7-Deazapurine and 8-Aza-7-deazapurine Nucleoside and Oligonucleotide Pyrene “Click” Conjugates: Synthesis, Nucleobase Controlled Fluorescence Quenching, and Duplex Stability

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Supporting Information

ABSTRACT: 7-Deazapurine and 8-aza-7-deazapurine nucleosides related to dA and dG bearing 7-octadiynyl or 7-tripropargylamine side chains as well as corresponding oligonucleotides were synthesized. “Click” conjugation with 1-azidomethyl pyrene (10) resulted in fluorescent derivatives. Octadiynyl conjugates show only monomer fluorescence, while the proximal alignment of pyrene residues in the tripropargylamine derivatives causes excimer emission. 8-Aza-7-deazapurine pyrene “click” conjugates exhibit fluorescence emission much higher than that of 7-deazapurine derivatives. They are quenched by intramolecular charge transfer between the nucleobase and the dye. Oligonucleotide single strands decorated with two “double clicked” pyrenes show weak or no excimer fluorescence. However, when duplexes carry proximal pyrenes in complementary strands, strong excimer fluorescence is observed. A single replacement of a canonical nucleobase by a pyrene conjugate stabilizes the duplex substantially, most likely by stacking interactions: 6−12 °C for duplexes with a modified “adenine” base and 2−6 °C for a modified “guanine” base. The favorable photophysical properties of 8-aza-7-deazapurine pyrene conjugates improve the utility of pyrene fluorescence reporters in oligonucleotide sensing as these nucleoside conjugates are not affected by nucleobase induced quenching.

INTRODUCTION

Fluorescence is a powerful tool for structural and functional studies of a diversity of molecules. Among the various fluorophores, pyrene derivatives are attractive fluorescent probes.3 Their fluorescence properties are utilized for the investigation of water-soluble polymers2a or to detect toxic metals in water,2b glucose,2c and even explosives.2d Pyrene has several photophysical properties that make it extraordinarily suitable as fluorescent reporter, such as high fluorescence quantum yield, chemical stability, and long fluorescence lifetime.2a,3,4 Moreover, the fluorescence of pyrene derivatives is sensitive to the polarity of the surrounding environment including solvent changes.5

In addition to monomer fluorescence, pyrene and its derivatives can show excimer emission. The excimer is formed when an excited pyrene monomer interacts in a specific manner with a proximal ground state (unexcited) pyrene.6 However, pyrene-modified oligonucleotide probes based on monomer emission intensity are often affected by fluorescence quenching through an electron-migration process between the excited pyrene and nucleoside bases.7 Pyrene excimer fluorescence is less sensitive to quenching than pyrene monomer emission.8 For this reason, pyrene excimer emission has been explored for monitoring RNA and DNA hybridization9 including studies on duplexes,10 triplexes,11 and quadruplexes,12 as well as on artificial DNAs, e.g., those with parallel chain orientation.13 For this, pyrene residues were linked to various positions of nucleic acids as well as to their constituents (nucleosides and nucleotides).14−16

The copper(I)-catalyzed Huisgen−Meldal−Sharpless azide−alkyne cycloaddition reaction (CuAAC)17 has emerged as an ideal bioorthogonal protocol for the preparation of rich chemical diversity. Our laboratory has already reported on the functionalization of nucleosides and oligonucleotides using the CuAAC reaction.18−21 This also includes the “double click” functionalization of oligonucleotides incorporating a branched side chain with fluorogenic 1-azidomethyl pyrene (10).22 The “double click” reaction brings the new ligands (pyrene) of the branched side chain into a proximal position.

Earlier, it was observed that the fluorescence of nucleoside dye conjugates, such as coumarin, is sensitive to nucleobase modification.18 The fluorescence is partially quenched when the dye is linked to a 7-deazapurine skeleton (purine numbering is used throughout the discussion). This prompted us to study the fluorescence behavior of nucleoside and oligonucleotide pyrene derivatives in artificial DNAs, e.g., those with parallel chain orientation.13 For this, pyrene residues were linked to various positions of nucleic acids as well as to their constituents (nucleosides and nucleotides).14−16

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conjugates and to identify nucleobases related to dA and dG that form stable Watson–Crick base pairs but do not show unwanted nucleobase induced fluorescence quenching.

This manuscript reports on 2′-deoxyadenosine and 2′-deoxyguanosine derivatives with a 7-deazapurine (pyrrolo[2,3-d]pyrimidine) and an 8-aza-7-deazapurine (pyrazolo[3,4-d]pyrimidine) skeleton as nucleobase surrogates bearing octadiynyl or tripropargylamine side chains, which were clicked to 1-azidomethylpyrene (10) to form the “click” conjugates 1–8 (Figure 1). The monomer fluorescence of 1–8 was compared, and the influence of the nucleobase on fluorescence quenching was studied on nucleoside and oligonucleotide level. Furthermore, concentration-dependent fluorescence measurements on nucleoside conjugates with tripropargylamine side chains were performed, which verified that the excimer emission of tripropargylamine pyrene conjugates results from intramolecular pyrene contacts and not from intermolecular interactions. The influence of nucleobase pyrene conjugates 1–8 on the DNA duplex stability was studied with regard to base modification.

RESULTS AND DISCUSSION

1. Synthesis of Phosphoramidites 14 and 18. For our study, eight different phosphoramidites based on the 7-deazapurine system and the related 8-aza-7-deazapurine congeners bearing octadiynyl and tripropargylamine side chains were synthesized. The phosphoramidites of nucleosides 19–24 were prepared according to earlier reported literature from our group,19–22 while the syntheses of phosphoramidites 14 and 18 are described below.

Phosphoramidite building block 14 was synthesized from nucleoside 11.19b For this, compound 11 was protected at the 6-amino group with an isobutyl residue affording the protected intermediate 12 in 86% yield. Then, compound 12 was converted to the S′-O-DMT derivative 13 under standard conditions. Further phosphitylation yielded the phosphoramidite 14 (63%) (Scheme 1).

Next, phosphoramidite 18 was prepared from the alkynylation nucleoside 1520b (Scheme 1). Amino group protection was performed on 15 with the N,N-dimethylaminomethylene group to afford the intermediate 16 (73%). Compound 16 was converted to the respective S′-O-DMT derivative under standard reaction conditions to yield 17 in 79%, and further phosphitylation gave the corresponding phosphoramidite 18 in 58%.

2. Synthesis and Characterization of Nucleoside Pyrene “Click” Conjugates with Octadiynyl and Tripropargylamine Side Chains. The Cu(I)-catalyzed “click” reaction was used for conjugation of the pyrene dye to the nucleobase. For this, 1-azidomethyl pyrene 10 was prepared from 1-pyrenemethanol following an already published procedure.23 The pyrene “click” conjugates of 7-octadiynyl-7-deaza-2′-deoxyguanosine (3) and 7-tripropargylamino-7-deaza-2′-deoxyguanosine (7) have already been reported by our laboratory.22 The 1,2,3-triazolyl pyrene nucleosides 1, 2, and 4 were synthesized from the 7-octadiynyl substituted nucleosides 19, 20, and 22 and 1-azidomethyl pyrene 10 in the presence of CuSO₄·5H₂O and sodium ascorbate in a 3:1:1 mixture of THF/t-BuOH/H₂O (Scheme 2).

The two terminal triple bonds of nucleosides 11, 15, 23, and 24 were functionalized with 1-azidomethyl pyrene (10), leading to the formation of the “double click” adducts 5–8 (Scheme 2). The reaction was performed by using the same procedure as that used for the synthesis of the octadiynyl pyrene “click” conjugates. In this reaction, a 2.7-fold excess of the pyrene azide was used to complete the “double click” conjugation. We found that both terminal triple bonds were functionalized simultaneously by the two pyrene reporters, even though they are space-demanding. “Double click” functionalization might result from the enhanced catalytic action of copper(I) being bound to the tripropargylamine side chain or to a monofunctionalized intermediate, thereby increasing the reaction rate for the second “click” reaction.17e

In order to determine the influence of the nucleobase on the pyrene fluorescence, the abasic pyrene conjugate 922 was prepared for comparison (Figure 1). This compound contains all necessary elements of the system except the nucleobase. All of the synthesized compounds were characterized by elemental analyses, 1H, 13C, and 1H−13C-gated-decoupled as well as DEPT-135 NMR spectra (Supporting Information). 13C NMR chemical shifts of “click” conjugates are summarized in Table 2 (Experimental Section). 13C NMR chemical shifts were assigned by 1H−13C gated-decoupled spectra and DEPT-135 NMR spectra (Table S1, Supporting Information).

The 1H NMR spectra of “click” conjugates show the disappearance of terminal C≡C hydrogen (singlet at δ ≈ 3.25 ppm), whereas the new methylene protons (δ ≈ 6.32 ppm) and singlets appearing around δ = 7.9−8.5 ppm are attributed to the protons of the newly formed triazole rings. The intensity of the proton signals of two triazole rings clearly

Figure 1. Pyrene “click” conjugates of 7-deazapurine and 8-aza-7-deazapurine nucleosides and abasic pyrene compound.
demonstrate the formation of the bis-dye adducts. Furthermore, 
$^{13}$C NMR spectra show the absence of the two terminal C=C carbon atom signals, while new double bond carbon signals of the 1,2,3-triazole moiety appear. As indicated in Table 2, they are located around 143–147 ppm (quaternary C-atom) and around 122 ppm (triazole-C)."
Scheme 3. Click Reaction Performed on Oligonucleotides Containing Tripropargylamine Nucleosides 11, 15, and 19–

Next, the “double click” reaction was performed on oligonucleotides 35–38 with 1-azidomethylpyrene (10) (Scheme 3) using the same procedure as for the synthesis of the octadiynyl pyrene “click” conjugates to yield the ODNs 39–42. For details, see the Experimental Section. All oligonucleotide pyrene “click” conjugates were purified by reversed-phase HPLC and characterized by MALDI-TOF mass spectroscopy (Table S2, Supporting Information). Oligonucleotide conjugates 33 and 41 were already described.22

Earlier, it was found that incorporation of a bulky tripropargylamine as well as an octadiynyl side chain does not perturb the DNA duplex structure.18,20–22 In accordance to earlier observations,23 tripropargylamino nucleosides 11 (35–26) and 15 (36–26) stabilize duplex DNA ($\Delta T_m = +3$ and +2 °C) as compared to the unmodified duplex 25–26 (Table 1 and Supporting Information, Figure S9). Consequently, the influence of dye modification with these linkers on the duplex stability was evaluated for the compounds described above. For this, the $T_m$ values of fully matched duplexes containing a pyrene dye were measured. More stable duplexes are formed by the pyrene-modified oligonucleotides compared to the unmodified duplex 25–26. A remarkable strong stabilization was observed for duplexes 31–26 and 32–26 ($\Delta T_m = +11$ and +12 °C) containing an octadiynyl modified “dA” pyrene “click” conjugate as compared to duplexes 39–26 and 40–26 containing the corresponding tripropargylamine modified “dA” pyrene “click” conjugate ($\Delta T_m = +6$ and +10 °C). The stabilization for duplexes 25–33, 25–34, 25–41, and 25–42 incorporating a “dG” pyrene “click” conjugate was still strong ($\Delta T_m = +2$ to +6 °C) but much less pronounced than for duplexes containing a “dA” pyrene conjugate. The DNA duplexes having one pyrene reporter group in each strand (31–33, 31–34, 32–33, and 32–34) are stable even to a greater extent ($\Delta T_m = +16$ to +19 °C) than duplexes (39–41, 39–42, 40–41, and 40–42) containing two pyrenes in each strand ($\Delta T_m = +8$ to +13 °C).

So, duplexes containing 7-deaza-dA and 8-aza-7-deaza-dA pyrene “click” conjugates (1, 2, 5, and 6) are much more stabilized than those incorporating 7-deaza-dG and 8-aza-7-deaza-dG pyrene “click” derivatives (3, 4, 6, and 8). Earlier, duplex stabilization by pyrene residues has been exemplified in several cases for residues attached to terminal or internal positions of the oligonucleotide chain.1e,5,24–25 However, to the best of our knowledge such a “dA” specific stabilization is unknown. This implies that probably the pyrene residue intercalates specifically with the “adenine” moiety ($\Delta T_m = +6$ to +12 °C), but intercalation is less efficient with the “guanine” residue ($\Delta T_m = +2$ to +6 °C). Such interactions are possible when the linker is long enough to form a fold back structure. Stabilization by pyrene intercalation seems to be most likely when compared to the $T_m$ values of the pyrene-modified oligonucleotides with those of the parent alkynylated oligonucleotides. Although the alkynyl linkers are already stabilizing, the contribution of the pyrene residues to duplex stabilization is predominant. The assumption of pyrene stacking is further supported by the absorption spectra of ss and ds oligonucleotides (Figures S2–S3, Supporting Information). Upon duplex formation, a small bathochromic shift of the absorption maxima and a slightly reduced peak-to-valley ratio of the absorption bands are observed. Participation of the triazole moieties and/or nearest neighbor nucleobases cannot be excluded.

4. Photophysical Properties of Nucleoside Pyrene “Click” Conjugates. Photoexcitable dyes such as pyrene, anthracene, or coumarin suffer from fluorescence quenching, when conjugated to nucleobases such as purines, 7-deazapurines or pyrimidines,18,20a,21a,22 caused by fluorescence resonance energy transfer (FRET) or charge separation (intramolecular electron transfer or hole transfer) between the dye and a base.2 To evaluate fluorescence quenching properties of pyrene “click” conjugates linked to various nucleobases via octadiynyl or tripropargylamine linkers and 1,2,3-triazole residues, fluorescence data of pyrene “click” conjugates were compared with those of the abasic alkyne side chain conjugate 9. It was anticipated that a simple octyne linker should have no significant influence on the fluorescence of pyrene, while the nucleobases might affect the fluorescence.

4.1. Monomer and Excimer Fluorescence of Nucleoside Pyrene “Click” Conjugates. At first, the monomeric pyrene “click” conjugates 1–4 were characterized by UV–vis spectra (Figure S1a, Supporting Information) measured in methanol. Based on the UV–vis spectra, the excitation wavelength for pyrene “click” conjugates 1–4 was chosen to be 340 nm. The tripropargylamine pyrene “click” conjugates 5–8 decorated with two pyrene residues show a UV absorbance (Figure S1b, Supporting Information) higher than that of the octadiynyl pyrene “click” conjugates 1–4 with no significant differences regarding the wavelength maxima and absorption pattern.

Then, fluorescence measurements were performed (Figure 2a). All “click” conjugates bearing one pyrene residue (1–4) show excitation maxima at 340 nm and monomeric pyrene emission maxima at 377 and 395 nm, while excimer emission was not observed. From Figure 2a, it is apparent that c′zC4d “click” conjugate 4 (Φ = 0.037) and the abasic pyrene
Table 1. $T_m$ Values of Octadiynyl and Tripropargylamine Substituted Oligonucleotides and Pyrene “Click” Conjugates

<table>
<thead>
<tr>
<th>octadiynyl modified oligonucleotides</th>
<th>$T_m$ [$^\circ$C]</th>
<th>$\Delta T_m$ [$^\circ$C]$^b$</th>
<th>tripropargylamine modified oligonucleotides</th>
<th>$T_m$ [$^\circ$C]</th>
<th>$\Delta T_m$ [$^\circ$C]$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-d(TAG GTC AAT ACT) (25)</td>
<td>49</td>
<td>-</td>
<td>5'-d(TAG GTC AAT ACT) (25)</td>
<td>49</td>
<td>-</td>
</tr>
<tr>
<td>3'-d(ATC CAG TTA TGA) (26)</td>
<td>52$^{13c}$</td>
<td>+3</td>
<td>3'-d(TAG GTC 11AT ACT) (35)</td>
<td>52</td>
<td>+3</td>
</tr>
<tr>
<td>5'-d(TAG GTC 19AT ACT) (27)</td>
<td>51$^{20a}$</td>
<td>+2</td>
<td>3'-d(TAG GTC 15AT ACT) (36)</td>
<td>51</td>
<td>+2</td>
</tr>
<tr>
<td>3'-d(ATC CAG TTA TGA) (26)</td>
<td>49$^{22}$</td>
<td>0</td>
<td>3'-d(TAG GTC AAT ACT) (25)</td>
<td>50$^{22}$</td>
<td>+1</td>
</tr>
<tr>
<td>5'-d(TAG GTC AAT ACT) (25)</td>
<td>49$^{21a}$</td>
<td>0</td>
<td>3'-d(TAG GTC AAT ACT) (37)</td>
<td>49$^{21b}$</td>
<td>0</td>
</tr>
<tr>
<td>3'-d(ATC CA21TTA TGA) (29)</td>
<td>60</td>
<td>+11</td>
<td>3'-d(TAG GTC 5AT ACT) (39)</td>
<td>55</td>
<td>+6</td>
</tr>
<tr>
<td>5'-d(TAG GTC 20AT ACT) (28)</td>
<td>61</td>
<td>+12</td>
<td>3'-d(TAG GTC 6AT ACT) (40)</td>
<td>59</td>
<td>+10</td>
</tr>
<tr>
<td>3'-d(ATC CAG TTA TGA) (26)</td>
<td>53$^{22}$</td>
<td>+4</td>
<td>3'-d(TAG GTC 6AT ACT) (41)</td>
<td>51$^{22}$</td>
<td>+2</td>
</tr>
<tr>
<td>5'-d(TAG GTC AAT ACT) (25)</td>
<td>54</td>
<td>+5</td>
<td>3'-d(ATC CA7 TTA TGA) (42)</td>
<td>55</td>
<td>+6</td>
</tr>
<tr>
<td>3'-d(ATC CA4 TTA TGA) (34)</td>
<td>65</td>
<td>+16</td>
<td>3'-d(ATC CA7 TTA TGA) (41)</td>
<td>57</td>
<td>+8</td>
</tr>
<tr>
<td>5'-d(TAG GTC 1AT ACT) (31)</td>
<td>68</td>
<td>+19</td>
<td>3'-d(ATC CA8 TTA TGA) (42)</td>
<td>60</td>
<td>+11</td>
</tr>
<tr>
<td>3'-d(ATC CA4 TTA TGA) (34)</td>
<td>66</td>
<td>+17</td>
<td>3'-d(ATC CA8 TTA TGA) (41)</td>
<td>57</td>
<td>+8</td>
</tr>
<tr>
<td>5'-d(TAG GTC 2AT ACT) (32)</td>
<td>67</td>
<td>+18</td>
<td>3'-d(ATC CA8 TTA TGA) (42)</td>
<td>62</td>
<td>+13</td>
</tr>
</tbody>
</table>

$^a$Measured at 260 nm in 1 M NaCl, 100 mM MgCl$_2$, and 60 mM Na-cacodylate (pH 7.0) with 2 $\mu$M + 2 $\mu$M single-strand concentration. $^b$Refers to the temperature difference of the modified duplex versus the unmodified reference duplex.

Conjugate 9 ($\Phi = 0.034$) show similar monomeric fluorescence intensity, whereas c"G$_5$d pyrene conjugate 3 ($\Phi = 0.004$) shows significant lower monomer fluorescence. Monomer fluorescence for octadiynyl “click” conjugates increases in the following order: octc"G$_5$ (3) < octc"A$_4$ (1) < octc"z"G$_5$ (2) < octc"z"G$_5$ (4) < abasic conjugate (9), as illustrated by the bar diagram (Figure 2b).

In contrast to octadiynyl “click” conjugates, the tripropargylamine “double click” conjugates 5–8 with two proximal pyrenes show strong excimer fluorescence (Figure 3a,b) with $\lambda_{max} \approx 465$ nm (band III) as well as monomer fluorescence at 377 nm (band I) and 395 nm (band II) upon excitation at 340 nm. From this, we conclude that the two pyrenes are stacking in all tripropargylamine nucleoside “double click” conjugates (5–8). The c"z"G$_5$d “click” conjugate 8 (excimer fluorescence, $\Phi = 0.059$) shows the highest value, while the c"G$_5$d “click” conjugate 7 (excimer fluorescence, $\Phi = 0.020$) shows low excimer emission. The monomer fluorescence intensity of tripropargylamine “click” conjugates 5–8 increases in the same order trpa"z"c"G$_4$d (7) < trpa"z"c"A$_4$d (5) < trpa"z"c"A$_4$d (6) < trpa"z"c"G$_4$d (8) (Figures 3a, 3b) as observed for the octadiynyl “click” conjugates 1–4 (Figure 2a,b). Interestingly, this order is changed for the “adenine click” conjugates 5 and 6: trpa"z"c"G$_4$d (7) < trpa"z"c"G$_4$d (8) (Figure 3a, 3b). These different dependencies of excimer versus monomer fluorescence are in line with earlier observations.

Excimer fluorescence of “double click” conjugates 5–8 could arise from either intramolecular or intermolecular interactions of two pyrene residues. To investigate this phenomenon concentration-dependent fluorescence measurements on the pyrene “double click” conjugates 7 and 8 were performed (Supporting Information, Figure S5). The c"z"G$_4$d “click” conjugate 8 develops the strongest eximer fluorescence, while the c"G$_5$d “double click” conjugate 7 shows low excimer...
emission (Figure 3a). Fluorescence intensity decreases almost linearly with decreasing concentration in both “click” conjugates (7 and 8). For “click” conjugate 8, the Ex/M intensity ratio was unchanged, i.e., about 1.0, for all measured concentrations, indicating that intramolecular excimer formation takes place. In “double click” conjugate 7, the excimer to monomer emission ratio is twice as high. In this case, the Ex/M ratio slightly decreases from 1.92 to 1.68 upon dilution (Supporting Information, Table S3). The observation that the monomer fluorescence is quenched to a greater extent than excimer fluorescence is in line with earlier findings of Yamana et al.9c

4.2. Fluorescence of 7-Deazapurine and 8-Aza-7-deaza-purine Oligonucleotide Pyrene “Mono-Click” Conjugates. The quenching effects of 7-deazapurine and 8-aza-7-deazapurine nucleobases on the pyrene moiety were also investigated on single-stranded oligonucleotide (ss) pyrene “click” conjugates 31–34 and corresponding duplexes (ds). Absorption spectra varied only slightly, with a small red shift observed upon duplex formation (Figure S2, Supporting Information).

Subsequently, fluorescence spectra of oligonucleotides 31–34 and their corresponding duplexes (Figure 4a,b) were performed. Complementary strands were always unmodified (25 or 26). All ss-oligonucleotides (31–34) as well as their duplexes show pyrene monomer fluorescence with \( \lambda_{\text{max}} \approx 382 \text{ nm} \) (band I) and 398 nm (band II) upon excitation at 340 nm. The fluorescence emission of \( c^7G_d \) pyrene modified ss-oligonucleotide 33 is quenched strongly compared to that of the \( c^2G_d \) pyrene modified ss-oligonucleotide 34 (Figure 4b).

These results correlate with the data found for the nucleoside conjugates 3 and 4. Hybridization with complementary strands (→ duplexes 25·33 and 25·34) leads to further fluorescence decrease.

The fluorescence intensity of pyrene modified 7-deazaadenine ss-oligonucleotide 31 is quenched (about 25%) compared to that of 8-aza-7-deazaadenine ss-oligonucleotide 32 (Figure 4a), which is in line with fluorescence data of the nucleosides. Surprisingly, the fluorescence intensity of the corresponding duplexes 31·26 and 32·26 containing 1 or 2 conjugates remains almost unchanged. This is different to the findings of the “dG” derivatives.

4.3. Fluorescence of 7-Deazapurine and 8-Aza-7-deaza-purine Oligonucleotide “Double Click” Conjugates with Pyrene Modifications in One Strand of Duplexes. Fluorescence spectra of “dA” modified ss-oligonucleotides 39 and 40 show strong monomer fluorescence and weak excimer fluorescence. Monomer emission of ss-oligonucleotide 39 is higher than that of ss-oligonucleotide 40, but excimer emission of 39 is slightly higher than that of 40 (Figure 5a). This result matches the fluorescence data obtained on nucleoside level (Figure 3a). When the corresponding duplexes are formed (39·26 and 40·26), monomer fluorescence intensity is increased strongly. Fluorescence spectra of “double clicked” dG” ss-oligonucleotides 41 and 42 show only monomer fluorescence, and when duplexes are formed (25·41 and 25·42), the fluorescence intensity strongly decreases (Figure 5b). Similar to the series of octadiynyl derivatives, the fluorescence emission of the “double clicked” pyrene and 7-deazaguanine modified ss-oligonucleotide 41 is quenched.

Figure 2. (a) Fluorescence spectra of octadiynyl nucleoside “click” conjugates 1–4 and abasic “click” conjugate 9 in methanol. (b) Bar diagram showing the monomer fluorescence intensity of “click” conjugates 1–4 and abasic “click” conjugate 9 in methanol. In all experiments the concentration of the dye conjugates was 6.8 × 10⁻⁶ M.

Figure 3. (a) Fluorescence spectra of tripropargylamine nucleoside “click” conjugates 5–8 in methanol. (b) Bar diagram showing the monomer emission and excimer fluorescence intensity of “click” conjugates 5–8 in methanol. The concentration of the dye conjugates was always 6.8 × 10⁻⁶ M.
and 8-aza-7-deazapurine modifications (31 exhibit fluorescence intensities in between. The duplexes containing deazapurine modifications (31-33, 34 and duplexes 25-33, 25-34 (2 μM of each strand) lie apart from each other. Fluorescence emission of duplex 31 and fluorescence emission spectra (Figure 6a) of duplexes 31-34, and 26-26 (2 μM of each strand). All spectra were measured in 1 M NaCl, 100 mM MgCl₂, and 60 mM Na-cacodylate (pH 7.0).

Figure 4. (a) Fluorescence emission spectra of the 2 μM ss-oligonucleotides 31, 32 and duplexes 31-26, 32-26 (2 μM of each strand). (b) Fluorescence emission spectra of the 2 μM ss-oligonucleotides 33, 34 and duplexes 25-33, 25-34 (2 μM of each strand). All spectra were measured in 1 M NaCl, 100 mM MgCl₂, and 60 mM Na-cacodylate (pH 7.0).

4.4. Fluorescence of 7-Deazapurine and 8-Aza-7-deaza-purine Oligonucleotide “Mono and Double Click” Conjugates with Pyrene Modifications in Both Strands of the Duplex. Tripropargylamine derivatives of 7-deaza-dG and 8-aza-7-deaza-dG develop excimer fluorescence neither in ss-oligonucleotides nor in ds DNA, when only one individual strand was decorated with two proximal pyrenes. Competing stacking interactions of one of the proximal pyrene residues with nucleobases accounted for this phenomenon. By that, one pyrene unit is not available for intramolecular pyrene interaction thus causing only monomer emission (Section 4.1 and Supporting Information).

Subsequently, UV–vis (Figure S4a, Supporting Information) and fluorescence emission spectra (Figure 6a) of duplexes 31-33, 31-34, 32-33, and 32-34 were measured containing one pyrene modification in each strand. All duplex combinations studied herein show only monomer fluorescence and lack excimer fluorescence, demonstrating that the pyrene residues lie apart from each other. Fluorescence emission of duplex 32-34 with 8-aza-7-deaza-dA (2) and 8-aza-7-deaza-dG (4) was found to be highest, while duplex 31-33 with only 7-deazapurine modifications (1 and 3) showed the lowest fluorescence. The duplexes containing “mixed” 7-deazapurine and 8-aza-7-deazapurine modifications (31-31 and 32-34) exhibit fluorescence intensities in between.

Figure 5. (a) Fluorescence emission spectra of the 2 μM ss-oligonucleotides 39 and 40 and duplexes 39-26, 40-26 (2 μM of each strand). (b) Fluorescence emission spectra of the 2 μM ss-oligonucleotides 41 and 42 and duplexes 25-41, 25-42 (2 μM of each strand). All spectra were measured in 1 M NaCl, 100 mM MgCl₂, and 60 mM Na-cacodylate (pH 7.0).

Next, UV–vis (Figure S4b, Supporting Information) and fluorescence emission spectra (Figure 6b) of the duplexes 39-41, 39-42, 40-41, and 40-42 containing one “double clicked” pyrene residue in each strand were investigated. All duplex combinations show strong excimer fluorescence and rather low monomer fluorescence (Figure 6b). Now, two pyrene residues lie in a close proximity, facilitating the π–π interaction between electronic clouds, thus giving rise to excimer fluorescence. It is noteworthy to mention that each pyrene residue involved in excimer formation comes from the opposite strand22,26 and that excimer fluorescence intensities are higher for the duplexes containing c7Ad pyrene “click” conjugate 39 (39-41 and 39-42) than for those incorporating c7zd pyrene “click” conjugate 40 (40-41 and 40-42).

Throughout our studies on oligonucleotides, we found that the fluorescence intensity of ss-oligonucleotides 33 and 41 as well as their duplexes 25-33 and 25-41 containing the c7Gd pyrene “click” conjugate 3 or 7 was quenched strongly.27,28 In contrast, it was observed that the c7zdGd pyrene “click” conjugates 4 (oct7zdc7Gd) and 8 (trpa7zdc7Gd) do not develop significant fluorescence quenching. The 7-deazaguanine conjugates 3 (oct7zdc7Gd) and 7 (trpa7zdc7Gd) on the other hand show low monomer fluorescence compared to the abasic “click” conjugate 9, pointing to the strong quenching of the pyrene fluorescence by the 7-deazaguanine moiety within both nucleoside “click” conjugates. Nucleobase-specific quenching by Förster resonance energy transfer (FRET) is ruled out as there is no overlap between excitation and emission spectra of the pyrene residue and nucleobases. So, quenching results from...
an intramolecular charge transfer between the nucleobase and the dye and subsequent formation of radical cations and radical anions.\(^7\),\(^27\).

Charge transfer between nucleobase and dye also depends upon the oxidation potential of the nucleobase. The 7-deazaguanine nucleoside has an oxidation potential lower than those of the other nucleosides.\(^29\),\(^31\) Hence during charge separation, the 7-deazaguanine nucleobase can be easily oxidized, forming the radical cation, and the dye yields a radical anion (Py\(^{\cdot -}\)).\(^30\) This type of charge separation, intramolecular hole transfer, leads to the quenching of pyrene fluorescence in “click” conjugates. The 8-aza-7-deazaguanine moiety and the 8-aza-7-deazaadenine moiety are not able to quench the fluorescence of the dye significantly. As a possible explanation, we anticipate a higher oxidation potential for the pyrazolo[3,4-\(\text{d}\)]pyrimidine compared to the pyrrolo[2,3-\(\text{d}\)]pyrimidine due to the additional ring nitrogen.

### CONCLUSIONS AND OUTLOOK

Functionalization of 7-octadiynyl or 7-tripropargylamine derivatives of 7-deazapurine and 8-aza-7-deazapurine nucleosides (19–22 and 11, 15, 23–24) with 1-azidomethyl pyrene (10) by the copper(I)-catalyzed azide alkyne “click” reaction yielded fluorescent nucleoside dye conjugates (1–8). The octadiynyl conjugates show only monomer fluorescence, whereas the proximal arrangement of the pyrene residues in the tripropargylated nucleosides leads to monomer and excimer emission. Excimer fluorescence results from intramolecular contacts of the pyrene residues as verified by concentration-dependent fluorescence studies. Monomer as well as excimer fluorescence intensity differs among the various nucleobases. 8-Aza-7-deazapurine pyrene nucleoside (2, 4, 6, 8) and oligonucleotide “click” conjugates (32, 40, 41) exhibit much higher fluorescence emission compared with that of 7-deazapurine derivatives (1, 3, 5, 7, 31, 33, 39, 41). The observed fluorescence quenching is attributed to charge separation among the nucleobases and pyrene residues, which is strong for 7-deazapurine nucleoside conjugates due to their lower oxidation potential. Similar quenching is observed on nucleosides as well as for single and double stranded oligonucleotides. Henceforth, 8-aza-7-deazaadenine and 8-aza-

### Table 2. \(^{13}\)C NMR Chemical Shifts of Nucleoside Derivatives and Pyrene “Click” Conjugates\(^a\)

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<th>C(5), C(7)</th>
<th>C(6a), C(7a)</th>
<th>C(4a), C(8a)</th>
<th>C=CH-CH(_2)/OMe</th>
<th>Triazole</th>
<th>C1'</th>
<th>C2'</th>
<th>C3'</th>
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\(\text{a}\) Measured in DMSO-d\(_6\) at 298 K. \(\text{b}\) Systematic numbering for 7-deazapurine derivatives. \(\text{c}\) Systematic numbering for 8-aza-7-deazapurine derivatives. \(\text{d}\) Purine numbering. \(\text{e}\) Tentative. \(\text{f}\) Superimposed by DMSO. \(\text{g}\) Not detected.
7-deaza-guanine nucleoside and oligonucleotide pyrene conjugates are perfect purine surrogates for canonical nucleobases as pyrene is not quenched by those derivatives. Also, these mimics base pair as strongly and specifically as canonical DNA constituents, while their pyrene conjugates form even more stable duplexes. A single replacement of a canonical nucleoside by a pyrene conjugate stabilizes corresponding duplexes substantially: 6–12 °C for duplexes with a modified “adenine” base and 2–6 °C for modified “guanine” base.

This improves the utility of pyrene fluorescence reporters for detection of oligonucleotides.

**EXPERIMENTAL SECTION**

**General Methods.** All chemicals and solvents were of laboratory grade as obtained from commercial suppliers and were used without further purification. Thin layer chromatography (TLC) was performed on TLC aluminum sheets coated with silica gel 60 F254 (0.2 mm). Flash column chromatography (FC): silica gel 60 (40–60 µM) at 0.4 bar. UV spectrophotometer: U-3000 spectrometer; J. Org. Chem. 2012, 77, 188–199.

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5.7 Hz, H4, HO-5), 5.29 (d, J = 4.2 Hz, H1, HO-3), 6.32 (s, 2H, pyrene-CH2), 6.46 (t, J = 6.7 Hz, H1H-1, 1.76 (s, 1H, H-8), 7.91–8.52 (m, 11H, Ar-H, H-5-triazole, H-2). Anal. Calc’d for C36H32N12O3 (977.37): C 71.13, H 4.73, N 18.74. Found: C 71.12, H 4.75, N 18.78.

6-Amino-1-[2-deoxy-β-β-erythro-pentofuranosyl]-1,5-dihydro-[3-(1-pyren-1-ylmethyl)-1H,1,2,3-triazole-4-yl]methylamino-prop-1-ynyl]-4H-pyrazolo[3,4-d]pyrimidine (4). Compound 8 was prepared using the general procedure II with compound 24° (0.100 g, 0.25 mmol), 1-azidomethyl pyrene 20° (0.175 g, 0.68 mmol), a freshly prepared solution of 1 M sodium ascorbate in water (101 μL, 0.10 mmol), and copper(II) sulfate pentahydrate 7.5% in water (80 μL, 0.03 mmol). Compound 8 was isolated as a colorless solid (0.178 g, 77%). TLC (CH3Cl/MeOH 90:10) Rf 0.3; UV λmax (MeOH)/nm 264 (ε/dm3 mol–1 cm–1) 53400, 275 (18700), 311 (20300), 325 (48100), 341 (71100). 1H NMR (DSMO-d6, 300 MHz) (δ ppm): 2.11–2.19 (m, 1H, H-2), 2.62–2.71 (m, 1H, H-2), 3.43–3.52 (m, 2H, H-3), 3.74–3.81 (m, 2H, CH2, H-4), 4.33–4.40 (m, 1H, H-3), 4.74 (t, J = 5.4 Hz, H1H-3), 5.24 (d, J = 4.2 Hz, H1H-3), 6.27–6.33 (m, 9H, 2H, pyrene-CH2), 6.78 (s, 2H, NH2), 7.93–8.50 (m, 2OH, Ar-H, 2H, H-5-triazole), 10.64 (s, 1H, NH). Anal. Calc’d for C36H32N12O3 (910.98): C 68.1, H 4.65. Found: C 68.9, H 4.75.

7-[2-deoxy-β-β-erythro-pentofuranosyl]-4-isobutylamino)-5-[3-(di[prop-2-yny]amino)-prop-1-ynyl]-7H-pyrrolo[2,3-d]pyrimidine (2). To a solution of compound 11° (0.430 g, 1.13 mmol) in anhydrous pyridine (6 mL) was added MeSiCl (1.5 mL, 11.6 mmol), and the mixture was stirred at room temperature. After 45 min, the reaction mixture was evaporated to dryness under reduced pressure, and the remaining residue was dissolved in dichloromethane (50 mL) and washed with 5% NaHCO3 solution (2 × 100 mL) and water (80 mL), dried over Na2SO4 and then concentrated. Purification by FC (silica gel column, 10 cm × 3 cm, CH2Cl2/acetone 7:3) gave a colorless foam of 13 (0.400 g, 80%). TLC (CH2Cl2/MeOH 94:6) Rf 0.65; UV λmax (MeOH)/nm 236 (ε/dm3 mol–1 cm–1) 34400, 277 (16000). 1H NMR (DSMO-d6, 300 MHz) (δ ppm): 1.14, 1.17 (s, 6H, 2 × CH3), 2.20–2.28 (m, 1H, H-2), 2.50–2.58 (m, 1H, H-2), 2.76–2.83 (m, 1H, CH2), 3.25 (s, 2H, 2 × CH2), 3.45 (s, 4H, 2 × CH2), 3.52–3.63 (m, 3H, 3H, CH2), 3.82–3.85 (m, 1H, H-4), 4.34–4.39 (m, 1H, H-3), 5.01 (s, J = 5.4 Hz, 1H, OH-5), 5.33 (d, J = 4.2 Hz, H1H-3), 6.63 (t, J = 6.6 Hz, 1H, H-1), 8.12 (s, 1H, H-6), 8.61 (s, 1H, H-2), 10.14 (s, 1H, NH). Anal. Calc’d for C45H38N12O2 (746.63): C 64.13, H 5.85, N 15.58. Found: C 64.0, H 5.85, N 15.50.

7-[2-deoxy-5-(4,4'-dimethoxytrityl)-β-β-erythro-pentofuranosyl]-4-isobutylamino)-5-[3-(di[prop-2-yny]amino)-prop-1-ynyl]-7H-pyrrolo[2,3-d]pyrimidine (13). Compound 12 (0.300 g 0.67 mmol) was dried by repeated co-evaporation with anhydrous pyridine (2 × 5 mL) before dissolving in anhydrous pyridine (8 mL). Then, 4,4'-dimethoxytrityl chloride (0.300 g, 0.88 mmol) was added in three portions to the remaining solution at room temperature under stirring for 6 h. Thereupon, MeOH (2 mL) was added, and the mixture was stirred for another 30 min. The reaction mixture was evaporated to dryness under reduced pressure, and the remaining residue was dissolved in dichloromethane (50 mL) and washed with 5% NaHCO3 solution (2 × 100 mL) and water (80 mL), dried over Na2SO4 and then concentrated. Purification by FC (silica gel column, 10 cm × 3 cm, CH2Cl2/acetone 7:3) gave a colorless foam of 13 (0.400 g, 80%). TLC (CH2Cl2/MeOH 94:6) Rf 0.65; UV λmax (MeOH)/nm 236 (ε/dm3 mol–1 cm–1) 34400, 277 (16000). 1H NMR (DSMO-d6, 300 MHz) (δ ppm): 1.00–1.17 (m, 6H, 2 × CH2), 2.17–2.35 (m, 1H, H-2), 2.62–2.69 (m, 1H, H-2), 2.80–2.89 (m, 1H, CH), 3.09–3.25 (m, 4H, 2 × CH2, CH2), 3.54 (s, 2H, CH3), 3.73 (s, 6H, 2 × CH2O), 3.95–3.98 (m, 1H, H-4), 4.34–4.41 (m, 1H, H-3), 5.38 (d, J = 4.5 Hz, HO-3), 6.63 (t, J = 6.3 Hz, H1H-1), 6.81–6.85 (m, 4H, Ar-H), 7.17–7.38 (m, 9H, Ar-H), 7.96 (s, 1H, H-6), 8.60 (s, 1H, H-2), 10.11 (s, 1H, H-1).

7-[2-Deoxy-5-O-(4,4′-dimethoxytrityl)-β-o-erythro-pentofuranosyl]-4-(isobutyryl)amino]-5-[3{(di-prop-2-ynyl)amino}prop-1-ynyl]-7H-pyrrolo[2,3-d]pyrimidine 3′-(2-cyanoethyl)-N,N-dimethylphosphoramidite (14). A stirred solution of 13 (0.200 g, 0.27 mmol) in anhydrous CH2Cl2 (5 mL) was preflushed with argon and treated with (i-Pr)2EtN (74 μL, 0.43 mmol) followed by 2-cyanoethyl-N,N-dimethylphosphoramidochloride (122 μL, 0.56 mmol). After stirring for 45 min at room temperature, the solution was diluted with CH2Cl2 (30 mL) and extracted with 3%aq NaHCO3 solution (20 mL). The organic layer was dried over Na2SO4 and concentrated. Evaporation of fractions containing UV activity afforded compound 16 (0.251 g, 73%) as a colorless foam. TLC (CH3Cl/MeOH 9:1) Rf 0.7; UV λmax (MeOH)/nm 260 (ε/dm3 mol-1 cm-1 ) 7800, 324 (28000). 1H NMR (DMSO-d6, 300 MHz) (δ, ppm): 2.22–2.30 (m, 1H, H-2′), 2.76–2.88 (m, 1H, H-2′), 3.19, 3.23 (s, 6H, 2C(CH3)), 3.36–3.38 (m, 2H, 2 × C(=CH)), 3.36–3.56 (m, 6H, H-5, 2 × C(CH3)), 3.69 (s, 2H, CH2), 3.79–3.84 (m, 1H, H-4′), 4.40–4.46 (m, 1H, H-3′), 4.78 (t, J = 5.7 Hz, 1H, H-5′), 5.29 (d, J = 4.5 Hz, 1H, OH-3′), 6.59 (t, J = 6.3 Hz, 1H, H-1′), 8.45 (s, 1H, H-2), 8.92 (s, 1H, CH). Anal. Calcd for C45H45N5O6 (751.87): C 71.89, H 6.03, N 9.31. Found: C 71.84, H 6.00, N 9.22.

The Journal of Organic Chemistry

18 (0.148 g, 58%). TLC (CH3Cl/acetone 95:15) Rf 0.40. 31P NMR (CDCl3, 121 MHz) (δ, ppm): 148.4, 148.2.

ASSOCIATED CONTENT

Supporting Information

1H−13C coupling constants of nucleoside derivatives and their pyrene “click” conjugates, molecular masses of modified oligonucleotides measured by MALDI-TOF mass spectrometry, concentration dependent UV and fluorescence spectra and Ex/M ratio of “click” conjugates 7 and 8, HPLC profiles of oligonucleotides determined at 260 nm, melting curves of oligonucleotide duplexes, UV−vis spectra of pyrene modified nucleosides and oligonucleotides, 1H NMR, 13C NMR, Deut-135 NMR, and 1H−13C gated-decoupled NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES


