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Improved Methodology for the Synthesis of a Cathepsin B Cleavable Dipeptide Linker, Widely Used in Antibody-Drug Conjugate Research

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ABSTRACT

Antibody-drug conjugates (ADCs) represent an emerging class of biopharmaceutical agents that deliver highly potent anticancer agents (payloads) selectively to tumors or components associated with the tumor microenvironment. The linker, responsible for the connection between the antibody and payload, is a crucial component of ADCs. In certain examples the linker is composed of a cleavable short peptide which imparts an additional aspect of selectivity. Especially prevalent is the cathepsin B cleavable Mc-Val-Cit-PABOH linker utilized in many pre-clinical ADC candidates, as well as the FDA approved ADC ADCETRIS® (brentuximab vedotin). An alternative route for the synthesis of the cathepsin B cleavable Mc-Val-Cit-PABOH linker is reported herein that involved six steps from *l*-Citrulline and proceeded with a 50% overall yield. In this modified route, the spacer (a *para*-aminobenzyl alcohol moiety) was incorporated via HATU coupling followed by dipeptide formation. Importantly, this route avoided undesirable epimerization and proceeded with improved overall yield. Utilizing this methodology, a drug-linker construct incorporating a potent small-molecule inhibitor of tubulin polymerization (referred to as KGP05), was synthesized as a representative example.

Keywords:

Dipeptide synthesis; Drug-linker construct; Cleavable linker; Epimerization

Antibody-drug conjugates (ADCs) represent a leading strategy for the targeted delivery of anticancer agents (payloads), and are one of the fastest growing classes of oncological therapeutics with over 60 conjugates in various stages of clinical trials.[1] ADCs are typically comprised of a monoclonal antibody (mAb) to which an anticancer therapeutic agent (payload) is conjugated through an appropriate cleavable or non-cleavable linker (Fig. 1). The use of a monoclonal antibody allows for a high degree of antigen selectivity, thereby potentially decreasing off-target effects often observed in standard chemotherapy.[2–5]

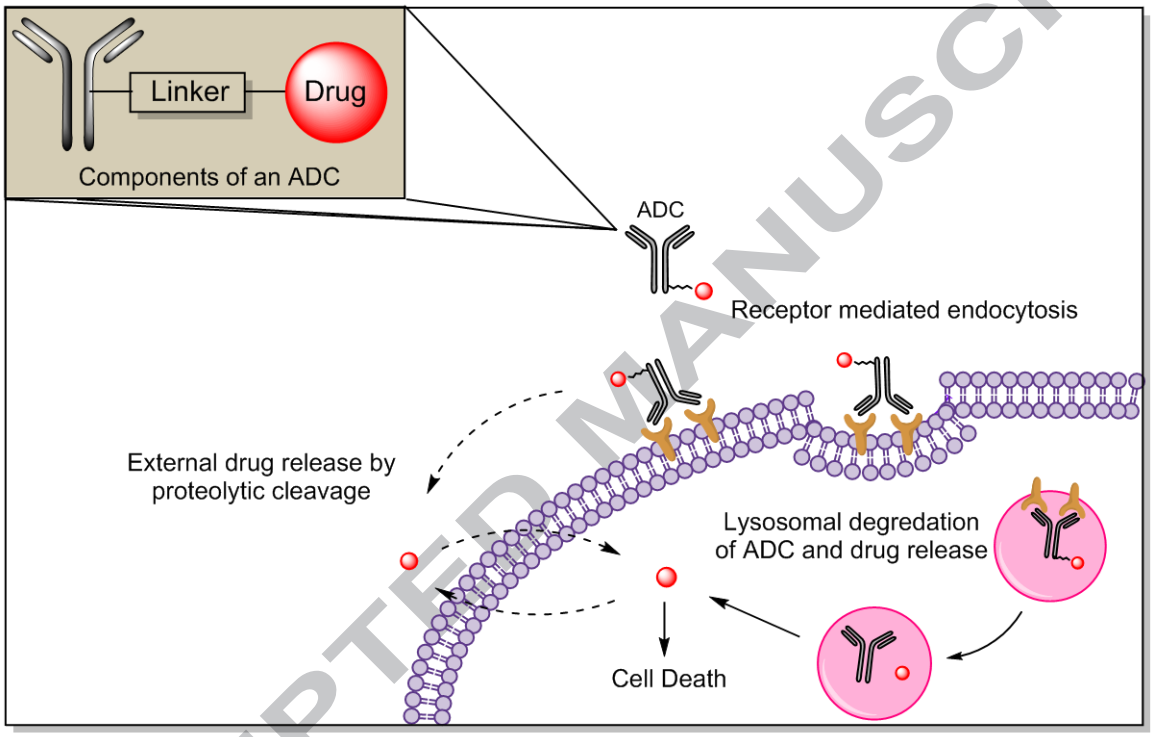


Figure 1: ADC mechanism of action

The linker plays a critical role in the stability of the ADC in systemic circulation, and is key for efficient and selective release of the payload at the tumor site. Linkers can be classified within two broad categories, cleavable and non-cleavable, based on the mechanism of drug release.[6,7] Cleavable linkers are designed to be sensitive to specific physiological conditions such as high glutathione concentration,[8–10] low pH,[11] or cleavage by protease enzymes highly expressed in cancer cells and/or the tumor microenvironment.[12,13] ADCs that contain non-cleavable linkers depend on lysosomal degradation of the monoclonal antibody in the targeted tumor cell for drug release.[6] Cleavable linkers can be broadly categorized into two types, chemically labile and enzymatically labile linkers. Chemically labile hydrazone and disulfide linkers are stable at the

neutral pH of blood.[14] Hydrazone linkers are cleaved in the acidic environment of endosomes (5.0-6.5) and lysosomes (4.5-5.0) associated with tumor cells, whereas disulfide linkages are cleaved by glutathione that is more highly concentrated (up to 1000 fold higher) in tumor cells versus normal tissue.[15] Chemically labile linkers often suffer from limited plasma stability.[6,13,16] Alternatively, peptide based linkers are designed as specific substrates for proteases that are highly expressed in cancer cells or the tumor microenvironment.[17] In Brentuximab vedotin (ADCETRIS®, Seattle Genetics), one of four FDA approved ADCs currently on the market, a cathepsin B cleavable maleimido-containing valine-citrulline based dipeptide linker (Mc-Val-Cit-PABOH) is utilized (Fig. 2).[18] The monomethylauristatin-E (MMAE)[16,19,20] payload (inspired by the natural product dolastatin 10,[21–24] representing a seminal contribution by Pettit and co-workers) is bonded through a self-immolative *para*-aminobenzyl alcohol spacer,[12,25–29] eloquently first advanced by Katzenellenbogen and co-workers.[30] Upon cleavage of the dipeptide by cathepsin B, the spacer undergoes a spontaneous 1,6-elimination resulting in release of the payload. The Mc-Val-Cit-PABOH linker (**1**, Fig. 2) displays high plasma stability (half-lives in mice and monkey of 6.0 and 9.6 days, respectively), and has been found to be superior over chemically labile linkers.[7,16]

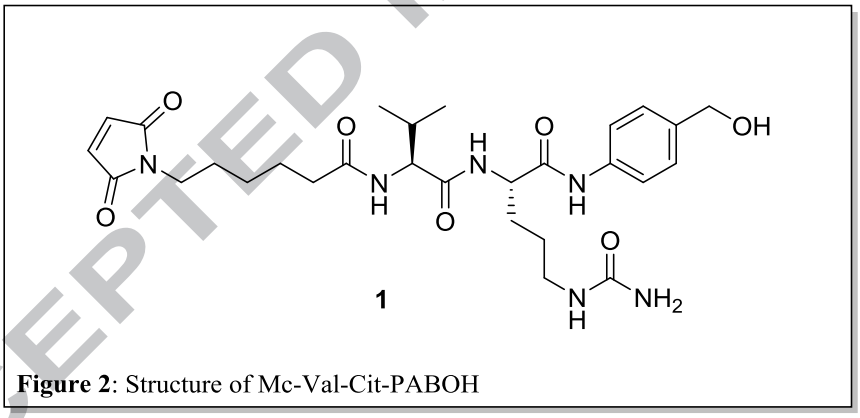


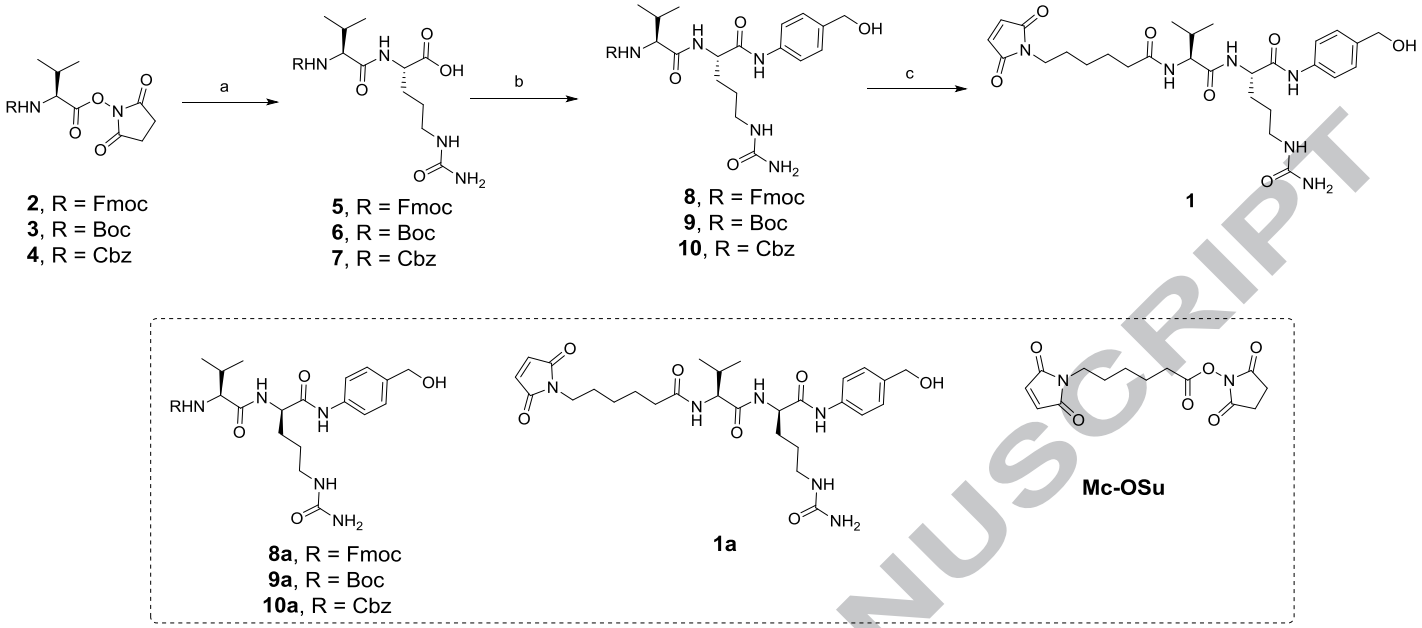
Figure 2: Structure of Mc-Val-Cit-PABOH

The Val-Cit dipeptide linker is commercially available, however it commands a high price (approximately $2,500/g).[31–34] This inspired us to undertake the synthesis of this linker to support our various drug-linker construct applications,[35] which utilize potent small-molecule inhibitors of tubulin polymerization as payloads.[36–41] While the Mc-Val-Cit-PABOH linker is certainly well-described in the literature,[42–45] we are aware of only two publications that provide a detailed experimental procedure for the synthesis of this specific linker.[13,46] The original contribution was provided by Dubowchick and co-workers,[13] however following that methodology (in our hands)

often led to fairly low overall yields along with unanticipated epimerization resulting in diastereomeric mixtures that proved difficult to separate. Therefore, we modified the methodology to provide a synthetic route with improved yield and no observable epimerization.[35] Subsequent to our patent publication on this methodology,[35] and during the late stages of manuscript preparation, we were encouraged to see that Wei and co-workers at Genentech reported a modified route to this Val-Cit dipeptide linker **1** utilizing similar, although not exactly identical, methodology.[46] Neri and co-workers have described related methodology, but for the synthesis of other short peptide constructs.[47]

The original reported synthesis by Dubowchick and co-workers (outlined in scheme 1)[13] was initially followed in our laboratory. The synthesis of Fmoc-Val-Cit **5** proceeded smoothly starting from commercially available Fmoc-Val-OSu **2**. The EEDQ coupling reaction used for amide bond formation between compound **5** and *para*-aminobenzyl alcohol in the next step was not only complicated by the formation of several side-products, but also suffered from low yields (average between 20-25%). Of most concern, compound **8** was always obtained as a mixture of diastereomers **8** and **8a** in variable ratios of 3:1 to 8:1 due to epimerization of the citrulline stereogentic center of compound **5** during the coupling reaction. In an effort to improve the yield and possibly reduce the rate of epimerization, EEDQ was replaced with HATU in the coupling reaction between compound **5** and 4-aminobenzyl alcohol. The yield was slightly improved, but the problem of epimerization persisted. It was then realized that the low yield and complicacy of this particular step was most likely due to Fmoc deprotection under basic reaction conditions. Therefore, to improve the yield, Fmoc was replaced with Boc and Cbz protecting groups which are stable under basic conditions (**6** and **7).** Both compounds **6** and **7** underwent HATU mediated coupling reactions with 4-aminobenzyl alcohol to generate the corresponding *para*-aminobenzyl alcohols **9** and **10** in good yield (70-80%). Unfortunately, even though a better yield was obtained, epimerization of the desired product still occurred resulting in a nearly 1:1 mixture of diastereomers for both compounds **9** and **10**.

Scheme 1: Original route used for the synthesis of MC-Val-Cit- PABOH

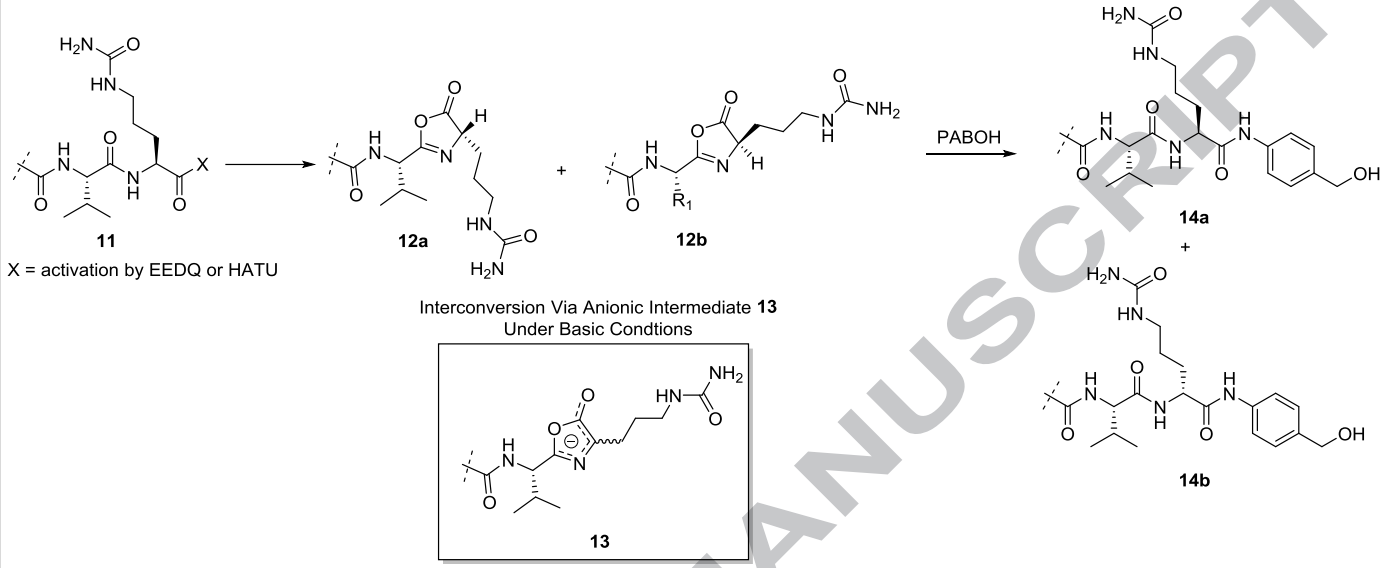


Reaction conditions: (a) **2** (1.0 equiv), l-Citrulline (1.1 equiv), $NaHCO_3$ (1.1 equiv), Water/THF, RT, 16 h. (b), **5** (1.0 equiv), 4-Aminobenzyl alcohol (2.0 equiv), EEDQ (2.0 equiv), CH_2Cl_2/MeOH (2:1), RT, in dark, 1.5 d. (c) (i) **8** (1.0 equiv), Diethylamine, NMP, RT, 16.0 h. (ii) Mc-OSu (1.1 equiv), NMP, RT, 16.0 h.

After exploring different protecting groups and coupling reagents (EEDQ and HATU) it was postulated that the reaction conditions were most likely not contributors to epimerization, but rather that the epimerization was due to the nature of the substrate used in the coupling step (step b, scheme 1). It is well known that coupling reactions between the carboxylic acid moiety of a peptide (di to polypeptide) and a nucleophile, such as an amine group of an amino acid, can be problematic due to epimerization of the α-chiral center with respect to the carboxylic acid group. A generalized example of such epimerization is detailed in scheme 2.[48–50] When the terminal carboxylic acid of a peptide is activated **11**, oxazolone **12a** can be formed as the leading intermediate. Under mildly basic conditions the oxazolone undergoes racemization via the formation of conjugate anionic intermediate **13**. The resulting oxazolone mixture of intermediates **12a** and **12b** then reacts with the nucleophile to generate products **14a** and **14b**, thus explaining epimerization of the stereogenic center. Therefore, peptides are usually grown at the *N*-terminus under mild activation conditions.[48–50] In this later approach, the carboxylic acid group is activated

on an *N*-protected α-amino acid and then reacted with a nucleophile such as *C*-protected amino acid or amine, thus avoiding oxazolone formation.[48,49]

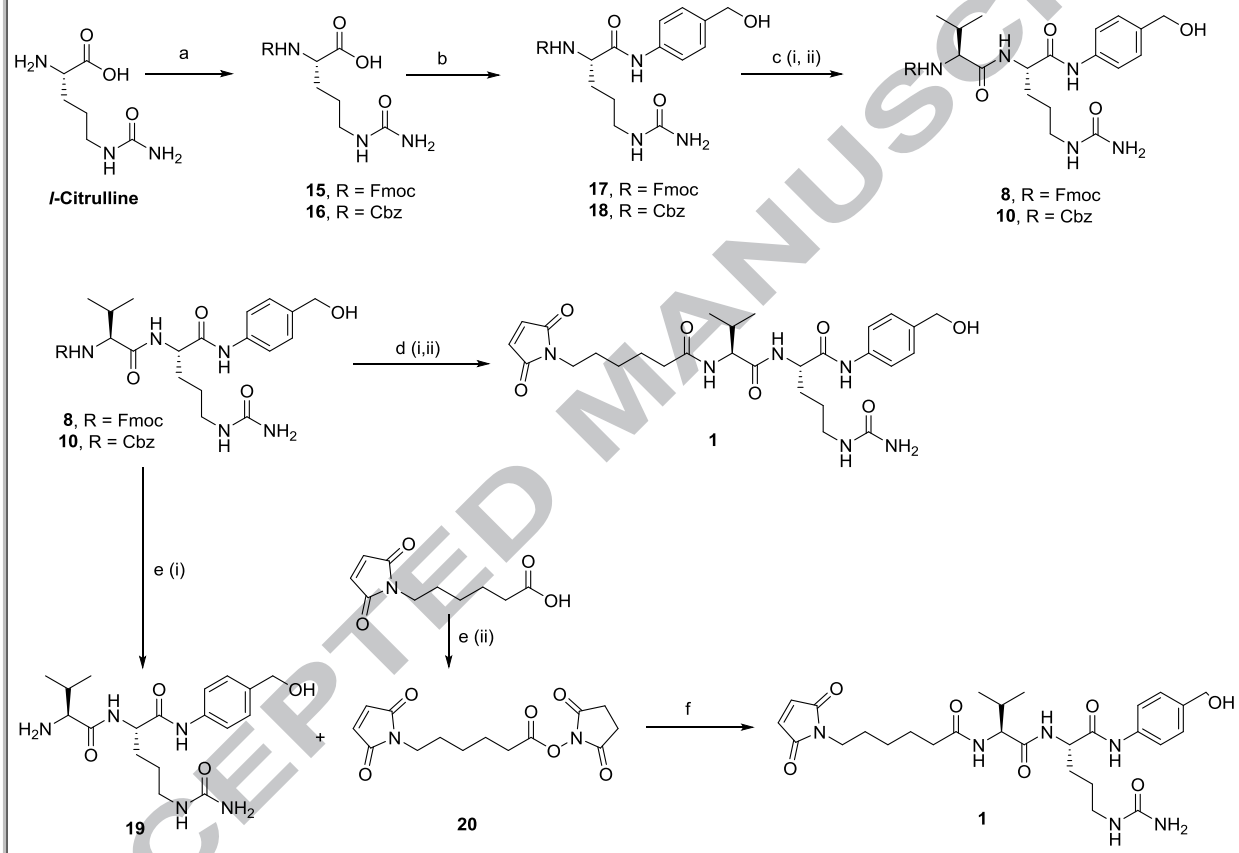
Scheme 2: Epimerization of α-chiral center via oxazolone formation during peptide coupling[48]



Oxazolone formation offers a rational explanation that accounts for compounds **8**, **9**, and **10** being obtained as a mixture of diastereomers. In order to avoid possible oxazolone formation a new route to compound **1** was developed (Scheme 3).

Our modified procedure (Scheme 3) worked well with both Fmoc and Cbz as protecting groups. Fmoc or Cbz protected *l*-citrullines **15** and **16** were synthesized through reaction of *l*-citrulline with Fmoc-Cl or Cbz-OSu in quantitative yield. Subsequent amide bond formation between the protected citrulline **15** or **16** and 4-aminobenzyl alcohol using HATU as the coupling reagent afforded Fmoc-Cit-PABOH **17** or Cbz-Cit-PABOH **18** in good yield (60-65% for **17** and 70-80% for **18**). In the case of Fmoc protected *l*-citrulline **15**, the reaction was carried out in the

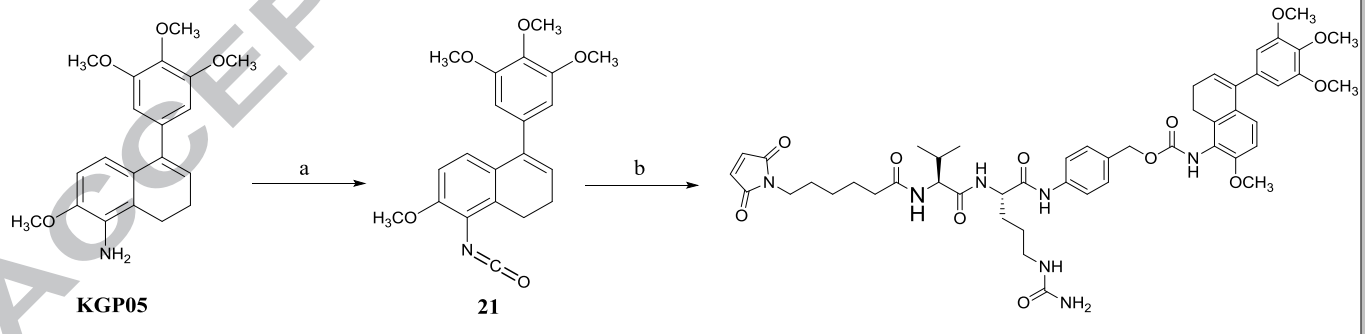
Scheme 3: Modified route to the Mc-Val-Cit-PABOH linker



Reaction conditions: (**a**) l-Citrulline (1.1 equiv), Fmoc-Cl or Cbz-OSu (1.0 equiv), $NaHCO_3$ (2.0 equiv), DME/Water (2:1), RT, 20 h, quant. (**b**), when R = Fmoc, **15** (1.0 equiv), 4-Aminobenzyl alcohol (3.0 equiv), HATU (1.2 equiv), DIPEA (1.0 equiv), DMF, RT, in dark, 48 h, 60-65% (17); and when R = Cbz, **16** (1.0 equiv), 4-aminobenzyl alcohol (1.2 equiv), HATU (1.2 equiv), DIPEA (4.0 equiv), DMF, RT, in dark, 48 h, 70-80% (**18**). (**c**) (**i**) When R = Fmoc, **17** (1.0 equiv), Triethylamine (20.0 equiv), DMF, RT, 24 h. (**ii**) Fmoc-Val-OSu (1.1 equiv), DMF, RT, 20 h, 85-95% (**8**); and when R = Cbz, (**i**) **18** (1.0 equiv), 10 % Pd-C (10 mol%), H_2, MeOH, RT, 5 h. (**ii**) Cbz-Val-OSu (1.2 equiv), DMF, RT, 20 h, 84-96% (**10**). (**d**) When R = Fmoc, (**i**) **8** (1.0 equiv), Triethylamine (20.0 equiv), DMF, RT, 24 h. (**ii**) Mc-OSu (1.1 equiv), DMF, RT, 20 h, 84-95% (1). When R = Cbz, (**i**) **10** (1.0 equiv), 10 % Pd-C (10 mol%), MeOH-CH_2Cl_2 (2:1), H_2, RT, 5 h. (**ii**) Mc-OSu (1.1 equiv), DMF, RT, 20 h, 88-98 % (**1**). (**e**) (i) When R = Fmoc, **8** (1.0 equiv), Triethylamine (20.0 equiv), DMF, RT, 24 h and when R = Cbz, **10** (1.0 equiv), 10 % Pd-C (10 mol%), MeOH-CH_2Cl_2 (2:1), H_2, RT, 5 h. (**ii**) 6-maleimidohexanoic acid (1.5 equiv), Triethylamine (2.0 equiv), N,N'-disuccinimidyl carbonate (1.2 equiv), DMF, RT, 6 h. (**f**) **19** (1.0 equiv), **20** (1.1 equiv), RT, DMF, 20 h, 85-97% (**1**).

presence of 1.0 equivalent of *N*,*N*-diisopropylethylamine (DIPEA) as significant Fmoc deprotection occurred in the presence of excess base thus resulting in a low yield. The synthesis of dipeptide **8** started with Fmoc deprotection of compound **17** using excess triethylamine (20 equivalents) in DMF at room temperature followed by the reaction of the generated Cit-PABOH with commercially available Fmoc-Val-OSu. Fmoc-Val-Cit-PABOH **8** was obtained in very good yield (85-95%) as a single diastereomer. Cbz-Val-Cit-PABOH **10** was also obtained in a similar manner in excellent yield (90-95%) as a single diastereomer. The Cbz deprotection was performed by Pd catalyzed hydrogenolysis. To obtain the final target compound **1 (**from **8**), Fmoc deprotection (triethylamine in DMF) of compound **8** afforded the free amine (Val-Cit-PABOH) which was subsequently reacted with the activated 6-maleimidohexanoic acid (Mc-OSu, **20**). Compound **1** was obtained in an excellent yield (up to 96%) as a single diastereomer. This final step was further optimized by generating the *o*-succinimide activated compound **20** *in situ.* In this modified procedure, 6-maleimidohexanoic acid was activated (*in situ*) by treatment with *N*,*N*'-disuccinimidyl carbonate in DMF and then reacted with the previously generated Val-Cit-PABOH compound **19**. Intermediate **10** was also converted to compound **1** in excellent yield (up to 98%) as a single diastereomer under similar conditions. Over 1 g of compound **1** has been synthesized in a single batch with an overall yield of 50% and intermediates **8** and **10** have been prepared in 3 g batches, with an overall yield of 55% and 65% respectively. Similar yields have been obtained through repeated syntheses (x10).

Scheme 4: Synthesis of KGP05 Drug-Linker Construct



KGP05 **21** **22**

Reaction Conditions: (a) KGP05 (1.0 eqiv), Triphosgene (2.0 equiv), CH_2Cl_2 / $NaHCO_3$ Sat [1:1], RT, 2 h, 85-90% (b) **21** (1.5 equiv), **1** (1.0 equiv), Dibutyltin Dilaurate (2.0 equiv), DMF, RT, 48 h, 60-80%

A new drug-linker construct (Scheme 4) incorporating the Mc-Val-Cit-PABOH linker **1** was synthesized, which utilized a promising aniline-based dihydronaphthalene inhibitor of tubulin

polymerization (colchicine binding site) as the payload (referred to as KGP05).[51,52] This unique payload demonstrates dual-mechanism of action functioning as both an antiproliferative agent (cytotoxin) and as a potent vascular disrupting agent (VDA). Small-molecule VDAs impart selective and irreversible damage to tumor-associated vasculature and are mechanistically distinct from the generally well-described angiogenesis inhibiting agents (AIAs).[53–56] Treatment of KGP05 with triphosgene generated the corresponding isocyanate intermediate **21**. The desired drug-linker construct **22** was obtained in a relatively high yield through the reaction of compound **1** with the generated isocyanate in the presence of dibutyltin dilaurate.

In conclusion, a modified route has been developed for the synthesis of the cathepsin B cleavable Val-Cit dipeptide linker **1** widely used in ADCs. The method proved to be straight-forward, consistent, and scalable. Above all, the methodology afforded the Val-Cit dipeptide linker **1** with exclusive diastereoselectivity, eliminating the potential problem of racemization associated with the citrulline stereogenic center.

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Highlights:

A revised synthesis was developed for the Val-Cit linker widely employed in ADCs.
The methodology proved to be high-yielding, reproducible, and devoid of epimerization.
A new drug-linker construct bearing a novel tubulin-active payload was synthesized.