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Salicylanilide diethyl phosphates: Synthesis, antimicrobial activity and cytotoxicity

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ABSTRACT

A series of 27 salicylanilide diethyl phosphates was prepared as a part of our on-going search for new antimicrobial active drugs. All compounds exhibited in vitro activity against *Mycobacterium tuberculosis*, *Mycobacterium kansasii* and *Mycobacterium avium* strains, with minimum inhibitory concentration (MIC) values of 0.5–62.5 μmol/L. Selected salicylanilide diethyl phosphates also inhibit multidrug-resistant tuberculous strains at the concentration of 1 μmol/L. Salicylanilide diethyl phosphates also exhibited mostly the activity against Gram-positive bacteria (MICs $\geqslant$1.95 μmol/L), whereas their antifungal activity is significantly lower. The IC_{50} values for Hep G2 cells were within the range of 1.56–33.82 μmol/L, but there is no direct correlation with MICs for mycobacteria.

1. Introduction

The worldwide increased number of multidrug-resistant tuberculosis (MDR-TB) cases, emergence of extensively drug-resistant tuberculosis (XDR-TB) and recently reported totally drug-resistant (TDR-TB) strains become a very serious health and social problem. Therefore the development of new drugs which should be able to shorten the treatment of TB and stop emerging resistance is required.

Nontuberculous (atypical) mycobacteria represent causative agents of various opportunistic human infections; their treatment is complicated, particularly due to the high level of antibiotic resistance. The problems with drug-resistance have been reported also for many other bacterial and fungal strains, including methicillin-resistant *Staphylococcus aureus* (MRSA), enterococci, *Pseudomonas aeruginosa* or the family of *Enterobacteriaceae*.

Salicylanilide esters with carboxylic acids[1–4] as well as benzenesulfonic acid[5] have been described sharing a significant antimycobacterial activity including against nontuberculous mycobacteria, MDR- and XDR-TB strains in micromolar or even in submicromolar range. Moreover, they have exhibited a mild inhibition of mycobacterial isocitrate lyase, an enzyme essential for the maintenance of latent TB infection.[1,4] Some of the salicylanilide esters were also reported as agents with a significant antimicrobial activity especially against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* and filamentous fungi.[2–4]

The modification of parent molecules leading to the prodrugs or more active derivatives may help to overcome undesired properties. Phosphate triesters (phosphotriesters) approach have been applied successfully e.g. in the design of nucleoside prodrugs.[6,7] Phosphate-based prodrugs have been developed, inter alia, for the modification of solubility, which represents one critical parameter for the drug administration. Many phosphate esters often show both good chemical stability and rapid and easy enzymatic hydrolysis.[8]

Substituted diethyl phenyl phosphates have been reported to exhibit various biological effects and applications including insecticides inhibiting acetylcholinesterase,[9–11] herbicides,[12] fungicidal,[13] antiviral and cytotoxic[14] agents. A well-known acetylcholinesterase inhibitor paraoxone, diethyl (4-nitrophenyl) phosphate, the member of diethyl phenyl phosphate group, has revealed also some interesting metabolic and physiological impacts.[15–17] However, as an organophosphorus compound, it displays a high toxicity for humans with a potential threat of misuse

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as military nerve poison. The main mechanism of action has been generally attributed to the central and peripheral inhibition of human acetylcholinesterase, but other additional mechanisms have been identified.[18]

Despite the organophosphate toxicity, we selected salicylanilide diethyl phosphates (diethyl [2-(phenylcarbamoyl)phenyl] phosphates) as a new group of salicylanilide phosphoric acid-derived molecules as potential inhibitors of mycobacterial and other microbes growth and enzymatic reactions. Despite of various reports dealing with particular molecular and cellular effects of salicylanilide derivatives (e.g.,[1,2,19]), the exact mechanism(s) of salicylanilide action as antimicrobial agents is not still completely elucidated, probably combining specific and non-specific aspects.

2. Results and discussion

2.1. Chemistry

Series of substituted diethyl [(2-phenylcarbamoyl)phenyl] phosphates **1** was prepared by two synthetic steps. Condensation of 4/5-chloro and 5-bromosalicylic acids with substituted anilines was done in a microwave reactor according to the lit.[20] Salicylanilides were esterified by diethyl chlorophosphate in the presence of triethylamine (TEA) in dichloromethane (Scheme 1). This procedure and subsequent isolation and purification gave yields within the range of 11–78%.

Based on the literature data,[6–9,21,22] we can propose two possible pathways of diethyl salicylanilide phosphates **1**, as possible salicylanilide prodrugs, decomposition in organisms (Fig. 1). Firstly, it could be cleft by a two-step process. After cleavage of two molecules of ethanol from phosphotriesters, resulting 2-(phenylcarbamoyl)phenyl phosphate as anion with two negative charges is much more soluble in water and it can be a convenient soluble transport form of parent salicylanilides. Then, the phosphate group may be removed hydrolytically by enzymes like phosphatase and released salicylanilide may cross through biological barriers.

In the case, when diethyl [2-(phenylcarbamoyl)phenyl] phosphate reaches the proximity of targeted cells unhydrolysed, it may penetrate their biomembranes and it should be cleaved into charged phosphate within the cell. This polar form should be concentrated in the cytoplasm due to potentially abolished salicylanilide membrane shuttling.

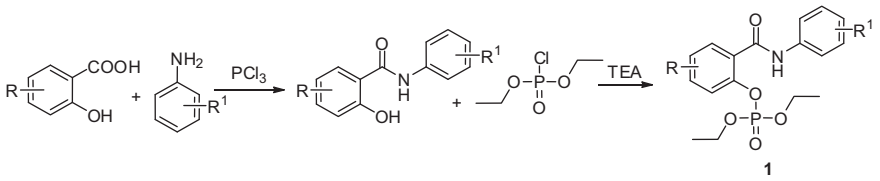
In the second way, the phosphotriester prodrug as a depot form could release salicylanilide directly by the hydrolysis of P–O(–Ar) bond. This step is catalysed by phosphotriesterase enzyme paraoxonase (PON1), which also inactivates highly toxic organophosphates like paraoxon, soman, tabun or sarin as well as other different substrates including aromatic esters. Paraoxonase is located in plasma, liver, brain, kidney and, importantly, in lungs.[22,23] However, it was described that replacement of the nitro group from paraoxon as well as electronic changes converted these analogues into inhibitors of PON1. Hydrophobicity was detrimental to hydrolysis by PON1.[24]

2.2. Antimycobacterial activities

All compounds were tested in vitro for their antimycobacterial activity against *Mycobacterium tuberculosis* (*Mtb.*) strain $H_{37}Rv$, *Mycobaterium avium* and two *Mycobacterium kansasii* strains; *M. kansasii* 6509/96 was isolated from a patient. The screened minimum inhibitory concentration (MIC) values are summarized in Table 1.

Results revealed that all tested compounds displayed an activity comparable with INH against *Mtb.* (five compounds—**1d**, **1f**, **1o**, **1r** and **1zz**—exhibited identical MIC as INH of 1 μmol/L), very high efficiency against *M. avium* (4–62.5 μmol/L, all esters superior to INH) and both strains of *M. kansasii* (0.5–32 μmol/L; all phosphates **1** showed a better inhibition of the strain 235/80 than INH and most of them were comparable or superior to isoniazid for the clinical isolate) and also much better activity against all evaluated mycobacteria than *para*-aminosalicylic acid (PAS), a second-line oral drug sharing a structural similarity.

The most active 5-chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl diethyl phosphate (**1zz**) has expressed the best activity against all tested strains (from 0.5 to 4 μmol/L), closely followed by its 4-chloro isomer **1r** and then 4-bromo derivative **1f**. In general, it can be postulated that activity is increased by 4-trifluoromethyl (**1f**, **1r**, **1zz**) and 3,4-dichloro (**1d**, **1o** and less **1y**) substitution on aniline part, followed by 3-CF_3 (especially **1e** and **1q**) and 4-Br. On the other site, fluorination of aniline led to the derivatives with the highest MICs (**1a**, **1i**, **1l**, **1m**, **1u**, **1v**) when compared to other monohalogen substitution patterns. When focused on position isomerism, derivatives of 4-chloroaniline showed a somewhat lower MICs than those of 3-chloro one (**1b** vs **1c**, **1j** vs **1k** and **1s** vs



Scheme 1. Synthesis of diethyl (2-phenylcarbamoyl)phosphates **1** (R for esters **1** = 4-Cl, 4-Br, 5-Cl, R^1 = 3-Cl, 4-Cl, 3,4-diCl, 3-Br, 4-Br, 3-F, 4-F, 3-CF_3, 4-CF_3).

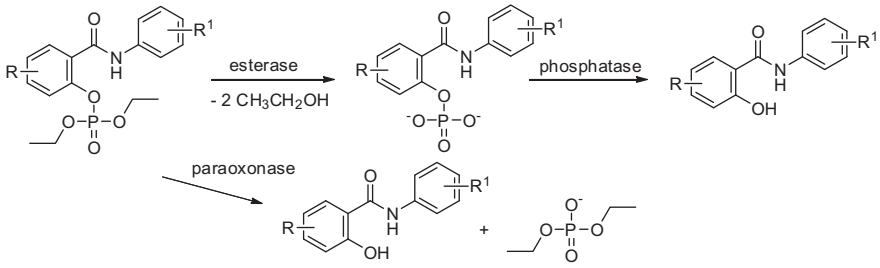
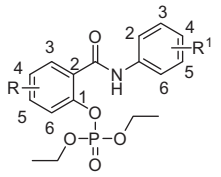


Figure 1. Two expected metabolic pathways of diethyl salicylanilide phosphates **1**.

Table 1
Antimycobacterial activity and cytotoxicity of salicylanilide diethyl phosphates **1**



1

				MIC (μmol/L)										IC_{50} (μmol/L) for HepG2
	R	R^1	C log*P*	*Mtb.* 331/88 (H_{37}Rv)		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96			
				14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d	
1a	4-Br	3-F	4.61	8	8	32	32	8	16	16	8	16	16	5.54
1b	4-Br	3-Cl	4.99	4	4	16	16	4	8	8	4	8	8	6.52
1c	4-Br	4-Cl	4.99	**2**	4	**8**	**8**	4	4	8	4	4	4	6.81
1d	4-Br	3,4-di-Cl	5.51	**1**	**1**	16	16	2	4	4	2	4	4	2.04
1e	4-Br	3-CF_3	5.36	**2**	**2**	16	16	4	4	8	4	8	8	1.84
1f	4-Br	4-CF_3	5.36	**1**	**1**	**8**	**8**	**1**	2	2	**2**	**2**	**2**	1.84
1g	4-Br	3-Br	5.27	**2**	**2**	16	16	4	8	8	4	8	8	4.41
1h	4-Br	4-Br	5.27	**2**	**2**	**8**	**8**	2	4	4	4	4	4	5.60
1i	4-Br	4-F	4.61	8	8	32	32	8	16	16	8	8	16	7.40
1j	4-Cl	3-Cl	4.72	4	4	16	16	8	8	8	8	16	16	2.68
1k	4-Cl	4-Cl	4.72	4	4	**8**	**8**	8	8	8	8	8	8	12.96
1l	4-Cl	3-F	4.34	8	8	32	32	16	32	32	16	32	32	17.17
1m	4-Cl	4-F	4.34	8	16	62.5	62.5	32	32	32	16	32	32	31.45
1n	4-Cl	4-Br	4.99	**2**	**2**	**8**	**8**	4	8	8	4	8	8	1.56
1o	4-Cl	3,4-di-Cl	5.24	**1**	**1**	16	16	4	4	4	4	4	4	2.84
1p	4-Cl	3-Br	4.99	4	4	16	16	4	8	8	8	8	8	1.81
1q	4-Cl	3-CF_3	5.08	**2**	**2**	16	16	8	8	8	8	8	8	5.68
1r	4-Cl	4-CF_3	5.08	**1**	**1**	**4**	**4**	**1**	**2**	**2**	**2**	**2**	**2**	2.33
1s	5-Cl	3-Cl	4.72	4	8	16	16	4	8	8	8	8	8	9.57
1t	5-Cl	3-Br	4.99	4	4	16	16	4	8	8	8	8	8	8.07
1u	5-Cl	3-F	4.34	8	8	16	16	8	16	16	8	16	16	11.17
1v	5-Cl	4-F	4.34	16	16	32	32	16	16	16	16	32	32	33.82
1w	5-Cl	4-Br	4.99	4	4	**8**	**8**	2	4	4	4	4	4	2.67
1x	5-Cl	4-Cl	4.72	4	4	**8**	**8**	4	4	8	4	8	8	5.60
1y	5-Cl	3,4-di-Cl	5.24	4	4	16	16	2	4	4	4	4	4	1.92
1z	5-Cl	3-CF_3	5.08	4	4	16	16	2	4	4	4	8	8	3.85
1zz	5-Cl	4-CF_3	5.08	**1**	**1**	**4**	**4**	**0.5**	**1**	**1**	**1**	**2**	**2**	2.51
INH				0.5–1	1	(250	>250	>250	>250	>250	2–4	4	4–8	NT
PAS				62.5	62.5	32	125	125	1000	>1000	32	125	500	NT

INH = isoniazid; PAS = *para*-aminosalicylic acid; NT = not tested.
The best MIC values for each strain are given in bold.

1x), similar results provided 3-/4-bromoanilines (**1g** vs **1h**, **1n** vs **1p**, **1t** vs **1v)**; for fluorine is this relationship in contrary (**1l** vs **1m**, **1u** vs **1v**). These data are in concordance with previously reported salicylanilide esters.[3–5,20] 5-Bromosalicylic acid produced derivatives (i.e., 4-bromo esters **1a**–**1i**) with the best average activity against *M. tuberculosis* and both *M. kansasii* strains, while compounds derived from 4-chlorosalicylic acid (i.e., 5-chloro esters) exhibit tst cytotoxicity was found forhe most favourable profile for *M. avium* and concomitantly with 5-bromosalicylic acid for *M. kansasii* 235/80.

Five most active phosphates (**1d**, **1f**, **1o**, **1r**, **1zz**) were evaluated at similar conditions and concentrations against four MDR-TB strains and one XDR-TB strain (dilution 10^{-3}) with different resistance patterns. All strains are resistant to INH, RIF, rifabutine, and streptomycin (STM); an additional resistance was present in some cases: 234/2005 resistant to INH, rifamycines, STM, ethambutol (EMB); 9449/2006 resistant to INH, rifamycines, and STM; Praha 1 resistant to INH, rifamycines, STM, EMB, and clofazimine; Praha 4 resistant to INH, rifamycines, STM, EMB, ofloxacin (OFX) and clofazimine; and Praha 131 resistant to INH, rifamycines, STM, EMB,

Table 2
MICs (in μmol/L) of diethyl salicylanilide phosphates **1** towards MDR- and XDR-TB

	Mtb. 234/2005		*Mtb.* 9449/2006		*Mtb.* Praha 1		*Mtb.* Praha 4		*Mtb.* Praha 131 (XDR-TB)	
	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
1d	1	1	1	1	1	1	1	1	1	1
1f	1	1	1	1	1	1	1	1	1	1
1o	1	1	1	1	1	1	1	1	1	1
1r	1	1	1	1	1	1	1	1	1	1
1zz	1	1	1	1	1	1	1	1	1	1
INH	16	16	16	16	16	16	16	16	16	16

INH = isoniazid.

OFX, gentamicin and amikacin (i.e., XDR-TB strain). All studied compounds **1** exhibited also notable and uniform activity against drug-resistant strains with MIC values of 1 μmol/L, although it does not exceed our carbamates[25] and benzoates[3] (Table 2), but being comparable (or somewhat better) to salicylanilide esters with *N*-acetyl-L-phenylalanine[20] or 4-(trifluoromethyl)benzoic acid.[4] The activity of salicylanilide diethyl phosphates **1** against drug-resistant mycobacterial strains does not depend on resistance pattern and MICs are identical as for drug-susceptible strain 331/88 ($H_{37}Rv$) indicating no cross-resistance with established drugs.

2.3. Antibacterial and antifungal activity

Investigating the biological activity, we evaluated salicylanilide diethyl phosphates **1** against eight bacterial and eight fungal strains (Tables 3 and 4).

Salicylanilide esters exhibited activity only against three Gram-positive strains: *S. aureus*, methicillin-resistant *S. aureus* and *S. epidermidis*; other species were resistant up to the concentration of 500 μmol/L. The diethyl phosphate esters can be separated into three groups. First, inactive derivatives or those with a very low antimicrobial activity with MICs ⩾250 μmoL/L for all strains: **1c**, **1d**, **1i**, **1m**, **1o**, **1q**, **1v**, **1w** and **1y**; second, molecules with a comparatively good antibacterial activity (MICs from 1.95 to 62.5 μmoL/L): **1e**, **1f**, **1h**, **1p**, **1r**, **1z**, **1zz**; and the remaining derivatives with moderate level (62.5–250 μmol/L) or exhibiting different efficacies for each strain: **1a**, **1b**, **1g**, **1j**–**1l**, **1n**, **1s**–**1u** and **1x**. In general, the lowest MICs displayed 4-bromo-2-[(4-bromophenyl)carbamoyl]phenyl diethyl phosphate **1h** (1.95–7.81 μmol/L). Benzylpenicillin showed a better in vitro activity than all molecules **1** against methicillin-susceptible *S. aureus*; on the other side, seven derivatives (**1f**, **1g**, **1h**, **1p**, **1r**, **1z**, **1zz**) expressed a significantly lower MICs than PNC against MRSA and thirteen diethyl phosphates (**1b**, **1e**–**1h**, **1j**, **1k**, **1n**, **1p**, **1r**, **1t**, **1z** and **1zz)** were superior against *S. epidermidis*.

With respect to structure–activity relationship, derivatives of 5-bromosalicylic acid showed the increased antibacterial activity than those synthesized from 4- a 5-chlorosalicylic acid. The introduction of trifluoromethyl group (**1e**, **1f**, **1z**, **1zz**) or bromine (**1g**, **1h**, **1p**) into aniline ring mostly enhanced the growth inhibition of *Staphylococci*. In contrast to antimycobacterial activity, dichloro derivatives were not evaluated as the most active esters (inactive or almost inactive ones: **1d**, **1o**, **1y**) and molecules containing fluorine were not worse than chlorinated esters. In general, salicylanilide diethyl phosphates **1** did not inhibited all bacterial strains non-specifically (five resistant strains from total eight in this assay) and their antimycobacterial activity is more uniform a homogeneous than those against other Gram-positive species.

Although salicylanilide phosphates **1** exhibited mostly significant antibacterial activity, based on the direct comparison of MIC values, it did not exceeded the action of salicylanilide 4-(trifluoromethyl)benzoates.[4]

Consequently, salicylanilide phosphates **1** revealed antifungal action only in part (Table 4). Two strains were completely resistant at 500 μmol/L (*Candida tropicalis*, *C. glabrata*), *C. albicans*, *Absidia corymbifera* and *Aspergillus fumigatus* were inhibited each only by one ester with MIC of 125 μmol/L (**1r** and **1h**, respectively) and 500 μmol/L (**1h** for *A. fumigatus*). Three derivatives (**1b**, **1h** and **1r**) affected the growth of *C. krusei* with MICs ⩾31.25 μmol/L and *Trichosporon asahii* strain was also inhibited by **1b**, **1h** and **1r** (MIC values from 15.62 to 250 μmol/L). Only *Trichophyton mentagrophytes* showed a higher rate of susceptibility in vitro. This strain was inhibited by eighteen phosphates **1** with MICs ⩾3.9 μmol/L;

Table 3
Antibacterial activity of diethyl salicylanilide phosphates **1**

	R	R^1	MIC/IC_{95} (μmol/L)					
			SA		MRSA		SE	
			24 h	48 h	24 h	48 h	24 h	48 h
1a	4-Br	3-F	125	250	250	250	250	250
1b	4-Br	3-Cl	125	250	125	250	125	250
1c	4-Br	4-Cl	500	>500	500	>500	500	>500
1d	4-Br	3,4-di-Cl	500	>500	500	>500	500	>500
1e	4-Br	3-CF_3	**7.81**	**7.81**	**15.62**	**15.62**	**7.81**	**7.81**
1f	4-Br	4-CF_3	**15.62**	31.25	**15.62**	**15.62**	**7.81**	15.62
1g	4-Br	3-Br	31.25	250	31.25	250	31.25	250
1h	4-Br	4-Br	**7.81**	**7.81**	**3.9**	**3.9**	**1.95**	**1.95**
1i	4-Br	4-F	>500	>500	500	500	>500	>500
1j	4-Cl	3-Cl	500	500	500	500	125	125
1k	4-Cl	4-Cl	62.5	125	125	250	125	250
1l	4-Cl	3-F	125	500	500	>500	250	500
1m	4-Cl	4-F	250	500	>500	>500	>500	>500
1n	4-Cl	4-Br	125	125	>500	>500	125	125
1o	4-Cl	3,4-di-Cl	>500	>500	>500	>500	>500	>500
1p	4-Cl	3-Br	**15.62**	31.25	31.25	62.5	15.62	31.25
1q	4-Cl	3-CF_3	>500	>500	500	500	250	>500
1r	4-Cl	4-CF_3	31.25	31.25	62.5	62.5	31.25	31.25
1s	5-Cl	3-Cl	500	500	500	500	250	500
1t	5-Cl	3-Br	125	500	250	500	125	250
1u	5-Cl	3-F	125	250	250	250	250	250
1v	5-Cl	4-F	250	500	>500	>500	>500	>500
1w	5-Cl	4-Br	500	>500	>500	>500	500	>500
1x	5-Cl	4-Cl	125	250	500	>500	250	500
1y	5-Cl	3,4-di-Cl	500	>500	500	>500	250	>500
1z	5-Cl	3-CF_3	31.25	62.5	31.25	62.5	15.62	31.25
1zz	5-Cl	4-CF_3	31.25	31.25	62.5	62.5	31.25	31.25
PNC			0.98	0.98	62.5	125	250	250

SA: *Staphylococcus aureus* CCM 4516/08; MRSA: methicillin-resistant *Staphylococcus aureus* H 5996/08; SE: *Staphylococcus epidermidis* H 6966/08. PNC: benzylpenicillin. The best MIC values for each strain are given in bold.

Table 4
Antifungal activity of salicylanilide diethyl phosphates **1**

	R	R[1]	MIC/IC_{80}/IC_{50} (μmol/L)											
			Candida albicans		*Candida krusei*		*Trichosporon asahii*		*Aspergillus fumigates*		*Absidia corymbifera*		*Trichophyton mentagrophytes*	
			24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	24 h	72 h	120 h
1a	4-Br	3-F	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	62.5	62.5
1b	4-Br	3-Cl	>500	>500	**31.25**	**125**	**15.62**	**62.5**	>500	>500	>500	>500	7.81	15.62
1c	4-Br	4-Cl	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1d	4-Br	3,4-di-Cl	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1e	4-Br	3-CF_3	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	62.5	62.5
1f	4-Br	4-CF_3	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	250	250
1g	4-Br	3-Br	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1h	4-Br	4-Br	>500	>500	**125**	**125**	**62.5**	**62.5**	**500**	**500**	**125**	**125**	**3.9**	**3.9**
1i	4-Br	4-F	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	500	500
1j	4-Cl	3-Cl	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1k	4-Cl	4-Cl	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	500	500
1l	4-Cl	3-F	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	62.5	62.5
1m	4-Cl	4-F	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1n	4-Cl	4-Br	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	250	250
1o	4-Cl	3,4-di-Cl	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	500	500
1p	4-Cl	3-Br	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	15.62	15.62
1q	4-Cl	3-CF_3	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1r	4-Cl	4-CF_3	**125**	>250	**125**	>250	125	250	>250	>250	>250	>250	31.25	31.25
1s	5-Cl	3-Cl	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1t	5-Cl	3-Br	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1u	5-Cl	3-F	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	500	500
1v	5-Cl	4-F	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	500	500
1w	5-Cl	4-Br	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
1x	5-Cl	4-Cl	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	500	500
1y	5-Cl	3,4-di-Cl	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	**7.81**	**7.81**
1z	5-Cl	3-CF_3	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	31.25	31.25
1zz	5-Cl	4-CF_3	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	250	250
FLU			1.0	2.0	>50	>50	4.00	9.00	>50	>50	>50	>50	17.0	26.0

FLU: fluconazole.
The best MIC values for each strain are given in bold.

four of them exceeded the in vitro efficacy of a triazole antimycotic drug fluconazole.

Among all esters, 4-bromo-2-[(4-bromophenyl)carbamoyl]phenyl diethyl phosphate **1h** displayed the highest antifungal potential similarly as in the case of Gram-positive bacteria. However, a wide range of diethyl phosphates avoided any shared notable antifungal activity and it is unfeasible to draw clear conclusions about dependency of the activity on the structure. However, the derivatives, which showed the most strong antitubercular activity, were inactive (**1d**, **1s**), almost inactive (**1f**) or intermediate active (**1r** and **1z**) against *T. mentagrophytes* under antifungal evaluation.

2.4. Cytotoxicity evaluation

In vitro cytotoxicity was investigated in the liver HepG2 cell model using a standard colorimetric method measuring a tetrazolium salt reduction. The cytotoxicity is expressed as IC_{50}, that is, concentration, which reduces the viability of the cells to 50% of the maximal viability. This parameter allowed the quantitative comparison of the toxicity among the tested compounds. The phosphorylation alleviated mildly the strong cytotoxicity of the parent salicylanilides to a bit more acceptable values of 1.56–33.82 μmol/L (Table 1).

The sharpest cytotoxicity was found for 2-[(4-bromophenyl)carbamoyl]-4-chlorophenyl diethyl phosphate (**1n**; IC_{50} = 1.56 μmol/L), on the other side, 5-chloro-2-[(4-fluorophenyl)carbamoyl]phenyl diethyl phosphate **1v** displayed the highest IC_{50} value being 33.82 μmol/L. Derivatives of 5-bromosalicylic acid showed increased cytotoxicity when compared to those synthesized from 4- and 5-chlorosalicylic acids. Trifluoromethyl, 3,4-dichloro and 4-bromo substitution patterns on the aniline ring resulted in enhanced cytotoxicity, whereas fluorine alleviated it in the best way.

Although most of the esters **1** share significant cytotoxicity for HepG2 cells, this property does not directly correlate with MICs for mycobacteria. It is obvious that the molecules exhibiting the lowest MIC values showed generally also low-level IC_{50} values and vice versa, but the relationship is not clear and simple. Lipophilicity may also play certain role. However, MICs of some derivatives are comparatively lower than corresponding IC_{50} values, for example **1k**, and some esters showed more pronounced cytotoxicity than antimycobacterial action—**1a**, **1e**, **1n**, **1p**, **1y** etc. Additionally, there are differences between MIC values for *M. tuberculosis* and atypical mycobacteria. These data indicate one or more specific mechanism(s) of action/target(s) for mycobacteria than a general cytotoxic impact on all cell types.

3. Conclusion

In sum, we have designed and synthesized a new series of salicylanilide diethyl phosphates with comparable and even better activity than INH, a first line anti-tuberculosis drug. In comparison to parent salicylanilides, their diethyl phosphates showed a decreased cytotoxicity and mostly an increased antimycobacterial activity resulting in improved selectivity indexes. Salicylanilide diethyl phosphates also inhibit multidrug-resistant *Mtb.* strains with identical MIC values as for susceptible ones. Temporary masking of free phenolic hydroxyl group increased the activity and also possibility for usage.

Salicylanilide diethyl phosphates also inhibited the growth of *Staphylococcus sp.* strains including MRSA and the growth of filamentous fungus *Trichophyton mentagrophytes*. However, these antimicrobial properties are heterogeneous and do not correlate identically with anti-tuberculosis and antimycobacterial activity. Additionally, five bacterial and two fungal strains were completely

resistant to presented salicylanilide phosphates, whereas mycobacteria including MDR- and XDR-TB strains displayed a homogenous susceptibility at low micromolar concentrations. It implies that the mechanism(s) and way(s) of the action are at least partly different and that the inhibition of mycobacteria is not a result of only a general cellular toxicity in spite of cell type.

4. Experimental part

4.1. Chemistry

Reagents and solvents were purchased from Sigma Aldrich (Darmstadt, Germany) and Penta Chemicals (Prague, the Czech Republic) and were used without further purification. Reactions and the purity of products were monitored by thin layer chromatography, plates coated with 0.2 mm silica gel 60 F_{254} (Merck), visualization by UV light (254 and 366 nm). Column chromatography purification was performed on silica gel 60 with particle size 0.063–0.2 mm (Fluka).

The melting points were determined on a Melting Point M-560 apparatus (Bűchi Labortechnik AG, Flawil, Switzerland) in open capillaries and are uncorrected. The IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) over the range of 400–4000 cm^{-1} using the ATR technique. The NMR spectra were measured in $CDCl_3$ solutions at ambient temperature on a Varian Mercury Vxbb 300 (300 MHz for ^1H and 75.5 MHz for ^{13}C, Varian Comp. Palo Alto, CA, USA) and Varian Mercury (500 MHz for ^1H and 125 MHz for ^{13}C, Varian Comp. Palo Alto, CA, USA). The chemical shifts (δ) are given in ppm, and tetramethylsilane was employed as the internal standard. Elementary analysis was performed on CE Instruments EA-1110 CHN analyser (CE Instruments, Wigan, UK).

The calculated log*P* values (C log*P*), that are the logarithms of the partition coefficients for octan-1-ol/water, were determined using the program CS ChemOffice Ultra version 12.0 (CambridgeSoft, Cambridge, MA, USA).

4.1.1. General procedure for the preparation of salicylanilide phosphates 1

The starting salicylanilides were prepared according to the literature.[20] Appropriate salicylanilide (2 mmol) was suspended at 20 °C in 10 mL of dichloromethane (DCM) and during intensive stirring 1.5 equiv of triethylamine was added. After 5 min, when the suspension became solution, 1.2 equiv of diethyl chlorophosphate was added in one portion. Then, the solution was stirred at ambient temperature for 2 h and the reaction was monitored using TLC (mixture of toluene and ethyl acetate 4:1). When the reaction was finished, the product was purified by column chromatography using silica gel and chloroform as mobile phase. After removing of chloroform obtained colourless oil was solidified by storing in freezer. Product was recrystallized from the mixture of acetone-hexane to obtain pure product, if necessary.

4.1.1.1. 4-Bromo-2-[(3-fluorophenyl)carbamoyl]phenyl diethyl phosphate (1a). Yield: 57%, white solid; mp 87 °C; IR (ATR): 1677 (amide I), 1611 (ν CC aromatic), 1551 (amide II), 1491, 1479 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.58 (1H, br s, NH), 8.04 (1H, dd, J = 2.6 Hz, J = 1.0 Hz, H3), 7.70 (1H, dt, J = 11.0 Hz, J = 2.3 Hz, H2′), 7.57 (1H, dd, J = 8.7 Hz, J = 2.6 Hz, H5), 7.38 (1H, ddd, J = 8.1 Hz, J = 2.0 Hz, J = 1.1 Hz, H6′), 7.29 (1H, td, J = 8.2 Hz, J = 6.3 Hz, H5′), 7.23 (1H, dd, J = 8.7 Hz, J = 1.4 Hz, H6), 6.83 (1H, tdd, J = 8.3 Hz, J = 2.5 Hz, J = 1.1 Hz, H4′), 4.30–4.15 (4H, m, CH_AH_B), 1.33 (6H, td, J = 7.1 Hz, J = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.96 (1C, d, J = 243.2 Hz, C3′), 161.83, 145.77 (1C, d, J = 7.1 Hz), 139.54 (1C, d, J = 10.8 Hz), 135.18 (1C, d, J = 1.8 Hz), 134.49 (1C, d, J = 1.2 Hz), 130.07 (1C, d, J = 9.2 Hz), 129.68 (1C, d, J = 5.7 Hz), 123.04 (1C, d, J = 2.3 Hz), 119.21 (1C, d, J = 2.1 Hz), 115.18 (1C, d, J = 3.0 Hz, C6′), 111.23 (1C, d, J = 21.3 Hz), 107.35 (1C, d, J = 26.3 Hz), 65.74 (2C, d, J = 6.1 Hz, CH_AH_B), 16.04 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}BrFNO_5P$ (446.20): C, 45.76; H, 4.07; N, 3.14. Found: C, 45.52; H, 4.21; N, 3.07.

4.1.1.2. 4-Bromo-2-[(3-chlorophenyl)carbamoyl]phenyl diethyl phosphate (1b). Yield: 47%, white solid; mp 64 °C; IR (ATR): 1698 (amide I), 1605 (ν CC aromatic), 1545 (amide II), 1479 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.54 (1H, br s, NH), 8.05 (1H, m, H3), 7.87 (1H, t, J = 2.1 Hz, H2′), 7.63–7.54 (2H, m), 7.31–7.20 (2H, m), 7.12 (1H, m), 4.30–4.15 (4H, m, CH_AH_B), 1.34 (6H, td, J = 7.1 Hz, J = 1.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.82, 145.78 (1C, d, J = 6.9 Hz), 139.19, 135.21 (1C, d, J = 1.7 Hz), 134.67, 134.51, 129.99, 129.60 (1C, d, J = 5.8 Hz), 124.58, 123.02 (1C, d, J = 2.3 Hz), 119.93, 119.22 (1C, d, J = 2.0 Hz), 117.87, 65.77 (2C, d, J = 6.0 Hz, CH_AH_B), 16.05 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}BrClNO_5P$ (462.66): C, 44.13; H, 3.92; N, 3.03. Found: C, 44.05; H, 4.01; N, 2.97.

4.1.1.3. 4-Bromo-2-[(4-chlorophenyl)carbamoyl]phenyl diethyl phosphate (1c). Yield: 58%, white solid; mp 64 °C; IR (ATR): 1675 (amide I), 1610 (ν CC aromatic), 1546 (amide II), 1493, 1482 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 9.54 (1H, br s, NH), 8.05 (1H, br s, H3), 7.75–7.67 (2H, m, H2′,H6′), 7.57 (1H, dd, J = 8.7 Hz, J = 2.2 Hz, H5), 7.36–7.28 (2H, m, H3′, H5′), 7.22 (1H, d, J = 8.7 Hz, H6), 4.29–4.15 (4H, m, CH_AH_B), 1.33 (6H, t, J = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 161.72, 145.73 (1C, d, J = 7.1 Hz), 136.70, 135.14 (1C, d, J = 1.8 Hz), 134.52, 129.79 (1C, d, J = 5.6 Hz), 129.46, 129.04, 123.06 (1C, d, J = 2.4 Hz), 121.06, 119.25 (1C, d, J = 2.0 Hz), 65.74 (2C, d, J = 6.4 Hz, CH_AH_B), 16.06 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}BrClNO_5P$ (462.66): C, 44.13; H, 3.92; N, 3.03. Found: C, 43.85; H, 3.81; N, 2.92.

4.1.1.4. 4-Bromo-2-[(3,4-dichlorophenyl)carbamoyl]phenyl diethyl phosphate (1d). Yield: 73%, white solid; mp 70 °C; IR (ATR): 1677 (amide I), 1593 (ν CC aromatic), 1528 (amide II), 1478 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.70 (1H, br s, NH), 8.02 (1H, dd, J = 2.5 Hz, J = 0.9 Hz, H3), 8.01 (1H, d, J = 2.4 Hz, H2′), 7.62–7.53 (2H, m, H5, H6′), 7.39 (1H, d, J = 8.8 Hz, H5′), 7.21 (1H, dd, J = 8.7 Hz, J = 1.4 Hz, H6), 4.30–4.15 (4H, m, CH_AH_B), 1.35 (6H, td, J = 7.1 Hz, J = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.86, 145.66 (1C, d, J = 6.9 Hz), 137.62, 135.30 (1C, d, J = 1.8 Hz), 134.52, 132.78, 130.50, 129.59 (1C, d, J = 5.6 Hz), 127.61, 123.16 (1C, d, J = 2.3 Hz), 121.46, 119.31 (1C, d, J = 2.1 Hz), 119.08, 65.83 (2C, d, J = 6.1 Hz, CH_AH_B), 16.06 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{17}BrCl_2NO_5P$ (497.10): C, 41.07; H, 3.45; N, 2.82. Found: C, 40.92; H, 3.55; N, 2.57.

4.1.1.5. 4-Bromo-2-{[3-(trifluoromethyl)phenyl]carbamoyl}phenyl diethyl phosphate (1e). Yield: 59%, white solid; mp 80 °C; IR (ATR): 1682 (amide I), 1618 (ν CC aromatic), 1564 (amide II), 1495, 1478, (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.74 (1H, br s, NH), 8.09–8.03 (2H, m, H3, H2′), 7.95 (1H, m, H4′), 7.59 (1H, dd, J = 8.7 Hz, J = 2.6 Hz, H5), 7.47 (1H, t, J = 7.9 Hz, H5′), 7.39 (1H, m, H6′), 7.24 (1H, dd, J = 8.7 Hz, J = 1.4 Hz, H6), 4.30–4.15 (4H, m, CH_AH_B), 1.34 (6H, td, J = 7.1 Hz, J = 1.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.01, 145.80 (1C, d, J = 6.9 Hz), 138.67, 135.28 (1C, d, J = 1.8 Hz), 134.52 (1C, d, J = 1.1 Hz), 131.38 (1C, q, J = 32.3 Hz, C3′), 129.56, 129.53 (1C, d, J = 5.8 Hz), 123.84 (1C, q, J = 270.8 Hz, CF_3), 123.07 (1C, d, J = 2.3 Hz), 122.91, 121.01 (1C, q, J = 3.8 Hz, C2′), 119.25 (1C, d, J = 2.0 Hz), 116.56 (1C, q, J = 4.0 Hz, C4′), 65.79 (2C,

d, J = 6.1 Hz, CH_AH_B), 16.01 (2C, d, J = 6.5 Hz, CH_3). Anal. Calcd for $C_{18}H_{18}BrF_3NO_5P$ (496.21): C, 43.57; H, 3.66; N, 2.82. Found: C, 43.23; H, 3.50; N, 2.49.

4.1.1.6. 4-Bromo-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl diethyl phosphate (1f). Yield: 52%, white solid; mp 70 °C; IR (ATR): 1698 (amide I), 1605 (ν CC aromatic), 1544 (amide II), 1479 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.76 (1H, br s, NH), 8.05 (1H, m, H3), 7.93–7.84 (2H, m, H3′,H5′), 7.66–7.56 (2H, m, H2′, H6′), 7.60 (1H, dd, J = 8.8 Hz, J = 2.7 Hz, H5), 7.23 (1H, dd, J = 8.8 Hz, J = 1.4 Hz, H6), 4.32–4.13 (4H, m, CH_AH_B), 1.34 (6H, td, J = 7.1 Hz, J = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.08, 145.73 (1C, d, J = 7.0 Hz), 141.15, 135.33 (1C, d, J = 1.8 Hz), 134.58 (1C, d, J = 1.2 Hz), 129.65 (1C, d, J = 5.6 Hz), 123.16 (1C, d, J = 2.3 Hz), 126.26 (2C, q, J = 3.8 Hz, C3′, C5′), 126.24 (1C, q, J = 32.5 Hz, C4′), 124.07 (1C, q, J = 269.9 Hz, CF_3), 119.56, 119.32 (1C, d, J = 2.0 Hz), 65.81 (2C, d, J = 6.1 Hz, CH_AH_B), 16.05 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{18}H_{18}BrF_3NO_5P$ (496.21): C, 43.57; H, 3.66; N, 2.82. Found: C, 43.44; H, 3.26; N, 2.92.

4.1.1.7. 4-Bromo-2-[(3-bromophenyl)carbamoyl]phenyl diethyl phosphate (1g). Yield: 74%, white solid; mp 85 °C; IR (ATR): 1682 (amide I), 1629, 1593 (ν CC aromatic), 1543 (amide II), 1480 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.54 (1H, br s, NH), 8.04 (1H, d, J = 2.8 Hz, H3), 8.01 (1H, t, J = 1.9 Hz, H2′), 7.64 (1H, m), 7.58 (1H, dd, J = 8.8 Hz, J = 2.7 Hz, H5), 7.32–7.17 (3H, m), 4.32–4.13 (4H, m, CH_AH_B), 1.35 (6H, td, J = 7.1 Hz, J = 1.0 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.80, 145.77 (1C, d, J = 7.0 Hz), 139.34, 135.21, 134.51, 130.29, 129.59 (1C, d, J = 5.9 Hz), 127.50, 123.03 (1C, d, J = 2.3 Hz), 122.73, 122.64, 119.22 (1C, d, J = 2.0 Hz), 118.33, 65.76 (2C, d, J = 6.0 Hz, CH_AH_B), 16.06 (2C, d, J = 6.5 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}Br_2NO_5P$ (507.11): C, 40.26; H, 3.58; N, 2.76. Found: C, 40.10; H, 3.33; N, 2.58.

4.1.1.8. 4-Bromo-2-[(4-bromophenyl)carbamoyl]phenyl diethyl phosphate (1h). Yield: 48%, white solid; mp 68 °C; IR (ATR): 1676 (amide I), 1606 (ν CC aromatic), 1544 (amide II), 1489, 1481 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.54 (1H, br s, NH), 8.04 (1H, d, J = 2.6 Hz, H3), 7.72–7.61 (2H, m, H2′, H6′), 7.57 (1H, dd, J = 8.8 Hz, J = 2.6 Hz, H5), 7.52–7.41 (2H, m, H3′, H5′), 7.22 (1H, dd, J = 8.8 Hz, J = 1.5 Hz, H6), 4.34–4.09 (4H, m, CH_AH_B), 1.34 (6H, td, J = 7.1 Hz, J = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.75, 145.71 (1C, d, J = 6.9 Hz), 137.18, 135.15 (1C, d, J = 1.7 Hz), 134.50, 131.98, 129.77 (1C, d, J = 5.5 Hz), 123.06 (1C, d, J = 2.3 Hz), 121.39, 119.25 (1C, d, J = 1.9 Hz), 117.12, 65.75 (2C, d, J = 6.1 Hz, CH_AH_B), 16.06 (2C, d, J = 6.5 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}Br_2NO_5P$ (507.11): C, 40.26; H, 3.58; N, 2.76. Found: C, 40.05; H, 3.28; N, 2.46.

4.1.1.9. 4-Bromo-2-[(4-fluorophenyl)carbamoyl]phenyl diethyl phosphate (1i). Yield: 77%, white solid; mp 81 °C; IR (ATR): 1672 (amide I), 1620 (ν CC aromatic), 1558 (amide II), 1509, 1476 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 9.45 (1H, br s, NH), 8.04 (1H, dd, J = 2.6 Hz, J = 1.0 Hz, H3), 7.77–7.66 (2H, m, H2′, H6′), 7.57 (1H, dd, J = 8.7 Hz, J = 2.6 Hz, H5), 7.23 (1H, dd, J = 8.7 Hz, J = 1.4 Hz, H6), 7.10–7.00 (2H, m, H3′, H5′), 4.31–4.11 (4H, m, CH_AH_B), 1.34 (6H, td, J = 7.1 Hz, J = 1.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 161.60, 159.42 (1C, d, J = 242.4 Hz, C4′), 145.77 (1C, d, J = 7.0 Hz), 135.02 (1C, d, J = 1.7 Hz), 134.45, 134.14 (1C, d, J = 2.7 Hz, C1′), 129.80 (1C, d, J = 5.8 Hz), 122.99 (1C, d, J = 2.2 Hz), 121.51 (2C, d, J = 7.7 Hz, C2′, C6′), 119.17 (1C, d, J = 2.0 Hz), 115.63 (2C, d, J = 22.3 Hz, C3′, C5′), 65.68 (2C, d, J = 6.0 Hz, CH_AH_B), 16.03 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}BrFNO_5P$ (446.20): C, 45.76; H, 4.07; N, 3.14. Found: C, 45.45; H, 3.88; N, 2.97.

4.1.1.10. 4-Chloro-2-{[3-(trifluoromethyl)phenyl]carbamoyl}phenyl diethyl phosphate (1j). Yield: 42%, white solid; mp 75 °C; IR (ATR): 1686 (amide I), 1617 (ν CC aromatic), 1565 (amide II), 1494, 1479, (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.75 (1H, br s, NH), 8.06 (1H, br s, H2′), 7.96 (1H, m, H4′), 7.91 (1H, dd, J = 2.7 Hz, J = 1.0 Hz, H3), 7.47 (1H, t, J = 8.2 Hz, H5′), 7.44 (1H, dd, J = 8.8 Hz, J = 2.7 Hz, H5), 7.40 (1H, m, H6′), 7.30 (1H, dd, J = 8.8 Hz, J = 1.4 Hz, H6), 4.30–4.16 (4H, m, CH_AH_B), 1.34 (6H, td, J = 7.1 Hz, J = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.12, 145.24 (1C, d, J = 6.9 Hz), 138.68, 132.32 (1C, d, J = 1.8 Hz), 131.79 (1C, d, J = 1.9 Hz), 131.59, 131.39 (1C, q, J = 32.2 Hz, C3′), 129.56, 129.25 (1C, d, J = 5.7 Hz), 123.85 (1C, q, J = 270.8 Hz, CF_3), 122.94, 122.79 (1C, d, J = 2.3 Hz), 121.01 (1C, q, J = 3.8 Hz, C2′), 116.57 (1C, q, J = 4.0 Hz, C4′), 65.79 (2C, d, J = 6.1 Hz, CH_AH_B), 16.02 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}BrFNO_5P$ (451.76): C, 47.86; H, 4.02; N, 3.10. Found: C, 47.53; H, 4.36; N, 3.24.

4.1.1.11. 4-Chloro-2-[(4-chlorophenyl)carbamoyl]phenyl diethyl phosphate (1k). Yield: 54%, white solid; mp 71 °C; IR (ATR): 1674 (amide I), 1609 (ν CC aromatic), 1546 (amide II), 1493 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 9.57 (1H, br s, NH), 7.89 (1H, d, J = 2.7 Hz, H3), 7.75–7.68 (2H, m, H2′, H6′), 7.42 (1H, dd, J = 8.8 Hz, J = 2.7 Hz, H5), 7.35–7.29 (2H, m, H3′, H5′), 7.28 (1H, dd, J = 8.8 Hz, J = 1.2 Hz, H6), 4.28–4.15 (4H, m, CH_AH_B), 1.33 (6H, t, J = 7.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 161.84, 145.15 (1C, d, J = 7.0 Hz), 136.71, 132.13 (1C, d, J = 1.7 Hz), 131.74 (1C, d, J = 1.9 Hz), 131.52, 129.50 (1C, d, J = 5.7 Hz), 129.45, 129.02, 122.73 (1C, d, J = 2.3 Hz), 121.05, 65.72 (2C, d, J = 6.3 Hz, CH_AH_B), 16.04 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}Cl_2NO_5P$ (418.21): C, 48.82; H, 4.34; N, 3.35. Found: C, 49.05; H, 4.24; N, 3.45.

4.1.1.12. 4-Chloro-2-[(3-fluorophenyl)carbamoyl]phenyl diethyl phosphate (1l). Yield: 64%, white solid; mp 86 °C; IR (ATR): 1678 (amide I), 1612 (ν CC aromatic), 1552 (amide II), 1491, 1482 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.60 (1H, br s, NH), 7.90 (1H, dd, J = 2.7 Hz, J = 1.0 Hz, H3), 7.71 (1H, dt, J = 10.9 Hz, J = 2.3 Hz, H2′), 7.43 (1H, dd, J = 8.8 Hz, J = 2.7 Hz, H5), 7.38 (1H, ddd, J = 8.1 Hz, J = 2.0 Hz, J = 1.1 Hz, H6′), 7.34–7.24 (2H, m, H6, H5′), 6.84 (1H, tdd, J = 8.3 Hz, J = 2.6 Hz, J = 1.1 Hz, H4′), 4.32–4.13 (4H, m, CH_AH_B), 1.34 (6H, td, J = 7.1 Hz, J = 1.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.97 (1C, d, J = 243.2 Hz, C3′), 161.94, 145.20 (1C, d, J = 6.9 Hz), 139.57 (1C, d, J = 10.9 Hz), 132.19 (1C, d, J = 1.8 Hz), 131.73 (1C, d, J = 2.0 Hz), 131.54 (1C, d, J = 1.1 Hz), 130.07 (1C, d, J = 9.3 Hz), 129.41 (1C, d, J = 5.7 Hz), 122.74 (1C, d, J = 2.4 Hz), 115.18 (1C, d, J = 3.0 Hz, C6′), 111.22 (1C, d, J = 21.2 Hz), 107.36 (1C, d, J = 26.3 Hz), 65.73 (2C, d, J = 6.1 Hz, CH_AH_B), 16.04 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}ClFNO_5P$ (401.75): C, 50.82; H, 4.52; N, 3.49. Found: C, 51.02; H, 4.57; N, 3.60.

4.1.1.13. 4-Chloro-2-[(4-fluorophenyl)carbamoyl]phenyl diethyl phosphate (1m). Yield: 56%, white solid; mp 74 °C; IR (ATR): 1673 (amide I), 1622 (ν CC aromatic), 1561 (amide II), 1509, 1479 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.48 (1H, br s, NH), 7.90 (1H, dd, J = 2.7 Hz, J = 1.0 Hz, H3), 7.77–7.66 (2H, m, H2′, H6′), 7.42 (1H, dd, J = 8.7 Hz, J = 2.7 Hz, H5), 7.28 (1H, dd, J = 8.7 Hz, J = 1.3 Hz, H6), 7.10–6.99 (2H, m, H3′, H5′), 4.31–4.12 (4H, m, CH_AH_B), 1.33 (6H, td, J = 7.1 Hz, J = 1.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.75, 159.43 (1C, d, J = 242.4 Hz, C4′), 145.20 (1C, d, J = 7.0 Hz), 134.15 (1C, d, J = 2.9 Hz, C1′), 132.05 (1C, d, J = 1.8 Hz), 131.70 (1C, d, J = 1.9 Hz), 131.50 (1C, d, J = 1.1 Hz), 129.54 (1C, d, J = 5.6 Hz), 122.69 (1C, d, J = 2.4 Hz), 121.52 (2C, d, J = 7.8 Hz, C2′, C6′), 115.65 (2C, d, J = 22.3 Hz, C3′, C5′), 65.69 (2C, d, J = 6.0 Hz, CH_AH_B), 16.04 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}ClFNO_5$-

P (401.75): C, 50.82; H, 4.52; N, 3.49. Found: C, 50.67; H, 4.68; N, 3.56.

4.1.1.14. 2-[(4-Bromophenyl)carbamoyl]-4-chlorophenyl diethyl phosphate (1n). Yield: 67%, white solid; mp 68 °C; IR (ATR): 1691 (amide I), 1605, 1592 (*ν* CC aromatic), 1541 (amide II), 1489 (*ν* CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): *δ* 9.58 (1H, br s, NH), 7.89 (1H, dd, *J* = 2.7 Hz, *J* = 1.0 Hz, H3), 7.70–7.61 (2H, m, H2′, H6′), 7.50–7.42 (2H, m, H3′, H5′), 7.42 (1H, dd, *J* = 8.7 Hz, *J* = 2.7 Hz, H5), 7.28 (1H, dd, *J* = 8.7 Hz, *J* = 1.4 Hz, H6), 4.32–4.12 (4H, m, CH_AH_B), 1.33 (6H, td, *J* = 7.1 Hz, *J* = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): *δ* 161.85, 145.11 (1C, d, *J* = 7.1 Hz), 137.20, 132.15 (1C, d, *J* = 1.8 Hz), 131.96, 131.74 (1C, d, *J* = 2.0 Hz), 131.53 (1C, d, *J* = 1.1 Hz), 129.49 (1C, d, *J* = 5.5 Hz), 122.76 (1C, d, *J* = 2.3 Hz), 121.37, 117.08, 65.72 (2C, d, *J* = 6.1 Hz, CH_AH_B), 16.05 (2C, d, *J* = 6.5 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}BrClNO_5P$ (462.66): C, 44.13; H, 3.92; N, 3.03. Found: C, 43.89; H, 4.07; N, 3.00.

4.1.1.15. 4-Chloro-2-[(3,4-dichlorophenyl)carbamoyl]phenyl diethyl phosphate (1o). Yield: 78%, white solid; mp 86 °C; IR (ATR): 1678 (amide I), 1594 (*ν* CC aromatic), 1529 (amide II), 1477 (*ν* CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): *δ* 9.72 (1H, br s, NH), 8.01 (1H, d, *J* = 2.5 Hz, H2′), 7.86 (1H, d, *J* = 2.7 Hz, H3), 7.57 (1H, dd, *J* = 8.6 Hz, *J* = 2.5 Hz, H6′), 7.47–7.34 (2H, m, H5, H5′), 7.26 (1H, d, *J* = 8.7 Hz, H6), 4.33–4.12 (4H, m, CH_AH_B), 1.35 (6H, t, *J* = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): *δ* 161.99, 145.09 (1C, d, *J* = 7.0 Hz), 137.64, 132.76, 132.31, 131.80 (1C, d, *J* = 2.0 Hz), 131.53, 130.49, 129.30 (1C, d, *J* = 5.6 Hz), 127.58, 122.86 (1C, d, *J* = 2.3 Hz), 121.45, 119.08, 65.81 (2C, d, *J* = 6.3 Hz, CH_AH_B), 16.06 (2C, d, *J* = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{17}Cl_3NO_5P$ (452.65): C, 45.11; H, 3.79; N, 3.09. Found: C, 45.34; H, 3.89; N, 3.24.

4.1.1.16. 2-[(3-Bromophenyl)carbamoyl]-4-chlorophenyl diethyl phosphate (1p). Yield: 64%, white solid; mp 65 °C; IR (ATR): 1679 (amide I), 1590 (*ν* CC aromatic), 1536 (amide II), 1478 (*ν* CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): *δ* 9.55 (1H, br s, NH), 8.02 (1H, t, *J* = 1.9 Hz, H2′), 7.90 (1H, d, *J* = 2.7 Hz, H3), 7.65 (1H, m), 7.44 (1H, dd, *J* = 8.7 Hz, *J* = 2.7 Hz, H5), 7.33–7.18 (3H, m), 4.31–4.15 (4H, m, CH_AH_B), 1.35 (6H, td, *J* = 7.1 Hz, *J* = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): *δ* 161.91, 145.21 (1C, d, *J* = 7.0 Hz), 139.36, 132.26 (1C, d, *J* = 1.7 Hz), 131.77 (1C, d, *J* = 1.8 Hz), 131.61, 130.31, 129.31 (1C, d, *J* = 6.0 Hz), 127.52, 122.74, 122.66, 118.35, 65.76 (2C, d, *J* = 6.0 Hz, CH_AH_B), 16.07 (2C, d, *J* = 6.5 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}BrClNO_5P$ (462.66): C, 44.13; H, 3.92; N, 3.03. Found: C, 43.87; H, 3.53; N, 2.88.

4.1.1.17. 4-Chloro-2-[(3-chlorophenyl)carbamoyl]phenyl diethyl phosphate (1q). Yield: 11%, white solid; mp 49 °C; IR (ATR): 1683 (amide I), 1609, 1596 (*ν* CC aromatic), 1541 (amide II), 1494 (*ν* CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): *δ* 9.58 (1H, br s, NH), 7.93–7.83 (2H, m, H3, H2′), 7.58 (1H, d, *J* = 8.7 Hz), 7.43 (1H, dd, *J* = 8.6 Hz, *J* = 2.8 Hz, H5), 7.35–7.23 (2H, m, H6, H5′), 7.11 (1H, d, *J* = 8.3 Hz), 4.32–4.14 (4H, m, CH_AH_B), 1.34 (6H, t, *J* = 7.0 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): *δ* 161.93, 145.20 (1C, d, *J* = 7.0 Hz), 139.22, 134.66, 132.22 (1C, d, *J* = 1.8 Hz), 131.73 (1C, d, *J* = 2.0 Hz), 131.55, 129.99, 129.34 (1C, d, *J* = 5.8 Hz), 124.55, 122.73 (1C, d, *J* = 2.3 Hz), 119.92, 117.85, 65.74 (2C, d, *J* = 6.1 Hz, CH_AH_B), 16.04 (2C, d, *J* = 6.5 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}Cl_2NO_5P$ (418.21): C, 48.82; H, 4.34; N, 3.35. Found: C, 48.64; H, 4.12; N, 3.42.

4.1.1.18. 4-Chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl diethyl phosphate (1r). Yield: 60%, white solid; mp 75 °C; IR (ATR): 1683 (amide I), 1605 (*ν* CC aromatic), 1540 (amide II), 1479 (*ν* CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): *δ* 9.78 (1H, br s, NH), 7.96–7.82 (3H, m, H3, H3′, H5′), 7.67–7.57 (2H, m, H2′, H6′), 7.45 (1H, dd, *J* = 8.7 Hz, *J* = 2.7 Hz, H5), 7.29 (1H, dd, *J* = 8.7 Hz, *J* = 1.4 Hz, H6), 4.32–4.13 (4H, m, CH_AH_B), 1.34 (6H, td, *J* = 7.1 Hz, *J* = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): *δ* 162.18, 145.15 (1C, d, *J* = 7.0 Hz), 141.17, 132.35 (1C, d, *J* = 1.8 Hz), 131.85 (1C, d, *J* = 2.0 Hz), 131.63 (1C, d, *J* = 1.2 Hz), 129.36 (1C, d, *J* = 5.6 Hz), 126.25 (2C, q, *J* = 3.8 Hz, C3′, C5′), 126.22 (1C, q, *J* = 32.6 Hz, C4′), 124.06 (1C, q, *J* = 269.9 Hz, CF_3), 122.87 (1C, d, *J* = 2.3 Hz), 119.55, 65.79 (2C, d, *J* = 6.1 Hz, CH_AH_B), 16.05 (2C, d, *J* = 6.4 Hz, CH_3). Anal. Calcd for $C_{18}H_{18}ClF_3NO_5P$ (451.76): C, 47.86; H, 4.02; N, 3.10. Found: C, 47.58; H, 3.78; N, 2.79.

4.1.1.19. 5-Chloro-2-[(3-chlorophenyl)carbamoyl]phenyl diethyl phosphate (1s). Yield: 77%, white solid; mp 56 °C; IR (ATR): 1673 (amide I), 1593 (*ν* CC aromatic), 1542 (amide II), 1478 (*ν* CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): *δ* 9.51 (1H, br s, NH), 7.93–7.85 (2H, m, H3, H2′), 7.58 (1H, br d, *J* = 8.0 Hz), 7.37 (1H, br s, H6), 7.32 (1H, br d, *J* = 8.6 Hz, H4), 7.27 (1H, t, *J* = 8.1 Hz, H5′), 7.11 (1H, br d, *J* = 8.2 Hz), 4.31–4.17 (4H, m, CH_AH_B), 1.36 (6H, t, *J* = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 162.31, 147.08 (1C, d, *J* = 6.9 Hz), 139.29, 137.84 (1C, d, *J* = 2.0 Hz), 134.65, 132.83, 129.96, 126.51 (1C, d, *J* = 1.5 Hz), 126.32 (1C, d, *J* = 5.5 Hz), 124. 46, 121.68 (1C, d, *J* = 2.3 Hz), 119.90, 117.82, 65.85 (2C, d, *J* = 6.0 Hz, CH_AH_B), 16.03 (2C, d, *J* = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}Cl_2NO_5P$ (418.21): C, 48.82; H, 4.34; N, 3.35. Found: C, 48.78; H, 4.07; N, 3.51.

4.1.1.20. 2-[(3-Bromophenyl)carbamoyl]-5-chlorophenyl diethyl phosphate (1t). Yield: 67%, white solid; mp 52 °C; IR (ATR): 1686 (amide I), 1591 (*ν* CC aromatic), 1539 (amide II), 1487, 1477 (*ν* CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): *δ* 9.50 (1H, br s, NH), 8.02 (1H, br s, H2′), 7.89 (1H, d, *J* = 8.4 Hz, H3), 7.64 (1H, d, *J* = 7.7 Hz), 7.37 (1H, br s, H6), 7.32 (1H, br d, *J* = 8.4 Hz, H4), 7.29–7.19 (2H, m), 4.34–4.14 (4H, m, CH_AH_B), 1.36 (6H, t, *J* = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): *δ* 162.30, 147.09 (1C, d, *J* = 7.3 Hz), 139.44, 137.86 (1C, d, *J* = 2.0 Hz), 132.83, 131.45, 130.27, 127.40, 126.51 (1C, d, *J* = 1.7 Hz), 126.29 (1C, d, *J* = 5.9 Hz), 122.72, 121.69 (1C, d, *J* = 2.3 Hz), 118.30, 65.85 (2C, d, *J* = 6.1 Hz, CH_AH_B), 16.05 (2C, d, *J* = 6.5 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}BrClNO_5P$ (462.66): C, 44.13; H, 3.92; N, 3.03. Found: C, 43.96; H, 4.08; N, 3.24.

4.1.1.21. 5-Chloro-2-[(3-fluorophenyl)carbamoyl]phenyl diethyl phosphate (1u). Yield: 62%, white solid; mp 65 °C; IR (ATR): 1672 (amide I), 1618, 1613, 1600 (*ν* CC aromatic), 1551 (amide II), 1492 (*ν* CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): *δ* 9.55 (1H, br s, NH), 7.89 (1H, br d, *J* = 8.5 Hz, H3), 7.71 (1H, br d, *J* = 10.9 Hz, H2′), 7.41–7.22 (4H, m, H4, H6, H5′, H6′), 6.83 (1H, dt, *J* = 8.5 Hz, *J* = 2.6 Hz, H4′), 4.32–4.16 (4H, m, CH_AH_B), 1.35 (6H, br t, *J* = 7.0 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 162.97 (1C, d, *J* = 243.2 Hz, C3′), 162.33, 147.08 (1C, d, *J* = 7.1 Hz), 139.65 (1C, d, *J* = 10.8 Hz), 137.81 (1C, d, *J* = 2.1 Hz), 132.81, 130.04 (1C, d, *J* = 9.3 Hz), 126.49 (1C, d, *J* = 1.3 Hz), 126.39 (1C, d, *J* = 5.8 Hz), 121.69 (1C, d, *J* = 2.4 Hz), 115.14 (1C, d, *J* = 2.9 Hz, C6′), 111.12 (1C, d, *J* = 21.2 Hz), 107.32 (1C, d, *J* = 26.4 Hz), 65.82 (2C, d, *J* = 6.1 Hz, CH_AH_B), 16.02 (2C, d, *J* = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}ClFNO_5P$ (401.75): C, 50.82; H, 4.52; N, 3.49. Found: C, 50.73; H, 4.41; N, 3.55.

4.1.1.22. 5-Chloro-2-[(4-fluorophenyl)carbamoyl]phenyl diethyl phosphate (1v). Yield: 55%, white solid; mp 93 °C; IR (ATR): 1683 (amide I), 1616, 1598 (*ν* CC aromatic), 1549 (amide II), 1511, 1485 (*ν* CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): *δ* 9.44 (1H, br s, NH), 7.89 (1H, dd, *J* = 8.4 Hz, *J* = 1.1 Hz, H3), 7.76–7.66 (2H, m, H2′, H6′), 7.36 (1H, dd, *J* = 2.0 Hz, *J* = 1.1 Hz, H6), 7.31 (1H, ddd, *J* = 8.4 Hz, *J* = 2.0 Hz, *J* = 1.0 Hz, H4), 7.09–6.99 (2H, m, H3′, H5′), 4.33–4.14 (4H, m, CH_AH_B), 1.35 (6H, td, *J* = 7.1 Hz, *J* = 1.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): *δ* 162.14, 159.38 (1C, d, *J* = 242.1 Hz, C4′), 147.09 (1C, d, *J* = 7.2 Hz), 137.65 (1C, d,

J = 2.0 Hz), 134.24 (1C, d, J = 2.9 Hz, C1′), 132.78, 126.56, 126.47 (1C, d, J = 1.6 Hz), 121.65 (1C, d, J = 2.3 Hz), 121.49 (2C, d, J = 7.8 Hz, C2′, C6′), 115.63 (2C, d, J = 22.2 Hz, C3′, C5′), 65.79 (2C, d, J = 6.2 Hz, CH_AH_B), 16.03 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}ClFNO_5P$ (401.75): C, 50.82; H, 4.52; N, 3.49. Found: C, 50.45; H, 4.32; N, 3.23.

4.1.1.23. 2-[(4-Bromophenyl)carbamoyl]-5-chlorophenyl diethyl phosphate (1w). Yield: 59%, white solid; mp 127 °C; IR (ATR): 1678 (amide I), 1601, 1589 (ν CC aromatic), 1536 (amide II), 1490 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.51 (1H, br s, NH), 7.88 (1H, d, J = 8.4 Hz, H3), 7.77–7.56 (2H, bm, H2′, H6′), 7.55–7.41 (2H, m, H3′, H5′), 7.35 (1H, br s, H6), 7.32 (1H, br d, J = 8.4 Hz, H4), 4.35–4.12 (4H, m, CH_AH_B), 1.35 (6H, t, J = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.26, 147.03 (1C, d, J = 7.3 Hz), 137.77 (1C, d, J = 1.9 Hz), 137.30, 132.82, 131.96, 126.64–126.38 (2C, m), 121.72 (1C, d, J = 2.2 Hz), 121.37, 116.99, 65.84 (2C, d, J = 6.1 Hz, CH_AH_B), 16.05 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}BrClNO_5P$ (462.66): C, 44.13; H, 3.92; N, 3.03. Found: C, 44.26; H, 4.14; N, 3.41.

4.1.1.24. 5-Chloro-2-[(4-chlorophenyl)carbamoyl]phenyl diethyl phosphate (1x). Yield: 72%, white solid; mp 118 °C; IR (ATR): 1683 (amide I), 1608, 1596 (ν CC aromatic), 1540 (amide II), 1494 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 9.52 (1H, br s, NH), 7.88 (1H, d, J = 8.4 Hz, H3), 7.74–7.68 (2H, m, H2′, H6′), 7.36 (1H, br s, H6), 7.34–7.29 (3H, m, H4, H3′, H5′), 4.30–4.17 (4H, m, CH_AH_B), 1.35 (6H, td, J = 7.1 Hz, J = 1.1 Hz, CH_3); ^{1313}C NMR ($CDCl_3$, 125 MHz): δ 162.23, 147.04 (1C, d, J = 7.1 Hz), 137.74 (1C, d, J = 2.0 Hz), 136.79, 132.79, 129.33, 129.00, 126.50 (1C, d, J = 1.5 Hz), 126.49 (1C, d, J = 6.1 Hz), 121.69 (1C, d, J = 2.3 Hz), 121.02, 65.82 (2C, d, J = 6.1 Hz, CH_AH_B), 16.03 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{18}Cl_2NO_5P$ (418.21): C, 48.82; H, 4.34; N, 3.35. Found: C, 48.52; H, 4.15; N, 3.06.

4.1.1.25. 5-Chloro-2-[(3,4-dichlorophenyl)carbamoyl]phenyl diethyl phosphate (1y). Yield: 63%, white solid; mp 106 °C; IR (ATR): 1672 (amide I), 1606, 1589 (ν CC aromatic), 1537 (amide II), 1475 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.66 (1H, br s, NH), 8.02 (1H, d, J = 2.5 Hz, H2′), 7.86 (1H, d, J = 8.3 Hz, H3), 7.57 (1H, dd, J = 8.8 Hz, J = 2.5 Hz, H6′), 7.39 (1H, d, J = 8.8 Hz, H5′), 7.37–7.29 (2H, m, H4, H6), 4.34–4.15 (4H, m, CH_AH_B), 1.36 (6H, td, J = 7.1 Hz, J = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.38, 146.99 (1C, d, J = 6.9 Hz), 137.96 (1C, d, J = 2.2 Hz), 137.72, 132.84, 132.77, 130.48, 127.50, 126.60 (1C, d, J = 1.6 Hz), 126.29 (1C, d, J = 5.5 Hz), 121.81 (1C, d, J = 2.4 Hz), 121.45, 119.05, 65.92 (2C, d, J = 6.1 Hz, CH_AH_B), 16.05 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{17}H_{17}Cl_3NO_5P$ (452.65): C, 45.11; H, 3.79; N, 3.09. Found: C, 45.18; H, 4.03; N, 3.23.

4.1.1.26. 5-Chloro-2-{[3-(trifluoromethyl)phenyl]carbamoyl}phenyl diethyl phosphate (1z). Yield: 55%, white solid; mp 62 °C; IR (ATR): 1693 (amide I), 1598, 1578 (ν CC aromatic), 1561 (amide II), 1490, 1446 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.67 (1H, s, NH), 8.07 (1H, br s, H2′), 7.93 (1H, d, J = 8.3 Hz, H4′), 7.89 (1H, d, J = 8.3 Hz, H3), 7.46 (1H, t, J = 8.00 Hz, H5′), 7.40–7.35 (2H, m, H6, H6′), 7.32 (1H, dd, J = 8.3 Hz, J = 1.4 Hz, H4), 4.31–4.18 (4H, m, CH_AH_B), 1.34 (6H, t, J = 7.0 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.48, 147.13 (1C, d, J = 7.1 Hz), 138.75, 137.96 (1C, d, J = 1.9 Hz), 132.83, 131.38 (1C, q, J = 32.2 Hz, C3′), 129.52, 126.54 (1C, d, J = 1.5 Hz), 126.22 (1C, d, J = 5.7 Hz), 123.85 (1C, q, J = 270.9 Hz, CF_3), 122.88, 121.74 (1C, d, J = 2.2 Hz), 120.91 (1C, q, J = 3.9 Hz, C2′), 116.55 (1C, q, J = 4.0 Hz, C4′), 65.87 (2C, d, J = 6.2 Hz, CH_AH_B), 15.98 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{18}H_{18}ClF_3NO_5P$ (451.76): C, 47.86; H, 4.02; N, 3.10. Found: C, 48.06; H, 4.17; N, 3.32.

4.1.1.27. 5-Chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl diethyl phosphate (1zz). Yield: 64%, white solid; mp 111 °C; IR (ATR): 1688 (amide I), 1605 (ν CC aromatic), 1541 (amide II), 1486 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.73 (1H, br s, NH), 7.96–7.80 (3H, m, H3, H3′, H5′), 7.67–7.55 (2H, m, H2′, H6′), 7.37 (1H, d, J = 1.4 Hz, H6), 7.34 (1H, br d, J = 8.3 Hz, H4), 4.34–4.14 (4H, m, CH_AH_B), 1.36 (6H, td, J = 7.1 Hz, J = 1.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 162.59, 147.06 (1C, d, J = 7.2 Hz), 141.25, 137.99 (1C, d, J = 2.1 Hz), 132.89 (1C, d, J = 1.1 Hz), 126.60 (1C, d, J = 1.6 Hz), 126.35 (1C, d, J = 1.7 Hz), 126.22 (2C, q, J = 3.8 Hz, C3′, C5′), 126.14 (1C, q, J = 32.5 Hz, C4′), 124.08 (1C, q, J = 269.9 Hz, CF_3), 121.82 (1C, d, J = 2.4 Hz), 119.52, 65.90 (2C, d, J = 6.1 Hz, CH_AH_B), 16.04 (2C, d, J = 6.4 Hz, CH_3). Anal. Calcd for $C_{18}H_{18}ClF_3NO_5P$ (451.76): C, 47.86; H, 4.02; N, 3.10. Found: C, 47.72; H, 3.69; N, 2.76.

4.2. Biological assays

4.2.1. In vitro antimycobacterial assay

The in vitro antimycobacterial activity of the synthesized compounds was determined against *Mycobacterium tuberculosis* My 331/88 ($H_{37}Rv$; dilution of strain 10^{-3}), *M. avium* My 330/88 (resistant to INH, RIF, EMB and OFX; dilution of strain 10^{-5}), *M. kansasii* My 235/80 (dilution of strain 10^{-4}) and *M. kansasii* 6509/96 (dilution of strain 10^{-4}). All of the strains were obtained from the Czech National Collection of Type Cultures (CNCTC) with the exception of *M. kansasii* 6509/96, which was clinically isolated from a patient. The antimycobacterial activity of the compounds was determined in a Šula's semisynthetic medium (SEVAC, Prague, Czech Republic) *via* the micromethod for the determination of the minimum inhibitory concentration (MIC) at 37 °C after 14 and 21 days and after 7, 14 and 21 days for *M. kansasii*.[26] The tested compounds and *para*-aminosalicylic acid were added to the medium in DMSO solutions, and INH was used as a standard in a sterile water solution. The concentrations of the tested compounds were used as follows: 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.064 and 0.032 μmol/L. The same concentrations within the range from 0.5 to 250 μmol/L were used for INH. The most active compounds were evaluated in the similar conditions and concentrations against five MDR-TB strains (dilution 10^{-3}) with different resistance patterns: 234/2005, 9449/2006, Praha 1, Praha 4 and Praha 131 (i.e., XDR-TB strain). All these strains were resistant to INH, rifamycines, and STM; in some cases additional resistance (EMB, aminoglycosides, OFX, clofazimine) was present.

4.2.2. In vitro antibacterial assay

The in vitro antibacterial activity was assayed against eight Gram-positive and Gram-negative strains: *Staphylococcus aureus* CCM 4516/08, methicillin-resistant *Staphylococcus aureus* H 5996/08 (MRSA), *Staphylococcus epidermidis* H 6966/08, *Enterococcus* sp. J 14365/08; *Escherichia coli* CCM 4517, *Klebsiella pneumoniae* D 11750/08, ESBL-positive *Klebsiella pneumoniae* J 14368/08, and *Pseudomonas aeruginosa* CCM 1961.

The microdilution broth method in Mueller–Hinton broth was used. The tested compounds were dissolved in DMSO to the final concentrations ranging from 500 to 0.49 mmol/L. Benzylpenicillin (penicillin G; PNC) was used as the comparative drug. The minimum inhibitory concentrations were assayed as 95% (IC_{95}) or higher reduction of growth compared to the control. The used method is described in Ref. 4

4.2.3. In vitro antifungal assay

The antifungal properties of all synthesized compounds were evaluated in vitro against four *Candida* strains (*Candida albicans* ATCC 44859, *Candida tropicalis* 156, *Candida krusei* E28, and *Candida glabrata* 20/I), *Trichosporon asahii* 1188 and three filamentous

fungi (*Aspergillus fumigatus* 231, *Absidia corymbifera* 272, and *Trichophyton mentagrophytes* 445). The microdilution broth method was used in RPMI 1640 with glutamine. Fluconazole (FLU) was used as the reference drug. The MICs were assayed as an 80% (IC_{80}) or higher reduction of growth in comparison to the control; for filamentous fungi, MICs are expressed as IC_{50} values. The used method is described in Ref. 27.

4.2.4. HepG2 cytotoxicity assay

The human hepatocellular liver carcinoma cell line HepG2 (p27–33) purchased from Health Protection Agency Culture Collections (ECACC, Salisbury, UK) was routinely cultured in Minimum Essentials Eagle Medium (Sigma Aldrich, Darmstadt, Germany) supplemented with 10% foetal bovine serum, 1% L-glutamine solution (Sigma Aldrich, Darmstadt, Germany) and non-essential amino acid solution (Sigma Aldrich, Darmstadt, Germany) in a humidified atmosphere containing 5% CO_2 at 37 °C. For subculturing, the cells were harvested after trypsin/EDTA (Sigma Aldrich, Darmstadt, Germany) treatment at 37 °C. The cells treated with the tested substances were used as experimental groups.

The cells were seeded in density 1×10^4 cells per well in a 96-well plate. Next day, the cells were treated with each of the tested substances dissolved in DMSO by dilution so that a final solution contained less than 1% DMSO in the medium. The most of the tested compounds were prepared at incubation concentrations 0.1–100 μM in triplicates. In addition, the compounds exerting higher toxicity were tested at the concentrations of 0.1–25 μmol/L. The following controls in triplicates were prepared for each experiment: 100% cell viability (untreated cells), 0% cell viability (cells treated by 10% DMSO), no cell control and vehiculum controls. After 24 h of incubation in a humidified atmosphere containing 5% CO_2 at 37 °C, the reagent from the kit CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) was added.[28] After 2 h of incubation at 37 °C, absorbance was recorded at 490 nm. A standard toxicological parameter IC_{50} was calculated for each of the tested substances using GraphPad Prism software 5.02 (GraphPad Software, San Diego, CA, USA).

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