G-Quadruplex Ligands

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Photo-Cross-Linking Probes for Trapping G-Quadruplex DNA**

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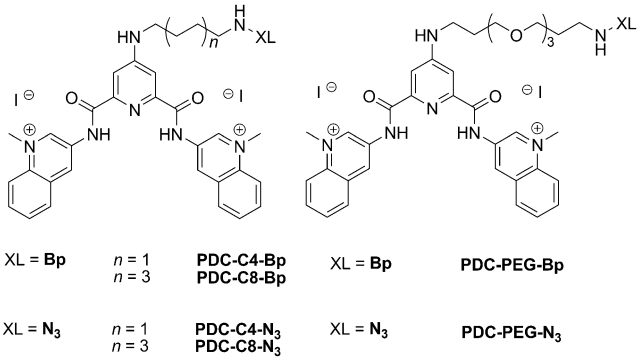
Abstract: *We have developed a straightforward synthetic pathway to a set of six photoactivatable G-quadruplex ligands with a validated G4-binding motif (the bisquinolinium pyridodicarboxamide PDC-360A) tethered through various spacers to two different photo-cross-linking groups: benzophenone and an aryl azide. The high quadruplex-versus-duplex selectivity of the PDC core was retained in the new derivatives and resulted in selective alkylation of two well-known G-quadruplexes (human telomeric G4 and oncogene promoter c-myc G4) under conditions of harsh competition. The presence of two structurally different photoactivatable functions allowed the selective alkylation of G-quadruplex structures at specific nucleobases and irreversible G4 binding. The topology and sequence of the quadruplex matrix appear to influence strongly the alkylation profile, which differs for the telomeric and c-myc quadruplexes. The new compounds are photoactive in cells and thus provide new tools for studying G4 biology.*

Oligonucleotides containing runs of three or four guanine residues may arrange into four-stranded DNA supramolecular structures called G-quadruplexes (G4s).[1] The discovery of these noncanonical structures in functional genomic regions suggests possible consequences for key processes, such as telomere maintenance, replication, and transcription.[2] Therefore, G4s have been the object of intense study with the aim of defining their potential as regulatory elements and/or therapeutic targets.[3] G4 DNA can be stabilized by small synthetic molecules, but so far most of these compounds bind in a reversible process through noncovalent interactions. Thus, these reversible binders have to exhibit high specificity for the G4 target to minimize off-target effects in a cellular context.[4] More recently, platinum complexes able to establish a stronger interaction by coordinating to nucleic bases of quadruplexes were developed.[5] These metal complexes form highly stable adducts with the human telomeric quadruplex, but the intrinsic reactivity of platinum may also induce the formation of nonspecific adducts. Recently, hybrid G4 binders bearing a classical alkylating agent (e.g. an oxirane)[6] and a chemically activatable alkylating moiety (e.g. a quinone methide) have been reported.[7] However, the formation of the resulting DNA adducts appeared to be reversible, which caused low cross-linking efficiency, below 15%. So far, the possibility of using light to trigger the alkylation of G4 structures has not been reported, although the photoalkylation of duplex DNA is rather well documented.[8]

In this context, we were keen to develop hybrid agents containing a validated G4-binding motif tethered to a photoreactive group. With this aim, we chose to link the bisquinolinum pyridodicarboxamide scaffold (abbreviated PDC and also known as 360A), one of the best G4 ligands reported,[9] to two motifs XL currently used for alkylating biomolecules, that is, benzophenone (**Bp**) and 4-azido-2,3,5,6-tetrafluorobenzoic acid ($\mathbf{N_3}$; Scheme 1). Both of these groups can be excited by UVA irradiation (see Figures S10 and S11 in the Supporting Information) at wavelengths close to the visible (330–365 nm) to generate highly reactive intermediates evolving through diverse photochemical pathways. For example, the excited triplet state of benzophenone undergoes [2+2] cycloaddition to alkenes to produce oxetanes by a Paternò–Buchi reaction[10] and may also abstract a hydrogen atom from accessible H donors to form a covalent bond between the resulting germinal alkyl and ketyl radicals.[11] On the other hand, photoexcited aryl azides form a nitrene, both in the singlet and triplet state, and this nitrene can initiate addition reactions with double bonds, insertion into C–H and



Scheme 1. Detailed chemical structure of the six PDC–XL derivatives synthesized in this study.

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N–H sites, or subsequent ring expansion and reaction with a nucleophile.[12] Therefore, both motifs have been exploited for photoaffinity labeling of protein–protein,[13] protein–ligand,[14] and DNA–ligand interactions.[15]

Six PDC–XL derivatives combining the two XL motifs and three different linkers were prepared and evaluated for their ability to form photoadducts with two well-known G4s, that is, the human telomeric sequence (22AG) and the oncogene promoter *c-myc* sequence (myc 22/G4T-G23T). Remarkably, most PDC–XL derivatives retained the high quadruplex-versus-duplex selectivity of the PDC core. As a result, selective alkylation of G4 was observed under competitive conditions. Furthermore, the alkylation occurred at nucleobases localized either in the loops or in the G-quartets, depending on the alkylating agent and on the topology of the quadruplex.

The photoalkylating motifs were introduced on the central pyridine ring, since the derivatization of this position[16] does not modify significantly the quadruplex-binding behavior.[17] To evaluate their influence, we chose three different connecting chains: two diaminoalkyl chains with four and eight carbon atoms (**C4** and **C8**, respectively) and a diaminopoly(ethylene glycol) chain (**PEG**; Scheme 1; see also Schemes S1–S3 in the Supporting Information). The affinity of the new derivatives for G4 and duplex DNA was first investigated by the G-quadruplex fluorescent intercalator displacement (G4-FID) assay, which is based on the competitive displacement of thiazole orange (TO).[18] All PDC–XL derivatives were found to displace TO with an efficacy comparable to that of PDC when *c-myc* was used as the G4 target (see Figure S1). In contrast, the displacement activity was more affected in the case of 22AG: It varied from a moderate to a more significant decrease in displacement (20–40%), depending on the spacer (see Figure S1). The influence of the XL moiety appeared negligible, except in the case of the **PDC-C4-Bp** derivative, which showed lower affinity than its $\mathbf{N_3}$ analogues. Nonetheless, all compounds showed quite low activity towards the duplex control (ds lac), thus indicating poor binding (see Figure S1). This result confirms the structural preference for quadruplex conformations.

We next investigated the ability of PDC–XL derivatives to form covalent adducts within the G4. Dose-dependent assays showed that a 2.5:1 ratio gave the highest photochemical yields, and these conditions were therefore applied throughout the study. The derivatives were treated with 22AG, both in NaCl and KCl buffers, and irradiated with a flash lamp ($\lambda =$ 360 nm).[14a] Analysis of the mixture by denaturing gel electrophoresis showed the formation of retarded bands attributed to alkylated 22AG, as the photoadducts provide additional mass and positive charges (Figure 1). The alkylation of the six compounds appeared significantly higher in K^+ buffer than in Na^+ buffer and was found to be strongly dependent on the nature of the spacer (Figure 1). Indeed, the highest yields were observed for the **C4** alkyl and **PEG** derivatives (26–36% in K^+, 13–29% in Na^+); however, the latter required stronger irradiation conditions (90 versus 150 flashes; see Table S1 in the Supporting Information). In stark contrast, alkylation was low or substantially absent for the **C8** derivatives (0–12%; Figure 1), irrespective of the irradiation conditions. Finally, the number of alkylated bands varied from one for **C4** derivatives to three for **PEG** chains (Figure 1). Altogether, these data indicate that **C4** and **PEG** are the most appropriate linkers for alkylation of the quadruplex. The low efficiency of the **C8** alkyl chain might be related to its strong lipophilic character: It is less prone to stay close to the highly hydrated DNA structure.

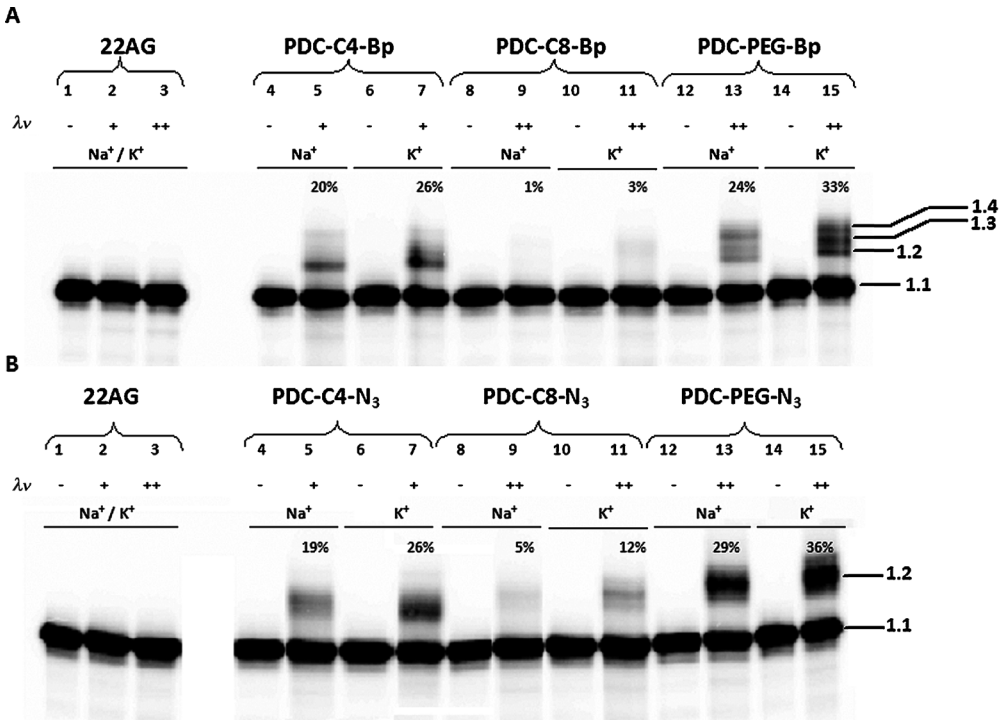

Figure 1. Denaturing gel electrophoresis (15% acrylamide) of the alkylation products of 22AG (10 μM) in the presence of Na^+ and K^+ buffer (100 mM) with A) **PDC-C4-Bp**, **PDC-C8-Bp**, and **PDC-PEG-Bp**, and B) **PDC-C4-N_3**, **PDC-C8-N_3**, and **PDC-PEG-N_3** (25 μM). Lanes 1, 2, 3, 4, 6, 8, 10, 12, and 14 are control experiments that show the effect of no irradiation (lane 1, 4, 6, 8, 10, 12, and 14) and irradiation for 90 flashes (lane 2) and 150 flashes (lane 3). Lanes 5 and 7 show the results of irradiation at 360 nm for 90 flashes; lanes 9, 11, 13, and 15 show the results of irradiation at 360 nm for 150 flashes. Bands have been numbered with respect to their migration as compared to a control (band 1.1: no migration; bands 1.2, 1.3, 1.4: retarded bands, numbered in the order of the extent of migration. $\lambda\nu$: − absence of irradiation, + 90 flashes, + + 150 flashes.

To assess whether the formation of adducts with 22AG was indeed selective for the G4 conformation, we reproduced the experiment with two control oligonucleotides, that is, the 17 bp duplex (ds lac) used in the FID assay and the single-stranded scrambled sequence 22Agmut. No adduct was observed in the presence of ds lac (see Figure S3), whereas low reactivity (8%) was observed with 22Agmut. This result is not surprising, as single-stranded DNA is known to trap cationic compounds through nonspecific electrostatic interactions. Subsequently, the photolysis experiments were reproduced in the presence of a large excess (8 mol equiv) of nonlabeled 22Agmut or ds lac. Under these conditions, G4 alkylation was retained with only a slight to moderate decrease in yield for the short-chain compounds (0–20%; Figure 2), whereas the two **PEG** derivatives were more affected by the presence of the competitors (ca. 30% decrease to 45% with 22Agmut). This result indicates that a long linker is deleterious to selectivity by favoring multiple positioning of the XL moiety, whereas a short linker that restricts dynamic motion[19] promotes alkylation with better G4 selectivity, thereby demonstrating the significance of proximity effects. Nonetheless, we cannot exclude the occurrence of static quenching of the excited triplet state of the cross-linker by electron-transfer and/or energy-transfer processes involving nucleic acid competitors.[20]

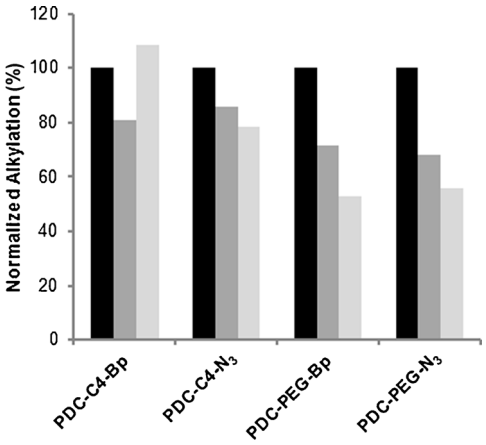

Figure 2. Quantitative analysis of the alkylation of the telomeric sequence 22AG (10 μM, 100 mM K^+-rich buffer) by the PDC–XL derivatives (25 μM) in the absence (black bars) or in the presence of a competitor (80 μM): ds lac (dark-gray bars), 22Agmut (light-gray bars).

With the aim of identifying the alkylation sites, we isolated the adducts from the gel and subjected them to two different sequencing procedures. For the PDC–$\mathbf{N_3}$ adducts, classical alkaline treatment, which induces cleavage on the 5′ side of alkylated bases, could be applied. Conversely, the PDC–**Bp** adducts reverted to the starting material at high temperature,[19b] thus making this sequencing method useless. In this case, the oligonucleotides were digested by 3′-exonuclease, which stops at alkylated sites.[21] The digested fragments were then isolated from the gel and treated with piperidine to remove the adducts, which affect their migration, and their size was determined by comparison with digested non-alkylated 22AG. The following alkylation sites were found for **PDC-PEG-Bp**: T6, T11, T12, T17, and T18, in both K^+ and Na^+ buffer (Figure 3 A,B; see also Figure S4 C). Interestingly, only two alkylation sites (T6 and T11) were identified for **PDC-C4-Bp** (see Figure S5 C). In conclusion, the benzophenone conjugates react exclusively with the loop thymine residues of 22AG: **PDC-C4-Bp** alkylates the first and second loop (starting from the 5′ end), whereas **PDC-PEG-Bp** alkylates the three loops (Figure 5 A; see also Figure S2).

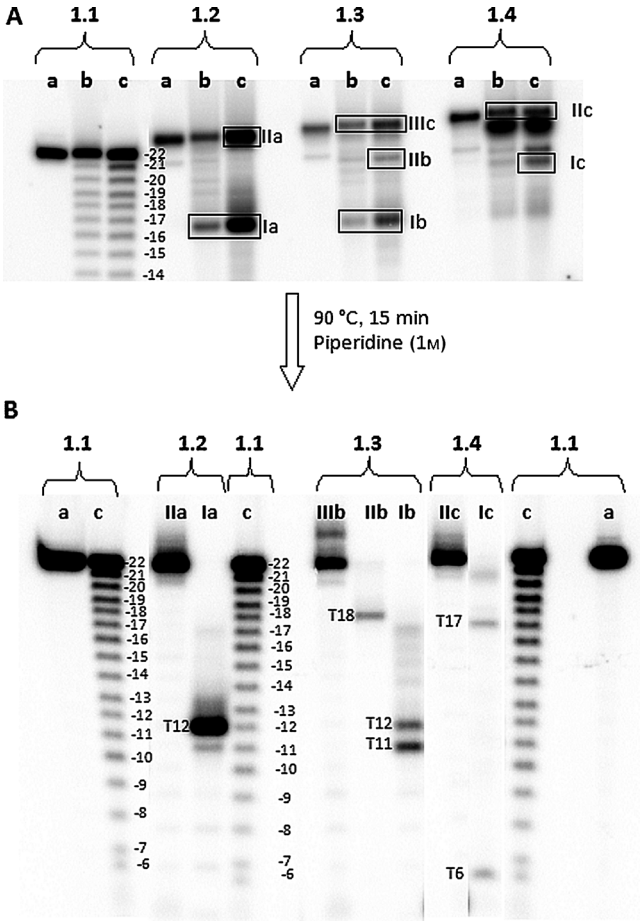

Figure 3. Denaturing gel electrophoresis of alkylated products of 22AG in K^+-rich buffer. A) 3′-Exonuclease digestion of bands 1.1–1.4 obtained by alkylation with **PDC-PEG-Bp** (see Figure 1 A): enzyme units/μL: lane a, 0; lane b, 0.04; lane c, 0.08. Band 1.1 corresponds to non-alkylated 22AG. B) Treatment of the digested fragments with piperidine. The numbered pyrimidine sites correspond to the digestion arrests.

In contrast, the direct treatment of **PDC-PEG-$\mathbf{N_3}$** adducts with piperidine revealed exclusive alkylation at guanine residues G10 and G14, regardless of the buffer cation (Figure 4; see also Figure S6). The same sites were observed for **PDC-C4-$\mathbf{N_3}$**, with one additional alkylation at G16 in K^+-rich buffer (see Figure S7 B). This result demonstrates that G10 and G14 are the only accessible and reactive residues for the electrophilic species produced by the photolysis of azide conjugates. As shown in Figure 5 A, G10 and G14 belong to

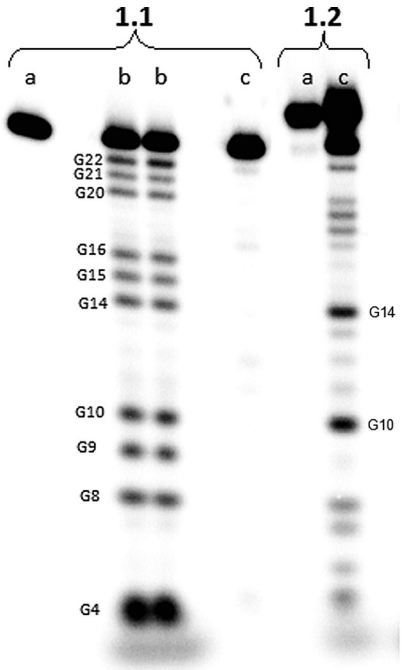

Figure 4. Denaturing gel electrophoresis of alkylated products of 22AG in K^+-rich buffer. Treatment with piperidine of bands 1.1 and 1.2 obtained by alkylation with **PDC-PEG-N_3** (see Figure 1 B): lane a: non-treated sample; lane b: sample (band 1.1, which corresponds to non-alkylated 22AG) treated with DMS (dimethyl sulfate) and piperidine to give a reference scale for fragment migration; lane c: piperidine-treated sample.

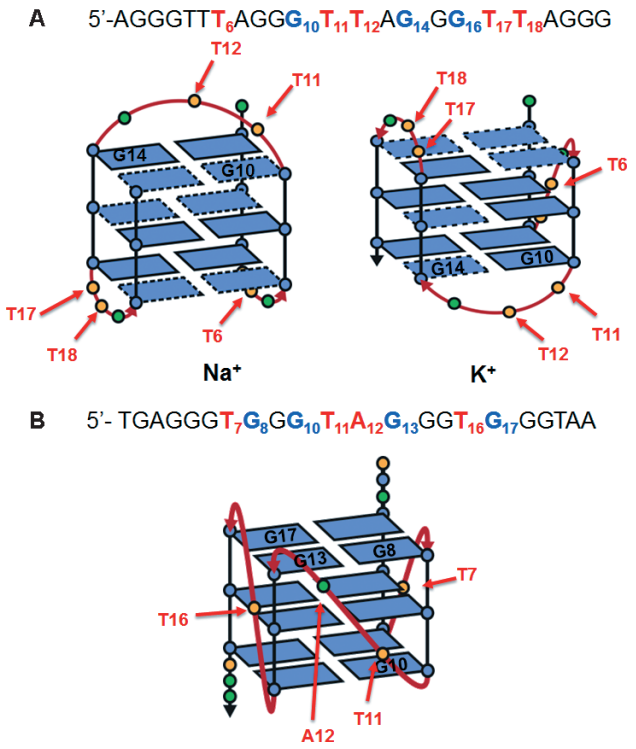

Figure 5. A) Schematic structure of the human telomeric G4 in Na^+- and in K^+-rich buffer, and sites alkylated by PDC–**Bp** and PDC–**N_3** derivatives. B) Schematic structure of the *c-myc* 22G4T-G23T G4 and sites alkylated by PDC–**N_3** derivatives. Guanine residues are represented by blue squares (G-quartets, dashed squares are *syn* and plain squares are *anti*) or blue spheres (G-loops); yellow and green spheres stand for T and A in the loops, respectively. Alkylated guanine residues are labeled in black; alkylated nucleobases in the loops are shown with red arrows.

the 5′ external quartet in almost all quadruplex conformations identified in solution (by NMR spectroscopy) for the telomeric sequence (see Figure S2).[22] Altogether, the results suggest that the quartet containing G10 and G14 may open transiently to expose the nucleophilic sites of the guanine residues (the ring N7 atoms), as previously observed with platinum complexes.[5b,23] Altogether, the alkylation profile for the **Bp** derivatives is consistent with a π-stacking of the ligands on the two external quartets to enable alkylation of the surrounding loops. The increased number of alkylation sites observed with the PEG derivative is in line with the longer length and higher flexibility of the spacer. In contrast, the two azide derivatives alkylate selectively the guanine residues located on the 5′ side with a high preference for G10 and G14, irrespective of the linker size. This result is again consistent with predominant π-stacking on the 5′ side but also suggests that the alkylation pattern is essentially determined by the nature of the reactive intermediate.

The four compounds were then evaluated with the *c-myc* sequence. Unexpectedly, the two **Bp** derivatives showed sluggish reactivity, which prevented analysis of the alkylation (see Figure S8). The near absence of reactivity of the **Bp** derivatives could reflect unfavorable conditions for photoadduct formation, possibly as a result of the high compactness of the *c-myc* G4 structure, which displays short loops that are arranged laterally and thus more difficult to access. The two azide derivatives induced the formation of a mixture of both retarded and accelerated bands (see Figure S8),[5b] which displayed alkylation of either the external G-quartet or the loops (Figure 5 B; see also Figure S9). However, the absence of reactivity of the **Bp** derivatives and the multiple alkylation pattern of the **N_3** derivatives could also result from the ligand-induced formation of dimeric G4 forms (see Figure S8), the presence of which may strongly impact the photoreactivity.[24]

Finally, the compounds were evaluated for their cytotoxicity with regard to two cancer cell lines (breast tumor MCF7 and non-small-cell lung carcinoma A549). All compounds showed no or low toxicity in the dark, whereas toxicity was triggered by UV/Vis irradiation (320–420 nm), with IC_{50} values ranging from 0.13 to 14 μM (see Table S1). Most importantly, the IC_{50} values are clearly dependent on the size of the linker (**C4** alkyl > **C8** alkyl > **PEG**) and on the nature of the alkylating agent **Bp** ≫ **N_3**, irrespective of the cell-line sensitivity. As a result, **PDC-C4-Bp** appears as the most active compound. Further studies devoted to analysis of the cellular targets (telomeres, transcription of proteins as controlled by G4-prone promoters) as well as cell-cycle analysis could indicate whether the activity of the compounds is mediated by G4 formation.

In conclusion, the use of two structurally different photoactivatable moieties linked to an efficient quadruplex-binding motif allowed us to selectively alkylate G4 structures in vitro,

which led to their irreversible trapping. Overall, the observed alkylation patterns reflect a subtle balance between the nature of the alkylating agent, the influence of the quadruplex topology, and the ligand spacer length. The new compounds, which display G4-binding selectivity combined with G4-topology-dependent photochemical behavior, are unique probes suitable for further investigations of quadruplex effects in various functional biological systems. Finally, the cellular results are promising, since only irradiated compounds were active, and in particular the benzophenone derivatives, which also reacted exclusively with the telomeric quadruplex in vitro. Whether the cellular target is a G4 structure remains to be evaluated. Nonetheless, our study already constitutes a step towards the discovery of specific G4 ligands that can be used to target preferentially certain G4-forming domains or subclasses of quadruplexes.

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[1] a) D. J. Patel, A. T. Phan, V. Kuryavyi, *Nucleic Acids Res.* **2007**, *35*, 7429 – 7455; b) J. B. Chaires, *FEBS J.* **2010**, *277*, 1098 – 1106.
[2] a) H. J. Lipps, D. Rhodes, *Trends Cell Biol.* **2009**, *19*, 414 – 422; b) J. L. Huppert, S. Balasubramanian, *Nucleic Acids Res.* **2007**, *35*, 406 – 413.
[3] a) D. Monchaud, M.-P. Teulade-Fichou, *Org. Biomol. Chem.* **2008**, *6*, 627 – 636; b) S. Balasubramanian, L. H. Hurley, S. Neidle, *Nat. Rev. Drug Discovery* **2011**, *10*, 261 – 275.
[4] S. Neidle, *FEBS J.* **2010**, *277*, 1118 – 1125.
[5] a) E. Largy, F. Hamon, F. Rosu, V. Gabelica, E. De Pauw, A. Guédin, J.-L. Mergny, M.-P. Teulade-Fichou, *Chem. Eur. J.* **2011**, *17*, 13274 – 13283; b) H. Bertrand, S. Bombard, D. Monchaud, E. Talbot, A. Guédin, J.-L. Mergny, R. Grünert, P. J. Bednarski, M.-P. Teulade-Fichou, *Org. Biomol. Chem.* **2009**, *7*, 2864 – 2871.
[6] F. Doria, M. Nadai, M. Folini, M. Scalabrin, L. Germani, G. Sattin, M. Mella, M. Palumbo, N. Zaffaroni, D. Fabris, M. Freccero, S. N. Richter, *Chem. Eur. J.* **2013**, *19*, 78 – 81.
[7] a) M. Nadai, F. Doria, M. Di Antonio, G. Sattin, L. Germani, C. Percivalle, M. Palumbo, S. N. Richter, M. Freccero, *Biochimie* **2011**, *93*, 1328 – 1340; b) F. Doria, M. Nadai, M. Folini, M. Di Antonio, L. Germani, C. Percivalle, C. Sissi, N. Zaffaroni, S. Alcaro, A. Artese, S. N. Richter, M. Freccero, *Org. Biomol. Chem.* **2012**, *10*, 2798 – 2806; c) M. Di Antonio, F. Doria, S. N. Richter, C. Bertipaglia, M. Mella, C. Sissi, M. Palumbo, M. Freccero, *J. Am. Chem. Soc.* **2009**, *131*, 13132 – 13141.
[8] a) 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; b) F. Doria, S. N. Richter, M. Nadai, S. Colloredo-Mels, M. Mella, M. Palumbo, M. Freccero, *J. Med. Chem.* **2007**, *50*, 6570 – 6579.
[9] G. Pennarun, C. Granotier, L. R. Gauthier, D. Gomez, F. Hoffschir, E. Mandine, J. F. Riou, J. L. Mergny, P. Mailliet, F. D. Boussin, *Oncogene* **2005**, *24*, 2917 – 2928.
[10] T. Bach, *Synthesis* **1998**, 683 – 703.
[11] P. J. Wagner, *Acc. Chem. Res.* **1971**, *4*, 168 – 177.
[12] a) W. Sander, D. Grote, S. Kossmann, F. Neese, *J. Am. Chem. Soc.* **2008**, *130*, 4396 – 4403; b) R. Poe, K. Schnapp, M. J. T. Young, J. Grayzar, M. S. Platz, *J. Am. Chem. Soc.* **1992**, *114*, 5054 – 5067.
[13] a) G. D. Prestwich, G. Dormán, J. T. Elliott, D. M. Marecak, A. Chaudhary, *Photochem. Photobiol.* **1997**, *65*, 222 – 234; b) G. Dorman, G. D. Prestwich, *Biochemistry* **1994**, *33*, 5661 – 5673.
[14] a) C. Dalhoff, M. Hüben, T. Lenz, P. Poot, E. Nordhoff, H. Köster, E. Weinhold, *ChemBioChem* **2010**, *11*, 256 – 265; b) L. R. Odell, N. Chau, A. Mariana, M. E. Graham, P. J. Robinson, A. McCluskey, *ChemMedChem* **2009**, *4*, 1182 – 1188.
[15] a) M. W. Berns, Z. Wang, A. Dunn, V. Wallace, V. Venugopalan, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 9504 – 9507; b) P. L. Gilbert, D. E. Graves, J. B. Chaires, *Biochemistry* **1991**, *30*, 10925 – 10931.
[16] P. Yang, A. De Cian, M.-P. Teulade-Fichou, J.-L. Mergny, D. Monchaud, *Angew. Chem.* **2009**, *121*, 2222 – 2225; *Angew. Chem. Int. Ed.* **2009**, *48*, 2188 – 2191.
[17] a) E. Largy, F. Hamon, M.-P. Teulade-Fichou, *Methods* **2012**, *57*, 129 – 137; b) A. Renaud de La Faverie, F. Hamon, C. Di Primo, E. Largy, E. Dausse, L. Delaurière, C. Landras-Guetta, J.-J. Toulmé, M.-P. Teulade-Fichou, J.-L. Mergny, *Biochimie* **2011**, *93*, 1357 – 1367.
[18] E. Largy, F. Hamon, M.-P. Teulade-Fichou, *Anal. Bioanal. Chem.* **2011**, *400*, 3419 – 3427.
[19] a) M. C. Cuquerella, V. Lhiaubet-Vallet, J. Cadet, M. A. Miranda, *Acc. Chem. Res.* **2012**, *45*, 1558 – 1570; b) K. Nakatani, T. Yoshida, I. Saito, *J. Am. Chem. Soc.* **2002**, *124*, 2118 – 2119.
[20] C. J. Burrows, J. G. Muller, *Chem. Rev.* **1998**, *98*, 1109 – 1152.
[21] S. Redon, S. Bombard, M.-A. Elizondo-Riojas, J.-C. Chottard, *Biochemistry* **2001**, *40*, 8463 – 8470.
[22] a) Y. Wang, D. J. Patel, *Structure* **1993**, *1*, 263 – 282; b) K. N. Luu, A. T. Phan, V. Kuryavyi, L. Lacroix, D. J. Patel, *J. Am. Chem. Soc.* **2006**, *128*, 9963 – 9970; c) A. T. Phan, K. N. Luu, D. J. Patel, *Nucleic Acids Res.* **2006**, *34*, 5715 – 5719.
[23] I. Ourliac-Garnier, M.-A. Elizondo-Riojas, S. Redon, N. P. Farrell, S. Bombard, *Biochemistry* **2005**, *44*, 10620 – 10634.
[24] H. T. Le, M. C. Miller, R. Buscaglia, W. L. Dean, P. A. Holt, J. B. Chaires, J. O. Trent, *Org. Biomol. Chem.* **2012**, *10*, 9393 – 9404.

