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Development of a diketopiperazine-forming dipeptidyl Gly-Pro spacer for preparation of an antibody–drug conjugate†

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We developed a novel diketopiperazine-forming dipeptidyl spacer aimed at application in antibody–drug conjugates. Enzymatic cleavage of a peptide linked to the Gly-Pro spacer resulted in formation of diketopiperazine, which was stable and non-toxic, and release of the parent drug.

Introduction

Antibody–drug conjugates (ADCs) play an increasingly important role in selective delivery of potent drugs to their intended site of action in the body.[1–3] There is a strong demand for efficient ADC molecule design, especially for cancer chemotherapy, because most chemotherapeutic medicines have a narrow safety margin, resulting in severe adverse effects. Recently, several ADCs have been developed, some of which have appeared promising in clinical trials.[4,5] An ADC consists of 3 components: a monoclonal antibody that targets a tumor antigen, a cytotoxic agent, and a linker (Fig. 1). The antibody is a pilot molecule for delivery of an anti-cancer agent. The linker is further divided into a specifier and a spacer. The linker moiety is located between the parent drug and the antibody and increases the rate of enzymatic cleavage. The specifier serves as a substrate for an enzyme with site-specific activity. In the absence of a spacer, enzymatic cleavage of the bond between the specifier and the parent drug may sometimes be inadequate, because of steric hindrance caused by the parent drug.[6,7] Once the bond between the carrier and the linker is cleaved, the spacer should spontaneously release the drug in its active form. In our development of ADCs,[8–10] we focused on a novel linker development.

The most widely used spacer is a *p*-aminobenzyloxycarbonyl spacer.[11] Once the amide bond between the specifier and the spacer has been cleaved, the spacer degrades in a self-immolative manner, and the parent drug is released. During degradation, a highly reactive iminoquinone methide intermediate is formed, which may interact with cellular nucleophiles, such as glutathione, a thiol (Scheme 1).[12] Some endopeptidases, such as prostate-specific antigen, only cleave the amide bonds between amino acids; therefore, the carbamate site in the *p*-aminobenzyloxycarbonyl spacer is sometimes not cleaved by endopeptidases.[13–15]

In this study, we designed a novel spacer for use in a versatile ADC strategy. The spacer consists of a proline-glycine (Gly-Pro) dipeptide, and easily formed diketopiperazine by cyclization. The rate of diketopiperazine formation depends on the proportion of *cis*/*trans* amide isomers of the dipeptide. Although most peptide sequences adopt only the energetically favorable *trans* conformation, proline is unique in that it has a *cis*-amide conformation.[16,17] Thus, proline greatly enhances the rate of diketopiperazine formation because of its great propensity to adopt the *cis*-amide conformation.

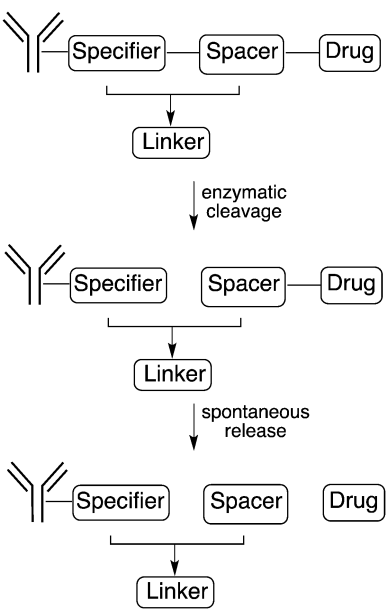

Fig. 1 Components of antibody–drug conjugates.

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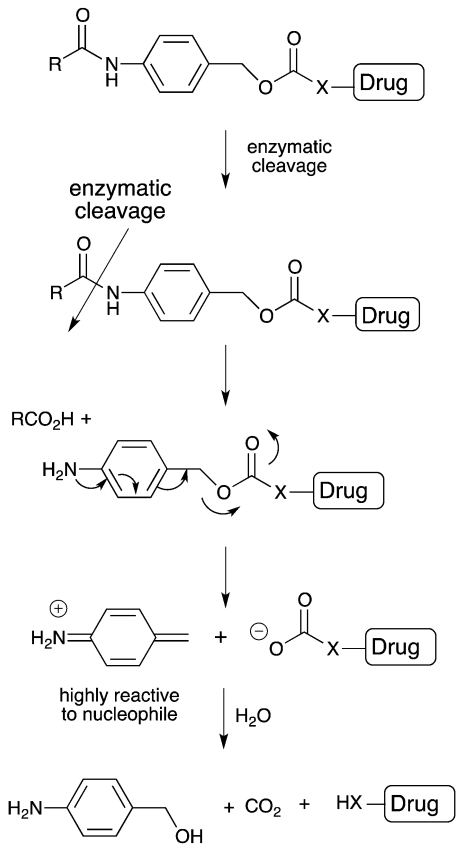
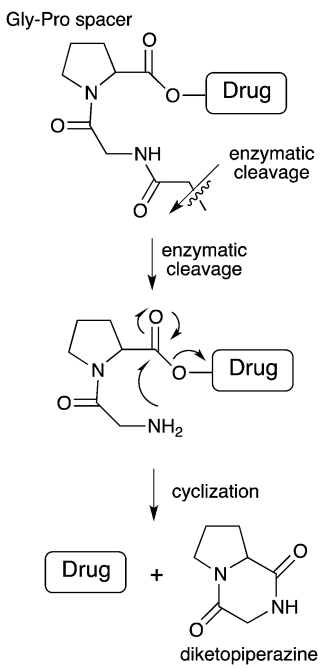
† Electronic supplementary information (ESI) available: Experimental details and ^{1}H-NMR and ^{13}C-NMR spectra. See DOI: 10.1039/c3md00075c



Scheme 1 The *p*-aminobenzyloxycarbonyl spacer degradation mechanism and formation of a highly reactive intermediate; X = NH or O.

In order to minimize the steric hindrance at the enzymatic cleavage site, Gly was chosen for the amino acid site near the cleavage site. The Gly-Pro sequence is well known as a "difficult sequence" for diketopiperazine formation during peptide synthesis.[18–22] Once an enzyme cleaves the peptide bond, the Gly-Pro sequence forms diketopiperazine to release the parent drug in its active form.[23] The diketopiperazine thus formed is normally stable and non-toxic (Scheme 2).



Scheme 2 Drug-release mechanism of the Gly-Pro spacer.

Using this novel spacer, we designed a plasmin-cleavable prodrug for chemotherapy. Plasmin, a serine protease, plays an important role in tumor invasion and metastasis.[24,25] The proteolytically active form of plasmin may be localized around the tumor tissue because it is formed from the inactive proenzyme plasminogen by the urokinase-type plasminogen activator produced by cancer and/or stroma cells.[7,26–30] Plasmin activity is very rapidly inhibited by inhibitors, such as α_2-anti-plasmin, present within the blood circulation. Thus, plasmin is a very promising enzyme for use in a tumor-specific prodrug approach. Since the recognition sequence for cleavage by plasmin is a Val-Leu-Lys tripeptide, we synthesized paclitaxel–peptide conjugates **1** and **2** (Fig. 2). These compounds have a Gly-Pro spacer-containing pentapeptide; unnatural (D)-proline was used for the linker in compound **2** in order to investigate the influence of stereochemistry on drug-release activity. In order to compare the efficacy of the novel linker, we synthesized compound **3**, containing the commonly used *p*-aminobenzyloxycarbonyl spacer, and compound **4**, without a spacer. We chose paclitaxel as the parent drug because it is one of the most useful anti-cancer agents clinically. The linker was attached to the 2-hydroxy group, because it is expected to decrease cytotoxic activity by modification of the 2-hydroxy functionality.[31–37] For an initial evaluation of linkers, an acetyl group was chosen for the N-terminus of paclitaxel-conjugated peptides, instead of using maleimide or bromoacetamide for immobilization to the antibody, in order to avoid the instability of maleimide or bromoacetamide during evaluation of drug-release.

Since paclitaxel is sensitive to both acidic and basic conditions, the methoxytrityl (Mmt) group, which is an acid-labile amino-protecting group,[38] was chosen for protecting the side-chain amino group of lysine. The synthesis was achieved as shown in Scheme 3. The sequential synthesis of a pentapeptide

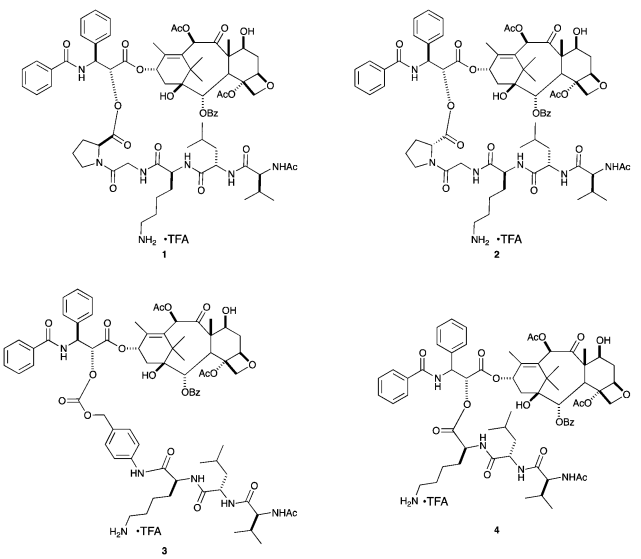
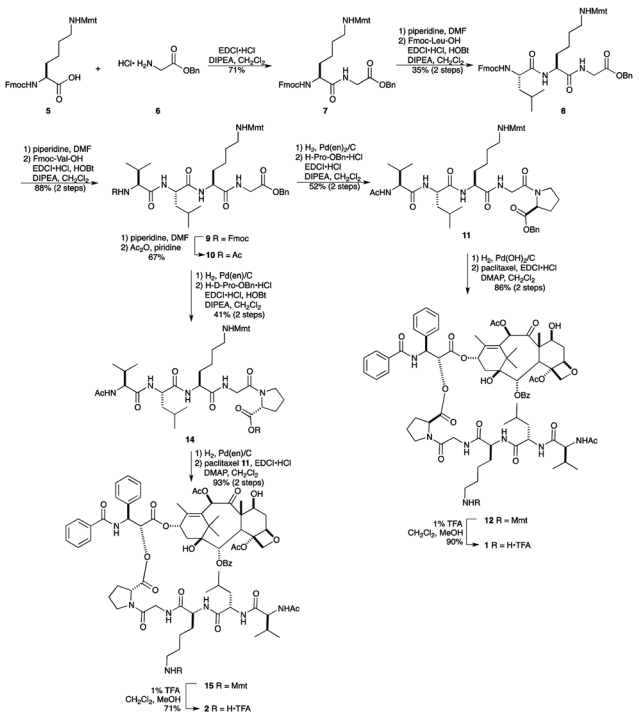


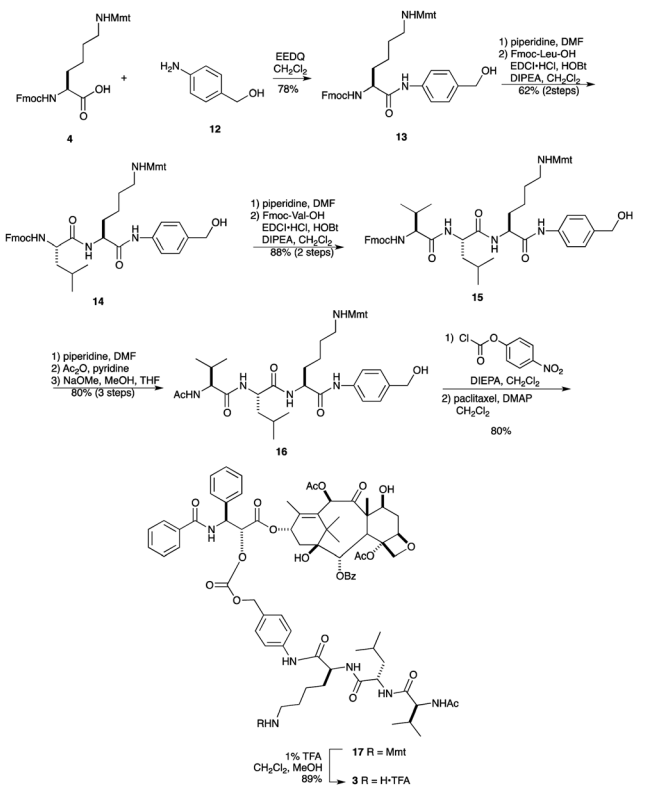
Fig. 2 Synthesized peptide–paclitaxel conjugates.



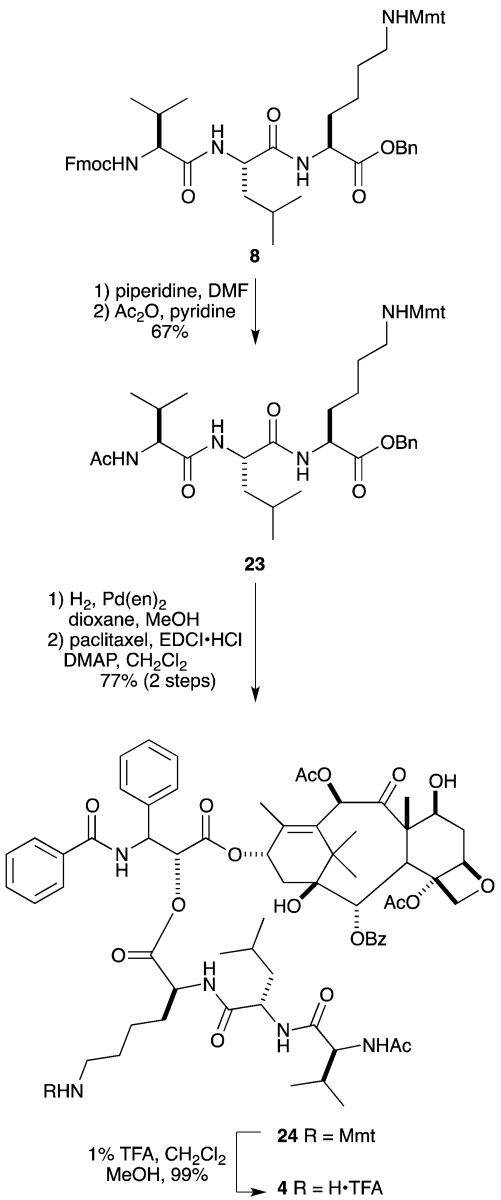
Scheme 3 Synthesis of compounds **1** and **2**.

from C-termini was difficult because of diketopiperazine formation at the dipeptide Fmoc-Gly-Pro-OBn-deprotection step. The pentapeptide is divided into 2 parts, the tetrapeptide and the proline. The Ac-Val-Leu-Lys(Mmt)-Gly-Pro-OBn sequence **10** was prepared in accordance with the Fmoc peptide synthesis strategy. After removal of the benzyl ester[39] of tetrapeptide **10**, (L)-proline was added to the C-terminus. Again, after the hydrogenolysis of the benzyl ester of pentapeptide **11**, the pentapeptide was attached to paclitaxel by water-soluble carbodiimide·hydrochloride (WSCDI·HCl) in the presence of 4-(dimethylamino)pyridine (DMAP). Finally, the Mmt group was removed without intact paclitaxel. The paclitaxel–peptide conjugate **1** was obtained in 71%. Unnatural (D)-proline-containing paclitaxel–peptide was prepared in a similar manner with a 27% overall yield from tetrapeptide **10**.

The less nucleophilic aminobenzyl alcohol **16** was introduced using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinone (EEDQ); then, the peptide was prepared from the C-termini in a sequential manner (Scheme 4). After activation of the hydroxyl



Scheme 4 Synthesis of *p*-aminobenzyloxy spacer-containing paclitaxel–peptide conjugate.



Scheme 5 Synthesis of paclitaxel–tripeptide conjugate **4**.

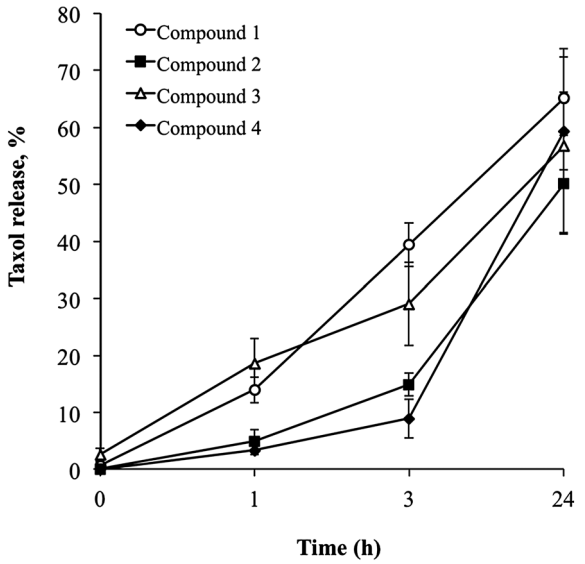


Fig. 3 Paclitaxel releasing ability of compounds **1–4** in the presence of plasmin.

group of the peptide by *p*-nitrophenylcarbonate, the peptide was introduced at the 2-position of paclitaxel.

The tripeptide Ac-Val-Leu-Lys(Mmt)-OH **24** was conjugated to paclitaxel without a linker, with high yield to synthesize compound **4** as shown in Scheme 5.

We next evaluated the ability of linkers to release the paclitaxel in the presence of plasmin (Fig. 3 and Table S1 in the ESI†). As expected, linker-conjugated paclitaxel **1–3** was cleaved in the presence of plasmin within 24 h. The Gly-Pro linker had almost the same efficacy as the compound *p*-aminobenzyloxycarbonyl spacer. Indeed, 39% of paclitaxel was released from compound **1** after 3 h. The unnatural (D)-proline-containing compound **2** was also cleaved by plasmin, although the cleavage rate was slower (15% after 3 h). The tripeptide-conjugated paclitaxel **4** was also cleaved, but the release rate was only 9%. After 24 h, half of the paclitaxel was released from compounds **1–3**.

Conclusions

In conclusion, we designed a novel dipeptidyl spacer to be used as a versatile ADC strategy. The spacer could be easily prepared using well-established peptide chemistry methods. The rate of diketopiperazine formation could be attenuated by choosing the appropriate peptide sequence. This strategy would be applicable to endopeptidases, which only recognize peptide bonds at particular cleavage sites. A detailed study related to the biological activity and pharmacokinetic profile will be reported in future.

Acknowledgements

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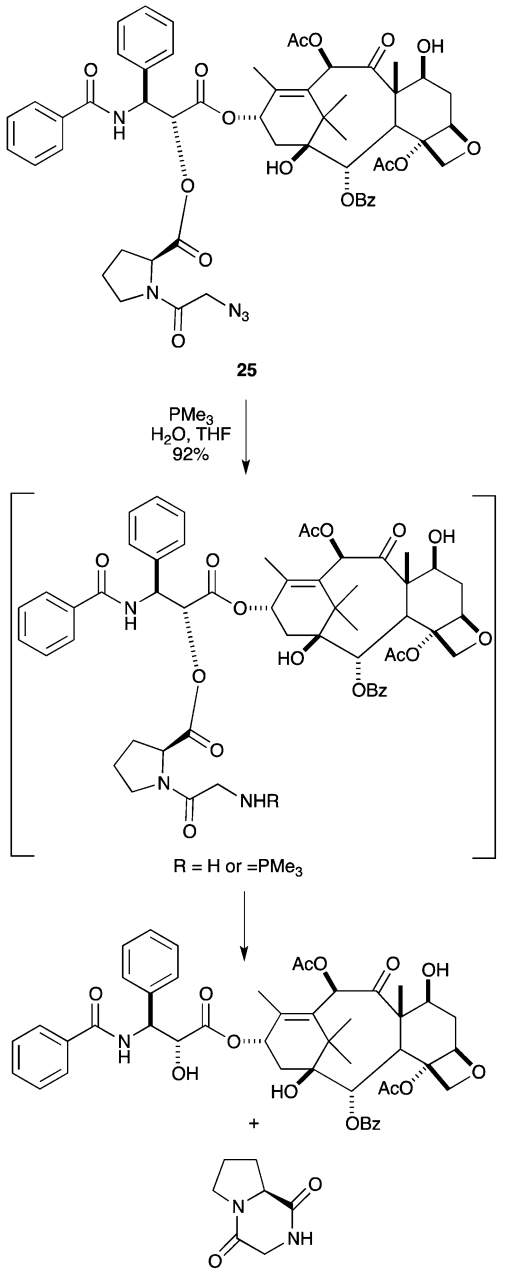
23 The reduction of compound **25** was released paclitaxel, and formation of diketopiperazine was confirmed.



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