CHEMISTRY
A EUROPEAN JOURNAL

ChemPubSoc
Europe

DOI: 10.1002/chem.201203667

Total Synthesis and Biological Evaluation of Grassypeptolide A

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Abstract: Herein, we describe in full our investigations into the synthesis of grassypeptolide A (**1**) in 17 linear steps with an overall yield of 11.3%. In particular, this work features the late-stage introduction of sensitive bis(thiazoline) heterocycles and 31-membered macrocyclization conducted at the sterically congested secondary amide site in superb conversion (72% yield). Biological evaluation indicated that grassypeptolide A significantly inhibited cancer cell proliferation in a dose-dependent manner. It induced cancer cell apoptosis, which was associated with increased cleavage of poly(ADP-ribose) polymerase (PARP) and decreased expression of bcl-2 and bcl-xL. Furthermore, grassypeptolide A also caused cell cycle redistribution by increasing cells in the G1 phase and decreasing cells in the S and G2 phases. In addition, cell cycle arrest was correlated with downregulation of cyclin D and upregulation of p27 and p21.

Keywords: cyclodepsipeptides · cytotoxicity · grassypeptolide · thiazolines · total synthesis

Introduction

Secondary metabolites produced by marine cyanobacteria are key synthetic targets in the quest for new leads in the pharmaceutical industry.[1] One particular species, *Lyngbya confervoides*,[2] is responsible for the production of a considerable number of bioactive peptide and depsipeptides. These compounds occur in either linear or cyclic forms with a variety of significant associated biological activities, including cytotoxicity (obyanamide[3]), trypsin inhibition (pompanopeptin A[4]), carboxypeptidase inhibition (pompanopeptin B[4]), cathepsin E inhibition (grassystatins A–C[5]), elastase inhibition (lyngbyastatins 5–7,[6] largamides A–C[7]), chymotrypsin inhibition (lyngbyastatin 4[8]), and antifungal activity (lobocyclamides A–C[9]). Some of the metabolic signatures of cyanobacterial peptides include a high degree of N-methylated amino acids, D-amino acids, and β-amino acids. We have been interested in marine secondary metabolites, especially the cyclopeptides and cyclodepsipeptides,[10] and view their syntheses as playing a key role in structural confirmation, structural modification, and subsequent activity control. We recently reported the first convergent total synthesis of grassypeptolide A.[10e] Herein, we describe our initial strategy for the total synthesis of grassypeptolide A and the discoveries that resulted from this approach. A detailed description of the successful route to grassypeptolide A and the establishment of a modality for its biological evaluation are also provided.

Grassypeptolide A (**1**, formerly named grassypeptolide), isolated from an extract of *Lyngbya confervoides* collected off Grassy Key in Florida by the Luesch group, is a potential anticancer cyclodepsipeptide.[11] It inhibited cancer cell growth with IC_{50} values from 1.0 to 4.2 μM. The chemical structure of **1** was established by using a combination of chemical and spectral techniques, and subsequently validated through X-ray analysis. Grassypeptolide A is composed of a number of unique, nonproteinogenic amino acid residues, such as the 2-methyl-3-aminobutyric acid (Maba), 2-aminobutyric acid (Aba), and several D- and N-methylated amino acids. Grassypeptolide A possesses a 31-membered macrolactone with a bis(thiazoline)-ring moiety. Although several bioactive cyclopeptides incorporating a single thiazoline have been reported,[12] the closest natural product related to **1** is lissoclinamide 7 (**2**),[13] which also contains two thiazoline rings (Figure 1). Unlike lissoclinamide 7, grassypeptolide A contains a free hydroxy group that is susceptible to cyclodehydration, leading to an oxazoline.

Since the first total synthesis of grassypeptolide, reported by us in 2010,[10e] other members of the grassypeptolide

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201203667.

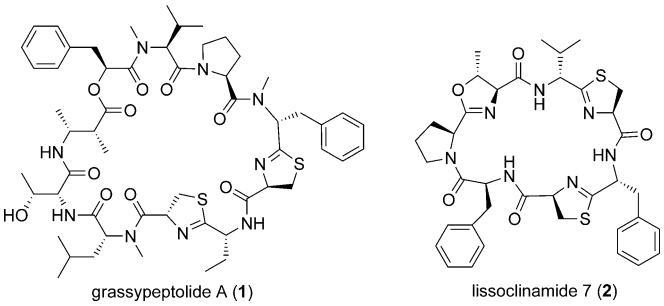


Figure 1. Structures of grassypeptolide A and lissoclinamide 7.

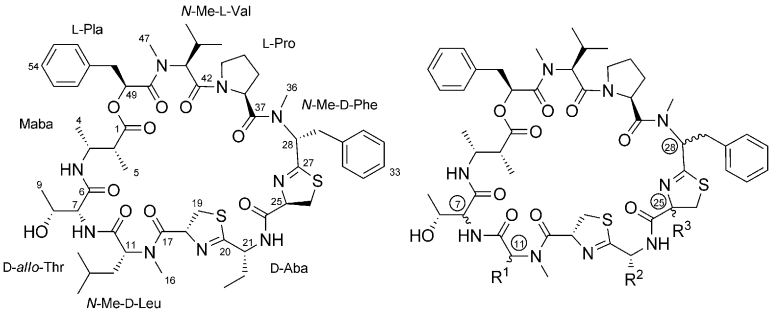


Figure 2. Structures of grassypeptolide A and additional analogues.

Table 1. Substituent and stereochemistry of grassypeptolides.

Grassypeptolides	R^1	R^2	R^3	Stereochemistry
A (**1**)	*i*Bu	Et	H	7*R*,11*R*,25*R*,28*R*
B (**3**)	*i*Bu	Me	H	7*R*,11*R*,25*R*,28*R*
C (**4**)	*i*Bu	Et	H	7*R*,11*R*,25*R*,28*S*
D (**5**)	*i*Bu	Et	Me	7*R*,11*R*,25*S*,28*S*
E (**6**)	*i*Bu	Et	Me	7*S*,11*S*,25*S*,28*S*
F (**7**)	Bn	Et	H	7*R*,11*R*,25*R*,28*R*
G (**8**)	Bn	Me	H	7*R*,11*R*,25*R*,28*R*

family were isolated (Figure 2 and Table 1). All of them are 31-membered cyclodepsipetides with a bis(thiazoline)-ring moiety. The structures of grassypeptolides B (**3**) and C (**4**)[14] are closely related to grassypeptolide A, with only minimal differences. When the ethyl substituent of **1** is changed to a methyl substituent in **3**, activity is slightly reduced (3–4-fold). Grassypeptolide C is the epimer of **1** at C28, with a L-Phe unit flanking the bis(thiazoline) moiety. Unlike trunkamide[15] and lissoclinamide 7 (**2**),[13b] the Phe moiety in both **4** and **1** cannot undergo base-induced interconversion, which may reflect less overall strain in the macrocycle of grassypeptolides.[14] Grassypeptolides D (**5**) and E (**6**),[16] which have identical methyl-substituted thiazoline subunits, were isolated from the marine cyanobacterium *Leptolyngbya sp.* collected from the Red Sea by the McPhail group. Both compounds were found to have significant cytotoxicity to HeLa and mouse neuro-2a blastoma cells. Grassypeptolides F (**7**) and G (**8**)[17] were isolated from an extract of the Palauan cyanobacterium *Lyngbya majuscula* by the McKee group in 2011. Both **7** and **8** show moderate inhibitory activity against the transcription factor AP-1.

Results and Discussion

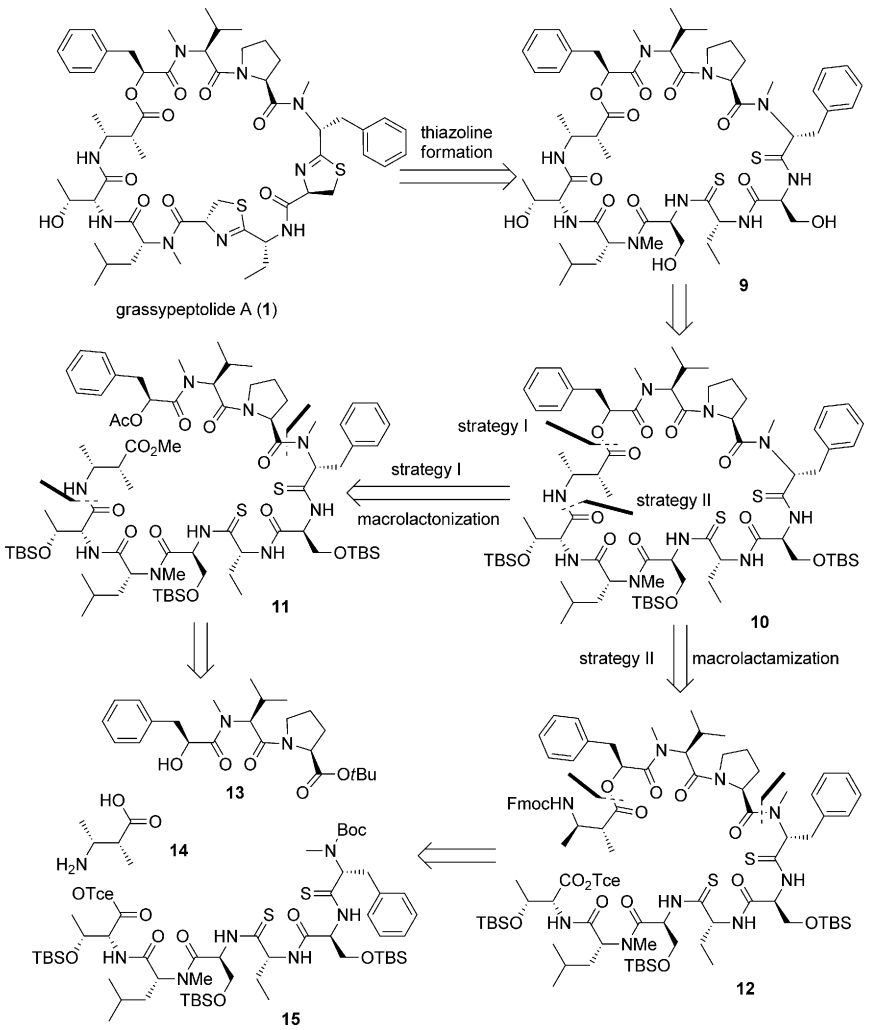
First-generation synthetic strategy for grassypeptolide A: The principal synthetic challenges associated with the preparation of grassypeptolide A are the efficient formation of the macrocyclic depsipeptide and selective assembly of the delicate bis(thiazoline) heterocycles, in the presence of a secondary hydroxy group. The bis(thiazoline) moiety was known to be highly sensitive and prone to epimerization at four of its stereogenic sites.[18]

In our retrosynthetic analysis, we envisioned macrocyclization and late-stage introduction of the labile bis(thiazoline) ring system (Scheme 1). Hence, grassypeptolide A was envisaged to be derived from macrocycle **9** by selective cyclodehydration of β-hydroxy thioamides. The regioselectivity is anticipated to stem from the chemical reactivity (thioamide vs. amide) and steric effect, favoring reaction of the primary over the secondary hydroxyl group. Macrocycle **9** can be derived from **10** by global deprotection of the TBS protecting groups. Macrocycle **10** could be prepared either by macrolactonization of **11** or macrolactamization of **12** (Scheme 1). Further disconnection of **11** and **12** affords three subunits (**13**, **14**, **15**). Both proposed strategies leading to the cyclization precursors (**11**, **12**) would require both intermediates **13** and **15** as the key coupling partners.

The synthesis of fragment **13** started from the condensation of *N*-Cbz-*N*-methyl-L-valine (**16**) with L-proline *tert*-butyl ester (**17**) to give rise to dipeptide **18** in 84% yield (Scheme 2). Hydrogenolytical removal of the Cbz protecting group in **18** followed by coupling with hydroxy acid **19**[19] by using BOPCl[20] in the presence of triethylamine afforded the corresponding tripeptide, which was deacetylated to furnish **13** in 95% yield. Further selective acid-catalyzed hydrolysis of the *tert*-butyl ester group in **13** produced the corresponding free acid **21**.

The 2-methyl-3-amino butyric acid (**14**) was prepared from *N*-Cbz-D-alanine by a three-step sequence involving the formation of a diazoketone[21] followed by a Wolff rearrangement[21,22] and a diastereoselective methylation.[10t,23]

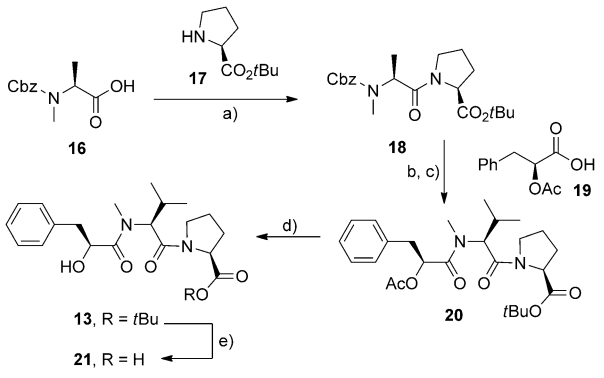
To devise an efficient synthetic approach towards bis(thioamide) **15**, we envisioned that **15** should be prepared from the C- to the N-terminus of the peptide sequence (Scheme 3). Literature precedent suggested that activation of the carboxylic acid (**15a**) adjacent to the thioamide bond would produce the corresponding thiazolinone (**15c**),[24]



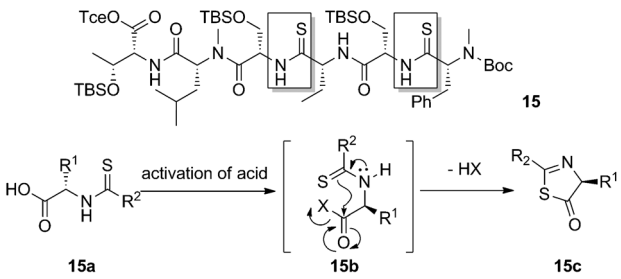
Scheme 1. Synthetic strategies to grassypeptolide A. Fmoc = fluorenylmethoxycarbonyl, TBS = *tert*-butyldimethylsilyl, Tce = trichloroethyl.

along with the epimerization of the alpha stereogenic center (Scheme 3). Bearing this analysis in mind, we devised a retrosynthetic strategy towards fragment **15** as illustrated in Scheme 4.

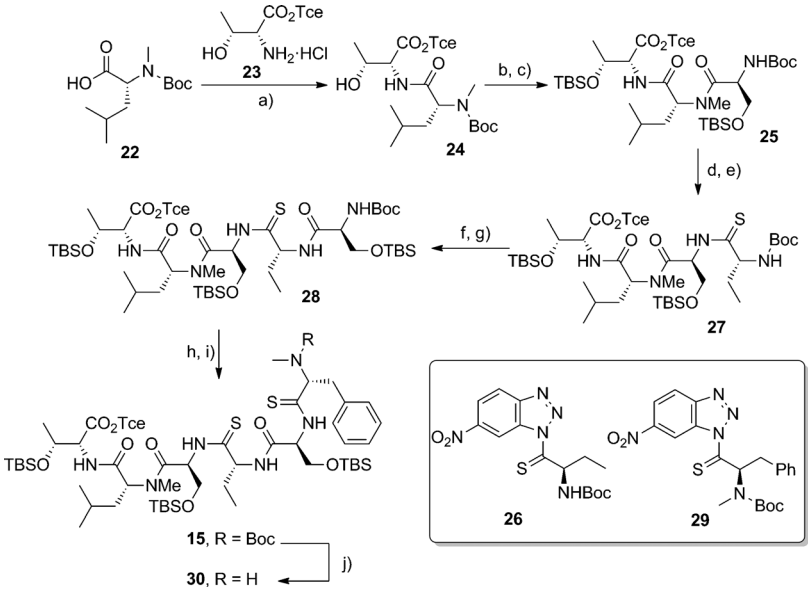
Thus, D-*allo*-threonine Tce ester (**23**) was condensed with *N*-Boc-*N*-methyl-D-leucine (**22**) employing EDCI and HOBt as the coupling reagent to furnish dipeptide **24** in 79% yield. Protection of the secondary hydroxy group in **24** as its silyl ether, followed by deprotection of the Boc group with TMSOTf[25] gave the corresponding free amine, which was then condensed with *N*-Boc-L-Ser(OTBS)-OH, employing BOPCl as the coupling reagent to give tripeptide **25** in 83% yield. The Boc protecting group in **25** was cleanly removed and the resulting free amine was coupled with the thioacylating agent **26**, prepared by using the method described by Rapoport,[26] to afford the thioamide **27** in 72% yield. To continue the linear chain elongation, after Boc deprotection the resulting free amine was condensed with *N*-Boc-L-Ser-(OTBS)-OH employing HATU as the coupling reagent. Intermediate **28** was then elaborated to the key fragment **15** in 82% yield by an identical strategy as described for **27**, including Boc deprotection and thioacylation with thioacylating agent **29** (Scheme 4). Further selective deprotection of Boc afforded the secondary amine **30**, which was ready for further coupling reactions.



Scheme 2. Synthesis of **13**: a) **17**, EDCI, HOBt, Et_3N, CH_2Cl_2, 0 °C to RT, 84%; b) 10% Pd/C, H_2, MeOH/EtOAc; c) **19**, BOPCl, Et_3N, CH_2Cl_2, 0 °C to RT, 92% from **18**; d) K_2CO_3, MeOH, 0 °C, 95%; e) H_2SO_4, CH_2Cl_2, 66%. BOPCl = bis(2-oxo-3-oxazolidinyl)phosphinic chloride, Cbz = carbobenzyloxy, EDCI = *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide, HOBt = 1-hydroxybenzotriazole.



Scheme 3. Analysis of the thioamide effect in peptide coupling processes. Boc = *tert*-butoxycarbonyl.



Scheme 4. Synthesis of **15**: a) **23**, EDCI, HOBt, Et_3N, CH_2Cl_2, 0 °C to RT, 79 %; b) TBSCl, imidazole, CH_2Cl_2, 0 °C to RT, 85 %; c) TMSOTf, NMM, CH_2Cl_2, RT; then Boc-L-Ser(OTBS)-OH, BOPCl, DIPEA, CH_2Cl_2, 0 °C to RT, 83 % (2 steps); d) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; e) **26**, THF, 0 °C, 72 % from **25**; f) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; g) Boc-L-Ser(OTBS)-OH, HATU, DIPEA, CH_2Cl_2, 0 °C to RT, 60 % from **27**; h) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; i) **29**, THF, 0 °C, 90 % from **28**; j) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT, 73 %. DIPEA = *N*,*N*-diisopropylethylamine, HATU = 2-(1-*H*-7-azabenzotriazol)-1,1,3,3-methyluronium hexafluorophosphate, NMM = *N*-methyl morpholine, TMSOTf = trimethylsilyl trifluoromethanesulfonate.

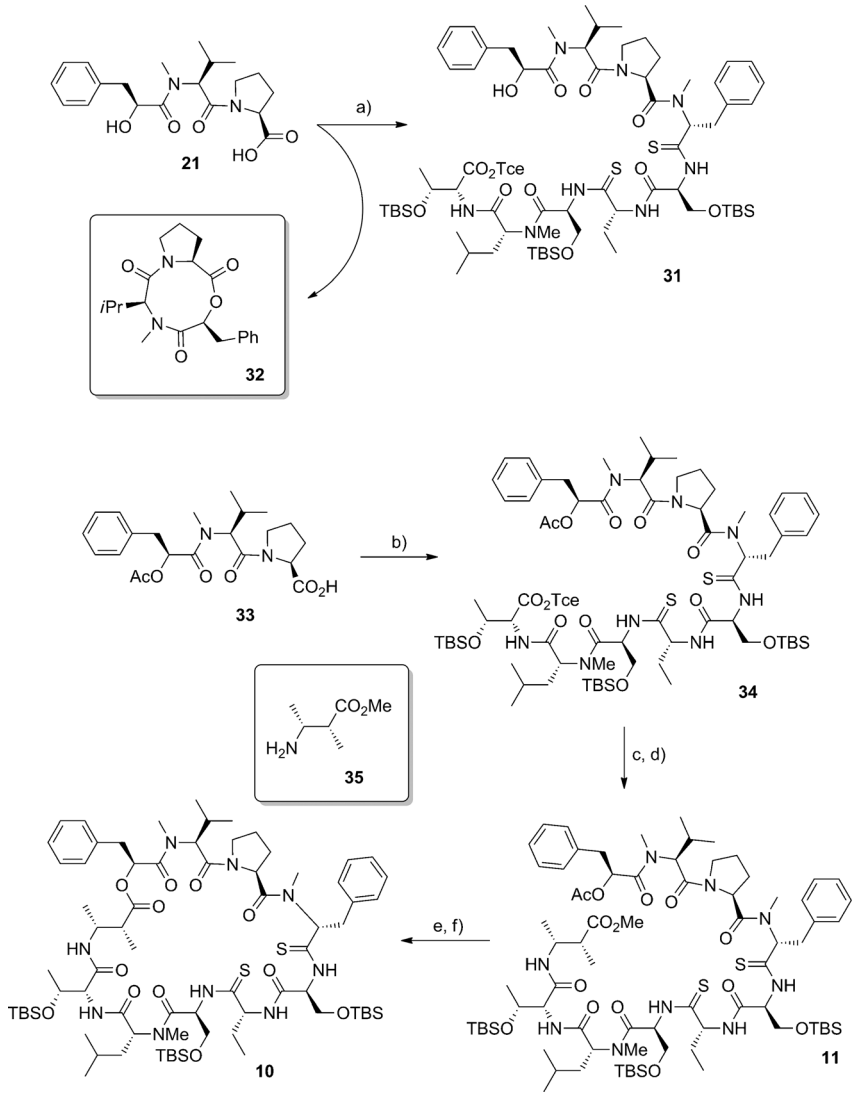
To our surprise, coupling of hydroxy acid **21** with the secondary amine **30** gave amide **31** in a low yield (44 %), even when using an excess amount of **21**. Examination of this coupling reaction unveiled that cyclodepsipeptide **32** was the major side product (Scheme 5). Formation of medium-sized rings has been considered as the inherent challenge.[27] Because the proline and *N*-Me amino acid could adopt the *cis*-amide configuration,[28] the hydroxy and carboxy termini in **21** would then reside in close proximity to each other, facilitating an intramolecular cyclization. To remedy the low-yield problem, we decided to employ acetyl-protected acid **33** as the coupling partner. Thus, treatment of **30** and **33** with BOPCl in the presence of diisopropylethylamine furnished the corresponding peptide **34** in 78 % yield. Reductive removal of the trichloroethyl ester under zinc-mediated[29] buffered conditions afforded the corresponding free acid, which was then condensed with β-amino acid methyl ester **35** (derived from **14**) to give linear precursor **11** in 21 % yield. It should be noted that both of the above reactions were sluggish, probably due to the steric effect of the adjacent TBS protecting group on the secondary hydroxy in theronine. Saponification of both the acetyl and methyl ester gave the corresponding *seco*-acid and set the stage for macrolactonization. Unfortunately, all attempts at this point to effect macrocyclization, by employing a variety of protocols, including EDCI/DMAP, *N*,*N*-dicyclohexylcarbodiimide (DCC)/DMAP/DMAP·HCl,[30] BOPCl,[20] and the Yamaguchi method[31] were unsuccessful.

To further explore the use of key intermediate **31**, we decided to install the ester moiety prior to the macrolactamization (Scheme 6). However, treatment of **31** with acid chloride **36** (prepared from **14**) under various conditions yielded only a trace amount of product **12**. This might be explained by assuming that the secondary hydroxy group of **31** formed a strong complex with the peptide backbone, which reduces its reactivity towards the esterification.

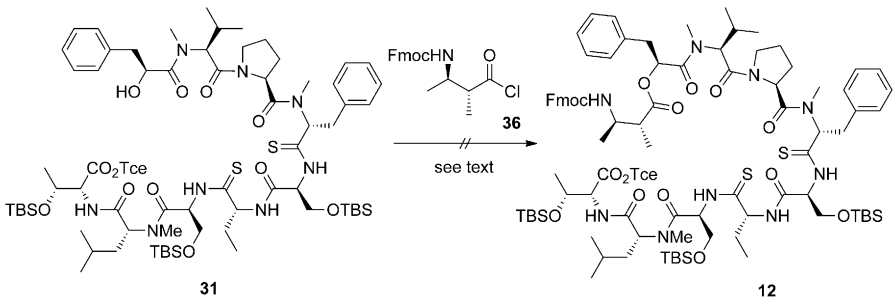
Second-generation synthetic strategy for grassypeptolide A: The failure to synthesize macrocycle **10** caused us to revise our strategy towards the macrocyclization. Conformational analysis of grassypeptolide A in solution and solid state by NMR spectroscopy and X-ray crystallography suggested that all the amide bonds were *trans* and the molecule had hydrogen bonds between the NH of Maba (C1–5) to the N of threonine (C6–9) and ester O of phenyllactic acid (C48–56), and an additional hydrogen bond between the NH of threonine (C6–9) to the carbonyl of Aba-thn-ca (C17–23).[11] We hypothesized that these aforementioned hydrogen bonds might facilitate the preorganization of a potential cyclization precursor that would in turn facilitate the final macrocyclization. According to this rationale, it was decided to close the macrocycle via the peptide bond between proline (C37–41) and *N*-Me-Phe-thn-ca (C24–36), despite the known problem of slower acylation of secondary amines. Further retrosynthetic analysis revealed that the linear precursor **9** may be most conveniently constructed by the assembly of the two fragments **38** and **39** with approximately equal complexity (Scheme 7).

Coupling of acid **40** (prepared from **14**) with the aforementioned alcohol **13**, promoted by various agents, including EDCI/DMAP, DCC/DMAP, and the Mukaiyama reagent,[32] failed to provide any useful quantities of product, presumably due to the steric bulk of the coupling partners. After extensive experimentation, we were delighted to find that the acyl chloride, derived from acid **40**, was superior in accelerating this sluggish reaction, and the key amide **38** was isolated in 90 % yield (Scheme 8).

With the key fragment **38** in hand, we next turned our attention to the synthesis of thioamide **39** (Scheme 9). The synthetic route is identical to the one employed previously for intermediate **15** (Scheme 4). Thus, the Boc group in **41** was removed with TMSOTf in the presence of 2,6-lutidine,



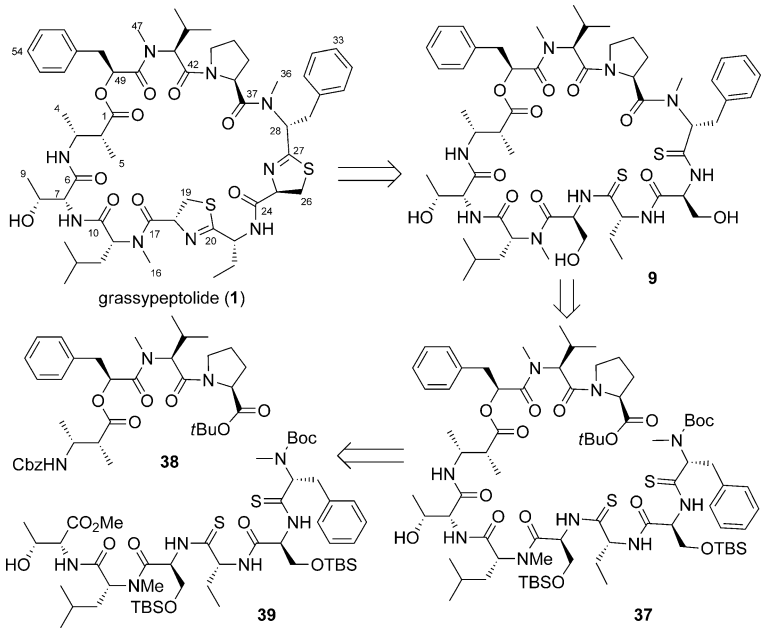
Scheme 5. Attempted macrolactonization of **11**: a) BOPCl, Et_3N, CH_2Cl_2, **30**, 0 °C to RT, 44 %; b) BOPCl, DIPEA, CH_2Cl_2, **30**, 78 %; c) Zn, NH_4OAc, THF; d) **35**, HATU, DIPEA, 21 %; e) LiOH, MeOH/THF; f) various conditions for macrolactonization.



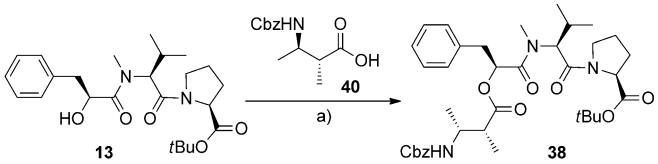
Scheme 6. Attempted synthesis of the macrolactamization precursor.

and the resulting free amine underwent a BOPCl-mediated coupling reaction with *N*-Cbz-L-serine(OTBS)-OH to afford tripeptide **42** in 89 % yield.[33] Upon hydrogenolysis of the Cbz group in **42**, the resulting free amine was coupled with the thioacylating agent **26** to give rise to thioamide **43** in 88 % yield.[33] After removal of the Boc protecting group in **43**, the free amine was condensed with *N*-Boc-L-serine(OTBS)-OH by employing various coupling reagents. Surprisingly, the condensation resulted generally in low yields when coupling reagents, such as EDCI/1-hydroxy-7-aza-benzotriazole (HOAt), HATU, BOPCl, and Mukaiyama reagent were employed. Gratifyingly, when PyAOP[34] was employed as a coupling agent, amide **44** was obtained in 88 % yield.[33] Intermediate **44** was then elaborated to the key fragment **39** in 82 % yield[33] by an identical strategy as described for **43**, including Boc deprotection and thiolation of the resulting free amine with thioacylating agent **29**.

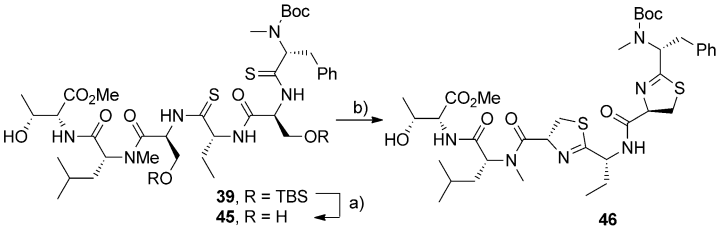
To test the feasibility of the selective construction of the bis(thiazoline) ring moiety from β-hydroxy thioamides **9** without the interfering oxazoline formation, a model study was undertaken by using segment **39** in the projected cyclodehydration (Scheme 10). Regioselectivity should be readily achieved when the alcohol moieties are in drastically different electronic or steric environments. This led to a simple hypothesis: if activation of two primary alcohols was truly occurring before the activation of a secondary alcohol, then selective formation of bis(thiazolines) in the presence of a secondary β-hydroxy amide might be achievable. It was with this hypothesis in mind that substrate **45** was synthesized. In the event, desilylation of **39** turned out to be somewhat problematic due to the sensitivity of the thioamide functionalities and protecting groups present in **39**. After some experimentation, it was found that the desired desilylated product **45** could be obtained in high yield by treatment of **39** with TBAF buffered with acetic acid. To our delight, treatment of **45** with the



Scheme 7. Retrosynthetic analysis of grassypeptolide A.



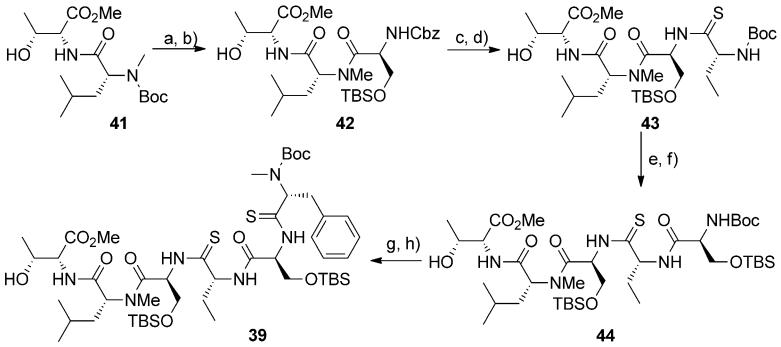
Scheme 8. Synthesis of fragment **38**: a) **40**, $(COCl)_2$, cat. DMF, CH_2Cl_2, 0 °C to RT; then **13**, cat. DMAP, NMM, CH_2Cl_2, 0 °C to RT, 90% from **13**. DMAP = 4-dimethylaminopyridine.



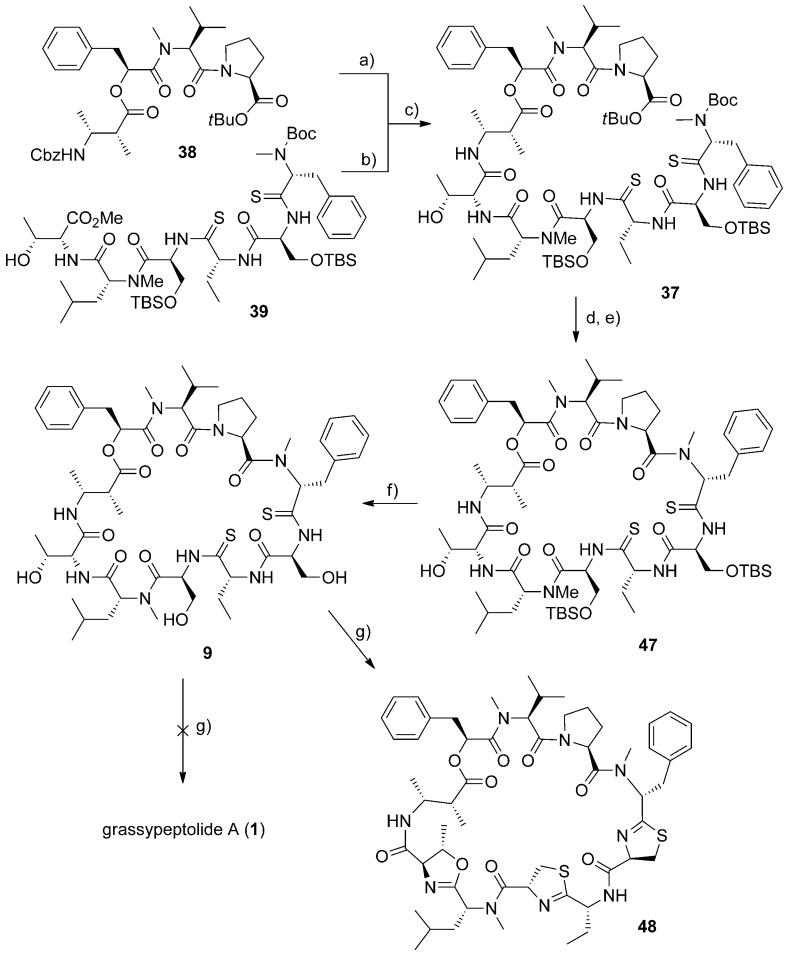
Scheme 10. Model studies on the selective formation of thiazolines: a) TBAF, HOAc, THF, 0 °C to RT, 97%; b) Burgess reagent, THF, 60–70 °C, 45%. Burgess reagent = methyl-*N*-(triethylammoniumsulfonyl)carbamate, TBAF = tetra-*n*-butylammonium fluoride.

Burgess reagent (inner salt)[35] at elevated temperature provided bis(thiazoline) **46** in 45% yield (Scheme 10). It was found that reagent purity and carefully controlled reaction times were critical to achieve optimal reaction yields, as formation of some unidentified byproducts might have been due to the presence of triethylamine derived from the decomposition over time of the Burgess reagent.

Encouraged by the success of the model study yielding the bis(thiazoline) derivative **46**, which is closely related to the grassypeptolide target molecule, we turned our attention to the required macrocycle **9** for the natural product. Thus, hydrogenolysis of the Cbz protecting group in **38** afforded the corresponding amine and saponification of the methyl ester of **39** gave the free acid, which underwent a PyAOP-mediated coupling reaction to provide the linear precursor **37** in 81% yield (Scheme 11). Simultaneous removal of the *tert*-butyl ester[36] and Boc protecting group was achieved by treatment of **37** with TMSOTf/2,6-lutidine at room temperature to produce the desired amino acid, which was immediately activated by BOPCl[20] in the presence of 2,6-lutidine under high-dilution conditions (0.001 M in CH_2Cl_2) to afford cyclodepsipeptide **47** in 72% yield[37] and without noticeable epimerization evident by NMR spectroscopic analysis. The primary TBS protecting groups in **47** were removed by using HOAc-buffered TBAF to give the triol **9**. This set the stage for our envisioned selective cyclodehydration of β-hydroxy thioamides that was expected to afford the



Scheme 9. Synthesis of fragment **39**: a) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; b) Cbz-L-Ser(OTBS)-OH, BOPCl, DIPEA, CH_2Cl_2, 0 °C to RT, 89% from **41**; c) Pd/C, H_2, MeOH, RT; d) **26**, THF, 0 °C, 88% from **42**; e) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; f) Boc-L-Ser(OTBS)-OH, PyAOP, DIPEA, CH_2Cl_2, 0 °C to RT, 88% from **43**; g) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; h) **29**, THF, 0 °C; then acidic workup, 82% from **44**. PyAOP = (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate)



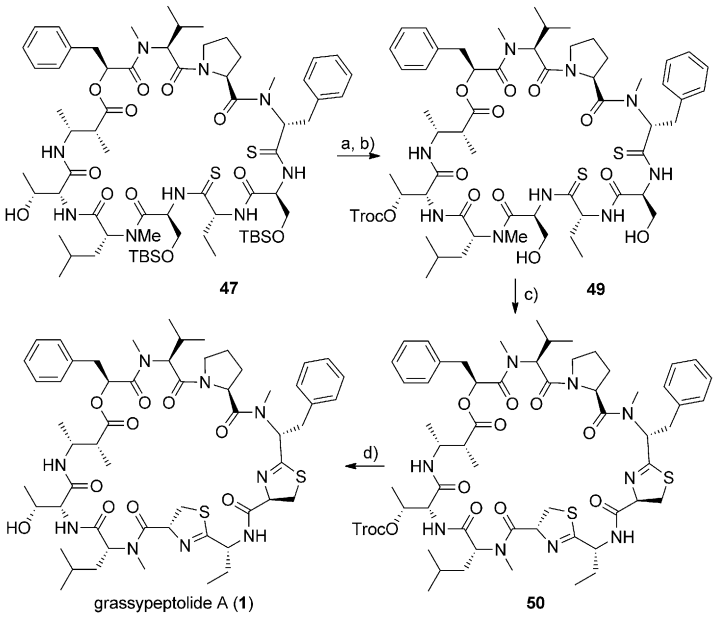
Scheme 11. Synthesis of cyclodepsipeptides **47** and **9** and attempts to complete the total synthesis: a) 10% Pd/C, H_2, EtOAc, RT; b) LiOH (1N), *t*BuOH/THF, 0°C; c) PyAOP, DIPEA, CH_2Cl_2, 0°C to RT, 81% from **39**; d) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; e) BOPCl, 2,6-lutidine, CH_2Cl_2 (0.001M), 0°C to RT, 72% from **37**; f) TBAF, HOAc, THF, 98%; g) Burgess reagent, THF, 60–70°C, 20%.

bis(thiazoline) of grassypeptolide A. Unfortunately, exposure of triol **9** to the same conditions as those shown in Scheme 10 failed to produce grassypeptolide A; instead, the major product isolated was the fully dehydrated product **48** along with trace amounts of monothiazoline-containing cyclodepsipeptide (Scheme 11). The lack of chemoselectivity in the cyclodehydration of β-hydroxy amide/thioamides might have been due to the increased conformational constraints of the macrocycle imposed by the formation of the first thiazoline skeleton. Several reaction conditions using DAST and various equivalents of reagents and reaction temperatures also failed to give rise to grassypeptolide A.

Re-evaluation of our synthetic strategy was required. To circumvent the difficulties associated with selective introduction of the labile bis(thiazoline) ring moiety, we elected to protect the secondary alcohol prior to cyclodehydration of β-hydroxy thioamides by taking advantage of the known propensity of the 2,2,2-trichloroethoxycarbonyl (Troc)[38] protecting group to undergo cleavage under neutral conditions. Thus, the secondary hydroxy group of **47** was protected as a Troc ester, and the existing two primary TBS protecting groups were selectively removed by using HOAc-buffered TBAF to give β-hydroxy thioamide **49**. Activation of the primary hydroxy groups of thioamide **49** with DAST[39] in CH_2Cl_2 at −78 to −50°C led to the cyclized product **50**, which was then treated with activated zinc and aqueous NH_4OAc in THF to afford the fully synthetic grassypeptolide A (**1**), in 37% yield (Scheme 12). Cyclodehydration of β-hydroxy thioamide **49** with Burgess reagent under the same conditions as for **46** (Scheme 10) was also attempted. In this case, only trace amounts of the desired product were observed by TLC analysis.

The spectral data for the synthetic material (^1H, ^{13}C NMR spectra, and HRMS) were identical to those published for the natural product and the optical rotation $[\alpha]_D^{20} = +87\ cm^3 g^{-1} dm^{-1}$ ($c = 0.12$ in CH_2Cl_2) was in close agreement with values reported in the literature for natural grassypeptolide A, $[\alpha]_D^{20} = +76\ cm^3 g^{-1} dm^{-1}$ ($c = 0.1$ in CH_2Cl_2), thus confirming the structure of the natural product. The synthetic method presented in this full paper gives the cyclodepsipeptide grassypeptolide A in 17 steps for the longest linear sequence, and an overall 11.3% yield (average of 88% per step).

Biological evaluation of grassypeptolide A and synthetic analogues: The anticancer effect of the synthetic grassypeptolide A (**1**) and its analogues **9**, **47**, and **48** was evaluated by cell proliferation in vitro by using cervical cancer cell line HeLa and colon cancer cell line HT29. Grassypeptolide A (**1**) significantly inhibited proliferation of HT29 and HeLa cells in a dose-dependent manner. The IC_{50} values of HeLa and of HT29 were 1.9 and 2.3 μM, respectively. Compound **48** also inhibited the growth of HeLa and HT29 with IC_{50} values of 9 and 3.6 μM, respectively (Figure 3). Compound **9** inhibited HeLa cell proliferation with an IC_{50} value at 23 μM, but it did not suppress the growth of HT29 cells at concentrations below 30 μM. In addition, compound **47** was not active towards the tested cell lines (Figure 3), which indicated the importance of the bis(thiazoline) system, which had been hypothesized to be responsible for the Zn^{2+} and



Scheme 12. Completion of the total synthesis: a) TrocCl, Py, CH_2Cl_2, 0 °C; b) TBAF/HOAc, THF, 0 °C to RT; c) DAST, CH_2Cl_2, −78 to −50 °C; d) Zn, NH_4OAc (1 M), THF, 0 °C, 37% from **47**. DAST = diethylaminosulfur trifluoride, Troc = 2,2,2-trichlorolethoxycarbonyl, TrocCl = trichloroethylchloroformate.

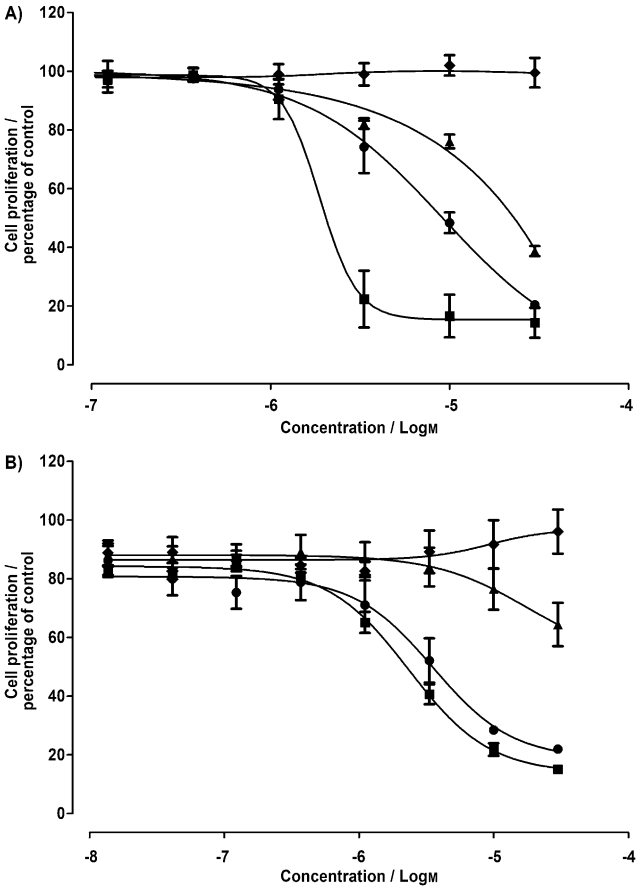


Figure 3. Effect of grassypeptolide A (**1**) on cell proliferation in A) HeLa and B) HT29 cell lines (●: **48**, ■: **1**, ▲: **9**, ◆: **47**). Cells were cultured for 48 h in the presence of various concentrations of grassypeptolide A (**1**) and its analogues. Proliferation was measured by MTS assay. Each point represents the mean ± SE from four determinations.

Cu^{2+} binding ability of grassypeptolides and to play a role in the antiproliferative activity.[14]

Among other hallmarks, cancer is characterized by the ability to evade apoptosis and aberrant cell cycle progression, leading to uncontrolled proliferation.[40] These acquired capabilities of cancer cells are the target of standard anticancer chemotherapy, and therefore we wanted to probe the effects of grassypeptolide A on related biochemical parameters and markers. For example, a desirable and standard downstream mechanism of action includes forcing cancer cells to undergo apoptosis. Furthermore, effective anticancer agents also commonly induce phase-specific cell cycle arrest, depending on the target and upstream mechanism. Antimitotic agents cause G2/M arrest, whereas agents interfering with growth factor signaling or DNA synthesis usually induce G1 or S arrest, respectively.

First, we validated the effect of grassypeptolide A (**1**) on cell cycle progression. As shown in Figure 4A, at concentrations near the IC_{50} **1** triggered a significant increase in the number of cells in the G0–G1 phase, with a corresponding decrease in the number of cells in the S and G2/M phase, consistent with previous observations for the natural product.[14] To elucidate the molecular mechanisms of **1** induction of cell cycle arrest in cancer cells, we measured the expression of cyclin D, which regulates the G1 to S transition, as well as cyclin-dependent kinase inhibitors p27 and p21 that suppress cell cycle progression in response to numerous stimuli. We observed for the first time a strong induction of p27 and p21 expression by the administration of **1** (Figure 4B). During tumor development, a lack of p21 and p27 expression was reported.[40] Therefore, up-regulation of p21 and p27 by **1** may contribute to its antitumor effect. Overexpression of cyclin D1 is another common feature in malignancy;[41] reduced cyclin D1 expression by **1** may be an important mechanism of its anticancer activity.

It was previously reported that at higher concentration this compound induces G2/M arrest.[14] This concentration-dependent effect on the cell cycle has precedence; for example, class I histone deacetylase (HDAC) inhibitors with Zn^{2+} chelating ability show the same effect.[42] In both cases, the IC_{50} correlates with the G1 arrest G1–S phase transition that is positively regulated by complexes of cyclins and cyclin-dependent kinases (CDKs) and negatively regulated by the endogenous inhibitors, that is to say, cyclin-dependent kinase inhibitors p21 and p27. CDKs themselves are promising anticancer targets[43] and the success of HDAC inhibitors in the clinic is partially attributed to cyclin D downregulation and the potent induction of p21, which serves as a biomarker.[44]

We further examined by flow cytometry whether grassypeptolide A (**1**) exhibits apoptosis-inducing activity. Apoptotic cells were defined as those stained with Annexin V but not with propidium iodide (PI). As shown in Figure 5A,

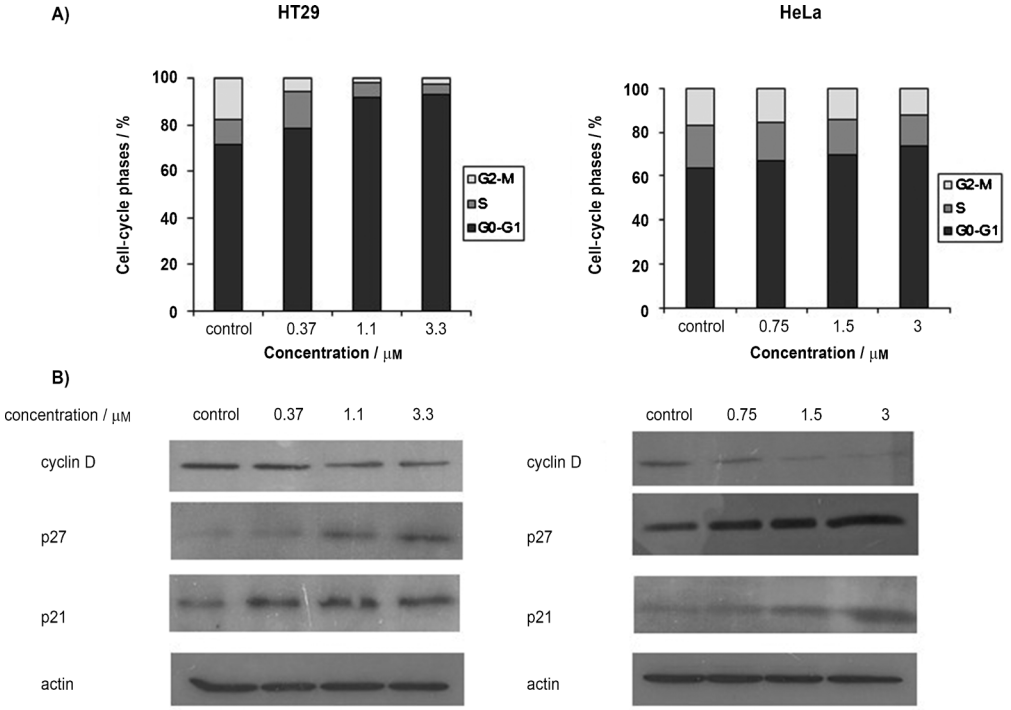


Figure 4. A) Effect of grassypeptolide A (**1**) on cell cycle distribution. HT29 and HeLa cells were pretreated with **1** at incremental concentrations for 24 h, and then stained with PI, followed by flow cytometry analysis. Relative to the control untreated cells, grassypeptolide A led to accumulation of cells in the G0–G1 phase and diminished cells in the S and G2M phases. b) The effect of grassypeptolide A treatment on cyclin D1, p27, and p21 protein expression in HT29 and HeLa cells. Expression of the proteins in cells treated with **1** at different concentrations for 24 h was analyzed by western blot. The results of a representative study are shown. Two additional experiments yielded similar results.

grassypeptolide A induced tumor cell apoptosis in a dose-dependent manner. To gain insight into the molecular mechanisms in conjunction with **1** induction of apoptosis, we investigated the expression of the key regulators of apoptosis, poly(ADP-ribose) polymerase (PARP), bcl-2, and bcl-xL, in vitro. Our study provided the first data demonstrating that the expression levels of both antiapoptotic bcl-2 and bcl-xL significantly decreased after treatment with **1** (Figure 5B). Both bcl-2 and bcl-xL have been regarded as potent therapeutic targets of cancer therapy based on their ability to disrupt apoptosis and confer resistance to chemotherapy and radiotherapy in cancer cells.[45,46] After grassypeptolide A administration, we also detected increased cleavage of PARP, a prominent marker of apoptosis. PARP cleavage is effected by caspase 3 and could be a sign that cells should undergo apoptosis because they were unable to repair the cellular injury triggered by the apoptosis inducers.[47]

Conclusions

We have developed a convergent total synthesis of grassypeptolide A (**1**). Key to the success of our synthetic route was the late-stage introduction of the sensitive tandem thiazoline heterocycles and 31-membered macrocyclization conducted at the sterically congested secondary amide site in superb conversion (72% yield). The efficiency of this transformation is noteworthy when viewed in the context of previously reported results for depsipeptide macrocyclization. Pre-organization of the cyclization precursor by potential hydrogen bonds resulting in a more favorable configuration could be attributed to the success of this transformation. The synthesis will provide a convenient access to a variety of grassypeptolide derivatives. Synthesis of other members of the grassypeptolide family and further structural modifications of grassypeptolides are currently in progress.

Experimental Section

Detailed experimental procedures have been moved into the Supporting Information.

Acknowledgements

We acknowledge financial support from the Hong Kong Research Grants Council (Projects: PolyU 5040/10P, PolyU 5037/11P, PolyU 5020/12P), Hong Kong Polytechnic University (PolyU 5636/08M, PolyU 5634/09M), Fong Shu Fook Tong Foundation, Joyce M. Kuok Foundation, National Science Foundation of China (21072007, 21133002, 21272011), and Shenzhen Bureau of Science, Technology, Information (JC200903160367A, JC201005260102A, JC201005260220A, ZYC201105170351A).

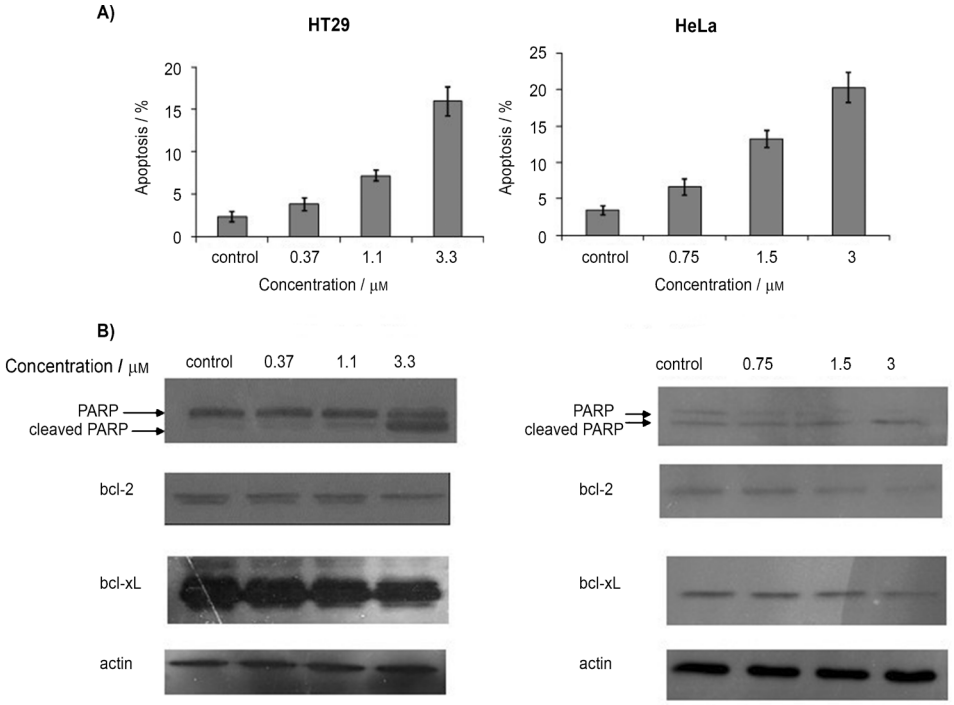


Figure 5. a) Effect of grassypeptolide A (**1**) on cell apoptosis in HT29 and HeLa cell lines. Cells were incubated with **1** at different concentrations for 24 h. Cells were harvested and double stained with an Annexin V-FITC antibody and with PI, and apoptosis was quantified as Annexin V positive and PI negative. Increased apoptosis following treatment with **1** in HT29 and HeLa cells was observed. A representative of each concentration was repeated twice in each experiment. Data are expressed as mean ± SE. b) The effect of grassypeptolide A treatment on PARP, bcl-2, and bcl-xL protein expression in HT29 and HeLa cells. Western blot was performed on total lysates from cells treated with different concentrations of **1** for 24 h. Cells treated with **1** showed several hallmarks of apoptotic cell death, including strong induction of PARP activity and reduction of bcl-2 and bcl-xL expression. The results of a representative study from three experiments are shown.

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Received: October 14, 2012
Revised: February 18, 2013
Published online: March 27, 2013

