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Synthetic Approaches Towards Tubulysins and Derivatives Thereof

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Abstract: Tubulysins, linear tetrapeptides produced by several strains of myxobacteria, show an extremely high toxicity towards a wide range of cancer cell lines, with IC_{50} values in the nano or even picomolar range. Therefore, tubulysins and their derivatives might be suitable candidates for the development of antitumor drugs. Several synthetic approaches for tubulysins and derivates have been developed, which will be discussed in the review.

Keywords: Antitumor drugs, myxobacteria, peptides, tubulin, tubulysin.

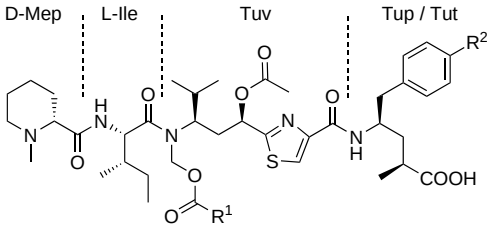
INTRODUCTION

The tubulysins are a family of tetrapeptides produced by several strains of myxobacteria in rather small quantities (< 4 mg/l culture broth). In 2000, Reichenbach and Höfle described the isolation of the first members of this family from the myxobacterial strains *Archangium gephyra* and *Angiococcus disciformis* [1]. The tubulysins showed no activity against bacteria and only little against fungi, but with IC_{50} in the picomolar range, they reveal extremely high cytotoxicity towards tumor cell lines. Several more tubulysins (Fig. **1**) have been described in 2004 and their structure in crystal and solution has been determined [2].

At the *N*-terminus an unusual *N*-methyl-(*R*)-pipecolic acid (Mep) is found, connected to the only proteinogenic amino acid L-isoleucine. A highly exotic building block is located in the middle of the molecule, called tubuvaline (Tuv). Biosynthetically, this building block is generated from valine *via N*-methylation, C_2-chain elongation, coupling to cysteine and subsequent heterocyclization/oxidation to form the thiazole moiety [3,4].

The acetoxy group and the highly unusual acylal side chains are biosynthetically introduced later on *via* oxidation of the *N*-methyl group and the α-position at the thiazole ring, followed by acylations. The different tubulysins mainly differ in the acylal side chain (R^1). A second difference is found in the *C*-terminal, also C_2-prolonged, amino acid, called tubuphenylalanine (Tup) or tubutyrosine (Tut), depending on the original amino acid incorporated. Recent biosynthetic studies by Müller *et al*. using a third producing strain *Cystobacter sp*. *SBCb004* and a mutant of *Angiococcus disciformis* (*An d48*) resulted in the identification of a wide range of tubulysin derivatives, mainly biosynthetic intermediates (Fig. **2**) [5].

In some examples the final stages of the proposed biosynthesis, the oxidation/acylation, are (in part) missing, others result from an abortive biosynthetic pathway. For example, in tubulysin U the *N*-methylation at the Tuv-unit is deleted, suppressing the incorporation of the acylal side chain. Pretubulysin was found to be the first enzyme-free intermediate in the pathway [5].



Mep: *N*-Methylpipecolic acid
Tuv: Tubuvaline
Tup: Tubuphenylalanine (R^2 = H)
Tut: Tubutyrosine (R^2 = OH)

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

Fig. (1). Structures of the tubulysins.

The high cytotoxic activity of the tubulysins results from their ability to bind to tubulin [6], disturbing the microtubule skeleton in the dividing cell, thus inducing apoptosis. This turns out the tubulysins into ideal candidates for the development of anti cancer drugs [7-9], as long as it is possible to target the tumor cells selectively [10]. Therefore, folic acid conjugates have been prepared, because high-affinity folate receptors (FR) are highly expressed on a wide range of human cancer cells. This approach allows to selectively address cancer cells in the presence of normal tissue cells [11,12]. Alternatively, the tubulysins can also be bound to dendrimers [13] or cyclodextrine-based nanoparticles, showing higher *in vivo* activity compared to the corresponding tubulysins alone [14]. Interestingly, also pretubulysin, the precursor of the tubulysins, shows a very high cytotoxicity towards a wide range of tumor cell lines [15,16]. With respect, that this compound is much easier synthetically available, and it

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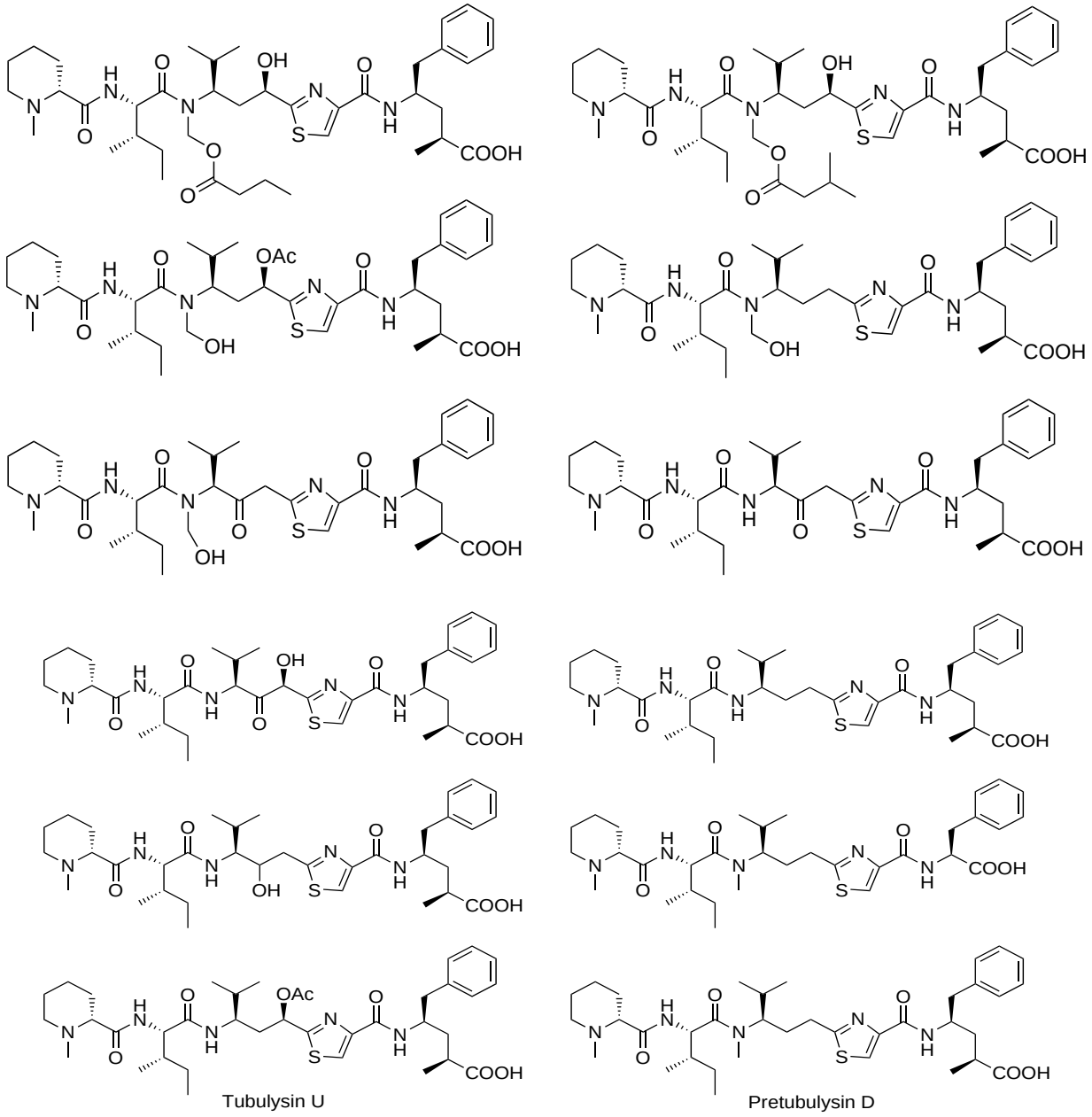
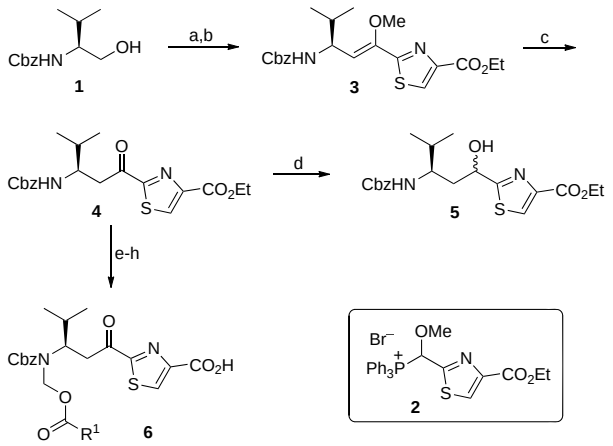


Fig. (2). New tubulysin derivatives isolated from *Angiococcus disciformis An d48.*

lacks labile structures, such as the acylal side chain and the acetoxy group, it is an ideal candidate for drug development as well [17]. For example, pretubulysin also shows a strong anti-angiogenic effect, both *in vitro* and *in vivo* [18]. As indicated by photoaffinity labeling experiments, pretubulysin binds to tubulin as the parent component [19]. In contrast to pretubulysin, containing an *N*-methyl group at the central amino acid, other derivatives such as tubulysin U and V, missing this *N*-alkyl substituent, are significantly less potent [20-22]. A wide range of other tubulysin derivatives has been prepared and investigated for their biological activity [23-25]. This review will focus on the synthetic strategies developed for the synthesis of tubulysins and simplified derivatives thereof. Stereoselective syntheses of the different building blocks will be discussed in chronic order, as well as the syntheses of the final compounds.

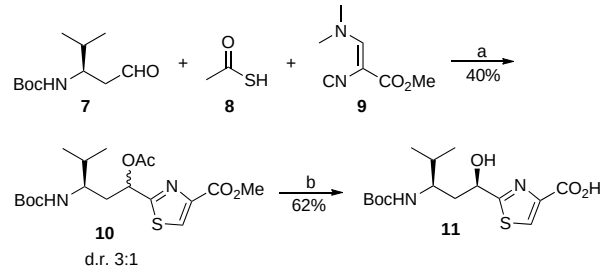
SYNTHESIS OF TUBUVALINE AND ITS DERIVATIVES

The first synthesis of the tubuvaline fragment was described by Höfle and Reichenbach in a patent in 2001 (Scheme **1**) [26]. Starting from protected (*S*)-valinol (**1**), oxidation and subsequent Wittig reaction with the ylide derived from **2** afforded the thiazolyl enol ether **3**, which was hydrolyzed towards the corresponding ketone **4**. Reduction with $NaBH_4$ gave rise to the secondary alcohol **5** as a diastereomeric mixture. For the incorporation of the side chain, the ethyl ester was converted into the selectively cleavable trimethylsilylethyl ester, before the side chain was introduced *via* alkylation of the Cbz-amide. Subsequent cleavage of the silyl ester provided the required building block **6**.



Scheme 1. Synthesis of Tuv fragment according to Höfle *et al.*: **a**) Swern oxidation; **b**) DBU, **2**; **c**) THF, HCl (35%); **d**) EtOH, $NaBH_4$; **e**) NaOH; **f**) TMSEtOH, DCC; **g**) NaH, $R^1CO_2CH_2Cl$; **h**) TBAF.

In 2004, also in patents, Dömling *et al.* described a straightforward synthesis of an *N*-protected tubuvaline-precursor based on a modified Passerini protocol (Scheme **2**) [27,28]. This approach was used in the synthesis of tubuvaline U and V as well [29,30]. In this case Schöllkopf isocyanide (**9**), accessible in one step from glycine isocyanide [31], reacting with Boc-protected homovaline aldehyde (**7**) and thioacetic acid (**8**) as an acid component. The α-acetoxy-substituted tubuvaline derivative **10** was obtained as a 3:1 diastereomeric mixture, albeit in moderate yield. The major diastereomer was found to be the required one. Subsequent saponification of the thiazole ester resulted in the simultaneous cleavage of the acetate group (**11**).



Scheme 2. Synthesis of Tuv fragment according to Dömling *et al.*: **a**) 1. $BF_3 \cdot Et_2O$, **7**, THF, –78 °C; 2. simultaneous addition of **8** and **9** in THF by syringe pump over 30 min; then room temperature overnight, major isomer separated; **b**) NaOH, THF/H_2O (3:1).

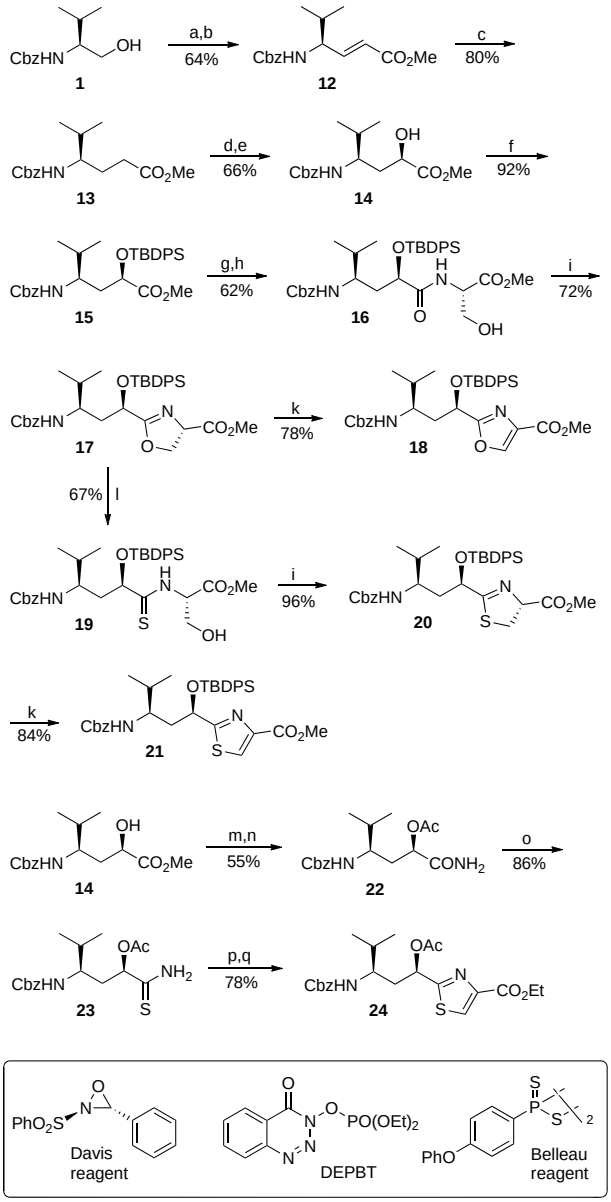
During their studies towards the synthesis of the Tuv-Tup dipeptide fragment, Wipf *et al.* developed an interesting approach using an asymmetric hydroxylation to introduce the α-hydroxy functionality (Scheme **3**) [32]. Starting from *N*-protected valinol **1**, TEMPO oxidation and subsequent Wittig reaction provided α,β-unsaturated ester **12**. This building block is also available directly from Cbz-valine methyl ester according to Knaus *et al.* [33]. Catalytic hydrogenation of **12** has been found to be not a trivial issue because of undesired γ-lactam formation. Finally, the double bond could be removed *via* Cu-catalyzed hydride addition, giving rise to **13**. Best results in the key step, the α-hydroxylation, were obtained by deprotonation of **13** with NaHMDS at –78 °C, followed by addition of Davis reagent. Herewith, the required α-hydroxylated ester **14** was formed as single diastereomer. Other bases or protecting groups gave lower yields. Subsequent *O*-silylation provided the fully protected ester **15** in 31% over the whole sequence (from valinol). Saponification of the ester and coupling with (*S*)-serine methyl ester using DEPBT [3-(Diethoxy-phosphoryloxy)-3H-benzo[d][1,2,3]triazin-4-one] [34] provided dipeptide **16**, which could be cyclized to oxazoline derivative **17**. This heterocycle has been found to be a suitable building block for several tubuvaline derivatives. Oxidation of **17** gave rise to the oxazole analogue **18**, while the reaction with H_2S resulted in a ring opening towards the thioamide **19**. Cyclization to **20** and oxidation provided the thiazole derivative **21** in high yield. Alternatively, the *O*-acetylated analogue **24** could be obtained from **14** *via* aminolysis of the ester followed by acetylation of the OH-group (**22**). Treatment with Belleau reagent [35] generated thioamide **23**, which could be subjected to a Hantzsch protocol [36] to provide thiazole **24**.

During their synthesis towards tubulysin D, Ellman *et al.* developed a convergent synthesis based on the addition of a metalloenamine, derived from ketimine **25**, to thiazoline aldehyde **26** (Scheme **4**) [37]. This aldehyde could be prepared in four steps according to the literature [38]. The stereoselectivity in the addition step strongly depends on the counter ion used. While almost 1:1 mixtures are obtained with Zn^{2+} and Mg^{2+} as counter ions, an excellent yield and diastereoselectivity was observed in the presence of $ClTi(O\textit{i}\text{-}Pr)_3$. Stereoselective reduction of the imine **27** at –78 °C proceeded with high stereoselectivity without reduction of the methyl ester. After chromatography, the *N*-protected amino alcohol **28** was obtained in diastereomerically pure form. Cleavage of the *N*-protecting group provided the salt **29** in quantitative yield. A similar approach was recently used for the synthesis of triazole analogs by Yang *et al.* [39].

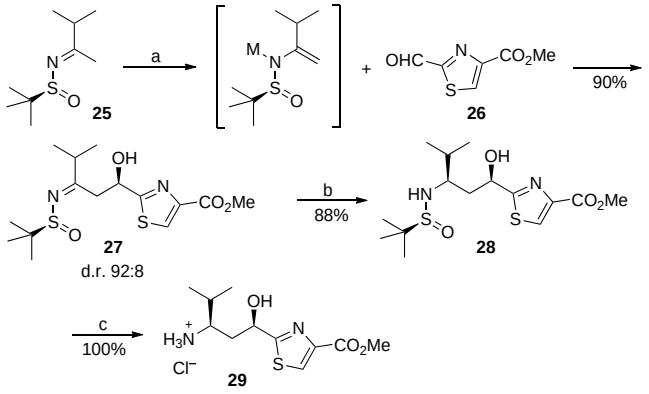
In 2007, Wipf *et al.* reported an alternative approach towards the *N*-methyl analog of tubuvaline (**33**) (Scheme **5**) [40]. The previously synthesized derivatives **21** and **24** evolved problems during the cleavage of the Cbz-protecting group which could not be removed by catalytic hydrogenation (e.g. Pd/C) because of the thiazole moiety. Therefore, the synthesis of a *N*-Boc-protected derivative **33** was envisaged. Starting from (*S*)-valine the Boc-protected *N*-methyl-homovaline aldehyde **30** was synthesized according to standard procedures. Addition of a thiazole Grignard reagent, generated from **31** by exchange with *sec*-butylmagnesium chloride, provided a separable 2:1 mixture of the two possible diastereomers of **32**. The desired major isomer was acetylated, deprotected and oxidized to give the *N*-Boc-protected tubuvaline derivative **33**.

In the same year, Zanda *et al.* reported a total synthesis of the simplified tubulysins U and V using another protocol towards *N*-desalkyltubuvaline **11** (Scheme **6**) [41]. Cysteine was condensed with pyruvaldehyde to thiazolidine derivative **34**, which was oxidized to thiazole **35** with MnO_2. Subsequent aldol condensation and aza-Michael addition of Boc-NH_2 gave rise to the protected β-amino ketone **36**. Stereose-

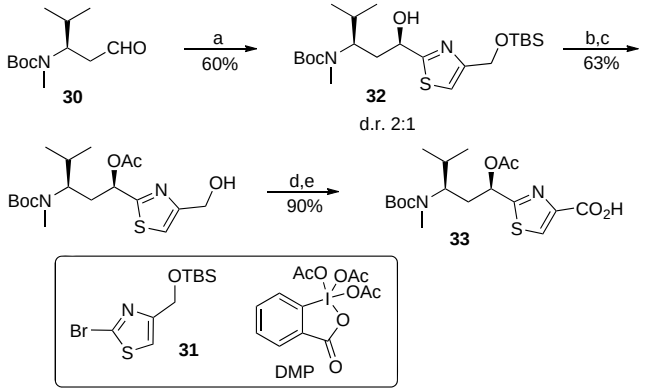
lective reduction of the keto group according to the Corey-Bakshi-Shibata (CBS) protocol [42] provided the epimeric alcohols **37**, which could easily be separated by flash chromatography. Saponification of the ethyl ester **37a** gave access to the free acid **11** in almost quantitative yield. Alternatively, ketone **36** can also be prepared by addition of ketone **35** to *N*-Boc-protected isobutylimine, generated *in situ* [20].



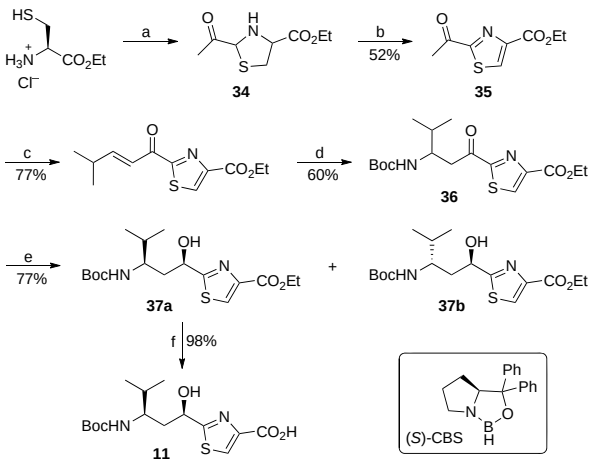
Scheme 3. Synthesis of Tuv derivatives **21** and **24** according to Wipf *et al.*: **a**) TEMPO, NaOCl, $NaHCO_3$, NaBr; **b**) Ph_3PCHCO_2Me; **c**) *rac*-Binap, NaOt-Bu, CuCl, PMHS (polymethylhydrosiloxane), toluene, rt, 3 d; **d**) NaHMDS; **e**) Davis reagent, THF, –78 °C, 60 min; **f**) TBDPSCl, imidazole, DMF, 60 °C; **g**) LiOH, H_2O; **h**) (*S*)-Ser-OMe, DEPBT, NEt_3; **i**) DAST, –78 °C; **k**) $BrCCl_3$, DBU, CH_2Cl_2, 0 °C, 7 h; **l**) H_2S, MeOH, NEt_3, rt, 3 d; **m**) NH_3, MeOH; **n**) Ac_2O, pyridine; **o**) Belleau reagent; **p**) $BrCH_2COCO_2Et$; **q**) TFA_2O, pyridine.



Scheme 4. Synthesis of Tuv derivative **29** according to Ellman *et al.*: **a**) LDA, $ClTi(Oi\text{-}Pr)_3$, Et_2O, –78 °C; **b**) $NaBH_4$, $Ti(OEt)_4$, –78 °C; **c**) HCl/dioxane, MeOH.

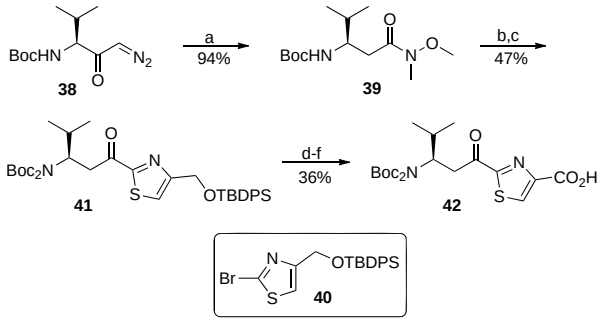


Scheme 5. Synthesis of Tuv derivative **33** according to Wipf *et al.*: **a**) *sec*-BuMgCl, **31**, THF; **b**) Ac_2O, pyridine; **c**) TBAF; **d**) Dess-Martin periodinane (DMP); **e**) $NaClO_2$, 2-methyl-2-butene.



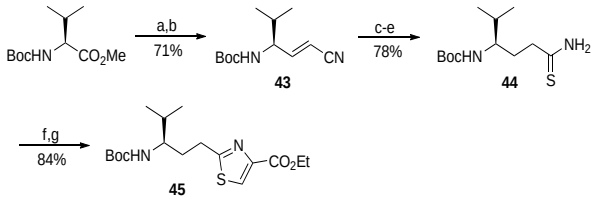
Scheme 6. Synthesis of Tuv derivative **11** according to Zanda *et al.*: **a**) Pyruvaldehyde, $NaHCO_3$, EtOH/H_2O (1:1), rt, 18 h; **b**) activated MnO_2, MeCN, 50 °C, 2 h; **c**) *i*-PrCHO, $TiCl_4$, NEt_3, dry THF, –78 °C to rt; **d**) $BocNH_2$, $Sn(OTf)_2$, MeCN, rt, 3 h; **e**) (*S*)-CBS, $BH_3{\cdot}Me_2S$, dry THF, 0 °C to rt, 2 h; **f**) LiOH, THF/H_2O 4:1, rt, 5 h.

A very similar approach was used by Fecik *et al.* for the synthesis of a keto analogue of tubuvaline **42** (Scheme **7**) [43]. A Wolff rearrangement of the valine derived diazoketone **38** was used to generate directly Weinreb amide **39**. For the next step, the addition of a lithiated thiazole, generated from bromothiazole **40**, the *N*-functionality was double protected. An excellent yield of the thiazolyl ketone **41** was obtained, which was converted into the simplified tubuvaline fragment **42** *via* desilylation and two-step oxidation of the primary alcohol functionality.



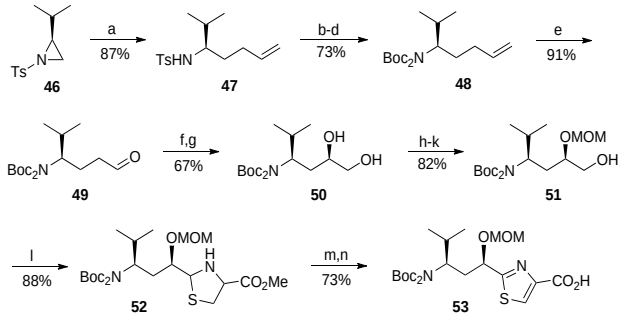
Scheme 7. Synthesis of Tuv derivative **42** according to Fecik *et al.*: a) CF_3CO_2Ag, (MeO)NHMe·HCl, NEt_3; b) LHMDS, Boc_2O; c) **40**, *n*-BuLi; d) HF·pyridine, pyridine; e) Dess-Martin periodinane (DMP); f) $NaClO_2$, 2-methyl-2-butene.

In 2009, Kazmaier *et al.* reported a straightforward synthesis of a desoxytubuvaline derivative **45** used in the synthesis of pretubulysin (Scheme **8**) [15,16]. Starting from *N*-Boc-protected valine ester, DIBAL-H reduction to the corresponding aldehyde and subsequent Wittig reaction provided unsaturated nitrile **43**. Catalytic hydrogenation, *N*-methylation and H_2S-addition towards the nitrile functionality afforded thioamide **44** which was subjected to a Hantzsch synthesis to give thiazole derivative **45**.



Scheme 8. Synthesis of Tuv derivative **45** according to Kazmaier *et al.*: a) DIBAL-H, toluene, –78 °C; b) Ph_3P=CHCN; c) H_2, Pd/C, MeOH; d) NaH, MeI, DMF, 0 °C; e) H_2S, NEt_3, $CHCl_3$, –78 °C to rt; f) $BrCH_2COCO_2Et$, acetone, –10 °C; g) TFA_2O, pyridine, CH_2Cl_2, –30 °C to rt.

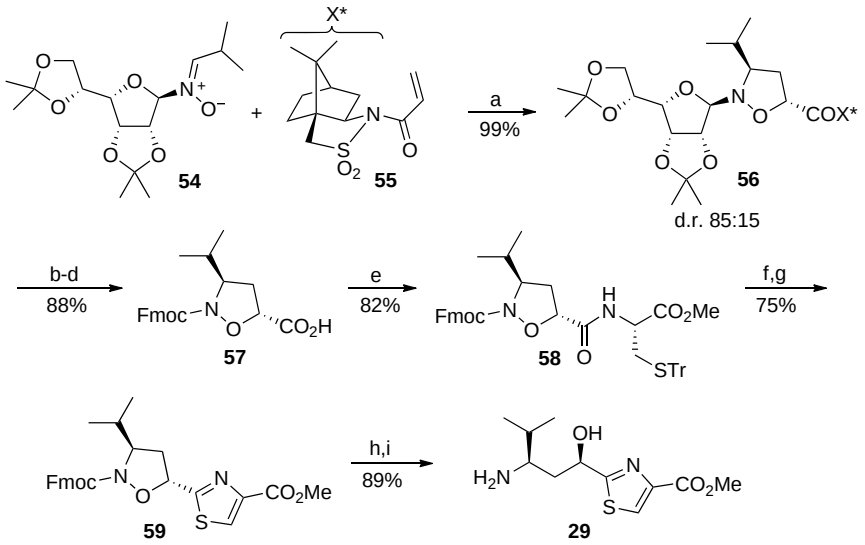
In the same year, Chandrasekhar *et al.* described a multigram scale synthesis of the Tuv-Tup fragment using a new approach for tubuvaline (Scheme **9**) [44]. Aziridine **46** was obtained from (*S*)-valine according to the literature [45]. **46** was opened regioselectively *via* Cu-catalyzed allyl Grignard addition. Sodium naphthalide was used to remove the Ts-protecting group from **47** and the resulting free amine was double Boc-protected (**48**). Oxidative cleavage of the double bond provided aldehyde **49**, which was subjected to an asymmetric α-hydroxylation. Rapid reduction with $NaBH_4$ furnished an unstable anilinoxy compound, which was cleaved with $CuSO_4$ to the corresponding diol **50**. A three step protocol was used to get access to the selectively MOM-protected alcohol **51**, which was oxidized to the aldehyde and directly converted into the thiazolidine **52**. Oxidation with MnO_2 and saponification of the ester provided MOM-protected tubuvaline derivative **53**.



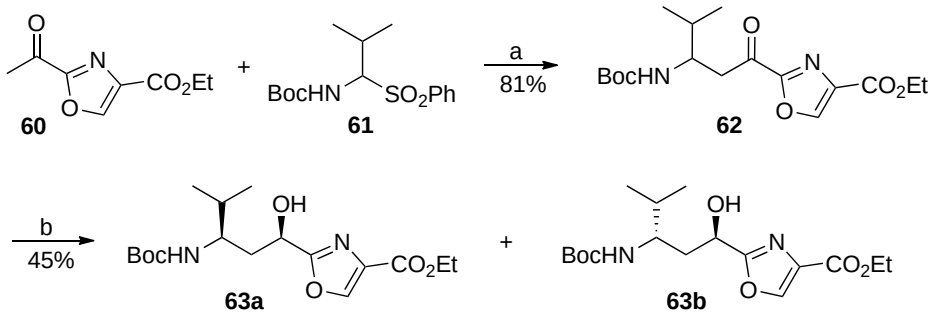
Scheme 9. Synthesis of Tuv derivative **53** according to Chandrasekhar *et al.*: **a**) AllylMgBr, CuCN, THF, 0 °C to rt, 4 h; **b**) Sodium naphthalide, THF, –20 °C, 1 h; **c**) Boc_2O, NEt_3, CH_2Cl_2, 0 °C to rt, 30 min; **d**) 1. *n*-BuLi, 2. Boc_2O, THF, –78 °C to rt; **e**) OsO_4, 2,6-lutidine, $NaIO_4$, dioxane/H_2O, 0 °C to rt, 30 h; **f**) 1. (*S*)-Pro, PhNO, DMSO; 2. $NaBH_4$, EtOH; **g**) $CuSO_4 \cdot 5\ H_2O$, MeOH; **h**) TBSCl, imidazole, CH_2Cl_2, 0 °C to rt; **i**) MOMCl, CH_2Cl_2, 0 °C to rt; **k**) TBAF, THF, 0 °C to rt; **l**) 1. $(COCl)_2$, DMSO, CH_2Cl_2, –78 °C, 2 h; 2. NEt_3, EtOH, (*R*)-Cys-OMe·HCl, rt, 2 h; **m**) MnO_2, MeCN, 50 °C, 20 h; **n**) LiOH, THF/H_2O.

An interesting approach towards tubuvaline derivative **29** was developed by Tamura *et al.* during the synthesis of several tubulysins (Scheme **10**) [46,47]. Key step of their synthesis is a [3+2]-cycloaddition of a *D*-gulose derived nitrone **54** with an α,β-unsaturated amide **55** containing camphor sultam as a second chiral auxiliary. Although the chiral induction of the nitrone was modest, a rather good diastereoselectivity (d.r. 85:15) for the coupling product **56** could be obtained due to double asymmetric induction conditions. The required stereoisomer was formed preferentially. The chiral auxiliaries could be removed stepwise by LiOH and $HClO_4$, respectively. Afterwards, the free amino functionality was Fmoc-protected (**57**). Coupling with *S*-tritylated cysteine provided the fully protected dipeptide **58**. The thiazole ring (**59**) was formed after cleavage of the *S*-protecting group, cyclization and subsequent MnO_2 oxidation. Finally, reductive cleavage of the N–O bond with $Mo(CO)_6$ and subsequent Fmoc-cleavage gave rise to tubuvaline derivative **29**.

Recently, Zanda *et al.* reported the synthesis of oxazole derivatives **63** of tubuvaline in analogy to their tubuvaline protocol (Scheme **11**) [22]. Ketooxazole **60** was obtained from lactic acid and serine and was subjected to an aldol-type addition using *N*-Boc-protected isobutyraldimine, generated *in situ* from amino sulfone **61**. β-Aminoketone **62** was obtained as a racemic mixture. Stereoselective reduction according to Corey-Bakshi-Shibata gave rise to a mixture of the diastereomeric alcohols **63** which could be separated by flash chromatography. The first step of the sequence could also be carried out in a highly diastereoselective fashion using chiral sulfimines [48].



Scheme 10. Synthesis of Tuv derivative **29** according to Tamura *et al.*: **a**) CH_2Cl_2, 40 °C, 48 h; **b**) LiOH, THF/H_2O; **c**) aq. $HClO_4$, MeCN; **d**) Fmoc-Cl, $NaHCO_3$, dioxane/H_2O; **e**) (*R*)-Cys(STr)-OMe, HATU, DIPEA, CH_2Cl_2; **f**) Ph_3P=O, Tf_2O, CH_2Cl_2; **g**) MnO_2, CH_2Cl_2; **h**) $Mo(CO)_6$, MeCN/H_2O; **i**) Et_2NH, MeCN.

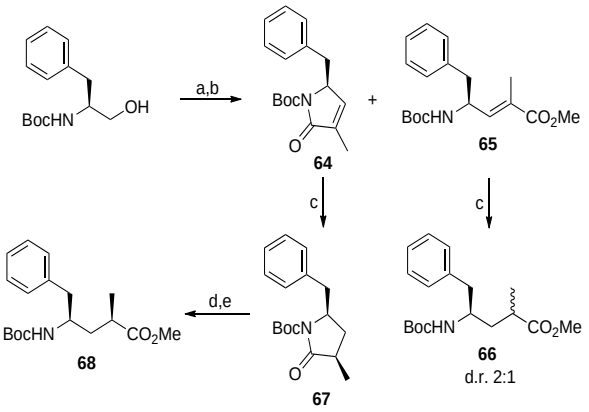


Scheme 11. Synthesis of Tuv derivatives **63** according to Zanda *et al.*: **a**) NaH, THF, 2–3 h; **b**) (*S*)-(–)-2-methyl-CBS-oxazaborolidine, $BH_3 \cdot SMe_2$, THF, 0 °C, 2–3 h.

SYNTHESES OF TUBUPHENYLALANINE AND ITS DERIVATIVES

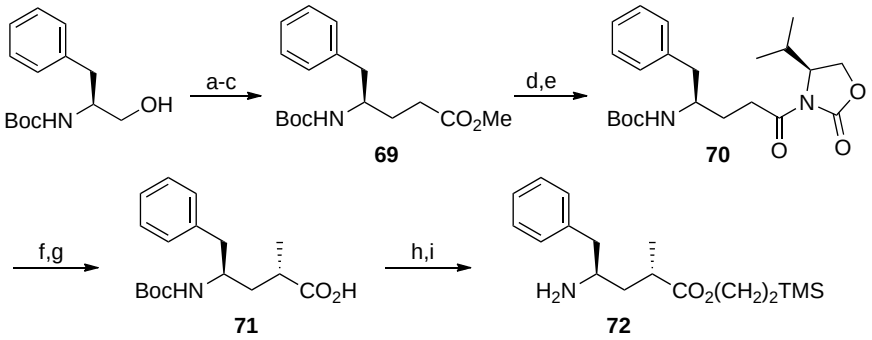
The first synthetic approaches towards tubuphenylalanine (Tup) were reported by Höfle *et al.* in their patent from 2001 (Scheme **12**) [26]. Boc-protected (*S*)-phenylalaninol was oxidized according to the Swern protocol, and the resulting aldehyde was subjected to a Horner-Wadsworth-Emmons reaction, providing a mixture of lactam **64** and unsaturated ester **65**. After separation, the two compounds were hydrogenated. While the open chain compound **60** gave rise to a 2:1 diastereomeric mixture of **66**, the unsaturated lactam **64** provided unfortunately the undesired stereoisomer of lactam **67** preferentially. Saponification and esterification yielded the undesired diastereomer **68** of Tup.

Therefore, a second approach was developed leading to the correct stereoisomer (Scheme **13**) [26]. In analogy to the previous approach (*S*)-*N*-Boc-phenylalaninol was subjected to oxidation, Wittig olefination and subsequent hydrogenation to give protected γ-amino acid ester **69**. Obviously no lactam formation was observed, also in the subsequent step, the formation of the *N*-acyloxazolidin-2-one **70**. This chiral auxiliary was used to introduce the α-methyl group stereoselectively, providing the requested Tup derivates **71** and **72**.

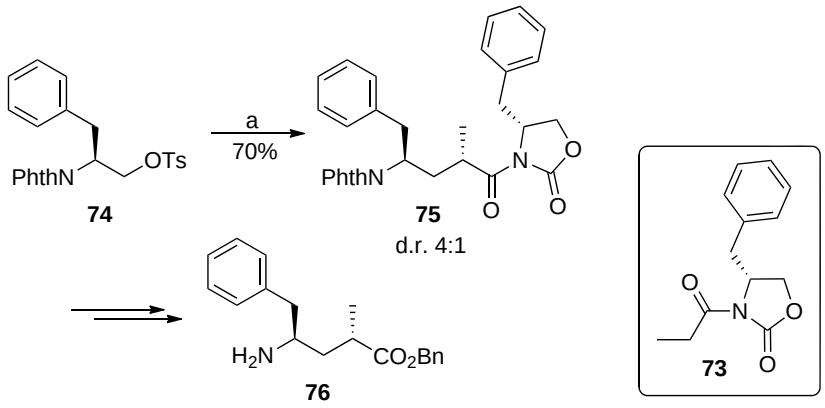


Scheme 12. Synthesis of Tup derivatives **66** and **68** according to Höfle *et al.*: **a**) Swern oxidation; **b**) *n*-BuLi, $(EtO)_2P(O)CH(CH_3)CO_2Me$; c) Pd/C, H_2; d) LiOH, H_2O_2; e) CH_2N_2.

Also in patents, researchers at Morphochem described a related approach based on an auxiliary-controlled *C*–*C*-coupling (Scheme **14**) [27,28]. Nucleophilic attack of deprotonated *N*-propionyloxazolidinone **73** on triflate **74**, easily



Scheme 13. Synthesis of Tup derivatives **71** and **72** according to Höfle *et al.*: **a**) Swern oxidation; **b**) Wittig reaction; **c**) H_2, Pd/C; **d**) NaOH, H_2O; **e**) 1. pivaloyl chloride, Et_3N; 2. (*S*)-4-isopropyloxazolidin-2-one; **f**) NaHMDS, MeI; **g**) H_2O_2, LiOH; **h**) $TMS(CH_2)_2OH$, DCC; **i**) TFA, CH_2Cl_2.



Scheme 14. Synthesis of Tup derivative **76** according to Dömling *et al.*: **a**) **73**, LHMDS, –40 °C, THF.

obtained from *N*-phthaloyl-(*S*)-phenylalanine, provided protected Tup-derivative **75** as a 4:1 diastereomeric mixture. Separation of the diastereomers and subsequent cleavage of protecting and auxiliary groups gave access to Tup derivative **76**.

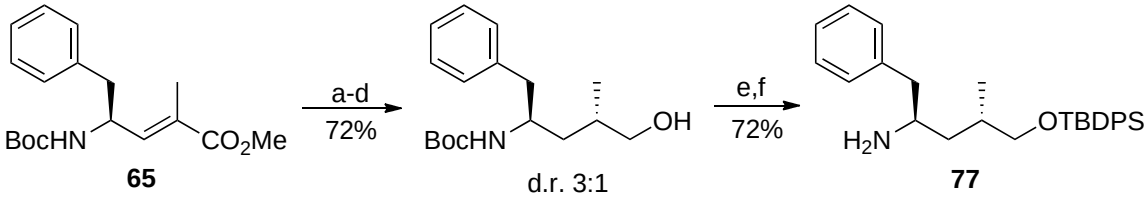
During their synthesis of the Tuv-Tup-fragment, Wipf *et al.* used an approach for Tup very similar to the Höfle group (Scheme **15**) [32]. They undertook a fairly stereocontrolled hydrogenation of α,β-unsaturated carboxylic acid obtained from **65**. However, they used the *O*-protected amine **77** in their peptide coupling step.

Also in 2004, Friestad *et al.* reported an interesting approach based on a Mn-mediated coupling of functionalized iodide **78** with a chiral modified hydrazone **79**, giving access to oxazolidinone **80** in excellent diastereoselectivity (Scheme **16**) [49]. The TFA-protected Tup derivative **81** was obtained after cleavage of the hydrazine and silyl ether and subsequent oxidation of the primary alcohol.

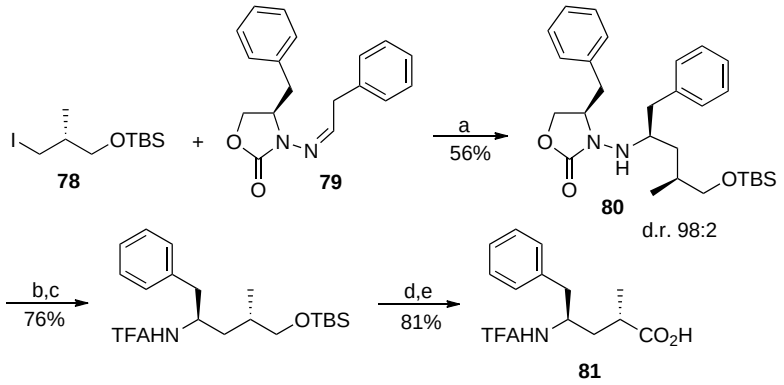
In 2006, Ellman *et al.* described a straightforward protocol towards unprotected Tup **84** in only three steps from commercially available starting materials (Scheme **17**) [37]. Key step was a SmI_2 mediated reductive coupling of methyl methacrylate and phenylacetaldimine **82**, obtained form (*R*)-*tert*-butane-sulfinamide and phenylacetaldehyde. Diastereomerically pure **83** could be obtained after chromatography. Subsequent ester hydrolysis and cleavage of the sulfinyl group gave rise to **84** in quantitative yield.

In the same year, Dömling and Wessjohann *et al.* described a synthesis *via* an asymmetric aziridine ring opening using pseudoephedrine derived propionamide **85** (Scheme **18**) [29]. Unfortunately, this approach generated the undesired configuration of the methyl group at the α-position (**87**) [50]. Cleavage of the auxiliary and the *N*-protecting group provided *epi*-Tup derivative **88**.

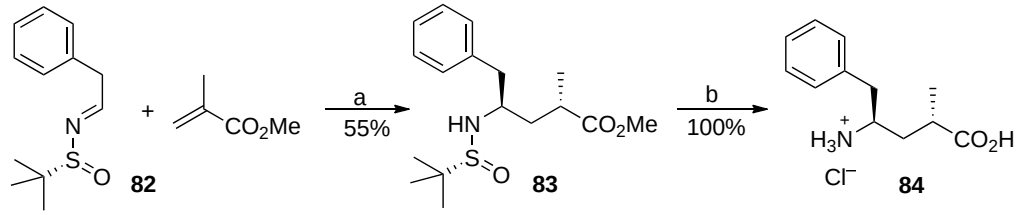
Shortly thereafter the authors presented another approach, based on Enders' SAMP auxiliary [51]. With



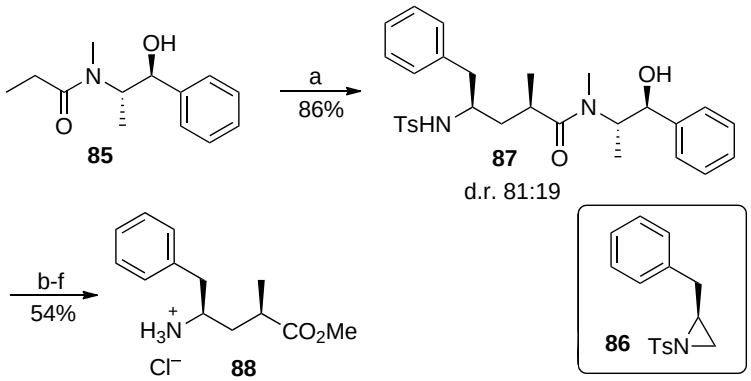
Scheme 15. Synthesis of Tup derivative **77** according to Wipf *et al.*: **a**) NaOH; **b**) H_2, Pd/C; **c**) *i*-BuOCOCl, Et_3N; **d**) $NaBH_4$; **e**) TBDPSCl, imidazole; **f**) TFA, PhSMe.



Scheme 16. Synthesis of Tup derivative **81** according to Friestad *et al.*: **a**) $Mn_2(CO)_{10}$, hν, $InCl_3$, CH_2Cl_2; **b**) TFA_2O, DMAP, pyridine; **c**) SmI_2, MeOH; **d**) TBAF, THF; **e**) $PhI(OAc)_2$, TEMPO.

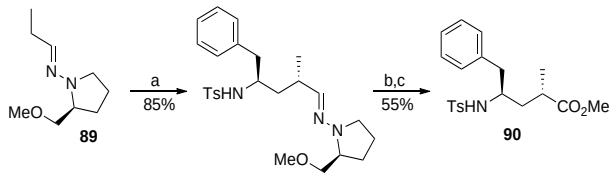


Scheme 17. Synthesis of Tup derivative **84** according to Ellman *et al.*: **a**) SmI_2, LiBr, H_2O, THF, –78 °C; **b**) HCl, dioxane/H_2O, Δ.



Scheme 18. Synthesis of *epi*-Tup derivative **88** according to Dömling and Wessjohann *et al.*: **a**) 1. LDA, LiCl, THF, –78 °C; 2. **86**, THF, –20 °C; **b**) 4M H_2SO_4/dioxane, reflux; **c**) MeOH, conc. HCl, reflux; **d**) Boc_2O, DMAP, MeCN; **e**) Mg (powder), MeOH, ultrasound; **h**) 4N HCl/dioxane.

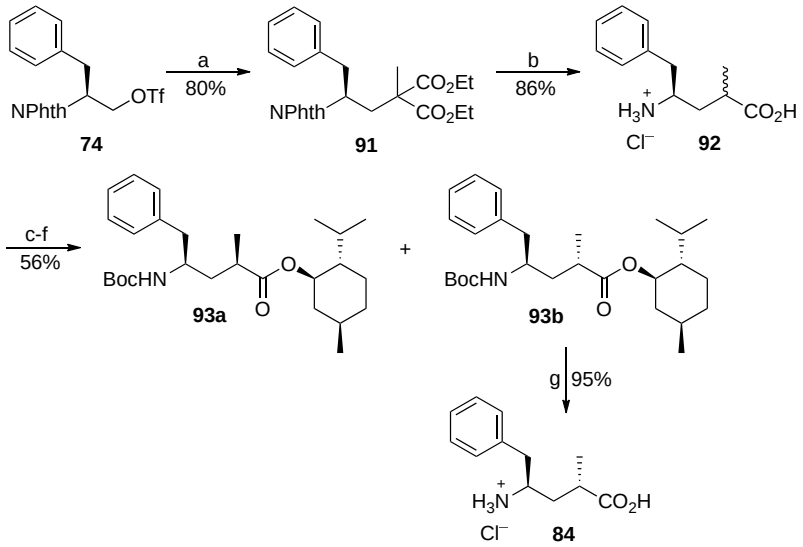
hydrazone **89**, the ring opening of the same aziridine **86** provided the desired product **90** with the correct configuration (Scheme **19**) [50].



Scheme 19. Synthesis of Tup derivative **90** according to Dömling and Wessjohann *et al.*: a) 1. LDA, 0 °C; 2. **86**, THF, –100 °C to rt; b) 1. O_3, acetone, –78 °C; 2. Jones reagent, –78 °C to rt; c) CH_2N_2, Et_2O/MeOH.

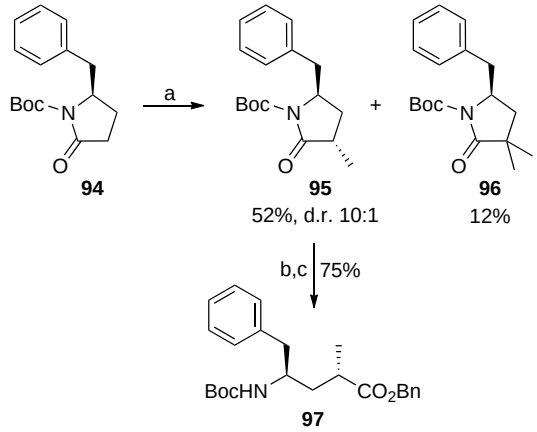
At the same time, Zanda *et al.* described the synthesis of enantiopure Tup **84** obtained by a chromatographic separation of the diastereomeric menthyl esters (Scheme **20**) [41]. Starting from the same *N*-protected triflate **74**, previously used by the Morphochem group, a S_N2 reaction of deprotonated methyl malonate generated the quaternary γ-amino malonate derivative **91**. Simultaneous cleavage of all protecting groups and decarboxylation provided unprotected Tup **92** as a 1:1 diastereomeric mixture. Conversion of **92** into the *N*-Boc-protected menthyl ester **93** allowed the separation of the stereoisomers by flash chromatography.

In 2008, Fecik *et al.* described the synthesis of Tup derivative **97** based on an asymmetric methylation of lactam **94** (Scheme **21**) [43]. Initial attempts using LDA or LHMDS and MeI resulted in low yields and diastereoselectivities in favor of the desired product **95**. The major side product was the dimethylated lactam. But with NaHMDS as a base, the yield and selectivity for **95** could be increased to a synthetically useful value. Saponification of the lactam ring and protection of the free carboxylic acid yielded the protected Tup derivative **97** as well as the corresponding dimethylated



Scheme 20. Synthesis of Tup **84** according to Zanda *et al.*: a) Diethyl 2-methylmalonate, NaH, 0 °C, rt, 7 h; b) 6N HCl, AcOH, 145 °C, 2 d; c) 2,2-dimethoxypropane, conc. HCl, MeOH, 60 °C, 1 d; d) Boc_2O, Et_3N, MeCN, 6 h; e) LiOH, H_2O/THF, 1 d; f) (–)-menthol, DCC, DMAP, CH_2Cl_2, 6 h; then flash chromatography to separate **93a** from **93b**; g) 6N HCl, 130 °C, 1.5 h.

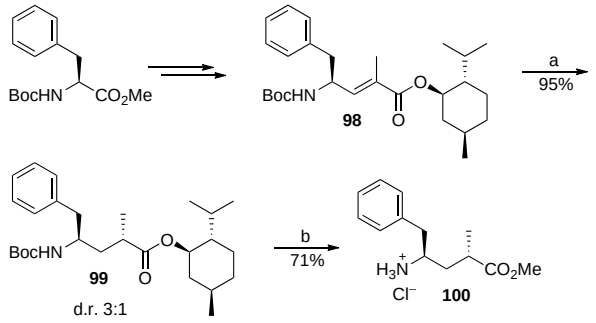
derivative, which were both incorporated into tubulysin derivatives [52].



Scheme 21. Synthesis of Tup **97** according to Fecik *et al.*: **a**) 1. NaHMDS, THF, –78 °C; 2. MeI, –78 °C to rt; **b**) LiOH; c) DBU, BnBr.

In 2009, Kazmaier *et al.* described an approach based on the previous work of Wipf [32] and Zanda [41]. Starting from *N*-Boc protected Phe-OMe, reduction, in situ Wittig olefination and subsequent transesterification provided α,β-unsaturated menthyl ester **98**, which was subjected to catalytic hydrogenation (Scheme **22**) [15,16]. The desired stereoisomer **99** was formed preferentially (diastereomeric ratio 3:1), and after chromatographic separation and deprotection, Tup ester **100** was obtained in almost diastereomerically pure form.

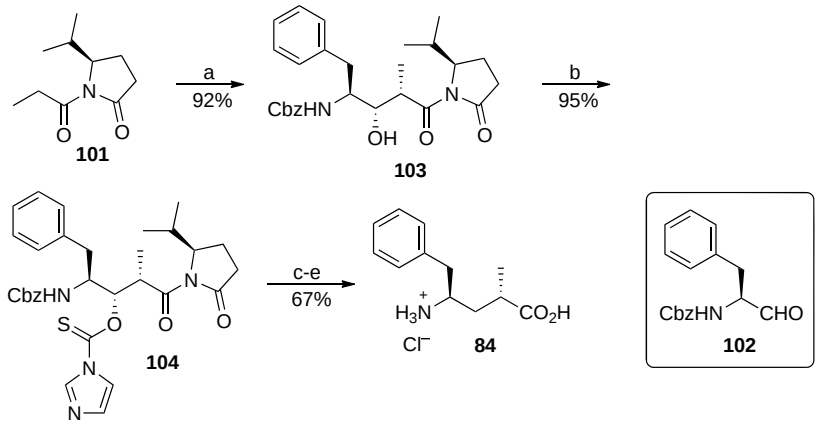
Tamura *et al.* took advantage of an Evans aldol reaction [53] of the (*Z*)-boron enolate of oxazolidinone **101** and *N*-protected phenylalaninal **102** (Scheme **23**) [47]. The expected aldol product **103** was obtained in good yield and as single stereoisomer, probably as the result of double stereoinduction. The OH-functionality formed was removed using the Barton-McCombie protocol [54]. Subsequent cleavage of the auxiliary and the Cbz-protecting group provided Tup **84**.



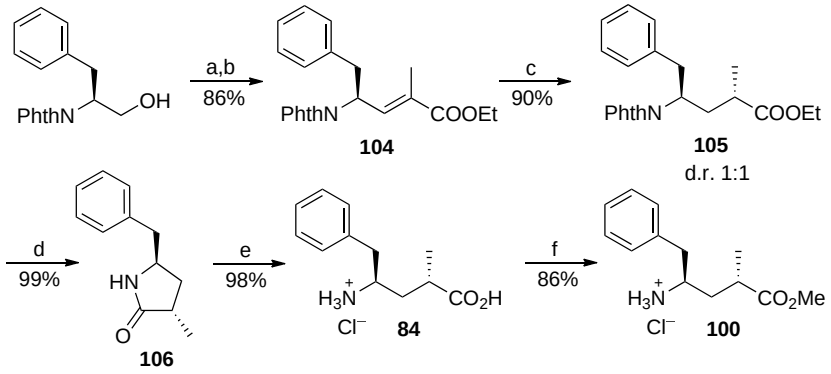
Scheme 22. Synthesis of Tup **100** according to Kazmaier *et al.*: **a**) H_2, Pd/C, MeOH; **b**) 1. chromatographic separation; 2. 6N HCl, 140 °C; 3. dimethoxypropane, cat. HCl, MeOH, 50 °C.

In 2011, Zanda *et al.* described the synthesis of Tup derivatives **84** and **100** similar to several previous approaches (Scheme **24**) [22]. Phthaloyl-protected phenylalaninol was oxidized and subjected to a Wittig olefination to give α,β-unsaturated ester **104**, which was subsequently hydrogenated to protected Tup **105**. The almost equimolar mixture of diastereomers was separated by flash chromatography. Cleavage of the phthaloyl protecting group resulted in the formation of lactam **106**, which was specified to Tup **84**.

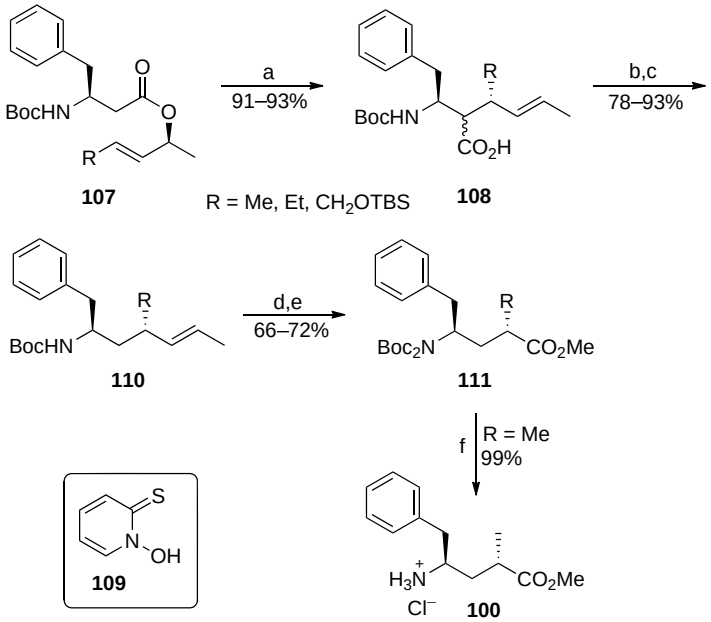
Recently, Kazmaier *et al.* described an approach towards Tup derivative **100** based on an Ireland-Claisen rearrangement (Scheme **25**) [54]. This approach allows easy variations of the α-substituent R by using the corresponding allylic esters. Ireland-Claisen rearrangement of β-amino acid allyl ester **107** provided the corresponding carboxylate **108** in excellent yield but moderate diastereoselectivity.



Scheme 23. Synthesis of Tup **84** according to Tamura *et al.*: **a**) 1. DIPEA, Bu_2BOTf, **102**, CH_2Cl_2, then 30% H_2O_2, MeOH; **b**) Im_2CS, THF; **c**) Bu_3SnH, AIBN, toluene; **d**) LiOH, 30% H_2O_2, THF/H_2O; **e**) H_2, 10% Pd/C, 4N HCl/ dioxane, THF.



Scheme 24. Synthesis of Tup-derivatives **84** and **100** according to Zanda *et al.*: **a**) Dess–Martin periodinane, $NaHCO_3$, CH_2Cl_2, 90 min; **b**) CH_2Cl_2, $Ph_3PCMeCO_2Et$, 16 h; **c**) H_2, Pd/C, EtOAc, 16 h, then chromatographic separation; **d**) $H_2NNH_2 \cdot H_2O$, EtOH, reflux; **e**) 6N HCl, 145 °C; **f**) dimethoxypropane, cat. HCl, MeOH, reflux.



Scheme 25. Synthesis of the Tup **100** according to Kazmaier *et al.*: **a**) LDA, TMSCl, THF, –78 °C to 60 °C, 2 h; **b**) DCC, **109**, DMAP, CH_2Cl_2, 0 °C to rt; **c**) *t*-BuSH, BEt_3, O_2, THF, 0 °C to rt; **d**) 1. *n*-BuLi; 2. Boc_2O, THF –78 °C to 60 °C; **e**) O_3, CH_2Cl_2, NaOH, MeOH, –78 °C to rt; **f**) HCl/dioxane, 0 °C.

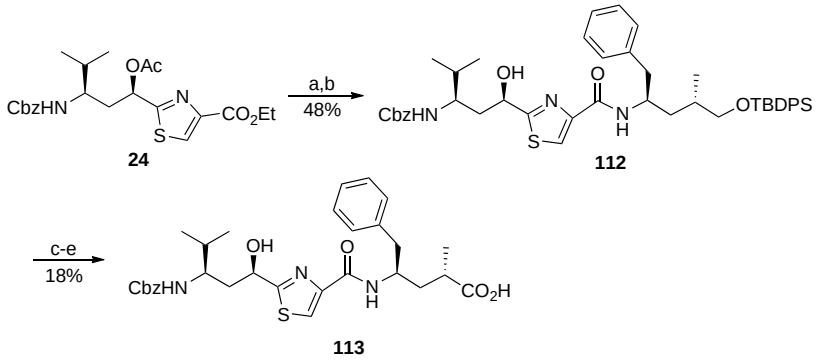
Fortunately, the configuration at the α-position does not play any role because the COOH group was removed, including this stereogenic centre, in the next step using the Barton method [55]. The carbamate (**110**) formed was double Boc-protected to avoid lactam formation in the next step, the ozonolysis using a protocol developed by Marshall *et al.* [56]. Cleavage of the Boc-protecting groups gave rise to the desired Tup-derivative **100**.

SYNTHESES OF TUV-TUP-FRAGMENTS

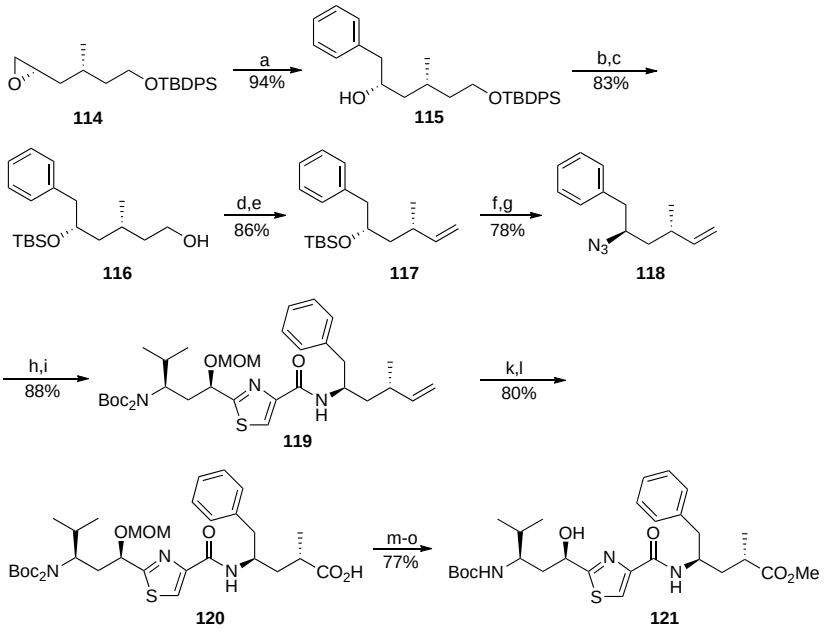
In principle, with the different building blocks in hand, the tubulysins and derivatives thereof can be obtained by standard peptide couplings using a wide range of coupling reagents. Nevertheless, in some cases, not the finished (unusual) amino acids but precursors thereof were used in the coupling steps, mainly to avoid undesired side reactions.

Wipf *et al.* subjected building block **24** to saponification, and subsequent DEPBT-coupling with silyl protected Tup-building block **77** led to the dipeptide fragment **112** (Scheme **26**) [32]. The secondary alcohol functionality was reacetylated in high yield. Cleavage of the silyl protecting group generated the free primary alcohol which was oxidized to the *N*-protected Tuv-Tup-fragment **113**. The generation of the terminal acid functionality after the peptide coupling step solved the problem of γ-lactam formation in the coupling step.

A comparable strategy was used by Chandrasekhar *et al.* (Scheme **27**) [44]. A suitable Tup precursor was obtained from a chiral protected epoxide **114** (synthesized from (-)-citronellol) in six steps. This epoxide was treated with



Scheme 26. Synthesis of Tuv-Tup fragment **113** according to Wipf *et al.*: **a**) NaOH, THF/H_2O; **b**) **77**, DEPBT, DIPEA; **c**) Ac_2O, pyridine; **d**) HF, pyridine; TEMPO, NaOCl, $NaOCl_2$, pH 6.7, MeCN.



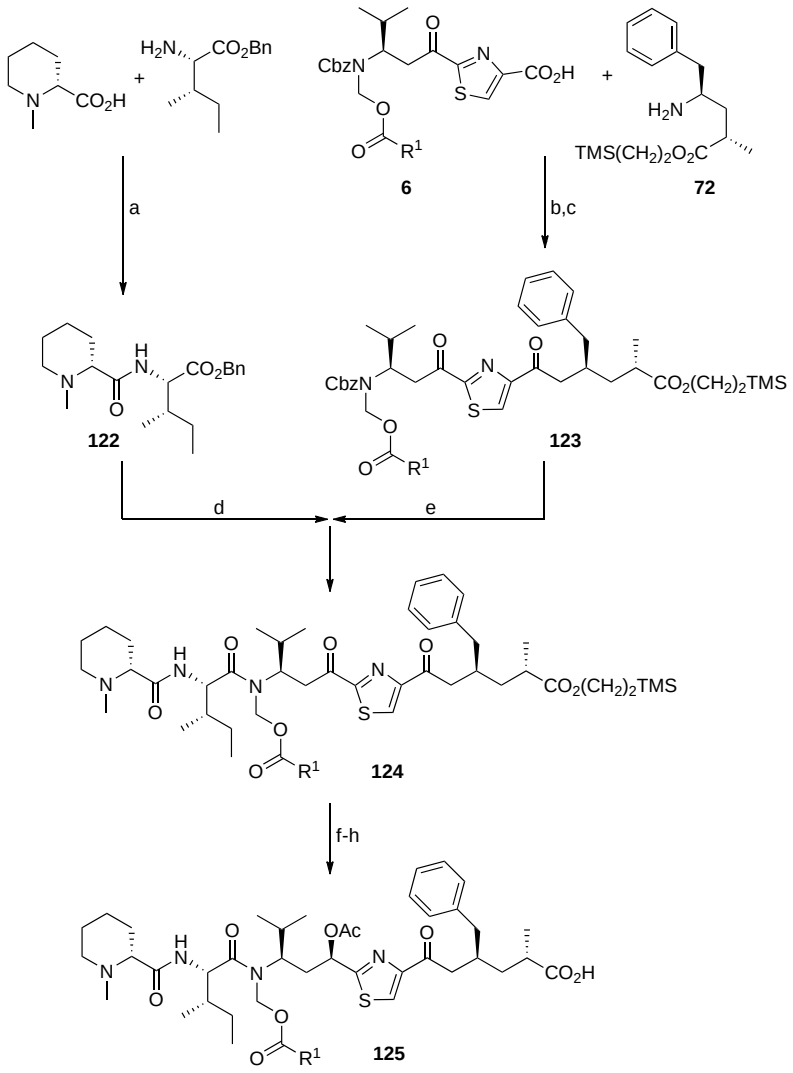
Scheme 27. Synthesis of Tuv-Tup fragment **119** according to Chandrasekhar *et al.*: **a**) PhMgBr, THF, 0 °C, 3 h; **b**) TBSCl, imidazole, CH_2Cl_2, 0 °C to rt, 6 h; **c**) NH_4F, MeOH, rt, 20 h; **d**) PPh_3, I_2, imidazole, Et_2O/MeCN, 0 °C, 15 min. **e**) KO*t*-Bu, THF, 0 °C to rt, 20 min; **f**) TBAF, THF, 0 °C to rt, 1 h; **g**) PPh_3, DIAD, DPPA, 0 °C, 45 min; **h**) $LiAlH_4$, THF, 0 °C, 30 min; **i**) **53**, HOBt, EDC, DIPEA, CH_2Cl_2, 0 °C to rt, 9 h; **k**) OsO_4, 2,6-lutidine, $NaIO_4$, dioxane/H_2O, 0 °C to rt, 20 h; **l**) Bis(acetoxy)iodobenzene, TEMPO, MeCN/ H_2O, rt, 1 h; **m**) CH_2N_2, Et_2O, 0 °C, 30 min; **n**) TFA/CH_2Cl_2, 0 °C, rt, 16 h, then NEt_3; **o**) Boc_2O, CH_2Cl_2, 0 °C to rt, 30 min.

phenyl magnesiumbromide to give the ring-opened product **115** as a single regioisomer in excellent yield. The secondary alcohol formed was silylated and the primary TBDPS group was selectively cleaved off (**116**). The resulting terminal alcohol was subjected to elimination giving rise to terminal alkene **117**. Cleavage of the silyl protecting group and Mitsunobu reaction with diphenylphosphoryl azide (DPPA) resulted in the formation of azide **118**. Reduction of the azide functionality and coupling with the double Boc-protected Tuv-fragment **53** delivered the dipeptide fragment **119**. The double bond was subjected to an oxidative cleavage and the corresponding aldehyde was directly oxidized to the triprotected Tuv-Tup fragment **120**, which was converted into the Boc-protected peptide ester **121**.

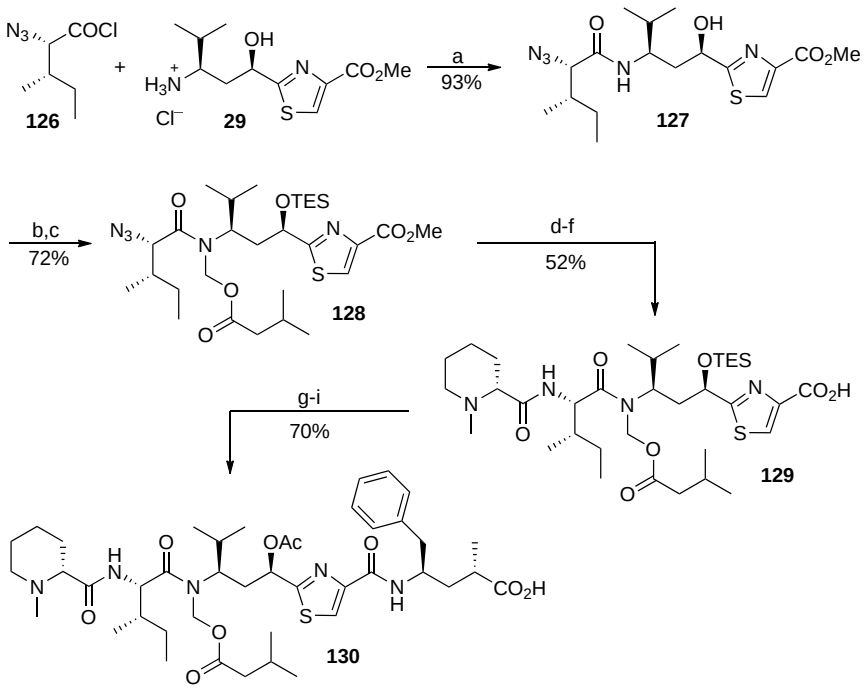
SYNTHESIS OF TUBULYSINS A – D

The first synthesis of tubulysins was reported by Höfle *et al.* in their patent from 2001, based on a fragment coupling strategy (Scheme **28**) [26]. Two dipeptides were formed, one from *N*-methyl (*R*)-pipecolic acid and (*S*)-lle-OBn (**122**), and the other one from the oxo form of tubuvaline **6** and the Tup fragment **72** (**123**). After hydrogenolysis of the benzylic protecting groups, peptide coupling *via* a pentafluorophenyl ester intermediate generated tetrapeptide **124**, which was reduced and subjected to functional group manipulations to provide the final tubulysin **125**.

Ellman *et al.* described the first stereoselective synthesis of tubulysin D, the most biologically active derivative of the whole family (Scheme **29**) [37,57]. Key step of their synthesis was the coupling of the α-azido acid chloride **126** with Tuv-derivative **29**. The azide masking group was chosen to allow a selective introduction of the *N*,*O*-acetal on the Tuv nitrogen of dipeptide **127**. Protection of the secondary alcohol as TES-ether and subsequent *N*-alkylation gave rise to dipeptide **128**. Advantageously, the azide group could be reduced under neutral conditions without affecting the sensitive *N*,*O*-acetal. Pd-catalyzed hydrogenation in the presence of the Mep pentafluorophenyl ester followed by deprotection of the secondary alcohol provided tripeptide **129**. The methyl ester could selectively be cleaved without affecting the



Scheme 28. Synthesis of tubulysins **125** according to Höfle *et al.*: **a**) diethyl cyanophosphonate, NEt_3; **b**) C_6F_5OH, DCC; **c**) NEt_3; **d**) $TFAOC_6F_5$; **e**) NEt_3, H_2, Pd/C; **f**) $NaBH_4$; **g**) Ac_2O; **h**) TBAF.



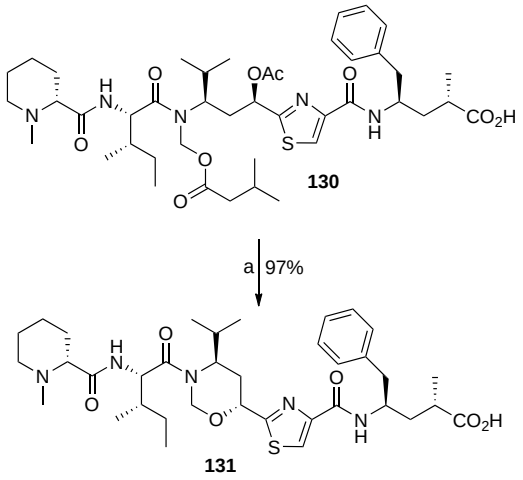
Scheme 29. Synthesis of tubulysin D (**130**) according to Ellman *et al.*: **a**) DIPEA, CH_2Cl_2; **b**) TESOTf, lutidine, CH_2Cl_2; **c**) 1. KHMDS, THF, –45 °C; 2. *i*-$BuCO_2CH_2Cl$; **d**) Mep-OC_6F_5, H_2, Pd/C, EtOAc; **e**) AcOH/THF/H_2O; **f**) Me_3SnOH, $Cl(CH_2)_2Cl$, 60 °C; **g**) C_6F_5OH, DIC, CH_2Cl_2; **h**) **84**, DIPEA, DMF; **i**) 1. Ac_2O, pyridine; 2. H_2O/dioxane.

sensitive *N,O*-acetal by using Me_3SnOH, according to a protocol described by Nicolaou *et al.* [58]. Activation of the free carboxylic acid as pentafluorophenyl ester and coupling with Tup **84** provided the required tetrapeptide which was finally *O*-acetylated to give tubulysin D (**130**).

An analogous strategy was used by Tamura *et al.* during their recent synthesis tubulysin epimers [47,59], as well as by Wessjohann *et al.* in their synthesis of tubulysin B [60].

SYNTHESES OF TUBULYSIN DERIVATIVES

If the tubulysins are treated with diluted acid, cyclization can occur giving rise to cyclo-tubulysins (**131**) (Scheme **30**) [47,60,61].

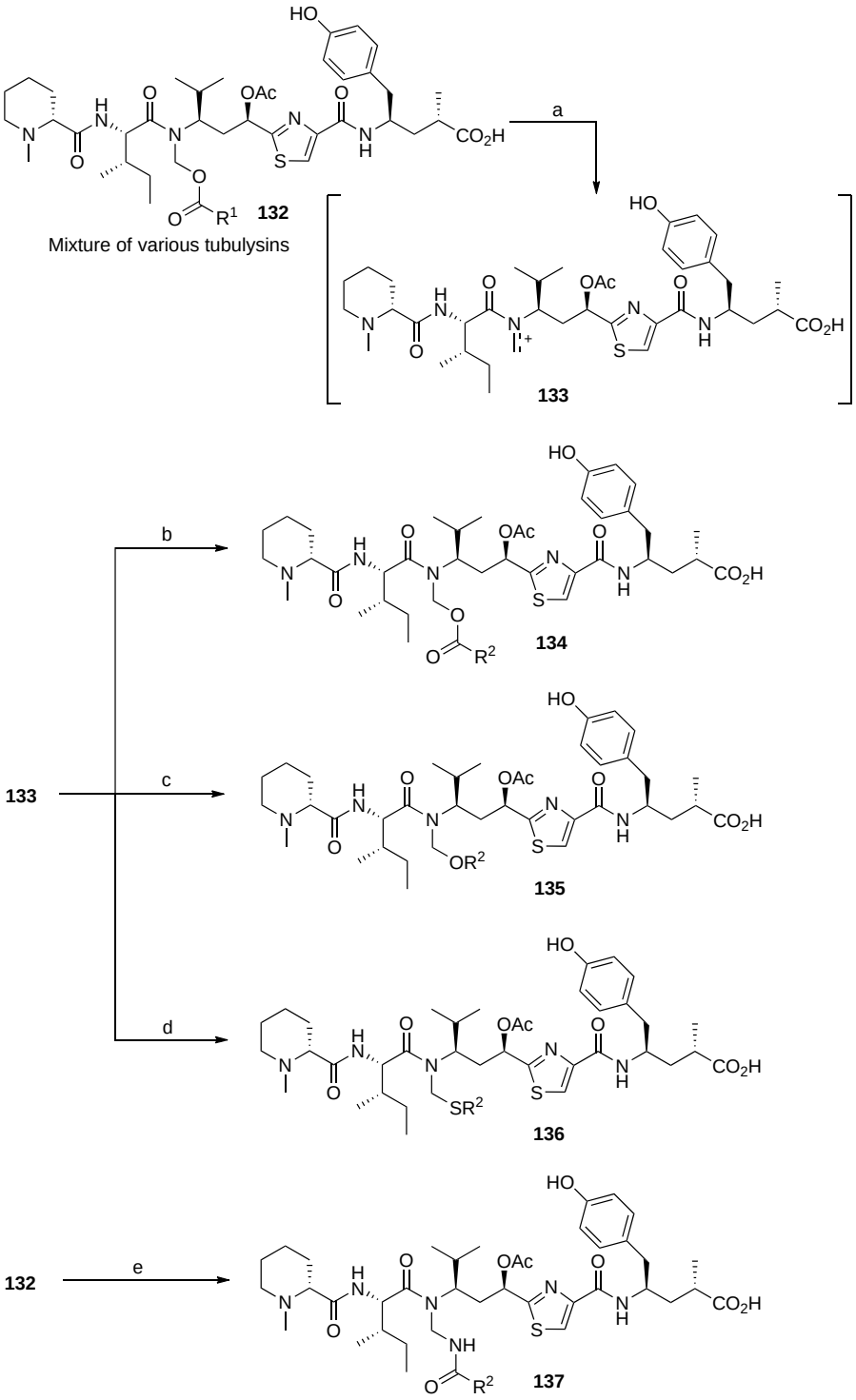


Scheme 30. Conversion of tubulysin D (**130**) into cyclo-tubulysin D (**131**): a) 1N HCl, THF.

Vlahov *et al.* reported the interconversion of tubulysins (**132**) into a wide range of *N*-functionalized derivatives *via* a stabilized *N*-acyliminium ion (Scheme **31**) [62]. Mixtures of different tubulysins (obtained by fermentation) were treated with trifluoroacetic acid, resulting in the cleavage of the different acyl side chains, forming the same *N*-acyl-iminium ion **133**. Addition of several carboxylic acids allows the synthesis of new tubulysins **134**, while the addition of alcohols and thiols gives rise to the corresponding *N*-acyl-*N,O*-acetals **135** and *N*-acyl-*N,S*-thioacetals **136**. Furthermore, the nitrogen of nitriles can also attack on the *N*-acyliminium ion in a Ritter reaction giving access to *N,N*'-diacyl-aminal derivatives **137**.

Wessjohann *et al.* described a very smart approach to highly potent tubulysin analogs named tubugis, in which the *N,O*-acetal moiety s replaced by a dipeptide element, which could easily be obtained in an Ugi reaction (Scheme **32**) [63]. Two of the four components required were themselves produced by other multicomponent reactions (MCRs). Key step of the synthetic sequence was the Ugi reaction of dipeptide **138**, silyl protected Tuv derivative **139**, formaldehyde and a range of alkyl isocyanides to give the key fragment **140** in one step in acceptable yield. Cleavage of the protecting groups, peptide coupling with Tup-OMe **100** under standard conditions and acetylation of the secondary alcohol provided the tubugis **141** in high yield. Their biological activity is comparable to tubulysin A and in the subnanomolar range.

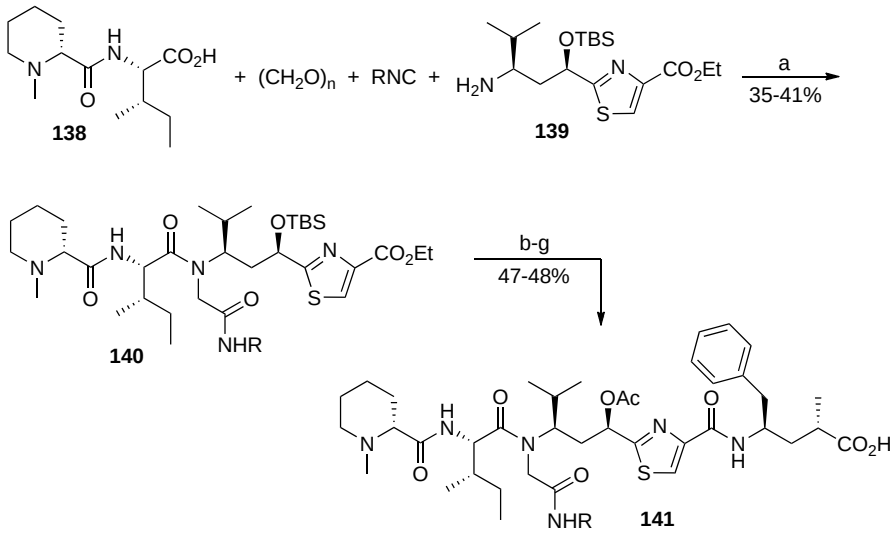
As a long *N*-side chain is not essential for good biological activity, it can be reduced to a *N*-methyl group without an extreme drop in activity. Wipf *et al.* reported the synthesis of *N*-desacyloxy tubulysin (*N*-methyl tubulysin) in 2007 (Scheme **33**) [40]. Deprotection of double protected



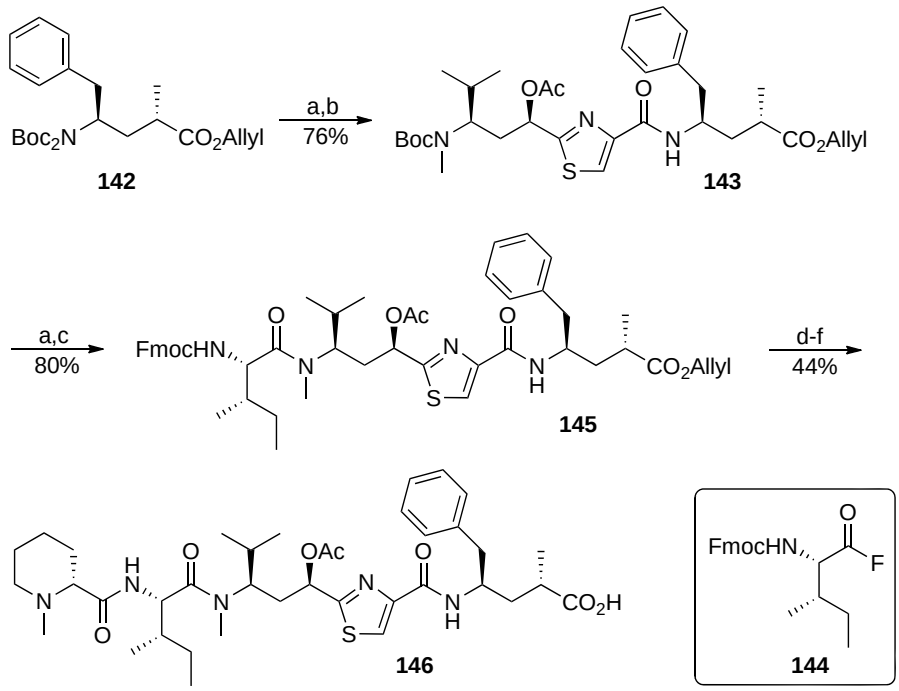
Scheme 31. Interconversion of tubulysins (**132**) according to Vlahov *et al.*: **a**) TFA, CH_2Cl_2, rt; **b**) R^2CO_2H; **c**) R^2OH; **d**) R^2SH; **e**) TFA, H_2SO_4, R^2CN.

Tup ester **142** and subsequent peptide coupling with Tuv-derivative **33** provided dipeptide **143** in good yield. Attempts to couple this dipeptide to a protected lle-derivative was found to be not a trivial issue. Attempts with most coupling reagents failed or gave low yields due to the congested steric environment and the reduced reactivity of the *N*-methylated amine. The acyl fluoride **144** was the reagent of choice providing the desired product **145** in up to 80% yield. After removing the Fmoc group, subsequent coupling with Mep and cleavage of the *C*-terminal allyl ester provided **146** in acceptable yield. The same approach was also used for the synthesis of other stereoisomers [26].

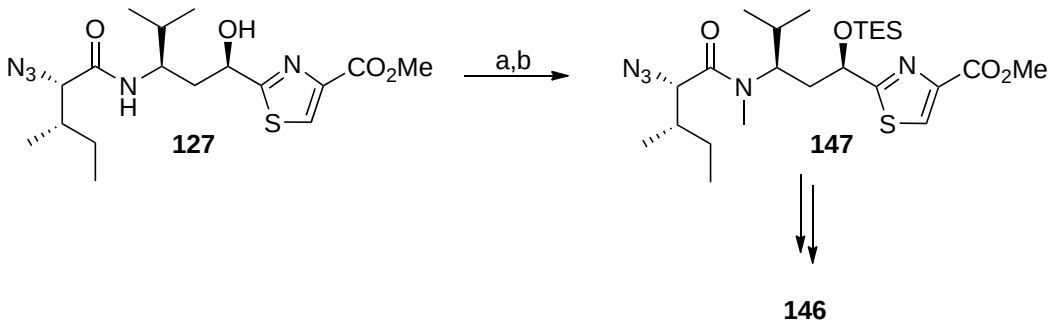
Ellman *et al.* described two independent approaches towards *N*-methyl analogs of the tubulysins (Scheme **34**).



Scheme 32. Synthesis of tubugis **141** according to Wessjohann *et al.*: **a**) MeOH; **b**) TFA, THF/H_2O; **c**) LiOH, THF/H_2O; **d**) C_6F_5OH, DIC, CH_2Cl_2; **e**) **100**, DIPEA, DMF; **f**) LiOH, THF/H_2O; **g**) Ac_2O/pyridine.



Scheme 33. Synthesis of *N*-methyl tubulysin **146** according to Wipf *et al.*: **a**) TFA, CH_2Cl_2; **b**) 1. *i*-BuOCOCl, NEt_3; 2. **33**, –20 °C to rt; **c**) **144**, DIPEA; **d**) $N(CH_2CH_2NH_2)_3$; **e**) Mep-OC_6F_5; **f**) $Pd(PPh_3)_4$, dimedone.



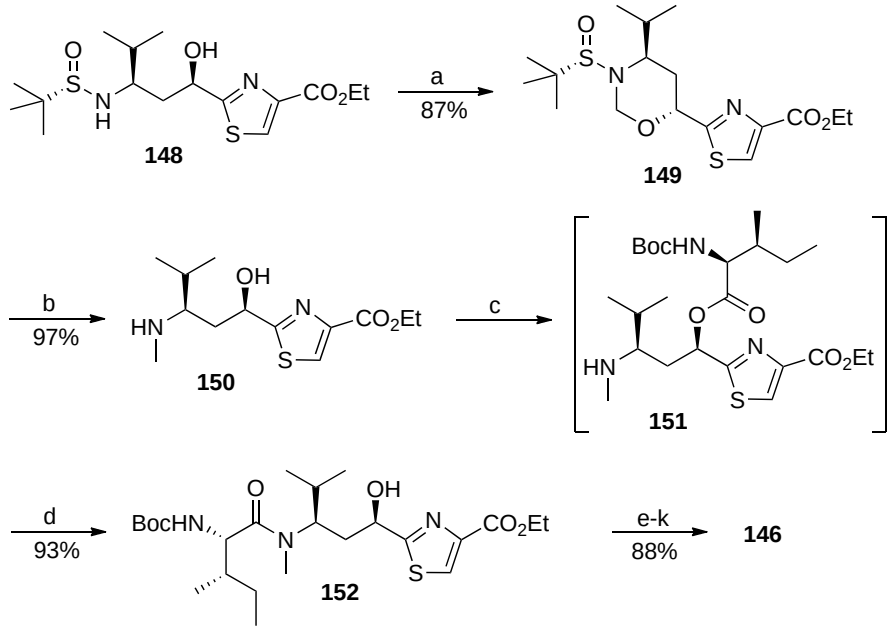
Scheme 34. Synthesis of *N*-methyl tubulysin **146** according to Ellman *et al.*: **a**) TESOTf, lutidine; **b**) 1. KHMDS, THF, –78 °C; 2: MeI.

According to their synthesis of tubulysin D, azido dipeptide **127** was subjected to *N*-methylation and the methylated dipeptide **147** was further converted into **146** [23].

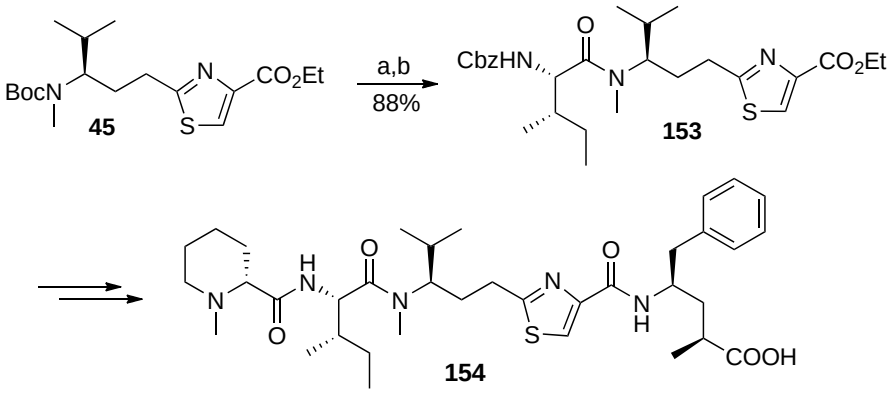
In a second approach, they started from *N*-sulfinyl-protected Tuv-derivative **148** (Scheme **35**) [23]. Simple heating of **148** with paraformaldehyde in toluene resulted in the formation of cyclic *N,O*-acetal **149**, which could be reduced with support-bound borohydride (MP-BH_3CN) to the required Tup-derivative **150**. In analogy to the previous example, the subsequent peptide coupling was the most critical step. A wide range of coupling agents were investigated, but all failed to give the desired dipeptide. Instead, the corresponding ester **151** was formed preferentially. On heating, ester **151** undergoes an O→N acyl shift providing the desired dipeptide **152** in excellent yield. The final synthesis of **146** provided no further problems, and several derivatives have been prepared by this protocol [64].

Not only the acyl moiety from the *N*-substituent can be removed without significant influence on activity, but also the acetoxy group in the Tuv fragment. Kazmaier *et al.* reported the synthesis of pretubulysin **154** (Scheme **36**) which shows cytotoxicity towards a wide range of tumor cells in the low or subnanomolar range [15,16]. With the desacetoxy-Tuv-derivative **45**, the peptide coupling (after Boc-deprotection) does not cause problems such as in the last examples, and especially good yields were obtained with BEP (2-Bromo-1-ethyl pyridinium tetrafluoroborate) as coupling reagent. Subsequent prolongation of the dipeptide **153** on both sides using standard peptide coupling reactions gave access to pretubulysin **154**. Several derivatives have been prepared where the central thiazole ring has been replaced by other aromatic or heteroaromatic ring systems, but these derivatives were significantly less active [18,25,65].

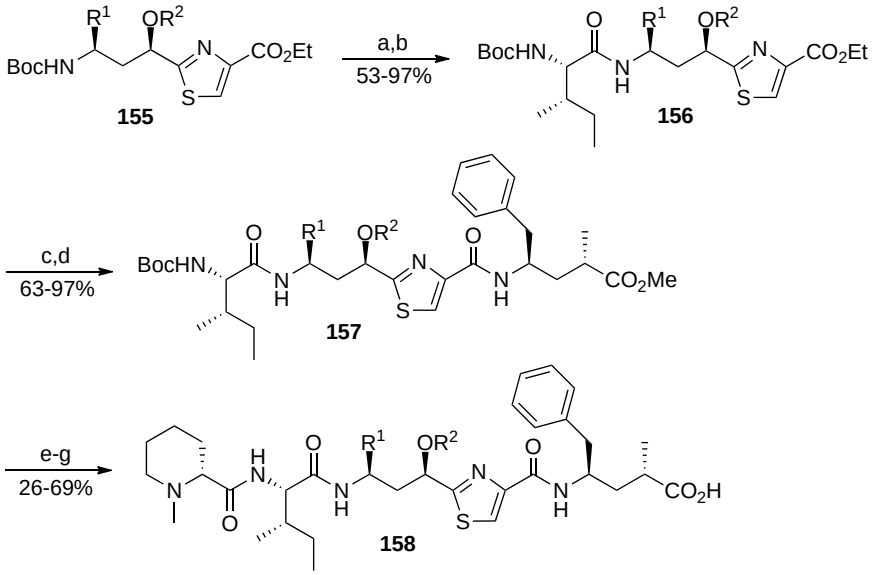
Also less active are tubulysins where the *N*-substituent is removed completely, such as in the tubulysins U and V. Several approaches towards these simplified analogs **158** have been reported, mainly based on standard peptide coupling protocols [20-22, 29, 30, 41, 52, 66]. A typical example leading to several modifications in the Tuv-fragment is shown in Scheme **37** [20].



Scheme 35. Synthesis of *N*-methyl tubulysin **146** according to Ellman *et al.*: **a**) $(CH_2O)_n$, toluene, 70 °C; **b**) MP-BH_3CN, MeCN/EtOH, HCl, dioxane, rt; **c**) Boc-IIe-OH, PS-DCCD, HOBt, CH_2Cl_2; **d**) toluene, 90 °C; **e**) TFA, CH_2Cl_2; **f**) Mep, PS-DCCD, HOBt, CH_2Cl_2; **g**) LiOH, H_2O/dioxane; **h**) Ac_2O, pyridine; **i**) C_6F_5OH, DIC, CH_2Cl_2; **k**) **84**, DIPEA, DMF.



Scheme 36. Synthesis of pretubulysin **154** according to Kazmaier *et al.*: **a**) 1. HCl, dioxane, 0 °C; **b**) Z-Ile, BEP, DIPEA, CH_2Cl_2, –10 °C.



Scheme 37. Synthesis of simplified tubulysin derivatives **158** according to Sani and Zanda *et al.* **a**) TFA, CH_2Cl_2, 0 °C to rt, 1 h; **b**) 1. HOBt, EDC·HCl; 2. Boc-Ile-OH, DIPEA, CH_2Cl_2, 0 °C to rt, 3 h; **c**) LiOH·H_2O, THF/H_2O, 0 °C to rt, 5 h; **d**) 1. HOAt, HATU; 2. **100**, NEt_3, CH_2Cl_2, 0 °C to rt, 3 h; **e**) TFA, CH_2Cl_2, 0 °C to rt, 1 h; **f**) 1. HOAt, HATU; 2. Mep-OH, NEt_3, 0 °C to rt, 3 h; **g**) 1 N LiOH, THF, 0 °C to rt, 2–3 days.

CONCLUSIONS

A wide range of synthetic protocols towards the synthesis of tubulysins, analogs and building blocks has been developed during the last years, allowing the synthesis of new derivatives in a straightforward manner. Structure-activity relationship (SAR) studies indicate that simplifications are tolerated in the *N*-side chain and on the Tuv motif, but at least an *N*-methyl group on the Tuv is required for good biological activity.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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