Optimized Fmoc-Removal Strategy to Suppress the Traceless and Conventional Diketopiperazine Formation in Solid-Phase Peptide Synthesis

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ABSTRACT: DKP (diketopiperazine) formation is a ubiquitous side reaction in SPPS (solid-phase peptide synthesis) that is highly sequence-dependent. Secondary amino acids are extremely prone to host such a side reaction. DKP formation is predominantly induced at the Fmoc (fluorenylmethyloxycarbonyl)-removal step mediated by a secondary amine, which conventionally employs piperidine/DMF (dimethylformamide). In this study, alternative Fmoc-removal solution 2% DBU (1,8-diazabicyclo[5.4.0]undec-7-ene)/5% piperazine/NMP (N-methyl-2-pyrrolidone) led to drastic DKP reduction relative to 20% piperidine/DMF.

INTRODUCTION

DKP (2,5-diketopiperazine) formation is a frequently occurring side reaction in SPPS (solid-phase peptide synthesis).[1] It is essentially a peptide fragmentation process induced by an intramolecular nucleophilic attack of the peptide N^{α}-group at the amide or ester moiety from the peptide backbone, leading to the formation of an N-terminal truncated peptide molecule by releasing a six-membered diketopiperazine (Scheme 1).

Scheme 1. Mechanism of DKP Formation
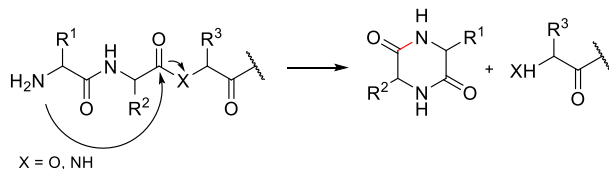
One of the most decisive effects facilitating DKP formation is the potential preference of the *cis*-configuration of the amide bond between the two constituting amino acids entangled in the DKP formation.[2] This is particularly the case when the second amino acid from the N-terminus is an amino acid with a secondary amino group like Pro,[3] proline analogues,[4] secondary amino group-bearing cyclic amino acids,[5] and N-alkyl amino acids.[6]

DKP formation is normally accelerated and intensified for the depsipeptide[7] (X=O in Scheme 1) since the hydroxyl derivative is a better leaving group than its amino counterpart. Such an inherent attribute is highly pertinent to the SPPS of

peptide acid as the growing peptide chains are immobilized on the solid supports through ester bonds, which are particularly susceptible to DKP formation at the dipeptide stage. This side reaction rationalizes the invention of CTC (2-chlorotrityl chloride) resin, which enables the suppression of DKP formation through its significant steric hindrance.[8]

Despite the introduction of CTC resin, severe DKP formation could still be induced at the step of Fmoc deblocking for Fmoc−Xaa¹−Xaa²−2-chlorotrityl resin. In such a circumstance, the C-terminal dipeptide will be cleaved from the resin through DKP formation when the subject dipeptide Fmoc−Xaa^penultimate−XaaC-terminal−2-chlorotrityl resin is subject to the Fmoc-deblocking treatment. The affected resin linker will be transformed to a 2-chlorotrityl hydroxyl moiety, which is inert and could not accommodate the further peptide chain growth, whereas the released DKP byproduct is removed from the reaction system through rinsing.[9] Contrary to the DKP formation in the middle of the peptide sequence, which generates the characteristic impurity des[Xaa^n−Xaa^{n+1}] void of the Xaa^n−Xaa^{n+1} moiety, the DKP side reaction that affects the C-terminal dipeptide will not be revealed by any related impurity from the crude product. A
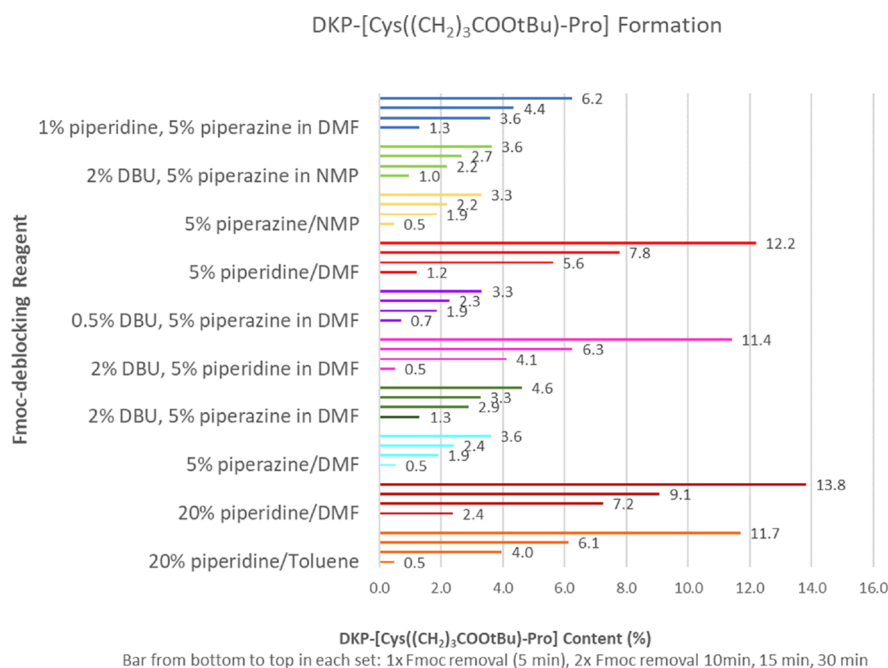
Figure 1. Progress of DKP-[Cys(CH₂)₃COOtBu-Pro] formation under various Fmoc-removal conditions.

highly pure crude product could be obtained with a significantly reduced production yield. In such a case, the C-terminal DKP formation is named "traceless DKP formation" since its occurrence could not be reflected by the impurity profile of the affected peptide product and is generally ignored.

Many strategies have been reported to be capable of suppressing DKP formation. Some of them exploit alternative N^α-protecting groups such as pNZ (p-nitrobenzyloxycarbonyl),[10] Trt (trityl),[11] and alloc (allyloxycarbonyl),[12] bypassing the base-directed deblocking of the N^α-protecting group. The dipeptide building block has also been verified as a viable strategy to preclude the transient existence of the DKP-susceptible intermediates.[13] Although effective in suppressing the DKP formation, these synthetic strategies are not highly compatible with the conventional Fmoc-chemistry-based peptide synthesis, particularly when industrial peptide production is concerned.

Alternative Fmoc-removal reagents such as TBAF (tetrabutylammonium fluoride) and DBU (1,8-diazabicyclo[5.4.0]-undec-7-ene)[9,14] have been tested to minimize the DKP formation. However, they all have respective intrinsic restrictions despite their effectiveness for DKP suppression. For instance, TBAF resulted in reduced Fmoc-removal kinetics,[14] whereas DBU could not quench the reactive byproduct dibenzofulvene, resulting in the undesired N^α-fluorenylmethylation.[15] It is thus preferably applied in continuous-flow syntheses or subjected to rigorous time control in batch syntheses.[16] Given these restrictions, an appropriate Fmoc-removal strategy that does not imperil the reaction kinetics and product integrity is highly desirable, particularly for the manufacturing of peptides with significant vulnerability to DKP formation including, but not limited to, traceless DKP.

■ RESULTS AND DISCUSSION

In the process of SPPS of a peptide API with a C-terminal sequence of H-Cys[(CH₂)₃COOtBu]-Pro-OH on CTC resin,

a highly pure crude product (97%) with a rather low yield was obtained. The reaction solution of Fmoc removal from Fmoc-Cys[(CH₂)₃COOtBu]-Pro-2-chlorotrityl resin was analyzed, and abundant DKP-[Cys(CH₂)₃COOtBu-Pro] was detected. Screening of the subject Fmoc removal under 10 various conditions was conducted to establish the cause-and-effect relationship for the DKP formation. The base, organic solvent, and base concentration vary in this screening study. It is to note that the Fmoc-deblocking solution was added to the resin twice with intervening drainage. The Fmoc removal was performed at room temperature for 5 and 30 min. Quantification of the DKP-[Cys(CH₂)₃COOtBu-Pro] byproduct released into the reaction mixture was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). The results are summarized in Figure 1.

The designed screening experiments evidently revealed that the propensity of the DKP formation from a certain peptide sequence was strongly subjected to the type of the base and solvent for the Fmoc removal. When piperidine was applied as the Fmoc-removal reagent, DKP would be rapidly formed regardless of the organic solvents. Total DKP formation soared to 13.8% after twofold Fmoc removal through the standard treatment with 20% (v/v) piperidine/DMF (dimethylformamide). Neither the reduction of the piperidine concentration to 5% (12.2% DKP) nor the substitution of the solvent by toluene (11.7% DKP) could effectively suppress the DKP formation. Nevertheless, replacing piperidine with 5% (w/v) piperazine could significantly reduce the DKP formation. Merely less than 4% DKP was generated by treating the peptide resin with 5% piperazine either in DMF or NMP (N-methyl-2-pyrrolidone). Despite such effectiveness, a large number of white precipitates were formed in the process of the Fmoc removal by piperazine, which evidently interfered with the following filtration operations. The precipitates were isolated and analyzed by LC/MS. The predominant component was identified as 1,4-bis(9H-fluoren-9-ylmethyl)-piperazine **3**. The mechanism of its formation is proposed in Scheme 2. Fmoc is eliminated by piperazine through the E1cB

Scheme 2. Mechanism of 1,4-Bis(9H-fluoren-9-ylmethyl)piperazine Formation through Piperazine-Mediated Fmoc Removal
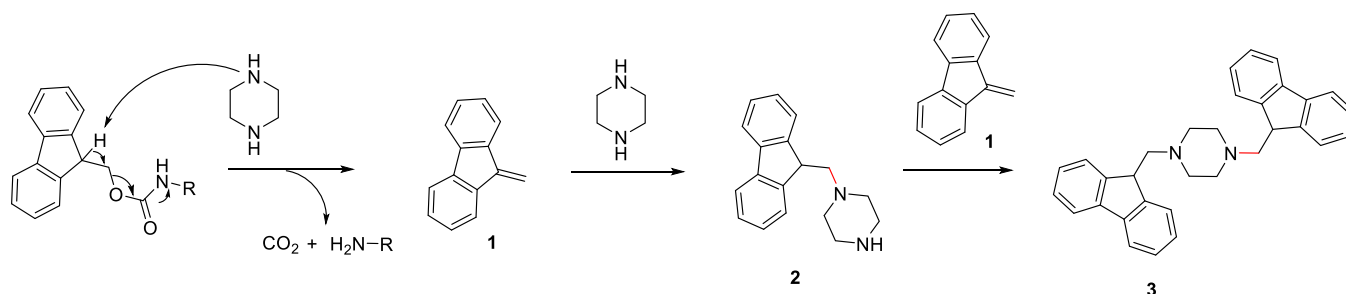
mechanism to form dibenzofulvene **1**. The latter is entrapped by piperazine to give 1-(9H-fluoren-9-ylmethyl)piperazine **2**, which can further function with another molecule of dibenzofulvene **1** and form 1,4-bis(9H-fluoren-9-ylmethyl)-piperazine **3**.

1,4-Bis(9H-fluoren-9-ylmethyl)piperazine **3** is insoluble in DMF and precipitates once formed at the piperazine-mediated Fmoc-deblocking step. It impeded the following filtration operations under the applied SPPS conditions. Nonetheless, it was noticed that the addition of 2% DBU and replacement of DMF by NMP could alleviate the formation of 1,4-bis(9H-fluoren-9-ylmethyl)piperazine **3** despite not being able to preclude its formation entirely. The operability for the topic SPPS has been evidently sustained by applying 2% (v/v) DBU, 5% piperazine (w/v) in NMP as the Fmoc-deblocking solution for the DKP formation-susceptible Fmoc removal. Such an improvement is probably attributed to the joint force of the varied population ratio of 1-(9H-fluoren-9-ylmethyl)piperazine **2** to 1,4-bis(9H-fluoren-9-ylmethyl)piperazine **3**, pH of the reaction solution, and the enhanced solubility of 1,4-bis(9H-fluoren-9-ylmethyl)piperazine **3** in NMP.

The extent of the total DKP-[Cys(CH$_2$)$_3$COOtBu-Pro] formation from 2% DBU, 5% piperazine/NMP-manipulated Fmoc removal was accomplished with 3.6% after twofold treatments in comparison with 13.8% from the conventional Fmoc removal with 20% piperidine/DMF. This beneficial effect has been verified in a scale-up with 24 mmol peptide synthesis, and 3.3% DKP was formed at the subject Fmoc-deblocking step. It is to note that 0.9% DKP was also detected in the first rinsing solution post-Fmoc removal, indicating that DKP formation could progress as long as the liberated N^α is not acylated by the incoming amino acid. This inherent artifact requests timely peptide resin rinse and amino acid coupling after the subject Fmoc deblocking.

A plethora of DKP-susceptible Fmoc−Xaa^1−Xaa^2−2-Cl trityl resins was applied as the substrates to verify the generality of the beneficial effects of 2% DBU, 5% piperazine/NMP in terms of the DKP suppression, with Xaa^2 occupied by a residue with a secondary amino group like Pro, Sar, and N-4-F-Bn-Gly in order to facilitate the DKP formation. The comparison of DKP development between 20% piperidine/DMF and 2% DBU, 5% piperazine/NMP treatment is charted in Figure 2. The substrate peptide resins were treated by the respective Fmoc-deblocking solution twice, with 5 min for the first and 30 min for the second. Note that the analytical errors are accounted for the DKP contents exceeding 100%. Indeed, DKP formation was pronouncedly intensified on these substrates, particularly when Xaa^2 was Sar or N-4-F-Bn-Gly. In almost all cases, 2% DBU, 5% piperazine/NMP evidently exhibited superior properties to suppress the

DKP formation compared with 20% piperidine/DMF, except when Xaa^1, instead of Xaa^2, was occupied by N-4-F-Bn-Gly.

Besides the improvement in suppressing the C-terminal traceless DKP formation, 2% DBU, 5% piperazine/NMP has also been tested for the ordinary DKP formation induced at the middle position of the peptide sequence. Substrate peptide resins Fmoc−Xaa^1−Sar/Pro−Xaa^3−2-Cl-trityl resin were applied to gauge the reduction of DKP formation accomplished by the 2% DBU, 5% piperazine/NMP strategy. The results are charted in Figure 3. 2% DBU, 5% piperazine/NMP exhibited superior DKP-suppressing attributes to 20% piperidine/DMF, particularly when Xaa^2 is occupied by Pro, even though the severities of DKP formation in the middle of the peptide sequences are generally alleviated compared to those C-terminal dipeptide-acid counterparts. These results expand the general applicability of 2% DBU, 5% piperazine/NMP as the Fmoc-deblocking solution for a middle position of the peptide in the context of DKP suppression.

It is also to note that the Fmoc-removal kinetics has been accelerated by 2% DBU, 5% piperazine/NMP compared with 20% piperidine/DMF (data in the Supporting Information). Such a beneficial effect should be attributed to the employment of the stronger base DBU.

■ CONCLUSIONS

In summary, we have reported a strategy of Fmoc removal with 2% DBU, 5% piperazine/NMP that could evidently minimize both the "traceless" and ordinary DKP formation in SPPS. This strategy could also enhance the Fmoc-removal kinetics compared to the conventional 20% piperidine/DMF treatment. The formation of the precipitate 1,4-bis(9H-fluoren-9-ylmethyl)piperazine was restrained by 2% DBU, 5% piperazine/NMP compared with 5% piperazine/DMF or NMP, which could also be employed to reduce the DKP formation. This solution could be readily implemented in industrial peptide manufacturing severely influenced by DKP formation.

■ EXPERIMENTAL SECTION

Materials. Fmoc-Gly-OH (≥98.0%), Fmoc-Gln(Trt)-OH (≥98.0%), Fmoc-Trp-OH (≥97.0%), Fmoc-Trp(Boc)-OH (≥97.0%), Fmoc-Pro-OH (≥99.0%), Fmoc-Tyr(tBu)-OH (≥98.0%), DIC (99%), Oxyma (97%), NMP (≥99.0%), toluene (≥99.5%), piperidine (99%), piperazine (99%), and DBU (98%) were purchased from Sigma-Aldrich; 2-Cl-trityl chloride resin (100−200 mesh, 1% DVB, 1.0−1.6 mmol/g), Fmoc-His(Trt)-OH (≥98.0%), and Fmoc-Asn(Trt)-OH were procured from Iris Biotech GmbH; Fmoc-Cys-[(CH$_2$)$_3$COOtBu]-OH (≥98.5%) was obtained from Flamma, Dalian HonKai Chemical Development and Fmoc−N-4-F-Bn-Gly-OH (≥98.5%) was purchased from PolyPeptide Group;
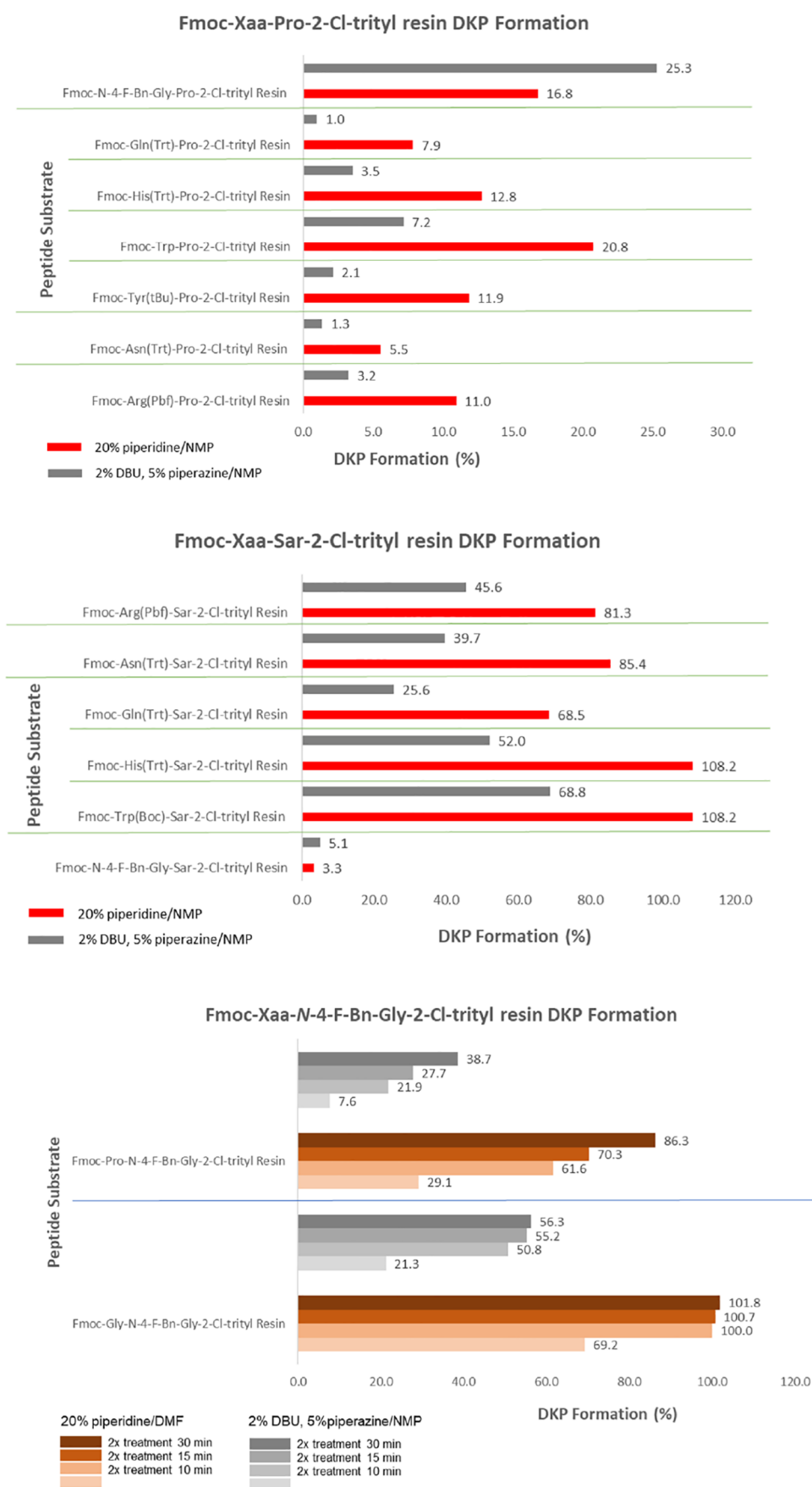
Figure 2. Comparison of DKP development by 20% piperidine/DMF and 2% DBU, 5% piperazine/NMP treatment of Fmoc−Xaa−Pro−2-Cl-trityl resin (top, total DKP formation), Fmoc−Xaa−Sar−2-Cl-trityl resin (middle, total DKP formation), and Fmoc−Xaa−N-4-F-Bn-Gly−2-Cl-trityl resin (bottom).
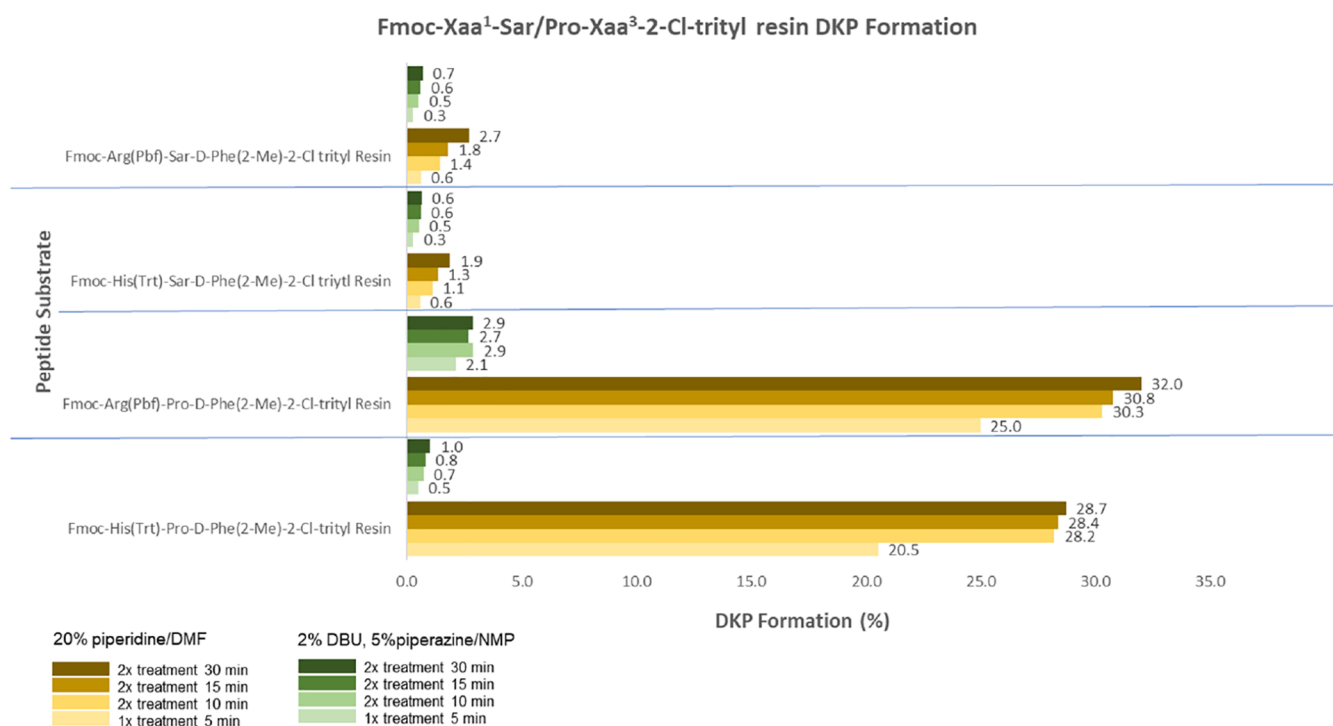
Figure 3. Comparison of DKP development by 20% piperidine/DMF and 2% DBU, 5% piperazine/NMP treatment of Fmoc—Xaa^1—Sar/Pro—Xaa^3—2-Cl-trityl resin.

Fmoc-Arg(Pbf)-OH (≥98.5%) was purchase from Flamma; Fmoc-Sar-OH (≥98.0%) was procured from Fluorochem; DMF (≥99.5%) was purchased from Merck; and Isolute C18 was procured from Biotage.

SPPS and Fmoc Deblocking. All the topic peptide resins were prepared by DIC/Oxyma-directed SPPS on 2-Cl-trityl chloride resin. The peptide assemblies were monitored by both the colorimetric test (ninhydrin test or chloranil test) and RP-HPLC. No incomplete amino acid couplings were encountered in the process of the peptide assemblies.

2.146 g of Fmoc-Cys[(CH$_2$)$_3$COOtBu]-Pro-2-Cl-trityl resin (0.28 mmol/g) was treated by using 2.21 mL of the subject Fmoc-deblocking solution (e.g., 20% piperidine/DMF, 2% DBU, 5% piperazine/NMP, etc.) at 25 °C for 5 min. The reaction solution was sampled, the resin was filtered, and another 2.21 mL of the Fmoc-deblocking solution was charged to the peptide resin. The reaction mixture was stirred by using a mechanical propeller at 25 °C for 30 min. The reaction solution was sampled at 10, 15, and 30 min. The samples were analyzed by RP-HPLC for the DKP-[Cys(CH$_2$)$_3$COOtBu-Pro] contents. The resin was rinsed with DCM (dichloromethane) and cleaved with 20% TFE (2,2,2-trifluoroethanol)/DCM. The derived cleavage solution was analyzed by RP-HPLC for the residual Fmoc-Cys[(CH$_2$)$_3$COOtBu]-Pro-OH to gauge the incomplete Fmoc removal. A reference Fmoc-Cys[(CH$_2$)$_3$COOtBu]-Pro-2-Cl-trityl resin with a known loading and weight was treated with 25% (v/v) piperidine/DMF solution overnight. The derived solution was applied as a 100% DKP metric to gauge the DKP contents from the samples in this study. It is to note that the treated resin was rinsed with DMF and DCM sequentially and cleaved with 20% (v/v) TFE/DCM for 10 min at room temperature. No dipeptide H-Cys[(CH$_2$)$_3$COOtBu]-Pro-OH could be detected in the cleavage solution (compared with the reference material). This result confirms that quantitative DKP formation has been accomplished through the overnight piperidine/DMF treatment, and the immobilized peptides have been unanimously released into the solution through DKP formation. All the other Fmoc removal and DKP quantification in this study followed the same procedures.

Preparation of DKP-[Cys(CH$_2$)$_3$COOtBu-Pro]. Dipeptide Fmoc-Cys[(CH$_2$)$_3$COOtBu]-Pro-OH was assembled on 1.0 g of CTC (1.2 mmol) resin through the SPPS strategy described above. 5.0 mL of 20% (v/v) piperidine/DMF solution was added to the resin, and the reaction mixture was stirred at room temperature for 24 h. The reaction solution was filtered, and 25 mL of H$_2$O was added to the filtrate, which was filtered again. The product solution was added to an SPE (solid phase extraction) column filled with ISOLUTE C18 (5 g/25 mL solution), which was pretreated with MeOH and 0.1% (v/v) TFA (trifluoroacetic acid)/H$_2$O solution sequentially. After loading the product solution, 20 mL of 0.1% (v/v) TFA/H$_2$O, 20% (v/v) acetonitrile/H$_2$O, 20% (v/v) acetonitrile/H$_2$O, 50% (v/v) acetonitrile/H$_2$O, and 50% (v/v) acetonitrile/H$_2$O were sequentially added to the SPE column to elute the product. The eluents were collected and analyzed by RP-HPLC. Fractions 2−4 were combined and concentrated to dryness. The obtained oily product was reconstituted in 20 mL of 20% (v/v) acetonitrile/H$_2$O solution and lyophilized. 185 mg of the oily product was obtained (100% purity, 45.1% yield). It is to note that racemization on the C$^\alpha$ from the Cys residue occurred in the reaction/workup detected by both LC/MS and NMR, and the (DKP-[Cys((CH$_2$)$_3$COOtBu-Pro]/DKP-[D-Cys(CH$_2$)$_3$COOtBu-Pro] ratio is ca. 61:39 and 67:32 as quantified by LC/MS and NMR, respectively). ^{1}H NMR (two sets of data with minor species in the bracket): (500 MHz, CDCl$_3$): δ 6.39 (6.46) (s, 1H), 4.24 (4.14) (dd, J = 10.2, 6.4 Hz, 1H), 4.12−4.06 (m, 1H), 3.74−3.65 (m, 1H), 3.57−3.50 (m, 1H), 3.01 (dd, J = 14.1, 3.7 Hz, 1H), 2.91 (dd, J = 14.1, 7.9 Hz, 1H), 2.60 (td, J = 7.3, 1.3 Hz, 2H), 2.46−2.40

(m, 1H), 2.32 (t, J = 7.1 Hz, 2H), 2.08−1.83 (m, 5H), 1.44 (s, 9H). $^{13}C\{^1H\}$ NMR (126 MHz, CDCl$_3$): δ 172.4, 168.8 (169.3), 164.3 (164.6), 80.7, 58.7 (59.4), 58.0 (53.2), 45.6, 37.3 (32.9), 34.1, 32.3 (31.0), 29.2 (28.4), 28.2, 24.8, 22.0 (22.6). $^{15}N\{^1H\}$ NMR (51 MHz, CDCl$_3$): δ 126.2, 112.6.

Preparation of DKP-[His(Trt)-Pro]. Dipeptide Fmoc-His(Trt)-Pro-OH was assembled on 1.0 g of CTC (1.2 mmol) resin through the SPPS strategy described above. 5.0 mL of 20% (v/v) piperidine/DMF solution was added to the resin, and the reaction mixture was stirred at room temperature for 24 h. The reaction solution was filtered, and 25 mL of H$_2$O was added to the filtrate to precipitate the DKP product. The filter cake was subjected to a slurry in 5 mL of DMF to remove the co-precipitated dibenzofulvenepiperidine. The suspension was filtered, and the filter cake was rinsed with 0.5 mL of DMF and 5 mL of H$_2$O sequentially and dried at 30 °C under vacuum overnight. 111 mg of the DKP-[His(Trt)-Pro] product was obtained. (98.9% purity, 20% yield).

^1H NMR: (500 MHz, CDCl$_3$): δ 7.66 (s, 1H), 7.37 (d, J = 1.4 Hz, 1H), 7.36−7.31 (m, 9H), 7.16−7.07 (m, 6H), 6.68 (d, J = 1.4 Hz, 1H), 4.19 (dt, J = 10.7, 2.5 Hz, 1H), 4.07 (ddd, J = 9.1, 6.9, 1.7 Hz, 1H), 3.66−3.50 (m, 2H), 3.37 (dd, J = 15.1, 3.1 Hz, 1H), 2.76 (dd, J = 15.1, 10.9 Hz, 1H), 2.37 (dtd, J = 13.1, 6.8, 2.5 Hz, 1H), 2.19−2.06 (m, 1H), 2.06−1.98 (m, 1H), 1.89 (ddtd, J = 12.8, 11.0, 8.8, 6.7 Hz, 1H). $^{13}C\{^1H\}$ NMR (126 MHz, CDCl$_3$): δ 169.4, 165.6, 142.4, 139.1, 137.2, 129.9, 128.3, 119.4, 75.6, 59.2, 56.0, 45.6, 28.6, 28.3, 22.7. $^{15}N\{^1H\}$ NMR (51 MHz, CDCl$_3$): δ 255.3, 190.0, 125.4, 116.6.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00214.

> Identification of DKP by LC/MS, DKP quantification, identification of the precipitate 1,4-bis(9H-fluoren-9-ylmethyl)piperazine by LC/MS and RP-HPLC, comparison of Fmoc-deblocking kinetics and DKP formation between 20% piperidine/DMF and 2% DBU, 5% piperazine/NMP-mediated Fmoc-dipeptide/tripeptide-2-Cl-trityl resin treatment, 1D ^1H NMR spectrum, 2D ^1H COSY spectrum, 2D ^1H TOCSY spectrum, 2D ^1H NOESY spectrum, 2D ^1H−^{13}C HSQC spectrum, 2D ^1H−^{13}C HMBC spectrum, and 2D ^1H−^{15}N HMBC spectrum of DKP-[Cys(CH$_2$)$_3$COOtBu-Pro], and DKP-[His(Trt)-Pro] (PDF)

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