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DNA interstrand crosslinking agents: Synthesis, DNA interactions, and cytotoxicity of dimeric achiral seco-amino-CBI and conjugates of achiral seco-amino-CBI with pyrrolobenzodiazepine (PBD)

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Abstract—The design and synthesis of three novel bisalkylating agents derived from the achiral seco-duocarmycin or CC-1065 analogs and pyrrolobenzodiazepines (PBDs) are described: achiral seco-CBI (cyclopropanebenz[*e*]indoline)-PBD **11**, achiral seco-CI-PBD **12**, and achiral seco-CBI dimer **13**. Compounds **11** and **12** demonstrated enhanced cytotoxicity over the monomer counterparts against the growth of P815 murine mastocytoma cells in culture. Conjugate **11** was found to covalently react with adenine-N3 positions within the minor groove at AT-rich sequences and to produce DNA interstrand crosslinks. Both compounds were found to induce apoptosis in P815 cells. Due to its poor water solubility, dimer **13** did not give any appreciable DNA binding or cytotoxicity.

Compounds that exhibit selectivity for binding to DNA sequences and covalently react with specific nucleobases continue to be important targets for the design and synthesis of novel medicinal agents.[1] Figure 1 gives examples of DNA minor groove binding and AT sequence-specific compounds that exhibit potent anticancer activities. It includes analogs of (+)-CC-1065 (**1**),[2] duocarmycin SA (**2**),[3] seco-CBI-TMI (**3**),[4] and the recently described achiral congeners from our laboratories, **4**[5] and **5**.[6] The latter class of compounds have been found to alkylate adenine-N3 and promote cell death through apoptosis. The achiral seco-amino-CBI-TMI **4** has low toxicity and strong in vivo anticancer activity against mouse B16 melanoma.[5]

Another class of DNA minor groove binding agents that are being extensively examined contain a pyrrolobenzodiazepine (PBD) moiety, and they are analogous to the anthramycins, for example, compound **6**.[7] PBD analogs display specificity of binding to 5′-Pu**G**Pu-3′ sequences, and they covalently react to form stable adducts between the imine group on the PBD with G-2-NH_2.[8]

In an effort aimed at enhancing the biological properties of CC-1065 (**1**) and PBD analogs, a number of dimers and conjugates between these two classes of compounds (Fig. 1) were designed and biologically tested. Bizelesin (**7**) was found to effectively produce interstrand DNA crosslinks at AT-rich sequences and it was endowed with potent cytotoxicity and anticancer activity. It has undergone clinical evaluation for cancer treatment.[9] Dimer compounds of PBD analogs were also developed, such as, DSB-120 (**8**)[10] and SJG-136 (**9**).[11] These compounds span six base pairs in the minor groove and produce DNA interstrand crosslinks between guanine-N2 positions at 5′-GATC-3′ sequences.[11] The latter

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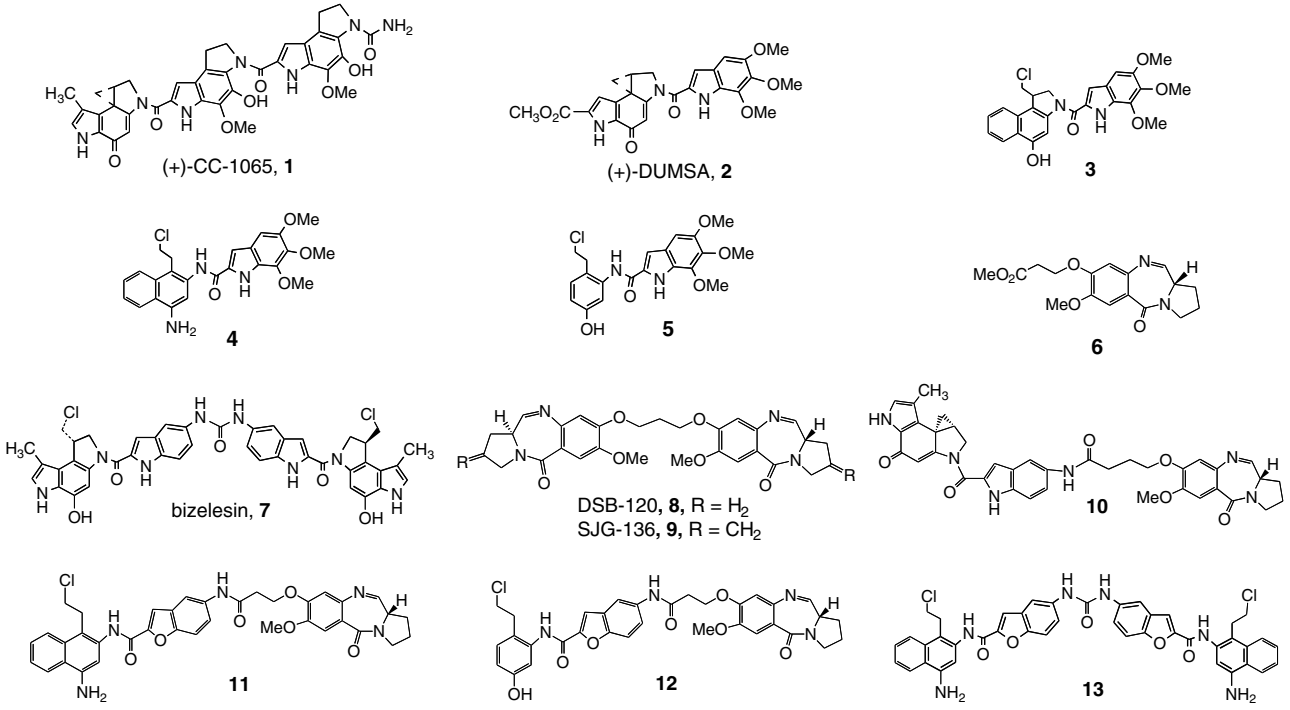


Figure 1. Structures of (+)-CC-1065 **1**, (+)-duocarmycin SA **2**, seco-CBI-TMI **3**, achral seco-amino-CBI-TMI **4**, achiral seco-CI-TMI **5**, PBD monomer **6**, bizelesin **7**, DSB-128 **8**, SJG-136 **9**, CPI-PBD **10**, achiral seco-amino-CBI-PBD **11**, achiral seco-CI-PBD **12**, Dimer of achiral seco-amino CBI **13**.

compound **9** is presently undergoing phase I clinical trial for cancer treatment in Europe and the USA.[12]

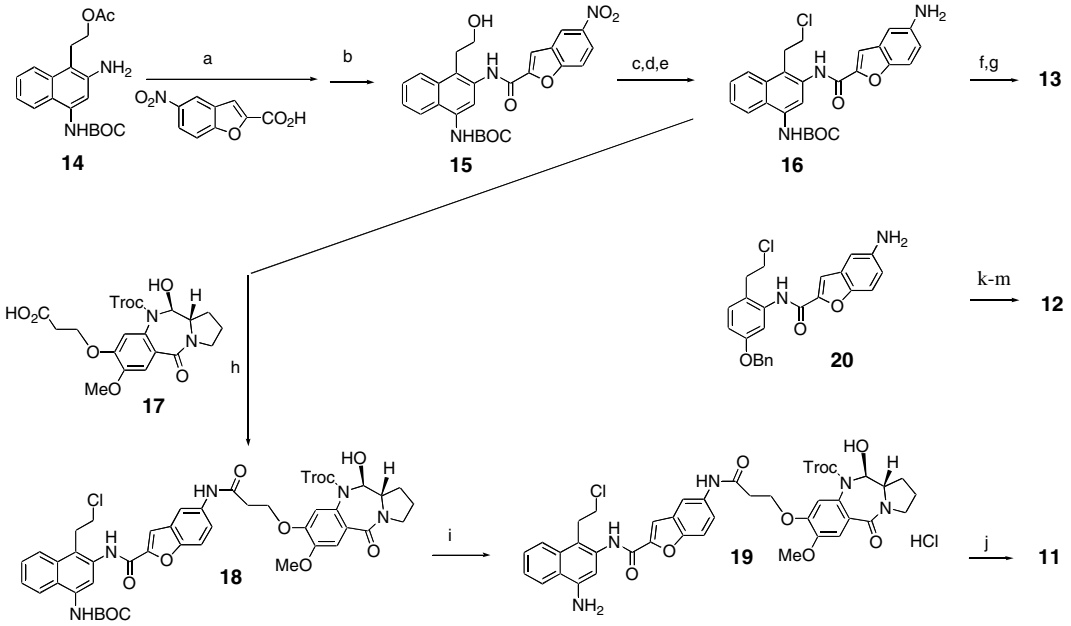
In addition to dimeric compounds, conjugates of these two classes of compounds have also been reported.[13] For example, the CPI (cyclopropylpyrolo[*e*]indolone)-PBD conjugate **10** was found to exhibit potent cytotoxicity against human cancer cells (such as breast MCF-7, colon SW480, and lung A549) grown in culture, and it also showed selectivity for producing interstrand crosslinks at a 5′-T[**A**]AATT**G**-3′ site (alkylation at the underlined and bold faced A and G residues).[13a]

With this background and the recent discovery that seco-achiral analogs of CC-1065 (**1**) are potent DNA interactive anticancer agents, we began to focus attention on preparing and testing the biological properties of hybrid PBD and achiral seco-amino-CBI conjugates. Accordingly, compounds **11** and **12**, which are conjugates of the PBD analog **6** and an achiral seco-amino-CBI analog of **4**, as well as a conjugate of compound **6** and an achiral seco-CI analog of **5**, respectively, were designed and prepared. These compounds offer a unique advantage over the published conjugates because they contain a single stereocenter that can be readily introduced early in the synthetic scheme through the use of 2(S)-(+)-pyrrolidinyl methanol as a synthon.[7] A dimeric compound of the achiral seco-CBI compound **13** was also prepared, and it is analogous to the bizelesin design. This communication describes the synthesis and the cytotoxicity of compounds **11**–**13**, along with an investigation of the sequence specificity, DNA alkylation, and interstrand crosslinking abilities of the compounds.

The target compounds were prepared following a synthetic strategy given in Scheme 1. Reaction of the aromatic amine functional group of compound **14** with 5-nitrobenzofuran-2-carboxylic acid proceeded readily using an EDCI and HOBt coupling method. The desired compound was isolated in 54% yield. The acetyl moiety was removed by treatment with a methanolic solution of potassium carbonate to yield compound **15** in 36% yield. Compound **15** was transformed into chloride **16** in three steps (mesylation of the primary alcohol, displacement of the mesylate group with LiCl, followed by catalytic hydrogenation to reduce the nitro group to an amine). The overall yield for converting alcohol **15** to chloride **16** was achieved at 5.4%.

The amino group of compound **16** was reacted with a PBD-carboxylic acid **17**[7] in the presence of EDCI, giving the desired product **18** in 62% yield. Final removal of the BOC-protecting groups with concd HCl, followed by elimination of the Troc protecting group using a Pb–Cd reaction, produced compound **19**, and target **11**, in 97% and 84% yields, respectively. A similar strategy was employed in the synthesis of target molecule **12**, except amine **20** was used.

Synthesis of the symmetrical dimer **13** was achieved in 30% yield by reaction of amine **16** with triphosgene. Removal of the BOC group of the intermediate yielded the desired compound **13** (68%). All compounds gave acceptable spectroscopic data (500 MHz ^{1}H NMR, FTIR, high resolution mass spectrometry, and melting points for solid samples).



Scheme 1. Reagents and conditions: (a) EDCI, HOBt, pyridine, 54%; (b) K_2CO_3, MeOH, 36%; (c) mesyl chloride, Et_3N, THF; (d) LiCl, DMF; (e) H_2, 10% Pd–C, THF, 5.4% from **16**; (f) triphosgene, DIPEA, CH_2Cl_2, THF, 30%; (g) concd HCl, THF, 68%; (h) EDCI, HOBt, DMF, 62%; (i) 3M HCl, EtOAc, 97%; (j) ammonium acetate, 10% Pb–Cd, THF, 84%; (k) **17**, EDCI, HOBt, DMF, 92%; (l) 10% Pd–C, THF, 50%; (m) ammonium acetate, 10% Pb–Cd, THF, 31%.

An MTT colorimetric study was used to determine the cytotoxic properties of compounds **11**–**13**.[4,5] P815 murine mastocytoma cells were continuously exposed to the compounds for 3 days. Viability was determined by the ability of the cells to convert MTT to a purple formazan dye. The results are shown in Table 1, and they are listed according to their ability to inhibit cell growth by 50% (IC_{50}) relative to an untreated control. It is worthy to note that for this class of compounds, the racemic compound **3**, which contained a stereocenter, was most cytotoxic, despite compounds **11**–**13** being theoretically capable of producing cytotoxic DNA interstrand crosslinks. However, the non-symmetrical conjugate **11** was more cytotoxic than its monomeric analogs: achiral-seco-amino-CBI **4** and PBD **6**, indicating that interstrand crosslink formation could increase the cytotoxic potency of these DNA reactive agents. The data, however, showed that dimer **13** was least active among the new compounds, and that could be due to the poor solubility of **13** in water and cell culture media.

Further cell-based studies were conducted to ascertain the mechanism by which compound **11** derived its cytotoxic activity. Cultured P815 cells treated at the IC_{50} dose for 18 h were fixed and treated with propidium iodine according to an earlier published procedure.[5] The cells were sorted on a flow cytometer, and the population of sub-G_0 cells was determined: 21% for treated versus 3% for an untreated control (data not shown). These results are consistent with earlier studies indicating that achiral CBI compounds are capable of inducing cancer cells to undergo apoptosis.[5,14]

Table 1. In vitro cytotoxicity studies in P815 cells for compounds **3**–**6** and **11**–**13**, following a continuous exposure for 3 days

Compound	Cytotoxicity IC_{50} (μM)
3	0.0086
4	0.068
5	5.6
6	55
11	0.026
12	0.56
13	42

An agarose DNA thermally induced cleavage study was conducted to determine the ability of compound **11** to induce DNA strand breaks. Supercoiled pBR322 DNA was treated with compound **11** at 6.6 μM for 40 h at 37 °C. DNA strand breaks were observed through the percent conversion of supercoiled DNA (Form I) to open circular DNA (Form II). In this case, the experiment gave 16 ± 2% Form II DNA, compared to the control DNA (0%), and free PBD monomer **6** (4%).

Since compound **11** was found to be more cytotoxic than its parent monomeric units (**4** and **6**), the interstrand crosslinking ability of compound **11** with a 5′-^{32}P-labeled linear pBR322 DNA was examined. As depicted in Figure 2, compound **9** effectively produced interstrand crosslinks, evidenced by formation of the DS or double stranded band in a sample of denatured DNA (SS).[15] This result is consistent with earlier reports, and the covalent adducts were thermally stable. For compound **11**, a crosslink DNA band appeared at 10 μM. It is worth noting that this gel crosslink study was performed using alkali denaturation because when the treated DNA sample was denatured by heating to 95 °C for 2 min no crosslink band was observed consistent with the presence of thermally labile adenine-N3 covalent adducts (data not shown).

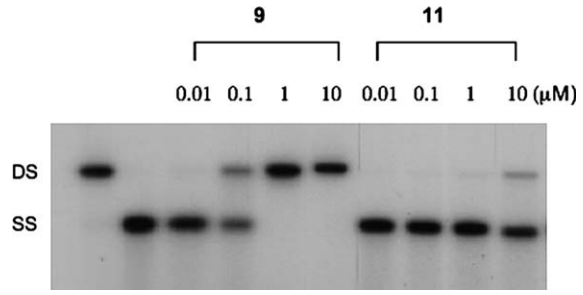


Figure 2. Agarose gel DNA crosslink studies. SS is single stranded DNAs, DS double stranded DNA. The 5′-^{32}P labeled DNA was treated with compounds for 24 h, and the sample denatured with alkali before it was analyzed by neutral agarose gel electrophoresis.

The covalent sequence specificity of duocarmycin analogs can be measured using a thermally induced cleavage-DNA sequencing method.[5,6] A singly 5′-^{32}P-radiolabeled linear fragment of pBR322 plasmid DNA was used as a template for these studies. A gel showing the sites of thermally induced cleavage for compounds **1**, **6**, and **11** is shown in Figure 3.

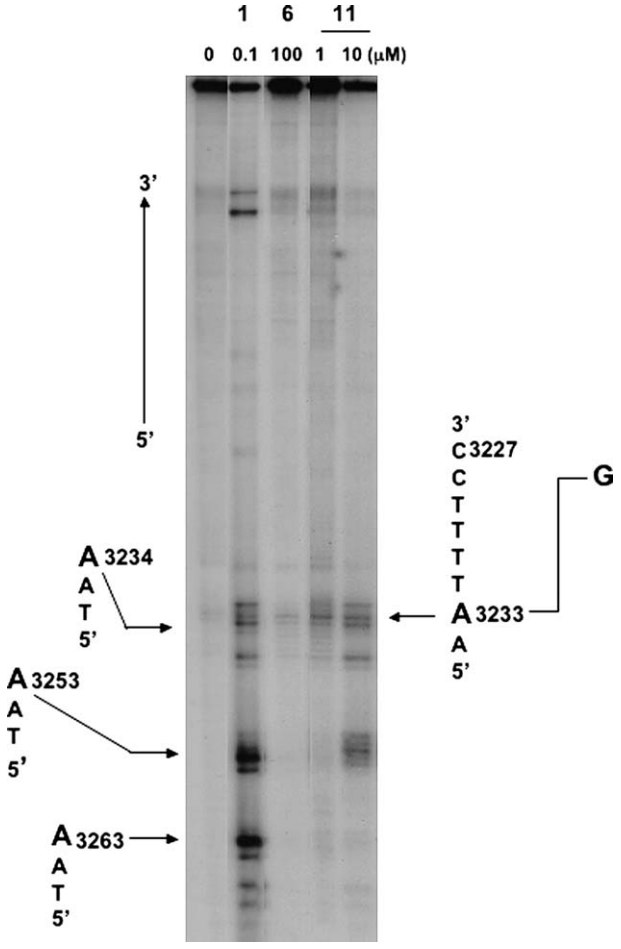


Figure 3. Thermally induced cleavage assay. Singly 5′-^{32}P end-labeled linearized plasmid pBR322 DNA was treated with compounds for 24 h, and the samples heated at 90 °C for 30 min prior to analysis on a denaturing polyacrylamide gel.

The results demonstrated that the DNA alone did not have any strand breaks. Consistent with literature reports,[2] at 0.1 μM, compound **1** (CC1065) demonstrated selectivity for 5′-TA**A** sequences, with alkylation at the underlined adenine-N3 position and consistent with the known sequence preference of compound **4**.[5] PBD ester **6** did not show evidence of DNA alkylation of this fragment, even at 100 μM, consistent with adducts at guanine-N2 not resulting in thermally induced strand breaks. In contrast, at a low concentration of 1 μM, molecule **11** exhibited a clear and selective alkylation band at A(3233). This is an interesting site because it lines up with the 5′-AA**A**(3233)TTTTC[**G**]C-3′, which is potentially an interstrand crosslink site due to the proximity between the alkylated adenine residue and a 5′-PuGPu site in the complementary strand.[13] However, when the concentration of compound **11** was increased to 10 μM, additional alkylation sites common to CC-1065 **1** became evident.

The results given in this communication demonstrate that conjugates of achiral analogs of CC-1065 or duocarmycins with PBDs are biologically active and are capable of producing interstrand crosslinks. Agent **11** has the potential of being developed as an anticancer agent. Further biological studies are underway and the results will be reported in due course.

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