COTUTELLE DOCTORAL THESIS

Synthesis, Evaluation and Mechanistic Studies of Bis-3-chloropiperidines as DNA Alkylating Agents

A Cumulative Dissertation Presented for the Joint Degree

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Dedicated to Martok

"Even the stones that have been placed in one's path can be made into something beautiful"

Johann Wolfgang von Goethe

Preface

This dissertation was completed as a cotutelle collaboration (joint supervision agreement) between the Justus Liebig University (JLU) of Giessen and the University of Padova (UNIPD). The thesis is based on experimental work carried out in Germany at the Institute of Organic Chemistry and Institute of Biochemistry of the JLU Giessen (from January 2010 till September 2013, under the supervision of Prof. Dr. R. Göttlich) as well as in Italy at the Department of Pharmaceutical and Pharmacological Sciences of the UNIPD (from October 2013 till March 2015, under the supervision of Prof. Dr. B. Gatto).

The present work is prepared as a cumulative doctoral thesis, consisting of several scientific articles already published or submitted for publication, which are incorporated into the thesis as successive chapters (Chapters 2–4). A general introduction to the theoretical background and an overall summary of the main findings are presented in Chapter 1. Moreover, this chapter describes the research aim and the relationships between the individual publications.

At this point, I would like to express my gratitude to the German National Academic Foundation (Studienstiftung des deutschen Volkes) for providing me a PhD scholarship. I am also very grateful to the International Giessen Graduate Centre for the Life Sciences (GGL) and the German Academic Exchange Service (DAAD) for awarding me a travel grant. The financial, educational and ideational support enabled me to successfully accomplish this binational, interdisciplinary dissertation.

List of Publications

This doctoral thesis is based on the following publications, which are referred to in the text by their respective chapter numbers:

Chapter 2:
I. Zuravka, R. Roesmann, A. Sosic, W. Wende, A. Pingoud, B. Gatto and R. Richard: Synthesis and DNA Cleavage Activity of Bis-3-chloropiperidines as Alkylating Agents; *ChemMedChem* **2014**, *9*, 2178–2185.

Chapter 3:
I. Zuravka, R. Roesmann, A. Sosic, R. Göttlich and B. Gatto: Bis-3-chloropiperidines Containing Bridging Lysine Linkers: Influence of Side Chain Structure on DNA Alkylating Activity; *Bioorg. Med. Chem.* **2015**, *23*, 1241–1250.

Chapter 4:
I. Zuravka, A. Sosic, B. Gatto and R. Göttlich: Synthesis and Evaluation of a Bis-3-chloro-piperidine Derivative Incorporating an Anthraquinone Pharmacophore; **2015**, *submitted for publication*.

Abstract

DNA alkylating agents represent a major class of first-line chemotherapeutic drugs in the fight against cancer. Among them, nitrogen mustards are the oldest and clinically most effective anticancer agents in use today. They exert their primary cytotoxic actions by covalently binding to DNA bases and thereby causing DNA strand cleavage, which can lead to the inhibition of cell division and ultimately cell death. However, adverse side effects and the emergence of nitrogen mustard drug resistance are apparent drawbacks that restrict their therapeutic utility. One approach to overcome these limitations is the design and subsequent structural optimisation of novel alkylating agents based on the mustard pharmacophore.

The objectives of the work described in this doctoral thesis are (a) the synthesis of nitrogen-linked bis-3-chloropiperidines as simplified analogues of the antibiotic 593A and (b) the biological evaluation of their DNA alkylating properties. These investigations aim at the elucidation of the molecular mechanism of action of this new class of piperidine mustards. The compounds examined in this research project are designed to explore the effect of different linker structures on alkylation activity, allowing the determination of structure–activity relationships.

Based on preliminary studies in our group, a series of bis-3-chloropiperidines with flexible alkyl groups, a conformationally restrained cyclohexyl moiety, rigid aromatic or lysine ester linkers was successfully prepared. The bidirectional synthetic strategy involves the reductive amination of the respective diamine linker with 2,2-dimethylpent-4-enal and subsequent chlorination, followed by iodide-catalysed cyclisation of the unsaturated bis-*N*-chloroamine to yield the target compound. Thus, a small library of bis-3-chloropiperidine derivatives has been assembled to provide an experimental proof-of-concept study.

The ability of the synthesised compounds to alkylate double-stranded DNA (plasmids pAT153 and pBR322; 22-mer oligonucleotide) was ascertained by comparative DNA cleavage assays using gel electrophoresis methods. The results demonstrate that the investigated bis-3-chloropiperidines exhibit significant DNA alkylation activities, superior to the nitrogen mustard antitumour drug chlorambucil. Our studies reveal that DNA cleavage occurs primarily through reactions with guanines via a DNA nicking mechanism. Experimental evidence indicates the presence of agent-induced DNA adducts. Furthermore, the reactive aziridinium ion formation is verified by ESI mass spectrometry.

The findings from this survey substantiate that the linker structure has a considerable impact on DNA alkylation efficiency of bis-3-chloropiperidines. On the basis of the obtained results, it is concluded that the flexibility and composition of the linker region are crucial features. In particular, the introduction of rigid aromatic motifs or an ester function leads to a reduced DNA cleavage activity, whereas DNA-affinic chromophores likely contribute to favourable DNA interactions. These established structure–activity relationships might offer the possibility for the modulation of reactivity of bis-3-chloropiperidines for the development of effective new DNA alkylating agents.

Kurzfassung

DNA-alkylierende Agenzien repräsentieren eine bedeutsame Gruppe von First-line Chemotherapeutika im Kampf gegen Krebs. Unter ihnen gehören die Stickstoff-Lost-Verbindungen zu den ältesten und wirkungsvollsten Zytostatika, welche in der heutigen Krebstherapie Verwendung finden. Die primäre zytotoxische Wirkung dieser Substanzklasse beruht auf kovalente Bindungen an DNA und daraus resultierenden DNA-Strangbrüchen, was zu einer Hemmung der DNA-Replikation und letztendlich zum Zelltod führen kann. Allerdings liegen ihre wesentlichen Nachteile in zahlreich auftretenden Nebenwirkungen und verstärkter Resistenzentwicklung, wodurch ihr therapeutischer Nutzen eingeschränkt wird. Ein vielsprechender Ansatz diese Defizite zu überwinden, besteht im Design sowie der anschließenden strukturellen Optimierung von neuartigen, auf dem Stickstoff-Lost-Pharmakophor basierenden Alkylanzien.

Die vorliegende Dissertation befasst sich mit den folgenden Zielsetzungen: (a) Synthese von Stickstoff-verbrückten Bis-3-chlorpiperidinen als strukturell vereinfachte Analoga des Antibiotikums 593A; (b) Biologische Evaluation der dargestellten Derivate hinsichtlich ihrer DNA-alkylierenden Eigenschaften. Das grundlegende Ziel der Forschungsarbeit ist die Aufklärung des molekularen Mechanismus dieser neuen Klasse von Piperidin-Lost-Verbindungen. Die im Rahmen des Vorhabens geplanten Zielstrukturen sind dahingehend gewählt, den Einfluss verschiedener Linker auf die Alkylierungsaktivität zu untersuchen und Struktur-Aktivitäts-Beziehungen abzuleiten.

Aufbauend auf eigenen sowie in unserer Arbeitsgruppe erfolgten Vorarbeiten, wurde eine Reihe von Bis-3-chlorpiperidinen mit flexiblen Kohlenwasserstoff-, einem konformationell eingeschränkten Cyclohexan-, starren aromatischen oder Lysinester-Linkern erfolgreich hergestellt. Die etablierte bidirektionale Synthesestrategie beinhaltet, neben der reduktiven Aminierung von 2,2-Dimethyl-4-pentenal mit dem jeweiligen Diamin-Linker und anschließender Chlorierung, als zentralen Schritt die Iodid-katalysierte Cyclisierung ungesättigter Bis-*N*-chloramine zu den gewünschten Zielverbindungen. Demgemäß wurde im Verlauf der Arbeit eine kleine Substanzbibliothek von Bis-3-chlorpiperidin-Derivaten aufgebaut, mit dem Ziel eine experimentelle Grundsatzstudie (Proof of Concept) durchzuführen.

Die Fähigkeit der synthetisierten Testsubstanzen doppelsträngige DNA (Plasmide pAT153 und pBR322; 22-mer Oligonukleotid) zu alkylieren, wurde in vergleichenden DNA-Spaltungsexperimenten mittels gelelektrophoretischer Methoden untersucht. Die Ergebnisse zeigen, dass die evaluierten Bis-3-chlorpiperidine signifikante, im Vergleich zu dem Antitumormittel Chlorambucil (Stickstoff-Lost-Derivat) deutlich überlegene, DNA-Alkylierungsaktivitäten aufweisen. Unsere Studien demonstrieren, dass die beobachtete DNA-Spaltung hauptsächlich durch Reaktionen mit Guanin nach einem DNA-Nicking-Mechanismus (Induktion von Einzelstrangbrüchen) erfolgt. Experimentelle Befunden belegen darüber hinaus die Generierung von DNA-Addukten. Weiterhin konnte die Bildung des reaktiven intermediären Aziridinium-Ions durch ESI-Massenspektrometrie eindeutig nachgewiesen werden.

Die durch diese Arbeit gewonnen Erkenntnisse bestätigen, dass die Linker-Struktur einen entscheidenden Einfluss auf die DNA-Alkylierungseffizienz von Bis-3-chlorpiperidinen ausübt. Zusammenfassend lässt sich anhand der Forschungsergebnisse schlussfolgern, dass die Flexibilität sowie die strukturelle Beschaffenheit des Linkers maßgebliche Faktoren darstellen. Insbesondere wird durch die Einführung von aromatischen Strukturmotiven oder einer Ester-Funktionalität die DNA-Spaltungsaktivität herabsetzt, wogegen DNA-affine Chromophore voraussichtlich zu günstigen DNA-Wechselwirkungen beitragen. Anhand der aufgestellten Struktur-Aktivitäts-Beziehungen besteht die Möglichkeit durch strukturelle Modifizierungen die Reaktivität von Bis-3-chlorpiperidinen gezielt zu steuern und infolgedessen neuartige, effektive DNA-Alkylanzien zu entwickeln.

Riassunto

Agenti alchilanti del DNA rappresentano una classe importante di chemioterapici di prima linea nella battaglia contro il cancro. Tra questi agenti antitumorali le mostarde azotate sono il gruppo clinicamente efficace e più antico attualmente in uso. Essi esercitano la loro azione citotossica primaria attraverso legami covalenti tra i nucleotidi, causando scissione del DNA. Questi danni al DNA possono portare all'inibizione della divisione cellulare ed infine alla morte cellulare. Gli effetti collaterali e l'emergere di resistenza alle mostarde azotate, tuttavia, sono gli svantaggi principali che limitano la loro utilità terapeutica. Un approccio che cerca di superare queste limitazioni è il design e la successiva ottimizzazione strutturale dei nuovi agenti alchilanti basati sul farmacoforo delle mostarde azotate.

Gli obiettivi del lavoro di ricerca descritti in questa tesi di dottorato sono (a) la sintesi di bis-3-cloropiperidine legate all'azoto mediante diversi linker come analoghi semplificati dell'antibiotico 593A e (b) la valutazione biologica delle loro proprietà alchilanti del DNA. Questi studi hanno lo scopo di chiarire il meccanismo molecolare d'azione di questa nuova classe di mostarde piperidiniche. I composti esaminati in questo lavoro sono progettati per esplorare l'effetto delle diverse strutture del linker sull'attività alchilante, permettendo la determinazione delle relazioni struttura-attività.

Sulla base degli studi preliminari nel nostro gruppo, è stata preparata con successo una serie di bis-3-cloropiperidine unite da gruppi alchilici flessibili, da altri conformazionalmente limitati come un gruppo cicloesilico, gruppi rigidi aromatici e altri uniti da una lisina esterificata con vari sostituenti. La sintesi bidirezionale prevede l'amminazione riduttiva del rispettivo diammina-linker con 2,2-dimetil-4-pentenale e successiva clorurazione, seguita da ioduro catalizzata ciclizzazione della bis-*N*-cloroamina insaturo a fornire il composto desiderato. In questo modo una piccola libreria di derivati bis-3-cloropiperidine è stata costituita per effettuare uno studio proof-of-concept.

La capacità dei composti sintetizzati di alchilare il DNA a doppio filamento (plasmidi pAT153 e pBR322, oligonucleotide 22-mer) è stata investigata da esperimenti di scissione del DNA (DNA cleavage assay) utilizzando metodi di elettroforesi su gel. I risultati dimostrano che le bis-3-cloropiperidine indagate presentano significative attività di alchilazione del DNA, superiori al chemioterapico clorambucile, un derivato della mostarda azotata. Infatti, i nostri studi rivelano che la scissione del DNA avviene principalmente attraverso reazioni con la base guanina, mediante un meccanismo di "nicking" (l'incisione di un filamento del DNA). Le evidenze sperimentali indicano che gli agenti sono capaci di indurre addotti di DNA. Inoltre, la formazione dello ione aziridinio reattivo è stata verificata con analisi di spettrometria di massa (ESI-MS).

I risultati di questo lavoro comprovano che la struttura del linker ha un considerevole impatto sull'efficienza alchilante delle bis-3-cloropiperidine. Sulla base dei dati ottenuti, si può concludere che la flessibilità e la composizione del linker sono fattori determinanti. In particolare, l'introduzione di gruppi aromatici rigidi oppure una funzione esterea porta ad una ridotta attività di clivaggio del DNA, mentre cromofori affini al DNA probabilmente contribuiscono alle interazioni favorevoli. Le relazioni tra struttura ed attività osservate per i derivati testati potrebbero offrire la possibilità di modulare la reattività di bis-3-cloropiperidine e quindi costituiscono una base per lo sviluppo di nuovi agenti alchilanti efficaci.

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CHAPTER 1:

Introduction and Synopsis

1.1 Chemical Warfare against Cancer

More than 40 years have passed since U.S. President Richard Nixon declared a "war on cancer" (National Cancer Act of 1971)[1] and "despite dramatic scientific gains, cancer remains an undaunted killer."[2] Today, one in every eight death is due to cancer and there are about 14 million new cancer cases globally each year.[3] In 2012 approximately 32.6 million people had been diagnosed with cancer. Within the next two decades, cancer-related deaths are predicted to rise from an estimated 8.2 million annually to 13 million a year.[4] These alarming statistics underscore the imperative need for continued research in preventing, detecting and treating cancer.

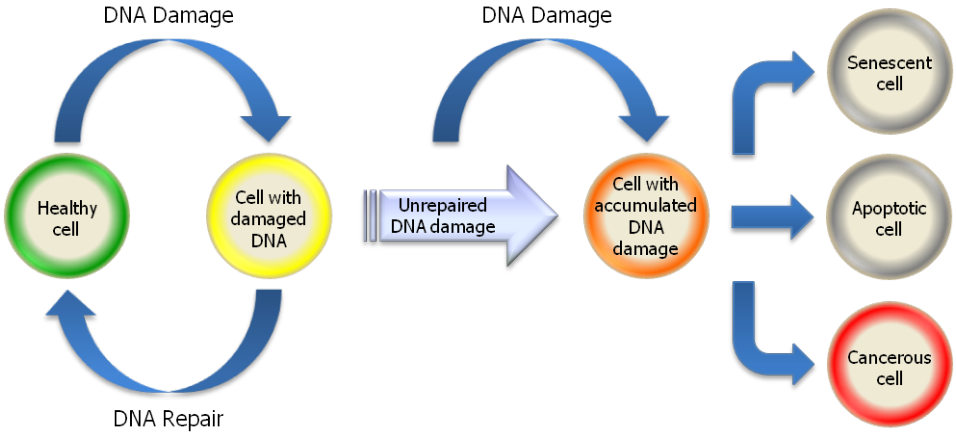


Figure 1: Schematic representation of the DNA damage pathway which can trigger a variety of cellular responses culminating in the development of cancer. Modified from Sigma-Aldrich®: DNA Damage and Repair, BioFiles 2007, 2.4, 20.[7]

Cancer is a general term for diseases characterised by uncontrolled multiplication of cells. These abnormal cells are able to invade surrounding tissue or spread throughout the body. This spreading process is called metastasis. All cancer types are caused by mutations in DNA which occur when there are alterations in the sequence of DNA bases.[5] DNA can be changed in many different ways, for example by environmental factors such as ultraviolet radiation or chemical mutagens as well as by random mistakes in DNA replication. To counteract these tens of thousands DNA damages that are produced each day inside every cell, all organisms have developed repair processes.[6] However any resulting damage, if not repaired correctly, may lead to severe consequences that range from the induction of cell death by apoptosis (programmed cell death) or senescence (cell growth arrest) to the emergence of cancer (Figure 1).

While DNA damage is a key factor in the origin of cancer cells, inhibition of DNA replication by DNA-damaging agents is used as part of clinical treatments to prevent the cancer cells from reproducing. In fact, many important chemotherapeutic drugs act by their ability to generate DNA lesions.[8] Among them, the DNA alkylators were the first anticancer therapeutics and they remain the most effective agents for cancer management.[9]

1.1.1 DNA Alkylating Agents in Cancer Therapy

Ironically, it were the effects of an alkylating chemical warfare agent during World Wars I and II that marked the beginning of modern cancer chemotherapy.[10] Following the use of the blistering "mustard gas" (Figure 2; bis(2-chloroethyl) sulphide is also known as: Sulphur mustard; Yperite; Senfgas; Lost) in 1917 it was recognised that besides its acute toxicity on exposed tissues, delayed systemic symptoms occurred in victims.[11] These disorders include leukopenia (deficiency of white blood cells), bone marrow aplasia (decrease or cessation of the production of blood cells), dissolving of lymphatic tissue and gastrointestinal ulceration.[12]

After the war, it was revealed that these delayed effects of the chemical agent were primarily against rapidly dividing cells, causing inhibition of cell division.[13] This suggested a logical approach to cancer treatment based on related compounds. A nitrogen analogue known as mechlorethamine (Mustargen®) was found to be a less toxic form of sulphur mustard and in 1942 the first clinical trials of this nitrogen mustard were initiated.[14,15] Noticeable regression of tumours and relief of symptoms were observed in patients with lymphoma. Encouraged by these findings, a large number of nitrogen mustards have been synthesised and studied in search of more effective anticancer agents. The derivatives, among others, chlorambucil and cyclophosphamide exhibited pharmacological improvements and they continue to have a prominent role in cancer therapy today (Figure 2).

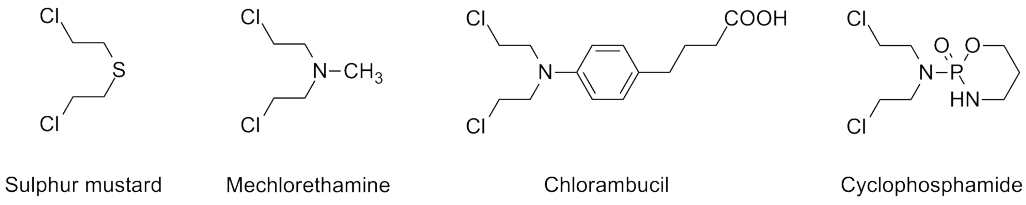


Figure 2: Chemical structures of the warfare agent sulphur mustard and therapeutically useful nitrogen mustard derivatives.

1.1.2 Chemistry of Nitrogen Mustards

Nitrogen mustards are classified as alkylating agents, as studies of the reaction mechanisms indicated that the cytotoxic effects were due to alkylation.[14,16] An alkylating agent is defined as a substance that can replace a hydrogen atom with an alkyl group under physiological conditions (aqueous solution, pH 7.4, 37 °C).[17] In particular, these alkylating reactions take place with important intracellular biomolecules, while DNA represents the principal target for the cytotoxic effect.

The general mechanism of action of the nitrogen mustards (**A**) involves the formation of a highly reactive aziridinium ion (**B**, Figure 3).[18] This electrophilic intermediate species (**B**) can react with susceptible nucleophilic sites within DNA to form covalent DNA adducts (see box in Figure 3).[19] In this regard, nitrogen mustards display a certain degree of target selectivity in their reactions with DNA bases. It was found that the most readily alkylated site on DNA is the N-7 position of guanine (**C**).[20] In addition to forming guanine monoadducts (**D**), the second chloroethyl side chain of the nitrogen mustard molecule can undergo the same activation process, followed by a nucleophilic attack on the generated alkylating moiety. Thus, these bifunctional agents are able to produce covalent biadducts (**E**) with guanine residues on both strands of DNA, affording interstrand cross-links.[20,21] As a consequence of DNA cross-linking and the resulting structural distortions of the double helix, DNA strand separation is prevented leading to inhibition of DNA replication and transcription.[22]

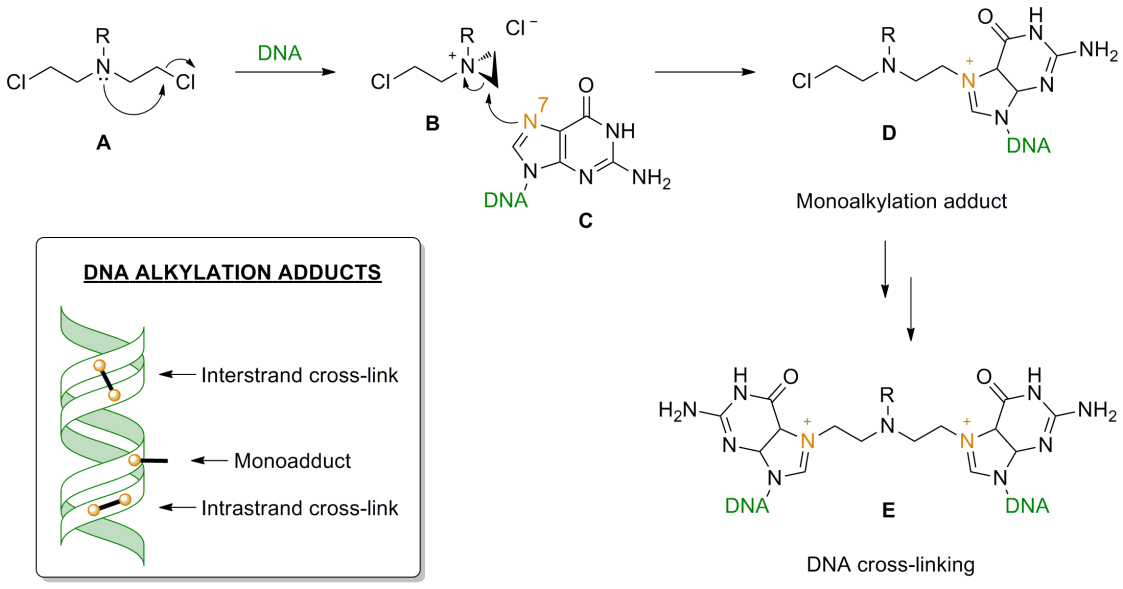


Figure 3: Mechanism of DNA alkylation by nitrogen mustards and formation of DNA adducts. Cross-linking can occur either within a DNA strand (intrastrand) or between two complementary strands of DNA (interstrand).

Blocking these essential DNA metabolic processes can eventually cause the induction of cytotoxic DNA double-strand breaks, which require complex repair mechanisms.[23] Furthermore, DNA alkylation can contribute to the destabilisation of the alkylated nucleobase (i.e., its glycosidic linkage). Thus, DNA becomes easily susceptible to hydrolytic reactions, facilitating subsequent deglycosylation (e.g., of alkylated guanine residues, Figure 4).[24,25] The loss of alkylated purine bases from the sugar-phosphate backbone (depurination) can eventually lead to single- or double-strand cleavage of DNA (see box in Figure 4). Alternatively, a ring-opening reaction may occur by attack of water at the C-8 position of an N-7 alkylated guanine, which is considered to be rather slow compared to the competing depurination.[25] Whether these strand breaks initiate cytotoxicity is ultimately determined by cellular responses to the alkylating agent and depends on the extent of DNA damage as well as how rapidly these lesions are removed by respective DNA repair pathways.

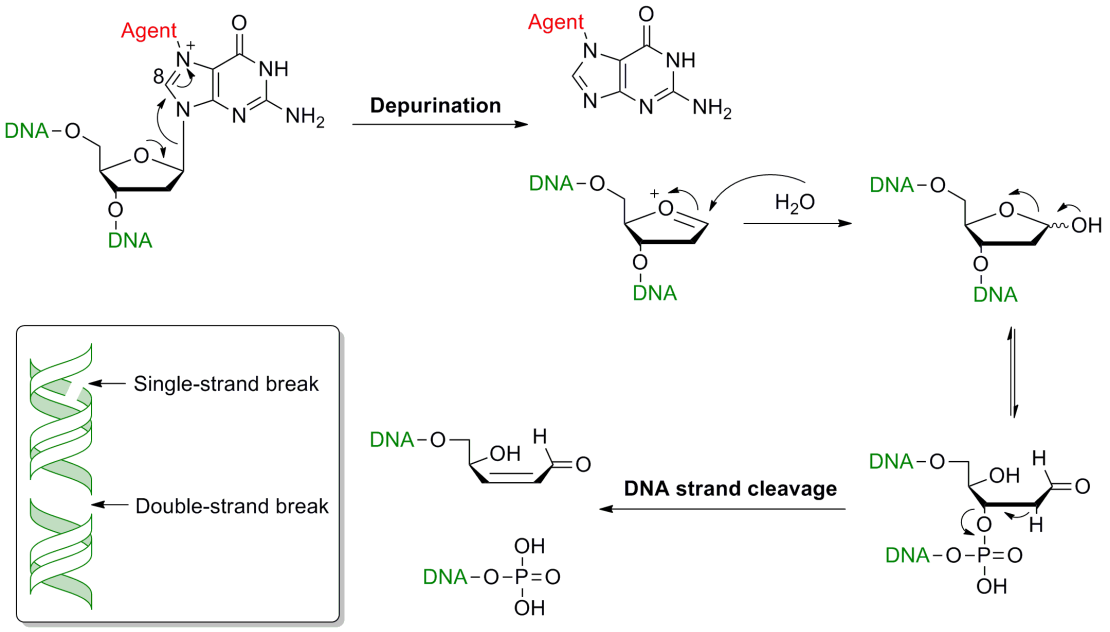


Figure 4: DNA strand cleavage triggered by depurination of N-7 alkylated guanine residues. Cleavage can occur on only one strand (single-strand break) or on both opposite strands of the DNA duplex (double-strand break).

However, these crucial cytotoxic alkylation reactions of nitrogen mustards do not differentiate between healthy and tumour cells, thereby leading to adverse side effects. An additional obstacle in cancer treatment, on the other hand, is the development of drug resistance which is attributed to enhanced DNA cross-link repair.[9,26] In fact, chemotherapeutic resistance and drug toxicity remain the most challenging issue for clinicians in managing cancer. Therefore, continued research in this area is required to develop more effective drugs for improved anticancer therapy.

1.2 Drug Development from Natural Products

Historically, nature has been ever since the source and inspiration for traditional remedies and novel therapeutic agents. However, only a small percentage of the world's biodiversity has been evaluated for potential pharmaceutical purposes.[27] It is apparent that many more valuable natural compounds with therapeutic potential are still awaiting discovery. Indeed, natural products continue to play a very significant role in modern drug discovery and development.[28] It has been estimated that approximately 50% of the newly approved drugs between the years 2000 and 2010 were derived from natural products.[29]

Generally, the term natural product refers to organic compounds that are produced by living systems such as plants, microorganisms and marine organisms and is usually used synonymously with secondary metabolite.[30] Whereas primary metabolites (nucleic acids, proteins, lipids, carbohydrates) are ubiquitous and essential to the organismal function, secondary metabolites are limited in their biological distribution and are not directly involved in the maintenance and development of an organism. Most often, the latter have different ecological roles, notably in specific defence mechanisms against herbivores, microbes, viruses or competing plants.[31]

Due to their known potential beneficial effects on human health, natural products have attracted sustained interest of scientists in this field, particularly with regard to cancer therapy. It is significant that today more than 60% of the anticancer agents in current use or clinical trials are of natural product origin.[32] Probably the most prominent example of a plant-based remarkably effective antitumour drug is paclitaxel (Taxol®), a highly functionalized diterpenoid, originally isolated from the bark of Pacific yew (*Taxus brevifolia*).[33] In spite of its great success in the treatment of advanced breast and ovarian cancer, paclitaxel was a subject of a major supply issue.[34] The problem of limited natural paclitaxel source was overcome by alternative production methods such as cell culture, semi-synthesis and biosynthesis in microorganisms.[35] As a matter of fact, the adequate resupply of natural products is a continuing challenge faced in drug discovery and development.[36] While efficient total synthesis can provide a potential route for bulk production of bioactive naturally occurring compounds, it can turn out to be an expensive and complicated procedure. Therefore, natural molecular scaffolds serve often as new structural leads for the design of simpler analogues with similar or optimised pharmacological properties. Also in case of the unique taxane skeleton, hundreds of new derivatives were synthesised and several drug candidates with improved therapeutic indices are currently in clinical trials.[37]

1.2.1 The Piperidine Mustard Pharmacophore

As highlighted in the previous section, rational drug design and syntheses of natural product-based analogues represent a key component with regard to the drug discovery process. From structural modifications of natural lead compounds, it is possible to determine the molecular features that are necessary for activity, the so-called pharmacophore.[38] One approach to improve the therapeutic efficacy of conventional anticancer drugs is by utilising the pharmacophore moiety as part of new bioactive molecules. Structural modifications might eventually lead to more suitable drug candidates with improved chemical stability and optimised side effect profiles. In recent years, a vast range of hybrid compounds incorporating the nitrogen mustard unit have been synthesised and tested to assess their potential as antitumour agents.[39]

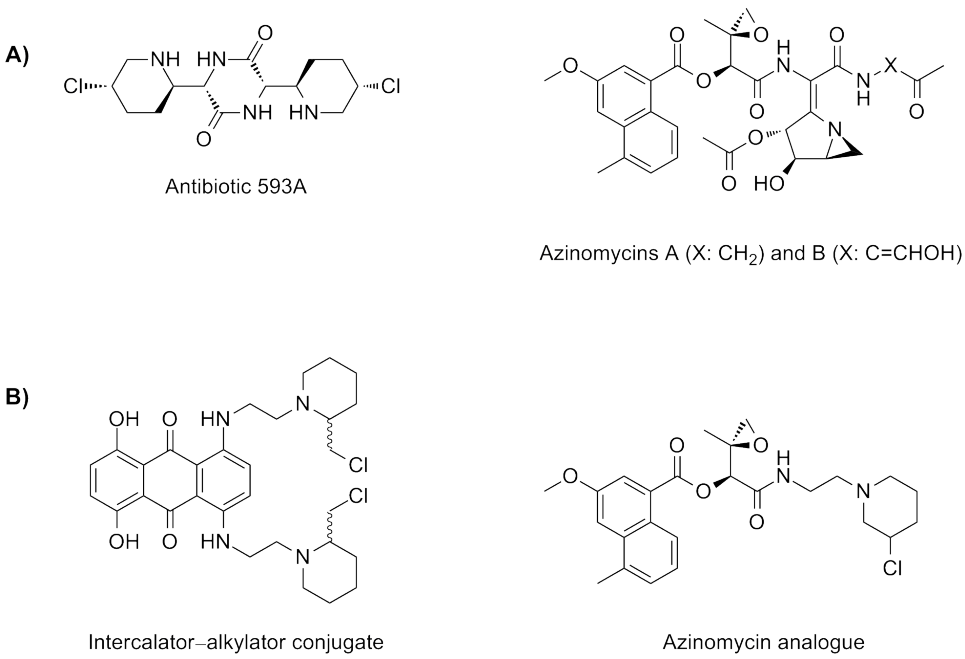


Figure 5: (A) Chemical structures of the antitumour antibiotics 593A, azinomycin A and azinomycin B. (B) Synthetic analogue of the azinomycins incorporating a 3-chloropiperiridine ring[46] as well as an example for an intercalator–alkylator conjugate[48] with piperidine mustard functionalities.

Interestingly, since nitrogen mustards are not found naturally in environment, the antibiotic 593A (NSC-135758, Figure 5A) represents the first mustard-related natural product isolated in 1970 from the soil organism *Streptomyces griseoluteus*.[40] The antibiotic is characterised by a unique 3-chloropiperdine ring that resembles the structure of the synthetic nitrogen mustard and therefore is supposed to have a similar mechanism of alkylation.[41]

The piperazinedione 593A displays high antineoplastic and antitumour activity, however, its potential use in chemotherapy was limited by its strong myelosuppressive effect (decrease in blood cell production).[42,43] In addition, the compound represents a synthetic challenge. To date, only one completed total synthesis has been reported.[44] As a result, the strong initial interest in 593A has been greatly diminished and over the last few decades no further research studies have been published on this compound. Though, the piperidine mustard pharmacophore has been used as alkylating moiety in the synthesis of new potential anticancer agents.[45] As an example, Searchey and co-workers replaced the densely functionalised DNA-alkylating aziridine fragment of the antitumour antibiotics azinomycin A and B with a more stable, therapeutically viable 3-chloropiperidine entity (Figure 5).[46,47] In an attempt to overcome drug resistance, piperidine-based mustards have also been incorporated in the design of DNA intercalator–alkylator conjugates (Figure 5B).[48] These DNA-affinic alkylating anthraquinones showed a broad spectrum of antitumour activity. Thus, the piperidine mustard pharmacophore has proven to be a promising structural scaffold in the search of more suitable anticancer drug candidates.

1.2.2 Molecular Scaffolds for DNA Recognition

Nature offers a diverse range of structural motifs for molecular recognition of DNA (e.g., intercalators, polypyrroles, polyimidazoles).[49] In fact, natural products have revealed interesting DNA-binding mechanisms which produce favourable pharmacological effects.[50] These molecular recognition processes involve electrostatic binding, interactions in the major and minor grooves of DNA as well as intercalation between the base pairs of the double helix.[51] Such noncovalent association of small molecules with nucleic acids can distort the regular structure of DNA and interfere with essential cellular functions. The ability of various natural compounds to interact with DNA via the above-mentioned binding modes is associated with their biological activities. For instance, it has been found that the naphthoate group of the azinomycin antibiotics (compare Figure 5A) plays a crucial role in sequence selective DNA-binding affinity and cytotoxic activity.[52] Of particular interest in the development of antitumour drugs are the DNA intercalators. This clinically important class of reversible DNA-binding agents is discussed in the following section of this chapter.

1.2.2.1 DNA Intercalators

A variety of natural and synthetic products bind to DNA by intercalating into the double helix (Figure 6).[53] DNA intercalators are mostly polycyclic, aromatic and planar molecules that are capable of inserting between DNA base pairs by means of noncovalent stacking interactions. As a result, intercalation causes conformational alterations in the helix geometry and local unwinding at the site of binding, leading to DNA damage which can trigger cytotoxic effects.

Due to their cytotoxic actions, intercalating agents have been widely investigated for their potential anticancer properties.[54] Representative and therapeutically interesting compounds include acridines and anthracyclines (Figure 7). The DNA-binding mechanism of the acridine proflavine was the first proposed model of intercalation described by Lerman in 1961.[55] To date, thousands of natural and synthetic acridine derivatives have been extensively explored as antitumour drugs and some have been approved for chemotherapy.[56]

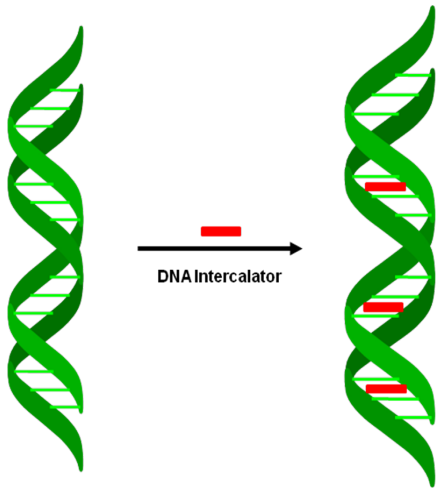


Figure 6: Schematic representation of the DNA intercalation process. The insertion of an intercalator between the base pairs of duplex DNA causes structural alterations in the DNA conformation.

In the late 1960s and early 1970s the anthracycline antibiotics doxorubicin and daunomycin have been introduced into clinical practice (Figure 7).[57] In particular, doxorubicin is among today's most effective anticancer drugs with broad-spectrum activity.[58] Structurally, anthracyclines consist of a common anthraquinone skeleton linked to a sugar moiety. This class of compounds exhibit antitumour efficacies through multiple mechanisms of action.[59] The tetracyclic anthraquinone-based chromophore is known to intercalate into the DNA double helix to form a stable ternary DNA–topoisomerase II–drug complex. The interference of anthracyclines with the function of topoisomerase II, an important enzyme that regulates DNA topology, and the consequent inhibition of DNA replication as well as DNA repair is considered as the primary mechanism of cytotoxic activity.[60] However, cellular resistance and life-threatening cardiotoxic side effects considerably limit the clinical value of the anthracycline antibiotics.[58,61] In order to overcome these obstacles, the simplified analogue mitoxantrone (Figure 7) was synthesised and shown to be an attractive alternative to the parent compounds.[62] In clinical studies, the antitumour activity of mitoxantrone was comparable to that of doxorubicin, but the analogue displayed an improved side effect profile.[63]

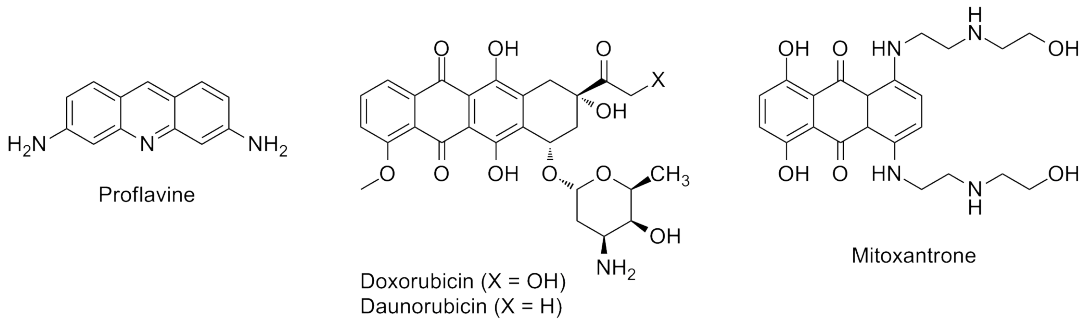


Figure 7. Structures of representative acridine and anthracycline DNA intercalators.

The development of such potent analogues with promising therapeutic properties has motivated the search for new compounds based on these intercalating pharmacophores. In fact, the acridine and anthraquinone scaffolds serve as privileged structural motifs for DNA-targeted drug design.[48,64]

1.3 Bis-3-chloropiperidines as DNA Alkylating Agents

1.3.1 Motivation, Aim and Objectives

The antibiotic 593A can be considered as a naturally occurring piperazinedione-bridged bis-3-chloropiperidine (compare Figure 8). Studies on the antitumour activity of this compound indicate that the antibiotic acts as an alkylating agent in inhibiting DNA synthesis and cell proliferation of tumour cells.[42] However, the exact mechanism of action is still unknown and remains to be elucidated; unfortunately, the difficulties encountered in the chemical synthesis of this natural product represent a challenge for mechanistic investigations.

In this context, synthetic studies[65,66] in our group towards the preparation of 3-chloropiperidines have led to our research interest in the synthesis of structurally simplified analogues of antibiotic 593A. Based on our promising preliminary results[67,68], the present research project focuses on the design and biological evaluation of nitrogen-linked bis-3-chloropiperidines with the overall aim to provide insight into the molecular mode of action of the related antibiotic.

The first part of the work is dedicated to the synthesis of a series of bis-3-chloropiperidines with various linker structures as proof-of-principle derivatives (Figure 8). To establish structure–activity relationships, linkers are defined in terms of the following structural features: linker length, conformation (i.e., flexible, rigid) and the chemical composition of a side-chain attached to the linker (e.g., ester and aromatic functional groups).

In the second part, mechanistic studies on the reaction of bis-3-chloropiperidines with double-stranded DNA are performed using gel electrophoresis methods. The synthesised compounds are investigated for their DNA alkylation properties in order to determine the effect of linker structure on activity.

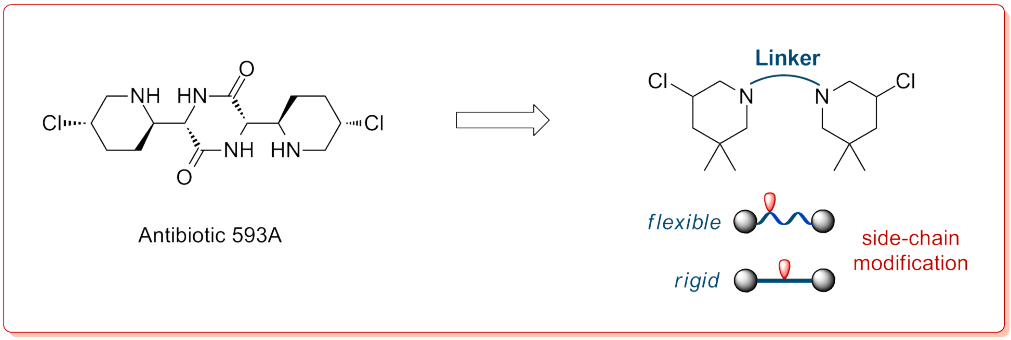
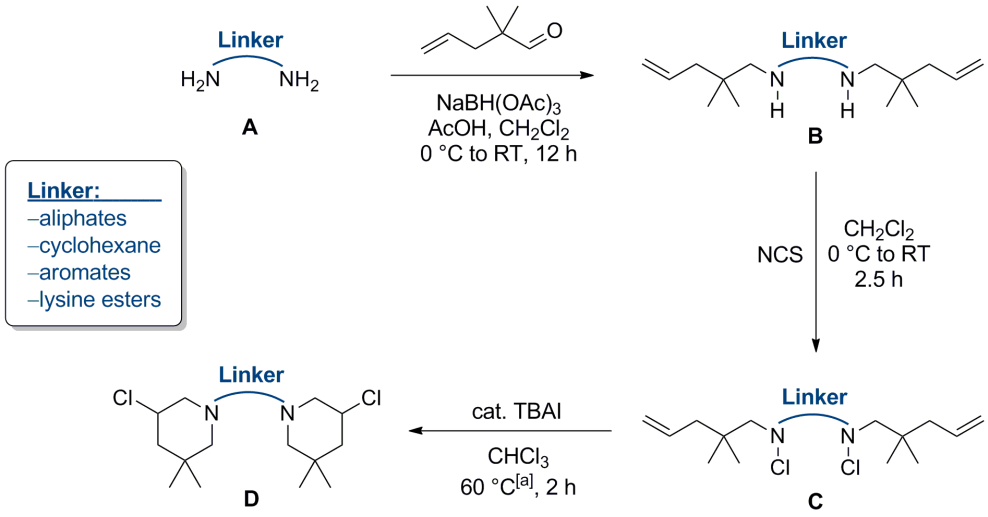


Figure 8: General structure of nitrogen-linked bis-3-chloropiperidines as simplified analogues of antibiotic 593A. Linker length, conformation (flexibility/rigidity) and composition (side-chain functional groups) is systemically varied to explore structure–activity relationships.

1.3.2 Synthesis of Bis-3-chloropiperidines

In recent years, our research group developed an efficient bidirectional method for the preparation of bis-3-chloropiperidines (see Chapter 2 and 3). As depicted in Scheme 1, the synthetic strategy permits the simultaneous formation of both piperidine ring systems by iodide-catalysed cyclisation of unsaturated bis-*N*-chloroamines (**C**).[65] We planned to synthesise a series of nitrogen-linked bis-3-chloropiperidines (**D**) from simple and easily accessible starting materials. With the aim to examine the impact of the linker structure on DNA alkylation activity we selected a variety of readily available diamine linkers (**A**). The syntheses of the precursor compounds were achieved following the well-established procedure in our laboratory. This approach is based on the double reductive amination of 2,2-dimethylpent-4-enal[69] with the appropriate diamine (**A**) using sodium triacetoxyborohydride[70]. Chlorination of the unsaturated diamines (**B**) with *N*-chlorosuccinimide (NCS) afforded the cyclisation precursors (**C**), which were transformed into the desired bis-3-chloropiperidines (**D**, mixture of diastereomers) by treatment with catalytic amounts of tetrabutylammonium iodide (TBAI)[65]. Accordingly, a set of bis-3-chloropiperidines containing different linker structures was successfully synthesised (all compounds selected for investigation are summarised in Table 1; compare Chapters 2–4).



Scheme 1: General strategy for the bidirectional synthesis of nitrogen-linked bis-3-chloropiperidines (**D**). The target compounds **D** were obtained as inseparable diastereomeric mixtures. [a] Oil bath temperature.

Preliminary work in our group initiated with the design of a series of derivatives incorporating flexible linkers with alkyl chain lengths ranging from 3–6 methylene units between the alkylating moieties.[67] Additionally, we chose to investigate conformationally restricted and rigid linkers by introducing a bridging cyclohexyl as well as aromatic linker structures.[71] In continuation of our previous research (see Chapter 2) on the impact of flexible and rigid linkers on DNA alkylating activity, we wanted to explore the effect of different aromatic motifs attached to the carboxylic acid side chain of a L-lysine linkage. Thus, a series of lysine ester bridged bis-3-chloropiperidines was prepared, enabling comparative studies on the structure–activity relationship (see Chapter 3 as well as Table 1). The results of these studies are discussed below.

1.3.3 DNA Alkylation by 3-Chloropiperidines

Generally, the reaction of 3-chloropiperidines (**A**) with nucleophiles has been found to proceed through the formation of a bicyclic aziridinium ion intermediate (**B,** Figure 9).[72] We were able to verify the rapid generation of **B** by electrospray ionisation mass spectrometry (ESI-MS; see Chapter 3). This electrophilic species (**B**) can subsequently alkylate nucleophilic centers in DNA to give five- (**C**) and six-membered (**D**) ring products. (Figure 9; see also Chapter 2).[48] The identity of the resulting DNA alkylation products depend on the steric effects of the residue R and the attacking nucleophile.

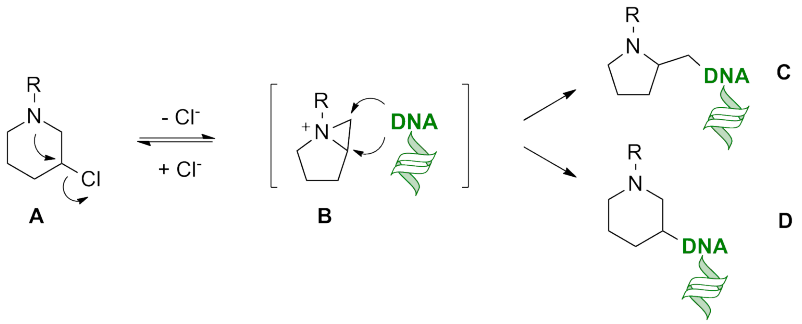


Figure 9: General alkylation mechanism of 3-chloropiperidines (**A**) via a bicyclic aziridinium ion intermediate (**B**). In a subsequent step, the electrophilic and highly reactive species (**B**) can be readily attacked by nucleophilic sites within the DNA double helix to form covalent DNA–pyrrolidine (**C**) and DNA–piperidine (**D**) adducts.

The main nucleophilic targets of alkylating agents are the purine and pyrimidine bases in the DNA molecule. Here, the preferred site of DNA attack depends on the accessibility of the reactive alkylating species to the DNA base in question and thus varies by agent and suitable chemical environment. As mentioned before, nitrogen mustards bind predominantly to the highly nucleophilic nitrogen atom on position 7 of the guanine ring system (see Section 1.1.2, Figure 3).[73] 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 destabilise the nucleobases, followed by deglycosylation and subsequent DNA strand scission (compare Figure 4).

1.3.4 DNA Cleavage Activity of Bis-3-chloropiperidines

In our studies on the reactivity of bis-3-chloropiperidines towards nucleic acids we analysed the DNA alkylating activities of the synthesised agents using gel electrophoresis techniques (compare Chapters 2–4). Consistent with our proposed mechanism of action for 3-chloropiperidines presented in the previous section, we could demonstrate that the examined compounds alkylate and cleave double-stranded DNA very efficiently. Furthermore, DNA-sequencing experiments identified guanine bases as the predominant site of alkylation by bis-3-chloropiperidines. These results are described in more detail below.

The ability of the synthesised compounds to cleave double-stranded DNA was studied in simple DNA plasmid system under physiological conditions (aqueous buffer at pH 7.4, 37 °C). Supercoiled DNA (pAT153 and pBR322, respectively) was incubated for 3 hours with increasing concentrations (0.5, 5, 50 μM) of appropriate agents. DNA strand cleavage can be detected by assessing the conversion from the naturally occurring supercoiled (SC) to the nicked open circular (OC) and linear form (L) of plasmid DNA by means of gel electrophoresis (Figure 10).[74]

These various DNA conformations migrate through the gel at different rates, depending on their size and charge as well as on the characteristics of the gel (i.e., concentration, gel type) and electrophoretic conditions (e.g., buffer system, applied voltage, duration of electrophoresis).[75] Generally, supercoiled DNA shows the highest electrophoretic mobility due to its compact conformation (compare band L, Figure 10B). If one DNA strand is cleaved (nicked), the supercoiled plasmid will relax into the open circular form, which can be monitored by the appearance of a slower migrating gel band (compare lane OC, Figure 10B). Double-strand cleavage will generate a linear DNA molecule that usually runs between the supercoiled and open circular forms (see Figure 10B, compare bands SC, L and OC).

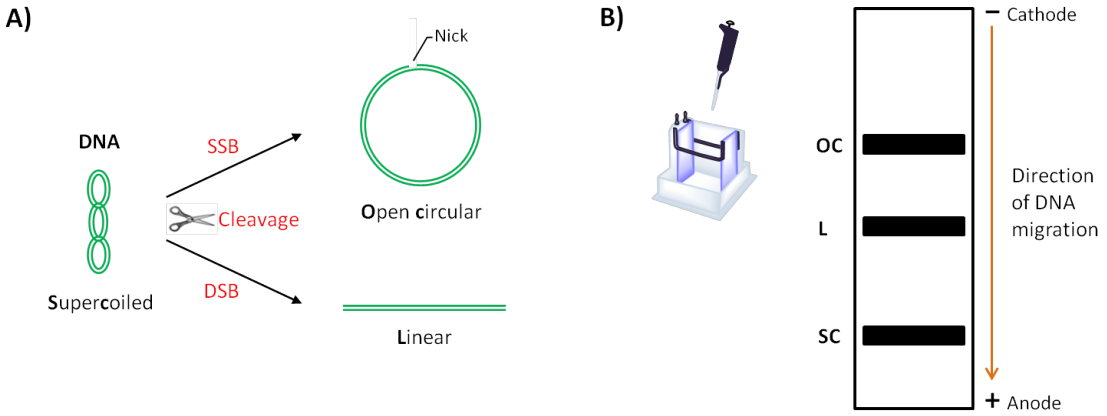


Figure 10: Schematics of plasmid DNA conformations. (A) DNA cleavage results in the conversion of supercoiled plasmid to the nicked open circular (single-strand break, SSB) or linear form (double-strand break, DSB). (B) Illustration of an electrophoresis gel showing the relative positions (in terms of relative migration rates) of the different plasmid DNA forms, visible as discrete bands. The typical order of electrophoretic mobility in agarose gels, from fastest to slowest, is as follows: supercoiled (SC) plasmid > linear (L) DNA > open circular (OC) form.

In an initial study[67], the agarose gel electrophoresis patterns for the cleavage of supercoiled plasmid DNA pAT153 by bis-3-chloropiperidines suggested the induction of double-strand breaks. Optimisation of the agarose concentration (1% agarose gel instead of 0.8%) and running buffer conditions (1 × Tris-borate-EDTA instead of 1 × Tris-phosphate-EDTA buffer) resulted in an improved separation of DNA fragments. Under these optimised electrophoretic conditions, the investigated compounds were found to nick supercoiled DNA to yield single-strand breaks, rather than cleavage of both strands. Significantly, the findings from our current studies reveal that bis-3-chloropiperidines exhibit effective DNA alkylating properties and promote guanine-specific cleavage through a DNA nicking mechanism (see Chapters 2–4).

In an attempt to estimate the relative cleavage activities of all bis-3-chloropiperidines tested, the effective concentrations causing 50% of the maximal DNA nicking (EC_{50}) were determined by measuring the ability of the agents to convert supercoiled DNA into its open circular form. Table 1 gives a summary of the approximate EC_{50} values (these data can be understood as visualization of results from the DNA cleavage assays; compare Chapters 2–4).

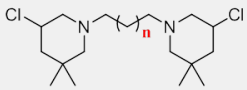
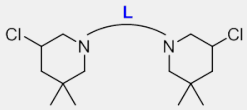
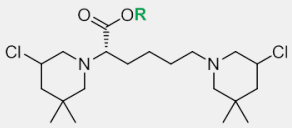
Scaffold	Linker/Substituent	EC_{50} [µM]
 *Flexible linker*	n = 1	< 1
	n = 2	< 1
	n = 3	< 1
	n = 4	< 1
 *Rigid linker*	L = 1,4-cyclohexane	1
	L = 1,4-xylene	2
	L = 1,3-xylene	1
	L = 3,5-xylyl-1-$COOCH_3$	8
 *Lysine ester linker*	R = methyl	3
	R = phenylmethyl	11
	R = phenylethyl	13
	R =phenylpropyl	> 50
	R = 1-naphthyl	> 50
	R = 1-naphthyl-4-OCH_3	> 50
	R = 2-methylanthraquinone	2
Chlorambucil		> 50

Table 1: Approximate half maximal effective concentration (EC_{50}) data for all investigated bis-3-chloropiperidines. EC_{50} values were calculated by comparing the intensity of supercoiled gel bands from the DNA cleavage assays (compare Chapters 2–4) with the value of DNA control lane as a function of compound concentration. Band intensities were quantified using GeneTools™ software from PerkinElmer®. [a] EC_{50} value was not determinable due to DNA fragmentation. [b] Unpublished data.[71] [c] Activity involves most likely DNA intercalation (see Chapter 4).

Our data indicate that the linker length has no apparent effect on DNA alkylating properties, whereas the flexibility of the linker region appears to be a key structural feature for bis-3-chloropiperidines with high activity (EC_{50} < 1 µM). The introduction of aromatic groups or an ester functionality into the linker structure, in general, leads to a decrease in DNA cleavage activity (compare Table 1).

Remarkably, although the naphthoate derivatives appear to have no apparent DNA alkylating capabilities (EC_{50} > 50 µM), our studies clearly show that these compounds interact with DNA (see Chapter 3). These results support our assumption that the incorporation of DNA-affinic moieties (such as naphthalene-type chromophores) can be a valuable concept for designing DNA-targeted agents.

Encouraged by these observations, a bis-3-chloropiperidine conjugate containing an anthraquinone pharmacophore was synthesised[76] and evaluated (compare Chapter 4). Based on the known DNA-binding characteristics of this anthracycline-derived structural feature (see Section 1.2.2.1), we hypothesised that the comparatively high DNA relaxation activity detected (EC_{50} = 2 μM) was likely due to intercalation of the anthraquinone component into the DNA double helix. Considerably, the tested bis-3-chloropiperidines have been shown to be significantly more reactive towards DNA than the antitumour drug chlorambucil (EC_{50} > 50 μM, see Table 1).

In order to identify the sites of DNA alkylation by bis-3-chloropiperidines, we used the technique of denaturing polyacrylamide gel electrophoresis (PAGE). This method permits a high-resolution separation of DNA fragments, based on their molecular weight, and is suited for nucleic acid sequence analysis.[77] Sequence-specific DNA alkylation and cleavage by the synthesised agents were explored (under physiological conditions at pH 7.4 and 37 °C) with a guanine-rich oligonucleotide (22-mer) duplex. The cleavage patterns obtained by denaturing PAGE revealed that the studied compounds alkylate and cleave DNA with a distinct preference for guanine residues. In fact, alkylation of DNA proceeds with a sequence selectivity similar to that observed for conventional nitrogen mustards (compare Section 1.1.2). In addition, the experimental findings imply that bis-3-chloropiperidines promote the formation of DNA adducts. The interaction of such chemically induced adducts with biological processes is associated with the therapeutic effectiveness of alkylating agents.

1.4 Summary and Future Directions

In the present research work, a series of nitrogen-linked bis-3-chloropiperidines was synthesised as structurally simplified analogues of the antitumour antibiotic 593A (compare Figure 8; all compounds discussed herein are summarised in Table 1). The ability of these nitrogen mustard-type compounds to alkylate DNA was studied with the aim to gain a better understanding of their molecular mechanism of action (see Chapters 2–4). Our approach was to survey the effect of various linker structures on DNA alkylation activity using electrophoretic methods for the detection of agent-induced DNA cleavage.

Thus, we have constructed a small library of bis-3-chloropiperidine derivatives with flexible alkyl, a conformationally constrained cyclohexyl, rigid aromatic or lysine ester linkers. The synthesis of these compounds was successfully carried out by means of an efficient bidirectional procedure developed in our laboratory. The key steps of the reaction sequence are the reductive amination of the respective diamine linker with 2,2-dimethylpent-4-enal, followed by chlorination and subsequent iodide-catalysed cyclisation of the unsaturated precursor to yield the corresponding desired bis-3-chloropiperidine (compare Scheme 1).

The synthesised compounds were evaluated in vitro for their alkylating properties towards double-stranded DNA (supercoiled plasmids pAT153 and pBR322 as well as a 22-mer oligonucleotide). For this purpose DNA cleavage assays were performed (under physiological conditions at pH 7.4 and 37 °C), allowing direct comparison of the relative efficiencies of DNA alkylation (see Table 1). The gel electrophoresis results demonstrated that the examined bis-3-chloropiperidines cleave DNA in a concentration-dependant manner (from 0.5 to 50 μM) via a DNA nicking mechanism, generating single-strand breaks.

Notably, we found that compounds containing flexible alkyl chain linkers exhibit significant DNA cleavage activities (EC_{50} < 1 μM), whereby chain length variation has no apparent influence. On the other hand, aromatic linkers are able to reduce the reactivity of bis-3-chloropiperidines with DNA, indicating that conformational flexibility of the linker region is an important structural feature for cleavage efficiency (compare Chapter 2). In addition, the introduction of an ester functionality to the side chain of the linker (i.e., xylyl ester, lysine ester) results in diminished activity, most likely depending on the size and nature of the ester. In general, activities decrease with increasing steric bulkiness of the ester unit (i.e., methyl group, simple phenyl derivatives, naphthalene and anthraquinone chromophores) in the lysine linker (see Chapters 3 and 4). An interesting exception to this observation is the anthraquinone conjugate that, despite its bulky shape, displays considerable activity (EC_{50} = 2 μM). Our data suggest a possible intercalative process in which the anthraquinone component inserts itself between the base pairs of the DNA double helix (compare Chapter 4). However, this mode of action remains to be clarified directly by appropriate intercalation experiments. Remarkably, although compounds with a naphthoate moiety appear to be inactive under the chosen incubation conditions (EC_{50} > 50 μM), we have ascertained that these agents are able to interact with double-stranded DNA (see Chapter 3). These findings indicate that such DNA-affinic chromophores can serve as DNA-recognizing structural elements that may contribute favourably to DNA-binding.

Furthermore, we analysed the alkylation sites of bis-3-chloropiperidines by DNA sequencing gel electrophoresis. Our results clearly demonstrated that DNA strand cleavage occurs primarily at guanine residues. Particularly striking was the detection of DNA adducts, which are currently being investigated using mass spectrometry (MS) techniques. Besides, we verified the formation of the reactive aziridinium ion intermediate by ESI-MS (see Chapter 3).

Taken together, these studies confirm our proposed mechanism of action (compare Section 1.3.3) and are consistent with the observed alkylation reactions of conventional nitrogen mustards. It is noteworthy that the examined bis-3-chloropiperidines proved to be more efficient DNA alkylating agents than the anticancer drug chlorambucil.

At present, studies are underway to evaluate the synthesised compounds for their in vitro cytotoxic effects on various human cancer cell lines (e.g., A375, HCT-15, Bx-PC3, 2008, MCF-7). Moreover, ongoing mechanistic investigations focus on identifying the alkylated positions in guanines as well as the characterisation (by means of MS) of the DNA alkylation products. In addition, we plan to further clarify the molecular mechanism underlying the observed interactions of the chromophore conjugates with double-stranded DNA. Due to our promising experimental results, future work on this project will be directed towards the design, synthesis and biological examination of novel bis-3-chloropiperidine derivatives attached to a DNA-affinic carrier molecule (e.g., intercalators, polypyrroles and polyimidazoles). This approach might provide a valuable strategy to enhance DNA-binding affinity and specificity of this new class of alkylating agents.

1.5 References

[1] M. B. Sporn, *Ann. NY. Acad. Sci.* **1997**, *833*, 137–146.

[2] T. Beardsley, *Sci. Am.* **1994**, *270*, 130–138.

[3] a) J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D. M. Parkin, D. Forman, F. Bray, *Int. J. Cancer* **2015**, *136*, E359–E386. b) R. Siegel, J. Ma, Z. Zou, A. Jemal, *CA-Cancer J. Clin.* **2014**, *64*, 9–29.

[4] J. Ferlay, I. Soerjomataram, M. Ervik, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. Lyon, France: International Agency for Research on Cancer, **2013**.

[5] L. B. Alexandrov, S. Nik-Zainal, D. C. Wedge, S. A. J. R. Aparicio, S. Behjati, A. V. Biankin, G. R. Bignell, N. Bolli, A. Borg, A.-L. Børresen-Dale et al., *Nature* **2013**, *500*, 415–421.

[6] a) T. Lindahl, D. Barnes, *Cold Spring Harbor Symposia on Quantitative Biology*, **2000**, *65*, 127–134. b) L. A. Mathews, S. M. Cabarcas, E. M. Hurt, *DNA repair of cancer stem cells*, Springer, Dordrecht, New York, **2013**.

[7] Sigma Aldrich®, *DNA Damage and Repair Mechanisms*, **2007**, can be found under: http://www.sigmaaldrich.com/technical-documents/articles/biofiles/dna-damage-and-repair.html.

[8] A. Skladanowski, P. Bozko, M. Sabisz, *Chem. Rev.* **2009**, *109*, 2951–2973.

[9] Z. H. Siddik in *The Cancer Handbook* (Ed.: M. R. Alison), John Wiley & Sons, Ltd, Chichester, UK, **2002**, 1295–1313.

[10] K. W. Kohn, *Cancer Res.* **1996**, *56*, 5533–5546.

[11] L. P. Bignold, *Anticancer Res.* **2006**, *26*, 1327–1336.

[12] E. B. Krumbhaar, H. D. Krumbhaar, *J. Med. Res.* **1919**, *40*, 497–509.

[13] G. P. Wheeler, *Cancer Res.* **1962**, *22*, 651–688.

[14] A. Gilman, F. S. Philips, *Science* **1946**, *103*, 409–436.

[15] R. B. Silverman, *The organic chemistry of drug design and drug action*, Elsevier Academic Press, Amsterdam, Boston, **2004**, 354 – 358.

[16] C. Golumbic, J. S. Fruton, M. A. Bergmann, *J. Org. Chem.* **1946**, *11*, 518–535.

[17] C. Avendaño, J. C. Menéndez, *Medicinal chemistry of anticancer drugs*, Elsevier, Amsterdam, Boston, **2008**, pp. 139.

[18] C. C. Price, *Ann. N. Y. Acad. Sci.* **1958**, *68*, 663–668.

[19] S. R. Rajski, R. M. Williams, *Chem. Rev.* **1998**, *98*, 2723–2796.

[20] P. Brookes, P. Lawley, *Biochem. J.* **1961**, *80*, 496–503.

[21] Kurt W. Kohn, Carlos L. Spears, Paul Doty.

[22] D. M. Noll, T. M. Mason, P. S. Miller, *Chem. Rev.* **2006**, *106*, 277–301.

[23] a) S. P. Jackson, *Carcinogenesis* **2002**, *23*, 687–696. b) L. Swift, R. Golsteyn, *IJMS* **2014**, *15*, 3403–3431.

[24] K. S. Gates, *Chem. Res. Toxicol.* **2009**, *22*, 1747–1760.

[25] K. S. Gates, T. Nooner, S. Dutta, *Chem. Res. Toxicol.* **2004**, *17*, 839–856; and references therein.

[26] L. Panasci, Z.-Y. Xu, V. Bello, R. Aloyz, *Anticancer Drugs* **2002**, *13*, 211–220.

[27] G. M. Cragg, D. J. Newman, *Pure Appl. Chem.* **2005**, *77*.

[28] D. A. Dias, S. Urban, U. Roessner, *Metabolites* **2012**, *2*, 303–336.

[29] D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2012**, *75*, 311–335.

[30] J. R. Hanson, *Natural products*. *The secondary metabolites*, Royal Society of Chemistry, Cambridge, **2003**.

[31] C. D. Amsler (Ed.) *Algal Chemical Ecology*, Springer Berlin Heidelberg, Berlin, Heidelberg, **2008**, 1–24.

[32] G. M. Cragg, P. G. Grothaus, D. J. Newman, *Chem. Rev.* **2009**, *109*, 3012–3043.
[33] M. C. Wani, H. L. Taylor, M. E. Wall, P. Coggon, A. T. McPhail, *J. Am. Chem. Soc.* **1971**, *93*, 2325–2327.
[34] G. M. Cragg, S. A. Schepartz, M. Suffness, M. R. Grever, *J. Nat. Prod.* **1993**, *56*, 1657–1668.
[35] G. M. Cragg, *Med. Res. Rev.* **1998**, *18*, 315–331.
[36] D. G. I. Kingston, *J. Nat. Prod.* **2011**, *74*, 496–511.
[37] D. G. I. Kingston, *J. Nat. Prod.* **2009**, *72*, 507–515.
[38] Q. Gao, L. Yang, Y. Zhu, *Curr. Comput. Aided Drug Des.* **2010**, *6*, 37–49.
[39] a) B. Marvania, P.-C. Lee, R. Chaniyara, H. Dong, S. Suman, R. Kakadiya, T.-C. Chou, T.-C. Lee, A. Shah, T.-L. Su, *Bioorg. Med. Chem.* **2011**, *19*, 1987–1998. b) L. F. Tietze, H. P. Bell, S. Chandrasekhar, *Angew. Chem. Int. Ed. Engl.* **2003**, *42*, 3996–4028. c) S. Fortin, G. Bérubé, *Expert Opin. Drug Discov.* **2013**, *8*, 1029–1047. d) S. Xu, L. Pei, C. Wang, Y.-K. Zhang, D. Li, H. Yao, X. Wu, Z.-S. Chen, Y. Sun, J. Xu, *ACS Med. Chem. Lett.* **2014**, *5*, 797–802. e) K. Pors, Z. Paniwnyk, K. C. Ruparelia, P. H. Teesdale-Spittle, J. A. Hartley, L. R. Kelland, L. H. Patterson, *J. Med. Chem* **2004**, *47*, 1856–1859.
[40] C. O. Gitterman, E. L. Rickes, D. E. Wolf, J. Madas, S. B. Zimmerman, T. H. Stoudt, T. C. Demny, *J. Antibiot.* **1970**, *23*, 305–310.
[41] B. Arison, J. Beck, *Tetrahedron* **1973**, *29*, 2743–2746.
[42] R. W. Brockman, S. C. Shaddix, M. Williams, R. F. Struck, *Cancer Treat. Rep.* **1976**, *60*, 1317–1324.
[43] a) F. M. Schabel, M. W. Trader, W. R. Laster, S. C. Shaddix, R. W. Brockman, *Cancer Treat. Rep.* **1976**, *60*, 1325–1333. b) G. P. Wheeler, V. H. Bono, B. J. Bowdon, D. J. Adamson, R. Wallace, *Cancer Treat. Rep.* **1976**, *60*, 1307–1316.
[44] T. Fukuyama, R. Frank, C. Jewell, *J. Am. Chem. Soc.* **1980**, *102*, 2122–2123.
[45] N. D. Henderson, S. M. Lacy, C. C. O'Hare, J. A. Hartley, S. McClean, L. P. Wakelin, L. R. Kelland, D. J. Robins, *Anticancer Drug Des.* **1998**, *13*, 749–768.
[46] M. A. Casely-Hayford, K. Pors, C. H. James, L. H. Patterson, J. A. Hartley, M. Searcey, *Org. Biomol. Chem.* **2005**, *3*, 3585.
[47] M. A. Casely-Hayford, K. Pors, L. H. Patterson, C. Gerner, S. Neidle, M. Searcey, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 653–656.
[48] K. Pors, S. D. Shnyder, P. H. Teesdale-Spittle, J. A. Hartley, M. Zloh, M. Searcey, L. H. Patterson, *J. Med. Chem.* **2006**, *49*, 7013–7023.
[49] a) W. A. Denny, *Frontiers in Medicinal Chemistry - Online* **2004**, *1*, 581–594. b) P. B. Dervan, B. S. Edelson, *Curr. Opin. Chem. Biol.* **2003**, *13*, 284–299.
[50] W. Tse, D. Boger, *Chem. Biol.* **2004**, *11*, 1607–1617.
[51] S. Neidle in *Principles of Nucleic Acid Structure*, Elsevier, **2008**.
[52] R. S. Coleman, C. H. Burk, A. Navarro, R. W. Brueggemeier, E. S. Diaz-Cruz, *Org. Lett*. **2002**, *4*, 3545–3548.
[53] a) M. J. Waring, *Nature* **1968**, *219*, 1320–1325. b) W. D. Wilson (Ed.) *Comprehensive Natural Products Chemistry*, Elsevier, **1999**.
[54] a) W. A. Denny, *Anticancer Drug Des.* **1989**, *4*, 241–263. b) R. Martínez, L. Chacón-García, *Curr. Med. Chem.* **2005**, *12*, 127–151.
[55] L. S. Lerman, *J. Mol. Biol.* **1961**, *3*, 18–30.
[56] a) M. Demeunynck, F. Charmantray, A. Martelli, *Curr. Pharm. Des.* **2001**, *7*, 1703–1724. b) B. Zhang, X. Li, B. Li, C. Gao, Y. Jiang, *Expert Opin. Ther. Pat.* **2014**, *24*, 647–664.
[57] G. Minotti, *Pharmacol. Rev*. **2004**, *56*, 185–229.
[58] C. Carvalho, R. X. Santos, S. Cardoso, S. Correia, P. J. Oliveira, M. S. Santos, P. I. Moreira, *Curr. Med. Chem.* **2009**, *16*, 3267–3285.

[59] a) D. A. Gewirtz, *Biochem. Pharmacol.* **1999**, *57*, 727–741. b) F. Z. Giovanni, L. Beretta in *Topics in Current Chemistry* (Ed.: K. Krohn), Springer Berlin Heidelberg, Berlin, Heidelberg, **2008**.
[60] A. Bodley, L. F. Liu, M. Israel, R. Seshadri, Y. Koseki, F. C. Giuliani, S. Kirschenbaum, R. Silber, M. Potmesil, *Cancer Res.* **1989**, *49*, 5969–5978.
[61] D. Nielsen, C. Maare, T. Skovsgaard, *Gen. Pharmacol.* **1996**, *27*, 251–255.
[62] D. Faulds, J. A. Balfour, P. Chrisp, H. D. Langtry, *Drugs* **1991**, *41*, 400–449.
[63] W. S. Dalton, A. E. Cress, D. S. Alberts, J. M. Trent, *Cancer Res.* **1988**, *48*, 1882–1888.
[64] a) T. A. Gourdie, K. K. Valu, G. L. Gravatt, T. J. Boritzki, B. C. Baguley, L. P. G. Wakelin, W. R. Wilson, P. D. Woodgate, W. A. Denny, *J. Med. Chem.* **1990**, *33*, 1177–1186. b) T. A. Gourdie, A. S. Prakash, L. P. G. Wakelin, P. D. Woodgate, W. A. Denny, *J. Med. Chem.* **1991**, *34*, 240–248.
[65] R. Göttlich, M. Noack, *Eur. J. Org. Chem*. **2002**, 3171–3178.
[66] a) J. Helaja, R. Göttlich, *Chem. Commun. (Camb.)* **2002**, 720–721. b) R. Göttlich, M. Noack, *Tetrahedron Lett.* **2001**, *42*, 7771–7774.
[67] R. Roesmann, *3-Chlorpiperidine als Reagenzien zur Spaltung von DNA*; Dissertation, Justus Liebig University, Giessen, **2008**.
[68] I. Zuravka, *Darstellung neuartiger Bis-3-chlorpiperidine als bifunktionelle Alkylanzien*; Diploma Thesis, Justus Liebig University, Giessen, **2009**.
[69] K. C. Brannock, *J. Am. Chem. Soc.* **1959**, *81*, 3379–3383.
[70] A. F. Abdel-Magid, K. G. Carson, B. D. Harris, C. A. Maryanoff, R. D. Shah, *J. Org. Chem.* **1996**, *61*, 3849–3862.
[71] With the collaboration of A. Francke, *Novel Synthesis of Bis-3-chloropiperidines with Rigid Aromatic Linkers as Bifunctional Alkylatings Agents*; Bachelor Thesis, Justus Liebig University, Giessen, **2010**.
[72] C. Hammer, S. Heller, J. Craig, *Tetrahedron* **1972**, *28*, 239–253; and references therein.
[73] W. Mattes, J. Hartley, K. Kohn, *Nucleic Acids Res.* **1986**, *14*, 2971–2987.
[74] P. Y. Lee, J. Costumbrado, C.-Y. Hsu, Y. H. Kim, *JoVE* **2012**.
[75] P. H. Johnson, L. I. Grossman, *Biochemistry* **1977**, *16*, 4217–4225.
[76] With the collaboration of I. Jülch, *Synthesis and Biochemical Assay of New 3-Chloropiperidines with DNA Intercalating Properties*; Bachelor Thesis, Justus Liebig University, **2011**.
[77] H. Summer, R. Grämer, P. Dröge, *JoVE* **2009**.

CHAPTER 2:

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

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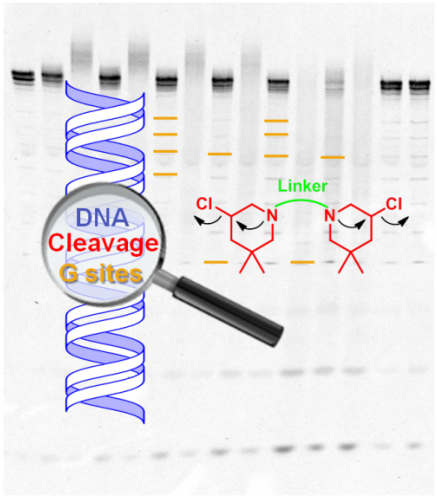
It's all about G's! An elegant and simple bidirectional synthetic approach to bis-3-chloropiperdines with different linker structures is reported. These new alkylating agents generate cleavage in double-stranded DNA, primarily through reactions towards guanine sites, via the formation of electrophilic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



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For Supporting Information see Appendix (pp. 55).

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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]

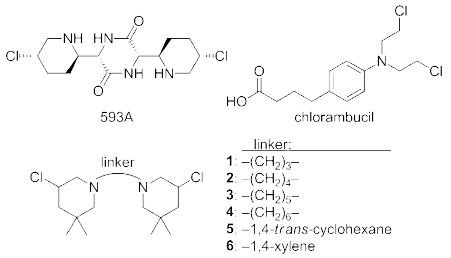

Figure 1. Structures of antibiotic 593A, the nitrogen mustard chlorambucil, and the synthesized bis-3-chloropiperidines as simplified analogues of 593A.

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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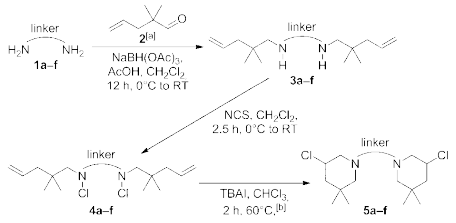
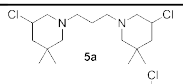
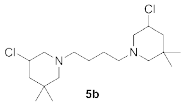
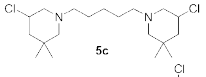
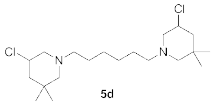
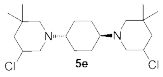
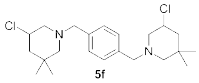
able structure, 593A has attracted considerable interest in synthetic organic and medicinal chemistry.[12] In 1979, Fukuyama et al. successfully achieved a challenging first total synthesis.[13] Studies of the biological effects of this compound suggest that the antibiotic acts as an alkylating agent 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).

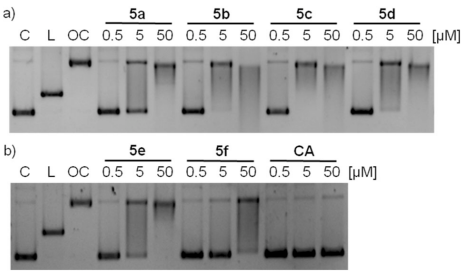
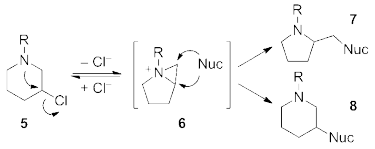


Figure 2. DNA cleavage activity of bis-3-chloropiperidine derivatives with a) flexible carbon chain linkers and b) restricted linkers. The supercoiled form of plasmid DNA pAT153 (120 ng) was incubated with the compounds at 37 °C for 3 h in BPE buffer, pH 7.4, at various concentrations (0.5, 5, 50 μM). 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]

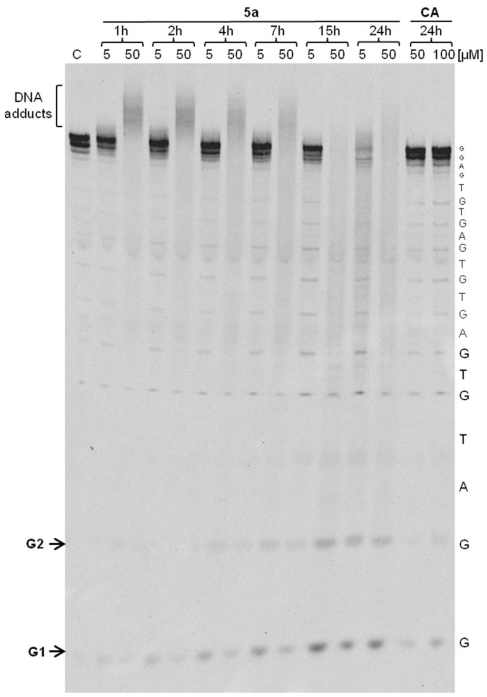


Figure 3. Representative denaturing polyacrylamide (20%) gel in 1× TBE, showing time- and concentration-dependent cleavage of a 22-mer double-stranded oligonucleotide caused via guanine alkylation by bis-3-chloropiperidine **5a**. The 5′-FAM-labeled scrambled duplex oligonucleotide (4 μM), GGA TGT GAG 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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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Keywords: antitumor agents · cyclization · DNA cleavage · nitrogen heterocycles · structure–activity relationships

[1] a) K. S. Gates, *Chem. Res. Toxicol.* **2009**, *22*, 1747–1760; b) N. Shrivastav, D. Li, J. M. Essigmann, *Carcinogenesis* **2010**, *31*, 59–70.
[2] a) L. H. Hurley, *Nat. Rev. Cancer* **2002**, *2*, 188–200; b) K. W. Kohn, *Cancer Res.* **1996**, *56*, 5533–5546.
[3] a) S. R. Rajski, R. M. Williams, *Chem. Rev.* **1998**, *98*, 2723–2796; b) Z. H. Siddik in *The Cancer Handbook* (Ed.: M. R. Alison), Wiley, Chichester, **2005**.
[4] a) D. M. Noll, T. M. Mason, P. S. Miller, *Chem. Rev.* **2006**, *106*, 277–301; b) E. W. Vogel, A. Barbin, M. J. Nivard, H. Stack, M. D. Waters, P. H. Lohman, *Mutat. Res. Fundam. Mol. Mech. Mutagen.* **1998**, *400*, 509–540.

[5] a) A. Gilman, F. S. Philips, *Science* **1946**, *103*, 409 – 436; b) C. C. Price, *Ann. N. Y. Acad. Sci.* **1958**, *68*, 663 – 668; c) G. P. Warwick, *Cancer Res.* **1963**, *23*, 1315 – 1333.
[6] a) W. Mattes, J. Hartley, K. Kohn, *Nucleic Acids Res.* **1986**, *14*, 2971 – 2987; b) P. Brookes, P. Lawley, *Biochem. J.* **1961**, *80*, 496 – 503; c) G. P. Wheeler, *Cancer Res.* **1962**, *22*, 651 – 688.
[7] a) Z. Rachid, F. Brahimi, Q. Qiu, C. Williams, J. M. Hartley, J. A. Hartley, B. J. Jean-Claude, *J. Med. Chem.* **2007**, *50*, 2605 – 2608; b) M. D. Urbaniak, J. P. Bingham, J. A. Hartley, D. N. Woolfson, S. Caddick, *J. Med. Chem.* **2004**, *47*, 4710 – 4715; c) P. G. Baraldi, I. Beria, P. Cozzi, C. Geroni, A. Espinosa, M. A. Gallo, A. Entrena, J. P. Bingham, J. A. Hartley, R. Romagnoli, *Bioorg. Med. Chem.* **2004**, *12*, 3911 – 3921; d) D. Verga, M. Nadai, F. Doria, C. Percivalle, M. Di Antonio, M. Palumbo, S. N. Richter, M. Freccero, *J. Am. Chem. Soc.* **2010**, *132*, 14625 – 14637; e) F. Doria, S. N. Richter, M. Nadai, S. Colloredo-Mels, M. Mella, M. Palumbo, M. Freccero, *J. Med. Chem.* **2007**, *50*, 6570 – 6579.
[8] a) K. Pors, S. D. Shnyder, P. H. Teesdale-Spittle, J. A. Hartley, M. Zloh, M. Searcey, L. H. Patterson, *J. Med. Chem.* **2006**, *49*, 7013 – 7023; b) M. A. Casely-Hayford, K. Pors, C. H. James, L. H. Patterson, J. A. Hartley, M. Searcey, *Org. Biomol. Chem.* **2005**, *3*, 3585; c) F. M. Anderson, C. C. O'Hare, J. A. Hartley, D. J. Robins, *Anticancer Drug Des.* **2000**, *15*, 119 – 126; d) N. D. Henderson, S. M. Lacy, C. C. O'Hare, J. A. Hartley, S. McClean, L. P. Wakelin, L. R. Kelland, D. J. Robins, *Anticancer Drug Des.* **1998**, *13*, 749 – 768.
[9] G. L. Patrick, J. Spencer in *An Introduction to Medicinal Chemistry*, Oxford University Press, New York, **2009**, pp. 234 – 237.
[10] C. O. Gltterman, E. L. Rickes, D. E. Wolf, J. Madas, S. B. Zimmerman, T. H. Stoudt, T. C. Demny, *J. Antibiot.* **1970**, *23*, 305 – 310.
[11] a) B. Arison, J. Beck, *Tetrahedron* **1973**, *29*, 2743 – 2746; b) G. R. Pettit, R. B. von Dreele, D. L. Herald, M. T. Edgar, H. B. Wood, *J. Am. Chem. Soc.* **1976**, *98*, 6742 – 6743.
[12] M. Slavik, *Recent Results Cancer Res.* **1978**, *63*, 282 – 287.
[13] T. Fukuyama, R. Frank, C. Jewell, *J. Am. Chem. Soc.* **1980**, *102*, 2122 – 2123.
[14] a) R. W. Brockman, S. C. Shaddix, M. Williams, R. F. Struck, *Cancer Treat. Rep.* **1976**, *60*, 1317 – 1324; b) G. P. Wheeler, V. H. Bono, B. J. Bowdon, D. J. Adamson, R. Wallace, *Cancer Treat. Rep.* **1976**, *60*, 1307 – 1316; c) F. M. Schabel Jr., M. W. Trader, W. R. Laster, S. C. Shaddix, R. W. Brockman, *Cancer Treat. Rep.* **1976**, *60*, 1325 – 1333.
[15] M. Noack, R. Göttlich, *Eur. J. Org. Chem.* **2002**, 3171 – 3178.
[16] A. F. Abdel-Magid, K. G. Carson, B. D. Harris, C. A. Maryanoff, R. D. Shah, *J. Org. Chem.* **1996**, *61*, 3849 – 3862.
[17] K. C. Brannock, *J. Am. Chem. Soc.* **1959**, *81*, 3379 – 3383.
[18] C. Hammer, S. Heller, J. Craig, *Tetrahedron* **1972**, *28*, 239 – 253, and references therein.
[19] a) C. E. Williamson, B. Witten, *Cancer Res.* **1967**, *27*, 33 – 38; b) A. Polavarapu, J. A. Stillabower, S. G. W. Stubblefield, W. M. Taylor, M.-H. Baik, *J. Org. Chem.* **2012**, *77*, 5914 – 5921.
[20] C. Avendaño, J. C. Menéndez, *Medicinal Chemistry of Anticancer Drugs*, Elsevier, Amsterdam, **2008**, pp. 141 – 145.

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CHAPTER 3:

Bis-3-chloropiperidines Containing Bridging Lysine Linkers: Influence of Side Chain Structure on DNA Alkylating Activity

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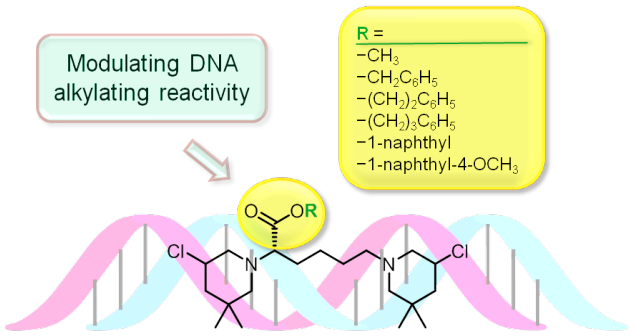
Abstract: A series of bis-3-chloropiperidines containing lysine linkers was synthesised as DNA alkylating model compounds by using a bidirectional synthetic strategy. These novel piperidine mustard based agents have been evaluated for their alkylating properties towards nucleic acids and were shown to 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.



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

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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]

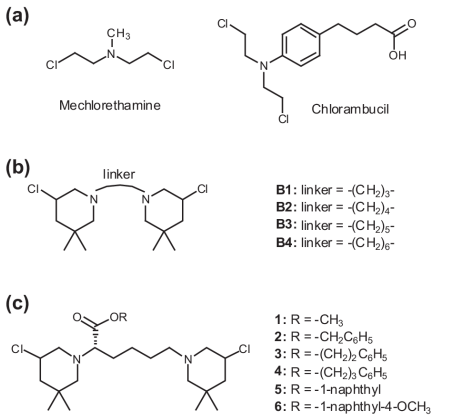

Figure 1. (a) Chemical structures of the nitrogen mustards mechlorethamine and chlorambucil. (b) Bis-3-chloropiperidines **B1**–**B4** investigated in a previous study.[14] (c) Series of newly synthesised compounds **1**–**6** with bridging lysine linkers.

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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

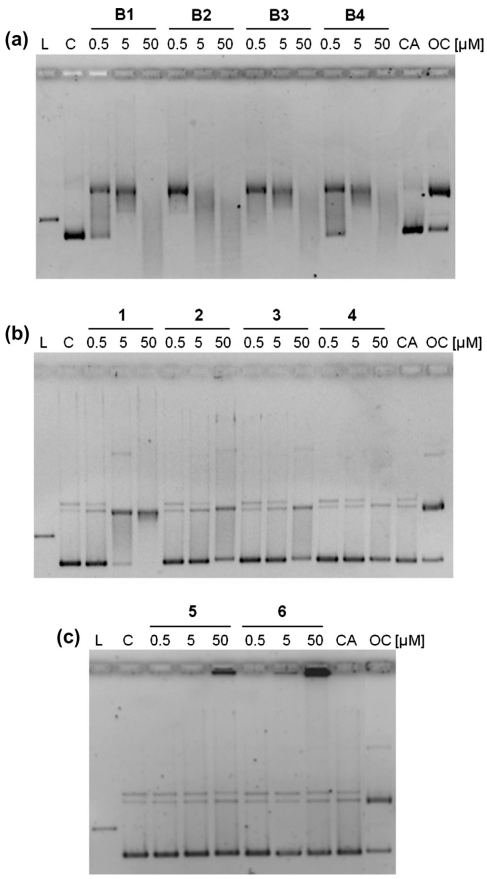
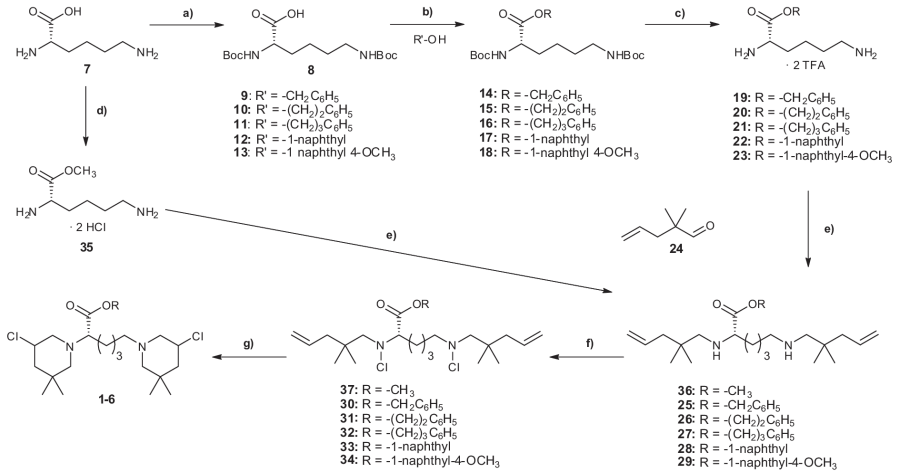


Figure 2. DNA cleavage activity of bis-3-chloropiperidines containing different flexible linker structures (a) increasing carbon chain length **B1**–**B4** (b) methyl and phenyl lysine esters **1**–**4** (c) naphthoate derivatives **5** and **6**. The supercoiled form of plasmid DNA pBR322 (3.5 nM) was 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.

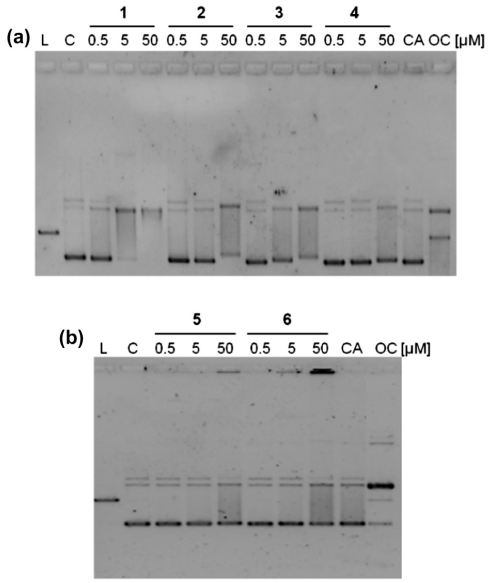


Figure 3. DNA cleavage activity of bis-3-chloropiperidines containing different lysine linker structures (a) methyl and phenyl lysine esters **1**–**4** (b) naphthoate derivatives **5** and **6**. The supercoiled form of plasmid DNA pBR322 (3.5 nM) was incubated with the compounds at ambient temperature 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.

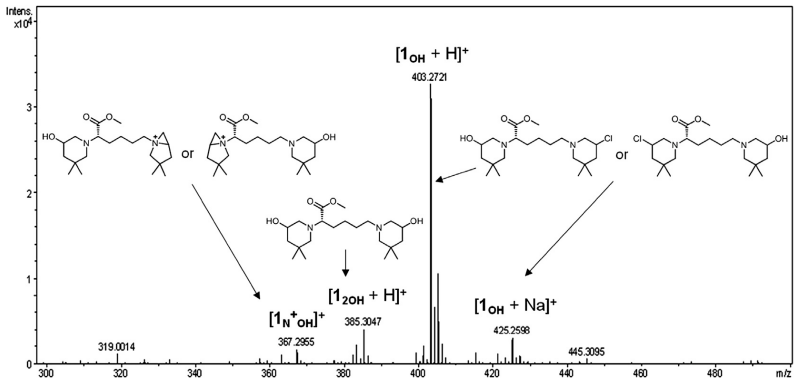


Figure 4. ESI-MS analysis of a sample taken after an incubation time of 15 min at 37 °C in a hydrolysis experiment of lysine methyl ester **1**. The ESI mass spectrum displays the hydroxyl substituted derivative ($\mathbf{1_{OH}}$ *m/z* 403 $[M+H]^+$ and *m/z* 425 $[M+Na]^+$) and the disubstituted hydrolysis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

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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.

CHAPTER 4:

Synthesis and Evaluation of a Bis-3-chloropiperidine Derivative Incorporating an Anthraquinone Pharmacophore

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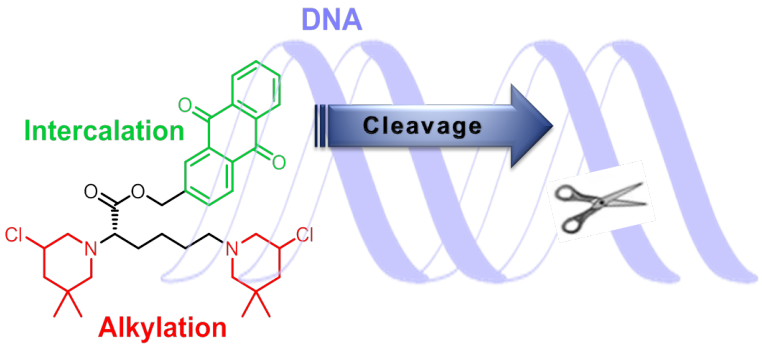
Combined attack on DNA: A novel bis-3-chloropiperidine tethered to an anthraquinone moiety was synthesised and investigated with regard to its reactivity toward double-stranded DNA. This conjugate has shown to alkylate and cleave DNA with a specificity for guanine bases. Our results suggest that besides DNA alkylation an intercalative interaction mode may contribute to the activity of the synthesised agent.



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Ivonne Zuravka, Alice Sosic, Barbara Gatto and Richard Göttlich: Synthesis and Evaluation of a Bis-3-chloropiperidine Derivative Incorporating an Anthraquinone Pharmacophore; **2015**, *submitted for publication*.

Synthesis and Evaluation of a Bis-3-chloropiperidine Derivative Incorporating an Anthraquinone Pharmacophore

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Abstract: With the aim to attain an alkylating agent with enhanced DNA-affinity, we have successfully synthesised lysine-linked bis-3-chloropiperidine **1** bearing a polycyclic aromatic moiety known to form stacking interactions with double-stranded DNA. The activity of conjugate **1** with plasmid DNA and with duplex oligonucleotide has been studied using gel electrophoretic analyses. The agent **1** was shown to alkylate and cleave DNA preferentially at guanine sites at a relatively low concentration (5 μM). In comparison, bis-3-chloropiperidines **B1a** and **B1b** carrying naphthalene chromophores proved to be less active than **1** in promoting DNA interactions and forming DNA adducts. Consistent with our expectations, the synthesised conjugate **1** appears to induce conformational changes of plasmid DNA suggesting intercalation-induced DNA unwinding. Overall, the efficiencies of DNA alkylation for tested compounds were considerably higher than those of the chemotherapeutic drug chlorambucil. Thus, the results of this work can provide a meaningful starting point for investigating the molecular mechanism of action of this novel DNA alkylating bis-3-chloropiperidine conjugate **1** with improved affinity for DNA.

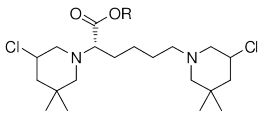
Keywords: alkylating agents • nitrogen mustard • anthraquinone • DNA cleavage • intercalation

Introduction

Alkylating and intercalating agents are important classes of DNA-interactive drugs that are used clinically in the field of chemotherapy.[1] The nitrogen mustard alkylators were among the earliest synthetic anticancer agents.[2] They react covalently with nucleophilic sites on DNA bases forming DNA adducts which contribute to their cytotoxic effects. On the other hand, the intercalators bind to DNA through non-covalent interaction by inserting their planar chromophore units between adjacent base pairs of the DNA duplex. The intercalation process causes conformational changes in DNA leading to the unwinding of the double helix.[3] In many instances, the DNA-binding abilities of intercalating drugs have been shown to correlate well to their respective biological properties.[4] However, several unpleasant side-effects and the emergence of drug resistance limit the clinical use of alkylating as well as intercalating agents.[5]

A valuable approach to improve affinity towards DNA and circumvent resistance mechanisms involves the design of DNA-directed alkylating agents by attaching the alkylators to DNA-affinic molecules.[6] Several lines of evidence have indicated that nitrogen mustards conjugated with intercalating carriers can exhibit up to 100-fold increase in potency and activity compared to the corresponding parent mustard.[7] In this context, the anthraquinone pharmacophore, which is present in a range of antitumour drugs such as the anthracycline antibiotic doxorubicin and its synthetic analogue mitoxantrone, can be an effective structural feature for the development of novel DNA-targeted agents.[8,9]

In our previous studies on the synthesis and biological evaluation of nitrogen-linked bis-3-chloropiperidines as DNA alkylating agents, we have demonstrated that these mustard-like compounds alkylate DNA very effectively.[10,11] The reaction with DNA proceeds through a bicyclic aziridinium ion intermediate, followed by attack of nucleophilic centers on DNA.[12] Our results revealed that the investigated alkylators were capable of inducing DNA strand cleavage preferentially at guanine sites, which is in accord with the observed alkylation patterns of conventional nitrogen mustards.[13] In addition, we have shown that the incorporation of a DNA-affinic naphthalene chromophore to the side chain carboxylate of lysine-bridged bis-3-chloropiperidines can provide favourable DNA interactions (compare compounds **B1a** and **B1b** in Figure 1).[11] These findings suggest that the attachment of a stacking moiety to the linker structure might reinforce the DNA recognition properties of these alkylators.



B1a: R = -1-naphthyl
B1b: R = -1-naphthyl-4-OCH_3
1: R = -2-methylanthraquinone

Figure 1. Chemical structures of lysine-bridged bis-3-chloropiperidines containing naphthalene chromophores (**B1a** and **B1b** were previously evaluated)[11] and of the presently investigated conjugate **1** incorporating an intercalating anthraquinone moiety.

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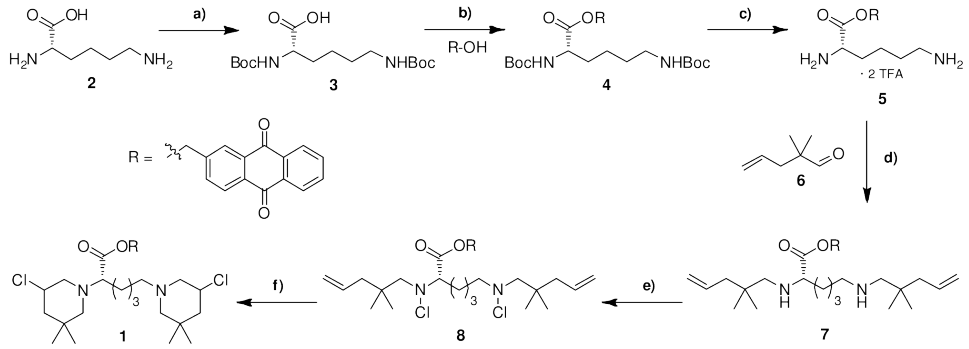

Scheme1. Synthesis of bis-3-chloropiperidine **1**. *Reactants and conditions*: (a) Boc_2O, 1 N NaOH, H_2O/Dioxan (1:1), rt, 12 h, 92%; (b) 2-(hydroxymethyl)-anthraquinone, acetonitrile, HOBt, Et_3N, dry CH_2Cl_2, rt, 23 h, 80%; (c) TFA, CH_2Cl_2, 0 °C to rt, 12 h, quant. (d) 2,2-dimethylpent-4-enal (**6**)[14] $NaBH(OAc)_3$, AcOH, dry CH_2Cl_2, 0 °C to rt, 12 h, 96%; (e) NCS, dry CH_2Cl_2, 0 °C to rt, 2.5 h, 53%; (f) TBAI (cat.), dry $CHCl_3$, 60 °C (oil bath temperature), 2 h, 49% (inseparable diastereomeric mixture)

Based on the above considerations, we reasoned that if an intercalator is linked to a bis-3-chloropiperidine compound it might be possible to design new DNA-directed bis-piperidine mustards. The DNA-intercalating ligand should serve as a carrier molecule to direct the alkylator to its biological target. It is expected that the carrier delivers the agent to DNA and locates the alkylating functionalities in the vicinity of DNA bases, which will presumably facilitate the formation of DNA adducts.[9] Accordingly, we have pioneered the synthesis of a lysine-bridged bis-3-chloropiperidine derivative (**1**) tethered to an anthraquinone intercalator (compare Figure 1). In the present paper we describe the synthetic preparation and results of investigations on DNA interactive properties by a novel anthraquinone-containing bis-3-chloropiperidine.

Results and Discussion

The synthesis of the DNA-targeted bis-3-chloropiperidine **1** was accomplished according to our previously reported method (Scheme 1).[11] The key steps in this bidirectional approach include reductive amination, followed by chlorination and iodide-catalysed cyclisation. Synthesis of the linker structure started with the bis-BOC protection of L-lysine **2** to furnish the pure compound **3** (yield 92%). As depicted in Scheme 1, the anthraquinone component can be covalently attached to the side chain of the lysine linker by esterification of **3** with 2-(hydroxymethyl)anthraquinone using dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt). This reaction proceeded smoothly to give the purified lysine ester **4** in 80% yield. After deprotection by treatment of **4** with trifluoroacetic acid (TFA), the resultant diamine linker **5** was successfully subjected to double reductive amination with 4-pentenal[14] **6** by means of sodium triacetoxyborohydride to generate compound **7** in 96% yield. Subsequently, the crude diamine **7** was chlorinated with *N*-chlorosuccinimide (NCS) to obtain the cyclisation precursor **8** in 53% isolated yield. Thus, **8** was readily converted into the title compound **1** in the presence of a catalytic amount of tetrabutylammonium iodide (TBAI). The desired bis-3-chloropiperidine **1** was isolated as an inseparable mixture of diastereomers in 49% yield upon purification by column chromatography.

With conjugate **1** in hand, we set about exploring its reactivity towards DNA. Alkylation activity was evaluated by a agarose gel electrophoresis assay using the supercoiled plasmid pBR322. In our studies, chlorambucil (CA), a chemotherapy drug of the nitrogen mustard type, was employed as a control. DNA cleavage experiments were carried out by incubating the supercoiled form of pBR322 with increasing concentrations (0.5, 5, 50 µM) of title compound **1** at 37 °C for 3 h in bisphosphate-EDTA (BPE) buffer at pH 7.4. Figure 2 A displays the agarose gel electrophoretic separation of the DNA reaction products. A gradual conversion of supercoiled plasmid into its open circular form can be observed in a concentration-dependent manner (0.5 µM to 5 µM, Figure 2 A) upon treatment of pBR322 with bis-3-chloropiperidine **1**. The generation of open circular DNA is indicative of single-strand breaks associated with base alkylation. Interestingly, the highest concentration of **1** tested (50 µM) resulted in retarded migration of the open circular plasmid band (Figure 2 A). The formation of this new slower migrating band may be ascribed to the intercalation of the anthraquinone-conjugate **1** into the DNA. Similar types of changes in electrophoretic mobility of plasmid DNA have been reported for other intercalating compounds.[15] In general, intercalation can lead to local DNA unwinding at the binding site contributing to a conformational distortion and lengthening of the DNA double helix. Such structural alterations in the DNA geometry affect the mobility of plasmid DNA in agarose gel matrix causing more slowly migrating bands.[16] Taken together, our findings indicate that bis-3-chloropiperidine **1** is capable of both alkylation and local intercalation to undergo nicking and unwinding reactions in double-stranded DNA.

In our preceding studies it has been shown that the preferential alkylation sites of bis-3-chloropiperidines are

guanine residues.[10,11] To examine whether the DNA alkylation pattern of the newly synthesized anthraquinone conjugate **1** is consistent, we next sought to perform a comparative analysis based upon our previously published experiment using a G-rich 22-mer oligonucleotide duplex.[10,11] Thus, recently investigated DNA-affinic bis-3-chloropiperidines[11] **B1a** and **B1b** were chosen for direct comparison in this study, since these compounds displayed potential DNA-interacting properties. Accordingly, the 5'-FAM-labelled double-stranded oligonucleotide was treated with agents **B1a**, **B1b** and **1** at 37 °C for 24 h in BPE buffer at two distinct concentrations (5 and 50 µM). After incubation samples were subjected to denaturing polyacrylamide sequencing gel electrophoresis.

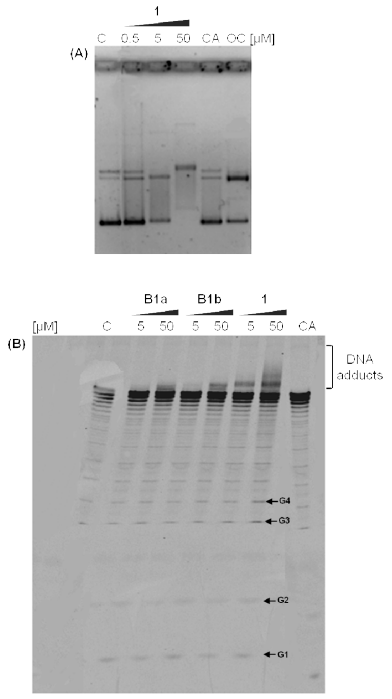

Figure 2. Representative electrophoresis gels displaying the extent of DNA alkylation by bis-3-chloropiperidine 1. Chlorambucil (CA, 50 µM)) was used as control substance. (A) DNA cleavage assay: The supercoiled form of plasmid DNA pBR322 (3.5 nM) was incubated with **1** at 37 °C for 3 h in BPE buffer, pH 7.4, at increasing concentrations (0.5, 5, 50 µM). Cleavage of DNA was analysed by agarose (1%) gel electrophoresis in 1 × TAE. C = supercoiled DNA control; OC = open circular DNA control. (B) Sequencing gel analysis for direct comparison in a sequencing-based analysis with **B1a** and **B1b**: Denaturing polyacrylamide (20%) gel in 1 × TBE showing the cleavage pattern of a 22-mer double-stranded oligonucleotide at guanine residues as well as formation of DNA adducts after exposure to compounds **B1a**, **B1b** and **1**. The 5'-FAM labelled scrambled duplex oligonucleotide (4 µM) GGA TGT GAG TGT GAG TGT GAG G was reacted with compounds at 37 °C for 24 h in BPE buffer, pH 7.4, at 5 and 50 µM. C = untreated duplex oligonucleotide control. Arrows correspond to exemplary cleavage bands at guanine residues.

Figure 2 B presents the obtained DNA fragments derived by cleavage reactions induced by tested compounds. Comparison of the cleavage patterns revealed that the bis-3-chloropipidines interacted with the double-stranded oligonucleotide in a site specific and concentration-dependent manner (compare Figure 2 B). In accordance with our earlier studies[10,11], guanine residues are to be the most predominant sites of alkylation (see cleavage bands at guanines residues G1 to G4 in Figure 2 B). This is particularly evident for compound **1**.

At a concentration of 50 µM the formation of new bands with an electrophoretic mobility less than the control band (lane C, Figure 2 B) can be observed for the highest concentrations of naphthalene compounds **B1a** and **B1b**. These relatively broad bands in the upper part of the gel presumably correspond to DNA adducts produced by the investigated agents (**B1a** and **B1b**, DNA adducts, Figure 2 B). This phenomenon of decreased electrophoretic DNA migration was more apparent for the anthraquinone conjugate **1**. Significantly, up-shifted gel bands probably representing DNA adducts have been detected for **1** at the lower agent concentration (5 µM) used in this experiment (compare Figure 2 B). Their intensity increased considerably at the higher concentration (50 µM) of compound **1** and a visible background smearing was observed, probably due to partial DNA degradation of the higher molecular weight adducts. These results suggest that bis-3-chloropiperidine **1** containing an anthraquinone moiety exerts higher potency and enhanced DNA binding activity toward the duplex oligonucleotide under the employed conditions compared to the naphthalene counterparts **B1a** and **B1b**. The strong interactions of **1** with DNA might be attributed to the intercalation ability of the anthraquinone unit. The obtained findings confirm the results and our conclusions drawn from the DNA cleavage assay (Figure 2 A). In agreement with our recent studies[10,11], the control compound chlorambucil (CA) was found to be less active than the tested bis-3-chloropiperidines (compare Figure 2 A and B).

In summary, we have presented the synthesis and evaluation of the DNA-alkylating properties of a novel bis-3-chloropiperidine conjugate **1** incorporating an intercalating anthraquinone pharmacophore. In keeping with our earlier work[10,11], the current electrophoretic studies reveal that the mustard type derivative **1** exhibits DNA cleavage activity on both supercoiled plasmid and duplex oligonucelotide primarily through reactions at guanine sites. Comparative examination of the effects of DNA-affinic groups attached to the bis-3-chloropiperidine molecule demonstrated that the anthraquinone conjugate **1** is a distinctively more potent DNA-interacting agents than the corresponding naphthalene derivatives **B1a** and **B1b**, apparently on account of its intercalative nature. Remarkably, our findings indicate that compound **1** is capable to induce structural changes in DNA, leading to the electrophoretic retardation of the double-stranded DNA helix. These observations support our hypothesis that **1** binds to DNA via an intercalative mode. However, this mode of action needs to be confirmed by a direct DNA intercalation assay in future investigations. Consequently, it appears to be worthwhile to explore further the potential of bis-3-chloropiperidine **1** as a DNA-targeted conjugate with alkylating and intercalating functionalities.

Experimental Section

Chemistry

General: 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). ^{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) and Bruker Avance II 400 spectrometer (^{1}H at 400 MHz; ^{13}C at 100 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.

2,2-Dimethylpent-4-enal (6): 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.[14]

***N*^{2},*N*^{6}-Bis(*tert*-butoxycarbonyl)-L-lysine (3):** 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).

(*S*)-(Anthraquinon-2-yl)methyl 2,6-bis((*tert*-butoxycarbonyl)-amino)-hexanoate (4): *N*$^{\varepsilon}$,*N*$^{\varepsilon}$-Bis(*tert*-butoxycarbonyl)-l-lysine **(3)** (146 mg, 0.42 mmol) was dissolved in acetonitrile (10 mL). Triethylamine (59 µL, 0.42 mmol, 2 equiv.) was added, followed by 2-(hydroxymethyl)anthraquinone (50 mg, 0.21 mmol). The mixture was stirred for a couple of minutes under nitrogen atmosphere and then cooled to 0 °C. Hydroxybenzotriazole (HOBt, 71 mg, 0.53 mmol, 2.5 equiv.) and dicyclohexyl carbodiimide (DCC, 109 mg, 0.53 mmol, 2.5 equiv.) were added simultaneously. The reaction mixture was stirred at room temperature for 23 h. 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) to yield **4** (95 mg, 0.17 mmol, 80%) as a yellow solid. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 8.28–8.34 (m, 3H), 8.22–8.26 (m, 1H), 7.79–7.85 (m, 2H), 7.75–7.77 (m, 1H), 5.25–5.40 (m, 2H) 5.05–5.18 (m, 1H), 4.62 (br s, 1H), 4.31–4.42 (m, 1H), 3.03–3.17 (m, 2H), 1.48–1.96 (overlapping signals, 4H), 1.44 and 1.42 (2 × s, total 18H), 1.10–1.29 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 182.7 (2 × C), 172.5, 156.1, 155.4, 141.9, 134.2 (2 × C), 133.6, 133.4, 133.1, 133.0, 127.8, 127.3 (2 × C), 126.1, 80.0, 79.1, 65.7, 63.4, 49.1, 39.9, 33.9, 32.0, 29.6, 28.4, 28.3, 25.6, 24.9, 22.5 ppm; HRMS (ESI): *m*/*z* calcd for $C_{31}H_{38}N_2NaO_8^+$: 589.2520; found: 589.2514 $[M+Na]^+$.

(*S*)-(Anthraquinon-2-yl)methyl 2,6-diaminohexanoate TFA salt (5): (*S*)-2,6-Bis(*tert*-butoxycarbonyl)-lysine ester **4** (88 mg, 0.16 mmol) was dissolved in dichloromethane (20 mL) and trifluoroacetic acid (4 mL) 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 **5** in quantitative yield. ^{1}H NMR (DMSO-d_6, 400.1 MHz): δ = 8.18–8.28 (m, 4H), 7.90–7.95 (m, 3H), 5.38–5.54 (m, 2H), 4.09–4.22 (m, 1H), 2.70–2.84 (m, 2H), 1.77–1.93 (m, 2H) 1.55–1.64 (m, 2H), 1.29–1.50 (m, 2H) ppm; ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ = 182.3, 182.1, 169.3, 142.0, 134.7, 134.6, 133.5, 133.1, 133.0, 132.7, 127.2, 126.8 (2 × C), 125.8, 66.1, 51.7, 38.3, 29.5, 26.4, 21.3; TFA salt was not observable in the spectrum; HRMS (ESI): *m*/*z* calcd for $C_{21}H_{23}N_2O_4^+$: 367.1652; found: 367.1655 $[M+H]^+$.

(*S*)-(Anthraquinon-2-yl)methyl 2,6-bis(2,2-dimethylpent-4-enyl-amino) -hexanoate (7): To a solution of the unsaturated aldehyde **6** (92 mg, 0.82 mmol, 2 equiv.) and TFA salt **5** (243 mg, 0.41 mmol) in anhydrous dichloromethane (20 mL) was added sodium triacetoxyborohydride (260 mg, 1.23 mmol, 3 equiv.) portion wise at 0 °C, followed by acetic acid (47 µL, 0.82 mmol, 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 diamine **7** (219 mg, 0.39 mmol, 96%), which was used for the next step without further purification. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 8.29–8.33 (m, 3H), 8.27–8.29 (m, 1H), 7.78–7.83 (m, 2H), 7.75–7.77 (m, 1H), 5.72–5.83 (m, 2H), 5.26–5.34 (m, 2H), 4.98–5.00 (m, 2H), 4.95–4.97 (m, 2H), 3.22–3.26 (m, 1H), 2.52–2.56 (m, 2H) 2.41 (d, J = 11.5 Hz, 1H), 2.31 (s, 1H), 2.14 (d, J = 11.5 Hz, 1H), 1.96–1.99 (m, 4H), 1.34–1.72 (several overlapping signals, 9H), 0.85 and 0.84 (2 × s, total 12 H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 182.8, 182.7, 175.6, 142.7, 135.5 (2 × C), 134.3, 134.2, 133.7, 133.4, 133.1, 133.0, 132.8, 127.7, 127.3, 126.2, 116.8, 116.7, 71.7, 65.1, 62.5, 58.3, 50.6, 44.7, 44.4, 43.4, 34.5, 34.2, 33.9, 25.6, 25.5, 25.3, 25.2, 23.8, 23.7 ppm; HRMS (ESI): *m*/*z* calcd for $C_{35}H_{47}N_2O_4^+$: 559.3530; found: 559.3531 $[M+H]^+$.

(*S*)-(Anthraquinon-2-yl)methyl 2,6-bis(*N*-chloro-*N*-(2,2-dimethyl-pent-4-enyl)amino)-hexanoate (8): *N*-Chlorosuccinimide (163 mg, 1.22 mmol, 2.2 equiv.) was added to a cooled (0 °C) solution of diamine **7** (310 mg, 0.56 mmol) in anhydrous dichloromethane (10 mL). 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 desired product **8** (185 mg, 0.30 mmol, 53%) was isolated from the residue by flash chromatography (pentane/TBME 10:1) as a yellow solid. ^{1}H NMR ($CDCl_3$, 400.1 MHz): δ = 8.29–8.35 (overlapping signals, 4H), 7.79–7.84 (m, 3H), 5.73–5.84 (m, 2H), 5.32–5.41 (m, 2H), 4.99–5.03 (m, 3H), 4.97–4.98 (m, 1H), 3.61 (t, J = 7.0 Hz, 1H), 3.16 (d, J = 15.0 Hz, 1H), 2.91–2.96 (m, 2H), 2.38 (s, 1H), 2.02–2.05 (m, 3H), 1.87–1.93 (m,

2H), 1.54–1.73 (several overlapping signals, 7H), 0.92, 0.93 and 0.94 (3 × s, total 12H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 182.8, 182.7, 175.6, 142.3, 135.2, 135.1, 134.3, 134.2, 133.70, 133.4, 133.3, 133.2, 133.1, 127.8, 127.3 (2 × C), 126.3, 117.4, 117.3, 74.8, 73.3, 72.4, 66.4, 64.4, 44.8, 44.6, 35.9, 35.6, 30.4, 27.7, 25.7, 25.5, 25.4, 23.3 ppm.

(2*S*)-(Anthraquinon-2-yl)methyl 2,6-bis(5-chloro-3,3-dimethyl-piperidin-1-yl)-hexanoate: The bis-*N*-chloroamine **1** (185 mg, 0.30 mmol) was dissolved in anhydrous chloroform (10 mL) and tetrabutylammonium iodide (11 mg, 0.03 mmol, 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 **1** (91 mg, 0.15 mmol, 49%, mixture of inseparable diastereomers) was obtained as a yellow oil. ^{1}H NMR ($CDCl_3$, 400.1 MHz) mixture of diastereomers: 8.30–8.33 (m, 3H), 8.29–8.30 (m, 1H), 7.80–7.84 (m, 2H), 7.75–7.79 (m, 1H), 5.25–5.35 (m, 2H), 3.25–3.33 (m, 1H), 3.18–3.24 (m, 1H), 3.08–3.17 (m, 1H), 2.27–2.50 (overlapping signals, 5H), 1.89–1.96 (m, 3H), 1.70–1.79 (m, 2H), 1.60–1.69 (m, 2H), 1.39–1.49 (m, 4H), 1.27–1.36 (m, 4H), 1.00 and 1.01 (2 × s, total 6H), 0.90 (2 × s, total 6H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, selected signals) major isomer: δ = 182.8, 182.7, 171.9, 142.6, 134.3, 134.2, 133.7, 133.4, 133.1, 127.7, 127.3, 126.3, 126.4, 66.7, 64.8, 64.0, 61.3, 57.7, 55.0, 54.3, 48.5, 33.7, 33.3, 33.2, 29.4, 29.1,29.0, 28.9, 25.2, 24.9, 23.9, 23.8 ppm; HRMS (ESI): *m/z* calcd for $C_{35}H_{45}Cl_2N_2O_4^+$: 627.2751; found: 627.2708 $[M+H]^+$.

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 $NaH_2PO_4{\cdot}2\ H_2O$, 6 mM $Na_2HPO_4{\cdot}12\ H_2O$, 1 mM $Na_2EDTA{\cdot}2\ H_2O$, 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.

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. In order to obtain the open circular form of pBR322, 250 ng of supercoiled plasmid was incubated on ice for 5 minutes 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).

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).

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References

[1] a) L. H. Hurley, *Nat. Rev. Cancer,* **2002**, *2*, 188–200. b) R. Martínez, L. Chacón-García, *Curr. Med. Chem.* **2005**, *12*, 127–151.
[2] L. P. Bignold, *Anticancer Res.*, **2006**, *26*, 1327–1336.
[3] M. J. Waring, *Nature*, **1968**, *219*, 1320–1325.
[4] S. Neidle in *Principles of Nucleic Acid Structure*, Elsevier, **2008**.
[5] a) L. Panasci, Z.-Y. Xu, V. Bello, R. Aloyz, *Anticancer Drugs,* **2002**, *13*, 211–220. b) C. Carvalho, R. X. Santos, S. Cardoso, S. Correia, P. J. Oliveira, M. S. Santos, P. I. Moreira, *Curr. Med. Chem.,* **2009**, *16*, 3267–3285.
[6] a) K. W. Kohn, J. A. Hartley, W. B. Mattes, *Nucleic Acids Res.,* **1987**, *15*, 10531–10549. b) W. A. Denny, *Anticancer Drug Des.,* **1989**, *4*, 241–263.
[7] T. A. Gourdie, K. K. Valu, G. L. Gravatt, T. J. Boritzki, B. C. Baguley, L. P. G. Wakelin, W. R. Wilson, P. D. Woodgate, W. A. Denny, *J. Med. Chem.,* **1990**, *33*, 1177–1186.
[8] a) J. William Lown, *Pharmacology & Therapeutics,* **1993**, *60*, 185–214. b) K. Pors, S. D. Shnyder, P. H. Teesdale-Spittle, J. A. Hartley, M. Zloh, M. Searcey, L. H. Patterson, *J. Med. Chem.,* **2006**, *49*, 7013–7023. c) K. Pors, Z. Paniwnyk, P. Teesdale-Spittle, J. A. Plumb, E. Willmore, C. A. Austin, L. H. Patterson, *Mol. Cancer Ther.,* **2003**, *2*, 607–610. d) D. Faulds, J. A. Balfour, P. Chrisp, H. D. Langtry, *Drugs,* **1991**, *41*, 400–449.
[9] D. Gibson, I. Binyamin, M. Haj, I. Ringel, A. Ramu, J. Katzhendler, *Eur. J. Med. Chem.,* **1997**, *32*, 823–831.
[10] I. Zuravka, R. Roesmann, A. Sosic, W. Wende, A. Pingoud, B. Gatto, R. Göttlich, *ChemMedChem,* **2014**, *9*, 2178–2185.
[11] I. Zuravka, R. Roesmann, A. Sosic, R. Göttlich, B. Gatto, *Bioorg. Med. Chem.,* **2015**, *23*, 1241–1250.
[12] C. Hammer, S. Heller, J. Craig, *Tetrahedron,* **1972**, *28*, 239–253.
[13] a) W. Mattes, J. Hartley, K. Kohn, *Nucleic Acids Res.,* **1986**, *14*, 2971–2987. b) A. Polavarapu, J. A. Stillabower, S. G. W. Stubblefield, W. M. Taylor, M.-H. Baik, *J. Org. Chem.,* **2012**, *77*, 5014–5021.

[14] K. C. Brannock, *J. Am. Chem. Soc.,* **1959**, *81*, 3379–3383.
[15] a) T. A. Gourdie, A. S. Prakash, L. P. G. Wakelin, P. D. Woodgate, W. A. Denny, *J. Med. Chem.,* **1991**, *34*, 240–248. b) D. L. Boger, M. W. Ledeboer, M. Kume, M. Searcey, Q. Jin, *J. Am. Chem. Soc.,* **1999**, *121*, 11375–11383.
[16] a) N. Osheroff, *DNA topology and enzymes*, Humana Press, Totowa, NJ, **1999**, *Vol. 1.* (in particular, chapter 2 and 3). b) A. Gianoncelli, S. Basili, M. Scalabrin, A. Sosic, S. Moro, G. Zagotto, M. Palumbo, N. Gresh, B. Gatto, *ChemMedChem.,* **2010**, *5*, 1080–1091.

APPENDIX

Abbreviations

aq.	Aqueous
BPE	Bisphosphate-EDTA buffer
C	Control
CA	Chlorambucil
cat.	Catalytic
d	Doublet
DCC	*N*,*N*'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia – for example
equiv.	Equivalent
ESI-MS	Electrospray ionisation mass spectrometry
FAM	Carboxyfluorescein
G	Guanine
h	Hour(s)
HOBt	Hydroxybenzotriazole
HRMS	High-resolution mass spectrometry
i.e.	Id est – that is
L	Linear DNA
M	Molar
m	Multiplet
NCS	*N*-Chlorosuccinimide
NMR	Nuclear magnetic resonance spectroscopy

OC	Open circular DNA
PAGE	Polyacrylamide gel electrophoresis
ppm	parts per million
RT	Room temperature
s	Singlet
satd.	Saturated
SC	Supercoiled DNA
SSB	Single-strand break
t	Triplet
TAE	Tris-acetate-EDTA buffer
TBAI	Tetrabutylammonium iodide
TBE	Tris-borate-EDTA buffer
TBME	*tert*-Butyl methyl ether
TFA	Trifluoroacetic acid
TMS	Tetramethylsilane
Tris	Tris(hydroxymethyl)aminomethane

Statement of Contribution

The following is a description of my contribution to the publications included in this thesis:

Chapter 2: Resynthesised the compounds for biological evaluation;
Performed a major part of the bioassays;
Drafted and wrote the manuscript.

Chapter 3: Performed the synthetic work and chemical characterisation;
Conducted the biological and ESI mass spectrometry analyses;
Drafted and wrote the manuscript.

Chapter 4: Contributed to the experimental work and interpretation of data;
Performed the biological studies;
Drafted and wrote the manuscript.

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Supporting Information

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

Ivonne Zuravka,[a, b] Rolf Roesmann,[a] Alice Sosic,[b] Wolfgang Wende,[c] Alfred Pingoud,[c] Barbara Gatto,*[b] and Richard Göttlich*[a]

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Table of contents:

General remarks:

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), 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 were determined by reference to the residual solvent resonances.

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 from Eppendorf (Hamburg, Germany) and Acrylamide-bis ready-to-use solution (40%, 19:1) from Merck (Darmstadt, Germany). Dilutions of bis-3-chloropiperidines and chlorambucil have been freshly prepared from a DMSO stock solution (8 mM) in water. Alkylation reactions were carried out in BPE buffer (2 mM NaH_2PO_4*2 H_2O, 6 mM Na_2HPO_4*12 H_2O, 1 mM Na_2EDTA*2 H_2O, pH 7.4).

Cleavage of pBR322 plasmid by 5a:

DNA cleavage assay was performed by incubating 120 ng of pBR322 plasmid with increasing concentrations (0.5, 5, 50 µM) of agent for 1, 6, 15 and 24 h at 37 °C in BPE buffer. The linearized standard was obtained by EcoRI (Fermentas) digestion, according to the supplier's instruction. In order to obtain the open circular form of pBR322, 250 ng of supercoiled plasmid was incubated on ice for 5 minutes with 0.001 U/µL RQ1 DNase RNase-free (Promega) in RQ1 buffer 1X in a total reaction volume of 10 µL. Nicking was stopped adding EGTA (ethylene glycol tetraacetic acid) 20 mM, pH 8.0. To all reaction tubes gel loading buffer (10 mM Tris-HCl, 50% glycerol, 0.025% bromophenol blue) was added and the samples were loaded on a 1% agarose gel. Electrophoresis was conducted in TBE 1X (Tris-HCl 89 mM, borate 89 mM, EDTA 2 mM). The DNA in the gel system were detected by staining with ethidium bromide (0.5 µg/mL) for 30 min and visualised by Geliance 600 Imaging System (PerkinElmer, Waltham, MA, USA).

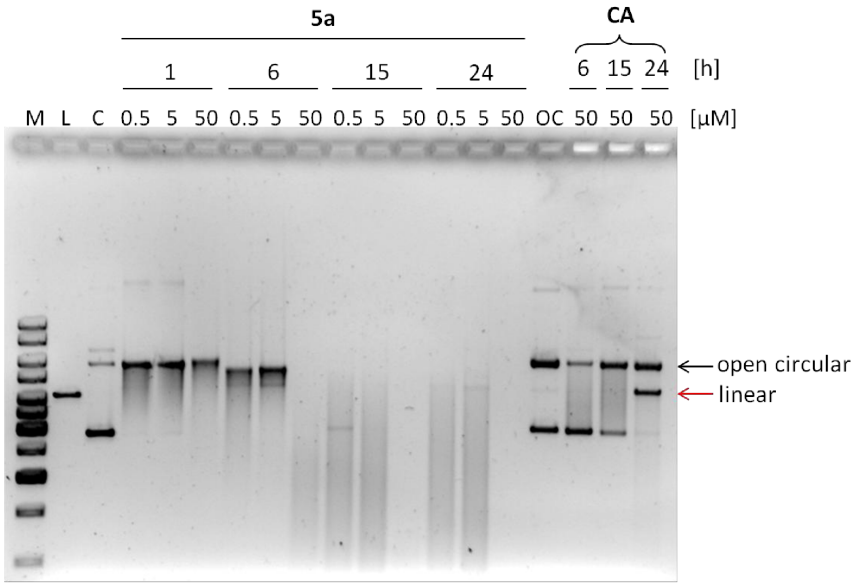
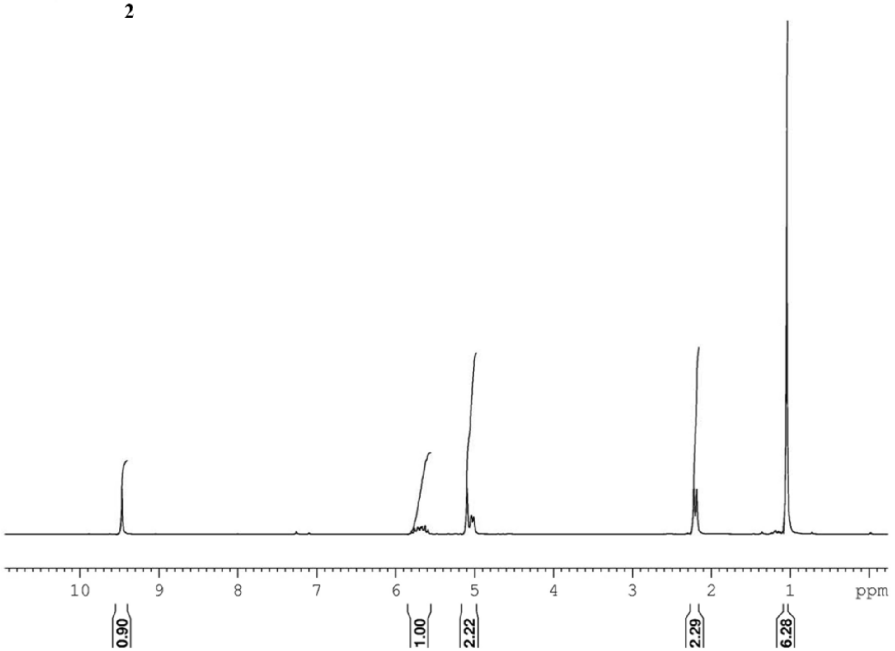


Figure S1. Time- and concentration-dependent cleavage of pBR322 plasmid caused by DNA alkylation by bis-3-chloropiperidine **5a**. The supercoiled form of plasmid DNA pBR322 (120 ng) was incubated with the compound at 37 °C for 3 h in BPE buffer, pH 7.4, at different concentrations (0.5, 5, 50 µM). Chlorambucil (CA) was used as control substance. Cleavage of DNA was analysed by agarose (1%) gel electrophoresis in TBE 1X. M = 1 kb Ladder; C = supercoiled DNA control; L = linear DNA control; OC = open circular (nicked) DNA control.

2,2-Dimethyl-4-pentenal

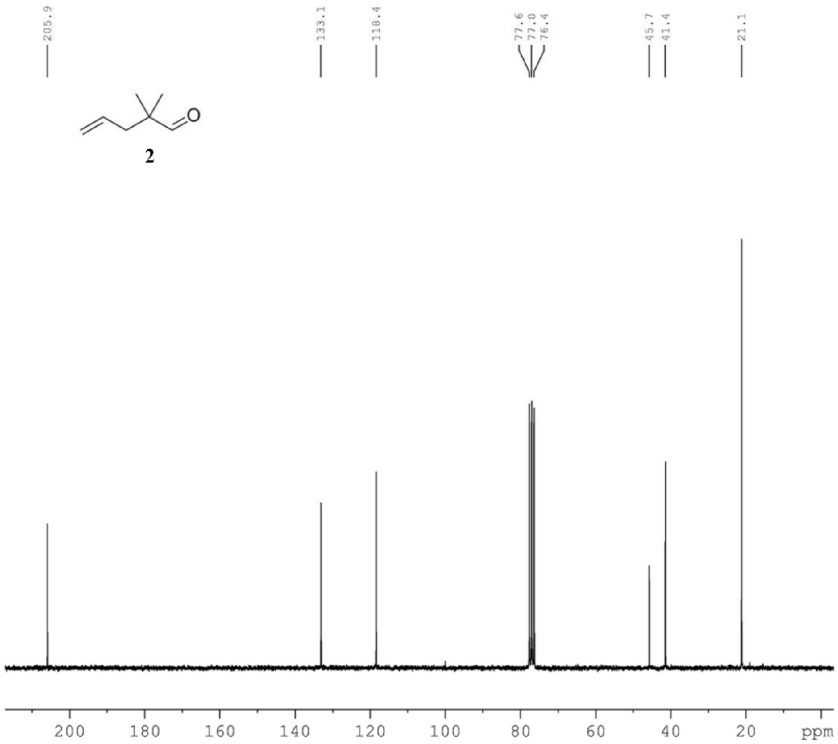
2



BRUKER

```
Current Data Parameters
NAME                  Jun08-2010
EXPNO                        130
PROCNO                         1

F2 - Acquisition Parameters
Date_                   20100609
Time                        0.44
INSTRUM                    spect
PROBHD    5 mm DUL 13C-1
PULPROG                     zg30
TD                         65536
SOLVENT                    CDCl3
NS                            16
DS                             2
SWH                     4132.231 Hz
FIDRES                  0.063053 Hz
AQ                      7.9299059 sec
RG                           287
DW                       121.000 use
DE                         6.00 use
TE                        296.0 K
D1                    1.00000000 sec
TD0                            1

======== CHANNEL f1 =======
NUC1                          1H
P1                         8.60 use
PL1                       -0.50 dB
SFO1                200.1312359 MHz

F2 - Processing parameters
SI                         32768
SF                  200.1300078 MHz
WDW                           EM
SSB                            0
LB                          0.30 Hz
GB                             0
PC                          1.00
```

2,2-Dimethyl-4-pentenal

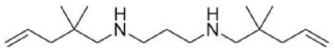


2

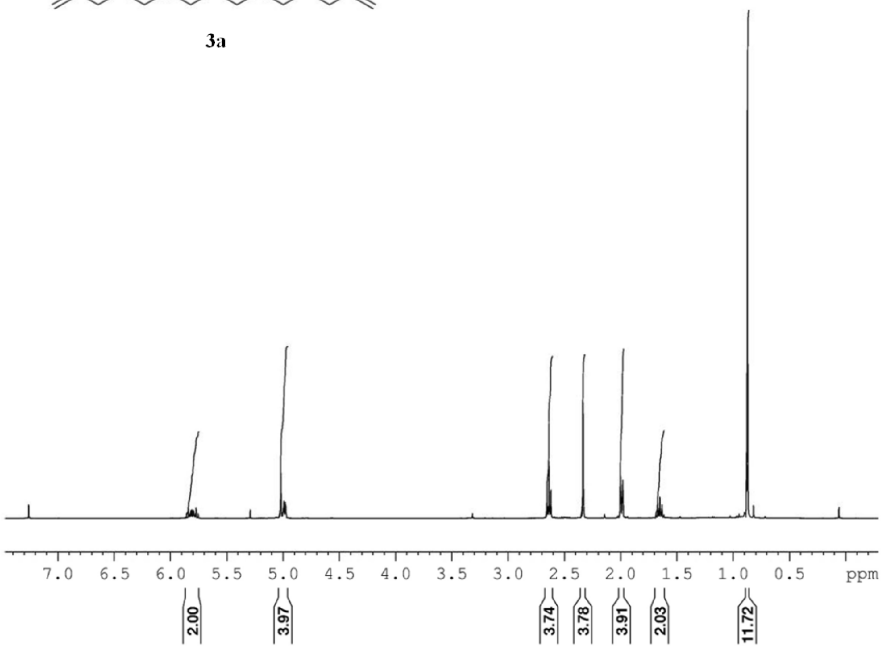
BRUKER

```
Current Data Parameters
NAME                 Jun08-2010
EXPNO                       131
PROCNO                        1

F2 - Acquisition Parameters
Date_                  20100609
Time                       6.12
INSTRUM                   spect
PROBHD    5 mm DUL 13C-1
PULPROG                zgpg30
TD                        65536
SOLVENT                   CDCl3
NS                         4096
DS                            4
SWH                   12019.230 Hz
FIDRES                 0.183399 Hz
AQ                     2.7263477 sec
RG                         6.35
DW                       41.600 usec
DE                        6.00 usec
TE                       296.9 K
D1                    2.00000000 sec
d11                   0.03000000 sec
DELTA                 1.89999998 sec
TD0                           1

======== CHANNEL f1 ========
NUC1                        13C
P1                        6.90 usec
PL1                      -1.00 dB
SFO1                50.3277608 MHz

======== CHANNEL f2 ========
CPDPRG2                 waltz16
NUC2                        1H
PCPD2                    80.00 usec
PL2                      -0.50 dB
PL12                     18.87 dB
PL13                     18.70 dB
SFO2               200.1308005 MHz

F2 - Processing parameters
SI                        32768
SF                 50.3227303 MHz
WDW                          EM
SSB                           0
LB                        1.00 Hz
GB                            0
PC                         1.40
```

N1,N3-Bis(2,2-dimethylpent-4-enyl)propane-1,3-diamine



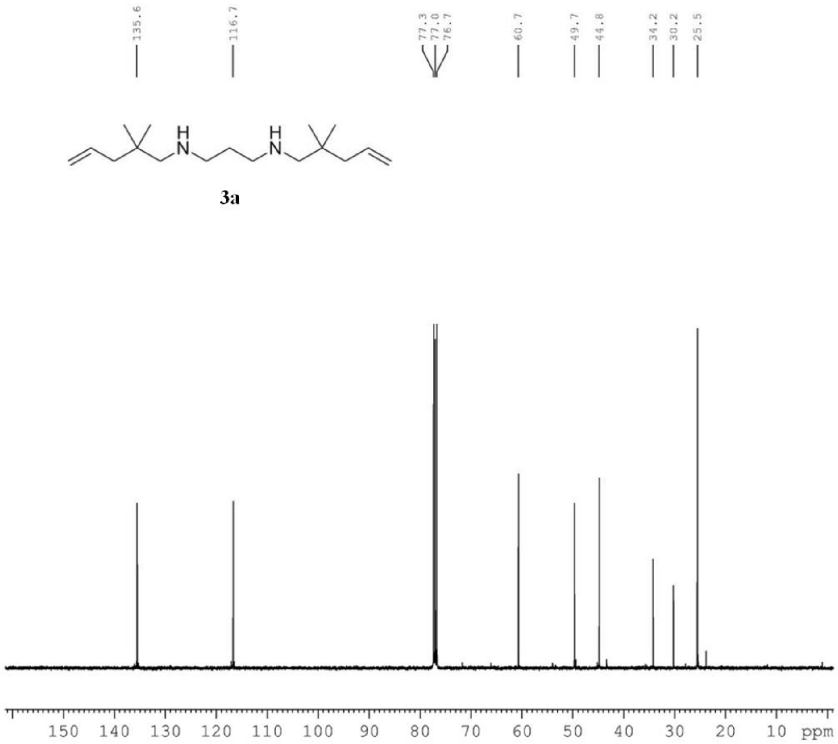
3a



BRUKER

```
Current Data Parameters
NAME        RRJul17-2007
EXPNO                160
PROCNO                 1

F2 - Acquisition Parameters
Date_           20070717
Time               14.56
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8223.685 Hz
FIDRES          0.125483 Hz
AQ              3.9846387 sec
RG                  71.8
DW                 60.800 use
DE                  6.00 use
TE                 296.9 K
D1              1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                 14.00 use
PL1                -2.50 dB
SFO1         400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF          400.1300101 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

N1,N3-Bis(2,2-dimethylpent-4-enyl)propane-1,3-diamine

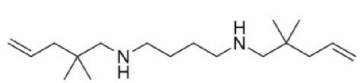


3a

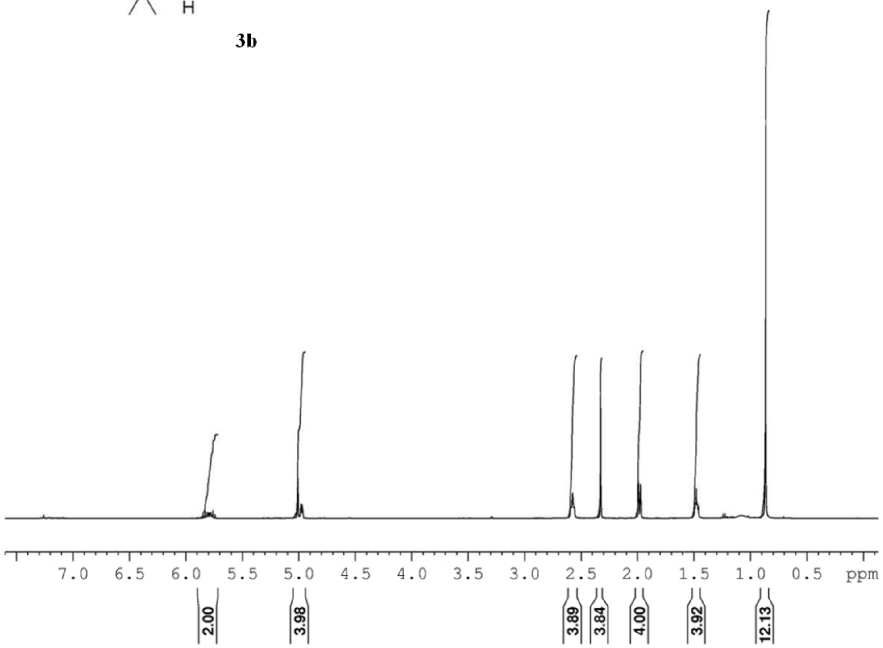
BRUKER

```
Current Data Parameters
NAME          RRJul17-2007
EXPNO              161
PROCNO               1

F2 - Acquisition Parameters
Date_           20070717
Time               19.06
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG          zgpg30
TD                65536
SOLVENT           CDCl3
NS                 1024
DS                    4
SWH           24038.461 Hz
FIDRES         0.366798 Hz
AQ             1.3631988 sec
RG                 2050
DW                20.800 usec
DE                 6.00 usec
TE                297.6 K
D1             2.00000000 sec
d11            0.03000000 sec
DELTA          1.89999998 sec
TD0                  1

======== CHANNEL f1 ========
NUC1                13C
P1                 8.80 usec
PL1               -2.60 dB
SFO1         100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2          waltz16
NUC2                1H
PCPD2             80.00 usec
PL2               -1.80 dB
PL12              13.30 dB
PL13              25.00 dB
SFO2         400.1316005 MHz

F2 - Processing parameters
SI                 32768
SF           100.6127711 MHz
WDW                  EM
SSB                   0
LB                 1.00 Hz
GB                    0
PC                 1.40
```

N1,N4-Bis(2,2-dimethylpent-4-enylbutane-1,4-diamine



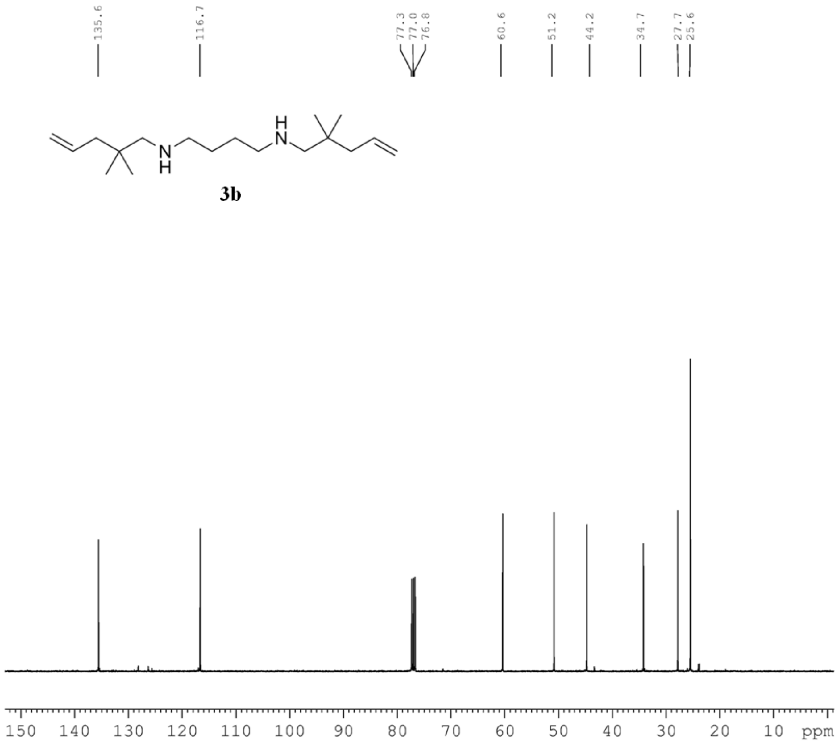
3b



BRUKER

```
Current Data Parameters
NAME        RRJul25-2007
EXPNO               530
PROCNO                1

F2 - Acquisition Parameters
Date_          20070726
Time               5.56
INSTRUM           spect
PROBHD   5 mm PABBO BB-
PULPROG            zg30
TD                65536
SOLVENT           CDCl3
NS                   16
DS                    2
SWH           8223.685 Hz
FIDRES        0.125483 Hz
AQ            3.9846387 sec
RG                 28.5
DW                60.800 use
DE                 6.00 use
TE                296.6 K
D1           1.00000000 sec
TD0                   1

======== CHANNEL f1 =======
NUC1                  1H
P1                14.00 use
PL1               -2.50 dB
SFO1        400.1324710 MHz

F2 - Processing parameters
SI                32768
SF          400.1300095 MHz
WDW                  EM
SSB                   0
LB                 0.30 Hz
GB                    0
PC                 1.00
```

N1,N4-Bis(2,2-dimethylpent-4-enyl)butane-1,4-diamine



3b

BRUKER

Current Data Parameters
NAME RRJul25-2007
EXPNO 531
PROCNO 1

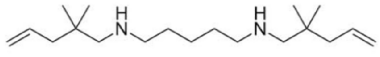
F2 - Acquisition Parameters
Date_ 20070726
Time 6.35
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 643
DS 4
SWH 24038.461 Hz
FIDRES 0.366798 Hz
AQ 1.3631988 sec
RG 2050
DW 20.800 usec
DE 6.00 usec
TE 297.2 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 8.80 usec
PL1 -2.60 dB
SFO1 100.6228298 MHz

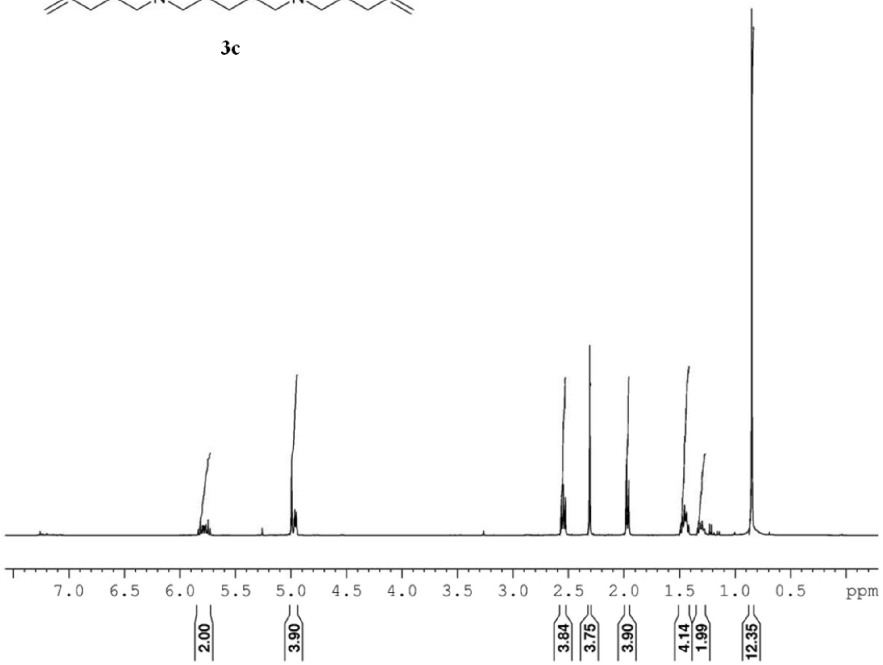
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -1.80 dB
PL12 13.30 dB
PL13 25.00 dB
SFO2 400.1316005 MHz

F2 - Processing parameters
SI 32768
SF 100.6127734 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

N1,N5-Bis(2,2-dimethylpent-4-enyl)pentane-1,5-diamine



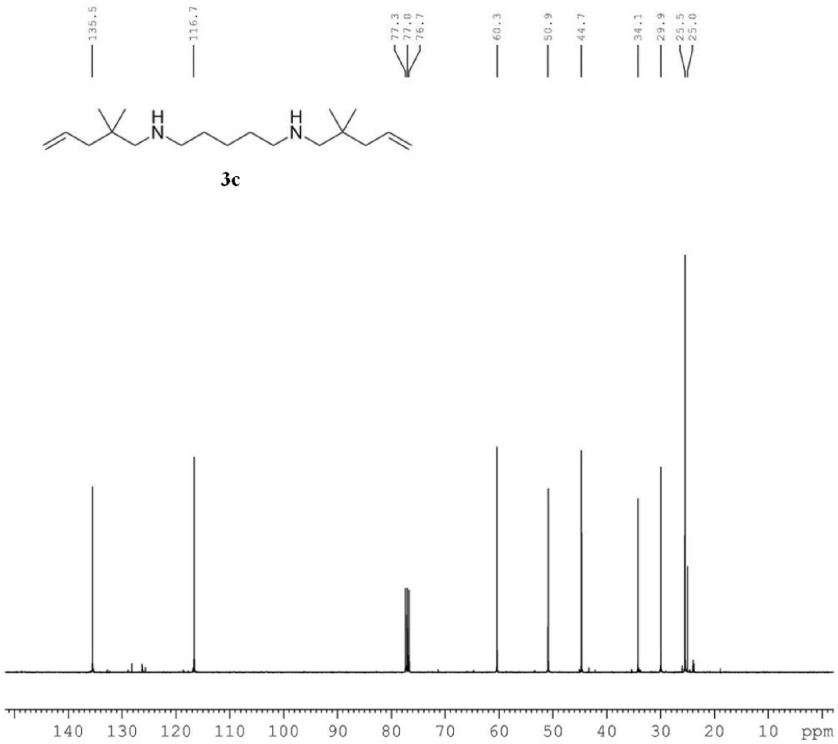
3c



BRUKER

```
Current Data Parameters
NAME        RRJul27-2007
EXPNO                330
PROCNO                 1

F2 - Acquisition Parameters
Date_           20070727
Time               15.37
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8223.685 Hz
FIDRES          0.125483 Hz
AQ             3.9846387 sec
RG                    18
DW                60.800 use
DE                 6.00 use
TE                296.6 K
D1            1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                14.00 use
PL1               -2.50 dB
SFO1         400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF           400.1300100 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

N1,N5-Bis(2,2-dimethylpent-4-enyl)pentane-1,5-diamine

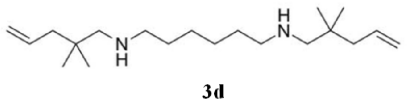


3c

BRUKER

```
Current Data Parameters
NAME          RRJul27-2007
EXPNO                  331
PROCNO                   1

F2 - Acquisition Parameters
Date_             20070727
Time                 16.38
INSTRUM              spect
PROBHD   5 mm PABBO BB-
PULPROG             zgpg30
TD                   65536
SOLVENT              CDCl3
NS                    1024
DS                       4
SWH              24038.461 Hz
FIDRES           0.366798 Hz
AQ               1.3631988 sec
RG                    2050
DW                20.800 usec
DE                 6.00 usec
TE                297.3 K
D1            2.00000000 sec
d11           0.03000000 sec
DELTA         1.89999998 sec
TD0                      1

======== CHANNEL f1 ========
NUC1                   13C
P1                  8.80 usec
PL1                -2.60 dB
SFO1         100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2            waltz16
NUC2                    1H
PCPD2              80.00 usec
PL2                -1.80 dB
PL12               13.30 dB
PL13               25.00 dB
SFO2         400.1316005 MHz

F2 - Processing parameters
SI                   32768
SF           100.6127750 MHz
WDW                     EM
SSB                      0
LB                  1.00 Hz
GB                       0
PC                    1.40
```

N1,N6-Bis(2,2-dimethylpent-4-enyl)hexane-1,6-diamine

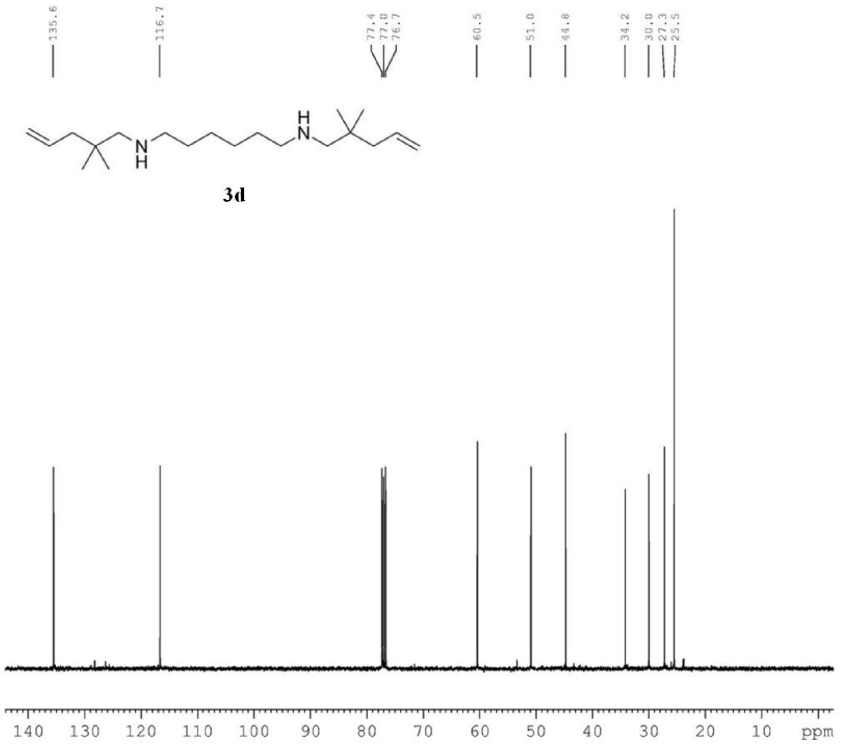


3d

BRUKER

```
Current Data Parameters
NAME        RRJul25-2007
EXPNO                512
PROCNO                 1

F2 - Acquisition Parameters
Date_           20070726
Time                6.46
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8223.685 Hz
FIDRES          0.125483 Hz
AQ              3.9846387 sec
RG                    36
DW                60.800 use
DE                 6.00 use
TE                296.6 K
D1             1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                14.00 use
PL1               -2.50 dB
SFO1         400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF           400.1300096 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

N1,N6-Bis(2,2-dimethylpent-4-enyl)hexane-1,6-diamine

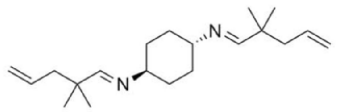


3d

BRUKER

```
Current Data Parameters
NAME          RRJul25-2007
EXPNO                  513
PROCNO                   1

F2 - Acquisition Parameters
Date_             20070726
Time                  6.56
INSTRUM              spect
PROBHD   5 mm PABBO BB-
PULPROG             zgpg30
TD                   65536
SOLVENT              CDCl3
NS                     227
DS                       4
SWH              24038.461 Hz
FIDRES           0.366798 Hz
AQ               1.3631988 sec
RG                    2050
DW                  20.800 usec
DE                   6.00 usec
TE                  297.2 K
D1             2.00000000 sec
d11            0.03000000 sec
DELTA          1.89999998 sec
TD0                      1

======== CHANNEL f1 ========
NUC1                   13C
P1                    8.80 usec
PL1                  -2.60 dB
SFO1          100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2             waltz16
NUC2                   1H
PCPD2                80.00 usec
PL2                  -1.80 dB
PL12                 13.30 dB
PL13                 25.00 dB
SFO2          400.1316005 MHz

F2 - Processing parameters
SI                    32768
SF            100.6127741 MHz
WDW                      EM
SSB                       0
LB                     1.00 Hz
GB                        0
PC                     1.40
```

trans-N1,N4-Bis(2,2-dimethylpent-4-enylidene)cyclohexane-1,4-diamine

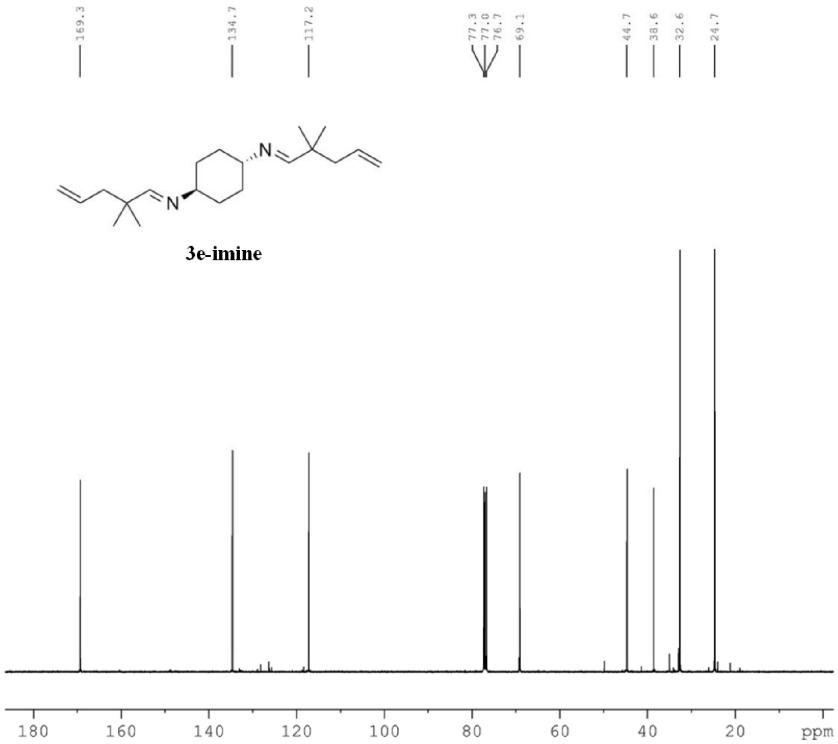


3e-imine

BRUKER

```
Current Data Parameters
NAME         RRJun25-2007
EXPNO                  90
PROCNO                  1

F2 - Acquisition Parameters
Date_            20070625
Time                21.36
INSTRUM             spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8223.685 Hz
FIDRES          0.125483 Hz
AQ              3.9846387 sec
RG                    36
DW                60.800 use
DE                 6.00 use
TE                296.6 K
D1             1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                 14.00 use
PL1                -2.50 dB
SFO1         400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF           400.1300096 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

trans-N1,N4-Bis(2,2-dimethylpent-4-enylidene)cyclohexane-1,4-diamine

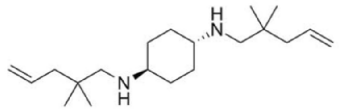


3e-imine

BRUKER

```
Current Data Parameters
NAME          RRJun25-2007
EXPNO                   91
PROCNO                   1

F2 - Acquisition Parameters
Date_              20070625
Time                  23.36
INSTRUM                spect
PROBHD   5 mm PABBO BB-
PULPROG             zgpg30
TD                   65536
SOLVENT              CDCl3
NS                    2048
DS                       4
SWH              24038.461 Hz
FIDRES           0.366798 Hz
AQ               1.3631988 sec
RG                    2050
DW                20.800 usec
DE                 6.00 usec
TE                 297.2 K
D1            2.00000000 sec
d11           0.03000000 sec
DELTA         1.89999998 sec
TD0                      1

======== CHANNEL f1 ========
NUC1                   13C
P1                  8.80 usec
PL1                -2.60 dB
SFO1         100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2            waltz16
NUC2                   1H
PCPD2              80.00 usec
PL2                -1.80 dB
PL12               13.30 dB
PL13               25.00 dB
SFO2         400.1316005 MHz

F2 - Processing parameters
SI                    32768
SF           100.6127739 MHz
WDW                     EM
SSB                      0
LB                  1.00 Hz
GB                       0
PC                    1.40
```

trans-N1,N4-Bis(2,2-dimethylpent-4-enyl)cyclohexane-1,4-diamine

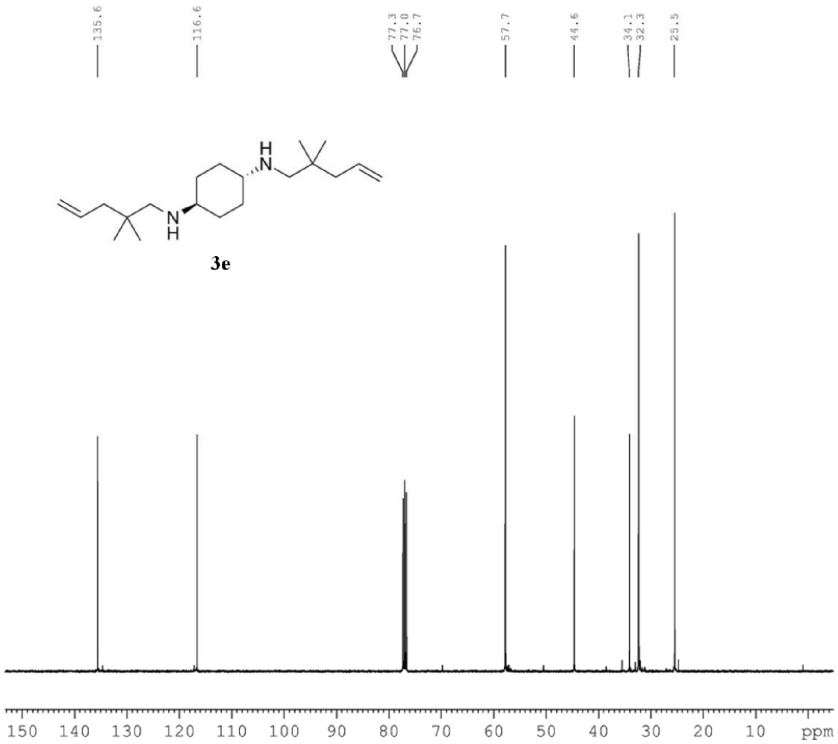


3e

BRUKER

```
Current Data Parameters
NAME        RRJun27-2007
EXPNO                130
PROCNO                 1

F2 - Acquisition Parameters
Date_           20070627
Time               17.48
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8223.685 Hz
FIDRES          0.125483 Hz
AQ              3.9846387 sec
RG                  28.5
DW                60.800 use
DE                 6.00 use
TE                296.5 K
D1             1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                 14.00 use
PL1                -2.50 dB
SFO1         400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF           400.1300096 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

trans-N1,N4-Bis(2,2-dimethylpent-4-enyl)cyclohexane-1,4-diamine

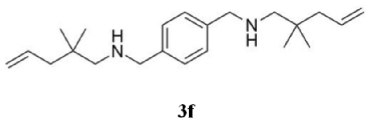


3e

BRUKER

```
Current Data Parameters
NAME          RRJun27-2007
EXPNO                  131
PROCNO                   1

F2 - Acquisition Parameters
Date_             20070627
Time                 18.50
INSTRUM              spect
PROBHD   5 mm PABBO BB-
PULPROG             zgpg30
TD                   65536
SOLVENT              CDCl3
NS                    1024
DS                       4
SWH              24038.461 Hz
FIDRES           0.366798 Hz
AQ               1.3631988 sec
RG                    2050
DW                 20.800 usec
DE                  6.00 usec
TE                 297.2 K
D1            2.00000000 sec
d11           0.03000000 sec
DELTA         1.89999998 sec
TD0                      1

======== CHANNEL f1 ========
NUC1                   13C
P1                    8.80 usec
PL1                  -2.60 dB
SFO1         100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2            waltz16
NUC2                   1H
PCPD2                80.00 usec
PL2                  -1.80 dB
PL12                 13.30 dB
PL13                 25.00 dB
SFO2         400.1316005 MHz

F2 - Processing parameters
SI                    32768
SF           100.6127736 MHz
WDW                      EM
SSB                       0
LB                     1.00 Hz
GB                        0
PC                     1.40
```

N,N´-(1,4-Phenylenebis(methylene))bis(2,2-dimethylpent-4-en-1-amine)

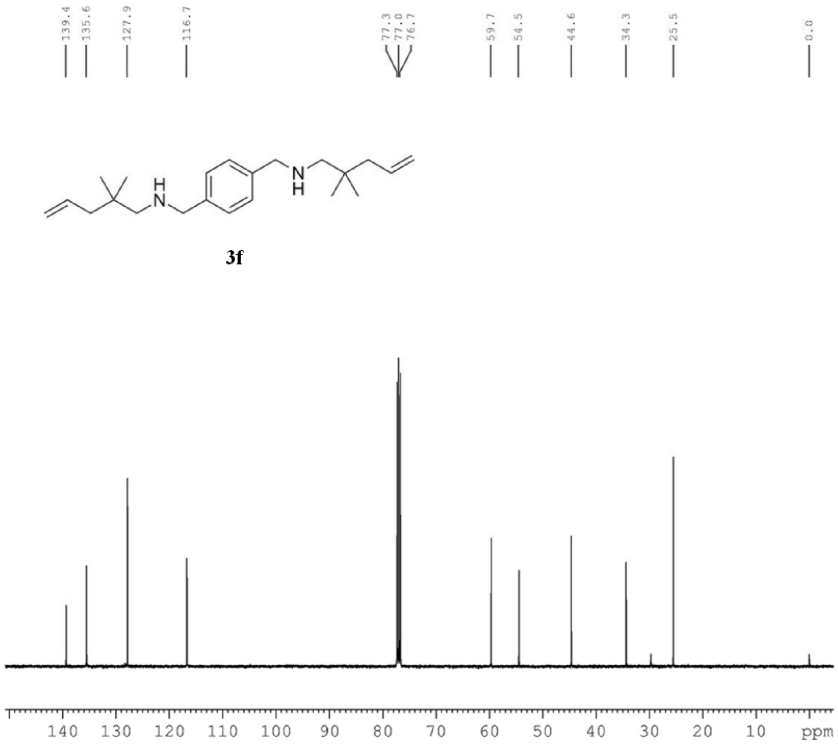


3f

BRUKER

```
Current Data Parameters
NAME          IZFeb22-2011
EXPNO                  370
PROCNO                   1

F2 - Acquisition Parameters
Date_             20110222
Time                 22.11
INSTRUM              spect
PROBHD   5 mm PABBO BB-
PULPROG               zg30
TD                   65536
SOLVENT              CDCl3
NS                      16
DS                       2
SWH               8223.685 Hz
FIDRES            0.125483 Hz
AQ                3.9846387 sec
RG                     161
DW                  60.800 use
DE                   6.00 use
TE                  300.0 K
D1                1.00000000 sec
TD0                      1

======== CHANNEL f1 =======
NUC1                   1H
P1                  13.60 use
PL1                 -2.50 dB
SFO1          400.1324710 MHz

F2 - Processing parameters
SI                   32768
SF            400.1300085 MHz
WDW                    EM
SSB                     0
LB                   0.30 Hz
GB                      0
PC                   1.00
```

N,N´-(1,4-Phenylenebis(methylene))bis(2,2-dimethylpent-4-en-1-amine)



3f

BRUKER

Current Data Parameters
NAME IZFeb22-2011
EXPNO 371
PROCNO 1

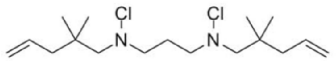
F2 - Acquisition Parameters
Date_ 20110222
Time 23.11
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1024
DS 4
SWH 24038.461 Hz
FIDRES 0.366798 Hz
AQ 1.3631988 sec
RG 2050
DW 20.800 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 8.60 usec
PL1 -2.50 dB
SFO1 100.6228298 MHz

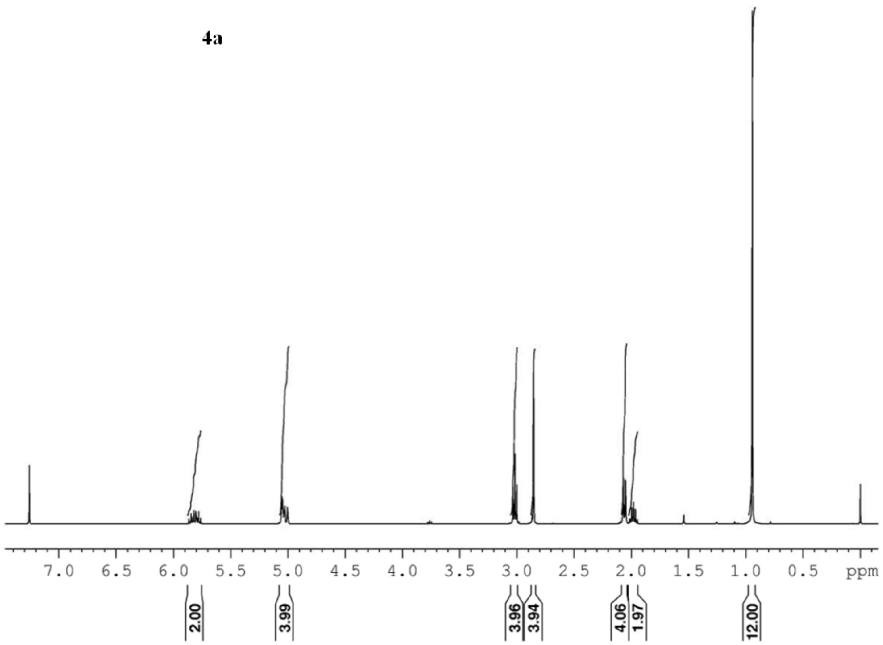
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 70.00 usec
PL2 -2.50 dB
PL12 10.60 dB
PL13 25.00 dB
SFO2 400.1316005 MHz

F2 - Processing parameters
SI 32768
SF 100.6127721 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

N1,N3-Dichloro-N1,N3-bis(2,2-dimethylpent-4-enyl)propane-1,3-diamine

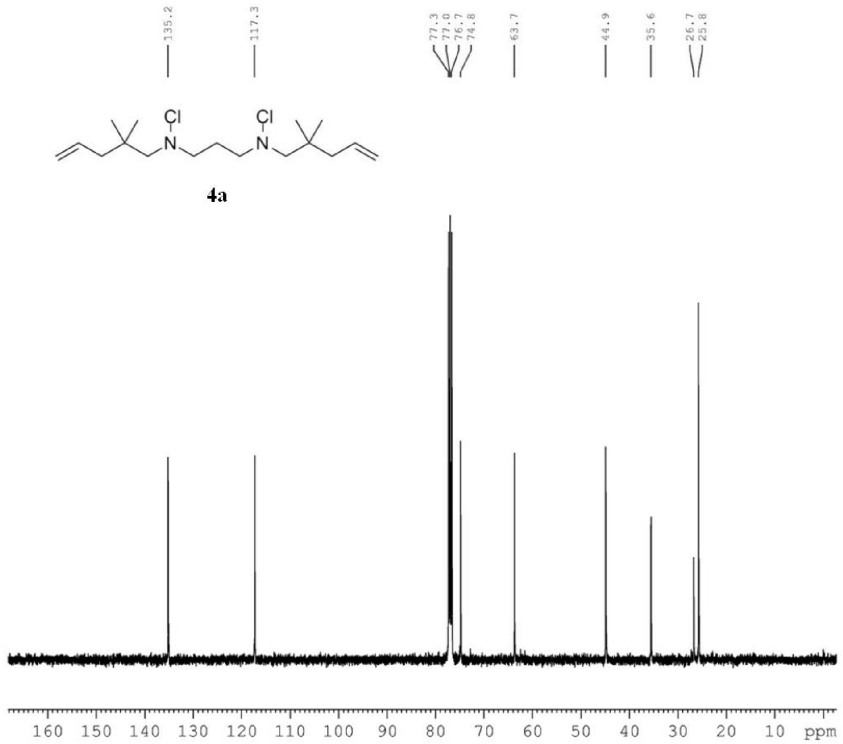


4a




```
Current Data Parameters
NAME        IZJul13-2011
EXPNO                240
PROCNO                 1

F2 - Acquisition Parameters
Date_           20110713
Time               11.07
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG            zg30
TD                65536
SOLVENT           CDCl3
NS                   16
DS                    2
SWH           8223.685 Hz
FIDRES        0.125483 Hz
AQ            3.9846387 sec
RG                  322
DW               60.800 use
DE                6.00 use
TE               300.0 K
D1           1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                13.60 use
PL1               -2.50 dB
SFO1        400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF          400.1300087 MHz
WDW                   EM
SSB                    0
LB                0.30 Hz
GB                     0
PC                1.00
```

N1,N3-Dichloro-N1,N3-bis(2,2-dimethylpent-4-enyl)propane-1,3-diamine

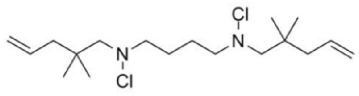


4a

BRUKER

```
Current Data Parameters
NAME          IZJul13-2011
EXPNO              221
PROCNO               1

F2 - Acquisition Parameters
Date_          20110713
Time              9.36
INSTRUM          spect
PROBHD   5 mm PABBO BB-
PULPROG         zgpg30
TD               65536
SOLVENT          CDCl3
NS                1024
DS                   4
SWH          24038.461 Hz
FIDRES        0.366798 Hz
AQ            1.3631988 sec
RG                2050
DW              20.800 usec
DE               6.00 usec
TE              300.0 K
D1           2.00000000 sec
d11          0.03000000 sec
DELTA        1.89999998 sec
TD0                  1

======== CHANNEL f1 ========
NUC1               13C
P1               8.60 usec
PL1             -2.50 dB
SFO1       100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2         waltz16
NUC2               1H
PCPD2           70.00 usec
PL2             -2.50 dB
PL12            10.60 dB
PL13            25.00 dB
SFO2       400.1316005 MHz

F2 - Processing parameters
SI                32768
SF         100.6127698 MHz
WDW                EM
SSB                 0
LB               1.00 Hz
GB                  0
PC               1.40
```

N1,N4-Dichloro-N1,N4-bis(2,2-dimethylpent-4-enyl)butane-1,4-diamine



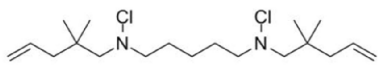
4b

BRUKER

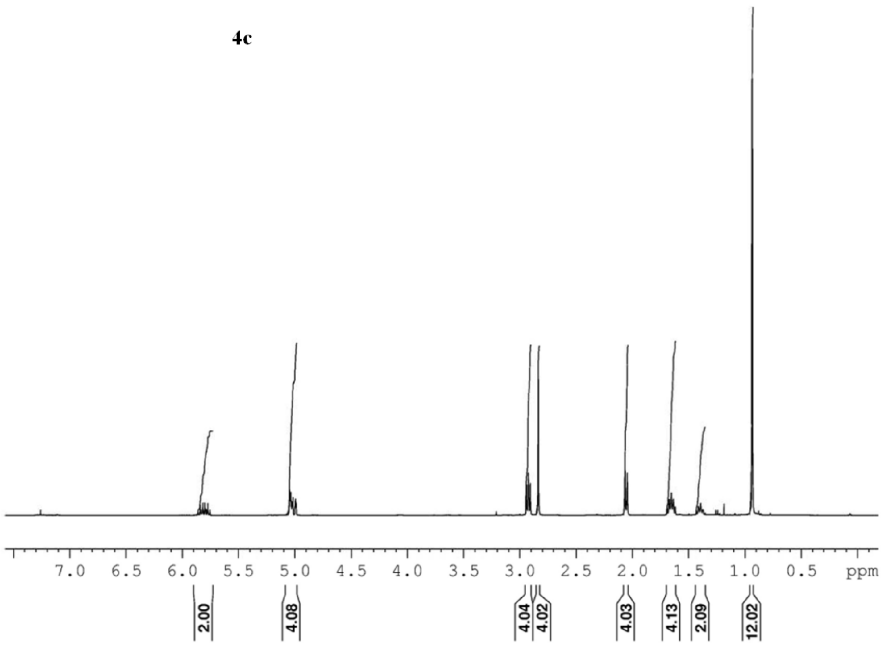
```
Current Data Parameters
NAME        RRJul27-2007
EXPNO                190
PROCNO                 1

F2 - Acquisition Parameters
Date_           20070727
Time                8.40
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8223.685 Hz
FIDRES          0.125483 Hz
AQ              3.9846387 sec
RG                    36
DW                60.800 use
DE                 6.00 use
TE                296.5 K
D1             1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                14.00 use
PL1               -2.50 dB
SFO1         400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF          400.1300365 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

N1,N4-Dichloro-N1,N4-bis(2,2-dimethylpent-4-enyl)butane-1,4-diamine
BRUKER
Current Data Parameters
NAME RBJul27-2007
EXPNO 191
PROCNO 1
F2 - Acquisition Parameters
Date_ 20070727
Time 14.03
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 512
DS 4
SWH 24038.461 Hz
FIDRES 0.366798 Hz
AQ 1.3631988 sec
RG 2050
DW 20.800 usec
DE 6.00 usec
TE 297.2 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
ID0 1
CHANNEL f1
NUC1 13C
P1 8.80 usec
PL1 -2.60 dB
SFO1 100.6228298 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -1.30 dB
PL12 13.30 dB
PL13 25.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6127706 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
135.2
117.3
77.3
77.0
76.7
74.8
66.4
44.9
35.6
29.7
25.2
Cl
N
N
Cl
4b
140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

N1,N5-Dichloro-N1,N5-bis(2,2-dimethylpent-4-enyl)pentane-1,5-diamine

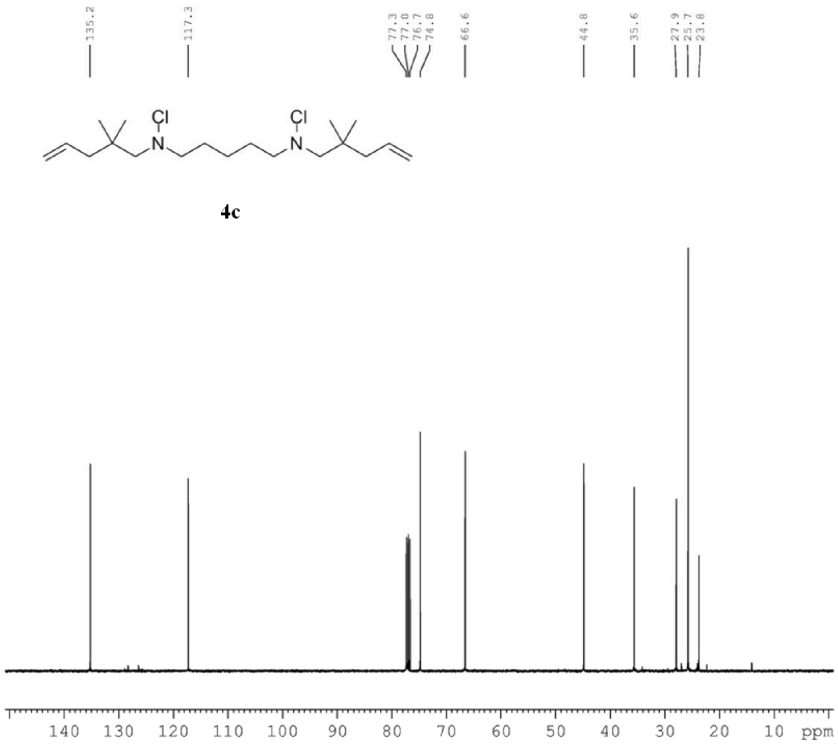


4c




```
Current Data Parameters
NAME        RRAug01-2007
EXPNO                340
PROCNO                 1

F2 - Acquisition Parameters
Date_           20070801
Time               11.11
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG            zg30
TD                65536
SOLVENT           CDCl3
NS                   16
DS                    2
SWH           8223.685 Hz
FIDRES        0.125483 Hz
AQ            3.9846387 sec
RG                 40.3
DW                60.800 use
DE                 6.00 use
TE                296.4 K
D1           1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                14.00 use
PL1               -2.50 dB
SFO1        400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF          400.1300098 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

N1,N5-Dichloro-N1,N5-bis(2,2dimethylpent-4-enyl)pentane-1,5-diamine

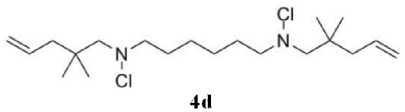


4c

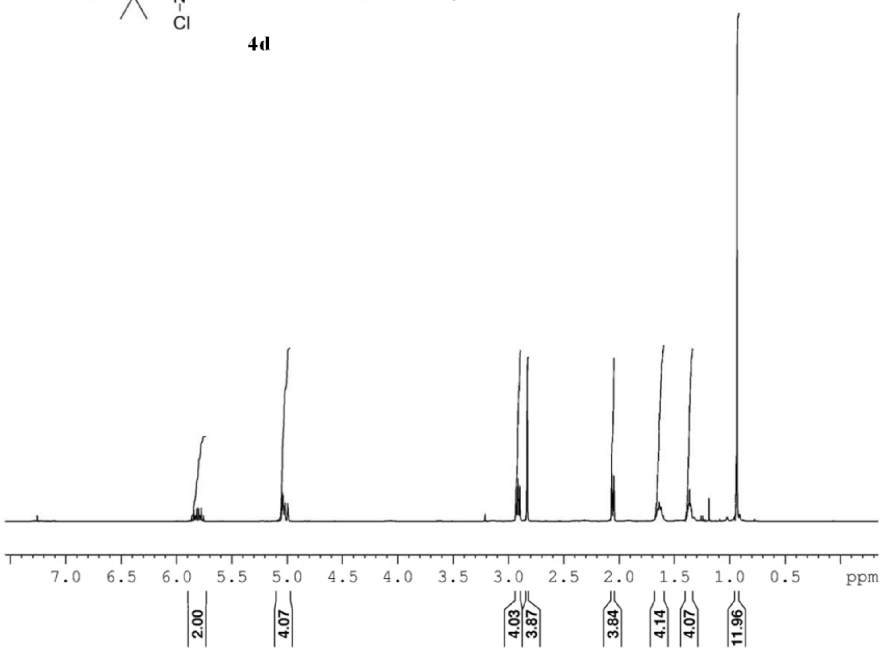
BRUKER

```
Current Data Parameters
NAME          RRAug01-2007
EXPNO                  341
PROCNO                   1

F2 - Acquisition Parameters
Date_           20070801
Time               22.08
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG          zgpg30
TD                65536
SOLVENT           CDCl3
NS                 1024
DS                    4
SWH           24038.461 Hz
FIDRES         0.366798 Hz
AQ             1.3631988 sec
RG                 2050
DW                20.800 usec
DE                 6.00 usec
TE                297.4 K
D1            2.00000000 sec
d11           0.03000000 sec
DELTA         1.89999998 sec
TD0                   1

======== CHANNEL f1 ========
NUC1                13C
P1                 8.80 usec
PL1               -2.60 dB
SFO1        100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2         waltz16
NUC2                1H
PCPD2             80.00 usec
PL2               -1.80 dB
PL12              13.30 dB
PL13              25.00 dB
SFO2        400.1316005 MHz

F2 - Processing parameters
SI                 32768
SF          100.6127700 MHz
WDW                   EM
SSB                    0
LB                 1.00 Hz
GB                     0
PC                 1.40
```

N1,N6-Dichloro-N1,N6-bis(2,2-dimethylpent-4-enyl)hexane-1,6-diamine



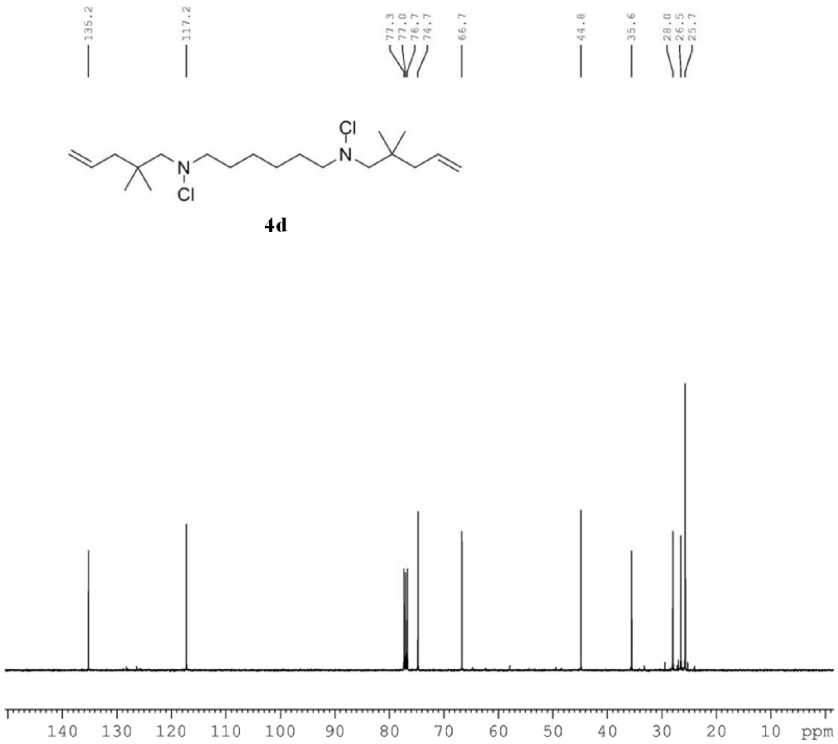
4d



BRUKER

```
Current Data Parameters
NAME        RRJul27-2007
EXPNO               180
PROCNO                1

F2 - Acquisition Parameters
Date_          20070727
Time               8.30
INSTRUM           spect
PROBHD   5 mm PABBO BB-
PULPROG            zg30
TD                65536
SOLVENT           CDCl3
NS                   16
DS                    2
SWH           8223.685 Hz
FIDRES        0.125483 Hz
AQ            3.9846387 sec
RG                   36
DW               60.800 use
DE                6.00 use
TE               296.5 K
D1           1.00000000 sec
TD0                   1

======== CHANNEL f1 =======
NUC1                 1H
P1               14.00 use
PL1              -2.50 dB
SFO1        400.1324710 MHz

F2 - Processing parameters
SI                32768
SF          400.1300096 MHz
WDW                  EM
SSB                   0
LB                 0.30 Hz
GB                    0
PC                 1.00
```

N1,N6-Dichloro-N1,N6-bis(2,2-dimethylpent-4-enyl)hexane-1,6-diamine

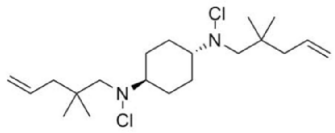


4d

BRUKER

```
Current Data Parameters
NAME          RRJul27-2007
EXPNO              181
PROCNO               1

F2 - Acquisition Parameters
Date_           20070727
Time              13.24
INSTRUM           spect
PROBHD   5 mm PABBO BB-
PULPROG          zgpg30
TD                65536
SOLVENT           CDCl3
NS                  471
DS                    4
SWH           24038.461 Hz
FIDRES         0.366798 Hz
AQ             1.3631988 sec
RG                 2050
DW                20.800 usec
DE                 6.00 usec
TE                297.2 K
D1            2.00000000 sec
d11           0.03000000 sec
DELTA         1.89999998 sec
TD0                  1

======== CHANNEL f1 ========
NUC1               13C
P1                8.80 usec
PL1              -2.60 dB
SFO1        100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2          waltz16
NUC2               1H
PCPD2            80.00 usec
PL2              -1.80 dB
PL12             13.30 dB
PL13             25.00 dB
SFO2        400.1316005 MHz

F2 - Processing parameters
SI                32768
SF          100.6127710 MHz
WDW                EM
SSB                 0
LB                1.00 Hz
GB                  0
PC                1.40
```

trans-N1,N4-Dichloro-N1,N4-bis(2,2-dimethylpent-4-enyl)cyclohexane-1,4-diamine

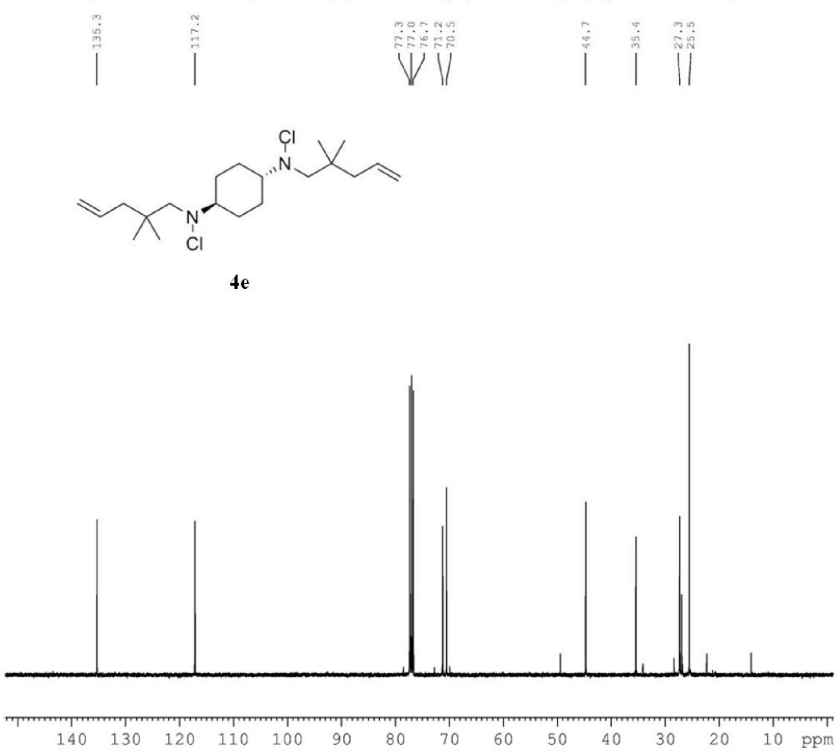


4e

BRUKER

```
Current Data Parameters
NAME          RRJun29-2007
EXPNO                   10
PROCNO                   1

F2 - Acquisition Parameters
Date_              20070629
Time                  11.05
INSTRUM               spect
PROBHD   5 mm PABBO BB-
PULPROG               zg30
TD                   65536
SOLVENT              CDCl3
NS                      16
DS                       2
SWH               8223.685 Hz
FIDRES            0.125483 Hz
AQ                3.9846387 sec
RG                      64
DW                  60.800 use
DE                   6.00 use
TE                  296.5 K
D1               1.00000000 sec
TD0                      1

======== CHANNEL f1 =======
NUC1                    1H
P1                  14.00 use
PL1                 -2.50 dB
SFO1           400.1324710 MHz

F2 - Processing parameters
SI                   32768
SF             400.1300097 MHz
WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.00
```

trans-N1,N4-Dichloro-N1,N4-bis(2,2-dimethylpent-4-enyl)cyclohexane-1,4-diamine



4e

BRUKER

Current Data Parameters
NAME RRJun29-2007
EXPNO 11
PROCNO 1

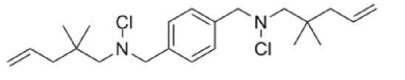
F2 - Acquisition Parameters
Date_ 20070629
Time 11.15
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 513
DS 4
SWH 24038.461 Hz
FIDRES 0.366798 Hz
AQ 1.3631988 sec
RG 2050
DW 20.800 usec
DE 6.00 usec
TE 297.1 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 8.80 usec
PL1 -2.60 dB
SFO1 100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -1.80 dB
PL12 13.30 dB
PL13 25.00 dB
SFO2 400.1316005 MHz

F2 - Processing parameters
SI 32768
SF 100.6127707 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

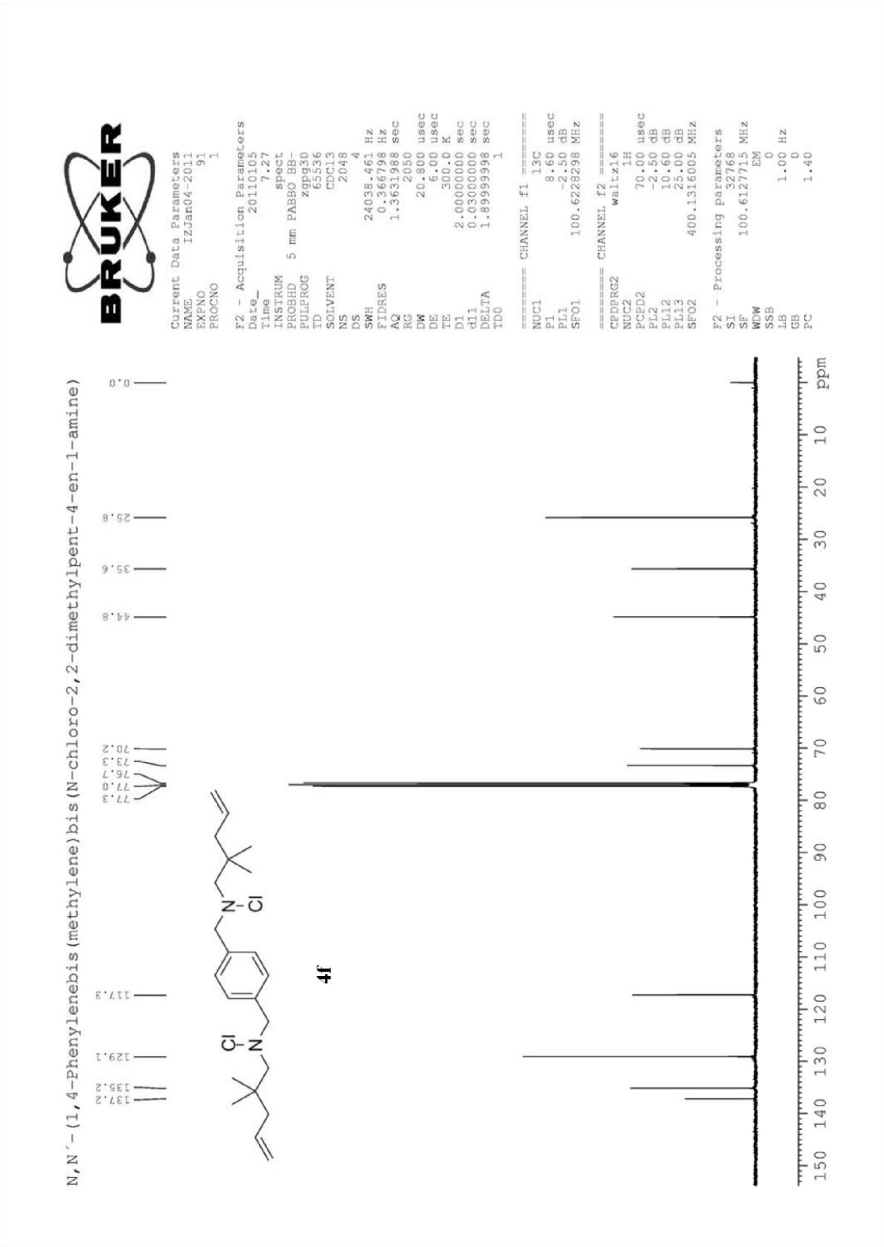
N,N´-(1,4-Phenylenebis(methylene))bis(N-chloro-2,2-dimethylpent-4-en-1-amine)



4f

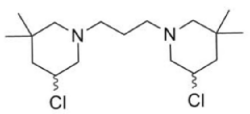
BRUKER

```
Current Data Parameters
NAME        IZJan04-2011
EXPNO                 90
PROCNO                 1

F2 - Acquisition Parameters
Date_           20110104
Time                8.40
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8223.685 Hz
FIDRES          0.125483 Hz
AQ              3.9846387 sec
RG                   228
DW                60.800 use
DE                 6.00 use
TE                300.0 K
D1             1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                13.60 use
PL1               -2.50 dB
SFO1         400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF           400.1300086 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

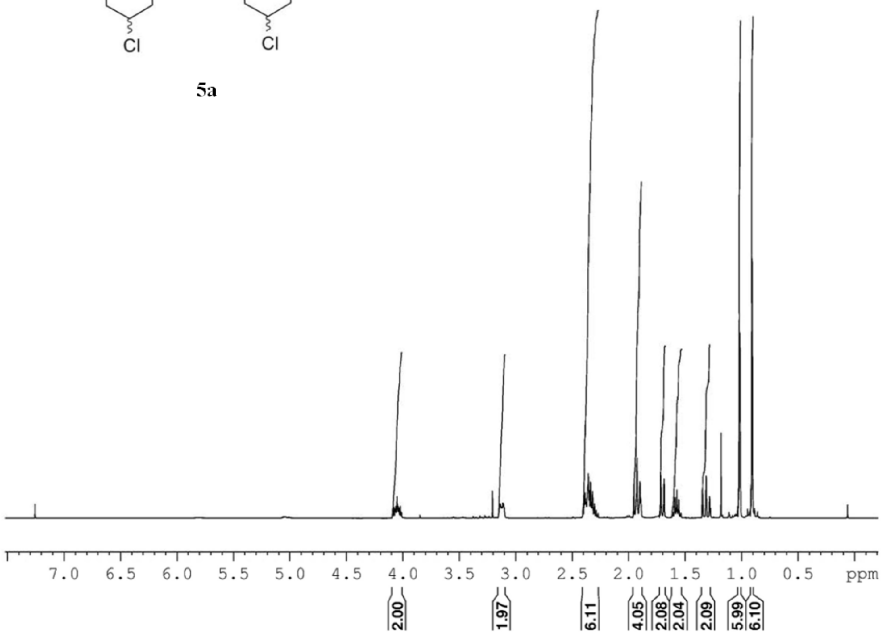

BRUKER
Current Data Parameters
NAME IZJan04-2011
EXPNO 91
PROCNO 1
F2 - Acquisition Parameters
Date_ 20110105
Time 7.27
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 2048
DS 4
SWH 24038.461 Hz
FIDRES 0.366798 Hz
AQ 1.3631988 sec
RG 2050
DW 20.800 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
ID0 1
CHANNEL f1
NUC1 13C
P1 8.60 usec
PL1 -2.50 dB
SFO1 100.6228298 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 70.00 usec
PL2 2.50 dB
PL12 15.60 dB
PL13 25.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6127715 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
N,N'-(1,4-Phenylenebis(methylene)bis(N-chloro-2,2-dimethylpent-4-en-1-amine)
4f
ppm

30

1,3-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)propane

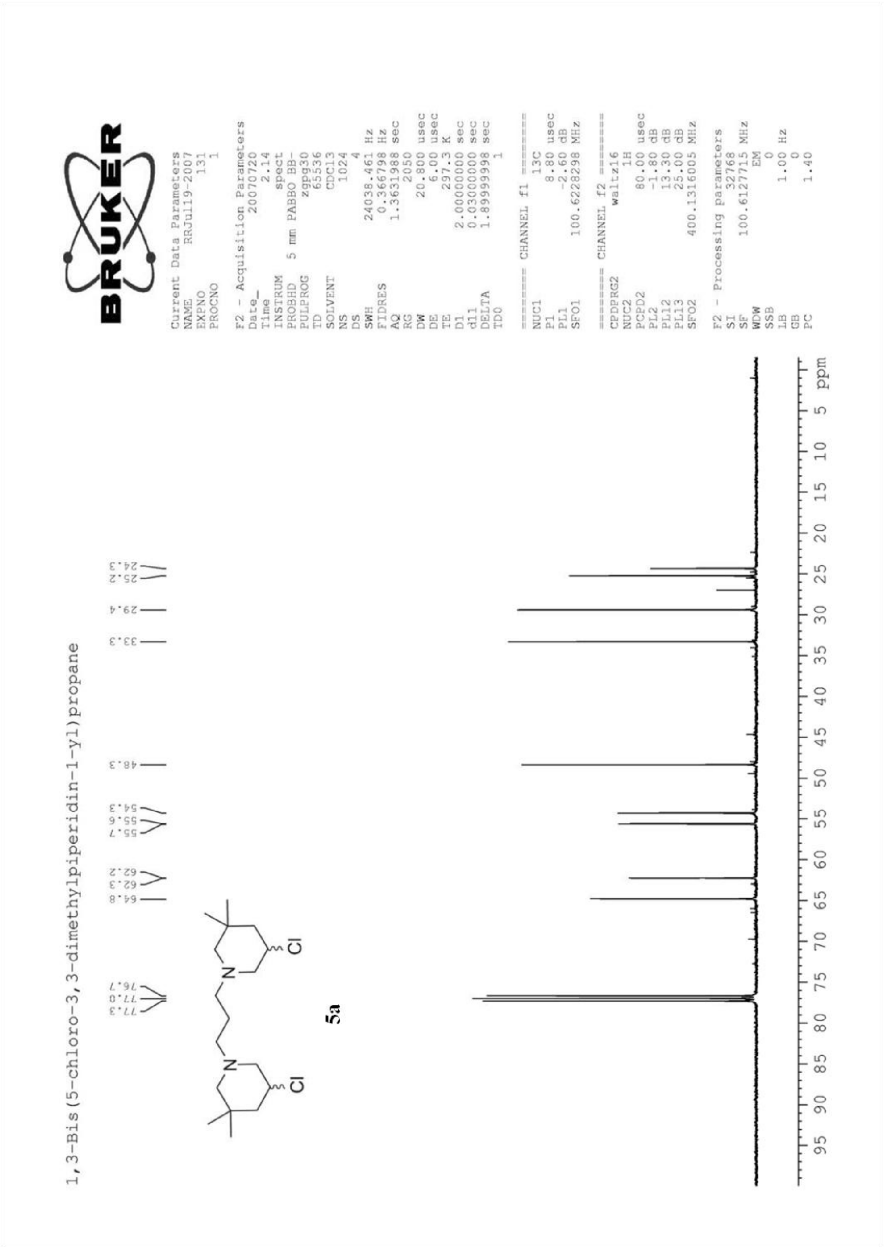


5a



BRUKER

```
Current Data Parameters
NAME        RRJul19-2007
EXPNO               130
PROCNO                1

F2 - Acquisition Parameters
Date_          20070720
Time               1.13
INSTRUM           spect
PROBHD   5 mm PABBO BB-
PULPROG            zg30
TD                65536
SOLVENT           CDCl3
NS                   16
DS                    2
SWH           8223.685 Hz
FIDRES        0.125483 Hz
AQ            3.9846387 sec
RG                 45.2
DW                60.800 use
DE                 6.00 use
TE                296.8 K
D1           1.00000000 sec
TD0                   1

======== CHANNEL f1 =======
NUC1                1H
P1                14.00 use
PL1               -2.50 dB
SFO1        400.1324710 MHz

F2 - Processing parameters
SI                32768
SF          400.1300095 MHz
WDW                  EM
SSB                   0
LB                 0.30 Hz
GB                    0
PC                 1.00
```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N
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76.7
64.8
62.3
62.2
55.7
54.3
48.3
33.3
29.4
25.2
24.3
BRUKER
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RG 2050
DW 20.800 usec
DE 6.00 usec
TE 297.3 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
ID0 1
CHANNEL f1
NUC1 13C
P1 8.80 usec
PL1 -2.60 dB
SFO1 100.6228298 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -1.90 dB
PL12 13.90 dB
PL13 25.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
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SF 100.6127715 MHz
WDW EM
SSB 0
LB 1.00 Hz
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PC 1.40
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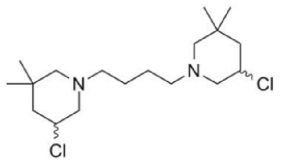
1,4-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)butane



5b

BRUKER

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PROCNO                 1

F2 - Acquisition Parameters
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Time               11.01
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG            zg30
TD                65536
SOLVENT           CDCl3
NS                   16
DS                    2
SWH           8223.685 Hz
FIDRES        0.125483 Hz
AQ            3.9846387 sec
RG                   32
DW               60.800 use
DE                6.00 use
TE               296.4 K
D1            1.00000000 sec
TD0                   1

======== CHANNEL f1 =======
NUC1                 1H
P1               14.00 use
PL1              -2.50 dB
SFO1        400.1324710 MHz

F2 - Processing parameters
SI                32768
SF          400.1300095 MHz
WDW                  EM
SSB                   0
LB                0.30 Hz
GB                    0
PC                 1.00
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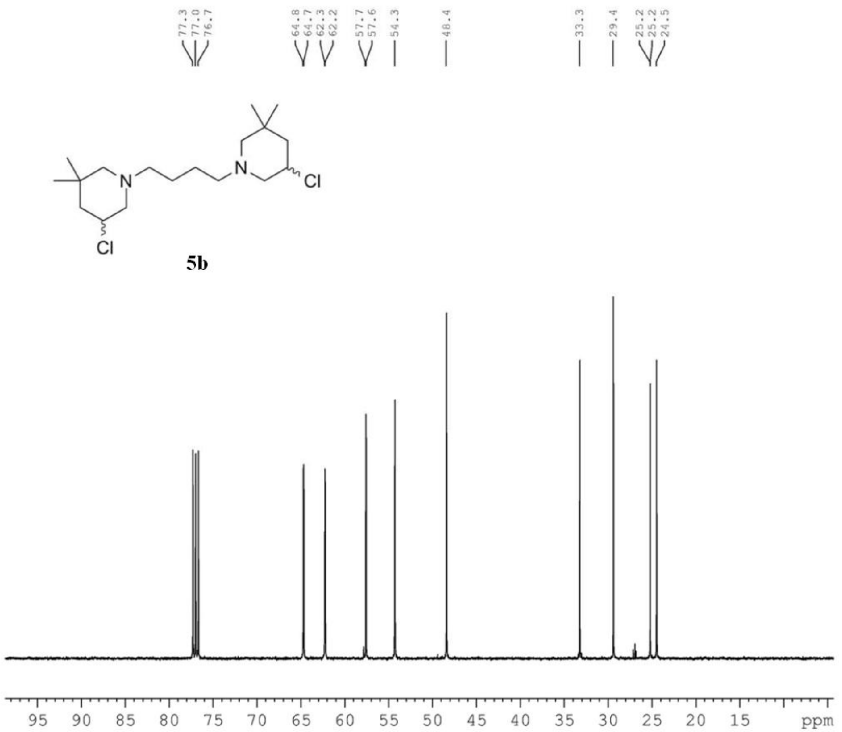
1,4-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)butane



5b

BRUKER

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PROCNO               1

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PULPROG         zgpg30
TD               65536
SOLVENT          CDCl3
NS                1024
DS                   4
SWH          24038.461 Hz
FIDRES        0.366798 Hz
AQ            1.3631988 sec
RG                2050
DW              20.800 usec
DE               6.00 usec
TE              297.5 K
D1           2.00000000 sec
d11          0.03000000 sec
DELTA        1.89999998 sec
TD0                  1

======== CHANNEL f1 ========
NUC1               13C
P1                8.80 usec
PL1              -2.60 dB
SFO1        100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2          waltz16
NUC2               1H
PCPD2            80.00 usec
PL2              -1.80 dB
PL12             13.30 dB
PL13             25.00 dB
SFO2        400.1316005 MHz

F2 - Processing parameters
SI                32768
SF          100.6127713 MHz
WDW                EM
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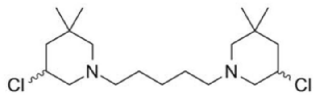
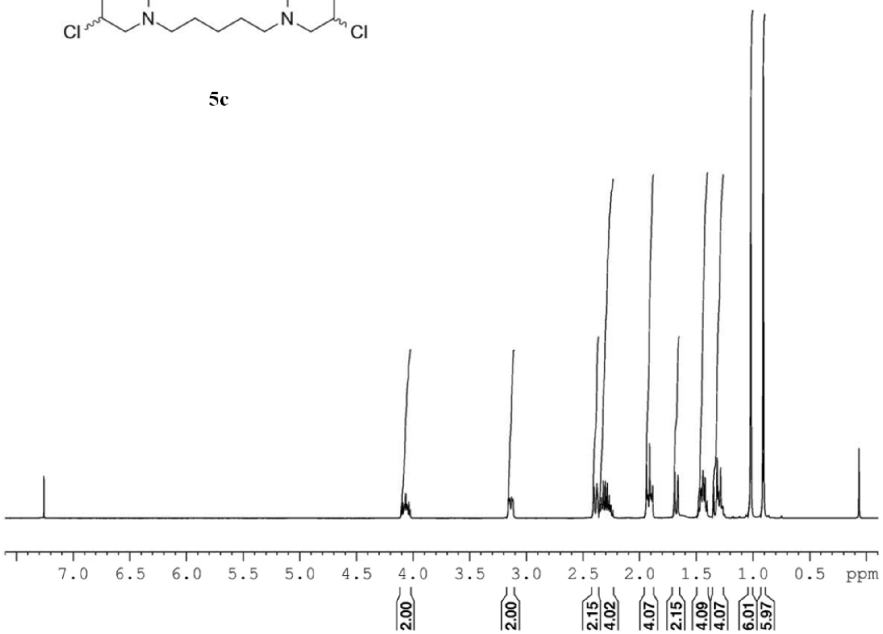
1,5-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)pentane



5c



BRUKER

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NAME        RRSep05-2007
EXPNO                280
PROCNO                 1

F2 - Acquisition Parameters
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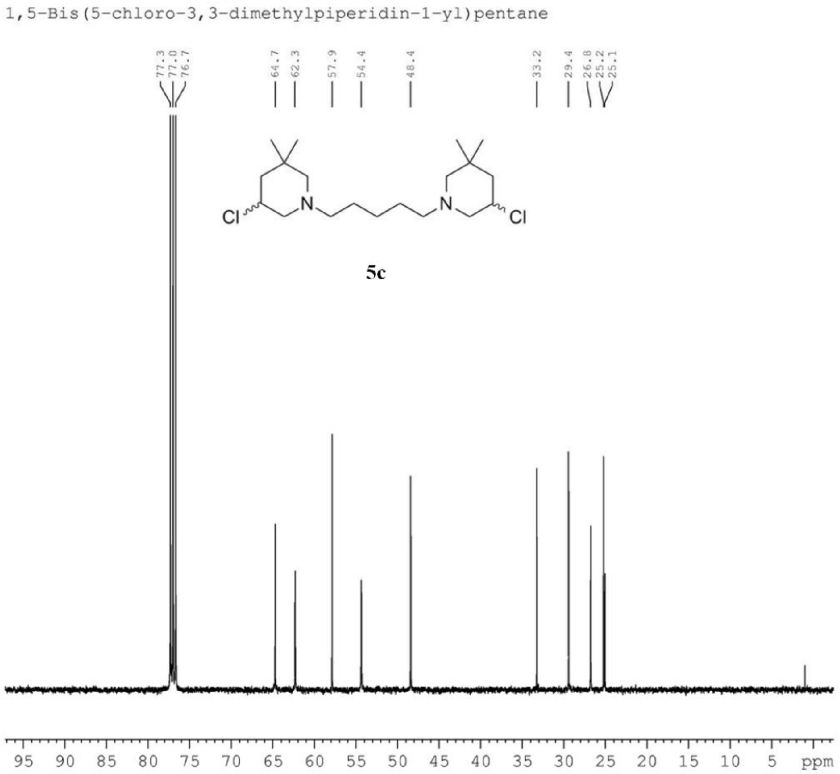
Time                5.03
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8223.685 Hz
FIDRES          0.125483 Hz
AQ              3.9846387 sec
RG                  71.8
DW                60.800 use
DE                 6.00 use
TE                296.7 K
D1             1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                14.00 use
PL1               -2.50 dB
SFO1         400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF           400.1300095 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

1,5-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)pentane



5c

BRUKER

```
Current Data Parameters
NAME          RRSep05-2007
EXPNO              281
PROCNO               1

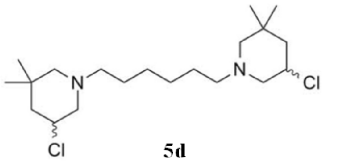
F2 - Acquisition Parameters
Date_          20070906
Time               6.04
INSTRUM           spect
PROBHD   5 mm PABBO BB-
PULPROG         zgpg30
TD               65536
SOLVENT          CDCl3
NS                1024
DS                   4
SWH          24038.461 Hz
FIDRES        0.366798 Hz
AQ            1.3631988 sec
RG                2050
DW              20.800 usec
DE               6.00 usec
TE              297.2 K
D1           2.00000000 sec
d11          0.03000000 sec
DELTA        1.89999998 sec
TD0                  1

======== CHANNEL f1 ========
NUC1               13C
P1                8.80 usec
PL1              -2.60 dB
SFO1        100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2         waltz16
NUC2               1H
PCPD2            80.00 usec
PL2              -1.80 dB
PL12             13.30 dB
PL13             25.00 dB
SFO2        400.1316005 MHz

F2 - Processing parameters
SI                32768
SF          100.6127710 MHz
WDW                EM
SSB                 0
LB                1.00 Hz
GB                  0
PC                1.40
```

1,6-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)hexane



5d

BRUKER

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Current Data Parameters
NAME        RRJul31-2007
EXPNO                140
PROCNO                 1

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Date_           20070731
Time                7.16
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8223.685 Hz
FIDRES          0.125483 Hz
AQ              3.9846387 sec
RG                  45.2
DW                60.800 use
DE                 6.00 use
TE                296.0 K
D1             1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                14.00 use
PL1               -2.50 dB
SFO1         400.1324710 MHz

F2 - Processing parameters
SI                 32768
SF           400.1300096 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```
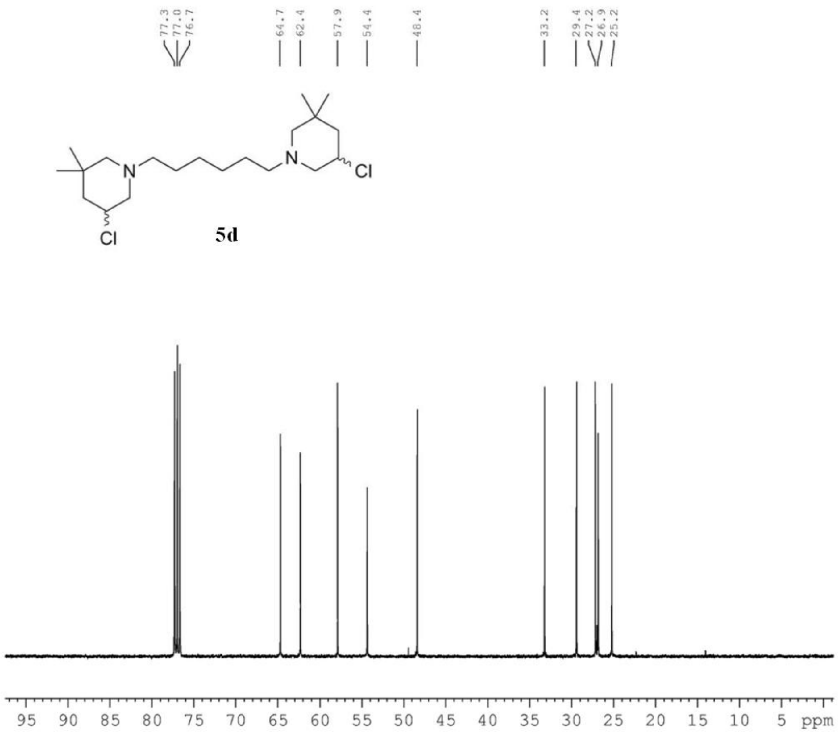

1,6-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)hexane



5d

BRUKER

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EXPNO 141
PROCNO 1

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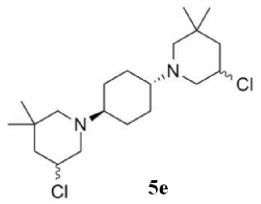
Time 10.51
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1024
DS 4
SWH 24038.461 Hz
FIDRES 0.366798 Hz
AQ 1.3631988 sec
RG 2050
DW 20.800 usec
DE 6.00 usec
TE 296.9 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 8.80 usec
PL1 -2.60 dB
SFO1 100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -1.80 dB
PL12 13.30 dB
PL13 25.00 dB
SFO2 400.1316005 MHz

F2 - Processing parameters
SI 32768
SF 100.6127712 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

trans-1,4-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)cyclohexane



5e

BRUKER

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PROCNO                  1

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Time                23.39
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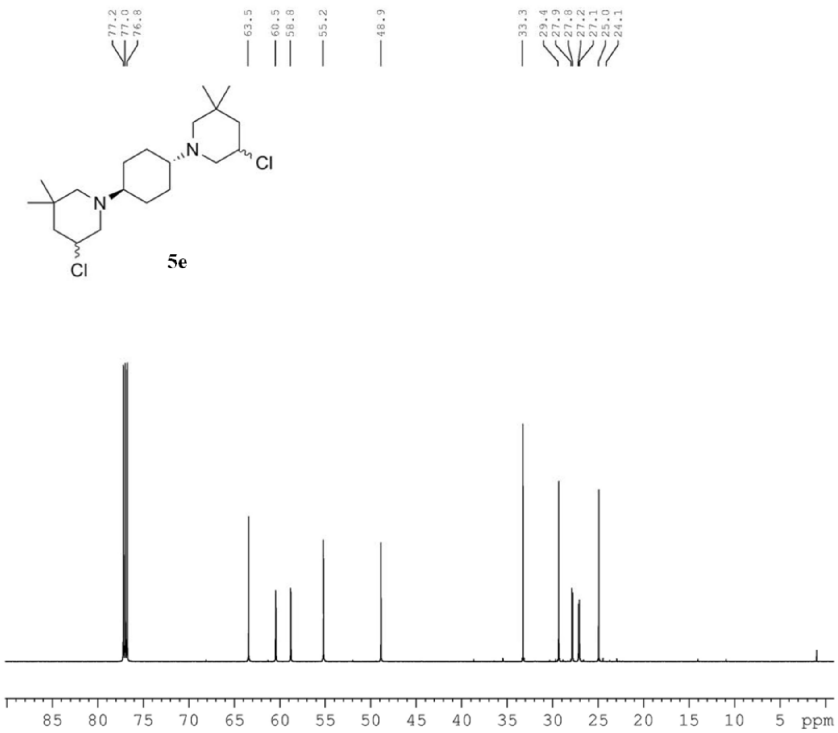
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH           12335.526 Hz
FIDRES         0.188225 Hz
AQ             2.6564426 sec
RG                  45.2
DW                40.533 use
DE                 6.50 use
TE                298.0 K
D1            1.00000000 sec
TD0                    1

======== CHANNEL f1 =======
NUC1                  1H
P1                12.50 use
PL1               -2.00 dB
SFO1         600.1937064 MHz

F2 - Processing parameters
SI                 32768
SF           600.1900138 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```

trans-1,4-Bis(5-chloro-3,3-dimethylpiperidin-1-yl)cyclohexane



5e

BRUKER

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EXPNO	21
PROCNO	1

F2 - Acquisition Parameters

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Time	8.22
INSTRUM	spect
PROBHD	5 mm PABBO BB-
PULPROG	zgig30
TD	65536
SOLVENT	CDCl3
NS	40000
DS	4
SWH	36057.691 Hz
FIDRES	0.550197 Hz
AQ	0.9088159 sec
RG	2050
DW	13.867 usec
DE	6.50 usec
TE	297.8 K
D1	2.00000000 sec
d11	0.03000000 sec
TD0	1

======== CHANNEL f1 ========

NUC1	13C
P1	9.50 usec
PL1	2.00 dB
SFO1	150.9329873 MHz

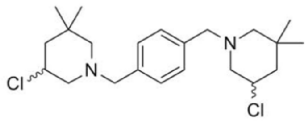
======== CHANNEL f2 ========

CPDPRG2	waltz16
NUC2	1H
PCPD2	77.00 usec
PL12	14.00 dB
PL2	-2.00 dB
SFO2	600.1924008 MHz

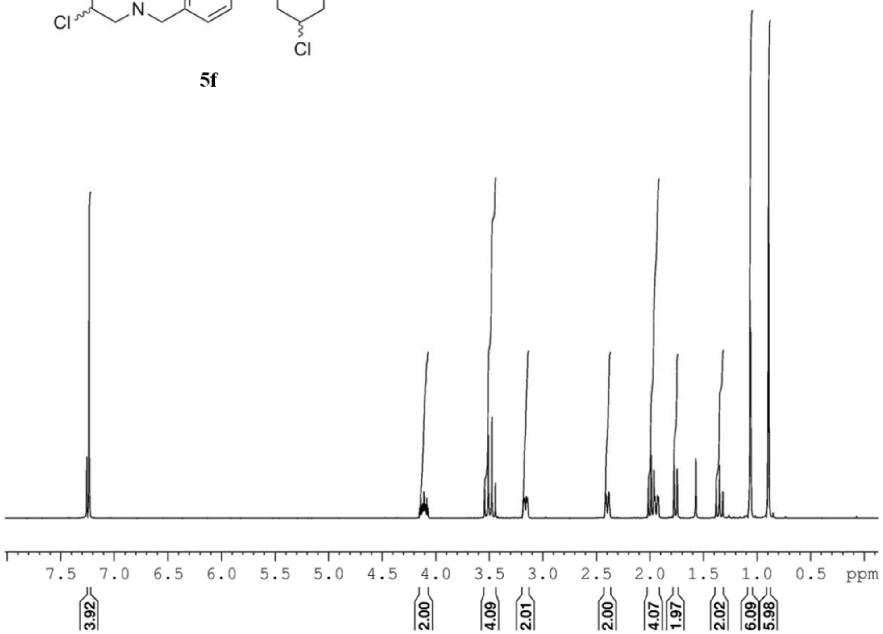
F2 - Processing parameters

SI	32768
SF	150.9179004 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	1.40

1,4-Bis((5-chloro-3,3-dimethylpiperidin-1-yl)methyl)benzene



5f



BRUKER

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PULPROG           zg30
TD               65536
SOLVENT          CDCl3
NS                  16
DS                   2
SWH           8223.685 Hz
FIDRES        0.125483 Hz
AQ            3.9846387 sec
RG                90.5
DW               60.800 use
DE                6.00 use
TE               296.7 K
D1           1.00000000 sec
TD0                  1

======== CHANNEL f1 =======
NUC1                 1H
P1               14.00 use
PL1              -2.50 dB
SFO1        400.1324710 MHz

F2 - Processing parameters
SI                32768
SF          400.1300094 MHz
WDW                  EM
SSB                   0
LB                 0.30 Hz
GB                    0
PC                 1.00
```

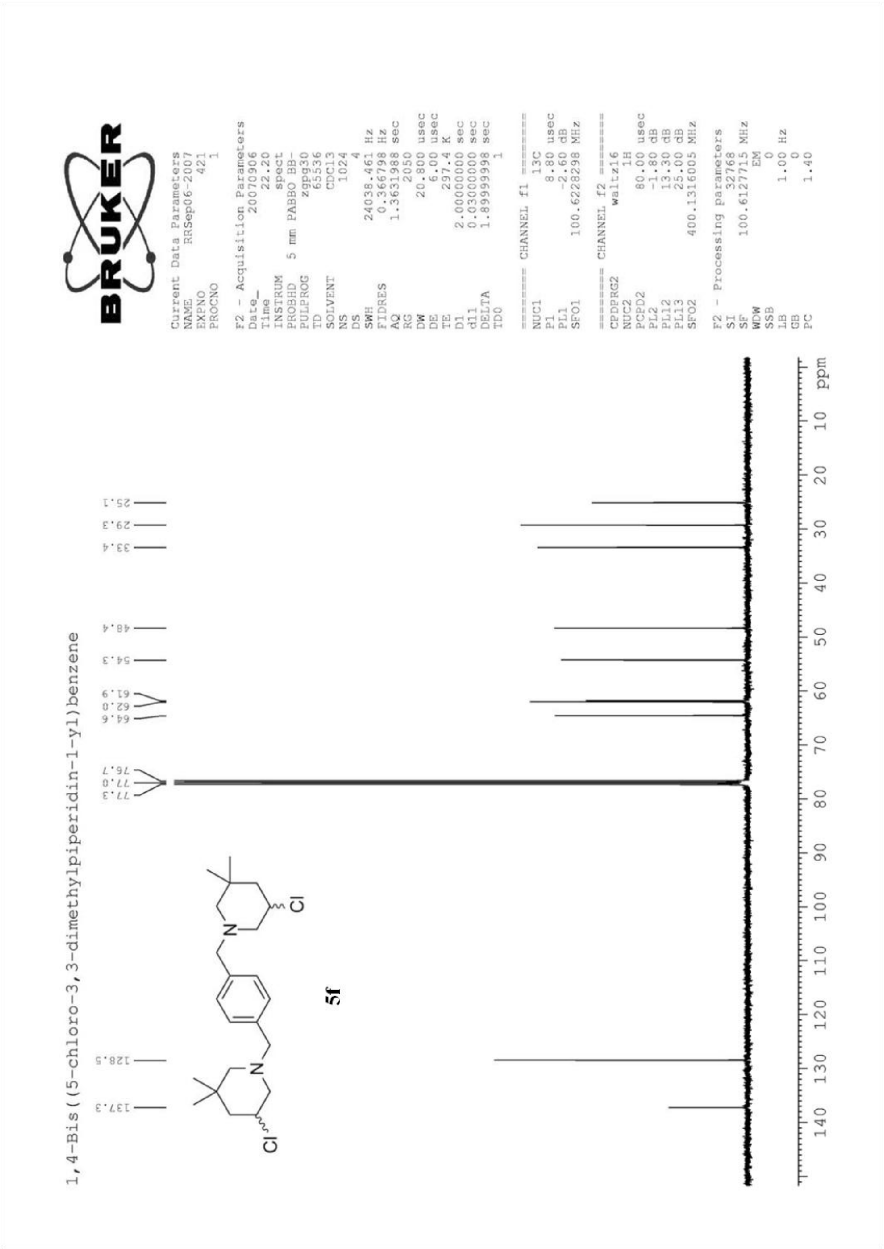
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Time 22.20
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PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1024
DS 4
SWH 24038.461 Hz
FIDRES 0.366798 Hz
AQ 1.3631988 sec
RG 2050
DW 20.800 usec
DE 6.00 usec
TE 297.4 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
ID0 1
CHANNEL f1
NUC1 13C
P1 8.80 usec
PL1 -2.60 dB
SFO1 100.6228298 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -1.90 dB
PL12 13.90 dB
PL13 25.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6127715 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1,4-Bis((5-chloro-3,3-dimethylpiperidin-1-yl)benzene
137.3
128.5
77.3
77.0
76.7
64.6
62.0
61.9
54.3
48.4
33.4
29.3
25.1
Cl
N
N
Cl
5f
140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

SUPPLEMENTARY DATA

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,*] and Barbara Gatto [a,*]

[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.

Sequencing gel analysis

The 5'-FAM-labelled duplex oligonucleotide (final concentration 2 µM) 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).

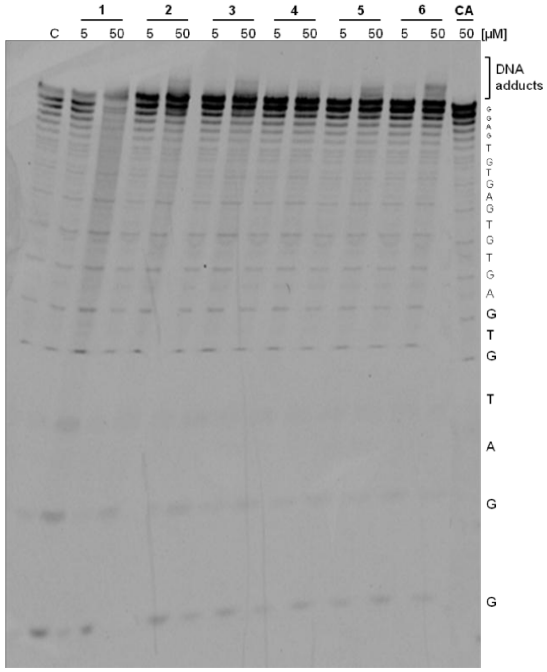

Figure S1. Denaturing polyacrylamide (20 %) gel electrophoresis in 1 × TBE (Tris-borate-EDTA) of a 5'-FAM-labeled scrambled duplex oligonucleotide (2 µM final), GGA TGT GAG TGT GAG TGT GAG G, incubated for 24 h at 37 °C in BPE buffer, pH 7.4, with bis-3-chloropiperidines **1–6** at two distinct concentrations (5 and 50 µM final). Chlorambucil (CA) was used as a control. C = untreated duplex oligonucleotide control.

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, Figure S2), 15 min (Figure 4, main text), 30 min (Figure S3), 45 min (Figure S4), 60 min (Figure S5) and 120 min (Figure S6), diluted with methanol, and analysed by ESI-MS. Measurements were performed in positive ion mode.

The ESI mass spectra verify the formation of the electrophilic aziridinium ion ($\mathbf{1_N^+{}_{OH}}$), detected already after 15 min of incubation (compare Figure 4, main text). Further hydrolysis leads to a subsequent increase in intensity of the dihydroxyl substituted product peak ($\mathbf{1_{2OH}}$) and the hydroxyl substituted derivative ($\mathbf{1_{OH}}$) coexisting with the reactive aziridinium species ($\mathbf{1_N^+{}_{OH}}$, see Figures S2–S6).

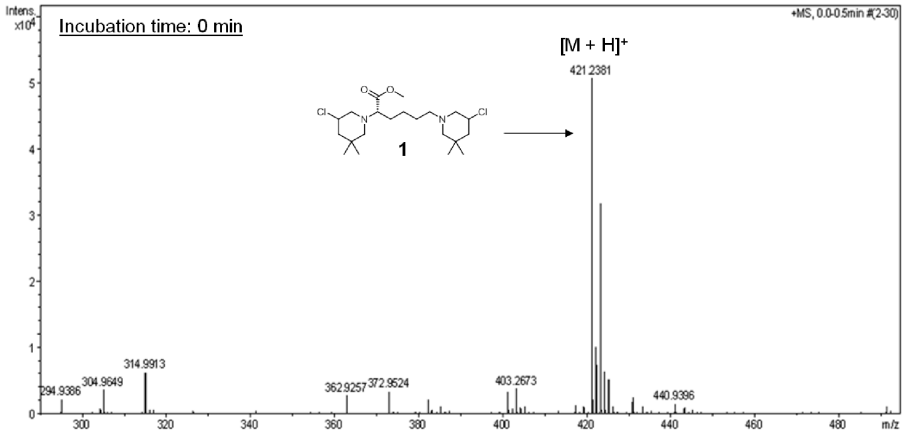

Figure S2. ESI-MS spectrum of compound **(1)** after 0 min of incubation: m/z calcd for $C_{21}H_{39}Cl_2N_2O_2^+$: 421.2383, found: 421.2381 $[M + H]^+$.

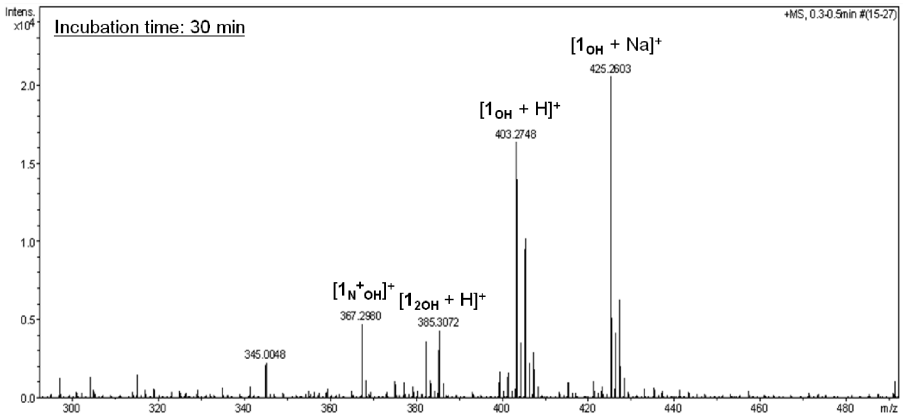

Figure S3. ESI-MS spectrum of compound **(1)** after 30 min of incubation at 37 °C in water: m/z calcd for $C_{21}H_{40}ClN_2O_3^+$: 403.2722, found: 403.2748 [$\mathbf{1_{OH}}$ + H]$^+$; m/z calcd for $C_{21}H_{39}ClN_2NaO_3^+$: 425.2541, found: 425.2603 [$\mathbf{1_{OH}}$ + Na]$^+$; m/z calcd for $C_{21}H_{39}N_2O_3^+$: 367.2955, found: 367.2980 [$\mathbf{1_{N^+OH}}$]$^+$; m/z calcd for $C_{21}H_{41}N_2O_4^+$: 385.3061, found: 385.3072 [$\mathbf{1_{2OH}}$ + H]$^+$.

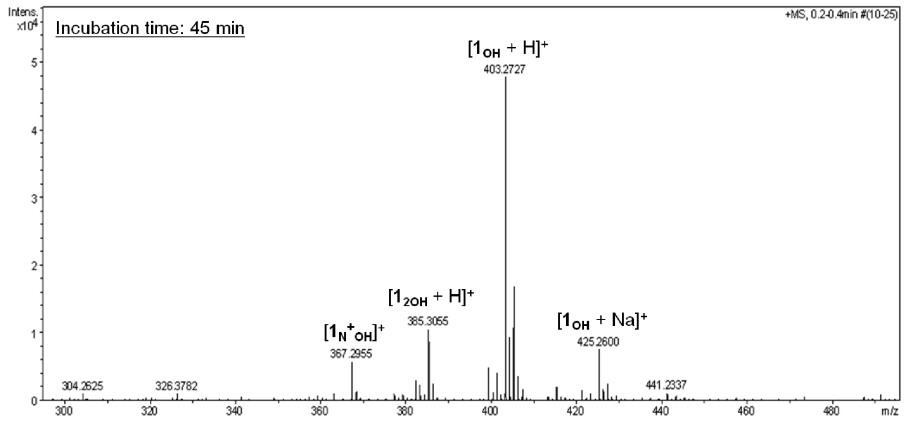

Figure S4. ESI-MS spectrum of compound **(1)** after 45 min of incubation at 37 °C in water.

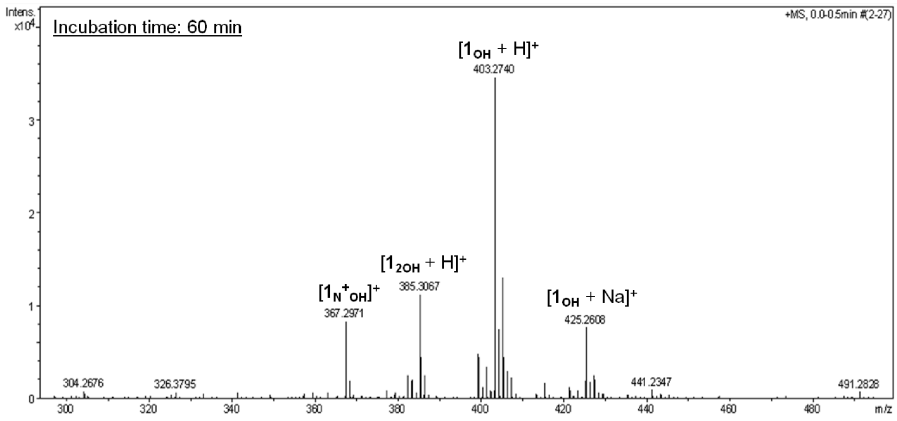

Figure S5. ESI-MS spectrum of compound **(1)** after 60 min of incubation at 37 °C in water.

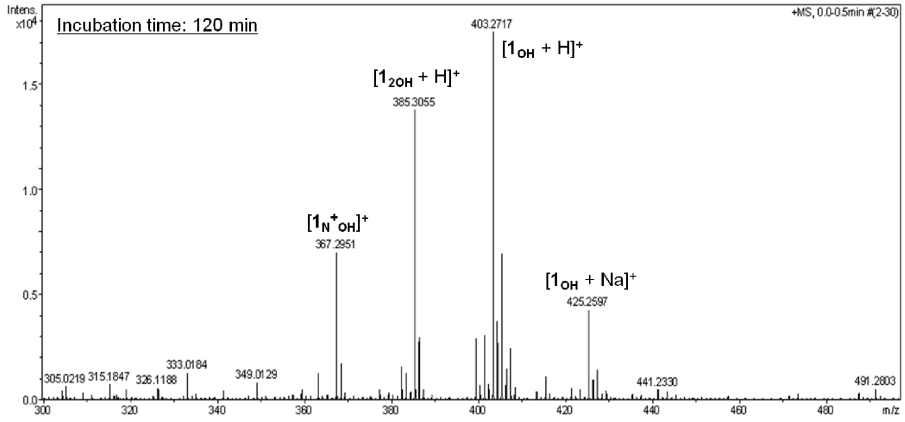

Figure S6. ESI-MS spectrum of compound **(1)** after 120 min of incubation at 37 °C in water.

Acknowledgments

This binational and interdisciplinary dissertation is the achievement of a unique and outstanding teamwork. I would like to convey my profound gratitude to each and every one of you who has participated in the successful realisation of this project...

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"Leider lässt sich eine wahrhafte Dankbarkeit mit Worten nicht ausdrücken"

"Unfortunately we are unable to express in words genuine gratitude"

Johann Wolfgang von Goethe