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Synthesis, DNA Interactions and Biological Activity of DNA Minor Groove Targeted Polybenzamide-Linked Nitrogen Mustards

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Abstract—A series of polybenzamide DNA minor groove binding ligands bearing either one or two monofunctional mustards have been synthesised, and their cytotoxicities and interactions with DNA have been studied. Analogues with two alkylating functions (e.g. compounds **7** and **14**) are the most cytotoxic, with **7** being 1000-fold more potent than the clinical mustard chlorambucil against P388 leukemia in culture, as well as being more potent *in vivo*. Monofunctional analogues were also significantly more cytotoxic than chlorambucil, despite bearing much less reactive mustard species. These results support the concept that targeting nitrogen mustard alkylating agents to DNA by attachment to DNA-affinic carriers can greatly enhance cytotoxicity due to alkylation, and that even for such DNA-targeted mustards, crosslinking is a more toxic event than monoalkylation. Close analogues of **7** differing only in their radius of curvature, appear to alkylate and crosslink DNA in similar fashion, yet have widely differing cytotoxicities. The most cytotoxic compound (**7**) possesses a geometry most complementary to that of duplex DNA, suggesting that the most toxic lesions are those which result in least DNA distortion, thus being less easily recognised by DNA repair systems.

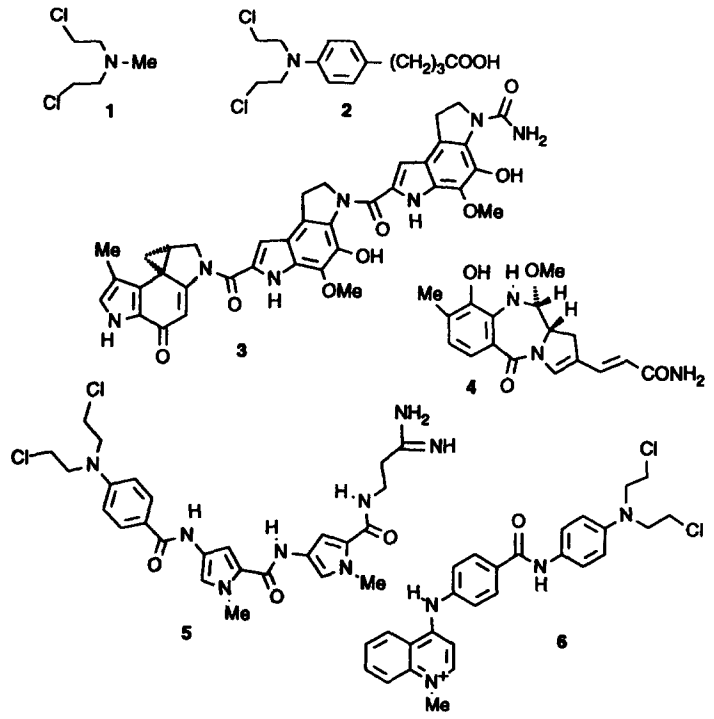
Introduction

The nitrogen mustard bisalkylating agents used in cancer chemotherapy, for example mechlorethamine (**1**) and chlorambucil (**2**), are reactive compounds with a 'bonding-dominated'[1] mechanism of DNA alkylation. Therefore they target DNA at the most electronegative sites, with monoadducts occurring primarily at the N7 of guanines in runs of guanines,[2] and interstrand crosslinks between the N7 positions of guanines in each strand at 5'-GNC sequences.[3]

However, simple mustards (and other alkylating agents) show only slight selectivity for longer DNA sequences, and their therapeutic properties are considerably due to cytokinetic effects. The guanine N7 adducts which they primarily form are among the most easily repaired. The development of alkylating agents with higher degrees of sequence specificity and/or altered sites of attack on DNA is of interest, because such compounds might possess alternative therapeutic mechanisms (for example, selective inhibition of oncogene expression).[4,5] One design for achieving this is to link the agents to (or incorporate them in) carrier structures (ligands) which also possess an intrinsic reversible 'DNA-binding' capability to the minor groove. Because most minor groove binding ligands cover relatively large binding sites, which occur on average less frequently, there is the potential for considerable sequence specificity (for example, a unique 8-base pair sequence occurs on average only once in every 6×10^5 base pairs). The binding orientation of the ligand may redirect alkylation to new sites on DNA, providing lesions which may be less readily repairable and thus more cytotoxic. Allied to this, the localisation due to reversible binding of the carrier to DNA may allow alkylating agents of lower reactivity to be used.

Most of the work to date in minor groove directed alkylating agents has been with non-mustard derivatives such as CC-1065 (**3**),[6] and anthramycin (**4**)[1,7] and its synthetic analogues.[8] Studies with these compounds have verified most of the above points, and in particular have demonstrated that drugs of this type can exhibit very high cytotoxicity. However, three classes of DNA minor groove targeted mustards have also been reported. The distamycin analogue FCE 24517 (**5**), bearing an aniline mustard of low chemical reactivity, has been shown to be a weak[9] but highly sequence-specific[10] DNA alkylating agent, reacting at adenines in AT-rich regions, probably in the minor groove.[11] Studies[12] of the kinetics of binding of the 4-anilinoquinolinium mustard **6**[13] and analogues to DNAs of varying sequence indicated that these compounds also react in the minor groove.

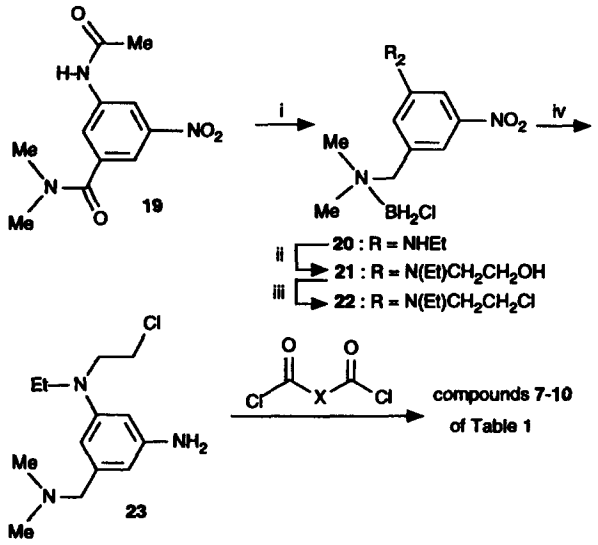
We have previously shown[14] that the novel polybenzamide 'split' bifunctional mustard **7** alkylates DNA preferentially in the minor groove at the N3 position of adenines in AT-rich sequences. Because of



the high cytotoxic potency and *in vivo* antitumor activity shown by this compound, we have now prepared further analogues, in order to explore relationships between compound structure and interaction with DNA in this series.

Results

The structures of the polybenzamide mustards studied here are recorded in Table 1. The symmetrical derivatives **7–10** were conveniently prepared by coupling the appropriate dicarbonyl dichloride with the amine **23**. The latter compound was prepared by the reported[14] route, but a different, boron-containing product (**20**) was obtained following reduction of **19** with BH_3·DMS complex (Scheme 1). This was identified as the mono-coordinated BH_2Cl complex by combustion analysis and by X-ray crystallographic analysis of the later intermediate **22** (Fig. 1). The complex **20** was consistently obtained, and the BH_2Cl moiety was retained in the next two steps of the synthesis, but was efficiently removed by treatment with $SnCl_2$/conc. HCl at 100 °C to give the known amine **23**. Use of the boron complexes was not intentional but proved beneficial, since these intermediates were much less polar than the corresponding free amines, facilitating their isolation and purification.



Scheme 1
i BH_3 Me_2S/THF/reflux/3.5 h, then MeOH/conc. HCl/20 °C/10 min.
ii Oxirane/THF/AcOH/20 °C/36 h.
iii $MsCl/CH_2Cl_2/NEt_3$ 20 °C/1 h, then LiCl/DMF/75 °C 30 min.
iv $SnCl_2$/conc. HCl/90 °C/1 h.

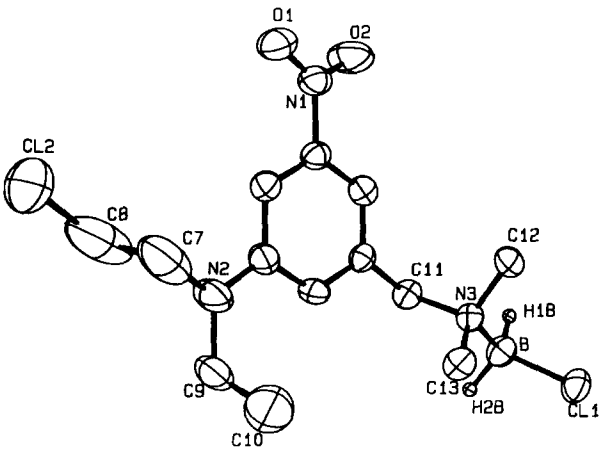
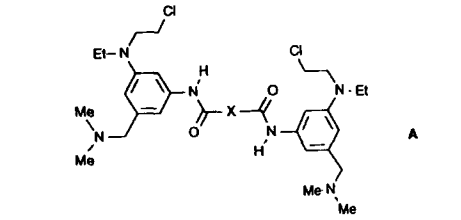


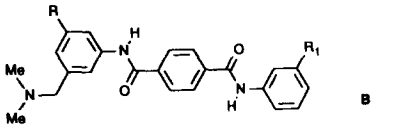
Figure 1. ORTEP diagram of *N*,*N*-dimethyl-3-[*N*-(2-chloroethyl)-*N*-ethylamino)-5-nitrobenzylamine·BH_2Cl complex (**22**).

The unsymmetrical derivative **12** was prepared from the acid **27**. This was elaborated from 3-(*N*,*N*-dimethylaminomethyl)aniline (**24**)[15] by coupling with 4-

(methoxycarbonyl)benzenecarbonyl chloride (**25**), followed by basic hydrolysis of the resulting ester **26** (Scheme 2). Coupling of **27** with **23** using diethyl cyanophosphonate gave **12**, coupling with **31** gave **15**, and coupling with **24** gave the non-mustard derivative **11**. A similar reaction between the amine **23** and 4-phenylcarbamoylbenzenecarboxylic acid gave **13**. The monocationic bis(mustard) **14** was prepared similarly

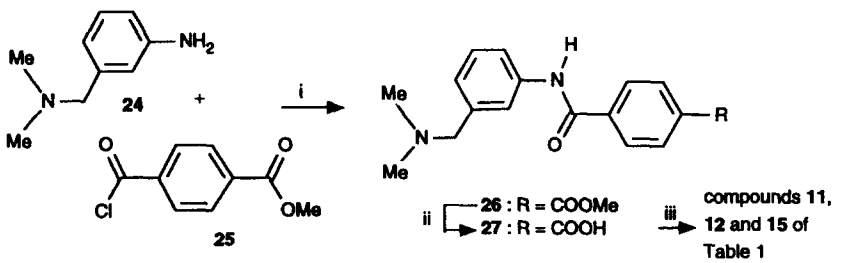
Table 1. Structural and biological data for polybenzamide mustards





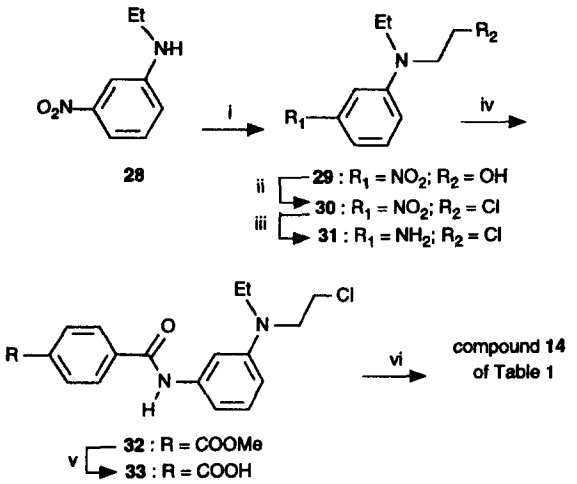
No.	form.	R	R_1	mp	formula	analyses	388 leukemia IC_{50}[a]	388 leukemia OD[b]	388 leukemia ILS[c]
7	A	X1		> 300	$C_{34}H_{46}Cl_2N_6O_2$	Ref. 14	0.007	8.9	37
8	A	X2		135–138	$C_{34}H_{46}Cl_2N_6O_2$	C,H,N,Cl	2.91	8.9	NA[d]
9	A	X3		142–143	$C_{32}H_{46}Cl_2N_8O_2$	C,H,N,Cl	0.17	5.9	NA
10	A	X4		> 250	$C_{36}H_{54}Cl_2N_6O_2$	C,H,N,Cl	0.43	5.9	NA
11	B	H	CH_2NMe_2	> 300	$C_{26}H_{30}N_4O_2 \cdot 2HCl$	C,H,N,Cl	0.39		
12	B	$N(Et)CH_2CH_2Cl$	CH_2NMe_2	162–165	$C_{30}H_{38}ClN_5O_2$	C,H,N,Cl	0.25	3.9	NA
13	B	$N(Et)CH_2CH_2Cl$	H	186–189	$C_{27}H_{31}ClN_4O_2$	C,H,N,Cl	0.37	5.9	NA
14	B	$N(Et)CH_2CH_2Cl$	$N(Et)CH_2CH_2Cl$	> 250	$C_{31}H_{39}Cl_2N_5O_2$	C,H,N,Cl	0.027	2.6	NA
15	B	H	$N(Et)CH_2CH_2Cl$	160–161	$C_{27}H_{31}ClN_4O_2$	C,H,N,Cl	0.33	2.6	NA
16				223–224	$C_{25}H_{26}N_4O_3$	C,H,N	15		
17				> 300	$C_{33}H_{42}Cl_2N_6O_3 \cdot H_2O$	C,H,N,Cl	0.63		
18				> 300	$C_{33}H_{42}Cl_2N_6O_3$	C,H,N,Cl	0.67		
2		chlorambucil					6.75[e]	225[e]	37[e]

[a] IC_{50}: concentration of drug (μM) to inhibit growth of P388 leukemia cells in culture to 50% of control values, after a 70 h exposure; Ref. 30. Values are the average of three determinations.
[b] OD: Dose of drug (mg kg^{-1} day^{-1}), administered intraperitoneally as a solution in 0.1 mL of 30% v/v EtOH–water as a single dose 24 h after intraperitoneal inoculation of 10^6 P388 leukemia cells (optimal dose for active compounds, maximum tolerated dose for inactive compound).
[c] ILS: percentage increase in lifespan of drug-treated, tumour-bearing animals compared with tumour-bearing controls, when treated at the optimal dose. Values of > 20% are considered significant (see Ref. 30).
[d] Inactive at all non-toxic doses.
[e] Data from Ref. 19.



Scheme 2
i Pyridine/0 °C.
ii NaOH/MeOH/H_2O/100 °C/1 h.
iii DMF/1-Meimidazole/DECP/25 °C/1.5 h.

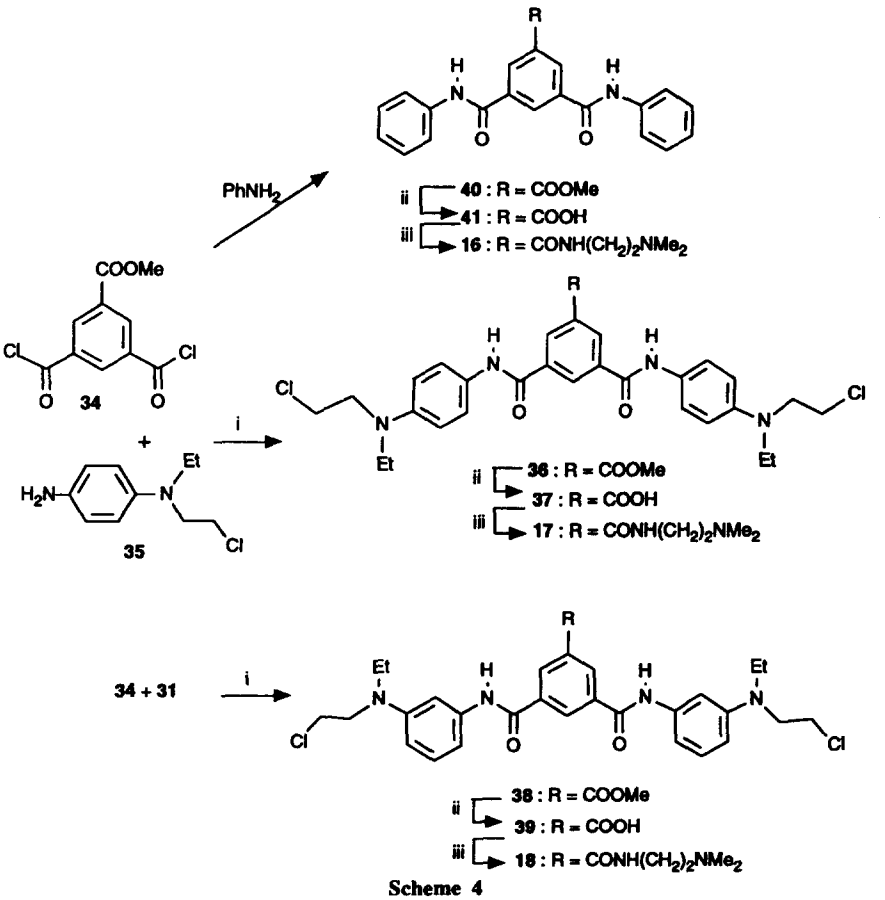
from the amine **23** and the acid **33**. The latter compound was obtained (Scheme 3) by reaction of *N*-ethyl-3-nitroaniline (**28**) with oxirane, and conversion of the resulting alcohol **29** to the chloride **30** by sequential treatment with MsCl/LiCl. Reduction of **30** with $SnCl_2$ at 90 °C for 1 h gave the amine **31**, which was coupled with 4-methoxycarbonylbenzenecarbonyl chloride, and the resulting ester **32** was hydrolysed with base.



Scheme 3
i Oxirane/THF/AcOH/20 °C/ 36 h.
ii $MsCl/CH_2Cl_2/NEt_3$ 20 °C/1 h, then LiCl/DMF/75 °C 30 min.
iii $SnCl_2$/conc. HCl/90 °C/1 h.
iv Acid chloride/CH_2Cl_2/0-20 °C/15 min.
v KOH/MeOH/25 °C/5 h.
vi DMF/1-Meimidazole/DECP/25 °C/1.5 h.

Compounds **17** and **18**, bearing central cationic units, and the corresponding non-mustard **16** were prepared by reaction of 5-methoxycarbonyl-1,3-benzenedicarbonyl dichloride (**34**) with the appropriate amines, followed by base hydrolysis of the ester in a two-phase system and coupling of the resulting acids with *N,N*-dimethylethylenediamine (Scheme 4).

By analogy with the polypyrrole antibiotics such as **5**, and from structural measurements,[16] the polybenzamide compounds prepared here are expected to adopt a flat, annular conformation complementary to that of the DNA minor groove. Compounds **8–10** retain the same terminal units as **7**, and thus the same relative positioning of cationic and mustard functions with respect to each other, but the different central unit result in differing annular dimensions. Compounds **12–15** retain the same overall structural geometry as **7**, and explore the effects of systematically varying the number and relative positioning of cationic and/or alkylating functions. Compounds **17** and **18** have a single cationic unit linked to the central aromatic ring. The non-alkylating 'parent' compounds **11** and **16** were prepared for comparative purposes.



Scheme 4
I CH_2Cl_2/EtN(iPr)$_2$/20 °C/1 h.
ii THF/1.0 N aqueous NaOH/20 °C/12 h.
iii CDI/DMF/20 °C/30 min, then $Me_2N(CH_2)_2NH_2$/20 °C/5 min.

Generally, the cytotoxicities and antitumor effects of simple mustards have been shown[17,18] to correlate closely with their chemical reactivities, since this largely dictates the rates of their bonding to DNA. One of the goals of the present work was to see whether localisation (in the most general sense) due to reversible binding of the carrier to DNA would allow the use of mustards of lower reactivity, as has been shown previously in other series of DNA-targeted alkylators.[19,20] Therefore the kinetics of both hydrolysis and DNA alkylation of the compounds were determined.

The kinetics of hydrolysis of the mustards under physiological-type conditions (pH 7.5 aqueous buffer, ionic strength 10 mM at 37 °C) were determined by HPLC. This method allows analysis of the kinetics of displacement of both chlorines to be followed from the difunctional compounds. Figure 2 shows typical plots of the peak areas of the hydrolysis products of **7** as a function of time, indicating the reaction follows a consecutive first order law. The integrated rate equations for such a series of first-order reactions:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \text{ where:}$$

$$A = A_0 e^{-k_1 t}$$

$$B = A_0 k_1/k_2 - k_1(e^{-k_1 t} - e^{-k_2 t})$$

$$C = A_0 - A - B$$

where A, B and C are the concentration at time t, A_0 is the initial concentration of mustard and k_1 and k_2 are the pseudo first-order rate constants for the first and second hydrolysis steps. Values of k_1 and k_2 were computed by iteration, and are recorded in Table 2.

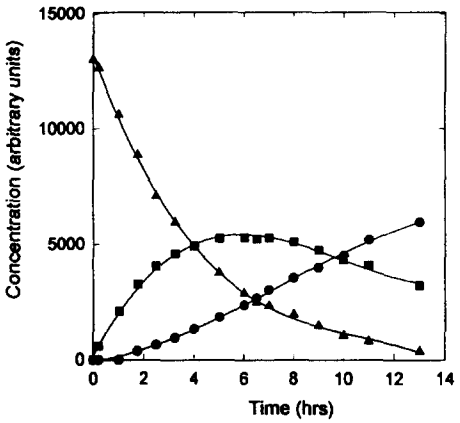


Figure 2. Time-course of hydrolysis of **7** in bistrispropane buffer (20 mM, pH 7.5, ionic strength 10 mM) at 37 °C, monitored by HPLC. Bismustard **7** (▲); corresponding half-mustard (■); corresponding diol (●).

As expected for symmetrical compounds, the first and second rate constants for the symmetrical bismustards **7–9** are almost identical at *ca* 7×10^{-5} s^{-1} (the reason for the slightly lower value of k_2 for **10** is not clear, since this is also a symmetrical compound). In contrast, the unsymmetrical bismustard **14** shows a significant difference between k_1 and k_2. The larger rate constant (14.6×10^{-5} s^{-1}) is similar to that of the monomustard **15** (19.9×10^{-5} s^{-1}), and is attributed to the more reactive mustard on the ring not bearing a charged group. The smaller rate constant (5.8×10^{-5} s^{-1}) is similar to those of compounds **7–9**, reflecting stabilisation of this mustard by the electron-withdrawing

Table 2. DNA interactions of the polybenzamide mustards of Table 1

No.	k_1[a] 10^5 k_{obs} (s^{-1})	k_2[a] 10^5 k_{obs} (s^{-1})	k_Σ[b]	X_{50}[c]
7	6.7 ± 0.1	5.7 ± 0.4	5.8 ± 0.2	0.07
8	7.6 ± 0.1	6.9 ± 0.5	11.6 ± 0.3	0.1
9	6.3 ± 0.2	5.3 ± 0.7	12.5 ± 0.1	0.07
10	7.06 ± 0.02	3.47 ± 0.03	18 ± 1	0.1
12	3.2 ± 0.2	-	3.1 ± 0.1	-
13	4.1 ± 0.3[d]	-	ND[e]	-
14	14.6 ± 0.2	5.8 ± 0.3	12 ± 1	0.1
15	19.9 ± 0.2	-	33 ± 1	-
17	51 ± 1[f]	29 ± 2[f]	g	0.3
18	26 ± 2[f]	23 ± 1[f]	g	*ca* 1.0[h]
2	32.5 ± 1.5	18.3 ± 2.9		*ca* 5

[a] k_1, k_2; rate constants for first and second hydrolysis of mustard units. Values are the average of 3 determinations ± SEM.
[b] k_Σ; rate constant for loss of parent compound in the presence of calf thymus DNA. Values are the average of 3 determinations ± SEM.
[c] Drug:base pair ratio causing approximately 50% crosslinking of linearised pBR322 DNA, determined by agarose gel electrophoresis.
[d] Data variable.
[e] Not determinable due to very variable data.
[f] Data determined at pH 6.2 (drug precipitates at pH 7.5).
[g] Compounds caused precipitation of DNA at pH 7.5 and 6.2; no measurements possible.
[h] Estimated by extrapolation.

$CH_2N^+Me_2$ group on the same ring in these units. These latter mustards are thus relatively unreactive in comparison with the clinically-used aromatic mustard chorambucil (**2**), which had a measured k_1 of 32.5×10^{-5} s^{-1}, comparable to that (38.6×10^{-5} s^{-1}) previously reported.[21] The rates of hydrolysis of **17** and **18**, bearing mustards on unsubstituted rings, are comparable to that of **15**, as expected (although these studies were conducted at pH 6.2 for solubility reasons).

The rate constants for loss of parent compound when exposed to DNA were determined by HPLC, and are given in Table 2. While this rate (k_Σ) is a composite of both hydrolysis and alkylation reactions, the degree of hydrolysis can be monitored by observation of the hydrolysed product. At a drug:base pair ratio of 1:10, no detectable hydrolysis products were observed for the dicationic bismustards **7–10** and monomustard **12**, suggesting that the loss of parent compound is due primarily to alkylation of DNA. This is in contrast to a recent study on the interaction of aniline mustards linked to DNA-intercalating ligands,[22] where the majority of the drug was hydrolysed and only a small fraction gave rise to DNA adducts. This was suggested[22] to be due to the well-known catalytic effect of DNA on the hydrolysis of reactive species which proceed through a cationic intermediate, by stabilisation of that intermediate.

However, hydrolysis products were detected in the incubation of the monocationic mustards **14** and **15** with DNA. With **15**, this comprised 1.8% of the total HPLC peak area after 25 min, and 71% after 3 h (by which time 95% of the parent compound had reacted). With **14**, the hydrolysis product was 4.6% of the total HPLC peak area after 1 h and 31% after 4 h. It is worth noting that, as well as possessing only one cationic centre, which may lower their level of reversible DNA binding, **14** and **15** also carry the more reactive mustards. Both of these effects are expected to favor hydrolysis over alkylation. For the dicationic, less reactive mustards **7–10** and **12**, rates of DNA alkylation were generally similar to, or faster than rates of hydrolysis. The monocationic, more reactive monomustard **15** had the largest k_Σ value (33×10^{-5} s^{-1}), but concomitant hydrolysis may be contributing to this. The analogues **17** and **18** with a centrally-attached cationic charge formed precipitates of a DNA/drug complex at both pH 7.5 and 6.2, and no data could be obtained for these derivatives.

The efficiency of DNA crosslinking by the bismustards **7–10**, **14**, **17** and **18** was studied by incubation of the compounds with linearised pBR322 DNA at varying drug:base pair ratios, followed by denaturation with methylmercury hydroxide.[23] The extent of crosslinking was then determined by comparison of the single- and double-strand species. The drug:base pair ratio required for 50% crosslinking were then estimated, and these are given in Table 2. All of the compounds with terminal cationic units gave comparable results (a drug:base pair ratio of 0.05–0.1), while the two compounds with centrally-positioned cationic functions (**17** and **18**) were much less effective cross-linkers. However, all were much more efficient than the untargeted mustard chlorambucil (**2**), despite the higher reactivity of the latter (Table 2).

The sites of alkylation by **7** have been shown to be at the N3 of adenines,[14] and it is therefore likely that DNA crosslinking by all these compounds occurs between adenines in AT-rich regions.

The cytotoxicities of the compounds were determined against P388 cells in a continuous exposure assay. The results confirmed earlier observations[14] of the very high potency of **7** (0.007 μM). This compares with a value of 6.75 μM for chlorambucil (**2**),[14] and 0.49 μM for the anilinoquinoline mustard **6**.[13] The pyrazole analogue **9** was 20-fold less cytotoxic (IC_{50} 0.17 μM), while the *meta*-substituted benzene derivative **8** was 20-fold less effective again (IC_{50} 2.9 μM). Activity thus declines with increasing radius of curvature of the molecule. Since the compounds all have mustards of comparable reactivity, and appear to alkylate DNA at approximately the same rate (Table 2), the very different levels of cytotoxicity suggest the compounds form lesions of widely differing cytotoxicity. Compounds **17** and **18**, bearing a single, centrally-attached cationic charge, were much more cytotoxic than the corresponding parent compound **16**. This is likely to be due to alkylating events, but no information on the interaction with DNA was obtained. Compounds **8–10** and **12–15** were inactive against P388 leukemia *in vivo* using a single dose schedule (Table 1), although **8–10** did show modest activity (ILS *ca* 40%) using a schedule of three daily doses.

The radii of curvature of compounds **7–10**, and the spatial separation of the mustard nitrogens in these compounds, were estimated from energy-minimised structures in conformations which had both the amide NH groups and alkylating units on the concave face (Table 3). The radius of curvature of DNA was similarly estimated from various oligodeoxynucleotide crystal structures[24] measured from the adenine N3 atoms in the floor of the minor groove. These calculations show that the radius of curvature of **7** (13.4 Å) most closely approximates that of DNA (11.1 Å), with the other compounds fitting less well.

Discussion

The results with compounds **12–15**, which share the basic structure of **7** but which have varying combinations of cationic and mustard functions, clearly show the importance of two alkylating units. Compound **14**, the only one with two mustard units, is by far the most cytotoxic (IC_{50} 0.027 μM), despite having only one cationic centre. In contrast, the other three monofunctional compounds are more than 10-fold less cytotoxic (IC_{50}s *ca* 0.3 μM; no different than the corresponding non-alkylating analogue **11**), with the

presence of one or two cationic centres having no effect. Compounds **16–18**, with a single, centrally-attached cationic charge, were much less cytotoxic.

Table 3. Analysis of fit of compounds **7–10** in the minor groove of DNA

Compound	d[a] (Å)	x[b] (Å)	Θ[c] (°)	r[d] (Å)
7	15.5	8.1	145	13.4
8	7.2	7.1	60	4.0
9	10.6	7.4	92	5.3
10	14.0	8.1	121	8.1
DNA[e]	-	8.0	138	11.2

[a]d: Distance (Å) between the nitrogen atoms of the mustard units, in energy-minimised drug conformations which had both the amide NH groups and alkylating units on the concave face. Model building and energy minimisations were carried out on a Silicon Graphics XS24-4000 Iris Indigo workstation, using Biosym Insight II/Discover software.
[b]x: For the (symmetric) drugs **7–10**; distance (Å) between a mustard nitrogen and the centre of the molecule (on the convex face); for the oligonucleotide, average distance between adenine N3 atoms in the floor of the minor groove (on the same strand).
[c]Θ: for drugs **7–10**; angle (°) between the nitrogen atoms of the mustard units and the centre of the molecule (on the convex face); for the oligonucleotide, the angle between three successive adenine N3 atoms in the floor of the minor groove (on the same strand).
[d]r: radius of curvature (A) = x.sin[Θ/2]/[sin(180-Θ)].
[e]d(CGCATATATGCG)$_2$ dodecamer; coordinates were taken from the Cambridge Crystal Structure Data File.

Despite the large variations in *in vitro* cytotoxicity, all of the compounds showed broadly similar potencies *in vivo*, with optimal doses (OD) in a single dose protocol of about 5–10 mg kg^{-1} (Table 1). Most of the bifunctional mustards (**7**, **8**, **10**) showed moderate *in vivo* activity (comparable to that of chlorambucil in this assay), but all the monofunctional compounds were inactive. While the compounds (even the monofunctional ones) are much more potent than chlorambucil (**2**) (OD 225 mg kg^{-1})[19] or the anilinoquinoline mustards related to **6** (ODs 30–100 mg kg^{-1}),[13] they fall well short of the potencies of CC-1065 (**3**) and analogues.[8]

These results further support the well-established[8,10,19] conclusion that targeting nitrogen mustard alkylating agents to DNA by attachment to DNA-affinic carriers can greatly enhance cytotoxicity due to alkylation. Despite bearing mustards less reactive than that of chlorambucil, even the monofunctional polybenzamide mustards showed much higher cytotoxic potencies. However, the analogues with two alkylating functions (**7** and **14**) are by far the most cytotoxic, suggesting that, even with these DNA-targeted mustards, crosslinking is a more toxic event than monoalkylation. Finally, close analogues of **7** differing only in their radius of curvature appear to alkylate and crosslink DNA in similar fashion, yet have widely differing cytotoxicities. The most cytotoxic compound (**7**) possesses a geometry closest to that of duplex DNA, suggesting that the most toxic lesions might be those which result in least distortion, thus being less well recognised by DNA repair enzymes.

Experimental

Analyses were carried out in the Microchemical Laboratory, University of Otago, NZ. Melting points were determined on an Electrothermal apparatus using the supplied, stem-corrected thermometer, and are as read. ^{1}H NMR spectra were obtained on a Bruker WP-60 spectrometer (Me_4Si), and ^{13}C NMR spectra on a Bruker AM-400. Mass spectra were obtained on an AEI MS-30 spectrometer at nominal 3000 resolution.

*N,N′-Bis[3-[N-(2-chloroethyl)-N-ethylamino]-5-(N,N-dimethylaminomethyl)phenyl]-1,4-bicyclo[2.2.2]octanedicarboxamide (**10**): example of method A (Scheme 1)*

$BH_3S(CH_3)_2$ (25 mL, 0.25 mol) was added to a suspension of *N,N*-dimethyl-3-acetamido-5-nitrobenzamide (**19**)[14] (17.55 g, 0.07 mol) in THF (350 mL) and the mixture was heated under reflux for 3.5 h, then cooled, slowly diluted with water (2 L) and left for 24 h at 4 °C. The precipitate was collected, dried, powdered, then suspended in MeOH (50 mL), cooled to 0 °C and stirred with conc. HCl (100 mL) at 20 °C until homogeneous and then for a further 10 min. The solution was poured into excess ice/NH_4OH and extracted with CH_2Cl_2, and the organic layer was washed twice with water, dried and evaporated under reduced pressure. The residue was dissolved in hot benzene, and the solution was diluted with a limited volume of petroleum ether to precipitate polar impurities, which were removed by filtration through a Celite pad. Concentration of the filtrate then yielded *N,N*-dimethyl-3-ethylamino-5-nitrobenzylamine·BH_2Cl complex (**20**) (9.98 g, 53%), which was used without further purification. A sample crystallised from iPr_2O gave orange prisms mp 90–91 °C. ^{1}H NMR ($CDCl_3$) δ 7.44 (*t*, *J* = 1.6 Hz, 1H, H-6), 7.42 (*t*, *J* = 2.2 Hz, 1H, H-4), 6.93 (*t*, *J* = 1.8 Hz, 1H, H-2), 4.10 (*br s*, 1H, NH), 4.03 (*s*, 2H, $C\underline{H}_2N(CH_3)_2$), 3.19–3.28 (*m*, 2H, $C\underline{H}_2CH_3$), 2.62 (*s*, 6H, $N(CH_3)_2$), 1.31 (*t*, *J* = 7.2 Hz, 3H, $CH_2C\underline{H}_3$). Anal. calcd for $C_{11}H_{17}N_3O_2{\cdot}BH_2Cl$: C, 48.7; H, 7.1; N, 15.5; Cl, 13.1. Found: C, 48.7; H, 7.0; N, 15.6; Cl, 13.5%.

Oxirane (25 mL, 0.51 mol) was added to a cooled solution of **20** (23.9 g, 0.088 mol) in THF (70 mL) and AcOH (70 mL), and the mixture was stirred at 20 °C for 36 h. Additional oxirane (20 mL, 0.41 mol) was added and the mixture was stirred for a further 36 h and then concentrated under reduced pressure. The residue was shaken with excess dilute Na_2CO_3, then kept at 0 °C for 20 h, and the precipitated solid was crystallised from CH_2Cl_2/petroleum ether to give *N,N*-dimethyl-3-[*N*-(2-hydroxyethyl)-*N*-ethylamino]-5-nitrobenzylamine·BH_2Cl complex (**21**) (15.8 g, 57%). A sample recrystallised from iPr_2O gave orange–red needles, mp 130–131 °C. ^{1}H NMR ($CDCl_3$) δ 7.57 (*t*, *J* = 2.3 Hz, 1H, H-6), 7.40 (*t*, *J* = 1.6 Hz, 1H, H-4) 7.19 (*m*, 1H, H-2), 4.03 (*s*, 2H, $C\underline{H}_2N(CH_3)_2$), 3.86 (*t*, *J* = 5.8 Hz, 2H, $CH_2C\underline{H}_2OH$), 3.56 (*t*, *J* = 6.0 Hz, 2H, $C\underline{H}_2CH_2OH$), 3.51 (*q*, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 2.64 (*s*, 6H, $N(CH_3)_2$), 1.80 (*br s*,

1H, OH), 1.23 (*t*, *J* = 7.1 Hz, 3H, $CH_2C\underline{H}_3$). Anal. calcd for $C_{13}H_{21}N_3O_3 \cdot BH_2Cl$: C, 46.8; H, 7.3; N, 13.3; Cl, 11.2. Found: C, 45.6; H, 7.4; N, 13.3; Cl, 11.5%.

Mesyl chloride (3.15 mL, 0.041 mol) was added dropwise to a stirred solution of **21** (11.70 g, 0.037 mol) in CH_2Cl_2 (250 mL) containing NEt_3 (6.14 mL, 0.044 mol) at 0 °C. The mixture was stirred at 20 °C for 1 h, then washed with dilute Na_2CO_3 (twice) water (twice), dried and concentrated under reduced pressure below 50 °C. The residue was stirred with a mixture of DMF (35 mL) and excess LiCl (5.6 g) at 75 °C for 30 min, then diluted with water. The resulting solid was purified by chromatography on silica gel, eluting with CH_2Cl_2, to give *N,N*-dimethyl-3-[*N*-(2-chloroethyl)-*N*-ethylamino]-5-nitrobenzylamine·BH_2Cl complex (**22**) (10.58 g, 85%), mp (CH_2Cl_2/petroleum ether) 148–149 °C. ^{1}H NMR ($CDCl_3$) δ 7.54 (*t*, *J* = 2.3 Hz, 1H, H-6), 7.45 (*t*, *J* = 1.4 Hz, 1H, H-4), 7.16 (*m*, 1H, H-2), 4.04 (*s*, 2H, $C\underline{H}_2N(CH_3)_2$), 3.71–3.77 (*m*, 2H, $C\underline{H}_2CH_2Cl$), 3.65–3.71 (*m*, 2H, $CH_2C\underline{H}_2Cl$), 3.53 (*q*, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 2.64 (*s*, 6H, $N(CH_3)_2$), 1.24 (*t*, *J* = 7.1 Hz, 3H, $CH_2C\underline{H}_3$. Anal. calcd for $C_{13}H_{20}ClN_3O_2 \cdot BH_2Cl$: C, 46.7; H, 6.6; N, 12.6; Cl, 21.2. Found: C, 46.8; H, 6.7; N, 12.4; Cl, 21.7%. The structure of **22** was confirmed by X-ray crystallography (Fig. 1).

A stirred solution of **22** (2.0 g, 6 mmol) in conc. HCl (20 mL) was treated portionwise at 25 °C with $SnCl_2 \cdot 2H_2O$ (6.0 g, 27 mmol), heated on the steam bath at 100 °C for 2 h, then concentrated under reduced pressure. The residue was shaken with a mixture of CH_2Cl_2, excess 2 N NH_4OH and ice, and filtered through a Celite pad. The organic layer was washed with water, dried and then evaporated under reduced pressure below 30 °C to give 3-[*N*-(2-chloroethyl)-*N*-ethylamino]-5-(*N,N*-dimethylaminomethyl)aniline (**23**)[14] (1.44 g, 94%) as an oil which was used directly. ^{1}H NMR ($CDCl_3$) δ 6.08 (*d*, *J* = 1.8 Hz, 1H, H-4), 6.07 (*d*, *J* = 2.0 Hz, 1H, H-6), 5.92 (*t*, *J* = 2.1 Hz, 1H, H-2), 3.58 (*s*, 6H, NH_2, CH_2CH_2Cl), 3.37 (*q*, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 3.27 (*s*, 2H, $C\underline{H}_2N(CH_3)_2$), 2.24 (*s*, 6H, $N(CH_3)_2$), 1.16 (*t*, *J* = 7.1 Hz, 3H, $CH_2C\underline{H}_3$).

A stirred solution of **23** (0.80 g, 3.13 mmol) in CH_2Cl_2 (25 mL) containing NEt_3 (0.35 g, 3.46 mmol) was treated dropwise at 0 °C with a solution of 1,4-bicyclo[2.2.2]octanedicarbonyl dichloride[25] (0.35 g, 1.49 mmol) in CH_2Cl_2 (5 mL). After being stirred for a further 30 min at 0 °C and for 30 min at 25 °C, the mixture was washed with 2 N Na_2CO_3 (2 ×) and water (12 ×) before being dried and concentrated under reduced pressure below 30 °C. The residue was purified by chromatography on a short column of alumina (activity II–III), eluting with EtOAc:MeOH (20:1) to give *N,N'*-bis[3-[*N*-(2-chloroethyl)-*N*-ethylamino]-5-(*N,N*-dimethylaminomethyl)phenyl]-1,4-bicyclo[2.2.2] octanedicarboxamide (**10**) (74% yield), mp (EtOAc/petroleum ether) > 250 °C. ^{1}H NMR ($CDCl_3$) δ 7.18 (*s*, 2H, 2 × NH), 7.17 (*s*, 2H, H-6',6"), 6.56 (*s*, 2H, H-2',2"), 6.37 (*s*, 2H, H-4',4"), 3.65 (*s*, 8H, 2 × CH_2CH_2Cl), 3.43 (*q*, *J* = 7.0 Hz, 4H, 2 × $C\underline{H}_2CH_3$), 3.33 (*s*, 4H, 2 × $C\underline{H}_2N(CH_3)_2$), 2.24 (*s*, 12H, 2 × $N(CH_3)_2$), 1.95 (*s*, 12H, 6 × CH_2), 1.18 (*t*, *J* = 7.0 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{36}H_{54}Cl_2N_6O_2$: C, 64.2; H, 8.1; N, 12.5; Cl, 10.5. Found: C, 63.9; H, 8.0; N, 12.5; Cl, 10.6%. Treatment as cold solution of the free base in EtOAc with cold MeOH/HCl (2.1 equiv.), followed by addition of EtOAc/petroleum ether, gave the dihydrochloride salt.

Similar reaction of **23** with 1,3-benzenedicarbonyl dichloride, followed by flash chromatography on alumina (activity II–III) in EtOAc gave *N,N'*-bis[3-[*N*-(2-chloroethyl)-*N*-ethylamino]-5-(*N,N*-dimethylaminomethyl)phenyl]-1,3-benzenedicarboxamide (**8**) (61%), mp (EtOAc/petroleum ether) 135–138 °C. ^{1}H NMR ($CDCl_3$) δ 8.40 (*s*, 1H, H-2), 8.35 (*s*, 2H, 2 × NH), 8.07 (*d*, *J* = 7.7 Hz, 2H, H-4,6), 7.57 (*t*, *J* = 7.8 Hz, 1H, H-5), 7.29 (*s*, 2H, H-6',6"), 6.78 (*s*, 2H, H-2',2"), 6.41 (*s*, 2H, H-4',4"), 3.64 (*s*, 8H, 2 × CH_2CH_2Cl), 3.45 (*q*, *J* = 7.0 Hz, 4H, 2 × $C\underline{H}_2CH_3$), 3.34 (*s*, 4H, 2 × $C\underline{H}_2N(CH_3)_2$), 2.23 (*s*, 12H, 2 × $NCH_3)_2$), 1.19 (*t*, *J* = 7.0 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{34}H_{46}Cl_2N_6O_2$: C, 63.6; H, 7.2; N, 13.1; Cl 11.1. Found: C, 63.4; H, 7.2; N, 13.2; Cl, 11.2%.

Similar reaction of **23** with 1-methyl-3,5-pyrazoledicarbonyl dichloride (prepared from 1-methyl-3,5-pyrazoledicarboxylic acid by treatment with $SOCl_2$/DMF) followed by flash chromatography as above gave *N,N'*-bis[3-[*N*-(2-chloroethyl)-*N*-ethylamino]-5-(*N,N*-dimethylaminomethyl) phenyl]- 1-methyl-3,5-pyrazoledicarboxamide (**9**) (62%), mp (EtOAc/petroleum ether) 142–143 °C. ^{1}H NMR ($CDCl_3$) δ 8.66 and 8.13 (2 × *s*, 2H, 2 × NH), 7.34 and 7.26 (2 × *s*, 2H, H-6',6"), 7.00 (*s*, 1H, H-4), 6.83 and 6.75 (2 × *s*, 2H, H-2',2"), 6.46 and 6.40 (*s*, 2H, H-4',4"), 4.26 (*s*, 3H, NCH_3), 3.62 and 3.61 (2 × *s*, 8H, 2 × CH_2CH_2Cl), 3.42 (*q*, *J* = 7.0 Hz, 4H, 2 × $C\underline{H}_2CH_3$), 3.33 (2 × *s*, 4H, 2 × $C\underline{H}_2N(CH_3)_2$), 2.24 (*s*, 12H, 2 × $N(CH_3)_2$, 1.17 (*t*, *J* = 7.0 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{32}H_{46}Cl_2N_8O_2$): C, 59.2; H, 7.2; N, 17.4; Cl, 11.0. Found: C, 59.2; H, 7.0; N, 17.4; Cl 11.2%.

Similar reaction of **23** with 1,4-benzenedicarbonyl dichloride, followed by flash chromatography as above, gave *N,N'*-bis[3-[*N*-(2-chloroethyl)-*N*-ethylamino]-5-(*N,N*-dimethylaminomethyl)phenyl]-1,4-benzenedicarboxamide (**7**) (61%), mp (EtOAc/petroleum ether) > 300 °C (lit.[14] mp 200 °C (dec.)).

N-[3-[N-(2-Chloroethyl)-N-ethylamino]-5-(N,N-dimethylaminomethyl)phenyl]-N'-[3-(N,N-dimethylaminomethyl)phenyl]-1,4-benzenedicarboxamide (12): example of method B (Scheme 2)

Equimolar amounts of 3-(*N,N*-dimethylaminomethyl)-aniline (**24**)[15] and 4-methoxycarbonylbenzenecarbonyl chloride (**25**) were reacted together in pyridine at 0 °C, to give methyl 4-[3-(*N,N*-dimethylaminomethyl)-phenyl]carbamoylbenzenecarboxylate (**26**) (73%

yield), mp (iPr_2O) 108–109 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 10.40 (*s*, 1H, NH), 8.09 (*s*, 4H, H-2,3,5,6), 7.75 (*s*, 1H, H-2'), 7.70 (*d*, *J* = 8.2 Hz, 1H, H-6'), 7.30 (*t*, *J* = 7.8 Hz, 1H, H-5'), 7.04 (*d*, *J* = 7.6 Hz, 1H, H-4'), 3.90 (*s*, 3H, CO_2CH_3) 3.38 (*s*, 2H, CH_2), 2.16 (*s*, 6H, $N(CH_3)_2$). Anal. calcd for $C_{18}H_{20}N_2O_3$: C, 69.2; H, 6.5; N, 9.0. Found: C, 69.1; H, 6.3; N, 8.9%.

A solution of **26** (5.30 g, 17 mmol) in MeOH (10 mL) was treated with one equivalent of NaOH (17.0 mL of 1.0 N aqueous solution), and the mixture was heated until the MeOH had boiled off, and for 1 h under reflux, then cooled and filtered. Exact neutralisation (with 17.0 mL of 1.0 N aqueous HCl) followed by refrigeration yielded 4-[3-(*N*,*N*-dimethylaminomethyl)phenyl]carbamoylbenzenecarboxylic acid (**27**) (4.73 g, 93%), mp (MeOH/EtOAc) 209–210 °C (dec.). ^{1}H NMR ($(CD_3)_2SO$) δ 10.38 (*s*, 1H, NH), 8.07 (*d*, *J* = 6.8 Hz, 2H, H-2,6), 8.02 (*d*, *J* = 6.9 Hz, 2H, H-3,5), 7.82 (*s*, 1H, H-2'), 7.72 (*d*, *J* = 8.1 Hz, 1H, H-6'), 7.33 (*t*, *J* = 7.8 Hz, 1H, H-5'), 7.09 (*d*, *J* = 7.6 Hz, 1H, H-4'), 3.57 (*s*, 2H, CH_2), 2.29 (*s*, 6H, $N(CH_3)_2$. Anal. calcd for $C_{17}H_{18}N_2O_3$: C, 67.4; H, 6.2; N, 9.3. Found: C, 67.6; H, 6.1; N, 9.2%.

An ice-cold solution of **27** (0.70 g, 2.35 mmol) in dry DMF (5 mL) containing 1-methylimidazole (0.21 g, 2.56 mmol) was added to freshly prepared **23** (0.61 g, 2.38 mmol) contained in a precooled flask. The mixture was stirred until homogeneous, and then treated dropwise with diethyl cyanophosphonate (93%, 0.43 g, 2.45 mmol) at 0 °C. The mixture was stirred at 25 °C for 1.5 h, then diluted with a large excess of 0.5 N Na_2CO_3 and the resulting solid collected and extracted into CH_2Cl_2. The CH_2Cl_2 solution was washed twice with water, dried (Na_2SO_4), and evaporated below 30 °C. The residue was chromatographed on a short column of alumina (activity II–III). Elution with EtOAc gave *N*-[3-[*N*-(2-chloroethyl)-*N*-ethylamino]-5-(*N*,*N*-dimethylaminomethyl)phenyl]-*N*^{1}-[3-(*N*,*N*-dimethylaminomethyl)phenyl]-1,4-benzenedicarboxamide (**12**) (0.59 g, 47%), mp (EtOAc/petroleum ether) 162–165 °C. ^{1}H NMR ($CDCl_3$) δ 8.27 and 8.15 (2 × *s*, 2H, 2 × NH), 7.86 (*s*, 4H, H-2,3,5,6), 7.66 (*d*, *J* = 8.0 Hz, 1H, H-6"), 7.56 (*s*, 1H, H-2"), 7.31 (*t*, *J* = 7.8 Hz, 1H, H-5"), 7.23 (*s*, 1H, H-6'), 7.10 (*d*, *J* = 7.6 Hz, 1H, H-4"), 6.78 (*s*, 1H, H-2'), 6.44 (*s*, 1H, H-4'), 3.63 (*s*, 4H, CH_2CH_2Cl), 3.43 (*q*, *J* = 7.0 Hz, 2H, $C\underline{H}_2CH_3$), 3.41 and 3.34 (2 × *s*, 4H, 2 × $C\underline{H}_2N(CH_3)_2$), 2.24 (*s*, 12H, 2 × $N(CH_3)_2$), 1.19 (*t*, *J* = 7.0 Hz, 3H, $CH_2C\underline{H}_3$). Anal. calcd for $C_{30}H_{38}ClN_5O_2$: C, 67.2; H, 7.1; N, 13.1; Cl, 6.6. Found: C, 67.1; H, 6.9; N, 13.1; Cl, 6.8%. Treatment of a cold solution of the free base in CH_2Cl_2 with cold MeOH/HCl (2.1 equiv.), followed by addition of EtOAc/petroleum ether gave the dihydrochloride salt.

Similar reaction of **27** with **24** gave bis-*N*,*N*^{1}-[3-(*N*,*N*-dimethylaminomethyl) phenyl] - 1 , 4 - benzenedicarboxamide (**11**). ^{1}H NMR (free base in $CDCl_3$) δ 8.40 (*s*, 2H, 2 × NH, 7.79 (*s*, 4H, H-2,3,5,6), 7.66 (*d*, *J* = 8.1 Hz, 2H, H-6',6"), 7.58 (*s*, 2H, H-2',2"), 7.30 (*t*, *J* = 7.8 Hz, 2H, H-5',5"), 7.11 (*d*, *J* = 7.6 Hz, 2H, H-4',4"), 3.39 (*s*, 4H, 2 × $C\underline{H}_2N(CH_3)_2$), 2.17 (*s*, 12H, 2 × $N(CH_3)_2$). Dihydrochloride salt, mp > 300 °C (MeOH/EtOAc). Anal. calcd for $C_{26}H_{30}N_4O_2 \cdot 2HCl$: C, 62.0; H, 6.4; N, 11.1; Cl, 14.1. Found: C, 61.7; H, 6.7; N, 11.2; Cl, 14.0%.

Similar reaction of **27** with **31** gave *N*-[3-[*N*-(2-chloroethyl)-*N*-ethylamino]phenyl]-*N*^{1}-[3-(*N*,*N*-dimethylaminomethyl)phenyl]-1,4-benzenedicarboxamide (**15**) (59% yield), mp (EtOAc/petroleum ether) 160–161 °C. ^{1}H NMR ($CDCl_3$) δ 8.34 and 8.26 (2 × *s*, 2H, 2 × NH), 7.80 (*s*, 4H, H-2,3,5,6), 7.66 (*d*, *J* = 8.2 Hz, 1H, H-6'), 7.57 (*s*, 1H, H-2'), 7.30 (*t*, *J* = 7.3 Hz, 1H, H-5'), 7.24 (*s*, 1H, H-2"), 7.18 (*t*, *J* = 8.1 Hz, 1H, H-5"), 7.10 (*d*, *J* = 7.6 Hz, 1H, H-4'), 6.89 (*d*, *J* = 7.7 Hz, 1H, H-6"), 6.49 (dd, *J* = 8.4, 2.4 Hz, 1H, H-4"), 3.61 (*s*, 4H, CH_2CH_2Cl), 3.42 (*q*, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 3.40 (*s*, 2H, $C\underline{H}_2N(CH_3)_2$), 2.23 (*s*, 6H, $N(CH_3)_2$), 1.18 (*t*, *J* = 7.0 Hz, 3H, $CH_2C\underline{H}_3$). Anal. calcd for $C_{27}H_{31}ClN_4O_2$: C, 67.7; H, 6.5; N, 11.7; Cl, 7.4. Found: C, 67.5; H, 6.4; N, 11.8; Cl, 7.7%.

Similar reaction of the amine **23** with 4-phenylcarbamoylbenzenecarboxylic acid gave *N*-[3-[*N*-(2-chloroethyl)-*N*-ethylamino]-5-(*N*,*N*-dimethylaminomethyl)phenyl]-*N*^{1}-phenyl-1,4-benzenedicarboxamide (**13**) (52% yield), mp (EtOAc/petroleum ether) 186–189 °C. ^{1}H NMR ($CDCl_3$) δ 8.08 and 7.98 (2 × *s*, 2H, 2 × NH), 7.91 (*s*, 4H, H-2,3,5,6), 7.67 (*d*, *J* = 7.8 Hz, 2H, H-2",6"), 7.38 (*t*, *J* = 7.9 Hz, 2H, H-3",5"), 7.26 (*s*, 1H, H-6'), 7.18 (*t*, *J* = 7.4 Hz, 1H, H-4"), 6.75 (*s*, 1H, H-2'), 6.50 (*s*, 1H, H-4'), 3.64 (*s*, 4H, CH_2CH_2Cl), 3.45 (*q*, *J* = 7.0 Hz, 2H, $C\underline{H}_2CH_3$), 3.37 (*s*, 2H, $C\underline{H}_2N(CH_3)_2$), 2.26 (*s*, 6H, $N(CH_3)_2$), 1.20 (*t*, *J* = 7.0 Hz, 3H, $CH_2C\underline{H}_3$). Anal. calcd for $C_{27}H_{31}ClN_4O_2$: C, 67.7; H, 6.5; N, 11.7; Cl 7.4. Found: C, 67.7; H, 6.4; N, 11.8; Cl 7.6%.

N-[3-[N-(2-Chloroethyl)-N-ethylamino]-5-(N,N-dimethylaminomethyl)phenyl]-N^1-[3-(N-(2-chloroethyl)-N-ethylamino)phenyl]-1,4-benzenedicarboxamide (14): example of method C (Scheme 3)

A solution of *N*-ethyl-3-nitroaniline (**28**) (13.3 g, 0.08 mol) in THF (50 mL) and AcOH (50 mL) was treated with oxirane (15 mL, 0.3 mol), and the mixture was stirred at 20 °C for 36 h. Additional oxirane (15 mL) was then added, and the mixture was stirred at 20 °C for an additional 36 h. Solvent was then removed under reduced pressure, and the residue was partitioned between CH_2Cl_2 and 1 N aqueous Na_2CO_3. The residue obtained from work-up of the organic layer was chromatographed on SiO_2 (EtOAc:petroleum ether, 1:3) to give *N*-ethyl-*N*-(2-hydroxyethyl)-3-nitroaniline (**29**) (12.3 g, 73%), mp (benzene/petroleum ether) 43 °C. ^{1}H NMR ($CDCl_3$) δ 7.53 (*t*, *J* = 2.3 Hz, 1H, H-2), 7.49 (*dd*, *J* = 8.2, 2.0 Hz, 1H, H-4), 7.30 (*dd*, *J* = 8.4, 8.2 Hz, 1H, H-5), 7.01 (*dd*, *J* = 8.4, 2.6 Hz, 1H, H-6), 3.84 (*d*, *J* = 3.6 Hz, 2H, $C\underline{H}_2OH$), 3.53 (*t*, *J* = 6.0 Hz, 2H, $NC\underline{H}_2CH_2OH$), 3.49 (*q*, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 1.72 (*br s*, 1H, OH), 1.20 (*t*, *J* = 7.1 Hz, 3H, CH_3). Anal. calcd for $C_{10}H_{14}N_2O_3$: C, 57.1; H, 6.7; N, 13.3. Found: C, 57.2; H, 6.5; N, 13.6%.

A stirred solution of **29** (4.0 g, 19 mmol) in CH_2Cl_2 (35 mL) containing NEt_3 (2.91 mL, 21 mmol) was treated dropwise at 0 °C with methanesulfonylchloride (1.62 mL, 21 mmol). After being stirred at 0 °C for a further 30 min and at 20 °C for 30 min, the reaction mixture was diluted with CH_2Cl_2 (35 mL) and washed successively with 1 N HCl, 1 N Na_2CO_3 and saturated NaCl, and worked-up to give the crude mesylate, which was immediately treated with LiCl (2 g) in dry DMF (20 mL) at 75 °C for 30 min. Removal of excess solvent under reduced pressure below 50 °C, and chromatography of the residue on SiO_2 (elution with petroleum ether:EtOAc, 4:1) gave *N*-(2-chloroethyl)-*N*-ethyl-3-nitroaniline (**30**) (3.58 g, 82%) as yellow prisms, mp (petroleum ether) 56–57 °C. ^{1}H NMR ($CDCl_3$) δ 7.51 (*dd*, *J* = 7.9, 1.9 Hz, 1H, H-4), 7.48 (*t*, *J* = 2.4 Hz, 1H, H-2), 7.33 (*t*, *J* = 8.2 Hz, 1H, H-5), 6.95 (*dd*, *J* = 8.4, 2.7 Hz, H-6), 3.70 (*m*, 2H, $C\underline{H}_2CH_2Cl$), 3.64 (*m*, 2H, CH_2Cl), 3.50 (*q*, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 1.22 (*t*, *J* = 7.1 Hz, 3H, CH_3). Anal. calcd for $C_{10}H_{13}ClN_2O_2$: C, 52.5; H, 5.7; N, 12.3; Cl, 15.5. Found: C, 52.3; H, 5.5; N, 12.4; Cl, 15.7%.

A solution of **30** (2.51 g, 11 mmol) in 12 N HCl (25 mL) was treated portionwise at 25 °C with $SnCl_2 \cdot 2H_2O$ (9.9 g, 44 mmol), heated on a steam bath at 90 °C for 1 h, then evaporated to dryness under reduced pressure. The residue was shaken vigorously with a mixture of CH_2Cl_2, 2 N NH_4OH and ice, and filtered through a Celite pad. Work-up of the organic layer (below 30 °C) gave essentially pure 3-[*N*-(2-chloroethyl)-*N*-ethylamino]aniline (**31**) (1.92 g, 88%) as an oil, which was used immediately.

A stirred solution of **31** (2.38 g, 12 mmol) in CH_2Cl_2 (30 mL) containing NEt_3 (1.80 mL, 13 mmol) was treated dropwise at 0 °C with a solution of 4-methoxycarbonylbenzenecarbonyl chloride (**25**) (2.18 g, 11 mmol) in CH_2Cl_2 (10 mL). After being stirred for a further 15 min at 0 °C and for 15 min at 25 °C, the mixture was washed with 1 N Na_2CO_3 and water, and the residue from the organic layer was chromatographed on SiO_2 (CH_2Cl_2) to give methyl 4-[3-(*N*-(2-chloroethyl)-*N*-ethylamino) phenyl] carbamoylbenzenecarboxylate (**32**) (3.36 g, 85%), mp (benzene/petroleum ether) 111–112 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 10.23 (*s*, 1H, NH), 8.03–8.13 (*m*, 4H, H-2,3,5,6), 7.11–7.21 (*m*, 3H, H-2',5',6'), 6.48 (*d t*, *J* = 7.5, 1.9 Hz, 1H, H-4'), 3.91 (*s*, 3H, CO_2CH_3), 3.73 (*t*, *J* = 7.0 Hz, 2H, $C\underline{H}_2CH_2Cl$), 3.61 (*t*, *J* = 7.1 Hz, 2H, $CH_2C\underline{H}_2Cl$), 3.41 (*q*, *J* = 7.0 Hz, 2H, $C\underline{H}_2CH_3$), 1.12 (*t*, *J* = 7.0 Hz, 3 H, $CH_2C\underline{H}_3$). Anal. calcd for $C_{19}H_{21}ClN_2O_3$: C, 63.2; H, 5.9; N, 7.8; Cl, 9.8. Found: C, 63.2; H, 6.1; N, 7.8; Cl, 10.0%.

A suspension of **32** (2.88 g, 8 mmol) in MeOH (100 mL) containing KOH (5.6 g) was stirred at 25 °C until homogeneous, and then for a further 5 h. The mixture was diluted with water, filtered, and acidified with AcOH to give 4-[3-(*N*-(2-chloroethyl)-*N*-ethylamino)-phenyl]carbamoylbenzenecarboxylic acid (**33**) (2.08 g, 75%), mp (EtOAc) 203 °C (dec.). ^{1}H NMR ($(CD_3)_2SO$) δ 13.3 (*br s*, 1H, CO_2H), 10.20 (*s*, 1H, NH), 8.07 (*d*, *J* = 8.5 Hz, 2H, H-3,5), 8.03 (*d*, *J* = 8.4 Hz, 2H, H-2,6,) 7.09–7.20 (*m*, 3H, H-2',5',6'), 6.48 (*d*, *J* = 9.1 Hz, 1H, H-4'), 3.73 (*t*, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH_2Cl$), 3.60 (*t*, *J* = 7.0 Hz, 2H, $CH_2C\underline{H}_2Cl$), 3.41 (*q*, *J* = 7.0 Hz, 2H, $C\underline{H}_2CH_3$), 1.12 (*t*, *J* = 7.0 Hz, 3H, $CH_2C\underline{H}_3$). Anal. calcd for $C_{18}H_{19}ClN_2O_3$: C, 62.3; H, 5.5; N, 8.0; Cl, 10.2. Found: C, 62.6; H, 5.4; N, 8.0; Cl, 9.7%.

Coupling of **33** with amine **23** using diethyl cyanophosphonate as above gave *N*-[3-[*N*-(2-chloroethyl)-*N*-ethylamino]-5-(*N*,*N*-dimethylaminomethyl)phenyl]-*N*^{1}-[3-(*N*-(2-chloroethyl)-*N*-ethylamino)phenyl]-1,4-benzenedicarboxamide (**14**) (61%) mp (EtOAc/petroleum ether) > 250 °C. ^{1}H NMR ($CDCl_3$) δ 8.11 and 8.07 (2 × *s*, 2H, 2 × NH), 7.87 (*s*, 4H, H-2,3,5,6), 7.24 (*s*, 2H, H-6',2"), 7.19 (*t*, *J* = 8.2 Hz, 1H, H-5"), 6.87 (*d*, *J* = 7.8 Hz, 1H, H-6"), 6.77 (*s*, 1H, H-2'), 6.50 (*dd*, *J* = 8.3, 2.4 Hz, 1H, H-4"), 6.44 (*s*, 1H, H-4'), 3.63 (*s*, 8H, 2 × CH_2CH_2Cl), 3.43 (2 × *q*, *J* = 7.0 Hz, 4H, 2 × $C\underline{H}_2CH_3$), 3.35 (*s*, 2H, $C\underline{H}_2N(CH_3)_2$), 2.24 (*s*, 6H, $N(CH_3)_2$), 1.19 (2 × *t*, *J* = 7.0 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{31}H_{39}Cl_2N_5O_2$: C, 63.7; H, 6.7; N, 12.0; Cl, 12.1. Found: C, 63.6; H, 6.8; N, 11.9; Cl, 12.0%.

N,N'-Bis[4-[N-(2-chloroethyl)-N-ethylamino]phenyl]-N"-[2-(dimethylamino)ethyl]-1,3,5-benzenetricarboxamide (17): example of method D (Scheme 4)

A suspension of 4-amino-*N*-(2-chloroethyl)-*N*-ethylaniline dihydrochloride[26] (**35**) (2.98 g, 11.0 mmol) in CH_2Cl_2 (25 mL) was stirred at 0 °C and treated with $EtN(iPr)_2$ (4.26 g, 33.0 mmol), then dropwise with a solution of 5-methoxycarbonyl-1,3-benzenedicarbonyl dichloride (**34**; prepared from the corresponding diacid[27] by treatment with $SOCl_2$/DMF) (1.31 g, 5.0 mmol) in CH_2Cl_2 (5 mL). The mixture was stirred at 20 °C for 1 h, then washed with dilute aqueous AcOH, dilute aqueous Na_2CO_3, water (twice), dried, and diluted with excess petroleum ether. The precipitated solid was purified by chromatography on silica gel, eluting with CH_2Cl_2:MeOH (20:1). Crystallisation from EtOAc gave *N,N*^{1}-bis[4-[*N*-(2-chloroethyl)-*N*-ethylamino]phenyl]-5-methoxycarbonyl-1,3-benzenedicarboxamide (**36**) (1.97 g, 67%), mp 187–188 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 10.36 (*s*, 2H, 2 × NH), 8.76 (*t*, *J* = 1.5 Hz, 1H, H-2), 8.65 (*d*, *J* = 1.6 Hz, 2H, H-4,6), 7.60 (*d*, *J* = 9.1 Hz, 4H, H-2',6',2",6"), 6.73 (*d*, *J* = 9.2 Hz, 4H, H-3',5',3",5"), 3.96 (*s*, 3H, CO_2CH_3), 3.73 (*t*, *J* = 7.0 Hz, 4H, 2 × $C\underline{H}_2CH_2Cl$), 3.61 (*t*, *J* = 6.8 Hz, 4H, 2 × $CH_2C\underline{H}_2Cl$), 3.41 (*q*, *J* = 7.0 Hz, 4H, 2 × $C\underline{H}_2CH_3$), 1.10 (*t*, *J* = 7.0 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{30}H_{34}Cl_2N_4O_4$: C, 42.0; H, 5.9; N, 9.6; Cl, 12.1. Found: C, 61.3; H, 6.0; N, 9.4; Cl, 12.3%.

A two-phase mixture of **36** (1.70 g, 2.9 mmol) in THF (35 mL) and 1.0 N aqueous NaOH (4.0 mL) was vigorously stirred for 12 h at 20 °C. Addition of 1.0 N aqueous HCl (5.0 mL) followed by water precipitated a solid which was crystallised from EtOAc/iPr_2O/

petroleum ether to give *N,N'*-bis-[4-[*N*-(2-chloroethyl)-*N*-ethylamino]phenyl]-5-carboxy-1,3-benzenedicarboxamide (**37**) (1.36 g, 82%) mp > 300 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 13.5 (*br s*, 1H, CO_2H), 10.34 (*s*, 2H, 2 × NH), 8.73 (*s*, 1H, H-2), 8.65 (*d*, *J* = 1.1 Hz, 2H, H-4,6), 7.60 (*d*, *J* = 8.9 Hz, 4H, H-2',6',2",6"), 6.73 (*d*, *J* = 9.0 Hz, 4H, H-3',5',3",5"), 3.72 (*t*, *J* = 6.8 Hz, 4H, 2 × $C\underline{H}_2CH_2Cl$), 3.61 (*t*, *J* = 6.8 Hz, 4H, 2 × $CH_2C\underline{H}_2Cl$), 3.41 (*q*, *J* = 6.9 Hz, 4H, 2 × $C\underline{H}_2CH_3$), 1.09 (*t*, *J* = 6.9 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{29}H_{32}Cl_2N_4O_4$: C, 60.9; H, 5.6; N, 9.8; Cl, 12.4. Found: C, 60.8; H, 5.8; N, 9.9; Cl, 12.2%.

A stirred solution of **37** (0.87 g, 1.52 mmol) in DMF (3 mL) was treated with 1,1'-carbonyldiimidazole (0.28 g, 1.72 mmol) at 20 °C for 30 min, then cooled to 0 °C and treated with *N,N*-dimethylethylenediamine (0.18 g, 2.04 mmol). The mixture was stirred at 20 °C for 5 min, then diluted with 10% aqueous Na_2CO_3. The resulting solid was purified by chromatography on alumina (activity II–III), eluting with CH_2Cl_2:MeOH (20:1) to give *N,N'*-bis[4-[*N*-(2-chloroethyl)-*N*-ethylamino]phenyl]-*N''*-[2-(dimethylamino)ethyl]-1,3,5-benzenetricarboxamide (**17**) (0.68 g, 69%) mp (CH_2Cl_2/*i*Pr_2O) > 300 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 10.27 (*s*, 2H, 2 × NHPh), 8.70 (*t*, *J* = 5.4 Hz, 1H, $NHCH_2$), 8.58 (*s*, 1H, H-2), 8.51 (*d*, *J* = 1.3 Hz, 2H, H-4,6), 7.60 (*d*, *J* = 9.0 Hz, 4H, H-2',6',2",6"), 6.73 (*d*, *J* = 9.1 Hz, 4H, H-3',5',3",5"), 3.73 (*t*, *J* = 6.9 Hz, 4H, 2 × $C\underline{H}_2CH_2Cl$), 3.61 (*t*, *J* = 6.8 Hz, 4H, 2 × $CH_2C\underline{H}_2Cl$), 3.34–3.46 (*m*, 6H, $NHCH_2$ 2 × $C\underline{H}_2CH_3$), 2.44 (*t*, *J* = 6.8 Hz, 2H, $C\underline{H}_2N(CH_3)_2$), 2.19 (*s*, 6H, $N(CH_3)_2$), 1.09 (*t*, *J* = 6.9 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{33}H_{42}Cl_2N_6O_3 \cdot H_2O$: C, 60.1; H, 6.7; N, 12.7; Cl, 10.8. Found: C, 60.1; H, 6.7; N, 12.5; Cl, 10.9%. Treatment of the free base with cold CH_2Cl_2/HCl (1 equiv.), followed by addition of EtOAc/petroleum ether, gave the hydrochloride salt.

N,N'-*Bis[3-[*N*-(2-chloroethyl)-*N*-ethylamino]phenyl]-*N''*-[2-(dimethylamino)ethyl]-1,3,5-benzenetricarboxamide (18)*

Reaction of **34** with freshly prepared amine **31** as for the preparation of **36** gave *N,N'*-bis[3-[*N*-(2-chloroethyl)-*N*-ethylamino]phenyl]-5-methoxycarbonyl-1,3-benzenedicarboxamide (**38**) (69% yield), mp (EtOAc/petroleum ether) 170–171 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 10.43 (*s*, 2 × NH), 8.77 (*s*, 1H, H-2), 8.66 (*d*, *J* = 0.9 Hz, 2H, H-4,6), 7.20 (*s*, 2H, H-2',2"), 7.13–7.20 (*m*, 4H, H-5',6',5",6"), 6.46–6.54 (*m*, 2H, H-4',4"), 3.97 (*s*, 3H, CO_2CH_3), 3.74 (*t*, *J* = 7.0 Hz, 4H, 2 × $C\underline{H}_2CH_2Cl$), 3.62 (*t*, *J* = 6.9 Hz, 4 H, 2 × $CH_2C\underline{H}_2Cl$), 3.42 (*q*, *J* = 6.9 Hz, 4H, 2 × $C\underline{H}_2CH_3$), 1.13 (*t*, *J* = 6.9 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{30}H_{34}Cl_2N_4O_4$: C, 61.5; H, 5.9; N, 9.6; Cl, 12.2. Found: C, 61.4; H, 6.1; N, 9.3; Cl, 12.2%.

Hydrolysis of **38** as for the preparation of **37** gave *N,N'*-bis[3-[*N*-(2-chloroethyl)-*N*-ethylamino]phenyl]-5-carboxy-1,3-benzenedicarboxamide (**39**) (80% yield), mp (EtOAc/*i*Pr_2O/petroleum ether) > 300 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 13.5 (*br s*, 1H, CO_2H), 10.41 (*s*, 2H, 2 × NH), 8.73 (*t*, *J* = 1.5 Hz, 1H, H-2), 8.66 (*d*, *J* = 1.6 Hz, 2H, H-4,6), 7.13–7.24 (*m*, 6H, H-2',5',6',2",5",6"), 6.50 (*d t*, *J* = 7.4, 2.1 Hz, 2H, H-4',4"), 3.74 (*t*, *J* = 7.1 Hz, 4H, 2 × $C\underline{H}_2CH_2Cl$), 3.62 (*t*, *J* = 7.0 Hz, 4H, 2 × $CH_2C\underline{H}_2Cl$), 3.42 (*q*, *J* = 7.0 Hz, 4H, 2 × $C\underline{H}_2CH_3$), 1.13 (*t*, *J* = 7.0 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{29}H_{32}Cl_2N_4O_4$: C, 60.9; H, 5.6; N, 9.8; Cl, 12.4. Found: C, 60.5; H, 6.2; N, 9.7; Cl, 12.5%.

Reaction of **39** with 1,1'-carbonyldiimidazole and *N,N*-dimethylethylenediamine as above, followed by chromatography on alumina (activity I–II) and eluting with EtOAc, gave *N,N'*-bis[3-[*N*-(2-chloroethyl)-*N*-ethylamino]phenyl]-*N''*-[2-(dimethylamino)ethyl]-1,3,5-benzenetricarboxamide (**18**) (82%) mp (CH_2Cl_2/*i*Pr_2O) > 300 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 10.35 (*s*, 2H, 2 × NHPh), 8.74 (*t*, *J* = 5.5 Hz, 1H, $N\underline{H}CH_2$), 8.60 (*s*, 1H, H-2), 8.55 (*d*, *J* = 1.4 Hz, 2H, H-4,6), 7.21 (*s*, 2H, H-2',2"), 7.12–7.20 (*m*, 4H, H-5',6',5",6"), 7.49 (*d t*, *J* = 6.6, 2.6 Hz, 2H, H-4',4"), 3.74 (*t*, *J* = 7.1 Hz, 4H, 2 × $C\underline{H}_2CH_2Cl$), 3.61 (*t*, *J* = 7.0 Hz, 4H, 2 × $CH_2C\underline{H}_2Cl$), 3.36–3.47 (*m*, 6H, $NHC\underline{H}_2$, 2 × $C\underline{H}_2CH_3$), 2.45 (*t*, *J* = 6.7 Hz, 2H, $C\underline{H}_2N(CH_3)_2$), 2.20 (*s*, 6H, $N(CH_3)_2$), 1.12 (*t*, *J* = 6.9 Hz, 6H, 2 × $CH_2C\underline{H}_3$). Anal. calcd for $C_{33}H_{42}Cl_2N_6O_3$: C, 61.8; H, 6.6; N, 13.1; Cl, 11.1. Found: C, 61.6; H, 6.4; N, 13.1; Cl, 11.5%. The hydrochloride salt was prepared as above.

N''-*[2-(Dimethylamino)ethyl]*-N,N'-*diphenyl-1,3,5-benzenetricarboxamide (16)*

5-Methoxycarbonylbenzene-1,3-dicarbonyl dichloride (**34**) was reacted with aniline in pyridine to afford *N,N'*-diphenyl-5-methoxycarbonyl-1,3-benzenedicarboxamide (**40**) (68%) mp (EtOAc) 226–226.5 °C (EtOAc). ^{1}H NMR ($(CD_3)_2SO$) δ 10.63 (*s*, 2H, 2 × NH), 8.81 (*t*, *J* = 1.6 Hz, 1H, H-2), 8.70 (*d*, *J* = 1.7 Hz, 2H, H-4,6), 7.81 (*d*, *J* = 7.8 Hz, 4H, H-2',6',2",6"), 7.40 (*t*, *J* = 7.9 Hz, 4H, H-3',5',3",5"), 7.15 (*t*, *J* = 7.4 Hz, 2H, H-4',4"), 3.34 (*s*, 3H, CH_3). Anal. calcd for $C_{22}H_{18}N_2O_4$: C, 70.6; H, 4.9; N, 7.5. Found: C, 70.5; H, 4.6; N, 7.4%.

This ester was heated under reflux in NaOH/MeOH/H_2O to yield 5-carboxy-*N,N'*-diphenyl-1,3-benzenedicarboxamide (**41**) (80%), mp (DMF/MeOH) > 320 °C. ^{1}H NMR ($(CD_3)_2SO$), δ 13.56 (*br s*, 1H, CO_2H), 10.61 (*s*, 2H, 2 × NH), 8.77 (*t*, *J* = 1.7 Hz, 1H, H-2), 8.70 (*d*, *J* = 1.7 Hz, 2H, H-4,6), 7.81 (*d*, *J* = 8.1 Hz, 4H, H-2',6',2",6"), 7.39 (*t*, *J* = 8.0 Hz, 4H, H-3',5',3",5"), 7.14 (*t*, *J* = 7.4 Hz, 2H, H-4',4"). Anal. calcd for $C_{21}H_{16}N_2O_4$: C, 70.0; H, 4.5; N, 7.8. Found: C, 69.7; H, 4.3; N, 7.7%.

This acid was reacted with *N,N*-dimethylethylenediamine under standard CDI-induced coupling conditions to give *N''*-[2-(dimethylamino)ethyl]-*N,N'*-diphenyl-1,3,5-benzenetricarboxam-ide (**16**), (71%), mp (EtOAc/*i*Pr_2O) 223–224 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 10.55 (*s*, 2H, 2 × NHPh), 8.73 (*t*, *J* = 5.5 Hz, 1H, $N\underline{H}CH_2$), 8.63 (*d*, *J* = 1.4 Hz, 1H, H-2), 8.57 (*d*, *J* = 1.5 Hz, 2H, H-4,6), 7.81 (*d*, *J* = 7.7 Hz, 4H, H-2',6',2",6"),

7.39 (*t*, *J* = 7.9 Hz, 4H, 3',5',3",5"), 7.14 (*t*, *J* = 7.4 Hz, 2H, H-4',4"), 3.38–3.47 (*m*, 2H, $NHC\underline{H}_2$), 2.45 (*t*, *J* = 6.8 Hz, 2H, $C\underline{H}_2N(CH_3)_2$), 2.20 (*s*, 6H, $N(CH_3)_2$. Anal. calcd for $C_{25}H_{26}N_4O_3$: C, 69.7; H, 6.1; N, 13.2. Found: C, 69.7; H, 5.9; N, 13.4%. Treatment with EtOAc/HCl gave the hydrochloride salt.

Crystallographic determination of 22

N,N-Dimethyl-3-[*N*-(2-chloroethyl)-*N*-ethylamino)-5-nitrobenzylamine·BH_2Cl complex (**22**) was crystallised from CH_2Cl_2/petroleum ether as red prisms, mp 148–149 °C, space group $P2_1/n$; cell constants *a* = 13.589(4), *b* = 6.674(7), *c* = 19.277(5) Å, β = 100.844(8)°; *z* = 4; V = 1716.9(9) Å^3. Lattice constants and intensity data were measured using graphite monochromated Mo Kα radiation, λ = 0.71069 Å, on a Nonius CAD-4 diffractometer. The data set consisted of 3002 unique reflections, of which 2286 were considered observed ($I > 3\sigma(I)$). The structure was solved using SHELXS and refined with SHELX-76.[28] All non-hydrogen atoms were found from the difference maps, as were the boron and ring hydrogens. The other hydrogens were placed in calculated positions and allowed to refine. R and R_w were 0.0577 and 0.0633. There was obviously some disorder in the chloroethyl side chain, as a peak of intensity 0.83 eÅ^{-3} could be observed near C7 and another of intensity 0.80 eÅ^{-3} near C8b. Carbon atoms at these positions of occupancy 0.27 combined with a reduction in the occupancy of C13 to 0.73 reduced R and R_w to 0.0483 and 0.0558 respectively. Further efforts to correct the disorder were not undertaken, as this did not significantly affect the geometry of the rest of the molecule. The largest shift/esd values during the final refinement were less than 0.08, and maximum and minimum peaks in the final difference map were 0.24 and 0.36 eÅ^{-3} respectively. Figure 1 shows an ORTEP projection of **22**.

Rates of hydrolysis in buffer

Stock solutions (2 mM) of the mustards were prepared in isopropanol (**7–10**, **12**, **14**, **15**) or DMSO (**17**, **18**), and stored at –20 °C. Reactions were initiated by addition of the stock solution (30 μL) to 20 mM bistrispropane buffer, pH 7.5, ionic strength 10 mM in NaCl (250 μL) held at 37 °C. Aliquots (10 μL) of the reaction mixture were withdrawn at varying time intervals and monitored for product formation by HPLC. The eluting solvent was a mixture of MeCN, MeOH and 0.5 M acetate buffer (AcOH/AcONa) containing 10 mM sodium 1-heptanesulfonate, in the ratio 50% MeCN:33% MeOH:17% AcOH/AcONa for compounds **9**, **14** and **15**, and 40% MeCN:40% MeOH:20% AcOH/AcONa for compounds **7**, **8**, **10**, **12**. Flow rate was 1 mL min^{-1} for compounds **7–10**, **12**, and 0.7 mL min^{-1} for compounds **14** and **15**, and the analytical wavelength was 260 nm. For compounds **17** and **18**, which precipitated at pH 7.5, the pH of bistrispropane buffer was lowered to 6.2, and elution was with MeCN:MeOH:acetate buffer (40:40:20) The flow rate was 0.7 mL min^{-1}, and analytical wavelengths of 268 nm and 262 nm were used for compounds **17** and **18** respectively. For hydrolysis of chlorambucil (**2**), aliquots (30 μL) of a 2 mM stock solution in isopropanol were diluted into buffer as above, and analysed by HPLC with detection at 254 nm and elution with MeCN:MeOH:0.5 M acetate buffer (35:35:15).

Rates of hydrolysis/alkylation with DNA

Aliquots (60 μL) of 2 mM stock solutions of the mustards in isopropanol were added to bistrispropane buffer (20 mM, pH 7.5, ionic strength 10 mM NaCl), at 37 °C containing double stranded calf thymus DNA (3.19 mM in base pairs, determined from absorbance at 260 nm).[29] The final volume in all cases was adjusted to 560 μL by addition of the appropriate volume of bistrispropane buffer. Aliquots (50 μL) of the reaction mixture were withdrawn at different time intervals, mixed well with 1-butanol (50 μL) and 3 M aqueous NaOAc (5 μL), and centrifuged for 2 min. A 20 μL aliquot was withdrawn from the top layer, injected directly onto an HPLC column, and analysed for loss of parent compound at the same wavelengths and flow rates as described above. The reactions were carried out at different base pair:mustard ratios. Compounds **17** and **18** formed precipitates of a DNA/drug complex at both pH 7.5 and 6.2, and no data could be obtained for these derivatives.

Assay for DNA cross-linking

Linearized pBR322 DNA (300 ng aliquots) were incubated with drugs at drug:base pair ratios of 0.01, 0.025, 0.05, 0.10, and 0.50 in TE buffer (10 mM Tris–HCl, 1 mM EDTA) at pH 7.5 in a total volume of 10 μL at 37 °C for 1 h. Samples were shielded from ambient light and incubated for various times at 20 °C, then denatured by the addition of 1 μL of 1% SDS and 5 μL of 50 mM methylmercury hydroxide, followed by incubation for 45 min at 20 °C. Renaturation of drug-treated DNA samples was carried out by incubation with 1.25 μL of 2-mercaptoethanol for 30 min on ice. This incubation time allowed negligible renaturation of non cross-linked DNA and up to 100% renaturation of cross-linked DNA.

Samples were prepared for electrophoresis by the addition of 2.5 μL of 40% sucrose and 0.25% bromophenol blue gel loading buffer. Electrophoresis was carried out on a non-denaturing 1% agarose 0.9 × TAE gel for 2 h at 60 V, using 0.9 × TAE, 10% DMSO running buffer. Both the gel and buffer were cooled to 4 °C before samples were loaded. Control denatured DNA was loaded first to check renaturation was not occurring in the wells before electrophoresis. Following electrophoresis, the gel was stained for 45 min in 1 × TAE containing 0.5 μg mL^{-1} ethidium bromide, followed by destaining in deionised water for 45 min. Gels were visualised on a Spectroline 302 nm

transilluminator, and photographed using a Polaroid MP 4 Land camera.

Biological evaluation

In vitro cytotoxicities were determined against exponentially-growing P388 cells in 96-well culture dishes, as described previously.[30] *In vivo* evaluations were carried out in mice inoculated intraperitoneally with 10^6 P388 leukemia cells. Drugs were given as solutions of the hydrochloride salts in 30% v/v aqueous ethanol as a single dose 24 h after tumor inoculation, at dose levels spaced 1.5-fold apart and covering the range from inactive to toxic.

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