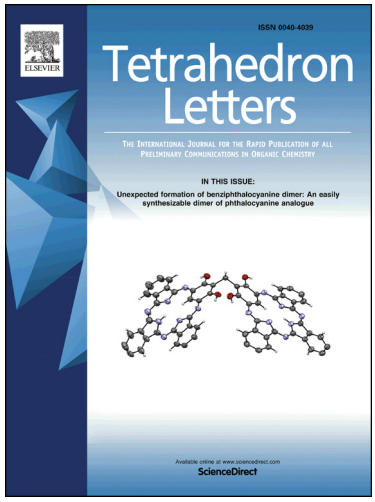

Green Solid-Phase Peptide Synthesis 4. γ-Valerolactone and *N*-Formylmorpholine as Green Solvents for Solid Phase Peptide Synthesis

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PII: S0040-4039(17)30789-X
DOI: http://dx.doi.org/10.1016/j.tetlet.2017.06.058
Reference: TETL 49050

To appear in: *Tetrahedron Letters*

Received Date: 3 May 2017
Revised Date: 15 June 2017
Accepted Date: 19 June 2017

Please cite this article as: Kumar, A., Jad, Y.E., El-Faham, A., de la Torre, B.G., Albericio, F., Green Solid-Phase Peptide Synthesis 4. γ-Valerolactone and *N*-Formylmorpholine as Green Solvents for Solid Phase Peptide Synthesis, *Tetrahedron Letters* (2017), doi: http://dx.doi.org/10.1016/j.tetlet.2017.06.058

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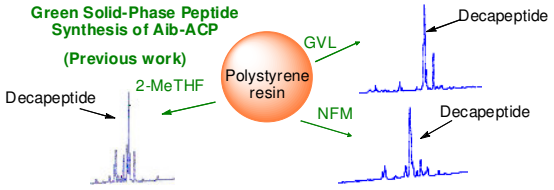

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Green Solid-Phase Peptide Synthesis 4. γ-Valerolactone and *N*-Formylmorpholine as Green Solvents for Solid Phase Peptide Synthesis

Ashish Kumar[a,b], Yahya E. Jad[b], Ayman El-Faham[c,d], Beatriz G. de la Torre*[b,e], Fernando Albericio*[a,b,c,f,g]

[a] *School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001, South Africa*
[b] *School of Health Sciences, University of KwaZulu-Natal, Durban 4001, South Africa*
[c] *Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*
[d] *Chemistry Department, Faculty of Science, Alexandria University, P.O. Box 426, Ibrahimia, 12321 Alexandria, Egypt*
[e] *School of Laboratory of Medicine and Medical Sciences, University of KwaZulu-Natal, Durban 4001, South Africa*
[f] *Department of Organic Chemistry, University of Barcelona, 08028-Barcelona, Spain*
[g] *CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Barcelona 08028, Spain*
Corresponding Authors: Beatriz G. de la Torre : e-mail: Garciadelatorreb@ukzn.ac.za; Fernando Albericio: phone: 0312603097; e-mail: Albericio@ukzn.ac.za

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Solid-phase peptide synthesis
Green solvents
Polystyrene resin
GVL
NFM

ABSTRACT

Herein, we report the use of γ-valerolactone (GVL) and *N*-formylmorpholine (NFM) as DMF substitutes in polystyrene based SPPS. The solubility of selected amino acids and coupling reagents were studied in GVL and NFM, followed by their use in the successful synthesis of Aib-enkephalin pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2) and Aib-ACP decapeptide (H-Val-Gln-Aib-Aib-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2).

Over the past decade, there have been a variety of governmental and industrial efforts to eliminate, replace, recycle or minimize the use of solvents in pharmaceutical and chemical industries. This effort has been driven by the desire to reduce human health impacts, process safety risks, and multiple impacts to the environment.[1, 2] The idea of using ''green'' solvents is the goal to minimize the environmental impact resulting from their use in chemical production, together with the use of bio-solvents derived from renewable resources. Therefore, substitution of hazardous solvents with green solvents is better for the environment as well as human, health and safety.[3, 4]

Since peptides play a crucial role in pharmaceutical and medicinal research, their synthesis has been a major focus for over a century.[5] Peptides can be synthesized using two main strategies: solution phase and solid-phase peptide synthesis (SPPS).[6-8] The protocol for SPPS, as initially developed by Merrifield,[9] is widely based on the use of a solid support, N^{α} protected amino acids, and the activation of carboxyl groups using coupling reagents.[5] The SPPS approach currently dominates peptide synthetic protocols because it allows the use of excess reactants to secure quantitative yields in each step. The excess reagents and/or by-products can easily be removed by filtration and washing steps using an appropriate solvent.[10] Therefore, the consumption of solvents is a key issue in SPPS. The most commonly used solvents are *N,N*-dimethylformamide (DMF), dichloromethane (DCM) and *N*-methylpyrrolidone (NMP). Several selection guides for green chemistry classify these as hazardous solvents.[11-15] Furthermore, the use of DMF in the pharmaceutical industry will probably be restricted by the Regulation for Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).[16] Therefore, the search for greener alternatives in SPPS is necessary.[7]

During the last few years, our group and others have investigated substitutes for DCM and DMF in SPPS.[7, 10, 17] However, swelling of the classical and most used, polystyrene (PS) resin limits the use of solvents other than DMF, DCM and NMP. On the other hand, the ChemMatrix resin, which was reported in 2006 by our group,[18] shows better swelling properties than the PS resin in polar as well as non-polar solvents, and therefore is compatible with a broader range of solvents. Thus in 2009, our group explored MeCN as an alternate for DMF using DIPCDI/OxymaPure as coupling reagents during ChemMatrix SPPS.[19] Later, we investigated the use of THF, however, MeCN and THF are not considered as green solvents.[11, 13] In the next screen of solvents, 2-methyl tetrahydrofuran (2-MeTHF), cyclopenthyl methyl ether (CPME) and isopropyl alcohol (IPA) were assayed. While CPME and IPA did not show compatibility with either of the two resins,[7] we proposed a successful green solid phase peptide synthesis (GSPPS) by employing DIPCDI/OxymaPure in 2-MeTHF as coupling conditions, 20% piperidine in 2-MeTHF for Fmoc removal and ChemMatrix resin as the solid support with additional washing steps using EtOAc.[7] The most problematic step in this strategy was Fmoc removal due to peptide aggregation. An optimized Fmoc removal was

achieved at a slightly higher temperature (40 °C). This strategy failed when the PS based resin was used.[7]

This prompted a parallel study of Fmoc removal using 20% piperidine employing different green solvents including γ-valerolactone (GVL), *N*-formylmorpholine (NFM), dimethyl carbonate, dimethyl isosorbide ether, EtOAc, and α,α,α-trifluorotoluene. As a result, it was found that GVL and NFM showed good results for Fmoc removal with the ChemMatrix resin and promising results with the PS resin.[20]

To expand the scope of the GSPPS strategy, herein we report the use of GVL and NFM with the PS resin. To the best of our knowledge, this represents the first report employing green solvents on PS; previous reports from our group and others dealt with ChemMatrix or PEG-PS resins.[7, 10, 17, 21-23]

GVL is a naturally available chemical[24] and has recently been described as an excellent green solvent candidate.[25] As demonstrated in detail by Horvath and co-workers,[24] GVL is renewable, has a low mp (-31°C), high bp (207°C), a definitive but acceptable smell for easy recognition of leaks and spills, non-toxicity and high solubility in water to assist biodegradation. Further, it does not hydrolyze under neutral conditions or form any measurable amount of peroxides in a glass flask kept under air for weeks, making it safe for large scale use. Due to the high solubility in water, GVL could be extracted from the reaction mixture using water and then separated *via* distillation since it does not form azeotrope with water.[24] Most importantly, GVL can be efficiently produced from biomass, preferentially from lignocelluloses.[26-28] On the other hand, NFM was reported as an alternative to the toxic solvent NMP.[29]

The solubility of reagents can limit the use of solvents in automatic peptide synthesizers.[7] Thus, we first investigated the solubility of different Fmoc-AA-OH compounds and OxymaPure in GVL or NFM and compared this with DMF. It was found that the amino acids (Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Aib-OH, Fmoc-Tyr(*t*Bu)-OH), showed good solubility (> 0.2 M) in GVL and NFM. The only exception was Fmoc-Phe-OH which showed a solubility of 0.16 M in GVL. It has been reported that peptide synthesizers generally use 0.1 or 0.2 M[7, 10] which is in fair agreement with our results. Furthermore, OxymaPure showed promising solubility in GVL and NFM (> 0.5 M).

Next, we investigated the performance of GVL and NFM in the key peptide coupling step Three different solvent system protocols were used as illustrated in Table 1: the standard DMF based protocol (A); GVL for coupling steps only (B) and NFM for coupling steps only (C).

Table 1. SPPS protocols used for this study.[a]

	Standard (A)	GVL (only for coupling) (B)	NFM (only for coupling) (C)
Washing	2×DMF 2×DCM 2×DMF	2×DMF 2×DCM 2×DMF	2×DMF 2×DCM 2×DMF
Deprotection	20% piperidine/DMF 7 min		
Washing	2×DMF 2×DCM 2×DMF	2×DMF 2×DCM 2×GVL	2×DMF 2×DCM 2×NFM
Coupling	Fmoc-AA-OH/DIC/OxymaPure (3 equiv) for 1 h		
	DMF	GVL	NFM
Washing	2×DMF	2×GVL	2×NFM

[a] Red indicates hazardous solvents, while green indicates green solvents.

The efficiency of GVL and NFM for SPPS was evaluated by synthesizing the relatively difficult model peptide Aib-enkephlin pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2) on the PS resin.[17] This short sequence containing two Aib residues in a row is known to increase the possibility of side-product (des-Aib-pentapeptide)[7] formation due to slow reaction as a result of steric hindrance.[30] Therefore, the peptide model Aib-enkephalin pentapeptide was prepared on the Fmoc-RinkAmide-AM-PS resin (0.1 g, 0.74 mmol/g) using the synthetic protocols described in Table 1. Excellent results were obtained using protocol B where GVL was used during the coupling steps, resulting in 99.2% purity of Aib-pentapeptide with 0.8% of des-Aib-pentapeptide by HPLC. Although protocol C rendered the product in lower purity than protocol A (93.1% *vs* 97.8%, Table 2, entries 1, and 3), it still can be considered as a promising result when the synthetic difficulty of this peptide is taken into consideration.

Table 2. HPLC purities of Aib-enkephalin pentapeptide assembled on the Fmoc-Rink Amide-AM-PS resin. [a]

Entry	Protocol	Pentapeptide (%)[b]	des-Aib (%)
1	A	97.8	2.1
2	B	99.2	0.8
3	C	93.1	3.6

[a] Same conditions and solvents used for the synthesis as in Table 1. [b] Determined by HPLC using the following conditions: linear gradient of 20 ⟶ 40% 0.1% TFA in a CH_3CN/0.1% TFA mixture in H_2O over 15 min, with a flow rate of 1.0 mL/min^{-1} and detection at 220 nm using a Phenomex C18 (3 µm, 4.6 × 50 mm).

Following the above results, the more demanding model peptide Aib-ACP decapeptide (H-Val-Gln-Aib-Aib-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2)[7, 17, 31] was synthesized using the PS resin. This modified version of model peptide ACP(65-74)[30] is the most used peptide for determining the efficiency of new SPPS protocols i.e., coupling reagents, resins; heating conditions or, as for this case, solvent systems. The results in Table 3 show excellent purities for the two greener syntheses, with protocol C proving the best (94.4%).

Table 3. HPLC purities of Aib-ACP decapeptide assembled on the Rink Amide-AM- PS resin. [a]

Entry	Protocol	Decapeptide (%)[b]	des-Aib (%)
1	A	93.8	6.2
2	B	89.7	1.4
3	C	94.4	5.6

[a] Same conditions and solvents used for the synthesis as in Table 1. [b] Determined by HPLC using the following conditions: linear gradient of 10 ⟶ 50% 0.1% TFA in a CH_3CN/0.1% TFA mixture in H_2O over 15 min, with a flow rate of 1.0 mL/min−1 and detection at 220 nm using a Phenomex C18 (3 µm, 4.6 × 50 mm).

The results obtained by performing coupling reactions with GVL or NFM encouraged us to completely avoid DMF and replace it by either GVL or NFM during washing and Fmoc removal steps. The peptide model Aib-ACP-decapeptide was manually assembled on the Fmoc-RinkAmide-AM-PS resin to evaluate these two protocols (Table 4). The HPLC purities of Aib-ACP decapeptide with GVL (protocol D) and NFM (protocol E) were almost identical (Table 5, entries 1 and 2,), but lower than that of protocol B and C (Table 3, entries 2, 3). However, the overall conclusion from this experiment is both GVL and NFM rendered this difficult peptide in purities higher than when 2-MeTHF and EtOAc (Table 5, entry 3) was

employed for the synthesis of the same peptide with the PS-resin.[7]

Table 4. Green SPPS protocols used for this study.

	GVL in all steps (D)	NFM in all steps (E)
Washing	2×GVL	2×NFM
Deprotection	20% piperidine/GVL 1×1 min + 1 ×7 min	20% piperidine/NFM 1×1 min + 1 ×7 min
Washing	3×GVL	3×NFM
Coupling	Fmoc-AA-OH/DIC/OxymaPure (3 equiv) for 1 h	
	GVL	NFM

Table 5. HPLC purities of Aib ACP- decapeptide in green solvent protocols.[a]

Entry	Protocol	Decapeptide (%)[b]
1	GVL in all steps	52.0
2	NFM in all steps	54.0
3	2-Me-THF in all steps	25.0[c]

[a] Same conditions and solvents used for the synthesis as in Table 4. [b] Determined by HPLC using the following conditions: linear gradient of 10 ⟶ 50% 0.1% TFA in a CH_3CN/0.1% TFA mixture in H_2O over 15 min, with a flow rate of 1.0 mL/min^{-1} and detection at 220 nm using a Phenomex C18 (3 µm, 4.6 × 50 mm). [c]Data extracted from ref 7.

In summary, we evaluated GVL and NFM as greener solvent alternatives in SPPS. GVL and NFM both show good solubility when compared to DMF. They also showed excellent coupling efficiencies when Aib-enkephalin pentapeptide and Aib-ACP decapeptide were synthesized in combination with the PS resin, DIC/OxymaPure as coupling reagent and DMF for washing and deprotection steps. Both solvents were then used to completely substitute DMF for successful synthesis of the same peptide models using the PS resin, resulting in higher purity than when 2-MeTHF was used. As reported in our previous publications,[7,20] and which was also confrmed here, we believe that the impurities come from the Fmoc-removal step, which is often the key step in the SPPS strategy.[20,32] Additionally, in the case of the GVL syntheses, we did not detect any major side-reaction associated with opening of the ring and further acylation of the amino function.[33] To the best of our knowledge, this is the first report applying a green solvent protocol with the PS resin. The overall conclusion drawn from this study is that GVL and NFM may be promising green solvents for SPPS in combination with the PS resin.

Acknowledgments

This work was funded in part by the following: National Research Foundation (NRF) and the University of KwaZulu-Natal (South Africa); and the International Scientific Partnership Program ISPP at King Saud University (ISPP# 0061) (Saudi Arabia). MEC (CTQ2015-67870-P), the *Generalitat de Catalunya* (2014 SGR 137), We thank Luxembourg Biotech for the gift of coupling reagents.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at

Highlights

- Green SPPS strategy using polystyrene (PS) resin
- GVL and NFM as excellent DMF substitutes in SPPS
- Solubility of reagents studied in GVL and NFM
- SPPS of Aib-enkephalin pentapeptide and Aib-ACP decapeptide using GVL and NFM