Journal of
Medicinal Chemistry

Article

Design, Synthesis, and Conformation–Activity Study of Unnatural Bridged Bicyclic Depsipeptides as Highly Potent HIF-1 Inhibitors and Antitumor Agents

Kota Koike, Masanobu Nagano, Masahiro Ebihara, Tasuku Hirayama, Mieko Tsuji, Hiroaki Suga, and Hideko Nagasawa

J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.9b02039 • Publication Date (Web): 23 Mar 2020

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Design, Synthesis, and Conformation–Activity Study of Unnatural Bridged Bicyclic Depsipeptides as Highly Potent HIF-1 Inhibitors and Antitumor Agents

Kota Koike[†], *Masanobu Nagano*[‡], *Masahiro Ebihara*[§], *Tasuku Hirayama*[†], *Mieko Tsuji*[†], *Hiroaki Suga*[‡] *and Hideko Nagasawa*[*,†]

[†]Laboratory of Pharmaceutical and Medicinal Chemistry, Gifu Pharmaceutical University, Gifu-city, Gifu 501-1196, Japan

[‡]Department of Chemistry, The University of Tokyo, Bunkyoku, Tokyo 113-0033, Japan.

[§]Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University, Gifu-city, Gifu 501-1193, Japan

KEYWORDS. Bicyclic peptide, HIF-1 inhibitor, antitumor agent, molecular modeling.

ABSTRACT

By carrying out structural modifications based on the bicyclic peptide structure of Echinomycin, we successfully synthesized various powerful antitumor derivatives. The ring conformation in the obtained compounds was restricted by cross-linking with an unnatural bond. The prepared derivatives were demonstrated to strongly suppress the hypoxia inducible factor (HIF)-1 transcriptional activation and hypoxia induction of HIF-1 protein expression. Particularly, alkene-bridged derivative **12** exhibited remarkably potent cytotoxicity (IC_{50} = 0.22 nM on the MCF-7 cell line) and HIF-1 inhibition (IC_{50} = 0.09 nM), which considerably exceeded those of echinomycin. Conformational analyses and molecular modeling studies revealed that the biological activities were enhanced following restriction of the conformation by cross-linking through a metabolically stable and rigid bridge bond. In addition, we proposed a new globular conformation stabilized by intramolecular π stacking that can contribute to the biological effects of bicyclic depsipeptides. The developments presented in the current study serve as a useful guide to expand the chemical space of peptides in drug discovery.

INTRODUCTION

With the growing interest in expanding the chemical space for exploring new therapies, synthetic macrocycles and constrained peptides have recently attracted significant attention.[1, 2] Such molecules are characterized by medium size and cyclic structures. They are also thought to combine the best properties of biologics and small molecules. Generally, cyclic peptides are considered to exhibit enhanced binding affinity, selectivity, and metabolic stability compared with their linear counterparts. Among them, bicyclic peptides display significantly enhanced conformational rigidity compared with monocyclic peptides, which is crucial for increasing the affinity of the target compound. Accordingly, as a result of greater conformational rigidity, metabolic stability, and higher target selectivity, bridged cyclic peptides are considered potential drug scaffolds for the next-generation therapeutics.[3-5]

Although recent advances in biotechnologies involving the preparation and screening of *de novo* designed bicyclic peptide libraries have made it possible to routinely discover a variety of biologically active peptides in a high-throughput platform,[6, 7] challenges in

optimizing the pharmacological properties such as cell permeability and oral bioavailability still exist.[2] Thus, lead optimization through the modification of biologically active natural membrane-penetrating peptides is still considered a promising strategy for drug development. Echinomycin (Ec, **1**) and related bisintercalating natural products characterized by unusual thioacetal bridged *N*-methylated cyclic depsipeptide scaffold with two pendant planar chromophores constitute a representative family of antitumor and anti-viral bicyclic peptides.[8, 9] Ec is known to exhibit highly potent antitumor activity through binding DNA in a sequence-specific fashion. The molecule intercalates into the consensus 5′-ACGT-3′ sequence of the hypoxia inducible factor (HIF)-1 recognition site, namely, hypoxia response element (HRE).[10-12] Consequently, Ec has been extensively studied in the medicinal chemistry and synthetic biology fields with the aim to expand the diversity of the structure and improve the bioactivity.[13-16] Ec therefore appears to be a promising candidate as the lead compound for structural modification studies of antitumor bicyclic peptides. Hypoxia is recognized as a hallmark of solid tumors and plays a crucial role in important physiological and pathological events including metabolism, proliferation, survival, invasion, metastasis, and angiogenesis.[17] Over the last two

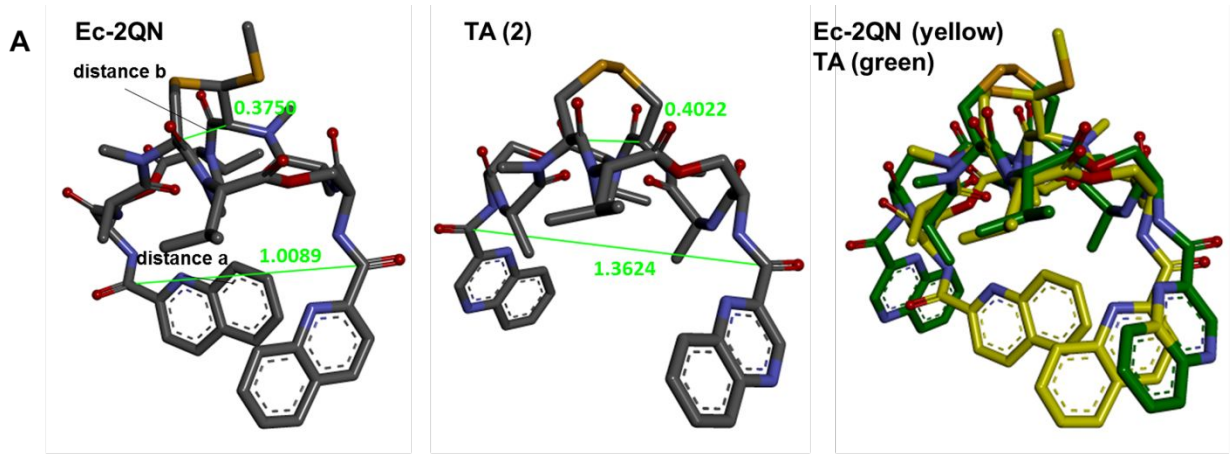
decades, HIF-1, a master transcription factor of hypoxic gene expression, has emerged as a promising therapeutic target for drug development not only in the field of cancer treatment but also for various other diseases related to ischemia and oxidative stress.[18-21] Considerable efforts are unceasingly made to develop HIF-1 inhibitors for cancer treatment.[22-24] Moreover, Ec has been subjected to a variety of clinical trials due to its highly potent tumor cytotoxicity; however, no significant effect was observed in the Phase II studies.[25, 26] Nevertheless, numerous preclinical studies focusing on the strong and specific HIF-1 inhibitory effect of Ec have been conducted.[27, 28] Recently, the importance of Ec has been highlighted again with the effect of specifically inhibiting cancer stem cells.[29, 30] In addition, the development of an antibody-drug conjugate using Ec analogs as payloads is also a promising approach.[31]

We have previously conducted structural development studies based on a unique bicyclic depsipeptide scaffold of quinomycin antibiotics, focusing on their potent antitumor effects and the specific inhibitory effect of the transcriptional activation of HIF-1α.[32] We established a valuable and practical liquid-phase procedure for the total synthesis of

Triostin A (TA, **2**) and its derivatives. We also found that TA exhibits moderate antitumor effects (IC_{50}: 2.0 μM) on the MCF-7 cells and a potent inhibiting effect of the HIF-1α transcriptional activity (IC_{50}: 26.7 nM) in a HRE-luciferase reporter gene assay (Figure 1). Intriguingly, it was found that quinomycin antibiotics such as Ec and TA containing a common depsipeptide ring displayed strong inhibitory effect on the hypoxia-induced protein expression of HIF-1α. This was a remarkable finding, because the protein expression of HIF-1α is an event upstream of DNA binding, suggesting the existence of a potential target molecule other than HRE.

Nonetheless, our previous structural modifications of the cyclic octadepsipeptide of TA did not lead to improvement of the biological activities, which remained 2-3 orders of magnitude lower than Ec.[32] Hence, to enhance the strong biological activities of Ec, we decided to focus on the bridge scaffold, which is the only dissimilarity from the structure of TA. TA contains a disulfide between cysteine residues, whereas Ec possesses an unusual dithioacetal bond.[33, 34] Overlaying the crystal structures of Echinomycin analog (Ec-2QN), which is a result of a substitution of the bis-quinoxaline moiety with a bis-

quinoline scaffold, and TA revealed that the two chromophore moieties are brought significantly closer together in Ec-2QN than in TA (Figure 1).[35, 36] As it can be seen, the distance between the carbonyl groups of the aromatic moiety shown as “distance a” and the distance between the two bridgehead carbons of Ec-2QN, i.e., “distance b” were significantly shorter than those of TA. This observation implied that the cross-bridge bond structure plays a critical role in the biological activities of quinomycin antibiotics and that the more constrained ring structure results in a more potent antitumor activity and HIF-1 inhibitory effect.



	Distance (nm in crystal structure)		IC_{50}	
	distance a	distance b	MTT [nM]	HIF-1 Luc. [nM]
Ec (1)	1.01[a]	0.375[a]	5.5 ± 0.2	0.35 ± 0.03
TA (2)	1.36	0.40	4100 ± 300	26.9 ± 1.3

Figure 1. Comparison of conformations and biological activities of Ec (**1**) and TA (**2**). (A) Crystal structure of Ec-2QN (CCDC no. 131833) and TA (**2**) (CCDC no. 131836) and overlay of Ec-2QN (yellow) and TA (**2**) (green). Distance a: the distance between the carbonyl groups of the quinoxaline moiety. Distance b: the distance between the two bridgehead position carbons.[35] (B) Conformation parameters, and half maximal inhibition concentration (IC_{50}) values of Ec (**1**) and TA (**2**) for cytotoxicity against MCF-7 cells and of inhibition of hypoxia-induced HIF-1 activation.[32] *a*. Measured at crystal structure of Ec-2QN.

With this preliminary analysis of the conformation–activity relationship in hand, we envisioned that stronger activity could be obtained by introducing unprecedented appropriate bridge structure to increase the rigidity of the octadepsipeptide ring. Consequently, we conducted structural modification of the bridged cyclic octadepsipeptide scaffold focusing on the structure of cross-bridge moiety. Although Ec can be biosynthesized from the precursor antibiotic TA by nonribosomal peptide

synthase,[36, 37] the total synthesis of Ec has not yet been achieved because of the difficulty of constructing the unusual dithioacetal bridge by chemical synthesis. In addition, several studies have been reported concerning the synthetic studies of the structure–activity relationship (SAR) of natural or synthetic quinomycin antibiotics.[13, 15, 38, 39] Nevertheless, only a few examples of oxidation or alkylation of the sulfur-containing moiety on the cross-bridge scaffold have been described.[32, 40] Thus, establishment of the structural requirements for the high potency of Ec by the 3-D SAR studies through the structural modification of the cross-bridge on the bicyclic peptide scaffold is expected to provide unique and valuable viewpoints for future peptide-based drug design.

Herein, we describe the design, synthesis, biological evaluation, and molecular modeling studies on the conformationally constrained cyclic octadepsipeptide derivatives with unnatural cross-bridge structures derived from quinoxaline antibiotics to elucidate the structural requirements for highly potent antitumor and HIF-1 inhibiting effects.

RESULTS AND DISCUSSION

Strategy for SAR study of bridged bicyclic octadepsipeptides.

As mentioned above, in our previous structural modification study of the disulfide-bridged octadepsipeptide scaffold, tumor cytotoxicity and inhibiting effect of HIF-1 transcriptional activation were significantly reduced for diastereoisomers containing one or two D-MeVal residues and a thiosulfonate group.[32] We therefore subsequently planned to investigate the relationship between conformation and activity by modifying the cross-bridge structure on the cyclic octadepsipeptide scaffold, as shown in Figure 2. We designed thioether- and selenoether-linked derivatives, since such bridge bonds were expected to constrain the depsipeptide ring to the same extent as the unusual dithioacetal bridge of Ec. The ring-closing metathesis (RCM), which is an effective orthogonal chemical cross-linking reaction, allowed the synthesis of an olefin-bridged derivative with a greater ring strain.[41] In addition to the constrained cyclic analogs, more flexible derivatives, including compounds containing a cleaved disulfide bond and an elongated thioacetal moiety, were also synthesized for comparison.

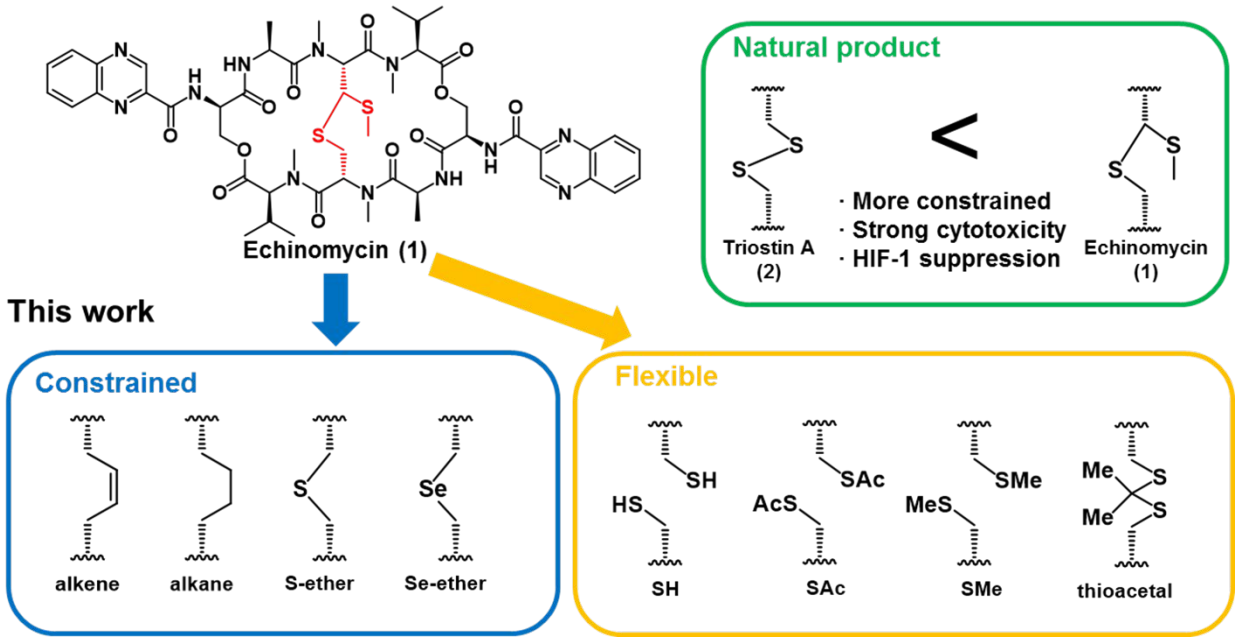


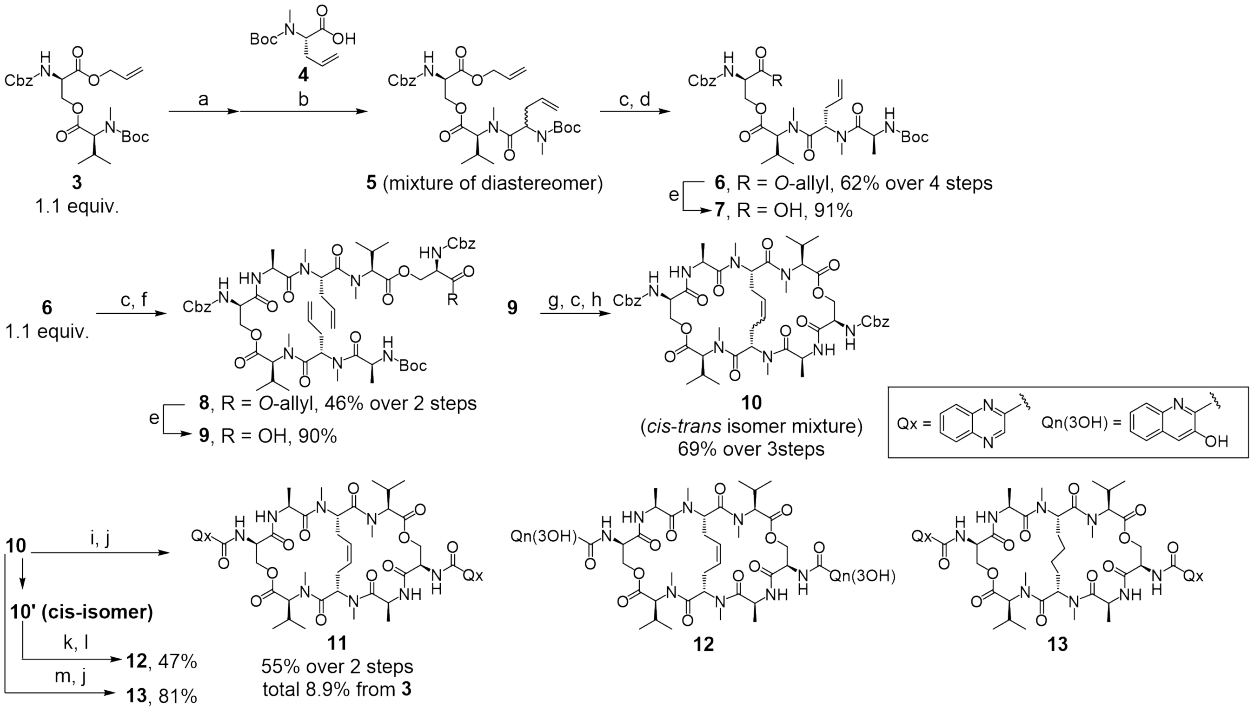
Figure 2. Strategy for molecular modification and SAR study of quinomycin antibiotics.

Chemical synthesis

In the first instance, alkene (**11**) and alkane (**13**) type derivatives, in which a disulfide bond was replaced with a C–C bond to improve the metabolic stability, were prepared (Scheme 1). We employed the RCM reaction, which enabled an efficient chemoselective and regioselective synthesis of complex bicyclic peptides.[41] The sequence started with the synthesis of Cbz-D-Ser(Boc-MeVal)-*O*All (**3**) according to an optimized procedure previously reported for the TA analogs.[32] Compound **3** was subsequently condensed with

N-methyl-allyl-glycine derivative **4** affording a mixture of diastereomers **5**. The next step involved conjugation of *N*-Boc-L-Ala-OH to **5** to give tetradepsipeptide **6** as a single isomer in four steps and a total yield of 62%. Half of the obtained compound **6** was then deallylated to give **7**. Subsequently, *N*-Boc deprotection of **6** was followed by condensation with compound **7** using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM)[42] to afford a linear octadepsipeptide **8**. Compound **9** obtained by deallylation of **8** was subjected to intramolecular cyclization (RCM) using Grubbs second-generation catalyst,[43] followed by intramolecular amide bond formation to produce olefin-bridged bicyclic peptide **10** as a mixture of (*E*) and (*Z*) isomers in three steps and 69% yield. Next, following the removal of the Cbz group by a catalytic reduction, **10** was condensed with quinoxaline-2-carboxylic acid to obtain the target alkene derivative **11** as a single isomer crystal in 55% yield (8.9% overall yield from **3** in 13 steps). Single crystal structure analysis of **11** revealed that the configuration of the alkene moiety is *Z* (Figure 3). The *Z* isomer of **10** was isolated, deprotected, and then condensed with 3-hydroxyquinoline-2-carboxylic acid to give **12**.[44] In addition, the alkane-

type derivative **13** was also obtained by hydrogenation of **10** under a pressure of 3 atm, followed by the introduction of quinoxaline-2-carboxylic acid.



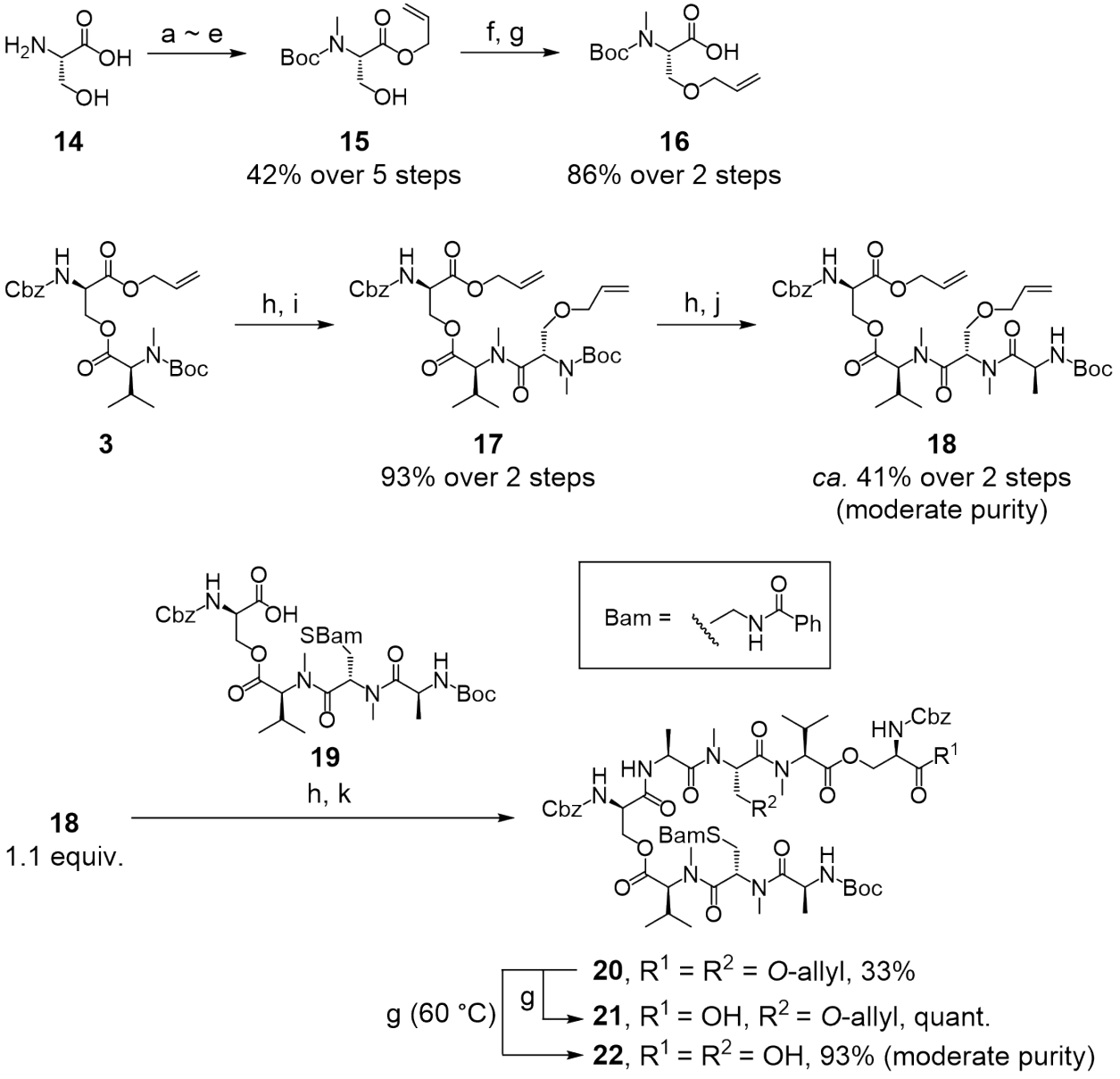
Scheme 1. Synthesis of alkene derivatives **11**, **12**, and alkane **13**.

a) HCl/AcOEt, (b) **4**, DMT-MM, AcOEt, (c) TFA, (d) Boc-Ala-OH, DMT-MM, NMM, DMF, (e) $Pd_2(dba)_3$, PPh_3, *N*-methylaniline, THF, (f) **7**, DMT-MM, AcOEt (g) Grubbs 2nd catalyst, CH_2Cl_2, (h) DMT-MM, NMM, DMF (1 mM), (i) $Pd(OH)_2/C$, H_2 (1 atm), MeOH, (j) quioxaline-2-carboxylic acid, DMT-MM, DMF, (k) thioanisole, TFA, (l) 3-hydroxyquinoline-2-carboxylic acid, EDCI·HCl, HOAt, $NaHCO_3$, DMF, (m) $Pd(OH)_2/C$, H_2 (3 atm), MeOH.

The synthetic procedures for thioether-bridged analog as a conformational mimic of Ec were subsequently investigated (Scheme 2 and 3). The linear octadepsipeptides **21** and **22** were synthesized by the condensation of half segments **18** and **19**[32] as shown in

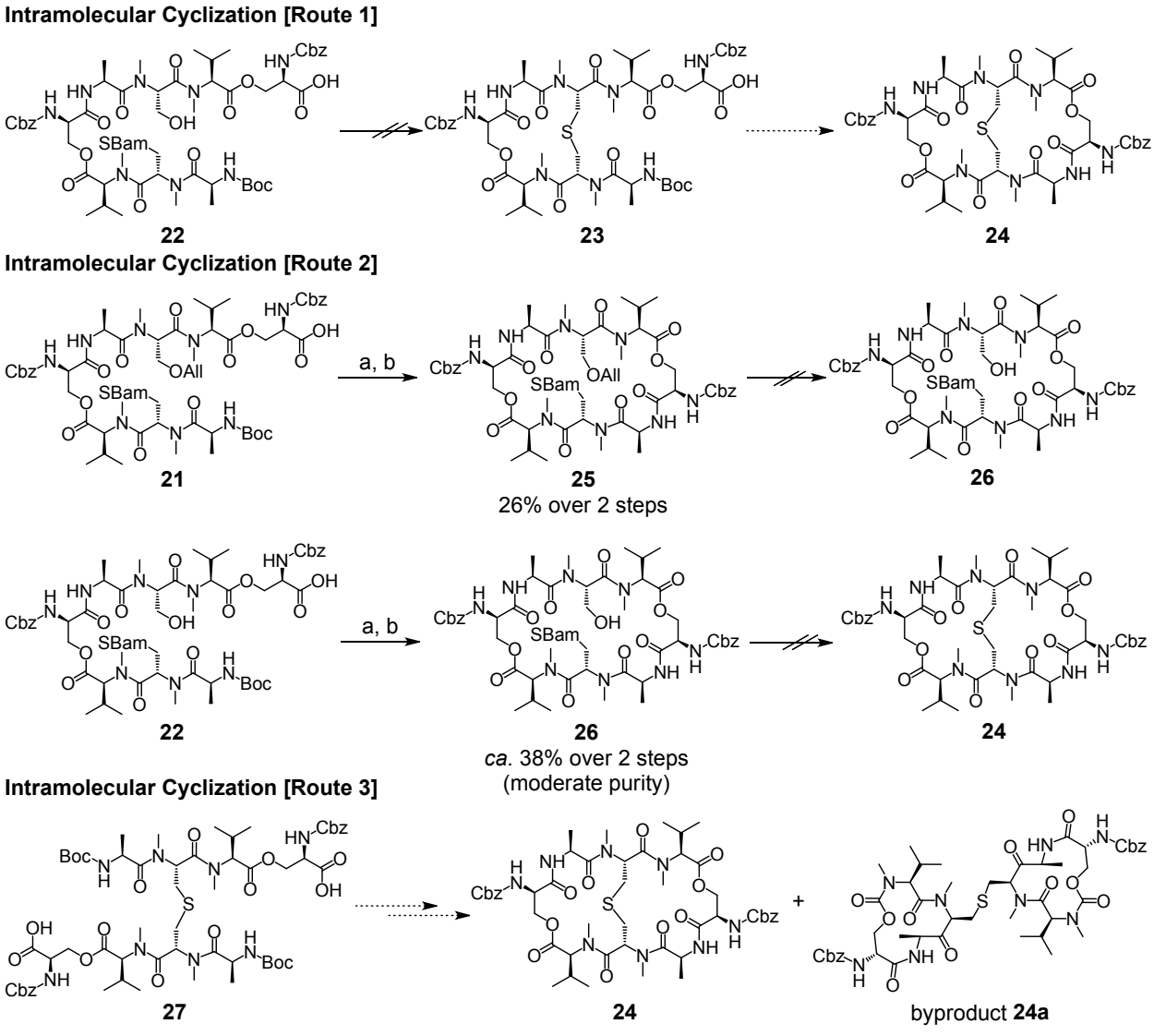
Scheme 2. Firstly, two different routes resulting in the cyclization of the linear octadepsipeptide **21** or **22** were examined (Scheme 3). Route 1 involved an initial thioether-bridged ring formation by intramolecular nucleophilic substitution or conjugate addition; however, the reaction failed under various conditions. As shown in route 2, the first cyclization of linear peptides **22** and **21** successfully yielded the intermediate cyclic octadepsipeptides **25** and **26**, respectively. Unfortunately, intramolecular thioether formation was also not successful in this case, because the presence of *N*-MeSer at the bridgehead position readily led to undesired β-elimination. Finally, we investigated route 3, which involved a double cyclization of a symmetric H-shaped intermediate **27** containing two carboxyl and two amino end groups. However, cyclization of the H-shaped intermediate was expected to form two isomers, **24** and **24a**, possessing theta- and manacle-shaped rings, respectively.[45] It has previously been reported that both theta- and manacle-shaped isomers of TA were obtained by a double cyclization of a H-shaped intermediate in a solid phase procedure.[46] In the study, double cyclization between *N*-Me-Cys and *N*-Me-Val afforded an unnatural conformer of TA. On the other hand, based on our earlier research, the synthetic strategy employed in the current study involved the

selection of compound **27** as an H-shaped key intermediate for the double cyclization sequence, because we previously determined that cyclization between D-Ser and Ala can suppress racemization during the conjugation step.[32]



Scheme 2. Synthesis of linear peptides **21** and **22**.

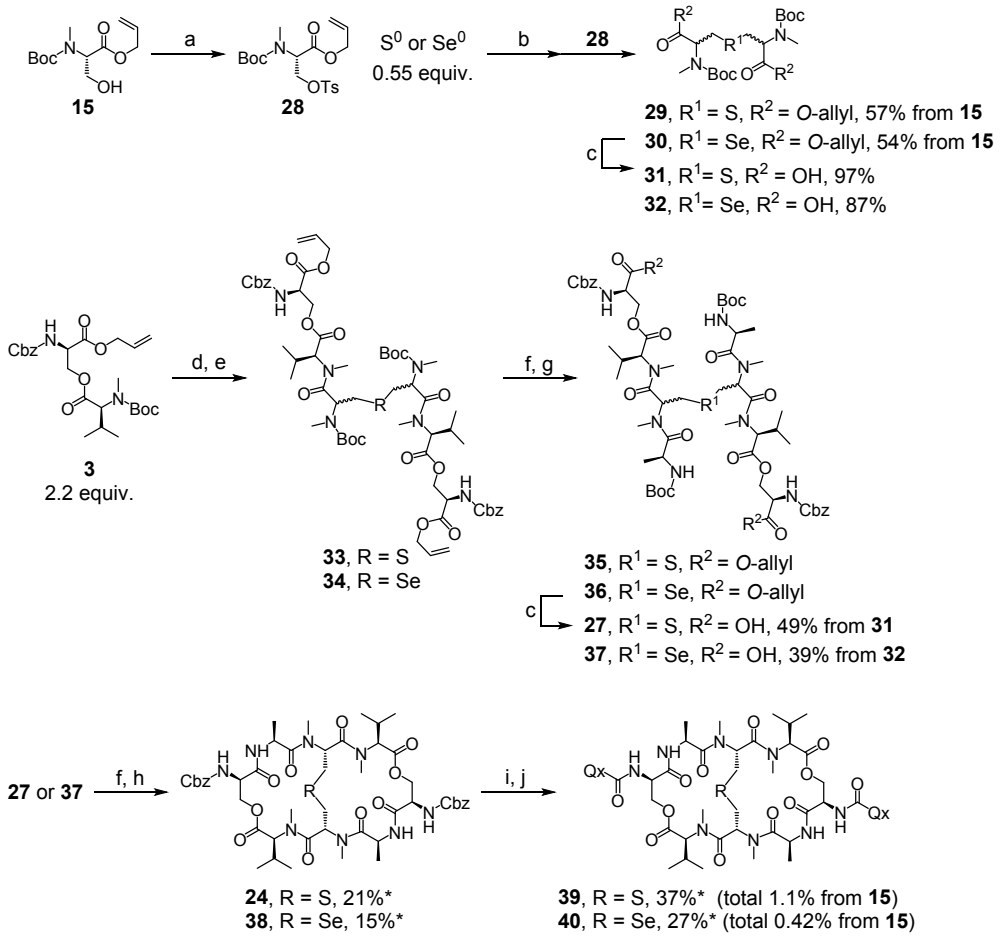
(a) Boc_2O, 1,4-dioxane, NaOH aq., (b) imidazole, TBDMSCl, THF, (c) NaH, MeI, dry THF, (d) allylbromide, K_2CO_3, DMF, (e) AcOH, THF, H_2O, (f) allyl ethyl carbonate, $Pd_2(dba)_3$, PPh_3, THF, (g) $Pd_2(dba)_3$, PPh_3, *N*-methylaniline, THF, (h) HCl/AcOEt, (i) **16**, DMT-MM, AcOEt, (j) Boc-Ala-OH, DMT-MM, NMM, DMF, (k) **19**, DMT-MM, AcOEt.



Scheme 3. Investigation of synthetic procedure for thioether bridged derivative **24**.

(a) TFA, (b) DMT-MM, NMM, DMF (1 mM).

Scheme 4 demonstrates the synthesis of thioether- and selenoether-linked derivatives **39** and **40** via the corresponding H-type depsipeptide intermediate (route 3). For the synthesis of the H-shaped intermediates, lanthionine derivatives **31** and **32** were first prepared, followed by the extension of the two C-terminals, and then the addition of Boc-Ala-OH to both of the N-terminals to obtain **27** and **37**.



Scheme 4. Synthesis of thioether and selenoether bridged derivatives, **39** and **40**.

(a) Ts-Cl, pyridine, DMAP, dry CH_2Cl_2, (b) $LiBHEt_3$, dry THF, (c) $Pd_2(dba)_3$, PPh_3, *N*-methylaniline, THF, (d) HCl/AcOEt, (e) **31** or **32**, DMT-MM, AcOEt, (f) TFA, (g) Boc-Ala-OH, DMT-MM, NMM, DMF, (h) DMT-MM, NMM, DMF (1 mM), (i) thioanisole, TFA, (j) quioxaline-2-carboxylic acid, DMT-MM, NMM, DMF. *The reaction yield was calculated from HPLC analysis.

As shown in Scheme 4, *N*-Boc-*N*-Me-L-Ser-*O*All (**15**) was obtained from L-Ser in five steps and 42% yield. Tosyl ester **28** derived from **15** was then converted to a thiol or selenol in the presence of elemental sulfur or selenium and $LiBHEt_3$.[47, 48] Subsequently, this intermediate was subjected to a nucleophilic substitution reaction with an equivalent of **28**, followed by deallylation to give *N*-Me-Lan derivative **31** or **32** as a diastereomeric mixture. After removal of the Boc group from dipeptide **3**, the resulting amine was condensed with dicarboxylic acid **31** or **32** to give hexadepsipeptide **33** or **34**. Compound **33** or **34** was subjected to a condensation with Boc-Ala-OH immediately after the removal of the Boc group, followed by deallylation to afford H-shaped octadepsipeptide **27** or **37** as a diastereomeric mixture. Subsequently, following the removal of the Boc group, the resulting crude product was treated with DMT-MM in DMF under highly diluted conditions to give a mixture of theta-shaped bicyclic peptide **24** and several isomers (Figure S16). These isomers were found to have the same molecular weight as **24** by liquid chromatography-mass spectrometry (LC-MS) analysis; however, they were challenging to separate and identify. Thus, without further purification, the mixture of isomers was treated with trifluoroacetic acid and thioanisole to remove the Cbz groups, followed by

condensation with quinoxaline-2-carboxylic acid to yield the target compound **39** as a single isomer by crystallization. Furthermore, the selenoether-bridged bicyclic peptide **40** was also successfully prepared from **37** using the same procedure. Pleasingly, the constrained bridged bicyclic octadepsipeptides **11**, **39**, and **40** all gave single crystals and could be subjected to X-ray structural analysis (Figure 3, Figure S1 and Table S1).

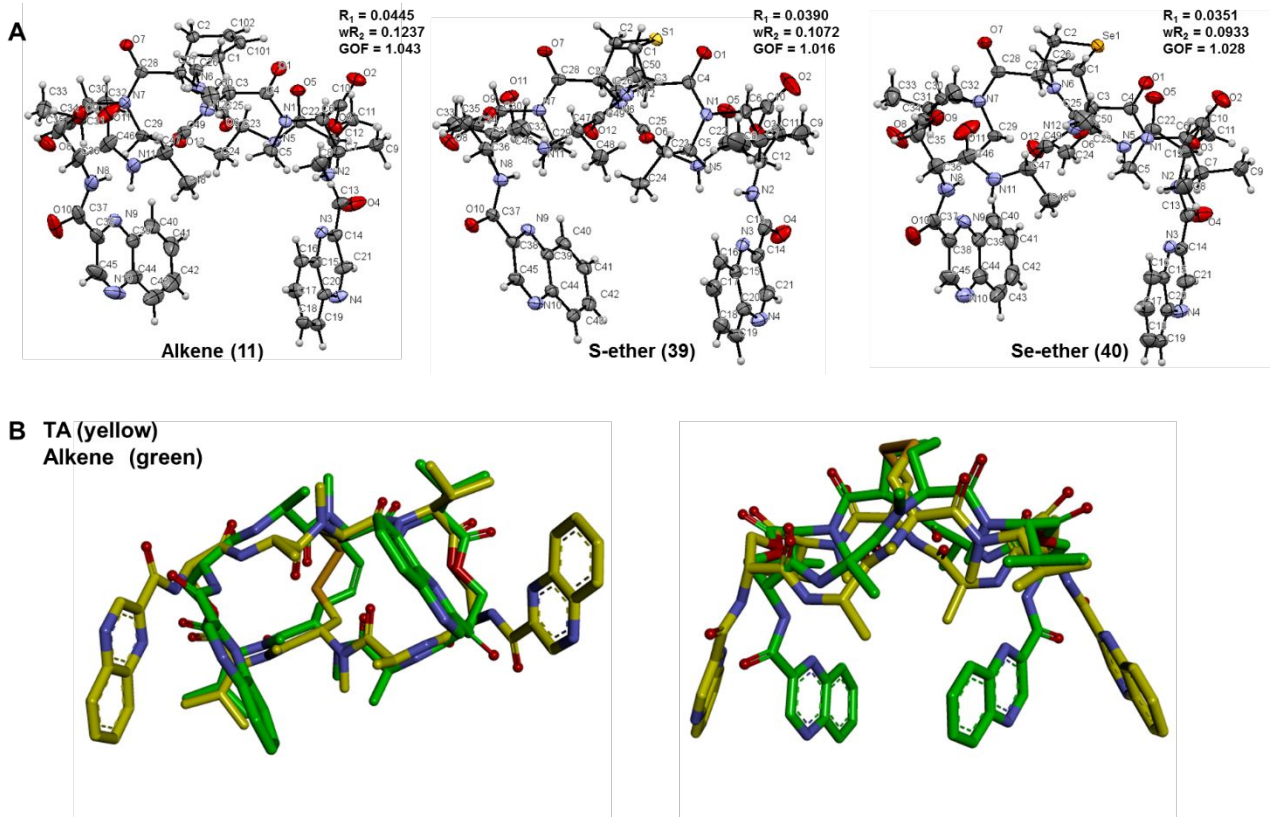
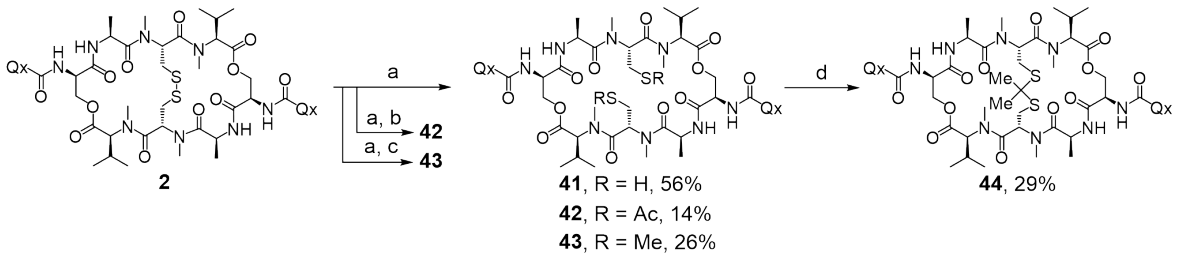


Figure 3. X-ray crystal structural analyses of constrained bridged bicyclic octadepsipeptides. (A) ORTEP drawing (50% probability ellipsoids) of **11**(CCDC no. 1885767), **39**(CCDC no. 1885766), and **40**(CCDC no. 1885765) with atom numbering

scheme. Crystal solvents were omitted and only one molecule in the asymmetric unit was shown. (B) Superposition of crystal structures of alkene (**11**) (structure in green) and TA (**2**) (structure in yellow, CCDC no. 131836).

Scheme 5 demonstrates the syntheses of flexible ring structure derivatives. The reduced form of monocyclic TA-SH (**41**) was obtained by the reductive cleavage of TA using tributylphosphine and was further converted to TA-SAc (**42**), TA-SMe (**43**), and TA-thioacetal (**44**).



Scheme 5. Syntheses of flexible cyclic derivatives **41**, **42**, **43**, and **44**.

(a) $P(^{n}Bu)_3$, H_2O, MeCN, (b) Ac_2O, NEt_3, CH_2Cl_2, (c) MeI, DIPEA, CH_2Cl_2, (d) $BF_3{\cdot}OEt_2$, CH_2Cl_2, acetone.

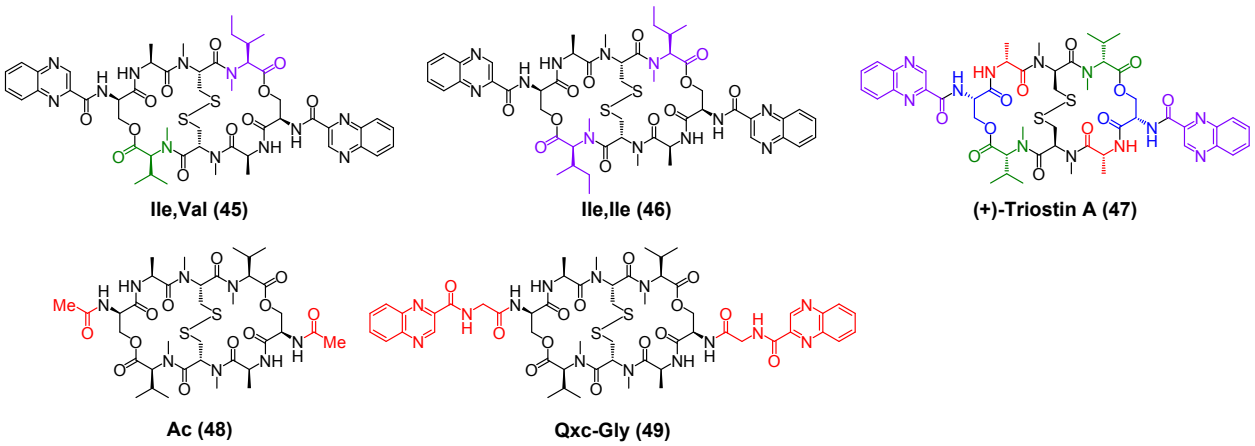
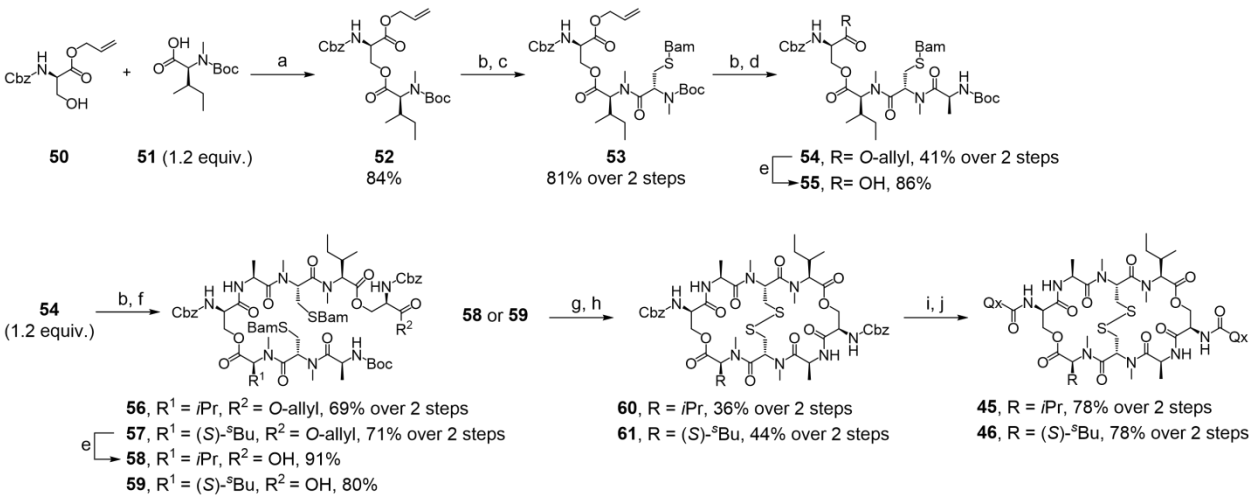


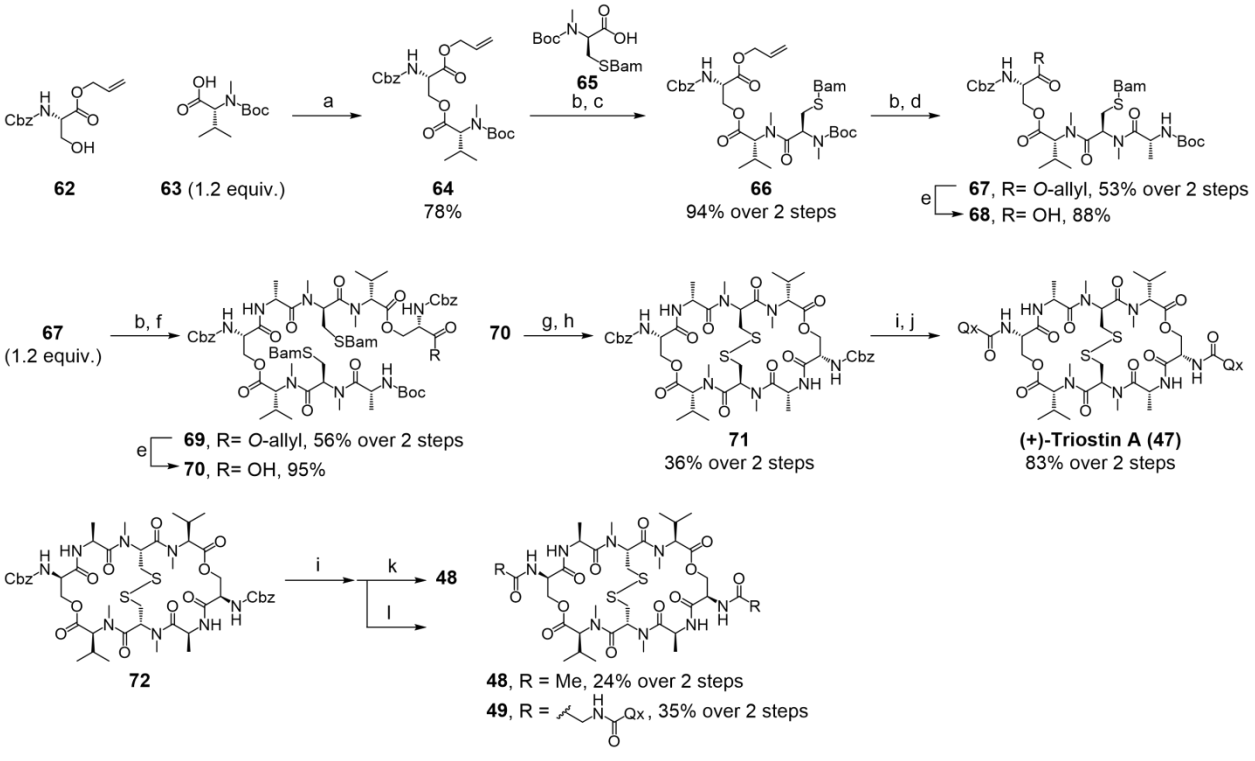
Figure 4. Structures of new TA derivatives.

As illustrated in Figure 4, TA derivatives TA-Ile, Val (**45**) and TA-Ile, Ile (**46**), in which one or two *N*-Me-Val moieties in the TA depsipeptide core were replaced with *N*-Me-Ile were also synthesized (Scheme 6). Moreover, the TA enantiomer, (+)-TA (**47**), TA-Ac (**48**) without pendant quinoxaline (Qx) and TA-Qx-Gly (**49**), in which Gly was inserted between Qx and the depsipeptide core, were prepared according to the procedure previously reported by our group (Scheme 7).[32]



Scheme 6. Syntheses of **45** and **46**.

(a) EDCI·HCl, HOAt, NEt_3,CH_2Cl_2, (b) HCl/AcOEt, (c) Boc-MeCys(Bam)-OH, DMT-MM, AcOEt, (d) Boc-Ala-OH, DMT-MM, NMM, DMF, (e) $Pd_2(dba)_3$, PPh_3, *N*-methylaniline, THF, (f) **19** or **55**, DMT-MM, AcOEt, (g) I_2, CH_3CN (1 mM), (h) EDCI·HCl, HOAt, CH_2Cl_2 (1 mM) (i) thioanisole, TFA, (j) quioxaline-2-carboxylic acid, DMT-MM, NMM, DMF.



Scheme 7. Syntheses of (+)-TA (**47**), **48** and **49**.

(a) EDCI·HCl, HOAt, NEt_3,CH_2Cl_2, (b) HCl/AcOEt, (c) **65**, DMT-MM, AcOEt, (d) Boc-D-Ala-OH, DMT-MM, NMM, DMF, (e) $Pd_2(dba)_3$, PPh_3, *N*-methylaniline, THF, (f) **68**, DMT-MM, AcOEt, (g) I_2, CH_3CN (1 mM), (h) EDCI·HCl, HOAt, CH_2Cl_2 (1 mM), (i) thioanisole, TFA, (j) quioxaline-2-carboxylic acid, DMT-MM, NMM, DMF, (k) AcOH, DMT-MM, NMM, DMF, (l) Qxc-Gly-OH, DMT-MM, NMM, DMF.

Biological evaluations

The biological evaluation of the obtained compounds involved the examination of the *in vitro* cytotoxicity using the MTT assay and the inhibitory effect of HIF-1 activity utilizing the luciferase reporter assay. The latter was performed using the stable transformants of the HEK293 cells previously established[49] by transfection of the luciferase-expressing reporter plasmid p2.1, an HRE reporter construct[50]. After the cells were incubated with various concentrations of the test compounds for 24 h under normoxic or hypoxic (1% O_2) conditions, luciferase activity was assessed according to the assay kit procedure. Cytotoxic effects on various cancer cell lines were evaluated by the cell viability assay using MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. For

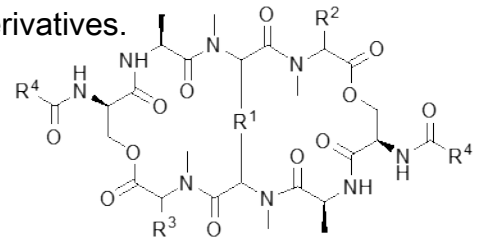
initial screening of the compounds synthesized here, half-maximal inhibitory concentration (IC_{50}) values of HIF-1 inhibition and cytotoxicity were determined from the HRE-luciferase reporter assay and MTT assay against MCF-7 (Table 1). As shown in our previous report, molecular modification of the depsipeptide core and the aromatic moiety of TA did not improve biological activities.[32] Altering the constituent amino acids of the depsipeptide core decreased both the cytotoxic activity and the HIF-1 inhibitory activity (Table 1a, runs 4-7). In addition, no activity was observed for the TA enantiomer, (+)-TA (**47**) (run 3). A quinoxaline pendant with fixed rotation was also essential for activity (run 8, 9). Regrettably, none of the above structural changes based on the disulfide-bridged bicyclic peptide scaffold resulted in an increase in the biological activities. These data suggest that a symmetrical cyclic octadepsipeptide core containing two chromophores is essential for the biological activity of quinomycin antibiotics. Subsequently, by introducing an unnatural cross-bridge bond, we ultimately succeeded in achieving strong antitumor and HIF-1 inhibitory activities at nM levels comparable with Ec (runs 10-14). When the alkene-bridge bond was reduced, the activities marginally decreased (run 12). This indicates that the rigidity of the macrocycle is a key factor for enhanced biological

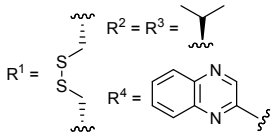
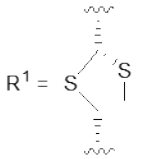
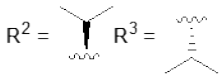
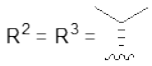
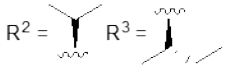
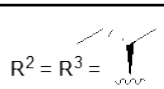
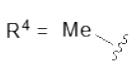
activities. Furthermore, the introduction of a hydroxyquinoline ring into the alkene-bridged depsipeptide core afforded the most potent compound **12**, which exhibited approximately 10 times stronger cytotoxicity (IC_{50} 220 pM) and four times higher inhibitory activity of HIF-1 transcription activation (IC_{50} 90 pM) than Ec **1** (run 11). Based on the observation of a rapid decrease in activity upon cleaving or extending the bridge bond, it is evident that the constrained macrocyclic conformation plays an essential role in potent biological activity (runs 15–18). Virtually analogous SAR trends were observed in cytotoxicity and HIF-1 inhibitory activity evaluations. It is noteworthy however that derivative **44** displayed relatively potent HIF-1 inhibitory activity, which was comparable with TA, whereas the cytotoxicity of **44** decreased. This suggests that the cytotoxicity and the HIF-1 inhibitory activity do not share the same target.

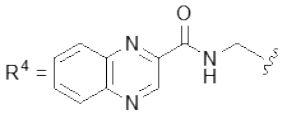
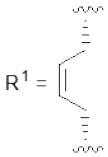
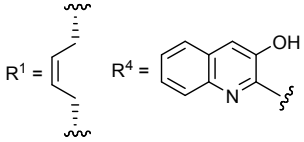
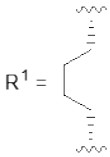
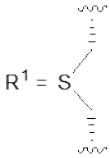
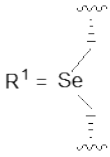
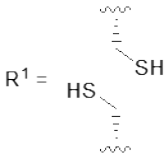
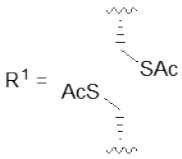
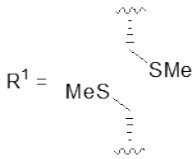
Rm values were shown for comparison of hydrophobicity[51] of cyclic octadepsipeptides (Table 1a). Furthermore, physicochemical parameters such as ALogP, polar surface area (PSA)[52] and solvent accessible surface area of the global minimum conformation (3D-SASA) of Ec (**1**), TA (**2**), **11**, **12**, **39** and **40** were calculated using Discovery Studio® 3.1 for

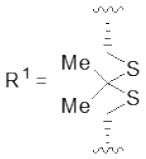
the prediction of bioavailability based on the 3D structure[53] (Table S18 in Supporting Information). Although it is known that there are quite a few compounds with favorable pharmacokinetic properties among cyclic peptides, these medium-sized molecules do not meet common physicochemical guidelines for cell permeability and bioavailability of drug-like small molecules due to their large and complex structures. Despite low water solubility of Ec (**1**), it is considered to have passive membrane permeability because it exhibits potent cytotoxicity at very low doses and acts as a DNA intercalator. Among the new cyclic peptides, alkene bridged molecules **11** and **12** had smaller PSA and 3D-SASA than those of Ec (**1**), especially **12** had the highest lipophilicity. These results suggest that compound **12** may have better pharmacokinetic profile than Ec (**1**). On the other hand, TA (**2**) was predicted to have a poor PK profile from all parameters.

Table 1a. Cytotoxic activity against MCF-7 and HIF-1 inhibitory activity of quinomycin derivatives.



run	Compound[a]		IC_{50}[b]			Rm value[f]
			cytotoxicity for 24 h[c][nM]	cytotoxicity for 72 h[d][nM]	HIF-1 Inhibitory activity[e] [nM]	
1	TA (**2**)		2000±500	212±14	26.9±1.3[g]	0.15
2	Ec (**1**)		5.5±0.2[f]	2.0±0.1	0.35±0.03[g]	0.28
Modification of depsipeptide core						
3	**47**	*ent*-TA	>100000	-	>100000	0.15
4	**73**[f]		24300±2000	-	2000±800	0.24
5	**74**[f]		6100±1000	-	590±120	0.25
6	**45**		4100±900	-	55.5±2.7	0.39
7	**46**		4000±700	-	60.0±5.6	0.45
Modification of aromatic moiety						
8	**48**		15500±1500	-	>100000	-0.3

9	**49**		13600±1000	-	>100000	-0.05
Modification of bridge bond						
10	**11**		13.4±1.5	2.9±0.3	1.0±0.2	0.25
11	**12**		-	0.22±0.03	0.09±0.01	0.52
12	**13**		123±5	23.5±0.1	6.3±1.2	0.32
13	**39**		-	9.7±1.2	2.4±0.3	0.2
14	**40**		-	7.1±0.6	2.0±0.1	0.2
15	**41**		2700±300	-	32.3±4.3	0.28
16	**42**		8700±600	-	235±28	0.23
17	**43**		4300±10	-	222±56	0.34

18	**44**		5900±1300	-	25.2±2.2	0.31

a. Unless otherwise specified, R^1 ~ R^4 are the same as TA. *b*. IC_{50}: 50% inhibitory concentration (mean ± SD, n = 3). *c*. Determined by MTT assay on MCF-7 cells following 24 h drug treatment. *d.* Determined by MTT assay on MCF-7 cells following 72 h drug treatment. *e*. Determined by HRE promoter luciferase assay. *f*. Rm: Hydrophobic parameter[51] calculated as shown in the Experimental section. *g.* Data from ref. 32.

Table 1b. Cytotoxic activity against MCF-7, MDA-MB-231, A549, and HT29 cells

compound	$IC_{50}{}^{a}$ [nM]			
	MCF-7	MDA-MB-231	A549	HT29
Ec (**1**)	2.0±0.1	2.3±0.3	4.7±0.6	3.5±0.2
TA (**2**)	212±14	178±3	322±85	340±42
Alkene (**11**)	2.9±0.3	3.2±0.3	5.3±0.4	6.2±0.5
Alkane (**13**)	23.5±0.1	25.3±0.5	71.9±4.5	57.4±7.5
S-ether (**39**)	9.7±1.2	8.0±0.4	16.2±0.2	16.2±0.6
Se-ether (**40**)	7.1±0.6	6.8±0.2	14.5±0.4	11.7±0.9

a. IC_{50}: 50% inhibitory concentration (mean ± SD, n = 3). Determined by MTT assay following 72 h drug treatment.

We subsequently evaluated the cytotoxicity of the new bridged bicyclic peptides **11**, **13**, **39**, **40**, TA (**2**), and Ec (**1**) against a variety of cancer cell lines. Table 1b shows the IC_{50} values determined using the MTT assay for MCF-7 and MDA-MB-231 breast cancer, A549 lung carcinoma, and HT29 colorectal adenocarcinoma cell lines treated with the

test compounds for 72 h. These compounds were also highly cytotoxic to normal cell lines, MCF-10A cells (mammary cell lines) and WRL68 cells (human embryonic liver cells)[54], indicating no tumor-selective toxicity (Table S17). Among the synthesized bridged octadepsipeptides, alkene-linked compound **11** exhibited the most potent cytotoxicity, which was comparable with Ec in all cell lines. The alkane-bridged derivative **13** obtained by hydrogenating compound **11** was an order of magnitude less active than **11** but 10 times more active than TA (**2**). These results also emphasized the importance of conformational rigidity for biological activity.

Analysis of HIF-1α protein expression

Ec has been shown to inhibit HIF-1α by DNA binding activity specific for the HRE.[11] On the other hand, we have previously demonstrated that Ec and TA suppress hypoxia-induced HIF-1α protein expression in the MCF-7 cells.[32] These results indicate that the Ec derivatives must have other mechanisms to suppress the accumulation of HIF-1α protein in addition to the DNA binding. Thus, Western blot analysis of HIF-1α was performed by treatment with the unnatural bridged derivatives, **11**, **12**, **39**, and **40**,

displaying potent HIF-1 inhibitory activity (Figure 5, see also Figure S22). All of the investigated compounds effectively suppressed the HIF-1 protein expression at very low concentrations below their cytotoxic dose in a concentration-dependent manner. In particular, derivative **12** significantly reduced hypoxia induction of HIF-1α protein expression at a dose of just 0.1 nM. This result places **12** among the most powerful known HIF-1 inhibitors.[23] In contrast, (+)-TA (**47**) had no effect on the HIF-1 protein expression. In reverse transcription polymerase chain reaction analysis, Ec and TA had no significant effect on the HIF-1α mRNA expression under hypoxia (data not shown).

Furthermore, we examined DNA binding interaction of new bicyclic depsipeptides by thermal denaturation method using differential scanning fluorimetry.[55, 56] Bis-intercalators, such as Ec, preferentially bind to specific sequences of DNA, resulting in stabilization of the DNA duplex and increasing its melting temperature (Tm). Then, melting curve analysis of a 11 bp double stranded DNA containing two Ec-binding ACGT sites or a fluorescently labelled hairpin-shaped DNA containing an ACGT site[57] was carried out in the presence or absence of bicyclic octadepsipeptides (Ec (**1**), **11**, **12**, or TA (**2**)) using

real-time PCR instruments[58] (Figure S21). As shown in Table 2, the order of Tm was Ec (**1**) > **12** > **11** > TA (**2**), indicating that Ec showed the strongest DNA binding. It was suggested that the potent cytotoxicity and HIF-1 inhibitory effect of these compounds could not be solely attributed to the ability of DNA binding.

Table 2. Binding affinity of bicyclic octadepsipeptides towards dsDNA containing ACGT site.

compound	Tm [°C][a]	Tm [°C][b]
non	44.2 ± 1.0	70.7 ± 0.0
Ec (**1**)	55.8 ± 0.5	81.6 ± 1.0
TA (**2**)	47.2 ± 0.6	78.0 ± 0.6
11	52.5 ± 1.0	81.3 ± 0.6
12	54.3 ± 2.2	44.2 ± 1.0

The melting temperatures (Tm) were determined (mean ± SD, n = 3) for the following oligonucleotides: *a*. d(ACGTAGAACGT) *b*. fluorescently labelled hairpin-shaped DNA, 5'–FAM–CCCTACGTATATGAAAATATACGTAGGG–BHQ1–3'. (See also Figure S21 and experimental section in Supporting Information).

These findings indicate the existence of targets involved in the translational step or degradation activation of HIF-1α, which is noteworthy for studying the mechanism of potent HIF-1 inhibitory activity of these compounds.

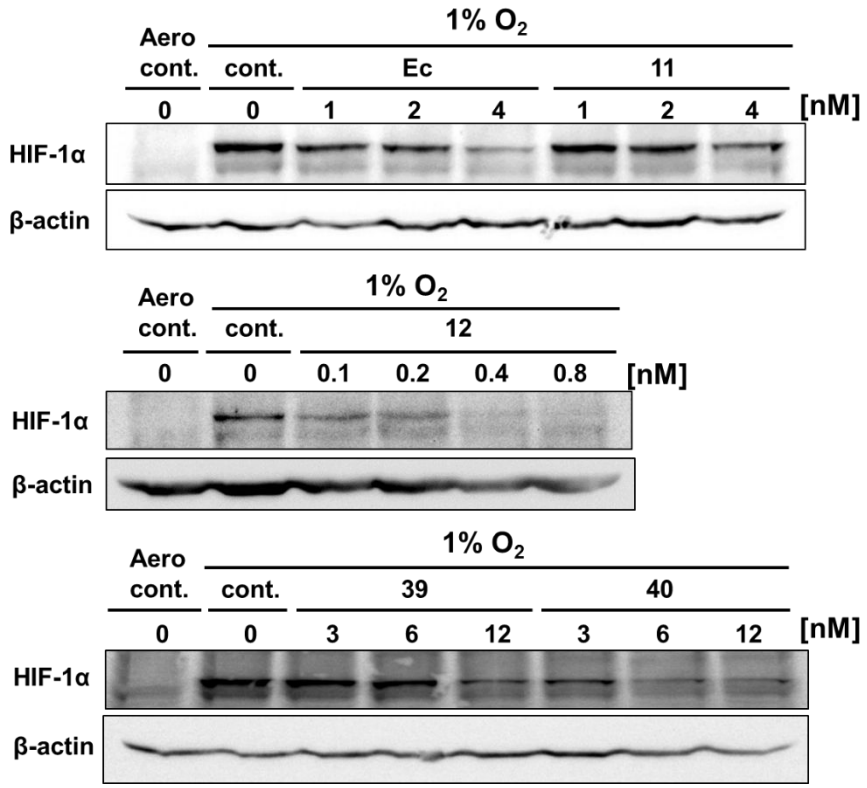


Figure 5. Western blot analyses of HIF-1α to evaluate the inhibition of Ec (**1**), **11**, **12**, **39**, and **40** on HIF-1α protein expression of MCF-7 cells induced by hypoxia. MCF-7 cells were incubated with several concentrations of test compounds for 16 h under aerobic (20% O_2) or hypoxic (1% O_2) conditions.

Conformational analysis of the synthesized compounds

To investigate the relationship between the conformation and cytotoxicity or the HIF-1 inhibitory effect of the presented cyclic depsipeptides, we examined their conformations

in detail. The nuclear magnetic resonance (NMR) structure of TA in $CDCl_3$ exists as a mixture of two symmetrical conformations, of which the preferred conformer in a polar solvent is similar to the conformation of Ec in $CDCl_3$.[59, 60] On the other hand, all of the novel bridged derivatives, alkenes (**11** and **12**), S-ether (**39**), and Se-ether (**40**), displayed a symmetrical conformation, similar to that of Ec in $CDCl_3$ on the NMR time scale at room temperature. In addition, the crystal structures of TA and Ec-2QN alone and of their complexes with a short self-complementary deoxyoligonucleotide have also been previously determined.[61-63] We successfully elucidated the crystal structures of the new derivatives **11**, **39**, and **40** (Figure 3, see also Figure S1 and Table S1). All new compounds exhibited a conformation of an octadepsipeptide ring, analogous to those of TA and Ec-2QN,[35] in which all peptide bonds were *trans*, the Cys–αCH and Cys–C=O groups were facing each other inside the ring, and the carbonyl oxygen atoms of all other amino acids were oriented in the same direction as the bridge bond protruding over the ring. Conversely, the orientation of the quinoxaline chromophores was different from that of TA. The chromophores protruded to the outside of the ring in TA, whereas in the alkene-bridged derivative (**11**) they rotated under the ring to face each other (Figure 3B).

Furthermore, in all of the analyzed crystals, the quinoxaline carboxylic acid and D-Ser formed a *trans*-amide, and the amide and C=N bonds of quinoxaline were in an s-*cis* relationship. The crystal packing structures of all evaluated compounds exhibited a regular dimeric stacking structure, in which two molecules face each other on the aromatic ring side, resulting in alternate π-stacking of the quinoxaline rings (Figure S1). The αC–N bond of D-Ser can be easily rotated to orient the chromophores in parallel to each other, as observed for the Ec-DNA complex.[63] Moreover, from the obtained crystal data for the new derivatives (**11**, **39**, and **40**), Ec-2QN, Ec-DNA bound (extracted from the Ec-DNA complex, 3GO3),[61] TA, and TA-D,D-Val,[32] the distances between the pendant quinoxaline carbonyl carbons (distance a) and the distance between the bridgehead carbon atoms (distance b) were measured as an indicator of ring strain (Table S1). Distances a, the major axis of the elliptical macrocycle, were close to that of Ec-2QN (run 1) and were significantly shorter than those of less active compounds TA and TA-D, D-Val (runs 6 and 7) for all of the synthesized compounds (runs 3–5). In addition, the torsion angles connecting two quinoxaline carbonyl carbon atoms as an index of the relative arrangement of the chromophores were almost identical for all new derivatives. The

crystal structures exhibit a more constrained ring conformation, which is found to be closer to the 3-D structure of Ec (**1**) than TA (**2**).

Based on the crystal data analysis, there appeared to be a correlation between the macrocycle conformations and the observed biological activities. Hence, we subsequently performed molecular mechanics calculations under aqueous conditions to predict the dominant conformations of the cyclic octadepsipeptides in living cells.[64-66] Using the corresponding solid-state structures as the initial structures, the conformational search in water was performed by Monte Carlo torsional sampling (20,000 steps) utilizing MacroModel. As a result, unlike the crystal structures, the global minimum (GM) energy conformations of both Ec and TA adopted more compact globular structures, which were stabilized by intramolecular face-to-face and/or edge-to-face π–π stacking interactions with the quinoxaline moieties (Figure 6A and Figure S23). Intriguingly, the conformation of Ec in cocrystals of Ec, biosynthetic enzyme Ecm18, and S-adenosyl-L-homocysteine, crystalized in aqueous buffer had a compact structure stabilized by intramolecular π stacking interactions and was similar to the obtained GM conformation (Figure 6A).

Notably, the other synthesized bicyclic depsipeptides **11**, **12**, **13**, **39**, and **40** gave nearly identical globular GM structures. However, they were classified into two groups with different relative arrangements of aromatic rings, presumably due to the steric effects of bridge bond structure (Figure 6B). Considering these observations, the calculated structures appear to be the expected forms under physiological conditions. In fact, previous structural analysis of Ec using NMR[60] and circular dichroism (CD)[67] spectroscopy revealed π–π stacking interactions between the quinoxaline rings. Hence, we measured the CD spectra of Ec, **11**, and **12** (100 μM phosphate buffer (pH 7.2) containing 40% (v/v) acetonitrile) at 25 ºC (Figure 6C see also Figure S25 to S28). A strong negative signal at approximately 240 nm (**11** and Ec) or 230 nm (**12**) was observed in the spectrum of each compound, which is characteristic for the π–π^* electronic transition of the quinoxaline or hydroxyquinoline chromophores, respectively. In addition, a small negative broad band in the higher wavelength region was also noted. Although the spectra of all bicyclic derivatives had a strong negative peak, similar to that of Ec, the signal of **43** with an opened bridge-bond exhibited a significantly smaller and broad negative band. In the absorption spectrum, the hypochromic effect was observed for all

compounds having quinoxaline chromophores with adding of phosphate buffer to the solvent (Figure S26). On the other hand, **12** with hydroxyquinolines showed a red shift in the absorption spectrum with the addition of phosphate buffer, probably due to the dissociation of hydroxyl groups. Furthermore, when the concentrations of **11** and **12** were diluted from 100 to 0.4 μM in phosphate buffer (pH 7.2) containing 40% (v/v) acetonitrile at 25 °C, the molar extinction coefficient of each compound was constant (Figure S27). This result suggests that the Cotton effects observed in the CD spectra under the treated conditions are unlikely to originate from the intermolecular association found in the crystal lattice (Figure S1). The obtained experimental data indicated that the bicyclic octadepsipeptides exhibited an intramolecular stacking interaction between the pendant chromophores in aqueous solution, supporting their GM energy conformations, in which the two aromatic amide groups were folded and stacked. The GM structures of alkene-bridged compounds **11** and **12** were nearly identical; however, in the GM structure of **12**, the planarity of the aromatic amide moiety was stabilized by an additional hydrogen bond between the carbonyl group at the 2-position and the hydroxyl moiety at the 3-position of quinoline. As a result, its potential energy was an order of magnitude lower than other

compounds, indicating high stability. (Figure 6B). The existence of this hydrogen bond was also confirmed by the NMR spectrum ($CDCl_3$), in which the chemical shift for the quinoline OH proton (11.3 ppm) of **12** clearly shifted downfield in comparison with the corresponding proton of methyl-3-hydroxyquinoline-2-carboxylate (10.4 ppm).[44] We then examined the CD and the ultraviolet (UV) spectra at temperatures in the range of 5–65 ºC (Figure S28). The signal intensity of **11**, **12**, and Ec in CD spectra gradually decreased upon heating, suggesting that the intramolecular π−π interaction was attenuated. On the other hand, the GM structures of the open bridged monocyclic derivatives **41** and **43** were unsymmetrical and irregular structures in the absence of the π–π stacking interactions. Overall, therefore, increasing the rigidity of the octadepsipeptide ring by introducing a more constrained bridge structure is essential for the formation of biologically active conformations of quinomycin derivatives. TA displays several orders of magnitude lower activity than Ec, which could be not only a result of the conformational restriction, but also due to the metabolic instability of the disulfide bridge bond. Generally, our analyses imply that the presence of the metabolically stable and rigid bond at the bridge position of cyclic octadepsipeptides is critical to restrict the particular conformation of the depsipeptides in

the cell and to display potent bioactivities. In particular, the unnatural alkene bridge was stable for 24 h in the serum containing a cell culture medium (Figure S20); hence, it is expected to be superior to the natural bridge bonds in maintaining active conformations by constraining the octadepsipeptide ring.

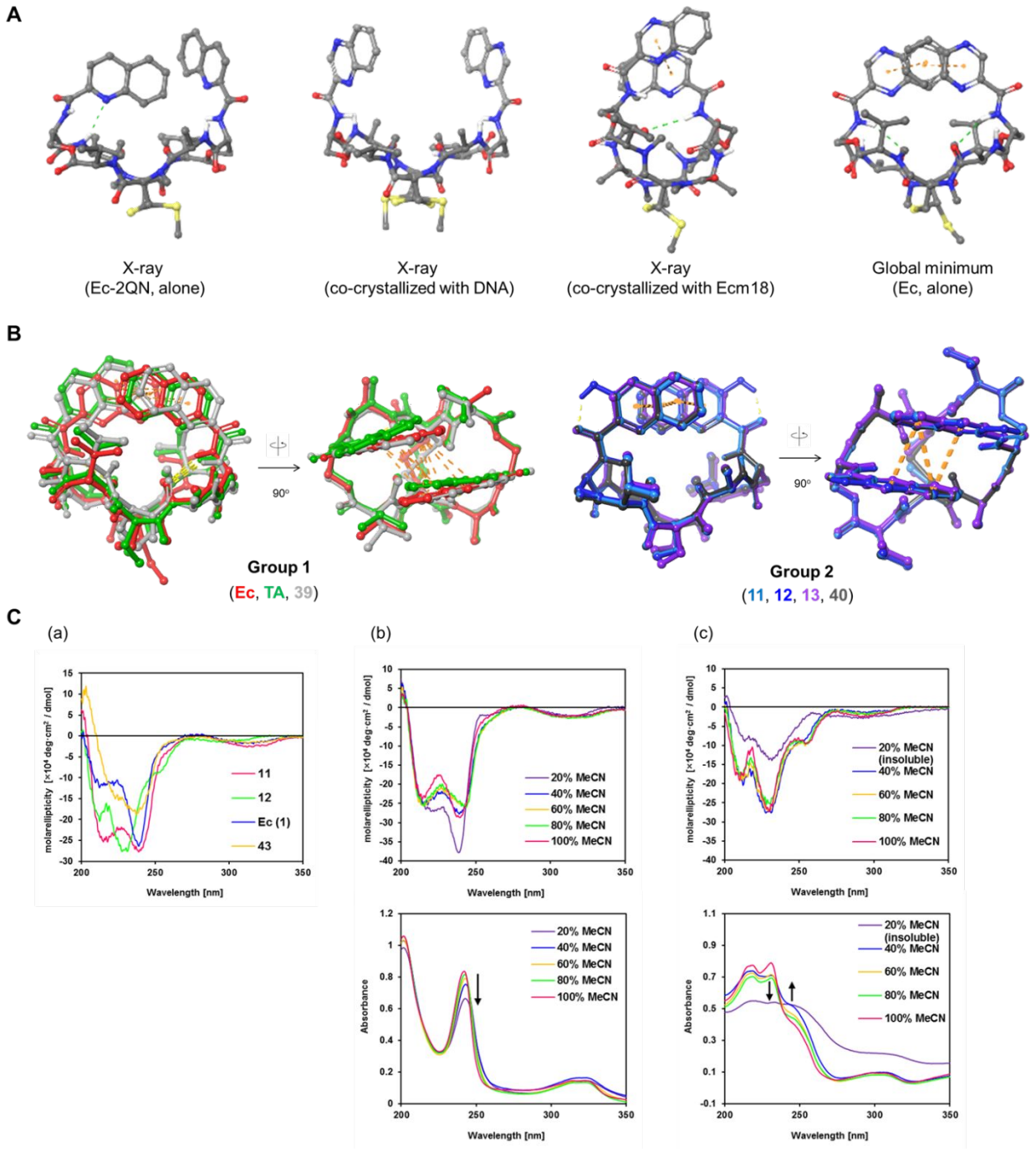


Figure 6. Conformational analysis of synthesized bicyclic octadepsipeptides. **A**, Alignment of X-ray structures and the global minimum structure of Ec (**1**) or Ec-2QN. X-

ray structure (Ec-2QN; CCDC no. 131833). X-ray structure (Ec (**1**) co-crystalized with DNA; PDB: 3GO3). X-ray structure (Ec (**1**) co-crystalized with Ecm18; PDB: 4NEC). Global minimum structure of Ec (**1**) selected by conformational search. **B**, Overlay of global minimum structures. Left: Ec (**1**), TA (**2**), and **39** as shown in group 1. Right: **11**, **12**, **13**, and **40** as shown in group 2. Superimposition was performed by selecting 4 atoms at Cα-carbons of bridge residues and carbonyl carbons directly connected to quinoxaline or 3-quinolinol, respectively. **C**, CD studies of bicyclic octadepsipeptides in aqueous solution. (a) CD spectra of compound Ec (**1**), **11**, **12**, and **43**. All spectra were obtained using 100 μM solution of the respective compound in 10 mM sodium phosphate buffer (pH 7.2) containing 40% (v/v) acetonitrile at 25 °C. (b) CD and UV spectra of compound **11** in 20–100% (v/v) acetonitrile. (c) CD and UV spectra of compound **12** in 20–100% (v/v) acetonitrile.

CONCLUSIONS

As previously mentioned, bridged bicyclic peptide scaffolds have shown remarkable potential for application in peptide drug development. Quinomycin antibiotics, natural bridged bicyclic peptides, are particularly attractive and promising lead compounds due to their potent antibacterial and antitumor effects. Crystal data of antitumor quinomycin antibiotics such as Ec and TA indicate that they exhibit a relatively simple and rigid structure with a symmetry property. Based on our previous SAR studies of the TA derivatives, it was considered that the symmetry property was one of the important structural requirements for the biological activity of this class of compounds. Moreover, introduction of ring strain into the macrocyclic compounds to reduce the structural flexibility was expected to improve the affinity toward the target substance in the biological environment. In the current study, constrained bicyclic octadepsipeptides containing various bridge structures were successfully synthesized. The obtained structures were demonstrated to fine-tune the ring strain without losing symmetry. Furthermore, to extract the active conformation in physiological conditions, we conducted conformation–activity relationship studies of these derivatives by conformational analysis using crystallography, spectrometry, and molecular modeling techniques. As a result, we proposed a new

globular conformation of bicyclic octadepsipeptides in aqueous conditions, in which two pendant chromophores fold each other and are stabilized by π–π stacking interactions. Such conformations can contribute to the potent biological activity of bicyclic peptides. By optimizing the bridge structure of the bicyclic depsipeptides, we finally succeeded in developing a promising new derivative **12** that surpasses the natural product Ec. Compound **12**, which contains an alkene-bridge bond and hydroxyquinoline chromophores, exhibits an excellent level of potency with 10-fold higher cytotoxicity and 4-fold higher HIF-1 inhibition than Ec. Until now, the intercalation of two pendant chromophores into the target DNA has been considered as the primary mechanism of cytotoxic activity of quinomycin antibiotics; however, present research suggests that there must be other target molecules interacting with the bicyclic depsipeptides developed herein in a unique fashion. As a result of their remarkable activity and straightforward synthesis, the unnatural bridged derivatives synthesized in the present study are very promising for application as new probes for target discovery and as payloads for bioconjugate drugs. Hence, the achievements in the development of cyclic peptides

constrained by cross-linking through unnatural chemical bonds serve as a useful guide for expansion of the chemical space of peptides in the field of drug discovery.

EXPERIMENTAL SECTION

General experimental conditions.

All commercially available reagents and solvents were used without further purification. All reactions were carried out under a nitrogen atmosphere. Normal-phase thin layer chromatography (TLC) was carried out on Silica gel 60 F254 (Merck, 1.05715.0009) using reagent grade solvents. TLC was detected by the absorption of UV light (254 nm) or using a visualization reagent (molybdophosphoric acid). Column chromatography was performed on silica gel (AP-300, Taiko-shoji) with mixed solvents as described. ^{1}H and ^{13}C NMR spectra were obtained for samples in the indicated solution at 25 °C utilizing the JEOL JNM-ECA500 spectrometer at 500 MHz frequency or the JNM-AL400 spectrometer at 400 MHz frequency in $CDCl_3$ or dimethylsulfoxide (DMSO)-d_6 with tetramethylsilane as an internal standard. ^{1}H NMR chemical shifts are reported in terms of the chemical shift (δ, ppm) relative to the singlet corresponding to tetramethylsilane at 0 ppm. Splitting

patterns are designated as follows: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; td, triplet of doublets; q, quartet; ddd, doublet of doublet of doublets; ddt, doublet of doublet of triplets; m, multiplet; br, broad. Coupling constants are reported in Hz. ^{13}C NMR spectra were fully decoupled and are reported in terms of the chemical shift (δ, ppm) relative to a triplet at δ = 77.0 ppm corresponding to $CDCl_3$ or a septet at δ = 39.5 ppm corresponding to DMSO-d_6. Melting points were obtained on cover glasses and were uncorrected. Electrospray ionization-mass spectrometry or direct analysis in real time-mass spectrometry measurements were carried out on the JEOL JMS-T100TD spectrometer. Optical rotations were measured using the JASCO P-1020 apparatus. The purity of the target compounds for biological evaluation was established by elemental analysis or RP-HPLC and HRMS analyses. RP-HPLC or LC-MS were performed on 20-AD series (Shimadzu) or LCMS-IT-TOF (Shimadzu) equipped with Waters Symmetry C18 analytical column (Waters, 3.5 μm, 4.6 × 75 mm, flow rate 0.5 mL/min). Solvent systems consisting of 0.1% (v/v) TFA or 0.05% (v/v) formic acid in ultra-pure water and MeCN were used and the eluting products were detected by light absorption at 220 nm. The purity (%) of the corresponding compound was determined by the ratio of the main peak

area to the total peak area (See the HPLC charts on pages S19–S21 in the Supporting Information).

N-Boc-*N*-Me-L-Gly(All)-OH (4) [cas: 136092-76-7]

H-L-Gly(allyl)-OH (3 g, 26.1 mmol) was dissolved in a mixture of 1,4-dioxane (20 mL) and 2 M NaOH aq. (50 mL) and a solution of Boc_2O (8.53 g, 39.1 mmol, 1.5 equiv.) in 1,4-dioxane (30 mL) was added to the solution at 0 ºC, and the resulting solution was warmed to room temperature and stirred at the same temperature for 3.5 h. Then, Boc_2O (2.84 g, 13.0 mmol, 0.5 equiv.) in 1,4-dioxane (10 mL) was added to the mixture and the mixture was stirred overnight. The mixture was then quenched with 10% citric acid aq. (200 mL), and extracted with AcOEt (200 mL × 3). The organic layer was washed with sat. NaCl aq. (200 mL), and dried over $MgSO_4$, solvent was removed under reduced pressure. Resulting crude was dissolved in dry THF (100 mL) and NaH (2.61 g, 60% in oil, 65.1 mmol, 2.5 equiv.) was added at 0 ºC, and the reaction mixture was stirred at 0 ºC for 30 min. Then methyl iodide (8.1 mL, 130.3 mmol, 5 equiv.) was added to the reaction mixture and the resulting solution was warmed to room temperature and stirred overnight. Then,

NaH (60% oil suspension, 5.22 g, 130.3 mmol, 5 equiv.) and methyl iodide (16.2 mL, 260.5 mmol, 10 equiv.) was added and the mixture was stirred until s.m. completely consumed monitored by TLC. The mixture was then quenched with 20% citric acid aq. (200 mL) and extracted with AcOEt (300 mL × 2). The combined organic layers were washed with sat. NaCl aq. (200 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (150 g) column chromatography eluted with *n*-hexane/CH_2Cl_2 (10:0, 5:5, 2:8), CH_2Cl_2/MeOH (10:0, 100:1, 50:1) to afford the target compound **4** (5.38 g, 23.5 mmol, 90% yield) as yellow oil; ^{1}H NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 5.70–5.79 (m, γ-CH), 5.09–5.20 (m, δ-CH_2), 4.80 (q, *J* = 5.2 Hz, α-CH), 4.47 (q, *J* = 5.0 Hz, α-CH), 2.87 (s, *N*-Me), 2.82 (s, *N*-Me), 2.70–2.76 (m, β-CH_2), 2.50–2.57 (m, β-CH_2), 1.47 (s, Boc), 1.45 (s, Boc); ^{13}C NMR (mixture of rotamers, 125 MHz, $CDCl_3$) δ 176.3 (COOH), 156.5 (Boc-C=O), 155.5 (Boc-C=O), 133.6 (All-=CH), 118.0 (All-=CH_2), 117.7 (All-=CH_2), 80.8 (Boc-C), 80.5 (Boc-C), 59.3 (β-CH_2), 57.8 (α-CH), 33.5 (β-CH_2), 33.0 (β-CH_2), 31.8 (*N*-Me), 31.2 (*N*-Me), 28.3 (Boc-CH_3); HRMS (DART) calcd for $C_{11}H_{20}NO_4^+$ $[M+H]^+$ 230.1387, found: 230.1393; $[\alpha]_D^{26.2}$ −2.5 ° (c 0.1, $CHCl_3$).

N-Cbz-D-Ser[*N*-Boc-*N*-Me-L-Gly(All)-*N*-Me-L-Val]-*O*All (5)

To a solution of *N*-Cbz-D-Ser(*N*-Boc-*N*-Me-L-Val)-*O*All (**3**)[32] (1.18 g, 2.4 mmol, 1.1 equiv.) in AcOEt (10 mL) was added 4 M HCl/AcOEt (10 mL, 40 mmol) at 0 ºC. Then the resulting solution was warmed to room temperature and stirred at the temperature for 2 h. The mixture was diluted with AcOEt (200 mL) and sat. $NaHCO_3$ aq. (100 mL). The two layers were separated and the organic layer was washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. Then a solution of the residue, *N*-Boc-*N*-Me-L-Gly(All)-OH **4** (500 mg, 2.2 mmol, 1 equiv.) and DMT-MM (0.91 g, 3.3 mmol, 1.5 equiv.) in AcOEt (20 mL) was stirred at room temperature overnight. The mixture was filtered, and the filtrate was diluted with AcOEt (150 mL) and water (150 mL). The two layers were separated and the organic layer was washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 9:1, 8:2) to afford the target compound **5** (mixture of diastereomers)

as pale yellow oil; HRMS (ESI) calcd for $C_{31}H_{45}N_3NaO_9^+$ $[M+Na]^+$ 626.3048, found: 626.3046.

N-Cbz-D-Ser[*N*-Boc-L-Ala-*N*-Me-L-Gly(All)-*N*-Me-L-Val]-*O*All (6)

N-Cbz-D-Ser[*N*-Boc-*N*-Me-DL-Gly(All)-*N*-Me-L-Val]-*O*All **5** was dissolved into TFA (5 mL) and the resulting solution was stirred at room temperature for 2 h. Then, solvent was removed under reduced pressure. A solution of the residue in DMF (15 mL) was added to the solution of NMM (0.49 mL, 4.8 mmol, 2 equiv.), *N*-Boc-L-Ala-OH (0.45 g, 2.4 mmol, 1 equiv.) and DMT-MM (1.00 g, 3.6 mmol, 1.5 equiv.) in DMF (5 mL) and the resulting mixture was stirred at room temperature overnight. The mixture was diluted with AcOEt (150 mL) and water (150 mL) and the two layers were separated. The organic layer was washed with sat. NaCl aq. (100 mL) and dried over $MgSO_4$, solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 9:1, 8:2, 7:3, 6:4) to afford the target compound **6** (1.00 g, 1.5 mmol, 62% yield on 4 steps) as pale yellow oil; ^{1}H NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 7.30–7.39 (m), 6.89 (d, J = 8.6 Hz), 5.83–5.93 (m), 5.79 (d, *J* = 8.0 Hz),

5.57–5.69 (m), 5.48 (dd, *J* = 8.6, 6.3 Hz), 5.44 (d, *J* = 8.0 Hz), 5.33 (d, *J* = 17.2 Hz), 5.25 (t, *J* = 9.5 Hz), 5.02–5.18 (m), 4.37–4.66 (m), 4.21 (d, *J* = 9.7 Hz), 4.01–4.04 (m), 3.04 (s), 2.95 (s), 2.94 (s), 2.85 (s), 2.44–2.55 (m), 2.14–2.27 (m), 1.43 (s), 1.42 (s), 1.26 (d, *J* = 6.9 Hz), 1.14 (d, *J* = 6.9 Hz), 0.95 (d, *J* = 6.3 Hz), 0.90 (d, *J* = 6.9 Hz), 0.79 (d, *J* = 6.9 Hz); ^{13}C NMR (mixture of rotamers, 125 MHz, $CDCl_3$) δ173.9, 173.4, 171.2, 171.1, 170.9, 170.3, 170.1, 169.6, 168.8, 156.2, 156.1, 155.6, 155.1, 155.0, 136.2, 136.1, 136.0, 133.2, 132.9, 131.4, 131.2, 131.1, 128.5, 128.4, 128.2, 128.1, 127.7, 119.1, 119.0, 118.7, 118.3, 118.2, 79.6, 67.1, 67.1, 66.6, 66.5, 66.4, 66.1, 65.8, 64.9, 64.8, 64.3, 63.1, 62.3, 60.3, 56.2, 54.5, 53.4, 53.3, 52.8, 51.6, 46.5, 46.3, 43.9, 33.3, 32.9, 31.4, 30.4, 30.2, 29.6, 28.4, 28.3, 27.7, 27.2, 21.0, 19.7, 19.5, 19.4, 19.0, 18.9, 18.7, 18.1, 15.2, 14.1; HRMS (ESI) calcd for $C_{34}H_{50}N_4NaO_{10}^+$ $[M+Na]^+$ 697.3419, found: 697.3396.

_N_-Cbz-D-Ser[_N_-Boc-L-Ala-_N_-Me-L-Gly(All)-_N_-Me-L-Val]-OH (7)

To a solution of _N_-Cbz-D-Ser[_N_-Boc-L-Ala-_N_-Me-L-Gly(All)-_N_-Me-L-Val]-_O_All **6** (1.83 g, 2.7 mmol), PPh_3 (28.5 mg, 0.11 mmol, 4 mol%) and _N_-methylaniline (0.35 mL, 3.3 mmol, 1.2 equiv.) in THF (15 mL) was added $Pd_2(dba)_3$ (12.4 mg, 0.014 mmol, 0.5 mol%). The

resulting solution was stirred at room temperature overnight in the dark. Then the mixture was diluted with AcOEt (100 mL) and sat. NH_4Cl aq. (100 mL). The two layers were separated and the water layer was extracted with AcOEt (100 mL). The combined organic layers were washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1) to afford the target compound **7** (1.56 g, 2.5 mmol, 91% yield) as pale yellow amorphous solid; ^{1}H NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 7.30–7.34 (m), 6.42 (d, *J* = 7.4 Hz), 6.06 (d, *J* = 8.0 Hz), 5.58–5.68 (m), 5.45–5.55 (m), 5.01–5.22 (m), 4.58–4.68 (m), 4.45–4.50 (m), 4.17 (d, *J* = 9.7 Hz), 2.97 (s), 2.95 (s), 2.80 (s), 2.43–2.53 (m), 2.09–2.27 (m), 1.95–2.05 (m), 1.61 (d, *J* = 7.4 Hz), 1.43 (s), 1.42 (s), 1.24–1.31 (m), 1.18 (t, *J* = 6.0 Hz), 0.99 (d, *J* = 6.3 Hz), 0.96 (d, *J* = 6.3 Hz), 0.86–0.92 (m), 0.84 (t, *J* = 7.4 Hz), 0.77–0.81 (m); ^{13}C NMR (mixture of rotamers, 125 MHz, $CDCl_3$) δ 173.9, 173.8, 171.5, 171.3, 171.2, 170.7, 170.1, 169.5, 156.0, 155.2, 136.1, 133.1, 133.0, 128.5, 128.2, 128.1, 118.5, 80.1, 79.8, 67.2, 67.1, 64.7, 64.5, 62.6, 53.1, 52.4, 46.6, 46.5, 33.4, 32.9, 31.9, 31.6, 30.4, 30.1, 29.7, 29.3,

29.1, 28.3, 27.3, 25.4, 22.7, 19.7, 19.6, 19.1, 18.8, 18.5, 18.3, 17.9, 14.1; HRMS (ESI) calcd for $C_{31}H_{45}N_4O_{10}^-$ $[M-H]^-$ 633.3141, found: 633.3147.

_N_-Cbz-D-Ser[_N_-Cbz-D-Ser(_N_-Boc-L-Ala-_N_-Me-L-Gly(All)-_N_-Me-L-Val)-L-Ala-_N_-Me-L-Gly(All)-_N_-Me-L-Val]-_O_All, _N_-Boc-octadepsipeptide-_O_All (Gly(All)) (8)

_N_-Cbz-D-Ser[_N_-Boc-L-Ala-_N_-Me-L-Gly(All)-_N_-Me-L-Val]-_O_All **6** (584.7 mg, 0.87 mmol, 1.1 equiv.) was dissolved into TFA (5 mL) and the resulting solution was stirred at room temperature for 2 h. The mixture was diluted with AcOEt (100 mL) and sat. $NaHCO_3$ aq. (100 mL). The two layers were separated and the organic layer was washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. Then a solution of the residue, _N_-Cbz-D-Ser[_N_-Boc-L-Ala-_N_-Me-L-Gly(All)-_N_-Me-L-Val]-OH **7** (500.0 mg, 0.79 mmol) and DMT-MM (261.6 mg, 0.95 mmol, 1.2 equiv.) in AcOEt (10 mL) was stirred at room temperature overnight. The mixture was then filtered, and the filtrate was diluted with AcOEt (100 mL) and water (100 mL). The two layers were separated and the water layer was extracted with AcOEt (100 mL). The combined organic layers were washed with sat. NaCl aq. (100 mL) and dried over $MgSO_4$,

the solvent was removed under reduced pressure. The residue was purified by silica gel (50 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 8:2, 6:4, 5:5, 4:6, 3:7, 2:8) to afford the target compound **8** (430.7 mg, 0.36 mmol, 46% yield on 2 steps) as colorless amorphous solid; HRMS (ESI) calcd for $C_{60}H_{86}N_8NaO_{17}^+$ $[M+Na]^+$ 1213.6003, found: 1213.5977.

N-Cbz-D-Ser[N-Cbz-D-Ser(N-Boc-L-Ala-N-Me-L-Gly(All)-N-Me-L-Val)-L-Ala-N-Me-L-Gly(All)-N-Me-L-Val]-OH, N-Boc-octadepsipeptide-OH (Gly(All)) (9)

To a solution of *N*-Boc-octadepsipeptide-*O*All (Gly(All)) **8** (420 mg, 0.35 mmol), PPh_3 (7.4 mg, 0.028 mmol, 8 mol%), *N*-methylaniline (0.046 mL, 0.42 mmol, 1.2 equiv.) in THF (5 mL) was added $Pd_2(dba)_3$ (3.2 mg, 0.0035 mmol, 1 mol%). The resulting solution was stirred at room temperature for 2 h in the dark. Then the mixture was diluted with AcOEt (100 mL) and sat. NH_4Cl aq. (100 mL). The two layers were separated and the organic layer was washed with sat. NaCl aq. (50 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (50 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 20:1, 10:1) to afford

the target compound **9** (361.1 mg, 0.31 mmol, 90% yield) as pale yellow amorphous solid; HRMS (ESI) calcd for $C_{57}H_{81}N_8O_{17}^-$ $[M-H]^-$ 1149.5725, found: 1149.5712.

Cbz-bicyclicpeptide (Gly(All)) (10)

To a solution of *N*-Boc-octadepsipeptide-*O*All (Gly(All)) **9** (360 mg, 0.31 mmol) in dry CH_2Cl_2 (300 mL, 1 mM) was added Grubbs 2nd catalyst (5.3 mg, 0.0062 mmol, 2 mol%). The resulting solution was stirred under reflux overnight. The reaction solution was concentrated under reduced pressure. Then the mixture was diluted with CH_2Cl_2 (30 mL) and sat. NH_4Cl aq. (30 mL). The two layers were separated and the water layer was extracted with CH_2Cl_2 (30 mL × 2). The combined organic layers were washed with sat. NaCl aq. (50 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was dissolved into TFA (5 mL) and the resulting solution was stirred at room temperature for 2 h. The mixture was concentrated under reduced pressure. To a solution of the residue in DMF (300 mL, 1 mM) was added NMM (0.16 mL, 1.6 mmol, 5 equiv.) and DMT-MM (432.6 mg, 1.6 mmol, 5 equiv.). The resulting solution was stirred at room temperature overnight. The mixture was concentrated under reduced pressure

and the residue was dissolved in AcOEt (70 mL) and washed with water (200 mL). The water layer was extracted with AcOEt (70 mL). The combined organic layers were washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, solvent was removed under reduced pressure. The residue was purified by silica gel (50 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 6:4, 4:6, 2:8, 1:9, 1:50, 0:10) to afford the target compound **10** (mixture of *cis-trans* isomers, 214.8 mg, 0.21 mmol, 69% yield on 3 steps) as colorless solid; HRMS (ESI) calcd for $C_{50}H_{68}N_8NaO_{14}^+$ $[M+Na]^+$ 1027.4753, found: 1027.4750.

TA-alkene (11)

A solution of Cbz-bicyclicpeptide (Gly(All)) **10** (200 mg, 0.20 mmol) and 20% $Pd(OH)_2/C$ (20 mg, 10 wt%) in MeOH (5 mL) was stirred at room temperature under H_2 atmosphere (1 atm) for 4 h. Then the mixture was filtered and the filtrate was concentrated under reduced pressure. To solution of the residue and 2-quinoxalinecarboxylic acid (140.2 mg, 0.80 mmol, 4 equiv.) in DMF (5 mL) was added DMT-MM (330.4 mg, 1.2 mmol. 6 equiv.). The resulting solution was stirred at room temperature overnight. The mixture was dissolved in CH_2Cl_2 (50 mL) and washed with water (50 mL). The organic layer was

separated and washed with sat. NaCl aq. (50 mL), and dried over $MgSO_4$, solvent was removed under reduced pressure. The residue was purified by silica gel (10 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1) to afford the target compound **11** (116.1 mg, 0.11 mmol, 55% yield on 2 steps) as colorless solid (recrystallized from Et_2O/MeOH/CH_2Cl_2 to afford colorless crystalline solid for X-ray crystallography); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.64 (s, 2H), 8.69 (br, d, *J* = 5.7 Hz, 2H), 8.20 (d, *J* = 8.6 Hz, 2H), 7.93 (d, *J* = 8.6 Hz, 2H), 7.85–7.88 (m, 2H), 7.77–7.80 (m, 2H), 6.88 (br, 2H), 6.09 (br, 2H), 5.49–5.55 (m, 2H), 5.19 (d, *J* = 10.3 Hz, 2H), 4.91–4.93 (m, 2H), 4.76–4.82 (m, 2H), 4.70 (br, 4H), 3.14 (s, 6H), 3.02 (s, 6H), 2.24–2.36 (m, 2H), 1.38 (d, *J* = 6.9 Hz, 6H), 1.10 (d, *J* = 6.9 Hz, 6H), 0.90 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.5, 171.2, 170.1, 167.4, 164.1, 144.2, 143.6, 142.3, 140.1, 132.1, 131.1, 129.7, 129.3, 64.5, 62.0, 54.3, 52.8, 46.5, 31.3, 30.0, 27.7, 20.4, 18.8, 17.6; HRMS (ESI) calcd for $C_{52}H_{64}N_{12}NaO_{12}^+$ $[M+Na]^+$ 1071.4659, found: 1071.4649; m.p. 273.8–275.9 °C (dec.); $[\alpha]_D^{26.9}$ −279.7 ° (c 0.1, $CHCl_3$); Anal. calcd for $C_{52}H_{66}N_{12}O_{13}$ $[M+1H_2O]$: C, 58.53; H, 6.23; N, 15.75, found: C, 58.44; H, 6.13; N, 15.73.

TA-alkene (Qn-3OH) (12)

A solution of Cbz-bicyclicpeptide **10'** (isolated *cis*-isomer, separated by silica gel column chromatography eluted with CH_2Cl_2/MeOH = 10:0, 200:1, 100:1, 50:1) (50 mg, 0.050 mmol) and thioanisole (0.058 mL, 0.50 mmol, 10 equiv.) in TFA (5 mL) was stirred at 50 ºC overnight. Then the mixture was concentrated under reduced pressure. To a solution of 3-hydroxyquinoline-2-carboxylic acid (37.6 mg, 0.20 mmol, 4 equiv.), HOAt (27.1 mg, 0.20 mmol, 4 equiv.) $NaHCO_3$ (41.8 mg, 0.50 mmol, 10 equiv.), and EDCI·HCl (38.1 mg, 0.20 mmol, 4 equiv.) in DMF (3 mL) was added the residue in DMF (2 mL). The resulting solution was stirred at room temperature for 5 h. The mixture was then dissolved in AcOEt (50 mL) and washed with water (50 mL). The organic layer was washed with 1 M HCl aq. (30 mL), sat. $NaHCO_3$ aq. (30 mL) and sat. NaCl aq. (30 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (20 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 30:1, 20:1) and YAMAZEN ULTRA PACK ODS-SM-50B 26 × 300 mm, 50 μm eluted with 0.1% TFA in MeCN/H_2O to afford the target compound **12** (25.2 mg, 0.023 mmol, 47% yield)

as pale yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.26 (s, 2H), 9.07 (d, *J* = 7.4 Hz, 2H), 7.78–7.81 (m, 2H), 7.67–7.70 (m, 2H), 7.64 (s, 2H), 7.45–7.48 (m, 4H), 6.75 (brs, 2H), 6.08 (t, *J* = 7.4 Hz, 2H), 5.48–5.54 (m, 2H), 5.17 (d, *J* = 10.3 Hz, 2H), 4.84–4.87 (m, 2H), 4.76–4.82 (m, 2H), 4.66–4.73 (m, 4H), 3.18 (s, 6H), 3.01 (s, 6H), 2.69 (brs, 2H), 2.47 (brs, 2H), 2.29–2.37 (m, 2H), 1.36 (d, *J* = 6.9 Hz, 6H), 1.10 (d, *J* = 6.3 Hz, 6H), 0.89 (d, *J* = 6.3 Hz, 6H) ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.5, 171.1, 170.4, 169.2, 167.3, 153.8, 141.6, 134.0, 132.4, 129.3, 128.9, 128.1, 127.5, 126.6, 120.9, 64.2, 62.2, 54.4, 52.7, 46.5, 31.5, 30.0, 27.9, 26.6, 20.4, 18.9, 17.6; HRMS (ESI) calcd for $C_{54}H_{66}N_{10}NaO_{14}^{+}$ $[M+Na]^{+}$ 1101.4652, found: 1101.4632; m.p. 201.2–203.9 °C; $[\alpha]_D^{26.4}$ −216.3 ° (c 0.1, $CHCl_3$); Anal. calcd for $C_{56}H_{67}F_3N_{10}O_{16}$ [M+1TFA]: C, 56.37; H, 5.66; N, 11.74, found: C, 55.97; H, 5.83; N, 11.57.

TA-alkane (13)

A solution of Cbz-bicyclicpeptide (Gly(All)) **10** (20 mg, 0.020 mmol) and 20% $Pd(OH)_2/C$ (8.0 mg, 40 wt%) in MeOH (10 mL) was stirred at room temperature under H_2 atmosphere (3 atm) for 1 day. Then the mixture was filtered and the filtrate was concentrated under

reduced pressure. To a solution of the residue and quinoxaline-2-carboxylic acid (14.0 mg, 0.080 mmol, 4 equiv.) in DMF (5 mL) was added DMT-MM (33.0 mg, 0.12 mmol, 6 equiv.). The resulting solution was stirred at room temperature overnight. The mixture was diluted with CH_2Cl_2 (50 mL) and water (50 mL) and the two layers were separated. The organic layer was washed with sat. NaCl aq. (50 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (10 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 20:1) to afford the target compound **10** (17.1 mg, 0.016 mmol, 81% yield on 2 steps) as colorless solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.55 (s, 2H), 9.08 (d, *J* = 9.7 Hz, 2H), 8.40 (d, *J* = 9.7 Hz, 2H), 8.15 (d, *J* = 7.4 Hz, 2H), 8.10 (d, *J* = 8.0 Hz, 2H), 7.88–7.94 (m, 4H), 5.72 (d, *J* = 13.2 Hz, 2H), 5.20 (dd, *J* = 9.7, 4.0 Hz, 2H), 5.14 (dd, *J* = 11.2, 3.7 Hz, 2H), 5.07 (dt, *J* = 15.5, 6.9 Hz, 2H), 4.45 (d, *J* = 10.9 Hz, 2H), 3.93 (d, *J* = 10.9 Hz, 2H), 3.42 (s, 6H), 2.95 (s, 6H), 2.36–2.40 (m, 4H), 1.67–1.72 (m, 2H), 1.53–1.56 (m, 2H), 1.40–1.48 (m, 2H), 1.02 (d, *J* = 5.2 Hz, 6H), 1.01 (d, *J* = 4.6 Hz, 6H), 0.19 (d, *J* = 6.3 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.1, 172.0, 170.3, 168.1, 163.9, 143.9, 143.8, 142.8, 140.3, 132.0, 131.3, 130.0, 129.3, 64.6, 64.2, 52.9, 51.2, 44.2, 32.2, 28.9, 28.6, 28.5, 24.1, 19.8, 19.3, 17.4;

HRMS (ESI) calcd for $C_{52}H_{66}N_{12}NaO_{12}^+$ $[M+Na]^+$ 1073.4815, found: 1073.4794; m.p. 144.5–149.3 °C; $[\alpha]_D^{27.1}$ −107.9 ° (c 0.05, $CHCl_3$); Purity: 95%, t_R = 9.7 min.

N-Boc-*N*-Me-L-Ser-*O*All (15)

H-L-Ser-OH **14** (5 g, 47.6 mmol) was dissolved in a mixture of 1,4-dioxane (50 mL) and 2 M NaOH aq. (100 mL) and a solution of Boc_2O (16.61 g, 76.1 mmol, 1.6 equiv.) in 1,4-dioxane (50 mL) was added dropwise to the solution at room temperature, and the resulting solution was stirred overnight. The mixture was then quenched with 1 M $KHSO_4$ aq. (200 mL), and extracted with AcOEt (200 mL × 2). The combined organic layers were washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, solvent was removed under reduced pressure. To a solution of the residue and imidazole (9.72 g, 142.7 mmol, 3 equiv.) in THF (130 mL) was added a solution of TBDMSCl (8.60 g, 57.1 mmol, 1.2 equiv.) in THF (70 mL) at 0 ºC. The mixture was warmed to room temperature, and stirred at the temperature overnight. The mixture was filtered and the filtrate was diluted with AcOEt (200 mL) and 1 M $KHSO_4$ (200 mL). The two layers were separated and the water layer was extracted with AcOEt (200 mL). The combined organic layers were washed with sat.

NaCl aq. (200 mL), and dried over $MgSO_4$, solvent was removed under reduced pressure. Resulting crude was dissolved in dry THF (200 mL) and NaH (60% in oil, 7.61 g, 190.3 mmol, 4 equiv.) was added at 0 ºC, and the reaction mixture was stirred at 0 ºC for 30 min. Then methyl iodide (17.8 mL, 285.5 mmol, 6 equiv.) was added to the reaction mixture and the resulting solution was warmed to room temperature. After 4 h, the reaction was quenched with 1 M $KHSO_4$ aq. (200 mL), and extracted with AcOEt (200 mL × 2). The combined organic layers were washed with sat. $Na_2S_2O_3$ aq. (100 mL) and NaCl aq. (200 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. Then a solution of the residue, allylbromide (6.1 mL, 71.4 mmol, 1.5 equiv.) and K_2CO_3 (7.89 g, 57.1 mmol, 1.2 equiv.) in DMF (150 mL) was stirred at room temperature for 1 h. After the filtration of an insoluble solid, DMF was removed under reduced pressure and the residue was diluted with AcOEt (200 mL) and water (200 mL). The two layers were separated and the water layer was extracted with AcOEt (200 mL). The combined organic layers were washed with sat. NaCl aq. (200 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was dissolved in the mixture of AcOH (70 mL), THF (35 mL), and water (35 mL). The mixture was stirred at 50 ºC overnight, and

diluted with AcOEt (200 mL) and water (200 mL). The two layers were separated and the water layer was extracted with AcOEt (200 mL). The combined organic layers were washed with sat. NaCl aq. (200 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 8:2, 7:3, 6:4) to afford the target compound **15** (5.06 g, 19.8 mmol, 42% yield on 5 steps) as yellow oil; ^{1}H NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 5.88–5.95 (m, allyl-=CH), 5.34 (d, *J* = 17.2 Hz, allyl-=CH_2 (*E*)), 5.26 (dd, *J* = 14.9, 5.7 Hz, allyl-=CH_2 (*Z*)), 4.64–4.69 (m, allyl-CH_2), 4.44 (br, α-CH), 4.22 (br, α-CH), 4.12 (br, β-CH_2), 3.92 (br, β-CH_2), 3.79 (br, β-CH_2), 2.96 (s, *N*-Me), 2.92 (s, *N*-Me), 2.74 (br-OH), 2.35 (br, OH), 1.47 (s, Boc), 1.43 (s, Boc); ^{13}C NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 170.3 (Ser-CO), 169.9 (Ser-CO), 156.3 (Boc-CO), 155.1 (Boc-CO), 131.5 (allyl-=CH), 131.3 (allyl-=CH), 118.7 (allyl-=CH_2), 118.3 (allyl-=CH_2), 80.7 (Boc-C), 80.4 (Boc-C), 65.6 (allyl-OCH_2), 62.2 (α-CH), 61.4 (α-CH), 60.8 (β-CH_2), 60.7 (β-CH_2), 33.7 (*N*-Me), 33.4 (*N*-Me), 28.1 (Boc-CH_3); HRMS (DART) calcd for $C_{12}H_{22}NO_5^+$ $[M+H]^+$ 260.1492, found: 260.1502; $[\alpha]_D^{25.7}$ −15.3 ° (c 0.1, $CHCl_3$).

Allyl Ethyl carbonate [cas: 1469-70-1]

To a solution of allyl alcohol (10 g, 172.2 mmol), pyridine (35.6 mL, 344.4 mmol, 2 equiv.) and DMAP (2.10 g, 17.2 mmol, 0.1 equiv.) in dry CH_2Cl_2 (400 mL) was added ethyl chloroformate (24.6 mL, 258.3 mmol, 1.5 equiv.) at 0 ºC. Then the resulting solution was warmed to room temperature and stirred overnight. The mixture was then washed with 10% citric acid aq. (100 mL × 5) and sat. NaCl aq. (200 mL). The organic layer was dried with $MgSO_4$, the solvent was removed under reduced pressure to afford target compound (22.13 g, 170.0 mmol, 99% yield) as pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.94 (qd, *J* = 11.1, 5.6 Hz, 1H, All-CH), 5.36 (dd, *J* = 17.2, 1.1 Hz, 1H, All-CH_2(*E*)), 5.27 (d, *J* = 10.9 Hz, 1H, All-CH_2(*Z*)), 4.63 (d, *J* = 5.7 Hz, 2H, All-OCH_2), 4.21 (q, *J* = 7.1 Hz, 2H, Et-OCH_2), 1.32 (t, *J* = 7.2 Hz, 3H, Et-CH_3).

N-Boc-*N*-Me-L-Ser(All)-OH (16)

A solution of *N*-Boc-*N*-Me-L-Ser-*O*All **15** (5 g, 19.3 mmol), PPh_3 (202.3 mg, 0.77 mmol, 4 mol%), allyl ethyl carbonate (5.02 g, 38.6 mmol, 2 equiv.) in THF (60 mL) was added $Pd_2(dba)_3$ (88.3 mg, 0.10 mmol, 1 mol%). The mixture was stirred at 60 ºC for 2 h in the

dark. Then the mixture was diluted with AcOEt (200 mL) and sat. NH_4Cl aq. (200 mL). The two layers were separated and the water layer was extracted with AcOEt (200 mL). The combined organic layers were washed with sat. NaCl aq. (200 mL), dried with $MgSO_4$ and the solvent was removed under reduced pressure. The residue, PPh_3 (202.3 mg, 0.77 mmol, 4 mol%) and *N*-methylaniline (2.5 mL, 23.1 mmol, 1.2 equiv.) in THF (60 mL) was added $Pd_2(dba)_3$ (88.3 mg, 0.10 mmol, 1 mol%). The mixture was stirred at room temperature overnight in the dark. Then the mixture was diluted with AcOEt (200 mL) and sat. NH_4Cl aq. (200 mL). The two layers were separated and the water layer was extracted with AcOEt (200 mL). The combined organic layers were washed with sat. NaCl aq. (200 mL), dried with $MgSO_4$ and the solvent was removed under reduced pressure. The residue was purified by silica gel (150 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 20:1) to afford the target compound **16** (4.31 g, 16.6 mmol, 86% yield on 2 steps) as pale yellow oil; ^{1}H NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 5.88 (qd, *J* = 11.0, 5.4 Hz, All-CH), 5.28 (d, *J* = 17.2 Hz, All-CH_2(*E*)), 5.20 (d, *J* = 10.3 Hz, All-CH_2(*Z*)), 4.84 (br, α-CH), 4.51 (br, α-CH), 3.79–4.07 (m, All-CH_2, β-CH_2), 2.97 (s, *N*-Me), 2.93 (s, *N*-Me), 1.47 (s, Boc), 1.46 (s, Boc), 1.43 (s, Boc).

N-Cbz-D-Ser[*N*-Boc-*N*-Me-L-Ser(All)-*N*-Me-L-Val]-*O*All (17)

To a solution of *N*-Cbz-D-Ser(*N*-Boc-*N*-Me-L-Val)-*O*All **3**[32] (3 g, 6.1 mmol) in AcOEt (10 mL) was added 4 M HCl/AcOEt (10 mL, 40 mmol) at 0 ºC. Then the resulting solution was warmed to room temperature and stirred at the temperature overnight. The mixture was diluted with AcOEt (200 mL) and sat. $NaHCO_3$ aq. (200 mL). The two layers were separated and the water layer was extracted with AcOEt (200 mL). The combined organic layers were washed with sat. NaCl aq. (200 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. Then a solution of the residue, *N*-Boc-*N*-Me-L-Ser(All)-OH **16** (1.74 g, 6.7 mmol, 1.1 equiv.) and DMT-MM (2.53 g, 9.1 mmol, 1.5 equiv.) in AcOEt (20 mL) was stirred at room temperature overnight. The mixture was filtered, and the filtrate was diluted with AcOEt (200 mL) and water (200 mL). The two layers were separated and the water layer was extracted with AcOEt (200 mL). The combined organic layers were washed with sat. NaCl aq. (200 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by silica gel (150 g) column

chromatography eluted with *n*-hexane/AcOEt (10:0, 9:1, 8:2) to afford the target compound **17** (3.43 g, 5.7 mmol, 93% yield on 2 steps) as colorless oil.

N-Cbz-D-Ser[*N*-Boc-L-Ala-*N*-Me-L-Ser(All)-*N*-Me-L-Val]-*O*All (18)

To a solution of *N*-Cbz-D-Ser[*N*-Boc-*N*-Me-L-Ser(All)-*N*-Me-L-Val]-*O*All **17** (3.4 g, 5.4 mmol) in AcOEt (10 mL) was added 4 M HCl/AcOEt 10 mL (40 mmol) at 0 ºC. Then the resulting solution was warmed to room temperature and stirred at the temperature for 3 h. The solvent was removed under reduced pressure. Then DMF (10 mL) solution of the residue was added to the solution of *N*-Boc-L-Ala-OH (1.22 g, 6.4 mmol, 1.2 equiv.), NMM (0.81 mL, 8.0 mmol, 1.5 equiv.) and DMT-MM (2.23 g, 8.0 mmol, 1.5 equiv.) in DMF (10 mL) was stirred at room temperature overnight. The mixture was diluted with AcOEt (200 mL) and water (200 mL). The two layers were separated and the water layer was extracted with AcOEt (200 mL).The combined organic layers were washed with sat. NaCl aq. (300 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by silica gel (150 g) column chromatography eluted with *n*-

hexane/AcOEt (10:0, 8:2, 7:3, 6:4) to afford the target compound **18** (1.55 g, 2.2 mmol, 41% yield on 2 steps, moderate purity) as pale yellow oil.

***N*-Cbz-D-Ser[*N*-Cbz-D-Ser(*N*-Boc-L-Ala-*N*-Me-L-Cys(Bam)-*N*-Me-L-Val)-L-Ala-*N*-Me-L-Ser(All)-*N*-Me-L-Val]-*O*All, *N*-Boc-octadepsipeptide-*O*All (Cys(Bam),Ser(All)) (20)**

Target compound **20** (pale yellow amorphous solid, 857.6 mg, 0.63 mmol, 33%) was synthesized according to the procedure for the synthesis of TA[32] by using *N*-Cbz-D-Ser[*N*-Boc-L-Ala-*N*-Me-L-Ser(All)-*N*-Me-L-Val]-*O*All **18** (1.50 g, 2.1 mmol, 1.1 equiv.), 4 M HCl/AcOEt (5 mL, 20 mmol) and AcOEt (5 mL) at step 1. *N*-Cbz-D-Ser[*N*-Boc-L-Ala-*N*-Me-L-Cys(Bam)-*N*-Me-L-Val]-OH **19**[32] (1.47 g, 1.9 mmol, 1 equiv.), DMT-MM (629.9 mg, 2.3 mmol, 1.2 equiv.) and AcOEt (10 mL) at step 2; LRMS (ESI) calcd for $C_{67}H_{93}N_9NaO_{19}S^+$ $[M+Na]^+$ 1382.6201, found: 1382.3.

***N*-Cbz-D-Ser[*N*-Cbz-D-Ser(*N*-Boc-L-Ala-*N*-Me-L-Cys(Bam)-*N*-Me-L-Val)-L-Ala-*N*-Me-L-Ser-*N*-Me-L-Val]-OH, *N*-Boc-octadepsipeptide-OH (Cys(Bam),Ser(OH)) (21)**

Target compound **21** (yellow amorphous solid, 1.19 g, 0.93 mmol, 93%, moderate purity) was synthesized according to the procedure for the synthesis of *N*-Boc-octadepsipeptide-*O*All (Cys(Bam),Ser(All)) **20** (1.4 g, 1.0 mmol), $Pd_2(dba)_3$ (47.1 mg, 0.051 mmol, 5 mol%), PPh_3 (108.0 mg, 0.41 mmol, 40 mol%), *N*-methylaniline (0.28 mL, 2.6 mmol, 2.5 equiv.) and THF (10 mL) at 60 °C; HRMS (ESI) calcd for $C_{61}H_{84}N_9O_{19}S^-$ $[M-H]^-$ 1278.5610, found: 1278.5603.

N-Cbz-D-Ser[N-Cbz-D-Ser(N-Boc-L-Ala-N-Me-L-Cys(Bam)-N-Me-L-Val)-L-Ala-N-Me-L-Ser(All)-N-Me-L-Val]-OH, N-Boc-octadepsipeptide-OH (Cys(Bam),Ser(All)) (22)

Target compound **22** (yellow amorphous solid, 831.8 mg, 0.63 mmol, quant.) was synthesized according to the procedure for the synthesis of TA by using *N*-Boc-octadepsipeptide-*O*All (Cys(Bam), Ser(All)) **20** (857.6 mg, 0.63 mmol), $Pd_2(dba)_3$ (5.8 mg, 0.0063 mmol, 1 mol%), PPh_3 (13.2 mg, 0.050 mmol, 8 mol%), *N*-methylaniline (0.082 mL, 0.76 mmol, 1.2 equiv.) and THF (5 mL); HRMS (ESI) calcd for $C_{64}H_{88}N_9O_{19}S^-$ $[M-H]^-$ 1318.5923, found: 1318.5922

Cbz-cyclicpeptide (Cys(Bam),Ser(All)) (25)

N-Boc-octadepsipeptide-OH (Cys(Bam),Ser(All)) **21** (500 mg, 0.38 mmol) was dissolved in TFA (5 mL). The resulting solution was stirred at room temperature for 2 h. The mixture was concentrated under reduced pressure. Then a solution of the residue, NMM 0.38 mL (3.8 mmol, 10 equiv.) and DMT-MM (1.05 g, 3.8 mmol, 10 equiv.) in DMF (300 mL) was stirred at room temperature overnight. The mixture was concentrated under reduced pressure and the residue was diluted with AcOEt (100 mL) and water (100 mL). The two layers were separated and the water layer was extracted with AcOEt (100 mL). The combined organic layers were washed with sat. NaCl aq. (100 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by silica gel (50 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1) to afford the target compound **25** (118.4 mg, 0.098 mmol, 26% yield on 2 steps) as yellow amorphous solid; HRMS (ESI) calcd for $C_{59}H_{79}N_9NaO_{16}S^+$ $[M+Na]^+$ 1224.5258, found: 1224.5273.

Cbz-cyclicpeptide (Cys(Bam),Ser(OH)) (26)

N-Boc-octadepsipeptide-OH (Cys(Bam),Ser(OH)) **22** (1.19 g, 0.93 mmol) was dissolved in TFA (10 mL). The resulting solution was stirred at room temperature for 1 h. The mixture was concentrated under reduced pressure. Then a solution of the residue, NMM (0.94 mL, 9.3 mmol, 10 equiv.) and DMT-MM (2.56 g, 9.3 mmol, 10 equiv.) in DMF (930 mL) was stirred at room temperature overnight. The mixture was concentrated under reduced pressure and the residue was diluted with AcOEt (150 mL) and water (150 mL). The two layers were separated and the water layer was extracted with AcOEt (150 mL).The organic layer was washed with sat. NaCl aq. (150 mL), and dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 20:1) to afford the target compound **26** (410.7 mg, 0.35 mmol, 38% yield on 2 steps, moderate purity) as yellow amorphous solid; HRMS (ESI) calcd for $C_{56}H_{75}N_9NaO_{16}S^+$ $[M+Na]^+$ 1184.4945, found: 1184.4937.

_N_-Boc-_N_-Me-Lan-_O_All (29)

To a solution of *N*-Boc-*N*-Me-L-Ser-*O*All **15** (1.5 g, 5.8 mmol), pyridine (2.4 mL, 23.1 mmol, 4 equiv.) and DMAP (141.3 mg, 1.2 mmol, 0.2 equiv.) in dry CH_2Cl_2 (15 mL) was added TsCl (3.31 g, 17.3 mmol, 3 equiv.) at 0 ºC and stirred overnight. The mixture was then diluted with AcOEt (200 mL) and water (100 mL) and the two layers were separated. The organic layer was washed with 10% citric acid aq. (100 mL) and sat. NaCl aq. (100 mL), and dried over $MgSO_4$, solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 10:1, 8:2) to afford tosyl ester **28**.

To a solution of sulphur (80.6 mg, 2.5 mmol, 0.55 equiv.) in dry THF (15 mL) were added $LiBHEt_3$ (1.0 M in THF, 5.5 mL, 5.5 mmol, 1.2 equiv.). After stirring for 10 min, the resulting solution was warmed and refluxed for 15 min, then cooled to room temperature. To the resulting solution was added the tosyl ester **28** (1.89 g, *ca.* 4.6 mmol) in dry THF (15 mL). The resulting solution was stirred overnight. The mixture was filtered through silica gel on celite and the filtrate was dried up under reduced pressure and the residue was purified by silica gel (100 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 20:1,

10:1, 8:2) to afford target compound **29** (856.2 mg, 1.7 mmol, 57% yield on 2 steps) as colorless oil; HRMS (ESI) calcd for $C_{24}H_{40}N_2NaO_8S^+$ $[M+Na]^+$ 539.2398, found: 539.2410.

N-Boc-*N*-Me-Lan-OH (31)

A solution of *N*-Boc-*N*-Me-Lan-*O*All **29** (850 mg, 1.6 mmol), PPh_3 (69.1 mg, 0.26 mmol, 16 mol%), *N*-Methylaniline (0.39 mL, 3.6 mmol, 2.2 equiv.) in THF (10 mL) was added $Pd_2(dba)_3$ (30.2 mg, 0.033 mmol, 2 mol%). The mixture was stirred at room temperature for 1 h in the dark. Then the mixture was dissolved in Et_2O (100 mL) and washed with 1 M NaOH aq. (25 mL × 2). To the combined water layer was added 2 M $KHSO_4$ aq. (50 mL) and was extracted with AcOEt (100 mL × 2). The organic layer was washed with sat. NaCl aq. (50 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 20:1, 10:1) to afford the target compound **31** (696.7 mg, 1.6 mmol, 97% yield) as yellow amorphous solid; HRMS (ESI) calcd for $C_{18}H_{31}N_2O_8S^-$ $[M-H]^-$ 435.1807, found: 435.1835.

N-Boc-*N*-Me-Lan-*N*-Me-L-Val-*O*-(*N*-Cbz-D-Ser-*O*All) (33)

To a solution of *N*-Cbz-D-Ser(*N*-Boc-*N*-Me-L-Val)-*O*All **3** (1.74 g, 3.5 mmol, 2.2 equiv.) in AcOEt (10 mL) was added 4 M HCl/AcOEt (10 mL, 40 mmol) at 0 ºC. Then the resulting solution was warmed to room temperature and stirred at the temperature for 2 h. The mixture was dissolved in AcOEt (100 mL) and washed with sat. $NaHCO_3$ aq. (100 mL). The water layer was extracted with AcOEt (100 mL). The combined organic layers were washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. Then a solution of the residue, *N*-Boc-*N*-Me-Lan-OH **31** (700 mg, 1.6 mmol, 1 equiv.) and DMT-MM (1.11 g, 4.0 mmol, 2.5 equiv.) in AcOEt (20 mL) was stirred at room temperature overnight. The mixture was filtered, and the filtrate was dissolved in AcOEt (100 mL) and washed with water (100 mL). The water layer was extracted with AcOEt (100 mL). The combined organic layers were washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 8:2, 7:3, 6:4) to afford the target compound **33** (1.66 g, *ca.* 1.4 mmol, 88% yield on 2 steps) as colorless amorphous solid; HRMS (ESI) calcd for $C_{58}H_{84}N_6NaO_{18}S^+$ $[M+Na]^+$ 1207.5455, found: 1207.5425.

***N*-Boc-L-Ala-*N*-Me-Lan-*N*-Me-L-Val-*O*-(*N*-Cbz-D-Ser-*O*All) (35)**

N-Boc-*N*-Me-Lan-*N*-Me-L-Val-*O*-(*N*-Cbz-D-Ser-*O*All) **33** (1.66 g, 1.4 mmol) was dissolved into TFA (5 mL) and the resulting solution was stirred at room temperature for 1 h. Then, solvent was removed under reduced pressure. A solution of the residue in DMF (10 mL) was added to the solution of NMM (1.4 mL, 14.0 mmol, 10 equiv.), *N*-Boc-L-Ala-OH (0.58 g, 3.1 mmol, 2.2 equiv.) and DMT-MM (0.97 g, 3.5 mmol, 2.5 equiv.) in DMF (5 mL). The resulting mixture was stirred at room temperature overnight. The mixture was diluted with AcOEt (100 mL) and water (200 mL). The two layers were separated and the water layer was extracted with AcOEt (100 mL). The combined organic layers were washed with sat. NaCl aq. (200 mL), and dried over $MgSO_4$, solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 7:3, 6:4, 5:5, 4:6) to afford the target compound **35** (1.03 g, *ca.* 0.78 mmol, 55% yield on 2 steps) as pale yellow amorphous solid; HRMS (ESI) calcd for $C_{64}H_{94}N_8NaO_{20}S^+$ $[M+Na]^+$ 1349.6197, found: 1349.6171.

***N*-Boc-L-Ala-*N*-Me-Lan-*N*-Me-L-Val-*O*-(*N*-Cbz-D-Ser-OH) (27)**

A solution of *N*-Boc-L-Ala-*N*-Me-Lan-*N*-Me-L-Val-*O*-(*N*-Cbz-D-Ser-*O*All) **35** (1.03 g, 0.78 mmol), PPh_3 (32.6 mg, 0.12 mmol, 16 mol%), *N*-Methylaniline (0.18 mL, 1.7 mmol, 2.2 equiv.) in THF (5 mL) was added $Pd_2(dba)_3$ (14.2 mg, 0.016 mmol, 2 mol%). The mixture was stirred at room temperature for 1 h in the dark. Then the mixture was diluted with AcOEt (100 mL) and 1 M $KHSO_4$ aq. (100 mL). The two layers were separated and the water layer was extracted with AcOEt (100 mL). The combined organic layers were washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The resulting crude was purified by silica gel (100 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 10:1) to afford the target compound **27** (967.8 mg, 0.78 mmol, quant., 49% yield from **31**) as yellow amorphous solid; HRMS (ESI) calcd for $C_{58}H_{85}N_8O_{20}S^-$ $[M-H]^-$ 1245.5606, found: 1245.5635.

Cbz-bicyclicpeptide (sulfide) (24)

N-Boc-L-Ala-*N*-Me-Lan-*N*-Me-L-Val-*O*-(*N*-Cbz-D-Ser-OH) **27** (960 mg, 0.78 mmol) was dissolved in TFA (10 mL). The resulting solution was stirred at room temperature for 1 h.

The mixture was concentrated under reduced pressure. Then a solution of the residue, NMM (0.79 mL, 7.8 mmol, 10 equiv.) and DMT-MM (1.07 g, 3.9 mmol, 10 equiv.) in DMF (780 mL) was stirred at room temperature overnight. The mixture was concentrated under reduced pressure and the residue was dissolved in AcOEt (100 mL) and washed with water (200 mL). The water layer was extracted with AcOEt (100 mL). The combined organic layers were washed with sat. NaCl aq. (100 mL), and dried over $MgSO_4$, solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 10:1) and YAMAZEN ULTRA PACK ODS-SM-50B 26 × 300 mm, 50 μm eluted with 0.1% TFA in MeCN/H_2O to afford pale yellow amorphous solid (mixture of isomers, 236.4 mg, the target compound **24**: 69% purity calculated from HPLC analysis, 163.5 mg, 0.16 mmol, 21% yield); HRMS (ESI) calcd for $C_{48}H_{66}N_8NaO_{14}S^+$ $[M+Na]^+$ 1033.4311, found: 1033.4324.

Ec-S-ether (39)

A solution of Cbz-bicyclicpeptide (sulfide) **24** (mixture of isomer, 100 mg, 0.10 mmol) and thioanisole (0.12 mL, 1.0 mmol, 10 equiv.) in TFA (5 mL) was stirred at 50 ºC overnight. Then the mixture was concentrated under reduced pressure. To a solution of the residue, NMM (0.060 mL, 0.59 mmol, 6 equiv.), and quinoxaline-2-carboxylic acid (69.7 mg, 0.40 mmol, 4 equiv.) in DMF (5 mL) was added DMT-MM (164.2 mg, 0.59 mmol, 6 equiv.). The resulting solution was stirred at room temperature for 4 h. The mixture was then dissolved in AcOEt (50 mL) and washed with sat. $NaHCO_3$ aq. (50 mL). The organic layer was washed with sat. NaCl aq. (50 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (20 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 20:1, 10:1) to afford pale blown amorphous solid (mixture of isomers, 50.3 mg, the target compound **39**: 54% purity calculated from HPLC analysis, 27.3 mg, 0.026 mmol, 26% yield). **39** was isolated by recrystallization from Et_2O/MeOH/CH_2Cl_2 as pale brown crystalline solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.62 (s, 2H), 8.71 (d, *J* = 6.9 Hz, 2H), 8.18 (d, *J* = 8.6 Hz, 2H), 7.91 (d, *J* = 8.0 Hz, 2H), 7.85–7.88 (m, 2H), 7.78–7.81 (m, 2H), 6.84 (d, *J* = 6.9 Hz, 2H), 6.27 (t, *J* = 6.6 Hz, 2H), 5.14 (d, *J* = 9.7 Hz, 2H), 4.87–4.89 (m, 2H), 4.79–4.84 (m, 4H), 4.68 (d, *J* =

12.0 Hz, 2H), 3.38 (dd, *J* = 14.6, 6.6 Hz, 2H), 3.17 (s, 5H), 2.95 (s, 6H), 2.52 (dd, *J* = 14.9, 6.9 Hz, 2H), 2.29–2.35 (m, 2H), 1.36 (d, *J* = 6.9 Hz, 6H), 1.10 (d, *J* = 6.3 Hz, 6H), 0.90 (d, *J* = 6.9 Hz, 6H) ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.0, 171.4, 169.9, 167.5, 164.3, 144.2, 143.6, 142.1, 140.0, 132.1, 131.1, 129.7, 129.3, 64.1, 62.0, 54.9, 53.5, 46.3, 36.1, 31.3, 30.1, 27.7, 20.4, 19.0, 17.5; HRMS (ESI) calcd for $C_{50}H_{62}N_{12}NaO_{12}S^+$ $[M+Na]^+$ 1077.4223, found: 1077.4204; m.p. 268.3–271.8 °C (dec.); $[\alpha]_D^{27.3}$ −256.5 ° (c 0.05, $CHCl_3$); Anal. calcd for $C_{50}H_{66}N_{12}O_{14}S$ $[M+2H_2O]$: C, 55.03; H, 6.10; N, 15.40, found: C, 55.09; H, 5.96; N, 15.37.

N-Boc-*N*-Me-*Se*-Lan-*O*All (30)

To a solution of *N*-Boc-*N*-Me-L-Ser-*O*All **15** (2 g, 7.7 mmol), pyridine (3.2 mL, 30.9 mmol, 4 equiv.) and DMAP (188.5 mg, 1.5 mmol, 0.2 equiv.) in dry CH_2Cl_2 (15 mL) was added TsCl (4.41 g, 23.1 mmol, 3 equiv.) at 0 ºC and stirred overnight. The mixture was then diluted with AcOEt (300 mL) and water (100 mL) and the two layers were separated. The organic layer was washed with 10% citric acid aq. (100 mL) and sat. NaCl aq. (100 mL), and dried over $MgSO_4$, solvent was removed under reduced pressure. The residue was

purified by silica gel (100 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 10:1, 8:2) to afford tosyl ester **28**.

To a solution of selenium (287.8 mg, 3.6 mmol, 0.55 equiv.) in dry THF (15 mL) were added $LiBHEt_3$ (1.0 M in THF, 8.0 mL, 8.0 mmol, 1.2 equiv.). After stirring for 10 min, the resulting solution was warmed and refluxed for 15 min, then cooled to room temperature. To the resulting solution was added the tosyl ester **28** (2.74 g, *ca.* 6.6 mmol) in dry THF (15 mL). The resulting solution was stirred overnight. The mixture was filtered through silica gel on celite and the filtrate was dried up under reduced pressure and the residue was purified by silica gel (100 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 20:1, 10:1, 8:2) to afford target compound **30** (1.45 g, 2.6 mmol, 78% yield on 2 steps) as colorless oil; HRMS (ESI) calcd for $C_{24}H_{40}N_2NaO_8Se^+$ $[M+Na]^+$ 587.1842, found: 587.1860

N-Boc-*N*-Me-*Se*-Lan-OH (32)

A solution of *N*-Boc-*N*-Me-*Se*-Lan-*O*All **30** (1.45 g, 2.6 mmol), PPh_3 (323.9 mg, 1.2 mmol, 48 mol%), *N*-Methylaniline (0.61 mL, 5.7 mmol, 2.2 equiv.) in THF (15 mL) was added

$Pd_2(dba)_3$ (141.4 mg, 0.15 mmol, 6 mol%). The mixture was stirred at room temperature overnight in the dark. Then the mixture was dissolved in Et_2O (200 mL) and washed with 1 M NaOH aq. (50 mL × 2). To the combined water layers was added 1 M $KHSO_4$ aq. (130 mL) and extracted with AcOEt (50 mL × 3). The organic layer was washed with sat. NaCl aq. (50 mL), and dried over $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel (100 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1, 20:1) to afford the target compound **32** (1.08 g, 2.2 mmol, 86% yield) as yellow amorphous solid; HRMS (ESI) calcd for $C_{18}H_{31}N_2O_8Se^-$ $[M-H]^-$ 483.1251, found: 483.1277.

N-Boc-*N*-Me-*Se*-Lan-*N*-Me-L-Val-*O*-(*N*-Cbz-D-Ser-*O*All) (34)

N-Boc-*N*-Me-*Se*-Lan-*N*-Me-L-Val-*O*-(*N*-Cbz-D-Ser-*O*All) **34** (colorless amorphous solid, 2.20 g, *ca.* 1.8 mmol, 89% yield on 2 steps) was synthesized according to the synthetic method of **33** by using *N*-Cbz-D-Ser(*N*-Boc-*N*-Me-L-Val)-*O*All **3** (2.20 g, 4.5 mmol, 2.2 equiv.), 4 M HCl/AcOEt (10 mL, 40 mmol), AcOEt (10 mL) (step 1), *N*-Boc-*N*-Me-*Se*-Lan-OH **32** (1000 mg, 2.0 mmol, 1 equiv.), AcOEt (20 mL), DMT-MM (1.40 g, 5.1 mmol, 2.5

equiv.) (step 2); HRMS (ESI) calcd for $C_{58}H_{84}N_6NaO_{18}Se^+$ $[M+Na]^+$ 1255.4900, found: 1255.4914.

_N_-Boc-L-Ala-_N_-Me-_Se_-Lan-_N_-Me-L-Val-_O_-(_N_-Cbz-D-Ser-_O_All) (36)

_N_-Boc-L-Ala-_N_-Me-_Se_-Lan-_N_-Me-L-Val-_O_-(_N_-Cbz-D-Ser-_O_All) **36** (colorless amorphous solid, 1.06 g, *ca.* 0.77 mmol, 43% yield on 2 steps) was synthesized according to the synthetic method of **35** by using TFA (5 mL), _N_-Boc-_N_-Me-_Se_-Lan-_N_-Me-L-Val-_O_-(_N_-Cbz-D-Ser-_O_All) **34** (2.2 g, 1.8 mmol) (step 1), _N_-Boc-L-Ala-OH (0.74 g, 3.9 mmol, 2.2 equiv.), NMM (1.8 mL, 17.9 mmol, 10 equiv.), DMT-MM (1.24 g, 4.5 mmol, 2.5 equiv.), DMF (15 mL) (step 2); HRMS (ESI) calcd for $C_{64}H_{94}N_8NaO_{20}Se^+$ $[M+Na]^+$ 1397.5642, found: 1397.5649.

_N_-Boc-L-Ala-_N_-Me-_Se_-Lan-_N_-Me-L-Val-_O_-(_N_-Cbz-D-Ser-OH) (37)

_N_-Boc-L-Ala-_N_-Me-_Se_-Lan-_N_-Me-L-Val-_O_-(_N_-Cbz-D-Ser-OH) **37** (yellow amorphous solid, 1.00 g, 0.77 mmol, quant.) was synthesized according to the synthetic method of **27** by using _N_-Boc-L-Ala-_N_-Me-_Se_-Lan-_N_-Me-L-Val-_O_-(_N_-Cbz-D-Ser-_O_All) **36** (1.06 g,

0.77 mmol), PPh_3 (97.1 mg, 0.37 mmol, 48 mol%), *N*-methylaniline (0.18 mL, 1.7 mmol, 2.2 equiv.), THF (5 mL), $Pd_2(dba)_3$ (42.4 mg, 0.046 mmol, 6 mol%); HRMS (ESI) calcd for $C_{58}H_{85}N_8O_{20}Se^-$ $[M-H]^-$ 1293.5051, found: 1293.5030.

Cbz-bicyclicpeptide (selenide) (38)

Pale yellow amorphous solid (mixture of isomers, 177.7 mg, the target compound **38**: 70% purity calculated from HPLC analysis, 123.9 mg, 0.12 mmol, 15% yield) was synthesized according to the synthetic method of **24** by using TFA 5 mL, *N*-Boc-L-Ala-*N*-Me-*Se*-Lan-*N*-Me-L-Val-*O*-(*N*-Cbz-D-Ser-OH) **37** (1000 mg, 0.77 mmol) (step 1), NMM (0.78 mL, 7.7 mmol, 10 equiv.), DMF (770 mL), DMT-MM (2.14 g, 7.7 mmol, 10 equiv.); HRMS (ESI) calcd for $C_{48}H_{66}N_8NaO_{14}Se^+$ $[M+Na]^+$ 1081.3756, found: 1081.3759.

Ec-Se-ether (40)

Pale blown amorphous solid (mixture of isomers, 53.2 mg, the target compound **40**: 37% purity calculated from HPLC analysis, 19.9 mg, 0.018 mmol, 19% yield) was synthesized according to the synthetic method of **39** by using Cbz-bicyclicpeptide (selenide) **38** (100 mg, 0.095 mmol), thioanisole (0.11 mL, 0.95 mmol, 10 equiv.), TFA (5 mL) (step 1), NMM

(0.057 mL, 0.57 mmol, 6 equiv.), quinoxaline-2-carboxylic acid (66.6 mg, 0.38 mmol, 4 equiv.), DMF (5 mL), DMT-MM (156.9 mg, 0.57 mmol, 6 equiv.). **40** was isolated by recrystallization from Et_2O/MeOH/CH_2Cl_2 as pale brown crystalline solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.63 (s, 2H), 8.71 (d, *J* = 6.9 Hz, 2H), 8.19 (d, *J* = 8.6 Hz, 2H), 7.93 (d, *J* = 8.6 Hz, 2H), 7.86–7.89 (m, 2H), 7.79–7.83 (m, 2H), 6.81 (d, *J* = 7.4 Hz, 2H), 6.30 (t, *J* = 6.9 Hz, 2H), 5.15 (d, *J* = 9.7 Hz, 2H), 4.88–4.90 (m, 2H), 4.80–4.84 (m, 2H), 4.77–4.80 (m, 2H), 4.68 (dd, *J* = 11.5, 1.1 Hz, 2H), 3.38 (dd, *J* = 13.7, 6.9 Hz, 2H), 3.17 (s, 6H), 2.95 (s, 6H), 2.49 (dd, *J* = 13.7, 6.9 Hz, 2H), 2.29–2.37 (m, 2H), 1.37 (d, *J* = 7.4 Hz, 6H), 1.10 (d, *J* = 6.9 Hz, 6H), 0.90 (d, *J* = 6.3 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.9, 171.3, 170.1, 167.5, 164.2, 144.2, 143.6, 142.1, 140.1, 132.1, 131.2, 129.7, 129.3, 64.1, 62.0, 55.0, 53.3, 46.4, 31.4, 30.0, 27.7, 27.0, 20.4 19.0, 17.5; HRMS (ESI) calcd for $C_{50}H_{62}N_{12}NaO_{12}Se^+$ $[M+Na]^+$ 1125.3668, found: 1125.3686; m.p. 271.0–274.8 °C (dec.); $[\alpha]_D^{27.4}$ −248.7 ° (c 0.05, $CHCl_3$); Anal. calcd for $C_{50}H_{68}N_{12}O_{15}Se$ $[M+3H_2O]$: C, 51.94; H, 5.93; N, 14.54, found: C, 51.99; H, 5.61; N, 14.36.

Triostin A derivative SH, TA-SH (41)

To a solution of Triostin A (**2**) (120 mg, 0.11 mmol) in CH_3CN (4 mL) was added $P(^nBu)_3$ (0.11 mL, 0.44 mmol, 4 equiv.). Reaction mixture was stirred at room temperature for 10 min, then H_2O (1 mL) was added and the resulting mixture was stirred at the same temperature overnight. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and H_2O (50 mL) and the two layers were separated. The organic layer was washed with sat. NaCl aq. (50 mL), dried over $MgSO_4$, and the solvent was removed under reduced pressure. The resulting crude was purified by MPLC eluted with H_2O/CH_3CN containing 0.1% TFA (90:10 ~ 30:70, Flow: 20 mL/min, Detect: 220 nm, silica gel 120 (Spherical) RP-18 40–50 mm 60 mL, KANTO) to afford the target compound **41** (67.3 mg, 0.062 mmol, 56% yield) as pale yellow solid including small amount of conformer; ^1H NMR (500 MHz, $CDCl_3$, see table S7); ^{13}C NMR (125 MHz, $CDCl_3$, see table S7); HRMS (ESI) calcd for $C_{50}H_{64}N_{12}NaO_{12}S_2^+$ $[M+Na]^+$ 1111.4100, found: 1111.4079; m.p. 187.5–190.0 °C; $[\alpha]_D^{19.6}$ −148.1 ° (c 0.101, $CHCl_3$); Purity: 92%, t_R = 14.5 min.

Triostin A derivative S-Ac, TA-SAc (42)

To a solution of Triostin A (**2**) (50 mg, 0.046 mmol) in CH_3CN (4 mL) was added $P(^nBu)_3$ (0.05 mL, 0.18 mmol, 4 equiv.). Reaction mixture was stirred at room temperature for 10 min, then H_2O (1 mL) was added and the resulting mixture was stirred at the same temperature overnight. The reaction mixture was dissolved in CH_2Cl_2 (50 mL) and washed with H_2O (50 mL). The organic layer was washed with sat. NaCl aq. (50 mL), dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by Sep-Pak C18 (Waters) eluted with H_2O/CH_3CN containing 0.1% TFA (10:90 ~ 0:100) or silica gel (10 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1). The resulting crude resolved into CH_2Cl_2 and then, NEt_3 (0.03 mL, 0.24 mmol, 6 equiv.) and AcO_2 (0.02 mL, 0.16 mmol, 4 equiv.) was added to the solution. The mixture was stirred at room temperature overnight, then the mixture was diluted with CH_2Cl_2 (50 mL) and H_2O (50 mL) and the two layers were separated. The organic layer was washed with sat. NaCl aq. (50 mL), dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by silica gel (10 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1) and Sep-Pak C18 (Waters) eluted with H_2O/CH_3CN containing 0.1% TFA (10:90 ~ 0:100). The resulting crude was purified

by liquid chromatography eluted with H_2O/CH_3CN containing 0.1% TFA (50:50 ~ 10:90, 45 min, Flow: 3 mL/min, Detect: 220 nm, nacalai COSMOSIL 5C18, 5 μm × 10 × 250 mm) to afford the target compound **42** (15.1 mg, 0.013 mmol, 14% yield on 2 steps) as colorless solid; ^{1}H NMR (mixture of conformers, 500 MHz, $CDCl_3$, see table S8); ^{13}C NMR (mixture of conformers, 125 MHz, $CDCl_3$, see table S8); HRMS (ESI) calcd for $C_{54}H_{68}N_{12}NaO_{14}S_2^+$ $[M+Na]^+$ 1195.4312, found: 1195.4335; m.p. 158.1–160.3 °C; $[\alpha]_D^{20.1}$ −180.8 ° (c 0.112, $CHCl_3$); Purity: ≥ 99%, t_R = 16.0 min.

Triostin A derivative S-Me, TA-SMe (43)

To a solution of Triostin A (**2**) (50 mg, 0.046 mmol) in CH_3CN (4 mL) was added P(^{n}Bu)$_3$ (0.05 mL, 0.18 mmol, 4 equiv.). Reaction mixture was stirred at room temperature for 10 min, then H_2O (1 mL) was added and the resulting mixture was stirred at the same temperature overnight. The reaction mixture was dissolved in CH_2Cl_2 (50 mL) and washed with H_2O (50 mL). The organic layer was washed with sat. NaCl aq. (50 mL), dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by Sep-Pak C18 (Waters) eluted with H_2O/CH_3CN containing 0.1% TFA (10:90 ~ 0:100).

The resulting crude was purified by HPLC twice eluted with H_2O/CH_3CN containing 0.1% TFA (70:30 ~ 20:80, 45 min, Flow: 3 mL/min, Detect: 220 nm, nacalai COSMOSIL 5C18, 5 μm × 10 × 250 mm). The resulting crude resolved into CH_2Cl_2 and then, DIPEA (17.7 μL, 0.10 mmol, 6 equiv.) and methyl iodide (4.2 μL, 0.068 mmol, 4 equiv.) was added to the solution. The mixture was stirred at room temperature overnight, then the mixture was dissolved in CH_2Cl_2 (50 mL) and washed with H_2O (50 mL). The organic layer was washed with sat. NaCl aq. (50 mL), dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by silica gel (10 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 200:1, 100:1, 50:1) to afford the target compound **43** (13.6 mg, 0.012 mmol, 26% yield on 2 steps) as colorless solid; ^{1}H NMR (mixture of conformers, 500 MHz, $CDCl_3$, see table S9); ^{13}C NMR (mixture of conformers, 125 MHz, $CDCl_3$, see table S9); HRMS (ESI) calcd for $C_{52}H_{68}N_{12}NaO_{12}S_2^+$ $[M+Na]^+$ 1139.4413, found: 1139.4391; m.p. 158.7–161.8 °C; $[\alpha]_D^{20.5}$ −131.1 ° (c 0.121, $CHCl_3$); Purity: ≥ 99%, t_R = 15.9 min.

TA dimethyl thioacetal, TA thioacetal (44)

To a solution of TA-SH **41** (50 mg, 0.046 mmol) in acetone (10 mL) and CH_2Cl_2 (30 mL) was added BF_3•OEt_2 (23.2 μL, 0.18 mmol, 4 equiv.) at room temperature. The resulting solution was stirred at the same temperature for 6.5 h. The mixture was dissolved in CH_2Cl_2 and washed with H_2O (50 mL). The organic layer was washed with sat. NaCl aq. (50 mL), and dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography eluted with CH_2Cl_2/MeOH (10:0, 100:1, 50:1) to afford the target compound **44** (15.1 mg, 0.013 mmol, 29% yield) as pale yellow solid; ^{1}H NMR and (500 MHz, $CDCl_3$, see table S10); ^{13}C NMR (125 MHz, $CDCl_3$, see table S10); HRMS (ESI) calcd for $C_{53}H_{69}N_{12}O_{12}S_2^+$ $[M+H]^+$ 1129.4594, found: 1129.4621; m.p. 192.3–196.5 °C; $[\alpha]_D^{27.0}$ −185.1 ° (c 0.05, $CHCl_3$); Purity: 91%, t_R = 13.7 min.

N-Boc-*N*-Me-L-Ile-OH (51) [cas: 52498-32-5]

Target compound **51** (4.42 g, 18.0 mmol, 83% yield) was synthesized according to the procedure for the synthesis of TA[32] by using *N*-Boc-L-Ile-OH (5 g, 21.6 mmol), NaH (60% in oil, 2.16 g, 54.0 mmol, 2.5 equiv.), MeI (6.7 mL, 54.0 mmol, 5 equiv.) and dry THF (100

mL); ^{1}H NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 4.25 (d, *J* = 8.6 Hz, α-CH), 4.04 (d, *J* = 9.7 Hz, α-CH), 2.88 (s, *N*-Me), 2.34–2.11 (m, β-CH), 2.11–1.88 (m, β-CH), 1.54–1.38 (m, Boc, γ-CH_2), 1.17–1.03 (m, γ-CH_2), 0.98 (d, *J* = 6.9 Hz, γ'-CH_3), 0.91 (t, *J* = 7.2 Hz, δ-CH_3); ^{13}C NMR (mixture of rotamers, 100 MHz, $CDCl_3$) δ 176.0, 175.8 (Ile-CO), 156.6, 155.7 (Boc-CO), 80.6 (Boc-C), 63.3, 63.1 (α-CH), 33.4 (*N*-Me), 31.4, 30.5 (β-CH), 28.2 (Boc-Me), 25.0, 24.9 (γ-CH_2), 15.9, 15.7 (γ'-CH_3), 10.6, 10.3 (δ-CH_3); HRMS (DART) calcd for $C_{12}H_{24}NO_4^+$ $[M+H]^+$ 246.1700, found: 246.1692; $[\alpha]_D^{20.2}$ −59.4 ° (c 0.1, $CHCl_3$).

_N_-Cbz-D-Ser(_N_-Boc-_N_-Me-L-Ile)-_O_All (52)

Target compound **52** (5.33 g, 10.5 mmol, 84% yield) was synthesized according to the procedure for the synthesis of TA by using *N*-Cbz-D-Ser-*O*All **50**[32] (3.5 g, 12.5 mmol), *N*-Boc-*N*-Me-L-Ile-OH **51** (1.49 g, 6.4 mmol, 1.2 equiv.), HOAt (2.56 g, 18.8 mmol, 1.5 equiv.), NEt_3 (2.6 mL, 18.8 mmol, 1.5 equiv.), CH_2Cl_2 (50 mL) and EDCI·HCl (3.60 g, 8.1 mmol, 1.5 equiv.); ^{1}H NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 7.43–7.29 (m, 5H, Cbz-Ar), 5.89 (ddd, *J* = 22.5, 11.0, 5.6 Hz, All-CH), 5.73 (d, *J* = 6.9 Hz, Ser-NH), 5.50 (d, *J* = 6.3 Hz, Ser-NH), 5.33 (d, *J* = 17.2 Hz, All-CH_2(*E*)), 5.26 (d, *J* = 10.3 Hz, All-CH_2(*Z*)),

5.12 (s, Cbz-CH_2), 4.76–4.58 (m, All-OCH_2, Ser-α-CH), 4.56 (d, J = 3.4 Hz, Ile-α-CH), 4.53 (d, J = 2.9 Hz, Ile-α-CH), 4.49–4.29 (m, Ser-β-CH_2), 4.19 (d, J = 9.7 Hz, Ser-β-CH_2), 2.79 (s, Ile-*N*-Me), 1.93 (br, Ile-β-CH), 1.53–1.33 (m, Boc-Me, Ile-γ-CH_2), 1.17–0.94 (m, Ile-γ-CH_2), 0.94–0.75 (m, Ile-γ-CH_3, δ-CH_3); ^{13}C NMR (mixture of rotamers, 125 MHz, $CDCl_3$) δ 171.3, 170.6 (Ile-CO), 168.9 (Ser-CO), 156.1, 155.8, 155.6, 155.3 (Boc-CO, Cbz-CO), 136.0, 135.9 (Cbz-Ar-C1), 131.2, 131.0 (All-CH), 128.4, 128.1 (Cbz-Ar-C2, C3, C4), 119.2, 119.0 (All-CH_2), 80.4, 80.2 (Boc-C), 67.2, 67.1, 66.5, 66.4, 64.2, 64.1 (Cbz-CH_2, All-OCH_2, Ser-β-CH_2), 63.1, 61.9 (Ile-α-CH), 53.3 (Ser-α-CH), 33.5, 33.3 (Ile-β-CH), 30.6, 30.2 (Ile-*N*-Me), 28.2 (Boc-Me), 25.0, 24.8 (Ile-γ-CH_2), 15.7, 15.6 (Ile-γ-CH_3), 10.6, 10.2 (Ile-δ-CH_3); HRMS (ESI) calcd for $C_{26}H_{38}N_2NaO_8^+$ $[M+Na]^+$ 529.2520, found: 529.2530.

_N_-Cbz-D-Ser[_N_-Boc-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile]-_O_All (53)

Target compound **53** (6.44 g, 8.5 mmol, 81% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using *N*-Cbz-D-Ser(*N*-Boc-*N*-Me-L-Ile)-*O*All **52** (5.33 g, 10.5 mmol), AcOEt (30 mL) and 4 M HCl/AcOEt (20 mL) in step 1 and *N*-Boc-*N*-

Me-L-Cys(Bam)-OH[32] (4.65 g, 12.6 mmol, 1.2 equiv.), DMT-MM (4.37 g, 15.8 mmol, 1.5 equiv.) and AcOEt (50 mL) in step 2; ^{1}H NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 8.04–7.69, 7.54–7.20, 7.12–6.98 (m, Bam-NH, Ar, and Cbz-Ar), 6.97–6.88 (br, Ser-NH), 5.92–5.75 (m, All-CH), 5.64–5.54 (br, Ser-NH), 5.37–5.01 (m, All-CH_2, Ser-NH and Cys-α-CH), 4.93–4.83 (m, Ile-α-CH) 4.83–4.28 (m, Bam-CH_2, Ser-α-CH, α-CH_2 and All-OCH_2), 4.12–4.08 (br, Ile-α-CH), 3.02-2.73 (m, Cys-β-CH_2), 2.73–1.88 (m, Cys-β-CH_2, *N*-Me and Val-*N*-Me), 2.04–1.88, 1.88–1.71 (m, Ile-β-CH), 1.51 (s, Boc-Me), 1.47 (s, Boc-Me), 1.45 (s, Boc-Me), 1.42 (s, Boc-Me), 1.37–1.23, 1.10–0.96 (m, Ile-γ-CH_2), 0.91 (d, *J* = 6.9 Hz, Ile-γ-CH_3), 0.89–0.77 (m, Ile-γ-CH_3 and δ-CH_3), 0.77–0.70 (m, Ile-γ-CH_3 and δ-CH_3), 0.68 (d, *J* = 6.9 Hz, Ile-γ-CH_3); ^{13}C NMR (mixture of rotamers, 125 MHz, $CDCl_3$) δ 170.6, 170.5, 170.2, 170.0, 169.6, 169.3, 168.9, 168.7, 168.6 (Bam-CO, Ala-CO, Cys-CO, Ile-CO, Ser-CO), 156.5, 156.3, 156.2, 155.8, 155.7, 155.6, 154.7, 154.6 (Cbz-CO, Boc-CO), 136.5, 136.0, 135.8, 133.9, 133.8, 131.6, 131.42, 131.36, 131.1, 131.0, 130.9, 128.4, 128.3, 128.2, 128.1, 127.8, 127.7, 1227.4, 127.3, 127.0, 119.4, 119.1, 119.0 (Bam-Ar, Cbz-Ar, All-CH and -CH_2), 82.0, 81.3, 81.0, 80.8 (Boc-C), 67.2, 67.1, 66.7, 66.6, 66.5, 66.3, 65.2, 64.2, 64.1, 63.6 (Cbz-CH_2, All-OCH_2, Ser-β-CH_2), 63.4, 60.6, 55.8, 55.6, 54.7,

54.4, 53.5, 53.2 (Ile-α-CH, Cys-α-CH, Ser-α-CH), 44.1, 43.9, 43.0, 42.8 (Bam-CH_2), 34.2, 34.1, 33.0, 32.8, 30.9, 29.4, 29.2, 28.4, 28.3, 28.2 (Cys-*N*-Me, Ile-*N*-Me and β-CH), 33.1, 31.7, 31.4, 30.2 (Cys-β-CH_2), 24.84, 24.80 (Ile-γ-CH_2), 15.6, 15.5, 15.2 (Ile-γ-CH_3), 11.2, 11.1, 10.4, 10.3 (Ile-δ-CH_3); HRMS (ESI) calcd for $C_{38}H_{52}N_4NaO_{10}S^+$ $[M+Na]^+$ 779.3296, found: 779.3306.

_N_-Cbz-D-Ser[_N_-Boc-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile]-_O_All (54)

Target compound **54** (2.86 g, 3.5 mmol, 41% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using *N*-Cbz-D-Ser[*N*-Boc-*N*-Me-L-Cys(Bam)-*N*-Me-L-Ile]-*O*All **53** (6.44 g, 8.5 mmol), AcOEt (20 mL) and 4 M HCl/AcOEt (20 mL) in step 1 and *N*-Boc-L-Ala-OH (1.92 g, 10.2 mmol, 1.2 equiv.), DMT-MM (3.53 g, 12.8 mmol, 1.5 equiv.) and DMF (50 mL) in step 2; ^{1}H NMR (mixture of rotamers, 500 MHz, $CDCl_3$) δ 7.93 (d, *J* = 7.4 Hz, Bam-Ar-C2), 7.87 (d, *J* = 7.4 Hz, Bam-Ar-C2), 7.62–7.28 (m, Bam-NH, Bam-Ar-C3, C4, Cbz-Ar), 6.32 (d, *J* = 8.6 Hz, Ser-NH), 5.97–5.76 (m, All-CH, Ser-NH), 5.76–5.57 (m, Cys-α-CH), 5.39 (d, *J* = 8.6 Hz, Ala-NH), 5.36 (d, *J* = 7.4 Hz, Ala-NH), 5.30 (dd, *J* = 17.2, 1.7 Hz, All-CH_2 (*E*)), 5.25 (dd, *J* = 10.3, 1.1 Hz, All-CH_2(*E*)), 5.10 (s,

Cbz-CH_2), 4.81 (d, *J* = 10.9 Hz, Ile-α-CH), 4.74 (dd, *J* = 13.5, 6.6 Hz, Bam-CH_2), 4.69–4.54 (m, All-*O*CH_2, Ala-α-CH, Ser-α-CH), 4.54–4.27 (m, Ser-β-CH_2, Bam-CH_2), 4.22 (d, *J* = 9.7 Hz, Ile-α-CH), 3.22 (dd, *J* = 14.3, 7.4 Hz, Cys-β-CH_2), 3.15 (dd, *J* = 14.3, 6.9 Hz, 0.76H, Cys-β-CH_2), 3.06 (s, Cys or Ile-*N*-Me), 2.96 (s, Cys or Ile-*N*-Me), 2.95–2.81 (m, Cys-β-CH_2, Cys and Ile-*N*-Me), 2.06–1.91 (m, Ile-β-CH), 1.90–1.77 (m, Ile-β-CH), 1.44 (s, Boc-Me), 1.43 (s, Boc-Me), 1.30, 1.29 (d, *J* = 6.9 Hz, Ala-β-CH_3), 1.27–1.18 (m, Ile-γ-CH_2), 1.03–0.93 (m, Ile-γ-CH_2), 0.91 (d, *J* = 6.3 Hz, Ile-γ-CH_3), 0.83 (t, *J* = 7.4 Hz, Ile-δ-CH_3), 0.81–0.75 (m, Ile-γ-CH_3, δ-CH_3); ^{13}C NMR (mixture of rotamers 125 MHz, $CDCl_3$) δ 174.0, 173.8, 172.6, 170.0, 169.5, 168.8, 168.7, 167.1, 166.7 (Ala-CO, Ile-CO, Cys-CO, Ser-CO, Bam-CO), 155.9, 155.7, 155.1, 154.9 (Boc-CO, Cbz-CO), 136.0, 133.8, 133.6 (Bam and Cbz Ar-C1), 131.6, 131.5 (Bam and Cbz Ar-C4) 131.1, 131.0 (All-CH), 128.5, 128.3, 128.2, 128.1, 127.7 (Bam and Cbz Ar-C2, C3), 127.3, 127.2 (All-CH_2), 79.8, 79.3 (Boc-C), 67.1, 66.5, 64.8, 64.2 (Cbz-CH_2, All-OCH_2, Ser-β-CH_2), 60.8, 55.8, 53.5, 53.2, 53.1, 53.0, 46.6, 46.5, 46.1 (Ile-α-CH, Cys-α-CH, Ser-α-CH, Ala-α-CH), 42.9, 42.4 (Cys-β-CH_2), 36.8, 35.6, 34.7, 32.7, 31.8, 31.1, 30.5, 30.14, 30.08 (Cys-*N*-Me, Ile-*N*-Me, Ile-β-CH), 31.8, 30.6 (Bam-CH_2), 28.28, 28.25 (Boc-Me), 25.2, 24.7 (Ile-γ-CH_2), 18.9, 18.7 (Ala-

β-CH_3), 15.6, 15.4 (Ile-γ-CH_3), 11.5, 10.3 (Ile-δ-CH_3); HRMS (ESI) calcd for $C_{41}H_{57}N_5NaO_{11}S^+$ $[M+Na]^+$ 850.3667, found: 850.3693.

N-Cbz-D-Ser[*N*-Boc-L-Ala-*N*-Me-L-Cys(Bam)-*N*-Me-L-Ile]-OH (55)

Target compound **55** (809.9 mg, 1.0 mmol, 86% yield) was synthesized according to the procedure for the synthesis of TA by using *N*-Cbz-D-Ser[*N*-Boc-L-Ala-*N*-Me-L-Cys(Bam)-*N*-Me-L-Ile]-*O*All **54** (1 g, 1.2 mmol), PPh_3 (12.7 mg, 0.048 mmol, 4 mol%), *N*-methylaniline (0.16 mL, 1.4 mmol, 1.2 equiv.), THF (5 mL) and $Pd_2(dba)_3$ 11.1 mg (0.012 mmol, 1 mol%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (d, *J* = 7.4 Hz, Bam-Ar-C2 and C6), 7.55 (t, *J* = 7.2 Hz, Bam-NH), 7.46 (t, *J* = 7.4 Hz, Bam-Ar-C3 and C5), 7.40–7.07 (m, Bam=Ar-C4, Cbz-Ar), 5.83 (dd, *J* = 10.3, 4.6 Hz, Cys-α-CH), 5.72 (d, *J* = 6.3 Hz, Ser-NH), 5.47 (d, *J* = 8.0 Hz, Ala-NH), 5.22 (dd, *J* = 14.3, 8.6 Hz, Bam-CH_2), 5.12 (s, Cbz-CH_2), 4.92 (d, *J* = 10.3 Hz, Ile-α-CH), 4.73–4.58 (m, Ala-α-CH, Ser-β-CH_2), 4.55 (t, *J* = 3.4 Hz, Ser-α-CH), 4.34 (dd, *J* = 11.2, 3.2 Hz, Ser-β-CH), 4.13 (dd, *J* = 14.6, 4.3 Hz, Bam-CH_2), 3.18 (dd, *J* = 15.2, 4.9 Hz, Cys-β-CH_2), 3.00–2.93 (m, Cys-β-CH_2), 2.90 (s, Cys-*N*-Me), 2.83 (s, Ile-*N*-Me), 2.07–1.87 (m, Ile-β-CH), 1.45 (s, Boc-Me), 1.31 (d, *J* = 6.9 Hz, Ala-β-

CH_3), 1.29–1.18 (m, Ile-γ-CH_2), 1.04–0.92 (m, Ile-γ-CH_2, γ-CH_3), 0.84 (t, J = 7.2 Hz, Ile-δ-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8 (Ser-CO), 170.4, 170.2, 169.8, 169.2 (Bam-CO, Ala-CO, Cys-CO, Ile-CO), 155.6, 155.0 (Ile-CO), 136.0, 132.9 (Bam-Ar-C1 and Cbz-Ar-C1), 132.1, 128.5, 128.4, 128.1, 128.0, 127.3, 127.2 (Bam-Ar and Cbz-Ar), 67.0, 64.4 (Cbz-CH_2 and Ser-β-CH_2), 60.8, 53.4, 52.6 46.7 (Ile-α-CH, Ser-α-CH, Cys-α-CH, Ala-α-CH), 41.8 (Bam-CH_2), 32.5, 31.1 (Cys-*N*-Me, Ile-*N*-Me), 30.0 (Cys-β-CH_2), 28.3 (Boc-Me and Ile-β-CH), 24.9 (Ile-γ-CH_2), 18.7 (Ala-β-CH_3), 15.7 (Ile-γ-CH_3), 10.2 (Ile-δ-CH_3); HRMS (ESI) calcd for $C_{38}H_{52}N_5O_{11}S^-$ $[M-H]^-$ 786.3390, found: 786.3387.

_N_-Cbz-D-Ser[_N_-Cbz-D-Ser(_N_-Boc-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Val)-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile]-_O_All, _N_-Boc-octadepsipeptide-_O_All (Val,Ile) (56)

Target compound **56** (936.8 mg, 0.63 mmol, 69% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using _N_-Cbz-D-Ser[_N_-Boc-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile]-_O_All **54** (900 mg,1.1 mmol, 1.2 equiv.), AcOEt (8 mL) and 4 M HCl/AcOEt (2 mL) in step 1 and _N_-Cbz-D-Ser[_N_-Boc-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Val]-OH **19**[32] (701.0 mg, 0.91 mmol), DMT-MM (300.8 mg, 1.1 mmol, 1.2 equiv.) and

AcOEt (10 mL) in step 2; HRMS (ESI) calcd for $C_{73}H_{98}N_{10}NaO_{19}S_2^+$ $[M+Na]^+$ 1505.6343, found: 1505.6350.

_N_-Cbz-D-Ser[_N_-Cbz-D-Ser(_N_-Boc-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Val)-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile]-OH, _N_-Boc-octadepsipeptide-OH (Val,Ile) (58)

Target compound **58** (790.1 mg, 0.55 mmol, 91% yield) was synthesized by using _N_-Boc-octadepsipeptide-_O_All (Val,Ile) **56** (886.8 mg, 0.60 mmol), PPh_3 (12.5 mg, 0.048 mmol, 8 mol%), _N_-methylaniline (0.08 mL, 0.72 mmol, 1.2 equiv.), THF (5 mL) and $Pd_2(dba)_3$ 10.9 mg (0.012 mmol, 2 mol%); HRMS (ESI) calcd for $C_{70}H_{93}N_{10}O_{19}S_2^-$ $[M-H]^-$ 1441.6065, found: 1441.6088.

(_N_-Cbz-D-Ser-L-Ala-_N_-Me-L-Cys-_N_-Me-L-Val)-(_N_-Cbz-D-Ser-L-Ala-_N_-Me-L-Cys-_N_-Me-L-Ile) (Serine-hydroxy)-Dilactone Disulfide, Cbz-bicyclicpeptide (Val,Ile) (60)

Target compound **60** (134.2 mg, 0.13 mmol, 36% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using _N_-Boc-octadepsipeptide-OH (Val,Ile) **58** (500 mg, 0.35 mmol), iodine (879.0 mg, 3.5 mmol, 10 equiv.) and MeCN (350

mL, 1 mM) in step 1 and HOAt (282.8 mg, 2.1 mmol, 6 equiv.), CH_2Cl_2 (350 mL, 1 mM) and EDCI·HCl (398.4 mg, 2.1 mmol, 6 equiv.) in step 2; ^{1}H NMR (mixture of conformers, 500 MHz, $CDCl_3$) δ 7.98 (d, *J* = 11.0 Hz, 1H, Ser-NH), 7.96 (d, *J* = 10.5 Hz, 1H, Ser-NH), 7.58 (d, *J* = 5.2 Hz, 1H, Ala-NH), 7.54 (d, *J* = 4.6 Hz, 1H, Ala-NH), 7.47–7.28 (m, 10H, Cbz-Ar), 5.83 (t, *J* = 7.7 Hz, 2H, Cys-α-CH), 5.55 (d, *J* = 4.6 Hz, 1H, Cbz-CH_2), 5.52 (d, *J* = 4.0 Hz, 1H, Cbz-CH_2), 5.00–4.84 (m, 6H, Ser-α-CH, Cbz-CH_2 and Ala-α-CH), 4.73–4.66 (m, 2H, Ser-β-CH_2), 4.47–4.32 (m, 2H, Ser-β-CH_2), 3.97 (d, *J* = 10.9 Hz, 1H, Ile-α-CH), 3.94 (d, *J* = 10.3 Hz, 1H, Val-α-CH), 3.21–2.97 (m, 4H, Cys-β-CH_2), 3.17 (s, 6H, Cys-*N*-Me) 2.95 (s, 3H, Val-*N*-Me), 2.93 (s, 3H, Ile-*N*-Me), 2.33–2.23 (m, 1H, Val-β-CH), 2.02–1.93 (m, 1H, Ile-β-CH), 1.47–1.35 (m, 1H, Ile-γ-CH_2), 1.05–0.82 (m, 13H, Ile-γ-CH_2, Val-γ-CH_3, Ile-δ-CH_3 and Ile-γ-CH_3), 0.75 (d, *J* = 6.9 Hz, 6H, Ala-β-CH_3); ^{13}C NMR (mixture of conformers, 125 MHz, $CDCl_3$) δ 174.2 (Ala-CO), 169.90 (Val-CO), 169.86 (Cys-CO), 169.76 (Ile-CO), 167.69 (Ser-CO), 167.66 (Ser-CO), 156.6 (Cbz-CO), 156.5 (Cbz-CO), 135.2, 129.6, 128.7, 128.5 (Cbz-Ar), 68.5 (Cbz-CH_2), 65.8 (Ser-β-CH_2), 65.7 (Ser-β-CH_2), 65.1 (Val-α-CH), 64.6 (Ile-α-CH), 53.8 (Ser-α-CH), 53.7 (Ser-α-CH), 50.7 (Cys-α-CH), 50.4 (Cys-α-CH), 44.8 (Ala-α-CH), 44.7 (Ala-α-CH), 40.5 (Cys-β-CH_2), 40.4 (Cys-β-CH_2),

35.9 (Ile-β-CH), 30.9 (Cys-*N*-Me), 29.34 (Val-*N*-Me), 29.30 (Ile-*N*-Me), 26.2 (Ile-γ-CH_2), 19.9 (Val-γ-CH_3), 19.7 (Val-γ-CH_3), 16.9 (Ala-β-CH_3), 16.8 (Ala-β-CH_3), 16.1 (Ile-γ-CH_3), 11.9 (Ile-δ-CH_3); HRMS (ESI) calcd for $C_{49}H_{68}N_8NaO_{14}S_2^+$ $[M+Na]^+$ 1079.4189, found: 1079.4215; m.p. 119.0–120.7 °C; $[\alpha]_D^{20.9}$ +41.5 ° (c 0.1, $CHCl_3$).

[*N*-(Quinoxaline-2-carbonyl)-D-Ser-L-Ala-*N*-Me-L-Cys-*N*-Me-L-Val]-[*N*-(Quinoxaline-2-carbonyl)-D-Ser-L-Ala-*N*-Me-L-Cys-*N*-Me-L-Ile) (Serine-hydroxy)-Dilactone Disulfide (TA-Val,Ile) (45)

Target compound **45** (40.3 mg, 0.037 mmol, 78% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using Cbz-bicyclicpeptide (Val,Ile) **60** (50 mg, 0.047 mmol), TFA (5 mL) and thioanisole (0.055 mL, 0.47 mmol, 10 equiv.) in step 1 and quinoxaline-2-carboxylic acid (33.3 mg, 0.19 mmol, 4 equiv.), *N*-methylmorpholine (0.029 mL, 0.28 mmol, 6 equiv.), DMT-MM (78.5 mg, 0.28 mmol, 6 equiv.) and DMF (5 mL) in step 2; ^1H NMR (small amount of conformer was contained, 500 MHz, $CDCl_3$, see table S11); ^{13}C NMR (mixture of conformers, 125 MHz, $CDCl_3$, see table S11); HRMS (ESI) calcd for $C_{51}H_{64}N_{12}NaO_{12}S_2^+$ $[M+Na]^+$ 1123.4100, found:

1123.4087; m.p. 242.2–245.0 °C (dec.); $[\alpha]_D^{27.7}$ −121.5 ° (c 0.05, $CHCl_3$); Purity: 93% t_R = 14.4 min.

_N_-Cbz-D-Ser[_N_-Cbz-D-Ser(_N_-Boc-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile)-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile]-_O_All, _N_-Boc-octadepsipeptide-_O_All (Ile,Ile) (57)

Target compound (971.1 mg, 0.65 mmol, 71% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using _N_-Cbz-D-Ser[_N_-Boc-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile]-_O_All **54** (900 mg, 1.1 mmol, 1.2 equiv.), AcOEt (8 mL) and 4 M HCl/AcOEt (2 mL) in step 1 and _N_-Cbz-D-Ser[_N_-Boc-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile]-OH **55** (713.7 mg, 0.91 mmol), DMT-MM (300.8 mg, 1.1 mmol, 1.2 equiv.) and AcOEt (10 mL) in step 2; HRMS (ESI) calcd for $C_{74}H_{100}N_{10}NaO_{19}S_2^+$ $[M+Na]^+$ 1519.6500, found: 1519.6480.

_N_-Cbz-D-Ser[_N_-Cbz-D-Ser(_N_-Boc-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile)-L-Ala-_N_-Me-L-Cys(Bam)-_N_-Me-L-Ile]-OH, _N_-Boc-octadepsipeptide-OH (Ile,Ile) (59)

Target compound **59** (707.6 mg, 0.49 mmol, 80% yield) was synthesized according to the procedure for the synthesis of TA by using *N*-Boc-octadepsipeptide-*O*All (Ile,Ile) **57** (921.1 mg, 0.61 mmol), PPh_3 (12.9 mg, 0.049 mmol, 8 mol%), *N*-methylaniline (0.08 mL, 0.74 mmol, 1.2 equiv.), THF (5 mL) and $Pd_2(dba)_3$ 11.3 mg (0.012 mmol, 2 mol%); HRMS (ESI) calcd for $C_{71}H_{95}N_{10}O_{19}S_2^-$ $[M-H]^-$ 1455.6222, found: 1455.6221.

(*N*-Cbz-D-Ser-L-Ala-*N*-Me-L-Cys-*N*-Me-L-Ile)$_2$ (Serine-hydroxy)-Dilactone Disulfide, Cbz-bicyclicpeptide (Ile,Ile) (61)

Target compound **61** (161.8 mg, 0.15 mmol, 44% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using *N*-Boc-octadepsipeptide-OH (Ile,Ile) **59** (500 mg, 0.34 mmol), iodine (870.6 mg, 3.4 mmol, 10 equiv.) and MeCN (350 mL, 1 mM) in step 1 and HOAt (280.1 mg, 2.1 mmol, 6 equiv.), CH_2Cl_2 (350 mL, 1 mM) and EDCI·HCl (394.5 mg, 2.1 mmol, 6 equiv.) in step 2; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (d, *J* = 9.7 Hz, 2H, Ser-NH), 7.55 (d, *J* = 8.6 Hz, 2H, Ala-NH), 7.49–7.29 (m, 10H, Cbz-Ar), 5.83 (t, *J* = 7.7 Hz, 2H, Cys-α-CH), 5.54 (d, *J* = 12.0 Hz, 2H, Cbz-CH_2), 4.98–4.83 (m, 6H, Ser-α-CH, Cbz-CH_2, Ala-α-CH), 4.69 (dd, *J* = 10.9, 2.3 Hz, 2H, Ser-β-CH_2), 4.37

(dd, J = 10.9, 1.1 Hz, 2H, Ser-β-CH_2), 3.97 (d, J = 10.3 Hz, 2H, Ile-α-CH), 3.17 (s, 6H, Cys-*N*-Me), 3.14–3.09 (m, 2H, Cys-β-CH_2), 3.05–2.98 (m, 2H, Cys-β-CH_2), 2.93 (s, 6H, Ile-*N*-Me), 2.02–1.92 (m, 2H, Ile-β-CH), 1.49–1.35 (m, 2H, Ile-γ-CH_2), 1.06–0.80 (m, 14H, Ile-γ-CH_2, γ-CH_3 and δ-CH_3), 0.75 (d, J = 6.9 Hz, 6H, Ala-β-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 1724.1 (Ala-CO), 169.9 (Cys-CO), 169.8 (Ile-CO), 167.6 (Ser-CO), 156.5 (Cbz-CO), 135.2, 129.6, 128.6, 128.5 (Cbz-Ar), 68.5 (Cbz-CH_2), 65.8 (Ser-β-CH_2), 64.5 (Ile-α-CH), 53.7 (Ser-α-CH), 50.5 (Cys-α-CH), 44.7 (Ala-α-CH), 40.3 (Cys-β-CH_2), 35.9 (Ile-α-CH), 30.8 (Cys-*N*-Me), 29.3 (Ile-*N*-Me), 26.2 (Ile-γ-CH_2), 16.8 (Ala-β-CH_3), 16.0 (Ile-γ-CH_3), 11.9 (Ile-δ-CH_3); HRMS (ESI) calcd for $C_{50}H_{70}N_8NaO_{14}S_2^+$ $[M+Na]^+$ 1093.4345, found: 1093.4350; m.p. 123.3–126.6 °C; $[\alpha]_D^{21.2}$ +55.4 ° (c 0.1, $CHCl_3$).

[*N*-(Quinoxaline-2-carbonyl)-D-Ser-L-Ala-*N*-Me-L-Cys-*N*-Me-L-Ile]$_2$ (Serine-hydroxy)-Dilactone Disulfide (TA-Ile,Ile) (46)

Target compound **46** (40.9 mg, 0.037 mmol, 78% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using Cbz-bicyclicpeptide (Ile,Ile) **61** (50 mg, 0.047 mmol), TFA (5 mL) and thioanisole (0.055 mL, 0.47 mmol, 10 equiv.) in

step 1 and quinoxaline-2-carboxylic acid (32.9 mg, 0.19 mmol, 4 equiv.), *N*-methylmorpholine (0.028 mL, 0.28 mmol, 6 equiv.), DMT-MM (77.6 mg, 0.28 mmol, 6 equiv.) and DMF (5 mL) in step 2; ^{1}H NMR (small amount of conformer was contained, 500 MHz, $CDCl_3$, see table S12); ^{13}C NMR (mixture of conformers, 125 MHz, $CDCl_3$, see table S12); HRMS (ESI) calcd for $C_{52}H_{66}N_{12}NaO_{12}S_2^+$ $[M+Na]^+$ 1137.4257, found: 1137.4264; m.p. 210.8–213.4 °C; $[\alpha]_D^{22.5}$ −159.2 ° (c 0.05, $CHCl_3$); Purity: 93%, t_R = 15.7 min.

N-Cbz-L-Ser-*O*All (62)

Target compound **62** (6.34 g, 22.7 mmol, 48% yield on 4 steps, *N*-Cbz-L-Ser(TBDMS)-*O*All 5.41 g, 13.7 mmol, 29% recovered) was synthesized according to the procedure for the synthesis of TA by using H-L-Ser-OH (5 g, 47.6 mmol), Cbz-Cl (8.0 mL, 57.1 mmol, 1.2 equiv.), $NaHCO_3$ (11.99 g, 142.7 mmol, 3 equiv.) and H_2O (100 mL) in step 1, TBDMSCl (8.38 g, 55.6 mmol, 1.2 equiv.), imidazole (9.46 g, 139.0 mmol, 3 equiv.) and THF (300 mL) in step 2, K_2CO_3 (7.68 g, 55.6 mmol, 1.2 equiv.), allylbromide (5.9 mL, 69.5 mmol, 1.5 equiv.) in step 3 and acetic acid (80 mL), THF (40 mL) and H_2O (40 mL) in step

4; HRMS (ESI) calcd for $C_{14}H_{17}NNaO_5^+$ $[M+Na]^+$ 302.0999, found: 302.1001; $[\alpha]_D^{20.7}$ +4.4 ° (c 0.205, $CHCl_3$).

N-Cbz-L-Ser(*N*-Boc-*N*-Me-D-Val)-*O*All (64)

Target compound **64** (2.07 g, 4.2 mmol, 78% yield) was synthesized according to the procedure for the synthesis of TA by using *N*-Cbz-L-Ser-*O*All **62** (1.5 g, 5.4 mmol), *N*-Boc-*N*-Me-D-Val-OH **63**[32] (1.49 g, 6.4 mmol, 1.2 equiv.), HOAt (1.10 g, 8.1 mmol, 1.5 equiv.), NEt_3 (1.1 mL, 8.1 mmol, 1.5 equiv.), CH_2Cl_2 (20 mL) and EDCI·HCl (1.54 g, 8.1 mmol, 1.5 equiv.); HRMS (ESI) calcd for $C_{25}H_{36}N_2NaO_8^+$ $[M+Na]^+$ 515.2364, found: 515.2358.

N-Boc-*N*-Me-D-Cys(Bam)-OH (65)

Target compound **65** (1.31 g, 3.5 mmol, 12% yield on 6 steps) was synthesized according to the procedure for the synthesis of TA by using H-D-Cys-OH•HCl•H_2O (5 g, 28.5 mmol), $MgSO_4$ (5 g), Trt-Cl (9.52 g, 34.2 mmol, 1.2 equiv.), AcOEt (25 mL) and DMF (25 mL) in step 1, Boc_2O (3.52 + 1.17 g, 16.1 + 5.4 mmol, 1.2 + 0.4 equiv.), 2 M NaOH aq. (30 mL) and 1,4-dioxane (30 mL) in step 2, NaH (60% in oil, 1.68 g, 42.0 mmol, 3 equiv.), MeI (5.2

mL, 84.0 mmol, 6 equiv.) and dry THF (50 mL) in step 3, TFA (4.9 mL, 65.3 mmol, 10 equiv.), TIPS (6.7 mL, 32.7 mmol, 5 equiv.) and CH_2Cl_2 (30 mL) in step 4, Bam-OH (1.18 g, 7.8 mmol, 1.2 equiv.) and dry TFA (30 mL) in step 5 and Boc_2O (2.14 + 2.14 g, 9.8 + 9.8 mmol, 1.5 + 1.5 equiv.), 2 M NaOH aq. (20 mL) and 1,4-dioxane (20 mL) in step 6; HRMS (ESI) calcd for $C_{17}H_{23}N_2O_5S^-$ $[M-H]^-$ 367.1333, found: 367.1330; $[\alpha]_D^{19.9}$ +45.8 ° (c 0.146, $CHCl_3$).

***N*-Cbz-L-Ser[*N*-Boc-*N*-Me-D-Cys(Bam)-*N*-Me-D-Val]-*O*All (66)**

Target compound **66** (2.10 g, 2.8 mmol, 94% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using *N*-Cbz-L-Ser(*N*-Boc-*N*-Me-D-Val)-*O*All **64** (1.5 g, 3.0 mmol), AcOEt (15 mL) and 4 M HCl/AcOEt (5 mL) in step 1 and *N*-Boc-*N*-Me-D-Cys(Bam)-OH **65** (1.35 g, 3.7 mmol, 1.2 equiv.), DMT-MM (1.26 g, 4.6 mmol, 1.5 equiv.) and AcOEt (20 mL) in step 2; HRMS (ESI) calcd for $C_{37}H_{50}N_4NaO_{10}S^+$ $[M+Na]^+$ 765.3140, found: 765.3138.

***N*-Cbz-L-Ser[*N*-Boc-D-Ala-*N*-Me-D-Cys(Bam)-*N*-Me-D-Val]-*O*All (67)**

Target compound **67** (1.16 g, 1.4 mmol, 53% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using *N*-Cbz-L-Ser[*N*-Boc-*N*-Me-D-Cys(Bam)-*N*-Me-D-Val]-*O*All **66** (2 g, 2.7 mmol), AcOEt (10 mL) and 4 M HCl/AcOEt (5 mL) in step 1 and *N*-Boc-D-Ala-OH (0.61 g, 3.2 mmol, 1.2 equiv.), DMT-MM (1.12 g, 4.0 mmol, 1.5 equiv.) and DMF (15 mL) in step 2; HRMS (ESI) calcd for $C_{40}H_{55}N_5NaO_{11}S^+$ $[M+Na]^+$ 836.3511, found: 836.3491.

_N_-Cbz-L-Ser[_N_-Boc-D-Ala-_N_-Me-D-Cys(Bam)-_N_-Me-D-Val]-OH (68)

Target compound **68** (504.2 mg, 0.65 mmol, 88% yield) was synthesized according to the procedure for the synthesis of TA by using *N*-Cbz-L-Ser[*N*-Boc-D-Ala-*N*-Me-D-Cys(Bam)-*N*-Me-D-Val]-*O*All **67** (600 mg, 0.74 mmol), PPh_3 (7.7 mg, 0.029 mmol, 4 mol%), *N*-methylaniline (0.10 mL, 0.88 mmol, 1.2 equiv.), THF (5 mL) and $Pd_2(dba)_3$ 6.8 mg (0.0074 mmol, 1 mol%); HRMS (ESI) calcd for $C_{37}H_{50}N_5O_{11}S^-$ $[M-H]^-$ 772.3233, found: 772.3250.

_N_-Cbz-L-Ser[_N_-Cbz-L-Ser(_N_-Boc-D-Ala-_N_-Me-D-Cys(Bam)-_N_-Me-D-Val)-D-Ala-_N_-Me-D-Cys(Bam)-_N_-Me-D-Val]-_O_All, _N_-Boc-*ent*-octadepsipeptide-_O_All (69)

Target compound **69** (417.8 mg, 0.28 mmol, 56% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using *N*-Cbz-L-Ser[*N*-Boc-D-Ala-*N*-Me-D-Cys(Bam)-*N*-Me-D-Val]-*O*All **67** (500 mg, 0.61 mmol, 1.2 equiv.), AcOEt (3 mL) and 4 M HCl/AcOEt (2 mL) in step 1 and *N*-Cbz-L-Ser[*N*-Boc-D-Ala-*N*-Me-D-Cys(Bam)-*N*-Me-D-Val]-OH **68** (396.2 mg, 0.51 mmol), DMT-MM (170.0 mg, 0.61 mmol, 1.2 equiv.) and AcOEt (10 mL) in step 2; HRMS (ESI) calcd for $C_{72}H_{96}N_{10}NaO_{19}S_2^+$ $[M+Na]^+$ 1491.6187, found: 1491.6160.

_N_-Cbz-L-Ser[_N_-Cbz-L-Ser(_N_-Boc-D-Ala-_N_-Me-D-Cys(Bam)-_N_-Me-D-Val)-D-Ala-_N_-Me-D-Cys(Bam)-_N_-Me-D-Val]-OH, _N_-Boc-*ent*-octadepsipeptide-OH (70)

Target compound **70** (351.9 mg, 0.25 mmol, 95% yield) was synthesized according to the procedure for the synthesis of TA by using *N*-Boc-*ent*-octadepsipeptide-*O*All **69** (380 mg, 0.26 mmol), PPh_3 (5.4 mg, 0.021 mmol, 8 mol%), *N*-methylaniline (0.034 mL, 0.31 mmol, 1.2 equiv.), THF (5 mL) and $Pd_2(dba)_3$ (4.7 mg, 0.0052 mmol, 2 mol%); HRMS (ESI) calcd for $C_{69}H_{91}N_{10}O_{19}S_2^-$ $[M-H]^-$ 1427.5909, found: 1427.5887.

(*N*-Cbz-L-Ser-D-Ala-*N*-Me-D-Cys-*N*-Me-D-Val)$_2$ (Serine-hydroxy)-Dilactone Disulfide *ent*-Cbz-bicyclicpeptide (71)

Target compound **71** (83.7 mg, 0.080 mmol, 36% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using *N*-Boc-*ent*-octadepsipeptide-OH **70** (320 mg, 0.22 mmol), iodine (568.1 mg, 2.2 mmol, 10 equiv.) and MeCN (225 mL, 1 mM) in step 1 and HOAt (182.8 mg, 1.3 mmol, 6 equiv.), CH_2Cl_2 (225 mL, 1 mM) and EDCI·HCl (257.5 mg, 1.3 mmol, 6 equiv.) in step 2; HRMS (ESI) calcd for $C_{48}H_{66}N_8NaO_{14}S_2^+$ $[M+Na]^+$ 1065.4032, found: 1065.4016; m.p. 138.0–142.0 °C; $[\alpha]_D^{20.4}$ −61.6 ° (c 0.105, $CHCl_3$).

(+)-Triostin A (47)

Target compound **47** (43.3 mg, 0.048 mmol, 83% yield on 2 steps) was synthesized according to the procedure for the synthesis of TA by using *ent*-Cbz-bicyclicpeptide **71** (50 mg, 0.048 mmol), TFA (5 mL) and thioanisole (0.056 mL, 0.48 mmol, 10 equiv.) in step 1 and quinoxaline-2-carboxylic acid (33.8 mg, 0.19 mmol, 4 equiv.), *N*-methylmorpholine (0.029 mL, 0.29 mmol, 6 equiv.), DMT-MM (79.6 mg, 0.29 mmol, 6

equiv.) and DMF (5 mL) in step 2; HRMS (ESI) calcd for $C_{50}H_{62}N_{12}NaO_{12}S_2^+$ $[M+Na]^+$ 1109.3944, found: 1109.3939; m.p. 205.0–209.5 °C (dec.); $[\alpha]_D^{26.2}$ +129.1 ° (c 0.094, $CHCl_3$); Purity: 91%, t_R = 12.4 min.

Ac-bicyclicpeptide, TA-Ac (48)

A solution of Cbz-bicyclicpeptide **72**[32] (50 mg, 0.048 mmol) and thioanisole (0.06 mL, 0.48 mmol, 10 equiv.) in TFA (5 mL) was stirred at 50 °C overnight. Then the mixture was concentrated under reduced pressure. To solution of the residue, *N*-methylmorpholine (0.03 mL, 0.29 mmol, 6 equiv.), and acetic acid (0.011 mL, 0.19 mmol, 4 equiv.) in DMF (5 mL) was added DMT-MM (80.2 mg, 0.29 mmol. 6 equiv.). The resulting solution was stirred at room temperature overnight. The mixture was extracted with CH_2Cl_2 (50 mL) and water (50 mL). The organic layer was washed with sat. NaCl aq. (50 mL), and dried with $MgSO_4$. The solvent was removed under reduced pressure. The residue was purified by silica gel (15 g) column chromatography eluted with CH_2Cl_2/MeOH (10:0, 100:1, 50:1, 20:1, 10:1). The resulting crude was purified by HPLC eluted with H_2O/CH_3CN containing 0.1% TFA (80:20 ~ 30:70, 60 min, Flow: 3 mL/min, Detect: 220 nm, nacalai COSMOSIL

5C18, 5 μm × 10 × 250 mm) to afford the target compound **48** (12.7 mg, 0.012 mmol, 24% yield on 2 steps) as pale yellow solid: ^{1}H NMR (500 MHz, DMSO-d_6, see table S15); ^{13}C NMR (125 MHz, DMSO-d_6, see table S15); HRMS (ESI) calcd for $C_{36}H_{58}N_8NaO_{12}S_2^+$ $[M+Na]^+$ 881.3508, found: 881.3482; m.p. 158.8–161.9 °C; $[\alpha]_D^{20.8}$ −19.0 ° (c 0.118, $CHCl_3$).

Quinoxaline-2-carbonyl-Gly-OH [cas: 5569-99-3]

To a solution of Gly-*O*Et•HCl (118.9 mg, 0.85 mmol, 1.5 equiv.), DMT-MM (235.6 mg, 0.85 mmol, 1.5 equiv.) and NMM (0.086 mL, 0.85 mmol, 1.5 equiv.) in DMF (5 mL) was added Quinoxaline-2-carboxylic acid (100 mg, 0.57 mmol). The resulting solution was stirred at room temperature overnight. The mixture was diluted with AcOEt (50 mL) and water (50 mL) and the two layers were separated. The organic layer was washed with sat. NaCl aq. (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by silica gel (10 g) column chromatography eluted with *n*-hexane/AcOEt (10:0, 9:1, 8:2, 7:3) to afford Quinoxaline-2-carbonyl-Gly-*O*Et (144.7 mg) as pale brown solid. Then it was dissolved into THF (3 mL) and 1 M NaOH

aq. (3 mL) and stirred for 30 min at room temperature. The mixture was diluted with AcOEt (50 mL) and 10% citric acid aq. (50 mL) and the two layers were separated. The organic layer was washed with sat. NaCl aq. (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. Quinoxaline-2-carbonyl-Gly-OH (111.4 mg, 0.48 mmol, 84% yield) was obtained as colorless solid; ^{1}H NMR (500 MHz, methanol-d_3) δ 9.42 (s, 1H, Qx-C3), 9.24 (br, 1H, Gly-NH), 8.13–8.16, 8.06–8.10, 7.83–7.89 (m, 1, 1, 2H, Qx-C5, C8, C7 and C6), 4.10–4.16 (m, 2H, Gly-α-CH_2).

TA-Qxc-Gly (49)

A solution of Cbz-bicyclicpeptide **72** (50 mg, 0.048 mmol) and thioanisole (0.06 mL, 0.48 mmol, 10 equiv.) in TFA (5 mL) was stirred at 50 °C overnight. Then the mixture was concentrated under reduced pressure. To solution of the residue, *N*-methylmorpholine (0.03 mL, 0.29 mmol, 6 equiv.), and Quinoxaline-2-carbonyl-Gly-OH (44.3 mg, 0.19 mmol, 4 equiv.) in DMF (5 mL) was added DMT-MM (80.2 mg, 0.29 mmol. 6 equiv.). The resulting solution was stirred at room temperature overnight. The mixture was diluted with CH_2Cl_2 (50 mL) and water (50 mL) and the two layers were separated. The organic layer

was washed with sat. NaCl aq. (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The resulting crude was purified by MPLC eluted with H_2O/CH_3CN containing 0.1% TFA (90:10 ~ 40:60, Flow: 20 mL/min, Detect: 220 nm, Silica gel 120 (Spherical) RP-18 40–50 mm 60 mL, KANTO) to afford the target compound **49** (19.9 mg, 0.017 mmol, 35% yield on 2 steps) as pale yellow solid; ^{1}H NMR (500 MHz, DMSO-d_6, see table S16); ^{13}C NMR (125 MHz, DMSO-d_6, see table S16); HRMS (ESI) calcd for $C_{54}H_{68}N_{14}NaO_{14}S_2^+$ $[M+Na]^+$ 1223.4373, found: 1223.4358; m.p. 277.0–282.5 °C (dec.); $[\alpha]_D^{18.8}$ −144.3 ° (c 0.101, DMSO).

Melting curve assay[58, 68, 69]

Melting curve assay was performed using Applied Biosystems® StepOnePlus™ Real-time PCR system (Thermo Fisher Scientific). All cyclic peptides (Ec (**1**), **11**, **12** or TA (**2**)) were dissolved in DMSO at 50 μM. To a solution of 0.125 μM dsDNA with or without 1 × SYBR green I (*ca.* 2 μM) in 50 mM Tris·HCl buffer added 1 μL of the DMSO stock solution of the cyclic peptide (final concentration 2.5 μM) to make a total volume of 20 μL. (SYBR green I was not added for fluorescently labeled hairpin DNA.) The mixture was heated from 25

to 95 °C at a rate of 1 °C per minute, while continuously monitoring the fluorescence intensity. The melting temperatures (Tm) were calculated using the StepOne Software version 2.3. Oligonucleotides of the following sequence were used: d(ACGTAGAACGT) or fluorescently labelled hairpin-shaped DNA, 5'–FAM–CCCTACGTATATGAAAATATACGTAGGG–BHQ1–3' (Hokkaido System Science Co., Ltd.).

Circular dichroism (CD) studies

A solution of each compound was prepared by dissolving in acetonitrile, followed by dilution to afford the desired concentration using 10 mM phosphate buffer (pH 7.2). CD spectra were acquired at 25 °C using a CD spectrometer (JASCO, J-820) equipped with a 1 mm path length quartz cell. Data pitch was set to 0.1 nm. The scanning speed was set to 100 nm/min and the spectra were averaged from four scans. The spectral baseline was recorded using 40% (v/v) acetonitrile in 10 mM phosphate buffer (pH 7.2). All data points were baseline subtracted, converted to a uniform scale of molar ellipticity, and plotted. Each plot was smoothed using nine data points per data point. For the

measurement of the temperature dependence, the temperature of the sample holder was set 1 min prior to the measurement.

Biological evaluation

Preparation of test compounds solutions.

All compounds were prepared as stock solutions in DMSO and stored in aliquots at −20 °C. The final concentration of DMSO in the biological assays was less than 1.0% (v/v).

Cell culture.

The HEK293 clone cell line was maintained in Eagle's minimum essential medium (E-MEM, Wako) containing 1% (v/v) nonessential amino acids (GIBCO) and 1 mmol/L sodium pyruvate (Wako), supplemented with 10% fetal bovine serum (FBS, GIBCO), 50 units/mL penicillin (Meiji), 50 μg/mL streptomycin (Meiji), and 50 μg/mL kanamycin (Meiji). Moreover, the MCF-7 and A549 cell lines were maintained in E-MEM (Wako) containing

1% nonessential amino acids (GIBCO) and 1 mmol/L sodium pyruvate (Wako), supplemented with 10% FBS (HyClone), 50 units/mL penicillin (Meiji), 50 μg/mL streptomycin (Meiji), and 50 μg/mL kanamycin (Meiji). The MDA-MB-231 cell line was maintained in the RPMI-1640 medium (Wako) containing 1 mmol/L sodium pyruvate (Wako), supplemented with 10% FBS (SIGMA), 50 units/mL penicillin (Meiji), 50 μg/mL streptomycin (Meiji), and 50 μg/mL kanamycin (Meiji). Lastly, the HT29 cell line was maintained in the RPMI-1640 medium (Wako), supplemented with 10% FBS (CCB), 50 units/mL penicillin (Meiji), 50 μg/mL streptomycin (Meiji), and 50 μg/mL kanamycin (Meiji). MCF-10A cell lines were maintained in MEGM™ BulletKit™ (Lonza) supplemented with 100 ng/mL cholera toxin (wako). WRL68 cell lines were maintained in D-MEM (low-glucose, Wako), supplemented with 10% FBS. All cells were incubated in a standard tissue culture incubator in 95% air and 5% CO_2 for normoxic conditions and in 1% O_2, 94% N_2, and 5% CO_2 for hypoxic conditions.

MTT assay

The cells were plated at a density of 4.5 × 10^3 cells/well (MCF-10A), 4.0 × 10^3 cells/well (MCF-7 and MDA-MB-231, and WRL68), 3.0 × 10^3 cells/well (A549), or 5.0 × 10^3 cells/well (HT29) (96-well plate, TPP Techno Plastic Products AG) in 100 μL of the medium overnight and then treated with the test compounds for 72 h. Each medium was then exchanged for a fresh one. The MTT reagent (0.5 mg/mL, Sigma-Aldrich) was added to the media. After 4 h of incubation at 37 °C, the media was removed and the cells were lysed with DMSO. The absorbance at 570 nm as measured using a plate reader (Multiskan JX, Thermo Fisher Scientific or GloMax®-Multi+, Promega).

For the first screening, MTT assay was conducted using MCF-7 (8.0 × 10^3 cells/well) cells treated with the test compounds for 24 h (Table 1).

HIF-1 dependent luciferase assay[49]

The HEK293 clone cells were seeded on a 24-well plate (8.0 × 10^4 cells/well) and then incubated for 24 h under normoxic conditions. Each medium was then exchanged for a fresh one containing 0.25% DMSO and several concentrations of the test compounds and incubated for 1 h. Subsequently, the cells were treated under normoxic or hypoxic

conditions (1% O_2) for 24 h. The luciferase assay was performed using the luciferase assay kit protocol (Roche). The activity was measured using a FB12 luminometer (Titertek-Berthold). The luciferase activity data were compensated by protein content.

Western Blotting

The proteins were extracted from the MCF-7 cells (2.5×10^6 cells/dish, 100 mm petri dish) or MDA-MB-231 cells (4.0×10^6 cells/dish) treated with the test compounds under normoxic or hypoxic (1% O_2) conditions for 16 h. In addition, the A549 cells (1.5×10^6 cells/dish) and the HT-29 cells (3.5×10^6 cells/dish) were treated with the test compounds under normoxic conditions for 12 h and then under hypoxic (1% O_2) conditions for 4 h.

The cell extracts were separated by SDS-PAGE (8% SDS) and transferred onto a nitrocellulose membrane. The membranes were blocked for 1 h at room temperature in 5% nonfat milk and then incubated for 1.5 h at room temperature with antibodies to HIF-1α (a dilution of 1:1000, Novus) and β-actin (a dilution of 1:3000, Sigma-Aldrich). Following washing, the membranes were incubated for 1 h at room temperature with 1:1000 and 1:3000 dilutions of horseradish peroxidase (HRP)-conjugated goat anti-

mouse IgG (Sigma-Aldrich). The proteins were visualized using chemiluminescence detection reagents (Pierce® Western Blotting Substrate or Immobilon™ Western Chemiluminescent HRP substrate) and measured utilizing a luminescent image analyzer (LAS3000, Fujifilm or ChemiDoc Touch, BIO-RAD).

Conformational search

The MacroModel version 10.7 module within Schrödinger Inc. was used for conformational calculations of bicyclic depsipeptides.[70] The structure was built within Maestro version 11.1.012 and initial structures were basically generated or derivatized from original X-ray structures due to the similarity of the analogous structures. The OPLS3 force field was used for modelling for all energies.[71] The conformational search was conducted in water. A torsional sampling (MCMM) approach was used to generate conformers, with enhanced torsional sampling, which is a simulating t*rans/cis* rotating of N-methyl-amide bonds. A total of 20,000 conformers were generated. All conformers were subjected to further minimization using the Powell-Reeves conjugate gradient (PRCG) method for a maximum of 1000 steps, and structures within 75 kJ/mol of the

lowest energy were saved for analysis. The three lowest energy structures from each calculation are shown in Figure S23 and S24 in Supporting Information. The lowest energy structures are shown in Figure 6 as a representative conformation.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Tables S1–S19, Figures S1–S28, experimental section for various spectroscopic analyses, biological evaluations, ^{1}H and ^{13}C NMR spectra characterization data, structural data of compounds **11**, **39** and **40** by X-ray crystallography; CCDC no; Authors will release the atomic coordinates upon article publication, analytical data of RP-HPLC and LC-MS, CD and UV, *in vitro* assay data, and conformational search method and data (PDF)

Crystallographic data for **11**(CCDC no. 1885767), **39**(CCDC no. 1885766), and **40**(CCDC no. 1885765) (CIF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: hnagasawa@gifu-pu.ac.jp Phone and Fax: +81-58-230-8112.

ORCID

Kota Koike: 0000-0003-3482-0747

Masanobu Nagano: 0000-0002-2901-7610

Masahiro Ebihara: 0000-0001-7183-326X

Tasuku Hirayama: 0000-0003-4541-7849

Mieko Tsuji: 0000-0003-2072-2118

Hiroaki Suga: 0000-0002-5298-9186

Hideko Nagasawa: 0000-0002-7662-0762

Author Contributions

The manuscript was written through contributions by all the authors. All authors have given approval to the final version of the manuscript. K.K, M.T., and T.H. synthesized compounds and performed spectroscopic analyses and biological assays. K.K and M.E. performed X-ray crystallography. K.K., and M.N. performed molecular modeling. K.K., M.N., H.S., and H.N. performed SAR studies. K.K, M.N., and H.N. wrote the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGEMENT

The authors are grateful to Dr. Gregg L. Semenza from Johns Hopkins University for generously providing us with the luciferase-expressing reporter plasmid p2.1. We would also like to thank Dr. Satoshi Ichikawa from Hokkaido University and Dr. Masumi Suzui from Nagoya City University for their insightful comments and helpful suggestions. We

also thank Dr. Hiroyasu Sato and Dr. Takashi Kikuchi of Rigaku corporation for X-ray crystallography. Dr. Yuji O. Kamatari from Gifu University provided valuable advice and assistance on measuring Tm using DSF. This research was supported by a Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (B) (24390029 and 16H05102 to H.N.) and the Collaborative Research Program of Institute for Chemical Research, Kyoto University (grant# 2019-90). This work was supported by JSPS A3 Foresight Program. This work was inspired by the international and interdisciplinary environments of the JSPS Core-to-Core Program, “Asian Chemical Biology Initiative.”

ABBREVIATIONS

Ala, alanine; All, allyl; Bam, benzamidomethyl; BSA, bovine serum albumin; Boc, *t*-butoxycarbonyl; Cbz, benzyloxycarbonyl; Cys, cysteine; D-MEM, Dulbecco's Modified Eagle's minimal essential medium; DIPEA, *N,N*-diisopropylethylamine; DMAP, *N,N*-dimethyl-4-aminopyridine; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; E-MEM, Eagle's minimal essential medium; Ec, Echinomycin; EDCI·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

hydrogenchloride; FBS, fetal bovine serum; Gly, glycine; HIF-1, hypoxia inducible factor-1; HOAt, 1-hydroxy-7-azabenzotriazole; HRE, hypoxia response element; HRMS, high resolution mass spectrometry; Ile, isoleucine; Lan, lanthionine; LC-MS, liquid chromatography-mass spectrometry; $LiBHEt_3$, lithium triethylborohydride; Luc, luciferase; MPLC, medium pressure liquid chromatography; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NMM, *N*-methylmorpholine; $Pd_2(dba)_3$, tries(dibenzylideneacetone)dipalladium(0); PSA, polar surface area; Qn(3OH), 3-hydroxyquinolone; Qx, quinoxaline; RCM, ring-closure metathesis; RP-HPLC, reverse-phase high performance liquid chromatography; SASA, solvent accessible surface area; Ser, serine; TA, Triostin A; TBDMSCl, *t*-butyldimethylsilyl chloride; TFA, trifluoroacetic acid; Tm, melting temperature; Ts, *p*-toluenesulfonyl; Val, valine;

REFERENCES

1. Morrison, C. Constrained peptides' time to shine? *Nat. Rev. Drug. Discov.* **2018**, *17*, 531–533.

2. Vinogradov, A. A.; Yin, Y.; Suga, H. Macrocyclic peptides as drug candidates: recent progress and remaining challenges. *J. Am. Chem. Soc.* **2019**, *141*, 4167–4181.

3. Bartoloni, M.; Jin, X.; Marcaida, M. J.; Banha, J.; Dibonaventura, I.; Bongoni, S.; Bartho, K.; Gräbner, O.; Sefkow, M.; Darbre, T. Bridged bicyclic peptides as potential drug scaffolds: synthesis, structure, protein binding and stability. *Chem. Sci.* **2015**, *6*, 5473–5490.

4. Rhodes, C. A.; Pei, D. Bicyclic peptides as next-generation therapeutics. *Chem. Eur. J.* **2017**, *23*, 12690–12703.

5. Di Bonaventura, I.; Jin, X.; Visini, R.; Probst, D.; Javor, S.; Gan, B. H.; Michaud, G.; Natalello, A.; Doglia, S. M.; Kohler, T.; van Delden, C.; Stocker, A.; Darbre, T.; Reymond, J. L. Chemical space guided discovery of antimicrobial bridged bicyclic peptides against Pseudomonas aeruginosa and its biofilms. *Chem. Sci.* **2017**, *8*, 6784–6798.

6. Itoh, H.; Tokumoto, K.; Kaji, T.; Paudel, A.; Panthee, S.; Hamamoto, H.; Sekimizu, K.; Inoue, M. Development of a high-throughput strategy for discovery of potent analogues of antibiotic lysocin E. *Nat. Commun.* **2019**, *10*, 2992.

7. Masuya, K. New trends in drug discovery and development by constrained peptides. *Nihon Yakurigaku Zasshi* **2016**, *148*, 322–328.

8. Yang, Z.; Shao, L.; Wang, M.; Rao, M.; Ge, M.; Xu, Y. Two novel quinomycins discovered by UPLC-MS from Stretomyces sp. HCCB11876. *J. Antibiot. (Tokyo)* **2019**, *72*, 164–168.

9. Fernandez, J.; Marin, L.; Alvarez-Alonso, R.; Redondo, S.; Carvajal, J.; Villamizar, G.; Villar, C. J.; Lombo, F. Biosynthetic modularity rules in the bisintercalator family of antitumor compounds. *Mar. Drugs* **2014**, *12*, 2668–2699.

10. Waring, M. J.; Wakelin, L. P. Echinomycin: a bifunctional intercalating antibiotic. *Nature* **1974**, *252*, 653–657.

11. Van Dyke, M. M.; Dervan, P. B. Echinomycin binding sites on DNA. *Science* **1984**, *225*, 1122–1127.

12. Kong, D.; Park, E. J.; Stephen, A. G.; Calvani, M.; Cardellina, J. H.; Monks, A.; Fisher, R. J.; Shoemaker, R. H.; Melillo, G. Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. *Cancer Res.* **2005**, *65*, 9047–9055.

13. Boger, D. L.; Ichikawa, S.; Tse, W. C.; Hedrick, M. P.; Jin, Q. Total syntheses of thiocoraline and BE-22179 and sssessment of their DNA binding and biological properties. *J. Am. Chem. Soc.* **2001**, *123*, 561–568.

14. Watanabe, K.; Hotta, K.; Praseuth, A. P.; Koketsu, K.; Migita, A.; Boddy, C. N.; Wang, C. C.; Oguri, H.; Oikawa, H. Total biosynthesis of antitumor nonribosomal peptides in Escherichia coli. *Nat. Chem. Biol.* **2006**, *2*, 423–428.

15. Tulla-Puche, J.; Auriemma, S.; Falciani, C.; Albericio, F. Orthogonal chemistry for the synthesis of thiocoraline–triostin hybrids. Exploring their structure–activity relationship. *J. Med. Chem.* **2013**, *56*, 5587–5600.

16. Ho, Y. T. C.; Leng, D. J.; Ghiringhelli, F.; Wilkening, I.; Bushell, D. P.; Kostner, O.; Riva, E.; Havemann, J.; Passarella, D.; Tosin, M. Novel chemical probes for the investigation of nonribosomal peptide assembly. *Chem. Commun. (Camb.)* **2017**, *53*, 7088–7091.

17. Macklin, P. S.; Yamamoto, A.; Browning, L.; Hofer, M.; Adam, J.; Pugh, C. W. Recent advances in the biology of tumour hypoxia with relevance to diagnostic practice and tissue-based research. *J. Pathol.* **2020**, in press, doi:10.1002/path.5402.

18. Semenza, G. L. Targeting hypoxia-inducible factor 1 to stimulate tissue vascularization. *J. Investig. Med.* **2016**, *64*, 361–363.

19. Subhani, S.; Vavilala, D. T.; Mukherji, M. HIF inhibitors for ischemic retinopathies and cancers: options beyond anti-VEGF therapies. *Angiogenesis* **2016**, *19*, 257–273.

20. Soni, S.; Padwad, Y. S. HIF-1 in cancer therapy: two decade long story of a transcription factor. *Acta Oncol.* **2017**, *56*, 503–515.

21. Pezzuto, A.; Perrone, G.; Orlando, N.; Citarella, F.; Ciccozzi, M.; Scarlata, S.; Tonini, G. A close relationship between HIF-1alpha expression and bone metastases in advanced NSCLC, a retrospective analysis. *Oncotarget* **2019**, *10*, 7071–7079.

22. Burslem, G. M.; Kyle, H. F.; Nelson, A.; Edwards, T. A.; Wilson, A. J. Correction: Hypoxia inducible factor (HIF) as a model for studying inhibition of protein-protein interactions. *Chem. Sci.* **2017**, *8*, 5214–5215.

23. Bhattarai, D.; Xu, X.; Lee, K. Hypoxia-inducible factor-1 (HIF-1) inhibitors from the last decade (2007 to 2016): a "structure-activity relationship" perspective. *Med. Res. Rev.* **2018**, 38, 1404–1442.

24. An, H.; Lee, S.; Lee, J. M.; Jo, D. H.; Kim, J.; Jeong, Y. S.; Heo, M. J.; Cho, C. S.; Choi, H.; Seo, J. H.; Hwang, S.; Lim, J.; Kim, T.; Jun, H. O.; Sim, J.; Lim, C.; Hur, J.; Ahn, J.; Kim, H. S.; Seo, S. Y.; Na, Y.; Kim, S. H.; Lee, J.; Lee, J.; Chung, S. J.; Kim, Y. M.; Kim, K. W.; Kim, S. G.; Kim, J. H.; Suh, Y. G. Novel hypoxia-inducible factor 1alpha (HIF-1alpha) inhibitors for angiogenesis-related ocular diseases: discovery of a novel scaffold via ring-truncation strategy. *J. Med. Chem.* **2018**, *61*, 9266–9286.

25. Gradishar, W. J.; Vogelzang, N. J.; Kilton, L. J.; Leibach, S. J.; Rademaker, A. W.; French, S.; Benson, A. B., 3rd. A phase II clinical trial of echinomycin in metastatic soft tissue sarcoma. An Illinois Cancer Center study. *Invest. New Drugs* **1995**, *13*, 171–174.

26. Chang, A. Y.; Kim, K.; Boucher, H.; Bonomi, P.; Stewart, J. A.; Karp, D. D.; Blum, R. H. A randomized phase II trial of echinomycin, trimetrexate, and cisplatin plus etoposide in patients with metastatic nonsmall cell lung carcinoma: an Eastern Cooperative Oncology Group study (E1587). *Cancer* **1998**, *82*, 292–300.

27. Yamaguchi, J.; Tanaka, T.; Saito, H.; Nomura, S.; Aburatani, H.; Waki, H.; Kadowaki, T.; Nangaku, M. Echinomycin inhibits adipogenesis in 3T3-L1 cells in a HIF-independent manner. *Sci. Rep.* **2017**, *7*, 6516.

28. Yao, Y.; Wang, L.; Zhou, J.; Zhang, X. HIF-1alpha inhibitor echinomycin reduces acute graft-versus-host disease and preserves graft-versus-leukemia effect. *J. Transl. Med.* **2017**, *15,* 28.

29. Huang, X.; Trinh, T.; Aljoufi, A.; Broxmeyer, H. E. Hypoxia signaling pathway in stem cell regulation: good and evil. *Curr. Stem Cell Rep.* **2018**, *4*, 149–157.

30. Ponnurangam, S.; Dandawate, P. R.; Dhar, A.; Tawfik, O. W.; Parab, R. R.; Mishra, P. D.; Ranadive, P.; Sharma, R.; Mahajan, G.; Umar, S.; Weir, S. J.; Sugumar, A.; Jensen, R. A.; Padhye, S. B.; Balakrishnan, A.; Anant, S.; Subramaniam, D. Quinomycin A targets Notch signaling pathway in pancreatic cancer stem cells. *Oncotarget* **2016**, 7, 3217–3232.

31. Ratnayake, A. S.; Chang, L. P.; Tumey, L. N.; Loganzo, F.; Chemler, J. A.; Wagenaar, M.; Musto, S.; Li, F.; Janso, J. E.; Ballard, T. E.; Rago, B.; Steele, G. L.; Ding, W.; Feng, X.; Hosselet, C.; Buklan, V.; Lucas, J.; Koehn, F. E.; O'Donnell, C. J.; Graziani, E. I. Natural product bis-intercalator depsipeptides as a new class of payloads for antibody-drug conjugates. *Bioconjug. Chem.* **2019**, *30*, 200–209.

32. Hattori, K.; Koike, K.; Okuda, K.; Hirayama, T.; Ebihara, M.; Takenaka, M.; Nagasawa, H. Solution-phase synthesis and biological evaluation of triostin A and its analogues. *Org. Biomol. Chem.* **2016**, *14*, 2090–2111.

33. Dell, A.; Williams, D. H.; Morris, H. R.; Smith, G. A.; Feeney, J.; Roberts, G. C. Structure revision of the antibiotic echinomycin. *J. Am. Chem. Soc.* **1975**, *97*, 2497–2502.

34. Otsuka, H.; Shoji, J.; Kawano, K.; Kyogoku, Y. Structure confirmation of triostin a by 1H and 13C magnetic resonance. *J. Antibiot. (Tokyo)* **1976**, *29*, 107–110.

35. Sheldrick, G. M.; Heine, A.; Schmidt-Base, K.; Pohl, E.; Jones, P. G.; Paulus, E.; Waring, M. J. Structures of quinoxaline antibiotics. *Acta Crystallogr. Sect. B* **1995**, *51*, 987–999.

36. Hotta, K.; Keegan, R. M.; Ranganathan, S.; Fang, M.; Bibby, J.; Winn, M. D.; Sato, M.; Lian, M.; Watanabe, K.; Rigden, D. J.; Kim, C. Y. Conversion of a disulfide bond into a thioacetal group during echinomycin biosynthesis. *Angew. Chem. Int. Ed.* **2014**, *53*, 824–828.

37. Sato, M.; Nakazawa, T.; Tsunematsu, Y.; Hotta, K.; Watanabe, K. Echinomycin biosynthesis. *Curr. Opin. Chem. Biol.* **2013**, *17*, 537–545.

38. Ichikawa, S.; Okamura, T.; Matsuda, A. Total synthesis of quinaldopeptin and its analogues. *J. Org. Chem.* **2013**, *78*, 12662–12670.

39. Katayama, K.; Okamura, T.; Sunadome, T.; Nakagawa, K.; Takeda, H.; Shiro, M.; Matsuda, A.; Ichikawa, S. Synthesis and biological evaluation of quinaldopeptin. *J. Org. Chem.* **2014**, *79*, 2580–2590.

40. Park, Y. S.; Kim, Y. H.; Kim, S. K.; Choi, S.-J. A new antitumor agent: methyl sulfonium perchlorate of echinomycin. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 731–734.

41. Cromm, P. M.; Schaubach, S.; Spiegel, J.; Furstner, A.; Grossmann, T. N.; Waldmann, H. Orthogonal ring-closing alkyne and olefin metathesis for the synthesis of small GTPase-targeting bicyclic peptides. *Nat Commun.* **2016**, *7*, 11300.

42. Kunishima, M.; Kawachi, C.; Iwasaki, F.; Terao, K.; Tani, S. Synthesis and characterization of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride. *Tetrahedron Lett.* **1999**, *40*, 5327–5330.

43. Scholl, M.; Ding, S.; Lee, C. W.; Grubbs, R. H. Synthesis and activity of a new generation of ruthenium-based olefin metathesis catalysts coordinated with 1,3-dimesityl-4,5-dihydroimidazol-2-ylidene ligands. *Org. Lett.* **1999**, *1*, 953–956.

44. Boger, D. L.; Chen, J.-H. A modified Friedlander condensation for the synthesis of 3-hydroxyquinoline-2-carboxylates. *The J. Org. Chem.* **1995**, *60*, 7369–7371.

45. Tezuka, Y.; Oike, H. Topological polymer chemistry: systematic classification of nonlinear polymer topologies. *J. Am. Chem. Soc.* **2001**, *123*, 11570–11576.

46. Sable, G. A.; Lim, D. Solid-Phase Synthesis of Triostin A Using a Symmetrical Bis(diphenylmethyl) Linker System. *J. Org. Chem.* **2015**, *80*, 7486–7494.

47. Schneider, A.; Rodrigues, O. E. D.; Paixão, M. W.; Appelt, H. R.; Braga, A. L.; Wessjohann, L. A. Stereoselective synthesis of Boc-protected l-seleno- and tellurolanthionine, l-seleno- and tellurocystine and derivatives. *Tetrahedron Lett.* **2006**, *47*, 1019–1021.

48. M. Stocking, E.; N. Schwarz, J.; Senn, H.; Salzmann, M.; A. Silks, L. Synthesis of L-selenocystine, L-[77Se]selenocystine and L-tellurocystine. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2443–2448.

49. Hattori, H.; Okuda, K.; Murase, T.; Shigetsura, Y.; Narise, K.; Semenza, G. L.; Nagasawa, H. Isolation, identification, and biological evaluation of HIF-1-modulating compounds from Brazilian green propolis. *Bioorg. Med. Chem.* **2011**, *19*, 5392–5401.

50. Semenza, G. L.; Jiang, B. H.; Leung, S. W.; Passantino, R.; Concordet, J. P.; Maire, P.; Giallongo, A. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J. Biol. Chem.* **1996**, *271*, 32529–32537.

51. Biagi, G. L.; Barbaro, A. M.; Gandolfi, O.; Guerra, M. C.; Cantelli-Forti, G. Rm values of steroids as an expression of their lipophilic character in structure-activity studies. *J. Med. Chem.* **1975**, *18*, 873–883.

52. Egan, W. J.; Merz, K. M., Jr.; Baldwin, J. J. Prediction of drug absorption using multivariate statistics. *J. Med. Chem.* **2000**, *43*, 3867–3877.

53. Ahlbach, C. L.; Lexa, K. W.; Bockus, A. T.; Chen, V.; Crews, P.; Jacobson, M. P.; Lokey, R. S. Beyond cyclosporine A: conformation-dependent passive membrane permeabilities of cyclic peptide natural products. *Future Med. Chem.* **2015**, 7, 2121–2130.

54. Suzui, M.; Masuda, M.; Lim, J. T.; Albanese, C.; Pestell, R. G.; Weinstein, I. B. Growth inhibition of human hepatoma cells by acyclic retinoid is associated with induction of p21(CIP1) and inhibition of expression of cyclin D1. *Cancer Res.* **2002**, *62*, 3997–4006.

55. Chaires, J. B. Thermal Denaturation of Drug–DNA Complexes. In *DNA-targeting Molecules as Therapeutic Agents*; Waring, M. J.; Royal Society of Chemistry: Cambridge, 2018; pp74–95.

56. Leng, F.; Chaires, J. B.; Waring, M. J. Energetics of echinomycin binding to DNA. *Nucleic Acids Res.* **2003**, *31*, 6191–6197.

57. Camunas-Soler, J.; Alemany, A.; Ritort, F. Experimental measurement of binding energy, selectivity, and allostery using fluctuation theorems. *Science* **2017**, *355*, 412–415.

58. Zipper, H.; Brunner, H.; Bernhagen, J.; Vitzthum, F. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res.* **2004**, *32*, e103.

59. Kalman, J. R.; Blake, T. J.; Williams, D. H.; Feeney, J.; Roberts, G. C. The conformations of triostin A in solution. *J. Chem. Soc., Perkin Trans. 1* **1979**, 1313–1321.

60. Cheung, H.; Feeney, J.; Roberts, G. C.; Williams, D.; Ughetto, G.; Waring, M. The conformation of echinomycin in solution. *J. Am. Chem. Soc.* **1978**, *100*, 46–54.

61. Wang, A. H.; Ughetto, G.; Quigley, G. J.; Hakoshima, T.; van der Marel, G. A.; van Boom, J. H.; Rich, A. The molecular structure of a DNA-triostin A complex. *Science* **1984**, *225*, 1115–1121.

62. Cuesta-Seijo, J. A.; Sheldrick, G. M. Structures of complexes between echinomycin and duplex DNA. *Acta Crystallogr. Sect. D: Biol. Crystallogr.* **2005**, *61*, 442–448.

63. Pfoh, R.; Cuesta-Seijo, J. A.; Sheldrick, G. M. Interaction of an echinomycin-DNA complex with manganese ions. *Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun.* **2009**, *65*, 660–664.

64. Mohamadi, F.; Richards, N. G.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. MacroModel—an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. *J. Comput. Chem.* **1990**, *11*, 440–467.

65. Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. Semianalytical treatment of solvation for molecular mechanics and dynamics. *J. Am. Chem. Soc.* **1990**, *112*, 6127–6129.

66. Onda, Y.; Masuda, Y.; Yoshida, M.; Doi, T. Conformation-based design and synthesis of apratoxin A mimetics modified at the alpha,beta-unsaturated thiazoline moiety. *J. Med. Chem.* **2017**, *60*, 6751–6765.

67. Costantino, P.; De Santis, P.; Ughetto, G. Circular dichroism studies on the echinomycin--DNA complex. *FEBS Lett.* **1978**, *88*, 349–352.

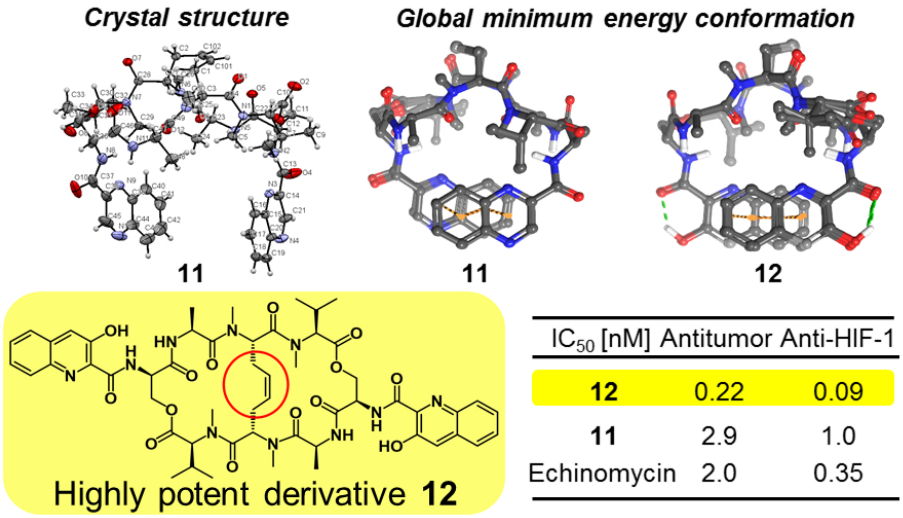
68. Darby, R. A.; Sollogoub, M.; McKeen, C.; Brown, L.; Risitano, A.; Brown, N.; Barton, C.; Brown, T.; Fox, K. R. High throughput measurement of duplex, triplex and

quadruplex melting curves using molecular beacons and a LightCycler. *Nucleic Acids Res.* **2002**, *30*, e39.

69. Kabir, A.; Honda, R. P.; Kamatari, Y. O.; Endo, S.; Fukuoka, M.; Kuwata, K. Effects of ligand binding on the stability of aldo-keto reductases: Implications for stabilizer or destabilizer chaperones. *Protein Sci.* **2016**, *25*, 2132–2141.

70. *Schrödinger Suite 2016: MacroModel*, version 10.7; Schrödinger, LLC, New York, 2016.

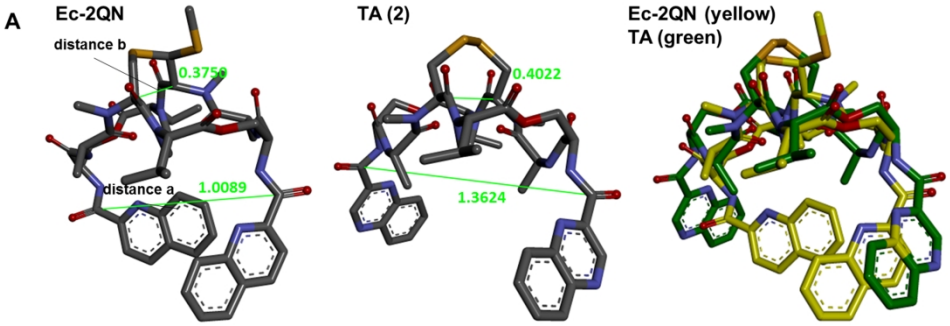
71. Harder, E.; Damm, W.; Maple, J.; Wu, C.; Reboul, M.; Xiang, J. Y.; Wang, L.; Lupyan, D.; Dahlgren, M. K.; Knight, J. L.; Kaus, J. W.; Cerutti, D. S.; Krilov, G.; Jorgensen, W. L.; Abel, R.; Friesner, R. A. OPLS3: a force field providing broad coverage of drug-like small molecules and proteins. *J. Chem. Theory Comput.* **2016**, *12*, 281–296.



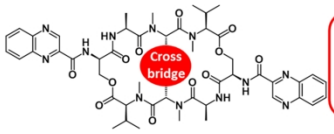
IC_{50} [nM]	Antitumor	Anti-HIF-1
12	0.22	0.09
11	2.9	1.0
Echinomycin	2.0	0.35

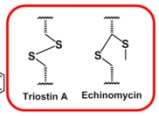
Table of Contents

82x44mm (300 x 300 DPI)



B





	Distance (nm in crystal structure)		IC_{50}	
	distance a	distance b	MTT [nM]	HIF-1 Luc. [nM]
Ec (1)	1.01[a]	0.375[a]	5.5 ± 0.2	0.35 ± 0.03
TA (2)	1.36	0.40	4100 ± 300	26.9 ± 1.3

Figure 1

489x275mm (300 x 300 DPI)

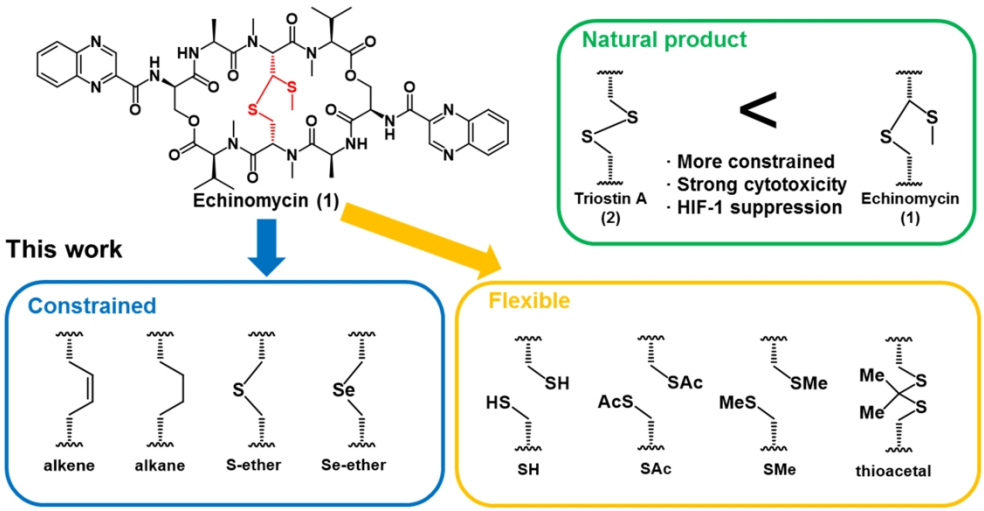


Figure 2

489x275mm (300 x 300 DPI)

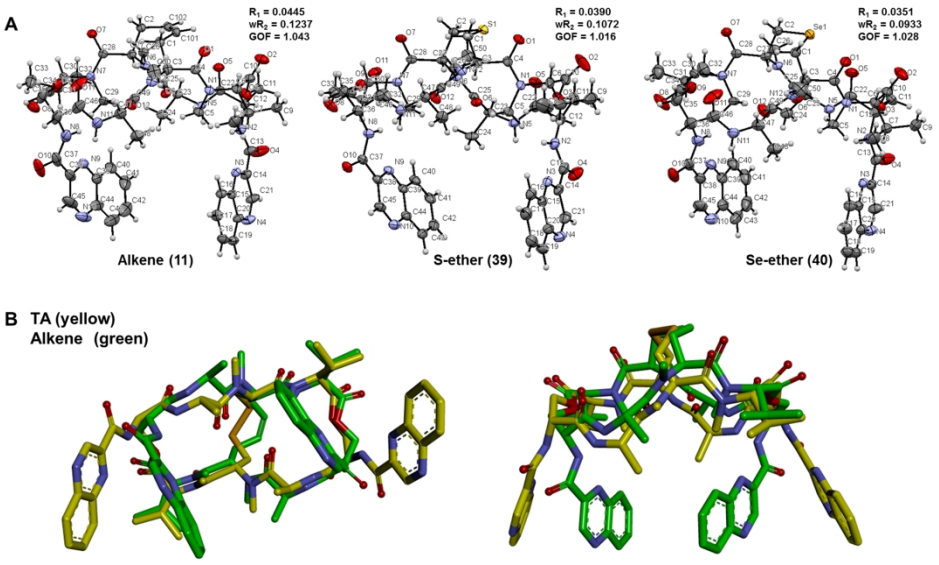


Figure 3

489x275mm (300 x 300 DPI)

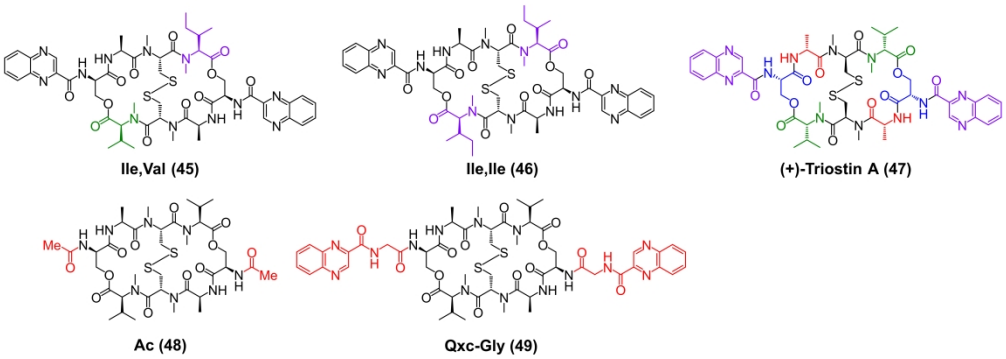


Figure 4

489x275mm (300 x 300 DPI)

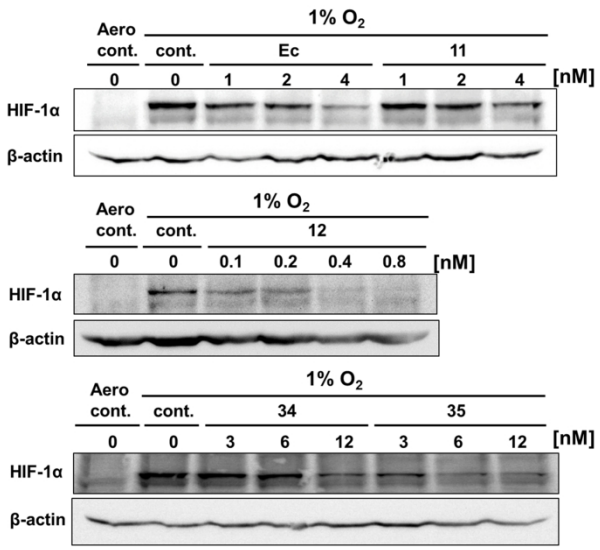


Figure 5

489x275mm (300 x 300 DPI)

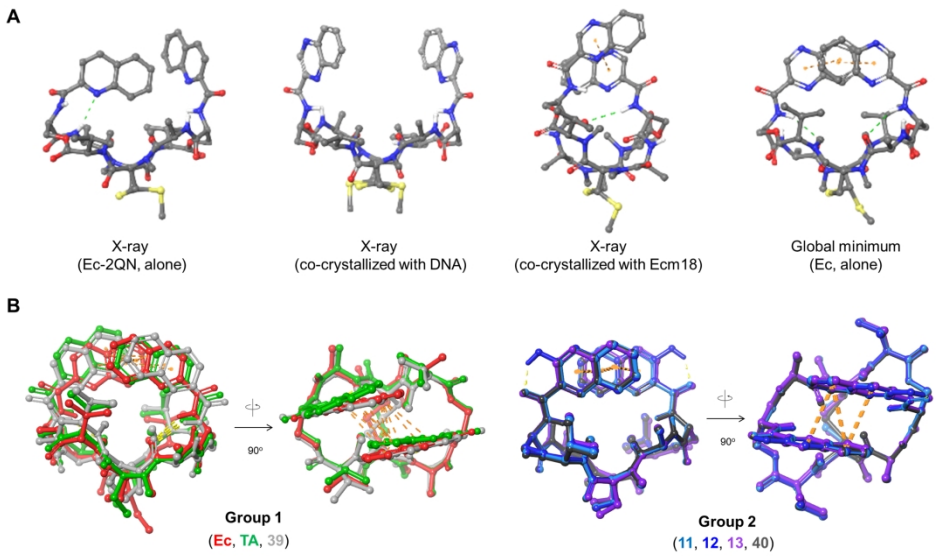


Figure 6A. 6B

489x275mm (300 x 300 DPI)

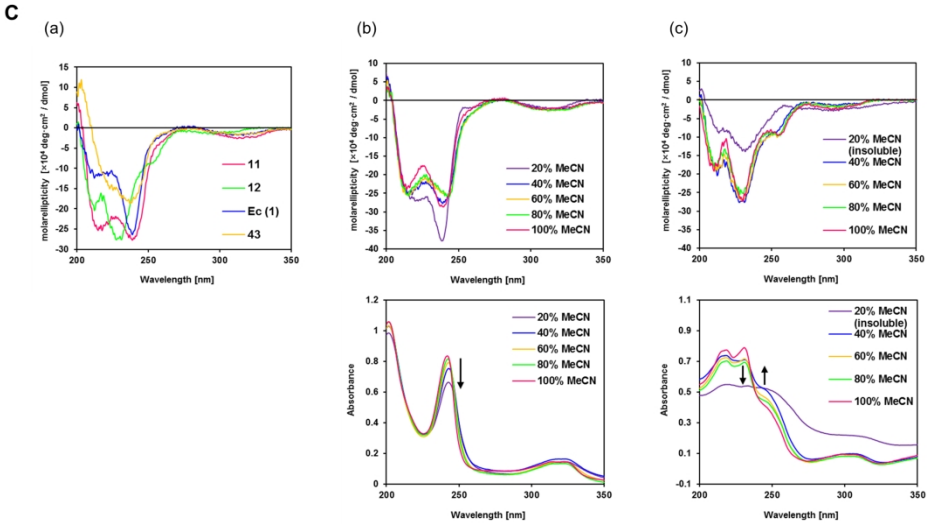
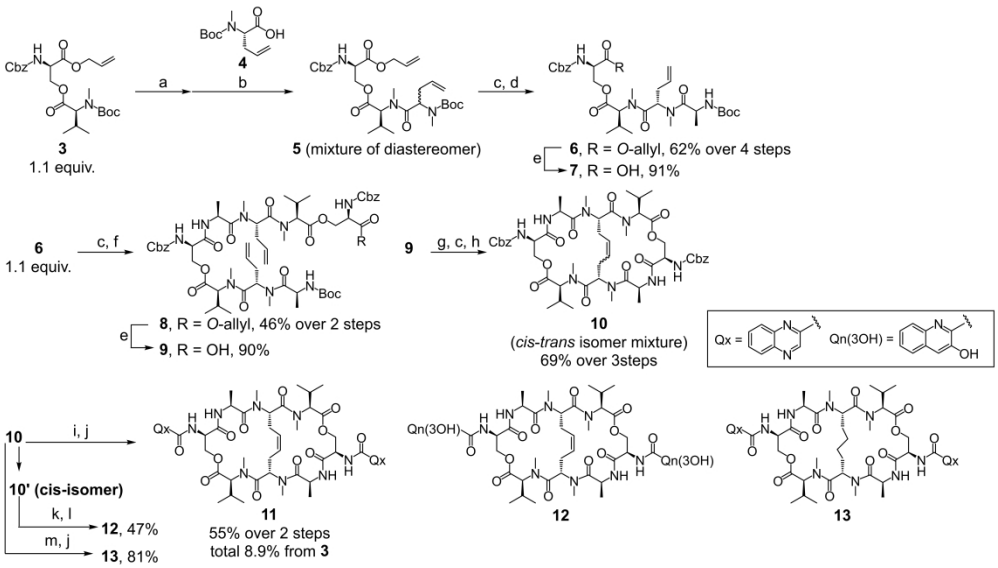


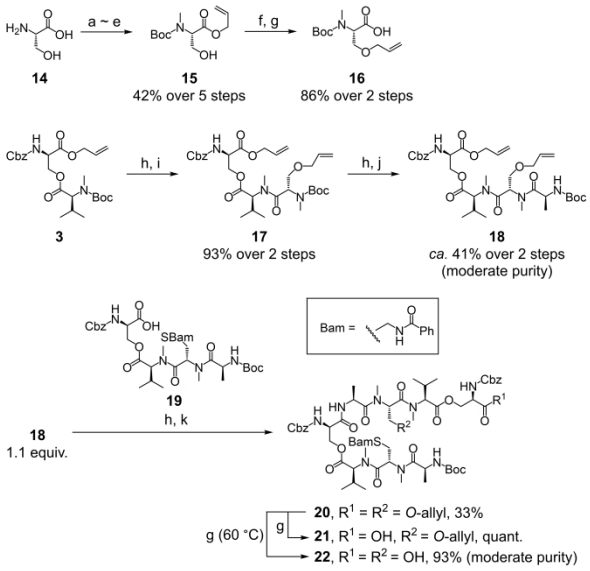
Figure 6C

489x275mm (300 x 300 DPI)



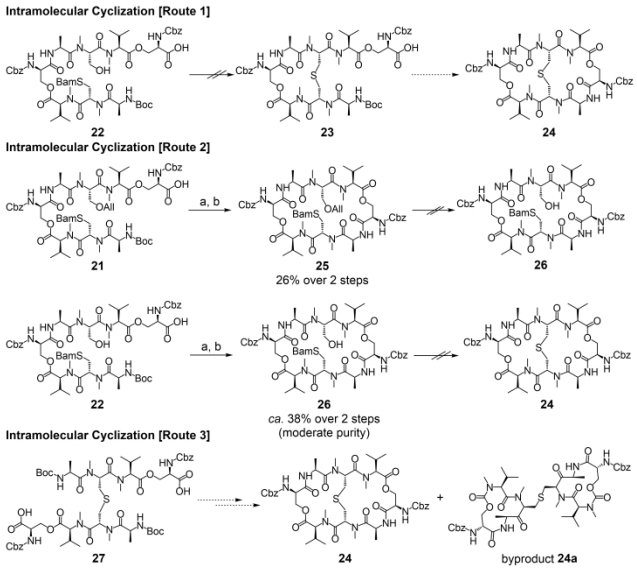
Scheme 1

489x275mm (300 x 300 DPI)



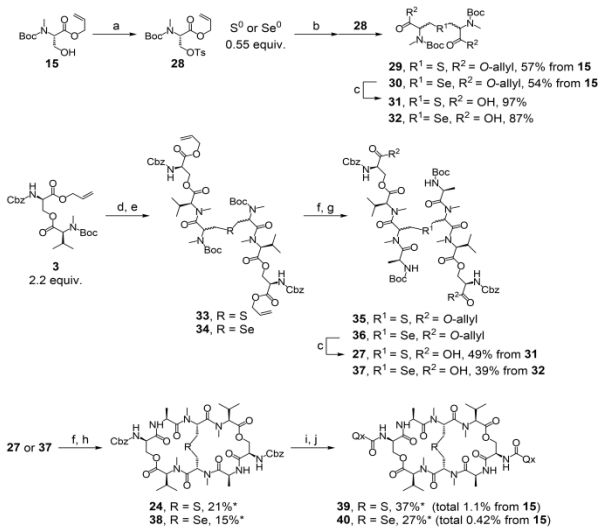
Scheme 2

489x275mm (300 x 300 DPI)



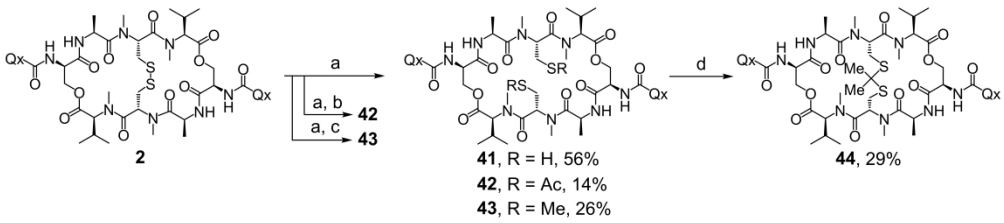
Scheme 3

489x275mm (300 x 300 DPI)



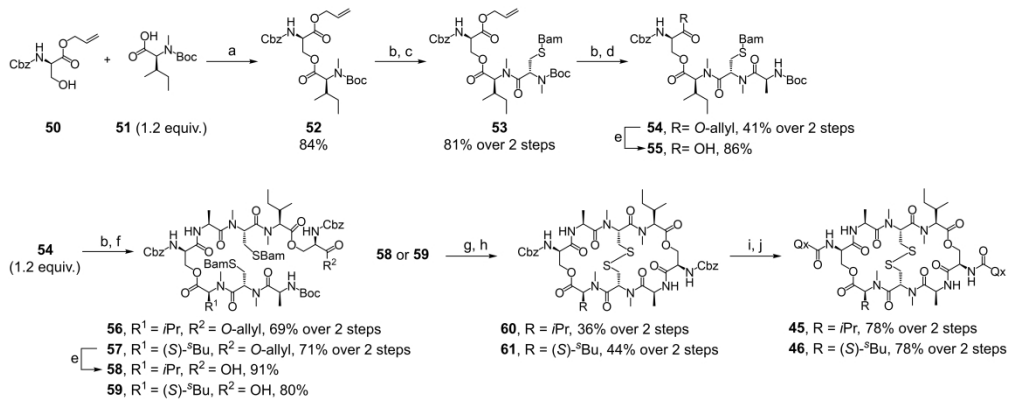
Scheme 4

489x275mm (300 x 300 DPI)



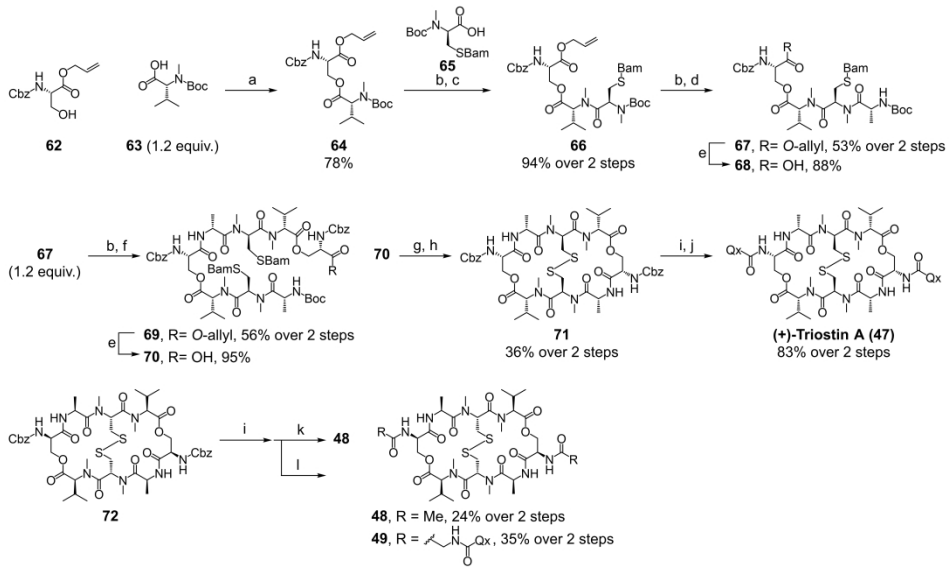
Scheme 5

489x275mm (300 x 300 DPI)



Scheme 6

489x275mm (300 x 300 DPI)



Scheme 7

489x275mm (300 x 300 DPI)