

Fluorinated Beta-diketo Phosphorus Ylides Are Novel Efflux Pump Inhibitors in Bacteria

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Abstract. *Background: One of the most important resistance mechanisms in bacteria is the increased expression of multidrug efflux pumps. To combat efflux-related resistance, the development of new efflux pump inhibitors is essential. Materials and Methods: Ten phosphorus ylides were compared based on their MDR-reverting activity in multidrug efflux pump system consisting of the subunits acridine-resistance proteins A and B (AcrA and AcrB) and the multidrug efflux pump outer membrane factor TolC (TolC) of Escherichia coli K-12 AG100 strain and its AcrAB-TolC-deleted strain. Efflux inhibition was assessed by real-time fluorimetry and the inhibition of quorum sensing (QS) was also investigated. The relative gene expression of efflux QS genes was determined by real-time reverse transcriptase quantitative polymerase chain reaction. Results: The most potent derivative was $Ph_3P=C(COC_2F_5)CHO$ and its effect was more pronounced on the AcrAB-TolC-expressing *E. coli* strain, furthermore the most active compounds, $Ph_3P=C(COCF_3)OMe$, $Ph_3P=C(COC_2F_5)CHO$ and $Ph_3P=C(COCF_3)COMe$, reduced the expression of efflux pump and QS genes. Conclusion: Phosphorus ylides might be valuable EPI compounds to reverse efflux related MDR in bacteria.*

Multidrug resistance (MDR) is a serious problem for the treatment of various diseases, such as bacterial and fungal infections and cancer, due to reduction or deficit response of

microorganisms as well as cancer cells to applied chemotherapeutic agents (1, 2).

One of the major mechanisms of MDR is the overexpression of efflux pumps (EPs), which reduces the accumulation of toxic agents. In bacteria the resistance nodulation division (RND) transporters have a great impact on MDR phenotype. The major cause for the MDR phenotype is due to overexpression of efflux pumps that are part of the RND family of transporters, for example the acridine-resistance proteins A and B (AcrA and AcrB) and the multidrug efflux pump outer membrane factor TolC (TolC) AcrAB-TolC system (1). These efflux pumps have the ability to recognize and extrude a large variety of unrelated antibiotics from the periplasmic space of the cell envelope, or from the cytoplasm. The energy required for the operation of the efflux pump is provided by the proton motive force created by the proton gradient resulting from electron transport (3).

The quorum-sensing system (QS) in bacteria is a regulatory system that controls gene expression depending on the density of the bacterial cell population. A transcriptional regulator (LuxR homolog), signal synthase (LuxI homolog) and autoinducer (acyl homoserine lactone) are crucial for the QS in most Gram-negative bacteria. SdiA, an *E. coli* LuxR homolog, has a great impact on the colonization of *E. coli* (4, 5). SdiA is a homolog of QS regulators that detects *N*-acylhomoserine lactone (AHL) signals from other bacteria. SdiA represses the expression of virulence factors by interacting with unknown stationary-phase signals in *E. coli* O157:H7, and enhances multidrug resistance by activating MDR efflux pumps in *E. coli* (6).

Organic compounds of phosphorus ylides (P-ylides) are a fascinating class of compounds (7). The biological activity of P-ylides related to their ATP-binding cassette subfamily B member 1 (ABCB1)-modulating activity in mouse lymphoma cells has already been described (8), however, additional information is needed to describe their valuable biological activities in other aspects.

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In this article, we report the MDR-modulating activities of P-ylides in bacteria related to the inhibition of efflux activity and QS.

Materials and Methods

Compounds. The synthesis of P-ylides was described previously (8) and the structures of the P-ylides (compounds **1-10**) screened for their MDR-modulating activities are shown in Table I. The compounds were dissolved in dimethyl sulfoxide (DMSO) for the experiments.

Acridine orange (AO), ethidium bromide (EB), and LB broth were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Modified LB agar (LB*) contained 5 g yeast extract, 10 g trypton, 10 g NaCl, 1 g K₂HPO₄, 0.3 g MgSO₄·7H₂O and 36 mg FeNaEDTA in 1.0 l of distilled water. Mueller Hinton broth was purchased from Scharlau Chemie S.A. (Barcelona, Spain).

Bacterial strains. Wild-type *E. coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44] expressing the AcrAB-TolC EP at its basal level and its AcrAB-TolC-deleted mutant strain (*E. coli* K-12 AG100A). The strains were kindly provided by Professor Dr. Hiroshi Nikaido (Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, CA, USA).

Strains used for QS tests: *Chromobacterium violaceum* 026 (CV026) as sensor strain; *Sphingomonas* spp. EZF 10-17 (*Sphingomonadaceae*) isolated from a grapevine crown gall tumor (used as AHL producer), this strain induced pigment production by CV026 and proved to be efficient for the study of QS interactions; *Enterobacter cloacae* 31298 (clinical wound isolate, used as AHL producer).

Determination of minimum inhibitory concentrations. The minimum inhibitory concentrations (MICs) of P-ylides were tested according to Clinical and Laboratory Standard Institute guidelines (9).

Real-time accumulation assay by Roche LightCycler real-time thermocycler. The effect of the studied compounds on the real-time accumulation of EB was assessed by an automated EB method (10), using a LightCycler real-time thermocycler (LightCycler 1.5; Roche, Indianapolis, IN, USA). Briefly, an aliquot of an overnight culture of the strain in LB medium was transferred to fresh LB medium and incubated until it reached an optical density (OD) of 0.6 at 600 nm. It was then washed with phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 13,000 × g for 3 min, the pellets re-suspended in PBS (pH 7.4) and the OD adjusted to 0.6 at 600 nm. The compounds were added individually at different concentrations (in double concentrated form) in 5 µl volumes of their stock solutions to 45 µl of EB solution of 2 mg/l in PBS. Then, 10 µl of the EB solution containing the compound were transferred into standard glass capillary tubes of 20 µl maximum volume (Roche) and 10 µl of bacterial suspension (OD of 0.6 at 600 nm) was added to the capillaries. The capillaries containing the samples were placed into a carousel (Roche) and the fluorescence was monitored at the FL-2 channel every minute on a real-time basis.

From the real-time data, the activity of the compound, namely the relative fluorescence index (RFI) of the last time point (minute

30) of the EB accumulation assay was calculated according to the formula:

$$RFI = \frac{RF_{treated} - RF_{untreated}}{RF_{untreated}}$$

where $RF_{treated}$ is the relative fluorescence at the last time point of the EB retention curve in the presence of an inhibitor, and $RF_{untreated}$ is the relative fluorescence at the last time point of the EB retention curve of the untreated solvent control (DMSO).

Assay for QS inhibition. LB* was used for these experiments. The sensor strain *C. violaceum* 026 (CV026) and the AHL producer strains EZF 10-17 *Sphingomonas* spp. (*Sphingomonadaceae*) or *E. cloacae* 31298 were inoculated as parallel lines and incubated at room temperature (20°C) for 24-48 h. QS inhibition was monitored by agar diffusion method. Filter paper discs (7.0 mm in diameter) were impregnated with 10 µl of stock solutions of the compounds in DMSO (10 mg/ml in DMSO). The discs were placed between the parallel lines of sensor and AHL producer strains on the surface of the nutrient agar. The plates were incubated at room temperature for a further 24-48 h and the interactions between the strains and compounds were evaluated for the reduction in size of the zone of pigment production and the zone of growth inhibition of the affected strains, in millimeters. AO was applied as positive control (11).

Expression analyses of genes by real-time reverse transcriptase quantitative polymerase chain (RT-qPCR) reaction. Bacteria were cultured in LB broth and were incubated overnight at 37°C with shaking. On the day of RNA isolation, the bacterial suspensions (OD of 0.6 at 600 nm) were transferred to 10 ml tubes in 3 ml aliquots and 50 µg/ml of compounds were added to the tubes which were incubated at 37°C. At intervals of 4 and 18 h of culture, the tubes were centrifuged at 12,000 × g for 2 min. Pellets were suspended in 100 µl TE buffer containing 1 mg/ml lysozyme by vigorous vortexing and were incubated at 37°C for 10 min. The total RNA was isolated in an RNase-free environment using NucleoSpin RNA kit (Macherey Nagel, Germany) according to the manufacturer's instructions. Purified RNA was stored in RNase-free water in nuclease-free collection tubes and was maintained at -20°C until quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry at 260 nm.

Expression of the *acrA*, *acrB*, multiple antibiotic resistance protein R (*marR*) and quorum-sensing transcriptional activator (*sdiA*) genes was studied by reverse transcription of total RNA. The data obtained for gene targets were normalized against the *E. coli* house-keeping gene glyceraldehyde-3-phosphate-dehydrogenase (*gapdh*) measured in the same sample. Real-time quantification of the RNA templates by real-time one-step RT-qPCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) strictly adhering to the manufacturer recommendations of the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany). Briefly, each well of 96-well microtiter plates contained, in a final volume of 20 µl, 10 µl of the 2x SensiFAST™ SYBR No-ROX One-Step Mix, 0.2 µl Reverse Transcriptase, 0.4 µl RiboSafe RNase Inhibitor, 5.4 µl diethylpyrocarbonate-treated water, 500 nM of each primer and approximately 20 ng of total RNA in RNAase-free water. Thermal cycling was initiated with a denaturation step of 5 min

Table I. Structures of P-ylides (compounds 1-10).

$$\text{Ph}_3\text{P}=\text{C}(\text{H})\text{R}^2 \xrightarrow{\text{R}^1\text{O}} \text{Ph}_3\text{P}=\text{C}(\text{R}^1)\text{R}^2$$

1-9

Compound	R ¹	R ²
1	COCF ₃	CN
2	COCF ₃	OMe
3	COCF ₃	COPh
4	COCF ₃	CHO
5	COCF ₃	COMe
6	COCF ₃	CO ₂ Et
7	COC ₂ F ₅	COPh
8	COC ₃ F ₇	COPh
9	COCH ₃	CN
10	H	CN

at 95°C, followed by 40 cycles each of 10 s at 95°C, 30 s at 57°C and 20 s at 72°C.

The forward and reverse primers used for assessment of the activity of the transporter genes *acrA*, *acrB*, the regulator *marR* and the QS regulator *sdiA* are shown in Table II.

Results

Compounds **1-10** did not have any antibacterial effect on the AcrAB-TolC-expressing *E. coli* AG100 (MDR) strain nor its AcrAB-TolC-deleted progeny *E. coli* AG100A (EP-deleted strain) (MIC above 100 µg/ml), except for **6**, which had a mild, non-significant effect on the EP-deleted strain (MIC=50 µg/ml).

The activity of the compounds was compared based on the relative final fluorescence index (RFI) of the real-time accumulation curves (Table III).

In the case of real-time EB accumulation by Light Cycler thermocycler, the amount of EB accumulated by cells is higher if the difference between RF_{treated} and RF_{untreated} is greater, therefore, the degree of inhibition of the EP system by the compound becomes greater.

The majority of the P-ylides were found to inhibit the AcrAB-TolC system of *E. coli* except Ph₃P=C(COCF₃)COPh (**3**), Ph₃P=C(COC₂F₅)COPh (**7**) and Ph₃P=C(COC₃F₇)COPh (**8**), which had little or no effect on the intracellular EB accumulation in the *E. coli* AG100 and the AG100A strains. Among the P-ylide series, Ph₃P=C(COCF₃)OMe (**2**), Ph₃P=C(COCF₃)CHO (**4**) and Ph₃P=C(COCF₃)COMe (**5**) exhibited strong AcrAB-TolC pump-inhibiting properties compared to the AcrAB-TolC pump-deficient mutant strain. The most potent derivative was **4** and its effect was more

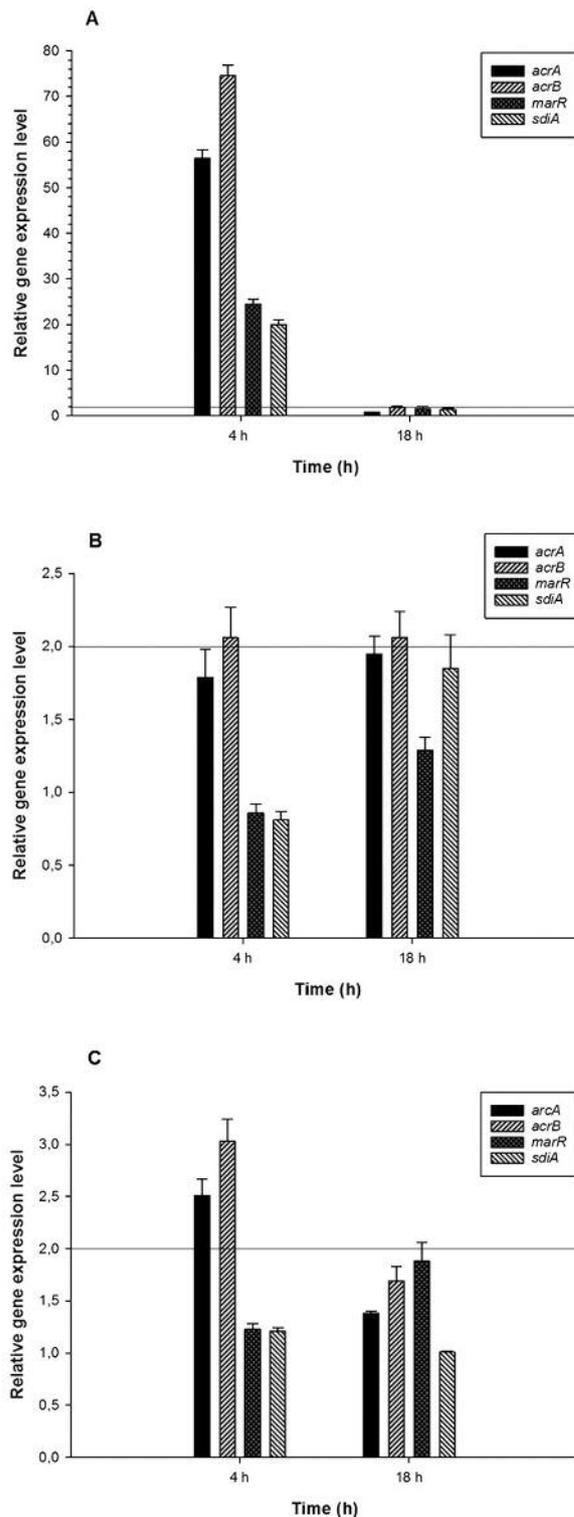


Figure 1. Relative gene expression levels of the genes of the acridine resistance protein A (*acrA*), acridine resistance protein B (*acrB*), multiple antibiotic resistance protein R (*marR*) and quorum-sensing transcriptional activator (*sdiA*) in the presence of compounds **2** (A), **4** (B), and **5** (C) after 4 and 18 h exposure in LB. The line denotes the threshold value, which was set at a two-fold increase in transcripts.

Table II. Forward and reverse primers used for assessment of the activity of the transporter genes *acrA* and *acrB*, the regulator *marR* and the quorum-sensing regulator *sdiA* of *Escherichia coli* AG100.

Gene	Full name	Primer sequence (5'-3')	Amplicon size (bp)	Reference
<i>acrA</i>	Acridine-resistance protein A	CTTAGCCCTAACAGGATGTGTTGAAATTACGCTTCAGGAT	189	(12)
<i>acrB</i>	Acridine-resistance protein B	CGTACACAGAAAGTGCTCAACGCTTCAACTTTGTTTTCTT	183	(12)
<i>marR</i>	Multiple antibiotic-resistance protein R	AGCGATCTGTCAATGAAATTCAGTTCAACCGGAGTAAT	170	(12)
<i>sdiA</i>	Quorum-sensing transcriptional activator	CTGATGGCTCTGATGCGTTTATCTGGTGGAAATTGACCGTATT	163	This study
<i>gapdh</i>	Glyceraldehyde-3-phosphate dehydrogenase	ACTTACGAGCAGATCAAAGCAGTTTCACGAAGTTGTCGTT	170	(12)

pronounced on the MDR *E. coli* strain compared to the pump-deleted *E. coli* strain. This activity suggests that the compound may interfere with the proton motive force because AcrB utilizes proton motive force as energy for its transport function.

The P-ylides were not able to inhibit the QS in the applied systems compared to the positive control AO (data not shown).

Regarding the effect of P-ylides on the relative expression of efflux pump and QS genes in *E. coli* AG100, the most effective compounds **2**, **4**, and **5** were selected for gene-expression studies. In the assay, the gene of the multidrug efflux pump subunit AcrB, the gene of the periplasmic AcrA subunit, the component of the *E. coli mar* locus (multiple antibiotic resistance), and the gene of the LuxR homolog SdiA were investigated. As shown in Figure 1A, compound **2** at 50 µg/ml up-regulated all the genes studied after 4 h of exposure, however, after 18 h, the gene expression returned to basal levels. Compound **4** also significantly up-regulated the secondary RND transporter gene *acrB* (approximately 2-fold increase) after 4 h and 18 h exposures as well. Surprisingly, there was up-regulation of *sdiA* expression after 18 h compared to the expression level after 4 h implicating the ability of compound **2** to influence the QS gene *sdiA*, however, this increase was not significant (Figure 1B). Compound **5** up-regulated the expression levels of *acrA* and *acrB* after 4 h, although after 18 h, the up-regulation of these genes was not significant (less than 2-fold increase) as presented by Figure 1C.

Discussion

Some phenothiazines and hydantoin are known to be EPIs against both bacteria and cancer cells (13). However, P-ylides **3**, **7** and **8** have been shown to have activity against the EPs of cancer cells (8), but not to have activity against the EP of bacteria. It is important to note that ABC transporters are primary efflux pumps deriving their energy from the hydrolysis of ATP, however, the AcrAB-TolC system is a three-component proton motive force-dependent multidrug efflux system. The most effective compounds in

Table III. Relative final fluorescence index (RFI) for the effect of P-ylides (compounds **1-10**) on *Escherichia coli* AG100 expressing acridine-resistance proteins A and B and the multidrug efflux pump outer membrane factor TolC, and the pump-deleted *E. coli* AG100A strain at 50 µg/ml.

Compound	RFI ^a	
	AG100	AG100A
1	0.36	0.46
2	0.64	0.49
3	0.04	0.12
4	0.73	0.46
5	0.42	0.27
6	0.25	0.49
7	0.02	0
8	0	0.19
9	0.29	0.32
10	0.42	0.44

^aRFI was calculated according to the formula:

$$RFI = \frac{RF_{treated} - RF_{untreated}}{RF_{untreated}}$$

where $RF_{treated}$ is the relative fluorescence at the last point (30 min) of the ethidium bromide (EB) retention curve in the presence an P-ylide and $RF_{untreated}$ is the relative fluorescence at the last point (30 min) of the EB retention curve of the untreated solvent control dimethyl sulfoxide.

the present bacterial system were compounds **2**, **4**, and **5**, which inhibited the AcrAB-TolC system and influenced the expression of the genes *acrA* and *acrB*, which are constituents of the AcrAB-TolC system. In addition, although the compounds are not QS inhibitors, compound **4** did increase the expression of *sdiA* after 18 h exposure.

It can be concluded that in the COCF₃ series (compounds **1-6**), the MDR-reverting activity in the MDR *E. coli* strain was intensified in the following order: CHO (**4**) > OMe (**2**), COMe (**5**) >> CO₂Et (**6**), CPh (**3**), CN (**1**).

Thus, some structurally related trifluoroacetylated P-ylides differ in their MDR-reversal activities between cancer cells and bacterial strains, indicating that the compounds differently act as inhibitors of primary (ABC_{B1}) and secondary (AcrB) efflux pumps because these pumps differ in their energy source for driving the pump (ATP and PMF, respectively).

The present study demonstrated that trifluoroacetylated P-ylides may be attractive lead EPIs for further development as a MDR-reversing agents, however, their mode of action should be elucidated by structure–activity relationship studies.

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