Journal of Medicinal Chemistry

Freie Universität Berlin

Brief Article

Structure-Cytotoxicity Relationships of Analogs of N14-Desacetoxytubulysin H

Dorin Toader, Fengjiang Wang, Lakshmaiah Gingipalli, Melissa Marie Vasbinder, Mark Roth, Shenlan Mao, Michael H Block, Jay Harper, Sambaiah Thota, Mei Su, Jianquo Ma, Vahe Bedian, and Adeela Kamal

J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.6b01023 • Publication Date (Web): 03 Nov 2016

ACS Publications

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Structure-Cytotoxicity Relationships of Analogs of N^{14}-Desacetoxytubulysin H

Dorin Toader,[a,c]* Fengjiang Wang,[a] Lakshmaiah Gingipalli,[a] Melissa Vasbinder,[a] Mark Roth,[a#] Shenlan Mao,[b] Michael Block,[a] Jay Harper[b], Sambaiah Thota,[a] Mei Su,[a] Jianquo Ma,[a] Vahe Bedian[a] and Adeela Kamal[b]

[a]Oncology iMED, AstraZeneca R&D Boston, 35 Gatehouse Drive, Waltham, Massachusetts 02451, United States [b]Oncology Research, MedImmune LLC, Gaithersburg, MD 20878, United States; [c]Antibody Discovery and Protein Engineering, MedImmune LLC, Gaithersburg, MD 20878, United States

KEYWORDS. Tubulysin, cytotoxicity, structure cytotoxicity relationships, solid phase synthesis.

ABSTRACT: Herein we report structure-cytotoxicity relationships for analogs of N^{14}-Desacetoxytubulyisn H **1**. A novel synthetic approach towards **1** enabled the discovery of compounds with a range of activity. Calculated basicity of the *N*-terminus of tubulysins was shown to be a good predictor of cytotoxicity. The impact of structural modifications at the C-terminus of **1** upon cytotoxicity is also described. These findings will facilitate the development of new tubulysin analogs for the treatment of cancer.

Tubulysins are a family of cytotoxic tetrapeptides of fungal origin that disrupt the microtubule network formation in cells[1]. Tubulysin D was reported to display 10-500 times higher cytotoxicity than paclitaxel and vinblastine[2] while the family of natural tubulysins A-I showed potency in the range of 0.3-8.4 nM against the multidrug-resistant human cell line KB-V1.[3] These cytotoxic compounds are poorly tolerated in vivo even in mice where the maximum tolerated dose of Tubulysin A was 25 μg/kg.[4] The highly toxic nature of naturally occurring tubulysins prevented their use as therapeutic agents. Conversely, evaluation of synthetic tubulysin analogs was slow due to their structural complexity and limited available synthetic approaches. Unlike other microtubule disrupting agents that have been co-crystallized with β-tubulin,[5a,b] the tubulysin field is lacking such a powerful drug-design tool. The only published structural study related to the interaction of tubulysin A with β-tubulin described the bioactive tubulin-bound conformation.[6] The main source of structure activity relationships is based on cytotoxicity data for a limited number of compounds.[7] Structural features of tubulysins (Figure 1) can be described as follows: (i) (R)-*N*-methylpipecolic acid (Mep); (ii) natural amino acid L-Ile; (iii) tubuvaline (Tuv) containing a thiazole, a potentially labile acyloxymethyl[8] and acetate ester groups and (iv) tubuphenylalanine (Tup) (R^2=H) or tubuphenyltyrosine (Tut) (R^2=OH) each displaying two defined chiral centers (Figure 1). Simplified synthetic analogs of natural tubulysins were described where the synthetically challenging acyloxymethyl group was replaced with a methyl group[9a-c] or was removed altogether.[10a-f] Alkyl substitution at the N^{14} nitrogen (following the positional assignment by Wipf[9a,11]) is favorable for potency. During our quest for compounds with potent activity against cancer cell we wanted to explore the effect of structural modifications of N^{14}-desacetoxytubulysin H **1** (Figure 1) upon cytotoxicity. The total synthesis of **1** was reported in independent disclosures by Wipf[9a] and Ellman,[9b] more recently, an elegant synthesis was published by Nicolau.[9d] In an effort to minimize the number of synthetic and purification steps we developed a solid phase synthesis for tubulysins that led to generation a set of cytotoxic compounds with a range of activity by modification of the

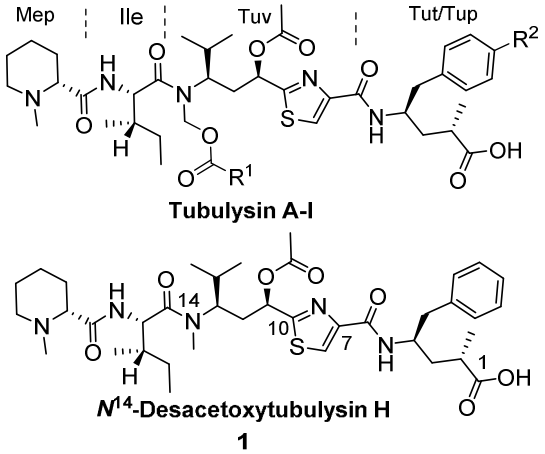
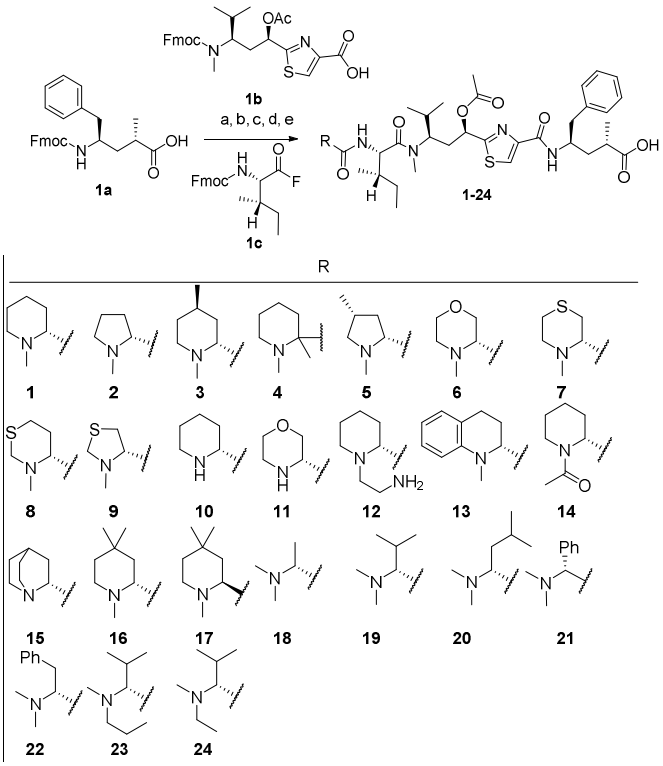


Figure 1. Generic Structure of Naturally Occurring Tubulysin A-I where R^1=alkyl or alkenyl; R^2 = H or OH (above) and structure of N^{14}–Desacetoxytubulysin H **1**. The positional numbering of **1** was made according to Wipf *et al*[9a] (below).

N-terminal amine and C-terminal amino acid. Cytotoxicity was impacted by structural modifications that modulated the basicity of the N-terminal amine. These results could offer a solution to the current challenges associated with making tubulysin analogs and provide guidance for the design of future generation of compounds with optimal therapeutic index.

Scheme 1. Synthetic approach to N-terminus analogs of N^{14}-Desacetoxytubulysin H[a]



a Reagents and conditions: (a) 2-chlorotrityl chloride resin, DIEA, DCM, rt, 4h then 20% piperidine in DMF 6 min; (b) **1b**, HATU, TMP, DMF 2h, rt, then 20% piperidine in DMF 6 min; (c) **1c**, DMAP, DIEA, DCM rt 6h, then 20% piperidine in DMF 6 min; (d) RCO_2H, HATU, TMP, DMF, rt, 2h; (e) TFA/DCM.

RESULTS AND DISCUSSION

Solid phase synthesis on acid labile 2-chlorotritylchloride resin (2-CTC) was implemented by synthesizing two previously unreported intermediates **1a** and **1b** for Fmoc-based peptide synthesis (Scheme 1) starting from the previously reported Boc-protected analogs[9a,d,10e](Supporting Information). The solid phase synthesis approach started with base-catalyzed immobilization of *N*-Fmoc-Tup **1a** on solid support. Deprotection followed by standard peptide coupling of *N*-Fmoc-*N*-desacyloxy-Tuv **1b** led to a solid-supported dipeptide. Deprotection with 20% piperidine in DMF for 6 min to prevent loss of acetyl ester followed by coupling of *N*-Fmoc-isoleucine fluoride **1c** in presence of DMAP and DIEA led to a precursor that was deprotected by brief treatment with 20% piperidine in DMF followed by coupling of the *N*-terminal amino acid. Acid cleavage of tetrapeptide followed by reverse phase HPLC purification afforded the desired tubulysin analogs in moderate overall yields based on loaded **1a** (Scheme 1). The *in vitro* cytotoxicity of synthesized tubulysin analogs was evaluated with an Alamar blue assay at a final concentration range from 0.3 nM to 3.3 μM. The following cancer cell lines were treated for 72h: human prostate cancer cell line DU-145, human colon carcinoma cell line HCT-116 and human breast cancer cell line MDA MB-231. Most compounds were tested at least twice under conditions that gave cytotoxicity variability between runs of less than 2 fold. Representative dose response curves are shown in Supporting Information. We evaluated a series of cyclic amines containing analogs that mimic the naturally occurring amino acid (R)-*N*-Me-pipecolic acid (Mep) **2-17** along with N^{14}-desacetoxytubulysin H **1** synthesized by our approach (Table 1). Compound **1**, made using the approach shown in Scheme 1, displayed potency consistent with the activity observed with a sample from the laboratory of Professor Wipf. Analogs displaying basic nitrogen not contained in a saturated heterocycle **18-24** were then evaluated (Table 1). Finally we explored the effect of modifications of the C-terminus of the molecule and also the effect of removal of the acetyl group from the Tuv residue (Table 2).

Table 1. *N*-terminus nitrogen SAR[a]

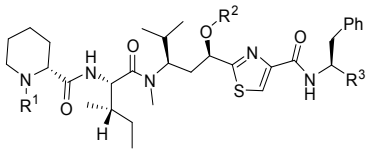
Cpd	GI_{50} (nM)[b] DU-145	HCT-116	MDA MB-231	Cpd	GI_{50} (nM)[b] DU-145	HCT-116	MDA MB-231
1	0.71	0.62	1.14	**13**	>3333	>3333	>3333
2	1[c]	0.6[c]	3.3[c]	**14**	>3333	798.2	>3333
3	0.9[c]	0.5[c]	3.4[c]	**15**	428	214	196
4	1.2	ND[d]	ND[d]	**16**	1.3	1.8	3.9
5	1.7[c]	0.8[c]	4.2[c]	**17**	50.6	88	118.3
6	808	408	998	**18**	4.5[c]	2.5[c]	3.9[c]
7	166	69.3	226	**19**	9.6	4.4	10.7
8	136.1	64.2	125	**20**	17	8.2	19.1
9	1116	352.4	816	**21**	82.3	52.5	111.4
10	46.2	21.3	19.62	**22**	134.4	102.9	224.8
11	2281	1384	>3333	**23**	>3333	>3333	>3333
12	176.3	173.8	243.1	**24**	2939	1644	3017

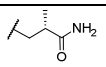
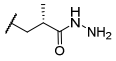
[a]structures of compounds are described in Scheme 1; [b]average of at least 2 distinct experiments; [c]average of one triplicate experiment; [d]ND not determined.

The cytotoxicity of N^{14}-desacetoxytubulysin H **1** was previously evaluated in several cancer cell lines and showed pM potency: L929 IC_{50} = 0.34 nM; SW-480 IC_{50} = 0.02 nM, KB-3-1 IC_{50} = 0.19 nM[9b] and T98 GI_{50} = 1.6 nM.[11] Consistent high potency was observed in our solid tumor cell line panel with compound **1**: GI_{50} = 0.71 nM (DU-145); 0.62 nM (HCT-116) and 1.14 nM (MDA-MB231). When *N*-Methyl-piperidine was replaced with *N*-Methyl-pyrrolidine minimal loss in cytotoxicity was observed as illustrated by compound **2** (Table 1). Interestingly, methyl substitution of piperidine and pyrrolidine as shown by compounds **3-5** was tolerated and led to low nM potency.

Compound **4** is of particular interest since it showed for the first time that a quaternary carbon at the site of the (R)-configuration in Mep leads to highly cytotoxic compounds even as racemate. Inclusion of oxygen or sulfur heteroatom into the heterocycle impaired the cytotoxicity significantly where compounds **6-9** showed weak potency in all cell lines tested. Compounds **10** and **11** that display secondary amine analogs of Mep showed a significant drop-off in potency with respect to the *N*-methyl counterparts. This observation showed that tertiary basic center at the *N*-terminus of the tubulysin peptide is a requirement for high potency. However substitution other than methyl group at the basic center can lead to reduced cytotoxicity as shown by compound **12**. A heteroatom, as is the case with nitrogen in the proximity of the piperidine basic center, led to >170 nM potency. The aniline analog **13** and the amide analog of Mep **14** showed significantly lower activity. The quinuclidine containing analog **15** was mildly cytotoxic (200-400 nM), indicating that the interaction of the Mep with tubulin is sensitive to the spatial orientation of the nitrogen lone pair of electrons. Finally, the importance of the chirality in Mep was illustrated by the 30-50 fold difference in cytotoxicity observed between the two enantiomers of Mep **16** and **17** with the (R) configuration being more potent. Compounds **16** also illustrated that di-substitution of the Mep residue was tolerated for the (R) enantiomer.

Table 2. C-terminus SAR and interdependencies



Cpd	R¹	R²	R³	GI_{50} (nM)[a] DU-145	HCT-116	MDA MB-231
25	CH_3	$COCH_3$		0.948	0.737	1.065
26	CH_3	$COCH_3$		6.63	6.09	5.24
27	H	$COCH_3$		11.52	3.68	9.39
28	CH_3	$COCH_3$		2.7[b]	5.9[b]	9[b]
29	CH_3	H		362.7	110.1	168.6

[a]average of at least 2 distinct experiments; [b]average of one triplicate experiment

Next we investigated analogs of Mep that lack a basic nitrogen in a heterocyclic ring. An *N*-methylsarcosine analog was reported to be much less cytotoxic than **1** in HeLa and T98G glioblastoma cells.[11] Building on the previously reported data we explored the substitution of the alpha-carbon in *N*-methylsarcosine to generate compounds **18-22**. Potent cytotoxicity was observed with *N,N*-dimethyl-alanine (**18**), *N,N*-dimethyl-(D)-valine (**19**) and *N,N*-dimethyl-(D)-leucine (**20**) while larger phenyl (**21**) and benzyl (**22**) substituents both showed less potent activity. Further modifications of the alkyl substituent of the tertiary nitrogen beyond a plain methyl led to complete loss of potency as illustrated by compounds **23-24**.

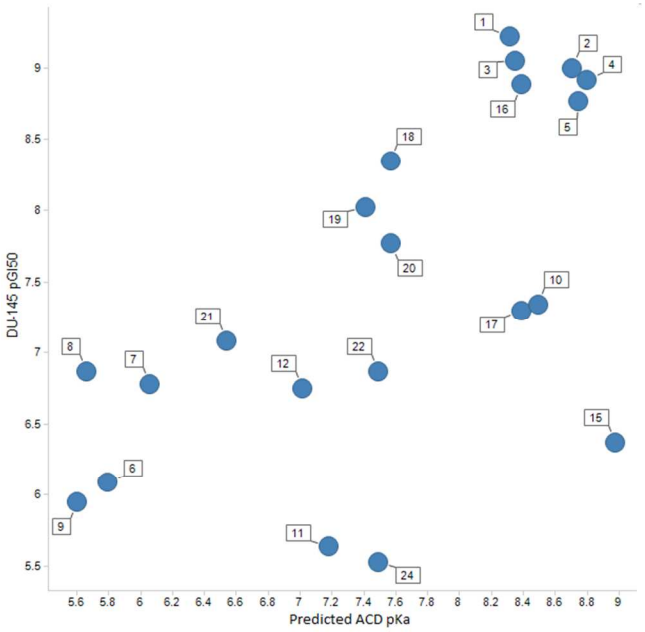


Figure 2. Cytotoxicity against DU 145 prostate cancer cells correlates well with the predicted basicity of the N-terminus nitrogen. This correlation applies to both cyclic and acyclic amines. The pKa prediction was made using ACD pKa prediction model. Each point on the plot is labeled with the corresponding compound number in Table 1.

Structural variants at the carbon end of the N^{14}-desacetoxytubulysin H peptide have been previously reported.[9d] Herein we showed that amide **25** displayed pM potency (Table 2) while the hydrazide **26** showed 5 fold lower cytotoxicity. To address the possibility that the slight increase in hydrophilicity of **10** resulted in reduced cytotoxicity, compound **27**, the methyl ester analog of **10**, was synthesized and tested. Increased cytotoxicity was observed relative to **10** but the activity was still significantly lower than **1** (Table 2). Surprisingly, removal of the carboxylate at the carbon end of tubulysin led to analog **28** that showed single digit nM activity in all cancer cell lines despite the dibasic nature of the compound. Analog **29**, that showed a large drop-off in cytotoxicity, underscored the importance of the acetate structural feature upon activity of the tubulysin class as a whole.[10a,c]

Several previous reports suggested that the presence of a basic center at the nitrogen terminus of the tubulysin peptide was required for potency.[12] This data set allowed us to further refine the relationships that exist between cytotoxicity and the basicity of the nitrogen end of the peptide (Figure 2). All compounds in Table 1 that showed a defined potency within the assay concentration range were used to generate a plot that interrogated the relationships between the cytotoxicity, as represented by

pGI_{50} against DU-145 cell line, and predicted pK_a of the basic nitrogen. Significant correlation was observed between the calculated pK_a and cytotoxicity for compounds **1**-**9**, **16**, and **18**-**21** (Figure 2). The common structural feature for these compounds was the presence of *N*-Me-heterocycle or *N*-methyl sarcosine moieties that lack bulky groups at the beta-position of the basic nitrogen. The outliers can be rationalized as follows: (i) the (S) regiochemistry for analog **17**; (ii) the secondary amines **10** and **11** follow the same pKa-dependence trend but with a much lower potency (iii) the quinuclidine analog (**15**) and (iv) three outliers compounds **12**, **22** and **24** suggest that in addition to the pKa, subtle steric shielding of the basic nitrogen can be a factor in the observed cytotoxicity. The aminoethyl moiety in compound **12**, the beta-phenyl moiety in compound **22** and the *N*-ethyl-sarcosine in compound **24** appear to impact potency. In summary this data set suggests that while pKa of the Mep nitrogen is a key driver for potency, the size and location of substituents in the vicinity of the basic nitrogen as well as the directionality of the nitrogen lone pair of electrons can impact the observed cytotoxicity. This SAR is consistent for the correlations drawn with HCT-116 and MDA MB-231 cytotoxicity data (Supporting Information).

In conclusion the newly developed synthetic approach to syntheses of N^{14}-desacetoxytubulysin H analogs enabled us to describe structure-cytotoxicity relationships that should facilitate further development of tubulysin analogs with potential applications in the clinic. By showing that solid phase synthesis is a viable and expeditious synthetic approach to tubulysins we lay the ground work for additional exploration of this class of unique natural products. We also demonstrated for the first time the interdependence between predicted basicity of the terminal nitrogen and cytotoxicity. This insight should allow for design of tubulysin analogs with desirable physicochemical properties and optimal cytotoxicity profile thus overcoming the current limitations reported with this family of compounds.

EXPERIMENTAL SECTION

General experimental

All tubulysin analogs evaluated in the cytotoxicity assay showed a ≥95% purity. The detailed methods for purity assessment are described in supporting information material along with all general methods.

Synthesis of (2S,4R)-4-(2-((1R,3R)-1-acetoxy-3-((2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methylpentyl)thiazole-4-carboxamido)-2-methyl-5-phenylpentanoic acid ***1***. To the dry resin-bound tripeptide **1d** (supporting information) (0.1 g, 0.101 mmol) was added a solution of (R)-1-methylpiperidine-2-carboxylic acid (0.360 g, 2 mmol), HATU (0.190 g, 0.50 mmol), 2,4,6-trimethylpyridine (0.100 mL, 0.75 mmol), and pyridine (0.040 mL, 0.50 mmol) in DMF (1.5 mL). The mixture was shaken at room temperature overnight. The resulting resin was filtered, washed with DMF (3×1 mL), MeOH (3×1 mL), and DCM (3×1 mL), dried in vacuo. To the dry resin (0.1 g, 0.13 mmol theoretical loading) was added 50% TFA in DCM (2 mL) at room temperature. The mixture was shaken at room temperature for 1 hour, then was filtered, and the resin was washed with 50% TFA in DCM (2 mL) 3 times. The combined filtrates were concentrated in vacuo, the residue was purified by reverse phase HPLC, acetonitrile/water (contained 0.1% FA), ACN from 5% to 55% in 10 min, the fractions containing the desired product were lyophilized to give **1** (0.015 g, FA salt, 97% pure (Method A), yield 16.2 %) as a white solid. LC-MS (ESI): m/z 728.6 $[M + H]^+$ (Calc. M=727.40). ^{1}H NMR (400 MHz, methanol-d_4) δ ppm 8.51 (d, *J* = 8.03 Hz, 1H), 7.95 - 8.02 (m, 1H), 7.11 - 7.19 (m, 4H), 7.03 - 7.11 (m, 1H), 5.62 (dd, *J* = 10.67, 2.64 Hz, 1H), 4.58 - 4.67 (m, 1H), 4.22 - 4.37 (m, 2H), 3.66 (dd, *J* = 12.30, 2.76 Hz, 1H), 3.32 - 3.42 (m, 1H), 3.02 (s, 3H), 2.92 - 3.00 (m, 1H), 2.77 - 2.85 (m, 2H), 2.65 (s, 3H), 2.45 (dd, *J* = 6.90, 2.64 Hz, 1H), 2.16 - 2.32 (m, 2H), 2.02 - 2.10 (m, 4H), 1.76 - 1.94 (m, 5H), 1.46 -1.73 (m, 4H), 1.03 - 1.14 (m, 4H), 0.93 (d, *J* = 2.01 Hz, 3H), 0.93 (d, *J*=15.31 Hz, 3H), 0.79 - 0.88 (m, 3H), 0.75 (d, *J* = 6.53 Hz, 3H).

Compounds **2**-**11**, **13** and **15**-**24** were made following a procedure analogous to the one described for compound **1** using the appropriate amino acid for the final coupling as described in Supporting information.

(2S,4R)-4-(2-((1R,3R)-1-acetoxy-3-((2S,3S)-2-((R)-1-(2-aminoethyl)piperidine-2-carboxamido)-N,3-dimethylpentanamido)-4-methylpentyl)thiazole-4-carboxamido)-2-methyl-5-phenylpentanoic acid **12**. Sodium triacetoxyborohydride (0.170 g, 0.80 mmol) was added in one portion to the resin precursor to **10** (supporting information) (0.1 g, 0.093 mmol) and tert-butyl 2-oxoethylcarbamate (0.255 g, 1.60 mmol) in DMF (1.5 mL) at 20 °C under nitrogen. The resulting mixture was stirred at room temperature for 2 hours. The resulting resin was filtered, washed with DMF (3×2 mL), MeOH (3×2 mL), DCM (3×2 mL), and was dried in vacuo. To the resin (0.1 g, 0.16 mmol) was added DCM (1 mL) and TFA (1.0 mL) at room temperature. The mixture was shaken at room temperature for 20 min, was filtered, and the resin was washed with DCM/TFA (2×3 mL). Then the solvent was evaporated, and the residue was purified by RP HPLC, ACN/water (contained 0.1% FA), ACN from 5% to 50% in 14 min. The pure fractions were lyophilized to give **12** (0.034 g, FA salt, 99% pure (Method B), yield 45.8 %) as a white solid. LC-MS (ESI): m/z 757.56 $[M + H]^+$ (Calc. M=756.42). ^{1}H NMR (400 MHz, methanol-d_4) δ ppm 8.54 (s, 2H), 8.08 (s, 1H), 7.30 – 7.10 (m, 5H), 5.80 – 5.62 (m, 1H), 4.73 (dd, *J* = 8.0, 2.5 Hz, 1H), 4.59 (s, 2H), 4.34 (m, 2H), 3.20 – 3.07 (m, 4H), 3.06 – 2.97 (m, 1H), 2.94 (d, *J* = 6.7 Hz, 2H), 2.89 – 2.81 (m, 1H), 2.67 – 2.56 (m, 1H), 2.55 – 2.47 (m, 1H), 2.46 – 2.31 (m, 2H), 2.25 (t, *J* = 11.3 Hz, 1H), 2.17 (s, 3H), 2.07 – 1.81 (m, 2H), 1.79 – 1.46 (m, 4H), 1.35 – 1.25 (m, 1H), 1.16 (d, *J* = 7.1 Hz, 3H), 1.04 (d, *J* = 6.6 Hz, 3H), 0.99 (dd, *J* = 6.8, 2.8 Hz, 3H), 0.93 (td, *J* = 7.4, 2.3 Hz, 3H), 0.86 (d, *J* = 6.6 Hz, 3H).

(2S,4R)-4-(2-((1R,3R)-1-acetoxy-3-((2S,3S)-2-((R)-1-acetylpiperidine-2-carboxamido)-N,3-dimethylpentanamido)-4-methylpentyl)thiazole-4-carboxamido)-2-methyl-5-phenylpentanoic acid **14**. To the

resin precursor to **10** (0.1 g, 0.093 mmol) was added a solution of acetyl chloride (0.057 mL, 0.80 mmol) in DMF (1 mL) followed by DMAP (9.77 mg, 0.08 mmol) and pyridine (0.078 mL, 0.96 mmol). The mixture was shaken at room temperature for 5 hours, and the resulting resin was filtered, washed with DMF (3×3 mL), MeOH (3×3 mL), DCM (3×3 mL), and then dried in high vacuum. To the resin (0.1 g, 0.16 mmol) was added DCM (1 mL) and HFIP (0.5 mL) at room temperature. The mixture was shaken at room temperature for 1 hour, was filtered, the resin was washed with MeOH/DCM (2×3 mL). The filtrates were evaporated in vacuo, and the residue was purified by RP HPLC, ACN/water (contained 0.1% TFA), ACN from 5% to 75% in 14 min. The fractions were combined to afford **14** (0.063 g, TFA salt, 96% pure (Method A), yield 77.8 %) as a white solid. LC-MS (ESI): m/z 756.0 $[M + H]^+$ (Calc. M=755.39). ^{1}H NMR (400 MHz, methanol-d4) δ ppm 8.04 - 8.13 (m, 2H), 7.46 - 7.60 (m, 1H), 7.21 - 7.30 (m, 4H), 7.11 - 7.22 (m, 1H), 5.65 - 5.81 (m, 1H), 5.50 (s, 2H), 5.20 (d, *J* = 4.80 Hz, 1H), 4.73 - 4.84 (m, 2H), 4.32 - 4.55 (m, 3H), 4.12 (q, *J* = 7.07 Hz, 2H), 3.81 (d, *J* = 13.39 Hz, 1H), 3.21 (br. s., 1H), 3.08 - 3.16 (m, 2H), 2.84 - 2.99 (m, 2H), 2.49 - 2.70 (m, 1H), 2.21 - 2.48 (m, 2H), 2.12 - 2.20 (m, 2H), 1.96 - 2.09 (m, 3H), 1.77 - 1.96 (m, 2H), 1.34 - 1.76 (m, 7H), 1.26 (t, *J* = 7.07 Hz, 3H), 1.07 - 1.22 (m, 4H), 0.88 - 1.02 (m, 6H), 0.78 - 0.87 (m, 3H).

Synthesis of (1R,3R)-1-(4-(((2R,4S)-5-amino-4-methyl-5-oxo-1-phenylpentan-2-yl)carbamoyl)thiazol-2-yl)-3-((2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methylpentyl acetate **25**. The synthesis was analogous to **1** except the synthesis was done on Rink amide resin (0.5 meq/g). To the resin (0.3 g, 0.15 mmol) was added DCM (2 mL) and TFA (2 mL) at room temperature. The mixture was shaken at room temperature for 1h, and the resin was filtered, washed with MeOH/DCM (3×2 mL). The filtrates were evaporated in *vacuo*, and the residue was purified by RP HPLC ACN/water (0.1% FA), ACN from 5% to 60% in 14 min, the pure fractions were lyophilized to give **25** as a solid (0.065 g, FA salt, 97% pure (Method B), yield 55.7 %). LC-MS (ESI): m/z 727.5 $[M + H]^+$ (Calc. M=726.41); ^{1}H NMR (400 MHz, methanol-d_4) δ ppm 8.09 (s, 1H), 7.23 (s, 2H), 7.22 (s, 3H), 7.19 – 7.13 (m, 1H), 5.72 (ddd, *J* = 13.6, 11.1, 2.8 Hz, 1H), 4.73 (dd, *J* = 7.8, 4.4 Hz, 1H), 4.51 – 4.27 (m, 2H), 3.12 (d, *J* = 3.0 Hz, 3H), 2.90 (dd, *J* = 6.9, 1.7 Hz, 2H), 2.80 – 2.65 (m, 1H), 2.59 – 2.45 (m, 4H), 2.44 – 2.19 (m, 2H), 2.15 (d, *J* = 3.8 Hz, 3H), 2.07 – 1.94 (m, 2H), 1.92 – 1.79 (m, 4H), 1.78 – 1.42 (m, 6H), 1.26 – 1.15 (m, 1H), 1.13 (dd, *J* = 7.0, 1.5 Hz, 3H), 1.02 (ddd, *J* = 14.2, 6.7, 2.0 Hz, 7H), 0.94 (td, *J* = 7.4, 2.1 Hz, 3H), 0.84 (d, *J* = 6.6 Hz, 3H).

(1R,3R)-3-((2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)-1-(4-(((2R,4S)-5-hydrazinyl-4-methyl-5-oxo-1-phenylpentan-2-yl)carbamoyl)thiazol-2-yl)-4-methylpentyl acetate **26**. Synthesis was analogous to **1** except the synthesis was done on 2-CTC that was treated with hydrazine to afford the corresponding hydrazine-resin; white solid (0.061 g, FA salt, 99% pure (Method B), yield 48.4 %). LC-MS (ESI): m/z 742.59 $[M + H]^+$ (Calc. M=741.42); ^{1}H NMR (400 MHz, methanol-d_4) δ ppm 8.52 (s, 1H), 8.09 (d, *J* = 1.2 Hz, 1H), 7.22 (d, *J* = 3.1 Hz, 4H), 7.18 – 7.10 (m, 1H), 5.73 (ddd, *J* = 25.4, 10.7, 3.1 Hz, 1H), 4.73 (dd, *J* = 17.3, 8.1 Hz, 1H), 4.64 – 4.38 (m, 1H), 4.30 – 4.16 (m, 1H), 3.12 (s, 2H), 3.09 (s, 2H), 2.94 – 2.82 (m, 3H), 2.48 – 2.36 (m, 2H), 2.33 (d, *J* = 10.2 Hz, 4H), 2.28 – 2.18 (m, 1H), 2.15 (d, *J* = 3.3 Hz, 3H), 2.05 – 1.95 (m, 1H), 1.92 – 1.76 (m, 4H), 1.76 – 1.52 (m, 6H), 1.46 – 1.28 (m, 1H), 1.25 – 1.14 (m, 1H), 1.10 (dd, *J* = 6.9, 3.0 Hz, 3H), 1.04 (t, *J* = 6.9 Hz, 3H), 0.99 (d, *J* = 6.8 Hz, 2H), 0.93 (td, *J* = 7.3, 2.1 Hz, 5H), 0.81 (t, *J* = 6.1 Hz, 3H).

Methyl (2S,4R)-4-(2-((1R,3R)-1-acetoxy-3-((2S,3S)-N,3-dimethyl-2-((R)-piperidine-2-carboxamido)pentanamido)-4-methylpentyl)thiazole-4-carboxamido)-2-methyl-5-phenylpentanoate **27**. The synthesis was analogous to **1** except of (R)-1-(tert-butoxycarbonyl)piperidine-2-carboxylic acid was used for the final step coupling. To the final resin was added DCM (1 mL) and HFIP (0.5 mL) at room temperature. The mixture was shaken at room temperature for 1h, and the resin was filtered, washed with MeOH/DCM (3×2 mL). The filtrates were evaporated in vacuo, and the residue was purified by RP HPLC ACN/water (0.1% TFA), ACN from 5% to 80% in 14 min, the pure fractions were lyophilized to give *N*-Boc-**27** (0.060 g, 46.1 %) as a white solid. LC-MS: 814 $(M+1)^+$. To the solution of *N*-Boc-**27** (59.7 mg, 0.07 mmol) in MeOH (1 mL) and toluene (2 mL) was added (diazomethyl)trimethylsilane (2.0 M in ether) (0.073 mL, 0.15 mmol) at room temperature. The mixture was stirred at room temperature for 30 min, and was evaporated *in vacuo*. The residue was dissolved in DCM (2 mL) and cooled to 0 °C and TFA (2 mL) was added. The mixture was stirred at 0 °C for 1 hour, and was evaporated in vacuo. The residue was purified by RP HPLC , ACN/water (0.1% TFA), ACN from 5% to 60% in 14 min. The fractions were lyophilized to give **27** (48.0 mg, TFA salt, 99% pure (Method A), 78 % yield) as a solid. LC-MS (ESI): m/z 728.5 $[M + H]^+$ (Calc. M=741.41); ^{1}H NMR (400 MHz, methanol-d_4) δ ppm 8.03 - 8.82 (m, 2H), 7.17 – 7.29 (m, 5H), 5.69 - 5.74 (m, 1H), 4.78 - 4.70 (m, 1H), 4.36 - 4.43 (m, 2H), 3.83 - 3.85 (m, 1H), 3.59 - 3.61 (m, 3H), 3.37 - 3.42 (m, 1H), 3.11 – 3.13 (m, 3H), 2.99 – 3.06 (m, 1H), 2.85 – 2.95 (m, 2H), 2.58 - 2.67 (m, 1H), 2.36 - 2.46 (m, 1H), 2.24 - 2.33 (m, 2H), 2.16 - 2.17 (m, 3H), 1.86 - 2.04 (m, 5H), 1.58 – 1.78 (m, 5H), 1.14 - 1.18 (m, 4H), 1.06 (d, *J* = 6.57 Hz, 3H), 1.02 (dd, *J* = 6.8, 2.8 Hz, 3H), 0.96 (td, *J* = 7.4, 5.0 Hz, 3H), 0.86 (d, *J* = 6.6 Hz, 1H).

(1R,3R)-1-(4-(((S)-1-amino-3-phenylpropan-2-yl)carbamoyl)thiazol-2-yl)-3-((2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methylpentyl acetate **28**. (1R,3R)-1-(4-(((S)-1-amino-3-phenylpropan-2-yl)carbamoyl)thiazol-2-yl)-3-((2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methylpentyl acetate **30**. 2-((1R,3R)-1-acetoxy-3-((2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methylpentyl)thiazole-4-carboxylic acid[9b] (0.050 g, 0.08 mmol) was added to a solution of 2,3,4,5,6-pentafluorophenol (25.4 mg, 0.138 mmol) and (0.022 mL, 0.138 mmol) in DCM (5 mL) at 0 °C. The solution was

allowed to reach room temperature, was stirred for 4 h, and the solvent was removed under vacuum. EtOAc (5 mL) was added to the mixture and the resulting suspension was filtered to afford the desired activated acid. The EtOAc was removed under vacuum and DMF (1 mL) was added, followed by the (S)-tert-butyl 2-amino-3-phenylpropylcarbamate (25.03 mg, 0.10 mmol) and DIEA (0.300 mL). The mixture was stirred at room temperature overnight and DMF was removed under high vacuum. The crude mixture was purified by reverse phase HPLC (Atlantis T3, 19×100 mm), 10 mM NH_4OAc/water, ACN from 10% to 80% in 20 minutes and the pure fractions were lyophilized to give N-Boc-**28** (0.025 g, 40.4 %) as solid. LC-MS (ESI): m/z 772.5 $[M + H]^+$; ^1H NMR (400 MHz, methanol-d_4) δ ppm 8.00 (s, 1H), 6.99 - 7.28 (m, 6H), 5.50 - 5.67 (m, 1H), 4.65 (d, *J* = 8.03 Hz, 1H), 4.37 (t, *J* = 9.66 Hz, 1H), 4.25 (t, *J* = 6.65 Hz, H), 3.16 (d, *J* = 6.27 Hz, 2H), 3.01 (s, 3H), 2.89 - 2.99 (m, 1H), 2.78 - 2.86 (m, 2H), 2.75 (dd, *J* = 11.42, 2.64 Hz, 1H), 2.09 - 2.34 (m, 7H), 1.99 - 2.08 (m, 4H), 1.65 - 1.83 (m, 5H), 1.42 - 1.65 (m, 5H), 1.19 - 1.34 (m, 12H), 1.09 (ddd, *J* = 13.55, 9.41, 7.15 Hz, 2H), 0.78 - 0.97 (m, 11H), 0.71 (d, *J* = 6.78 Hz, 4H). To a 25 mL round bottom flask with a magnetic stirring bar was added *N*-Boc-**28** (21.5 mg, 0.028 mmol) and DCM (3 mL) at 0 °C. Pre-cooled 50% TFA/DCM (2 mL) was added and the mixture was stirred at 0 °C for 2 h. The volatiles were removed under reduced pressure and triturated with diethyl ether and dried under high vacuum to give **28** (0.022 g, di-acetate salt, 96% pure (Method A), 99 % yield) as solid. LC-MS (ESI): m/z 671.5 $[M + H]^+$ (Calc. M=670.39); ^1H NMR (400 MHz, methanol-d_4) δ ppm 8.54 (d, *J* = 8.2 Hz, 0.5H), 8.26 (d, *J* = 8.9 Hz, 0.5H), 8.07 (s, 1H), 7.30 – 6.95 (m, 5H), 5.59 (dd, *J* = 11.5, 2.4 Hz, 1H), 4.63 (dd, *J* = 7.8, 5.2 Hz, 1H), 4.36 - 4.46 (m, 3H), 3.75 – 3.55 (m, 1H), 3.45 – 3.29 (m, 1H), 3.08 (t, *J* = 7.0 Hz, 1H), 3.02 (s, 3H), 2.90 (d, *J* = 7.3 Hz, 2H), 2.65 (s, 3H), 2.28 (ddd, *J* = 14.7, 11.4, 3.0 Hz, 1H), 2.17 – 2.06 (m, 2H), 2.04 (s, 3H), 1.91 – 1.59 (m, 3H), 1.56 – 1.41 (m, 2H), 1.23 – 1.01 (m, 1H), 0.93 (t, *J* = 6.6 Hz, 6H), 0.84 (t, *J* = 7.3 Hz, 2H), 0.74 (d, *J* = 6.6 Hz, 3H).

(2S,4R)-4-(2-((1R,3R)-3-((2S,3S)-N,3-dimethyl-2-((R)-1-methylpiperidine-2-carboxamido)pentanamido)-1-hydroxy-4-methylpentyl)thiazole-4-carboxamido)-2-methyl-5-phenylpentanoic acid **29**. To compound **1** (50 mg, 0.06 mmol) was added ammonium hydroxide (7.0 M in MeOH) (2 mL, 14.00 mmol) at room temperature. The mixture was stirred at room temperature overnight when LC-MS indicated the reaction was completed. The solvent was evaporated in vacuo, and the residue was purified by RP HPLC (Gilson), ACN/water (0.1% FA), ACN from 0% to 55% in 14 min. The fractions were lyophilized to give **29** (0.021 g, FA, 99% pure (Method C), 56.3 %) as a solid. LC-MS (ESI): m/z 686.49 $[M + H]^+$(Calc. M=685.39); ^1H NMR (400 MHz, methanol-d4) δ ppm 8.42 (s, 1H), 7.91 (s, 1H), 7.20 – 7.11 (m, 4H), 7.09 – 7.02 (m, 1H), 4.61 (d, *J* = 8.7 Hz, 1H), 4.56 (dd, *J* = 10.4, 2.4 Hz, 1H), 4.50 (s, 1H), 4.31 – 4.19 (m, 1H), 3.07 (s, 2H), 3.05 – 2.97 (m, 1H), 2.87 (d, *J* = 10.4 Hz, 1H), 2.82 (d, *J* = 6.6 Hz, 2H), 2.42 (s, 1H), 2.27 (d, *J* = 8.5 Hz, 2H), 2.25 (s, 3H), 1.96 – 1.46 (m, 11H), 1.38 – 1.24 (m, 1H), 1.21 – 1.09 (m, 1H), 1.09 – 1.03 (m, 3H), 1.03 – 0.97 (m, 1H), 0.93 – 0.86 (m, 6H), 0.81 (t, *J* = 7.4 Hz, 3H), 0.75 (t, *J* = 6.0 Hz, 3H).

ASSOCIATED CONTENT

Supporting Information. General information and detailed experimental procedures for preparation of intermediates **1a**, **1b** and resin bound tripeptide **1d**, ^1H NMR traces for all compounds **1-29**, representative dose response curves for cytotoxicity assay, and full experimental details and characterization of compounds **2-11**, **13** and **15-24**, table with data containing pIC_{50} in HCT-116 and MDA MB-231 and predicted pKa, and Figures S1–S2 showing the correlations plots. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +1 301-398-1094. E-mail: toaderd@medimmune.com.

Present Addresses

The Broad Institute, Cambridge, MA

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This study was supported by MedImmune and AstraZeneca.

ACKNOWLEDGMENT

We are indebted to Professor Peter Wipf for a sample of N^{14}-Desacetoxytubulysin H. The authors thank Changshou Gao for critical review of the manuscript.

ABBREVIATIONS

DIEA:N,N-Diisopropylethylamine; HATU: [Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium -3-oxid hexafluorophosphate; TMP:2,4,6-Trimethylpyridine; DIC=N,N′-Diisopropylcarbodiimide; HFIP=hexafluoroisopropanol.

REFERENCES

(1) Sasse, F.; Steinmetz, H.; Heil, J.; Hofle, G.; Reichenbach, H. Tubulysins, new cytostatic peptides from myxobacteria acting on microtubuli. Production, isolation, physico-chemical and biological properties. *J. Antibiot.*, **2000**, *53*, 879–885.

(2) Höfle, G.; Glaser, N.; Leibold, T.; Karama, U.; Sasse, F. ; Steinmetz, H. Semisynthesis and degradation of the tubulin inhibitors epothilone and tubulysin. *Pure Appl. Chem.*, **2003**, *75* (2-3), 167–178.

(3) 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.

(4) Schluep, T.; Gunawan, P.; Ma, L.; Jensen, G. S.; Duringer, J.; Hinton, S.; Richter, W.; Hwang, Polymeric Tubulysin-peptide nanoparticles with potent antitumor activity. *J. Clin. Cancer Res.* **2009**, *15*,181–189.

(5) (a) Cormier, A.; Marchand, M.; Ravelli, R. B. G.; Knossow, M.; Benoit, G. Structural insight into the inhibition of tubulin by vinca domain peptide ligands. *EMBO Rep.* **2008**, *9*, 1101–1106. (b) Maderna, A.; Doroski, M.; Subramanyam, C.; Porte, A.; Leverett, C. A.; Vetelino, B. C.; Chen, Z.; Risley, H.; Parris, K.; Pandit, J.; Varghese, A. H.; Shanker, S.; Song, C.; Sukuru, S. C. K.; Farley, K. A.; Wagenaar, M. M.; Shapiro, M. J.; Musto, S.; Lam, M.-H.; Loganzo, F.; O'Donnell, C. J. Discovery of cytotoxic dolastatin 10 analogues with N-terminal modifications. *J. Med. Chem.* **2014**, 57, 10527–10543.

(6) Kubicek, K.; Grimm, S. K.; Orts, J.; Sasse, F.; Carlomagno, T. The tubulin-bound structure of the antimitotic drug tubulysin. *Angew. Chem. Int. Ed.* **2010**, *49*, 4809–4812.

(7) Murray, B. C.; Peterson, M. T.; Fecik, R. A. Chemistry and biology of tubulysins: antimitotic tetrapeptides with activity against drug resistant cancers. *Nat. Prod. Rep.*, **2015**, 32, 654-662.

(8) Iley, J.; Moreira, R.; Rosab E. J. Acyloxymethyl as a drug protecting group. Kinetics and mechanism of the hydrolysis of *N*-acyloxymethylbenzamides. *Chem. Soc., Perkin Trans. 2* **1991**, 563-570.

(9)(a) Wipf, P.; Wang, Z. Total synthesis of N14-Desacetoxytubulysin H. *Org. Lett.* **2007**, *9*(8), 1605-1607. (b) 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. (c) Peterson, A. W.; Peltier, H. M.; Ellman, J.A. Expedient synthesis of N-methyl tubulysin analogues with high cytotoxicity. *J. Org. Chem.* **2008**, *73*(12), 4362–4369. (d) Nicolaou, K. C.; Yin, J.; Mandal, D. ; Erande, R. D.; Klahn, P.; Jin, M.; Aujay, M.; Sandoval, J.; Gavrilyuk, J.; Vourloumis, D. Total synthesis and biological evaluation of natural and designed tubulysins. *J. Am. Chem. Soc.* **2016**, *138*(5), 1698-1708.

(10)(a) 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. (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.* **2007**, 46, 2347–2348 (Corrigendum). (c) Balasubramanian, R.; Raghavan,B.; Begaye, A.; Sackett, D. L.; Fecik, R. A. Total synthesis and biological evaluation of tubulysin U, tubulysin V, and their analogues. *J. Med. Chem.* **2009**, 52(2), 238–240. (d) Shankar, S. P.; Jagodzinsk, M.; Malpezzi, L.; Lazzari, P.; Manca, I.; Greig, I. R. ; Sani, M.; Zanda, M. Synthesis and structure–activity relationship studies of novel tubulysin U analogues – effect on cytotoxicity of structural variations in the tubuvaline fragment. *Org. Biomol. Chem.*, **2013**, *11*, 2273-2287. (e) Raghavan, B.; Balasubramanian, R.; Steele, J. C.; Sackett, D. L.; Fecik, R. A. Cytotoxic simplified tubulysin analogues. *J. Med. Chem.* **2008**, *51*(6), 1530–1533. (f) Sani, M.; Fossati, G.; Huguenot, F; Zanda, M. Total Synthesis of Tubulysins U and V *Angew. Chem. Int. Ed.* **2007**, *46*, 3526–3529. (g) Shibue, T.; Hirai, T.; Okamoto, I.; Morita, N.; Masu, H.; Azumaya, I.; Tamura, O. Total syntheses of tubulysins *Chem. Eur. J.* **2010**, *16*, 11678–11688.

(11) 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.

(12) Shankar, P. S.; Bigotti, S.; Lazzari, P.; Manca, I.; Spiga, M.; Sani M.; Zanda, M. Synthesis and cytotoxicity evaluation of diastereoisomers and *N*-terminal analogues of tubulysin-U. *Tetrahedron Lett.* **2013**, *54*, 6137–6141.

Insert Table of Contents artwork here