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Labeling Deoxyadenosine for the Preparation of Functional Conjugated Oligonucleotides

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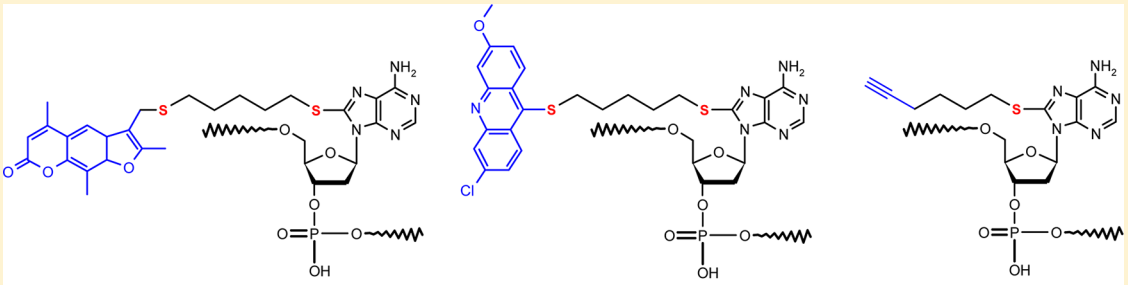
Supporting Information



ABSTRACT: Herein we present a versatile synthetic method for the 8-thioalkylation of (deoxy)adenosine with a short carbon linker having on the other side a variety of molecules (psoralen, acridine) and functional groups (alkyne). After conventional protections, the modified adenosine can be phosphytylated and inserted into an oligonucleotide without affecting the standard protocols for supported oligonucleotide synthesis. The hybridization properties of a generic oligonucleotide containing the above conjugated moieties toward both DNA and RNA are evaluated both in the case of a perfectly complementary strand and in the case of a single mismatch. This methodology is suitable for the preparation of several types of derivatives and—through the alkynyl moiety—provides fast access to click-chemistry transformations.

INTRODUCTION

In the past thirty years, oligonucleotides have been chemically bound (i.e., conjugated) to a variety of: fluorophores,[1,2] drugs,[3−5] or other active molecules such as alkylating[6] or cleaving groups[7,8] for biochemical and structural studies; they have been bound to a variety of materials, such as metals,[9] glass,[10] polymers,[11] and peptides, for the realization of biochemical probes and biosensors. Oligonucleotides have also been actively employed as down-regulators of genetic expression in cellular studies, where they have been conjugated to a plethora of derivatives to enhance the cellular uptake[12−16] or increase the binding affinity toward their RNA target[17−19] (antisense methodology) or to form more efficient triple helices on the double-stranded DNA[20,21] (antigene approach). More recently, oligonucleotides have also been used as molecular scaffolds to exploit their autoassembly properties for the realization of supramolecular aggregates with potential applications in the field of electronics devices.[22−26] For each goal it is important to be sure that the chosen methodology does not hamper the hybridization properties of the native oligonucleotide on which are based the forecasted applications.

Since mid 1980's almost all the synthetic oligonucleotides are prepared through the automated supported synthesis; hence, any functionalization must be introduced during this procedure, or performed as postsynthetic treatment on a suitable modified oligonucleotide carrying a reactive group, introduced during the automated synthesis.

The most common derivatizations of the oligonucleotides are done at their 5′ or 3′ ends or both, as in the case of the so-called "molecular beacons";[27,28] however, for some purposes it is important to be able to link a tether in the middle of a sequence, by modifying a base, a sugar, a phosphate, or an entire internucleotidic region.[29] Derivatization of nucleobases enables the incorporation of one or more suitable functionalities to the desired position of the oligonucleotide,[2] leaving the 5′ and 3′ ends available for further modifications. The labeling of the base has been used for the preparation of several derivatives ranging from artificial endonucleases[7,30] to the control of the correct base pairing.[31−33] The functionalization of the nucleobase is usually performed linking the chosen moiety to the C-6 position of pyrimidines, and on the C-8 position of purine bases.[34−38]

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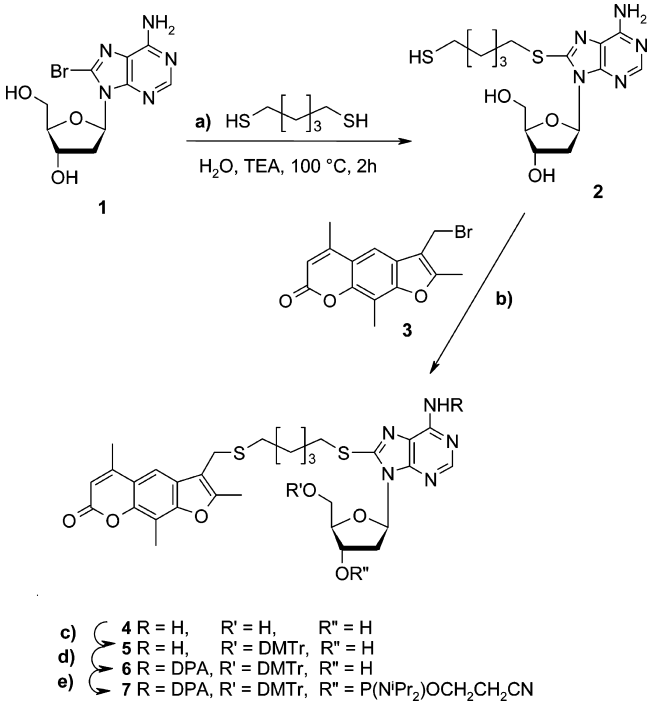
Here, we report a study on the preparation of thioderivatives bound to the C-8 position of the deoxyadenosine containing different functional molecules, and we describe the synthesis and the hybridization properties of oligonucleotides containing these modifications in the middle of a generic sequence.

RESULTS

Synthesis of Psoralen-Deoxyadenosine Conjugate and Its Amidite. We started this study looking for an easy procedure to prepare oligonucleotides conjugated with psoralen for antisense applications. Starting from the paper of Pieles et al.,[34] we applied our previous knowledge of the preparation of 8-thioadenosine derivatives[26,39] to improve his procedure.

The preparation of the psoralen derivative followed the pathway described in Scheme 1.

Scheme 1. Synthesis of Protected Phosphoramidite of PsodA (7) from 8-Bromodeoxyadenosine (1)[a]



[a](a) 1,5-Pentanedithiol in water and TEA, 100 °C, 2 h (80%); (b) bromomethylpsoralen (**3**) in DMF and Ca_2CO_3, 80 °C, 4 h, (85%); (c) DMTrCl, Py, 1 h rt (80%); (d) TMSiCl in Py 30 min, then DPACl, 2 h, then MeOH 15 min (75%); (e) $ClP(N^iPr_2)$-OCH_2CH_2CN in CH_2Cl_2 and DIPEA, 30 min (60%).

With this approach we demonstrate for the first time the usefulness of a bifunctional thiol as a nucleophile reagent for the displacement of bromine from the C-8 position of deoxyadenosine. This reaction can be smoothly performed in water and leads to a simple purification of the thioalkylated nucleoside **2** that only requires the extraction of the compound with warm EtOAc from the aqueous crude mixture and the removal of the solvent by evaporation under reduced pressure. The nucleoside **2** is kept under vacuum and preferably used in a short time to prevent its dimerization. In the next step compound **2** is coupled with the bromomethylpsoralen **3** synthesized according to a published procedure.[34] The coupling is performed in warm DMF and calcium carbonate for around 4 h. The derivative **4** is recovered by extraction with EtOAc and washed with water. The crude compound can be used for the next step without any further purification. Step **c** is the protection of the 5′-hydroxyl group of the nucleoside with DMTr. This reaction is performed in standard condition in pyridine at room temperature using fresh DMTrCl. After workup compound **5** is chromatographed on silica gel with 2% MeOH in dichloromethane. According to our consolidated procedure, the amino group of deoxyadenosine is protected with diphenylacetyl chloride[40] (DPACl) following the transient protection of the 3′-hydroxyl group as trimethylsilyl derivative.[41] This two-step protection is performed in dry pyridine, and compound **6** is recovered following a short treatment with MeOH in the same pot used to cleave the silyl protection and the bis-DPA amide derivative. This compound needs to be chromatographed on silica gel with some TEA to prevent the removal of the trityl group. Step **e**, corresponding to the preparation of the phosphoramidite, is performed in standard condition using some excess of cyanoethyl-diisopropyl-chlorophosphite in anhydrous dichloromethane and DIPEA. The crude reaction mixture is then diluted with dichloromethane, and washed with aqueous bicarbonate. The crude compound, obtained after the removal of the solvent, is chromatographed on silica gel with a gradient of EtOAc/cycloehexane/TEA from 20/70/10 to 82/9/9. Compound **7** was found stable in freezer for several weeks as brittle foam in dry conditions.

Synthesis of Acridine-Deoxyadenosine Derivative and Its Amidite. Having established an easy protocol for the synthesis of the functionalized derivative of **2** we challenged the value of our procedure for the preparation of other conjugates. Looking for a fluorescent and intercalative moiety, we chose to bind an acridine as our new target. To the best of our knowledge a deoxyadenosine conjugated with an acridine has never been described before.

This synthesis parallels that of compound **7** and is outlined in Scheme 2.

The acridine derivative **8** is reacted with the free thiol **2** in a sealed tube at 80 °C in DMF and TEA. The solvent is removed and the compound chromatographed on silica gel with 10% of methanol in dichloromethane. Steps **c** to **e** are performed as above for the psoralen derivative, with a somewhat lesser yield at each step.

Synthesis of Alkynyl-Deoxyadenosine Derivative and Its Amidite. So far we have demonstrated that the pentamethylenic arm could be derivatized with at least two different substituents, coupling the free thiol function with an organic moiety containing a reactive halogen either bromine or chloride. With the aim to explore the possibility to change the nature of the arm, we performed the thioalkylation with an alkynyl thiol, leading to a conjugate with a terminal triple C−C bond—a powerful tool for the click-chemistry. We also check its suitability for the preparation of phosphoramidite and oligonucleotides. The synthesis is depicted in Scheme 3.

The alkylation of 8-bromodeoxyadenosine with hexyne thiol **13** in the same conditions used to prepare the previous conjugates, afforded compound **14** in somewhat lower yield, probably due to the reduced hydrophilicity of the hexyne thiol **13** with respect to that of the 1,5-pentane dithiol. Compound **14** was then transformed to the corresponding protected amidite following the already described procedures. Perhaps it is worth noting that both the tritylation step **c** and the following amidation **d** gave higher yields than those obtained for the previously described derivatives, probably because of the lesser

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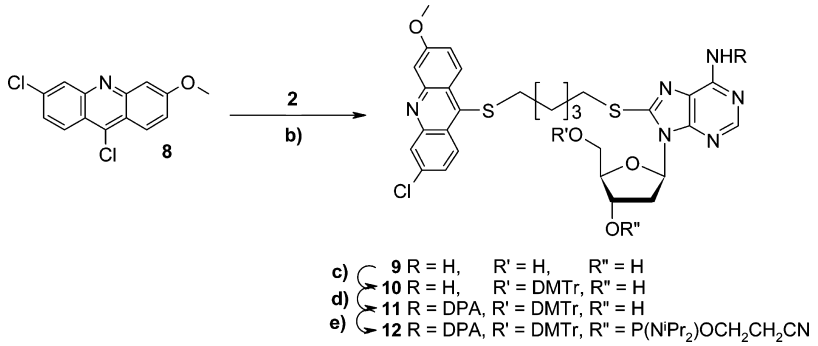
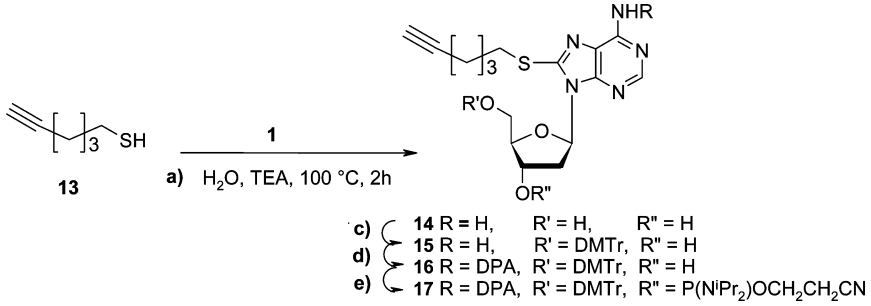
Scheme 2. Synthesis of Protected Phosphoramidite of Acr-dA (12) from 8-Thiopentyl-5-thiol-deoxyadenosine (2)[a]



[a](b) 2,9-Dichloro-6-methoxyacridine (**8**) in DMF and TEA, 80 °C, 17 h, in a stoppered vial (70%); (c) DMTrCl, Py, 1 h rt (65%); (d) TMSiCl in Py 30 min, then DPACl, 2 h, then MeOH 15 min (70%); (e) $ClP(N^iPr_2)OCH_2CH_2CN$ in CH_2Cl_2 and DIPEA, 30 min (60%).

Scheme 3. Synthesis of Protected Phosphoramidite of Alkyne-dA (17) from 8-Bromo-deoxyadenosine (1).[a]



[a](a) Hex-5-yne-1-thiol (**13**) in water and TEA, 100 °C, 2 h (30%); (c) DMTrCl, Py, 1 h rt (78%); (d) TMSiCl in Py 30 min, then DPACl, 2 h, then MeOH 15 min (90%); (e) $ClP(N^iPr_2)OCH_2CH_2CN$ in CH_2Cl_2 and DIPEA, 30 min (60%).

Table 1. Oligonucleotides Utilized in This Study

entry code	sequence	notes
P-norm	$^{5'}$CGTGC X TCCTAGC$^{3'}$	normal Probe with X= A
P-pso	$^{5'}$CGTGC X TCCTAGC$^{3'}$	Probe with X = comp. **4**
P-acr	$^{5'}$CGTGC X TCCTAGC$^{3'}$	Probe with X = comp. **9**
P-yne	$^{5'}$CGTGC X TCCTAGC$^{3'}$	Probe with X = comp. **14**
T1-DNA	$^{3'}$GCACG T AGGATCG$^{5'}$	DNA Target 1 with no mismatches
T2-DNA	$^{3'}$GCACG T AG**A**ATCG$^{5'}$	DNA Target 2 with one mismatch (**A**)
T1-RNA	$^{3'}$gcacg u aggaucg$^{5'}$	RNA Target 1with no mismatches *
T2-RNA	$^{3'}$gcacg u ag**a**aucg$^{5'}$	RNA Target 2 with one mismatch (**a**) *

[a]**T1**- and **T2-RNA** were from a commercial source.

steric hindrance of the alkyne moiety compared with the psoralen and acridine ones.

We anticipate that the modified deoxyadenosine **14**[42] as well as their derived oligonucleotides (this work) were then used for the preparation of more complex derivatives via click-chemistry. To the best of our knowledge there are no commercially available derivatives of adenosine for this scope.

Preparation of the Oligodeoxynucleotides. Up to now we have described the labeling of deoxyadenosine with at least three different moieties (psoralen, acridine, and C≡C triple bond) binding them to the C-8 position of the base by a short alkylic linker, through a sulfur atom, and the transformation of the modified deoxyadenosines into phosphoramidites suitable to be employed in the automated synthesis. In this paragraph we report about the use of the above-mentioned amidites in real syntheses, checking for coupling efficiency, cleavability of the introduced moieties to the deblocking conditions, and purification issues.

The syntheses of the oligodeoxynucleotides were performed with a Pharmacia Gene Assembler II plus, on a 1.3 μM scale, using commercial amidites, supports (CPG), and standard protocols. The coupling time of the modified adenosine was increased by 2 min, as the only concession to the modified monomers. They were charged on the synthesizer at the usual concentration of 0.1 M in anhydrous acetonitrile (0.08 M for the less soluble acridine derivative). We prepared the

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oligodeoxynucleotides listed in Table 1. The length of the probe was chosen to maximize the effects of modifications on the duplex stability, expecting at the same time a good degree of duplex formation at room temperature, to facilitate HPLC analysis, and to allow the formation of a full helix turn, in order to extend our finding to real application cases. The arbitrary sequence was chosen to avoid secondary structures, with the modified adenosine (indicated with X) located in such a way to provide a unique site for psoralen binding at the center of the duplex, and locating a transition mismatch in a sensitive position.

The coupling efficiency of the synthesis was evaluated based on spectroscopic readings of the DMTr cation, performed by the synthesizer. In the same manner we also checked the efficiency of the consecutive coupling. (See Table in SI).

In all the syntheses the efficiency figures fitted with the normal range of functioning of the machine. Thus we can affirm that the coupling efficiency of the modified amidites, with the precautionary elongation of the coupling time, is identical to that of a commercial one, and the introduction of a modified base does not affect the coupling of the subsequent amidite.

At the end of the syntheses (DMTr-off), the supports were sealed in Eppendorf tubes filled with 30% aqueous ammonia, and heated at 50 °C for 16 h. After this time the tubes were cooled, and the supports were filtered off and washed with 1 mL of water. The aqueous phases were combined and lyophilized. The crude products were then purified on a preparative RP-18 column in a 0.1 M aqueous solution of TEAA with a gradient of acetonitrile from 0 to 30%. The yields in pure compounds (fractions with a purity of more than 97% from HPLC at 260 nm) are reported in the Table in SI. All the oligonucleotides showed the expected mass demonstrating that no unexpected modifications were induced by the synthesis and purification procedures.

Hybridization Experiments. Having the pure compounds in our hands, we were eager to see the possible differences of the hybridization properties. To this aim we envisaged to test the probes against their complementary strand. As some modifications can have different effects on the melting temperatures depending on the DNA or RNA nature of the opposite strand,[43,44] we planned to measure the melting temperatures of our probes against both **T1-DNA** and **-RNA**, and also against targets containing a single mismatch: an adenosine instead of a guanosine in position 5: **T2-DNA** and **-RNA**.

The results of these experiments are summarized in Table 2.

These values will be discussed later on, together with the results of following experiments. At this point we only anticipate that all compounds but **P-acr** and **P-pso** with **T2-RNA** do form duplexes stable enough to be studied at room temperature.

Irradiation of P-pso with Their Targets. Having demonstrated that **P-pso** does form duplexes of sufficient stability at room temperature except perhaps in its combination with **T2-RNA**, we thought it was worth testing if it was possible to covalently bind their target upon irradiation. Therefore we irradiated the same cuvettes, containing the **P-Pso** mixtures used for the melting experiment, with a mercury lamp equipped with a pass filter at 350 nm. We followed the experiments taking samples at various times and analyzing them in HPLC. The results obtained with **T1-DNA** as a target are shown in Figure 1.

Table 2. T_m Values of the Four Sets of UV Melting Experiments Performed in 100 mM NaCl, 10 mM Sodium Cacodylate at pH 7.0 Following the Absorbance at 260 nm in the Range 15−90 °C at 0.4 °C/min[a]

P vs T1-DNA		P vs T2-DNA	
P-acr	48.9 °C	**P-acr**	28.6 °C
P-pso	49.5 °C	**P-pso**	30.8 °C
P-yne	49.8 °C	**P-yne**	32.7 °C
P-norm	56.2 °C	**P-norm**	40.3 °C
P vs T1-RNA		**P vs T2-RNA**	
P-acr	42.3 °C	**P-acr**	19.4 ± 1.0 °C
P-pso	46.5 °C	**P-pso**	22.4 °C
P-yne	50.1 °C	**P-yne**	26.2 °C
P-norm	54.9 °C	**P-norm**	32.2 °C

[a]The concentration of the strands was 2 μM. T_ms were determined by the first derivative method. The extimated error of the measurement is around ±0.4 °C if not otherwise stated.

The events represented by the chromatograms of Figure 1 left panel can be interpreted as follows [6] (Figure 1 right panel). The initial duplex formed by **P-pso** with **T1-DNA** that we know to be present at room T is split by the HPLC chromatography on a RP-18 column, performed at 30 °C in TEAA in a gradient of ACN. At time 0, the two strands are visible as two separate peaks, one with a retention time (R_t) of around 4.5 min corresponding to **T1-DNA**, and the other with R_t of around 7.8 min corresponding to the more lipophilic **P-pso**. At the beginning of the irradiation (5−10 min), a new peak appears at R_t = 7.5 min, it increases in intensity at the expenses of the peak corresponding to **P-pso**. We can assume that in the initial duplex the psoralen moiety is able to intercalate, as planned, between the ApT steps of both strands, one of the A being A(6) of the **P-pso**. Psoralen is known to undergo a cycloaddition reaction with the C5−C6 bond of thymine bases upon radiation, with both its furanosidic and pyranosidic rings, one at a time, on the two strands to make a cross-linking.[45] In our assembly the first cycloaddition is made on the thymidine in position 7 of the **P-pso** strand with the proximal furanose ring of the conjugated psoralen. This newly formed compound explains the new peak at R_t = 7.5 min. Later on (20−120 min), a new chromatographic peaks at R_t = 5.5 min appears, corresponding to the formation of the cross-linking product in which the pyranose ring of the psoralen makes a cycloaddition on the thimidine in position 8 of the **T1-DNA** strand. This cross-linking product has a retention time shorter than that of the first adduct, being less lipophilic upon the binding with the hydrophilic **T1-DNA** strand. Over time this peak increases at the expenses of both **T1-DNA** and **P-pso**. At 310 min the peak at R_t = 5.5 min is the predominant one, meaning that the psoralen has linked together the two strands. The cross-linking induced by psoralen is known to be reversible upon irradiation at 260 nm; indeed, we found that our system reverted to the original situation (with the formation of some byproducts due to the prolonged UV exposition) upon irradiation at this new frequency (figure in SI). The behavior so far described was observed also with the **T1-RNA** target, while both **T2** strands (**-DNA** and **-RNA**) did not show any cycloaddition product (data not shown).

We can conclude this paragraph affirming that **P-pso** was able to form a duplex with its complementary strand (either DNA or RNA), albeit with a lower T_m than the natural **P-norm** oligonucleotide. In the formed duplex, upon irradiation, the

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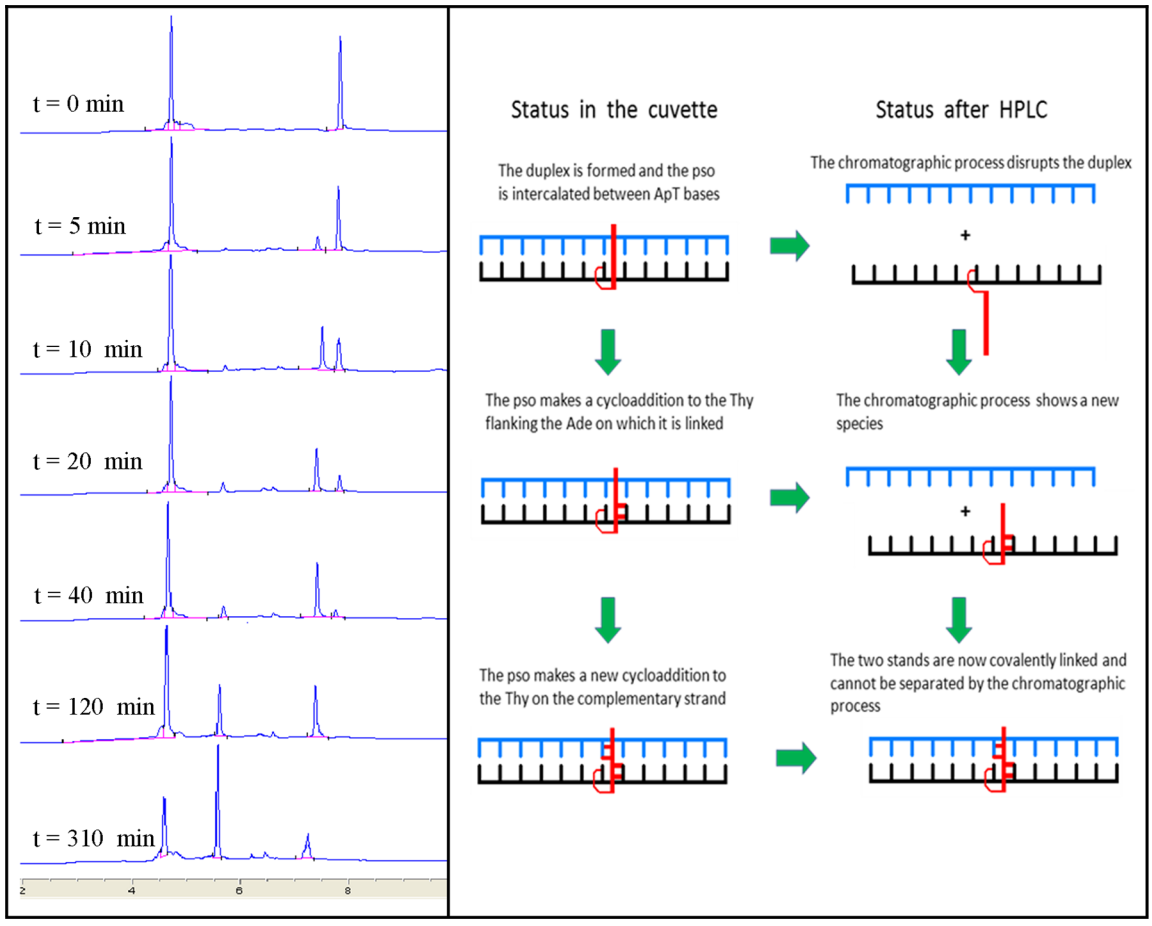

Figure 1. Left panel: HPLC chromatograms of samples taken from the mixture **P-pso** with **T1-DNA** upon irradiation at 350 nm, at the indicated times. Right panel: a cartoon with our explanation of the underlying events.

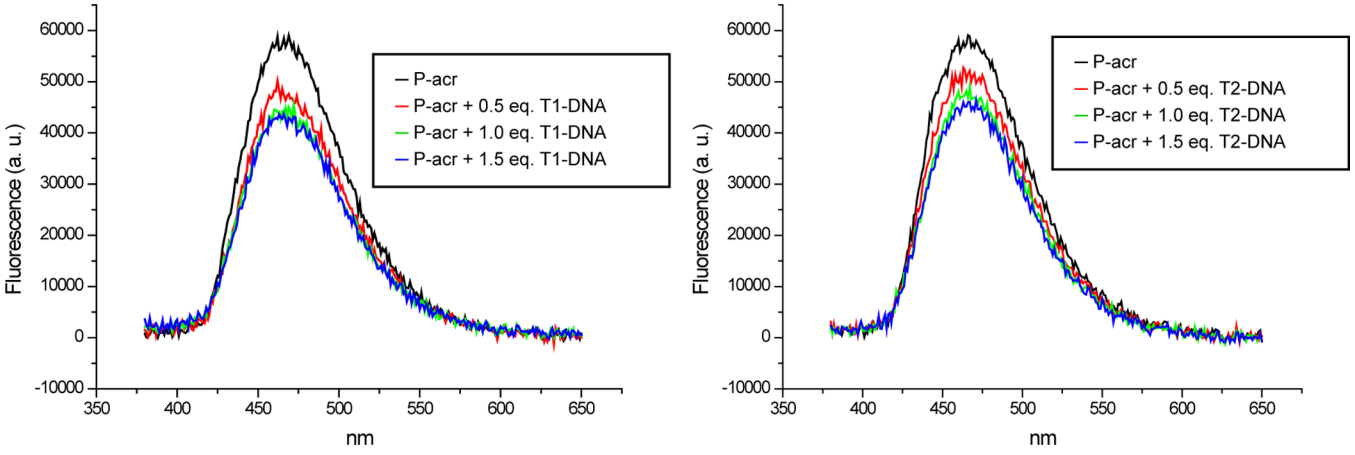

Figure 2. Emitted fluorescence of **P-acr** alone (black lines) and after addition of 0.5 (red line), 1 (green line), and 1.5 eq (blue line) of **T1-DNA** (left panel) or **T2-DNA** (right panel). The mixtures were irradiated at 370 nm with a cutoff at 375 nm and a temperature of 24 °C.

psoralen moiety was able to make cycloaddition products with the nearer thymidines on both the strands, as planned. This behavior was suppressed by the presence of a mismatch in the proximity of the presumed intercalation site with both DNA and RNA targets. This finding means that the conjugate with the modified adenosine is able to exert its programmed biochemical activity, and also that in this kind of "recognition" the activity of the **P-pso** is impaired by the presence of a near mismatch. In other words, this conjugate shows a good degree of selectivity toward its complementary strand.

Fluorescence Variation of P-acr upon Hybridization. Acridine can intercalate between DNA base-pairs in duplex and triplex, contributing to stabilize the adducts.[46,47] Following intercalation, its emitted fluorescence changes. We set up a simple titration experiment to check this point registering the fluorescence signal of **P-acr** alone and upon addition of 0.5, 1,

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and 1.5 equivalents (eq) of **T1-DNA** (Figure 2 left panel) or **T2-DNA** (Figure 2 right panel).

In the left panel (Figure 2) we observe that the fluorescence signal of **P-acr** changes from around 5900 to 4200 a.u. upon addition of 1 eq of the matching complementary strand **T1-DNA**; moreover, this signal does not change with the further addition of another 0.5 eq of target. In the right panel experiment, instead, the maximum of the fluorescence signal decreases from 5900 to 4700 upon addition of 1 eq of **T2-DNA** and reaches the value of 4500 upon a further addition of 0.5 eq of the mismatched complementary strand. This means that the acridine moiety of the **P-acr** conjugate maintains its ability to interact with the formed duplex as planned for these conjugates.

Click Chemistry on P-yne Oligonucleotide. To show the suitability of the alkyne moiety present on the **P-yne** strand to be exploited in a further derivatization using the Copper Azido Alkyne Cyclization (CuAAC) methodology, we prepared the 4′-azidomethyl-4,5′,8-trimethylpsoralen (**azidopsoralen**) derivatizing compound **3** with sodium azide. Then we used it in a test reaction with **P-yne** in a sealed tube. After a 3 min heating at 80 °C, then further 16 h at room temperature, we found indeed the complete transformation of **P-yne** into a new derivative, corresponding to the product of the cycloaddition: **P-yne-pso**. This compound showed the expected mass and the same fluorescence of **P-pso** upon irradiation at 350 nm (Figure 3).

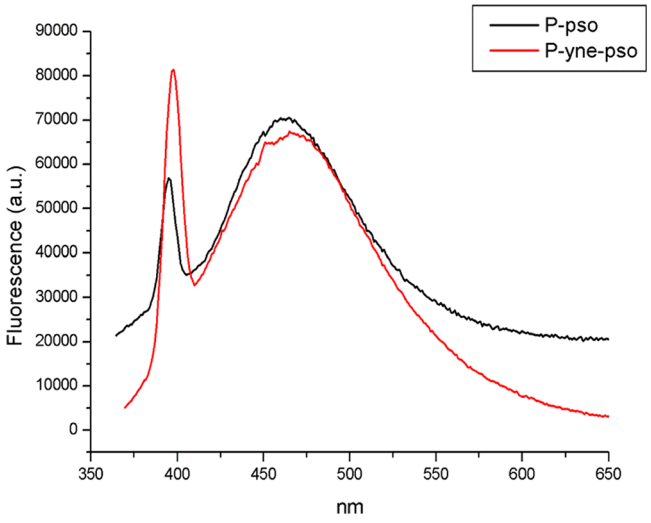

Figure 3. Emitted fluorescence of a a 0.8 μM solution of **P-pso** (black line) and **P-yne-pso** (red line) in 0.1 M NaCl and 0.01 M sodium cacodylate upon irradiation at 350 nm.

The successful synthesis of this derivative proved that the **P-yne** probe is suitable to be employed for postsynthetic derivatization, at least with the **azidopsoralen**, employing the CuACC methodology. In principle this result paves the way for the preparation of large range of different conjugates.

■ DISCUSSION AND CONCLUSIONS

Having reported the synthesis of different conjugated oligonucleotide probes, we are ready to briefly discuss about their hybridization properties.

Regarding the stability, we can observe that within each series (DNA and RNA) the order of stability is acr < pso < yne ≪ norm, i.e., all the modifications are weakly destabilizing.

With respect to **DNA-T2**, the difference between the T_m of **P-norm** and **-yne** remains 7 °C, whereas with all the other conjugates the variations are less than 2 °C. The presence of the mismatch lowers the melting temperature of the duplex of 16 °C in the case of **P1-norm**. A similar trend was observed in the case of hybridization toward **RNA-T2** with a lowering of the 23 °C showed by the **P-norm**.

In this light we can state that the conjugations with psoralen and acridine does not increase the stability of the native oligonucleotide (**P-norm**), despite the presence of the intercalators and their possible contribute to the overall stability of the complexes. Two different hypotheses can be formulated to explain these findings: the first one is that the intercalation arises but the overall effect is still weakly destabilizing; the second one is that the modification at the C-8 position of the base hampers the correct pairing of the modified adenosine, preventing the correct positioning of the intercalator moieties.

We have some good reasons to favor the first hypothesis:

- The T_m difference between **P-norm/T1-DNA** and **P-norm/T2-DNA** is of 16 °C (13 °C with the respective RNA targets): this means that a real mismatch leads to a much bigger difference from that found with the conjugate selected.
- In the absence of intercalation we can hardly explain the fluorescence variations observed with the **P-acr** or the alkylation of the matched complementary strands observed with the **P-pso**.

It is also interesting to note that the **P-pso** does not bind, upon irradiation, with the **T2-DNA**, despite the fact that at the experimental temperature of 22 °C the duplex is formed for more than 80% (a figure obtained from the melting profile). We explain this finding by the fact that the duplex distortion due to the near mismatch prevents the correct intercalation of the psoralen inside the duplex. This can be considered a bonus in terms of selectivity in the case of use of **P-pso** as an antisense agent. In the same way the mismatch prevents correct positioning of the **P-acr** as demonstrated by a lesser variation of the fluorescence signal after the addition of 1 eq of **T2-DNA**.

In conclusion, we have found a direct way to conjugate a variety of moieties to the position C-8 of deoxyadenosine, which we can confidently predict to also be applicable to adenosine. The method allows both the direct linkage of the moiety, as in the case of the alkyne, and a second step to derivatize the other thiol end of the linker introduced by the thioalkylation, with moieties having a reactive halogen, as demonstrated for the psoralen and acridine. The thio-alkylation procedure is of particular interest because it can be performed in water and can save at least one tedious chromatographic step of purification. The labeled derivatives can be transformed into amidites with conventional procedures. These amidites are fully compatible with the conventional supported synthesis of oligonucleotides and standard deblocking methodologies. These conjugates, as well as all the others based on the labeling of the nucleobases, can be used to synthesize multiple labeled oligonucleotides, with the same or different moieties, still leaving place for terminal modifications. The conjugates so obtained are still functionally active; therefore, this methodology is demonstrated to be a precious tool for the precise positioning of the tethered moiety both in biological assays and in supramolecular assembly. The suitability of **P-yne** to be employed for the synthesis of more complex conjugates via

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CuAAC, one of the most used click-chemistry procedures to synthesize bioconjugate molecules, extends the applicable range of our methodology. Considering the enormous potential of oligonucleotide−ligand conjugates for delivery of nucleic acid therapeutics, we are evaluating click-chemistry for preparation of oligonucleotide conjugates that can be more easily internalized in cells.

EXPERIMENTAL PROCEDURES

General. HPLC-MS analyses were performed on a Agilent 1100 HPLC system and an Esquire 3000 Plus Bruker mass spectrometer using a Zorbax C8 column (4.6 × 150 mm, 5 μm) (linear gradient water/CH_3CN at a 0.5 mL/min flow rate, detection at λ 260 nm). HPLC analysis were performed on a Agilent 1260 with a diode array detector and a Varian Pro Star 363 fluorescence detector. NMR spectra were recorded with a Varian Mercury 400 MHz instrument. The synthesis of the oligonucleotides was achieved with a Pharmacia Gene Assembler 2 instrument. UV melting studies were performed with a Cary 100 spectrophotometer. A LOT-Oriel instrument equipped with a 100 W mercury lamp and interference filters was used to irradiate the mixture with the **P-pso** probe.

Synthesis of 8-Thiopentanethiol-2′-deoxyadenosine (Compound 2). In 500 mL flask equipped with a condenser were added: a 1.6 mM suspension of commercial 8-bromo-2′-deoxyadenosine (220 mg, 0.67 mmol) in water, 1,5-pentanedithiol (276 mg, 2.2 mmol), and triethylamine (0.92 mL, 6.7 mmol). The resulting solution was heated at 100 °C for 2 h. The warm reaction mixture (40−50 °C) was extracted with ethylacetate (2 × 100 mL), the solvent was evaporated under reduced pressure, and the residue used without purification (80% yield on HPLC).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.02 (1H, s, H2), 6.20 (1H, dd, $J_1 = J_2 = 6.4$ Hz; collapsing to d upon irradiation at δ 2.10; H1′), 5.45 (1H, m; ex with D_2O; C5′−OH), 5.31 (1H, d, J = 4.0 Hz m; ex with D_2O; C3′−OH), 4.43 (1H, br d; H4′), 3.82 (1H, br m; H3′), 3.65 (1H, dd, part A of an ABX system, J_{AB} = 12 Hz, J_{AX} = 4.4 Hz; collapsing to d, J_{AB} = 4.4 Hz upon irradiation at δ 3.87; H5′), 3.50 (1H, dd, part B of an ABX system, J_{AB} = 12 Hz, J_{BX} = 4.4 Hz; collapsing to d, J_{AB} = 4.4 Hz upon irradiation at δ 3.87; H5″), 3.10 (1H, m; collapsing to dd, J_1 = 6.0 Hz, J_2 = 13.0 Hz, upon irradiation at δ 6.20; collapsing to dd, J_1 = 6.4 Hz, J_2 = 13.0 Hz, upon irradiation at δ 2.10; H2′), 2.67 (2H, m), 2.45 (3H, m changing to 2H, t J = 4 Hz upon D_2O shake, CH_2, SH), 2.10 (1H, m; H2″), 1.80−1.60 (6H, m).

^{13}C NMR (100 MHz, DMSO-d_6) δ: 24.1 (CH_2), 27.4 (CH_2), 28.6 (CH_2), 28.9 (CH_2), 32.7 (CH_2), 33.3 (CH_2), 62.8 (CH_2), 71.8 (CH), 85.5 (CH), 88.8 (CH), 118.8 (q), 151.1 (q), 151.9 (q), 154.9 (CH) 167.6 (q).

ESI-MS (m/z): 386.1 $[M+H]^+$ (calc. 385.1 for M = $C_{15}H_{23}N_5O_3S_2$).

8-Thiopentanethiomethylpsoralensoralen-2′-deoxyadenosine (Compound 4). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.02 (1H, s, H2), 7.55 (1H, s), 6.29 (1H, s), 6.25 (1H, dd, $J_1 = J_2$ = 6.0 Hz; H1′), 5.42 (1H, m, ex with D_2O; C3′−OH), 5.30 (2H, d, J = 4 Hz, ex with D_2O; NH_2), 4.47 (1H, br s, H3′), 3.88 (1H, br s, H4′), 3.62 (1H, m, H5′), 3.50 (1H, m, H5″), 3.23 (2H, m), 3.10 (1H, m, H2′), 2.50−2.30 (12H, m), 1.75−1.40 (6H, m).

^{13}C NMR (100 MHz, DMSO-d_6): δ 9.0 (CH_3), 12.7 (CH_3), 19.4 (CH_3), 24.1 (CH_2), 28.1 (CH_2), 29.0 (CH_2), 29.1 (CH_2), 31.3 (CH_2), 32.7 (CH_2), 38.0 (CH_2), 62.9 (CH_2), 72.0 (CH), 85.5 (CH), 88.9 (CH), 112.9 (CH), 113.2 (CH), 116.2 (q), 120.2 (q), 123.6 (q), 125.5 (q), 148.8 (q), 151.1 (q), 151.9 (q), 154.4 (CH), 155.1 (q), 160.1 (q), 160.7 (q).

ESI-MS (m/z): 648.3 $[M+Na]^+$ and 626.3 $[M+H]^+$ (calc. 625.2 for M = $C_{30}H_{35}N_5O_6S_2$).

5′-Dimethoxytrityl-8-thiopentanethiomethylpsoralensoralen-2′-deoxyadenosine (Compound 5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (1H, s, H2), 7.65 (1H, s), 7.26 (9H, m), 6.75 (4H m), 6.29 (1H, dd, $J_1 = J_2$ = 6.4 Hz; H1′), 6.24 (1H, s), 5.36 (1H, br s, ex with D_2O; C3′−OH), 4.89 (1H, m, H3′), 4.06 (1H, m, H4′), 3.80−3.70 (7H, m; OCH_3, H5′), 3.47 (1H, m, H2′), 3.40 (1H, m, H5″), 3.23 (2H, m), 2.56 (3H, s; CH_3), 2.49 (3H, s; CH_3), 2.41 (5H, m; CH_3, CH_2), 2.28 (1H, m H2′), 1.75−1.40 (6H m).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 8.8 (CH_3), 12.4 (CH_3), 19.6 (CH_3), 25.3 (CH_2), 28.1 (CH_2), 28.3 (CH_2), 28.9 (CH_2), 31.4 (CH_2), 32.5 (CH_2), 37.2 (CH_2), 55.4 (CH_3), 64.0 (CH_2), 73.3 (CH), 84.4 (CH), 85.9 (CH), 86.4 (q), 109.5 (q), 111.6 (q), 111.9 (CH), 113.2 (q), 125.1 (q), 127.0 (CH), 128.0 (CH), 128.4 (CH), 130.2 (CH), 136.1 (q), 145.0 (q), 151.6 (q), 151.8 (q), 153.6 (q), 154.0 (CH), 158.6 (q).

ESI-MS (m/z): 950.5 $[M+Na]^+$ and 928.5 $[M+H]^+$ (calc. 927.3 for M = $C_{51}H_{53}N_5O_8S_2$).

5′-Dimethoxytrityl-8-thiopentanethiomethylpsoralensoralen-N^6-diphenylacetyl-2′-deoxyadenosine (Compound 6). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.35 (1H, s, H2), 7.65 (1H, s), 7.50−7.10 (19H, m), 6.75 (4H m), 6.24 (1H, dd, $J_1 = J_2$ = 6.4 Hz; H1′), 6.20 (1H, s), 4.95 (1H s), 4.85 (1H, m, H3′), 4.10 (1H, m, H4′), 3.85−3.65 (7H, m; OCH_3, H5′), 3.44 (1H, m, H2′), 3.39 (1H, d, J = 4 Hz; H5″), 3.22 (2H, m), 2.56 (3H, s; CH_3), 2.49 (3H, s; CH_3), 2.41 (5H, m; CH_3, CH_2), 2.25 (1H, m H2′), 1.80−1.40 (6H m).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 8.7 (CH_3), 12.5 (CH_3), 19.6 (CH_3), 25.3 (CH_2), 28.2 (CH_2), 28.8 (CH_2), 31.5 (CH_2), 32.2 (CH_2), 37.2 (CH_2), 38.8 (CH_2), 55.5 (CH_3), 57.5 (CH), 64.2 (CH_2), 73.0 (CH), 84.7 (CH), 86.1 (CH), 86.5 (q), 109.4 (q), 111.6 (q), 111.9 (CH), 113.0 (q), 113.2 (CH), 125.1 (q), 127.0 (CH), 127.6 (CH), 127.9 (CH), 128.4 (CH), 128.9 (CH), 129.1 (CH), 129.4 (CH), 130.2 (CH), 136.1 (q), 139.0 (q), 145.9 (q), 146.1 (q), 153.4 (q), 153.7 (q), 154.0 (CH), 154.7 (q), 154.9 (q), 158.6 (q), 161.9 (q).

ESI-MS (m/z): 1122.5 $[M+H]^+$ and 1144.5 $[M+Na]^+$ (calc. 1121.4 for M = $C_{65}H_{63}N_5O_9S_2$).

5′-Dimethoxytrityl-8-thiopentanethiomethylpsoralensoralen-N^6-diphenylacetyl-2′-deoxyadenosine-3′-cyanoethyl diisopropylphosphoramidite (Compound 7). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.50 (1H, br s), 8.35 (1H, s, H2), 7.65 (1H, s), 7.50−7.10 (19H, m), 6.75 (4H, m), 6.24 (1H, dd, $J_1 = J_2$ = 6.4 Hz; H1′), 6.20 (1H, s), 4.95 (1H, s), 4.85 (0.5H, m, H3′), 4.83 (0.5H, m, H3′), 4.10 (1H, m, H4′), 3.85−3.65 (9H, m), 3.44 (1H, m, H2′), 3.40 (3H, m), 3.22 (2H, m), 2.65 (1H, m), 2.56 (3H, s; CH_3), 2.52 (1H, m), 2.49 (3H, s; CH_3), 2.41 (5H, m; CH_3, CH_2), 2.25 (0.5H, m H2′), 2.23 (0.5H, m H2″), 1.80−1.40 (6H m), 1.21(3H, s), 1.19 (6H, s), 1.17 (3H, s).

^{31}P NMR (160 MHz, $CDCl_3$) δ: 149.9, 149.6.

Synthesis of 8-Thiopentanethiomethylacridine-2′-deoxyadenosine (Compound 9). A solution of **2** (385 mg, 1.0 mmol), acridine **8** (600 mg, 2 mmol), and triethylamine (1.8 mL, 10 mmol) was heated in dry dimethylformamide (10 mL) at 80 °C in a sealed tube for 17 h. The reaction mixture was diluted with 5 mL of CH_2Cl_2/CH_3OH 80/20. The crude compound was precipitated with 80 mL of petroleum ether/

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ethyl ether 70/30. The precipitate was purified on silica gel column. Elution with CH_2Cl_2/CH_3OH 90:10 led to the target product **9** (440 mg, 70% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.66 (1H, d, J = 9.2), 8.18 (1H, d, J = 2.4), 8.13 (1H, s, H2), 8.09 (1H, d, J = 9.6 Hz), 7.93 (1H, d, J = 2.8), 7.49 (2H, m), 6.28 (1H, dd, J_1 = 5.6 Hz, J_2 = 9.6 Hz; collapsing to d J = 9.6 Hz upon irradiation at δ 2.2; H1′), 4.82 (2H, br s; ex with D_2O; NH_2), 4.70 (1H, d, J = 5.6; collapsing to s upon irradiation at δ 2.90; H3′), 4.17 (1H br s, H4′), 4.01 (3H, s), 3.90 (1H, dd, part A of an ABX system, J_{AB} = 12.8 Hz, J_{AX} = 1.6 Hz; H5′), 3.73 (1H, dd, part B of an ABX system, J_{AB} = 12.8 Hz, J_{BX} = 1.6 Hz; H5″), 3.10 (2H, m), 2.90 (3H, m), 2.19 (2H, dd, J_1 = 5.6 Hz, J_2 = 13.6 Hz; H2″), 2.63 (2H, m), 1.48 (4H, m).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 27.8 (CH_2), 28.6 (CH_2), 29.9 (CH_2), 32.6 (CH_2), 36.9 (CH_2), 40.3 (CH_2), 56.0 (CH_3), 63.6 (CH_2), 73.6 (CH), 86.9 (CH), 89.9 (CH), 126.3 (CH), 127.9 (q), 128.1 (CH), 128.2 (CH), 128.7 (CH), 130.6 (q), 131.9 (CH), 146.6 (q), 147.2 (q), 151.2 (q), 153.9 (CH), 158.5 (q), 160.2 (q).

ESI-MS (m/z): 627.1 and 629.1 $[M+H]^+$. (calc. 626,1 M = $C_{29}H_{31}ClN_6O_4S_2$).

5′-Dimethoxytrityl-8-thiopentanethiomethylacridine-2′-deoxyadenosine (Compound 10). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.68 (1H, d, J = 8.7), 8.20 (1H, d, J = 2.2), 8.10 (1H, d, J = 9.5 Hz), 8.05 (1H, s, H2), 7.94 (1H, d, J = 2.9 Hz), 7.50 (2H, m), 7.40−7.10 (9H, m), 6.75 (4H, m), 6.26 (1H, dd, J = 6.9; H1′), 4.88 (1H, m; H3′), 4.05 (1H, m; H4′), 4.01 (3H, s), 3.76 (3H, s), 3.75 (3H, s), 3.72 (1H, m; H5′), 3.47 (1H, m, H2′), 3.39 (1H, m; H5″), 3.14 (2H, m), 2.90 (2H, m), 2.27 (1H, m; H2″), 1.63 (2H, m), 1.45 (4H, m).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 27.8 (CH_2), 28.9 (CH_2), 29.9 (CH_2), 32.6 (CH_2), 37.0 (CH_2), 40.3 (CH_2), 55.6 (CH_3), 56.1 (CH_3), 63.6 (CH_2), 73.8 (CH), 86.4 (q), 86.9 (CH), 89.9 (CH), 120.0 (CH), 126.3 (CH), 127.2 (CH), 127.9 (q), 128.1 (CH), 128.2 (CH), 128.5 (CH), 128.7 (CH), 130.0 (CH), 130.6 (q), 131.9 (CH), 136.1 (q), 145.0 (q), 146.6 (q), 147.2 (q), 151.5 (q), 153.9 (CH), 158.6 (q), 160.2 (q).

ESI-MS (m/z): 951.1 and 953.1 $[M+Na]^+$. (Calc. 928.3 for M = $C_{50}H_{49}ClN_6O_6S_2$).

5′-Dimethoxytrityl-8-thiopentanethiomethylacridine-N^6-diphenylacetyl-2′-deoxyadenosine (Compound 11). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.68 (1H, d, J = 8.7), 8.35 (1H, s, H2), 8.20 (1H, d, J = 2.2), 8.10 (1H, d, J = 9.2 Hz), 7.94 (1H, d, J = 2.6 Hz), 7.49 (2H, m), 7.36−7.15 (19 H, m), 6.75 (4H, m), 6.20 (1H, dd, J = 6.8; H1′), 4.95 (1H, s), 4.84 (1H, m; H3′), 4.74 (2H, br s; ex with D_2O; NH_2), 4.10 (1H, m; H4′), 4.01 (3H, s), 3.75 (3H, s), 3.74 (3H, s), 3.72 (1H, m; H5′), 3.40 (1H, m, H2′), 3.36 (1H, m; H5″), 3.12 (2H, m), 2.90 (2H, m), 2.27 (1H, m; H2″), 1.61 (2H, m), 1.45 (4H, m).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 27.9 (CH_2), 29.0 (CH_2), 30.0 (CH_2), 32.6 (CH_2), 37.0 (CH_2), 40.3 (CH_2), 55.6 (CH_3), 56.1 (CH_3), 57.5 (CH), 63.6 (CH_2), 73.7 (CH), 86.4 (q), 86.9 (CH), 89.9 (CH), 120.0 (CH), 126.3 (CH), 127.2 (CH), 127.6 (CH), 128.0 (q), 128.2 (CH), 128.4 (CH), 128.5 (CH), 128.7 (CH), 129.1 (CH), 129.6 (CH), 130.0 (CH), 130.6 (q), 131.9 (CH), 136.1 (q), 139.0 (q), 145.0 (q), 146.6 (q), 147.2 (q), 151.5 (q), 153.9 (CH), 158.6 (q), 160.2 (q), 162.0 (q).

ESI-MS (m/z): 1123.3 $[M+H]^+$, 1145.4 $[M+Na]^+$. (Calc. 1122.4 for $C_{64}H_{59}ClN_6O_7S_2$).

5′-Dimethoxytrityl-8-thiopentanethiomethylacridine-N^6-diphenylacetyl-2′-deoxyadenosine-3′-cyanoethyl diisopropylphosphoramidite (Compound 12). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.68 (1H, d, J = 8.7), 8.45 (1H br s), 8.36 (0.5H, s; H2), 8.35 (0.5H, s; H2), 8.20 (1H, br s), 8.10 (1H, d, J = 9.2 Hz), 7.94 (1H, d, J = 2.6 Hz), 7.49 (2H, m), 7.36−7.15 (19 H, m), 6.75 (4H, m), 6.20 (1H, dd, J = 6.8 Hz; H1′), 5.0 (1H s), 4.85 (0.5H, m; H3′), 4.82 (0.5H, m; H3′), 4.21 (1H, m; H4′), 4.01 (3H s), 4.81 (1H, m; H5′), 3.75 (1.5 H, s), 3.74 (3H, s), 3.73 (1.5H, s) 3.70 (1H, m; H5″), 3.60 (2H, m), 3.50 (1H, m, H2′), 3.40 (2H, m), 3.30 (2H, m), 2.95 (2H, m), 2.60 (1H, m), 2.45 (1H, m), 2.40 (0.5 H, m; H2″), 2.35 (0.5 H, m; H2″), 1.40−1.10 (18 H, m).

^{31}P NMR (160 MHz, $CDCl_3$) δ: (147.4, 147.1).

Synthesis of 8-Thiohex-5-yne-2′-deoxyadenosine (Compound 14). Compound **1** (220 mg, 0.67 mmol) and thiol **13** (430 mg, 4 mmol) were reacted as reported above for the preparation of **2**. The target compound **14** (243 mg) was obtained in 30% yield, and used without purification.

^{1}H NMR (400 MHz, CD_3OD) δ: 8.10 (1H, s, H2), 6.39 (1H, dd, J_1 = J_2 = 6.0 Hz; collapsing to d upon irradiation at δ 2.25; H1′), 4.60 (2H, m), 4.08 (1H, m; H4′), 3.86 (1H, dd, part A of an ABX system, J_{AB} = 12.4 Hz, J_{AX} = 2.4 Hz; H5′), 3.75 (1H, dd, part B of an ABX system, J_{AB} = 12.4 Hz, J_{BX} = 2.4 Hz; H5″), 3.36 (3H, m), 3.02 (1H, m; H2′), 2.25 (1H, m; H2″), 2.21 (2H, m), 1.90 (2H, m), 1.68 (2H, m).

^{13}C NMR (100 MHz, CD_3OD) δ: 21.3 (CH_2), 31.3 (CH_2), 32.2 (CH_2), 35.8 (CH_2), 41.9 (CH_2), 42.9 (CH_2), 67.0 (CH), 76.5 (CH), 87.2 (q), 90.4 (CH), 93.1(CH), 124.2 (q), 153.8 (q), 154.4 (q), 154.8 (CH), 158.6 (q).

ESI-MS (m/z): 364.0 $[M+H]+$. (Calc. 363.1 for M = $C_{16}H_{21}N_5O_3S$).

5′-Dimethoxytrityl-8-thiohex-5-yne-2′-deoxyadenosine (Compound 15). ^{1}H NMR (400 MHz, CD_3OD) δ: 7.93 (1H, s, H2), 7.35 (2H, m), 7.24−7.12 (7H, m), 6.73 (4H, m), 6.40 (1H, dd, J_1 = J_2 = 6.4 Hz; collapsing to d upon irradiation at δ 2.26; H1′), 4.74 (1H, m, H3′), 4.08 (1H, m; collapsing to t, J = 4.8 Hz upon irradiation at δ 4.74 and to d, J = 4.0 Hz upon irradiation at δ 3.33; H4′), 3.75 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.45 (1H, m H2′), 3.37−3.29 (5H, m), 2.24 (1H, m; H2″), 2.16 (2H, m), 1.90 (2H, m), 1.63 (2H, m).

^{13}C NMR (100 MHz, CD_3OD) δ: 21.3 (CH_2), 31.3 (CH_2), 32.2 (CH_2), 35.8 (CH_2), 40.7 (CH_2), 49.8 (CH_2), 58.5 (CH_3), 67.9 (CH), 75.7 (CH), 88.7 (CH), 90.2 (q), 90.5 (CH), 116.6 (CH), 130.3 (CH), 131.3 (CH), 132.1 (CH), 133.9 (CH), 140.2 (q), 149.3 (q), 154.7 (CH), 158.2 (q), 162.7 (q).

ESI-MS (m/z): 666.4 $[M+H]^+$ 668.4 $[M+Na]^+$. (Calc. 665.3 for M = $C_{37}H_{39}N_5O_5S$).

5′-Dimethoxytrityl-8-thiohex-5-yne-N^6-diphenylacetyl-2′-deoxyadenosine (Compound 16). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.31 (1H, s, H2), 7.45−7.07 (19 H, m), 6.70 (4H, m), 6.36 (1H, dd, J_1 = J_2 = 6.8 Hz; H1′), 5.45 (1H, s), 4.76 (1H, m, H3′), 4.10 (1H, m; collapsing to d, J = 4.4 Hz upon irradiation at δ 3.27 and to t, J = 6.0 Hz upon irradiation at δ 4.76; H4′), 3.68 (3H, s, OCH_3), 3.65 (3H, s, OCH_3), 3.48 (1H, m; H2′), 3.32−3.24 (5H, m), 2.28 (1H, m; H2″), 2.16 (2H, m), 1.90 (2H, m), 1.63 (2H, m).

^{13}C NMR (100 MHz, CD_3OD) δ: 21.5 (CH_2), 31.4 (CH_2), 32.1 (CH_2), 35.6 (CH_2), 40.7 (CH_2), 49.8 (CH_2), 58.4 (CH_3), 62.3 (CH), 67.9 (CH), 75.6 (CH), 88.9 (CH), 90.0 (q), 90.7 (CH), 116.6 (CH), 128.2 (q), 131.0 (CH), 131.3 (CH), 132.0 (CH), 132.3 (CH), 132.6 (CH), 132.9 (CH), 134.0 (CH), 140.1 (q), 143.4 (q), 143.9 (q), 149.2 (q), 150.4 (q), 154.0 (CH), 159.6 (q), 162.7 (q), 175.5 (q).

ESI-MS (m/z): 860.4 $[M+H]^+$. (Calc. 859.3 for M = $C_{51}H_{49}N_5O_6S$).

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5′-Dimethoxytrityl-8-thiohex-5-yne-N^6-diphenylacetyl-2′-deoxyadenosine-3′-cyanoethyl diisopropylphosphoramidite (Compound 17). **^{1}H NMR** (400 MHz, CD_3OD) δ: 8.43 (1H br s), 8.38 (0.5 H s), 8.37(0.5 H s), 7.45−7.07 (19 H, m), 6.70 (4H, m), 6.27 (1H, m; H1′), 6.00 (1H, s), 4.94 (0.5 H, m; H3′), 4.86 (0.5 H, m; H3′), 4.22 (1H m; H4′) 3.85 (1H m; H5′), 3.74 (1.5 H; OCH_3), 3.75 (3 H; OCH_3), 3.76 (1.5 H; OCH_3), 3.67 (1H m; H5″), 3.60 (2H m), 3.50 (1H m; H2′), 3.45 (2H, m), 3.30 (3H m), 2.61 (1H m), 2.47 (1H m), 2.40 (0.5H m; H2″), 2.35 (0.5 H, m; H2″), 1.90 (2H m), 1.60 (2H m), 1.25 (2H m), 1.21 (3H s), 1.19 (6H s), 1.17 (3H, s).

^{31}P NMR (160 MHz, $CDCl_3$) δ: (149.3, 149.0).

Synthesis and Purification of Oligonucleotides. All the oligonucleotides were synthesized on a Pharmacia Gene Assembler 2 on a 1.3 μM scale with standard protocols with a 2 min longer coupling time for the conjugated adenosines. All the oligonucleotides were deblocked by treatment with 30% aqueous ammonia at 50 °C for 16 h, then lyophilized. The targets were purified on a preparative C-18 reverse phase column using 0.1 M aqueous solution of triethylammonium acetate (TEAA) in a gradient of acetonitrile from 0 to 30%. The collected fractions were analized in HPLC to give final lots with purity higher than 97% at 260 nm, and then lyophilized.

Synthesis of P-yne-pso. To a solution of **P-yne** (18 OD c.a. 0.15 μmol) in 310 μL of H_2O/triethylammonium acetate buffer solution (2 M, pH 7) /DMSO mixture 1/0.5/1.6 v/v, a solution of **azidomethylpsoralen** (0.3 μmol, 30 μL of a 10 mM DMSO solution) and ascorbic acid aq. solution freshly prepared (40 μL of 5 mM) was added . The mixture was briefly stirred with a vortex mixer and deaerated by bubbling nitrogen. To the resulting preparation, 20 μL of a $CuSO_4$·$5H_2O$−TBTA complex (10 mM solution in H_2O/DMSO 45/55 mixture) was added, and the tube was flushed with nitrogen and sealed. The mixture was heated for 3 min at 80 °C and kept at room temperature overnight. The reaction was diluted with 4-fold volume of cold ethanol and kept at −20 °C for 20 min. The cold mixture was centrifuged for 10 min; then, the supernatant was removed and the resulting pellet washed (2 × 1 mL) of cold ethanol each, and finally dried. 5 OD (c.a. 0.05 μmol) of a compound with purity higher than 90% at 260 nm in HPLC analysis was recovered.

ESI-MS Analysis. **T1-DNA**: 3998.9 (calc. 3999.7 for $C_{127}H_{159}N_{53}O_{75}P_{12}$). **T2-DNA**: 3983.1 (calc. 3983.6 for $C_{127}H_{159}N_{53}O_{74}P_{12}$). **P-norm**: 3908.8 (calc. 3908.7 for $C_{125}H_{160}N_{46}O_{77}P_{12}$). **P-pso**: 4283.0 (calc. 4282.8 for $C_{145}H_{182}N_{46}O_{80}P_{12}S_2$). **P-acr**: 4284.4 (calc. 4283.7 for $C_{144}H_{178}ClN_{47}O_{78}P_{12}S_2$). **P-yne**: 4020.7 (calc. 4020.7 for $C_{131}H_{168}N_{46}O_{77}P_{12}S$). **P-yne-pso**: 4303.6 (calc. 4303.0 for $C_{146}H_{181}N_{49}O_{80}P_{12}S$).

The synthetic methodology described in this work is based on the patent: M. L. Capoibianco and M. L. Navacchia PCT Int. Appl. WO 2012164484 A1 2012.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures for the preparation of compounds: **1**−**7**, **10**−**12**, and **15**−**17**; detailed synthetic procedures and the full characterization of compond **13** and **azidopsoralen**; Table: efficiency of coupling for oligonucleotides and final recovery based on CPG loading. Figure: HPLC study of reversion of adducts formed upon cross-linking of **P-pso** with **T1-DNA**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Wojczewski, C., Stolze, K., and Engels, J. W. (1999) Fluorescent oligonucleotides - Versatile tools as probes and primers for DNA and RNA analysis. *Synlett. 10*, 1667−1678.

(2) Capobianco, M. L., Barbarella, G., and Manetto, A. (2012) Oligothiophenes as fluorescent markers for biological applications. *Molecules 17*, 910−933.

(3) Garbesi, A., Bonazzi, S., Zanella, S., Capobianco, M. L., Giannini, G., and Arcamone, F. (1997) Synthesis and binding properties of conjugates between oligodeoxynucleotides and daunorubicin derivatives. *Nucleic Acids Res. 25*, 2121−2128.

(4) Arya, D. P., and Willis, B. (2003) Reaching into the major groove of B-DNA: synthesis and nucleic acid binding of a Neomycin-Hoechst 33258 conjugate. *J. Am. Chem. Soc. 125*, 12398−12399.

(5) Duca, M., Oussedik, K., Ceccaldi, A., Halby, L., Guianvarc'h, D., Dauzonne, D., Monneret, C., Sun, J. S., and Arimondo, P. B. (2005) Triple helix-forming oligonucleotides conjugated to new inhibitors of topoisomerase II: Synthesis and binding properties. *Bioconjugate Chem. 16*, 873−884.

(6) Murakami, A., Yamayoshi, A., Iwase, R., Nishida, J., Yamaoka, T., and Wake, N. (2001) Photodynamic antisense regulation of human cervical carcinoma cell growth using psoralen-conjugated oligo-(nucleoside phosphorothioate). *Eur. J. Pharm. Sci. 13*, 25−34.

(7) Moser, H. E., and Dervan, P. B. (1987) Sequence-specific cleavage of double helical DNA by triple helix formation. *Science 238*, 645−650.

(8) Boutorine, A. S., Tokuyama, H., Takasugi, M., Isobe, H., Nakamura, E., and Helene, C. (1995) Fullerene-oligonucleotide conjugates: Photoinduced sequence-specific DNA cleavage. *Angew. Chem. 33*, 2462−2465.

(9) Du, H., Strohsahl, C. M., Camera, J., Miller, B. L., and Krauss, T. D. (2005) Sensitivity and specificity of metal surface-immobilized "molecular beacon" biosensors. *J. Am. Chem. Soc. 127*, 7932−7940.

(10) Jung, A., Stemmler, I., Brecht, A., and Gauglitz, G. (2001) Covalent strategy for immobilization of DNA-microspots suitable for microarrays with label-free and time-resolved optical detection of hybridization. *Fresenius J. Anal. Chem. 371*, 128−136.

(11) Alemdaroglu, F. E., and Herrmann, A. (2007) DNA meets synthetic polymers - highly versatile hybrid materials. *Org. Biomol. Chem. 5*, 1311−1320.

(12) Bijsterbosch, M. K., Rump, E. T., De Vrueh, R. L. A., Dorland, R., van Veghel, R., Tivel, K. L., Biessen, E. A. L., van Berkel, T. J. C., and Manoharan, M. (2000) Modulation of plasma protein binding and in vivo liver cell uptake of phosphorothioate oligodeoxynucleotides by cholesterol conjugation. *Nucleic Acids Res. 28*, 2717−2725.

(13) Ballico, M., Drioli, S., Morvan, F., Xodo, L., and Bonora, G. M. (2001) Triple, MPEG-conjugated, helix-forming oligonucleotides (TRIPEGXs): Liquid-phase synthesis of natural and chimeric "all-purine" sequences linked to high molecular weight poly(ethylene glycols). *Bioconjugate Chem. 12*, 719−725.

(14) Weyermann, J., Lochmann, D., Georgens, C., and Zimmer, A. (2005) Albumin-protamine-oligonucleotide-nanoparticles as a new antisense delivery system. Part 2: cellular uptake and effect. *Eur. J. Pharm. Biopharm. 59*, 431−438.

(15) Wu, R. P., Youngblood, D. S., Hassinger, J. N., Lovejoy, C. E., Nelson, M. H., Iversen, P. L., and Moulton, H. M. (2007) Cell-penetrating peptides as transporters for morpholino oligomers: effects of amino acid composition on intracellular delivery and cytotoxicity. *Nucleic Acids Res. 35*, 5182−5191.

(16) Youngblood, D. S., Hatlevig, S. A., Hassinger, J. N., Iversen, P. L., and Moulton, H. M. (2007) Stability of cell-penetrating peptide-morpholino oligomer conjugates in human serum and in cells. *Bioconjugate Chem. 18*, 50−60.
(17) Chen, J. K., Carlson, D. V., Weith, H. L., Obrien, J. A., Goldman, M. E., and Cushman, M. (1992) Synthesis of an oligonucleotide-intercalator conjugate in which the linker chain is attached via the phenolic hydroxyl group of fagaron. *Tetrahedron Lett. 17*, 2275−2278.
(18) Asseline, U., Thuong, N. T., and Helene, C. (1997) Synthesis and properties of oligonucleotides covalently linked to intercalating agents. *New J. Chem. 21*, 5−17.
(19) Byrn, S. R., Carlson, D. V., Chen, J. K., Cushman, M. S., Goldman, M. E., Ma, W. P., Pidgeon, C. L., Ray, K. A., Stowell, J. G., and Weith, H. L. (1991) Drug-oligonucleotide conjugates. *Adv. Drug Delivery Rev. 6*, 287−308.
(20) Vinogradov, S., Roig, V., Sergueeva, Z., Nguyen, C. H., Arimondo, P., Thuong, N. T., Bisagni, E., Sun, J. S., Helene, C., and Asseline, U. (2003) Synthesis and binding properties of oligo-2′-deoxyribonucleotides conjugated with triple-helix-specific intercalators: benzo[e] and benzo[g] pyridoindoles. *Bioconjugate Chem. 14*, 120−135.
(21) Stierle, V., Duca, M., Halby, L., Senamaud-Beaufort, C., Capobianco, M. L., Laigle, A., Jolles, B., and Arimondo, P. B. (2008) Targeting MDR1 gene: Synthesis and cellular study of modified daunomycin-triplex-forming oligonucleotide conjugates able to inhibit gene expression in resistant cell lines. *Mol. Pharmacol. 73*, 1568−1577.
(22) Rothemund, P. W. K. (2006) Folding DNA to create nanoscale shapes and patterns. *Nature 440*, 297−302.
(23) Gu, H. Z., Chao, J., Xiao, S. J., and Seeman, N. C. (2010) A proximity-based programmable DNA nanoscale assembly line. *Nature 465*, 202−U286.
(24) Alesi, S., Brancolini, G., Melucci, M., Capobianco, M. L., Venturini, A., Camaioni, N., and Barbarellala, G. (2008) Water-soluble, electroactive, and photoluminescent quaterthiophene-dinucleotide conjugates. *Chem.—Eur. J. 14*, 513−521.
(25) Alesi, S., Brancolini, G., Viola, I., Capobianco, M. L., Venturini, A., Camaioni, N., Gigli, G., Melucci, M., and Barbarella, G. (2009) Self-organization, optical, and electrical properties of alpha-quinquethiophene-dinucleotide conjugates. *Chem.—Eur. J. 15*, 1876−1885.
(26) Navacchia, M. L., Favaretto, L., Treossi, E., Palermo, V., and Barbarella, G. (2010) Self-complementary nucleoside-thiophene hybrid systems: synthesis and supramolecular organization. *Macromol. Rapid Commun. 31*, 351−355.
(27) Tyagi, S., and Kramer, F. R. (1996) Molecular beacons: Probes that fluoresce upon hybridization. *Nat. Biotechnol. 14*, 303−308.
(28) Cazzato, A., Capobianco, M. L., Zambianchi, M., Favaretto, L., Bettini, C., and Barbarella, G. (2007) Oligothiophene molecular beacons. *Bioconjugate Chem. 18*, 318−322.
(29) Akiyama, T., and Hogan, M. E. (1997) Structural analysis of DNA bending induced by tethered triple helix forming oligonucleotides. *Biochemistry 36*, 2307−2315.
(30) Dreyer, G. B., and Dervan, P. B. (1985) Sequence-specific cleavage of single-stranded DNA: oligodeoxynucleotide- EDTA X Fe(II). *Proc. Natl. Acad. Sci. U. S. A. 82*, 968−972.
(31) Hurley, D. J., Seaman, S. E., Mazura, J. C., and Tor, Y. (2002) Fluorescent 1,10-phenanthroline-containing oligonucleotides distinguish between perfect and mismatched base pairing. *Org. Lett. 4*, 2305−2308.
(32) Hwang, G. T., Seo, Y. J., and Kim, B. H. (2004) A highly discriminating quencher-free molecular beacon for probing DNA. *J. Am. Chem. Soc. 126*, 6528−6529.
(33) Capobianco, M. L., Cazzato, A., Alesi, S., and Barbarella, G. (2008) Oligothiophene-5-labeled deoxyuridines for the detection of single nucleotide polymorphisms. *Bioconjugate Chem. 19*, 171−177.
(34) Pieles, U., Sproat, B. S., Neuner, P., and Cramer, F. (1989) Preparation of a novel psoralen containing deoxyadenosine building block for the facile solid-phase synthesis of psoralen-modified oligonucleotides for a sequence specific crosslink to a given target sequence. *Nucleic Acids Res. 17*, 8967−8978.
(35) Manfredini, S., Baraldi, P. G., Bazzanini, R., Marangoni, M., Simoni, D., Balzarini, J., and Declercq, E. (1995) Synthesis and cytotoxic activity of 6-vinyl- and 6-ethynyluridine and 8-vinyl- and 8-ethynyladenosine. *J. Med. Chem. 38*, 199−203.
(36) Sagi, G., Otvos, L., Ikeda, S., Andrei, G., Snoeck, R., and Declercq, E. (1994) Synthesis and antiviral activities of 8-alkynyl-, 8-alkenyl-, and 8-alkyl-2′-deoxyadenosine analogues. *J. Med. Chem. 37*, 1307−1311.
(37) Tierney, M. T., and Grinstaff, M. W. (2000) Synthesis and stability of oligodeoxynucleotides containing C8-labeled 2 ′-deoxyadenosine: Novel redox nucleobase probes for DNA-mediated charge-transfer studies. *Org. Lett. 2*, 3413−3416.
(38) Hwang, G. T., Seo, Y. J., and Kim, B. H. (2005) Pyrene-labeled deoxyuridine and deoxyadenosine: fluorescent discriminating phenomena in their oligonucleotides. *Tetrahedron Lett. 46*, 1475−1477.
(39) Chatgilialoglu, C., Navacchia, M. L., and Postigo, A. (2006) A facile one-pot synthesis of 8-oxo-7,8-dihydro-(2 ′-deoxy)adenosine in water. *Tetrahedron Lett. 47*, 711−714.
(40) Capobianco, M. L., Carcuro, A., Tondelli, L., Garbesi, A., and Bonora, G. M. (1990) One pot solution synthesis of cyclic oligodeoxyribonucleotides. *Nucleic Acid Res. 18*, 2661−2669.
(41) Ti, G. S., Gaffney, B. L., and Jones, R. A. (1982) Transient protection: efficient one-flask synthesis of protected deoxynucleosides. *J. Am. Chem. Soc. 104*, 1316−1319.
(42) Perrone, D., Bortolini, O., Fogagnolo, M., Marchersi, E., Mari, L., Massarenti, C., Navacchia, M. L., Sforza, F., Varani, K., and Capobianco, M. L. Synthesis and in vitro cytotoxicity of deoxyadenosine-bile acid conjugates linked with 1,2,3-triazole. *Submitted*.
(43) Morvan, F., Porumb, H., Degols, G., Pompon, A., Sproat, B. S., Rayner, B., Malvy, C., Lebleu, B., and Imbach, J. L. (1993) Comparative analysis of seven oligonucleotide analogs as potential antisense agents. *J. Med. Chem. 36*, 280−287.
(44) Pandolfi, D., Rauzi, F., and Capobianco, M. L. (1999) Evaluation of different types of end-capping modifications on the stability of oligonucleotides toward 3 ′- and 5 ′-exonucleases. *Nucleosides Nucleotides 18*, 2051−2069.
(45) Kanne, D., Rapoport, H., and Hearst, J. E. (1984) 8-Methoxypsoralen-nucleic acid photoreaction - effect of methyl substitution on pyrone vs furan photoaddition. *J. Med. Chem. 27*, 531−534.
(46) Asseline, U., Delarue, M., Lancelot, G., Toulme, F., Thuong, N. T., Montenaygarestier, T., and Helene, C. (1984) Nucleic-acid binding-molecules with high-affinity and base sequence specificity - intercalating agents covalently linked to oligodeoxynucleotides. *Proc. Natl. Acad. Sci. U.S.A. 81*, 3297−3301.
(47) Sun, J. S., Francois, J. C., Montenay-Garestier, T., Saison-Behmoaras, T., Roig, V., Thuong, N. T., and Helene, C. (1989) Sequence-specific intercalating agents: intercalation at specific sequences on duplex DNA via major groove recognition by oligonucleotide-intercalator conjugates. *Proc. Natl. Acad. Sci. U.S.A. 86*, 9198−9202.