Coronarin D conjugated to methylene homologues of chlorambucil: synthesis and evaluation of their cytotoxicity

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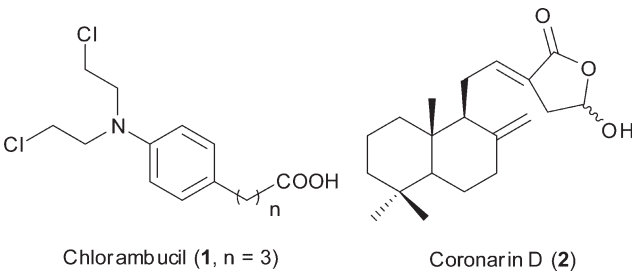
Methylene homologues of chlorambucil were synthesised and conjugated to the labdane diterpene coronarin D. The products were evaluated for their *in vitro* cytotoxicity, and were found to exhibit selective activity against MOLT-3 cell line. Two homologues of chlorambucil showed a comparable cytotoxic effect to their parent. However, as compared with the non-derivatised chlorambucil and its homologues, their conjugation with coronarin D through ester linkage did not enhance *in vitro* cytotoxicity against the tested cancer cell lines.

Keywords: chlorambucil, methylene homologues, coronarin D, cytotoxicity

Chlorambucil (**1**, Fig. 1), 4-[4-(*N*,*N*-bis(2-chloroethyl)amino)-phenyl]butyric acid is an aromatic nitrogen mustard alkylating agent. It was developed by Everett *et al*.,[1] and is still used as a chemotherapy for the treatment of chronic lymphocytic leukaemia. The anti-cancer effects of chlorambucil result from its interference with DNA replication and RNA transcription through the alkylation and cross-linking of DNA strands, leading to DNA damage and cellular death.[2] Coronarin D (**2**, Fig. 1) is a labdane diterpene that is easily isolated in high yield from the rhizomes of *Hedychium coronarium*, and displays significant cytotoxicity against various cancer cell lines.[3–5] Recently, Kunnumakkara *et al*.[6] reported the anti-inflammatory mechanism of coronarin D. They found that coronarin D inhibited both constitutive and inducible nuclear factor-κB pathway activation. They hypothesised that the anti-inflammatory and cytotoxic effects of coronarin D were due to its ability to inhibit NF-κB and NF-κB-regulated gene expression. They also found that coronarin D potentiated the cytotoxic effect of different chemotherapeutic agents against different tumor cell types. The goal of the current study was to evaluate the cytotoxicity of coronarin D linked as an ester to chlorambucil and homologues, with a varied methylene chain length. The masking of the ionizable carboxylic group of the chlorambucil and homologues, through an ester linkage with a labdane moiety, should increase the overall lipophilicity of the nitrogen mustard and possibly enhance the ability of the compound to traverse the cell membrane. Furthermore, the dual cytotoxic mechanism of conjugated compounds, DNA damaging effects of the nitrogen mustards and the anti-inflammatory effect of coronarin D, may induce some unique cytotoxicity. Varying the chain length of methylene of chlorambucil also allowed an examination of the effect of spacer arm between coronarin D and the nitrogen mustard moiety on their cytotoxicity.



Chlorambucil (**1**, n = 3) Coronarin D (**2**)

Fig. 1 Structures of chlorambucil (**1**) and coronarin D (**2**).

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Results and discussion

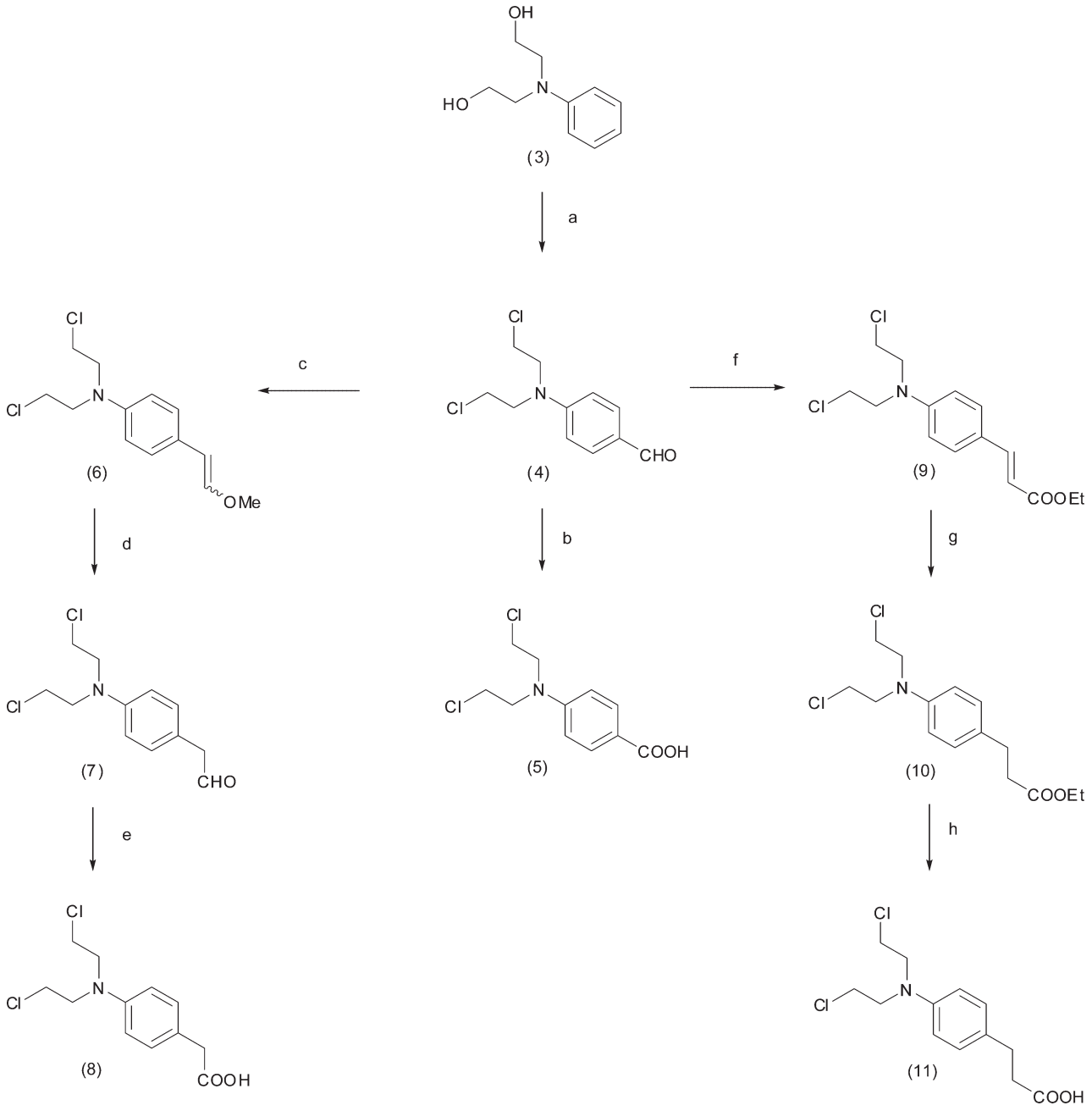
Chemistry

The syntheses of methylene homologues of chlorambucil, containing zero to two methylene groups (n=0,1,2), are outlined in Scheme 1. 4-(*N,N*-Bis(2-chloroethyl)amino)benzalde hyde (**4**),[7] the precursor of all homologues, was obtained in good yield by the treatment of *N*-phenyldiethanolamine (**3**) with phosphoryl chloride in dimethylformamide.[8] Oxidation of **4** with potassium permanganate in pyridine gave 4-(*N,N*-bis(2-chloroethyl)amino)benzoic acid (**5**, n=0) in moderate yield.[7] To prepare 4-(*N,N*-bis(2-chloroethyl)amino)phenylacet ic acid (**8**, n=1),[1] **4** was subjected to a Wittig reaction with (m ethoxymethyl)triphenylphosphonium chloride[9] in the presence of 1.0 M sodium bis(trimethylsilyl)amide in tetrahydrofuran to form the enol ether **6** as an inseparable mixture of *E* and *Z* isomers. The ratio of *E* and *Z* isomers, as deduced from the integration of vinylic proton signals, is approximately 1:1.25. Hydrolysis of the resulting **6** in acidic condition produced aldehyde **7**, which was oxidized with sodium chlorite[10] to yield the desired carboxylic acid **8**. Preparation of 3-[4-(*N,N*-bis(2-chloroethyl)amino)phenyl]propanoic acid (**11**, n=2)[1] was accomplished in three steps. First, olefination of **4** with triethyl phosphonoacetate[11] in the presence of sodium hydride yielded the *E* isomer of ethyl aminophenylprop-2-enoate **9**. Catalytic hydrogenation of **9** over Pd/C catalyst then provided the ester **10**.[1] Finally, acidic hydrolysis of **10** gave the desired carboxylic acid **11**. Hydrolysis of **10** under basic condition (1M aq. NaOH) produced only a low yield of **11**, together with many by-products, probably due to the nucleophilicity of hydroxide ion from base.

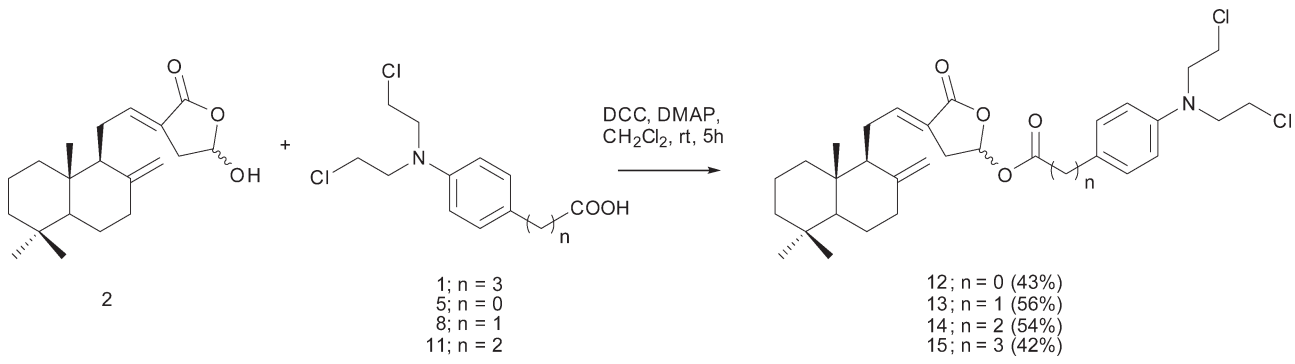
Coronarin D was esterified by the chlorambucil homologues using dicyclohexylcarbodiimide in the presence of 4-(dimethylamino)pyridine in dichloromethane (Scheme 2). The conjugated products, coronarin D *N,N*-bis(2-cholroethyl)-4-aminobenzoate (**12**), coronarin D *N,N*-bis(2-cholroethyl)-4-aminophenylacetate (**13**), coronarin D *N,N*-bis(2-chloroethyl)-4-aminophenylpropanoate (**14**), and coronarin D *N,N*-bis(2-chloroethyl)-4-aminophenylbutanoate (**15**), were obtained in 43%, 56%, 54%, and 42% yields, respectively. The sensitive lactol group of coronarin D might undergo tautomerization in the reaction solution. This may cause undesired side reactions leading to the moderate yields of the conjugated products.

Cytotoxicity studies

The cytotoxic activity of all synthesised coronarin D-linked chlorambucil homologues (**12–15**), as well as chlorambucil (**1**) and its homologues (**5**, **8**, and **11**) against T-lymphoblast: acute lymphoblastic leukaemia (MOLT-3), hepatocarcinoma



Scheme 1 *Regagents and conditions*: (a) $POCl_3$, DMF, $ClCH_2CH_2Cl$, reflux, 3h (94%); (b) $KMnO_4$, H_2O, pyridine, rt, 20h (50%); (c) $ClPPh_3CH_2OCH_3$, NaHMDS, THF, 0 °C, 0.5h (99%); (d) 2N HCl, THF, reflux, 15 min (52%); (e) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, t-BuOH, rt, 1h (64%); (f) $(EtO)_2P(O)CH_2CO_2Et$, NaH, THF, rt, 1.5h (99%); (g) H_2/Pd(C), EtOAc, rt, 1.5h (84%); (h) Conc.HCl, 100 °C, 4h (98%).



Scheme 2 Synthesis of coronarin D-linked homologues of chlorambucil.

(HepG2), cholangiocarcinoma (HuCCA-1) and lung carcinoma (A549) cell lines, is shown in Table 1. All these compounds exhibited selective activity against MOLT-3, but were inactive against other tested cell lines. Among the homologues of chlorambucil, homologues **8** and **11** displayed a comparable cytotoxicity to chlorambucil (**1**) (IC_{50} = 1.49, 1.18 and 1.42 μM, for **8**, **11**, and **1**, respectively), whereas homologue **5** showed the weakest cytotoxicity against MOLT-3 with IC_{50} of 5.98 μM. A possible explanation is that the presence of electron-withdrawing carboxyl group attached at the *para* position of the aromatic ring diminished the electron density on nitrogen, which affected the formation of the aziridinium ion, the active intermediate for alkylating nitrogen mustards. This result concurred with previously reported data.[12–13] Among the coronarin D-chlorambucil homologues conjugates, conjugates **13** (n=1) and **14** (n=2) showed strong cytotoxicity against MOLT-3, whereas conjugate **15** exhibited moderate cytotoxicity, and conjugate **12** demonstrated only weak cytotoxicity. The weak cytotoxicities of compounds **5** and **12** could be attributed to poor aziridinium formation. Comparing the cytotoxicities of the non-derivatised nitrogen mustards with their corresponding conjugates, **1** (IC_{50} = 1.42 μM) showed better cytotoxicity than **15** (IC_{50} = 4.92 μM). Likewise, **5** (IC_{50} = 5.98 μM) showed better cytotoxicity than **12** (IC_{50} = 22.65 μM). Compound **8** (IC_{50} = 1.49 μM) exhibited comparable cytotoxicity to **13** (IC_{50} = 1.41 μM), and **11** (IC_{50} = 1.18 μM) demonstrated slightly better cytotoxicity than **14** (IC_{50} = 1.81 μM). The conjugation of chlorambucil and its homologues to coronarin D through ester linkage apparently did not improve their *in vitro* cytotoxicity toward the tested cancer cell lines. Calculated log P (CLOGP; partition coefficient octanol/water) values of all compounds (Table 1) were used as a rough estimate of lipophilicity. The CLOGP values of the corresponding conjugated compounds increased more than twice compared with the non-conjugated compounds. However, their cytotoxicities did not improve significantly. The result indicated that the increased lipophilicity had no effect on their cytotoxicity, and no correlation between CLOGP and cytotoxicity was observed for this case. On the other hand, there was a significant correlation between the calculated amine pKa (Table 1) and the cytotoxicity of the compounds. Compounds **5** and **12** had low calculated amine pKa values of nitrogen atoms (-0.08 and 0.14, respectively), indicating weak basicity, which resulted in poor aziridinium ion formation and then weak cytotoxicity. In addition, when the calculated amine pKa values of compounds **1**, **8**, **11**, and **13–15** were similar (1.69–1.72), their cytotoxicities were comparable (IC_{50} = 1.18–1.81 μM), except for compound **15** (IC_{50} = 4.92 μM). Therefore, the calculated amine pKa values might serve as a useful tool for the prediction of *in vitro* cytotoxicity against MOLT-3 of the alkylating mustards. Finally, the cytotoxicities of coronarin D-chlorambucil homologues conjugates against MOLT-3 cell line were higher than that of coronarin D (except for compound **12**), but comparable to those of their corresponding non-derivatised nitrogen mustards. Therefore, it could be postulated that the cytotoxicity of the conjugated compounds is due mainly to the nitrogen mustards.

Conclusion

A series of chlorambucil homologues and their ester-linked conjugates with coronarin D were synthesised and evaluated for their cytotoxicity. The result showed that the conjugation of chlorambucil and its homologues to coronarin D through ester linkage did not enhance *in vitro* cytotoxicity against the tested cancer cell lines compared with the non-derivatised nitrogen mustards. Regarding the comparable cytotoxicities of the conjugates and their corresponding nitrogen mustards, it could be postulated that the cytotoxicity of the conjugates is due mainly to the nitrogen mustards, not coronarin D.

Experimental

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 spectrometer (200 MHz for ^{1}H and 50 MHz for ^{13}C) and on a Bruker AVANCE 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C). Chemical shifts were reported as δ values in ppm relative to tetramethylsilane. Mass spectra were obtained on a Finnigan Polaris GCQ mass spectrometer, whereas accurate masses (HRMS) were obtained using a Bruker Micro TOF in APCI positive mode. The IR spectra were recorded in terms of cm^{-1} on a Perkin Elmer Spectrum One FT-IR spectrometer. Melting points were determined on a SMP3 melting point apparatus and reported in °C. Column chromatography was performed on Merck silica gel 60 (70–230 mesh). Chlorambucil was purchased from Fluka. Coronarin D was isolated from the rhizomes of *Hedychium coronarium* according to the previous report[4] and its structure was confirmed by the spectroscopic methods (^{1}H and ^{13}C NMR, MS, FTIR).

4-(N,N-Bis(2-chloroethyl)amino)benzaldehyde (**4**): $POCl_3$ (7.50 mL, 80.46 mmol) was added dropwise to a solution of *N*,*N*-dimethylformamide (7.50 mL, 96.87 mmol) in 1,2-dichloroethane (30 mL) at 0 °C, and the mixture was stirred at 0 °C for 10 min. A solution of *N*-phenyldiethanolamine (3.0 g, 16.57 mmol) in *N*,*N*-dimethylformamide (7.50 mL, 96.87 mmol) was added to the above mixture at 0 °C, and the mixture was refluxed for 3 h. The reaction mixture was poured onto ice and saturated aqueous potassium acetate was added to adjust the pH to 6, and the mixture was extracted with CH_2Cl_2. The

Table 1 Cytotoxicity against cancer cell lines

Compound	IC_{50}/μM[a]				CLOGP[b]	Amine pKa[c]
	MOLT-3	HepG2	HuCCA-1	A549		
1	1.42±0.13	%C=19.5[d]	%C=42.0	%C=45.0	3.81	1.72
2	8.71±1.95	60.79±6.54	29.37±1.67	33.02±2.22	4.41	ND[e]
5	5.98±0.31	%C=11.2	%C=26.0	%C=26.0	1.72	–0.08
8	1.49±0.15	%C=4.7	154.00±4.06	%C=34.0	1.60	1.68
11	1.18±0.03	%C=25.9	%C=30.0	%C=22.0	1.66	1.72
12	22.65±0.86	%C=38.3	%C=0.0	%C=18.0	6.91	0.14
13	1.41±0.05	%C=26.4	%C=47.0	%C=11.0	6.95	1.69
14	1.81±0.08	%C=0.0	%C=0.0	%C=16.0	7.10	1.72
15	4.92±0.30	%C=4.5	%C=4.0	%C=0.0	7.28	1.72
Etoposide	0.04±0.01	19.11±3.01	–	–	ND	ND
Doxorubicin	–	0.34±0.03	0.74±0.18	0.38±0.05	ND	ND

[a] Results are expressed as mean ± standard error; average of three independent experiments.
[b] CLOGP = calculated log P [Marvin 5.6.0.0, 2011, ChemAxon (http://www.chemaxon.com)]
[c] Calculated amine pKa [Marvin 5.6.0.0, 2011, ChemAxon (http://www.chemaxon.com)]
[d] Inactive (IC_{50}≥50 μg mL^{-1}); reported in %cytotoxicity at the substance concentration of 50 μg mL^{-1}.
[e] Not determined

combined organic layer was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (15% EtOAc/hexane) to produce **4** (3.83 g, 94%) as a white solid; m.p. 87–88 °C (lit.[7] 87–88 °C); FTIR (KBr), ν_{max}: 1667, 1589, 1522, 1404, 1361, 1165, 714 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 9.81 (s, 1H, CHO), 7.78 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$), 6.74 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$), 3.65–3.89 (m, 8H, 2×NC*H*$_2$C*H*$_2$Cl); ^{13}C NMR (50 MHz, $CDCl_3$): δ 190.1, 151.0, 132.3, 126.8, 111.3, 53.3, 40.0 ppm; MS (EI), *m/z* (relative intensity): 245(M^+, 45), 196(100), 132(32), 117(13), 91(5), 77(14), 63(6), 51(10); HRMS (APCI) Calcd for $C_{11}H_{14}NOCl_2$ $[M+H]^+$ 246.0447; found 246.0435.

4-(N,N-Bis(2-chloroethyl)amino)benzoic acid (**5**): A solution of $KMnO_4$ (0.71 g, 4.50 mmol) in water (15 mL) was added dropwise to a refluxing, stirred solution of **4** (0.46 g, 1.88 mmol) in pyridine (7.50 mL). The mixture was then stirred at room temperature for 20 h. The MnO_2 was removed by filtration and washed with hot water. The filtrate was concentrated under reduced pressure. The residue was then added with water and cooled in an ice bath and neutralised with 10% aq. HCl to pH 6. The brown precipitate acid was collected by filtration and purified by silica gel column chromatography (20% EtOAc/hexane) to provide **5** as a white solid (0.30 g, 50%); m.p. 166–168 °C, lit.[7] m.p. (168–169 °C); FTIR (KBr), ν_{max}: 1667, 1599, 1183, 833, 720 cm^{-1}; ^1H NMR (200 MHz, acetone-d_6): δ 7.79 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$), 6.90 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$), 4.00–3.70 (m, 8H, 2×NCH$_2$C*H*$_2$Cl); ^{13}C NMR (50 MHz, acetone-d_6): δ 168.0, 151.3, 132.6, 119.5, 112.1, 53.6, 41.4 ppm; MS (EI), *m/z* (relative intensity): 261(M^+, 37), 212(100), 148(12), 132(18), 117(10), 104(4), 77(6), 63(5); HRMS (APCI) Calcd for $C_{11}H_{14}NO_2Cl_2$ $[M+H]^+$ 262.0396; found 262.0389.

(E)- and (Z)-N,N-Bis(2-chloroethyl)-4-(2-methoxyvinyl)aniline (**6**): Sodium hexamethyldisilazide (1.0 M in tetrahydrofuran, 14.7 mL, 14.7 mmol) was added dropwise to a 0 °C cooled suspension of (met hoxymethyl)triphenylphosphonium chloride (4.20 g, 12.25 mmol) in anhydrous tetrahydrofuran (20 mL). The deep red mixture was stirred for 20 min at that temperature to ensure complete ylid formation. A solution of **4** (2.00 g, 8.16 mmol) in anhydrous tetrahydrofuran (15 mL) was then added dropwise to the red ylid solution, and the mixture was stirred for another 1 h under N_2 atmosphere at 0 °C. Water was added to the reaction mixture, and the tetrahydrofuran was removed under reduced pressure. The residue was extracted with CH_2Cl_2. The combined organic layer was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (5% EtOAc/hexane) to provide an inseparable mixture of *E*- and *Z*-isomers of enol ether **6** in a ratio of 1:1.25 (2.20 g, 98%) as colourless oil. FTIR (KBr), ν_{max}: 1641, 1608, 1516, 1352, 1240, 1177, 1093, 823 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): 7.49 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$ of *Z* isomer), 7.14 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$ of *E* isomer), 6.91 (d, *J* = 12.8 Hz, 1H, CH=C*H* of *E* isomer), 6.62 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$ of *Z* isomer), 6.61 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$ of *E* isomer), 6.03 (d, *J* = 7.0 Hz, 1H, CH=C*H* of *Z* isomer), 5.75 (d, *J* = 12.8 Hz, 1H, C*H*=CH of *E* isomer), 5.15 (d, *J* = 7.0 Hz, 1H, C*H*=CH of *Z* isomer), 3.75–3.57 (m, 8H, 2×NC*H*$_2$C*H*$_2$Cl of *E* and *Z* isomers), 3.74 (s, 3H, OC*H*$_3$ of *Z* isomer), 3.66 (s, 3H, OC*H*$_3$ of *E* isomer); ^{13}C NMR (50 MHz, $CDCl_3$): δ 147.0$_E$, 145.9$_Z$, 144.2$_E$, 143.9$_Z$, 129.6$_Z$, 126.5$_E$, 126.2$_E$, 126.0$_Z$, 112.6$_E$, 112.0$_Z$, 105.3$_Z$, 104.7$_E$, 60.4$_Z$, 56.5$_Z$, 53.6$_{E\text{ and }Z}$, 40.5$_{E\text{ and }Z}$ ppm; MS (EI), *m/z* (relative intensity): 273(M^+, 22), 224(100), 210(9), 161(33), 146(18), 118(31), 91(31), 63(10); HRMS (APCI) Calcd for $C_{13}H_{18}NOCl_2$ $[M+H]^+$ 274.0760; found 274.0758.

2-(4-(N,N-Bis(2-chloroethyl)amino)phenyl)acetaldehyde (**7**): 2N HCl (1 mL) was added to a solution of **6** (1.0 g, 66.4 mmol) in tetrahydrofuran (10 mL), and the solution was refluxed for 15 min. The mixture was cooled to room temperature, and water was added. The mixture was concentrated under reduce pressure to remove tetrahydrofuran, and the residue was extracted with dichloromethane. The combined organic layer was washed with saturated aqueous $NaHCO_3$ and water, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (10% EtOAc/hexane) to provide **7** (500 mg, 52%) as a colourless oil. FTIR (KBr), ν_{max}: 1718, 1614, 1518, 1353, 1180, 810, 737 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 9.71 (t, *J* = 2.3 Hz, 1H, C*H*O), 7.10 (d, *J* = 8.7 Hz, 2H, C*H*$_{aro}$), 6.69 (d, *J* = 8.7 Hz, 2H, C*H*$_{aro}$), 3.76–3.59 (m, 10H, 2×NC*H*$_2$C*H*$_2$Cl and C*H*$_2$CHO); ^{13}C NMR (75 MHz, $CDCl_3$): δ 199.7, 145.4, 130.9, 120.5, 112.4, 53.4, 49.5, 40.4 ppm; MS (EI), *m/z* (relative intensity): 259(M^+, 18), 230(28), 210(100), 181(6), 146(6), 132(9), 118(27), 75(6); HRMS (APCI) Calcd for $C_{12}H_{16}NOCl_2$ $[M+H]^+$ 260.0603; found 260.0600.

2-(4-(N,N-Bis(2-chloroethyl)amino)phenyl)acetic acid (**8**): A solution of sodium chlorite (384 mg, 4.25 mmol) in NaH_2PO_4 pH 3.5 buffer (3.4 mL) was added dropwise to a stirred solution of **7** (880 mg, 3.4 mmol) and 2-methyl-2-butene (3.6 mL, 34 mmol) in *tert*-butanol (15 mL) at room temperature and the mixture was stirred for 1 h. Then the mixture was basified to pH 10 with 6N NaOH, and the tert-butanol was removed under reduced pressure. The residue was dissolved in water and extracted with hexane. The aqueous layer was acidified to pH 3 with 10% HCl and extracted with diethyl ether. The organic layer was washed with water, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (50% EtOAc/hexane) to provide **8** (600 mg, 64%) as a white solid, m.p. 103.5–104.5 °C (lit.[8] 105 °C); FTIR (KBr), ν_{max}: 1704, 1616, 1520, 1354, 1249, 1181, 805 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.17 (d, *J* = 8.7 Hz, 2H, C*H*$_{aro}$), 6.65 (d, *J* = 8.7 Hz, 2H, C*H*$_{aro}$), 3.74–3.59 (m, 8H, 2×NC*H*$_2$C*H*$_2$Cl), 3.55 (s, 2H, C*H*$_2$COOH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.5, 145.3, 130.7, 122.2, 112.1, 53.5, 40.4, 39.8 ppm; MS (EI), *m/z* (relative intensity): 275(M^+,8), 226(100), 144(8), 132(8), 118(45), 90(9), 77(7), 63(12); HRMS (APCI) Calcd for $C_{12}H_{16}NO_2Cl_2$ $[M+H]^+$ 276.0553; found 276.0553.

Ethyl 3-[4-(N,N-bis(2-chloroethyl)amino)phenyl]prop-2-enoate (**9**): Triethyl phosphonoacetate (0.88 mL, 4.41 mmol) was slowly added to a 0 °C cooled stirred suspension of NaH (60% oil dispersion, 0.26 g, 6.61 mmol) in dry tetrahydrofuran (5 mL), and the reaction mixture was stirred at 0 °C for 30 min under N_2 atmosphere. A solution of **4** (0.90 g, 3.67 mmol) in dry tetrahydrofuran (10 mL) was then added, and the reaction mixture was stirred at room temperature for an additional 1.5 h. The reaction mixture was added with water and extracted with ethyl acetate. The combined organic layer was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (20% EtOAc/hexane) to yield **9** (1.15 g, 99.3%) as a pale yellow solid; m.p. 78–79 °C; FTIR (KBr), ν_{max}: 1698, 1597, 1518, 1153, 813 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 7.61 (d, *J* = 16.0 Hz, 1H, C*H*=CH), 7.44 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$), 6.68 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$), 6.26 (d, *J* = 16.0 Hz, 1H, CH=C*H*), 4.25 (q, *J* = 7.3 Hz, 2H, C*H*$_2$CH$_3$), 3.60–3.80 (m, 8H, 2×NC*H*$_2$C*H*$_2$Cl), 1.33 (t, *J* = 7.3 Hz, 3H, CH$_2$C*H*$_3$); ^{13}C NMR (50 MHz, $CDCl_3$): δ 167.5, 147.6, 144.2, 130.0, 124.0, 114.1, 111.9, 60.2, 53.3, 40.2, 14.3 ppm; MS (EI), *m/z* (relative intensity): 315(M^+, 84), 266(100), 157(4); HRMS (APCI) Calcd for $C_{15}H_{20}NO_2Cl_2$ $[M+H]^+$ 316.0866; found 316.0872.

Ethyl 3-[4-(N,N-bis(2-chloroethyl)amino)phenyl]propanoate (**10**): A mixture of **9** (1.00 g, 4.08 mmol) and 10% Pd/C (0.01 g) in ethyl acetate (5.0 mL) was evacuated and flushed thrice with hydrogen gas. After stirring under H_2 atmosphere for 1.5 h, the Pd/C was removed by filtration through Celite and washed with ethyl acetate. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (5% EtOAc/hexane) to provide **10** (0.84 g, 83.5%) as a pale yellow oil (lit.[8] 40.5 °C); FTIR (KBr), ν_{max}: 1731, 1519, 1353, 1180, 811 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 7.09 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$), 6.67 (d, *J* = 8.8 Hz, 2H, C*H*$_{aro}$), 4.13 (q, *J* = 7.3 Hz, 2H, C*H*$_2$CH$_3$), 3.79–3.55 (m, 8H, 2×NC*H*$_2$C*H*$_2$Cl), 2.87 (t, *J* = 7.3 Hz, 2H, C*H*$_2$CH$_2$), 2.57 (t, *J* = 7.3 Hz, 2H, CH$_2$C*H*$_2$), 1.24 (t, *J* = 7.3 Hz, 3H, CH$_2$C*H*$_3$); ^{13}C NMR (50 MHz, $CDCl_3$): δ 173.0, 144.0, 129.6, 122.4, 112.7, 60.4, 53.8, 40.2, 36.1, 29.9, 14.2 ppm; MS (EI), *m/z* (relative intensity): 317(M^+, 33), 268(100), 240(10), 230(5), 179(3), 132(6), 118(7), 77(3); HRMS (APCI) Calcd for $C_{15}H_{22}NO_2Cl_2$ $[M+H]^+$ 318.1022; found 318.1017.

3-[4-N,N-Bis(2-chloroethyl)amino)phenyl]propanoic acid (**11**): A solution of **10** (100 mg, 0.32 mmol) in conc. HCl (5 mL) was heated at 100 °C for 4 h. After cooling to room temperature, the reaction mixture was poured into ice water and neutralised to pH 6 with saturated aqueous $NaHCO_3$. The reaction mixture was extracted with ethyl acetate. The combined organic layer was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (50% EtOAc/hexane) to yield **11** (89 mg, 97.8%) as a white solid, m.p. 113.5–114.5 °C, lit.[8] 114–115 °C); FTIR (KBr), ν_{max}: 1710, 1524, 1210, 816 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 7.14 (d, *J* = 8.4 Hz, 2H, C*H*$_{aro}$), 6.62 (d, *J* = 8.4 Hz, 2H, C*H*$_{aro}$), 3.80–3.54 (m, 8H, 2×NC*H*$_2$C*H*$_2$Cl), 2.89 (t, *J* = 7.4 Hz, 2H, C*H*$_2$CH$_2$), 2.64 (t, *J* = 7.4 Hz, 2H, CH$_2$C*H*$_2$); ^{13}C NMR

(50 MHz, $CDCl_3$): δ 178.7, 144.7, 129.5, 129.4, 112.4, 53.6, 40.5, 35.8, 29.5 ppm; MS (EI), *m/z* (relative intensity): 289(M^+, 27), 240(100), 180(10), 144(6), 132(6), 118(17), 91(5), 77(6); HRMS (APCI) Calcd for $C_{13}H_{18}NO_2Cl_2$ $[M+H]^+$ 290.0709; found 290.0704.

*Synthesis of coronarin D linked mustard agents (***12–15***); general procedure*

A solution of the nitrogen mustard agent (1 mmol) and dimethylaminopyridine (0.3 mmol) in CH_2Cl_2 (5 mL) was added to a solution of coronarin D (1 mmol) in CH_2Cl_2 (5 mL). The mixture was stirred at room temperature for 5 min with guard tube; a solution of dicyclohexylcarbodiimide (1 mmol) in CH_2Cl_2 (10 mL) was then added, and the reaction mixture was stirred at room temperature for a further 5 h. Water was then added, and the reaction mixture was extracted with CH_2Cl_2. The combined organic layer was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to provide the desired coronarin D linked mustard agent.

Coronarin D N,N-bis(2-chloroethyl)-4-aminobenzoate (**12**): FTIR (KBr), ν_{max}: 2930, 1768, 1720, 1604, 1521, 1270, 1178, 1096, 957 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 7.90 (d, *J* = 8.8 Hz, 2H), 6.90–6.80 (m, 2H), 6.65 (d, *J* = 8.8 Hz, 2H), one set (1H) of 4.84 (s)/4.41 (s) and one set (1H) of 4.83 (s)/4.35 (s), 3.84–3.62 (m, 8H), 3.30–3.10 (m, 1H), 3.00–2.85 (m, 1H), 0.88 (s, 3H), 0.81 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 168.9, 164.5, 150.4, 148.1/148.0, 144.5/144.4, 132.3, 122.6, 117.2, 110.9, 107.4, 92.9, 56.1, 55.4, 53.2, 42.0, 40.0, 39.5, 39.3, 37.8, 33.5, 32.3, 32.2, 25.7, 24.1, 21.7, 19.3, 14.3 ppm; MS (EI), *m/z* (relative intensity): 486(25), 394(27), 358(15), 290(100), 277(16), 215(31), 164(9), 152(28); HRMS (APCI) Calcd for $C_{31}H_{42}NO_4Cl_2$ $[M+H]^+$ 562.2485; found 562.2481.

Coronarin D N,N-bis(2-chloroethyl)-4-aminophenylethanoate (**13**): FTIR (KBr), ν_{max}: 2931, 1770, 1520, 1131, 973, 960 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.14 (d, *J* = 8.2 Hz, 2H), 6.85–6.75 (m, 1H), 6.70–6.60 (m, 3H), one set (1H) of 4.84 (s)/4.38 (s) and one set (1H) of 4.82 (s)/4.33 (s), 3.74–3.52 (m, 10H), 3.20–3.05 (m, 1H), 2.85–2.75 (m, 1H), 0.89 (s, 3H), 0.82 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3, 168.7, 148.0, 145.3, 144.8/144.7, 130.5, 122.2, 121.6, 112.1, 107.4/107.3, 92.7, 56.1, 55.3, 53.4, 42.0/41.9, 40.4, 39.9/39.8, 39.4, 39.3/39.2, 37.7, 33.5, 32.0, 31.9, 25.7, 24.0, 21.7, 19.3/19.2, 14.3 ppm; MS (EI), *m/z* (relative intensity): 575(M^+, 4), 526(10), 230(100), 226(52), 118(52), 81(43), 69(72), 55(50); HRMS (APCI) Calcd for $C_{32}H_{44}NO_4Cl_2$ $[M+H]^+$ 576.2642; found 576.2660.

Coronarin-D N,N-bis(2-chloroethyl)-4-aminophenylpropanoate (**14**): FTIR (KBr), ν_{max}: 2929, 1764, 1519, 1134, 958 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 7.09 (d, *J* = 8.8 Hz, 2H), 6.86–6.77 (m, 1H), 6.69–6.67 (m, 1H), 6.62 (d, *J* = 8.8 Hz, 2H), one set (1H) of 4.83 (s)/4.38 (s) and one set (1H) of 4.82 (s)/4.32 (s), 3.75–3.57 (m, 8H), 3.20–3.02 (m, 1H), 2.90–2.56 (m, 5H), 0.89 (s, 3H), 0.82 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 171.2, 168.7, 148.1, 144.7, 144.6, 129.5, 129.1, 122.2, 112.4, 107.5, 92.3, 56.2, 55.4, 53.6, 42.0, 40.5, 39.5, 39.4, 37.8, 36.0, 33.6, 32.0, 31.9, 29.4, 25.8, 24.1, 21.7, 19.3, 14.4 ppm; MS (EI), *m/z* (relative intensity): 589(M^+, 85), 553(14), 540(100), 498(4), 337(9), 289(5), 240(18), 91(6); HRMS (APCI) Calcd for $C_{33}H_{46}NO_4Cl_2$ $[M+H]^+$ 590.2798; found 590.2789.

Coronarin D N,N-bis(2-chloroethyl)-4-aminophenylbutanoate (**15**): FTIR (KBr), ν_{max}: 2932, 1762, 1519, 1387, 1134, 959 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 7.05 (d, *J* = 8.8 Hz, 2H), 6.88–6.75 (m, 1H), 6.70–6.60 (m, 3H), one set (1H) of 4.82 (s)/4.37 (s) and one set (1H) of 4.82 (s)/4.32 (s), 3.75–3.57 (m, 8H), 3.20–3.02 (m, 1H), 2.86–2.70 (m, 1H), 2.62–2.50 (m, 2H), 0.88 (s, 3H), 0.81 (s, 3H), 0.71 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 171.7, 168.7, 148.0, 144.7/144.6, 144.1, 130.1, 129.7, 122.2, 112.7, 107.5/107.4, 92.3, 56.1, 55.4, 53.8, 42.0, 40.4, 39.5, 39.3, 37.8, 33.8, 33.6, 33.3, 32.0, 31.9, 26.2, 25.7, 24.1, 21.7, 19.3, 14.4 ppm; MS (EI), *m/z* (relative intensity): 603(M^+,12), 554(46), 302(32), 286(100), 254(87), 238(38), 230(22), 224(35); HRMS (APCI) Calcd for $C_{34}H_{48}NO_4Cl_2$ $[M+H]^+$ 604.2955; found 604.2948.

Cytotoxicity studies

The cytotoxic activity of compounds **1**, **5**, **8**, and **11–15** was evaluated against a panel of cancer cell lines, namely, MOLT-3, HepG2, HuCCA-1, and A549, using previously reported procedures.[14] Briefly, the cells in the logarithmic growth phase were seeded in 96-well plates (Costar, 3599, USA) at a density of 10,000–15,000 cells/well, and incubated at 37 °C in a humidified atmosphere with 95% air and 5% CO_2. After 24 h, an additional medium containing either the test compounds or the vehicle was added at the desired final concentrations, and the cultures were further incubated for 48 h. Afterward, the cell monolayer was washed with phosphate-buffered saline PBS (pH 7.2), fixed with 95% ethanol, stained with crystal violet solution, and lysed with 0.1 N HCl in methanol. The absorbance of the lysate was measured at 540 nm on an automatic microtiter plate reader (Multiskan Ascent, Labsystem). All tests were carried out in triplicate, and the mean value was calculated. The activity was expressed as IC_{50} (concentration of substances that inhibits 50% of cell growth) using etoposide and doxorubicin (Sigma Aldrich) as the standard drugs.

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References

1 J. Everett, J. Roberts and W. Ross, *J. Chem. Soc.*, 1953, 2386.
2 L. Hurley, *Nat. Rev. Cancer*, 2002, **2**, 188.
3 H. Itokawa, H. Morita, I. Katou, K. Takeya, A.J. Cavalheiro, R.C.B. de Oliveira, M. Ishige and M. Motidome, *Planta Med.*, 1988, **54**, 311.
4 N. Chimnoi, S. Pisutcharoenpong, L. Ngiwsara, D. Dechtrirut, D. Chokchaichamnankit, N. Khunnawutmanotham, C. Mahidol and S. Techasakul, *Nat. Prod. Res.*, 2008, **22**, 1255.
5 N. Chimnoi, C. Sarasuk, N. Khunnawutmanotham, P. Intachote, S. Seangsai, B. Saimanee, S. Pisutjaroenpong, C. Mahidol and S. Techasakul, *Phytochem. Lett.*, 2009, **2**, 184.
6 A.B. Kunnumakkara, H. Ichikawa, P. Anand, C.J. Mohankumar, P.S. Hema, M.S. Nair and B.B. Aggarwal, *Mol. Cancer Ther.*, 2008, **7**, 3306.
7 W.C.J. Ross, *J. Chem. Soc.*, 1949, 183.
8 R.M. Silverstein, E.E. Ryskiewicz and C. Willard, *Org. Synth.*, 1963, Coll. **4**, 831.
9 M. Treu and U. Jordi, *Molecules*, 2002, **7**, 374.
10 G.A. Kraus and B. Roth, *J. Org. Chem.*, 1980, **45**, 4825.
11 A.K. Dutta, C. Xu and M.E.A. Reith, *J. Med. Chem.*, 1996, **39**, 749.
12 T.J. Bardos, N. Datta-Gupta, P. Hebborn and D.J. Triggle, *J. Med. Chem.*, 1965, **8**, 167.
13 A. Sunters, C.J. Springer, K.D. Bagshawe, R.L. Souhami and J.A. Hartley, *Biochem. Pharmacol.*, 1992, **44**, 59.
14 T. Tengchaisri, R. Chawengkirttikul, N. Rachaphaew, V. Reutrakul, R. Sangsuwan and S. Sirisinha, *Cancer Lett.*, 1998, **133**, 169.