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Synthesis, antimicrobial, and alkylating properties of 3-phosphonic derivatives of chromone

Dimethyl 2,6-dimethyl-4-oxo-4*H*-chromen-3-yl-phosphonate (**1a**) and dimethyl 6-methyl-2-phenyl-4-oxo-4*H*-chromen-3-yl-phosphonate (**1b**) were synthesized and reacted with primary aliphatic amines to yield title compounds **4–6**. Their antibacterial properties against Gram-positive and Gram-negative bacteria strains were tested by the MIC method. Four of seventeen tested compounds (**1d**, **3**, **4a**, and **4b**) exhibit detectable activity against *S. aureus*. Some representative examples of newly synthesized compounds were tested for their alkylating properties *in vitro* in the Preussmann test. Compounds **1a**, **1c**, **1d**, **3**, **5d**, and **6a** possess highly alkylating activity toward standard derivative 4-(4′-nitrobenzyl)pyridine (NBP).

Key Words: Chromone; Phosphonic acids; Antibacterial and alkylating activity

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Introduction

The phosphorus-containing antibiotics, especially phosphonic acids, steadily increasing in number, represent an interesting group of antimicrobial agents. Some of these compounds are produced by total chemical synthesis, but many represent products of microbial origin. Up to the seventies there were no major developments concerning antimicrobial aminophosphonic acids[1–4]. However for the last three decades numerous aminophosphonic and aminophosphinic acid derivatives have been identified as biologically active compounds [5]. Fosmidomycin [HCON(OH)-$(CH_2)_3$-PO_3H_2], one of four structurally related phosphonic acid derivatives, isolated from *Streptomyces,* is the most interesting agent among natural phosphonate antibiotics for use in therapy [6]. On the other hand, the derivatives of chromones or flavones are another group of biologically interesting compounds. They exhibit diverse biological properties including anticonvulsant [7], antimicrobiological [8], and antitumor activities [9]. Little is known about the biological activity of new chromone derivatives containing phosphorus [10]. Synthesis of such benzo-γ-pyrone derivatives containing phosphonic acid residue was previously presented by us [11] and others [12]. This paper is a continuation of our research on the derivatives of chromone modified with phosphonic acid residue (**1a** and **1b**), their chemical synthesis and reactions with primary amines, as well the studies on antimicrobial and alkylating properties of the obtained compounds.

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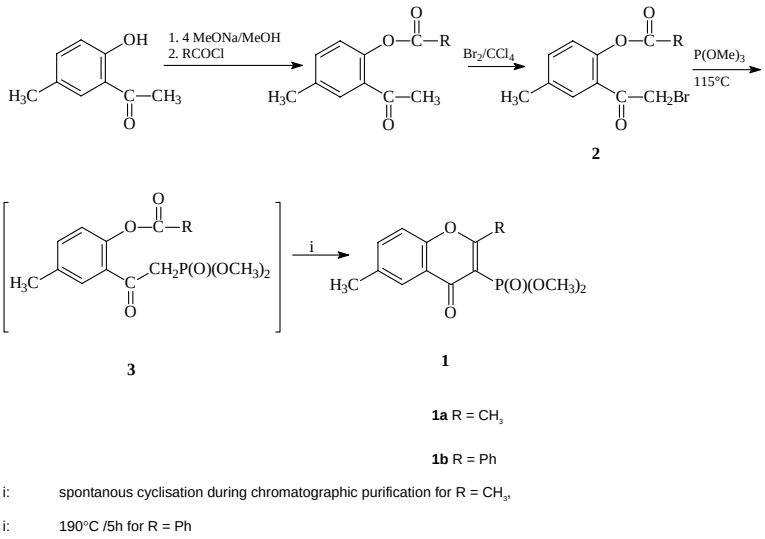
Results

Chemistry

Synthesis of dimethyl 2,6-dimethyl-4-oxo-4*H*-chromen-3-yl-phosphonate **1a** and dimethyl 6-methyl-2-phenyl-4-oxo-4*H*-chromen-3-yl-phosphonate **1b** was achieved by acylation of 2′-hydroxyacetophenone in basic conditions, subsequent bromination of keto-methyl group and heating of bromo-derivative **2** [13, 14] with trimethyl phosphite under conditions of Arbuzov reaction [15] (Scheme 1). When acetyl chloride was used as an acylating agent, and a crude



2

3

1

1a R = CH_3

1b R = Ph

i: spontanous cyclisation during chromatographic purification for R = CH_3,

i: 190°C /5h for R = Ph

Scheme 1

reaction product was purified on a silica gel column, the title compound **1a** was isolated as the only reaction product with overall yield of 29%. In the case of acylation of 2′-hydroxyacetophenone with benzyl chloride we were able to isolate by-product **3**, which underwent further cyclisation into desired product **1b** after additional heating at 190 °C for 5 h.

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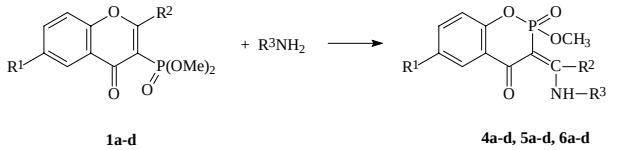


1a-d 4a-d, 5a-d, 6a-d

Compd	R^1	R^2	R^3
1a	CH_3	CH_3	–
1b	CH_3	Ph	–
1c	H	CH_3	–
1d	H	Ph	–
4a	CH_3	CH_3	CH_2Ph
4b	CH_3	Ph	CH_2Ph
4c	H	CH_3	CH_2Ph
4d	H	Ph	CH_2Ph
5a	CH_3	CH_3	CH_3
5b	CH_3	Ph	CH_3
5c	H	CH_3	CH_3
5d	H	Ph	CH_3
6a	CH_3	CH_3	$(CH_2)_2OH$
6b	CH_3	Ph	$(CH_2)_2OH$
6c	H	CH_3	$(CH_2)_2OH$
6d	H	Ph	$(CH_2)_2OH$

Scheme 2

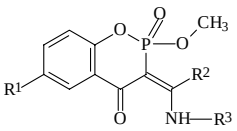
Compound **1b** was isolated with 47% yield. The structure of **1a** and **1b** was unequivocally confirmed by spectral analysis detailed in Experimental. Compounds **1a** and **1b,** as well as previously obtained derivatives **1c** and **1d** [11] , were reacted with primary aliphatic amines i.e. benzylamine, methylamine and ethanolamine, as previously described [16]. Several derivatives of 2-methoxy-3-[1-alkylamino)ethylidene or benzylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[*e*][1,2]oxaphosphinane (**4–6)** were obtained (Scheme 2). ^{1}H, ^{31}P and ^{13}C NMR, mass spectrometry, IR, and elemental analysis elucidated the structure of compounds **4**, **5**, and **6**. All spectral data were in accordance with those expected for structures **4**, **5**, and **6**. The IR spectra of all the investigated derivatives exhibited γ-pyrone C=O stretching bonds at 1580–1618 cm^{-1}. In the ^{31}P NMR spectra characteristic signals of phosphonic phosphorus atom appeared at 19.13–24.73 ppm. Reaction yields and characteristics of derivatives **4–6** are collected in Table 1.

In addition the structure of the derivatives **4b** and **4d** was confirmed by X-ray analysis (Fig. 1a and Fig. 1b, respectively). Although the oxaphosphinane rings of both derivatives are not ideally planar (the atom deviating most from weighted least-squares plane passing through O1, P2, C3, C4, C10, C9 is O1–0.242 (2) Å in **4b** and C3–0.058 (3) Å in **4d**), the C3, C4, C10, C9 fragment is perfectly planar, which indicates structural similarity to nalidixic acid. Moreover, the intramolecular hydrogen bonds N32-H32...O4 also leads to planarity of the oxaphosphinane ring. Geometry of hydrogen bond: N32-H32: 0.82 (3) Å **3b**, 0.93 (4) Å **4d**; H32...O4: 1.89 (3)Å **4b**, 1.76 (4)Å **3d**; N32...O4: 2.59 (1) Å **4b**, 2.56 (1) Å **4d**, and N32-H32...O4: 144 (2)° **4b**, 142 (4)° **4d**.

Pharmacology

In these studies we determined antibacterial activity of all the new phosphonic acid chromone derivatives, including compounds described in our previous paper [11]. Thus, compounds **1a–d**, **3**, **4a–d**, **5a–d** and **6a–d** were investigated with regard to their antibacterial activity against standard Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, and against two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, the strains of different drug resistance recommended by National Committee for Laboratory Standards (NCCLS) [17]. Minimum inhibitory concentrations (MIC) were measured using a standard method of dilution on solid support (Mueller-Hinton agar, Difco). We used nalidixic acid, an active therapeutic against Gram-negative bacteria, as a control reference. The results were collected after incubation of bacteria strain with solutions of the investigated compounds on the agar plate for 18 h at 35 °C. Subsequent dilutions in average range from 1000 to 31 μg/mL were applied for preparation of the samples. Used concentrations of nalidixic acid were much lower and ranged from 125 to 3.1 μg/mL.

Table 1. Data of the synthesized compounds.



Comp.	R^1	R^2	R^3	Mp [°C]	Yield [%]	Solvent for cryst.	Formula*	Molecular weight
4a	CH_3	CH_3	CH_2Ph	94.0–95.0	62.0	methanol	$C_{19}H_{20}NO_4P$	357.33
4b	CH_3	Ph	CH_2Ph	169.0–171.0	57.1	methanol	$C_{24}H_{22}NO_4P$	419.39
4d	H	Ph	CH_2Ph	192.0–194.0	90.0	methanol	$C_{23}H_{20}NO_4P$	405.37
5a	CH_3	CH_3	CH_3	157.0–160.0	46.3	methanol	$C_{13}H_{16}NO_4P$	281.24
5b	CH_3	Ph	CH_3	123.0–125.0	75.0	methanol	$C_{18}H_{18}NO_4P$	343.30
6a	CH_3	CH_3	$(CH_2)_20H$	144.0–146.0	69.6	methanol:ethyl ether	$C_{14}H_{18}N0_5P$	311.26
6b	CH_3	Ph	$(CH_2)_20H$	147.5–150.0	64.5	benzene	$C_{19}H_{20}N0_5P$	373.33
6d	H	Ph	$(CH_2)_20H$	176.5–178.0	57.9	acetone:ethyl ether	$C_{18}H_{18}N0_5P$	359.30

*All new compounds gave satisfactory elemental analyses (±0.4%).

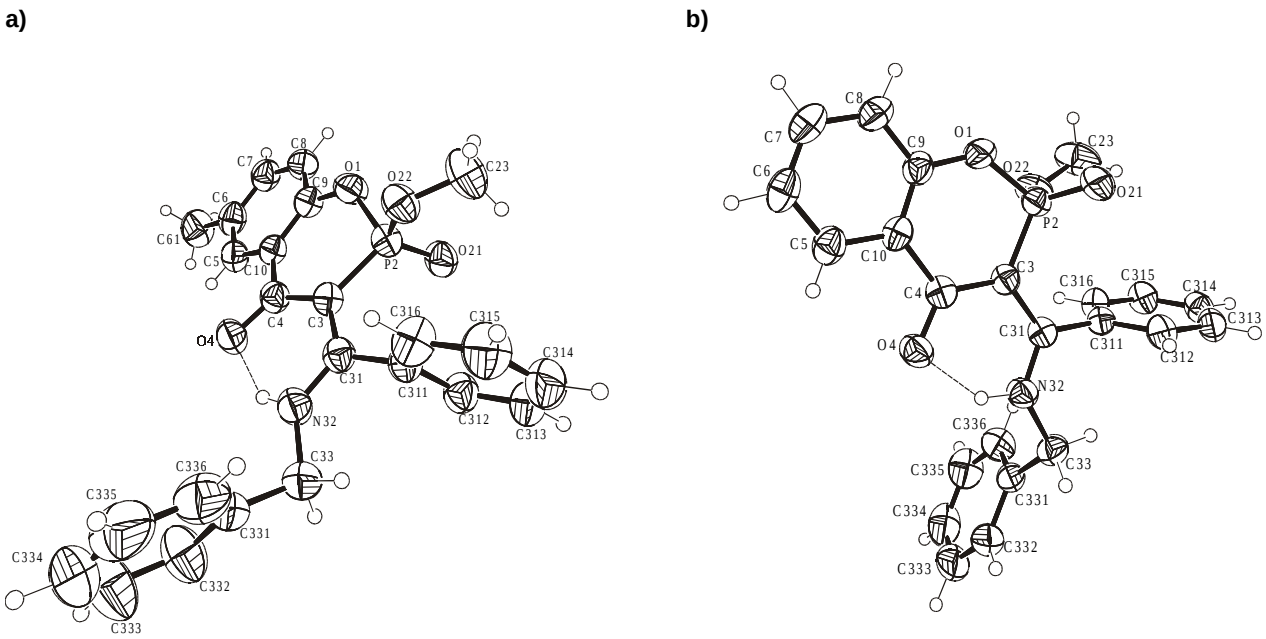


Figure 1. The molecular structure and atomic numbering scheme of compounds **4b** (a) and **4d** (b). Displacement ellipsoids are drawn at 40% probability level.

Table 2.

Cmpd	Conc. µg/mL	Minimum inhibitory concentrations (MIC), µg/mL							
		Gram-positive					Gram-negative		
		S. aureus ATCC 6538P	*S. aureus* ATCC* 29213	*S. aureus* ATCC * 25923	*E. faecalis* ATCC* 29212	*B. subtilis* ATCC 6633	*E. coli* ATCC* 35218	*E. coli* ATCC 25922	*Ps. aeruginosa ATCC* 27853
1d	700–31	500	600	600	–	–	–	–	–
3	1000–31	250	250	250	–	250	–	–	–
4a	500–31	62	62	250	> 500	125	–	–	–
4b	700–31	125	125	250	–	250	–	–	–
nalidixic acid	125–3.1	62	62	62	62	≤3.1	≤3.1	≤3.1	55

(–) Lack of inhibiting activity.
* Strains of high drug-resistance.

The obtained results of minimum inhibitory concentrations are listed in Table 2. Only very few of the investigated derivatives (**1d**, **3**, **4a** and **4b**) showed any inhibitory effect in used concentrations. The remaining investigated compounds showed no inhibitory effect against any of the microorganisms tested. The most sensitive species towards the tested compounds are two strains of *Staphylococcus aureus* (ATCC 6538P and 29213). The values of MIC for **4a** are close to those ones determined for nalidixic acid. However, none of the investigated compounds showed any inhibitory effect toward Gram-negative bacteria.

Alkylating agents belong to the first class of cytostatics used for therapy [18]. In *in vivo* conditions these agents alkylate nucleophilic centres of nucleobases and of amino acids, resulting in cross-linking of a double stranded DNA molecules, and of proteins. Such covalent cross-link of DNA prevents unwinding of nucleic acids functionally important in various biological processes, including replication and transcription [19]. One of the most known and useful methods for determination of alkylating properties of tested compounds is the *in vitro* test of Preussmann [20]. In this test 4-(4′-nitrobenzyl)pyridine (NBP) is used as alkylating target molecule. Nitrogen atom of pyridine ring of NBP is alkylated giving respective quarternary salt, which in alkaline conditions transforms into neutral coloured compound. The level of alkylation can be quantified spectrophotometrically in the range of 560 nm. Thus, compounds **1–6** of the **a**, **c**, and **d** series (Table 3) were screened for their alkylating activity toward NBP. The screening was carried out at a concentration of 0.005 mM/mL in 2-methoxyethanol. The obtained results are presented in Table 3.

Table 3. NBP test results

No	Cmpds	Absorbance [A][a]	Alkylation activity[b]
1	**1a**	0.8916	+++
2	**1c**	1.3118	+++
3	**1d**	0.6819	+++
4	**3**	1.2191	+++
5	**4a**	0.1251	++
6	**4c**	0.2263	++
7	**4d**	0.3476	++
8	**5a**	0.2588	++
8	**5c**	0.2083	++
9	**5d**	0.9821	+++
10	**6a**	0.7487	+++
11	**6c**	0.1213	++
12	**6d**	0.0893	+
13	**Cyclo-phosphamide**[b]	0.1200	++

[a] means from 3 determinations, [b] according to ref.[20] : (–) A < 0.05, (+) A = 0.05–0.1, (++) A = 0.1– 0.5, (+++) A > 0.5

Discussion

Several new derivatives of chromone modified with phosphonic acid residue were chemically synthesized by the three-step procedure outlined in Scheme 1. We obtained 3-phosphonic derivatives of chromone substituted with various alkyl or aryl substituents at position 2 and/or 6 of the benzo-γ-pyrone ring. These derivatives were further functionalised by their reaction with aliphatic amines (Scheme 2). Nucleophilic addition of nitrogen atom on C-2 of a pyrone ring resulted in formation of an open intermediate, which after subsequent oxy-anion nucleophilic attack on phosphorus atom resulted in oxaphosphinane ring formation. The detailed mechanism of this reaction was discussed in our previous paper [16]. All the newly synthesized compounds (**1a, 1b, 3, 4a, 4b, 4d, 5a, 5b, 6a, 6b**, and **6d**) as well as previously described ones (**1c, 1d, 4c, 5c, 5d**, and **6c**) [11, 16] were tested for their antibacterial activity toward standard Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, and against two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, the strains of different drug resistance. Most of the investigated derivatives showed no inhibitory effect in the concentrations used. Only compounds **1d**, **3**, **4a**, and **4b** show selective antibacterial activity against Gram-positive bacteria of different drug-resistance strains. The values of MIC (Table 2) are in the range of 600 to 62 μg/mL and among the lowest values close to those determined for nalidixic acid. We used nalidixic acid as a control reference

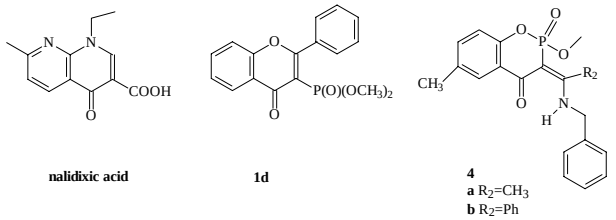


Figure 2.

due its structural proximity to the investigated phosphonic acid derivatives (Figure 2). Nowadays therapeutic application of nalidixic acid is remarkably limited due to the fast development of drug-resistant bacterial strains. This compound, however, is still used as a common reference for comparison of antibacterial activity of newly investigated compounds [21, 22, 23]. Antibacterial activity of nalidixic acid is coupled with its high affinity to double stranded DNA. The presence of C-4 carbonyl group and C-3 carboxyl group, both positioned in one plane, in nalidixic acid structure seems to be responsible for its biological activity in respect of the nucleic acid biomolecule [24]. In our studies compound **1d** is a close structural analogue of nalidixic acid in which phopsphonic acid residue is introduced into the chinolone system instead of carboxylic acid group. However, the two other compounds, **4a** and **4b**, posses the *cis*-enone structural motif with enamine at its C-terminus. X-Ray crystallographic data of compound **4b** support an almost planar structure of the oxaphosphinane ring, indicating a close structural similarity between the nalidixic acid and derivatives **4a** and **4b**.

Alkylating properties of compounds **1–6** of the **a**, **c**, and **d** series (Table 3) were determined by the *in vitro* Preussmann test [20]. The level of alkylation was quantified spectrofotometrically in the range of 560 nm. Most of the tested compounds possess very high (+++) and high (++) alkylation activity toward NBP. For comparison the activity of cyclophosphamide, the well know DNA alkylating agent, used in the clinical treatment of some kinds of cancer, is shown [20, 25].

In summary, we present here the chemical synthesis, and spectral characteristics of new chromone analogues possessing an aminophosphonic acid residue. Biological activity of the obtained compounds was tested in two aspects. As determined, the tested derivatives are very efficient alkylating agants; however, they do not exhibit any significant antibacterial activity. Further screenings for cytotoxicity of compounds **1**–**6** in *in vivo* systems are in progress.

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Experimental

The melting points were determined using an Electrothermal 1A9100 apparatus and they are uncorrected. The IR spectra were recorded on a Pye-Unicam 200G Spectrophotometer in KBr. The ^{1}H NMR spectra were registered at 300 MHz on a Varian Mercury spectrometer. ^{31}P NMR spectra were recorded on a Varian 75 MHz spectrometer. Positive chemical shift values are assigned to compounds absorbing downfield of phosphoric acid. The MS data were obtained on a LKB 2091 mass spectrometer (70 eV ionisation energy). Satisfactory elemental analyses (±0.4% of the calculated values) were obtained for the new compounds. The Microanalytical Laboratory

of the Institute of Chemistry performed elemental analyses using a Perkin Elmer PE 2400 CHNS analyser.

Dimethyl 2-methyl-4-oxo-4*H*-chromen-3-yl-phosphonate (**1c**), dimethyl 2-phenyl-4-oxo-4*H*-chromen-3-yl-phosphonate (**1d**), 2-methoxy-3-[1-(alkylamino)ethylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[*e*][1,2]oxaphosphinane **3c, 4c, 5c**, and 2-methoxy-3-[1-(methylamino)benzylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[*e*][1,2]oxaphosphinane **4d** were prepared as described [11, 16]. All solvents were purified using standard methods.

*Dimethyl 2,6-dimethyl-4-oxo-4H-chromen-3-yl-phosphonate (***1a***)*

To 10 mmol of 2′-bromo-2-acyloxy-5-methylacetophenone melted in a flask, 12 mmol of trimethyl phosphite were added dropwise at 110–115 °C. After 30 min heating the excess of phosphite was removed by distillation and the resulting yellow oil was applied on a silica gel column. The column was eluted with a mixture of solvents chloroform: acetone = 5:1. The crude product was crystallised by addition of a little methanol to yield 0.96 g of pure **1a** (29.0%), mp 73.5–75.0 °C. R_f = 0.45 in ethyl acetate:acetone 5:1.

IR (KBr): ν (cm^{-1}) = 1650 (C=O), 1237 (P=O), 1026 (C-O-C), **^{1}H NMR** ($CDCl_3$): δ (ppm) = 2.44 (s, 3H, CH_3), 2.86 (s, 3H, CH_3), 3.18 (d, 6H, OCH_3, $^3J_{PH}$=11.7 Hz), 7.27–7.93 (m, 3H, aromat.); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 21.04 (s, CH_3), 21.37 (s, CH_3), 53.07 (d, OCH_3, $^2J_{PC}$ = 5.73 Hz), 109.46 (d, C=*C*-P, $^1J_{PC}$ =191.2 Hz), 176.25 (C=O, $^2J_{PC}$ = 25.19 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm) = 18.191; **MS** (70 eV): *m/z* (%) = 282 (100) [M$^+$], 267 (23), 236 (11), 188 (27.56), 174 (52), 158 (15.85), 134 (24.0), 78 (6.03). Anal. (C, H, N) $C_{13}H_{15}O_5P$ (282.22).

*Dimethyl 5-methyl-2-oxo-phenylethyl-phosphonate (***3***)*

To the 10 mmol of 2′-bromo-5-methyl-2-benzyloxyacethophenone melted in a flask, 1.5 g (12 mmol) of trimethyl phosphite were added dropwise at 110–120 °C. After about 30 minutes of heating the excess of phosphite was distilled off. The obtained yellow oil was purified by column chromatography with chloroform: acetone 5:1, to yield 1.99 g of compound **3** (55%, mp 126.5–128.7 °C).

IR (KBr): ν (cm^{-1}) = 1730, 1677 (C=O), 1274 (P=O), 1060 (C-O-C); **^{1}H NMR** ($CDCl_3$): δ (ppm) = 2.43 (s, 3H, CH_3), 3.59 (d, 2H, CH_2, $^2J_{PH}$ = 22.2 Hz), 3.7 (d, 6H, O-CH_3, $^3J_{PH}$ = 11.31 Hz), 7.13–8.21 (m, 8H, aromat.); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 21.14(CH_3), 40.47 (d, CH_2, $^1J_{PC}$ =131.19 Hz), 53.26 (d, OCH_3, $^2J_{PC}$ = 6.59 Hz), 165.10 (s, C=O), 191.44 (d, C=O, $^2J_{PC}$ = 6.87 Hz), **^{31}P NMR** ($CDCl_3$): δ (ppm) = 23.54, **MS** (70eV): *m/z*(%) = 362 (20.3) [M$^+$], 105 (100), 77 (28.67). Anal. (C, H, N) $C_{18}H_{19}O_6P$ (362.30).

*Dimethyl 6-methyl-2-phenyl-4-oxo-4H-chromen-3-yl-phosphonate (***1b***)*

Compound **3** (6 mmol) was heated in an oil bath at 190–200 °C for 5 h. The obtained dense brownish oil was purified on a silica gel column with chloroform: acetone 9:1. The product was crystallised by addition of a small quantity of methanol. 0.98g of **1b** (47.4% yield) was obtained (mp 143.5–145 °C. R_f = 0.46 ethyl acetate:acetone 9 : 1).

IR (KBr): ν (cm^{-1}) = 1164 (C=O), 1618 (C=C), 1242 (P=O), 1027 (C-O-C); **^{1}H NMR** ($CDCl_3$): δ (ppm) = 2.47 (s, 3H, CH_3), 3.64 (d, 2H, OCH_3, $^3J_{PH}$ = 11.5 Hz), 7.26–8.01 (m, 8H, aromat.); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 20.97 (s, CH_3), 53.27 (d, OCH_3, $^2J_{PC}$ = 5.76 Hz), 100.76 (d, C=*C*-P, $^1J_{PC}$ = 196.2 Hz), 175.25 (C=O, $^2J_{PC}$ = 24.19 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm) = 16.61; **MS** (70 eV): *m/z* (%) = 344 (32.72) [M$^+$], 329 (37.73), 249 (100), 134 (35.43), 106 (14.90), 78 (15.30). Anal. (C, H, N) $C_{18}H_{17}O_5P$ (344.29).

*General procedure for synthesis of 2-methoxy-3-[1-(alkylamino)ethylidene or benzylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[e][1,2]oxaphosphinane (***4–6***)*

To the solution of respective derivative of chromone (**1a**, **1b**, **1c** or **1d)** (10 mmol), in methanol (5 mL) a solution of benzylamine, methylamine or ethanolamine (10 mmol) in methanol (0.5 mL) were added. The mixture was left overnight at room temperature, and then cooled to –10 °C. The precipitated crude solid was filtered off, dried, and crystallised. Colourless products **4–6** were obtained in 57–95% yields. The properties of the compounds **4–6** are listed in Table 1.

*2-Methoxy-6-methyl-3-[1-(benzylamine)ethylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[e][1,2]oxaphosphinane (***4a***)*

IR (KBr): ν (cm^{-1}) = 3432 (NH), 1579 (C=O), 1265 (P=O), 1014 (COC); **^{1}H NMR** ($CDCl_3$): δ (ppm) = 2,35 (s, 3H, CH_3), 2.58 (s, 3H, CH_3), 3.73 (d, 6H, OCH_3, $^3J_{PH}$ = 11.7 Hz), 4.65 (d, 2H, CH_2, *J* = 5.95), 6.92–7.83 (m, 8H, aromat.), 13.71 (b_{road}, 1H, NH); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 18.57 (d, C=C-*C*H$_3$, $^3J_{PC}$ = 3.15 Hz), 20.97 (CH_3), 48.00 (CH_2), 52.80 (d, OCH_3, $^2J_{PC}$ = 7.16 Hz), 91.32(d, *C*=C-CH_3, $^1J_{PC}$ = 196.10 Hz), 172.78 (d, C=*C*-CH_3, $^2J_{PC}$ = 18.61 Hz), 182.85 (d, *C*=O, $^2J_{PC}$ = 14.87 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm) = 21.85; **MS** (70 eV): *m/z* (%) 357 (100%) [M$^+$], 340 (16.97), 266 (30.70), 131 (29.82), 106 (29.65), 91 (67.68).

*2-Methoxy-6-methyl-3-[1-(benzylamine)benzylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[e][1,2]oxaphosphinane (***4b***)*

IR (KBr): ν (cm^{-1}) = 3417 (NH), 1618 (C=O), 1240 (P=O), 1053 (COC); **^{1}H NMR** ($CDCl_3$): δ (ppm) = 2.36 (s, 3H, CH_3), 3.32 (d, 6H, CH_3, $^3J_{PH}$ = 11.7 Hz), 4.29 (d, 2H, CH_2, *J* = 6.15 Hz), 6.96–7.85 (m, 13H, aromat.), 13.64 (s, 1H, NH); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 20.98 (s, CH_3), 49.04 (CH_2), 52.69 (d, OCH_3, $^2J_{PC}$ = 6.87 Hz), 93.84 (d, *C*=C-CH_3, $^1J_{PC}$ = 197.82 Hz), 172.86 (d,C=*C*-CH_3, $^2J_{PC}$ = 18.04 Hz), 183.5(d, C=O, $^2J_{PC}$ = 5.17 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm) = 19.24; **MS** (70 eV): *m/z* (%) = 419 (100%) [M$^+$], 402 (10.32), 313 (12.34), 193 (53.18), 106 (17.23), 91 (34.15).

*2-Methoxy-3-[1-(benzylamine)benzylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[e][1,2]-oxaphosphinane (***4d***)*

IR (KBr): ν (cm^{-1}) = 3414.0 (NH), 1590 (C=O), 1261 (P=O); **^{1}H NMR** ($CDCl_3$): δ (ppm) = 3.32 (d, 3H, OCH_3, $^3J_{PH}$ = 11.9 Hz), 4.30 (d, 2H, CH_2, *J* = 6.15 Hz), 7.06–8.08 (m, 14H, aromat.), 13.65 (s_{broad}, 1H, NH); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 49.02 (CH_2), 52.75 (d, $^2J_{PC}$ = 7.16 Hz), 93.78 (d, $^2J_{PH}$ =196.64 Hz), 118.94 (d, *J* = 8.90), 172.93 (d, *J* = 18.03 Hz), 183.26 (d, *J* = 15.07 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm) = 19.13 Hz; **MS** (70 eV): *m/z* (%) = 405 (100) [M$^+$], 388 (9.60), 299 (10.68), 193 (52.57), 106 (18.96), 91 (38.74).

*2-Methoxy-6-methyl-3-[1-(methylamine)ethylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[e][1,2]oxaphosphinane (***5a***)*

IR (KBr): ν (cm^{-1}) = 3433 (NH), 1590 (C=O), 1257 (P=O), 1010 (COC); **^{1}H NMR** ($CDCl_3$): δ (ppm) = 2.36 (s, 3H, CH_3), 2.54 (d, 3H, CH_3, $^4J_{PH}$ = 0.99 Hz), 3.14 (d, 3H, NCH_3, *J* = 5.16 Hz), 3.73 (d, 3H, O-CH_3, $^3J_{PH}$ = 11.7 Hz), 6.99–7.82 (m, 3H, aromat.), 13.29 (s_{broad}, 1H, NH); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 18.21 (d, C=C-CH_3, $^3J_{PC}$ = 2.86 Hz), 21.00 (s, *C*H$_3$), 30.23 (d, N*C*H$_3$, $^4J_{PC}$ = 1.15 Hz), 52.79 (d, O-CH_3, $^2J_{PC}$ = 7.16 Hz), 91.00 (d, -*C*=C, $^1J_{PC}$ = 196.39 Hz), 182.50 (d, C=O, $^2J_{PC}$ = 14.89 Hz), 173.50 (d, C=*C*-CH_3, $^2J_{PC}$ = 18.61 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm) =

21.97 Hz. **MS** (70 eV): *m/z* (%) = 281 (100%) [M^+], 264 (40.55), 237 (11.49), 234 (7.87), 134 (12.0), 56 (23.49).

2-Methoxy-6-methyl-3-[1-(methylamine)benzylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[e][1,2]oxaphosphinane (**5b**)

IR (KBr): ν (cm^{-1}) = 3433 (NH), 1589 (C=O), 1257 (P=O), 1010 (COC); ^{1}H NMR ($CDCl_3$): δ (ppm) = 2.47 (s, 3H, CH_3), 2.82 (d, 3H, NCH_3, J = 5.16 Hz), 3.64 (d, 3H, OCH_3, $^3J_{PH}$ = 11.5 Hz), 6.95–8.00 (m, 8H, aromat.), 13.22 (s_{broad}, 1H, NH); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 21.00 (s, *C*H_3), 30.23 (d, N-*C*H_3, $^4J_{PC}$ = 1.15 Hz), 53.39 (d, O-CH_3, $^2J_{PC}$ = 7.15 Hz), 96.70 (d, *C*=C, $^1J_{PC}$ = 195.37 Hz), 184.50 (d, C=O, $^2J_{PC}$ = 15.81 Hz), 172.32 (d, C=*C*-CH_3, $^2J_{PC}$ = 19.53 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm) = 19.60 Hz; **MS** (70 eV): *m/z* (%) = 343 (29.35) [M^+], 326 (100), 282 (54.23), 234 (13.78), 77 (18.96).

2-Methoxy-6-methyl-3-[1-(ethanolamine)ethylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[e][1,2]oxaphosphinane (**6a**)

IR (KBr): ν (cm^{-1}) = 3371 (NH), 1594 (C=O), 1257 (P=O), 1013 (CO-C); **^{1}H NMR** ($CDCl_3$): δ (ppm) = 2.34 (s, 3H, CH_3), 2.56 (d, 3H, CH_3, $^4J_{PH}$ = 0.99 Hz), 2.84(s_{broad}, 1H, OH), 3.67 (t, 2H, CH_2, J_{HH} = 3.17), 3.71 (d, 3H, OCH_3, $^3J_{PH}$ = 11.9 Hz), 3.91(t, 2H, CH_2, J_{HH} = 5.35), 7.00–7.82 (m, 3H, aromat.), 13.54 (s_{broad}, 1H, NH); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 18.61 (d, C=C-*C*H_3, $^3J_{PC}$ = 3.15 Hz), 20.98 (s, *C*H_3), 46.26 (d, CH_2), $^4J_{PC}$ = 1.15 Hz), 52.90 (d, OCH_3, $^2J_{PC}$ = 7.16 Hz), 91.00 (d, -*C*=C, $^1J_{PC}$ = 196.39 Hz), 182.50 (d, C=O, $^2J_{PC}$ = 14.89 Hz), 173.50 (d, C=*C*CH_3, $^2J_{PC}$ = 18.61 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm) = 22.04 Hz; **MS** (70 eV): *m/z* (%) = 311 (25.76) [M^+], 280 (42.91), 268 (100), 251 (99.37), 227 (8.99), 145 (9.16).

2-Methoxy-6-methyl-3-[1-(ethanolamine)benzylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[e][1,2]oxaphosphinane (**6b**)

IR (KBr): ν (cm^{-1}) = 3311 (NH, OH), 1617 (C=O), 1230 (P=O), 1029 (COC); **^{1}H NMR** ($CDCl_3$): δ (ppm) = 1,26 (s_{broad}, 1H, OH), 2,37 (s, 3H, CH_3), 3.27 (d, 2H, CH_3), 3.33 (d, 6H, OCH_3, $^3J_{PH}$=11.7 Hz), 3.74 (t, 2H, CH_2, J = 5.35), 6.96–8.11 (m, 8H, aromat.), 13.46 (s_{broad}, 1H, NH); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 21.03 (CH_3), 47.34 (CH_2), 52.75 (d, O*C*H_3, $^2J_{PC}$ = 7.16), 49.04 (d, *C*-O-C, ^2J=4.87 Hz), 61.16 (CH_2), 93.44 (d, *C*=C-Ph, $^1J_{PC}$ = 198.11 Hz), 173.14 (d, C=*C*-Ph, $^2J_{PC}$ = 18.32 Hz), 183.22 (d, C=O, $^2J_{PC}$ = 15.46 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm) = 24.73; **MS** (70 eV): *m/z* (%): 373 (20.69) [M^+], 342 (55.35), 330 (100), 313 (17.93), 227 (10.4), 179 (11.17), 104 (7.49).

2-Methoxy-6-methyl-3-[1-(ethanolamine)benzylidene]-2,3-dihydro-2,4-dioxo-2λ^5-benzo[e][1,2]oxaphosphinane (**6d**)

IR (KBr): ν (cm^{-1}) = 3337.0 (NH, OH), 1583 (C=O), 1220 (P=O), 1056 (C-O-C); **^{1}H NMR** ($CDCl_3$): δ (ppm) = 2.43 (s_{broad}, 1H, OH), 3.25 (t, 2H, CH_2, J = 5.36), 3.32 (d, 3H, OCH_3, $^3J_{PH}$ = 11.9 Hz), 3.71 (t, 2H, CH_2, J = 5.36), 7.05–8.24 (m, 9H, aromat.), 13.45 (s, 1H, NH); **^{13}C NMR** ($CDCl_3$): δ (ppm) = 47.39 (s, *C*H_2-CH_2-OH), 52.82 (d, OCH_3, $^2J_{PC}$= 6.87 Hz), 61.06 (s, CH_2-*C*H_2OH), 93.34 (d, C=*C*Ph, J_{PC} = 198.1 Hz), 151.04 (d, $^2J_{PC}$ = 4.88 Hz), 173.18 (d, C=*C*-Ph, $^3J_{PC}$ = 18.32 Hz), 182.90 (d, C=O, $^2J_{PC}$ = 15.17 Hz); **^{31}P NMR** ($CDCl_3$): δ (ppm): 19.42; **MS** (70 eV): *m/z* (%) = 359 (17.26) [M^+], 315 (78.40), 283 (13.51), 235 (100), 120 (28.40), 92 (25.78), 77 (38.48).

X-ray crystallographic details for **4b** *compound* [29]*, Fig. 1a*

$C_{24}H_{22}NO_4P$, *Mr* = 419.39, triclinic, space group P-1, *a* = 10.645(1) *b* = 11.520(1) *c* = 9.643(1)Å α = 110.91(1) β = 97.39(1) γ = 98.58(1)°, *V* = 1070.9(1) Å^3 , *Z* = 2, *Dx* = 1.301 Mg m^{-3}, *F*(000) = 440, *T* = 293 K μ (CuKα) = 1.390 mm^{-1}, colourless crystal of dimensions 0.2 × 0.3 × 0.4 mm, cell dimension from 18 reflections in the range θ = 39.85–39.97°. The intensities were collected on a Rigaku AFC5S diffractometer using graphite-monochromated CuKα radiation, λ = 1.54178Å, ω scans. The intensities were corrected for the absorption [23] effect with T_{min} = 0.5664 and T_{max} = 0.7892 and the Lorentz and polarization efect. Intensities of 3 standard reflections checked every 150 reflections: no decay, θ range 4.2–72.6°, 4277 measured reflections of which 4052 were unique (R_{int}= 0.022) The structure was solved by direct methods using SHELXS86[27], which revealed the positions of non-H atoms. All H-atoms were located in a difference Fourier map. The structure was refined on F^2 by full-matrix least-squares methods using SHELX97[28] non-H atoms refined anisotropically, H-atoms fixed in calculated positions excluding H32. The refinement was carried out on 304 parameters using 2840 observed reflections with $I>2\sigma$ (I) gave $R1$ = 0.048 $wR2$ = 0.1465 (w =1/[$\sigma^2(F_o^2)$+(0.0876P)^2], where P = (F_o^2+2F_c^2)/3, S = 1.018, max and min residual electron density 0.280, –0.289 e Å^{-3}

X-ray crystallographic details for **4d** *compound* [29]*, Fig. 1b*

$C_{23}H_{20}NO_4P$, *Mr* = 405.39, triclinic, space group P-1, *a* = 9.994(5) *b* = 11.682(3) *c* = 9.085(1)Å, α = 95.24(1) β = 101.90(2) γ = 70.20(3) °, *V* = 976.3(6) Å^3, *Z* = 2, *Dx* = 1.379 Mg m^{-3}, *F*(000) = 424, *T* = 293 K, μ (CuKα) = 1.506 mm^{-1}, colourless crystal of dimensions 0.5 × 0.2 × 0.1mm, AFC5S Rigaku four-circle diffractometer, graphite-monochromated CuKα radiation, λ = 1.54178Å, ω scans, cell constants from 12 reflections in the range θ = 38.36–39.81°, intensities of 3 standard reflections checked every 150 reflections, no decay, θ range 4.79–72.63°, 3924 measured reflections of which 3691 were unique (R_{int}= 0.013). The intensities were corrected for absorption [22] efect with T_{min} = 0.5382 and T_{max} = 0.8705. Structure solution by direct methods using SHELXS86[23], and refined 296 parameters on F^2 by full-matrix least-square methods using SHELX97[20], non-H atoms refined anisotropically, H-atoms fixed in calculated positions and refined using a riding model. Final $R1$ = 0.0501 and $wR2$ = 0.1293 (w = 1/[$\sigma^2(F_o^2)$ + (0.0731 P)^2], where P = (F_o^2 + 2F_c^2)/3, S = 0.786, max and min residual electron density 0.47, –0.408 e Å^{-3}

Pharmacology

Antibacterial activity

The new synthesised compounds were tested. Minimal inhibitory concentrations (MIC) were measured using standard method of dilution on solid support (Mueller-Hinton agar, Difco). Bacterial suspension (10^4 CFU/mL) was spotted on the support with Steer's replicator. Simultaneously, MIC for nalidixic acid was measured. MHA plates with bacterial suspension alone were used as a control. The plates were analysed after 18 h incubation at 35 °C. DMSO solutions of all analysed compounds were prepared with the concentration of 1000 to 31 μg/mL.

Alkylating properties (NBP test)

The tested compounds were dissolved in 2-methoxyethanol (1 mL, 0.005 mmol) and NBP (1 mL, 5% 2130-methoxyethanol solution) was added. The samples were heated at 100 ± 0.5 °C for 1 h and cooled quickly to temp. 20 °C. To the samples 2-methoxyethanol (2.5 mL) and piperidine (0.5 mL) were added

to give a total volume of 5 mL. The final concentration was 1.0×10^{-3} mol/L. After 90 s the absorbance was measured at λ = 560 nm in glass cells (1 cm), in the presence of 2-methoxyethanol (Table 3)

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[29] Crystallographic data for the structure of **4b** and **4d** have been deposited with the Cambridge Crystallographic Data Base as deposition No CCDC 161016 for **4b** and 161017 for **4d** Copies of the data can be obtained free of charge on application to the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44-1223-336033; e-mail:deposit@ccdc.cam.ac.uk).