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New diaryl ω-(isothiocyanato)alkylphosphonates and their mercapturic acids as potential antibacterial agents

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ABSTRACT

Thirty-four novel, diaryl ω-(isothiocyanato)alkylphosphonates with chlorine atom and methoxy, dimethoxy, methylsulfanyl, or methoxycarbonyl groups at *ortho*, *meta*, or *para* positions of the phenyl ring, and with an unbranched alkyl chain (n = 2–6) were designed and synthesized in a one-pot reaction in 11–76% yields. All isothiocyanates thus generated were evaluated for the first time for antibacterial activity on *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacterial strains, and had satisfactory antibacterial activity in most cases. The highest activity, similar to that of reference gentamicin activity against *S. aureus*, was seen in compounds **9** and **13** (1.5 ± 0.1 and 2.5 ± 0.2 μM, respectively), whereas for *P. aeruginosa* more than half of tested compounds proved to be more effective than gentamicin. Additionally, selected isothiocyanates (**9**, **13**, **18**, and **23**) were transformed in 52–73% yields into mercapturic acids **42–45**, which also exhibited satisfactory antibacterial effect against *S. aureus* strain.

1. Introduction

Cruciferous vegetables, such as broccoli, Brussels sprouts, and radish [1,2], are rich in glucosinolates [3]. When these plants are crushed or chewed, these biologically inactive compounds are hydrolyzed by a myrosinase enzyme to intermediate thiohydroximate-*O*-sulfonates that subsequently undergo the Lossen rearrangement to become biologically active isothiocyanates (Fig. 1) [4]. There is much clinical evidence that the intake of a large quantity of cruciferous vegetables reduces the risk of different cancers (*e.g.* breast [5] or prostate cancer) [6,7]. Plant-derived isothiocyanates as phenethyl isothiocyanate (PEITC) [8,9], or the most thoroughly studied sulforaphane (SFN) [4,10,11] exhibit antiproliferative properties in all steps of carcinogenesis [12–14]. The PEITC has an effect on modulation of Toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent signaling pathway of TLRs on chronic inflammatory diseases [15]. The presence of a highly electrophilic −N═C═S functional group in ITCs makes them good candidates for interactions with potential molecular targets.

Large number of plant-derived isothiocyanates have been proven to have antimicrobial activity, as has been recently elaborated in two elegant and comprehensive reviews [16,17]. Isothiocyanates shown in Fig. 2 have a wide spectrum of action against both gram-negative strains, such as, for example *Pseudomonas aeruginosa*, and gram-positive strains, such as *Staphylococcus aureus*. The mechanism of action of isothiocyanates depends on the strain that is tested.

Thus, SFN and erucin (ER) – a sulfide analog of SFN (Fig. 2) – inhibit bacterial quorum sensing (QS) [18], biofilm formation, and pyocyanin production of *P. aeruginosa*. PEITC, benzyl isothiocyanate (BITC), and allyl isothiocyanate (AITC; Fig. 2) were tested with regard to inhibition of biofilm production, and PEITC significantly reduced the biofilm development in both *P. aeruginosa* and *S. aureus* [19]. Additionally, AITC and PEITC were capable of altering the integrity of the bacterial cell membrane in a dose-dependent manner in *P. aeruginosa* and *S. aureus* strains.

The antimicrobial activity of plant-derived isothiocyanates prompt a search for non-natural isothiocyanates with potential antibacterial action. Some current examples of such studies are presented in Fig. 2. The

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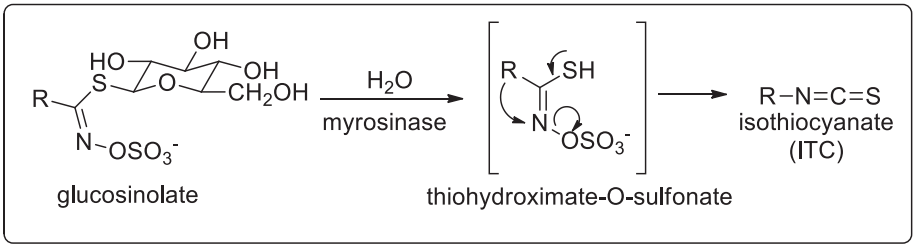


Fig. 1. Myrosinase-induced degradation of glucosinolates to isothiocyanates.

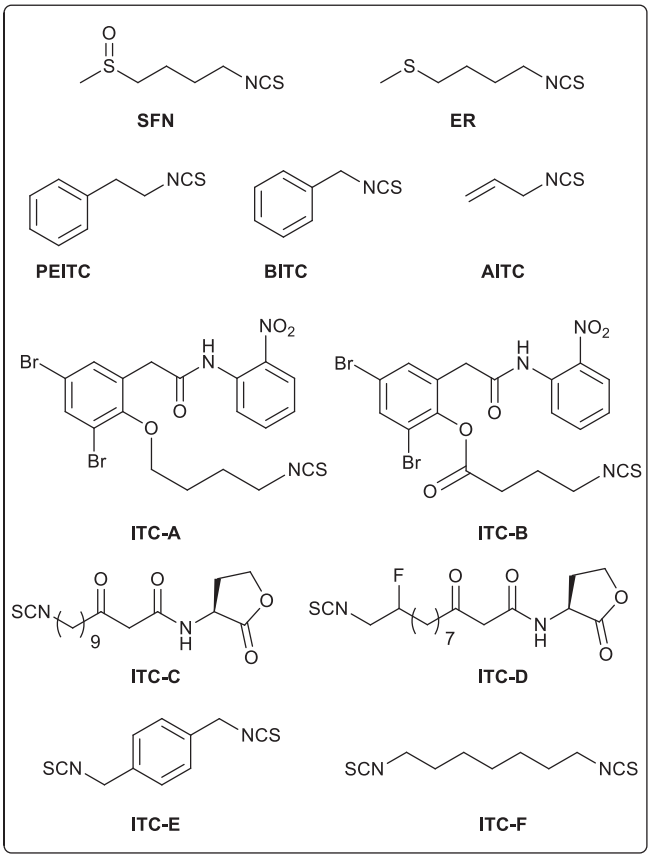


Fig. 2. Selected natural and synthetic analogs of isothiocyanates with antimicrobial activity.

2-(3,5-dibromo-2-(4-isothiocyanatobutoxy)phenyl)-*N*-(2-nitrophenyl) acetamide (ITC-A) derived from phenylcetic acid and its analog 2,4-dibromo-6-(2-((2-nitrophenyl)amino)-2-oxoethyl)phenyl 4-isothiocyanatobutanoate (ITC-B; Fig. 2) have proved to be transcriptional regulator (LasR) antagonists of QS in *P. aeruginosa* [20]. Moreover, (*S*)-12-isothiocyanato-3-oxo-*N*-(2-oxotetrahydrofuran-3-yl)dodecanamide (ITC-C) derived from homoserine lactone (Fig. 2) was an antagonist of LasR QS in *P. aeruginosa* and *E. coli* [21]. In 2016, Meijler's group [22] synthesized β-halogenated isothiocyanate probes derived from homoserine lactone – second-generation inhibitors of bacterial QS in *P. aeruginosa* and *E. coli*. Among these probes 11-fluoro-12-isothiocyanato-3-oxo-*N*-((*S*)-2-oxotetrahydrofuran-3-yl)dodecanamide (ITC-D), covalently bound to LasR, showed more complete inhibition of LasR than the first-generation isothiocyanate probe ITC-C. Mustaev et al. [23] tested the antimicrobial activity of ω-hydroxyalkylisothiocyanates and bis-isothiocyanates, wherein the isothiocyanate moieties were separated by a phenylene or alkyl linker (1,4-bis(isothiocyanatomethyl)benzene (ITC-E) and 1,7-diisothiocyanatoheptane (ITC-F), respectively, Fig. 2) as well as isothiocyanates containing an aromatic or heterocyclic ring and structurally diverse alkyl isothiocyanates. Studies demonstrated that the tested isothiocyanates showed moderate activity; however, they were more active against gram-positive than gram-negative strains.

Numerous studies show that the presence of secondary, polar functional group in isothiocyanates backbone is critical for their high bioactivity compared to the unsubstituted analogs [24,25]. On the other hand phosphonic acid derivatives with their single C—P bond belong to unique class of natural compounds in terms of stability and mimic properties. These compounds are resistant to hydrolytic cleavage, phosphonyl moiety is a useful analog of transition-states, as well as a useful mimic of intermediates and primary metabolites [26]. There is only a limited number of research work describing biological activity of phosphorus containing isothiocyanates. Posner et al. [24] were the first who described phosphorus analog of sulforaphane, in which methylsulfinyl group was replaced by dimethylphosphine oxide moiety. Also our group synthesized and evaluated *in vitro* antiproliferative activity of dialkyl α- and β-(isothiocyanato)alkylphosphonates [27], diphenyl α-(isothiocyanato)alkylphosphonates [28], and recently published dialkyl and diphenyl ω-(isothiocyanato)alkylphosphonates [29]. Moreover, we have proven antiproliferative potential of phosphonates, phosphinates and phosphine oxides isothiocyanate-derived mercapturic acids [30].

In the context of known antimicrobial activity of many plant-derived and synthetic isothiocyanates, as well as high biological activity of phosphorus containing isothiocyanates, the key aim of this study was to synthesize and evaluate the antibacterial activity of diaryl ω-(isothiocyanato)alkylphosphonates (DiArP-ITCs) against selected gram-negative (*P. aeruginosa*) and gram-positive (*S. aureus*) bacterial strains. The objective of this research was also to investigate whether the introduction of additional substituents in the esters aryl rings and their electronic properties increase the antibacterial potential of isothiocyanatoalkylphosphonates. In this regard, we have designed and obtained a library of 34 novel DiArP-ITCs with unbranched alkyl chains containing 2–6 carbon atoms, and with the chlorine atom, or methoxy, methylsulfanyl, or methoxycarbonyl groups at *ortho*, *meta*, or *para* positions of the phenyl ester ring. Additionally, preliminary assessment of antibacterial effect of diaryl ω-(isothiocyanato)alkylphosphonates-derived mercapturic acids (DiArP-ITC-NACs) has been undertaken. The reason to undertake this study was to examine these compounds as a rational alternative for the parent isothiocyanates. It is well documented that the reversibility of formation of ITCs-NAC conjugates from parent ITCs and NAC plays a key role in their biological activity [31]. To our knowledge, the antimicrobial potential of this class of phosphorus isothiocyanates and their merapturic acids has never been investigated.

2. Material and methods

2.1. General procedure of synthesis for compounds 8–41

In a 25 mL round bottom flask combined with septum, magnetic bar and protected against the moisture with $CaCl_2$ tube diethyl ω-(isothiocyanato)alkylphosphonate (**1–5**) (1 mmol, 1 eq) was dissolved in dry DCM (1 mL) and BTMS (0.46 mL, 3.5 mmol, 3.5 eq) was added

dropwise. Reaction was stirred for 24 h at rt. After that solvent and excess of BTMS was evaporated under reduce pressure and volatile materials were co-evaporated with DCM (2 × 2 mL). Residue oil was dissolved in dry DCM (1.5 mL) and catalyst amount of DMF (2 drops) and oxalyl chloride (0.63 mL, 7.3 eq) was dropped inside. The solution stirred for 1 h at rt. and 30 min at 40 °C. Solvent and excess of volatile materials were evaporated and co-evaporated under reduce pressure with DCM (2 × 2 mL), and residual oil was again dissolved in dry DCM (1.5 mL) and cooled in ice bath to 5 °C. In the second round bottom flask substituted phenol (4 mmol, 4 eq), triethylamine (0.83 mL, 6 mmol, 6 eq) and catalyst amount of DMAP (5 mg, 5% mol) were dissolved in dry DCM (1.5 mL) and dropwise to first flask. The reaction stirred 20 min at 5 °C and 20 h at rt. Next DCM (50 mL) was added to the reaction mixture and the solution was washed by 1 M NaOH (3 × 8 mL), H_2O (8 mL), 1 N HCl (3 × 8 mL), H_2O (8 mL), brine (8 mL) and dry over anhydrous $MgSO_4$. Product was purified by flash chromatography and in some cases by preparative thin layer chromatography. Purity of synthesized compounds **8–41** was confirmed by HPLC.

2.2. General procedure of synthesis for compounds 42–45

Isothiocyanates (**9**, **13**, **18** or **23**, 0.5 mmol, 1 eq) was added to a suspension of *N*-Acetyl-L-Cysteine (NAC) (0.073 g, 0.45 mmol, 0.9 eq) and $NaHCO_3$ (0.040 g, 0.48 mmol, 0.95 eq) in a mixture of EtOH (2 mL) and water (0.5 mL). The mixture was stirred 72 h at rt. Crude product was dissolved in H_2O (3 mL) and extracted with hexane (3 × 10 mL) to removed unreacted isothiocyanate. Next, water layer was acidified with 0.1 M HCl to pH 1–2 until white precipitate was formed. Product was extracted with EtOAc (5 × 20 mL). Combined organic layers were washed with brine (10 mL) and dried over anhydrous $MgSO_4$. Solvent was evaporated under reduce pressure to give analytically pure products **42–45**.

2.3. Antibacterial activities

S. aureus strain PCM 2602 and *P. auroginosa* strain PCM 2563 were purchased from the Polish Collection of Microorganisms in Wrocław. The assay was performed using 96-well microtiter plates (Sarstedt, Stare Babice, Poland) in a final 200 μL volume of Mueller-Hinton Broth (MHB, Merck, Warsaw, Poland), pH 7.5. Compounds and antibiotics (gentamycin, vancomycin, penicillin G, sulfamethoxazole and ampicillin) were dissolved in DMSO and diluted with MHB just before addition to the plate. *S. aureus* inoculum was prepared from an agar plate, cultured for 24 h at 37 °C and diluted with medium to 0.5 of the McFarland turbidity standard (1×10^8 c.f.u. mL^{-1}). Then, serially diluted compounds and antibiotics were added into the wells containing 190 μL of *S. aureus* or *P. auroginosa* suspension and incubated at 37 °C for 24 h. The number of survivors were measured (OD_{600}) and IC_{50} values were calculated using nonlinear regression of variable slope model (Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC$_{50}$-X)*HillSlope)). All measurements were performed in duplicate and represent the average of at least four independent experiments. The results are presented as the mean ± SEM.

2.4. Antiproliferative activities

Three human cancer cell lines were used to evaluate antiproliferative activity of obtained compounds: human colon adenocarcinoma cell lines sensitive and resistant to doxorubicin (LoVo) and (LoVo/DX) respectively and lung (A549) cancer. A549 and LoVo cell lines were purchased from the American Type Culture Collection (ATCC Rockville, Maryland, USA), the LoVo/DX by courtesy of Prof. E. Borowski (Technical University of Gdańsk, Poland). Additionally, murine normal fibroblasts Balb/3T3 purchased from ATCC were used as a control cell line. All the cell lines are maintained at the Institute of Immunology and Experimental Therapy (HIIET), Wrocław, Poland.

Human colon adenocarcinoma (LoVo and LoVo/DX) and lung cell line (A549) were cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (OptiMEM from Gibco, RPMI 1640 from PAA, Austria), supplemented with 5% fetal bovine serum (PAA, Austria), 2 mM L-glutamine, 1 mM sodium puryvate (Sigma-Aldrich, Germany) and 10 μg/100 mL doxorubicin for LoVo/DX (Sigma-Aldrich, Germany). The Balb/3T3 cell line was cultured in DMEM (Thermo-Fisher Scientific, Warsaw, Poland) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. All culture media contained antibiotics: 100 U/mL penicillin and 100 μg/mL streptomycin (Polfa-Tarchomin, Poland). All cell lines were cultured during entire the experiment in humid atmosphere at 37 °C and 5% CO_2.

2.4.1. The antiproliferative assays in vitro

Twenty four hours before adding the tested compounds, all cell lines were seeded in 96-well plates (Sarstedt, Germany) in appropriate media with 10^4 cells per well (except A549 seeded 0.25×10^4). All cell lines were exposed to each tested agent at four different concentrations in the range 100 to 0.1 μM for 72 h. Cells were also exposed to the reference drug cisplatin (Ebewe, Austria) and doxorubicin (IBA, Poland). Additionally, all cell lines were exposed to DMSO (solvent used for tested compounds) (Sigma-Aldrich, Germany) at concentrations corresponding to these present in tested agents' dilutions.

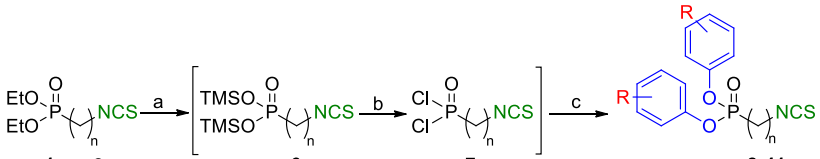
2.4.2. SRB

After 72 h of incubation with the tested compounds, cells were fixed *in situ* by gently addition of 50 μL per well of cold 50% trichloroacetic acid TCA (POCh, Poland) and were incubated at 4 °C for an hour. Following, wells were washed four times with water and 50 μL of 0.1% solution of sulforhodamine B (Sigma-Aldrich, Germany) in 1% acetic acid (POCh, Poland) was added to each well and plates were incubated at room temperature for 30 min. After incubation time, unbound dye was removed by washing plates four times with 1% acetic acid whereas stain bound to cells was solubilized with 10 mM Tris base (Sigma-Aldrich, Germany). Absorbance of each solution was read at Synergy H4 (BioTek Instruments USA) at the 540 nm wavelength. Entire washing procedure was performed on Biotek EL-406 washing station [32].

Results are presented as mean IC_{50} (concentration of the tested compound, that inhibits cell proliferation by 50%) ± standard deviation. IC_{50} values were calculated in Prolab-3 system based on Cheburator 0.4, Dmitry Nevozhay software for each experiment [33]. Compounds at each concentration were tested in triplicates in single experiment and each experiment was repeated at least three times independently.

3. Results and discussion

The target compounds – structurally diverse DiArP-ITCs **8–41** – were prepared using a one-pot protocol [29] from diethyl ω-(isothiocyanato)alkylphosphonates **1–5**, which are easily accessible from the parent azides by a procedure recently established in our laboratory [29]. Such a scenario enables the use of a few best available diethyl esters **1–5** in the synthesis of the series of DiArP-ITCs. The scope and general nature of such an approach is shown in Scheme 1. Thus, diethyl ω-(isothiocyanato)alkylphosphonates **1–5** are quantitatively converted in the reaction with bromotrimethylsilane (BTMS) [34,35] to bis(trimethylsilyl) ω-(isothiocyanato)alkylphosphonates **6**. The latter, in the reaction with oxalyl chloride $(COCl)_2$, generates intermediate ω-(isothiocyanato)alkylphosphonic dichlorides **7**; these, after coupling with the appropriately substituted phenols in the presence of triethylamine and the catalytic amounts of 4-dimethylaminopyridine (DMAP), produce the target ω-(isothiocyanato)alkylphosphonates **8–41** with high purity (≥97%) and, in moderate-to-good yields, after flash chromatography and, in some cases, after preparative thin-layer chromatography [29]. All steps in the production were monitored by ^{31}P NMR



1, n= 2
2, n= 3
3, n= 4
4, n= 5
5, n= 6

6 **7** **8-41**

Compd	n	R	Y[%]
8	3	4-Cl	65
9	4		65
10	5		60
11	6		36
12	2	3-Cl	44
13	3		70
14	4		48
15	5		59
16	6		43
17	2	2-Cl	24
18	3		47
19	4		46
20	5		51
21	6		43

Compd	n	R	Y[%]
22	2	4-MeO	45
23	3		63
24	4		50
25	5		65
26	6		66
27	2	4-MeS	20
28	3		60
29	4		75
30	5		52
31	6		51
32	2	4-MeOC(O)	41
33	3		60
34	4		65
35	5		62
36	6		58

Compd	n	R	Y[%]
37	2	3,4-diMeO	11
38	3		69
39	4		76
40	5		74
41	6		43

Scheme 1. Synthesis of diaryl ω-(isothiocyanato)alkylphosphonates. *Reagents and conditions*: (a) **1–5** (1 mmol), BTMS (3.5 equiv.), DCM, rt., 24 h; (b) $(COCl)_2$ (7.3 equiv.), $DMF_{(cat)}$ (two drops), DCM, 1 h, rt. then 30 min at 40 °C; (c) R-C_6H_4OH for compounds **8–36**, or 3,4-diMeO-C_6H_3OH for compounds **37–41** (4 equiv.), Et_3N (6 equiv.), $DMAP_{(cat)}$ (5 mol%), DCM, 20 min then 0 °C, followed by 20 h at rt.

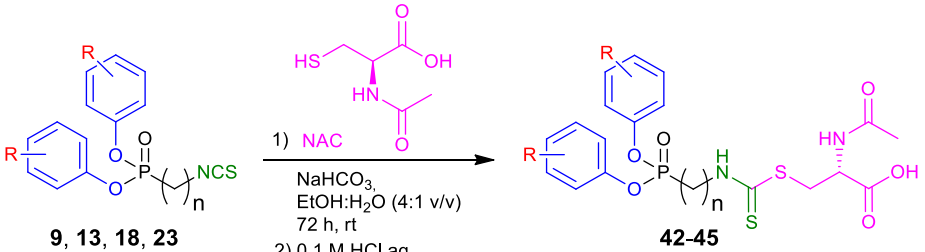
spectroscopy on model compound **11** (see Supplementary Materials, Fig. S1). Using the aforementioned methodology, a series of 34 novel, structurally diverse diaryl ω-(isothiocyanato)alkylphosphonate compounds **8–41** containing a chlorine atom; methoxy, dimethoxy, methylsulfanyl, or methoxycarbonyl groups at *ortho*, *meta*, or *para* position of the phenyl ester; and with an unbranched alkyl chain (n = 2–6) have been prepared (Scheme 1). Unfortunately, some isothiocyanates (**17**, **27**, and **37**) with two carbon atom chains were isolated in poor 11–24% yields – a result of the competitive β-elimination leading to a side formation of the substantial amounts of vinylphosphonates under the specified reaction conditions. All DiArP-ITCs **8–41** were fully characterized by ^{1}H, ^{31}P, ^{13}C NMR, IR, high resolution mass spectrometry and HPLC (the copies of NMR spectra and HPLC traces are included in the Supplementary Materials).

Finally, selected diaryl ω-(isothiocyanato)alkylphosphonates **9** and **13** (R = 4-Cl and 3-Cl, respectively) with three and four carbon atoms chains - the most active against *S. aureus* strain, as well as three carbon atoms analogs **18** and **23** (R = 2-Cl and 4-MeO, respectively) were converted to mercapturic acids **42–45** (DiArP-ITC-NACs) using protocol developed by Vermeulen et al. [36] (Scheme 2). In this straightforward synthesis, DiArP-ITCs react with a mixture of *N*-acetyl-L-cysteine (NAC) and sodium bicarbonate in an aqueous ethanol solution for 72 h at rt. The final products, after evaporation of solvents and acidification of the residue with 0.1 M HCl, are isolated as white solids in moderate to high yields (52–72%), and with high purity (≥98%).

Target mercapturic acids **42–45** were used for further study without any additional purification. All novel DiArP-ITC-NACs **42–45** were fully characterized by ^{1}H, ^{31}P, ^{13}C NMR, IR, high resolution mass spectrometry and HPLC. As other phosphorus isothiocyanate-derived mercapturic acids [30], DiArP-ITC-NACs **42–45** are the mixtures of the rotamers (1.0:0.05–1.0:0.11 ratios) in $CDCl_3$ solution (the copies of NMR spectra, and HPLC traces are included in the Supplementary Materials).

Mercapturic acids **42–45** were also subjected to stability studies. Evaluation of chemical stability was performed in phosphate buffer at physiologically relevant pH 7.2 at 37 °C, and decomposition products were studied using HPLC (Table 1).

Compounds **42–45** show comparable chemical stability, with half-



9, **13**, **18**, **23**

42-45

Compd	n	R	Y[%]
42	4	4-Cl	54
43	3	3-Cl	61
44	3	2-Cl	72
45	3	4-MeO	52

Scheme 2. Preparation of phosphonates isothiocyanate-derived mercapturic acids. *Reagents and conditions*: Isothiocyanates **9**, **13**, **18** or **23** (0.5 mmol), NAC (0.9 equiv.), $NaHCO_3$ (0.95 equiv.), EtOH: H_2O (4: 1 v/v) (2.5 ml), 72 h, at rt.; then, 0.1 M HCl aq.

Table 1
Stability of **42–45** in phosphate buffer at pH 7.2.

Compd	Half-life $t_{1/2}$ [h]
42	3.04
43	2.59
44	2.87
45	3.28

life ($t_{1/2}$) values in the range 2.59–3.28 h. We have also found that decomposition of ITC–NACs adducts **42–45** proceed with cleavage of the thiocarbamoyl linkage, resulting in regeneration of the parent isothiocyanates. Attempts to assess the chemical stability of diaryl ω-(isothiocyanato)alkylphosphonates in phosphate buffer at pH 7.2 failed, as tested ITCs were not soluble in water solutions.

The DiArP-ITCs **8–41** that had substitutions at *ortho*, *meta*, or *para* positions of the phenyl ester ring with a chlorine atom, or methoxy, methylsulfanyl, or methoxycarbonyl groups as well as with unbranched alkyl chains (n = 2–6) were evaluated for antibacterial activity on *S. aureus* and *P. aeruginosa* strains, respectively, in comparison with natural PEITC and with gentamicin used as a reference antibiotic. Our results showed that the variation in chemical structure implies different grade and type of activity (SAR) of tested DiArP-ITCs (Table 2).

In general, the calculated IC_{50} (the concentration that inhibits the growth of a half of the inoculum) values of the studied compounds range from 2 to 30 μM. However, for isothiocyanates **31** and **38**, the concentration necessary to inhibit bacterial growth of both the tested strains was so high (IC_{50} > 50 μM) that they were defined as weak-antibacterial.

Among the chloro-derivatives (**8–9**, **12–14**, and **17–19**) with short alkyl chains (n = 2–4), some influence on the antibacterial activity has been observed with regard to the position of the chlorine atom at the phenyl ester ring. However, such an influence is not observed for chloro-derivatives with longer 5- and 6-carbon chains (compounds **10**, **11**, **15**, **16**, **20**, and **21**). The elongation of the unbranched alkyl chain to six carbon atoms as in the *ortho*- and *meta*-chloro-substituted ω-(isothiocyanato)alkylphosphonates **16** and **21**, respectively, increases their selectivity. Both compounds, **16** and **21**, show average antimicrobial activity against *S. aureus* (IC_{50} = 15.0 ± 2.7 and 19.0 ± 1.1 μM, respectively), and are found to be weakly active toward *P. aeruginosa*.

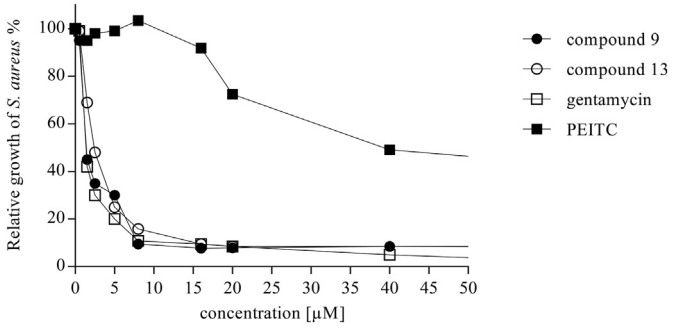
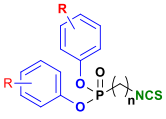


Fig. 3. Relative growth of *S. aureus*.

The most active chloro-substituted isothiocyanates (**9** and **13**) inhibit *S. aureus* growth in a range of action of gentamicin (IC_{50} = 1.0 ± 0.1 μM); moreover, they are more active than the reference gentamicin (IC_{50} = 12.0 ± 1 μM) against *P. aeruginosa* strains – in particular, the concentration of *para*-chloro-derivative **9** (R = 4-Cl, n = 4) that inhibits the growth of a half of the inoculum of *S. aureus* (Fig. 3) and *P. aeruginosa* (Fig. 4) is 1.5 ± 0.1 and 7.0 ± 1.1 μM, respectively. The compounds **9** and **13** were more active on *S. aureus* than vancomycin (IC_{50} = 17.0 ± 5.2 μM), while their antibacterial activity was slightly less effective than other antibiotics - penicillin G, sulfamethoxazole and ampicillin (IC_{50} = 0.5 ± 0.2 μM; 0.8 ± 0.2 μM; 0.01 ± 0.002, respectively). In turn, the introduction of a chlorine atom at the *meta*-position, and shortening of the alkyl chain to three carbon atoms simultaneously, as in compound **13** (R = 3-Cl, n = 3), almost doubled the antimicrobial activity toward *P. aeruginosa* (IC_{50} = 4.0 ± 1.6 μM, Fig. 4), and slightly increased the IC_{50} value relative to *S. aureus* (IC_{50} = 2.5 ± 0.2 μM, Fig. 3).

Taking into account the SAR of the investigated DiArP-ITCs **27–31** with methylsulfanyl substituents at the *para* position of the phenyl ester ring, their action against *S. aureus* and *P. aeruginosa* seems to be more

Table 2
Antibacterial activity of isothiocyanates **8–41**.



Compd	R	n	IC_{50} [μM] ± SD		Compd	R	n	IC_{50} [μM] ± SD	
			S. aureus	*P. aeruginosa*				*S. aureus*	*P. aeruginosa*
8	4-Cl	3	18.0 ± 1.3	5.0 ± 1.3	**27**	4-MeS	2	16.0 ± 0.6	9.0 ± 1.5
9		4	1.5 ± 0.1	7.0 ± 1.1	**28**		3	9.0 ± 0.4	> 50
10		5	9.0 ± 0.1	17.0 ± 2.7	**29**		4	8.0 ± 1.9	> 50
11		6	13.0 ± 0.2	4.0 ± 0.1	**30**		5	9.0 ± 0.1	4.5 ± 3.0
12	3-Cl	2	11.0 ± 0.2	9.0 ± 0.5	**31**		6	> 50	> 50
13		3	2.5 ± 0.2	4.0 ± 1.6	**32**	4-MeOC(O)	2	> 50	8.5 ± 4.6
14		4	7.5 ± 0.3	3.0 ± 2.3	**33**		3	19.0 ± 2.2	6.0 ± 0.8
15		5	8.0 ± 0.4	8.0 ± 0.5	**34**		4	12.5 ± 1.2	7.0 ± 1.3
16		6	15.0 ± 2.7	> 50	**35**		5	7.0 ± 2.0	7.0 ± 1.2
17	2-Cl	2	20.0 ± 0.5	18.0 ± 3.4	**36**		6	14.0 ± 0.5	11.0 ± 3.2
18		3	8.5 ± 0.6	4.0 ± 0.7	**37**	3,4-diMeO	2	> 50	16.0 ± 0.1
19		4	29.0 ± 0.2	4.0 ± 1.2	**38**		3	> 50	> 50
20		5	8.0 ± 1.1	6.7 ± 0.7	**39**		4	17.0 ± 0.1	> 50
21		6	19.0 ± 1.1	> 50	**40**		5	15.0 ± 1.5	5.0 ± 0.5
22	4-MeO	2	17.0 ± 0.8	14.0 ± 2.1	**41**		6	12.0 ± 0.1	> 50
23		3	10.0 ± 0.1	14.5 ± 0.5	Gentamicin			1.0 ± 0.1	12.0 ± 1
24		4	7.5 ± 0.3	14.0 ± 3.3	PEITC			45.0 ± 2.0	> 50
25		5	7.0 ± 0.8	> 50	SD – standard deviation				
26		6	10.0 ± 0.9	> 50					

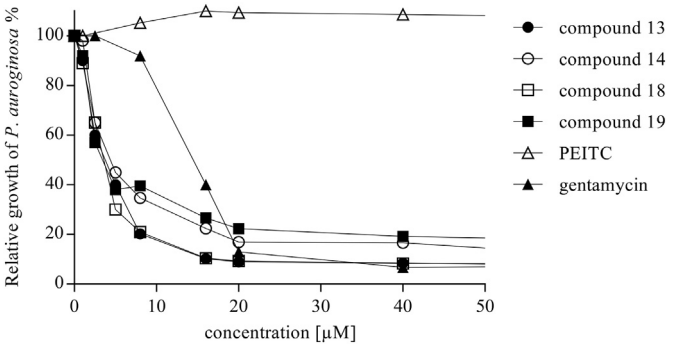


Fig. 4. Relative growth of *P. aeruginosa*.

selective than nearly all other ω-(isothiocyanato)alkylphosphonates. A high selectivity of action is observed for compounds **28** and **29** toward *S. aureus* (IC_{50} = 9.0 ± 0.4 and 8.0 ± 1.9 μM, respectively), whereas there was very weak antibacterial activity toward *P. aeruginosa* (gram-negative bacteria). The *para*-methylsulfanyl derivatives **30** and **31** with longer alkyl chains (n = 5 and 6) are noticeable exceptions here. Compound **30** (R = 4-MeS, n = 5) exhibits antibacterial activity against *S. aureus* (IC_{50} = 9.0 ± 0.1 μM) and *P. aeruginosa* (IC_{50} = 4.5 ± 3.0 μM), whereas compound **31** (R = 4-MeS, n = 6) displays weak antibacterial activity toward both of the tested strains.

The presence of two methoxy groups, such as in compounds **37–41** (R = 3,4-diMeO), does not increase their antibacterial activity as compared to mono-methoxy derivatives **22–26** although it changes the selectively of action. Among the 3,4-dimethoxy series, only compound **40** demonstrates activity against both bacterial strains, with some selectivity toward *P. aeruginosa* (IC_{50} = 5.0 ± 0.5 μM). Isothiocyanate **37** was moderately active only against *P. aeruginosa* (IC_{50} = 16.0 ± 0.1 μM), whereas compounds **39** and **41** showed moderate selectivity toward *S. aureus* (IC_{50} = 17.0 ± 0.1 and 12.0 ± 0.1 μM, respectively); moreover, as mentioned earlier, isothiocyanate **38** displayed weak antibacterial activity against both strains. A high selectivity is observed for the *para*-methoxy derivatives **25** and **26** with five and six carbon chains, respectively. Compounds **25** and **26** exhibit IC_{50} of 7.0 ± 0.8 and 10.0 ± 0.9 μM, respectively, toward *S. aureus*, whereas both isothiocyanates are weakly active in inhibiting *P. aeruginosa* growth.

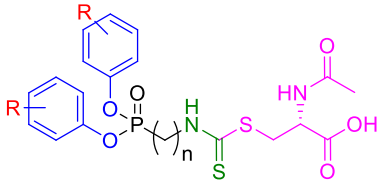
In turn, the *para*-methoxycarbonyl derivatives **32–36** demonstrated moderate antibacterial activity against both strains concomitantly, with better activity against *P. aeruginosa*.

In general, for all tested compounds with long alkyl chains (n = 6), some decrease in the antibacterial activity against gram-positive and gram-negative bacteria was observed. The majority of compounds (**16**, **21**, **26**, **31**, and **41**) revealed IC_{50} > 50 μM toward *P. aeruginosa*; in some cases, their antibacterial activity was weaker than that of the others (n = 2–5) against *S. aureus*.

Finally, diaryl ω-(isothiocyanato)alkylphosphonates-derived mercapturic acids **42–45** were evaluated for antibacterial activity against *S. aureus* strain. Surprisingly, none of the studied DiArP-ITC-NACs proved to be substantially more active than others (Table 3).

Thus, antibacterial activity of mercapturic acids **42–45** on *S. aureus* strain is within the range of 8.0 ± 0.4–11.0 ± 0.2 μM, and does not exceed the activity of parent isothiocyanates. The best **43** (IC_{50} = 8.0 ± 0.4 μM) is eight times less active than reference gentamicin (IC_{50} = 1.0 ± 0.1 μM; see, Table 2 for details). It seems that neither the position (*para*, *meta* or *ortho*) nor electronic properties of substituents (chlorine atom *vs.* methoxy group) have significant impact on activity. Although antibacterial activity of DiArP-ITC-NACs compared unfavorably with parent isothiocyanates, in-depth studies on antimicrobial activity of DiArP-ITC-NACs are necessary to withdraw unambiguous conclusions. Antifungal and antiviral activity has not been tested yet.

Table 3
Antibacterial activity of mercapturic acids **42–45** against *S. aureus*.



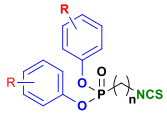
Compd	R	n	IC_{50} [μM] ± SD
42	4-Cl	4	10.0 ± 0.1
43	3-Cl	3	8.0 ± 0.4
44	2-Cl	3	11.0 ± 0.2
45	4-MeO	3	10.0 ± 0.2

SD – standard deviation

In comparison to extensively studied ITCs mode of action on cancer cells, relatively little is known about the mechanisms underlying their direct influence on bacteria cells growth. Available data [17] indicate that naturally occurring isothiocyanates (like benzyl, phenethyl and allyl isothiocyanates as well as SFN) exhibit some similarities in this matter with inhibition of bacterial quorum sensing, inhibition of pyocyanin production as well as membrane integrity disruption, reported most often. Thus, it is highly plausible that DiArP-ITC antibacterial activity relies on analogical mechanisms. Furthermore, the mode of action of ω-(isothiocyanato)alkylphosphonates and their mercapturic acids in their antibacterial activity may pertain to the inhibition of bacterial proteases. The mode of action of the diaryl ω-(isothiocyanato) alkylphosphonates and their mercapturic acids needs clarification. Studies on this subject are ongoing in our laboratories.

Apart from antibacterial activity, all of the synthesized DiArP-ITCs **8–41** were also evaluated for antiproliferative activity on human colon adenocarcinoma cell lines sensitive and resistant to doxorubicin (LoVo) and (LoVo/DX) respectively and compared to natural SFN as well as cisplatin (CDDP) and doxorubicin (Doxo) used as reference compounds. The synthesized DiArP-ITCs **8–41** showed antiproliferative activity, with IC_{50} values in the range of 1.0–28.2 μM on LoVo and 3.4–26.1 μM on LoVo/DX cell lines, respectively. Their activity was higher or in some cases similar to natural SFN (IC_{50} on LoVo 22.9 μM and on LoVo/DX 18.1 μM). The most active were *para*-methoxy-substituted and dimethoxy-substituted compounds (**22–26** and **37–41**, respectively) with compound **37** (n = 2) being the most active among all of the isothiocyanates tested on the LoVo cell line; its activity (IC_{50} = 1.0 ± 0.1 μM) was nearly 3 times higher than that of cytostatic CDDP, and > 22 times higher than SFN. At the same time, isothiocyanates **22** and **39** showed the highest activity (IC_{50} = 3.4 μM), similar to CDDP, on the LoVo/DX subline. Almost all studied isothiocyanatophosphonates exhibited higher antiproliferative activity on the LoVo/DX sub-line than doxorubicin. Furthermore, we have proved that the position of chlorine atom in ester phenyl group of isothiocyanates has no impact on their antiproliferative activity. Chloro-substituted isothiocyanates **8–21** as well as *para*-methylsulfanyl substituents **27–31** and *para*-methoxycarbonyl derivatives **32–36** demonstrated lower actiproliferative activity than compounds **22–26** and **37–41**. Evaluation of antiproliferative mechanism of action of novel diaryl ω-(isothiocyanato)alkylphosphonates is ongoing in our laboratories. However, it can be assumed that due to the structural similarity of the obtained compounds DiArP–ITC to natural SFN resulting from the presence of the primary –NCS group, it is very likely that DiArP–ITC in the same way as SFN can activating the Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) antioxidant pathway. The possible mechanism does not occur directly in bacteria due to the absence of Nrf2. However, Harvey et al. [37] demonstrate the importance of Nrf2 in improving

Table 4
Toxicity of selected isothiocyanates **9** and **13**.



Compd	R	*n*	IC_{50} ± SD [μM]			
			LoVo	LoVo/DX	A549	Balb/3T3
9	4-Cl	4	4.6 ± 0.1	8.1 ± 4.1	7.5 ± 4.4	6.7 ± 1.1
13	3-Cl	3	2.5 ± 0.3	4.9 ± 1.6	10.9 ± 2.6	8.5 ± 3.3
SFN			22.9 ± 2.0	18.1 ± 0.3	–	–
CDDP			3.1 ± 1.1	3.7 ± 0.8	3.0 ± 0.7	6.1 ± 0.7
Doxo			0.3 ± 0.1	12.2 ± 2.1	–	–

SD – standard deviation.

antibacterial defenses and provide a rationale for targeting this pathway, *via* pharmacological agents such as sulforaphane, to prevent bacterial infection. The similarity of DiArP-ITC to SFN can also significant influence on increase of antibacterial defence in host organism. It is good starting point of new investigations toward activity of DiArP-ITC in host organism during infection.

The most active compounds on *S. aureus* strain **9** and **13** were evaluated for toxicity on murine normal fibroblasts Balb/3T3 and compare to activity on LoVo and LoVo/DX cancer cell lines. Their activity were also tested on lung cancer (A549) (Table 4).

Results showed that both compounds **9** and **13** were more toxic on LoVo cancer cell line than on Balb/3T3. DiArP-ITC **9** was almost 1.5 times more toxic and compound **13** was 3.4 times more toxic on LoVo than on Balb/3T3. Compound **13** was also more toxic on LoVo/DX than on Bal/3T3, while the toxicity of **9** was higher on Balb/3T3 than on LoVo/DX. Additionally, DiArP-ITC **13** was slightly more active on LoVo than reference CDDP. High antibacterial activity on *S. aureus* and *P. aeruginosa* (IC_{50} = 2.5 μM and IC_{50} = 4.0 μM, respectively, Table 2), high antiproliferative activity on LoVo (IC_{50} = 2.5 μM) and low toxicity on Balb/3 T3 (IC_{50} = 8.5 μM) as well as 1.2 times higher antiproliferative activity than CDDP on LoVo confirm that compound **13** could be a potential candidate to further *in vivo* trials.

On A549 compounds **9** and **13** showed lover antiproliferative activity than for LoVo cancer cell line and were less toxic than on Balb/3T3.

4. Conclusions

In summary, a library of novel, structurally diverse diaryl ω-(isothiocyanato)alkylphosphonates with chlorine atom or methoxy, dimethoxy, methylsulfanyl, or methoxycarbonyl groups, at different positions in the ester aryl ring, and with carbon chains of various length were designed and synthesized with moderate-to-good yields and purity up to 97%. For the first time diaryl ω-(isothiocyanato)alkylphosphonates were tested *in vitro* for the antibacterial activity on selected gram-positive strain (*S. aureus*) and gram-negative strain (*P. aeruginosa*). All compounds exhibited better antimicrobial action than plant-derived PEITC. The most active isothiocyanates **9** (IC_{50} = 1.5 ± 0.1 μM) and **13** (IC_{50} = 2.5 ± 0.2 μM) exhibited similar activity on *S. aureus* to reference gentamicin. Among all the DiArP-ITCs tested on *P. aeruginosa*, more than half of the compounds presented higher antibacterial activity than gentamicin. Of these, the chlorine analogs **11**, **13**, **14**, **18**, and **19** with the highest activity in the range of 3–4 μM were 3 and 4 times more active than gentamicin. When antimicrobial activity was compared, several DiArP-ITCs were inactive on both bacterial strains, and some isothiocyanates showed selectivity only toward one strain. All DiArP-ITCs were also evaluated on LoVo and LoVo/DX cancer cell lines and were showed higher and comparable antiproliferative activity than natural SFN. Selected isothiocyanates **9** and **13** were tested for toxicity on murine normal fibroblasts Balb/3 T3. Compounds were more toxic on LoVo than on Balb/3T3. In all tests compound **13** was exhibited high antibacterial and antiproliferative activity as well as low toxicity and could be a potential candidate to other clinic tests. Additionally, selected diaryl ω-(isothiocyanato)alkylphosphonates-derived mercapturic acids **42–45** were synthesized in high yields and with purity up to 98%. Preliminary evaluation of their *in vitro* antibacterial activity on *S. aureus* strain showed that mercapturic acids **42–45** exhibited moderate antibacterial activity – lower or similar to the parent isothiocyanates. Our study on DiArP-ITCs as well as their mercapturic acids address a pressing need of finding a new class of antimicrobial agents, especially in the context of growing multi-drug-resistance of many human pathogens. We believe that DiArP-ITCs and DiArP-ITC-NACs can be considered as potential candidates for further development in regard to antibacterial activity.

Abbreviations

AITC allyl isothiocyanate
BITC benzyl isothiocyanate
BTMS bromotrimethylsilane
CDDP cisplatin
DiArP-ITCs diaryl ω-(isothiocyanato)alkylphosphonates
DiArP-ITC-NACs diaryl ω-(isothiocyanato)alkylphosphonates-derived mercapturic acids
DMAP 4-dimethylaminopyridine
Doxo doxorubicin
ER erucin
ITCs isothiocyanates
LasR transcriptional regulator
LoVo human colon adenocarcinoma cell line
LoVo/DX human colon adenocarcinoma cell lines resistant to doxorubicin
MRSA methicillin-resistant strains of *S. aureus*
NAC *N*-acetyl-L-cysteine
NQO1 NAD(P)H quinone oxidoreductase 1
Nrf2 Nuclear factor (erythroid-derived 2)-like 2
PEITC phenethyl isothiocyanate
QS quorum sensing
SFN sulforaphane

Conflict of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.01.020.

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