

Total Synthesis and Biological Evaluation of Tubulysin U, Tubulysin V, and Their Analogues

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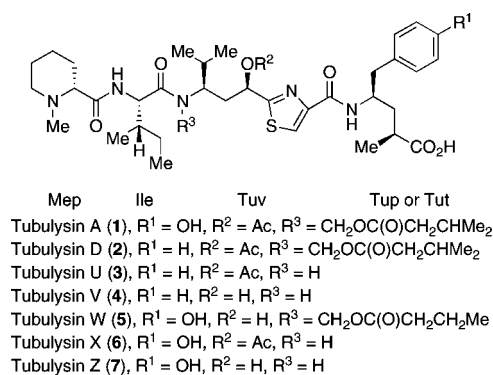
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Abstract: A stereoselective total synthesis of the cytotoxic natural products tubulysin U, tubulysin V, and its unnatural epimer epitubulysin V, is reported. Simplified analogues containing *N,N*-dimethyl-*D*-alanine as a replacement for the *N*-terminal *N*-Me-pipecolic acid residue of the tubulysins are also disclosed. Biological evaluation of these natural products and analogues provided key information with regard to structural and stereochemical requirements for antiproliferative activity and tubulin polymerization inhibition.

The tubulysins (**1–7**, Chart 1) are a group of extremely potent tubulin polymerization inhibitors that rapidly disintegrate the cytoskeleton of dividing cells and induce apoptosis.¹ Tubulysins are produced in very limited quantities by fermentation of the myxobacteria *Angiococcus disciformis* and *Archangium gephyra*.^{1a,b} Biosynthesis of tubulysins is accomplished by an unusual mixed nonribosomal peptide synthetase–polyketide synthase (NRPS–PKS)^a system.² This has led to extensive investigations into their total synthesis and the synthesis of novel analogues.^{3–6} The most active natural product known so far in this family is tubulysin D (**2**), which retains its anticancer activity against the multidrug resistant P-glycoprotein-expressing human KB-V1 cervix carcinoma cell line (IC₅₀ = 0.31 nM).^{1b} This activity profile makes the tubulysins exciting leads for the treatment of multidrug resistant cancers.

Structurally, tubulysins are tetrapeptides comprising of *N*-methylpipecolic acid (Mep) at the *N*-terminus, isoleucine (Ile, the only proteinogenic amino acid) as the second residue, the unique thiazole-containing tubuvaline (Tuv) as the third residue, and two possible γ -amino acids at the *C*-terminus (tubutyrosine (Tut) or tubuphenylalanine (Tup)). An acetylated alcohol and an *N,O*-acetal in tubuvaline are found in most members of this natural product family. Tubulysins U–X (**3–6**) and Z (**7**) are the only natural tubulysins in which one or both of these functional groups are not present. Although these are relatively simple molecules to envision a total synthesis of, there are a number of challenging synthetic issues as summarized in a recent review:³ (1) the installation of the extremely sensitive *N,O*-acetal, (2) the synthesis of the complex thiazole-containing

Chart 1



Tuv, and (3) the assembly of the tetrapeptide, as a consequence of the sterically congested Tuv region.

The total synthesis of tubulysins D,⁴ U, and V⁵ and numerous analogues⁶ has allowed for determination of structure–activity relationship (SAR) studies for these natural products. In a report by Wipf and co-workers, it was established that the Mep residue at the *N*-terminus of the tubulysins could be replaced with the acyclic amino acid *N*-methylsarcosine.^{6b} This observation was corroborated by Ellman and co-workers who reported that incorporation of *N*-methylsarcosine in the place of Mep provided a tubulysin analogue with equal antiproliferative activity to tubulysin D.^{6c} The latter group extended the SAR of this family of peptides by stating that a basic tertiary amine was required at the Mep residue for activity, a finding that was confirmed by our research group.^{6d,e}

We have recently reported the synthesis and initial biological activity of a series of simplified tubulysin analogues lacking the *N,O*-acetal and having the acetate group of Tuv replaced with a ketone.^{6d,e} Systematic modifications of the Mep residue (*N*-desmethyl, stereochemistry, and ring size) and Tup residue (desmethyl and dimethyl substituents in place of the stereogenic α -methyl group) have enabled us to establish the minimal structural requirements for cytotoxicity. Our simplified analogues having a ketone in the Tuv residue are perfect precursors to tubulysins U and V (**3** and **4**). Usually, this latter series of tubulysins is less active, but tubulysins U and V were reported to have bioactivity similar to paclitaxel and the epothilones.^{1b} Herein, we report the total synthesis and biological activity of tubulysins U and V and a series of simplified analogues in which the Mep residue has been replaced with *N,N*-dimethyl-*D*-alanine. These data extend our SAR studies of these important natural products.

Before attempting the reduction of a full-length tetrapeptide precursor to tubulysin V, we performed a nonstereoselective NaBH₄ reduction on dipeptide **8**^{6d} (Scheme 1). This reaction was used as a model to assess the selectivity of reduction and to establish the stereochemistry of the two alcohol products. A mixture of alcohols **9** and **10** were obtained with 1:3 selectivity and were readily separable by chromatography. The stereochemistry of the major product **10** was determined by modified Mosher analysis⁷ of the ¹H NMR spectrum of MTPA esters **11** and **12**, formed by reaction of alcohol **10** with (*R*)- and (*S*)-MTPA-Cl, respectively.

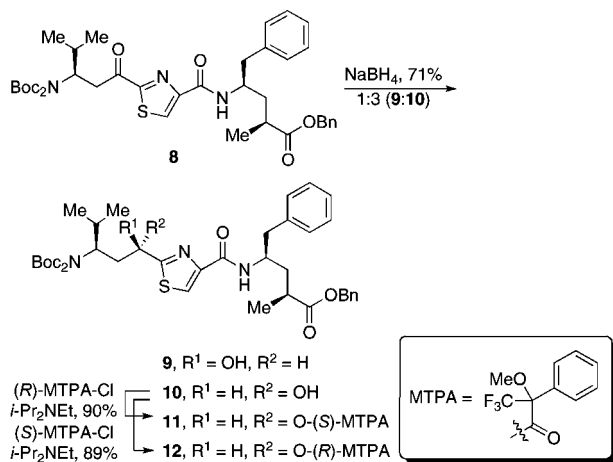
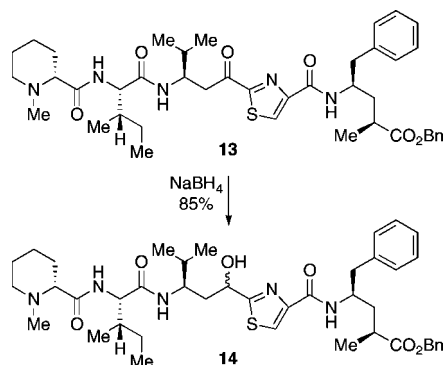
We next sought to examine the reduction of tetrapeptide **13** to give a protected precursor of tubulysin V. Reduction of tetrapeptide **13** with NaBH₄ also afforded a mixture of the two diastereomeric

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^a Abbreviations: Ala, alanine; CBS, Corey–Bakshi–Shibata; Ile, isoleucine; Mep, *N*-methylpipecolic acid; MTPA, α -methoxy- α -trifluoromethylphenylacetic acid; NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; Pip, pipecolic acid; SAR, structure–activity relationship; Tup, tubuphenylalanine; Tut, tubutyrosine; Tuv, tubuvaline.

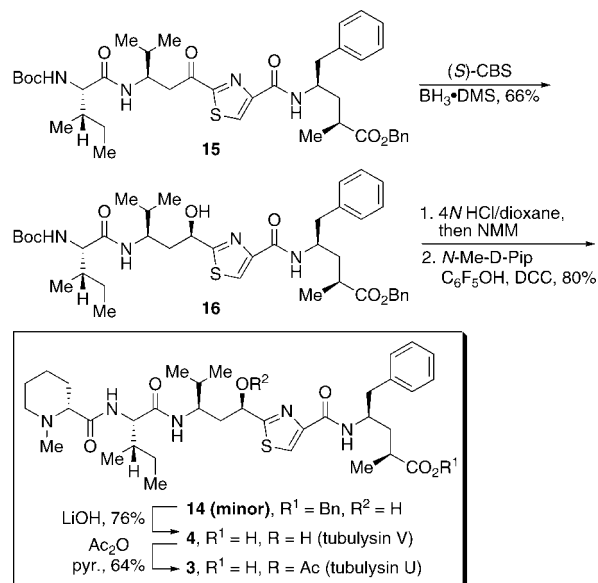
Scheme 1. Nonselective Reduction of Dipeptide **8**Scheme 2. Nonstereoselective Reduction of Tetrapeptide **13**

alcohol products **14** with little selectivity (2.4:1, Scheme 2). Each diastereomer was isolated and individually characterized. The stereochemistry of each diastereomer was later determined by spectral comparison to the immediate precursor to tubulyisin V. CBS reduction⁸ of tetrapeptide **13** gave intractable mixtures, so we then attempted the reduction of a tripeptide intermediate.

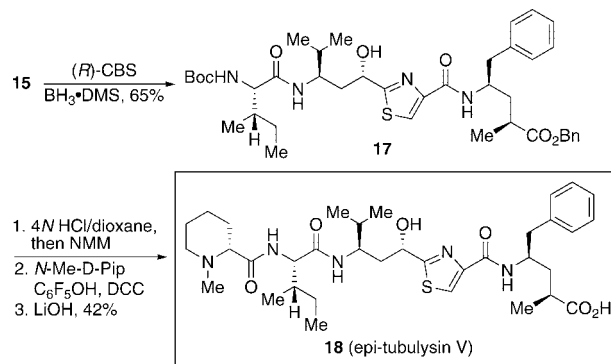
Tripeptide intermediate **15** was found to be optimal for achieving stereoselective reduction of the Tuv ketone. Reduction of tripeptide **15** with NaBH₄ and the Luche reagent⁹ failed to produce any significant diastereoselectivity. We were gratified to find that treatment of tripeptide **15** with the *(S)*-CBS or *(R)*-CBS reagent in the presence of BH₃·DMS almost exclusively afforded the reduced tripeptides **16** and **17**, respectively (Schemes 3 and 4). The stereochemical outcome of these reactions was supported by the following: (1) the ¹H NMR spectrum of **4** matched that of tubulyisin V as previously reported^{5a} and (2) the findings of Zanda and co-workers who reported that the *(S)*-CBS reagent with BH₃·DMS reacted nearly exclusively from the *si* face of the ketone of a racemic Tuv intermediate to give the alcohol products possessing the natural configuration as established by X-ray crystallography.^{5a} The ¹H NMR spectrum of the immediate tetrapeptide benzyl ester precursor of tubulyisin V was identical to the minor diastereomer **14**, enabling us to assign the stereochemistry of the purified diastereomers **14**.

The total synthesis of tubulyisin V (**4**) was completed by amino group deprotection of reduced tripeptide **16**, followed by coupling with *N*-Me-D-Pip and saponification of the benzyl ester (Scheme 3). Interestingly, purification of tubulyisin V by reverse-phase HPLC under the conditions reported by Dömling and co-workers^{5b} using MeOH as the mobile phase resulted in

Scheme 3. Total Synthesis of Tubulyisins U and V



Scheme 4. Synthesis of Epi-Tubulyisin V



isolation of the methyl ester to a significant extent (38%). Using acetonitrile as the mobile phase smoothly afforded the natural product in an analytically pure form. Acetylation of the secondary hydroxyl group of Tuv contained in tubulyisin V (**4**) under standard conditions afforded a second natural product, tubulyisin U (**3**, Scheme 3). Epi-tubulyisin V (**18**) was synthesized and isolated in an identical fashion starting from reduced tripeptide **17** (Scheme 4).

A small series of simplified analogues in which the *N*-terminal residue of the tubulyisins (Mep) was replaced with the acyclic *N,N*-dimethyl-D-alanine was synthesized to extend our previous findings and those of Wipf and Ellman. Synthesis of these analogues follows our published route for the synthesis of other series of simplified analogues.^{6d,e} Tripeptides **15**, **19**, and **20** were deprotected, coupled with *N,N*-dimethyl-D-Ala, and saponified to afford tetrapeptides **21–23** (Scheme 5).

Tubulyisins U and V and analogues **18** and **21–23** were evaluated for antiproliferative activity in IA9 ovarian cancer cells and MCF-7 breast cancer cells and for *in vitro* inhibition of tubulin polymerization (Table 1).^{6d,e,8} The hemisterlin analogue HTI-286 (SPA110) was used as a positive control in all assays.

Tubulyisin V is about 30- to 45-fold more active than epi-tubulyisin V in the antiproliferative assays, suggesting that the absolute configuration of the secondary alcohol in tubulyisin V is important for activity. Acetylation of this alcohol to produce tubulyisin U caused a dramatic improvement in potency by 3 orders of magnitude, possibly because of its increased lipophilicity and

Scheme 5. Synthesis of Simplified Tubulysin Analogues 21–23

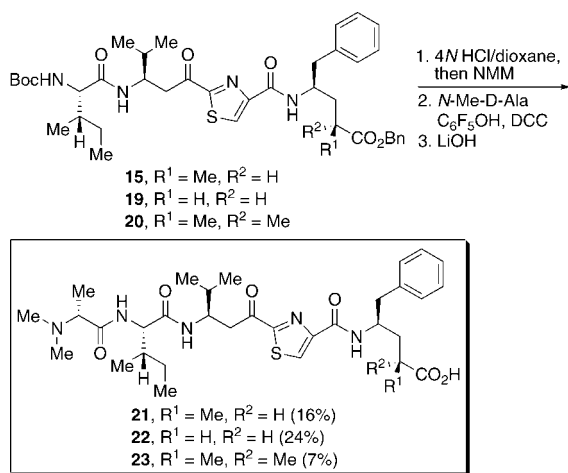


Table 1. Biological Activity of Tubulysins U and V and Analogues

compd ^a	inhibition of proliferation (IC ₅₀ , μM) ^b		tubulin inhibition (IC ₅₀ , μM) ^c
	1A9	MCF-7	
3 (tubulysin U)	0.00065	0.0004	1.9
4 (tubulysin V)	0.12	0.24	1.1
18 (epi-tubulysin V)	5.1	8.1	1.3
21 (FT-040)Tc	> 50	ND ^d	3.6
22 (FT-039)	0.3	0.17	12.5
23 (FT-038)	14.2	12.2	8.5
HTI-286 (SPA110)	0.0002	0.00015	0.7

^a Compounds were isolated and tested as their HCl salts. ^b Values are the mean of two independent IC₅₀ determinations with a maximum drug concentration of 50 mM in DMSO. ^c In vitro inhibition of tubulin polymerization. ^d ND: not determined.

potential to cross cell membranes more effectively. The simplified analogues 21–23, which contain an acyclic Mep variant, vary widely in their antiproliferative activity. Compound 22 has sub-micromolar activity similar to that of tubulysin V, whereas compound 21 was not active at the highest concentration tested (50 μM). Analogue 23 has intermediate activity that is less than that of epi-tubulysin V. The antiproliferative assay results for analogues 21–23 differ from the trends observed for other series, where the incorporation of dimethyltubuphenylalanine increased activity.^{6d,e}

Tubulysins U and V and epi-tubulysin V approach the potency of HTI-286 in the in vitro tubulin polymerization inhibition assay. Interestingly, among all compounds evaluated in Table 1, inhibition of tubulin polymerization does not correlate with antiproliferative activity. The reasons for this are not clear at this time and are the subject of ongoing investigations.

We report here a stereoselective total synthesis of tubulysins U and V, epi-tubulysin V, and a series of simplified analogues. Biological evaluation of the natural products established the importance of the acetate and hydroxyl groups in the Tuv residue. The extremely potent antiproliferative activity of tubulysin U indicates that a tertiary amide between the Ile and Tuv residues, exemplified by the *N,O*-acetal found in tubulysins A and D, is not required. Data obtained from the simplified analogues 21–23 suggest that a cyclic constraint at the N-terminal Mep residue is not a requirement for activity. The results presented here suggest ways to improve the antiproliferative activity of simplified tubulysin analogues. These and other modifications to the tubulysin scaffold and further biological evaluation of select analogues will be reported in due course.

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Supporting Information Available: Experimental procedures, spectroscopic data, and NMR spectra for all new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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