Chemistry and Biology of Tubulysins: Antimitotic Tetrapeptides with Activity against Drug Resistant Cancers

Journal:	*Natural Product Reports*
Manuscript ID:	NP-HIG-03-2014-000036.R2
Article Type:	Highlight
Date Submitted by the Author:	15-Jan-2015
Complete List of Authors:	Murray, Bryan; University of Minnesota, Medicinal Chemistry Peterson, Michael; University of Minnesota, Medicinal Chemistry; Memorial Sloan-Kettering Cancer Center, Molecular Pharmacology and Chemistry Program Fecik, Robert; University of Minnesota, Medicinal Chemistry

Natural Product Reports

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Chemistry and Biology of Tubulysins: Antimitotic Tetrapeptides with Activity against Drug Resistant Cancers.

Bryan C Murray, Michael T Peterson and Robert A Fecik*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Since their first report in 2000, tubulysins have sparked great interest for development as anti-cancer agents due to their exceptionally potent anticancer activity. Progress in the discovery and development of tubulysins, especially tubulysin conjugates, has quickly advanced despite limitations in their availability from Nature. In this *Highlight*, the key research on the isolation and structure determination, biosynthesis, bioactivity, structure activity relationships (SAR), synthesis, and conjugates of tubulysins is presented.

Introduction

Tubulysins (**1–14**, **Figure 1**) are antimitotic tetrapeptides isolated from myxobacteria reported by Höfle and co-workers in 2000.[1] They have potent antiproliferative activity against human cancer cells, including drug-resistant cells, by inhibiting tubulin polymerization.[1] A key issue that has stalled extensive study of tubulysins is the extreme difficulty in obtaining the natural products from myxobacteria.

Despite the scarce supply of tubulysins, their biological activity and mechanism of action has been investigated. Extensive synthetic efforts on the total synthesis of tubulysins and analogs have yielded important insights on structure-activity relationships (SAR). The more recent identification of the tubulysin biosynthetic gene cluster has also spurred research into metabolic engineering of the producing organisms.

Since tubulysins effect their activity through a validated mechanism, they hold great clinical promise for the treatment of drug-resistant cancers. We review here the isolation and structure determination, biosynthesis, biological activity, SAR, synthesis, and conjugates of this fascinating class of natural products.

Isolation and Structure Determination

Höfle and co-workers first reported in 2000 the isolation of four members of the tubulysin family (A, B, D, and E) while screening myxobacterial culture extracts for biologically active compounds.[1] Tubulysins have extremely potent antiproliferative activity against cancer cells, including multidrug resistant KB-V1 cervix carcinoma cells (tubulysin D, IC_{50} = 0.08 ng/mL).[1] *Archangium gephyra* Ar 315 produces tubulysins A, B, C, G, and I, while *Angiococcus disciformis* An d48 produces tubulysins D, E, F, and H.[1,2] More recently a third producing myxobacterium, *Cystobacter* SBCb004, was identified and also produces tubulysins A, B, C, G, and I.[3] Metabolite production in these wild-type strains is low, however. For instance, An d48 produces only up to 1 mg/L of tubulysin D,[1] while Ar 315 and SBCb004 produce up to 4[1] and 0.48[3] mg/L, respectively.

A combination of degradation, NMR, and ^{13}C-enriched biosynthetic precursor feeding experiments were used to determine the structures of tubulysins A–I and the absolute configuration of the seven stereogenic centers.[2,4] All tubulysins are comprised of *N*-methyl-D-pipecolinic acid (Mep), L-isoleucine (Ile), and tubuvaline (Tuv), which contains an unusual *N,O*-acetal and a secondary alcohol or acetoxy group. Tubulysins A–C, G, and I contain the C-terminal tubutyrosine (Tut) γ-amino acid, while D, E, F and H instead have tubuphenylalanine (Tup) at this position (**Figure 1**). The X-ray structure of tubulysin A confirmed the original structure and absolute configuration assignments.[2]

The X-ray and solution structures of tubulysin A in d_6-DMSO and CD_3OD show the molecule in an extended conformation.[2] Using transferred NOE data from the NOESY spectrum of an aqueous tubulysin A-tubulin solution, the tubulin-bound conformation was found to be compact with the aromatic thiazole and phenyl rings collapsed into a hydrophobic core with the *N,O*-acetal and Ile side chain.[5] The crystal structure of a tubulysin bound to tubulin has yet to be reported.

Biosynthesis

Identification of the tubulysin biosynthetic gene cluster in *A. disciformis* An d48 was reported in 2004[6] and in *Cystobacter* sp. SBCb004 in 2010,[3] spurred by the potential to increase fermentation titers and discover novel analogs through metabolic engineering and combinatorial biosynthesis. Tubulysins are biosynthesized by a mixed nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) system that incorporates many unique and rare features. Many aspects of their biosynthesis remain to be elucidated. The Tub NRPS-PKS comprises 7 modules (2 PKS and 5 NRPS) on 5 proteins (TubB–F) that produces pretubulysin A (**15**) or D (**16**), which undergo tailoring

oxidations and acylations to afford tubulysins and a host of other minor metabolites (**Figure 1**). TubZ, responsible for synthesis of the starter unit, L-pipecolinic acid, is also expressed in the biosynthetic gene cluster, as well as TubA, which is likely an acyltransferase.[6,7]

Identification of the oxidases and other possible acyl transferases that carry out the tailoring reactions has proven difficult since there are no additional candidate genes expressed within the tubulysin biosynthetic gene cluster. Gene *633P1* in SBCb004 encodes for a P450 monooxygenase that may carry out both tailoring oxidations; SBCb004 with mutant *633P1* only produced non-oxidized pretubulysin metabolites.[7]

There are several unusual aspects to tubulysin biosynthesis that deserve mention. The order of the domains in the two PKS modules (KS-AT-KR-DH-ER-ACP) is different from all other known PKS systems (KS-AT-DH-ER-KR-ACP).[6] Further, the identification of over 20 new tubulysin metabolites shows that many components of the pathway are imperfect in the processing of chain elongation intermediates.[3] Among these new metabolites are ones lacking the *N*-methyl group on tubuvaline, as previously observed with tubulysins U, V, X, and Z.[3] Remarkably, skipping of the NMT domain of module 3 in many cases leads to further domain skipping of one or more of the β-carbon processing domains (KR, DH, ER) of PKS module 4 (see representative compound **17**, *N*-desmethyl 12-keto pretubulysin A, **Figure 1**).[3] Metabolite **18** (**Figure 1**), tyrosine pretubulysin A, also indicates that the second PKS module 7 can be skipped in its entirety, suggesting that the intermediate following extension with phenylalanine or tyrosine by module 6 can be directly hydrolyzed by the final TE domain.[3] Lastly, the tailoring oxidation and acylation reactions are imperfect; all of the new metabolites identified had bypassed one or more of these reactions.[3] These findings suggest that the Tub NRPS-PKS system has enormous potential for expanding the combinatorial biosynthetic toolbox.

A new family of *N*-terminal docking domains in TubCdd from *A. disciformis* An d48 was described in 2008.[8] TubCdd is homodimeric, with an unusual fold containing an exposed β-hairpin that appears to be a key determinant of the interaction of partner polypeptides that could be a docking code.[8] This docking code can be used to alter binding affinity. Structure elucidation by NMR of TubCdd revealed a new αββαα fold and a corresponding electrostatic specificity code for this docking interaction.[8] The TubCdd docking domains were found at the NRPS–NRPS interface between subunits TubB and TubC in the tubulysin assembly line, and it is believed that this and TubBdd are representative of recognition elements that operate at NRPS–NRPS and PKS–NRPS junctions.[8]

In an effort to boost production titers of tubulysins, the biosynthetic gene cluster from SBCb004 was successfully reconstituted in *Pseudomonas putida* and *Myxococcus xanthus* DK1622 for heterologous expression.[7] When introduced into *P. putida*, two separate plasmid constructs expressed low levels of pretubulysin A (0.04 and 0.2 μg/L) and only trace amounts of tubulysin A. Supplementing the cultures with D,L-pipecolinic acid increased production to 0.66 and 1.76 μg/L. Pretubulysin A production in *M. xanthus* (supplemented with D,L-pipecolinic acid) was much higher (190 μg/L), and additional metabolites could also be detected.

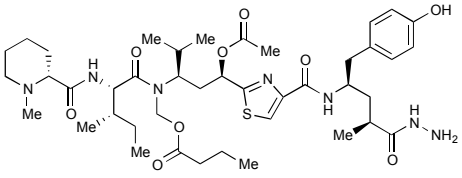
Bioactivity

The exceptional antiproliferative activity of tubulysins has led to a great deal of interest in evaluating their clinical potential and studying their mechanism of action. In growth inhibition assays tubulysins were inactive against bacteria and yeasts, weakly active against fungi, and highly active against human cancer cells, including multidrug resistant cells that overexpress P-glycoprotein.[1,10] The tubulysins have shown potent growth inhibition against breast,[9] cervix,[1,2] colon,[10] leukemia,[1] lung,[11] melanoma,[10] ovarian,[9] and prostate[10] cancer cells. In the NCI 60-human cancer cell line screen tubulysin A had an estimated GI_{50} for all cells of 12 nM; the lowest concentration tested (10 nM) was too high to establish a GI_{50} for all cell lines.[10] Additionally, tubulysins exhibit a degree of selectivity towards cancer cells due to their rapid division rates.[10,12]

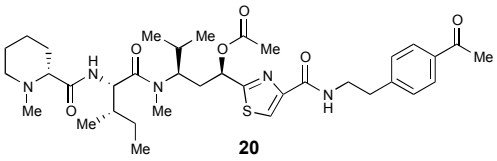
It was quickly established that the mechanism of action of tubulysins is inhibition of polymerization of the cytoskeletal protein tubulin and induction of apoptosis.[1,10] Binding competition experiments with radiolabeled vinblastine and colchicine revealed that tubulysin A binds to the peptide binding site located on β-tubulin near the *Vinca* alkaloid binding site.[12] Other natural products that bind to the peptide site include dolastatin 10, hemiasterlin, cryptophycins, phomopsin A, and others. A contributing mechanism of action may be anti-angiogenic effects. Tubulysin A exhibited strong anti-angiogenic effects, and was more potent than both paclitaxel and TNP-470 in cord formation and cell migration assays.[10]

The potential of tubulysin A for clinically relevant drug-drug interactions has also been evaluated. Tubulysin A does not inhibit CYP1A2 and CYP2D6 *in vitro* (IC_{50}s > 100 μM), and inhibits CYP3A4 moderately (IC_{50} = 82 μM).[10] Thus, the potential for drug-drug interactions involving these cytochrome P450s is low.

Despite their exciting *in vitro* bioactivity profile, all tubulysins examined to date have proven to be extremely toxic *in vivo*. Two natural tubulysins have been evaluated *in vivo*, as well as two analogs. The maximum tolerated dose of tubulysin A in nude mice administered in three weekly doses is 0.05 mg/kg; doses above this (0.3–3 mg/kg) resulted in treatment-related deaths of all animals.[13] In an HT29 colorectal carcinoma mouse xenograft model, tubulysin A at 0.1 mg/kg three times a week showed no efficacy and a 50% mortality rate.[13] No efficacy was also observed when the dose of tubulysin A was reduced to 0.025 mg/kg in an H460 non-small cell lung carcinoma xenograft.[13] Tubulysin B was evaluated in a KB nasopharyngeal carcinoma xenograft mouse model, and showed no efficacy at 0.1, 0.2, and 0.5 μmol/kg with limited tolerability due to weight loss.[14] Two tubulysin analogs (**19** and **20)** have proven similarly toxic *in vivo*.[15,16] Efforts to increase their *in vivo* safety and efficacy through targeted delivery are described below.



Tubulysin B hydrazide (**19**)



20

Structure-Activity Relationships

Total synthesis of tubulysins, and their modular tetrapeptide composition, has aided greatly in defining SAR, which are summarized in **Figure 2**. The search for novel tubulysin analogs has largely been driven by efforts to improve physico-chemical properties, such as increased aqueous solubility, and to discover simplified analogs that are synthetically more accessible than the natural products.

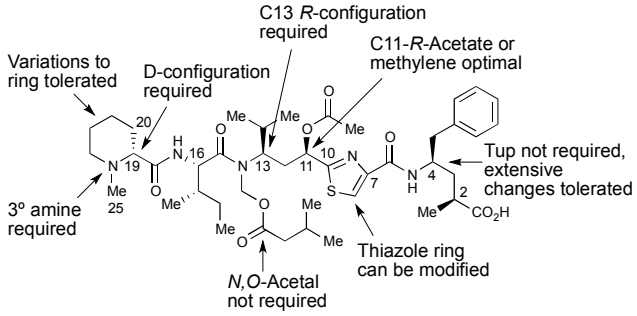
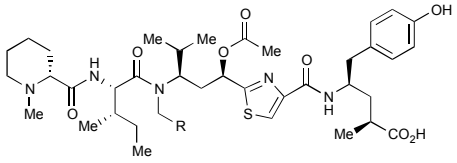


Figure 2. Summary of tubulysin SAR.

At the N-terminus, the 6-membered ring of the Mep residue can be effectively replaced with the 5-membered and acyclic residues *N*-methyl-D-proline,[17–19] *N*-methylsarcosine,[11,20,21] and *N,N*-dimethyl-D-alanine[9] with minimal loss of activity in most cases. The D-configuration of this residue is required, however, as L-amino acids at this position abolish anticancer activity.[17,18] A tertiary amine is also required on this residue; desmethyl[17,18] and acetyl analogs,[19] removal of Mep,[21] and replacement of Mep with an acetyl group[21] results in loss of activity.

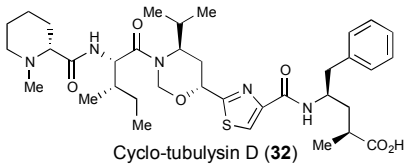
The residue most rich in functional groups, Tuv, has been subject to extensive modification. Natural tubulysins have two variations of this residue: the absence or presence of the labile *N,O*-acetal and acetyl groups. Activity increases as the lipophilicity of the *N,O*-acetal increases,[2] although this functional group is not a requirement for potent anticancer activity as demonstrated with tubulysins U and V.[9] The *N,O*-acetal has successfully been replaced with a –H[9,17–19,22] or –Me group,[20,23,24] as found in the pretubulysin biosynthetic intermediates. Another successful modification at this position is replacement of the *N,O*-acetal with *N*-alkylamide groups in a small series of so-called tubugis.[25] A group at Endocyte has demonstrated that extensive modifications to the *N,O*-acetal are tolerated. A mixture of fermentation-derived tubulysins was reacted with TFA to afford an intermediate *N*-acyliminium ion that could be trapped with a variety of *C*-, *O*-, and *S*-based nucleophiles.[26] All of the resulting analogs (**21**–**31**) had antiproliferative activity comparable to or better than tubulysins A and B, with *N,S*-thioacetals **28** and **29** being the most potent.[26] Interestingly, an analog designated as cyclo-tubulysin D (**32**), which contains a cyclic *N,O*-acetal formed with the C11-alcohol, was nearly as potent as tubulysin U.[27]



R = –$CH_2CH{=}CH_2$ (**21**)

R = –OMe (**22**), –O^{n}Pr (**23**), –O-isopentyl (**24**), –OCH_2CH_2OH (**25**)

R = –SEt (**26**), –S^{n}Pr (**27**), –S^{n}Bu (**28**), –S-*n*-pentyl (**29**), –SCH_2CH_2OH (**30**), –SCH_2CH_2SH (**31**)



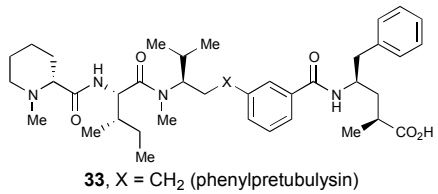
Cyclo-tubulysin D (**32**)

While the *N,O*-acetal is quite tolerant of changes, modifications to the C11-acetyl group of Tuv can have dramatic effects on activity, and minimal variations are tolerated. Hydrolysis of the acetyl group to the secondary alcohol results in up to a 1000-fold loss of activity, exemplified by tubulysins U and V[9] and a tubulysin D analog.[21] Stereochemistry of the C11 position is also important; inversion of this stereocenter from the natural *R*-configuration to the *S*-configuration as either the acetate[19,20,27,28] or alcohol[9] reduces activity. Oxidation of C11 to a ketone reduces activity, with the best compounds from this series only as potent as tubulysin V.[9,17,18] Only a modest loss of activity was observed when the acetyl group was replaced with a methyl or MOM ether, however, a benzoyl analog was inactive.[29] The most striking exception to the *R*-acetyl group being optimal at C11 is found with pretubulysin D (**16**), which is fully reduced to the methylene group at this position yet retains anticancer potency on the level of tubulysin A.[11,23,24]

Stereochemistry at the C13 position of Tuv has a more profound impact on activity than the C11 position. Inversion of the C13-isopropyl group of tubulysin D from the *R*- to *S*-configuration results in a dramatic loss of antiproliferative activity.[19,27,28] The substituent at C13 also appears to be intolerant of changes. Replacement of the isopropyl group with cyclohexyl or aryl (phenyl and *p*-methoxyphenyl) groups gave inactive analogs.[29]

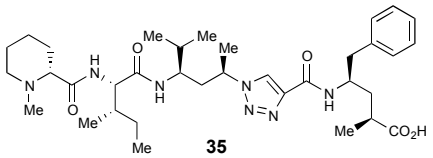
Lastly, a limited number of modifications to the thiazole ring of Tuv have been recently explored. The oxazole analog of tubulysin U is slightly more potent than the natural product.[30] Drawing inspiration from pretubulysin D, replacement of the thiazole ring with a *meta*-substituted phenyl group to give phenylpretubulysin (**33**) and phenoxypretubulysin (**34**) resulted in potent analogs although 2–4 orders of magnitude less potent than pretubulysin D.[23] An analog (**35**) with a 1,2,3-triazole replacing the thiazole has been reported to have antiproliferative activity similar to tubulysin V.[22] Triazolyl analog **35** also replaces the

C11-alcohol of tubulysin V with a methyl group due to decomposition of an intermediate with an alcohol at this position.[22]



33, X = CH_2 (phenylpretubulysin)

34, X = O (phenoxypretubulysin)



35

Natural tubulysins with Tup at the C-terminus (such as tubulysins D–F and H) instead of Tut (tubulysins A–C, G, and I) are more potent, which may be due to their enhanced lipophilicity and cell membrane permeability.[2] The entire Tup residue is not essential for activity; the Mep–Ile–Tuv tripeptide of tubulysin D was only one order of magnitude less potent than tubulysin D, and analogs that lack the phenyl and carboxylic acid groups (such as analog **20**), or both, retained similar levels of activity as the tripeptide.[16,21] Not all replacements of Tup are tolerated. The L-phenylalanine analog of pretubulysin D was substantially less potent,[11] as were *N*-alkyl amides of Tup and Tup analogs incorporating a benzophenone group designed as imaging and labeling probes.[24]

Stereochemistry of the Tup residue is less important than that observed with Tuv. Inversion of the C2-methyl or C4-benzyl groups results in only small decreases in activity.[19,31,32] Elimination of the C2 stereocenter in a series of C2-desmethyl[9,11,18] and C2-dimethyl analogs[9,18] generally resulted in slight loses in activity compared to the respective analogs with a C2-methyl group in the natural *S*-configuration.[9,11,17] Finally, the C-terminal carboxylic acid is a common site of conjugation (see below), and therefore has usually been modified in this context. Formation of amides seems to be well tolerated, as both tubulysin B hydrazide (**19**),[15] the 2-mercaptoethyl amide of tubulysin A, as well as its disulfide dimer,[13] were only slightly less potent that the respective natural products. The methyl ester of phenylpretubulysin (**33**), however, exhibited decreased activity compared to **33**, and is proposed to likely be a prodrug.[23]

The SAR studies described above and summarized in **Figure 2** demonstrate the flexibility in modifications of key residues of tubulysins. Tuv is clearly the most critical to the anticancer potency of tubulysin analogs, yet extensive changes are tolerated, particularly to the *N,O*-acetal. A limited number of modifications to Mep are tolerated, and the tertiary amine is essential. In contrast, a broad range of major changes can be made to Tup, and the entire residue is not essential for activity; this is especially important since Tup is a common site of conjugation for targeted delivery approaches. It is reasonable to assume that simplified tubulysin analogs that are more accessible than the natural products, and that possess more favorable physico-chemical properties to improve drug product formulation and *in vivo* pharmacokinetics, is a realistic goal of medicinal chemists.

Synthesis

Due to the limited availability of tubulysins from fermentation, total synthesis of tubulysins has been widely investigated. Total syntheses of tubulysins B,[31] D,[27,33] U,[9,27,32,34–36] V,[9,27,34–36] and pretubulysin D[11] have been reported, in addition to the analogs above, which are typically obtained through modification of these methods. The tetrapeptide scaffold is usually assembled by sequential peptide coupling of the amino acids or two dipeptide fragments. Most synthetic work has focused on the more complex tubuvaline and tubuphenylalanine residues, and this section highlights the various approaches to these two amino acids.

Tubuvaline (Tuv). Many syntheses of tubuvaline utilize protected L-valine (in varying oxidation states) as the starting material for the C-13 stereocenter,[9,17,34,35,37–39] which is typically used for a one-[17,34,35,38] or two-carbon[11,37] homologation prior to installation of the thiazole ring and C-11 alcohol. In one case, a protected aziridine derived from L-valine was opened with allylMgBr.[39] Dömling and co-workers elegantly designed a one-step multicomponent reaction of protected tubuvaline **39** from Boc-β-homovalinal **36**, Schöllkopf isonitrile **37**, and thioacetic acid (**38**) in 40% yield and 3:1 dr of the C-11 acetate (**Figure 3a**).[34,35] Metal-halogen exchange of a protected bromothiazole followed addition to β-homovalinal[38] or the β-homovaline Weinreb amide[17] furnishes the tubuvaline framework with the C11-hydroxy group installed directly (as a 2:1 mixture of separable diastereomers)[38] or through CBS reduction of the resulting C-11 ketone.[9,17] The C-11 alcohol can also be obtained stereoselectively by α-hydroxylation using the methods of Davis[37] and MacMillan (**Figures 3b** and **3c**).[39]

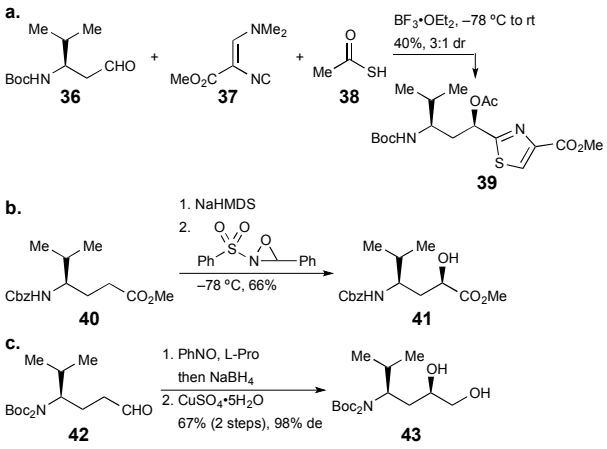


Figure 3. Key reactions in the synthesis of Tuv from L-valine.

A variety of other strategies have been used in the synthesis of tubuvaline, often to showcase the utility of stereoselective reactions. Tamura and co-workers accomplished installation of both the C11- and C13-stereocenters with a 1,3-cycloaddition of gulose-derived nitrone **44** with Oppolzer's camphor sultam **45** to generate isoxazolidine **46** in quantitative yield and 85:15 dr (**Figure 4a**).[40] Construction of the thiazole using cysteine and reductive cleavage of the *N-O* bond furnishes the Tuv residue **48**.[40] Alternatively, peptide couplings prior to a late-stage *N-O* bond cleavage takes advantage of this bond as a protecting group and has led to natural tubulysins and analogs.[27]

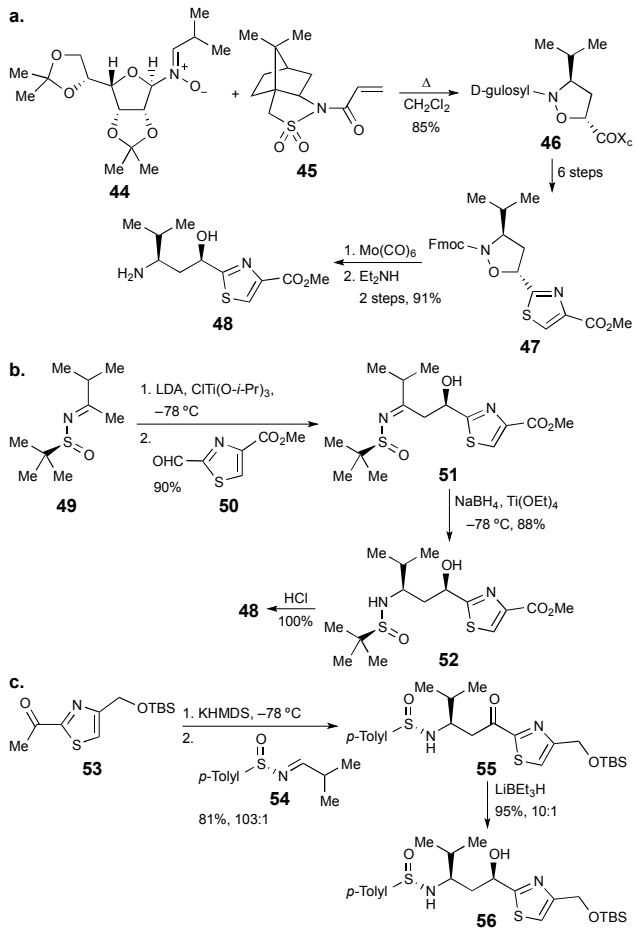


Figure 4. Other key reactions in representative syntheses of Tuv.

The key sequence to tubuvaline in Ellman's tubulysin D total synthesis involves use of chiral sulfinamide **49**, which first stereoselectively adds into thiazole aldehyde **50** as a metalloenamine to give the C-11 alcohol (**51**) in 90% yield, and then directs a diastereoselective reduction of the ketamine with $NaBH_4$ and $Ti(OEt)_4$ to give the C-13 stereocenter (**52**) with high selectivity (91:1 dr) and isolated yield (88%) (**Figure 4b**).[33] A slight modification of this sequence (using the ethyl ester of the thiazole aldehyde **50**) was used in the synthesis of tubulysin analogs with the *N,O*-acetal replaced with an *N*-methyl group since a methyl ester was not required for selective hydrolysis.[41]

Another synthesis utilized a chiral sulfinamide (**54**) in reaction with the enolate of thiazoyl ketone **53** to set the C-13 stereocenter (81% yield, 103:1 dr), and the C-11 alcohol was then introduced by selective (10:1 dr) reduction of the resulting ketone **55** with $LiBEt_3H$ in 95% yield (**Figure 4c**).[32] Zanda and co-workers used a non-selective Michael addition of *t*-butyl carbamate to a tubuvaline intermediate for the C-13 stereocenter in their total synthesis of tubulysins U and V, followed by selective CBS reduction of the resulting thiazoyl ketone and separation of the diastereomers by flash chromatography.[36] Stereoselective Michael additions have been studied using chiral oxazolidinones as a nitrogen source.[42] The same racemic thiazoyl ketone intermediate can also be obtained by reaction of the enolate of a thiazoyl methyl ketone with an α-amino sulfone.[29]

Arguably the most difficult challenge in the total synthesis of tubulysins is the installation of the *N,O*-acetal due to its lability and incompatibility with a variety of reagents for selective functional group transformations. Currently, Ellman's method developed for the synthesis of tubulysin D is the only one that has been reported.[33] Using an α-azido-protected Ile–Tuv dipeptide (**57**), *N*-alkylation of the amide bond is possible using a strong base (KHMDS) and chloromethyl ester **58** to install the *N,O*-acetal (**59**) in excellent yield (73%) (**Figure 5**).[33] The authors note that protection of the C-11 alcohol as the TES silyl ether was key to the reaction's success; a sterically demanding TIPS group at this position resulted in less than 10% yield.[33] A subsequent neutral azide reduction and other carefully chosen reaction conditions and protecting groups enabled completion of the tubulysin D synthesis without hydrolysis of the sensitive *N,O*-acetal.[33] This method has been utilized by others in the total synthesis of tubulysin B,[31] D,[27] and analogs,[21,28,41] and side reactivity of the *N,O*-acetyl under similar reaction conditions has been noted.[28,31]

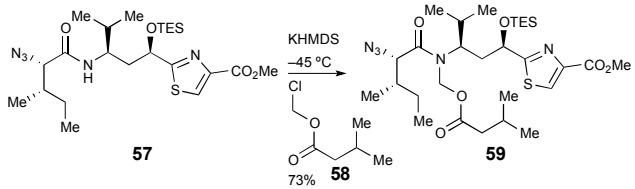


Figure 5. Installation of the *N,O*-acetal of Tuv.

Installation of the *N,O*-acetal requires particular alkylation conditions, and the *N,O*-acetal itself suffers from instability upon further molecular manipulations. Incorporation of an *N*-methyl group at this position of tubuvaline, as seen in pretubulysins, is straightforward. The same *N*-alkylation conditions for the *N,O*-acetal at the tripeptide stage using MeI can be followed,[21,41] however, other bases (NaH and NaHMDS) are suitable at earlier stages of the synthesis.[11,23,38,43] Ellman and co-workers also reported *N*-methylation of a tubuvaline intermediate by formation of 1,3-tetrahydrooxazine with paraformaldehyde, followed by reduction with solid-supported cyanoborohydride.[41] Reaction of tubuvaline in an Ugi reaction afforded analogs with *N*-alkylamides that varied according to the isocyanide used.[25]

Tubuphenylalanine (Tup). Most syntheses of tubuphenylalanine are derived from the chiral pool, with L-phenylalanine serving as the source of the C4-benzyl group. In this synthetic strategy, the most common approach is a two-carbon homologation of protected phenylalanal by a Wittig reaction followed by hydrogenation of the resulting alkene (see representative example in the conversion of alkene **60** to alcohol **61**,[37] **Figure 6a**). Although in the first report of this approach by Höfle the hydrogenation was non-selective,[4] its synthetic ease has encouraged optimization by other groups. Despite the variations employed, the diastereoselectivity of the hydrogenation to install the C2-methyl stereocenter is low (1–3:1) and all methods require chromatographic separation of the diastereomers.[11,29–31,37]

Other groups have also used phenylalanine derivatives as the starting material for the synthesis of tubuphenylalanine. Homologation of phenylalaninol with α-methyl diethylmalonate gave Tup as a mixture of diastereomers with respect to the C2-methyl group, which necessitated resolution by flash chromatography of an *N*-Boc menthyl ester derivative.[36] *N*-Boc protected γ-lactam **62**, obtained from a three-step, two-carbon homologation of *N*-Boc-phenylalanine with Meldrum's acid,[44] was employed for a diastereoselective (10:1) α-methylation to afford tubuphenylalanine upon hydrolysis of lactam **63** (**Figure**

6b).[17] Tubuphenylalanine can also be synthesized by Evans aldol reaction of oxazolidinone **65** with *N*-Boc-phenylalaninal, followed by Barton–McCombie deoxygenation of the resulting alcohol **66** with 1,1'-thiocarbonyldiimidazole (TCDI), *n*-Bu_3SnH, and AIBN, and hydrolysis of the chiral auxiliary (**Figure 6c**).[40]

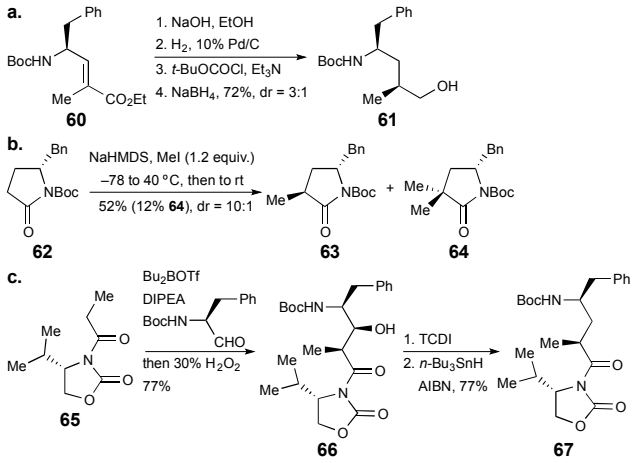


Figure 6. Key reactions in the synthesis of Tup from L-phenylalanine.

An early approach to tubuphenylalanine by Friestad and co-workers featured a stereoselective Mn-mediated addition to chiral hydrazone **68** by alkyl iodide **69** with high selectivity (dr >49:1) and in good yield (56%) to form the C4-stereogenic center (**70**, **Figure 7a**).[45] Ellman and co-workers elegantly installed both stereogenic centers at C2 and C4 by SmI_2 reductive coupling of chiral sulfinimine **71** with methyl methacrylate (**72**) in 99% yield and 80:15:3:2 diastereoselectivity (**73**, **Figure 7b**).[33]

In another method, the C2-methyl stereocenter was set with a hydrazone derivatized with Enders' SAMP auxiliary (**74**) that was alkylated with chiral aziridine **75** in the key step (**Figure 7c**).[34,35] Beginning with (–)-citronellol, a sequence featuring addition of PhMgBr to a chiral epoxide furnished tubuphenylalanine upon further functional group manipulations.[39] One last, recently reported method was highlighted by diastereoselective addition of BnMgCl to chiral sulfinyl imine **77** to give tubuphenylalanine as its γ-lactam **78** (90% yield, 6.6:1 dr, **Figure 7d**).[32]

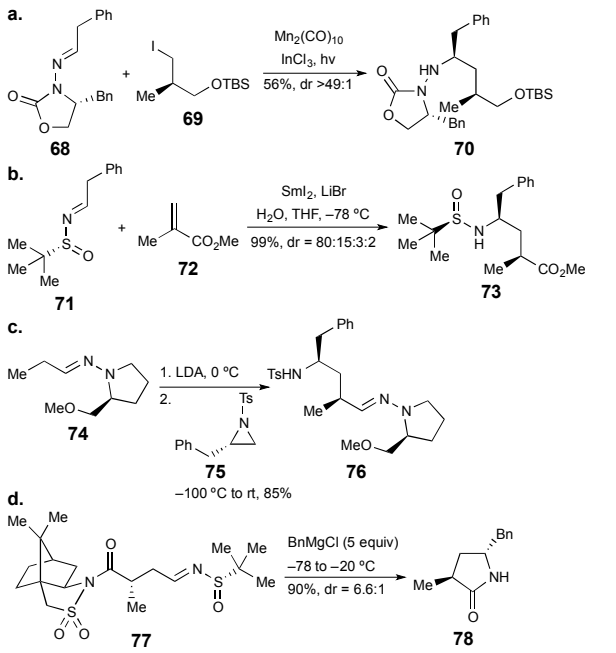


Figure 7. Other key reactions in representative syntheses of Tup.

The large number of diverse methods for the synthesis of two key residues, Tuv and Tup, is indicative of the intense interest in the potential therapeutic applications of tubulysins. Accordingly, these methods have provided medicinal chemists with a wealth of synthetic opportunities for analog synthesis.

Conjugates

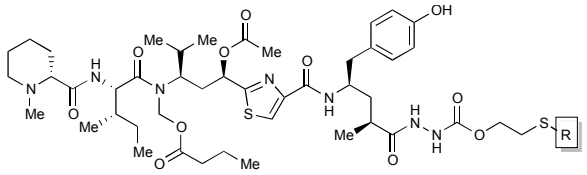
The extreme toxicity and poor aqueous solubility of tubulysins has spurred efforts to address these limitations through the development of targeted delivery conjugates. Numerous conjugates capable of selective delivery of tubulysins to cancer cells have been disclosed, thus avoiding toxic effects on non-cancerous cells.

Taking advantage of the high expression of the folate receptor in many cancers, the first targeted tubulysin disclosed was a tubulysin B-folic acid conjugate (EC0305, **79**) by researchers at Endocyte. A linker region was attached to the carboxylic acid of the Tup residue of tubulysin B as a hydrazide, and a disulfide bond connected the linker to folic acid.[46] Following selective uptake into cancer cells, the disulfide bond is reduced in endosomes to release tubulysin B hydrazide (**19**), which has antiproliferative activity comparable to tubulysin B.[15] Whereas tubulysin B hydrazide showed no efficacy, with dose-limiting toxicities in mice implanted with folate-receptor positive KB nasopharyngeal tumors at doses of 0.5–1 μmol/kg, EC0305 was effective and safe at all doses tested (0.5–2 μmol/kg).[15]

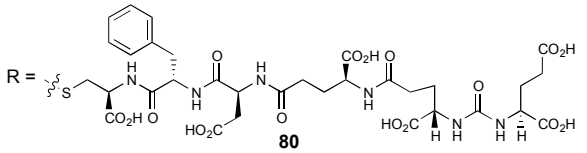
Comparison of EC0305 to two other tubulysin hydrazide-folic acid conjugates and a tubulysin B ester-folic acid conjugate established the superiority of EC0305 in a KB mouse xenograft model.[14] Dosing EC0305 at 1 μmol/kg for three times a week for 2 weeks resulted in all mice being tumor-free at the end of the study (80 days).[14] The ester-linked conjugate was substantially more toxic *in vivo*,[14] presumably due to the increased lability of the ester. EC0305 was also more efficacious than a desacetylvinblastine hydrazide-folic acid conjugate (EC145),[14] currently in phase II human trials, in two additional mouse

xenograft models. Endocyte initiated a phase I clinical trial of the tubulysin B hydroazide–folic acid conjugate EC1456 in advanced solid tumors in December 2013 (ClinicalTrials.gov Identifier: NCT01999738).

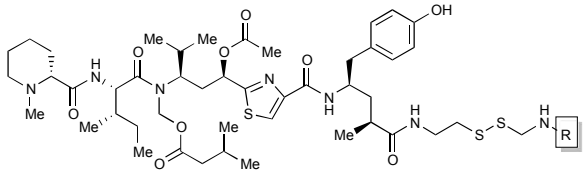
Success has also been achieved in conjugating tubulysin B hydrazide with peptide spacers of varying length to DUPA (2-[3-(1,3-dicarboxypropyl)ureido] pentanedioic acid), a molecule that binds to the cell surface glycoprotein prostate-specific membrane antigen (PSMA) overexpressed in prostate carcinomas and other tumors. The tubulysin-DUPA conjugates (such as **80**) exhibited PSMA-mediated delivery to LNCaP human prostate cancer cells, and released tubulysin B hydrazide (**19**) intracellularly to afford anti-proliferative activity in cell culture in the low nM range (IC_{50} = 5–24 nM).[47]



R = Folate (EC0305, **79**)



80



R = CDP (**81**)

Another approach described the linkage of tubulysin A with a linear copolymer of β-cyclodextrin and polyethylene glycol (CDP). CDP-polymer conjugates improve distribution to tumor site, increase aqueous solubility, and result in cellular uptake by endocytosis.[48,49] The tubuphenylalanine residue of tubulysin A was modified to incorporate a disulfide-containing amide that was linked to CDP through a disulfide bond designed to release the active thiol derivative intracellularly (**81**).[13] When conjugated, this complex self-assembled into nanoparticles. CDP-TubA increased the maximum tolerated dose to 6 mg/kg in nude mice, compared to 0.05 mg/kg for tubulysin A.[13] In two mouse xenograft models (HT29 human colorectal and H460 human non-small cells lung carcinomas) CDP-TubA (3 or 6 mg/kg) was as effective or superior to paclitaxel and vinblastine with minimal toxicity observed.[13]

Yet another study described a polymeric tubulysin conjugate that involved attachment of tubulysin analog **20** to a dendrimer via a hydrazone linker with the ketone of the phenethylamine that replaces Tup.[16] Dendrimer drug delivery systems target tumors by selective uptake due to the enhanced permeability and retention (EPR) effect, and other favorable effects on pharmacokinetics and toxicity.[50] When injected into mice bearing C26 murine colon carcinoma tumors, the tubulysin-dendrimer conjugate caused a tumor growth delay of 172% when dosed at 165 mg/kg (of the tubulysin analog), extending median survival time from 20 (control) to 38 days.[16] Non-conjugated tubulysin analog **20** did not delay tumor growth or increase median survival time at 10 or 20 mg/kg, and the higher dose caused two treatment-related deaths.[16] The tubulysin-dendrimer conjugate exhibited high aqueous solubility, and demonstrated increased efficacy and safety *in vivo*.[16]

Tubulysin conjugates show great potential for advancement to clinical trials, as the vast majority of data confirm that they retain substantial efficacy *in vivo* while reducing toxicity when compared to the parent tubulysins.

Conclusion

Tubulysins have emerged as promising anticancer leads due to their powerful activity in drug-resistant cells through a validated mechanism of action. Studies from numerous labs have revealed a great deal about tubulysin chemistry and biology, including their biosynthesis, bioactivity, structure-activity relationships and methods for synthesis. Although conjugates with the natural products seem to be the most promising means by which tubulysins may evolve into practical pharmaceutical agents, other studies deserve additional exploration. Further understanding of tubulysin biosynthesis holds promise for efficient production of tubulysins, perhaps far surpassing chemical total synthesis. Further analog design offers hope to improve the bioactivity and drug-like properties of tubulysins. Lastly, development of conjugates and other drug delivery systems may alleviate safety concerns, thus overcoming one of the most serious limitations of tubulysins. With the first tubulysin drug product (EC1456) now in human clinical trials, translation of these exquisitely potent natural products is now a reality.

References

1 F. Sasse, H. Steinmetz, J. Heil, G. Höfle and H. Reichenbach, *J. Antibiot.*, 2000, **53**, 879–885.
2 H. Steinmetz, N. Glaser, E. Herdtweck, F. Sasse, H. Reichenbach and G. Höfle, *Angew. Chem. Int. Ed.*, 2004, **43**, 4888–4892.
3 Y. Chai, D. Pistorius, A. Ullrich, K. J. Weissman, U. Kazmaier and R. Müller, *Chem. Biol.*, 2010, **17**, 296–309.
4 G. Höfle, N. Glaser, T. Leibold, U. Karama, F. Sasse and H. Steinmetz, *Pure Appl. Chem.*, 2003, **75**, 167–178.
5 K. Kubicek, S. K. Grimm, J. Orts, F. Sasse and T. Carlomagno, *Angew. Chem. Int. Ed.*, 2010, **49**, 4809–4812.
6 A. Sandmann, F. Sasse and R. Müller, *Chem. Biol.*, 2004, **11**, 1071–1079.
7 Y. Chai, S. Shan, K. J. Weissman, S. Hu, Y. Zhang and R. Muller, *Chem. Biol*., 2012, **19**, 361–371.
8 C. D. Richter, D. Nietlispach, R. W. Broadhurst and K. J. Weissman, *Nat. Chem. Biol.*, 2008, **4**, 75–81.
9 R. Balasubramanian, B. Raghavan, A. Begaye, D. L. Sackett and R. A. Fecik, *J. Med. Chem.*, 2009, **52**, 238–240.
10 G. Kaur, M. Hollingshead, S. Holbeck, V. Schauer–Vukašinović, R. Camalier, A. Dömling and S. Agarwal, *Biochem. J.*, 2006, **396**, 235–242.
11 A. Ullrich, J. Herrman, R. Müller and U. Kazmaier, *Eur. J. Org. Chem.*, 2009, **36**, 6367–6378.
12 M. W. Khalil, F. Sasse, H. Lünsdorf, Y. A. Elnakady and H. Reichenbach, *ChemBioChem*, 2006, **7**, 678–683.
13 T. Schluep, P. Gunawan, L. Ma, G. Jensen, J. Duringer, S. Hinton, W. Richter and J. Hwang, *Clin. Cancer Res.*, 2009, **15**, 181–189.
14 J. A. Reddy, R. Dorton, A. Dawson, M. Vetzel, N. Parker, J. S. Nicoson, E. Westrick, P. J. Klein, Y. Wang, I. R. Vlahov and C. P. Leamon, *Mol. Pharmaceut.*, 2009, **6**, 1518–1525.
15 C. P. Leamon, J. A. Reddy, M. Vetzel, R. Dorton, E. Westrick, N. Parker, Y. Wang and I. Vlahov, *Cancer Res.*, 2008, **68**, 9839–9844.

16 W. C. Floyd III, G. K. Datta, S. Imamura, H. M. Kieler–Ferguson, K. Jerger, A. W. Patterson, M. E. Fox, F. C. Szoka, J. M. J. Frechet and J. A. Ellman, *ChemMedChem*, 2011, **6**, 49–53.

17 B. Raghavan, R. Balasubramanian, J. C. Steele, D. L. Sackett and R. A. Fecik, *J. Med. Chem.*, 2008, **51**, 1530–1533.

18 R. Balasubramanian, B. Raghavan, J. C. Steele, D. L. Sackett and R. A. Fecik, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 2996–2999.

19 P. S. Shankar, S. Bigotti, P. Lazzari, I. Manca, M. Spiga, M. Sani, and M. Zanda, *Tetrahedron Lett.*, 2013, **54**, 6137–6141.

20 Z. Wang, P. A. McPherson, B. S. Raccor, R. Balachandran, G. Zhu, B. W. Day, A. Vogt and P. Wipf, *Chem. Biol. Drug Des.*, 2007, **70**, 75–86.

21 A. W. Patterson, H. M. Peltier, F. Sasse and J. A. Ellman, *Chem. Eur. J.*, 2007, **13**, 9534–9541.

22 X. Yang, C. Dong, J. Chen, Q. Liu, B. Han, Q. Zhang, and Y. Chen, *Tetrahedron Lett.*, 2013, **54**, 2986–2988.

23 J. L. Burkhart, R. Müller and U. Kazmaier, *Eur. J. Org. Chem.*, 2011, **16**, 3050–3059.

24 J. Eirich, J. L. Burkhart, A. Ullrich, G. C. Rudolf, A. Vollmar, S. Zahler, U. Kazmaier, and S. A. Sieber, *Mol. BioSyst.*, 2012, **8**, 2067–2075.

25 O. Pando, S. Stark, A. Denkert, A. Porzel, R. Preusentanz and L. A. Wessjohann, *J. Am. Chem. Soc.*, 2011, **133**, 7692–7695.

26 I. R. Vlahov, Y. Wang, M. Vetzel, S. Hahn, P. J. Kleindl, J. A. Reddy and C. P. Leamon, *Bioorg. Med. Chem. Lett*., 2011, **21**, 6778–6781.

27 T. Shibue, T. Hirai, I. Okamoto, N. Morita, H. Masu, I. Azumaya, and O. Tamura, *Chem. Eur. J.*, 2010, **16**, 11678–11688.

28 T. Shibue, I. Okamoto, N. Morita, H. Morita, Y. Hirasawa, T. Hosoya and O. Tamura, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 431–434.

29 P. S. Shankar, M. Jagodzinska, L. Malpezzi, P. Lazzari, I. Manca, I. R. Greig, M. Sani, and M. Zanda, *Org. Biomol. Chem.*, 2013, **11**, 2273–2287.

30 S. Shankar P., M. Sani, F. R. Saunders, H. M. Wallace and M. Zanda, *Synlett*, 2011, **12**, 1673–1676.

31 O. Pando, S. Dörner, R. Preusentanz, A. Denkert, A. Porzel, W. Richter and L. Wessjohann, *Org. Lett.*, 2009, **11**, 5567–5569.

32 X.-d. Yang, C.-m. Dong, J. Chen, Y.-h. Ding, Q. Liu, X.-y. Ma, Q. Zhang, and Y. Chen, *Chem. Asian J.*, 2013, **8**, 1213–1222.

33 H. M. Peltier, J. P. McMahon, A. W. Patterson and J. A. Ellman, *J. Am. Chem. Soc.*, 2006, **128**, 16018–16019.

34 A. Dömling, B. Beck, U. Eichelberger, S. Sakamuri, S. Menon, Q.-Z. Chen, Y. Lu and L. Wessjohann, *Angew. Chem. Int. Ed.*, 2006, **45**, 7235–7239.

35 A. Dömling, B. Beck, U. Eichelberger, S. Sakamuri, S. Menon, Q.-Z. Chen, Y. Lu and L. Wessjohann, *Angew. Chem. Int. Ed.*, 2007, **46**, 2347.

36 M. Sani, G. Fossati, F. Huguenot and M. Zanda, *Angew. Chem. Int. Ed.*, 2007, **46**, 3526–3529.

37 P. Wipf, T. Takada and M. J. Rishel, *Org. Lett.*, 2004, **6**, 4057–4060.

38 P. Wipf and Z. Wang, *Org. Lett.*, 2007, **9**, 1605–1607.

39 S. Chandrasekhar, B. Mahipal and M. Kavitha, *J. Org. Chem.*, 2009, **74**, 9531–9534.

40 T. Shibue, T. Hirai, I. Okamoto, N. Morita, H. Masu, I. Azumaya and O. Tamura, *Tetrahedron Lett.*, 2009, **50**, 3845–3848.

41 A. W. Patterson, H. M. Peltier and J. A. Ellman, *J. Org. Chem.*, 2008, **73**, 4362–4369.

42 S. Shankar P., M. Sani, G. Terraneo and M. Zanda, *Synlett*, 2009, **8**, 1341–1345.

43 J. L. Burkhart and U. Kazmaier, *RSC Adv.*, 2012, **2**, 3785–3790.

44 M. Smrcina, P. Majer, E. Majerová, T. A. Guerassina and M. A. Eissenstat, *Tetrahedron*, 1997, **53**, 12867–12874.

45 G. K. Friestad, J.–C. Marie and A. M. Deveau, *Org. Lett.*, 2004, 6, 3249–3252.

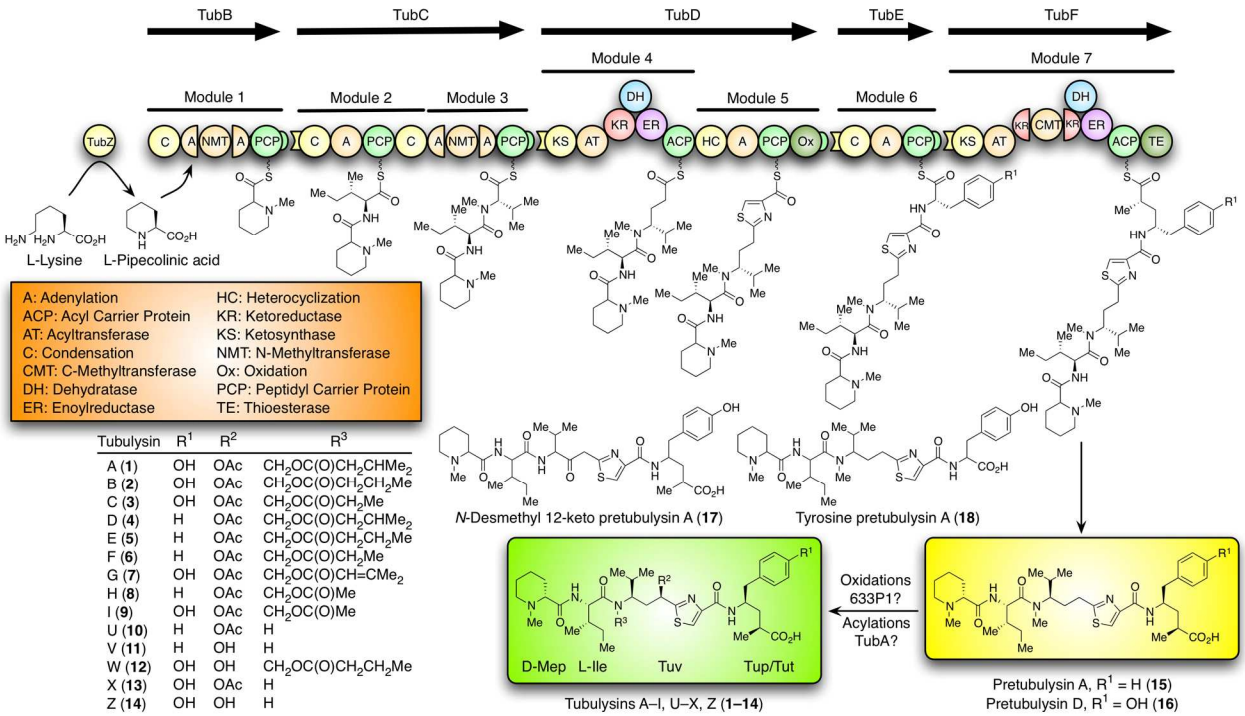
46 I. R. Vlahov, Y. Wang, P. J. Kleindl and C. P. Leamon, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 4558–4561.

47 S. A. Kularatne, C. Venkatesh, H. – K. R. Santhapuram, K. Wang, B. Vaitilingam, W. A. Henne and P. S. Low, *J. Med. Chem.*, 2010, **53**, 7767–7777.

48 J. Cheng, K. T. Khin, G. S. Jensen, A. Liu, M. E. Davis, *Bioconjug. Chem.*, 2003, **14**, 1007–1017.

49 J. Cheng, K. T. Khin, M. E. Davis, *Mol. Pharmaceut.*, 2004, **1**, 183–193.

50 S. H. Medina and M. E. H. El-Sayed, *Chem. Rev.*, 2009, **109**, 3141–3157.



Tubulysin	R^1	R^2	R^3
A (**1**)	OH	OAc	$CH_2OC(O)CH_2CHMe_2$
B (**2**)	OH	OAc	$CH_2OC(O)CH_2CH_2Me$
C (**3**)	OH	OAc	$CH_2OC(O)CH_2Me$
D (**4**)	H	OAc	$CH_2OC(O)CH_2CHMe_2$
E (**5**)	H	OAc	$CH_2OC(O)CH_2CH_2Me$
F (**6**)	H	OAc	$CH_2OC(O)CH_2Me$
G (**7**)	OH	OAc	$CH_2OC(O)CH{=}CMe_2$
H (**8**)	H	OAc	$CH_2OC(O)Me$
I (**9**)	OH	OAc	$CH_2OC(O)Me$
U (**10**)	H	OAc	H
V (**11**)	H	OH	H
W (**12**)	OH	OH	$CH_2OC(O)CH_2CH_2Me$
X (**13**)	OH	OAc	H
Z (**14**)	OH	OH	H

Figure 1. Tubulysin biosynthetic pathway and structures of pretubulysin and tubulysin metabolites.