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

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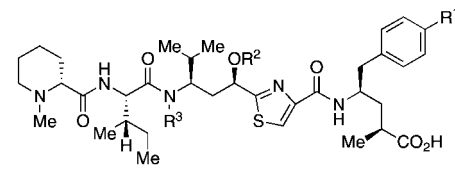
Abstract: A stereoselective total synthesis of the cytotoxic natural products tubulysin U, tubulysin V, and its unnatural epimer epi-tubulysin V, is reported. Simplified analogues containing *N*,*N*-dimethyl-D-alanine as a replacement for the N-terminal *N*-Me-pipecolinic acid residue of the tubulysins 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 (IC_{50} = 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*-methylpipecolinic 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 Tuv, and (3) the assembly of the tetrapeptide, as a consequence of the sterically congested Tuv region.

Chart 1



Tubulysin A (**1**), R^1 = OH, R^2 = Ac, R^3 = $CH_2OC(O)CH_2CHMe_2$
Tubulysin D (**2**), R^1 = H, R^2 = Ac, R^3 = $CH_2OC(O)CH_2CHMe_2$
Tubulysin U (**3**), R^1 = H, R^2 = Ac, R^3 = H
Tubulysin V (**4**), R^1 = H, R^2 = H, R^3 = H
Tubulysin W (**5**), R^1 = OH, R^2 = H, R^3 = $CH_2OC(O)CH_2CH_2Me$
Tubulysin X (**6**), R^1 = OH, R^2 = Ac, R^3 = H
Tubulysin Z (**7**), R^1 = OH, R^2 = H, R^3 = H

The total synthesis of tubulysins D,[4] U, and V[5] and numerous analogues[6] 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 $NaBH_4$ 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 analysis[7] 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 $NaBH_4$ also afforded a mixture of the two diastereomeric

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

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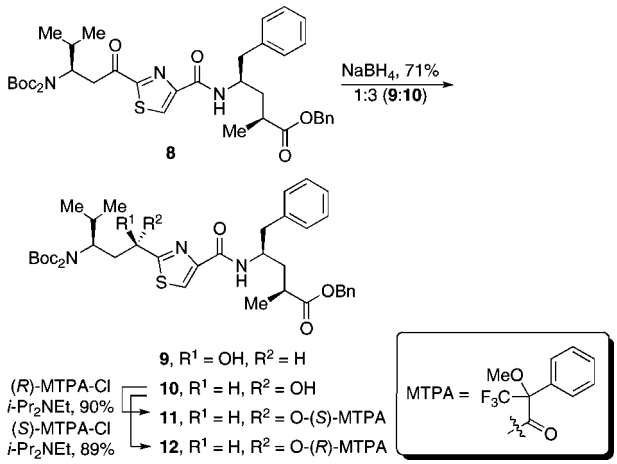
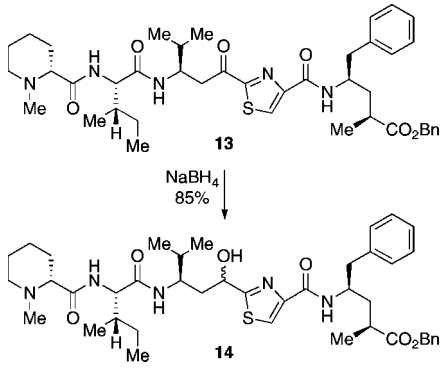
Scheme 1. Nonselective Reduction of Dipeptide **8**



Scheme 2. Nonstereoselective Reduction of Tetrapeptide **13**

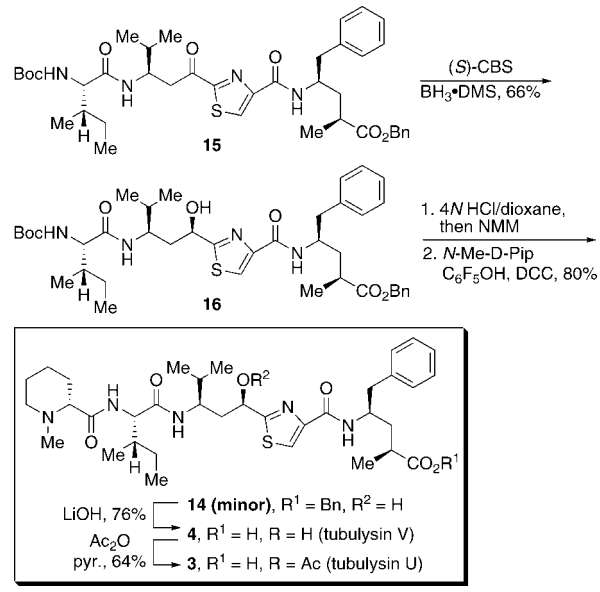


alcohol products **14** with little selectivity (2.4:1, Scheme 2). Each diastereomer was isolated and individually characterized. The stereochemistry of each diastereomer was later determined by spectral comparison to the immediate precursor to tubulysin V. CBS reduction[8] of tetrapeptide **13** gave intractable mixtures, so we then attempted the reduction of a tripeptide intermediate.

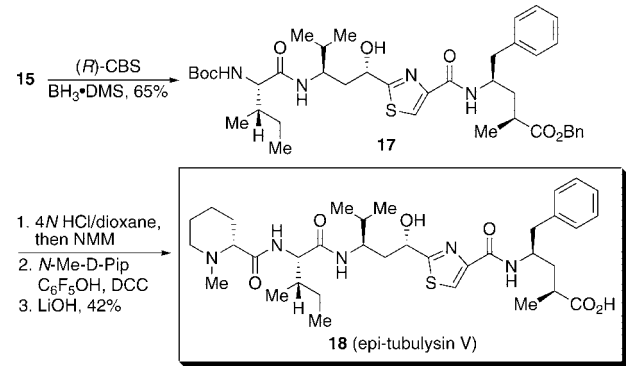
Tripeptide intermediate **15** was found to be optimal for achieving stereoselective reduction of the Tuv ketone. Reduction of tripeptide **15** with $NaBH_4$ and the Luche reagent[9] failed to produce any significant diastereoselectivity. We were gratified to find that treatment of tripeptide **15** with the (*S*)-CBS or (*R*)-CBS reagent in the presence of $BH_3 \cdot DMS$ 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 tubulysin V as previously reported[5a] and (2) the findings of Zanda and co-workers who reported that the (*S*)-CBS reagent with $BH_3 \cdot DMS$ 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 tubulysin V was identical to the minor diastereomer **14**, enabling us to assign the stereochemistry of the purified diastereomers **14**.

The total synthesis of tubulysin 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 tubulysin 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 Tubulysins U and V



Scheme 4. Synthesis of Epi-Tubulysin 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 tubulysin V (**4**) under standard conditions afforded a second natural product, tubulysin U (**3**, Scheme 3). Epi-tubulysin 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 tubulysins (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).

Tubulysins U and V and analogues **18** and **21**−**23** were evaluated for antiproliferative activity in 1A9 ovarian cancer cells and MCF-7 breast cancer cells and for in vitro inhibition of tubulin polymerization (Table 1).[6d,e,8] The hemiasterlin analogue HTI-286 (SPA110) was used as a positive control in all assays.

Tubulysin V is about 30- to 45-fold more active than epi-tubulysin V in the antiproliferative assays, suggesting that the absolute configuration of the secondary alcohol in tubuvaline is important for activity. Acetylation of this alcohol to produce tubulysin 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**

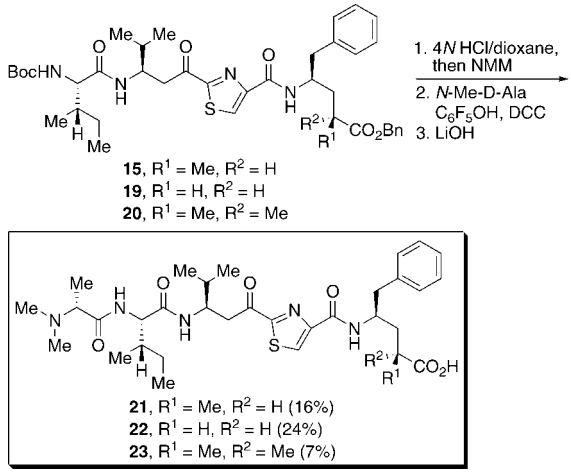


Table 1. Biological Activity of Tubulysins U and V and Analogues

compd[a]	inhibition of proliferation (IC_{50}, μM)[b] 1A9	MCF-7	tubulin inhibition (IC_{50}, μM)[c]
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 IC_{50} 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 subnanomolar 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.

References

(1) (a) Sasse, F.; Steinmetz, H.; Heil, J.; Höfle, G.; Reichenbach, H. Tubulysins, new cytostatic peptides from myxobacteria acting on microtubuli. *J. Antibiot.* **2000**, *53*, 879–885. (b) Steinmetz, H.; Glaser, N.; Herdtweck, E.; Sasse, F.; Reichenbach, H.; Höfle, G. Isolation, crystal and solution structure determination, and biosynthesis of tubulysins—powerful inhibitors of tubulin polymerization from myxobacteria. *Angew. Chem., Int. Ed.* **2004**, *43*, 4888–4892. (c) Khalil, M. W.; Sasse, F.; Lünsdorf, H.; Elnakady, Y. A.; Reichenbach, H. Mechanism of action of tubulysin, an antimitotic peptide from myxobacteria. *ChemBioChem* **2006**, *7*, 678–683. (d) Kaur, G.; Hollingshead, M.; Holbeck, S.; Schauer-Vukasinovic, V.; Camalier, R. F.; Dömling, A.; Agarwal, S. Biological evaluation of tubulysin A: a potential anticancer and antiangiogenic natural product. *Biochem. J.* **2006**, *396*, 235–242.

(2) Sandmann, A.; Sasse, F.; Müller, R. Identification and analysis of the core biosynthetic machinery of tubulysin, a potent cytotoxin with potential anticancer activity. *Chem. Biol.* **2004**, *11*, 1071–1079.

(3) Neri, D.; Fossati, G.; Zanda, M. Efforts toward the total synthesis of tubulysins: new hopes for a more effective targeted drug delivery to tumors. *ChemMedChem* **2006**, *1*, 175–180.

(4) Peltier, H. M.; McMahon, J. P.; Patterson, A. W.; Ellman, J. A. The total synthesis of tubulysin D. *J. Am. Chem. Soc.* **2006**, *128*, 16018–16019.

(5) (a) Sani, M.; Fossati, G.; Huguenot, F.; Zanda, M. Total synthesis of tubulysins U and V. *Angew. Chem., Int. Ed.* **2007**, *46*, 3526–3529. (b) Dömling, A.; Beck, B.; Eichelberger, U.; Sakamuri, S.; Menon, S.; Chen, Q.-Z.; Lu, Y.; Wessjohann, L. A. Total synthesis of tubulysin U and V. *Angew. Chem., Int. Ed.* **2006**, *45*, 7235–7239. (c) Dömling, A.; Beck, B.; Eichelberger, U.; Sakamuri, S.; Menon, S.; Chen, Q.-Z.; Lu, Y.; Wessjohann, L. A. Total synthesis of tubulysin U and V. *Angew. Chem., Int. Ed.* **2007**, *46*, 2347–2348 (Corrigendum).

(6) (a) Wipf, P.; Wang, Z. Total synthesis of N^{14}-desacetoxytubulysin H. *Org. Lett.* **2007**, *9*, 1605–1607. (b) Wang, Z.; McPherson, P. A.; Raccor, B. S.; Balachandran, R.; Zhu, G.; Day, B. W.; Vogt, A.; Wipf, P. Structure−activity and high-content imaging analyses of novel tubulysins. *Chem. Biol. Drug Des.* **2007**, *70*, 75–86. (c) Patterson, A. W.; Peltier, H. M.; Sasse, F.; Ellman, J. A. Design, synthesis, and biological properties of highly potent tubulysin D analogues. *Chem.—Eur. J.* **2007**, *13*, 9534–9541. (d) Raghavan, B.; Balasubramanian, R.; Steele, J. C.; Sackett, D. L.; Fecik, R. A. Cytotoxic simplified tubulysin analogues. *J. Med. Chem.* **2008**, *51*, 1530–1533. (e) Balasubramanian, R.; Raghavan, B.; Steele, J. C.; Sackett, D. L.; Fecik, R. A. Tubulysin analogs incorporating desmethyl and dimethyl tubuphenylalanine derivatives. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2996–2999. (f) Friestad, G. K.; Marié, J.-C.; Deveau, A. M. Stereoselective Mn-mediated coupling of functionalized iodides and hydrazones: a synthetic entry to the tubulysin γ-amino acids. *Org. Lett.* **2004**, *6*, 3249–3252.

(7) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. High-field FT NMR application of Mosher's method. The absolute configurations of marine terpenoids. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.

(8) Corey, E. J.; Helal, C. J. Reduction of carbonyl compounds with chiral oxazaborolidine catalysts: a new paradigm for enantioselective catalysis and a powerful synthetic method. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 1986–2012.

(9) Gemal, A.; Luche, J. L. Lanthanoids in organic synthesis. 6. Reduction of α-enones by sodium borohydride in the presence of lanthanoid chlorides: synthetic and mechanistic aspects. *J. Am. Chem. Soc.* **1981**, *103*, 5454–5459.

(10) Hamel, E. Evaluation of antimitotic agents by quantitative comparisons of their effects on the polymerization of purified tubulin. *Cell Biochem. Biophys.* **2003**, *38*, 1–21.

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