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APPLICATION OF REVERSIBLE DETECTION METHOD FOR N-TERMINUS AMINO GROUPS: SOLID PHASE SYNTHESIS OF STYLISSATIN B

Ao Tan, Keigo Takamatsu, Fusheng Xu, Seren Osanai, and Hiroyuki Konno*

Department of Biological Engineering, Graduate School of Science and Engineering, Yamagata University, Yonezawa, Yamagata 992-8510, Japan. *E-mail address: konno@yz.yamagata-u.ac.jp

This paper is dedicated to Professor Somsak Ruchirawat on celebration of his 80th Birthday.

Abstract – The first synthesis of the proline-rich cyclic heptapeptide stylissatin B is described. Reversible detection method of N-terminus amino groups using tetrachloro-*N*-hydroxyphthalimide was applied as the Fmoc-solid phase peptide synthesis. The end points of all reactions of solid support could be pursued by the detection methods.

INTRODUCTION

Solid phase peptide synthesis (SPPS) is an indispensable technique for the modern life sciences,[1] facilitating the construction of peptide libraries much more quickly and concisely compared to solution phase synthesis.[2] However, in almost cases, the reactions used to elongate the peptide chains on solid supports cannot be directly monitored, and are usually driven to completion using large excesses of reagents, resulting in wastage. The Kaiser test, wherein ninhydrin (2,2-dihydroxyindane-1,3-dione)[3,4] can be used to check the end point of the coupling reaction between amino groups and activated esters. But most secondary and tertiary amino groups such as proline and N-methyl-amino acids and additionally primary amino groups, serine and asparagine, are not reactive under the Kaiser conditions. Moreover, the small amount of resin submitted to the Kaiser test is not recoverable, decreasing isolated yield.[5]

Recently, we reported a novel and reversible detection method for the amino groups (ReD-A) using *N*-hydroxyphthalimide (NHPI).[6-8] This method has three advantages over the Kaiser test: (i) it is reversible (i.e., samples of resin can be used to next reaction after testing); (ii) it is conducted at room temperature – the resin turns red if it bears unreacted amines and pale yellow if it does not, without having to be heated;

and (iii) it can be used to detect primary, secondary, and tertiary amino groups – including those that do not react under Kaiser test conditions.[8] The ReD-A method is therefore particularly well–suited to the preparation of proline rich peptides.

Stylissatins A-D are proline-rich, cyclic peptides isolated from the marine sponge *Stylissa massa* by Lin,[9] and Kigoshi.[10] Stylissatin B (**1**) incorporates three proline residues which adopt *cis* and *trans* rotamers, and a histidine residue which is associated with catalytic activity, and was found to exert potent cytotoxicity against tumor cell lines with IC_{50} values in the sub μM range. Whereas the synthesis, structure, and evaluation of stylissatin A were reported in detail by Shaheen in 2016,[11] synthetic studies and structural analyses of stylissatins B (**1**), C and D have yet to be reported. Here, to demonstrate the utility of our ReD-A method, we disclose the synthesis of stylissatin B (**1**).

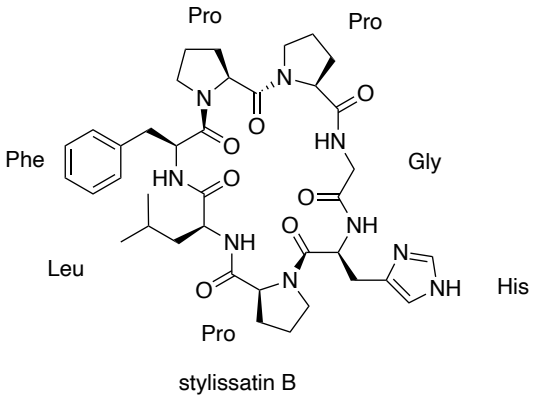
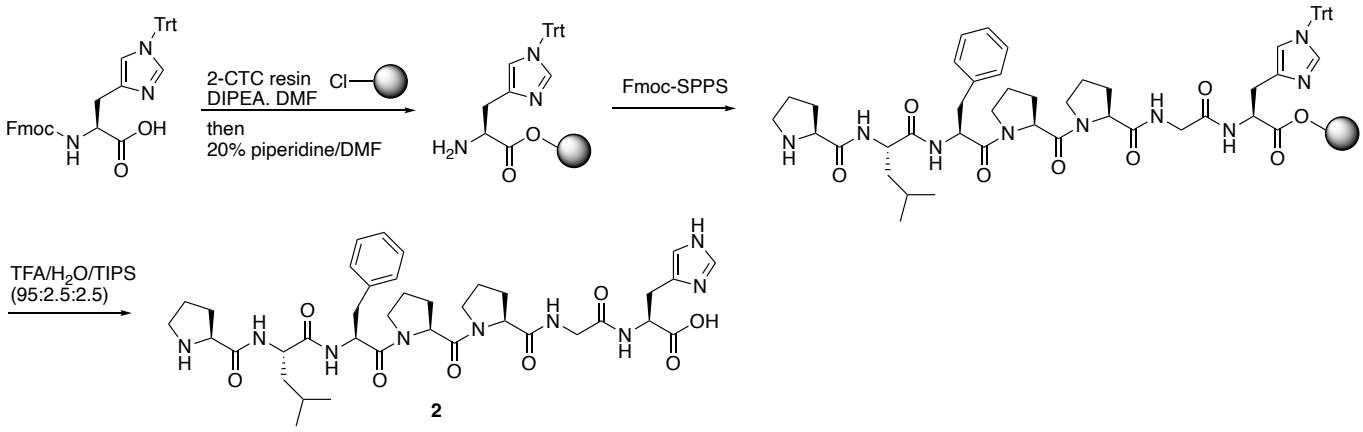


Figure 1. Chemical structure of stylissatin B (**1**)

RESULTS AND DISCUSSION

Our synthetic plan for the synthesis of stylissatin B (**1**) included the synthesis of all seven of its possible linear precursors (**2**-**8**), so that final cyclization step could be optimized, Scheme 1 and Table 1. For each peptide precursor, the Kaiser test and ReD-A methods were used to monitor the peptide chain elongation reactions on solid support. Linear precursors (**2**-**8**) were prepared by standard Fmoc-SPPS using 2-CTC resin; each coupling reaction was carried out using the appropriate Fmoc-amino acid in combination with DIPCI, HOBt, and DIPEA in DMF for 2 h, having removed the Fmoc group using 20% piperidine/DMF (Figure 2). In our previous paper, the ReD-A method relied on NHPI, which turned a read color in the presence of amino groups, allowing their visualization. In this paper, we studied the alternative detection reagent, 3,4,5,6-tetrachloro-*N*-hydroxyphthalimide (TCNHPI),[12] with view to the development of a reversible detection method. Addition of their TCNHPI or Kaiser reagent to a sample of Fmoc-His(Trt)-resin did not result in any reaction (the resin remained pale yellow) because the amino groups were protected by the Fmoc group. However, after Fmoc deprotection with 20% piperidine/DMF, the resin turned deep purple (Kaiser test) and deep brown (ReD-A method). Resin samples that reacted in the Kaiser

test were permanently stained, but the resin that reacted with TCNHPI could be returned to a yellow color (from brown) by simply washing it with DMF, ready for the next coupling reaction. As expected, similar results were obtained for Fmoc-Gly-His(Trt)-resin and Gly-His(Trt)-resin. The Kaiser condition did not react to proline and occasionally amino acid residues on N-terminus and therefore the resins employed by SPPS from Fmoc-Pro-Gly-His(Trt)-resin to Pro-Leu-Phe-Pro-Pro-Gly-His(Trt)-resin except for Leu-Phe-Pro-Pro-Gly-His(Trt)-resin could not be stained and solutions were maintained to pale yellow. In contrast, our ReD-A method was very helpful to visualize the end point of the coupling reaction – the brown color (indicating reaction completion) being in stark contrast to the pale yellow of unreacted resin). Therefore, the ReD-A method using TCNHPI was proven to be applicable to the synthesis of proline rich peptides (Figure 2).



Scheme 1. Preparation of proline-rich linear peptide PLFPPGH (**2**)

Cleavage and removal of the Trt group protecting the His side chain of peptides (**2**-**8**) was accomplished by stirring the resin with TFA/H_2O/TIPS (95:2.5:2.5) for 2 h yielded. HPLC profiles of linear peptides (**2**-**6**) had multiple peaks arising from *cis* and *trans* rotamers of their respective Pro-Pro motif, whereas those of two other linear peptides (**7**-**8**) were each single peak corresponding to the *trans*/*trans* configuration of the same Pro-Pro motifs in the case of eluting with MeCN/H_2O (see supporting information Figure S1). The NMR spectra of linear peptides (**2**-**8**) in DMSO-d_6 showed that the ratios of these isomers were different. It means that these conformations change in water and/or organic solvents (Table 1).

Treatment of linear peptides (**2**-**8**) with DIPCI/HOBt/DIPEA in DMF for 24 h at room temperature gave the desired peptide stylissatin B (**1**) and its dimer as by product. The ratio of these peptides was determined by HPLC and ESIMS analysis (see supporting information Figure S2). Peptide FPPGHPL (**7**) cyclized to the dimer of stylissatin B (**1**) preferentially. Use of the peptides PLFPPGH (**2**), HPLFPPG (**3**), GHPLFPP (**4**) and LFPPGHP (**8**) gave stylissatin B (**1**) and its dimer in a ca 1:1 ratio. Fortunately, the peptide PGHPLFP (**5**) converted to stylissatin B (**1**) and its dimer in a 5:1 ratio. Furthermore, use of linear peptide

PPGHPLF (**6**) gave stylissatin B (**1**) and its dimer in a ratio of 6:1 by HPLC. The chemical yield of stylissatin B (**1**) was 37% from resin loading after the purification on preparative HPLC. The ^{1}H- and ^{13}C-NMR spectra of our synthetic stylissatin B (**1**) were consistent with those reported in the literature, including with the peaks at 32.3 ppm and 22.3 ppm indicative of the *cis* configuration of one of the three Pro residues.[13] In addition, the retention time of our synthetic stylissatin B (**1**) on HPLC were identical to that of a natural sample (see supporting information Figure S3). Thus, the first synthesis and assignment of stylissatin B (**1**) were achieved (Scheme 2).

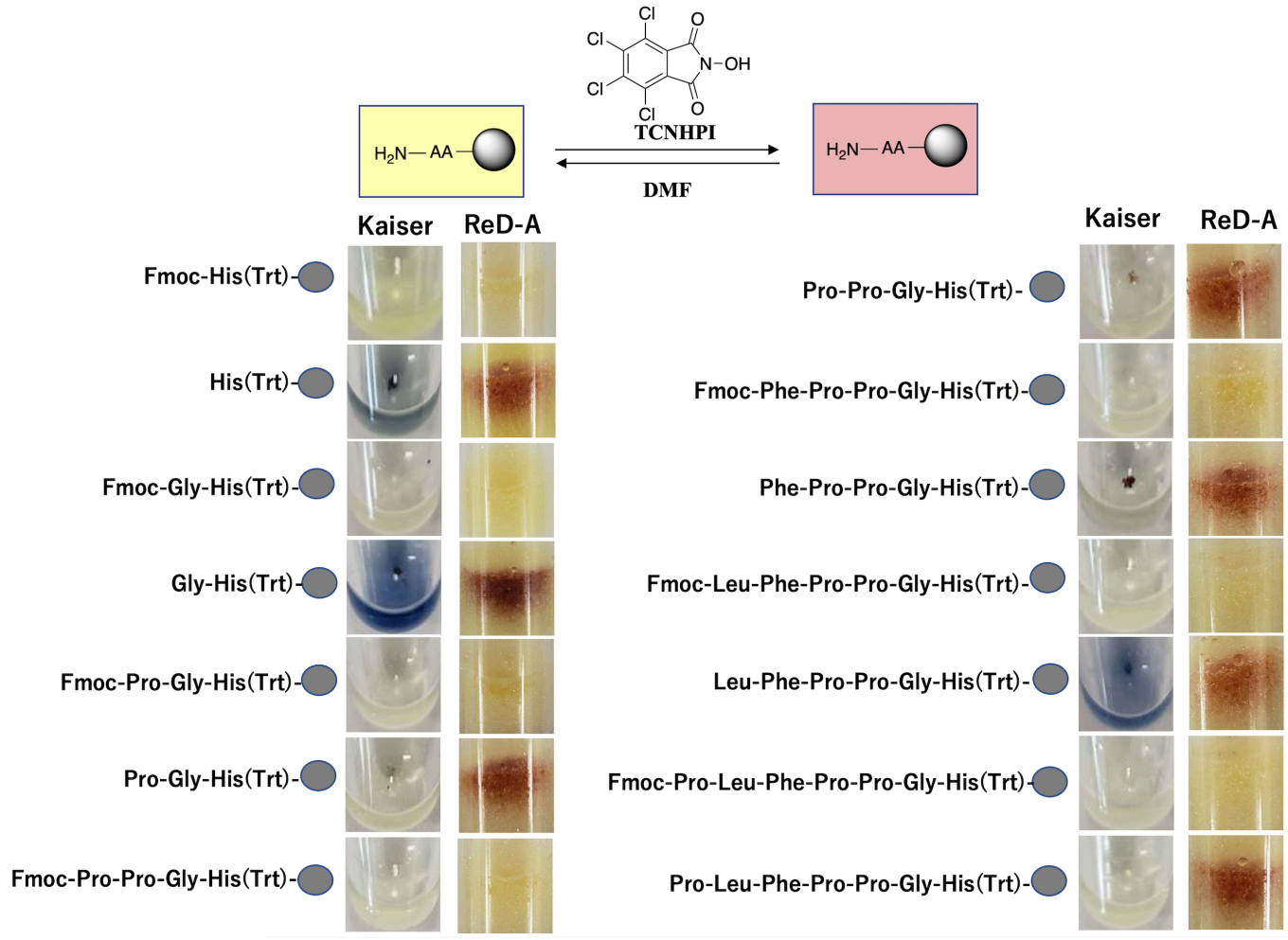


Figure 2. Reaction monitoring of the elongation of PLFPPGH (**2**) by the Kaiser test and ReD-A methods. Kaiser test: a sample of the resins was heated in a solution of ninhydrin, KCN, phenol, pyridine in EtOH at 95 °C for 5 min; ReD-A method: a DMF solution of TCNHPI (3 M) is dropped to a sample of the resins at room temperature. Having visualized the staining (if any), the resin sample was washed with DMF and advanced to the next step in the reaction sequence.

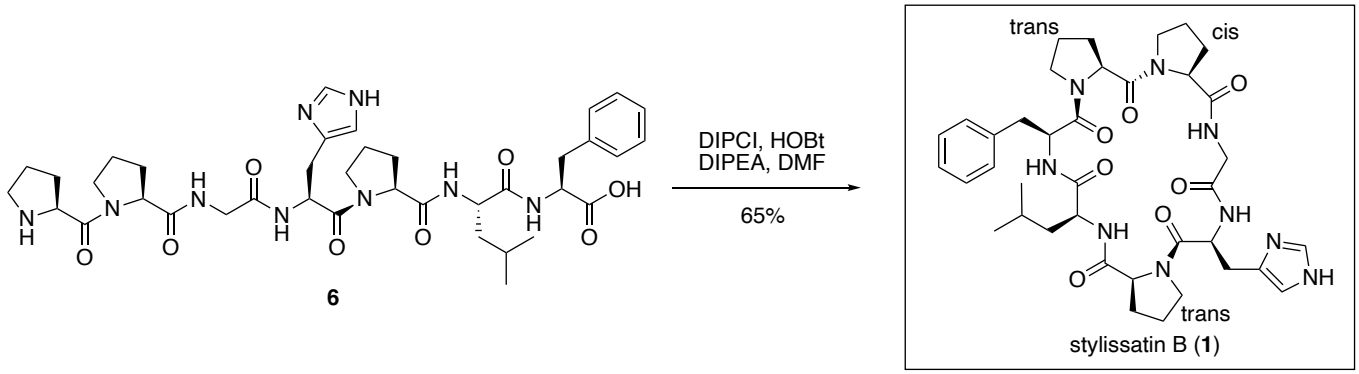
In conclusion, the first synthesis of the Pro-rich peptide stylissatin B (**1**) has been accomplished using Fmoc-SPPS, and the ReD-A method for the visualization of free, resin-supported amines. Further studies to understand the scope of this method and use it to prepare a variety of peptides are now underway.

Table 1. Linear peptides by Fmoc-SPPS

entry	sequence	yield (%)	ESIMS $[M+H]^{+}$ [a]	Rt (min)[b]
1	PLFPPGH (**2**)	33	764.29	9.61
2	HPLFPPG (**3**)	65	764.29	6.36
3	GHPLFPP (**4**)	32	764.29	9.17
4	PGHPLFP (**5**)	53	764.38	8.18
5	PPGHPLF (**6**)	54	764.38	7.98
6	FPPGHPL (**7**)	37	764.38	6.22
7	LFPPGHP (**8**)	35	764.32	5.27

[a] MS (ESI) shows *m/z* 764.40 based on calculated for $C_{38}H_{54}N_9O_8$ $[M+H]^{+}$.

[b] RP-HPLC (flow rate of 1 mL/min): linear gradient from 10% to 90% MeCN over 30 min.



Scheme 2. Synthesis of stylissatin B (**1**)

EXPERIMENTAL

General All solvents were reagent grade. All commercial reagents were of the highest purity available. Optical rotations were determined with a JASCO P-2200 polarimeter at the sodium D line. IR spectra were recorded on a Spectrum Two (PerkinElmer, Waltham, MA, USA). ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on JEOL JNM-ECA600 spectrometer. Chemical shifts are reported in ppm with reference to solvent signals [^{1}H NMR: DMSO-d_6 (2.50); ^{13}C NMR: DMSO-d_6 (39.5)]. Mass spectra were obtained using JEOL AccuTOF JMS-T100LC (ESI-MS). Analytical HPLC was carried out using a COSMOSIL 5C_{18}-AR-II column (4.6ID×150 mm) eluting with a linear gradient of MeCN (0.1% TFA) in H_2O (0.1% TFA) over a run time of 30 min (flow rate of 1 mL/min), a HITACHI L-2400 as a UV-Vis

detector, and a HITACHI L-2130 pump. Preparative HPLC was performed with a COSMOSIL 5C_{18}-AR-II column (10ID x 250 mm) eluting with a linear gradient of MeCN (0.1% TFA) in H_2O (0.1% TFA) at a run time of 30 min (flow rate of 2 mL/min), on a HITACHI L-2400 as a UV-Vis detector, HITACHI L-2130 Pump. UV measurements were recorded at a wavelength of 220 nm for the peptide analyses.

Linear peptide PPGHPLF (6) To 2-chlorotrityl chloride resin (50 mg, 80 μmol) pre-swollen in DMF for 30 min was added a solution of Fmoc-Phe-OH (124 mg, 0.32 mmol) and DIPEA (27 μL, 0.32 mmol) in DMF (2 mL). The reaction mixture was gently agitated at room temperature. After 2 h, the resin was filtered and washed with DMF, then added to a solution of 20% piperidine in DMF. The mixture agitated at room temperature. After 30 min, the resin was filtered and washed with DMF. After 2 h, the resin was filtered and washed with DMF, then added to a solution of 20% piperidine in DMF. The mixture was agitated at room temperature. After 30 min, the resin was filtered and washed with DMF. To the peptidyl resin was added a mixture of Fmoc-Leu-OH (113 mg, 0.32 mmol), DIPCI (27 μL, 0.32 mmol), HOBt (24 mg, 0.32 mmol) and DIPEA (27 μL, 0.32 mmol) in DMF (2 mL), and the reaction mixture was agitated at room temperature. After 2 h, the resin was filtered and washed with DMF, then a solution of 20% piperidine in DMF was added and the mixture agitated at room temperature. After 30 min, the resin was filtered and washed with DMF. To the peptidyl resin was added a mixture of Fmoc-Pro-OH (108 mg, 0.32 mmol), DIPCI (27 μL, 0.32 mmol), HOBt (24 mg, 0.32 mmol) and DIPEA (27 μL, 0.32 mmol) in DMF (2 mL). The reaction mixture was agitated at room temperature for 2 h, after which the resin was filtered and washed with DMF, then a solution of 20% piperidine in DMF was added and the mixture agitated at room temperature. After 30 min, the resin was filtered and washed with DMF. To the peptidyl resin was added a mixture of Fmoc-D-His(Trt)-OH (198 mg, 0.32 mmol), DIPCI (27 μL, 0.32 mmol), HOBt (24 mg, 0.32 mmol) and DIPEA (27 μL, 0.32 mmol) in DMF (2 mL) and the reaction mixture was agitated at room temperature. After 2 h, the resin was filtered and washed with DMF, then a solution of 20% piperidine in DMF was added and the mixture agitated at room temperature. After 30 min, the resin was filtered and washed with DMF. The resin was filtered and washed with DMF. To the peptidyl resin was added a mixture of Fmoc-Gly-OH (95 mg, 0.32 mmol), DIPCI (27 μL, 0.32 mmol), HOBt (24 mg, 0.32 mmol) and DIPEA (27 μL, 0.32 mmol) in DMF (2 mL), and the reaction mixture was agitated at room temperature. After 2 h, the resin was filtered and washed with DMF, then a solution of 20% piperidine in DMF was added and the mixture agitated at room temperature. After 30 min, the resin was filtered and washed with DMF. To the peptidyl resin was added a mixture of Fmoc-Pro-OH (108 mg, 0.32 mmol), DIPCI (27 μL, 0.32 mmol), HOBt (24 mg, 0.32 mmol) and DIPEA (27 μL, 0.32 mmol) in DMF (2 mL) was added and the mixture agitated at room temperature. After 2 h, the resin was filtered and washed with DMF, then added to a solution of 20% piperidine in DMF. The mixture was agitated at room temperature. After 30 min, the resin

was filtered and washed with DMF. To the peptidyl resin was added a mixture of Fmoc-Pro-OH (108 mg, 0.32 mmol), DIPCI (27 μL, 0.32 mmol), HOBt (24 mg, 0.32 mmol) and DIPEA (27 μL, 0.32 mmol) in DMF (2 mL) was added and the mixture agitated at room temperature. After 2 h, the resin was filtered and washed with DMF, then added to a solution of 20% piperidine in DMF. The mixture was agitated at room temperature. After 30 min, the resin was filtered and washed with DMF. To the peptidyl resin was added TFA (6.0 mL), H_2O (0.15 mL) and TIPS (0.15 mL) and agitated at room temperature. After 2 h, the mixture was filtered and concentrated under reduced pressure. The residue was redissolved in Et_2O, centrifuged and decanted to give crude linear peptide **6** (34 mg, 43 μmol, 53.5%) as a white powder. $[\alpha]_D^{27}$ -46.1 (c 1.52, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.94 (1H, s), 8.33 (1H, t, J = 6.6 Hz), 8.22 (1H, d, J = 9.6 Hz), 8.17 (1H, d, J = 9.0 Hz), 8.04 (1H, d, J = 9.6 Hz), 7.40 (1H, s), 7.30 (2H, m), 7.24 (1H, m), 7.23 (2H, m), 4.86 (1H, q, J = 9.0 Hz), 4.45 (1H, d, J = 6.0 Hz), 4.40 (1H, dt, J = 9.6, 6.6 Hz), 4.36 (1H, dd, J = 9.6, 6.0 Hz), 4.29 (1H, dd, J = 10.2, 5.4 Hz), 4.23 (1H, q, J = 8.4 Hz), 3.76 (2H, m), 3.75 (2H, m), 3.55 (5H, m), 3.10 (1H, dd, J = 10.2, 6.0 Hz), 3.07 (1H, dd, J = 10.2, 6.0 Hz), 2.97 (1H, dd J = 17.4, 9.0 Hz), 2.91 (1H, dd, J = 17.4, 9.0 Hz), 2.45 (1H, m), 2.15 (1H, m), 2.05 (1H, m), 1.93 (1H, m), 1.85 (2H, m), 1.80 (5H, m), 1.75 (1H, m), 1.66 (1H, m), 1.45 (2H, m), 0.91 (3H, d, J = 8.4 Hz), 0.85 (3H, d, J = 7.8 Hz). ^{13}C NMR (150 MHz, DMSO-d_6) δ 173.3, 172.3, 171.94, 171.88, 169.0, 168.7, 167.3, 137.9, 134.3, 129.6, 129.4, 128.6, 126.9, 117.9, 60.3, 59.9, 58.8, 53.7, 51.6, 50.1, 47.5, 47.3, 46.3, 42.2, 41.2, 37.1, 29.8, 29.7, 28.4, 27.1, 25.0, 24.9, 24.5, 24.1, 23.5, 22.1. IR (ATR) ν max cm^{-1}: 2959, 1628, 1532, 1447, 1182, 1130, 833, 798, 720, 703, 628. HRMS (ESI) *m/z*: calcd. for $C_{38}H_{54}N_9O_8$ $[M+H]^+$ 764.4088, found 764.4095. RP-HPLC (flow rate of 1 mL/min): t_R = 8.18 min (linear gradient from 10% to 90% MeCN over 30 min).

Cyclic peptide stylissatin B (1) DIPCI (9 μL, 100 μmol), HOBt·H_2O (8 mg, 100 μmol) and DIPEA (9 μL, 100 μmol) were added to a solution of linear peptide **6** (20 mg, 26 μmol) in DMF (1.0 mL) and the mixture was stirred for 24 h. The resulting mixture was purified by preparative HPLC to give stylissatin B (**1**) (8 mg, 17 μmol, 65%) as a colorless solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.07 (1H, s), 8.52 (1H, t, J = 6.0 Hz), 7.90 (1H, brs), 7.35 (1H, d, J = 9.0 Hz), 7.30 (1H, s), 7.22 (2H, m), 7.18 (1H, m), 7.16 (2H, m), 6.96 (1H, brs), 5.02 (1H, brs), 4.68 (1H, dd, J = 13.8, 7.8 Hz), 4.37 (1H, dd, J = 8.4, 4.8 Hz), 4.31 (1H, d, J = 8.4 Hz), 4.20 (1H, brs), 4.08 (1H, t, J = 8.4 Hz), 3.93 (1H, m), 3.76 (1H, m), 3.93 (1H, m), 3.57 (1H, m), 3.55-3.48 (2H, m, overlap), 3.46 (2H, m, overlap), 3.35-3.37 (2H, m, overlap), 3.08 (1H, dd, J = 13.8, 7.2 Hz), 2.77 (1H, m), 2.64 (1H, dd, J = 13.8, 5.4 Hz), 2.21 (1H, m), 2.20 (1H, m), 2.18 (1H, m), 2.07 (1H, m), 1.98 (1H, m), 1.93 (1H, m), 1.85 (2H, m), 1.77 (m, 3H), 1.68 (1H, m), 1.51 (2H, m), 1.45 (1H, m), 0.88 (3H, d, J = 5.4 Hz), 0.78 (3H, d, J = 6.6 Hz). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.9, 172.2, 171.9, 170.6, 170.4, 168.6, 167.9, 138.3, 134.8, 129.6, 129.3, 128.4, 126.5, 117.6, 62.8, 60.2, 59.4, 52.1, 51.4, 49.7,47.8, 47.3, 47.2, 44.2, 39.4, 37.6, 32.3, 29.5, 28.8, 27.8, 25.3, 25.1, 24.7, 23.4, 22.3, 21.2. IR (ATR) ν max cm^{-1}:

1627, 1517, 1446, 1201, 720. HRMS (ESI) *m/z*: calcd. for $C_{38}H_{51}N_9O_7Na$ $[M+Na]^+$ 768.3809, found 768.3792. RP-HPLC (flow rate of 1 mL/min): t_R = 16.7 min (linear gradient from 10% to 50% MeCN over 30 min). $[\alpha]_D^{28}$ -54.6 (c 0.71, MeOH).

Detection protocol for amino groups using ReD-A method After each reaction, 3 M TCNHPI/DMF is dropped to the resins in reaction tube at room temperature. After the checking by visual observations (yellow or brown), the resin was filtered and washed with DMF three times. The resin color returned to pale yellow (original color) and stained resin is proceeded to next reactions (coupling or deprotection).

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