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Synthesis and DNA Cleavage Activity of Bis-3-chloropiperidines as Alkylating Agents

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Nitrogen mustards are an important class of bifunctional alkylating agents routinely used in chemotherapy. They react with DNA as electrophiles through the formation of highly reactive aziridinium ion intermediates. The antibiotic 593A, with potential antitumor activity, can be considered a naturally occurring piperidine mustard containing a unique 3-chloropiperidine ring. However, the total synthesis of this antibiotic proved to be rather challenging. With the aim of designing simplified analogues of this natural product, we developed an efficient bidirectional synthetic route to bis-3-chloropiperidines joined by flexible, conformationally restricted, or rigid diamine linkers. The key step involves an iodide-catalyzed double cyclization of unsaturated bis-*N*-chloroamines to simultaneously generate both piperidine rings. Herein we describe the synthesis and subsequent evaluation of a series of novel nitrogen-bridged bis-3-chloropiperidines, enabling the study of the impact of the linker structure on DNA alkylation properties. Our studies reveal that the synthesized compounds possess DNA alkylating abilities and induce strand cleavage, with a strong preference for guanine residues.

Introduction

Electrophiles readily attack nucleophilic centers of intracellular biomolecules like DNA and proteins. Their reactivity with DNA involves the formation of covalent adducts through base alkylation that cause DNA damage, leading to inhibition of DNA replication and, eventually, to cell death.[1] This cytotoxic mode of action has been exploited in cancer treatment. In fact, DNA alkylating agents are the oldest class of anticancer drugs and are still widely used in chemotherapy.[2] The most potent and effective drugs, such as the nitrogen mustards chlorambucil (Figure 1), cyclophosphamide, and melphalan, react as bifunctional alkylating agents.[3] Compared with monoalkylators, they are capable of forming covalent bonds at two nucleophilic sites within DNA to induce intra- and interstrand cross-links, which are considered responsible for the cytotoxic effects.[4] The common mechanism of alkylation by nitrogen mustards is based on the formation of an electrophilic aziridinium ion, which is a highly reactive alkylating species.[5] In general, nitrogen mustards interact in a relatively nonspecific way with DNA. In addition, most of them have been found to preferentially alkylate the N7 position of guanine.[6]

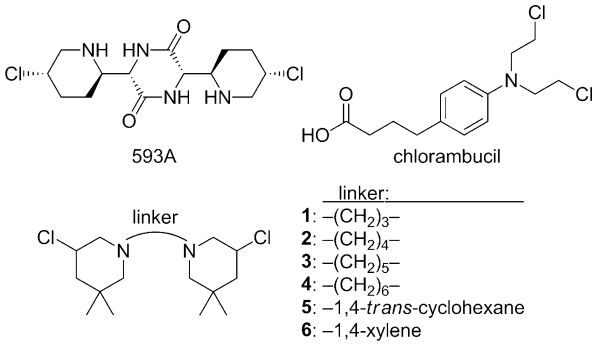

Figure 1. Structures of antibiotic 593A, the nitrogen mustard chlorambucil, and the synthesized bis-3-chloropiperidines as simplified analogues of 593A.

Over the last decades, several bifunctional nitrogen mustard analogues and alkylating agents have been synthesized and investigated for their biological properties.[7] In this context, piperidine- and pyrrolidine-based mustards have shown potential as alkylating antitumor agents via bicyclic aziridinium ion formation.[8] Due to the ring system, the alkylating moiety is sterically restricted, which in turn may contribute to a more controlled reactivity and enhance DNA interactions in vivo. Introducing conformational rigidity by means of ring constraints is a popular strategy in medicinal chemistry to improve affinity and selectivity of drug candidates toward defined biological targets.[9]

A structurally related natural product to the synthetic nitrogen mustards containing a restricted ring system is the antibiotic 593A (Figure 1), isolated from *Streptomyces griseoluteus* in 1970.[10] This compound is a symmetrical piperazinedione composed of two unique 3-chloropiperidine rings.[11] Due to its antineoplastic and antibiotic properties, coupled with its remark-

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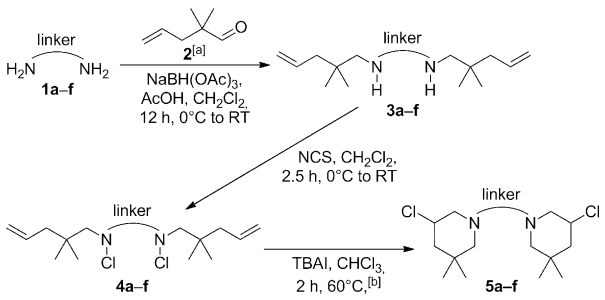
able structure, 593A has attracted considerable interest in synthetic organic and medicinal chemistry.[12] In 1979, Fukuyama et al. successfully achieved a challenging first total synthesis.[13] Studies of the biological effects of this compound suggest that the antibiotic acts as an alkylating agent but with a different mode of action, as 593A was shown to be active in tumors resistant to cyclophosphamide.[14] However, in the recent past, no further research has been undertaken in this area. Thus, to gain a more detailed understanding of this interesting mechanism of action, we synthesized a set of structurally simplified analogues of antibiotic 593A. Bis-3-chloropiperidines **5a–f** (Figure 1 and Table 1) were selected as proof-of-principle compounds and further characterized with respect to their alkylating activity toward DNA.

Previously, our research group established a mild, catalytic, general procedure for the cyclization of unsaturated *N*-chloroamines to 3-chloropiperidines.[15] This synthetic strategy could be successfully extended to the synthesis of bis-3-chloropiperidines. Herein we report a new method for a facile bidirectional synthesis of novel bis-3-chloropiperidines, followed by evaluation of their alkylating activity as well as their DNA sequence selectivity.

To study the impact of the linker structure on DNA alkylation, we prepared bis-3-chloropiperidine derivatives with flexible hydrocarbon linkers, as well as with a more conformationally constrained cyclohexyl and a rigid aromatic linker. The results demonstrate that the synthesized compounds react with DNA very efficiently and that alkylation takes place primarily at guanine residues, in line with what has been published for conventional nitrogen mustards.[6]

Table 1. Bidirectional synthesis of nitrogen-linked bis-3-chloropiperidines **5a–f** by reductive amination, followed by chlorination and subsequent iodide-catalyzed cyclization.



Yield of Diamine [%]	Yield of bis-*N*-chloroamine [%]	Yield of bis-3-chloropiperidine [%]
3a 95	**4a** 94	**5a** 88
3b 91	**4b** 87	**5b** 88
3c 92	**4c** 77	**5c** 78
3d 95	**4d** 80	**5d** 77
3e 37[c]	**4e** 78	**5e** 63
3f 88	**4f** 91	**5f** 88

[a] See Experimental Section for the preparation of **2**. [b] Oil bath temperature. [c] For diamine linker **1e**, isolation of the imine and its subsequent reduction was found to be a more effective method: Na_2SO_4, RT, 24 h, then $NaBH_4$, 2-propanol/MeOH, RT, 12 h, 82% over two steps. NCS = *N*-chlorosuccinimide, TBAI = tetrabutylammonium iodide.

Results and Discussion

Chemistry

Structurally, the antibiotic 593A is a dimer consisting of a piperazinedione linker connecting two 3-chloropiperidines. With the aim of obtaining simplified analogues, our original synthetic plan focused on the linkage of two 3-chloropiperidine monomers via an alkyl spacer to the 6-position of each piperidine ring. However, this strategy did not lead to satisfactory results, due to poor yields, and was abandoned after several attempts. One major problem with this synthetic approach can be attributed to the high reactivity and consequently low stability of the 3-chloropiperidine ring.

Thus, it appeared ideal to prepare both heterocyclic rings at the end of the synthesis. We also reasoned that the preparation of nitrogen-linked bis-3-chloropiperidines might provide a more convenient strategy, as the introduction of protecting groups would be avoided. To take advantage of the symmetry of the desired bis-3-chloropiperidines, we proposed a bidirectional route. Accordingly, our synthetic plan involved the simultaneous formation of the piperidine rings following a well-established procedure in our laboratory for iodide-catalyzed cyclization of unsaturated *N*-chloroamines.[15]

A facile and efficient bidirectional route was designed to enable the synthesis of a series of nitrogen-linked bis-3-chloropiperidines from easily accessible starting materials, as outlined in Table 1. We selected a variety of bridging diamine linkers to examine the effect of linker length and flexibility on the DNA alkylating properties of the derivatives. To study these features, we first constructed a set of compounds with three to six carbon atoms between the two piperidine rings. Furthermore, as the antibiotic 593A is connected by a rather rigid piperazinedione system, we targeted the synthesis of bis-3-chloropi-

peridines with a conformationally restricted cyclohexyl as well as a rigid aromatic spacer unit, linking the alkylating moieties through the 1,4-positions, similarly to the natural product.

The precursors could be readily prepared by double reductive amination of 2,2-dimethylpent-4-enal **2** with the appropriate commercially available diamines **1a–f** using sodium triacetoxyborohydride.[16] Aldehyde **2** was obtained from isobutyraldehyde and allylic alcohol according to the reported procedure.[17] However, a single-stage reductive amination reaction was not as suitable for *trans*-1,4-cyclohexanediamine **1e**, affording the product in rather low yields. The unsaturated cyclohexyl diamine was therefore prepared by imine formation in a prior step and subsequent reduction in a separate step with sodium borohydride. Chlorination of **3a–f** with *N*-chlorosuccinimide (NCS) yielded the unsaturated bis-*N*-chloroamines **4a–f**, which were cyclized using a catalytic amount of tetrabutylammonium iodide (TBAI) to give the desired bis-3-chloropiperidines **5a–f** as an inseparable mixture of stereoisomers.

Bis-3-chloropiperidines induce nicking of supercoiled plasmid DNA

The activity of the synthetic bis-3-chloropiperidines toward DNA was tested analyzing the conversion of supercoiled DNA in its different topological forms. DNA strand cleavage can be ascertained from the different electrophoretic migration of supercoiled, open circular and linear forms of the plasmid DNA in agarose gels. The anticancer drug chlorambucil (CA), which serves as a representative of the bifunctional nitrogen mustards, was used as a control. In our experiment, the supercoiled form of pAT153 was incubated with increasing concentrations (0.5, 5, 50 μM) of alkylating agents at 37 °C for 3 h in bisphosphate-EDTA (BPE) buffer at pH 7.4. The results shown in Figure 2 demonstrate that the tested compounds efficiently nick the plasmid, a feature connected to DNA alkylation, followed by destabilization of the nucleobases, resulting in strand cleavage.[1a] Bis-3-chloropiperidines **5a–e** induced efficient nicking of supercoiled plasmid at a concentration of 5 μM. Compound **5c**, with a flexible five-carbon linker chain (Figure 2a), displayed total conversion of the supercoiled form to the open circular form under these conditions, whereas compound **5f** (Figure 2b), with an aromatic linker, had no appreciable effect. An increase in the concentration of alkylating agents led to DNA fragmentation, resulting in smears or diffused bands in the agarose gel. In clear contrast, DNA cleavage by the control substance CA (Figure 2b) could not be detected at the highest concentration (50 μM). Similar results were observed with different plasmids (not shown). When time course studies were conducted, complete fragmentation of plasmid DNA was observed at the highest concentration after 15 h of incubation, while under the same experimental conditions, partial plasmid linearization was detected for CA (Supporting Information, Figure S1).

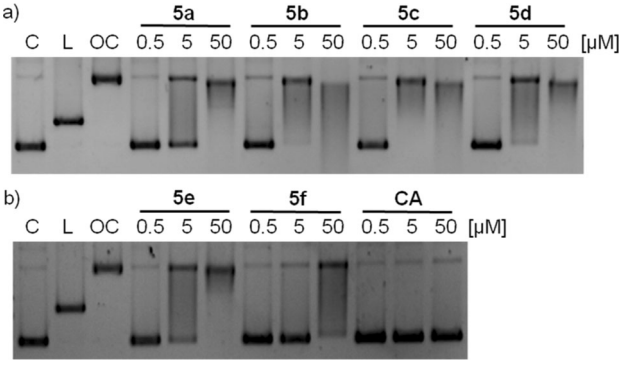
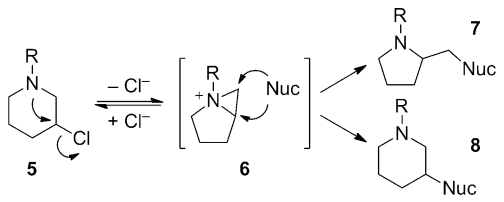


Figure 2. DNA cleavage activity of bis-3-chloropiperidine derivatives with a) flexible carbon chain linkers and b) restricted linkers. The supercoiled form of plasmid DNA pAT153 (120 ng) was incubated with the compounds at 37 °C for 3 h in BPE buffer, pH 7.4, at various concentrations (0.5, 5, 50 μM). Chlorambucil (CA) was used as a control. Cleavage of DNA was analyzed by agarose (1%) gel electrophoresis in 1× TBE. C = supercoiled DNA control, L = linear DNA control, OC = open circular (nicked) DNA control.

By comparing the DNA cleavage activities of the investigated bis-3-chloropiperidines shown in Figure 2, it appears that the linker length had no apparent influence on alkylating properties, as a similar level of DNA nicking could be observed within the group of compounds **5a–d**, which vary in linker length (Figure 2a). However, it should be noted that **5a** was slightly less active than the corresponding derivatives with an increased linker length (compare **5a** with **5b–d**). Likewise, the introduction of a more conformationally restricted cyclohexane linker did not alter the alkylating efficiency. In contrast, the incorporation of a rigid aromatic linker resulted in a decrease of activity (compare compounds **5e** and **5f**, Figure 2b). This different reactivity might be attributed to the fixed geometry of the planar aromatic linker and potential stacking interactions. These findings indicate that the flexibility of the linker component is an important structural feature of bis-3-chloropiperidines with regard to DNA alkylation efficiency.

Mechanism of DNA alkylation by 3-chloropiperidines

3-Chloropiperidines **5** react with DNA via a highly electrophilic bicyclic aziridinium intermediate **6**, formed from intramolecular nucleophilic displacement of chloride by nitrogen as indicated in Scheme 1. Detailed mechanistic studies have confirmed the existence of the aziridinium ion.[18] This reactive species can be readily attacked by nucleophilic centers of the DNA bases to give five-membered **7** and six-membered **8** ring adducts (Scheme 1). The favored alkylation product depends on the



Scheme 1. Proposed alkylation mechanism of 3-chloropiperidines **5**. The reaction proceeds via a bicyclic aziridinium ion intermediate **6**, which is readily attacked by nucleophiles (Nuc). The resulting product can be a pyrrolidine **7** or a piperidine **8** adduct, depending on the steric influence of both the R group and the nucleophile.

bulkiness of the R-group attached to the nitrogen, as well as on the nature of the attacking nucleophile.[8a,18] Steric hindrance almost certainly diverts the alkylation reaction to the sterically less hindered side of the aziridinium ion **6**, affording the pyrrolidine adduct **7**.

In addition, the N-substituent (R) influences the basicity of the nitrogen atom, which in turn has a severe impact on the formation of the respective aziridinium ions. Accordingly, electron-donating groups should lead to an increased formation rate and stabilization of the reactive species, as the R-group will push electron density into the aziridinium moiety. On the other hand, electron-withdrawing substituents should have the opposite effect by destabilizing the positive charge of the aziridinium ion. As a result, the nitrogen center in aromatic nitrogen mustards like CA is less basic than in the aliphatic analogues. Thus, the latter alkylating agents are much more readily activated.[19]

Consequently, the formation of DNA alkylation products depends mainly on the reactivity and the nature of the alkylating agent. However, due to a general instability of these covalent DNA adducts in solution, the structural characterization is difficult. Previous studies established that guanine is the most reactive site for DNA alkylation by many cytotoxic alkylating agents.[1a,6] One effect of guanine alkylation is an increase in the electrophilicity of neighboring positions, which can lead to hydrolytic DNA cleavage reactions. Alternatively, alkylation can destabilize the nucleobases, followed by deglycosylation and subsequent DNA strand scission.[1a,20]

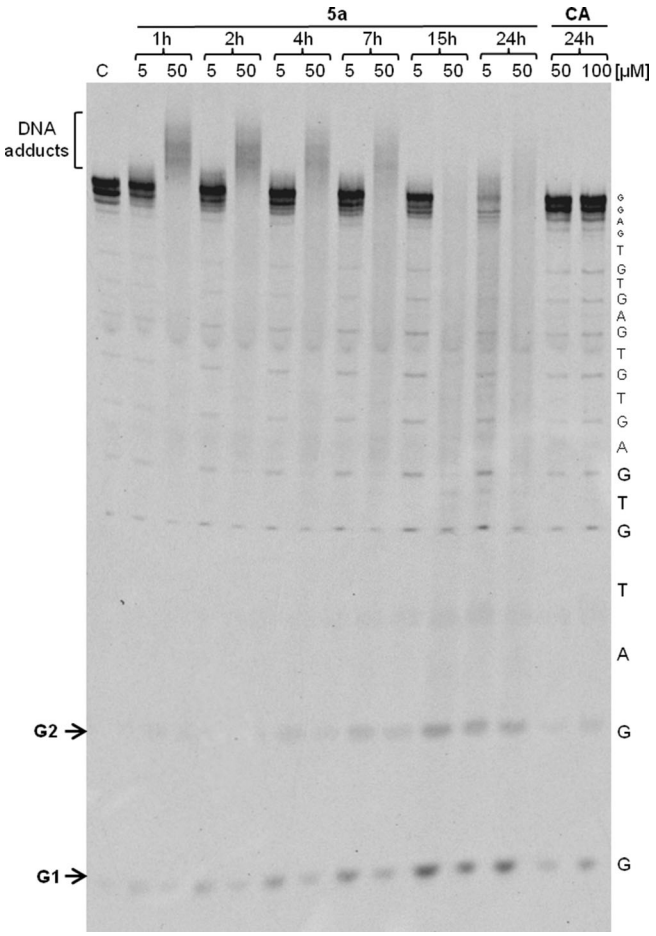


Figure 3. Representative denaturing polyacrylamide (20%) gel in 1× TBE, showing time- and concentration-dependent cleavage of a 22-mer double-stranded oligonucleotide caused via guanine alkylation by bis-3-chloropiperidine **5a**. The 5′-FAM-labeled scrambled duplex oligonucleotide (4 μM), GGA TGT GAG TGT GAG TGT GAG G, was treated with compound **5a** at 37 °C in BPE buffer, pH 7.4, at 5 and 50 μM for incubation times as indicated. Chlorambucil (CA) was used as a control. Arrows indicate the position of fast-migrating fragment bands. C = untreated duplex oligonucleotide control.

DNA alkylation by 3-chloropiperidines

To obtain experimental evidence of DNA alkylation by the synthesized bis-3-chloropiperidines, we examined their effects on a 22-mer oligonucleotide duplex containing a G-rich sequence. After incubation, the reaction products were resolved by polyacrylamide gel electrophoresis (PAGE), enabling identification of the specific alkylated bases. Alkylation reactions were carried out in BPE buffer, pH 7.4, at 37 °C for a range of incubation times from 1 h to 24 h at two distinct concentrations (5 and 50 μM). Sequence specificities were analyzed by high-resolution PAGE. Figure 3 shows the results for bis-3-chloropiperidine **5a**, which was selected as a representative example for this study, toward the 5′-FAM-labelled double-stranded oligonucleotide. The tested compound, **5a**, showed alkylation followed by cleavage at guanines. The effect is dependent on time and concentration, with the oligonucleotide being degraded after 24 h alkylation at a concentration of 50 μM. In comparison, the control substance, CA, displayed no appreciable effect on the oligonucleotide at this concentration or even at a higher concentration of 100 μM. This finding supports the above-mentioned assumption that formation of the aziridinium ion in the case of the bis-3-chloropiperidines with aliphatic N-substituents is more favorable than the activation of CA. The nitrogen lone pair in CA is delocalized into the aromatic ring and is therefore less available for electrophilic attack.[19] Accordingly, it can be expected that the bis-3-chloropiperidines in this study possess higher alkylating activities than the aromatic mustard CA, which is in agreement with our experimental observations.

Interestingly, in addition to DNA fragmentation, the appearance of diffuse gel bands with lower mobility than the control band corresponding to the untreated duplex oligonucleotide (C, Figure 3) were observed at the highest dose of compounds. The shifted bands may result from formation of DNA adducts (DNA adducts, Figure 3). These lower mobility adducts were transformed in fragmented oligonucleotides over time, as evidenced by the smearing of the DNA band concurrent with the occurrence of fast migrating bands corresponding to shorter DNA fragments (arrows, Figure 3) cleaved at guanines. Although a certain degree of precipitation may have occurred, the band diffusion and the smear in the lanes moving to lower molecular weight with time was consistent with fragmentation of DNA, which was observed in all our experimental conditions. At the same G position in the gel, relatively weak fragment bands were observed for CA.

The alkylation patterns from the sequencing gel analysis revealed that bis-3-chloropiperidines preferentially induce DNA cleavage through reactions with guanines, sustaining our proposed mechanism of reaction. In general, the sequence selectivity is similar to that observed previously for chemotherapeutic nitrogen mustards.[6] It should be pointed out here that the alkylating potency of the set of compounds examined follow the same pattern observed in the supercoiled DNA nicking assay (data not shown).

Conclusions

With the aim of designing simplified analogues of the antineoplastic antibiotic 593A, we successfully synthesized a series of bis-3-chloropiperidines by a three-step route using a bidirectional strategy. Relationships between the linker structure of compounds and DNA alkylation activity were determined by a DNA cleavage assay with a supercoiled plasmid and sequencing gel analysis with a 22-mer duplex oligonucleotide. The results demonstrate that examined bis-3-chloropiperidines **5a–e** alkylate DNA with high efficiency, involving the induction of strand cleavage primarily at guanine sites. In addition, our studies reveal that linker length does not affect the alkylating properties, whereas a change in flexibility by insertion of an aromatic rigid linker structure causes a decrease in reactivity (**5f**). Significantly, all of the tested bis-3-chloropiperidines were more reactive toward DNA than the anticancer drug CA. Further investigations into the reactivity of bis-3-chloropiperidines with DNA are underway to provide a better understanding of the molecular mechanism of action, useful for directing the synthesis of new molecules with therapeutic effect.

Experimental Section

Chemistry

Commercially available reagents were used as supplied. All solvents were purified by distillation and dried, if necessary, prior to use. Reactions requiring the use of anhydrous solvents were carried out in heat gun-dried glassware under an argon atmosphere (Schlenk technique). Products were purified by flash chromatography on silica gel 60 (Merck). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 200 spectrometer (^{1}H at 200 MHz; ^{13}C at 50 MHz), a Bruker Avance II 400 spectrometer (^{1}H at 400 MHz; ^{13}C at 100 MHz), and a Bruker Avance III 600 spectrometer (^{1}H at 600 MHz; ^{13}C at 150 MHz) in the stated deuterated solvent using TMS as an internal standard. Chemical shifts were determined by reference to the residual solvent resonances. High-resolution EI mass spectrometry data were obtained with a Finnigan MAT 95 (70 eV); all ESI mass spectra were obtained with a Finnigan LCQDuo mass spectrometer. NMR spectra of all synthesized compounds are included in the Supporting Information.

2,2-Dimethylpent-4-enal (2): Freshly distilled isobutyraldehyde (108 g, 1.5 mol) and allyl alcohol (58.0 g, 1.0 mol) were added to a solution of *p*-toluenesulfonic acid (0.25 g) in *p*-cymene (200 g). The mixture was heated at reflux for 32 h under a Dean–Stark trap until no more water was separated and the sump temperature reached ~140 °C. After vacuum distillation (76 °C at 200 mbar) through a 50 cm Vigreux column, aldehyde **2** (81.4 g, 0.73 mol, 73%) was obtained as a colorless liquid. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 9.47$ (s, 1H; CH=O), 5.70 (m, 1H), 5.05 (m, 2H), 2.21 (d, $J =$ 7.3 Hz, 2H), 1.05 ppm (s, 6H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 205.9$, 133.1, 118.4, 45.7, 41.4, 21.1 ppm. These data are consistent with published data.[16,18]

General procedure for the synthesis of diamines 3a–f: Sodium triacetoxyborohydride (2.5 equiv) was added portionwise to a solution of the unsaturated aldehyde **2** (2 equiv) and the appropriate diamine in anhydrous CH_2Cl_2 (7 mL mmol^{-1} of diamine) at 0 °C, followed by addition of acetic acid (1–2 equiv). The reaction mixture was stirred at room temperature under argon atmosphere for 12 h and was then quenched with 20% NaOH solution. The phases were separated, and the aqueous layer was extracted three times with 20 mL CH_2Cl_2. The combined organic phases were first washed with brine, then with water, and were dried over Na_2SO_4. The solvent was removed under reduced pressure to afford the corresponding product, which was used in the next step without further purification.

***N*^{1},*N*^{3}-Bis(2,2-dimethylpent-4-enyl)propane-1,3-diamine (3a)**: Compound **3a** was obtained from **2** and 1,3-diaminopropane as a colorless liquid (2.22 g, 8.32 mmol, 95%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.80$ (ddt, $J = 15.8$ Hz, $J = 11.5$ Hz, $J = 7.5$ Hz, 2H), 4.99 (m, 4H), 2.64 (t, $J = 6.8$ Hz, 4H), 2.33 (s, 4H), 1.99 (d, $J = 7.5$ Hz, 4H), 1.65 (quin, $J = 6.8$ Hz, 2H), 0.87 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 135.6$, 116.7, 60.7, 49.7, 44.8, 34.2, 30.2, 25.5 ppm; HRMS (EI): *m/z* calcd for $C_{17}H_{34}N_2$: 266.2722; found: 266.2714.

***N*^{1},*N*^{4}-Bis(2,2-dimethylpent-4-enyl)butane-1,4-diamine (3b)**: Compound **3b** was obtained from **2** and 1,4-diaminobutane as a colorless liquid (5.84 g, 20.8 mmol, 91%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 5.76 (ddt, $J = 16.1$ Hz, $J = 11.9$ Hz, $J = 7.5$ Hz, 2H), 4.95 (m, 4H), 2.54 (t, $J = 6.3$ Hz, 4H), 2.30 (s, 4H), 1.95 (d, $J = 7.5$ Hz, 4H), 1.44 (m, 4H), 0.83 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 135.6$, 116.7, 60.6, 51.2, 44.2, 34.7, 27.7, 25.6 ppm; HRMS (EI): *m/z* calcd for $C_{18}H_{36}N_2$: 280.2878; found: 280.2847.

***N*^{1},*N*^{5}-Bis(2,2-dimethylpent-4-enyl)pentane-1,5-diamine (3c)**: Compound **3c** was obtained from **2** and 1,5-diaminopentane as a colorless liquid (3.81 g, 12.9 mmol, 92%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.75$ (m, 2H), 4.93 (m, 4H), 2.52 (t, $J = 7.1$ Hz, 4H), 2.28 (s, 4H), 1.94 (d, $J = 7.5$ Hz, 4H), 1.43 (dt, $J = 14.7$ Hz, $J = 7.4$ Hz, 4H), 1.28 (ddd, $J = 12.1$ Hz, $J = 7.3$, $J = 2.4$ Hz, 2H), 0.82 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 135.5$, 116.7, 60.3, 50.9, 44.7, 34.1, 29.9, 25.5, 25.0 ppm; HRMS (EI): *m/z* calcd for $C_{19}H_{38}N_2$: 294.3035; found: 294.3015.

***N*^{1},*N*^{6}-Bis(2,2-dimethylpent-4-enyl)hexane-1,6-diamine (3d)**: Compound **3d** was obtained from **2** and 1,6-diaminohexane as a colorless liquid (8.43 g, 27.3 mmol, 95%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.77$ (m, 2H), 4.96 (m, 4H), 2.53 (t, $J = 7.5$ Hz, 4H), 2.30 (s, 4H), 1.96 (d, $J = 7.5$ Hz, 4H), 1.42 (m, 4H), 1.27 (m, 4H), 0.84 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 135.6$, 116.7, 60.4, 50.9, 44.8, 34.2, 30.0, 27.3, 25.5 ppm; HRMS (EI): *m/z* calcd for $C_{20}H_{40}N_2$: 308.3191; found: 308.3195.

trans*-*N*^{1},*N*^{4}-Bis(2,2-dimethylpent-4-enyl)cyclohexane-1,4-diamine (3e): Compound **3e** was obtained from **2** and *trans*-1,4-cyclohexanediamine as a colorless liquid (1.02 g, 3.34 mmol, 37%). *Isolation of the **3e-imine** and its subsequent reduction afforded a significantly higher yield of **3e** (82% over two steps) than the single-stage reductive amination procedure. The method for synthesis of **3e-imine** and its reduction for preparation of corresponding bis-*N*-diamine **3e** is below:

***trans*-N^1,N^4-Bis(2,2-dimethylpent-4-enylidene)cyclohexane-1,4-diamine (3e-imine)**: *trans*-1,4-Cyclohexanediamine (1.50 g, 13.1 mmol, 1 equiv) was added portionwise to a solution of 2,2-dimethyl-4-pentenal **2** (2.94 g, 26.2 mmol, 2 equiv) in 20 mL anhydrous CH_2Cl_2 at 0 °C, followed by one spatula (~1 g) of Na_2SO_4. The reaction mixture was stirred at room temperature for 24 h. After removing the Na_2SO_4 by filtration, the solvent was removed under reduced pressure to afford pure product **3e-imine** in 93% yield (3.69 g, 12.2 mmol) as a colorless liquid: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.51 (s, 2H), 5.69 (m, 2H), 4.95 (m, 4H), 2.90 (m, 2H), 2.10 (d, J = 7.4 Hz, 4H), 1.57 (m, 8H), 0.98 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.3, 134.7, 117.2, 69.1, 44.7, 38.6, 32.6, 24.7 ppm; HRMS (EI): *m/z* calcd for $C_{20}H_{34}N_2$: 302.2722; found: 302.2749.

***trans*-N^1,N^4-Bis(2,2-dimethylpent-4-enyl)cyclohexane-1,4-diamine (3e)**: Sodium borohydride (462 mg, 1 equiv) was added portionwise to a solution of **3e-imine** (3.69 g, 12.2 mmol) in 30 mL 2-propanol and 5 mL MeOH at 0 °C. The reaction mixture was stirred at room temperature for 12 h and was then hydrolyzed with 20% NaOH solution. The phases were separated, and the aqueous layer was extracted three times with 20 mL diethyl ether. The combined organic phases were washed with brine and dried over Na_2SO_4. The solvent was removed under reduced pressure to obtain the desired compound **3e** as a colorless liquid (3.27 g, 10.7 mmol, 88%), which was used without further purification for the next step: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.71 (m, 2H), 4.95 (m, 4H), 2.31 (s, 4H), 2.27 (m, 2H), 1.94 (d, J = 7.5 Hz, 4H), 1.85 (d, J = 6.2 Hz, 4H), 1.05 (m, 4H), 0.82 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 135.6, 116.6, 57.7, 44.6, 34.1, 32.3, 25.5 ppm; HRMS (EI): *m/z* calcd for $C_{20}H_{38}N_2$: 306.3035; found: 306.3021.

***N*,*N*′-(1,4-Phenylenebis(methylene))bis(2,2-dimethylpent-4-en-1-amine) (3f)**: Compound **3f** was obtained from **2** and 1,4-bis(aminomethyl)benzene according to the general procedure as a colorless liquid (4.18 g, 12.7 mmol, 88%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.29 (s, 4H) 5.79 (m, 2H), 4.99 (m, 4H), 3.78 (s, 4H), 2.37 (s, 4H), 2.02 (d, J = 7.5 Hz, 4H), 0.89 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 139.4, 135.6, 127.9, 116.7, 59.7, 54.5, 44.6, 34.3, 25.5 ppm; HRMS (EI): *m/z* calcd for $C_{22}H_{36}N_2$: 328.2878; found: 328.2888.

General procedure for the synthesis of bis-*N*-chloroamines 4a–f: *N*-Chlorosuccinimide (2.2 equiv) was added to a cooled (0 °C) solution of the corresponding bis-*N*-diamine in anhydrous CH_2Cl_2 (10 mL mmol^{-1} of bis-*N*-diamine). The reaction mixture was stirred first for half an hour at 0 °C, and then for an additional 2 h at room temperature. After removal of the solvent in vacuo, the product was isolated from the residue by flash chromatography (pentane/TBME, 10:1).

***N*^{1},*N*^{3}-Dichloro-*N*^{1},*N*^{3}-bis(2,2-dimethylpent-4-enyl)propane-1,3-diamine (4a)**: Compound **4a** was obtained from **3a** according to the general procedure as a colorless oil (354 mg, 1.06 mmol, 94%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.81 (ddt, J = 16.7 Hz, J = 10.5 Hz, J = 7.5 Hz, 2H), 5.03 (m, 4H), 3.02 (t, J = 6.7 Hz, 4H), 2.85 (s, 4H), 2.06 (d, J = 7.5 Hz, 4H), 1.98 (quin, J = 6.7 Hz, 2H), 0.95 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 135.2, 117.3, 74.8, 63.7, 44.9, 35.6, 26.7, 25.8 ppm.

***N*^{1},*N*^{4}-Dichloro-*N*^{1},*N*^{4}-bis(2,2-dimethylpent-4-enyl)butane-1,4-diamine (4b)**: Compound **4b** was obtained from **3b** according to the general procedure as a colorless oil (1.73, 4.94 mmol, 87%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.74 (ddt, J = 16.7 Hz, J = 10.5 Hz, J = 7.5 Hz, 2H), 4.95 (m, 4H), 2.88 (m, 4H), 2.77 (s, 4H), 2.00 (d, J = 7.5 Hz, 4H), 1.63 (m, 4H), 0.87 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 135.2, 117.3, 74.8, 66.4, 44.9, 35.6, 25.7, 25.2 ppm.

***N*^{1},*N*^{5}-Dichloro-*N*^{1},*N*^{5}-bis(2,2-dimethylpent-4-enyl)pentane-1,5-diamine (4c)**: Compound **4c** was obtained from **3c** according to the general procedure as a colorless oil (1.45 g, 3.99 mmol, 77%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.80 (ddt, J = 16.7 Hz, J = 10.4 Hz, J = 7.5 Hz, 2H), 5.02 (m, 4H), 2.93 (t, J = 7.1 Hz, 4H), 2.84 (s, 4H), 2.06 (d, J = 7.5 Hz, 4H), 1.66 (q, J = 7.3, 4H), 1.40 (m, 2H), 0.94 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 135.2, 117.3, 74.8, 66.6, 44.8, 35.6, 27.9, 25.7, 23.8 ppm.

***N*^{1},*N*^{6}-Dichloro-*N*^{1},*N*^{6}-bis(2,2-dimethylpent-4-enyl)hexane-1,6-diamine (4d)**: Compound **4d** was obtained from **3d** according to the general procedure as a colorless oil (1.51 g, 3.99 mmol, 80%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.81 (ddt, J = 16.7 Hz, J = 10.4 Hz, J = 7.5 Hz, 2H), 5.03 (m, 4H), 2.91 (t, J = 6.9 Hz, 4H), 2.83 (s, 4H), 2.06 (d, J = 7.5 Hz, 4H), 1.64 (m, 4H), 1.36 (m, 4H), 0.94 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 135.2, 117.2, 74.7, 66.7, 44.8, 35.6, 28.0, 26.5, 25.7 ppm.

***trans*-N^1,N^4-Dichloro-N^1,N^4-bis(2,2-dimethylpent-4-enyl)cyclohexane-1,4-diamine (4e)**: Compound **4e** was obtained from **3e** according to the general procedure as a colorless viscous oil (3.13 g, 8.34 mmol, 78%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.81 (m, 2H), 5.02 (m, 4H), 2.79 (s, 4H), 2.63 (m, 2H), 2.05 (d, J = 7.5 Hz, 4H), 2.00 (d, J = 6.5 Hz, 4H), 1.53 (m, 4H), 0.93 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 135.3, 117.2, 71.2, 70.5, 44.7, 35.4, 27.3, 25.5 ppm.

***N*,*N*′-(1,4-Phenylenebis(methylene))bis(*N*-chloro-2,2-dimethylpent-4-en-1-amine) (4f)**: Compound **4f** was obtained from **3f** according to the general procedure as a colorless oil (1.61 g, 4.06 mmol, 91%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.35 (s, 4H), 5.76 (ddt, J = 15.3 Hz, J = 11.0 Hz, J = 7.5 Hz, 2H), 5.00 (m, 4H), 4.11 (s, 4H), 2.92 (s, 4H), 2.07 (d, J = 7.5 Hz, 4H), 0.94 ppm (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 137.2, 135.2, 129.1, 117.3, 73.3, 70.2, 44.8, 35.6, 25.8 ppm.

General procedure for the synthesis of bis-3-chloropiperidines 5a–f: The bis-*N*-chloroamine was dissolved in anhydrous chloroform (10 mL mmol^{-1} of bis-*N*-chloroamine), and tetrabutylammonium iodide (10 mol%) was added to the solution. The resulting mixture was then heated at 60 °C (oil bath temperature) for 2 h. After removal of the solvent under reduced pressure, the residue was purified by flash chromatography (pentane/TBME, 10:1). The bis-3-chloropiperidine was obtained as a mixture of diastereomers.

1,3-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)propane (5a): Compound **5a** was obtained from **4a** according to the general procedure as a colorless oil (310 mg, 0.92 mmol, 88%, mixture of diastereomers): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.06 (ttd, J = 8.9 Hz, J = 4.4 Hz, J = 1.0 Hz, 2H), 3.13 (dd, J = 10.4 Hz, J = 4.3, 2H), 2.35 (m, 6H), 1.93 (m, 4H), 1.71 (d, J = 11.0 Hz, 2H), 1.58 (quin, J = 7.2 Hz, 2H), 1.32 (t, J = 12.3 Hz, 2H), 1.02 (s, 6H), 0.91 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 64.8, 62.3, 62.2, 55.7, 55.6, 54.3, 48.4, 33.3, 29.4, 25.2, 24.3 ppm; HRMS (ESI): *m/z* calcd for $C_{17}H_{32}N_2Cl_2 + H^+$: 335.2015; found: 335.2015.

1,4-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)butane (5b): Compound **5b** was obtained from **4b** according to the general procedure as a colorless oil (1.51 g, 4.33 mmol, 88%, mixture of diastereomers): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.06 (ttd, J = 11.8 Hz, J = 4.4 Hz, J = 1.1 Hz, 2H), 3.13 (dd, J = 10.6 Hz, J = 4.3, 2H), 2.38 (d, J = 11.0 Hz, 2H), 2.29 (m, 4H), 1.91 (m, 4H), 1.67 (dd, J = 11.1 Hz, J = 2.7 Hz, 2H), 1.44 (m, 4H), 1.31 (t, J = 12.3 Hz, 2H), 1.02 (s, 6H), 0.90 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 64.8, 64.7, 62.3, 62.2, 57.7, 57.6, 54.3, 48.4, 33.3, 29.4, 25.2, 24.5 ppm; HRMS (ESI): *m/z* calcd for $C_{18}H_{34}N_2Cl_2 + H^+$: 349.2172; found: 349.2172.

1,5-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)pentane (5c): Compound **5c** was obtained from **4c** according to the general procedure as a colorless oil (1.24 g, 3.40 mmol, 78%, mixture of diastereomers): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.07 (tt, J = 11.3 Hz, J = 4.4 Hz, 2H), 3.14 (dd, J = 10.6 Hz, J = 4.4, 2H), 2.39 (d, J = 11.1 Hz, 2H), 2.30 (m, 4H), 1.91 (m, 4H), 1.68 (d, J = 11.1 Hz, 2H), 1.44 (quin, J = 7.1 Hz, 4H), 1.31 (m, 4H), 1.02 (s, 6H), 0.91 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 64.7, 62.3, 57.9, 54.4, 48.4, 33.2, 29.4, 26.8, 25.2, 25.1 ppm; HRMS (ESI): *m/z* calcd for $C_{19}H_{36}N_2Cl_2+H^+$: 363.2328; found: 363.2328.

1,6-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)hexane (5d): Compound **5d** was obtained from **4d** according to the general procedure as a colorless oil (1.16 g, 3.07 mmol, 77%, mixture of diastereomers): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.06 (ddt, J = 11.9 Hz, J = 10.8 Hz, J = 4.4 Hz, 2H), 3.13 (dd, J = 10.6 Hz, J = 4.4, 2H), 2.39 (d, J = 11.0 Hz, 2H), 2.29 (m, 4H), 1.91 (m, 4H), 1.67 (d, J = 11.1 Hz, 2H), 1.43 (m, 4H), 1.30 (m, 6H), 1.02 (s, 6H), 0.90 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 64.7, 62.4, 57.9, 54.4, 48.4, 33.2, 29.4, 27.2, 26.9, 25.2 ppm; HRMS (ESI): *m/z* calcd for $C_{20}H_{38}N_2Cl_2+H^+$: 377.2485; found: 377.2485.

***trans*-1,4-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)cyclohexane (5e)**: Compound **5e** was obtained from **4e** according to the general procedure as a colorless solid (176 mg, 0.47 mmol, 63%, mixture of diastereomers): ^{1}H NMR (600 MHz, $CDCl_3$): δ = 4.01 (ddd, J = 15.7 Hz, J = 11.0 Hz, J = 4.4 Hz, 2H), 3.09 (dd, J = 10.4 Hz, J = 4.1, 2H), 2.30 (d, J = 11.0 Hz, 2H), 2.26 (m, 2H), 2.20 (t, J = 10.5 Hz, 2H), 1.97 (d, J = 11.0 Hz, 2H), 1.91 (dd, J = 12.5 Hz, J = 4.0, 2H), 1.80 (m, 4H), 1.26 (m, 2H), 1.23 (m, 4H), 0.99 (s, 6H), 0.89 ppm (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 63.5, 60.5, 58.8, 55.2, 48.9, 33.3, 29.4, 27.9, 27.8, 27.2, 27.1, 25.0 ppm; HRMS (ESI): *m/z* calcd for $C_{20}H_{36}N_2Cl_2+H^+$: 375.2328; found: 375.2328.

1,4-Bis((5-chloro-3,3-dimethylpiperidin-1-yl)methyl)benzene (5f): Compound **5f** was obtained from **4f** according to the general procedure as a colorless oil (1.41 g, 3.55 mmol, 88%, mixture of diastereomers): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.24 (s, 4H), 4.11 (ddd, J = 16.0 Hz, J = 11.3 Hz, J = 4.4 Hz, 2H), 3.49 (q, J = 13.4 Hz, 4H), 3.16 (dd, J = 10.0 Hz, J = 4.0, 2H), 2.39 (d, J = 10.9 Hz, 2H), 1.97 (m, 4H), 1.76 (d, J = 11.0 Hz, 2H), 1.35 (t, J = 12.3 Hz, 2H), 1.06 (s, 6H), 0.89 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 137.3, 128.5, 64.6, 62.0, 61.9, 54.3, 48.4, 33.4, 29.3, 25.1 ppm; HRMS (ESI): *m/z* calcd for $C_{22}H_{34}N_2Cl_2+H^+$: 397.2172; found: 397.2172.

Bioassays

The water used in all biochemical experiments was prepared from the Milli-Q Synthesis (Millipore) water purification system. Chlorambucil and chemicals for preparing buffer solutions were purchased from Sigma–Aldrich (St. Louis, MO, USA), Agarose D-1 Low EEO was purchased from Eppendorf (Hamburg, Germany), and acrylamide-bis ready-to-use solution (40%, 19:1) was purchased from Merck (Darmstadt, Germany). Oligonucleotides were purchased from Eurogentec (Seraing, Liège, Belgium) and stored at −20 °C in TE (10 mM Tris-HCl, 1 mM EDTA). The sequence of the scrambled oligonucleotide used for 5′-FAM labeling was: 5′-FAM-GGA TGT GAG TGT GAG TGT GAG G-3′; the complementary co-scrambled oligonucleotide sequence was: 5′-CCT CAC ACT CAC ACT CAC ATC C-3′. The 5′-FAM labeled scrambled oligonucleotide was mixed with equimolar amounts of its complementary co-scrambled oligonucleotide in BPE buffer (2 mM $NaH_2PO_4{\cdot}2H_2O$, 6 mM $Na_2HPO_4{\cdot}12H_2O$, 1 mM $Na_2EDTA{\cdot}2H_2O$, pH 7.4), denatured at 95 °C for 5 min, and then left to cool to room temperature (slow annealing). This step ensured the formation of the duplex DNA through annealing of scrambled and co-scrambled oligonucleotides. Dilutions of bis-3-chloropiperidines and chlorambucil were freshly prepared from a DMSO stock solution (8 mM) in water. Alkylation reactions were carried out in BPE buffer.

Preparation of plasmid DNA: Plasmid pAT153 was transformed into *E. coli* strain JM109 (Promega). After fermentation, the plasmid DNA was isolated using the Promega PureYield Plasmid Midiprep System according to the instructions of the supplier. The concentration of plasmid was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

DNA cleavage assay: DNA cleavage assays were performed using pAT153 plasmid obtained as described above. pAT153 (120 ng) was incubated with increasing concentrations (0.5, 5, 50 μM) of agent for 3 h at 37 °C in BPE buffer. Plasmid pAT153 nicked by Nb·Bpu10I (Fermentas) was used as a marker for the open circular DNA form, while the linearized standard was obtained by HindIII (NEB) digestion, according to the manufacturer's instructions. Gel loading buffer (10 mM Tris-HCl, 50% glycerol, 0.025% bromophenol blue) was added to all reaction tubes, and the samples were loaded onto a 1% agarose gel. Electrophoresis was conducted in TBE 1X (Tris-HCl 89 mM, borate 89 mM, EDTA 2 mM). DNA in the gel system was detected by staining with ethidium bromide (0.5 μg mL^{-1}) for 30 min with visualization by a Geliance 600 imaging system (PerkinElmer, Waltham, MA, USA).

Sequencing gel analysis: The 5′-FAM-labelled duplex oligonucleotide (4 μM) was incubated with each alkylating agent (final concentrations of 5 and 50 μM) in BPE buffer. The reaction was analyzed at different incubation times: 1, 2, 4, 7, 15, and 24 h at 37 °C. The samples were dried in a vacuum centrifuge (UNIVAPO 100H, UniEquip), resuspended in 5 μL of denaturing gel loading buffer (10 mM Tris-HCl, 80% formamide, 0.025% bromophenol blue), and loaded on a 20% denaturing polyacrylamide gel (7 M urea) in TBE 1X. The fluorescence of the oligonucleotide bands were detected by scanning using Storm Scanner Control (STORM 840, Molecular Dynamics).

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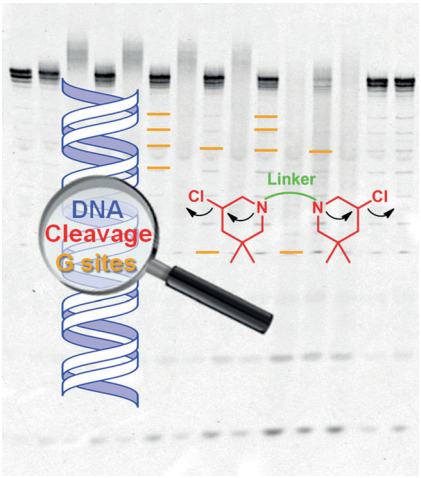
FULL PAPERS

It's all about Gs! An elegant and simple bidirectional synthetic approach to bis-3-chloropiperdines with various linker structures is reported. These new alkylating agents induce cleavage of double-stranded DNA, primarily through reactions toward guanine sites via the formation of electrophilic aziridinium ion intermediates.



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Synthesis and DNA Cleavage Activity of Bis-3-chloropiperidines as Alkylating Agents