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Graphical Abstract

Development of a practical and scalable route for the preparation of the deacetoxytubuvaline (dTuv) fragment of Pretubulysin and analogues

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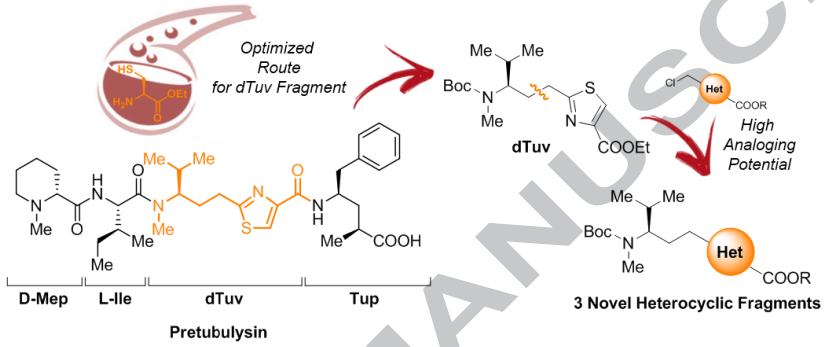

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Development of a practical and scalable route for the preparation of the deacetoxytubuvaline (dTuv) fragment of Pretubulysin and analogues

Margherita Brindisi,[a,#] Samuele Maramai,[a,#] Alessandro Grillo,[a] Simone Brogi,[a] Stefania Butini,[a,*] Ettore Novellino,[b] Giuseppe Campiani[a,*] and Sandra Gemma[a]

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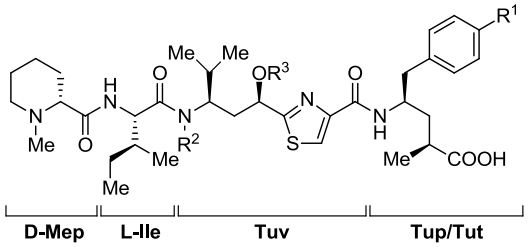
ABSTRACT

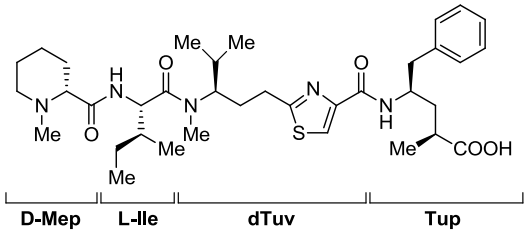
We present herein a novel and convenient route for the scaling-up of the dTuv fragment of pretubulysin. The newly conceived chemical path involves a practical and efficient one-step procedure for the preparation of a key thiazole intermediate, followed by a high-yielding Wittig olefination/reduction step. The optimized route, starting from the inexpensive and non-toxic *L*-cysteine encompasses five synthetic steps and only two chromatographic purifications, thus displaying a dramatically increased overall yield. The versatility of the proposed approach also provides new hints for the exploration of pretubulysin derivatives bearing diverse heterocyclic portions.

Natural products have proven to be a valuable source of new and effective anticancer agents and some of the mostly used antitumor drugs are naturally occurring compounds or (semi)synthetic derivatives of them.[1] However, their supply remains one of the main issues: the natural sources from which they are obtained are often difficult to handle and their complex structures and stereochemistry do not allow an easy and scalable industrial synthesis. Thus, there is a continuous need for novel, chemically accessible routes for the synthesis of natural antitumor agents or their simplified derivatives or precursors. Most of the currently available drugs for the treatment of cancer are based on the inhibition of cell proliferation by induction of apoptosis.[2] Among the various mechanisms of action of natural products, microtubules represent an attractive target, since they play a pivotal role in cell viability, growth, morphology maintenance and mitosis, cell motility and homing.[3-5] Microtubules are composed of continuously assembling and disassembling α- and β-tubulin heterodimers and the interference with these dynamics results in mitotic arrest and apoptosis. Compounds able to interfere with microtubule dynamics possess specific binding domains on both α- and β-tubulin and can be divided into two main classes: microtubule-depolymerizing agents (e.g. colchicine, nocodazole, and Vinca alkaloids) and microtubule-stabilizing agents (e.g. taxanes and epothilones).[6]

A novel group of microtubule-depolymerizing agents has been recently isolated from the microorganisms of the genus *Myxobacterium*, namely the tubulysins (**1a,b**, Figure 1).[7]



1a, Tubulysin A, R^1 = OH, R^2 = CH_2COO*i*Bu, R^3 = Ac
1b, Tubulysin D, R^1 = H, R^2 = CH_2COO*i*Bu, R^3 = Ac



2, Pretubulysin

Figure 1. Tubulysin A and D (**1a,b**) and their simplified analogue Pretubulysin (**2**).

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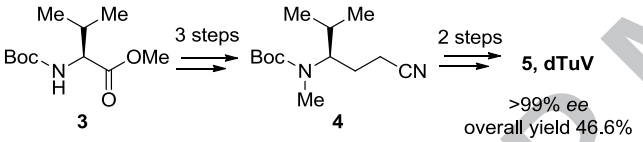
Tubulysins are linear cytotoxic tetrapeptides which can be structurally subdivided into four amino acid building blocks (Figure 1): *N*-methyl-*D*-pipecolic acid (*D*-Mep), *L*-isoleucine (*L*-Ile), and three unnatural amino acids derivatives, tubuvaline (Tuv) and either tubutyrosine (Tut) for tubulysin A (**1a**), or tubuphenylalanine (Tup) for tubulysin D, (**1b**). Tubulysin A turned out to be highly potent against various tumour cell lines[8] and its incubation with cultured mammalian cells led to microtubule depletion.[9] More recently, pretubulysin (**2**, Figure 1), a putative biosynthetic intermediate of tubulysins, was isolated from the extracts of the producing strain *Angiococcus disciformis*. It displayed only a slight loss of potency when compared to tubulysin itself,[10,11] induced apoptotic cell death in various cancer cell lines at nanomolar concentrations and inhibited metastasis and tumor growth in vitro and in vivo.[12]

Pretubulysin (**2**) possesses a simplified structure with respect to tubulysins, in which the tubuvaline moiety is replaced by its deacetoxy analogue (dTuv, Figure 1). A large-scale synthesis of this unnatural peptide has been recently proposed[10] together with a small series of derivatives which retained a good activity against some tumor cell lines.[13] Most of these analogues bear little modifications in the structure of dTuv. To date, only a phenyl ring bioisoster was explored and no other heterocyclic analogues have been described[14] possibly due to limitations of the synthetic accessibility.

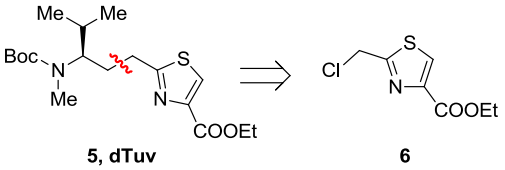


Scheme 1. The reported synthesis of **5**.[10]

The current synthesis of the dTuv fragment of **2**, carried out by Ullrich and co-workers, encompassed the preparation of the nitrile intermediate **4** from Boc-*L*-valine methylester in 3 steps, followed by the nasty hydrolysis of the cyano group with the hazardous gaseous hydrogen sulfide and subsequent cyclization with ethyl bromopyruvate.[10] The overall yield of this 5-steps procedure is 46.6 % and the enantiomeric excess for compound **5** is > 99 % (Scheme 1).

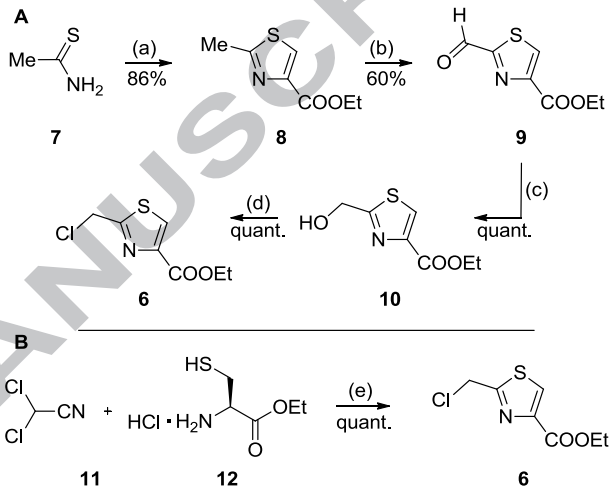
In the frame of our research activity involving the development of innovative antitumor agents,[15-21] peptidomimetics[22-24] and natural compounds,[25-27] we sought to investigate the possibility of generating a novel route towards the dTuv fragment of pretubulysin characterized by higher scalability, safety and a higher analoging potential for the synthesis of a small set of derivatives.

Here we report an easier, high-yielding, and versatile procedure to obtain large amounts of dTuv (**5**). Our disconnection approach envisaged the chloromethylthiazole derivative **6** as the key intermediate for the synthesis of dTuv, to be employed in the Wittig-type coupling with the Val fragment (Eq. 1).



Our first approach toward the synthesis of the thiazole derivative **6** (Scheme 2A) started from one molar equivalent of thioacetamide (**7**), which was condensed with ethyl bromopyruvate providing methylthiazole **8**. Oxidation to the corresponding aldehyde **9** with selenium dioxide in acidic medium and subsequent reduction in the presence of sodium borohydride, afforded the alcohol **10**. Finally, reaction with thionyl chloride provided derivative **6**.

Even though reduction (step c) and chlorination (step d) occurred in nearly quantitative yields and without needing further purification, the oxidation stage (step b) turned out rather inefficient, displaying only 60% yield, without the possibility of starting material recovery.



Scheme 2. A) First approach to the synthesis of thiazole derivative **6**. Reagents and conditions: (a) Ethyl bromopyruvate, EtOH, 80 °C, 12 h; (b) SeO_2, acetic acid, 120 °C, 12 h; (c) $NaBH_4$, dry THF, 25 °C, 2 h; (d) $SOCl_2$. Dry DCM, 25 °C, 1 h; B) Optimization of the route for the preparation of intermediate **6**. (e) *i.* MeONa, dry MeOH, dry DCM, 0 °C, 1 h then 25 °C, 12 h; *ii.* DIEA, dry DCM, 50 °C, 6 h then rt, 12 h.

In order to further improve our synthetic strategy, we employed an alternative and safer procedure, in which the derivative **6** was obtained in a practical one-step procedure (Scheme 2B).[28] Accordingly, *L*-cysteine ethylester hydrochloride (**12**) and dichloroacetonitrile were reacted in the presence of sodium methoxide and then treated with *N*,*N*-diisopropylethylamine, to afford pure **6** in nearly quantitative yield.[29]

Notably, this route to compound **6** avoids the use of dangerous and hazardous reagents for the synthesis of thiazole ring, namely hydrogen sulphide, as proposed in the original synthesis of dTuv fragment, or thioacetamide, oxidizing/reducing agents and thionyl chloride, as in our previous synthetic approach.

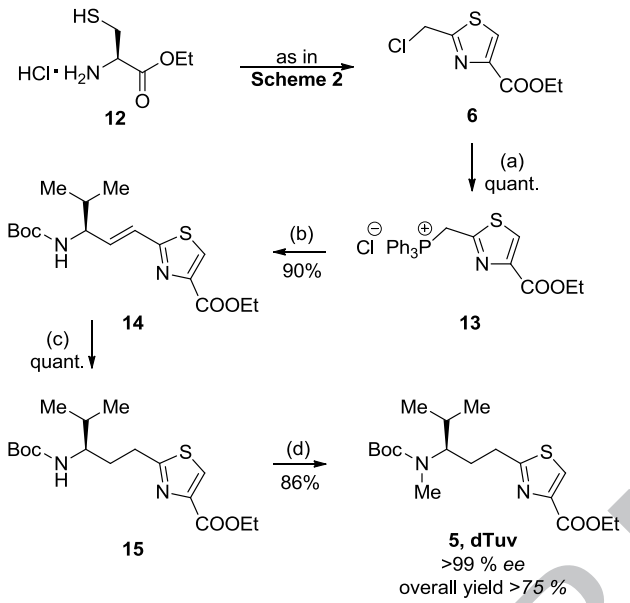
With a consistent amount of intermediate **6** in hands we proceeded with the preparation of the corresponding Wittig salt. Gratifyingly, treatment of **6** with triphenylphosphine in toluene allowed the total conversion in the salt **13** recoverable from the reaction mixture by a simple filtration step.

The route proceeded with one-pot reduction/Wittig olefination starting from *N*-Boc-*L*-valine methyl ester, first reacted with DIBAL-H and then treated with Wittig salt **13** and potassium *tert*-butoxide as the base. No racemization was observed in this one-pot reaction, while attempts of isolation of the Boc-

valinal intermediate resulted in nearly complete racemization, according to reports by Ullrich et al.[10]

The unsaturated product **14** was subsequently reduced by means of catalytic hydrogenation and *N*-alkylated using sodium hydride and methyl iodide, fully tracing out the reported procedure.[10] HPLC analysis (Chiralcel-OD chiral column, *n*-Hex/*i*PrOH 90:10, 1 mL/min) confirmed the enantiomeric purity of the dTuv **5** (t_R [(*R*)-**5**] = 9.36 min).

The new optimized synthesis of dTuv is reported in Scheme 3.[30-33]



Scheme 3. New synthesis of dTuv. Reagents and conditions: (a) PPh_3, dry toluene, 85 °C, 24 h; (b) *N*-Boc-*L*-valine methylester, DIBAL, dry DCM, -78 °C, 1 h then KO*t*Bu, dry DCM, 25 °C, 12 h; (c) H_2, Pd/C, MeOH, 25 °C, 1 h; (d) NaH, MeI, dry DMF, 0 °C to 25 °C, 12 h;

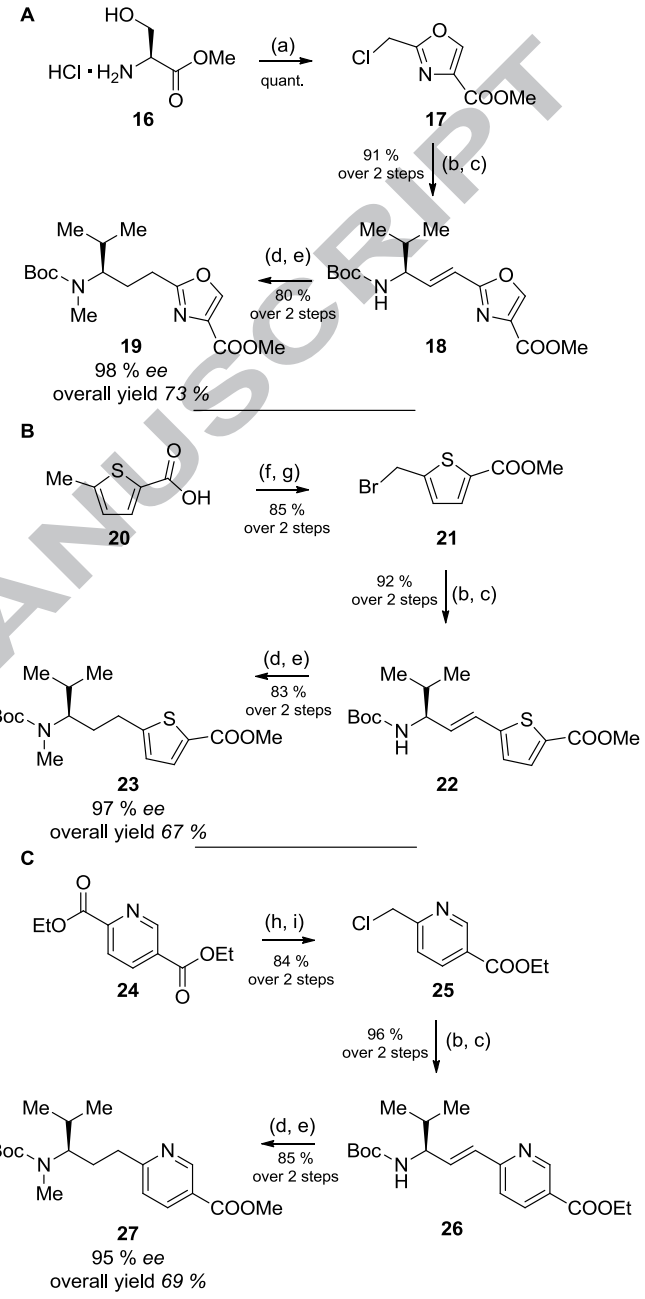
For improving analoging of dTuv with diverse heterocycle-containing fragments, and for testing the versatility of our protocol, we prepared three new derivatives bearing an oxazole (**19**), a thiophene (**23**), and a pyridine (**27**) system. The exploited synthetic strategies were the same reported for dTuv and are outlined in Scheme 4.

The preparation of the key intermediate **17** used in the synthesis of the oxazole derivative **19** traced out the same high-yielding protocol used for the thiazole counterpart, starting in this case from *L*-serine methyl ester hydrochloride (**16**). Chloro-derivative **17** was quantitatively converted into the corresponding Wittig salt leading, after sequential Wittig reaction, hydrogenation and *N*-methylation, to fragment **19** (Scheme 4A).

Intermediates **21** and **25**, were obtained starting from the corresponding commercially available heterocycles (compounds **20** and **24**). Esterification of 5-methyl-2-thiophenecarboxylic acid **20**, followed by radical bromination of the methyl functionality provided bromide **21**, while monoreduction of nicotinate **24** and successive treatment with thionyl chloride afforded chloride **25**.

The path towards fragments **23** (Scheme 4B) and **27** (Scheme 4C) is completely superimposable to the path described for **19** (Scheme 4A). It has to be noticed that in the *N*-alkylation step for the preparation of **27**, a parallel and complete conversion to methyl ester took place, but this issue did not affect the reaction effectiveness.

The newly realized fragments **19**, **23** and **27** not only display a good overall yield, comparable to that registered for dTuv **5**, but also confirm the versatility of our proposed synthetic approach.



Scheme 4. New dTuv derivatives. Reagents and conditions: (a) *i*. MeONa, dry MeOH, dry DCM, 0 °C, 1 h then 25 °C, 12 h; *ii*. DIEA, dry DCM, 50 °C, 6 h then rt, 12 h; (b) PPh_3, dry toluene, 85 °C, 24 h; (c) *N*-Boc-*L*-valine methylester, DIBAL, dry DCM, -78 °C, 1 h then KO*t*Bu, dry DCM, 25 °C, 12 h; (d) H_2, Pd/C, MeOH or EtOAc (for **19**), 25 °C, 1 h; (e) NaH, MeI, dry DMF, 0 °C, 6 h. (f) $SOCl_2$, MeOH, 25 °C, 2 h; (g) NBS, AIBN, CCl_4, 80 °C, 2 h; (h) $NaBH_4$, $CaCl_2$, THF/EtOH 1:1, 0 °C, 12 h; (i) $SOCl_2$, dry DCM, 25 °C, 2 h.

In conclusion, we developed a novel and convenient route for scaling-up the dTuv fragment of pretubulysin. The newly conceived chemical path encompassed a practical, highly scalable and efficient one-step procedure for the preparation of a key thiazole intermediate starting from the inexpensive *L*-

cysteine, followed by a high-yielding Wittig olefination step. Additionally, three quantitative non-chromatographic steps were involved, with an overall yield of the entire route >75 %. The versatility of the proposed approach also provides new hints for the exploration of pretubulysin derivatives. Accordingly, we herein synthesized three novel fragments bearing different heterocyclic moieties thus supporting the broad analoging potential of our proposed approach.

Supplementary data

Supplementary data (experimental methods for the preparation of intermediates, NMR and ESI-MS data) associated with this article can be found, in the online version, at http://dx.doi.org/XXXXXXXXX

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29. *Ethyl 2-(chloromethyl)thiazole-4-carboxylate* (**6**). To a stirred solution of MeONa (25.00 mg, 0.46 mmol) in dry MeOH (4.50 mL) and dry DCM (30.00 mL) cooled to 0 °C, dichloroacetonitrile (5.00 g, 45.50 mmol) was added and the mixture was stirred at 0 °C under N_2 atmosphere for 1 h. Then, *L*-cysteine ethyl ester hydrochloride salt (8.50 g, 45.50 mmol) was added and the reaction was stirred at 25 °C for 12 h. Water was added and the aqueous phase was extracted with DCM (3 x 25.00 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated. The crude was taken up with DCM (50.00 mL) and DIEA (11.90 mL, 68.30 mmol) was added. The mixture was stirred at 50 °C under N_2 atmosphere for 5 h and at 25 °C for further 12 h. Water was added and the aqueous phase was extracted with DCM (3 x 25.00 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated. No further purification was needed (quantitative yield). ESI-MS *m/z* 206 $[M+H]^+$, 228 $[M+Na]^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.39 (t, *J* = 7.1 Hz, 3H), 4.41 (q, *J* = 7.1 Hz, 2H), 4.87 (s, 2H), 8.19 (s, 1H).

30. *((4-(Ethoxycarbonyl)thiazol-2-yl)methyl)triphenylphosphonium chloride* (**13**). To a solution of compound **6** (9.00 g, 43.90 mmol) in dry toluene (25.00 mL), PPh_3 (11.50 g, 43.90 mmol) was added and the reaction was stirred at 85 °C under N_2 atmosphere for 24 h. The precipitate was filtered off to afford pure salt **13** (quantitative yield) as an off-white powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (t, *J* = 7.1 Hz, 3H), 4.29 (q, *J* = 7.1 Hz, 2H), 6.39 (d, *J* = 14.4 Hz, 2H), 7.57-7.69 (m, 6H), 7.70-7.80 (m, 3H), 7.87-7.99 (m, 6H), 8.03 (s, 1H).

31. *(S)-(E)-Ethyl 2-(3-((tert-butoxycarbonyl)amino)-4-methylpent-1-en-1-yl)thiazole-4-carboxylate* (**14**). To a stirred solution of *N*-Boc-(*L*)-valine methyl ester (2.50 g, 10.80 mmol) in dry DCM (15.00 mL) cooled to -78 °C, a solution of Dibal-H in DCM (21.60 mL, 21.60 mmol) was slowly added dropwise and the mixture was stirred at -78 °C under N_2 atmosphere for 1 h. Then, a suspension of salt **13** (10.11 g, 21.60 mmol) and KO*t*Bu (2.42 g, 21.60 mmol) in dry DCM (15.00 mL) was added and the reaction was slowly warmed to 25 °C and stirred for 12 h. A saturated solution of potassium

sodium tartrate was added and the aqueous phase was extracted with DCM (3 x 20.00 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated. The crude was purified by means of chromatography on silica gel (20% ethyl acetate in petroleum ether) to afford pure compound **14** (90 % yield) as a slightly yellow oil. ESI-MS *m/z* 355 $[M+H]^+$, 377 $[M+Na]^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.77-1.08 (m, 6H), 1.31-1.52 (m, 12H), 1.78-1.98 (m, 1H), 4.23 (br s, 1H), 4.42 (q, *J* = 7.1 Hz, 2H), 4.58 (br s, 1H), 6.56 (dd, *J* = 15.8, 5.5 Hz, 1H), 6.82 (d, *J* = 16.2 Hz, 1H), 8.04 (s, 1H).

32. *(R)-Ethyl 2-(3-((tert-butoxycarbonyl)amino)-4-methylpentyl)thiazole-4-carboxylate* (**15**). Compound **14** (3.45 g, 9.75 mmol) was dissolved in MeOH (30.00 mL) and the solution was degassed several times with N_2 before adding a catalytic amount of Pd/C 10%. The atmosphere was filled with H_2 and the reaction was stirred 2 h at 25 °C. The mixture was filtered on filter paper to remove the excess of Pd/C and the solvent was removed under reduced pressure. No further purification was needed (quantitative yield). ESI-MS *m/z* 357 $[M+H]^+$, 379 $[M+Na]^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.77-0.97 (m, 6H), 1.31-1.51 (m, 12H), 1.63-1.84 (m, 2H), 1.91-2.11 (m, 1H), 2.97-3.29 (m, 2H), 3.48-3.61 (m, 1H), 4.41 (q, *J* = 7.2 Hz, 2H), 8.03 (s, 1H).

33. *(R)-Ethyl 2-(3-((tert-butoxycarbonyl)(methyl)amino)-4-methylpentyl)thiazole-4-carboxylate* (**5**). To a stirred solution of compound **15** (3.47 g, 9.75 mmol) and MeI (2.43 mL, 39.00 mmol) in dry DMF (40.00 mL) cooled to 0 °C in ice-bath, NaH (0.59 g, 24.38 mmol) was added portionwise over a period of 1 h. The reaction was stirred at 25 °C under N_2 atmosphere for 12 h. A saturated solution of NH_4Cl was added and volatiles were removed under reduced pressure. The aqueous phase was extracted with DCM (3 x 25.00 mL) and the combined organic layers were dried over Na_2SO_4, filtered, and evaporated. The crude was purified by means of chromatography on silica gel (30 % ethyl acetate in petroleum ether) to afford pure compound **5** (86 % yield) as a transparent oil. HPLC: Chiralcel OD, *n*-Hex/*i*PrOH, 90:10, 1 mL/min, t_R[(*R*)-**5**] = 9.36 min; ESI-MS *m/z* 371 $[M+H]^+$, 393 $[M+Na]^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85 (d, *J* = 6.6 Hz, 3H), 0.94 (d, *J* = 3.1 Hz, 3H, rotamer 1), 0.96 (d, *J* = 3.1 Hz, 3H, rotamer 2), 1.35-1.49 (m, 12H, rotamers 1+2), 1.63-1.93 (m, 2H), 2.03-2.24 (m, 1H), 2.64 (s, 3H, rotamer 1), 2.69 (s, 3H, rotamer 2), 2.96 (t, *J* = 8.1, Hz, 2H), 3.55-3.74 (m, 1H, rotamer 1), 3.76-3.92 (m, 1H, rotamer 2), 4.41 (q, *J* = 7.1, Hz, 2H), 8.03 (s, 1H, rotamer 1), 8.04 (s, 1H, rotamer 2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.5, 19.8, 20.1, 20.3, 20.4, 28.6, 30.1, 30.2, 30.6, 30.8, 60.5, 61.4, 61.5, 79.3, 79.7, 126.9, 127.0, 146.9, 147.0, 156.5, 156.7, 161.5, 161.6, 171.5, 171.8 (rotamers 1+2).

Research Highlights

- Set-up of a novel route for the scaling-up of the dTuv fragment of Pretubulysin.

- The optimized route starts from the inexpensive and non-toxic *L*-cysteine.

- The novel approach displays a dramatically increased overall yield.

- The newly conceived path allowed the preparation of three novel fragments.