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Phosphate-specific fluorescence labeling with BO-IMI: reaction details

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Abstract

Previously we reported that BO-IMI, a reagent which contains a BODIPY fluorophore linked to an imidazole group, can be used to covalently label a phosphomonoester in a single step under aqueous conditions [P. Wang, R.W. Giese, Anal. Chem. 65 (1993) 3518]. The reaction was conducted in the presence of a water-soluble carbodiimide 1-ethyl-3-(3′-N,N′-dimethylaminopropyl)carbodiimide [EDC] to activate the phosphomonoester, and the coupling took place onto both the N1 and N3 imidazole nitrogens of BO-IMI. Whether the two BO-IMI-phosphomonoester regioisomers migrated separately or together during capillary electrophoresis depended on the pH, due to a difference in their pK_a values. Since then, we have studied the reaction in more detail leading to the information reported here. First, we have learned that the regioisomer ratio changes during the course of the reaction, and found that the mechanism involves both spontaneous and BO-IMI-catalyzed hydrolysis of the less stable isomer. Second, there is a background reaction in which BO-IMI becomes attached to EDC. Third, the BO-IMI-phosphomonoester product (a mixture of two isomers), that is observed by capillary electrophoresis at an alkaline pH, is found to no longer contain the two fluorine atoms present in the starting BO-IMI reagent. This is because they are replaced by hydroxy groups at high pH. Finally, an event was discovered which complicates the detection of less than about 60 fmol of a phosphomonoester with BO-IMI: hydrolysis of a tiny fraction of the BO-IMI takes place during the coupling reaction, which leads to chemical noise in the capillary electropherogram.

Keywords: Derivatization, electrophoresis; BO-IMI; Nucleotides

1. Introduction

Recently we introduced a new reagent and technique which achieves phosphate-specific fluorescence labeling under aqueous conditions [1,2]. The new reagent is ‘‘BO-IMI’’, a fluorescent derivative of N-acetyl-histidine. In our technique the phosphate moiety of a target compound is activated with a water soluble carbodiimide for single-step coupling to the imidazole moiety of BO-IMI. The method is phosphate specific since carboxylic acids are labeled only transiently by BO-IMI during the reaction. Due to our interest in detecting a class of trace analytes called DNA adducts, we primarily tested nucleotides. Diluted standards of BO-IMI labeled nucleotides were detected at the low attomole level (polarity-switching injection of 4.4 μl containing 2.2 amol of each BO-IMI-nucleotide) by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF)

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[1]. We have also specifically labeled a phosphate group on pepsin, a phosphoprotein, with BO-IMI [3].

Our basic technique for labeling a target phosphate compound with BO-IMI consists of three steps: (1) BO-IMI coupling at pH 6; (2) filtration through a cation exchange silica packing at pH 7.0; and (3) CE-LIF at pH 10.4. The filtration step removes the residual BO-IMI and water soluble carbodiimide along with related decomposition products. A high pH is typically used for the subsequent separation by capillary electrophoresis in order to enhance selectivity, since some nucleobases have pK_a values in the vicinity of this pH.

Here we report the results of experiments which lead to a more detailed understanding of our new reagent and technique. This work was motivated primarily by our interest in extending the method to labeling of trace quantities of nucleotides. Thus far we have detected 60 femtomoles of dAMP, but have encountered chemical noise at this level, partly because of a defect in the current reagent.

2. Experimental

2.1. Materials

BO-IMI[1],C8-[N-acetyl-N-(2-fluorenyl)]amino-5′-dGMP [4] (C8-AAF-5′-dGMP), C8-oxo-5′-dAMP [5], and C8-benzyloxy-5′-dAMP [5] were prepared as described. 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl hydrazide (BODIPY FL C_3 hydrazide) was from Molecular Probes (Eugene, OR, USA). Sodium tetraborate (BORAX), tris[hydroxymethyl]-aminomethane (TRIS), 1-ethyl-3-(3′-N,N-dimethylaminopropyl)carbodiimide (EDC), 2-(N-morpholino)-ethanesulfonic acid (MES), 5′-dAMP, 5′-dCMP, 5′-dGMP, and 5′-TMP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and propyl sulfonic acid silica (40 μm, 60 Å) were from J.T. Baker Inc. (Phillipsburg, NJ, USA). Ammonium acetate (NH_4Ac) was from Fluka (Buchs, Switzerland). 5′-pCAAAGCTTG (a DNA oligomer) was from Oligos Etc. Inc. (Wilsonville, OR, USA). Microcentrifuge tubes (1.5 ml, 05-407-10, Fisher Scientific, Pittsburgh, PA, USA) were used for the BO-IMI reactions.

2.1.1. Buffers

Buffer A (pH 6.0): 0.5 ml of 0.2 *M* MES, 0.38 ml of 0.1 *M* NaOH, and 9.1 ml of water.

Buffer B (pH 6.0): 2.0 ml of 0.2 *M* MES, 0.28 ml of 0.5 *M* TRIS, 4.0 ml methanol, and 34 ml of water.

Buffer C (pH 8.7): 0.8 ml of 0.5 *M* boric acid, 0.8 ml of 0.5 *M* TRIS, 4 ml acetonitrile, and 34.4 ml of water.

Buffer D (pH 10.4): 2.0 ml of 0.05 *M* BORAX, 1.8 ml of 0.1 *M* NaOH, 5 ml of acetonitrile, and 41.2 ml of water.

2.1.2. Equipment

A home-built capillary electrophoresis (CE) apparatus with laser-induced fluorescence detection (Ar ion laser with excitation at 488 nm) was used [6]. The CE unit was interfaced to a Macintosh Centris 610 computer through DYNAMAX MacIntegrator I (Rainin Instrument Co., Inc., MA, USA). One of the contact input ports was used to trigger the data acquisition, and one of the contact outputs was used to interlock and trigger the regulated high-voltage d.c. power supply (Glassman High Voltage Inc., NJ, USA). CE was performed in a 70 cm long fused-silica capillary (75 μm I.D.) with the detection window 45 cm from the injection end. Samples were injected hydrodynamically: anode end 5 cm higher for 20 s (about 10 nl).

2.2. Fluoride measurement of hydrolyzed BODIPY hydrazide

An Orion 501 digital ionalyzer and an Orion F^- ion-selective electrode (Orion Research Inc., Cambridge, MA, USA) were used for F^- measurement. Fluoride standard solutions, which gave a linear calibration curve, were prepared by weighing KF (MCB, Norwood, OH, USA), dissolving it to 0.1 *M* in 0.1 *M* potassium phosphate, pH 6.5, and making dilutions in this buffer to 10^{-5} *M* fluoride. After 1.0 ml of a 8.9×10^{-4} *M* solution of BODIPY hydrazide in 0.01 *M* NaOH was kept for 0.5 h at room temperature, it was diluted with 9 ml of the 0.1 *M* phosphate buffer and tested, revealing a fluoride concentration of 1.8×10^{-4} *M* (1.8×10^{-3} *M* in the original solution).

2.3. Cation exchange filtration of a BO-IMI/nucleotide reaction mixture

BO-IMI (1.2×10^{-2} *M*), 5′-dAMP (1.2×10^{-1} *M*), and EDC (1.2×10^{-1} *M*) in buffer A, 10 μl each, were briefly mixed and then kept at room temperature in the dark for 15 min. The starting BO-IMI was removed by loading the reaction mixture onto a Pasteur pipet column containing ~140 mg propyl sulfonic acid silica retained on glass wool, and eluting (with air pressure from a rubber bulb, including a terminating flow of air through the column) with 2×0.3 ml of buffer A. Before this separation was performed, bulk cation exchanger in this buffer was adjusted to pH 6 with 0.1 *M* NaOH, and, after the column was prepared, it was washed with 10 ml of buffer A.

2.4. Detection of 60 and 600 fmol of 5′-dAMP

BO-IMI (1.2×10^{-2} *M*), 5′-dAMP, and EDC (9×10^{-2} *M*), 1 μl each in buffer A, were mixed, and the resulting solution was kept dark at room temperature for 2 h. After the reaction mixture was diluted with 0.5 ml of pH 7.0 0.01 *M* NH_4Ac (pH adjusted with NH_4OH), it was loaded onto a cartridge column (see below). A rubber bulb was employed to push all the liquid out of the cartridge into a collection vial. A second 0.5 ml NH_4Ac was applied and similarly pushed into the vial. The collected solution was evaporated to dryness in a Speed-Vac (Savant Instruments). Fifty μl of buffer D was added followed after 30 min by injection into the CE capillary. To prepare the cartridge column, bulk cation exchanger in 0.01 *M* NH_4Ac was adjusted to pH 7.0 with 0.1 *M* NaOH, and loaded (140 mg) into a pasteur pipet plugged with glass wool followed by washing with 10 ml of pH 7.0 0.01 *M* NH_4Ac.

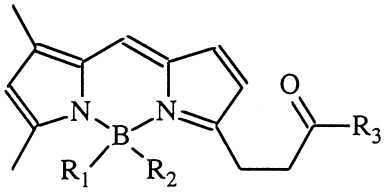
3. Results and discussion

Previously we pointed out that both the N1 and N3 nitrogens of the imidazole moiety of BO-IMI (**1**; the structures of our compounds are presented in Table 1) react with a phosphomonoester [1]. For 5′-dAMP and 3′-dAMP (and the other corresponding mononucleotides that were studied), the ratio of the major (**3**) to minor (**2**) product was 97:3 and 98:2, respectively. It was assumed (but not proved) that this product ratio arose kinetically as a consequence of steric effects, tentatively making **3**=N1 regioisomer and **2**=N3 regioisomer for BO-IMI-5′-dAMP (see Table 1 for a definition of the N1 and N3 positions). To obtain this data, we conducted the labeling reaction at pH 6.0 for 3 h, and then subjected the product mixture to capillary electrophoresis at the same pH. This separated the pair of isomeric products, apparently due to a difference in the pK_a values of their imidazole moieties in this pH region. For example, the imidazole moiety of guanosine 5′-phosphoimidazolide has a pK_a of 6.07 at 37°C [7]. At an elevated pH (≥8.7 was examined) the isomeric products comigrated, conveniently allowing a single peak to be observed for each target phosphate compound that was tested.

When the reaction mixture (at pH 6.0) is directly examined by CE-LIF after a shorter time period, e.g., 0.3 h, as shown in Fig. 1A, a higher ratio of **2** relative to **3** is seen relative to what is observed after 3 h (Fig. 1B). Thus there is a shift with time from a kinetically to a more thermodynamically-controlled product mixture. Further observations, about to be described, suggest that the primary mechanism for this shift involves preferential hydrolysis at pH 6.0 of the less stable isomer (**2**), which releases the 5′-dAMP for a second round of coupling to BO-IMI (excess EDC, a water soluble carbodiimide, is present).

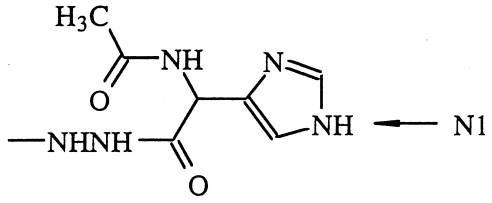
We subjected a reaction mixture (containing a 10-fold molar excess of 5′-dAMP over BO-IMI) after 15 min to cation exchange filtration, which removed the residual EDC (but apparently not any preformed EDC-5′-dAMP, since it lacks a net charge). The collected sample was divided into two parts (A and B). Part A was immediately treated with an amount of BO-IMI equivalent to what was present initially; this adjustment was done without changing the pH. Buffer A was added to part B to keep the volume the same. The samples were stored at room temperature in the dark, while aliquots of each were tested periodically by CE to monitor the ratio of isomeric products. Thus we were testing the effect of BO-IMI on the ratio of **2** to **3** in the absence of intact EDC, to avoid the complication of hydrolyzed **2** or **3**

Table 1
Structures of BODIPY derivatives



1=BO-IMI

	R_1	R_2	R_3
1,1′	F, OH	F, OH	X
2,2′	F, OH	F, OH	X-5′-dAMP[a]
3,3′	F, OH	F, OH	X-5′-dAMP[a]
4,4′	F, OH	F, OH	$NHNH_2$
5,5′	F, OH	F, OH	see Fig. 5
6′	OH	OH	$NHNHCOCH_3$ [b]
7′	OH	OH	X-C8-benzyloxy-5′-dAMP[c]
8′	OH	OH	X-C8-AAF-5′-dGMP[c]
9′	OH	OH	X-C8-oxo-5′-dAMP[c]
10′	OH	OH	OH
11′	OH	OH	X-pCAAGCTTG

X = 

[a] Phosphoimidazolides where one is N1 and one is N3 with respect to the imidazole moiety. Which isomer is the major product is unknown, however.
[b] Postulated structure.
[c] Mixture of N1 and N3 phosphoimidazolides with respect to the imidazole moiety.

re-reacting with EDC. After 20 h, the pH of the A and B parts were 6.23 and 6.40, respectively.

As the absolute peak area for **2** decreased progressively in B (the part not supplemented with BO-IMI) throughout this storage period (first order kinetics; $t_{1/2}$=2.3 h), the peak for BO-IMI increased correspondingly, while that for **3** decreased to a lesser degree. (Previously we determined that the half-life for **3** at pH 6.0 is 19.7 h [1]). In part A (supplemented with BO-IMI), fourfold less **2**, and slightly more **3** (about 10%), was present at the first time point (1.1 h after the filtration step) than in part B containing no added BO-IMI. Apparently the added BO-IMI in part A was doing two things: reacting with EDC-5′-dAMP (which accounts for the initial, slight increase of **3** in A) and also catalyzing the hydrolysis of **2** (which both lowers the pH and the yield of **2** in A relative to what happens in B). Thus, both spontaneous and BO-IMI-catalyzed hydrolysis of the less stable isomer **2** in the ordinary reaction mixture (where excess EDC is present) appear to drive its conversion, via reactivation with EDC, to **3**. In the ordinary reaction (which is usually conducted for 3 h), this thereby leads to the 97:3 product ratio of **3:2**. Other known catalysts for hydrolysis of a phosphoimidazolide of a nucleotide include both $H_2PO_4^-$ and HPO_4^{2-} [7].

Assuming, as before, that **2** is the N3 isomer, then why does this isomer hydrolyze more rapidly at pH 6.0? It is known that a phosphoimidazolide hydrolyzes more rapidly when the imidazole moiety is protonated [7]. We speculate that the postulated N3 isomer hydrolyzes faster since protonation at its free N1 site can be stabilized by solvation with little interference from the remote C4 alkyl group, in contrast to the behavior of the N1 isomer.

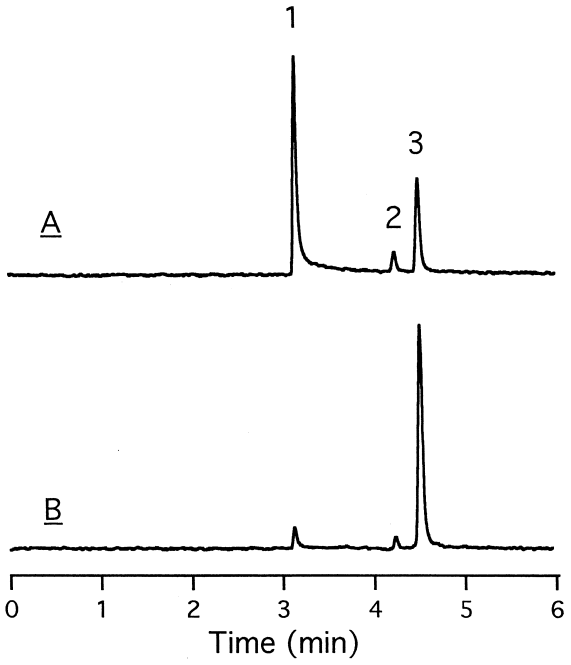


Fig. 1. Electropherograms at pH 6.0 (buffer B) showing two BO-IMI-5′-dAMP isomers (**2** and **3**) as a function of reaction time, which was either 0.3 (A) or 3.0 h (B). Peak **1** is BO-IMI. Sample preparation: BO-IMI (2.4×10^{-2} *M*), dAMP (2.4×10^{-2} *M*), and EDC (1.8×10^{-1} *M*) in buffer A, 10 μl each, were combined.

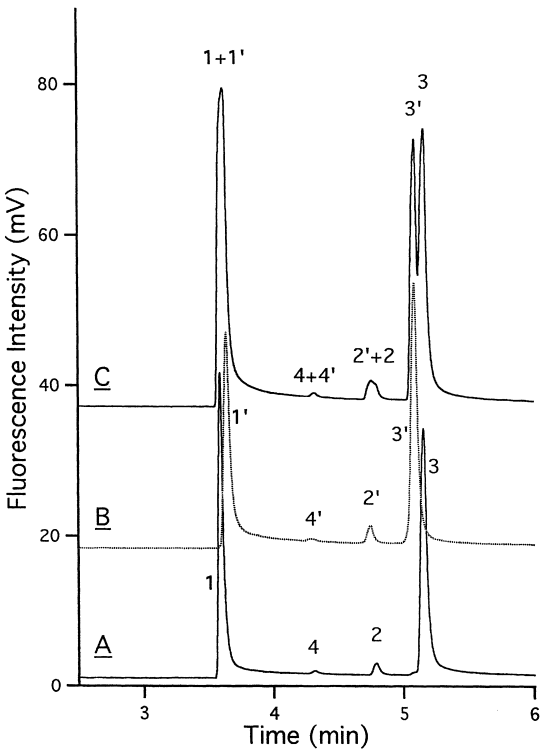


Fig. 2. Effect of pH on BO-IMI-5′-dAMP. (A) Fresh reaction mixture of BO-IMI, dAMP and EDC. (B) Sample after incubation in Buffer D (pH 10.4) for 0.5 h at room temperature. (C) Coinjection of B and C. Peaks: **1**=BO-IMI; **2** and **3**=BO-IMI-5′-dAMP; **4**=BODIPY FL C_3 hydrazide. **1′**, **2′**, **3′**, **4′** are the corresponding dihydroxy compounds. The separation was done at pH 6.0 (buffer B).

The electropherogram shown in Fig. 2A basically corresponds to those shown in Fig. 1 except that a small amount of BODIPY FL C_3 hydrazide, **4**, is present as a neutral marker. When the pH of this reaction mixture is raised to 10.4 and then, one hour later, returned to pH 6.0, the electropherogram shown in Fig. 2B is observed. Coinjecting the two reaction mixtures gives the electropherogram shown in Fig. 2C. Considering the relative peak positions as well as areas, it certainly appears that compounds **2** and **3** are converted to **2′** and **3′**, respectively, by the intermediate exposure to high pH. (Shortly we will also discuss compounds **1′** and **4′**).

What happens at an elevated pH is that the two fluorine atoms attached to the boron are replaced by hydroxy groups. This was demonstrated in two ways. First, a fluoride ion selective electrode was used to determine that two equivalents of fluoride are formed when hydrazide **4**, as a pure sample, is exposed to alkaline pH. Second, appropriate protonated molecule and fragment ions were observed for compound **4′** by fast atom bombardment mass spectrometry (data not shown). Compound **4** and its hydrolysis product **4′** comigrate at both pH 6.0 and 10.4 by CE (data not shown), demonstrating the neutrality of the dihydroxy product **4′**. Also seen in Fig. 2 is a peak for **1′**, the dihydroxy version of BO-IMI.

Some of the BO-IMI forms a conjugate with EDC during the course of the reaction. To confirm the identity of this side product, **5**, a sample of a reaction mixture was subjected to fast atom bombardment mass spectrometry, leading to the mass spectrum shown in Fig. 3. No incubation at pH 10.4 was done for the latter sample, so the species detected was the difluoro compound. The peak at *m*/*z* 174 apparently arises from reaction of the released *m*/*z* 156 moiety,

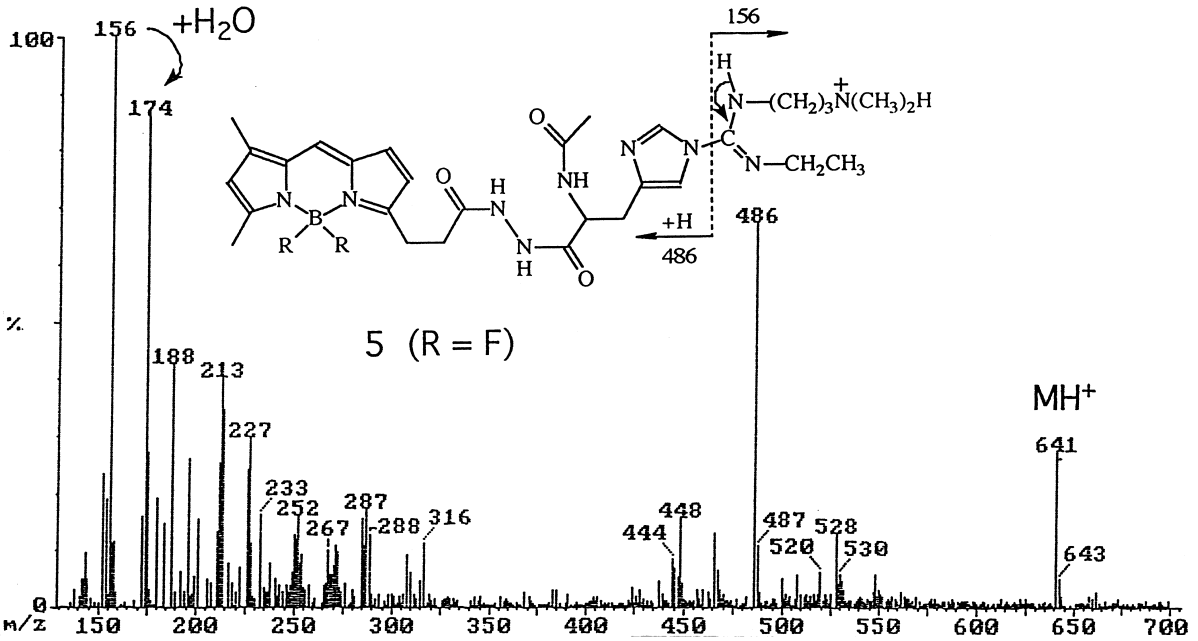


Fig. 3. Mass spectrum (capillary liquid chromatography continuous-flow fast atom bombardment) of BO-IMI-EDC, **5**. The solvent was 1% glycerol in 0.01 *M* ammonium acetate, and the instrumentation and technique were described before [9].

a carbodiimide, with water to form the corresponding urea, as indicated in the figure. Compound **5** partly reforms **1** when exposed to 0.1 *M* HCl for 5 h, consistent with the known hydrolytic instability of an imidazole–carbodiimide conjugate at low pH [8].

Both compounds **5′** and **1′** migrate as anions at pH 10.4. There is no known pK_a for imidazole in this pH region. BODIPY FL C_3 hydrazide and its dihydroxy analog have essentially the same migration times at this pH, so the charge cannot arise from the dihydroxyboron moiety. Thus some ionization of the dicarbohydrazide component of **5′** and **1′** must be taking place. To support this hypothesis, we acetylated BODIPY FL C_3 hydrazide in acetonitrile with acetic anhydride, and observed that the product, **6′**, unlike dihydroxy BODIPY hydrazide, **4′**, also migrates as a negatively charged species at pH 10.4. The alkaline pK_a values of dihydroxy BO-IMI and **6′** were found to be 10.0 and 10.8, respectively, by monitoring their electrophoretic mobility relative to that of BODIPY hydrazide (neutral marker of electroendosmosis) as a function of pH (data not shown).

In Fig. 4 is shown the separation by CE of BO-IMI conjugates of normal deoxynucleotides, two DNA adducts (compounds **8** and **9**) and also a synthetic precursor, **7**, for the preparation of **9**. The inset in this figure shows the electropherogram of a BO-IMI labeled DNA oligomer. This latter result was reported before [1], but the electropherogram was not shown. Each conjugate gives a single peak since the N1 and N3 isomers for each one comigrate at alkaline pH.

With the existing reagent and conditions we achieved the detection of 60 femtomoles of 5′-dAMP as shown by the electropherogram in Fig. 5B. Corresponding electropherograms for the detection of 600 femtomoles of 5′-dAMP, and for a blank reaction, are shown in Fig. 5A,C, respectively. While these samples were cation-exchange filtered prior to the CE separation, a peak for dihydroxy BO-IMI (**1′**) is observed. Apparently there are two origins of this peak: hydrolysis of some **2′** and **3′** (especially of the less stable **2′**), and incomplete removal of BO-IMI (a limited amount of cation-exchange packing was employed). The presence of a significant peak for compound **10′**, dihydroxy BODIPY FL C_3 carboxylic acid, reveals some hydrolytic instability of BO-IMI under the reaction conditions, along with the

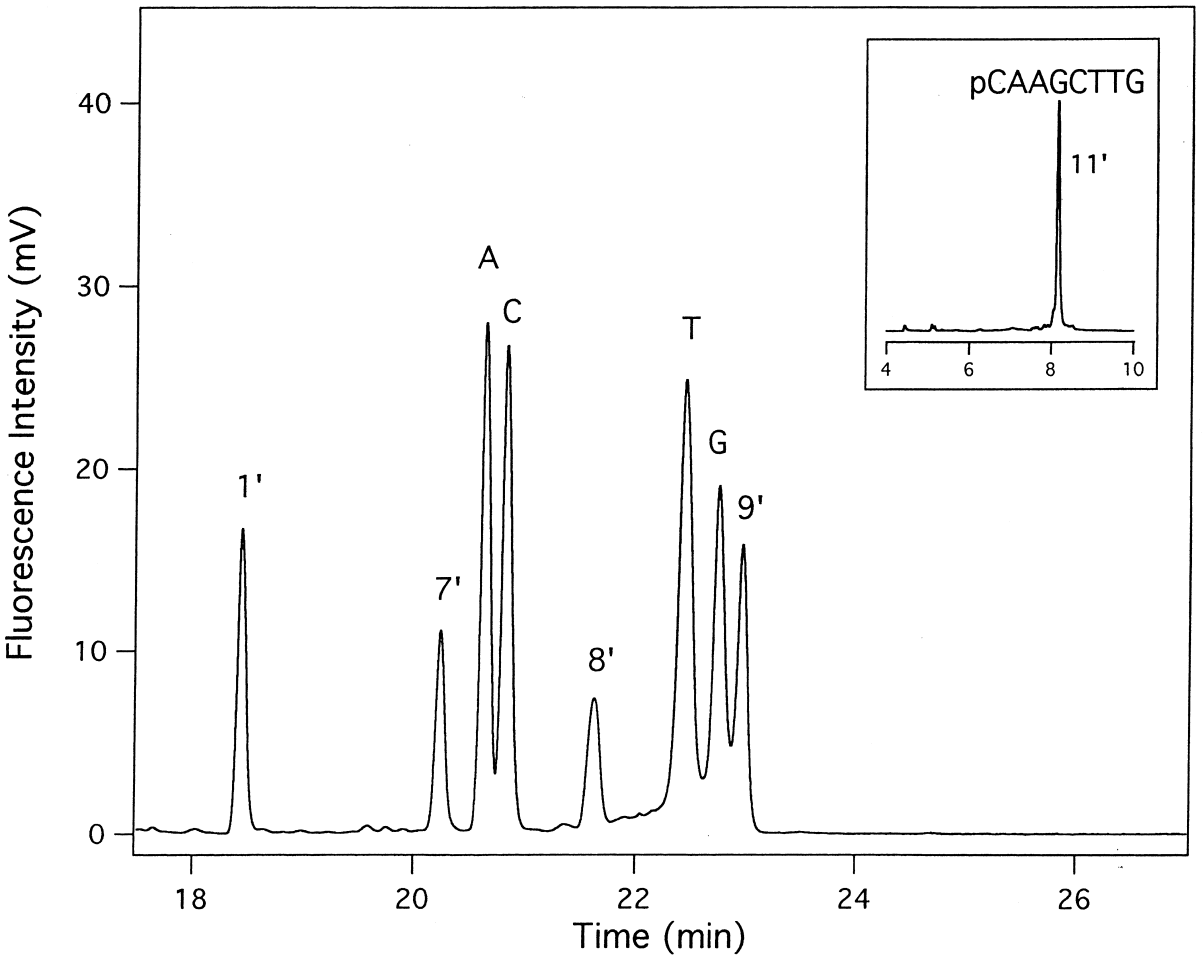


Fig. 4. Electropherogram at pH 10.4 (buffer D) of dihydroxy-BO-IMI conjugates of 5′-dAMP (A), 5′-dCMP (C), TMP (T), 5′-dGMP (G), C8-benzyloxy-5′-dAMP (**7′**), C8-[N-acetyl-N-(2-fluorenyl)]amino-5′-dGMP (**8′**), and C8-hydroxy-5′-dAMP (**9′**). Peak **1′** is dihydroxy-BO-IMI. Inset, corresponding dihydroxy BO-IMI conjugate of the oligodeoxynucleotide 5′-pCAAGCTTG (**11′**), at pH 8.7 (buffer C, which was in use at the outset of our project). Sample preparation: BO-IMI (1.2×10^{-2} *M*), total adducts (1.2×10^{-2} *M*), and EDC (9×10^{-2} *M*), all in buffer A, 10 μl each, were mixed, and the resulting solution was kept unstirred in the dark at room temperature for 2 h followed by filtration through a pasteur pipet column containing ~140 mg propyl sulfonic acid silica retained on glass wool.

failure of this acid to retain, of course, on the cation exchanger. Unfortunately, compound **10′**, in turn, can undergo activation by EDC in the reaction mixture to label amino contaminants. We assume that this accounts for at least some of the other background peaks that are present in this electropherogram. This inherent limitation of BO-IMI, arising from the instability of the leash between the dye and the imidazole moiety, suggests that a leash with greater hydrolytic stability would make it easier to achieve higher sensitivity.

4. Conclusion

BO-IMI is a useful reagent, as demonstrated here (e.g., Figs. 4 and 5) and in our prior publications. While the labeling reactions of phosphomonoesters is complex, the reaction is specific and, overall, can lead to single peaks by CE-LIF from such analytes. We are working on improving the reagent by establishing a more stable leash between the imidazole and dye moieties, towards a goal of extending the sensitivity below the current femtomole level.

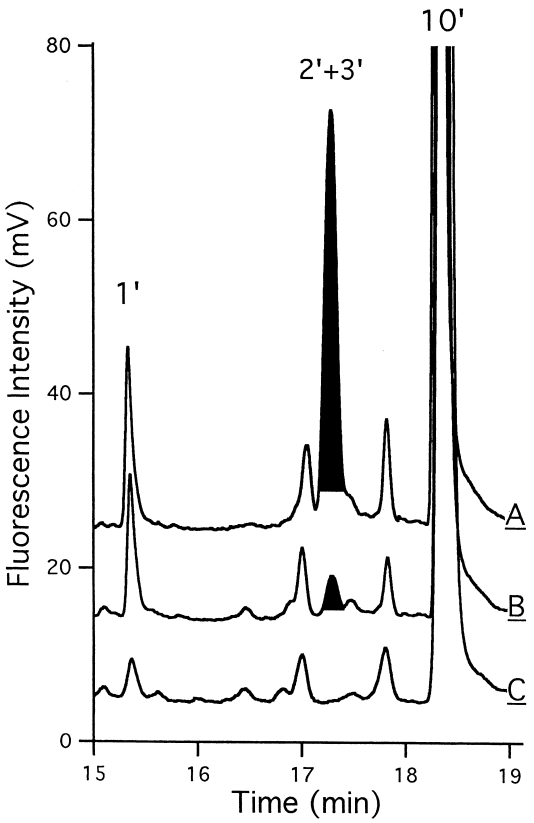


Fig. 5. Electropherograms obtained by reacting 600 (A), 60 (B) and 0 (C) femtomoles of 5′-dAMP with BO-IMI followed by cation exchange filtration, evaporation, addition of buffer D, incubation for 30 min, and injection. Peak assignments: see Table 1.

Acknowledgements

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References

[1] P. Wang, R.W. Giese, Anal. Chem. 65 (1993) 3518.
[2] R. Giese, P. Wang, US Patent 5 512 486, 1996.
[3] P. Wang, R.W. Giese, Anal. Biochem. 230 (1995) 329.
[4] S.M. Wolf, P. Vouros, Anal. Chem. 67 (1995) 891.
[5] S. Abdel-Baky, R.W. Giese, Synth. Commun. 23 (1993) 861.
[6] E.S. Yeung, P. Wang, W. Li, R.W. Giese, J. Chromatogr. 608 (1992) 73.
[7] A. Kanavarioti, M.T. Rosenbach, J. Org. Chem. 56 (1991) 1513.
[8] M. Bodanszky, J. Martinez, Synthesis 0 (1981) 333.
[9] S.M. Wolf, P. Vouros, Chem. Res. Toxicol. 7 (1994) 82.