table 7. Green ethers for peptide precipitation. The information is recompiled from [113,142,148].

		Polymeric Support	
#*	**Solvent**	**PS**	**CM**

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3	2-MeTHF	**Good**	**Good**
4	CPME	**Good**	**Good**
5	4-Methyltetrahydropyran ether	**Good**	**NP**
	Green binary mixture		
56	ACN:EtOAc (1:1)	**Good**	**NP**
57	4-Methyltetrahydropyran ether in n-heptane	**Good**	**NP**

* # refers to those indicated in Figure 2
Good (Green)-efficient precipitation; NP (Gray)-not performed

To pursue the idea of recycling the CTC resin, our group studied the effect of each ether on the morphological structure of the support, because the quality of the beads has to be assured prior to their reuse in further syntheses. CPME showed excellent performance in preserving the morphological characteristics of PS (Figure 6). This was not the case with the other ethers, such as DEE and MTBE. The positive result with CPME could be explained by the similarity of its solubility parameter with that of PS [144]. In this respect, the solubility parameters are derived mainly from the cohesive energy of the chemical compounds, hence their ability to mix with each other [149].

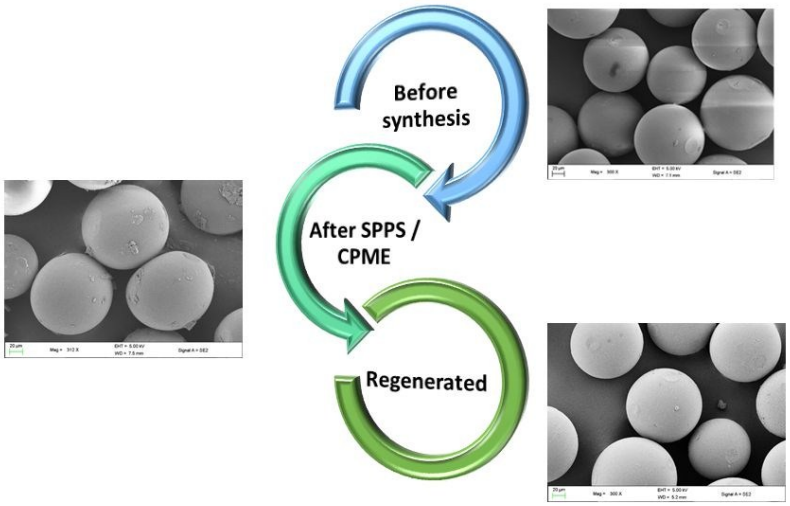

Figure 6. Schematic representation of CTC resin recycling after green CPME precipitation.

11. Water-based SPPS

Water can be considered the optimal green solvent. In this regard, the first efforts to use water in the SPPS context, and therefore to make the procedure greener, were made by Kawasaki and co-workers, who described water-soluble active esters [150]. Later, they evaluated the efficacy of water-soluble coupling reagents [151] and introduced several N^{α}-amino water-soluble protecting groups: 2-[phenyl(methyl)sulfonio]ethyloxycarbonyl tetrafluoroborate (Pms) [152-154],

ethanesulfonylethoxycarbonyl (Esc) [155], and 2-(4-sulfophenylsulfonyl)ethoxycarbonyl (Sps) [156] (Figure 7). All these protecting groups are removable in the presence of a base (piperidine, piperazine, NaOH) through a β-elimination pathway similar to those used to remove the Fmoc group.

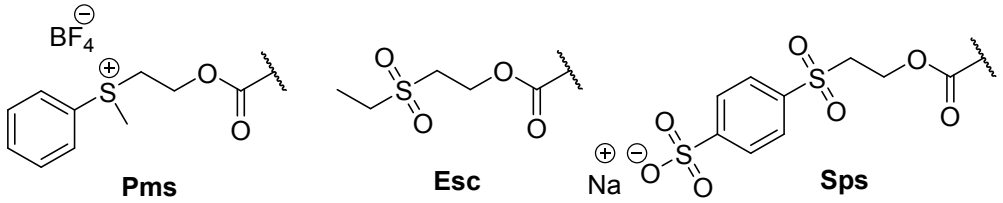

Figure 7. Water-soluble N^{α}-amino protecting groups for SPPS.

As mentioned earlier, reactions in SPPS are diffusion-dependent. Therefore, the swelling of the polymeric support is one of the most important prerequisites for a successful reaction[97]. In this regard, for water-based SPPS, polymeric supports with high hydrophilic components should be used, accompanied by water-soluble protected amino acids and coupling reagents. For this purpose, PS-PEG polymeric supports (TentaGel, Argogel, among others) or PEG-based CM ones are compatible with water as they show greater swelling in this medium than the more conventional PS-based supports. Kawasaki and co-workers studied all the protecting groups introduced by themselves (Figure 7). In this regard, they successfully synthesized Leu-Enkephalin and Met-Enkephalin pentapeptides via SPPS using PEG-PS-based resins [152-154]. Coupling was achieved using water-soluble carbodiimide EDC.HCl in combination with N-hydroxy-5-norbornene-endo-2,3-dicarboximide (HNOB) for Pms [152-154]. The addition of DIEA was considered for Esc and Sps [155,156]. In all cases, water was used as the washing solvent, while an aqueous 0.2% Triton-X solution [157] was also used to enhance the swelling of the polymeric support, as well as the solubility of Sps-/Esc-amino acids [152-156]. The protecting groups were removed using aqueous NaOH for Pms and Esc [152-155], and NaOH/EtOH for Sps [156]. However, it should be noted that although these protecting groups were very well designed, they had a number of drawbacks, these relating mainly to a lack of stability (Pms) or excess stability (Esc) [158]. The synthesized peptides were released from the resin using TFA. The target pentapeptide was achieved in final yields of 61, 32, 71, and 61% when Pms, Pms-ONp, Esc, and Sps were used, respectively.

With the same idea in mind, in 2016, Knauer and co-workers introduced the SO_3^- group onto Fmoc, Trt, *t*Bu, Boc, and Cbz groups to enhance the water solubility of the those protecting groups (Figure 8) [159]. The resulting compounds of this sulfonation reaction are called 9-(2-sulfo)fluorenylmethyloxycarbonyl (Smoc) amino acid derivatives. Smoc amino acids have been successfully used to synthesize peptides by means of SPPS. The aforementioned strategy was

previously used by Merrifield for purification purposes. In this regard, he added the Smoc group to the free amine of growing peptide chains at the end of a solid-phase synthesis and then separated the Smoc-peptide chromatographically after the cleavage [160].

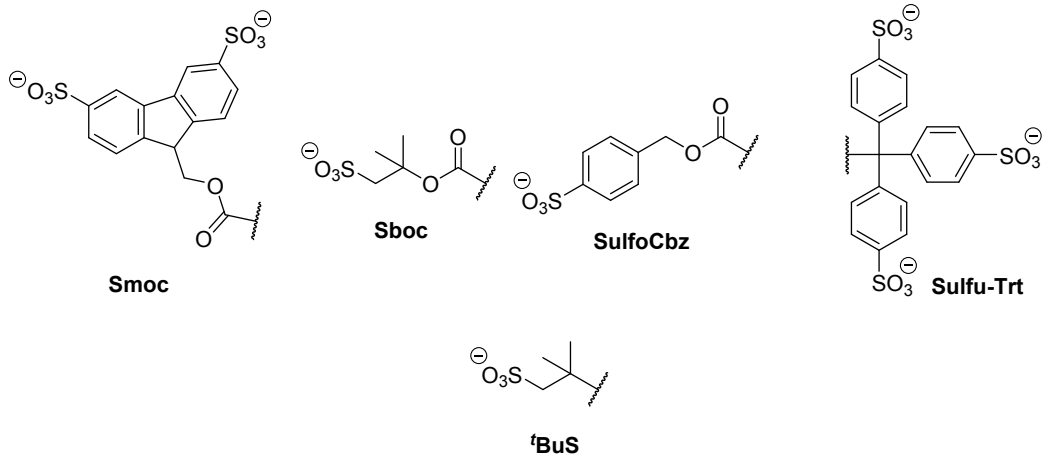

Figure 8. Sulfonated protecting groups.

In 2009, SPPS in water was reported by Grøtli M. and co-workers [161]. They described the synthesis of Leu-enkephalin penta-peptide using water on Tentagel- and CM-based resins and using Boc, Fmoc and azido amino acids. After studying several coupling reagents, those authors concluded that the ideal selection was EDC.HCl in combination with HNOB. Washing was done with water and Boc group removal was accomplished with 1N HCl. The subsequent neutralization was then performed with 10% DIEA in water [161]. Boc-amino acids rendered higher yields than Fmoc or azido derivatives. The performance of the former is attributable to the lesser hydrophobic character of the Boc group in comparison with Fmoc and azido groups. As envisaged, lower coupling efficiency was observed in the case of PS-based resins, which is explained by the poor swelling of this support in water. Grøtli M. and co-workers observed rapid formation of the active ester in the presence of HNOB [161]. Thus, a short activation time (1 min) and short reaction time are recommended. It should be pointed out that HNOB undergoes a Loosen rearrangement after the attack of the nucleophile on the protected-AA-ONB active ester [161]. A side product with a mass of the desired peptide+135, which corresponded to a β-alanine derivative, was detected during the synthesis of Leu-enkephalin in water using EDC.HCl along with HNOB as additive [161].

In 2007, Hojo and co-workers introduced innovative technology to overcome the water solubility issues of Fmoc- and Boc-amino acids [162-165]. They successfully processed these amino acids in water-dispersible nanoparticles through dispersion-based processes. This approach thus enhanced the mixing of the amino acids with the resin [165]. Their work was exemplified by the synthesis of Leu-Enkephalin in water using Tentagel-based resin. Coupling was achieved using water-soluble

carbodiimide EDC.HCl, HONB, and DIEA. Washing was done with water, and the Fmoc group was then removed by treatment with 0.1 N NaOH in 90% ethanol. Two syntheses were carried out in parallel, the first with nanodispersed Fmoc-amino acids and the second with the unprocessed ones. The former achieved a 67% yield of the pentapeptide, while no peak was observed by HPLC for the latter. Furthermore, the technology was also tested in Boc SPPS, which achieved a 89% yield of Leu-Enkephalin [165]. In Boc mode, the coupling reaction was performed using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) along with 4-methylmorpholine (NMM). The Boc group was removed with the aid of TFA and 4M HCl in dioxane. As expected, these results can be explained by the lower hydrophobicity of the Boc vs. Fmoc group.

Those authors extended this technology and synthesized more difficult longer peptides using a microwave-assisted mode [166]. However, in this case, and after testing several coupling reagents, they found that DMTMM with NMM was the best combination [166,167]. They successfully synthesized Leu-Enkephalin using nanodispersed Boc-amino acids [168]. Of note, these authors has also demonstrated that the aqueous microwave-assisted paradigm renders lower epimerization in comparison to the common paradigm with organic solvents. In this regard, the epimerization of Cysteine (Acm) was as low as 0.50% vs. 10.5% [167], and in case of Histidine (Trt) was 3.3% vs. 13.8% [169].

12. Concluding Remarks and Future Perspectives

There is a clear consensus among analysts that SPPS is the only synthetic methodology that can ensure a large number of peptides in the market. Although the individual steps of the SPPS addressed here cannot be considered green due to the low atom economy (presence of protecting groups) and, more importantly, to the large amount of waste generated, overall SPPS shows some key green characteristics. In this regard, in SPPS it is feasible to run a large number of synthetic steps in the same reactor, with the possibility of automation, with excellent yields, without isolation of the intermediates and therefore without their purification, and in a relatively short period of time when compared with the chemistry in solution. In this context, it is important for academic and industrial groups to channel further efforts into greening this process. A first but key step towards this goal involves finding replacements for hazardous solvents currently in use.

Overall, in this regard, the work reported to date on green solvents has demonstrated that these alternatives match or even improve on the performance of widely used hazardous solvents. However, fine-tuning of the synthetic procedure is sometimes required when using the new green alternatives.

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In some respects, 2-MeTHF could be considered the most "complete" green solvent to date. It works efficiently in the incorporation step of the first amino acid [142], in Fmoc removal and subsequent amide bond formation [126], and in the precipitation step [142]. Of note, the use of a single solvent for the whole synthetic process could be beneficial from a cost point of view.

However, the largest peptides have been synthesized using GVL assisted by microwave heating with CM. This polymeric support has usually given better results than PS, provided that conventional or microwave heating overcomes some of the limitations encountered at room temperature [141].

NBP, which has a very similar structure to NMP and even belongs to the same family as DMF, has emerged as a promising solvent. The high viscosity of NBP, which would explain its slightly lower performance when compared with DMF, is easily overcome by heating at 40 °C, which is an acceptable temperature even for industrial applications [111].

The emergence of binary solvent mixtures has recently opened up new avenues in the field. The use of a mixture will allow chemists to tailor "new" solvents with optimal properties. Furthermore, a mixture could facilitate the use of relatively non-expensive solvents, thereby having a favorable economic impact. If the satisfactory performance of immiscible solvents in SPPS is confirmed, it could open the possibility of more efficient recycling. In this context, the development of more binary and even tertiary solvents should be further investigated. In this regard, the concourse of machine-learning techniques may allow important advances in the field.

When peptide cleavage from CTC or Sieber resins is carried out with low content of TFA, finding a replacement for CH_2Cl_2 is the main priority. In general, the selection of a green solvent or mixture should also take into consideration the ease of the recycling step.

Although GCIPR has defined several greener coupling reagents, this is a field that is relatively unexplored and worthy of extensive study. In this regard, the development of recyclable catalysts with the idea of replacing hazardous coupling reagents and/or reducing the excess of reagents the of could have a great impact on the field [170]. The use of enzymes for peptide coupling during the stepwise elongation of the chain of amino acids on solid-phase is probably still far from being implemented. However, enzymes for the ligation of unprotected peptide fragments will be helpful for greening the whole process [171].

From an engineering perspective, the implementation of technologies such as flow chemistry [21] and "on line" real-time monitoring [22] are expected make a significant to contribution to reducing the excesses of reagents and/or the reaction times. In this regard, it is anticipated that these technologies and also microwave-assisted chemistry could be incorporated at the production level.

However, the major revolution in the green arena in the coming years is expected to come from a complete change in the protection scheme, leading to a reduction or even absence of protecting groups. Alternatively, more work, like that initiated by Kawasaki [152-156] and followed years later by Knauer [159], to replace the Fmoc group is needed. The development of side-chain protecting groups that facilitate the solubility of amino acids in water or in a broader range of solvents is also expected to contribute to attempts to green the SPPS approach.

As a final conclusion, amazing research opportunities await scientists in the field who are pursuing a more sustainable world.

Conflicts of interest

There are no conflicts to declare.

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