Total Synthesis

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Total Synthesis of Tubulysin U and V**

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In memory of Ivar Ugi

Tubulysins (**1**) are compounds of extraordinary potency, rapidly degrading the tubulin cytoskeleton, with tubulysin D being the most active tubulin-modifier known so far.[1,2] The tubulysins were first described by Höfle, Reichenbach, and co-workers in 2000.[3,4] Several representatives are active with GI_{50} values (growth inhibition of 50%) in the low picomolar range against the NCI-60 cancer cell-line panel, and some are highly antiangiogenic.[5] Semisynthetic tubulysins, derived from isolated material, show promising in vivo anticancer properties and are candidates for antibody conjugates. Thus, tubulysins are attractive leads as novel anticancer agents.[5] However, so far, tubulysins can only be produced by a fermentation process that yields less than 10 mg L^{-1} by several rather tedious chromatographic purification steps. Thus, and

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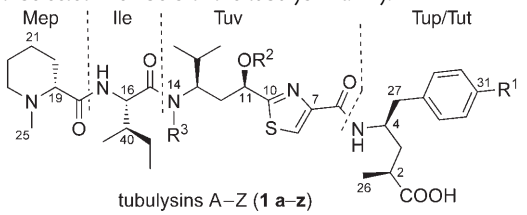
in order to reveal the structure–activity relationship for these compounds, a total synthesis is highly desirable.

Tubulysins are structurally related to the marine natural product class of dolastatins and the anticancer drug LU 103793. Similar to dolastatins 10[6] and 15,[7] tubulysins are unusual peptides, mainly composed of the uncommon or hydrophobic amino acids *N*-methylpipecolic acid (Mep), isoleucine (Ile), tubuvaline (Tuv), and tubuphenylalanine (Tup) or tubutyrosine (Tut), with an N- to C-terminal distance of ≈18 Å, an aromatic amino acid at the C terminus, and a tertiary amino terminus. Two major series of tubulysins can be distinguished (Table 1). Those with labels from the beginning

Table 1: Selected members of the tubulysin family.[a] [8]



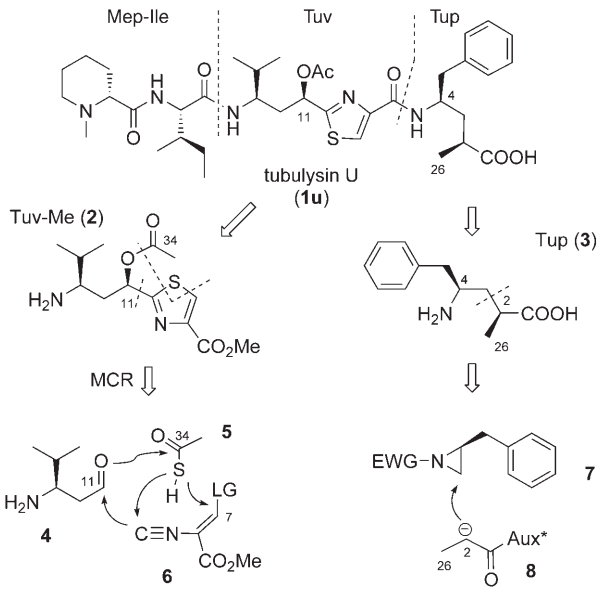
Tubulysin	R^1	R^2	R^3
A	OH	Ac	$CH_2O-(CO)-CH_2CH(CH_3)_2$
...			
D	H	Ac	$CH_2O-(CO)-CH_2CH(CH_3)_2$
E	H	Ac	$CH_2O-(CO)-CH_2CH_2CH_3$
F	H	Ac	$CH_2O-(CO)-CH_2CH_3$
G	H	Ac	$CH_2O-(CO)-CH_3$
...			
U	H	Ac	H
V	H	H	H
...			
Z	OH	H	H

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

of the alphabet (for example, the tubulysin A or D series, with Tut or Tup, respectively) have both the Tuv hydroxy group (R^2) and an *N*,*O*-acetal involving the amide nitrogen atom of tubuvaline (R^3) acylated. The series with labels from later in the alphabet (for example, tubulysins U–Z) has these two functions partially or completely missing (Table 1).[9] Usually, this latter series is less active, but the members studied so far (tubulysins W–Z) reach the activity range of, for example, taxol or the epothilones.

Despite several attempts, no complete synthesis of a tubulysin has been described so far.[10,11] The total synthesis is challenging for several reasons. Only one amino acid of the tetrapeptide (Ile) occurs naturally as a separate compound. The central fragment, Tuv, constitutes a complex, thiazole-containing amino acid with two stereocenters. In the series labeled with earlier alphabet letters, the bis-acyl *N*,*O*-acetal at Tuv makes a total synthesis more difficult, since this functional group is not compatible with most protecting-group strategies. The C-terminal amino acid, Tup, constitutes a γ-amino acid comprising two stereocenters as well. Most importantly, the middle part of all tubulysins is sterically highly crowded, and peptide-bond formation often fails to properly connect either the Ile or Mep residues to the rest of the molecule.

Herein we report the first stereoselective total synthesis of two members of the tubulysin family. A retrosynthetic analysis of the "pentapeptidic" tubulysin U (**1u**, Scheme 1)

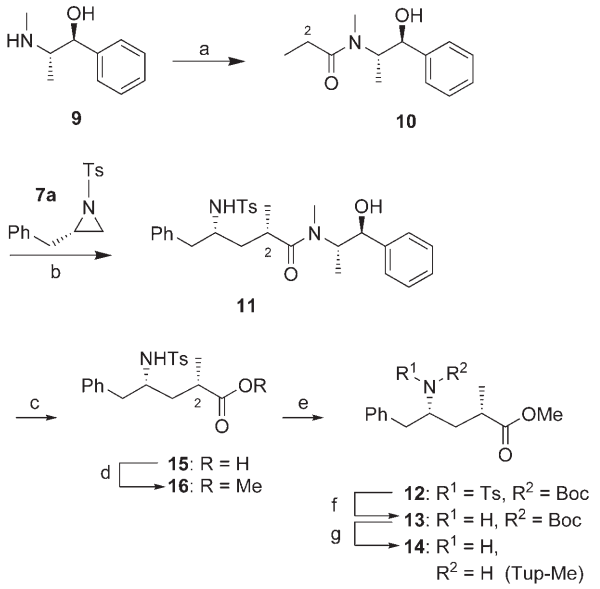


Scheme 1. Retrosynthesis of tubulysin U (**1u**). LG = leaving group, EWG = electron-withdrawing group, Aux* = chiral auxiliary, MCR = multicomponent reaction.

leads to the four amino acids Mep, Ile, Tuv, and Tup, of which Tuv is biosynthetically derived from valine, an acetate equivalent, and cysteine as a hidden fifth amino acid. For the chemical synthesis, we introduced two disconnections to yield three equally complex and large substructures. For the C-terminal moiety, the stereocontrolled alkylation of aziridine **7** by using a chiral auxiliary (such as **8**) appeared most useful. Tubuvaline, the complex-looking centerpiece of all tubulysins, resolves unexpectedly into a set of three easily available building blocks (**4**–**6**) if one considers a recently discovered thiazole synthesis,[12] an isonitrile-based multicomponent reaction (MCR) based on the multiple reactivity of Schöllkopf type isonitriles, **6**.[13] The N terminus contains the commercially available amino acids pipecolinic acid and Ile. When earlier attempts toward the synthesis of tubulysins are considered, the formation of the Ile–Tuv amide bond is expected to constitute the most severe problem. According to the established C- to N-terminus peptide-assembly routine, the successive coupling of Ile and Mep should be preferred, due to the lower propensity for racemization. However, coupling of a preformed Mep–Ile dipeptide is more convergent and may be more attractive if racemization is not an issue or if the central Ile–Tuv amide bond formation reaction gives only a low yield.

According to the retrosynthesis, tubuphenylalanine (**3**) is available from commercial (*S*)-*N*-Ts-2-benzylaziridine (**7a**; Ts = toluene-4-sulfonyl), by ring opening with a propanoyl anion under the control of pseudoephedrine as a chiral auxiliary connected to propionic acid, as in amide **10**

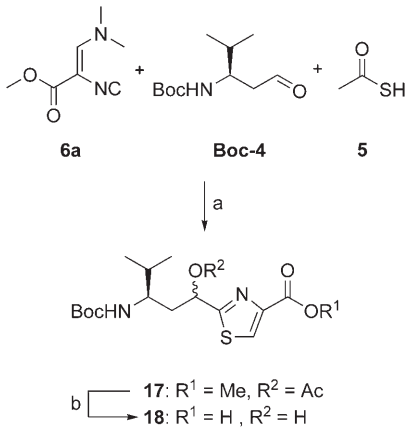
(Scheme 2).[14,15] The desired *syn* diastereomer is formed in 70% relative yield and is separated from the minor *anti* isomer by chromatography. Amide hydrolysis and removal of the tosylate paves the way to Tup and its various protected



Scheme 2. Synthesis of differently protected tubuphenylalanine (Tup) derivatives by the pseudoephedrine route. a) Propionic anhydride, Et_3N, CH_2Cl_2, 93%; b) 1. LDA, LiCl, THF, −78°C; 2. (*S*)-(+)-2-benzyl-1-(toluene-4-sulfonyl)-aziridine, THF, −20°C, 86% (81:19 d.r. mixture of C-2 diastereomers, major isomer shown separated and processed further); c) 4 M H_2SO_4/dioxane, reflux, 91%; d) MeOH, conc. HCl, reflux, 85%; e) Boc_2O, DMAP, CH_3CN, 100%; f) Mg (powder), MeOH, ultrasound, 70%; g) 4 N HCl in dioxane, 100%. Boc = *tert*-butoxycarbonyl, DMAP = 4-dimethylaminopyridine, LDA = lithium diisopropylamide, THF = tetrahydrofuran.

forms **12**–**16**, of which the Tup methyl ester hydrochloride, **14**·HCl, was used for the further synthesis. Racemization was not observed. Several alternative routes to this building block, for example, the use of Evans methodology, were tried but did not lead to the product at all or gave lower yields and significantly lower *de* values (see also reference [3]).

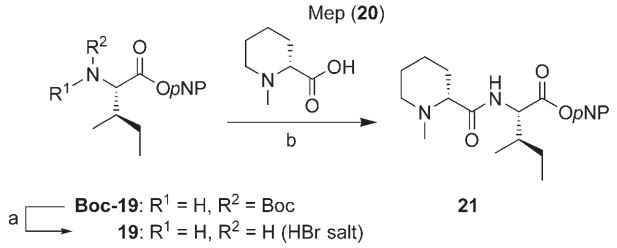
Tubuvaline is available as its Boc-protected methyl ester **17** in a very straightforward one-pot process following a newly discovered three-component synthesis of 2-hydroxymethylthiazoles (Scheme 3).[12] Thus, Schöllkopf isonitrile (**6a**, available in one step from glycine isonitrile),[13] Boc-protected L-homovaline aldehyde (Boc-**4**),[16] and thioacetic acid (**5**) gave **17** in 40% yield, with a 3:1 ratio of diastereomers. These values constitute considerable improvements over both the first thiazole MCRs[12] and the alternative routes.[11] The major diastereomer shows the right configuration at C-11, as determined by comparison with data for the Cbz-Tuv ethyl ester (Cbz = benzyloxycarbonyl) published by Wipf et al.[11] and the original NMR data for tubulysin A.[2] Basic hydrolysis allows cleavage of the ester to give building block **18**, which is also suitable for the synthesis of tubulysins V and U (see below). The selective hydrolysis of the methyl ester (R^1), while leaving the acetate (R^2) intact, is possible by enzymatic



Scheme 3. One-pot synthesis of protected L-tubuvaline by a new MCR to form 2-hydroxymethyl thiazoles. a) 1. $BF_3{\cdot}Et_2O$, Boc-L-homovaline aldehyde, THF, −78°C; 2. simultaneous addition of thioacetic acid and Schöllkopf isonitrile in THF by syringe pump over 30 min; then room temperature overnight; 40%, 3:1, major isomer separated; b) NaOH, THF/H_2O (3:1), 62%.

catalysis (see below), as has been demonstrated with tubulysin A.[8]

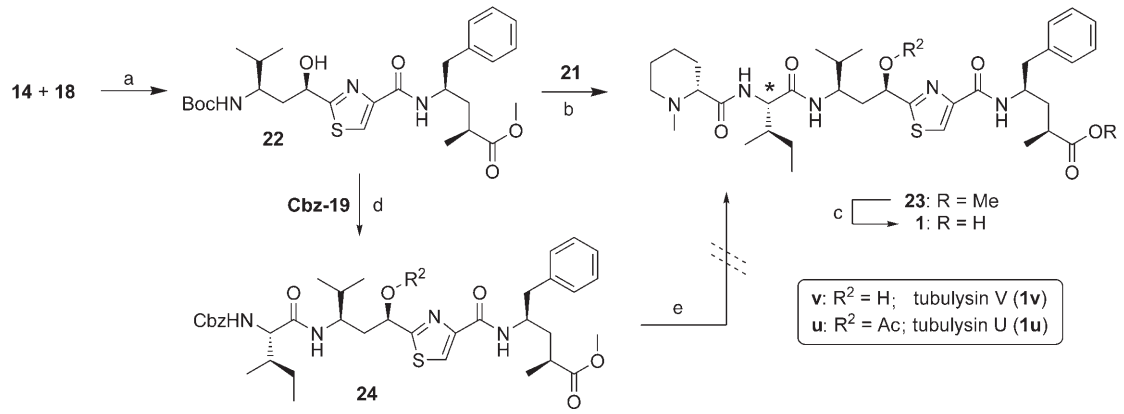
D-Mep-L-Ile is the third major building block (Scheme 4). The commercial isoleucine 4-nitrophenyl (*p*NP) ester, Boc-



Scheme 4. Synthesis of D-Mep–L-Ile as the activated *p*NP ester. a) HBr in acetic acid (33%), 0°C, 91%; b) 1. *N*-methyl D-pipecolic acid, Et_3N, CH_2Cl_2, −15°C; 2. ethyl chloroformate, −10°C; 3. L-Ile-*p*NP, −10°C, 43%. *p*NP = 4-nitrophenyl.

19, can be *N*-deprotected. Activated as a mixed anhydride, D-Mep[17] reacts chemoselectively and without detectable racemization with **19** to give the Mep–Ile dipeptide already activated at the C terminus. The coordinated levels of carbonyl activation and careful control of the reaction temperature save several steps of protection–deprotection–activation, by utilizing *p*NP as a carboxyl-protecting moiety in the first coupling and as an activation moiety in the second, upcoming amide coupling.

Finally, suitably monoprotected Tup **14** and Tuv **18** were coupled without racemization with 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4-(3*H*)-one (DEPBT, Scheme 5).[18] After Boc deprotection of **22**, the second coupling step (Scheme 5) with Cbz-Ile activated as a *p*NP-ester, Cbz-**19**, also proceeds in very good yield and without racemization. The subsequent deprotection of the benzyloxycarbonyl group is clean. However, the final coupling step with D-Mep (**20**), activated by various methods (for example, TBTU, 2(-7-aza-



Scheme 5. Assembly of tubulysin U (**1u**) by prolongation of the N terminus. a) 1. **18**, DIPEA, DEPBT, DMF, 0°C; 2. **14**, DMF, 78%; b) 3-step 1-pot procedure: 1. TFA, CH_2Cl_2; 2. D-Mep–L-Ile-*p*NP (**21**), Et_3N, DMF; 3. Ac_2O, pyridine, 83%, 1:1 mixture of diastereomers; c) porcine liver esterase, phosphate buffer (pH 7.2), 36°C, 61%. Alternative route: d) 3-step procedure (b) with Cbz-Ile-*p*NP (step 2), only one diastereomer detected, 85%; e) 1. Pd/C/H_2, MeOH, 93%; 2. TBTU, DIPEA, DMF, D-Mep, only one diastereomer detected, 10%. DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, TBTU = 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, TFA = trifluoroacetic acid

1*H*-benzotriazole)-1,1,3,3-tetramethyluronium tetrafluoroborate (TATU), or 1-hydroxy-1*H*-benzotriazole (HOBt)), gave protected tubulysins as complex mixtures with a maximum 10% yield of isolated product. These were separable by GC but hardly by preparative LC. This was not satisfactory, and thus the more convergent direct coupling of Boc-Tuv–Tup-Me, **22**, with Mep–Ile-*p*NP, **21**, was tried. A one-pot procedure, including amino deprotection of the Tuv–Tup part, coupling, and reacetylation of the resulting methyl ester of tubulysin V, **23v**, gave the corresponding tubulysin U ester **23u** in an excellent 83% yield. Unfortunately, but not unexpectedly, quantitative epimerization occurs at the α-carbon atom of Ile during dipeptide transfer (indicated by an asterisk in Scheme 5). Nevertheless, separation of the 1:1 diasteromeric mixture gives over 40% of the desired tubulysin U ester **23u** with the correct stereochemistry; this more convergent process is thus much more efficient than the less-racemization-prone stepwise route.

Finally, selective methyl ester hydrolysis in the presence of the acetate and, most importantly, without epimerization or other acid- or base-induced deterioration of tubulysin U was achieved by selective enzymatic hydrolysis with porcine liver esterase to give the title compound **1u** in 61% yield. Tubulysin V (**1v**), the deacetylated derivative, is likewise obtainable by either full ester hydrolysis, and indeed it forms as a minor byproduct even under controlled enzyme hydrolysis conditions, or by omitting the reacetylation step after the first coupling.

Overall, we have developed a short, stereoselective, and convergent route to selected tubulysin family members. The rapid access will form the basis for further structure–activity relationship and biological studies of this promising class of anticancer natural products.

Experimental Section

MCR synthesis of **17**: Boc-**4** (1 g, 4.64 mmol) in THF (1.5 mL) was added to $BF_3{\cdot}Et_2O$ (1.16 mL, 9.29 mmol) in THF (2.5 mL) at −78°C. The resulting mixture was stirred for 5 min at −78°C. Thioacetic acid (**5**, 0.33 mL, 4.64 mmol) in THF (1.5 mL) and a solution of **6a**[13] (0.72 g, 4.64 mmol) in THF (1.5 mL) were added simultaneously by using a double-syringe pump over a period of 30 min. The reaction mixture was stirred for 1 h at −78°C, allowed to come to room temperature, and stirred overnight. The reaction was quenched with water (15 mL) and, after addition of Et_2O (15 mL), the resulting mixture was stirred for 30 min. After separation of the phases, the aqueous layer was washed with Et_2O (3 × 10 mL). The combined organic layers were washed with a solution of citric acid (5%, 2 × 10 mL), sat. $NaHCO_3$ solution (2 × 10 mL), and brine (2 × 10 mL), dried over Na_2SO_4, filtered, and concentrated in vacuo. The obtained oil was purified by flash chromatography on silica (hexanes/EtOAc 7:3) to yield two diastereomers: **17a** (0.60 g) and **17b** (0.21 g) in 43% combined yield (diastereomeric ratio 3:1). For further data, see the Supporting Information.

Synthesis of **1u/v** by coupling of Tup–Tuv-Me with Mep–Ile-pNP and selective enzymatic methyl ester hydrolysis:

Synthesis of **23**: TFA (300 μL, 1.77 mmol) was added to **22** (66 mg, 0.12 mmol) in CH_2Cl_2 (3.5 mL). The mixture was stirred for 1 h at room temperature and all volatile matter was removed in vacuo. The residue was dissolved in DMF (3.5 mL) and this was followed by subsequent addition of Et_3N (150 μL, 1 mmol) and **21** (60 mg, 0.16 mmol). After stirring for 38 h at room temperature, the solvent was evaporated under high vacuum ($p < 1$ mbar, 25°C). The crude residue was dissolved in pyridine (3.5 mL) and, after addition of acetic acid (0.7 mL), the mixture was stirred for 3 h at room temperature. Evaporation of all volatile matter in vacuo yielded the crude product, which was purified by reversed-phase HPLC (RP-18, MeOH/H_2O/TFA 65:35:0.1). Yield = 71 mg (83%).

Synthesis of **1u**: A phosphate buffer (2 mL, pH 7.3, 0.02 M) was added to **23** (6 mg, 8.2 μmol) in dimethylsulfoxide (0.15 mL) and the mixture was vigorously stirred for 5 min. The temperature was kept constant at 36°C and porcine liver esterase (0.15 mL, 250 units mg^{-1}) was added. After stirring for 4 h (detection of only one product; careful TLC control), the mixture was extracted with EtOAc (2 × 15 mL) and the combined organic extracts were freed of solvent in vacuo without heating. The crude product was purified by reversed-phase HPLC (RP-18, MeOH/H_2O/TFA 65:35:0.1). Yield = 3.6 mg (61%) of **1u**. Longer treatment with porcine liver esterase yielded increasing amounts of **1v**.

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