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Synthesis and biological evaluation of echinomycin analogues as potential colon cancer agent

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Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer-related death, thus a novel chemotherapeutic agent for colon cancer therapy is needed. In this study, analogues of echinomycin, a cyclic peptide natural product with potent toxicity to several human cancer cell lines, were synthesized, and their biological activities against human colon cancer cells were investigated. Analogue 3 as well as 1 inhibit HIF-1 α -mediated transcription. Notably, transcriptome analysis indicated that the cell cycle and its regulation were involved in the effects on cells treated with 3. Analogue 3 exhibited superior *in vivo* efficacy to echinomycin without significant toxicity in mouse xenograft model. The low dose of 3 needed to be efficacious *in vivo* is also noteworthy and our data suggest that 3 is an attractive and potentially novel agent for the treatment of colon cancer.

Cancer is a leading cause of death from disease in many countries, and 9.9 million people died of cancer in 2020 worldwide¹. Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer-related death among men and women. The distant metastasis of cancer cells is also an important issue, a colorectal cancer tends to metastasize to the liver and lungs. In particular, the 5-year survival rate for stage IV colorectal cancer patients with distant metastases to the liver and lungs is still extremely low. Current treatments for colorectal cancer include surgical resection and chemotherapy. However, even after successful surgical resection followed by chemotherapy, the 5-year recurrence rate is still high. Therefore, a novel chemotherapeutic agent for colon cancer therapy is urgently needed.

Echinomycin² (1, Fig. 1), a bicyclic octadepsipeptide possessing two quinoxaline chromophores, is in a class of natural products that can bind to double-stranded DNA due to bisintercalation by the chromophores^{3–6}. Because of its potent toxicity against human cancer cells, several phase I and II clinical trials with this compound were conducted in the 1990s^{3–14}. However, drug development was halted because minimal therapeutic effects were observed for terminal patients with solid tumors. Migration, angiogenesis, and invasion are important for tumor progression and metastasis and are regulated by the transcription factor, hypoxia-inducible factor-1 (HIF-1)^{15,16}. In 2015, it was reported that echinomycin suppresses the HIF-1 pathway by inhibiting the binding of HIF-1 α to the hypoxia-responsive element (HRE), which is the *cis*-element of HIF-1 α ¹⁷. In addition, it has been suggested that 1 suppresses the Notch signaling pathway, resulting in potent inhibitory activity against pancreatic cancer by targeting stem cells¹⁸. It has also been reported that 1 can selectively kill leukemia-initiating cells in patients with relapsed acute myeloid leukemia (AML) without causing normal stem cell toxicity¹⁹. In these studies, the dose of 1 demonstrating efficacy was quite low compared to those used in previous clinical trials. It has been suggested that 1 is still a promising anticancer drug lead compound because of its ability to alter the clinical

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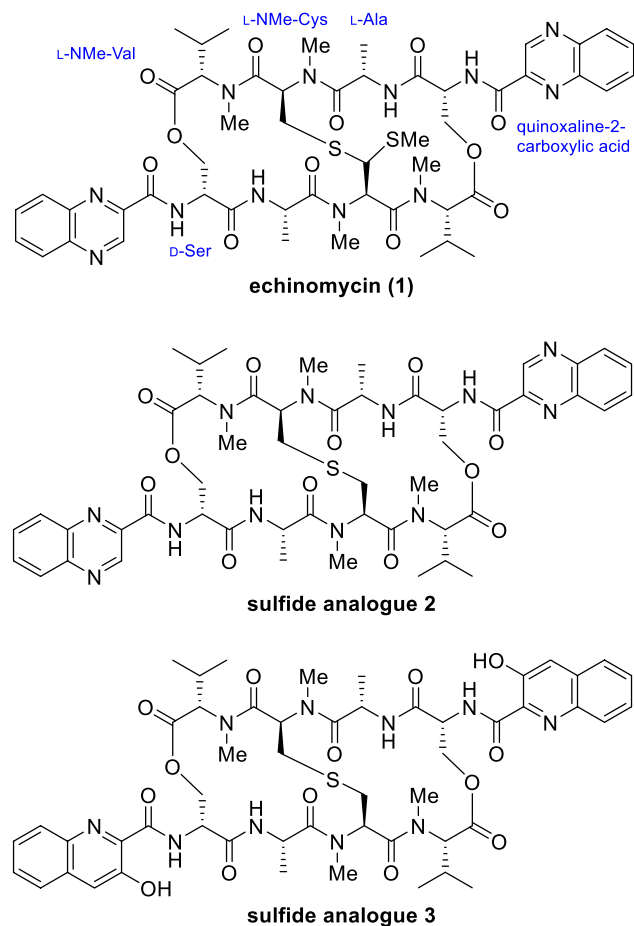


Figure 1. Chemical structures of echinomycin and its analogues.

protocol. We accomplished the first total synthesis of **1** by constructing the characteristic thioacetal moiety via a Pummerer rearrangement of **2** in the late stage of the synthesis²⁰. A brief structure–activity relationship study revealed that **2** reduces but still retains the toxicity of **1** to human pancreatic cancer MIA PaCa-2 cells. We further found that the chromophore analogue **3**, which possesses 3-hydroxyquinoline-2-carbonyl groups, exhibited a similar cytotoxic potency to that of **1**. The fact that sulfide analogues **2** and **3** exhibit potencies similar that of **1** is advantageous because these analogues are more synthetically accessible than **1**. The toxicity of these analogues to human colorectal cells has not yet been tested, and the structural requirements of the chromophores remain to be investigated from our previous study. In this study, the synthesis of chromophore analogues of **2** and an in vitro and in vivo evaluation of their antiproliferative activity against SW620 cell lines, which are human colon cancer cells derived from highly invasive lymph node metastases, are described.

Results

Synthesis of echinomycin analogues

First, the impact of other pendant chromophores was investigated to expand our current knowledge of the structure–activity relationships for **2** (Fig. 2). Analogues **4–7** were designed to determine the impact of the number and position of each nitrogen in the quinoxalines of **2** on the cytotoxic activity. Quinoxaline is a fused aromatic group composed of two six-membered rings. Similarly, to determine the effect of chromophore shape, analogues **8–17**, which have five-membered aromatic rings fused to benzene as the chromophores, **18** and **19**, which have extended chromophores, and **20**, where the phenyl rings of **2** were truncated, were designed. One of the characteristics of our synthetic strategy toward **2** and **3** is the installation of chromophores at the late stage of synthesis, which allows us to access a range of chromophore analogues efficiently. Analogues **4–20** were synthesized as shown in Fig. 3. Analogues **4–7** were synthesized by deprotection of the Cbz groups on the amines of the D-Ser residues of **21** using TFA in the presence of thioanisole followed by acylation of the resulting amines with the corresponding carboxylic acids using EDCI, HOAt, and Et₃N in DMF. As for the synthesis of **8–20**, the liberated diamine obtained from **21** was once protected with the Boc group to provide **22** in 39% yield over two steps. There are two advantages for the changing the protecting group from Cbz to Boc. The first is to reduce the time for the synthesis of multiple compounds, because the Boc group is more quickly removed by under an acidic conditions than the Cbz group. The second is to simplify the purification of the products. Specifically, the benzylmethylphenylsulfonium trifluoroacetate was generated as a byproduct during Cbz deprotection of **21** using TFA and thioanisole. The sulfonium salt is difficult to separate from the liberated amine, whereas the removal of

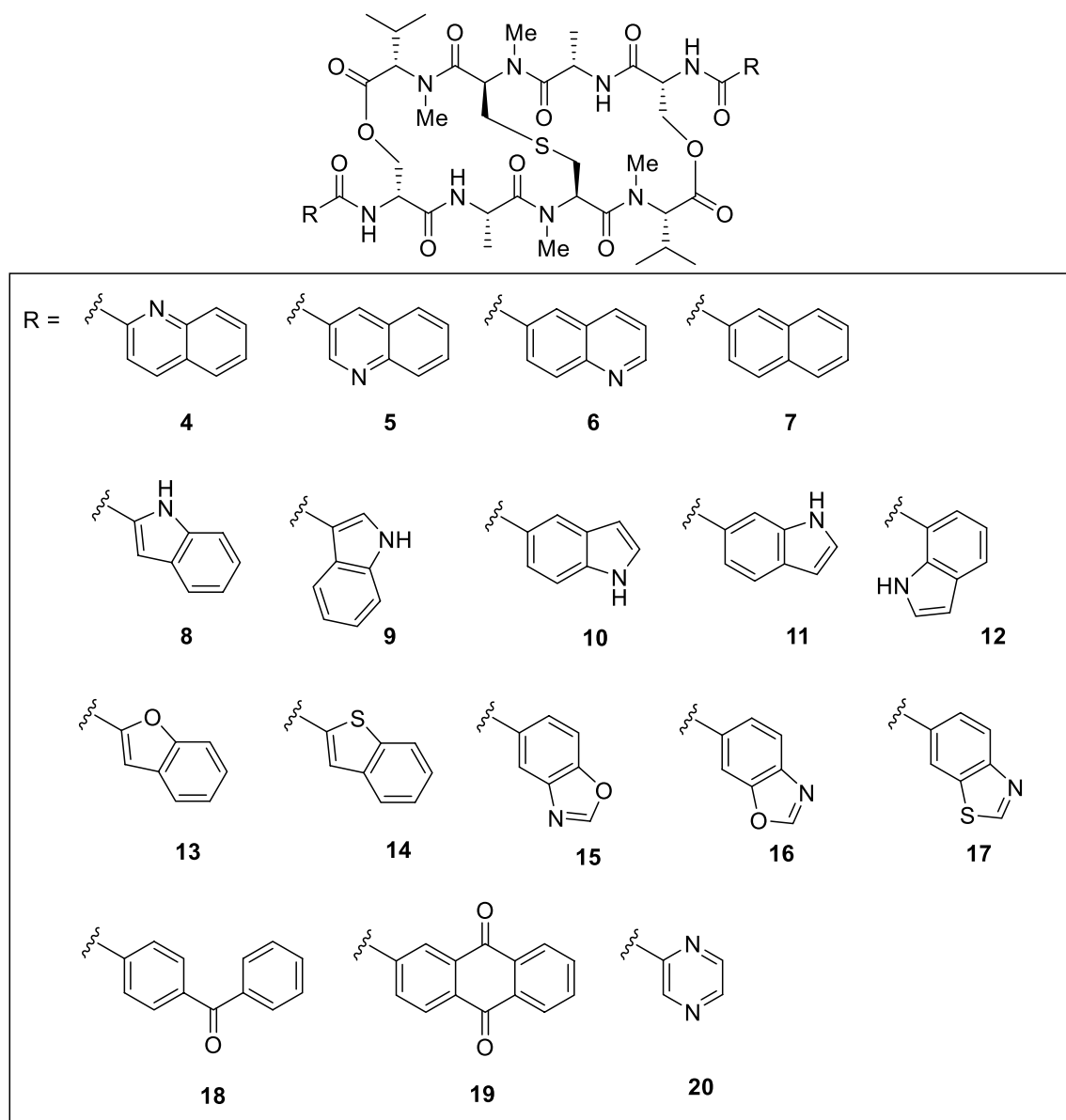


Figure 2. Chemical structures of echinomycin analogues.

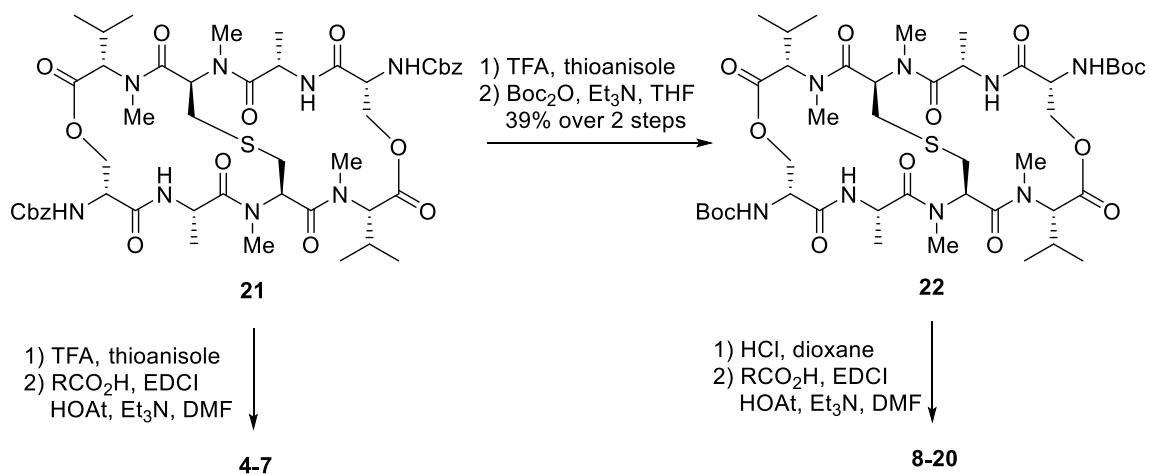


Figure 3. Synthesis of echinomycin analogues.

the Boc group does not generate such problematic byproducts. This change largely improved the synthetic access to a range of analogues. Deprotection of the Boc groups of **22** by HCl in dioxane followed by condensation with the corresponding carboxylic acids afforded **8–20**.

Toxicity of echinomycin analogues to human colon cancer cells

With a range of chromophore analogues in hand, their toxicity to SW620 cells was investigated by WST assays. Additionally, the 50% inhibitory concentration (IC₅₀) values were determined and compared to those of **2**, which has 2-quinoxaline chromophores, as a positive control for **3–20** (Table 1). To compare the toxicities of the new analogues to pancreatic cancer cells with the toxicity of **1** side by side, their activity against SW620 cells and MIA PaCa-2 cells is also shown in Table 1. Similar to a previous investigation with MIA PaCa-2 cells²⁰, **2** retained its potent toxicity to SW620 cells with an IC₅₀ value of 26 nM although a tenfold reduction in the activity was observed. Analogue **3**, which has 3-hydroxyquinoline chromophores, exhibited cytotoxicity equipotent to that of **1** (2.5 nM for **1** vs. 0.85 nM for **3**). Under these conditions, the 2-quinoline analogue **4** showed slightly better cytotoxicity than **2** (IC₅₀ 26 nM), indicating that the nitrogens at the 4-positions of the quinoxaline chromophores in **2** are not necessary for cytotoxicity. In contrast to **4**, analogues **5** and **6**, where the nitrogens at the 1-positions of the chromophores in **4** are transposed, displayed largely reduced activity, with IC₅₀ values of 1210 and 1193 nM, respectively. Naphthalene analogue **7**, which lacks all the nitrogens in **2**, showed completely diminished activity. Analogues **8–17** possess 5-membered heterocycles fused to the benzene ring. This structural alteration has a great impact on cytotoxicity, and all of these analogues completely lost their activity (IC₅₀ > 10,000 nM). Extended chromophore analogues **18** and **19** as well as truncated analogue **20** also lost their cytotoxicity.

Induction of apoptosis by **3**

Compound **3** exhibits very potent toxicity to SW620, as expected. Therefore, the expression of cleaved caspase-3 in SW620 cells was subsequently evaluated to clarify the effects of **3** on cancer cell apoptosis. Western blotting analysis of SW620 cells treated with **3** revealed that the expression of cleaved caspase-3 was significantly greater than that in untreated cells (Fig. 4). TUNEL staining was then performed. The number of apoptotic SW620 cells increased after treatment with **3** compared to the number of apoptotic control cells. These data indicated that **3** exhibited a tumor-suppressive effect by inducing colon cancer cell apoptosis. In both cases, **3** exhibited better activity than **1**.

Inhibition of the HIF-1 pathway

Echinomycin is known to inhibit the HIF-1 pathway by inhibiting the binding of HIF-1 α to HRE^{15,21,22}. The ability of **3** to inhibit HIF-1 α -mediated transcription was investigated by using an HRE reporter gene assay in SW620 cells transiently overexpressing HIF-1 α . As shown in Figs. 3 and 5 markedly decreased HIF-1 α -induced

Compounds	IC ₅₀ (nM) ^a	
	SW620 ^b	MIA PaCa-2 ^c
1	2.5 ± 0.8	0.75 ± 0.02
2	25.6 ± 6.0	8.1 ± 1.0
3	0.85 ± 0.13	1.4 ± 0.1
4	20.5 ± 3.1	5.8 ± 0.9
5	1210 ± 35	399 ± 76
6	1193 ± 187	327 ± 66
7	> 10,000	> 10,000
8	> 10,000	> 10,000
9	> 10,000	> 10,000
10	> 10,000	> 10,000
11	> 10,000	> 10,000
12	> 10,000	> 10,000
13	> 10,000	> 10,000
14	> 10,000	> 10,000
15	> 10,000	> 10,000
16	> 10,000	> 10,000
17	> 10,000	> 10,000
18	> 10,000	> 10,000
19	> 10,000	> 10,000
20	45% inhibition at 10 μ M	55% inhibition at 10 mM

Table 1. Cytotoxicity of **1** and its analogues. ^a50% inhibitory concentration. Each data is shown as mean \pm SE (n = 3). ^bHuman colon cancer cell. ^cHuman pancreatic carcinoma cell.

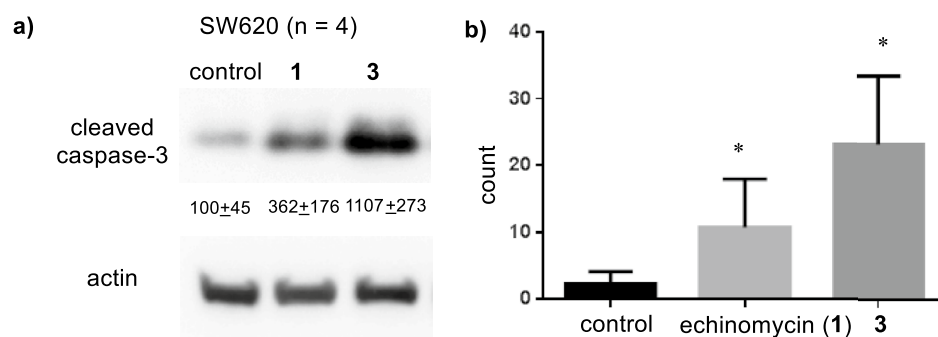


Figure 4. Apoptotic effect of echinomycin and **3** on SW620 cells evaluated by (a) Western blotting to measure cleaved caspase-3 and (b) TUNEL assay. *Indicates $P < 0.005$ by Student's t test. The error bars show the s.d. ($n = 4$).

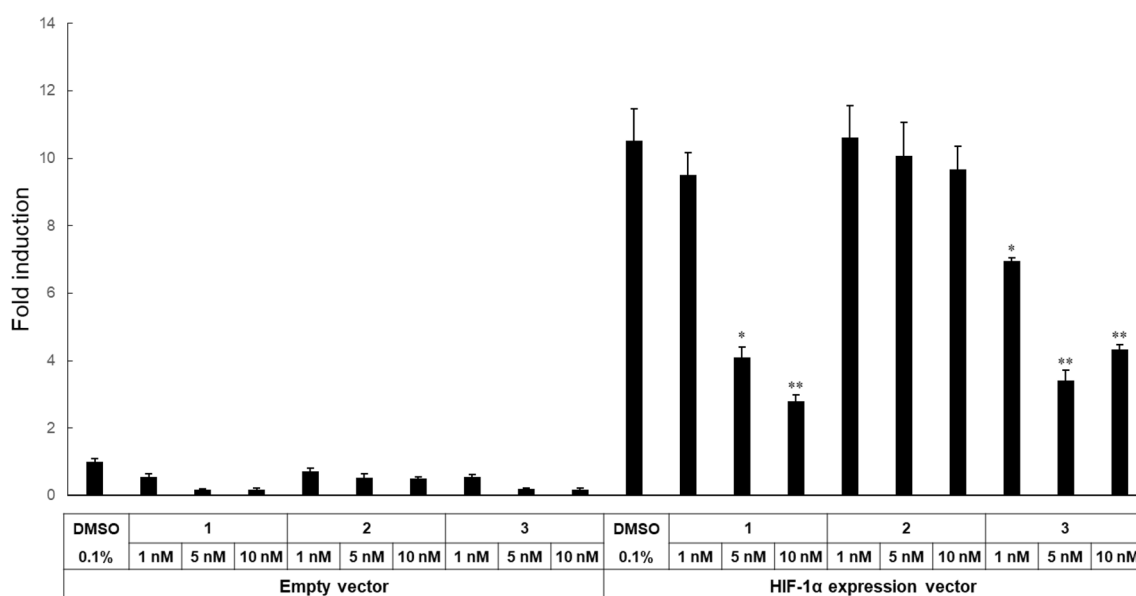


Figure 5. Effect of echinomycin (**1**), **2** and **3** on HIF-1 α -dependent transcription in SW620 cells. SW620 cells were transiently transfected with empty vector pCI-neo-3 \times FLAG or HIF-1 α expression vector pCI-neo-3 \times FLAG-HIF-1 α , together with pGL3-5 \times HRE-Luc and pGL4.75 [hRluc/CMV]. One day after transfection, echinomycin (**1**), **2**, or **3** was added to the cell culture media and incubated for an additional 16 h. The cells were lysed, and a dual luciferase assay was performed. Firefly luciferase activity was normalized to *Renilla* luciferase activity, and the data are presented as fold induction relative to the values obtained in cells DMSO-treated cells transfected with empty vector. The error bars represent standard deviations ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ compared to DMSO-treated cells transfected with HIF-1 α (Student's test).

HRE reporter activity to a similar extent as echinomycin, indicating that **3** also has an inhibitory effect on HIF-1 α -mediated transcription (Fig. 4).

Transcriptome analysis

Although modulation of the HIF-1 pathway has been reported to be one of the modes of action of **1**, it has also been suggested that **1** inhibits other signaling pathways including the Notch pathway¹⁹. Therefore, transcriptome analysis was then performed to gain insight into the mode of action of **3**. Namely, to determine the changes in mRNA expression in SW620 cells treated with **3**, a high-throughput sequencing analysis was conducted in a manner similar to our previous study²³.

Seven thousand and three hundred seventy mRNAs exhibited more than twofold changes in expression levels that were significant ($p < 0.05$) in comparison to their expression levels in control cells (Supplementary Table 1). Pathway analysis was performed using the software program MetaCore, and the results are shown in Table 2. These results indicated that the cell cycle and its regulation were involved in the changes in the expression of these mRNAs in the cells treated with **3**. Notably the HIF-1 pathway was not indicated by this analysis, suggesting that the HIF-1 pathway may not be the primary target of **3**.

No.	Maps
1	Cell cycle_Initiation of mitosis
2	DNA damage_ATM/ATR regulation of G2/M checkpoint
3	Cell cycle_Chromosome condensation in prometaphase
4	Cell cycle_The metaphase checkpoint
5	Cell cycle_Role of 14-3-3 proteins in cell cycle regulation
6	DNA damage_ATM/ATR regulation of G1/S checkpoint
7	Cell cycle_Role of APC in cell cycle regulation
8	Cell cycle_Cell cycle (generic schema)
9	Cell cycle_Start of DNA replication in early S phase
10	Development_Positive regulation of STK3/4 (Hippo) pathway and negative regulation of YAP/TAZ function
11	Development_Negative regulation of STK3/4 (Hippo) pathway and positive regulation of YAP/TAZ function
12	Cell cycle_Role of SCF complex in cell cycle regulation
13	Cell cycle_Spindle assembly and chromosome separation
14	Cell cycle_Nucleocytoplasmic transport of CDK/Cyclins
15	Cell cycle_Influence of Ras and Rho proteins on G1/S Transition
16	Apoptosis and survival_Role of PKR in stress-induced apoptosis
17	Cell cycle_Role of Nek in cell cycle regulation
18	Signal transduction_mTORC1 downstream signaling
19	Cell cycle_Regulation of G1/S transition (part 1)
20	Cell cycle_Sister chromatid cohesion

Table 2. The pathway analysis performed using the MetaCore software program. The cell cycle and its regulation pathway were markedly altered in the cells treated with **3**.

To identify potential target molecules of echinomycin, two types of probe compounds were designed (Fig. 6). The first one was biotinylated probe **27** with an ethylene glycol linker. Target identification using this type of probe is one of the most frequently used techniques. The other one was photochemical probe **29** with a diazirine photo cross-linker containing an alkyne handle, which was developed by Yao et al.²⁴ The diazirine moiety forms a covalent bond with the target molecule by the generation of a carbene upon UV irradiation, while the alkyne is used for subsequent immobilization on the beads via a click reaction. The most important feature of this linker is that it can minimize structural changes due to the introduction of the linker, and it has been reported to be used even in living cells. These compounds were synthesized from sulfoxide **23** in a manner similar to the total synthesis of **1**, via a scheme that was developed by our group²⁰. Namely, Pummerer rearrangement of **23** by AcCl in CH₂Cl₂ gave the corresponding chloride, which was sequentially treated with **24** as a nucleophile and ZnCl₂ as a promoter in THF at room temperature for 15 h to provide **25** in 26% yield over two steps from **23**.

After removal of the *t*-Bu group of **25** by *aq.* TFA, the resulting carboxylic acid was condensed with biotinylated amine **26** to afford the desired biotinylated probe **27**. Chemical probe **29** was synthesized using the minimalist linker **28**. Identification of the target molecule(s) of echinomycin using **27** and **29** is currently underway.

Metabolic stability of **3**

Before investigating the *in vivo* efficacy of **3**, its metabolic stability was evaluated by treatment with human or mouse liver microsomes at 37 °C for 30 min, and the remaining analogues were analyzed by LC-MS/MS (Table 3). The clearance value of **3** were 83.7 and 386 mL/min/kg for human and mouse liver microsomes, respectively, in the presence of NADPH. This means that approximately 67% of **3** was unaffected by human liver microsomes. Thus, it was revealed that these analogues were metabolically stable, especially in human liver microsomes. Furthermore, the metabolic clearance values in the absence of NADPH were 45.1 and 118 mL/min/kg in human and mouse liver microsomes, respectively, suggesting that in liver microsomes in both species, approximately half of the metabolic reactions are oxidative reactions and the other half are hydrolytic reactions involving the peptide and/or ester bonds in **3**.

Investigation of the maximum tolerated dose of **3**

Toxicity Body weight change is an indicator of systemic toxicity. Accordingly, the body weights of mice intraperitoneally treated with different doses (0.04 and 0.4 µg/mouse) of either **1** or **3** were measured (Fig. S1). After treatment with dosages of 0.04 and 0.4 µg/mouse, the body weights did not differ greatly from those of the control, indicating that **1** and **3** did not exhibit severe systemic toxicity at these dosages administered over 14 days. A blood hematological examination (including nineteen parameters) was also conducted on these mice on Day 14 (Fig. S2). There were no significant changes in almost any of the parameters in the mice treated with either **1** or **3** at doses of 0.04 or 0.4 µg/mouse. At a dose of 0.4 µg/mouse, a decrease in total bilirubin (T-BIL) and an increase in aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were observed in both groups treated with **1** and **3** (indicated by arrows). These results indicate that **1** and **3** do not

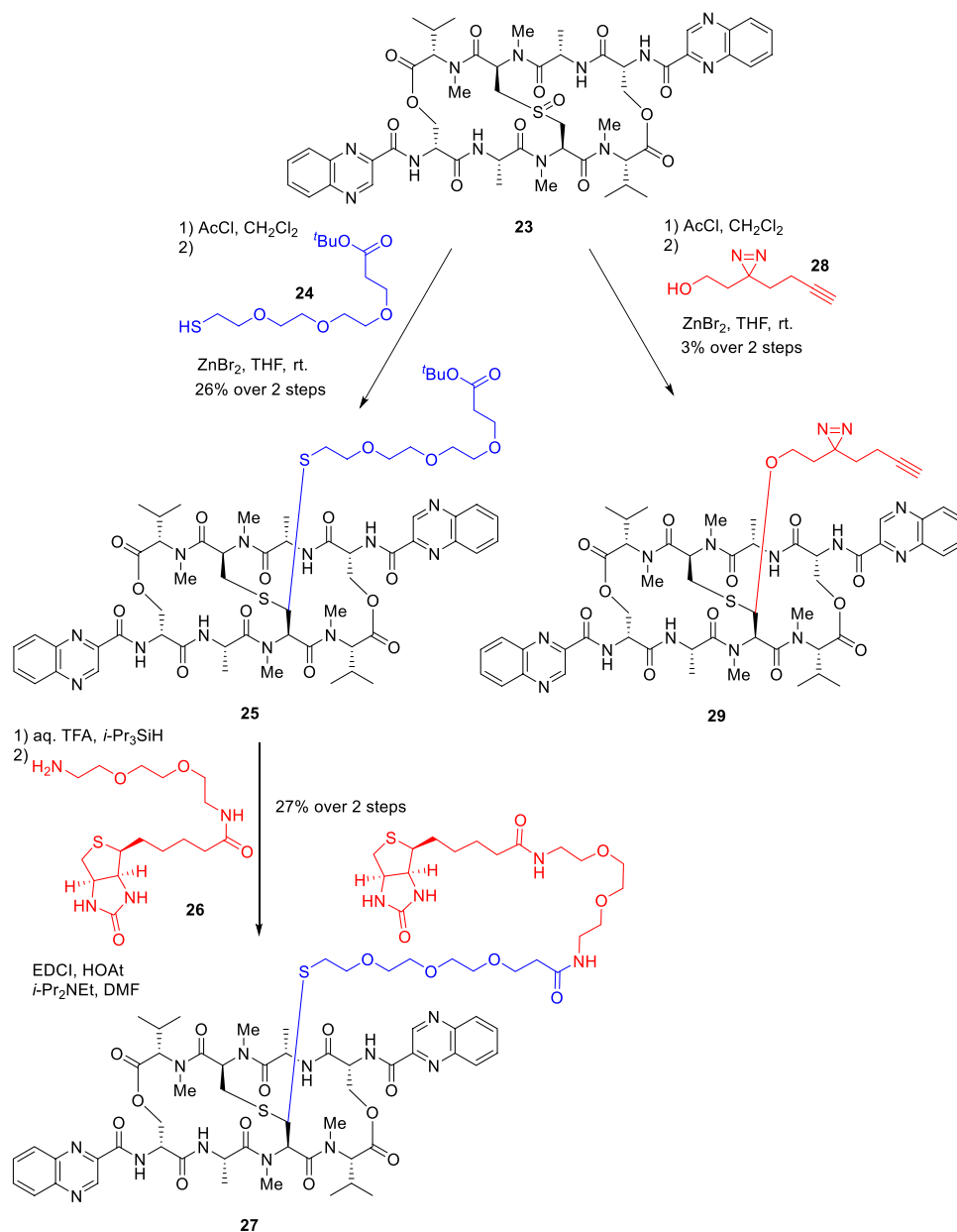


Figure 6. Synthesis of echinomycin probes.

	1		3		Midazolam	
	NADPH(+)	NADPH(-)	NADPH(+)	NADPH(-)	NADPH(+)	NADPH(-)
Human	<22.0	<22.0	83.7	45.1	435	<22.0
Mouse	<69.8	<69.8	386	118	4006	<69.8

Table 3. Metabolic stability of echinomycin (**1**) and **3** (mL/min/kg).

show significant toxicity at least up to a dose of 0.04 µg/mouse, which is expected to be the maximum tolerated dose of **3** for the subsequent *in vivo* evaluations.

Tumor growth suppression by **3** *in vivo*

Since the metabolic stability and identification of the maximum tolerated dose of **3** were confirmed, the effect of **3** on colon cancer progression *in vivo* was then investigated (Fig. 7). A xenograft model was generated by injecting a suspension of 2×10^6 SW620 cells into the backs of nude mice. The doses of the compounds were 0.04

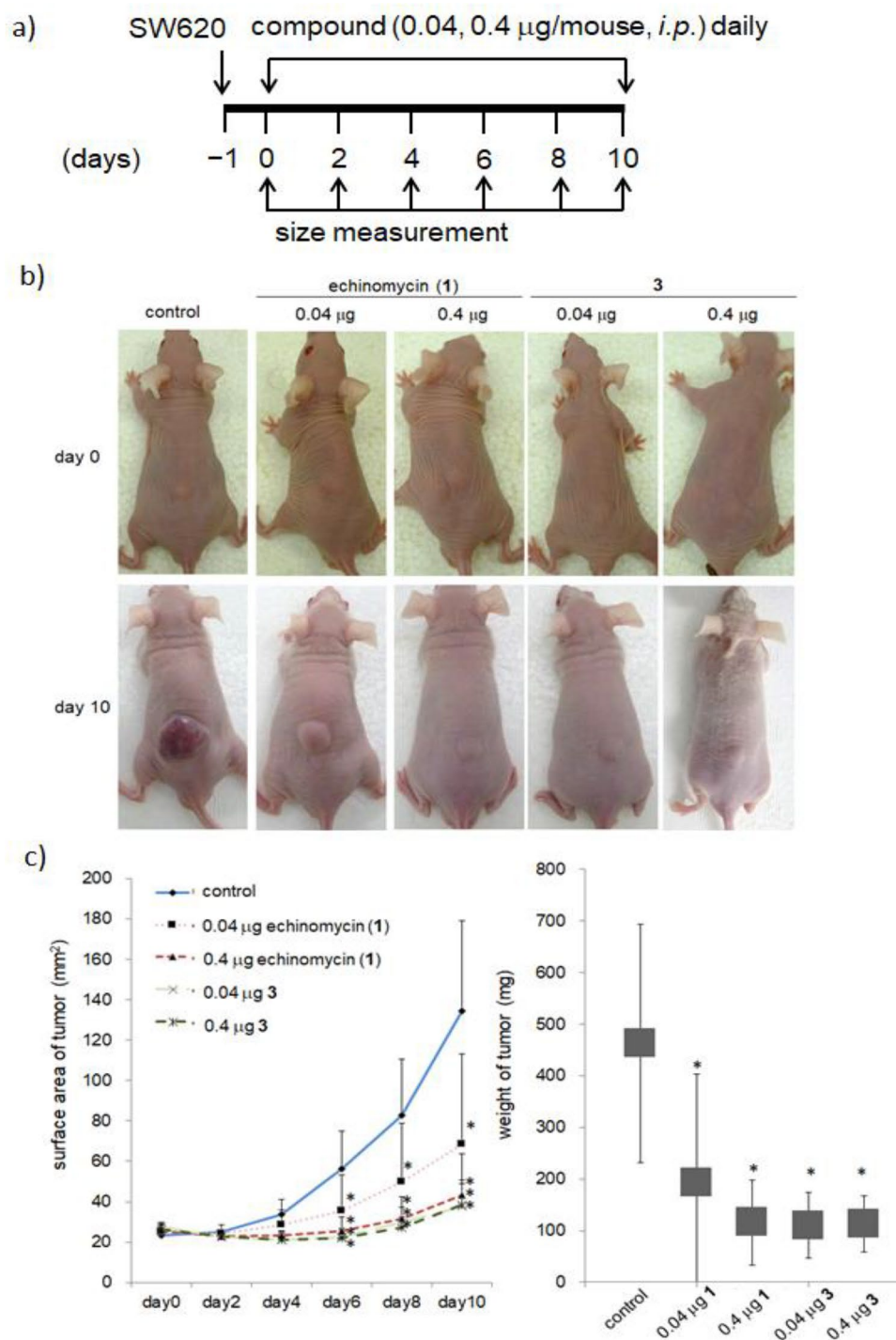


Figure 7. In vivo anticancer activity of echinomycin and 3 on the SW620 xenograft mice model. Male BALB/C nude mice were administered by intraperitoneal injection with the vehicle, echinomycin and 3, at dosages of 0.04 and 0.4 $\mu\text{g}/\text{mice}$ ($n = 10$ each group), every day for 10 days (a, b). Average tumor volume (c) was measured. Data are expressed as the mean \pm standard error ($n = 8$). *Indicates $P < 0.05$ by Student's t test. The error bars show the s.d. ($n = 10$).

and 0.4 $\mu\text{g}/\text{mouse}$, which correspond to approximately 1.6 and 16 $\mu\text{g}/\text{kg}$, respectively, according to the results obtained in Fig. S1. A solution of 1, 3, or PBS was injected intraperitoneally into each mouse, and tumor sizes were measured each day. Tumor growth in the groups treated with 1 and 3 was significantly suppressed compared to that in the PBS-injected group. Of note is the efficacy of tumor growth inhibition with 3. The efficacy of 3 at a dose of 0.04 $\mu\text{g}/\text{mouse}$ is equivalent to that of 1 at a dose of 0.4 $\mu\text{g}/\text{mouse}$, and it is revealed that 3 possesses superior tumor growth inhibition properties to 1.

Discussion

Echinomycin analogues were synthesized and biologically evaluated in this study. Evaluating the impact of other pendant chromophores was considered the first goal of this study, with which we aimed to significantly expand our current knowledge on the structure–activity relationship of **2**. Analogue **3** was designed inspired by the fact that congener natural products of **1**, such as SW-163C²⁵, thiocoraline²⁶, sandramycin²⁷, and quinaldopeptin²⁸, share 3-hydroxyquinoline-2-carbonyl groups as chromophores and display strong toxicity to a range of human cancer cell lines. An investigation of the cytotoxicity of these analogues to SW620 cells revealed the following structure–activity relationship for the chromophore. (1) The nitrogens at the 4-positions of the quinoxaline chromophores in **2** are not necessary. (2) The nitrogens at the 1-position contribute significantly to the cytotoxicity. (3) Naphthalene analogue **7**, which lacks all the nitrogens of **2**, displayed completely diminished activity. (4) The shape of the 6-membered heterocycles fused to the benzene ring is important for cytotoxicity. The SARs of echinomycin elucidated in this study in conjunction with those previously reported²⁰ are summarized in Fig. 8.

Analogue **3** as well as **1** inhibited HIF-1 α -mediated transcription (Fig. 4). It has also been suggested that **1** inhibits other signaling pathways including the Notch pathway¹⁹. Our transcriptome analysis indicated that treatment with **3** altered the expression of 7370 mRNAs in SW620 cells. Pathway analysis revealed that the cell cycle and its regulation were involved in these changes in mRNA expression in the cells treated with **3** (Table 2). Interestingly, the HIF-1 and NOTCH signaling pathways were not identified as the main pathways in this analysis. Further studies will be necessary to elucidate the detailed modes of action of **3** as well as **1** by using chemical probes **27** and **29**.

Analogue **3** showed lower clearance values with mouse and human liver microsomes. Although the observed difference in metabolic stability could arise from differences in the chemical stability of a thioacetal and thioether moieties or from conformational changes throughout the entire cyclic peptide caused by changes in the thioacetal linkage, more detailed studies are needed to determine which contribution is greater.

Echinomycin analogue **3** exhibited superior in vivo efficacy to echinomycin without significant toxicity in mouse xenograft model. The low dose of **3** needed in vivo efficacy is also noteworthy. The chemical structures of echinomycin and **3** are not very different and their in vitro cytotoxic activities are similar. However, the in vivo activity of **3** was better than that of echinomycin, even though the clearance of **3** was worse. One possible reason is the difference in the mechanism of **1** and **3**, which was suggested by our transcriptome analysis. Although the details are not apparent, the HIF-1 pathway likely influences the difference between the in vivo efficacy of **1** and **3**. Another reason for this difference is the difference in protein binding rates. Namely, in vivo bioactivity tends to be worse for protein binding rates that are higher at the same plasma concentration. On the other hand, the higher the protein binding rate is, the slower the drug is lost from the body and the longer it stays in the body, so its contribution to the effect of the drug is greater. It is thought that the difference in in vivo activity may be due to a difference in the balance between the two. A dose of 0.04 $\mu\text{g}/\text{mouse}$ corresponds to 1.6 $\mu\text{g}/\text{kg}$ for mice, and a calculated human equivalent dose of 0.13 $\mu\text{g}/\text{kg}$ (4.8 $\mu\text{g}/\text{m}^2$)²⁹. No obvious toxicity was observed at this dose in the mice judging from body weight loss and the blood hematological examination, while there was significant efficacy in the xenograft model. As previously noted, human clinical trials of **1** have suggested that echinomycin at doses ranging from 1200 to 2128 $\mu\text{g}/\text{m}^2$ given intravenously is toxic. However, there was no observed toxicity at the lower doses of 60 and 120 $\mu\text{g}/\text{m}^2$ ³⁰. Sulfide **3** retains the cytotoxic activity of **1** both in vitro and in vivo and is more accessible and scalable than **1**. Our data suggest that echinomycin is an attractive and potentially novel agent for the treatment of colon cancer in addition to pancreatic cancer and AML.

Experimental section

General experimental methods

Compounds **4–29** were prepared using methods previously reported by our group²⁰. Namely, all reactions except those carried out in the aqueous phase were performed under an argon atmosphere unless otherwise noted. Materials were purchased from commercial suppliers and used without further purification unless otherwise noted. Solvents were distilled according to the standard protocol. Isolated yields were calculated by weighing products. The weight of the starting materials and the products were not calibrated. Analytical thin layer chromatography

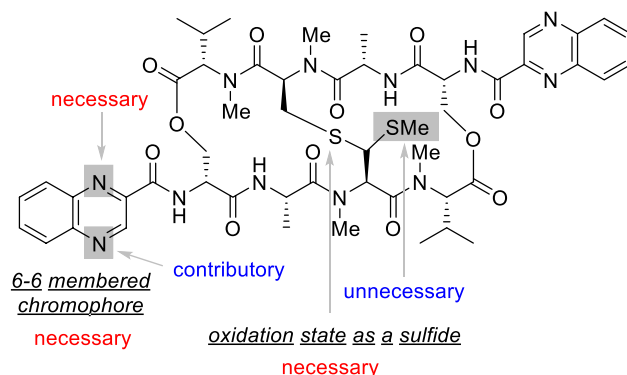


Figure 8. Summary of SAR of echinomycin.

(TLC) was performed on Merck silica gel 60F₂₅₄ plates. Normal-phase column chromatography was performed on Merck silica gel 5715 or Wakogel 60N. Flash column chromatography was performed on Kanto Chemical Silica Gel 60N (spherical, neutral, 40–50 μm). Hi-flash column chromatography was performed on YAMAZEN Hi-Flash™ column silica gel (40 μm) or Fuji Silysia Chromatorex MB/PSQ (50–200 μm). ¹H NMR spectra were measured in CDCl₃, DMSO-*d*₆ or CD₃OD solution and reported in parts per million (δ) relative to tetramethylsilane (0.00 ppm) as an internal standard or referenced to residual solvent peak of DMSO-*d*₆ (2.49 ppm) or CD₃OD (3.31 ppm) using JEOL ECS400 (400 MHz) and ECX400P (400 MHz) spectrophotometers, unless otherwise noted. ¹³C NMR spectra were measured in CDCl₃, DMSO-*d*₆ or CD₃OD solution and referenced to residual solvent peaks of CDCl₃ (77.0 ppm), DMSO-*d*₆ (39.52 ppm) or CD₃OD (49.00 ppm) using JEOL ECS400 (100 MHz) and ECX400P (100 MHz) spectrophotometers. The coupling constant (*J*) was reported in hertz (Hz). Abbreviations of multiplicity were as follows; s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, br: broad. Data were presented as follows; chemical shift (multiplicity, integration, coupling constant). The assignment was based on ¹H–¹H COSY, HMBC and HMQC NMR spectra. Mass spectra were obtained on Waters MICRO MASS LCT–premier or Advion expression CMS and the mass analyzer type used for the HRMS measurements was TOF. Optical rotation was measured on a Rudolph Research Analytical Autopol IV automatic polarimeter. Liquid chromatography-mass spectrometry (LC–MS) was performed on the systems as follows: Shimadzu Prominence-i LC-2030C Plus as an HPLC system; FCV-20AH2 as a flow-line selection valve; LCMS-8040 as a liquid chromatograph mass spectrometer, and LabSolutions as a system controller.

General procedure for the synthesis of 4–7

Compounds were prepared using methods previously reported by our group²⁰. Namely, compound **22** (25.3 mg, 25.0 μmol) in TFA (2.5 mL) was treated with thioanisole (117.2 μL, 1.00 mmol) at room temperature for 24 h. The reaction mixture was concentrated *in vacuo*, and then washed with Et₂O (5 mL × 3) to afford a pale yellow solid. The residue, ^tPr₂NEt (25.5 μL, 125 μmol), carboxylic acid (100 μmol) and HOAt (17.0 mg, 125 μmol) in DMF (1.0 mL) was treated with EDCI (24.0 mg, 100 μmol) at 0 °C, and the whole mixture was stirred at room temperature for 5 h. The reaction mixture was diluted with EtOAc (5 mL), and washed with H₂O (5 mL), 1 M *aq.* HCl (5 mL), *sat. aq.* NaHCO₃ (5 mL) and brine (5 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The residue was purified to afford 4–7.

{N-(Quinoline-2-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val}₂ sulfide (4)

Compound **4** (5.3 mg, 5.0 μmol, 20%) was synthesized using methods previously reported by our group²⁰, from quinaldic acid (100 μmol), and purified with flash silica gel column chromatography (Φ 0.7 × 5.2 cm, CHCl₃/MeOH: 100/0 → 99.5/0.5 → 99/1 → 98.5/1.5) as a white amorphous solid. ¹H NMR (CDCl₃, 400 MHz) δ 8.95 (d, 2H, Ser-NH, *J*_{Ser-NH, Ser-α-CH} = 7.2 Hz), 8.30 (d, 2H, Ar-H, *J* = 8.2 Hz), 8.24 (d, 2H, Ar-H, *J* = 8.2 Hz), 7.89–7.84 (m, 4H, Ar-H), 7.70 (ddd, 2H, Ar-H, *J* = 1.3, 6.9, 8.2 Hz), 7.58 (ddd, 2H, Ar-H, *J* = 1.1, 6.9, 8.0 Hz), 6.84 (d, 2H, Ala-NH, *J*_{Ala-NH, Ala-α-CH} = 7.4 Hz), 6.26 (dd, 2H, Cys-α-CH, *J*_{Cys-α-CH, Cys-β-CH} = *J*_{Cys-α-CH, Cys-β-CH} = 6.6 Hz), 5.13 (d, 2H, Val-α-CH, *J*_{Val-α-CH, Val-β-CH} = 10.0 Hz), 4.89–4.78 (m, 6H, Ala-α-CH, Ser-α-CH, Ser-β-CH), 4.67 (dd, 2H, Ser-β-CH, *J*_{Ser-β-CH, Ser-α-CH} = 1.4, *J*_{Ser-β-CH, Ser-β-CH} = 11.4 Hz), 3.38 (dd, 2H, Cys-β-CH, *J*_{Cys-β-CH, Cys-α-CH} = 6.6, *J*_{Cys-β-CH, Cys-β-CH} = 14.8 Hz), 3.24 (s, 6H, NCH), 2.95 (s, 6H, NCH), 2.52–2.45 (m, 2H, Cys-β-CH), 2.36 (qqd, 2H, Val-β-CH, *J*_{Val-β-CH, Val-γ-CH} = *J*_{Val-β-CH, Val-γ-CH} = 6.9, *J*_{Val-β-CH, Val-α-CH} = 10.0 Hz), 1.34 (d, 6H, Ala-β-CH, *J*_{Ala-β-CH, Ala-α-CH} = 6.9 Hz), 1.10 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.9 Hz), 0.92 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.9 Hz); ¹³C NMR (CDCl₃, 100 MHz) δ 173.2, 171.3, 170.3, 168.0, 165.6, 148.5, 146.6, 138.0, 130.5, 129.8, 129.5, 128.5, 128.1, 118.9, 64.2, 62.3, 55.0, 53.5, 46.3, 36.1, 31.7, 30.3, 28.1, 20.5, 19.4, 17.6; ESIMS-LR *m/z* 1075.2 [(M + Na)⁺]; ESIMS-HR calcd for C₅₂H₆₅N₁₀O₁₂S 1053.4499, found 1053.4515; [α]²²_D –172.8 (*c* 0.14, CHCl₃).

{N-(Quinoline-3-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val}₂ sulfide (5)

Compound **5** (4.6 mg, 4.4 μmol, 17%) was synthesized using methods previously reported by our group²⁰, from 3-quinolinecarboxylic acid (17.3 mg, 100 μmol), and purified with flash silica gel column chromatography (Φ 0.7 × 5.2 cm, CHCl₃/MeOH: 100/0 → 99/1 → 98.5/1.5 → 98/2 → 97.5/2.5 → 97/3) as a white amorphous solid. ¹H NMR (DMSO-*d*₆, 400 MHz) δ 9.26 (d, 2H, Ar-H, *J* = 2.2 Hz), 8.83 (d, 2H, Ar-H, *J* = 2.2 Hz), 8.80 (d, 2H, Ser-NH, *J*_{Ser-NH, Ser-α-CH} = 7.9 Hz), 8.14 (d, 2H, Ar-H, *J* = 7.6 Hz), 8.10 (d, 2H, Ar-H, *J* = 8.8 Hz), 7.91–7.86 (m, 4H, Ala-NH, Ar-H), 7.72 (td, 2H, Ar-H, *J* = 1.4, 7.7 Hz), 6.29 (dd, 2H, Cys-α-CH, *J*_{Cys-α-CH, Cys-β-CH} = *J*_{Cys-α-CH, Cys-β-CH} = 6.6 Hz), 4.84 (d, 2H, Val-α-CH, *J*_{Val-α-CH, Val-β-CH} = 10.8 Hz), 4.71 (ddd, 2H, Ser-α-CH, *J*_{Ser-α-CH, Ser-β-CH} = 2.9, *J*_{Ser-α-CH, Ser-β-CH} = 7.7, *J*_{Ser-α-CH, Ser-NH} = 7.9 Hz), 4.58 (dd, 2H, Ala-α-CH, *J*_{Ala-α-CH, Ala-β-CH} = *J*_{Ala-α-CH, Ala-NH} = 6.6 Hz), 4.50–4.45 (m, 2H, Ser-β-CH), 4.37 (dd, 2H, Ser-β-CH, *J*_{Ser-β-CH, Ser-α-CH} = 2.9, *J*_{Ser-β-CH, Ser-β-CH} = 13.0 Hz), 3.17–3.07 (m, 2H, Cys-β-CH), 3.03 (s, 6H, NCH), 2.81 (s, 6H, NCH), 2.53–2.49 (m, 2H, Cys-β-CH), 2.23 (qqd, 2H, Val-β-CH, *J*_{Val-β-CH, Val-γ-CH} = 6.4, *J*_{Val-β-CH, Val-α-CH} = 6.8, *J*_{Val-β-CH, Val-α-CH} = 10.8 Hz), 1.27 (d, 6H, Ala-β-CH, *J*_{Ala-β-CH, Ala-α-CH} = 6.6 Hz), 1.00 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.4 Hz), 0.76 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.8 Hz); ¹³C NMR (DMSO-*d*₆, 100 MHz) δ 172.3, 170.3, 169.8, 167.7, 165.3, 149.0, 148.5, 136.1, 131.4, 129.2, 128.8, 127.5, 126.7, 126.4, 64.7, 61.5, 54.1, 53.1, 46.1, 35.2, 30.5, 29.7, 26.5, 20.2, 18.8, 16.6; ESIMS-LR *m/z* 1075.4 [(M + Na)⁺]; ESIMS-HR calcd for C₅₂H₆₅N₁₀O₁₂S 1053.4499, found 1053.4506; [α]²³_D –175.3 (*c* 0.20, DMSO).

{N-(Quinoline-6-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val}₂ sulfide (6)

Compound **6** (5.0 mg, 4.8 μmol, 19%) was synthesized using methods previously reported by our group²⁰, from 6-quinolinecarboxylic acid (17.3 mg, 100 μmol), and purified with flash silica gel column chromatography (Φ 0.7 × 6.3 cm, CHCl₃/MeOH: 100/0 → 99/1 → 98.5/1.5 → 98/1 → 97.5/2.5 → 97/3 → 95/5) as a white amorphous solid. ¹H NMR (DMSO-*d*₆, 400 MHz) δ 9.00 (dd, 2H, Ar-H, *J* = 1.8, 4.2 Hz),

8.70 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 7.8$ Hz), 8.53–8.51 (m, 4H, Ar-H), 8.17–8.11 (m, 4H, Ar-H), 7.85 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 5.2$ Hz), 7.63 (dd, 2H, Ar-H, $J = 4.2, 8.6$ Hz), 6.28 (dd, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.8$ Hz), 4.86 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 10.4$ Hz), 4.71 (ddd, 2H, Ser- α -CH, $J_{\text{Ser-}\alpha\text{-CH, Ser-}\beta\text{-CH}} = 3.0$, $J_{\text{Ser-}\alpha\text{-CH, Ser-NH}} = 7.8$, $J_{\text{Ser-}\alpha\text{-CH, Ser-}\beta\text{-CH}} = 8.0$ Hz), 4.59–4.47 (m, 4H, Ala- α -CH, Ser- β -CH), 4.33 (dd, 2H, Ser- β -CH, $J_{\text{Ser-}\beta\text{-CH, Ser-}\alpha\text{-CH}} = 3.0$, $J_{\text{Ser-}\beta\text{-CH, Ser-}\beta\text{-CH}} = 11.4$ Hz), 3.09 (dd, 2H, Cys- β -CH, $J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 6.8$, $J_{\text{Cys-}\beta\text{-CH, Cys-}\beta\text{-CH}} = 14.8$ Hz), 3.01 (s, 6H, NCH), 2.81 (s, 6H, NCH), 2.54–2.49 (m, 2H, Cys- β -CH), 2.22 (qqd, 2H, Val- β -CH, $J_{\text{Val-}\beta\text{-CH, Val-}\gamma\text{-CH}} = 6.4$, $J_{\text{Val-}\beta\text{-CH, Val-}\gamma\text{-CH}} = 7.2$, $J_{\text{Val-}\beta\text{-CH, Val-}\alpha\text{-CH}} = 10.4$ Hz), 1.27 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 7.2$ Hz), 0.99 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz), 0.76 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 7.2$ Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 172.3, 170.2, 169.7, 167.8, 166.0, 152.3, 148.8, 137.1, 131.6, 129.1, 128.4, 127.8, 127.0, 122.3, 64.8, 61.4, 54.1, 53.2, 46.0, 35.3, 30.4, 29.8, 26.5, 20.2, 18.6, 16.6; ESIMS-LR m/z 1075.4 [(M + Na) $^+$]; ESIMS-HR calcd for $\text{C}_{52}\text{H}_{65}\text{N}_{10}\text{O}_{12}\text{S}$ 1053.4499, found 1053.4536; $[\alpha]_{\text{D}}^{23} -208.5$ (c 0.20, DMSO).

{N-(Naphthalene-2-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (7)

Compound 7 (8.8 mg, 8.4 μmol , 33%) was synthesized using methods previously reported by our group²⁰, from 2-naphthoic acid (17.2 mg, 100 μmol) with flash silica gel column chromatography (Φ 0.7 \times 8.4 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 99/1 \rightarrow 98.5/1.5) as a white amorphous solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.60 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 7.8$ Hz), 8.44 (s, 2H, Ar-H), 8.07–7.98 (m, 6H, Ar-H), 7.91 (dd, 2H, Ar-H, $J = 1.6, 8.6$ Hz), 7.82 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 5.6$ Hz), 7.65–7.59 (m, 4H, Ar-H), 6.29 (dd, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.6$, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.8$ Hz), 4.86 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 10.4$ Hz), 4.71 (ddd, 2H, Ser- α -CH, $J_{\text{Ser-}\alpha\text{-CH, Ser-}\beta\text{-CH}} = 2.8$, $J_{\text{Ser-}\alpha\text{-CH, Ser-NH}} = 7.8$, $J_{\text{Ser-}\alpha\text{-CH, Ser-}\beta\text{-CH}} = 8.0$ Hz), 4.57 (dd, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = J_{\text{Ala-}\alpha\text{-CH, Ala-NH}} = 6.4$ Hz), 4.52–4.47 (m, 2H, Ser- β -CH), 4.32 (dd, 2H, Ser- β -CH, $J_{\text{Ser-}\beta\text{-CH, Ser-}\alpha\text{-CH}} = 2.8$, $J_{\text{Ser-}\beta\text{-CH, Ser-}\beta\text{-CH}} = 11.0$ Hz), 3.10 (dd, 2H, Cys- β -CH, $J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 6.6$, $J_{\text{Cys-}\beta\text{-CH, Cys-}\beta\text{-CH}} = 14.8$ Hz), 3.01 (s, 6H, NCH), 2.81 (s, 6H, NCH), 2.53–2.49 (m, 2H, Cys- β -CH), 2.22 (qqd, 2H, Val- β -CH, $J_{\text{Val-}\beta\text{-CH, Val-}\gamma\text{-CH}} = 6.4$, $J_{\text{Val-}\beta\text{-CH, Val-}\gamma\text{-CH}} = 6.8$, $J_{\text{Val-}\beta\text{-CH, Val-}\alpha\text{-CH}} = 10.4$ Hz), 1.27 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 7.2$ Hz), 0.99 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.8$ Hz), 0.76 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 173.2, 173.1, 169.7, 168.3, 167.9, 135.2, 132.7, 129.3, 129.0, 128.5, 128.4, 128.3, 127.9, 127.1, 123.3, 65.7, 61.9, 55.1, 54.9, 46.6, 36.5, 30.7, 30.3, 27.0, 20.5, 18.3, 17.6; ESIMS-LR m/z 1073.2 [(M + Na) $^+$]; ESIMS-HR calcd for $\text{C}_{54}\text{H}_{67}\text{N}_8\text{O}_{12}\text{S}$ 1051.4594, found 1051.4565; $[\alpha]_{\text{D}}^{24} -235.0$ (c 0.20, CHCl_3).

(N-Boc-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val) $_2$ sulfide (22)

Compounds were prepared using methods previously reported by our group²⁰. Namely, compound 21 (20.0 mg, 19.8 μmol) was treated with thioanisole (185 μL , 1.58 mmol) and TFA (3.9 mL) at room temperature, and the whole mixture was stirred at 40 $^\circ\text{C}$ for 15 h. The resulting mixture was concentrated *in vacuo*, and washed with Et_2O (15 mL \times 3) to afford a pale yellow solid. A solution of the residue and Boc_2O (18.2 μL , 79.2 μmol) in THF (0.4 mL) was treated with Et_3N (21.9 μL , 158 μmol) at room temperature, and the whole mixture was stirred at 40 $^\circ\text{C}$ for 5 h. The resulting mixture was diluted with AcOEt (15 mL), and the solution was washed with 1 M *aq.* HCl (5 mL \times 2), brine (5 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (Φ 1 \times 10 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 99/1 \rightarrow 98/2 \rightarrow 97/3) to afford 22 (7.3 mg, 7.7 μmol , 39% over 2 steps) as a colorless solid. ^1H NMR (CDCl_3 , 400 MHz) δ 6.81 (s, 2H, Ala-NH), 6.22 (br s, 2H, Cys- α -CH), 5.28 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 6.4$ Hz), 5.05 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 10.6$ Hz), 4.81 (br s, 2H, Ala- α -CH), 4.65 (dd, 2H, Ser- β -CH, $J_{\text{gem}} = 11.9$, $J_{\text{Ser-}\beta\text{-CH, Ser-}\alpha\text{-CH}} = 4.1$ Hz), 4.53 (d, 2H, Ser- β -CH, $J_{\text{gem}} = 11.9$ Hz), 4.38 (br s, 2H, Ser- α -CH), 3.30 (m, 2H, Cys- β -CH), 3.07 (s, 6H, N-CH), 2.92 (s, 6H, N-CH) 2.48 (br s, 2H, Cys- β -CH), 2.23 (m, 2H, Val- β -CH), 1.47 (s, 18H, Boc), 1.37 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 7.3$ Hz), 1.080 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz), 0.82 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.9$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 173.2, 171.6, 169.9, 168.4, 155.6, 81.5, 64.3, 62.1, 55.0, 54.8, 46.1, 36.2, 31.4, 30.3, 28.3, 27.5, 20.4, 18.9, 17.7; ESIMS-LR m/z 943.87 [(M + H) $^+$]; ESIMS-HR calcd for $\text{C}_{42}\text{H}_{71}\text{N}_8\text{O}_{14}\text{S}$ 943.4805; found 943.4833; $[\alpha]_{\text{D}}^{17} -187.33$ (c 0.73, CHCl_3).

General procedure for the synthesis of 8–20

Compounds were prepared using methods previously reported by our group²⁰. Namely, compound 22 (100 mg, 106 μmol) was treated with 4 M HCl/1,4-dioxane (8 mL) at room temperature for 1 h. The resulting mixture was concentrated *in vacuo*, and the residue was washed with Et_2O (15 mL \times 3) to afford a white solid. The residue was divided into 5 portions. A suspension of each portion, carboxylic acid (63.6 μmol), HOAt (8.7 mg, 63.6 μmol) and Et_3N (20.6 μL , 148 μmol) in DMF (0.3 mL) was treated with EDCI (12.2 mg, 63.6 μmol) at 0 $^\circ\text{C}$, and the whole mixture was stirred at room temperature for several hours. The resulting mixture was diluted with AcOEt , and the solution was washed with H_2O , 1 M *aq.* HCl, *sat. aq.* NaHCO_3 and, brine, dried over Na_2SO_4 , filtered, and concentrated *in vacuo*. The residue was purified to afford 8–20.

{N-(Indol-2-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (8)

Compound 8 (6.5 mg, 6.3 μmol , 30% over 2 steps) was synthesized using methods previously reported by our group²⁰, from indole-2-carboxylic acid (10.2 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 2 \times 6 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 94/6) as a white solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 11.7 (d, 2H, Ar-NH), 8.40 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 7.3$ Hz), 7.84 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 4.1$ Hz), 7.65 (d, 2H, Ar, $J = 7.8$ Hz), 7.44 (d, 2H, Ar, $J = 7.8$ Hz), 7.21–7.04 (m, 6H, Ar), 6.28 (t, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.0$ Hz), 4.83 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 8.7$ Hz), 4.65 (m, 2H, Ser- α -CH), 4.55 (qd, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-NH}} = 5.0$, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = 4.1$ Hz), 4.45 (m, 2H, Ser- β -CH),

4.31 (d, 2H, Ser- β -CH, $J_{\text{gem}} = 11.0$ Hz), 3.08 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 14.6$, $J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 5.5$ Hz), 3.03 (s, 6H, N -CH), 2.80 (s, 6H, N -CH), 2.50–2.45 (overlap, 2H, Cys- β -CH), 2.23 (m, 2H, Val- β -CH), 1.27 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 5.0$ Hz), 0.99 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 4.6$ Hz), 0.76 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 5.0$ Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 172.3, 170.3, 169.8, 167.8, 161.2, 136.6, 130.8, 126.9, 123.8, 121.7, 119.9, 112.4, 103.7, 64.8, 61.5, 54.2, 52.7, 46.0, 30.5, 29.8, 29.0, 26.6, 20.3, 18.8, 16.6; ESIMS-LR m/z 1029.53 [(M + H) $^+$]; ESIMS-HR calcd for $\text{C}_{50}\text{H}_{65}\text{N}_{10}\text{O}_{12}\text{S}$ 1029.4499, found 1029.4497; $[\alpha]_{\text{D}}^{22} - 177.67$ (c 1.08, DMSO).

{N-(Indol-3-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (9)

Compound **9** (4.2 mg, 4.1 μmol , 19% over 3 steps) was synthesized using methods previously reported by our group²⁰, from N -Boc-indole-3-carboxylic acid (16.6 mg, 63.6 μmol). After amide coupling, the residue was treated with 50% TFA/ CH_2Cl_2 (1 mL) at room temperature, and the whole mixture was stirred at 40 $^\circ\text{C}$ for 5 h. The resulting mixture was concentrated *in vacuo*, and the residue was purified by high flash silica gel column chromatography (Φ 2 \times 6 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 94/6) to afford compound **9** as a white solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 11.7 (d, 2H, Ar-NH), 8.06 (d, 2H, Ar, $J = 8.2$ Hz), 8.05 (s, 2H, Ar- C^2H), 7.82 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 7.3$ Hz), 7.78 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 5.0$ Hz), 7.18–7.11 (m, 4H, Ar), 6.28 (dd, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.9$, 5.9 Hz), 4.81 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 10.5$ Hz), 4.69 (m, 2H, Ser- α -CH), 4.57 (qd, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = 6.0$, $J_{\text{Ala-}\alpha\text{-CH, Ala-NH}} = 5.0$ Hz), 4.43 (m, 2H, Ser- β -CH), 4.30 (d, 2H, Ser- β -CH, $J_{\text{gem}} = 10.5$ Hz), 3.08 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 14.6$, $J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 5.9$ Hz), 3.02 (s, 6H, N -CH), 2.80 (s, 6H, N -CH), 2.50–2.45 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 14.6$, $J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 6.9$ Hz), 2.21 (m, 2H, Val- β -CH), 1.26 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 6.4$ Hz), 0.99 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 5.5$ Hz), 0.76 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.0$ Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 172.3, 170.3, 169.9, 168.5, 164.5, 136.2, 129.0, 125.8, 122.1, 120.6, 112.1, 109.8, 65.1, 61.6, 54.2, 52.4, 46.0, 35.3, 30.5, 29.7, 26.6, 20.2, 18.9, 16.7; ESIMS-LR m/z 1029.88 [(M + H) $^+$]; ESIMS-HR calcd for $\text{C}_{50}\text{H}_{65}\text{N}_{10}\text{O}_{12}\text{S}_1$ 1029.4499, found 1029.4497; $[\alpha]_{\text{D}}^{23} - 113.26$ (c 0.19, DMSO).

{N-(Indol-5-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (10)

Compound **10** (12.4 mg, 12.1 μmol , 57% over 2 steps) was synthesized using methods previously reported by our group²⁰, from indole-5-carboxylic acid (10.2 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 2 \times 6 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 94/6) as a white solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 11.4 (d, 2H, Ar-NH), 8.29 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 7.3$ Hz), 8.12 (s, 2H, Ar), 7.79 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 5.0$ Hz), 7.61 (d, 2H, Ar, $J = 8.7$ Hz), 7.46 (d, 2H, Ar, $J = 8.7$ Hz), 7.45 (s, 2H, Ar), 6.56 (s, 2H, Ar), 6.27 (dd, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.6$, 6.2 Hz), 4.83 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 10.5$ Hz), 4.66 (m, 2H, Ser- α -CH), 4.56 (qd, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = 6.9$, $J_{\text{Ala-}\alpha\text{-CH, Ala-NH}} = 5.0$ Hz), 4.44 (m, 2H, Ser- β -CH), 4.28 (d, 2H, Ser- β -CH, $J_{\text{gem}} = 10.6$ Hz), 3.09 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 15.1$, $J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 6.2$ Hz), 3.01 (s, 6H, N -CH), 2.80 (s, 6H, N -CH), 2.50 (overlap, 2H, Cys- β -CH), 2.24–2.18 (m, 2H, Val- β -CH), 1.26 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 6.9$ Hz), 0.98 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz), 0.76 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 172.3, 170.3, 169.8, 168.3, 167.5, 136.7, 127.0, 124.6, 120.7, 120.3, 111.1, 102.2, 65.0, 61.5, 54.2, 53.1, 45.9, 35.3, 30.5, 29.8, 26.6, 20.2, 18.8, 16.7; ESIMS-LR m/z 1051.95 [(M + Na) $^+$]; ESIMS-HR calcd for $\text{C}_{50}\text{H}_{65}\text{N}_{10}\text{O}_{12}\text{S}$ 1029.4499, found 1029.4497; $[\alpha]_{\text{D}}^{24} - 234.83$ (c 0.81, DMSO).

{N-(Indol-6-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (11)

Compound **11** (6.1 mg, 5.9 μmol , 28% over 2 steps) was synthesized using methods previously reported by our group²⁰, from indole-6-carboxylic acid (10.2 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 2 \times 6 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 94/6) as a white solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 11.5 (d, 2H, Ar-NH), 8.30 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 7.4$ Hz), 7.92 (s, 2H, Ar), 7.78 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 5.5$ Hz), 7.62 (d, 2H, Ar, $J = 8.5$ Hz), 7.54 (s, 2H, Ar), 7.49 (d, 2H, $J = 8.5$ Hz), 6.50 (s, 2H, Ar), 6.28 (t, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.4$ Hz), 4.83 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 10.1$ Hz), 4.66 (m, 2H, Ser- α -CH), 4.57 (qd, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = 7.3$, $J_{\text{Ala-}\alpha\text{-CH, Ala-NH}} = 5.5$ Hz), 4.45 (m, 2H, Ser- β -CH), 4.28 (d, 2H, Ser- β -CH, $J_{\text{gem}} = 10.1$ Hz), 3.08 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 15.1$, $J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 6.4$ Hz), 3.01 (s, 6H, N -CH), 2.79 (s, 6H, N -CH), 2.50 (overlap, 2H, Cys- β -CH), 2.20 (m, 2H, Val- β -CH), 1.25 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 7.3$ Hz), 0.98 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.0$ Hz), 0.75 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 172.3, 170.3, 169.8, 168.2, 167.4, 135.1, 130.2, 128.4, 126.3, 119.6, 118.2, 111.6, 101.4, 64.9, 61.5, 54.2, 53.2, 45.9, 35.4, 30.5, 29.8, 26.6, 20.3, 18.8, 16.7; ESIMS-LR m/z 1051.91 [(M + Na) $^+$]; ESIMS-HR calcd for $\text{C}_{50}\text{H}_{65}\text{N}_{10}\text{O}_{12}\text{S}$ 1029.4499, found 1029.4497; $[\alpha]_{\text{D}}^{24} - 216.52$ (c 0.26, DMSO).

{N-(Indol-7-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (12)

Compound **12** (6.1 mg, 5.9 μmol , 28% over 2 steps) was synthesized using methods previously reported by our group²⁰, from indole-7-carboxylic acid (10.2 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 2 \times 6 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 94/6) as a white solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 11.2 (d, 2H, Ar-NH), 8.45 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 7.3$ Hz), 7.84 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 5.9$ Hz), 7.78 (d, 2H, Ar, $J = 8.2$ Hz), 7.62 (d, 2H, Ar, $J = 7.3$ Hz), 7.36 (t, 2H, $J = 2.7$ Hz), 7.12 (t, 2H, $J = 7.6$ Hz), 6.51 (dd, 2H, Ar, $J = 3.2$, 2.3 Hz), 6.27 (dd, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.9$, 6.4 Hz), 4.84 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 10.5$ Hz), 4.72 (ddd, 2H, Ser- α -CH, $J_{\text{Ser-}\alpha\text{-CH, Ser-}\beta\text{-CH}} = 8.2$, $J_{\text{Ser-}\alpha\text{-CH, Ser-NH}} = 7.3$, $J_{\text{Ser-}\alpha\text{-CH, Ser-}\beta\text{-CH}} = 2.6$ Hz), 4.56 (qd, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = 6.8$, $J_{\text{Ala-}\alpha\text{-CH, Ala-NH}} = 5.9$ Hz), 4.47 (dd, 2H, Ser- β -CH, $J_{\text{gem}} = 11.2$, $J_{\text{Ser-}\beta\text{-CH, Ser-}\alpha\text{-CH}} = 8.2$ Hz), 4.34 (dd, 2H, Ser- β -CH, $J_{\text{gem}} = 11.2$, $J_{\text{Ser-}\beta\text{-CH, Ser-}\alpha\text{-CH}} = 2.6$ Hz), 3.09 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 14.6$, $J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 6.4$ Hz), 3.01 (s, 6H, N -CH), 2.79 (s, 6H, N -CH), 2.50 (overlap,

2H, Cys- β -CH), 2.21 (m, 2H, Val- β -CH), 1.25 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 6.8$ Hz), 0.98 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz), 0.75 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.9$ Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 172.3, 170.4, 169.8, 168.1, 167.0, 134.1, 129.3, 127.0, 124.4, 120.4, 118.1, 116.2, 101.2, 64.9, 61.5, 54.2, 52.9, 46.0, 35.4, 30.5, 29.8, 26.6, 20.3, 18.7, 16.7; ESIMS-LR m/z 1051.71 [(M + Na) $^+$]; ESIMS-HR calcd for $\text{C}_{50}\text{H}_{65}\text{N}_{10}\text{O}_{12}\text{S}$ 1029.4499, found 1029.4497; $[\alpha]_D^{25} - 207.55$ (c 0.61, DMSO).

{N-(Benzofurane-2-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (13)

Compound 13 (18.5 mg, 18.0 μmol , 85% over 2 steps) was synthesized using methods previously reported by our group²⁰, from benzofuran-2-carboxylic acid (10.3 mg, 63.6 μmol), and purified with silica gel column chromatography (Φ 1 \times 10 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 99/1 \rightarrow 98/2 \rightarrow 97/3) as a white solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.38 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 8.7$ Hz), 7.98 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 6.0$ Hz), 7.80 (d, 2H, Ar, $J = 8.2$ Hz), 7.67 (d, 2H, Ar, $J = 8.2$ Hz), 7.64 (s, 2H, Ar), 7.49 (m, 2H, Ar), 7.35 (m, 2H, Ar), 6.28 (dd, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.9, 6.4$ Hz), 4.84 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 10.5$ Hz), 4.69 (m, 2H, Ser- α -CH), 4.57 (qd, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = 6.9, J_{\text{Ala-}\alpha\text{-CH, Ala-NH}} = 6.0$ Hz), 4.46 (dd, 2H, Ser- β -CH, $J_{\text{gem}} = 11.4, J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 7.8$ Hz), 4.31 (d, 2H, Ser- β -CH, $J_{\text{gem}} = 11.4$ Hz), 3.09 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 15.1, J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 6.4$ Hz), 3.03 (s, 6H, N-CH), 2.80 (s, 6H, N-CH), 2.46 (overlap, 2H, Cys- β -CH), 2.20 (m, 2H, Val- β -CH), 1.28 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 6.9$ Hz), 0.98 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.9$ Hz), 0.77 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.9$ Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 172.3, 170.3, 169.8, 168.1, 167.0, 134.1, 129.3, 126.9, 124.4, 120.4, 118.1, 116.2, 101.2, 64.9, 61.5, 54.2, 52.9, 46.0, 35.4, 30.5, 29.8, 26.6, 20.2, 18.7, 16.7; ESIMS-LR m/z 1053.41 [(M + Na) $^+$]; ESIMS-HR calcd for $\text{C}_{50}\text{H}_{63}\text{N}_8\text{O}_{14}\text{S}$ 1031.4179, found 1031.4124; $[\alpha]_D^{25} - 238.12$ (c 0.97, DMSO).

{N-(Benzothiophene-2-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (14)

Compound 14 (19.6 mg, 18.5 μmol , 87% over 2 steps) was synthesized using methods previously reported by our group²⁰, from benzothiophene-2-carboxylic acid (11.3 mg, 63.6 μmol), and purified with silica gel column chromatography (Φ 1 \times 10 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 99/1 \rightarrow 98/2 \rightarrow 97/3) as a white solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.71 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 7.8$ Hz), 8.14 (s, 2H, Ar), 8.06–8.00 (m, 4H, Ar), 7.85 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 5.5$ Hz), 7.51–7.44 (m, 4H, Ar), 6.29 (t, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.4$ Hz), 4.84 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 10.5$ Hz), 4.64 (m, 2H, Ser- α -CH), 4.56 (dq, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = 6.9, J_{\text{Ala-}\alpha\text{-CH, Ala-NH}} = 5.5$ Hz), 4.50 (m, 2H, Ser- β -CH), 4.31 (m, 2H, Ser- β -CH), 3.08 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 15.1, J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 6.4$ Hz), 3.02 (s, 6H, N-CH), 2.80 (s, 6H, N-CH), 2.50 (overlap, 2H, Cys- β -CH), 2.22 (m, 2H, Val- β -CH), 1.28 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 6.9$ Hz), 0.99 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz), 0.75 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 172.3, 170.2, 169.8, 161.7, 140.3, 138.9, 138.6, 128.4, 126.5, 126.0, 125.4, 125.1, 122.9, 64.6, 61.5, 54.2, 53.1, 46.1, 35.4, 30.5, 29.8, 26.6, 20.3, 18.8, 16.6; ESIMS-LR m/z 1085.44 [(M + Na) $^+$]; ESIMS-HR calcd for $\text{C}_{50}\text{H}_{63}\text{N}_8\text{O}_{12}\text{S}_3$ 1063.3722, found 1063.3690; $[\alpha]_D^{26} - 246.77$ (c 0.58, DMSO).

{N-(Benzoxazole-5-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (15)

Compound 15 (7.2 mg, 7.0 μmol , 33% over 2 steps) was synthesized using methods previously reported by our group²⁰, from benzoxazole-5-carboxylic acid (10.4 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 2 \times 6 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 99/1 \rightarrow 98/2 \rightarrow 97/3) as a white solid. ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.88 (d, 2H, Ar, $J = 2.1$ Hz), 8.57 (d, 2H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 6.9$ Hz), 8.33 (s, 2H, Ar), 7.96 (d, 2H, Ar, $J = 8.7$ Hz), 7.92 (d, 2H, Ar, $J = 8.7, 2.1$ Hz), 7.81 (d, 2H, Ala-NH, $J_{\text{Ala-NH, Ala-}\alpha\text{-CH}} = 4.6$ Hz), 6.27 (dd, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 6.0, J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 5.7$ Hz), 4.82 (d, 2H, Val- α -CH, $J_{\text{Val-}\alpha\text{-CH, Val-}\beta\text{-CH}} = 9.2$ Hz), 4.65 (m, 2H, Ser- α -CH), 4.56 (qd, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = 5.5, J_{\text{Ala-}\alpha\text{-CH, Ala-NH}} = 4.6$ Hz), 4.44 (m, 2H, Ser- β -CH), 4.32 (d, 2H, Ser- β -CH, $J_{\text{gem}} = 11.0$ Hz), 3.08 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 15.1, J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 5.7$ Hz), 3.02 (s, 6H, N-CH), 2.80 (s, 6H, N-CH), 2.50 (overlap, 2H, Cys- β -CH), 2.19 (m, 2H, Val- β -CH), 1.26 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 5.5$ Hz), 0.99 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 5.0$ Hz), 0.75 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 5.5$ Hz); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ 172.3, 170.3, 169.8, 167.9, 166.1, 155.6, 151.3, 139.5, 130.8, 125.8, 119.7, 111.2, 64.8, 61.5, 54.1, 53.3, 46.0, 35.3, 30.5, 29.8, 26.5, 20.3, 18.7, 16.6; ESIMS-LR m/z 1033.57 [(M + H) $^+$]; ESIMS-HR calcd for $\text{C}_{48}\text{H}_{61}\text{N}_{10}\text{O}_{14}\text{S}$ 1033.4084, found 1033.4098; $[\alpha]_D^{26} - 412.43$ (c 0.21, DMSO).

{N-(Benzoxazole-6-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val} $_2$ sulfide (16)

Compound 16 (11.9 mg, 11.5 μmol , 54% over 2 steps) was synthesized using methods previously reported by our group²⁰, from benzoxazole-6-carboxylic acid (10.4 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 2 \times 6 cm, $\text{CHCl}_3/\text{MeOH}$: 100/0 \rightarrow 99/1 \rightarrow 98/2 \rightarrow 97/3) as a white solid. ^1H NMR (CD_3OD , 400 MHz) δ 8.63 (s, 2H, Ar), 8.25 (s, 2H, Ar), 7.98 (dd, 2H, Ar, $J = 8.2, 1.8$ Hz), 7.87 (d, 2H, Ar, $J = 8.2$ Hz), 6.24 (dd, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 7.3, 6.4$ Hz), 4.93–4.89 (overlap, 4H, Val- α -CH, Ser- α -CH), 4.70 (q, 2H, Ala- α -CH, $J_{\text{Ala-}\alpha\text{-CH, Ala-}\beta\text{-CH}} = 7.3$ Hz), 4.63 (dd, 2H, Ser- β -CH, $J_{\text{gem}} = 11.9, J_{\text{Ser-}\beta\text{-CH, Ser-}\alpha\text{-CH}} = 5.4$ Hz), 4.54 (dd, 2H, Ser- β -CH, $J_{\text{gem}} = 11.9, J_{\text{Ser-}\beta\text{-CH, Ser-}\alpha\text{-CH}} = 2.3$ Hz), 3.34 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 15.1, J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 6.4$ Hz), 3.16 (s, 6H, N-CH), 3.01 (s, 6H, N-CH), 2.65 (dd, 2H, Cys- β -CH, $J_{\text{gem}} = 15.1, J_{\text{Cys-}\beta\text{-CH, Cys-}\alpha\text{-CH}} = 7.3$ Hz), 2.25 (m, 2H, Val- β -CH), 1.39 (d, 6H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 7.3$ Hz), 1.04 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz), 0.84 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.9$ Hz); ^{13}C NMR (CD_3OD , 100 MHz) δ 175.3, 172.2, 171.9, 170.2, 169.5, 157.4, 151.1, 143.9, 133.1, 125.7, 121.0, 112.0, 65.4, 63.9, 56.1, 54.8, 35.6, 32.2, 30.8, 28.4, 20.5, 19.6, 16.6, 7.9; ESIMS-LR m/z 1033.63 [(M + H) $^+$]; ESIMS-HR calcd for $\text{C}_{48}\text{H}_{61}\text{N}_{10}\text{O}_{14}\text{S}$ 1033.4084, found 1033.4098; $[\alpha]_D^{24} - 257.59$ (c 0.45, DMSO).

{N-(Benzthiazole-6-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val}₂ sulfide (17)

Compound **17** (11.5 mg, 10.8 μmol, 51% over 2 steps) was synthesized using methods previously reported by our group²⁰, from benzothiazole-6-carboxylic acid (11.4 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 2 × 6 cm, CHCl₃/MeOH: 100/0 → 99/1 → 98/2 → 97/3) as a white solid. ¹H NMR (DMSO-*d*₆, 400 MHz) δ 9.56 (s, 2H, Ar), 8.69 (d, 2H, Ar, *J* = 1.4 Hz), 8.62 (d, 2H, Ser-NH, *J*_{Ser-NH, Ser-α-CH} = 8.2 Hz), 8.20 (d, 2H, Ar, *J* = 8.7 Hz), 8.00 (d, 2H, Ar, *J* = 8.7, 1.4 Hz), 7.84 (d, 2H, Ala-NH, *J*_{Ala-NH, Ala-α-CH} = 5.5 Hz), 6.27 (dd, 2H, Cys-α-CH, *J*_{Cys-α-CH, Cys-β-CH} = 6.9, 6.4 Hz), 4.84 (d, 2H, Val-α-CH, *J*_{Val-α-CH, Val-β-CH} = 10.6 Hz), 4.66 (ddd, 2H, Ser-α-CH, *J*_{Ser-α-CH, Ser-β-CH} = 9.2, *J*_{Ser-α-CH, Ser-NH} = 8.2, *J*_{Ser-α-CH, Ser-β-CH} = 2.8 Hz), 4.55 (qd, 2H, Ala-α-CH, *J*_{Ala-α-CH, Ala-β-CH} = 6.9, *J*_{Ala-α-CH, Ala-NH} = 5.5 Hz), 4.45 (dd, 2H, Ser-β-CH, *J*_{Ser-β-CH, Ser-α-CH} = 9.2 Hz), 4.31 (dd, 2H, Ser-β-CH, *J*_{Ser-β-CH, Ser-α-CH} = 2.8 Hz), 3.08 (dd, 2H, Cys-β-CH, *J*_{Cys-β-CH, Cys-α-CH} = 6.4 Hz), 3.00 (s, 6H, N-CH), 2.80 (s, 6H, N-CH), 2.54–48 (overlap, 2H, Cys-β-CH), 2.24–2.17 (m, 2H, Val-β-CH), 1.26 (d, 6H, Ala-β-CH, *J*_{Ala-β-CH, Ala-α-CH} = 6.9 Hz), 0.99 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.4 Hz), 0.75 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.4 Hz); ¹³C NMR (DMSO-*d*₆, 100 MHz) δ 172.3, 170.3, 169.8, 167.9, 166.1, 159.3, 154.9, 133.7, 131.0, 125.6, 122.8, 122.6, 64.8, 61.4, 54.1, 53.2, 46.1, 35.3, 30.5, 29.8, 26.5, 20.3, 18.7, 16.6; ESIMS-LR *m/z* 1065.53 [(M + H)⁺]; ESIMS-HR calcd for C₄₈H₆₁N₁₀O₁₂S₃ 1065.3627, found 1065.3638; [α]_D²⁵ – 224.92 (c 0.95, DMSO).

{N-(4-Benzoylphenylcarbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val}₂ sulfide (18)

Compound **18** (17.4 mg, 15.0 μmol, 71% over 2 steps) was synthesized using methods previously reported by our group²⁰, from *p*-benzoylbenzoic acid (14.4 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 2 × 6 cm, CHCl₃/MeOH: 100/0 → 99/1 → 98/2) as a white solid. ¹H NMR (DMSO-*d*₆, 400 MHz) δ 8.62 (d, 2H, Ser-NH, *J*_{Ser-NH, Ser-α-CH} = 7.8 Hz), 7.99 (d, 4H, Ar, *J* = 8.2 Hz), 7.84 (d, 4H, Ar, *J* = 8.2 Hz), 7.82 (m, 2H, Ala-NH), 7.77–7.69 (m, 6H, Ar), 7.60–7.57 (m, 4H, Ar), 6.29 (t, 2H, Cys-α-CH, *J*_{Cys-α-CH, Cys-β-CH} = 6.4 Hz), 4.83 (d, 2H, Val-α-CH, *J*_{Val-α-CH, Val-β-CH} = 10.6 Hz), 4.66 (m, 2H, Ser-α-CH), 4.56 (m, 2H, Ala-α-CH), 4.45 (m, 2H, Ser-β-CH), 4.31 (d, 2H, Ser-β-CH, *J*_{Ser-β-CH, Ser-α-CH} = 11.0 Hz), 3.08 (dd, 2H, Cys-β-CH, *J*_{Cys-β-CH, Cys-α-CH} = 14.6, *J*_{Cys-β-CH, Cys-α-CH} = 6.4 Hz), 3.00 (s, 6H, N-CH), 2.79 (s, 6H, N-CH), 2.50 (overlap, 2H, Cys-β-CH), 2.22 (m, 2H, Val-β-CH), 1.25 (d, 6H, Ala-β-CH, *J*_{Ala-β-CH, Ala-α-CH} = 6.9 Hz), 0.99 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.4 Hz), 0.75 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.4 Hz); ¹³C NMR (DMSO-*d*₆, 100 MHz) δ 195.4, 172.3, 170.3, 169.8, 167.7, 165.9, 139.6, 137.1, 136.6, 133.1, 129.7, 129.6, 128.7, 127.8, 64.7, 61.4, 54.1, 53.2, 46.0, 35.3, 30.5, 29.8, 26.5, 20.3, 18.7, 16.6; ESIMS-LR *m/z* 1181.50 [(M + Na)⁺]; ESIMS-HR calcd for C₆₀H₇₁N₈O₁₄S 1159.4805, found 1159.4802; [α]_D²⁴ – 189.17 (c 0.23, DMSO).

{N-(Anthraquinone-2-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val}₂ sulfide (19)

Compound **19** (16.0 mg, 13.2 μmol, 62% over 2 steps) was synthesized using methods previously reported by our group²⁰, from anthraquinone-2-carboxylic acid (16.0 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 2 × 6 cm, CHCl₃/MeOH: 100/0 → 99/1 → 98/2) as a pale yellow solid. ¹H NMR (DMSO-*d*₆, 400 MHz) δ 8.87 (d, 2H, Ser-NH, *J*_{Ser-NH, Ser-α-CH} = 7.8 Hz), 8.60 (s, 2H, Ar), 8.32 (s, 4H, Ar), 8.23 (dd, 4H, Ar, *J* = 4.6, 3.2 Hz), 7.96–7.94 (m, 4H, Ar), 7.91 (d, 2H, Ala-NH, *J*_{Ala-NH, Ala-α-CH} = 5.5 Hz), 6.30 (t, 2H, Cys-α-CH, *J*_{Cys-α-CH, Cys-β-CH} = 6.9 Hz), 4.85 (d, 2H, Val-α-CH, *J*_{Val-α-CH, Val-β-CH} = 10.5 Hz), 4.68 (m, 2H, Ser-α-CH), 4.59 (m, 2H, Ala-α-CH), 4.49 (m, 2H, Ser-β-CH), 4.35 (d, 2H, Ser-β-CH, *J*_{Ser-β-CH, Ser-α-CH} = 9.6 Hz), 3.10 (dd, 2H, Cys-β-CH, *J*_{Cys-β-CH, Cys-α-CH} = 14.2, *J*_{Cys-β-CH, Cys-α-CH} = 6.9 Hz), 3.05 (s, 6H, N-CH), 2.81 (s, 6H, N-CH), 2.54 (overlap, 2H, Cys-β-CH), 2.26 (m, 2H, Val-β-CH), 1.29 (d, 6H, Ala-β-CH, *J*_{Ala-β-CH, Ala-α-CH} = 6.9 Hz), 0.99 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.4 Hz), 0.77 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.9 Hz); ¹³C NMR (DMSO-*d*₆, 100 MHz) δ 182.1, 172.3, 170.3, 169.8, 167.6, 165.2, 138.7, 134.9, 134.8, 133.3, 133.1, 133.0, 127.2, 126.9, 125.8, 64.6, 61.5, 54.2, 53.3, 46.1, 35.3, 30.5, 29.8, 26.5, 20.3, 18.8, 16.6; ESIMS-LR *m/z* 1233.60 [(M + Na)⁺]; ESIMS-HR calcd for C₆₂H₆₇N₈O₁₆S 1211.4390, found 1211.4396; [α]_D²⁴ – 192.98 (c 0.79, DMSO).

{N-(Pyridine-2-carbonyl)-D-Ser-L-Ala-N-Boc-N-Me-L-Cys-N-Me-L-Val}₂ sulfide (20)

Compound **20** (8.3 mg, 8.7 μmol, 49% over 2 steps) was synthesized using methods previously reported by our group²⁰, from pyridine-2-carboxylic acid (7.4 mg, 63.6 μmol), and purified with high flash silica gel column chromatography (Φ 1 × 10 cm, CHCl₃/MeOH: 100/0 → 98/2 → 96/4 → 94/6) as a white solid. ¹H NMR (CDCl₃, 400 MHz) δ 9.41 (d, 2H, Ar, *J* = 1.4 Hz), 8.80 (d, 2H, Ar, *J* = 2.3 Hz), 8.50 (dd, 2H, Ar, *J* = 2.3, 1.4 Hz), 8.45 (d, 2H, Ser-NH, *J*_{Ser-NH, Ser-α-CH} = 7.3 Hz), 6.72 (d, 2H, Ala-NH, *J*_{Ala-NH, Ala-α-CH} = 7.3 Hz), 6.23 (dd, 2H, Cys-α-CH, *J*_{Cys-α-CH, Cys-β-CH} = 6.9, 6.4 Hz), 5.10 (d, 2H, Val-α-CH, *J*_{Val-α-CH, Val-β-CH} = 10.1 Hz), 4.88–4.85 (m, 2H, Ser-α-CH), 4.80 (dq, 2H, Ala-α-CH, *J*_{Ala-α-CH, Ala-β-CH} = 7.3, *J*_{Ala-α-CH, Ala-β-CH} = 7.3 Hz), 4.75 (dd, 2H, Ser-β-CH, *J*_{Ser-β-CH, Ser-α-CH} = 11.4, *J*_{Ser-β-CH, Ser-α-CH} = 4.6 Hz), 4.63 (dd, 2H, Ser-β-CH, *J*_{Ser-β-CH, Ser-α-CH} = 1.4 Hz), 3.36 (dd, 2H, Cys-β-CH, *J*_{Cys-β-CH, Cys-α-CH} = 15.1, *J*_{Cys-β-CH, Cys-α-CH} = 6.4 Hz), 3.11 (s, 6H, N-CH), 2.93 (s, 6H, N-CH), 2.47 (dd, 2H, Cys-β-CH, *J*_{Cys-β-CH, Cys-α-CH} = 15.1, *J*_{Cys-β-CH, Cys-α-CH} = 6.9 Hz), 2.24 (m, 2H, Val-β-CH), 1.35 (d, 6H, Ala-β-CH, *J*_{Ala-β-CH, Ala-α-CH} = 7.3 Hz), 1.06 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.9 Hz), 0.84 (d, 6H, Val-γ-CH, *J*_{Val-γ-CH, Val-β-CH} = 6.9 Hz); ¹³C NMR (CDCl₃, 100 MHz) δ 173.2, 171.4, 170.0, 167.5, 164.1, 148.2, 144.8, 143.4, 142.9, 64.0, 32.0, 54.9, 53.3, 46.4, 36.1, 31.4, 30.3, 27.7, 20.5, 19.0, 17.5; ESIMS-LR *m/z* 955.48 [(M + H)⁺]; ESIMS-HR not obtained; [α]_D¹⁹ – 201.55 (c 0.83, CHCl₃).

Echinomycin probe 27

Compound **27** was prepared using methods previously reported by our group²⁰. Namely, **25** (5.9 mg, 4.4 μmol) was treated with triisopropylsilane (0.96 μL, 4.96 μmol) and 80% *aq.* TFA (400 μL) at room temperature for 2.5 h. The reaction mixture was concentrated *in vacuo*, and then azeotroped with toluene to afford a crude carboxylic acid. A mixture of the residue and biotin-PEG2-amine **26** (2.2 mg, 5.9 μmol) in 50% CHCl₃/MeOH (100 μL) was treated with DMT-MM (1.8 mg, 6.5 μmol) at room temperature for 3.5 h. The reaction mixture was concentrated *in vacuo*, and the crude was purified by preparative TLC (20 × 20 cm, 10% MeOH in CHCl₃)

to afford an inseparable mixture containing unreacted carboxylic acid (3.6 mg). Then the crude carboxylic acid (3.6 mg), $^i\text{Pr}_2\text{NEt}$ (1.90 μL , 11.2 μmol), EDCI (0.8 mg, 4.2 μmol) and HOAt (0.6 mg, 4.2 μmol) in DMF (100 μL) was treated with biotin-PEG2-amine (**26**) (2.1 mg, 5.6 μmol) at room temperature for 14 h. The reaction mixture was concentrated *in vacuo*, and then azeotroped with toluene to afford a white solid. The crude was purified by preparative TLC (20 \times 20 cm, 10% MeOH in CHCl_3) to afford compound **27** (1.94 mg, 1.18 μmol , 27% over 3 steps) as a white amorphous solid. ^1H NMR (CDCl_3 , 400 MHz) δ 9.64 (s, 1H, Ar-H), 9.64 (s, 1H, Ar-H), 8.83 (d, 1H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 6.4$ Hz), 8.69 (d, 1H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 7.6$ Hz), 8.20 (d, 1H, Ar-H, $J = 4.8$ Hz), 8.18 (d, 1H, Ar-H, $J = 5.2$ Hz), 8.00 (d, 1H, Ar-H, $J = 7.6$ Hz), 7.96 (d, 1H, Ar-H, $J = 8.0$ Hz), 7.90–7.80 (m, 4H, Ar-H), 7.01–6.97 (m, 3H, Ala-NH, biotin-NH), 6.56 (s, 1H, biotin-NH), 6.43 (d, 1H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 8.4$ Hz), 6.19 (d, 1H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 10.4$ Hz), 5.59 (s, 1H, CONH), 5.59 (s, 1H, CONH), 5.16–5.11 (m, 3H, Val- α -CH, Cys- β -CH), 4.98–4.91 (m, 2H, Ser- α -CH, Ala- α -CH), 4.85–4.79 (m, 2H, Ser- α -CH, Ala- α -CH), 4.72–4.59 (m, 4H, Ser- β -CH), 4.51–4.48 (m, 1H, biotin-NHCH), 4.31–4.29 (m, 1H, biotin-NHCH), 3.78–3.40 (m, 25H, Cys- β -CH, $\text{H}_b, \text{H}_c, \text{H}_d, \text{H}_e, \text{H}_f, \text{H}_g, \text{H}_h, \text{H}_i, \text{H}_j, \text{H}_k, \text{H}_l, \text{H}_m, \text{H}_n$), 3.16–3.11 (m, 7H, NCH, biotin-SCH), 3.00 (s, 3H, NCH), 2.99 (s, 3H, NCH), 2.94–2.80 (m, 3H, Cys- β -CH, H_h), 2.74–2.68 (m, 2H, biotin-SCH₂), 2.51 (t, 2H, H_a, H_b , $J_{\text{H}_a, \text{H}_b} = 6.0$ Hz), 2.38–2.28 (m, 2H, Val- β -CH), 2.23 (t, 2H, H_o, H_p , $J_{\text{H}_o, \text{H}_p} = 7.2$ Hz), 1.79–1.63 (m, 2H, H_q), 1.49–1.43 (m, 2H, H_r), 1.40 (d, 3H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 6.4$ Hz), 1.39 (d, 3H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 6.0$ Hz), 1.32–1.25 (m, 2H, H_s), 1.09 (d, 3H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz), 1.07 (d, 3H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.0$ Hz), 0.92 (d, 3H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.8$ Hz), 0.87 (d, 3H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.4$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 173.8, 173.5, 173.3, 172.0, 171.2, 170.9, 170.4, 169.0, 167.8, 167.5, 164.3, 164.1, 163.2, 144.4, 144.3, 143.8, 143.8, 142.5, 142.5, 140.3, 140.3, 132.3, 132.2, 131.3, 131.2, 129.9, 129.8, 129.6, 129.5, 77.4, 70.6, 70.5, 70.4, 70.3, 70.0, 67.5, 65.2, 65.1, 62.9, 62.1, 61.9, 60.2, 60.0, 55.4, 53.7, 53.5, 52.4, 46.8, 46.4, 40.7, 39.3, 37.1, 35.9, 32.5, 31.5, 31.0, 30.4, 30.0, 29.8, 28.2, 27.9, 27.8, 25.5, 20.6, 20.5, 19.3, 18.9, 18.3, 17.4; ESIMS-LR m/z 1669.6 [(M + Na)⁺]; ESIMS-HR calcd for $\text{C}_{75}\text{H}_{106}\text{N}_{16}\text{NaO}_{20}\text{S}_3$ 1669.6824, found 1669.6798; $[\alpha]_D^{23} - 184.42$ (c 0.19, CHCl_3).

Echinomycin probe **29**

Compound **29** was prepared using methods previously reported by our group²⁰. Namely, **23** (27.8 mg, 26.0 μmol) in CH_2Cl_2 (519 μL) was treated with acetyl chloride (5.6 μL , 77.9 μmol) at room temperature for 1.5 h. Acetyl chloride (5.6 μL , 77.9 μmol) was added to the mixture, and the whole mixture was further stirred for 1 h. The reaction mixture was concentrated *in vacuo*, and then azeotroped with toluene to afford a pale yellow solid. A mixture of the residue, 2-(3-but-3-ynyl-3H-diazirin-3-yl)-ethanol (**28**) (35.9 mg, 259.0 μmol) in THF (200 μL) was treated with zinc bromide (29.2 mg, 130 μmol) at room temperature for 20 h. The reaction mixture was diluted with EtOAc (50 mL) and CHCl_3 (10 mL), and washed with H_2O (20 mL \times 2) and brine (20 mL). The organic layer was dried over Na_2SO_4 , filtered, concentrated *in vacuo* and washed with Et_2O . The crude was purified by preparative TLC (20 \times 20 cm, 5% MeOH in CHCl_3) to afford compound **29** (0.9 mg, 0.76 μmol , 3%) as a white amorphous solid. ^1H NMR (CDCl_3 , 400 MHz) δ 9.64 (s, 1H, Ar-H), 9.64 (s, 1H, Ar-H), 8.82 (d, 1H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 6.0$ Hz), 8.74 (d, 1H, Ser-NH, $J_{\text{Ser-NH, Ser-}\alpha\text{-CH}} = 6.9$ Hz), 8.20 (dd, 1H, Ar-H, $J = 3.2, 1.6$ Hz), 8.18 (dd, 1H, Ar-H, $J = 3.4, 1.1$ Hz), 8.04 (d, 1H, Ar-H, $J = 6.9$ Hz), 8.02 (d, 1H, Ar-H, $J = 7.8$ Hz), 7.90–7.81 (m, 4H, Ar-H), 7.07–7.04 (m, 2H, Ala-NH), 6.46 (d, 2H, Cys- α -CH, $J_{\text{Cys-}\alpha\text{-CH, Cys-}\beta\text{-CH}} = 7.3$ Hz), 5.18–5.13 (m, 3H, Val- α -CH, Cys- β -CH), 4.96–4.82 (m, 4H, Ser- α -CH, Ala- α -CH), 4.71–4.56 (m, 4H, Ser- β -CH), 3.76 (td, 1H, H-1, $J_{\text{gem}} = 9.7, J_{\text{H-1, H-2}} = 6.6$ Hz), 3.24–3.19 (m, 1H, H-1), 3.12 (s, 3H, NCH), 3.08 (s, 3H, NCH), 3.04–3.02 (m, 1H, Cys- β -CH), 2.97 (s, 3H, NCH), 2.96 (s, 3H, NCH), 2.78–2.71 (m, 1H, Cys- β -CH), 2.40–2.27 (m, 2H, Val- β -CH), 2.03–2.00 (m, 2H, H-3, H-5), 1.93–1.86 (1H, m, H-2), 1.78–1.71 (m, 1H, H-2), 1.66–1.62 (m, 1H, H-3), 1.44–1.41 (m, 1H, H-4), 1.43 (d, 3H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 6.9$ Hz), 1.42 (d, 3H, Ala- β -CH, $J_{\text{Ala-}\beta\text{-CH, Ala-}\alpha\text{-CH}} = 6.9$ Hz), 1.36–1.25 (m, 1H, H-4), 1.08 (d, 6H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.0$ Hz), 0.90 (d, 3H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.9$ Hz), 0.86 (d, 3H, Val- γ -CH, $J_{\text{Val-}\gamma\text{-CH, Val-}\beta\text{-CH}} = 6.9$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 174.3, 173.6, 171.2, 170.2, 168.8, 164.3, 164.1, 144.3, 143.8, 143.8, 142.6, 140.4, 140.3, 132.2, 131.2, 131.2, 129.8, 129.7, 129.7, 83.0, 77.4, 69.7, 65.3, 65.1, 64.8, 62.4, 62.0, 59.5, 53.6, 53.3, 52.5, 46.9, 46.7, 32.8, 32.1, 32.0, 31.2, 30.8, 29.9, 29.8, 27.6, 27.5, 26.9, 20.5, 20.5, 18.9, 18.6, 18.2, 18.0, 13.3; ESIMS-LR m/z 1213.6 [(M + Na)⁺]; ESIMS-HR calcd. for $\text{C}_{57}\text{H}_{70}\text{N}_{14}\text{NaO}_{13}\text{S}$ 1213.4860, found 1213.4882; $[\alpha]_D^{24} - 239.91$ (c 0.42, CHCl_3).

Evaluation of cytotoxicity

Basically, the evaluation was performed using methods previously reported by our group^{20,23}. Cytotoxic activities of compounds against MIA PaCa-2 (American Type Culture Collection), SW620 (European Collection of Authenticated Cell Cultures) were measured by WST-8 assay according to manufacturer's protocol. Briefly, cells (1×10^4 cells/well) in a 96-well plate were cultured in medium (for MIA PaCa-2, D-MEM (Wako, Japan) containing 10% fetal bovine serum (Funakoshi, Japan), 100 units/mL penicillin G and 100 $\mu\text{g}/\text{mL}$ streptomycin (Wako, Japan), for SW620, RPMI-1640 (Wako, Japan) containing 10% fetal bovine serum, 100 units/mL penicillin G and 100 $\mu\text{g}/\text{mL}$ streptomycin) were cultured at 37 $^\circ\text{C}$ for 24 h under 5% CO_2 atmosphere. Then, cells were treated with test compounds (final DMSO concentration was 1%) at 37 $^\circ\text{C}$ for 72 h under 5% CO_2 atmosphere. Then, cells were treated with a solution of Cell Counting Kit-8[®] (Dojindo, Japan) for 3 h. After that, cell viabilities were determined based on the measurement of absorption at 450 nm of the wells by using TECAN Infinite[®] M200 microplate reader. The results were expressed as a percentage of the negative control (cells treated with DMSO only), and that value was fixed at 100%. IC_{50} values were calculated by using the Microsoft Excel 15.24 software and the GraphPad Prism 8.3.0/9.0 software and each data is shown as mean \pm SE ($n = 3$) in Table 3.

Western blotting

Basically, the analysis was performed using methods previously reported by our group²³. Proteins were extracted from samples using NP-40 Cell Lysis Buffer (Thermo Fisher Scientific, MA, USA) supplemented with Complete protease inhibitor (Sigma Aldrich) and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Equal amounts of protein were separated by SDS-PAGE (12.5%), transferred onto a nitrocellulose membrane, and blocked with SuperBlock T-20 (PBS; ThermoFisher Scientific). Primary antibody of cleaved caspase-3 (Cell Signaling Technology, Inc, MA, USA, #9661) were diluted to 1:1000 in SuperBlock T-20 (PBS) and incubated with blots overnight at 4 °C. After washing in T-PBS, blots were probed with HRP-conjugated secondary antibodies (R&D Systems, Inc., MN, USA), washed in T-PBS, and then developed using the Super-Signal West Pico enhanced chemiluminescence system (ThermoFisher Scientific). Protein expression levels were normalized to the actin expression (BD Transduction Laboratories, KY, USA).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

Basically, the analysis was performed using methods previously reported by our group²³. SW-620 cells were plated on a Lab-Tek[®] Chamber Slide[™] (ThermoFisher Scientific) 24 h prior to stimulation. After fixing the slides in 4% paraformaldehyde and through PBS washing, an In Situ Cell Death Detection Kit and TMR red (Roche Diagnostic, Indianapolis, IN, USA) were used for staining according to the manufacturer's instructions. The cells were mounted with an anti-fade mounting medium, and fluorescence microscopy (KEYENCE Corporation, Osaka, Japan) was employed for visualizing TUNEL-positive cells.

Reporter gene assay

Basically, the assay was performed using methods previously reported by our group²¹. SW620 cells were transfected with either HIF-1 α expression vector pCI-neo-3 \times FLAG-HIF-1 α or empty vector pCI-neo-3 \times FLAG, a derivative of pCI-neo (Promega, Madison, WI, USA), together with pGL3-5xHRE-Luc and pGL4.75 [hRluc/CMV] (Promega), by using PEI MAX (Polysciences Inc., Warrington, PA, USA). At one day after transfection, the cells were exposed to the test compounds for 16 h, and then cell lysates were prepared. A dual-luciferase assay was performed with a dual-luciferase reporter assay system (Promega) and GloMax20/20 luminometer (Promega).

Transcriptome analysis

Basically, the analysis was performed using methods previously reported by our group²³. RNA libraries were generated using an Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) according to the manufacturer's instructions. Emulsion PCR was carried out with an Ion OneTouch[™] system and an Ion OneTouch 200 Template Kit v3 (Thermo Fisher Scientific). Template-positive Ion Sphere[™] particles were enriched and purified for the sequencing reaction with an Ion OneTouch[™] ES system (Thermo Fisher Scientific). The template-positive Ion Sphere[™] Particles were loaded onto Ion PI[™] Chips (Thermo Fisher Scientific) and for high throughput sequencing with an Ion Proton[™] Semiconductor sequencer (Thermo Fisher Scientific). Sequencing data were mapped on a human reference genome sequence (GRCh38/hg38) using the Torrent Suite software program (Life Technologies). The expression analysis was performed in the CLC Genomics Workbench software program (CLC bio, Aarhus, Denmark), and differences among the samples were determined using an unpaired Student's t-test. The gene list describing fold change and p-value was uploaded to the MetaCore software (Clarivate Analytics, PA, USA, URL; <https://portal.genego.com/>, version 6.33.69110.), and then pathway analysis was performed.

Hepatic microsomal stability assay

Basically, the analysis was performed using methods previously reported³¹. Disappearance of the parent compound over time was measured by using the amount of drug at time zero as a reference. After 5 min of preincubation, 1 mM NADPH (final concentration, the same applies to the following, NADPH (+)) or 0.1 M phosphate buffer (pH 7.4, NADPH(-)) was added to a mixture containing 1 μ M of the test compound, 0.2 mg/mL of human or mouse liver microsomes (Sekisui XenoTech LLC (Kansas City, KS)), 1 mM EDTA and 0.1 M phosphate buffer (pH 7.4) and incubated at 37 °C for 30 min by rotation at 60 rpm. An aliquot of 50 μ L of the incubation mixture was sampled and added to 250 μ L of chilled acetonitrile/internal standard (IS). After centrifuging for 15 min at 3150 \times g (4 °C), the supernatants were analyzed by LC-MS/MS. Hepatic microsomal stability (mL/min/kg, CLint) was calculated according to the previous report³², using 48.8 and 45.4 mg MS protein/g liver and 25.7 and 87.5 g liver/kg body weight as scaling factors for human and mouse, respectively.

LC-MS/MS quantification method

Basically, the analysis was performed using methods previously reported³¹. An LC-MS8060 instrument equipped with a Shimadzu Nexera series LC system (Shimadzu, Kyoto, Japan) was used. All compounds were analyzed in multi-reaction monitoring mode under electron spray ionization conditions. The analytical column used was a CAPCELLPAK C18 MGIII (3 μ m \times 2.0 mmID \times 35 mm; OSAKA SODA, Osaka, Japan) at 50 °C. The gradient mobile phase consisted of 0.1% formic acid in water (mobile phase A), and 0.1% formic acid in acetonitrile (mobile phase B) at a total flow rate of 1 mL/min. The initial mobile phase composition was 10% B, which was held constant for 0.2 min, increased in a linear fashion to 90% B over 1 min, then held constant for 0.8 min, and finally brought back to the initial condition of 10% B over 0.01 min and re-equilibrated for 1 min. The transitions (precursor ion > product ion) of echinomycin, **3** and IS (methyl testosterone) are 1101.45 > 1053.5 (positive), 1083.5 > 523.95 (negative), and 303.1 > 109.1 (positive), respectively.

Xenografts

Basically, the experiments were performed using methods previously reported³³. Male BALB/c nude mice (6 weeks old) were purchased from Charles River Laboratories Japan, Inc. SW-620 cells (2×10^6 cells/35 μ L PBS/tumor) were mixed with matrigel (15 μ L/tumor) (BD Transduction Laboratories). Subsequently, 50 μ L of cell suspension was subcutaneously injected into the back of BALB/c nude mice. Daily intraperitoneal injections of each compound began on the day following the SW-620 cell injection. The tumor size was calculated by the following formula: Tumor size (mm^2) = (major diameter) \times (minor diameter).

Serum biochemistry

Male BALB/c mice (7 weeks old) were purchased from Charles River Laboratories Japan, Inc. Each compound was intraperitoneally injected daily for 14 days. The whole blood of mice was obtained from the inferior vena cava under anesthesia with isoflurane by inhalation. Serum was collected by centrifuging blood at 5000 rpm for 10 min. Biochemistry was performed in Oriental Yeast Co., Ltd, Japan.

Ethics declaration

The study received ethical approval for the use of an opt-out methodology from the Medical Ethics Committee of Asahikawa Medical University (Approval No. 16069, 20057). All methods were carried out in accordance with relevant guidelines and regulations, and they are reported in compliance with the ARRIVE guidelines.

Data availability

Data supporting the findings of this manuscript are available from the corresponding author upon request.

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Author contributions

K.K., H.K., M.F., and S.I. designed the research, and K.K., H.K., K.N., M.F., and S.I. designed the experiments. K.K., K.M., and Y.K. synthesized compounds. K.M. and F.Y. performed a cytotoxicity assay. H.K. performed a TUNEL assay, transcriptome analysis, and in vivo evaluation. K.N. performed assays associated with HIF inhibition. K.K. performed a hepatic microsomal stability assay. S.I. wrote the paper. All authors discussed the results and commented on the paper and have given approval to the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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