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Short communication

Preparation of an IMI dye (imidazole functional group) containing a 4-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole fluorophore for labeling of phosphomonoesters

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Abstract

We are studying dye–imidazole conjugates (''IMI dyes'') as reagents for labeling phosphomonoesters such as nucleotides. Previously we have employed a BODIPY dye in our IMI reagents, and analyzed the labeled products by capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) involving an argon ion laser. (The BODIPY fluorophore is a 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene). Here we broaden the technology by preparing a DBD–IMI dye [DBD=4-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole], and using a helium–cadmium laser. While DBD–IMI (IMI3) is about 50× more stable photolytically than a BODIPY–IMI dye (IMI2, a conjugate of a BODIPY dye with histamine, was tested), the detection limit for IMI2 ($5\cdot10^{-11}$ *M*; *S*/*N*=5, CE–LIF with an argon ion laser) is tenfold better than that for IMI3 ($5\cdot10^{-10}$ *M*, *S*/*N*=5, helium–cadmium laser). IMI3 conjugates of the four major DNA nucleotides were prepared and detected by CE–LIF.

Keywords: Detection, electrophoresis; Derivatization, electrophoresis; Dyes; Nucleotides

1. Introduction

Previously we introduced fluorescent dye–imidazole conjugates (''IMI dyes'') as derivatization reagents for the detection of phosphomonoesters [1–4]. These dyes incorporate an imidazole reactive group which covalently links to a phosphomonoester once the latter is activated with a carbodiimide reagent. The dye-labeled phosphomonoester then can be detected based on its fluorescence, e.g., by means of capillary electrophoresis with laser-induced fluorescence detection (CE–LIF). In this way we have detected standards of some nucleotides, glucose-6-phosphate, and *O*-phosphotyrosine. Glucose-1-phosphate and *O*-phosphoserine did not label apparently due to electrostatic or steric effects [1]. Also we have detected pepsin (a phosphoprotein) via phosphate-specific labeling with an IMI tag [2]. The labeling reaction is specific for phosphomonoesters because the side reaction with carboxylic acids leads to *N*-acylimidazoles which are hydrolytically unstable. Mechanistic details of the reaction were studied [3], and a slight hydrolytic instability of IMI1 (a conjugate of BODIPY FL C_3 hydrazide with N-acetylhistidine that we originally called ″BO–IMI [1]) was overcome by the preparation of IMI2, a conjugate of

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BODIPY FL SE with histamine [4]. (The BODIPY fluorophore is a 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene).

Thus far our IMI reagents have incorporated a BODIPY dye, which has an absorption maximum near the 488 nm line of the argon ion laser. This is the type of laser that we have employed to date in our CE–LIF system. Since the helium–cadmium laser also is employed frequently in CE–LIF systems, we sought to develop an IMI dye suitable for this alternative equipment.

We have selected a DBD dye for this purpose, comprising a 4-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD) fluorophore. This type of dye was introduced and further developed by Imai and coworkers (e.g., Refs. [5,6]) in the form of chiral reagents for the analysis of various racemates by derivatization–HPLC–fluorescence detection. An excitation wavelength of 450 nm was employed [6] which is close to the 442 nm line of a helium–cadmium laser. Conveniently, the dye is available commercially as an acid chloride, suitable for conversion to an IMI reagent. Two additional features that made us select this dye are its large Stokes shift of about 120 nm, and its lack of functional groups that could react with the carbodiimide and thereby complicate our labeling reaction for a phosphomonoester.

2. Experimental

2.1. Chemicals

4 - (*N*, *N* - Dimethylaminosulfonyl) - 7 - (2 - chloro - formylpyrrolidin-1-yl)-2,1,3-benzoxadiazole [(*R*)-(+)-DBD-ProCl] was purchased from Tokyo Kasei (Tokyo, Japan). Histamine was from Fluka (Buchs, Switzerland) and was recrystallized in chloroform before use. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), 2-(*N*-morpholino)ethanesulfonic acid (MES) and the sodium salts of 2′-deoxyadenosine 5′-monophosphate, thymidine 5′-monophosphate, 2′-deoxycytidine 5′-monophosphate, and 2′-deoxyguanosine 5′-monophosphate (dGMP) were from Sigma (St. Louis, MO, USA). Electrophoresis purity grade sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Hercules, CA, USA). ACS grade sodium bicarbonate was from Baker (Phillipsburg, NJ, USA). Distilled deionized water was used in the preparation of all aqueous solutions. All other chemicals and organic solvents were of analytical-reagent grade and were used without further purification. IMI2 was prepared as described previously [4].

2.1.1. DBD–Pro-IMI (IMI3)

To a 1 ml chloroform solution of (*R*)-(+)-DBD-ProCl (2.23 mg, 6.22 μmol) was added 1.5 ml of chloroform containing histamine (1.73 mg, 15.6 μmol) and the mixture was stirred at room temperature overnight. The residue from evaporation was subjected to silica flash chromatography (10 ml pipet column) using methanol: chloroform (5:95, v/v) as mobile phase. The fraction containing the product (second band) was dried in a Savant SpeedVac concentrator (Holbrook, NY, USA) and then was further purified by submarine gel electrophoresis using the technique that we have described previously [3]. Matrix assisted laser desorption ionization time-of-flight mass spectrometry was used to confirm the molecular mass of the product, with 2,4,6-trihydroxyacetophenone as the matrix.

2.2. Nucleotide labeling with IMI3

IMI3 was concentrated to $5 \cdot 10^{-4}$ *M* in a Savant SpeedVac concentrator. Nucleotides (10^{-6} *M*) and EDAC (0.10 *M*) were prepared in 0.10 *M* pH 7.0 (adjusted with NaOH) MES buffer. Five μl aliquots each of the nucleotide mixture, EDAC, and IMI3 were added to a glass conical vial and then mixed thoroughly by swirling. The reaction was allowed to stand in the dark overnight. An aliquot of 1.5 μl was removed and subjected to immobilized metal affinity chromatography (IMAC) as described before [4].

2.3. CE–LIF

The CE–LIF system was the same as that reported before [1], except that a different laser, wavelength, and set of filters were used: helium–cadmium laser (Liconix, Santa Clara, CA, USA), 14 mW, 442 nm excitation, 560 nm detection; 442 nm laser line filter/560 nm interference filter (Oriel, Stratford, CT, USA). The separation was done in a 65 cm (40 cm to

detector)×75 μm I.D. capillary at 16 kV (35 μA) with anodic siphoning injection (anode elevated 10 cm for 20 s to load about 10 nl). The CE buffer was 10 m*M* pH 10.60 sodium carbonate with 50 m*M* SDS.

2.4. Exposure to UV

A solution of IMI3 (100 μl of $3.7 \cdot 10^{-4}$ *M*) in methanol was placed in a conical glass vial and placed 1.5 cm under a spectraline Model ENF-24 UV lamp (365 nm) from Spectronics (Westburn, NY, USA) for 30 min at room temperature. After IMAC was done as described previously [4], the resulting sample (50 μl, 10% methanol) was subjected to CE–LIF. A control sample was processed in the same way, except for the UV irradiation. Also corresponding samples and controls of IMI2 were treated in the same way, except that the initial samples were 50 μl of $5.8 \cdot 10^{-5}$ *M* IMI2.

3. Results and discussion

IMI3, the structure of which is shown in Fig. 1, was prepared by reacting histamine with *R*-(+)-DBD-Pro-COCl. The reaction conditions were the same as what we used previously to prepare IMI2 [4]. It is conceivable that an *N*-acylimidazole intermediate might have formed in the reaction and then reacted further to form the desired product, but we did not study this. During the subsequent purification step by silica flash chromatography, there was one, unidentified colored band that moved faster than the analyte. The collected product gave a single, colored band during the subsequent purification step by submarine gel electrophoresis, which was conducted as described before [4].

The detection of $5 \cdot 10^{-10}$ *M* IMI3 by CE–LIF with a helium–cadmium laser is shown in Fig. 1, where the *S*/*N* is about 5. A similar *S*/*N* is observed for $5 \cdot 10^{-11}$ *M* IMI2 by CE–LIF with an argon ion laser (data not shown). Thus IMI3 is tenfold less sensitive

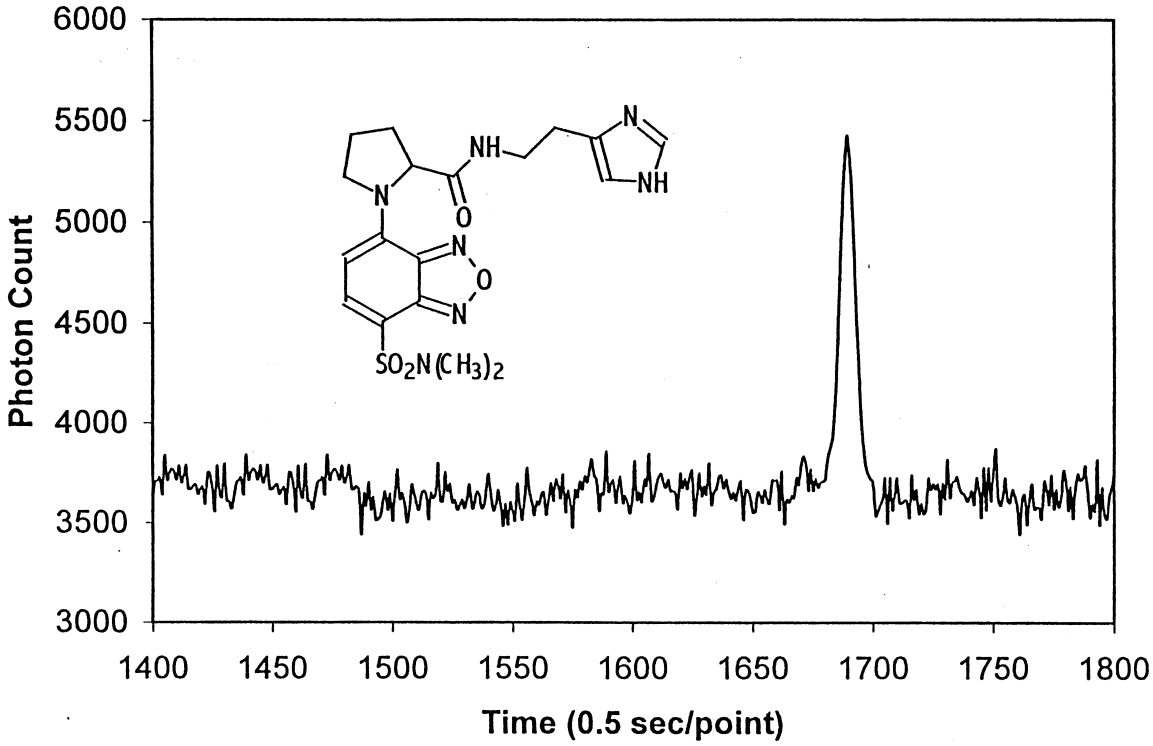

Fig. 1. Structure of IMI3, and detection of a $5 \cdot 10^{-10}$ *M* sample of this reagent by CE–LIF with a helium–cadmium laser. Electrolyte: 10 m*M* pH 10.60 sodium carbonate with 50 m*M* SDS.

than IMI2 in this comparison, which involves an optimum excitation wavelength for each. When IMI3 is measured with an argon ion laser, which is not optimum for it, a similar S/N is observed for $1 \cdot 10^{-8}$ M.

It is well known that organic compounds in general, and especially fluorophores, tend to degrade when exposed to light. Some fluorophores seem to be more susceptible than others to such degradation. We decided to compare IMI2 and IMI3 in this respect, using conditions relevant to our analytical procedure. The dye moiety in IMI2 is BODIPY whereas it is DBD in IMI3.

We exposed methanol solutions of IMI2 ($5.8 \cdot 10^{-5}$ M) and IMI3 ($3.7 \cdot 10^{-4}$ M) to UV radiation (365 nm) for 30 min. (A higher concentration of IMI3 was used since its fluorescence signal is about tenfold lower than that of IMI2; as just pointed out.) After the samples were subjected to IMAC, the electropherograms shown in Fig. 2 were obtained. Residual IMI3 is seen in both the exposed (a) and nonexposed (b) samples of this dye (Fig. 2B), apparently because its higher concentration prevented its complete removal on the small IMAC column employed. Taking into account the 13-fold higher concentration of the IMI3-derived (B) vs.

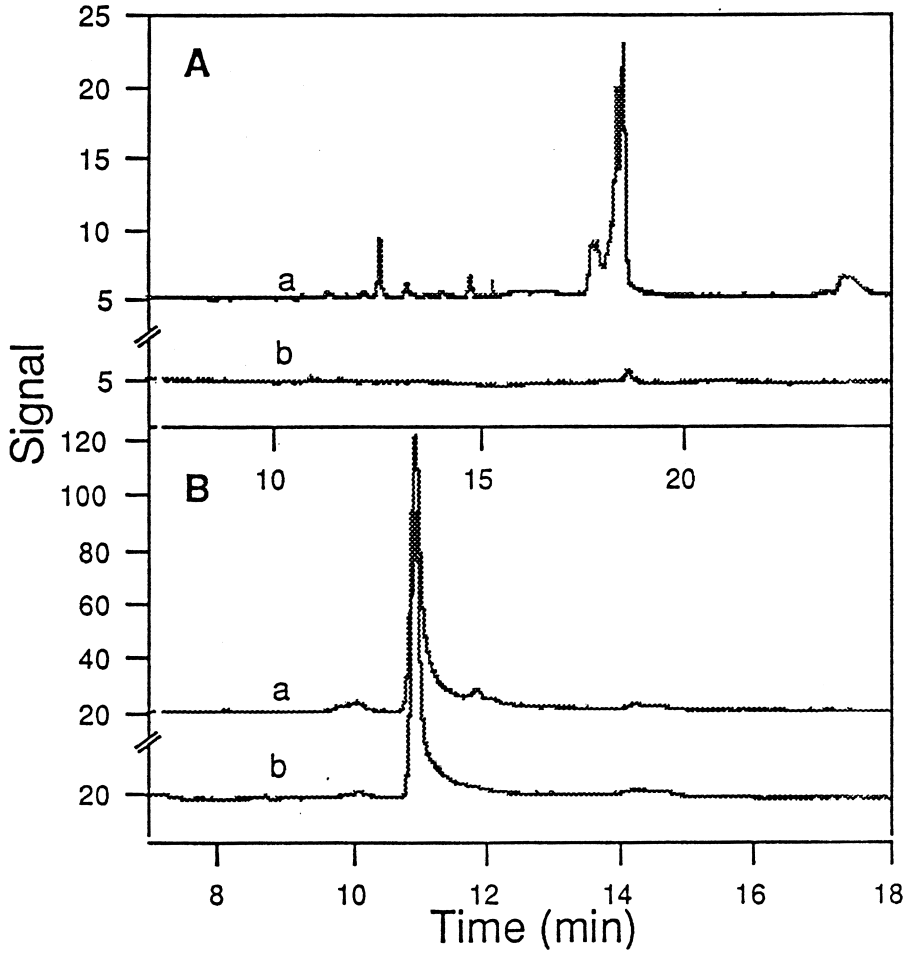

Fig. 2. Electropherograms of IMI2 (*A*) and IMI3 (*B*) before (b) and after (a) exposure to UV radiation followed by elution through an IMAC column. The concentration of the final sample is 13× higher in B than in A.

IMI2-derived (A) samples that are analyzed in Fig. 2, we see that the level of fluorescent impurities not retained on IMAC is about 50-fold higher in the IMI2-derived sample. Assuming that these impurities have undergone photolytic damage to the imidazole moiety (because they are unretained on IMAC), then the microenvironment of the imidazole moiety is quite different in the two dyes. Perhaps the richer array of functional groups in DBD relative to BODIPY causes a tighter intramolecular interaction of the dye and imidazole moieties in IMI3 and this reduces photolytic damage to the imidazole in this compound. On the other hand, the dye moieties might differ in the degrees to which they are causative rather than protective, since imidazole can be oxidized upon exposure to light in the presence of a photosensitizing dye [7]. Obviously the true mechanism is totally obscure.

Reaction of IMI3 with a mixture of the four, normal nucleotides as 5′-monophosphates, leads, after IMAC, to the electropherogram shown in Fig. 3. The separation mechanism is complex, in part because SDS micelles are present, but the effective mobility of IMI3–dGMP may be the highest mainly because guanine has the lowest acidic pK_a of the four DNA nucleobases. A comparable experiment with IMI2 (data not shown) gives about tenfold higher sensitivity, consistent with the above comparison of the IMI2 and IMI3 dyes.

4. Conclusion

The technique of labeling a phosphomonoester with an IMI dye and detecting the products by CE–LIF has been extended to a CE system incorporating a helium–cadmium laser. This was accomplished by preparing IMI3, which incorporates a DBD dye. Relative to IMI2, which incorporates a BODIPY dye, IMI3 affords tenfold poorer sensitivity

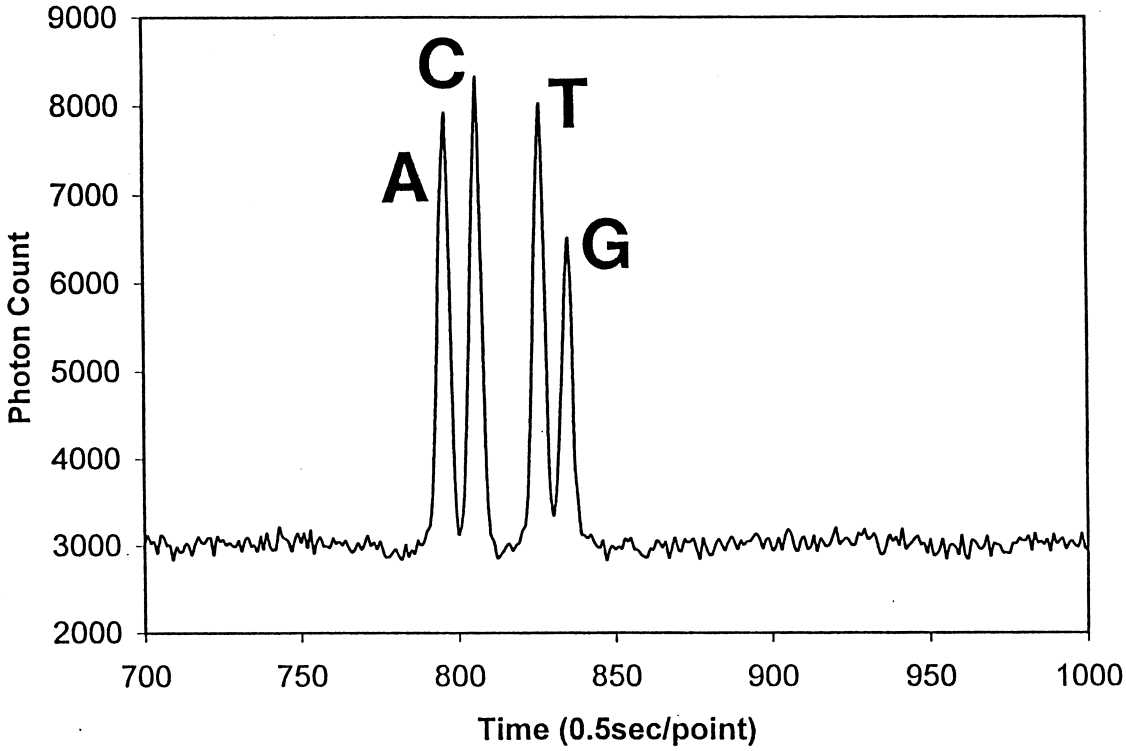

Fig. 3. Separation of IMI3 conjugates of nucleoside 5′-monophosphates by CE–LIF using the same conditions as in Fig. 1.

(argon ion laser for IMI2, helium–cadmium for IMI3, which is optimal for each).

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