Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 705–708
Bioorganic & Medicinal Chemistry Letters

Pyrenyldiazomethane, a versatile reagent for nucleotide phosphate alkylation

Mitsuharu Kotera,[a,*,†] Marie-Louise Dheu,[a] Anne Milet,[a] Jean Lhomme[a] and Ali Laayoun[b]

[a]*LEDSS, UMR5616 CNRS-BP53, Université Joseph Fourier, 38041 Grenoble, France*
[b]*BioMérieux, Advanced Technology/Molecular Diagnostics, Chemin de l'Orme, 69280 Marcy l'Etoile, France*

Received 4 June 2004; revised 6 November 2004; accepted 10 November 2004
Available online 2 December 2004

Abstract—Pyrenyldiazomethane was shown to react quantitatively and selectively at phosphate with 2′-, 3′-, and 5′-nucleotide phosphates incorporating the different nucleic bases.

Introduction of reporter molecules (haptens, fluorescent dyes,...) into nucleotides or nucleic acids is key to a number of biological and biotechnological applications. Most frequently the reporter group is introduced as an electrophilic reagent leading to alkylation at the nucleobase or less frequently on the sugar moiety.[1–3] Only a few methods have been reported for introduction of groups on phosphate in aqueous media. Most published studies[4–12] of nucleotide phosphate alkylation have been carried out for preparative purposes so that the site selectivity of the alkylation is not well understood. Yields are generally poor. Also these alkylation reactions have been usually carried out in non-aqueous organic media or in heterogeneous two-phase system. Furthermore they require tedious purification procedure so these methods are not appropriate for nucleotide labeling. Site selective alkylation of phosphate group is considered to be difficult due to the poor nucleophilicity of phosphate and to the presence of competitive nucleophilic sites in nucleotides (e.g., 2-NH_2 group of guanosine etc.).

Recently we developed a new labeling procedure based on diazo compounds bearing a biotin moiety for site-selective phosphate alkylation in mono and polynucleotides.[13,14] Major drawback of this procedure is that it requires an additional step (affinity labeling with streptavidine-fluorescent conjugate) for detection. Here we wish to report the alkylation reactions of mononucleotides with pyrenyldiazomethane (PDAM **1**), a fluorescent reagent that is described for derivatizing carboxylic acids.[15] We show that pyrenyldiazomethane is a convenient reagent for quantitative and selective fluorescent labeling at phosphate in 2′, 3′-, and 5′-nucleotides incorporating the four natural bases (Scheme 1).

Pyrenyldiazomethane was prepared from 1-formylpyrene using a described procedure.[15] First, we examined the reaction of PDAM **1** with 3′-UMP **2** using conditions similar to those reported for reaction with carboxylic acids. Reaction of PDAM **1** (2 mM) with 3′-uridine monophosphate (3′-UMP) **2** (40 μM) in homogeneous solution (H_2O/CH_3CN/MeOH/AcOEt = 1:1:2:1) containing borate buffer (2 mM, pH 8.3) at room temperature overnight yielded quantitatively the 3′-adduct **3** corresponding to alkylation of the phosphate group. This reaction was monitored conveniently by capillary electrophoresis as shown in Figure 1. Several factors influencing the reaction were studied.

Effects of medium and temperature: Selection of an appropriate reaction medium was critical as this reaction involves charged ionic substrates (nucleotides) and a lipophilic reagent (PDAM). No reaction was observed using two-phase systems (H_2O–CH_2Cl_2). Using described solvent mixture, the reaction proceeded smoothly at room temperature in homogeneous

Keywords: Pyrenyldiazomethane; Nucleotide; Phosphate alkylation.
* Corresponding author. Tel.: +33 390 244 175; fax: +33 390 244 306; e-mail: kotera@bioorga.u-strasbg.fr
† Address: Laboraoire de Chimie Génétique, Associé CNRS/Université Louis Pasteur de Strasbourg, Faculté de Pharmacie, BP 24, 67401 Illkirch, France.

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.11.024

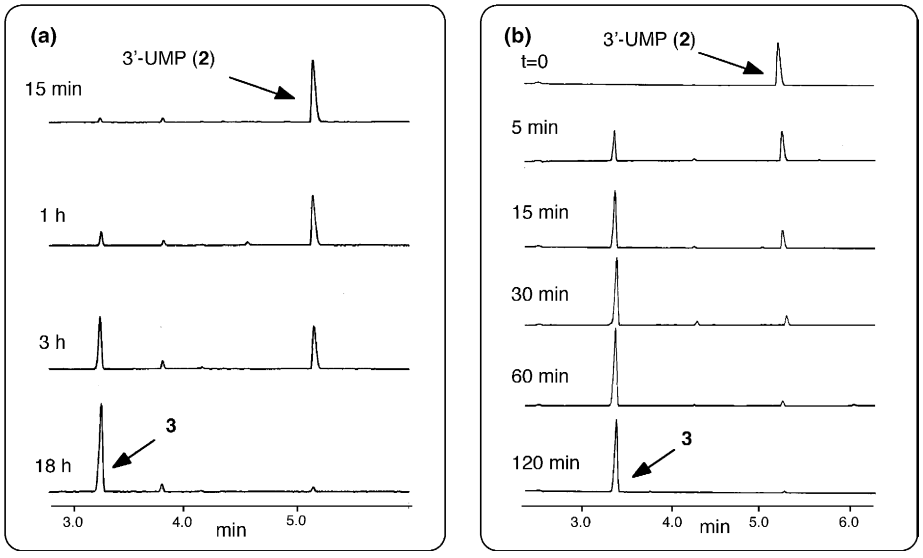
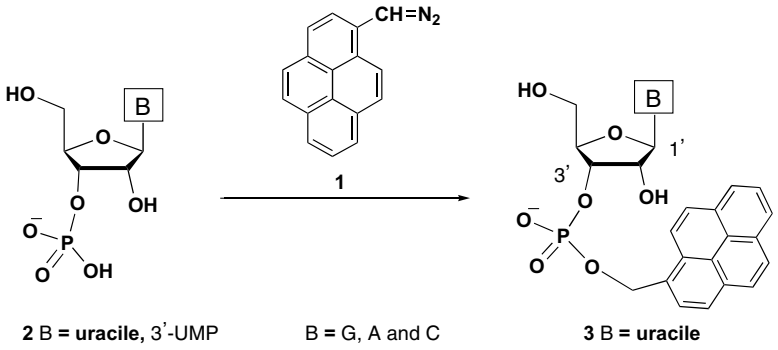


Figure 1. Alkylation of 3′-UMP 2 (40 μM) with PDAM **1** (2 mM) monitored by capillary electrophoresis: (a) rt, in AcOEt/CH_3CN/MeOH/H_2O (1/1/2/1), pH 8.3 (borate buffer: 2 mM). (b) 60 °C, in AcOEt/CH_3CN/H_2O (1/3/1), pH 6 (H_3BO_3: 2 mM).



Scheme 1. Site selective alkylation of nucleotides with PDAM (**1**). Same reactivity was observed from 2′-UMP, 5′-UMP, and 3′-TMP.

solution. We found that the same reaction can be carried out without methanol in homogeneous solution (H_2O/CH_3CN/AcOEt = 1:3:1). In the latter conditions, increasing the temperature from 20 to 60 °C allowed the reaction to proceed 20 times faster (Fig. 1b). The alkylation proceeded similarly in the mixture DMSO/H_2O (1:1), however in DMSO–H_2O (1/9) the reaction was about five times slower. In this latter case, we observed some precipitates in the reaction mixture.

Effect of pH and buffer: Alkylation rate at pH = 8.3 was about five times slower as compared to pH 7.3. At lower pH (<6.0), no more acceleration was observed. The borate buffer was found to be most appropriate. For example using the HEPES buffer, significant amounts of secondary products probably arising from sulfonate alkylation were observed.

Effect of concentration: Although the reaction kinetics were not studied in detail, it was shown qualitatively that alkylation of 3′-UMP is a second-order reaction in competition with PDAM **1** decomposition that is a first-order reaction. Thus alkylation of 3′-UMP **2** in higher concentrations (0.2 mM) with PDAM **1** (2 mM) proceeded similarly in the first stages of the reaction, but 3′-UMP **2** remained unchanged after about 80% transformation.

Structure determination of adduct 3: Adduct **3** was isolated from a 20 μmol-scale reaction and its structure was confirmed by NMR spectroscopy.[16] Phosphate alkylation was clearly proven by observation of the CH_2-pyrenyl resonance at 5.5 ppm as a complex multiplet, which transformed into a simplified AB system upon decoupling irradiation of phosphorous (Fig. 2). In the ^1H NMR spectrum, the H-1′, H-2′ H-3′, H-5, and H-6 resonances of **3** were significantly shifted (0.7–2.2 ppm, Table 1) to high fields as compared to those of 3′-UMP **2**. These observations indicate that the pyrenyl ring is in stacked conformation with the uracile ring, which is confirmed by the theoretical study. In the minimum energy structure of compound **3** (Fig. 3),[17] the H-2′ atom is pointing toward the pyrenyl ring and situated at a 3.17 Å distance from the pyrenyl ring. The H-1′, H-3′, H-5, and H-6 atoms were equally positioned within short distances (3.47, 3.57, 4.79, and

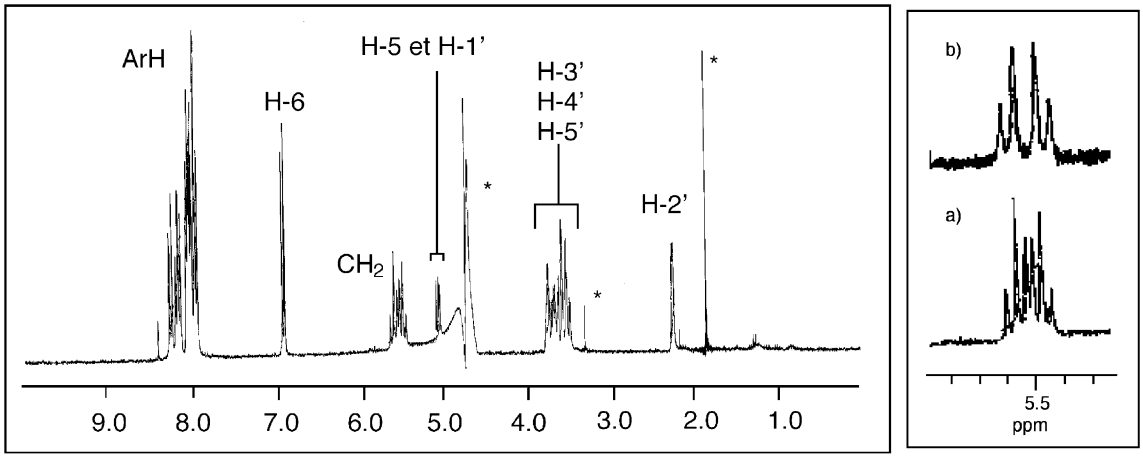


Figure 2. ^{1}H NMR Spectrum (300 MHz) of adduct **3** in D_2O. ∗: Solvent resonances and other small impurities. On right: CH_2 resonance at 5.5 ppm without (a) and with (b) irradiation on phosphorous.

Table 1. Experimental and calculated ^{1}H NMR of **2** and **3**

	2	**3**	$\Delta\delta$ (ppm) exp.	$\Delta\delta$ (ppm) calc.
H-1′	5.88	5.03	0.85	0.83
H-2′	4.50	2.25	2.25	2.73
H-3′	4.37	3.67	0.70	0.54
H-4′	4.18	3.73	0.45	0.32
H-5′	3.83	3.54	0.29	0.25
H-5	5.85	5.06	0.79	0.32
H-6	7.85	6.90	0.95	0.74

4.28 Å) from the pyrenyl plane while H-4′ and H-5′ are located at longer distances from the pyrenyl ring. To confirm this hypothesis, we have computed the NMR shifts, which are very sensitive to the geometry. And indeed, the computed NMR shifts exhibit a good agreement with the experimental data. Because of the aromatic character of the pyrenyl group, it is reasonable to assign the quite important deviations, going from **2** to **3**, of both experimental and computed shift, to this approach of the H atoms in the shielding cone.

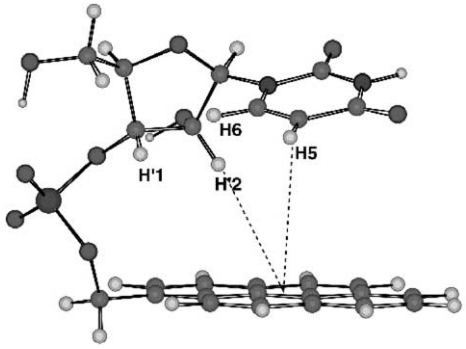


Figure 3. Optimized structure of compound **3**.

Alkylation of other nucleotides: We next examined the alkylation reactions of other nucleotides by PDAM **1** [standard conditions: PDAM (2 mM), nucleotide (40 μM), H_3BO_3 (2 mM), 60 °C, 2 h, in H_2O/CH_3CN/AcOEt = (1:3:1)]. The reactions were monitored as previously by capillary electrophoresis (Fig. 4). The

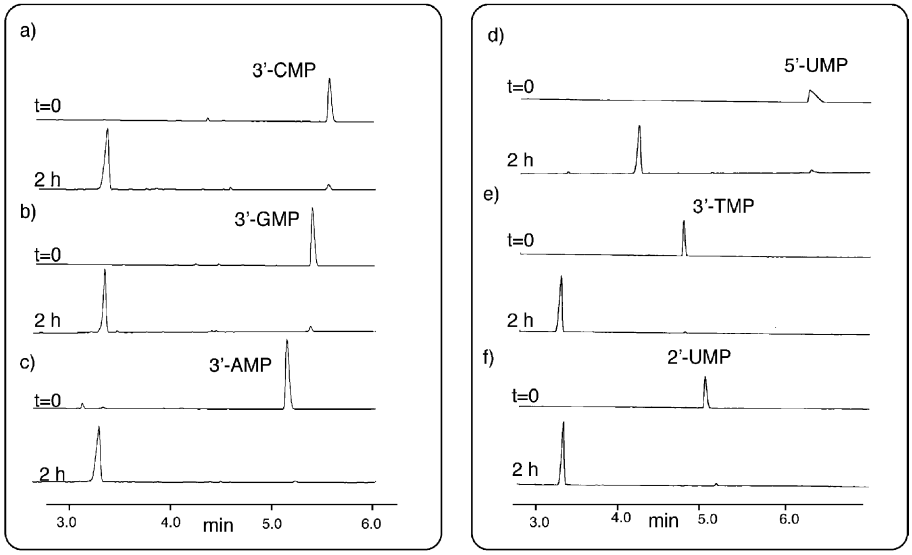


Figure 4. Alkylation of (a) 3′-CMP, (b) 3′-GMP, (c) 3′-AMP, (d) 5′-UMP (e) 3′-TMP, and (f) 2′-UMP (40 μM) with PDAM **1** (2 mM) monitored by capillary electrophoresis, 60 °C in AcOEt/CH_3CN/H_2O (1/3/1), pH < 6 (H_3BO_3: 2 mM).

nucleotides studied were 3′-AMP, 3′-GMP, 3′-CMP, 5′-UMP, 2′-UMP (ribonucleotides), and 3′-TMP (2′-deoxyribonucleotide). In all studied cases, we observed high chemoselective alkylation of the phosphate group. The reaction rates were similar for all nucleotides and the quasi-complete conversion was achieved within 2 h in these conditions. Quite remarkable is that reaction at the base is not observed in all cases studied, even with guanine as the base, which is however known for its high reactivity with usual alkylating agents (halides, epoxides, sufonates,...).

All those results indicate that pyrenyldiazomethane is a reagent of choice for rapid, quantitative, and selective labeling and derivatization of nucleotides at the phosphate. It can be used independently of the nature of the nucleic base and of the position of phosphate in the sugar residue.

Acknowledgements

The calculations were performed in Grenoble, and at the CECIC (Centre d'expérimentations pour le calcul intensif en Chimie de grenoble). The University Joseph Fourier of Grenoble and CNRS are acknowledged for providing the computer facilities.

References and notes

1. Wojczewski, C.; Stolze, K.; Engels, J. W. *Synlett* **1999**, 1667–1678.
2. Davies, M. J.; Shah, A.; Bruce, I. J. *Chem. Soc. Rev.* **2000**, *29*, 97–107.
3. O'Donnel, M. J.; Laughlin, L. W. Reporter Groups for the Analysis of Nucleic Acid Structure. In *Bioorganic Chemistry: Nucleic Acids*; Hecht, S. M., Ed.; Oxford Univ. Press: New York, 1996; pp 216–243.
4. Ayukawa, H.; Ohuchi, S.; Ishikawa, M.; Hata, T. *Chem. Lett.* **1995**, 81.
5. Furuta, T.; Torigai, H.; Osawa, T.; Iwamura, M. *J. Chem. Soc., Perkin Trans. 1* **1993**, 3139–3142.
6. Walker, J. W.; Reid, G. P.; McCray, J. A.; Trentham, D. R. *J. Am. Chem. Soc.* **1988**, *110*, 7170–7177.
7. Engels, J.; Reidys, R. *Experientia* **1978**, *34*, 14–15.
8. Engels, J.; Schlaeger, E. J. *J. Med. Chem.* **1977**, *20*, 907–911.
9. Mizuno, Y.; Endo, T.; Miyaoka, T.; Ikeda, K. *J. Org. Chem.* **1974**, *39*, 1250–1255.
10. Endo, T.; Ikeda, K.; Kawamura, Y.; Mizuno, Y. *J. Chem. Soc., Chem. Commun.* **1973**, 673–674.
11. Nishimura, S.; Saneyoshi, M. *Chem. Pharm. Bull.* **1980**, *28*, 1695–1703.
12. Sato, E.; Yoshikawa, M.; Kanaoka, Y. *Chem. Pharm. Bull.* **1990**, *38*, 2287–2289.
13. Bourget, C.; Trévisiol, E.; Lhomme, J.; Kotera, M.; Tora, C.; Bernal-Méndez, E.; Laayoun, A. *Nucleoside, Nucleotides Nucleic Acids* **2003**, *22*, 1371–1373.
14. Laayoun, A.; Kotera, M.; Sothier, I.; Trévisiol, E.; Bernal-Méndez, E.; Bourget, C.; Menou, L.; Lhomme, J.; Troesch, A. *Bioconj. Chem.* **2003**, *14*, 1298–1306.
15. Nimura, N.; Kinoshita, T.; Yoshida, T.; Uetake, A.; Nakai, C. *Anal. Chem.* **1988**, *60*, 2067–2070.
16. 1-Pyrenylmethyl uridine-3′-phosphate (3): A mixture of 8.8 mg (20.0 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 50 mg (200 μmol) of PDAM was stirred at rt for 5 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 to dryness. The crude product was purified by reverse-phase silica gel chromatography (LiChroprep RP 18 silica gel, 40–63 μm; eluant: 030% MeOH/H_2O) to afford 8.6 mg (77%) of solid. ^{1}H NMR (300 MHz, D_2O); 2.25 (m, 1H, H-2′), 3.54 (m, 2H, H-5′), 3.67 (m, 1H, H-3′), 3.73 (m, 1H, H-4′), 5.03 (d, $J = 1.8$, 1H, H-1′), 5.06 (d, $J = 8.1$, 1H, H-5), 5.43–5.62 (m, 2H, CH_2), 6.90 (d, $J = 8.1$, 1H, H-6), 7.92–8.39 (m, 9H). ^{31}P NMR (121 MHz, D_2O); 0.3. ES-MS 537.0 (calcd for $C_{26}H_{22}N_2O_9P$: 537.11 Da). UV [λ_{max} nm ($\varepsilon \times 10^3$) in H_2O]; 343 (19.4), 327 (13.6), 275 (21.6), 264 (16.2), 241 (35.4).
17. The structures were built and displayed by INSIGHT II version 2000, the simulations involving molecular minimizations (MM) using the CVFF force field and the BFGS minimizer of the DISCOVER program of Accelrys.[18–20] Molecular dynamic simulations were performed by 1 fs steps during 200 ps at $T = 800$ K with dielectric constant $\varepsilon_r = 1$. The more stable geometries were fully optimized through analytic gradient calculations using the hybrid Hartree–Fock density functional theory approach B3LYP[21,22] with the 6-31+G** basis set. The NMR shielding tensors have been computed with the gauge independent atomic orbital (GIAO) method. All the quantum calculations were carried out using the Gaussian98 program package.[23]
18. Biosym Technologies, Inc. 9685 Scranton Road San Diego, CA 92121-2777, USA.
19. Gilson, M. K.; Sharp, K. A.; Honig, B. H. *J. Comput. Chem.* **1987**, *9*, 327–335.
20. Gilson, M. K.; Honig, B. *Proteins: Struct. Funct., Genet.* **1988**, *4*, 7–18.
21. Becke, A. D. *J. Chem. Phys.* **1992**, *97*, 9173.
22. Lee, C.; Yang, W.; Parr, R. G. *Phys. Rev.* **1988**, *B37*, 785.
23. Gaussian 98, Revision A.6, Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Andres, J. L.; Gonzalez, C.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. Gaussian, Inc., Pittsburgh PA, 1998.