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Analogues of DNA minor groove cross-linking agents incorporating aminoCBI, an amino derivative of the duocarmycins: synthesis, cytotoxicity, and potential as payloads for antibody-drug conjugates

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Abbreviations: ADC, antibody-drug conjugate; BINAP, 2,2'-bis(diphenylphosphino)-1,1'-binaphthalene; CBI, 1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one; EDCI, *N*-ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide hydrochloride; MMAE, monomethyl auristatin E; PBD, pyrrolobenzodiazepine; Pgp, P-glycoprotein.

Keywords: DNA cross-linking agent; AminoCBI; Duocarmycin; Pyrrolobenzodiazepine; Antibody-drug conjugate

Abstract

A Pd-catalysed amination method is used to convert *seco*-CBI, a synthetic analogue of the alkylating subunit of the duocarmycin natural products, from the phenol to amino form. This allows efficient enantioselective access to the more potent *S* enantiomer of aminoCBI and its incorporation into analogues of DNA minor groove cross-linking agents. Evaluation in a panel of nine human tumour cell lines shows that the bifunctional agents containing aminoCBI are generally less cytotoxic than their phenolCBI analogues and more susceptible to P-glycoprotein-mediated resistance. However, all bifunctional agents are potent cytotoxins, some in the sub-pM IC_{50} range, with *in vitro* properties that compare favourably with established microtubule-targeted ADC payloads.

1. Introduction

Antibody-drug conjugates (ADCs) are an emerging class of antitumour agents in which cytotoxic small molecules are covalently linked to an antibody that recognises a tumour-associated antigen.[1-3] The recent approvals of Adcetris® (brentuximab vedotin) and Kadcyla® (ado-trastuzumab emtansine) have stimulated research in this area, and there are now about 50 ADCs in clinical development.[3] At least 35 of these candidates employ microtubule-targeted compounds as the cytotoxic species, typically from the auristatin or maytansine classes (see for example monomethyl auristatin E (MMAE) **1** and the maytansine DM1 **2**, Figure 1).

More recently there has been interest in exploring payloads with a different mode of action. DNA minor groove alkylating agents are of particular interest because they tend to be very cytotoxic, active at all phases of the cell cycle, and insensitive to drug resistance mechanisms. Pyrrolobenzodiazepines (PBDs, see substructure in Figure 1) are derived from natural products that alkylate in the minor groove at the C2-NH_2 group of guanine. As simple monomeric agents they are not generally considered cytotoxic enough to be effective ADC payloads, but linking two PBDs together creates

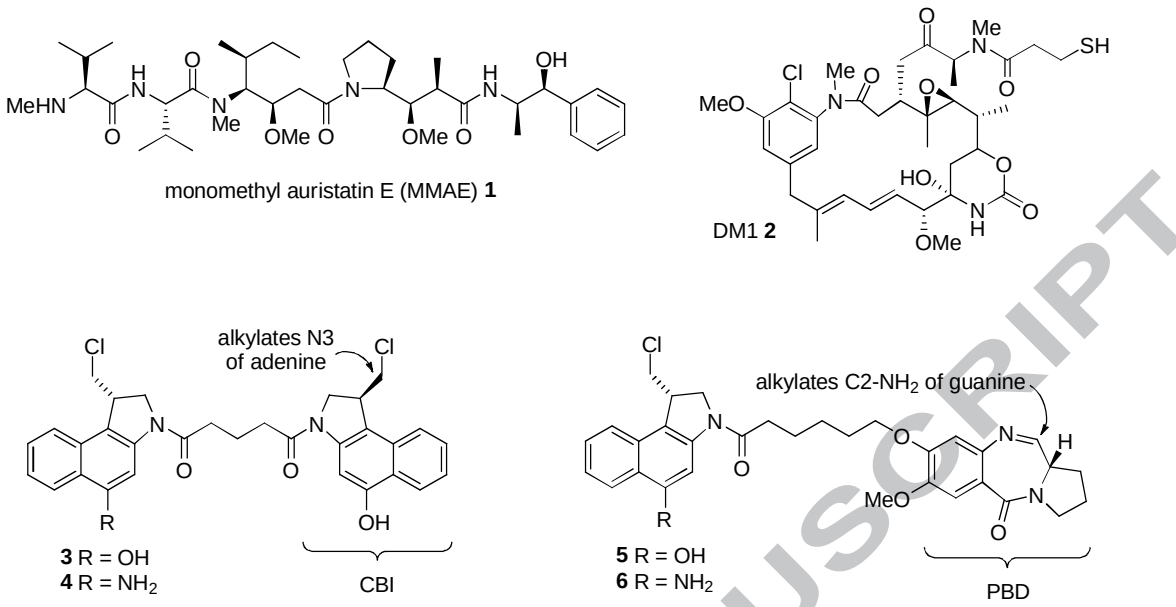


Figure 1. Structures of some known and proposed ADC payloads. For simplicity CBI is here applied to the *seco* or open-chain form of the alkylating subunit, and the term phenolCBI used to distinguish R = OH from aminoCBI (R = NH_2).

cross-linking agents which can exhibit IC_{50}s in the sub-nM range.[4] ADCs with PBD dimer payloads have shown promising preclinical activity[5,6] and six such conjugates recently entered clinical trial. Duocarmycins are another class of natural products that alkylate DNA in the minor groove, targeting the N3 position of adenine. Even monoalkylating agents of this type e.g. those employing the synthetic CBI alkylating subunit[7] (substructure in Figure 1) can be highly cytotoxic compounds, and these have also shown promise as ADC payloads.[8,9]

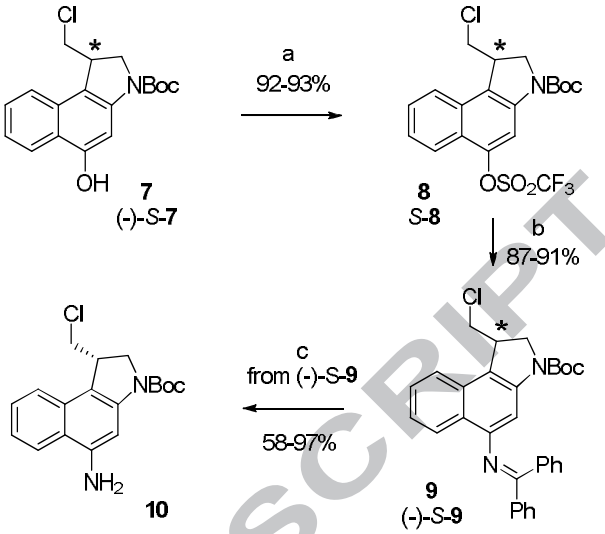
As for PBDs, it has been known for some time that linking duocarmycin analogues together to make dimers can further enhance their already marked cytotoxicity.[10] We have investigated bis-CBI dimers[11] and also CBI-PBD heterodimers,[12] establishing that cytotoxicity is clearly a function of DNA cross-linking ability, and that this can be optimised with appropriate linker chain length and enantiomeric form. Other examples of CBI-PBD heterodimers have also been reported.[13,14] Despite the exceptional potency of particular exemplars (e.g. **3**[11] and **5**[12]) there have been few reports on these agents as ADC payloads.[15,16] We have also described aminoCBIs, a structural variant of the CBI alkylating subunit where the crucial phenol is replaced with an amino substituent. AminoCBIs are the active form of nitroCBI hypoxia-activated prodrugs which have demonstrated selective activity against hypoxic cells in human tumour xenografts.[17] AminoCBIs and phenolCBIs share the same mechanism of action and have remarkably similar properties, including equivalent potency as monoalkylating agents, even though they exhibit quite different stability under physiological conditions.[18] The much higher reactivity of aminoCBIs has potential advantages in an ADC setting in that rapid hydrolysis might act to limit the toxicity of any systemically released agent, as has been proposed for a particular phenolCBI payload which undergoes unusually rapid hydrolysis in plasma.[8] AminoCBIs also lend themselves to alternative chemical methods for linking to an antibody that may not be accessible or sufficiently stable with their phenol analogues.

In this study we report the synthesis of two new bifunctional agents **4** and **6** that incorporate aminoCBI. We compare their cytotoxicity across an extensive cell line panel with that of their phenolCBI analogues and with microtubule-targeted agents, with a view to the utility of **3**-**6** as novel ADC payloads.

2. Results and discussion

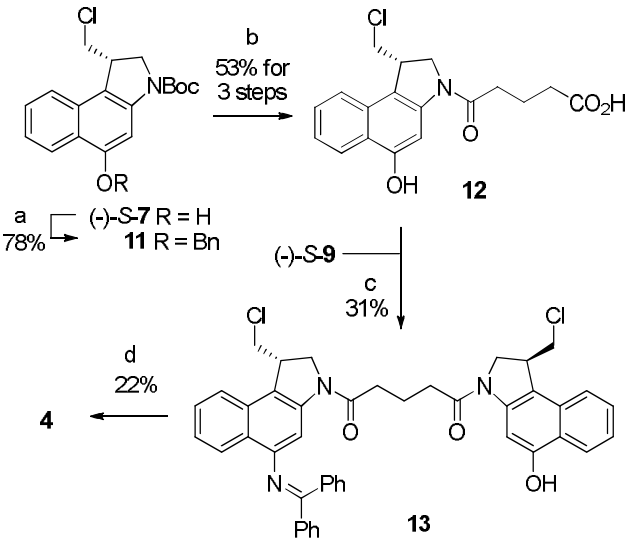
2.1. Synthesis

For the synthesis of **4** and **6** we needed access to the *S* enantiomer of aminoCBI **10** (Scheme 1) which had not been reported previously. We chose to explore a Pd-catalysed amination route from the corresponding *S*-phenolCBI (-)-*S*-**7** for which an efficient enantioselective synthesis was available.[19] The method was first tested with racemic **7**[20,21] which was converted to the triflate **8** and reacted with benzophenone imine in the presence of catalytic $Pd(OAc)_2$ and BINAP ligand. A good yield of the desired **9** was obtained, although using somewhat higher catalyst loadings and a longer reaction time than reported.[22] Similarly high yields for both steps were obtained on a multigram scale starting with the corresponding *S*-enantiomer (-)-*S*-**7**. Deprotection of the imine via transamination with hydroxylamine[22] was slow at room temperature and messy on heating, although aminoCBI **10** was the major product as determined by TLC comparison with authentic racemic material.[23] Acid-mediated deprotection was explored using both aqueous HOAc and methanolic HCl, with the latter providing a surprisingly selective reaction in the presence of the Boc protecting group and providing **10** in almost quantitative yield.



Scheme 1. Conversion of phenolCBI to aminoCBI. Compounds **7**, **8**, and **9** are racemic at the optically active site marked with an asterisk. Reagents and conditions: (a) Tf_2O, Et_3N, CH_2Cl_2, 0 °C; (b) benzophenone imine, $Pd(OAc)_2$, BINAP, THF, 65 °C; (c) HOAc, aqueous THF (58%) or HCl, MeOH, CH_2Cl_2 (97%).

For the preparation of the bifunctional agents **4** and **6** it was found advantageous to leave the imine in place as a protecting group until the last stage of the synthesis. This required orthogonal deprotection of the Boc group of (-)-*S*-**9** in the presence of the imine which was achieved under acidic conditions in non-protic solvents, as shown in Schemes 2 and 3. Firstly however, for the aminoCBI-phenolCBI dimer **4**, a synthesis of acid **12** was required (Scheme 2). This was accomplished by temporary benzyl protection of phenol (-)-*S*-**7**, removal of the Boc protecting group and reaction with glutaric anhydride, followed by hydrogenolysis to cleave the benzyl ether. Imine (-)-*S*-**9** was treated with HCl in dioxane and the crude product used directly in an EDCI (*N*-ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide hydrochloride)-mediated coupling with **12** to give dimer **13** in moderate yield. Final deprotection of the imine using aqueous HOAc, followed by HPLC purification, gave the desired dimer **4**.

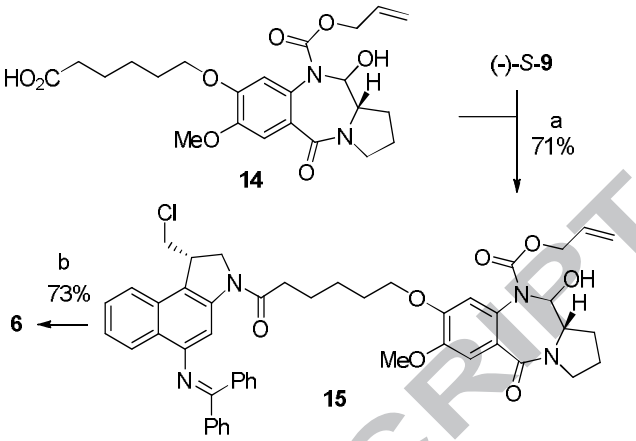


Scheme 2. Synthesis of aminoCBI-phenolCBI dimer **4**. Reagents and conditions: (a) BnBr, K_2CO_3, KI, DMF; (b) HCl then glutaric anhydride, DMAP, pyridine then Pd/C, NH_4HCO_2; (c) (-)-*S*-**9**, HCl then **12**, EDCI; (d) HOAc then HPLC purification.

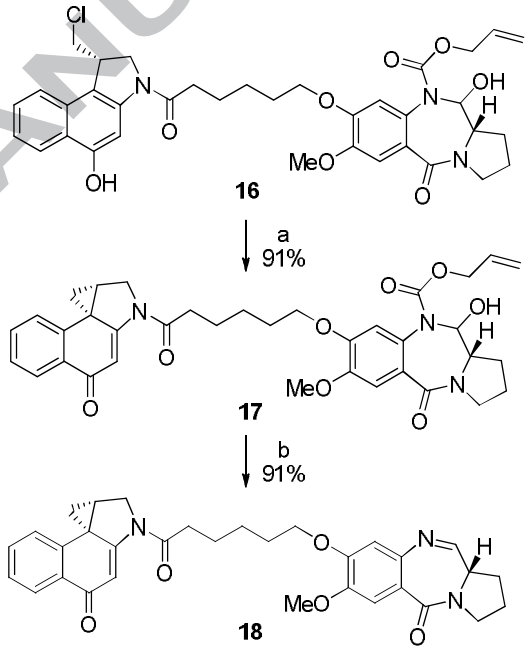
The preparation of the aminoCBI-PBD heterodimer **6** used the previously reported PBD acid **14**[12] as a starting material (Scheme 3). In this case selective Boc deprotection of (-

)-*S*-**9** was performed using TFA rather than HCl which resulted in a much higher-yielding and cleaner subsequent amide formation. The imine and Alloc protecting groups of **15** were sequentially removed using HCl and a brief exposure to $Pd(PPh_3)_4$ and pyrrolidine respectively, and the product **6** was isolated after column chromatography.

The spirocyclic analogue of **5** was also prepared (**18** in Scheme 4). Starting from the known Alloc-protected phenolCBI-PBD dimer **16**[12] spirocyclisation was induced by exposure to K_2CO_3 in DMA, followed by removal of the Alloc protecting group as described above. The phenolCBI-containing dimers **3** and **5** were prepared as previously reported. Some other structural variants of the exceptionally potent **3** were also considered, namely extending the polymethylene linker from three to five carbons, or substituting the central methylene with an NH group. The synthesis of these dimers in racemic form is described in the Supporting Information.



Scheme 3. Synthesis of aminoCBI-PBD dimer **6**. Reagents and conditions: (a) (-)-*S*-**9**, TFA, CH_2Cl_2 then Na_2CO_3 then **14**, EDCI, TsOH, DMA; (b) HCl, dioxane then $Pd(PPh_3)_4$, pyrrolidine.



Scheme 4. Synthesis of cyclopropyl form of phenolCBI-PBD dimer. Reagents and conditions: (a) K_2CO_3, DMA; (b) $Pd(PPh_3)_4$, pyrrolidine, CH_2Cl_2.

2.2. In vitro cytotoxicity

Cytotoxicity was determined by inhibition of proliferation after 96 h exposure to the compounds in a panel of nine human tumour cell lines of diverse origin (Table 1). The established payloads MMAE (**1**) and DM1 (examined as the S-methylated analogue **2**-Me) exhibited IC_{50}s in sub-nM range, as previously reported,[24] but for several cell lines an IC_{50} could not be established for either compound because proliferation inhibition reached a plateau at high compound concentrations. This behaviour, which could be a consequence of cell cycle arrest under the assay conditions used, is illustrated for MMAE in Figure 2a. Both microtubule-targeted payloads were also considerably less active in MES-SA/Dx5, a resistant sub-line of MES-SA that overexpresses P-glycoprotein (Pgp).[25]

We previously reported that the phenolCBI dimer **3** exhibits IC_{50}s in the 0.1 to 2 pM range against a panel of six human tumour cell lines.[11] This remarkable cytotoxicity was confirmed in the present study with **3** showing IC_{50}s generally in the 0.5 to 2 pM range, including in MES-SA/Dx5. Even in the most 'resistant' cell line, HCC1937, the IC_{50} of **3** was 29 pM (16 pg/mL). The corresponding compound **4** in which one end of the dimer was changed from phenolCBI to aminoCBI showed a loss in potency of on average 24-fold (range 2 to >55-fold), and an increased sensitivity to overexpression of Pgp, but retained low pM-level cytotoxicity in most of the cell lines examined.

We have also reported that the phenolCBI-PBD heterodimer **5** displays IC_{50}s in the pM range against a selection of human tumour cell lines, particularly after extended (5-day) compound

Table 1. *In vitro* cytotoxicity of known and proposed ADC payloads

Cell line	IC_{50} (nM)						
	1	**2**-Me[a]	**3**	**4**	**5**	**6**	**18**
MES-SA (uterine, Pgp -ve)	0.67	0.11	0.00054	0.0058	0.033	0.91	0.017
MES-SA/Dx5 (uterine, high Pgp)	>30	>2	0.0024	0.11	0.11	20.8	0.052
BJAB (B-cell)	0.32	0.086	0.00063	0.015	0.011	1.2	0.0043
KPL-4 (breast)	0.099	0.023	0.0015	0.021	0.036	0.74	0.016
HCC1569 X2 (breast)	nd[b]	nd	0.018	>1	0.13	6.2	0.048
T-47D (breast)	nd	nd	0.0021	0.081	0.016	2.5	0.0077
HCC1937 (breast)	nd	nd	0.029	0.066	0.11	9.5	0.064
NCI-H1781 (NSCLC)	nd	nd	0.0047	0.0098	0.0098	0.53	0.0043
SW 900 (NSCLC)	nd	nd	0.0088	0.033	0.051	3.7	0.024

[a]S-methylated analogue of DM1. [b]IC_{50} not determined because of incomplete inhibition of proliferation.

exposure.[12] In the current study **5** was approximately equipotent with **4**, and similar levels of cytotoxicity were observed whether the CBI was in the *seco* form (**5**) or spirocyclised prior to cell treatment (**18**). This latter observation is consistent with previous reports on CBI monoalkylating agents and has been attributed to facile spirocyclisation under physiological conditions.[7] In all cell lines, exposure to a sufficiently high concentration of **5** led to complete inhibition of proliferation (Figure 2b), behaviour that was common to all the bifunctional agents examined and in marked contrast to **1** and **2**-Me. Once again, swapping the phenolCBI end of the dimer to an aminoCBI led to a reduction in potency, with **6** being on average 85-fold less cytotoxic than **5**, and also more sensitive than **5** to Pgp overexpression (IC_{50} ratio in MES-SA/Dx5 compared to MES-SA 3.3 for **5** but 23 for

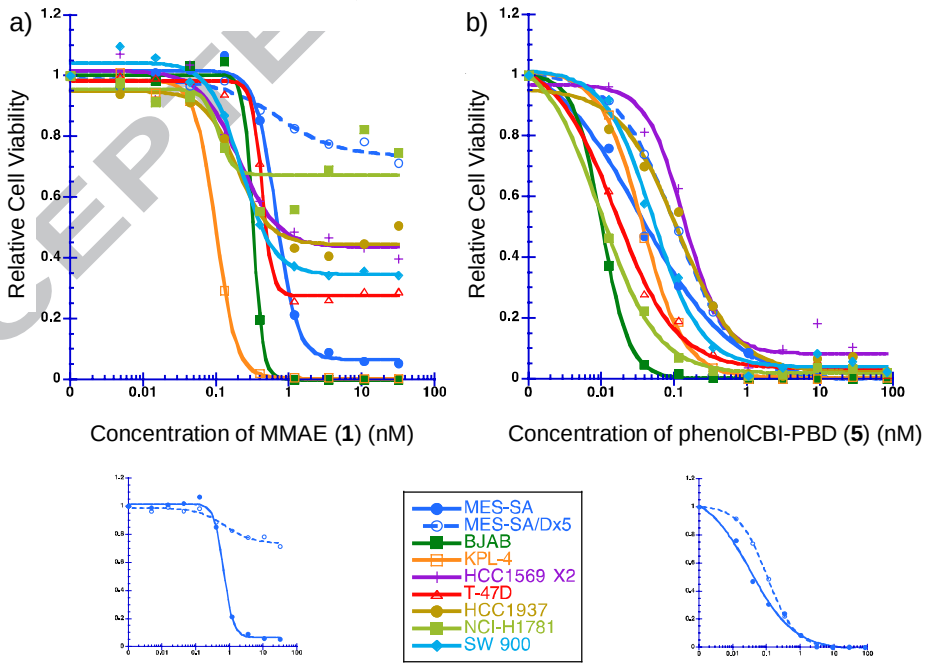


Figure 2. Representative cell viability curves for a) a microtubule-targeted cytotoxin, MMAE (1) and b) a DNA minor groove cross-linking agent, phenolCBI-PBD heterodimer (5). The smaller graphs below reproduce the cell viability curves for the MES-SA and MES-SA/Dx5 pair of cell lines, illustrating the substantial resistance of the Pgp-overexpressing subline to 1 (IC_{50} ratio > 45) but not 5 (IC_{50} ratio 3.3).

6). Overall the cytotoxicity of the bifunctional agents followed the order **3** > **4** ~ **5** > **6** spanning 2-3 orders of magnitude in the pM to nM range.

Given that **3** is clearly the most potent of the cytotoxins examined some other variants of this structure were also investigated. However it was found that extending the polymethylene linker from three to five carbons, or substituting the central methylene with an NH group led to a reduction in potency, as assessed for racemic compounds in the KPL-4 breast cancer cell line (see Supporting Information). Similar observations have been reported for *S,S*-enantiomers[26] and for an analogue containing a central *N*-propargyl unit,[27] and these structural modifications were not pursued further.

3. Conclusions

The Pd-catalysed amination method described here gives easy access to the more active *S* enantiomer of the aminoCBI alkylating subunit **10**. This route is considerably more efficient than alternative chiral HPLC-based resolutions that have been applied to intermediates in various aminoCBI and nitroCBI syntheses.[28,29] Moreover intermediate (-)-*S*-**9** is synthetically very useful since highly selective removal of either the imine or the Boc protecting group can be achieved via appropriate choice of acidic reaction conditions.

Straightforward access to (-)-*S*-**9** has allowed the synthesis of **4** and **6**, novel bifunctional agents that are analogues of the exceptionally cytotoxic CBI dimer **3** and CBI-PBD heterodimer **5**. Surprisingly, given that DNA monoalkylating agents incorporating phenolCBI or aminoCBI are of equivalent cytotoxicity,[18] swapping the phenolCBI of **3** or **5** for the corresponding aminoCBI in **4** and **6** leads to a substantial reduction in potency, ranging from 2- to 190-fold depending on the particular compound and cell line. The bifunctional agents incorporating aminoCBI are also unexpectedly more sensitive to overexpression of Pgp than their phenolCBI analogues.

Nevertheless, dimer **4** retains notable low pM level cytotoxicity in most of the cell lines examined, and, in common with **3**, **5**, and **6**, completely inhibits the proliferation of all cell lines at sufficiently high concentrations. This stands in contrast to MMAE (**1**) and S-methylated DM1 (**2**-Me) which gave, for example, maximum inhibition of proliferation of only 40-45% in NCI-H1781 and 50-55% in HCC1569 X2 even at ≥10 nM concentration. These observations reinforce the potential of DNA minor groove alkylating agents, especially ultrapotent bifunctional agents, to overcome some of the limitations of microtubule-targeted toxins as ADC payloads.

4. Experimental

4.1. Chemistry

Solvents were distilled prior to use by common laboratory methods. Petroleum ether refers to the fraction with a bp = 40-60 °C. Organic solutions were dried over anhydrous Na_2SO_4 or $MgSO_4$. Column chromatography was carried out on silica gel (Merck 230-400 mesh). Melting points were determined on an Electrothermal 2300 Melting Point Apparatus. NMR spectra were obtained on a Bruker Avance 400 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C spectra. HRMS was performed with a Bruker micrOTOF-QII mass spectrometer. Low resolution mass spectra were acquired by direct injection of methanolic solutions into a Surveyor MSQ mass spectrometer using atmospheric pressure chemical ionization (APCI). Combustion analyses were carried out in the Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand.

4.1.1. *tert*-Butyl 1-(chloromethyl)-5-(((trifluoromethyl)sulfonyl)oxy)-1*H*-benzo[*e*]indole-3(2*H*)-carboxylate (8)

Trifluoromethanesulfonic anhydride (69 μL, 0.41 mmol) was added dropwise to a solution of **7**[20,21] (125 mg, 0.37 mmol) and Et_3N (63 μL, 0.45 mmol) in CH_2Cl_2 (15 mL) at 0 °C. After 5 min the mixture was diluted with water and extracted with CH_2Cl_2 (×2). The extracts were dried and evaporated and the residue was purified by column chromatography (eluting with EtOAc:petroleum ether 1:9) to give **8** as a very pale yellow oil (160 mg, 92%): ^{1}H NMR ($CDCl_3$) δ 8.31 (v br s, 1H), 8.03 (d, *J* = 8.5 Hz, 1H), 7.76 (d, *J* = 8.4 Hz, 1H), 7.60 (dt, *J* = 7.6, 1.0 Hz, 1H), 7.51 (dt, *J* = 7.7, 1.0 Hz, 1H), 4.32 (br s, 1H), 4.22-4.14 (m, 1H), 4.09-4.01 (m, 1H), 3.91 (dd, *J* = 11.2, 2.7 Hz, 1H), 3.51 (dd, *J* = 11.0, 10.3 Hz, 1H), 1.61 (s, 9H).

4.1.2. (*S*)-*tert*-Butyl 1-(chloromethyl)-5-(((trifluoromethyl)sulfonyl)oxy)-1*H*-benzo[*e*]indole-3(*2H*)-carboxylate (*S*-8)

The same procedure (20 min reaction time) was used to convert (-)-S-**7**[19] (1.70 g) to the corresponding triflate *S*-**8** (2.20 g, 93%): ^{1}H NMR identical to that for the racemic material.

4.1.3. *tert*-Butyl 1-(chloromethyl)-5-((diphenylmethylene)amino)-1*H*-benzo[*e*]indole-3(*2H*)-carboxylate (9)

A mixture of **8** (170 mg, 0.36 mmol), benzophenone imine (79 mg, 0.44 mmol), Cs_2CO_3 (0.17 g, 0.52 mmol), $Pd(OAc)_2$ (0.8 mg, 3.6 μmol), and BINAP (2,2'-bis(diphenylphosphino)-1,1'-binaphthalene) (3.4 mg, 5.5 μmol) in THF (6 mL) was degassed by bubbling with nitrogen and then stirred at reflux under nitrogen overnight. More portions of $Pd(OAc)_2$ (total 4%) and BINAP (total 6%) were added and the reaction was monitored by TLC until the starting material was consumed. After 5 days the mixture was diluted with CH_2Cl_2 and filtered through Celite®. The filtrate was evaporated and the residue was purified by column chromatography (eluting with EtOAc:petroleum ether 1:9 and then CH_2Cl_2) to give **9** as a yellow oil (158 mg, 87%): ^{1}H NMR ($CDCl_3$) δ 7.99 (d, *J* = 8.3 Hz, 1H), 7.87-7.81 (m, 2H), 7.63 (d, *J* = 8.4 Hz, 1H), 7.54-7.41 (m, 4H), 7.34-7.29 (m, 1H), 7.24-7.13 (m, 3H), 7.10-7.05 (m, 2H), 4.21-4.13 (m, 1H), 4.04-3.98 (m, 1H), 3.95-3.86 (m, 2H), 3.36 (t, *J* = 10.7 Hz, 1H), 1.51 (s, 9H), 1H not observed; ^{13}C NMR δ 168.7, 152.3, 149.9, 139.3, 135.9, 130.9, 129.8, 129.5, 128.7, 128.2, 127.8, 127.2, 125.3, 124.2, 123.3, 122.1, 117.7, 104.3, 81.1, 52.6, 46.4, 41.7, 28.4. A sample was recrystallised from MeOH giving a yellow solid: mp 172-174 °C. Anal. calcd for $C_{31}H_{29}ClN_2O_2$: C, 74.91; H, 5.88; N, 5.64. Found: C, 74.79; H, 6.18; N, 5.64.

4.1.4. (-)-(*S*)-*tert*-Butyl 1-(chloromethyl)-5-((diphenylmethylene)amino)-1*H*-benzo[*e*]indole-3(*2H*)-carboxylate ((-)-*S*-9)

The same procedure (15% $Pd(OAc)_2$ and 15% BINAP, sealed tube, 60-65 °C, 4 days) was used to convert *S*-**8** (2.15 g) to the corresponding imine (-)-*S*-**9** (2.09 g, 91%): ^{1}H NMR (DMSO-d_6) δ 7.85 (d, *J* = 8.4 Hz, 1H), 7.80 (d, *J* = 8.4 Hz, 1H), 7.75 (d, *J* = 7.3 Hz, 2H), 7.61-7.57 (m, 1H), 7.54-7.49 (m, 3H), 7.37-7.33 (m, 1H), 7.30-7.23 (m, 3H), 7.06 (d, *J* = 6.7 Hz, 2H), 4.14-4.02 (m, 2H), 3.99-3.94 (m, 2H), 3.77 (dd, *J* = 10.8, 7.4 Hz, 1H), 1.46 (s, 9H), 1H not observed. HRMS calcd for $C_{31}H_{30}ClN_2O_2$ (MH^+) *m/z* 497.1990, found 497.1984. $[\alpha]_D^{28}$ = -101.5° (*c* = 0.995, CH_2Cl_2).

4.1.5. (*S*)-*tert*-Butyl 5-amino-1-(chloromethyl)-1*H*-benzo[*e*]indole-3(*2H*)-carboxylate (10)

(a) Imine deprotection using aqueous HOAc. HOAc (65 mL) was added to a solution of (-)-*S*-**9** (1.30 g, 2.62 mmol) in THF (195 mL) and water (98 mL) and the mixture was stirred at room temperature for 18 h. The mixture was concentrated under vacuum at <30 °C to remove most of the THF and then extracted with EtOAc (200 mL). The extract was washed with saturated aqueous $NaHCO_3$ (×4, until washings were pH 8), dried, and the solvent was removed under vacuum. Purification of the residue by column chromatography (eluting with petroleum ether and then with

EtOAc:petroleum ether 1:9) gave **10** as an unstable solid (503 mg, 58%): ^{1}H NMR (DMSO-d_6) δ 8.01 (d, *J* = 8.4 Hz, 1H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.40 (ddd, *J* = 8.1, 6.8, 0.9 Hz, 1H), 7.36 (br s, 1H), 7.20 (ddd, *J* = 8.1, 6.8, 1.1 Hz, 1H), 5.91 (s, 2H), 4.11-3.91 (m, 4H), 3.66 (dd, *J* = 10.6, 8.2 Hz, 1H), 1.53 (s, 9H); consistent with that previously reported for the racemate.[23]

(b) Imine deprotection using methanolic HCl. HCl (4M in dioxane, 0.63 mL, 2.5 mmol) was added to a solution of (-)-*S*-**9** (497 mg, 1.00 mmol) in dry CH_2Cl_2 (10 mL) and MeOH (10 mL) under nitrogen and the mixture was stirred at room temperature for 1 h. The solvents were evaporated at 20 °C and the resulting yellow solid was stirred with a mixture of EtOAc-petroleum ether (1:10) (200 mL) for 30 min. The solvents were separated and the process was repeated. The solid was then stirred at 0 °C with cold aqueous Na_2CO_3 (2N, 200 mL) and CH_2Cl_2 (200 mL) for 15 min. The CH_2Cl_2 layer was separated, washed with water (100 mL), and then dried and evaporated at 25 °C to give **10** as an unstable solid (322 mg, 97%): ^{1}H NMR consistent with that previously reported for the racemate.[23] HRMS (ESI) calcd for $C_{18}H_{22}ClN_2O_2$ (MH^+) *m/z* 333.1364, found: 333.1355; calcd for $C_{18}H_{21}ClN_2NaO_2$ (MNa^+) *m/z* 355.1184, found: 355.1179; calcd for $C_{18}H_{21}ClKN_2O_2$ (MK^+) *m/z* 371.0923, found: 371.0920.

4.1.6. (*S*)-*tert*-Butyl 5-(benzyloxy)-1-(chloromethyl)-1*H*-benzo[*e*]indole-3(2*H*)-carboxylate (11)

Benzyl bromide (7.13 mL, 60 mmol), KI (50 mg, 0.30 mmol) and K_2CO_3 (4.14 g, 30 mmol) were added to a solution of (-)-*S*-**7** (2.00 g, 5.99 mmol) in DMF (5 mL) and the mixture was stirred at room temperature for 2 h. EtOAc was added, the insoluble material was filtered off, and the filtrate was partitioned with water. The organic layer was separated and the aqueous phase was extracted with more EtOAc (×3). The combined organic extracts were dried and evaporated and the residue was purified by column chromatography (eluting with EtOAc: petroleum ether 1:10) to give **11** as a white solid (1.97 g, 78%): mp 186-188 °C (lit[7] mp 184.5-185.5 °C); ^{1}H NMR ($CDCl_3$) identical to that reported.[7]

Further elution (EtOAc: petroleum ether 1:1) gave spirocyclised starting material (8b*R*,9a*S*)-*tert*-butyl 4-oxo-9,9a-dihydro-1*H*-benzo[*e*]cyclopropa[*c*]indole-2(4*H*)-carboxylate[7] (345 mg, 19%).

4.1.7. (*S*)-5-(1-(Chloromethyl)-5-hydroxy-1*H*-benzo[*e*]indol-3(2*H*)-yl)-5-oxopentanoic acid (12)

HCl in dioxane (4N, 40 mL) was added to a solution of **11** (1.60 g, 3.77 mmol) in CH_2Cl_2 (15 mL) cooled in an ice bath. The cooling bath was removed and the mixture was stirred for 3 h, then evaporated to dryness. The residue was stirred with THF (15 mL) in an ice bath and glutaric anhydride (646 mg, 5.66 mmol), DMAP (46 mg, 0.38 mmol) and pyridine (5 mL) were added. The cooling bath was removed and the mixture was stirred for 4 h, then evaporated to dryness. The residue was dissolved in dilute aqueous $NaHCO_3$ and washed 3 times with EtOAc. The aqueous phase was acidified using 1N HCl to a pH of 2 and extracted with EtOAc (×3). The combined extracts were washed with brine, dried, and filtered through a silica gel pad washing with a mixture MeOH:EtOAc (1:10). The solvent was evaporated to give benzyl-protected **12** as an off-white solid (978 mg, 59%).

Aqueous NH_4HCO_2 (25%, 23 mL) and Pd-C catalyst (10%, 1.5 g) were added to a solution of benzyl-protected **12** (2.00 g, 4.57 mmol) in THF (60 mL) at -20 °C. The mixture was stirred at -10 to -15 °C for 22 h, then diluted with MeOH at 0 °C and filtered through Celite®, washing with MeOH. The solvents were concentrated under vacuum until a solid began to precipitate. Water (150 mL) and petroleum ether (150 mL) were added and the mixture was stirred at room temperature while being acidified to pH 1 with conc. HCl. The mixture was stirred for a further 30 min and then the solid was filtered off, washed with water and petroleum ether, and dried to give **12** as a beige solid (1.43 g, 90%): ^{1}H NMR (DMSO-d_6) δ 12.07 (br s, 1H), 10.35 (s, 1H), 8.08 (d, *J* = 8.0 Hz, 1H), 7.98 (s, 1H), 7.77 (d, *J* = 8.3 Hz, 1H), 7.50-7.46 (m, 1H), 7.33-7.29 (m, 1H), 4.30 (t, *J* = 10.4 Hz, 1H), 4.14-4.12

(m, 2H), 3.98 (dd, *J* = 10.9, 2.8 Hz, 1H), 3.78 (dd, *J* = 10.8, 7.8 Hz, 1H), 2.63-2.45 (m, 2H), 2.35 (t, *J* = 7.4 Hz, 2H), 1.89-1.78 (m, 2H); LRMS (APCI) calcd for $C_{18}H_{19}ClNO_4$ (MH^+) *m/z* 348.1, found 348.6.

4.1.8. 1-((*S*)-1-(Chloromethyl)-5-((diphenylmethylene)amino)-1*H*-benzo[*e*]indol-3(2*H*)-yl)-5-((*S*)-1-(chloromethyl)-5-hydroxy-1*H*-benzo[*e*]indol-3(2*H*)-yl)pentane-1,5-dione (13)

HCl (g) was bubbled through a solution of (-)-*S*-**9** (425 mg, 0.855 mmol) in dry dioxane (12 mL) at room temperature. A solid precipitated and after 15 min the solvent was removed under vacuum. To the residue was added **12** (327 mg, 0.941 mmol), EDCI (573 mg, 2.99 mmol), and DMA (10 mL). The mixture was stirred under nitrogen for 3 days and then the solvent was removed under vacuum. The residue was purified by column chromatography (first column eluting with CH_2Cl_2:MeOH 10:0 then 9:1; second column Et_2O:CH_2Cl_2 10:0 to 5:5 to 0:10; third column CH_2Cl_2:MeOH 99:1 then 98:2) to give **13** (193 mg, 31%): ^{1}H NMR (DMSO-d_6) δ 10.35 (s, 1H), 8.08 (d, *J* = 8.1 Hz, 1H), 8.00 (s, 1H), 7.84 (t, *J* = 9.0 Hz, 2H), 7.79-7.74 (m, 3H), 7.62-7.57 (m, 2H), 7.54-7.46 (m, 4H), 7.40-7.36 (m, 1H), 7.33-7.29 (m, 1H), 7.27-7.22 (m, 3H), 7.09-7.08 (m, 2H), 4.34-4.26 (m, 2H), 4.22-4.12 (m, 4H), 4.01-3.97 (m, 2H), 3.83-3.76 (m, 2H), 2.69-2.50 (m, 4H), 1.93-1.86 (m, 2H). HRMS calcd for $C_{44}H_{38}Cl_2N_3O_3$ (MH^+) *m/z* 726.2285; found 726.2264.

4.1.9. 1-((*S*)-5-Amino-1-(chloromethyl)-1*H*-benzo[*e*]indol-3(2*H*)-yl)-5-((*S*)-1-(chloromethyl)-5-hydroxy-1*H*-benzo[e]indol-3(2*H*)-yl)pentane-1,5-dione (4)

HOAc (8 mL) was added to a solution of **13** (190 mg, 0.261 mmol) in THF (24 mL) and water (12 mL) and the mixture was stirred at room temperature. After 19 h the mixture was diluted with water, causing a solid to precipitate. The THF was removed under vacuum and the aqueous suspension treated with i-Pr_2NEt until neutral. The solid was filtered off, washed with water and dried. Attempts to purify the crude product by trituration with various solvent mixtures, or by column chromatography (eluting with CH_2Cl_2:MeOH 10:0 then 9:1) were only partially successful. The material was instead purified by preparative HPLC (Synergi-MAX RP 4 μ column, 21.20 × 250 mm; 13 mL/min; mobile phase: solvent A: H_2O/TFA pH 2.5, solvent B: MeCN/H_2O 9:1; solvent A:solvent B 20:80) to give **4** (30 mg, 22%) as a white solid: ^{1}H NMR (DMSO-d_6) δ 10.36 (s, 1H), 8.08 (d, *J* = 8.1 Hz, 1H), 8.04-8.02 (m, 2H), 7.79-7.77 (m, 2H), 7.69 (d, *J* = 8.3 Hz, 1H), 7.51-7.47 (m, 1H), 7.42 (t, *J* = 7.5 Hz, 1H), 7.34-7.30 (m, 1H), 7.23 (t, *J* = 7.6 Hz, 1H), 5.91 (s, 2H), 4.36-4.26 (m, 2H), 4.19-4.13 (m, 3H), 4.08-4.04 (m, 1H), 4.01-3.93 (m, 2H), 3.79 (dd, *J* = 10.7, 8.2 Hz, 1H), 3.70 (dd, *J* = 10.5, 8.9 Hz, 1H), 2.75-2.66 (m, 2H), 2.63-2.54 (m, 2H), 2.00-1.93 (m, 2H). HRMS calcd for $C_{31}H_{29}Cl_2N_3NaO_3$ (MNa^+) *m/z* 584.1478, found 584.1456. $[\alpha]_D^{28}$ = -37.6° (*c* = 0.559, DMSO).

4.1.10. (11a*S*)-Allyl 8-((6-((*S*)-1-(chloromethyl)-5-((diphenylmethylene)amino)-1*H*-benzo[*e*]indol-3(2*H*)-yl)-6-oxohexyl)oxy)-11-hydroxy-7-methoxy-5-oxo-2,3,11,11a-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepine-10(5*H*)-carboxylate (15)

Trifluoroacetic acid (2 mL) was added to a solution of (-)-*S*-**9** (166 mg, 0.33 mmol) in dry CH_2Cl_2 (2 mL) at 0 °C under nitrogen and the mixture was stirred at this temperature for 2 h 30 min. The mixture was diluted with cold EtOAc and stirred with excess aqueous Na_2CO_3 (2N). The EtOAc layer was separated, washed with water, and then dried and evaporated at 20 °C. To the residue was added acid **14**[12] (155 mg, 0.33 mmol), EDCI (128 mg, 0.67 mmol), toluenesulfonic acid (11.5 mg, 0.067 mmol), and dry DMA (1 mL) and the mixture was stirred at room temperature under nitrogen for 18 h. Water was added and after 15 min the preciptated solid was filtered off, washed with water and then petroleum ether (3×20 mL) and dried to give **15** as a yellow solid (200 mg, 71%); mp 124-127 °C; ^{1}H NMR (DMSO-d_6) δ 7.83 (t, *J* = 8.0 Hz, 2H), 7.79-7.00 (m, 2H), 7.64-7.47 (m, 5H), 7.37

(t, *J* = 7.6 Hz, 1H), 7.30-7.17 (m, 3H), 7.13-7.09 (m, 3H), 6.77 (s, 1H), 6.49 (poorly resolved d, *J* = 5.2 Hz, 1H), 5.88-5.72 (m, 1H), 5.45 (dd, *J* = 8.9 and 6.0 Hz, 1H), 5.15-4.99 (m, 2H), 4.66-4.52 (m, 1H), 4.46-4.33 (m, 1H), 4.32-4.05 (m, 3H), 4.05-3.87 (m, 3H), 3.85-3.71 (m, 4H), 3.53-3.43 (m, 1H), 3.40-3.19 (m, partially obscured by water peak, 2H), 2.61-2.34 (m, partially obscured by DMSO peak, 2H), 2.10-1.68 (m, 6H), 1.68-1.52 (m, 2H), 1.52-1.35 (m, 2H). HRMS (ESI) calcd for $C_{49}H_{50}ClN_4O_7$ (MH^+) *m/z* 841.3363, found 841.3380; calcd for $C_{49}H_{49}ClN_4NaO_7$ (MNa^+) *m/z* 863.3182, found 863.3205. $[\alpha]_D$ = +2.1° (c 0.48, $CHCl_3$).

4.1.11. (*S*)-8-((6-((*S*)-5-Amino-1-(chloromethyl)-1*H*-benzo[*e*]indol-3(2*H*)-yl)-6-oxohexyl)oxy)-7-methoxy-2,3-dihydro-1H-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-5(11a*H*)-one (6)

HCl in dioxane (4M, 83 μL, 0.33 mmol) was added to a solution of **15** (56 mg, 0.065 mmol) in dry CH_2Cl_2 (1.5 mL) and dry MeOH (1.5 mL) under nitrogen and the mixture was stirred at room temperature for 1 h 20 min. The mixture was evaporated under reduced pressure at 20 °C to give a red-brown residue which was triturated with EtOAc:petroleum ether (1:10, 2×25 mL) and then dried. To this solid were added pyrrolidine (55 μL, 0.65 mmol) and $Pd(PPh_3)_4$ (3.8 mg, 9.8% Pd) in dry CH_2Cl_2 (5 mL) under nitrogen. After 3 min at room temperature the mixture was transferred directly to a silica column. Chromatography (eluting with EtOAc:MeOH 50:1), with concentration of product-containing fractions at 20 °C, gave **6** as a pale yellow solid (27.3 mg, 73%); mp 207-211 °C (dec); ^{1}H NMR (DMSO-d_6) δ 7.93 (s, 1H), 7.79 (d, *J* = 8.4 Hz, 1H), 7.70-7.62 (m, 2H), 7.55-7.45 (m, 2H), 7.39-7.31 (m, 1H), 6.81 (s, 1H), 4.48-3.87 (m, 11H, 2H exchange with D_2O), 3.87-3.77 (m, 1H), 3.77-3.68 (m, 1H), 3.64-3.52 (m, 1H), 3.38 (t, *J* = 10.8 Hz, 1H), 2.67-2.55 (m, 1H), 2.55-2.43 (m, 1H), 2.38-2.25 (m, 2H), 2.14-1.78 (m, 6H), 1.70-1.55 (m, 2H). HRMS (ESI) calc for $C_{32}H_{36}ClN_4O_4$ (MH^+) *m/z* 575.2420, found 575.2409; calcd for $C_{32}H_{35}ClN_4NaO_4$ (MNa^+) *m/z* 597.2239, found 597.2254. $[\alpha]_D$ = +516.1° (c 0.062, $CHCl_3$).

4.1.12. (11a*S*)-Allyl 11-hydroxy-7-methoxy-5-oxo-8-((6-oxo-6-((8b*R*,9a*S*)-4-oxo-9,9a-dihydro-1*H*-benzo[*e*]cyclopropa[*c*]indol-2(4*H*)-yl)hexyl)oxy)-2,3,11,11a-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepine-10(5*H*)-carboxylate (17)

K_2CO_3 (48 mg, 0.35 mmol) was added to a solution of **16**[12] (34 mg, 0.05 mmol) in dry DMA (1 mL) and the mixture was stirred at room temperature for 3 h. The mixture was partitioned between EtOAc (150 mL) and cold aqueous Na_2CO_3 (1N, 50 mL). The EtOAc layer was separated, washed with water (2×30 mL), and then dried and evaporated. The glassy solid residue was triturated with EtOAc:petroleum ether (1:1) (2×3 mL) to give **17** as a beige solid (29.2 mg, 91%); ^{1}H NMR ($CDCl_3$) δ 8.22 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.52 (td, *J* = 7.6, 1.4 Hz, 1H), 7.42 (td, *J* = 8.1, 1.0 Hz, 1H), 7.23 (s, 1H), 6.87 (d, *J* = 7.5 Hz, 1H), 6.71 (s, 1H), 5.89-5.71 (m, 1H), 5.64 (dd, *J* = 8.0, 3.4 Hz, 1H), 5.14 (d, *J* = 11.6 Hz, 2H), 4.67 (dd, *J* = 13.9, 5.5 Hz, 1H), 4.52-4.40 (m, 1H), 4.19-3.96 (m, 4H), 3.90 (s, 3H), 3.79 (d, *J* = 4.5 Hz, 1H), 3.74-3.66 (m, 1H), 3.62-3.45 (m, 2H), 2.83-2.75 (m, 1H), 2.63-2.50 (m, 2H), 2.19-2.10 (m, 2H), 2.10-1.95 (m, 2H), 1.95-1.64 (m, 5H), 1.64-1.50 (m, 2H), 1.44 (t, *J* = 4.8 Hz, 1H), 1 H not observed. HRMS (ESI) calcd for $C_{36}H_{40}N_3O_8$ (MH^+) *m/z* 642.2810, found 642.2823; calcd for $C_{36}H_{39}N_3NaO_8$ (MNa^+) *m/z* 664.2629, found 664.2652. $[\alpha]_D$ = +257.1° (c 0.14, $CHCl_3$).

4.1.13. (8b*R*,9a*S*)-2-(6-(((*S*)-7-Methoxy-5-oxo-2,3,5,11a-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy)hexanoyl)-9,9a-dihydro-1*H*-benzo[*e*]cyclopropa[*c*]indol-4(2*H*)-one (18)

Pyrrolidine (35 μL, 0.43 mmol) and $Pd(PPh_3)_4$ (2.5 mg, 9.8% Pd) were added to a solution of **17** (27.3 mg, 0.043 mmol) in dry CH_2Cl_2 (2 mL) under nitrogen and the mixture was stirred at room temperature. After 3 min the mixture was transferred directly to a silica column. Chromatography (eluting with EtOAc:MeOH 50:1) gave **18** as a glassy solid (20.6 mg, 91%); ^{1}H NMR ($CDCl_3$) δ 8.22

(dd, *J* = 7.9, 1.1 Hz, 1H), 7.66 (d, *J* = 4.4 Hz, 1H), 7.56-7.46 (m, 2H), 7.40 (td, *J* = 8.1, 1.1 Hz, 1H), 6.87 (d, *J* = 7.5 Hz, 1H), 6.80 (s, 1H), 4.18-3.99 (m, 4H), 3.94 (s, 3H), 3.88-3.77 (m, 1H), 3.76-3.69 (m, 1H), 3.63-3.53 (m, 1H), 2.62-2.74 (m, 1H), 2.63-2.49 (m, 2H), 2.39-2.25 (m, 2H), 2.12-1.98 (m, 2H), 1.98-1.72 (m, 4H), 1.67 (dd, *J* = 7.6, 4.6 Hz, 1H), 1.64-1.50 (m, 2H), 1.43 (t, *J* = 4.8 Hz, 1H), 1 H not observed. HRMS (ESI) calcd for $C_{32}H_{34}N_3O_5$ (MH^+) *m/z* 540.2493, found 540.2504; calcd for $C_{32}H_{33}N_3NaO_5$ (MNa^+) *m/z* 562.2312, found 562.2325. $[\alpha]_D$ = +880.8° (c 0.15, $CHCl_3$).

4.2 In vitro cytotoxicity

Cells were seeded in 384-well plates and treated with drug 24 h later. After 4 days of drug incubation, the cell viability was determined using Promega CellTiter-Glo luminescent reagent, which measures ATP level (an indirect measure of cell number). The luminescent intensity was measured on PerkinElmer Envision reader. The relative cell viability was calculated by normalizing to non-drug treatment control and was graphed using KleidaGraph software package. IC_{50} value was determined as the concentration to obtain 50% of the maximum cell killing. Each IC_{50} was determined by curve-fitting from 10 data points, and each data point was the average of quadruplicates.

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Supplementary Information

Supplementary data associated with this article (describing the synthesis and properties of two racemic phenolCBI dimers and an iminodiacetic acid-linked phenolCBI dimer, and ^{1}H NMR spectra of **4**, **6**, **18**, and four compounds of the Supporting Information) can be found, in the online version.

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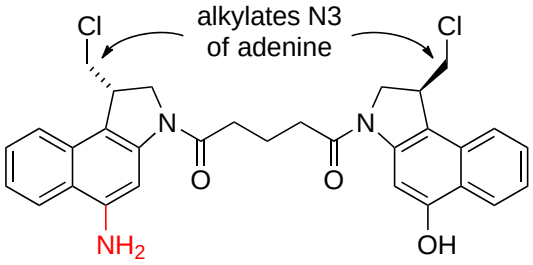
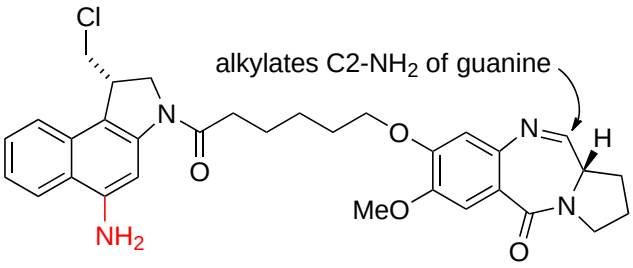
***Graphical Abstract (for review)**



IC_{50} (MES-SA) = 5.8 pM



IC_{50} (MES-SA) = 0.91 nM