ANALYSIS OF ORGANOPHOSPHORUS COMPOUNDS.
1. APPLICATION OF IODINE-AZIDE REACTION FOR DETECTION OF THIOPHOSPHOROORGANIC COMPOUNDS IN THIN-LAYER CHROMATOGRAPHY

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The application of organophosphorus compounds as inducing agents in the iodine-azide reaction was investigated. Their induction activity was exhibited by thiophosphoryl compounds; their induction coefficients were dependent on the number and nature of sulphur atoms in the $P(S)_n$ function. These relationships can be used for the group differentiation of organophosphorus compounds, for example phosphates, thiophosphates and dithiophosphates. On the basis of their induction activity, thiophosphoryl inductors determination methods (titrimetric, coulometric and spectrophotometric methods) based on determination of the quantity of consumed iodine (μmol to nmol scale), was elaborated. The correlation between induction factors (F_i) in the iodine-azide reaction and detection limits (DL_s) using the iodine-azide reagent has been established. The iodine-azide reagent has been used for the selective thin-layer chromatographic detection of several phosphorothioates, including sugar and/ nucleosides phosphorothioates and related compounds. Comparison of TLC detection systems for phosphorothioates using iodine-azide procedures and other representative procedures are presented.

Keywords: induced iodine-azide reaction; organophosphorus inductors; phosphorothioates; sugar phosphorothioates; thiophosphoryl nucleotides; micro-determination of thiophophoryl inductors; detection; TLC.

1. Introduction

Thiophosphoroorganic compounds represent an abundant and structurally diverse group of organophosphoro-derivatives of great industrial [1-

4] and synthetic importance [5,6,7]. Structural formulae of the basic types of thiophosphoroorganic compounds are presented in Table 1.

Table 1. Basic types of thiophosphoroorganic compounds.

No	Structure	Type of compounds[a]	No	Structure	Type of compounds[a]
1	R^3-O, $R^2-O-P=O$, R^1-O	Phosphates	11	R^3-S, $R^2-S-P=S$, R^1-S	Phosphoro-tetrathiolates
2	R^3-O, $R^2-O-P=O$, R^1-S	Phosphoro-thiolates	12	R^3-O, $R^2-O-P=O$, R^1	Phosphonates
3	R^3-O, $R^2-O-P=S$, R^1-O	Phosphoro-thionates	13	R^3-O, $R^2-O-P=S$, R^1	Phosphono-thionates
4	R^2-O, $R^1-O-P=S$, R_2N	Thiophosphor-amidates	14	R_2N, $R_2N-P=S$, R^1	Diamidothio-phosphonates
5	R_2N, $R_2N-P=S$, R^1-O	Thiophosphor-diamidates	15	R^3-O, $R^2-P=S$, R^1	Phosphino-thionates
6	R^2-O, R^1-O P(O) O P(S) $O-R^2$, $O-R^1$	Monothiopyro-phosphates	16	R_2N, $R^2-P=S$, R^1	Amidothio-phosphinates
7	R^2-O, R^1-O P(S) O P(S) $O-R^2$, $O-R^1$	Dithiopyro-phosphates	17	R^3-S, $R^2-P=S$, R^1	Phosphino-dithionates
8	R^3-O, $R^2-O-P=S$, R^1-S	Phosphoro-thiolothionats	18	R^2, R^1 P(S) S-S P(S) R^2, R^1	Dithiophos-phinates disulfides
9	R^2-O, R^1-O P(S) S-S P(S) $O-R^2$, $O-R^1$	Dithiophos-phates disulfides	19	R^3, $R^2-P=O$, R^1	Phosphine oxides
10	R^3-O, $R^2-S-P=S$, R^1-S	Phosphoro-trithiolates	20	R^3, $R^2-P=S$, R^1	Phosphine sulfides

[a]Acids: **n** ($R^1 = R^2 = R^3 = H$); **nA** ($R^1 = R^2 =$ H); **nB** ($R^1 =$ H); **nC** (R ≠ H); **a** (Me); **b** (Et); **c** (Bu); **d** (Hex); **e** (Oct); **f** (Ph); etc.; [e.g. **1** = phosphoric acid; **1A** = alkyl (or aryl, or aralkyl) phosphate; **1B** = dialkyl phosphate; **1C** = trialkyl phosphate; and correspondingly - **1Ca** = trimethyl phosphate].

Following Schroder's discovery of insecticidal properties of thiophosphoroorganic compounds in the 1930's [8,9], the number of thiophosphoroorganic insectides has been growing continuously [8-12]. It is estimated that, to date, over 500 different thiophosphoroorganic derivatives have been synthesised and tested over 100 of which are commercially available as plant protection agents. Such dynamic development of this agrochemistry domain presents an indicator of the effectiveness of thiophosphoroorganic insecticidals. Moreover, unlike chloroorganic compounds, thiophosphoroorganic do not accumulate in the environment and their fast biodegradation makes them environmentally-friendly [12].

Structurally varied thiophosphoroorganic compounds exhibit diverse biological activity and apart from being used as insectidals they have found other agrochemical applications [12]. Thus, thiophosphoroorganic compounds are widely used as insecticides (Table 2), acaricides, nematocides, fungicides, bactericides, herbicides, rodenticides, growth regulators, insect chemosterilants and insecticide synergists. Several thiolophosphates $[(RO)_2P(O)S^-]$ were found as very useful tool in exploring enzymatic reaction mechanisms since they react considerably slower than their oxygen analogs.

Table 2. Representative thiophosphoryl insecticides [11,12]

Thiophosphoryl insecticides			
Structure	Name	Structure	Name
	Malathion		Iso-Malathion
	Dimethoate		Methaamido-phos
	Fenthion		Fenitrothion
	Iprobenfos		Acephate
	Fonofos		Phorate
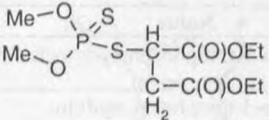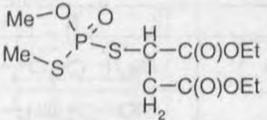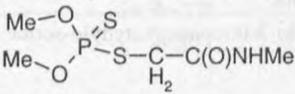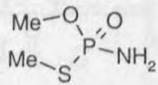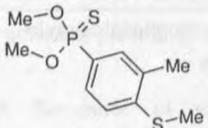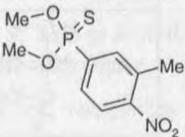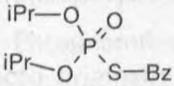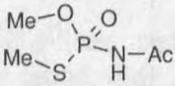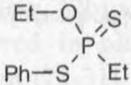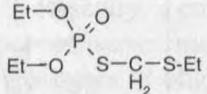
Biological activity of phosphorothioates is attributed to their thiono-thiolo isomerisation (Fig. 1) occurring in aqueous solutions.

Fig. 1. Thiono-thiolo rearrangement

More recently, phosphorothioate analogues of numerous biophosphates, such as phospholipids (Table 3), sugars phosphates (Fig. 2) or nucleotides (Fig. 3), were synthesized and used as important tools for basic research in biochemistry and molecular biology.

Thiophospholipids (Table 3), isosteric with natural phospholipids were found to exhibit diverse pharmacological activity, including in anticancer, antivirus, antipyretic, antiallergic and immunomodulatory area [13,14]. These compounds also play an important role in the investigations on a polymorphism of phospholipid based bio-membranes [13,15,16] and also in enzyme action mechanisms of the phospholipase class [17-19].

Table 3. Main types of thiophospholipids (exemplified by 1,2-distearylo-3-thiophosphatydylolipids) (X: O or S; Y: O or S)

Thiophospholipids		
Structure	R	Name
	Me₃N⁺—CH₂CH₂—	1,2-distearylo-3-thiophosphatydylocholine
	HO—CH₂CH₂—	1,2-distearylo-3-thiophosphatydylo-ethanoloamine
	HO—C(=O)—CH(H)—NH₂	1,2-distearylo-3-thiophosphatydylo-serine
	H₂C—, HC—OH, C—OH H₂	1,2-distearylo-3-thiophosphatydylo-glycerol
	H₂C—, HC—OH, C— H₂	1,2-distearylo-di-3-thiophosphatydylo-glycerol
	OH ... OH OH OH (inositol ring)	1,2-distearylo-3-thiophosphatydylo-myo-inositol

Sugar phosphorothioates and/or phosphorodithioates are applied as anti-glaucoma and/or anti-parasite drugs [20,21] and also as plant protection agents [22]. Sugar phosphorothioates are also used as tools in investigations of enzymatic reactions mechanisms [23,24].

X: O or S; Y: O or S; R, R': H, alkyl, aryl or aralkyl moiety
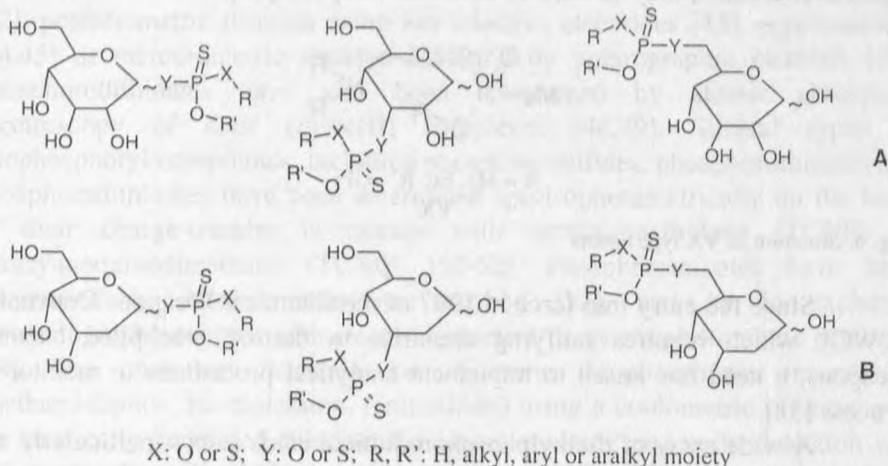
Fig. 2. Structures of representative phosphorothioates of sugars exemplified by glucose (A) and deoxyglucose (B) derivatives.
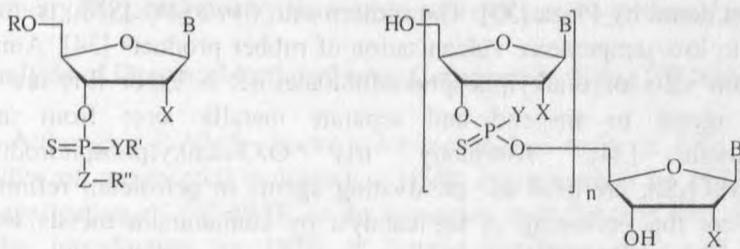
B: purine or pirimidine base; X: H or OH; Y: O or S; Z: O or S; R, R', R'': alkyl, aryl or carbonate moiety; n: polymerisation degree

Fig. 3. Structures of phosphorothioate analogues of nucleotides and oligonucleotides (polinucleotides).

Phosphorothioate analogues of nucleotides and oligonucleotides, introduced originally by Eckstein [25], have found wide application in both biochemistry [26] and molecular biology [27]. Recently, a considerable interest is observed in oligonucleotides as potential antisense modulators of gene expression [28-31]. Thus, phosphorothioate analogues of oligonucleotides were

found to be good candidates for antiviral and/or antitumor drugs (antisense strategy) [28].

Some thiophosphoroorganic compounds such as VX [O-ethyl-S-(2-diisopropylamino-ethyl)-methylthiophosphonate], the most lethal nerve gas agent ever created, may be used as chemical weapons [32].

$$\text{Me}-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^{\diagdown R}}{\diagup}}{P}}\diagdown S\diagtriangledown \diagdown N\diagtriangledown\overset{R'}{\underset{R'}{}}$$

R = Me, Et; R'' = iPr

VX
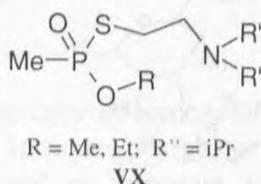
Fig. 4. Structure of VX type agents

Since the entry into force in 1997 of the Chemical Weapons Convention (CWC), which requires ratifying countries to destroy stockpiled chemical weapons, a need has arisen to implement analytical procedures to monitor the process [32].

A wide array of dialkylphosphorodithioic acids salts – particularly zinc salts $[(RO)_2P(S)S^-]_2Zn$ – are used as anti-wear and anti-corrosion lubricating oil additives. These salts react with metal surfaces of gears and moving engine parts to improve smoothness and provide excellent resistance to rust and corrosion. Various applications of dialkylphosphorodithioic acids salts $[RO)_2P(S)S^-]$ were reported in detail by Płaza [33]. The sodium salt, $(iPrO)_2P(S)SNa$, is used as an activator in low-temperature vulcanisation of rubber products [34]. Ammonium and sodium salts of dialkylphosphorodithioates (R = Et or iPr) are used as flotation agents to suspend and separate metallic ores from unwanted contamination [34]. Antimony tris O,O-dialkylphosphorodithioates, $[(RO)_2P(S)S^-]_3Sb$, are used as passivating agents in petroleum refining since they prevent the poisoning of the catalyst by contaminant metals which are present in oil feeds [34,35].

2. Analysis of Organothiophosphorus Compounds

Due to numerous applications of thiophosphates on the one hand and difficulties with their analysis [36] on the other hand, it is crucial to improve their determination methods in terms of selectivity and sensitivity. In spite of many publications on the subject, quantitative and qualitative determinations of the majority of organothiophosphorus derivatives still pose a challenge for contemporary analysis. Up to now these compounds have been analysed chiefly using chromatographic methods with only a narrow range of other techniques [37,38].

Phosphorothioates have been determined by quantitative TLC followed by bromometric titration [39], voltammetric determination [40] or densitometric determination preceded by reaction with palladium reagent [41]. Phosphorothioates as well as phosphorodithioates have been determined by means of acidimetric titration after reaction with chloromethylpyridinum iodide [42], potentiometric titration using ion-selective electrodes [43], argentometric [44,45] or mercurometric titration [46] and by polarographic methods [47]. Phosphorodithioates have also been determined by atomic absorption spectroscopy of their cupric(II) complexes [48,49]. Several types of thiophosphoryl compounds, including phosphine sulfides, phosphorothioates and phosphorodithioates have been determined spectrophotometrically on the basis of their charge-transfer complexes with tetracyanoethylene (TCNE) or tetracyanoquinodimethane (TCNQ) [50-52]. Phosphorothioates have been determined by chemiluminescence method [53], and by ion-exchange chromatography coupled with electroconductive or spectrophotometric detectors [54]. The analytical determination of some thiophosphorus insecticides (methamidophos, iso-malathion, fenitrothion) using a coulometric titration with the anodically generated chlorine and biamperometric end-point detection was elaborated by Ciesielski and co-workers [55]. Phosphorothioates and phosphorodithioates have also been selectively detected using immunoassay [56]. Thiophosphoryl compounds are also analized using Gas Chromatography [57], Mass Spectrometry [58], Flow-Injection-Analysis, Atomic Absorption Spectrometry [59], and also by Fourier-transform Raman Spectroscopy [60].

2.1. Analysis of Organothiophosphorus Compounds Using ^{31}P NMR

Although ^{31}P NMR spectra were reported as early as 1951 it was the availability of commercial multinuclear NMR spectrometers by 1955. That led to the application of ^{31}P NMR as an important tool for structure elucidation. With the introduction by 1970 of Fourier-transform (FT) and high-field superconducting magnet NMR spectrometers, ^{31}P NMR spectroscopy expanded to the study of biological phosphorous compounds. The ^{31}P nucleus has convenient properties suitable for FT NMR: spin ½, 100% of natural abundance, moderate relaxation time and a wide range of chemical shift [δ(^{31}P) > 600 ppm], which recommend this technique for organophosphorus compounds analysis. With substantial increase of sensitivity of modern FT NMR spectrometers, analytical applications of this technique grew dramatically during the last decades [61].

For analysis (identification and/or determination) of phosphorus-containing compounds, including thiophosphoryl derivatives, ^{31}P NMR technique is widely used in analytical as well as organic and related chemistry

[61,62]. The analytical potential of this technique is reflected by results compiled in Table 4.

Table 4. Representative ^{31}P NMR spectra of phosphorus compounds [62]

No	Compound structure	δ (^{31}P) [ppm][a]	No	Compound structure	δ (^{31}P) [ppm][a]
1	H_3PO_4	0.0	21	$(EtO)_3P=S$	68
2	NaH_2PO_4	0.5	22	$(EtS)(EtO)_2P=S$	94
3	Na_2HPO_4	3.5	23	$(EtO)_2P(S)-SH$	86
4	Na_3PO_4	6.0	24	$[(EtO)_2P(S)-S-]_2$	84
5	Na_3PO_3S	32	25	$(EtS)_3P=S$	92
6	$Na_3PO_2S_2$	61	26	H_3PO_3	5
7	Na_3POS_3	86	27	NaH_2PO_3	4
8	Na_3PS_4	87	28	Na_2HPO_3	4
9	$(EtO)_3P=O$	0.0	29	$(EtS)_3P$	115
10	$(EtS)(OEt)_2P=O$	26	30	$(EtO)_2P(S)H$	61
11	$(EtS)_2(OEt)P=O$	54	31	$(EtO)_2P(O)H$	7.5
12	$(EtS)_3P=O$	61	32	$(EtO)_2P(S)SH$	67
13	$Me_3P=O$	36	33	$Me_3P=S$	59
14	$Bu_3P=O$	41	34	$Bu_3P=S$	48
15	$Ph_3P=O$	25	35	$Ph_3P=S$	40
16	$Me-P(O)(OEt)_2$	30	36	$Me-P(O)(SEt)_2$	57
17	$Me-P(S)(OEt)_2$	95	37	$Me-P(S)(SEt)_2$	78
18	$Me_2(PrS)P=S$	53	38	$(H_2N)_3P=O$	22
19	$Et_2(PrS)P=S$	61	39	$(Me_2N)_3P=O$	23.5
20	$(Me_2N)_3P=S$	81	40	$(H_2N)_3P=S$	61

[a] Positive chemical shift values are reported for compounds absorbing at lower fields than H_3PO_4.

The dependence of chemical shifts of ^{31}P nuclei in acidic organophosphorus compounds on pH (Table 4, [63-65]), causes that supplementary techniques in their analysis (including TLC supplied with selective detection) are required.

2.2. Thin-Layer Chromatographic Detection of Organophosphorus Compounds

Thin-layer chromatography (TLC) combined with chemoselective detection has been considered as the method of choice, especially for non-volatile and thermally unstable organophosphorus derivatives [37-40,66,67]. Thus, phosphorothioates and phosphorodithioates have been detected by TLC using silver nitrate solution alone [68] or in conjunction with chelating indicators (e.g., bromocresol green) [68-70] and using copper(II) chloride solution and potassium hexacynoferrate(III) solution [71] as subsequent spray reagents. Also, potassium iodoplatinate [38], palladium(II) chloride [38,41,72] and palladium(II) complexes with fluorescent indicators [e.g., palladium(II)-calcein] [73] have been widely used for the detection of thiophosphoryl insecticides. Phosphinothioate metal complexes have been localized on TLC plates by means of a dithizone reagent [40,74]. The detection of thiophosphoryl compounds has also been achieved using 2,6–dibromo-benzoquinone-4-chlorimine (DCQ) [75,76], an ammonia solution of 4-methyl umbelliferone preceded by bromine vapour treatment [76], fluorescein [77], ammonium molybdate reagent [71,78,79] and potassium iodate solution [80] as spray reagents.

The thiophosphoryl compounds are visible in the UV region (254 nm) using fluorescent-chromatoplates [81]. Several reports have described the use of the TLC-enzyme inhibition (TLC-EI) technique for the detection of a variety of organophosphorus compounds, including the P-S type compounds [38,82-84].

Most of the procedures cited above seem to employ general rather than specific detection reagents for thiophosphoryl compounds therefore we turned our attention to the iodine-azide reaction which is known to be induced by various sulfur compounds.

3. Application of the Iodine-Azide Reagent

Iodine-azide reagent has been used in the analysis of divalent sulfur compounds for a number of years after the iodine-azide reaction was first described by Raschig at the beginning of the twentieth century [85]. In the course of the induced iodine-azide reaction iodine is consumed and nitrogen is evolved [86].

$$I_2 + 2N_3^- \xrightarrow{\text{S(II)}} 2I^- + 3N_2$$
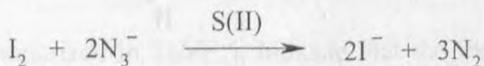
S(II) – inductor containing sulfur (II)

Fig. 4. General scheme of the induced iodine-azide reaction

The sensitivity of sulfur compounds determinations heavily depends on the induction coefficient defined by the equation (1):

<div align="right">(eq. 1)</div>

$$F_i = n_I / n_i$$

where: n_I is moles of iodine consumed in the induced reaction and n_i is moles of the inductor.

This implies straight forward relationship – the higher the induction coefficient, the more sensitive the determination of a given inductor [86-95].

3.1. Application of the Iodine-Azide Reagent in Analysis of Organophosphorus Compounds

The first report on the application of the iodine-azide reagent for TLC detection of phosphorothioate based herbicides (parathion, malathion, chlorthion, metasystox, diazinon, thiometon) was published by Fischer and Otterback in 1959 [96], and later by Cserhati and Orsi [97]. In the last two decades we have been exploring the phenomenon of induction activity, exhibited in the iodine-azide reaction by the thiophosphoryl compounds [98-108]. As the result we determined induction factors F_i of the major class of organophosphorus compounds which are presented in Table 5.

We proposed the tentative mechanism of induction effect exhibited by thiophosphoryl inductors [99,101], on the basis of earlier mechanistic works published by Strickland [90], Mayerstein [91] and Kurzawa [92], and concerning the reaction with the use of thiolic inductors (Fig. 4).

$$I_3^- + N_3^- \rightleftharpoons I_2N_3^- + I^-$$

$$\underset{R'}{\overset{R}{\diagdown}}\underset{}{\overset{X}{\diagup}}P\!-\!S\text{-}H + I_2 \longrightarrow \underset{R'}{\overset{R}{\diagdown}}\underset{}{\overset{X}{\diagup}}P\!-\!S\text{-}I + HI$$

$$\underset{R'}{\overset{R}{\diagdown}}\underset{}{\overset{X}{\diagup}}P\!-\!S\text{-}I + N_3^- \longrightarrow \underset{R'}{\overset{R}{\diagdown}}\underset{}{\overset{X}{\diagup}}P\!-\!S\!-\!N_3 + I^-$$

$$2\ \underset{R'}{\overset{R}{\diagdown}}\underset{}{\overset{X}{\diagup}}P\!-\!S\!-\!N_3 + I_2N_3^- \longrightarrow 2\ \underset{R'}{\overset{R}{\diagdown}}\underset{}{\overset{X}{\diagup}}P\!-\!S\text{-}I + 3\,N_2 + I^-$$
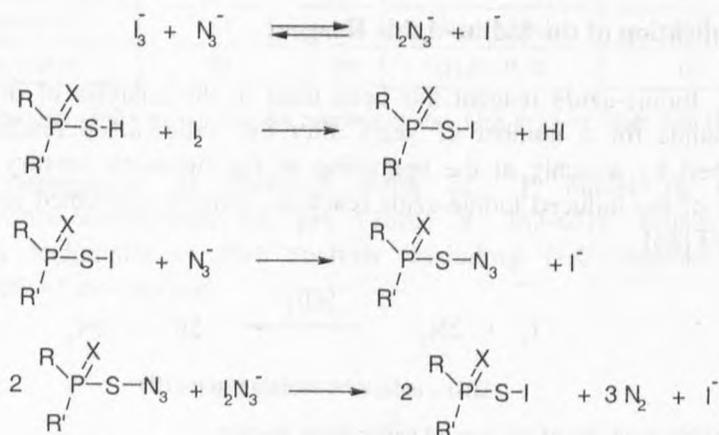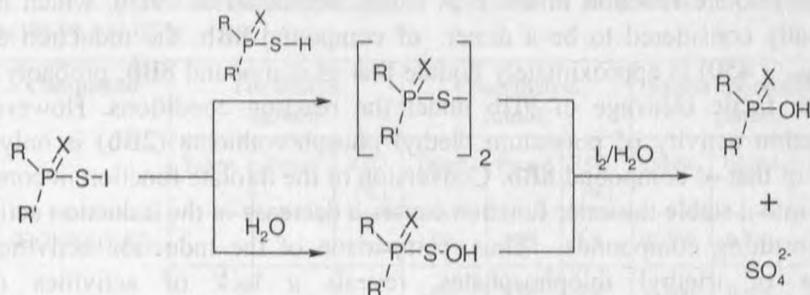
Fig. 4. Postulated mechanism of the iodine-azide induced reaction with the use of phosphorothiolates [99,101].

Table 5. Induction coefficients of the representative organophosphorus compounds [98,100,102]

Compound			Compound		
No	Structure	F_i^a	No	Structure	F_i^a
1	H_3PO_4	0	9Db	$[(EtO)_2P(S)-S-]_2$	450
1Bb	$(EtO)_2P(O)-OH$	0	11Cb	$(EtS)_3P=S$	8
2Bb	$(EtO)_2P(S)-O^- K^+$	40	14f	$Ph(H_2N)_2P=S$	142
2Cb	$(EtO)_2P(O)-SEt$	0	15Ab	$Ph_2(EtO)P=S$	105
3Cb	$(EtO)_3P=S$	6	16f	$Ph_2(NH_2)P=S$	152
6Db	$(EtO)_2P(O)-O-P(S)(OEt)_2$	6	19f	$Ph_3P=O$	0
7Db	$(EtO)_2P(S)-O-P(S)(OEt)_2$	12	20a	$Me_3P=S$	158
8Bb	$(EtO)_2P(S)-S^- K^+$	220[b]	20c	$Bu_3P=S$	133 190[b]
8Bc	$(BuO)_2P(S)-S^- K^+$	210[b]	20e	$Oct_3P=S$	92[b]
8Cd	$(HexO)_2P(S)-S^- K^+$	137[b]	20f	$Ph_3P=S$	213[b]
8Be	$(OctO)_2P(S)-S^- K^+$	89[b]	20g	$MePh_2P=S$	197[b]
8Cb	$(EtO)_2P(S)-SEt$	20	20h	$Me_2PhP=S$	195[b] 154

[a] Determined by iodometric titration. [b] Determined by spectrophotometric method.

The data summarized in Table 5, indicate that the induction activity of thiophosphoryl compounds is strongly dependent on the structure, especially on the nature of the P-S bonds. Thus, potassium diethyl phosphorodithioate (8Bb) exhibits a high induction activity ($F_{i(8Bb)}$ = 220), apparently due to the presence

of the thiolate function in the P-S- anion. For disulfide **9Db**, which may be formally considered to be a dimer of compound **8Bb**, the induction activity ($F_{i(9Db)}$ = 450) is approximately double that of compound **8Bb**, probably owing to the facile cleavage of **9Db** under the reaction conditions. However, the induction activity of potassium diethyl phosphorothioate (**2Bb**) is only about 20% of that of compound **8Bb**. Conversion of the thiolate function in compound **2Bb** into a stable thioester function causes a decrease in the induction activity of the resulting compounds. Thus, comparison of the induction activities of a series of triethyl thiophosphates, reveals a lack of activities of the phosphorothiolate **2Cb** ($F_{i(2Cb)}$ = 0), low activity of triethyl phosphorothionate (**3Cb**) ($F_{i(3Cb)}$ = 6) and triethyl phosphorotetrathiolate (**11Cb**) ($F_{i(11Cb)}$ = 8) and a slightly higher activity of triethyl phosphorodithioate (**8Cb**) ($F_{i(8Cb)}$ = 20). In contrast, phosphine sulfides **20**, containing the P-S bond, exhibit remarkably high induction effects, strongly dependent, however, on the structure. Thus, sulfides **20** with alkyl substituents exhibit induction coefficients (F_i) on the range levels 92 ($F_{i(20e)}$) to 158 ($F_{i(20a)}$). Phosphine sulfides with aryl substituents exhibit F_i on the level 195 ($F_{i(20g)}$, $F_{i(20h)}$) to 213 ($F_{i(20f)}$). The replacement of the phenyl group in phosphine sulfide **20f** by the ethoxy group leads to ethyl diphenylphosphinothionate (**20f** $\rightarrow$ **15Ab**) and to substantial decrease in the activity of these compounds ($F_{i(15Ab)}$ = 105). Similar replacement the phenyl by the amide function affords diphenylphosphinoamidethionate (**20f** $\rightarrow$ **16f**) and to decrease in the activity of this compounds ($F_{i(16f)}$ = 152). Surprisingly, the substitution of the second phenyl by the amidate functions (**16f** $\rightarrow$ **14f**) does not cause significant change in the induction activity ($F_{i(14f)}$ = 142). Tetraethyl monothiopyrophosphate (**6Db**) and tetraethyl dithiopyrophosphate (**7Db**) also exhibit low induction activities ($F_{i(6Db)}$) = 6 and $F_{i(7Db)}$) = 12, respectively).

Taking into account a high induction potency of phosphine sulfides, and other thiophosphoryl compounds, and the lack of convenient methods of their determination, the method based on the iodine-azide reaction can be considered as the method of choice.

Three procedures for indirect determination of thiophosphoryl compounds based on the induced iodine consumption were elaborated and their representative results are presented in Table 6. These include: (a) titrimetric method (on μmol scale) - performed via the induced iodine-azide reaction and the subsequent titrimetric determination of the consumed quantity of iodine (b) coulometric method (on nmol scale) - performed via the induced iodine-azide reaction and the subsequent coulometric determination of the consumed quantity of iodine; and (c) spectrophotometric method (on nmol scale) – performed via the induced iodine-azide reaction and the subsequent spectrophotometric determination of the consumed quantity of iodine.

Table 6. Results of the determination of representative thiophosphoryl compounds – inductors [98,99,100,102]

No	Compound	Titrimetric [μmol]			Coulometric [nmol]			Spectrophotometric [nmol]		
		Taken	Found	RSD [%]	Taken	Found	RSD [%]	Taken	Found	RSD [%]
8Bb	$(EtO)_2P(S)S^- K^+$				1.00	1.03	8.5	0.200	0.204	2.6*
					2.00	1.96	4.8	0.400	0.404	1.1*
					5.00	5.06	2.7	0.500	0.520	4.6
					10.00	10.00	2.4	2.00	1.97	1.4
					20.00	19.70	2.0	4.00	3.98	2.9
8Bc	$(BuO)_2P(S)S^- K^+$				1.00	1.01	7,7	0.50	0.475	8.4
					5.00	4.92	3.8	2.00	2.06	3.2
					20.00	19.60	2.0	4.00	3.97	2.1
8Be	$(OctO)_2P(S)S^- K^+$							2.00	2.01	3.2
								6.00	6.06	2.5
								10.00	10.00	0.8
9Db	$[(EtO)_2P(S)S-]_2$	0.050	0.051	2.5	0.050	0.048	8.2	0.050	0.047	7.3*
		0.125	0.124	2.0	2.50	2.45	2.3	0.150	0.153	3.1*
		0.375	0.370	1.6	10.0	9.90	1.8	0.250	0.245	2.4*
14	$PhP(S)(NH_2)_2$	0.200	0.197	3.1	1.00	1.03	7.4	1.00	1.02	5.2
		0.400	0.408	2.4	5.00	4.95	2.6	2.00	2.02	4.3
		1.2	1.19	1.9	25.0	24.6	1.9	6.00	5.97	2.1
16	$Ph_2P(S)NH_2$	0.150	0.153	3.8	1.00	0.98	6.8	0.70	0.72	4.5
		0.300	0.304	2.9	5.00	4.95	2.4	1.50	1.47	3.0
		25.0	24.9	1.5	25.0	24.9	1.5	6.00	5.95	2.5
20a	$Me_3P=S$	0.150	0.146	2.8	1.00	0.98	7.1	0.50	0.52	4.2
		1.00	1.01	1.6	5.00	4.96	2.3	5.0	4.92	2.4
					20.0	20.2	1.7			

20c	Bu$_3$P=S	0.150	0.148	3.1	1.00	0.98	7.1	0.50	0.52	3.1
		1.00	1.01	1.6	10.0	9.8	2.1	5.0	4.90	1.6
					25.0	25.2	2.4			
20h	Me$_2$PhP=S	0.150	0.153	3.1	1.00	0.97	7.2	0.50	0.51	3.3
		1.00	0.99	1.2	5.00	4.95	2.1	5.0	5.04	1.8
					20.0	20.2	1.5			
20g	MePh$_2$P=S	0.150	0.147	2.8	1.00	1.03	7.6	0.50	0.49	3.2
		1.00	0.955	1.7	5.00	4.92	2.4	5.0	5.04	1.8
					20.0	19.7	2.1			
20f	Ph$_3$P=S							0.50	0.52	3.4
								2.00	2.01	2.7
								4.00	3.92	2.1

* UV measurements carried out in a 5 cm long cuvette.

Different values of the induction coefficients found for the same compound depending on the method of determination were due to different reaction conditions applied in each analytical technique. The induction coefficient has been found to be independent of pH in the range of $5.5 < pH < 7.5$ for all determined sulfides. However, in the case of phosphorothioate **2B** and/or phosphorodithioate **8B** salts, as well as their disulfides **9D**, induction coefficient increased with decrease of the pH values. The use of solutions whose pH is lower than 6 (optimal) is not recommended because of the emission of the poisonous, volatile hydrazoic acid.

3.2. TLC Detection of Thiophosphoryl Compounds Using the Iodine-Azide Reagent

The results of the detection of various thiophosphoryl compounds by means of UV (254 nm), the iodine detection, the molibdate reagent and using iodine-azide reagent are summarized in Table 7.

Detection limits (DL) of organophosphorus compounds, resulting from their induction activity are strongly dependent on their structures and element contributions [103-105]. The activity of thiophosphoryl derivatives depends on the nature of the P(S)$_n$ function.

Table 7. Detection limits of representative organophosphorus compounds with UV detection, using iodine vapour, the molybdate reagent and iodine-azide procedure [105]

No	Structure	Detection limits [nmol]						
		$F_i^{/a}$	UV [254 nm]	$I_2^{/b}$	— $I_2\text{-}N_3^{-/c}$ A	B^d	Molybdate^e C^f	D^g
1Cb	Et—O—P(=O)(O—Et)(O—Et)	-	-^{h}	50	-^{h}	-^{h}	-^{h}	30
1Ci	Ph—O—P(=O) (cyclic O)	-	25	0.5	-^{h}	-^{h}	-^{h}	50
1Cf	Ph—O—P(=O)(O—Ph)(O—Ph)	-	10	30	-^{h}	-^{h}	-^{h}	30
2Ck	Ph—O—P(=O)(S, cyclic)	22	25	0.5	2.5	>5	25	
2Ci	Ph—S—P(=O) (cyclic O)	13	25	5	25^i	>25	50	
3Ci	Ph—O—P(=S) (cyclic O)	156	25	0.5	2.5		5.0	
8Ci	Ph—S—P(=S) (cyclic O)	190	2.0	0.5	0.5	>5	1.5	
8Ck	Ph—O—P(=S)(S, cyclic)	163	25	0.5	0.5		1.5	
10Cl	Ph—O—P(=S)(S, cyclic S)	193	20	0.5	0.5		1.5	
10Ck	Ph—S—P(=S)(S, cyclic)	220	5	0.5	0.5		1.5	

11Ck	Ph—S—P with S structure	208	2.0	0.5	0.5	0.5	1.5

[a] Determined in solution after 15 min. [b] Brown spots on yellow background. [c] White spots on yellow background. [d] After pre-treatment with ammonia vapour. [e] Blue spots. [f] Standard molybdate procedure. [g] Molybdate procedure (D) with prior mineralization of chromatographed compounds by perchloric acid at 180 °C. [h] Not detectable at the level of 50 nmol per spot. [i] After 15 min of exposure.

Our results indicate that the induction activity (resulting from this detection) of thiophosphoryl compounds is to be attributed rather to the thiolate (P-S⁻) function than to the thiol ester (P-S-R) or thiono (P=S) functions. Consequently, phosphorodithioates and their salts, disulfides and both alkyl and aralkyl phosphine sulfides have the lowest detection limits. Triphenylphosphine sulfides and ethoxydiphenylphosphine sulfides have high detection limits – above 150 nmol presumably owing to the fact, that the iodine-azide reaction is hampered by iodide ions present in the spraying solution.

The determinability of organophosphorus thioamides has been found to depend on their amide radicals: primary and secondary thioamides have low or average detection limits (0.1–2.5 nmol) whereas tertiary thioamides remain inactive. Monothioacids, phosphorothioate and tetraethyl monothiopyrophosphate exhibit moderate detection limits (10-30 nmol), whereas thiophosphate, dithiophosphate and perthiophosphate are extremely difficult to detect (>200 nmol). A similar trend is observed for thiophosphoryl derivatives of trivalent phosphorus. Dialkylthiophosphites have medium detection limits (20-30 nmol) this is to be explained by their isomerisation from the thiono [P(S)H], to the thiolic, [P-S-H], forms whereas triethylthiophosphites and triethyltrithiophosphites have very high detection limits (>100 nmol).

The iodine-azide reagent allows the microdetection of aliphatic and mixed alkyl-aryl phosphine sulfides. Organophosphorus thioacids, their salts, partial esters and primary and secondary thioamides as well as disulfides, in general, compounds with the P=S or P-S-R functions exhibit low detection limits. All the other tested compounds with the P=S or P-S-R functions, exhibit poor to moderate detection limits and the application of the iodine-azide reagent requires their prior hydrolytic activation to the P-S⁻ function.

It has to be noted, that the iodine-azide test reaction applied for sulfur derivatives of phosphorus acids may be interfered by the presence of some sulfur compounds, without phosphorus in the molecule. A similar interference problem also exists in the case of molybdate test, which is the most common test for phosphorus compounds [109]. Thus, the application of the iodine-azide reaction

or the molybdate reagent gives indiscriminately positive results for several thiophosphoryl and sulfur derivatives. To overcome this lack of selectivity, we additionally developed a modified molybdate procedure, based on the phosphoro-molybdate reaction with prior oxidative elimination of potential sulfur reductants of molybdate. As a consequence, the combination of the iodine-azide test with the modified molybdate test permits the differentiation of thiophosphoryl, phosphoryl and sulfur compounds.

Table 8. Detection of sulfur compounds and thiophosphoryl compounds by means of the iodine-reagent, the molybdate reagent and the pre-oxidation-molybdate procedure [103,105]

Compound			Detection procedure[a]				
Entry (No)	Structure [No]	Amount (µg per spot)	I_2-N_3^{-}[b]	Mo[c]	(1) $Br_{2(aq.)}$ (2) Mo	(1) HNO_3 (2) Mo	(1) H_2O_2 (2) Mo
1	HOCH$_2$CH$_2$SH	1	+	+	−	−	−
		10	++	++	−	−	−
2	HSC$_6$H$_4$CO$_2$H-m	1	+	−	−	−	−
		10	++	++	+[d]	+[d]	+[d]
3	PhSSPh	1	+	−	−	−	−
		10	+	+	+[d]	+[d]	+[d]
4	PhNHC(S)NH$_2$	1	+	+	+[d]	+[d]	−
		10	++	++	+[d]	+[d]	+[d]
5	(EtO)$_2$P(S)ONa [2Bb]	1	+−	++	+−[c]	+−[c]	+−[c]
		10	+	++	+[c]	+[c]	+[c]
6	(EtO)$_2$P(S)SNa [8Bb]	1	+	+	+[b]	+−[c]	+−[c]
		10	++	++	+[c]	+[c]	+[c]
7	[(EtO)$_2$P(S)S]$_2$ [9Db]	1	+	+	+[c]	+[c]	+[c]
		10	++	++	++[c]	++[c]	++[c]
8	PhP(S)(NHMe)$_2$ [14f]	1	+	−	−	−	−
		10	+	+	+[c]	+[c]	+[c]
9	[Ph$_2$P(S)S]$_2$ [18Df]	1	+	+	−	−	−
		10	++	+	+[c]	+[d]	+[d]

[a] Detection: ++ = strong, + = distinct, +/− = spot detectable, - = spot not detectable.
[b] White spots. [c] Blue spots. [d] Brown spots with or without molybdate spray.

The best results are achieved when bromine water is used for preliminary oxidation. Only thiophosphoroorganic derivatives appear as blue spots in the reaction with molybdate. Compounds that contain only sulphur become visible as brown spots.

In the light of the results presented here, it is clear that only the iodine-azide reagent allows the selective detection of thiophosphates. Other detection systems (UV, iodine$_{(v)}$, HCl$_{(v)}$) routinely used in the TLC of organophosphorus compounds give a positive test for both phosphates and thiophosphates.

The iodine-azide reagent can be used for the detection of phosphorothioate analogues of nucleotides in the presence of phosphate and/or phosphonate derivatives. The combination of the iodine-azide and molybdate detection procedures can be successfully applied for the chromatographic detection and subsequent TLC differentiation of nucleotides and their phosphorothioate analogues.

The encouraging results on the use of the iodine–azide reaction for TLC detection of some phosphorothioate derivatives of nucleotides [104] prompted us to perform some additional investigations on the structural factors influencing the reaction course. We focused on the induction activity of various types of thiophosphate derivatives and the analytical repercussions, especially within the context of their selective TLC detection. In the course of these studies, we have determined the induction coefficients (F_i) of several representative phosphates and phosphorothioates, in order to establish their molecular structure–induction and detectability–induction activity relationships. The results clearly demonstrate the lack of induction activity exhibited by phosphates and substantial induction activity exhibited by phosphorothioates. The latter increases with the number of sulfur atoms in the inductor's molecule, clearly illustrating the higher induction potency of derivatives with the thiono (P=S) rather than the thiolo (P–S–R) functions.

The F_i parameters determined for aqueous solutions of phosphorothioates three times; after 5, 15 and 60 min exhibit strong time dependence, with a plateau usually 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 correlation between detection limits and induction coefficients is better for shorter periods of exposure time (1 to 5 min). The results illustrate the reasonably good correlation between the induction potency (F_i) of phosphates and phosphorothioates and their detectability.
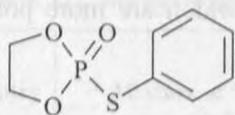
$$F_i(5') = 9$$
(DL = 25 nmol)
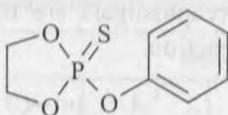
$$F_i(5') = 141$$
(DL = 2,5 nmol)
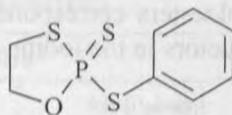
$$F_i(5') = 199$$
(DL = 0,5 nmol)

Phosphates exhibit no induction potency and are not detectable on TLC plates. Sugar phosphorothioates (Fig. 2) exhibit even more specific induction properties. Their induction activity is dependent on their molecular structure, and increases strongly with reaction time [105]. Thus, the deoxyglucose phosphorothioate $C_{(1)}$-S-P has moderate induction activity ($F_{i(5')} = 47$), glucose derivatives have medium induction activity ($90 < F_{i(5')} < 107$), and the compound with the $C_{(3)}$-S-P linkage pattern exhibits the highest induction activity ($F_{i(5')} = 280$) of the series. These results suggest that S-alkylphosphoro(thio)ates with the $C_{(1)}$-S-P linkage pattern exhibit lower induction activities than those with other [e.g. at $C_{(3)}$] alignments of phosphorothioate in the sugar ring. The deoxysugar derivative has lower induction potency than the glucose phosphorothioate. The induction activity of all sugar phosphorothioates exhibits a strong dependence on the reaction time as the P-S-C bond breaks. The induction coefficient (F_i) after 60 min is five times higher than after 5 minutes.

The correlation between DL and F_i appears to be yet more complex in the case of sugar derivatives than in the case of the series of phosphorothioates. Surprisingly, the high induction potency that these compounds exhibit in solution is not reflected in their TLC detection on silica gel plates. Prolongation of the exposure time does not influence the detectability. This may be due to the fact that the mechanism of the iodine–azide induced reaction is more complex on silica gel plates.

The relatively poor detection of certain phosphorothioates in the iodine-azide method can be greatly enhanced by hydrolytic pre-treatment of the chromatographic plate. Such a pre-treatment 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 with a phosphorothioate moiety at the $C_{(1)}$ carbon of the sugar unit [the $C_{(1)}$-S-P linkage], are difficult to detect using the iodine–azide reagent (DL = 100 nmol). Their detection rate increases strongly if the plate is pre-exposed to ammonia vapour (DL = 3 nmol) or HCl vapour (DL = 2 nmol). Such high increase in the detectability suggests, that during the hydrolytic pre-treatment of sugars

thioloesters corresponding mercaptosugars are formed, which are more potent inductors in the iodine–azide reaction.
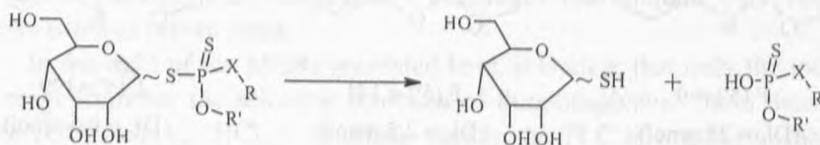
Fig. 6. Hypothetical course of the digestion of 1-thiophosphorylsugars, occurring during the treatment with HCl or ammonia vapour.

Phosphorothioates with a thiophosphoryl function at the $C_{(3)}$ atom of the sugar unit [the $C_{(3)}$–S–P linkage] have been found to have substantially higher detectability. The iodine-azide method allows detect these compounds at the level of 20 nmol per spot. Moreover, when alkaline hydrolysis or acidic hydrolysis is used the detection level increases to 2 nmol and to 0.5 nmol respectively.

The results of some other representative procedures applied for the TLC detection of phosphates and phosphorothioates, are summarized below.

All tested compounds give a positive test reaction when exposed to iodine vapour (brown spots on a yellow background) and their detection limits range between 0.2 to 0.5 nmol per spot for compounds thiophosphates and at 2.5–10.0 nmol per spot for sugar derivatives. UV detection (at 254 nm) leads to ambiguous results. Thus, the phosphorothioates with an aromatic ring and/or the phosphorothiono (P=S) moiety exhibit detection limits at the level 2.0 to 25 nmol per spot, whereas phosphorothioates with aliphatic substituents are only poorly detected, with DL below 100 nmol per spot. The detection of phosphates and/or phosphorothioates using molybdate reagents depends on the stability of their phosphoester or phosphothioester functions. In the case of application of a milder procedure A – without prior hydrolysis with perchloric acid – the DL_s vary from 1.5–5.0 nmol for compounds with the P=S linkage, and 25–50 nmol for compounds with the P-S-C linkage [104]. In the sugar series, the compound bearing with the $C_{(3)}$–S–P linkage has a DL of 0.5 nmol, whereas compounds with the $C_{(1)}$–S–P linkage are very difficult to detect ($DL_s > 100$ nmol per spot). These detectabilities substantially increase when chromatographed compounds have been subjected to prior hydrolysis with perchloric acid and the plate has been heated to 180°C (procedure B) [103,109,110].

The application of the iodine-azide procedure, combined with the molybdate procedure, for the selective TLC analysis of phosphates and thiophosphates mixtures (entries 1-3) is illustrated in Table 9.

Table 9. TLC analysis of the mixtures of phosphates and phosphorothioates [105]

Entry (No)	Mixture of compounds[a]	Detection system[b]				
		UV /R_F[c]	I_2[d] /R_F[c]	I_2-N_3^-[e] /R_F[c]	Molibdate[f]	
					A[g]/R_F[c]	B[h]/R_F[c]
1	Ph–O–P (cyclic, O) and	+/0.35	++/0.35	–	–	+/0.35
	Ph–O–P (cyclic, S,O) and	+/0.46	++/0.46	++/0.46	+/0.46	++/0.46
	Ph–O–P (cyclic, S,S,O)	+/0.51	++/0.51	++/0.51	++/0.51	++/0.51
2	Ph–O–P (cyclic, O) and	+/0.35	++/0.35	–	–	+/0.35
	Ph–O–P (cyclic, S,O) and	+/0.46	++/0.46	++/0.46	+/0.46	++/0.46
	Ph–S–P (cyclic, S,S)	++/0.58	++/0.58	++/0.58	++/0.58	++/0.58
3	Ph–O–P(=O)(O–Ph)(O–Ph) and	++/0.41	+/0.41	++/0.41[d]	–	+/0.41
	Ph–O–P (cyclic, S,O) and	+/0.51	++/0.51	++/0.51	++/0.51	++/0.51
	Ph–S–P (cyclic, S,S)	++/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.

Thus, the cyclic phosphates were chosen for our research so as to differentiate hydrolysis susceptibility. We chose three mixtures containing phosphates and two with sulfur atoms in different positions. As we had expected, only thiophosphates gave positive tests with the iodine-azide reagent. Phosphates were not visible also when the molybdate reagent without prior hydrolysis was applied (procedure A). The molybdate used in the more drastic procedure B was unselective and all derivatives appeared as blue spots.

3.2.1. TLC Detection of Phosphorothioate Analogues of Nucleotides

The group of thiophosphoryl compounds of increasing importance both in pure and applied chemistry, includes phosphorothioate analogues of nucleotides and oligonucleotides presented by the general structure given on Fig. 3. Some representative structures are presented on Fig. 7.

OTP(T$_{Ac}$)　　　　DMT(T)ODMP　　　DMT(T)DTP　　　　(T)DTP
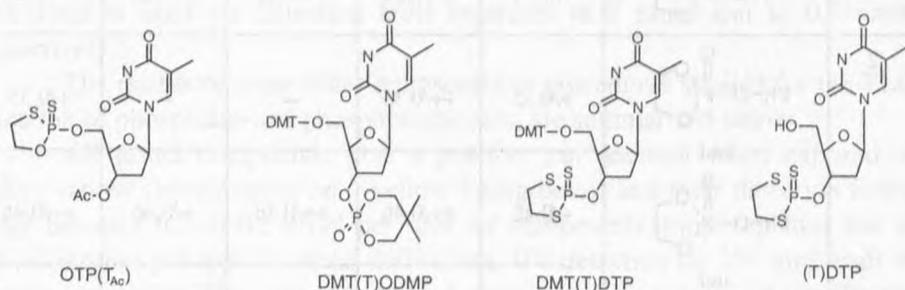
Fig. 7. Representative structures of thiophosphate nucleosides applied.

Despite the fact that all nucleosides and their derivatives are easily detectable by UV spectroscopy it was crucial to find a method for the selective identification of phosphorothioate analogues of nucleotide analogues in mixtures containing also "non-sulfurized" nucleic acid components.

Table 10. Detection limits of phosphorothioate analogues of nucleotides with UV detection (254 nm), using iodine (vapour), the molibdate reagent and the iodine-azide detection reagent [104]

Entry (No)	Compound	Detection system (nmol per spot)					TLC [R$_F$]
	Structure [a]	I$_2$ [b]	UV	I$_2$-N$_3$ [-/c]	Molybdate [d-f]	HCl [e]	
1	PhO-OTP	0.1	1.5	0.3	1.5[d]	-[g]	0.73[h]
2	5'-CMP	2.0	6.0	-	2.0[d]	-[g]	0.37[i]
3	DMT(T)ODPP	25	1.3	25	0.1[e]; 10[f,d]	0.1	0.37[k]

4	DMT($_{Si}$U)OTP	0.3	0.6	1.2	0.1^e; 10^{f,d}	0.1	0.50^k
5	DMT($_{Si}$A^{Bz})OTP	0.3	0.6	1.0	0.1^e	0.1	0.73^i
6	DMT(T)OTP	0.15	0.6	0.5	0.1^e	0.1	0.69^i
7	DMT(dA^{Pya})OTP	0.5	0.6	1.3	0.4^e	0.3	0.52^i
8	OTP($_{Ac}$T)	0.2	1.2	0.6	12^d	-^{g}	0.69^i
9	OTP(d$_{Ac}$G^{iBr})	0.2	0.2	0.6	2.0^d	-^{g}	0.60^i
10	DMT(T)DTP	0.15	0.6	0.5	0.15^e; 10^{f,d}	0.15	0.67^i
11	DMT(dA^{Bz})DTP	0.15	0.6	0.5	0.15^e	0.15	0.64^i

[a] Abbreviations: A, G, U, T: adenosine, guanosine, uridine, thymidine; $_{Si}$A, $_{Si}$G, $_{Si}$U; corresponding ribonucleosides blocked at 2' position with [t]BuMe$_2$Si group; dA, dG: corresponding deoxyribonucleosides; Ac: acetyl; iBr: isobutyryl; Ph: phenyl; Bz: benzoyl; Pya: N-methylpyrrolidin-2-ylidenyl; DMT dimethoxytrityl; OTP: 2-thio-1,3,2-oxathiaphospholane; DTP: 2-thio-1,3,2-dithiaphospholane; ODPP: diphenylphos-phate (detailed structures of representative compounds are given in Fig. 7). [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 μg 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$_2$O (80:15:5).

In the light of the results presented in Table 10, only the iodine-azide detection reagent allows the selective detection of the thiophosphate (phosphorothioate) systems. Other applied detection systems, routinely used for the TLC of organophosphorus compounds (UV, iodine, HCl(v), the molibdate reagent) gave a positive test for both phosphates and thiophosphates.

In contrast, the iodine-azide reagent detects sulfur-containing molecules and therefore can be useful for the detection of phosphorothioate analogues 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 analogues (Table 11).

As stated above, detection limits of thiophosphoryl compounds correspond to their induction potency in Raschig's reaction which in turn depends on the inductor's structure. Therefore it was of great interest to examine the structure-induction activity relationship for more complex compounds namely phosphorothioate nucleotide analogs. Nucleotide derivatives containing 2-thio-1,3,2-oxathiaphosphalane (OTP) or a 2-thio-1,3,2-dithiaphosphalane (DTP) ring were analysed using the iodine-azide reagent [104].

Table 11. TLC analysis of the mixtures of nucleotides and their thiophosphoryl analogues [104]

Entry (No)	Mixture of compounds [a]	Detection system [b] [Detection/(R_F)[h]]				
		UV [254 nm]	Iodine(v)	Molybdate		I_2-N_3[-/f]
				A	B	
1	PhO-OP	+(0.35)	++(0.35)	-	++(0.35)	-
	and					
	PhO-TOP	+/(0.46)	++/(0.46)	+/(0.46)	++/(0.46)	++/(0.46)
	and					
	PhO-TDTP	+/(0.51)	++/(0.51)	++/(0.51)	++/(0.51)	++/(0.51)
2	ODPP	++/(0.41)	+/(0.41)	-/(0.41)	+/(0.41)	++/(0.41)
	and					
	PhO-TOTP	+/(0.51)	++/(0.51)	++/(0.51)	++/(0.51)	++/(0.51)
	and					
	PhO-TDTP	++/(0.58)	++/(0.58)	++/(0.58)	++/(0.58)	++/(0.58)
3	T(DTP)	++/(0.13)	++/(0.13)	++d/(0.13)	++d/(0.13)	++(0,13)
	and					
	DMT(T)ODMP	++/(0.20)	++/(0.20)	++c/(0.20)	++d/(0.20)	—g
					++c/(0.71)	—
4	OTP(TAc)	++/(0.17)	++/(0.17)	++d/(0.17)	++d/(0.17)	++(0.17)
	and					
	DMT(T)DTP	++/(0.37)	++/(0.37)	++c/(0.37)	++d/(0.37)	++(0.37)
					++c/(0.76)	

[a] Taken amount: 10 nmoles of each nucleotide per spot (abbreviations as in Table 10). [b] Detection: ++ = strong, + = distinct, +/- = spot detectable, - = spot 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-ethyl acetate (9:1) (line 1and 2), benzene-MeOH (9:1) (line 3 and 4).

Iodine detection of phosphorothioates is more sensitive than that exhibited for their phosphate analogs. Phosphorothioates with aromatic system are visible in UV light. Iodine-azide detection of phosphorothioates also proves to be a highly sensitive method (0.15 to 1.3 nmol per spot) and the detection sensitivity, as in the case of simple derivatives, depends on the structure of the molecule. O-aryl phosphorothioates are detectable at the level of ca. 0.3 nmol. The substitution of sulfur by selenium atom does not affect the DL value. The detection limits for phosphorodithioate nucleotide analogues are in the range of

0.5–1.3 nmol. No significant differences are observed in the detection of phosphorothioate nucleotides with various nucleo-bases and only small differences between the series of ribonucleotides and deoxyribonucleotides. More distinct differences are observable in the series of nucleotides with phosphorothioate functions attached at the 2' and 5' positions of the sugar ring of nucleosides. The detectability of phosphorothioate nucleotide analogues tends to increase with the increase of sulfur content in the phosphorothioate function and in the case of phosphorotrithioate DMT(dG)DTP the detection level even reaches the level of 0.15 nmol per spot.

The iodine-azide reagent does not exhibit any activity towards phosphate compounds which do not contain sulfur such as DMT(T)ODPP, DMT(T)ODMP, (see Fig. 7) cytidine 5'-monophosphate and guanosine 5'–monophosphate. These compounds cannot be detected even at the level of 100 nmol per spot.

The detection of phosphorothioate nucleotide analogues by means of the molybdate procedure leads to ambiguous results as both phosphorothioates and phosphates react with the molybdate reagent forming blue spots on a white background [110]. However, since this procedure requires strong acid medium, the dimethoxytrityl protected nucleotides (DMT) appear on chromatographic plates as pink spots which is due to dimethoxytrityl cation formation. The strong absorbance of dimethoxytrityl cation at ca. 500 nm sufficiently masks characteristic blue spots that result from the reaction of the molybdate reagent with the phosphates and phosphorothioates. Therefore, the application of the molybdate procedure for the TLC detection of DMT-nucleotides requires some modification.

The developed chromatographic plate has to be sprayed with a 10% solution of trifluoroacetic acid then it has to be redeveloped before the molybdate agent is used. Detritylated nucleotides appear as blue spots and DMT-TFA derivatives appear above as pink spots.

3.2.2. TLC Detection of Sulfur-Containing Aminophosphonic Acids

The detection limits (DL) for cysteines (Cys and Cys^P), homocysteines (Hcys and $Hcys^P$) and methionines (Met and Met^P), by means of the iodine-azide reagent and the other representative detection procedures are summarized in Table 12.

The results of the reaction of the examined amino acids with the iodine-azide reagent, carried out both in solution and on a TLC plate, generally presented poor correlation.

Thus, Cys ($F_i = 325$), Cys^P ($F_i = 138$) and $Hcys^P$ ($F_i = 60$) exhibit a DL = 30 nmol, whereas Hcys ($F_i = 95$) is detectable at DL = 30 nmol per spot. Both

methionines (Met and Met^P) have not been characterized by induction coefficients due to their reaction with iodine alone, occurring in a solution [101]. However, their treatment with the iodine-azide reagent on TLC plates gave typical positive test results by formation of characteristic white spots on a yellow background with the DL = ca. 20 nmol per spot.

All examined aminophosphonic acids, as well as the thiolic amino acids, can be detected by means of the molybdate detection reagent. Thus, for Met^P the detection limit was as the order as 5 nmol, and for Cys^P and Hcys^P of the order of 20 nmol. Surprisingly, for Cys and Hcys (amino acids without phosphonic moiety) the detection limits was 30 nmol, whereas Met and Gly are not detectable up to a level of 50 nmol per spot. This phenomenon can be explained by the interaction of the sulfydryl groups of these amino acids with the molibdate reagent. The application of the iodine-azide detection allows visualization of the sulfur-containing amino acids on TLC plates in mixtures with other amino acids. The application of the pre-oxidation step prior to the molibdate detection allow on the differentiation of Cys^P, Hcys^P and Met^P from their carboxylic analogues (Cys, Hcys and Met) [107].

Table 12. Comparison of the TLC detection properties of sulfur-containing amino acids and their aminophosphonic analogues [107]

Amino acids		F_i	TLC Detection Limits [nmol per spot]					R_F^g
Abbrev.	Structure		UV	I_2^b	I_2-$N_3^{-/c}$	Ninh.^{d}	Mo^e	
Cys	H·S–C–C(=O)OH, H$_2$, NH$_2$	325	$-^i$	20	20	20	30^f	0.19
Hcys	H·S–[CH$_2$]$_2$–C(=O)OH, NH$_2$	95	$-^i$	5	30	5	30^f	0.45
Met	Me–S–[CH$_2$]$_2$–C(=O)OH, NH$_2$	$-^h$	$-^i$	5	20	20	$-^j$	0.51
Cys^P	H–S–C–C–P(=O)(OH)(OH), H$_2$, NH$_2$	138	$-^i$	20	20	20	20	0.25

Hcys[P]	H-S-[CH$_2$]$_2$-C(H)(NH$_2$)-P(=O)(OH)(OH)	60	–[i]	5	30	5	30	0.40
Met[P]	Me-S-[CH$_2$]$_2$-C(H)(NH$_2$)-P(=O)(OH)(OH)	–[h]	–[i]	5	20	1	5	0.46

[a] Induction factor of amino acids (F$_i$) determined in solution. [b] Iodine procedure: brown spots on yellow background. [c] Iodine-azide procedure: white spot on yellow back-ground. [d] Ninhydrine procedure: blue spots. [e] Molybdate procedure: blue spots. [f] Spots appeared after 15 min of exposition time. [g] TLC system: cellulose plates DC vs. n-BuOH–AcOH–water (12 : 3 : 5). [h] Does not induce the iodine-azide reaction in solution. [i] Not detectable in UV up to the level of 100 nmol of amino acid. [j] Not detectable up to the level of 50 nmol of amino acid.

3.2.3. Unusual Induction in the Iodine-Azide Induced Reaction

Our further research on the application of the iodine-azide reagent for organophosphorus compounds analysis has shown that non-sulfur compounds induce the iodine-azide reaction [106]. The results supporting this thesis are presented in Table 13.

Table 13. Comparison of the TLC detection limits (μg per spot) of phosphorus and organophosphorus compounds by means of UV (254 nm), iodine vapour, the iodine-azide reagent other representative reagents [106]

No	Compound Structure	Detection Limits (μg per spot) [a]						R$_F$
		UV	I$_2$ [b]	I$_2$-N$_3^{-/c}$	Mn [e]	Mo [g]	Ninh. [h]	
1	(HO)$_3$P=O	–	–	–	1 [f]	0.2	10	0.51 [m]
1Cb	(EtO)$_3$P=O	–	10	10 [b]	–	–	–	0.53 [n]
1Cf	(PhO)$_3$P=O	3	10	3 [b]	–	–	–	0.68 [n]
12	H-P(=O)(O-H)(O-H)	–	10	3 [b,d]	1 [f]	10	20	0.66 [m]
12Ba	(MeO)$_2$P(O)H	–	10	3	3	–	10	0.48 [n]
12Bb	(EtO)$_2$P(O)H	–	10	10	3	–	10	0.55 [n]
12Cf	(PhO)$_2$P(O)H	10	10	10	3	3	10	0.70 [n]
12Ca	(MeO)$_3$P	–	3	10	3	3	10	0.50 [n]
12Cb	(EtO)$_3$P	–	3	10	3	3	10	0.55 [n]
12Cf	(PhO)$_3$P	10	10	3	3	30	1 [i]	0.72 [n]

12b	$Et-P$ with O, $O-H$, $O-H$	–	–	–	1^f	1	20^k	0.72^m
12n	$Et-C-P$ with H, O, OH, $O-H$, $O-H$	–	–	–	1^f	10	20^k	0.64^m
12r	$Et-C-P$ with H, O, NH_2, $O-H$, $O-H$	–	–	–	1^f	0.2	0.1^i	0.30^m
21	H, H, $P-O-H$, O	–	10	$30^{d,b}$	1	10	30	0.36^m
22fb	Ph_2P-OEt	10	10	10^b	3	3	3	0.51^n
23f	Ph_3P	1	1	3	3	3	1	0.71^n

[a] "—" = not detectable at 50 μg per spot. [b] Brown spots. [c] White spots on yellow background. [d] Appear after 15 min. [e] Light green-grey spots on light-brownish background. [f] Pink spots without heating or after preheating to 100 °C . [g] Blue spots – after preheating to 100 °C or after irradiation at 360 nm. [h] Pink spots after preheating to ca. 100°C. [i] Pink spots without heating. [k] White spots on faint-pink background. [m,n] TLC systems: [m]cellulose/iBuOH-THF-water-acetone-Tos-OH (8 ml : 6:5 ml : 1 ml : 0.3 g); [n]silica gel/acetone.

However, until now, there have not been any reports in the chemical literature describing the induction of the iodine–azide reaction caused by compounds which do not contain sulfur.

The results of the TLC detection of the broad spectrum of tri- and pentavalent phosphorus compounds illustrate the possibility of differentiation of these compounds. The majority of di- and trialkyl phosphonic acid esters [$(RO)_2PHO$ and $(RO)_3P$] as well as triphenyl phosphine give a positive reaction with the iodine-azide reagent. Other derivatives of trivalent phosphorus with P-C bonds appear as brown spots. Phosphonic and phosphoric acids are inactive. The molybdate reagent unlike the iodine-azide reagent does not allow to differentiate between tri- and pentavalent phosphorus compounds.

Such unexpectedly good detection of trivalent phosphorus compounds, which is comparable to that of the sulfur compounds, can be explained by their reactivity. Detection sensitivity depends both on the structure of the phosphorus compounds and on the type of TLC plates used. Thus, triphenyl phosphine is detected as white spots on silica oxide plates, or brown spots on alumina oxide or on reversed-phase plates. However, in spite of the distinct induction activity of these phosphoryl derivatives on the TLC plates, they are totally inactive in Raschig's reactions carried out in solution. The determination of the induction

coefficients can also be used to distinguish between phosphates, phosphorothioates and phosphoro-dithioates and also phosphine sulfides from phosphine oxides.

3. Conclusions

Detection systems, routinely used for the TLC of organophosphorus compounds (UV, iodine, molybdate reagents) give positive test results both for phosphates and phosphorothioates. In the light of the results presented here, the iodine–azide detection reagent can be used for the sensitive detection of a broad spectrum of phosphorothioates. In the case of several sugars thiophosphate derivatives the detectability can be substantially enhanced by hydrolytic pre-treatment prior to the iodine–azide detection. Since the iodine–azide reagent gives a negative test reaction with phosphates and phosphonates, this procedure can be used as a method for the selective detection of phosphorothioates in mixtures with other phosphoroorganic derivatives.

To sum up, the selectivity of the iodine–azide reagent in the TLC detection of thiophosphates is diminished by interference of sulfur-containing compounds and also by some phosphoryl derivatives. The fact that the scope of action of the iodine–azide reagent is not limited to sulphur containing compounds should be taken into consideration in further analytical applications of this reagent.

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