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Evaluation of greener solvents for solid-phase peptide synthesis

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ABSTRACT

Polar aprotic solvents such as *N,N*-Dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), *N,N'*-dimethylacetamide (DMAc) and chlorinated solvent such dichloromethane (DCM) are the most widely used solvents for Fmoc solid-phase peptide synthesis (SPPS). These solvents are considered hazardous chemicals but are normally used in large amounts for washing, deprotection, and coupling steps during SPPS. DMF, DMAc and NMP are classified as toxic for reproduction in accordance with Article 57(c) of REACH (Registration, Evaluation Authorization and Restriction of CHemicals) and were identified as SVHC (Substance Very High Concern). The aim of this study was to find a greener solvent alternative which could replace DMF in SPPS manufacturing processes at Ipsen. Greener solvents which demonstrated efficient resin swelling and solubility were selected as candidates for SPPS trials for the small-scale synthesis of commercial and developmental peptides.

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Green chemistry; greener solvents; solvent evaluation; peptides; solid-phase peptide synthesis

Introduction

First reported by R.B. Merrifield in early 1963, solid-phase peptide synthesis (SPPS) is the most commonly used method for the production of peptides in both research laboratories and in the pharmaceutical industry today (*1*). SPPS involves the use of a solid-phase support, typically a resin, which acts as an anchor for the addition of the first Nα protected C-terminal amino acid that is coupled to the solid support, followed by the removal of the Nα protecting group. This process is repeated until the desired peptide sequence is synthesized (Figure 1) (*2–5*).

Sustainable solvents are a topic of growing interest in both the research community and the chemical industry due to a growing awareness of the impact of solvents on pollution, energy usage, and contributions to air quality and climate change. Solvent losses represent a major portion of organic pollution, and solvent removal represents a large proportion of process energy consumption which increases the overall cost of the manufacturing process (*6*).

SPPS requires the use of excess reagents and solvents to ensure that each coupling step goes to completion and one of the main advantages of this method is that the excess reagents and by-products are easily removed by incorporating resin washing steps followed by filtration. Generally, hazardous polar aprotic solvents such as *N,N'*-dimethylformamide (DMF), 1-methyl-2-pyrrolidone (NMP), *N,N'*-dimethylacetamide (DMAc) and the hazardous chlorinated solvent dichloromethane (DCM) are employed for SPPS and the excessive use of these solvents during peptide synthesis generates a high volume of hazardous waste (*7–9*). The main uses and hazards associated with these solvents are detailed in Table 1. Currently, there are 50 peptide drugs on the market, approximately 170 in clinical trials, and >200 in preclinical development (*9*). Peptides are an important class of APIs because they show specific and high biological activities with low toxicity (*10*). These peptide-based APIs are generally synthesized using legacy manufacturing methods with little focus on Green Chemistry and engineering. Waste generated

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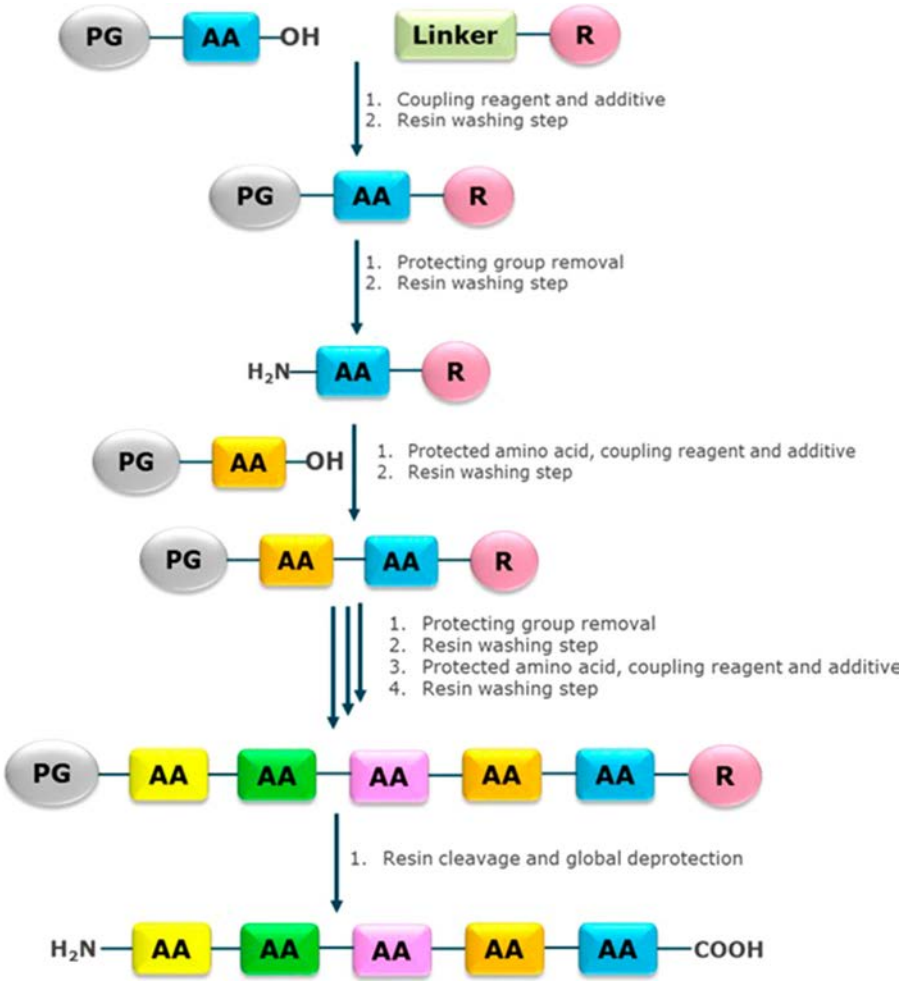

Figure 1. Solid-phase peptide synthesis (SPPS) overview.

from current peptide and oligonucleotide processes is typically 3000–15,000 kg per kg API produced (10–50-mer products) (*11*, *12*).

Table 1. Summary of hazardous polar aprotic solvents currently used for SPPS.

Solvent	Hazardous statement(s)
N,N'-dimethylformamide (DMF)	**H360D***** May damage the unborn child. **H332** Acute toxicity. **H312** Harmful in contact with skin or if inhaled. **H319** Causes serious eye irritation.
N,N'-dimethylacetamide (DMAc)	**H360D***** May damage the unborn child. **H312** Harmful in contact with skin or if inhaled. **H332** Acute toxicity
1-methyl-2-pyrrolidone (NMP)	**360D***** Reproductive toxicity may damage the unborn child **H319** Serious eye irritation **H315** Causes skin irritation **H335** Specific target organ toxicity – single exposure, may cause respiratory irritation
Dichloromethane (DCM)	**H351** Suspected of causing cancer.

*** Classified by REACH (Registration, Evaluation Authorization and Restriction of Chemicals) as a substance of very high concern (SVHC).

Background

Greener solvents

The field of Green Chemistry is defined as the 'design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances' (*13*). Over the past 10 years there has been a significant increase in the number of publications detailing alternative or greener approaches to peptide synthesis with the aim of reducing the large volumes of hazardous waste produced by the conventional SPPS approach. Included in these alternative approaches is the introduction of flow chemistry into SPPS and the use of microreactors. However, while these can reduce the amount of hazardous waste, neither of these alternative technologies eliminate the use of hazardous solvents.

Mechanochemistry is a promising new technology which is solvent free (*14*), however, this technology is still in its infancy for peptide synthesis and at present is not feasible for the large-scale commercial manufacturing of peptides (*15–17*). Another approach reported by

Rasmussen et al. is the use of chemo-enzymatic peptide synthesis (CEPS) as a more efficient and sustainable method to manufacture therapeutic peptides (*18*). The use of enzymatic methodologies, such as CEPS, in peptide synthesis greatly reduces the quantity of hazardous reagents required compared to standard SPPS methods. Rasmussen et al. employed CEPS methods to manufacture exenatide, a 39-mer synthetic GLP-1 agonist, which is the API found in the antidiabetic drugs, Byetta® and Bydureon®. They report that CEPS is an easily scalable, highly efficient strategy for sustainable manufacturing of complex peptide therapeutics, with the potential to emerge as a more cost-efficient alternative to standard peptide manufacturing methods (*18*).

Albericio et al. also reported that the substitution of the hazardous solvent DMF with γ-valerolactone (GVL), combined with the application of microwave-assisted automated SPPS, allowed the synthesis of peptides with a wide range of lengths and high purity using polystyrene- and polyethylene-glycol-based resins. They also reported that they were able to attain yields that were comparable to those obtained with standard methodologies (*19*).

Another exciting advancement in this area is the ability to perform peptide synthesis in water using surfactant as demonstrated by Lipshutz et al. (*20*). They report the synthesis of polypeptides under mild aqueous micellular conditions by introducing the designer surfactant TPGS-750-M. This attractive approach which avoids the use organic solvents has provided encouraging results (*21*).

The most reported method for the reduction of hazardous solvents in peptide synthesis involves investigating alternative, greener solvents for peptide synthesis that provide the desired function (solubility and separability) without the undesirable chemical properties that cause environmental, health and safety issues (*22*–*25*). Currently, the most commonly used solvent in SPPS is DMF, which due to its highly reprotoxic nature, has been classified as a Substance of Very High Concern (SVHC). This classification has influenced the scientific community to investigate greener solvents to replace hazardous polar aprotic solvents which are still widely used for SPPS today (*26*). The EPA state that *N,N*-dimethylformamide has been determined to be a systemic toxicant. An Acceptable Daily Intake (ADI), defined as the amount of a chemical to which humans can be exposed on a daily basis over an extended period of time (usually a lifetime) without suffering a deleterious effect, for *N,N*-dimethylformamide is 0.096 mg/kg/day for oral exposure (*27, 28*).

Over the past 10 years several papers have reported the use of less hazardous solvents for SPPS which include the following greener solvents: water, cyclopentyl methyl ether (CPME), methyl *tert*-butyl ether (MTBE), tetrahydrofuran (THF), acetonitrile (ACN), 2-methyltetrahydrofuran (Me-THF) (*4, 29*), ethyl acetate (EtOAc) (*4, 30*), dimethyl carbonate (DMC), γ-valerolactone (GVL) (*31, 32*), *N*-formylmorpholine (*33*) and most recently the use of *N*-butylpyrrolidinone (NBP), which is characteristically similar to NMP but is not classified as either reprotoxic or mutagenic (*34, 35*). The use of NBP has been reported by Novartis for the synthesis of octreotide, an eight amino acid peptide. Octreotide was successfully synthesized by Lopez et al. who report that with further optimization NBP could replace DMF in the manufacturing process of octreotide (*22, 36*). Rasmussen and Pawlas have also suggested that mixtures of greener solvents in peptide synthesis could enhance reagent and amino acid solubility and resin swelling properties in comparison with a single solvent system. In a recent publication, they detail the synthesis of a crude 6-mer which was synthesized in a higher yield and purity in DMSO/EtOAc (1:9 ratio) than in DMF and in addition, the EtOAc was recycled, by distillation, for further synthetic use (*37*). Cabri and co-workers also describe the benefits of binary solvent mixtures such as Cyrene/diethyl carbonate (30:70 ratio), sulfolane/diethyl carbonate (30:70 ratio), and anisole/dimethyl carbonate (70:30 ratio) which all exhibited good swelling properties for polystyrene (PS) and polyethylene glycol (PEG) resins as well as having the capability to dissolve a large proportion of amino acids (*23*).

Some of these greener solvent alternatives are still in the early stages of development and their toxicity and stability are not yet fully understood. Albericio et al. recently reported stability issues with GVL when used with base during the Fmoc removal step in peptide synthesis. Albericio et al. observed a competing ring-opening reaction when a solution of piperidine or 4-methyl piperidine in γ-Valerolactone was prepared. This competing ring-opening reaction led to a reduction in the concentration of base in the solution over time and could impact the efficiency of the Fmoc removal step. Their findings indicate that a solution of either piperidine or 4-methyl piperidine in GVL should be prepared daily to ensure optimal performance of the Fmoc removal solutions. It was also suggested that the concentration of base in these solutions could be increased to overcome this issue (*38*).

Although there are greener alternatives to solvents currently used in peptide synthesis, the CHEM21 selection guide of classical- and less classical-solvents does not recommend ACN, MTBE or THF for pilot or production-scale manufacturing (*39*). There are alternative solvent guides available and these include; the Pfizer

Table 2. CHEM21 guide for solvent selection (*37*).

Recommended	water, ethanol, *i*-propanol, *n*-butanol, ethyl acetate, *i*-propyl acetate, *n*-butyl acetate, anisole, sulfolane
Recommended or problematic	methanol, *t*-butyl alcohol, benzyl alcohol, ethylene glycol, acetone, methyl ethyl ketone, methyl *i*-butyl ketone, cyclohexanone, methyl acetate, acetic acid, acetic anhydride
Problematic	methyl-tetrahydrofuran, heptane, methyl-cyclohexane, toluene, xylenes, chlorobenzene, acetonitrile, *N,N′*-dimethylpropyleneurea, dimethyl sulfoxide
Problematic or hazardous	methyl-*t*-butyl ether, tetrahydrofuran, cyclohexane, dichloromethane, formic acid pyridine
Hazardous	diisopropyl ether 1,4-dioxane, *N,N′*-dimethylformamide, pentane, hexane, *N,N′*-dimethylacetamide, 1-methyl-2-pyrrolidone, methoxy-ethanol, triethylamine
Highly hazardous	diethyl ether, benzene, chloroform, 4-chloromethan, dichloroethane, nitromethane, carbon disulfide, hexamethylphosphoramide

Recommended: solvents to be tested first in a screening exercise.
Problematic: these solvents can be used in the lab or in the Kilolab but cause high energy consumption.
Hazardous: the constraints on scale-up are very strong.
Highly hazardous: solvents to be avoided, even in the laboratory.

'traffic light' solvent selection guide, the Sanofi solvent selection guide and the GSK solvent selection guide (Table 2) (*40–44*).

REACH regulations

The common polar aprotic solvents used in peptide synthesis, such as DMF, NMP and DMAC, are classified as toxic for reproduction in accordance with Article 57(c) of REACH (Registration, Evaluation Authorization and Restriction of CHemicals) and were identified as SVHC (Substance Very High Concern). All of them are included in the Candidate list for Authorization Article 57 (c) of Regulation (EC) 1907/2006 (REACH) in 2011–2012.

REACH legislation regulates the use of potentially harmful and environmentally damaging substances in the European Union (EU) and the European Economic Area, i.e. Norway, Iceland and Liechtenstein (EEA). This regulation was established to improve the protection of human health and the environment from the risk that can be posed by chemicals. The legislation applies

Figure 2. REACH process diagram.

to all chemicals used in industrial processes and covers all sectors which manufacture, import, distribute or use chemicals as raw materials or finished products. REACH also promotes alternative methods for performing hazard assessment of substances with the aim of reducing the number of tests carried out on animals. The REACH process includes Registration, Evaluation and Regulatory Risk Management (Figure 2). Initially, REACH was focused on the Registration, Evaluation and Classification of thousands of chemicals. By 2011, the focus shifted to deciding which chemicals should be Authorized or Restricted and moreover, to encourage the substitution process for the more hazardous chemicals. Currently, all chemicals manufactured or imported into the EU or EEA at or over one tonne a year must be registered (*45*).

A Risk Management Options Analysis (RMOA) is conducted on the SVHCs to determine how best to control the continued use of that substance. The RMOA may show that there is already community legislation in place to control the use of that substance, or that the substance requires further control (such as Authorization or Restriction) and so the substance is placed on the Candidate List of SVHC for authorization. Authorization and Restriction are lengthy and expensive processes that require extensive stakeholder consultation. If the substance is to be restricted, then the manufacturer, importer and downstream user must apply a control limit to protect employees or customers. If the substance is to be authorized the company needs to: apply for Application for Authorization (AfA) and await approval or rejection; if AfA is approved, the company must actively seek out a substitute chemical to replace the SVHC; perform the socio-economic analysis and pay a fee for use of the substance for a limited time. If Authorization or Restriction is not granted, then the substance will be banned for use after a period of time 'sunset date' (Figure 3).

Several substances commonly used in manufacturing processes are at varying stages in the authorization process. Recently, several substances proposed for Annex XIV (REACH Authorization list) have been put on hold to decide if Authorization is the best Risk Management Option for the substance. This hold is largely attributed to concerns raised by industry and other stakeholders about the suitability of the Authorization process.

DMF was included on the Candidate List as a SVHC for Authorization in Dec 2012 and it was proposed for the Authorization process in 2014. In 2016, it was proposed that polar aprotic solvents such as DMF with NMP and DMAC should be grouped together under the same classification and this decision was confirmed in Feb

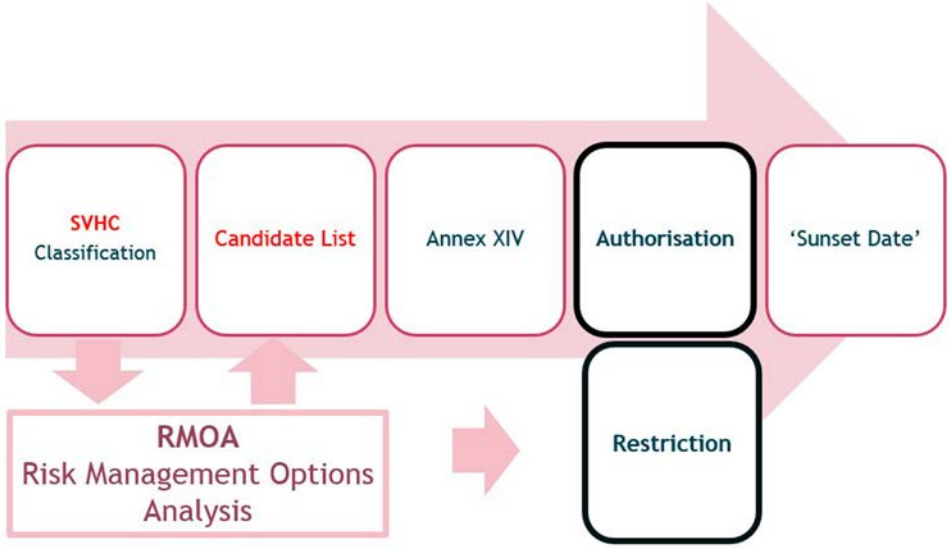

Figure 3. REACH regulatory risk management.

2018 (*46*). In Oct 2018, the new Restriction proposal for DMF was initiated and the public consultation occurred. The final deadline for comments on the restriction report was June 2019. According to the DMF restriction proposal from Oct 2018, DMF may only be manufactured and used in industrial and professional settings in concentrations at or below 0.3%; meaning that under normal operating conditions the exposure will remain below the derived no effect levels (DNEL). The DNEL for DMF for workers has a calculated value of 3.2 mg/m^3 for long-term inhalation exposure, and a calculated value of 0.79 mg/kg/day for dermal exposure (*47*, *48*). The Socio-economic analysis was performed, and the public consultation was concluded in Sep 2019. Changes to the DNEL for DMF were proposed and the new limits for workers for long-term inhalation exposure were set at 6 mg/m^3 and for dermal exposure the level is now 1.1 mg/kg/day (Figure 4) (*49*).

After 9 May 2020, NMP shall not be placed on the market as a substance on its own or in mixtures with a concentration equal to or greater than 0.3%, unless manufacturers, importers and downstream users have included all relevant chemical safety reports and safety data sheets. The DNEL for NMP has a calculated value of 14.4 mg/m^3 for exposure by inhalation and a calculated value of 4.8 mg/kg/day for dermal exposure (*50*).

DMAc was placed on the Candidate List of SVHC for Authorization in Dec 2011 and grouped with NMP and DMF in 2018. In Dec 2019 DMAc was proposal for restriction and start of Call for Evidence public consultation. Deadline for comments on the Call of Evidence is 13 Mar 2020 (*51*).

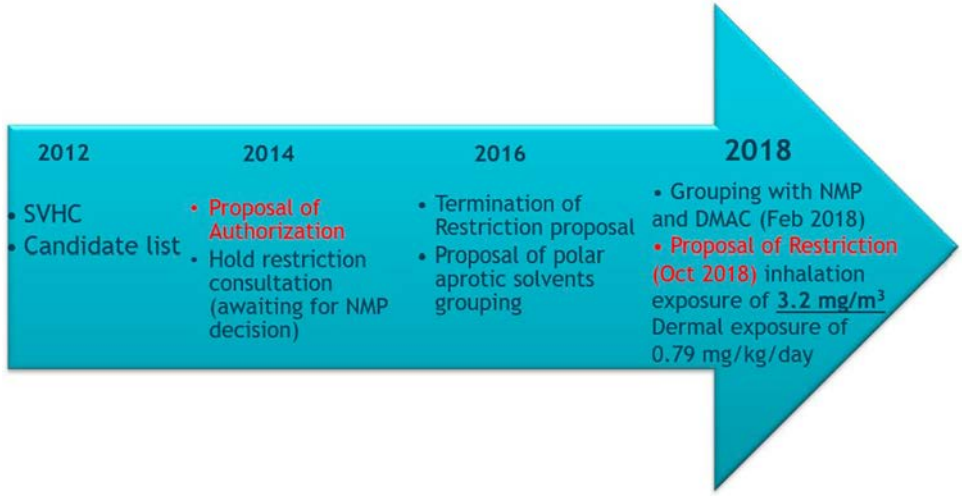

Figure 4. Map of authorization/restriction process of DMF.

Case study: alternative solvents to DMF for manufacturing Ipsen peptide products

The aim of this study was to find an alternative solvent to DMF for solid-phase peptide synthesis manufacturing processes commonly used at Ipsen. The initial screening involved the evaluation of resin swell factor. Solvents substitution studies were performed on optimized manufacturing conditions for all the peptides. The resin's evaluation is one of the first part of these optimization studies. Based on the yield, impurity profile, but also cost evaluation for commercial scale, safety of supply process and commercial manufacturing handling the resin is then chosen for further evaluation. Resin X, Y and Z are 3 different polystyrene resins, from 3 different suppliers, commonly used in peptide synthesis. The loading of all the resins were evaluated in the range from 0.4–1.2 mmol/g, and for all the projects the loading between 0.65 and 0.95 mmol/g was chosen. These studies were performed in standard DMF conditions. The resins are carboxylic and amide resins.

Those greener solvents which showed promising swell factors were further evaluated by completing solubility trials with the amino acids (AAs) and reagents currently used to manufacture commercial and developmental peptide products at Ipsen. Greener solvents which demonstrated efficient resin swelling and solubility were selected as candidates for SPPS trials for the small-scale synthesis of commercial and developmental peptides. The yield and purity profile of the peptides manufactured using greener solvents was then compared to the yield and purity achieved using the current manufacturing processes.

An investigation into the use of alternative solvents for SPPS for Projects A, B and C was conducted at IMIL (Ipsen Manufacturing Ireland Ltd.). The manufacture of peptides A, B and C involved SPPS on common polystyrene resins (resin X, Y and Z) using standard Fmoc (9-Fluorenylmethoxycarbonyl) and/or Boc (tert-Butyloxycarbonyl) methodology.

Process performance, industrialization and safety factors were assessed to provide solvents as suitable solvent candidates for Projects A, B and C. Process performance factors include the swell properties of a resin in the solvent, as well as the solubility of amino acids, coupling reagents and additives in the solvent (*52*). Industrialization factors include the bulk availability and cost of the solvent, while safety factors consider available toxicity data and REACH implications.

Swelling studies were carried out on Resins X, Y and Z as a preliminary evaluation of solvents for each project. The ability of a solvent to swell the resin adequately is critical in SPPS as it ensures availability of the active sites for subsequent coupling steps. Following the resin swell test for each solvent, the swell factor was calculated using the formula;

$$\textbf{Swell factor} = \frac{\textbf{Volume of resin (mL)}}{\textbf{Weight of resin (g)}}$$

Based on the results from the aforementioned swelling studies,* the most suitable solvent candidates were chosen for solubility studies.† An ideal solvent should exhibit good solubility for all reagents used in SPPS to ensure coupling reactions go to completion and result in an acceptable yield and purity for the final peptide. The solubility of each amino acid in the peptide sequence was tested in each solvent candidate, as well as the coupling reagents and additives used in the SPPS. Following this, SPPS was carried out using the solvent candidates and the purity and yield of the peptides were compared to when the SPPS was performed in DMF. The yields are displayed as a percentage of the standard DMF manufacturing process. The control experiment was performed in parallel to the study at the same scale, with the same equipment and raw materials. Analysis was run at the same time on calibrated GMP (Good manufacturing practice) equipment and the yields were calculated based on a standard HPLC (High performance liquid chromatography) assay.

Project A

Peptide A is cyclic octapeptide and consists of three unnatural amino acids, five natural amino acids and a small molecule residue (Figure 5).

All amino acids were protected with an Fmoc group. A total of seven greener solvents were considered as alternatives to DMF for SPPS for Project A. Swelling studies were carried out for Resin X with these solvents and the results are compiled in Table 3. Resin X had an acceptable swell factor only in DCM, NMP and 2-MeTHF, with intermediate results obtained in DMC, EtOAc, 2-MeTHF, CPME, trifluorotoluene and DMF.

Based on the results from the aforementioned swelling studies, the most suitable solvents candidates

*Swell test methodology: 1g of resin was added to a graduate cylinder, followed by 10 ml of solvent. The volume of resin was measured after 30 min and 1, 2 and 4 h.

†Solubility study's methodology: The amino acids/small molecule was dissolved in the solvent (0.1–0.3 M concentration) and visual check of solubility was performed (max time of dissolution 5–15 min). If the amino acids were not dissolved the coupling reagent, additives and /or base were added as protocol related with the project and the visual check was repeated.

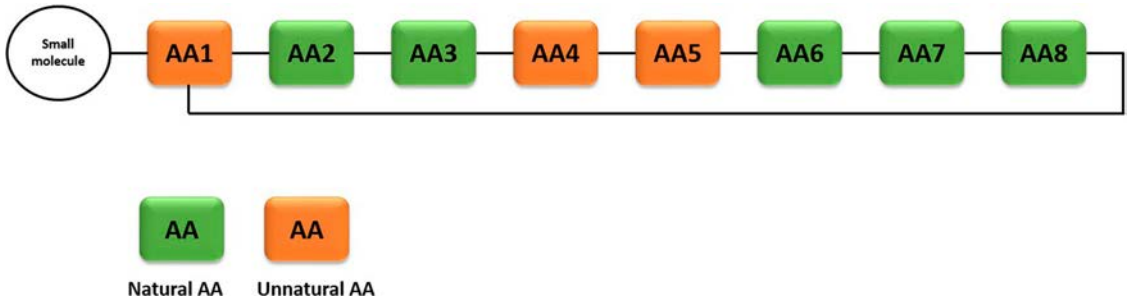

Figure 5. Structure of peptide A.

Table 3. Swell test results for Resin A.

Resin A	
Solvent	Swell factor (ml/g)
DMC	2.4
EtOAc	2.1
IPAc	0.7
2-MeTHF	4.1
CPME	2.4
MTBE	0.7
trifluorotoluene	2
DCM	5
DMF	3.5
NMP	4.8

Acceptable Result >4, Intermediate result 2–4, Unacceptable result <2.

(DMC, 2-MeTHF, CPME and EtOAc) were chosen for further evaluation for Project A. The solubility of each amino acid in the peptide sequence was tested, the results of which are detailed in Table 4. All amino acids exhibited poor or intermediate solubility in the solvent candidates (0.1-0.3M concentration). The addition of base had no impact on the solubility of the amino acids. The coupling agents and additives were also insoluble in the solvent candidates.

Due to the poor solubility of the amino acids used in project A, only DMC was evaluated for use in SPPS (Table 5). When SPPS was performed using DMC the yield was considerably lower at only 14%, compared to the yield for the original process performed in DMF. The purity was significantly lower at 4% compared to 82% for the peptide manufactured with DMF.

Table 4. Solubility of Fmoc protected amino acids used in Project A.

	Project A			
	DMC	2-MeTHF	CPME	EtOAc
8th AA	±	-	-	-
7th AA	-	-	-	-
6th AA	-	-	-	-
5th AA	-	-	-	-
4th AA	-	-	-	-
3rd AA	-	-	-	-
2nd AA	-	-	-	-
1st AA	-	-	-	-

Acceptable Result: Dissolution after addition of solvents (+), Intermediate result: Dissolution after addition of coupling reagent and base (±), Unacceptable result: AAs not dissolved after addition of solvent and base (–).

Table 5. SPPS results in alternative solvents for Project A.

	Project A	
	Yield %	Crude purity %
DMF (Standard)	X	82
DMC	14% of X	4
DMC (Optimized Conditions)	36% of X	11

To increase the yield and solubility the SPPS protocol was changed. The original coupling reagent was changed to COMU/Oxyma Pure/base system and the concentration of coupling solution was reduced from 0.3–0.1 M. Automatic recoupling for all amino acids was performed. Despite attempts to optimize the SPPS in DMC, the yield could only be increased to 36% of the yield for the original process performed in DMF. In addition, the purity remained low at 11% with elevated levels of deletion impurities observed particularly with later couplings. Based on these results the use of DMC was not recommended for further evaluation in Project A.

Project B

A cyclic octapeptide manufactured for Project B contains two unnatural amino acids, and six natural amino acids (Figure 6). The manufacture of peptide B utilizes a mixture of Boc and Fmoc protected amino acids. For project B, a total of nine solvents were considered as DMF substitute for SPPS. The results from the swelling studies indicated that Resin Y had an acceptable swell factor in a number of solvents including DMC, 2-MeTHF, CPME, NBP and EtOAc, which were chosen for further evaluation (Table 6).

The results for the solubility studies for Project B are detailed in Table 7. 2-MeTHF and NBP gave acceptable results for all seven amino acids, while DMC, CPME,

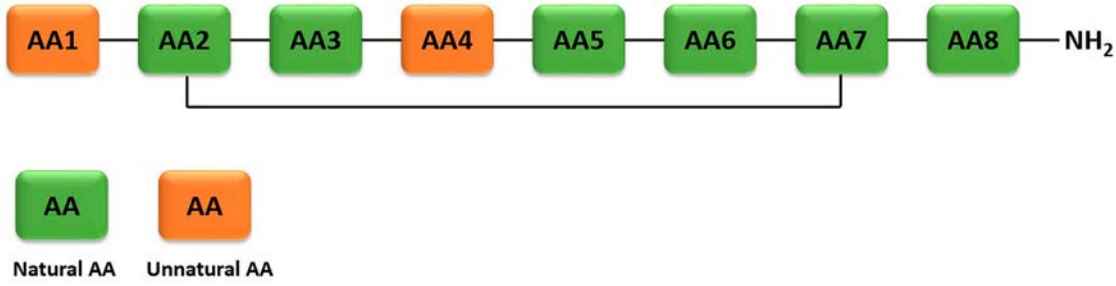

Figure 6. Structure of peptide B.

EtOAc showed a mixture of acceptable and intermediate results for the swelling studies

All five solvents used in the solubility studies were evaluated for use in SPPS for Project B (Table 8). When SPPS was performed using CPME and EtOAc the yield was considerably lower at 26 and 46% of the original process performed in DMF. The purity of the peptides manufactured in CPME and EtOAc was also lower but still acceptable at 70% and 65% of the original process respectively. SPPS in DMC also resulted in a significant drop in yield at only 13% of original process performed in DMF with a low purity of 43%.

When SPPS was performed using NBP the yield remained low at 59% of original DMF process; however, the purity was adequate at 70%. Similarly, SPPS in 2-MeTHF gave the peptide in a yield of 65% of original DMF conditions and a purity of 65%. In an attempt to increase these results, the SPPS was carried out in a mixture of NBP and 2-MeTHF; however, the yield remained at 56% of original results and purity at 71%.

The use of greener solvents as a replacement for DMF for SPPS in project B gave significantly lower yield results. The best result was obtained using 2-MeTHF (65% yield of the original yield for process performed in DMF). The use of the alternative solvents resulted in a higher formation of impurities, mostly deletion impurities, when compared to the DMF synthesis. Predictably, the efficiency of the coupling and deprotection reactions during the SPPS was found to be lower in the greener solvents when compared to the DMF standard. Additionally, the 1st and 6th amino acids started '"gelating'" during activation step in 2-MeTHF.

Table 6. Swell test results for Resin B.

Resin B Solvent	Swell factor
DMC	9
EtOAc	6.2
IPAc	6
2-MeTHF	9.7
CPME	9
MTBE	4.1
Trifluorotoluene	6
NBP	9
Cyrene	3
DCM	9
DMF	6.9
NMP	10

Acceptable Result >6, Intermediate result 4–6, Unacceptable result <4.

Table 7. Solubility of Fmoc and Boc protected amino acids used in Project B.

	Project B				
	DMC	2-MeTHF	CPME	NBP	EtOAc
7th AA	±	+	±	+	±
6th AA	+	+	+	+	+
5th AA	±	+	±	+	±
4th AA	±	+	+	+	±
3rd AA	±	+	+	+	+
2nd AA	±	+	±	+	±
1st AA	+	+	+	+	+

Acceptable Result: Dissolution after addition of solvents (+), Intermediate result: Dissolution after addition of coupling reagent and base (±), Unacceptable result: AAs not dissolved after addition of solvent and base (–).

Project C

Project C involved the manufacture of cyclic octapeptide, composed of five unnatural amino acids, three natural amino acids and a small molecule residue (Figure 7). The amino acids used for the manufacture of peptide C were Fmoc protected. For project C, nine

Table 8. SPPS results in alternative solvents for Project B.

Project B	Yield %	Crude Purity %
DMF (Standard)	X	82
CPME	26% of X	70
EtOAc	46% of X	65
NBP	59% of X	70
2-MeTHF	65% of X	65
DMC	13% of X	43
2-MeTHF (NBP only for 1st and 2nd amino acids)	56% of X	71

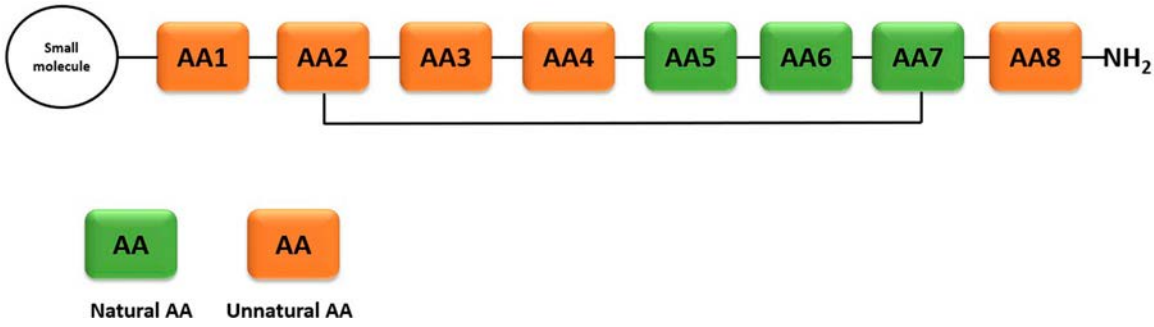

Figure 7. Structure of peptide C.

Table 9. Swell factors for Resin C.

Resin C	
Solvent	Swell factor (ml/g)
DMC	4.4
EtOAc	4.0
2-MeTHF	6.4
CPME	5.7
MTBE	2.0
γ-Valerolactone	5.0
NBP	7.1
Cyrene	2.0
MTHP	6.3
DMF	5.4
NMP	7.3

Acceptable Result >6, Intermediate result 2–6, Unacceptable result ≤2.

solvents were preliminarily examined as replacements for DMF in SPPS.

The results from the swelling studies carried out with Resin Z are outlined in Table 9. Resin Z had an intermediate swell factor in DMC, EtOAc, CPME and γ-Valerolactone, while 2-MeTHF, NBP, NMP and MTHP (4-Methyltetrahydropyran) showed an acceptable resin performance.

Based on the results from the aforementioned swelling studies, the most suitable solvent candidates were chosen for further evaluation in solubility studies. The solubility of each amino acid used in project C was tested in these solvents, the results of which are outlined in Table 10. DMC, 2-MeTHF, CPME, MTHP, EtOAc showed unacceptable results for the future evaluation for this project. Two solvents, NBP and γ-Valerolactone, showed a combination of both acceptable and intermediate results for the solubility studies and these were chosen for further evaluation for project C.

Table 10. Solubility of Fmoc protected amino acids used in Project C.

Project C							
	DMC	2-MeTHF	CPME	NBP	MTHP	EtOAc	GVL
8th AA	-	+	-	+	+	+	+
7th AA	-	-	-	+	-	-	±
6th AA	+	+	+	+	+	+	+
5th AA	-	+	-	+	+	+	+
4th AA	-	-	-	±	-	-	+
3rd AA	-	-	-	±	-	-	±
2nd AA	-	+	-	+	-	-	±
1st AA	-	-	-	+	-	-	±
Small Molecule	-	-	-	±	-	-	+

Acceptable Result: Dissolution after addition of solvents (+), Intermediate result: Dissolution after addition of coupling reagent and base (±), Unacceptable result: AAs not dissolved after addition of solvent and base (-).

Table 11. SPPS results in alternative solvents for Project C.

Project C		
	Yield %	Purity %
DMF (Standard)	X	60
NBP	68% of X	48
NBP (for washes, deprotection) /DMF (for coupling reactions)	92% of X	55

Table 12. Use of DMF vs. NBP in SPPS for Project C.

Project C		
	DMF additions	NBP additions
SPPS in DMF	108	-
SPPS in NBP (for washes, deprotection) /DMF (for coupling reactions)	19	89

* Based on a pilot-scale GMP campaign.

SPPS was performed at a small scale in NBP and γ-Valerolactone. When SPPS was performed using γ-Valerolactone, the purity of peptide C was significantly lower at 18% of the purity of peptide C generated in DMF. Large amounts of deletion impurities for two amino acids (AA8 and AA3) were observed and this was potentially due to the inferior swelling results of Resin Z in γ-Valerolactone. Based on these findings, γ-Valerolactone was not recommended for larger scale studies for Project C. However, the solvent remains a potentially viable greener solvent for other resins and requires further evaluation for future projects.

Small scale studies using NBP for project C gave promising results and it was decided to proceed to a larger scale manufacture of peptide C using the

solvent. When SPPS was performed using NBP the yield was lower at 68% of the original yield for the process performed in DMF. The purity of peptide C manufactured in DMF at a large scale was 60% while that of NBP was slightly lower at 48%. Evidently, the use of NBP in the SPPS had an impact on the yield and purity of peptide C; therefore, it was decided to investigate a strategy whereby DMF was used for the coupling reactions and NBP was used for all washes carried out. Following the DMF/NBP combination strategy for SPPS, peptide C was obtained in a yield at 92% of the original process performed in DMF (Table 11). Similarly, the purity for the DMF/NBP SPPS was comparable to the DMF SPPS at 55% vs. 60%.

This strategy using a combination of DMF and NBP for the manufacture of peptide C results in an 82% reduction in DMF use for the SPPS (Table 12). While further investigations are required, this study provides promising results indicating that NBP may be a viable alternative for DMF in SPPS for project C and for future projects.

Discussion

This work has further highlighted the need to find alternative greener solvents to use in SPPS in place of DMF and has reaffirmed that these solvents can be a viable substitution. A systematic approach was carried out for the evaluation of greener solvents for three projects at Ipsen. Whilst each solvent gave varied results for the different resins, a number were carried through to the final evaluation of performance during SPPS.

For Project A, all solvents were deemed to be incompatible due on their poor ability to swell resin X as well as their inability to dissolve the SPPS reagents. As a result, only DMC was chosen for evaluation during SPPS which, even when conditions were optimized, gave a poor yield (36% of original yield for process performed in DMF) and purity (11%) for peptide A.

Five solvents (2-MeTHF, NBP, DMC, CPME, EtOAc) progressed to the SPPS evaluation for Project B based on the results from the swell test and solubility studies. From the experimental results, 2-MeTHF and NBP stood out as potential alternatives for DMF in SPPS (59 and 65% of original yield for process performed in DMF). However, further optimization using these solvents would be needed to increase the yield and purity of peptide B before they can be considered as viable substitutions.

Based on the resin swell test and solubility studies, NBP and γ-Valerolactone were evaluated as DMF alternatives in SPPS for Project C. The discovery of deletion impurities in peptide C generated in γ-Valerolactone indicated that the solvent was not suited for the manufacture process used in Project C. The use of NBP for the generation of peptide C furnished a lower yield (68% of original yield for process performed in DMF). In addition, SPPS performed in NBP produced peptide C in slightly lower purity (48%) when compared to DMF (60%); however, the impurity profile is similar. This lower yield and purity are potentially caused by a slower coupling reaction in NBP, which is more viscous than DMF. This could be alleviated by using longer reaction times or by using heated SPPS and requires further investigation. In line with other examples in literature, NBP is a promising candidate to replace the reprotoxic DMF in SPPS for Project C (*34*).

In an effort to reduce the quantity of DMF used for the manufacture of peptide C, a strategy utilizing both NBP and DMF was considered. Using DMF for the coupling reactions and NBP for wash steps, peptide C was produced in a yield (92% of original yield for process performed in DMF) and purity (55%) almost comparable to that of DMF alone. This strategy represents a reduction in the amount of DMF used during the SPPS by 82%.

Conclusion

Based on these case studies it is clear that, while there is no 'gold standard' solvent that can replace DMF in SPPS, viable options are available. Each resin and peptide sequence will need to be evaluated prior to deciding on a possible DMF substitution. Selecting alternative solvents is highly challenging and common problems include unacceptable resin swelling, insolubility of raw materials and lower yields and purities of the peptide. Furthermore, regulatory filling remains a barrier for implementing such changes in current commercial processes.

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