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Efforts toward the Total Synthesis of Tubulysins: New Hopes for a More Effective Targeted Drug Delivery to Tumors

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Cancer chemotherapy relies on the expectation that anticancer drugs will preferentially kill rapidly dividing tumor cells rather than normal cells. As a large portion of the tumor cells must be killed to obtain and maintain a complete remission, large drugs doses are typically used, with significant toxicity toward proliferating nonmalignant cells. Indeed, the majority of pharmacological approaches for the treatment of solid tumors suffers from poor selectivity, thus limiting dose escalation (that is, the doses of drug required to kill tumor cells cause unacceptable toxicities to normal tissues).

The development of more selective anticancer drugs with an improved ability to discriminate between tumor cells and normal cells is possibly the most important goal of modern anticancer research. For this reason, there is much current interest in the development of new cytotoxic chemical entities with improved selectivity toward tumor cells and lower systemic toxicity. A highly promising strategy is based on the discovery that many tumors overexpress specific protein markers at the site of neoplasia, suggesting the possibility of implementing a ligand-based selective targeted delivery of drugs to the cancer cells and/or the tumor stroma.[1] Indeed, a tumor-associated antigen can often be targeted by a monoclonal antibody (mAb) with suitable affinity and specificity. The pharmacokinetic properties of mAbs can be modulated by the choice of the recombinant antibody format, which ranges from the small scFv antibody fragments (which typically exhibit 90% clearance from the circulatory system within 1 hour) to the full immunoglobulins IgG (which display a half-life of weeks for the beta phase of blood clearance). Furthermore, mAbs and their fragments can be engineered to carry functional groups suitable for chemical modification such as C-terminal cysteine residues for selective coupling with thiol-reactive compounds. These features make mAbs excellent vehicles for the targeted delivery of cytotoxic drugs to the tumor in the form of an antibody–drug conjugate, whereas mAbs themselves are often only weakly cytotoxic and therefore not therapeutically useful.[2] Mylotarg (Wyeth) is the first example of a recently approved mAb–cytotoxic drug conjugate for cancer therapy.[3] Considering the difference in molecular weight between antibodies and cytotoxic molecules, and the fact that the injection of large quantities of antibody (>100 mg) into patients is not desirable for cost-of-goods considerations, it is intuitive to see that ideal drugs for targeted delivery applications should be capable of killing cells in the sub-nanomolar concentration range and should carry suitable functional groups for coupling to antibody molecules. Such highly potent drugs may allow the use not only of internalizing antibodies, but also of antibodies directed against the more abundant and stable stromal antigens, provided that a suitable hydrolytic mechanism is available for the liberation of the drug at the tumor site. Maytansanoids, auristatins, taxanes, epothilones, and other highly potent natural compounds have attracted considerable interest in this respect.[4] However, the recent discovery of tubulysins by Höfle, Reichenbach, and co-workers at the Helmholtz research center GBF (Braunschweig, Germany) generated the unprecedented hope of finally having a sufficiently powerful weapon in hand that allows full exploitation of the spectacular selectivity of mAbs.[5] Recently, the German biotechnology company Morphochem and GBF set up a cooperation agreement with the objective to develop new therapeutics against cancer by using tubulysins.

Tubulysins (Figure 1) are a family of tetrapeptides produced in rather small quantity (<4 mg L^{-1} culture broth) by two different species of myxobacteria: *Archangium gephyra* and *Angiococcus disciformis*. The structure, stereochemistry, and biosynthetic pathway of tubulysins were recently determined by Höfle, Reichenbach, and co-workers, who also reported the potent cytotoxic activity (in the nanomolar concentration range) of these compounds.[6] The cytotoxic activity of tubulysins stems from their ability to bind tubulin and disintegrate microtubules of dividing cells, thus inducing apoptosis. As cancer cells show high cell-division rates, this property of tubulysins could be used to selectively target cancer cells, although their extremely high toxicity renders unlikely the therapeutic use of tubulysins alone.

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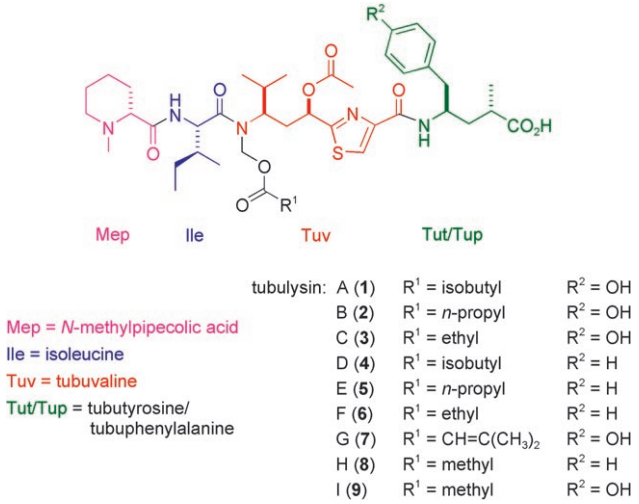
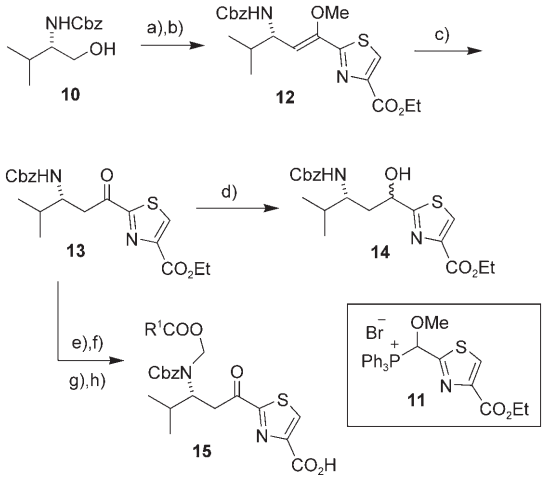


Figure 1. Structure of tubulysins.

From the structural point of view, tubulysins have 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 that features two stereogenic centers dubbed tubuvaline (Tuv) 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 functions (Figure 1). Tubulysins are practically water-insoluble, and the most cytotoxic forms are also the most lipophilic, namely tubulysins D, E, F, and H (listed in decreasing order of lipophilicity), which contain Tup instead of Tut.

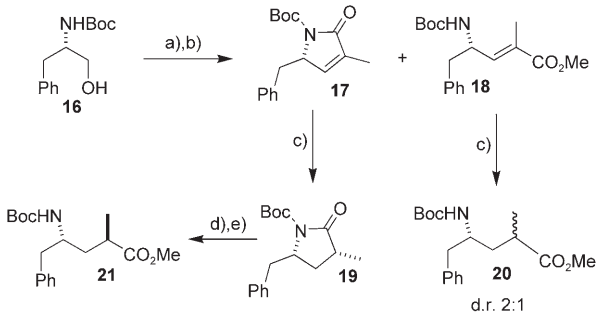
Although the synthesis of tubulysins might not appear to be an extremely challenging endeavor upon initial observation, to date, there are no published reports of total syntheses, suggesting that several important challenges are present. Indeed, a number of challenging synthetic issues have been described in a recent review by Dömling and Richter (Morphochem), who report that a major hurdle is the installation of the acid- and base-labile *N*,*O*-acetal ester and the configurationally and chemically sensitive thiazole fragment.[4b] However, two research groups, one led by Höfle and the other by Dömling, have claimed two different total syntheses of tubulysins, so far described only in patents.

The synthesis of the Tuv unit **15**, reported by Höfle and coworkers[7] (Scheme 1), starts from L-valinol **10**, which was oxidized to the corresponding α-amino aldehyde and subjected to a Wittig reaction with the phosphorane generated from the phosphonium salt **11**. The reaction provided the thiazolyl enol ether **12**, which hydrolyzed in acidic conditions to the thiazolyl ketone **13**. Hydride reduction of the latter afforded carbinol **14**. Alternatively, the thiazolyl ester function of the ketone **13** could be hydrolyzed to the acid, which could then be reprotected as the TMS-ethyl ester. The Cbz-amino function was alkylated, and the TMS-ester was hydrolyzed to provide the targeted *N*-Cbz *N*,*O*-acetal groups of Tuv **15**.



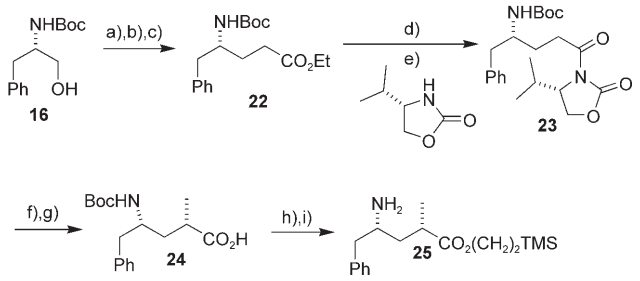
Scheme 1. Synthesis of the Tuv fragment by Höfle et al.: a) Swern oxidation; b) DBU, **11**; c) THF, HCl (35%); d) EtOH, $NaBH_4$; e) NaOH; f) TMSEtOH, DCC; g) NaH, $R^1CO_2CH_2Cl$; h) TBAF. Cbz = benzyloxycarbonyl, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DCC = *N*,*N*′-dicyclohexylcarbodiimide, TBAF = tetrabutylammonium fluoride, TMSEtOH = 2-(trimethylsilyl)ethanol.

The Tup unit was synthesized according to two different strategies. In the first (Scheme 2) Tup was obtained predominantly as the undesired diastereomer. *N*-Boc-protected L-phenylalaninol **16** was subjected to Swern oxidation, and the resulting intermediate aldehyde underwent a Horner–Emmons reaction to afford a mixture of lactam **17** and γ-amino ester **18**, with different configurations about the double bond. The two compounds were separately hydrogenated to afford the corresponding saturated products **19** and **20**. Lactam **19** was hydrolyzed to the carboxylic acid, which was treated with diazomethane to provide methyl ester **21**.



Scheme 2. First synthesis of the Tup fragment by 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. Boc = *tert*-butoxycarbonyl.

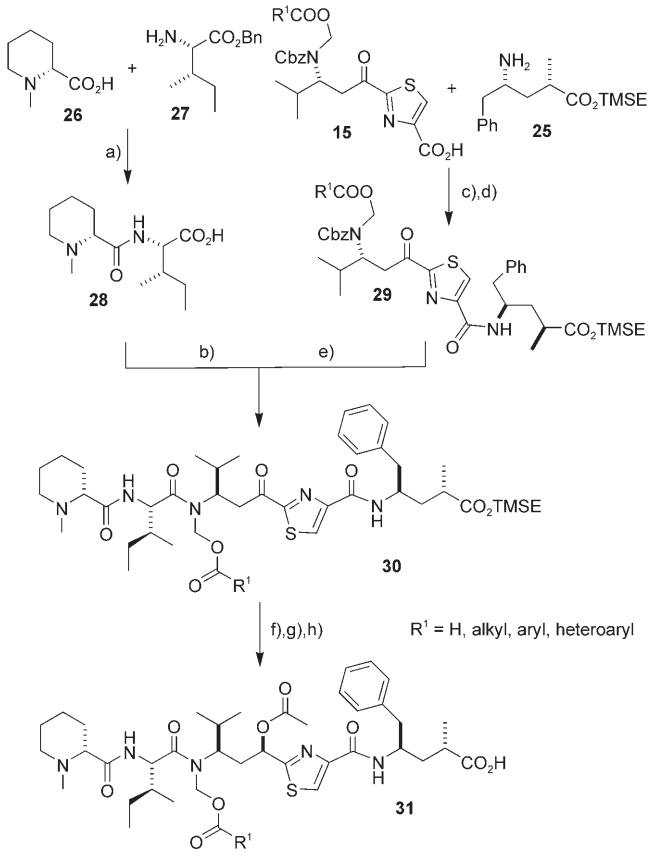
In the second approach to the Tup unit (Scheme 3), which in this case was obtained with the correct stereochemistry, **16** was oxidized and submitted to Wittig-type two-carbon homologation into the γ-amino ester **22**. The methyl group was installed on the stereogenic α-carbon of the final C-protected Tup **25** by conversion of **22** into the *N*-acyloxazolidin-2-one **23**



Scheme 3. Second synthesis of the Tup fragment by Höfle et al.: a) Swern oxidation; b) Wittig reaction; c) H_2/Pd-C; d) NaOH, H_2O; e) pivaloyl chloride, Et_3N; f) NaHMDS, MeI; g) H_2O_2, LiOH; h) TMSEtOH, DCC; i) TFA, CH_2Cl_2. HMDS = hexamethyldisilazane, TFA = trifluoroacetic acid.

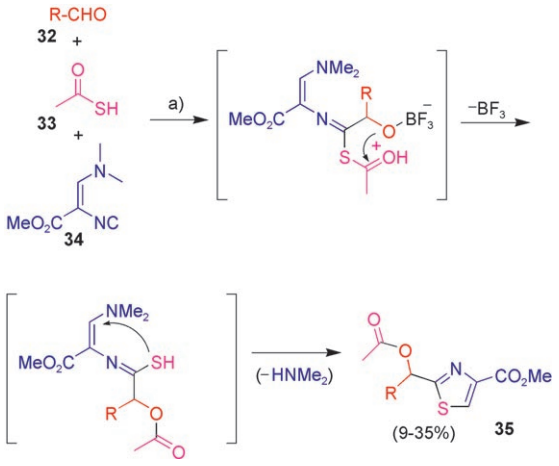
and subsequent stereoselective methylation, followed by exocyclic cleavage to **24** and protection of the carboxyl group.

The tubulysin skeleton was assembled next (Scheme 4). Interestingly, the oxo form of the thiazolyl amino acid *N*-Cbz–Tuv–H **15**, which already bears the *N*,*O*-acetal function, was used in the coupling with H–Tup–OTMSE **25** to deliver the key dipeptide **29**. After hydrogenolysis of the *N*-Cbz group, the R^1COOCH_2–(oxo)Tuv–Tub–OTMSE fragment was coupled with the Mep–Ile–OH dipeptide **28** to afford the protected oxotubulysin **30**. This was reduced and subjected to functional-group manipulations to attain the final tubulysin **31**.



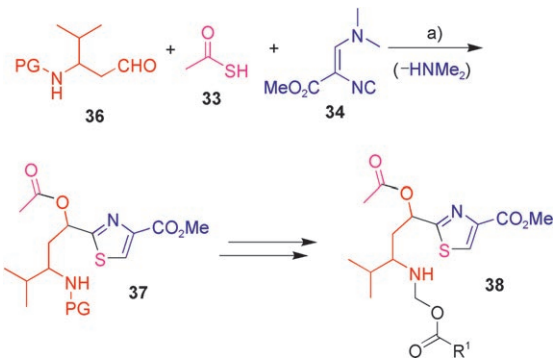
Scheme 4. Completion of the synthesis of tubulysins by Höfle et al.: a) diethyl cyanophosphonate, Et_3N; b) pentafluorophenol trifluoroacetate; c) pentafluorophenol, DCC; d) Et_3N; e) Et_3N, Pd/C, H_2; f) $NaBH_4$; g) Ac_2O; h) TBAF. TMSE = 2-(trimethylsilyl)ethyl.

The Morphochem approach[8] to tubulysins is based on a novel multicomponent reaction (Scheme 5) that allows a versatile combinatorial approach to the Tuv 2-acyloxymethylthiazole fragment **35**.[9] Thus, aldehyde **32**, thioacetic acid **33**, and 3-(*N*,*N*-dimethylamino)-2-isocyanoacrylate **34** in the presence of a Lewis acid afforded the complex target thiazolyl framework **35** smoothly, albeit in rather low yields. The proposed mechanism of the multicomponent process is portrayed in Scheme 5.



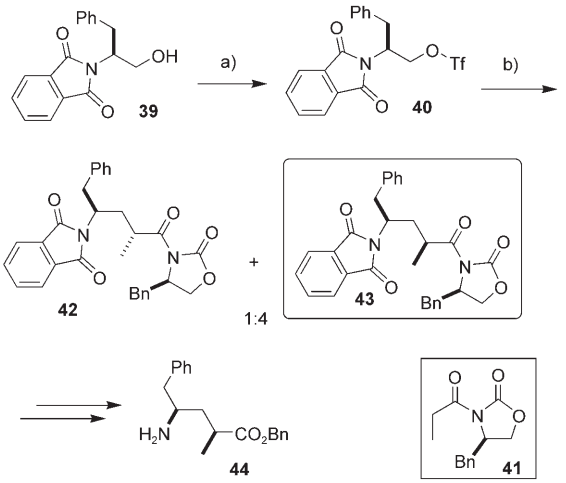
Scheme 5. Multicomponent synthesis (Morphochem) of the Tuv 2-acyloxymethyl-thiazole fragment: a) $BF_3{\cdot}OEt_2$, THF.

The entire Tuv unit **38** can therefore be assembled by using the β-amino aldehyde **36** (Scheme 6) as starting material, followed by attachment of the *N*,*O*-acetal moiety. Low yields and scarce stereochemical control are the main drawbacks of this otherwise very direct and flexible approach to the complex Tuv structure.



Scheme 6. Multicomponent synthesis (Morphochem) of the Tuv unit: a) $BF_3{\cdot}OEt_2$, THF. PG = protecting group.

The Morphochem approach to the Tup fragment relies on the C–C bond-forming nucleophilic attack by the *N*-propionyloxazolidin-2-one **41** (Scheme 7) at the triflate **40**, obtained from *N*-phthaloyl-L-phenylalaninol **39**. The reaction provides

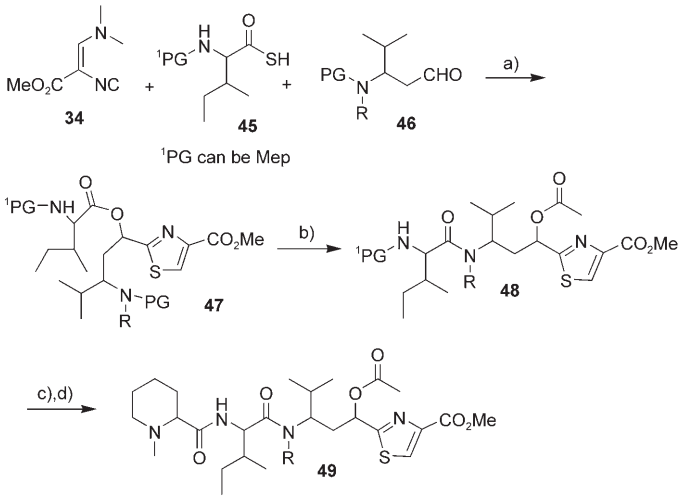


Scheme 7. Stereocontrolled synthesis (Morphochem) of the Tup unit: a) Py, Tf_2O, CH_2Cl_2, −78 °C (60%); b) **41**, LiHMDS, −40 °C, THF (70%). Bn = benzyl, Py = pyridine, Tf = trifluoromethanesulfonyl.

the diastereomer **43** with the desired stereochemistry (d.r. 4:1). Exocyclic oxazolidinone cleavage and protecting group manipulation leads to the appropriately protected Tup unit **44**.

A coupling sequence (with 1-hydroxybenzotriazole, diisopropylcarbodiimide, or DCC) that involves the Mep–Ile–OH dipeptide **28**, the Tuv fragment **38**, and the Tup unit **44**, permits the assembly of the whole tubulysin structure. Unfortunately, little stereochemical information is provided in the patents from Morphochem; it is therefore difficult to understand whether or not this approach allows control of stereochemistry of the final tubulysin products.

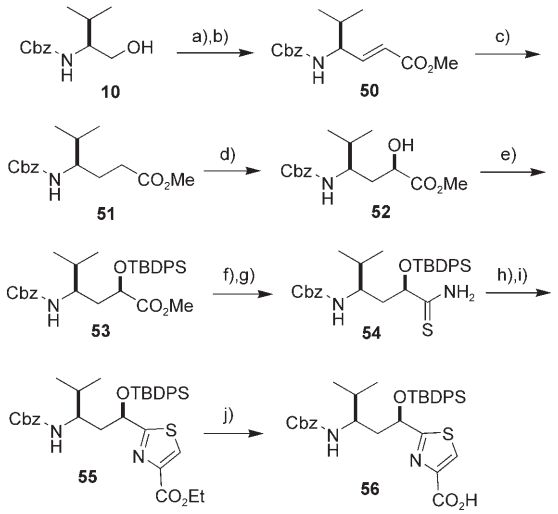
In a variation of the same multicomponent strategy (Scheme 8), the thiol acid derivative **45** of Ile is used with the β-amino aldehyde **46** and the isocyanoacrylate **34** to provide an intermediate ester **47** that isomerizes to the corresponding Ile–Tuv dipeptide **48** upon cleavage of the Tuv amino group. Coupling with Mep–OH gives the Mep–Ile–Tuv tripeptide **49**.



Scheme 8. Alternative partial synthesis (Morphochem) of the tubulysin structure: a) $BF_3{\cdot}OEt_2$, THF; b) Cleavage of PG and isomerization; c) Cleavage of PG^1; d) Coupling with **26**.

Also in this case, little stereochemical information about the protocol is provided.

Recently, Wipf and co-workers developed an interesting approach to the Tuv–Tup unit.[10] In the optimized synthesis of Tuv (Scheme 9), the γ-amino ester precursor **51** was obtained by Wittig reaction and subsequent selective double-bond hydrogenolysis of **50**, obtained through the aldehyde derived from *N*-Cbz-L-valinol (**10**). α-Hydroxylation to **52** was carried



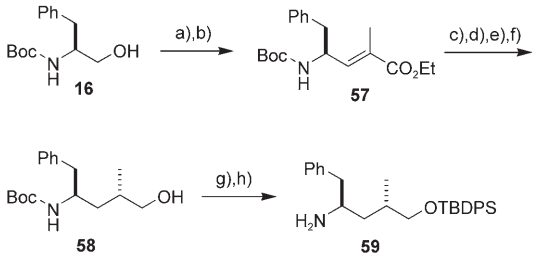
Scheme 9. Synthesis of orthogonally protected, enantiomerically pure Tuv–OH by Wipf et al.: a) TEMPO, NaOCl, $NaHCO_3$, NaBr; b) $Ph_3P{=}CHCO_2Me$ (64%); c) *rac*-BINAP, *t*BuNa, CuCl, polymethylhydroxysiloxane (80%); d) NaHMDS, sulfonyloxaziridine (66%); e) TBDPSCl, imidazole (92%); f) NH_3, MeOH; g) Belleau's reagent (2,4-bis(4-phenoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide) (60%); h) $BrCH_2COCO_2Et$; i) TFAA, Py (70%); j) NaOH. BINAP = 2,2′-bis(diphenylphosphino)-1,1′-binaphthalene, TBDPSCl = *tert*-butyl(chloro)diphenylsilane, TEMPO = 2,2,6,6-tetramethylpiperidin-1-yloxyl, TFAA = trifluoroacetic anhydride.

out by the Davis reagent, and the protected α-silyloxy ester **53** was then transformed into the thioamide **54**, which was submitted to condensation with bromopyruvate to afford the orthogonally protected Tuv ester **55** and then converted into the acid **56**.

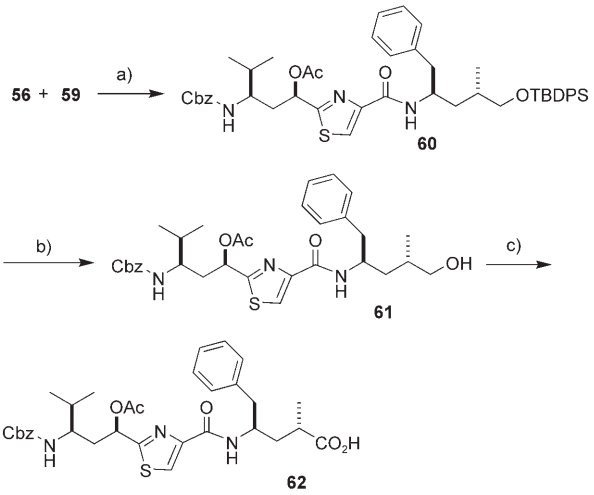
The Tup unit was prepared with a strategy similar to that described by Höfle and co-workers (see Scheme 2), namely by installation of a three-carbon fragment onto the aldehyde derived from *N*-Boc-L-phenylalaninol **16** (Scheme 10) by Wittig reaction. This was followed by a fairly stereocontrolled hydrogenation of the resulting α,β-unsaturated ester **57** to **58**. However, in the Wipf approach the primary carbinol derivative **59** of Tup is used for the coupling.

Coupling of **56** with **59** (Scheme 11) afforded the *O*-silylcarbinolic dipeptide precursor **60**, which was deprotected to **61** and oxidized to the target Cbz–Tuv–Tup–OH dipeptide **62**.

A diastereoselective approach to both Tuv and Tup γ-amino acid frameworks, based on a Mn-mediated coupling of functionalized iodides and hydrazones, has been recently reported by Friestad and co-workers.[11] The Tuv precursor **68** (Scheme 12) was obtained by coupling isopropyl iodide with the chiral hydrazone **65**, followed by samarium(II)-promoted

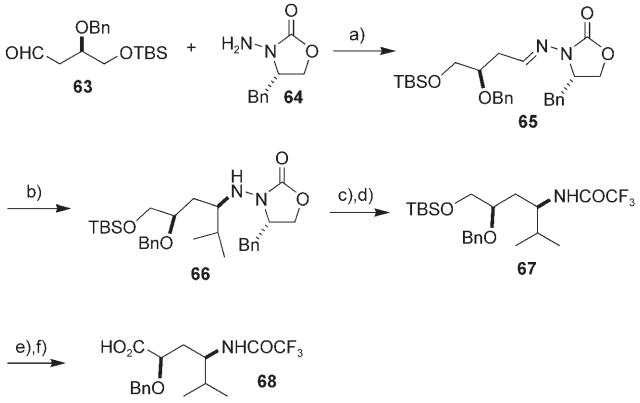


Scheme 10. Synthesis of an enantiomerically pure Tup derivative by Wipf et al.: a) TEMPO, NaOCl, $NaHCO_3$, NaBr; b) $Ph_3P{=}C(CH_3)CO_2Et$ (69%); c) NaOH; d) H_2, Pd/C; e) *i*BuOCOCl, Et_3N; f) $NaBH_4$ (72%, d.r. 3:1) ; g) TBDPSCl, imidazole; h) TFA, PhSMe (72%).



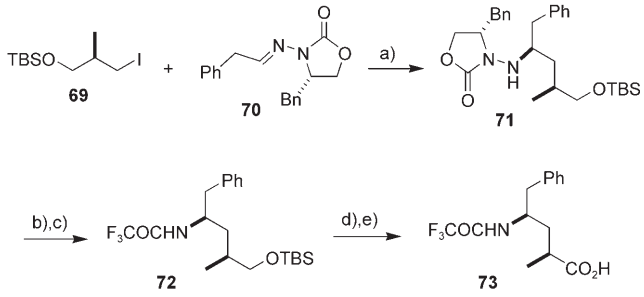
Scheme 11. Synthesis of the Tuv–Tub fragment by Wipf et al.: a) 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4-(3*H*)-one, Hunig's base; b) HF, Py; c) TEMPO, NaOCl, NaO_2Cl, pH 6.7 (23% isolated).

cleavage of the hydrazine N–N bond of **66** to afford the intermediate silylether **67**. This was deprotected and oxidized to yield the target compound **68**.



Scheme 12. Approach to a Tup precursor by Friestad et al.: a) *p*-toluenesulfonic acid, M.S. (99%); b) *i*Pr-I, $Mn_2(CO)_{10}$, *hν*, $InCl_3$, CH_2Cl_2 (77%, d.r. > 98:2); c) TFAA, DMAP, Py; d) SmI_2, MeOH (71%); e) TBAF, THF; f) $PhI(OAc)_2$, TEMPO (96%). DMAP = 4-dimethylaminopyridine.

Analogously, the iodide **69** (Scheme 13) was stereoselectively coupled to the chiral hydrazone **70** to afford the hydrazine **71**, which was transformed into the target TFA–Tup–OH **73** through cleavage and oxidation of the silylether precursor **72**.



Scheme 13. Approach to TFA–Tup–OH by Friestad et al.: a) $Mn_2(CO)_{10}$, *hν*, $InCl_3$, CH_2Cl_2 (56%, d.r. > 98:2); b) TFAA, DMAP, Py; c) SmI_2, MeOH (76%); d) TBAF, THF; e) $PhI(OAc)_2$, TEMPO (81%).

Conclusions and Perspectives

Although at first sight tubulysins present a relatively simple linear tetrapeptide structure, the presence of several chemically and configurationally sensitive functions together with a certain density of reactive groups render their total synthesis a challenging endeavor. In fact, despite their considerable interest and potential as powerful cytotoxic substrates for the therapeutic targeting of tumors, no total synthesis has been published yet. Future synthetic approaches to tubulysins will have to meet the needs of life scientists, providing sufficiently large quantities of pure compounds to test the performance of mAb–tubulysin conjugates for targeted delivery to tumors. Furthermore, chemical modification strategies are badly needed for the site-specific coupling of tubulysins to macromolecular carriers, which are compatible with the cleavage and liberation of cytotoxic moieties either in intracellular compartments or in the tumor stroma, possibly by proteolytic activation. Bioproduction of tubulysins through genetically engineered microorganisms might also represent a viable approach,[12] but this does not appear to be a solution for the near future.

Acknowledgements

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Keywords: antibodies · antitumor agents · drug delivery · peptides · synthesis design

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