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Piperazine and DBU: a safer alternative for rapid and efficient Fmoc deprotection in solid phase peptide synthesis†

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In Solid Phase Peptide Synthesis (SPPS), contamination with deletion sequences which often co-elute with the target peptide continues to be a major challenge as these impurities can significantly affect the target peptide's properties. Here, we report an efficient Fmoc-deprotection solution containing piperazine and DBU which can cause complete removal of the Fmoc group in less than a minute. This combination rivals piperidine in speediness as revealed by kinetic studies. We demonstrate the efficiency of the piperazine/DBU solution by synthesizing the polyAla stretch with a significant reduction of deletion products occurring due to partial Fmoc deprotection. We verify the utility of the deprotection solution by successfully synthesizing four aggregation prone difficult peptide sequences. We further demonstrate that this combination can also be used to synthesize aspartimide and epimerization prone sequences when supplemented with 1% formic acid and is compatible with 2-chlorotrityl chloride resin. We conclude that piperazine/DBU can be used as a safer and effective alternative to piperidine in Fmoc-SPPS.

Introduction

The successful assembly of "difficult peptides" by Solid-Phase Peptide Synthesis (SPPS) largely hinges upon two key reactions: (a) unmasking of the amine functionality on peptidyl resin; (b) acylation *i.e.* coupling of an N^{α}-protected amino acid to the freshly exposed amine. Less than 100% efficiency for either of the reactions results in the occurrence of deletion sequences which often co-elute with the target peptide, a highly undesirable outcome especially for peptides with therapeutic indications. Several strong yet racemization free coupling reagents have been developed in last decade to overcome incomplete acylation.[1] In combination with microwave heating which reduces on-resin peptidyl aggregation, very high coupling efficiencies have been realized.[2,3] In contrast, amine deprotection methods have not evolved much and even after 37 years since the first report by Atherton *et al.*,[4] 20% piperidine in DMF continues to be the universal choice for standard Fmoc-SPPS.

However, piperidine is a controlled substance finding usage in the synthesis of narcotic drugs and psychotropic substances and leads to large amounts of toxic waste (20–50% solutions are required) thus increasing the manufacturing costs of peptides. Piperidine solution in a polar aprotic solvent such as DMF or NMP has been the reagent of choice for Fmoc-deprotection owing to its high basicity (pK_a 11.12) and strong nucleophilic character attributed to unhindered access. A few weaker but safer bases have been reported such as piperazine (pK_a 9.73) and 4-methyl piperidine (pK_a 10.78) but in the absence of robust kinetic data, doubts remain over their efficacy.[5,6] Similarly DBU has found extensive usage in flow synthesis but has not entered mainstream batch Fmoc-SPPS as being a strong base (pK_a 13.5) it promotes side reactions such as aspartimide formation.[7]

Synthesis of peptides containing highly hydrophobic regions or repeat sequences continues to be challenge given their propensity to aggregate on-resin during synthesis itself leading to deletion and truncated sequences. Peptides containing more than 6 Ala repeats represent one of the toughest sequences for SPPS assembly where incomplete Fmoc-deprotection is frequently observed resulting in a mixture of deletion sequences.[8] After a critical threshold of 5 Ala is reached, extensive inter chain hydrogen bond formation causes transition from random coil to β sheet leading to on resin aggregation which could adversely affect subsequent sequence elongation.[9] Few attempts have been made to distinguish whether the problem arises due to incomplete acylation or insufficient Fmoc-deprotection.[2,10,11] Previous synthesis attempts using 20% piperidine yielded a complex mix of desired product and the deletion peptides having up to 5 deleted Ala residues.[9] Frequently high temperatures are employed to ensure full deprotection but this worsens base catalysed side reactions.[12] Alternatively, addition of a strong base such as 2% DBU to this deprotection solution for synthesis of KA_6RA, a more hydrophilic variant of polyAla, has been shown to improve the product profile but complete eradication of deletion impurities were not seen.[11] There is an urgent need for an efficient Fmoc-

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deprotection method that can reliably yield milligram quantities of target difficult peptides without deletion sequences.

In the present study, we compare the kinetics of a few Fmoc-deprotection reagents and their combinations. Our results show that 5% piperazine + 2% DBU is faster than 20% piperidine and significantly reduces the occurrence of deletion sequences arising due to incomplete Fmoc-deprotection in model sequence $YA_{10}K$. We further demonstrate that addition of 1% formic acid to piperazine/DBU minimizes aspartimide formation in model hexapeptide VKDGYI and this solution can be safely used with 2-chlorotrityl chloride resin, an acid sensitive support.

Results and discussion

Kinetics of Fmoc deprotection

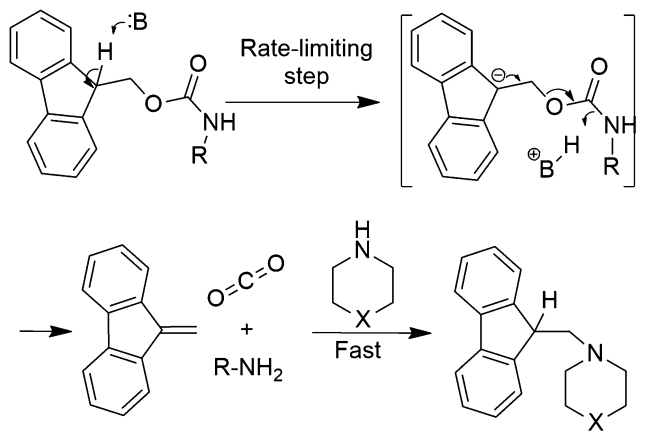
Fmoc-deprotection in SPPS by an unhindered nucleophilic base such as piperidine in polar solvents (*e.g.* DMF) proceeds through a two-step mechanism: removal of Fmoc group followed by quenching of resultant by-product dibenzofulvene (DBF). The detachment of Fmoc follows E_1C_B mechanism with the abstraction of proton from the bulky 9-fluorenylmethyl ring being the rate determining step (Scheme 1).[13] Since an excess of nucleophile is available *in situ* and DBF is a highly reactive electrophile, it is safe to assume that quenching occurs instantaneously. First detailed study on Fmoc-deprotection was reported by Atherton *et al.*,[4] who determined half-lives ($t_{1/2}$) based on amino-acid quantification and found $t_{1/2}$ for Fmoc-Val to be 6 s for 20% piperidine in DMF. Since then only a few studies have reported $t_{1/2}$ for similar combinations but their estimates range anywhere from half a minute to tens of minutes probably due to differences in the experimental protocols.[6,14]

As we set out to find an optimal substitute for piperidine, our first challenge was to devise a robust yet simple protocol for monitoring Fmoc-deprotection kinetics providing reproducible data. Qualitatively, Fmoc release in SPPS is routinely monitored by measuring the absorbance of DBF–nucleophile adduct in deprotection solution at 301 nm.[15] We posited that the same method could provide time-point data if the on-going reaction was quenched instantaneously without perturbing the rate determining step. To achieve this, an aliquot including the resin was withdrawn from the reaction and immediately filtered to remove resin; thus quenching the Fmoc-deprotection by physical separation (Fig. 1). The absorbance data at 301 nm was recorded for each aliquot, which represented the extent of Fmoc released *i.e.* the progress of the reaction at a specific time-point and used to calculate $t_{1/2}$ for Fmoc-deprotection (ESI Fig. 1†). We used resin bound Fmoc-Val as the model system and validated this protocol by measuring $t_{1/2}$ for Fmoc-deprotection using 20% piperidine in DMF. We found that $t_{1/2}$ for above combination was 7 s indicating that 99.99% deprotection was achieved within 1.5 min (Table 1). This was in close agreement with $t_{1/2}$ of 6 s reported by Atherton *et al.*,[4] for the same setup albeit in solution phase.

The simplest approach to mitigate negative impacts of piperidine could be to use a lower concentration as 5% solution has been shown to be nearly as effective as 20% solution.[14] However, in our hands a decrease in concentration of piperidine from 20% to 10% and 5% increased $t_{1/2}$ to 20 s and 39 s respectively which translated into needing 4.4 min and 8.6 min to achieve full deprotection (Table 1). This result was unsurprising as the decrease in concentration of piperidine is expected to slow down the rate of deprotection. It is likely that aforementioned study did not capture the early stages of fast Fmoc-deprotection as the experimental set up allowed recording of first data at 2 min and by then deprotection was largely over for all measured concentrations. This further demonstrated the utility of the newly devised protocol for kinetic measurements. The kinetic data presented in Table 1 is on a relative scale.

Piperazine is a structurally and chemically similar nucleophilic base which has lesser toxicity and offers price advantage over piperidine. Wade *et al.*, demonstrated that using 6% piperazine solution for Fmoc-deprotection reduced base catalysed side reactions such as aspartimide formation and epimerization.[5]

However authors cautioned against the broad utility of piperazine in SPPS as lower basicity could slow down Fmoc release and thus exacerbate problem of incomplete N^{α}-deprotection for difficult syntheses.[5,16] We tested 2% piperazine in DMF for Fmoc removal and found deprotection to be sluggish with a $t_{1/2}$ of 139 s. Upon increasing concentration to 5%



Scheme 1 Base induced cleavage of Fmoc and subsequent quenching of dibenzofulvene by a nucleophile. X = CH_2 or NH.

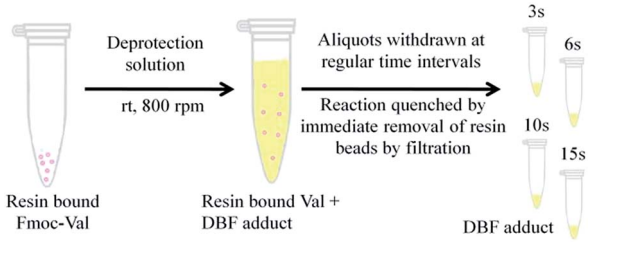


Fig. 1 Schematics of methodology adopted for estimating the half-life of Fmoc-deprotection for resin bound Fmoc-Val. Progress of reaction was monitored by absorbance at 301 nm.

Table 1 Half-life ($t_{1/2}$) and the time required for 99.99% Fmoc-deprotection ($t_{99.99}$) for resin bound Fmoc-Val

Deprotection solution	$t_{1/2}$ (s)	$t_{99.99}$[a] (min)
20% piperidine	7	1.5
10% piperidine	20	4.4
5% piperidine	39	8.6
2% piperazine	139	31
5% piperazine	50	11
10% piperazine[b]	21	4.5
5% piperazine + 0.5% DBU	11	a
5% piperazine + 1% DBU	7	1.5
5% piperazine + 2% DBU	4	<1

[a] $t_{99.99} = -(\ln 0.0001)(t_{1/2}/0.693)$. [b] 10% piperazine solution in EtOH : NMP (10 : 90).

piperazine, $t_{1/2}$ was reduced to 50 s, thus requiring 11 min for complete Fmoc removal which was expectedly longer than 8.6 min required with 5% piperidine (Table 1). Although piperazine forms a saturated solution at 6% (w/v) in DMF, up to 10% solubility can be achieved in EtOH : NMP (10 : 90).[2] This combination resulted in $t_{1/2} = 21$ s which was similar to 10% piperidine. However, for further optimization we chose DMF over NMP as Atherton *et al.*[4] have previously shown that continued exposure of building blocks to NMP causes degradation of amino acids over time.

Previous reports have indicated that dilute solutions of DBU (2% or less) in DMF can effectively deprotect Fmoc in flow-synthesis.[7] Since DBU is a strong non-nucleophilic base, DBF intermediate remains unquenched and can attach itself to the freshly unmasked amine functionality. Thus a quenching nucleophile is highly desirable while using DBU in batch mode. To accelerate the Fmoc-deprotection, we supplemented 5% piperazine in DMF with 0.5% DBU and observed that $t_{1/2}$ reduced to 12 s. An increase in DBU concentration to 1% resulted in $t_{1/2}$ of 7 s which was same as observed for 20% piperidine. Addition of 2% DBU further reduced $t_{1/2}$ to 4 s which effectively meant complete Fmoc removal in less than a minute (Table 1). A direct correlation between DBU concentration and rate of Fmoc deprotection was observed and these results indicated that here primarily DBU was functioning as the base leading to the release of Fmoc and piperazine's role could be limited to DBF quenching only.

Efficiency of deprotection solution

After establishing that 5% piperazine solution supplemented with 2% DBU provided faster deprotection than 20% piperidine we set out to test the effectiveness of this combination for difficult peptide syntheses. For these tests we chose a polyAla sequence $YA_{10}K$ consisting of a continuous tract of ten Ala residues with a very high degree of hydrophobic and high tendency for on resin aggregation.[10]

As a positive control, we synthesized polyAla sequence $YA_{10}K$ on rink amide; C-terminal Lys was added to improve solubility of final peptide in aqueous medium while N-terminus was derivatized with Tyr, a chromophoric residue to enhance visibility and enable quantification during LC-MS analysis. Fmoc-deprotection protocol consisted of two incubations for 3 min and 12 min each with fresh deprotection solution followed by an additional wash with the same solution to ensure complete removal of any residual by-product. For quantitative analysis peptides were cleaved using 95% TFA followed by direct injection onto LC-MS. As probable deletion sequences exhibit varying hydrophobicity, elimination of precipitation step helped avoid any potential biases in characterization arising due to incomplete ether precipitation of more hydrophobic sequences. This also helped us get a full product profile and detect any side products formed during synthesis.

Our first attempt with 20% piperidine resulted in 70% desired 10-mer peptide, $YA_{10}K$ while deletion sequences consisting of 9-mer, 8-mer and 7-mer accounted for the rest of the peptide product (Fig. 2a and Table 2). The 6-mer sequence was present in trace amount with no other detectable peptide peak. When we replaced 20% piperidine with 5% piperazine + 2% DBU and re-synthesized $YA_{10}K$ using the exactly same protocol the yield for the desired 10-mer product increased to 89%. Accordingly deletion sequences were significantly reduced with 9-mer accounting for 10% product and only trace amount of 8-mer could be detected (Fig. 2b and Table 2). At this juncture we could not conclude whether the 9-mer deletion product was arising due to inadequate acylation or incomplete Fmoc-deprotection as both can be caused by on-resin aggregation. Since we carried out double acylation using highly potent coupling agent DIC/Oxyma along with microwave assisted heating, secondary structures were broken and coupling was fully achieved. However incomplete Fmoc-deprotection could

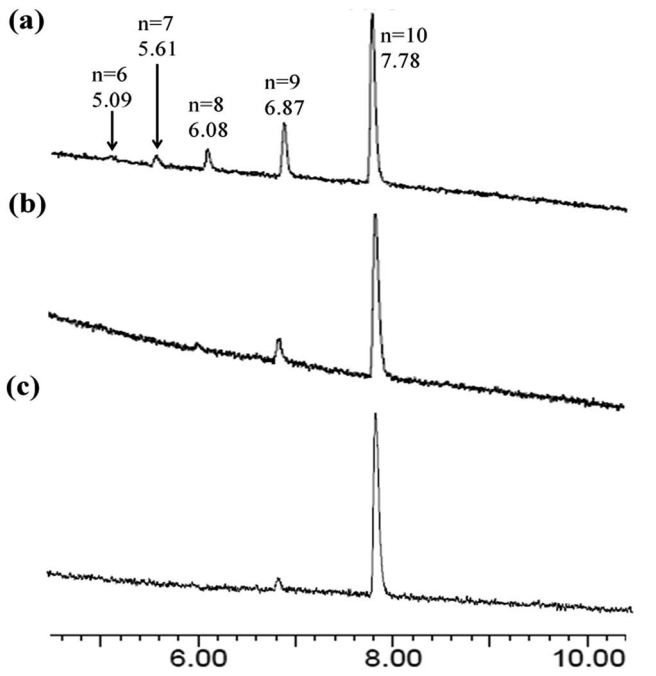


Fig. 2 RP-HPLC chromatogram of $YA_{10}K$ synthesized using (a) 20% piperidine, (b) 5% piperazine + 2% DBU and (c) 5% piperazine + 2% DBU with additional deprotection at 65 °C. Detection at 274 nm.

Table 2 Deletion sequences observed in the synthesis of $YA_{10}K$

		Relative yields[b]%		
YA_nK	m/z	20% piperidine	5% piperazine + 2% DBU	5% piperazine + 2% DBU, Δ
$n = 10$	1019.8	70	89	96
$n = 9$	948.8	20	10	<4
$n = 8$	877.7	7	<1	n.d.[a]
$n = 7$	806.6	3	n.d.	n.d.
$n = 6$	735.5	<1	n.d.	n.d.

[a] n.d. – not detected. [b] Determined by HPLC, detection at 274 nm.

also hinder the subsequent acylation reaction resulting in deletion sequences and truncated products.[17] To test this hypothesis, during Fmoc-deprotection step we replaced wash with deprotection solution with incubation under microwave irradiation at 65 °C for 3 min. This modification resulted in 96% of target 10-mer peptide and amount of 9-mer was reduced to less than 4% (Fig. 2c and Table 2). This indicated that majority of Fmoc deprotection occurred at rt itself and some of the Fmoc protected peptide which was inaccessible could be made available by additional microwave heat assisted deprotection step.

Since DBU is a stronger base than piperidine and if we assume that piperazine acts only as a nucleophile when mixed with DBU, it can be derived that faster kinetics led to higher degree of Fmoc deprotection and thus improved target peptide to deletion sequence ratios. This was only possible if Fmoc removal was being hindered by aggregation induced by Fmoc release, else both reagents should yield complete Fmoc removal given the time provided is 10 times than as required. If on-resin aggregation of freshly deprotected peptide was faster than the Fmoc removal, some of the residual Fmoc-protected sequences could get trapped in the core of the aggregate and thus became inaccessible. Allowing more time for deprotection was not useful and even though microwave heating during acylation broke aggregates, residual Fmoc-protected sequence did not undergo acylation and led to deletion sequence.

As we introduced additional microwave heating step for Fmoc-deprotection, probably trapped Fmoc-protected sequences were released and immediately deprotected. This translated into much lesser amount of deletion sequences. Perhaps performing Fmoc deprotection fully under microwave heating could have eliminated deletion sequences entirely but those harsh conditions are not recommended for regular SPPS.

We further synthesised four known aggregation prone difficult peptides sequences using piperazine/DBU deprotection solution. Peptides consisting of glutamine repeats are often used as model peptides to study protein aggregation in Huntington's disease.[18,19] Hexapeptides VQIVYK and KVQIIN are the primary nucleating sequences for the onset of tau protein aggregation due to the peculiar arrangement of amino acids and their ability to promote steric zippers between adjacent β sheets.[20] Similarly, $A\beta_{25\text{-}35}$ is the most amylodygenic 11-mer fragment in Aβ peptide which is extremely prone to aggregation.[21] We successfully assembled these difficult peptides sequences using 5% piperazine + 2% DBU as Fmoc cleavage solution without any detectable deletion by LC-MS (Table 3). This further supported our claim that piperazine/DBU can be universally used for synthesis of difficult sequences with very little or no deletion products and racemization.

Minimization of side products in SPPS

The most notable base induced side reactions in Fmoc-SPPS is aspartimide formation which occurs by the attack of amide nitrogen on β carbonyl moiety of protected aspartic acid side chain leading to the formation of cyclic aminosuucinate.[22] This imide ring can be opened by neucleophiles such as piperidine, piperazine or residual water giving rise to a mixture of α-aspartyl and β-aspartyl peptides[23] (Scheme 2). Aspartimide and its derivatives imbedded in the peptidyl backbone often co-elute with the target peptide making the separation and characterization very difficult.

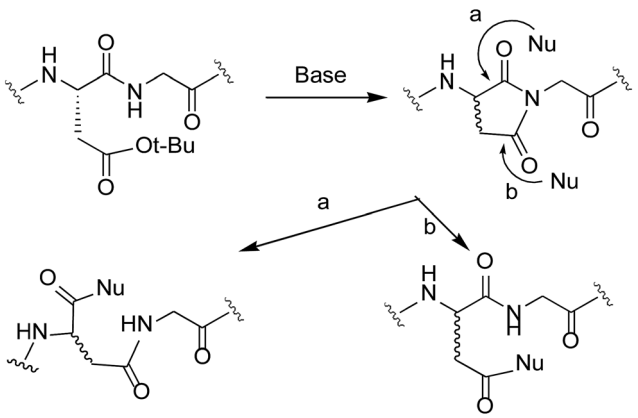
Cyclization is prevalent in Asp–X motif where X can be Gly, Asn, Ala or Gln but Asp-Gly is most vulnerable to ring formation due to minimal steric hindrance.[24] One such aspartimide prone model sequence VKDGYI a hexapeptide originally derived from toxin II of scorpion *Androctonusaustralis hector* was chosen to test the compatibility of deprotection solution.[25] According to literature reports, piperazine as N^{α} deprotection reagent reduces base induced side reactions while DBU exacerbates the problem.[5] To minimize negative impact of strong basicity of DBU we limited its concentration to 1%. When we synthesized the model peptide using a mixture of 5% piperazine + 1% DBU, we observed massive aspartimide and related by-product formation with less than 5% of target peptide (data not shown).

Several approaches have been reported to circumvent the problem of aspartimide formation in Fmoc/*t*Bu based SPPS such as using a sterically hindered side chain protections such as OMpe and OEpe[25,26] for aspartic acid or addition of acidic additives such as HOBt,[27] formic acid (FA)[27] or Oxyma[28] to

Table 3 Aggregation prone sequences assembled using 5% piperazine + 2% DBU

Peptide name	Sequence	Molecular mass	m/z (observed)	% yield[d]
PolyQ	$K_2Q_8K_2$	1553.86	777.95[a]	n.d.
PHF6	VQIVYK	747.45	748.43[b]	80
PHF6*	KVQIINY	876.04	876.47[b]	82
$A\beta_{25\text{–}35}$	GSNKGAIIGLM	1058.58	1075.52[c]	n.d.

[a] For $(M + 2H^+)/2$. [b] For $(M + H^+)$. [c] For $(M + H^+ + 16)$ for Met oxidation. [d] Based on HPLC chromatogram of crude peptides at 274 nm, n.d. = not determined.



Scheme 2 Base catalyzed aspartimide formation and byproducts from nucleophilic ring opening. Nu = OH, piperidine, piperazine.

deprotection solution. Addition of inorganic acids such as HCl resulted in low solubility of their piperazinium salts in piperazine. Similarly, solid additives such as HOBt and Oxyma could not be used owing to the gelation which causes problem during washing. We chose 1% FA as the additive and modified the deprotection solution to 5% piperazine, 1% DBU and 1% FA to effectively mitigate the formation of aspartimide. We re-synthesized VKDGYI using our modified deprotection solution and found desired peptide to be 97.6% with only 2.4% aspartimide and no detectable piperazides (Fig. 3a and Table 4).

To simulate the conditions of prolonged peptide synthesis and further evaluate the extent of aspartimide formation, the resin bound peptide synthesized as above was incubated at 50 °C for 60 min in the presence of various deprotection solutions (Fig. 3b–f and Table 4). Post-cleavage, direct analysis of peptide products by HPLC revealed that prolonged exposure to 20% piperidine alone resulted in 24.4% aspartimide and 20% piperidide formation but addition of 1% FA significantly reduced aspartimide and piperidide formation to 22.5%. When the peptidyl resin was incubated with 5% piperazine + 1% DBU, only 9.2% of target peptide could be observed with the rest being aspartimide and piperazide. Exposure to 5% piperazine along with 1% FA resulted in 7.2% aspartimide formation. Inclusion of 1% DBU, *i.e.* the same deprotection solution as used for the solid phase assembly of the peptide resulted in slightly poorer product profile with 14% aspartimide and no detectable piperizide formation (Fig. 3).

These results indicated that for all combinations studied in simulated conditions, addition of FA lowered aspartimide formation and led to higher recovery of target peptide as compared to no FA solutions. We also observed that 5% piperazine + 1% DBU + 1% FA caused lower aspartimide formation than 20% piperidine + 1% FA. Although the exact mechanism of aspartimide suppression is unknown, it can be argued that protonation by acid may modulate the basicity of piperidine or DBU just enough to hinder aspartimide formation but does not stop Fmoc removal. We further measured the kinetics of Fmoc deprotection in the presence of acidic additives and found that addition of 1% FA to 5% piperazine + 1% DBU resulted in $t_{1/2}$ of 29 s which was nearly same as $t_{1/2}$ (=27 s) observed for 20% piperidine + 1% FA. This indicates that the addition of acidic additives slows down deprotection. This decrease can be attributed to general acid inhibition and seems to be independent of the deprotection solution used for the study. Even though we did not observe complete suppression of aspartimide with the modified deprotection solution (entry 1, Table 4) and prolonged exposure could further lower the target peptide yield (entry 6, Table 4), the model sequence we chose

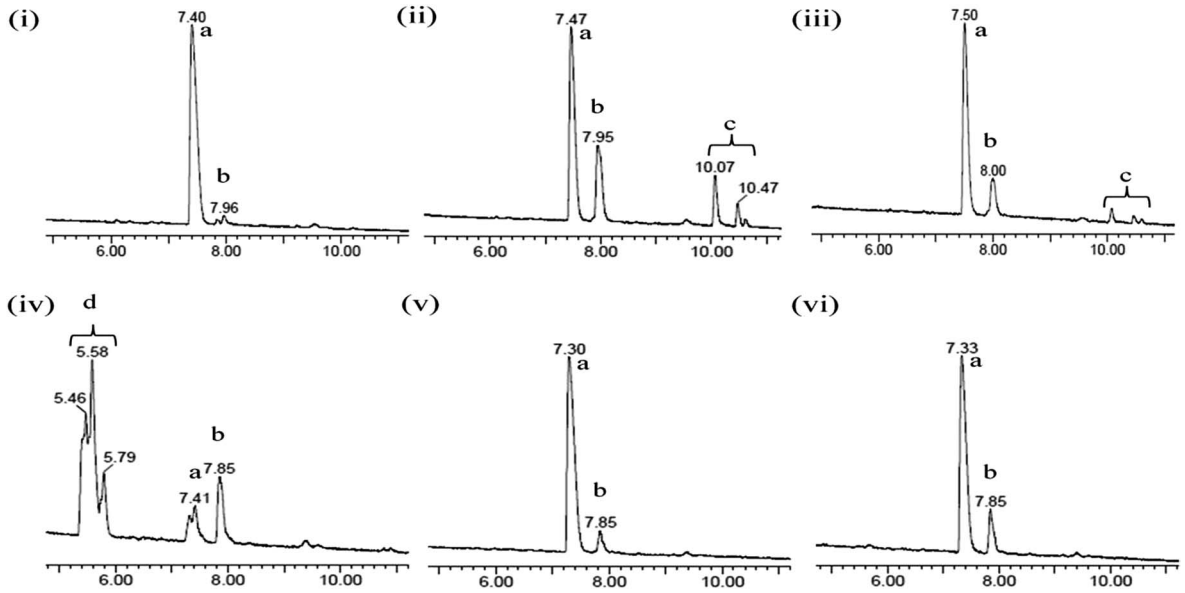


Fig. 3 RP-HPLC analysis of aspartimide and other side products formed following the incubation of resin bound VKDGYI with deprotection solution for 1 h at 50 °C: (i) no treatment, (ii) 20% piperidine, (iii) 20% piperidine + 1% FA, (iv) 5% piperazine + 1% DBU, (v) 5% piperazine + 1% FA and (vi) 5% piperazine + 1% DBU + 1% FA. (a) Target peptide, (b) D/L aspartimide, (c) D/L-α/β-piperidide, (d) D/L-α/β-piperazide. Detection at 274 nm.

Table 4 Effect of addition of DBU and/or formic acid on aspartimide and piperidide/piperazide formation in resin bound VKDGYI

	Relative yield[b]%		
Incubation solution[c]	Target peptide[d]	D/L-Aspartimide	Piperidides or piperazides
No treatment	97.6	2.4	n.d.[a]
20% piperidine	55.6	24.4	20.0
20% piperidine + 1% FA	77.5	17.2	5.3
5% piperazine + 1% DBU	9.2	13.7	77.1
5% piperazine + 1% FA	92.8	7.2	n.d.
5% piperazine + 1% DBU + 1% FA	86.0	14.0	n.d.

[a] n.d. = not detected. [b] Determined by HPLC, detection at 274 nm. [c] At 50 °C for 1 h. [d] Includes co-eluting β-peptide if any.

represents an extreme case and unlikely to be found in practice. We propose that FA modified piperazine/DBU solution shall provide enough suppression of base induced side reaction to yield aspartimide free peptides for most SPPS applications.

Another major base induced side reaction in Fmoc-SPPS is epimerization of cysteine and histidine residues. While a majority of epimerization occurs while activating amino acids for acylation, some has been attributed to repeated exposure to strong bases used for Fmoc removal.[29] To assess the effect of addition of DBU to the Fmoc deprotection solution on epimerization of embed cysteine residue, we chose a model peptide H_2N-Gly-Cys-Phe-OH. This tripeptide contains an internal cysteine residue prone to epimerization and the resulting tripeptide containing D-cysteine can be visualized as a well resolved separate peak eluting after the original product.[29] We synthesized the tripeptide with DIC/OXYMA as coupling reagent and 5% piperazine + 1% DBU + 1% FA for Fmoc deprotection. To simulate prolonged peptide synthesis with repeated exposure to strongly basic Fmoc deprotection reagents, the resin bound peptide was exposed to different deprotection solutions at 50 °C for 60 min. Post cleavage, LC-MS analysis revealed that prolonged exposure to 20% piperidine and 5% piperazine + 2% DBU resulted in 1.6% and 1.9% epimerized product respectively (Table 5). However, virtually no increase in epimerized product was observed for peptide incubated with 5% piperazine + 1% DBU + 1% FA when compared with non-incubated control sample (Table 5). These results suggest that there is only a minor change in the extent of epimerization upon switching from piperidine to piperazine/DBU and this can be nearly eliminated by the addition of 1% FA.

Table 5 Effect of deprotection solutions on levels of epimerization in resin bound model tripeptide GCF

Incubation solution[b]	Level of epimerization (D-peptide/L-peptide) × 100[a]
None	1.39%
20% piperdine	1.56%
5% piperazine + 2% DBU	1.91%
5% piperazine + 1% DBU + 1% FA	1.41%

[a] Determined by HPLC, detection at 274 nm. [b] At 50 °C for 1 h.

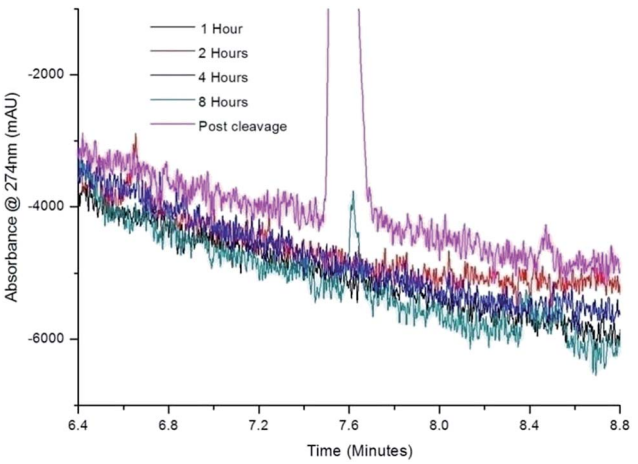


Fig. 4 Detection of premature release of Gly-Tyr dipeptide from 2-chlorotrityl chloride resin induced by piperazine/DBU solution supplemented with 1% FA. "Post-cleavage" sample as positive control was obtained by 5 min treatment with 5% TFA.

To check if the addition of 1% FA to deprotection solution could result in premature cleavage during SPPS on acid sensitive resins, 2-CTC resin carrying protected di-peptide (Tyr(O-*t*Bu)-Gly) was incubated with 5% piperazine + 1% DBU + 1% FA solution. LC-MS analysis of filtrates showed that there was no peptide present at 4 hours and only trace amount could be detected after 8 hours of incubation (Fig. 4). This result indicated that even though inclusion of 1% formic acid was expected to render the deprotection solution slightly less basic, the change in acidity was not enough to trigger any notable premature release of peptide and above optimized deprotection solution was compatible with peptide assembly on 2-CTC resin.

Conclusion

We have developed piperazine/DBU combination as an effective alternative to piperidine exhibiting rapid kinetics that aids in complete Fmoc removal in SPPS. With this solution we could successfully assemble aggregation prone difficult peptides without any deleterious effects occurring due to incomplete deprotection. Addition of 1% formic acid can further reduce

aspartimide formation and epimerization in sequences containing aspartic acid and cysteine residues respectively. We believe that incorporation of piperazine/DBU combination as Fmoc-deprotection reagent in SPPS can greatly enhance the overall yield and purity of target peptides by eliminating deletion and truncation peptide sequences.

Experimental

Peptide synthesis

All amino acids (AA) and reagents were purchased from Sigma Aldrich unless specified otherwise and used as received. All side chains of trifunctional Fmoc–AA were protected as follows: *tert*-butyloxycarbonyl (Boc) for Lys, *tert*-butyl ester for Asp, *tert*-butyl ether for Tyr and trityl for Gln. Peptide sequences were assembled on rink amide aminomethyl polystyrene resin (loading 1.1 mmol g^{-1}) in a microwave-assisted peptide synthesizer Initiator$^+$ SP wave (Biotage, Uppsala, Sweden) using Fmoc method. All compositions are reported as % volume unless specified otherwise.

The resin was weighed into a 5 mL polypropylene fritted syringe and swelled in anhydrous DCM (2 mL) for 2.5 hours. After draining, the resin was washed with DCM (3 × 2 mL) and subjected to Fmoc removal followed by coupling of first Fmoc–AA as described below. Each subsequent Fmoc–AA was coupled to the peptidyl resin using the same two-step procedure:

1. Fmoc group from the peptidyl resin was removed by incubation with 2 mL of deprotection solution (5% piperazine + 2% DBU in DMF unless specified) for 3 min and 12 min at ambient temperature with continuous vortexing at 900 rpm. The resin was additionally washed with 2 mL of deprotection solution followed by alternate washes with DMF (3 × 2 mL) and DCM (3 × 2 mL).

2. All Fmoc–AA were coupled twice by DIC/Oxyma method. Briefly, Fmoc–AA (5 equiv., 0.1 M in dry DMF) was mixed with Oxyma (5 equiv.), preactivated with DIC (5 equiv.) for 3 min with mild shaking and added to the peptidyl resin manually. After incubation for 5 min at rt in the MW reactor, the reaction temperature was increased to 65 °C (~40 watt output) and maintained for 15 min with continuous vortexing at 700 rpm. The resin was drained, washed with DMF (2 mL) and the coupling reaction was repeated one additional time to ensure complete acylation. Post-coupling, the resin was washed with DMF (3 × 2 mL) and DCM (3 × 2 mL) alternately.

Peptide cleavage and characterization

Post-synthesis the peptidyl resin was washed with methanol (3 × 2 mL) and DCM (3 × 2 mL) alternately and dried under reduced pressure at 4 °C for 2 h. The peptide was released from the resin with 95% TFA, 2.5% water and 2.5% Tis (50 μL TFA solution per mg of peptidyl resin) at rt for 3 h with continuous vortexing at 900 rpm. The resin was removed by filtration and washed with TFA. All filtrates were combined, evaporated under argon flow to nearly 1/5th of the volume and directly used for characterization by LC-MS unless specified otherwise. If required, fully unprotected peptide was precipitated from ice-cold diethyl ether and pelleted by centrifugation. After decanting ether, peptide pellet was dried under argon flow and stored at −80 °C until further characterization.

All peptide samples were analysed by UPLC-MS (Synapt G2Si, Waters) equipped with a reverse phase C18 column (X Bridge C18, 100 × 4.6 mm, 2.5 μm particle size, Waters) using ESI method for mass spectrometry. For direct characterization of peptides without precipitation, 5 μL of TFA solution carrying fully deprotected peptide was diluted 100 fold with water/acetonitrile and 5 μL was injected onto UPLC-MS. Peptides bound to the column were eluted with a linear gradient from 0 to 40% acetonitrile/water containing 0.1% formic acid over 15 min at a flow rate of 0.4 mL min^{-1} and monitored at 220 and 274 nm unless specified otherwise. For post-precipitation characterization, peptide pellet was dissolved in appropriate water/acetonitrile solution and analyzed as above.

Fmoc deprotection kinetics

Rink amide-AM resin carrying Fmoc protected Val was used to measure kinetics of Fmoc deprotection reaction. Briefly, 5 mg of resin bound Fmoc-Val was placed in a 2 mL microcentrifuge tube and mixed with 1 mL of test deprotection solution in a thermomixer (Eppendorf) with continuous vortexing at 900 rpm. From the reaction mixture 40 μL were withdrawn at a predefined set time point (3 s, 6 s, 10 s, 15 s, 30 s and so on) and immediately filtered through a 200 μL filter tip to remove the resin. The filtrate was analyzed for dibenzofulvene (DBF) adduct by UV-Vis spectrometer (Tecan M200 pro Infinite equipped with Nanoquant). For each time point, absorbance spectrum was recorded and after subtraction of blank (spectrum for deprotection solution itself), absorbance at 301 nm (A_t) was noted. Collection and analysis of aliquots was continued until the absorbance at 301 nm reaches a plateau (A_{max}). Assuming first order kinetics, $\ln[1 - (A_t/A_{max})]$ was plotted against time and from the slope of the best-fit straight line, half-life ($t_{1/2}$) of the reaction was calculated as shown in the ESI.†

Synthesis of aggregation prone difficult sequences

Poly-Ala peptide ($YA_{10}K$) was synthesized on rink amide-AM resin at 20 μmole scale using two different deprotection schemes separately: 20% piperidine in DMF and 5% piperazine + 2% DBU in DMF. After cleavage, peptide solutions in TFA were analysed directly by UPLC-MS for the desired product as well as truncated sequences and product ratios were calculated from the chromatogram at 274 nm using the MassLynx software (Waters); $t_R(YA_{10}K) = 7.8$ min, $m/z = 1019.80$ for $[M + H^+]$; $t_R(YA_9K) = 6.9$ min, $m/z = 948.75$ for $[M + H^+]$; $t_R(YA_8K) = 6.1$ min, $m/z = 877.68$ for $[M + H^+]$; $t_R(YA_7K) = 5.6$ min, $m/z = 806.63$ for $[M + H^+]$, $t_R(YA_6K) = 5.0$ min, $m/z = 735.58$ for $[M + H^+]$. PolyQ ($K_2Q_8K_2$), PHF6 (VQIVYK), PHF6* (KVQIINY) and $A\beta_{25-35}$ (GSNKGAIIGLM) were synthesized on rink amide-AM resin at 20 μmole scale using 5% piperazine + 2% DBU in DMF, cleaved and peptide solutions in TFA were analysed directly by UPLC-MS with detection at 220 nm; $t_R(K_2Q_8K_2) = 2.6$ min, $m/z = 777.95$ for $[M + 2H^+]/2$; t_R(VQIVYK) = 6.06 min, $m/z = 747.45$; t_R(KVQIINY) = 6.55 min, $m/z = 876.47$; $t_R(A\beta_{25-35}) = 6.83$ min,

m/z = 1075.52 for [M + H^+ + 16]. Yields for crude peptides with tyrosine residues were directly calculated from HPLC chromatogram at 274 nm by comparison with Boc-Tyr-OH as standard.

Aspartimide and related side-products

Model aspartimide prone peptide VKDGYI was synthesized on rink amide-AM resin at 50 μmole scale using 5% piperazine + 1% DBU + 1% FA in DMF as Fmoc deprotection solution. The resin bound peptide was divided into 10 aliquots, each representing ~5 μmoles of peptide. Each aliquot of resin bound peptide was incubated with 500 μL of a specific deprotection solution for 60 min at 50 °C. Post-incubation, the peptide was cleaved and TFA solution was analysed directly by UPLC-MS. The desired peptide as well as aspartimide and related side-products were identified from the chromatogram at 274 nm and relative amounts were calculated using the MassLynx software (Waters); t_R(desired peptide) = 7.3–7.5 min, m/z = 693.50 for [M + H^+]; t_R(aspartimide) = 7.85–8.0 min, m/z = 675.47 for [M + H^+]; t_R(piperazide, multiple peaks) = 5.4–5.8 min, m/z = 761.59 for [M + H^+]; t_R(α + β piperidide) = 10.1 and 10.5 min, m/z = 760.62 for [M + H^+].

Compatibility with 2-chloro trityl chloride resin

Fmoc-Tyr(O-*t*Bu) was coupled to 2-chlorotritylchloride resin preloaded with Gly (loading 0.51 mmol g^{-1}) using standard coupling method as above and Fmoc group was removed with 5% piperazine + 1% DBU + 1% FA. Reference protected dipeptide (Tyr(O-*t*Bu)-Gly) was obtained by treating the resin with 5% TFA in DCM for 1 h with continuous vortexing at 900 rpm. To determine the premature cleavage of the dipeptide, the peptidyl resin was incubated with deprotection solution (5% piperazine + 1% DBU + 1% FA) at rt. Aliquots were withdrawn at 1, 2, 4, 8 and 24 h and injected onto UPLC-MS for analysis. All chromatograms at 274 nm were analyzed for the appearance of a peak at 274 nm (t_R = 7.6 min, m/z = 295.15 [M + H^+]) corresponding to the prematurely cleaved protected dipeptide.

Cysteine epimerization studies

To determine the extent of epimerization, model tripeptide NH_2-Gly-Cys-Phe-OH was assembled on rink amide resin (0.6 mmol g^{-1}) at 20 mg scale using DIC/OXYMA for acylation and Fmoc was removed using 5% piperazine + 1% DBU + 1% FA. The resin bound peptide was divided into 4 aliquots; each containing ~5 mg of peptide. Aliquots were incubated with 500 μL of specific deprotection solution for 60 min at 50 °C. One aliquot with no incubation served as negative control. Post-incubation, the peptide was cleaved and TFA solution was analysed directly by UPLC-MS. A positive control was obtained for this experiment by synthesizing tripeptide using HOAt/HATU/DIPEA as coupling reagent which resulted in 10.2% epimerized peptide. The ratio of L-peptide to D-peptide was calculated using MassLynx software (Waters); t_R (L-peptide) = 8.14 min, m/z = 325.38; t_R (D-peptide) = 9.78, m/z = 325.38 for [M + H^+].

Abbreviations

AcN	Acetonitrile
Boc	Butyloxycarbonyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DBF	Dibenzofulvene
DCM	Dichloromethane
DIC	*N*,*N*′-Diisopropylcarbodiimide
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	Dimethylformamide
EtOH	Ethanol
FA	Formic acid
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo [4,5-*b*]pyridinium-3-oxid-hexafluorophosphate
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	Hydroxybenzotriazole
Oxyma	2-Ethyl(hydroxyimino)cyanoacetate
PolyA	Poly alanine
PolyQ	Poly glutamine
rt	Room temperature
t-Bu	*tert*-Butyl
Tis	Triisopropylsilane

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