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Biotin-phenyldiazomethane conjugates as labeling reagents at phosphate in mono and polynucleotides

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Abstract—Molecules **2**–**5** that include in their structure a biotin moiety as detectable unit and differently substituted phenyl diazo functions as reactive group were prepared as reagents for labeling the phosphate group in mono and polynucleotides. These molecules were shown to react selectively and quantitatively with the model nucleotide 3′-UMP. They were used successfully in the labeling step of DNA and RNA analysis using high-density DNA-chips (or microarrays) technology.

1. Introduction

Labeling of nucleotides, oligonucleotides, and nucleic acids is essential in many applications, notably in nucleic acid based technologies. Most described methods involve labeling reagents of electrophilic nature, halides, sulfonates, isocyanates, isothiocyanates, metal (platinum) derivatives… that react essentially at the nucleophilic sites of the nucleic bases.[1–5] This generally leads to modifications in the hybridization properties of the labeled bases, which constitutes severe limitation for many applications, for example, those using DNA-chips (or microarrays) requiring a high specificity level. Labeling at the phosphate represents an attractive alternative to avoid this problem. However selective reaction at phosphate is poorly documented, due essentially to the weak nucleophilicity of such a group.[6–14] In the course of a program aimed at devising new labeling reagents for DNA-chip analysis,[15–18] an application in which it is of utmost importance to preserve the hybridization properties of the labeled nucleic acid targets, we became interested in diazo compounds by analogy with their use for alkylation of carboxylate groups.[19]

We recently explored the use of diazo chemistry to alkylate the phosphate group. We showed notably that pyrenyldiazomethane, a reagent described for derivatizing carboxylic acids, is a reagent of choice to alkylate selectively the phosphate group in nucleotide monophosphates. Thus 3′-UMP, 3′-CMP, 3′-AMP, 3′-GMP, 3′-TMP, as well as the 2′- or 5′- analogs 2′-UMP and 5′-UMP were selectively and quantitatively converted to the corresponding pyrenyl phosphate esters by treatment with pyrenyldiazomethane in buffered borate aqueous solutions. No reaction at the nucleophilic sites of the nucleic bases could be detected.[20] These observations seemed of great interest to introduce labels or reporter molecules at phosphate in polynucleotides. Molecule *m*-BioPMDAM **1** that includes in its structure the biotin moiety as detectable unit and the diazo function as reactive group was thus designed and shown to react selectively and efficiently with phosphate in nucleotide monomers, oligonucleotides, DNA, and RNA. The molecule fulfills the requirements for hybridization-based analysis and especially for detection on high-density DNA chips.[21,22] In order to evaluate further the possible effects of substitution on the properties of the newly designed label *m*-BioPMDAM in terms of reactivity, selectivity, stability, and water solubility… notably for applications using DNA-chip technology we prepared analogs **2**–**5**. Molecules *o*-BioPMDAM **2** and *p*-BioPMDAM **3** are the *ortho*- and *para*-isomers of *m*-BioPMDAM **1**. *m*-BioDPDAM **4** was designed to study the effect of phenyl substitution. We had previously seen that replacement of the methyl group by hydrogen led to instability of the diazo function.[20] The

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stabilizing effect of a strong electron-withdrawing group was thus evaluated with the nitro-substituted analog BioNPDAM **5**. In the present paper we describe the synthesis of molecules **2**–**5**, we show that they react selectively at phosphate with the model nucleotide phosphate 3′-UMP with significant differences in the reaction kinetics. All give positive results using amplified nucleic acid targets and DNA-chips analysis.

2. Results and discussion

2.1. Synthesis of the labeling molecules 2–5

o-BioPMDAM **2**, *p*-BioPMDAM **3**, and *m*-BioDPDAM **4** were prepared using the same synthetic route (Scheme 1) by coupling D-biotin with the respective commercial aminoketones in DMF using isobutyl chloroformate and *N*-methylmorpholine as condensing agent (yield: 50–70%). The resulting ketones were transformed into the hydrazones, by warming with hydrazine monohydrate in ethanol, in 80–90% yields. Oxidation of the hydrazones into the diazo compounds proved quite delicate. The reaction was best achieved by stirring the hydrazones with MnO_2 at room temperature for a short period (10–15 min). Poor results were observed when using AgO,[23] $BaMnO_4$,[24] or NiO_2[25] as oxidizing agents in different solvents. The diazo compounds, as pink powders, were characterized notably by their ^1H and ^{13}C NMR spectra. They exhibited a strong characteristic band at 2038 cm^{-1} in the IR spectra (see e.g., NMR and IR spectra of *o*-BioPMDAM in Fig. 1).

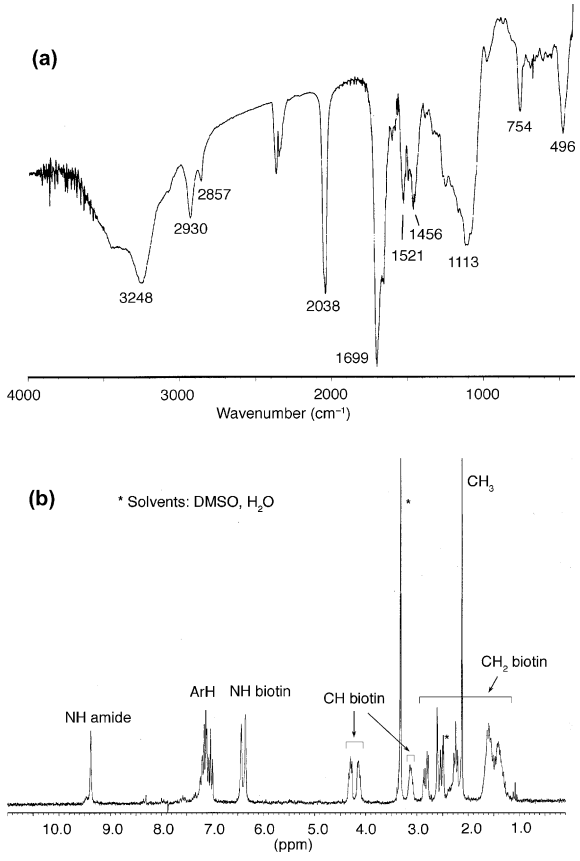
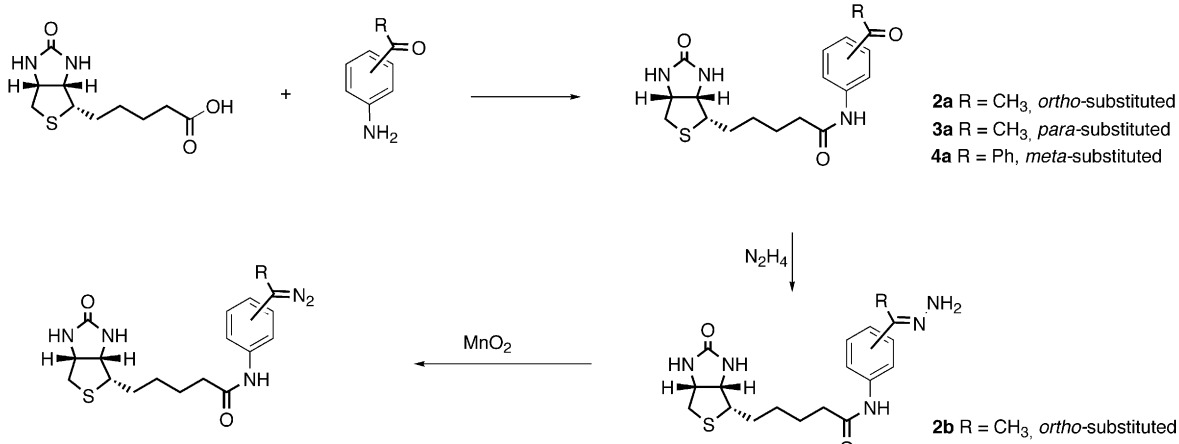


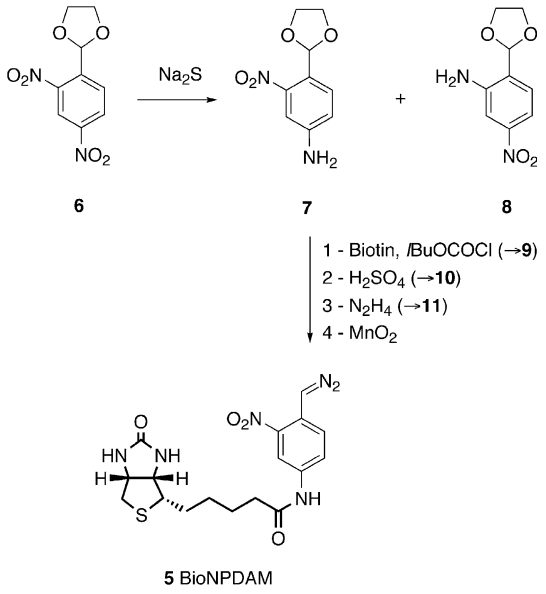
Figure 1. IR (a) and ^1H NMR (b) 200 MHz, in DMSO-d_6 spectra of *o*-BioPMDAM **2**.

The nitro analog BioNPDAM **5** was obtained (Scheme 2) starting from 2,4-dinitrobenzaldehyde ethylene acetal **6**. Controlled reduction with sodium sulfide in ethanol gave a mixture of the two mono reduced derivatives from which the major 2-nitro-4-aminoacetal **7** was isolated and purified in 45% yield. The minor 2-amino-4-nitro derivative **8** was obtained in 9% yield. The two isomers were characterized by their ^1H NMR spectrum. The 2-nitro-4-aminoethylene acetal **7** was coupled to D-biotin and the acetal function was hydrolyzed with dilute sulfuric acid (90% yield). Hydrazonation followed by MnO_2 oxidation gave the diazo compound BioNPDAM **5** characterized as previously by the NMR and IR data.[21,22]



1 *m*-BioPMDAM, R = CH_3, *meta*-substituted
2 *o*-BioPMDAM, R = CH_3, *ortho*-substituted
3 *p*-BioPMDAM, R = CH_3, *para*-substituted
4 *m*-BioDPDAM, R = Ph, *meta*-substituted

Scheme 1. Synthesis of the labeling molecules **2**–**4**.



Scheme 2. Synthesis of the labeling molecule BioNPDAM **5**.

Simple diazo alkanes are unstable compounds while aryl substitution generally increases the stability. It was thus necessary to test the stability of the new diazo compounds. Stability was evaluated in solution by monitoring the ^{1}H NMR spectra of **2**–**5** as a function of time in DMSO-d_6 at room temperature from which the half-lives for decomposition were determined. They average to 3 days for molecules **1**, **2**, and **5**. Molecule **3** is slightly less stable with a half-life evaluated to 1 day.

2.2. Reactivity with the model nucleotide 3′-UMP

We have reported that the marker *m*-BioPMDAM **1** reacts with nucleotides to form unique adduct corresponding to alkylation at the phosphate.[21,22] This was observed with all nucleotides studied, independently of the nature of the base and of the position of the phosphate group. The same observations had been done for reaction of the pyrenyldiazomethane molecule.[20] Using 3′-UMP as representative nucleotide, capillary electrophoresis had proved to be the method of choice to monitor the reaction. We used the same methodology to examine the reaction of the new labeling molecules **2**–**5**. Reactions were run in an homogenous solvents mixture (DMSO–CH_3CN–H_2O) to dissolve both the hydrophilic 3′-UMP nucleotide and the lipophilic diazo reagents, at pH 7.3 using borate buffer, at 60 °C. The reaction profiles were quite comparable for the four labels **2**–**5**. One very major (if not unique) peak appeared in the electropherograms with concomitant disappearance of the nucleotide peak (Fig. 2). The reaction products corresponding to the observed peaks were isolated in the case of the *o*- and *p*-BioPMDAM reagents. The ^{1}H NMR spectra (Fig. 3) of corresponding adducts **12** and **13** exhibited all peaks corresponding to the indicated structures with alkylation at phosphate, notably a multiplet at 5.4 ppm corresponding to the CH proton vicinal to the phosphate. The presence of two diastereoisomers in a 1/1 ratio was indicated by the splitting of the sugar protons. These data are quite comparable to those reported for the adduct formed in the reaction with the *meta*-isomer *m*-BioPMDAM **1**.[21,22]

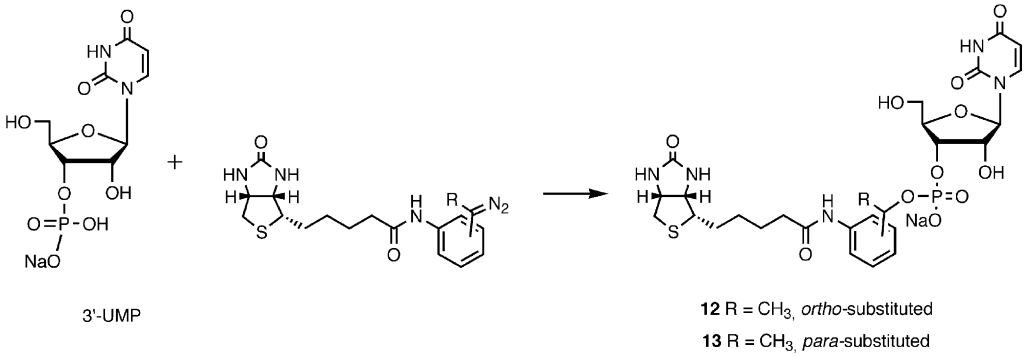
Capillary electrophoresis also allowed comparison of the kinetics of the reactions with the different diazo molecules. Half-lives for disappearance of the UMP nucleotide in identical reaction conditions as indicated could be evaluated from the evolution of the peaks as a function of time. Quite different velocities were measured for the different labels (Table 1). Taking the already reported *m*-BioPMDAM label **1** as reference, it appears that replacing the methyl substituent by phenyl (in DPDAM **4**) or introducing the electroattracting nitro group (in BioNPDAM **5**) decreases the reaction kinetics. The *ortho* substituted molecule *o*-BioPMDAM **2** exhibits unexpected high reactivity compared to the *para*-isomer *p*-BioPMDAM (this acceleration could hypothetically be due to rapid internal proton transfer from the NH amido group to the neighboring diazo carbon atom C=N_2 in the first step of the alkylation reaction).[26]

2.3. Hydrolytic stability of adducts

In the course of studies on DNA-chips aimed at evaluating the efficiency of molecules **2**–**5** to label RNA targets (see infra), we noticed that the signals measured on the chip exhibited intensity decrease with time. This phenomenon was variable depending on the structure of the labeling molecule. Decrease was particularly rapid for the *o*- and *p*-BioPMDAM labels **2** and **3**. This was not observed with DNA targets, which suggests hydrolysis of the RNA adducts assisted by participation of the 2′-OH with loss of the biotin unit. We thus studied hydrolysis in basic conditions of the UMP-adducts formed with *ortho*-, *meta*-, *para*-BioPMDAM, and *meta*-BioDPDAM. Capillary electrophoresis appeared again as a method of choice to monitor the reaction by measuring the kinetics for disappearance of the adducts. The half-lives for hydrolysis in 0.2 M aqueous NaOH are given in Table 2. The hydrolytic stability of the *m*-BioPMDAM adduct is clearly higher than the *ortho*- and *para*-isomers. These observations could account for the instability of labeled RNA fragments for their chip analysis.

2.4. DNA and RNA labeling

DNA and RNA labeling by molecules **2**–**5** was examined using targets generated by enzymatic amplification, PCR and post-PCR in vitro transcription, of 16S rDNA hypervariable region of *Mycobacterium tuberculosis* (*Mtb*, 202 nt).[27] Following the procedure previously used to test the labeling efficiency of *m*-BioPMDAM, the amplified targets were reacted with the diazo reagents **2**–**5** and fragmented prior to their hybridization to the *Mycobacterium* DNA-chip. Cleavage of labeled DNA and RNA targets into smaller fragments is a prerequisite for uniformity and hybridization specificity on the DNA-chip. RNA targets were cleaved with Mn^{2+} and imidazole. The DNA targets were fragmented by acidic treatment that promoted random depurination



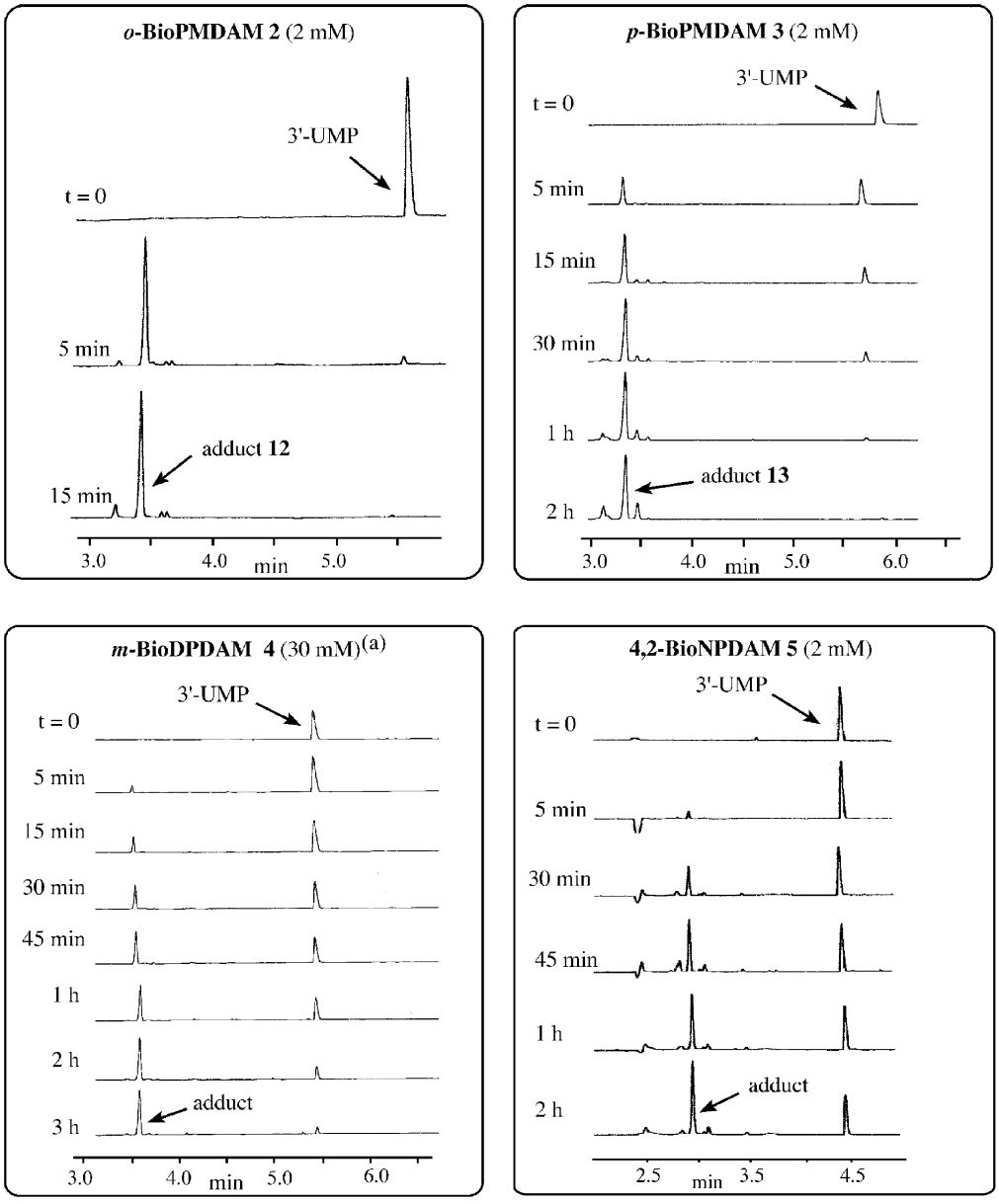


Figure 2. Adduct formation between 3′-UMP (0.04 mM) and labels **2**–**5** (concentrations as indicated) in DMSO–CH_3CN–H_2O (1:3:1), H_3BO_3 (2 mM) at 60 °C monitored by capillary electrophoresis. (a) Only traces of adduct are detected when 4 is used at 2 mM concentration.

and cleavage. After hybridization on DNA-chip, DNA and RNA fragments were stained with streptavidin bearing fluorescent R-phycoerythrin.[21] The chip results (Table 3) indicate that the two target types, the DNA amplicons and the RNA transcripts were successfully labeled. The signal intensities (rfu) were strong with signal/background ratios high. With the exception of diphenyl diazomethane **4**, base-calling values, that

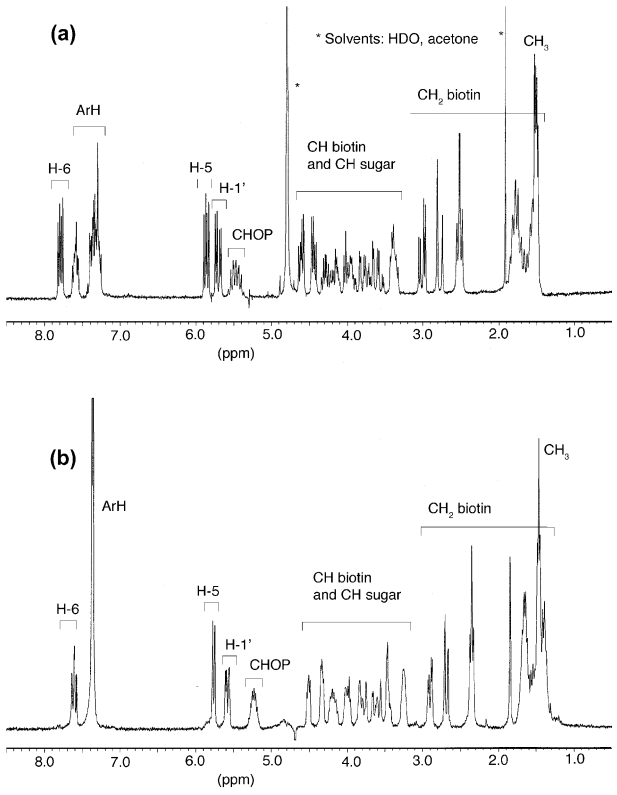


Figure 3. ^{1}H NMR spectra (in D_2O) of adducts **12** (a, 200 MHz) and **13** (b, 300 MHz, HDO peak suppressed by PRESAT), formed between 3′-UMP and, respectively, *o*-BioPMDAM **2** and *p*-BioPMDAM **3**.

Table 1. Kinetics for adduct formation between labeling molecules **1**–**5** and 3′-UMP

Labeling molecule	$t_{1/2}$
m-BioPMDAM **1**	15 min
o-BioPMDAM **2**	≪5 min
p-BioPMDAM **3**	5 min
m-BioDPDAM **4**	≫2 h
BioNPDAM **5**	45 min

Half-lives for transformation of 3′-UMP (0.04 mM) in the presence of labels (2 mM), H_3BO_3 (2 mM) in DMSO-CH_3CN-H_2O (1:3:1) at 60 °C.

Table 2. Hydrolysis of adducts formed between 3′-UMP and labels **1**–**4**

Labeling molecule	$t_{1/2}$
m-BioPMDAM **1**	198 min
o-BioPMDAM **2**	30 min
p-BioPMDAM **3**	8 min
m-BioDPDAM **4**	54 min

Half-lives for disappearance of adducts (1.6 mM) in aqueous NaOH (200 mM) at 60 °C.

measure the percent homology between the experimentally derived sequence and the 202 nt sequence tiled on the chip were superior or equal to 95% for all four molecules. This latter observation indicates that the hybridization properties of labeled targets have not been altered by the presence of the label. The low BC (%) value observed with diphenyl compound **4** is probably due to the steric hindrance of such a structure affecting the hybridization properties. It is also to be noted that the signal intensities are in all cases too high to be solely attributed to the labeling of the terminal phosphate generated during the cleavage treatment. As already reported for *m*-BioPMDAM these two observations, the signal intensities and the base-calling values, are strong indication that labeling also occurred at the internucleotidic phosphates.[28]

Table 3. Chip analysis of DNA amplicons labeled with molecules **1**–**5**

Labeling molecule	BC (%)[a]	Median intensity (rfu)	Signal/ background
m-BioPMDAM **1**	97	22,960	39.5
o-BioPMDAM **2**	97	25,951	31.6
p-BioPMDAM **3**	95	43,785	36.3
m-BioDPDAM **4**	93	32,359	9.1
BioNPDAM **5**	100	24,392	27.1

[a] In this model study, BC (%) ⩾ 95% correspond to a correct identification of DNA target.

3. Conclusion

All these results show that diazo chemistry opens the route to the design of new, versatile reagents for labeling of nucleotides, oligonucleotides, and nucleic acids at phosphate. Depending on the application, target of interest (DNA or RNA), detection format (high complexity or low complexity chip), ...the reactivity of the reagents can be modulated by introducing structural modifications to the diazo moiety. The stability of labeled fragments can be controlled as well. The nature of the label can be easily varied following the indicated scheme, and such physical properties as water solubility... can be controlled by varying the nature and/or the length of the linking chain between the diazo group and the detectable moiety. The improvement of the stability in water of these labels is also a major goal. Work is in progress in the design of new reagents that fulfill the solubility and stability requirements.

4. Experimental

4.1. General remarks

All commercially available chemical reagents were used without purification unless otherwise indicated. TLC: Merck Kieselgel 60 F_{254}, layer thickness 0.25 mm. Visualization by UV light (254 nm) or by phosphomolybdic acid solution. Preparative column chromatographies: Macherey-Nagel Kieselgel, 230–400 mesh. Mp: Reichert Thermovar (uncorrected). IR: Nicolet Impact 400. UV/Vis: Varian Cary 400 scan. NMR: Bruker AC 200 Avance 300 and Varian U+500 spectrometers. NMR spectra were referenced to the residual solvent peak, chemical shifts δ in ppm, apparent scalar coupling constants J in Hz. MS: Esquire 3000+ (ES-MS) and Thermofinningan Polaris Q (DCI and EI)—Elemental analysis were performed by 'Service central de microanalyse du CNRS'.

4.2. 2-(*N*-Biotinoylamino)acetophenone (2a)

To a solution of D-biotin (1.0 g, 4.1 mmol) in dry DMF (45 mL) cooled at 0 °C under argon, were added successively *N*-methylmorpholine (590 μL, 5.33 mmol) and isobutyl chloroformate (840 μL, 6.60 mmol). The solution was stirred for 30 min, and then 2-aminobenzophenone (824 mg, 6.10 mmol) was added. The solution was stirred at room temperature for 3.5 h and then the solvent was removed in vacuo. The residue was triturated with cold water (50 mL). The resulting precipitate was filtered, washed with water, and recrystallized from MeOH to give **2a** (1.1 g, 72%) as a white powder. Mp 105–110 °C. IR (KBr): 3248, 2930, 2857, 1700, 1670, 1652, 1582, 1528, 1448, 1354, 1310, 1245, 1165, 962, 860, 759, 606 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ = 11.24 (br s, 1H), 8.33 (d, J = 7.9 Hz, 1H), 7.97 (d, 2H, J = 6.9 Hz), 7.57 (t, J = 7.7 Hz, 1H), 7.18 (t, J = 7.0 Hz, 1H), 6.44 (br s, 1H), 6.35 (br s, 1H), 4.30 (m, 1H), 4.14 (m, 1H), 3.12 (m, 1H), 2.80 (dd, J = 5.0, 12.5 Hz, 1H), 2.61 (s, 3H), 2.55 (d, J = 12.5 Hz, 1H), 2.37 (t, J = 7.2 Hz, 2H), 1.67–1.30 (m, 6H). MS-DCI (NH_3/isobutane): m/z = 361.9 $[M+H]^+$. $C_{18}H_{23}N_3O_3S$–$0.5H_2O$ (370.5): calcd C 58.36, H 6.53, N 11.34; found C 58.29, H 6.54, N 11.25.

4.3. 2-(*N*-Biotinoylamino)acetophenone hydrazone (2b)

To a solution of the ketone **2a** (500 mg, 1.38 mmol) in absolute ethanol (8 mL), hydrazine monohydrate (572 μL, 11.1 mmol) was added. The solution was refluxed for 50 min and then solvents were evaporated. The residue was suspended in water, filtered, washed with water and with ether, and dried in vacuo to give **2b** (416 mg, 80%). Mp 156–158 °C. IR (KBr): 3412, 3241, 2931, 2909, 2857, 1706, 1677, 1590, 1532, 1444, 1303, 1270, 1169, 1114, 751, 643, 606 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ = 11.97 (br s, 1H), 8.35 (d, J = 7.9 Hz, 1H), 7.45 (d, J = 6.9 Hz, 1H), 7.19 (t, J = 7.7 Hz, 1H), 7.04 (t, J = 7.2 Hz, 1H), 6.61 (s, 2H), 6.42 (br s, 1H), 6.35 (br s, 1H), 4.32 (m, 1H), 4.14 (m, 1H), 3.12 (m, 1H), 2.81 (dd, J = 5.0, 12.5 Hz, 1H), 2.56 (d, J = 12.5 Hz, 1H), 2.31 (t, J = 7.4 Hz, 2H), 2.09 (s, 3H), 1.72–1.30 (m, 6H). MS-DCI (NH_3/isobutane): m/z = 376.1 $[M+H]^+$. $C_{18}H_{25}N_5O_2S$–$0.5H_2O$ (384.5): calcd C 56.23, H 6.82, N 18.21; found C 56.12, H 6.77, N 18.09.

4.4. 2-(*N*-Biotinoylamino)phenylmethyldiazomethane (2)

To a solution of hydrazone **2b** (200 mg, 0.53 mmol) in DMF (10 mL), MnO_2 (800 mg) was added and the suspension was stirred for 15 min at room temperature. The mixture was suction filtered through Celite and molecular sieves 3 Å (powder, 0.5 cm thickness). After evaporation in vacuo (0.5 mmHg, T < 40 °C), the oily residue was triturated with ether to afford **2** (130 mg) as a pink powder. IR (KBr): 3248, 2930, 2857, 2038, 1699, 1521, 1456, 1113, 754, 469 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ = 9.37 (br s, 1H), 7.26–7.00 (m, 4H), 6.43 (br s, 1H), 6.35 (br s, 1H), 4.30 (m, 1H), 4.15 (m, 1H), 3.12 (m, 1H), 2.82 (dd, J = 5.0, 12.5 Hz, 1H), 2.54 (d, J = 12.5 Hz, 1H), 2.24 (t, J = 7.2 Hz, 2H), 2.12 (s, 3H), 1.70–1.29 (m, 6H).

4.5. 4-(*N*-Biotinoylamino)acetophenone (3a)

The compound **3a** was prepared by the same procedure as described for **2a** starting from D-biotin (1.0 g, 4.1 mmol) and 4-aminoacetophenone (824 mg, 6.10 mmol). Yield: 888 mg (60%). Mp 235–236 °C. IR (KBr): 1706, 1688, 1667, 1608, 1589, 1526, 1461, 1401, 1276, 1262, 1180, 833, 700, 680, 595 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ = 10.18 (br s, 1H), 7.89 (d, J = 8.7 Hz, 2H), 7.69 (d, J = 8.8 Hz, 2H), 6.40 (br s, 1H), 6.33 (br s, 1H), 4.27 (m, 1H), 4.11 (m, 1H), 3.11 (m, 1H), 2.80 (dd, J = 5.0, 12.3 Hz, 1H), 2.55 (d, J = 12.3 Hz, 1H), 2.50 (s, 3H), 2.35 (t, J = 7.4 Hz, 2H), 1.67–1.28 (m, 6H). MS-DCI (NH_3/isobutane): m/z = 361.9 $[M+H]^+$. $C_{18}H_{23}N_3O_3S$–$1.5H_2O$ (388.5): calcd C 55.65, H 6.75, N 10.82; found C 55.37, H 6.35, N 11.26.

4.6. 4-(*N*-Biotinoylamino)acetophenone hydrazone (3b)

To a solution of the ketone **3a** (870 mg, 2.4 mmol) in absolute ethanol (8 mL), hydrazine monohydrate (995 μL, 19.5 mmol) was added. The solution was refluxed for 3 h. After cooling at room temperature, resulting precipitate was filtered, washed, and dried in vacuo to give **3b** (820 mg, 90%). Mp 283–285 °C. IR (KBr): 3336, 3282, 3183, 2931, 2857, 1701, 1659, 1593, 1521, 1459, 1405, 1325, 1264, 1187, 1111, 828, 719 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ = 9.86 (br s, 1H), 7.52 (s, 4H), 6.43 (br s, 1H), 6.35 (br s, 1H), 6.21 (br s, 2H), 4.29 (m, 1H), 4.12 (m, 1H), 3.12 (m, 1H), 2.81 (dd, J = 5.0, 12.5 Hz, 1H), 2.56 (d, J = 12.5 Hz, 1H), 2.32 (t, J = 7.2 Hz, 2H), 1.97 (s, 3H), 1.69–1.30 (m, 6H). MS-DCI (NH_3/isobutane): m/z = 376.1 $[M+H]^+$. $C_{18}H_{25}N_5O_2S$ (375.5): calcd C 57.58, H 6.71, N 18.65; found C 57.68, H 6.74, N 18.59.

4.7. 4-(*N*-Biotinoylamino)phenylmethyldiazomethane (3)

The compound **3** was prepared by the same procedure as described for **2** starting from the hydrazone **3b** (200 mg, 0.53 mmol). Yield: 190 mg. IR (KBr): 3257, 2930, 2857, 2032, 1698, 1597, 1524, 1510, 1457, 1404, 1307, 1259, 1180, 1114, 1071, 828 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ = 9.82 (br s, 1H), 7.57 (d, J = 8.7 Hz, 2H), 6.83 (d, J = 8.7 Hz, 2H), 6.41 (br s, 1H), 6.34 (br s, 1H), 4.28 (m, 1H), 4.12 (m, 1H), 3.11 (m, 1H), 2.80 (dd, J = 4.8, 12.7 Hz, 1H), 2.55 (d, J = 12.3 Hz, 1H), 2.25 (t, J = 7.0 Hz, 2H), 2.10 (s, 3H), 1.70–1.25 (m, 6H).

4.8. 3-(*N*-Biotinoylamino)benzophenone (4a)

The compound **4a** was prepared by the same procedure as described for **2a** starting from D-biotin (1.0 g, 4.1 mmol) and 3-aminobenzophenone (1.6 mg, 8.1 mmol). Yield: 1.3 g (75%). Mp 97–100 °C. IR (KBr): 3269, 3079, 2920, 2857, 1698, 1595, 1555, 1428, 1325, 722, 642 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ = 10.10 (br s, 1H), 8.00–7.39 (m, 9H), 6.43 (br s, 1H), 6.35 (br s, 1H), 4.27 (m, 1H), 4.13 (m, 1H), 3.12

(m, 1H), 2.84 (dd, $J = 5.0$, 12.3 Hz, 1H), 2.55 (d, $J = 12.3$ Hz, 1H), 2.31 (t, $J = 7.3$ Hz, 2H), 1.70–1.30 (m, 6H). MS-DCI (NH_3/isobutane): $m/z = 424.1$ $[M+H]^+$.

4.9. 3-(*N*-Biotinoylamino)benzophenone hydrazone (4b)

To a solution of the ketone **4a** (400 mg, 0.94 mmol) in absolute ethanol (8 mL), hydrazine monohydrate (366 μL, 7.5 mmol) was added. The solution was refluxed overnight. After evaporation, the residue was purified by flash chromatography (elution with MeOH/CH_2Cl_2 5–7%). Yield: 207 mg (50%). Mp 110–112 °C. IR (KBr): 3285, 3071, 2920, 2857, 1690, 1539, 1484, 1420, 1333, 1253, 1063, 777, 698 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): $\delta = 9.99$ and 9.80 (two br s, 1H), 7.70–6.75 (m, 9H), 6.42 (br s, 1H), 6.35 (br s, 1H), 6.26 and 6.22 (two br s, 1H), 4.28 (m, 1H), 4.13 (m, 1H), 3.12 (m, 1H), 2.78 (dd, $J = 5.0$, 12.0 Hz, 1H), 2.59 (d, $J = 12.0$ Hz, 1H), 2.30 and 2.24 (two t, $J = 7.3$ Hz, 2H), 1.67–1.26 (m, 6H). MS-DCI (NH_3/isobutane): $m/z = 438.1$ $[M+H]^+$.

4.10. 3-(*N*-Biotinoylamino)diphenyldiazomethane (4)

To a solution of the hydrazone **4b** (50 mg, 0.11 mmol) in THF (2 mL), MnO_2 (80 mg) was added and the suspension was stirred for 5 min. The mixture was filtered through Celite and molecular sieves 3 Å (powder, 0.5 cm thickness). After evaporation in vacuo (0.5 mmHg, $T < 40$ °C), the residue was triturated with ether to afford **4** (45 mg) as a pink powder. IR (KBr): 3253, 2928, 2039, 1698, 1611, 1547, 1500, 1436, 1261, 753, 690 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): $\delta = 9.95$ (br s, 1H), 7.60–7.20 (m, 8H), 6.92 (m, 1H), 6.42 (br s, 1H), 6.35 (br s, 1H), 4.28 (m, 1H), 4.14 (m, 1H), 3.12 (m, 1H), 2.83 (dd, $J = 5.0$, 12.0 Hz, 1H), 2.59 (d, $J = 12.0$ Hz, 1H), 2.27 (t, $J = 8$ Hz, 2H), 1.68–1.25 (m, 6H).

4.11. 2,4-Dinitrobenzaldehyde ethyleneacetal (6)

A solution of 2,4-dinitrobenzaldehyde (5.0 g, 25.5 mmol), ethylene glycol (20 mL), and *p*-toluenesulfonic acid (150 mg) in toluene (250 mL) was refluxed with Dean–Stark apparatus for 6 h. After cooling at room temperature, the reaction mixture was diluted with AcOEt (150 mL) and washed with water. Organic phase was dried over $MgSO_4$ and evaporated to give yellow oil (6.4 g). This crude product was used without purification. ^1H NMR (200 MHz, $CDCl_3$): $\delta = 8.70$ (d, $J = 2.1$ Hz, 1H), 8.44 (dd, $J = 2.1$, 8.6 Hz, 1H), 8.02 (d, $J = 8.6$ Hz, 1H), 6.49 (s, 1H), 4.13–3.96 (m, 4H).

4.12. 4-Amino-2-nitrobenzaldehyde ethyleneacetal (7)

To a solution of dinitroacetal **6** (6.4 g, 25.5 mmol) in EtOH–H_2O (6:1, 350 mL), Na_2S–$9H_2O$ (12.3 g, 51.1 mmol) was added. The solution was refluxed for 30 min and then concentrated to about 100 mL. The aqueous residue was diluted with 50 mL of water and then extracted three times with CH_2Cl_2. Combined extracts were washed with brine and dried over $MgSO_4$. After evaporation of solvents, the residue was purified by flash chromatography (elution with cyclohexane/ethyl acetate 60:40). From the faster eluting fractions, the isomer **8** was obtained (0.5 g, 9%) and from the following fractions, the isomer **7** (2.42 g, 45%).

Isomer (**7**): ^1H NMR (200 MHz, $CDCl_3$): 7.49 (d, $J = 8.6$ Hz, 1H), 7.09 (d, $J = 2.4$ Hz, 1H), 6.80 (dd, $J = 2.4$, 8.6 Hz, 1H), 6.27 (s, 1H), 4.05 (br s, 2H), 3.99 (m, 4H). MS-DCI (NH_3/isobutane): $m/z = 211.0$ $[M+H]^+$. $C_9H_{10}N_2O_4$ (210.2): calcd C 51.43, H 4.80, N 13.33; found C 50.96, H 4.46, N 13.72.

Isomer (**8**): ^1H NMR (200 MHz, $CDCl_3$): 7.60–7.40 (m, 3H), 5.84 (s, 1H), 4.50 (m, 2H), 4.09–4.07 (m, 4H).

4.13. 4-Biotinoylamino-2-nitrobenzaldehyde ethyleneacetal (9)

To a suspension of D-biotin (1.0 g, 4.1 mmol) in dry DMF (20 mL) cooled at 0 °C under argon, were added successively *N*-methylmorpholine (600 μL, 6.40 mmol) and isobutyl chloroformate (700 μL, 5.5 mmol). The solution was stirred for 30 min, and the nitroamine (1.0 g, 4.75 mmol) was added. The solution was stirred at room temperature for 4 h and then the solvent was removed in vacuo. The oily residue was purified by flash chromatography (elution with MeOH/CH_2Cl_2 7–10%) to afford **9** (1.1 g, 62%) ^1H NMR (200 MHz, DMSO-d_6): $\delta = 10.40$ (br s, 1H), 8.31 (d, $J = 2.0$ Hz, 1H), 7.77 (dd, $J = 2.0$, 7.2 Hz, 1H), 7.68 (d, $J = 7.2$ Hz, 1H), 6.43 (br s, 1H), 6.36 (br s, 1H), 6.23 (s, 1H), 4.28 (m, 1H), 4.14 (m, 1H), 3.92 (m, 4H), 3.12 (m, 1H), 2.85 (dd, $J = 5.0$, 12.0 Hz, 1H), 2.76 (d, $J = 12.0$ Hz, 1H), 2.34 (t, $J = 7.0$ Hz, 2H), 1.69–1.30 (m, 6H). MS-DCI (NH_3/isobutane): m/z: 437.0 $[M+H]^+$. $C_{19}H_{24}N_4O_6S$–$0.5H_2O$ (445.50): calcd C 51.23, H 5.66, N 12.58; found C 50.98, H 5.57, N 12.22.

4.14. 4-Biotinoylamino-2-nitrobenzaldehyde (10)

To a solution of the acetal **9** (768 mg, 1.76 mmol) in THF (25 mL), H_2SO_4 aqueous solution (2 N, 4 mL) was added. After stirring at room temperature for 4 h, the reaction mixture was concentrated and the residue was triturated with water. The resulting yellow powder was filtered, washed, and dried in vacuo. Yield: 694 mg (76%). IR (KBr): 3369, 3259, 2928, 2849, 1697, 1587, 1531, 1405, 1319, 1248, 837, 751 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): $\delta = 10.69$ (br s, 1H), 10.09 (s, 1H), 8.43 (d, $J = 2.0$ Hz, 1H), 7.91 (s, 2H), 6.42 (br s, 1H), 6.35 (br s, 1H), 4.29 (m, 1H), 4.13 (m, 1H), 3.12 (m, 1H), 2.84 (dd, $J = 5.0$, 12.0 Hz, 1H) 2.78 (d, $J = 12.0$ Hz, 1H), 2.39 (t, $J = 7.0$ Hz, 2H), 1.69–1.30 (m, 6H). MS-DCI (NH_3/isobutane): $m/z = 392.9$ $[M+H]^+$.

4.15. 4-Biotinoylamino-2-nitrobenzaldehyde hydrazone (11)

To a suspension of the aldehyde **10** (694 mg, 1.77 mmol) in ethanol (12 mL), hydrazine monohydrate (686 μL, 14.1 mmol) was added. The resulting orange solution

was refluxed for 1 h. After cooling, the resulting precipitate was filtered, washed, and dried in vacuo to give **11** (700 mg, 97%). Mp 165–167 °C. IR (KBr): 3442, 3388, 3251, 2936, 2852, 1694, 1584, 1529, 1478, 1338, 1250, 1090 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): $\delta = 10.31$ (br s, 1H), 8.31 (d, $J = 1.9$ Hz, 1H), 7.96 (s, 1H), 7.87 (d, $J = 8.8$ Hz, 1H), 7.68 (dd, $J = 1.9$, 8.8 Hz, 1H), 7.31 (br s, 2H), 6.42 (br s, 1H), 6.34 (br s, 1H), 4.29 (m, 1H), 4.13 (m, 1H), 3.12 (m, 1H), 2.84 (dd, $J = 5.0$, 12.2 Hz, 1H), 2.78 (d, $J = 12.2$ Hz, 1H), 2.33 (t, $J = 7.2$ Hz, 2H), 1.69–1.30 (m, 6H). MS-DCI (NH_3/isobutane): $m/z = 407.0$ $[M+H]^+$. $C_{17}H_{22}N_6O_4S$–$0.5H_2O$ (415.5): calcd C 49.15, H 5.58, N 20.23; found C 49.35, H 5.55, N 19.81.

4.16. 4-Biotinoylamino-2-nitrophenyldiazomethane (5)

To a solution of the hydrazone **11** (200 mg, 0.492 mmol) in DMF (8 mL), MnO_2 (341 mg) was added and the suspension was stirred for 10 min at room temperature. The mixture was filtered through Celite and molecular sieves 3 Å (powder, 0.5 cm thickness). After evaporation in vacuo (0.5 mmHg, <40 °C), the residue was triturated with ether to afford **5** (180 mg) as a pink powder. IR (KBr): 3281, 2922, 2848, 2367, 2073, 1697, 1583, 1525, 1460, 1330, 1297, 1158, 831, 759 cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): $\delta = 10.21$ (br s, 1H), 8.60 (d, $J =$ 2.2 Hz, 1H), 7.77 (dd, $J = 2.2$, 8.8 Hz, H_5); 7.22 (d, $1H_{aro}$, $J = 8.8$ Hz, H_6), 6.60 (s, 1H), 6.41 (br s, 1H), 6.33 (br s, 1H), 4.29 (m, 1H), 4.13 (m, 1H), 3.12 (m, 1H), 2.84 (dd, $J = 5.1$, 12.5 Hz, 1H), 2.78 (d, $J =$ 12.3 Hz, 1H), 2.29 (t, $J = 7.3$ Hz, 2H), 1.69–1.30 (m, 6H).

4.17. Adduct formation between 3′-UMP and labels 2–5

Analytical: 3′-UMP (0.04 mM) was incubated with labels **2**–**5** (2 mM for **2**, **3** and **5**; 30 mM for **4**) in a mixture of DMSO–CH_3CN–H_2O (1:3:1, 2.5 mL total) containing 2 mM H_3BO_3 (pH 7.3) at 60 °C. Aliquots (250 µL) in appropriate time intervals were washed three times with CH_2Cl_2 and the analyzed using capillary electrophoresis.

Capillary electrophoresis (CE) procedure: CE experiments were performed with a Beckman P/ACE 5000 capillary electrophoresis instrument (Beckman Coulter, Fullerton, CA). An untreated fused silica capillary (75 µm × 50 cm) was used. The applied voltage was 30 kV (normal polarity) and the capillary temperature maintained at 23 °C. The electrophoregrams were recorded at 254 nm. Borate buffer solution (0.1 M, pH 8.3) was prepared from boric acid by adjusting pH with NaOH solution and filtered through 0.2 µm filter. Samples were injected by pressure (0.5 psi, 5 s). Before each run, the capillary was regenerated by using successively NaOH solution (0.1 N, 2 min), pure water (2 min) and borate buffer (2 min) by pressure (20 psi).

*Preparative run (Adduct **12**)*: A mixture of 8.8 mg (20 µmol) of 3′-UMP (disodic salt tetrahydrated), 2 mL of H_3BO_3 solution (0.1 M), 2 mL of CH_3CN, 6 mL of methanol, and 24 mg (63 µmol) of *o*-BioPMDAM (**2**) was stirred at room temperature for 4 h. To the resulting mixture was added CH_2Cl_2 (30 mL) and H_2O (3 mL). Aqueous phase was separated and further washed twice with CH_2Cl_2 (30 mL) and then concentrated. The crude product was purified by reverse-phase silica gel chromatography (LiChroprep RP 18 silica gel, 40–63 µm, eluant: 0–20% MeOH/H_2O) to afford 9 mg (65%) of solid **12**. Two sets of signals for two diastereoisomers were observed in the ^1H NMR spectrum. ^1H NMR (300 MHz, D_2O): $\delta = 7.80$ and 7.77 (2d, $J = 7.9$ Hz, 1H), 7.58 (m, 1H), 7.40–7.30 (m, 3H), 5.88 and 5.83 (2d, $J = 7.9$ Hz, 1H), 5.72 (d, $J = 4.9$ Hz, 0.5H), 5.68 (d, $J = 3.7$ Hz, 0.5H), 5.48 (m, 1H), 4.62 (m, 1H), 4.58 (m, 1H), 4.35–3.30 (m, 6H), 3.00 (dd, $J = 13.1$ and 4.9 Hz, 1H), 2.78 (d, $J = 12.8$ Hz, 1H), 2.52 (t, $J = 7.2$ Hz, 2H), 1.50 and 1.48 (2d, $J = 6.4$ Hz, 3H), 1.35–1.75 (m, 6H).

*Adduct **13***: The compound **13** was obtained by the same procedure as described for **12** starting from 3′-UMP (8.8 mg) and *p*-BioPMDAM (**3**, 75 mg, 0.20 mmol). Yield: 8.4 mg (61%). ^1H NMR (300 MHz, D_2O): $\delta = 7.72$ and 7.69 (2d, $J = 7.8$ Hz, 1H), 7.46 (s, 4H), 5.86 (d, $J = 7.8$ Hz, 1H), 5.70 (d, $J = 4.5$ Hz, 0.5H), 5.66 (d, $J = 2.6$ Hz, 0.5H), 5.46 (m, 1H), 4.62 (m, 1H), 4.58 (m, 1H), 4.30–3.52 (m, 5H), 3.33 (m, 1H), 3.00 (dd, $J = 12.7$ and 4.5 Hz, 1H), 2.78 (d, $J = 12.7$ Hz, 1H), 2.45 (t, $J = 7.0$ Hz, 2H), 1.58 and 1.53 (2d, $J = 5.4$ Hz, 3H), 1.35–1.75 (m, 6H).

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Supplementary data

IR and ^1H NMR spectra of **3**, **4**, and **5**, electrophoregrams monitoring hydrolysis of adducts formed between 3′-UMP and labels **1**–**4**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2004.12.046.

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28. In these conditions, it was shown that approximately 3 of every 10 phosphates were labeled.[21]