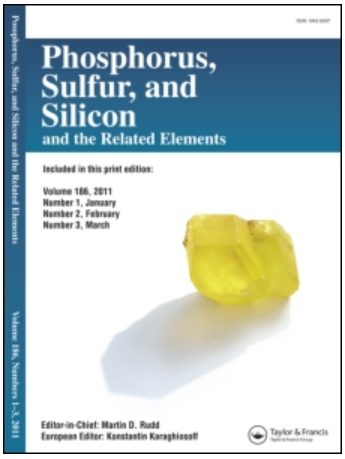

Phosphorus, Sulfur, and Silicon and the Related Elements

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

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To cite this Article Onway, Nancy E. , Fidanza, Jacqueline A. and McLaughlin, Larry W.(1990) 'Reaction of Internucleotidic Phosphorothioate Diesters with Fluorescent Reporter groups', Phosphorus, Sulfur, and Silicon and the Related Elements, 51: 1, 27 — 30
To link to this Article: DOI: 10.1080/10426509008040674
URL: http://dx.doi.org/10.1080/10426509008040674

Phosphorus, Sulfur, and Silicon, 1990, Vols. 51/52, pp. 27–30
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REACTION OF INTERNUCLEOTIDIC PHOSPHOROTHIOATE DIESTERS WITH FLUORESCENT REPORTER GROUPS

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Abstract The substitution of a non-bridging oxygen by sulfur in a DNA internucleotidic phosphorus residue creates a nucleophilic site which is amenable to labeling by reporter groups such as fluorophores or spin labels. Placement of a single phosphorothioate diester at a selected position allows site-specific attachment of the reporter group. Incorporating a phosphorothioate at each and every internucleotidic phosphorous linkage allows the incorporation of multiple reporter groups, ideally one for each nucleoside residue.

INTRODUCTION

Phosphorothioate diesters can be incorporated into nucleic acids in place of native phosphate diesters by both chemical[1,2] and enzymatic procedures[3]. Such derivatives have been used for studies of enzyme mechanisms,[3,4,5] selective cleavage of DNA during site-directed mutagenesis[6] and the protection of DNA against nucleases[7]. The chemical reactivity of phosphorothioates has been less thoroughly examined. Phosphorothioate monoesters will react with the fluorophore monobromobimane[8] and allow terminal labeling of RNA and DNA. Recently it has been reported that DNA sequencing can be performed by degrading phosphorothioate containing DNA[9,10].

We have developed a simple protocol which allows the covalent introduction of single or multiple fluorescent markers[11] or other probes into DNA fragments via alkylation of the sulfur residue of phosphorothioate diesters.

RESULTS

This study began with an examination of the reactivity of the simplest phosphorothioate diester Tp(s)T. This diester was treated with fluorphores containing a variety of functional groups and we observed that γ-bromo-α,β-unsaturated carbonyls, iodo(or bromo)acetamides and azirdinyl-sulfonamides could be employed to covalently label the phosphorothioate diester. These reactions typically proceeded in aqueous or largely aqueous solutions

at 25 and/or 50 °C for 18 to 30 h at which time conversion of the charged diester to the neutral triester (Fig. 1) was 85-95% complete. The reactions were commonly monitored by HPLC although ^{31}P-NMR could also be effectively used. Control reactions indicated that in the absence of the phosphorothioate diester no significant modification was observed.

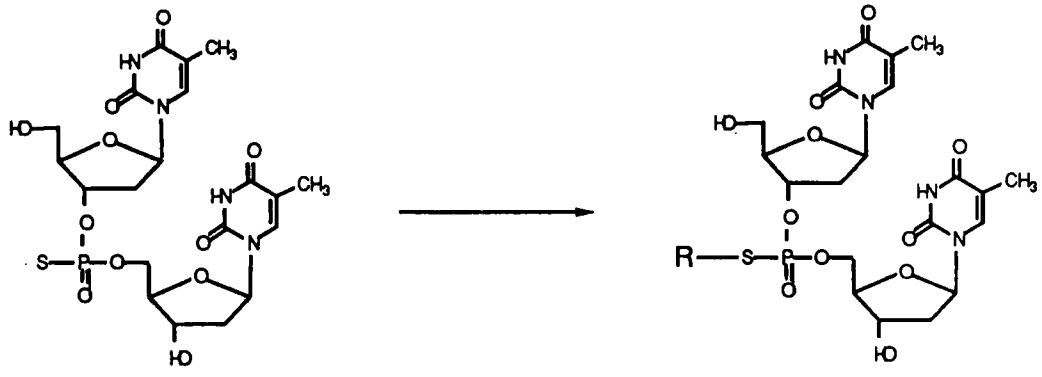
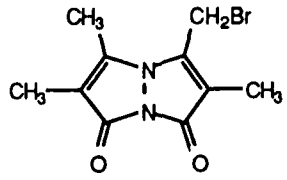


Figure 1. Alkylation of the phosphorothioate diester Tp(s)T forming the corresponding triester which carries the desired reporter group R.

Further studies with either the dodecamer, d(CGCAsAAAAAGCG), or the eicosomer, d(CGTACTAGTTsAACTAGTACG) confirmed that specific modification of the phosphorothioate diester proceeded in the absence of nonspecific base modification. The products of these reactions could be isolated by HPLC (ODS-Hypersil, 20 mM KH_2PO_4, pH 5.5 and 0-70% methanol in 60 min) and obtained in high purity.

We have additionally examined the stability of the various phosphorothioate triesters with respect to pH. The triesters were generally stable for long periods of time in neutral or mildly acidic solutions but hydrolyzed (with P-S bond cleavage) under basic conditions. The rate of hydrolysis increased with increasing pH as expected.

The most intensively investigated aspect of this process involves the introduction of multiple fluorescent markers into DNA molecules to ultimately allow non-radiative detection of nucleic acids at levels comparable to those generally achieved with radioisotopes. In order to obtain high detection sensitivity using fluorography it was necessary to attach multiple fluorophores to the DNA fragments. Using the internucleotidic phosphate (as the phosphorothioate derivative) it was theoretically possible to attach one fluorophore for each nucleoside residue present. However, since DNA fragments are generally analyzed by gel electrophoresis (and alkylation of the charged phosphorothioate diester alters the charge of the DNA fragment, thus its electrophoretic mobility), it was necessary to introduce the label to the DNA after the electrophoretic procedure, that is, while the nucleic acid was still imbedded within the polyacrylamide gel matrix. "Post assay" fluorescent labeling in this manner employs the fluorophore monobromobimane:



Monobromobimane

The bimane labeled phosphorothioate exhibits an excitation maximum of 390 nm and an emission maximum of 480 nm.

The desired DNA fragments containing phosphorothioate diesters were prepared either chemically using phosphoramidite solid-phase DNA synthesis or enzymatically by employing the appropriate α-thio nucleoside triphosphates. After resolution of DNA fragments using polyacrylamide gel electrophoresis, the gel matrix was soaked overnight in 4 mM monobromobimane in 50% aqueous acetonitrile. Under these conditions the fluorophore diffuses through the gel and efficiently alkylates the phosphorothioate diesters (Fig 2). Post-assay labeling in this manner relies upon the observation that monobromobimane itself is largely non-fluorescent but upon alkylation of a sulfur residue a highly fluorescent derivative results.

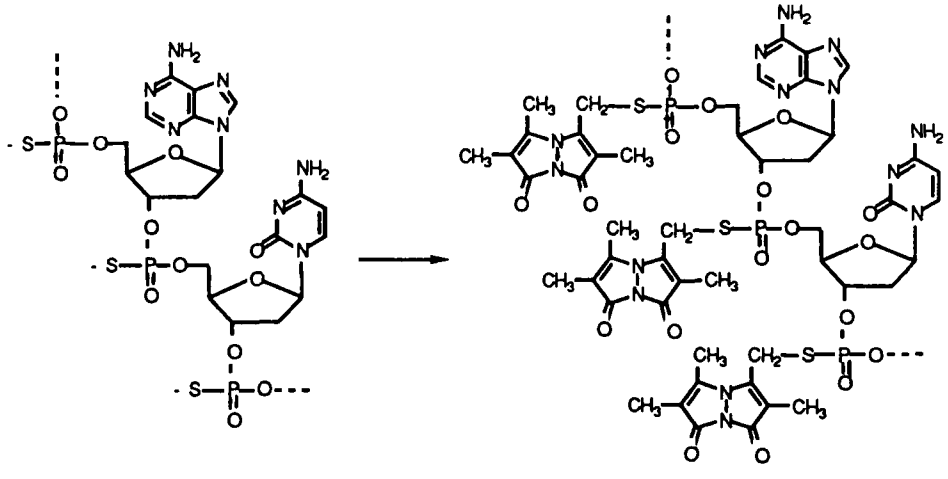


Figure 2. Post-assay labeling of phosphorothioate containing DNA introduces multiple fluorophores at potentially every internucleotidic phosphorus residue.

The sensitivity of detection (to the naked-eye) of DNA fragments labeled in manner has been determined by post-assay labeling of differing amounts of DNA of varying lengths (or varying numbers of phosphorothioate diesters) and visualizing the fluorescent bands using a standard transilluminator (λmax = 366 nm). Based upon this analysis, an increasing number of bimane fluorophores results in an approximately linear

increase in the detection sensitivity of the DNA fragments (Table 1). Fragments containing hundreds of potential labeling sites (phosphorothioate diesters) can be visualized in the low femtomolar range (10^{-15} moles) which is very near the sensitivity commonly achieved with the use of radioisotopes.

TABLE I Detection of DNA Containing Multiple Bimane Fluorophores

Fragment Length	Phosphorothioate Diesters (Bimane Labels)	Detection Limit
Dimer	1	25 pmol
15-mer	3	7 pmol
21-mer	20	1 pmol
444-mer	104	40 fmol
444-mer	223	27 fmol
444-mer	328	14 fmol

Labeling of the internucleotidic phosphorus residue in the manner described provides high detection sensitivity for DNA fragments. This approach should be applicable to hybridization techniques using membrane-bound nucleic acids as well as DNA sequencing procedures using enzymatic (Sanger) sequencing protocols.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Institutes of Health (GM 37065).

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