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Expedient Synthesis of *N*-Methyl Tubulysin Analogues with High Cytotoxicity

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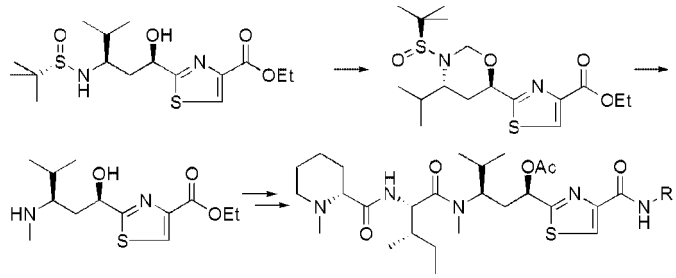

An optimized and highly efficient synthesis of potent, bioactive *N*-methyl tubulysin analogues **2** and **4** has been achieved with > 40% overall yields. This synthesis represents a significant improvement over previously reported syntheses of these and related tubulysin analogues. The stereoselective synthesis of the unnatural amino acid tubuvaline is accomplished using *tert*-butanesulfinamide chemistry. *N*-Alkylation to form *N*-methyl tubuvaline is performed without protection of the tubuvaline alcohol by implementing a unique *N*-methylation strategy via formation and reduction of a 1,3-tetrahydrooxazine heterocycle. Acylation of the hindered *N*-methyl tubuvaline amine utilizes a novel sequence of *O*-acylation followed by an *O*- to *N*-acyl transfer to form the hindered amide bond between *N*-methyl tubuvaline and isoleucine. This high-yielding synthesis should enable the production of large quantities of material for biological studies.

Introduction

The tubulysins are exceptionally potent cell growth inhibitors that act by inhibiting tubulin polymerization, thus inducing apoptosis.[1] With activity exceeding that of vinblastine, paclitaxel, and the epothilones,[2] the tubulysins are exciting leads for new anticancer therapeutics.

The tubulysin scaffold can be divided into four amino acid segments as defined in Figure 1: D-Mep (D-*N*-methyl pipecolinic acid), Ile (L-isoleucine), Tuv (tubuvaline), and Tup/Tut (tubuphenylalanine/tubutyrosine). The most potent tubulysins (tubulysins A-I) incorporate a rare *O*-acyl *N*,*O*-acetal moiety on the Tuv-amide,[2,3] while the less potent tubulysins (tubulysins U-Z) lack this functionality.[4,5]

The isolation of the tubulysins from myxobacterial cultures[3] produces only small quantities of these natural products; therefore, efficient synthesis routes to the natural tubulysins as well as their analogues are needed to advance biological studies.

Indeed, much effort has been devoted to the synthesis of these complex tetrapeptides. Partial syntheses of the unnatural amino acid segments Tuv and Tup/Tut have been reported,[6] as have syntheses of unnatural tubulysin analogues[7–10] and the less potent tubulysins U and V.[11] In 2006, our group reported the

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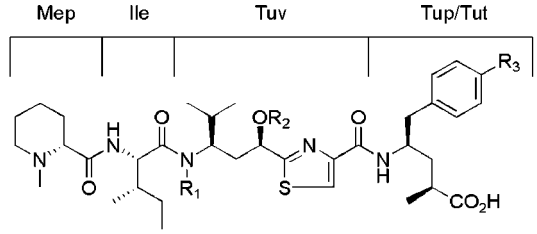
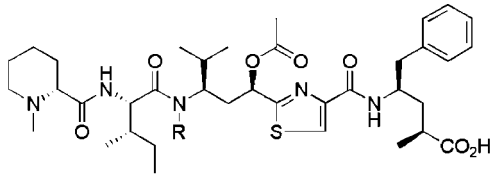
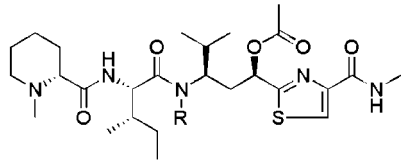


tubulysin A: $R_1 = CH_2OC(O)CH_2CH(CH_3)_2$; R_2 = Ac; R_3 = OH
B: $R_1 = CH_2OC(O)CH_2CH_2CH_3$; R_2 = Ac; R_3 = OH
C: $R_1 = CH_2OC(O)CH_2CH_3$; R_2 = Ac; R_3 = OH
D: $R_1 = CH_2OC(O)CH_2CH(CH_3)_2$; R_2 = Ac; R_3 = H
E: $R_1 = CH_2OC(O)CH_2CH_2CH_3$; R_2 = Ac; R_3 = H
F: $R_1 = CH_2OC(O)CH_2CH_3$; R_2 = Ac; R_3 = H
H: $R_1 = CH_2OC(O)CH_3$; R_2 = Ac; R_3 = H
I: $R_1 = CH_2OC(O)CH_3$; R_2 = Ac; R_3 = OH
U: R_1 = H; R_2 = Ac; R_3 = H
V: R_1 = H; R_2 = H; R_3 = H

FIGURE 1. Structures of selected tubulysins.



1 (tubulysin D): R = $CH_2OC(O)CH_2CH(CH_3)_2$; IC_{50} = 0.022 ng/mL
2: R = CH_3; IC_{50} = 0.016 ng/mL



3: R = $CH_2OC(O)CH_2CH(CH_3)_2$; IC_{50} = 0.35 ng/mL
4: R = CH_3; IC_{50} = 0.50 ng/mL

FIGURE 2. Structure and cell-growth inhibitory activity of tubulysin D (**1**) and selected analogues (**2**−**4**) against human colon adenocarcinoma cell line SW-480 (ATCC CCL-228).[8,14]

first total synthesis of any naturally occurring tubulysin, tubulysin D (Figure 2, **1**),[12] which is the most potent of the tubulysin family.[2] This synthesis represents the only route capable of incorporating the *O*-acyl *N*,*O*-acetal, which is extremely labile to both acid and base, thus presenting many synthetic challenges.[13]

We also prepared a number of unnatural tubulysin analogues that had modifications in the Mep and Tup regions, as well as analogues that replaced the *O*-acyl *N*,*O*-acetal with a simplified *N*-methyl amide.[8] Upon biological evaluation of this focused library, the SAR surprisingly revealed that compounds containing the Tuv *N*-methyl amide (Figure 2, **2** and **4**) had activity similar to that of the analogous compounds containing the *O*-acyl *N*,*O*-acetal (Figure 2, **1** and **3**). This finding is important because exclusion of the labile *O*-acyl *N*,*O*-acetal should result in compounds that are more biologically stable and easier to prepare than the parent tubulysins. In parallel efforts, Peter Wipf and co-workers have also synthesized and reported on the potent cytotoxicity of **2**.[7,9]

Our previously reported sequence for the preparation of tubulysin D and analogues (Scheme 1)[8,12] was predicated on a synthetic strategy and protecting group scheme that was compatible with the *O*-acyl *N*,*O*-acetal moiety present in tubulysin D, which is labile under both acidic and basic conditions. Herein, we report a new sequence for the preparation of *N*-methyl tubulysin analogues that is considerably more efficient than our previously reported syntheses of *N*-methyl tubulysin derivatives **2** and **4**[8] as well as the other reported syntheses of **2**[15] and related analogues. This sequence, which provides *N*-methyl tubulysin analogues **2** and **4** in >40% overall yields from commercial materials, should enable the straightforward production of sufficient quantities of *N*-methyl tubulysin analogues for biochemical and pharmacological studies.

Results and Discussion

The initial route to tubulysin D and analogues containing the *O*-acyl *N*,*O*-acetal, which was also followed in the production of *N*-methyl tubulysins, is shown in Scheme 1. β-Hydroxy ketimine **7** was obtained by stereoselective addition of the metalloenamine[16] of *N*-*tert*-butanesulfinyl ketimine **5**[17] to thiazole aldehyde **6**, which was prepared in four steps according to literature procedure.[12,18] Reduction of imine **7** then efficiently provided sulfinyl-protected tubuvaline methyl ester **8** with high selectivity.[16,19] Acidic cleavage of the sulfinyl group, followed by acylation with α-azido isoleucine acid chloride (**10**), afforded azide **11**. The tubuvaline alcohol was then protected as a triethylsilyl ether (TES), followed by alkylation of the tubuvaline amide with either chloromethyl isobutyl carbonate (**13**) or methyl iodide, to produce *N*-alkyl amides **14** and **15**. Reduction of the azide and in situ acylation with the pentafluorophenyl ester of D-Mep (Mep-PFP) afforded Mep-coupled products **16** and **17**. Use of the azide masking group was necessary for two reasons: (1) typical carbamate protecting groups would be competitively alkylated during the amide alkylation step, and (2) unmasking the protected amine under mild conditions with in situ acylation was essential given the highly labile nature of the newly formed *O*-acyl *N*,*O*-acetal. Cleavage of the silyl protecting group under mild conditions then provided **18** and **19**. Selective hydrolysis of the thiazole methyl ester over the more highly activated acyl group present in the *O*-acyl *N*,*O*-acetal was next accomplished using Me_3SnOH, which is selective for sterically unhindered esters.[20] The syntheses were completed by coupling carboxylic acids **20** and **21** with either

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(9) Wang, Z.; McPherson, P. A.; Raccor, B. S.; Balachandran, R.; Zhu, G.; Day, B. W.; Vogt, A.; Wipf, P. *Chem. Biol. Drug Des.* **2007**, *70*, 75–86.
(10) Reference 4 originally reported the total synthesis of naturally occurring tubulysins U and V, but was actually the synthesis of epimeric compounds. Correction: Dömling, A.; Beck, B.; Eichelberger, U.; Sakamuri, S.; Menon, S.; Chen, Q.-Z.; Lu, Y.; Wessjohann, L. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 2337–2348.
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(12) Peltier, H. M.; McMahon, J. P.; Patterson, A. W.; Ellman, J. A. *J. Am. Chem. Soc.* **2006**, *128*, 16018–16019.
(13) Iley, J.; Moreira, R.; Calheiros, T.; Mendes, E. *Pharm. Res.* **1997**, *14*, 1634–1639.
(14) Other cancer cell lines such as human cervix carcinoma KB-3-1 (DSMZ ACC 158) and mouse fibroblast L929 (DSMZ ACC 2) also showed comparable biological activities.
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(16) Kochi, T.; Tang, T. P.; Ellman, J. A. *J. Am. Chem. Soc.* **2003**, *125*, 11276–11282.
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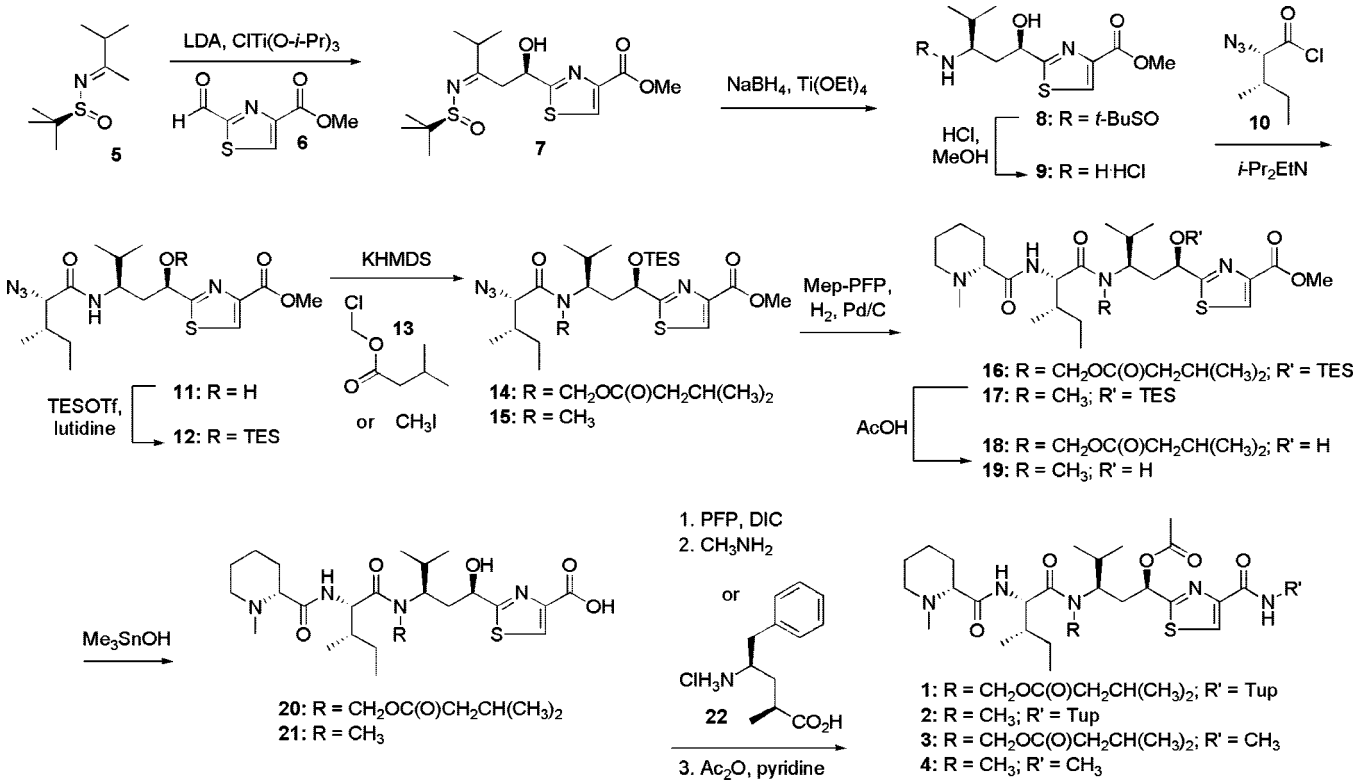
SCHEME 1. Initial Synthesis of Tubulysin D (1) and Related Analogues 2−4

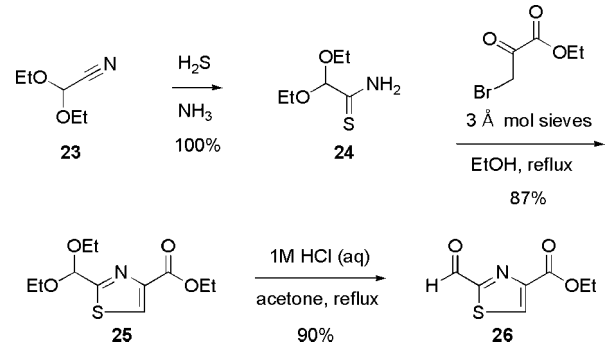


tubuphenylalanine hydrochloride (**22**)[21] or methylamine to provide tubulysin D (**1**) and analogues **2**−**4**.

The high lability of the *O*-acyl *N*,*O*-acetal functionality was an overarching design feature of our initial total synthesis of tubulysin D, and as overviewed above, the same synthetic route could also be employed to prepare *N*-methyl tubulysin analogues. However, because these *N*-methyl analogues no longer incorporate the labile *O*-acyl *N*,*O*-acetal, substantial synthetic constraints were removed, providing the opportunity to modify the initial route to achieve a considerably more efficient synthesis. The use of α-azido acid chloride **10**, which requires two steps to synthesize using highly reactive triflyl azide,[22] could be eliminated by installing the *N*-methyl substituent at an earlier stage. Additionally, it would be ideal if the tedious tubuvaline alcohol silyl ether protection and deprotection steps could be eliminated. Also, in the absence of the *O*-acyl *N*,*O*-acetal, a less toxic and more practical reagent could be used in place of Me_3SnOH for hydrolysis of the late-stage thiazole ester intermediate. More straightforward means for directly coupling the amino acid subunits would also be preferred, for example, direct coupling of *N*-methyl pipecolinic acid rather than coupling via the pentafluorophenyl ester, which is readily epimerized and cannot be stored.

The optimized synthesis of *N*-methyl tubulysin analogues **2** and **4** began with the preparation of thiazole **26** in 78% yield over three steps from commercially available diethoxyacetonitrile (**23**), according to literature methods (Scheme 2).[18] This pathway easily gives large quantities of thiazole precursor **26**. In contrast, the initial synthesis relied on thiazole methyl ester **6** (Scheme 1), which requires an additional step to prepare, to

SCHEME 2. Synthesis of Thiazole Aldehyde 26



enable selective late-stage hydrolysis of the thiazole methyl ester in the presence of the labile *O*-acyl *N*,*O*-acetal. Addition of the metalloenamine derived from *N*-*tert*-butanesulfinyl ketimine **5**[12,16] to thiazole aldehyde **26** then provided β-hydroxy imine **27** (Scheme 3). Excess LDA (3.5 equiv) and $ClTi(O\text{-}i\text{-}Pr)_3$ (3 equiv) were both necessary to achieve high conversion and diastereoselectivity (93:7). Stereoselective reduction of imine **27** was accomplished with 92:8 dr using $NaBH_4$ and $Ti(OEt)_4$.[12,16,19] The reaction was performed at low temperatures to prevent reduction of the ethyl ester and to increase selectivity. After chromatography, *N*-*tert*-butanesulfinyl tubuvaline ethyl ester (**28**) was isolated in diastereomerically pure form in 74% yield over the two steps.

We next sought to install the *N*-methyl group without protection of the tubuvaline alcohol. Not surprisingly, alkylation of **28** with methyl iodide was not selective and provided a mixture of products. We therefore envisioned an alternative approach that proceeded through 1,3-tetrahydrooxazine **29** or **30**, which could potentially be reduced to give *N*-methyl tubuvaline ethyl ester **31** (Scheme 4).

(20) Nicolaou, K. C.; Estrada, A. A.; Zak, M.; Lee, S. H.; Safina, B. S. *Angew. Chem., Int. Ed.* **2005**, *44*, 1378–1382.
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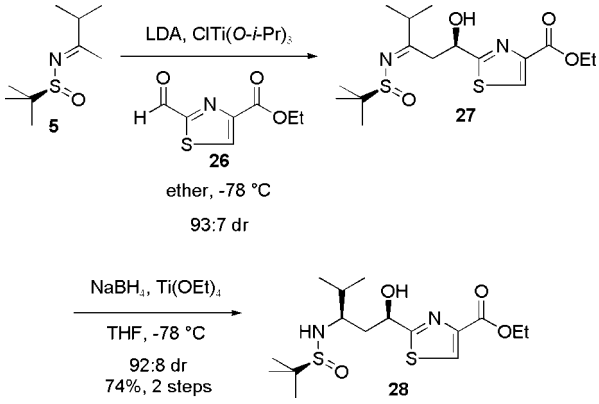
(22) Lundquist, J. T., IV.; Pelletier, J. C. *Org. Lett.* **2001**, *3*, 781–783.

SCHEME 3. Stereoselective Synthesis of *N-tert*-Butanesulfinyl Tubuvaline Ethyl Ester (28)



The direct alkylation of *N*-sulfinyl-1,3-amino alcohol **28** under basic conditions was first attempted using dihalogenated methylene reagents such as CH_2Br_2. This type of reactivity is precedented for *N*-acyl- and *N*-alkyl-1,2-amino alcohol scaffolds,[23] as well as for *N*-alkyl-1,3-amino alcohols,[24] but to our knowledge, not for *N*-acyl-1,3-amino alcohols. Exploration of the alkylation of **28** with CH_2Br_2 using a variety of solvents and bases as precedented for *N*-acyl-1,2-amino alcohol systems (Table 1, entries 1−4) proved unsuccessful. We therefore explored condensation of **28** with paraformaldehyde under acidic conditions to yield tetrahydrooxazine **30** (Scheme 4). Formation of tetrahydrooxazines and oxazolidines by reaction of formaldehyde with 1,3- and 1,2-amino alcohols with *N*-aryl- and *N*-alkyl substituents is common. Reactions with *N*-acyl- and *N*-sulfonyl-1,2-amino alcohols have also been reported in a few cases,[25] but there are no reports using *N*-acyl-1,3-amino alcohols. In our initial attempt to condense **28** with paraformaldehyde, 1 equiv of TsOH in benzene with heating at 70 °C resulted in complete conversion to sulfinyl-deprotected tetrahydrooxazine **30** in less than 2 h (Table 1, entry 5). Unfortunately, **30** was prohibitively unstable and hydrolyzed upon purification via silica gel or reverse-phase chromatography. Attempts to convert the crude product mixture directly to the desired *N*-methyl tubuvaline **31** via reduction with $NaCNBH_3$[26] gave instead the undesired dimethylated product due to contaminating amounts of paraformaldehyde that remained after workup. Therefore, our focus turned to the preparation of tetrahydrooxazine **29**, which should be more stable and isolable.

Incrementally decreasing the amount of TsOH from stoichiometric amounts to 0.001 equiv (Table 1, entries 6−9) increased the ratio of tetrahydrooxazines **29** and **30** to 88:12. An examination of solvents using 0.01 equiv of TsOH established that more polar solvents such as THF and dioxane gave decomposition products (entries 11 and 12), while toluene gave a slightly better ratio than benzene with similar conversions after 5 h (entries 8 and 13). Because even low concentrations of acid catalyzed not only tetrahydrooxazine formation but also undesired sulfinyl group cleavage, the condensation reaction with paraformaldehyde was performed in the absence of any acid (Table 1, entry 14). Gratifyingly, heating **28** with paraformaldehyde in toluene resulted in complete conversion to the desired *N*-sulfinyl-protected tetrahydrooxazine **29**.

When the reaction was performed on a preparative scale, **29** proved to be stable to silica gel chromatography and was obtained in 87% yield (Scheme 5). Reduction of **29**[27] with support-bound cyanoborohydride (MP-BH_3CN) resin under acidic conditions then readily provided **31** in 97% yield as the free amine after silica gel chromatography in the presence of NH_4OH. Accordingly, *N*-methyl tubuvaline ethyl ester (**31**) was prepared in 49% yield over seven steps and serves as a versatile and scalable intermediate for the construction of *N*-methyl tubulysin analogues with variation at the Mep, Ile, or Tup/Tut positions.

Coupling of *N*-Boc-isoleucine (Boc-Ile-OH) with *N*-methyl tubuvaline **31** to form the hindered tertiary amide linkage was next attempted. However, using a variety of coupling reagents and reaction parameters, ester **32** was surprisingly the predominant product (Table 2). Bisacylated product **33** was also observed in some examples, but no more than 8% of the desired amide product **34** was observed in any case. The predilection for *O*-acylation with a secondary alcohol was quite unexpected, although it is not without precedent.[28,29] For amino alcohol **31**, selective esterification is possibly aided by intramolecular general base catalysis provided by the proximal secondary amine. EDC, which exists as an HCl salt, HOAt, and excess base gave primarily ester **32** contaminated with significant amounts of bisacylated product **33**, but no amide product **34** was observed (entry 1). Decreasing the amount of base used in the reaction decreased the amount of **33** (entry 2), as did using the less active HOBt in place of HOAt (entry 3). EDC with HOBt and 1.2 equiv of base provided only monoacylated products with a 97:3 ratio of ester **32** to desired amide **34**. Using these conditions in DMF, however, failed to give a significantly improved product ratio (entry 4). Similar base and HOBt/HOAt dependence was observed when support-bound cyclohexylcar-

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(27) To our knowledge no examples exist of reducing 1,3-*N*-acyl-, 1,3-*N*-sulfonyl-, or 1,3-*N*-sulfinyl-tetrahydrooxazines to the corresponding 1,3-amino alcohol. For examples with 1,2-*N*-acyl-oxazolidines, see refs 25f, i, and 25j, as well as: (a) Reddy, G. V.; Rao, G. V.; Iyengar, D. S. *Chem. Commun.* **1999**, *4*, 317–318. (b) Reddy, G. V.; Rao, G. V.; Sreevani, V.; Iyengar, D. S. *Tetrahedron Lett.* **2000**, *41*, 953–954.

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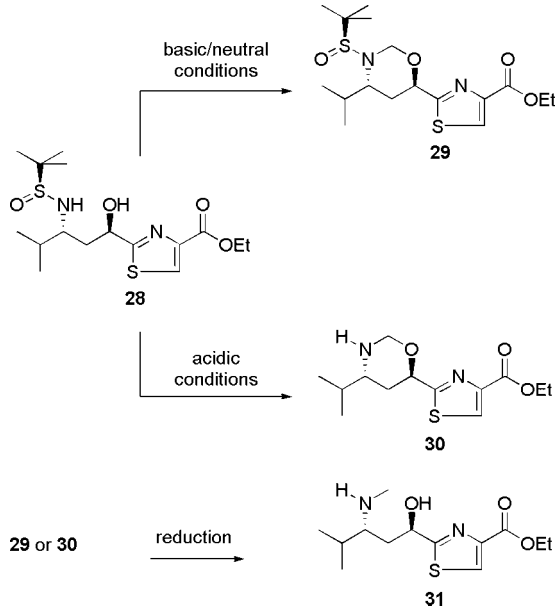
(29) Welsh, L. H. *J. Am. Chem. Soc.* **1947**, *69*, 128–134.

TABLE 1. Conditions for Formation of 1,3-Tetrahydrooxazines 29 and 30

entry	solvent	temp (°C)	time (h)	reagent (equiv)	acid/base (equiv)	product ratio[a] (**28:29:30**)
1	THF	60	24	CH_2Br_2 (8)	K_2CO_3 (2)	100:0:0
2	THF/DMSO (9:1)	60	24	CH_2Br_2 (8)	K_2CO_3 (2), TBAC[b](0.2)	100:0:0
3	pyridine	60	20	CH_2Br_2 (5)	pyridine	100:0:0
4	CH_2Br_2	100	24	CH_2Br_2	*i*-Pr_2EtN (10)	100:0:0
5	benzene	70	2	$(CH_2O)_n$ (20)	TsOH (1)	0:0:100
6	benzene	70	2	$(CH_2O)_n$ (20)	TsOH (0.2)	0:28:72
7	benzene	70	4	$(CH_2O)_n$ (20)	TsOH (0.05)	0:78:22
8	benzene	70	5	$(CH_2O)_n$ (20)	TsOH (0.01)	10:77:13[c]
9	benzene	70	22	$(CH_2O)_n$ (20)[d]	TsOH (0.001)	0:88:12
10	benzene	23	48	$(CH_2O)_n$ (10)	TsOH (0.2)	42:47:11[c]
11	THF	70	17	$(CH_2O)_n$ (20)[d]	TsOH (0.01)	decomp.[e]
12	dioxane	70	17	$(CH_2O)_n$ (20)[d]	TsOH (0.01)	decomp.[e]
13	toluene	70	5	$(CH_2O)_n$ (20)	TsOH (0.01)	13:84:3[c]
14	toluene	70	50	$(CH_2O)_n$ (20)		0:100:0

[a] Product ratio of crude material determined by NMR. [b] Tetrabutylammonium chloride (TBAC) used as a phase-transfer catalyst. [c] Reaction progress halted prior to completion. [d] Two additions of 10 equiv. [e] Complex mixture of decomposition products observed.

SCHEME 4. Outline of 1,3-Tetrahydrooxazine Formation and Reduction from *N*-Sulfinyl-1,3-amino alcohol 28



bodiimide (PS-CCD) was used (entries 5−7). Other coupling conditions such as PyBOP with HOBt (entry 8), acid fluoride (Boc-Ile-F,[30] entry 9), or isobutyl mixed anhydride (Boc-Ile-O-CO_2-*i*-Bu, entry 10) also failed to give appreciable amounts of amide **34**.

Because **32** could be prepared very cleanly without any of the bisacylated product **33** using PS-CCD or PyBOP, we next sought to transform ester **32** to *N*-methyl amide **34** (Scheme 6).[29,31] Ester **32** was cleanly obtained on a preparative scale from **31** using PS-CCD and HOBt in the absence of base. After purification of **32** only by filtration and aqueous extraction, heating at 90 °C in toluene under anhydrous conditions provided the desired amide product **34** in 93% yield over the two steps.

Acidic cleavage of the Boc-group of **34**, followed by condensation with D-Mep and saponification of the ethyl ester with LiOH, gave tripeptide **21** in 99% yield over the three steps with only a single chromatographic purification (Scheme 6). This is an improvement over the prior synthesis (Scheme 1),[8,12] which required preparation of the pentafluorophenyl ester of D-Mep, whose byproducts were nontrivial to remove, and the use of Me_3SnOH for selective saponification of the thiazole ester. Acetylation of the free alcohol then afforded **35** in 97% yield.

In our biological evaluation of tubulysin D analogues, we not only demonstrated that the *O*-acyl *N*,*O*-acetal is unnecessary for potent biological activity, but also that broad modifications at the Tup position have little effect on cell-based activity (Figure 2).[8] Therefore, compound **35**, which was prepared in 44% yield over 13 steps, is a versatile intermediate for the preparation of *N*-methyl tubulysin analogues with variation at the Tup/Tut position, as demonstrated by the preparation of bioactive compounds **2** and **4**.

Compound **4** was prepared from **35** via PS-CCD conditions with methylamine hydrochloride in 98% yield (Scheme 7). For the formation of **2**, the carboxylic acid of **35** was first activated as the PFP-ester followed by the addition of tubuphenylalanine hydrochloride (**22**) to afford **2** in 92% yield (Scheme 7).[32]

Conclusion

In summary, we have developed the asymmetric total synthesis of *N*-methyl tubulysin **2** which proceeds in 15 linear steps and in 40% overall yield. This synthesis represents by far the most efficient and scalable preparation of this important tubulysin analogue.[7,8] *N*-Methyl tubulysin **4** was also prepared in 43% overall yield over 14 steps through a nearly identical route. Additionally, the synthesis is highly modular, enabling modification at numerous positions for the preparation of diverse *N*-methyl tubulysin analogues. Biochemical studies of *N*-methyl tubulysin analogues are currently underway and will be reported in due course.

Experimental Section

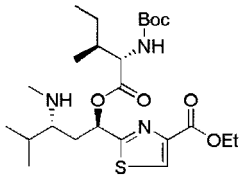
(+)-2-[(*S*$_s$,1*R*)-1-Hydroxy-4-methyl-3-(2-methylpropane-2-sulfinylimino)pentyl]thiazole-4-carboxylic acid ethyl ester (27). A 0.9 M solution of *i*-Pr_2NH (0.669 mL, 4.76 mmol) in Et_2O (5.5 mL) was cooled to 0 °C, and *n*-BuLi (1.69 mL, 4.17 mmol, 2.47 M solution in hexanes) was added. The solution was stirred for 20

(30) Carpino, L. A.; Sadat-Aalaee, D.; Chao, H. G.; DeSelms, R. H. *J. Am. Chem. Soc.* **1990**, *112*, 9651–9652.

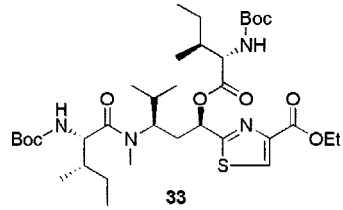
(31) Facile *O*- to *N*-acyl transfer has been reported, most notably in the *N*-methyl-1,2-amino alcohol pseudoephedrine series. See ref 29 and: (a) Myers, A. G.; Gleason, J. L.; Yoon, T.; Kung, D. W *J. Am. Chem. Soc.* **1997**, *119*, 656–673.

(32) Due to the unprotected carboxylic acid present in **22**, PFP-ester activation was used in preference to carbodiimide coupling procedures that would cause oligomerization.

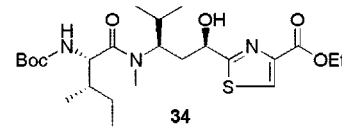
TABLE 2. Acylation of *N*-Methyl Tubuvaline Ethyl Ester (31)



32



33

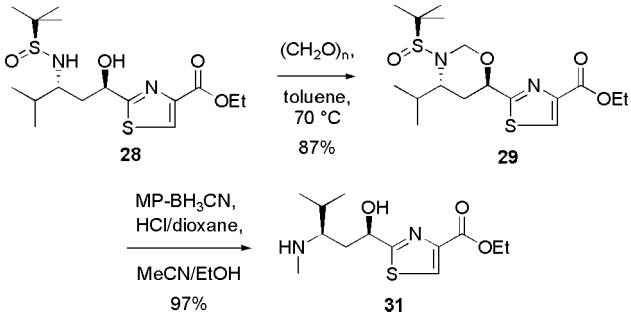


34

entry[a]	solvent	coupling reagents (equiv)	*i*-Pr$_2$EtN (equiv)	Boc-Ile-X (equiv)	product ratio[b] (**32:33:34**)
1	CH_2Cl_2	EDC (1.1), HOAt (1.1)	3	Boc-Ile-OH (1.1)	70:30:0
2	CH_2Cl_2	EDC (1.2), HOAt (1.1)	1.2	Boc-Ile-OH (1.05)	86:8:6
3	CH_2Cl_2	EDC (1.1), HOBt (1.0)	1.2	Boc-Ile-OH (1.1)	97:0:3
4	DMF	EDC (1.2), HOBt (1.1)	1.2	Boc-Ile-OH (1.05)	86:6:8
5	CH_2Cl_2	PS-CCD (1.2), HOBt (1.05)	1.2	Boc-Ile-OH (1.05)	96:2:2
6	CH_2Cl_2	PS-CCD (1.2), HOBt (1.05)	0	Boc-Ile-OH (1.1)	100:0:0
7	CH_2Cl_2	PS-CCD (1), HOAt (1)	0	Boc-Ile-OH (1.05)	96:4:0
8	CH_2Cl_2	PyBOP (1.1), HOBt (1.1)	1.2	Boc-Ile-OH (1.1)	100:0:0
9	CH_2Cl_2		1.1	Boc-Ile-F (1.1)	decomp.[c]
10	CH_2Cl_2		0	Boc-Ile-O-CO_2-*i*-Bu (1.2)	83:17:0[d]

[a] All reactions were performed at room temperature, with reaction times ranging from 12 to 24 h. [b] Product ratio of crude material determined by reverse-phase HPLC. [c] A complex product mixture was observed, with **32** still the major product. [d] A complex mixture of other acylation and Boc-deprotected products was also observed.

SCHEME 5. Synthesis of *N*-Methyl Tubuvaline Ethyl Ester (31)



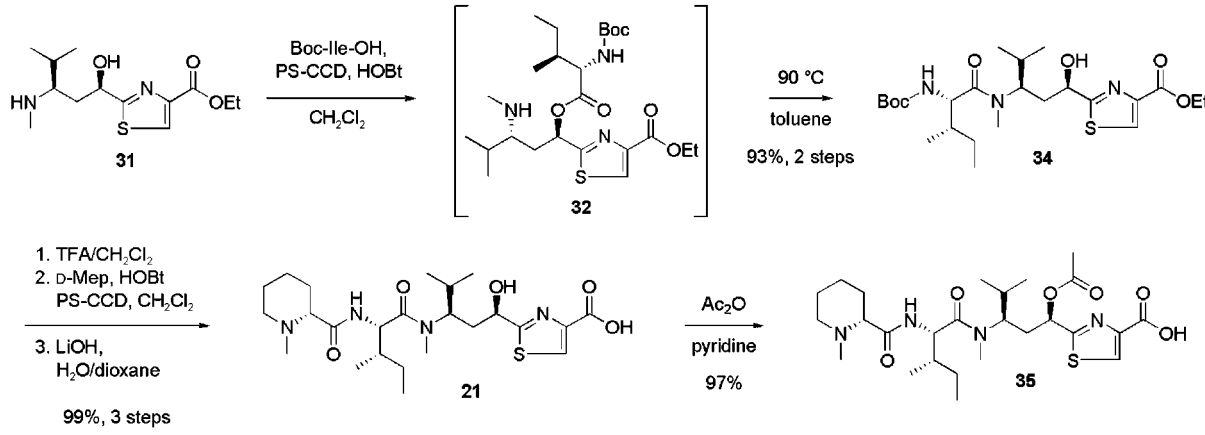
min at 0 °C and then cooled to −78 °C. A solution of sulfinyl ketimine **5**[17] (0.339 g, 1.79 mmol) in Et_2O (3.6 mL) was added, and the reaction mixture was stirred for 30 min at −78 °C. Chlorotitanium triisopropoxide (0.744 mL, 3.57 mmol) was then added, and the reaction mixture was stirred for an additional 45 min at −78 °C. Aldehyde **26**[18] (0.220 g, 1.19 mmol) was added in one portion, and the solution was stirred at −78 °C for 46 h. The solution was neutralized using a 4:1 (v/v) solution of THF/AcOH (2.5 mL) followed by addition of H_2O (15 mL). The resulting mixture was warmed to rt and filtered through Celite, washing the filter cake thoroughly with EtOAc. The solution was washed once with brine, dried over $MgSO_4$, filtered, and concentrated. NMR analysis on the unpurified material established a 93:7 ratio of diastereomers. HPFC purification (98:2 to 80:20 CH_2Cl_2:MTBE) produced a ∼5.3:1 mixture of the pure major diastereomer **27** and starting sulfinyl ketimine **5** as a yellow oil. The mixture was further reacted without additional purification.

A small sample was further purified by HPFC (90:10 to 80:20 toluene:EtOAc) for characterization purposes and was isolated as a yellow oil: $[\alpha]^{25}_D = +67.3$ ($c = 1.0$, $CHCl_3$); IR (film) 1476, 1625, 1732, 2871, 2931, 2970, 3220 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.15 (app t, 6H, $J = 8.5$ Hz), 1.25 (s, 9H), 1.34 (t, 3H, $J = 7.0$ Hz), 2.75−2.85 (m, 1H), 3.22−3.32 (m, 2H), 4.35 (q, 2H, $J = 7.0$ Hz), 5.08 (m, 1H), 6.56 (d, 1H, $J = 8.5$ Hz), 8.06 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.2, 20.0, 20.8, 22.3, 38.8, 43.7, 58.3, 61.2, 67.3, 127.6, 147.1, 161.3, 178.1, 187.4; HRMS (FAB) calcd for $C_{16}H_{26}N_2O_4NaS_2$ (M + Na) 397.1232, found 397.1229.

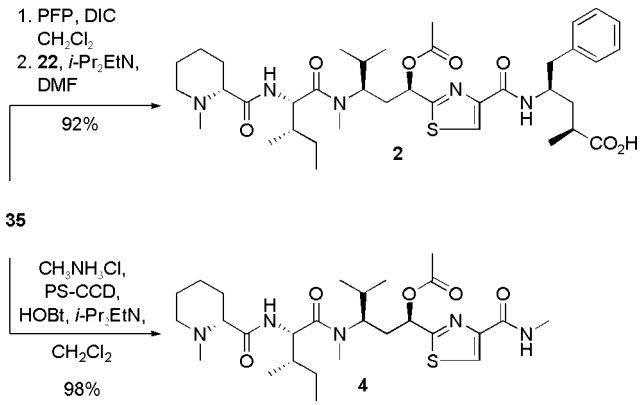
(+)-2-[(S_s,1*R*,3*R*)-1-Hydroxy-4-methyl-3-(2-methylpropane-2-sulfinylamino)pentyl]thiazole-4-carboxylic acid ethyl ester (28). A solution of the product mixture containing β-hydroxy imine **27** (∼0.943 mmol) and imine **5** (∼0.178 mmol) in THF (3.50 mL) was cooled to −78 °C. $Ti(OEt)_4$ (0.469 mL, 2.24 mmol) was added, followed by $NaBH_4$ (0.0847 g, 2.24 mmol), and the solution was stirred at −78 °C for 12 h. The solution was acidified using a 4:1 (v/v) solution of THF/AcOH (3.2 mL) followed by addition of EtOH (2 mL) and H_2O (10 mL). The solution was warmed to rt and diluted with EtOAc. The mixture was washed once with brine, and the aqueous fraction was extracted once with EtOAc. The combined organic fractions were dried with Na_2SO_4, filtered, and concentrated. NMR analysis on the unpurified material established a 92:8 ratio of diastereomers. HPFC purification (100% EtOAc) produced 0.332 g (74%, over two steps) of the pure major diastereomer **28** as a colorless oil: $[\alpha]^{25}_D = +103.2$ ($c = 1.0$, $CHCl_3$); IR (film) 1474, 1718, 2868, 2929, 2960, 3283 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.90 (d, 3H, $J = 6.8$ Hz), 0.93 (d, 3H, $J = 6.8$ Hz), 1.29 (s, 9H), 1.40 (t, 3H, $J = 7.1$ Hz), 1.70 (doublet of septets, 1H, $J = 2.3$, 6.8 Hz), 1.91 (ddd, 1H, $J = 3.6$, 11.5, 14.7 Hz), 2.30 (ddd, 1H, $J = 3.0$, 11.8, 14.7 Hz), 3.30 (d, 1H, $J = 8.5$ Hz), 3.40−3.47 (m, 1H), 4.38−4.45 (m, 2H), 5.18 (ddd, 1H, $J = 3.1$, 6.7, 11.6 Hz), 5.48 (d, 1H, $J = 6.7$ Hz), 8.11 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.4, 17.3, 19.7, 23.0, 33.9, 40.6, 56.3, 58.5, 61.3, 67.8, 127.4, 147.0, 161.6, 177.6; HRMS (FAB) calcd for $C_{16}H_{28}N_2O_4NaS_2$ (M + Na) 399.1388, found 399.1389.

(+)-2-[(S_s,1*R*,3*R*)-4-Isopropyl-3-(2-methylpropane-2-sulfinyl)-[1,3]oxazinan-6-yl]thiazole-4-carboxylic acid ethyl ester (29). A 0.1 M solution of *N*-sulfinyl amino alcohol **28** (0.575 g, 1.53 mmol) in toluene (15.3 mL) with paraformaldehyde (0.917 g, 30.5 mmol) was heated in a sealed vessel at 70 °C with stirring for 50 h. After cooling to rt, the mixture was filtered through Celite, washing the filter cake thoroughly with toluene. The solution was concentrated and purified via HPFC (88:12 to 30:70 CH_2Cl_2:EtOAc) to afford 0.518 g of tetrahydrooxazine **29** (87%) as a foamy yellow solid: $[\alpha]^{25}_D = +108.4$ ($c = 1.0$, $CHCl_3$); IR (film) 1011, 1076, 1087, 1163, 1195, 1474, 1729, 2957, 2979 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.01 (d, 3H, $J = 6.8$ Hz), 1.02 (d, 3H, $J = 6.8$ Hz), 1.17 (s, 9H), 1.35 (t, 3H, $J = 7.1$ Hz), 2.08−2.16 (m, 1H), 2.28−2.40 (m, 1H), 2.31 (d, 1H, $J = 14.3$ Hz), 3.02 (dd, 1H, $J = 4.7$, 10.1 Hz), 4.37 (q, 2H, $J = 7.1$ Hz), 4.72 (d, 1H, $J = 11.6$ Hz), 5.14 (d, 1H, $J = 11.6$ Hz), 5.20 (dd, 1H, $J = 2.5$, 11.5 Hz), 8.11 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.3, 20.5, 20.7, 22.8, 27.5, 32.6, 59.2, 61.3, 62.7, 71.4, 71.6, 127.8, 146.5, 161.3, 173.3; HRMS (FAB) calcd for $C_{17}H_{29}N_2O_4S_2$ (M + H) 389.1569, found 389.1566.

SCHEME 6. Synthesis of Carboxylic Acid Intermediate 35



SCHEME 7. Synthesis of *N*-Methyl Tubulysins 2 and 4



(+)-(1*R*,3*R*)-2-(1-Hydroxy-4-methyl-3-methylaminopentyl)thiazole-4-carboxylic acid ethyl ester (31). To a 0.18 M solution of tetrahydrooxazine **29** (0.200 g, 0.514 mmol) in 9:1 MeCN/EtOH (2.9 mL) with MP-BH_3CN (2.52 mmol/g, 0.204 g, 0.514 mmol) was added dropwise HCl (4.0 M in 1,4-dioxane, 0.515 mL, 2.06 mmol) with stirring. Stirring was continued for 3 h at rt, and then the solution was concentrated and purified by HPFC (95:5:1 to 90:10:1 CH_2Cl_2:EtOH:NH_4OH). The product fractions were concentrated, diluted with EtOAc, and washed once with saturated $NaHCO_3$(aq) to remove excess ammonia salts. The aqueous fraction was back-extracted once with EtOAc. The combined organic fractions were dried with Na_2SO_4 and concentrated to afford 0.143 g of *N*-methyl tubuvaline ethyl ester **31** (97%) as a foamy solid: mp 85−88 °C; $[\alpha]^{25}_D = +70.5$ ($c = 0.8$, $CHCl_3$); IR (film) 1087, 1232, 1315, 1492, 1713, 2947, 3119 cm^{-1}; ^1H NMR (500 MHz, d_4-MeOD) δ 0.89 (d, 3H, $J = 6.8$ Hz), 0.90 (d, 3H, $J = 6.8$ Hz), 1.39 (t, 3H, $J = 7.0$ Hz), 1.88−2.04 (m, 3H), 2.38 (s, 3H), 2.43−2.48 (m, 1H), 4.38 (q, 2H, $J = 7.0$ Hz), 5.14−5.18 (m, 1H), 8.32 (s, 1H); ^{13}C NMR (125 MHz, d_3-MeCN) δ 14.6, 17.3, 20.1, 28.8, 33.1, 33.9, 61.9, 63.5, 72.1, 128.6, 148.2, 162.4, 180.8; HRMS (FAB) calcd for $C_{13}H_{23}N_2O_3S$ (M + H) 287.1429, found 287.1427.

2-{(1*R*,3*R*)-1-[(2*S*,3*S*)-2-*tert*-Butoxycarbonylamino-3-methylpentanoyloxy]-4-methyl-3-methylaminopentyl}thiazole-4-carboxylic acid ethyl ester (32). A 0.1 M solution of *N*-methyl tubuvaline ethyl ester **31** (0.305 g, 1.06 mmol), 1-hydroxybenzotriazole (HOBt) (0.146 g, 1.08 mmol), and Boc-Ile-OH (0.267 g, 1.11 mmol) in CH_2Cl_2 (10.6 mL) was cooled in a salted ice−water bath. PS-CCD (1.38 mmol/g, 0.920 g, 1.27 mmol) was added with stirring, and the bath was warmed to rt. Stirring was continued for 14 h, the solution was filtered, and the resin was washed with CH_2Cl_2. The filtrate was then concentrated, diluted with EtOAc, and washed once with saturated $NaHCO_3$(aq). The aqueous fraction was back-extracted twice with EtOAc, and the combined organic fractions were dried with Na_2SO_4, filtered, and concentrated to afford intermediate **32**, which was taken on without further purification.

A small sample of ester **32** was further purified for characterization purposes. The intermediate was first isolated as the formic acid salt via reverse-phase HPFC (80:20 to 0:100 H_2O:MeCN with 0.1% formic acid). The product fractions were concentrated, diluted with EtOAc, and washed once with saturated $NaHCO_3$(aq) to provide the free amine. The organic fraction was dried with Na_2SO_4 and concentrated to afford pure ester **32** as an amorphous solid: ^1H NMR (400 MHz, $CDCl_3$) δ 0.81 (d, 3H, $J = 6.8$ Hz), 0.86−0.93 (m, 6H), 0.95 (d, 3H, $J = 6.8$ Hz), 1.11−1.25 (m, 1H), 1.37 (t, 3H, $J = 7.1$ Hz), 1.34−1.48 (m, 2H), 1.40 (s, 9H), 1.82−1.99 (m, 3H), 2.04−2.14 (m, 1H), 2.29−2.36 (m, 1H), 2.34 (s, 3H), 4.39 (q, 2H, $J = 7.1$ Hz), 4.30−4.36 (m, 1H), 5.07 (d, 1H, $J = 9.2$ Hz), 6.37 (dd, 1H, $J = 3.1$, 9.9 Hz), 8.11 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.5, 14.3, 15.5, 16.6, 19.1, 24.7, 28.2, 28.6, 33.6, 36.1, 37.9, 58.2, 60.4, 61.4, 72.0, 79.7, 127.6, 147.1, 155.4, 161.1, 170.9, 171.5; HRMS (FAB) calcd for $C_{24}H_{42}N_3O_6S$ (M + H) 500.2794, found 500.2787.

(−)-2-{(1*R*,3*R*)-3-[(2*S*,3*S*)-(2-*tert*-Butoxycarbonylamino-3-methylpentanoyl)methylamino]-1-hydroxy-4-methylpentyl}thiazole-4-carboxylic acid ethyl ester (34). Crude ester **32** (~1.06 mmol) in toluene (10.6 mL, 0.1 M) was heated to 90 °C in a sealed vessel with stirring for 30 h. HPFC purification (88:12 to 0:100 hexanes:EtOAc) afforded 0.492 g of *N*-methyl carbamate **34** (93%, two steps) as an amorphous solid. The ^1H NMR spectrum indicated that the product existed as a 7:1 mixture of rotamers, with the major isomer reported: $[\alpha]^{25}_D = -11.7$ ($c = 1.0$, $CHCl_3$); IR (film) 1016, 1094, 1166, 1206, 1234, 1366, 1495, 1617, 1713, 2875, 2934, 2965, 3325 cm^{-1}; ^1H NMR (500 MHz, d_4-MeOD) δ 0.84 (d, 3H, $J = 7.4$ Hz), 0.91 (t, 3H, $J = 7.4$ Hz), 0.94−0.99 (m, 6H), 1.39 (t, 3H, $J = 7.1$ Hz), 1.42 (s, 9H), 1.58−1.69 (m, 1H), 1.77−1.87 (m, 2H), 1.88−1.95 (m, 1H), 2.14−2.24 (m, 1H), 3.15 (s, 3H), 4.38 (q, 2H, $J = 7.1$ Hz), 4.41 (d, 1H, $J = 8.8$ Hz), 4.44−4.57 (br s, 1H), 4.63−4.68 (m, 1H), 8.30 (s, 1H); ^{13}C NMR (125 MHz, d_4-MeOD) δ 11.3, 14.7, 16.2, 20.3, 20.6, 25.7, 28.8, 31.1, 37.6, 38.7, 56.8, 58.1, 62.5, 69.9, 80.5, 129.1, 147.8, 158.0, 162.8, 176.5, 180.5; HRMS (FAB) calcd for $C_{24}H_{41}N_3O_6NaS$ (M + Na) 522.2614, found 522.2605.

(−)-2-[(1*R*,3*R*)-1-Hydroxy-4-methyl-3-(methyl-{(2*S*,3*S*)-3-methyl-2-[((1*R*)-1-methylpiperidine-2-carbonyl)amino]pentanoyl}amino)pentyl]thiazole-4-carboxylic acid (21). To a 0.1 M solution of carbamate **34** (0.445 g, 0.891 mmol) in CH_2Cl_2 (9.00 mL) was added an equal volume of TFA (9.00 mL) with stirring for 1 h. The solution was then concentrated, diluted with EtOAc, and washed once with saturated $NaHCO_3$(aq). The aqueous fraction was back-extracted twice with EtOAc, and the combined organic fractions were dried with Na_2SO_4, filtered, and concentrated to afford the Boc-deprotected free amine of **34**, which was taken on to the next step without further purification. To this amine

in CH_2Cl_2 (0.1 M, 9.00 mL) were added HOBt (0.122 g, 0.908 mmol) and D-Mep[12] (0.134 g, 0.935 mmol). The mixture was then cooled in a salted ice−water bath with stirring, and PS-CCD (1.38 mol/g, 0.775 g, 1.07 mmol) was added. The bath was warmed to rt, and stirring was continued for 14 h. The mixture was then filtered, the resin was washed with CH_2Cl_2, and the filtrate was concentrated. The crude mixture was then diluted with EtOAc and washed once with saturated $NaHCO_3$(aq). The aqueous fraction was back-extracted twice with EtOAc, and the combined organic fractions were dried with Na_2SO_4, filtered, and concentrated to afford the Mep-coupled intermediate, which was taken on without further purification. To the crude intermediate diluted in 1,4-dioxane (0.1 M, 9.00 mL) was added a 0.4 M solution of LiOH (85.3 mg, 3.56 mmol) in degassed H_2O (9.00 mL). After stirring for 5 h, the solution was concentrated and HPFC purified (90:10:1 to 70:30:1 CH_2Cl_2:MeOH:NH_4OH) to afford 0.438 g of carboxylic acid **21** (99%, three steps) as an amorphous solid. Spectral data of **21** matched that previously reported by us for the preparation of **21** via Scheme 1.[8]

(−)-2-[(1*R*,3*R*)-1-Acetoxy-4-methyl-3-(methyl-{(2*S*,3*S*)-3-methyl-2-[((1*R*)-1-methylpiperidine-2-carbonyl)amino]pentanoyl}amino)-pentyl]thiazole-4-carboxylic acid (35). A 0.1 M solution of alcohol **21** (23.0 mg, 0.0463 mmol) in pyridine (0.460 mL) was cooled in an ice−water bath, and Ac_2O (21.9 μL, 0.232 mmol) was added with stirring. The bath was warmed to rt, and stirring was continued for 24 h. The solution was then cooled in an ice−water bath, and a 1:1 (v/v) solution of degassed H_2O/dioxane (2 mL) was added. The bath was warmed to rt, and stirring was continued for 22 h. Concentration, followed by HPFC purification (80:20:1 CH_2Cl_2:MeOH:NH_4OH), afforded 24.3 mg of acetate **35** (97%) as an amorphous solid: $[\alpha]^{25}_D = -14.8$ ($c = 0.8$, MeOH); IR (film) 1222, 1369, 1470, 1593, 1639, 1747, 2874, 2961 cm^{-1}; ^1H NMR (500 MHz, d_6-DMSO) δ 0.67 (d, 3H, $J = 6.6$ Hz), 0.82 (t, 3H, $J = 7.4$ Hz), 0.86 (d, 3H, $J = 6.7$ Hz), 0.91 (d, 3H, $J = 6.5$ Hz), 0.99−1.10 (m, 1H), 1.10−1.22 (m, 1H), 1.29−1.40 (m, 1H), 1.40−1.51 (m, 2H), 1.52−1.58 (m, 1H), 1.58−1.67 (m, 2H), 1.71−1.82 (m, 2H), 1.97−2.04 (m, 1H), 2.08 (s, 3H), 2.11 (s, 3H), 2.12−2.17 (m, 1H), 2.17−2.25 (m, 1H), 2.52−2.56 (m, 1H), 2.84−2.89 (m, 1H), 2.97 (s, 3H), 4.23−4.43 (br s, 1H), 4.60 (app t, 1H, $J = 8.8$ Hz), 5.53 (dd, 1H, $J = 2.4$, 11.0 Hz), 7.69 (d, 1H, $J = 9.2$ Hz), 8.32 (s, 1H); ^{13}C NMR (125 MHz, d_6-DMSO) δ 10.6, 15.4, 19.4, 20.0, 20.6, 22.4, 23.9, 24.3, 28.9, 29.4, 33.9, 35.8, 43.4, 52.5, 54.5, 55.1 (br s), 67.7, 69.6, 126.8, 150.1, 162.9, 169.1, 169.7, 171.5, 172.7; HRMS (FAB) calcd for $C_{26}H_{43}N_4O_6S$ (M + H) 539.2903, found 539.2884.

***N*-Methyl Tubulysin 2.** A solution of carboxylic acid **35** (29.0 mg, 53.8 μmol) and pentafluorophenol (14.9 mg, 80.7 μmol) in 540 μL of CH_2Cl_2 was cooled in an ice−water bath followed by addition of PS-CCD (1.38 mmol/g, 24.1 mg, 33.3 μmol). The bath was warmed to rt and stirred for 15 h. The mixture was then filtered, the resin was washed with CH_2Cl_2, and the filtrate was concentrated. The crude PFP-ester was used without further purification. DMF (215 μL, 0.25 M) was added to the crude product followed by addition of the hydrochloride salt of tubuphenylalanine (**22**)[12] (39.2 mg, 161 μmol) and *i*-Pr_2EtN (47.0 μL, 269 μmol). The reaction mixture was stirred for 24 h and concentrated. HPFC purification (80:20 to 20:80 MeCN:H_2O) afforded 36.1 mg (92%) of **2** as an amorphous solid. Spectral data of **2** matched that previously reported by us for the preparation of **2** via Scheme 1.[8]

***N*-Methyl Tubulysin 4.** To a 0.1 M solution of carboxylic acid **35** (12.8 mg, 23.8 μmol), *i*-Pr_2EtN (25.0 μL, 143 μmol), HOBt (3.9 mg, 29 μmol), and CH_3NH_3Cl (6.4 mg, 95 μmol) in CH_2Cl_2 (238 μL) was added PS-CCD (1.38 mmol/g, 24.1 mg, 33.3 μmol) in a sealed vessel. After stirring for 10 h, 6.4 mg (95 μmol) of CH_3NH_3Cl and 25.0 μL (143 μmol) of *i*-Pr_2EtN were added and stirring was continued for an additional 10 h. The mixture was then filtered, the resin was washed with CH_2Cl_2, and the filtrate was concentrated. HPFC purification (80:20 to 20:80 H_2O:MeCN) afforded 12.9 mg of *N*-methyl tubulysin **4** (98%) as an amorphous solid. Spectral data of **4** matched that previously reported by us for the preparation of **4** via Scheme 1.[8]

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Supporting Information Available: General experimental procedures and copies of ^1H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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