Natural Products

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Pretubulysin, a Potent and Chemically Accessible Tubulysin Precursor from *Angiococcus disciformis*

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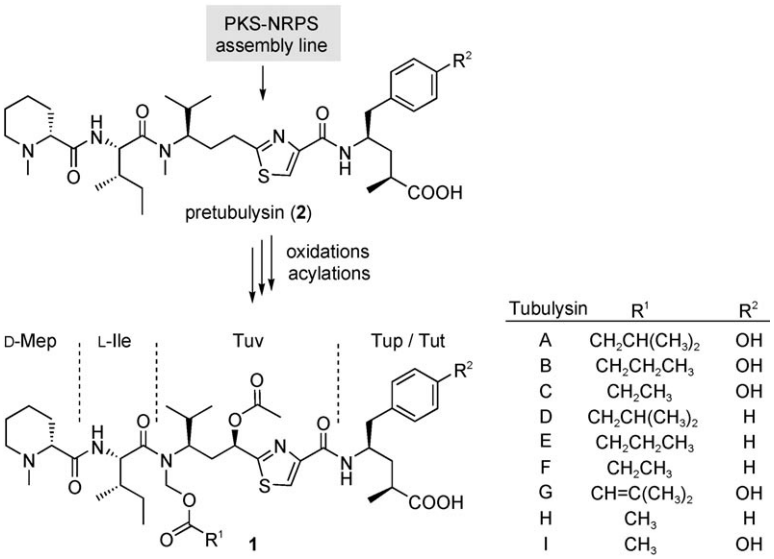
Dedicated to Prof. Heinz G. Floss on the occasion of his 75th birthday

The tubulysins (**1**) are a family of nine secondary metabolites (Scheme 1) produced by several strains of myxobacteria, including *Angiococcus disciformis* An d48 and *Archangium gepyhra* Ar315.[1] The compounds share a linear tetrapeptide core, consisting of *N*-methylpipecolic acid (Mep), isoleucine (Ile), a novel amino acid called tubuvaline (Tuv), and a chain-extended analogue of either phenylalanine or tyrosine, referred to as tubuphenylalanine (Tup) or tubutyrosine (Tut), respectively. An acetoxy moiety is common to all structures, while the acyl group within the bis-acyl *N*,*O*-acetal substituent varies in size from acetate in tubulysins H and I, to 3-methyl butyrate in tubulysins A and D. The tubulysins are among a handful of natural products that interact with the eukaryotic cytoskeleton, inhibiting the polymerization of tubulin at very low concentrations ($< 50\ pg\,mL^{-1}$).[1,2] Notably, their ability to suppress the growth of cancer cells exceeds that of other tubulin modifiers, including the epothilones, vinblastine, and Taxol, by 20- to 100-fold. The clear potential to deploy the tubulysins against multidrug-resistant tumors has stimulated significant research into the chemistry and biology of these compounds. Structure–activity relationship (SAR) studies using synthetic analogues of tubulysin D,[3–6] the most active metabolite, have begun to identify the essential structural features underlying its cytotoxicity, and also suggest strategies for optimizing the metabolite's pharmacological properties. Together, the data reveal a surprising tolerance to structural modification; for example, only a minor loss of activity was observed when the chemically labile *N*,*O*-acetal was replaced with simple alkyl groups.



Tubulysin	R^1	R^2
A	$CH_2CH(CH_3)_2$	OH
B	$CH_2CH_2CH_3$	OH
C	CH_2CH_3	OH
D	$CH_2CH(CH_3)_2$	H
E	$CH_2CH_2CH_3$	H
F	CH_2CH_3	H
G	$CH{=}C(CH_3)_2$	OH
H	CH_3	H
I	CH_3	OH

Scheme 1. Proposed biosynthetic pathway to the nine known tubulysins A–I.

Our complementary approach to pure chemical synthesis has been to elucidate in detail the biosynthesis of myxobacterial natural products such as the tubulysins, in order to identify novel metabolites and enable the genetic engineering of derivatives.[7] We report here the structure of a new compound, pretubulysin, from *A. disciformis*, which was elucidated by feeding studies, high-resolution mass spectrometry, and comparison to synthetic material. Pretubulysin, whose structure is identical to that of a previously postulated biosynthetic intermediate,[7a] retains the high tubulin-degrading activity of its more complex tubulysin relatives.

Sequencing of the tubulysin gene cluster in *A. disciformis*, which is known to produce tubulysins D, E, F, and H (Scheme 1),[1b] showed that the metabolites are assembled on a hybrid system made up of polyketide synthases (PKSs) and non-ribosomal polypeptide synthetases (NRPSs); this multienzyme "assembly line" consists of five NRPS modules and two PKS modules.[7a] The approximately 40 kbp cluster also contains a cyclodeaminase-encoding gene, *tubZ*, whose protein product is likely to be involved in the biosynthesis of

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pipecolic acid, the presumed starter unit for tubulysin assembly.

To demonstrate directly the involvement of *tubZ* in the pathway, we inactivated the gene by insertional mutagenesis. As this mutation was expected to interrupt the supply of pipecolic acid, we anticipated that the resulting strain would no longer produce tubulysins. To our surprise, analysis by HPLC-MS revealed tubulysin D in extracts of the mutant An d48-*tubZ*$^-$ at approximately 3 % of the wild-type level, but in addition, substantial amounts of the novel metabolite **2** (*m*/*z* 670.4; Figure 1); tubulysins E, F, and H were not detected. This result strongly implicates TubZ in provision of the pipecolic acid moiety, but suggests there is a second lysine cyclodeaminase function in *A. disciformis*. We confirmed the relationship of **2** to the tubulysins by supplementation of culture broths with commercially available [D_8]L-valine; the resulting incorporation pattern in **2** was analogous to that in tubulysin D (see Figure S1 in the Supporting Information).

Accurate mass determination of **2** gave *m*/*z* 670.39873, consistent with a molecular formula of $C_{36}H_{55}N_5O_5S$ (calcd. $[M+H]^+ = 670.400215$, $\Delta = -0.939$ ppm). As the metabolite appeared to have the structure of the proposed first enzyme-free intermediate in the pathway[7a]—that is, the polyketide-nonribosomal polypeptide core minus the four post-assembly line oxidative and acylation reactions—we designated it as pretubulysin (**2**) (Scheme 1). Furthermore, reanalysis of extracts of wild-type *A. disciformis* An d48 revealed low levels of pretubulysin (Figure 1), supporting its intermediacy in the pathway. We therefore reasoned that pretubulysin might represent a stable analogue of the more complex tubulysins, and would thus be well suited for evaluation as a drug candidate.

We aimed to prove the structure of pretubulysin by NMR spectroscopy. However, despite growth of *A. disciformis* on a 20 L scale, we were unable to purify sufficient quantities of the compound. We therefore used tandem mass spectrometry (MS^2-MS^6) to probe the structure, and compared the fragmentation pattern of **2** to that of tubulysin D (the MS^2 data are presented in Figure 1). The patterns showed little similarity, suggesting that the presence of the acyl groups in

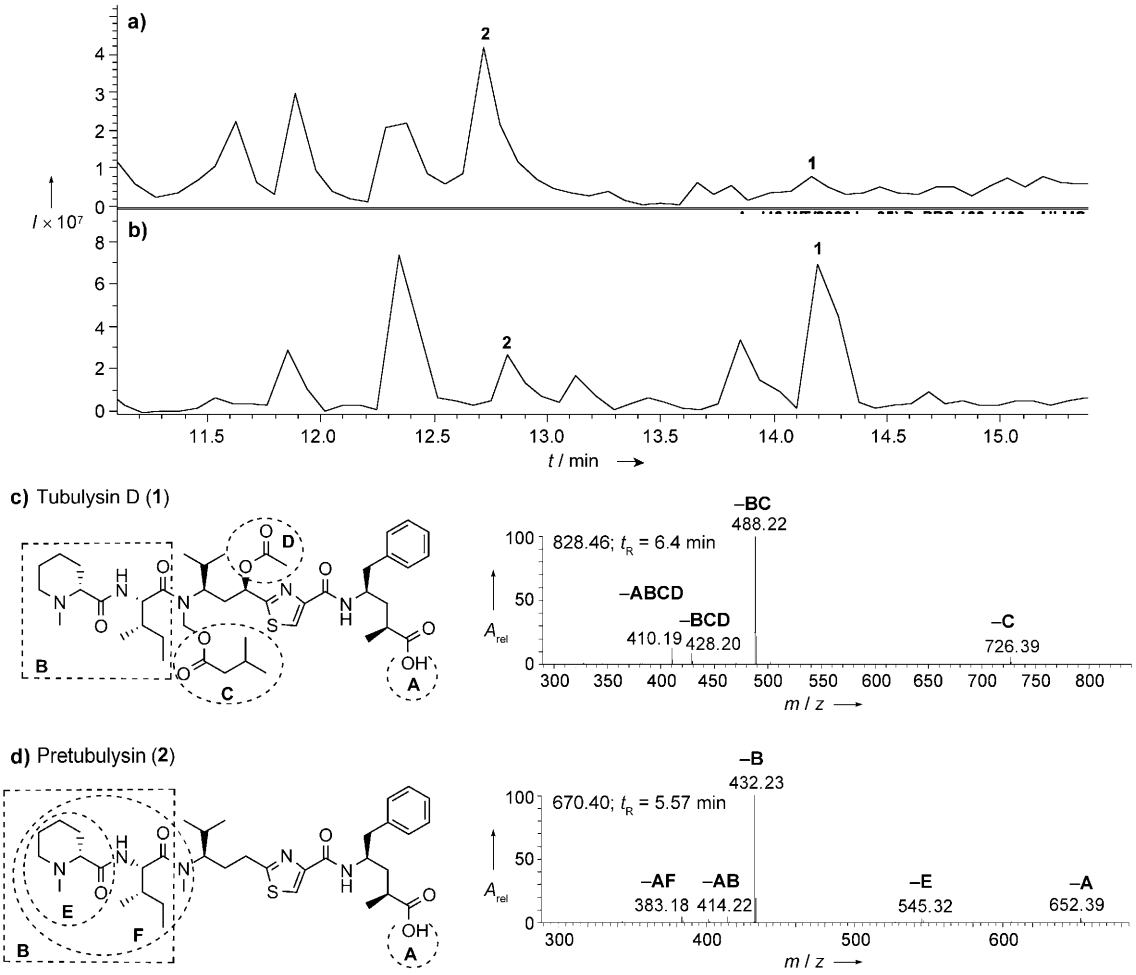
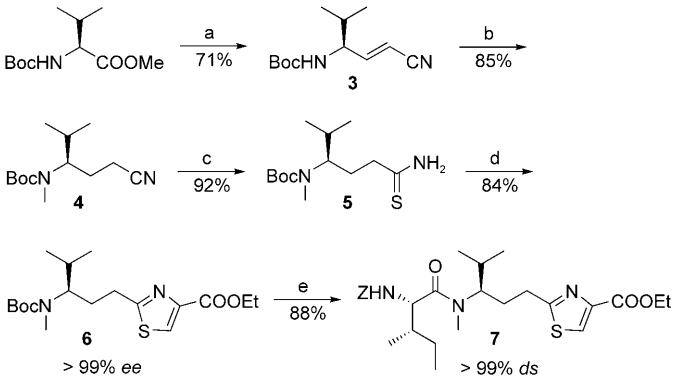


Figure 1. Identification of pretubulysin. a) HPLC-MS analysis (base peak chromatogram (BPC)) of mutant An d48-tubZ$^-$. Peaks corresponding to tubulysin D (**1**) and pretubulysin (**2**) are indicated. b) HPLC-MS analysis (BPC) of extracts of wild-type *A. disciformis* An d48, showing peaks corresponding to **1** and **2**. Comparative analysis of the MS^2 fragmentation patterns of **1** (c) and **2** (d). Comparable fragments lost from each metabolite are indicated. The mass spectra include the retention time and molecular mass of the respective parent ions and are labeled to indicate the fragments lost to generate each daughter peak. All data were obtained on a Thermo LTQ Orbitrap Hybrid FT mass spectrometer.

tubulysin D significantly influence the mode of fragmentation. Nonetheless, accurate mass analysis of pretubulysin fragments revealed molecular formulas consistent with our proposed structure (see Figure S2 in the Supporting Information).

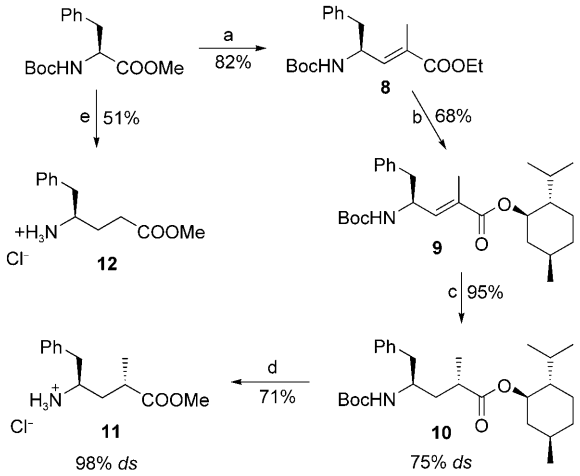
To provide final proof for our biosynthetic hypothesis and to investigate the biological activity of pretubulysin in more detail, however, we aimed to develop an efficient synthesis of the compound.[8] The synthesis of the central Ile-dTuv unit (dTuv: desacetoxytubuvaline) is shown in Scheme 2. Starting



Scheme 2. Synthesis of the Ile-dTuv fragment. Reagents and conditions: a) 1. DIBAL, toluene, −78 °C; 2. $Ph_3P{=}CHCN$; b) 1. H_2, Pd/C, MeOH; 2. NaH, MeI, DMF, 0 °C; c) H_2S, NEt_3, $CHCl_3$, −78 °C→RT; d) 1. $BrCH_2COCOOEt$, acetone, −10 °C; 2. TFAA, pyridine, CH_2Cl_2, −30 °C→RT; e) 1. HCl, dioxane, 0 °C; 2. *Z*-Ile, BEP,[10] diisopropylethylamine, CH_2Cl_2, 10 °C. DIBAL = diisobutylaluminum hydride, BEP = 2-bromo-1-ethylpyridinium tetrafluoroborate, TFAA = trifluoroacetic anhydride.

from the *N*-Boc-protected valine ester, a DIBAL reduction and in situ Wittig reaction of the resulting aldehyde gave rise to the unsaturated nitrile **3** in enantiomerically pure form. Catalytic hydrogenation and subsequent *N*-methylation to **4** proceeded without racemization. The nitrile functionality was then converted into the thioamide **5**, which was subjected to a Hantzsch thiazole synthesis. Trifluoroacetanhydride (TFAA) was added to the hydroxythiazoline intermediate to form the thiazole **6**. Cleavage of the Boc protecting group and coupling with *Z*-Ile gave rise to the required dipeptide **7**.

Despite the apparent simplicity of the Tup moiety, the stereoselective introduction of the α-methyl group is not a trivial issue.[5,9] As our first attempts to introduce the methyl group stereoselectively by means of enolate alkylation were unsuccessful, we decided to use the more straightforward approach of catalytic hydrogenation. The required α,β-unsaturated ester **8** was easily obtained from protected phenylalanine by DIBAL reduction/Wittig olefination, as reported for dTuv (Scheme 3). No epimerization was observed in this one-pot reaction, whereas isolation of the aldehyde intermediate resulted in nearly complete racemization. Catalytic hydrogenation gave rise to the saturated Tup derivative as a 2:1 mixture of diastereomers. Hydrogenation of the free acid (as performed by Wipf et al.[5]) or the corresponding allyl alcohol unfortunately brought no improvement in selectivity. As Zanda et al. had reported the



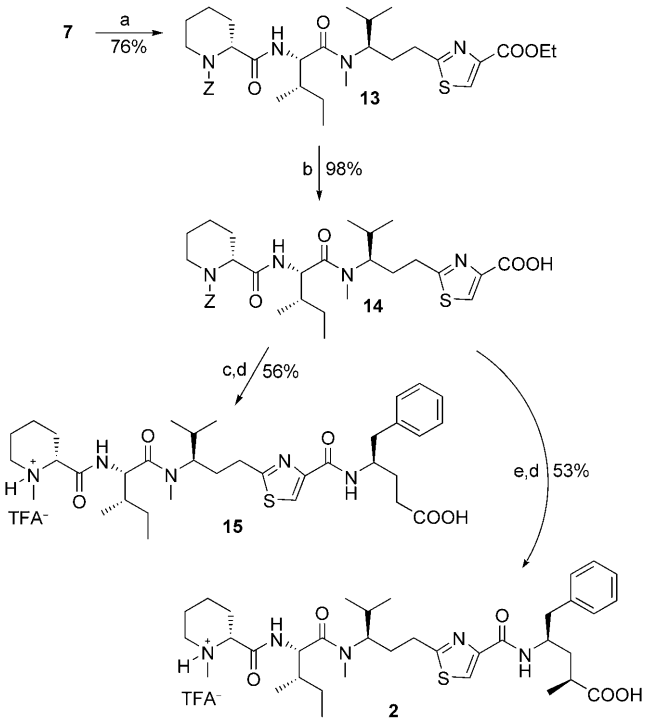
Scheme 3. Synthesis of Tup (**11**) and dTup (**12**). Reagents and conditions: a) 1. DIBAL, CH_2Cl_2, −78 °C; 2. $Ph_3P{=}C(CH_3)COOEt$; b) 1. NaOH, dioxane, 80 °C; 2. menthol, DCC, DMAP, Et_2O, 0 °C; c) H_2, Pd/C, MeOH; d) 6 N HCl, 140 °C; 2. DMP, cat. HCl, MeOH, 50 °C; e) 1. DIBAL, CH_2Cl_2, −78 °C; 2. $Ph_3P{=}CHCOOEt$; 3. H_2, Pd/C, MeOH; 4. HCl, dioxane, 0 °C. DCC = dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine.

separation of the corresponding menthyl esters by chromatography,[9] we converted **8** into menthyl ester **9**. Its hydrogenation provided **10** with a slightly better selectivity with respect to the required diastereomer. After separation of the diastereomers, cleavage of the protecting groups yielded **11** in enantiomerically pure form.

To evaluate if the α-methyl group has any influence on the biological activity, we also synthesized the desmethyl derivative (dTup) **12**.[10] This readily available analogue was further used to establish the final steps of our synthesis (Scheme 4). Cleavage of the *Z* protecting group from dipeptide **7** and subsequent peptide coupling with protected D-Pip gave rise to tripeptide **13**, which could be saponified to give the free acid **14** in quantitative yield. Coupling with dTup (**12**) again proceeded without difficulty. The sequence was finalized by cleavage of the *Z* protecting group and reductive methylation of the pipecolic acid. Finally, saponification and acidification gave rise to desmethylpretubulysin (**15**). With this route in hand, we achieved the synthesis of pretubulysin (**2**) in an analogous manner.

All mass spectrometric data obtained on the biosynthetic material were essentially identical to those from analysis of authentic, synthetic pretubulysin (see Figure S3 in the Supporting Information), confirming the compound's identity. In addition, the biological activity of the synthetic compound towards human acute myeloid leukemia cells (HL-60) was comparable to that of the natural metabolite.

We next used the HL-60 cells to compare the cytotoxicity of **2** to that of the synthetic variant **15**, as well as to tubulysins A (**1**A) and D (**1**D) (Table 1). Tubulysin D is known to be the more toxic of the two tubulysins, with a potency approximately six times greater than that of tubulysin A.[2] As predicted from the earlier SAR studies, pretubulysin (**2**) retained good activity relative to tubulysins A and D (3- and 5-fold lower cytotoxicity, respectively), while removal



Scheme 4. Synthesis of pretubulysin (**2**) and the desmethyl analogue **15**. Reagents and conditions: a) 1. HBr/HOAc; 2. *Z*-(D)-Pip, ClCOO*i*Bu, NMM, THF, −20 °C; b) NaOH, dioxane, 0 °C; c) **12**, ClCOO*i*Bu, NMM, THF, −20 °C; d) 1. HBr/HOAc; 2. $(CH_2O)_n$, $NaBH_3CN$, MeOH; 3. NaOH, dioxane, 0 °C; 4. TFAA, e) **11**, ClCOO*i*Bu, NMM, THF, −20 °C. NMM = *N*-methylmorpholine.

Table 1: Cytotoxicity of tubulysins and analogues as evaluated by the MTT assay (IC_{50} [ng ml^{-1}]).[a]

Cell lines	**1**A	**1**D	**2**	**15**
HL-60	0.01	0.006	0.03	0.39
L929	0.19	0.015	6.5	74
U937	0.003	0.0004	0.08	0.41

[a] Values represent the average of two measurements; incubation time: 5 d.

of the C2 methyl group (as in compound **15**) led to a 13-fold relative reduction in cytotoxicity. Based on these results, tubulysins A and D, and compounds **2** and **15** were evaluated against two more cell lines, L929 (mouse connective tissue fibroblast) and U937 (human histiocytic lymphoma). Both **2** and **15** exhibited activity against both cell lines, albeit less than that of tubulysin A, with **2** being reproducibly the more potent of the synthetic compounds.

These results reveal several important structure–activity relationships, in addition to the data reported by the other groups.[3–6] Notably, the good potency of pretubulysin (**2**) confirms that neither the *N*,*O*-acetal nor the acetoxy functionality of Tuv are necessary for cytotoxicity, although there is a modest reduction in activity (ca. 10-fold) relative to a previously characterized analogue which retained the acetoxy group. Comparison of the data obtained on **2** and its C2-desmethyl analogue **15** reveals that the methyl group in Tup also provides an approximately 10-fold enhancement in biological activity.

In conclusion, we have shown that the structural complexity of the tubulysins can be significantly reduced without a dramatic drop in the biological activity. Pretubulysin (**2**), which was identified as a direct biosynthetic precursor of the tubulysins, is less reactive than tubulysins A and D but retains subnanomolar activity. Taken together, these findings should aid in future efforts to design simplified, yet highly potent analogues of the tubulysins for evaluation as anticancer agents.

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