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## Graphical Abstract

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**Synthesis and *in vitro* evaluation of SG3227, a pyrrolobenzodiazepine dimer antibody-drug conjugate payload based on sibiromycin**

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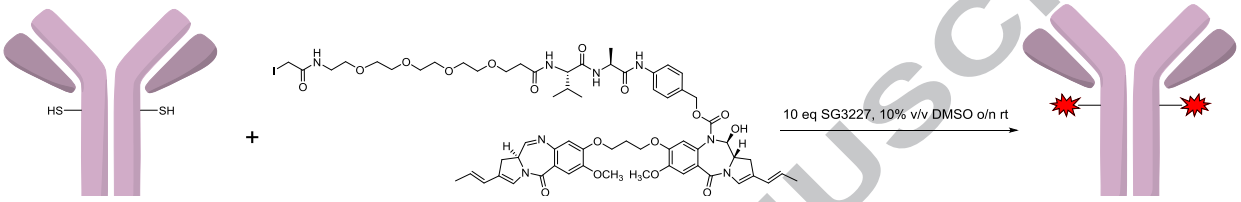

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# Synthesis and *in vitro* evaluation of SG3227, a pyrrolobenzodiazepine dimer antibody-drug conjugate payload based on sibiromycin

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ABSTRACT

A novel pyrrolobenzodiazepine dimer payload, SG3227, was rationally designed based on the naturally occurring antitumour compound sibiromycin. SG3227 was synthesised from a dimeric core in an efficient fashion. An unexpected room temperature Diels-Alder reaction occurred during the final step of the synthesis and was circumvented by use of an iodoacetamide conjugation moiety in place of a maleimide. The payload was successfully conjugated to trastuzumab and the resulting ADC exhibited potent activity against a HER2-expressing human cancer cell line *in vitro*.

The pyrrolobenzodiazepines (PBDs) are a family of naturally occurring antitumour agents isolated from various *Streptomyces* species.[1-3] The PBD monomers target purine-guanine-purine motifs in the minor groove of DNA. Once situated in the minor groove, an aminal bond is formed between the C11 position of the PBD and the N2 of guanine. PBD dimers can bind to both opposing strands of DNA, creating covalent crosslinks at purine-GATC-pyrimidine sequences. Their ability to form such DNA interstrand crosslinks makes PBD dimers highly potent anticancer agents.[4-6]

Due to their high potency, PBD dimers have been used as warheads in antibody-drug conjugates (ADCs).[7, 8] Currently, there are two PBD warheads being evaluated in various phase I, II and III clinical trials.[9-11]

Sibiromycin is the most potent naturally occurring PBD ($IC_{50}$ L1210, leukaemia = 2.9 nM).[12] It contains an amino sugar derivative at the C7 position and a *trans*-propenyl group at the C2 position that interacts favourably with the minor groove of DNA. We have previously synthesized a sibiromycin-like PBD dimer[13], SG2219, a more potent cytotoxic agent than stand-alone clinical agent SG2000[6] (**Figure 1, Table 1**).

In this letter we report the synthesis, conjugation and *in vitro* activity of an ADC with a sibiromycin-like PBD dimer payload, SG3227. It was envisioned that the SG2219 warhead (pharmacologically-active unit) would be linked to the antibody by a cathepsin-cleavable "trigger" peptide *via* the N10 position of the PBD, masking one of the imine functionalities. This strategy imparts two important benefits - one is that the payload has more of a pro-drug like character, since as long as the peptide trigger remains in place, the drug cannot crosslink DNA. Another advantage is that there are fewer potentially reactive groups in the compound that can cause complications during synthesis.

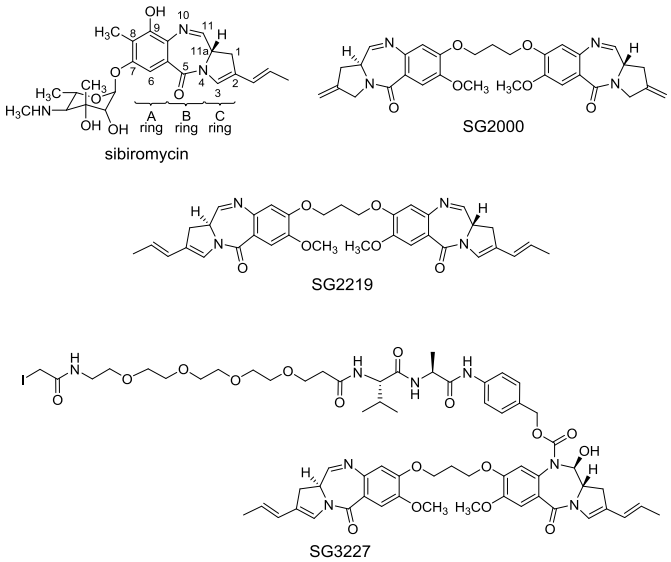



**Figure 1.** Sibiromycin, a naturally-occurring PBD; SG2000, a clinical stand-alone PBD dimer; SG2219, the PBD warhead released by SG3227 ADCs; and SG3227, a PBD dimer payload for ADC synthesis.

**Table 1.** *In vitro* activity of PBD dimers SG2219 and SG2000

| Cell lines | K562 | NCIN87 | BT474 | SKBR3 | MDA-MB-468 |
|---|---|---|---|---|---|
| SG2219 | 1 | 5 | 641 | 121 | 6.4 |
| SG2000 | 735 | 66 | 7000 | 520 | 78 |

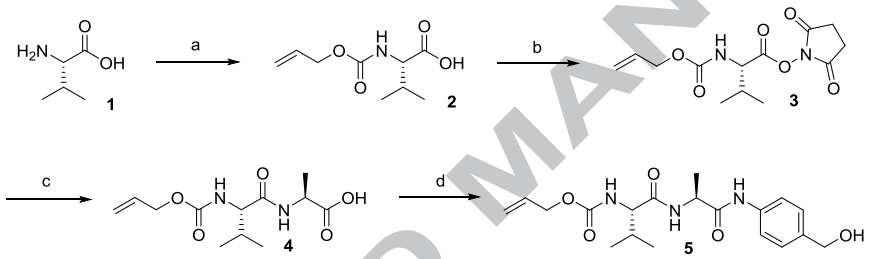
$IC_{50}$ values in pM (mean of three independent experiments) were determined using CellTiter96 (MTS) following 96 h incubation.

We have previously reported a monomeric approach to the synthesis of PBD payloads *via* TIPS-protected phenol building blocks.[14] We herein report an alternative synthesis with fewer synthetic steps starting from dimeric core **6** (**Scheme 2**).

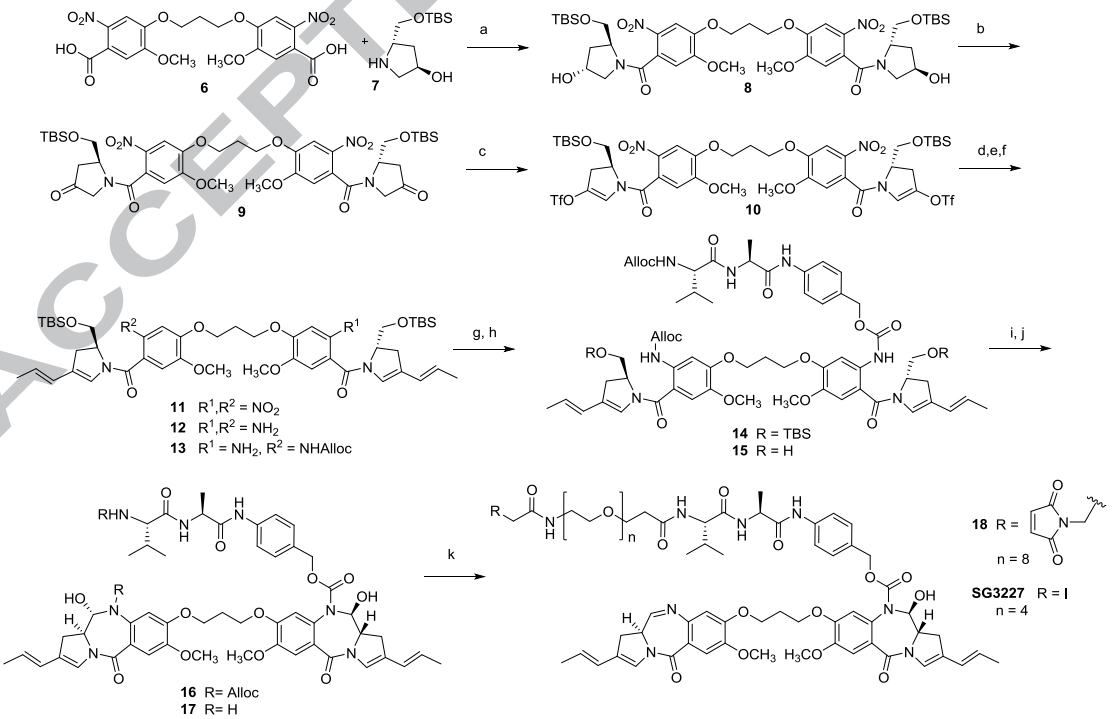
**Scheme 1** shows the synthesis of the alloc-protected linker building-block **5**.[15] L-valine was Alloc-protected, then activated as the succinimide ester, reacted with L-alanine, followed by coupling with *p*-aminobenzyl alcohol to yield **5**. The use of activating agent EEDQ avoided any undesired esterification and potential epimerisation.[16]

The dimer core **6** was converted to its acyl chloride and coupled to **7** (**Scheme 2**),[4, 5] to form bis-secondary alcohol **8**. Oxidation in the presence of TCCA and TEMPO gave the bis-ketone **9** in quantitative yield. Subsequent triflation with triflic anhydride in the presence of 2,6-lutidine selectively led to the desired C2-C3 bis-enol triflate regioisomer.[17, 18] Suzuki coupling with *trans*-propen-1-ylboronic acid, followed by reduction of the nitro groups with zinc in formic acid/methanol, resulted in bis-aniline **12**.

The symmetry of **12** was broken by Alloc protection of one of the two aniline groups in a statistical manner using allyl chloroformate. The remaining free aniline was then converted to the isocyanate with triphosgene and subsequently quenched with benzyl alcohol **5** to give carbamate **14**. The TBS protecting groups were then efficiently removed with TBAF. The resulting unsymmetrical *bis*-alcohol **15** was subjected to Swern oxidation, with concomitant ring closure of both B-rings present in the dimer to give **16**. The Alloc groups were then removed and the resulting intermediate **17** was immediately coupled to Mal-$PEG_8$-acid.




**Scheme 1.** Synthesis of the peptide linker. Reagents and conditions: (a) Allyl chloroformate, $K_2CO_3$, $H_2O$/THF 0 °C to rt; (b) *N*-hydroxysuccinimide, DCC, DCM, rt; (c) L-Ala-OH, $NaHCO_3$, THF/$H_2O$, rt, 50% (3 steps); (d) *p*-Aminobenzyl alcohol, EEDQ, THF, rt, 87%.




**Scheme 2.** Synthesis of SG3227. Reagents and conditions: (a) Oxalyl chloride, DMF, DCM, -40 °C, 96%; (b) TCCA, TEMPO, DCM, 0 °C, 100%; (c) $(CF_3SO_2)_2O$, 2,6-lutidine, DCM, -50 °C, 99%; (d) $Pd(PPh_3)_4$, propenyl boronic acid, $K_3PO_4$, dioxane, rt, 54% (3 steps); (e) Zn, 5% $HCO_2H$/MeOH, 64%; (f) Allyl chloroformate, pyridine, DCM, 52%; (g) Triphosgene, TEA, THF, Alloc-Val-Ala-PABA **5**, 67%; (h) TBAF (1M in THF), 66%; (i) DMSO, oxalyl chloride, TEA, THF, 56%; (j) $Pd(PPh_3)_4$, pyrrolidine, DCM; (k) **23**, DIC, DCM, 74% (2 steps, **SG3227** only).

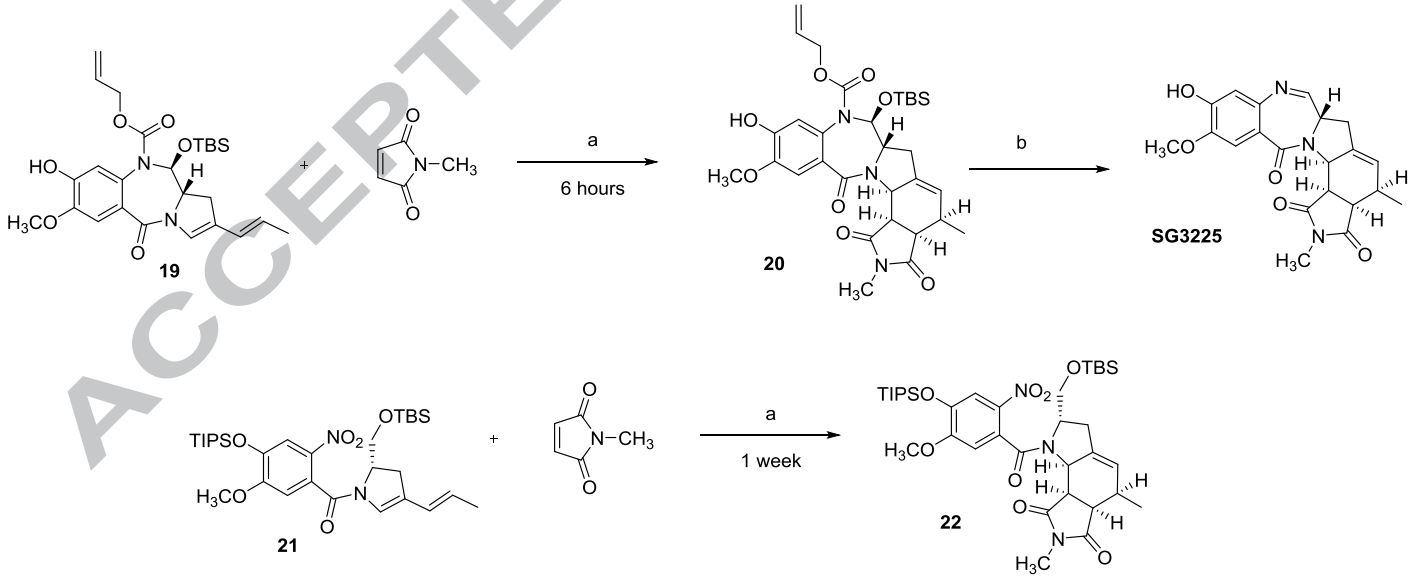
However, a significant amount of by-products were formed during the coupling reaction and pure **18** was not isolated. It was hypothesized that a Diels-Alder [4+2] cycloaddition had occurred between the maleimide conjugation moiety and the trans-propenyl/C-ring diene. Test reactions on monomeric intermediates[19] confirmed this observation (**Scheme 3**). The carbamate ring-closed intermediate **19** underwent full conversion to the Diels-Alder product **20** within 6 hours at room temperature. However, the ring-open intermediate **21** took one week to fully react. The lower reactivity of **21** is thought to be due to the electron-withdrawing A-ring nitro group remotely conjugated to the diene.

It is thought that the Diels-Alder reaction is facilitated by the electron-withdrawing carbonyl groups in the maleimide dienophile, lowering the LUMO energy level. In addition, the enamide nitrogen conjugated to the diene raises the energy level of the HOMO sufficiently high to allow the reaction to proceed at ambient temperature.[20] Compound **20** was deprotected[21] to unmask imine monomer **SG3225.** *In vitro* cytotoxicity assay of **SG3225** showed significant lack of activity in K562 cells (>1000 nM). It is hypothesised that steric clashes of the maleimide ring with the DNA minor groove walls prevent efficient binding (see proposed 3D model in the supporting information).

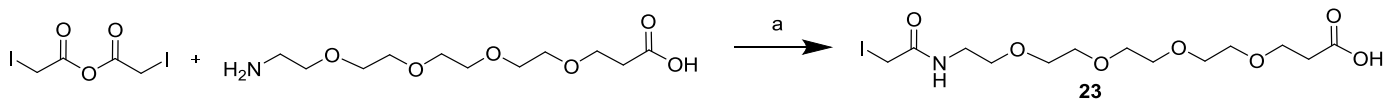
In order to circumvent the Diels-Alder side-reactions, it was decided that an iodoacetamide would be used in place of the maleimide, in a modification of the approach suggested by Alley et al.[22] which discussed the stability of maleimide and bromoacetamide linkers. Interestingly, this strategy was used independently by Puthenveetil et al.[23] when faced with a similar challenge. An acetamide linkage has the additional advantage of avoiding deconjugation *via* a retro-Michael reaction following attachment to an antibody.[24] Iodoacetic anhydride was coupled to amino-$PEG_4$-acid yielding iodoacetamide-$PEG_4$-acid **23** (**Scheme 4**), which was coupled to amine **17** using DIC, to give ADC payload **SG3227** in 74% yield.

A number of precautions were necessary to avoid undesired side-reactions of the highly labile iodoacetamide group. Firstly, the use of DIC as a coupling agent in place of EDCI.HCl. Indeed, it was found that chloride ions present in the reagent substitute with the iodoacetamide, forming the less reactive chloroacetamide. This also meant that brine (saturated sodium chloride solution) had to be excluded during work-up. Other precautions included protection from light and heat, as both can result in iodoacetamide degradation.

In order to evaluate the biological potency of **SG3227**, the payload was conjugated stochastically to trastuzumab (an anti-HER2 monoclonal antibody), and in a site-specific manner to an engineered trastuzumab variant. Stochastic ADC production involves partial reduction of the trastuzumab inter-chain disulfide bonds to release cysteine thiols, which can then react with the iodoacetamide-containing payload. The resulting trastuzumab-SG3227 conjugate had an average drug-to-antibody ratio (DAR) of 2.22. In contrast, with the engineered variant, a full reduction followed by re-oxidation of the inter-chain disulfide bonds is required to selectively free the engineered cysteine thiols for conjugation. Consequently, the drug distribution and DAR of such 'site-specific' ADCs are much more homogeneous than that for stochastic conjugates, and DAR 2 is usually the predominant species generated. In this instance, the average DAR was 1.68. Several process modifications, such as the use of propylene glycol, avoidance of chloride salts and prolonged conjugation times, were implemented to achieve conjugation (see supporting information). Whilst conjugation with iodoacetamide was found challenging, this moiety represents a viable alternative to maleimides when they are incompatible with the ADC warhead.




**Scheme 3.** Diels-Alder reactions of *N*-methyl maleimide with PBD intermediates **19** and **21**. Reagents and conditions: (a) DCM, rt, 60%, (b) $Pd(PPh_3)_4$, pyrrolidine, DCM. (Tentative structure elucidation for Diels Alder products consistent with selective endo stereochemistry, see two dimensional COSY and NOESY NMRs of **SG3225** in supporting information).



**Scheme 4.** Synthesis of the iodoacetamide PEG acid **23**. Reagents and conditions: (a) DCM, rt, 77%.

Both the stochastic Her-SG3227 (trastuzumab-SG3227) and engineered Her-SG3227 ADCs exhibited high cytotoxic potency in the HER2-expressing gastric carcinoma cell line NCI-N87 (**Figure 2**), with $IC_{50}$ values of 1.48 ng/mL and 4.29 ng/mL respectively. The same ADCs were about 3 orders of magnitude less potent in the HER2-negative cell line MDA-MB-468 with $IC_{50}$ values of 2130 ng/mL and 1220 ng/mL, respectively.

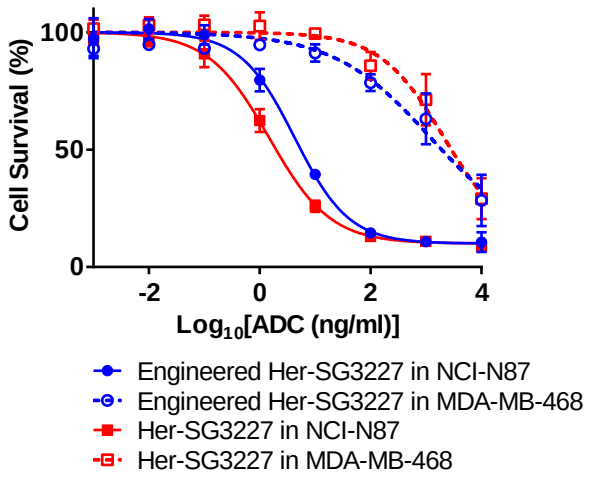



**Figure 2.** *In vitro* cytotoxicity of HER2-targeted SG3227 ADCs in NCI-N87 (HER2-positive) and MDA-MB-468 (HER2-negative) cell lines.

Since the two cell lines have similar sensitivity to the naked warhead, SG2219 (**Table 1**), the observed significant reduction in cytotoxic sensitivity of MDA-MB468 to both ADCs is likely to reflect the lack of HER2 expression in this cell line and indicate HER2-mediated binding and internalisation of these ADCs in the NCI-N87 cell line. The approximately three-fold greater potency of stochastic Her-SG3227 relative to engineered Her-SG3227 in NCI-N87 is consistent with its higher DAR.

In conclusion, a novel iodoacetamide ADC payload, SG3227, has been synthesized from a dimeric core in an expedient synthesis (1.61% overall yield for the 13 steps sequence starting from vanillin). The payload is designed to release the PBD warhead SG2219 upon cathepsin-mediated linker cleavage inside the lysosomes of target cancer cells. Two trastuzumab-ADCs were produced, by stochastic and site-specific conjugations. Both ADCs showed good selectivity and potency against a HER2-expressing cell line, demonstrating the potential of SG3227 as an effective anti-cancer ADC payload.

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## Supplementary Material

2D NMRs for SG3225. Experimental procedures for Iodoacetamide 23, SG3227, and conjugation protocols. (PDF).

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