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Oligonucleotide conjugation by tyrosine-click reaction

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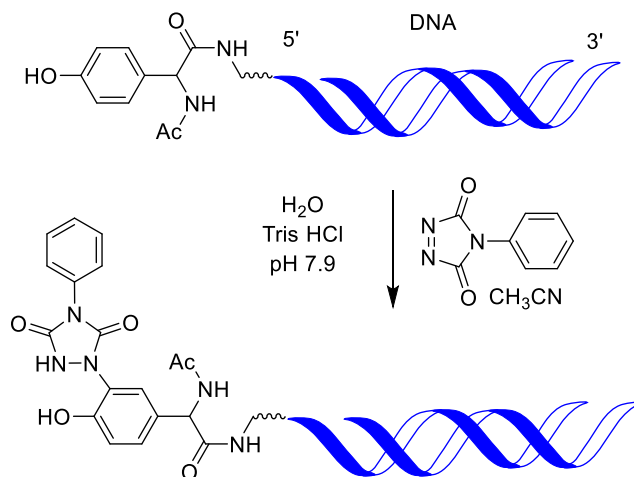
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<https://ibmm.umontpellier.fr/Oligonucleotides-Modifies,262.html>

Abstract: *N,O*-diacetyl tyrosine and 3-(4-hydroxy-phenyl)-propanoic acid were converted into phosphoramidite derivatives and introduced to the 5'-end of oligonucleotides. The resulting oligonucleotides exhibiting a 4-hydroxyphenyl alkyl group were conjugated with 4-phenyl-1,2,4-triazoline-3,5-dione as a model of a Y-Click reaction. The reaction is fast (< 1h) and efficient (> 90%). A partial side reaction occurred on the C8 position of deoxyguanosine in single-stranded oligonucleotides. This side reaction does not take place on the G quadruplex and double-stranded oligonucleotides and when N2 position of guanine is protected. This Y-Click reaction could be applied to 1,2,4-triazoline-3,5-dione derivatives displaying a fluorescent dye, a carbohydrate or orthogonal functions (alkyne, azide, ketone or maleimide) for the labelling of oligonucleotides.

Modified oligonucleotides

Graphical abstract



1 Tyrosine-click reaction was established for the conjugation of oligonucleotides exhibiting a
2 tyrosine moiety and using phenyl-1,2,4-triazoline-3,5-dione as a model. The reaction can be
3 applied in solution or on solid support.
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7 **Introduction**

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9 Conjugation of oligonucleotides to various molecules has attracted significant research interest
10 as it allows an easy way to modulate their biological properties. Most of the modifications of
11 the oligonucleotides are introduced either at the 5' or the 3' extremity, but can also be introduced
12 in the middle of the sequence.^[1, 2] To this end, several chemistries that rely on so-called "Click
13 Chemistry"^[3-5] have been developed including Diels-Alder ligation,^[6, 7] oxime ligation,^[8-16]
14 copper-catalyzed alkyne-azide cycloaddition (CuAAC),^[17-23] copper-free strain-promoted
15 alkyne-azide cycloaddition (SPAAC),^[24-27] alkyne-nitrile oxide^[28] or cyclooctyne-nitrile
16 oxide^[29] click cycloadditions, Staudinger ligation,^[30] thiol-Michael addition,^[31-35] thioether
17 ligation,^[36] or inverse-electron-demand Diels-Alder reaction.^[37, 38]

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19 In the search of other biorthogonal conjugations,^[4, 39] the use of 1,2,4-triazoline-3,5-dione
20 (TAD) derivatives able to selectively react with tyrosine moiety has recently gained some
21 interest as a selective method to conjugate peptides, proteins, or antibodies.^[40, 41] This reaction
22 also called tyrosine click reaction or Y-Click allowed the decoration of peptides, proteins, or
23 antibodies with glycans,^[42-44] oligonucleotides,^[45] fluorescent dyes,^[40, 42, 44-46] PEG-tags,^[42] or
24 with an antiviral agent.^[42] The use of TAD derivatives was also proposed for radiolabeling with
25 ¹⁸F.^[47] This Y-Click was associated most of the time to another click reaction (CuAAC,
26 SPAAC, oxime ligation, maleimide-thiol Michael addition).^[40, 44-46, 48] According to this
27 strategy, the TAD derivative bearing a clickable function (e.g. ketone, alkyne azide
28 maleimide...) was first selectively conjugated to peptides, proteins or antibodies on a tyrosine
29 residue and the second click reaction allowed the introduction of the decoration of interest. To
30 our knowledge, the use of TAD derivatives for the conjugation of oligonucleotides is limited to
31 only one publication reported by Luedtke.^[49] In this case, the reaction does not involve tyrosine
32 but rather a 5-vinyl-2'-deoxyuridine in the sequence of an oligonucleotide reacting with 4-
33 phenyl-1,2,4-triazoline-3,5-dione. The efficiency of the conjugations depends of the structure
34 of the nucleic acid (60% for an antiparallel guanosine quadruplex, 30% for a single-stranded
35 oligonucleotide, 15% for a (3+1) G-quadruplex (G4), and no reaction for a duplex).
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58 Herein, we report the evaluation of the conjugation of oligonucleotides with phenyl-1,2,4-
59 triazoline-3,5-dione (PTAD) as a model of TAD derivatives. The oligonucleotides were
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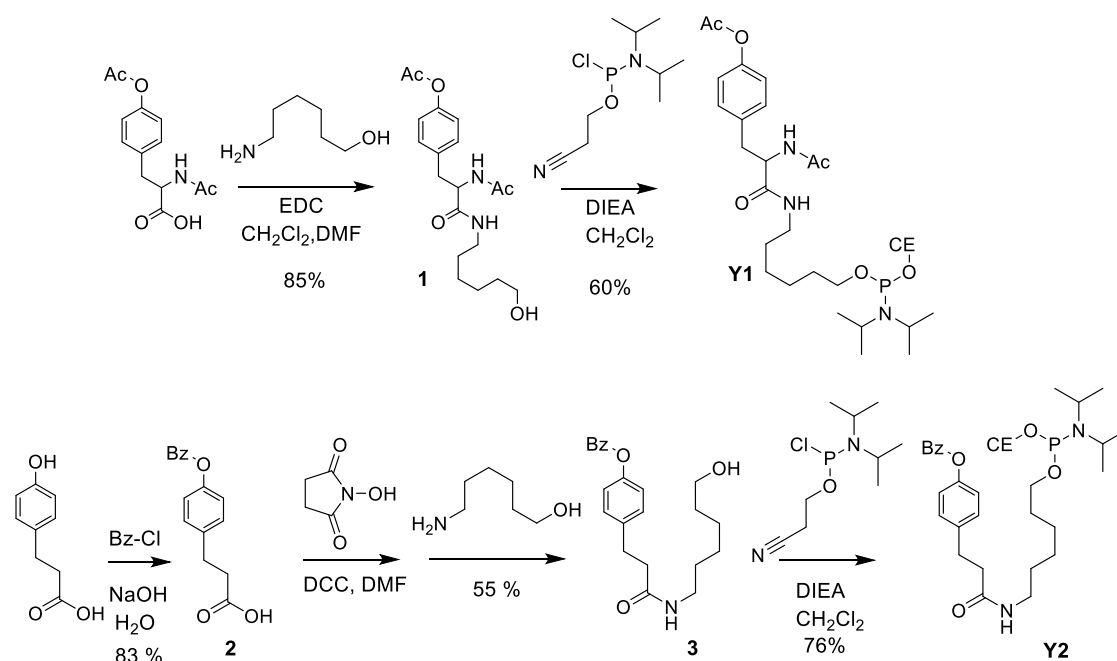
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modified in 5' with a tyrosine **Y1** or tyrosine-like **Y2** motif thanks to the synthesis of two new phosphoramidites exhibiting a 4-hydroxyphenyl alkyl group. The corresponding modified oligonucleotides allowed quick conjugation of PTAD with high efficiency (> 90%) using the tyrosine click reaction. However, a partial side reaction on position 8 of deoxyguanosine was observed on single-stranded oligonucleotides. In contrast, when oligonucleotides were either double-stranded or of G-quadruplex structure, the side reaction was eliminated.

Results and Discussion

Synthesis of tyrosine-inspired phosphoramidites

Two phosphoramidite derivatives **Y1** and **Y2**^[50] exhibiting a 4-hydroxyphenyl alkyl motif were synthesized (Scheme 1). Compound **Y1** corresponds to the *N,O*-diacetyl tyrosine phosphoramidite with a hexyl linker. Meanwhile, compound **Y2** is an analogue of **Y1** deprived of the *N*-acetyl moiety, and where a benzoyl protecting group was introduced on the phenol instead of an acetyl, to gain better stability. **Y1** was prepared in two steps starting from the commercially available *N,O*-diacetyl-tyrosine. First, 6-amino-hexanol was coupled with *N,O*-diacetyl tyrosine and then the phosphoramidite function was introduced with a 51% overall yield. For **Y2**, first the benzoyl group was introduced on the hydroxyl function of 4-hydroxyphenyl propionic acid, followed by the coupling with 6-amino hexanol, and lastly the phosphoramidite function was introduced with a 35% overall yield.

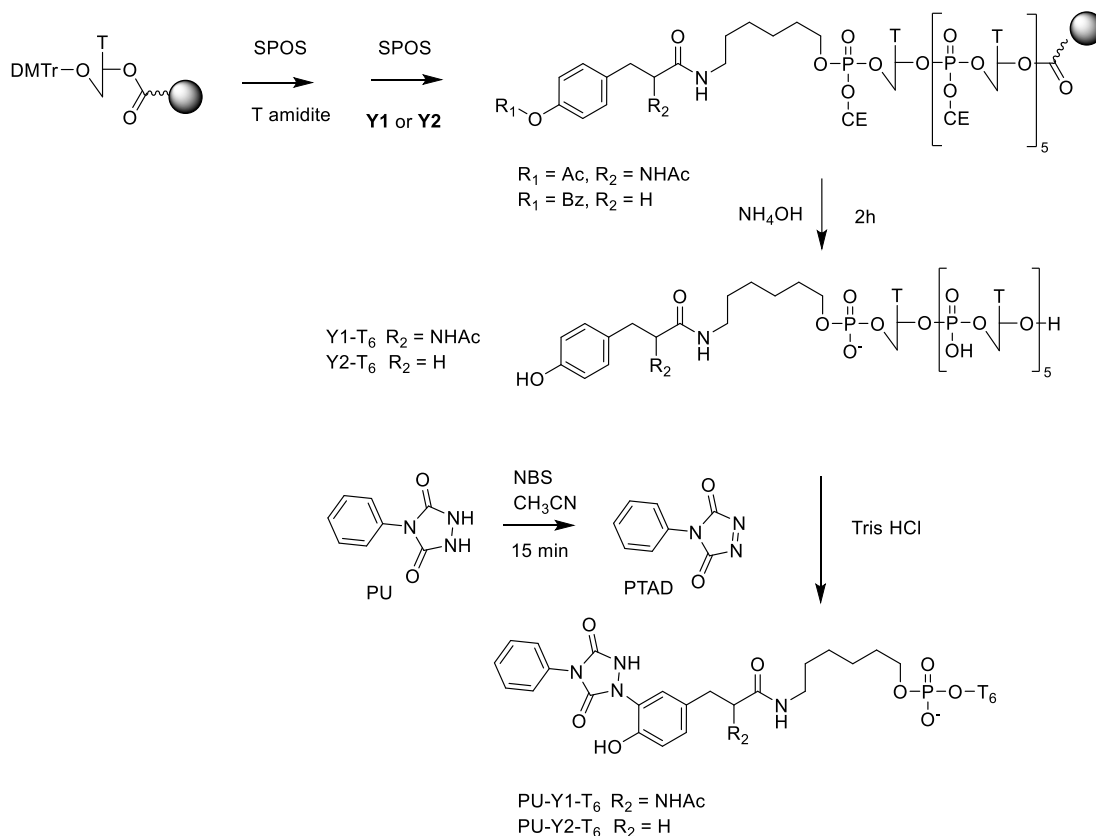


Scheme 1. Synthesis of **Y1** and **Y2** phosphoramidites. CE: 2-cyanoethyl.

Y-Click conjugations

The coupling efficacy was first evaluated on a hexathymidylate (T_6) model. After the elongation of the T_6 on solid support by standard phosphoramidite chemistry, a final coupling was performed with **Y1** or **Y2** with an additional coupling time of 30 sec to ensure efficacy. After release and deprotection, both $Y1-T_6$ and $Y2-T_6$ were analyzed by HPLC and characterized by MALDI-TOF MS showing the exclusive formation of the expected modified oligonucleotides. To test the Y-Click reaction, we used the 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) prepared by oxidation of 4-phenylurazole (PU) with *N*-bromosuccinimide (NBS) in acetonitrile (Scheme 2). This oxidation is easily followed since after the addition of NBS, the colorless solution turns very quickly to a deep red which is characteristic of PTAD formation. Alternatively, *N*-chlorosuccinimide (NClS) can be used even if the oxidation is slower (~ 5 min).

Several conditions of the Y-Click conjugation were tested (Table 1). PTAD is poorly stable in water since the deep red color vanishes within a few minutes. For this reason, sequential additions of smaller portions were more effective than only one large portion added of an equal number of equivalents. In a first experiment, four additions of 5 eq. of PTAD every hour to a solution of $Y2-T_6$ in tris-HCl buffer led to 84% coupling (entry 1, Fig. S1-4). Double addition of PTAD (10 eq. every 30 min) was somewhat less efficient with 75% coupling. Further addition of 5 eq. gave a small increase (80%, entry 2). Since it was reported that this reaction is fast, a third experiment was performed adding five times 5 eq. every 5 min leading to 86% after 25 min. Likewise the addition of 5 more eq. led to a small increase (90%, entry 3). An attempt to increase the yield using microwave activation at 60°C was unsuccessful and resulted in only 66% coupling (entry 4). Finally, the reaction was followed more closely on $Y1-T_6$ (Entry 5). Ten eq. of PTAD were added every 10 min and the coupling was monitored every 10 min leading to a 91% coupling within 40 min (Fig. S5-6). This study showed that the Y-Click reaction can be applied to the conjugation of oligothymidylates leading to a high coupling efficiency (~90 %) using 30 to 40 equivalents of PTAD. The same reactivity was observed for the $Y1$ and $Y2$ derivatives.



28 **Scheme 2.** Synthesis of Y1-T₆ and Y2-T₆ and their conjugates.

31 **Table 1.** Coupling efficiency of Y1-T₆ and Y2-T₆ with PTAD.

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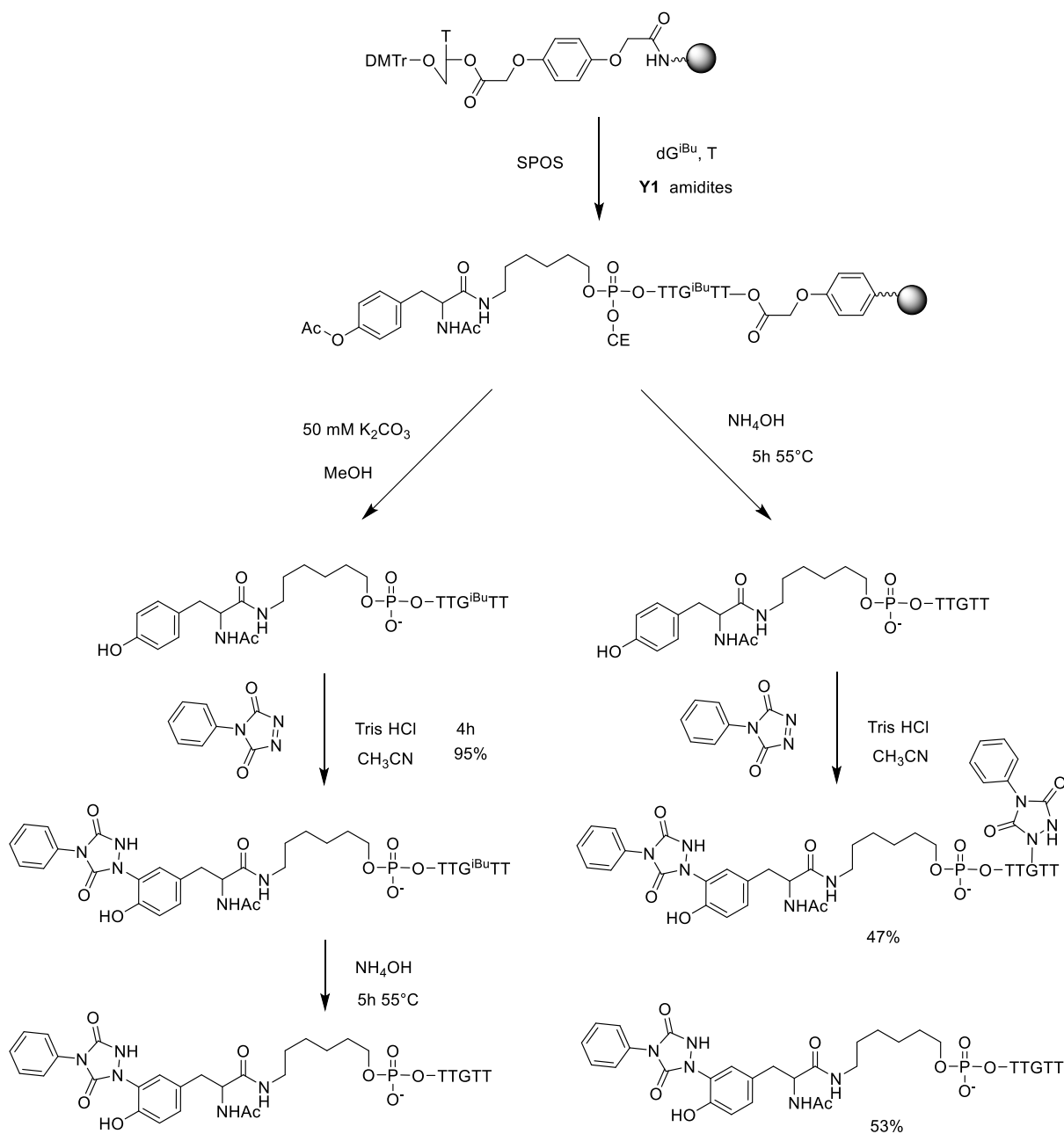
Entry	Oligo ^[a]	PTAD in CH ₃ CN (25 mM)	Time (min)	Yield ^[b]
1	Y2-T ₆	4 x 5 eq/60 min Σ 20 eq	240	84%
2	Y2-T ₆	2 x 10 eq/30 min Σ 20 eq	150	75%
		+ 5eq Σ 25 eq	240	80%
3	Y2-T ₆	5 x 5 eq/5 min Σ 25 eq	25	86%
		+ 5eq Σ 30 eq	60	90%
4	Y2-T ₆ ^[c]	2 x 10 eq/30 min Σ 20 eq	60	66%
5	Y1-T ₆	10 eq	10	47%
		+10 eq Σ 20 eq	20	72%
		+10 eq Σ 30 eq	30	82%
		+10 eq Σ 40eq	40	91%

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^[a]Reaction performed at room temperature with 50 nmol of oligonucleotide (initial concentration 500 μ M) in 200 mM Tris HCl buffer pH 7.9 (100 μ L), ^[b]Yield of conversion calculated from HPLC profiles, ^[c]60 °C under microwaves.

The Y-Click coupling was then tested on a heteropolymer Y2-TAGCT exhibiting the four nucleosides. The Y-Click coupling was applied using five times 10 eq. of PTAD every 5 min. Surprisingly, the HPLC profile showed the formation of the expected conjugated oligonucleotide as a minor product (33%) and a side product corresponding to the incorporation of two PU units (M+175) as a major product (67%) (Fig. S7-8). This result was unexpected since it was reported by Luedtke that PTAD did not react with nucleosides.^[49] The reaction performed in cacodylate buffer as described by Luedtke gave the same result. To investigate this phenomenon, we synthesized three oligonucleotides TCGTCA, GTCAGT and TCATCA exhibiting different nucleoside compositions and without the phenol moiety. To each oligonucleotide, 50 eq of PTAD was added. The formation of one and two adducts was observed on TCGTCA and GTCAGT respectively, while TCATCA remained intact (data not shown). This result strongly suggested that the side reaction occurred on the deoxyguanosine.

To gain more information, Y1-TTG^{iBu}TT was synthesized using solid support with a Q-linker^[51] allowing a mild and fast release of the oligonucleotide from the support while keeping the iBu protecting group on dG (Scheme 3). Y1-TTG^{iBu}TT was treated four times with 10 eq. of PTAD every 10 min and the reaction was monitored by HPLC and MALDI-TOF MS for 4 h. A coupling of around 95% was observed without the formation of a side compound (Fig. S9-10). A final ammonia treatment afforded the PU-Y1-TTGTT (Fig. S11). In comparison, the fully deprotected Y1-TTGTT treated with PTAD in the same conditions showed the formation of the expected PU-Y1-TTGTT (53%) contaminated with the side compound PU-Y1-TTG^{PU}TT (47%) (Scheme 3, Fig. S12-13).



43 **Scheme 3.** Synthesis of PU-Y1-TTGTT and side product PU-Y1-TTG^{PU}TT. SPOS: Solid Phase
44 Oligonucleotide Synthesis.

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48 Since the presence of the *i*Bu protecting group prevents the side reaction, it was envisaged that
49 the addition of the urazole occurred on the guanine nucleobase either on the exocyclic amine in
50 position 2 or on the nitrogen atom in position 1, through a formation of a N-N bond (Figure 1).
51 However, PTAD is not supposed to form N-N but rather N-C bonds^[40]. The only possibility for
52 an N-C bond formation is the coupling on the C8 atom of the guanine. It is interesting to note
53 that PTAD can react on the 2-position of furan leading to the formation of a N-C bond.^[52]
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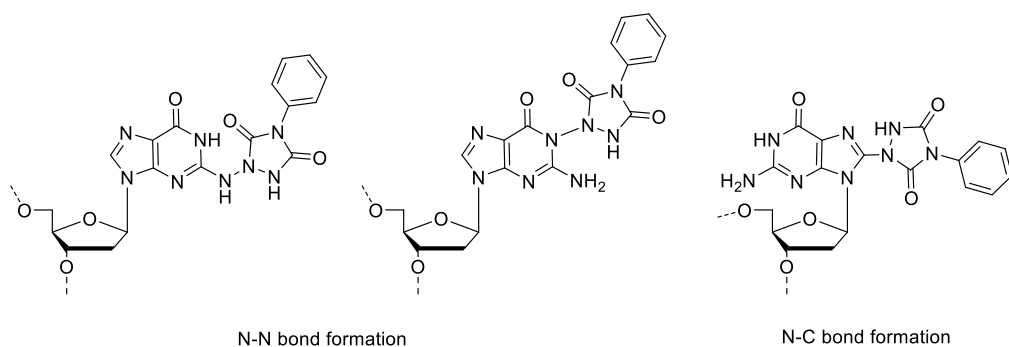
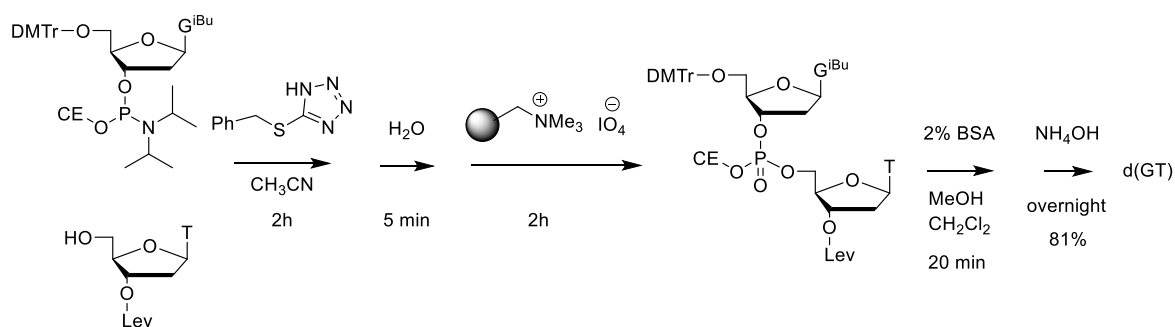
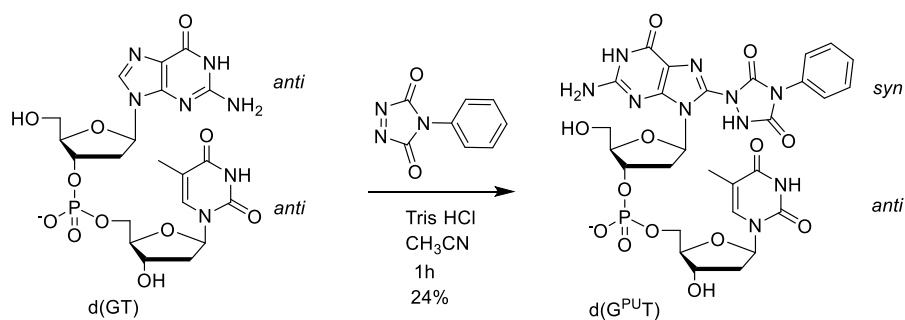


Figure 1. Hypothetical N-N versus N-C deoxyguanosine adducts with PTAD.

To determine what type of coupling occurred, we decided to study by NMR the product of the reaction of PTAD on d(GT) as a model. In order to have enough material for the NMR study, the dimer was prepared in solution (Scheme 4). The commercially available deoxyguanosine phosphoramidite was activated by 5-(benzylthio)-tetrazole in presence of 3'-levulinyl-thymidine to form the phosphite linkage. The excess of phosphoramidite was hydrolyzed by addition of water and the oxidation was performed by the addition of solid-supported IO_4^- .^[53] The fully protected dimer was directly detritylated with a 2% solution of benzene sulfonic acid. Lastly the basolabile protecting groups were removed with a concentrated solution of ammonia, affording the d(GT) dimer. The d(GT) dimer was treated with PTAD and the reaction, monitored by HPLC, showed full conversion to a new compound of higher retention time (Scheme 5).



Scheme 4. Synthesis of d(GT) in solution. The grey ball represents a polystyrene resin. CE: cyanoethyl, Lev: levulinyl, BSA: benzene sulfonic acid.



Scheme 5. Coupling of PTAD on d(GT).

Both dimers d(GT) and d(G^{PU}T) were studied by NMR showed the addition of PU on the C8 position with the formation of an N-C bond. The analysis of the NMR spectra for the modified dimer first highlighted the disappearance of the C8 proton. The comparison of the ¹H and ¹³C resonances (Table S1) showed that the thymidine remained mostly unchanged from d(GT) to d(G^{PU}T). In contrast, the deoxyguanosine chemical shifts were significantly modified, particularly on the nucleobase where the C8 chemical shift of d(G^{PU}T) was shifted downfield (+3.3 ppm) due to the change from a C-H bond to a C-N bond. The minor differences observed for the ¹H-¹H coupling constants of the sugar rings indicated a limited impact of the modification on the conformation of the sugar, where the South form largely predominates. In contrast, the C8 modification on the guanine nucleobase induced a change in its orientation relative to the sugar, as supported by a qualitative comparison of the heteronuclear ³J_{H1'-C4/C2} and ³J_{H1'-C6/C8} couplings.^[54] As expected for d(GT), ³J_{H1'-C6} coupling was superior to ³J_{H1'-C2} for T and ³J_{H1'-C8} was superior to ³J_{H1'-C4} for G which demonstrate a preference for *anti* orientation for both nucleobases. For d(G^{PU}T), the *anti*-orientation remained for T but for G^{PU} the ³J_{H1'-C8} coupling constant was inferior to the ³J_{H1'-C4} coupling revealing a shift of the *syn/anti* equilibrium to the *syn* orientation (Figure 2). This change of orientation can be explained by the presence of the bulky PU group on C8 that induces a steric hindrance with the ribose as already described in literature.^[55] The strong deshielding observed for H_{2'} in G^{PU} (+0.53 ppm) relative to unmodified G, was comparable to the values reported for other guanosines substituted in C8 with bulky substituents, e.g. 8-dimethylamino (0.57 ppm in DMSO-d₆),^[56] 8-tert-butyl (0.65 ppm in DMSO) and 8-(α-hydroxyisopropyl) (0.51 ppm in D₂O) guanosines^[55] and supported the change of the nucleobase orientation.

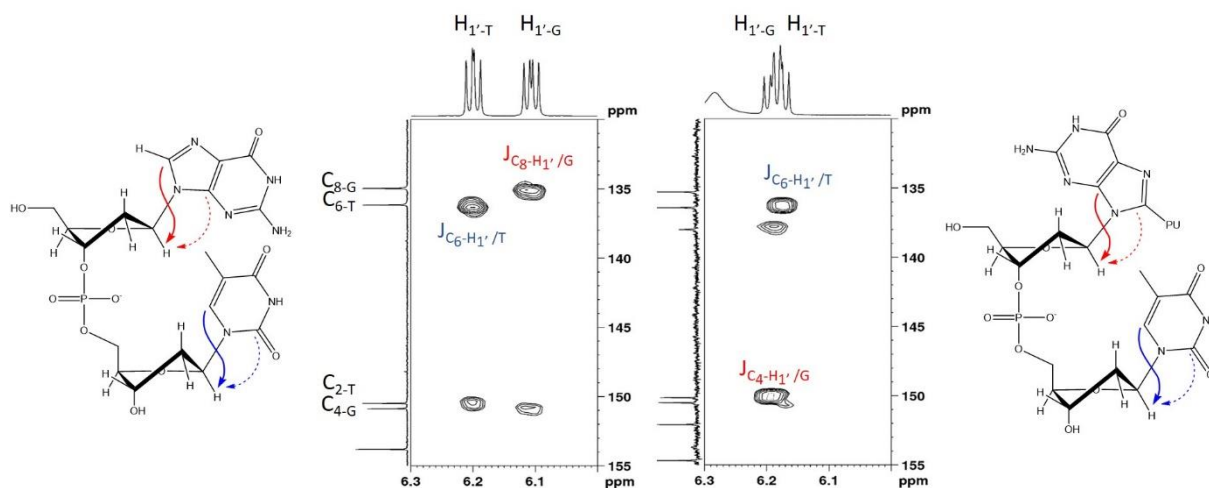


Figure 2. Selected part of the long-range heteronuclear ^1H - ^{13}C spectrum (optimized for a long-range coupling constant of 8 Hz): left d(GT) and right d(G^{PU} T). Full and dotted arrows represent the strong and weak correlations respectively.

We further investigated the reactivity of PTAD on different structures of oligonucleotides like guanosine quadruplex (G4), double-stranded oligonucleotides as hairpins or duplexes, and on a single-stranded oligonucleotide (Table 2).

Table 2. Sequences of oligonucleotides.

Structures	Name	Sequences	PTAD	Yield ^[a]
G4	Y1-TBA	5'-Y1-GGT TGG TGT GGT TGG-3'	2 x 25 eq	91%
Hairpin	Y1-h16	5'-Y1- <u>ATA CTT</u> -TTTT <u>AAG TAT</u> -3'	5 x 10 eq	100%
Duplex	Y1-16mer c16mer	5'-Y1-ATT TTC ATT GTT ATA A-3' 3'-TAA AAG TAA CAA TAT T-5'	5 x 10 eq	92%
Single Strand In solution	Y1-16mer	5'-Y1-ATT TTC ATT GTT ATA A-3'	5 x 10 eq	73%
Single strand On solid support	Y1-12mer	5'-Y1-TCG TCA GTC AGT-3'	7 x 5 eq	84%

Underlined sequences are complementary. ^[a]Yield of conversion calculated from HPLC profiles.

Tyrosine phosphoramidite **Y1** was introduced at the 5'-end of the G4 oligonucleotide GGT TGG TGT GGT TGG corresponding to the thrombin binding aptamer (TBA).^[57] The modified Y1-TBA was fully deprotected by ammonia and treated with PTAD (2 x 25 eq.). The Y-Click

1 conjugation was efficient to 91% conversion with almost no formation of side reactions on
2 deoxyguanosines. Indeed, the side-product was not observed in the HPLC chromatogram and
3 the M+175 ion was in the low background of the MALDI-TOF spectrum (Fig. S14-16).
4 Likewise, when the hairpin structure Y1-h16 was treated with PTAD (5 x 10 eq.) full
5 conversion of the starting oligonucleotide to PU-Y1-h16 was observed, with less than 5%
6 formation of the side-product (Fig. S17-19). For the Y1-16mer/c16mer duplex, the reaction was
7 performed in 1 M NaCl to stabilize it. Y1-16mer was converted into its PU-Y1-16mer conjugate
8 (>92%) and no side reaction occurred on its deoxyguanosine nor on that of the complementary
9 c16mer strand (Fig. S19-20). In contrast, when single-stranded Y1-16mer, was treated with 50
10 eq. of PTAD 73% of PU-Y1-c16mer was observed with 23% of Pu-Y1-16mer^{G^{PU}} as side-
11 product (Fig. S21-22). This single-stranded Y1-16mer has the same nucleoside composition as
12 Y1-h16 but is unable to form a hairpin.

13 For comparison, the Y-Click reaction was performed on solid support, on the 12-mer 5'-Y1-
14 TCG TCA GTC AGT fully protected except on the phenol. To this end, after elongation of the
15 oligonucleotide and the coupling of Y1, a short treatment with K₂CO₃/MeOH allowed the *O*-
16 deacetylation of the tyrosine. The Y-Click reaction was performed with PTAD formed by
17 oxidation with NCIS.^[58] The conjugation was performed by addition of PTAD (5 eq.) every 30
18 min, seven times. Then, solid support was washed and treated with concentrated ammonia
19 affording the PU-Y1-12mer conjugate (84 % yield) with no trace of urazole adduct on the
20 guanine residues (Fig. S23-24). These experiments showed that when deoxyguanosines are *N*-
21 protected or involved in Hoosteen (G4) or Watson-Crick (dsDNA) hydrogen bonds, the C8
22 position is not reactive to PTAD. In contrast, a side reaction occurs partially at the C8 position
23 when the exocyclic amino is free.

24 Conclusion

25 In summary, the reaction of PTAD with 4-hydroxy-phenyl residues (tyrosine or tyrosine-like)
26 at the 5'-end of oligonucleotides is very efficient and allowed their chemoselective
27 bioconjugation. The Y-Click was rapid and efficient with more than 90% of conversion within
28 1h. However, PTAD can react on the C8 position of deoxyguanosine of single-stranded
29 oligonucleotide leading to 30-50% of side-reaction. This side-reaction is almost avoided on G4
30 structure or hairpin and totally suppressed on duplex or when the guanine is protected on the
31 N2 position. Since the TAD derivatives are relatively unstable in water, it is more efficient to
32 add them in small portions, several times, rather than using all of the reagent at once. In
33 comparison to other click reactions, the Y-Click requires a larger amount of reagent (~50 eq.)

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than CuAAC or ligation oxime (2 to 20 eq) for example. However, since the scale of the reaction is low, the use of 50 eq of reagent for conjugation remains reasonable. TAD derivatives with fluorescent dyes^[40] or carbohydrates^[44] or exhibiting an alkyne, azide, ketone, maleimide functions^[40, 43-45, 48, 49] have been reported and can be used for conjugation of oligonucleotides exhibiting a 4-hydroxyphenyl alkyl group through the Y-Click reaction. This Y-Click reaction applied to oligonucleotides is bioorthogonal with other click reactions and increases the possibilities of multiple oligonucleotide conjugations.

Experimental Section

General Methods: All commercial chemicals were reagent grade and were used without further purification. Flash column chromatography was performed on silica gel 60 (40–63 μm). NMR analyses were made on Bruker spectrometers ^1H NMR (400–600 MHz), ^{13}C NMR spectra (101–151 MHz), and ^{31}P NMR (162 MHz) in the stated solvents at room temperature. Chemical shifts were reported in parts per million (ppm) referenced to the solvent residual peaks (δ ^1H = 7.26 and δ ^{13}C = 77.16 ppm for CDCl_3 ; δ ^1H = 1.94 and δ ^{13}C = 1.32 ppm for CD_3CN ; δ ^1H = 2.50 and δ ^{13}C = 39.5 ppm for DMSO-d_6). Both d(GT) and d(G^{PU}T) dimers were completely characterized and their ^1H and ^{13}C resonances were unambiguously assigned using homo- and heteronuclear correlations. HRMS were recorded on a Micromass Q-TOF spectrometer with an electrospray ionization (ESI) in negative or positive mode. MALDI-TOF MS studies were performed on an Axima Assurance spectrometer (Shimadzu Biotech). Spectra were recorded, in negative mode, using 2,4,6-trihydroxyacetophenone (THAP) with 10 % of ammonium citrate as a matrix in water CH_3CN , 1:1, v/v. Samples were mixed with the matrix as 1:1 or 1:5 v/v ratio and 1 μL of the solution was deposited on the stainless-steel plate for drying.

***N,O*-Diacetyl-L-tyrosine *N*-(6-hydroxyhexyl) amide (1):** To a solution of *N,O*-diacetyl-L-tyrosine (500 mg, 1.88 mmol) and EDC (427 mg, 2.6 mmol) in CH_2Cl_2 (10 mL) and DMF (1 mL), 6-amino-hexanol (242 mg, 2.07 mmol) was added and the reaction was stirred overnight. The solution was applied on a silica gel column and the compound **1** was eluted with methanol (up to 7%) in CH_2Cl_2 (580 mg, 85%). ^1H NMR (500 MHz, CDCl_3) δ 7.21 (d, J = 8.5 Hz, 2H, Ar), 6.99 (d, J = 8.5 Hz, 2H, Ar), 6.63 (bs, 1H, -NH-), 6.23 (bs, 1H, -NH-), 4.59 (q, J = 7.5 Hz, 1H, -CH-), 3.58 (t, J = 6.4 Hz, 2H, - CH_2O), 3.17-3.07 (m, 2H, - NCH_2 -), 3.05 & 2.98 (2xdd, J = 13.5, 8.4 and 6.2 Hz, 2x1H, - CH_2 -(Ph)), 2.28 (3H, CH_3CO), 1.96 (3H, CH_3CO -), 1.50 (quint., J = 7.0 Hz, 2H, - CH_2 -), 1.36 (m, 2H, - CH_2 -), 1.31 (m, 2H, - CH_2 -), 1.20 (m, 2H, - CH_2 -)

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) . ^{13}C $\{^1\text{H}\}$ NMR (126 MHz, CDCl_3) δ 170.9, 170.3, 169.8, 149.7, 134.5, 130.4, 121.8, 62.6, 54.8, 39.4, 38.1, 32.4, 29.1, 26.3, 25.2, 23.3, 21.3. HRMS (ESI⁺): calcd. for $\text{C}_{19}\text{H}_{29}\text{N}_2\text{O}_5$ [$\text{M} + \text{H}$]⁺ 365.2076; found 365.2086.

(*N,O*-Diacetyl-L-tyrosine amide hexyl)-(2-cyanoethyl)-*N*-diisopropyl-phosphoramidite

(Y1): To a solution of *N,O*-diacetyl-L-tyrosine *N*-(6-hydroxyhexyl) amide **1** (580 mg, 1.59 mmol) and DIEA (390 μL , 2.22 mmol) in dry CH_2Cl_2 (16 mL) and dry CH_3CN (4 mL) under argon, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (415 μL , 1.75 mmol) was added, and the mixture was stirred for 2 h. After addition of water (1 mL) and 15 min stirring, CH_2Cl_2 (50 mL) was added, and the solution was washed with a 0.2 M TEAAc pH 7 aqueous solution (100 mL). The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The crude residue was purified by silica gel chromatography [pyridine (10%) and EtOAc (up to 90%) in cyclohexane] to afford **Y1** (540 mg, 60%). ^1H NMR (400 MHz, CDCl_3) δ 7.19 (d, 2H, $J = 8.3$ Hz, H_{Ar}), 6.99 (d, 2H, $J = 8.3$ Hz, H_{Ar}), 6.39 (d, 1H, $J = 7.9$ Hz, NH-Ac), 5.98 (t, 1H, $J = 5.4$ Hz, NH-CO), 4.56 (q, $J = 7.0$ Hz, 1H, -CH-), 3.88 – 3.73 (m, 2H, - CH_2O -), 3.73 – 3.51 (m, 4H, - CH_2O - & $2\times\text{CH}_{\text{iPr}}$), 3.12 (m, 2H, - CH_2 -), 3.06– 2.94 (m, 2H, - CH_2 -), 2.63 (t, $J = 6.4$ Hz, 2H, - CH_2 -CN), 2.27 (s, 3H, CH_3 -Ac), 1.96 (s, 3H, CH_3 -Ac), 1.57 (quint., $J = 7.0$ Hz, 2H, - CH_2 -), 1.36 (m, 2H, - CH_2 -), 1.31 (m, 2H, - CH_2 -), 1.21 (m, 2H, - CH_2 -), 1.17 (d, 6H, $\text{CH}_{3\text{iPr}}$), 1.16 (d, 6H, $\text{CH}_{3\text{iPr}}$). ^{13}C $\{^1\text{H}\}$ NMR (101 MHz, CDCl_3) δ 170.7, 170.1, 169.5, 149.7, 134.5, 130.4, 121.8, 117.9, 63.7, 63.6, 58.4, 58.2, 54.8, 43.2, 43.0, 39.5, 38.2, 31.2, 31.1, 29.3, 26.6, 25.6, 24.8, 24.7, 24.6, 23.3, 21.2, 20.5, 20.4. ^{31}P $\{^1\text{H}\}$ NMR (162 MHz, CDCl_3) δ 147.25 and 147.24 ppm. HRMS (ESI⁺): calcd. for $\text{C}_{28}\text{H}_{48}\text{N}_4\text{O}_7\text{P}$ [$\text{M} + \text{H}_2\text{O} + \text{H}$]⁺ 583.3261; found 583.3262.

3-(4-Benzoyloxyphenyl)-propionic Acid (2): 3-(4-Hydroxyphenyl)-propionic acid (2.0 g, 12 mmol) was dissolved in 10 mL of icy water containing sodium hydroxide (960 mg, 24 mmol). Benzoyl chloride (1.5 mL, 13.2 mmol) was added dropwise over 5 min to the solution with an ice bath and the mixture was stirred for 15 min. Ice bath was removed and the mixture was stirred for another 15 min at rt. Ice-cold hydrochloric acid (0.12 M) was then added up to acidic pH. The resulting white solid was filtered off, washed with water, and recrystallized from ethanol (2.7 g, 83%). ^1H NMR (500 MHz, CD_3CN) δ 8.16 (dd, $J = 8.4$ and 1.3 Hz, 2H, H_{Ar}), 7.70 (tt, $J = 7.5$ Hz and 1.3 Hz, 1H, H_{Ar}), 7.57 (t, $J = 7.7$, 2H, H_{Ar}), 7.32 (d, $J = 8.7$, 2H, H_{Ar}), 7.15 (m, 2H, H_{Ar}), 2.92 (t, $J = 7.6$ Hz, 2H, CH_2), 2.63 (t, $J = 7.6$ Hz, 2H, CH_2). ^{13}C $\{^1\text{H}\}$ NMR (126 MHz, CD_3CN) δ 174.3, 166.2, 150.4, 139.8, 134.7, 130.8, 130.6, 130.4, 129.8, 122.7, 35.7, 30.7. HRMS (ESI⁺): calcd. for $\text{C}_{16}\text{H}_{15}\text{O}_4$ [$\text{M} + \text{H}$]⁺ 271.0965; found 271.0964.

1 ***N*-(6-Hydroxyhexyl)-3-(4-benzoyloxyphenyl)-propionamide (3)**: To a solution of 3-(4-
2 benzoyloxyphenyl)-propionic Acid **2** (1.5 g, 5.5 mmol) and *N*-hydroxysuccinimide (703 mg, 6
3 mmol) in dry DMF (15 mL) was added dicyclohexylcarbodiimide (1.24 g, 6 mmol). After 1.5
4 h, 6-amino-hexanol (702 mg, 6 mmol) was added and the reaction was stirred for 2 h. After
5 filtration, the solution was evaporated to dryness and the residue was dissolved in CH₂Cl₂ and
6 washed with water. The organic layer was dried (Na₂SO₄) and concentrated in vacuo. The crude
7 product was purified by silica gel chromatography (Methanol in CH₂Cl₂, 7%) to afford **2** as a
8 white solid (1.12 g, 55%). ¹H NMR (400 MHz, CDCl₃) δ 8.19 (dd, *J* = 8.5 and 1.3 Hz, 2H, H_{Ar}),
9 7.64 (tt, *J* = 7.5 and 1.3 Hz, 1H, H_{Ar}), 7.51 (t, *J* = 8.0 Hz, 2H, H_{Ar}), 7.25 (d, *J* = 8.5 Hz, 2H,
10 H_{Ar}), 7.12 (d, *J* = 8.5 Hz, 2H, H_{Ar}), 5.43 (bt, 1H), 3.62 (t, *J* = 6.5 Hz, 2H, OCH₂), 3.22 (q, *J* = 1
11 7.0 Hz, 2H, NCH₂), 2.99 (t, *J* = 7.5 Hz, 2H, CH₂), 2.46 (t, *J* = 7.6 Hz, 2H, CH₂), 1.65 (bs, 1H),
12 1.55 (quint., *J* = 7.0 Hz, 2H, CH₂), 1.45 (quint., *J* = 7.0 Hz, 2H, CH₂), 1.37 (m, 2H, CH₂), 1.29
13 (m, 2H, CH₂). ¹³C {¹H} NMR (101 MHz, CDCl₃) δ 172.0, 165.5, 149.5, 138.7, 133.8, 130.3,
14 129.7, 129.6, 128.7, 121.8, 62.8, 39.5, 38.7, 32.6, 31.3, 29.7, 26.6, 25.4. HRMS (ESI⁺): calcd.
15 for C₂₂H₂₈NO₄ [M + H]⁺ 370.2018; found 370.2027.

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31 **6-[[3-(4-benzoyloxyphenyl)-propionamido]-hexyl]-(2-cyanoethyl)-*N*-diisopropyl-**
32 **phosphoramidite (Y2)**: *N*-(6-Hydroxyhexyl)-3-(4-benzoyloxyphenyl)-propionamide (**3**, 1.12
33 g, 3 mmol) was added to a solution of DIEA (730 μL, 4.2 mmol) in dry CH₂Cl₂ (15 mL) under
34 argon. 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (780 μL, 3.3 mmol) was then
35 added, and the mixture was stirred for 2 h. After addition of water (1 mL) and 15 min stirring,
36 CH₂Cl₂ (50 mL) was added, and the solution was washed with saturated aqueous NaHCO₃. The
37 organic layer was dried (Na₂SO₄) and concentrated in vacuum. The crude residue was purified
38 by silica gel chromatography [NEt₃ (4%) and EtOAc (up to 50%) in cyclohexane] to afford **Y2**
39 (1.3 g, 76%). ¹H NMR (400 MHz, CDCl₃) δ 8.19 (dd, *J* = 8.5 and 1.3 Hz, 2H, H_{Ar}), 7.63 (tt, *J*
40 = 7.5 and 1.3 Hz, 1H, H_{Ar}), 7.50 (t, *J* = 7.8 Hz, 2H, H_{Ar}), 7.25 (d, *J* = 8.3 Hz, 2H, H_{Ar}), 7.12 (m,
41 2H, H_{Ar}), 5.48 (bt, 1H), 3.88-3.73 (m, 2H, -OCH₂-), 3.69-3.52 (m, 4H, -OCH₂- & 2xCH_iPr),
42 3.21 (q, *J* = 6.7 Hz, 2H, -NCH₂-), 2.98 (t, *J* = 7.7 Hz, 2H, -CH₂-), 2.62 (t, *J* = 6.4 Hz, 2H, -CH₂-
43), 2.46 (t, 2H, -CH₂-), 1.60 (quint., *J* = 7.0 Hz, 2H, -CH₂-), 1.46 (quint., *J* = 7.1 Hz, 2H, -CH₂-
44), 1.37 (m, 2H, -CH₂-), 1.30 (m, 2H, -CH₂-), 1.17 (d, *J* = 6.8 Hz, 6H, -CH_{3-i}Pr), 1.16 (d, *J* = 6.8
45 Hz, 6H, -CH_{3-i}Pr). ¹³C {¹H} NMR (101 MHz, CDCl₃) δ 172.0, 165.4, 149.4, 138.8, 133.7, 130.3,
46 129.7, 129.5, 128.7, 121.8, 117.9, 63.8, 63.6, 58.4, 58.2, 43.2, 43.0, 39.6, 38.7, 31.3, 31.2, 31.1,
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2 29.6, 26.7, 25.7, 24.8, 24.73, 24.66, 20.54, 20.47. ^{31}P { ^1H } NMR (162 MHz, CDCl_3) δ 147.25
3 ppm. HRMS (ESI $^+$): calcd. for $\text{C}_{31}\text{H}_{45}\text{N}_3\text{O}_5\text{P}$ [$\text{M} + \text{H}$] $^+$ 570.3068; found 570.3091.
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5 **Oligonucleotide synthesis:** All conventional reagents, solvents for DNA synthesis and
6 nucleoside phosphoramidites with benzoyl protection for dC and dA, and isobutyryl or tert-
7 butylphenoxyacetyl protection for dG were purchased from Link $^{\circledR}$ technologies, ChemGenes $^{\circledR}$
8 Corporation and Biosolve $^{\circledR}$ Chimie. The solid-supported synthesis of oligonucleotides was
9 performed on a 394 ABI DNA synthesizer starting from commercially available solid supports
10 with succinyl or Q-linker at 1 μmol scale according to standard phosphoramidite chemistry
11 protocols. The detritylation step was performed for 65 s using 3 % TCA in CH_2Cl_2 . For the
12 coupling step, benzylmercaptotetrazole (0.3 M in anhydrous CH_3CN) was used as the activator
13 with modified **Y1** and **Y2** phosphoramidites (0.1 M in CH_3CN , 60 sec coupling time) and
14 nucleoside phosphoramidites (0.1 M in CH_3CN , 30 sec coupling time). The capping step was
15 performed with phenoxyacetic anhydride using commercially available solutions (Cap A: acetic
16 anhydride:pyridine:THF 10:10:80 v/v/v; Cap B: 10 % *N*-methylimidazole in THF) for 60 sec.
17 The oxidation step was performed with a standard, diluted iodine solution (0.1 M I_2 ,
18 THF:pyridine:water 90:5:5, v/v/v) for 15 sec. Oligomers were deprotected and released from
19 the CPG support by treatment with a 30% aqueous ammonia for 4-6 h at 55 $^{\circ}\text{C}$.
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34 **General method for coupling with PTAD:** In solution: A 50 mM solution of phenyl-urazole
35 in CH_3CN was added a 50 mM solution of NBS or NCIS in CH_3CN to form the 25 mM deep
36 red solution of PTAD within 5-15 min. Oxidation by NCIS required more time up to 15 min.
37 To the oligonucleotide (50 nmol, 333 μM) in solution in 200 mM Tris HCl buffer pH 7.9 was
38 sequentially added the solution of PTAD 20 μL , 10 eq. every 10 min, five times. On solid
39 support: 0.5 μmol of solid-supported Y1-oligonucleotide was treated with a 50 mM K_2CO_3
40 methanol solution for 15 min at rt. After filtration and wash with methanol and CH_2Cl_2 , CPG
41 beads were dried. PTAD solution obtained by NCIS oxidation (25 mM, 100 μL) was added 7
42 times every 30 min to the solid-supported Y1-12mer in Tris HCl buffer pH 7.9 (100 μL). After
43 3 h 30, the beads were filtered and washed with CH_3CN and finally treated with concentrated
44 aqueous ammonia for 5 h at 55 $^{\circ}\text{C}$ affording PU-Y1-12mer (84% spectrometric purity).
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56 **Synthesis of d(GT):** Deoxyguanosine phosphoramidite (500 mg, 0.59 mmol) and 3'-levulinyl-
57 thymidine (200 mg, 0.59 mmol) were dried by co-evaporation with dry acetonitrile (2 x 20 mL).
58 The residue was dissolved in dry acetonitrile (12 mL) and benzylthiotetrazole (283 mg, 2.5
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1 mmol) was added. The mixture was stirred for 2 h and water (0.5 mL) was added, after 5 min
2 solid supported IO_4^- (500 mg, 1.25 mmol)^[53] was added. After 2h, the solution was filtered off
3 and diluted with CH_2Cl_2 (100 mL) and washed with a saturated aqueous solution of $\text{Na}_2\text{S}_2\text{O}_3$
4 and water. Organic layer was dried (Na_2SO_4) and evaporated affording a syrup. After
5 solubilisation in $\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}$ (18 mL, 6:3, v/v), 2 mL of a 10% benzene sulfonic acid
6 solution in $\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}$ (6:3, v/v) were added. After 20 min the reaction was quenched by
7 addition of polyvinylpyridine (1g, 8 mmol). After filtration the solution was evaporated to
8 dryness and the dimer was purified by silica gel chromatography for 2 to 10 % of methanol in
9 CH_2Cl_2 affording 370 mg, 79% of protected dimer with free 5'-OH function.

10 **Deprotection:** The partially protected dimer (370 mg, 0.47 mmol) was dissolved in methanol
11 (4 mL) and concentrated aqueous ammonia (10 mL) was added. The solution was stirred
12 overnight at rt and evaporated. The dimer was purified by flash chromatography on C18 reverse
13 phase column (4 g) from 1% to 15% CH_3CN in 30 mM TEAAC buffer pH 7 over 20 min.
14 TEAAC was removed by coevaporation and lyophilization. Triethylammonium ions were
15 exchanged to sodium by elution on DOWEX 50 W X8 Na^+ form affording 225 mg, 81% of
16 d(GT). ^1H (600 MHz ; 298K, DMSO-d_6): 11.89 (bs, 2H, $\text{NH}_{1\text{G}}$ & $\text{NH}_{3\text{T}}$), 7.85 (s, 1H, $\text{H}_{8\text{G}}$), 7.80
17 (bq, 1H, $\text{H}_{6\text{T}}$), 7.27 (bs, 2H, $\text{NH}_{2\text{G}}$), 6.20 (dd, 1H, $^3\text{J}_{1'2'} = 7.7$ Hz; $^3\text{J}_{1'2''} = 6.2$ Hz, $\text{H}_{1'\text{T}}$), 6.10
18 (dd, 1H, $^3\text{J}_{1'2'} = 8.5$ Hz; $^3\text{J}_{1'2''} = 5.8$ Hz, $\text{H}_{1'\text{G}}$), 5.60 (bs, 2H, $\text{OH}_{3'\text{T}}$ & $\text{OH}_{5'\text{G}}$), 4.70 (m, 1H, $\text{H}_{3'\text{G}}$),
19 4.30 (m, 1H, $\text{H}_{3'\text{T}}$), 4.00 (m, 1H, $\text{H}_{4'\text{G}}$), 3.86 (m, 1H, $\text{H}_{4'\text{T}}$), 3.83 (m; 2H, $\text{H}_{5'\text{T}}$ & $\text{H}_{5''\text{T}}$), 3.52 (m,
20 2H, $\text{H}_{5'\text{G}}$ & $\text{H}_{5''\text{G}}$), 2.52 (m, 1H, $\text{H}_{2'\text{G}}$), 2.38 (m, 1H, $\text{H}_{2''\text{G}}$), 2.12 (ddd, 1H, $^2\text{J}_{2'2''} = 13.2$ Hz, $^3\text{J}_{3'2'}$
21 = 6.1 Hz, $^3\text{J}_{1'2'} = 7.8$ Hz, $\text{H}_{2'\text{T}}$), 2.03 (ddd, 1H, $^2\text{J}_{2'2''} = 13.2$ Hz, $^3\text{J}_{3'2''} = 3.0$ Hz, $^3\text{J}_{1'2''} = 6.2$ Hz,
22 $\text{H}_{2''\text{T}}$), 1.80 (d, 3H, $^4\text{J}_{67} = 1.0$ Hz, $\text{H}_{7\text{T}}$). ^{13}C (125 MHz ; 298K, DMSO-d_6): 164.1 ($\text{C}_{4\text{T}}$, CO),
23 158.4 ($\text{C}_{6\text{G}}$, CO), 155.2 ($\text{C}_{2\text{G}}$, Cq), 151.1 ($\text{C}_{4\text{G}}$, Cq), 150.7 ($\text{C}_{2\text{T}}$, CO), 136.4 ($\text{C}_{6\text{T}}$, CH), 134.7
24 ($\text{C}_{8\text{G}}$, CH), 116.6 ($\text{C}_{5\text{G}}$, Cq), 109.9 ($\text{C}_{5\text{T}}$, Cq), 86.4 ($\text{C}_{4'\text{G}}$, CH, $^3\text{J}_{4'\text{P}} = 4.2$ Hz), 86.0 ($\text{C}_{4'\text{T}}$, CH,
25 $^3\text{J}_{4'\text{P}} = 7.5$ Hz), 83.8 ($\text{C}_{1'\text{T}}$, CH), 82.6 ($\text{C}_{1'\text{G}}$, CH), 75.1 ($\text{C}_{3'\text{G}}$, CH, $^2\text{J}_{3'\text{P}} = 4.5$ Hz), 70.9 ($\text{C}_{3'\text{T}}$,
26 CH), 64.6 ($\text{C}_{5'\text{T}}$, CH_2 , $^2\text{J}_{5'\text{P}} = 4.8$ Hz), 61.9 ($\text{C}_{5'\text{G}}$, CH_2), 39.3 ($\text{C}_{2'\text{T}}$, CH_2), 38.6 ($\text{C}_{2'\text{G}}$, CH_2), 12.2
27 ($\text{C}_{7\text{T}}$, CH_3). HRMS (ESI): calcd. for $\text{C}_{20}\text{H}_{25}\text{N}_7\text{O}_{11}\text{P}$ [M - H]⁻ 570.1355; found 570.1356.

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51 **Reaction of PTAD on d(GT):** One mL of 50 mM phenyl-urazole in CH_3CN and 1 mL of 50
52 mM NCIS in CH_3CN were mixed. After 15 min, the red solution (2 mL) was added to a solution
53 of dimer d(GT) (10 mg, 17.5 μmol) in 1 mL of 400 mM Tris HCl buffer pH 7.9. After 1h, the
54 solution was extracted with ethyl acetate and the aqueous layer was evaporated. The dimer
55 adduct was purified by HPLC on a DNAPac PA200 column from Thermo Scientific (quaternary
56 amine functionalized NanoBeads) using 25 mM Tris HCl buffer with 5% CH_3CN with an
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increasing concentration of 400 mM NaClO₄. The pure dimer was desalted on C₁₈ reverse phase column using TEAAC buffer pH 7. After removal of TEAAC by multiple coevaporations and lyophilization, triethylammonium salts were exchanged to Na⁺ by elution on DOWEX 50 W X8 sodium form affording 3.1 mg 24% of d(G^{Pu}T).

¹H (600 MHz ; 298K, DMSO-d₆): 11.16 & 10.90 (bs, 2x1H, NH_G & NH_T), 7.82 (q, 1H, ³J₆₋₇ = 1 Hz, H_{6T}), 7.57 (dd, 2H, ³J₁₃₋₁₄ = 8.4 Hz, ⁴J₁₃₋₁₅ = 1.1 Hz, H_{13T}), 7.38 (t, 2H, ³J₁₃₋₁₄ ~ ³J₁₄₋₁₅ = 7.4 Hz, H_{14T}), 7.22 (tt, 1H, ³J₁₄₋₁₅ = 7.4 Hz, ⁴J₁₃₋₁₅ = 1.1 Hz, H_{15T}), 6.28 (bs, 2H, NH_{2-G}), 6.19 (dd, 1H, ³J_{1'2'} = 8.4 Hz; ³J_{1'2''} = 6.1 Hz, H_{1'G}), 6.18 (dd, 1H, ³J_{1'2'} = 8.0 Hz; ³J_{1'2''} = 6.1 Hz, H_{1'T}), 5.80 (d, 1H, ³J_{3'-OH3'} = 3.8 Hz, OH_{3'}), 5.70 (bs, 1H, OH_{5'G}), 4.71 (m, 1H, H_{3'G}), 4.29 (m, 1H, H_{3'T}), 3.98 (m, 1H, H_{4'G}), 3.83 (m, 1H, H_{4'T}), 3.82 & 3.79 (m, 2x1H, H_{5'T} & H_{5''T}), 3.58 & 3.47 (m, 2x1H, H_{5'G} & H_{5''G}), 3.05 (m, 1H, H_{2'G}), 2.28 (ddd, 1H, ²J_{2'2''} = 13.0 Hz, ³J_{3'2''} = 1.9 Hz, ³J_{1'2''} = 6.0 Hz, H_{2''G}), 2.10 (ddd, 1H, ²J_{2'2''} = 13.1 Hz, ³J_{3'2'} = 6.0 Hz, ³J_{1'2'} = 8.0 Hz, H_{2'T}), 2.03 (ddd, 1H, ²J_{2'2''} = 13.1 Hz, ³J_{3'2''} = 2.9 Hz, ³J_{1'2''} = 6.1 Hz, H_{2''T}), 1.80 (d, 3H, ⁴J₆₇ = 1.1 Hz, H_{7T}). ¹³C (150 MHz ; 298K, DMSO-d₆): 163.8 (C_{4T}, CO), 154.7 (C_{6G}, CO), 152.1 (C_{2G}, Cq), 150.5 (C_{2T}, CO), 150.1 (C_{4G}, Cq), 138.0 (C_{8G}, Cq), 136.4 (C_{6T}, CH), 135.3 (C₁₂, Cq), 128.0 (C₁₄, CH), 125.3 (C₁₅, CH), 125.2 (C₁₃, CH), 114.7 (C_{5G}, Cq), 109.7 (C_{5T}, Cq), 86.4 (C_{4'G}, CH, ³J_{4'-P} = 3.7 Hz), 86.1 (C_{4'T}, CH, ³J_{4'-P} = 7.2 Hz), 84.0 (C_{1'G}, CH), 83.7 (C_{1'T}, CH), 75.5 (C_{3'G}, CH, ²J_{3'-P} = 4.3 Hz), 71.0 (C_{3'T}, CH), 64.7 (C_{5'T}, CH₂, ²J_{5'-P} = 5.1 Hz), 62.4 (C_{5'G}, CH₂), 39.0 (C_{2'T}, CH₂), 36.1 (C_{2'G}, CH₂), 12.0 (C_{7T}, CH₃). HRMS (ESI⁻): calcd. for C₂₈H₃₀N₁₀O₁₃P [M - H]⁻ 745.1737; found 745.1748.

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Bio



In 1982, Albert Meyer joined the French National Center of scientific Research (CNRS) to work at the University of Montpellier (France) in the laboratory of Prof. Jean-Louis Imbach. He is Chemical Engineer, graduated from CNAM in organic chemistry in 1998. In 2007, he was a visiting scientist at Caruthers laboratory (CO, USA) to obtain new skills in mass spectrometry around the construction of DNA and RNA. This research lies various aspects of nucleic acid chemistry focusing notably on the design of chemically modified DNAs and their conjugates for therapeutic and diagnostic applications. This year, he received the Cristal award from CNRS.



Carine Baraguey was educated at the Paris VI University (nowadays Sorbonne University, France). She received her Ph.D. in 1999, under the supervision of Prof. B. Bodo from the National Museum of Natural History. In 1999, she obtained a European fellowship and worked on the molecular dynamics of carbohydrates by ^{13}C NMR in the team of Prof. M. Zeidler of the Rheinisch-Westfälische Technische Hochschule in Aachen (Germany). Interested in molecular modeling of biomolecules, she joined the Prof. A. Xavier's group at the Institute of Chemical and Biological Technology of the University of Lisbon (Portugal) from 2000 to 2002. Back in France, she became contractual Assistant Professor in the Structure and Function of Bioactive Molecules Laboratory directed by Dr G. Chassaing (Paris VI University) in 2003 and in the Structure and Recognition of Biomolecules Laboratory directed by Prof. F. Toma (Evry) in 2004. Since 2005, she is Assistant Professor in the ChemBioNAC team of the Institute of Biomolecules Max Mousseron in Montpellier where she brings her expertise in the field of the nuclear magnetic resonance spectroscopy.



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