Design of a triple-helix-specific cleaving reagent

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Background: Double-helical DNA can be recognized sequence specifically by oligonucleotides that bind in the major groove, forming a local triple helix. Triplex-forming oligonucleotides are new tools in molecular and cellular biology and their development as gene-targeting drugs is under intensive study. Intramolecular triple-helical structures (H-DNA) are expected to play an important role in the control of gene expression. There are currently no good probes available for investigating triple-helical structures. We previously reported that a pentacyclic benzoquinoquinoxaline derivative (BQQ) can strongly stabilize triple helices.

Results: We have designed and synthesized the first triple-helix-specific DNA cleaving reagent by covalently attaching BQQ to ethylenediaminetetraacetic acid (EDTA). The intercalative binding of BQQ should position EDTA in the minor groove of the triple helix. In the presence of Fe^{2+} and a reducing agent, the BQQ–EDTA conjugate can selectively cleave an 80 base pair (bp) DNA fragment at the site where an oligonucleotide binds to form a local triple helix. The selectivity of the BQQ–EDTA conjugate for a triplex structure was sufficiently high to induce oligonucleotide-directed DNA cleavage at a single site on a 2718 bp plasmid DNA.

Conclusions: This new class of structure-directed DNA cleaving reagents could be useful for cleaving DNA at specific sequences in the presence of a site-specific, triple-helix-forming oligonucleotide and also for investigating triple-helical structures, such as H-DNA, which could play an important role in the control of gene expression *in vivo*.

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Introduction

Oligonucleotides can bind to the major groove of double-helical DNA in a sequence-specific manner [1,2]. Recognition involves the formation of Hoogsteen or reverse Hoogsteen hydrogen bonds between the bases in the oligonucleotide and the purine bases of the DNA sequence. Stable complexes are formed at oligopyrimidine•oligopurine sequences of DNA, where the third strand winds smoothly around the double helix, within the major groove, to form a local triple helix. This sequence-specific recognition forms the basis of the 'antigene strategy' aimed at controlling gene expression at the transcriptional level by triplex-forming oligonucleotides [3]. Triple-helical structures may also form in DNA at oligopyrimidine•oligopurine sequences with mirror symmetry that undergo a conformational rearrangement when subject to constraints such as torsional stress (reviewed in [4]). This so-called H-DNA structure involves a triple-helical and a single-stranded region. The formation of H-DNA might play a role in the control of gene expression even though this has not been demonstrated yet under physiological conditions, in large part because we lack appropriate reagents to probe for such structures in long DNA fragments.

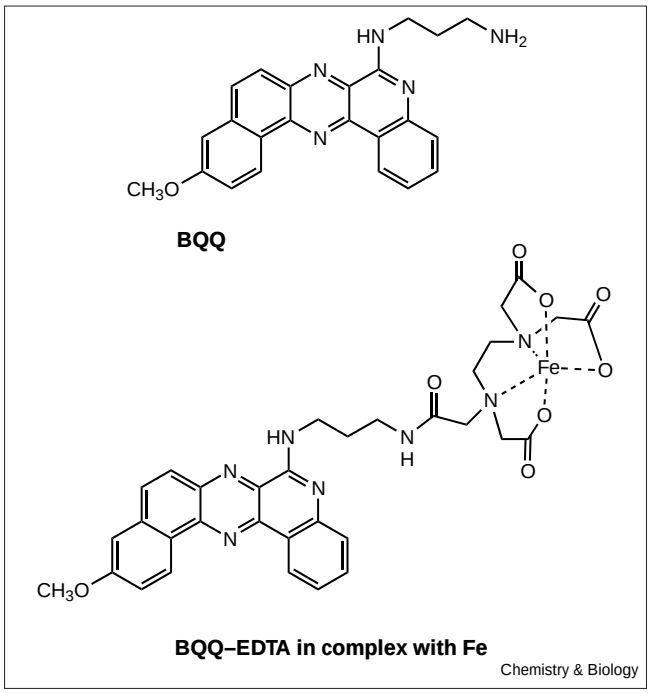
Much effort has been devoted to stabilizing triple-helical complexes either by introducing chemical modifications into the third-strand oligonucleotide (reviewed in [5]) or by designing triplex-specific ligands [6–14]. Among the latter are benzopyridoindole, benzoindoloquinoline and benzopyridoquinoxaline derivatives [6,10–13], which stabilize triple-helical complexes by intercalating between the base triplets [11]. We previously reported that a crescent-shaped, pentacyclic molecule, a benzoquinoquinoxaline derivative (BQQ; Figure 1) was a highly specific triplex-binding ligand [14]. Here we describe the first triplex-specific cleaving reagent, which consists of a highly stabilizing intercalator covalently linked to the well-known cleaving agent ethylenediaminetetraacetic acid (EDTA). This conjugate strongly discriminates between triplex and duplex structures and induces a selective cleavage of double-stranded DNA at the site where the triple helix structure is formed. Selectivity for the triplex structures is clearly manifested on an 80 base pair (bp) DNA fragment, as well as on a plasmid DNA.

Results and discussion

Design of the BQQ–EDTA conjugate

We recently reported the design of BQQ as a triplex stabilizing agent [14]. The specificity of BQQ for a triplex

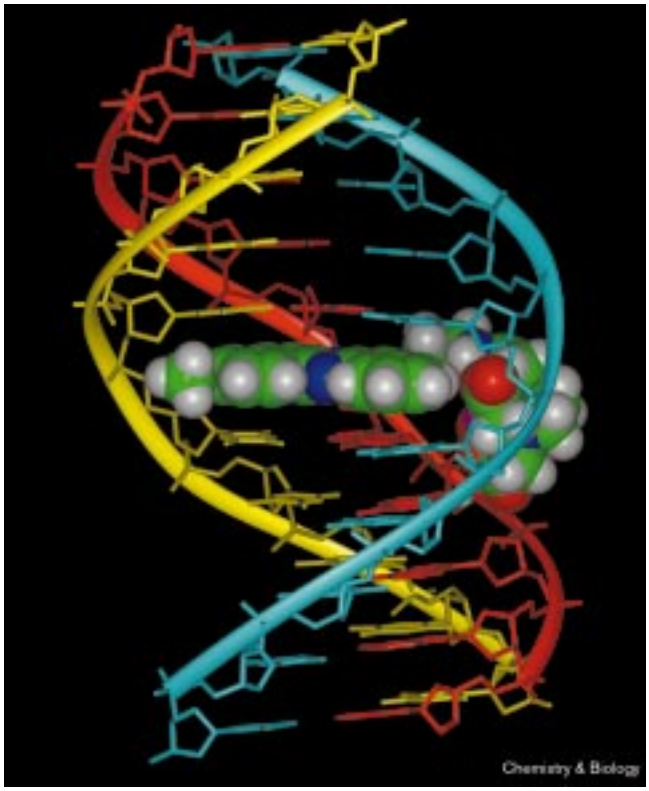
Figure 1



Chemical structures of (a) benzoquinoquinoxaline (BQQ) and (b) BQQ–EDTA conjugate. The syntheses of BQQ and BQQ–EDTA are described in the Materials and methods section (see Figures 6,7).

structure (as compared with a duplex structure) was evaluated from melting temperature (T_m) measurements on a 26 bp duplex (5′-TGTCAATTCTTCTTTTTTCTAAC-TCG-3′/3′-ACAGTTAAGAAGAAAAAAGATTGAGC-5′), which was used as a template to form a triple-helical complex of 14 base triplets with the third strand oligonucleotide d5′-TTCTTCTTTTTTCT-3′. When a solution of the complex formed by an oligonucleotide with a duplex DNA is heated, two successive transitions are usually observed: triplex ↔ duplex + third strand and duplex ↔ single strands. In the presence of a triplex-specific ligand, the first transition is shifted to higher temperatures. If the ligand also binds to the duplex, the second transition is also shifted to higher temperatures. The changes in melting temperatures ($\Delta T_m^{3\rightarrow2}$ and $\Delta T_m^{2\rightarrow1}$, respectively) give an indication of the affinity of the ligand for the triplex and duplex structures, respectively. For the sequence indicated above, we measured a $\Delta T_m^{3\rightarrow2}$ of 51°C and a $\Delta T_m^{2\rightarrow1}$ of 4°C in the presence of BQQ, which demonstrates that the ligand stabilizes triple-helical complexes strongly and also is highly selective for a triplex versus the corresponding duplex. No triplex-specific cleaving reagent has been described to date, however. Such a reagent might be used to provide evidence for triple-helical structures such as H-DNA in chromatin and to probe triple-helical complexes during transcription processes. We chose to conjugate the triplex-specific compound BQQ to EDTA, which is known

Figure 2



Energy-minimized model of BQQ–EDTA•Fe intercalated within a triple helix composed of T•AXT base triplets. The oligopyrimidine and oligopurine strands of Watson–Crick double helix are blue and red, respectively, and the third strand oligonucleotide is yellow. The hydrogen atoms in the triple helix are omitted for clarity. BQQ–EDTA•Fe is shown in space-filled representation with the aromatic rings intercalated within the triplex and the EDTA•Fe moiety located in the minor groove. Molecular modeling was carried out using JUMNA program package [21] and Insight II software (MSI, San Diego).

for its ability to cleave DNA. OH• radicals generated by the EDTA•Fe complex attack the deoxyribose sugars in the minor groove and lead to strand-cleavage reactions. This property was previously exploited to synthesize a methidiumpropyl–EDTA conjugate (MPE) that can cleave double-helical DNA upon intercalation of methidium between the base pairs [15].

Molecular modeling, in addition to experimental data, indicated that BQQ intercalates into the triple helix with its aminopropyl substituent located in the minor groove [14]. We therefore synthesized a BQQ–EDTA conjugate (Figure 1) in which the EDTA was attached to the aminopropyl sidechain of BQQ, which should position EDTA in the minor groove once the pentacyclic aromatic moiety is intercalated in the triple helix. The syntheses of BQQ and BQQ–EDTA are described in the Materials and methods section. Figure 2 shows a molecular model of BQQ–EDTA

intercalated in a triple-helical complex with the EDTA•Fe moiety located in the minor groove.

We have investigated whether this BQQ–EDTA can cleave DNA in a triple-helical complex and whether the cleavage reaction is specific for the triple-helical site embedded in a large double-helical DNA fragment.

Triplex-directed cleavage of DNA

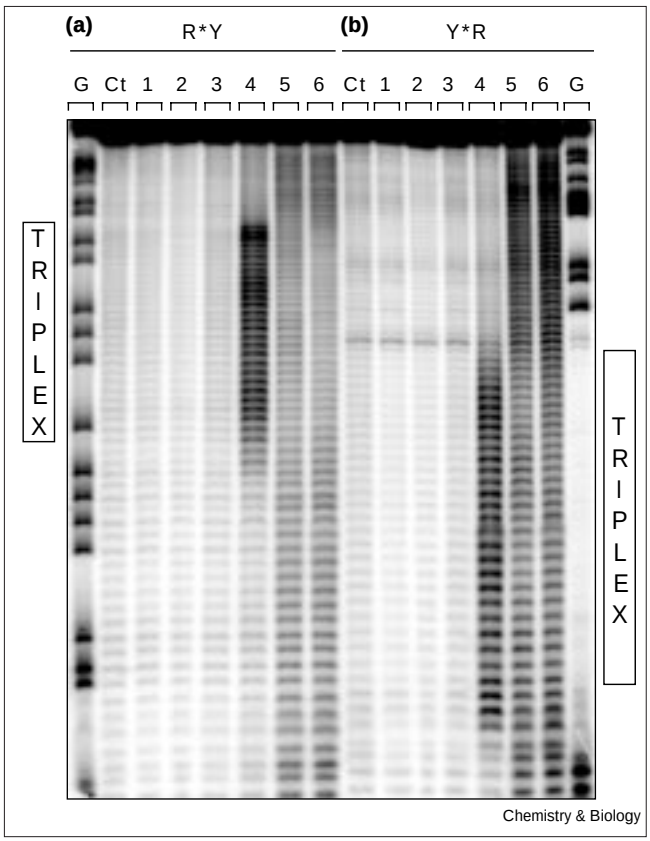
An 80 bp DNA fragment was used as a substrate for a 27 nucleotide (nt)-long pyrimidine oligonucleotide that can form a triple helix with a 27 bp oligopyrimidine•oligopurine segment of the 80 bp DNA fragment. The 27 nt oligonucleotide was a repeat of the 14 nt oligonucleotide used in the stability studies (see above), except for a missing T in the middle. As shown in Figure 3, in the presence of Fe^{2+} and a reducing agent, selective cleavage by BQQ–EDTA was observed in the presence of the triplex-forming oligonucleotide (lanes 4) within the triple-helical region on both strands of the DNA. In the presence of a control oligonucleotide (lanes 3), which could not form a triplex, no sequence-specific cleavage was observed. We also tested MPE on the same systems (lanes 5 and 6). As expected, MPE induced cleavage on both strands all along the duplex DNA fragment. In the presence of the triplex-forming oligonucleotide, weak inhibition of MPE cleavage was observed at the oligonucleotide binding site, indicating that MPE was more weakly bound to the triplex region than to the duplex.

A quantitative analysis of these gels was carried out. As shown in Figure 4, cleavage by BQQ–EDTA occurred throughout the region covered by the triplex-forming oligonucleotide. The cleavage sites on both strands were compared (Figure 4b). Although the cleavage intensity was not equal on the two strands, a shift of about two nucleotides was observed towards the 3′-end. This is a signature of cleavage occurring within the minor groove of DNA [16,17], indicating that the EDTA moiety of BQQ–EDTA is indeed located in the minor groove as shown in Figure 2.

Sequence-selective cleavage of plasmid DNA

The 80 bp DNA fragment was cloned into a pUC19 plasmid between *Eco*RI and *Hind*III sites. Cleavage experiments were carried out on the 2718 bp plasmid linearized by the restriction enzyme *Xmn*I. If cleavage by BQQ–EDTA occurs at the triplex site in the presence of a triplex-forming oligonucleotide, two fragments of ~1901 and 817 bp should be generated (these lengths were calculated as if cleavage occurred in the center of the triple-helix site; even though it was shown above that cleavage occurred all along the triplex domain). The gel shown in Figure 5 clearly shows that the expected fragments were observed in the presence of the triplex-forming oligonucleotide, but not in the presence of a nonspecific oligonucleotide. These experiments provide clear evidence for triplex-directed cleavage of the 2718 bp plasmid. The yield of the reaction was time- and concentration-dependent (Figure 5 and data not shown).

Figure 3

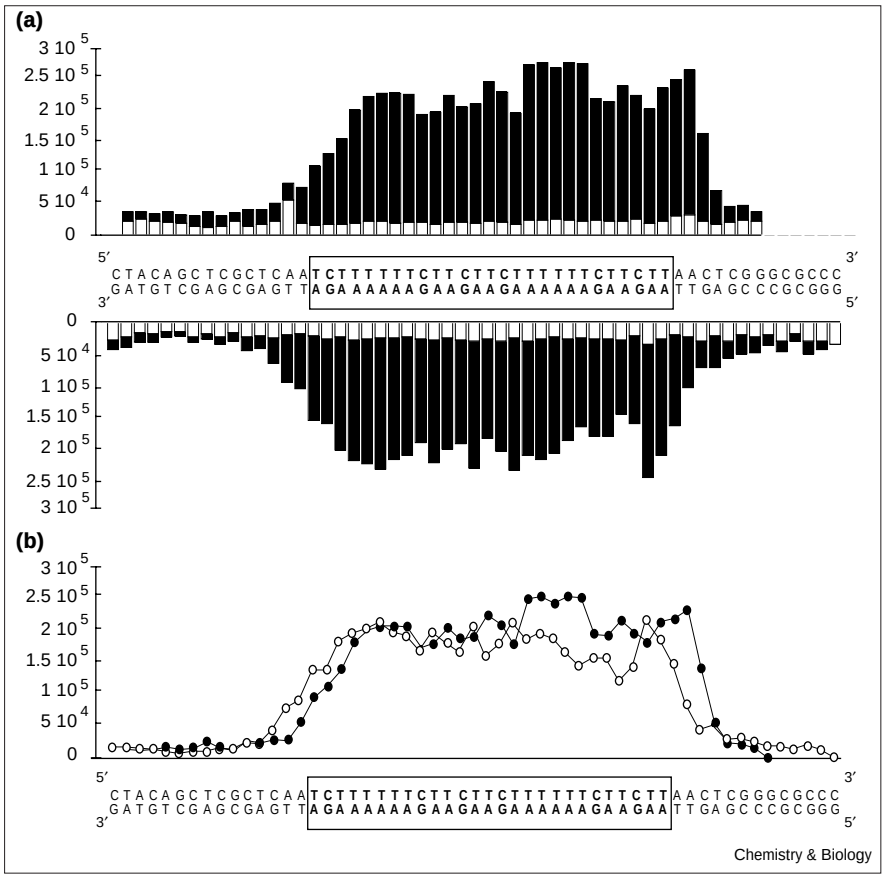


DNA cleavage by BQQ–EDTA•Fe and MPE•Fe on an 80 bp DNA fragment containing a 27 bp oligopyrimidine•oligopurine sequence. (a) and (b) show the DNA cleavage pattern when the oligopurine- or oligopyrimidine-containing strand was 3′-end radiolabeled (R*Y or Y*R), respectively. Two G-sequence lanes are shown on each side (lane G) and the triple-helix site is indicated. Samples were incubated in the presence of either a nonspecific (lanes 1,3 and 5) or a specific (lanes 2,4 and 6), triplex-forming oligonucleotide. Lanes 1 and 2, BQQ (5 μM) + EDTA (5 μM); lanes 3 and 4, BQQ–EDTA (5 μM); lanes 5 and 6, MPE (5 μM); Ct lanes serve as a control in the presence of 5 μM $Fe(NH_4)_2(SO_4)_2$ and 5 mM dithiothreitol (DTT).

Significance

It is thought that triple-helical structures could play an important role in the control of gene expression. There are currently no good probes available for investigating such structures. We previously reported a pentacyclic benzoquinoquinoxaline derivative (BQQ) that can strongly stabilize triple helices. Using molecular modeling and experimental studies of triplex-specific intercalating reagents we have now designed and synthesized the first triple-helix-specific DNA-cleaving reagent by covalently attaching BQQ to ethylenediaminetetraacetic

Figure 4

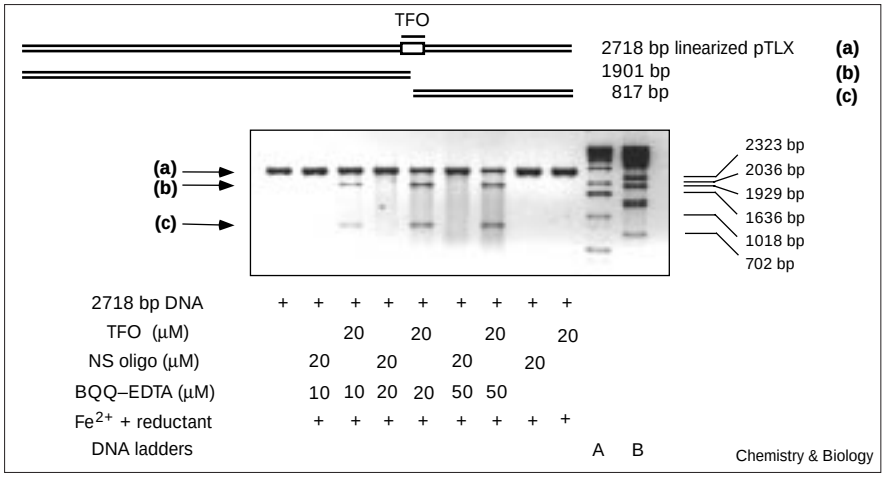


Analysis of DNA cleavage by BQQ–EDTA•Fe in the presence of nonspecific (lanes 3, Figure 3a,b) and specific (triplex-forming) oligonucleotide (lanes 4, Figure 3a,b). (**a**) Histogram of the radioactivity of each band as quantitated by PhosphorImager (Molecular Dynamics). The sequence is shown in the middle. The open and filled bars indicate the DNA-cleavage profiles in the presence of nonspecific and specific oligonucleotides, respectively, on the oligopyrimidine-containing (top) and oligopurine-containing (bottom) strands. The control lanes (Ct, Figure 3) gave a pattern similar to the open bars. (**b**) Overlay plot of the triple-helix-directed DNA cleavage on the oligopyrimidine strand (filled circles) and oligopurine strand (open circles), in the presence of the specific triplex-forming oligonucleotide.

acid (EDTA). The weak binding of BQQ to double-helical DNA and the strong stabilization of triple-helical complexes provide BQQ–EDTA with high selectivity for triple helices. This new compound possesses two important properties: it enhances the stability of the oligonucleotide-directed triple-helical structure and it cleaves DNA specifically at the triplex site.

In addition, conjugation of BQQ to a cleaving moiety (EDTA) provides structural information concerning the

Figure 5



Triplex-directed cleavage on a 2718 bp DNA fragment by BQQ–EDTA. TFO and NS oligo are 27-mer specific and nonspecific oligonucleotides, respectively (see the Materials and methods section). The concentration of oligonucleotides and BQQ–EDTA are indicated. Lanes A and B are DNA ladders taken from 1 kb ladder (GibcoBRL) and λDNA *Bst*II digestions, respectively. The electrophoresis was carried out on a 1% agarose gel in Tris-borate-EDTA buffer. Arrows indicate full-length plasmid (**a**) and the two fragments (**b**,**c**) obtained after sequence-specific cleavage.

triplex–ligand complex. Molecular modeling had suggested that BQQ intercalates in the triplex with its aminopropyl substituent in the minor groove [14]. The cleavage studies presented here demonstrate the presence of the aminopropyl sidechain in the minor groove.

Triple-helix formation requires oligopyrimidine•oligopurine sequences on DNA. Such sequences are overrepresented in eukaryotic genomes [18]. Furthermore, the range of recognition sequences by oligonucleotides can be extended (reviewed in [5]). This new reagent should, therefore, find a wide range of applications in site-specific cleavage of DNA. Its selectivity for triple-helical structures should make it a good probe for the presence of intramolecular triple helices, such as H-DNA. Studies along this line are in progress.

Materials and methods

Oligonucleotides (OligoGold grade) were synthesized by Eurogentec (Seraing, Belgium) or by Genosys and were used after ethanol precipitation. The length of oligonucleotides was controlled by electrophoresis. The syntheses of 6-methoxy-1-naphthylamine (**1**) and 4-chloro-3-nitro-quinoline (**2**) are described elsewhere [19,20]. All other chemical reagents including methidiumpropyl–EDTA (MPE) were purchased from either Sigma or Aldrich.

Synthesis of BQQ and the BQQ–EDTA conjugate (Figures 6, 7)

*m4-[1-(6-methoxy)naphthyl]-amino-3-nitro-quinolin-2-(1H)-one (**3**)*. 6-methoxy-1-naphthylamine (2.7 g) (**1**) [19] was added to a solution of **2** [20] (3.65 g) in ethanol (180 ml) and triethylamine (2.8 ml). The mixture was refluxed under nitrogen atmosphere for 18 h. It was concentrated and left to precipitate for 24 h at 20°C. The obtained solid was filtered, washed with ethanol (7 × 2 ml), and dried to provide brown microcrystals (3 g, 51% yield), which were directly used in the next reaction step.

*11-Methoxy-5H-benzo[f]quino[3,4-b]quinoxalin-6-one (**4**)*. 5.3 g of compound **3** and 1.7 g of sodium borohydride were added to a methanol solution (480 ml) of sodium methoxide (152 g). The mixture was refluxed for 18 h under mechanical stirring. 1.5 l of water was added and the methanol was evaporated. More water (1.4 l) was added and the mixture was cooled on ice (5–10°C). 5 N hydrochloric acid (565 ml) was added slowly followed by 14% aqueous ammonia in order to reach pH 7. The obtained precipitate was filtered, washed with water (1.2 l) and dried (4.5 g, 93% yield). 1H-NMR (DMSO-d_6), δ (ppm): 9.34 (d, 1H, H-1, J = 9.1 Hz); 8.97 (d, 1H, H-13, J = 10.3 Hz); 8.26 (d, 1H, H-8, J = 9.5 Hz); 8.1 (d, 1H, H-9, J = 9.2 Hz); 7.7 (m, 2 H, H-10, H-12); 7.54 (dd, 1H, H-2, J = 8.8 Hz, 2.6 Hz); 7.46 (m, 2H, H-3, H-4); 4.04 (s, 3 H, OCH_3).

*6-Chloro-11-methoxy-benzo[f]quino[3,4-b]quinoxaline (**5a**)*. A mixture of **4** (1.4 g), benzyltriethylammonium chloride (3.9 g), phosphorus oxychloride (22 ml) and acetonitrile (50 ml) was refluxed under nitrogen atmosphere for 4.5 h. The excess of phosphorus oxychloride was evaporated together with the solvent under reduced pressure. Ice (100 g) was added to the residue and the mixture was neutralized by the addition of 14% aqueous ammonia and then left overnight at room temperature. This afforded a precipitate, which was filtered, washed with water and dried under reduced pressure over P_2O_5. The product was converted to **5b** without any further purification (1.23 g, 82% yield). Analytical sample was obtained by recrystallisation from ethanol to provide yellow microcrystals, mp 266–267°C. Anal. calc'd for $C_{20}H_{12}N_3ClO$: C, 69.46; H, 3.47; N, 12.16; Cl, 10.27. Found: C, 69.37; H, 3.38; N, 12.29; Cl, 10.17. NMR data could not be obtained because of the very low solubility of this compound.

*6-[3-(Dimethylamino)propyl]amino-11-methoxy-benzo[f]quino-[3,4-b]quinoxaline (BQQ) (**5b**)*. Compound **5a** (0.6 g) was heated for 2 h at 100°C in the presence of a large excess of 1,3-diaminopropane (14 ml). The excess of the diamine was evaporated under reduced pressure. Water (50 ml) and then 28% aqueous ammonia (1 ml) were added to the residue. The obtained solid was filtered, washed with water and then dried. It was directly transformed into the water-soluble maleate salt (93% yield), mp 206°C. Anal. calc'd for

Figure 6

Synthesis of BQQ derivatives. (i) Reflux 18 h in EtOH/triethylamine. (ii) CH_3ONa (5N) in CH_3OH, $NaBH_4$ (3 eq.), reflux 18 h. (iii) $POCl_3$ (large excess), $BnNEt_3Cl$ (4 eq.) in CH_3CN, reflux 4.5 h. (iv) $H_2N(CH_2)_3NH_2$ (large excess), 100°C, 2 h.

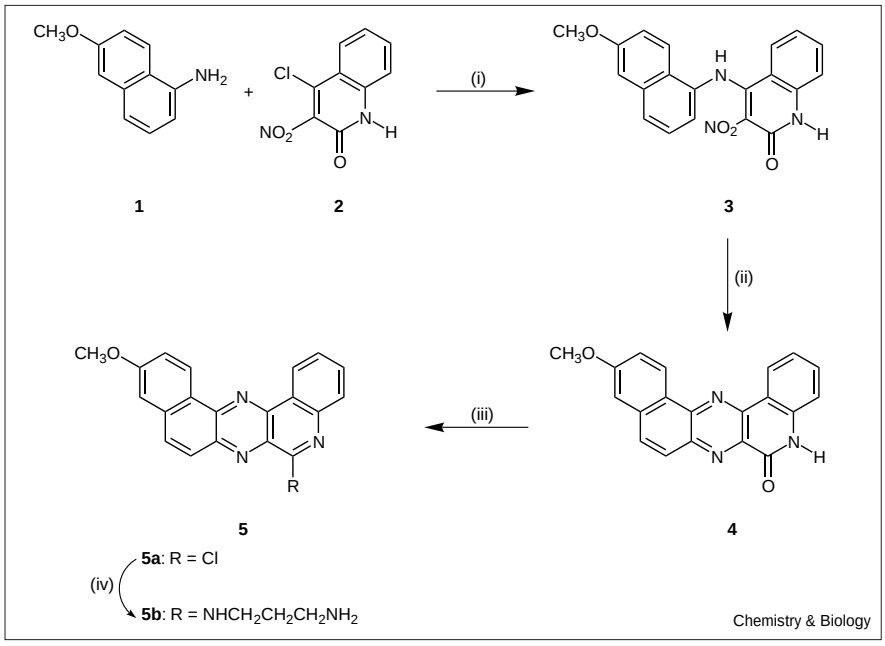
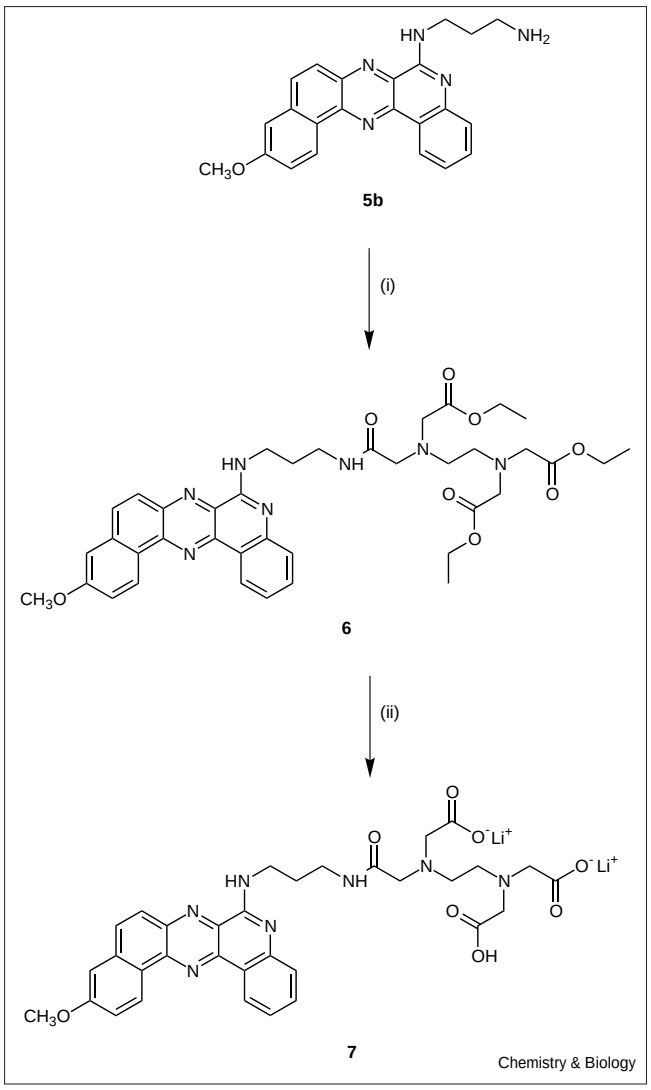


Figure 7



Synthesis of the BQQ–EDTA conjugate. (i) EDTA–TEE (1 eq.), DCC (2.1 eq.), HOBt (1.7 eq.) in DMF, 25 h. (ii) 0.2 M LiOH (6 eq.), in MeOH/H_2O 4:1, –5°C.

$C_{33}H_{33}N_5O_5$. 0.25 H_2O: C, 61.16; H, 5.17; N, 10.82. Found: C, 61.02; H, 5.08; N, 10.88. ^{1}H NMR (DMSO-d_6) δ (ppm): 9.39 (d,1H, H-1, J = 8.9 Hz); 9.05 (d, 1H, H-13, J = 8.4 Hz); 8.40 (broad s, 1H, NH-6); 8.28 (d, 1H, H-8, J = 9.3 Hz); 8.06 (d, 1H, H-9); 7.81 (broad s, 2H, NH_2); 7.80–7.65 (m, 3H, H-3, H-4, H-10); 7.54 (dd, 1H, H-12, J = 2.3Hz); 7.55–7.45 (m, 1H, H-2); 6.13 (s, CH = CH-maleate); 4.01 (s, 3H, OCH_3); 3.88 (m, 2H, CH_2-α); 3.48 (m, 2H, CH_2-γ); 2.05 (m, 2H, CH_2-β).

*BQQ–EDTA-triethyl ester (**6**).* Ethylenediaminetetraacetic acid triethyl ester (EDTA-TEE; 0.137 g) was activated with dicyclohexylcarbodiimide (2.1 eq.) and 1-hydroxybenzotriazole (1.7 eq.) in dimethylformamide (8 ml, 30 min) under N_2-atmosphere. BQQ-$NH(CH_2)_3NH_2$ (free base, 0.167 g, 1.2 eq.) **5b** was added and the mixture was stirred for 25 h. Purification of **6** on neutral alumina column using dichloromethane as eluent afforded a yellow-orange powder with a yield of 61%. ^{1}H NMR ($CDCl_3$) δ (ppm): 9.35–7.20 (10H, Ar-H); 4.10 (m, 6H, CH_2-ethyl, J = 7.2 Hz); 4.0 (s, 3H, C-OCH_3); 3.86 (m, 2H, CH_2-α, J = 6.0 Hz); 3.53 (s, 4H, CH_2-CO); 3.50 (m, 2H, CH_2-γ); 3.44 (s, 2H, CH_2-CO); 3.37 (s, 2H, CH_2-CO); 2.82 (s, 4H, N-CH_2-CH_2-N); 2.06 (m, 2H, CH_2-β, J = 6.4 Hz); 1.22 (t, 3H, CH_3-ethyl, J = 7.2 Hz); 1.18 (t, 6H, CH_3-ethyl, J = 7.2 Hz).

*BQQ–EDTA (**7**).* The triethyl ester (**6**; 0.164 g) was dissolved in methanol (6 ml) and then hydrolyzed using a 0.2 M solution of LiOH (6 eq.) in methanol-water (4:1, 10 ml). Purification of the lithium salt of the triacid compound **7** was accomplished by silanized silica gel chromatography using a gradient of 0–20% acetone in water. The pure product was isolated by methanol/diethyl ether trituration as the decahydrate with a yield of 70%. Anal. calc'd for $C_{33}H_{52}N_7O_{18}Li_2$: C, 46.70; H, 5.94; N, 11.55; Li, 1.64. Found: C, 46.89; H, 5.23; N, 11.33; Li, 1.21. ^{1}H-NMR (CD_3OD) δ (ppm): 9.0–7.09 (Ar, 10H), 3.90 (s, 3H), 3.75 (t, J= 6.0 Hz, 2H), 3.45 (t, J = 6.3 Hz, 2H), 3.30 (s), 3.24 (s), 3.15 (s); (8H), 2.69 (s), 2.65 (s); (4H), 2.04 (m, J = 7.0 Hz, 2H); MALDI–TOF MS, 658.0 (658.2 calc'd for M+H).

Cleavage assay on an 80 bp DNA fragment

The oligopurine- and oligopyrimidine-containing strands of the 80 bp DNA fragment were 3′-end radiolabeled separately (R*Y or Y*R). Samples of this fragment were incubated for 30 min at room temperature in 20 mM cacodylate buffer (pH 6.0, 100 mM NaCl) in the presence of 20 μM nonspecific or specific oligonucleotide, respectively. Two different sequences of a 27 nt nonspecific oligonucleotide were used: d5′-GGGTTTTTTTTTGGGTTTGGGTTGGGG-3′ and d5′-TCT-CTCTCTCTCTCTCTCTCTCTCT-3′. The sequence of the 27 nt specific oligonucleotide was d5′-TTCTTCTTTTTTCTTCTTCTTTTT-TCT-3′. The reaction was carried out by the addition of either BQQ (5 μM) + EDTA (5 μM), BQQ–EDTA (5 μM), or MPE (5 μM). To each sample, 5 μM $Fe(NH_4)_2(SO_4)_2$ was added and the reaction was initiated by adding 5 mM DTT. It was left to proceed for 15 min at room temperature then stopped by ethanol precipitation and analyzed using acrylamide gel electrophoresis.

Triplex-directed cleavage on a 2718 bp DNA fragment

The 2718 bp DNA fragment was incubated for 30 min in the same buffer indicated above in the presence of 20 μM of nonspecific or specific oligonucleotide (see previous section). Different concentrations of BQQ–EDTA were used (10, 20 and 50 μM). The reaction was carried out in the presence of 1 equivalent of $Fe(NH_4)_2(SO_4)_2$ and initiated by the addition of 5 mM dithiothreitol. It proceeded for 1 h at 37°C then all samples were analyzed using 1% agarose gel electrophoresis. DNA ladders taken from 1 kb ladder (GibcoBRL) and λDNA *Bst*II digestions were loaded on the same gel.

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