Letters

Cytotoxic Simplified Tubulysin Analogues

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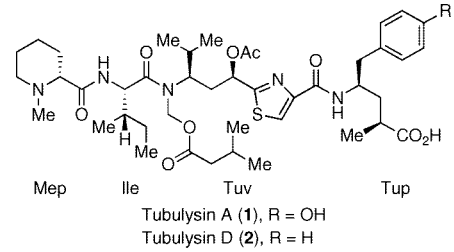
Abstract: An efficient route for the synthesis of the tubulysin family of antimitotic peptides was developed. Simplified tubulysin analogues were synthesized to define the minimum pharmacophore required for cytotoxicity. Simplified tubulysin analogues retain significant cytotoxicity and reveal important preliminary structure–activity relationships.

Tubulysins A and D (**1** and **2**, Chart 1) are representative members of a family of antimitotic peptides isolated from the myxobacteria *Archangium gephyra* and *Angiococcus disciformis* by Höfle and co-workers.[1,2] Tubulysin D retains potent anticancer activity against the P-glycoprotein-expressing human KB-V1 (multidrug-resistant cervix carcinoma) cell line ($IC_{50} = 0.31$ nM),[2] and tubulysin A is active against the HCT-15 (multidrug-resistant colon carcinoma) cell line ($GI_{50} = 0.12$ nM).[3] Preclinical studies with tubulysin A have also shown it to have promising antiangiogenic effects.[3] Tubulysin A noncompetitively inhibits vinblastine binding to tubulin,[4] suggesting that the tubulysins bind to the peptide binding site located near the *Vinca* alkaloid binding site of β-tubulin. The extraordinary anticancer activity of the tubulysins against a validated target makes them exciting leads for the development of novel drugs for multidrug-resistant cancers.

Clinical development of the tubulysins has been hampered by the extremely limited supply available from natural sources. Fermentation of *Angiococcus disciformis* An d48 yields 0.25–1 mg/L tubulysin D,[1,2] and fermentation of *Archangium gephyra* Ar315 yields 3 mg/L tubulysin A.[2] We sought to develop efficient methods for the total synthesis of tubulysins that would provide convenient access to synthetic analogues of potential therapeutic interest and that would enable the study of their interaction with tubulin. Various methods have been disclosed for the total synthesis of tubulysin natural products,[5–8] tubulysin analogues,[9–11] and key intermediates.[12] We report here an efficient total synthesis of a series of simplified tubulysin analogues that were designed to establish the minimum structural requirements for cytotoxicity. We hypothesized that the labile acetyl and *N,O*-acetal functionalities of tubuvaline (Tuv) were unnecessary for activity, and we also sought to examine the effects of changes to the *N*-methylpipecolinic acid (Mep) fragment.

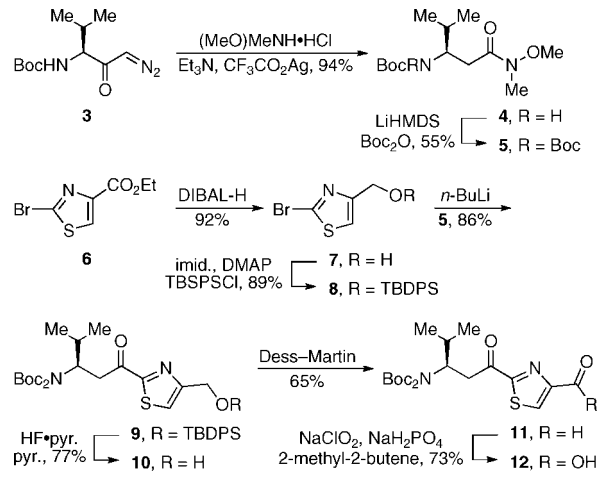
Our synthetic route to tubuvaline, the central thiazole-containing amino acid of the tubulysins, is depicted in Scheme

Chart 1[a]



[a] Mep, *N*-methylpipecolinic acid; Ile, isoleucine; Tuv, tubuvaline; Tup, tubuphenylalanine.

Scheme 1. Synthesis of Tubuvaline Fragment **12**



1. Wolff rearrangement of diazomethylketone **3**[13] under the conditions of Limal and co-workers directly afforded Weinreb amide **4**.[14] This was further protected as its bis-carbamate derivative, Weinreb amide **5**, for use as a potential acylating agent. Our initial attempts at treating bromothiazole **6**[15] with organometallic reagents (including *n*-BuLi and *i*-PrMgCl) at low temperature (−78 °C) followed by addition of Weinreb amide **5** led to complex mixtures resulting from addition of the organometallic reagent to the ester of thiazole **6**. Bromothiazole **6** was therefore reduced to alcohol **7**[9] and protected as silyl ether **8**. We were gratified to find that treatment of silyl ether **8** with *n*-BuLi and Weinreb amide **5** smoothly afforded thiazolyl ketone **9**. Deprotection to alcohol **10**, followed by a two-step oxidation to aldehyde **11** and carboxylic acid **12** completed the synthesis of a simplified tubuvaline fragment.

For the synthesis of tubuphenylalanine (Tup), we envisioned that lactam **13**[16] could be diastereoselectively alkylated. Lactam **13**[17,18] and related lactams[19] have previously been alkylated with alkyl and benzyl bromides to give the trans products with modest diastereoselectivity (∼4:1, trans/cis). Our initial attempts to mimic these conditions with LDA and MeI gave modest yields of monoalkylated lactam **14** with low diastereoselectivity (∼2:1, trans/cis). Optimization of this alkylation using LiHMDS as the base significantly improved the diastereoselectivity. Lactam **13** was treated successively with LiHMDS and MeI at −78 °C, and the temperature and length of the reaction were varied (Table 1). The major side product from these reactions was

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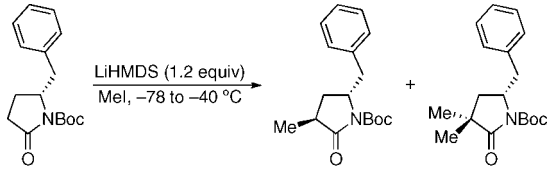
Table 1



entry	MeI (equiv)	time (h)	**14** (%, dr)	**15** (%)
1[a]	5	2	45, 2:1	ND[b]
2	3	2	36, 2.3:1	26
3[c]	2	6, 10	35, 7:1	27
4	1.1	15	24, 12:1	30
5[d]	1.1	6	14, 3:1	27
6	5	4	29, 3:1	27
7[e]	2	1, 2	30, 2:1	28
8[c,f]	1.2	1, 2	52, 10:1	12

[a] −78 to −20 °C. [b] ND: not determined. [c] −78 to −40 °C, warm to 23 °C. [d] 0.95 equiv of LiHMDS. [e] −78 to −40 °C, warm to −20 °C. [f] 1.2 equiv of NaHMDS (2.0*M* in THF).

dialkylated lactam **15**, whose yield was not affected by changes in the reaction temperature, time, or equivalents of LiHMDS and MeI. A possible explanation for the significant yield of dialkylated lactam **15** is that the intermediate enolate deprotonates the newly formed monoalkylated lactam **14** in competition with alkylation by MeI. Under the optimal conditions for yield and diastereoselectivity found here (entry 8) with NaHMDS, the desired monoalkylated lactam **14** was obtained in 52% yield and 10:1 dr, along with a 12% yield of dialkylated lactam **15**. The diastereoselectivity of the alkylations was determined by analysis of the ^{1}H NMR spectra of monoalkylated lactam **14**. These results compare favorably to the method of Wipf and co-workers for installation of this stereogenic center by reduction of an alkene with 3:1 diastereoselectivity.[12]

Monoalkylated lactam **14** (as a 12:1 diastereomeric mixture) was saponified to open the lactam and afford carboxylic acid **16**, which was recrystallized to a single diastereomer (Scheme 2). The reduction of carboxylic acid **16** to alcohol **17** and comparison of its spectral data to literature values[12] confirmed the stereochemistry of monoalkylated lactam **14** and carboxylic acid **16**. Alkylation of carboxylic acid **16** to give benzyl ester **18** followed by deprotection and coupling with the protected carboxylic acid **12** yielded dipeptide **19**. Removal of the *N*-Boc groups and coupling with *N*-Boc-L-isoleucine gave the key tripeptide **20**. Alternative coupling conditions were explored but these afforded tripeptide **20** in reduced yields.

To examine the effects of changes to the ring size, stereochemistry, and methyl group of the *N*-methylpipecolinic acid fragment on biological activity, tripeptide **20** was N-deprotected and coupled to amino acids **21a–h** (Scheme 2). Amino acids **21a–h** that were not commercially available were synthesized by published methods.[5,20] Under the coupling conditions required for *N*-methylamino acids **21a–d**, the resulting tetrapeptides were contaminated with small amounts of dicyclohexylurea (DCU). The partially purified products were subsequently treated with LiOH to afford tetrapeptides **22a–d**. *N*-Boc amino acids **21e–h** were coupled with tripeptide **20** under different conditions to give intermediate tetrapeptides **22e–h**. Cleavage of the esters and *N*-Boc groups afforded tetrapeptides **23a–d**. This route provided a series of simplified tubulysin analogues that lack the *N,O*-acetal and have a ketone in place of the acetate found in the natural products.

The initial cytotoxicity of tubulysin analogues **22a–d** and **23a–d** as IC_{50} values was measured against 1A9 ovarian cancer cells (Table 2). Two tubulin polymerization inhibitors were used as positive controls: the hemiasterlin analogue SPA110/HTI-286 and podophyllotoxin. Several important structure–activity relationships for the *N*-methylpipecolinic acid and tubuvaline positions were revealed.

Scheme 2. Synthesis of Tubulysin Analogues

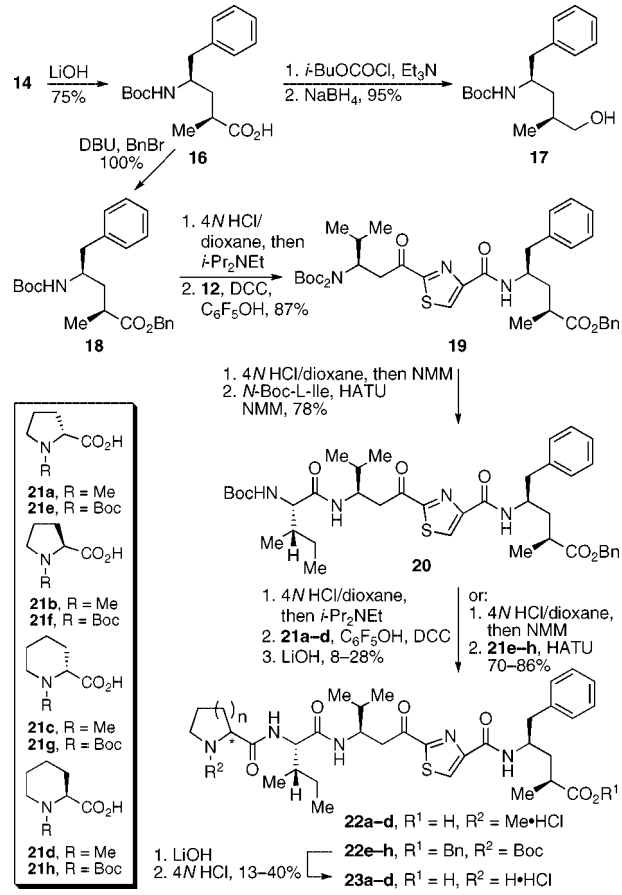
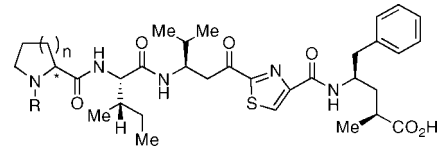


Table 2. Cytotoxicity of Tubulysin Analogs Against 1A9 Ovarian Cancer Cells.[a]



compd	R	*n*	abs config. (*)	1A9 IC_{50} (μM)
22a (FT-023)	Me•HCl	1	*R*	0.8
22b (FT-025)	Me•HCl	1	*S*	18
22c (FT-022)	Me•HCl	2	*R*	0.2
22d (FT-024)	Me•HCl	2	*S*	25
23a (FT-018)	H•HCl	1	*R*	>33
23b (FT-020)	H•HCl	1	*S*	>33
23c (FT-019)	H•HCl	2	*R*	23
23d (FT-021)	H•HCl	2	*S*	24
podophyllotoxin				0.003
HTI-286 (SPA110)				0.03 nM

[a] Data are the average of two independent IC_{50} value determinations with a maximum drug concentration of 33 μM in DMSO.

Comparison of **22a–d** to **23a–d** clearly shows a requirement for a *N*-methyl group or tertiary amine at the N-terminus. There is also a stereochemical requirement for the *R*-configuration (D-amino acid configuration found in the natural products) at the *N*-methylpipecolinic acid position. A potency difference of 2 orders of magnitude is observed between analogues that incorporate a D-amino acid at this position (**22a** and **22c**) versus

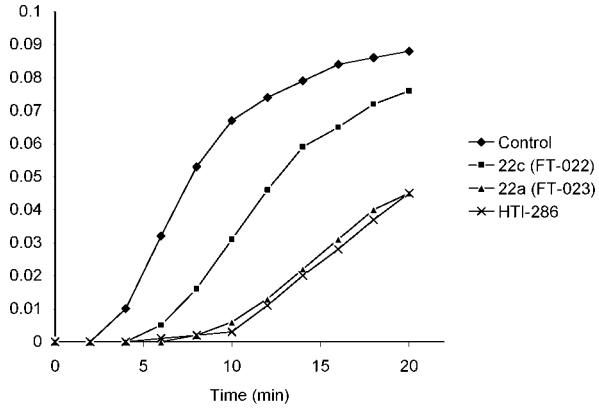


Figure 1. Inhibition of tubulin polymerization by **22a** and **22c**. Rat brain tubulin (10 μM) was mixed with various concentrations (0.5, 1, 2, and 4 μM) of test compounds on ice in 0.8 M sodium glutamate, pH 6.6, and 0.4 mM GTP. Samples were warmed to 33 °C, and optical density at 350 nm was followed with time in a SpectraMax Plus plate reading spectrophotometer (Molecular Devices, Sunnyvale, CA). Only one concentration is shown for clarity, 1 μM for all compounds: ◆ = control, ▲ = **22a**, ■ = **22c**, × = HTI-286. Combretastatin A4 is not shown.

those with an L-amino acid (**22b** and **22d**). The ring size of *N*-methylpipecolinic acid can be varied, provided the *N*-methyl group and stereochemical requirements are followed. Compounds incorporating *N*-methyl-D-proline (**22a**) and *N*-Me-D-pipecolinic acid (**22c**) are roughly equipotent.

At the tubuvaline position, significant modifications are tolerated. The cytotoxicity of **22a** and **22c** shows that a tertiary amide is not required, and the acetoxy or hydroxy groups present in the natural products can be replaced with a ketone. The latter change is significant, since it eliminates the need for a stereogenic center that is challenging to install.

The most active compounds in this series, **22a** (FT-023) and **22c** (FT-022), have potent cytotoxicity against 1A9 ovarian cancer cells; however, they are notably less cytotoxic than HTI-286, a hemiasterlin analogue presently in phase I clinical trials.

To confirm their mechanism of action, analogues **22a** and **22c** were also tested for the ability to inhibit tubulin polymerization in vitro (Figure 1).[21] Under these conditions, the IC_{50} obtained was approximately 1.0 μM for **22a**, 1.8 μM for **22c**, 1.0 μM for HTI-286, and 1.1 μM for combretastatin A4. These results establish inhibition of tubulin polymerization as the mechanism of tubulysin analogues **22a** and **22c**.

Interestingly, the relatively equivalent IC_{50} values of **22a** and **22c** to HTI-286 in the tubulin polymerization assay do not correlate to their cytotoxicity. The reasons for this discrepancy are not known at this time. One potential explanation is that **22a** and **22c** have decreased permeability across the cell membrane.

These results confirm and extend the preliminary structure–activity data reported for two small series of tubulysin D[11] and H[10] analogues. The cytotoxicity and tubulin inhibition data for **22a** and **22c** are comparable to those found for two other tubulysin analogues, although different cell lines and assay conditions were used.[10] We show here that additional modifications to tubuvaline are tolerated. These changes, the lack of a tertiary amide, and elimination of a stereogenic center simplify the processes required for the synthesis of tubulysin analogues. Synthesis of **22a** and **22c** was achieved in 12 linear steps from commercially available materials, which compares favorably to the total syntheses of natural tubulysins.[22]

In summary, we report here the synthesis and initial biological evaluation of eight tubulysin analogues that incorporate changes at the *N*-methylpipecolinic acid and tubuvaline positions. The modifications to tubuvaline are notable, since they further simplify its structure, and the *N*-methylpipecolinic acid changes demonstrate the effects of stereochemistry and the *N*-methyl group on activity. Two analogues, **22a** (FT-023) and **22c** (FT-022), retain a potent level of cytotoxicity, and their antitubulin activity is equipotent to HTI-286 and combretastatin A4. Additional modifications to the tubulysin scaffold and further biological properties of analogues **22a** and **22c** will reported in due course.

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Supporting Information Available: Experimental procedures and spectral data for **4**, **5**, **7**–**12**, **14**–**16**, **18**–**20**, **22a**–**h**, and **23a**–**d**; ^{1}H and ^{13}C NMR spectra for **4**, **5**, **8**–**12**, **14**–**16**, **18**–**20**, **22a**–**h**, and **23a**–**d**; and experimental procedures for biological testing. This material is available free of charge via the Internet at http://pubs.acs.org.

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(22) The total synthesis of tubulysin D was completed in 16 linear steps (ref 5). Tubulysins U and V required 15 and 16 linear steps, respectively (ref 8). These natural products were also synthesized in 10 linear steps; however, the penultimate intermediate completely epimerized (ref 6).

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