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# Design, Synthesis, and Biological Properties of Highly Potent Tubulysin D Analogues

**Andrew W. Patterson,[a] Hillary M. Peltier,[a] Florenz Sasse,*[b] and Jonathan A. Ellman*[a]**

**Abstract:** Ten analogues of tubulysin D were synthesized and assayed against established mammalian cell lines, including cancer cells measuring inhibition of cell growth by an MTT assay. These experiments establish for the first time the essential features for the potent cytotoxicity of tubulysin D. The activities of analogues **2** to **5** demonstrate that numerous modifications may be introduced at the C-terminus of the natural product with only modest loss in activity, while the activities of analogues **6** to **8** suggest that a basic amine must be present at the N-terminus to maintain activity. Most surprisingly, analogue **10** establishes that replacement of the chemically labile *O*-acyl *N*,*O*-acetal with the stable *N*-methyl group results in almost no loss in activity. In aggregate, these structure–activity relationships enable the design of analogues such as **11** that are smaller and considerably more stable than tubulysin D but that maintain most of its potent cell-growth inhibitory activity.

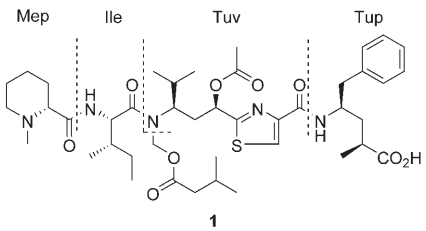
**Keywords:** cytotoxicity · natural products · total synthesis · tubulin · tubulysin

## Introduction

The tubulysins, first isolated by the Höfle/Reichenbach group from myxobacterial cultures,[1] are exceptionally potent cell-growth inhibitors that act by inhibiting tubulin polymerization and thereby induce apoptosis.[2] The tubulysins, of which tubulysin D is the most potent, have activity that exceeds other tubulin modifiers including, the epothilones, vinblastine, and paclitaxel (Taxol), by 20- to 1000-fold.[3] Paclitaxel and vinblastine are current drugs for the treatments for a variety of cancers, and epothilone derivatives are under active evaluation in clinical trials.[4] Synthetic derivatives of tubulysin D would provide essential information about the mechanism of inhibition and key binding interactions, and could have superior properties as anticancer agents either as isolated entities or as chemical warheads on targeted antibodies or ligands.

Tubulysin D (**1**) is a complex tetrapeptide that can be divided into four regions as defined in Figure 1: Mep (D-*N*-methyl pipecolinic acid), Ile (L-isoleucine), Tuv (tubuvaline), and Tup (tubuphenylalanine). All of the more potent derivatives of tubulysin, including tubulysin D, also incorporate the interesting *O*-acyl *N*,*O*-acetal functionality, which has rarely been observed in natural products. This reactive functionality is documented to be quite labile to both acidic and basic reaction conditions, and therefore may play a key role in the function of the tubulysins.[5]

Recently, we reported the total synthesis of tubulysin D,[6] which represents the first synthesis of any member of the tubulysin family that incorporates the *O*-acyl *N*,*O*-acetal functionality.[7] Herein, we report the synthesis and for the first




**1**

Figure 1. Tubulysin D (**1**).

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time the biological evaluation of tubulysin analogues providing key insights into the structural features necessary for binding. These studies have also resulted in the identification of lower molecular weight and considerably more stable analogues that maintain the majority of tubulin polymerization inhibitory activity.

## Results and Discussion

As shown in Figure 2, nine analogues were initially prepared. Analogues **2–5** were designed to probe the Tup position at the *C*-terminus of the peptide natural product, while analogues **6–8** were designed to probe the Mep position at the N-terminus. Analogues **9** and **10** were designed to test the importance of the two most labile sites in the molecule. Analogue **9** serves to test the importance of the acetyl group present in the tubulysins. In contrast **10**, which incorporates a methyl group in place of the reactive *O*-acyl *N*,*O*-acetal, like the natural product should still be able to access both *cis*- and *trans*-amide conformations because **10** retains the tertiary amide at the site of modification.

Compounds **2–10** were assayed against established mammalian cell lines, including cancer cells, by measuring inhibition of cell growth by an MTT assay[1] (Table 1). The activities of the tubulysin analogues varied from 0.05–120 ng mL$^{-1}$ in L929 mouse fibroblast cells, with a number of simplified analogues maintaining significant activity.

Table 1. Biological activity of compounds **1–11**.

| Analogue | $IC_{50}$ [ng mL$^{-1}$] L929[a] | SW-480[b] | KB-3-1[c] |
|---|---|---|---|
| **1** (Tub D)[d] | 0.056[e] | 0.022 | 0.070[f] |
| **2** | 0.24 | 0.30 | 0.25 |
| **3** | 3.5 | 0.91 | 1.8 |
| **4** | 0.30 | 0.35 | 0.22 |
| **5** | 2.2 | 0.35 | 1.5 |
| **6** | 0.040[g] | 0.010 | 0.029 |
| **7** | 120 | 15 | 80 |
| **8** | 45 | 8.8 | 40 |
| **9** | 0.25 | 0.057 | 0.22 |
| **10** | 0.23[h] | 0.016[g] | 0.13[g] |
| **11** | 1.7 | 0.50 | 1.2 |

[a] Mouse fibroblasts (DSMZ ACC 2). [b] Human colon adenocarcinoma (ATCC CCL-228). [c] Human cervix carcinoma (DSMZ ACC 158). [d] Synthetic tubulysin D prepared previously.[6] [e] The $IC_{50}$ of isolated tubulysin D was previously determined to be 0.01–0.03 ng mL$^{-1}$ with cell line L929.[1,3] [f] The $IC_{50}$ of isolated tubulysin D was previously determined to be 0.02 ng mL$^{-1}$ with cell line KB-3–1.[1,3] [g] Average of two experiments. [h] Average of four experiments.

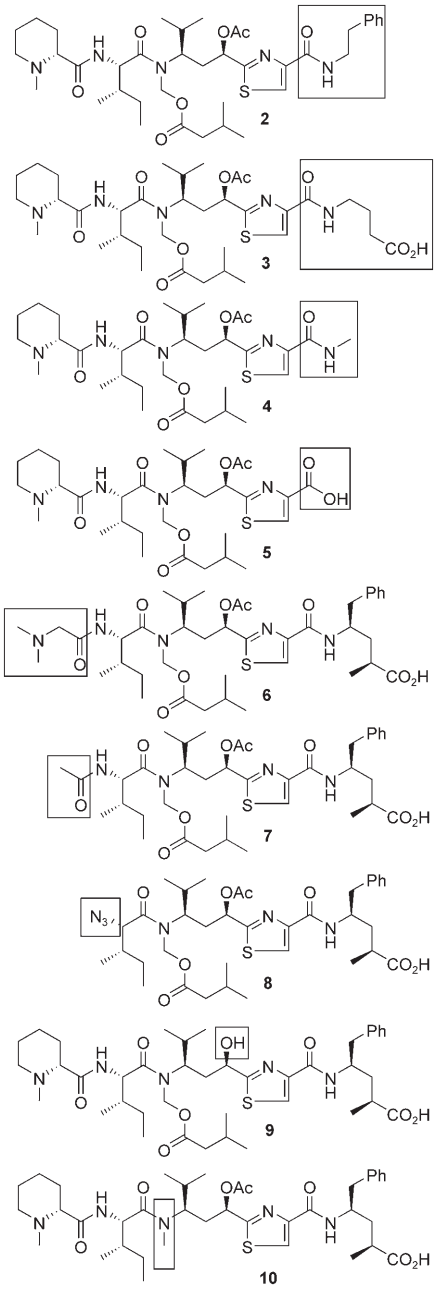



Figure 2. Tubulysin analogues **2–10**.

Upon examination of the Tup position at the C-terminus of tubulysin D (**2–5**), we found that a wide range of modifications were tolerated (Table 1). Analogues designed to retain only the phenethyl or γ-carboxy group showed comparable cytoxicity. Even the greatly simplified *N*-methyl derivative **4** and the truncated tripeptide **5** maintained good activity. The considerable tolerance at this site for large and small as well as hydrophobic and hydrophilic functionality is of considerable significance because it suggests that this site would be appropriate for attachment of functionality in the design of targeted antibodies and for the incorporation of probe molecules such as fluorescent agents.

Examination of the Mep position at the N-terminus of the natural product established the importance of maintaining a basic amine at this position. Removal of the Mep group (**8**) and replacement of the Mep group with a simple acetyl group (**7**) caused a drastic decrease in activity (Table 1). However, compound **6**, which retains the tertiary amine functionality, was essentially equipotent with tubulysin D. In

addition to defining the importance of the basic amine, the simplified nature and lower molecular weight of the *N*,*N*-dimethyl glycine present in **6** is also noteworthy.

The activities observed for analogues **9** and **10** were most surprising. Analogue **9** showed a minimal drop in cytotoxicity relative to the natural product demonstrating that the *O*-acetyl group is not important for activity. The high cytotoxicity of **10**, which is only four-fold less active than tubulysin D, is even more surprising, and clearly indicates that the *N*,*O*-acetal is not necessary for cytoxicity.

To discern whether the structure–activity relationships observed for the analogue series were additive, analogue **11** (Figure 3), which combines the truncations present in both

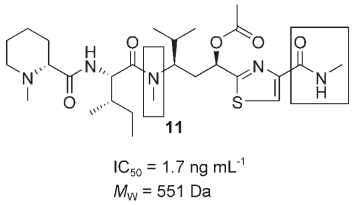



Figure 3. Analogue **11**.

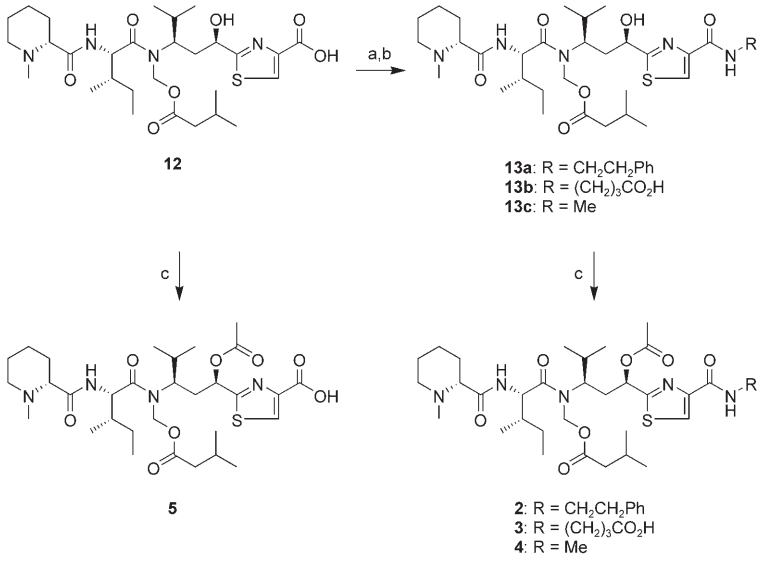
analogues **4** and **10**, was also prepared. Potent cytotoxicity (2.0 ng mL$^{-1}$) was observed for **11** (Table 1). This result is particularly exciting because **11** at 551 Da is considerably lower in molecular weight than tubulysin D (827 Da). In addition, analogue **11** incorporates the stable *N*-methyl group in place of the reactive *O*-acyl *N*,*O*-acetal functionality.

All analogues were also analyzed in human colon and cervix cancer lines, with structure–activity relationships that largely parallel SAR for the L929 cell line. The activities of analogue **6**, which possesses a Mep modification, and analogue **10**, which lacks the *N*,*O*-acetal, in the colon cancer cell line SW-480 were also notable, with both analogues proving to be more active than tubulysin D.

To check for the mode of action of the analogues, $PtK_2$ potoroo cells were incubated with the analogues at concentrations above the $IC_{50}$ with L929, and stained for microtubule cytoskeleton by immunofluorescence methods after 18 h. In each case we observed a disturbance in the microtubule system, either an interference with the microtubular network in non-dividing cells or abnormal mitotic spindles in dividing cells. These results show that the activity of all of the analogues can be attributed to an action on the tubulin/microtubule system, and is not a result of nonspecific cytotoxicity.

The synthesis of the aforementioned analogues **2**–**11** was accomplished in a highly efficient manner. Analogues **2**–**5** were prepared from intermediate **12** (Scheme 1) that we had previously reported in the synthesis of tubulysin D.[6] Activation of the acid as the pentafluorophenyl ester followed by addition of phenethylamine, 4-aminobutyric acid, or methylamine hydrochloride provided amides **13a**–**c**, respectively. Acetylation of the Tuv alcohol then provided analogues **2**–**4**. Compound **5**, which is terminated by the Tuv carboxylic acid, was directly prepared by acetylation of alcohol **12** (Scheme 1).

For compounds **6**–**8** in which Mep at the N-terminus has been replaced, an earlier azido intermediate in the synthesis of tubulysin D (**14**) was used as the common precursor.[6] Silyl ether deprotection and then selective cleavage of the methyl ester over the reactive *O*-acyl *N*,*O*-acetal with $Bu_3SnOH$ provided acid **16** (Scheme 2). Activation of the carboxylic acid as the pentafluorophenyl ester followed by addition of tubuphenylalanine hydrochloride (**17**) and acetylation of the Tuv alcohol afforded analogue **8**. This analogue also serves as the penultimate intermediate to analogues **6**

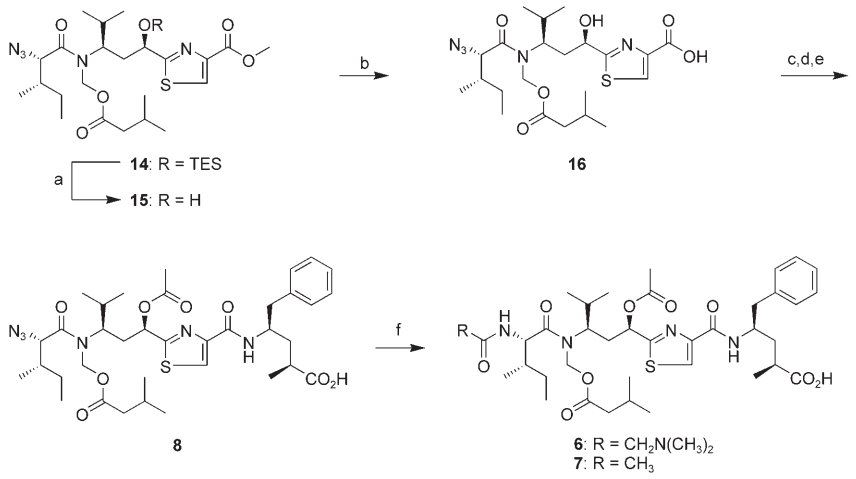



Scheme 1. Preparation of **2**–**5**. a) pentafluorophenol, 1,3-diisopropylcarbodiimide, $CH_2Cl_2$; b) 2-phenylethylamine, 4-aminobutyric acid, or methylamine hydrochloride, *i*$Pr_2EtN$, DMF, 49% (**13a**), 70% (**13b**), or 68% (**13c**) yield for two steps; c) $Ac_2O$, pyridine, then (for **4** and **6**) $H_2O$/dioxane, 99% (**3**), 81% (**4**), 90% (**5**), or 97% (**6**).

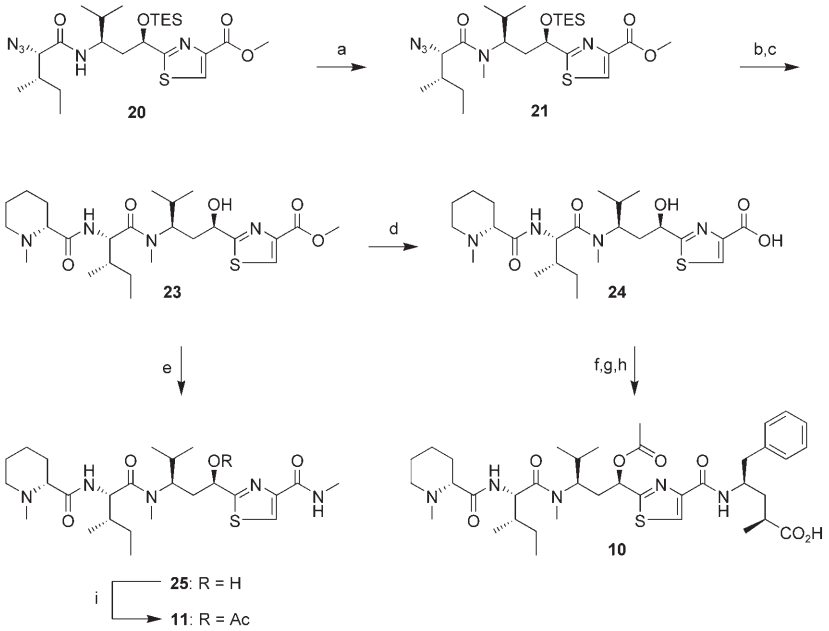
and **7**, which were prepared by reduction of the azide in the presence of the pentafluorophenyl ester of *N*,*N*-dimethylglycine (**18**) or acetic acid (**19**), respectively.

Compound **10**, which is directly analogous to tubulysin D with the *O*-acyl *N*,*O*-acetal being replaced by an *N*-Me amide, was synthesized starting from azide **20**, which served as an early intermediate in the synthesis of tubulysin D (Scheme 3).[6] Deprotonation of the Tuv-amide with KHMDS followed by addition of methyl iodide provided *N*-




Scheme 2. Preparation of **6**–**8**. a) AcOH/THF/$H_2O$, 73%; b) $Me_3SnOH$, $Cl(CH_2)_2Cl$, 60°C, 36%; c) pentafluorophenol, 1,3-diisopropylcarbodiimide, $CH_2Cl_2$; d) **17**, $iPr_2EtN$, DMF; e) $Ac_2O$, pyridine, then $H_2O$/dioxane, 39% yield for three steps; f) **18** or **19**, $H_2$, Pd/C, EtOAc then $H_2O$/dioxane, 48% (**7**) or 77% (**8**).




Scheme 3. Preparation of **10** and **11**. a) potassium bis(trimethylsilyl)amide, THF, −45°C then methyl iodide, 82%; b) **22**, $H_2$, Pd/C, EtOAc; c) AcOH/THF/$H_2O$, 87% for two steps; d) $Me_3SnOH$, $Cl(CH_2)_2Cl$, 60°C, 88%; e) methylamine, THF/MeOH, 100°C, 52%; f) PFP, DIC, $CH_2Cl_2$; g) **17**, $iPr_2EtN$, DMF; h) $Ac_2O$, pyridine, then $H_2O$/dioxane, 56% for three steps; i) $Ac_2O$, pyridine, 72%.

Me amide precursor **21**. Silyl group deprotection followed by reductive coupling in the presence of the pentafluorophenyl ester of D-*N*-methyl pipecolinic acid (**22**) then provided Mep-coupled product **23**. Cleavage of the methyl ester, followed by coupling with tubuphenylalanine and acetylation of the Tuv alcohol afforded descarboxy analogue **10**.[8] Compound **11** was prepared in a similar manner to **10**. Heating ester **23** in the presence of methylamine directly provided amide **25** (Scheme 3). Acetylation then afforded analogue **11**.

## Conclusion

In conclusion, we have constructed a series of analogues of tubulysin D containing variations at the Tup, Mep, and *N*,*O*-acetal positions that has for the first time established the essential features of these regions of the natural product necessary for biological activity against a series of human and animal cancer cell lines. The biological data indicate that a wide range of modifications at the Tup position, for example, **2**–**5**, are well-tolerated indicating that this could be a key location for conjugation to antibodies or for the incorporation of fluorescent and other probe molecules. The biological data also indicates that while a basic amine in the Mep region of tubulysin is necessary for biological activity, very simple and low molecular weight substituents, for example, **6**, are acceptable at this site. Notably, neither of the most labile sites in the natural product, the *O*-acetyl group and the *O*-acyl *N*,*O*-acetal, are necessary for biological activity (see analogues **9** and **10**, respectively). This finding enables the design of highly potent tubulysin analogues that are of considerably greater stability than the natural product. Further biological characterization of these new tubulysin analogues is in progress as are the synthesis and biological evaluation of additional tubulysin analogues. Even more efficient and high yielding syntheses of the most promising of the simplified analogues will also be reported in due course.

## Experimental Section

**General**: Compounds **9**, **12**, **14**, **17**, **20**, and **22** were prepared as previously described.[6] Unless otherwise noted, all starting materials were obtained from commercial suppliers and used without further purification. Methyl iodide was filtered through a plug of basic alumina (Brockman activity 1) immediately prior to use. Toluene, THF, ether, dioxane, and $CH_2Cl_2$ were dried over alumina under a nitrogen atmosphere. Methanol, $iPr_2NH$, $iPr_2EtN$, dichloroethane, and pyridine were distilled from $CaH_2$

immediately prior to use. Where noted, water and acetic acid were degassed using three consecutive freeze–pump–thaw cycles. Reactions were carried out in flame or oven-dried glassware under a $N_2$ atmosphere. Extracts were dried over $Na_2SO_4$. Products were concentrated using a Büchi rotary evaporator under reduced pressure. Chromatography was carried out either with Merck 60 Å 230–400 mesh silica gel or via HPFC purification on a Biotage SP1 instrument (Charlottesville, VA) equipped with a normal-phase Biotage Si flash column or reverse-phase Biotage C18 column. Where noted, water was removed from samples by lyophilization using a Labconco Corp. freeze-dry system (Kansas City, MO). Optical rotation measurements were performed on a Perkin-Elmer 241 polarimeter. Optical rotations ($[\alpha]$) are measured in deg $cm^3\,g^{-1}\,dm^{-1}$. Concentration ($c$) is measured in $g\,dL^{-1}$. IR spectra were recorded on a Nicolet Avatar 360 FTIR spectrometer equipped with an attenuated total reflectance accessory and only partial data are listed. $^1H$ NMR and $^{13}C$ NMR spectra were obtained at room temperature with Bruker AV-400 and DRX-500 spectrometers. Chemical shifts are expressed in ppm relative to internal solvent. High-resolution mass spectra were performed by the University of California at Berkeley Micro-Mass Facility.

**Intermediate 13a**: Acid **12** (40.0 mg, 0.0670 mmol) was added to a solution of pentafluorophenol (19.0 mg, 0.101 mmol) and 1,3-diisopropylcarbodiimide (11.5 μL, 0.0737 mmol) in $CH_2Cl_2$ (0.51 mL) at 0 °C. The reaction mixture was warmed to RT, stirred for 24 h, and concentrated. EtOAc (10 mL) was added, and the crude product was filtered, with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated, and the crude material was used without further purification. DMF (0.270 mL) was added to the crude product at 0 °C, followed by phenethylamine (10.2 μL, 0.0804 mmol) and $i$Pr$_2$EtN (23.0 μL, 0.134 mmol). The reaction mixture was allowed to warm to RT, stirred for 24 h, and concentrated. Normal-phase HPFC purification ($CH_2Cl_2$/MeOH 100:0 → 90:10) afforded **13a** (23.0 mg, 49 %). $[\alpha]_D^{23} = +15.0$ ($c = 1.5$ in MeOH); $^1H$ NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.80–0.92 (m, 12H), 0.97 (d, 3H, $J = 6.9$ Hz), 1.00 (d, 3H, $J = 6.7$ Hz), 1.19–1.30 (m, 3H), 1.49–1.65 (m, 5H), 1.69–1.75 (m, 2H), 1.98–2.18 (m, 7H), 2.14 (s, 3H), 2.58 (app d, 1H, $J = 10.4$ Hz), 2.91 (m, 1H), 2.92 (app t, 2H, $J = 7.5$ Hz), 3.55–3.66 (m, 2H), 4.58 (d, 1H, $J = 9.8$ Hz), 4.75 (d, 1H, $J = 10.2$ Hz), 5.51 (d, 1H, $J = 12.2$ Hz), 6.17 (d, 1H, $J = 12.2$ Hz), 7.18–7.21 (m, 1H), 7.24–7.30 (m, 4H), 8.09 ppm (s, 1H); $^{13}C$ NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 10.7, 16.2, 20.7, 22.77, 22,81, 24.3, 25.96, 25.97, 26.1, 26.7, 31.5, 36.71, 36.73, 37.6, 38.8, 42.0, 44.4, 44.7, 55.4, 56.6, 69.5, 70.3, 124.7, 127.5, 129.6, 129.8, 140.3, 150.7, 163.5, 173.4, 175.5, 178.2, 179.1 ppm; IR: $\tilde{\nu}$ = 1497, 1544, 1661, 1738, 2793, 2874, 2934, 2961, 3301, 3370 $cm^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{37}H_{58}N_5O_6S$: 700.4108; found: 700.4105 $[M+H]^+$.

**Intermediate 13b**: Acid **12** (25.0 mg, 0.0419 mmol) was added to a solution of pentafluorophenol (12.0 mg, 0.0628 mmol) and 1,3-diisopropylcarbodiimide (7.22 μL, 0.0461 mmol) in $CH_2Cl_2$ (0.31 mL) at 0 °C. The reaction mixture was warmed to RT, stirred for 24 h, and concentrated. EtOAc (10 mL) was added, and the crude product was filtered, with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated and the crude material was used without further purification. DMF (0.170 mL) was added to the crude product at 0 °C, followed by the 4-aminobutyric acid (5.18 mg, 0.0503 mmol) and $i$Pr$_2$EtN (18.2 μL, 0.105 mmol). The reaction mixture was allowed to warm to RT, stirred for 24 h, and concentrated. Normal-phase HPFC purification ($CH_2Cl_2$/MeOH 100:0 → 90:10) afforded **13b** (20.0 mg, 70 %). $[\alpha]_D^{23} = +10.3$ ($c = 1.0$ in MeOH); $^1H$ NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.81–0.85 (m, 3H), 0.88–0.91 (m, 9H), 0.97 (d, 3H, $J = 7.0$ Hz), 1.00 (d, 3H, $J = 6.1$ Hz), 1.19–1.26 (m, 1H), 1.27–1.34 (m, 1H), 1.53–1.66 (m, 5H), 1.70–1.77 (m, 2H), 1.91 (m, 3H), 1.99–2.06 (m, 4H), 2.13–2.19 (m, 3H), 2.19 (s, 3H), 2.28 (app t, 2H, $J = 7.3$ Hz), 2.70 (app d, 1H, $J = 11.4$ Hz), 2.96 (app d, 1H, $J = 11.1$ Hz), 3.42 (app t, 2H, $J = 7.2$ Hz), 4.59 (d, 1H, $J = 9.2$ Hz), 4.76 (d, 1H, $J = 9.0$ Hz), 5.51 (d, 1H, $J = 11.9$ Hz), 6.16 (d, 1H, $J = 12.2$ Hz), 8.08 ppm (s, 1H); $^{13}C$ NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 10.7, 16.2, 20.7, 22.77, 22.81, 24.1, 25.9, 26.7, 27.3, 31.4, 35.6, 37.6, 38.89, 38.90, 40.4, 43.0, 44.44, 44.48, 55.4, 56.6, 58.7, 69.9, 70.1, 124.5, 150.9, 163.6, 173.4, 174.92, 174.95, 174.96, 180.9 ppm; IR: $\tilde{\nu}$ = 1451, 1550, 1643, 1658, 1736, 2795, 2872, 2925, 2961, 3365 $cm^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{33}H_{56}N_5O_8S$: 682.3850; found: 682.3861 $[M+H]^+$.

**Intermediate 13c**: Acid **12** (30.0 mg, 0.0503 mmol) was added to a solution of pentafluorophenol (14.0 mg, 0.0754 mmol) and 1,3-diisopropylcarbodiimide (8.70 μL, 0.0553 mmol) in $CH_2Cl_2$ (0.38 mL) at 0 °C. The reaction mixture was warmed to RT, stirred for 24 h, and concentrated. EtOAc (10 mL) was added, and the crude product was filtered, with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated, and the crude material was used without further purification. DMF (0.201 mL) was added to the crude product at 0 °C, followed by the hydrochloride salt of methylamine (18.0 mg, 0.151 mmol) and $i$Pr$_2$EtN (44.0 μL, 0.251 mmol). The reaction mixture was allowed to warm to RT, stirred for 24 h at RT, and concentrated. Normal-phase HPFC purification ($CH_2Cl_2$/MeOH 100:0 → 90:10) afforded **13c** (31.0 mg, 68 %). $[\alpha]_D^{23} = +15.5$ ($c = 1.0$ in MeOH); $^1H$ NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.83 (d, 3H, $J = 5.9$ Hz), 0.90 (app t, 9H, $J = 7.5$ Hz), 0.98 (app t, 6H, $J = 7.5$ Hz), 1.20–1.30 (m, 3H), 1.49–1.63 (m, 5H), 1.69–1.72 (m, 2H), 1.98–2.06 (m, 5H), 2.13 (s, 3H), 2.14–2.17 (m, 2H), 2.55 (app d, 1H, $J = 11.5$ Hz), 2.88–2.91 (m, 1H), 2.93 (s, 3H), 4.59 (d, 1H, $J = 9.9$ Hz), 4.75 (d, 1H, $J = 10.8$ Hz), 5.51 (d, 1H, $J = 12.1$ Hz), 6.18 (d, 1H, $J = 12.1$ Hz), 8.08 ppm (s, 1H); $^{13}C$ NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 10.6, 16.2, 20.7, 22.7, 22.8, 24.3, 25.9, 26.1, 26.3, 26.6, 31.56, 31.64, 36.9, 37.6, 38.8, 44.4, 44.7, 55.3, 56.6, 69.5, 70.4, 124.4, 150.7, 164.2, 164.9, 173.4, 175.7, 179.1 ppm; IR: $\tilde{\nu}$ = 1420, 1498, 1654, 1737, 2791, 2872, 2934, 2961, 3322, 3367 $cm^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{30}H_{52}N_5O_6S$: 610.3638; found: 610.3634 $[M+H]^+$.

**Tubulysin analogue 2**: A solution of **13a** (15.5 mg, 0.0221 mmol) in pyridine (0.221 mL) was cooled to 0 °C, and acetic anhydride (10.0 μL, 0.111 mmol) was added. The reaction mixture was allowed to warm to RT over 2 h and was stirred at RT for 24 h. The solvent was removed under reduced pressure. Column chromatography ($CH_2Cl_2$/MeOH 100:0 → 90:10) afforded **2** as an amorphous solid (16.4 mg, 99 %). $[\alpha]_D^{23} = +50.0$ ($c = 0.4$ in MeOH); $^1H$ NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.79 (d, 3H, $J = 6.3$ Hz), 0.83 (d, 3H, $J = 6.5$ Hz), 0.86 (d, 3H, $J = 6.6$ Hz), 0.90 (app t, 3H, $J = 7.3$ Hz), 0.96 (d, 3H, $J = 7.17$ Hz), 1.05 (d, 3H, $J = 6.4$ Hz), 1.16–1.22 (m, 1H), 1.28–1.36 (m, 2H), 1.53–1.68 (m, 4H), 1.75–1.86 (m, 3H), 1.91–2.08 (m, 3H), 2.10–2.14 (m, 1H), 2.13 (s, 3H), 2.17–2.26 (m, 1H), 2.24 (s, 3H), 2.48 (app t, 1H, $J = 13.1$ Hz), 2.72 (app t, 1H, $J = 10.8$ Hz), 2.91 (app t, 2H, $J = 7.5$ Hz), 2.99 (app d, 1H, $J = 11.7$ Hz), 3.62 (m, 2H), 4.43 (br s, 1H), 4.60 (d, 1H, $J = 9.3$ Hz), 5.40 (d, 1H, $J = 12.7$ Hz), 5.85 (d, 1H, $J = 11.3$ Hz), 6.16 (d, 1H, $J = 12.5$ Hz), 7.18–7.21 (m, 1H), 7.25–7.30 (m, 4H), 8.17 ppm (s, 1H); $^{13}C$ NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 10.7, 16.4, 20.3, 20.7, 20.8, 22.71, 22.73, 24.0, 25.6, 25.8, 26.7, 31.4, 32.2, 35.7, 36.7, 37.3, 42.0, 44.2, 44.4, 55.1, 56.5, 70.0, 70.6, 125.7, 127.5, 129.6, 129.9, 140.3, 150.7, 163.1, 170.6, 171.9, 173.2, 174.6, 176.5 ppm; IR: $\tilde{\nu}$ = 1229, 1370, 1426, 1455, 1497, 1544, 1667, 1742, 2848, 2874, 2934, 2961, 3306, 3383 $cm^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{39}H_{60}N_5O_7S$: 742.4213; found: 742.4206 $[M+H]^+$.

**Tubulysin analogue 3**: A solution of **13b** (15.0 mg, 0.0220 mmol) in pyridine (0.220 mL) was cooled to 0 °C, and acetic anhydride (10.4 μL, 0.110 mmol) was added. The reaction mixture was allowed to warm to RT over 2 h and was stirred at RT for 24 h. The reaction mixture was then cooled to 0 °C, and a 1:1 mixture of dioxane/water (0.630 mL) was added. The mixture was allowed to warm to RT and was stirred for 12 h at RT. The solvent was removed under reduced pressure. Column chromatography ($CH_2Cl_2$/MeOH 100:0 → 90:10) afforded **3** as an amorphous solid (13.0 mg, 81 %). $[\alpha]_D^{23} = +24.3$ ($c = 0.7$ in MeOH); $^1H$ NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.79 (d, 3H, $J = 6.7$ Hz), 0.85 (d, 3H, $J = 6.9$ Hz), 0.87–0.92 (m, 6H), 0.96 (d, 3H, $J = 6.6$ Hz), 1.05 (d, 3H, $J = 6.3$ Hz), 1.15–1.23 (m, 1H), 1.35–1.42 (m, 1H), 1.57–1.65 (m, 3H), 1.69–1.72 (m, 1H), 1.77–1.81 (m, 1H), 1.84–1.93 (m, 4H), 1.95–2.01 (m, 2H), 2.08–2.18 (m, 2H), 2.13 (s, 3H), 2.28–2.39 (m, 4H), 2.33 (s, 3H), 2.48–2.53 (m, 1H), 2.94 (app d, 1H, $J = 10.1$ Hz), 3.08 (app d, 1H, $J = 11.3$ Hz), 3.40–3.43 (m, 2H), 4.41 (br s, 1H), 4.60 (d, 1H, $J = 9.5$ Hz), 5.40 (d, 1H, $J = 12.4$ Hz), 5.87 (d, 1H, $J = 10.8$ Hz), 6.13 (d, 1H, $J = 10.8$ Hz), 8.18 ppm (s, 1H); $^{13}C$ NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 10.7, 16.4, 20.3, 20.70, 20.76, 22.73, 22.74, 23.7, 25.54, 22.56, 26.7, 26.9, 31.1, 32.2, 34.7, 35.9, 37.4, 40.2, 44.18, 44.25, 55.2, 56.5, 69.7, 70.7, 125.6, 150.8, 163.2, 170.7, 171.9, 173.3, 173.8, 176.4, 180.5 ppm; IR: $\tilde{\nu}$ = 1371, 1420, 1497, 1544, 1667, 1741, 2342, 2360, 2840, 2872, 2929, 2961, 3293, 3368 $cm^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{35}H_{58}N_5O_9S$: 724.3955; found: 724.3937 $[M+H]^+$.

**Tubulysin analogue 4**: A solution of **13c** (10.5 mg, 0.0172 mmol) in pyridine (0.172 mL) was cooled to 0 °C, and acetic anhydride (8.10 μL, 0.0861 mmol) was added. The reaction mixture was allowed to warm to RT over 2 h and was stirred at RT for 24 h. The solvent was removed under reduced pressure. Column chromatography ($CH_2Cl_2$/MeOH 100:0 → 90:10) afforded **4** as an amorphous solid (10.1 mg, 90 %). $[\alpha]_D^{23} = +60.5$ ($c = 0.6$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.78 (d, 3H, $J = 7.0$ Hz), 0.84 (d, 3H, $J = 6.7$ Hz), 0.87–0.91 (m, 6H), 0.96 (d, 3H, $J = 6.7$ Hz), 1.04 (d, 3H, $J = 6.3$ Hz), 1.25–1.15 (m, 1H), 1.24–132 (m, 1H), 1.48–1.65 (m, 4H), 1.72–1.75 (m, 2H), 1.79–1.86 (m, 1H), 1.93–2.18 (m, 5H), 2.13 (s, 3H), 2.15 (s, 3H), 2.22–2.29 (m, 1H), 2.47–2.57 (m, 2H), 2.89–2.94 (m, 1H), 2.92 (s, 3H), 4.43 (brs, 1H), 4.60 (d, 1H, $J = 10.1$ Hz), 5.39 (d, 1H, $J = 12.2$ Hz), 5.87 (d, 1H, $J = 12.0$ Hz), 6.18 (d, 1H, $J = 12.5$ Hz), 8.16 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 10.7, 16.4, 20.3, 20.69, 20.74, 22.68, 22.69, 24.3, 25.6, 26.1, 26.3, 26.6, 31.5, 32.2, 35.9, 37.3, 44.2, 44.7, 55.0, 56.6, 70.4, 70.7, 125.5, 150.7, 163.8, 170.8, 171.9, 173.3, 175.5, 176.7 ppm; IR: $\tilde{\nu}$ = 1229, 1371, 1420, 1466, 1499, 1549, 1665, 1742, 2792, 2848, 2875, 2934, 2961, 3305, 3380 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{32}H_{54}N_5O_7S$: 652.3744; found: 652.3719 $[M+H]^+$.

**Tubulysin analogue 5**: A solution of **12** (25.0 mg, 0.0419 mmol) in pyridine (0.420 mL) was cooled to 0 °C, and acetic anhydride (19.8 μL, 0.209 mmol) was added. The reaction mixture was allowed to warm to RT over 2 h and was stirred at RT for 24 h. The reaction mixture was then cooled to 0 °C, and a 1:1 mixture of dioxane/water (1.50 mL) was added. The mixture was allowed to warm to RT and was stirred for 12 h at RT. The solvent was removed under reduced pressure. Column chromatography ($CH_2Cl_2$/MeOH 100:0 → 90:10) afforded **5** as an amorphous solid (26.0 mg, 97 %). $[\alpha]_D^{23} = +12.0$ ($c = 2.6$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.82–0.84 (m, 3H), 0.88–0.92 (m, 9H), 0.96 (d, 3H, $J = 6.7$ Hz), 1.01 (d, 3H, $J = 6.3$ Hz), 1.15–1.22 (m, 1H), 1.52–1.70 (m, 4H), 1.75–1.82 (m, 2H), 1.93–2.05 (m, 4H), 2.12 (s, 3H), 2.20–2.23 (m, 4H), 2.39–2.55 (m, 6H), 3.15–3.22 (m, 1H), 4.64 (d, 1H, $J = 9.6$ Hz), 5.39 (d, 1H, $J = 12.1$ Hz), 5.83 (d, 1H, $J = 11.7$ Hz), 5.98 (brs, 1H), 8.02 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 10.9, 16.3, 20.6, 20.8, 20.9, 22.8, 23.2, 25.1, 25.4, 26.6, 30.9, 31.8, 36.1, 37.5, 44.0, 44.1, 54.8, 55.5, 56.3, 69.1, 71.0, 125.3, 155.1, 169.0, 171.8, 172.0, 173.5, 175.7, 178.4 ppm; IR: $\tilde{\nu}$ = 1371, 1422, 1471, 1499, 1597, 1666, 1743, 2874, 2934, 2962, 3384 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{31}H_{51}N_4O_8S$: 639.3428; found: 639.3439 $[M+H]^+$.

**Intermediate 15**: A solution of **14** (475 mg, 0.759 mmol) in deoxygenated AcOH/$H_2O$/THF (3:1:1, 38.0 mL) was stirred at RT for 27 h. Addition of toluene (400 mL) followed by concentration and normal-phase HPFC purification (hexanes/EtOAc 95:5 → 60:40) afforded **15** as an amorphous solid (283 mg, 73 %). $[\alpha]_D^{23} = +55.1$ ($c = 1.0$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.88–0.99 (m, 15H), 1.02 (d, 3H, $J = 6.5$ Hz), 1.25–1.35 (m, 1H), 1.72–1.79 (m, 1H), 1.80–1.90 (m, 1H), 1.98–2.09 (m, 2H), 2.10–2.25 (m, 2H), 2.34 (d, 2H, $J = 7.0$ Hz), 3.74 (d, 1H, $J = 9.5$ Hz), 3.89 (s, 3H), 4.47–4.70 (brs, 1H), 4.79 (d, 1H, $J = 10.5$ Hz), 5.48 (d, 1H, $J = 12.5$ Hz), 5.58 (d, 1H, $J = 10.5$ Hz), 8.30 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 11.0, 16.1, 20.4, 20.8, 22.88, 22.91, 26.1, 26.7, 32.0, 36.7, 39.3, 44.2, 52.8, 64.6, 69.6, 129.3, 147.7, 163.3, 173.6, 173.7, 180.2 ppm; IR: $\tilde{\nu}$ = 1095, 1212, 1652, 1735, 2099, 2963 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{23}H_{37}N_5O_6SNa$: 534.2362; found: 534.2367 $[M+Na]^+$.

**Intermediate 16**: $Me_3SnOH$ (736 mg, 4.07 mmol) was added to a solution of methyl ester **15** (260 mg, 0.509 mmol) in dichloroethane (25.0 mL). The reaction mixture was heated to 55 °C for 22 h and then concentrated. Normal-phase HPFC ($CH_2Cl_2$/MeOH/AcOH 100:0 → 90:10:1) followed by reverse-phase HPFC (MeCN/$H_2O$ 20:80 → 100:0) and lyophilization afforded **16** as an amorphous solid (90.0 mg, 36 %). $[\alpha]_D^{23} = +51.5$ ($c = 1.0$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.88–0.94 (m, 9H), 0.97 (app t, 6H, $J = 6.8$), 1.03 (d, 3H, $J = 6.5$), 1.31 (sept, 1H, $J = 7.4$), 1.72–1.81 (m, 1H), 1.82–1.90 (brs, 1H), 2.00–2.09 (m, 2H), 2.10–2.17 (m, 1H), 2.18–2.28 (m, 1H), 2.31 (app t, 2H, $J = 7.5$), 3.75 (d, 1H, $J = 9.5$), 4.44–4.68 (brs, 1H), 4.77 (d, 1H, $J = 9.5$), 5.48 (d, 1H, $J = 12.5$), 5.58 (d, 1H, $J = 12.5$), 8.24 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 10.8, 16.0, 20.3, 20.6, 22.71, 22.73, 26.0, 26.5, 32.0, 36.7, 39.1, 44.1, 64.6, 69.5, 128.5, 149.2, 164.7, 173.58, 173.61, 179.5 ppm; IR: $\tilde{\nu}$ = 1088, 1217, 1651, 1735, 2100, 2964 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{22}H_{35}N_5O_6SNa$: 520.2206; found: 520.2200 $[M+Na]^+$.

**Tubulysin analogue 8**: Acid **16** (39.0 mg, 0.0784 mmol) was added to a solution of pentafluorophenol (2.0 mg, 0.118 mmol) and 1,3-diisopropylcarbodiimide (13.4 μL, 0.0862 mmol) in $CH_2Cl_2$ (1.12 mL) at 0 °C. The reaction mixture was warmed to RT, stirred for 24 h, and concentrated. EtOAc (10 mL) was added, and the crude product was filtered with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated, and the crude material was used without further purification. DMF (1.00 mL) was added to the crude product at 0 °C, followed by **17** (57.0 mg, 0.235 mmol) and $i$Pr$_2$EtN (68.0 μL, 0.392 mmol). The reaction mixture was allowed to warm to RT, stirred for 24 h at RT, and concentrated. Normal-phase HPFC purification (EtOAc/MeOH 100:0 → 95:5) afforded the product containing trace amounts of $i$Pr$_2$EtN. The product mixture (32.0 mg, 0.181 mmol) was dissolved in pyridine (1.80 mL), cooled to 0 °C, and acetic anhydride (0.140 mL, 1.45 mmol) was added. The reaction mixture was allowed to warm to RT over 2 h and was stirred at RT for 22 h. The reaction mixture was then cooled to 0 °C, and a 1:1 mixture of deoxygenated $H_2O$/dioxane (0.5 mL) was added. The mixture was allowed to warm to RT and was stirred for 14 h at RT. The solvent was removed under reduced pressure. Reverse-phase HPFC (MeCN/$H_2O$ 20:80 → 100:0) followed by lyophilization afforded **8** as an amorphous solid (51.0 mg, 39 %, over three steps). $[\alpha]_D^{23} = +61.4$ ($c = 1$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.87 (app t, 6H, $J = 6.8$ Hz), 0.92 (d, 3H, $J = 6.5$ Hz), 0.93 (d, 3H, $J = 6.5$ Hz), 0.98 (t, 3H, $J = 7.3$ Hz), 1.10 (d, 3H, $J = 6.5$ Hz), 1.17 (d, 3H, $J = 7.0$ Hz), 1.25–1.35 (m, 1H), 1.62–1.69 (m, 1H), 1.72–1.80 (m, 1H), 1.86–1.94 (m, 1H), 1.94–2.04 (m, 2H), 2.06–2.16 (m, 3H), 2.17 (s, 3H), 2.28–2.40 (m, 1H), 2.48–2.58 (m, 2H), 2.91 (d, 2H, $J = 6.5$ Hz), 3.72 (d, 1H, $J = 9.5$ Hz), 4.32–4.41 (m, 1H), 4.41–4.54 (brs, 1H), 5.46 (d, 1H, $J = 12.5$ Hz), 5.59 (d, 1H, $J = 12.5$ Hz), 5.90 (dd, 1H, $J = 2.0$, 11.0 Hz), 7.16 (app sextet, 1H, $J = 4.5$ Hz), 7.23 (app d, 4H, $J = 4.5$ Hz), 8.10 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 10.9, 16.2, 18.7, 20.1, 20.8, 20.9, 22.8, 22.9, 26.1, 26.8, 32.2, 35.8, 36.3, 38.0, 39.4, 42.3, 44.3, 50.9, 64.6, 70.7, 125.6, 127.5, 129.4, 130.6, 139.5, 150.9, 162.8, 170.9, 172.0, 173.21, 173.24, 180.0 ppm; IR: $\tilde{\nu}$ = 1218, 1669, 1739, 2099, 2964 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{36}H_{52}N_6O_8NaS$: 751.3465; found: 751.3456 $[M+Na]$.

***N*,*N*-dimethylglycine pentafluorophenyl ester (18)**: To a solution of *N*,*N*-dimethylglycine (82.0 mg, 0.800 mmol) in EtOAc (2.00 mL, filtered through activated alumina) were added pentafluorophenol (162 mg, 0.880 mmol) and 1,3-dicyclohexylcarbodiimide (182 mg, 0.88 mmol). The reaction mixture was stirred for 12 h at RT at which time it was filtered (washing with EtOAc) and concentrated. Ester **18** was used immediately without further purification.

**Tubulysin analogue 6**: Pd/C (10 wt %, 8.7 μg) and azide **8** (18.0 mg, 0.0247 mmol) were added to a solution of **18** (0.0988 mmol) in EtOAc (0.40 mL, filtered through activated alumina). The reaction mixture was stirred under a hydrogen atmosphere for 26 h and then filtered through a plug of Celite with washing of the filter pad with EtOAc. The filtrate was concentrated, and a 1:1 mixture of deoxygenated $H_2O$/dioxane (4.0 mL) was added. The mixture was stirred for 20 h at RT and concentrated. Reverse-phase HPFC (MeCN/$H_2O$ 20:80 → 100:0) followed by lyophilization afforded **6** as an amorphous solid (9.3 mg, 48 %). $[\alpha]_D^{23} = -2.0$ ($c = 0.6$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.81 (d, 3H, $J = 6.5$ Hz), 0.87 (d, 3H, $J = 6.5$ Hz), 0.89 (d, 3H, $J = 7.0$ Hz), 0.92 (t, 3H, $J = 7.5$ Hz), 0.98 (d, 3H, $J = 6.5$ Hz), 1.06 (d, 3H, $J = 6.5$ Hz), 1.12–1.21 (m, 1H), 1.17 (d, 3H, $J = 7.0$ Hz), 1.58–1.70 (m, 2H), 1.77–1.91 (m, 2H), 1.92–2.05 (m, 3H), 2.06–2.17 (m, 2H), 2.16 (s, 3H), 2.35 (s, 6H), 2.46–2.57 (m, 2H), 2.92 (d, 2H, $J = 5.5$ Hz), 3.11 (q, 2H, $J = 16.2$ Hz), 4.28–4.50 (brs, 1H), 4.32–4.38 (m, 1H), 4.70 (d, 1H, $J = 8.5$ Hz), 5.46 (d, 1H, $J = 12.0$ Hz), 5.87 (d, 1H, $J = 11.0$ Hz), 6.05 (d, 1H, $J = 12.0$ Hz), 7.12–7.18 (m, 1H), 7.19–7.26 (m, 4H), 8.09 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 11.1, 16.6, 19.0, 20.3, 20.8, 20.9, 22.9, 25.5, 26.2, 26.9, 32.4, 35.9, 37.6, 39.1, 39.7, 42.1, 44.5, 45.8, 51.2, 55.2, 62.7, 70.8, 125.6, 127.5, 129.4, 130.6, 139.7, 150.9, 162.7, 170.8, 171.7, 172.0, 173.3, 176.5, 181.8 ppm; IR: $\tilde{\nu}$ = 1226, 1496, 1542, 1741, 2964 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{40}H_{62}N_5O_9S$: 788.4268; found: 788.4256 $[M+H]^+$.

**Acetic acid pentafluorophenyl ester (19)**: To a solution of acetic acid (46 μL, 0.800 mmol) in EtOAc (2.00 mL, filtered through activated alumina) were added pentafluorophenol (162 mg, 0.880 mmol) and 1,3-dicyclohexylcarbodiimide (182 mg, 0.88 mmol). The reaction mixture was stirred for 12 h at RT at which time it was filtered (washing with EtOAc) and concentrated. Ester **19** was used immediately without further purification.

**Tubulysin analogue 7**: Pd/C (10 wt%, 7.0 μg) and azide **8** (15.0 mg, 0.021 mmol) were added to a solution of **19** (0.082 mmol) in EtOAc (0.40 mL, filtered through activated alumina). The reaction mixture was stirred under a hydrogen atmosphere for 21 h and then filtered through a plug of Celite with washing of the filter pad with EtOAc. The filtrate was concentrated, and a 1:1 mixture of deoxygenated $H_2O$/dioxane (2.0 mL) was added. The mixture was stirred for 7 h at RT and concentrated. Normal-phase HPFC ($CH_2Cl_2$/MeOH 99:1 → 90:10) afforded **7** as an amorphous solid (12.0 mg, 77%). $[\alpha]_D^{23} = -7.0$ ($c = 0.4$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.81 (d, 3H, $J = 7.0$ Hz), 0.86 (d, 3H, $J = 7.0$ Hz), 0.88 (d, 3H, $J = 7.0$ Hz), 0.92 (t, 3H, $J = 7.5$ Hz), 0.95 (d, 3H, $J = 7.0$ Hz), 1.07 (d, 3H, $J = 6.5$ Hz), 1.17 (d, 3H, $J = 7.5$ Hz), 1.12–1.22 (m, 1H), 1.26–1.36 (m, 1H), 1.57–1.69 (m, 2H), 1.80–1.91 (m, 1H), 1.95 (s, 3H), 1.96–2.08 (m, 4H), 2.09–2.16 (m, 1H), 2.16 (s, 3H), 2.25–2.34 (m, 1H), 2.45–2.57 (m, 2H), 2.91 (d, 2H, $J = 7.0$ Hz), 4.32–4.50 (brs, 1H), 4.34–4.42 (m, 1H), 4.62 (d, 1H, $J = 9.5$ Hz), 5.42 (d, 1H, $J = 12.0$ Hz), 5.89 (d, 1H, $J = 9.5$ Hz), 6.13 (d, 1H, $J = 12.0$ Hz), 7.12–7.18 (m, 1H), 7.19–7.25 (m, 4H), 8.10 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 11.0, 16.4, 18.8, 20.0, 20.8, 20.9, 22.2, 22.87, 22.88, 25.6, 26.9, 32.5, 35.8, 37.2, 38.1, 39.5, 42.3, 44.5, 50.9, 55.6, 70.8, 125.7, 127.5, 129.4, 130.6, 139.5, 150.7, 162.7, 170.9, 172.0, 173.0, 173.3, 176.6, 180.3 ppm; IR: $\tilde{\nu}$ = 1225, 1554, 1647, 1740, 2876, 2963 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{38}H_{56}N_4O_9NaS$: 767.3666; found: 767.3648 $[M+Na]^+$.

**Intermediate 21**: A solution of **20** (905 mg, 1.77 mmol) in THF (6.0 mL) was cooled to −45°C and KHMDS (6.02 mL, 3.01 mmol, 0.50 M in toluene) was added. The resulting mixture was stirred for 20 min at −45°C. Methyl iodide (754 mg, 5.31 mmol, filtered through activated alumina) was added, and the reaction mixture was allowed to warm to RT over 4.5 h at which time the reaction was quenched with MeOH (5.0 mL). The crude product was diluted with EtOAc (250 mL) and washed with brine (100 mL). The aqueous layer was extracted with EtOAc (2×100 mL). The organic portions were dried, filtered, and concentrated. Normal-phase HPFC (hexanes/EtOAc 95:5 → 60:40) yielded **21** as an amorphous solid (761 mg, 82%). The $^1$H NMR corresponded to a 10:1 mixture of rotamers, with the major isomer reported. $[\alpha]_D^{23} = +67.5$ ($c = 1.0$ in $CHCl_3$); $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ = 0.55–0.70 (m, 6H), 0.84 (d, 3H, $J$ = 6.8 Hz), 0.85–0.93 (m, 15H), 0.95 (d, 3H, $J = 6.8$ Hz), 1.17–1.29 (m, 1H), 1.60–1.79 (m, 2H), 2.00–2.20 (m, 3H), 2.92 (s, 3H), 3.50 (d, 1H, $J$ = 9.6 Hz), 3.89 (s, 3H), 4.37–4.45 (m, 1H), 4.90 (dd, 1H, $J = 3.6$, 6.4 Hz), 8.07 ppm (s, 1H); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ 4.6, 6.7, 10.6, 15.9, 19.1, 20.0, 25.0, 30.2, 34.9, 40.1, 52.2, 57.3, 63.9, 71.0, 127.4, 146.4, 161.8, 169.5, 178.3 ppm; IR: $\tilde{\nu}$ = 1094, 1210, 1238, 1645, 1736, 2098, 2877, 2960 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{24}H_{44}N_5O_4SiS$: 526.2883; found: 526.2877 $[M+H]^+$.

**Intermediate 23**: Pd/C (10 wt%, 242 mg) and azide **21** (359 mg, 0.683 mmol) were added to a solution of **22** (2.17 mmol) in EtOAc (6.80 mL, filtered through activated alumina). The reaction mixture was stirred under a hydrogen atmosphere for 26 h and then filtered through a plug of Celite, with washing of the filter pad with EtOAc. Normal-phase HPFC purification (EtOAc/MeOH 99:1 → 95:5) provided 483 mg of Mep-coupled product. The product was dissolved in deoxygenated AcOH/$H_2O$/THF (35.0 mL, 3:1:1) and stirred at RT for 28 h. Concentration followed by normal-phase HPFC purification (EtOAc/MeOH 98:2 → 85:15) afforded **23** as an amorphous solid (302 mg, 87%, over two steps). The $^1$H NMR corresponded to a 7.5:1 mixture of rotamers, with the major isomer reported. $[\alpha]_D^{23} = -4.8$ ($c = 1.0$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.81 (d, 3H, $J = 6.5$ Hz), 0.91 (t, 3H, $J$ = 7.5 Hz), 0.97 (d, 3H, $J = 6.5$ Hz), 0.99 (d, 3H, $J = 6.5$ Hz), 1.16–1.34 (m, 2H), 1.49–1.66 (m, 4H), 1.75 (d, 2H, $J = 10.5$ Hz), 1.77–1.86 (brs, 1H), 1.89–2.01 (m, 2H), 2.02–2.09 (m, 1H), 2.17 (s, 3H), 2.18–2.26 (m, 1H), 2.57 (d, 1H, $J = 9.0$ Hz), 2.85–2.95 (m, 1H), 3.17 (s, 3H), 3.91 (s, 3H), 4.40–4.55 (brs, 1H), 4.68 (d, 1H, $J = 9.5$ Hz), 4.75 (d, 1H, $J = 9.0$ Hz), 8.32 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 11.2, 16.2, 20.5, 20.7, 24.4, 25.9, 26.3, 31.1, 31.6, 32.1, 37.7, 38.8, 44.9, 52.8, 55.0, 56.7, 69.9, 70.6, 129.3, 147.5, 163.2, 175.4, 175.7, 180.6; IR: $\tilde{\nu}$ = 1095, 1212, 1238, 1495, 1622, 1722, 2936 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{25}H_{43}N_4O_5S$: 511.2954; found: 511.2947 $[M+H]^+$.

**Intermediate 24**: $Me_3SnOH$ (496 mg, 2.74 mmol) was added to a solution of methyl ester **23** (175 mg, 0.343 mmol) in dichloroethane (17.0 mL). The reaction mixture was heated to 60°C for 20 h and then concentrated. Column chromatography (100% $CH_2Cl_2$ to elute tin containing materials followed by $CH_2Cl_2$/MeOH/$NH_4OH$ 80:20:1 to elute the product) afforded **24** as an amorphous solid (150 mg, 88%). The $^1$H NMR corresponded to a 6:1 mixture of rotamers, with the major isomer reported. $[\alpha]_D^{23} = -17.4$ ($c = 1.0$ in MeOH); $^1$H NMR ([D$_6$]DMSO, 500 MHz): $\delta$ = 0.70 (m, 3H), 0.75–0.82 (m, 3H), 0.83–0.90 (m, 6H), 1.04–1.16 (m, 1H), 1.17–1.28 (m, 2H), 1.37–1.55 (m, 3H), 1.56–1.72 (m, 3H), 1.73–1.91 (m, 3H), 2.00–2.24 (m, 2H), 2.22 (s, 3H), 2.84 (brs, 1H), 2.94–3.00 (m, 1H), 3.04 (s, 3H), 4.16–4.60 (brs, 1H), 4.49 (d, 1H, $J = 10.5$), 4.56 (app t, 1H, $J = 9.0$), 5.93–6.40 (brs, 1H), 8.05 (s, 1H), 8.25 ppm (s, 1H); $^{13}$C NMR ([D$_6$]DMSO, 125 MHz): $\delta$ = 10.0, 14.7, 19.1, 19.5, 19.6, 21.8, 23.58, 23.62, 28.75, 28.8, 35.2, 36.7, 42.6, 52.3, 54.1, 67.1, 67.5, 126.7, 147.8, 162.2, 170.7, 172.0, 177.6 ppm; IR: $\tilde{\nu}$ = 1276, 1368, 1471, 1616, 2874, 2961 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{24}H_{41}N_4O_5S$: 497.2798; found: 497.2793 $[M+H]^+$.

**Tubulysin analogue 10**: Acid **24** (34.0 mg, 0.0684 mmol) was added to a solution of pentafluorophenol (19.0 mg, 0.103 mmol) and 1,3-diisopropylcarbodiimide (12.0 μL, 0.0752 mmol) in $CH_2Cl_2$ (0.52 mL) at 0°C. The reaction mixture was warmed to RT, stirred for 24 h, and concentrated. EtOAc (10 mL) was added, and the crude product was filtered with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated, and the crude material was used without further purification. DMF (0.270 mL) was added to the crude product at 0°C, followed by **17** (50.0 mg, 0.205 mmol) and $i$Pr$_2$EtN (60.0 μL, 0.342 mmol). The reaction mixture was allowed to warm to RT, stirred for 24 h at RT, and concentrated. Normal-phase HPFC purification ($CH_2Cl_2$/MeOH 98:2 → 80:20) followed by reverse-phase HPFC (MeCN/$H_2O$ 20:80 → 100:0) afforded 34.0 mg of product containing trace amounts of $i$Pr$_2$EtN. The product mixture (34.0 mg, 0.496 mmol) was dissolved in pyridine (0.50 mL), cooled to 0°C, and acetic anhydride (38.0 μL, 0.397 mmol) was added. The reaction mixture was allowed to warm to RT over 2 h and was stirred at RT for 22 h. The reaction mixture was then cooled to 0°C, and a 1:1 mixture of deoxygenated $H_2O$/dioxane (1.6 mL) was added. The mixture was allowed to warm to RT and was stirred for 20 h at RT. The solvent was removed under reduced pressure. Normal-phase HPFC ($CH_2Cl_2$/MeOH 95:5 → 80:20) followed by lyophilization afforded **10** as an amorphous solid (28.0 mg, 56%, over three steps). The $^1$H NMR corresponded to a 16:1 mixture of rotamers, with the major isomer reported. $[\alpha]_D^{23} = -19.2$ ($c = 0.9$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.81 (d, 3H, $J = 6.5$ Hz), 0.92 (t, 3H, $J = 7.3$ Hz), 0.98 (d, 3H, $J = 6.5$ Hz), 1.03 (d, 3H, $J = 6.5$ Hz), 1.16 (d, 3H, $J = 7.0$ Hz), 1.09–1.23 (m, 1H), 1.37–1.41 (m, 1H), 1.56–1.74 (m, 5H), 1.75–1.92 (m, 4H), 1.96–2.05 (m, 1H), 2.15 (s, 3H), 2.31 (s, 3H), 2.23–2.41 (m, 3H), 2.51 (brs, 1H), 2.85 (d, 1H, $J$ = 10.5 Hz), 2.92 (d, 2H, $J = 6.5$ Hz), 3.05 (d, 1H, $J = 11.5$ Hz), 3.10 (s, 3H), 4.30–4.50 (m, 2H), 4.73 (d, 1H, $J = 8.0$ Hz), 5.71 (dd, 1H, $J = 2.5$, 11.0 Hz), 7.13–7.18 (m, 1H), 7.19–7.25 (m, 4H), 8.08 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 11.3, 16.4, 19.1, 20.4, 20.6, 20.9, 23.7, 25.5, 25.5, 30.9, 31.0, 31.1, 35.6, 37.6, 39.5, 39.6, 42.0, 44.2, 51.2, 55.2, 56.4, 69.7, 71.2, 125.1, 127.4, 129.3, 130.6, 139.8, 151.1, 162.7, 171.6, 171.8, 173.6, 175.0, 182.5 ppm; IR: $\tilde{\nu}$ = 1220, 1495, 1541, 1643, 1712, 2964 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{38}H_{57}N_5O_7S$: 728.4057; found: 728.4053 $[M+H]^+$.

**Intermediate 25**: In a sealed tube, a solution of methylamine (5.00 mmol, 2 M in THF) was added to a solution of **23** (27.0 mg, 0.0529 mmol) in MeOH (2.50 mL). The reaction solution was heated to 100°C for 21 h. After the solution cooled to RT, the solvent was removed under reduced pressure. Reverse-phase HPFC (MeCN/$H_2O$ 20:80 → 100:0) followed by lyophilization provided compound **25** (14.0 mg, 52%) as an amorphous

solid. The $^1$H NMR corresponded to a 6:1 mixture of rotamers, with the major isomer reported. $[\alpha]_D^{23} = -2.9$ ($c = 1.0$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.83 (d, 3H, $J = 6.5$ Hz), 0.90 (t, 3H, $J = 7.5$ Hz), 0.966 (d, 3H, $J = 6.5$ Hz), 0.974 (d, 3H, $J = 6.5$ Hz), 1.15–1.25 (m, 1H), 1.25–1.32 (m, 1H), 1.48–1.66 (m, 4H), 1.72 (d, 2H, $J = 10.5$ Hz), 1.81–1.99 (m, 3H), 2.00–2.10 (m, 1H), 2.16 (s, 3H), 2.19–2.37 (m, 1H), 2.53–2.58 (m, 1H), 2.87–2.94 (m, 1H), 2.92 (s, 3H), 3.17 (s, 3H), 4.16–4.58 (br s, 1H), 4.64 (dd, 1H, $J = 2.3$, 10.3 Hz), 4.72 (d, 1H, $J = 9.0$ Hz), 8.06 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 11.1, 16.1, 20.5, 20.6, 24.4, 25.9, 26.3, 26.4, 31.3, 31.7, 37.8, 38.8, 44.8, 55.4, 56.7, 70.0, 70.6, 124.2, 151.0, 164.4, 175.3, 175.7, 179.4 ppm; IR: $\tilde{\nu}$ = 1070, 1499, 1551, 1646, 2876, 2961 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{25}H_{44}N_5O_4S$: 510.3114; found: 510.3098 $[M+H]^+$.

**Tubulysin analogue 11**: A solution of **25** (12.0 mg, 0.0235 mmol) in pyridine (0.500 mL) was cooled to 0 °C, and acetic anhydride (18.0 μL, 0.188 mmol) was added. The reaction mixture was allowed to warm to RT over 2 h and was stirred at RT for 21 h. The solvent was removed under reduced pressure. Reverse-phase HPFC (MeCN/$H_2O$ 20:80 → 100:0) followed by lyophilization afforded **11** as an amorphous solid (9.3 mg, 72 %). The $^1$H NMR corresponded to a 23:1 mixture of rotamers, with the major isomer reported. $[\alpha]_D^{23} = -2.2$ ($c = 0.6$ in MeOH); $^1$H NMR ([D$_4$]methanol, 500 MHz): $\delta$ = 0.80 (d, 3H, $J = 7.0$ Hz), 0.92 (t, 3H, $J = 7.5$ Hz), 0.98 (d, 3H, $J = 7.0$ Hz), 1.02 (d, 3H, $J = 6.5$ Hz), 1.13–1.22 (m, 1H), 1.24–1.34 (m, 1H), 1.49–1.67 (m, 4H), 1.72–1.78 (m, 2H), 1.79–1.91 (m, 2H), 2.07 (dt, 1, $J = 3.0$, 11.5 Hz), 2.15 (s, 3H), 2.18 (s, 3H), 2.22–2.31 (m, 1H), 2.34–2.41 (m, 1H), 2.56 (dd, 1H, $J = 2.5$, 11.0 Hz), 2.90–2.95 (m, 1H), 2.94 (s, 3H), 3.11 (s, 3H), 4.40–4.51 (br s, 1H), 4.74 (d, 1H, $J = 8.0$ Hz), 5.70 (dd, 1H, $J = 2.5$, 11.5 Hz), 8.14 ppm (s, 1H); $^{13}$C NMR ([D$_4$]methanol, 125 MHz): $\delta$ = 11.2, 16.4, 20.4, 20.6, 20.9, 24.4, 25.6, 26.3, 26.4, 31.1, 31.7, 35.7, 37.7, 44.9, 54.9, 56.7, 70.6, 71.2, 125.0, 150.9, 163.9, 171.82, 171.83, 175.3, 175.6 ppm; IR: $\tilde{\nu}$ = 1221, 1498, 1549, 1643, 1755, 2937 cm$^{-1}$; HRMS (FAB): $m/z$: calcd for $C_{27}H_{46}N_5O_5S$: 552.3220; found: 552.3218 $[M+H]^+$.

**Biological assays**

**Cell culture and growth inhibition assay**: Cell lines were obtained from the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ). All cell lines were cultivated under conditions recommended by their respective depositors. Growth inhibition was measured in microtiter plates. Aliquots of 120 μL of the suspended cells (50000 mL$^{-1}$) were given to 60 μL of a serial dilution of the inhibitor and incubated at 37 °C and 10 % $CO_2$. After 5 d, when control cells had grown to confluence state, the metabolic activity in each well was determined using an MTT assay.[9] $IC_{50}$ values were defined as the analogue concentration that showed only 50 % of the activity of the control wells.

**Fluorescence staining**: $PtK_2$ cells (ATCC CCL-56) were grown on glass coverslips (13 mm diameter) in four-well plates. Exponentially growing cells were incubated with the analogues for 18 h. Cells were then fixed with cold (−20 °C) acetone/methanol 1:1 for 10 min. For labeling the microtubules, cells were incubated with a primary monoclonal antibody against α-tubulin (1:500; Sigma), then with a secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 488 (1:200; Molecular Probes) at 37 °C for 45 min. Nuclei and chromosomes were stained with DAPI (1 μg mL$^{-1}$). The cells were washed with PBS between all incubations. The coverslips were mounted using Prolong Antifade Gold (Molecular Probes), and viewed with a Zeiss Axiophot fluorescence microscope using appropriate filter sets.

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