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Total Synthesis and Biological Evaluation of Natural and Designed Tubulysins

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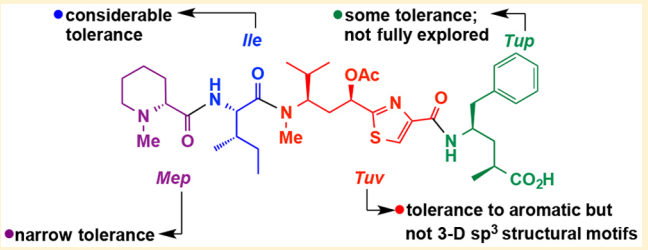
Supporting Information

ABSTRACT: A streamlined total synthesis of N^{14}-desacetoxytubulysin H (**Tb1**) based on a C−H activation strategy and a short total synthesis of pretubulysin D (**PTb-D43**) are described. Applications of the developed synthetic strategies and technologies to the synthesis of a series of tubulysin analogues (**Tb2**−**Tb41** and **PTb-D42**) are also reported. Biological evaluation of the synthesized compounds against an array of cancer cells revealed a number of novel analogues (e.g., **Tb14**), some with exceptional potencies against certain cell lines [e.g., **Tb32** with IC_{50} = 12 pM against MES SA (uterine sarcoma) cell line and 2 pM against HEK 293T (human embryonic kidney) cell line], and a set of valuable structure−activity relationships. The highly potent cytotoxic compounds discovered in this study are highly desirable as payloads for antibody−drug conjugates and other drug delivery systems for personalized targeted cancer chemotherapies.



1. INTRODUCTION

The tubulysins were originally isolated from strains of the myxobacteria *Archangium gephyra* and *Angiococcus disciformis* and shown to be potent cytotoxic compounds.[1−4] Members of this class of natural products include tubulysins A−I and pretubulysin D (**PTb-D43**) (Figure 1). Interestingly, while some of these compounds exhibit extremely high cytostatic properties (IC_{50} values in the picomolar range) against human cancer cell lines, they show little, if any, activity against bacteria or filamentous fungi.[1−9] Their mechanism of action involves depolymerization of microtubules leading to collapse of the cytoskeleton.[5−7,10] Their killing properties extend over a wide range of cancer cells, including ovarian, breast, colon, lung, and leukemia. Their impressive biological properties coupled with their natural scarcity stimulated intense research activities directed toward their chemical synthesis, structural modification, and biological evaluation. As a result of this surge, total syntheses of several naturally occurring tubulysins[11−13] (e.g., A, B, C, G and I,[14−17] U[15,18−20] and V,[15,19−21] and pretubulysin D;[8,27−29] Figure 1) and analogues thereof have been reported.[22−40] Among the most interesting and potent of the latter is N^{14}-desacetoxytubulysin H (**Tb1**) (Figure 1).[22,26,33]

Because of their impressive activities against cancer cells, the tubulysins are of particular interest as payloads for antibody−drug conjugates (ADCs)[41] and probody−drug conjugates (PDCs),[42] where extremely high potencies are desirable. In this article, we describe our approaches to the total synthesis of **Tb1**, **PTb-D43**, and several of their designed analogues (**Tb2**−**Tb41** and **PTb-D42**; Figure 2) and their biological evaluation, which led to the discovery of extremely potent cytotoxic compounds and useful structure−activity relationships (SARs).

2. RESULTS AND DISCUSSION

2.1. Retrosynthetic Analysis of N^{14}-Desacetoxytubulysin H. The peptide nature of the tubulysin structure clearly points to a synthetic strategy based on the three amide bond couplings shown in Figure 3, as exemplified for the case of N^{14}-desacetoxytubulysin H (**Tb1**). Thus, three amide bond disconnections lead to the fragments *N*-methyl-D-pipecolic acid (Mep), L-isoleucine (Ile), tubuvaline (Tuv), and tubuphenylalanine (Tup), as indicated in Figure 3a. The most interesting disconnection of the tubulysin molecule, however, is that based on the C−H activation coupling (C10−C11 bond) of an aldehyde (**A**) with a thiazole system (**B**) to afford a thiazolyl ketone (**C**), whose asymmetric reduction would lead to a thiazolyl alcohol (**D**) corresponding to the desired structural motif of the target molecule (see Figure 3b).

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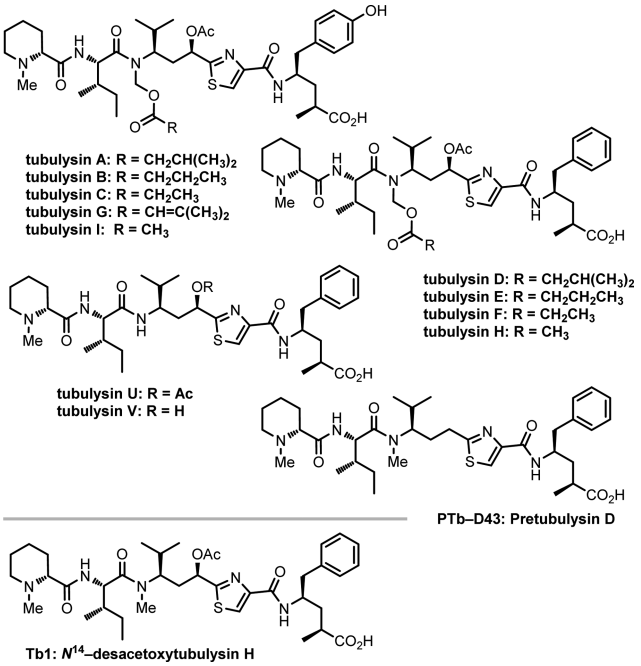

Figure 1. Molecular structures of naturally occurring tubulysins A, B, C, G, I; D, E, F, H; U, V; pretubulysin D (**PTb-D43**); and N^{14}-desacetoxytubulysin H (**Tb1**).

2.2. Total Synthesis of N^{14}-Desacetoxytubulysin H (Tb1) and Its Methyl Ester (Tb2). Schemes 1A and 1B summarize the streamlined total synthesis of N^{14}-desacetoxytubulysin H (**Tb1**) starting from the known and readily available aldehyde **1** [Scheme 1A,a; prepared from (*S*)-Boc-valine in multigram quantities].[43] Thus, a brief optimization study of the C−H-activation-based coupling of aldehyde **1** with a suitable thiazolyl moiety (**2**, **2a**−**f**; Scheme 1A,b) led to the finding that thiazolyl acetate **2** performed the best as a substrate suitable for this reaction, furnishing the coupling product, ketone **3**, in 81% yield under the previously reported conditions [$PhI(OCOCF_3)_2$, $TMSN_3$].[44] Reduction of thiazolyl ketone **3** with (*S*)-CBS in the presence of $BH_3{\cdot}SMe_2$[45] then produced alcohol **4** in 82% yield as a single diastereoisomer after chromatographic purification. Elaboration of hydroxy compound **4** to acetoxycarboxylic acid **5** was achieved through a sequence involving deacetylation (K_2CO_3, MeOH), selective oxidation of the primary alcohol (TEMPO, BAIB; then $NaClO_2$), and acetylation (Ac_2O, py) of the resulting secondary alcohol in 66% overall yield. Coupling of carboxylic acid **5** and amino ester **6**[34] in the presence of *i*-BuOCOCl and Et_3N afforded amide **7** in 91% yield.

The Boc group was cleaved from **7** using TFA, and the resulting amine was coupled with acid fluoride **8**[22] to afford peptide **9** (*i*-Pr_2NEt, 92%), as shown in Scheme 1B. Removal of the Fmoc protecting group from **9** [$N(CH_2CH_2NH_2)_3$] followed by coupling of the so-generated amine with *N*-methyl-D-pipecolic acid (**10**) provided tubulysin H methyl ester (**Tb2**) in 62% overall yield. Conversion of **Tb2** to **Tb1** required sequential treatment with Me_3SnOH[46] (cleavage of both the methyl ester and the acetate) and reacetylation (Ac_2O, py)/aqueous workup; this was achieved in 56% overall yield, as shown in Scheme 1B.

2.3. Total Synthesis of Pretubulysin D (PTb-D43) and Its Methyl Ester (PTb-D42). Scheme 2 summarizes the total synthesis of pretubulysin D (**PTb-D43**) starting from known aldehyde **1**.[43] Reduction of **1** with $NaBH_4$ followed by bromination of the resulting alcohol using CBr_4 and PPh_3 furnished bromide **11** in 74% overall yield. Coupling of thiazole TBS ether **12** with bromide **11** in the presence of *n*-BuLi gave product **13** in 76% yield. Elaboration of compound **13** to carboxylic acid **14** was achieved through desilylation (TBAF) and oxidation of the resulting alcohol (DMP then $NaClO_2$) in 92% overall yield. Coupling of carboxylic acid **14** with amino ester **6**[34] in the presence of HATU and Et_3N led to amide **15** in 81% yield. Boc group removal from the latter compound (TFA) followed by coupling of the resulting amine with acid fluoride **8**[22] furnished peptide **16** (*i*-Pr_2NEt) in 94% yield, as shown in Scheme 2. Removal of the Fmoc protecting group from **16** [$N(CH_2CH_2NH_2)_3$] and coupling of the resulting amine with **10** provided pretubulysin D analogue **PTb-D42** in 72% overall yield, whose conversion to **PTb-D43** was accomplished with Me_3SnOH[46] (82% yield) or LiOH (90% yield), as shown in Scheme 2.

2.4. Synthesis of N^{14}-Desacetoxytubulysin H Analogues (Tb3−Tb41). With a practical and efficient synthesis of tripeptide Fmoc derivative **9** available to us (see Scheme 1B), we turned our attention to its application to the construction of designed tubulysin analogues **Tb3**−**Tb10**, **Tb34**, and **Tb35** (for structures, see Figure 2) with varying amino acid residues at the "left side" (Mep) of the molecule. The required building blocks for these analogues (**17**−**26**; Scheme 3) were prepared as described in the Supporting Information (SI). Removal of the Fmoc protecting group from **9** [$N(CH_2CH_2NH_2)_3$] followed by coupling of the resulting amine with carboxylic acids **20**−**25**, ester **17**, and isocyanates **18**, **19**, and **26** furnished the corresponding tetrapeptides, whose appropriate functional group manipulations led to the targeted tubulysin analogues **Tb3**−**Tb10**, **Tb34**, and **Tb35** in yields ranging from 46 to 90%, as summarized in Scheme 3.

Tubulysin analogues **Tb11**−**Tb16** (for structures, see Figure 2), in which the "right end" amino acid (Tup) has been replaced with various amino acid residues, were synthesized through three sequential peptide coupling reactions from unnatural amino acid derivative **5** (see Scheme 1A) and building blocks **17**, **27**, and **28** (Scheme 4; synthesized as described in the SI), as summarized in Scheme 4. Thus, coupling of **5** with either cubane (**29**)[47] or [1.1.1]bicyclopentane methyl ester amine (**30**)[47] (see the SI for further details) in the presence of HATU and HOAt furnished dipeptides **31** (55% yield) and **32** (54% yield), respectively. Exposure of the so-formed dipeptides to TFA resulted in removal of the Boc group to afford the corresponding amines, whose coupling with acid fluoride **8** in the presence of *i*-Pr_2NEt in DMF led to the formation of tripeptides **33** (76% overall yield) and **34** (71% overall yield), respectively. Finally, removal of the Fmoc group from **33** and **34** [$N(CH_2CH_2NH_2)_3$] followed by coupling of the resulting amines with pentafluorophenyl esters **17**, **27**, and **28** gave tubulysin analogues **Tb11**−**Tb16** in 58−73% overall yields, as shown in Scheme 4 (see the SI for further details).

Scheme 5 summarizes the synthesis of analogues **Tb17**−**Tb19** possessing the rigid cubane or [1.1.1]bicyclopentane functionality instead of the thiazole moiety. Thus, amino esters **29**[47] and **30**[47] were coupled with the amino acid residues **35**, **6**, and **8** and then with **10** or *N*,*N*-dimethylglycine (**36**) to furnish the desired N^{14}-desacetoxytubulysin derivatives **Tb17**, **Tb18**, and **Tb19** in overall yields of 75%, 58%, and 72%, respectively, as depicted in Scheme 5.

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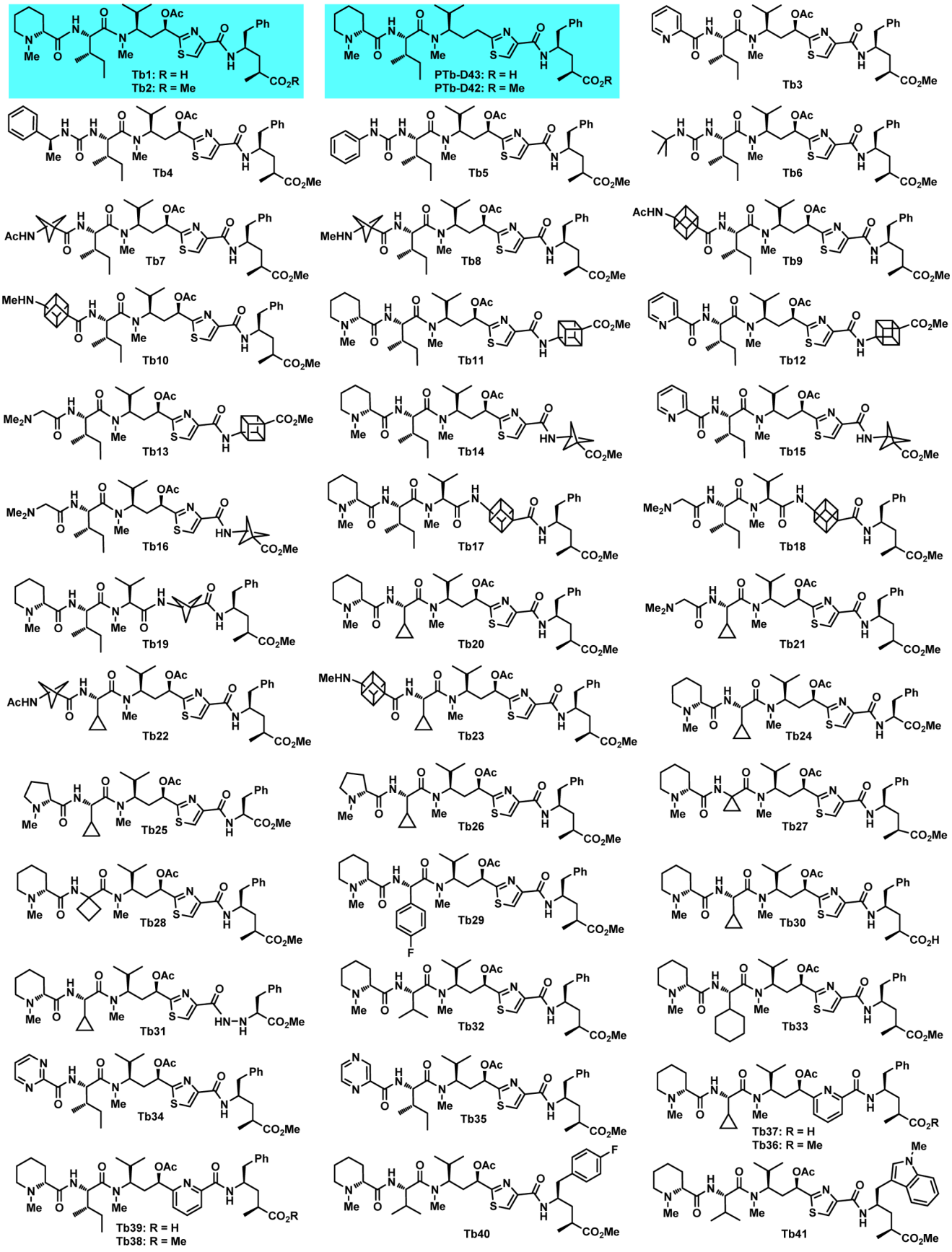

Figure 2. Molecular structures of N^{14}-desacetoxytubulysin H (**Tb1**), pretubulysin D (**PTb-D43**), and designed analogues (**Tb2**–**Tb41** and **PTb-D42**).

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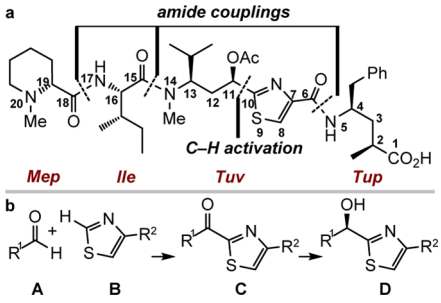
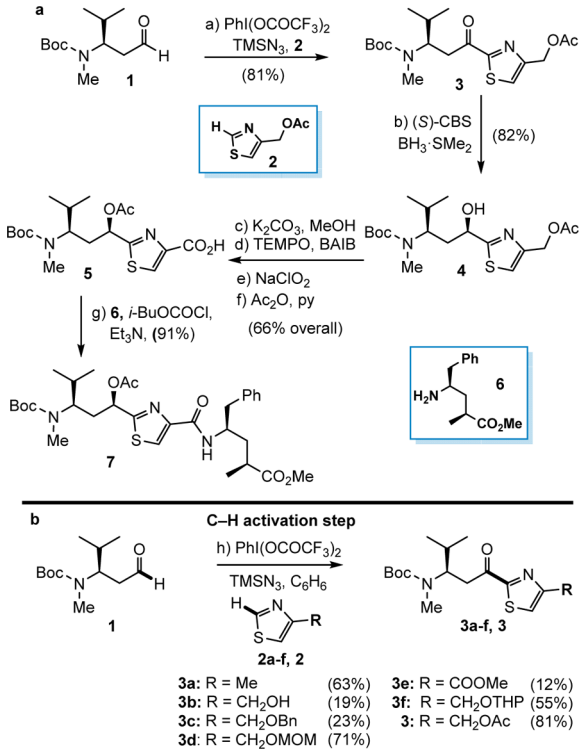


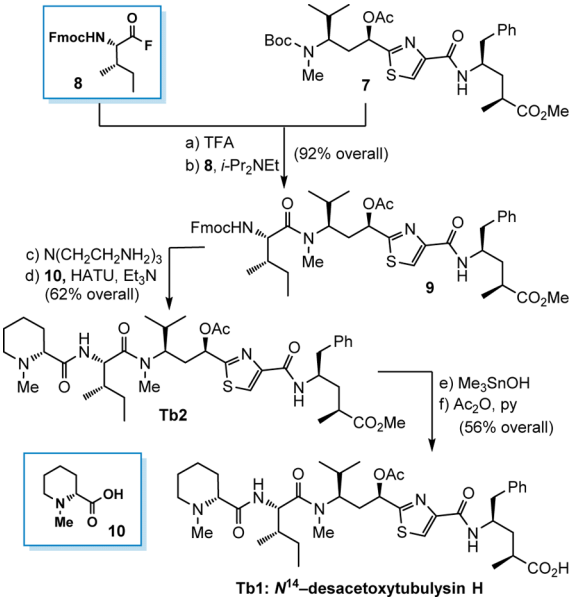
Figure 3. (a) Retrosynthetic analysis of **Tb1**. (b) C−H activation step to form the C10−C11 bond.

Scheme 1A. Synthesis of Tb1 Intermediate 7[a]



[a]Reagents and conditions: Panel (a): (a) **1** (2.0 equiv), **2** (1.0 equiv), $TMSN_3$ (2.0 equiv), PIFA (2.0 equiv), benzene, 23 °C, 16 h, then **1** (2.0 equiv), $TMSN_3$ (2.0 equiv), PIFA (2.0 equiv), 23 °C, 12 h, 81%; (b) (*S*)-CBS (0.2 equiv), BH_3·SMe_2 (1.0 equiv), 0 → 23 °C, 18 h, 82%; (c) K_2CO_3 (4.0 equiv), MeOH, 23 °C, 3 h, 93%; (d) TEMPO (0.1 equiv), BAIB (1.0 equiv), CH_2Cl_2, 23 °C, 16 h, 96%; (e) $NaClO_2$ (5.0 equiv), NaH_2PO_4·H_2O (12 equiv), 2-methyl-2-butene (7.5 equiv), *t*-BuOH, THF, H_2O, 23 °C, 12 h; (f) Ac_2O (3.2 equiv), py (3.5 equiv), CH_2Cl_2, 0 → 23 °C, 15 h, 74% for the two steps; (g) *i*-BuOCOCl (2.0 equiv), Et_3N (4.0 equiv), THF, −20 °C, 30 min, then **6** (2.1 equiv), −20 → 23 °C, 24 h, 91% or **6** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 18 h, 74%. Panel (b): Screening of thiazole substrates for C−H/C−H coupling: (h) **1** (2.0 equiv), **2** (1.0 equiv), $TMSN_3$ (2.0 equiv), PIFA (2.0 equiv), benzene, 23 °C, 16 h; then **1** (2.0 equiv), $TMSN_3$ (2.0 equiv), PIFA (2.0 equiv), 23 °C, 12 h, 12−81% yield. Abbreviations: THF = tetrahydrofuran; TMS = trimethylsilyl; PIFA = phenyliodine(III) bis(trifluoroacetate); TEMPO = 2,2,6,6-tetramethylpiperidin-1-oxyl; BAIB = bis(acetoxy)iodobenzene; DMF = dimethylformamide; HATU = 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluoro-phosphate; (*S*)-CBS = (*S*)-(−)-methyl-CBS-oxazaborolidine; Boc = *tert*-butyloxycarbonyl; TBS = *tert*-butyldimethylsilyl; THP = tetrahydropyran-2-yl; Bn = benzyl; MOM = methoxymethyl.

Scheme 1B. Total Synthesis of N^{14}-Desacetoxytubulysin H (Tb1) and Its Methyl Ester (Tb2)[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 3 h; (b) **8** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 92% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 3 h; (d) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 62% for the two steps; (e) Me_3SnOH (20 equiv), 1,2-dichloroethane, reflux, 12 h; (f) Ac_2O (4.0 equiv), py, 0 → 23 °C, 12 h, 56% for the two steps. Abbreviations: TFA = trifluoroacetic acid; Fmoc = fluorenylmethyloxycarbonyl; py = pyridine; Ac = acetyl.

Scheme 6 summarizes the synthesis of tubulysin analogues **Tb20**−**Tb23**, **Tb26**−**Tb30**, **Tb32**, and **Tb33**, which incorporate varying combinations of structural motifs in the place of the *N*-methyl-D-pipecolic acid and isoleucine residues. Their synthesis began with removal of the Boc group from dipeptide **7** (see Scheme 1A) followed by coupling with Fmoc-protected acid fluorides **43**−**48** [prepared from their amino acid counterparts **43a**−**48a**[22] by sequential exposure to Fmoc-Cl and DAST (76−95% yield for the two steps); see Scheme 6 (top)] to give tripeptides **49**−**54** in 72−92% yield for the two steps, as shown in Scheme 6 (see the SI for further details). Cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] from these intermediates followed by coupling with building blocks **10**, **22**, **25**, **36**, and **55** and further standard elaborations of the resulting products led to tubulysin analogues **Tb20**−**Tb23**, **Tb26**−**Tb30**, **Tb32**, and **Tb33** in overall yields of 39−91%.

The synthesis of tubulysin analogues **Tb24** and **Tb25** (for structures, see Figure 2), in which the "right end" amino acid residue (Tup), isoleucine (Ile), and "left end" (Mep) have been replaced with **56**, **43**, and **10** or **55**, respectively, as shown in Scheme 7. Thus, coupling of **5** with **56** in the presence of HATU furnished dipeptide **57** in 93% yield. Exposure of the so-formed dipeptide to TFA resulted in removal of the Boc group to afford the corresponding amine, whose coupling with acid fluoride **43** in the presence of *i*-Pr_2NEt in DMF led to the formation of tripeptide **58** in 85% overall yield. Finally, removal of the Fmoc group from **58** [$N(CH_2CH_2NH_2)_3$] followed by coupling of the resulting amine with **10** or **55** under HATU conditions furnished tubulysin analogues **Tb24** (82% yield) and **Tb25** (97% yield), as shown in Scheme 7 (see the SI for further details).

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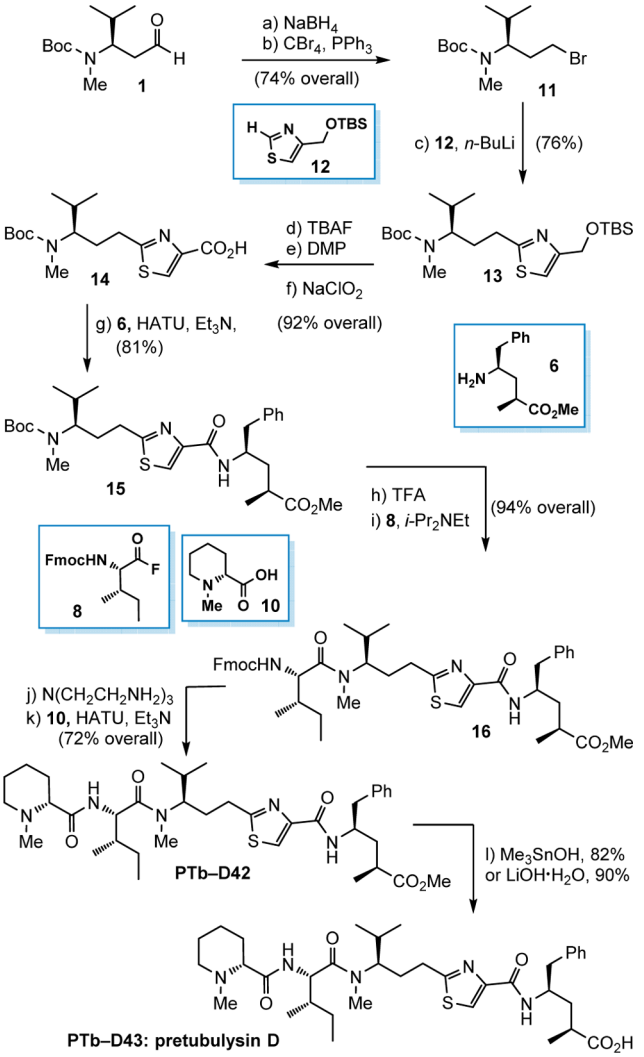
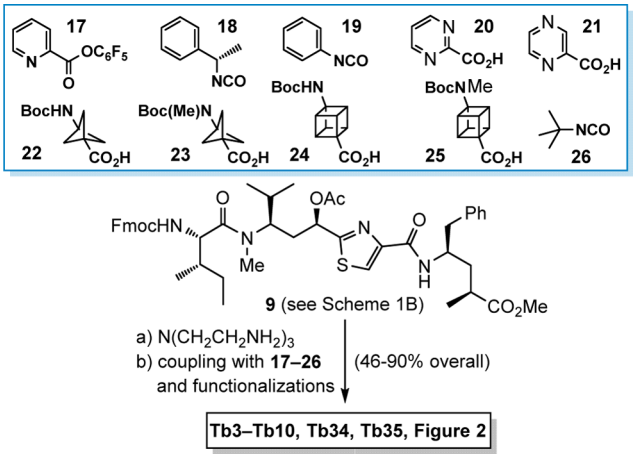
Scheme 2. Total Synthesis of Pretubulysin D (PTb-D43) and Its Methyl Ester (PTb-D42)[a]



[a]Reagents and conditions: (a) $NaBH_4$ (1.5 equiv), MeOH, 0 → 23 °C, 1 h, 92%; (b) CBr_4 (2.0 equiv), PPh_3 (2.0 equiv), 0 → 10 °C, 1 h, 80%; (c) **12** (1.1 equiv), *n*-BuLi (1.2 equiv; 2.6 M in hexane), THF, −78 → 0 °C, 3 h, 76%; (d) TBAF (2.0 equiv; 1 M in THF), THF, 0 °C, 1 h, 94%; (e) DMP (1.5 equiv), CH_2Cl_2, 23 °C, 1 h, 90%; (f) $NaClO_2$ (5.4 equiv), $NaH_2PO_4·H_2O$ (12 equiv), 2-methyl-2-butene (7.5 equiv), *t*-BuOH, THF, H_2O, 23 °C, 1 h; 92%; (g) **6** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 18 h, 81%; (h) TFA (45 equiv), CH_2Cl_2, 23 °C, 2 h; (i) **8** (4.1 equiv), *i*-Pr_2NEt (6.2 equiv), DMF, 0 → 23 °C, 18 h, 94% for the two steps; (j) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (k) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 72% for the two steps; (l) Me_3SnOH (20 equiv), 1,2-dichloroethane, reflux, 12 h, 82% or $LiOH·H_2O$ (5.0 equiv), THF, H_2O, 23 °C, 24 h, 90%. Abbreviations: TBAF = tetra-*n*-butylammonium fluoride; DMP = Dess−Martin periodinane.

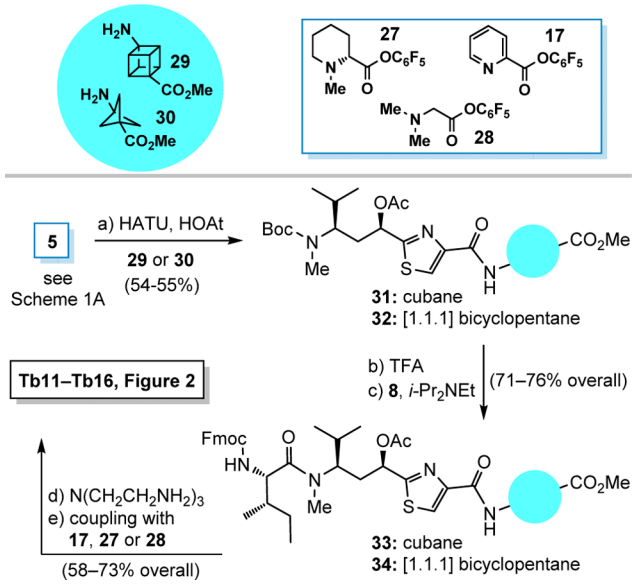
Tubulysin analogue **Tb31**, in which the "right side" amide linkage has been replaced by a hydrazide bond, was synthesized from amino acid derivative **5** (see Scheme 1A) through the sequence shown in Scheme 8. Thus, **5** was converted to its methyl ester **59** ($TMSCHN_2$, 73% yield) and thence to dipeptide **60** by removal of the Boc group (TFA) followed by coupling of the resulting amine with acid fluoride fragment **43** (*i*-Pr_2NEt, 75% overall yield). Compound **60** was treated with

Scheme 3. Synthesis of N^{14}-Desacetoxytubulysin H Analogues Tb3−Tb10, Tb34, and Tb35[a]



[a]Reagents and conditions: (a) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 3 h; (b) **17** (6.0 equiv), DMF, 0 → 23 °C, 24 h, 58% for the two steps for **Tb3**; or **18** or **19** or or **26** (6.0 equiv), *i*-Pr_2NEt (6.0 equiv), CH_2Cl_2, 0 → 23 °C, 24 h, 80% for the two steps for **Tb4**, 74% for the two steps for **Tb5**, 83% for the two steps for **Tb6**; or **22** or **24** or **23** or **25** (1.5 equiv), HATU (1.3 equiv), HOAt (1.3 equiv), *i*-Pr_2NEt (3.0 equiv), DMF, 0 → 23 °C, 24 h, 48% for the two steps for Boc-protected **Tb7**, 52% for the two steps for Boc-protected **Tb9**, 46% for the two steps for Boc-protected **Tb8**, 52% for the two steps for Boc-protected **Tb10**; then TFA (30 equiv), CH_2Cl_2, 0 → 23 °C, 12 h, 90% for **Tb8**, 66% for **Tb10**; then Ac_2O (10 equiv), py, 0 → 23 °C, 12 h, 56% for the two steps for **Tb7**, 55% for the two steps for **Tb9**; or **20** or **21** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 79% for the two steps for **Tb34**, 77% for the two steps for **Tb35**.

Scheme 4. Synthesis of Tubulysin Analogues Tb11−Tb16[a]



[a]Reagents and conditions: (a) **29** or **30** (1.5 equiv), HATU (1.3 equiv), HOAt (1.3 equiv), *i*-Pr_2NEt (4.0 equiv), DMF, 23 °C, 24 h, 55% for **31**, 54% for **32**; (b) TFA (45 equiv), CH_2Cl_2, 23 °C, 18 h; (c) **8** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 23 °C, 24 h, 76% for the two steps for **33**, 71% for the two steps for **34**; (d) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 23 °C, 3 h; (e) **17** or **27** or **28** (6.0 equiv), DMF, 0 → 23 °C, 24 h, 58−73% for the two steps for **Tb11−Tb16** (for further details, see the SI). HOAt = 1-hydroxy-7-azabenzotriazole.

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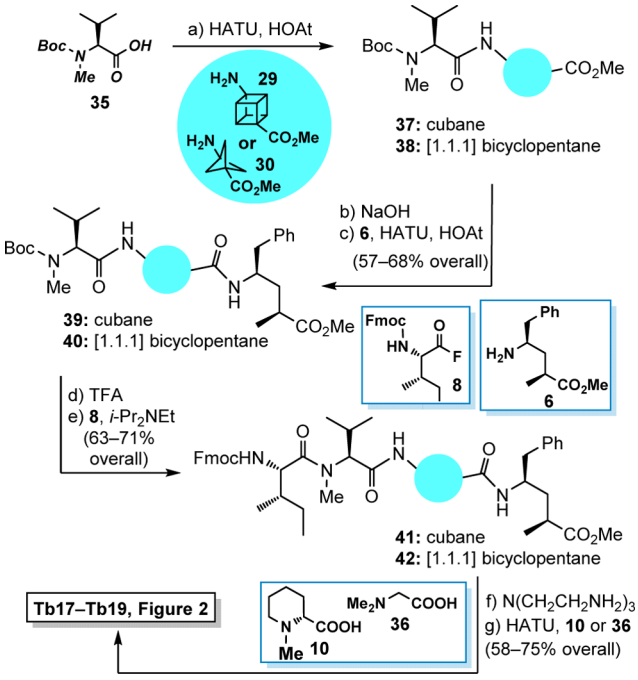
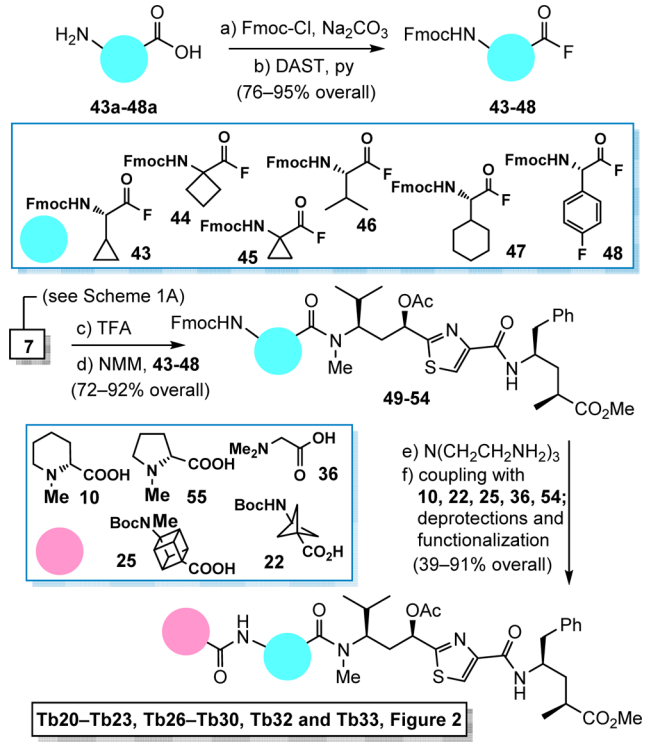
Scheme 5. Synthesis of Tubulysin Analogues Tb17–Tb19[a]



[a]Reagents and conditions: (a) **29** or **30** (1.2 equiv), HATU (1.5 equiv), HOAt (0.1 equiv), Et_3N (8.0 equiv), DMF, 0 → 23 °C, 4 h; (b) 1 M NaOH(aq) (2.3 equiv), THF, 23 °C, 11 h; (c) **6** (1.0 equiv), HATU (1.2 equiv), HOAt (0.1 equiv), Et_3N (6.5 equiv), DMF, 0 → 23 °C, 15 h, 57% for the three steps for **39**, 68% for the three steps for **40**; (d) TFA (45 equiv), CH_2Cl_2, 23 °C, 12 h; (e) **8** (3.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 24 h, 63% for the two steps for **41**, 71% for the two steps for **42**; (f) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 3 h; (g) **10** (6.0 equiv) or *N*,*N*-dimethylglycine (**36**) (6.0 equiv), HATU (6.0 equiv), Et_3N (6.5 equiv), DMF, 0 → 23 °C, 12 h, 75% for the two steps for **Tb17**, 58% for the two steps for **Tb18**, 72% for the two steps for **Tb19**.

Scheme 6. Synthesis of Tubulysin Analogues Tb20–Tb23, Tb26–Tb30, Tb32, and Tb33[a]



[a]Reagents and conditions: (a) Fmoc-Cl (1.1 equiv), Na_2CO_3 (2.5 equiv), H_2O, 1,4-dioxane, 23 °C, 6 h; (b) DAST (1.2 equiv), py (1.0 equiv), CH_2Cl_2, 23 °C, 1 h, 76–95% for the two steps for **43**–**48**; (c) TFA (45 equiv), CH_2Cl_2, 23 °C, 12 h; (d) **43**–**48** (4.0 equiv), NMM (8.0 equiv), DMF, 23 °C, 18 h, 72–92% for the two steps; (e) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 3 h; (f) **10** (2.0 equiv) or **36** (1.5 equiv) or **22** (1.5 equiv) or **25** (1.5 equiv) or **55** (1.5 equiv), HATU (1.3 equiv), HOAt (1.3 equiv), NMM (3.0 equiv), DMF, 23 °C, 24 h, 49% for the two steps for **Tb20**; then Me_3SnOH (20 equiv), 1,2-dichloroethane, reflux, 12 h, then Ac_2O (4.0 equiv), py, 0 → 23 °C, 12 h, 54% for the two steps for **Tb30**, 39% for the two steps for **Tb21**, 49% for the two steps for Boc-protected **Tb22**; then TFA (30.0 equiv), CH_2Cl_2, 23 °C, 12 h, then Ac_2O (10 equiv), py, 23 °C, 6 h, 91% for the two steps for **Tb22**, 47% for the two steps for Boc-protected **Tb23**; then TFA (30 equiv), CH_2Cl_2, 23 °C, 12 h, 73% for **Tb23**, 85% for the two steps for **Tb26**, 76–81% for the two steps for **Tb27**–**Tb29**, 75–80% for the two steps for **Tb32** and **Tb33**. Abbreviations: DAST = diethylaminosulfur trifluoride; NMM = *N*-methylmorpholine.

$[N(CH_2CH_2NH_2)_3]$ to cleave the Fmoc group, and the resulting amine was coupled with **10** (HATU, Et_3N, 82% overall yield) to furnish tripeptide **61**. Tripeptide **61** was converted to its carboxylic acid counterpart **62** (Me_3SnOH;[46] Ac_2O, py, 75% overall yield). This compound was then transformed into its pentafluorophenyl ester (C_6F_5OH, DIC), and the latter was coupled with hydrazine derivative **63**[50] in the presence of *i*-Pr_2NEt to afford tubulysin analogue **Tb31** in 73% overall yield.

Scheme 9 depicts the synthesis of tubulysin analogues **Tb36**–**Tb39**, in which the thiazole moiety has been replaced with a pyridine structural motif (within the Tuv amino acid unit) and various isoleucine substitutes. Aldehyde **1**[43] was converted to Weinreb amide **64** through a sequence involving methyl ester formation (NaCN, MnO_2, MeOH, 82% yield) followed by reaction with $MeNHOCH_3$·HCl and *i*-PrMgCl (86% yield). Coupling of **64** with the lithioderivative of bromopyridine **65** (see the SI for preparation) furnished ketone **66** in 72% yield; asymmetric reduction of **66** with (*S*)-CBS and BH_3·SMe_2 afforded hydroxy compound **67** in 84% yield. The latter compound was elaborated to acetoxycarboxylic acid **68** through a sequence involving acetylation (Ac_2O, py), desilylation (TBAF), and oxidation (DMP; $NaClO_2$) in 93% overall yield. Coupling of **68** with amino methyl ester **6** gave dipeptide **69** (94% yield), whose Boc group cleavage (TFA) led to the corresponding secondary amine, which was coupled with acid fluoride fragments **8** and **43** to afford tripeptides **70** and **71**, respectively (90–96% overall yield). Removal of Fmoc $[N(CH_2CH_2NH_2)_3]$ from the latter intermediates followed by coupling of the resulting amines with **10** under HATU conditions furnished tubulysin analogues **Tb36** and **Tb38**, respectively, in 62–66% yield. The latter were converted to their carboxylic acid counterparts **Tb37** and **Tb39**, respectively, through the sequential action of Me_3SnOH[46] (cleavage of the methyl ester and acetate moieties) and Ac_2O, py (reacetylation of the hydroxy group) in 65–68% overall yield, as shown in Scheme 9.

Tubulysin analogues **Tb40** and **Tb41** (for structures, see Figure 2), in which the "right end" amino acid residue (Tup) and isoleucine have been replaced with structural motifs represented by **75** or **76** and **46**, respectively, were obtained as shown in Scheme 10. Thus, removal of the Boc group from **59** (TFA) followed by coupling of the resulting amine with **46** in

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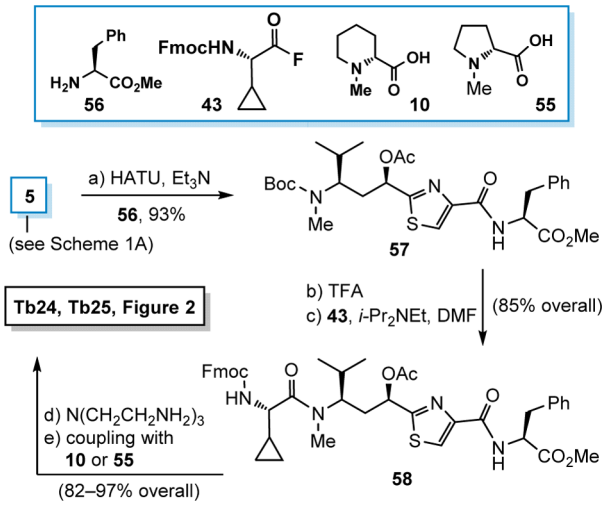
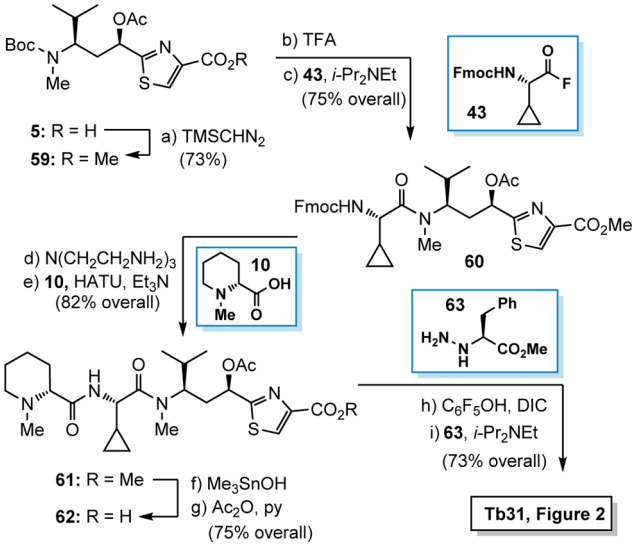
Scheme 7. Synthesis of Tubulysin Analogues Tb24 and Tb25[a]



[a]Reagents and conditions: (a) **56** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 23 °C, 18 h, 93%; (b) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 6 h; (c) **43** (4.0 equiv), *i*-Pr_2NEt (5.0 equiv), DMF, 0 → 23 °C, 18 h, 85% for the two steps; (d) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 3 h; (e) **10** (2.0 equiv) or **55** (2.0 equiv), HATU (1.5 equiv), Et_3N (3.0 equiv), DMF, 0 → 23 °C, 24 h, 82% for the two steps for **Tb24**, 97% for the two steps for **Tb25**.

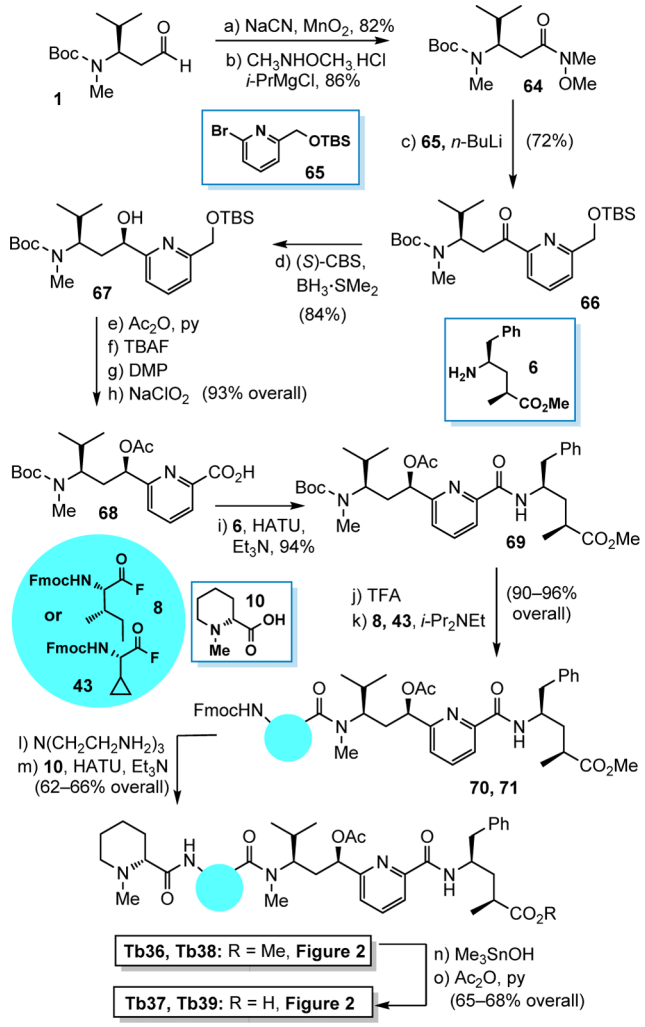
Scheme 8. Synthesis of Hydrazide Tubulysin Analogue Tb31[a]



[a]Reagents and conditions: (a) $TMSCHN_2$ (2 M in Et_2O, 1.2 equiv), toluene/methanol (3:2), 23 °C, 30 min, 73%; (b) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 12 h; (c) **43** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 75% for the two steps; (d) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 3 h; (e) **10** (1.5 equiv), HATU (1.5 equiv), Et_3N (3.0 equiv), DMF, 0 → 23 °C, 24 h, 82% for the two steps; (f) Me_3SnOH (10 equiv), 1,2-dichloroethane, reflux, 12 h; (g) Ac_2O (4.0 equiv), py, 0 → 23 °C, 12 h, 75% for the two steps; (h) C_6F_5OH (1.5 equiv), DIC (1.2 equiv), CH_2Cl_2, 0 → 23 °C, 24 h; (i) **63** (1.2 equiv), *i*-Pr_2NEt (3.0 equiv), DMF, 23 °C, 20 h, 73% for the two steps. DIC = *N*,*N*′-diisopropylcarbodiimide.

the presence of *i*-Pr_2NEt in DMF led to the formation of dipeptide **72** in 73% overall yield. The latter was treated with $[N(CH_2CH_2NH_2)_3]$ to cleave the Fmoc group, and the resulting amine was coupled with **10** (HATU, Et_3N, 78% overall yield) to furnish tripeptide **73**. Tripeptide **73** was then converted to its carboxylic acid counterpart **74** (Me_3SnOH; Ac_2O, py, 74% overall yield). Finally, coupling of **74** with **75** and **76** under HATU conditions furnished tubulysin analogues **Tb40** (75% yield) and **Tb41** (76% yield), respectively, as shown in Scheme 10 (see the SI for further details).

Scheme 9. Synthesis of Tubulysin Analogues Tb36–Tb39[a]



[a]Reagents and conditions: (a) NaCN (2.0 equiv), MnO_2 (17 equiv), MeOH, 0 → 23 °C, 24 h, 82%; (b) CH_3NHOCH_3·HCl (2.1 equiv), *i*-PrMgCl (4.0 equiv), THF, −20 → 0 °C, 3 h, 86%; (c) *n*-BuLi (2.5 M in hexanes, 1.44 equiv), **65** (1.2 equiv), THF, −78 → −50 °C, 3 h, 72%; (d) (*S*)-CBS (0.1 equiv), BH_3·SMe_2 (1.0 equiv), 0 → 23 °C, 24 h, 84%; (e) Ac_2O (3.0 equiv), Et_3N (4 equiv), 0 → 23 °C, 2 h, 93%; (f) TBAF (1 M in THF, 2.0 equiv), THF, 0 → 23 °C, 30 min, 96%; (g) DMP (1.5 equiv), CH_2Cl_2, 23 °C, 1 h, 89%; (h) $NaClO_2$ (5.4 equiv), NaH_2PO_4·H_2O (12 equiv), 2-methyl-2-butene (7.5 equiv), *t*-BuOH, THF, H_2O, 23 °C, 1 h, 95%; (i) **6** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 94%; (j) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (k) **8** or **43** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 96% for the two steps for **70**, 90% for the two steps for **71**; (l) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 3 h; (m) **10** (1.5 equiv), HATU (1.5 equiv), Et_3N (3.0 equiv), DMF, 0 → 23 °C, 24 h, 62% for the two steps for **Tb36**, 66% for the two steps for **Tb38**; (n) Me_3SnOH (20 equiv), 1,2-dichloroethane, reflux, 12 h; (o) Ac_2O (4.0 equiv), py, 0 → 23 °C, 12 h, 65% for the two steps for **Tb37**, 68% for the two steps for **Tb39**.

2.5. Biological Evaluation of Tubulysin Analogues. A number of the synthesized compounds (i.e., **Tb1**–**Tb23**) were

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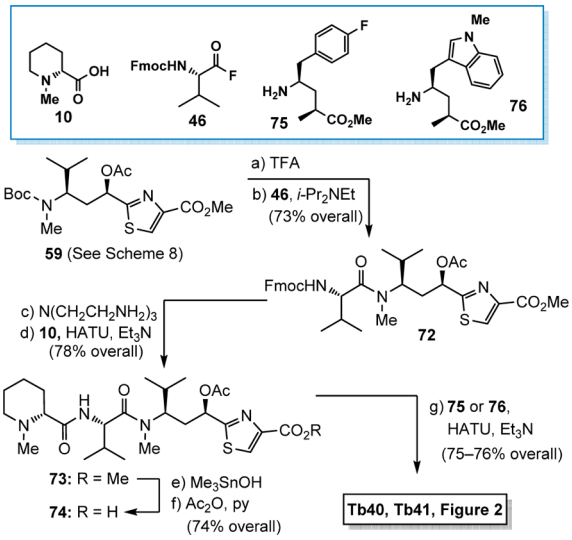
Scheme 10. Synthesis of Analogues Tb40 and Tb41[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 12 h; (b) **46** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 73% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 3 h; (d) **10** (1.5 equiv), HATU (1.5 equiv), Et_3N (3.0 equiv), DMF, 0 → 23 °C, 24 h, 78% for the two steps; (e) Me_3SnOH (20 equiv), 1,2-dichloroethane, reflux, 12 h; (f) Ac_2O (4.0 equiv), py, 0 → 23 °C, 12 h, 74% for the two steps; (g) **75** or **76** (1.2 equiv), HATU (1.2 equiv), Et_3N (2.4 equiv), DMF, 23 °C, 18 h, 75% for the two steps for **Tb40**, 76% for the two steps for **Tb41**.

evaluated for their activities against a variety of cancer cell lines, namely, the NCI-60 human cancer cell line panel representing leukemia, melanoma, lung, colon, brain, ovary, breast, prostate, and kidney types of cancer through the In Vitro Cell Line Screening Project (IVCLSP) of the National Cancer Institute (NCI), and a summary of the results is shown in Table 1 (for detailed data, see the SI). As can be seen from these data, tubulysins **Tb12**, **Tb15**, **Tb17**−**Tb19**, and **Tb22** failed to pass the one-dose test (10 μM), while the rest (**Tb1**−**Tb11**, **Tb13**, **Tb14**, **Tb16**, **Tb20**, **Tb21**, and **Tb23**) advanced to further testing at lower concentrations. Among the most potent were **Tb1**, **Tb2**, **Tb11**, **Tb14**, and **Tb20**, which exhibited consistently potent activities against leukemia, non-small-cell lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast cancer cell lines, as shown by their selected GI_{50} values (highlighted in blue in Table 1). The most impressive activities were exhibited by tubulysin **Tb11** (leukemia, GI_{50} = 159 pM; non-small-cell lung cancer, GI_{50} = 331 pM; colon cancer, GI_{50} = 1140 pM; CNS cancer, GI_{50} = 1150 pM; melanoma, GI_{50} = 349 pM; ovarian cancer, GI_{50} = 489 pM; renal cancer, GI_{50} = 768 pM; prostate cancer, GI_{50} = 1130 pM; breast cancer, GI_{50} = 428 pM), **Tb14** (leukemia, GI_{50} = 64 pM; non-small-cell lung cancer, GI_{50} = 156 pM; colon cancer, GI_{50} = 433 pM; CNS cancer, GI_{50} = 382 pM; melanoma, GI_{50} = 137 pM; ovarian cancer, GI_{50} = 256 pM; renal cancer, GI_{50} = 364 pM; prostate cancer, GI_{50} = 449 pM; breast cancer, GI_{50} = 267 pM), and **Tb20** (leukemia, GI_{50} = 98 pM; non-small-cell lung cancer, GI_{50} = 247 pM; colon cancer, GI_{50} = 263 pM; CNS cancer, GI_{50} = 345 pM; melanoma,

Table 1. Selected NCI-60 Cytotoxicity Human Cancer Cell Line Panel Data (GI_{50} Values[a] in nM) for Tubulysins Tb1−Tb23[b]

compound	one dose[c]	leukemia	non-small cell lung cancer	colon cancer	CNS cancer	melanoma	ovarian cancer	renal cancer	prostate cancer	breast cancer
Tb1	-	0.264	0.347	0.269	0.305	0.074	0.234	0.548	0.339	0.147
Tb2	-	0.078	0.280	0.158	0.219	0.030	0.042	0.218	0.162	0.043
Tb3	-	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Tb4	-	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Tb5	-	>1000	>1000	>1000	>1000	416	>1000	>1000	>1000	514
Tb6	-	286	164	339	439	83.9	127	154	689	71.4
Tb7	-	17.6	57.8	61.3	219	31.4	48.5	134	105	42.8
Tb8	-	9.62	96.7	121	105	33.3	75.0	164	125	52.1
Tb9	-	24.2	83.6	331	246	46.6	61.0	238	459	53.2
Tb10	-	61.3	60.2	355	271	49.5	92.6	413	500	66.4
Tb11	-	0.159	0.331	1.14	1.15	0.349	0.489	0.768	1.13	0.428
Tb12	84.90	-	-	-	-	-	-	-	-	-
Tb13	-	13.8	142	122	117	40.2	111	167	167	136
Tb14	-	0.064	0.156	0.433	0.382	0.137	0.256	0.364	0.449	0.267
Tb15	94.38	-	-	-	-	-	-	-	-	-
Tb16	-	15.1	85.0	98.1	127	46.1	99.1	186	303	123
Tb17	97.37	-	-	-	-	-	-	-	-	-
Tb18	98.48	-	-	-	-	-	-	-	-	-
Tb19	100.82	-	-	-	-	-	-	-	-	-
Tb20	-	0.098	0.247	0.263	0.345	0.035	0.049	0.270	0.211	0.044
Tb21	-	1.65	6.62	23.9	24.1	2.23	5.79	23.2	34.6	4.04
Tb22	49.38	-	-	-	-	-	-	-	-	-
Tb23	-	16.3	84.5	204	119	45.1	54.9	181	308	44.7

[a]GI_{50} is the concentration at which the compound inhibits growth by 50%. [b]See the SI for complete NCI-60 data. [c]Mean growth %.

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Table 2. Cytotoxicity Data against the Cancer Cell Lines MES SA, MES SA DX, and HEK 293T[a] for Tubulysins Tb1−Tb41, PTb-D42, and PTb-D43 (IC_{50} Values[b] in nM)

compound	MES SA	MES SA DX	HEK 293T
Tb1	0.34	>10	0.02
Tb2	0.20	>10	0.03
Tb3	>50	>50	>50
Tb4	>50	>50	>50
Tb5	>50	>50	>50
Tb6	>50	>50	>50
Tb7	>50	>50	>50
Tb8	>50	>50	>50
Tb9	>50	>50	>50
Tb10	>50	>50	>50
Tb11	0.84	>50	0.26
Tb12	>50	>50	>50
Tb13	>50	>50	>50
Tb14	0.35	>50	0.11
Tb15	>50	>50	>50
Tb16	>50	>50	>50
Tb17	>50	>50	>50
Tb18	>50	>50	>50
Tb19	>50	>50	>50
Tb20	0.046	<200	0.047
Tb21	16.830	>50	>10
Tb22	>50	>50	>50
Tb23	>50	>50	>50
Tb24	13.1	>100	9.69
Tb25	>50	>100	>50
Tb26	0.20	>100	0.12
Tb27	2.46	>100	1.96
Tb28	1.13	81.72	0.69
Tb29	2.61	499.8	2.24
Tb30	4.09	>100	0.35
Tb31	8.16	>100	7.28
Tb32	0.012	1.29	0.002
Tb33	0.066	101	0.048
Tb34	>1,000	>1,000	>500
Tb35	>1,000	>1,000	>500
Tb36	0.308	>1,000	0.631
Tb37	14.430	>1,000	3.524
Tb38	0.357	<500	0.574
Tb39	0.891	<500	0.194
Tb40	15.56	>1000	>1000
Tb41	>1000	>1000	21.12
PTb-D42	>1000	>1000	>1000
PTb-D43	408.8	>1000	109.0

[a]MES SA = uterine sarcoma cell line; MES SA DX = MES SA cell line with marked multidrug resistance; HEK 293T = human embryonic kidney cell line. [b]IC_{50} is the 50% inhibitory concentration of the compound against cell growth; see the SI for further details.

GI_{50} = 35 pM; ovarian cancer, GI_{50} = 49 pM; renal cancer, GI_{50} = 270 pM; prostate cancer, GI_{50} = 211 pM; breast cancer, GI_{50} = 44 pM). As we were also interested in certain other cell lines, we subjected these and the remaining compounds (**Tb1**−**Tb41**, **PTb-D42**, and **PTb-D43**) to further testing against MES SA (uterine sarcoma cells), MES SA DX (multidrug-resistant uterine sarcoma cells), and HEK 293T (human embryonic kidney cells). As shown in Table 2, several of these compounds exhibited picomolar potencies, with the most potent (highlighted in blue) being **Tb1** (MES SA, IC_{50} = 340 pM; HEK 293T, IC_{50} = 20 pM), **Tb2** (MES SA, IC_{50} = 200 pM; HEK 293T, IC_{50} = 30 pM), **Tb11** (MES SA, IC_{50} = 840 pM; HEK 293T, IC_{50} = 260 pM), **Tb14** (MES SA, IC_{50} = 350 pM; HEK 293T, IC_{50} = 110 pM), **Tb20** (MES SA, IC_{50} = 46 pM; HEK 293T, IC_{50} = 47 pM), **Tb26** (MES SA, IC_{50} = 200 pM; HEK 293T, IC_{50} = 120 pM), **Tb32** (MES SA, IC_{50} = 12 pM; HEK 293T, IC_{50} = 2 pM), **Tb33** (MES SA, IC_{50} = 66 pM; HEK 293T, IC_{50} = 48 pM), **Tb36** (MES SA, IC_{50} = 308 pM; HEK 293T, IC_{50} = 631 pM), **Tb38** (MES SA, IC_{50} = 357 pM; HEK 293T, IC_{50} = 574 pM), and **Tb39** (MES SA, IC_{50} = 891 pM; HEK 293T, IC_{50} = 194 pM). Particularly impressive are the potencies exhibited by compounds **Tb20**, **Tb32**, and **Tb33**. Tubulysins are known to be substrates for Pgp pumps,[11,48,49] as supported by the observed low activities of all of the tubulysins tested except for **Tb28**, **Tb32**, and **Tb33**. In that regard, the latter analogues are notable for their relatively high potencies against the MDR cell line MES SA DX (**Tb28**, IC_{50} = 81.72 nM; **Tb32**, IC_{50} = 1.29 nM; **Tb33**, IC_{50} = 101 nM).

2.6. Structure−Activity Relationships. The biological data obtained in these studies provide important SARs within the tubulysin family of compounds. Figure 4 summarizes the

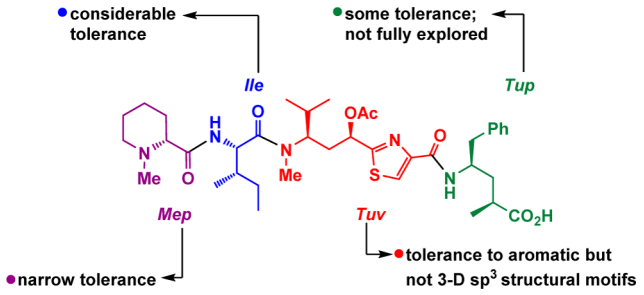

Figure 4. Structure−activity relationships.

conclusions of these studies of the effect of structural changes in the four domains *N*-methyl-D-pipecolinic acid (Mep), L-isoleucine (Ile), tubuvaline (Tuv), and tubuphenylalanine (Tup) of N^{14}-desacetoxytubulysin H (**Tb1**). Thus, replacing the Mep amino acid residue with a variety of other amino acid derivatives led to significant loss of activity, except in the case of **Tb26**, in which the substitution of the six-membered-ring picolinic acid residue for its five-membered-ring counterpart allowed the molecule to retain its potency (see the IC_{50} values for **Tb1** vs **Tb26** in Table 2). These results indicate rather little tolerance for structural modifications in this domain. In contrast, the isoleucine domain of the molecule exhibits considerable tolerance to changes (see, e.g., the IC_{50} values for **Tb20**, **Tb24**, **Tb26**, **Tb27**, **Tb28**, and **Tb29** in Table 2). The tubuvaline segment of tubulysin does not seem to tolerate three-dimensional sp^3 structural motifs (see the potencies for **Tb17**−**Tb19** in Table 2) but accommodates other aromatic moieties such as pyridine in place of the thiazole unit with only a small loss of potency (see the potencies for **Tb36**−**Tb39** in Table 2). The effects of substitution of the thiazole unit with other aromatic moieties remain to be explored. Finally, the

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modifications made in the Tup moiety of the tubulysin molecule (e.g., shorter carboxylate chain, fluorophenyl, and *N*-methylindolyl) led to significant loss of activity, suggesting considerable sensitivity of the molecule toward modification of this domain (see the potencies of **Tb24**, **Tb25**, **Tb40**, and **Tb41** in Table 2). Some of these results were unexpected [e.g., the loss of potency of **Tb40** (MES SA) and **Tb41** (HEK 293T)] and require further experimentation and understanding before meaningful and reliable SARs can be derived for these cases. However, substituting Tup with the cubane or [1.1.1]-bicyclopentane structural motif resulted in comparable potencies to those of **Tb1** and **Tb2** (i.e., **Tb14**; see Tables 1 and 2), indicating tolerance of the receptor to these substitutions.

3. CONCLUSION

The described synthetic endeavors culminated in short and streamlined chemical processes for the synthesis of N^{14}-desacetoxytubulysin H and pretubulysin D and allowed rapid and efficient syntheses of a number of analogues within the tubulysin class of cytotoxic agents. The new synthetic strategy devised for N^{14}-desacetoxytubulysin H and a number of its analogues features a novel C−H activation reaction that allows high-yielding coupling of an aldehyde intermediate with a thiazole moiety to give the required tubuvaline structural motif of the molecule. This C−C coupling reaction may be of considerable scope and generality beyond thiazoles and quinolones.[44] Biological evaluation of the synthesized molecules led to the identification of extremely potent tubulysin analogues (e.g., **Tb14**, **Tb20**, **Tb32**, and **Tb33**) equipped with carboxylate handles for conjugation to appropriate delivery systems such as antibodies[41] and probodies.[42] The resulting conjugates (ADCs and PDCs) would be highly desirable for personalized targeted chemotherapy, a recently emerged paradigm for cancer treatment. Such efforts are currently in progress, while new molecular entities within the tubulysin class are being designed and pursued synthetically. The newly developed structure−activity relationships are particularly path-pointing in this regard.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b12557.

Experimental procedures, compound characterization data, and biological evaluation and data (PDF)

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Notes

The authors declare no competing financial interest.

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