Iodine–azide reagent for the detection of biologically oriented thiophosphoryl compounds in thin-layer chromatography systems

Andrzej Kotyński[a], Witold Ciesielski[b], Zbigniew H. Kudzin[b,*], Andrzej Okruszek[c], Danuta Krajewska[c], Paweł Lipka[a]

[a]*Institute of Chemistry, Medical University of Łódź, Muszyńskiego 1, Łódź 90-151, Poland*
[b]*Faculty of Chemistry, University of Łódź, Narutowicza 68, Łódź 90-136, Poland*
[c]*Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Sienkiewicza 112, Łódź 90-363, Poland*

Abstract

The correlation between induction factors (F_i) of phosphorothioates in the iodine–azide reaction and their detection limits (DLs) using the iodine–azide reagent has been established. The iodine–azide reagent has been used for the selective thin-layer chromatographic detection of several sugar phosphorothioates and related compounds. In several cases, hydrolytic treatment of phosphorothioates, prior to their detection on thin-layer chromatography (TLC) plates with the iodine–azide reagent, was accompanied by a dramatic increase in the detectability of the starting compounds. Comparison of TLC detection systems for phosphorothioates using iodine–azide procedures and other representative procedures are presented. The application of the iodine–azide procedure combined with the molybdate procedure for selective TLC detection of phosphates and phosphorothioates is illustrated.   © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Organic phosphorothioates have found wide application in agrochemistry as potent pesticides [1,2]. More recently, phosphorothioate analogs of numerous biophosphates, such as nucleotides, sugars or phospholipids, were synthesized and used as important tools for basic research in biochemistry and molecular biology [3,4]. Among these, phosphorothioate analogs of oligonucleotides were found to be good candidates for antiviral or antitumor drugs (antisense strategy) [4]. Because of the numerous applications of thiophosphates and the difficulty

involved in analyzing them [5], there was a need to develop selective and sensitive methods of determination and/or detection for this class of derivatives [2–4].

We have published our results on the induction activity of thiophosphoryl compounds in the iodine–azide reaction [6,7], illustrated by Eq. (1).

$$I_2 + 2N_3^- \xrightarrow{\text{P-S inductor}} 2I^- + 3N_2 \qquad (1)$$
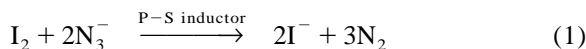
On this basis, methods for the analytical determination of thiophosphoryl inductors [7] and their detection in thin-layer chromatography (TLC) systems [8] have been developed. These studies revealed that the detection limits of thiophosphoryl

*Corresponding author.

compounds correspond to their induction potency, expressed by the induction factor (F_i), which, in turn, was found to be directly dependent on the inductor's structure, especially on the type and polarity of the P–S bond.

Thus, numerous phosphorothioate compounds, especially those of type I, containing P–S–R bonds (phosphorothiolates), are characterized by low induction activity (low F_i value) [6].

$$R'O\backslash \overset{\overset{\displaystyle O}{\parallel}}{\underset{\underset{\displaystyle \mathbf{I}}{}}{P}}-S-R$$
$$R''O/$$
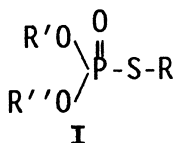
We have found that the TLC detection of phosphorothioates by means of the iodine–azide reagent can be greatly enhanced by preactivating them by chemically converting them into derivatives with higher F_i values.

Such a transformation can be performed easily by hydrolytic conversion of the thiol ester (P–S–R) functions, which exhibit low induction activities in the iodine–azide reaction (Eq. (1)), into free thiol (R–SH) (Eq. (2)), which exhibits a relatively stronger induction effect.

$$P-S-R \rightarrow P-O^- + R-S^- \tag{2}$$
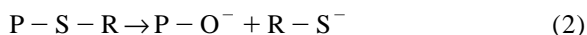
As a result, the hydrolytic pretreatment of chromatographed thiophosphoryl derivatives containing thiol ester groups should lead to an increase in their detection by TLC using the iodine–azide reagent.

In this paper, we wish to present our results on the TLC detection of a series of phosphorothioates containing the P–S–R function, including phosphorothioate analogs of sugar phosphates, using the iodine–azide reagent either alone or in combination with hydrolytic pretreatment.

These results are correlated with the induction potency of the initial thiophosphate derivatives and are compared to the detection limits (DLs) obtained using other representative detection reagents.

2. Experimental

2.1. Materials

Phosphates (**1**) and phosphorothioates (**2–14**)

were prepared according to refs. [9–11]. Other reagents and chemicals were purchased from Aldrich (Milwaukee, WI, USA).

2.2. Solutions and reagents

Methanol solutions of phosphorothioates were used (concentrations ranged from 5×10^{-2} to 1×10^{-3} M).

2.2.1. Solutions for iodometric titration

A 0.25 M aqueous solution of sodium azide was buffered to pH 4.75 with hydrochloric acid before use. A 0.01 M aqueous solution of iodine was used, containing 4 g/l of potassium iodide. A 0.01 M aqueous solution of sodium arsenite was used. The indicator solution used was a 0.5% aqueous solution of starch indicator.

2.2.2. Solutions for the TLC detection

A 1 M aqueous solution of sodium azide and a 1 M solution of iodine (as a 1 M aqueous solution of potassium iodide) were used for the TLC detection. Molybdate reagent was prepared by dissolving 1 g of ammonium molybdate in 40 ml of water, followed by the addition of 3 ml of concentrated hydrochloric acid and 5 ml of 70% perchloric acid. This solution was finally diluted with 100 ml of cold acetone [13].

The perchloric acid used was a 20% solution in ethanol–water (2:1, v/v). Tin(II) chloride reagent was prepared by heating 1 g of tin(II) chloride dihydrate in 10 ml of concentrated hydrochloric acid until dissolved, followed by dilution with 40 ml of water and 50 ml of acetone.

2.3. Determination of the inductive effect on the iodine–azide reaction

The efficiency of the compounds (**1–14**) as inductors was characterized and the results were compared on the basis of their induction factors (F_i), defined by Eq. (3):

$$F_i = n_I / n_i \tag{3}$$

where n_I is the amount of iodine consumed in the induced iodine–azide reaction and n_i is the amount of the P–S inductor (both given in millimoles).

The consumption of iodine in the induced iodine–azide reaction was determined by iodometric titration. Thus, the reaction flask (a 100 ml Erlenmayer flask, equipped with a glass stopper and a magnetic stirrer) was charged with 50 ml of 0.25 M sodium azide, followed by the addition of the inductor sample. To the resulting solution, 0.01 M iodine (10 ml) was added, with stirring, the flask was tightly stoppered and the reaction mixture was allowed to stand for the time indicated in Table 1. Excess iodine was then titrated back using 0.01 M sodium arsenite in the presence of starch indicator.

2.4. Thin-layer chromatography

Precoated silica gel 60 F_{254} aluminum sheets (10×5 cm, 0.2 mm thick layer) or precoated cellulose plates (10×5 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 using the appropriate detection system (for details, see Table 1).

2.4.1. Detection of phosphorothioate compounds by the iodine–azide procedure

Indirect detection using iodine–azide reagent was carried out using a freshly prepared mixture of sodium azide and iodine solutions (1:1, v/v). The phosphorothioate derivatives, inductors of the iodine–azide reaction, appeared as white spots on a yellow background and were stable for more than 0.5 h.

If prehydrolysis of the thiolester bond was required, the plates were exposed to hydrochloric acid or conc. aqueous ammonia vapors for 5 min prior to the final treatment with the iodine–azide reagent.

2.4.2. Detection of phosphates and phosphorothioates

2.4.2.1. Molybdate procedure A
The chromatographic plates were air dried in a fume hood, sprayed with the molybdate reagent and, while still wet, were irradiated using a 254-nm ultraviolet source for 3 to 5 min. The plate was then further exposed to air and light for 1 to 2 h, to allow complete color development.

2.4.2.2. Molybdate procedure B
The chromatographic plates were air dried in a fume hood, sprayed with perchloric acid and heated in an oven to 180°C for 30 min. The plates were removed from the oven, cooled to room temperature, sprayed with molybdate reagent and, while still wet, were replaced in the oven for 5 min. After cooling, the plates were sprayed with a solution of tin(II) chloride and placed in a tank with ammonia vapors.

In both procedures, phosphate and phosphorothioate derivatives appeared as blue spots on a white background.

3. Results and discussion

The results obtained by us previously on the use of the iodine–azide reaction for TLC detection of some phosphorothioate derivatives of nucleosides [13] have prompted us to perform some additional investigations on the structural factors influencing the course of the reaction.

These investigations were focused on the induction activity of various types of thiophosphate derivatives and the analytical repercussions, especially in the context of their selective TLC detection.
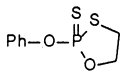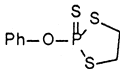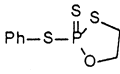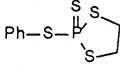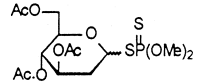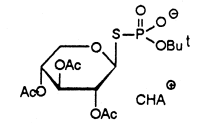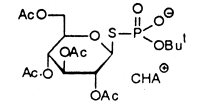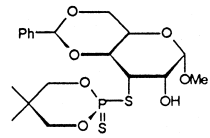
In the course of these studies, we have determined the induction coefficients (F_i) of a series of representative phosphates (**1**) and phosphorothioates (**2**–**10**), in order to establish their molecular structure–induction and detectability–induction activity relationships. The data on the induction activity (F_i) of these derivatives and their TLC DLs, using the iodine–azide detection procedure, are presented in Table 1.

The results clearly demonstrate the lack of induction activity exhibited by phosphates (**1**), contrasting with the substantial induction activity (F_i) of the phosphorothioates (**2**–**10**). These increased with the number of sulphur atoms in the inductor's structure, clearly illustrating the higher induction potency for derivatives with thiono (P=S) rather than thiolo (P–S–R) functions. The phosphorothioate sugar derivatives (**11**–**14**) exhibited characteristic induction activity, which was quite different from that

Table 1

Detection limits of phosphates (**1**) and phosphorothioates (**2–14**) with UV detection, using iodine vapors, the molybdate reagent and the iodine–azide procedures

Nr	Phosphates and phosphorothioates Structure[a]	Induction Factor[b] [F_i] 5'	15'	60'	UV [254 nm] (nmol)	I_2[c] (nmol)	$I_2\text{-}N_3^{-}$[d] (nmol)	$I_2\text{-}N_3^{-}$[e,d] (nmol)	$I_2\text{-}N_3^{-}$[f,d] (nmol)	Molybdate reagent[g] (nmol) A[h]	B[i]	TLC [R_F]
1a	Et–O–P(=O)(O–Et)(O–Et)	—	—	—	—[k]	50	—[k]	—[k]	—[k]	—[k]	30	0.53[m]
1b	Ph–O–P(=O)(O–Ph)(O–Ph)	—	—	—	10	30	—[k]	—[k]	—[k]	—[k]	30	0.41[n] 0.68[m]
1c	Ph–O–P(=O)(cyclic O–O)	—	—	—	25	0.5	—[k]	—[k]	—[k]	—[k]	50	0.75[p] 0.35[n] 0.14[r]
2	Ph–O–P(=O)(cyclic S–O)	16	22	30	25	0.5	2.5	>5		25		0.74[p] 0.37[n] 0.14[r]
3	Ph–S–P(=O)(cyclic O–O)	9	13	22	25	5	25[l]	>25		50		0.75[p] 0.36[n]
4	Ph–O–P(=S)(cyclic O–O)	141	156	160	25	0.5	2.5			5.0		0.72[p] 0.46[n] 0.52[r]
5	Ph–S–P(=S)(cyclic O–O)	187	190	200	2.0	0.5	0.5	>5		1.5		0.72[p] 0.42[n] 0.50[r]
6	cholesteryl O–P(=S)(cyclic S–O)				20	0.2	0.5			2.0		<0.03[p] 0.76[m]

	Structure											
7	Ph–O–P(=S)(S)(O) cyclic (1,3,2-oxathiaphospholane)	150	163	172	25	0.5	0.5			1.5		0.71^p 0.51^n 0.62^r
8	Ph–O–P(=S)(S)(S) cyclic	160	193	211	20	0.5	0.5			1.5		0.70^p 0.54^n 0.63^r
9	Ph–S–P(=S)(S)(O) cyclic	199	220	255	5	0.5	0.5			1.5		0.72^p 0.52^n 0.63^r
10	Ph–S–P(=S)(S)(S) cyclic	170	208	223	2.0	0.5	0.5	0.5	0.5	1.5		0.73^p 0.58^n 0.65^r
11	AcO, OAc, AcO sugar –S–P(=S)(OMe)$_2$	47	106	252	100	2.5	50	3.0	50	100	10	0.65^p
12	AcO, OAc, OAc sugar –S–P(=O)(O$^\ominus$)(OBu^t) CHA$^\oplus$	107	325	522	100	10	100	2.0	2.0	100	10	0.67^p
13	AcO, OAc, OAc sugar –S–P(=O)(O$^\ominus$)(OBu^t) CHA$^\oplus$	90	212	520	100	2	100	5.0	2.0	100	10	0.70^p
14	Ph, OMe, OH sugar –S–P(=S)(O)(O) cyclic	280	370	400	20	4.0	20	2.0	0.5	0.5		0.80^p 0.73^m

[a]Abbreviations: Ac=acetyl, Bu^t=*tert.*-butyl, CHA$^+$=cyclohexylammonium cation, Me=methyl, Ph=phenyl. [b]Determined in solution. [c]Brown spots on yellow background. [d]White spots on yellow background. [e]After pretreatment with NH$_3$ vapors. [f]After pretreatment with HCl vapors. [g]Blue spots. [h]Molybdate procedure A. [i]Molybdate procedure B, with prior mineralization of chromatographed compounds by perchloric acid at 180°C. [k]Not detectable at the level of 50 nmol per spot. [l]After 15 min of exposure. [m]Silica gel/acetone. [n]Silica gel/benzene–ethyl acetate (9:1, v/v). [p]Silica gel/methanol. [r]Reversed-phase plate (RP)/benzene–cyclohexane (1:1, v/v).

shown by the series of non-sugar phosphorothioates (**2–10**).

The F_i parameters were determined for aqueous solutions of phosphorothioates and exhibited strong time dependence, with a plateau usually being reached after a reaction time of 1 h.

However, due to the more complex nature of the iodine–azide reaction during the TLC detection of phosphorothioates, the DL=f(F_i) correlation can be applied better for shorter periods of exposure time, of between 1 and 5 min.

The results (Table 1) illustrate the reasonably good correlation between the induction potency (F_i) of phosphates (**1**) and phosphorothioates (**2–14**) and their detectability using the iodine–azide reagent.

Thus, phosphates (**1**) exhibited no induction potency in solution and were not detectable on TLC plates. Compound **2**, with $F_{i(5')}=16$ was detected at the level of 2.5 nmol per spot, compound **3** ($F_{i(5')}=9$) at 25 nmol, compounds **4–10** [$140 < F_{i(5')} < 200$] at 0.5 to 2.5 nmol, and compounds **11–13** [$50 < F_{i(5')} < 100$] were detected at the level of 50–100 nmol per spot. In contrast, compound **14**, with a high value of $F_{i(5')}$ (280) exhibited only moderate detectability, having a DL value ca. 20 nmol per spot.

Compounds **2** and **3**, characterized by similar values of $F_{i(5')}$, demonstrated substantial differences in their detectability, with DL=2.5 and 25 nmol per spot, respectively. In addition, the efficient detection of compound **3** required a prolonged exposure time (15 min), which may imply the influence of secondary processes occurring at the silica oxide surface.

These examples illustrate the differences between the kinetic course of the iodine–azide-induced reaction carried out in solution and on the SiO_2 surface. The detection of derivative **3** occurred immediately after ammonia pretreatment of the plate.

The group of phosphorothioates (**11–14**) was found to exhibit more specific induction properties, presumably due to the presence of sugar units in their molecules. The induction activity of compounds **11–14** was found to be dependent on their molecular structures, increasing strongly with reaction time. Thus, the phosphorothioate derivative of deoxyglucose (compound **11**) exhibited moderate induction activity [$F_{i(5')}=47$], compounds **12** and **13** exhibited medium induction activity [$90 < F_{i(5')} < 107$] and

compound **14** exhibited the highest induction activity ($F_{i(5')}=280$) of the series. These results suggest that the phosphorothioates (**11–14**) containing the C(1)–S–P linkage exhibited lower induction activities than those with other [e.g. at C(3)] alignments of phosphorothioate in the sugar ring. The deoxysugar derivative (**11**) has a lower induction potency than derivatives **12** and **13**.

The induction activity of all of the phosphorothioate derivatives (**11–14**) exhibited a strong dependence on the reaction time. Thus, for compounds **11–13**, the induction coefficients (F_i) increased fivefold in going from 5 to 60 min, reaching a level above 500 in the latter case.

The correlation between DL and F_i found in the series of phosphorothioates (**2–10**) appears to be more complex when applied to sugar derivatives (**11–14**).

Surprisingly, the high induction potency that compounds **11–14** exhibited in solution is not reflected in their TLC detection on silica gel plates. Thus, compounds **12** and **13**, with $F_{i(5')} \geq 90$, exhibited low DLs, ca. 100 nmol per spot. Prolongation of the exposure time did not influence the detectability of derivatives **11–13**. These effects may be due to a more complex mechanism of the iodine–azide induced reaction carried out at the silica oxide surface.

The relatively poor detection of phosphorothioates **11–14** using the iodine–azide reagent can be greatly enhanced by hydrolytic pretreatment of the chromatographic plate. Such a pretreatment step most probably involves the hydrolytic splitting of the P–S–C(sugar) bonds and can occur, depending on the structure, either under basic or acidic conditions.
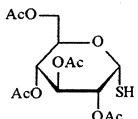
Thus, compounds **11** (DL=50 nmol), and **12** and **13** (DL=100 nmol), having a phosphorothioate moiety at the C-1 carbon of the sugar unit [the C(1)–S–P linkage], cannot be detected well using the iodine–azide reagent. The detection rate increases strongly when the plate is pre-exposed to aqueous ammonia vapors (**11**; DL=3 nmol) or aqueous HCl vapors (**12** and **13**; DL=2 nmol).

Such a high increase in the detectability of derivatives **11–14** suggests that, during the hydrolytic pretreatment, mercaptosugars are formed that are more active as inductors in the iodine–azide reaction (see Table 2).

Higher detectability was found for phosphoro-

Table 2
Induction factors (F_i) of sulphydryl compounds (**15–18**) in the iodine–azide reaction and their detection limits (DLs) using the iodine–azide detection reagent

Nr	Sulphydryl derivative	Induction Factor (F_i)[a]					TLC[b,c]	
		0.5'	5'	15'	60'	120'	DL (nmol)	R_F
15	$HOCH_2CH_2SH$	30 63[d]	33	34	35	35	10	0.70
16	$HSCH_2CH_2SH$	14[d]				9.2[d]	10	0.01
17	PhSH	31[d]				39[d]	100	0.71
18		36	52	59	74	78	1[e]	0.70

[a]Determined in solution.
[b]Silica gel/methanol.
[c]White spots on yellow background.
[d]Reported in Ref. [14].
[e]The same DL value after pretreatment with NH_3 and/or HCl vapors.

thioate **14**, having a thiophosphoryl function at the C-3 atom of the sugar unit [the C(3)–S–P linkage]. This compound is detectable with the iodine–azide reagent at the level of 20 nmol per spot and this detection level increases to DL=2 nmol during basic or to DL=0.5 nmol per spot during acidic pretreatment.

The results of the other representative procedures applied for the TLC detection of phosphates **1** and phosphorothioates **2–14** are summarized in Table 1. Thus, compounds **1–14** gave a positive test reaction when exposed to the action of iodine vapor (brown spots on a yellow background at 0.2 to 0.5 nmol per spot for compounds **1–10** and at 4.0–15.0 nmol per spot for sugar derivatives **11–14**).

Their detection under UV light (at 254 nm) leads to ambiguous results. Thus, the phosphorothioates containing an aromatic ring (**2–5** and **7–10**) and/or the phosphorothiono (P=S) moiety (**4, 5** and **7–10**) exhibit detection limits at the level 2.0 to 25 nmol per spot, whereas phosphorothioates with aliphatic substituents (**11–13**) were only poorly detected, with DLs>100 nmol per spot.

The detection of phosphates (**1**) and/or phosphorothioates (**2–14**) using molybdate reagents was dependent on the stability of their phosphoester or phosphothioester functions. Thus, the DLs of phosphorothioates (**2–10**) using the molybdate reagent (procedure A, [13]) vary from 1.5–5.0 nmol (**4–10**) to 25 nmol (**2**) and 50 nmol (**3**). In the sugar series, compound **14** [with the C(3)–S–P linkage] had a DL of 0.5 nmol and compounds **11–13** [with the C(1)–S–P linkage] were very difficult to detect, having DLs>100 nmol per spot. These detectabilities substantially increased when chromatographed compounds were subjected to hydrolytic treatment prior to detection (procedure B, [7,12]).
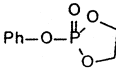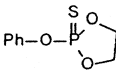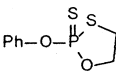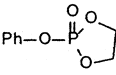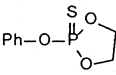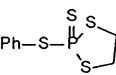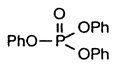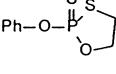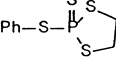
In conclusion, these detection systems, which are routinely used for the TLC of organophosphorus compounds (UV, iodine, molybdate reagents; Table 1) gave positive test results for both phosphates and phosphorothioates.

In light of these results, the iodine–azide detection reagent can be used for the sensitive detection of a broad spectrum of phosphorothioates. In several cases, their (thiophosphate derivatives of sugars) detectability can be substantially increased by performing hydrolytic pretreatment prior to detection using the iodine–azide reagent.

Taking into account the well documented fact of negative test reaction of the iodine–azide reagent with phosphates and phosphonates [7,13], this pro-

Table 3
TLC analysis of the mixtures of phosphates and phosphorothioates

Mixture of compounds[a]	Detection system[b]				
	UV_{254}/R_F[c]	I_2[d]$/R_F$[c]	I_2-N_3^{-}[e]$/R_F$[c]	Mo[f,g]$/R_F$[c]	Mo[f,h]$/R_F$[c]
Ph—O—P(=O)(O)(O) cyclic (phosphate)	+/0.35	++/0.35	−	−	+/0.35
&	&	&			&
Ph—O—P(=S)(O)(O) cyclic	+/0.46	++/0.46	++/0.46	+/0.46	++/0.46
&	&	&	&	&	&
Ph—O—P(=S)(S)(O) cyclic	+/0.51	++/0.51	++/0.51	++/0.51	++/0.51
Ph—O—P(=O)(O)(O) cyclic	+/0.35	++/0.35	−	−	+/0.35
&	&	&			&
Ph—O—P(=S)(O)(O) cyclic	+/0.46	++/0.46	++/0.46	+/0.46	++/0.46
&	&	&	&	&	&
Ph—S—P(=S)(S)(O) cyclic	++/0.58	++/0.58	++/0.58	++/0.58	++/0.58
PhO—P(=O)(OPh)(OPh)	++/0.41	+/0.41	++[d]/0.41	−	+/0.41
&	&	&	&		&
Ph—O—P(=S)(S)(O) cyclic	+/0.51	++/0.51	++/0.51	++/0.51	++/0.51
&	&	&	&	&	&
Ph—S—P(=S)(S)(S) cyclic	++/0.58	++/0.58	++/0.58	++/0.58	++/0.58

[a]Taken 10 μg of each compound. [b]+ + =strongly detected, + =distinct detection, − =not detectable. [c]Silica gel/benzene–ethyl acetate (9:1, v/v). [d]Brown spots on yellow background. [e]White spots on yellow background. [f]Blue spots. [g]Molybdate reagent, procedure A. [h]Molybdate reagent, procedure B.

cedure can be used as a method of choice for the selective detection of phosphorothioates in mixtures with other phosphoroorganic derivatives.

The application of the iodine–azide procedure, combined with the molybdate procedure, for the selective TLC detection of phosphates and phosphorothioates is illustrated in Table 3.

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