Triple-Helix Directed Cleavage of Double-Stranded DNA by Benzoquinoquinoxaline-1,10-phenanthroline Conjugates

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In memory of Professor Claude Hélène

Oligonucleotide-directed triple-helix formation provides a rational means to interfere with genomic DNA targets and to direct modifications at specific sites. We have developed a new class of compounds that, at low concentrations, efficiently targets and damages double-stranded DNA specifically at the site where a triple-helical structure is formed. In these new compounds, a triple-helix-specific intercalator—benzoquinoquinoxaline (BQQ)—was coupled to one of two isomeric 1,10-phenanthrolinecarboxaldehyde derivatives. 1,10-Phenanthroline derivatives are known to cleave DNA in the presence of copper ions. The obtained BQQ-1,10-phenanthroline (BQQ–OP) conjugates were compared with regard to their ability to cleave triple-helix DNA. Both conjugates displayed a sequence preference inside the triple-helical site, as judged from the more pronounced cleavage obtained at stretches of T·A×T base triplets.

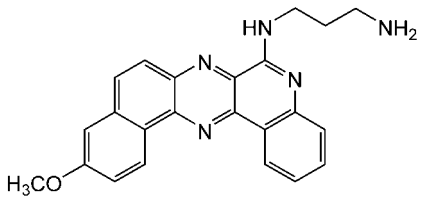
Introduction

Triple-helical structures of DNA have been extensively studied during the past two decades, in particular in the context of the antigene strategy for the control of gene expression.[1] Sequence-specific DNA recognition by an oligonucleotide that leads to the formation of a triple helix has largely been exploited in regulating gene expression at the transcriptional level, and in directing modifications of genomic DNA at selected sites through mutagenesis or homologous recombination.[2] Triple-helix forming oligonucleotides (TFOs) are therefore powerful gene-specific tools that can be employed in a wide range of applications in experimental biology as well as gene-based biotechnology and therapeutics. The formation of intermolecular triple-helical structures is based on the sequence-specific recognition, by an oligonucleotide, of an oligopyrimidine·oligopurine sequence of double-stranded DNA.[3,4] The third-strand oligonucleotide binds to the major groove of the double helix, forming hydrogen bonds with the purine bases.

Intramolecular triple-helical DNA structures, on the other hand, may be formed when oligopyrimidine·oligopurine mirror repeat sequences undergo conformational rearrangements while subjected to physical constraints and/or acidic conditions.[5] Thanks to the completion of the sequencing of the human genome and of those of several other organisms, and also to the rapid development of visualizing and imaging techniques, there is increasing interest in the study of intramolecular triple-helical structures that may be formed in living cells.[6]

DNA sequences capable of adopting any of the triple-helical structures described above constitute potentially attractive therapeutic targets. It therefore follows that synthetic molecules possessing specific affinity towards such triple-helical structures should be highly valuable tools for examining cellular mechanisms that regulate the expression of certain disease-related genes and the DNA damage-repair machinery.[6] Triplex-specific binding compounds should thus function as structural probes, specifically targeting the sites where DNA triple helices are formed.

We have recently reported the design and synthesis of a series of DNA triplex-cleaving conjugates in which analogues of the triple-helix-specific intercalating compound benzoquinoquinoxaline BQQ **1** carrying different aminoalkyl side chains were covalently linked to ethylenediaminetetraacetic acid



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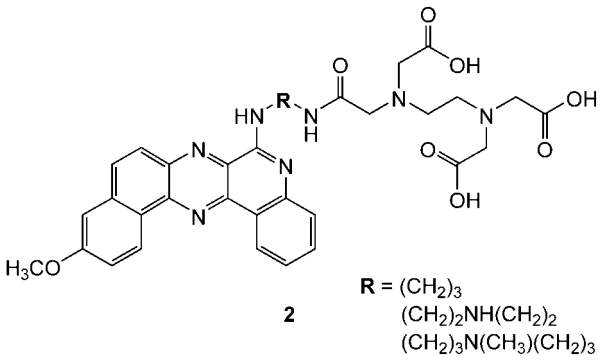
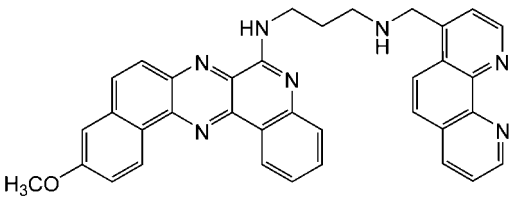
[EDTA·Fe^{II}].[7–9] In the presence of a reducing agent, EDTA·Fe^{II} complexes are believed to generate freely diffusible hydroxyl radicals, which in turn cause nucleic acid strand scission at solvent-accessible sites.[10,11] It was demonstrated that benzoquinoquinoxaline–EDTA·Fe^{II} conjugates **2** act as triplex-specific



2 **R** = $(CH_2)_3$, $(CH_2)_2NH(CH_2)_2$, $(CH_2)_3N(CH_3)(CH_2)_3$

cleaving agents, causing cleavage of double-stranded DNA at sites where a triple-helix structure is formed. Although these derivatives proved to have an efficient double-stranded DNA cleavage yield (72% at 5 μM BQQ-EDTA), their application in cells is expected to be limited. Indeed, it has been reported that EDTA·Fe^{II}-mediated cleavage of DNA was inhibited by the presence of Mg^{II}, among other metal ions, when present in stoichiometric amounts.[12]

In our search for triple-helix specific binding compounds that would function under physiological conditions, we have undertaken the synthesis of a new class of triplex-intercalating cleaving agents incorporating 1,10-phenanthroline (OP) units in their structures. 1,10-Phenanthroline derivatives are mainly known for their nucleic acid cleavage activity and can also act as antifungal agents.[13–18] Here we describe the first 1,10-phenanthroline-based intercalating probes—**3** and **4**—capable of recognising and binding triple-helical structures of DNA and subsequently directing double-stranded DNA damage specifically at the triplex site. Furthermore, quantitative analysis of the cleavage results showed that the new agents display an additional sequence-specific binding preference towards stretches located inside the targeted triple-helical structures.



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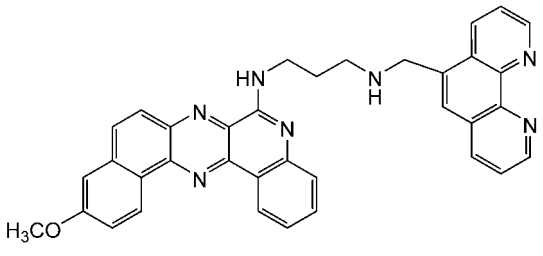

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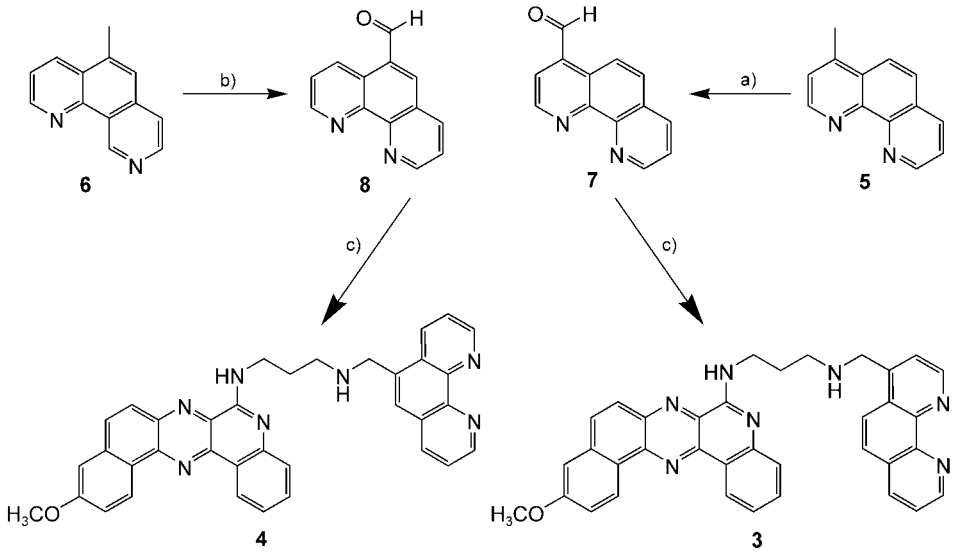
Results and Discussion

Design and synthesis of BQQ–OP derivatives 3 and 4

The ability of copper(II) complexes of 1,10-phenanthroline to cleave the phosphodiester backbones of DNA has been extensively studied for nearly two decades.[14,19–20] It has been shown that both $[(OP)_2Cu]^{2+}$ and oligonucleotide- or protein-conjugated OP-Cu^{II} are able to generate radical species that cause DNA and RNA cleavage even in the presence of Mg^{II} ions.[21–22] This "chemical nuclease" property has been widely exploited in the structural analysis of nucleic acids, such as in footprinting, and in the determination of secondary structures of ribosomal RNA.[21,23–27] Moreover, radical cleavage by Cu^{II}-1,10-phenanthroline has recently been used to probe RNA tertiary structures in cell cultures.[28] In contrast to those involved in EDTA·Fe-promoted cleavage of nucleic acids, the radical species generated in a copper(II)-phenanthroline reaction is not diffusible and will therefore only target the sugars located in the immediate vicinity of the DNA or RNA binding site. Consequently, conjugation of 1,10-phenanthroline to a structure-specific DNA-ligand, such as a triplex-intercalating compound, should provide an excellent way to cause distinct site-specific modifications and to probe the formation of DNA triple-helical structures. Here we have chosen to explore the chemical nuclease properties of benzoquinoquinoxaline-OP conjugates in targeting triple-helical structures of DNA and thereby to determine their potential as triplex-specific probes. Different methods of conjugating phenanthrolines with oligonucleotides or proteins, by using either 5-iodoacetamido- or 5-nitro-1,10-phenanthrolines, have appeared in the literature.[14,29] For our purposes we needed a different functional group on the 1,10-phenanthroline skeleton that would react efficiently with the primary aliphatic amino group in benzoquinoquinoxaline (BQQ, **1**) in order to give a stable, readily purified and easily handled product. We also planned to examine the two isomeric compounds derived from conjugation of BQQ to either the 4- or the 5-position of 1,10-phenanthroline so as to compare their triplex-binding specificity and cleavage efficiency. For these reasons, two 1,10-phenanthroline carboxaldehydes **7** and **8** (Scheme 1) were synthesized and condensed with BQQ **1** by a versatile and simple reductive amination protocol.

Oxidation of 4-methylphenanthroline **5** (Scheme 1) to give aldehyde **7** was achieved according to literature methods by use of selenium dioxide (SeO_2) in dioxane/water.[30] Unfortunately, these conditions were not applicable to the preparation of aldehyde **8** from 5-methylphenanthroline **6** (Scheme 1). Instead, the required aldehyde **8** was obtained by bromination of the methyl group in **6** by treatment with *N*-bromosuccinimide, followed by conversion of the derived bromomethyl



Scheme 1. *Synthesis of BQQ–OP conjugates* ***3*** *and* ***4****: a) SeO_2 in dioxane-water (96:4), b) SeO_2 in* ortho*-dichlorobenzene (please see safety recommendations in ref. [31]), c) 1) BQQ* ***1*** *in methanol/dimethylformamide (2:1), 2) $NaBH_4$ in dichloromethane/methanol (1:1).*

intermediate to the corresponding alcohol under hydrolytic conditions, and subsequent oxidation with MnO_2 (see Experimental Section). Despite the success of this method in providing the desired carboxaldehyde, it required three steps and the overall yield was low. We therefore reconsidered the use of SeO_2 under more drastic conditions, and indeed, when compound **6** and SeO_2 were heated for two hours at reflux in *ortho*-dichlorobenzene,[31] the 5-carboxaldehyde derivative **8** was obtained in 62% yield.

Benzoquinoquinoxaline **1** has been shown to intercalate and stabilize triple-helical DNA structures.[7] We have recently examined the effect of varying the length and character of the aminoalkyl side chain of BQQ **1** on the stabilizing properties of BQQ–EDTA derivatives **2**.[9] Triplex-directed cleavage was obtained in all three cases, although differences in the DNA cleavage efficiencies of these agents were observed. Most importantly, it was shown that the two conjugates with six- and eight-membered aminoalkyl linkers retain an affinity toward triple-helix structures comparable to that of **1** itself. So as to maintain a six-atom separation between the phenanthroline and BQQ units in our new conjugates, compounds **7** and **8** were condensed with a benzoquinoquinoxaline derivative possessing an aminopropyl side chain (**1**). Previous triplex-directed DNA cleavage experiments and modelling studies on conjugates **1** and **2** had indicated that the aminoalkyl side chain adopts a position in the minor groove.[7,8] It is anticipated on this basis that the 1,10-phenanthroline component in our new conjugates should similarly reside in, or close to, the minor groove.

The terminal amino group in the side chain of BQQ **1** reacted smoothly with both phenanthrolinecarboxaldehydes **7** and **8** (Scheme 1), and monitoring of the reaction mixture by ^1H NMR showed the gradual disappearance of the aldehyde proton signal. The derived imine intermediates were then reduced by sodium borohydride ($NaBH_4$), which provided BQQ–OP conjugates **3** and **4**, respectively, in pure form.

BQQ–OP-mediated double-stranded cleavage of DNA

Conjugation of 1,10-phenanthroline to TFOs had been reported earlier, and sequence-specific cleavage of double-stranded DNA at triplex sites was achieved even though these triple-helix structures are only moderately stable. In contrast, the intercalating-cleaving BQQ–OP complexes **3** and **4** would be expected to promote and stabilize the triple-helix structure formed in the presence of a TFO. We have demonstrated the specificity and efficiency of DNA cleavage by BQQ–OPs **3** and **4**, in the presence of a cognate TFO, on the following targets: an 80 bp restriction fragment, in order to study the cleavage pattern with nucleotide resolution, a 2719 bp double-stranded DNA fragment, and the corresponding supercoiled DNA plasmid, in order to assess the selectivity of the BQQ-mediated cleavage.

Triplex-directed cleavage of a short DNA fragment

We examined the ability of BQQ–OPs **3** and **4** to direct DNA double-stranded cleavage specifically to the triplex site and analysed the cleavage patterns of the two compounds on both oligopurine and oligopyrimidine strands (Figure 1 A and B). In separate experiments, we radiolabelled the 3′-end of either strand of an 80 bp DNA fragment. This fragment possesses a 27 bp oligopyrimidine·oligopurine sequence capable of forming an intermolecular triple-helix structure in the presence of a specific TFO.[32] The cleavage experiments were initiated by hybridization of the double-stranded DNA with the specific 27-nt TFO (S) thus forming a triple helix. In parallel, incubation of the DNA with a non-specific oligonucleotide (NS) of equal length was used as a control. The different BQQ–OP derivatives **3** and **4** were evaluated for their ability to perform triplex-directed cleavage. In the presence of TFO (S), Cu^{II} ion and 3-mercaptopropionic acid (MPA), both BQQ–OP derivatives caused double-stranded cleavage of the 80 bp DNA fragment specifically at the site where a triple-helix structure was formed (Figure 1 A and B, lanes 2 and 4). On the other hand, replacement of the specific triplex-forming third strand with a control oligonucleotide (NS) that is not capable of forming a triple-helix structure did not lead to any cleavage under the same experimental conditions (Figure 1 A and B, lanes 1 and 3).

Analysis of the resulting double-stranded cleavage patterns by BQQ–OP derivatives was carried out by use of the Image

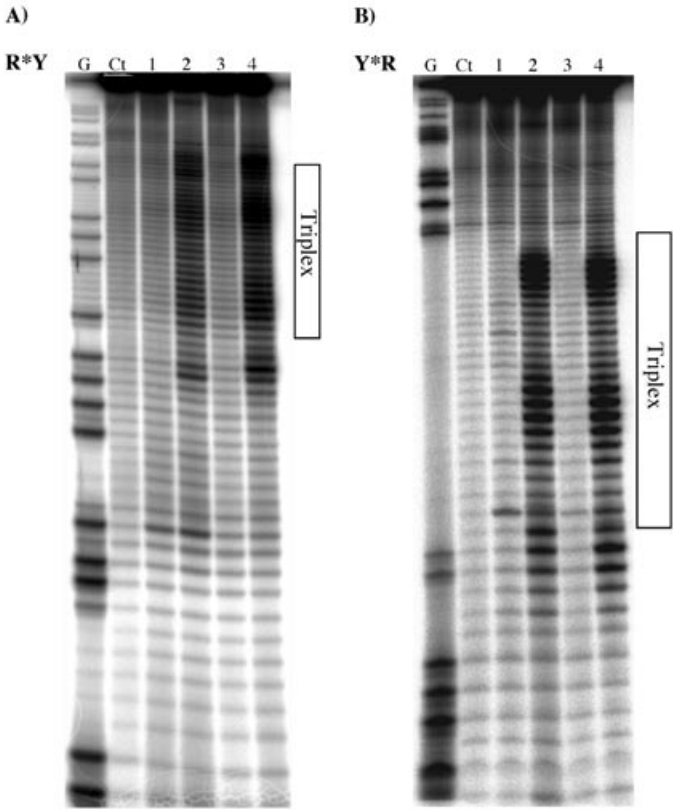


Figure 1. *Triplex-directed cleavage of an 80-mer double-stranded DNA by BQQ–OPs **3** and **4**. **A**) The purine-rich strand (R*) of the double helix is ^{32}P-radiolabelled at the 3′-end. Lanes 1 and 2: cleavage by BQQ–OP **4** in the presence of a non-specific (NS) and a specific (S) third strand oligonucleotide. Lanes 3 and 4: cleavage by BQQ–OP **3** in the presence of a non-specific (NS) and a specific (S) third strand oligonucleotide. G-sequence ladder of R*-strand is marked G. Ct is the control untreated 80 bp double-stranded DNA. **B**) The pyrimidine-rich strand (Y*) is ^{32}P-radiolabelled at the 3′-end. Lanes 1 and 2: cleavage by BQQ–OP **4** in the presence of a non-specific and a specific third strand oligonucleotide. Lanes 3 and 4: cleavage by BQQ–OP **3** in the presence of non-specific and specific third strand oligonucleotide, respectively. G-sequence ladder of Y*-strand is marked G. Ct is the control untreated 80 bp double-stranded DNA.*

Gauge (Fujifilm) quantification program. Interestingly, as judged from the intensities of the bands inside the triple-helix site, we observed that the double-stranded cleavage by the two reagents **3** and **4** was not of equal strength over the entire triplex site (Figure 1). We identified the nucleotides in the oligopyrimidine•oligopurine sequence that had been exposed to the highest rate of cleavage by use of G-sequencing ladders. Figure 2 shows overlay plots representing the quantified cleavage of the two DNA strands by BQQ–OP **4**. Analysis of the plots indicated the presence of two major cleavage sites, which correlated to two stretches of T•A×T base triplets (• and × represent Watson–Crick and Hoogsteen hydrogen bonding, respectively). DNA strand scission by metal complexes of 1,10-phenanthroline is known to be a localized event, owing to the character of the radical species produced in situ. This could, in part, explain the detected sequence-specific cleavage pattern of double-stranded DNA by BQQ–OP derivatives. DNA cleavage by BQQ-EDTA-Fe^{II} derivatives, on the other hand, indicated a similar but only weak sequence-specific preference in the triplex site under the same experimental conditions.[8] It is further noteworthy that conjugation of BQQ to either the 4- or the 5-position of 1,10-phenanthrolinecarboxaldehyde did not seem to affect the sequence-specific intercalation of these cleaving agents, as they showed similar cleavage patterns (Figure 1 A and B) for both DNA strands. These data highlight a new and interesting aspect of triplex-specific probing of nucleic acids: the two newly designed cleaving agents could preferentially target certain regions inside the triple-helix site. However, triple helices with a broad diversity of sequence composition will have to be investigated in order to confirm the versatility of these findings. Studies along these lines are in progress in our laboratories.

Triplex-directed cleavage of longer DNA fragments by BQQ–OPs **3** and **4**

We initially examined the triplex-specific cleavage of long DNA fragments by BQQ–OP derivatives **3** and **4** through the use of a 2718 bp linearized plasmid (pTLX).[32] This plasmid is a construct derived from a pUC12 vector where a 27 bp oligopyrimidine•oligopurine sequence has been cloned. The linear DNA fragment was obtained by digestion of pTLX with the unique-site restriction enzyme Xmn I, and was purified by precipitation. Hybridization with TFO(s) led to the formation of a triple-helix structure. This was followed by the addition of the copper-chelated BQQ–OP compound **3** or **4**, and the cleavage reaction was initiated by addition of 3-mercaptopropionic acid. Different concentrations of **3** and **4** were examined in order to determine the optimum concentration leading to the most efficient triplex-directed cleavage. Double-stranded cleavage of DNA at the triplex site would produce two distinct fragments of approximately 1901 and 817 bp, if the cleavage were to occur on average in the middle of the formed triplex. Indeed, cleavage by the two BQQ–OP derivatives **3** and **4** at all the examined concentrations generated two fragments that correlated with the expected size of fragments, as shown in Figure 3 (lanes 2, 4, 6, and 8). This indicates that Cu^{2+} complexes of BQQ–OP were able to recognise, bind and cleave a rather long DNA fragment selectively at the site where a triple helix was formed. Quantitative analysis of the separated bands revealed that BQQ–OP **4** was the more efficient cleaving agent of the two synthesized derivatives, since it gave a 61% yield of cleavage at 0.5 μM, while on the other hand BQQ–OP **3** gave only 45% under the same experimental conditions. In addition, the new BQQ–OP **4** exhibits a much higher cleavage efficiency than the previously studied BQQ-EDTA conjugates **2** under similar reaction conditions.[8,9] Indeed, more elevated concentrations (5–50 μM) of BQQ-EDTA derivatives **2** are necessary to obtain comparable yields of DNA cleavage.

Control experiments were carried out in which TFO (S) was replaced by a non-complementary oligonucleotide (NS). No cleavage could be detected in any of these experiments, as shown in Figure 3 (lanes 1, 3, 5 and 7). Our results clearly indicate that the observed DNA scission by BQQ–OP derivatives is indeed an event specific to the presence of a triple-helix structure and that BQQ–OP **4** is the most efficient triplex-specific

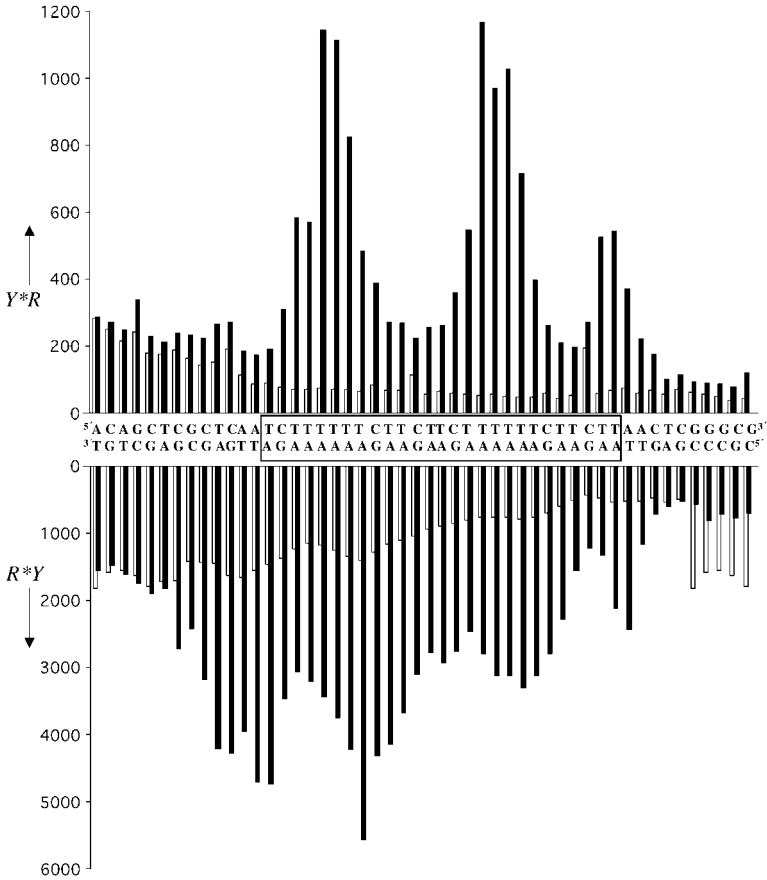


Figure 2. *Quantitative analysis of DNA cleavage by BQQ–OP* ***4*** *(Figure 1* ***A*** *and* ***B****, lanes 1 and 2). Radioactivity of each of the bands obtained from cleavage of R* (bottom) and Y* strand (top) is quantified by phosphorimager. The sequence is shown in the middle. The open and filled bars represent the DNA-cleavage profiles in the presence of a non-specific and a specific oligonucleotide, respectively. Major cleavage sites correspond to stretches of T·A×T base triplets (· and × represent Watson–Crick and Hoogsteen hydrogen bonding, respectively).*

cleaving agent among all BQQ-cleaving conjugates studied until now.

Cleavage of plasmid DNA by BQQ–OP derivative 4

In order to investigate the outcome of the cleavage reaction by BQQ–OP **4** when a supercoiled DNA plasmid was targeted, we carried out triplex formation and triplex-directed cleavage with supercoiled plasmid pTLX. In the presence of a specific TFO (S), Cu^{2+} and a reducing agent, BQQ–OP **4** generated a major linear fragment that was quantified to 66% of the total treated DNA (Figure 4, lane 3). When this fragment was cleaved by a unique-site restriction enzyme (Xmn I), two shorter fragments were produced, corresponding to approximately 1901 and 817 bp (lane 5). Furthermore, the total value of these two bands upon quantification also gave 66% of the treated DNA, indicating that the linear fragment generated by BQQ–OP **4** (lane 3) was indeed the result of a specific cleavage at the triplex site. Incubation of the DNA plasmid with a non-specific oligonucleotide (NS) followed by treatment with BQQ–OP derivative **4** and Cu^{2+} under the same cleavage conditions was used as a control. In the absence of a triple-helical structure, only a weak linear fragment was detected (Figure 4, lane 2). The only product generated from the subsequent cleavage of that fragment by Xmn I corresponded to a linear fragment of almost the same size as the linearized 2718 bp pTLX (Figure 4, lane 4). Similar results were obtained when the plasmid was first linearized with Xmn I and then cleaved with BQQ–OP **4** (Figure 4, lanes 6 and 7). These results indicate that the new cleaving agent BQQ–OP **4** is able to intercalate in the supercoiled DNA plasmid, discriminating between double and triple-helix structures, and to cause efficient double-stranded cleavage at quite low concentrations.

Conclusion

Two major related topics are described in this work. Firstly, the employment of a 1,10-phenanthrolinecarboxaldehyde functional group in the synthesis of phenanthroline conjugates offers a very distinct advantage over other known methods for the coupling of BQQ or other DNA-binding compounds, as both the synthetic procedure and product purification are simple and efficient. This strategy will very likely find a general application in the conjugation of 1,10-phenanthroline derivatives to different desired carrier ligands.

Secondly, examination of the obtained BQQ-1,10-phenanthroline (BQQ–OP) conjugates as cleaving agents specific to triple-helix sites represents a key step in the search for suitable triplex-specific probes that might act in a structure-dependent manner under physiological conditions. We showed that, in the presence of Cu^{II} ions and a reducing agent, BQQ–OP conjugates were able to cleave double-stranded DNA specifically at the site where a triple helix was formed. In particular, BQQ–OP conjugate **4** showed the highest cleavage efficiency of all BQQ-cleaving agents so far studied, at low concentration. These compounds may therefore prove to be potential candidates for probing the formation of both inter- and intramolecular DNA triple-helical structures in cells, particularly as DNA cleavage by OP derivatives is not inhibited by the presence of magnesium(II) ions. Further studies will hopefully show that BQQ–OP derivatives can be used as biologically active molecules able to direct DNA damage at selected genomic sites and as valuable tools in the examination of triplex-related biological processes.

Experimental Section

Synthesis of 1,10-phenanthroline-4-carboxaldehyde (7): Oxidation of 4-methyl-1,10-phenanthroline (**5**) was achieved according to the literature.[30] A solution of this compound (1 g) in dioxane/water (30 mL, 96:4) was slowly added to a warm homogenous solution of selenium dioxide SeO_2 (1.22 g) in dioxane/water (30 mL, 96:4). The reaction mixture was heated at reflux for 2 h and was

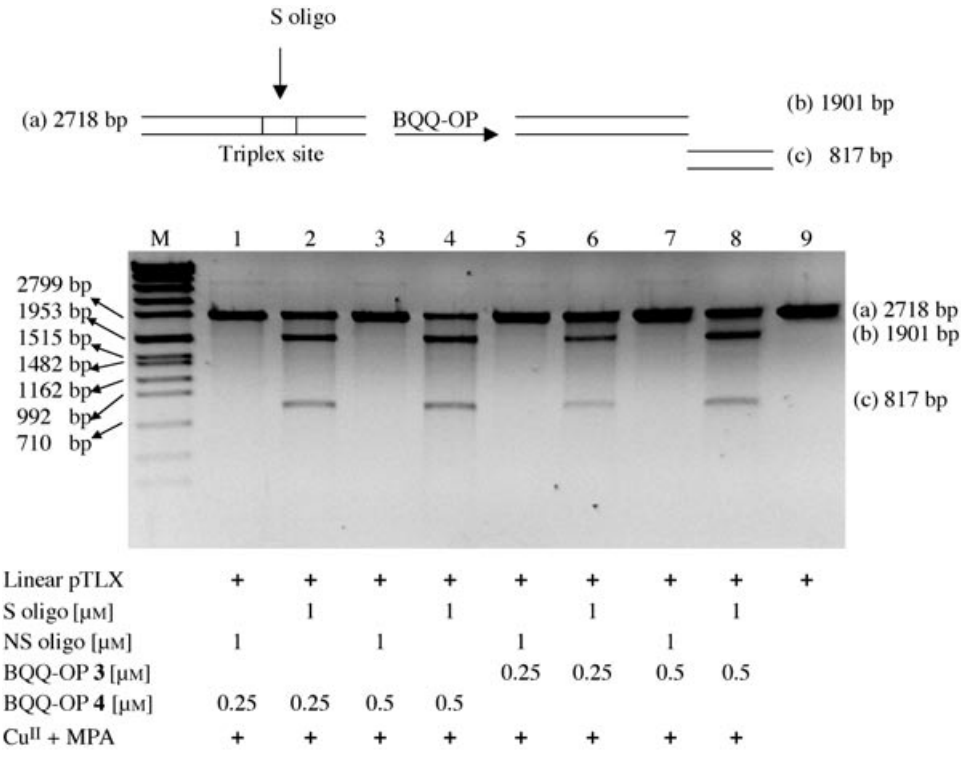


Figure 3. *Triplex-directed cleavage of a 2718 bp DNA fragment by BQQ–OPs **3** and **4**. S and NS oligo are 27-mer specific and nonspecific oligonucleotides, respectively. M is a DNA molecular weight marker VII (Roche Diagnostics). Concentration of oligonucleotides and conjugate **3** and **4** are indicated. a) Full length DNA fragment; b) and c) are the two fragments produced by the triplex-directed cleavage.*

then passed through a pad of celite 524. The solvent was concentrated at reduced pressure, and the residue was dissolved in dichloromethane and washed by repeated extraction with water. The organic layers were collected, and the solvent was evaporated. The crude product was purified by neutral Al_2O_3 gel column chromatography (7% water) with dichloromethane/ethanol (95:5) as eluent (50% yield). ^{1}H NMR ($CDCl_3$): $\delta = 10.62$ (s, 1H; Ar–CHO), 9.52 (d, $J = 4.4$ Hz, 1H; Ar–H), 9.31 (dd, $J = 4.4$, 1.7 Hz, 1H; Ar–H), 9.08 (d, $J = 9.2$ Hz, 1H; Ar–H), 8.38 (dd, $J = 8.1$, 1.7 Hz, 1H; Ar–H), 8.06 (d, $J = 4.4$ Hz, 1H; Ar–H), 8.03 (d, $J = 9.2$ Hz, 1H; Ar–H), 7.77 ppm (dd, $J = 8.2$, 4.5 Hz, 1H; Ar–H).

Synthesis of 1,10-phenanthroline-5-carboxaldehyde (8): Two different methods were employed for the oxidation of 5-methyl-1,10-phenanthroline.

A) Three-step procedure: 5-Methyl-1,10-phenanthroline (**6**, 0.415 g) was suspended in dry carbon tetrachloride (CCl_4, 40 mL) and the solution was maintained under a nitrogen atmosphere. *N*-Bromosuccinimide (0.456 g) and benzoyl peroxide (0.052 g) were added, and the solution was heated at reflux for 45 min. A reddish solid that formed was filtered off and washed twice with CCl_4. The supernatant was evaporated at reduced pressure. In situ hydrolysis of the obtained bromomethyl derivative was achieved by treatment of the crude product (0.8 g), in dioxane (6 mL), with

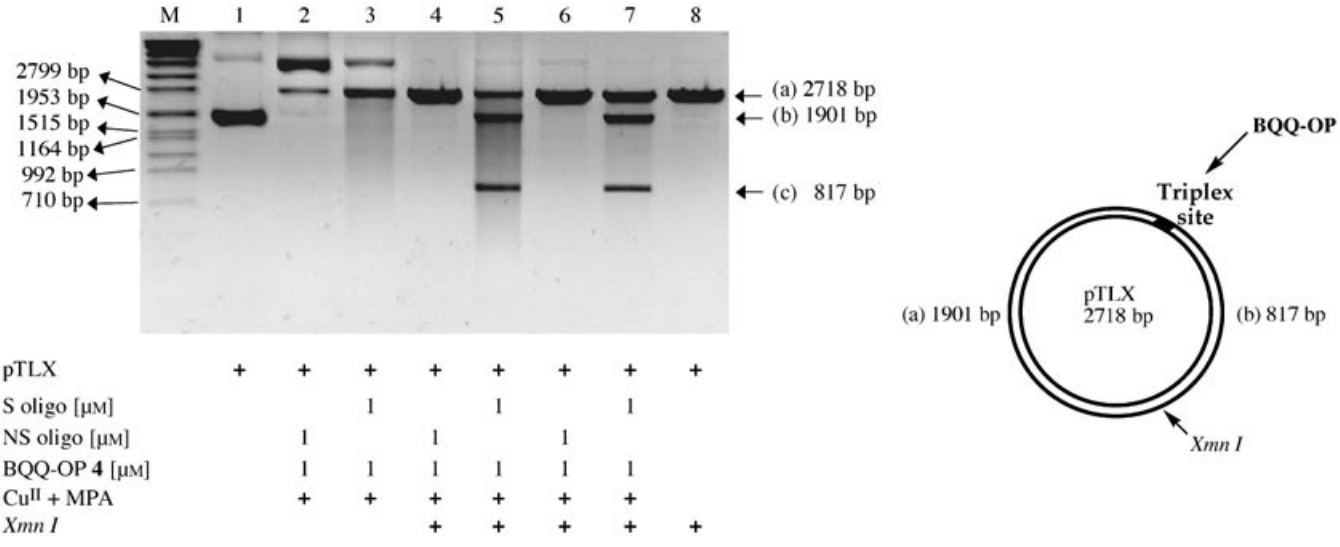


Figure 4. *Triplex-directed cleavage of supercoiled and linearized plasmid (2718 bp) by BQQ–OP **4.** S and NS oligo are 27-mer specific and non-specific oligonucleotides, respectively. M is a DNA molecular weight marker VII (Roche Diagnostics). Concentration of oligonucleotides and conjugate **4** are indicated. Lanes 1 and 8 are supercoiled and linearized plasmid, respectively. Lanes 3 and 7 are triplex-directed cleavage of supecoiled and linearized plasmid by conjugate **4**, respectively. Lanes 2 and 6 are control cleavage reactions of supecoiled and linearized plasmid, respectively, in the presence of a non-specific oligonucleotide. Lanes 4 and 5 are the respective products of lanes 2 and 3 after treatment with Xmn I. a) Full-length 2718 bp linear fragment; b) and c) are the two fragments obtained by cleavage mediated by BQQ–OP **4**.*

potassium hydroxide (2 N, 60 mL) for 28 h with stirring at room temperature. The mixture was then concentrated to a small volume, and dichloromethane was added. After several aqueous washings, the organic layer was dried over magnesium sulfate and the solvent was evaporated. The crude hydrolyzed product (0.185 g) was dissolved in dichloromethane (10 mL) and treated with manganese dioxide (0.6 g) for 43 h with stirring at room temperature. The precipitate was filtered off, and the solvent was evaporated. The pure compound was obtained in 20% overall yield after purification by neutral Al_2O_3 gel column chromatography (7% water) with dichloromethane as eluent. MS [CI]: 209.0718 $[M+H]^+$; $C_{13}H_9N_2O$ calcd 209.0715.

B) Oxidation with selenium dioxide: 5-Methyl-1,10-phenanthroline **6** (0.3 g) and SeO_2 (0.38 g) were suspended in *ortho*-dichlorobenzene (50 mL) and the mixture was heated at reflux for two hours (because of the incompatibility of *o*-dichlorobenzene and oxidising agents[31] the use of a shield to protect the experimenter is recommended), and then cooled to room temperature. Filtration through a celite pad was followed by the addition of hydrochloric acid (1 N) to the filtrate. The aqueous phase was collected and washed with dichloromethane and then neutralized by the addition of saturated aqueous sodium bicarbonate. Dichloromethane was added, and extraction was repeated several times. The organic layers were dried over magnesium sulfate, and the solvent was evaporated at reduced pressure. The pure product was isolated as a white powder after purification by neutral Al_2O_3 gel column chromatography (7% water) with dichloromethane as eluent (62% yield). ^{1}H NMR ($CDCl_3$): $\delta = 10.15$ (s, 1H; Ar–CHO), 9.48 (dd, $J = 8.5$, 1.6 Hz, 1H; Ar–H), 9.10 (m, $J = 11.4$, 4.2, 1.5 Hz, 2H; Ar–H), 8.18 (dd, $J = 8.1$, 1.6 Hz, 1H; Ar–H), 8.09 (s, 1H; Ar–H), 7.56 ppm (m, 2H; Ar–H); MS [CI]: 209.0712 $[M+H]^+$; $C_{13}H_9N_2O$ calcd. 209.0715.

Synthesis of 4- and 5-methyl-1,10-phenanthroline conjugates of 6-((3-aminopropyl)amino)-11-methoxy-benzo[f]quino[3,4-b]quinoxaline (3 and 4): BQQ **1** (0.05 g) and 1,10-phenanthroline-4-(or 5)-carboxaldehyde (**7** or **8**, respectively, 0.022 g) were suspended in dried methanol/dimethylformamide (12 mL, 2:1), and the mixture was heated for 6 h at 70 °C. The precipitate was filtered and dried at reduced pressure. The crude imine intermediate was reduced without further purification according to the following protocol. The crude imine was dissolved in dichloromethane/methanol (12 mL, 1:1) and the mixture was cooled to 0 °C. Sodium borohydride ($NaBH_4$, 10 mg) was added in small portions, and the reaction mixture was stirred at room temperature for 1.5 h. The reaction was stopped by the addition of hydrochloric acid (0.5 M, 2 mL), and dichloromethane was added. After repeated washings with saturated aqueous sodium carbonate, the organic layers were dried over magnesium sulfate, and the solvent was evaporated at reduced pressure. The pure product was obtained after purification by neutral Al_2O_3 gel column chromatography (7% water) with dichloromethane/ethanol (99:1) as eluent.

BQQ–OP **3** (55% yield): ^{1}H NMR ($CDCl_3$): $\delta = 9.34$ (d, $J = 9.0$ Hz, 1H; Ar–H), 9.08 (m, 2H; Ar–H), 8.95 (d, $J = 7.8$ Hz, 1H; Ar–H), 7.91(m, 4H; Ar–H), 7.68 (d, $J = 9.2$ Hz, 2H; Ar–H), 7.39 (m, 6H; Ar–H), 4.43 (s, 2H; –N–CH_2–Ar), 4.03 (m, 5H; OCH_3 + CH_2-α), 3.13 (t, $J = 6.1$ Hz, 2H; CH_2-χ), 2.25 ppm (q, $J = 5.9$ Hz, 2H; CH_2-β); MS [CI]: 576.2512 $[M+H]^+$; $C_{36}H_{30}N_7O$ calcd. 576.2512.

BQQ–OP **4** (51% yield): ^{1}H NMR ($CDCl_3$): $\delta = 9.36$ (d, $J = 9.1$ Hz, 1H; Ar–H), 9.1 (m, 2H; Ar–H), 9.0 (d, $J = 8.8$ Hz, 1H; Ar–H), 8.53 (d, $J = 8.8$ Hz, 1H; Ar–H), 8.03 (d, $J = 8.8$ Hz, 1H; Ar–H), 7.84 (d, $J = 9.5$ Hz, 2H; Ar–H), 7.66 (d, $J = 8.9$ Hz, 2H; Ar–H), 7.5 (m, 7H; Ar–H), 4.34 (s, 2H; N–CH_2–Ar), 4.0 (m, 5H; OCH_3 + CH_2-α), 3.06 (t, $J = 6.6$ Hz, 2H; CH_2-χ), 2.17 ppm (q, $J = 6.3$, 2H; CH_2-β). MS [CI]: 576.2517 $[M+H]^+$; $C_{36}H_{30}N_7O$ calcd. 576.2512.

Triplex-directed cleavage of an 80 bp DNA fragment by BQQ–OPs 3 and 4: Two probe samples of an 80 bp DNA fragment incorporating an oligopyrimidine·oligopurine target were prepared as follows.[32] The double-stranded DNA fragment was obtained from plasmid pTLX by restriction enzyme digestion with Hind III and EcoR I. The probes were ^{32}P-radiolabelled at the 3′-end of either the oligopurine- (R*Y) or the oligopyrimidine-rich strand (Y*R), respectively. The probes were incubated overnight at +4 °C in 20 mM cacodylate buffer (pH 7.0, 50 mM NaCl) with either a specific (S) or non-specific (NS) oligonucleotide (Eurogentec). The sequences of the 27-nt specific and nonspecific oligonucleotides were 5′-TTCTTCTTTTTTCTTCTTCTTTTTTCT-3′ and 5′-TCTCTCTCTCTC-TCTCTCTCTCTCTCT-3′, respectively. BQQ–OP **3** or **4** (1 μM) was pre-chelated with $CuSO_4$ (2 μM, 15 min) prior to addition to each of the probes. The DNA probes were incubated with the intercalator/cleaving metal complex for 45 min at room temperature, and MPA (2 mM) was added in order to initiate the reaction. The cleavage reactions proceeded for 1 h at 37 °C and were stopped by ethanol precipitation. The samples were analysed by 15% denaturing acrylamide gel electrophoresis (7 M urea).

Triplex-directed cleavage of supercoiled and linear pTLX: A 2718 bp supercoiled DNA plasmid (pTLX) or a linearized 2718 bp fragment was incubated with a specific (S) or a nonspecific (NS) third-strand oligonucleotide in cacodylate buffer (20 mM, pH 7.0, 50 mM NaCl) at +4 °C overnight. Different final concentrations of BQQ–OP **3** or BQQ–OP **4** were used: 0.25 μM, 0.5 μM and 1 μM. The BQQ–OP derivatives were pre-incubated with $CuSO_4$ (2 μM) at room temperature for 15 min. The mixtures of BQQ–OP **3** or **4** and $CuSO_4$ were added to the supercoiled plasmid or the linear DNA fragment, and intercalation was allowed to proceed for 45 min at room temperature. The cleavage reactions were initiated by the addition of MPA (2 mM). The reactions were allowed to proceed for 7 h at 37 °C and were analysed by standard 1% agarose gel electrophoresis and ethidium bromide staining. Quantification of the separated bands was carried out by use of Image Quant software.

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