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# The Development and Scale-Up of an Antibody Drug Conjugate Tubulysin Payload

Jeremy S. Parker,*[,†] Marc McCormick,[†] David W. Anderson,[‡] Beatrice A. Maltman,[‡] Lakshmaiah Gingipalli,[§] and Dorin Toader[§]

[†]AstraZeneca, Pharmaceutical Sciences, Silk Road Business Park, Macclesfield, SK10 2NA, U.K.
[‡]Almac Sciences, 5 The Fleming Building, Edinburgh Technopole, Milton Bridge, Midlothian, EH26 0BE, U.K.
[§]AstraZeneca, R&D Boston, 35 Gatehouse Drive, Waltham, Massachusetts 02451, United States

Supporting Information

**ABSTRACT:** Significant development and scale-up work was completed on the synthesis of an antibody drug conjugate payload based on the tubulysin natural products. This work included the development of new routes to the tubuvaline and tubuphenylaniline portions of the molecules, as well as extensive optimization of the solid phase peptide synthesis used to assemble the molecule. The initial route (21 steps longest linear sequence, 0.01% overall yield) was improved to a new, more robust route (19 steps longest linear sequence, 2.4% overall yield) affording a 240-fold increase in overall yield and allowing delivery of over 86 g of the required molecule.

## INTRODUCTION

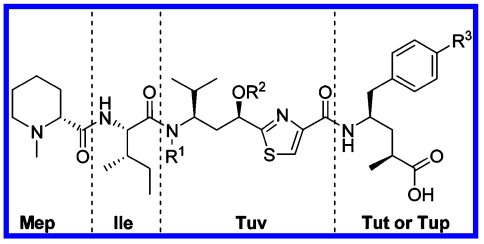
Antibody drug conjugates (ADCs) represent an exciting new class of therapeutics, combining a tumor targeting antibody with a cell-killing cytotoxic drug (payload).[1] Recent advances in this field include the launches of brentuximab vedotin (Adcetris) for the treatment of relapsed or refractory Hodgkin's lymphoma and relapsed or refractory systemic anaplastic large cell lymphoma[2] and trastuzumab emtansine (Kadcyla) for the treatment of advanced HER2 positive breast cancer.[3]

One of the key challenges in developing new ADCs is the identification of suitable cytotoxic payloads. To find molecules with the necessary potency, researchers have focused on natural product based cytotoxic molecules, for example the use of auristatins[4] based on dolastatin 10[5] which have been used in Adcetris, and maytansinoids[6] based on maytansine[7] which have been used in Kadcyla. The molecules that have been selected are typically highly complex natural product based molecules, necessitating an important contribution to ADC projects from synthetic organic chemistry.

As part of ongoing work to develop new ADCs, we identified the tubulysins as a promising class of molecules for use as payloads. The tubulysins were originally isolated from a culture broth of strains of myxobacteria *Archangium gephyra* Ar 315 and *Angiococcus disciformis* An d48,[8] with further examples from *Cystobacter* sp. SBCb004.[9] The molecules were shown to be potent inhibitors of tubulin polymerization,[10] causing disintegration of the cytoskeleton and leading to apoptosis. This activity has led to significant interest in the tubulysins as small molecule anticancer agents[11] and more recently in conjugates with antibodies,[12] dendrimers,[13] and nanoparticles.[14]

Structural characterization of the tubulysins[15] showed the molecules to be tetrapeptides, containing *D*-*N*-methyl-pipecolic acid (Mep), *L*-isoleucine (Ile), tubuvaline (Tuv), and either tubutyrosine (Tut) or tubuphenylalanine (Tup) (Table 1).

**Table 1. Structure of Tubulysins**




| tubulysin | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| A | $CH_2OCOCH_2CHMe_2$ | Ac | OH |
| B | $CH_2OCOCH_2CH_2CH_3$ | Ac | OH |
| C | $CH_2OCOCH_2CH_3$ | Ac | OH |
| D | $CH_2OCOCH_2CHMe_2$ | Ac | H |
| E | $CH_2OCOCH_2CH_2CH_3$ | Ac | H |
| F | $CH_2OCOCH_2CH_3$ | Ac | H |
| G | $CH_2OCOCH{=}CMe_2$ | Ac | OH |
| H | $CH_2OCOCH_3$ | Ac | H |
| I | $CH_2OCOCH_3$ | Ac | OH |
| U | H | Ac | H |
| V | H | H | H |
| W | $CH_2OCOCH_2CH_2CH_3$ | H | OH |
| X | H | Ac | OH |
| Z | H | H | OH |

The variability in structure of the tubulysins is mostly observed in the Tuv portion of the molecule. The first series (A−I) contains an unusual and synthetically challenging *N*,*O*-acetal group ($R^1$), as well as an acetylated hydroxyl group ($R^2$). A second series (U−Z) is missing the *N*,*O*-acetal group and/or the acetyl group and is less active. On the basis of their complex structure, limited availability, and potential as anticancer

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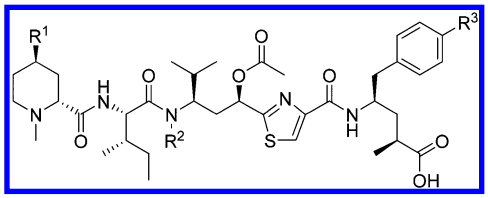
agents, significant efforts have been undertaken on the synthesis of tubulysins,[16] including total syntheses of Tubulysin B,[17] D,[18] U,[19] and V.[20] Work has also been completed on evaluating the key structural features[21] that impart the tubulysins with their activity, looking to deliver structurally simplified versions of these molecules. Of particular interest in simplified analogues have been pretubulysins[22] and $N^{14}$-desacetoxytubulysin H[23] (**1**), both of which replace the Tuv *N,O*-acetal group with a simple *N*-methyl group without significant loss of activity.

Initial work[24] to identify an effective ADC payload focused on evaluation of structure activity relationship (SAR) of the Mep and Tup residues of **1**, with the new compounds being evaluated using an Alamar blue assay against human prostate cancer cell line DU-145, human colon carcinoma cell line HCT-116, and human breast cancer cell line MDA MB-231[24] (Table 2). A review of all the produced data led to the selection

**Table 2. SAR of Tubulysin Analogues of $N^{14}$-Descetoxytubulysin H**



| | | | | $GI_{50}$ (nM) | | |
|---|---|---|---|---|---|---|
| Cpd | $R^1$ | $R^2$ | $R^3$ | DU-145 | HCT-116 | MDA MB-231 |
| **1** | H | Me | H | 0.7 | 0.6 | 1.1 |
| **2** | Me | Me | H | 0.5 | 0.9 | 3.4 |
| **3** | H | Me | $NH_2$ | 16.4 | 12.4 | 51.6 |
| **4** | Me | Me | $NH_2$ | 4.0 | 1.9 | 6.6 |
| **5** | Me | Et | $NH_2$ | 1.0 | 0.6 | 2.5 |

of **2**, where the Mep residue is changed to a (2*R*,4*R*)-1,4-dimethylpiperidine-2-carboxylic acid, as the starting point for further design work. The next step was to identify a suitable attachment point for a linker which would allow conjugation to an antibody. The conversion of the phenolic group of Tut residues to an aniline (**3**) had been exemplified previously,[25] although this modification was found to significantly reduce potency compared to **1**. This approach was evaluated for **2** affording **4**, which had a reduced potency compared to **2**. Further structural modifications showed the replacement of the *N*-methyl group of Tuv with an *N*-ethyl group was effective in restoring potency that matches **2**, and this molecule (**5**) was selected for use as the warhead for the new ADC payload. Design work was completed by evaluating the inclusion of amino acids in the linker attached to 4-amino Tup (to provide cleavable linkers) and looking at the length and type of spacer that could be used to join the warhead to a conjugatable maleimide. Following a evaluation of the performance of the different linker options, mc-Lys-MMETA (methyl Mep *N*-ethyl tubulysin aniline) (**6**) was identified[26] as an effective ADC payload.[27]

With the successful identification and application of **6**, further supplies of material were required to support toxicology and clinical studies. Conducted on a similar scale to all previous tubulysin syntheses, the initial work[24,26] had only provided milligram quantities of material, whereas multigram quantities of **6** were required to progress the development program, requiring significant improvements to the synthesis of this molecule.

In this Article, we describe the development and scale-up of an efficient and robust synthesis of **6**. The synthesis improvements were completed over four successive campaigns of development and scale-up, during which the overall yield improved from 0.01% to 2.4% (a 240 fold increase), ultimately affording over 86 g of the required compound.

## RESULTS AND DISCUSSION

The initial work on the project had established solid phase peptide synthesis (SPPS) using 2-chlorotrityl resin (CTR) as an effective method of assembling tubulysins, and this approach was used as the start point for the development and scale-up. This approach required the separate synthesis of each of the components of **6**, with the use of *N*-Fmoc protecting groups to facilitate the SPPS (Figure 1).

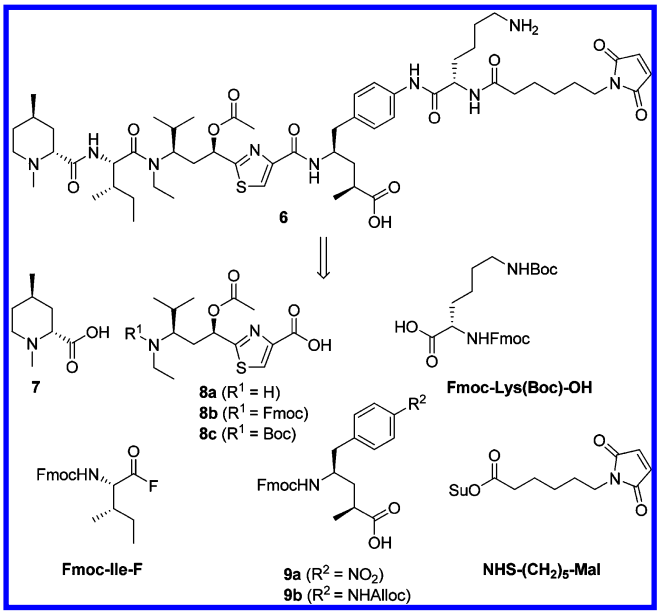



**Figure 1.** Retrosynthetic analysis of mc-Lys-MMETA (**6**).

**Tup Synthesis and Coupling.** Tup derivatives were initially prepared using the methodology reported by Wipf,[28] but for larger amounts, with a focus on preparing *N*-Fmoc 4-nitro-Tup (**9a**), key elements of the approach reported by Zanda[19b] were adopted.[24,26] This methodology was effective at supplying material for early development campaigns and was scaled-up directly affording 22 g of **9a**. The overall yield for the sequence (8 steps) was 2%; thus for later development and scale-up, it was decided to review the route, looking to improve both the yield and the efficiency of the synthesis. In the previous sequence, the majority of the structure had been derived from 4-nitro-L-phenylalanine, with the remaining three carbons being introduced from Meldrum's acid (2 carbons) and methyl iodide (1 carbon). Thus, to improve the efficiency of the synthesis, it was decided to investigate the introduction of all three carbon atoms in a single step using a 2-methylmalonate derivative.

To introduce the 2-methylmalonate derivative, it was decided to use cyclic sulfamidate chemistry.[29] *N*-Boc 4-nitro-L-phenylalanine was converted to the corresponding alaninol (**10**) by preparing a functionalized acid and reduction. Initial work used CDI to prepare the imidazolide, followed by reduction with sodium borohydride, but this was found to cause racemization. Similar results were obtained with isobutylchloroformate and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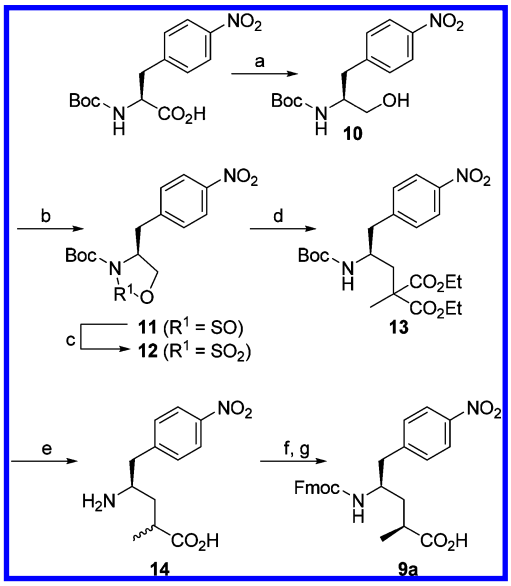
sodium borohydride, but conversion to the methyl ester followed by reduction with sodium borohydride gave high conversion and good quality product without racemization. The alaninol (**10**) was then cyclized to the sulfamidite (**11**) with thionyl chloride and oxidized to the sulfamidate (**12**) using standard procedures. This was then reacted with the anion of diethyl methylmalonate to successfully afford the diester (**13**). Ensuring good purity of input materials and low water content ensured good conversion. The product of the reaction was directly telescoped into the decarboxylation/saponification step affording racemic **14** which was Fmoc protected using Fmoc-OSu to afford racemic **9a**. Development work showed that this was the optimum point to separate the diastereomers, which was achieved using supercritical fluid chromatography (SFC) allowing the required diastereomer (**9a**) to be recovered efficiently from the separation. The improved overall yield for the seven-step sequence was 19% (Scheme 1).

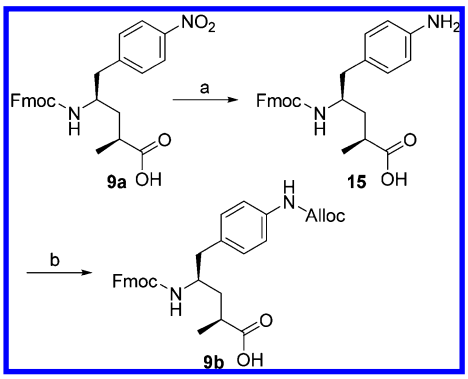
**Scheme 1. Improved Synthesis of *N*-Fmoc 4-Nitro-Tup (9a)**[a]




[a]Reagents and conditions: (a) $Me_2SO_4$, $Na_2CO_3$, THF then $NaBH_4$, MeOH/THF, 87%; (b) $SOCl_2$, DCM; (c) $RuCl_3{\cdot}3H_2O$, $NaIO_4$, $MeCN/H_2O$, 74% (2 steps); (d) diethyl methylmalonate, NaH, THF; (e) HCl; (f) Fmoc-OSu, $NaHCO_3$, acetone/water, 72% (three steps); (g) SFC, 40%.

Reviewing the use of **9a** in the SPPS identified the tin mediated reduction of the nitro group as a major issue, with loss of material from the solid phase as well as significant deacetylation. Thus, during the first development and scale-up campaign, it was decided to change the timing of the reduction of the nitro group, completing this on the Tup prior to the peptide assembly. Thus, **9a** was reduced to the aniline (**15**) using a Pd/C mediated hydrogenation. Some care was required with the reaction, as hydrogenolysis of Fmoc protecting group could occur with extended reaction times.[30] However, this issue could be overcome by careful dosing of the hydrogen or by the use of Pt/C catalysts. The resultant **15** was then protected with an Alloc protecting group affording **9b** (Scheme 2). The Alloc protecting group was initially introduced using Alloc-Cl/citrate buffer, but this was later changed to Alloc-Cl/sodium hydrogen carbonate in aqueous

**Scheme 2. Synthesis of *N*-Fmoc *N*′-Alloc Tup (9b)**[a]




[a]Reagents and conditions: (a) $H_2$, Pd/C, MeOH; (b) AllocCl, $NaHCO_3$, $THF/H_2O$, 56% (2 steps).

THF, which gave an improved reaction profile and crude product purity.

Overall the new route (Schemes 1 and 2) was used to supply 527 g of **9b** (9 steps, 10% overall yield) for the final development and scale-up campaign.

With the successful preparation of **9b**, SPPS was started using 2-chlorotrityl resin affording resin-bound Tup (**16**). In early development and scale-up campaigns, loading was allowed to vary between 0.5 mmol/g and 0.75 mmol/g, but a more detailed study demonstrated that the maximum reliable capacity of the resin was 0.50 mmol/g, and this value was used to provide consistency as well as ensuring efficient use of **9b**. The Fmoc protecting group was then removed using piperidine affording **17**, ready for Tuv coupling.

**Tuv Synthesis and Coupling.** *N*-Alkyl Tuv derivatives were initially prepared starting from protected valine as demonstrated by Wipf,[23a] but to circumvent the use of diazomethane, a simple synthesis of Weinreb amides analogous to those used by Fecik[31] was developed from L-*β*-leucine. This synthesis afforded *N*-Boc *N*-Et Tuv (**8c**) whch was deprotected and the resultant amino acid (**8a**) *N*-Fmoc protected to afford *N*-Fmoc *N*-Et Tuv (**8b**).[26] This sequence was directly scaled-up, supplying all four campaigns of development and scale-up, with the largest scale preparation affording 760 g of material (13 steps, 1% overall yield).

Although effective at supplying material, the key issue with the route was the low overall yield. Particularly of note is the low yielding final *N*-Fmoc protection (32% yield), mostly likely due to the steric hindrance around the secondary amine center (presence of *N*-ethyl and *α*-isopropyl groups). The route represents poor efficiency in terms of redox economy,[32] with an initial reduction of a carboxylic acid group followed by a final oxidation, as well as an inefficient nonchiral reduction of a ketone to a secondary alcohol (even with a recycle demonstrated as part of the 760 g campaign). These issues were tackled by the development of a new, more efficient route to **18**. Thus, carboxylic acid (**19**) was protected as the *tert*-butyl ester, and this molecule (**20**) could be metalated and reacted with the previously prepared Weinreb amide affording **21**. An asymmetric hydrogenation screen was completed which identified (Mes)RuCl(*R*,*R*-MsDPEN)[33] as a suitable catalyst, affording the chiral alcohol (**22**) in >99% chiral purity. The alcohol could be acetylated using the previously demonstrated conditions affording **18**. This molecule can be converted to **8a** by removing both the *N*-Boc and *O*-*tert*-butyl ester protecting groups in a single step, improving the overall efficiency of the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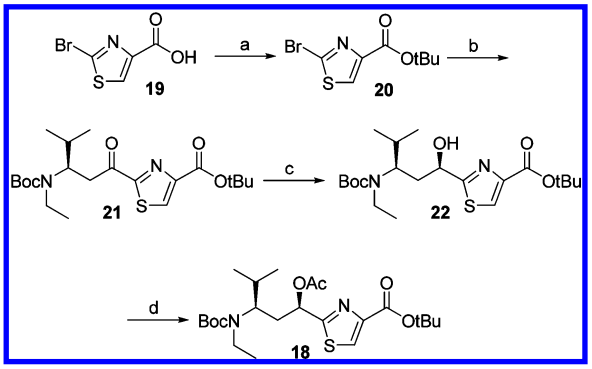
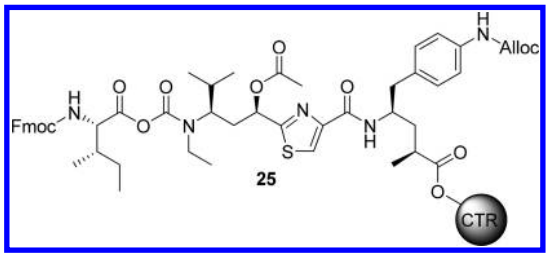
Scheme 3. Improved Synthesis of Protected *N*-Et Tuv (18)[a]




[a]Reagents and conditions: (a) CDI, DBU, tBuOH, DMF, 85%; (b) (*R*)-*tert*-butyl ethyl(1-(methoxy(methyl)amino)-4-methyl-1-oxopentan-3-yl)carbamate, iPrMgBr, 2-MeTHF, 35%; (c) (Mes)RuCl-(*R*,*R*-MsDPEN), HCOOH:TEA (5:2), THF/water; (d) AcCl, py, 82% (two steps).

protecting group strategy. The route (Scheme 3) was successfully scaled-up to afford 2.00 kg of **18** (7 steps, 20% overall yield) for use in later campaigns.

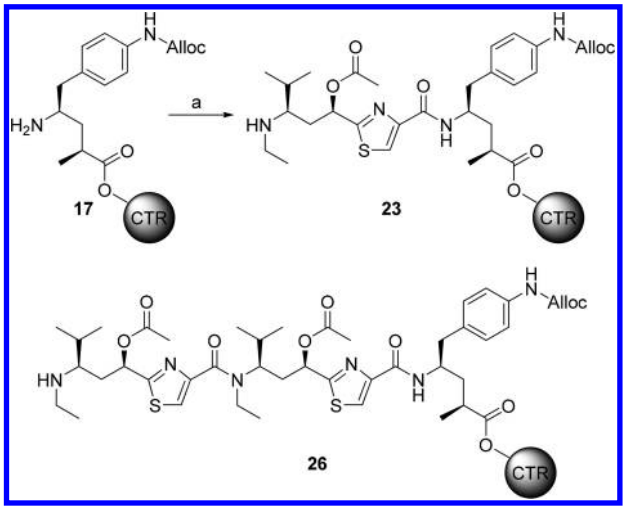
Following the procedures developed in the initial work,[24,26] *N*-Fmoc *N*-Et Tuv (**8b**) was used in the SPPS, coupling the material with **17** using HATU/TMP, followed by Fmoc deprotection using piperidine to afford **23**. However, significant issues were observed when the subsequent coupling of Fmoc-Ile-F was undertaken, with a significant new impurity being observed at 50% HPLC area. Investigations showed that, under a range of conditions, the impurity could be converted back to **23** and that the impurity had a *m*/*z* of 982, 44 units higher than the mass of the expected product (**24**). On the basis of this information, a tentative structural assignment of a mixed anhydride (**25**) was made, with a carbon dioxide group inserted between the secondary amine of **23** and the Fmoc-Ile. The mixed anhydride is proposed to form from a carbamic acid on the secondary amine due an incomplete removal of the Fmoc protecting group.




The reason and mechanism for the formation of the impurity are not fully understood, but experimental work found that treating the batch containing the mixture of **24** and **25** with a range of acids, bases, and nucleophiles was effective at converting the impurity to **23**, and the use of 3% HOBT in DMF was selected for convenience. The resulting mixture of **23** and **24** could all be converted to **24** using Fmoc-Ile-F.

With both the issue of the formation of the new impurity (**25**) and the low yielding introduction of the Fmoc protecting group (**8a** to **8b**), it was decided to investigate whether unprotected *N*-Et Tuv (**8a**) could be used in the SPPS, with the low reactivity of secondary amine center limiting the amount of double inclusion product that could potentially be generated. A laboratory investigation demonstrated that **8a** could be successfully coupled to **17** with only 4% HPLC area of double inclusion product (**26**) observed (Scheme 4). The double inclusion products have significantly different retention times on HPLC and the final impurity that this material produces can be removed in the final purification.

Scheme 4. Coupling of Unprotected Tuv (8a)[a]




[a]Reagents and conditions: (a) **8a**, HATU, DIPEA, DMF.

With the SPPS now requiring *N*-Et Tuv (**8a**), the deprotection of **8c** was examined in more detail. Analysis showed that the deprotection conditions used previously caused significant deacetylation (30−40%) during processing. A simpler process using a controlled amount of TFA and DCM was found to be effective at removing the Boc group without loss of the acetyl group. Following solvent removal the product could be dissolved in diethyl ether and precipitated by the addition of diisopropylethylamine. The resultant solid, containing both **8a**·DIPEA and DIPEA·TFA, could be used directly in the SPPS after the w/w assay of **8a** had been accurately determined. The coupling of **8a** onto **17** was then investigated. It was found that the use of HATU/DIEA as the coupling agents caused both deacetylation of the product (**23**) and trifluoroacetylation of **17**. Changing the coupling reagents to OxymaPure/DIC[34] eliminated the trifluoroacetylation and significantly reduced the amount of deacetylation. This procedure was investigated on an intermediate scale, and although the coupling was shown to be effective, significant deacetylated product was also present. The formation of this was traced to the use of a methanol wash of the resin, and removing this (from this and subsequent stages) ensured excellent conversion to **23** without loss of the acetyl group.

**Ile Synthesis and Coupling.** The Ile residue was introduced using Fmoc-Ile-F[35] prepared by reacting DAST with Fmoc-Ile-OH. The chemistry was directly scaled-up with the largest scale preparation affording 6.45 kg of material.

The initial SPPS issues encountered with the formation of the mixed anhydride impurity (**25**) were resolved by introducing unprotected *N*-Et Tuv (**8a**) and further development started from **23**, using the previous conditions of 3 cycles of 8 equiv of Fmoc-Ile-F. A more detailed study was completed looking at the efficiency of the three cycles of Fmoc-Ile-F coupling. This showed that, after the first coupling cycle, a conversion of 70% **23** to **24** had been achieved; after the second coupling cycle 90% and after the third coupling cycle 95%. As noted previously, the coupling onto the secondary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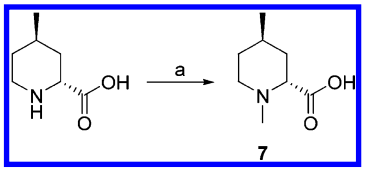
amine center of Tuv is challenging due to steric hindrance, and this study clearly demonstrated the need for three cycles of Fmoc-Ile-F coupling. As this stage generates an acid byproduct (HF), there were concerns about this resulting in the loss of the peptide from the resin; thus, bis(trimethylsilyl)acetamide was introduced as a fluoride scavenger[36] following which a range of coupling conditions showed no significant loss of material. The Fmoc group was removed from **24** using two cycles of piperidine/DMF affording **27**.

**Mep Synthesis and Coupling.** (2*R*,4*R*)-1,4-Dimethylpiperidine-2-carboxylic acid (**7**) was prepared by a reductive amination of (2*R*,4*R*)-4-methylpiperidine-2-carboxylic acid (Scheme 5).

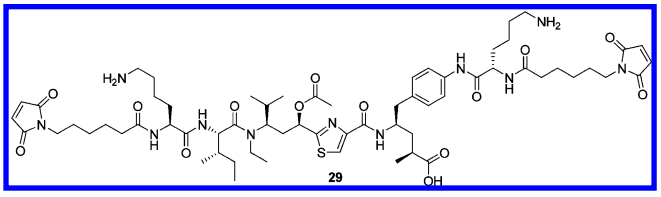
**Scheme 5. Synthesis of (2*R*,4*R*)-1,4-Dimethylpiperidine-2-carboxylic Acid (7)[a]**



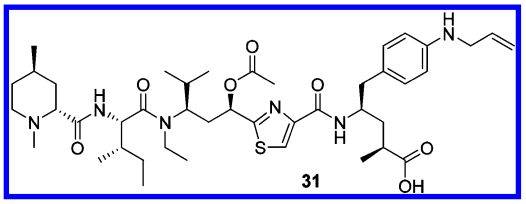

[a]Reagents and conditions: (a) $CH_2O_{(n)}$, $H_2$, Pd/C, MeOH/$H_2O$, 85%.

This compound had initially been coupled in the SPPS using HATU/TMP, but following an evaluation of the reaction the coupling was changed to the preferred conditions of OxymaPure and DIC to afford **28**.

Related to this transformation, at the end of the SPPS, an impurity with a similar retention time (RRT 0.97) to **6** was observed, meaning that it was not possible to purify the product to the required purity of >95%. The impurity was identified as a dimaleimide (**29**), causing significant concern as the molecule contained two maleimide groups and thus had the potential to cross-link two antibodies. After a careful review of the synthesis, it was concluded that the formation of **29** could be traced back to the low solubility of **7**. This low solubility had resulted in incomplete dissolution of **7**, and when the resultant solution was charged to the SPPS, less than the required 1.60 equiv was present, leaving a portion of **27** present. Following Alloc deprotection, Fmoc-Lys(Boc)−OH and the maleimide linker could be added to both ends of this molecule resulting in **29**. To circumvent this problem, a more detailed check was introduced after the coupling of **7** to ensure complete coupling, with the option for an additional coupling cycle to be used if required.



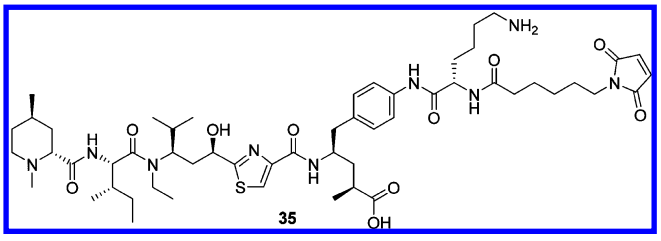

**Alloc Deprotection.** Deprotection of the Alloc protecting group on **28** was initially achieved using using 3 cycles of tetrakis(triphenylphosphine) palladium (0) with triphenylsilane as the allyl-scavenger[37] affording **30**. More detailed analysis of the process showed the deprotection to have proceeded to 40%, 80% and 90% after first, second and third cycles respectively confirming the requirement for 3 cycles. As part of the ongoing analysis at the end of the SPPS, another impurity with similar retention time (RRT 0.97) to **6** was observed which was identified as *N*-Allyl MMETA (**31**). To avoid the issues with the formation and removal of this impurity the scavenging agent for the deprotection was changed from triphenylsilane to dimethylamine borane complex,[38] which completely eliminated the formation of **31**.




**Fmoc-Lys(Boc)-OH and NHS-$(CH_2)_5$-Mal Coupling.** Following optimization studies, the coupling of Fmoc-Lys(Boc)-OH was changed from the initial conditions of HATU/TMP to OxymaPure/DIC affording **32**, and the two cycles of piperidine/DMF were retained to deprotect the Fmoc group affording **33**. Finally the NHS-$(CH_2)_5$-Mal was coupled using DIPEA in DMF affording the resin-bound Boc-protected product (**34**).

**Detachment from Resin and Purification.** Detachment of the product and deprotection were initially achieved using trifluoroacetic acid affording crude **6**, which could be purified by reverse-phase HPLC.[24,26] Initial deliveries were hindered by the low purity of the crude material (HPLC purity of ca. 30% area), which could only be purified to an HPLC purity of 88% area even with two sequential reverse-phase HPLC runs.

To resolve this issue, significant process development work was carried out on all aspects of the detachment, purification, and isolation. This work was aided by the development of the SPPS (described previously), which improved the HPLC purity of the crude material to 60−70% area. Detachment and deprotection of **34** were found to be reliably achieved using 25% trifluoroacetic acid in dichloromethane affording a solution of crude **6**. Stability data were generated on the various solutions of **6** postcleavage and during purification. This work showed that solutions held for extended periods should be stored at −20 °C to avoid the formation of deacetylated mc-Lys-MMETA (**35**).




An investigation into whether precipitation of this material would improve purity was initiated, and isolated crude material was azeotroped with toluene, DCM, and diethyl ether, affording a gummy solid. Isolation of the product by precipitation from diethyl ether was investigated, but a filterable solid could not be obtained due to the extremely hygroscopic nature of the product.

Thus, purification was undertaken using reverse-phase HPLC followed by a further reverse-phase HPLC concentration step, with a focus on improving the system resolution to enable separation of the product from impurities, as well as improving product loading, recovery and stability.

Control of pH was found to be critical during the purification. Following purification, the pH of product fractions was increased from pH 2. However, the adjustment to pH 6 resulted in the formation of a significant impurity, with levels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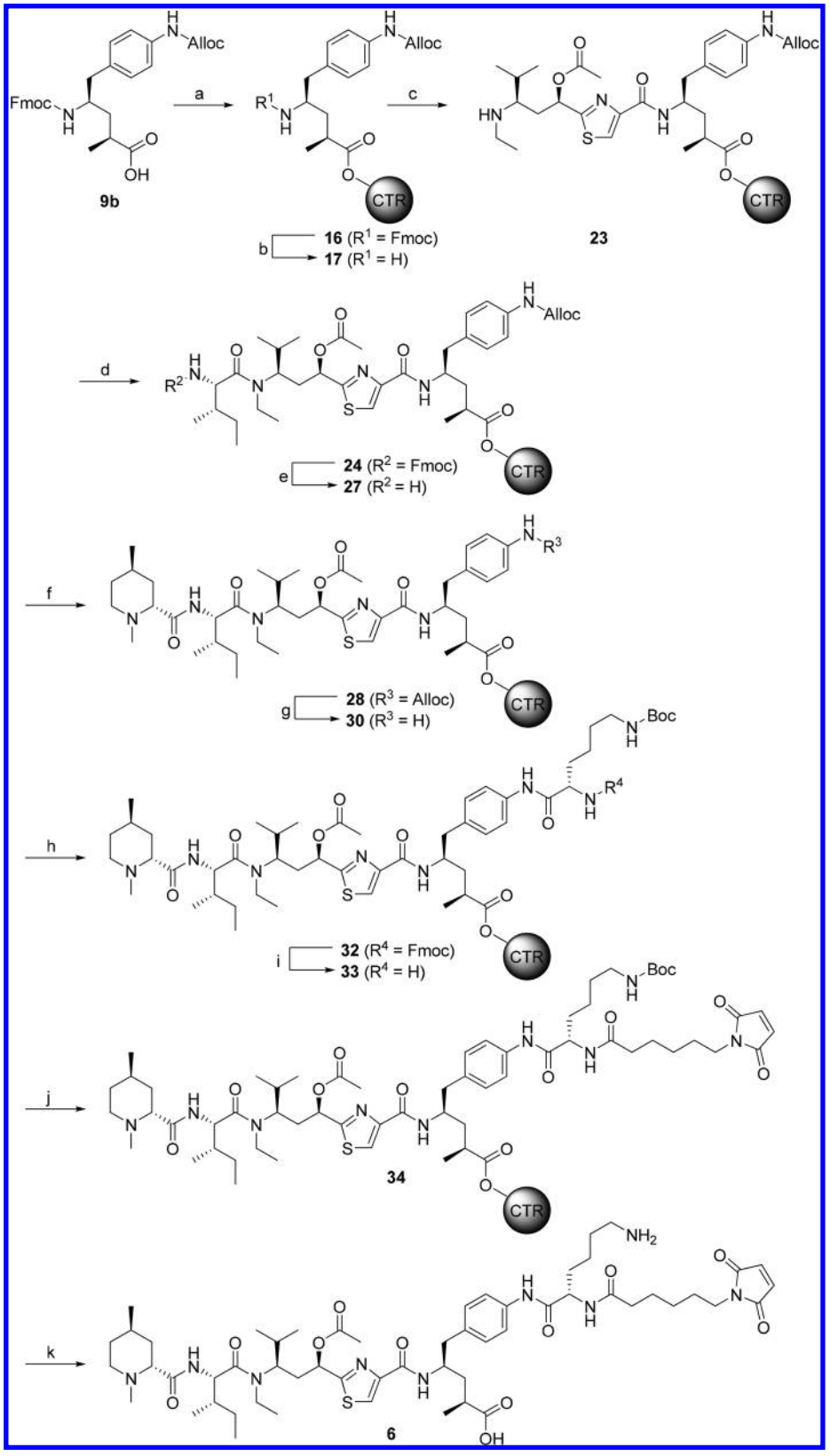
**Scheme 6. Improved Synthesis of mc-Lys-MMETA (6)**[a]




[a]Reagents and conditions: (a) chlorotrityl resin, $Et_3N$, DMF/DCM; (b) piperidine, DMF; (c) **8a**, OxymaPure, DIC, DMF; (d) Fmoc-Ile-F, BSA, DIPEA, DMF; (e) piperidine, DMF; (f) **7**, OxymaPure, DIC, DMF; (g) $Pd(PPh_3)_4$, $BH_3.HNMe_2$, DCM; (h) Fmoc-Lys(Boc)-OH, OxymaPure, DIC, DMF; (i) piperidine, DMF; (j) NHS-$(CH_2)_5$-Mal, DIPEA, DMF; (k) TFA, DCM, 24% (11 steps).

up to 50% in the product fractions. The new impurity was identified as the product where the amino group of the lysine had cyclized onto the maleimide group (**36**). Further studies confirmed that the formation of this impurity occurs at pH 6,



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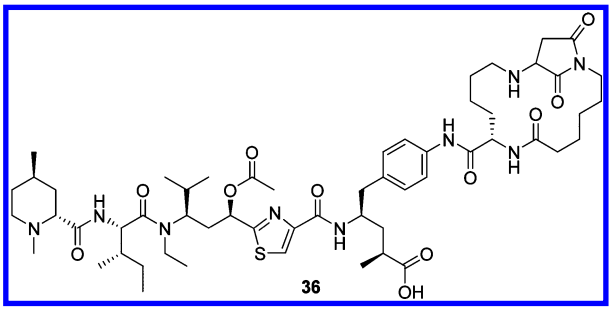
but not at lower pH (4–5). It was not possible to reverse the Michael addition of the lysine to the maleimide, and the impurity ran very close to **6** in the reverse-phase HPLC method, making it impossible to purify the remaining product present to the required purity of >95%. As result of this work, careful control of both mixing and pH control of all product fractions was introduced.




Following the reverse-phase HPLC purification and concentration, **6** was isolated by lyophilization, affording the product as a white solid with an assay of ca. 73% w/w (corresponding to the tri-TFA salt) and an HPLC purity of >95% area.

**Synthesis Scale-Up.** The improvements described above were introduced over a number of campaigns of development, during which material was provided for toxicology and early clinic studies. The largest delivery was completed using two SPPS runs (Scheme 6), using 300 g of 2-chlorotrityl chloride resin for each run. The two runs were completed with a synthesis scale of 170 and 158 mmol, and the purity of the crude **6** following cleavage from the resin was 64 and 70%, respectively. Following the two reverse-phase HPLC procedures, 122 g of product was obtained with a HPLC purity of 95.8%. The assay of the material was 71% w/w; thus 86.4 g of **6** was present (24% yield for SPPS and purification).

## CONCLUSIONS

An efficient and robust synthesis of an ADC payload based on tubulysin natural products has been successfully developed and scaled-up. This work has included the development of new, more efficient routes to tubuphenylalanine and tubuvaline portions of the molecule, as well as improvements in the robustness of the SPPS assembly. A first scale-up of the chemistry had afforded a yield of 1% for the synthesis of the tubuphenylaniline portion (**9a**) and a yield of 1% for the SPPS, giving an overall yield of 0.01% for the longest linear sequence of the synthesis. Following the development work, **9a** was synthesized in a 10% yield, and with a 24% yield for the SPPS, an overall yield of 2.4% for the longest linear sequence was achieved (a 240-fold increase) delivering over 86 g of **6**. This work has supported toxicology and early clinic studies, and further development is planned in anticipation of the progression of the molecule into later clinical trials.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.oprd.7b00232.

Full description of experimental procedures including large-scale preparation of **8c** using original procedures (PDF)

## AUTHOR INFORMATION

### Corresponding Author

*E-mail: jeremy.parker@astrazeneca.com.

### ORCID

Jeremy S. Parker: 0000-0002-4758-3181

Dorin Toader: 0000-0001-6330-5280

### Present Address

Dorin Toader: Mersana Therapeutics Inc. 840 Memorial Drive, Cambridge, MA 02139, United States.

### Notes

The authors declare no competing financial interest.

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