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Improved Total Synthesis of Tubulysins and Design, Synthesis, and Biological Evaluation of New Tubulysins with Highly Potent Cytotoxicities against Cancer Cells as Potential Payloads for Antibody−Drug Conjugates

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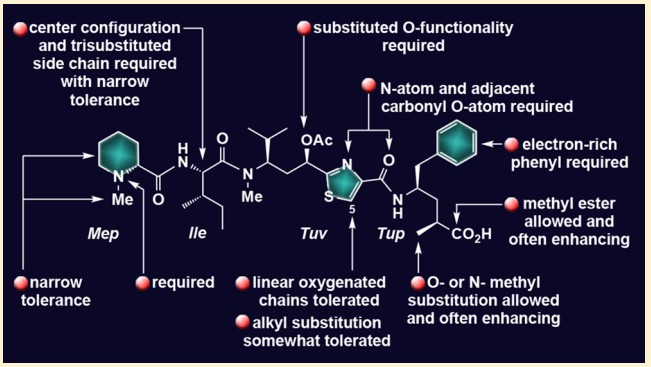
Supporting Information

ABSTRACT: Improved, streamlined total syntheses of natural tubulysins such as V (**Tb45**) and U (**Tb46**) and pretubulysin D (**PTb-D43**), and their application to the synthesis of designed tubulysin analogues (**Tb44**, **PTb-D42**, **PTb-D47−PTb-D49**, and **Tb50−Tb120**), are described. Cytotoxicity evaluation of the synthesized compounds against certain cancer cell lines revealed a number of novel analogues with exceptional potencies [e.g., **Tb111**: IC_{50} = 40 pM against MES SA (uterine sarcoma) cell line; IC_{50} = 6 pM against HEK 293T (human embryonic kidney cancer) cell line; and IC_{50} = 1.54 nM against MES SA DX (MES SA with marked multidrug resistance) cell line]. These studies led to a set of valuable structure−activity relationships that provide guidance to further molecular design, synthesis, and biological evaluation studies. The extremely potent cytotoxic compounds discovered in these investigations are highly desirable as potential payloads for antibody−drug conjugates and other drug delivery systems for personalized targeted cancer chemotherapies.



1. INTRODUCTION

The tubulysins are among the most potent cytotoxic compounds ever discovered from Nature.[1−3] Their mechanism of action involves depolymerization of microtubules with disintegration of the cytoskeleton as a consequence.[4−6] Isolated from the myxobacteria *Archangium gephyra* and *Angiococcus disciformis*,[7,8] these natural products elicited intense research efforts directed toward their total synthesis, analogue design and synthesis, and biological investigations as part of anticancer drug discovery and development programs.[9−14] Thus, total syntheses of the naturally occurring tubulysins A,[15] B,[15] C,[16] D,[16−18] G, I, U (**Tb46**, Figure 1),[16,19−22] and V (**Tb45**, Figure 1)[16,20−24] and pretubulysin D (**PTb-D43**, Figure 1),[8a,11] as well as numerous analogues, have been accomplished.[25−49] From the latter, N^{14}-desacetoxytubulysin H (**Tb1**, Figure 1) is distinguished for its methyl, instead of the acyl methyl, substituent on N14 of tubulysins A−I[11,15−18,25] and its high potency.[6,25] We have recently published a total synthesis of N^{14}-desacetoxytubulysin H (**Tb1**, Figure 1) and several of its analogues (e.g., **Tb32**, Figure 1) and their biological evaluation.[11] In this Article we report on a more extensive study that included (a) streamlined total syntheses of the natural tubulysins V (**Tb45**, Figure 1) and U (**Tb46**, Figure 1) and pretubulysin D (**PTb-D43**, Figure 1); (b) design and synthesis of numerous novel tubulysin analogues (i.e., **PTb-D42**, **Tb44**, **PTb-D47−PTb-D49**, and **Tb50−Tb120**, Figure 2); and (c) biological evaluation of the synthesized compounds. These investigations led to the discovery of a number of exceptionally potent antitumor agents particularly suited as payloads for antibody−drug conjugates (ADCs)[50,51] and other delivery systems.[52]

2. RESULTS AND DISCUSSION

Having developed our C−H activation-based strategy for the synthesis of the tubuvaline residue,[11] and in order to devise a practical synthesis of tubulysins V (**Tb45**) and U (**Tb46**), pretubulysin D (**PTb-D43**), and their analogues, we decided to

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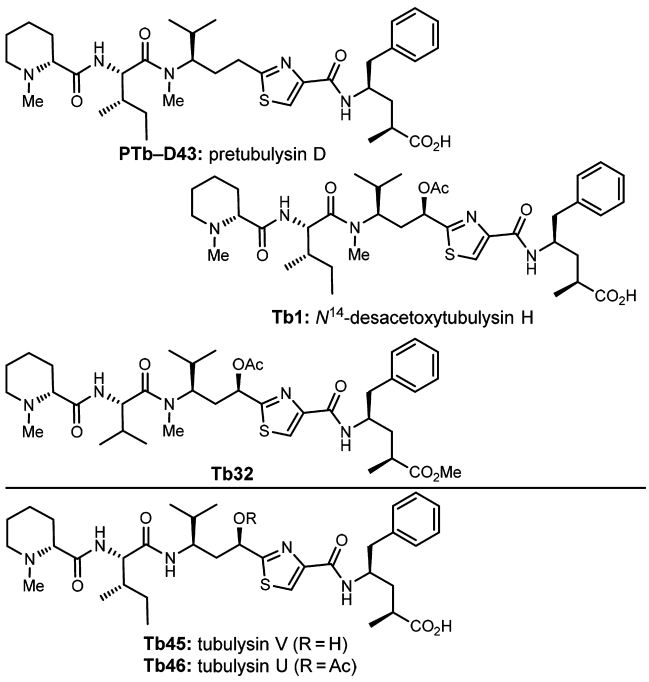

Figure 1. Molecular structures of naturally occurring tubulysins V (**Tb45**) and U (**Tb46**), pretubulysin D (**PTb-D43**), N^{14}-desacetoxytubulysin H (**Tb1**), and previously synthesized potent tubulysin analogue (**Tb32**).

improve and apply our synthetic technologies to that end. In pursuing new tubulysin analogues, we applied rational ligand design based on our previously developed preliminary structure–activity relationships (SARs)[11] and the recently reported X-ray crystallographic analysis regarding the binding requirements of tubulysin-like molecules to microtubules, their biological target.[53]

2.1. Total Synthesis of Tubulysin V (Tb45), Tubulysin U (Tb46) and Its Methyl Ester (Tb44). Our newly developed, streamlined total synthesis of the naturally occurring tubulysins V (**Tb45**) and U (**Tb46**), and the methyl ester of the latter (**Tb44**), proceeded along an appropriately modified and improved synthetic route as shown in Scheme 1.[11] Thus, aldehyde **1**[11,54] was subjected to C–H activation coupling with thiazole derivative **2** [$PhI(OCOCF_3)_2$, $TMSN_3$],[11,55] furnishing coupling product **3** in 56% yield. Stereoselective reduction of the thiazolyl ketone moiety within **3** with (*S*)-CBS catalyst in the presence of $BH_3{\cdot}Me_2S$[11,56] produced alcohol **4** in 83% yield and as a single diastereoisomer after chromatographic purification. Elaboration of intermediate **4** to acetoxy carboxylic acid **5** was achieved through a sequence involving deacetylation (K_2CO_3, MeOH), two-step selective oxidation of the so generated primary alcohol (TEMPO, BAIB; then $NaClO_2$), and acetylation of the secondary alcohol (Ac_2O, Et_3N) in 78% overall yield. Coupling of carboxylic acid **5** with ammonium salt **6**[11] in the presence of HATU and Et_3N led to amide **7** (94% yield). Removal of the Boc group from the latter through the action of TFA, followed by coupling of the resulting amine with carboxylic acid **8**,[11] produced peptide **9** (HATU, Et_3N, 92% yield) as shown in Scheme 1. Cleavage of the Boc protecting group from **9** (TFA) and coupling of the resulting amine with *N*-methyl-D-pipecolic acid (**10**) afforded tubulysin U methyl ester (**Tb44**, 85% overall yield). Conversion of **Tb44** to tubulysin U (**Tb46**) via tubulysin V (**Tb45**) required sequential treatment with Me_3SnOH[11,57] (cleavage of both methyl ester and acetate moieties, 68% yield), and re-acetylation of the resulting hydroxy carboxylic acid (Ac_2O, pyridine, 79% yield) as shown in Scheme 1.

2.2. Improved Total Synthesis of Pretubulysin D (PTb-D43) and Its Methyl Ester (PTb-D42). In an effort to streamline our original synthesis of pretubulysin D (**PTb-D43**), and since the adjacent to the thiazole carbonyl moiety was not needed in this case [cf. intermediates **3** (Scheme 1) and **14** (Scheme 2)], we decided to employ the commercially available valine derivative **11** as the starting material. Thus, and as shown in Scheme 2, exposure of **11** to $TMSCHN_2$ followed by $LiAlH_4$ reduction of the resulting methyl ester furnished the corresponding primary alcohol, whose reaction with CBr_4 and PPh_3 led to bromide **12** in 62% overall yield for the three steps. Coupling of the anion generated from thiazole **13** (see Supporting Information for preparation), through the action of *n*-BuLi, with bromide **12** furnished **14** in 78% yield.[58] Transformation of TBS-ether **14** to the desired carboxylic acid (**15**) was achieved through desilylation (TBAF) followed by two-step oxidation of the resulting alcohol (DMP; then $NaClO_2$), in 78% overall yield. Coupling of carboxylic acid **15** with aminoester **6**[11] in the presence of HATU and Et_3N led to amide **16** (82% yield). Removal of the Boc protecting group from the latter (TFA) followed by coupling of the resulting amine with acid fluoride **17**[11] furnished peptide **18** (*i*-Pr_2NEt, 95% overall yield for the two steps) as shown in Scheme 2. Cleavage of the Fmoc-group from **18** under basic conditions [$N(CH_2CH_2NH_2)_3$] and coupling of the so formed amine with *N*-methyl-D-pipecolic acid (**10**)[11] provided pretubulysin D precursor **PTb-D42** in 72% overall yield. Conversion of **PTb-D42** to pretubulysin D (**PTb-D43**) was accomplished using the previously reported conditions (LiOH, 91% yield)[11] as presented in Scheme 2.

2.3. Synthesis of Pretubulysin D Analogues PTb-D47, PTb-D48, and PTb-D49. Scheme 3 summarizes the synthesis of pretubulysin analogues **PTb-D47**, **PTb-D48**, and **PTb-D49** from known intermediates **18**[11] and **16**,[11] respectively. Thus, removal of the Fmoc protecting group from **18** [$N(CH_2CH_2NH_2)_3$] and coupling of the resulting amine with carboxylic acid **19** (for its synthesis see Supporting Information) provided pretubulysin analogue **PTb-D47** in 82% overall yield, as shown in Scheme 3A. Removal of the Boc group from **16** (TFA) followed by coupling of the resulting amine with acid fluoride **20**[11] gave peptide **21** (*i*-Pr_2NEt, 95% overall yield) as shown in Scheme 3B. Cleavage of the Fmoc group from **21** through the action of $N(CH_2CH_2NH_2)_3$ and coupling of the resulting amine with either *N*-methyl-D-pipecolic acid (**10**)[11] or *n*-butyl-substituted pipecolic acid **19** provided pretubulysin D analogues **PTb-D48** and **PTb-D49** in 81% and 76% overall yields, respectively.

2.4. Synthesis of N^{14}-Desacetoxytubulysin H Analogues Tb50–Tb120. Given that N^{14}-methyl-substituted tubulysins (such as N^{14}-desacetoxytubulysin H,[11,25] **Tb1**) have been proven more potent than their N^{14}-H and N^{14}-acetoxymethyltubulysin (such as tubulysin H) counterparts, we focused considerable efforts on designing and synthesizing a number of N^{14}-methyl-substituted tubulysins. Scheme 4 summarizes the synthesis of N^{14}-methyl-substituted tubulysins **Tb50** and **Tb51**, in which the pipecolic acid residue of the molecule is replaced with *N*-Me-substituted pyrrole and imidazole structural motifs, respectively. Thus, cleavage of the Fmoc protecting group from previously synthesized inter-

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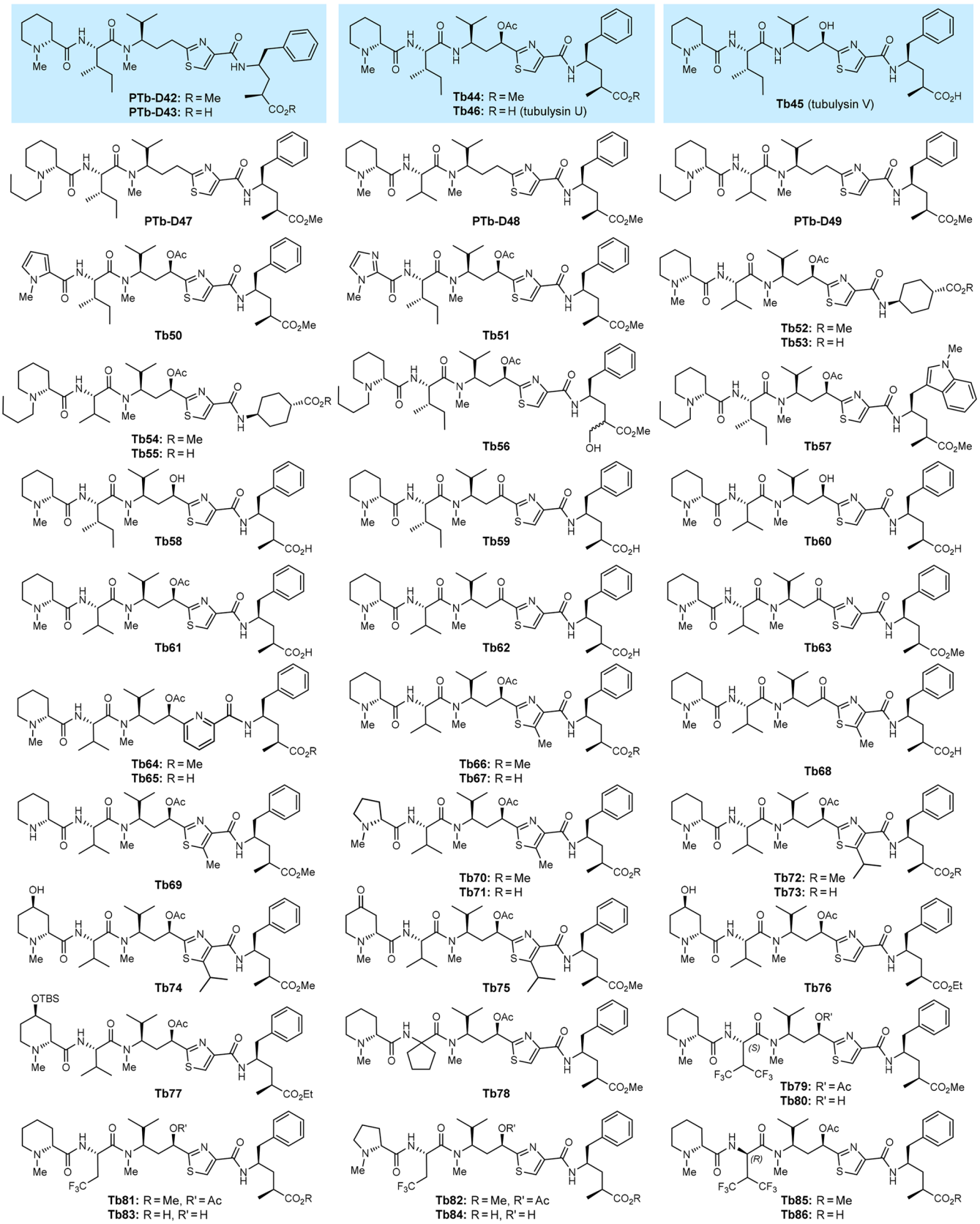

Figure 2. continued

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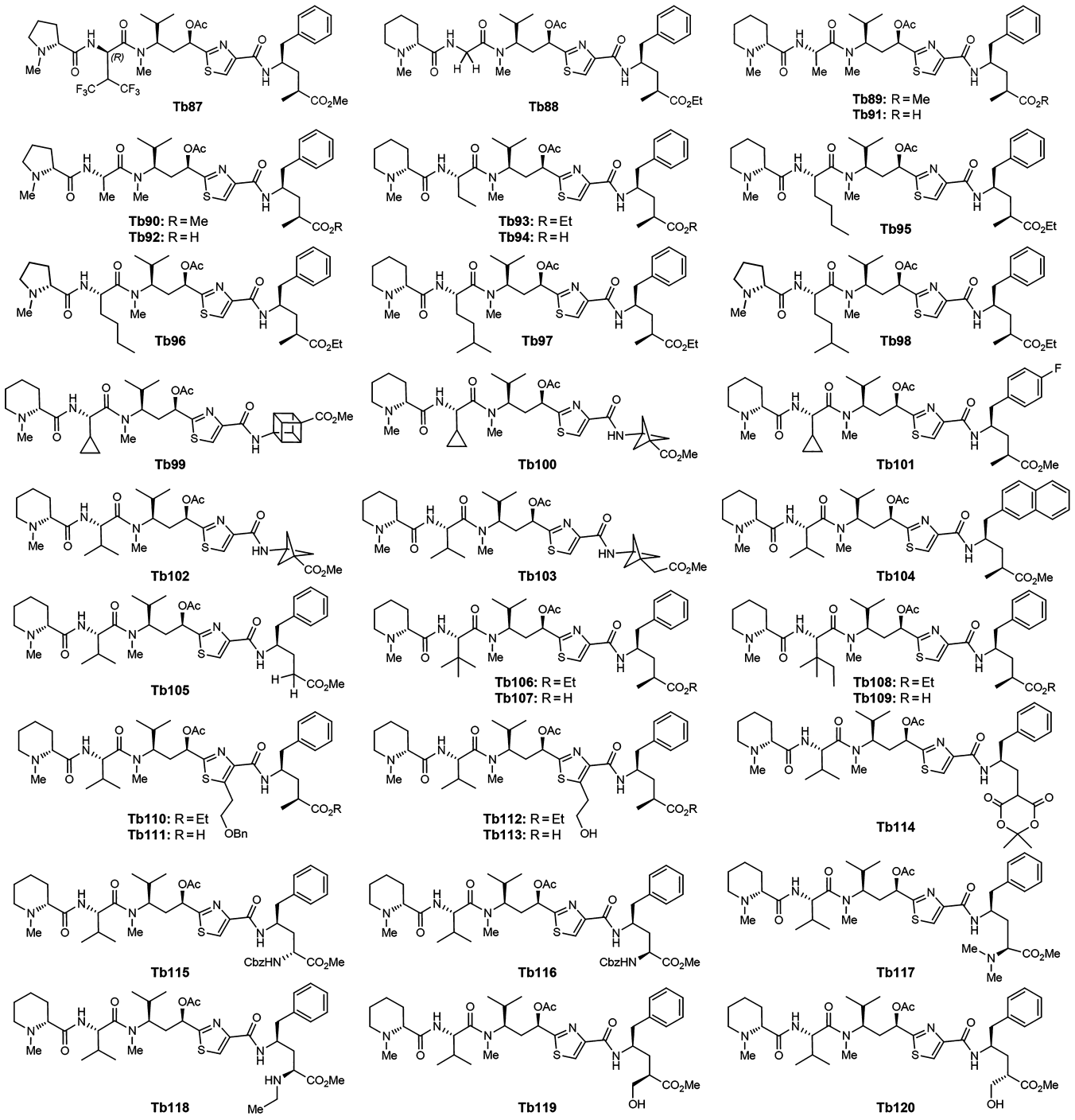

Figure 2. Molecular structures of synthesized naturally occurring tubulysins [pretubulysin D (**PTb-D43**), tubulysins U (**Tb46**) and V (**Tb45**)] and synthesized designed tubulysin analogues (**Tb44**, **PTb-D42**, **PTb-D47**, **PTb-D48**, **PTb-D49**, and **Tb50**–**Tb120**).

mediate **22**[11] through the action of *N*,*N*-bis(2-aminoethyl)-1,2-ethanediamine followed by coupling of the so generated amine with 1-methyl-1*H*-pyrrole-2-carboxylic acid (**23**) and 1-methyl-1*H*-imidazole-2-carboxylic acid (**24**) provided N^{14}-desacetoxytubulysin analogues **Tb50** and **Tb51**, in 74% and 76% yields, respectively, as summarized in Scheme 4.

Tubulysin analogues **Tb52**–**Tb55**, in which changes in the two end structural motifs were made while keeping the proven to be desirable N^{14}-Me and the *i*-Pr moieties on the isoleucine residue, were synthesized as shown in Scheme 5. Thus, coupling of carboxylic acid **25**[11] with commercially available ammonium salt **26** in the presence of HATU furnished dipeptide **27** (84% yield). Exposure of this protected dipeptide to TFA resulted in removal of the Boc group to afford the corresponding amine, whose coupling with acid fluoride **20** in the presence of *i*-Pr_2NEt in DMF led to the formation of tripeptide **28** (92% overall yield). Removal of the Fmoc group from **28** [$N(CH_2CH_2NH_2)_3$], followed by coupling of the resulting amine with *N*-methyl-D-pipecolic acid (**10**) and *n*-butyl-substituted pipecolic acid **19** (see Supporting Information for preparation) under HATU conditions, resulted in the formation of tubulysin analogues **Tb52** (72% yield) and **Tb54** (77% yield), respectively, as shown in Scheme 5 (see Supporting Information for further details). Finally, the

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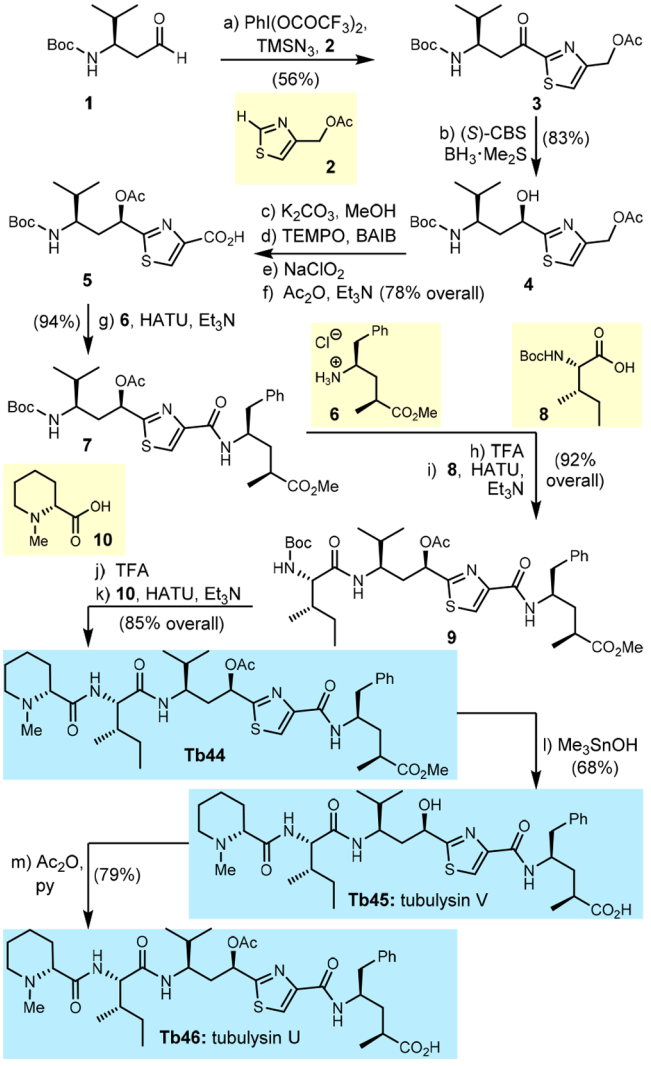
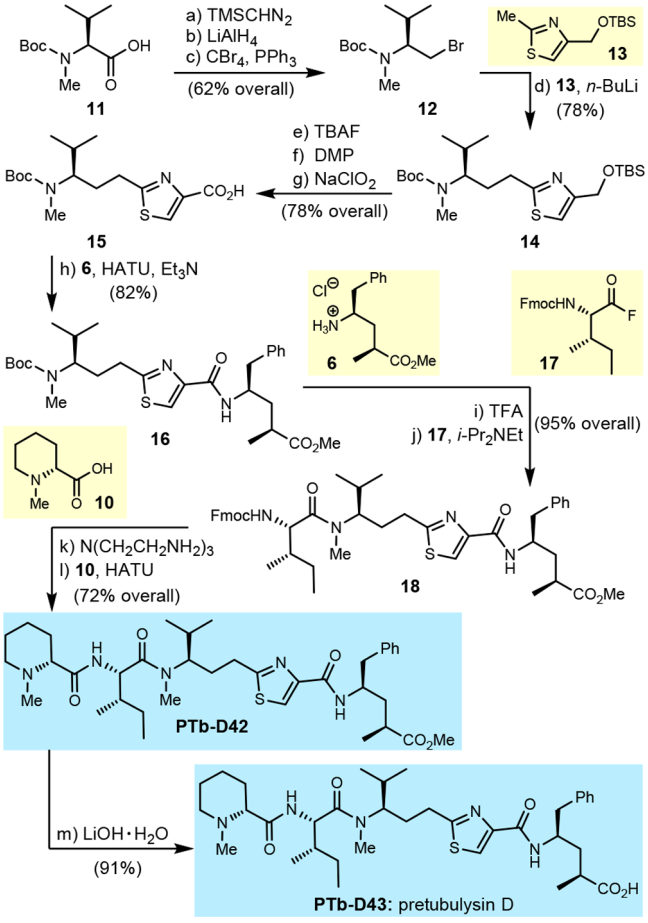
Scheme 1. Syntheses of Tubulysin U Methyl Ester Tb44 and Tubulysins V (Tb45) and U (Tb46)[a]



[a]Reagents and conditions: (a) **1** (2.0 equiv), **2** (1.0 equiv), $TMSN_3$ (1.5 equiv), PIFA (1.5 equiv), benzene, 23 °C, 12 h; then **1** (2.0 equiv), $TMSN_3$ (1.5 equiv), PIFA (1.5 equiv), 23 °C, 12 h, 56%; (b) (*S*)-CBS (0.2 equiv; 1.0 M in toluene), $BH_3{\cdot}Me_2S$ (5.0 equiv; 2.0 M in THF), 0 → 23 °C, 18 h, 83%; (c) K_2CO_3 (4.0 equiv), MeOH, 23 °C, 3 h, 95%; (d) TEMPO (0.1 equiv), BAIB (1.0 equiv), CH_2Cl_2, 23 °C, 16 h, 98%; (e) $NaClO_2$ (5.4 equiv), $NaH_2PO_4{\cdot}H_2O$ (12.5 equiv), 2-methyl-2-butene (7.5 equiv), *t*-BuOH, THF, H_2O, 23 °C, 12 h; (f) Ac_2O (3.0 equiv), Et_3N (3.0 equiv), CH_2Cl_2, 0 → 23 °C, 15 h, 78% for the two steps; (g) **6** (2.0 equiv), HATU (3.2 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 14 h, 94%; (h) TFA (1.75 equiv), CH_2Cl_2, 0 → 23 °C, 6 h; (i) **8** (2.0 equiv), HATU (4.0 equiv), Et_3N (10 equiv), DMF, 0 → 23 °C, 12 h, 92% for the two steps; (j) TFA (1.75 equiv), CH_2Cl_2, 0 → 23 °C, 6 h; (k) **10** (2.2 equiv), HATU (3.2 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 85% for the two steps; (l) Me_3SnOH (10 equiv), CH_2Cl_2, reflux, 12 h, 68%; (m) Ac_2O (7.5 equiv), pyridine, 0 → 23 °C, 12 h, 79%. TMS = trimethylsilyl; PIFA = phenyliodine(III)bis(trifluoroacetate); (*S*)-CBS = (3a*S*)-tetrahydro-1-methyl-3,3-diphenyl-1*H*,3*H*-pyrrolo[1,2-*c*][1,3,2]oxazaborole; TEMPO = (2,2,6,6-tetramethylpiperidin-1-yl)oxyl; BAIB = bis-(acetoxy)iodo benzene; Ac = acetyl; py = pyridine; THF = tetrahydrofuran; HATU = 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; DMF = *N*,*N*-dimethylformamide; TFA = trifluoroacetic acid; Boc = *tert*-butyloxycarbonyl.

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



[a]Reagents and conditions: (a) $TMSCHN_2$ (1.2 equiv; 2.0 M in diethyl ether), toluene:methanol (2:1, *v*/*v*), 0 → 23 °C, 0.5 h, 74%; (b) $LiAlH_4$ (2.0 equiv; 2.0 M in THF), THF, 0 °C, 1 h, 98%; (c) CBr_4 (2.0 equiv), PPh_3 (2.0 equiv), benzene, 0 → 10 °C, 1 h, 81%; (d) **13** (1.2 equiv), *n*-BuLi (1.2 equiv; 2.5 M in hexanes), THF, −78 → 0 °C, 2.5 h, 78%; (e) TBAF (2.0 equiv; 1.0 M in THF), THF, 0 °C, 1 h, 94%; (f) DMP (1.5 equiv), CH_2Cl_2, 23 °C, 15 min, 90%; (g) $NaClO_2$ (5.4 equiv), $NaH_2PO_4{\cdot}H_2O$ (12.5 equiv), 2-methyl-2-butene (7.5 equiv), *t*-BuOH, THF, H_2O, 23 °C, 1 h; 92%; (h) **6** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 82%; (i) TFA (45 equiv), CH_2Cl_2, 23 °C, 2 h; (j) **17** (4.1 equiv), *i*-Pr_2NEt (6.2 equiv), DMF, 0 → 23 °C, 18 h, 95% for the two steps; (k) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (l) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 72% for the two steps; (m) $LiOH{\cdot}H_2O$ (5.0 equiv), THF, H_2O, 23 °C, 24 h, 91%. TBAF = tetra-*n*-butylammonium fluoride; DMP = Dess−Martin periodinane; Fmoc = fluorenylmethyloxycarbonyl.

corresponding methyl esters were converted to their carboxylic acid counterparts **Tb53** and **Tb55**, respectively, through the sequential action of Me_3SnOH[11,57] (cleavage of methyl ester and acetate moieties) and Ac_2O/pyridine (re-acetylation of hydroxy group) in 68% and 74% overall yield, respectively, as presented in Scheme 5.

The syntheses of tubulysin analogues **Tb56** and **Tb57**, where the "right end" (Tup) and the "left end" (Mep) amino acid residues of N^{14}-desacetoxy tubulysin **Tb1**[11] were replaced with structural motifs represented by fragments **33** or **34**[11] and **19** (for their synthesis see Supporting Information), respectively, are presented in Scheme 6. Thus, removal of the Boc group

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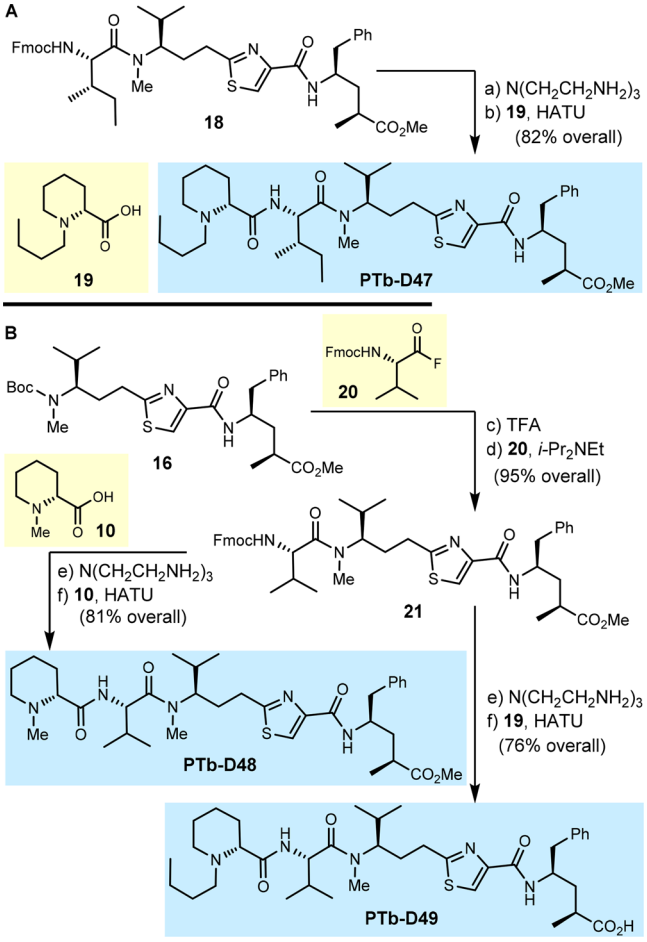
Scheme 3. Synthesis of Pretubulysin D Analogues PTb-D47, PTb-D48, and PTb−D49[a]

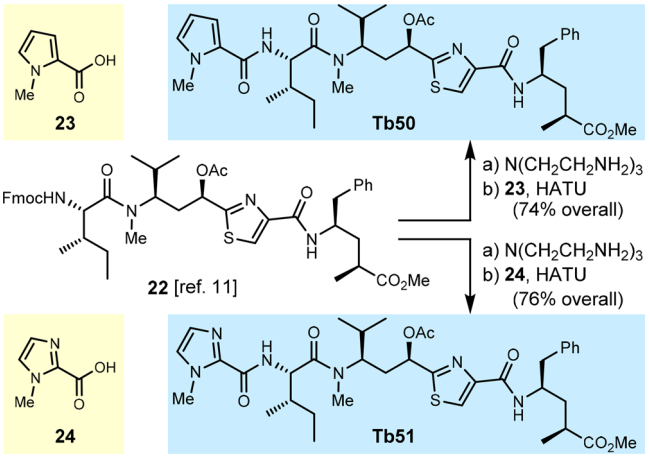


[a]Reagents and conditions: (a) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **19** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 82% for the two steps; (c) TFA (40 equiv), CH_2Cl_2, 23 °C, 2 h; (d) **20** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 95% for the two steps; (e) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (f) **10** or **19** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 81% for the two steps for **PTb-D48** and 76% for the two steps for **PTb-D49**.

from **29**[11] (TFA) followed by reaction of the resulting amine with acid fluoride **17** in the presence of *i*-Pr_2NEt in DMF led to the formation of dipeptide **30** (75% overall yield). The latter was further treated with $N(CH_2CH_2NH_2)_3$ to remove the Fmoc group, and the resulting amine was coupled with pipecolic acid derivative **19** (HATU, Et_3N) to furnish tripeptide **31** in 82% overall yield. Tripeptide **31** was then converted to its carboxylic acid counterpart (**32**) through sequential treatment with Me_3SnOH and Ac_2O as described above for the conversion of **Tb52** to **Tb53**, in 78% overall yield. Finally, coupling of **32** with ammonium salt **33** or **34** under HATU conditions furnished tubulysin analogues **Tb56** (71% yield) and **Tb57** (76% yield), respectively, as shown in Scheme 6 (see Supporting Information for further details).

Tubulysin analogue **Tb59** in which the acetoxy group of **Tb2** was replaced with a carbonyl group, was synthesized from the previously reported acetoxy ester analogue **Tb2**[11] through hydroxy tubulysin **Tb58** as summarized in Scheme 7. Thus, exposure of **Tb2** to Me_3SnOH furnished **Tb58** in 78% yield.

Scheme 4. Synthesis of N^{14}-Desacetoxytubulysin Analogues Tb50 and Tb51[a]



[a]Reagents and conditions: (a) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **23** or **24** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 74% for the two steps for **Tb50** and 76% for the two steps for **Tb51**.

The latter was converted to the desired keto acid analogue **Tb59** (81% yield) through the action of DMP, as shown in Scheme 7.

Tubulysin analogues **Tb60**, **Tb61**, **Tb62**, and **Tb63**, containing a valine instead of an isoleucine residue, were synthesized as summarized in Scheme 8. Specifically, the previously reported analogue **Tb32**[11] was converted to its carboxylic acid counterpart **Tb60** through exposure to Me_3SnOH[11,S7] (cleavage of methyl ester and acetate moieties, 70% yield) followed by re-acetylation of the hydroxy acid to **Tb61** (Ac_2O/pyridine, 61% yield). **Tb60** was converted to its keto acid counterpart **Tb62**, in 78% yield, by DMP oxidation as shown in Scheme 8. Methyl ester formation from the latter using $TMSCHN_2$ furnished **Tb63** in 71% yield.

Scheme 9 summarizes the synthesis of tubulysin analogues **Tb64** and **Tb65**, in which the thiazole moiety was replaced with a pyridine structural motif (while maintaining all the other structural features of **Tb32**, one of our most promising tubulysin analogues).[11] Their synthesis was initiated with the removal of the Boc group from dipeptide **35**[11] (TFA) followed by coupling of the liberated amine with Fmoc-protected acid fluoride **20**[11] to provide tripeptide **36** (99% yield for the two steps) as shown in Scheme 9. Cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] from this intermediate followed by coupling of the so generated amine with *N*-methyl-D-pipecolic acid (**10**) led to tubulysin analogue **Tb64** (75% overall yield). Finally, analogue **Tb64** was converted to its carboxylic acid counterpart **Tb65** through the sequential action of Me_3SnOH[11,S7] and Ac_2O/pyridine in 68% overall yield, as shown in Scheme 9.

Tubulysin analogues **Tb66**, **Tb67**, and **Tb68**, in which the thiazole moiety carries a methyl group, were synthesized from the known and readily available aldehyde **37**[11,S4] as summarized in Scheme 10. Thus, C−H activation-based coupling of aldehyde **37** with methyl thiazole acetate **38**,[11] under the previously reported conditions [$PhI(OCOCF_3)_2$, $TMSN_3$],[11,S5] provided ketone **39** in 75% yield. Reduction of thiazolyl ketone **39** with (*S*)-CBS catalyst in the presence of $BH_3{\cdot}Me_2S$[11,S6] produced alcohol **40** in 72% yield as a single diastereoisomer

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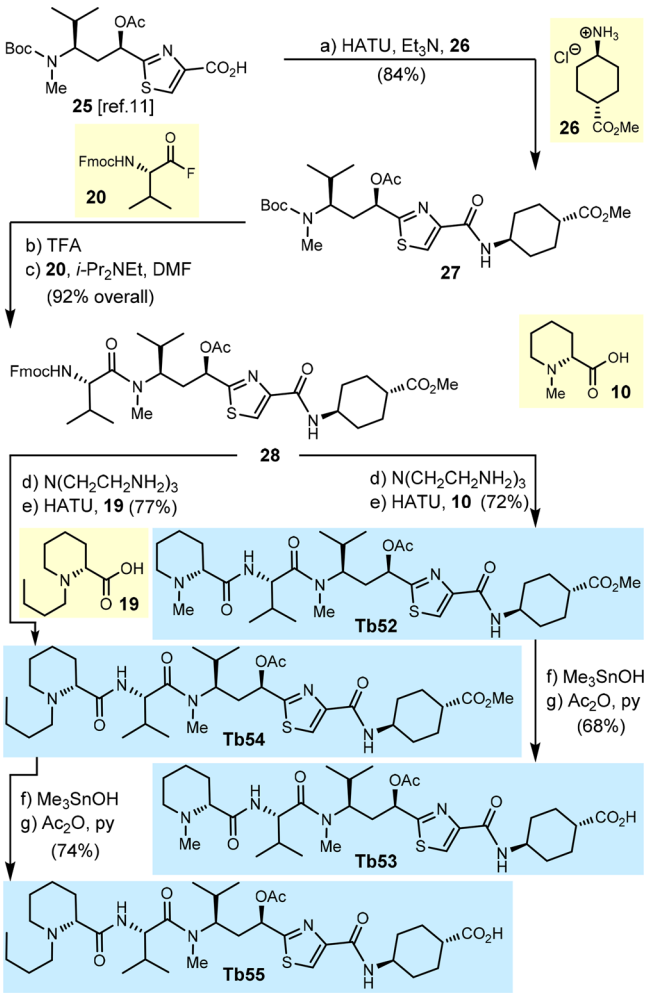
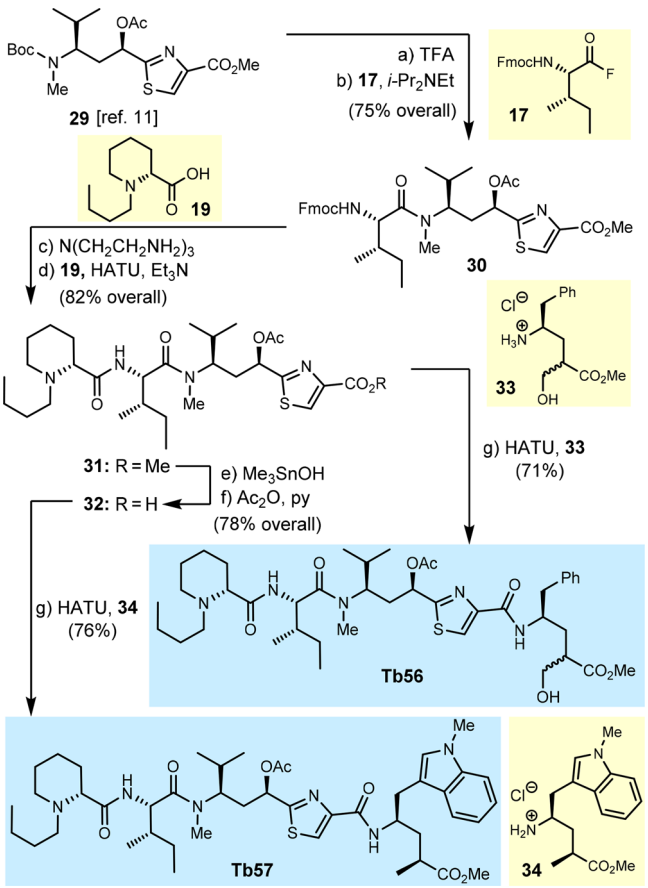
Scheme 5. Synthesis of Tubulysin Analogues Tb52–Tb55[a]



[a]Reagents and conditions: (a) **26** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 23 °C, 18 h, 84%; (b) TFA (40 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (c) **20** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 92% for the two steps; (d) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (e) **10** (3.0 equiv) or **19** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 72% for the two steps for **Tb52**, 77% for the two steps for **Tb54**; (f) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (g) Ac_2O (6.0 equiv), pyridine, 0 → 23 °C, 12 h, 68% for the two steps for **Tb53**, 74% for the two steps for **Tb55**.

Scheme 6. Synthesis of Tubulysin Analogues Tb56 and Tb57[a]



[a]Reagents and conditions: (a) TFA (40 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **17** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 75% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (16 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **19** (1.5 equiv), HATU (1.5 equiv), Et_3N (3.0 equiv), DMF, 0 → 23 °C, 24 h, 82% for the two steps; (e) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (f) Ac_2O (6.0 equiv), pyridine, 0 → 23 °C, 12 h, 78% for the two steps; (g) **33** or **34** (1.2 equiv), HATU (1.2 equiv), Et_3N (2.4 equiv), DMF, 0 → 23 °C, 18 h, 71% for the two steps for **Tb56** and 76% for the two steps for **Tb57**.

after chromatographic purification. The required elaboration of alcohol **40** to acetoxy carboxylic acid **41** was achieved through a sequence involving deacetylation (K_2CO_3, MeOH), selective oxidation of the resulting primary alcohol (TEMPO, BAIB; then $NaClO_2$), and acetylation (Ac_2O, pyridine) of the remaining secondary alcohol, in 61% overall yield for the four steps. Coupling of carboxylic acid **41** and ammonium salt **6**[11] in the presence of HATU and Et_3N led to amide **42** (88% yield). The Boc protecting group was cleaved from the latter compound (TFA), and the resulting amine was coupled with acid fluoride **20**[11] (*i*-Pr_2NEt, 91%) to afford peptide **43** as shown in Scheme 10. Removal of the Fmoc group from **43** [$N(CH_2CH_2NH_2)_3$] followed by coupling of the so generated amine with *N*-methyl-D-pipecolic acid (**10**) provided tubulysin analogue **Tb66** (65% overall yield). Tubulysin analogue **Tb67** was formed from **Tb66** through methyl ester hydrolysis (Me_3SnOH) and acetylation (Ac_2O, pyridine) of the resulting hydroxy acid (62% overall yield), as shown in Scheme 10. Keto acid tubulysin analogue **Tb68** was obtained from **Tb66** by treatment with Me_3SnOH followed by oxidation of the resulting hydroxy acid with Dess–Martin periodinane in 64% overall yield (Scheme 10).

Scheme 11 summarizes the synthesis of tubulysin analogues **Tb69** (lacking the *N*-Me substituent), **Tb70**, and **Tb71**, the latter two containing the *N*-methyl pyrrolidine structural motif as a substitution for the piperidine residue. Thus, advanced intermediate **43** (for preparation see Scheme 10) was converted to its amino counterpart through the action of $N(CH_2CH_2NH_2)_3$, and the latter was coupled with Fmoc-protected pipecolic acid **44** and *N*-methyl-D-proline (**45**) to afford tubulysin analogues **Tb69** and **Tb70** in 62% and 82% overall yields, respectively. Finally, methyl ester **Tb70** was converted to its carboxylic acid counterpart **Tb71** through the sequential action of Me_3SnOH[11,57] and Ac_2O/pyridine in 74% overall yield, as shown in Scheme 11.

Scheme 12 depicts the synthesis of tubulysin analogues **Tb72** and **Tb73**, both of which feature an isopropyl group on the

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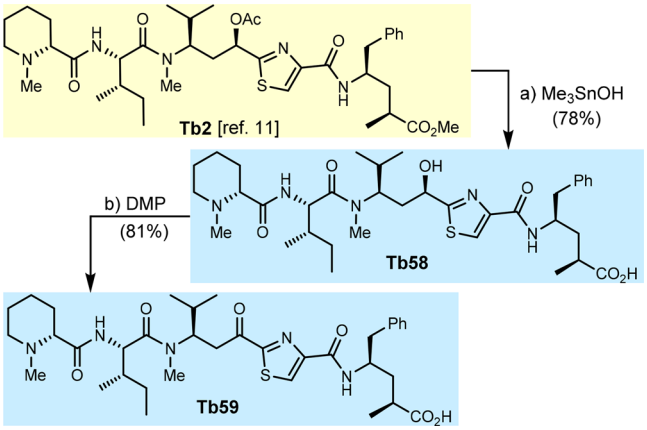
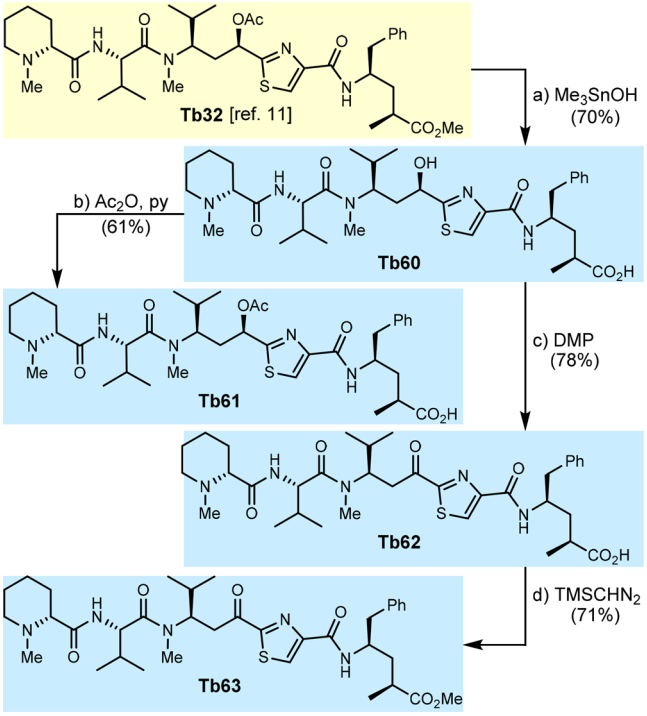
Scheme 7. Synthesis of Tubulysin Analogues Tb58 and Tb59[a]



[a]Reagents and conditions: (a) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h, 78%; (b) DMP (1.5 equiv), CH_2Cl_2, 23 °C, 30 min, 81%.

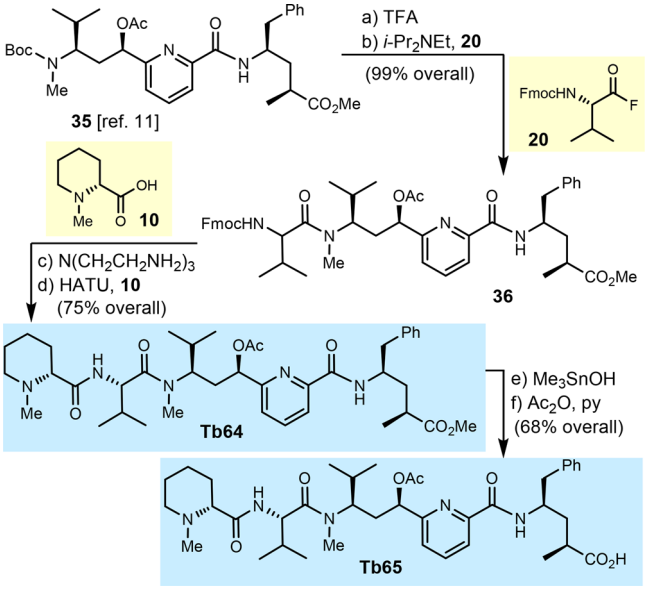
Scheme 8. Synthesis of Tubulysin Analogues Tb60−Tb63[a]



[a]Reagents and conditions: (a) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h, 70%; (b) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 61%; (c) DMP (1.5 equiv), CH_2Cl_2, 0 → 23 °C, 30 min, 78%; (d) $TMSCHN_2$ (1.2 equiv; 2.0 M in diethyl ether), toluene:methanol (2:1, *v*/*v*), 0 → 23 °C, 1 h, 71%.

thiazole structural motif. Thus, commercially available bromothiazole ester derivative **46** was reduced to the corresponding primary alcohol ($LiBH_4$) and the latter was silylated (TBSCl, imidazole, 86% yield for the two steps) to afford bromothiazole **47**. The lithio derivative generated from bromide **47** and *n*-BuLi was then reacted with Weinreb amide **48** forming ketone **49**, whose asymmetric reduction with (*S*)-CBS catalyst and $BH_3{\cdot}Me_2S$ gave, stereoselectively, hydroxy compound **50**. The latter was elaborated to acetoxy carboxylic

Scheme 9. Synthesis of Tubulysin Analogues Tb64 and Tb65[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 23 °C, 2 h; (b) **20** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 99% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 75% for the two steps; (e) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (f) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 68% for the two steps.

acid **51** through a sequence involving acetylation (Ac_2O, pyridine, 82% yield), desilylation (TBAF, 98% yield), and oxidation (DMP, 89% yield; then $NaClO_2$, 2-methyl-2-butene, 98% yield). Carboxylic acid **51** was coupled with ammonium salt **6** under HATU conditions furnishing Boc-protected segment **52**, whose deprotection (Boc removal, TFA) and union of the resulting amine with acid fluoride **20** under standard conditions led to fragment **53** in 84% overall yield. Deprotection of the latter [$N(CH_2CH_2NH_2)_3$] followed by coupling of the resulting amine with *N*-methyl-D-pipecolic acid (**10**) under HATU conditions furnished coveted tubulysin analogue **Tb72** (81% overall yield), and its carboxylic acid counterpart **Tb73** (72% overall yield) upon sequential ester cleavage and re-acetylation under the standard conditions mentioned above and summarized in Scheme 12.

Tubulysins **Tb74** and **Tb75** carry oxygenated pipecolic acid residues as well as an isopropyl group on their thiazole moiety, as shown in their structures (see Scheme 13). They were synthesized from advanced intermediate **53** (for preparation, see Scheme 12) as shown in Scheme 13. Thus, Fmoc derivative **53** was deprotected [$N(CH_2CH_2NH_2)_3$], and the resulting amine was reacted with hydroxy *N*-methyl pipecolic acid **54** under HATU conditions to afford **Tb74** in 69% overall yield. Analogue **Tb75** was generated from **Tb74** by DMP oxidation in 78% yield as shown in Scheme 13.

Retaining the valine moiety instead of the isoleucine residue just like their **Tb74** and **Tb75** siblings but lacking the isopropyl group on their thiazole ring, tubulysins **Tb76** and **Tb77** feature oxygenated *N*-methyl pipecolic acid structural motifs and an ethyl, rather than a methyl, ester group at the other end of the molecule. Their synthesis proceeded from *N*-Boc-protected thiazolyl carboxylic acid **25**[11] as summarized in Scheme 14.

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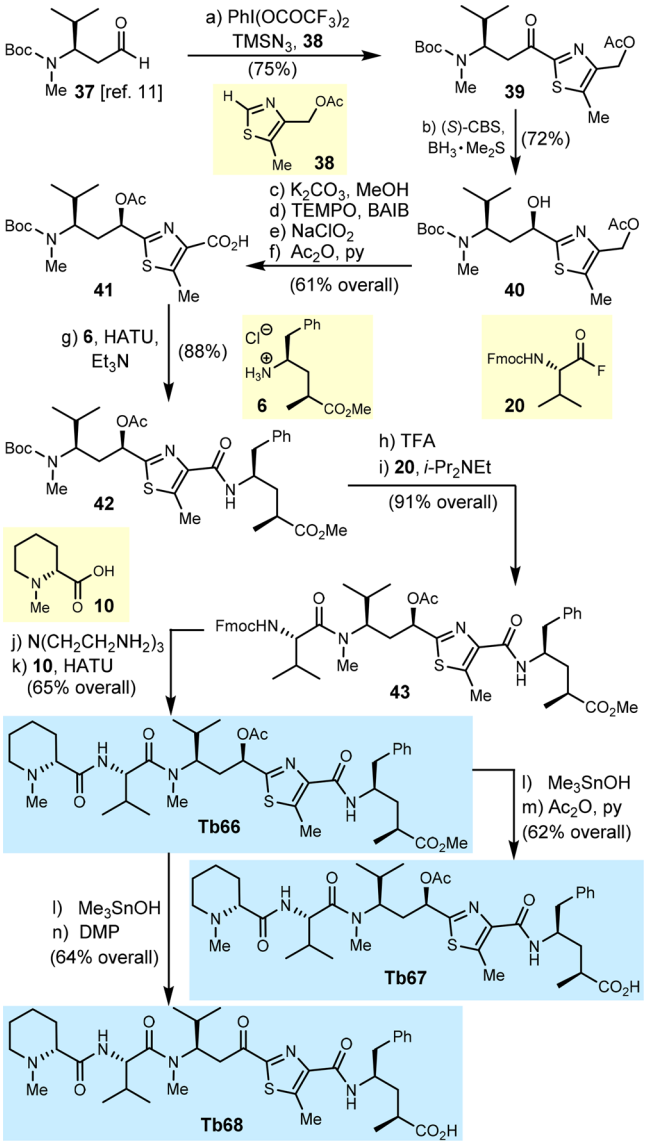
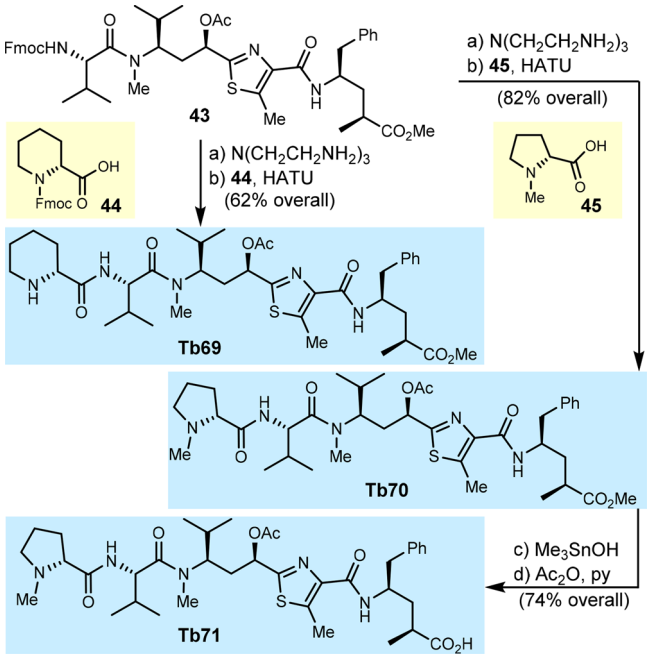
Scheme 10. Synthesis of Tubulysin Analogues Tb66–Tb68[a]



[a]Reagents and conditions: (a) **37** (2.0 equiv), **38** (1.0 equiv), $TMSN_3$ (2.0 equiv), PIFA (2.0 equiv), benzene, 23 °C, 16 h; then **37** (2.0 equiv), $TMSN_3$ (2.0 equiv), PIFA (2.0 equiv), 23 °C, 12 h, 75%; (b) (*S*)-CBS (0.2 equiv; 1.0 M in toluene), $BH_3{\cdot}Me_2S$ (1.0 equiv; 2.0 M in THF), 0 → 23 °C, 18 h, 72%; (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, 91%; (e) $NaClO_2$ (5.4 equiv), $NaH_2PO_4{\cdot}H_2O$ (12.2 equiv), 2-methyl-2-butene (7.5 equiv), *t*-BuOH, THF, H_2O, 23 °C, 12 h; (f) Ac_2O (3.0 equiv), pyridine (3.0 equiv), CH_2Cl_2, 0 → 23 °C, 15 h, 72% for the two steps; (g) **6** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 88%; (h) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (i) **20** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 91% for the two steps; (j) $N(CH_2CH_2NH_2)_3$ (15 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, 65% for the two steps; (l) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (m) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 62% for the two steps; (n) DMP (1.5 equiv), CH_2Cl_2, 0 → 23 °C, 30 min, 64% for the two steps.

Thus, **25** was coupled to ammonium salt **55** in the presence of HATU and Et_3N to afford dipeptide **56** (81% yield), whose exposure to TFA led to the corresponding amine. Coupling of the latter with acid fluoride **20** was facilitated by *i*-Pr_2NEt leading to tripeptide **57**. Analogue **Tb76** was smoothly generated from **57**, upon liberation of its amino group [$N(CH_2CH_2NH_2)_3$] and union of the resulting amine substrate with hydroxypipecolic acid **54** under the influence of HATU and Et_3N (96% yield for the two steps). Finally, silylation of the resulting alcohol with TBDMSOTf and 2,6-lutidine furnished analogue **Tb77** in 87% overall yield, as presented in Scheme 14. The latter analogue was meant to test the effect of increased lipophilicity of the TBS-bearing pipecolic acid residue.

Scheme 11. Synthesis of Tubulysin Analogues Tb69–Tb71[a]



[a]Reagents and conditions: (a) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **44** or **45** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 62% for the two steps for **Tb69** and 82% for the two steps for **Tb70**; (c) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (d) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 74% for the two steps.

Tubulysin analogue **Tb78**, whose novel structural motif is the pentyl spirocycle moiety instead of the isoleucine residue, was synthesized as shown in Scheme 15. Thus, removal of the Boc group from previously reported dipeptide **58**[11] (TFA) and coupling of the liberated amine with Fmoc-protected acid fluoride **59** (see Supporting Information for preparation) under standard conditions provided tripeptide **60** (56% yield for the two steps). Cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] from the latter followed by coupling of the generated amine with *N*-methyl-D-pipecolic acid (**10**) led to the targeted tubulysin analogue **Tb78** in 69% overall yield.

Scheme 16 summarizes the synthesis of tubulysin analogues **Tb79** and **Tb80**, both of which feature a hexafluoro isopropyl unit[59] as opposed to their isoleucine residue. Their synthesis began with removal of the Boc group from the previously reported dipeptide **58**[11] and proceeded with coupling of the liberated amine with Fmoc-protected acid fluoride **61** (prepared from its amino acid counterpart by sequential exposure to FmocCl and DAST; see Supporting Information for details) followed by cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] to afford amine **62** (27% yield for the three steps) as shown in Scheme 16 (see Supporting Information for further details). Coupling of the so generated

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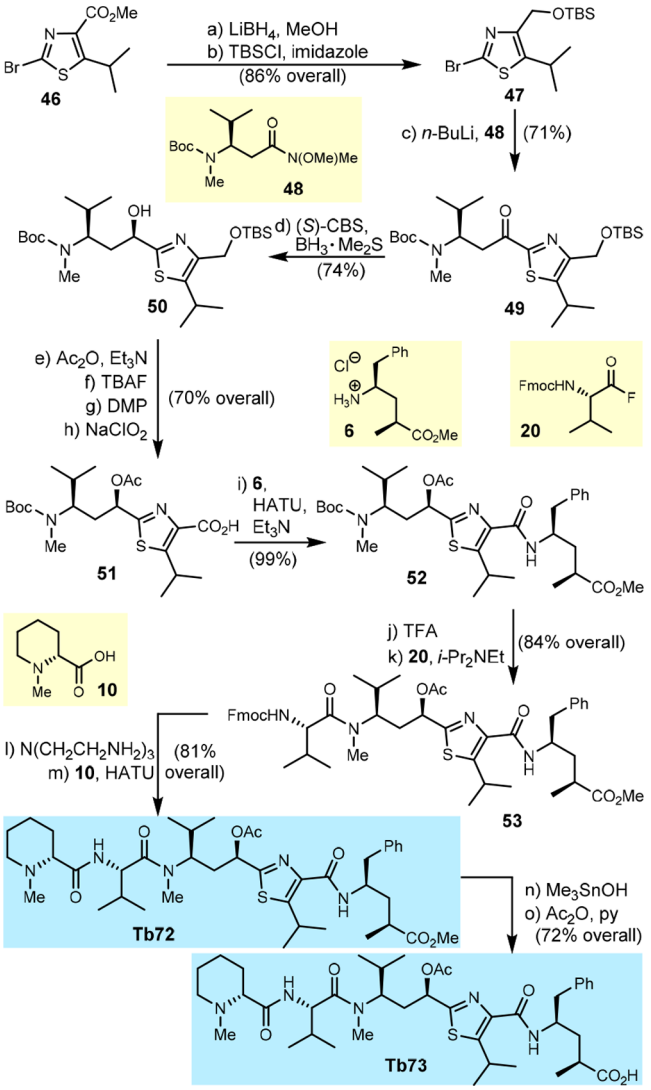
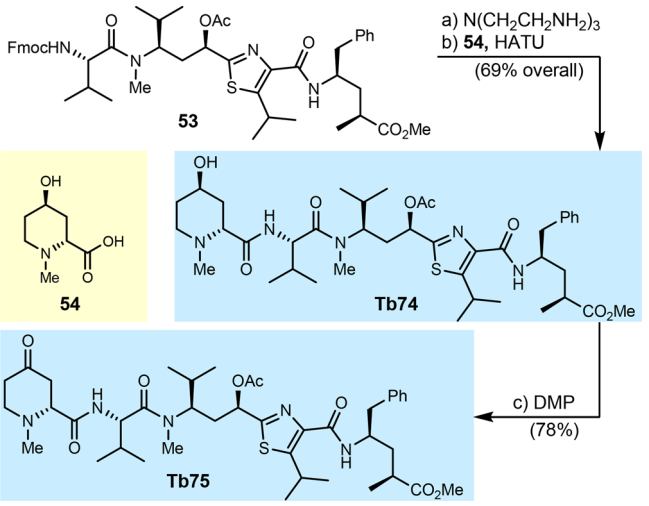
Scheme 12. Synthesis of Tubulysin Analogues Tb72 and Tb73[a]



[a]Reagents and conditions: (a) $LiBH_4$ (1.53 equiv; 2 M in THF), MeOH (1.55 equiv), THF, 0 → 23 °C, 12 h; (b) TBSCl (1.23 equiv), imidazole (1.23 equiv), CH_2Cl_2, 0 → 23 °C, 0.5 h, 86% for the two steps; (c) *n*-BuLi (1.44 equiv; 2.5 M in hexanes), **48** (1.0 equiv), THF, −78 → −50 °C, 3 h, 71%; (d) (*S*)-CBS (0.1 equiv, 1.0 M in toluene), $BH_3{\cdot}Me_2S$ (1.0 equiv; 2.0 M in THF), 0 → 23 °C, 36 h, 74%; (e) Ac_2O (3.0 equiv), Et_3N (4.0 equiv), 0 → 23 °C, 2 h, 82%; (f) TBAF (2.0 equiv; 1 M in THF), THF, 0 → 23 °C, 30 min, 98%; (g) DMP (1.5 equiv), CH_2Cl_2, 23 °C, 1 h, 89%; (h) $NaClO_2$ (5.4 equiv), $NaH_2PO_4{\cdot}H_2O$ (12.2 equiv), 2-methyl-2-butene (7.5 equiv), *t*-BuOH, THF, H_2O, 23 °C, 1 h, 98%; (i) **6** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 99%; (j) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (k) **20** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 84% for the two steps; (l) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (m) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 81% for the two steps; (n) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (o) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 72% for the two steps.

amine **62** with *N*-methyl-D-pipecolic acid (**10**) resulted in the formation of tubulysin analogue **Tb79** (87% yield). Finally, time controlled exposure of **Tb79** to Me_3SnOH[11,57] (5 h; cleavage of acetate only) furnished analogue **Tb80** in 87% yield, as shown in Scheme 16.

Scheme 13. Synthesis of Tubulysin Analogues Tb74 and Tb75[a]



[a]Reagents and conditions: (a) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **54** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 69% for the two steps; (c) DMP (1.5 equiv), CH_2Cl_2, 23 °C, 30 min, 78%.

Scheme 17 summarizes the synthesis of tubulysin analogues **Tb81**, **Tb82**, **Tb83**, and **Tb84**, which incorporate a trifluoro ethyl moiety,[59] instead of the isoleucine residue. Their synthesis began with removal of the Boc group from the previously reported dipeptide **58**[11], and coupling of the liberated amine with Fmoc-protected acid fluoride **63** (prepared from its amino acid counterpart by sequential exposure to FmocCl, and DAST; see Supporting Information for details) to provide tripeptide **64** (70% yield for the two steps), as shown in Scheme 17 (see Supporting Information for further details). Cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] from this intermediate afforded free amine **65** (82% yield), which was coupled with either *N*-methyl-D-pipecolic acid (**10**) or *N*-methyl-D-proline (**45**) to give tubulysin analogues **Tb81** (79% yield) or **Tb82** (66% yield), respectively. Finally, exposure of **Tb81** and **Tb82** to Me_3SnOH[11,57] furnished analogues **Tb83** (82% yield) and **Tb84** (79% yield), respectively, as shown in Scheme 17.

Scheme 18 summarizes the synthesis of tubulysin analogues **Tb85**, **Tb86**, and **Tb87**, which incorporate an (*R*)-hexafluoro isopropyl moiety[59] instead of the (*S*)-isoleucine residue found in many of the other designed analogues. Their synthesis began with removal of the Boc group (TFA) from the previously reported dipeptide **58**[11] followed by coupling of the so obtained amine with Fmoc-protected acid fluoride **66** (prepared from its amino acid counterpart by sequential exposure to FmocCl and DAST) to provide tripeptide **67**, upon cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] (38% yield for the three steps), as shown in Scheme 18 (see Supporting Information for further details). Coupling of the latter with either *N*-methyl-D-pipecolic acid (**10**) or *N*-methyl-D-proline (**45**) under HATU conditions led to tubulysin analogues **Tb85** (89% yield) and **Tb86** (88% yield), respectively. Sequential treatment of **Tb85** with Me_3SnOH[11,57] and Ac_2O/pyridine then gave analogue **Tb87** in 89% overall yield as shown in Scheme 18.

Tubulysin analogue **Tb88**, lacking isoleucin's side chain, was constructed as shown in Scheme 19. Thus, removal of the Boc group from dipeptide **56** (for preparation, see Scheme 14) with

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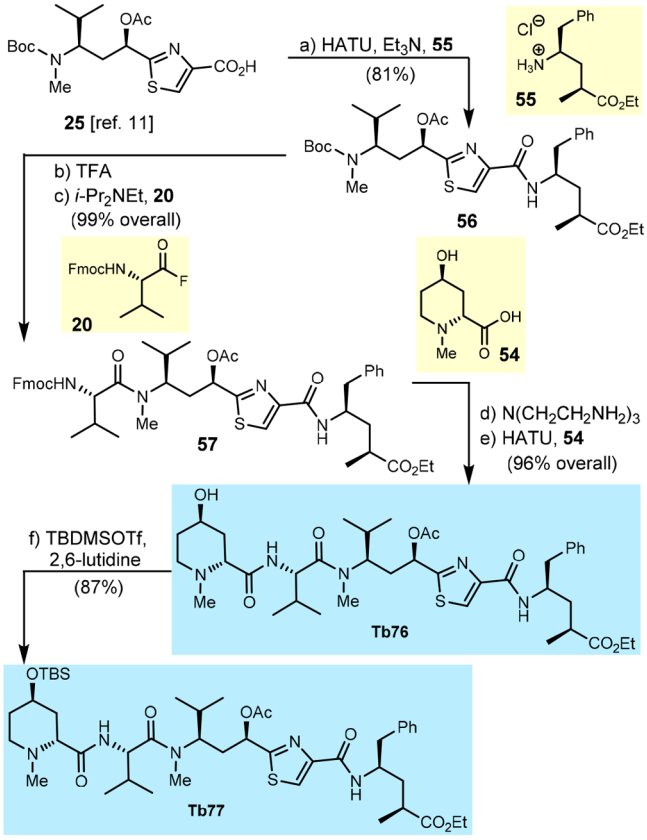
Scheme 14. Synthesis of Tubulysin Analogues Tb76 and Tb77[a]



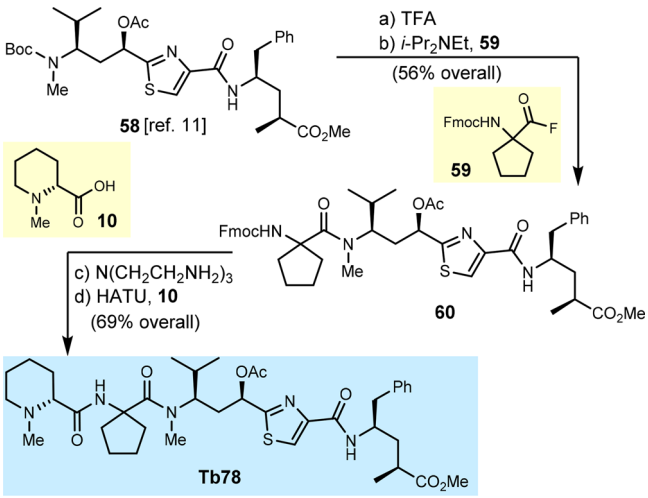
[a]Reagents and conditions: (a) **55** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 23 °C, 24 h, 81%; (b) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (c) **20** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 99% for the two steps; (d) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (e) **54** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 96% for the two steps; (f) TBDMSOTf (2.0 equiv), 2,6-lutidine (3.0 equiv), CH_2Cl_2, 0 → 23 °C, 0.5 h, 87%.

TFA followed by coupling of the resulting amine with Fmoc-protected acid fluoride **68** in the presence of *i*-Pr_2NEt afforded tripeptide **69** (86% yield for the two steps). Cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] from the latter followed by coupling of the generated amine with *N*-methyl-D-pipecolic acid (**10**) under HATU conditions led to the targeted tubulysin analogue **Tb88** (72% overall yield).

Scheme 20 summarizes the synthesis of tubulysin analogues **Tb89** and **Tb91**, both featuring an alanine in place of their isoleucine residue, and **Tb90** and **Tb92**, which furthermore feature the proline counterpart (as represented by building block **45**) of the pipecolic acid residue. The synthesis of these tubulysin analogues started with Boc-protected dipeptide **58**[11] and proceeded through tripeptide **71**. Thus, exposure of **58** to TFA generated the corresponding free amine, which was coupled with acid fluoride **70** (prepared from its amino acid precursor by sequential treatment with FmocCl and DAST; see Supporting Information for further details) in the presence of *i*-Pr_2NEt to furnish **71** in 92% overall yield. Removal of the Fmoc group [$N(CH_2CH_2NH_2)_3$] from this intermediate followed by union of the resulting amine with either *N*-methyl-D-pipecolic acid (**10**) or *N*-methyl-D-proline (**45**) in the presence of HATU led to tubulysin analogues **Tb89** (89% overall yield) and **Tb90** (88% overall yield), respectively. Finally, **Tb89** and **Tb90** were converted to their carboxylic acid counterparts **Tb91** and **Tb92** through the sequential action of Me_3SnOH[11,57] and Ac_2O/pyridine in 82% and 85% overall yield, respectively, as presented in Scheme 20.

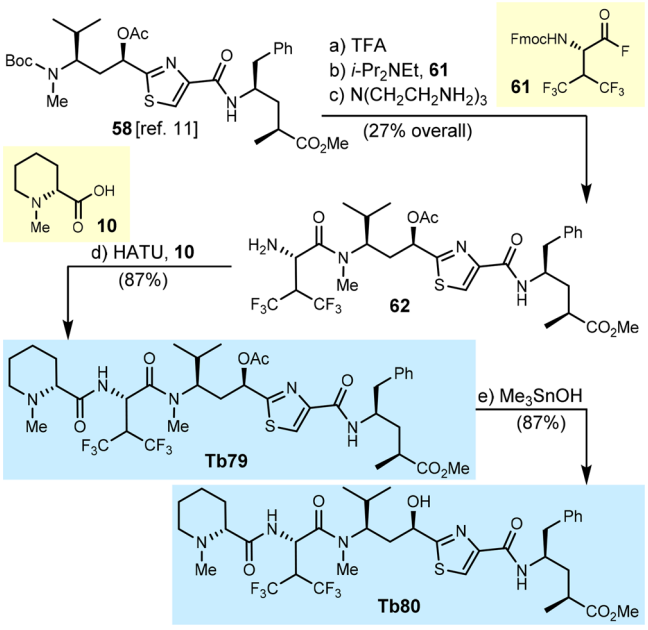
Tubulysin analogues **Tb93** and **Tb94**, featuring an ethyl group instead of the isobutyl group at their isoleucine residue,

Scheme 15. Synthesis of Tubulysin Analogue Tb78[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **59** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 56% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 69% for the two steps.

Scheme 16. Synthesis of Tubulysin Analogues Tb79 and Tb80[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **61** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h, 27% for the three steps; (d) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 87%; (e) Me_3SnOH (20 equiv), 1,2-dichloroethane, reflux, 5 h, 87%.

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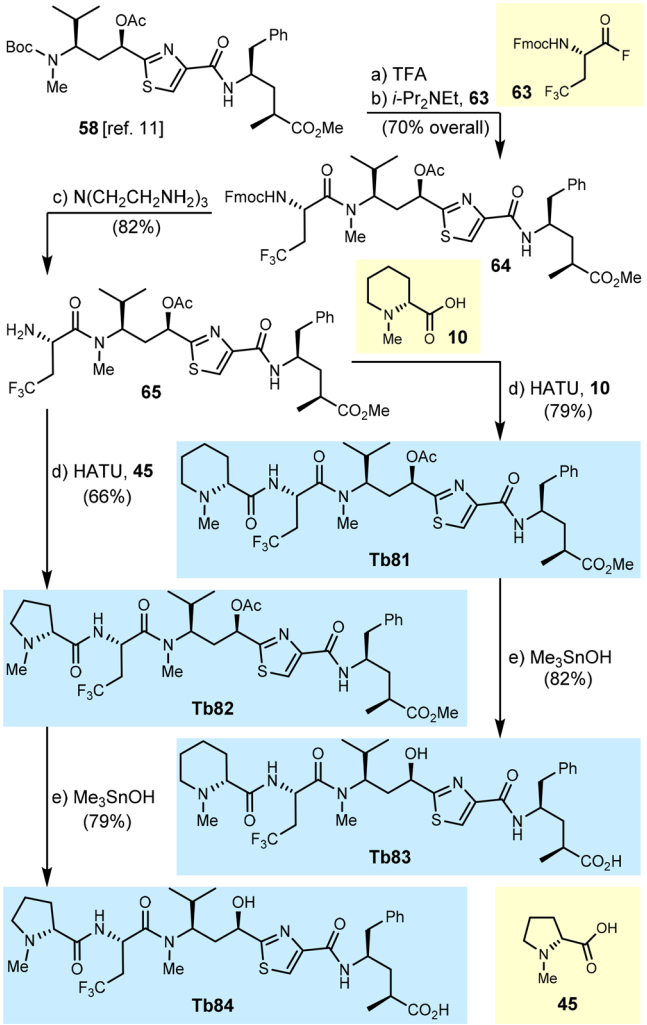
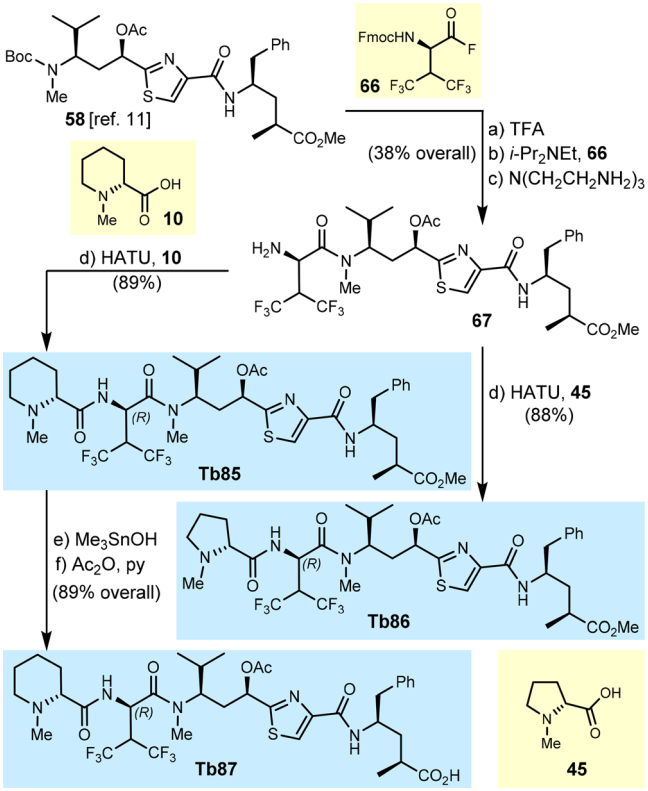
Scheme 17. Synthesis of Tubulysin Analogues Tb81–Tb84[a]



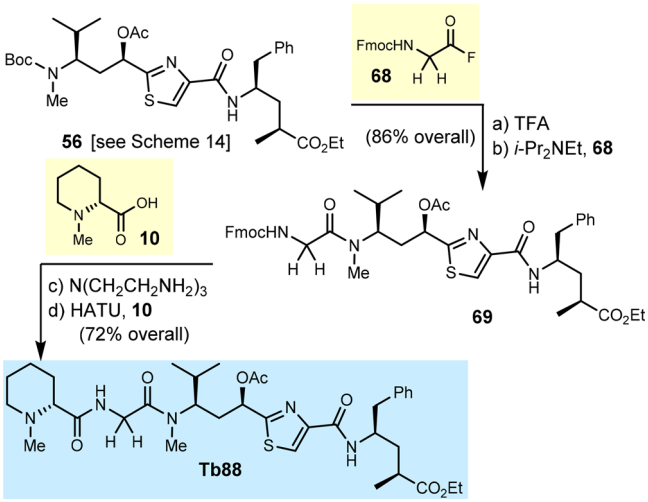
[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **63** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 70% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h, 82%; (d) **10** or **45** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 79% for **Tb81** and 66% for **Tb82**; (e) Me_3SnOH (20 equiv), 1,2-dichloroethane, reflux, 5 h, 82% for the **Tb83** and 79% for the **Tb84**.

Scheme 18. Synthesis of Tubulysin Analogues Tb85–Tb87[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **66** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h, 38% for the three steps; (d) **10** or **45** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 89% for **Tb85** and 88% for **Tb86**; (e) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (f) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 89% for the two steps.

Scheme 19. Synthesis of Tubulysin Analogue Tb88[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **68** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 86% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 72% for the two steps.

were synthesized as summarized in Scheme 21. Thus, removal of the Boc group from fragment **56** (for preparation, see Scheme 14) with TFA and coupling of the so obtained amine with Fmoc-protected acid fluoride **72** in the presence of *i*-Pr_2NEt provided tripeptide **73** (85% yield for the two steps). Cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] from the latter followed by coupling of the resulting amine with *N*-methyl-D-pipecolic acid (**10**) gave tubulysin analogue **Tb93** (86% overall yield). Methyl ester **Tb93** was then converted to its carboxylic acid counterpart **Tb94** through sequential use of Me_3SnOH[11,57] and Ac_2O/pyridine in 76% overall yield, as shown in Scheme 21.

Scheme 22 summarizes the synthesis of tubulysin analogues **Tb95** and **Tb96**, whose primary feature is the *n*-butyl group in place of their isoleucine side chain. Their synthesis began with removal of the Boc group from intermediate **56** (for preparation, see Scheme 14) and coupling of the liberated amine with Fmoc-protected acid fluoride **74** (prepared from its amino acid counterpart by sequential exposure to FmocCl and DAST) to provide tripeptide **75** (98% yield for the two steps)

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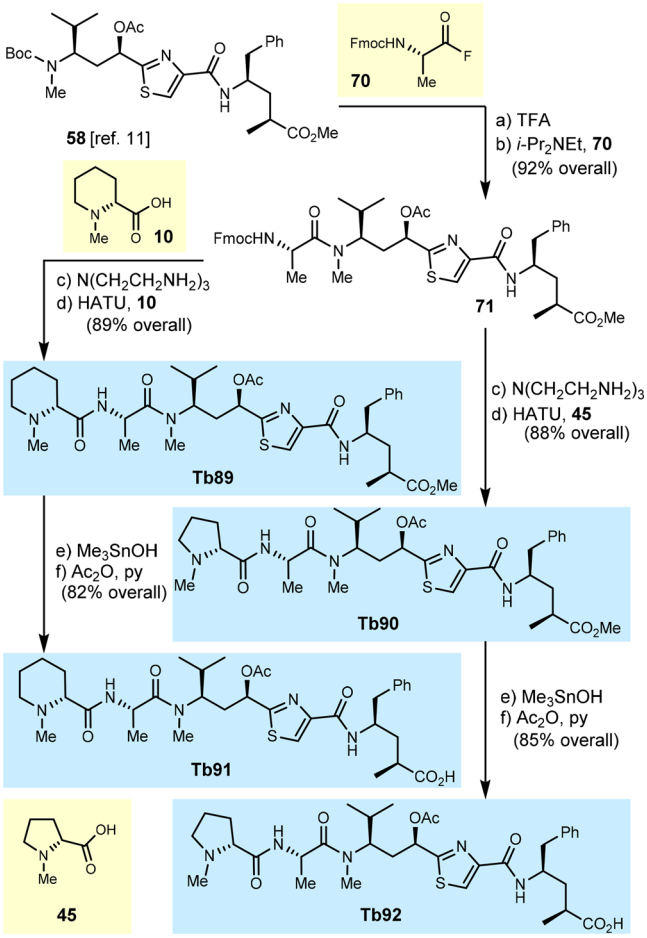
Scheme 20. Synthesis of Tubulysin Analogues Tb89–Tb92[a]

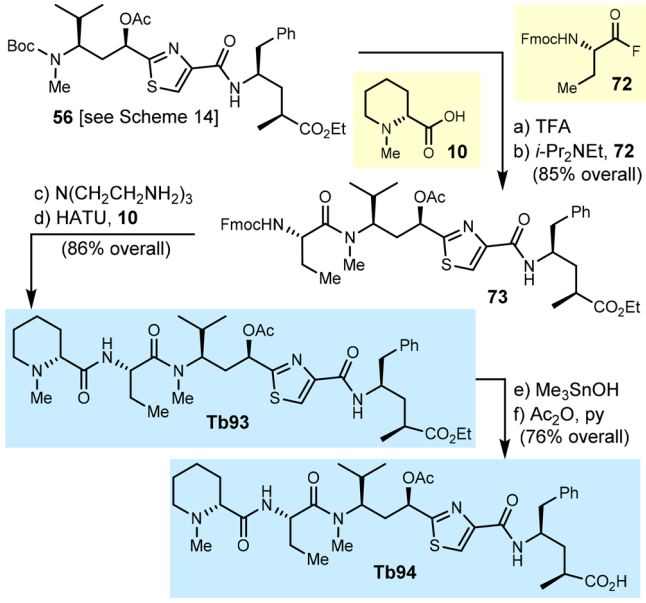


[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **70** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 92% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **10** or **45** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 89% for the two steps for **Tb89** and 88% for the two steps for **Tb90**; (e) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (f) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 82% for the two steps for **Tb91** and 85% for the two steps for **Tb92**.

as shown in Scheme 22 (see Supporting Information for further details). Cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] from this intermediate, followed by coupling with either *N*-methyl-D-pipecolic acid (**10**) or its proline sibling **45** led to tubulysin analogues **Tb95** (90% overall yield) and **Tb96** (87% overall yield), respectively, as shown in Scheme 22.

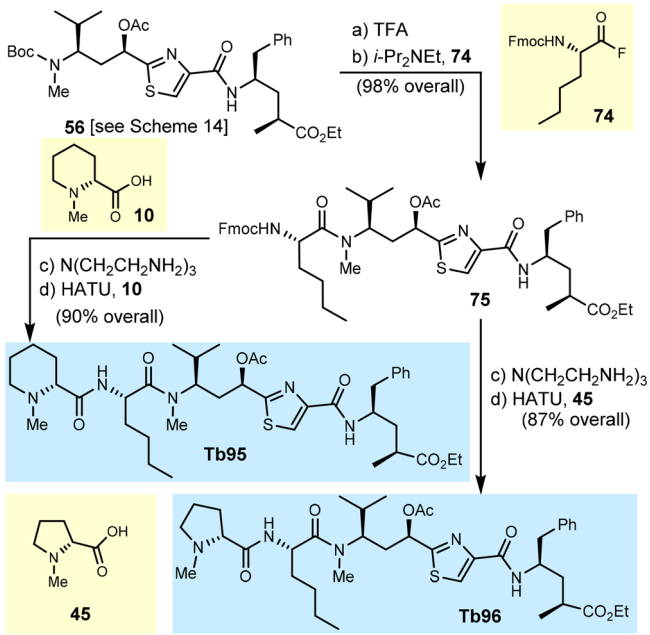
Tubulysin analogues **Tb97** and **Tb98**, whose novel feature is their 3-methylbutyl moiety as opposed to their isoleucine residue, were constructed from dipeptide fragment **56** (for preparation see Scheme 14). Thus, removal of the Boc group from dipeptide **56** and coupling of the so generated amine with Fmoc-protected acid fluoride **76** (prepared from its amino acid counterpart by sequential exposure to FmocCl and DAST) provided tripeptide **77** (93% yield for the two steps) as shown in Scheme 23 (see Supporting Information for further details). Cleavage of the Fmoc group [$N(CH_2CH_2NH_2)_3$] from the latter followed by coupling with either *N*-methyl-D-pipecolic acid (**10**) or *N*-methyl-D-proline (**45**) led to tubulysin analogues **Tb97** (79% overall yield) or **Tb98** (74% overall yield), as summarized in Scheme 23.

Scheme 21. Synthesis of Tubulysin Analogues Tb93 and Tb94[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **72** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 85% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 86% for the two steps; (e) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (f) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 76% for the two steps.

Scheme 22. Synthesis of Tubulysin Analogues Tb95 and Tb96[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **74** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 98% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **10** or **45** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 90% for the two steps for **Tb95** and 87% for the two steps for **Tb96**.

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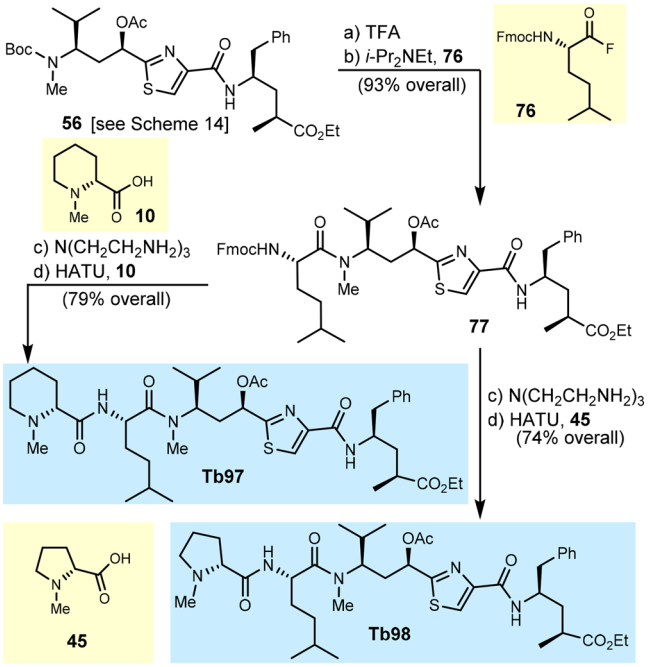
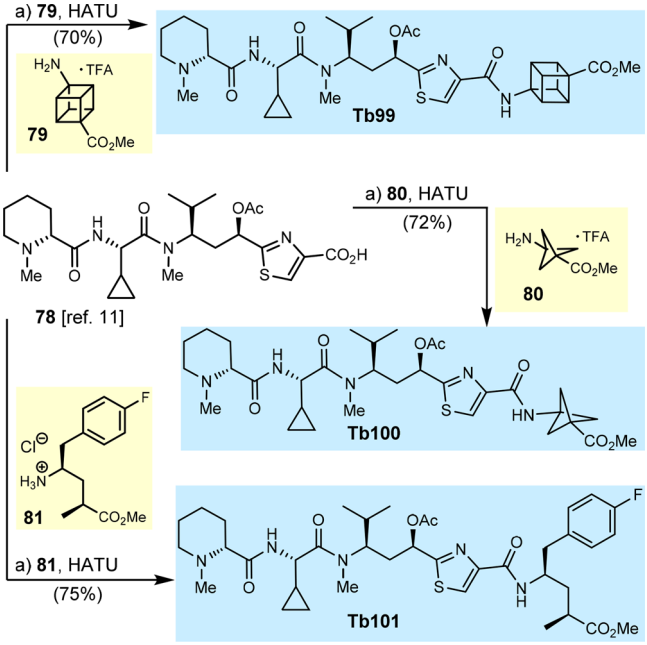
Scheme 23. Synthesis of Tubulysin Analogues Tb97 and Tb98[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **76** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 93% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **10** or **45** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 79% for the two steps for **Tb97** and 74% for the two steps for **Tb98**.

Scheme 24. Synthesis of Tubulysin Analogues Tb99–Tb101[a]



[a]Reagents and conditions: (a) **79** or **80** or **81** (5.0 equiv), HATU (5.0 equiv), Et_3N (10 equiv), DMF, 0 → 23 °C, 16 h, 70% for **Tb99**, 72% for **Tb100**, and 75% for **Tb101**.

Tubulysins **Tb99–Tb101** are characterized with rather drastic modifications at their isoleucine and tubuphenylalanine residues (i.e., cyclopropyl, cubane, and [1.1.1]bicyclopentane moieties). Their syntheses are shown in Scheme 24. Thus, the previously synthesized cyclopropyl-carrying intermediate **78**[11] was coupled with amino esters **79**,[11,60] **80**,[11,60] and **81**[11] under the influence of HATU and Et_3N to afford amides **Tb99** (70% yield), **Tb100** (72% yield) and **Tb101** (75% yield), respectively.

Tubulysin analogues **Tb102** and **Tb103** (both featuring a [1.1.1]bicyclopentane structural motif at the "right edge" of the molecule instead of the tubuphenylalanine residue), **Tb104** (featuring the bulkier naphthalene instead of the phenyl moiety on its tubuphenylalanine residue), and **Tb105** (missing the methyl group on its tubuphenylalanine residue) were synthesized as highlighted in Scheme 25. Thus, key intermediate **82**[11] was coupled with amino acid methyl ester **83** (see Supporting Information for its preparation) under the influence of HATU and Et_3N to afford **Tb103** (79% yield). Similarly carboxylic acid **82**[11] was joined with amino acid methyl ester **80**[11,60] leading to **Tb102** (75% yield). **Tb104** and **Tb105** were synthesized through similar couplings of **82**[11] with fragments **84** (see Supporting Information for preparation) (78% yield) and **85** (see Supporting Information for preparation) (78% yield) as summarized in Scheme 25.

The next series of tubulysin analogues (i.e., **Tb106–Tb109**, Schemes 26 and 27) were intended to probe the effect of shape, but mainly volume of the lipophilic substituent of the isoleucine residue on the potency of the tubulysin molecule. Thus, tubulysins **Tb106** and **Tb107**, carrying a tertiary butyl group on their isoleucine residue, were synthesized from dipeptide fragment **56** (prepared as shown in Scheme 14) as summarized in Scheme 26. Thus, deprotection of the amino group (TFA) of **56** and coupling of the resulting amine with acid fluoride **86** (see Supporting Information for preparation) in the presence of *i*-Pr_2NEt afforded tripeptide **87** (81% overall yield for the two steps). Removal of the Fmoc group from the latter [$N(CH_2CH_2NH_2)_3$] and coupling of the so generated amine with carboxylic acid **10**[11] (HATU, Et_3N) led first to **Tb106** (76% overall yield) and then to **Tb107** upon ester hydrolysis (Me_3SnOH)[11,57] and re-acetylation (Ac_2O, pyridine), in 84% yield for the two steps.

Tubulysin analogues **Tb108** and **Tb109**, carrying a 3,3-dimethylpentanoic group at their isoleucine residue were similarly synthesized from **56** as depicted in Scheme 27. Thus, deprotection of **56** as described above (i.e., TFA) followed by *i*-Pr_2NEt facilitated coupling of the resulting amine with acid fluoride **88** (see Supporting Information for preparation) furnished intermediate tripeptide **89** (72% overall yield from **56**). Fmoc removal from **89** with [$N(CH_2CH_2NH_2)_3$] and coupling of the so obtained amine with carboxylic acid **10** facilitated by HATU and Et_3N led, in 74% overall yield, to analogue **Tb108**. Finally, exposure of **Tb108** to Me_3SnOH furnished the corresponding hydroxy carboxylic acid, which was acetylated (Ac_2O, pyridine) to afford analogue **Tb109**, in 70% overall yield from **Tb108**, as shown in Scheme 27.

In an attempt to decipher further SARs within the tubulysin family of compounds we designed and synthesized tubulysin analogues **Tb110–Tb113** (Scheme 28) equipped with benzyloxy ethyl and hydroxy ethyl groups on the thiazole ring. To this end, bromothiazole methyl ester **90**[61] (for preparation, see Supporting Information) was reduced with DIBAL-H to the corresponding alcohol, which was silylated (TBSOTf, 2,6-lutidine) to afford TBS-ether **91** (86% overall

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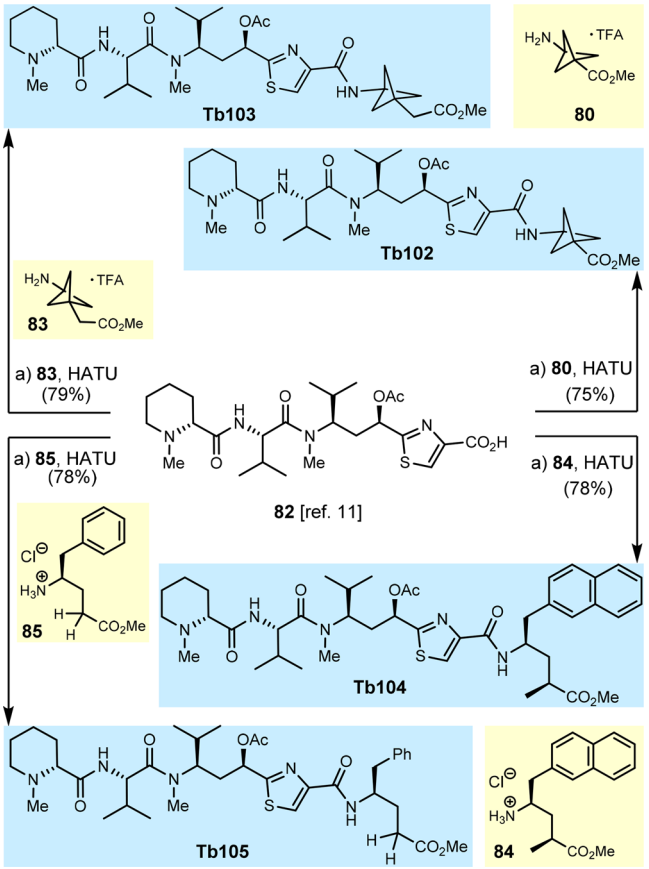
Scheme 25. Synthesis of Tubulysin Analogues Tb102–Tb105[a]

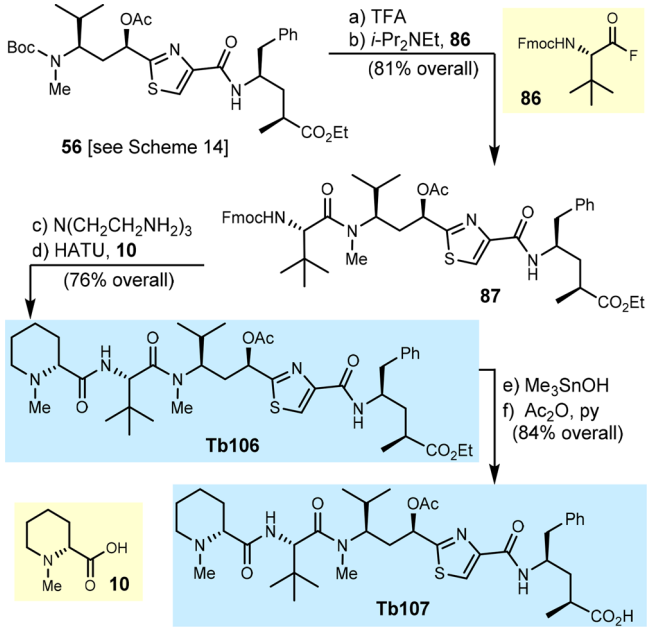


[a]Reagents and conditions: (a) **80** or **83** or **84** or **85** (1.2 equiv), HATU (1.2 equiv), Et_3N (2.4 equiv), DMF, 0 → 23 °C, 18 h, 75% for **Tb102**, 79% for **Tb103**, 78% for **Tb104**, and 78% for **Tb105**.

yield). Bromide **91** was treated with *n*-BuLi, and to the resulting lithio derivative was added Weinreb amide **92**[11] to afford ketone **93** in 70% yield. Reduction of the latter with $BH_3{\cdot}Me_2S$ in the presence of CBS catalyst[11,56] furnished stereoselectively hydroxy compound **94** (66% yield). The latter compound was elaborated to acetoxy carboxylic acid **95** through a sequence involving acetylation (Ac_2O, Et_3N, 88% yield), desilylation (TBAF, 99% yield), and oxidation (DMP, 91% yield; then $NaClO_2$, 99% yield). Coupling carboxylic acid **95** with ammonium salt **55** through the action of HATU and Et_3N furnished fragment **96** in 93% yield. Removal of the Boc group (TFA) from the latter followed by coupling of the so generated amine with acid fluoride **20** gave tripeptide **97** (92% overall yield). Finally, cleavage of the Fmoc group from **97** and coupling of the resulting amine with carboxylic acid **10** facilitated by HATU and Et_3N led to analogue **Tb110** in 75% overall yield. Tubulysin analogue **Tb111** was obtained from **Tb110** through the standard procedure of hydrolysis (acetate and ethyl ester) with LiOH[11] followed by re-acetylation of the resulting hydroxy acid with Ac_2O/pyridine (77% overall yield). Finally, **Tb110** and **Tb111** were converted to **Tb112** and **Tb113** in 71% and 65% yield, respectively, through hydrogenolysis [$Pd(OH)_2$/C catalyst, H_2] as shown in Scheme 28.

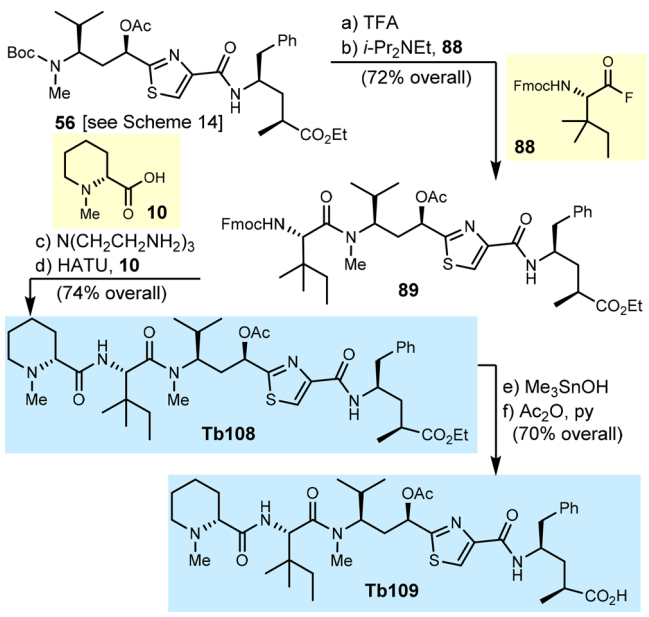
We finally explored modifications at the tubuphenylalanine (Tup) site of the tubulysin molecule as demonstrated with the

Scheme 26. Synthesis of Tubulysin Analogues Tb106 and Tb107[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **86** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 81% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 76% for the two steps; (e) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (f) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 84% for the two steps.

Scheme 27. Synthesis of Tubulysin Analogues Tb108 and Tb109[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (b) **88** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 72% for the two steps; (c) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (d) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 74% for the two steps; (e) Me_3SnOH (50 equiv), 1,2-dichloroethane, reflux, 12 h; (f) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 70% for the two steps.

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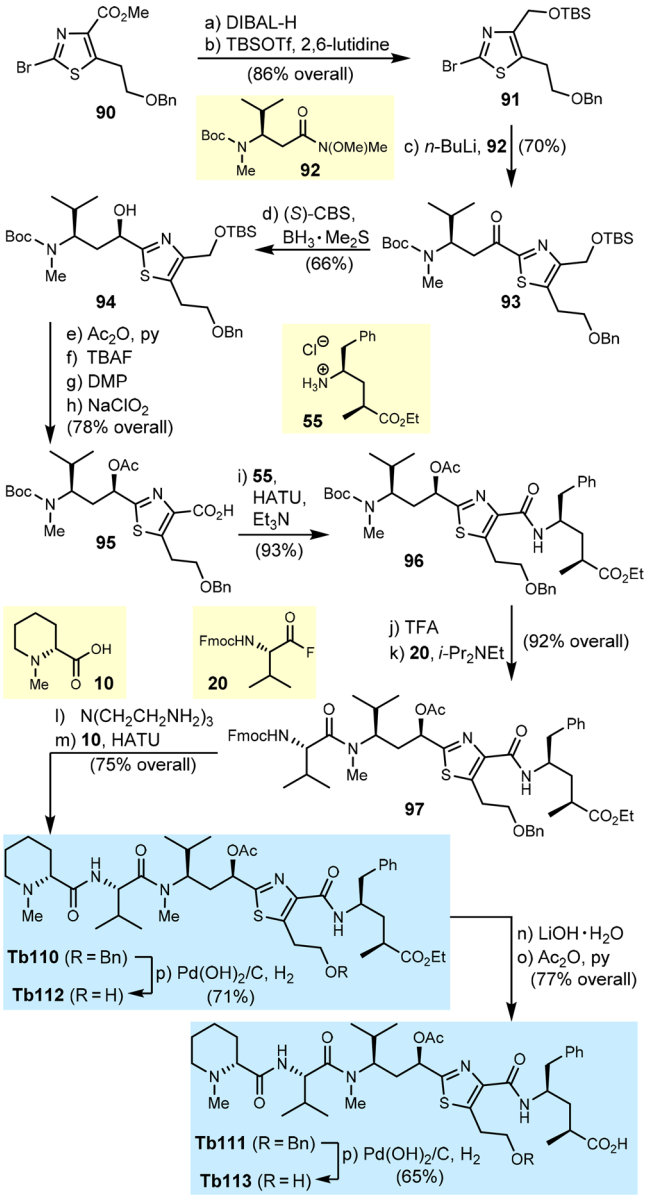
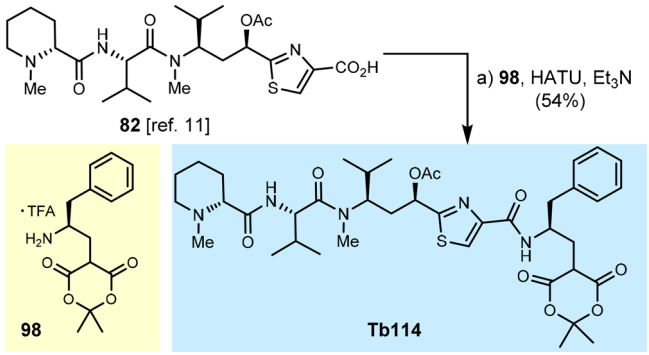
Scheme 28. Synthesis of Tubulysin Analogues Tb110–Tb113[a]



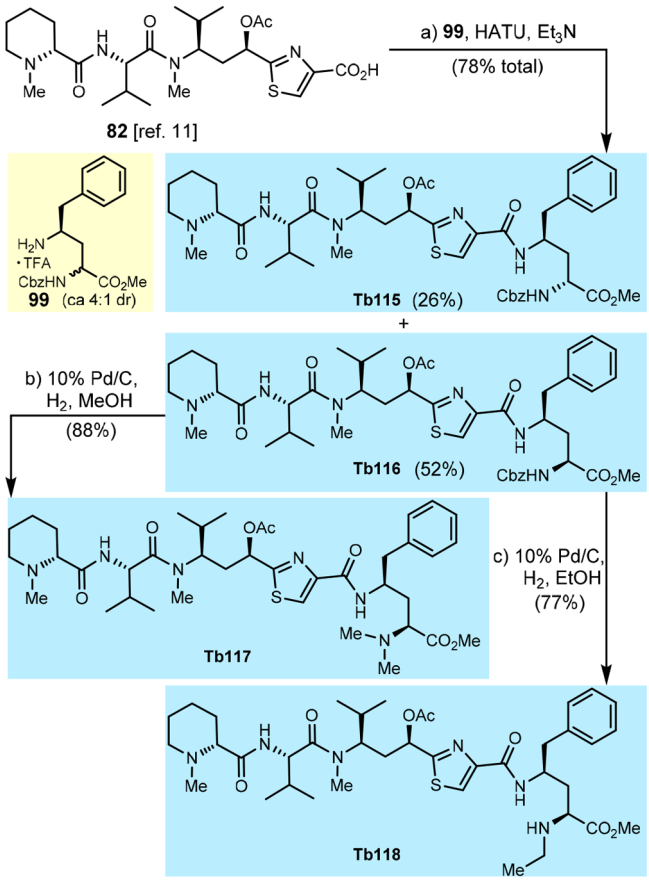
[a]Reagents and conditions: (a) DIBAL-H (3.0 equiv; 1.0 M in diethyl ether), diethyl ether, −78 → 23 °C, 1 h, 87%; (b) TBSOTf (1.2 equiv), 2,6-lutidine (2.0 equiv), CH_2Cl_2, 0 → 23 °C, 0.5 h, 99%; (c) *n*-BuLi (1.44 equiv; 2.5 M in hexanes), **92** (1.0 equiv), THF, −78 → −50 °C, 3 h, 70%; (d) (*S*)-CBS (0.1 equiv; 1.0 M in toluene), BH_3·Me_2S (1.0 equiv; 2.0 M in THF), 0 → 23 °C, 36 h, 66%; (e) Ac_2O (3.0 equiv), Et_3N (4.0 equiv), 0 → 23 °C, 2 h, 88%; (f) TBAF (2.0 equiv; 1.0 M in THF), THF, 0 → 23 °C, 30 min, 99%; (g) DMP (1.5 equiv), CH_2Cl_2, 23 °C, 0.5 h, 91%; (h) $NaClO_2$ (5.4 equiv), NaH_2PO_4·H_2O (12.2 equiv), 2-methyl-2-butene (7.5 equiv), *t*-BuOH, THF, H_2O, 23 °C, 1 h, 99%; (i) **55** (1.5 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 93%; (j) TFA (45 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (k) **20** (4.0 equiv), *i*-Pr_2NEt (6.0 equiv), DMF, 0 → 23 °C, 18 h, 92% for the two steps; (l) $N(CH_2CH_2NH_2)_3$ (15 equiv), CH_2Cl_2, 0 → 23 °C, 2 h; (m) **10** (3.0 equiv), HATU (3.0 equiv), Et_3N (6.0 equiv), DMF, 0 → 23 °C, 24 h, 75% for the two steps; (n) LiOH·H_2O (5.0 equiv), THF:H_2O (5:1, *v*/*v*), 23 °C, 24 h; (o) Ac_2O (4.0 equiv), pyridine, 0 → 23 °C, 12 h, 77% for the two steps; (p) $Pd(OH)_2$/C (20 wt%), H_2, MeOH, 23 °C, 18 h, 71% for **Tb112** and 65% for **Tb113**. DIBAL-H = diisobutylaluminum hydride.

Scheme 29. Synthesis of Tubulysin Analogue Tb114[a]



[a]Reagents and conditions: (a) **98** (1.2 equiv), HATU (1.2 equiv), Et_3N (2.4 equiv), DMF, 0 → 23 °C, 18 h, 54%.

Scheme 30. Synthesis of Tubulysin Analogues Tb115–Tb118[a]



[a]Reagents and conditions: (a) **99** (ca. 4:1 dr, 1.2 equiv), HATU (1.2 equiv), Et_3N (2.4 equiv), DMF, 0 → 23 °C, 18 h, 26% for **Tb115** and 52% for **Tb116**; (b) 10% Pd/C (50 wt%), H_2, MeOH, 23 °C, 20 h, 88%; (c) 10% Pd/C (50 wt%), H_2, wet EtOH, 23 °C, 20 h, 77%.

structures of analogues **Tb114**–**Tb118** (Schemes 29 and 30). Tubulysin analogue **Tb114**, possessing a C_2-symmetric malonic acid type structural motif at its "right end" (resembling Meldrum's acid structure) was synthesized from carboxylic acid **82**[11] and ammonium salt **98**[62] (see Supporting Information for details) through the action of HATU in the presence of Et_3N in 54% yield, as shown in Scheme 29.

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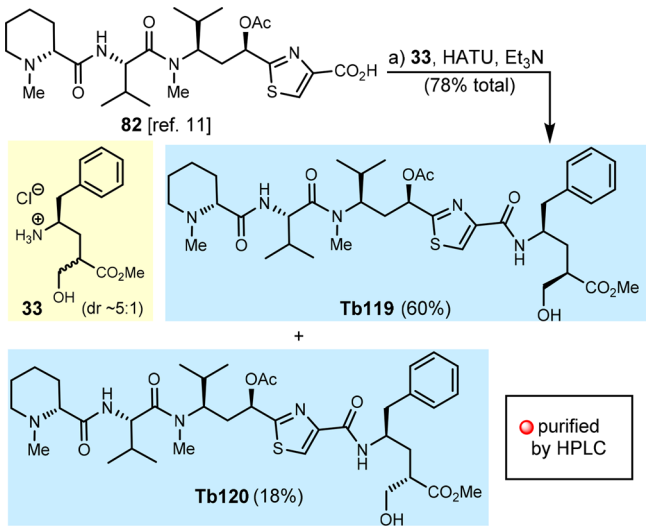
The amino containing tubulysin analogues **Tb115**–**Tb118** were prepared from the previously synthesized fragment **82**[11] and amino acid derivative **99**[63] (diastereomeric mixture ca. 4:1, see Supporting Information for preparation) as summarized in Scheme 30. Thus, coupling of carboxylic acid **82** with ammonium salt **99** in the presence of HATU and Et_3N yielded **Tb115** and **Tb116** as a mixture of diastereoisomers (78% yield, ca. 1:2, separated by silica gel column chromatography). **Tb116** was subjected to hydrogenolysis in MeOH (10% Pd/C, 50 wt%, H_2, 23 °C, 20 h) to afford dimethylamino tubulysin analogue **Tb117** in 88% yield. Similar treatment of **Tb116** in EtOH led to ethyl amino tubulysin analogue **Tb118** in 77% yield. Apparently, this is a known outcome of hydrogenolytic Cbz group cleavage from primary amines under strong catalyst and prolonged time conditions.[64] As demonstrated here, this reaction provides practical means for accessing substituted amines of considerable complexity.

Scheme 31 summarizes the synthesis of **Tb119** and **Tb120**, both containing a hydroxymethyl group adjacent to their carboxylate moiety (instead of a methyl group) of the tubuphenylalanine residue. Thus, tripeptide carboxylic acid **82**[11] was coupled with ammonium salt **33** (ca. 4:1 dr, see Supporting Information for preparation) under the influence of HATU and Et_3N to afford tubulysin analogues **Tb119** and **Tb120** as a mixture of diastereoisomers, which were separated by HPLC to give pure **Tb119** (60% yield) and **Tb120** (18% yield).[64]

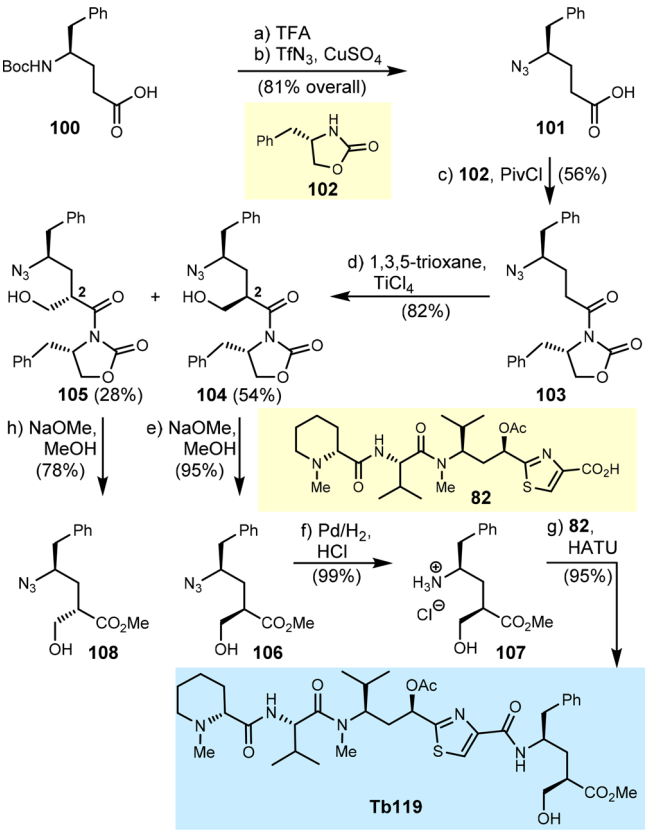
Stereoselective syntheses of tubulysin analogues **Tb119** and **Tb120** were also developed starting with readily available phenylalanine derivative **100**, as shown in Schemes 32 and 33. Thus, substrate **100** was converted to its azide counterpart **101** through a two-step sequence (TFA; TfN_3, $CuSO_4$ catalyst, 81% overall yield, Scheme 32). The latter was reacted with chiral auxiliary **102** [(*S*)-4-benzyl-2-oxazolidinone] in the presence of PivCl, Et_3N, and LiCl to afford oxazolidinone **103** (56% yield), which was hydroxymethylated stereoselectively with trioxane in the presence of $TiCl_4$, leading to hydroxy azide oxazolidinone **104** (54% yield) and, unexpectedly, its C2 epimeric sibling **105** (28% yield). Besides NMR spectroscopic analyses revealing its general structure, the absolute stereochemistry of the latter compound was established by converting it to hydroxy ester **108** (NaOMe; 78% yield), whose data matched those obtained from another sample of the same compound prepared from oxazolidinone **111** as shown in Scheme 33. Hydroxy azide methyl ester **106** was then generated from oxazolidinone **104** through the action of NaOMe (95% yield). Reduction (H_2/Pd catalyst, HCl, MeOH, 99% yield) of the azide group within the latter provided hydroxy ammonium salt **107**, which was smoothly coupled with tripeptide carboxylic acid **82**[11] under HATU conditions to afford the targeted tubulysin analogue **Tb119** in 95% yield, as summarized in Scheme 32. The stereoselective synthesis of diastereomeric analogue **Tb120** proceeded through the same sequence, starting with carboxylic acid **101** and via intermediates **110**, **111**, **108**, and **112**, by utilizing the enantiomeric chiral auxiliary [**109**: (*R*)-4-benzyl-2-oxazolidinone] in similar yields and without the formation of

Scheme 31. Synthesis of Tubulysin Analogues Tb119 and Tb120[a]



[a]Reagents and conditions: (a) **33** (1.2 equiv), HATU (1.2 equiv), Et_3N (2.4 equiv), DMF, 0 → 23 °C, 18 h, 60% for **Tb119** and 18% for **Tb120**.

Scheme 32. Synthesis of Tubulysin Analogues Tb119[a]



[a]Reagents and conditions: (a) TFA (45 equiv), CH_2Cl_2, 0 °C, 3 h, quant; (b) TfN_3 (3.0 equiv; 0.57 M in CH_2Cl_2), $CuSO_4{\cdot}5H_2O$ (0.1 equiv), K_2CO_3 (2.0 equiv), MeOH, H_2O, 23 °C, 12 h, 81% yield for the two steps; (c) Et_3N (1.8 equiv), LiCl (1.7 equiv), PivCl (1.5 equiv), (*S*)-4-benzyl-2-oxazolidinone **102** (1.7 equiv), −20 °C, 2 h, 56%; (d) $TiCl_4$ (1.1 equiv), *i*-Pr_2NEt (1.1 equiv), 1,3,5-trioxane (1.1 equiv), 0 °C, 3.5 h, 54% for **104** and 28% for **105**; (e) NaOMe (1.0 equiv), MeOH, CH_2Cl_2, −78 → 0 °C, 2 h, 95%; (f) 10% Pd/C (50 wt %), H_2, HCl (1.2 equiv; 1 M in MeOH), MeOH, 23 °C, 0.5 h, 99%; (g) **82** (0.9 equiv), HATU (1.2 equiv), Et_3N (2.4 equiv), DMF, 0 → 23 °C, 18 h, 95%; (h) NaOMe (1.0 equiv), MeOH, CH_2Cl_2, −78 → 0 °C, 2 h, 78%.

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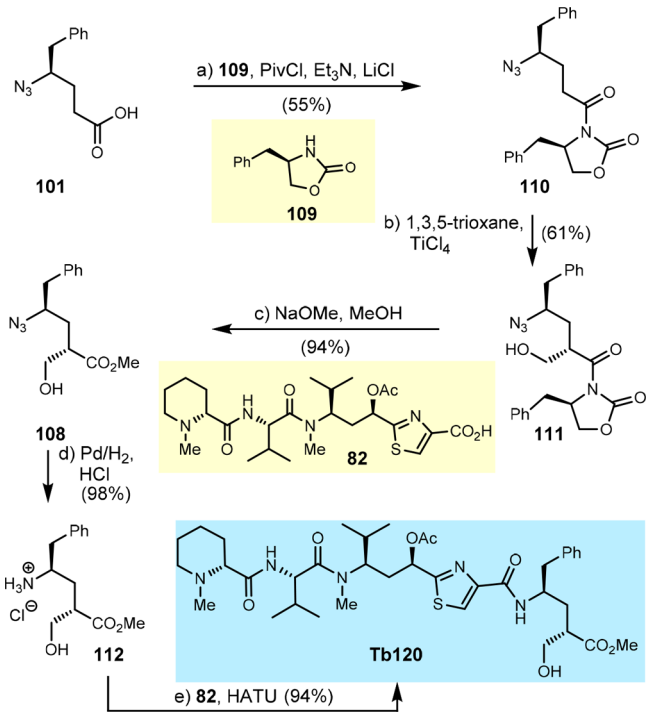
Scheme 33. Synthesis of Tubulysin Analogues Tb120[a]



[a]Reagents and conditions: (a) Et_3N (1.8 equiv), LiCl (1.7 equiv), PivCl (1.5 equiv), **109** (1.7 equiv), −20 °C, 2 h, 55%; (b) $TiCl_4$ (1.1 equiv), *i*-Pr_2NEt (1.1 equiv), 1,3,5-trioxane (1.1 equiv), 0 °C, 3.5 h, 61%; (c) NaOMe (1.0 equiv), MeOH, CH_2Cl_2, −78 → 0 °C, 2 h, 94%; (d) 10% Pd/C (50 wt%), H_2, HCl (1.2 equiv; 1 M in MeOH), MeOH, 23 °C, 0.5 h, 98%; (e) **82** (0.9 equiv), HATU (1.2 equiv), Et_3N (2.4 equiv), DMF, 0 → 23 °C, 18 h, 94%.

epimeric side products during the stereoselective hydroxymethylation step (**110** → **111**) as shown in Scheme 33.

2.5. Biological Evaluation of Tubulysin Analogues and Structure−Activity Relationships. The synthesized analogues were evaluated for their activity against MES SA (uterine sarcoma cells), MES SA DX (multidrug-resistant uterine sarcoma cells), and HEK 293T (human embryonic kidney cancer cells). As shown in Table 1, several of these compounds exhibited picomolar potencies, with the most notable (highlighted with yellow background) being **Tb94**, **Tb95**, and **Tb102**, and even more potent (highlighted with green background) **Tb64**, **Tb67**, **Tb73**, **Tb93**, **Tb106**, **Tb108**, **Tb112**, **Tb117**, **Tb119**, **Tb120**, and most potent (highlighted with blue background) **Tb107**, **Tb109**, **Tb111**, **Tb115**, **Tb116**, and **Tb118**. The latter group (i.e., **Tb107**, **Tb109**, **Tb111**, **Tb115**, **Tb116**, and **Tb118**) is also notable for their relatively potent (low nanomolar) cytotoxicities against the marked drug-resistant cancer cell line MES SA DX.

With the large number of tubulysins synthesized and tested in our laboratories, and guided by the insights recently obtained through X-ray crystallographic studies[53] on tubulin binding molecules, we were in a position to explain and develop clearer SARs within the tubulysin family of compounds. The X-ray-derived structures of N^{14}-desacetoxytubulysin H (**Tb1** in this study; tubulysin M in ref 53) and its peptide-like relatives HTI-286[53] and monomethyl auristatin E (MMAE)[53] revealed a binding model that included a number of binding sites on the tubulysin molecule, including (from "left" to "right", see Figure 3): (1) basic nitrogen on the "left domain" (protonated form, binding through a salt bridge to a carboxylate moiety of tubulin); (2) one amide NH moiety binding through H-bonding to a carbonyl O of the receptor; (3) a hydrophobic moiety (i.e., 2-methyl butyl group) binding to a hydrophobic pocket within tubulin; (4) carbonyl O binding through H-bonding to an amide NH moiety of tubulin; (5) a second hydrophobic group (i.e., isopropyl moiety) binding to a different hydrophobic pocket within the tubulin unit; (6) the thiazole N and the adjacent carbonyl O, both serving as H-bond acceptors from a H-bond donor on the tubulin unit; (7) the phenyl moiety of the tubuphenyl alanine residue fitting snuggly into a hydrophobic cavity within a tubulin unit; and (8) the carboxylate unit forming a salt bridge with a counterpart within the tubulin receptor. This model seems to be, more or less, in accordance with our findings, correlating well structural motifs with potencies within the family of compounds synthesized and tested in this study (see Figure 2 and Table 1).

As the pipecolic acid residue of the tubulysin molecule perfectly occupies its binding site on tubulin, according to the X-ray-generated model,[53] it was not surprising that certain modifications made to this fragment led to only insignificant or low biological activity. This phenomenon is clearly demonstrated by tubulysin analogues **Tb50** and **Tb51** (in which the pipecolic acid was exchanged for 1-methyl-1*H*-pyrrole-2-carboxylic acid). It was also evident in the cases of **PTb-D47**, **PTb-D49**, **Tb54**, **Tb55**, and **Tb77**, in which the pipecolic acid residue was modified at the N-atom with the larger *n*-butyl in place of the methyl group or oxygenated on one of the carbons of the ring, changing its steric and/or hydrophobic requirements that apparently do not fit the binding site of the tubulin receptor. In addition, when the pipecolic acid moiety was replaced with its five-membered proline counterpart, as in **Tb70** and **Tb71**, significant loss of potency was observed (see Table 1), providing further support for the strict and crucial requirements of the pipecolic acid binding site within the tubulin receptor, although other novel substituents on this nitrogen-bearing residue may prove fruitful.

As can be seen in Figure 3, the isoleucine (Ile) residue of the tubuysin ligand provides a hydrogen-bonding opportunity (one acceptor and one donor) and one hydrophobic moiety (the 3-methybutyl group) that fits snuggly into the α2 tubulin subunit. As shown from our results, the tolerance of the hydrophobic acceptor site is rather limited. Thus, tubulysins equipped with an isopropyl (e.g., **Tb61**), tertiary butyl (e.g., **Tb106**, **Tb107**), and the one-carbon higher 1,1-dimethylpropyl moieties (e.g., **Tb108**, **Tb109**) exhibited exceptional potencies. On the other hand, the longer *n*-butyl (e.g., **Tb95**, **Tb96**) groups and 3-methylbutyl (e.g., **Tb97**, **Tb98**) instead of the isoleucine side chain are not tolerated, as evident from the lack of or significantly lower cytotoxicities of the corresponding analogues (see Table 1). Furthermore, smaller size group substitutions at the isoleucine side chain position, as in **Tb88** (hydrogen) and **Tb89−Tb92** (methyl group), led, surprisingly, to no significant activity. Interestingly, the ethyl group containing tubulysin analogues **Tb93** and **Tb94** exhibited significant potencies, indicating perhaps the lower limit of lipophilicity and steric demand required at that position for potent activity. The fluorinated tubulysin analogues **Tb79−Tb87** carrying substituted ethyl or isopropyl moieties on the isoleucine residue, were disappointing in that none exhibited subnanomolar potencies, although some had significant activities (see Table 1). This observation may be attributed to the polarization of

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Table 1. Cytotoxicity Data against Cancer Cell Lines MES SA, MES SA DX, and HEK 293T[a] for Tubulysins PTb-D47−PTb-D49 and Tb50−Tb120

compound	MES SA	MES SA DX	HEK 293T	compound	MES SA	MES SA DX	HEK 293T
Tb1	0.34	>10	0.02	**Tb83**	>1000	>1000	>1000
Tb32	0.012	1.29	0.002	**Tb84**	>1000	>1000	>1000
PTb-D47	>1000	>1000	>1000	**Tb85**	37.32	>1000	32.4
PTb-D48	>1000	>1000	>1000	**Tb86**	>1000	>1000	>1000
PTb-D49	>1000	>1000	>1000	**Tb87**	233.5	>1000	>1000
Tb50	>1000	>1000	>1000	**Tb88**	457.70	>2500	493.80
Tb51	>1000	>1000	>1000	**Tb89**	>1000	>1000	>1000
Tb52	6.164	66.55	3.83	**Tb90**	>1000	>1000	636.4
Tb53	>1000	>1000	>1000	**Tb91**	>1000	>1000	>1000
Tb54	>1000	>1000	>1000	**Tb92**	>1000	>1000	>1000
Tb55	>1000	>1000	>1000	**Tb93**	0.15	31.93	0.20
Tb56	>1000	>1000	>1000	**Tb94**	0.46	89.77	0.40
Tb57	>1000	>1000	>1000	**Tb95**	0.937	>1000	0.53
Tb58	>1000	>1000	>1000	**Tb96**	10.5	>1000	5.26
Tb59	>1000	>1000	>1000	**Tb97**	4.65	>1000	2.87
Tb60	14.02	>2500	6.34	**Tb98**	41.8	393.2	26.5
Tb61	6.07	>1000	5.99	**Tb99**	>1000	>1000	>1000
Tb62	>1000	>1000	>1000	**Tb100**	15.16	>1000	16.51
Tb63	>1000	>1000	>1000	**Tb101**	>1000	>1000	>1000
Tb64	0.22	108.70	0.10	**Tb102**	0.926	54.12	0.355
Tb65	2.44	278.80	2.04	**Tb103**	2.74	22.57	1.793
Tb66	1.034	>1000	0.773	**Tb104**	9.5	>1000	12.83
Tb67	0.836	71.52	0.1435	**Tb105**	2.94	>1000	1.53
Tb68	>1000	>1000	>1000	**Tb106**	0.12	2.73	0.13
Tb69	30.82	>1000	>1000	**Tb107**	0.01	4.05	0.02
Tb70	3.113	>1000	2.32	**Tb108**	0.95	6.08	0.38
Tb71	1.422	>1000	0.408	**Tb109**	0.36	1.39	0.01
Tb72	11.70	>70	4.02	**Tb110**	3.091	>400	1.87
Tb73	1.28	44.69	0.16	**Tb111**	0.04	1.54	0.006
Tb74	5.32	>400	2.46	**Tb112**	0.10	>400	0.09
Tb75	400.00	>400	126.60	**Tb113**	5.79	>400	0.315
Tb76	4.88	>2500	1.37	**Tb114**	1.225	>400	0.524
Tb77	848	>2500	>400	**Tb115**	0.020	13.850	0.010
Tb78	7.18	780.5	5.77	**Tb116**	0.007	6.002	0.003
Tb79	39.09	>1000	36.37	**Tb117**	0.099	4.629	0.059
Tb80	>1000	>1000	>1000	**Tb118**	0.14	5.60	0.015
Tb81	5.84	>1000	4.98	**Tb119**	0.093	>14	0.041
Tb82	47.53	>1000	51.60	**Tb120**	0.059	>14	0.039

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

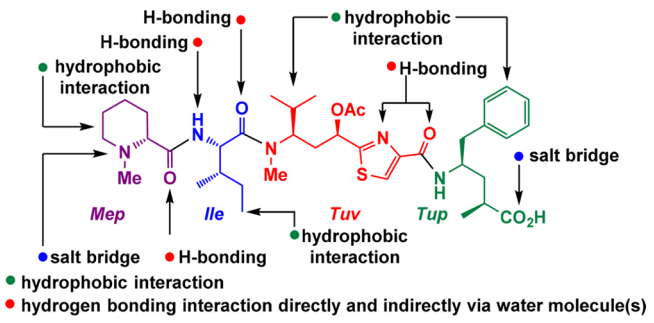

Figure 3. Binding interactions of N^{14}-desacetoxytubulysin H [**Tb1**, (tubulysin M)[53a]] as deciphered from X-ray crystallographic analysis.[53a]

the bonds within these moieties that contribute negatively to their fitting into the hydrophobic pocket of the receptor.

A more complex explanation may be necessary for the effect of the spiro cyclopentyl moiety within the isoleucine residue leading to the lower potency of **Tb78**, the latter structural motif most likely changing the overall tertiary conformation of the tubulysin molecule, thereby decreasing its complementarity to its binding pocket.

According to the crystallographic analysis,[53] the acetate group of the Tuv fragment sits at a narrow channel within the interface of the α and β tubulin subunits, with no recognizable H-bonding interactions. The significant reduction in potency that accompanies the removal of this acetate (as in **PTb-D42**, **PTb-D43**, and **DTb-D48**), its deprotection to the naked hydroxyl group (as in **Tb58** and **Tb60**), and its oxidation to the corresponding ketone (as in **Tb59**, **Tb62**, **Tb63**, and **Tb68**), may suggest an unknown structural or biochemical function of this moiety (e.g., facilitating entrance of the molecule into the cell).

The thiazole component of Tuv forms two H-bonding interactions emanating from the thiazole nitrogen atom and the adjacent carbonyl to the backbone of the β1 tubulin subunit, thus stabilizing the overall conformation of the central region of the bound tubulysin molecule. Consequently, any aromatic functionality that maintains these interactions should be tolerated as long as it does not contribute to additional steric or electronic constraints within the binding channel, as demonstrated by analogues **Tb64** and **Tb65** (pyridine instead of thiazole). The 5-position (i.e., H-substituted position) of the thiazole ring is oriented toward an open space[53] and away from the interface of the two tubulin monomers, although some steric constraints could be imposed by substituents in the near neighborhood of the thiazole ring.[53] The recognition of this open space inspires and provides guidance for further refinement of the tubulysin molecule as potential payloads for ADCs. Thus, methyl substitution at the 5-position in analogues **Tb66** and **Tb67** results in some loss of potency, whereas the presence of an isopropyl group in analogues **Tb72** and **Tb73** led to significant loss of potency. In contrast, the longer linear chains, as in **Tb110**−**Tb113**, proved beneficial as demonstrated by their generally increased cytotoxicity potency.

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The tubulysin phenylalanine (Tup) domain positioned as it is at the "right end" of the molecule binds, according to the X-ray data,[53] at the edge of the binding channel of tubulin. As such, it is free to rotate as long as structural changes do not disturb other binding interactions. Thus, the [1.1.1]-bicyclopentane containing analogues **Tb100**, **Tb102**, and **Tb103** and the cyclohexyl carrying analogue **Tb52** are marginally tolerated, with **Tb102** showing subnanomolar potencies against two of the cell lines tested (see Table 1). The bulkier naphthalene substituent at this position, as in **Tb104**, is also barely tolerated as concluded from its modest potency compared to the most active compounds (see Table 1). Although *para*-substitution of the aromatic ring of the Tup phenylalanine residue is tolerated as evidenced by a number of active natural tubulysins (e.g., A–C, G, and I)[15−18] that contain a phenolic moiety at that position, the presence of a fluorine residue in this aromatic ring, as in analogue **Tb101**, is not, leading to loss of activity (see Table 1). These data suggest the importance of a potential $\pi-\pi$ interaction of this moiety with a binding site in the receptor.

The Tup carboxylic acid moiety is involved in a very important interaction with Arg278 of the tubulin receptor, forming a salt bridge that provides additional stabilization of the ligand–receptor complex. The adjacent methyl group appears to be in an open space region not limited by any apparent steric or electronic constraints. Removal of this methyl group, as in **Tb105**, results in considerable loss of activity (see Table 1). Most importantly, however, the replacement of this methyl group of the Tup residue with nitrogen-containing substituents, as in **Tb115**–**Tb118**, translates into high potencies, with **Tb117** and **Tb118** being the most impressive.

Figure 4 summarizes the conclusions drawn from these studies on the effect of structural changes within the four

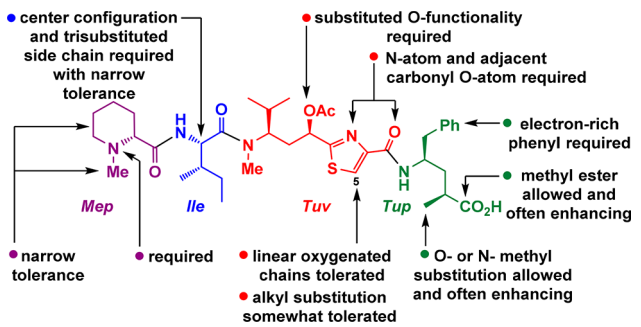

Figure 4. Structure–activity relationships (SARs).

domains Mep [(*N*-methyl-D-pipecolic acid or pipecolic acid)], Ile (L-isoleucine), Tuv (tubuvaline), and Tup (tubuphenyl alanine) on the cytotoxicity potencies of the N^{14}-desacetoxytubulysin H molecule (**Tb1**). These SARs are consistent with the X-ray crystallographic studies of the tubulysin–tubulin complex and provide guidance for the design of new optimized tubulysin analogues.

3. CONCLUSION

The described synthetic endeavors culminated in short and efficient chemical processes for the synthesis of natural tubulysins V (**Tb45**) and U (**Tb46**) and pretubulysin D (**PTb-D43**) and allowed rapid and efficient syntheses of a number of tubulysin analogues (**Tb44**, **PTb-D42**, **PTb-D47**–**PTb-D49**, and **Tb50**–**Tb120**). Biological evaluation of the synthesized compounds led to the identification of extremely potent tubulysin analogues (e.g., **Tb107**, **Tb111**, **Tb115**, and **Tb116**, see Table 1 and Figure 2) equipped with hydroxyl, amino, and carboxylate handles for conjugation to appropriate targeting systems such as antibodies[50,51] and other delivery systems.[52] Such conjugates are highly desirable for personalized targeted chemotherapies, a currently intensely pursued and rapidly emerging paradigm for cancer treatment. The newly developed structure–activity relationships described herein are in line with recently reported X-ray crystallographic analysis results obtained from a tubulysin–tubulin complex,[53] and provide strong pathpointing guidance for further optimization of the latest tubulysin analogues.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b12692.

Experimental procedures and characterization data for all compounds; biological evaluation and data, HEK 293T, MES SA, and MES SA DX (AbbVie Stemcentrx) (PDF)

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Notes

The authors declare no competing financial interest.

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