Modifying Solid-Phase Peptide and Azapeptoid Synthesis Procedures Using Green Solvents and Coupling Reagents

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ABSTRACT

In recent years, there has been an increasing interest in implementing eco-friendly practices within Solid Phase Peptide Synthesis (SPPS) procedures. SPPS is a synthetic process used in peptide synthesis and peptidomimetics, which are essential to drug development as peptide drugs have the advantage of specificity and easily recyclable byproducts within the body. However, SPPS requires an extensive amount of solvents, many of which are harsh on both human and environmental health. For example, the widely used solvent Dimethylformamide (DMF) can cause damage to the liver upon chronic exposure, has mutagenic properties, and is a reproductive toxin. This solvent is also incredibly troublesome during the disposal process. This research focuses on evaluating the resin swelling, solubility, and product yield of the green solvents, Dimethyl Sulfoxide (DMSO):Ethyl Acetate, Gamma-Valerolactone (GVL), and 4-Methyltetrahydrofuran in comparison to DMF while using the eco-friendly coupling reagent, (1-Cyano-2-Ethoxy-2-Oxoethylidenumenooxy) Dimethylamino Morpholino Carbenium Hexafluorophosphate (COMU). The effectiveness of the different green solvents will be presented in addition to the crude purities and overall product yields. This can be beneficial to the scientific community as the implementation of eco-friendly procedures in SPPS, and peptidomimetics, would allow for a less hazardous working environment, decreased environmental impact, and more sustainable practices.

INTRODUCTION

Solid-Phase Peptide Synthesis (SPPS) is a synthetic process used for the creation of peptides, which are essential to drug development due to their unique advantages of specificity and low toxicity (Malonis, Ryan J., et al., 2019). Peptidomimetics, specifically azapeptides, are also a valued tool for therapeutic drug development due to their increased stability. In SPPS, resin, a solid support typically composed of polymeric beads, allows impurities to be easily dissolved away rather than requiring the use of the recrystallization procedures typically used in Liquid-Phase Peptide Synthesis (Merrifield, R.B., 1963). The process is composed of three major steps: deprotection, activation and coupling (FIGURE 1). Deprotection is the removal of the N-terminus protecting group and is followed by coupling, which is the addition of a single Fmoc-amino acid. However, the procedure requires the excessive use and disposal of solvents, which makes up one of the largest sources of waste associated with chemical production (Lawrenson, Stefan, et al., 2017), with roughly 80-90% of SPPS waste being composed of solvents (Constable, David J. C., et al., 2007). Many of these solvents can have significant adverse effects on both human and environmental health, one of which is N,N-Dimethylformamide (DMF). DMF is an attractive industrial solvent that is often utilized in peptide synthesis due to its high reagent solubility, affordability, and resin swelling capabilities. However, due to DMF's classification as a substance of high concern under Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) regulations, the replacement of DMF with greener solvents is highly prioritized (Lawrenson, Stefan, et al., 2017).

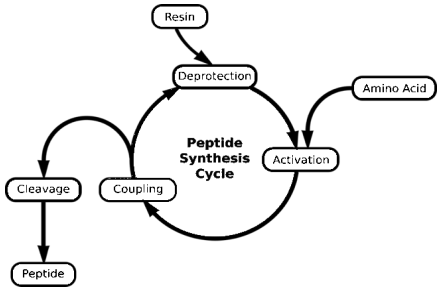


FIGURE 1. General Scheme of Solid-Phase Peptide Synthesis Procedure (Pampel, Julian)

Repeated cycles of (1) Deprotection, (2) Activation of the amino acid, and (3) Coupling; followed by the removal of the peptide.

The effectiveness of each of the green solvents will be measured by its solubility of the reagents, resin swelling and deprotection capabilities, crude purity, and product yield. The effectiveness of the green coupling reagent, (1-Cyano-2-Ethoxy-2-Oxoethylidenuminooxy) Dimethylamino Morpholino Carbenium Hexafluorophosphate (COMU), will be measured by similar standards, mainly by its coupling capabilities, crude

purity, and product yield. COMU is an ideal greener alternative for benzotriazole-based coupling reagents due to its less hazardous safety profile, decreased likelihood of causing allergic reactions, and because of its high water-solubility (El-Faham, Ayman, et al., 2010). The coupling capabilities of the reagent will be evaluated based on whether the reagent causes specific deletions or additions of Fmoc-amino acids.

This research investigates the effectiveness of the green solvents Dimethyl Sulfoxide:Ethyl Acetate (DMSO:EtOAc, 6:4), Gamma Valerolactone (GVL), and Dimethyl Sulfoxide:2-Methyl Tetrahydrofuran (DMSO:2-Me THF, 1:1). Furthermore, the synthesis utilizes the green coupling reagent COMU and the greener base alternative to Piperidine: 4-Methyl Piperidine. A 2015 study assessed the effectiveness of a completely DMF free synthesis, instead substituting the solvent with 2-Me THF and EtOAc using various rinsing procedures (Jad, Yahya E., et al., 2015). The experiment concluded that the most efficient procedure utilized 2-Me THF for Fmoc removal, washing, and coupling procedures in addition to washing with EtOAc. The use of 2-Me THF has also been supported in a separate study that concluded that the most effective synthesis protocol involved 2-Me THF as the main solvent with DMF deprotections and washings (Jad, Yahya E., et al., 2016). In addition to 2-Me THF's limited toxicity, it can be derived from renewable resources like furfural and levulinic acid (Pace, Vittorio, et al., 2012), is remarkably stable in comparison to other solvents like THF due to its low miscibility in water, and a significant number of industries have begun to assess the 2-Me THF in various synthetic procedures, often with acceptable results (Pace, Vittorio, et al., 2012). The use of DMSO as a binary solvent throughout this research is largely based upon prior experiments in which it has been established that the chemical is most effective during coupling when paired with another green solvent like 2-Me THF, EtOAc, or GVL (Jad, Yahya E., et al., 2015). Additionally, the use of a 20% 4-Methyl Piperidine rather than a 20% Piperidine solution for the deprotection procedure was decided based on two major factors: the toxicity of Piperidine (Committee on

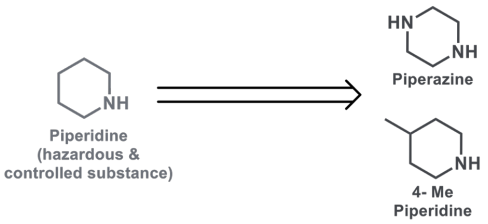


FIGURE 2. Eco-Friendly Piperidine Alternatives (Created by Molly Helton, used with permission)

Instead of using the toxic controlled substance Piperidine, alternatives like Piperazine and 4-Me Piperidine should be implemented due to their less hazardous nature.

Toxicology, 2012) and the difficulty associated with obtaining the chemical due to its status as a controlled substance (FIGURE 2). Although benzotriazole-based reagents are more widely utilized, COMU has proven itself to be a highly effective coupling reagent, especially during azapeptoid synthesis. The morpholine group likely contributes to this effectiveness by influencing the solubility, stability, and reactivity of the reagent (El-Faham, Ayman, et al., 2010).

Former studies have been incredibly enlightening throughout the process of determining how to modify SPPS procedures to be more environmentally-conscious, and some questions posed by the previous results may be resolved throughout this research. For instance, while some prior studies have experienced difficulties with incomplete deprotections (Pace, Vittorio, et al., 2012), this issue will be avoided by performing one deprotection for 5 minutes and then another using a fresh 4-Me Piperidine solution for 15 minutes. Another topic addressed throughout this paper will be how COMU performs along with a variety of green solvents. Once this study is concluded, the application of green procedures in azapeptoid synthesis will also be tested.

The research presented throughout this paper will build upon previous experiments in order to outline the unique procedures used to create more eco-friendly SPPS processes. Firstly, the non-green solvent DMF will be used in combination with the non-green coupling reagent HBTU to synthesize the peptide LYRAG (FIGURE 3), conditions which typically produce very good yields. The non-green procedure will be used throughout the experiment as a comparison. Then, DMF will be used in tandem with COMU, allowing a clear comparison to HBTU. Finally, each of the green solvents will be used individually along with the COMU coupling reagent to synthesize LYRAG. Then, the green conditions used for the LYRAG synthesis will be applied to the Ac-Phenylalanine (Fmoc-L-Phenylalanine, Chem Impex Int'l Inc., 100% purity)-azaGlycine-Phenylalanine-NH_2 azapeptoid synthesis.

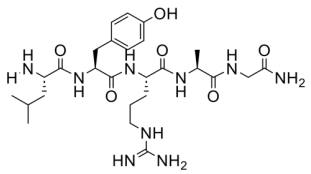


FIGURE 3. Structure of the Peptide LYRAG (Created by student researcher using ChemDraw, 2025)

The peptide LYRAG is composed of 5 amino acids: Leucine, Tyrosine, Arginine, Alanine, and Glycine. Each amino acid is Fmoc protected on the N-Terminus, but Arginine and Tyrosine have additional side chain protecting groups.

The chosen subject focuses on modifying Solid-Phase Peptide Synthesis because Liquid-Phase Peptide

Synthesis is a convoluted process that is typically only used for shorter peptide sequences in larger quantities (Merrifield, R.B., 1963). Solid-Phase Peptide Synthesis is much more straightforward and effective. Therefore, if Solid-Phase Peptide Synthesis is the most efficient method, then creating a more environmentally conscious procedure would be greatly beneficial to both future advancements and should be considered a high priority. The main topic addresses the implementation of green solvents in addition to the reagent COMU because solvents make up the majority of the waste produced in Solid-Phase Peptide Synthesis and many of the chemicals currently utilized not only create a hazardous working environment, but can also have a harmful impact on ecosystems upon disposal. The experiment outlined throughout this paper is essential because the effectiveness of eco-friendly modifications must be assessed before full implementation, ensuring any shortcomings are addressed. Due to DMF and HBTU's high performance and affordable pricing, the substitutions for the solvent and coupling reagent must justify the added cost and procedural complexity. By the end of this experiment, the advantages and disadvantages of each solvent as well as the effectiveness of the COMU coupling reagent will be clearly identified along with possible improvements and future questions.

This paper investigates which green solvents can produce a synthesis and yield comparable to DMF, and whether the green coupling reagent COMU is a viable substitute for HBTU. Research suggests COMU may be sufficient, though green solvents may present challenges with solubility, resin swelling, purity, and overall yield. The results will provide insight into the next steps for eco-friendly chemical processes, helping assess the peptide industry's current environmental consciousness and its future direction.

MATERIALS AND METHODS

Throughout experimentation, the standard safety procedures were strictly followed, including the use of Personal Protective Equipment (PPE), chemical safety training, proper waste disposal, and careful handling of hazardous materials. Before entering the lab, an extensive chemical safety course was completed and the appropriate PPE was obtained. Additionally, the handling of strong acids was done by an experienced professional. All experiments were conducted within the labs fume hood and treated with the utmost caution.

The first full LYRAG synthesis was a completely non-green procedure, using the standard DMF (N,N-Dimethylformamide, Fisher Chemical, 99.8% purity) solvent and the benzotriazole-based coupling reagent, HBTU (Hexafluorophosphate Benzotriazole Tetramethyl Uronium, Advanced ChemTech, 100% purity). The reagents used in this synthesis include Fmoc Leucine (Fmoc-L-Leucine, Chem-Impex Int'l Inc., 99.8% purity), Fmoc Tyrosine (Fmoc-*O-tert*-butyl-L-tyrosine, Chem-Impex Int'l, Inc., 100% purity), Fmoc Arginine (N^{ω}-Fmoc- N^{ω}-L-Arginine, Chem-Impex Int'l Inc., 99.5% purity), Fmoc Alanine (Fmoc-L-Alanine, Chem-Impex Int'l Inc., 99.91% purity), Fmoc Glycine (Fmoc-Glycine, Chem-Impex Int'l Inc., 99.74% purity), HBTU, and DIEA (N,N-Diisopropylethylamine, Chem-Impex, >99% purity), in addition to the Rink-Amide – MBHA Resin (Iris Biotech, 100% purity). The first step in the synthesis was weighing out the 200 mg of resin into a 3 mL SPPS cartridge. Then, the DMF solvent was added and the cartridge was placed on an IKA MS 3 Basic Shaker for 30 minutes to swell the resin. Once swelling was complete, the DMF was drained. For deprotection, 2 mL of a 20% 4-Me Piperidine in DMF solution was added to the resin, where it was then shaken for 5 minutes. The resin was then drained and a fresh 4-Me Piperidine solution was added. The resin was then shaken for 15 more minutes. Once complete, the resin was drained and washed three times with DMF. The resin was then set aside and Fmoc Glycine (135 mg, 0.456 mmol, 3 equivalence) was weighed into a 20 mL vial and then dissolved in 2 mL of DMF. To complete the activation of the solution, HBTU (173 mg, 0.456 mmol, 3 equivalence) and DIEA (0.159 mL, 0.912 mmol, 6 equivalence) was added to the Fmoc Glycine to be stirred for 5 minutes. To couple on the amino acid, the solution was added to the resin cartridge and put on the shaker for one hour at room temperature. After an hour, the resin was drained for a final time and rinsed thrice with DMF. The same procedure was done for each of the amino acid couplings. A test cleavage was performed after the Fmoc Arginine and Leucine couplings and a final cleavage after the

completed peptide was analyzed on the Agilent Technologies 1260 Infinity II Liquid Chromatography Mass Spectrometer (LC/MS).

The full cleavage consists of a 20 mL vial, a Biotage V-10 Touch, water, TIPS (Triisopropylsilane, Chem-Impex Int'l Inc., 99.58% purity), TFA (Trifluoracetic Acid, Chem-Impex Int'l Inc., 99.9% purity), Ethyl Ether (Ethyl Ether Anhydrous, Fisher Chemical, 99.0%), and Acetonitrile (Acetonitrile, Fisher Chemical, 99.9%)., and a Thermo Scientific Sorvall ST 8 Centrifuge for the ether precipitation. First, the 200 mg of resin were transferred to a new 20 mL vial and 8 mL of the 2.5% H_2O, 2.5% TIPS, and 95% TFA solution was added. Then the 20 mL was placed onto the shaker for 2 hours. The solution was then filtered into a 30 mL conical tube of cold ether. Then, the tube of ether was centrifuged until the solid was completely separated from the ether. The ether was then disposed of and the solid was redissolved in Acetonitrile and collected into a new 20 mL vial for evaporation. The purification procedure includes the use of an Agilent Technologies ProStar Prep High Performance Liquid Chromatographer (HPLC). Once purified, the sample was evaporated and placed on a Labconco FreeZone 4.5 Plus Lyophilizer to remove any remaining water.

The first green synthesis procedure used the non-green solvent DMF and the green coupling reagent COMU ((1-Cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate, Chem-Impex Int'l Inc., 99.6% purity). This experiment was used to identify the effectiveness of COMU as a coupling reagent in comparison to HBTU. The same synthesis procedure used for the DMF and the HBTU experiment was applied to the DMF and COMU procedure. However, the HBTU (173 mg, 0.456 mmol, 3 equivalence) was swapped with COMU (195 mg, 0.456 mmol, 3 equivalence). For the implementation of the green solvents DMSO:EtOAc (6:4) (Dimethyl Sulfoxide, Fisher Chemical, 99.9% purity; Ethyl Acetate, Fisher Chemical, 99.9% purity), GVL (Gamma-Valerolactone, Thermo Scientific, 98% purity), and DMSO:2-MeTHF (1:1) (Dimethyl Sulfoxide, Fisher Chemical, 99.9% purity; 2-Methyltetrahydrofuran, TCI America, >98% purity), the same general procedure for the DMF and COMU synthesis was used.

After the results of the green LYRAG syntheses were collected, the green solvents were then used alongside COMU to test the possible application of green procedures in azapeptoid synthesis. The trimer made for each of the solvents was composed of the following Fmoc-amino acids: Fmoc Phenylalanine, Fmoc Hydrazine, and another Fmoc Phenylalanine (FIGURE 4).

Before implementing the green solvents, the standard non-green procedure was conducted using DMF as the solvent and HBTU for the first coupling. The results were used to evaluate the effectiveness of each green solvent and the substitution of HBTU with COMU. The standard procedure consists of five major steps: HBTU coupling, N,N'-Disuccinimidyl Carbonate (DSC) (N,N'-Disuccinimidyl carbonate, Chem Impex Int'l Inc., 99.5% purity) coupling, COMU coupling, acetylation, and late-stage alkylation. The HBTU coupling, DSC coupling, COMU coupling, and acetylation typically utilize the standard DMF solvent while late-stage alkylation uses THF as the solvent.

The first step in the non-green azapeptoid synthesis used HBTU to couple on the first Fmoc Phenylalanine in addition to the reagent DIEA. This coupling used the same procedure previously described, only with Fmoc Phenylalanine (176 mg, 0.456 mmol, 3 equivalence) as the amino acid. The second step in the standard azapeptoid synthesis used DSC to couple on the Fmoc Hydrazine. Before the Fmoc Hydrazine was coupled on, another 4-Me Piperidine in DMF deprotection was performed. After deprotection, Fmoc Hydrazine (116 mg, 0.456 mmol, 3 equivalence) and DSC (117 mg, 0.456 mmol, 3 equivalence) were weighed into two separate 20 mL vials. Then, 1.6 mL of DMF was added to each vial and both were cooled to 0°C to prevent dimerization. Then, the DSC solution was added dropwise into the Fmoc Hydrazine solution and stirred for 5 more minutes. Then, the solution was warmed to room temperature and added to the resin. The reaction was then placed on the shaker for 16 hours. The third step of the azapeptoid synthesis used COMU and DMF to couple on the final Fmoc Phenylalanine. The procedure was the same as the previous DSC coupling, only with COMU (195 mg, 0.456 mmol, 3 equivalence).

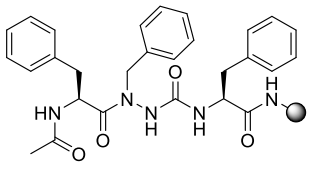


FIGURE 4. Structure of the Azapeptoid Ac-Phe-azaGly-Phe (Created by student researcher with ChemDraw, 2025)

The azapeptoid Ac-Phe-azaGly-Phe is composed of two Fmoc-amino acids, Phenylalanine, Hydrazine, and another Phenylalanine.

The fourth step in the standard azapeptoid synthesis procedure was the acetylation. The purpose of the acetylation is to protect the N-terminus, ensuring that the reaction only occurs on the aza residue. However, prior to the full non-green acetylation procedure, a separate experiment was conducted to evaluate the effectiveness of each green solvent in this particular step. This procedure was

first done by weighing out a fresh 200 mg of resin into a 3 mL SPPS cartridge. Then, the resin was swelled in DMF for 30 minutes and then drained. A 2 mL solution of 4-Me Piperidine in DMF was then added to the resin and shaken for 5 minutes. The resin was then drained and a fresh 4-Me Piperidine solution was added and the reaction was put back on the shaker for another 15 minutes. Once fully deprotected, the resin was drained and rinsed thrice with DMF. Then, Fmoc Phenylalanine (176 mg, 0.456 mmol, 3 equivalence) was weighed into a 20 mL vial where it was dissolved in 2 mL of DMF. Once dissolved, COMU (195 mg, 0.456 mmol, 3 equivalence) was added. Then, DIEA (0.159 mL, 0.912 mmol, 6 equivalence) was added to the Fmoc-amino acid solution and it was stirred for 5 minutes. The Fmoc-amino acid solution was then added to the resin and shaken for 1 hour. Once fully coupled, the resin was drained and washed three more times with DMF. Another Fmoc deprotection was then performed. For the acetylation tests, the 200 mg of resin was weighed out evenly into four separate SPPS cartridges. Each cartridge used a different solvent. The solvents tested were DMF, DMSO:EtOAc (6:4), GVL, and DMSO:2-MeTHF (1:1). Once weighed out, each of the cartridges were shaken in 2 mL of 20% 4-Me Piperidine in the chosen solvent for 5 and 15 minutes. Then, the cartridges were drained and washed with their respective solvents. The resin was then shaken in 2 mL of 20% Acetic Anhydride in the solvent for 30 minutes. Once complete, the resin was drained for a final time. The results of these acetylation tests determined which solvent would be used for the full green acetylation procedures for the green azapeptoid syntheses. Once these tests were complete, the non-green acetylation procedure was conducted. First, the resin was deprotected in a 4-Me Piperidine in DMF solution for 5 and 15 minutes. Then, the resin was washed with DMF. The cartridge was then shaken for 30 minutes in 2 mL of a 20% Acetic Anhydride (Acetic Anhydride, Fisher Chemical, 97.0% purity) in DMF solution.

The last step in the azapeptoid synthesis procedure was late-stage alkylation. This step is typically conducted using the toxic solvent, THF. However, prior to full synthesis, a small-scale late-stage alkylation test was performed to determine whether THF could be substituted for any of the greener solvents. The solvents tested in this procedure were DMSO:EtOAc (6:4), GVL, DMSO:2-MeTHF (1:1), and 2-MeTHF. The reagents used in the procedure were Tetraethylammonium Hydroxide (TEAH) (Tetraethylammonium Hydroxide, Thermo Scientific, 99.9% purity) and Benzyl Bromide (BnBr) (Benzyl Bromide, TCI America, >98.0%). First, 50 mg of resin was weighed out into 4 separate SPPS cartridges. The resin was then swelled in its

respective solvent for 30 minutes. Then, the solvent was drained and 500 microliters of the solvent was added into the resin. The solvent was not drained before the next step. TEAH (0.025 mL, 0.034 mmol, 0.9 equivalence) was then added to each cartridge and the solution was shaken for 30 minutes. Then, Benzyl Bromide (0.045 mL, 0.38 mmol, 10 equivalence) was added to the solution and the resin was kept on the shaker for another 16 hours.

The full late-stage alkylation procedure consisted of TEAH (0.102 mL, 0.14 mmol, 0.9 equivalence), Benzyl Bromide (0.180 mL, 1.52 mmol, 10 equivalence), and THF as the solvent. This process used the same procedure as the previously outlined alkylation, only on a larger scale. The full cleavage for the standard azapeptoid synthesis will slightly differ from the previously-outlined procedure because the peptoid lacks any major protecting groups. Rather than 95% TFA, 2.5% TIPS, and 2.5% H_2O, the cleavage solution will consist of 8 mL of 95% TFA and 5% H_2O. The cleavage will not include an ether precipitation and rather than being redissolved in Acetonitrile, the peptoid will be redissolved in Ethyl Acetate and then prepped for the lyophilizer.

Once the full non-green azapeptoid synthesis was complete, there were two green azapeptoid syntheses performed using the same general procedures. The first green synthesis used DMSO:EtOAc and the second used DMSO:2-MeTHF (1:1), both of which substituted HBTU for COMU and the original alkylation solvent THF for 2-MeTHF.

RESULTS

Throughout this project, the effectiveness of various green solvents and the coupling reagent COMU was evaluated over a wide range of experiments. These experiments included a resin swelling test, reagent solubility tests for the LYRAG and azapeptoid syntheses, small-scale acetylation and alkylation optimizations, the full synthesis of LYRAG (TABLE 1), and the azapeptoid Ac-Phe-azaGly-Phe (TABLE 2) under various green conditions. For the full syntheses, the crude purity, purified purity, product yield, and percent yield were all taken into consideration when evaluating the effectiveness of a procedure.

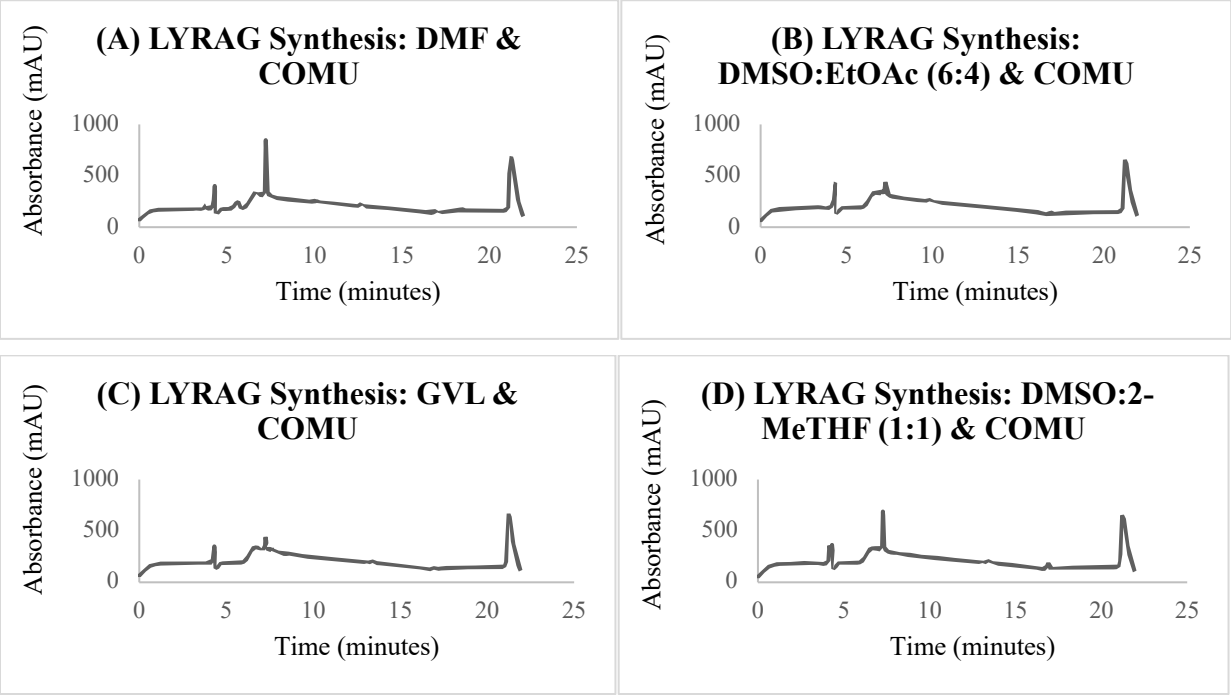
The first full LYRAG synthesis utilized standard SPPS procedures and was used as a control for all of the following green LYRAG syntheses. The DMF solvent and HBTU coupling reagent produced the most efficient results with an 86% crude purity, 99% purified purity, and an 84% product yield. The second full synthesis was used to assess the effectiveness of the green coupling reagent COMU. This procedure used the DMF solvent and the COMU coupling reagent. The results were comparable to the standard synthesis with an 84% crude purity, 99% purified purity, and 73% product yield. Based on these results, COMU can be classified as an effective alternative to HBTU because the two coupling reagents produced an equal purified purity and there was only a minor decrease in product yield.

The first fully green LYRAG synthesis used DMSO:EtOAc (6:4) and COMU. This synthesis resulted in a 58% crude purity, 84% purified purity, and 25% product yield. The crude purity and product yield were much lower than that of DMF and COMU, but the purified purity was adequate. The second green synthesis, using GVL and COMU, produced the poorest results with a 51% crude purity, 83% purified purity, and 22% product yield. Although the crude purity and product yield were the lowest of all the solvents, GVL's purified purity was on par with that of DMSO:EtOAc (6:4). The most comparable results of the green solvents were yielded from DMSO:2-MeTHF (1:1), which produced a 97% crude purity, 94% purified purity, and 34% product yield. While the product yield of DMSO:2-MeTHF (1:1) was much lower than that of DMF, the green solvent produced the highest crude purity and a satisfactory purified purity.

TABLE 1. Results of the LYRAG Syntheses (Created by student researcher using Microsoft Word, 2025)

The purification data of each LYRAG synthesis using various solvents. The DMF syntheses were used to evaluate the effectiveness of each of the green procedures.

Solvent	Coupling Agent	Crude Purity (%)	Purified Purity (%)	Yield (mg)	Yield (%)
DMF	HBTU	86	99	74	84
DMF	COMU	84	99	65	73
DMSO: EtOAc (6:4)	COMU	58	84	22	25
Gamma-Valerolactone	COMU	51	83	19	22
DMSO: 2-Me THF (1:1)	COMU	97	94	30	34



GRAPH 1. LC/MS Trace for the LYRAG Syntheses (Created by student researcher using Microsoft Excel with data collected from LC/MS, 2025)

The LC/MS trace measures the absorbance (mAU) of the sample over time (minutes). The product for each synthesis is located at ~7 minutes.

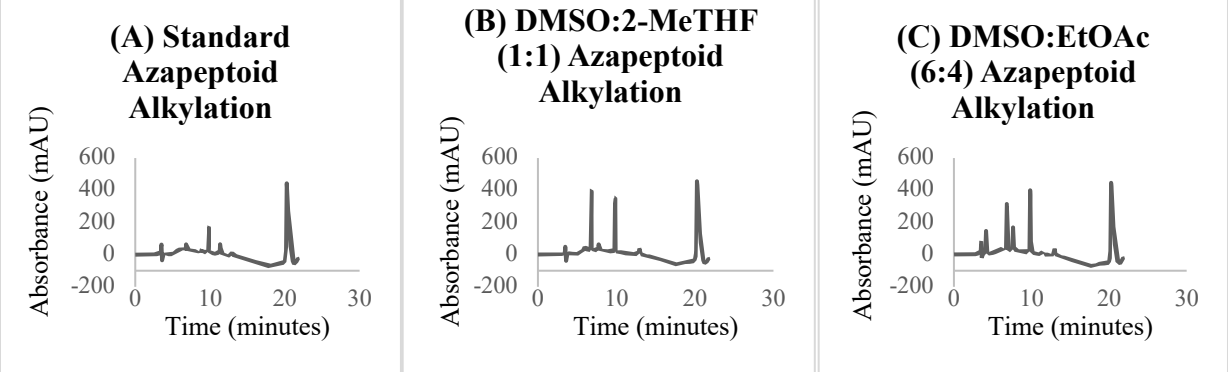
A small-scale acetylation synthesis in each of the green solvents was also performed prior to full syntheses, in which each solvent produced a conversion greater than 99%. Another step in the azapeptoid synthesis that required further optimization prior to the full synthesis was the late-stage alkylation. The standard THF solvent produced 13% starting material, 70% monoalkylated material, and 17% dialkylated material. DMSO:EtOAc (6:4) produced 83% starting material, 17% monoalkylated material, and no dialkylated material. GVL produced 72% starting material, 28% monoalkylated material, and no dialkylated material. DMSO:2-MeTHF (1:1) produced 27% starting material, 66% monoalkylated material, and 7% dialkylated material. 2-MeTHF produced 20% starting material, 72% monoalkylated material, and 8% dialkylated material. Because the goal of this particular azapeptoid synthesis was to produce mainly monoalkylated product, the green solvent with the highest monoalkylation was used for the full green syntheses. Therefore, 2-MeTHF was used for the alkylation of each of the green azapeptoid syntheses because it had the highest amount of monoalkylated product, a lower amount of dialkylated product than THF, and only a slight amount of starting material.

The first full Ac-Phe-azaGly-Phe azapeptoid synthesis used the previously outlined standard conditions. This synthesis produced an acetylated crude purity of 84%, a final alkylated crude purity of 43%, a purified purity of 99%, and a product yield of 17%. The product yield for azapeptoid synthesis tends to be lower than that of natural peptide synthesis, so an 8-30% yield was to be expected. The standard synthesis was used to evaluate the effectiveness of the green solvents and green coupling reagent COMU. The first green azapeptoid synthesis used DMSO:2-MeTHF (1:1) and COMU. This procedure produced an acetylated crude purity of 84%, a final alkylated crude purity of 40%, a purified purity of 99%, and a product yield of 13%. The second green azapeptoid synthesis used DMSO:EtOAc (6:4) and COMU. This synthesis produced an acetylated crude purity of 74%, a final alkylated crude purity of 35%, a purified purity of 99%, and a product yield of 13%. As the DMSO:2-MeTHF and DMSO:EtOAc both produced similar results, they are promising candidates for green azapeptoid synthesis. However, the DMSO:EtOAc (6:4) synthesis also produced several unidentified impurities after the DSC coupling.

TABLE 2. The Results of Various Azapeptoid Syntheses (Created by student researcher using Microsoft Word, 2025)

The purified purity, yield, and percent yield of various azapeptoid syntheses using the green solvents DMSO:EtOAc (6:4) and DMSO:2-MeTHF (1:1) in comparison to DMF.

Starting Material Solvent	Alkylation Solvent	Acetylated Crude Purity (%)	Alkylated Crude Purity (%)	Purified Purity (%)	Yield (mg)	Yield (%)
DMF	THF	84	43	99	13	17
DMSO:EtOAc (6:4)	2-MeTHF	74	35	99	10	13
DMSO:2-MeTHF (1:1)	2-MeTHF	84	40	99	10	13



GRAPH 2. LC/MS Traces for the Azapeptoid Syntheses (Created by student researcher using Microsoft Excel with data from LC/MS, 2025)

The LC/MS trace measures the absorbance (mAU) of the sample over time (minutes). The product for each synthesis is located at ~10 minutes.

In former studies, the green solvent 2-MeTHF was found to be the most effective (Jad, Yahya E., et al., 2015). However, it was often used in combination with EtOAc or the non-green solvent DMF. Additionally, previous research utilized the coupling reagent OxymaPure, so it is likely that the DMSO:2-MeTHF (1:1) solvent combination was not implemented because reagent solubility was not an issue for the OxymaPure like it was for the COMU (Jad, Yahya E., et al.,

2016). In contrast with these former studies, the research outlined throughout this paper also used a 4-Me Piperidine base rather than a Piperidine base during the Fmoc deprotection. The use of 4-Me Piperidine for deprotections was already implemented as standard practice in the lab in which these experiments were conducted.

CONCLUSION

To conclude, the hypothesis that certain green solvents could yield results comparable to DMF was supported. In previous studies, 2-MeTHF and EtOAc were the most promising for green SPPS, especially when combined with DMSO. While DMSO:2-MeTHF (1:1) wasn't fully comparable to DMF, it was the most effective overall. DMSO:EtOAc (6:4) also showed good results for azapeptoid synthesis. Although current green solvent yields aren't ideal, further optimization could improve them. The hypothesis that COMU could substitute HBTU was also supported, as COMU had only slightly lower yields and similar purities, suggesting it may be a viable green alternative.

A potential error that could have occurred during the green LYRAG syntheses was the ether precipitations during the full cleavage procedures. For the green solvents, the ether precipitation was less effective than it was with the standard procedure. As a result, the ether had to be evaporated and recombined with the purified product, which required an additional purification. This instance could have caused a decrease in the product yield for the green syntheses. Additionally, it may have been the reason that there was a slight decrease between the crude purity and the purified purity.

An improvement that could be implemented for future reference is a more in-depth investigation on why the ether precipitation was ineffective for the green solvents and how the issue could be addressed. The possibility of changes to the ether precipitation procedure or an entirely new procedure to replace the ether precipitation step for the green solvents should be explored. It may also be insightful to carry out the LYRAG syntheses with the green solvents in combination with HBTU and then compare each of the results to that of COMU so that there could be a more precise evaluation of COMU's effectiveness. For the azapeptoid syntheses, the solvent GVL was not used because it failed the small-scale reagent solubility test prior to the full synthesis. It may be useful to test out different green solvent combinations with GVL to see if the reagent solubility could be improved.

If green conditions can be applied to SPPS for both natural peptides and azapeptoids, it would increase the future possibility for the large-scale implementation of eco-friendly procedures in medicinal advancements. Although green procedures need further optimizations, these experiments have yielded useful information concerning eco-friendly chemical processes.

With more in-depth investigations, a broader understanding can be gained of where the chemical industry currently stands and how it can proceed in the direction of environmental consciousness.

BIBLIOGRAPHY

Committee on Toxicology. (2012, December 28). *Piperidine.* National Academies Press (US). https://www.ncbi.nlm.nih.gov/books/NBK241478/

Constable, D. J. C., Curzons, A. D., & Cunningham, V. L. (2007). Perspective on solvent use in the pharmaceutical industry. *Organic Process Research & Development, 11*(1), 133–37, https://doi.org/10.1021/op060170h

El-Faham, A., & Albericio, F. (2010). COMU: A third generation of uronium-type coupling reagents. *Journal of Peptide Science, 16*(1), 6–9, https://doi.org/10.1002/psc.1204

Favatella, T. (2025). *LC/MS traces for the azapeptoid syntheses* [Unpublished graph]. Created by student researcher using Microsoft Excel with data from LC/MS.

Favatella, T. (2025). *LC/MS traces for the LYRAG syntheses* [Unpublished graph]. Created by student researcher using Microsoft Excel with data from LC/MS.

Favatella, T. (2025). *Results of the LYRAG syntheses* [Unpublished table]. Created by student researcher using Microsoft Word.

Favatella, T. (2025). *Structure of the azapeptoid Ac-Phe-azaGly-Phe* [Unpublished figure]. Created by student researcher using ChemDraw.

Favatella, T. *Structure of the peptide LYRAG* [Unpublished figure]. Created by student researcher using ChemDraw.

Favatella, T. (2025). *The results of various azapeptoid syntheses* [Unpublished table]. Created by student researcher using Microsoft Word.

Helton, M. (2025). *Eco-friendly piperidine alternatives* [Unpublished figure]. Used with permission.

Jad, Y. E., El-Faham, A., & Albericio, F. (2015). 2-Methyltetrahydrofuran and cyclopentyl methyl ether for green solid-phase peptide synthesis. *Amino Acids, 48*(2), 419–426. https://doi.org/10.1007/s00726-015-2095-x

Jad, Y. E., El-Faham, A., & Albericio, F. (2016). Green solid-phase peptide synthesis 2. 2-Methyltetrahydrofuran and ethyl acetate for solid-phase peptide synthesis under green conditions. *ACS Sustainable Chemistry & Engineering, 4*(12), 6809–6814, https://doi.org/10.1021/acssuschemeng.6b01765

Knowledge Sourcing Intelligence LLP. (2025). *Dimethylformamide market size & forecast 2025-2030 | Free sample.* https://www.knowledge-sourcing.com/report/dimethylformamide-market

Lawrenson, S., Aricò, F., & North, M. (2017). Greener solvents for solid-phase synthesis. *Green Chemistry, 19*(4), 952–962. https://doi.org/10.1039/c6gc03147a

Malonis, R. J., Lai, J. R., & Vergnolle, O. (2019). Peptide-based vaccines: Current progress and future challenges. *Chemical Reviews, 120*(6), 3210–3229. https://doi.org/10.1021/acs.chemrev.9b00472

Merrifield, R. B. (1963). Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *Journal of the American Chemical Society, 85*(14), 2149–2154. https://doi.org/10.1021/ja00897a025

Pace, V., Hoyos, P., Fernandez, S., & Alcántara, A. R. (2012). 2-Methyltetrahydrofuran (2-MeTHF): A biomass-derived solvent with broad application in organic chemistry. *ChemSusChem, 5*(8), 1369–1379. https://doi.org/10.1002/cssc.201100780

Pampel, J. (n.d.). *Peptide synthesis - Methods and reagents*. Antibodies Online. https://www.antibodies-online.com/resources/17/5034/peptide-synthesis-methods-and-reagents/