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Solid-phase Total Synthesis of (−)-Apratoxin A and Its Analogues and Their Biological Evaluation

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Abstract: Two approaches for the solid-phase total synthesis of apratoxin A and its derivatives were accomplished. In synthetic route A, the peptide was prepared by the sequential coupling of the corresponding amino acids on trityl chloride SynPhase Lanterns. After cleavage from the polymer-support, macrolactamization of **10**, followed by thiazoline formation, provided apratoxin A. This approach, however, resulted in low yield because the chemoselectivity was not sufficient for the formation of the thiazoline ring though its analogue **33** was obtained. However, in synthetic route B, a cyclization precursor was prepared by solid-phase peptide synthesis by using amino acids **13**–**15** and **18**. The final macrolactamization was performed in solution to provide apratoxin A in high overall yield. This method was then successfully applied to the synthesis of apratoxin analogues. The cytotoxic activity of the synthetic derivatives was then evaluated. The epimer **34** was as potent as apratoxin A, and *O*-methyl tyrosine can be replaced by 7-azidoheptyl tyrosine without loss of activity. The 1,3-dipolar cycloaddition of **38** with phenylacetylene was performed in the presence of a copper catalyst without affecting the thiazoline ring.

Keywords: cytotoxicity · peptides · solid-phase synthesis · structure–activity relationships · total synthesis

Introduction

Apratoxin A (**1**), isolated from the marine cyanobacterium *Lyngbya majuscula*, exhibits potent cytotoxic activity against KB and LoVo cancer cells with IC_{50} values of 0.52 and 0.36 nM, respectively.[1] This 25-membered cyclic depsipeptide is comprised of proline, three methylated amino acids (*N*-methylisoleucine, *N*-methylalanine, and *O*-methyltyrosine), α,β-unsaturated modified cysteine residue (moCys), a dihydroxylated fatty acid moiety, and 3,7-dihydroxy-2,5,8,8-tetramethylnonanoic acid (Dtena) (Figure 1). Apratoxin A has a thiazoline ring in its macrocycle and epimerization at the α position of the thiazoline at the C34 position as well as β-elimination of the hydroxy group at the C35 position were observed under weakly acidic conditions, such as in $CDCl_3$ and the resulting dehydroapratoxin A (**2**), lost the cytotoxicity.[2] Apratoxin B–G (**3**–**8**), which are structurally related to apratoxin A, were also isolated and their biological activity was investigated.[2–5] After Forsyth and Chen reported the first total synthesis of apratoxin A (**1**),[6] two groups, including our group, have achieved the total synthesis of **1** and its related derivatives.[7a,8a] Oxoapratoxin A (**9**), an oxazoline analogue, is more accessible and has been synthesized by three groups.[7b,8b,9] In particular, Cavelier et al. recently reported the solid-phase total synthesis of **9**, which enabled the synthesis of various analogues of oxoapratoxin derivatives.[9] They prepared two linear precursors on a polymer support, and macrolactamization was successfully performed between *O*-MeTyr and a modified serine, and also between the modified serine and Dtena. The oxazoline was finally obtained from the modified serine moiety using dehydrocyclization. They also mentioned that the formation of oxazo-

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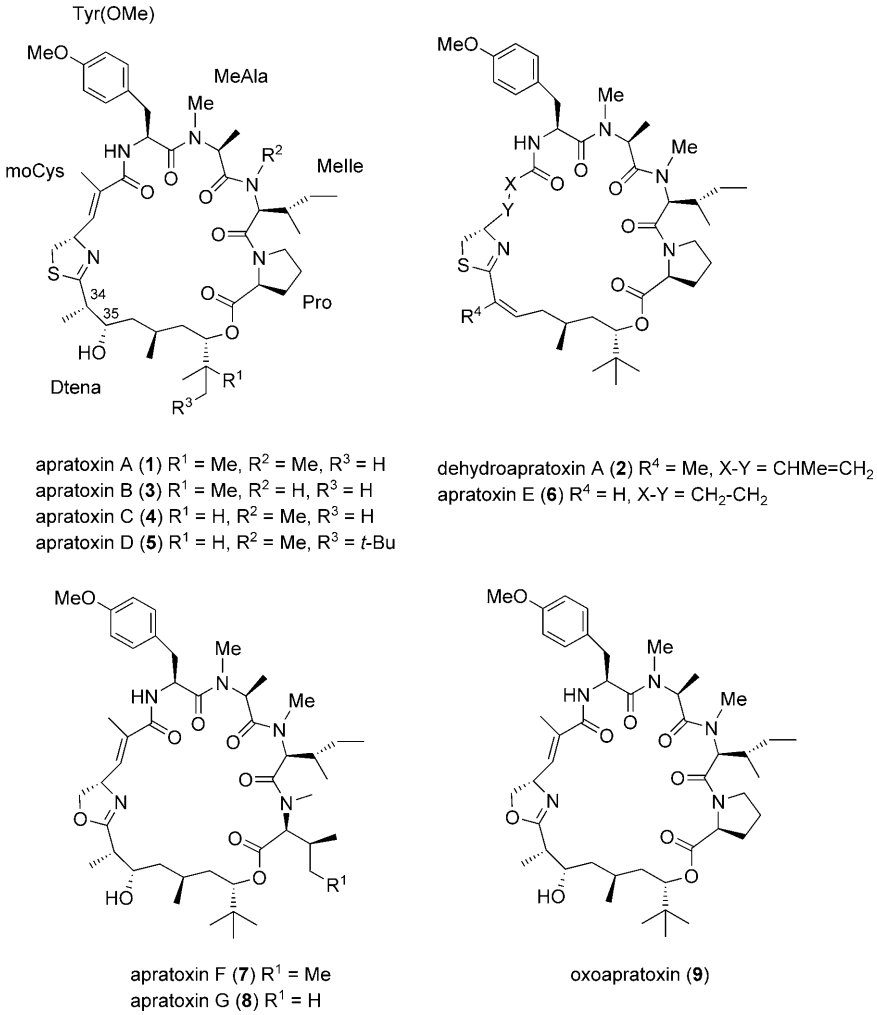


Figure 1. Structure of apratoxin A–G and oxoapratoxin A.

line prior to macrocyclization led to **9** without any adverse affect.

As apratoxin A (**1**) exhibits potent cytotoxicity of cancer cells, elucidation of its biological mode of action would be an interesting development and could lead to a new anticancer agent. In 2006, Luesch et al. proposed the mode of action of apratoxin A by functional genomics, where apratoxin A induces pronounced G_1 cell cycle arrest and apoptosis by the inhibition of phosphorylation of the signal transducer and the activator of transcription (STAT) 3.[10a] Upstream events were recently reported in which apratoxin A inhibits Janus kinase (JAK)/STAT signaling through rapid down-regulation of interleukin 6 signal transducer (gp130), and also depletes cancer cells of several cancer-associated receptor tyrosine kinases by preventing their N-glycosylation, which led to their rapid proteasomal degradation.[10b] On the other hand, Shen et al. have proposed that oxoapratoxin A (**9**) modulates degradation of Hsp90 client proteins through the chaperone-mediated autophagy (CMA) pathway.[11] This feature was proposed on the basis of affinity pull down assays by using a biotin probe of an oxoapratoxin derivative. In view of our independent approach to elucidate the biological mechanism of apratoxin A, we report the solid-phase total synthesis of apratoxin A (**1**) and its analogues, evaluation of their biological activity, and structure–activity relationships.

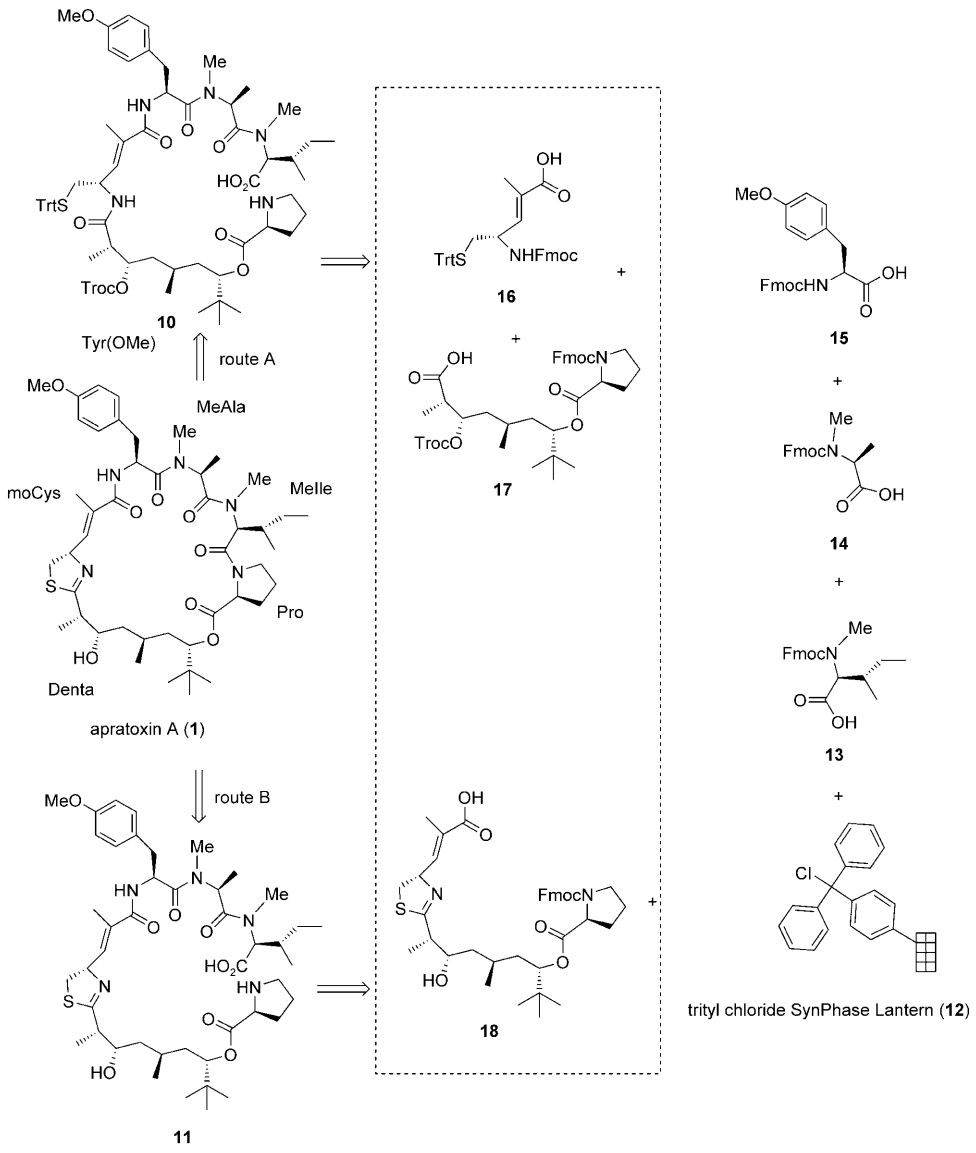
Abstract in Japanese:

ヒトの腫瘍細胞に対し強力な細胞毒性を有するアプラトキシンAの誘導体を効率よく合成するために固相合成を用いた全合成を行った。チアゾリン環を有する合成フラグメントを用いて，固相上で全てのフラグメントをカップリングすることに成功し,固相からの切り出し,および溶液中でマクロラクトン化を経てアプラトキシンAおよびアジド基を側鎖に有する誘導体の合成を行った。さらにそれら誘導体について細胞毒性評価を行い，構造活性相関を明らかにした。

Results and Discussion

Retrosynthetic Analysis

Our synthetic strategy has been illustrated in Scheme 1. We planned two synthetic routes using solid-phase peptide synthesis (SPPS). For route A, thiazoline formation was performed in the final step of the synthesis after macrolactamization of **10**, because the C34 position easily epimerizes and dehydration occurs under weakly acidic conditions. The formation of a thiazoline ring from the modified cysteine moiety must be chemoselective in the presence of the five amide bonds. For route B, the thiazoline ring was formed prior to the peptide coupling reaction, as previously reported in the solution phase synthesis. Macrolactamization will be the final step in this synthetic route. The thiazoline formed should be stable under the peptide coupling/deprotection conditions on a polymer-support.

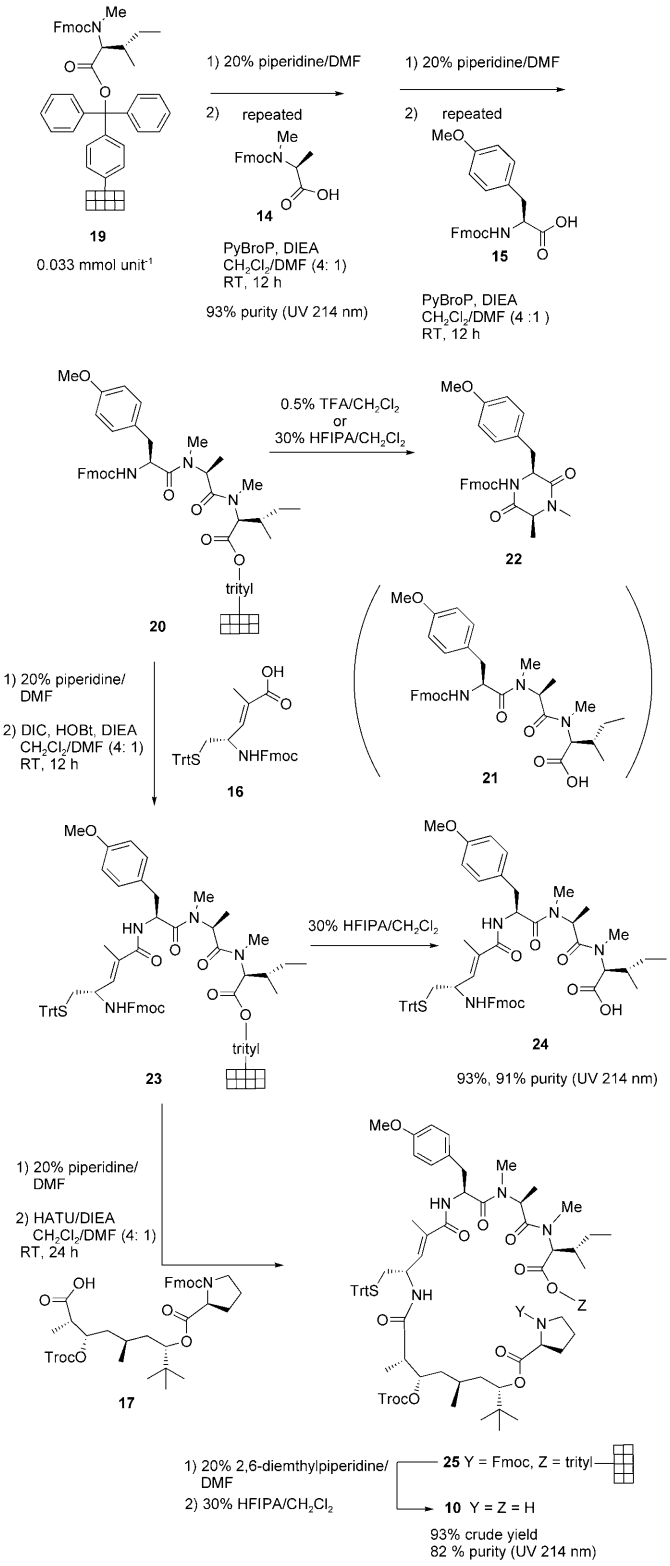


Scheme 1. The two synthetic routes toward solid-phase synthesis of apratoxin A (**1**).

Synthetic Approach of Route

A To synthesize various derivatives, SPPS was performed by using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy connected by a trityl linker. The cyclization precursors can be cleaved under mild conditions, such as using hexafluoroisopropyl alcohol (HFIPA). The attachment of Fmoc-Melle-OH (**13**) to trityl chloride SynPhase Lanterns, **12** preformed from commercially available trityl alcohol SynPhase Lanterns (0.037 mmol/lantern) with AcCl, was performed by using diisopropylethylamine (DIEA)/CH_2Cl_2 (Scheme 2).[12] After cleavage of **13** from the polymer-supported **19** with 0.5% TFA/CH_2Cl_2, the loading amount of **13** was determined to be 0.033 mmol per lantern. After removal of the Fmoc group with 20% piperidine/DMF, acylation with Fmoc-MeAla-OH (**14**) was repeatedly performed by using PyBroP/DIEA.[13] The purity was determined to be 93% by high-performance liquid chromatography (HPLC) analysis (UV 214 nm) after acid cleavage. PyBroP is known as a good condensation reagent for *N*-methylamino acids, and it actually gave better results than other reagents, such as diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) or HATU.[14] After Fmoc cleavage, acylation with Fmoc-Tyr(OMe)-OH (**15**) was also repeatedly performed by using PyBroP. Surprisingly, acid cleavage with either 0.5% TFA/CH_2Cl_2 or 30% hexafluoroisopropyl alcohol (HFIPA)/CH_2Cl_2[15] did not provide the desired tripeptide **21**, but rather provided diketopiperadine **22** in quantitative yield. As we did not observe acid free dipeptide cleaved between two *N*-methylamino acids, MeAla-Melle,[16] it is thought that the diketopiperadine would be formed, thereby releasing H-Melle-OH as a leaving group, only after acid cleavage from the polymer support. Therefore, we continued the next acylation with Fmoc-moCys-OH (**16**), after removal of the Fmoc group on the polymer support. The condensation reaction was performed by using DIC/HOBt to afford polymer-supported tetrapeptide **23** in 93% yield with 91% purity (UV 214 nm) determined by cleavage with 30% HFIPA/CH_2Cl_2. After removal of the Fmoc group, peptide coupling with Fmoc-Pro-Dtena-(OTroc)-OH (**17**)[7b] was performed by using HATU as a condensation reagent, the reaction was completed in 24 h, as monitored by the Kaiser test.[17] We were then able to reduce the amount of **17** to 2 equivalents. To prepare the cyclization precursor **10**, the polymer-supported peptide **25** was treated with 20% piperidine/DMF, followed by 30% HFIPA/CH_2Cl_2. However, the product obtained was not the desired compound **10**, but rather a piperidine adduct to the *N*-trichloroethoxycarbonyl (Troc) group. To avoid this problem, 2,6-dimethylpiperidine was utilized instead of piperidine.[18] The desired compound **10** was then obtained in 93% crude yield with purity of 82%, as detected by HPLC (UV 214 nm), and was then used for macrocyclic formation without purification.

Macrolactamization of **10** was achieved by using a solution of HATU/DIEA prior to the formation of a thiazoline



Scheme 2. Solid-phase synthesis of cyclization precursor **10** (route A).

ring (Scheme 3). After purification by silica gel chromatography, the cyclic peptide **26** was isolated in 60% overall yield from **19**. The crucial thiazoline ring formation was investigated by Kelly and co-workers.[19] Treatment of **26** with trifluoromethanesulfonic anhydride/triphenylphosphine oxide (Tf_2O/Ph_3PO), followed by cleavage of the Troc group with Zn/NH_4OAc provided a complex mixture. Repeatedly separated by preparative thin-layer chromatography (TLC), apratoxin A (**1**) was isolated in 10% overall yield over two steps. Treatment with an excess amount of Tf_2O/Ph_3PO decreased the yield because an undesired reaction with other amido groups also occurred. Although apratoxin A (**1**) was synthesized by using a solid-phase method, route A was not suitable for the synthesis of various derivatives of **1**. Therefore, we investigated the synthetic route B.

Synthetic Approach of Route B

Coupling of polymer-supported tetrapeptide **23** with thiazoline-containing **18** (2.5 equiv) was investigated as we previously reported, in solution-phase synthesis (Scheme 4).[7] PyAOP[20] was an effective condensation reagent to complete the reaction in DMF/CH_2Cl_2 in 24 h. After removal of the Fmoc group with 20% piperidine/DMF, the cyclization precursor **11** was cleaved from the polymer support by using 30% HFIPA/CH_2Cl_2 in 90% crude yield and 80% purity (UV 214 nm). This product was used in the next reaction without purification. Macrolactamization was performed by using HATU/DIEA as previously reported. After simple purification by silica gel chromatography, a diastereomeric mixture of apratoxin A (**1**) and 34-epi-apratoxin A was provided in 53% combined yield from **19** in a ratio of 85:15. The diastereomers were easily separated by preparative TLC, and their spectral data were identical to those previously reported.[7b] As previously reported, the epimerization at position 34 does not occur in the solution-phase synthesis. This partial epimerization could take place during removal of the Fmoc group in the treatment with 20% piperidine on the polymer support (Scheme 5).

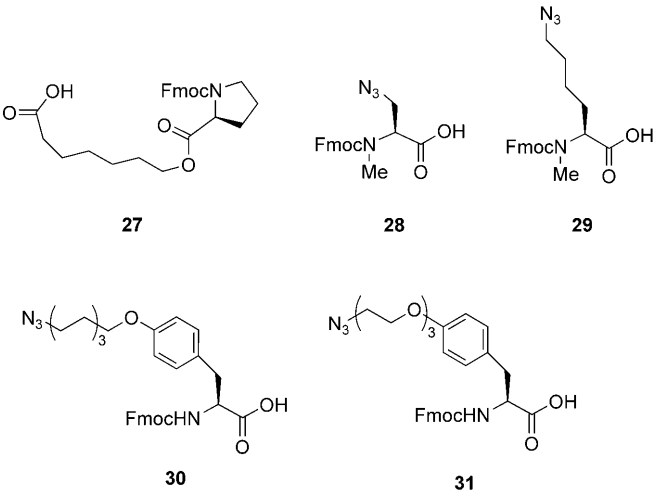
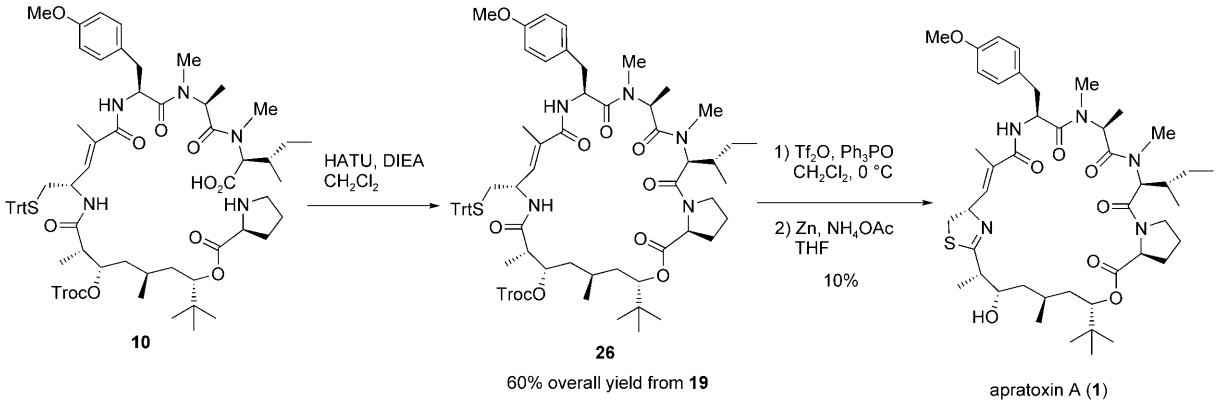
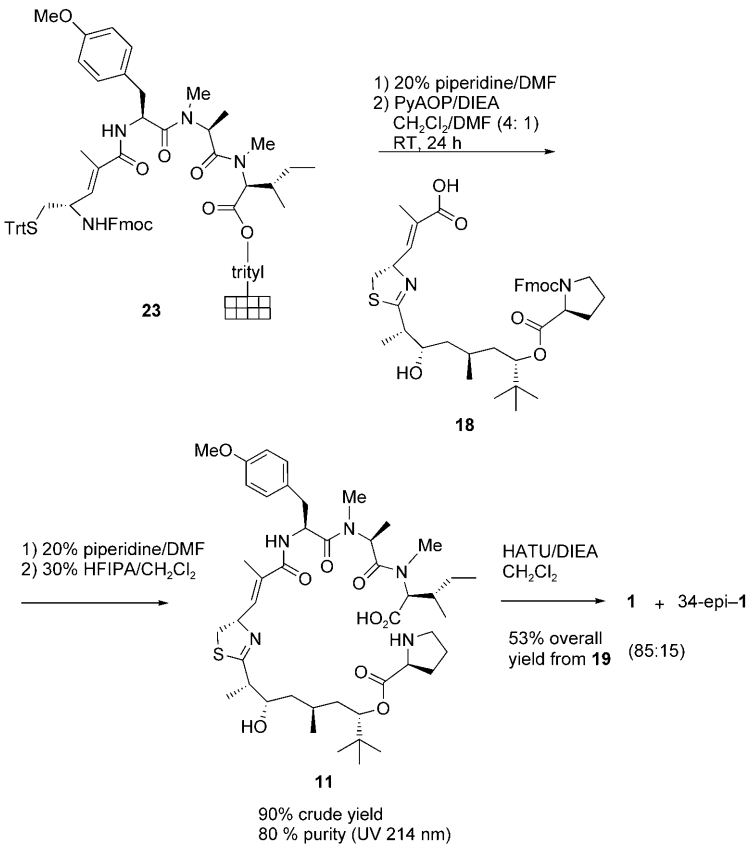


Figure 2. Synthetic fragments for the synthesis of apratoxin analogues.



Scheme 3. Thiazoline formation performed after macrolactamization in the total synthesis of apratoxin A (**1**) through route A.



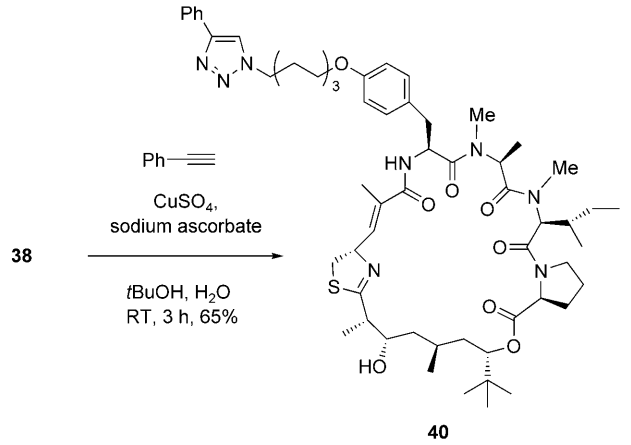
Scheme 4. Solid-phase total synthesis of apratoxin A (**1**) by route B.

Synthesis of Apratoxin Analogues

Because we have established the solid-phase total synthesis of apratoxin A, various analogues can now be prepared and applied to the precursor of a molecular probe to elucidate the structure–activity relationships (Figures 2 and 3). Silylation of **1** with triethylsilyl triflate provided triethylsilyl ether **32** in 50% yield, whereas acylation of the hydroxy group in **1** did not proceed. According to route A, simple amino acid **27**, instead of **17**, was utilized in the synthesis of **33**. Syntheses of azido-containing apratoxin derivatives **34–39** were also investigated. On the basis of the Huisgen 1,3-dipolar cycloaddition[21] or "click chemistry",[22] an azide is a suitable functional group for the attachment of a detection tag in activity-based small-molecular probes.[23] When azido-containing amino acids **28–31** were utilized in place of MeAla, Tyr(OMe) residue through route B, the desired products **36–39** were provided in 31–47% overall yields in diastereomeric ratios of 90:10–70:30 (Table 1, entries 8–11). The major diastereomers were isolated by preparative TLC or preparative reversed-phase HPLC. In the attempted synthesis of **34** in which Melle was replaced by **28** (entry 6), no product was detected because the linear peptide had been cleaved off from the polymer support; this led to the formation of diketopiperadine. The compound **35** (Figure 3) was obtained in a small amount, which was detected by LC-MS when **29** was used in place of Melle (entry 7).

Cytotoxic Activity of Apratoxin Analogues

We have now synthesized several apratoxin analogues and the cytotoxic activity of these against HeLa cells was evaluated, and the results are depicted in Table 1. Synthetic apratoxin A (**1**) was potent with an IC_{50} value of 0.19 μM (entry 1).[24] The 34-epimer was, interestingly, as potent as apratoxin A (entry 2). The stereochemistry of the methyl group at position 34 does not affect the potency. Oxoapra-



Scheme 5. Copper-catalyzed 1,3-dipolar cycloaddition of **38** with phenylacetylene.

toxin (**9**) was slightly less active than apratoxin A, and the result is in good agreement with that previously reported by Ma et al. (entry 3).[8] When the hydroxy group at position 35 was protected by a triethylsilyl group (**32**, entry 4), and there was no substituent on the fatty acid moiety (**33**, entry 5), there was an evident lack of cytotoxic activity. Thus, the substituents on the fatty acid moiety are important in inducing potent activity. Azido-containing analogues **35**, **38**, and **39** retained the activity (entries 7, 10, and 11), whereas the potency of **36** and **37** in which the MeAla was replaced by azido-containing MeAla and MeLys derivatives, respectively, decreased to one-tenth of that of **1** (entries 8 and 9). Substitution of the *O*-methyl group in a tyrosine residue with a 7-azidoheptyl group resulted in potent activity (**38**, entry 10). Thus, **38** could be a good precursor in the preparation of a molecular probe, which can be utilized for identification of the target molecule of apratoxin A.[25]

Coupling Reaction of an Azido-containing Apratoxin Analogue with Phenylacetylene

To study a molecular probe synthesis, we performed copper(I)-catalyzed 1,3-dipolar cycloaddition of **38** with phenyl acetylene (Scheme 5).[26,27] The reaction proceeded smoothly at room temperature in the presence of $CuSO_4$ and sodium ascorbate in *t*BuOH/H_2O, thereby leading to triazole **40** in 65% yield without affecting the thiazoline ring. Compound **40** retained potent cytotoxic activity (IC_{50} 0.03 μM; Table 1, entry 12). As the triazole formed did not affect the biological activity, **38** could be a good precursor for the preparation of an activity-based molecular probe having a detection tag.

Conclusions

We have demonstrated two routes for the solid-phase synthesis of apratoxin A analogues. In the synthetic route A, the peptide **25** was prepared by sequential coupling of the

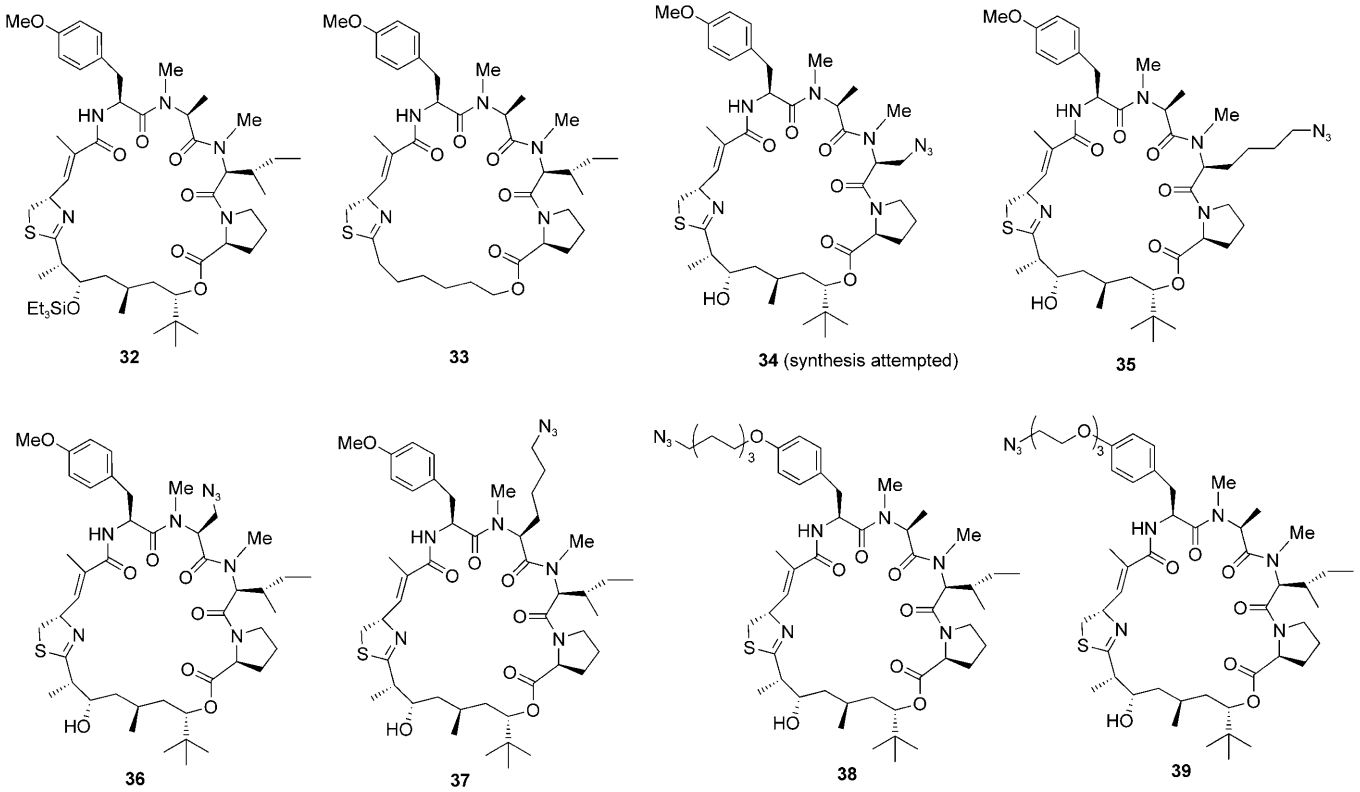


Figure 3. Synthetic apratoxin analogues **32**–**39**.

Table 1. Synthetic analogues of apratoxin A and their cytotoxic activity.

Entry	Compound	Yield[a] [%]	Ratio of diastereomers	Cytotoxicity[b] IC_{50} [μM]
1	**1**	53	85:15	0.19
2	34-epi-**1**[c]			0.19
3	**9**[c]			0.38
4	**32**			34
5	**33**	25[d]		28
6	**34**	0[e]		–
7	**35**	<3[f]	70:30	0.45
8	**36**	31	90:10	2.1
9	**37**	47	70:30	1.7
10	**38**	36	90:10	0.08
11	**39**	42	80:20	0.35
12	**40**			0.03

[a] Overall yield from polymer-supported **19** through method B. [b] Against HeLa cells. [c] Previously synthesized in our group (Ref. [7b]). [d] Through route A. [e] The desired cyclization precursor was not obtained after cleavage from the polymer-support. [f] Detected by LC-MS.

amino acids **13**–**17** on trityl chloride SynPhase Lanterns (**12**). After cleavage from the polymer support, macrolactamization of **10** followed by thiazoline formation, provided apratoxin A (**1**). Its analogue **33** was similarly synthesized. This approach, however, resulted in low yield because chemoselectivity was insufficient in the formation of a thiazoline ring. In contrast, in the synthetic route B, the cyclization precursor **11** was prepared by solid-phase peptide synthesis by using **13**–**15**, and **18**. The final macrolactamization was performed in solution to provide apratoxin A in high overall yield. This method was successfully applied to the synthesis of apratoxin analogues **35**–**39**. The cytotoxic activity of the synthetic derivatives was then evaluated. The 34-epimer was as potent as apratoxin A and *O*-methyl tyrosine could be replaced by *O*-7-azidoheptyl tyrosine with no loss of activity. The 1,3-dipolar cycloaddition of **38** with phenylacetylene was performed in the presence of a copper-catalyst without the thiazoline ring being affected. This method could be applied to the synthesis of a molecular probe to identify the target molecule of apratoxin A. Further studies are in progress in our laboratories.

Experimental Section

General procedure: NMR spectra were recorded on a JEOL Model ECP-400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) instrument in the indicated solvent. The spectra were referenced internally according to residual solvent signals of CD_2Cl_2 (^{1}H NMR, δ 5.3 ppm; ^{13}C NMR, δ 53.8 ppm). Data for ^{1}H NMR were recorded as follows: chemical shift (δ, ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br. = broad), coupling constant (Hz), and integration. Data for ^{13}C NMR were recorded in terms of chemical shift (δ, ppm). Optical rotations were measured with a JASCO P-1020 polarimeter. All reactions were monitored by thin-layer chromatography carried out on 0.2 mm E. Merck silica gel plates (60F-254) with UV light, visualized by *p*-anisaldehyde solution, ceric sulfate, or 10% ethanolic phosphomolybdic acid. Merck silica gel was used for column chromatography. ESI-TOF mass spectra were measured with a Waters LCT Premier™ XE spectrometer. HRMS (ESI-TOF) were calibrated with leucine enkephalin (Sigma) as an external standard. HPLC analysis was performed on a Waters 2695 Separation Module equipped with Waters 2996 Photodiode Array detector (Inertsil ODS-3, 3 μm, 4.6 × 75 mm) with a linear gradient of solvent I = 0.1% HCOOH in H_2O and solvent II = 0.1% HCOOH in CH_3CN with a flow rate of 1.0 mL min^{-1}. Method A: 10% of solvent II in solvent I (0–1 min), 10–100% of solvent II in solvent I (1–10 min), 100% of solvent II (10–12 min) or Method B: 50% of solvent II in solvent I (0–1 min), 50–100% of solvent II in solvent I (1–10 min), 100% of solvent II (10–12 min).

General procedure for loading of *N*-Fmoc protected amino acids to the trityl SynPhase Lanterns: Trityl alcohol SynPhase Lanterns (D-series, 0.037 mmol unit^{-1}) were treated with 10% AcCl in CH_2Cl_2 at room temperature for 3 h.[12] After completion of the reaction, the lanterns were washed with CH_2Cl_2 (2 min × 5). A solution of Fmoc amino acid (0.074 mmol unit^{-1}) and DIEA (44 μL unit^{-1}) in CH_2Cl_2 (1 mL unit^{-1}) at room temperature was added to the lanterns. After being left for 12 h, the lanterns were washed with DMF (3 min × 3), CH_2Cl_2 (3 min × 3), THF/H_2O = 4:1 (3 min × 3), MeOH (3 min × 2), and ether (3 min × 2), and then dried in vacuo to give Fmoc amino acid-supported lanterns.

General procedure for cleavage of polymer-supported peptidic acids: The peptide supported lantern (1 unit) was treated with HFIPA/CH_2Cl_2 (3:7). After being left for 30 min, the lantern was removed and the reaction mixture was concentrated in vacuo to give the corresponding acid.

General procedure for removal of the *N*-Fmoc group on the polymer support: The *N*-Fmoc protected peptide supported lanterns were treated with a solution of 20% piperidine or 2,6-dimethylpiperidine in case of **25** in DMF (10 min × 3). After the reaction was complete, the lanterns were washed with DMF (3 min × 5) and were then used for the next reaction.

General procedure for acylation with 14–15 and 28–31 on the polymer support: The amino acid-supported lanterns (0.033 mmol unit^{-1}) were treated with a solution of Fmoc amino acid **14**–**15** or **28**–**31** (0.148 mmol unit^{-1}), PyBroP (0.148 mmol unit^{-1}), and DIEA (0.296 mmol unit^{-1}) in CH_2Cl_2/DMF = 4:1 (1 mL unit^{-1}). After being left for 12 h, the lanterns were washed with DMF (3 min × 3), and the above coupling reaction was repeated. After the reaction was complete, the lanterns were washed with DMF (3 min × 3), CH_2Cl_2 (3 min × 3), THF/H_2O = 4:1 (3 min × 3), MeOH (3 min × 2), and ether (3 min × 2).

General procedure for acylation with 16 on the polymer support: The amino acid-supported lanterns (0.033 mmol unit^{-1}) were treated with a solution of Fmoc-MoCys-OH (**16**) (0.10 mmol unit^{-1}), DIC (15.5 μL, 0.10 mmol unit^{-1}), HOBt (13.5 mg, 0.10 mmol unit^{-1}), and DIEA (0.043 mL, 0.25 mmol unit^{-1}) in CH_2Cl_2/DMF = 4:1 (1 mL unit^{-1}). After being left for 12 h, the lanterns were washed with DMF (3 min × 3), CH_2Cl_2 (3 min × 3), THF/H_2O = 4:1 (3 min × 3), MeOH (3 min × 2), and ether (3 min × 2).

General procedure for acylation with 17 or 27 on a polymer support (route A): The amino acid-supported lanterns (0.033 mmol unit^{-1}) were treated with a solution of Fmoc amino acid **17** or **27** (0.066 mmol unit^{-1}), HATU (32.0 mg, 0.10 mmol unit^{-1}), and DIEA (0.043 mL, 0.25 mmol unit^{-1}) in CH_2Cl_2/DMF = 4:1 (1 mL unit^{-1}). After being left for 24 h, the lanterns were washed with DMF (3 min × 3) and CH_2Cl_2 (3 min × 3).

General procedure for acylation with 18 on the polymer support (route B): The tetrapeptide supported lanterns (0.033 mmol unit^{-1}) were treated with a solution of carboxylic acid **18** (0.0825 mmol unit^{-1}), PyAOP (0.0825 mmol unit^{-1}), and DIEA (0.165 mmol unit^{-1}) in CH_2Cl_2/DMF = 1:1 (0.8 mL unit^{-1}). After being left for 24 h, the lanterns were washed with DMF (3 min × 3) and CH_2Cl_2 (3 min × 3).

General procedure for macrolactamization: To a solution of the crude cyclization precursor in CH_2Cl_2 (25 mL), DIEA (0.15 mmol) and HATU

(0.050 mmol) were added at 0 °C under argon. After being stirred at 0 °C to room temperature for 24 h, the reaction mixture was concentrated in vacuo. The residue was used in the next reaction without purification.

26: ^{1}H NMR (400 MHz, CD_2Cl_2: δ = 7.17–7.38 (m, 15H, Trt), 7.14 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.3 Hz, 1H), 6.79 (d, J = 8.8 Hz, 2H), 6.33 (dq, J = 9.3, 1.0 Hz, 1H), 6.04 (d, J = 10.2 Hz, 1H), 5.25 (m, 1H), 5.03 (d, J = 11.2 Hz, 1H), 4.92 (d, J = 12.2 Hz, 1H), 4.80–4.89 (m, 3H), 4.65 (q, J = 6.8 Hz, 1H), 4.57 (d, J = 12.2 Hz, 1H), 4.48 (dd, J = 8.8, 3.9 Hz, 1H), 3.91 (m, 1H), 3.74 (s, 3H), 3.68 (m, 1H), 3.19 (dd, J = 13.2, 8.8 Hz, 1H), 2.87 (dd, J = 13.2, 6.4 Hz, 1H), 2.73 (dq, J = 6.8, 3.4 Hz, 1H), 2.60 (s, 3H), 2.57 (s, 3H), 2.45 (dd, J = 11.7, 7.3 Hz, 1H), 2.27 (dd, J = 11.7, 5.4 Hz, 1H), 2.22 (m, 1H), 2.05 (m, 1H), 1.76–2.00 (m, 4H), 1.71 (d, J = 1.0 Hz, 3H), 1.70 (m, 1H), 1.23–1.55 (m, 4H), 1.06 (d, J = 6.8 Hz, 3H), 1.01 (d, J = 6.8 Hz, 3H), 0.97 (d, J = 6.8 Hz, 3H), 0.96 (m, 1H), 0.86 (d, J = 6.8 Hz, 3H), 0.80 (t, J = 7.4 Hz, 3H), 0.78 ppm (s, 9H); HRMS (ESI-TOF): m/z (%) calcd for $C_{67}H_{86}N_5O_{11}SCl_3$: 1274.5188 [$M$+H$^+$]; found: 1274.5182.

Synthesis of apratoxin A (1) and 33 (route A): To a solution of **26** or a related compound composed of **27** instead of **17** (0.010 mmol) and triphenylphosphine oxide (16.5 mg, 0.060 mmol) in CH_2Cl_2 (0.4 mL), Tf_2O (5.0 mL, 0.030 mmol) was added dropwise at 0 °C under argon. After being stirred at the same temperature for 2 h, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ at 0 °C, and the aqueous layer was extracted with ethyl acetate. The organic layers were combined and washed with brine, then dried over Na_2SO_4, and concentrated in vacuo. The residue was used for the next reaction without further purification. To the solution of crude thiazoline in THF (0.4 mL) and aqueous NH_4OAc (1.0 M, 0.1 mL) zinc dust (10 mg, 0.15 mmol) was added at room temperature. After being stirred at room temperature for 30 min, the reaction mixture was partitioned between ethyl acetate and brine. The solution was extracted five times with ethyl acetate. The organic layers were combined, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography with silica gel (1 to 4% MeOH in $CHCl_3$) and preparative TLC on silica gel (90% ethyl acetate in hexane) to give **1** (0.9 mg, 0.001 mmol, 10% in 2 steps). Spectral data of **1** were identical to those previously reported. **33** was obtained according to the above procedure but without treatment with Zn/NH_4OAc. **33**: $[\alpha]_D^{22}$ = −159 (c = 0.705, MeOH); IR (neat): $\tilde{\nu}$ = 3431, 2932, 1736, 1639, 1512, 1442, 1247, 1177 cm^{-1}; ^{1}H NMR (400 MHz, CD_2Cl_2, major rotamer): δ = 7.13 (d, J = 8.8 Hz, 2H), 6.81 (d, J = 8.8 Hz, 2H), 6.47 (dq, J = 9.8 Hz, 1.0 Hz, 1H), 6.35 (d, J = 9.8 Hz, 1H), 5.36 (ddd, J = 9.8, 9.3, 5.4 Hz, 1H), 5.25 (ddd, J = 9.8, 8.8, 4.9 Hz, 1H), 4.92 (d, J = 11.7 Hz, 1H), 4.81 (q, J = 6.3 Hz, 1H), 4.34 (m, 1H), 4.22 (m, 1H), 4.04 (m, 1H), 3.93 (m, 1H), 3.75 (s, 3H), 3.59 (m, 1H), 3.47 (dd, J = 11.2, 8.8 Hz, 1H), 3.15 (dd, J = 12.7, 9.3 Hz, 1H), 3.10 (dd, J = 11.2, 4.9 Hz, 1H), 2.89 (dd, J = 12.7, 5.4 Hz, 1H), 2.77 (s, 3H), 2.59 (s, 3H), 2.54 (ddd, J = 15.6, 9.8, 5.9 Hz, 1H), 2.41 (ddd, J = 15.6, 8.8, 4.9 Hz, 1H), 2.18 (m, 1H), 1.84–2.09 (m, 4H), 1.94 (d, J = 1.0 Hz, 3H), 1.26–1.71 (m, 9H), 0.98 (m, 1H), 0.95 (d, J = 6.8 Hz, 3H), 0.83 (t, J = 7.8 Hz, 3H), 0.71 ppm (d, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2, major rotamer): δ = 172.3, 172.2, 171.9, 170.0, 169.7, 166.6, 158.8, 137.6, 130.6, 128.9, 128.7, 114.0, 73.4, 64.3, 59.6, 57.7, 55.3, 54.6, 50.0, 47.5, 39.5, 38.7, 34.1, 29.8, 29.6, 29.4, 28.8, 28.5, 27.7, 25.5, 25.4, 25.3, 25.1, 15.6, 14.1, 12.7, 9.9 ppm; HRMS (ESI-TOF): m/z (%) calcd for $C_{39}H_{57}N_5O_7S$: 740.4057 [M+H$^+$]; found: 740.4061.

35-*O*-Triethylsilyl apratoxin A (32): To a solution of apratoxin A (**1**) (2.3 mg, 2.7 μmol) in CH_2Cl_2 (0.4 mL), 2,6-lutidine (3.2 μL, 27.4 μmol) and TESOTf (3.1 μL, 13.7 μmol) were added at −50 °C under argon. After being stirred at the same temperature for 30 min, the reaction mixture was quenched with methanol at −50 °C, and diluted with ethyl acetate. The organic layers were combined and washed first with saturated aqueous $NaHCO_3$, then with brine, and then dried over Na_2SO_4, and concentrated in vacuo. The residue was purified twice by preparative TLC on silica gel (50% ethyl acetate in hexane) to give 35-*O*-triethylsilyl apratoxin A (**32**) (1.3 mg, 1.4 μmol, 50%). ^{1}H NMR (400 MHz, CD_2Cl_2): δ = 7.11 (d, J = 8.3 Hz, 2H), 6.80 (d, J = 8.3 Hz, 2H), 6.63 (dq, J = 9.3, 1.4 Hz, 1H), 6.34 (d, J = 9.8 Hz, 1H), 5.39 (m, 1H), 5.06 (ddd, J = 10.7, 9.3, 8.8 Hz, 1H), 4.88 (d, J = 11.2 Hz, 1H), 4.87 (q, J = 6.8 Hz, 1H), 4.71 (dd, J = 6.4, 3.9 Hz, 1H), 4.18 (m, 1H), 4.11 (dd, J = 8.3, 7.8 Hz, 1H), 3.99 (m, 1H), 3.74 (s, 3H), 3.51 (m, 1H), 3.33 (dd, J = 10.7, 8.8 Hz, 1H), 3.13 (dd, J = 13.2, 9.3 Hz, 1H), 2.86 (dd, J = 13.2, 5.8 Hz, 1H), 2.84 (dd, J = 10.7, 10.7 Hz, 1H), 2.79 (s, 3H), 2.77 (m, 1H), 2.57 (s, 3H), 2.20 (m, 1H), 2.00 (m, 1H), 1.91 (d, J = 1.4 Hz, 1H), 1.79–1.89 (m, 3H), 1.69 (m, 1H), 1.49 (m, 1H), 1.24–1.38 (m, 4H), 1.19 (d, J = 7.3 Hz, 3H), 0.99 (m, 1H), 0.98 (d, J = 6.3 Hz, 3H), 0.93 (t, J = 7.8 Hz, 9H), 0.92 (d, J = 6.8 Hz, 3H), 0.84 (s, 9H), 0.81 (t, J = 7.8 Hz, 3H), 0.66 (d, J = 6.8 Hz, 3H), 0.58 ppm (q, J = 7.8 Hz, 6H); HRMS (ESI-TOF): m/z (%) calcd for $C_{51}H_{83}N_5O_8SSi$: 954.5810 [M+H$^+$]; found: 954.5811.

Synthesis of apratoxin A (1), 34-epi-1, and 35–39 (route B): After macrolactamization of the corresponding cyclization precursor obtained by the above procedure, the residue was purified by column chromatography on silica gel (MeOH in $CHCl_3$) to provide the corresponding macrolactams as a diastereomeric mixture. When necessary, further purification was performed by preparative TLC on silica gel (ethyl acetate in *n*-hexane), or reversed phase preparative HPLC (CH_3CN-H_2O) to isolate the diastereomers in pure form.

35 major diastereomer: t_R 29.3 min (Inertsil C_{18} ODS, 3 μm, 4.6×250 mm, 1.0 mL min^{-1}, UV detection at 220 nm, an isocratic system of 65% aqueous CH_3CN); HRMS (ESI-TOF): m/z (%) calcd for $C_{45}H_{68}N_8O_8S$: 881.4959 [M+H$^+$]; found: 881.4958.

36 major diastereomer: t_R 9.13 min (method B); $[\alpha]_D^{23}$ = −169 (c = 0.130, MeOH); ^{1}H NMR (400 MHz, CD_2Cl_2): δ = 7.14 (d, J = 8.8 Hz, 2H), 6.83 (d, J = 8.8 Hz, 2H), 6.23 (dq, J = 9.3, 1.0 Hz, 1H), 5.93 (d, J = 9.2 Hz, 1H), 5.22 (m, 1H), 5.11 (d, J = 11.7 Hz, 1H), 5.07 (m, 1H), 4.93 (dd, J = 13.7, 2.9 Hz, 1H), 4.46 (d, J = 11.2 Hz, 1H), 4.16 (t, J = 7.8 Hz, 1H), 4.10 (m, 1H), 3.97 (dd, J = 12.2, 5.4 Hz, 1H), 3.75 (s, 3H), 3.44–3.61 (m, 2H), 3.42 (dd, J = 10.7, 8.8 Hz, 1H), 3.20–3.36 (m, 2H), 3.02–3.08 (m, 2H), 2.98 (s, 3H), 2.89 (dd, J = 13.2, 5.9 Hz, 1H), 2.65 (s, 3H), 2.57 (m, 1H), 2.00–2.26 (m, 4H), 1.91 (d, J = 1.0 Hz, 3H), 1.72–1.90 (m, 3H), 1.50 (m, 1H), 1.22–1.32 (m, 2H), 1.09 (m, 1H), 1.03 (d, J = 6.8 Hz, 3H), 0.93 (d, J = 6.8 Hz, 3H), 0.90 (m, 1H), 0.85 (d, J = 6.8 Hz, 3H), 0.85 (s, 9H), 0.81 ppm (t, J = 6.8 Hz, 3H); HRMS (ESI-TOF): m/z (%) calcd for $C_{45}H_{68}N_8O_8S$: 881.4959 [M+H$^+$]; found: 881.4965.

37 major diastereomer: t_R 9.53 min (method B); $[\alpha]_D^{23}$ = −150 (c 0.10, MeOH); ^{1}H NMR (400 MHz, CD_2Cl_2): δ = 7.16 (d, J = 8.8 Hz, 2H), 6.80 (d, J = 8.8 Hz, 2H), 6.22 (dq, J = 9.8, 1.0 Hz, 1H), 5.93 (d, J = 9.3 Hz, 1H), 5.21 (m, 1H), 5.12 (d, J = 11.7 Hz, 1H), 5.07 (m, 1H), 4.93 (dd, J = 12.2, 1.5 Hz, 1H), 4.48 (d, J = 11.2 Hz, 1H), 4.15 (t, J = 7.8 Hz, 1H), 4.12 (m, 1H), 3.75 (s, 3H), 3.43–3.63 (m, 3H), 3.43 (dd, J = 10.7, 8.3 Hz, 1H), 2.98–3.20 (m, 4H), 2.96 (s, 3H), 2.89 (dd, J = 13.2, 5.4 Hz, 1H), 2.64 (s, 3H), 2.57 (m, 1H), 2.00–2.28 (m, 4H), 1.92 (d, J = 1.0 Hz, 3H), 0.82–1.90 (m, 14H), 1.03 (d, J = 6.8 Hz, 3H), 0.94 (d, J = 6.3 Hz, 3H), 0.86 (d, J = 6.8 Hz, 3H), 0.85 (s, 9H), 0.81 ppm (t, J = 6.8 Hz, 3H); HRMS (ESI-TOF): m/z (%) calcd for $C_{48}H_{74}N_8O_8S$: 923.5429 [M+H$^+$]; found: 923.5422.

38 major diastereomer: t_R 11.0 min (method A); $[\alpha]_D^{25}$ = −162 (c = 0.150, MeOH); ^{1}H NMR (400 MHz, CD_2Cl_2): δ = 7.11 (d, J = 8.8 Hz, 2H), 6.78 (d, J = 8.8 Hz, 2H), 6.21 (dq, J = 9.8, 1.0 Hz, 1H), 5.98 (d, J = 8.3 Hz, 1H), 5.23 (m, 1H), 5.12 (d, J = 11.7 Hz, 1H), 5.00 (m, 1H), 4.93 (dd, J = 12.2, 1.5 Hz, 1H), 4.49 (d, J = 11.2 Hz, 1H), 4.11–4.16 (m, 2H), 3.90 (t, J = 6.8 Hz, 2H), 3.42–3.62 (m, 3H), 3.26 (m, 1H), 3.24 (t, J = 6.8 Hz, 2H), 2.99–3.10 (m, 2H), 2.86 (dd, J = 13.2, 5.4 Hz, 1H), 2.79 (s, 3H), 2.62 (s, 3H), 2.56 (m, 1H), 1.97–2.28 (m, 4H), 1.92 (d, J = 1.0 Hz, 3H), 1.70–1.90 (m, 5H), 1.57 (m, 2H), 1.32–1.62 (m, 7H), 1.19–1.29 (m, 2H), 1.11 (d, J = 6.8 Hz, 3H), 1.05 (m, 1H), 1.03 (d, J = 6.8 Hz, 3H), 0.94 (d, J = 6.8 Hz, 3H), 0.89 (m, 1H), 0.86 (d, J = 6.8 Hz, 3H), 0.85 (s, 9H), 0.82 ppm (t, J = 7.3 Hz, 3H); HRMS (ESI-TOF): m/z (%) calcd for $C_{51}H_{80}N_8O_8S$: 965.5898 [M+H$^+$]; found: 965.5900.

39 major diastereomer: t_R 10.8 min (method A); $[\alpha]_D^{24}$ = −134 (c = 0.110, MeOH); ^{1}H NMR (400 MHz, CD_2Cl_2): δ = 7.13 (d, J = 8.8 Hz, 2H), 6.81 (d, J = 8.8 Hz, 2H), 6.20 (dq, J = 8.8, 1.0 Hz, 1H), 5.98 (d, J = 9.8 Hz, 1H), 5.21 (m, 1H), 5.12 (d, J = 11.7 Hz, 1H), 5.01 (m, 1H), 4.93 (dd, J = 12.2, 2.0 Hz, 1H), 4.48 (d, J = 11.2 Hz, 1H), 4.12–4.16 (m, 2H), 4.05 (brt, J = 4.6 Hz, 2H), 3.79 (brt, J = 4.6 Hz, 2H), 3.58–3.66 (m, 7H), 3.48 (m, 1H), 3.42 (dd, J = 11.2, 3.9 Hz, 1H), 3.35 (t, J = 5.4 Hz, 2H), 3.25 (brs, 1H), 3.07 (dd, J = 11.2, 3.9 Hz, 1H), 3.04 (dd, J = 12.2, 11.2 Hz, 1H), 2.86 (dd, J = 12.2, 4.9 Hz, 1H), 2.79 (s, 3H), 2.62 (s, 3H), 2.60 (m, 1H), 2.00–2.24 (m, 4H), 1.92 (d, J = 1.0 Hz, 3H), 1.75–1.91 (m, 3H), 1.50 (m, 1H), 1.23–

1.29 (m, 2H), 1.12 (d, J=6.4 Hz, 3H), 1.03 (m, 1H), 1.02 (d, J=7.3 Hz, 3H), 0.94 (d, J=6.8 Hz, 3H), 0.86 (d, J=6.8 Hz, 3H), 0.85 (s, 9H), 0.84 (m, 1H), 0.82 ppm (t, J=7.3 Hz, 3H); HRMS (ESI-TOF): m/z (%) calcd for $C_{50}H_{78}N_8O_{10}S$: 983.5640 [M+H$^+$]; found: 983.5640.

Formation of triazole 40 by 1,3-dipolar cycloaddition of 38 with phenyl acetylene: To a solution of apratoxin A derivative **38** (0.7 mg, 0.7 μmol) and phenylacetylene (1.0 μL, 3.6 μmol) in 2-methyl-2-propanol (0.1 mL) and H_2O (0.1 mL), 1 M sodium ascorbate (1.0 μL, 1.0 μmol) and 0.5 M $CuSO_4$ (1.0 μL, 0.5 μmol) was added, respectively, at room temperature under argon. After being stirred at room temperature for 3 h, the reaction mixture was quenched with saturated aqueous $NaHCO_3$, and the aqueous layer was extracted with ethyl acetate. The organic layers were combined and washed with brine, then dried over Na_2SO_4, and concentrated in vacuo. The residue was purified twice by preparative TLC on silica gel (90% EtOAc in hexane) to afford triazole **40** (0.5 mg, 0.46 μmol, 65%). t_R 10.2 min (method B); $[\alpha]_D^{24}=-145$ (c=0.070, MeOH); ^{1}H NMR (400 MHz, CD_2Cl_2): δ=7.80 (dd, J=6.8, 1.5 Hz, 2H), 7.77 (s, 1H), 7.41 (dd, J=6.8, 6.8 Hz, 2H), 7.31 (m, 1H), 7.11 (d, J=8.8 Hz, 2H), 6.77 (d, J=8.8 Hz, 2H), 6.20 (dq, J=9.3, 1.0 Hz, 1H), 5.97 (d, J=9.8 Hz, 1H), 5.21 (m, 1H), 5.12 (d, J=11.2 Hz, 1H), 5.00 (m, 1H), 4.93 (dd, J=12.2, 2.0 Hz, 1H), 4.48 (d, J=10.8 Hz, 1H), 4.37 (t, J=7.3 Hz, 2H), 4.12–4.16 (m, 2H), 3.89 (t, J=6.4 Hz, 2H), 3.43–3.62 (m, 2H), 3.42 (dd, J=11.2, 8.8 Hz, 1H), 3.25 (brs, 1H), 3.06 (dd, J=11.2, 3.9 Hz, 1H), 3.02 (dd, J=13.6, 11.2 Hz, 1H), 2.85 (dd, J=13.6, 5.4 Hz, 1H), 2.78 (s, 3H), 2.62 (s, 3H), 2.57 (m, 1H), 1.98–2.25 (m, 4H), 1.92 (m, 2H), 1.91 (d, J=1.0 Hz, 3H), 1.80–1.90 (m, 2H), 1.68–1.77 (m, 3H), 1.20–1.52 (m, 9H), 1.11 (d, J=6.8 Hz, 3H), 1.04 (m, 1H), 1.03 (d, J=6.8 Hz, 3H), 0.94 (d, J=6.4 Hz, 3H), 0.87 (m, 1H), 0.86 (d, J=6.8 Hz, 3H), 0.85 (s, 9H), 0.82 ppm (t, J=7.4 Hz, 3H).

Cytotoxic activity: Cytotoxicity against human cervical carcinoma HeLa cells was determined by a colorimetric assay by using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt]. Cells were cultured in Dulbecco's Modification of Eagle's **Me**dium (DMEM; Wako Pure Chemical Industries, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (GIBCO, Carlsbad, CA), penicillin (100 units mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 °C in a humidified incubator under a 5% CO_2 atmosphere. The 384-well plates were seeded with aliquots of a 20 μL medium containing 1.0×10^3 cells per well and were incubated overnight before being treated with compounds at various concentrations for 48 h. Plates were incubated for 1 h at 37 °C after the addition of 2 μL WST-8 reagent solution (Cell Counting Kit; Dojindo, Kumamoto, Japan) per well. The absorption of formazan dye was measured at 450 nm. The vehicle solvent (DMSO) was used as a negative control.

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[24] Based on the above result, the IC_{50} value of synthetic apratoxin A measured in our hands was not as small as that previously reported. In our collaborative study with Professor Gerwick's group, however, it was found that synthetic apratoxin A exhibited the same potency as the natural product in vitro. Furthermore, the synthetic one had a significant in vivo antitumor effect in conventional mice bearing syngeneic solid tumors. See Ref. 5.
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