Total Synthesis

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Total Synthesis of Tubulysins U and V**

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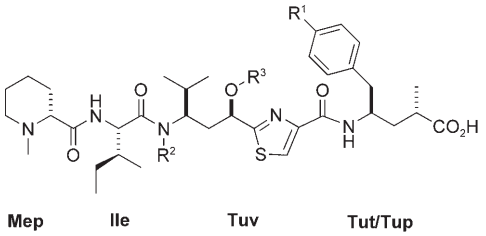
Tubulysins (Scheme 1) are a family of tetrapeptides produced in rather small quantities ($< 4\ mg\,L^{-1}$ culture broth) by two different species of myxobacteria: *Archangium gephyra* and *Angiococcus disciformis*.[1] The structure, stereochemistry, and biosynthetic pathway of the tubulysins were recently determined by Höfle, Reichenbach, and co-workers, who also reported the potent cytotoxicity (in the nanomolar concentration range) of these compounds.[2] The cytotoxicity of the tubulysins stems from their ability to bind tubulin and disintegrate microtubules of dividing cells, thus inducing apoptosis.[3]

From a structural point of view, tubulysins are derived from an *N*-methylpipecolic acid residue (Mep) at the N terminus, isoleucine (the only proteinogenic amino acid) at the second position, an unusual thiazole-containing amino acid, tubuvaline (Tuv), which features two stereogenic centers at the third position, and two possible γ-amino acids at the C terminus: either tubutyrosine (Tut, tubulysins A, B, C, G, and I) or tubuphenylalanine (Tup, tubulysins D, E, F, and H). Additionally, the N-terminal residue of Tuv is functionalized with a highly unusual N,O-acetal substituent with different ester groups (Scheme 1).

Although at first sight the synthesis of tubulysins might not appear to be extremely challenging, to date there are only two published reports of their total syntheses, which suggests that several important challenges must be present.[4] Indeed, a number of challenging synthetic issues have been described in a recent review by Dömling and Richter,[5] who reported that the major hurdles are: 1) the installation of the acid and base-labile N,O-acetal ester, 2) the synthesis of the configurationally and chemically sensitive thiazole fragment, and 3) the assembly of the peptide framework, as a consequence of the steric congestion of the central Tuv region. However, the research groups of Höfle and Dömling have claimed two different total syntheses of tubulysins that are so far described only in patents.[6] The synthesis of the Tuv, Tup, and dipeptide fragments of tubulysins were also reported by the groups of Wipf[7] and Friestad.[8]



Tubulysin A	R^1 = OH	R^2 = CH_2OOC-isobutyl	R^3 = Ac
B	R^1 = OH	R^2 = CH_2OOC-*n*-propyl	R^3 = Ac
C	R^1 = OH	R^2 = CH_2OOC-ethyl	R^3 = Ac
D	R^1 = H	R^2 = CH_2OOC-isobutyl	R^3 = Ac
U (**1**)	R^1 = H	R^2 = H	R^3 = Ac
V (**2**)	R^1 = H	R^2 = H	R^3 = H

Scheme 1. Structure of tubulysins.

Very recently, Dömling, Wessjohann, and co-workers described the first total synthesis of tubulysins U (**1**) and V (**2**) (Scheme 1) without the N,O-acetal ester fragment.[9] According to these authors, the series of tubulysins lacking this synthetically challenging function is less active, but the cytotoxicity of some of these tubulysins still reaches the activity range of, for example, taxol or the epothilones. This synthesis allowed the synthesis of just a few milligrams of the target tubulysins. Furthermore, it is affected by several drawbacks, such as 1) the use of a hard to cleave *N*-tosyl protecting group in the synthesis of Tup; 2) several epimerizations occurring at various levels in the synthesis of the Tuv unit and peptide fragments thereof; and 3) several rather inefficient and complex synthetic steps, such as the assembly of the tetrapeptide framework and the final removal of the protecting groups. Thus, we felt that a more direct, flexible, and modular synthesis was needed that would allow the preparation of different tubulysin stereoisomers and structurally modified tubulysin-like structures.

We describe herein an effective synthesis of tubulysins U (**1**) and V (**2**) that can be used to prepare hundreds of milligrams of pure compounds. We also show that the tubulysins U and V previously obtained by Dömling et al.[9] are actually stereoisomers of the natural molecules.

The γ-amino acid tubuphenylalanine (Tup) was synthesized in a multigram amount as a mixture of epimers at the α-carboxy stereocenter (Scheme 2). L-Phenylalaninol (**3**) was transformed into the phthaloylimide **4**, and then treated with triflic anhydride to afford the corresponding triflate **5**. Nucleophilic substitution by the sterically hindered sodium salt of α-methyl diethylmalonate produced a clean S_N2 reaction to give the quaternary γ-amino malonate **6**. A one-

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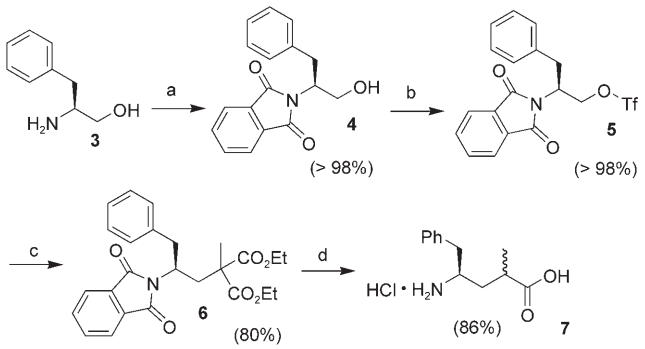
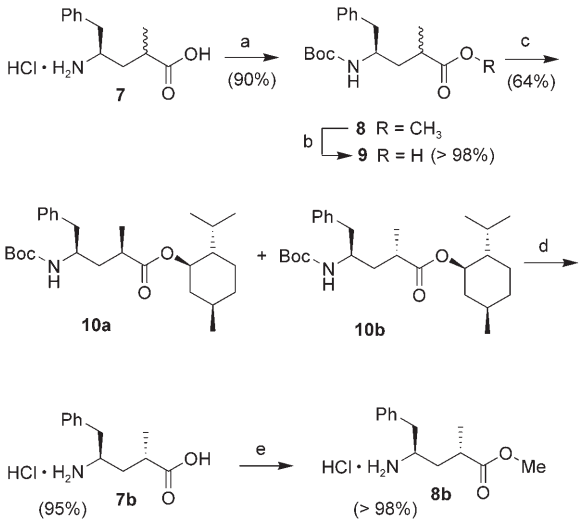
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



Scheme 2. Synthesis of epimeric Tup. a) Phthalic anhydride, Et_3N, toluene, Dean–Stark trap, 145 °C, 40 h; b) $(CF_3SO_2)_2O$, Py, CH_2Cl_2, −78 °C to RT, 3 h; c) diethyl 2-methylmalonate, NaH, 0 °C, RT, 7 h; d) 6 N HCl, AcOH, 145 °C, 2 days. Py = pyridine, Tf = trifluoromethanesulfonyl.

pot simultaneous cleavage of all the protecting groups and decarboxylation provided a 1:1 epimeric mixture of H-Tup-OH hydrochloride (**7**) in just four straightforward steps and an overall yield of 68% from **3**.

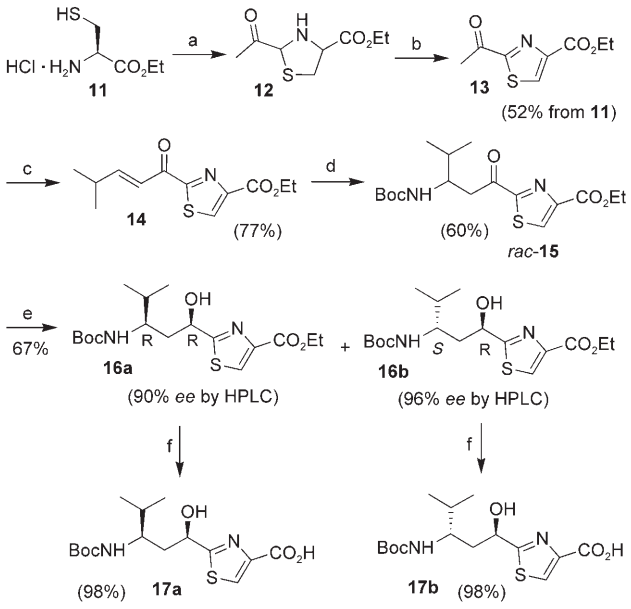
We performed extensive experimentation to obtain **7** in a stereochemically pure form. Eventually, we found that the *N*-Boc-protected (−)-menthyl esters **10a**,**b** (Scheme 3) were separable by flash chromatography. These compounds were obtained by conversion of **7** into Boc-Tup-OMe (**8**), saponification of the methyl ester to give Boc-Tup-OH **9**, and conversion of the carboxylic acid epimers (**9**) into the (−)-menthyl esters **10a** and **10b**. X-ray analysis of **10a** allowed unambiguous assignment of the stereochemistry of these compounds.[10] Cleavage of all the protecting groups by acidic hydrolysis gave the stereochemically pure H-Tup-OH·HCl (**7b**), which was resubjected to esterification to give stereo-



Scheme 3. Preparation of stereopure Tup-OMe. a) 1. 2,2-dimethoxypropane, conc. HCl, dry MeOH, 60 °C, 1 day; 2. Boc_2O, Et_3N, acetonitrile, 6 h; b) LiOH, H_2O/THF (1:1), 1 day; c) (−)-menthol, DCC, DMAP, CH_2Cl_2, 6 h; then flash chromatography to separate **10b** from **10a**; d) 6 N HCl, 130 °C, 1.5 h; e) 2,2-dimethoxypropane, conc. HCl, dry MeOH, 60 °C, 1 day. Boc = *tert*-butoxycarbonyl, DCC = *N*,*N*′-dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine.

pure H-Tup-OMe·HCl (**8b**), which was suitable for the subsequent coupling reactions.

The synthesis of stereochemically pure tubuvaline (Tuv) is shown in Scheme 4. L-Cysteine (**11**) was condensed with pyruvaldehyde to afford the 2-acetyl-4-ethoxycarbonylthiazolidine **12** which was oxidized in a multigram amount to



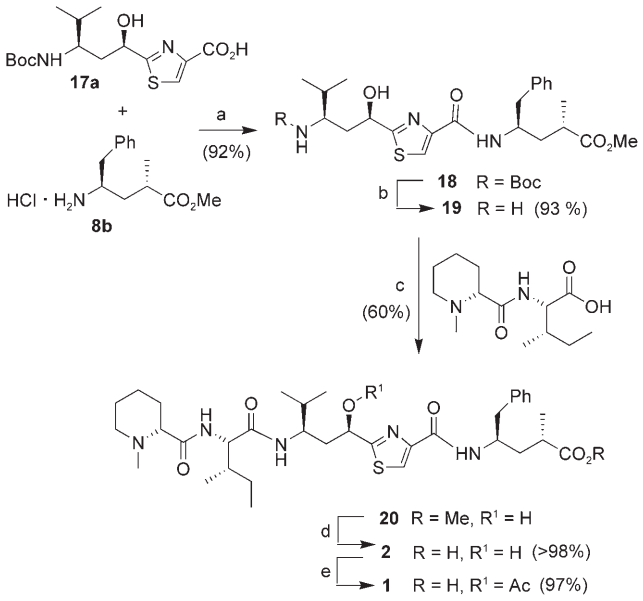
Scheme 4. Synthesis of stereopure Tuv. a) Pyruvaldehyde, $NaHCO_3$, EtOH/H_2O (1:1), 18 h, RT; b) activated MnO_2, acetonitrile, 50 °C, 2 h; c) isobutyraldehyde, $TiCl_4$, Et_3N, dry THF, −78 °C to RT; d) $BocNH_2$, $Sn(OTf)_2$, acetonitrile, 3 h, RT; e) (*S*)-CBS, BH_3·Me_2S, dry THF, 0 °C to RT, 2 h; f) LiOH, THF/H_2O 4:1, 5 h, RT.

thiazole **13** by using MnO_2.[11] The thiazole **13** gave rise to a rather efficient crotonic condensation with isobutyraldehyde under Lewis acid catalysis, particularly with $TiCl_4$. The intermediate aldol could only be detected by TLC when the reaction was carried out at low temperature, since dehydration to the α,β-unsaturated thiazolyl ketone **14** took place spontaneously upon heating the reaction mixture at room temperature. The amino group was installed by an aza-Michael addition[12] of Boc-NH_2 to **14** catalyzed by tin(II) triflate to give the β-amino ketone **15**.[13]

To obtain stereochemically pure Tuv derivatives we used the Corey, Bakshi, and Shibata oxazaborolidine (CBS catalyst) mediated reduction.[14] (*S*)-CBS, in the presence of BH_3·Me_2S, reacted nearly exclusively with the *Si* face of **15** to give the diastereomeric alcohol (*R*,*R*)-**16a** in 90% *ee* from (*R*)-**15**, and to the (*S*,*R*)-**16b** diastereomer in 96% *ee* from (*S*)-**15**. The epimeric alcohols **16a** and **16b** could be easily separated by flash chromatography. The relative stereochemistry could be assigned unambiguously by X-ray analysis of racemic **16b**.[10] The stereopure target Boc-Tuv-OH (**17a**) was obtained after mild saponification of the methyl ester **16a**. The epimeric alcohol **17b** was obtained by the same protocol. To prove the feasibility of the method for the preparation of the whole set of stereoisomers of Tuv, we also performed the reaction of (*R*)-CBS with **15**, which afforded, as expected, *ent*-

16a and *ent*-**16b**. The absolute stereochemistry of alcohols **16** (whose relative configuration was determined by X-ray diffraction, see above) was assessed by chemical correlation with the enantiopure *O*-Ac-Cbz-Tuv-OEt described by Wipf et al. (see the Supporting Information).[7]

Completion of the synthesis of tubulysins U and V is shown in Scheme 5. Initial attempts to assemble the peptide by standard stepwise methodology, namely by coupling the



Scheme 5. Synthesis of tubulysins U and V. a) HOAt, HATU, TMP, DMF, 3 h; b) CH_2Cl_2, 20% TFA, 1 h; c) $isoBuO_2CCl$, NMM, AcOEt, 8 h; d) LiOH, THF/H_2O (4:1), 3 d, then TFA; e) Ac_2O, Py, 14 h. HOAt = 7-aza-1-hydroxy-1*H*-benzotriazole, HATU = *N*-[dimethylamino)-1*H*-1,2,3-triazole[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate, TMP = 2,4,6-trimethylpyridine, TFA = trifluoroacetic acid.

Tuv-Tup dipeptide with Ile and then with Mep, failed because the latter coupling did not occur. The coupling of the two dipeptides Mep-Ile-OH and H-Tuv-Tup-OMe was therefore explored. The former was obtained by standard methods (see the Supporting Information). The coupling of Boc-Tuv-OH (**17a**) with H-Tup-OMe (**8b**) using HATU/HOAt delivered the dipeptide Boc-Tuv-Tup-OMe (**18**) without any detectable loss of stereochemical purity and very satisfactory yields. Interestingly, comparison of the ^{1}H NMR spectrum of **18** (see the Supporting Information) with that reported by Dömling et al. under identical conditions showed subtle but clear differences. Furthermore, polarimetric analysis showed nearly opposite rotations, namely $[\alpha]_D^{23} = +15.2\ deg\,cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.7\ g\,cm^{-3}$, $CHCl_3$) for **18**, and $[\alpha]_D^{23} = -20.67\ deg\,cm^3\,g^{-1}\,dm^{-1}$ ($c = 1.0\ g\,cm^{-3}$, $CHCl_3$) for Dömling's dipeptide. These findings strongly suggest that the Boc-Tuv-Tup-OMe dipeptide previously described by Dömling et al.[9] should actually be a different stereoisomer, with the incorrect stereochemistry.[15]

Treatment of **18** with TFA delivered H-Tuv-Tup-OMe (**19**) in a stereopure form and excellent yield. Unfortunately, the key coupling between Mep-Ile-OH and H-Tuv-Tup-OMe (**19**) was found to be extremely challenging, because of the proclivity of the Ile amino acid center to undergo epimerization. The same problem was observed by Dömling et al. who could not suppress the epimerization.[9] A number of different conditions were tried, including a HATU/HOAt-promoted coupling, but the formation of the tetrapeptide methyl ester **20** was always accompanied by at least 40% epimerization of the Ile stereocenter. Eventually, we found that the use of ethyl chloroformate and *N*-methylmorpholine (NMM) in ethyl acetate afforded the target **20** in very good yields, with very little epimerization (< 5%).[16] Simple flash chromatographic purification allowed **20** to be obtained in a purity of greater than 98%. Careful hydrolysis of the methyl ester with LiOH gave the target tubulysin V (**2**) in good yield and again without any detectable epimerization. Finally, acetylation of the Tuv hydroxy group afforded the other target tubulysin U (**1**) in a chemically and stereochemically pure form. So far, the method has allowed the preparation of up to 100 mg of pure tubulysin U (**1**) from a single reaction sequence. Not surprisingly, both the ^{1}H and ^{13}C NMR spectra did not match those previously described in the literature,[9] which must therefore belong to a different stereoisomer, and not to the natural molecule.

In summary, we have developed an effective synthesis of tubulysins U and V in chemically/stereochemically pure form and with the correct stereochemistry. This route should allow the synthesis of all the possible stereoisomers in amounts suitable for a thorough investigation of the biological properties of these peptides. The efficient access to tubulysins U and V should also allow for further structure–activity relationships and biological studies, to investigate possible applications in anticancer therapy.

Experimental Section

Synthesis of **6**: Diethyl methylmalonate (6.00 mL, 34.89 mmol) was added dropwise to a suspension of NaH (1.37 g, 60% dispersion in mineral oil, 34.15 mmol) in dry THF (30 mL), cooled at 0 °C and under nitrogen. The reaction was warmed to RT and stirred for 15 min. The resulting solution was added over a period of 30 min to a solution of **5** (6.41 g, 15.52 mmol) in dry THF (70 mL), cooled at 0 °C and in a nitrogen atmosphere. The resulting mixture was stirred for 6 h at the same temperature and then warmed at 30 °C for 1 h. The reaction was quenched by adding a saturated aqueous solution of NH_4Cl (20 mL) and water (20 mL). The layers were separated and the aqueous phase was extracted with AcOEt (4 × 30 mL). The collected organic layers were dried over anhydrous Na_2SO_4, filtered, and the solvent was removed in vacuo. The residue was purified by flash chromatography (*n*-Hex/AcOEt 3:1) to give 5.48 g of **6** as a yellowish oil (80% yield).

Synthesis of **15**: $Sn(OTf)_2$ (1.10 g, 2.64 mmol) and $BocNH_2$ (1.55 g, 13.2 mmol) were added to a solution of **14** (3.38 g, 13.2 mmol) in CH_3CN (50 mL). The resulting mixture was stirred for 3 h at RT. The solvent was removed in vacuo and the crude product was purified by flash chromatography (*n*-Hex/AcOEt 8:2) to give 2.87 g of **15** (60% yield) as a white solid.

Synthesis of **20**: Trifluoroacetic acid (2 mL) was added to a solution of **18** (350 mg, 0.64 mmol) in CH_2Cl_2 (8 mL). The solution was stirred for 1 h and all the volatile compound removed in vacuo to give 352 mg of **19**. *N*-methylmorpholine (NMM) (90 μL, 0.82 mmol) was added to a suspension of Mep-Ile-OH (210 mg, 0.82 mmol) in dry AcOEt (5 mL). The mixture was cooled at −10 °C and $isoBuO_2CCl$ (106 μL, 0.82 mmol) was added. After 5 min, a solution of **19** (185 mg,

0.41 mmol) in dry AcOEt (2 mL) was added. The resulting suspension was allowed to warm to RT and the stirring was continued for 8 h. The reaction was diluted with H_2O (10 mL) and the phases were separated. After drying the organic phase over anhydrous Na_2SO_4, and subsequent filtration, the solvent was removed in vacuo. The residue was purified by flash chromatography (CH_2Cl_2/MeOH 96:4) to afford 170 mg of **20** (60% yield) as a white foam.

Synthesis of **2**: A 1N aqueous solution of $LiOH{\cdot}H_2O$ (184 μL, 0.18 mmol) was added to a solution of **20** (84 mg, 0.12 mmol) in THF (5 mL). The reaction was stirred for 3 days and then acidified with TFA to pH 1–2. The resulting mixture was washed with H_2O (5 mL) and extracted whit AcOEt (10 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and the solvent removed in vacuo to give 92 mg of pure **2** (> 98% yield) as a white foam.

Synthesis of **1**: Ac_2O (1 mL) was added to a solution of **2** (79 mg, 0.10 mmol) in pyridine (2 mL) and the solution was stirred overnight. The mixture was quenched with H_2O (10 mL) and extracted with AcOEt (1 × 10 mL). The organic phase was washed with brine (3 × 10 mL) and the solvent removed in vacuo. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 9:1) to give 70 mg of **1** (97% yield) as a white powder.

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