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Formation of a Novel C11-Acetone Adduct of a Pyrrolobenzodiazepine (PBD) With Loss of Cytotoxicity

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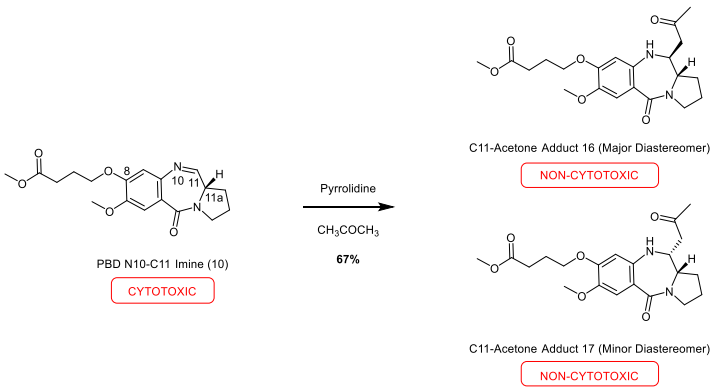

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Abstract The pyrrolidine-catalysed formation of novel diastereomeric C11-acetone adducts was observed during the chromatographic purification of pyrrolobenzodiazepine (PBD) compounds in the presence of acetone. The mechanism of this reaction was explored, and the adducts obtained fully characterised. Talirine, the cytotoxic payload element of the Antibody-Drug Conjugate (ADC) vadastuximab talirine, was also found to form this adduct under similar conditions. A cellular cytotoxicity evaluation of the modified PBD compounds confirmed their lack of cytotoxicity, consistent with loss of the DNA-interactive N10-C11 imine functionality. As well as the new chemistry reported here, given the number of PBD-based ADCs presently in the clinic, this observation may be important for the larger scale manufacture of PBD-based products.

Key words Pyrrolobenzodiazepines, PBDs, Stork Enamine, DNA-Binding, Talirine, ADC.

Originally discovered in *Streptomyces* bacteria, pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) and their derivatives are of interest as chemotherapeutic agents[1]. Examples of PBD structures include anthramycin, SJG-136 and talirine (Figure 1). One PBD-based agent, SJG-136[2], reached Phase II clinical trials in ovarian cancer and leukaemia, and a clinical trial in canine lymphoma is still underway[3]. PBD dimers are also being used as cytotoxic payloads to attach to antibodies to form Antibody-Drug Conjugates (ADCs), and examples containing the PBD dimers talirine and tesirine are presently in the clinic[4-6]. The presence of an electrophilic imine/carbinolamine at the N10-C11 position of the PBD structure is regarded as essential for their biological activity. This 'soft' imine electrophile undergoes nucleophilic attack by the nucleophilic C2-NH_2 groups of guanine bases within the DNA minor groove, forming covalent adducts (Figure 2)[7-10]. These adducts are thought to inhibit the activity of a number of DNA processing proteins including RNA polymerase, endonucleases and transcription factors, ultimately leading to cell death[11,12,13,14].

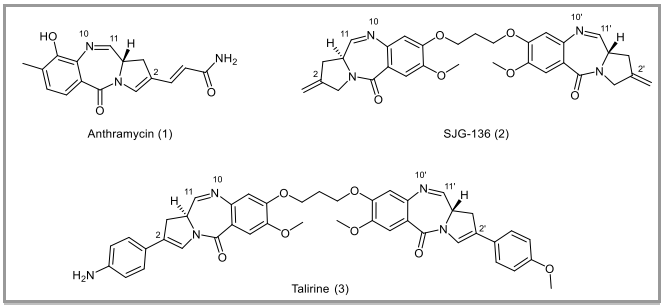


Figure 1 Structures of Anthramycin (**1**), a naturally occurring PBD monomer, and the synthetic PBD dimers SJG-136 (**2**) and talirine (**3**).

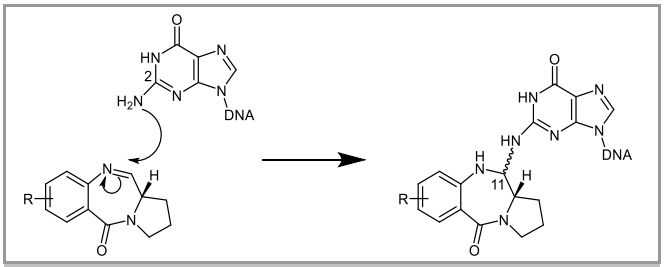


Figure 2 Mechanism of binding of a PBD to the C2-amino group of a guanine base within the DNA minor groove.

In addition to the significance of the N10-C11 imine moiety of the PBD core from a biological perspective, this inherently reactive functional group can also be manipulated synthetically in order to enhance stability or alter toxicity profiles. For example, water or methanol can add to the imine to form carbinolamine (**4**) or carbinolamine methyl ether (**5**) species, respectively. The former is thought to form in aqueous solution and has been postulated

to be the ultimate biologically active species for all PBD-based molecules (Figure 3)[15]. The imine, carbinolamine and carbinolamine methyl ether forms have been shown to be interconvertible, and some PBDs are more stable in their methyl ether forms when stored in powder form (*e.g.*, anthramycin methyl ether[16]).

Recently, several groups have sought to reversibly modify the N10-C11 imine to develop more selectively targeted PBD pro-drugs. Examples include the conjugation of enzymatically cleavable agents such as sugars[17] and nitroimidazole carbamate[18] moieties to the N10-position of the imine. Furthermore, in the construction of ADCs containing PBD-based payloads, control of the reactivity of the N10-C11 imine is often important to improve overall stability and toxicity of the ADC. Accordingly, the antibody is sometimes connected to the PBD through a carbamate linkage to the N10-position, allowing release of the electrophilic N10-C11 imine moiety only upon carbamate cleavage[19].

Irreversible modifications of the N10-C11 imine have also been reported. For example, thiol nucleophiles such as thiophenol and glutathione have also been shown to add to the C11-position of the imine functionality (e.g., **6**)[20, 21]. In the case of carbon nucleophiles, the stereoselective addition of an indole to the C11-position of a PBD core structure has been reported by Mori and co-workers[22] to produce the natural product tilivalline (**7**), a good example of the potential for modification and diversification at this position. Recently, the biosynthesis of this compound by *Klebsiella oxytoca*[16] has been implicated as a possible cause of human antibiotic-associated colitis[23] However, in comparison with traditional, imine-containing PBDs, **7** displays relatively weak cytotoxicity[24] which may derive from a non-DNA-binding mechanism. The similarly synthesised C11-cyano derivative (**8**)[25] appears to be more cytotoxic, and may be able to convert to the N10-C11 imine form under biological conditions, although this has not been demonstrated experimentally. Therefore, based on the above, the formation of potentially irreversible C-C bonds at the C11-position of the PBD structure has been avoided in the development of PBD-based therapeutic agents in an effort to maintain DNA-binding properties and cytotoxicity.

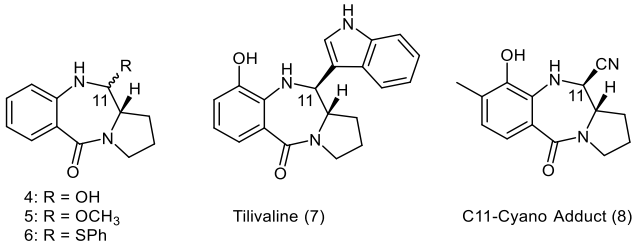
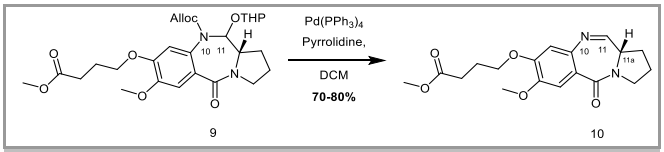


Figure 3 Examples of structures of products from the attack of various nucleophiles on the N10/C11-imine position of a PBD core, including C11-adducts formed from water (**4**), methanol (**5**) and thiophenol (**6**), the natural product tilivalline (**7**) and a C11-cyano-PBD derivative (**8**; major diastereomer shown).

Here, we report that acetone can add to the C11-position of a PBD core *via* an apparent Stork enamine reaction. This was initially observed to occur during the purification of PBD compounds by column chromatography when acetone was used as a component of the solvent system. The acetone adducts are devoid of cytotoxicity due to the loss of the DNA-interactive N10-C11 imine moiety. Therefore, the observation of this reaction has implications for the manufacture of PBD-based therapeutic agents, as well as being of chemical interest.

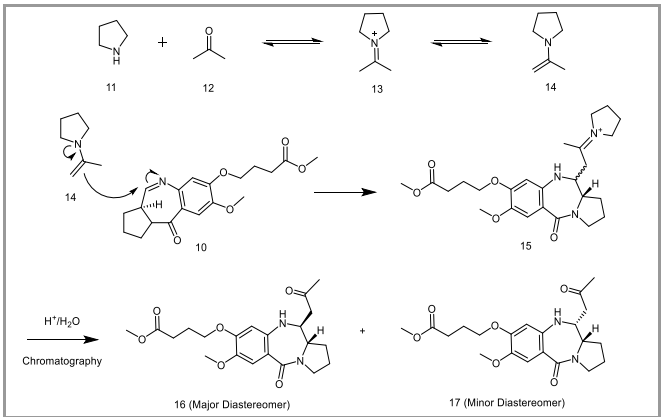
PBDs are mostly synthesized *via* a multi-step route starting from commercially available building blocks such as vanillin and pyrrolidinemethanol, and many typical routes have been reported and reviewed[15]. The N10-C11 imine moiety (in its carbinolamine form) is protected during a typical synthesis using Alloc (at the N10) and THP (at the C11) as protecting groups (*e.g.*, **9**, Scheme 1). In particular, the C11-O-THP protection avoids loss of the relatively vulnerable C11-OH group when exposed to numerous reagents during multistep synthetic routes[1, 15].

The final step of the synthesis involves removal of the N10-Alloc group *via* a palladium catalysed Tsuji-Trost reaction, using pyrrolidine as a nucleophile. This leads to a concerted loss of the THP group to yield the imine **10** with typical yields of 70-80% (Scheme 1).



Scheme 1 The final step of a commonly utilized synthetic route for PBDs. The biologically-active N10-C11 imine functionality of **10** is produced through removal of the N10-allyl carbamate and C11-OTHP protecting groups of the penultimate intermediate **9**.

Progress of the reaction is typically monitored through TLC or HPLC-MS and, in order to remove the palladium catalyst and other impurities, flash column chromatography is frequently used. In the observation reported here, a dichloromethane/acetone solvent system was used for the final purification of a PBD of type **9**. However, following purification of the concentrated reaction mixture after chromatography, an increase in mass was observed by HPLC-MS analysis, with two new compounds produced for which NMR analysis indicated a loss of the imine functionality. Further chromatography allowed the two products to be isolated and characterised as the diastereomeric C11-acetone adducts **16** and **17**[26] (Scheme 2).



Scheme 2 Proposed Stork enamine mechanism for the formation of the C11-acetone adducts **16** and **17** from PBD imine **10**.

This proposed Stork enamine reaction mechanism is feasible because the unpurified PBD imine was exposed to residual

pyrrolidine during the final deprotection step. The combination of acetone (used as a component of the eluent during chromatography) and pyrrolidine could generate a reactive enamine **14** which may act as a carbon nucleophile, attacking the PBD imine at the C11-position and forming intermediate **15**. Subsequent hydrolysis of the enamine would yield the final products **16** and **17** as a mixture of two diastereomers. One diastereomer (**16**) formed preferentially (*i.e.*, ~70% of total product), although it was possible to separate both diastereomers by further flash column chromatography, obtaining compounds of identical molecular weight as identified by UPLC-MS (see *S1, Supporting Information*). It proved difficult to conclusively confirm the diastereomeric ratio through NMR due to the complexity of the spectra. However, based upon COSY NMR and the observed multiplicity of the C11a-proton (ddd, J = 10.6, 8.9, 4.9 Hz, see *S2, Supporting Information*) in the NMR spectrum of the dominant diastereomer, it was evident that the C11(*S*)/C11a(*S*)-diastereomer forms preferentially. This would involve attack of the enamine from the most sterically-favoured direction, *trans* to the pyrrolidine ring of the PBD (see Figure 4).

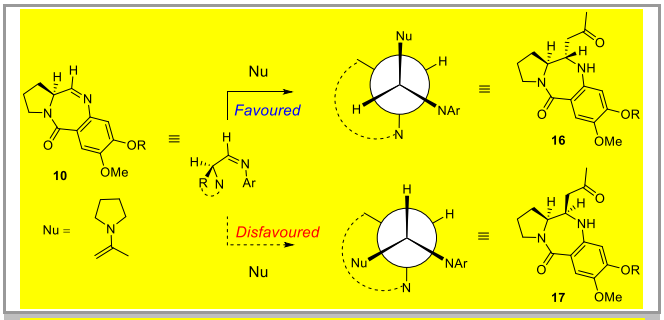


Figure 4 Rationalisation of the stereochemistry of the dominant diastereomer **16** based on the most sterically-favoured approach of the nucleophile to PBD **10** (R = $CH_2CH_2CH_2$-$COOCH_3$). The dotted line in the Newman Projection represents the pyrrolidine ring of the PBD.

To provide more evidence for the proposed mechanism (*i.e.*, Scheme 2), pyrrolidine (0.1 equiv.) was added to the purified PBD imine **10** dissolved in acetone. After 72 hours at room temperature, conversion to **16** and **17** in good yield (67% overall) was observed (see *S3, Supporting Information*). The experiment was repeated in the presence of silica gel (0.05 equiv.) to simulate the chromatographic conditions, however no significant difference in the rate or extent of formation of **16** and **17** was observed, indicating that only pyrrolidine and acetone are required for reaction to occur.

To investigate the vulnerability of more clinically-relevant PBD compounds to enamine attack, talirine (**3**, Figure 1), a PBD-based ADC payload of significant current clinical interest[4-6], was exposed to similar acetone/pyrrolidine conditions. Evidence of mono and *bis* acetone adducts were observed using LC-MS (see *S4, Supporting Information*). Given the asymmetric nature of this dimer molecule, up to eight possible adducts with different stereochemistries may have formed, and this mixture was too complex to resolve chromatographically. An example of a potentially preferred adduct is shown in Figure 5.

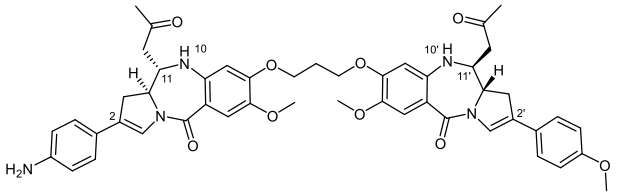


Figure 5 One of eight potential *bis*-acetone adducts of talirine (**3**) formed upon treatment with acetone/pyrrolidine. Acetone adducts may form at both N10-C11 loci in either *S* or *R* configurations.

Formation of **16** and **17** was similarly observed when piperidine was used in place of pyrrolidine, albeit in more modest yield (16%). To eliminate the possibility that this reaction proceeded through the base catalysed enolization of acetone, pyrrolidine was substituted with the hindered base 2,2,6,6-tetramethylpiperidine (TMP) of equal basicity. No formation of **16** and **17** was observed in the presence of this base, suggesting that an enamine intermediate is required for adduct formation. Further reactions were attempted using more complex ketone structures as well as diethylamine and morpholine as base (see Table 1). However, these reactions were unsuccessful, suggesting that acetone and pyrrolidine may be the optimal reagents. The lack of reactivity of the more complex ketones could be due to their increased steric bulk and/or lower nucleophilicity of their corresponding bases. For example, it has been reported that morpholine-containing enamines consistently show poorer nucleophilicity compared to their pyrrolidine-containing counterparts [27].

The major isolated diastereomer **16** and a 50:50 mixture of both diastereomers (**16**/**17**) were evaluated for cytotoxicity in a breast tumour cell line (MDA-MB-231) using the MTT assay (48 hours incubation). The unmodified imine-containing PBD **10** was also evaluated as a control (Table 2; and *S5, Supporting Information*). PBD **10** was cytotoxic in the low micromolar range, consistent with DNA-binding due to the presence of the N10-C11 imine. In contrast, the C11-modified PBD **16**, and the mixture of **16**/**17**, were non-cytotoxic at concentrations of up to 100 μM (the maximum concentration tested), consistent with the absence of the electrophilic N10-C11 imine moiety and an inability to react covalently with DNA. The lack of cytotoxicity for both **16** and **17** provides further evidence for the importance of the N10-C11 imine for the biological activity of PBD molecules.

Table 1 Investigation of the use of various ketones and different bases for the formation of **16** and **17**.

Ketone	Base	Adduct Formation	Yield (%)
Acetone	Pyrrolidine	Yes	67
Acetone	Piperidine	Yes	16
Acetone	TMP	No	N/A
Acetone	Diethylamine	No	N/A
Acetone	Morpholine	No	N/A
Butan-2-one	Pyrrolidine	No	N/A
Pentan-3-one	Pyrrolidine	No	N/A
Acetophenone	Pyrrolidine	No	N/A

Table 2 Cytotoxicity of the unmodified PBD (**10**) and the C11-acetone adducts (**16** and **17**) in the MDA-MB-231 breast tumour cell line.

Compound	IC_{50} (μM)[a]
10	6.67 (±1.08)
16	>100
16 & **17** (1:1)	>100

[a]Cytotoxicity toward the MDA-MB-231 breast tumour cell line measured using an MTT assay after 48 hours incubation.

In summary, the formation of C11-acetone adducts of PBD molecules is reported, most likely mediated through a Stork enamine-type mechanism. Interest in PBDs as therapeutic agents has increased in recent years due to the development of highly potent PBD-based transcription factor inhibitors[14] and Antibody-Drug Conjugates (ADCs) containing PBD-based payloads[4, 5, 28]. Therefore, the observation reported here that a biologically inactive C11-acetone adduct can form during chromatographic purification has potential implications for the manufacture of PBD-based therapeutic agents. Awareness of the possibility of this reaction may allow its prevention by using alternative amines to pyrrolidine for protecting group removal during the final synthetic steps. From a medicinal chemistry perspective, development of novel non-DNA-interactive PBD-based libraries could also be produced through this reaction for other purposes. Finally, the cytotoxicity evaluation carried out on compounds **16** and **17** provides further evidence for the importance of the N10-C11 imine functionality for the biological activity of PBDs.

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Supporting Information

YES

Primary Data

NO

References and Notes

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26. **Experimental procedure for the synthesis of 16 and 17**. Pyrrolidine (2.3 µl, 0.1 equiv.) was added to a solution of **10** (100 mg, 1 equiv.) in acetone (1 ml), and the solution allowed to stir at room temperature for 72 hours. The excess acetone and pyrrolidine were removed under reduced pressure, and the resulting oil purified using flash column chromatography (20:80 acetone/dichloromethane to resolve the two diastereomers **16** and **17** (both yellow oils) in ~67% overall yield.

Methyl 4-(((11*S*,11a*S*)-7-methoxy-5-oxo-11-(2-oxopropyl)-2,3,5,10,11,11a-hexahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy)butanoate (16; Major Diastereomer): ^{1}HNMR (400 MHz, $CDCl_3$): 7.29 (s, 1H), 6.19 (s, 1H), 4.02 (t, 2H, J= 6.2 Hz), 3.84 (s, 3H), 3.79-3.66 (m, 2H), 3.70 (s, 3H), 3.69-3.65 (m, 1H), 3.44 (ddd, 1H, J= 10.6, 8.9, 4.9 Hz), 2.57-2.52 (m, 4H), 2.24 (s, 3H) 2.19-2.07 (m, 2H), 2.05-1.92 (m, 3H), 1.75-1.64 (m, 1H). ^{13}CNMR (100 MHz, $CDCl_3$): 208.3, 173.6, 168.4, 151.2, 144.6, 138.3, 119.4, 113.1, 106.7, 67.8, 61.7, 60.0, 56.2, 53.7, 51.7, 46.9, 30.8, 30.3, 30.0. 24.7, 23.2.

Methyl 4-(((11*R*,11a*S*)-7-methoxy-5-oxo-11-(2-oxopropyl)-2,3,5,10,11,11a-hexahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy)butanoate (17, Minor Diastereomer): ^{1}HNMR (400 MHz, $CDCl_3$): 7.45 (s, 1H), 5.97 (s, 1H), 4.05-3.99 (m, 2H), 3.95 (t, 2H, J= 6.3 Hz), 3.92-3.85 (m, 1H), 3.77 (s, 3H), 3.64 (s, 3H), 3.61-3.52 (m, 1H), 2.79 (dd, 1H, J= 18.0, 10.4 Hz), 2.51-2.44 (m, 3H), 2.29-2.18 (dt, 1H, J= 13.2, 7.2 Hz) 2.15-2.04 (m, 5H), 1.93-1.80 (m, 2H), 1.72-1.61 (m, 1H).

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