Benzoquinazoline Derivatives as Substitutes for Thymine in Nucleic Acid Complexes. Use of Fluorescence Emission of Benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione in Probing Duplex and Triplex Formation†

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ABSTRACT: Triple helix formation obeys structural features that do not allow accommodation of every double-stranded sequence; it requires the occurrence of homopurine stretches. A further constraint comes from the weak energy of interaction between the third strand and the double-stranded target. In an attempt to design bases leading to increased stability of triplexes, we explored the ability of modified bases with an extended aromatic domain to increase third strand binding through stacking interactions. We report here the use of benzo[*g*]- and benzo[*f*]quinazoline-2,4-dione-(1*H*,3*H*)-dione as substitutes for thymine in the canonical TA*T triplet. The synthesis and characterization of the β nucleoside derivatives of benzoquinazolines are described. Triplex-forming oligonucleotides containing these modified bases have been prepared, and their ability to form triplexes has been evaluated by UV absorption-monitored thermal denaturation measurements. Benzo[*g*]quinazoline and benzo[*f*]quinazoline formed triple-stranded structures with slightly decreased stabilities. In addition, benzo[*g*]quinazoline revealed strong fluorescence emission properties which can be used to monitor selectively the formation of triple-helical structures. Annealing of benzo[*g*]quinazoline to complementary strands did not produce any fluorescence modification. But when it was introduced into the Hoogsteen strand of PyPu*Py complexes, the fluorescence intensity was reduced and the emission maximum was shifted to short wavelengths.

The antisense strategy is based on the inhibition of gene expression by synthetic oligonucleotides complementary to single-stranded RNA targets (*1*). Although mRNAs are made of single chains, they cannot be considered single-stranded nucleic acids. They fold back into secondary and tertiary structures, giving rise to various motifs (hairpins, bulges, and pseudoknots) formed through intramolecular pairing of complementary sequences scattered along the RNA strand (*2*). Many of these RNA motifs play a role in gene expression. The TAR hairpin of HIV-1 interacts with virus and host proteins to enhance transcription (*3*). In *Trypanasoma*, a hairpin located 3′ of the stop codon stabilizes the procyclin message (*4*). Thus, structured RNA regions constitute valid targets for oligonucleotides which may interfere with regulatory processes. However, structures will compete with antisense oligonucleotide binding (*5*). Therefore, alternative strategies should be considered (*6*). It has been demonstrated that RNA (or DNA) hairpins can be targeted by complementary oligos, leading to the formation of local triple helices (*7*, *8*). Such complexes were able to impair in vitro translation in cell-free media (R. Le Tinévez and J.-J. Toulmé, unpublished results).

Nucleic acid triplexes are formed by binding a third strand in the major groove of a homopurine-homopyrimidine duplex (*9*). Two structural motifs for triplexes exist which differ in their sequence composition and third strand orientation relative to the homopurine strand of the duplex (*10*). In parallel triplexes, the third strand is made of pyrimidines and binds parallel to the purine strand of the duplex. Sequence specificity arises from the recognition of TA[1] pairs by thymine (TA*T triplets) and of CG pairs by protonated cytosine (CG*C^+ triplets) via Hoogsteen hydrogen bonding.[2] In antiparallel triplexes, the third strand contains purines and binds antiparallel to the purine strand of the duplex. The CG and TA pairs are recognized by a guanine and adenine, respectively, forming CG*G and TA*A triplets via reverse Hoogsteen hydrogen bonding. Mixed purine and pyrimidine sequences can be constructed to read the purine strand, leading to TA*A, TA*T, or CG*G triplets. In this latter case, the orientation of the third strand depends on the number GT steps (*11*).

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[1] Abbreviations: T_m, melting temperature; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; NOE, nuclear Overhauser effect; s, singlet; d, doublet; t, triplet; br, broad; UV, ultraviolet; ROESY, rotating frame Overhauser effect spectroscopy; COSY, correlation spectroscopy; 2D NMR, two-dimensional NMR spectroscopy; HMQC, heteronuclear multiple-quantum correlation; HMBC, heteronuclear multiple-bonding connectivity; MS, mass spectrum; HRMS, high-resolution mass spectrum; FAB, fast atom bombardment; IR, infrared; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; HMDS, hexamethyldisilazane; RT, room temperature; DMT, dimethoxytrityl; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; A, adenine; C, cytosine; G, guanine; T, thymine; Py, pyrimidine; Pu, purine.

[2] In this notation, the third strand is written in the last position and the asterisk indicates the Hoogsteen interaction type.

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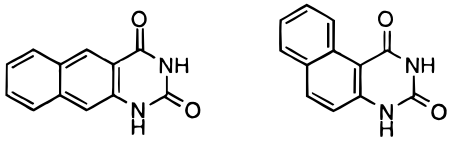
Besides the sequence constraint, another limitation for triplex formation comes from a weak energy of interaction between the third strand and the double-stranded target. Thermodynamic analysis revealed that both enthalpy and free energy of triple helix formation (from duplex) are lower than that of the duplex (*12*). Furthermore, parallel triplexes formed by different combinations of RNA or DNA strands are highly sensitive to the nature of the nucleic acid backbone (*13*−*17*). Although these studies led the authors to a slightly different order for the relative stabilities of triplex combinations, they all indicated that triplexes composed of DR*D or RR*D strands are not stable (D and R stand for DNA and RNA strands, respectively).

Modified bases have been proposed to overcome the first limitation (i.e., the sequence constraint) (*18*). In an attempt to design bases allowing increased stability of triplexes, we explored the ability of modified bases with an extended aromatic domain to increase third strand binding through stacking interactions. Quinazoline-2,4-(1*H*,3*H*)-dione and modified quinazolines were subsituted for T's in TA*T triplets (*19*, *20*). While maintaining base pairing properties of thymine, these heterocycles may allow an increased contribution of van der Waals forces to stacking interactions. These derivatives did not stabilize triple helices, but improved the specificity for triple helix compared to that for double strand formation. A preferential binding of modified oligomers to double strands compared to single strands was demonstrated by UV absorption-monitored melting curves of the modified complexes (triplexes and duplexes). Positive values for $\Delta\Delta T_m$ [ΔT_m(triplex) − ΔT_m(duplex)] were observed for most of the quinazoline derivatives (*20*).

We report here a further extension of this approach using polycyclic aromatic bases to increase the area of overlapping domains. This led us to synthesize benzo[*g*]- (**1**) and benzo-[*f*]quinazoline-2,4-(1*H*,3*H*)-dione (**2**).



1 **2**

Although the areas of the aromatic domain of the two compounds are very similar, they differ primarily in the orientation and shape of the hydrophobic extension which may lead to different overlapping regions with the adjacent bases along the third strand, and hence to different contributions to stacking interactions. **2** shows an enriched π electron domain in a position similar to that of 5-propynyl derivatives of pyrimidines (*18*) which proved to stabilize third strand interactions in triple helices (*21*). These nonpolar areas should stack favorably with the polar nucleic acid bases (*22*, *23*). The β deoxyribonucleosides of these heterocycles were introduced in oligonucleotides. The oligonucleotide sequences used here are the same as the ones reported in previous studies, allowing careful comparison of experimental data (*19*, *20*). The stabilities of deoxyribonucleotide triple-stranded structures were reached by thermal denaturation experiments.

Furthermore, **1** displays a strong fluorescence emission with a quantum yield of about 0.82 compared to that of quinine sulfate (0.51). It can be used to monitor triple-stranded structure formation. The formation of triple-helical structure when **1** was incorporated into an oligopyrimidine third strand resulted in a shift of the fluorescence emission maximum to short wavelengths and in a decreased fluorescence intensity (F. Godde et al., submitted). We report here data on the use of emission properties of **1** when introduced in pH-dependent triple-stranded structures incorporating both TA*T and CG*C^+ triplets.

EXPERIMENTAL PROCEDURES

General

Thin layer chromatography (TLC) was performed on Merck silica gel 60F254 alumimnium-backed plates; visualization was done by UV illumination and staining with a 10% perchloric acid solution. Flash chromatography refers to column chromatography performed with Merck silica gel 60 (0.04−0.063 mm).

The NMR spectra were recorded on a Brüker AC 200 spectrometer working at 200 MHz for ^{1}H, 50.32 MHz for ^{13}C, and 81.02 MHz for ^{31}P. The ROESY, HMQC, and HMBC experiments were performed on a Brüker AMX 500 spectrometer. The chemical shifts are expressed in parts per million using TMS as an internal standard (^{1}H and ^{13}C data) and 85% H_3PO_4 as an external standard (^{31}P data). The IR data and UV spectra were recorded on a Brüker UFS-25 spectrofluorimeter and on a Kontron Uvikon 940 spectrophotometer, respectively. All chemical reagents were obtained from Aldrich except ammonium sulfate (Fluka) and sodium metal (Prolabo).

Chemical Procedures

The reaction mixtures were extracted with dichloromethane. The combined organic phases were washed with a saturated solution of $NaHCO_3$, and when copper ions were used in the reactions, an additional washing step with a 5% EDTA solution was included. The final organic phases were washed with brine, dried over $MgSO_4$, and concentrated.

*Benzo[g]quinazoline-2,4-(1H,3H)-dione (**1**).* 3-Amino-2-naphthoic acid (**3**) (20 g, 106.8 mmol) and urea (40 g, 666 mmol) were refluxed for 2 days in 400 mL of phenol. The solution was cooled to 100 °C and poured into water (400 mL). The product was filtered and washed with water. The product was dissolved in 200 mL of DMF at 100 °C, and then $CHCl_3$ (1000 mL) was added and the mixture cooled overnight at 4 °C. Yield: 17.6 g of an ochre powder (88%). R_f = 0.42 (1/9 methanol/chloroform). MS (FAB$^+$): 213.2 $(M + H)^+$. IR (KBr): 3219, 3031, 2842, 1701, 1668, 1634, 1517, 1475, 1433 cm^{-1}. ^{1}H NMR (200 MHz, DMSO-d_6): δ 7.45 (td, 1H, H_7, J_1 = 8 Hz, J_2 = 1 Hz), 7.50 (s, 1H, H_{10}), 7.60 (td, 1H, H_8, J_1 = 8 Hz, J_2 = 1.2 Hz), 7.85 (d, 1H, H_9, J = 8 Hz), 8.05 (d, 1H, H_6, J = 8 Hz), 8.60 (s, 1H, H_5), 11.20 (br s, 1H, H_1*), 11.30 (br s, 1H, H_3*). ^{13}C NMR (50.32 MHz, DMSO-d_6): δ 110.20 (C_{10}), 115.15 (C_{4a}), 124.90 (C_7), 126.70 (C_{10}), 128.40 (C_{5a}), 129.00 (C_5*), 129.20 (C_8), 129.50 (C_6*), 136.30 (C_{9a}), 136.50 (C_{10a}), 150.30 (C_2), 162.90 (C_4).

*1-(3,5-Di-O-p-toluoyl-2-deoxy-α,β-D-erythro-pentofuranosyl)benzo[g]quinazoline-2,4-(3H)-dione (**13**).* Benzo[*g*]-quinazoline-2,4-(1*H*,3*H*)-dione (**1**) (10.34 g, 48.7 mmol) was refluxed with a few crystals of ammonium sulfate and

acetamide in hexamethyldisilazane (HMDS, 400 mL) for 2 days under anhydrous conditions. Excess HMDS was removed under vacuum by coevaporation with dry toluene. The residue was dissolved in dry toluene (100 mL) and added to a solution of 2-deoxy-3,5-di-*O*-*p*-toluoyl-D-pentofuranosyl chloride (**10**) (18.95 g) in dry acetonitrile (600 mL) with 1.1 equiv of CuI (10.2 g). This mixture was stirred at room temperature (RT) for 3 days. The reaction was monitored by TLC. The solution was filtered through Celite, and then acetonitrile was removed under vacuum. The protected nucleoside was purified by flash chromatography along a gradient of MeOH/CH_2Cl_2 (0 to 20%). The yield was 56% (15.49 g) of an anomeric mixture (yellow and amorphous). R_f = 0.57 (4/6 ethyl acetate/cyclohexane). MS (FAB^+): 587.5 $(M + Na)^+$. ^1H NMR (200 MHz, mixture of anomers, $CDCl_3$): δ 2.30 and 2.40 (s, 6H, CH_3 Tol), 3.05 (t, 1H, $H_{2'}$, J = 7 Hz), 3.40 (m, 1H, $H_{2''}$, J = 7 Hz), 4.50−5.15 (m, 3H, $H_{4'}$, $H_{5'}$, and $H_{5''}$), 6.85 (bt, 1H, $H_{1'}$, J = 7 Hz), 7.05−8.05 (m, 13H, Har), 8.70 and 8.75 (s, 1H, $H_{5\alpha}$ and $H_{5\beta}$), 9.90 (br s, 1H, H_3). ^{13}C NMR (50.32 MHz, $CDCl_3$): δ 21.55 (CH_3 Tol), 33.90−34.45 ($C_{2'}$), 64.15−64.95 ($C_{5'}$), 74.20−75.25 ($C_{3'}$), 81.05−81.25 ($C_{1'}$), 85.10−85.65 ($C_{4'}$), 112.40−112.80 (C_{10}), 115.95 (C_{4a}), 125.55, 126.10, 126.40, 127.40, 128.00, 128.70, 129.7 (Car Tol, C_{5-9}), 134.30−135.20 (C_{10a}), 136.2 (C_{9a}), 143.7−144.1 (Car Tol), 149.9 (C_2), 162.0 (C_4), 166.0−166.3 (carbonyl Tol).

*1-(2-Deoxy-*α*,*β*-*D*-erythro-pentofuranosyl)benzo[g]quinazoline-2,4-(3H)-dione (**15**).* A solution of 2.05 equiv of sodium methylate (100 mL) was poured into a suspension of product **13** (15.4 g, 27.3 mmol) in dry methanol (400 mL) and the mixture stirred for 1 h. The solution was neutralized by 1 N HCl, concentrated to 100 mL, poured into diethyl ether (500 mL), and filtered to remove the precipitate. Yield: 7.25 g (81%).

*1-[2-Deoxy-5-(4,4′-dimethoxytrityl)-*β*-D-erythro-pentofuranosyl]benzo[g]quinazoline-2,4-(3H)-dione (**17**).* The mixture of nucleoside (**15**) (6.20 g, 18.9 mmol) was dried by three coevaporations with anhydrous pyridine and then dissolved in anhydrous pyridine (20 mL). 4,4′-Dimethoxytrityl chloride (1.2 equiv, 7.68 g) in anhydrous pyridine (40 mL) was then added. The reaction mixture was stirred for 5 h at RT under argon; the reaction was stopped with methanol (5 mL) and the mixture concentrated. The anomers were separated by flash chromatography along a gradient of methanol/dichloromethane (0 to 15% with 1% triethylamine). Yield: 2.54 g (β anomer, 20%, amorphous) and 5.28 g (α anomer, 42%, amorphous). β anomer R_f = 0.22 in methanol/chloroform (5/95). MS (FAB^+): 631.4 $(M + H)^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 2.20 (m, 1H, $H_{2'\alpha}$), 3.00 (m, 1H, $H_{2'\beta}$), 3.70 (2s, 6H, OCH_3), 3.40−3.80 (m, 2H, $H_{5'}$ and $H_{5''}$), 4.05 (m, 1H, $H_{4'}$), 4.75 (m, 1H, $H_{3'}$), 6.65−6.85 (m, 5H, Har and $H_{1'}$), 7.10−7.50 (m, 13H, Har and H_{5-9}), 7.90 (s, 1H, H_{10}), 8.75 (m, 1H, H_5). ^{13}C NMR (50.32 MHz, $CDCl_3$): δ 36.50 ($C_{2'}$), 54.75 (CH_3O-DMT), 63.65 ($C_{5'}$), 77.20 ($C_{3'}$), 84.45 ($C_{1'}$), 84.75 ($C_{4'}$), 86.05 (Cq DMT), 112.80 (C_{10} and Car), 116.30 (C_{4a}), 125.50 (C_7), 126.50 (C_9), 127.45, 128.15, 128.55, 128.65, 128.90, 130.0, 135.00 (Car, $C_{5,5a,6,8}$), 135.65 (C_{9a}*), 136.00 (C_{10a}*), 144.50 (Cq DMT), 150.20 (C_2), 158.20 (Cq DMT), 162.35 (C_4).

*1-(2-Deoxy-*β*-D-erythro-pentofuranosyl)benzo[g]quinazoline-2,4-(3H)-dione (**21**).* **17** (970 mg, 1.43 mmol) was stirred for 1 h in aqueous acetic acid (80%, 30 mL) at RT. The mixture was then evaporated, loaded onto a short silica gel column, and eluted with methanol/dichloromethane (1/1), yielding 210 mg (94% yield) of β nucleoside (white powder). UV (EtOH): 253 (ϵ = 49 800), 282 (ϵ = 4900), 294 (ϵ = 5300), 306 (ϵ = 2850), 360 nm (ϵ = 2000). UV (5% EtOH in H_2O): 219 (ϵ = 21 250), 253 (ϵ = 50 300), 294 nm (ϵ = 4950). MS (FAB^+): 329.2 $(M + H)^+$. HRMS: 329.1129 $(M + H)^+$ calcd for $C_{17}H_{17}N_2O_5$, found 329.1137. ^1H NMR (500 MHz, DMSO-d_6): δ 1.95 (m, 1H, $H_{2'\alpha}$), 2.75 (m, 1H, $H_{2'\beta}$), 3.75 (m, 2H, $H_{5''}$ and $H_{5''}$), 3.80 (m, 1H, $H_{4'}$), 4.55 (m, 1H, $H_{3'}$), 5.15 (t, 1H, $OH_{5'}$, J = 4.8 Hz), 5.25 (d, 1H, $OH_{3'}$, J = 4.9 Hz), 6.80 (t, 1H, $H_{1'}$, J = 7.8 Hz), 7.50 (t, 1H, H_7, J = 8.3 Hz), 7.65 (t, 1H, H_8, J = 8.3 Hz), 7.95 (d, 1H, H_9, J = 8.3 Hz), 8.10 (d, 1H, H_6, J = 8.3 Hz), 8.45 (s, 1H, H_{10}), 8.70 (s, 1H, H_5), 11.60 (br s, 1H, H_3). ^{13}C NMR (125.8 MHz, DMSO-d_6): δ 35.30 ($C_{2'}$), 60.10 ($C_{5'}$), 68.90 ($C_{3'}$), 83.30 ($C_{1'}$), 86.00 ($C_{4'}$), 113.40 (C_{10}), 116.10 (C_{4a}), 125.10 (C_7), 127.00 (C_9), 127.70 (C_{5a}), 128.40 (C_5, C_6, and C_8), 133.90 (C_{10a}), 135.40 (C_{9a}), 149.60 (C_2), 161.10 (C_4).

*1-[2-Deoxy-3-O-[2-cyanoethoxy(diisopropylamino)-phosphino]-5-(4,4′-dimethoxytrityl)-*β*-D-erythro-pentofuranosyl]benzo[g]quinazoline-2,4-(3H)-dione (**23**).* **17** (500 mg, 0.79 mmol) was dissolved in anhydrous dichloromethane (stabilized with 0.002% amylene) (4 mL). Four equivalents of diisopropylethylamine (552 μL) and 1.5 equiv of 2-cyanoethyl diisopropylchlorophosphoramidite (262 mg) in anhydrous dichloromethane (0.5 mL) were added. The reaction was stopped after 3 h at RT with 30 μL of methanol and the mixture extracted with ethyl acetate (50 mL). The phosphoramidite was purified twice by flash chromatography with ethyl acetate/dichloromethane/triethylamine (45/45/10) as the eluent, yielding 326 mg of pure phosphoramidite (50% yield). R_f = 0.63 (5/95 methanol/chloroform). HR-FAB: calcd for $C_{47}H_{50}N_4O_8P$ $(M - H)^-$ 829.336629, found 829.337641. ^1H NMR (200 MHz, $CDCl_3$): δ 1.25 (m, 12H, CH_3 ^{i}Pr), 2.35 (m, 1H, $H_{2'\alpha}$), 2.60 (t, 2H, CH_2CN, J = 6.5 Hz), 3.15 (m, 1H, $H_{2'\beta}$), 3.40−3.90 (m, 6H, CH_2OP, CH ^{i}-Pr, $H_{5'}$, and $H_{5''}$), 3.65 and 3.70 (2s, 6H, CH_3O-DMT), 4.20 (m, 1H, $H_{4'}$), 4.90 (m, 1H, $H_{3'}$), 6.60−6.75 (m, 6H, Har), 6.85 (t, 1H, $H_{1'}$, J = 7 Hz), 7.10−7.55 (m, 14H, Har and H_{6-8}), 7.90 (d, 1H, H_9, J = 7.8 Hz), 8.05 (s, 1H, H_{10}), 8.80 (s, 1H, H_5). ^{13}C NMR (50.32 MHz, $CDCl_3$): δ 20.15 (CH_2-CN), 24.45 (CH_3 ^{i}Pr), 35.65 ($C_{2'}$), 43.00 and 43.25 (CH ^{i}-Pr), 54.15 (CH_3O-DMT), 58.25 (d, $^2J_{CP}$ = 18.7 Hz, CH_2OP), 63.00 ($C_{5'}$), 72.95 (d, $^2J_{CP}$ = 16.7 Hz, $C_{3'}$), 84.35 ($C_{1'}$*), 84.70 ($C_{4'}$*), 86.25 (Cq DMT), 112.90 (Car), 113.15 (C_{10}), 116.15 (C_{4a}), 117.40 (CN), 125.85 (C_7), 126.70 (C_9), 127.60, 127.80, 128.15, 128.40, 128.45, 128.80, 129.25, 130.20, 130.45 (Car and $C_{5,5a,6,8}$), 134.95 (C_{10a}), 135.60 (Car), 136.30 (C_{9a}), 144.35 (Cq DMT), 149.90 (C_2), 158.35 (Cq DMT), 162.05 (C_4). ^{31}P NMR (81.02 MHz, $CDCl_3$): δ 147.18, 147.51.

*Pyridinium 2-(Acetylamino)-1-naphthalenesulfonate (**5**).* A suspension of 2-amino-1-naphthosulfonic acid (**4**) (20 g, 89.6 mmol) in acetic anhydride (30 mL) was added to 100 mL of pyridine/acetic anhydride (1/1) and the mixture stirred at RT for 2 days. The pyridinium salt was filtered and washed with glacial acetone. A white powder was obtained (18.08 g, 59%). MS (FAB^-): 264.1 $(M - H)^-$. IR (KBr): 3449, 3240, 3073, 2089, 1684, 1609, 1576, 1534, 1500 cm^{-1}.

*1-Bromo-2-(acetylamino)naphthalene (**6**).* Product **5** (2.66 g, 65.9 mmol) was dissolved in H_2O (200 mL). Bromine

(1.1 equiv, 3.70 mL) dissolved in acetic acid/H_2O (15/1) was added and the mixture stirred for 2 h at RT. The product was filtered and crystallized in EtOH as a white powder, and finally washed with cold EtOH/H_2O (1/1). Yield: 11.08 g (64%). $R_f = 0.59$ (1/1 ethyl acetate/hexane). MS (FAB^-): 262.0 $(M - H)^-$. IR (KBr): 3261, 3052, 1659, 1592, 1567, 1525, 1500 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 2.25 (s, 3H, CH_3), 7.40 (t, 1H, H_7, $J = 8.3$ Hz), 7.55 (t, 1H, H_6, $J = 8.3$ Hz), 7.75 (d, 1H, H_3, $J = 8.5$ Hz), 7.90 (br s, 1H, H_8), 8.10 (d, 1H, H_5, $J = 8.3$ Hz), 8.35 (d, 1H, H_4, $J = 8.5$ Hz). ^{13}C NMR (50.32 MHz, $CDCl_3$): δ 24.75 (CH_3), 111.68 (C_1), 121.00 (C_4), 125.50 (C_8), 126.50 (C_6), 127.60 (C_5*), 128.05 (C_7*), 128.10 (C_3*), 131.45 (C_{4a}**), 131.80 (C_{8a}**), 168.45 (carbonyl).

*1-Cyano-2-(acetylamino)naphthalene (**7**).* **6** (13.88 g, 52.59 mmol) was dissolved in a suspension of 1.1 equiv of copper cyanide (5.18 g) in dimethylformamide (150 mL) and the mixture refluxed for 4 h. The reaction was monitored by TLC. The DMF was concentrated under vacuum, and then poured into a solution of chloroform/methanol (8/2). **7** was crystallized in ethanol (pink crystals). Yield: 7.82 g (71%). $R_f = 0.51$ (1/1 ethyl acetate/hexane). MS (FAB^-): 198.9 $(M - H)^-$. IR (KBr): 3293, 3042, 2226, 1697, 1672, 1630, 1597, 1521, 1496 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6 at 60 °C): δ 2.20 (s, 3H, CH_3), 7.60 (t, 1H, H_7, $J = 8$ Hz), 7.70 (t, 1H, H_6, $J = 8$ Hz), 7.8 (d, 1H, H_3, $J = 9$ Hz), 8.0 (d, 1H, H_8, $J = 8$ Hz), 8.05 (d, 1H, H_5, $J = 8$ Hz), 8.15 (d, 1H, H_4, $J = 9$ Hz), 10.25 (br s, 1H, NH). ^{13}C NMR (50.32 MHz, DMSO-d_6 at 60 °C): δ 23.05 (CH_3), 101.35 (C_1), 115.20 (CN), 121.55 (C_4), 122.95 (C_8), 126.20 (C_6), 128.25 (C_7), 128.75 (C_5), 129.55 (C_{4a}*), 131.80 (C_{8a}*), 133.20 (C_3), 168.60 (carbonyl).

*1-Cyano-2-aminonaphthalene (**8**).* **7** (9 g, 42.8 mmol) was dissolved in a 1 N sodium hydroxyde solution in methanol (400 mL) and the mixture stirred for 2 days at RT. The mixture was concentrated twice. A white powder was obtained (6.90 g, 96%). $R_f = 0.67$ (1/1 ethyl acetate/cyclohexane). MS (FAB^+): 169.1 $(M + H)^+$. IR (KBr): 3429, 3324, 3240, 3031, 2194, 1927, 1642, 1626, 1601, 1567, 1509 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 4.80 (br s, 2H, NH_2), 6.85 (d, 1H, H_3, $J = 8.5$ Hz), 7.30 (t, 1H, H_7, $J = 8.5$ Hz), 7.50 (t, 1H, H_6, $J = 8.5$ Hz), 7.65 (d, 1H, H_8, $J = 8.5$ Hz), 7.75 (d, 1H, H_5, $J = 8.5$ Hz), 7.90 (d, 1H, H_4, $J = 8.5$ Hz). ^{13}C NMR (50.32 MHz, $CDCl_3$): δ 87.40 (C_1), 116.60 (C_4), 117.20 (CN), 123.05 (C_8*), 123.60 (C_6*), 126.55 (C_{4a}), 128.35 (C_5**), 128.80 (C_7**), 133.20 (C_{8a}), 150.30 (C_2).

*Benzo[f]quinazoline-1-amino-3-hydroxy (**9**).* **8** (5.91 g, 35.2 mmol) was mixed with urea (10.53 g, 175.5 mmol) and the mixture heated to 180 °C for 1 h. A pale solid was isolated and washed with ethanol. The white powder was dried under vacuum. The reaction yield was 96% (7.12 g). IR (KBr): 3324, 3135, 1718, 1684, 1651, 1584 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6 at 60 °C): δ 6.80 (br s, 2H, NH_2), 7.35 (d, 1H, H_5, $J = 9$ Hz), 7.50 (t, 1H, H_8, $J = 8$ Hz), 7.65 (t, 1H, H_9, $J = 8$ Hz), 7.95 (d, 1H, H_7, $J = 8$ Hz), 8.05 (d, 1H, H_6, $J = 9$ Hz), 8.72 (br s, 1H, H_{10}).

*Benzo[f]quinazoline-1,3-(2H,4H)-dione (**2**).* **9** (7.12 g, 33.7 mmol) was refluxed in DMF/12 N HCl (3/2) for 6 days. The reaction mixture was poured into water (800 mL), filtered, and washed with ethanol (200 mL) and diethyl ether (200 mL). $R_f = 0.52$ (1/9 methanol/chloroform). Yield: 4.0 g (56%, white powder). MS (FAB^+): 213.2 $(M + H)^+$. IR (KBr): 3418, 3167, 3104, 3042, 2811, 1705, 1655, 1622, 1588, 1530 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 7.35 (d, 1H, H_5, $J = 8.3$ Hz), 7.50 (t, 1H, H_8, $J = 8.3$ Hz), 7.65 (t, 1H, H_9, $J = 8.3$ Hz), 7.90 (d, 1H, H_7, $J = 8.3$ Hz), 8.10 (d, 1H, H_6, $J = 8.3$ Hz), 9.60 (d, 1H, H_{10}, $J = 8.3$ Hz), 11.15 (br s, 2H, H_2 and H_4). ^{13}C NMR (50.32 MHz, DMSO-d_6): δ 105.15 (C_{10b}), 115.65 (C_6), 124.25 (C_{10}*), 124.50 (C_8*), 128.20 (C_9**), 128.50 (C_7**), 128.95 (C_{6a}***), 130.50 (C_{10a}***), 135.75 (C_5), 142.45 (C_{4a}), 149.60 (C_3), 163.40 (C_1).

*1-(3,5-Di-O-p-toluoyl-2-deoxy-α,β-D-erythro-pentofuranosyl)benzo[f]quinazoline-1,3-(2H)-dione (**14**).* Benzo[*f*]quinazoline-2,4-(1*H*,3*H*)-dione (**2**) (199 mg, 0.939 mmol) was refluxed with a few crystals of ammonium sulfate and acetamide in hexamethyldisilazane (HMDS, 20 mL) for 2 days under anhydrous conditions. Excess HMDS was removed in vacuo by coevaporation with dry toluene. The residue was dissolved in dry toluene (2 mL) and added to a solution of 2-deoxy-3,5-di-*O*-*p*-toluoyl-D-pentofuranosyl chloride (**10**) (364.7 mg) in dry acetonitrile (10 mL) with 1.1 equiv of CuI (196 mg). The mixture was stirred at RT for 2 days. The mixture of anomers was concentrated twice and then purified by flash chromatography along a gradient of methanol/dichloromethane (0 to 10%). Yield: 258 mg (48%, amorphous). $R_f = 0.66$ (1/1 ethyl acetate/cyclohexane). ^1H NMR (200 MHz, DMSO-d_6, mixture of anomers): δ 2.35 (s, 6H, CH_3 Tol), 3.05 (m, 0.1H, $H_{2'}$), 3.20−3.30 (m, 2H, $H_{2'}$ and $H_{2''}$), 4.45−5.10 (m, 3H, $H_{4'}$, $H_{5'}$, and $H_{5''}$), 5.85 (m, 1H, $H_{3'}$), 6.80 and 7.05 (t, 1H, $H_{1'}$, $J = 7$ Hz, $J = 7.7$ Hz), 7.25 (t, 4H, Har Tol, $J = 6.2$ Hz), 7.50 (t, 1H, H_8, $J = 8$ Hz), 7.60 (t, 1H, H_9, $J = 8$ Hz), 7.75−8.05 (m, 7H, Har Tol, H_7, H_6, H_5), 9.65 (m, 1H, $H_{10'}$, $J = 8.6$ Hz), 10.45 and 11.70 (br s, 1H, H_2). ^{13}C NMR (50.32 MHz, DMSO-d_6, mixture of anomers): δ 20.95 (CH_3 Tol), 34.60−34.80 ($C_{2'}$), 63.50−63.80 ($C_{5'}$), 74.00−74.90 ($C_{3'}$), 80.65−81.35 ($C_{1'}$), 84.80−85.90 ($C_{4'}$), 108.30 (C_{10b}), 115.00 (C_6), 125.45 (C_{10}*), 126.10 (C_8*), 126.35 (Car* Tol), 126.55 (C_7*), 128.10 (C_9*, Car Tol), 129.00 (C_{6a}**), 130.60 (C_{10a}**), 135.50 (C_5), 142.00 (C_{4a}), 143.55−143.70 (Car Tol), 149.45 (C_3), 162.50 (C_1), 163.30 (carbonyl Tol).

*1-(2-Deoxy-α,β-D-erythro-pentofuranosyl)benzo[f]quinazoline-1,3-(2H)-dione (**16**).* **14** (2.76 g, 4.41 mmol) was dissolved in anhydrous methanol (150 mL) and the mixture stirred at RT for 2 h with 2.1 equiv of a sodium methanoate solution (213 mg of sodium, 20 mL of anhydrous methanol). The mixture of nucleosides was purified by a short flash chromatography and eluted with methanol/dichloromethane (1/1). Yield: 1.23 g (85%).

*1-[2-Deoxy-5-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]benzo[f]quinazoline-1,3-(2H)-dione (**18**).* The mixture of nucleosides **16** (309 mg, 0.94 mmol) was dried by three coevaporations with anhydrous pyridine and then dissolved in anhydrous pyridine (10 mL). 4,4′-Dimethoxytrityl chloride (1.1 equiv, 400 mg) dissolved in a minimum of anhydrous pyridine was added. The reaction mixture was stirred at RT for 3 h; the reaction was stopped by addition of 30 μL of water and the solution concentrated. The two anomers were separated by flash chromatography along a gradient of methanol/dichloromethane (0 to 3% with 1% triethylamine). Yield: 380 mg [59%, β anomer, amorphous, $R_f = 0.24$ (5/95 methanol/chloroform)] and 29 mg (5%, α anomer, amorphous $R_f = 0.48$). ^1H NMR (200 MHz,

$CDCl_3$): δ 2.25 (m, 1H, $H_{2'\alpha}$), 2.95 (m, 1H, $H_{2'\beta}$), 3.45 (m, 2H, $H_{5'}$ and $H_{5''}$), 3.65 (s, 6H, OCH_3), 4.05 (m, 1H, $H_{4'}$), 5.30 (m, 1H, $H_{3'}$), 6.70 (d, 4H, H aromatic DMT, $J = 8.7$ Hz), 7.00 (t, 1H, $H_{1'}$, $J = 7.8$ Hz), 7.05−7.60 (m, 13H, H_6, H_7, H_8, H_9, H aromatic DMT), 8.20 (d, 1H, H_5, $J = 9.3$ Hz), 9.70 (d, 1H, H_{10}, $J = 8.7$ Hz). ^{13}C NMR (50.32 MHz, $CDCl_3$): δ 37.50 ($C_{2'}$), 54.85 (OCH_3), 62.45 ($C_{5'}$), 70.05 ($C_{3'}$), 84.25 ($C_{1'}$*), 85.00 ($C_{4'}$*), 86.20 (Cq DMT), 109.05 (C_{10b}), 116.40 (C_6), 125.45, 125.75, 126.60, 127.55, 127.85, 128.25, 128.90, 129.35, 130.1, 131.10 (Car and $C_{6a,7,8,9,10,10a}$), 135.45 (Cq DMT), 135.80 (C_5), 141.95 (C_{4a}), 144.50 (Cq DMT), 150.60 (C_3), 158.25 (Cq DMT), 163.05 (C_1).

*1-(2-Deoxy-β-*D*-erythro-pentofuranosyl)benzo[f]quinazoline-1,3-(2H)-dione (**22**).* **18** (118 mg, 0.174 mmol) was stirred for 1 h in aqueous acetic acid (80%, 30 mL) at RT. Next the solution was evaporated, loaded onto a short silica gel column, and eluted with methanol/dichloromethane (1/1), yielding 52.5 mg (yield 92%) of β nucleoside. HRMS: 329.1119 $(M + H)^+$ calcd for $C_{17}H_{17}N_2O_5$, found 329.1137. ^{1}H NMR (500 MHz, DMSO-d_6, β anomer): δ 2.00 (m, 1H, $H_{2'\alpha}$), 2.70 (m, 1H, $H_{2'\beta}$), 3.70 (m, 2H, $H_{5'}$ and $H_{5''}$), 3.80 (m, 1H, $H_{4'}$), 4.50 (m, 1H, $H_{3'}$), 5.05 (br s, 1H, $OH_{5'}$), 5.30 (br s, 1H, $OH_{3'}$), 6.80 (t, 1H, $H_{1'}$, $J = 7.8$ Hz), 7.55 (m, 1H, H_8), 7.65 (m, 1H, H_9), 7.95 (d, 1H, H_7, $J = 7.8$ Hz), 8.10 (d, 1H, H_6, $J = 9.4$ Hz), 8.20 (d, 1H, H_5, $J = 9.4$ Hz), 9.65 (d, 1H, H_{10}, $J = 8.7$ Hz), 11.70 (br s, 1H, H_2). ^{13}C NMR (125.8 MHz, DMSO-d_6): δ 36.30 ($C_{2'}$), 60.40 ($C_{5'}$), 69.10 ($C_{3'}$), 83.60 ($C_{1'}$), 86.10 ($C_{4'}$), 107.90 (C_{10b}), 116.20 (C_6), 124.70 (C_{10}), 125.05 (C_8), 127.80 (C_7), 128.40 (C_9, C_{10a}*), 128.70 (C_{6a}*), 134.75 (C_5), 141.50 (C_{4a}), 149.40 (C_3), 162.20 (C_1).

*1-[2-Deoxy-3-O-[2-cyanoethoxy(diisopropylamino)-phosphino]-5-(4,4′-dimethoxytrityl)-β-*D*-erythro-pentofuranosyl]benzo[f]quinazoline-1,3-(2H)-dione (**24**).* The β anomer **18** (234 mg, 3.45 mmol) was dissolved in anhydrous dichloromethane (stabilized with 0.002% amylene) (3 mL). Four equivalents of diisopropylethylamine (236 μL) and 1.5 equiv of 2-cyanoethyl diisopropylchlorophosphoramidite (126 mg) in anhydrous dichloromethane (0.75 mL) were added. The reaction was stopped after 1 h at RT with 30 μL of methanol and the mixture extracted with ethyl acetate (50 mL). The phosphoramidite was purified twice by flash chromatography with ethyl acetate/dichloromethane/triethylamine (45/45/10) as the eluent, yielding 189 mg of pure phosphoramidite (66% yield). $R_f = 0.79$ (45/45/10 ethyl acetate/hexane/triethylamine). MS (FAB^-): 829.0 $(M - H)^-$. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.90−1.20 (m, 12H, CH_3 ^{i}Pr), 2.20−2.70 (m, 3H, $H_{2'\alpha}$ and CH_2CN), 2.90 (m, 1H, $H_{2'\beta}$), 3.30−3.80 [m, 7H, $H_{5'}$, $H_{5''}$, and $H_{4'}$, CH_2OP and $CH(CH_3)_2$], 3.70 (s, 6H, CH_3O), 4.85−5.15 (m, 1H, $H_{3'}$), 6.65−6.80 (m, 6H, H aromatic), 6.95 (t, 1H, $H_{1'}$, $J = 7.6$ Hz), 7.10−7.70 (m, 14H, H aromatic), 8.20 (d, 1H, H_5, $J = 9.3$ Hz), 9.70 (d, 1H, H_{10}, $J = 8.7$ Hz). ^{13}C NMR (50.32 MHz, $CDCl_3$): δ 24.05 (CH_2CN), 24.35 (CH_3 ^{i}Pr), 36.30 ($C_{2'}$), 43.05−43.10 (CH ^{i}Pr), 54.90 (OCH_3), 58.10 (d, CH_2OP, $^2J_{CP} = 19.1$ Hz), 61.80 ($C_{5'}$), 71.80−72.25 (2d, $C_{3'}$, $^2J_{CP} = 17.8$ Hz, $^2J_{CP} = 23.6$ Hz), 84.20 and 84.25 ($C_{4'}$ and $C_{1'}$), 86.35 (Cq DMT), 109.05 (C_{10b}), 112.90 (C aromatic DMT), 116.30 (C_6), 117.25 (CN), 125.70, 125.85, 126.75, 127.65, 127.95, 128.35, 129.15, 129.50 (C aromatic and $C_{7,8,9,10}$), 130.20 (C_{10a}*), 131.20 (C_{6a}*), 136.35 (C_5), 137.35 (Cq aromatic), 141.85 (C_{4a}), 144.40 (Cq DMT), 150.15 (C_3), 158.40 (Cq DMT), 162.50 (C_1). ^{31}P NMR (81.02 MHz, $CDCl_3$): δ 147.27, 147.67.

Oligonucleotide Synthesis

All oligonucleotides were synthesized on a 0.2 μmol scale on a Millipore Expedite 8909 DNA synthesizer using conventional β-cyanoethyl phosphoramidite chemistry. The modified and standard bases were dissolved in anhydrous acetonitrile (0.1 M final concentration). The modified phosphoramidites were used with a coupling time of 15 min. The coupling efficiency was the same as that of unmodified amidites (>98%). All oligomers were synthesized "trityl on". After synthesis, the solid supports were treated overnight at 55 °C with fresh, concentrated NH_4OH (3 mL). The solution was then collected and concentrated to dryness. The crude tritylated oligonucleotide was purified by reverse-phase HPLC (Nucleoside 300-5 C18 column) using the following gradient system: A (0.1 M triethylammonium acetate at pH 7); B (0.1 M triethylammonium acetate at pH 7 in 80% acetonitrile). A linear gradient of 0 to 60% buffer B over 60 min at a flow rate of 1 mL/min was used. Elution was monitored by UV absorption at 260 nm for analytical runs and 290 nm for preparative ones. After collection, pooled fractions were lyophilized, resuspended in 1 mL of water, and extracted with ethyl acetate. Finally, oligonucleotides were precipitated using *n*-butanol. When required, aliquots of purified oligonucleotides were analyzed by gel electrophoresis to confirm expected length and purity. When necessary, oligomers were purified by preparative electrophoresis, and were visualized by the standard "UV Shadow" technique.

UV Thermal Melting Experiments

Purified oligonucleotides (0.5 nmol of each strand) were dissolved in 0.5 mL of the appropriate buffer, and the mixture was boiled for 2 min. The double helix studies used a buffer containing 10 mM sodium cacodylate (pH 7), 50 mM NaCl, and 1 mM magnesium acetate. The buffer for the triple helix studies was 10 mM sodium cacodylate (pH 7), 100 mM NaCl, 10 mM magnesium acetate, and 1 mM spermine. Melting temperature experiments were performed on a Cary 1E UV−visible spectrophotometer with a controller unit. Samples were kept at 4 °C for at least 30 min and then heated from 4 to 90 °C at a rate of 0.5 °C/min. The absorbance at 260 nm was measured every 2 min. A molar exctinction coefficient of 4.98×10^4 mol^{-1} L cm^{-1} at 260 nm was used for base **1**.

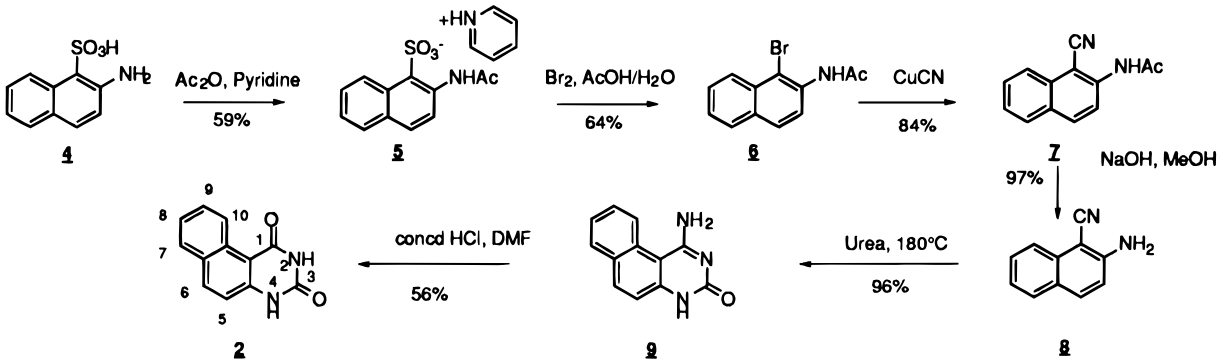
Fluorescence Measurements

The buffers for fluorescence measurements were the same as the ones used for T_m experiments (see above). The fluorescence spectra were recorded on a Kontron SFM 25 spectrofluorimeter using a 5 mm × 5 mm cuvette that was thermostated with a Huber cryothermostat.

RESULTS

The incorporation of either of the two benzoquinazoline derivatives **1** or **2** as a substitute for thymine in triplex-forming oligonucleotides required the synthesis of their β

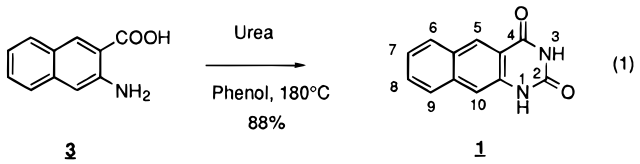
Scheme 1: Chemical Synthetic Pathway for Benzo[*f*]quinazoline-2,4-(1*H*,3*H*)-dione



deoxynucleoside derivatives as well as the corresponding phosphoramidites used in automatic solid-phase synthesis.

Synthesis of Benzo[g]quinazoline-2,4-(1H,3H)-dione (1)

An efficient synthesis of **1** was previously reported by Curd et al. (*24*) according to eq 1.



Condensation of the commercially available 3-aminonaphthoic acid (**3**) with urea in phenol yielded the desired pyrimidine-2,4-dione moiety. **1** was isolated by crystallization from a DMF/$CHCl_3$ mixture and fully characterized by ^1H and ^{13}C NMR spectroscopy.

Synthesis of Benzo[f]quinazoline-2,4-(1H,3H)-dione (2)

The synthetic route for **2** was adapted from Rosowsky et al. (*25*) (Scheme 1). The commercially available 2-amino-1-naphthalenesulfonic acid was acetylated (**5**) and then converted into the bromonaphthalene derivative **6**. Treatment of this compound with cuprous cyanide in DMF yielded the 2-(acetylamino)-1-cyanonaphthalene **7** from which **8** was readily obtained upon removal of *N*-acetyl protecting group by brief alkaline hydrolysis. Condensation of **8** with urea quantitatively yielded the 1-aminobenzoquinazoline-3-(4*H*)-one **9** which by acid hydrolysis led to the expected benzo-[*f*]quinazoline-1,3-(2*H*,4*H*)-dione (**2**).

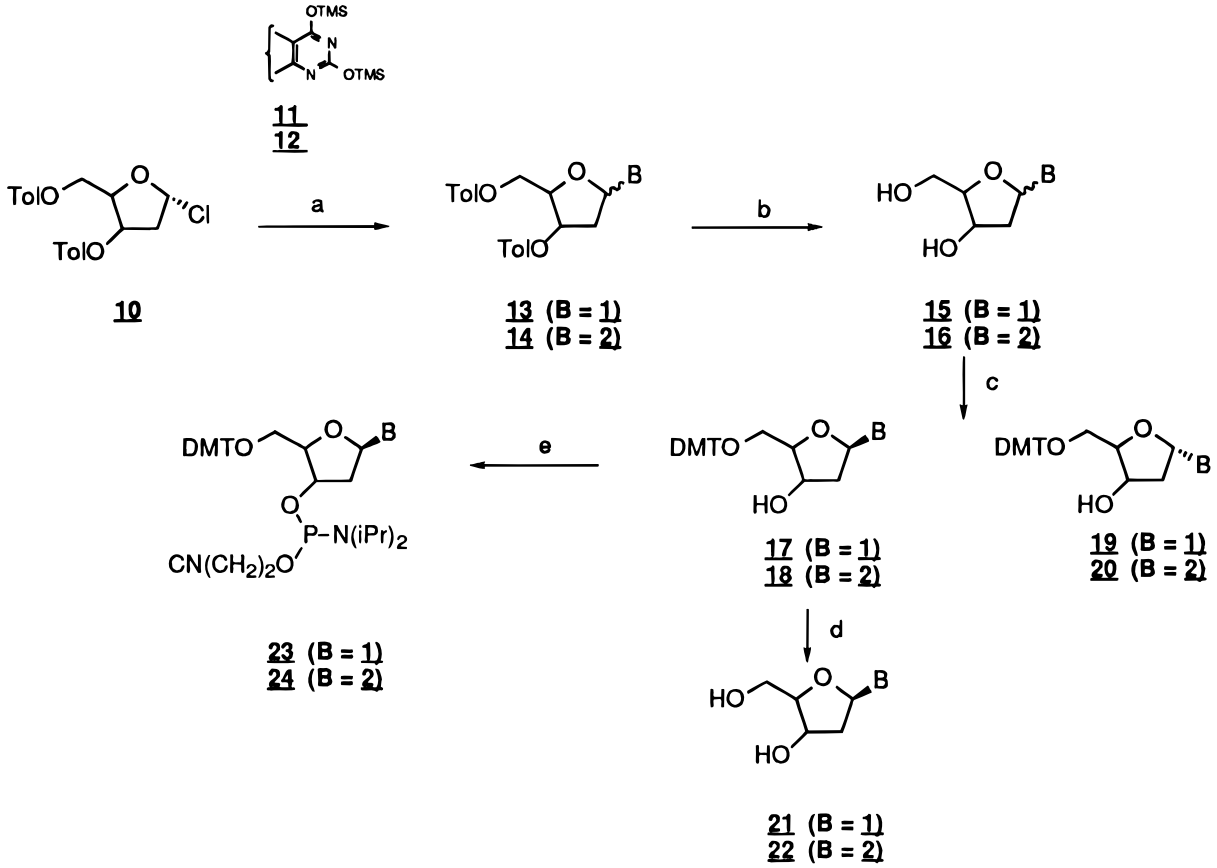
Synthesis of Deoxynucleoside Derivatives of Benzoquinazolines

The synthesis of the deoxynucleoside derivatives of **1** and **2** had not been reported previously. We used the synthetic route established previously for other quinazoline derivatives (*19*, *20*). This route (Scheme 2) relies mainly on the resolution of the anomeric mixture obtained from direct glycosylation of 2-deoxyribofuranoside chloride, at the 5′-monotrityl derivative step.

Silylated derivatives of **1** and **2** (**11** and **12**, respectively) obtained from treatment with hexamethyldisilazane (HMDS) in the presence of catalytic amounts of $(NH_4)_2SO_4$ and CH_3CONH_2 were then condensed with 1-(chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl)-α-D-*erythro*-pentofuranose (**10**) via CuI catalysis in dry acetonitrile at room temperature. Such conditions are known to favor the β introduction of the heterocyclic ring (*26*). The unresolvable anomeric mixtures of ditoluoyl nucleosides (**13** and **14**) were isolated through flash chromatography over a silica gel column in 56 and 48% yields, respectively. Debenzoylation with sodium methoxide in methanol afforded the free nucleosides **15** (81%) and **16** (85%). The 5′ positions of the α/β mixtures were then tritylated with 4,4′-dimethoxytrityl chloride in anhydrous pyridine. The resulting 5′-ODMT derivatives were separated on a flash silica gel column. Monotrityl derivatives of benzo[*g*]quinazoline nucleosides **17** (β) and **19** (α) were obtained in 62% overall yield in a 1/2 ratio. The benzo[*f*]quinazoline compounds **18** (β) and **20** (α) were obtained in 64% overall yield with a much more favorable ratio of β/α (10/1). The β anomers were then converted to phosphoramidites using the conventional phosphitylation reaction with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in dichloromethane in the presence of *N*,*N*-diisopropylethylamine (*27*). Phosphoramidites **25** (50% yield) and **26** (66% yield) were isolated as white foam after two purification steps by silica gel flash chromatography using ethyl acetate/dichloromethane/triethylamine (45/45/10, v/v/v) as the elution solvent.

The structures of the various compounds reported above were checked by ^1H and ^{13}C NMR spectroscopy which clearly revealed the presence of the two anomeric configurations for **13**−**16**. The configurations were determined on detritylayed compounds **21**−**24** obtained after aqueous acetic acid treatment. The assignments of ^1H and ^{13}C NMR spectra of these compounds were reached by $^1H-^1H$ and $^1H-^{13}C$ correlation methods [COSY, HMQC (*28*), and HMBC (*29*)] (data not shown). The assignment of configuration at the anomeric carbon was reached through ROESY 2D NMR experiments. We have previously shown (*19*) that the analysis of the dipolar interaction of nearby protons was the most reliable method for unambiguously determining the configuration at C1 in the quinazoline series. The same method was used here, and clear NOE correlation cross-peaks were detected between H-1′ and H-4′ of **21** and **22** as expected for β anomers. The ROESY map for **21** is shown in Figure 1. Along with the cross-peak between H-1′ and H-4′, additional correlations were detected between H-10 and H-1′, H-3′, and H-2′β. These cross-peaks led us to propose a mixture of syn and anti conformers. NOE correlation

Scheme 2: Chemical Synthetic Route to the Nucleoside and Phosphoramidite Derivatives of **1** and **2**[a]



[a] (a) CuI, dry CH_3CN; (b) $CH_3O^-Na^+$, CH_3OH; (c) DMTrCl, Pyr; (d) CH_3COOH, H_2O; (e) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphite, *N*,*N*-diisopropylamine.

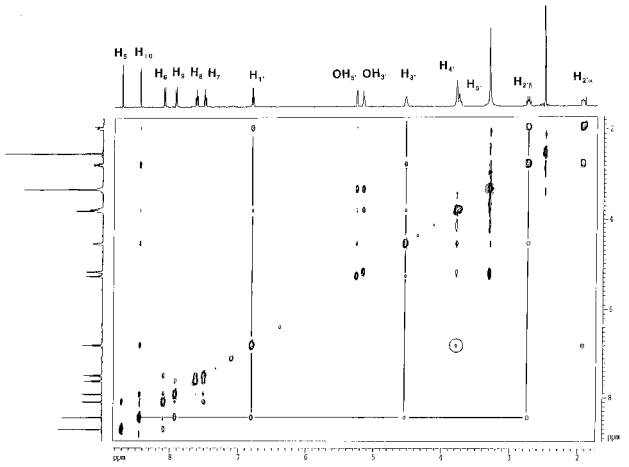


FIGURE 1: Part of the 2D ROESY correlation map of the β anomer **17** (mixing time of 300 ms). The open circle shows the correlation spot for H-1′ and H-4′, as expected for the β configuration. Lines show the correlation spots for H-10 and H-1′, H-3′, and H-2′β.

between H-10 and H-1′ is consistent with a syn conformation, whereas H-3′ and H-2′β cross-peaks support an anti conformation. Furthermore, the ^{1}H chemical shift pattern of 2′ and 2″ protons (two clearly distinct multiplets) is fully consistent with the pattern observed for the β anomers of quinazoline derivatives (*20*).

Oligonucleotide Synthesis

Phosphoramidites **25** and **26** were incorporated into oligonucleotides using standard phosphoramidite chemistry. The coupling time of the modified monomers was increased to 15 min compared to 1 min for unmodified ones. Under these conditions, the coupling yields were similar to those of A, C, G, or T phosphoramidites, as estimated from detritylation profiles.

Oligonucleotide sequences are shown in Table 1. Single-stranded or double-stranded targets were designed so we could evaluate the ability of the modified bases to elicit duplex or triplex formation. In triplexes, the Hoogsteen third strand was bridged by a pentanucleotide loop to one of the strands of the target duplex, leading to the formation of bimolecular complexes characterized by fast kinetics (*30*).

Thermal Denaturation Studies

Single-Stranded Targets. We first checked the ability of benzoquinazoline-modified oligomers to bind to a single-standed DNA target. As shown in Table 1, the substitution of one T by **1** or **2** led to a significant destabilization of duplexes **I-1** and **I-2** ($\Delta T_m = -5$ and -6.5 °C, respectivey). These results are very similar to that obtained previously with the same sequences containing quinazoline derivatives (*19*, *20*).

*Parallel Triplexes (Py·Pu*Py Triplexes).* As expected, the melting curves of these complexes showed a single transition from the bound to the dissociated state (*31*, *32*). The formation of triplex **II-TT** resulted in a 10.5 °C increase in T_m, compared to that of **IV-a**, the control duplex constituted of the Watson−Crick part of triplex **II**, indicating third strand contribution to the overall complex stability. Single or

Table 1: Oligonucleotide Sequences[a]

I - Duplexes

```
5' A C A A C A C A C C A C
   | | | | | | | | | | | |
3' T G T T G X G T G G T G
```

Cplx	Seq.	Tm (°C)	ΔTm (°C)
I-T	X=T	50.5	
I-1	X=1	45.5	-5
I-2	X=2	44.0	-6.5

II - Parallel hairpin triplexes

```
   C C T T T T T T T T T T 5'
A    | | | | | | | | | | |
C    G A A A A A A A A A A 3'
A    • • • • • • • • • • •
   C C T T T X Y T T T T T 3'
```

Cplx	Seq.	Tm (°C)	ΔTm (°C)
II-TT	X=Y=T	43.5	
II-1T	X=1, Y=T	41.5	-2
II-2T	X=2, Y=T	41.0	-2.5
II-11	X=1, Y=1	40.5	-3
II-22	X=2, Y=2	38.5	-5
II-GT	X=G, Y=T	36.0	-8

III - Antiparallel hairpin triplexes

```
   5' C T C C T C C C T C C T 3'
      | | | | | | | | | | | |
A  C  G A G G A G G G A G G A 5'
C    • • • • • • • • • • • •
A  C  G T G G X G G G T G G T 3'
```

Cplx	Seq.	Tm (°C)	ΔTm (°C)
III-T	X=T	62.0	
III-1	X=1	59.5	-2.5
III-2	X=2	57.0	-5

IV - Control duplexes

```
5' C T T T T T T T T T T T
   | | | | | | | | | | | |
3' G A A A A A A A A A A A
```

Cplx	Tm (°C)
IV-a	33.0

```
5' C T C C T C C C T C C T
   | | | | | | | | | | | |
3' G A G G A G G G A G G A
```

Cplx	Tm (°C)
IV-b	54.0

[a] Vertical bars and dots indicate Watson−Crick and Hoogsteen hydrogen bonding, respectively. The T_ms were obtained under the conditions described in Experimental Procedures. T_m is the melting temperature (±0.5 °C). $\Delta T_m = T_m$(modified oligomers) − T_m(unmodified oligomers).

double substitution of T by benzoquinazoline **1** or **2** in the Hoogsteen domain of sequence II led to a slight destabilization (ΔT_m = ca. −2 °C) of the corresponding triplexes **II-1T** and **II-2T**. Double susbtitution did not lead to an additive effect for **1** but induced further destabilization for **2** (ΔT_m = −5 °C). These data can be compared to those of triplex **II-GT** which includes a substitution of T by G, leading to a TA*G triplet, the least stable triplet mismatch in this context (*33*) (ΔT_m = −8 °C).

*Antiparallel Triplexes (Py·Pu*Pu Triplexes).* Similar experiments were performed within the purine motif series. Complexes **III** were designed to form CG*G or TA*T triplexes, the Hoogsteen third strand exhibiting an antiparallel orientation relative to the purine strand. Triplex formation was accompanied by a 8 °C increase in T_m compared to that of the control double-stranded **IVb**. Introduction of benzoquinazoline **1** and **2** in this context led to a more pronounced destabilization with a single substitution (ΔT_m = −2.5 to −5 °C) than for parallel triplexes.

Fluorescence Studies

The purification of **1** by TLC was easily monitored due to the strong fluorescence emission of this derivative, suggesting that it could be used to monitor complex formation. Preliminary results demonstrated that benzo[*g*]-quinazoline fluorescence was sensitive to TA*T triplet formation (F. Godde et al., submitted). This prompted us to investigate double- and triple-stranded complex formation with **1**-containing oligonucleotides in which the benzo[*g*]-quinazoline was introduced in different surroundings (Table 2a). The 11mers (**TC** and **TC1**) form Watson−Crick duplexes upon binding to the **GA** oligo, and the 27mers (**H**, **H1**, and **W1**)[3] were designed to give rise to triple-stranded structures through the formation of TA*T and CG*C^+ triplets. The modified residue (**1**) was placed either in the

[3] In this notation, H1 stands for incorporation of **1** in the Hoogsteen strand and W1 for incorporation in the Watson−Crick ones.

Table 2: Sequences Used in Fluorescence Studies and Melting Temperatures (T_ms)

(a) Names and Sequences

Oligonucleotides	Sequences
GA	5′ G A A G A A A A G G A 3′
TC	3′ C T T C T T T T C C T 5′
TC1	C T T C **1** T T T C C T 5′
H	C T T C T T T T C C T 3′ T_5 C T T C T T T T C C T 5′
H1	C T T C **1** T T T C C T 3′ T_5 C T T C T T T T C C T 5′
W1	C T T C T T T T C C T 3′ T_5 C T T C **1** T T T C C T 5′

(b) T_ms of the Various Complexes and Hyperchromicities of the Observed Transitions[a]

	T_m (°C), hyperchromicity (%)	
oligonucleotide	pH 6.0	pH 7.0
TC + GA	37.5, 12	37, 12
TC1 + GA	NC, 17	NC, 17
H + GA	47.5, 31	41.0, 25
H1 + GA	44.5, 31	38.4, 30
W1 + GA	45.0, 28	39.0, 25

[a] NC, noncooperative transition.

Watson−Crick or in the Hoogsteen strand. In every case, **1** was adjacent to a C residue on one side and to a T on the other side. UV absorbance-monitored thermal denaturation studies were first conducted on these complexes (Table 2b). The same observation as in the above study (Table 1) can be made: a clear destabilizing contribution of **1** on double strands, and a limited destabilization for triple-stranded structures. In this case, the transition TC1−GA duplex displayed a broad transition indicative of low cooperativity (not shown). As expected in contrast to duplexes, the triplexes formed with the GA strand revealed a pH-dependent stability (Table 2); a 6 °C decrease in T_m was observed when the pH was raised from 6 to 7, consistent with the requirement of protonated C for triplex formation (*34*, *35*). The introduction of **1** either in the Hoogsteen (**H1**+**GA**) or in the Watson−Crick part of the triplex (**W1**+**GA**) induced a weak destabilizing effect ($\Delta T_m \approx -3$ °C) at both acidic and neutral pH.

The incorporation of a single **1** residue into these oligopyrimidines led to oligomers displaying fluorescent properties similar to that of the isolated nucleoside (F. Godde et al., submitted); a broad emission spectrum ($\lambda = 434$ nm) (Figure 2) was observed upon excitation either at 260 or at 360 nm which corresponded to the two major excitation maxima (F. Godde, submitted). Similar fluorescence intensities were observed for oligonucleotides **TC1**, **H1**, and **W1** which revealed they were insensitive to pH (pH range of 5.0−8.0). The fluorescence quantum yield of the modified oligopyrimidines was dependent on neighbor bases; it was lowered only by 15% compared to that of the free nucleoside for **TC1**, indicative of limited stacking interactions in this oligomer, but when **1** was inserted between two G's, a 100-fold reduction of the fluorescence intensity was observed

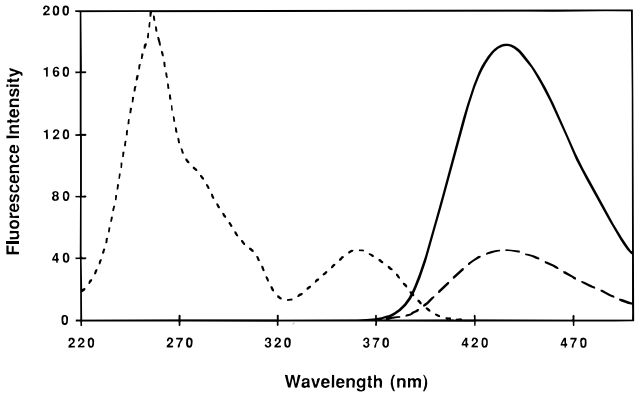


FIGURE 2: Excitation fluorescence spectrum (- - -) and emission spectrum (—) of oligonucleotide **H1**. The fluorescence was recorded for an emission wavelength set at 434 nm for 0.1 μM H1 in the following buffer: 10 mM sodium cacodylate, 100 mM NaCl, 10 mM magnesium acetate, and 1 mM spermine (pH 5). The emision spectrum ($\lambda_{exc} = 250$ nm) was obtained under the same conditions.

Table 3: Fluorescence Data of Duplexes and Triplexes ($\lambda_{exc} = 250$ nm)[a]

complex	pH	Mg^{2+} spermine	t (°C)	λ_{em} (nm)	fluorescence ratio
TC1 and **GA**	6	with	10	434	0.8
W1 and **GA**	6	with	10	428	0.9
H1 and **GA**	5	with	10	428	0.2
H1 and **GA**	5	with	25	428	0.2
H1 and **GA**	5	without	10	434	1
H1 and **GA**	8	with	25	434	0.5

[a] The fluorescence ratio is the relative fluorescence intensity of the complex compared to that of **TC1** (under the same experimental conditions; each oligo at 0.1 μM).

(not shown). However, the maximum remained in the same position (434 nm).

Binding of the 11mer **TC1** to its **GA** complementary sequence resulted only in a weak inhibition of the fluorescence (20%) without any shift of the emission maximum (Table 3). In contrast, the fluorescence properties of the oligomer **H1** were strongly affected upon binding to the oligopurine 5′-$GA_2GA_4G_2A$ (**GA**); mixing these oligonucleotides at low pH in the presence of Mg^{2+} resulted in a 6 nm shift of the maximum position to the short wavelength and in a 80% decrease of the fluorescence quantum yield (Figure 3a). Titration of **H1** by the 11mer **GA** led to the formation of a 1/1 complex as fluorescence variation plateaued at about the stoichiometric concentration of the two oligomers (Figure 3b).

The association between the oligopurine and the oligopyrimidine was dependent on the ionic and pH conditions; fluorescence modifications were no longer observed in the absence of Mg^{2+} or spermine (Table 3). Raising simultaneously the pH and the temperature resulted also in reduced fluorescence alterations compared to what was observed at pH 5. This is characteristic of Py·Pu*Py triple helices involving $CG*C^+$ triplets.

A weaker effect was observed when **1** was inserted into the second strand of the complex. Mixing **W1** with the oligomer **GA** also induced a 6 nm shift of the maximum emission, but this was accompanied by only a slight quenching effect (Table 3). Therefore, this fluorescent T analogue senses different helical conformations, making it

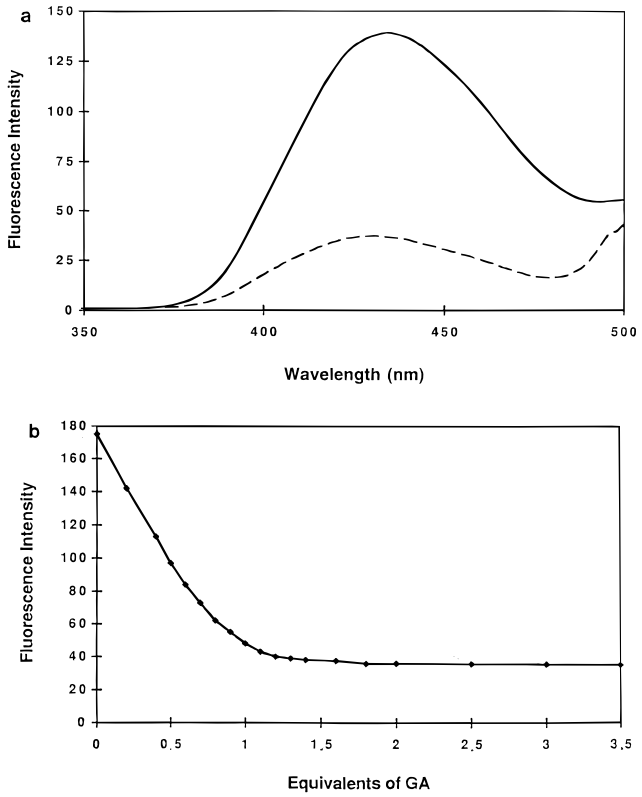


FIGURE 3: (a) Emission spectra of **H1** and **GA** complexes (λ_{exc} = 250 nm). Emission spectra of **H1** (0.1μM) either in the absence (—) or in the presence of an equimolar amount of **GA** (- - -) were recorded at 10 °C in a 10 mM sodium cacodylate buffer (pH 5) containing 100 mM NaCl, 10 mM magnesium acetate, and 1 mM spermine. (b) Fluorimetric titration curve of **H1** by **GA** at 0.1 μM H1 with 10 mM sodium cacodylate (pH 5), 100 mM NaCl, 10 mM magnesium acetate, and 1 mM spermine at 25 °C.

a sensitive reporter group for monitoring nucleic acid interactions.

DISCUSSION

Two general methods are being used for the preparation of 2′-deoxy-β-D-nucleoside, namely, the direct glycosylation and the deoxygenation procedures. The first approach leads to a low diastereoselectivity due to the lack of a 2′-*O*-acyl function which allows control by neighboring group participation. However, the second method is lengthy and suffers also from tedious purification steps. In our previous work on the conception of modified bases with extended aromatic domain, we developed the strategy used in this study, which generally enables the easy purification of 5′-tritylated anomers (*19*). This method also proved to be successful with the benzoquinazoline compounds described here.

Nevertheless, careful assignment of the 1′ configuration had to be done. The use of the splitting pattern of H-1′ observed in ^{1}H NMR may lead to erroneous conclusions (*36*). Detection of dipolar relaxation proccesses between nearby protons through NOESY or ROESY 2D NMR experiments is the most reliable method. In general, the β anomers display intensive cross-peaks between H-1′ and H-4′ (*37*−*39*, and references therein). NOE cross-peaks between protons of the aromatic heterocycle and assigned deoxyribose protons allow us to determine the conformation of the glycosidic bond. The mixture of syn and anti conformers of **1** is similar to the observation previously reported for the β anomers of quinazoline (*19*). The chemical shift pattern of H-2′,2″ may be also correlated with the syn and/or anti conformation of the base (*19*, *38*).

Targeting nucleic acid structures with oligonucleotides requires the design of sequences that are able to interact with non-single-stranded regions, for instance, through triple helix formation. We developed a strategy in which an antisense sequence forms simultaneously a double strand with a single-stranded region at the bottom of a hairpin and a triple strand against the stem region (*7*). Such a strategy would ideally need specific bases for single strand and double strand recognition to increase the overall specificity of the antisense oligonucleotide. The recognition properties of target sequences by **1** or **2** containing antisense oligomers do not fulfill completely the above-mentioned requirements. We actually observed some selectivity since **1** and **2** although leading to slighty destabilized triplexes ($\Delta T_m = -2$ to -2.5 °C) induced a more pronounced destabilizing effect with single-stranded targets ($\Delta T_m = -5$ to -6.5 °C). The results reported here display the same trends as previously observed for quinzoline-2,4-dione derivatives (*20*). Although the benzoquinazoline compounds have an extended hydrophobic domain compared to quinazoline, we were unable to detect any increase in stacking interactions with the adjacent base triplets. This might mean that the structural parameters of consecutive triplets do not allow a favorable overlap of aromatic rings. Alternatively, we could surmise that the benzoquinazoline ring induces an increase in the stacking surfaces with the two neighbor triplets, thus disrupting the regular helical parameters of the triple helix, leading to an overall destabilizing effect. Hunter showed recently that the helical twist angle between adjacent base pairs of double helices is most likely determined by the geometric constraints of the backone (*40*). Whether such a conclusion can be extended to triple helices and to modified nucleic acid bases is not known.

Benzo[*g*]quinazoline displayed strong fluorescence emission properties which can be used to monitor the formation of nucleic acid complexes. Several fluorescent analogues of nucleic acid bases have been proposed for investigating duplex formation (*41*, *42*). Benzo[*g*]quinazoline emission seems particularly sensitive to the helical conformation, allowing preferential detection of triplex formation. Annealing of benzo[*g*]quinazoline-containing oligonucleotides to complementary strands led to weak if any fluorescence modification upon duplex formation, suggesting a limited variation of the fluorophore environment. However, incorporation of **1** in the Hoogsteen strand of Py·Pu*Py complexes drastically reduced the fluorescence intensity and shifted the emission maximum, an indication of changes in both stacking interactions and hydrophobic environment. Furthermore, when **1** was incorporated in the Watson−Crick part of a triplex, its maximum emission was also shifted but fluorescence quenching was more limited compared to the incorporation in the thrid strand. This behavior of the fluorophore suggests that stacking with adjacent base triplets involves much more the extended aromatic domain of quinazoline than the stacking with neighboring base pairs in duplexes. Benzo[*g*]quinazoline can be used to selectively investigate third strand interaction in Py·Pu*Py complexes. Variations of fluorescence properties may reflect subtle local structural alteration; indeed, we demonstrated in a preliminary report

(F. Godde et al., submitted) that **1** inserted in a "T" third strand displayed also a 6 nm shift of the emission maximum, but that the fluorescence quenching was only 20% upon binding to the cognate duplex. It should be mentionned that the fluorescence of **1** was not sensitive to the titration of adjacent C residues.

Benzoquinazoline derivatives were originally conceived as substitutes for thymine in triplex-forming oligonucleotides. They effectively allow the formation of triple-stranded structures, the stabilities of which are only slightly altered by substitution in the Hoogsteen third strand of Py·Pu*Py complexes. Further developments along this line will consider the targeting of the RNA purine strand using triple strand-forming oligomers made of 2′-OMe-RNA strands which were able to form stable RR*M structures (*43*) (in this notation, M stands for the 2′-Ome backbone and R for the RNA strands).

The high fluorescence quantum yield of benzo[*g*]quinazoline, at an excitation wavelength of either 260 or 360 nm, could also prove to be useful for monitoring the uptake and compartmentalization of antisense oligonucleotides in intact cells.

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