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Synthesis of the Deacetoxytubuvaline Fragment of Pretubulysin and its Lipophilic Analogues for Enhanced Permeability in Cancer Cell Lines

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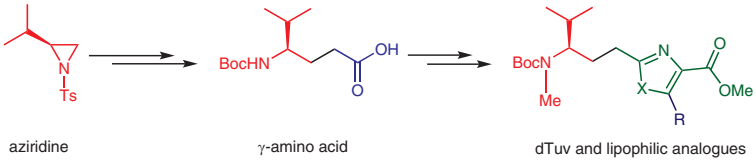

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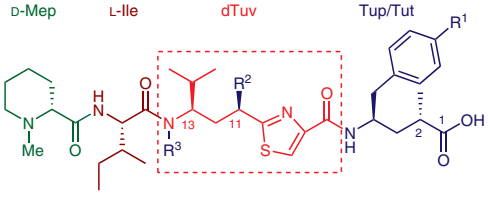
Abstract In the last two decades, tubulysins have emerged as alternatives to microtubule depolymerizing agents such as colchicine and vinblastine, which are well-established anticancer agents. However, the complex structure of tubulysins has always posed a challenge for synthetic chemists to scale up the production of these compounds. We report a new strategy for the practical gram-scale synthesis of a (*4R*)-4-[(*tert*-butoxycarbonyl)amino]-5-methylhexanoic acid through regioselective cleavage of a chiral aziridine ring with a vinyl Grignard reagent to afford *tert*-butyl [(1*R*)-1-isopropylbut-3-en-1-yl]carbamate, which was subjected to regioselective hydroboration–oxidation with 9-BBN. The resulting (*4R*)-4-[(*tert*-butoxycarbonyl)amino]-5-methylhexanoic acid was successfully transformed into the deacetoxytubuvaline fragment of pretubulysin or its highly lipophilic methyl-substituted thiazole and oxazole analogues for incorporation into pretubulysins. Increasing the lipophilicity of tubulysin or pretubulysin molecules should enhance their cell permeability and cytotoxicity in cancer cell lines.

Key words amino acid, deacetoxytubuvaline, tubulysin, pretubulysin, medicinal chemistry

Vinca alkaloids such as vinblastine, vincristine, or vinorelbine have been used to treat various types of cancer. More recently, the taxanes, especially paclitaxel and docetaxel as lead compounds for this family of drugs, have brought about a revolution in anticancer therapy. The *Vinca* alkaloids and taxanes disrupt microtubule polymerization dynamics in the cell by targeting tubulin proteins, consequently inducing apoptosis. If we take into account the current number of drugs of these classes in preclinical and clinical treatments, microtubules undoubtedly emerge as important targets for cancer chemotherapy.[1]

Höfle and co-workers first identified a series of antimitotic tetrapeptides called tubulysins from myxobacterial culture broths.[2] Later, Müller and co-workers discovered the gene cluster responsible for biosynthesis of tubulysins.[3] Structurally, tubulysins are linear tetrapeptides containing four amino acid fragments: *N*-methylpipecolic acid (D-Mep), L-isoleucine (Ile), and two unusual amino acids, tubuvaline (Tuv) and tubutyrosine (Tut) or tubuphenylalanine (Tup). Moreover, during the biosynthesis of tubulysins, D-Mep, L-Ile, deacetoxytubuvaline (dTuv), and Tut or Tup are sequentially assembled to form pretubulysin A or D, respectively, as precursor. Subsequently, a biocatalyzed oxidation/acylation reaction transforms the pretubulysins into tubulysins through the introduction of an acetate group at C-11.[3]



R^1 = OH, R^2 = OAc, R^3 = $CH_2OCOCH_2CH(CH_3)_2$, Tubulysin A
R^1 = H, R^2 = OAc, R^3 = $CH_2OCOCH_2CH(CH_3)_2$, Tubulysin D
R^1 = OH, R^2 = H, R^3 = Me, Pretubulysin A
R^1 = R^2 = H, R^3 = Me, Pretubulysin D

Figure 1 Structures of pretubulysins (Prt) A and D with the dTuv fragment highlighted

Mechanistically, tubulysins interact with the eukaryotic cytoskeleton and prevent the formation of microtubules by binding with tubulin proteins, leading to cell death.[4] Their mode of action has been clinically validated, and their high potency against multidrug-resistant tumors has made tubulysins a first choice in the development of targeted drug-delivery systems. Their subnanomolar cytotoxicity and remarkable antiangiogenic properties have led to significant research activity[5] in developing amenable synthetic procedures in the laboratory. However, the clinical development of the tubulysin family of compounds into a marketable

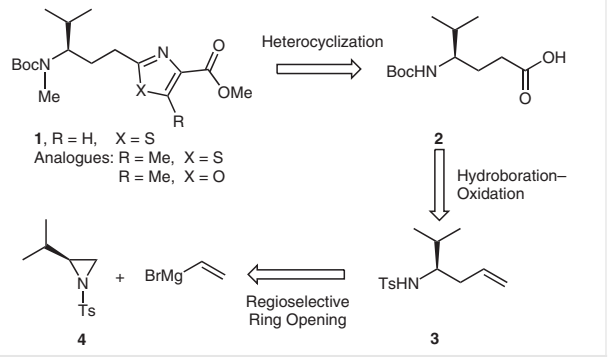
drug has been hampered by their structural complexity and by their instability during biological assays. Staben and co-workers[6] have recently demonstrated that during in vivo metabolism of antibody–tubulysin conjugates, the labile C-11 acetate group compromises activity. They therefore successfully replaced the acetate moiety with a moderately stable propyl ether functionality.

Structure–activity relationship studies of natural and synthetic analogues of tubulysins have revealed that these tetrapeptides are surprisingly tolerant of structural modifications in several regions. Chemical and stereochemical changes are tolerated moderately at the C-11 hydroxy group. Although, hydrolysis of the C11-acetyl group led to a 1000-fold decrease in biological activity, complete reduction of the secondary alcohol at C-11 to a methylene group has been reported to restore the anticancer activity to nanomolar concentrations.[7] Investigations revealed that the antiangiogenic and antiproliferative activities and the overall potency of pretubulysin are comparable to those of tubulysin A (Tub-A).[8] Furthermore, the mode of action of pretubulysin was found to be similar to that of Tub-A in inducing apoptosis through DNA laddering and nuclear fragmentation.[8,9] The interesting biological profile, stability, and easier chemical accessibility make pretubulysin an attractive target. In addition, several molecular conjugates containing pretubulysin or its derivatives have been studied, for example, as photoaffinity probes for tubulin imaging studies[10] or as folate receptor-targeted oligoamides.[11]

Existing methods for synthesizing the deacetoxytubuvaline (dTuv) and its analogues generally use protected L-valine for the incorporation of the C-13 stereocenter of pretubulysin. This protocol is typically carried out by a two-carbon homologation of a protected L-valine ester, followed by installation of the heterocyclic ring through either nucleophilic substitution/heterocyclization or by standard C–C coupling. For example, in the former approach, Ullrich and co-workers elegantly transformed methyl L-valinate into its corresponding nitrile homologue through a Wittig reaction, and they constructed the thiazole ring by a Hantzsch thiazole methodology.[12] In the latter case, Nicolaou and co-workers reduced the C-terminus of L-valine to form the corresponding *N*-methyl bromo derivative, which underwent C–C bond formation with lithiated methylthiazole.[13] Similarly, Brindisi and co-workers prepared a chloromethylthiazole derivative from L-serine methyl ester and coupled this to L-valinal through a Wittig reaction.[14] In contrast to these methods, Colombo and co-workers used a *tert*-butylsulfinamide chiral auxiliary handle to install the C-13 stereocenter of pretubulysin through stereoselective nucleophilic addition of a terminal alkyne onto a *tert*-butylsulfinimine, followed by Sonogashira C–C coupling with a bromothiazole and selective hydrogenation of the resultant heterocyclic alkyne.[15]

Despite these developments in methods for synthesizing pretubuvaline and its analogues, there are no proven synthetic protocols for large-scale preparations of these biologically active entities. Here, we report a novel and practical route that permits gram-scale synthesis of the γ-amino acid intermediate **2** by regioselective ring opening of chiral *N*-tosylaziridine **4** with vinyl Grignard reagent followed by hydroboration–oxidation to afford *N*-Boc-*N*-tosyl-δ-amino alcohol **6**. Derivative **6** is then oxidized and detosylated to give *N*-Boc-γ-amino acid **2**, which is converted into dTuv (**1**) and two of its methyl-substituted analogues in good yields. The methyl substituent in the heterocyclic ring increases the lipophilicity and cell permeability of the tubulysin and pretubulysin derivatives, thereby enhancing their cytotoxic potency. In future, this large-scale synthesis of **2** should permit the construction of a diverse set of pretubulysin architectures for cytotoxic evaluation in various cancer cell lines.

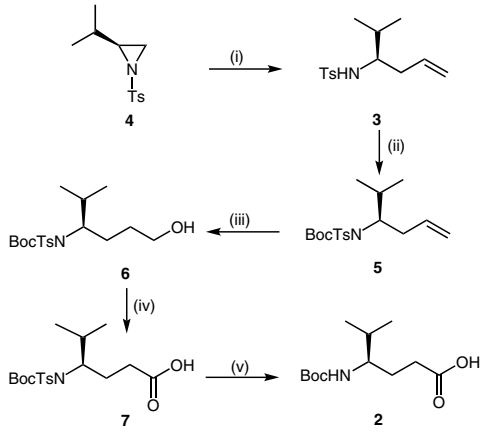
A retrosynthetic analysis of the close precursor, the *N*-Boc-γ-amino acid **2**, for the synthesis of dTuv (**1**) and its analogues (R = Me, X = S, O) is depicted in Scheme 1. According to this strategy, the key intermediate **2** might be obtained from aminoalkene **3** through a regioselective hydroboration/oxidation sequence. Aminoalkene **3** might, in turn, be prepared by regioselective ring opening of (*S*)-2-isopropyl-1-tosylaziridine (**4**) with vinylmagnesium bromide.



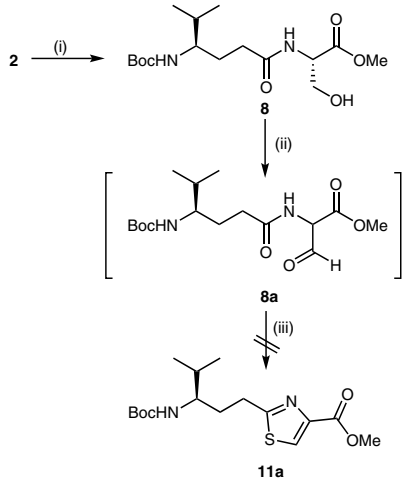
Scheme 1 Retrosynthetic analysis of deacetoxytubuvaline (**1**; dTuv) and its analogues

γ-Amino acids are versatile building blocks in synthetic organic chemistry. Their wide range of applications[16] as peptidomimetics, in the treatment of nervous-system disorders, and as anticancer drugs have led to significant efforts to achieve their stereoselective synthesis.[17] In this context, we have developed a novel method for preparing the required *N*-Boc-γ-amino acid **2** by a two-carbon homologation of enantiomerically pure aziridine substrate **4** with a vinyl Grignard reagent (Scheme 2).

(*S*)-2-Isopropyl-1-tosylaziridine (**4**) was prepared on a multigram scale (see Supporting Information)[22] and treated with vinylmagnesium bromide in the presence of catalytic amount of CuCN. Tosylaziridine **4** underwent ring-opening



Scheme 2 Synthesis of the *N*-Boc γ-substituted γ-amino acid **2**. *Reagents and conditions*: (i) vinylmagnesium bromide, CuCN, THF, 0 °C to r.t., 2 h, 72%; (ii) Boc_2O, DMAP, CH_2Cl_2, r.t., 2 h, 87%; (iii) 9-BBN, THF, r.t., 12 h, then H_2O_2, 2 N aq NaOH, 0 °C to r.t., 12 h, 93%; (iv) PDC, DMF, r.t., 24 h, 85%; (v) Mg, NH_4Cl, MeOH, reflux, 2 h, 81%.



Scheme 3 Unsuccessful attempt at the synthesis of deacetoxytubuvaline derivative **11a**. *Reagents and conditions*: (i) Methyl L-serinate, HOBt, EDC, DIPEA, CH_2Cl_2, 0 °C to r.t., 12 h, 73%; (ii) IBX, EtOAC, reflux, 12 h; (iii) Lawesson's reagent, THF, reflux, 12 h.

nucleophilic attack by the Grignard reagent on the less-hindered site of aziridine to give the 1-tosylamino alkene **3** in 72% yield. N-Boc protection of **3** was carried out by treatment with Boc_2O in the presence of DMAP to afford *N*-Boc-1-(tosylamino)alkene **5** in 93% yield.

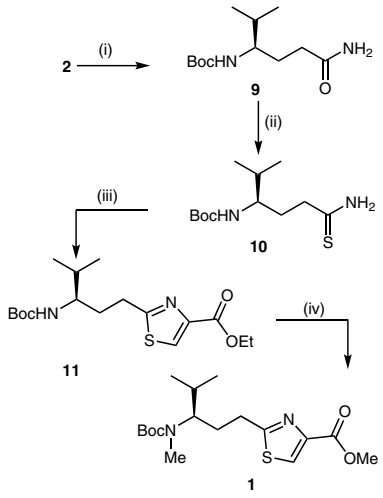
Regioselective hydroboration of **5** with 9-BBN, followed by alkaline oxidation of the resulting alkyl borane with H_2O_2, resulted in the formation of alcohol **6** in 93% yield. Pyridinium dichromate (PDC) oxidation of **6** afforded the *N*-Boc-*N*-tosyl-γ-amino acid **7** in 85% yield. This was detosylated with magnesium metal in the presence of ammonium chloride to provide the key intermediate *N*-Boc-γ-amino acid **2** (Scheme 2). Intermediate **2** is a critical intermediate in the proposed synthesis of dTuv (**1**) and its methyl-substituted thiazole and oxazole analogues, as outlined in Schemes 4–6 below.

Initially, the *N*-Boc-γ-amino acid **2** was coupled with L-serine by using *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling agent in the presence of benzotriazol-1-ol (HOBt) to obtain dipeptide **8**, which was transformed into dTuv (**1**) by heterocyclization of intermediate **8a**. However, all our attempts to convert the 2-iodoxybenzoic acid (IBX)-oxidation product of **8** into **1** in situ via the unstable intermediate **8a** by using Lawesson's reagent were unsuccessful, giving no trace of the required derivative **11a** (Scheme 3).

Consequently, we chose to prepare the *N*-Boc-γ-amino carboxamide **9**, which could be converted into *N*-Boc-γ-amino thioamide **10** under standard reaction conditions. The thioamide **10** should subsequently be readily transformed into *N*-Boc-protected dTuv through heterocyclization by treatment with ethyl bromopyruvate (Scheme 4). We therefore treated the *N*-Boc-γ-amino acid **2** with ammonium chloride in the presence of a base with 3-[bis(dimethylamino)methyliumyl]-3*H*-benzotriazole 1-oxide hexafluorophosphate (HBTU) as the coupling agent, to furnish *N*-Boc-γ-aminocarboxamide **9** in 95% yield. Intermediate **9** was then converted into thioamide **10** in 89% yield by treatment with Lawesson's reagent in refluxing THF. The thioamide **10** was subsequently converted into the 2-alkyl-substituted thiazole ethyl ester **11** in an excellent yield of 86% by following the procedure reported by Menche and co-workers.[18] Finally, N-methylation of **11** by methyl iodide in the presence of sodium hydride in *N*,*N*-dimethylformamide (DMF) as solvent gave dTuv (**1**) in 87% yield (Scheme 4). Note that during the N-methylation reaction of **11**,[23] a parallel and complete conversion of the ethyl ester of **11** into methyl ester **1** took place.

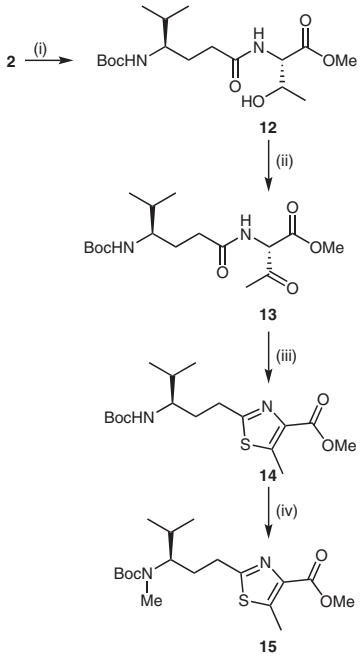
Studies by Stark and Assaraf[19] have shown that increasing the lipophilicity of tubulysin derivatives can enhance their cell diffusion, leading to lower IC_{50} values. We are currently focusing on synthesizing more-lipophilic derivatives of tubulysins and pretubulysins. Pretubulysins are naturally more lipophilic than the corresponding tubulysins due to the absence of the C-11 acetoxy group. Furthermore, we were interested in designing novel dTuv analogues and enhancing the lipophilicity of the heterocyclic ring by the introduction of alkyl substituents as simple as a methyl group. Subsequently, we intended to incorporate the methyl-substituted dTuv analogues into a series of modern pretubulysin architectures.

To achieve the synthesis of the 5-methylthiazole-subunit-containing dTuv analogues, the *N*-Boc-γ-amino acid **2** was coupled with L-threonine by using EDC as a coupling agent in the presence of HOBt, with DIPEA as a base, to furnish dipeptide **12** in 70% yield. The secondary alcohol functionality in dipeptide **12** was oxidized to a ketone by using



Scheme 4 Synthesis of deacetoxytubuvaline (dTuv; **1**). *Reagents and conditions*: (i) HOBt, HBTU, NH_4Cl, DIPEA, CH_2Cl_2, 0 °C to r.t., 4 h, 95%; (ii) Lawesson's reagent, THF, 0 °C to r.t., 12 h, 89%; (iii) ethyl bromopyruvate, EtOH, 65 °C, 1 h, 86%; (iv) MeI, NaH, DMF, r.t., 12 h, 87%.

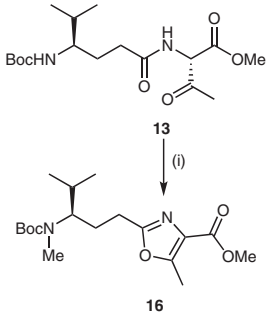
Dess–Martin periodinane (DMP) to afford keto amide ester **13** in 82% yield. Intermediate **13** was treated with Lawesson's reagent for *in situ* thionation–cyclization to obtain the *N*-Boc-protected 5-methylthiazole ester **14**. N-Methylation of **14** with MeI in the presence of NaH provided the target dTuv analogue **15** in 80% yield (Scheme 5).



Scheme 5 Synthesis of the 5-methylthiazole analogue of deacetoxytubuvaline **15**. *Reagents and conditions*: (i) L-threonine methyl ester hydrochloride, EDC, HOBt, DIPEA, CH_2Cl_2, 0 °C to r.t., 12 h, 70%; (ii) DMP, CH_2Cl_2, r.t., 4 h, 82%; (iii) Lawesson's reagent, THF, reflux, 6 h, 70%; (iv) MeI, NaH, DMF, r.t., 12 h, 80%.

The oxazole analogue of the Tuv fragment in the tubulysin series was first introduced by Wipf et al.[20] and, subsequently, Shankar and co-workers[21] found that oxazole-containing tubulysin U analogues are even more cytotoxic than their thiazole counterparts.

With this in mind, we also synthesized the dTuv 5-methyloxazole analogue **16**. The keto amide ester **13** was treated with triphenylphosphine (PPh_3) and iodine to form the 5-methyloxazole subunit through an oxidative cyclization reaction. Without further purification of the intermediate and after workup, N-methylation gave the final dTuv oxazole analogue **16** in 80% yield (Scheme 6).



Scheme 6 Synthesis of 5-methyloxazole analogue of deacetoxytubuvaline **16**. *Reagents and conditions*: (i) PPh_3, I_2, THF, –40 °C, 3 h, then MeI, NaH, DMF, r.t., 12 h, 80%.

In summary, we have developed a simple protocol for the synthesis of the deacetoxytubuvaline (dTuv) fragment **1** of pretubulysin and its methyl-substituted thiazole and oxazole analogues, **15** and **16**, respectively, in high yields. The synthetic design is suitable for scaled-up synthesis of these highly desirable fragments. Total syntheses of new pretubulysins, containing these lipophilic fragments, and an in vitro evaluation of their cytotoxicity in a range of cancer cell lines is currently underway in our laboratory and will be reported in due course.

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0037-1611359.

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(23) **Methyl 2-{(3*R*)-3-[(*tert*-Butoxycarbonyl)(methyl)amino]-4-methylpentyl}-1,3-thiazole-4-carboxylate (1)**
Anhyd DMF (2 mL) was added to the thiazole ethyl ester **11** (0.080 g, 0.22 mmol) in a 10 mL round-bottomed flask equipped with a magnetic stirrer bar. MeI (0.05 mL, 0.89 mmol) was then added, and the mixture was cooled to 0 °C. NaH (0.013 g, 0.55 mmol) was added portionwise over 15 min with constant stirring, and the mixture was stirred at 25 °C under N_2 for a further 12 h. When **11** was completely consumed (TLC), the reaction was quenched with sat. aq NH_4Cl (10 mL). The aqueous layer was extracted with EtOAc (3 × 25 mL), and the combined organic extracts were washed with brine (3 × 10 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The resulting crude residue was purified by column chromatography [silica gel, hexane–EtOAc (80:20)] to give a colorless liquid; yield: 70.0 mg (87%); TLC: R_f = 0.25 (hexane–EtOAc, 70:30). $[\alpha]_D^{20}$ –20.4 (*c* 0.1, CH_2Cl_2).
IR (CH_2Cl_2): 2963, 2920 (C–H), 1722, 1694 (C=O), 1685 (C=N), 1484, 1392 (C–H), 1170 (C–O), 1155 (S–O), 867 (=C–H), 775, 720 (C–H) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 8.08 (s, 1 H, rotamer 1), 8.06 (s, 1 H, rotamer 2), 3.95 (s, 3 H, rotamer 1), 3.94 (s, 3 H, rotamer 2), 2.99–2.95 (m, 4 H, rotamers 1 + 2), 2.70 (s, 3 H, rotamer 1), 2.65 (s, 3 H, rotamer 2), 2.18–2.11 (m, 2 H, rotamers 1 + 2), 1.89–1.81 (m, 2 H, rotamers 1 + 2), 1.70–1.67 (m, 4 H, rotamers 1 + 2), 1.46 (s, 9 H, rotamer 1), 1.43 (s, 9 H, rotamer 2), 0.96 (t, *J* = 6.0 Hz, 6 H, rotamer 1), 0.85 (d, *J* = 6.5 Hz, 6 H, rotamer 2). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 171.9, 162.0, 156.6, 146.5, 127.2, 79.6, 60.3, 52.5, 30.73, 30.5, 30.0, 29.7, 28.4, 20.2, 19.9. Diagnostic signals of minor rotamer ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 171.5, 161.9, 146.4, 79.2, 52.4, 20.1, 19.6. HRMS (ESI): *m/z* $[M + H]^+$ calcd for $C_{17}H_{29}N_2O_4S$: 379.1662; found: 379.1675.