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# Iodine–azide reagent in detection of thiophosphoryl nucleotides in thin-layer chromatography systems[1]

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**Abstract**

The TLC detection limits of the phosphorothioate analogs of nucleotides and related compounds by means of UV, iodine, HCl vapours, the iodine–azide reagent and the molybdate reagent have been determined. The iodine–azide reagent has been applied for the selective TLC detection of the phosphorothioate analogs of nucleotides.

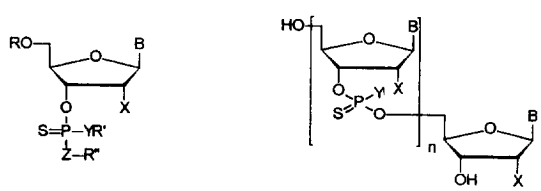
*Keywords:* Detection, TLC; Thiophosphoryl nucleotides; Nucleotides; Iodine–azide

## 1. Introduction

Thiophosphoryl compounds constitute a class of organophosphorus derivatives of great industrial [1–3] and synthetic [4,5] importance. Many of them exhibit significant biological activity and therefore are employed in agrochemistry [4] and in related areas [3,6].

The group of thiophosphoryl compounds of increasing importance, both in pure and applied chemistry, includes phosphorothioate analogs of nucleotides and oligonucleotides [7,8], presented by general structures I and II (Scheme 1).

In spite of the fact that all nucleosides and their derivatives are easily detected by UV spectroscopy, it was of great interest to find a method for the selective identification of phosphorothioate analogs of nucleotides in mixtures containing non-sulfurized nucleic acid components. In our opinion to find such a method was an important challenge for contempor-




I II

Scheme 1. B: purine or pirymidine base; X: H or OH; Y: O or S; Z: O or S; R, R', R": alkyl, aryl or carbohydrate moiety; n: degree of polymerization.

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[1] Dedicated to Prof. F. Plenat on the occasion of her retirement from the Montpellier University.


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ary analytical chemistry of organophosphorus compounds [9].

Recently we have published our results on the induction activity of thiophosphoryl compounds in the iodine–azide reaction [10–12], illustrated by Eq. (1).

$$I_2 + 2N_3^- \overset{\text{P–S inductor}}{\rightarrow} 2I^- + 3N_2 \quad (1)$$

On this basis, methods for the analytical determination of thiophosphoryl inductors [10,12] and their detection in TLC systems [11], have been elaborated. These studies revealed that the detection limits of thiophosphoryl compounds correspond to their induction potency, which in turn was found to be directly dependent on the inductor's structure.

Therefore, it was interesting to investigate the aforementioned structure–induction relationship in more complex structures, such as those presented in phosphorothioate analogs of nucleotides.

In the present paper we would like to present our results on the TLC detection of nucleoside derivatives containing 2-thio-1,3,2-oxathiaphosphalane or a 2-thio-1,3,2-dithiaphosphalane ring (precursors in the synthesis of oligonucleotides II [13–15]) and related derivatives using the iodine–azide reagent.

## 2. Experimental

### 2.1. Materials

Phosphorothioates were prepared according to Refs. [13–15]. Other organophosphorus compounds were prepared according to Ref. [16] or purchased from Aldrich (Milwaukee, WI, USA). Other reagents and chemicals were purchased from Aldrich.

### 2.2. Solutions and reagents

The concentrations of the compounds chromatographed were ca. $5\times10^{-2}$ to $1\times10^{-3}$ *M* in methanol (or in water: compounds 23 and 24).

An aqueous solution (1 *M*) of sodium azide and 1 *M* solution of iodine (in 1 *M* aqueous solution of potassium iodide) were employed.

Molybdate reagent was prepared by dissolving 1 g of ammonium molybdate in 40 ml of water, followed by 3 ml of concentrated hydrochloric acid and 5 ml of 70% perchloric acid. This solution was finally diluted with 100 ml of cold acetone [17].

### 2.3. Thin-layer chromatography

Precoated silica gel 60 $F_{254}$ aluminum sheets (10 cm×5 cm, 0.2 mm thick layer) or precoated cellulose plates (10 cm×5 cm, 0.1 mm thick layer) (Merck, Darmstadt, Germany) were used for TLC experiments.

The plates were spotted with an appropriate amount of compound (deposition area ca. 0.2 $cm^2$) developed for a distance of 8 cm with the eluent, air dried and detected with the appropriate detection system (for details see Table 1).

#### 2.3.1. Detection of thiophosphoryl compounds by the iodine–azide procedure

The indirect detection by means of the iodine–azide reagent was carried out using a freshly prepared 1:1 (v/v) mixture of sodium azide and iodine solutions. The phosphorothioate derivatives – inductors of iodine–azide reaction – appeared (due to the catalytic effect) as white spots in a yellow background, and were stable for more than 1 h.

#### 2.3.2. Detection of thiophosphoryl compounds by the molybdate procedure

The chromatographic plates were air dried in a fume hood (approximately 30 min), sprayed with the molybdate reagent and, while still wet, irradiated using a 254-nm ultraviolet source for 3 to 5 min. The plate channel was then further exposed to air and light for 1 to 2 h to assure complete color development. The phosphate and phosphorothioate derivatives appeared as blue spots on a white background.

#### 2.3.3. Detection of DMT-protected nucleotides by the molybdate procedure

The chromatographic plates were sprayed with a 10% solution of trifluoroacetic acid (TFA) in methanol, exposed at room temperature for 10 min to complete the detritylation, air dried and redeveloped with the appropriate solvent system. The plates were again air dried and sprayed with the molybdate reagent. The plates, still wet, were irradiated using a 254-nm ultraviolet source for 3 to 5 min. The plate

Table 1
Detection limits of phosphorothioate analogs of nucleotides with UV detection (254 nm), using iodine (vapor), and the molybdate and the iodine-azide detection reagent

| Compound Nr | Structure[a] | Detection limits (nmol per spot) | | | | | TLC $R_F$ |
|---|---|---|---|---|---|---|---|
| | | $I_2$ [b] | UV | $I_2$-$N_3^-$/c | Molybdate reagent | $HCl_{(v)}^e$ | |
| 1 | PhO-OTP | 0.1 | 1.5 | 0.3 | 1.5[d] | –[g] | 0.73[h] |
| 2 | NphO-OTP | 0.1 | 1.5 | 0.3 | 1.5[d] | –[g] | 0.74[h] |
| 3 | NphO-OTP* | 0.3 | 1.5 | 0.3 | 3.0[d] | –[g] | 0.74[h] |
| 4 | NphO-(Se)OTP | 0.1 | 1.5 | 0.3 | 1.5[d] | –[g] | 0.74[h] |
| 5 | DMT($U_{Si}$)OTP | 0.3 | 0.6 | 1.2 | 0.1[e] | 0.1 | 0.50[j] |
| | | | | | 10[f,d] | | 0.50[j,k] |
| 6 | DMT($C_{Si}^{Bz}$)OTP | 0.3 | 0.3 | 1.0 | 0.1[e] | 0.1 | 0.65[j] |
| | | | | | 10[f,d] | | 0.65[j,k] |
| 7 | DMT($A_{Si}^{Bz}$)OTP | 0.3 | 0.6 | 1.0 | 0.1[e] | 0.1 | 0.73[i]; 0.59[j] |
| 8 | DMT($G_{Si}^{iBu}$)OTP | 0.3 | 0.5 | 1.0 | 0.1[e] | 0.1 | 0.22[j] |
| 9 | DMT(T)OTP | 0.15 | 0.6 | 0.5 | 0.1[e] | 0.1 | 0.69[i] |
| 10 | DMT($dC^{Pya}$)OTP | 0.5 | 0.6 | 1.3 | 0.4[e] | 0.3 | 0.52[i] |
| 11 | DMT($dA^{Pya}$)OTP | 0.5 | 0.6 | 1.3 | 0.4[e] | 0.3 | 0.52[i]; 0.03[h] |
| 12 | DMT($dG^{Pya}$)OTP | 0.5 | 0.6 | 1.3 | 0.4[e] | 0.3 | 0.57[i]; 0.07[h] |
| | | | | | 10[f,d] | | 0.57[i,k] |
| 13 | OTP($T_{Ac}$) | 0.2 | 1.2 | 0.6 | 12.0[d] | –[g] | 0.69[i]; 0.17[j] |
| 14 | OTP($dC_{Ac}^{Bz}$) | 0.2 | 0.2 | 0.6 | 2.0[d] | –[g] | 0.64[i]; 0.31[j] |
| 15 | OTP($dG_{Ac}^{iBu}$) | 0.2 | 0.2 | 0.6 | 2.0[d] | –[g] | 0.6[i]; 0.03[j] |
| 16 | (T)DTP | 0.15 | 1.2 | 0.5 | 2.0[d] | –[g] | 0.67[i]; 0.13[j] |
| 17 | DMT(T)DTP | 0.15 | 0.6 | 0.5 | 0.15[e] | 0.15 | 0.67[i]; 0.37[j] |
| | | | | | 10[f,d] | | 0.37[j,k] |
| 18 | DMT($dC^{Pya}$)DTP | 0.15 | 0.6 | 0.5 | 0.15[e] | 0.15 | 0.53[i] |
| 19 | DMT($dA^{Bz}$)DTP | 0.15 | 0.6 | 0.5 | 0.15[e] | 0.12 | 0.64[i] |
| 20 | DMT($dG^{Pya}$)DTP | 0.15 | 0.6 | 0.15 | 0.15[e] | 0.15 | 0.55[i] |
| | | | | | 10[f,d] | | |
| 21 | DMT(T)ODPP | 25 | 1.3 | 25[b] | 0.1[c] | 0.1 | 0.37[j] |
| | | | | | 10[f,d] | | 0.37[j,k] |
| 22 | DMT(T)ODMP | 25 | 1.3 | 25[b] | 0.1[e] | 0.1 | 0.20[j] |
| 23 | 5′-CMP | 2.0 | 6.0 | –[g] | 2.0[d] | –[g] | 0.37[l] |
| 24 | 5′-GMP | 2.0 | 6.0 | –[g] | 2.0[d] | –[g] | 0.19[l] |

[a] Abbreviations: A, G, C, U, T: adenosine, guanosine, cytidine, uridine, thymidine; $U_{Si}$, $C_{Si}$, $A_{Si}$, $G_{Si}$: corresponding ribonucleoside blocked at 2′ position with t-Bu$Me_2$Si group; dA, dG, dC: corresponding deoxyribonucleosides; Ac: acetyl; iBu: isobutyryl; Ph: phenyl; Nph: naphthyl; Bz: benzoyl; Pya: N-methylpyrrolidin-2-ylidenyl; DMT: dimethoxytrityl; OTP: 2-thio-1,3,2-oxathiaphospholane; DTP: 2-thio-1,3,2-dithiaphospholane; OTP*: 2-thio-4,4-dimethyl-1,3,2-oxathiaphospholane; ODPP: diphenylphosphate; ODMP: 2-oxo-4,4-dimethyl-1,3,2-dioxaphosphorinane; (detailed structures of representative compounds are given in Table 3).

[b] Brown spots on yellow background.

[c] White spots on brown background.

[d] Blue spots on buff background.

[e] Pink spots.

[f] After treatment with a TFA solution (10%) in MeOH and redevelopment with appropriate solvent system.

[g] Not detectable up to level of 50 $\mu$g per spot.

[h] Silica gel/MeCN.

[i] Silica gel/MeOH.

[j] Silica gel/benzene–MeOH (9:1).

[k] A pink spot containing DMT cation was shifted up.

[l] Cellulose/MeOH–formic acid–$H_2O$ (80:15:5).

channel was then exposed to air and light for 1 to 2 h to assure complete color development. The phosphate and phosphorothioate compounds appeared as blue spots, whereas dimethoxytrityl products were visible as separate pink spots at higher $R_F$.

## 3. Results and discussion

The results of the procedures applied for TLC detection of phosphorothioate analogs of nucleotides and related compounds are summarized in Table 1.

Thus, the examined compounds gave a positive test reaction (brown spots on yellow background) when exposed to the action of iodine vapor at the level of 0.1 to 0.5 nmol per spot for compounds 1–20. This iodine detection of phosphorothioates was more sensitive than that exhibited for their phosphate analogs. Thus, the detection limits (DL) of nucleotides (23 and 24) and protected nucleotides (21 and 22) were estimated as 2.0 nmol and 25 nmol, respectively; i.e. 10 to 100 times higher than he DL of their phosphorothioate analogs.

Phosphorothioates exhibit also good detection in UV light (DL: 0.6 to 6.0 nmol), due to the presence of the aromatic systems in their molecules.

The detection of phosphorothioates by means of the iodine–azide reagent allows their visualization as white spots on a brown background, on the DL level of 0.15 to 1.3 nmol per spot. The detection sensitivity of phosphorothioates in this detection system exhibited the characteristic structure–induction activity dependence. Thus, *O*-aryl phosphorodithioates were detectable at the level of ca. 0.3 nmol. The substitution of sulfur by selenium atom did not affect the DL value of compound 4 in comparison with

Table 2
TLC analysis of the mixtures of nucleotides and their thiophosphoryl analogs

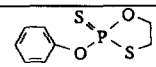
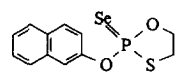
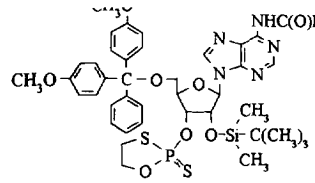
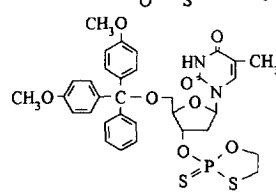
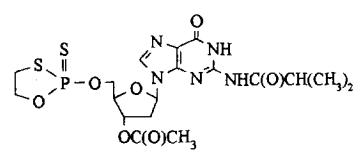
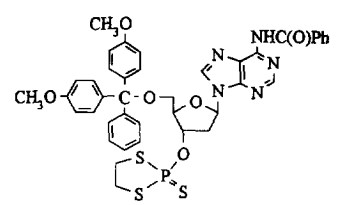
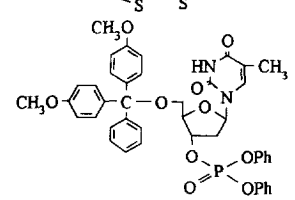
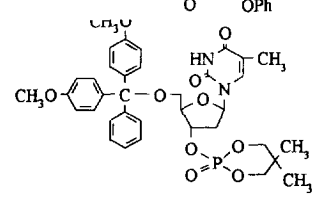
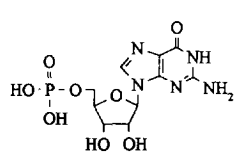
| Mixture of nucleotides[a] | Detection system[b] | | | |
|---|---|---|---|---|
| | UV [254 nm] detection/$R_F^h$ | Molybdate[c,d] detection/$R_F^h$ | Molybdate[e] detection/$R_F^h$ | $I_2$-$N_3^-$[f] detection/$R_F^h$ |
| DMT(T)ODMP and (T)DTP | ++/0.20 and ++/0.13 | ++[c]/0.20 and ++[d]/0.13 | ++[d]/0.20 and ++[d]/0.13 and ++[c]/0.71 | –[g] ++[f]/0.13 |
| DMT(T)ODPP and DMT($C_{Si}^{Bz}$)OTP | ++/0.40 and ++/0.62 | ++[c]/0.40 and ++[c]/0.62 | ++[d]/0.40 and ++[d]/0.62 and ++[c]/0.76 and ++[c]/0.83 | –[g] ++[f]/0.62 |
| OTP($T_{Ac}$) and DMT(T)DTP | ++/0.17 and ++/0.37 | ++[d]/0.17 and ++[c]/0.37 | ++[d]/0.17 and ++[d]/0.37 and ++[c]/0.76 | ++[f]/0.17 ++[f]/0.37 |

[a] Taken amount: 10 nmoles of each nucleotide per spot.
[b] ++ = strong detection, + = distinct detection, +/− = detectable, − = not detectable.
[c] Pink spots.
[d] Blue spots on buff background.
[e] After treatment with TFA and re development with an appropriate solvent system.
[f] White spots on yellow background.
[g] Taken at amount 50 μg per spot.
[h] Silica gel/benzene–MeOH (9:1).

those determined for derivatives 1–3. Phosphorodithioate analogs of nucleotides were detected at the range of DL between 0.5 to 1.3 nmol per spot. No substantial changes have been observed in the detection of phosphorothioate nucleotides with different nucleobases (see compounds 5–8, 10–12, 13–15 and 16–19) with only small differences between the series of ribonucleotides and deoxyribonucleotides (see derivatives 5–8 vs. 10–12). More distinct differences appeared in the series of nucleotides 10–12 vs. 13–15 bearing the phosphorothioate functions attached at the 2′ and 5′ position of the sugar ring of nucleosides. The detectability of phosphorothioate analogs of nucleotides generally increases with increase of sulfur content in the phosphorothioate function (see series 9–12 vs. 16–20); in the case of phosphorotrithioate nucleotide 20 the DL value reached a level of 0.15 nmol per spot.

The iodine–azide reagent does not exhibit any activity towards phosphate compounds. This has been clearly demonstrated during the treatment of the chromatographic plates charged with DMT(T)ODPP (21) and/or DMT(T)ODMP (22) and/or cytidine 5′-monophosphate (CMP, 23) and/or guanosine 5′-monophosphate (GMP, 24) with this reagent. These compounds could not be detected even at the level of ca. 50 nmol per spot – it means at a level exceeding 40–300 times the DL values determined for phosphorothioates 1–20. The detection of phosphorothioate analogs of nucleotides by means of the molybdate procedure leads to ambiguous results. Thus, phosphorothioates and phosphates react with the molybdate reagent usually forming blue spots on a white background [17]. However, since this procedure requires strongly acidic conditions, the dimethoxytrityl protected nucleotides (DMT-nucleotides) appeared on chromatographic plates as pink spots, obviously due to the formation of dimethoxytrityl cation. This effect also occurs during exposure of chromatographic plates charged with DMT-nucleotides to HCl vapor. The strong absorbance of dimethoxytrityl cation at ca. 500 nm sufficiently masks characteristic blue spots resulting from the reaction of the molybdate reagent with the phosphate and/or phosphorothioate functions. Therefore the application of the molybdate procedure for the TLC detection of DMT-nucleotides required some modification. Thus, subsequent treatment of chromato-

Table 3
The representative structures of derivatives used in this work

| Nr. | Abbreviation | Structure |
|---|---|---|
| 1 | PhO-OTP |  |
| 4 | NphO-(Se)OTP |  |
| 7 | DMT($A^{Bz}_{Si}$)OTP |  |
| 9 | DMT(T)OTP |  |
| 15 | OTP($dG^{iBu}_{Ac}$) |  |
| 19 | DMT($dA^{Bz}$)DTP |  |
| 21 | DMT(T)ODPP |  |
| 22 | DMT(T)ODMP |  |
| 24 | 5′-GMP |  |

graphed DMT-nucleotides with a 10% solution of TFA, followed by the TLC redevelopment and subsequent treatment of the chromatographic plate with the molybdate reagent, enables the visualization of detritylated nucleotides as blue spots. Pink spots of released DMT–TFA derivatives were shifted up in comparison with blue spots of detritylated phosphate and/or phosphorothioate nucleotides (Table 1 and 2).

In the light of the results presented here, only the iodine–azide detection reagent allows the selective detection of the thiophosphate (phosphorothioate) systems. The other applied detection systems, routinely used in the TLC of organophosphorus compounds (UV, iodine, $HCl_{(v)}$, the molybdate reagent), gave a positive test for both phosphates and thiophosphates (Table 1).

In contrast, the iodine–azide reagent only detects sulfur-containing molecules and therefore can be useful for the detection of phosphorothioate analogs of nucleotides in the presence of the phosphate and/or phosphonate type derivatives. As a consequence, the combination of the iodine–azide detection procedure and the molybdate detection procedure can be applied for the chromatographic detection and subsequent TLC differentiation of nucleotides and their phosphorothioate analogs (Table 2). The representative structures of the derivatives applied in this work are given in Table 3.

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