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Application of a new green protocol in Solid-Phase Peptide Synthesis: identification of a new green solvent mixture compatible with ETT/TBEC

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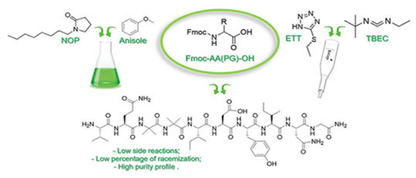
Introduction

Solid Phase Peptide Synthesis (SPPS) is the preferred technique for synthesizing bioactive peptides. However, traditional SPPS generates significant waste and employs hazardous solvents like DMF and DCM. The aim of this research is to investigate solvents that align with the green chemistry and are suitable for all stages of SPPS.

Some solvents, such as p-cymene and anisole, taken into consideration in this work, can be derived from renewable sources like plants and biomass, rendering them environmentally sustainable choices. However, many of these alternative solvents possess different physicochemical properties compared to DMF. To overcome this challenge, solvent mixtures are employed. In this study, we identified a novel green solvent mixture by combining anisole with NOP; its ability to swell different resins and its capability to solubilize all Fmoc protected amino acids was investigated. The same mixture was also assessed in combination with a pair of coupling reagents, TBEC and ETT and model peptides Aib-enkephalin and Aib-ACP were synthesized resulting in favorable outcomes in terms of peptide synthesis efficiency.



Methods

The solubility test was performed by adding 1 mL of either mixture into 10 mL test tubes containing 0.2 mmol of Fmoc-AA(PG)-OH or coupling agents. The tubes were then placed on a shaker at room temperature until complete dissolution.

For swelling test, 0.1 g of several resins were weighed and placed in a 5 mL reactor equipped with a polypropylene filter, which occupied a volume of 0.4 mL. The desired solvents, was added to the resin, and the mixture was stirred at room temperature for 45 minutes.

Deprotection kinetics test

Fmoc-Val-OH (0.2 mmol) was dissolved in a solution consisting of 20% piperidine and anisole/NOP (75:25). The reaction mixture was stirred at room temperature, and 2 µL were extracted at t=0 (before adding the base), and after 2, 4, 6, 8 minutes. These samples were analyzed using HPLC system.

Racemization study

The synthesis of Z-Phg-Pro-NH2 was performed by dissolving Z-Phg-OH (0.05 mmol) in a glass vial with 0.625 mL of DMF or anisole/NOP (75:25). The desired reagents were then added to the following order: H-Pro-NH2 (0.05 mmol), and ETT/TBEC (0.05 mmol, 1:2). The solution was stirred at room temperature for 3 h.

SPPS of Aib-enkephalin and Aib-ACP

Peptides synthesis was performed according to the solid phase approach using standard Fmoc methodology by Biotage Initiator + Alstra automated microwave synthesizer.

Results

The main goal is to replace the most common solvent used, DMF, with solvents or solvent mixtures that have a high score in the GSK Green Solvents Guide and, at the same time, are effective in all the steps of SPPS. Among these, the green solvents such as p-cymene and anisole[1] have been selected. As shown in Table 1, both anisole and p-cymene exhibit a low dielectric constant and dipole moment value, making them poor candidates for dissolving all amino acids and coupling agents. To address this issue, it was necessary to use mixtures with other green solvents to enhance these properties. Indeed, ethanol, butanol, ethyl acetate, and NOP[2], besides being classified as green solvents, exhibit better hydrophilic characteristics.

Table 1. Physical-chemical properties and cost of p-cymene, anisole, and co-solvents.

Physical-chemical properties	**Solvent**			**Co-solvent**			
	DMF	**p-Cymene**	**Anisole**	Ethyl acetate	Butanol	Ethanol	NOP
Boiling point	153°C	178°C	154°C	77°C	83°C	78°C	172°C
Density	0.95 g/cm³	0.86 g/cm³	0.99 g/cm³	0.90 g/cm³	0.77 g/cm³	0.78 g/cm³	0.92 g/cm³
Viscosity	0.85 mm²/s	0.81 mm²/s	0.78 mm²/s	0.44 mm²/s	2.95 mm²/s	1.1 mm²/s	6.6 mm²/s
Dielectric constant	36.7	2.24	4.33	6.02	10.90	24.5	32.0
Dipole moment	3.86	0.18	1.38	1.88	1.7	1.69	-
Melting point	-31°C	-68°C	-37°C	-84°C	25°C	-114°C	-25°C
Flash point	58°C	47°C	51°C	-4°C	35°C	12°C	142°C
Cost	72.50 €/L	49.00 €/L	97.10 €/L	55.60 €/L	109.00 €/L	169.00 €/L	195.00 €/L

Solubility test

Mix	P-CYMENE/ EtOAc	P-CYMENE/ANISOLE	P-CYMENE/ BuOH	P-CYMENE/ EtOH	P-CYMENE/NOP	ANISOLE/ EtOH	ANISOLE/BuOH	ANISOLE/ EtOAc	ANISOLE/NOP
	% EtOAc	% Anisole	% BuOH	% EtOH	% NOP	% EtOH	% BuOH	% EtOAc	% NOP
%	10 15 20 25 30	10 15 20 25 30	10 15 20 25 30	10 15 20 25 30	10 15 20 25 30	10 15 20 25 30	10 15 20 25 30	10 15 20 25 30	10 15 20 25
Gly									
Ile									
Leu									
Val									
Ala									
Phe									
Pro									
Met									
Tyr (tBu)									
Thr (tBu)									
Ser (tBu)									
Asp (OtBu)									
Glu (OtBu)									
Cys (Trt)									
Asn (Trt)									
Gln (Trt)									
His (Trt)									
Lys (Boc)									
Trp (Boc)									
Arg (Pbf)									

To evaluate the effectiveness of the chosen solvents in solid-phase peptide synthesis (SPPS), we first examined their ability to dissolve amino acids and coupling agents. Some amino acids were not completely soluble in mixtures tested, only mixture anisole/NOP (75:25) was able to solubilize all Fmoc protected amino acids and coupling agents, such as, TBEC[3] and ETT.[4]

Swelling test

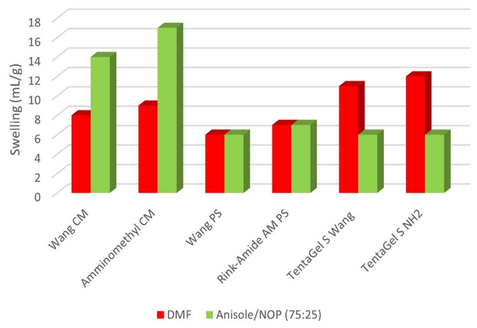


Figure 1. Swelling results of several resins in DMF and anisole/NOP (75:25).

A suitable solvent should have the ability to effectively swell the most used resins. In this study, we selected different types of resins and linkers. Subsequently, after removing the solvent and drying the resin, we evaluated the degree of swelling using the following formula: (ml/g) = (measured − volume 0.4)/0.1 g. As illustrated in Figure 1, TG resins showed a less degree of swelling than DMF, while PS resins behaved similarly in both solvents. On the other hand, CM resins showed better swelling in the green mixture than DMF.

Therefore, PS based resin and ChemMatrix resin showed a good degree of swelling in anisole/NOP (75:25) so the morphology of polystyrene and ChemMatrix resin beads was analyzed through scanning electron microscopy (SEM) using a Carl Zeiss EVOMA10 microscope (Figure 2). In particular, our SEM images at higher magnification show that the ChemMatrix® resin swells significantly in the green selected mixture.

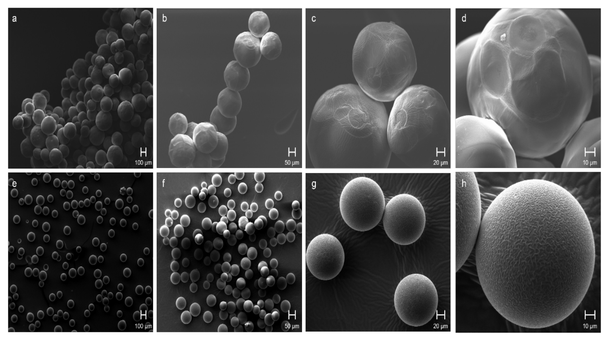


Figure 2. SEM pictures of PS and CM resin: (**a**) CM resin beads before swelling, (**b-d**) CM resin beads after swelling, and (**e**) polystyrene resin beads before swelling (**f-h**) polystyrene resin beads after swelling.

Deprotection kinetics test

In SPPS, it is common to use a solution of piperidine in DMF to remove the Fmoc group from the α-amino function. The experiment involved a 20% piperidine solution in anisole/NOP (75:25) and the formation of dibenzofulvene (DBF) was monitored using HPLC at time points of 0, 2, 4, 6 min. The peak corresponding to Fmoc-Val-OH disappeared after 4 minutes, so the Fmoc cleavage reaction is complete in few minutes, and this is very similar to when performed in DMF.

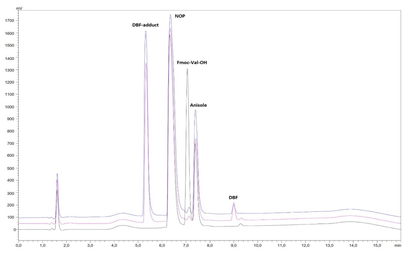


Figure 3. Chromatogram of Fmoc-Val-OH deprotection Kinetics test. t_0=0 min (black), t_1=2 min (purple), t_2=4 min (blue).

Racemization study

Racemization represents a fundamental side reaction in peptide synthesis. The couplings were performed using (L)-amino acids and the crude was analyzed by HPLC-MS injection after 3 h, in order to determine the formation of the dipeptide and the degree of racemization. Table 2 illustrates the degree of racemization. The green solvent mixture, Anisole/ NOP (75:25), provided only about 0.16% of racemization (entry **1**), which is lower than that observed in DMF (entry **2**).

Table 2. Racemization ratio (%) in the synthesis of Z-Phg-Pro-NH_2.[a]

Entry	**Solvent**	**Agents of coupling**	**Dipeptide (%)**	**DL[b] (%)**
1	Anisole/ NOP (75:25)	TBEC/ETT (2:1)	99.84	0.16
2	DMF	TBEC/ETT (2:1)	99.6	0.40

[a] Racemization was calculated by HPLC.
[b] Defined as (Z-D-Phg-Pro-NH_2/ Z-L-Phg-Pro-NH_2) x 100.

SPPS of Aib-enkephalin and Aib-ACP

As mentioned before, to apply the selected mixture to SPPS, it is important to test it throughout the synthetic process. In this case, a reference pentapeptide, Aib-enkephalin (H-Tyr-Aib-Aib-Phe-Leu-OH/NH2) (16), was chosen. The effectiveness of the new method is evaluated by measuring the amount of des-Aib. The synthesis was carried out on Wang PS and Rink-Amide AM PS, using microwave-assisted synthesis and mixture anisole/ NOP (75:25) and DMF in all steps..

Table 3. HPLC purities of Aib-enkephalin pentapeptide assembled on different resins with Anisole/ NOP (75:25) as solvent for all steps.

Entry	**Solvent**	**Agents of coupling**	**Resin**	**Pentapeptide (%)**	**Des-Aib (%)**	**Other (%)**
1	DMF	TBEC/ETT (2:1)	Wang PS	91.56	-	29.69
2	Anisole/ NOP (75:25)	DIC/ETT (2:1)	Wang PS	99.63	-	0.37
3	Anisole/ NOP (75:25)	TBEC/ETT (2:1)	Wang PS	97.81	-	2.19
4	DMF	TBEC/ETT (2:1)	Rink-Amide PS	85.35	-	14.65
5	Anisole/ NOP (75:25)	DIC/ETT (2:1)	Rink- Amide PS	66.20	-	33.8
6	Anisole/ NOP (75:25)	TBEC/ETT (2:1)	Rink-Amide PS	89.64	-	10.36

Purity values of Aib-enkephalin were calculated by HPLC. As shown in Table 3, the highest values of purity were obtained for pentapeptide synthesized on Wang PS resin with new selected green mixture (entries **2** and **3**) compared to that obtained with the synthesis performed in standard solvent, DMF (entry **1**). Another interesting result was obtained using TBEC/ETT, as coupling agents, and Rink-Amide PS, as resin (entry **6**), in fact, the percentage of side reactions was lower than that obtained during the synthesis performed in DMF (entry **4**). Considering these results, entry **3** and **6** can be considered the best method for further investigations.

The same protocol was applied for the synthesis of a longer and difficult sequence, Aib-ACP decapeptide (H-Val-Gln-Aib-Aib-Ile-Asp-Tyr-Ile-Asn-Gly-OH/NH_2). HPLC purities of Aib-ACP are shown in Table 4. The highest values of purity were obtained for decapeptide synthesized on Rink-Amide PS resin with new selected green mixture (entry **4)**. Its value is approximately double that of the decapeptide synthesized with standard solvent (entry **3**).

Table 4. HPLC purities of Aib-ACP decapeptide assembled on Rink-Amide CM.

Entry	**Agents of coupling**	**Solvent**	**Resin**	**Decapeptide (%)**	**Des-Aib (%)**	**Other (%)**
1	TBEC/ETT (2:1)	DMF	Wang PS	96.64	3.36	-
2	TBEC/ETT (2:1)	Anisole/ NOP (75:25)	Wang PS	77.21	22.79	-
3	TBEC/ETT (2:1)	DMF	Rink-Amide PS	48.40	50.01	1.59
4	TBEC/ETT (2:1)	Anisole/ NOP (75:25)	Rink-Amide PS	98.86	1.14	-

Conclusion

In this work, we identified a new green solvent mixture for solid-phase peptide synthesis as a valid alternative to standard solvents, DMF. In particular, mixture of Anisole/NOP (75:25) was able to solubilize all Fmoc amino acids and showed a good property of swelling for Rink amide PS and Wang PS resins. Furthermore, we used a pairs of coupling agents, TBEC and ETT, able to reduce side reaction and improve the percentage of purity of synthetized peptides. We obtained satisfactory results synthesizing the pentapeptide, Aib-enkephalin and a longer peptide, Aib-ACP. Thus demonstrating that the new green protocol may replace the traditional DMF-based one for industrial peptide synthesis.

References

1. Delolo, F. G.; dos Santos, E. N.; Gusevskaya, E. V. Anisole: a further step to sustainable hydroformylation. *Green Chem*. **2019**, 21, 109.
2. Martelli, G.; Cantelmi, P.; Tolomelli, A.; Corbisiero, D.; Mattellone, A.; Ricci, A.; Fantoni, T.; Cabri, W.; Vacondio, F.; Ferlenghi, F.; Mor, M.; Ferrazzano, L. Steps towards sustainable Solid Phase Peptide Synthesis: use and recovery of N-octyl pyrrolidone. *Green Chemistry*. **2021**, 23, 4095.
3. Manne, S. R.; Luna, O.; Acosta, G. A.; Royo, M.; El-Faham, A.; Orosz, G.; De la Torre, B.; Albericio, F. Amide Formation: Choosing the Safer Carbodiimide in Combination with OxymaPure to Avoid HCN Release. *Org. Lett.* **2021**, 23, 6900−6904.
4. Nzama, H.; Manne, S. R.; Akintayo, D. C.; de la Torre, B. G.; Albericio, F. Ethylthio-1H-tetrazole (ETT) as coupling additive for the solid-phase synthesis (SPS) of hindered amino acid-containing peptides. Tetrahedron Letters **2024**, 142, 155108.

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