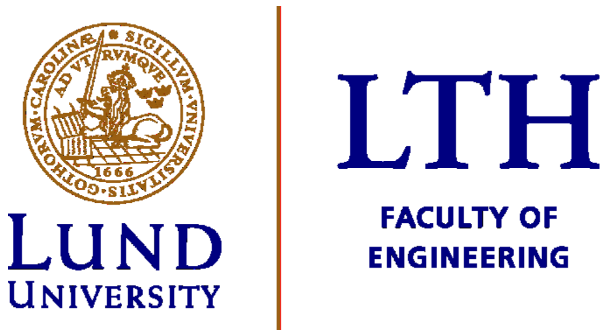

Greening peptide chemistry by using NBP as solvent for SPPS

by

Andrea Öhlander

Master Thesis 2022

Department of Chemistry Lund University,
Faculty of Engineering,
SE-221 00, Lund, Sweden

Andrea Öhlander

Diploma work, Organic Chemistry 30 ECTS
Master of Science in Engineering, Biotechnology

Supervisor	Professor **Ulf Nilsson**	Lund University Centre for Analysis and Synthesis SE-221 00 Lund, Sweden
Co-supervisors	MSc **Artur Sahakjan**	Red Glead Discovery SE-223 81 Lund, Sweden
	Associate Professor **Richard E. Johnsson**	Red Glead Discovery SE-223 81 Lund, Sweden
Examiner	Professor **Ulf Ellervik**	Lund University Centre for Analysis and Synthesis SE-221 00 Lund, Sweden

Abstract

The preferred method for synthesising therapeutic peptides today is by Solid-Phase Peptide Synthesis (SPPS). SPPS is a wasteful process, where the main component of the waste is hazardous solvents, i.e., *N,N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP) or dichloromethane (DCM). [3] It is therefore desirable to search for new and greener solvents to use for SPPS, which is what has been studied in this project. Five different solvents were chosen for this study: dihydrolevoglucosenone (Cyrene), ethyl acetate (EtOAc), 1,3-dioxolane (DOL), 2-methyl tetrahydrofuran (2-Me-THF) and *N*-butylpyrrolidinone (NBP). By systematic evaluation of the solvents, or binary mixtures of these, for their resin swelling capacity and ability to dissolve amino acids and reagents, it was possible to find solvents and solvent systems to be used in SPPS. Eight solvents and binary mixtures of varying "greenness" were identified as possible replacements for today's solvents. Three of these solvents and binary mixtures were found to perform better than DMF by producing higher yield and purity in synthesis of [Asp^5]-vasopressin, i.e., NBP, NBP:EtOAc (1:1), NBP:2-Me-THF (1:1). NBP was further investigated for its utility in SPPS and was found to be successful with various coupling reagents and resins in synthesis of [Asp^5]-vasopressin. NBP was lastly used in synthesis of [Asp^{26}]-calcitonin, which gave good yield of 28.7% and high purity of 97.9%. This further proved that NBP shows great potential for replacing DMF in SPPS.

Populärvetenskaplig sammanfattning

Den föredragna metoden för att syntetisera terapeutiska peptider är idag genom fastfas peptidsyntes (SPPS). SPPS är en process som genererar mycket avfall, där huvudkomponenten i avfallet är de toxiska lösningsmedlen *N,N*-dimetylformamid (DMF), *N*-metylpyrrolidon (NMP) och diklormetan (DCM).[3] Därför är det önskvärt att söka efter nya och grönare lösningsmedel att använda för SPPS, vilket är vad som har studerats i detta projekt.

År 1963 introducerade Robert Bruce Merrifield konceptet fastfas peptidsyntes, vilket senare gav honom Nobelpriset i kemi. Tanken med SPPS är en stegvis koppling av aminosyror till ett fast material, kallat resin, i upprepade cykler av avlägsnande av skyddsgrupper och kopplingsreaktioner. Kraven på ett lösningsmedel i SPPS är många. För att ett lösningsmedel ska vara brukbart i SPPS måste det lösa alla reagens och aminosyror, det måste svälla resinet och helst ha en hög kokpunkt för att kunna genomföra reaktioner vid högre temperatur.

I denna studie var det första kriteriet för ett grönare lösningsmedel att lösningsmedlet inte klassificeras som ett ”ämne som inger mycket stora betänkligheter” av Europeiska kemikaliemyndigheten (ECHA). Utöver detta gjordes ett urval på lösningsmedel baserat på deras kemiska likheter med DMF, såsom polaritet och viskositet, samt deras presterande i SPPS-relaterade experiment genomförda i tidigare rapporter. [1,2,7-13] Fem olika lösningsmedel valdes därav: dihydrolevoglukosenon (Cyrene), etylacetat (EtOAc), 1,3-dioxolan (DOL), 2-metyltetrahydrofuran (2-Me-THF) och *N*-butylpyrrolidinon (NBP). Ingen av dessa lösningsmedel klassificeras av ECHA, även om DOL för närvarande är under utredning.

De fem lösningsmedlen, eller binära blandningar av dessa, utvärderades systematiskt för deras resinsvällningsförmåga, och förmåga att lösa upp aminosyror och reagens. Genom detta var det sedan möjligt att hitta lösningsmedel och binära blandningar som skulle kunna användas i SPPS. Åtta lösningsmedel och binära blandningar av varierande "grönhet" identifierades som möjliga att ersätta dagens lösningsmedel. Tre av dessa lösningsmedel och binära blandningar visade sig fungera bättre än DMF, genom att producera högre utbyte och renhet vid syntes av den nio aminosyror långa peptiden [Asp^{5}]-vasopressin. Dessa var NBP, NBP:EtOAc (1:1) och NBP:2-Me-THF (1:1). NBP undersöktes sedan ytterligare för dess användbarhet i SPPS, och visade sig fungera bra även med olika typer av kopplingsreagens och resin. NBP användes slutligen vid syntes av den 32 aminosyror långa peptiden [Asp^{26}]-calcitonin, vilket gav bra utbyte och hög renhet. Detta bevisade ytterligare att NBP visar stor potential för att ersätta DMF i SPPS.

Abbreviations

2-Me-THF	2-Methyl tetrahydrofuran
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
Boc	*Tert*-butoxycarbonyl
Cyrene	Dihydrolevoglucosenone
Cys (C)	Cysteine
DCM	Dichloromethane
DIC	*N,N′*-Diisopropylcarbodiimide
DIPEA	*N,N*-Diisopropylethylamine
DMF	*N,N*-Dimethylformamide
DOL	1,3-Dioxolane
EDT	Ethane-1,2-dithiol
EtOAc	Ethyl acetate
Fmoc	Fluoren-9-ylmethyloxycarbonyl
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Gly (G)	Glycine
HBTU	*N,N,N′,N′*-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
HOBt	1-Hydroxybenzotriazole hydrate
HPLC	High-performance liquid chromatography
His (H)	Histidine
LCMS	Liquid chromatography–mass spectrometry
Leu (L)	Leucine
Lys (K)	Lysine
NBP	*N*-Butylpyrrolidinone
NMP	*N*-Methylpyrrolidone
Oxyma	Ethyl 2-cyano-2-(hydroxyimino)acetate
PEG	Polyethylene glycol
HPLC-PREP	Preparative high performance liquid chromatography
PS	Polystyrene
Phe (F)	Phenylalanine
Pro (P)	Proline
SPPS	Solid-phase peptide synthesis
Ser (S)	Serine
TFA	Trifluoroacetic acid
TIPS	Triisopropyl silane
Thr (T)	Threonine
Tyr (Y)	Tyrosine
Val (V)	Valine

Acknowledgments

This project was performed at Red Glead Discovery as a master thesis for the Centre for Analysis and Synthesis at the Faculty of Engineering, Lund University (LU). This project makes up 30 credits within Organic chemistry and lasted from January to June 2022.

I would like to express a special thank you to my two supervisors at Red Glead, Artur Sahakjan and Richard E. Johnsson. Artur, for always being there and giving me your time whenever I needed it. Throughout this project you have helped, supported and motivated me. Richard, for presenting this project to me and guiding me along the way with valuable insights.

Thank you to Johan Evenäs and the skilled people of Red Glead for giving me this opportunity and sharing your knowledge with me. A special thanks to the peptide group, which contributed with many great insights and useful tips during my laboratory work. Thank you to Klara Jonasson for helping me with important NMR-analysis. I would also like to extend a thank you to Ulf Nilsson for being my supervisor and Ulf Ellervik for being my examiner.

Table of Contents

Background and Introduction ... 8
Solid-phase peptide synthesis ... 8
Green chemistry and selection of solvents ... 10
Aim ... 13
Results and Discussion ... 14
Resin swelling tests ... 14
Amino acids and coupling reagents solubility ... 17
ChemMatrix resin swelling test ... 19
Synthesis of [Asp^5]-Vasopressin with DIC/Oxyma ... 19
Synthesis of [Asp^5]-Vasopressin with HBTU/DIPEA/HOBt in NBP ... 23
Synthesis of [Asp^5]-Vasopressin using ChemMatrix resin in NBP ... 24
Synthesis of [Asp^{26}]-Calcitonin in NBP ... 25
Conclusions and Future perspectives ... 28
Experimental section ... 29
Resin swelling tests ... 29
Solubility of amino acids and coupling reagents ... 30
Synthesis of [Asp^5]-Vasopressin ... 30
Synthesis of [Asp^{26}]-Calcitonin ... 30
Cleavage off resin ... 31
Purification ... 31
Analysis ... 32
References ... 33
Appendix ... 36
Mechanisms ... 36
Raw data ... 38

Background and Introduction

In recent years, the interest in therapeutic peptides have steadily been growing as significant advances have been made to improve the efficiency of peptide synthesis. The preferred method for synthesising therapeutic peptides is Solid-Phase Peptide Synthesis (SPPS), as the method enables synthesis of peptides with high purity and reproducibility.[1]

The focus of improvement in SPPS has so far been on optimising reaction parameters such as coupling reagents, protecting groups, and reaction conditions.[2] Coupling reagents and amino acids are used in excess, and large amounts of solvents are used for washing the solid support, isolating and purifying the produced peptide. This makes SPPS a highly wasteful process, where the main component of the waste is solvents.[3] The main solvents used in SPPS today are *N,N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), or dichloromethane (DCM). These three solvents are all hazardous, where DMF and NMP are reprotoxic and DCM carcinogenic. It is therefore desirable to search for new and greener solvents to use for SPPS, which is what has been studied in this project.

Solid-phase peptide synthesis

In 1963, Robert Bruce Merrifield introduced the concept of Solid-phase peptide synthesis, which later granted him the Nobel Prize in chemistry. The idea of SPPS is a stepwise coupling of amino acids to a solid material in repeated cycles, as demonstrated in Figure 1.[4]

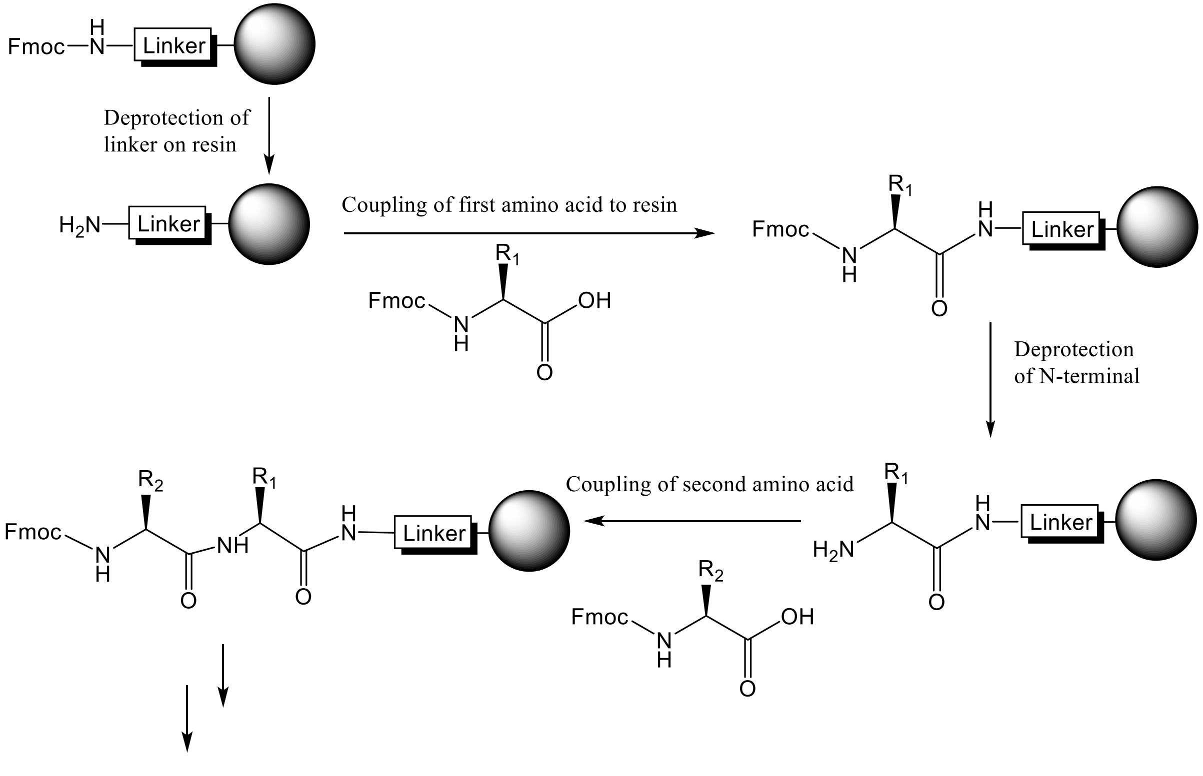

Figure 1: Schematic overview of SPPS. The grey sphere represents the solid support resin.

Amino acids consist of an amine group (N), a carboxylic group (C) and a side chain (R), as demonstrated in Figure 1. In biosynthesis, proteins and peptides are synthesised from the N- to C-terminal. In SPPS however, peptides are traditionally synthesised from C to N, even though research have shown that synthesis in the opposite direction is possible as well.[5]

SPPS utilises amino acids with a protecting group on the N-terminal, either fluoren-9-ylmethyloxycarbonyl (Fmoc) or *tert*-butoxycarbonyl (Boc). The choice of N-terminal protecting group decides the strategy for the synthesis, as Fmoc is removed by basic conditions and Boc by acidic. In this study the Fmoc-protecting group strategy was chosen. The Fmoc-group is often preferred since it can be removed under milder conditions compared to the Boc-group and offers more options when it comes to side-chain protecting groups.[4]

The solid material used in SPPS are resins, which is polymer matrices with functional groups, i.e., linkers, which the amino acids are coupled to. There are three main classes of resins for peptide synthesis: polystyrene (PS), polystyrene with polyethylene glycol (PEG) chains and crosslinked PEG chains. The loading of the resin is determined by the numbers of linkers present, in mmol/g. To achieve sufficient coupling of amino acids to the resin, it is essential that the swelling properties of the resin are good enough to facilitate mass transportation. Due to this, the first step in SPPS is to swell the resin using a suitable solvent, traditionally either DMF, NMP, or DCM.[4]

As the resin contains functional groups, these are often protected like the N-terminal of the amino acids using Fmoc (Figure 1), and hence the second step of synthesis after swelling is to deprotect the resin. Deprotection of Fmoc was in this study done using 20% piperidine in suitable solvent. After deprotection, the resin is exposed to the first amino acid to be coupled to the resin. Next step is for the N-terminal of the coupled amino acid to be deprotected and the second amino acid added. The cycle of deprotection and coupling of amino acids is repeated until the peptide is completed. Once the peptide is done it is cleaved from the resin. In this study the peptide was cleaved using trifluoroacetic acid (TFA) and isolated by precipitation in cold diethyl ether. When the peptide is cleaved from the resin but not yet purified it is referred to as the crude.

To achieve coupling of amino acids, the carboxylic group needs to be activated by an electrophile to enable reaction with the amine group of another amino acid. Prevention of side reactions, such as racemisation of the C-terminal of the amino acid to be coupled, is done with addition of auxiliary nucleophiles which form activated esters to maintain the stereochemistry. Coupling reagents used in this study was the carbodiimide electrophile *N,N′*-diisopropylcarbodiimide (DIC), combined with the auxiliary nucleophile ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma). Reaction was also done using the in-situ reagent HBTU, which is an electrophile/nucleophile salt, in presence of the base DIPEA and auxiliary nucleophile HOBt. For mechanisms of coupling and deprotection, see Appendix Figure A1-2.[4]

Green chemistry and selection of solvents

The requirements of a solvent in SPPS are many. For a solvent to be sufficient in SPPS it must dissolve all reagents and amino acids, it must swell the resin and preferably have a high boiling point to suffice reactions with increased temperatures. Today, the main solvents that meets these requirements are DMF, NMP, and DCM. However, DMF and NMP have been classified as 'Substances of Very High Concern' (SVHC) by the European Chemical Agency (ECHA), and DCM are under investigation for the same classification. The classification aims at ensuring that SVHCs are replaced by safer alternatives in the near future.[6] ECHA imposes restrictions on hazardous chemicals through the regulation 'Registration, Evaluation, Authorisation and Restriction of Chemicals' (REACH), and SVHCs concludes the list of candidate chemicals for future European bans. This calls for a change to greener solvents to be used in SPPS.

To find a greener solvent for SPPS, the term "green" needs to be defined. Today, no absolute definition of a green solvent seems to exist, and for many solvents sufficient data for this type of classifications are missing. However, there are several solvent selection guides available, proposing ways to determine whether a solvent can be regarded as green or not. Ideally, a green solvent should be derived from renewable feedstock, require low energy consumption during production, be nontoxic, be biodegradable and have the possibility to be re-cycled – and at the same time, perform better or equally to the solvents used today. However, finding such solvent is a great challenge.

In this study, the first criterion for a friendlier solvent was for the solvent not to be SVHC classified. Then a selection of solvents was chosen based on their chemical similarities to DMF, such as polarity, viscosity, and boiling point. Their performance in SPPS-related experiments reported in previous reports, such as their ability to swell resin, dissolve amino acids and efficiency in coupling reactions was also taken into consideration. [1,2,7-13] From this, five different solvents were chosen: dihydrolevoglucosenone (Cyrene), ethyl acetate (EtOAc), 1,3-dioxolane (DOL), 2-methyl tetrahydrofuran (2-Me-THF) and *N*-butylpyrrolidinone (NBP). None of these solvents are classified as SVHC, even though DOL are presently under investigation by the ECHA. The chemical properties and structures of the solvents are presented in Table 1. All solvents are aprotic polar substances.

Table 1: Chemical properties for the solvents DMF, Cyrene, DOL, EtOAc, 2-Me-THF and NBP. Data retrieved from ECHA.[14]

Solvent	Structure	MW (g/mol)	Viscosity (mPa s)	Boiling point (°C)	Polarity[7,12,15,16] (E_N^T)
DMF		73.1	0.80	153	0.386 High polarity
Cyrene		128.1	13.80	227	0.346 High polarity
DOL		74.1	0.59	76	0.383 High polarity
EtOAc		88.1	0.45	77	0.228 Medium polarity
2-Me-THF		86.1	0.58	78	0.179 Low polarity
NBP		141.2	4.30	241	0.349 High polarity

To determine a solvents greenness however, more parameters than ECHA's classification can be taken into consideration. As no "perfect" green solvent seems to yet exist, the greenness of the solvents will be discussed in comparison to the solvents used today, and more specifically to DMF. The solvents can be regarded greener than DMF in either toxicity or environmental impact, or preferably both. There are a few solvent selection guides available which addresses these aspects, and these have been used to further investigate the solvents' greenness.

Several pharmaceutical companies have their own solvent selection guides, including Pfizer, AstraZeneca, Sanofi, and GSK.[17-21] In addition to these, the CHEM21 (Innovative Medicines Initiative) and the ACS Green Chemistry Institute Pharmaceutical Roundtable (GCIPR) have developed selection tools aiming at greening chemistry – where solvents are one area of interest.[22] They have based their selection guide on data from pharmaceutical companies, i.e., Pfizer and AstraZeneca, as well as several universities. In these guides, the solvents are

generally classified based on three criteria: safety, health, and environment, and ranked in each category from 1 to 10, where 10 represents the highest hazard level. The safety criterion takes aim at the flammability of the substance, the health criterion on occupational hazard and the environmental criterion on ozone layer impact, ecotoxicity, bioaccumulation, volatility, and recyclability. In Table 2 are the scoring of the five solvents investigated in this study, as well as DMF for comparison. None of these selection guides have yet classified NBP.

Table 2: Summary of solvent selection guide classifications.

Solvent	**Pfizer**[17]	**Sanofi**[20]	**GSK**[21]	**CHEM21 and AstraZeneca (SHE)**[18,22]
DMF	Undesirable	Substitution requested	Major known issues	3, 9, 5: Hazardous
Cyrene	-	-	Some known issues	1, 2, 7: Problematic
NBP	-	-	-	-
2-Me-THF	Usable	Recommended	Some known issues	6, 5, 3: Problematic
EtOAc	Preferred	Recommended	Few known issues	5, 3, 3: Recommended
DOL	-	-	Some known issues	7, 10, 5: Hazardous

Cyrene is a renewable solvent derived from cellulose. It is non-mutagenic, non-genotoxic and biodegradable, decomposing to carbon dioxide and water.[16] However, its high boiling point contributes to both GSK and CHEM21 classifying it as a solvent with some issues. This because the solvent requires a higher energy input to be recycled. Cyrene's high boiling point was however regarded as an advantage in this study, as it enables for SPPS to be performed at higher temperatures.

NBP is a relatively new solvent and has therefore limited data and classifications. However, Sherwood et al. concludes that NBP is a non-reprotoxic, non-mutagenic and biodegradable solvent.[23,24] NBP also has a negligible dose toxicity and lower skin and eye irritability than DMF.[25] NBP does however hold the same concerns as Cyrene regarding its high boiling point and recyclability, and NBP is not retrieved from a renewable source.

2-Me-THF is a solvent renewably sourced from furfural or levulinic acid and is ranked between a problematic and preferred solvent by the reviewed solvent guides.[27] This because its high flammability and corrosive properties, earning it a somewhat higher safety and health scoring compared to for example Cyrene and EtOAc.

EtOAc is defined as a preferred solvent by all reviewed solvent guides. This because of its low toxicity, few safety concerns and low environmental impact. In addition, Fergal et al. reported the possibility to produce EtOAc renewably.[26] However, this is not commonly done today.

DOL is to this date still regarded as a greener alternative to DMF. In 2014 Moscoso et al. defined DOL as a "nontoxic, odourless, easy to evaporate and environmentally friendly" solvent.[28] However, data presented since then suggests otherwise, and due to this is the ECHA investigating DOL for a possible inclusion to the SVHC list and CHEM21 classifies DOL as a hazardous substance.[29] This because DOL is a highly flammable compound and is suspected to be reprotoxic. DOL was studied in this project, as it is still regarded as greener than DMF, but the use was determined to be kept at a minimum.

Aim

The aim of this project was to find a suitable single solvent or binary solvent mixture that is regarded greener than today's solvents for SPPS and is equal or better performing.

The solvent or solvent system needs to be generally applicable to several different amino acids, coupling reagents, resins, reaction temperatures, and peptides. There are high demands on the solvent to be used and hence several challenges need to be addressed.

1. The resin needs to swell to make all the reactive sites available for the peptide to grow.
   - Place a certain amount of resin together with the candidate solvent and stir for a certain period of time. Measure the final volume in mL/g.
2. All amino acids and coupling reagents need to be soluble.
   - Investigate the solubility of amino acids and coupling reagents at 0.5 M in the candidate solvent by subsequent visual observation of the state of the mixture.
3. Peptide synthesis steps need to have a high yield to be viable.
   - Synthesize chosen peptides with the coupling reagents DIC/Oxyma or HBTU/HOBt/DIPEA in the candidate solvent. Analyse the purity and yield of the synthesis with different analytical techniques. Compare with syntheses completed in DMF to determine the solvent's performance.

Results and Discussion

The outset of this study was to find a neat solvent or binary solvent mixture through a series of experiments, to end up with one or a few alternatives to be analysed in greater detail. First the solvents' resin swelling was to be evaluated, and then their ability to dissolve amino acids and coupling reagents. The solvents performing well in these initial studies was then to be studied in synthesis of chosen peptides.

Resin swelling tests

Multiple resin swelling tests were performed using a standardised method, described in the Experimental section: **Resin swelling tests**. The swelling was measured in mL/g increase of the resin after being incubated with selected solvent for 30 min with shaking at 500 rpm on a shaker. The resin used was the PS-PEG resin TentaGel S RAM with a loading of 0.22 mmol/g. TentaGel is a commonly used resin and decided to be a good resin model. Generally, less resin swelling was observed compared to previous studies[7,12], but differences in method as well as shorter incubation time could explain this difference. The results presented here are however comparable since they have been achieved using the same method and with replicates (see **Raw data**).

The five selected green solvents were first evaluated for their resin swelling capacity using DMF as a reference, presented in Figure 2. Two neat solvents, NBP and DOL, proved to be comparable with DMF in swelling the resin. The other three solvents (EtOAc, 2-Me-THF and Cyrene) reached about 2.0 mL/g swelling, which was about 50% of the swelling achieved by the other solvents. 2.0 mL/g was selected as cut-off level for further investigation and hence solvents and solvents mixtures that did not reach more swelling than 2.0 mL/g under these conditions was not further investigated.

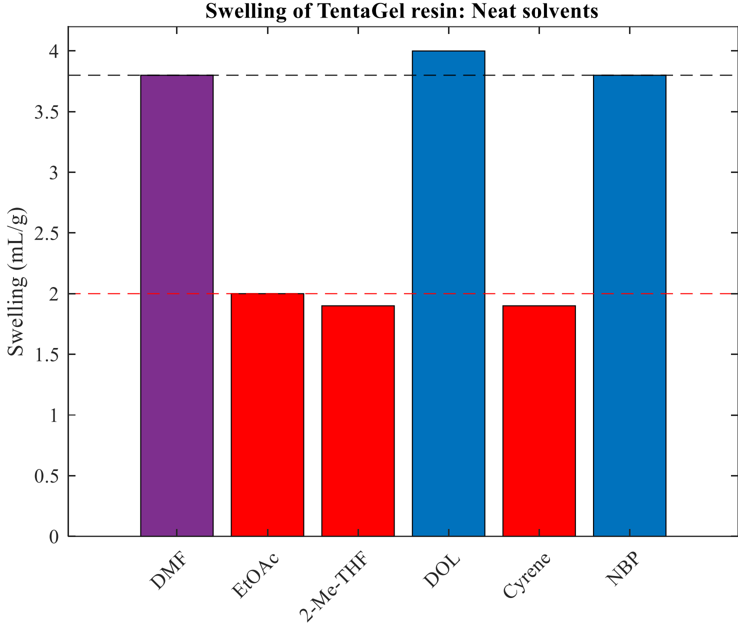

Figure 2: Swelling of TentaGel S RAM resin (0.22 mmol/g) in mL/g using DMF, EtOAc, 2-Me-THF, DOL, Cyrene and NBP, respectively. The black dotted line denotes DMF's swelling at 3.8 mL/g and the red dotted line the cut-off level for resin swelling at 2.0 mL/g.

The swelling performance of the green solvents were also tested in 1:1 mixture with DMF. The hypothesis was that any reduction of DMF is an improvement. The result is presented in Figure 3.

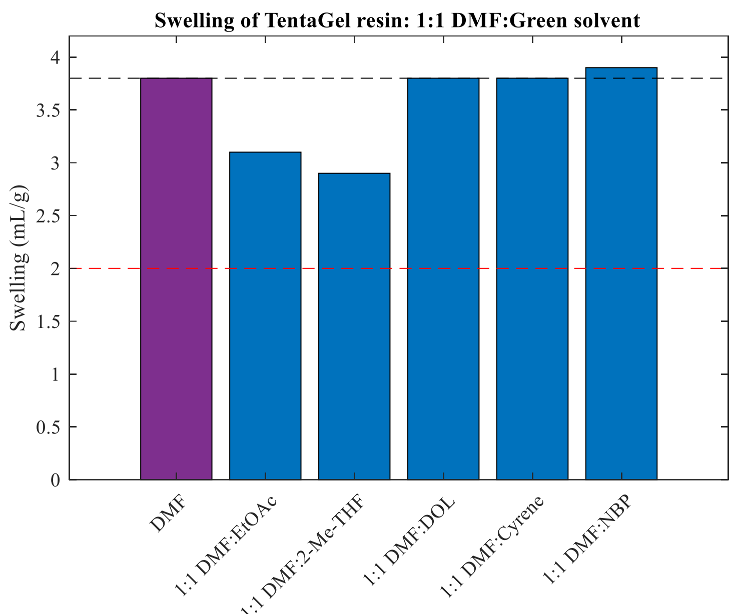

Figure 3: Swelling of TentaGel S RAM resin (0.22 mmol/g) in mL/g using 1:1 DMF with a green solvent, and neat DMF as reference. The black dotted line denotes DMF's swelling at 3.8 mL/g and the red dotted line the cut-off level of resin swelling at 2.0 mL/g.

It can be seen that the swelling got considerably improved for the solvents that previously showed poor resin swelling, i.e., EtOAc, 2-Me-THF and Cyrene. Also, the solvents that previously showed good swelling performance had similar swelling in mixture with DMF. Hence, it could be possible to achieve good swelling by mixing "poor resin swelling"-solvents (EtOAc, 2-Me-THF, and Cyrene) with "good resin swelling"-solvents (NBP and DOL) to get green binary mixtures. Figure 4 illustrates the results of the green binary mixtures.

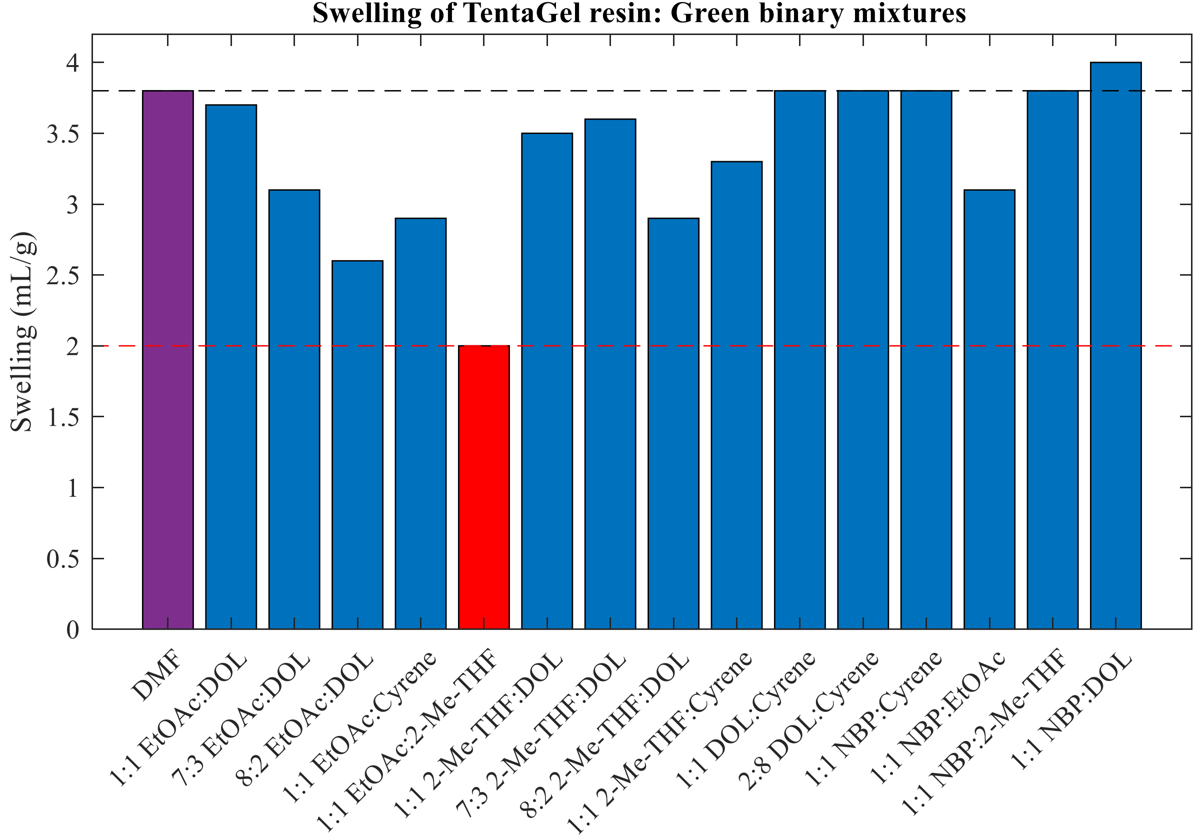

Figure 4: Swelling of TentaGel S RAM resin (0.22 mmol/g) in mL/g using green binary mixtures and DMF as reference. The black dotted line denotes DMF's swelling at 3.8 mL/g and the red dotted line the cut-off level of resin swelling at 2.0 mL/g.

First the swelling of 1:1 mixtures was evaluated: EtOAc:DOL, EtOAc:Cyrene, EtOAc:2-Me-THF, 2-Me-THF:DOL, 2-Me-THF:Cyrene, DOL:Cyrene, NBP:Cyrene, NBP:EtOAc, NBP:2-Me-THF and NBP:DOL. It was preferable to keep the DOL ratio low, as previously discussed, so mixtures with minimised DOL was also evaluated: (7:3) EtOAc:DOL, (8:2) EtOAc:DOL, (7:3) 2-Me-THF:DOL, (8:2) 2-Me-THF:DOL and (2:8) DOL:Cyrene. The assumption of combining "poor resin swelling"-solvents with "good resin swelling"-solvents proved to be effective, as most of the green binary mixtures showed feasible swelling performance. In addition, mixtures of "bad resin swelling"-solvents were analysed to see if it would could improve the swelling, i.e., 1:1 of EtOAc:Cyrene, EtOAc:2-Me-THF and 2-Me-THF:Cyrene. It turned out that this was the case for all mixtures except for EtOAc:2-Me-THF.

An in-dept analysis of the resin swelling with NBP and Cyrene in mixture with DMF was also completed (Figure 5 A and B). NBP and Cyrene were chosen because both have a high boiling point, Cyrene is regarded to be one of the "greenest" solvents and NBP has in previous studies showed great potential.[12,13,16,23] This analysis further proved that a "poor resin swelling"-solvent in combination with DMF could produce better swelling, i.e., Cyrene, while a "good resin swelling"-solvent in combination with DMF reached the same amount of swelling, i.e., NBP. Neat Cyrene proved to be poor at swelling resin, and a clear trend of less resin swelling as the amount of Cyrene was increased was observed.

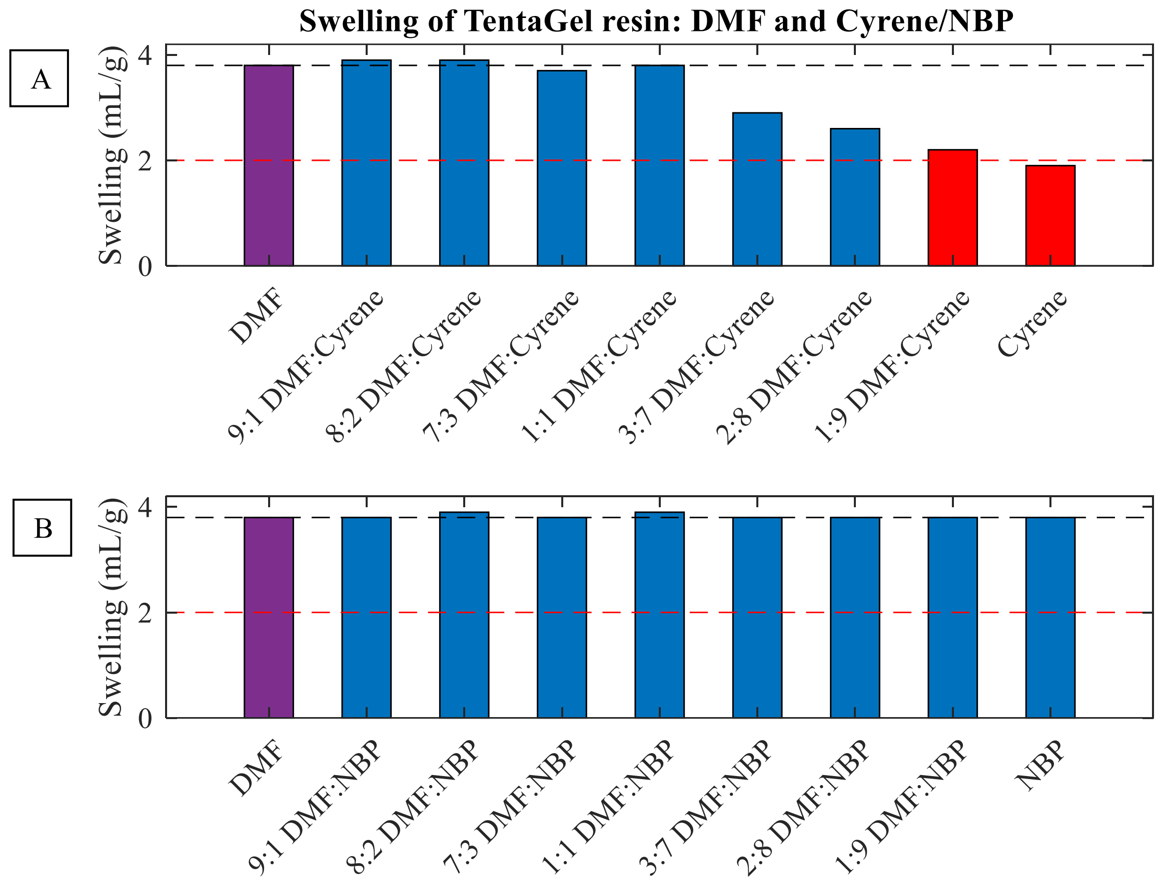

Figure 5: Swelling of TentaGel S RAM resin (0.22 mmol/g) in mL/g Cyrene (A) or NBP (B) in mixture with DMF, and neat DMF as reference. The black dotted line denotes DMF's swelling at 3.8 mL/g and the red dotted line the cut-off level of resin swelling at 2.0 mL/g.

Lastly, the solvents which showed promising resin swelling in room temperature was studied using micro-wave heating as well. Depending on the boiling point of the solvent, the resin was incubated in the selected solvent or solvent mixture in 25 min at 70°C or 30 min at 40°C (ratios containing EtOAc, 2-Me-THF or DOL), as described in the Experimental section: **Resin swelling tests**. The result can be seen in Figure 6.

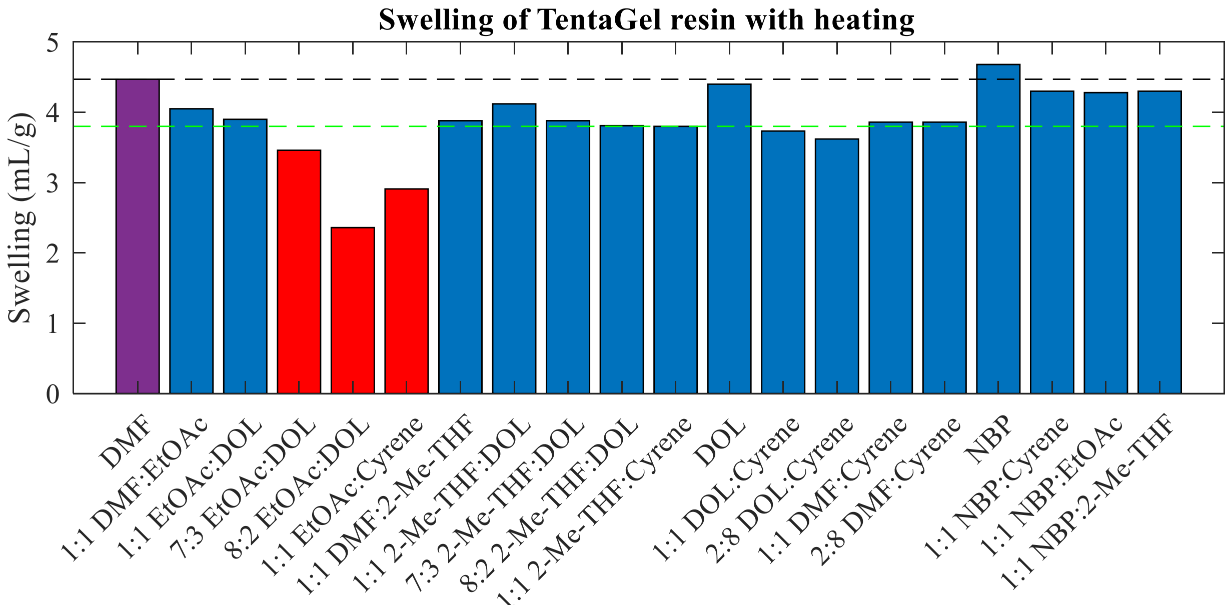

Figure 6: Swelling of TentaGel S RAM resin (0.22 mmol/g) in mL/g in selected neat solvent or binary mixture using micro-wave heating, with DMF as reference. The black dotted line denotes DMF's swelling using heating at 4.47 mL/g and the green dotted line DMF's swelling in room temperature at 3.8 mL/g.

If the solvents showed similar or better swelling than DMF in room temperature (3.8 mL/g) it was decided to be sufficient swelling. The ratios (7:3) EtOAc:DOL, (8:2) EtOAc:DOL and (1:1) EtOAc:Cyrene did not show a substantial increase in swelling between heating and room temperature, and did not reach close to 3.8 mL/g swelling, and were therefore not further investigated.

All neat solvents and solvent system with a swelling above 2.0 mL/g in room temperature as well as increased swelling (around 3.8 mL/g) in heating were further investigated for solubility of amino acids and coupling reagents. Even though Cyrene had showed poor resin swelling it was included in the solubility tests as well.

Amino acids and coupling reagents solubility

Eight common Fmoc-protected amino acids: cysteine, tyrosine, phenylalanine, glutamine, aspartic acid, proline, arginine, glycine, the coupling reagents DIC and Oxyma, and piperidine were to be investigated for their solubility in selected solvent. The visual state of the mixture determined whether the compound was soluble or not. All amino acids, DIC and Oxyma was dissolved to a concentration of 0.5 M, and piperidine to 20 %, in selected solvent, as described in Experimental section: **Solubility of amino acids and coupling reagents**. The result of the solubility test is presented in Table 3.

Table 3: Solubility of 0.5 M of cysteine, tyrosine, phenylalanine, glutamine, aspartic acid, proline, arginine, glycine, DIC and Oxyma, and 20% piperidine, in selected solvent/solvent mixture. Green illustrates fully dissolved compound and red insoluble compound. Yellow represents dissolved compound, but which precipitates a few minutes after removal from ultrasound bath. (*) denotes compounds that do not stay dissolved over-night and (-) data not collected.

Solvent	**Cys**	**Tyr**	**Phe**	**Gln**	**Asp**	**Pro**	**Arg**	**Gly**	**DIC**	**Oxyma**	**20% Piperidine**
Cyrene									-	-	-
DOL											
NBP											
1:1 DMF:Cyrene											
2:8 DMF:Cyrene			*								
1:1 DMF:2-Me-THF									-	-	-
1:1 DMF:EtOAc									-	-	-
1:1 2-Me-THF:Cyrene			*								
7:3 DOL:Cyrene	-	-	*	-	-	-	-	-	-	-	-
6:4 DOL:Cyrene	-	-		-	-	-	-	-	-	-	-
1:1 DOL:Cyrene											
3:7 DOL:Cyrene				-		-	-		-	-	-
2:8 DOL:Cyrene									-	-	-
1:1 DOL:2-Me-THF									-	-	-
2:8 DOL:2-Me-THF											
1:1 DOL:EtOAc			*		*			*			
1:1 2-Me-THF:EtOAc					*				-	-	-
1:1 NBP:Cyrene											
1:1 NBP:EtOAc											
1:1 NBP:2-Me-THF											

In total, 14 of the analysed 20 ratios showed sufficient solubility of all tested amino acids and coupling reagents. Generally, phenylalanine posed the biggest solubility challenge. This was however expected since phenylalanine often is hard to dissolve in DMF as well.

The solvents and solvent systems Cyrene, (3:7) DOL:Cyrene, (2:8) DOL:Cyrene, (1:1) DOL:Cyrene, (6:4) DOL:Cyrene and (1:1) 2-Me-THF:EtOAc were decided to be too poor at dissolving the amino acids and reagents, and were excluded from further investigations. The possibility to dissolve most amino acids in (1:1) DOL:Cyrene but have phenylalanine in (7:3) DOL:Cyrene was considered, but other more promising ratios with less usage of DOL were favoured. The mixture (1:1) DOL:EtOAc, could dissolve all amino acids, but did not keep them dissolved for longer than a few hours. This could be inconvenient for longer syntheses as the amino acids then needs to be prepared right before addition to the reaction. Hence this ratio was also excluded.

From this, 12 ratios were found to be possible solvents/solvent systems to use for synthesis of peptides. Of these, eight were chosen for further investigation. (1:1) DMF:EtOAc and (1:1) DMF:2-Me-THF were excluded as the greener alternatives with EtOAc/2-Me-THF in combination with NBP were favoured. The same reasoning went for (1:1) DOL:2-Me-THF, as

the greener alternative (2:8) DOL:2-Me-THF also showed promising results. Lastly, (1:1) NBP:Cyrene was excluded as well. This because the high viscosity of the mixture was believed to pose problems for synthesis on the automated peptide synthesizer. The eight solvent systems/neat solvents chosen were therefore: (1:1) DMF:Cyrene, (2:8) DMF:Cyrene, DOL, NBP, (1:1) 2-Me-THF:Cyrene, (8:2) 2-Me-THF:DOL, (1:1) NBP:EtOAc and (1:1) NBP:2-Me-THF.

ChemMatrix resin swelling test

The swelling of the resin H-Rink amide ChemMatrix, with a loading of 0.40 mmol/g, was also tested using the eight solvent ratios to be used in synthesis. ChemMatrix resin consists only of crosslinked PEG, and is known for its good swelling properties[30], which was why this resin was used for additional resin swelling tests. The results are presented in Figure 7.

The resin swelling tests were performed using a standardised method, described in the Experimental section: **Resin swelling tests**. The swelling was measured in mL/g increase of the resin after being incubated with selected solvent for 60 min with shaking at 500 rpm on a shaker.

All solvents and solvent ratios showed more swelling of the ChemMatrix resin compared to the TentaGel resin. Surprisingly, (1:1) DMF:Cyrene showed the highest degree of swelling, and in the other end NBP less swelling compared to the other solvents. This in contrast to the swelling of the TentaGel resin where (1:1) DMF:Cyrene showed less swelling and NBP more.

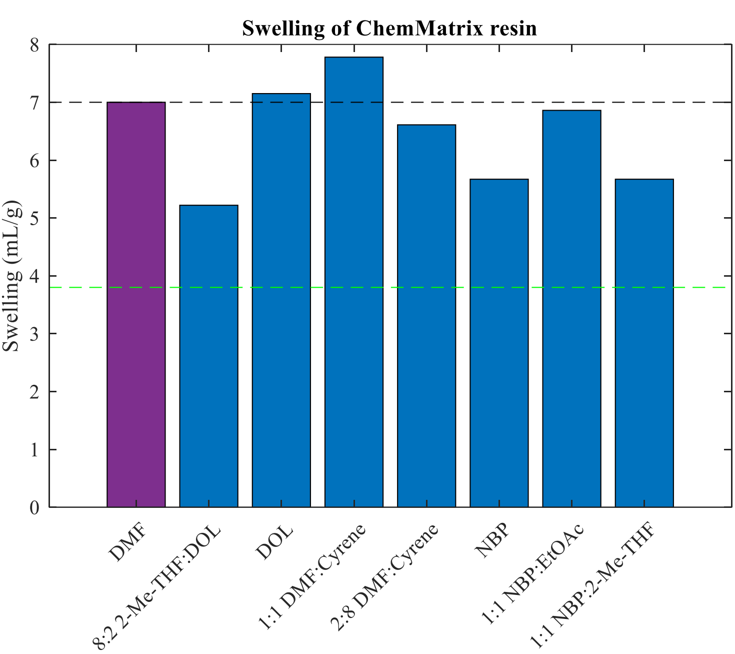

Figure 7: Swelling of H-Rink amide ChemMatrix resin (0.40 mmol/g) in mL/g in selected neat solvent or binary mixture, with DMF as reference. The black dotted line denotes DMF's swelling at 7.00 mL/g and the green dotted line DMF's swelling of TentaGel resin at 3.8 mL/g.

Synthesis of [Asp^5]-Vasopressin with DIC/Oxyma

To assess the eight solvent and solvent systems' utility for SPPS, the peptide [Asp^5]-vasopressin was synthesised in each solvent and in DMF for reference. [Asp^5]-Vasopressin is a nine amino acid long peptide with a molecular weight of 1086.4 Da and the sequence:

$$H_2N\text{-CYFQDCPRG-}CONH_2$$

Vasopressin is a peptide with an internal disulphide bridge, see Figure 8. This disulphide bridge is, in our lab, usually formed after the peptide is cleaved from the resin and hence this work has focused on the SPPS part of vasopressin. Vasopressin is an antidiuretic hormone and one

of the top ten sold peptide drugs.[31] The peptide contains challenging amino acids, such as the sterically hindered arginine which typically has a large protecting group, and temperature sensitive cysteine which is prone to oxidise.[32] The sterically hindered couplings between glutamine, phenylalanine and tyrosine also poses challenges. Vasopressin has several similar analogues, which further broaden the application of the solvents used to synthesize vasopressin. The theory was that if the solvent worked in synthesis of vasopressin, it could be applied for its analogues as well. Due to this, vasopressin was determined to be a good model peptide.

Figure 8: Molecular structure of [Asp^5]-vasopressin.

The synthesis of [Asp^5]-vasopressin was performed on a Biotage Alstra Initiator using micro-wave meditated heating. The coupling reagents used were DIC in combination with Oxyma, and deprotection of the Fmoc-protecting group was done using 20% piperidine in selected solvent. All coupling reagents and amino acids were used in excess (4 eq.) to get sufficient coupling. It was observed that for solvents with higher viscosity, i.e., NBP and Cyrene, the time for emptying the reaction vial using vacuum needed to be increased to make sure that reagents and amino acids in excess were removed after each coupling. The reaction conditions are described in detail in the Experimental section: **Synthesis of [Asp^5]-Vasopressin**. The syntheses were checked using LCMS (**Analysis**) to see if the correct peptide was obtained. If product was found, the peptide was cleaved from the resin using 95% TFA, 2% TIPS, 2% water and 1% EDT.

In Figure 9, the HPLC profile and purity of the crude from each synthesis is presented. It is desirable to achieve a high crude purity since this could reduce the number of purification steps, which also contributes to the greenness of the solvent as this reduces the amount of solvents used.

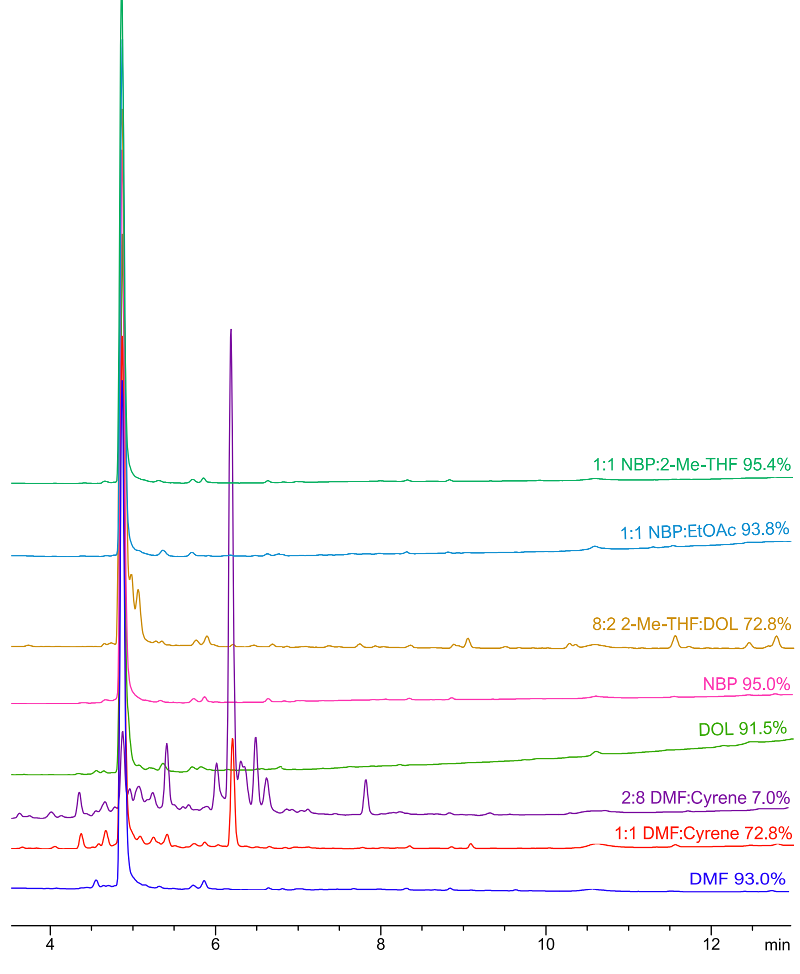

Figure 9: HPLC profiles (220 nm) of [Asp⁵]-vasopressin synthesised in selected solvent/solvent system, and DMF for reference. The solvent system and crude purity are presented by each HPLC profile.

Varying crude purity could be observed, where the reference synthesis in DMF attained a crude purity of 93.0%. Highest crude purity was achieved in synthesis using NBP (95.0%) or mixtures with NBP, i.e., (1:1) NBP:2-Me-THF (95.4%) and (1:1) NBP:EtOAc (93.8%). The synthesis in DOL gave a crude purity of 91.5%, which is comparable to DMF. (1:1) DMF:Cyrene and (8:2) 2-Me-THF:DOL both gave a crude purity of 72.8%.

The synthesis in (2:8) DMF:Cyrene gave a very small amount of product (7.0%), as can be seen in Figure 10. The largest peak observed in the HPLC profile (36.5%) had a molecular weight of 1197.4 m/z instead. The synthesis in (1:1) 2-Me-THF:Cyrene almost only generated this molecule, and because of this was the peptide never cleaved for the resin. The same mass was observed in (1:1) DMF:Cyrene as well, but in much smaller amount (7.1%). Hence, it is believed that this mass is a by-product generated in synthesis of [Asp^5]-vasopressin when Cyrene is present. The by-product agrees with [Asp^5]-vasopressin, plus a molecular weight of 110 m/z. This corresponds to Cyrene minus water. Initial NMR studies showed that the cyclic structure of Cyrene was intact, however the sp^2-carbon of Cyrene was not observed. The methine in the alpha position of the Cyrene showed NOE-correlations to the amide proton and side-chain of the following amino acid tyrosine. This could indicate the formation of an imine between the Cyrene and the peptides N-terminal. However, the NMR analysis was noisy and unstable, making the reading more difficult. It was observed that if the reacted peptide was left

dissolved in water for certain period of time (> 1 day), more of the parental [Asp^5]-vasopressin peptide was formed, and the mass of 1197.4 m/z got reduced, see Figure A3 in Appendix. It therefore seems that the formed structure is not stable. As it is now, it cannot be said for certain what the mass of 1197.4 m/z is, even though some suggestions have been made. This is therefore a subject for future studies.

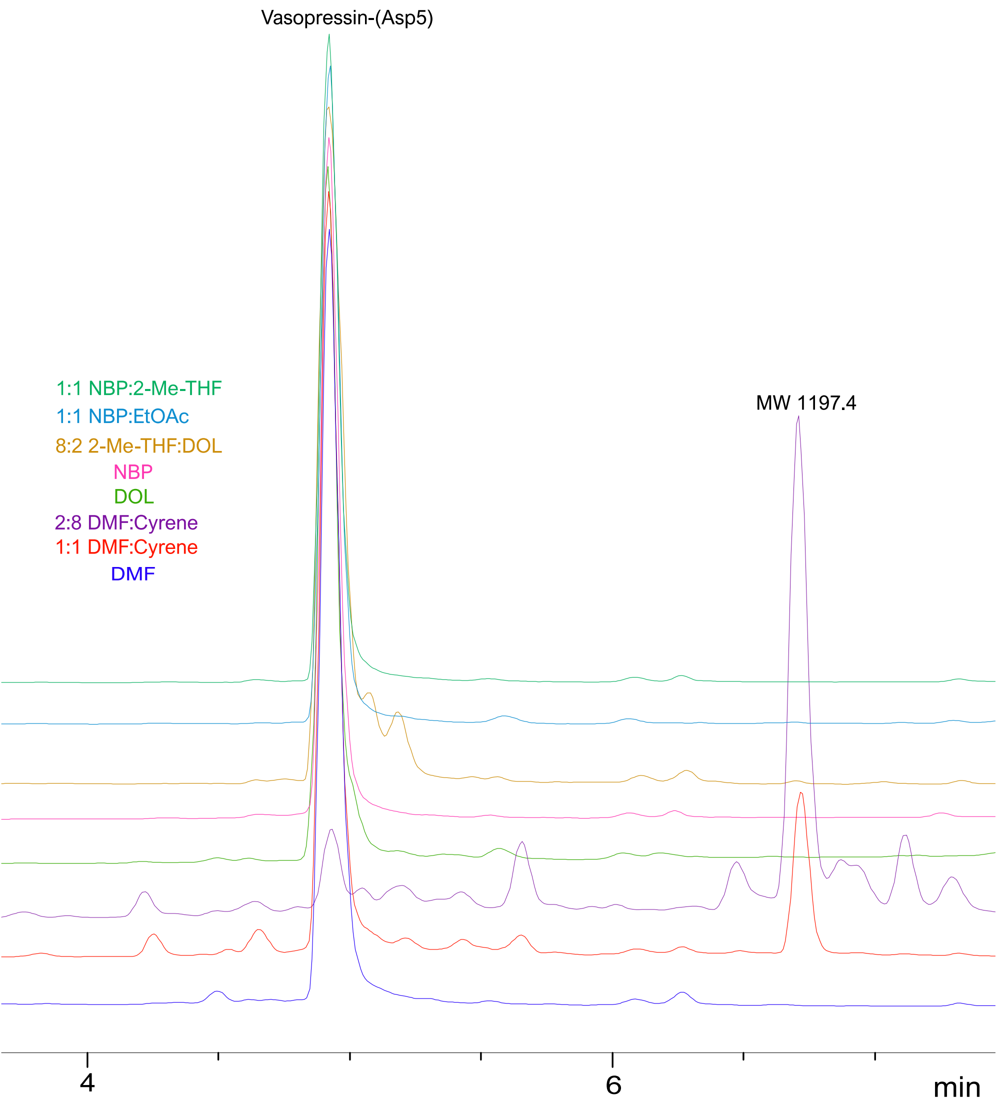

Figure 10: Zoom in on HPLC profiles (220 nm) of [Asp^5]-vasopressin synthesised in selected solvent/solvent system, and DMF for reference. The solvent systems are presented on the left. The peak identified as [Asp^5]-vasopressin are denoted as such, and the peak with molecular weight of 1197.4 m/z is denoted as well.

The crude was then purified using flash chromatography (**Purification**). For batches that did not reach >95% purity after flash chromatography, additional purification with preparative HPLC (HPLC-PREP) was performed. After purification, the yield was calculated according to Equation 1, see Appendix Table A2 for data.

$$yield = \frac{actual\ yield\ [mmol]}{theoretical\ yield\ based\ on\ resin\ loading\ [mmol]} \quad (1)$$

The final purity and yield are presented in Table 4. Syntheses in NBP or NBP-mixtures resulted in very high yield and purity, comparable or better to the synthesis in DMF. The synthesis in (1:1) DMF:Cyrene resulted in lower yield, even though high purity was achieved after only flash chromatography. The synthesis in DOL resulted in lower yield compared to the synthesis in DMF, and did also require an extra purification step with HPLC-PREP. The same was observed for the synthesis in (8:2) 2-Me-THF:DOL, even though higher yield was achieved compared to the synthesis in only DOL.

Table 4: The yield (Equation 1) and final purity achieved for [Asp^5]-vasopressin in selected solvent. All syntheses have been purified using flash chromatography, (*) represents the batches which has had additional purification using HPLC-PREP. The (-) represents where data is not applicable.

Solvent ratios	Yield	Final purity
DMF	85.1%	97.4%
DMF:Cyrene (1:1)	39.0%	97.2%
DMF:Cyrene (2:8)	-	-
DOL	51.1%	98.4%*
NBP	88.2%	99.1%
2-Me-THF:Cyrene (1:1)	-	-
2-Me-THF:DOL (8:2)	74.9%	99.1%*
NBP:EtOAc (1:1)	86.8%	98.1%
NBP:2-Me-THF (1:1)	86.8%	98.7%

In terms of yield and purity, both neat NBP and NBP-mixtures, (1:1) NBP:EtOAc and (1:1) NBP:2-Me-THF, performed better than DMF. However, neat NBP was chosen for further, more in-dept analysis. The difference in boiling point between NBP and EtOAc and 2-Me-THF might give rise to an azeotrope, which is something to be investigated in future studies.

Synthesis of [Asp^5]-Vasopressin with HBTU/DIPEA/HOBt in NBP

To replace DMF in SPPS it is necessary for the replacing solvent to suffice different reaction conditions, for example make various coupling reagents work. To assess this, [Asp^5]-vasopressin was synthesised using HBTU in combination with the base DIPEA and the auxiliary nucleophile HOBt in NBP. It was however found that NBP could dissolve all amino acids and coupling reagents to an acceptable concertation (0.5 M) except for HBTU. Concentration of HBTU down to 0.125 M was tested, but HBTU remained insoluble. Therefore, HBTU was kept dissolved in DMF during synthesis.

The synthesis was, as previously syntheses, performed on a Biotage Alstra Initiator using micro-wave heating and in overall same conditions as for the syntheses using DIC/Oxyma to make them comparable (**Synthesis of [Asp^5]-Vasopressin**). The synthesis was checked using LCMS (**Analysis**) to see if the correct peptide was obtained. The peptide was cleaved from resin using 95% TFA, 2% TIPS, 2% water and 1% EDT. In Figure 11, the HPLC profile and crude purity of the synthesis using HBTU/DIPE/HOBt and DIC/Oxyma in NBP is presented.

It can be seen that the crude purity is similar between the two syntheses, even though slightly higher when using DIC and Oxyma. The yield of the synthesis using HBTU, DIPEA and HOBt was determined to 75.8% (Equation 1), with a final purity of 98.8%, obtained after flash chromatography. When using DIC/Oxyma the yield was 88.2% and final purity 99.1% after flash chromatography, see Appendix Table A3 for data.

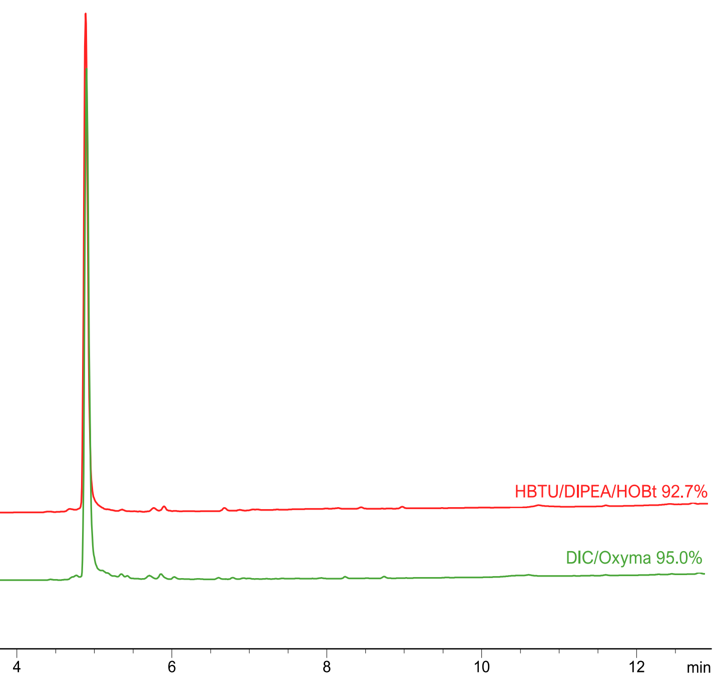

Figure 11: HPLC profiles (220 nm) of [Asp^5]-vasopressin synthesised in NBP using DIC/Oxyma and HBTU/DIPEA/HOBt respectively. The crude purity is presented by the HPLC profile.

The synthesis using HBTU, DIPEA and HOBt in NBP showed comparable yield and purity to when using DIC and Oxyma. The results showed that other couplings reagents in NBP can produce a synthesis with good purity and yield. The fact that HBTU was not soluble in NBP can be seen as a disadvantage of using NBP. However, it was reasoned that any reduction of DMF is good, and therefore to keep HBTU dissolved in DMF and all other compounds in NBP was a viable solution. There is a possibility to investigate the solubility of HBTU in NBP-mixtures as well, which is a subject for a future study.

Synthesis of [Asp^5]-Vasopressin using ChemMatrix resin in NBP

It was further investigated how NBP could perform in synthesis of [Asp^5]-vasopressin when using another resin, i.e., H-Rink amide ChemMatrix resin with a loading of 0.40 mmol/g. The synthesis was performed on a Biotage Alstra Initiator using micro-wave meditated heating. The coupling reagents used were DIC/Oxyma, and deprotection of Fmoc was done using 20% piperidine. All coupling reagents and amino acids were used in excess (4 eq.) to get sufficient coupling (**Synthesis of [Asp^5]-Vasopressin**). The synthesis was checked using LCMS (**Analysis**) to see if the correct peptide was obtained. The peptide was cleaved from resin using 95% TFA, 2% TIPS, 2% water and 1% EDT. In Figure 12, the HPLC profile and purity of the crude from the synthesis is presented with DMF as a reference.

NBP proved to give higher crude purity than DMF when using ChemMatrix resin similar to when using TentaGel resin. After flash chromatography, the synthesis done in NBP gave a final purity of 98.6% and in DMF 98.4%.

The yield was calculated according to Equation 1 to 59.6% for the DMF synthesis and 64.7% for the NBP synthesis. Hence, NBP showed better performance in terms of yield and purity of the synthesis than DMF using another type of resin as well.

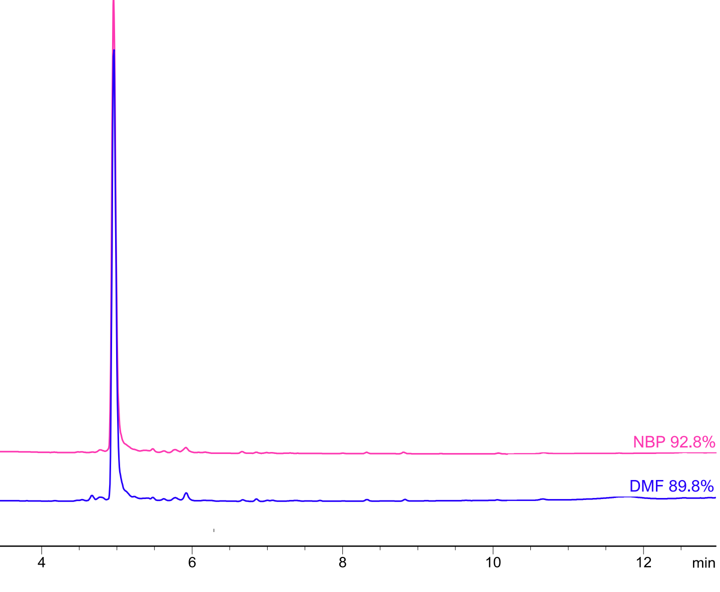

Figure 12: HPLC profiles (220 nm) of [Asp^5]-vasopressin synthesised in NBP and DMF using ChemMatrix resin. The crude purity is presented by the HPLC profile.

Synthesis of [Asp^{26}]-Calcitonin in NBP

Lastly, to investigate NBP's utility in SPPS, a longer and more complex peptide was to be synthesised. [Asp^{26}]-Calcitonin is a 32 amino acid long peptide with a molecular weight of 3432.7 Da and the sequence:

H_2N-CSNLSTCVLGKLSQELHKLQTYPRTDTGSGTP-$CONH_2$

Calcitonin is a peptide hormone which affects the calcium levels in the blood. Synthetic analogues of calcitonin are used in treatment of diseases which weakens the bones, i.e., osteoporosis.[33] As for the case with vasopressin, calcitonin is a peptide with an internal disulphide bridge, see Figure 13. This disulphide bridge is, in our lab, usually formed after the peptide is cleaved from the resin and hence this work has focused on the SPPS part.

Figure 13: Molecular structure of [Asp^{26}]-calcitonin.

Since [Asp^{26}]-calcitonin is a long peptide, the synthesis is more complex and challenging than that of vasopressin. The peptide [Asp^{26}]-calcitonin also contains challenging amino acids, such as the sterically hindered arginine and temperature sensitive cysteine and histidine.[32] Several sterically hindered couplings, i.e., between glutamine, glutamic acid and leucine, also poses challenges. [Asp^{26}]-Calcitonin contains a number of amino acids with hydrophobic side-chains (leucine and valine) and these are so-called β-branched amino acids. In combination with glycine, these are known to generate β-sheet packing, which could form insoluble peptide aggregates.[34] Due to these challenges, [Asp^{26}]-calcitonin was determined to be a good model peptide.The synthesis of calcitonin was performed on a Biotage Alstra Initiator using micro-wave meditated heating. The coupling reagents used were DIC/Oxyma, and deprotection of the Fmoc-protecting group was done using 20% piperidine. All coupling reagents and amino acids were used in excess (4 eq.) to get sufficient coupling. The method is described in detail in the Experimental section: **Synthesis of [Asp^{26}]-Calcitonin**. The synthesis was checked using LCMS (**Analysis**) to see if the correct peptide was obtained. The peptide was then cleaved from resin using 95% TFA, 2% thioanisole, 2% water and 1% EDT. In Figure 14, the HPLC profiles and purity of the crude and final product are presented.

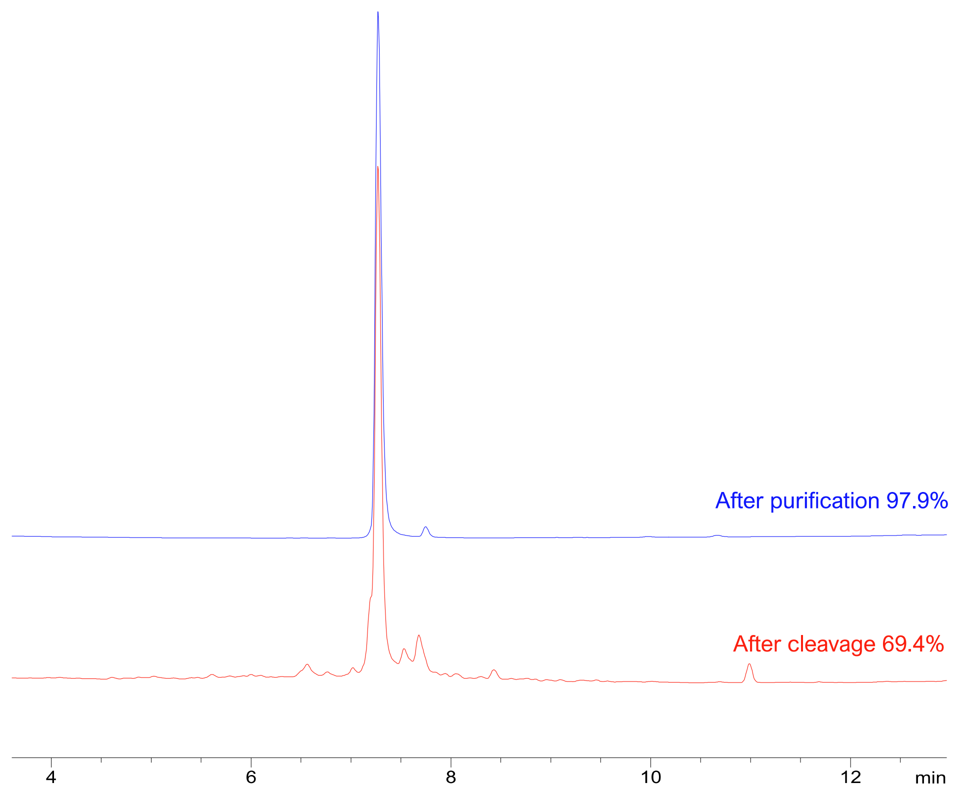

Figure 14: HPLC profiles (220 nm) of [Asp^{26}]-calcitonin synthesised in NBP using DIC/Oxyma as crude and final product, respectively. The purity is presented by the HPLC profile.

The final purity was achieved by purification first by flash chromatography (to 94.0%) and then by HPLC-PREP (97.9%), see **Purification**. The yield was calculated to 28.7% (Equation 1, data in Table A3 Appendix). This gives an average synthesis step yield of 98%, indicating very good coupling efficiency. The synthesis faced no major challenges, as coupling reactions and deprotection of Fmoc were sufficient at reasonable times. Cleavage from resin were done with 95% TFA, 2% thioanisole, 2% water and 1% EDT after 2 h, but additional 1h and 45 minutes with 70% TFA, 13.5% thioanisole, 13.5% H_2O and 3% EDT were needed for complete deprotection of amino acid-protecting groups. After cleavage, the crude purity was relatively high, and a high final purity was achieved. Therefore, the synthesis was determined to be successful, which further demonstrated that NBP shows promise for SPPS.

Conclusions and Future perspectives

By systematic evaluation of solvents/binary solvent mixtures for their resin swelling capacity and ability to dissolve amino acids and reagents, it was possible to find solvents/solvent systems which could be used in SPPS. Eight solvents/binary mixtures of varying "greenness" were identified as possible replacements for DMF. Six of the solvent systems offering a complete reduction of DMF, and two a partial reduction.

From synthesis of the nine amino acid long peptide [Asp^5]-vasopressin using DIC/Oxyma and TentaGel resin, three solvents/solvent systems were found to perform better than DMF. This by producing higher yield and purity, and these were NBP, (1:1) NBP:EtOAc and (1:1) NBP:2-Me-THF. Three solvent systems produced acceptable yield and purity, (1:1) DMF:Cyrene, DOL and (8:2) 2-Me-THF:DOL, and two solvent ratios failed in producing sufficient amount of the peptide product, (2:8) DMF:Cyrene and (1:1) 2-Me-THF:Cyrene.

NBP was further investigated for its function in SPPS. Synthesis of [Asp^5]-vasopressin using HBTU/DIPEA/HOBt showed that other couplings reagents in NBP can produce good purity and yield. HBTU was not soluble in NBP but having HBTU dissolved in DMF and all other compounds in NBP was a viable solution. [Asp^5]-Vasopressin was also synthesised in NBP using DIC/Oxyma and ChemMatrix resin. Here, both purity and yield were slightly higher compared to the synthesis in DMF.

Lastly, the 32 amino acid long peptide [Asp^{26}]-calcitonin was synthesised in NBP. The synthesis faced no major challenges, the crude purity was relatively high, and a high final purity was achieved. Hence was the synthesis deemed successful. This further proved that NBP shows great potential for replacing DMF in SPPS.

The future of NBP in SPPS looks promising. To fully investigate NBP in SPPS, several complex peptides could be synthesised, as well as using other types of resins and optimising reaction conditions. This would broaden the application of NBP in peptide synthesis. It would also be possible to further investigate NBP-mixtures. Binary mixtures with NBP could enable peptide specific fine-tuning in terms of polarity and viscosity, as well as solubility of coupling reagents. NBP in combination with renewable solvents such as 2-Me-THF could also increase the greenness of the solvent.

Experimental section

Resin swelling tests

TentaGel resin in room temperature

100 mg of TentaGel S RAM resin with a loading of 0.22 mmol/g was placed in a 5 mL vial and the volume of resin before swelling was noted. 2 mL of selected solvent was added, and the mixture shaken at 500 rpm for 30 min and then the solvent was removed with vacuum. Resin was left to settle for 5 min and then the volume after swelling was noted. The swelling was calculated and reported as mL/g. Two replicates were done for each solvent system. If a large difference between the two replicates were found (around 1 mL/g in difference), a third replicate was done.

TentaGel resin with heating

200 mg of TentaGel S RAM resin with a loading of 0.22 mmol/g was placed in a 10 mL vial with a radius of 0.755 cm, and the height of resin before swelling was noted. 4 mL of selected solvent was added, and the mixture was placed in Biotage Initiator Alstra. The mixture was stirred for 25 min at 70°C or 30 min at 40°C, depending on the solvents boiling point (40°C for solvents with boiling point <80°C), and then the solvent was removed with vacuum. Resin was left to settle for 5 min and then the height after swelling was noted. The swelling was calculated and reported as mL/g.

ChemMatrix resin

50 mg of H-Rink amide ChemMatrix resin with a loading of 0.40 mmol/g was placed in a 2 mL vial with a radius of 0.449 cm, and the height of resin before swelling was noted. 1 mL of selected solvent was added, and the mixture was stirred at 500 rpm for 60 min, and then the solvent was removed with vacuum. Resin was left to settle for 5 min and then the height after swelling was noted, see Figure 15. The swelling was calculated and reported as mL/g.

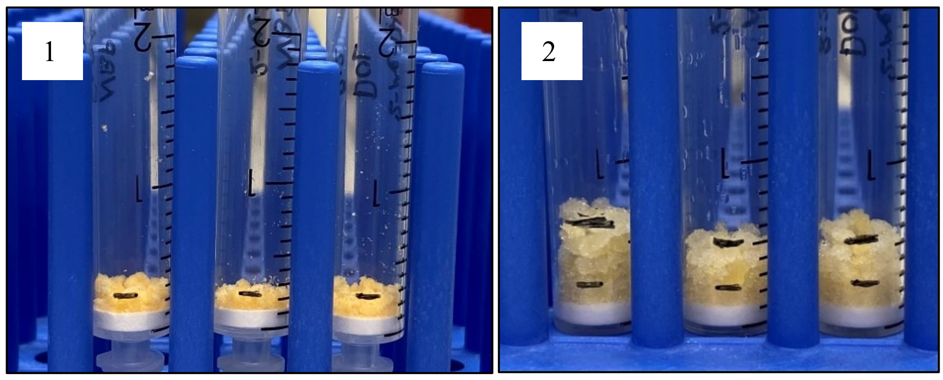

Figure 15: Before addition of solvent (1), after addition and removal of solvent (2). Solvent used left to right: NBP:EtOAc (1:1), NBP:2-Me-THF (1:1), 2-Me-THF:DOL (8:2).

Solubility of amino acids and coupling reagents

1 mmol of each amino acid or reagent was added to 2 mL of selected solvent (0.5 M). The solution was mixed using vortex. If dissolution did not take place right away the mixture was placed in an ultrasound bath (Branson 2510) until dissolved. If not dissolved after 60 min the compound was deemed to be insoluble in selected solvent. The solubility was determined by subsequent visual observation of the state of the mixture.

Synthesis of [Asp^5]-Vasopressin

Synthesis of the nine amino acid long peptide [Asp^5]-vasopressin was done on the automated peptide synthesizer Biotage Alstra Initiator. The resin used was TentaGel S RAM resin with 0.22 mmol/g loading, or H-Rink amide ChemMatrix resin with loading of 0.40 mmol/g.

Swelling of resin with the selected solvent (4.5 mL for 10 mL vial, or 9 mL for 30 mL vial) was done for 25 min with micro-wave heating of 70°C or 30 min in 40°C, depending on the boiling point of the solvent.

Removal of Fmoc protecting group was done for 6 and 15 min with 4.5 mL in 10 mL vial, or 9 mL in 30 mL vial, using 20% piperidine in selected solvent. If difficult deprotection an extra step of 15 min was added. After deprotection with piperidine the resin was washed four times with the selected solvent.

Coupling reactions were done using 4 equivalents of DIC/Oxyma (0.5 M) in selected solvent, or 3.92 eq. HBTU/8 eq. DIPEA/4 eq. HOBt (0.5 M) in selected solvent, except for HBTU which was dissolved in DMF. 4 eq. of amino acids (0.5 M) was used. Reaction was carried out for 12 min with micro-wave heating of 70°C or 40°C, depending on the boiling point of the solvent. Reaction times were increased from the fifth amino acid to 20 min. Arginine-coupling was performed in 45°C or 40°C, for 45 min and Cysteine-coupling in room temperature; first one for 1 h and second one for 2 h. Emptying times of the reaction vial was increased from 30 s to 2 min for solvents with higher viscosity, i.e., NBP and Cyrene.

Synthesis of [Asp^5]-vasopressin using DIC/Oxyma as coupling reagents and TentaGel resin were done in; DMF, (1:1) DMF:Cyrene, (2:8) DMF:Cyrene, DOL, NBP, (1:1) 2-Me-THF:Cyrene, (8:2) 2-Me-THF:DOL, (1:1) NBP:EtOAc and (1:1) NBP:2-Me-THF. Synthesis of [Asp^5]-vasopressin using HBTU/DIPEA/HOBt as coupling reagents and TentaGel resin was done in NBP. Synthesis of [Asp^5]-vasopressin using ChemMatrix resin and DIC/Oxyma was done in NBP and DMF.

Synthesis of [Asp^{26}]-Calcitonin

Synthesis of the 32 amino acid long peptide [Asp^{26}]-calcitonin was done on the automated peptide synthesizer Biotage Alstra Initiator with NBP as solvent. The synthesis was done using 0.57 g of TentaGel S RAM resin with loading of 0.22 mmol/g.

Swelling of resin with 4.5 mL NBP in a 10 mL Biotage vial was done for 25 min with micro-wave heating of 70°C.

Removal of Fmoc protecting group for 6 min with 4.5 mL 20% piperidine in NBP and 15 min with 4.5 mL 20% piperidine in NBP. After the deprotection, the resin was washed four times with 4.5 mL NBP.

Coupling reactions were done using 4 equivalents of DIC/Oxyma (0.5 M) in NBP and 4 eq. of amino acids (0.5 M) in NBP. Reaction was carried out for 12 min at 70°C for the first seven couplings. Then reaction times was increased to 15 min for amino acid eight to 16, and from amino acid 17 to 32 reaction times were increased to 20 min. Arginine-coupling was performed in 45°C for 45 min and Cysteine- and Histidine-coupling in room temperature for 1 h – the second Cysteine was left to react for 2 h. Emptying of the reaction vial was done with vacuum for 2 min.

Cleavage off resin

[Asp^5]-Vasopressin

The peptide was cleaved from resin using 15 mL (for 0.57 g resin) or 20 mL (for 0.77-1 g resin) of 95% TFA, 2% TIS, 2% H_2O and 1% EDT, for 1.5-2h. The resin was filtered off and washed with TFA. TFA was evaporated until the peptide precipitated. The peptide was precipitated in cold diethyl ether, centrifuged and the diethyl ether discarded. The peptide was then dissolved in water and freeze-dried.

[Asp^{26}]-Calcitonin

The peptide was cleaved from resin using 20 mL of 95% TFA, 2% thioanisole, 2% H_2O and 1% EDT, for 2h. The resin was filtered off and washed with TFA. TFA was evaporated until the peptide precipitated. The peptide was precipitated in cold diethyl ether, centrifuged and the diethyl ether discarded. The peptide was then dissolved in water and freeze-dried.

A second cleavage of protecting groups was done with 15 mL of 70% TFA, 13.5% thioanisole, 13.5% H_2O and 3% EDT, for 1h 45 min.

Purification

Purification of the synthesized peptides was done using flash chromatography presented in Table 5 using suitable gradient. If the peptide did not reach 95% purity after flash purification, it was further purified using HPLC-PREP presented in Table 6.

Table 5: Flash chromatography set-up

Instrument	Biotage Selekt (Jöns Jacob Berzelius)
Solvent	A: 0.1 % TFA in water B: ACN
Detection	UV 210-254 nm
Flowrate	50 mL/min
Column	Biotage Sfär C18 D Duo 100 Å 30 µm Weight 60 g

Table 6: HPLC Preparative set-up

Instrument	Agilent Technologies 1260 Infinity (Pluto)
Solvent	A: 0.1 % TFA in water B: ACN
Gradient	15-35% B, 20 min – Vasopressin 17-42% B, 25 min – Calcitonin
Detection	UV 220 and 254 nm
Flowrate	12 mL/min
Column	Kromasil 100-5-C18 Size: 21.2 × 250 mm

Analysis

Analysis of synthesized peptides was done using LCMS and HPLC, with set-up presented in Table 7-9.

Table 7: LCMS set-up (1)

Instrument	Agilent 1100 Series (Merrifield)
Solvent	A: 0.1 % TFA in water B: 0.1% TFA in ACN
Detection	UV 220 and 254 nm, and mass spectrometer
Method	5-95% B, 6 min
Flowrate	0.8 mL/min
Column	Symmetry C18 5µm Size: 2.1 × 50 mm

Table 8: LCMS set-up (2)

Instrument	Agilent Technologies 1260 Infinity II (Titan)
Solvent	A: 0.1 % FA in 95% water/5% ACN B: 0.1% FA in ACN
Detection	UV 220 and 254 nm, and mass spectrometer
Method	10-95 % B, 8 min
Flowrate	0.6 mL/min
Column	Agilent Poroshell 120 EC-C18 2.7 µm Size: 3.0 × 50 mm

Table 9: Analytical HPLC set-up

Instrument	Agilent 1100 Series (Moon)
Solvent	A: 0.1 % TFA in water B: 0.1% TFA in ACN
Detection	UV 220 nm
Method	10-80 % B, 16.1 min
Flowrate	1 mL/min
Column temperature	40°C
Column	Aeris 3.6 µm PEPTIDE XB-C18 100 Å Size: 150 × 4.6 mm

References

1. Jadhav, S. et al. Replacing DMF in solid-phase peptide synthesis: varying the composition of green binary solvent mixtures as a tool to mitigate common side-reactions. *Green Chemistry* 23, 3312-3321 (2021).

2. Egelund, P. et al. Fmoc-Removal with Pyrrolidine Expands the Available Solvent Space in Green Solid-Phase Peptide Synthesis. *ACS Sustainable Chemistry & Engineering* 9, 14202-14215 (2021).

3. Ferrazzano, L. et al. Sustainability in peptide chemistry: current synthesis and purification technologies and future challenges. *Green Chemistry* 24, 975-1020 (2022).

4. Jensen, K., Tofteng Shelton, P. & Pedersen, S. *Peptide Synthesis and Applications*. 1-18 (Humana Press, 2013).

5. Thieriet, N., Guibé, F. & Albericio, F. Solid-Phase Peptide Synthesis in the Reverse (N → C) Direction. *Organic Letters* 2, 1815-1817 (2000).

6. REACH - Chemicals - Environment - European Commission. *Ec.europa.eu* (2022). at <https://ec.europa.eu/environment/chemicals/reach/reach_en.htm>

7. Martin, V. et al. Harnessing polarity and viscosity to identify green binary solvent mixtures as viable alternatives to DMF in solid-phase peptide synthesis. *Green Chemistry* 23, 3295-3311 (2021).

8. Martelli, G. et al. Steps towards sustainable solid phase peptide synthesis: use and recovery of *N*-octyl pyrrolidone. *Green Chemistry* 23, 4095-4106 (2021).

9. MacMillan, D., Murray, J., Sneddon, H., Jamieson, C. & Watson, A. Evaluation of alternative solvents in common amide coupling reactions: replacement of dichloromethane and *N,N*-dimethylformamide. *Green Chemistry* 15, 596 (2013).

10. Wilson, K., Murray, J., Jamieson, C. & Watson, A. Cyrene as a bio-based solvent for HATU mediated amide coupling. *Organic & Biomolecular Chemistry* 16, 2851-2854 (2018).

11. Pawlas, J. et al. 2D green SPPS: green solvents for on-resin removal of acid sensitive protecting groups and lactamization. *Green Chemistry* 21, 2594-2600 (2019).

12. Lopez, J., Pletscher, S., Aemissegger, A., Bucher, C. & Gallou, F. *N*-Butylpyrrolidinone as Alternative Solvent for Solid-Phase Peptide Synthesis. *Organic Process Research & Development* 22, 494-503 (2018).

13. Kumar, A., Alhassan, M., Lopez, J., Albericio, F. & Torre, B. *N*-Butylpyrrolidinone for Solid-Phase Peptide Synthesis is Environmentally Friendlier and Synthetically Better than DMF. *ChemSusChem* 13, 5288-5294 (2020).

14. ECHA. *Echa.europa.eu* (2022). at <https://echa.europa.eu/home>

15. Spange, S., Lienert, C., Friebe, N. & Schreiter, K. Complementary interpretation of $E_T(30)$ polarity parameters of ionic liquids. *Physical Chemistry Chemical Physics* 22, 9954-9966 (2020).

16. Sherwood, J. et al. Dihydrolevoglucosenone (Cyrene) as a bio-based alternative for dipolar aprotic solvents. *Chem. Commun.* 50, 9650-9652 (2014).

17. Alfonsi, K. et al. Green chemistry tools to influence a medicinal chemistry and research chemistry based organisation. *Green Chem.* 10, 31-36 (2008).

18. Diorazio, L., Hose, D. & Adlington, N. Toward a More Holistic Framework for Solvent Selection. *Organic Process Research & Development* 20, 760-773 (2016).

19. Solvent Selection Tool - American Chemical Society. *American Chemical Society* (2022). at <https://www.acs.org/content/acs/en/greenchemistry/research-innovation/tools-for-green-chemistry/solvent-tool.html>

20. Prat, D. et al. Sanofi's Solvent Selection Guide: A Step Toward More Sustainable Processes. *Organic Process Research & Development* 17, 1517-1525 (2013).

21. Alder, C. et al. Updating and further expanding GSK's solvent sustainability guide. *Green Chemistry* 18, 3879-3890 (2016).

22. Prat, D. et al. CHEM21 selection guide of classical- and less classical-solvents. *Green Chemistry* 18, 288-296 (2016).

23. Sherwood, J., Parker, H., Moonen, K., Farmer, T. & Hunt, A. *N*-Butylpyrrolidinone as a dipolar aprotic solvent for organic synthesis. *Green Chemistry* 18, 3990-3996 (2016).

24. Bryan, M. et al. Key Green Chemistry research areas from a pharmaceutical manufacturers' perspective revisited. *Green Chemistry* 20, 5082-5103 (2018).

25. Gao, F. et al. Replacement strategies for non-green dipolar aprotic solvents. *Green Chemistry* 22, 6240-6257 (2020).

26. Byrne, F. et al. Tools and techniques for solvent selection: green solvent selection guides. *Sustainable Chemical Processes* 4, (2016).

27. Pace, V., Hoyos, P., Castoldi, L., Domínguez de María, P. & Alcántara, A. 2-Methyltetrahydrofuran (2-MeTHF): A Biomass-Derived Solvent with Broad Application in Organic Chemistry. *ChemSusChem* 5, 1369-1379 (2012).

28. Moscoso, R., Carbajo, J. & Squella, J. 1,3-Dioxolane: A green solvent for the preparation of carbon nanotube-modified electrodes. *Electrochemistry Communications* 48, 69-72 (2014).

29. 1,3-dioxolane. *ECHA Substance Information* (2022). at <https://echa.europa.eu/substance-information/-/substanceinfo/100.010.422>

30. ChemMatrix® Resin for solid phase peptide synthesis. *Sigmaaldrich.com* (2022). at <https://www.sigmaaldrich.com/SE/en/technical-documents/technical-article/protein-biology/protein-labeling-and-modification/chemmatrix-resin>

31. Muttenthaler, M., King, G., Adams, D. & Alewood, P. Trends in peptide drug discovery. *Nature Reviews Drug Discovery* 20, 309-325 (2021).

32. Denton, E. Using double coupling to improve your peptide synthesis. *Selekt.biotage.com* (2022). at <https://selekt.biotage.com/peptideblogs/using-double-coupling-to-improve-your-peptide-synthesis>

33. calcitonin | Definition, Production, & Function. *Encyclopedia Britannica* (2022). at <https://www.britannica.com/science/calcitonin-hormone>

34. Mueller, L., Baumruck, A., Zhdanova, H. & Tietze, A. Challenges and Perspectives in Chemical Synthesis of Highly Hydrophobic Peptides. *Frontiers in Bioengineering and Biotechnology* 8, (2020).

Appendix

Mechanisms

The Fmoc-removal mechanism is presented in Figure A1.[4]

Fmoc - Amino acid - Linker - Resin

Piperidine

Figure A1: The mechanism of Fmoc deprotection using piperidine

The mechanism for the formation of activated ester and coupling of amino acid to resin using DIC/Oxyma is presented in Figure A2.[4]

Fmoc - Amino acid

DIC

O-Acylurea derivative

Oxyma

Peptidyl-resin

Activated ester

N,N'-Diisopropylurea

Figure A2: The mechanism for the formation of an activated ester and coupling of amino acid to resin using DIC/Oxyma.

Raw data

Raw data from resin swelling tests and yield calculations is presented as follows. Last is the HPLC-profile of a sample taken three days in a row. First peak has the molecular weight of 1087.4 m/z ([Asp^5]-vasopressin, $[M+H]^+$) and the second peak a molecular weight of 1197.4 m/z.

Resin swelling

Table A1: Recorded resin swelling in mL/g. The resin used was TentaGel S RAM (0.22 mmol/g) (TG) and H-Rink amide ChemMatrix resin (0.40 mmol/g) (CM). (*) stronger vacuum applied.

Solvent	TG swelling 1 (r.t.)	TG swelling 2 (r.t.)	TG swelling 3 (r.t.)	Avg. TG swelling (r.t.)	TG swelling with heat 1	TG swelling with heat 2	TG swelling with heat 3	Avg. TG swelling with heat	CM swelling (r.t.)
DMF	3.8	2.8	3.8	3.8	4.47			4.47	7.00
1:1 DMF:EtOAc	2.8	3.4		3.1	4.05			4.05	
EtOAc	2.0	2.0		2.0					
1:1 EtOAc:DOL	3.7	3.7		3.7	3.90			3.90	
7:3 EtOAc:DOL	3.3	2.9		3.1	3.46			3.46	
8:2 EtOAc:DOL	1.9	2.8	2.4	2.6	2.36			2.36	
1:1 EtOAc:Cyrene	2.9	2.8		2.9	2.91			2.91	
1:1 EtOAc:2-Me-THF	1.9	2.0		2.0					
1:1 DMF:2-Me-THF	2.9	2.8		2.9	3.88			3.88	
2-Me-THF	1.9	1.9		1.9					
1:1 2-Me-THF:DOL	3.7	3.3		3.5	4.12			4.12	
7:3 2-Me-THF:DOL	3.7	3.4		3.6	3.88			3.88	
8:2 2-Me-THF:DOL	2.9	1.9	2.8	2.9	3.81			3.81	5.22
1:1 2-Me-THF:Cyrene	3.3	3.2		3.3	3.71	3.89		3.80	
1:1 DMF:DOL	3.8	3.8		3.8					
DOL	4.0	3.9		4.0	4.40			4.40	7.15
1:1 DOL:Cyrene	3.8	3.8		3.8	2.98*	4.26	3.96	3.73	
2:8 DOL:Cyrene	2.8	3.8	3.7	3.8	3.27	3.97		3.62	
9:1 DMF:Cyrene	3.9	3.9		3.9					
8:2 DMF:Cyrene	3.9	3.8		3.9					
7:3 DMF:Cyrene	3.6	3.8		3.7					
1:1 DMF:Cyrene	3.9	3.7		3.8	3.86			3.86	7.78
3:7 DMF:Cyrene	2.9	2.9		2.9					
2:8 DMF:Cyrene	2.4	2.8		2.6	3.86			3.86	6.61
1:9 DMF:Cyrene	2.3	2.0		2.2					
Cyrene	1.9	1.9		1.9					
9:1 DMF:NBP	3.7	3.8		3.8					
8:2 DMF:NBP	3.8	3.9		3.9					
7:3 DMF:NBP	3.8	3.8		3.8					
1:1 DMF:NBP	3.8	3.9		3.9					
3:7 DMF:NBP	3.8	3.8		3.8					
2:8 DMF:NBP	3.8	3.8		3.8					
1:9 DMF:NBP	3.8	3.8		3.8					
NBP	3.3	3.8	3.7	3.8	4.68			4.68	5.67

1:1 NBP:Cyrene	3.8	3.7		3.8	4.30			4.30	
1:1 NBP:EtOAc	3.2	2.9		3.1	4.28			4.28	6.86
1:1 NBP:2-Me-THF	2.9	3.7	3.8	3.8	4.30			4.30	5.67
1:1 NBP:DOL	4.1	3.9		4.0					

Yields

Table A2: Weight of TentaGel S RAM resin (0.22 mmol/g) used in synthesis using DIC/Oxyma for each solvent system. Weight of [Asp^5]-vasopressin (MW 1086.4) after final purification, and calculated yield. (*) oxidised product (MW 1084.4).

Solvent	Resin (g)	Final product (mg)	Yield
DMF	0.57	115.5	85.1%
1:1 DMF:Cyrene	1.00	93.0*	39.0%
2:8 DMF:Cyrene	0.77	10.0 (83.1% purity)	-
DOL	0.77	93.9	51.1%
NBP	0.77	162.0	88.2%
1:1 2-Me-THF:Cyrene	0.77	-	-
8:2 2-Me-THF:DOL	0.77	137.6	74.9%
1:1 NBP:EtOAc	0.57	118.0	86.8%
1:1 NBP:2-Me-THF	0.57	118.0	86.8%

Table A3: Weight and loading of the resin used in synthesis for each solvent system. In brackets are the "unique conditions" for that particular synthesis. Weight in mg of [Asp^5]-vasopressin (MW 1086.4) or [Asp^{26}]-calcitonin) (MW 3432.7) after final purification, and calculated yield.

Solvent (unique conditions)	Resin (g)	Loading (mmol/g)	Final product (mg)	Yield
NBP ([Asp^5]-vasopressin, HBTU/DIPEA/HOBt, TentaGel S RAM resin)	0.57	0.22	103.0	75.8%
NBP ([Asp^5]-vasopressin, DIC/Oxyma, H-Rink amide ChemMatrix resin)	0.21	0.40	59.0	64.7%
DMF ([Asp^5]-vasopressin, DIC/Oxyma, H-Rink amide ChemMatrix resin)	0.21	0.40	52.0	59.6%
NBP ([Asp^{26}]-calcitonin, DIC/Oxyma, TentaGel S RAM resin)	0.57	0.22	123.7	28.7%

HPLC profile

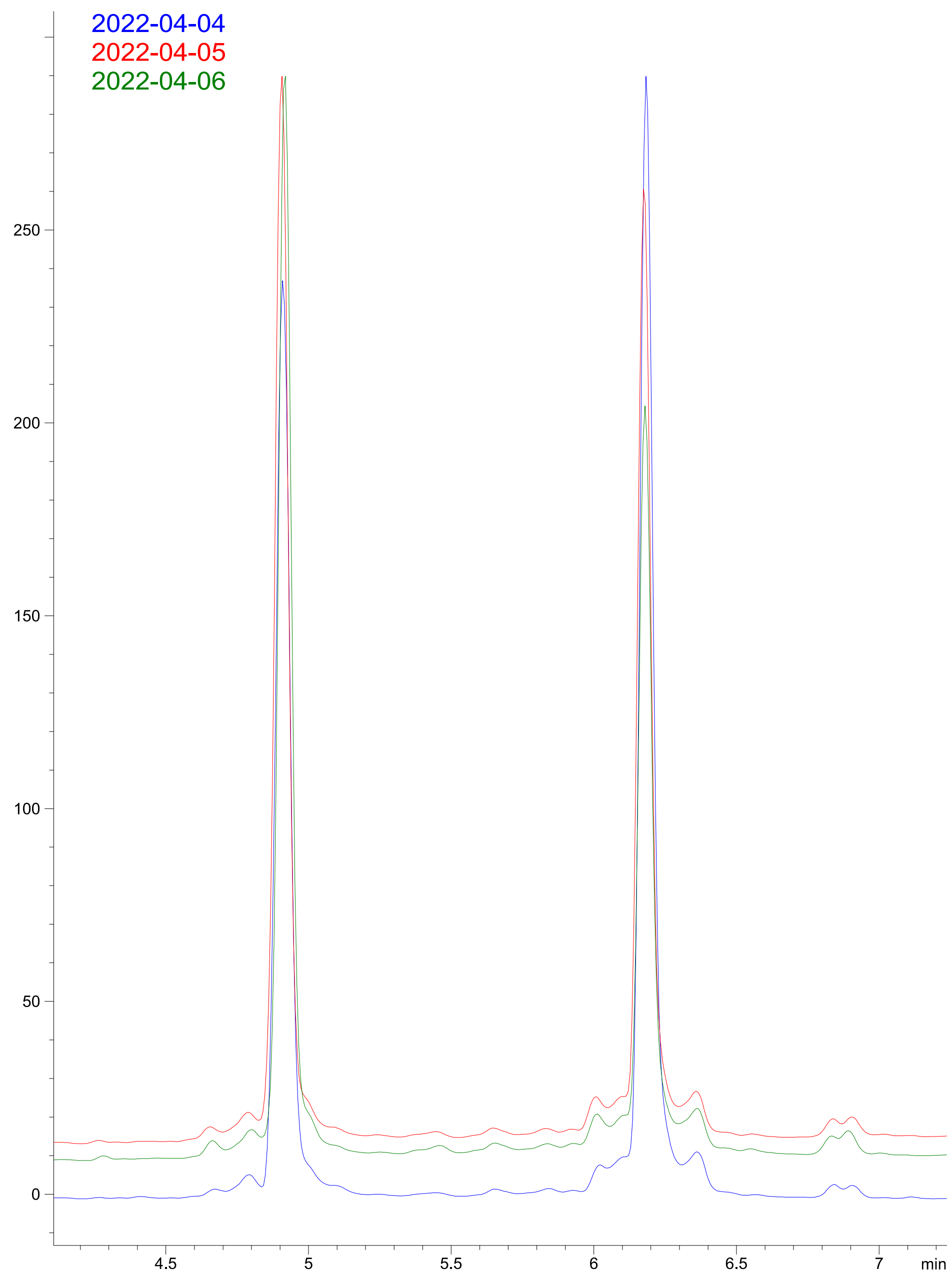

Figure A3: HPLC profile (220 nm) taken of the same sample three days in a row. First peak has the molecular weight of 1087.4 m/z ([Asp^5]-vasopressin, $[M+H]^+$) and the second peak a molecular weight of 1197.4 m/z.