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Expanding the scope of sustainable peptide synthesis through post-linear synthesis reactions

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The replacement of *N*,*N*-dimethylformamide (DMF) in solid-phase peptide synthesis (SPPS) is critical for improving the sustainability and safety of peptide research. While *N*-butyl-2-pyrrolidinone (NBP) has emerged as a viable alternative for linear peptide synthesis, its applicability to post-linear synthesis modifications remains underexplored. Here, we demonstrate the use of NBP as an alternative solvent for key on-resin transformations required in peptidomimetic synthesis. Orthogonal protecting group strategies were successfully implemented, enabling selective removal of Cys(SIT), Cys(Acm), Cys(Trt), and Lys(Dde) under mild conditions. Notably, SIT deprotection achieved 92% conversion in a single 4-hour treatment, while Acm and Trt were selectively removed using iodine or *N*-halosuccinimides, respectively. Lys(Dde) was efficiently cleaved in the presence of Cys(Acm) using 4% hydrazine in NBP. Furthermore, on-resin cyclisation *via* disulfide bond and 1,4-disubstituted triazole formation proceeded effectively in NBP, as did N-terminal acetylation under mild conditions. These findings establish NBP as a practical and greener alternative to DMF for diverse post-linear synthesis modifications, supporting the development of sustainable SPPS protocols.

Introduction

The development of solid phase peptide synthesis (SPPS) is used to access a wide variety of peptidomimetic structures through the addition of natural and synthetic amino acid moieties. Currently, *N*,*N*-dimethylformamide (DMF) is the gold standard solvent associated with SPPS, providing optimal swelling of the solid support and solvation of reagents at high concentrations.[1] However, DMF is a known liver toxin in addition to being a reproductive hazard, with evidence indicating a high risk to foetal development.[2,3] Subsequently, regulations sanctioned by the European Commission in 2023 have imposed DMF use restrictions; mandating lower levels of short and long term exposures.[4] Thus, there is a requirement for safer solvent alternatives compatible with SPPS.[5]

For a solvent to be suitable for SPPS, it must meet several key criteria: (i) provide adequate swelling of the solid support, (ii) effectively solubilise SPPS reagents, and (iii) maintain compatibility with coupling and deprotection chemistries.[1] However, to be considered as a long-term DMF replacement, the solvent(s) must also comply with the safety standards set by the European Chemicals Agency under the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation.[6] Beyond DMF, a number of solvents and binary solvent systems have been identified that satisfy these requirements.[7–12]

Binary solvent systems allow for the adjustment of chemical and physical features of solvents such as viscosity or polarity.[11,13,14] Of recent note, a binary system of EtOAc/DMSO implemented by Polypeptide, BaChem, and Novo Nordisk has proven successful in synthesising longer peptide sequences.[10,15]

For singular solvents, *N*-butyl-2-pyrrolidinone (NBP) has drawn attention in several studies as a non-reprotoxic and biodegradable alternative to DMF (Fig. 1).[16] NBP was found to limit side reactions common to linear SPPS, such as racemisa-

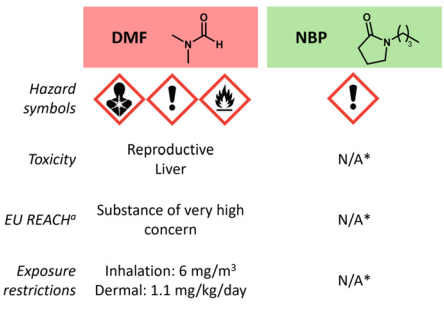

Fig. 1 Comparison of hazard symbols, toxicity and regulation surrounding DMF and NBP. [a] European Union Registration, Evaluation, Authorisation and Restriction of Chemicals. *Not applicable at time of writing Oct 2025.

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tion and aspartimide formation, becoming a top candidate for the synthesis of short peptides.[17,18] However, potential drawbacks include a high boiling point and viscosity, while also being prone to peroxide formation.[19] In order to reduce the viscosity and cost associated with NBP, binary solvent mixtures of NBP with EtOAc or DOL have also been investigated.[10,20]

Thus far, solvent investigations have focussed on the linear synthesis of peptides. While this is an essential advancement towards sustainable technologies, further investigations into post-linear synthesis modifications, to the best of our knowledge, have not been published. Orthogonal protecting group removal, subsequent peptide functionalisation and other post-synthesis modifications are typically achieved utilising DMF.[21–23] Therefore, methodologies to facilitate on-resin peptidyl transformations, abating DMF, are essential for achieving completely sustainable SPPS protocols.

We sought to develop new methods for the post-linear synthesis modifications of peptides using a non-reprotoxic solvent. As this study was not focussed on automated linear SPPS, we elected to investigate a singular solvent rather than one of the previously mentioned binary systems. Despite the potential drawbacks of NBP, our main research interest is in relatively short peptides and so we elected to expand the reaction profile of NBP.

Herein, we describe the use of NBP as a replacement for DMF in several important post-synthesis modifications, including the selective deprotection of orthogonal cysteine and lysine protecting groups, N-terminal capping and on-resin cyclisation methods, *via* disulfide bond or 1,4-triazole formation. Each reaction was optimised for highest conversion to product while also considering the 12 principles of green chemistry.[24,25]

Experimental

General procedure for peptide synthesis

Peptides were synthesised batchwise as required on a 0.3 mmol scale using Fmoc-rink amide AM resin (0.53 mmol g^{-1} loading, 0.566 g). Resin swelling was achieved in NBP (5 mL) for 1 hour. Peptides were elongated in cycles of amino acid coupling followed by Fmoc removal. Fmoc-protected amino acid (3 equiv., 0.1 M in NBP) coupling was achieved by treatment with DIC (3 equiv., 0.1 M in NBP) and Oxyma (3 equiv., 0.1 M in NBP) at r.t. for 2 × 40 min. Fmoc removal was achieved by treatment with 20% piperidine in NBP (8 mL, v/v/v) at r.t. for 1 × 5 min followed by r.t. 1 × 15 min. The resin was washed with NBP following Fmoc removals (2 × 5 mL), MeOH (2 × 5 mL), and CH_2Cl_2 (2 × 5 mL), and after coupling NBP (2 × 5 mL).

General procedure for SIT deprotections

The peptidyl resin (1 equiv.) was swollen in NBP (200 μL) at 50 °C for 20 min. The peptide was cooled to room temperature and treated with a solution of DTT (15 mg, 0.1 mmol, 10 equiv.) dissolved in 4% DIPEA, 4% H_2O in NBP (1 mL, v/v/v) for 4 hours. Following DTT treatment, the resin was thoroughly washed with NBP (3 × 1 mL), MeOH (3 × 1 mL), CH_2Cl_2 (3 × 1 mL), and dried under vacuum.

General procedure for I_2 Trt/Acm removal

The peptidyl resin (1 equiv.) was swollen in NBP (200 μL) at 50 °C for 20 min. The peptide was cooled to room temperature and treated with a solution of iodine (10 mg, 0.04 mmol, 4 equiv.) dissolved in NBP (200 μL) and $Na_2S_2O_8$ (5 mg, 0.02 mmol, 2 equiv.) dissolved in NBP (200 μL) for 1 hour. Following iodine treatment, the resin was thoroughly washed with NBP (3 × 1 mL), MeOH (3 × 1 mL), CH_2Cl_2 (3 × 1 mL), and dried under vacuum.

General procedure for *N*-halosuccinimide Trt/Acm removal

The peptidyl resin (1 equiv.) was swollen in NBP (200 μL) at 50 °C for 20 min. The peptide was cooled to room temperature and treated with a solution of *N*-halosuccinimide (NCS, NBS or NIS) (4 mg, 0.022 mmol, 2.2 equiv.) dissolved in 0.5% TFA, 20% H_2O in NBP (600 μL, v/v/v) for 30 min. Following NBS treatment, the resin was thoroughly washed with NBP (3 × 1 mL), MeOH (3 × 1 mL), CH_2Cl_2 (3 × 1 mL), and dried under vacuum.

General procedure for Lys(Dde) removal

The peptidyl resin (1 equiv.) was swollen in NBP (200 μL) at 50 °C for 20 min. The peptide was cooled to room temperature and treated with a solution of 4% NH_2NH_2 in NBP (1 mL, v/v) for 3 × 3 min. Following NH_2NH_2 treatment, the resin was thoroughly washed with NBP (3 × 1 mL), MeOH (3 × 1 mL), CH_2Cl_2 (3 × 1 mL), and dried under vacuum.

General procedure for *N*-chlorosuccinimide disulfide formation

The peptidyl resin (1 equiv.) was swollen in NBP (200 μL) at 50 °C for 20 min. The peptide was cooled to room temperature and treated with a solution of *N*-chlorosuccinimide (2 equiv.) in NBP (800 μL) for 2 × 15 min. Upon reaction completion, the resin was thoroughly washed with NBP (3 × 1 mL), MeOH (3 × 1 mL), CH_2Cl_2 (3 × 1 mL), and dried under vacuum.

General procedure for 1,4-triazole formation

The peptidyl resin (1 equiv.) was swollen in NBP/DMSO, 9 : 1, (200 μL, v/v) at 50 °C for 20 min. To this, copper iodide (0.9 equiv.), DIPEA (5 equiv.), 2,6-lutidine (5 equiv.), NaAsc (1.5 equiv.) and TBTA (1.2 equiv.) in NBP/DMSO, 9 : 1 (800 μL, v/v) was added and the reaction heated (60 min, 55 °C). Upon reaction completion, the resin was washed with NBP (3 × 1 mL), MeOH (3 × 1 mL), CH_2Cl_2 (3 × 1 mL), and dried under vacuum.

General procedure for N-terminal capping

The peptidyl resin (1 equiv.) was swollen in NBP (200 μL) at 50 °C for 20 min. The peptide was cooled to room temperature. To this acetic anhydride (3 equiv.) and DIPEA (6 equiv.) in NBP (800 μL) were added, and the reaction was agitated at r.t. for 1 hour. Upon reaction completion, the resin was thoroughly washed with NBP (3 × 1 mL), MeOH (3 × 1 mL), CH_2Cl_2 (3 × 1 mL), and dried under vacuum.

Results and discussion

Synthesis of linear model peptides

To investigate a variety of post-synthesis modifications, model peptides with the generalised sequence *LeuTyrArgAlaLys*, where * represents a varied amino acid, were synthesised (Fig. 2).[17] The choice of the model peptide was based on the LYRAX sequence reported by Hackeng *et al.*, where X in this case is a Lys to allow for side chain protecting group variability.[26] The peptides investigated herein were synthesised manually on a 0.3 mmol scale using a Fmoc SPPS strategy. The resin was swollen with NBP (5 mL) at room temperature for 1 hour. Fmoc protecting groups were removed with two treatments of 20% piperidine in NBP (5 and 15 min). Standard amino acids were coupled twice, employing 3 equivalents of amino acid, DIC and Oxyma (2 × 45 minutes) at a final concentration of 0.1 M. Arginine couplings were performed for extended time periods (2 × 1 hour) and unnatural amino acids were coupled once for 3 hours. Unless otherwise stated, the N-terminal Fmoc remained intact for orthogonal protecting group investigations using NBP. Peptides **1** and **2** contain sec-isoamyl mercaptan (SIT) and triphenylmethyl (Trt) protected cysteines respectively in the varied position, and all other amino acids utilise standard acid-labile protecting groups. Peptide **3** contains acetamidomethyl (Acm) protected cysteines in the variable position along with a 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protected lysine in place of the standard *tert*-butoxycarbonyl (Boc) protection. Finally, an N-terminal propargylglycine (Pra) residue and a C-terminal azidohomoalanine (Aha) residue constitute peptide **4**. The crude purity of all linear peptides was determined by analytical HPLC to be greater than 90%.

Orthogonal peptidyl transformations using sustainable solvent

Sustainable removal of Cys(SIT) protecting group. A widely used strategy for cysteine protection involves masking the free thiol group *via* an acid- and base-stable disulfide bond. These protecting groups are typically removed using thiol-based reducing agents such as 1,2-ethanedithiol (EDT) or dithiothreitol (DTT). An example of this class, sec-isoamyl mercaptan (SIT), was recently reported by Chakraborty *et al.*[27]

Standard SIT removal is performed in DMF employing 5 equivalents of DTT per SIT-protected cysteine in a DMF/DIPEA/H_2O mixture (92 : 4 : 4, v/v/v) for three 20 minute treatments.[27] After confirming the miscibility of water with NBP, SIT removal was attempted utilising NBP while maintaining the ratio of cleavage cocktail components.[28]

Treatment of peptide **1** under these conditions resulted in near-complete consumption of the starting material, with two major products observed: the desired product **5** and the single SIT deprotected peptide, in a 7 : 56 : 37 ratio (Fig. 3). To improve the efficiency of the reaction, the peptide was treated with the deprotection solution in a single step for one hour. This approach yielded a comparable conversion to product **5** (47%). Encouraged by this result, the reaction was extended to 2 and 4 hours using a single set of reagents. Treatment for 2 hours resulted in an 82% conversion to product **5**, with no starting material peak observed by RP-HPLC and only one of the SIT group positions being harder to remove (Fig. 3C). Extending the treatment of peptide **1** with the deprotection cocktail to 4 hours afforded the highest conversion, yielding 92% of the desired product **5**.

Sustainable disulfide bond formation using Cys(Acm) and Cys(Trt). The acetamidomethyl (Acm) protecting group is commonly employed in on-resin disulfide bond formation as removal can be coupled with peptide cyclisation in a concerted manner. Acm displays orthogonality to multiple cysteine protecting groups, facilitating selective disulfide bond formation

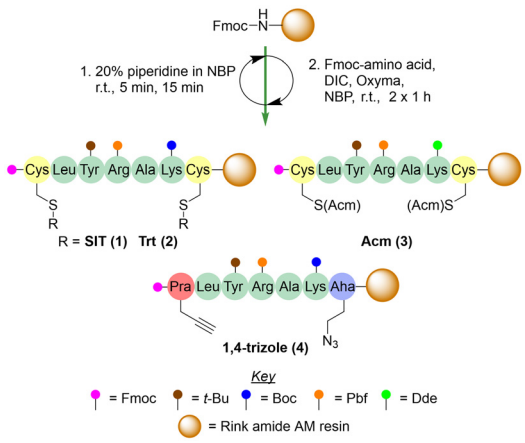
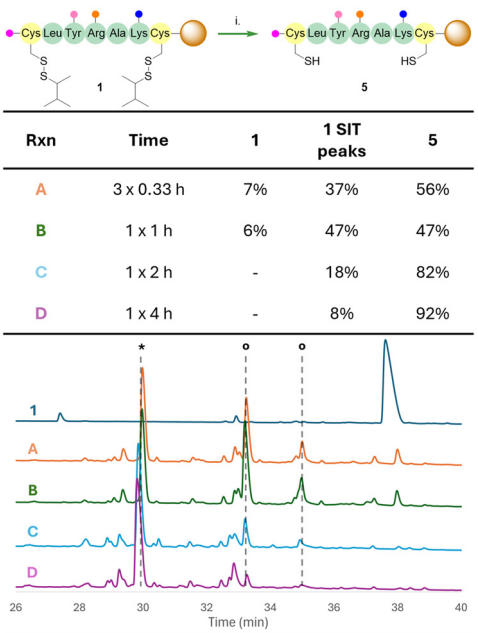


Fig. 2 Sequences of peptides (**1–4**) manually synthesised for post-linear synthesis modification tests in NBP.



Rxn	Time	1	1 SIT peaks	5
A	3 x 0.33 h	7%	37%	56%
B	1 x 1 h	6%	47%	47%
C	1 x 2 h	-	18%	82%
D	1 x 4 h	-	8%	92%

Fig. 3 Removal of SIT protecting groups to liberate the free thiols of cysteine. (i) DTT (10 eq.), NBP/DIPEA/H_2O (92 : 4 : 4), r.t. *indicates desired product peak. °Indicates the two different peaks from peptides in which one SIT group remains attached.

within disulfide-rich peptides.[29] The triphenylmethyl (Trt) protecting group is conventionally employed with the intention of being removed during global deprotection and resin cleavage. However, orthogonal conditions for Trt removal without affecting other acid labile functionalities have been reported. There are also reports indicating variability in selectivity, with either Acm or Trt being preferentially removed under certain conditions.[30,31] Solvated in DMF, Acm can be fully removed and cyclised using 3 equivalents of *N*-chlorosuccinimide or I_2 with $Na_2S_2O_8$ (4 eq., 2 eq., respectively).[32,33] Similarly, Trt can be selectively deprotected with the same conditions utilising iodine in DMF.[33] To assess both the efficiency and potential cooperativity of Acm and Trt removal in NBP, a series of deprotection conditions were systematically evaluated.

Subjecting peptides **2** and **3** to I_2 in the presence of $Na_2S_2O_8$ (Fig. 4, entry A) resulted in preferential Acm removal over Trt. Peptide **3** (Acm) showed near-complete consumption of starting material. On the contrary, peptide **2** (Trt) remained largely intact despite these conditions leading to complete deprotection in DMF. Subsequently, oxidation using *N*-halosuccinimides was explored. Peptide **3** exhibited partial conversion for each succinimide, with the highest yield of product **7** achieved using NCS for 30 minutes (Fig. 4, entry C). Interestingly, Trt deprotection and subsequent oxidation of peptide **2** was more efficient with NIS and NBS, affording 85% and 82% of product **6** formation, respectively. Thus, in contrast to entry A, NIS and NBS provided high selectivity for Trt removal over Acm. These findings demonstrate that NBP is compatible with selective removal and oxidation of both Acm and Trt protecting groups.

Orthogonal deprotection of Lys(Dde). Due to many peptidomimetics requiring multiple post-synthesis modifications, orthogonal protection strategies must be employed. To demonstrate NBP's suitability for orthogonal chemistry, we installed a 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protected lysine in place of Lys(Boc) in peptide **3**. Under the Acm removal and oxidation conditions, lysine remained protected with the Dde group. Due to the base sensitivity of the N-terminal Fmoc protecting group, peptide **3** was first Fmoc deprotected to allow for a more cohesive analysis of Dde removal in NBP (**8**). Complete DDE removal is achieved employing 3 × 2 minutes treatments of 2–4% NH_2NH_2 in DMF (1 mL, v/v), thus, we initially screened a range of hydrazine based deprotection conditions.[34–36] Peptide **8** was treated with a 4% hydrazine solution (3 × 3 minutes) which resulted in the complete removal of Dde to give peptide **9** (Fig. 5A). Using this as a starting point, we then sought to improve the efficiency of the reaction by decreasing the number of hydrazine treatments required for complete removal of the protecting group. We found that one treatment of 3 minutes with the 4% hydrazine solution (Fig. 5B) was sufficient to completely remove Dde. We confirmed the orthogonality of this approach with Cys(Acm) as the protecting group remained stable following the hydrazine treatments.

Peptidomimetic cyclisation methods using sustainable solvent

On-resin disulfide bond formation. Following the successful removal of the SIT protecting groups in peptide **1** to liberate free thiols **5**, we next aimed to form the disulfide bond linkage on-resin, using the green solvent NBP. Standard literature conditions report the use of two treatments with *N*-chlorosuccinimide (NCS) (2.2 equiv.) for 15 min in DMF to form the cyclic disulfide product.[37] Replicating this in NBP, complete oxidation of the free thiols in peptide **5** to disulfide peptide **6** matched the literature condition for DMF (Table 1, entry I) (for full HPLC spectra, see SI Fig. S24).

Formation of 1,4-disubstituted triazole mimetics. The formation and use of 1,4-disubstituted triazoles as part of a di-

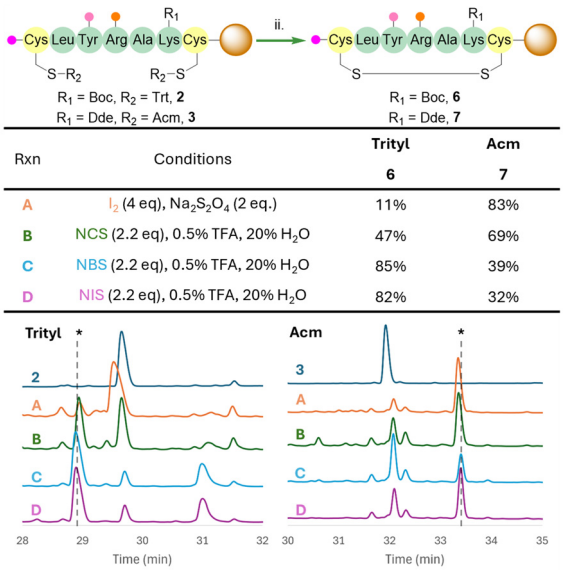


Fig. 4 Removal of the Trt and Acm protecting group and subsequent oxidation to form a disulfide bond. (ii) NBP, r.t., 1 h, see table for other reagents used. *Indicates desired product peak.

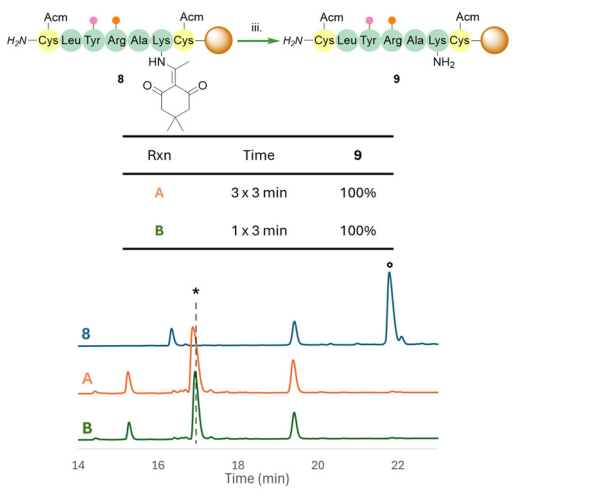
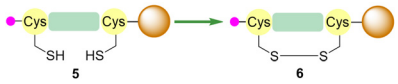
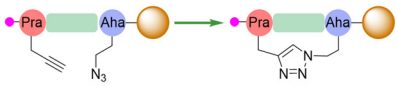


Fig. 5 Removal of the Lys(Dde) protecting group to liberate the primary amine. (iii) Hydrazine/NBP (4 : 96, v/v), r.t., see table for time. °Indicates starting material peak. *Indicates product peak.

Table 1 Summary of optimal conditions for cyclisation methods and N-terminal acetylation

Entry	Reaction	Conditions for full conversion
I		NCS (2.2 eq.), NBP, r.t., 2 × 15 min
II		CuI (0.9 eq.), TBTA (1.2 eq.), DIPEA (5 eq.), 2,6-lutidine (5 eq.), NaAsc (1.5 eq.), NBP : DMSO (9 : 1), 55 °C, 1 h
III		Acetic anhydride (3 eq.), DIPEA (6 eq.), NBP, r.t., 1 h

sulfide bridge mimetic functionality has been widely reported in literature.[38–43] On-resin formation of the triazole utilising a copper source in a binary solvent system of DMF and trifluoroethanol (TFE) (1 : 1, v/v) has been previously reported by our group.[43] Due to the toxicity of TFE, we looked to adapt these conditions by searching for literature examples using other co-solvents in this reaction. We selected dimethyl sulfoxide (DMSO) as the most sustainable co-solvent and employed this at a lower ratio (NBP/DMSO, 9 : 1, v/v). Our initial investigations subjected peptide **4** to triazole forming conditions at 55 °C for 10 minutes which resulted in a 55% conversion to expected product **10**. To obtain maximum yield, we repeated the reaction, increasing the time to 30 minutes or 1 hour at 55 °C. HPLC and LC/MS analysis confirmed that a 30 minutes reaction time resulted in 84% conversion, while 1 hour resulted in full conversion of starting material **4** to product **10** (Table 1, entry II) (for full HPLC spectra, see SI Fig. S25).

Sustainable N-terminal acetylation

Following the success in protecting group removal, we aimed to investigate the effects of NBP towards peptidyl acetylation. Standard protocols employ acetic anhydride and base in DMF, depending on number of equivalents used, reaction times vary from 5 minutes (50 eq.) to multiple hours (3 eq.). Keeping in line with the reaction efficiency theme of this paper, we decided to keep the equivalents of acetic anhydride and *N*,*N*-diisopropylethylamine low (3 eq., 6 eq., respectively), trialling the reaction at three lengths. After 5 minutes, we observed a 45% conversion of the free N-terminal peptide (**11**) to product **12**, which increased to 75% if left for 30 minutes. Following 60 minutes of treatment, full conversion to the capped peptide was observed (Table 1, Entry III) (for full HPLC spectra, see SI Fig. S26).

Conclusions

This work expands the reaction profile demonstrated by NBP in peptidyl post-linear synthesis reactions. With the advantages of being non-reprotoxic and biodegradable, NBP has been identified as an appropriate sustainable alternative to DMF. Despite the challenges faced for automated synthesis due to its viscosity, NBP remains user-friendly for manual linear and post-linear synthesis workflows. Thus, we elected to develop generalised protocols for standard post-translational peptidyl reactions in NBP.

First, orthogonal protecting group chemistries involving cysteine were investigated. It was determined that SIT, Acm, and Trt protecting groups can be successfully removed employing NBP as the solvent. Optimal SIT group removal was achieved in a single treatment, thus addressing one of the 12 principles of green chemistry by improving the overall reaction efficiency. Furthermore, we report orthogonal conditions for selective Acm and Trt removal in NBP. Acm groups are removed selectively *via* I_2, while Trt is cleaved with *N*-bromo or *N*-iodosuccinimide.

Orthogonal removal of the Lys(Dde) protecting group in the presence of Cys(Acm) was achieved with a single 1 minute treatment of 4% hydrazine, without compromising the peptides alternative protecting groups.

On-resin peptide cyclisation and N-terminal acylation were successfully demonstrated in NBP. Disulfide bond formation using *N*-chlorosuccinimide in NBP proceeded with efficiency comparable to literature-reported DMF based conditions. Additionally, cyclic 1,4-disubstituted triazole peptides were obtained in NBP, albeit requiring extended reaction times compared to DMF. Finally, N-terminal acetylation was achieved under mild conditions, employing three equivalents of acetic anhydride for 1 hour at room temperature.

Overall, these findings establish NBP as a practical sustainable solvent for diverse post-linear synthesis modifications in peptide chemistry, offering a viable alternative to DMF. By enabling orthogonal protecting group strategies, selective transformations, key cyclisation and acylation reactions under mild conditions, this work supports the broader adoption of greener methodologies in peptidomimetic synthesis.

Author contributions

M. A. M.: experimental work, data analysis, writing original draft, M. D. F. C.: experimental work, K. I. M. A.: project conception, experimental work; A. G. J.: funding acquisition, draft editing; N. W.: funding acquisition, project conception, experimental work, data analysis, writing original draft, draft editing.

Conflicts of interest

All of the authors in this study declared no conflicts of interest.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. SI contains general protocols, detailed experimental procedures and characterisation data for each crude peptide. See DOI: https://doi.org/10.1039/d5ob01890k.

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