Bioorganic & Medicinal Chemistry 23 (2015) 1241–1250

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Bis-3-chloropiperidines containing bridging lysine linkers: Influence of side chain structure on DNA alkylating activity

Ivonne Zuravka [a,b], Rolf Roesmann [a], Alice Sosic [b], Richard Göttlich [a,*], Barbara Gatto [b,*]

[a] Institute of Organic Chemistry, Justus Liebig University Giessen, Heinrich-Buff-Ring 58, 35392 Giessen, Germany
[b] Dipartimento di Scienze del Farmaco, Università di Padova, via Marzolo 5, 35131 Padova, Italy

ARTICLE INFO

Article history:
Received 20 November 2014
Revised 27 January 2015
Accepted 28 January 2015
Available online 7 February 2015

Keywords:
Bis-3-chloropiperidines
Alkylating agents
Nitrogen mustards
Aziridinium ion
DNA cleavage

ABSTRACT

A series of bis-3-chloropiperidines containing lysine linkers was synthesised as DNA alkylating model compounds by using a bidirectional synthetic strategy. These novel piperidine mustard based agents have been evaluated for their alkylating properties towards nucleic acids and were shown to alkylate and cleave DNA with strong preference for guanine residues. Our studies reveal that the introduction of aromatic groups in the side chain of the lysine linker has an impact on DNA alkylating activity. Analysis by ESI mass spectrometry enabled the verification of the reactive aziridinium ion formation. Overall, the results confirm our recently proposed reaction mechanism of bis-3-chloropiperidines.

1. Introduction

Alkylating agents which interact directly with DNA to form covalent bonds have an important therapeutic role in anticancer treatment.[1–3] Nitrogen mustards were the first effective antineoplastic drugs and are still commonly used in chemotherapy.[4] Their mechanism of action is based on the formation of a highly reactive electrophilic aziridinium ion.[5,6] This reactive species can be readily attacked by multiple nucleophilic sites in DNA. Previous studies established that most alkylating agents react particularly with the N7 position of guanine.[7–9] In the first place base alkylation prevents DNA replication and induces DNA fragmentation by hydrolytic reactions, which ultimately leads to cell death. However, the emergence of resistance to this class of drugs is also a substantial challenge in cancer therapy.[10,11] As a result there are ongoing research efforts to develop more effective antitumour drugs.

An important strategy in drug design is based on gaining a deeper insight into the underlying mechanism of action. In this regard mechanistic understanding can be derived from chemical analogues of the original drug which can serve as model compounds. As an example, the well-known chemotherapeutic drug chlorambucil was developed as a more stable analogue of mechlorethamine, the prototype of the nitrogen mustards (Fig. 1a).[12]

In the search for more effective alkylating agents with less systemic toxicity, various mustard analogues have been investigated.[7]

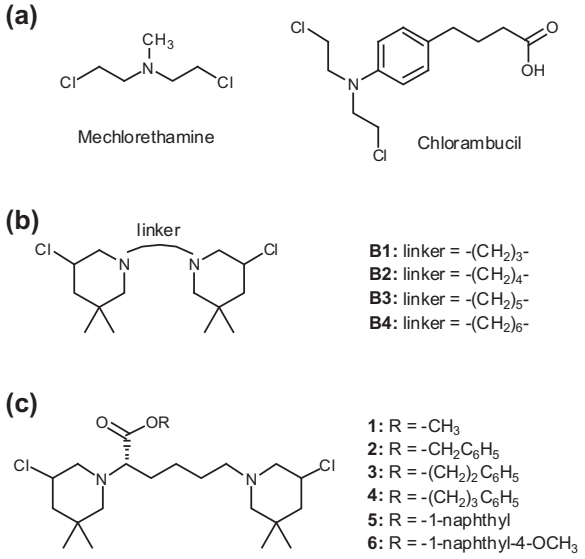


Figure 1. (a) Chemical structures of the nitrogen mustards mechlorethamine and chlorambucil. (b) Bis-3-chloropiperidines **B1**–**B4** investigated in a previous study.[14] (c) Series of newly synthesised compounds **1**–**6** with bridging lysine linkers.

* Corresponding authors. Tel.: +49 641 993 4340; fax: +49 641 993 4349 (R.G.); tel.: +39 049 827 5717; fax: +39 049 827 5366 (B.G.).
E-mail addresses: richard.goettlich@uni-giessen.de (R. Göttlich), barbara.gatto@unipd.it (B. Gatto).

http://dx.doi.org/10.1016/j.bmc.2015.01.050
0968-0896/

In particular the aromatic nitrogen mustards have taken a significant role in cancer therapy.[13] Still, further identification of the molecular interactions between DNA and agents is needed to better understand their mechanisms of action.

As discussed in the previous paragraph, it is apparent that structural modifications of leading compounds are a fundamental concept in order to increase biological activity and potency. In a recent proof-of-principle study we demonstrated that nitrogen-linked bis-3-chloropiperidines (**B1**–**B4**, Fig. 1b), which can be considered as piperidine-based analogues of nitrogen mustards, are more reactive towards DNA than chlorambucil.[14]

These new alkylating agents induce highly efficient cleavage in double-stranded DNA with dominating preference for guanine sites. In addition, investigations of linker structure on DNA alkylation activity revealed that the flexibility of the linker is important for alkylation efficiency, whereas the direct introduction of an aromatic group as linker leads to a decrease in activity.[14] Due to our experimental observations it appeared promising to attempt the synthesis of novel bis-3-chloropiperidines containing a flexible linker which offers the possibility of placing the aromatic moiety in the side chain (**1**–**6**, Fig. 1c). The attachment of an aromatic unit to a side chain of the linker as in the case of chlorambucil instead of its placement in the linker structure itself may enable a control of the biochemical reactivity of the agents and enhance as well as modulate DNA affinity.

In our previous study it has been shown that the bis-3-chloropiperidine **B3** with a flexible pentyl hydrocarbon linker exhibited highly efficient DNA alkylating activity (see also gel electrophoresis data below, Fig. 2a).[14] The amino acid L-lysine **7** is a suitable linker structure for the preparation of a new set of bis-3-chloropiperidines. The carboxylic acid functionality of lysine provides a position at which the side chain can be modified. For instance, chemical derivatisation can be easily achieved through esterification of the carboxyl group with various alcohols. Consequently, lysine represents a flexible linker which allows an experimental comparison between the bis-3-chloropiperidines of the present study **1**–**6** with the previous series of compounds **B1**–**B4** (in particular **B3**).

To examine the influence of aromatic motifs on the bridging lysine esters we analysed DNA alkylating activity of the set of compounds **1**–**6**. The lysine derivatives were chosen as it is relatively easy to prepare different esters and thereby study the impact of different groups on the reactivity towards DNA. Thus, we first planned the synthesis of bis-3-chloropiperidines **2**, **3** and **4** by introducing a simple phenyl group tethered through flexible hydrocarbon spacers to the lysine linker. The lysine methyl ester **1** was selected for comparison purposes in the biochemical assays. Design of compounds **5** and **6** was based on the knowledge that naphthalene chromophores, present in the antitumour antibiotics neocarzinostatin and azinomycin A and B, contribute significantly to reinforce the affinity for DNA.[15–17] Additionally, the methoxy group in the 4-position of the naphthalene moiety **6** can participate in hydrogen bonding interactions.

In the present paper we report the synthesis and the evaluation of DNA alkylating properties of these novel lysine-linked bis-3-chloropiperidines as prototype model compounds to better understand the molecular mechanism of action of bis-3-chloropiperidine derivatives.

2. Results and discussion

2.1. Synthesis of bis-3-chloropiperidines

The synthesis of bis-3-chloropiperidines **B1**–**B4** has been reported elsewhere.[14] The synthetic route (Scheme 1) for the preparation of the target compounds **1**–**6** started with the amino-protection of readily available L-lysine **7** by using di-*tert*-butyl dicarbonate (Boc_2O) to afford the di-Boc-L-lysine **8**. In order to introduce aromatic groups to the carboxylic acid side chain of lysine we planned the synthesis of a series of lysine ester derivatives. Thus, coupling of **8** with the appropriate aromatic alcohols **9**–**13** in the presence of hydroxybenzotriazole (HOBt) and dicyclohexyl carbodiimide (DCC) furnished the corresponding lysine esters **14**–**18**. Subsequently, the BOC protecting groups were cleaved with trifluoroacetic acid (TFA) to yield the free diamines **19**–**23** as their TFA salt.

Following the three-step procedure previously reported, the desired bis-3-chloropiperidines **2**–**6** were readily prepared from the precursors **19**–**23**.[14] The bidirectional method involves the

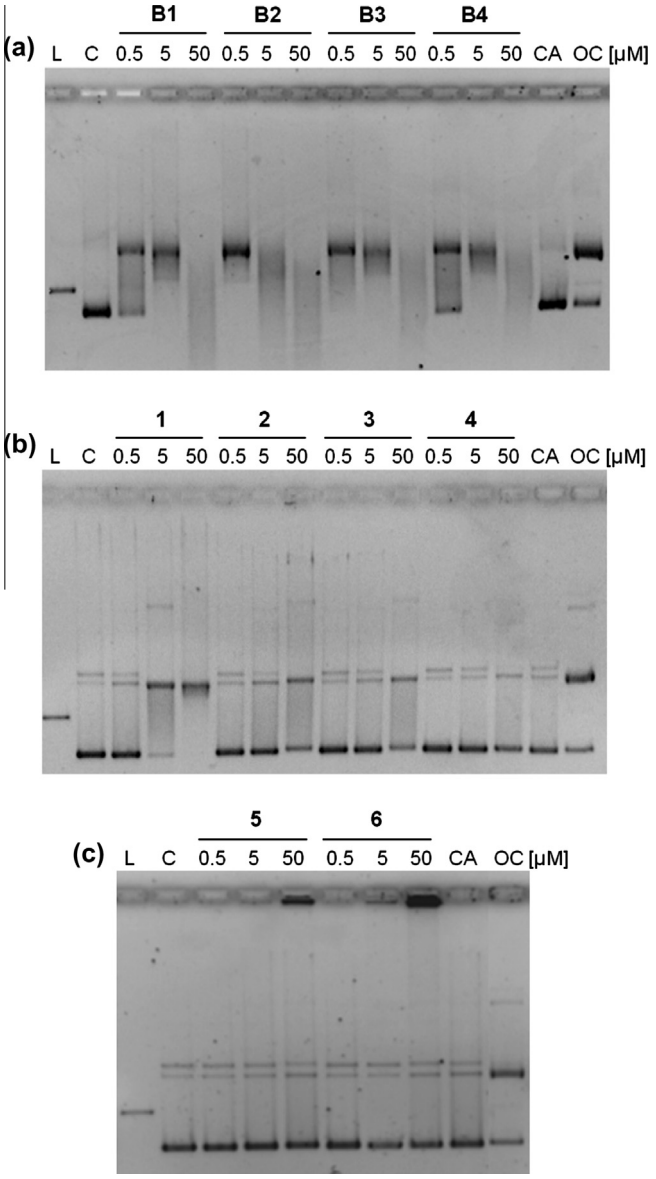
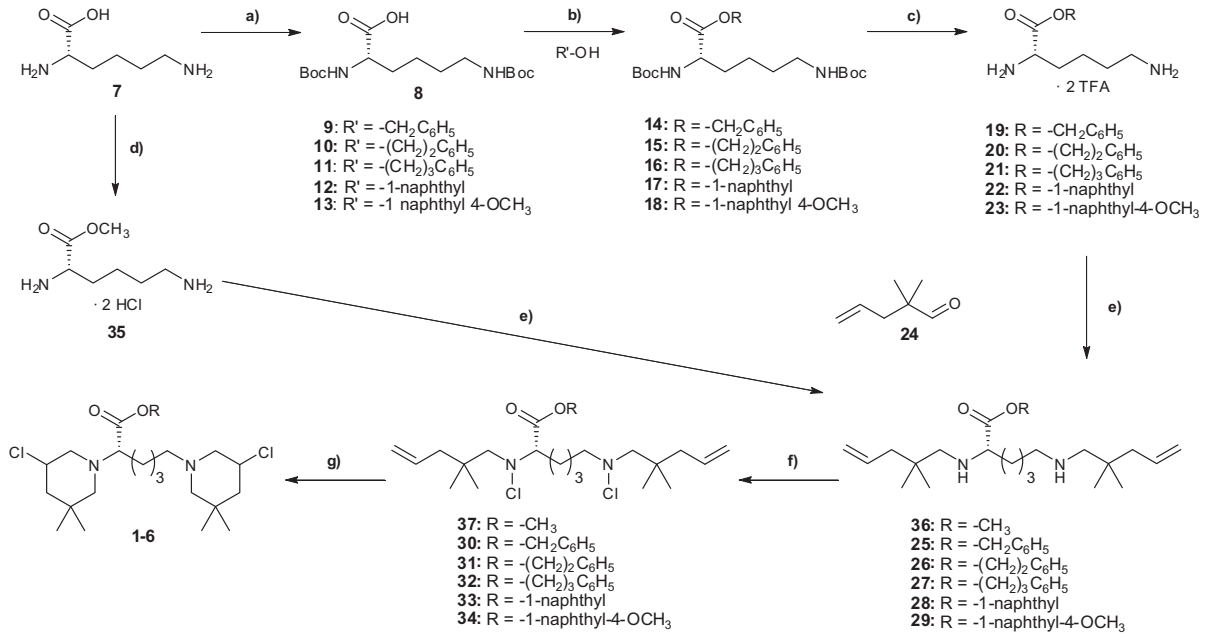


Figure 2. DNA cleavage activity of bis-3-chloropiperidines containing different flexible linker structures (a) increasing carbon chain length **B1**–**B4** (b) methyl and phenyl lysine esters **1**–**4** (c) naphthoate derivatives **5** and **6**. The supercoiled form of plasmid DNA pBR322 (3.5 nM) was 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, 50 μM) was used as a control. Cleavage of DNA was analysed by agarose (1%) gel electrophoresis in 1× TAE (Tris–acetate–EDTA). C = supercoiled DNA control, L = linear DNA control, OC = open circular (nicked) DNA control.



Scheme 1. Synthesis of bis-3-chloropiperidines **1**–**6** (see Fig. 1c for the residues R). Reactants and conditions: (a) Boc_2O, 1 N NaOH, H_2O/dioxan (1:1), rt, 12 h, 92%; (b) aromatic alcohols (**9**–**13**, respectively) DCC, HOBt, Et_3N, dry CH_2Cl_2, rt, 1–3 d, 88% (**14**), 89% (**15**), 72% (**16**), 84% (**17**), 86% (**18**); (c) TFA, CH_2Cl_2, 0 °C to rt, 12 h, quant.; (d) 2,2-dimethoxypropane, MeOH, concd HCl, reflux to rt, 15 h, 88%; (e) 2,2-dimethylpent-4-enal (**24**),[18] $NaBH(OAc)_3$, AcOH, dry CH_2Cl_2, 0 °C to rt, 12 h, 89% (**36**), 65% (**25**), 89% (**26**), 81% (**27**), 90% (**28**) 84% (**29**); (f) NCS, dry CH_2Cl_2, 0 °C to rt, 2.5 h, 29% (**37**), 29% (**30**), 30% (**31**), 28% (**32**), 21% (**33**), 36% (**34**); (g) TBAI (cat.), dry $CHCl_3$, 60 °C (oil bath temperature), 2 h, 54% (**1**), 64% (**2**), 68% (**3**), 72% (**4**), 56% (**5**)50% (**6**) (inseparable diastereomeric mixture).

double reductive amination of 2,2-dimethylpent-4-enal[18] **24** with the appropriate lysine ester **19**–**23** using sodium triacetoxyborohydride, followed by chlorination of diamines **25**–**29** with *N*-chlorosuccinimide (NCS). The resulting unsaturated bis-*N*-chloroamines **30**–**34** were eventually converted into the cyclisation products **2**–**6** in the presence of a catalytic amount of tetrabutylammonium iodide (TBAI). The desired bis-3-chloropiperidines were obtained as an inseparable mixture of diastereomers.

For the synthesis of compound **1** a different approach has been chosen. The L-lysine methyl ester **35** was prepared according to a published method by treatment of L-lysine **7** with 2,2-dimethoxypropane and concentrated hydrochloric acid.[19] The bis-3-chloropiperidine derivative **1** was then generated via the precursor compounds **36** and **37** using the same three-step procedure as described before (Scheme 1).

2.2. Bis-3-chloropiperidines exhibit DNA nicking activity

We previously demonstrated that compounds **B1**–**B4** induced highly efficient nicking of supercoiled plasmid pAT153.[14] In our present study we wanted to explore if the incorporation of an aromatic group into the side chain of a bridging lysine linker as well as the side chain spacer length would affect the reactivity of the agents with DNA. Thus, the DNA alkylating activities of the synthesised bis-3-chloropiperidines **1**–**6** were analysed by electrophoretic assays in agarose gels using the supercoiled plasmid pBR322. For comparison also the control compounds **B1**–**B4** were tested in these conditions. The nitrogen mustard chlorambucil (CA, 50 μM) was used as a control. In our experiment, the supercoiled form of pBR322 was incubated with gradually 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.

As seen in Figure 2, that depicts the results after gel electrophoresis, bis-3-chloropiperidines **B1**–**B4** displayed significant nicking of the supercoiled plasmid at the lowest tested concentration of 0.5 μM (Fig. 2a). Increasing concentrations of agents **B1**–**B4** resulted in DNA fragmentation giving rise to smeary diffuse bands on the agarose gel, as expected. Within the series, varying the linker chain length did not alter the reactivity, since compounds **B1**–**B4** exert similar alkylating activity (Fig. 2a), with **B2** and **B3** being slightly more active. These data are consistent with our findings in recently published DNA cleavage studies employing a different plasmid (pAT153).[14]

Figure 2b and c show the agarose gel electrophoresis pattern for the novel lysine-bridged bis-3-chloropiperidines **1**–**6**. Compounds **1**–**3** generated single-strand nicks in supercoiled DNA, whereas **4** showed no significant effect (Fig. 2b). Compound **1**, lacking the aromatic group in the side chain of the lysine linker, proved to be the most active agent in this series of bis-3-chloropiperidines (compare **1** with **2**–**6** in Fig. 2b and c) and displayed almost complete conversion of the supercoiled plasmid to the open circular form at the relatively low concentration of 5 μM and 3 h (Fig. 2b). However, it seems that the incorporation of the ester functionality in the lysine linker reduces the alkylation potency, for compound **1** displayed less activity than the corresponding derivative **B3** (compare **B3** in Fig. 2a with **1** in Fig. 2b). Moreover, a correlation between the spacer length and alkylating properties could be ascertained within the group of bis-3-chloropiperidines **2**–**4**, where the alkyl chain joining the lysine linker and the aromatic moiety was varied from one to three carbons. As can be seen in Figure 2b, increasing the spacer length resulted in a loss of DNA nicking activity (compare **2**–**4**), which may be related to entropic effects. Yet there was no apparent conversion of the pBR322 plasmid into an open circular form by the naphthoate derivatives **5** and **6** (Fig. 2c), indicating that the compounds exert no DNA nicking activity under these conditions. However, at a concentration of 50 μM the occurrence of DNA precipitation can be observed (visible ethidium bromide-stained bands in the gel wells, Fig. 2c). This might be attributed to possible binding of the agents **5** and **6** to DNA, thereby increasing its molecular weight and consequently leading to precipitation in the wells.

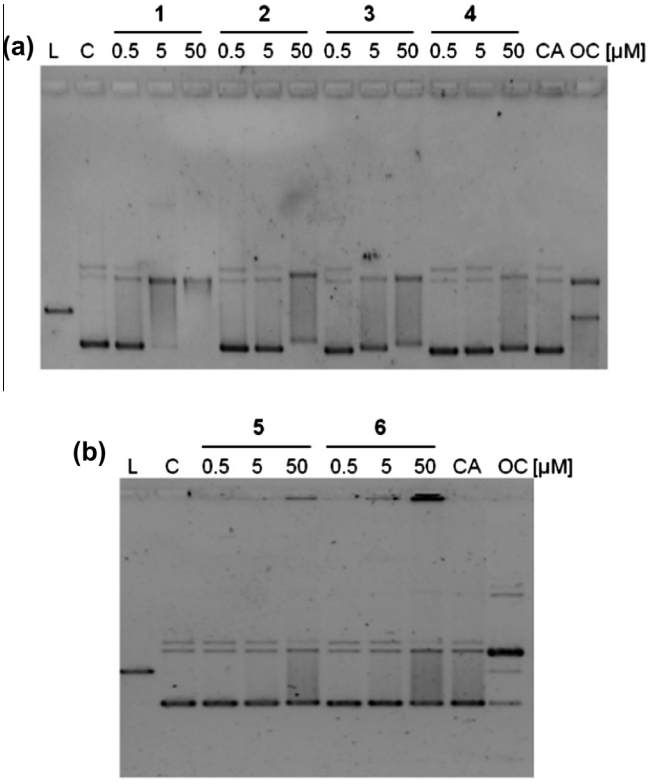


Figure 3. DNA cleavage activity of bis-3-chloropiperidines containing different lysine linker structures (a) methyl and phenyl lysine esters **1**–**4** (b) naphthoate derivatives **5** and **6**. The supercoiled form of plasmid DNA pBR322 (3.5 nM) was incubated with the compounds at ambient temperature for 24 h in BPE buffer, pH 7.4, at various concentrations (0.5, 5, 50 μM). Chlorambucil (CA, 50 μM) was used as a control. Cleavage of DNA was analysed by agarose (1%) gel electrophoresis in 1× TAE (Tris–acetate–EDTA). C = supercoiled DNA control, L = linear DNA control, OC = open circular (nicked) DNA control.

We repeated the experiment for compounds **1**–**6** at lower temperatures and increasing the incubation time to 24 h to verify DNA cleavage and to enhance possible stacking interactions between DNA bases and naphthyl groups. The obtained electrophoretic patterns, shown in Figure 3, were similar to those seen after 3 h of incubation at 37 °C for **1**–**4** (compare Fig. 2b with Fig. 3a), further validating our results. Gradually increasing the concentrations of the naphthoate derivatives **5** and **6** resulted again in precipitation of DNA, confirming our suggestion that **5** and **6** interact with DNA.

This phenomenon became significantly obvious with the methoxy-substituted naphthoate **6** at a concentration of 50 μM (Fig. 2c and Fig. 3b), implying that the incorporation of a methoxy group can possibly improve DNA interactions. Compounds **5** and **6** seemed to be somewhat more active under the chosen conditions (compare Fig. 2c with Fig. 3b). These findings might be explained by the prolonged contact of the agents with DNA due to longer incubation time. Therefore, the slightly higher activity of **5** and **6** may be related to an improved positioning of the alkylating species with respect to the DNA. This supports the idea that combining alkylating agents with DNA-affinity moieties is a promising possibility to design DNA-recognizing hybrid molecules with nucleic acids alkylation capability.

In summary, these findings revealed that aromatic groups at the linker side chain are compatible with DNA alkylating activity, although its positioning influences the reactivity with DNA. Consistent with our previous data, the antitumour drug chlorambucil (CA) had no detectable activity on plasmid DNA under the chosen experimental conditions (Figs. 2 and 3). In addition, our results suggest that the size of the ester substituent has a perceptible effect on alkylation activity and that naphthoate analogues are able to interact with DNA.

2.3. Bis-3-chloropiperidines react with guanines in DNA

We recently demonstrated by polyacrylamide gel electrophoresis (PAGE) that bis-3-chloropiperidines preferentially induce DNA cleavage through reactions with guanines.[14] In order to clarify whether the alkylating patterns of this new class of agents are consistent, we carried out the same experiment used in our previous evaluation of bis-3-chloropiperidines, exploring their effects on a 22-mer oligonucleotide duplex containing a G-rich sequence.[14] Subsequent sequence specificity analysis by high-resolution PAGE revealed that the alkylating potency of the set of test compounds correlates with the DNA nicking activity (compare Figs. 2 and S1†). In fact, the results for bis-3-chloropiperidines **1**–**6** toward the 5′-FAM-labelled double-stranded oligonucleotide demonstrated that the lysine methyl ester **1** displayed significantly higher alkylation activity than its aromatic counterparts (compare **1** with **2**–**6** in Fig. S1†).

The shifted gel bands with lower mobility than the control band (C, Fig. S1†) are comparable to those obtained from our previous study and suggest the formation of DNA adducts (DNA adducts, Fig. S1†).[14] In addition, there were no marked differences in selectivity of DNA alkylation compared to our previous analysis.[14] Our current results confirm that bis-3-chloropiperidines induce DNA strand cleavage primarily at guanine sites (compare Fig. S1†).

2.4. ESI-MS analysis of reactive aziridinium ion formation

The proposed mechanism of DNA alkylation by bis-3-chloropiperidines involves an intramolecular nucleophilic displacement of chloride by nitrogen to afford a reactive bicyclic aziridinium ion. Once formed, this electrophilic intermediate can be rapidly attacked by nucleophiles.[14] To prove this hypothesis and to investigate the chemical behaviour of the lysine-linked bis-3-chloropiperidines described, a 80 μM solution of the most active compound (**1**) in distilled water was prepared and incubated for a time consistent with the reaction with DNA. Small samples were taken at different incubation times (0 min, 15 min, 30 min, 45 min, 60 min and 120 min, at 37 °C) and analysed by ESI-MS (Figs. 4 and S2–S6†).

The results of ESI-MS analysis demonstrate the fast formation of the reactive species: while the major peak in the ESI mass spectrum of Figure 4 is assigned to the hydroxyl substituted derivative ($\mathbf{1_{OH}}$), we observe the appearance of the electrophilic aziridinium ion ($\mathbf{1_N{}^+{}_{OH}}$), detected already after 15 min of incubation. A minor peak assigned to the dihydroxyl substituted compound ($\mathbf{1_{2OH}}$) is also apparent at 15 min (Fig. 4). Further hydrolysis leads to a subsequent increase in intensity of the dihydroxyl substituted product peak ($\mathbf{1_{2OH}}$) coexisting with the reactive aziridinium species ($\mathbf{1_N{}^+{}_{OH}}$, compare Figs. S2–S6† corresponding to longer incubation time). These observations, and the rapid formation of the reactive species followed by the nucleophilic attack of water, are in good correlation with our hypothesis and with studies on DNA reactivity shown here and in previous paper.[14]

Consequently, the present investigations are further supporting our proposed mechanism of reaction. While more work is needed to determine the reactivity in complex biological conditions reflecting in vivo environment, the current studies serve as an experimental model system to demonstrate the efficacy of bis-3-chloropiperidines as DNA alkylating agents.

† See Supplementary data S1–S6.

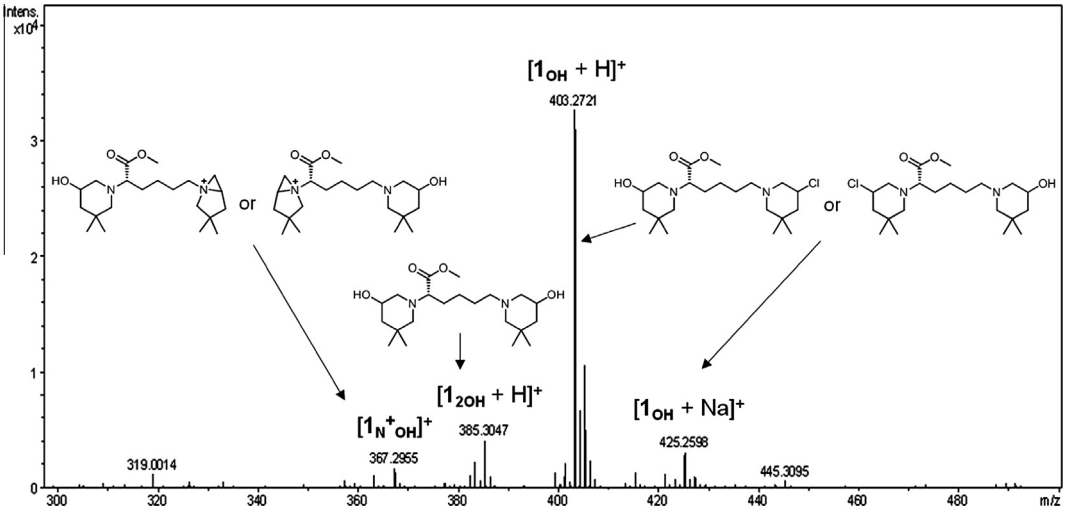


Figure 4. ESI-MS analysis of a sample taken after an incubation time of 15 min at 37 °C in a hydrolysis experiment of lysine methyl ester **1**. The ESI mass spectrum displays the hydroxyl substituted derivative ($\mathbf{1_{OH}}$ *m/z* 403 $[M+H]^+$ and *m/z* 425 $[M+Na]^+$) and the disubstituted hydrolysis product ($\mathbf{1_{2OH}}$ *m/z* 385 $[M+H]^+$) as well as the reactive aziridinium species ($1_{N^+\ OH}$ *m/z* 367 $[M]^+$).

3. Conclusions

A series of new lysine-bridged bis-3-chloropiperidines were prepared as model compounds in order to explore the influence of aromatic moieties on DNA alkylating activity. Accordingly, a phenyl group was attached by esterification to the lysine side chain via hydrocarbon spacers of different chain length (compounds **2**–**4**). In addition, two derivatives (**5** and **6**) containing a naphthoate unit were synthesised to analyse whether the incorporation of this known DNA-binding chromophore might increase reactivity toward DNA. Thus, we evaluated DNA alkylation activity by a DNA cleavage assay with a supercoiled plasmid and sequencing gel analysis with a 22-mer duplex oligonucleotide. Results with nucleic acids are consistent with ESI-MS analysis, confirming the fast formation of the reactive species in solution. Our studies revealed a clear correlation between linker structure and extent of DNA alkylation.

As a proof-of-principle, the results demonstrated that the introduction of aromatic groups to the linker side chain curtail the reactivity of bis-3-chloropiperidines in comparison to their related counterparts containing symmetrical non-aromatic linkers. Furthermore, it was shown that an increase in the length of the alkyl spacer chain attached to the linker also lowers the activity (compounds **2**–**4**). Within the current series, the most active compound was the derivative **1** suggesting that bis-3-chloropiperidines without an aromatic group seem to be significantly more potent alkylating agents. These findings are consistent with recently published data and can be particularly advantageous in terms of modulating the reactivity of bis-3-chloropiperidines towards DNA.[14]

It is noteworthy that although the naphthoate analogues **5** and **6** proved to be less potent DNA alkylating agents our experiments indicate that these compounds interact with DNA. This suggests that the presence of a DNA-affinity moiety in the bis-3-chloropiperidine molecule can contribute to DNA recognition and might direct the location of the alkylating unit near to suitable DNA bases.

Moreover, the present results also confirm those from our previous study showing that the examined compounds induce cleavage of double-stranded DNA, primarily through reactions toward guanine residues through a DNA nicking mechanism.[14] Currently, additional biochemical investigations and measurements of cytotoxicity are in progress to provide further insight into the mechanism of action. Utilizing the knowledge obtained from these studies might represent valuable starting points for further development and optimisation of novel alkylating derivatives based on the bis-3-chloropiperidine scaffold.

4. Experimental section

4.1. General remarks

Commercially available reagents were used as supplied. All solvents were purified by distillation and dried, if necessary, by standard methods. Reactions requiring the use of anhydrous solvents were carried out in heat-gun-dried glassware under a nitrogen atmosphere (Schlenk technique). Products were purified by flash chromatography on silica gel 60 (Merck). Melting points were measured using Digital Melting Point Analyzer KSP1N apparatus (Krüss Optronic) and are uncorrected. ^{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), Bruker Avance II 400 spectrometer (^{1}H at 400 MHz; ^{13}C at 100 MHz) and Bruker Avance III 600 spectrometer (^{1}H at 600 MHz; ^{13}C at 150 MHz) in the deuterated solvent stated using TMS as internal standard. Chemical shifts (δ is expressed in part per million) were determined by reference to the residual solvent resonances. High-resolution ESI mass spectrometry data were obtained with ESImicrOTOF (Bruker Daltonics) mass spectrometer. The samples were dissolved in methanol and analysed in positive ion mode. All elemental analysis (CHN) were performed on a Carlo Erba Modell 1106 instrument.

4.2. Synthetic procedures

4.2.1. 2,2-Dimethylpent-4-enal (24)

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 to reflux for 32 h under a Dean–Stark trap until no more water was separated and a sump temperature of about 140 °C was reached. After vacuum distillation (76 °C at 200 mbar) through a 50 cm Vigreux column the aldehyde (81.4 g, 0.73 mol, 73%) was obtained as a colourless liquid. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 9.47 (s, 1H), 5.60–5.70 (m, 1H), 5.01–5.05 (m, 2H), 2.21 (d, *J* = 7.3 Hz, 2H), 1.05 (s, 6H) ppm; ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 205.9, 133.1, 118.4, 45.7, 41.4, 21.1 ppm. These data are consistent with published data.[18]

4.2.2. L-Lysine methyl ester dihydrochloride (35)

2,2-Dimethoxypropane (70 mL) and concentrated hydrochloric acid (18 mL) were added to a suspension of L-lysine monohydrochloride (10.0 g, 54.7 mmol) in methanol (110 mL). The reaction mixture was heated under reflux for 3 h and stirred 12 h at room temperature. The solvent was removed under reduced pressure and the resulting oil was dissolved in a small amount of methanol. Addition of ice-cold *tert*-butyl methyl ether (450 mL) resulted in crystallisation of the desired product. The compound was recrystallised from methanol/*tert*-butyl methyl ether (11.2 g, 48.0 mmol, 88%); mp 204 °C (lit.,[19] 203–205 °C); ^{1}H NMR (CD_3OD, 400.1 MHz): δ = 4.06 (t, *J* = 6.5 Hz, 1H), 3.82 (s, 3H), 2.94 (t, *J* = 7.5 Hz, 2H), 1.86–2.01 (m, 2H), 1.71 (dt, *J* = 15.3 Hz, *J* = 7.7 Hz, 2H), 1.44–1.61 (m, 2H) ppm; ^{13}C NMR (CD_3OD, 100.6 MHz): δ = 170.8, 53.7, 53.6, 40.2, 30.9, 27.9, 12.1 ppm. These data are consistent with published data.[19]

4.2.3. N^2,N^6-Bis(*tert*-butoxycarbonyl)-L-lysine (8)

To a solution of L-lysine monohydrate (5.09 g, 31.0 mmol) in water/dioxane (1:1, 100 mL) were added di-*tert*-butyl dicarbonate (16.9 g, 78.0 mmol) and 1 N NaOH aq (35 mL). The reaction mixture was stirred at room temperature 12 h and then concentrated in vacuo until approximately 50 mL remained. The pH was adjusted to 1–2 by careful addition of an aqueous $KHSO_4$ solution (150 g/L). The suspension was extracted three times with ethyl acetate. The combined organic layers were dried over $MgSO_4$ and the solvent was removed under reduced pressure to afford the product (9.89 g, 28.5 mmol, 92%) as an oil. ^{1}H NMR (DMSO-d_6, 200.1 MHz): δ = 12.40 (s, 1H, O*H*), 6.98 (d, *J* = 7.9 Hz, 1H), 6.76 (t, *J* = 5.4 Hz, 1H), 3.75–3.86 (m, 1H), 2.50–2.83 (m, 2H), 1.37 (s, 18H), 1.14–1.68 (m, 6H) ppm; ^{13}C NMR (DMSO-d_6, 50.3 MHz): δ = 173, 155.1, 77.4, 76.8, 52.9, 29.9, 28.6, 27.7, 22.4 ppm (one signal, probably around 40 ppm, is hidden by the solvent signal).

4.2.4. General synthetic procedure for compounds 14–18

N^2,N^6-Bis(*tert*-butoxycarbonyl)-L-lysine **(8)** (2 equiv) was dissolved in anhydrous dichloromethane (10 mL/mmol of **8**). Triethylamine (2 equiv) was added, followed by the appropriate alcohol (1 equiv). The mixture was stirred for a couple of minutes under nitrogen atmosphere and then cooled to 0 °C. Hydroxybenzotriazole (HOBt, 2 equiv) and dicyclohexyl carbodiimide (DCC, 2 equiv) were added simultaneously. The reaction mixture was stirred at room temperature for the times indicated below. The precipitate was removed by filtration and discarded. The filtrate was washed successively with $NaHCO_3$ aq (satd), then $NaHSO_4$ aq (150 g/L), $NaHCO_3$ aq (satd) and finally with water. The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting crude product was purified by flash chromatography (pentane/ethyl acetate 1:1).

4.2.4.1. (*S*)-Benzyl 2,6-bis(*tert*-butoxycarbonylamino)-hexanoate (14). Prepared according to the general procedure from **8** (405 mg, 1.17 mmol) and benzyl alcohol (63.2 mg, 0.59 mmol) yielding **14** (225 mg, 0.52 mmol, 88%) after 21 h of reaction time as a colourless oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.31–7.39 (m, 5H), 5.11–5.22 (m, 2H), 4.29–4.35 (m, 1H), 3.03–3.09 (m 2H), 1.77–1.89 (m, 2H), 1.59–1.69 (m, 2H), 1.43 (s, 18H), 1.29–1.37 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 172.6, 156.0, 135.4, 128.6, 128.4, 128.3, 79.6, 78.8, 66.9, 60.4, 40.1, 32.3, 29.5, 28.4, 28.3, 22.4 ppm; HRMS (ESI): *m*/*z* calcd for $C_{23}H_{36}N_2O_6Na^+$: 459.2471; found: 459.2466 $[M+Na]^+$.

4.2.4.2. (*S*)-Phenethyl 2,6-bis(*tert* butoxycarbonylamino)-hexanoate (15). Prepared according to the general procedure from **8** (8.80 g, 25.4 mmol) and 2-phenylethyl alcohol (1.55 g, 12.7 mmol) yielding **15** (5.10 g, 11.3 mmol, 89%) after 48 h of reaction time as a pale yellow oil; (Found: C, 63.85; H, 8.66; N, 6.18. $C_{24}H_{38}N_2O_6$ requires C, 63.98; H, 8.50; N, 6.22%); ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.27–7.31 (m, 2H), 7.19–7.24 (m, 3H), 4.20–4.40 (m, 3H), 3.01– 3.11 (m, 2H), 2.94 (t, *J* = 7.0 Hz, 2H), 1.51–1.75 (m, 2H), 1.43 (s, 18H), 1.06–1.38 (m, 4H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 172.5, 155.8, 155.2, 137.3, 128.8, 128.7, 126.5, 79.6, 78.9, 65.4, 63.5, 39.9, 34.8, 32.1, 29.3, 28.2, 22.2 ppm; HRMS (ESI): *m*/*z* calcd for $C_{24}H_{38}N_2O_6Na^+$: 473.2628; found: 473.2623 $[M+Na]^+$.

4.2.4.3. (*S*)-3-Phenylpropyl 2,6-bis(*tert*-butoxycarbonylamino)hexanoate (16). Prepared according to the general procedure from **8** (2.00 g, 5.77 mmol) and 3-phenylpropyl alcohol (393 mg, 2.89 mmol) yielding **16** (969 mg, 2.09 mmol, 72%) after 43 h of reaction time as a colourless oil; (Found: C, 64.68; H, 8.67; N, 5.97. $C_{25}H_{40}N_2O_6$ requires C, 64.63; H, 8.68; N, 6.03%); ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.26–7.31 (m, 2H), 7.16–7.21 (m, 3H), 4.24–4.29 (m, 1H), 4.14 (t, *J* = 6.5 Hz, 2H), 3.07–3.14 (m, 2H), 2.66–2.73 (m, 2H), 1.76–2.08 (m, 4H), 1.59–1.68 (m, 2H), 1.44 (d, *J* = 3.7 Hz, 18H), 1.32–1.40 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 172.8, 156.0, 140.9, 128.4, 128.3, 126.0, 79.8, 64.5, 62.2, 40.0, 34.2, 32.0, 30.0, 29.5, 28.4, 28.3, 22.4 ppm; HRMS (ESI): *m*/*z* calcd for $C_{25}H_{41}N_2O_6^+$: 487.2784; found: 487.2736 $[M+H]^+$.

4.2.4.4. (*S*)-Naphthalen-1-yl 2,6-bis((*tert*-butoxycarbonyl)-amino)hexanoate (17). Prepared according to the general procedure from **8** (2.00 g, 5.77 mmol) and naphthalen-1-ol (416 mg, 2.89 mmol) yielding **17** (1.91 g, 2.41 mmol, 84%) after 68 h of reaction time as a yellow solid; ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.85–7.91 (m, 2H), 7.74 (d, *J* = 7.8 Hz, 1H), 7.50–7.54 (m, 2H), 7.45 (t, *J* = 7.8 Hz, 1H), 7.22 (d, *J* = 7.0 Hz, 1H), 5.24 (br s, 1H), 3.18 (br s, 2H), 2.10–2.17 (m, 1H), 1.91–2.00 (m, 1H), 1.57–1.64 (m, 4H), 1.49 (s, 9H), 1.45 (m, 9H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 171.7, 171.1, 156.1, 155.7, 146.4, 134.6, 127.9, 126.6, 126.5, 126.3, 125.3, 121.2, 117.8, 80.2, 79.2, 53.7, 40.0, 32.1, 29.7, 28.4, 28.3, 22.7 ppm; HRMS (ESI): *m*/*z* calcd for $C_{26}H_{36}N_2NaO_6^+$: 495.2471; found: 495.2471 $[M+Na]^+$.

4.2.4.5. (*S*)-4-Methoxynaphthalen-1-yl 2,6-bis((*tert*-butoxy-carbonyl)amino)hexanoate (18). Prepared according to the general procedure from **8** (2.00 g, 5.77 mmol) and 4-methoxynaphthalen-1-ol (503 mg, 2.89 mmol) yielding **18** (1.25 g, 2.49 mmol, 86%) after 68 h of reaction time as a pale red, gel-like solid; ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 8.24–8.27 (m, 1H), 7.79 (d, *J* = 7.9 Hz, 1H), 7.47–7.55 (m, 2H), 7.13 (d, *J* = 8.5 Hz, 1H), 6.75 (d, *J* = 8.3 Hz, 1H), 5.27 (br s, 1H), 4.64–4.65 (m, 1H), 3.99 (s, 3H), 3.17 (s, 2H), 2.08–2.16 (m, 1H), 1.70–1.85 (m, 1H), 1.53–1.63 (m, 4H), 1.48 (s, 9H), 1.44 (m, 9H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 172.0, 171.1, 156.1, 155.7, 153.6, 139.7, 127.3, 127.1, 126.2, 125.8, 122.3, 120.0, 117.4, 80.1, 55.7, 53.7, 32.2, 29.7, 28.4, 28.3, 21.0 ppm; HRMS (ESI): *m*/*z* calcd for $C_{27}H_{38}N_2NaO_7^+$: 525.2577; found: 525.2574 $[M+Na]^+$.

4.2.5. General synthetic procedure for compounds 19–23

(*S*)-2,6-Bis(*tert*-butoxycarbonyl)-lysine ester was dissolved in dichloromethane (5 mL/mmol) and trifluoroacetic acid (1 mL/mmol) was slowly added at 0 °C. The reaction mixture was stirred 12 h at room temperature. Removal of the solvent under reduced pressure gave the pure product in quantitative yield.

4.2.5.1. (*S*)-Benzyl 2,6-diaminohexanoate TFA salt (19). Following the general procedure, **14** (210 mg, 0.48 mmol) was deprotected to provide the corresponding TFA salt **19** in quantitative yield. ^{1}H NMR (DMSO-d_6, 400.1 MHz): δ = 8.47 (s, 2H), 7.79 (s, 2H), 7.35–7.43 (5H), 5.24 (s, 2H), 4.08–4.10 (m, 1H), 2.67–2.74

(m, 2H), 1.73–1.84 (m, 2H), 1.48–1.55 (m, 2H), 1.23–1.44 (m, 2H) ppm; ^{13}C NMR (DMSO-d_6, 50.3 MHz): δ = 168.8, 134.6, 128.0, 127.9, 127.8, 66.6, 51.2, 51.2, 28.9, 25.8, 20.7 ppm. HRMS (ESI): m/z calcd for $C_{13}H_{21}N_2O_2^+$: 237.1603; found: 237.1590 $[M+H]^+$ (one signal, probably around 40 ppm, is hidden by the solvent signal).

4.2.5.2. (*S*)-Phenethylhexane 2,6-diaminohexanoate TFA salt (20). Following the general procedure, **15** (925 mg, 2.05 mmol) was deprotected to provide the corresponding TFA salt **20** in quantitative yield. ^{1}H NMR (DMSO-d_6, 400.1 MHz): δ = 8.44 (s, 2H), 7.83 (s, 2H), 7.22–7.34 (m, 5H), 4.32–4.81 (m, 2H), 3.87–4.04 (m, 1H), 2.95 (t, J = 6.5 Hz, 2H), 2.66–2.73 (m, 2H), 1.64–1.70 (m, 2H), 1.42–1.48 (m, 2H), 1.13–1.36 (m, 2H) ppm; ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ = 169.0, 137.1, 128.4, 127.9, 126.1, 65.6, 51.2, 37.9, 33.6, 29.0, 26.6, 20.7 ppm; HRMS (ESI): m/z calcd for $C_{14}H_{23}N_2O_2^+$: 251.1760; found: 251.1715 $[M+H]^+$.

4.2.5.3. (*S*)-3-Phenylpropyl 2,6-diaminohexanoate TFA salt (21). Following the general procedure, **16** (190 mg, 0.41 mmol) was deprotected to provide the corresponding TFA salt **21** in quantitative yield. ^{1}H NMR (DMSO-d_6, 400.1 MHz): δ = 8.44 (s, 2H), 7.78 (s, 2H), 7.28–7.31 (m, 2H), 7.18–7.22 (m, 3H), 4.16 (t, J = 6.5 Hz, 2H), 3.99–4.05 (m, 1H), 2.73–2.80 (m, 2H), 2.66 (t, J = 7.5 Hz, 2H), 1.89–1.96 (m, 2H), 1.75–1.81 (m, 2H), 1.51–1.58 (m, 2H), 1.30–1.47 (m, 2H) ppm; ^{13}C NMR (DMSO-d_6, 50.3 MHz): δ = 169.1, 140.6, 128.0, 127.9, 125.6, 64.6, 59.4, 51.4, 30.8, 29.2, 29.1, 26.0, 20.4 ppm; HRMS (ESI): m/z calcd for $C_{15}H_{25}N_2O_2^+$: 265.1916; found: 265.1902 $[M+H]^+$.

4.2.5.4. (*S*)-Naphthalen-1-yl 2,6-diaminohexanoate TFA salt (22). Following the general procedure, **16** (190 mg, 0.41 mmol) was deprotected to provide the corresponding TFA salt **21** in quantitative yield. ^{1}H NMR (DMSO-d_6, 400.1 MHz): δ = 8.44 (s, 2H), 7.78 (s, 2H), 7.28–7.31 (m, 2H), 7.18–7.22 (m, 3H), 4.16 (t, J = 6.5 Hz, 2H), 3.99–4.05 (m, 1H), 2.73–2.80 (m, 2H), 2.66 (t, J = 7.5 Hz, 2H), 1.89–1.96 (m, 2H), 1.75–1.81 (m, 2H), 1.51–1.58 (m, 2H), 1.30–1.47 (m, 2H) ppm; ^{13}C NMR (DMSO-d_6, 50.3 MHz): δ = 169.1, 140.6, 128.0, 127.9, 125.6, 64.6, 59.4, 51.4, 30.8, 29.2, 29.1, 26.0, 20.4 ppm; HRMS (ESI): m/z calcd for $C_{15}H_{25}N_2O_2^+$: 265.1916; found: 265.1902 $[M+H]^+$.

4.2.5.5. (*S*)-4-Methoxynaphthalen-1-yl 2,6-diamino-hexanoate TFA salt (23). Following the general procedure, **18** (968 mg, 1.93 mmol) was deprotected to provide the corresponding TFA salt **23** in quantitative yield. ^{1}H NMR (DMSO-d_6, 400.1 MHz): δ = 8.19–8.23 (m, 1H), 7.93–7.96 (br s, 2H), 7.88–7.90 (m, 1H), 7.56–7.65 (m, 2H), 7.32 (d, J = 8.4 Hz, 1H), 7.01 (d, J = 8.5 Hz, 1H), 4.55–4.60 (m, 1H), 4.17–4.22 (m, 4H), 4.00 (s, 1H), 2.84–2.86 (m, 2H), 2.03–2.18 (m, 2H), 1.55–1.72 (m, 4H); ^{13}C NMR (DMSO-d_6, 50.3 MHz): δ = 168.8, 158.6, 158.3, 153.2, 138.7, 127.2, 126.5, 126.1, 125.3, 121.0, 118.2, 115.2, 103.5, 55.9, 51.9, 33.7, 29.6, 26.6, 21.5 ppm.

4.2.6. General synthetic procedure for compounds 25–29 and 36

To a solution of the unsaturated aldehyde **24** (2.4 equiv) and the appropriate diamine in anhydrous dichloromethane (7 mL/mmol of diamine) was added sodium triacetoxyborohydride (3 equiv) portion wise at 0 °C, followed by acetic acid (1.2 equiv). The reaction mixture was stirred at room temperature under an nitrogen 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 dichloromethane. The combined organic phases were first washed with brine, then with water and dried over $MgSO_4$. The solvent was removed under reduced pressure to afford the corresponding product, which was used for the next step without further purification.

4.2.6.1. (*S*)-Methyl 2,6-bis(2,2-dimethylpent-4-enylamino)-hexanoate (36). Prepared according to the general procedure from 2,2-dimethyl-4-pentenal **24** (5.57 g, 49.6 mmol) and L-lysine methyl ester dihydrochloride **35** (4.82 g, 20.7 mmol) yielding **36** (6.46 g, 18.3 mmol, 89%) as a pale yellow liquid; (Found: C, 71.16; H, 11.53; N, 7.82. $C_{21}H_{40}N_2O_2$ requires C, 71.54; H, 11.44; N, 7.95%); ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 5.73–5.87 (m, 2H), 4.97–5.06 (m, 4H), 3.69 (s, 3H), 3.12 (t, J = 6.8 Hz, 1H), 2.52 (t, J = 6.8 Hz, 2H), 2.34 (d, J = 11.3 Hz, 1H), 2.32 (s, 2H), 2.07 (d, J = 11.5 Hz, 1H), 1.95–2.02 (m, 4H), 1.29–1.65 (m, 6H), 0.84 and 0.88 (2× s, total 12H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 176.2, 135.3, 116.6, 116.5, 62.3, 60.2, 58.0, 51.3, 50.5, 44.5, 44.1, 34.2, 34.0, 33.3, 29.6, 25.3, 25.1, 25.0, 23.6 ppm.

4.2.6.2. (*S*)-Benzyl 2,6-bis(2,2-dimethylpent-4-enylamino)-hexanoate (25). Prepared according to the general procedure from **19** (850 mg, 1.83 mmol) and 2,2-dimethyl-4-pentenal **24** (493 mg, 4.39 mmol) yielding **25** (510 mg, 1.19 mmol, 65%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.30–7.38 (m, 5H), 5.70–5.81 (m, 2H), 5.12–5.16 (m, 2H), 4.95–5.10 (m, 4H), 3.10–3.13 (m, 1H), 2.86–2.90 (m, 2H), 2.38 (d, J = 11.3 Hz, 1H), 2.15 (d, J = 7.8 Hz, 2H), 2.01–2.03 (m, 1H), 1.93–1.96 (m, 2H), 1.76–1.84 (m, 2H), 1.15–1.69 (m, 6H), 1.07 (s, 6H), 0.82 (2× s, total 6H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 175.3, 135.8, 134.4, 133.3, 128.5, 128.3, 118.9, 116.8, 66.3, 62.2, 58.1, 57.3, 49.1, 44.3, 43.5, 34.4, 33.5, 32.7, 29.6, 25.2, 25.1, 23.8 ppm; HRMS (ESI): m/z calcd for $C_{27}H_{45}N_2O_2^+$: 429.3481; found: 429.3454 $[M+H]^+$.

4.2.6.3. (*S*)-Phenethyl 2,6-bis(2,2-dimethylpent-4-enylamino)hexanoate (26). Prepared according to the general procedure from **20** (980 mg, 2.05 mmol) and 2,2-dimethyl-4-pentenal **24** (551 mg, 4.92 mmol) yielding **26** (810 mg, 1.83 mmol, 89%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.28–7.33 (m, 2H), 7.10–7.25 (m, 3H), 5.73–5.88 (m, 2H), 4.97–5.07 (m, 4H), 4.35 (t, J = 7.0 Hz, 2H), 3.03–3.15 (m, 1H), 2.90–2.97 (m, 2H), 2.72 (s, 1H), 2.07–2.36 (m, 5H), 1.95–2.03 (m, 4H), 1.74–1.83 (m, 2H, 3-H), 1.30–1.65 (m, 4H), 0.84 and 0.89 (2× s, total 12H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 175.3, 137.1, 134.9, 134.7, 128.3, 127.9, 126.0, 116.5, 116.2, 71.1, 64.2, 50.1, 44.2, 43.8, 42.8, 34.6, 33.8, 33.6, 24.7, 24.6, 23.2 ppm; HRMS (ESI): m/z calcd for $C_{28}H_{47}N_2O_2^+$: 443.3638; found: 443.3620 $[M+H]^+$.

4.2.6.4. (*S*)-3-Phenylpropyl 2,6-bis(2,2-dimethylpent-4-enylamino)hexanoate (27). Prepared according to the general procedure from **21** (1.00 g, 2.03 mmol) and 2,2-dimethyl-4-pentenal **24** (547 mg, 4.87 mmol) yielding **27** (755 mg, 1.65 mmol, 81%) as a yellow-brown oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.26–7.30 (m, 2H), 7.16–7.21 (m, 3H), 5.73–5.81 (m, 2H), 4.96–5.07 (m, 4H), 4.09–4.15 (m, 2H), 3.08 (dd, J = 7.7 Hz, J = 5.5 Hz, 1H), 2.92–2.96 (m, 2H), 2.66–2.69 (m, 4H), 2.16–2.43 (m, 4H), 1.82–2.09 (m, 8H), 1.32–1.69 (m, 4H), 1.09 (s, 6H), 0.85 (2× s, total 6H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 175.4, 140.9, 135.4, 135.2, 128.4, 128.3, 126.0, 117.1, 116.9, 63.9, 62.3, 58.2, 49.1, 44.3, 44.2, 43.3, 34.4, 33.5, 32.8, 32.1, 25.3, 25.2, 23.7, 23.4 ppm; HRMS (ESI): m/z calcd for $C_{29}H_{49}N_2O_2^+$: 457.3794; found: 457.3758 $[M+H]^+$.

4.2.6.5. (*S*)-Naphthalen-1-yl 2,6-bis((2,2-dimethylpent-4-en-1-yl)amino)hexanoate (28). Prepared according to the general procedure from **22** (765 mg, 1.53 mmol) and 2,2-dimethyl-4-pentenal **24** (412 mg, 3.67 mmol) yielding **28** (638 mg, 1.37 mmol, 90%) as a yellow-orange oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.85–7.90 (m, 2H), 7.74–7.76 (m, 1H), 7.45–7.54 (m, 3H), 7.25 (d, J = 7.2 Hz, 1H), 5.77–5.91 (m, 2H), 4.99–5.06 (m, 4H), 3.54–3.58 (m, 1H), 2.64–2.69 (m, 3H), 2.32–2.38 (m, 3H), 1.97–2.09 (m, 5H), 1.81–1.89 (m, 1H), 1.56–1.73 (m, 4H), 1.32–1.55 (m, 2H), 0.94 (2× s, total 6H),

0.90 (s, 6H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 174.6, 146.6, 135.5, 135.4, 134.7, 128.1, 126.8, 126.4, 126.0, 125.4, 121.1, 118.0, 117.0, 116.8, 62.9, 60.4, 58.5, 50.8, 44.8, 44.5, 34.6, 34.2, 33.7, 29.9, 25.5, 25.4, 25.3, 23.9 ppm; HRMS (ESI): *m*/*z* calcd for $C_{30}H_{45}N_2O_2^+$: 465.3481; found: 465.3484 $[M+H]^+$.

4.2.6.6. (*S*)-4-Methoynaphthalen-1-yl 2,6-bis((2,2-dimethyl-pent-4-en-1-yl)amino)hexanoate (29). Prepared according to the general procedure from **23** (1.00 g, 1.88 mmol) and 2,2-dimethyl-4-pentenal **24** (508 mg, 4.52 mmol) yielding **29** (785 mg, 1.59 mmol, 84%) as a pale brown oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 8.24–8.28 (m, 1H), 7.74–7.79 (m, 1H), 7.48–7.55 (m, 2H), 7.11 (d, *J* = 8.5 Hz, 1H), 6.76 (d, *J* = 8.3 Hz, 1H), 5.76–5.91 (m, 2H), 4.99–5.07 (m, 4H), 4.00 (s, 3H), 3.51–3.55 (m, 1H), 2.62–2.67 (m, 2H), 2.38 (s, 2H), 2.31–2.35 (m, 1H), 1.93–2.11 (m, 5H), 1.78–1.87 (m, 1H), 1.56–1.74 (m, 5H), 0.90 and 0.93 (2× s, total 12H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 174.9, 153.4, 139.9, 135.5, 127.5, 126.9, 126.2, 125.7, 122.5, 120.8, 117.6, 116.9, 116.8, 102.8, 71.7, 62.8, 60.3, 58.5, 55.7, 50.7, 44.8, 44.5, 43.4, 34.6, 34.2, 25.5, 25.4, 25.3, 23.9, 23.8 ppm; HRMS (ESI): *m*/*z* calcd for $C_{31}H_{47}N_2O_3^+$: 495.3587; found: 495.3581 $[M+H]^+$.

4.2.7. General procedure for the synthesis of bis-*N*-chloroamines 30–34 and 37

N-Chlorosuccinimide (2.2 equiv) was added to a cooled (0 °C) solution of the corresponding diamine in anhydrous dichloromethane (10 mL/mmol of diamine). The reaction mixture was stirred first for half an hour at 0 °C and for 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).

4.2.7.1. (*S*)-Methyl 2,6-bis(*N*-chloro-*N*-(2,2-dimethylpent-4-enyl)amino)hexanoate (37). Prepared according to the general procedure from **36** (2.50 g, 7.09 mmol) yielding **37** (880 mg, 2.09 mmol, 29%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 5.75–5.85 (m, 2H), 4.99–5.04 (m, 4H), 3.75 (s, 3H), 3.49 (t, *J* = 7.3 Hz, 1H), 3.11–3.17 (m, 1H), 2.88–2.95 (m, 3H), 2.83 (s, 2H), 2.04 (d, *J* = 7.5 Hz, 4H), 1.81–1.87 (m, 2H), 1.63–1.71 (m, 2H), 1.27–1.45 (m, 2H), 0.92, 0,93 and 0.94 (3× s, total 12H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 171.3, 135.2, 135.1, 117.3, 117.2, 74.8, 73.3, 72.2, 66.4, 44.8, 44.5, 35.8, 35.6, 30.3, 27.7, 25.7, 25.5, 25.4, 23.3 ppm; HRMS (ESI): *m*/*z* calcd for $C_{21}H_{39}Cl_2N_2O_2^+$: 421.2389; found: 421.2320 $[M+H]^+$.

4.2.7.2. (S)-Benzyl 2,6-bis(*N*-chloro(2,2-dimethylpent-4-en-1-yl)amino)hexanoate (30). Prepared according to the general procedure from **25** (188 mg, 0.28 mmol) yielding **30** (40 mg, 0.80 mmol, 29%) as a clear and colourless viscous liquid. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.31–7.39 (m, 5H), 5.73–5.85 (m, 2H), 5.19–5.23 (m, 2H), 5.00–5.05 (m, 3H), 4.97–5.00 (m, 1H), 3.53 (t, *J* = 6.8 Hz, 1H), 3.11–3.15 (m, 1H), 2.86–2.92 (m, 3H), 2.83 (s, 1H), 2.04 (t, *J* = 7.3 Hz, 3H), 1.83–1.89 (m, 2H), 1.62–1.70 (m, 2H), 1.50–1.59 (m, 1H), 1.25–1.43 (m, 3H), 0.91 (2× s, total 12H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 170.7, 135.6, 135.2, 135.1, 128.5, 128.3, 117.3, 117.2, 74.8, 73.3, 72.3, 66.5, 66.4, 44.8, 44.7, 36.8, 35.6, 30.4, 27.7, 25.7, 25.5, 25.4, 23.3 ppm; HRMS (ESI): *m*/*z* calcd for $C_{27}H_{43}Cl_2N_2O_2^+$: 497.2702; found: 497.2763 $[M+H]^+$.

4.2.7.3. (*S*)-Phenethyl 2,6-bis(*N*-chloro(2,2-dimethylpent-4-en-1-yl)amino)hexanoate (31). Prepared according to the general procedure from **26** (810 mg, 1.83 mmol) yielding **31** (281 mg, 0.55 mmol, 30%) as a clear and colourless viscous liquid. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.30–7.34 (m, 2H), 7.22–7.27 (m, 3H), 5.74–5.87 (m, 2H), 4.99–5.06 (m, 4H), 4.37–4.46 (m, 2H), 3.44 (t, *J* = 7.0 Hz, 1H), 3.00–3.07 (m, 2H), 2.92 (t, *J* = 6.5 Hz, 2H), 2.85 (s, 2H), 2.06 (d, *J* = 7.5 Hz, 2H), 2.04 (d, *J* = 8.5 Hz, 2H), 1.77–1.85 (m, 2H), 1.60–1.69 (m, 2H), 1.49–1.57 (m, 1H), 1.31–1.41 (m, 1H), 0.95 (s, 6H), 0.91 (2× s, total 6H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 170.9, 141.0, 135.2, 135.1, 128.5, 128.4, 126.1, 117.3, 117.2, 74.8, 73.4, 66.4, 64.1, 44.8, 44.6, 35.9, 35.6, 32.3, 30.4, 30.3, 27.8, 27.0, 25.7, 25.6, 25.4, 23.3 ppm; HRMS (ESI): *m*/*z* calcd for $C_{28}H_{45}Cl_2N_2O_2^+$: 511.2858; found: 511.2828 $[M+H]^+$.

4.2.7.4. (*S*)-3-Phenylpropyl 2,6-bis(*N*-chloro(2,2-dimethyl-pent-4-en-1-yl)amino)hexanoate (32). Prepared according to the general procedure from **27** (750 mg, 1.64 mmol) yielding **32** (245 mg, 0.45 mmol, 28%) as a clear and colourless viscous liquid. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.32–7.37 (m, 2H), 7.24–7.29 (m, 3H), 5.81–5.92 (m, 2H), 5.05–5.11 (m, 4H), 4.20–4.30 (m, 2H), 3.54 (t, *J* = 7.3 Hz, 1H), 2.97–3.03 (m, 3H), 2.90 (s, 2H), 2.79 (t, *J* = 7.5 Hz, 2H), 2.10–2.13 (m, 4H), 2.05–2.10 (m, 2H), 1.88–1.93 (m, 2H), 1.70–1.78 (m, 2H), 1.43–1.52 (m, 1H), 1.25 (s, 2H), 0.99 and 1.01 (2× s, total 12H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 170.9, 141.0, 135.2, 135.1, 128.5, 128.4, 126.1, 117.3, 117.2, 74.8, 73.4, 66.4, 64.1, 44.8, 44.6, 35.9, 35.6, 35.4, 32.3, 30.4, 30.3, 27.8, 27.0, 25.7, 25.6, 25.4, 23.3 ppm; HRMS (ESI): *m*/*z* calcd for $C_{29}H_{47}Cl_2N_2O_2^+$: 525.3015; found: 525.2954 $[M+H]^+$.

4.2.7.5. (*S*)-Naphthalen-1-yl 2,6-bis(chloro(2,2-dimethyl-pent-4-en-1-yl)amino)hexanoate (33). Prepared according to the general procedure from **28** (620 mg, 1.33 mmol) yielding **33** (150 mg, 0.28 mmol, 21%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 7.98–8.00 (m, 1H), 7.87–7.90 (m, 1H), 7.76 (d, *J* = 8.3 Hz, 1H), 7.51–7.56 (m, 2H), 7.48 (t, *J* = 7.8 Hz, 1H), 7.29–7.31 (m, 1H), 5.77–5.91 (m, 2H), 5.00–5.08 (m, 4H), 3.92–4.30 (m, 1H), 3.32–3.36 (m, 1H), 3.12–3.16 (m, 1H), 3.01 (t, *J* = 6.2 Hz, 2H), 2.88 (s, 2H), 2.12–2.14 (m, 3H), 2.07–2.09 (m, 2H), 1.74–1.82 (m, 3H), 1.58–1.69 (m, 2H), 1.00 (2× s, total 6H), 0.96 (s, 6H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 169.6, 146.4, 135.2, 135.1, 134.7, 128.0, 126.7, 126.6, 126.5, 126.2, 125.3, 121.3, 118.0, 117.3, 117.2, 74.9, 73.7, 66.4, 44.8, 36.0, 35.6, 30.6, 27.8, 25.7, 25.6, 25.5, 23.5 ppm; HRMS (ESI): *m*/*z* calcd for $C_{30}H_{42}Cl_2N_2NaO_2^+$: 555.2521; found: 555.2517 $[M+Na]^+$.

4.2.7.6. (*S*)-4-Methoxynaphthalen-1-yl 2,6-bis(chloro(2,2-dimethylpent-4-en-1-yl)amino)hexanoate (34). Prepared according to the general procedure from **29** (769 mg, 1.55 mmol) yielding **34** (320 mg, 0.57 mmol, 36%) as a colourless oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 8.24–8.31 (m, 1H), 7.85–7.92 (m, 1H), 7.47–7.59 (m, 2H), 7.17 (d, *J* = 8.3 Hz, 1H), 6.78 (d, *J* = 8.5 Hz, 1H), 5.75–5.91 (m, 2H), 4.98–5.08 (m, 4H), 4.01 (s, 3H), 3.91 (t, *J* = 7.5 Hz, 1H), 3.29–3.35 (m, 1H), 3.09–3.17 (m, 1H), 3.00 (t, *J* = 6.5 Hz, 2H), 2.87 (s, 2H), 2.08 (dd, *J* = 10.2 Hz, 7.0 Hz, 6H), 1.72–1.82 (m, 2H), 1.59 (br s, 2H), 0.99 (2× s, total 6H), 0.95 (s, 6H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 170.0, 153.6, 139.8, 135.2, 135.1, 127.4, 127.1, 126.2, 125.8, 122.4, 121.1, 117.7, 117.5, 117.3, 102.8, 74.9, 73.7, 72.4, 66.4, 55.7, 44.8, 44.7, 36.0, 30.6, 27.0, 25.7, 25.6, 25.5, 23.5 ppm; HRMS (ESI): *m*/*z* calcd for $C_{31}H_{45}Cl_2N_2O_3^+$: 563.2807; found: 563.2807 $[M+H]^+$.

4.2.8. General procedure for the synthesis of bis-3-chloropiperidines 1–6

The bis-*N*-chloroamine was dissolved in anhydrous chloroform (10 mL/mmol 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 inseparable diastereomers.

4.2.8.1. (*S*)-Methyl 2,6-bis(5-chloro-3,3-dimethylpiperidin-1-yl)hexanoate (1). Prepared according to the general procedure from **37** (750 mg, 1.78 mmol) yielding **1** (406 mg, 0.96 mmol, 54%) as a pale yellow oil; (Found: C, 59.65; H, 9.09; N, 6.31. $C_{21}H_{38}Cl_2N_2O_2$ requires C, 59.85; H, 9.09; N, 6.65%); ^{1}H NMR ($CDCl_3$, 400.1 MHz) mixture of diastereomers: δ = 3.92–4.10 (m, 2H), 3.68 (s, 3H), 3.08–3.19 (m, 3H), 2.36–2.46 (m, 2H), 2.22–2.27 (m, 2H), 1.87–1.97 (m, 4H), 1.53–1.74 (m, 4H), 1.26–1.49 (m, 6H), 0.99 and 1.01 (2× s, total 6H), 0.90 and 0.91 (2× s, total 6H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, selected signals) major isomer: δ = 172.7, 66.8, 64.7, 64.1, 62.3, 61.5, 57.6, 55.0, 54.3, 51.0, 48.6, 48.3, 33.2, 29.3, 26.4, 25.2, 24.9, 23.8 ppm; HRMS (ESI): *m*/*z* calcd for $C_{21}H_{39}Cl_2N_2O_2^+$: 421.2389; found: 421.2388 $[M+H]^+$.

4.2.8.2. (2*S*)-Benzyl 2,6-bis(5-chloro-3,3-dimethylpiperidin-1-yl)hexanoate (2). Prepared according to the general procedure from **30** (125 mg, 0.25 mmol) yielding **2** (80 mg, 0.16 mmol, 64%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz) mixture of diastereomers: δ = 7.32–7.40 (m, 5H), 5.11–5.14 (m, 2H), 3.92–4.05 (m, 2H), 3.10–3.12 (m, 1H), 2.24–2.47 (m, 6H), 1.85–1.96 (m, 4H), 1.50–1.73 (m, 4H), 1.29–1.48 (m, 6H), 0.97–1.00 (overlapping signals, 6H), 0.86–0.90 (overlapping signals, 6H) ppm; ^{13}C NMR ($CDCl_3$, 50.3 MHz, selected signals) major isomer: δ = 171.8, 135.9, 128.5, 128.4, 128.3, 66.8, 65.9, 63.9, 62.8, 61.4, 57.6, 54.4, 54.0, 48.6, 48.5, 33.6, 33.2, 29.6, 26.9, 25.2, 24.9, 23.8 ppm; HRMS (ESI): *m*/*z* calcd for $C_{27}H_{43}Cl_2N_2O_2^+$: 497.2702; found: 497.2698 $[M+H]^+$.

4.2.8.3. (2*S*)-Phenethyl 2,6-bis(5-chlor-3,3-dimethyl-piperidin-1-yl)hexanoate (3). Prepared according to the general procedure from **31** (250 mg, 0.49 mmol) yielding **3** (169 mg, 0.33 mmol, 68%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz) mixture of diastereomers: δ = 7.29–7.33 (m, 2H), 7.21–7.25 (m, 3H), 4.28–4.42 (m, 2H), 3.88–4.10 (m, 2H), 3.01–3.14 (m, 3H), 2.94–2.98 (m, 2H), 2.12–2.39 (m, 6H), 1.59–1.94 (m, 6H), 1.19–1.35 (m, 6H), 0.84–1.01 (overlapping signals, 12H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, selected signals) major isomer: δ = 171.9, 137.3, 128.5, 128.2, 126.3, 66.5, 64.4, 64.1, 63.6, 62.0, 57.3, 54.0, 48.2, 48.1, 34.8, 32.9, 29.6, 26.4, 25.2, 24.9, 23.5 ppm; HRMS (ESI): *m*/*z* calcd for $C_{28}H_{45}Cl_2N_2O_2^+$: 511.2858; found: 511.2851 $[M+H]^+$.

4.2.8.4. (*S*)-3-Phenylpropyl 2,6-bis(5-chlor-3,3-dimethyl-piperidin-1-yl)-hexanoate (4). Prepared according to the general procedure from **32** (220 mg, 0.42 mmol) yielding **4** (158 mg, 0.30 mmol, 72%) as a pale yellow oil; (found: C, 66.20; H, 8.83; N, 5.19. $C_{29}H_{46}Cl_2N_2O_2$ requires C, 66.27; H, 8.82; N, 5.33%); ^{1}H NMR ($CDCl_3$, 400.1 MHz) mixture of diastereomers: δ = 7.27–7.31 (m, 2H), 7.18–7.22 (m, 3H), 4.12 (t, *J* = 6.2 Hz, 2H), 3.94–4.08 (m, 2H), 3.12–3.21 (m, 3H), 2.68–2.72 (m, 2H), 2.26–2.51 (m, 6H), 1.90–2.01 (m, 6H), 1.58–1.70 (m, 8H), 1.39–1.49 (m, 4H), 1.00–1.02 (overlapping signals, 6H), 0.90 (s, 6H) ppm; ^{13}C NMR ($CDCl_3$, 50.3 MHz, selected signals) major isomer: δ = 171.9, 141.0, 128.4, 128.3, 126.0, 66.8, 64.7, 63.5, 61.6, 57.6, 54.1, 48.5, 48.3, 33.2, 32.3, 30.3, 29.4, 26.5, 25.2, 24.0, 23.9 ppm; HRMS (ESI): *m*/*z* calcd for $C_{29}H_{47}Cl_2N_2O_2^+$: 525.3015; found: 525.2999 $[M+H]^+$.

4.2.8.5. (2*S*)-Naphthalen-1-yl 2,6-bis(5-chloro-3,3-dimethyl-piperidin-1-yl)hexanoate (5). Prepared according to the general procedure from **33** (138 mg, 0.26 mmol) yielding **5** (77 mg, 0.14 mmol, 56%) as a colourless oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz) mixture of diastereomers: δ = 7.84–7.92 (m, 2H), 7.74 (d, *J* = 7.5 Hz, 1H), 7.51–7.53 (m, 2H), 7.47 (t, *J* = 8.5 Hz, 1H), 7.24–7.26 (m, 1H), 3.99–4.20 (m, 2H), 3.61–3.67 (m, 1H), 3.34–3.42 (m, 1H), 3.14–3.23 (m, 1H), 2.49–2.64 (m, 2H), 2.28–2.48 (m, 3H), 1.89–2.04 (m, 4H), 1.71–1.77 (m, 1H), 1.58 (br s, 6H), 1.26 (s, 2H), 1.03–1.11 (overlapping signals, 6H), 0.88–0.99 (overlapping signals, 6H) ppm; ^{13}C NMR ($CDCl_3$, 50.3 MHz, selected signals) major isomer: δ = 171.0, 146.3, 134.7, 128.1, 126.7, 126.5, 126.0, 125.3, 121.1, 118.1, 67.0, 64.8, 63.9, 62.3, 57.7, 54.3, 48.6, 48.4, 33.3, 29.1, 26.5, 25.2, 24.1 ppm; HRMS (ESI): *m*/*z* calcd for $C_{30}H_{43}Cl_2N_2O_2^+$: 533.2702; found: 533.2702 $[M+H]^+$.

4.2.8.6. (2*S*)-4-Methoxynaphthalen-1-yl 2,6-bis(5-chloro-3,3-dimethylpiperidin-1-yl)hexanoate (6). Prepared according to the general procedure from **34** (320 mg, 0.57 mmol) yielding **6** (160 mg, 0.28 mmol, 50%) as a pale green oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz) mixture of diastereomers: δ = 8.23–8.31 (m, 1H), 7.74–7.79 (m, 1H), 7.49–7.56 (m, 2H), 7.11–7.17 (m, 1H), 6.78 (d, *J* = 8.3 Hz, 1H), 4.04–4.18 (m, 2H), 4.01 (s, 3H), 3.58–3.65 (m, 1H), 3.32–3.41 (m, 1H), 3.16–3.19 (m, 1H), 2.51–2.61 (m, 2H), 2.28–2.43 (m, 3H), 1.89–2.00 (m, 4H), 1.68–1.75 (m, 1H), 1.52–1.58 (m, 4H), 1.23–1.48 (m, 4H), 1.03–1.11 (overlapping signals, 6H), 0.92–0.96 (overlapping signals, 6H) ppm; ^{13}C NMR ($CDCl_3$, 50.3 MHz, selected signals) major isomer: δ = 171.3, 153.5, 139.7, 127.4, 126.9, 126.2, 125.7, 122.5, 120.8, 117.7, 102.8, 67.0, 64.9, 62.9, 62.3, 61.3, 55.7, 55.2, 54.3, 48.4, 33.8, 33.3, 29.1, 26.5, 25.2, 24.1 ppm; HRMS (ESI): *m*/*z* calcd for $C_{31}H_{45}Cl_2N_2O_3^+$: 563.2807; found: 563.2804 $[M+H]^+$.

4.3. 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; used at 1:5 dilution), 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.

4.3.1. DNA cleavage assay

DNA cleavage assays were performed using pBR322 plasmid. pBR322 (3.5 nM) was incubated with increasing concentrations (0.5, 5, 50 mM) of agent for 3 h at 37 °C in BPE buffer. The linear DNA standard was generated by EcoRI (Promega) digestion of pBR322, according to the manufacturer's instructions. In order to obtain the open circular form of pBR322, 250 ng of supercoiled plasmid was incubated on ice for 5 min with 0.001 U/μL RQ1 DNase–RNase-free (Promega) in 1× RQ1 buffer in a total reaction volume of 10 μL. Nicking reaction was stopped adding 5 μL EGTA (ethylene glycol tetraacetic acid) 20 mM (pH 8.0). 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 1× TAE (Tris–HCl 40 mM, acetic acid 20 mM, EDTA 1 mM). DNA in the gel system was detected by staining with ethidium bromide (0.5 μg/mL) for 30 min with visualization by a Geliance 600 imaging system (PerkinElmer, Waltham, MA, USA).

4.3.2. Sequencing gel analysis

The 5′-FAM-labelled duplex oligonucleotide (2 μM final concentration) was incubated with each alkylating agent (final concentrations of 5 and 50 μM) in BPE buffer (final dilution 1:5) for 24 h at 37 °C. The samples were dried in a vacuum centrifuge (UNIVAPO 100H, UniEquip), resuspended in 5 mL 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 1× TBE (Tris–HCl 89 mM, borate 89 mM, EDTA 2 mM). The fluorescence of the oligonucleotide bands were detected by scanning using Storm Scanner Control (STORM 840, Molecular Dynamics).

4.4. ESI-MS analysis of reactive aziridinium ion formation

A 80 μM solution of lysine methyl ester (**1**) in distilled water was prepared from a 8 mM stock solution in DMSO and incubated at 37 °C for 2 h. Small samples were taken after 0 min (immediately after compound (**1**) was dissolved in water), 15 min, 30 min, 45 min, 60 min and 120 min, diluted with methanol, and analysed by ESI-MS. Measurements were performed in positive ion mode.

Acknowledgments

This work was supported financially by MIUR, Progetti di Ricerca di Interesse Nazionale (Grant 2010W2KM5L_006) to B.G. The authors would like to acknowledge the assistance of Dr. Erwin Röcker with mass spectrometric measurements. I.Z. thanks the German National Academic Foundation (Studienstiftung des deutschen Volkes) for providing a PhD scholarship, as well as the International Giessen Graduate Centre for the Life Sciences (GGL) and the German Academic Exchange Service (DAAD) for a travel grant.

Supplementary data

Supplementary data (sequencing gel analysis and ESI-MS spectra of the hydrolysis reaction of compound **1**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.01.050.

References and notes

1. Avendaño, C.; Menéndez, J. C. *Medicinal Chemistry of Anticancer Drugs*; Elsevier: Amsterdam, Boston, 2008.
2. Siddik, Z. H. In *The Cancer Handbook*; Alison, Malcolm R., Ed.; John Wiley & Sons Ltd: Chichester, UK, 2005; p 139.
3. Hurley, L. H. *Nat. Rev. Cancer* **2002**, *2*, 188.
4. Kohn, K. W. *Cancer Res.* **1996**, *56*, 5533.
5. Warwick, G. P. *Cancer Res.* **1963**, *23*, 1315.
6. Price, C. C.; Gaucher, G. M.; Koneru, P.; Shibakawa, R.; Sowa, J. R.; Yamaguchi, M. *Ann. N.Y. Acad. Sci.* **1969**, *163*, 593 (2 Biological Ef).
7. Wheeler, G. P. *Cancer Res.* **1962**, *22*, 651.
8. Polavarapu, A.; Stillabower, J. A.; Stubblefield, S. G. W.; Taylor, W. M.; Baik, M.-H. *J. Org. Chem.* **2012**, *77*, 5014.
9. Mattes, W.; Hartley, J.; Kohn, K. *Nucleic Acids Res.* **1986**, *14*, 2971.
10. Noll, D. M.; Mason, T. M.; Miller, P. S. *Chem. Rev.* **2006**, *106*, 277.
11. Panasci, L.; Xu, Z.-Y.; Bello, V.; Aloyz, R. *Anticancer Drugs* **2002**, *13*, 211.
12. Silverman, R. B. *The Organic Chemistry of Drug Design and Drug Action*; Elsevier Academic Press: Amsterdam, Boston, 2004. pp 353–358.
13. Williamson, C. E.; Witten, B. *Cancer Res.* **1967**, *27*, 33.
14. Zuravka, I.; Roesmann, R.; Sosic, A.; Wende, W.; Pingoud, A.; Gatto, B.; Göttlich, R. *ChemMedChem* **2014**, *9*, 2178.
15. Coleman, R. S.; Burk, C. H.; Navarro, A.; Brueggemeier, R. W.; Diaz-Cruz, E. S. *Org. Lett.* **2002**, *4*, 3545.
16. Casely-Hayford, M. A.; Pors, K.; Patterson, L. H.; Gerner, C.; Neidle, S.; Searcey, M. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 653.
17. Yang, X. L.; Wang, A. H. *Pharmacol. Ther.* **1999**, *83*, 181.
18. Brannock, K. C. *J. Am. Chem. Soc.* **1959**, *81*, 3379.
19. Davies, C. E.; Heightman, T. D.; Hermitage, S. A.; Moloney, M. G. *Synth. Commun.* **1996**, *26*, 687.