N-Butylpyrrolidinone for Solid-Phase Peptide Synthesis is Environmentally Friendlier and Synthetically Better than DMF

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Solid-phase peptide synthesis (SPPS) is the method of choice for the preparation of peptides in both laboratory scale and large production. Although the methodology has been improved during the last decades allowing the achievement of long peptides and challenging sequences in good yields and purities, the process was not revised from an environmental point of view. One of the main problems in this regard is the large amount of solvents used, and therefore the tons of generated waste. Moreover, the solvent of choice for the SPPS is *N,N*-dimethylformamide (DMF), which is considered as reprotoxic; thus, there is an urgent necessity to replace it with safer solvents. The DMF substitution by a green solvent is not a trivial task, because it should solubilize all the reagents and byproducts involved in the process, and, in addition to

facilitating the coupling of the different amino acids, it should not favor the formation of side-reactions compared with DMF. Herein, it was demonstrated that the use of the green solvent *N*-butylpyrrolidinone (NBP) as a replacement of DMF was beneficial in two well-documented side reactions in peptide synthesis, racemization and aspartimide formation. The use of NBP rendered a lower or equal level of racemization in the amino acids more prone to this side reaction than DMF, whilst the aspartimide formation was clearly lower when NBP was used as solvent. Our findings demonstrate that the use of a green solvent does not hamper the synthetic process and could even improve it, making it environmentally friendlier and synthetically better.

Introduction

In the past, the development of new drugs was dominated mainly by the so-called small molecules. However, the higher selectivity and potency of peptides is now attracting the increasing interest of the pharmaceutical industry.^[1] Given this change in paradigm, the development of environmentally friendlier solid-phase peptide synthesis (SPPS) approaches is

urgent,^[2] since this methodology is the most efficient way to prepare these key molecules. The success of this synthetic strategy lies in a solid polymeric protecting group that allows the use of reagents in excess. These are later removed by simple filtration and extensive washing of the solid support, achieving a quantitative yield. However, to carry out the reactions and washes, large amounts of solvents are required, the most widely used being the hazardous catalogued^[3] DMF (*N,N*-dimethylformamide),^[4] NMP (*N*-methylpyrrolidone),^[5] and DCM (dichloromethane).^[6] In this context, the need to find replacements for these solvents is a priority, as recently pointed out by the ACS Green Chemistry Institute® Pharmaceutical Round Table (GCIPR).^[7] In recent years, our groups and others have focused on the search for green alternatives to the solvents currently in use.^[8] This is not a trivial task since the solvent has to meet several requirements. First, it should swell the resin properly and show good diffusion into it, thus viscosity is an important parameter to be considered. Second, it should have the capacity to dissolve protected amino acids and coupling reagents at manageable concentrations, thereby facilitating the coupling reaction and also fluorenylmethoxycarbonyl (Fmoc) removal, the latter a more demanding reaction than expected.^[9]

As a result of these studies, we have found several alternatives for each chemical step in Fmoc/tBu SPPS. Thus, 2-MeTHF,^[10] γ -valerolactone (GVL),^[11] and *N*-butylpyrrolidinone (NBP) (Tamisolve® NxG)^[12] have proved suitable for the elongation of the peptide chain (coupling and removal of temporary protecting group). When the target peptide is a C-

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terminal carboxylic acid and Wang-resin is used, the incorporation of the first amino acid is possible in either 2-MeTHF^[13] or GVL^[14] while 2-MeTHF is the solvent of choice for 2-chlorotriyl chloride (CTC)-resin.^[15]

Nevertheless, studies on new solvents for SPPS do not usually address the frequent side reactions that occur during the synthetic process,^[16] which are crucial, especially for the production of peptides on a multi-gram scale. In this regard, we have recently reported the minimization of δ -lactam formation during the incorporation of Fmoc-Arg(Pbf)-OH into the peptide chain when NBP is used as solvent.^[17] The high extension of this reaction is detrimental for coupling efficiency, generating sequences with Arg deletions and thus having a high impact on the purity of the final product. However, this is not the only obstacle to tackle during SPPS. Despite great progress in peptide synthesis with respect to resins, coupling reagents, and protecting groups, unsolved side reactions such as racemization^[18] and aspartimide formation continue to be an issue.^[19] These two reactions trouble the large-scale production of peptides since the by-products obtained are often difficult to detect (same molecular weight and similar polarity). Also, in the case of aspartimide formation, often results in poor yield or even unaffordable sequences. Thus, in light of the importance and economic impact of these reactions and given that NBP has emerged as a suitable substitute for DMF, here we analyzed and compared the racemization and aspartimide formation in SPPS using the solvents DMF and NBP.

Results and Discussion

Among the green solvents mentioned in the introduction, NBP looks very appealing because of its chemical structure resemblance mostly to the NMP, which is one of the most used solvents in SPPS. NBP is a highly boiling point (243 °C), dipolar aprotic solvent, considered non-toxic because it has been identified as being non-mutagenic (OECD 471), non-reproductively toxic (OECD 414), and potentially biodegradable (OECD 302B).^[20] On the other hand, when orally administered in rats, NBP shows more acute toxicity than NMP (LD₅₀ rat oral, 300–2000 mg kg⁻¹ vs. $\approx$ 4000 mg kg⁻¹).^[20a] Although its price in bulk (8.5 € kg⁻¹) is still more expensive than DMF (1.5 € kg⁻¹), the increase in its consumption suggest that the difference between both prices will shorten as happens with all commodities.

As it has been pointed above, to consider a solvent for SPPS it should accomplish some requirements. One of them is the ability to solubilize at least the most current protected amino acids and coupling reagents as well. Table 1 shows that NBP is able to solubilize all Fmoc-protected natural amino acids, the coupling additives and COMU^[21] please define^[22] in a concentration range higher than the standard used in SPPS.

Furthermore, the swelling of the polystyrene and ChemMatrix resins are slightly inferior in NBP than in DMF, but of the same order as it is showed in Table 2. Interestingly, the polystyrene in NBP reach the maximum swelling after 12 h showing the role of the viscosity in this physical parameter.

Table 1. Solubility of reagents (Fmoc-AA-OH and additives/coupling reagents) in NBP at RT.^[a]

Reagent	Solubility [m]
Fmoc-Ala-OH	> 0.8
Fmoc-Cys(Trt)-OH	> 0.8
Fmoc-Asp(OtBu)-OH	> 0.8
Fmoc-Glu(OtBu)-OH	> 0.8
Fmoc-Phe-OH	> 0.8
Fmoc-Gly-OH	> 0.8
Fmoc-His(Trt)-OH	> 0.8
Fmoc-Ile-OH	> 0.8
Fmoc-Lys(Boc)-OH	> 0.8
Fmoc-Leu-OH	> 0.8
Fmoc-Met-OH	> 0.8
Fmoc-Asn(Trt)-OH	0.54
Fmoc-Pro-OH	> 0.8
Fmoc-Gln(Trt)-OH	0.72
Fmoc-Arg(Pbf)-OH	> 0.8
Fmoc-Ser(tBu)-OH	> 0.8
Fmoc-Thr(tBu)-OH	> 0.8
Fmoc-Val-OH	> 0.8
Fmoc-Trp(Boc)-OH	> 0.8
Fmoc-Tyr(tBu)-OH	> 0.8
HOAt	> 0.8
HOBt	> 0.8
OxymaPure	> 0.8
COMU	0.33
HATU	0.11
HBTU	0.07

[a] AA: amino acid. ^[21] please define HOAt, HOBt, HATU, and HBTU^[22]

Table 2. Swelling of resins in NBP and DMF at RT.^[a]

Resin	Time [h]	NBP [mL g ⁻¹]	DMF [mL g ⁻¹]
Fmoc-Rink amide AMPS	1	3.25	4.25
	3	3.75	4.75
	12	5.25	4.75
H-Rink amide ChemMatrix	1	5.25	5.75
	3	5.25	5.75
	12	5.25	5.75

[a] ^[21] please define AMPS^[22]

Racemization study

In SPPS, racemization occurs mainly during the coupling step when the α -carboxylic group of the incoming protected amino acid is activated. There are two plausible mechanisms, one through the formation of an enol intermediate and the other through an oxazolone (Scheme 1). The rate of racemization depends on many factors, such as the amino acid side chain, the coupling reagents used, the presence of base, and also the solvent.^[21]

Of the 20 proteinogenic amino acids, it is recognized that Ser, Cys, and His are the residues most prone to racemization,^[22] and therefore we focused on these. To quantify the presence of diastereomers, the sequences H-Gly-Xxx-Phe-NH₂, where Xxx was Ser(tBu), Cys(Trt), ^[21] please define Trt^[22] or His(Trt), were synthesized under distinct conditions (discussed below), but in all cases using 1.5 equiv. of reagent and 2 h of coupling reaction using DIC^[21] please define DIC^[22]/OxymaPure as coupling reagents, since they are known as racemization

Scheme 1. Racemization pathways during amide bond formation.

minimizer.^[23] To ensure the identification of the DL $\blacksquare\blacksquare$ please define DL $\blacksquare\blacksquare$ peptides, the diastereomers were previously synthesized and the best elution method for each DD $\blacksquare\blacksquare$ please define DD $\blacksquare\blacksquare$ /DL pair was adjusted (Figures S1–S18).

The first set of syntheses was carried out in DMF or NBP, at RT with a Fmoc-amino acid pre-activation time of 3 min. After cleavage, the crude peptides were analyzed by HPLC, and the amount of DL peptide was quantified by integration of the peaks. Table 3, entry 1 shows that under these conditions, no racemization was detected in case of Cys, very low racemization for Ser, and the highest values were observed for His, as expected. Moreover, these results indicated that the percentage of racemization was lower when NBP was used during the coupling step. To confirm this observation, a new set of syntheses was done with temperature being the only parameter changed. The whole process was conducted at 45 °C because previous studies by our groups showed this temperature to be optimal when NBP is used.^[17] As expected, an increment in temperature increased the racemization ratio, mostly for the case of His (Table 3, entry 2). Data analysis confirmed the previous results, namely the epimerization tendency was Cys < Ser < His, and epimerization was significantly lower when NBP was used. This is very clear again for the His.

Table 3. Percentage of epimerization of Ser, Cys, and His under different conditions.^[a]

Entry	Coupling condition	Solvent	H-GsF-NH ₂ [%]	H-GcF-NH ₂ [%]	H-GhF-NH ₂ [%]
1	pre-activation, RT	DMF	0.18	ND	2.71
		NBP	ND	ND	2.49
2	pre-activation, 45 °C	DMF	1.42	ND	19.53
		NBP	0.23	ND	7.24
3	in situ activation, 45 °C	DMF	0.14	ND	8.83
		NBP	0.11	ND	3.93

[a] ND: Not detected

Finally, another set of syntheses was performed in which the temperature was kept at 45 °C but no pre-activation step was carried out. In this case, a solution mixture of Fmoc-amino acid and OxymaPure was added first, followed by the required amount of DIC (in situ activation). Under these conditions (Table 3, entry 3), the racemization values dropped off close to those obtained at RT with pre-activation (Table 3, entry 1), thereby indicating that pre-activation at 45 °C conditions (Table 3, entry 2) should not be carried out. As in the two previous assays, the use of NBP showed to be beneficial to minimize the amino acids racemization.

To favor fair competition between peptide bond formation and racemization, the rationale after the use of this in situ activation was to minimize the time that the carboxylic group is activated in the absence of the nucleophile (peptide resin), because these conditions only racemization can occur.

Aspartimide formation

Aspartimide formation is a well-known side reaction in SPPS.^[19] It involves cyclization by nucleophilic attack of the α -nitrogen of the preceding residue over the carboxylic carbon of the Asp side chain. The reaction extension depends on the residue located at the C-terminus of Asp (Asp-AA). When the amino acid is Gly there is a greater tendency toward aspartimide formation because it is the least hindered residue.^[24] Although aspartimide formation was reported early under the *tert*-butoxycarbonyl (Boc)/Bzl $\blacksquare\blacksquare$ please define Bzl $\blacksquare\blacksquare$ methodology in acidic conditions,^[25] it became dramatic when the use of Fmoc/*t*Bu methodology became widely extended.^[26] Under basic conditions, aspartimide formation occurs easily during piperidine treatment to remove the Fmoc group, and it is accumulative along the synthetic cycles. Therefore, the greater the number of cycles after addition of the Asp residue, the greater the formation of aspartimide. Nevertheless, the biggest issue with this side reaction is the high number of by-products that can be generated. On the one hand, the aspartimide form

is prone to racemization, and on the other, it is susceptible to nucleophilic attack at both carbonyl groups, leading to the α - and β -peptide derivatives (with water, α - and β -Asp peptides, and with piperidine, α - and β -piperidides) many times difficult to isolate from the desired sequence (Scheme 2).^[27]

Many efforts have been channeled into strategies to prevent aspartimide formation during the synthetic process. In this regard, work has led to the development of different side chain protecting groups for Asp^[26b,28] or backbone protecting groups for the previous residue NH.^[29] However, none of these approaches has been successful to date.^[26b] Beside the basicity, it has been also demonstrated that the solvent plays a key role in determining the extent of aspartimide formation. In general, this side reaction is reduced when non-polar solvents are used during the treatments with bases.^[30] Therefore, to study and quantify aspartimide formation in NBP compared with DMF, we synthesized the sequence H-Ala-Lys-Asp-Gly-Tyr-Ile-NH₂ (1), corresponding to fragment 1–6 of the toxin II from scorpion *Androctonus australis Hector*, since it is one of the most widely used model for this purpose.^[30]

First, two simultaneous syntheses of sequence (1)/peptide A were carried out under the same conditions, one using DMF and the other NBP. The whole synthetic procedure was performed at 45 °C because it is the optimal temperature for NBP as this solvent has greater viscosity than DMF. For coupling, an equimolar amount of Fmoc-AA-OH, DIC and OxymaPure at a ratio of 1.5 equiv. with respect to the peptide resin was allowed to react for 2 h. Fmoc removal was achieved by treatment with a 20% piperidine solution in the respective solvent for 7 min. At the same time and following a standard protocol at RT, the β -peptide [H-Ala-Lys-Asp (Gly-Tyr-Ile-NH₂)-OH, 1B] was synthesized as reference to recognize this impurity on the crude peptides. Thus, after cleavage, the crude peptides obtained were analyzed by HPLC (Figure 1) in a smooth linear gradient method, from 5 to 20% of B in 15 min, which allowed the resolution of the different components in the mixtures, namely α -peptide, β -peptide, and aspartimide, although not their respective epimers. Comparison of the three HPLC profiles revealed that the syntheses of the α -

Figure 1. HPLC profile: A) peptide (1) synthesized in DMF at 45 °C; B) peptide (1B) synthesized in DMF at RT; C) peptide (1) synthesized in NBP at 45 °C.

and β -peptides ran in DMF rendered poorer quality crude peptides than those performed in NBP. In the case of the α -peptide synthesized in DMF, a content of 7% of β -peptide and a small peak (< 1%) identified as aspartimide (Asi, in Figure 1)

Scheme 2. Aspartimide formation and resulting by-products formed during the Fmoc/tBu synthetic strategy.

were observed. Interestingly, the same kinds of impurities were found in the β -peptide synthesis, it means the α -peptide counterpart and the aspartimide, but in reverse proportion 1 and 7% respectively. This difference might suggest the lowest stability of the aspartimide ring at higher temperature, 45 °C versus RT. In contrast, the chromatogram corresponding to the synthesis using NBP did not show any of these impurities. This observation indicates the capacity of this solvent to minimize aspartimide formation. Additionally, an extra peak, identified as desGly peptide (* in Figure 1), was detected in the crude corresponding to α -peptide in DMF. This peak was negligible when NBP was used as solvent.

After this encouraging result, we studied aspartimide formation under stress conditions. To this end, four equal portions of resin-bound peptide from the synthesis using NBP were taken and aspartimide formation was forced by a 4 h treatment with a 20% piperidine solution in DMF at RT and at 45 °C, as well as with a 20% piperidine solution in NBP at RT and at 45 °C. These treatments simulate more than 30 deprotection cycles of 7 min. After cleavage, the crude peptides obtained were analyzed by HPLC (Figure 2). To ensure the maximum resolution of the components in the mixture, the same smooth linear gradient method as before was used, namely 5 to 20% of B in 15 min, but extended for 2 min at 20% of B to allow elution of the piperidide derivatives. These

analyses showed that at RT, after 4 h of treatment with a 20% piperidine solution in DMF, the conversion of α -peptide to piperidide derivatives was moderate, around 15%. However, interestingly, when the solution of piperidine was in NBP almost no piperidides were detected. The same trend was observed at 45 °C. Of note, in DMF, the conversion to piperidides and β -peptide was > 65% whilst in NBP it was only around 30%.

As mentioned previously, the polarity of the solvent greatly influences aspartimide formation: the higher the polarity of the solvent, the greater the rate of aspartimide formation. With regard to the polarity of the two solvents addressed in this study, our results are consistent with this general rule, since the value for DMF on the Reichardt polarity scale^[31] is 0.386,^[31] which is significantly higher than that reported for NBP (0.336).^[20a]

Conclusions

Previous reports^[12,17] demonstrated that *N*-butylpyrrolidinone (NBP) had the necessary characteristics to replace *N,N*-dimethylformamide (DMF) and *N*-methylpyrrolidone (NMP) as solvent for the elongation of the peptide chain in solid-phase peptide synthesis (SPPS), including the advantage of not being reprotoxic. The results from the present study reinforce NBP as a promising candidate for this purpose. This solvent caused a

Figure 2. HPLC profile of aspartimide formation under forced conditions: 4 h treatment with a 20% piperidine solution in DMF or NBP at RT and 45 °C compared with peptide (1) synthesized in NBP.

lower racemization rate than DMF in all the scenarios tested: Ser, Cys, and His at different temperatures and distinct coupling strategy, pre-activation or in situ activation. This is due probably to the lower polarity of NBP compared to DMF. As it occurs when DMF is used, the incorporation of protected His in NBP must be optimized for each sequence. Furthermore, and because again of its lower polarity, NBP prevented aspartimide formation. In this regard, even under forced conditions in the presence of 20% piperidine at 45 °C, NBP produced 50% fewer by-products, piperidides, and β -peptide than DMF. In all the cases discussed in this work, NBP has been showed superiority in front of DMF for reducing the side-reaction formation. This reinforces the use of NBP as substitute of DMF in SPPS and complements the findings in the same direction of other groups. Thus, Pawlas and co-workers^[32] have recently demonstrated that NBP could reduce the reaction of DIC with OxymaPure which could render hydrogen cyanide previously described by Kolis and co-workers.^[33] Based on our own work and other carried out by other research groups, we conclude, once again, that the use of green solvents in SPPS does not hamper the synthetic process but might even enhance it, as has been demonstrated herein.

Experimental Section

All reagents and solvents were purchased from commercial suppliers and used without further purification. Fmoc amino acids, Fmoc-Rink Amide AM resin (loading 0.74 mmol g⁻¹) was supplied by Iris Biotech. NBP (TamiSolveNxG) was purchased from Eastman, OxymaPure and DIC were a gift from Luxembourg Biotech. Piperidine and 4-methylpiperidine were supplied by Sigma-Aldrich. DMF, other organic solvents, and HPLC-quality acetonitrile (CH₃CN) were purchased from Merck. Milli-Q water was used for RP-HPLC analyses. Analytical HPLC was performed on an Agilent 1100 system using a Phenomenex AerisTMC18 (3.6 μ m, 4.6 $\times$ 150 mm) column, with a flow rate of 1.0 mL min⁻¹ and UV detection at 220 nm. Chemstation software was used for data processing. Buffer A: 0.1% trifluoroacetic acid (TFA) in H₂O; buffer B: 0.1% TFA in CH₃CN. LC-MS was performed on a Thermo Scientific Dionex UltiMate 3000 using Phenomenex AerisTMC18 (3.6 μ m, 4.6 $\times$ 150 mm) column. Buffer A: 0.1% formic acid in H₂O; buffer B: 0.1% formic acid in CH₃CN.

General SPPS: All peptides were synthesized on a Rink amide AM resin (loading 0.74 mmol g⁻¹) manually in polypropylene syringes fitted with a polyethylene porous disc, following a Fmoc/tBu strategy using DIC/OxymaPure as coupling method for 2 h. Fmoc was removed by treatment with a solution of 20% of piperidine or 4-methylpiperidine in NBP or DMF (1 $\times$ 7 min). Before and after removal and coupling reactions, the resin was washed with NBP or DMF (4 $\times$ 1 min). The whole process was carried out at RT or 45 °C, specified in each case. Global deprotection and cleavage were done with TFA-triisopropylsilane (TIS)-H₂O (95:2.5:2.5) for 1 h at RT, and after filtration, the peptide was precipitated with cold ether. After centrifugation, the solvent was removed by decantation and further cold ether was added, and the whole process was repeated three times. Finally, the peptide was dissolved in water and lyophilized.

Solubility: 1 mL of NBP was added to 0.1 mmol of either the protected amino acid/coupling reagent (reagent) and stirred until dissolution. Then, successive addition of 0.1 mmol of the reagent was added with stirring until to reach at 0.8 M concentration or the

compound was not able to be solubilized. In this case, successive additions of 0.1 mL of NBP were added with stirring until total solubilization.

Resin swelling:^[10b] Resins (200 mg) were placed in a 5-mL syringe, treated with enough solvent to swell the resin, and allowed to stand for 5 min. The swollen resin was compressed with the piston until no more solvent could be extracted. The piston was pulled slowly until the resin recuperated its maximum volume in the syringe, and the volume of the resin was read (the void volume of the tip and the syringe was averaged to 0.15 mL). The swelling was calculated according to the following formula: (volume of the swelled resin–0.15 mL)/0.2 g = $\times$ [mL g⁻¹]. **please check formula**

Racemization study: Fmoc removal was carried out with 4-methylpiperidine. Pre-activation coupling: Fmoc-AA–OH/DIC/OxymaPure (1:1:1, 1.5 equiv.) was dissolved in NBP or DMF, and after 3 min the solution was added to the peptide resin (already swelled with the corresponding solvent) and left to react for 2 h. In situ activation: first Fmoc AA–OH/OxymaPure (1:1, 1.5 equiv.) was dissolved in the corresponding solvent, added to the peptide resin, and heated at 45 °C. DIC (1.5 equiv.) was then added and left to react for 2 h.

Crude peptides were analyzed by HPLC. HPLC gradients were: 0–40% B into A in 15 min for H–GCF–NH₂, 0–30% B into A in 15 min for H–GSF–NH₂, and 5–20% B into A in 15 min for H–GHF–NH₂. **please define GCF, GSF, and GHF**

Aspartimide formation study: Peptides 1 and 1B were synthesized in DMF or NBP as previously described, using the in situ activation coupling method and piperidine for Fmoc removal. The resin for peptide 1 was kept with 20% piperidine in DMF/NBP for 4 h at RT or at 45 °C with stirring. It was then washed with the respective solvent and dried. Peptides were cleaved from the resin and analyzed by HPLC as gradient 5–20% B into A in 15 min. t_R (β -peptide) = 7.22 min, m/z = 665.38 [M + H], t_R (α -peptide) = 7.51 min, m/z = 665.38 [M + H], t_R (piperidide) = 13.00, 13.23, 14.81, 15.52 min, m/z = 732.47 [M + H] (Figure S19).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: aspartimide formation • green solvent • N-butylpyrrolidinone • racemization • solid-phase peptide synthesis

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FULL PAPERS

A greener route: Solid-phase peptide synthesis suffers from the required large amounts of *N,N*-dimethylformamide (DMF) solvent. Here, DMF is replaced with the green solvent *N*-butylpyrrolidinone (NBP), which is beneficial in two well-documented side reactions in peptide synthesis, racemization and aspartimide formation. The use of NBP renders a lower or equal level of racemization in the amino acids more prone to this side reaction than DMF, and the aspartimide formation is clearly lower when NBP is used as solvent.

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***N*-Butylpyrrolidinone for Solid-Phase Peptide Synthesis is Environmentally Friendlier and Synthetically Better than DMF**

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