Phosphate-specific fluorescence labeling under aqueous conditions

ARTICLE *in* ANALYTICAL CHEMISTRY · MAY 1993

Impact Factor: 5.64 · DOI: 10.1021/ac00071a032

2 AUTHORS:

Poguang Wang
Northeastern University
33 PUBLICATIONS **487** CITATIONS
SEE PROFILE

Roger Giese
Northeastern University
143 PUBLICATIONS **1,502** CITATIONS
SEE PROFILE

Phosphate-specific fluorescence labeling under aqueous conditions

Poguang. Wang, and Roger W. Giese

Anal. Chem., **1993**, 65 (23), 3518-3520• DOI: 10.1021/ac00071a032 • Publication Date (Web): 01 May 2002

CORRESPONDENCE

Phosphate-Specific Fluorescence Labeling under Aqueous Conditions

Poguang Wang and Roger W. Giese*

Department of Pharmaceutical Sciences in the Bouve College of Pharmacy and Health Professions and Barnett Institute of Chemical Analysis, Northeastern University, Boston, Massachusetts 02115

INTRODUCTION

The phosphate group is important in biochemistry. It contributes to the structure and function of all major classes of organic biomolecules comprising proteins, lipids, carbohydrates, and nucleic acids, along with precursors, combinations, and metabolites of these substances. As biomolecules function under aqueous conditions, it is useful to have techniques for the specific detection of phosphate compounds under such conditions.

One general approach to the detection of organic phosphate compounds is to derivatize them with a molecular label such as a fluorophore. For example, nucleotides and oligonucleotides have been labeled under aqueous conditions by activating the terminal phosphate moiety with a water-soluble carbodiimide for coupling to an alcohol or amine reagent possessing a label, or possessing a second functional group permitting subsequent attachment to a label.[1-4] However, this general technique is subject to interferences since carboxylic acids also will be labeled. A convenient method for efficiently removing the residual reagents at the end of the reaction is not always available, which can compromise the usefulness of a labeling reaction in chemical analysis.

Here we introduce a technique which achieves phosphate-specific fluorescence labeling under aqueous conditions. The method is convenient since it is carried out in a single step and the residual reagents are removed by cation-exchange trapping.

EXPERIMENTAL SECTION

BO-IMI Synthesis. 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl hydrazide (BODIPY FL C_3 hydrazide) 5 mg, from Molecular Probes (Eugene, OR) in 0.75 mL of dimethyl sulfoxide, 30 mg of *N*-acetyl-L-histidine, and 50 mg of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) in 2.1 mL of buffer A (see below) were stirred for 1.5 h at room temperature in the dark. The reaction mixture was applied to a column containing 500 mg of propanesulfonic acid silica (J. T. Baker Inc., Phillipsburg, NJ) that had been washed with buffer C (see below) until the eluent was pH 7.2 (~15 mL). After the column was washed with 30 mL of buffer D, the product was eluted with 7 mL of the supernate obtained by combining 9 mL of brine and 3 mL of methanol and extracted into 5 × 6 mL of ethyl acetate/acetonitrile (1:1 v/v). The latter solution (BO-IMI stock) of pure product (it was a single peak both by CE in buffer B, which demonstrated the absence of BODIPY FL C_3 hydrazide, and by HPLC, which also showed the absence of *N*-acetyl-L-histidine) was stored at −20 °C in the dark. The yield (based on diluting 1:20 into methanol and using ϵ = 71 000 at 503 nm[5]) was 95%. This absorptivity value is for BODIPY FL C_3 hydrazide. HPLC conditions: Microsorb 86-200-C5, C18-Si column, 5 μm, 4.6 mm i.d. × 25 cm length (Rainin, Woburn, MA); 0.05 M phosphate, pH 7.1/acetonitrile, 70:30, 1 mL/min; *N*-acetyl-L-histidine (1.9 min), BO-IMI (9.5 min), BODIPY FL C_3 hydrazide (15.1 min). FAB-MS, *m/z* 486 $(M + H)^+$.

Buffers: (A) 0.5 mL of 0.2 M 2-*N*-(morpholino)ethanesulfonic acid (MES), 0.06 mL of 0.1 M NaOH, and 9.5 mL of water (pH 5.0); (B) 2.0 mL of 0.2 M MES, 0.28 mL of 0.5 M tris-(hydroxymethyl)aminomethane (TRIS) (pH 6.0), 4.0 mL of methanol, and 36 mL of water; (C) 0.1 M NaOH added to 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), (pH 7.2); (D) 0.1 M NaOH added to 0.01 M HEPES (pH 7.2); (E) 0.5 mL of 0.2 M MES, 0.38 mL of 0.1 M NaOH, and 9.1 mL of water (pH 6.0); (F) 0.8 mL of 0.5 M boric acid, 0.8 mL of 0.5 M TRIS, 4 mL of acetonitrile, and 34.4 mL of water (pH 8.7); (G) 2.0 mL of 0.05 M sodium borate, 1.8 mL of 0.1 M NaOH, 5 mL of acetonitrile, and 41.2 mL of water (pH 10.4).

Analytical Procedure. An aliquot of the BO-IMI stock was evaporated and redissolved in buffer E to a concentration of 12 mM. To a 0.3-mL conical vial was added 10 μL each of the analyte solution (dAMP, dCMP, dGMP, and TMP, 0.3 mM each) fresh (≤1 h old) 12 mM BO-IMI, and fresh (≤10 min) 90 mM EDC (each in buffer E). The reaction mixture was kept dark for 2.75 h at room temperature (unstirred), and then 5 μL was applied to a Pasteur pipet column containing 200 mg of propanesulfonic acid silica retained on glass wool that had been washed with 5 mL of buffer C and 4 mL of buffer D. All elutions were done under pressure from a rubber bulb. The analyte–BO-IMI conjugates were eluted with 2 × 0.5 mL of buffer D, and the combined sample was diluted 50-fold into buffer G prior to hydrostatic injection (5-cm height difference for 20 s; ca. 10 nL) into the CE column. The CE equipment, including an argon ion laser for on-column detection (488 nm) was described before.[6,7] The separation was performed in a 100-cm-long fused silica capillary (75 μm i.d.; PolyMicro Technologies, Phoenix, AR) with the detection window (burned-off polyimide) 60 cm from the injection (anode) end of the capillary, at 18 kV. Yields were calculated based on peak areas using a solution of BODIPY FL C_3 hydrazide as a calibrant, where the concentration of the latter solution was determined by relying on the absorptivity value (see above) of this compound.

Polar-Switching Injection. The technique of "polarity-switching injection", introduced by Chien and Burgi[8] was utilized for an attomole-level CE separation. A coupling reaction was performed and applied to the ion-exchange cartridge as above. The ion-exchange cartridge was washed with 4 mL of water (instead of 4 mL of buffer D as above), the BO-IMI conjugates were eluted with 2 × 0.5 mL of water, and the resulting sample was diluted (5 × 10^5)-fold with water. The entire capillary (4.4-μL volume) was filled with the sample, and the buffer reservoirs were filled with buffer G as usual. Voltage (18 kV) was applied

* Address reprint requests to this author.

(1) Jain, R.; Sharma, M. *Cancer Res.* **1993**, *53*, 2771–2774.
(2) Al-Deen, A. N.; Cecchini, D. C.; Abdel-Baky, S.; Moneam, N. M. A.; Giese, R. W. *J. Chromatogr.* **1990**, *512*, 409–414.
(3) Kricka, L. J., Ed. *Nonisotopic DNA Probe Techniques*; Academic Press: San Diego, CA, 1992.
(4) Ivanovskaya, M. G. *Nucleosides Nucleotides* **1987**, *6*, 913–939.
(5) Haugland, R. P. *Handbook of Fluorescent Probes and Research Chemicals*; Molecular Probes: Eugene, OR, 1992; p 46.
(6) Li, W.; Moussa, A.; Giese, R. W. *J. Chromatogr.* **1992**, *608*, 171–174.
(7) Yeung, E. S.; Wang, P.; Li, W.; Giese, R. W. *J. Chromatogr.* **1992**, *608*, 73–77.
(8) Chien, R.-L.; Burgi, D. S. *Anal. Chem.* **1992**, *64*, 1046–1050.

0003-2700/93/0365-3518$04.00/0

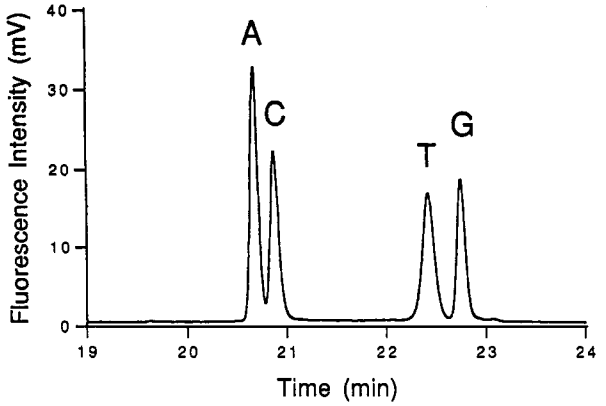
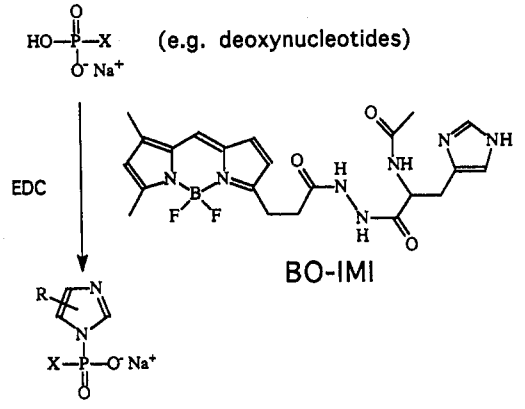


Figure 1. Capillary electropherogram (buffer G) of the BO-IMI phosphorimidazolide conjugates of 5′-dAMP (A), 5′-dCMP (C), 5′-TMP (T), and 5′-dGMP (G). This electropherogram was obtained by reacting a mixture of the four deoxynucleotides (3 nmol each) with BO-IMI and injecting ca. 10 nL of the final sample, corresponding to 3×10^{-8} of the initial sample.

(anode at the detector end of the capillary) until the current reached 3.4 μA (95% of its normal value of 3.6 μA). The eletrical leads were reversed, and the separation was begun at the same voltage. The yield was estimated by polarity-switching injection of authentic BO-IMI–dAMP as a calibrant (5.8×10^{-11} M in water, based in turn on calibration by CE-LIF, as above, against BODIPY FL C_3 hydrazide).

RESULTS AND DISCUSSION

By taking advantage of the known ability of a water-soluble carbodiimide such as EDC to activate a carboxylic acid for nucleophilic coupling,[9] we prepared BO-IMI by coupling *N*-acetyl-L-histidine to the nucleophilic fluorescent dye BODIPY FL C_3 hydrazide. We then labeled some compounds bearing a terminal phosphate group with BO-IMI, as indicated, similarly relying on the known ability of EDC to activate a phosphate group of this type for coupling.[10,11] For example, reaction of a mixture of 3 nmol each of 5′-dAMP, 5′-dCMP, 5′-TMP, and 5′-dGMP with BO-IMI in the presence of EDC under aqueous conditions, followed by filtration through a cation-exchange cartridge (which nicely removes both residual BO-IMI and EDC), and then injection into a capillary electrophoresis column with laser-induced fluorescence detection (CE-LIF) gives the electropherogram shown in Figure 1. The yields of the A, C, T, and G products in Figure 1 are 64, 47, 48, and 38%, respectively, from a 2.75-h reaction at room temperature. In this reaction, the molar ratio of the total deoxynucleotides to BO-IMI to EDC was 1:10:75. We did not attempt to increase the yield by increasing the concentrations of the reagents, but a somewhat higher yield could be obtained by lowering the temperature and extending the reaction time; e.g., 73, 71, 72, and 50% yields, respectively, were obtained after 24 h at 2 °C.



Reaction of each of these deoxynucleotides individually with BO-IMI, in a 1:1 molar ratio, shows that 5′-dAMP reacts faster (~2× at the outset) than the others for unknown reasons. Perhaps 5′-dAMP provides a favorble microenvironment for the coupling reaction. For each of these compounds, the total peak area by CE-LIF (decreasing BO-IMI and increasing BO-IMI–dNMP) is constant throughout the reaction, establishing that BO-IMI and these conjugates have the same fluorescence yields. In part, we selected a BODIPY dye for this purpose due to its anticipated inertness to EDC.

Early in our studies, 5′-dAMP was selected arbitarily as a model analyte to develop the labeling reaction. It was found that this nucleotide is labeled to the same extent with BO-IMI, with no evidence of side products by CE-LIF, in the presence or absence of glycine (10-fold molar excess over 5′-dAMP) or in 1% albumin. This result is consistent with the hydrolytic instability of acylimidazoles,[12] accounting for the specificity of BO-IMI for labeling a phosphate as opposed to a carboxyl group.

BO-IMI–5′-dAMP is relatively stable at alkaline pH but hydrolyzes to re-form BO-IMI under acidic conditions (e.g., $t_{1/2}$ = 2.7, 2.8, 4.7, and 19.7 h at pH 2, 4, 5, and 6, respectively; no hydrolysis after 5 h at pH 7–10.4; 2% hydrolysis after 1 week at pH 8.7; solutions stored at room temperature in the dark). Further, this conjugate is stable in 0.1 M glycinamide, carbohydrazide, ethylenediamine, and mercaptoethylamine, and in 1% albumin, but re-forms 24% BO-IMI in 0.1 M imidazole, all after 2 h at room temperature, pH 8.7.

When BO-IMI–5′-dAMP is determined by CE-LIF at pH 6.0, two peaks are observed in a ratio of 3:97 with migration times of 4.05 and 4.26 min, respectively (data not shown; 40-cm migration in a 70-cm capillary at 23 kV). The other nucleotide conjugates behave the same, as do corresponding 3′-dNMPs (except the product ratio is 2:98 for the latter). Apparently the two peaks arise from attachment of the nucleotide to both the *N*3 and *N*1 positions of the imidazole moiety of BO-IMI. Perhaps the major product is the *N*1 isomer due to a steric effect. These products comigrate at alkaline pH (e.g., pH 8.7 and above; pH 10.4 with buffer G was used in Figure 1).

BO-IMI also labels some other phosphate compounds: 5′-dADP (33% yield after 2 h of reaction), 5′-dATP (11%), 5′-dGTP (15%), *O*-phospho-L-tyrosine (17%), α-D-glucose 6-phosphate (12%), and CAAGCTTG-5′-phosphate (25%). Other phosphate compounds (α-D-glucose 1-phosphate, phosphocreatine, phosphoenolpyruvate, *O*-phospho-L-serine) failed to react with BO-IMI under the current conditions, apparently due to electrostatic or steric constraints. No additional efforts were made at this stage to achieve labeling of the latter compounds, or to enhance the yields of the prior compounds, by changing the conditions of the reaction.

To demonstrate the practical sensitivity potentially available by this technique, a reaction mixture was formed as in Figure 1, but then the ion-exchange cartridge was eluted with water, and the subsequent 1-mL sample was diluted (5×10^5)-fold with water prior to injection of 4.4 μL into the CE column by polarity switching, leading to the electropherogram

(9) Hermanson, G. T.; Mallia, A. K.; Smith, P. K. *Immobilized Affinity Ligand Techniques*; Academic Press: San Diego, CA, 1992.
(10) Chu, B. C. F.; Wahl, G. M.; Orgel, L. E. *Nucleic Acids Res.* **1983**, *11*, 6513–6529.
(11) Morrison, L. E.; Halder, T. C.; Stols, L. M. *Anal. Biochem.* **1989**, *183*, 231–244.
(12) Anjaneyulu, P. S. R.; Staros, J. V. *Int. J. Pept. Protein Res.* **1987**, *30*, 117–124.

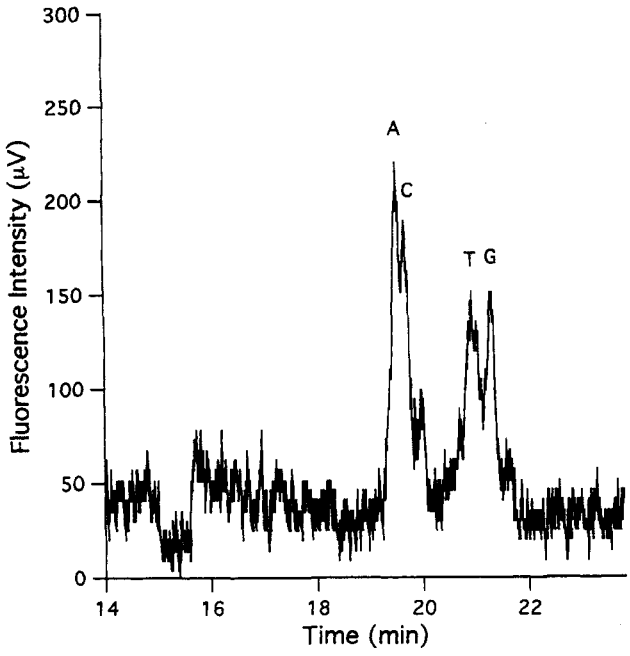


Figure 2. Capillary electropherogram as in Figure 1, except that the reaction mixture was purified and diluted (5×10^5)-fold in water (to an average concentration of 5.0×10^{-13} M per conjugate, assuming 100% recovery after the ion exchange step) prior to polarity-switching injection of 4.4 μL (containing ~2.2 amol per conjugate) into the capillary.

shown in Figure 2. This injection technique was introduced by Chien and Burgi[8] and provides, in a simple way, a means to achieve trace detection by CE-LIF starting with dilute sample. Taking into account the coupling yields established above for this reaction (38-64%; averaging to 49%) and assuming a 100% recovery of the products after the ion-exchange step, the 4.4-μL volume contained an average of 2.2 amol per conjugate, corresponding to 5.0×10^{-13} M per conjugate. A comparison of peak areas when 10 nL of a given sample is injected normally (syphoning) vs when 4.4 μL of a 10^3-fold diluted sample is injected with polarity switching reveals that ~75% of the sample is lost in the latter case. Since the peak areas relative to sample concentration are the same when the 5.0×10^{-13} M vs a 100-fold higher concentration is injected, the 75% loss is apparently due entirely to sample backout. Overall, the enrichment observed is about 50- and 100-fold in terms of peak height (reduced by peak broadening) and peak area, respectively, under the current conditions, including the negative contribution from the sample loss.

We are mainly interested in BO-IMI labeling/CE-LIF for the trace detection of covalent damage to DNA, termed "DNA adducts". For this purpose it would be similar in concept to ^{32}P-postlabeling-TLC,[13-15] a radioenzymatic technique for this purpose. In related work by Sharma and co-workers (ref 1 and references cited therein), DNA adducts have been detected by the analogous sequence: coupling with ethylenediamine, reaction with dansyl chloride, and separation by HPLC with fluorescence detection.

Our work should also encourage the more general application of the phosphorimidazolide linkage, as in conjugation work with biomolecules, given that it can be formed specifically (aside from carbodiimide side reactions) under mild, aqueous conditions and that its stability can be controlled by pH or the presence of imidazole. Previously phosphorimidazolides have been utilized as reaction intermediates (involving imidazole or a methylimidazole) rather than as products.[2,10,16]

Conclusion. The analytical methodology introduced here has several attractive features: (1) specificity of BO-IMI for some terminal-phosphate compounds; (2) single-step, efficient labeling under mild aqueous conditions; (3) simple removal by ion-exchange filtration of the coupling reagents at the end of the reaction; (4) practical (low column cost, easy column cleaning, polarity-switching injection of a multimicroliter sample volume) and sensitive (low-attomole level) separation/detection of the products by CE-LIF; (5) use of the 488-nm line of an argon ion laser for the LIF to achieve relatively high wavelength excitation to minimize sample noise with an inexpensive laser with stability in intensity; and (6) opportunity to reverse the labeling and recover the intact phosphate–analyte by exposure to slightly acid pH or imidazole. These collective advantages make the methodology worthy of further attention as a means to detect DNA adducts and some other trace phosphate–analytes.

ACKNOWLEDGMENT

This work was funded by the Office of Health and Environmental Research Division, Department of Energy, as Grant DE-FG02-90ER60964. Publication number 595 from the Barnett Institute.

RECEIVED for review June 4, 1993. Accepted August 23, 1993.

(13) Randerath, K., Randerath, E., Garner, R. C., Farmer, P. B., Steel, G. T., Wright, A. S., Eds. *Human Carcinogen Exposure*; IRL Press: Oxford, UK, 1991; pp 25–46.

(14) Geneste, O.; Camus, A. M.; Castegnaro, M.; Petruzzelli, S.; Macchiarini, P.; Angeletti, C. A.; Giuntini, C.; Bartsch, H. *Carcinogenesis* **1991**, *12*, 1301–1305.

(15) Manchester, D. K.; Wilson, V. L.; Hsu, I.-C.; Choi, J.-S.; Parker, N. B.; Mann, D. L.; Weston, A.; Harris, C. C. *Carcinogenesis* **1990**, *11*, 553–559.

(16) Kanavarioti, A.; Rosenbach, M. T. *J. Org. Chem.* **1991**, *56*, 1513–1521.