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Semisynthesis and degradation of the tubulin inhibitors epothilone and tubulysin*

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Abstract: The structure–activity relationships of epothilones indicate that major modifications are only tolerated in the western ring segment. In particular, C2 methyl of the thiazole ring appears to be most flexible. Its broad modification started from epothilone F, which was obtained from natural epothilone B by hydroxylation via the *N*-oxide. Some of the prepared derivatives exhibit improved esterase stability in addition to high cytotoxic activity. For these and other favorable properties, amine (BMS-310705) was recently introduced in clinical trials. In an alternative approach, modified side chains were introduced by replacement of the C12,C15 ring segment via ring-opening olefin metathesis (ROM) of epothilone C in the presence of ethylene to 12,13-*seco*-epothilone C, introduction of a synthetic building block followed by ring-closing olefin metathesis (RCM), and epoxidation to the 16-alkyne analog of epothilone A.

The structure of the tetrapeptide tubulysin D was confirmed by total hydrolysis to *N*-methyl D-pipecolic acid, L-isoleucine, tubuvaline (Tuv), tubuphenylalanine (Tup), formaldehyde, and 3-methylbutyric acid. Mild acidic hydrolysis to cyclo-tubulysin and oxidative degradation to L-valine allowed the assignment of the stereocenters of Tuv, hydrazinolysis, and comparison with synthetic reference samples to that of Tup. The absolute configuration of tubulysin D is: (*R*)-Mep, (2*S*,3*S*)-Ile, (1′*R*,3′*R*)-Tuv, and (2*S*,4*R*)-Tup.

INTRODUCTION

Tubulin is a major component of all nucleated (eucaryotic) cells and comprises, for example, 10–20 % of the protein inventory of the brain. Soluble tubulin is a noncovalent dimer of two similar subunits, α- and β-tubulin, each with a molecular weight of about 50 kDa. In the presence of guanosine 5′-triphosphate (GTP), these heterodimers polymerize in a head-to-tail fashion to give fibers known as protofilaments, which, in turn, by lateral forces form sheets and eventually tubes normally containing 13 protofilaments, the well-known microtubules. Functional microtubules are decorated with a number of further proteins and show a highly dynamic behavior: They grow and shrink by addition and dissociation of α/β-tubulin heterodimers, a process that is also designated as polymerization and depolymerization. This process is highly regulated and vital for a variety of functions such as migration of cells, intracellular transport of vesicles, and formation of the mitotic spindle [1]. Not surprisingly, during evolution, Nature has developed a number of inhibitors and promoters of the complex processes. Most of these compounds are produced as secondary metabolites by microorganisms, marine organisms, and higher plants [1,2]. Targeting the tubulin system of other species, they became powerful

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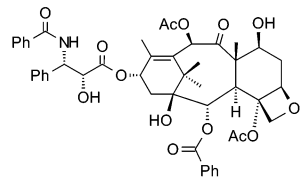
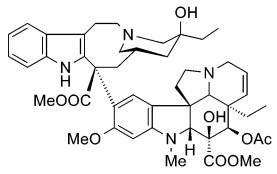
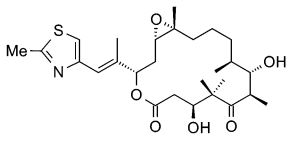


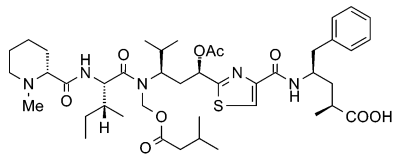
Taxol^{R} (**1**)



Vinblastin (**2**)



Epothilone B (**3**)



Tubulysin D (**4**)

weapons by inhibition of cell division, including rapidly dividing human cancer cells. Prototypes of tubulin polymerization inhibitors are colchicine and the vinca alkaloids such as vinblastine (**2**), whereas Taxol® (**1**) was the first compound that inhibits depolymerization of microtubules and is a promoter of tubulin polymerization. Both vinca alkaloids and Taxol were developed to clinically most successful anticancer drugs. Nevertheless, they are far from ideal drugs, showing toxic side effects and impaired activity for resistant tumors. Apart from optimization of the existing drugs by semisynthesis, screening for new tubulin inhibitors, in particular from microorganisms and marine organisms, was quite successful in the past decade [1–3]. Among others, three highly cytotoxic metabolites from myxobacteria, epothilone (**3**) [4,5], tubulysin (**4**) [6], and disorazole [7] (structure not shown) were identified as tubulin inhibitors. In 1995, Bollag et al. at the Merck Research Laboratories discovered that epothilones A and B (**3**), like Taxol (**1**), induce tubulin polymerization and stabilize microtubules. Both groups of compounds apparently occupy the same or overlapping binding sites on β-tubulin. The observation that epothilones retain their activity for resistant cancer cells [5,8] and other favorable properties such as increased water solubility [4] inspired many total syntheses [9], and simple production by fermentation facilitated extensive semisynthetic modification [10].

Table 1 Cytotoxic activity of Taxol (**1**), vinblastine (**2**), epothilone B (**3**), tubulysin D (**4**), and dolastatin 10 (**34**) for sensitive and resistant cell lines (IC_{50} ng/ml).

Compound	Cell line			
	L 929[a]	K-562[b]	KB-3.1[c]	KB-V1[d]
Taxol (**1**)	80	10	10	150
Vinblastine (**2**)	15	6	7	120
Epothilone B (**3**)	0.7	0.3	0.6	0.3
Tubulysin D (**4**)	0.03	0.02	0.02	0.08
Dolastatin 10 (**34**)	0.1	0.1	0.2	1.2

[a]Fibroblast cell line from connective tissue of a mouse.

[b]Human myelogenous leukemia cell line.

[c]Human cervix carcinoma cell line.

[d]Multidrug-resistant KB clone.

Years later, the tubulysins were isolated from the myxobacteria *Archangium gephyra* and *Angiococcus disciformis* for their cytotoxic activity [6]. They soon turned out to be the microbial counterparts of the vinca alkaloids, inhibiting tubulin polymerization and inducing depolymerization of microtubules. Moreover, tubulysins appear to occupy the vinca binding site on β-tubulin and, like the epothilones, retain their activity for vinblastine and multidrug-resistant tumor cells [6,11]. Table 1 gives an overview of the cytotoxic activities for some sensitive and resistant cell lines. Both epothilone B (**3**) and tubulysin D (**4**) are by a factor of 10–500 more active than the plant-derived Taxol (**1**) and vinblastine (**2**), although their structures are much smaller and by far less complex. However, these figures should not be overemphasized because for clinical application as an antitumor agent, in vivo efficacy at a tolerable dose is needed and not sheer cytotoxicity. To improve the desirable properties of epothilones and tubulysins, great efforts in total synthesis and chemical derivatization have been undertaken and are still ongoing. Recent results of our work on the modification of the epothilone side chain and on the degradation and elucidation of the absolute configuration of tubulysin will be presented.

STRUCTURE–ACTIVITY RELATIONSHIPS OF EPOTHILONE

Since the discovery of the anti-tubulin activity of epothilones A and B (**3**), several hundred derivatives have been prepared by derivatization of the functional groups [10], and an even greater variety of derivatives and analogs have been obtained by total synthesis [9]. In addition, 37 structural variants of biosynthesis were isolated from the bacterial cultures [12], which, in most cases, would not have been easily accessible by chemical synthesis. Although all these compounds were not tested under the same conditions and assessed by different standards, a fairly consistent picture of the structure–activity relationship was obtained (Fig. 1). Whereas most functional groups of the macrocycle have to stay in place (shaded area), the lactone can be changed to a lactam [10g] and the C6 methyl can be replaced by small alkyl or alkenyl groups [13]. Quite unexpectedly, the epoxide is not essential for biological activity. It may be replaced by an episulfide [10i], aziridine [10h], cyclopropane [10f], or even a *Z* double bond [14,15]. Apparently, all these groups provide the same rigid conformation of the C11/C14 ring segment, which, in turn, stabilizes the required conformation of the entire macrocycle. Hydrogenation of the C12, C13 double bond is accompanied by complete loss of activity. The double bond in the side chain and its *E* configuration is also essential for biological activity [16], and its methyl substituent increases activity by a factor of 5 [12]. This observation suggested the replacement of the linker region by a benzene ring, and, in fact, a benzothiazole side chain confers excellent biological activity [13,17]. Replacement of the thiazole by an oxazole [12,14,15] or pyridine [13,18] retains activity as long as the nitrogen stays in its place. Remarkably, *N*-oxidation in the epothilone B series reduces activity only slightly; however, in the epothilone A series it reduces activity by a factor of 50 [10d]. The methyl substituent on the thiazole turned out to be the most flexible part of the molecule. Provided the modification does not demand more space than three carbon atoms, high activity is observed with most lipophilic and polar groups. However, replacement of the methyl by a carboxy group reduces activity by a factor

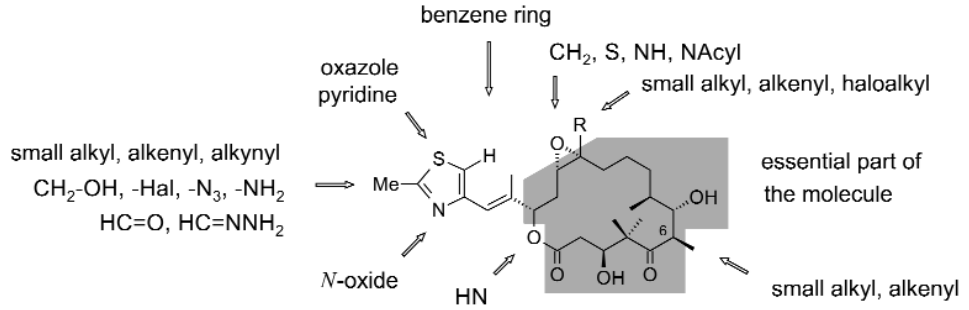
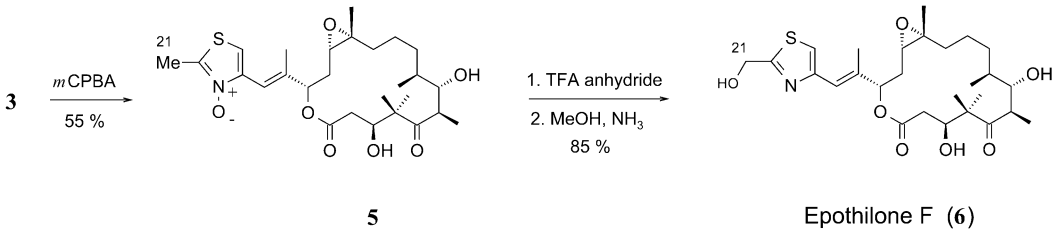


Fig. 1 Structure–activity relationships of epothilone.

of 25 [19]. In the following section, some of these modifications of the side chain and the linker region will be described in more detail.

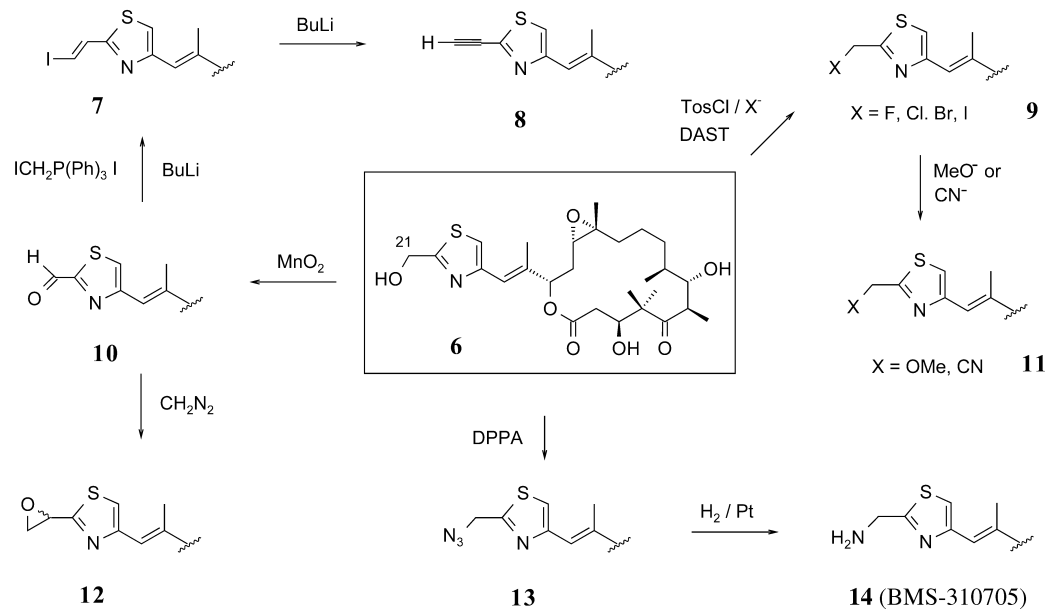
DERIVATIZATION OF THE C21 METHYL OF EPOTHILONES A AND B

Starting from natural epothilones produced by fermentation, functionalization was initiated by *N*-oxidation using *m*-chloroperbenzoic acid to give, for example, epothilone B-*N*-oxide (**5**) in 55 % yield. Treatment of **5** with trifluoroacetic acid anhydride induced a Polonovsky-type rearrangement that transposes the oxygen from nitrogen to the side chain (Scheme 1). Work-up with methanolic ammonia gave the hydroxymethyl derivative epothilone F (**6**) in 85 % yield [10d].



Scheme 1 Hydroxylation at C21 of epothilone B (**3**).

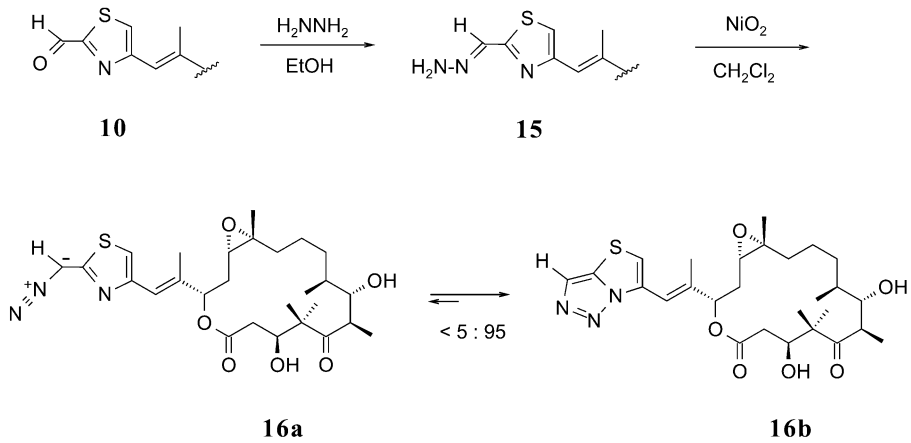
Epothilone F has been isolated before in trace amounts as a by-product of fermentation [20] and later was also obtained by biotransformation of epothilone B (**3**) using *Streptomyces* and *Amycolata* strains [21]. Obviously, the hydroxymethyl group is extremely versatile, leading to a great variety of further derivatives [19] (Scheme 2). As "benzylic" alcohol, it was, for example, smoothly oxidized by manganese dioxide to aldehyde **10**, and to the corresponding carboxylic acid by subsequent treatment with silver oxide. Carbon chain extension of aldehyde **10** was achieved by Grignard- and Wittig-type reactions (e.g., to the iodovinyl compound **7**, which on treatment with *n*-butyl lithium gave the alkyne analog **8**). Diazomethane transformed the aldehyde **10** to a 1:1 mixture of diastereomeric epoxides **12**.



Scheme 2 Derivatization of the C21 hydroxy group of epothilone F (**6**).

Tosylation of alcohol **6** and nucleophilic displacement with halide ions gave the chloro, bromo, and iodo derivatives **9**, whereas reaction with diethylaminosulfurtrifluoride (DAST) introduced fluorine. Also, by nucleophilic displacement, methyl ether and cyano derivatives **11** were obtained from the bromomethyl derivative **9**. Treatment of alcohol **6** with diphenylphosphoryl azide gave the azidomethyl derivative **13**, which, by catalytic hydrogenation, yielded the amine **14**.

Alternatively, nitrogen may be introduced via the aldehyde **10** to give, for example, hydrazone **15**. This, following literature precedence, was oxidized with nickel peroxide to give the mixture of diazo and triazolo compounds **16a** and **16b** (Scheme 3). According to NMR, the equilibrium is far in favor of the cyclo form **16b**, however, an IR bond at 2078 cm^{-1} indicates presence of the diazo form **16a**. From the high cytotoxicity, good tubulin binding can be expected, which makes these compounds ideal candidates for photoaffinity labeling of the epothilone binding site [22].



Scheme 3 Synthesis of 21-diazo epothilone B (**16a**) and equilibration with its cyclo-form (**16b**).

During the preliminary evaluation of the derivatives prepared, we investigated also the stability of the lactone bond. In our early work on epothilones A and B, we had noted that the lactone is easily cleaved by esterases and hypothesized that this is responsible for the short half-life of epothilones A and B in the early animal studies. Colleagues at Bristol-Myers Squibb (BMS) confirmed this finding and

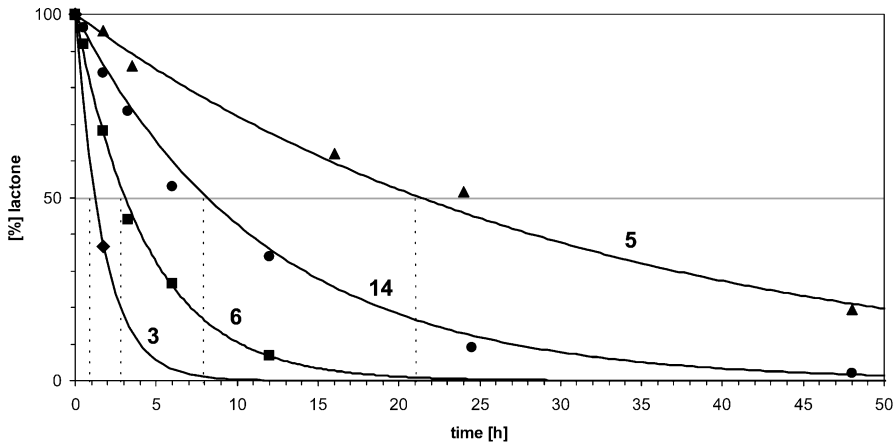


Fig. 2 Cleavage of the lactone bond of epothilones **3**, **5**, **6**, and **14** by pig liver esterase.

developed a semisynthesis of the epothilone B lactam that is resistant to esterases [10g]. This compound (BMS-247550) is presently in phase II clinical studies as an anticancer drug [23]. When the side chain-modified epothilones were investigated for esterase stability in a model system using pig liver esterase, remarkable differences were observed (Fig. 2).

Whereas epothilone B (**3**) had a half-life of 1.2 h, that of the more polar epothilone F (**6**) and the amine **14** was 4.0 and 8.1 h, and that of the *N*-oxide **5** even 20.9 h [19]. The origin of this remote control of esterase activity is not known. Owing to the improved stability and other favorable properties, epothilone B amine (**14**, BMS-310705) was introduced by BMS in phase I clinical trials [23].

REPLACEMENT OF THE SIDE CHAIN C16 ALKENE FOR AN ALKYNE

Replacement of the thiazole ring of epothilones for other heterocycles revealed that for good activity the nitrogen has to stay in its place (Fig. 3) [13,18]. This is in good agreement with our earlier findings [10c,e] that any substituents in the 5-position of the thiazole abolish biological activity.

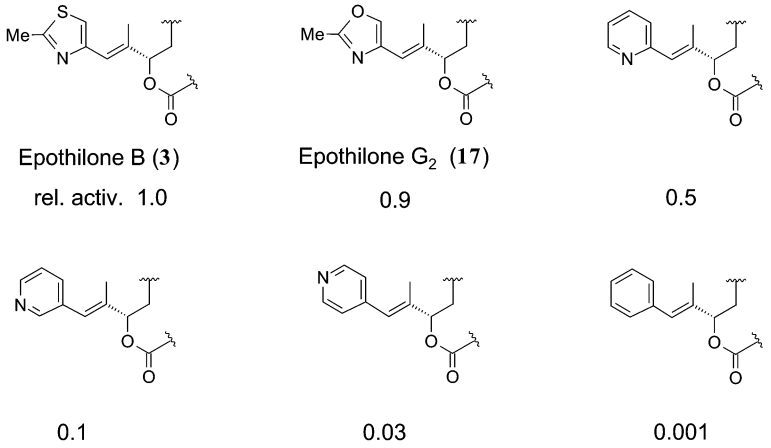


Fig. 3 Relative biological activity of thiazole-modified epothilones.

This was explained by rotation of the thiazole due to repulsion of the C16 methyl and the substituent X in structure **A**, with the result that the nitrogen is not available for a hydrogen bridge to the tubulin protein. When the original conformation is locked by a benzene ring in the original conformation (structure **B**), highly active derivatives are obtained. With this information in mind, we wondered what the activity of hitherto unknown alkyne analogs **C** would be. As in **B**, there is only one axis of rotation of the side chain, with the difference that the nitrogen in C is closer to that axis and slightly moved away from the macrocycle.

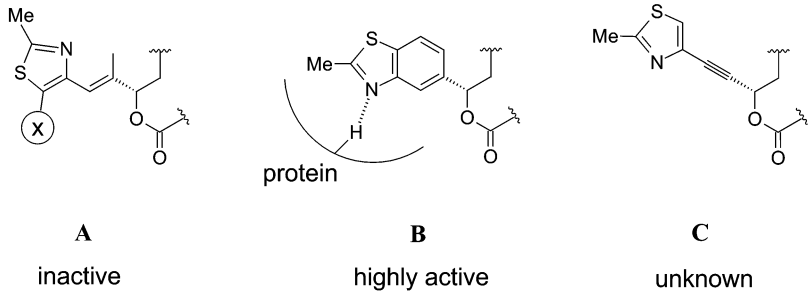
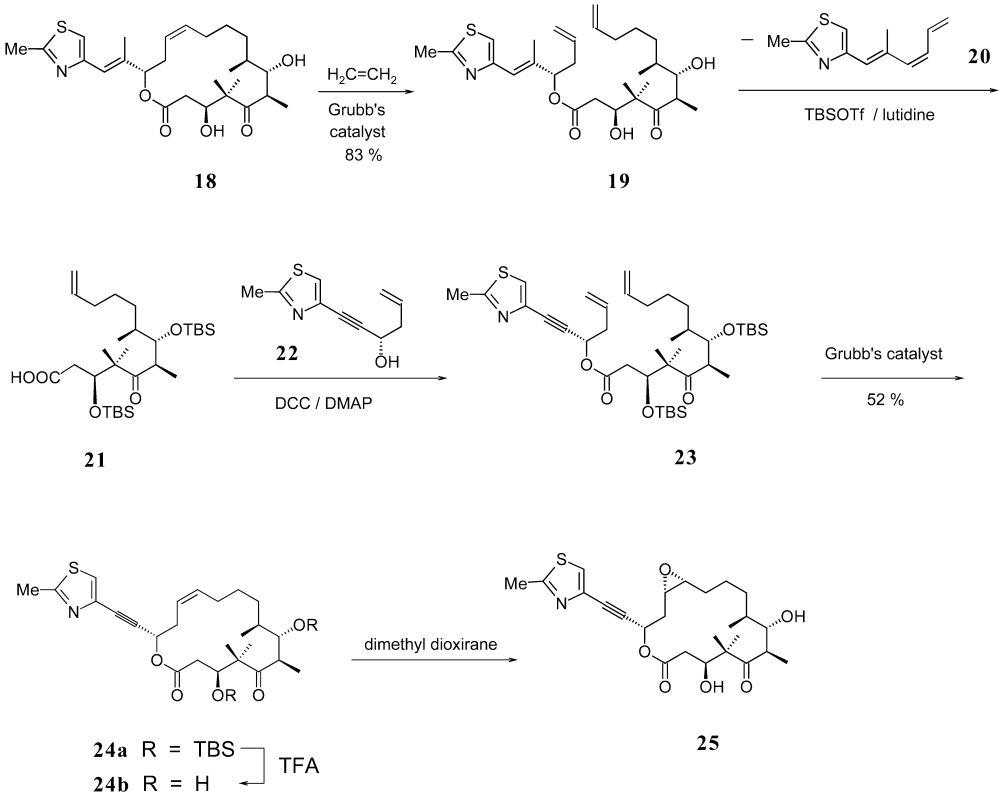


Fig. 4 Placement of the nitrogen in epothilone derivatives **A** and analogs **B** and **C**.

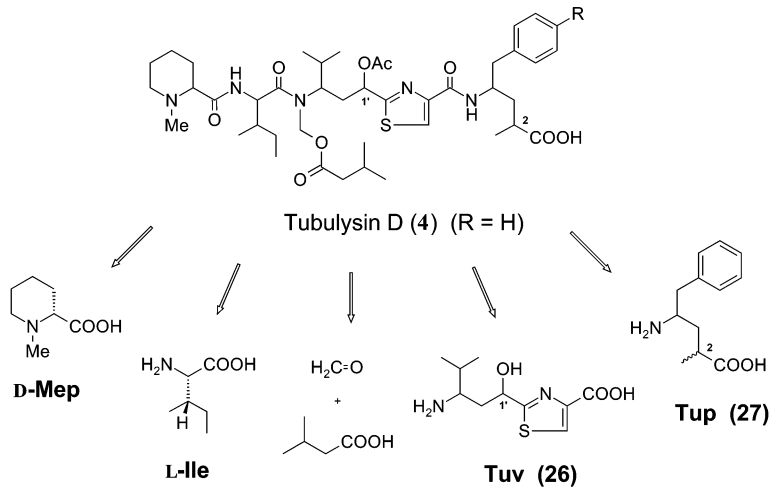
After some experimentation, it was concluded that the shortest access to type C compounds would be exchange of a whole ring segment carrying the side chain. Starting with epothilone C (**18**), produced by fermentation, we found that with an excess of ethylene and second-generation (imidazolidine) Grubb's catalysts ring-opening olefin metathesis (ROM) to **19** occurred in good yield [16]. Silylation with an excess of reagents not only protected the hydroxy groups of **19** but also cleaved the ester by elimination of trien **20** to give carboxylic acid **21**. The new ring segment **22** with the desired triple bond was obtained by (*S*)-BINOL catalyzed allylation of propynal as described by Keck et al. [24], followed by Sonogashira coupling with 4-bromo-2-methylthiazole. Esterification of **21** and **22** with 1,3-dicyclohexylcarbodiimide/4-dimethylaminopyridine (DCC/DMAP) gave **23**, which underwent RCM with Grubb's catalyst to **24a** as described in Nicolaou's [25] and Schinzer's [26] total syntheses of epothilone C (**18**). After deprotection and separation of the 12*E*/*Z* isomers, the 12*Z* isomer was treated with dimethyl dioxirane to give a mixture of α and β epoxide from which **25** with the natural epoxide configuration was separated by chromatography (Scheme 4). Although this synthetic approach has the serious drawback that in the RCM and epoxidation, inevitably mixtures of isomers are formed it allows us to synthesize analogs of epothilone A in only six steps from allylic alcohols and natural epothilone C (**18**) for biological testing. The alkyne analogs **24b** and **25** were by a factor of more than 100 less active than their natural counterparts.



Scheme 4 Side chain replacement of epothilone C (**18**) by ROM/RCM.

DEGRADATION AND ABSOLUTE CONFIGURATION OF TUBULYSIN D

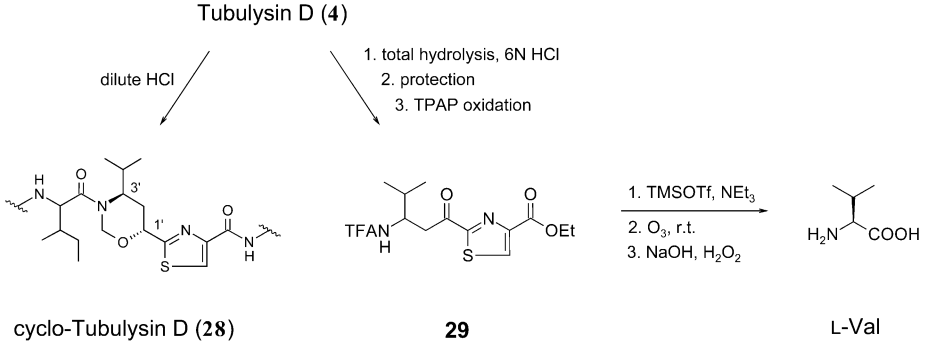
In our continued screening of myxobacteria, two *A. gephyra* and *A. disciformis* strains were identified by their high cytotoxic activity [6]. Small samples of several pure compounds responsible for this activity were obtained only after multistep chromatographic enrichment. On closer inspection, it turned out



Scheme 5 Total hydrolysis of tubulysin D (**4**) with hydrochloric acid.

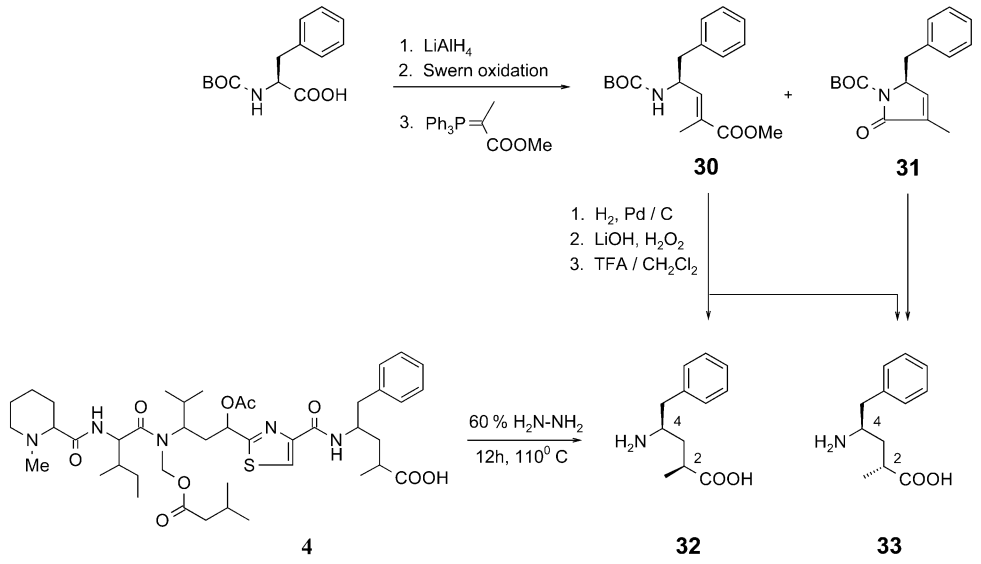
that in cell culture they induce degradation of the tubulin cytoskeleton and consequently were named tubulysins.

Their structures were derived from the elemental composition and 2D NMR spectra of the basic components, tubulysin A (R = OH) and tubulysin D (**4**, R = H). Both are tetrapeptides from *N*-methyl pipecolic acid, isoleucine, and the novel amino acids named Tuv (**26**), Tup (**27**), or tubutyrosine (Tut), R = OH, respectively [27] (Scheme 5). Another unusual feature of tubulysins is a *N*,*O*-acetal at the Tuv amino group, which is *O*-acylated by various short-chain fatty acids. Total acidic hydrolysis of **4** yielded the expected amino acids, 3-methylbutyric acid, and formaldehyde, which was identified as methylene-bis(2,4-dinitrophenyl hydrazine). After trifluoracetylation and esterification with ethanol, gas chromatography (GC) analysis on chiral columns identified *N*-methyl-D-pipecolic acid and L-isoleucine. To assign the two stereocenters of Tuv (**20**), the hydrolysis mixture from tubulysin D (**4**), after protection, was oxidized with tetrapropylammonium perruthenate (TPAP). The mixture obtained containing ketone **29** after silylation to the enol ether was degraded by ozonation to give L-valine. Mild acidic hydrolysis of tubulysin D (**4**), on the other hand, cleaves both acyl groups followed by cyclization to cyclo-tubulysin D (**28**) through an intermediate *N*-acyl methylene-immonium ion (Scheme 6). NMR analysis of **28** revealed *anti* configuration of C1′ and C3′, and thus Tuv has 1′*R*,3′*R* configuration.



Scheme 6 Partial hydrolysis of tubulysin D (**4**) to cyclo-tubulysin D (**28**) and degradation of **4** to L-valine.

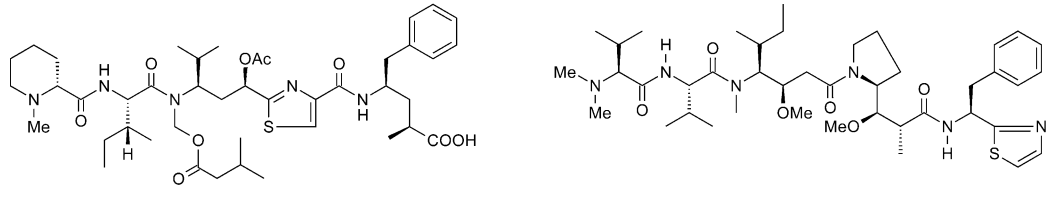
Tup was obtained from acidic hydrolysis of tubulysin D (**4**) as a 1:1 mixture of diastereomers. Assuming that racemization had occurred at the C2 center, we started a synthesis of reference compounds from L-BOC-Phe by alanate reduction, Swern oxidiation to the aldehyde, and chain extension by Wittig reaction. Whereas the 2*E* olefin **30** was stable, the 2*Z* isomer spontaneously cyclized to the γ-lactam **31**. On catalytic hydrogenation of **30**, a 2:1 mixture of diastereomers was formed identical to that obtained in the ratio of 1:1 by acidic hydrolysis of **4** (Scheme 7). Hydrogenation of **31** gave only one isomer with the absolute configuration shown in **33**. Hydrazinolysis of tubulysin D (**4**) also gave only one isomer identified as **32** with 2*S*,4*R* configuration.



Scheme 7 Hydrazinolysis of tubulysin D (**4**) and stereoselective synthesis of the Tup epimers **32** and **33**.

From these results the tetrapeptide tubulysin D (**4**) is constructed from *N*-methyl-D-pipecolic acid, L-isoleucine, (1′*R*,3′*R*)-tubuvaline, and (2*S*,4*R*)-tubuphenylalanine.

Most remarkably, the tubulysins show great similarity to dolastatin 10 (**34**) first isolated by Pettit et al. [28] from the sea hare *Dolabella auricularia*. Both compounds are unusual peptides of similar size and composition, and both are potent inhibitors of tubulin polymerization [11,29] (Table 1). Their biosynthesis enzymes are apparently polypeptide-polyketide synthase hybrids incorporating valine, isoleucine, cysteine, phenylalanine, acetate, and propionate building blocks, though in different sequence. Furthermore, the cysteines are cyclized to thiazoles, and the first and third amino acids are *N*-methylated. These similarities are a strong indication for a common ancestor of the biosynthesis genes, which have been optimized independently for production of two different molecules with high tubulin binding affinity. This is even more probable since Moore's [30] group discovered that the actual producer of dolastatin 10 (**34**) and the related symplostatins are marine cyanobacteria.



Tubulysin D (**4**) Dolastatin 10 (**34**)

However, as long as the genes for both pathways have not been cloned and analyzed, an independent invention and convergent evolution of the structures cannot be excluded.

CONCLUSIONS

Epothilones (**3**) and tubulysins (**4**) are recognized as powerful promoters and inhibitors of tubulin polymerization like their plant-derived counterparts Taxol (**1**) and vinblastine (**2**). We have demonstrated that the methyl group in the thiazole ring of epothilones can be modified extensively, with retention of activity and improvement of pharmacological properties. Access to more deep-seated modifications was achieved by replacement of the C12,C15 ring segment of epothilones using a ROM/RCM strategy. Whereas RCM is widely used in natural product synthesis, to our knowledge ROM or cleavage by olefin metathesis of complex natural products has not yet been described. Apart from semisynthesis, cleavage of natural products by olefin metathesis may be useful in the structure analysis of olefinic natural products.

The absolute configuration of the seven stereogenic centers of the tetrapeptide tubulysin D (**4**) was determined by degradation and comparison with authentic samples. Interestingly, in the structurally related dolastatin 10 (**34**) the isoleucine, valine, and phenylalanine building blocks have the same absolute configuration as in tubulysin D, whereas the first amino acids, *N*-methyl pipecolic acid and *N*,*N*-dimethylvaline, are homologous but have different configuration. From the structural similarity, it can be expected that both compounds occupy the same binding site on tubulin, leaving those groups open for modification, which are different, for example, in tubulysin D, the carboxy group, and the acyloxymethylene side chain.

ACKNOWLEDGMENTS

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