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Original article

In vivo antitumor, in vitro antibacterial activity and alkylating properties of phosphorohydrazine derivatives of coumarin and chromone

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Abstract

The aim of this research was to examine chemical and biological properties of the products (**4a–c**/**5a–c**, **8b–c**, **9a–b**) of the reaction of methyl chromone-3-carboxylate (**2**), 3-formyl-4-hydroxycoumarin (**3**), 3-formylchromone (**6**) and chromone 3-carbonyl chloride (**7**) with phosphorus hydrazides (**1a–c**). For structure and keto-enol tautomerism analyses ^{1}H, ^{13}C, ^{31}P NMR spectroscopy was used. The ring transformation species (**4a–c**/**5a–c**) containing the coumarin ring (**5a–c**) were predominant in the solution. The chromone series **8b–c** and **9a–b** was obtained in reaction of phosphorus hydrazides (**1a–c**) with 3-formylchromone (**6**) and chromone-3-carbonyl chloride (**7**). Alkylating activity of phosphorohydrazides of coumarin and chromone was determined with in vitro Preussmann test (NBP test). Some of the compounds were examined towards antitumor and antibacterial activity. Compounds **4b–c**/**5b–c** and **9a** demonstrated in vitro antitumor activity against P388 leukemia. Antineoplastic activity of the compounds **4b**/**5b** and **9a** combined with methotrexate was showed using L1210 murine leukemia.

Keywords: Phosphorohydrazine; Coumarin; Chromone; Tautomerism; Antitumor activity; Alkylating properties; Antibacterial activity

1. Introduction

The structure of 4-oxo-4*H*-1-benzopyrane (chromone) and 2-oxo-2*H*-1-benzopyrane (coumarin) are present in natural and synthetic compounds possessing biological activity [1]. Chromone and coumarin derivatives are of great interest because of their antimicrobial [2,3], antitumor [4,5] and antiviral [6,7] activity. Alkylating properties of benzopyranes have been widely described in many studies [8–10]. Moreover, chemistry of phosphorohydrazones and their derivatives has become a subject of great interest in the recent years. Especially coordination chemistry of the compounds has been widely studied [11–14]. Complexes of phosphorohydrazones with transition metals show significant biological activity. In our study we used novel derivatives of phosphorus hydrazides as examples of coumarin and chromone-like compounds.

Obtained compounds were investigated towards their antineoplastic activity [15]. Palladium (II) and platinum (II) complexes of the compounds were tested towards antibacterial activity (unpublished results). We intended to continue the earlier studies of the chromone structure and its phosphorohydrazide derivatives [15–23]. In previous work from our laboratory the novel potential anticancer drug *cis*-bis (3-aminoflavone) dichloroplatinum(II) (*cis*-Pt(II) complex of 3-aminoflavone) has been reported [24]. The observation that this compound prolong the survival fine of mice with lymphoid leukemia L1210 [25] prompted a study of the genotoxicity [26], the genotoxicity in A 549 cells [27], the genotoxicity in lymphocytes [28], induction of apoptosis and necrosis in lymphocytes and induction of apoptosis and necrosis in A 549 cells [29] by the *cis*-bis(3-aminoflavone)dichloroplatinum (II) in comparison with cisplatin.

Here we present a detailed investigation of seven compounds **4a–c**/**5a–c**, **6a**, **8b–c** and **9a–b** using ^{1}H, ^{13}C, ^{31}P NMR spectroscopy. The crystallographic results clearly indi-

Abbreviations: ALD, approximate lethal dose; DMSO, dimethyl sulfoxide; ILS, increase of the lifespan; MIC, minimum inhibitory concentrations; MST, median survival time; NBP 4-(4′-nitrobenzyl)pyridine; S.D., standard deviation; TMS, tetramethylsilane.

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cate three different structures: 4-hydroxycoumarin (**5a**, **c**) [21, 23], benzopyran-2,4-dione (**4b**) [18] and chromone (**8b**, **c**; **9a**, **b**) [18,21–23]. We reported the keto-enol tautomerism of **4a–c**/**5a–c** in the solution that was not reported in literature. In the previous preliminary study we reported antitumor activity of this phosphorohydrazides against leukemia L1210 [15]. In current work we present their in vitro alkylating and antibacterial activity as well as in vivo cytostatic activity. Antineoplastic activity against P388 (**4b–c**/**5b–c**, **9a**) and L1210 leukemia of **4b**/**5b**, **9a** combined with methotrexate was examined.

2. Chemistry

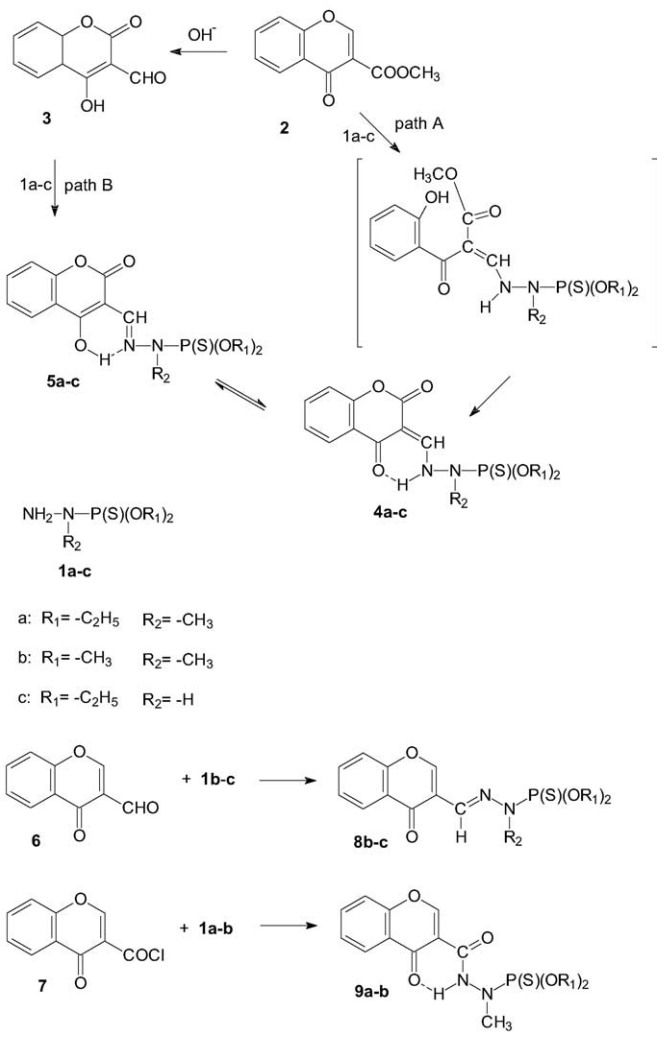
2.1. Spectroscopic analysis

The general procedures for preparation of **4a–c**/**5a–c** in the reaction of methyl 4-oxo-4H-1-benzopyran-3-carboxylate (**2**) with phosphorohydrazides (**1a–c**) were described in our previous reports [18,19]. Here the compounds **4a–c**/**5a–c** were prepared starting from 4-hydroxy-2-oxo-2H-1-benzopyran-3-carboxaldehyde (**3**) (Scheme 1). It has been found that in reaction of chromone-3-carboxylic acid esters or 3-formyl-4-hydroxycoumarin with hydrazine and its derivatives there were obtained products containing the chromone [30] and coumarin [31] structure. The formation of both structure resulted from the transformation of the chromone ring to coumarin under the influence of bases [32,33] or other nucleophilic reagents [34–36]. In our investigations the reaction of methyl chromone-3-carboxylate with phosphorus hydrazine derivatives led to obtain new products with the structure of chromon-2,4-dione [18]. The crystallographic results clearly indicate two different tautomeric forms for products with **1a–c**. Their crystal structures were assigned as **4b** (ketoenamine form) [18] and **5a**, **c** (iminoenol form) [21,23].

In this study, due to application of ^{1}H, ^{13}C, ^{31}P NMR high resolution spectroscopy it has been demonstrated that these compounds exist in solutions, irrespectively of their structures in solid state, in both tautomeric forms. Although we used various separation methods (crystallization, column chromatography) it was not possible to identify pure tautomers. Spectroscopic study was performed in solid state (^{31}P NMR) and in two solvent with different polarity (^{31}P, ^{1}H, ^{13}C NMR). Spectral data for all compounds are listed in Section 5 and the ^{31}P NMR spectra of **4a–c**/**5a–c** are shown in Fig. 1. In the ^{31}P NMR in solid only one signal appeared while after dissolving of these compounds two signals of phosphorus have been observed.

In the $CDCl_3$ solution of **5a–c**/**4a–c** the ratio of two signals was about 8:1 (**a**), 9:1 (**b**) and 5:1 (**c**), respectively, and in DMSO-d_6 solution in underwent significant changes in the case of (**a**) 4:1, (**b**) 3:1 and (**c**) 3.5:1 (Fig. 1). Analogous solvent effect we observed in ^{1}H NMR spectra. All these spectral data in $CDCl_3$ and DMSO-d_6 revealed the predominant signals for iminoenol (**5a–c**) and minor for ketoenamine forms (**4a–c**). In ^{1}H NMR spectra ($CDCl_3$) of **4a–c**/**5a–c** more intense signals of the OH derived protons exchangeable with D_2O are singlets

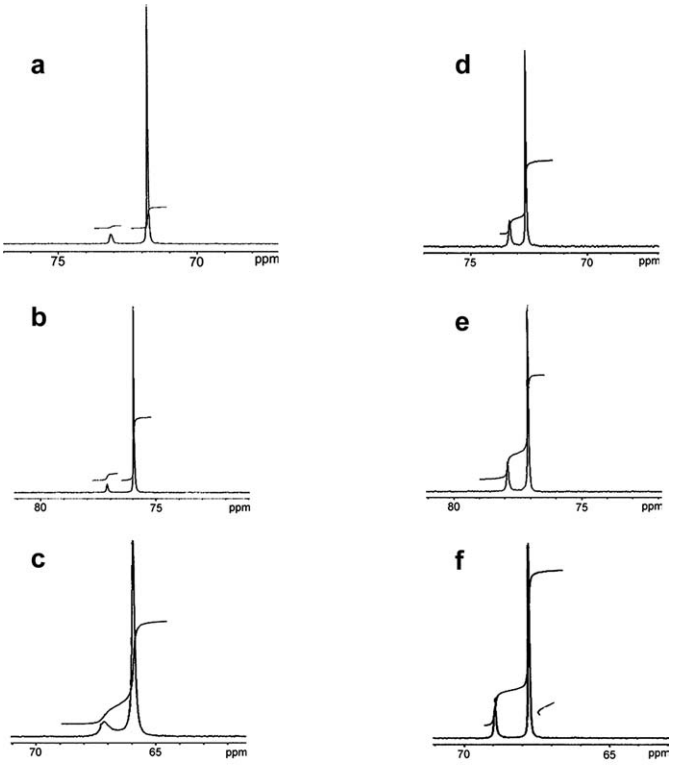
Synthetic route to the phosphorohydrazides of coumarin (**5a-c**), benzopyran-2,4-dione (**4a-c**) and chromone (**8b-c**, **9a-b**)



Scheme 1. Synthetic route to the phosphorohydrazides of coumarin (**5a–c**), benzopyran-2,4-dione (**4a–c**) and chromone (**8b–c**, **9a–b**).

with the chemical shifts 13.60 ppm (**5a**, **b**) and 13.00 (**5c**). In abolition to this singlets for compound **4c**/**5c** we observed a doublet centered at δ 7.10 ($^{2}J_{PH}$ = 25 Hz) indicating of NH–P functionality presence. At the same time their spectra showed the minor signals due to NH (d, J_{HH} = 12.1 Hz) at δ 10.75 (**4a**), 10.71 (**4b**), 10.94 (**4c**) coupled to CH (d, J_{HH} = 12.1 Hz) which resonates at 8.59 (**4a**), 8.58 (**4b**) and 8.67 (**4c**). If D_2O is added to a solution the CH protons resonated as clear singlets. The major signals of CH protons are also singlets centered at δ 8.13 (**5a**), 8.14 (**5b**) and 8.42 (**5c**). These data are consistent with the formation of CH=N–N(R) (major) and CH–NH–N(R) (minor).

The NCH_3 signals of **5a–b** (major) and **4a–b** (minor) resonated as doublets centered at δ 3.12 ($^{3}J_{PH}$ = 9.1 Hz), 3.23 ($^{3}J_{PH}$ = 9.2 Hz) and 3.15 ($^{3}J_{PH}$ = 10.9 Hz), 3.17 ($^{3}J_{PH}$ = 10.7 Hz), respectively. The signals of the alkoxy group and aromatic ring protons are equivalent for both tautomeric forms.



The ^{31}P NMR spectra of 4a/5a, 4b/5b, 4c/5c, in $CDCl_3$ (a, b, c) and in DMSO-d_6 (d, e, f) respectively.

Fig. 1.

Generally the values of shifts in resonance spectra of the compounds **4a**–**b**/**5a**–**b** in DMSO-d_6 were similar to these received in $CDCl_3$. The relative proportions of iminoenol (**5a**–**b**) and ketoenamine form (**4a**–**b**) in this solution were about 4:1. This observation may be explained with stability of ketoenamine system interaction of this tautomer and DMSO-d_6.

The spectra of **4c**/**5c** in both solvents are quite similar, however, in DMSO-d_6 the signal of proton assigned to NH–P is shifted downfield.

In ^{13}C NMR spectra of the compounds **4a**–**c**/**5a**–**c**, the signals of all carbon atoms in both enol (**5a**–**c**) and keton. forms (**4a**–**c**) (see Section 5) appeared. In each case, characteristic signals of C-2 atoms were found for both forms at around 170 ppm, and for C-4 significant differences were observed at about 160 ppm for **5a**–**c**, and about 175 ppm for **4a**–**c**. The determined values of C-4 shifts, being in compliance with the literature [33,37], confirm the presence of –OH group (4-hydroxycoumarin system) for **5a**–**c** and the C = O group (benzopyran-2,4-dione system for **4a**–**c**).

In the reaction of chromone **6** with phosphorohydrazides **1b**–**c**, regardless of the conditions of the reaction, the compounds **8b**–**c** were always obtained, whose structure was determined by means of the X-ray method [21,22]. Fitton et al. [38], who investigated reactions of 3-formylchromone with primary aromatic amines, found out that besides usually formed aldimines products of the chromone structure appeared due to addition of 1,4 of the consecutive amine molecule. Chromone derivatives underwent keto-enol tautomerism.

In the reactions studied, obtained Schiff's bases (**8b**–**c**) (Scheme 1) did not undergo reactions with an excess of phosphorohydrazides. In the ^{31}P NMR spectrum of the products the single phosphorus signals were always present (see Section 5). The analysis of ^{1}H and ^{13}C NMR spectra of the compounds **8b**–**c** enabled to determine the structure of tautomers of the compounds **4a**–**c**/**5a**–**c**. In ^{1}H NMR spectrum in $CDCl_3$ of the compound **8b** a doublet signal of N–CH_3 group protons occurred at 3.30 ppm with a coupling constant of $^{3}J_{PH} = 9.5$ Hz. In the spectrum of tautomers **4b**/**5b** there were two doublet signals present in the same solvent, one weaker at 3.17 ppm ($^{3}J_{PH} = 10.7$ Hz), and another stronger at 3.23 ppm ($^{3}J_{PH} = 9.2$ Hz). Conformity of the shift values as well as the coupling constant occurs between the latter signal of **4b**/**5b** and the values given for **8b**.

On the basis of the achieved results, it can be assumed that the predominant tautomer posses hydrazone structure identical as in **8b**. In ^{13}C NMR spectrum the shifts value of C-4 of the compounds **8b**–**c**, characteristic for γ-pyrone, 176.08 (**b**) and 175.88 (**c**), respectively, is in accordance with the values observed for the tautomer in the minor form 178.15 (**4b**) and 176.92 (**4c**).

Compounds **9a**–**b** were obtained using the previously described method [18] from chromone-3-carbonyl chloride and phosporohydrazide **1a**–**b**. The structures of this compounds in the solid state were determined by the X-ray method [18,23], in the current study we present ^{1}H, ^{13}C and ^{31}P NMR spectroscopic data in solutions (see Section 5).

2.2. Alkylating activity

In recent years, the 4-(4′-nitrobenzyl)pyridine (NBP-test or Preussmann test) [39] has been used to determine the alkylating properties of potentially antineoplastic compounds [40]. In vitro alkylating compounds react with the nucleophilic nitrogen atom of the pyridine ring of NBP. The reaction product in alkaline media gives a colored solution, whose absorbance is measurable. The NBP test was also used for the evaluation of alkylating properties of the new synthesized compounds and their platinum (II) and palladium (II) complexes [8–10,25,41].

The results of NBP test for compounds **4a**–**c**/**5a**–**c**, **8b**–**c**, **9a**–**b** at a concentration of 5×10^{-3} M in 2-methoxyethanol are presented in Table 1. The data were related to the alkylating activity of cyclophosphamide and trimethylthiophosphate [39].

3. Pharmacology

3.1. Antitumor activity

The two phosphorohydrazone derivatives of coumarin and chromone **4b**/**5b** and **9a** demonstrate antitumor activity against leukemia L1210 [15]. Here we present the results of the influ-

Table 1
NBP test results

Compounds	Absorbance (A)[a]	Alkylating activity[b]
4a/**5a**	0.420	++
4b/**5b**	0.470	++
4c/**5c**	0.395	++
8b	0.320	++
8c	0.380	++
9a	0.280	++
9b	0.210	++
Cyclophosphamid	0.120	++
Trimethylthiophosphat	0.350	++

[a] Means from three determinations.
[b] According to [39]: $A < 0.005$; (+) $A = 0.005$–0.1; (++) $A = 0.1$–0.5; (+++) $A > 0.5$.

ence of combination of these compounds with methotrexate on mice with the L1210 leukemia survival. The compounds of **4a**/**5a** and **8b**–**c** were not absorbed from the peritoneal cavity [15]. Therefore these compounds have not been further examined in this study.

The influence of compounds **4b**/**5b** and **9a** in monotherapy and in combination with methotrexate on survival of mice L1210 leukemia is presented in Table 2. Compounds **4b**/**5b** and **9a** were administered at a total dose of 0.5 and 0.1 of approximate lethal dose (ALD_{50}), respectively. Methotrexate was administrated i.p. at a total dose of 21 mg kg^{-1} (0.5 ALD_{10}) [42].

Increase of the lifespan (ILS) of the L1210 inoculated mice is estimated at 69% (**4b**/**5b**) or 30% (**9a**) and after administration of these compounds with methotrexate: 84% or 60%, respectively.

The influence of compounds **4b-c**/**5b-c** and **9a** in monotherapy on survival of mice with P388 leukemia is presented in Table 3. ILS 53% (**4b**/**5b**) and 30% (**9a**) was reached. Compound **4c**/**5c** did not inhibit the neoplastic cells growth in any of the administered doses (ILS was 0% and 6%).

3.2. Antibacterial screening

Compounds signed as (**4a**/**5a**, **4b**/**5b**, **8b** and **9a-b**) were screened for in vitro antibacterial activity against Gram-positive (*Staphylococcus aureus*, *Enterococcus faecalis*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*). Nalidixic acids and phosphomycin were used as reference standard in the test. The minimum inhibitory concentrations (MIC, μg ml^{-1}) were determined using standard agar dilution method [43].

Table 2
Antitumor activity of **4b**/**5b**, **9a** in monotherapy and in combination with methotrexate against L1210 leukemia

Compounds	Dose[a] (mg kg^{-1})	MST[b] (days)	ILS[c] (%)	ALD_{50}[d] (mg kg^{-1})
4b/5b	150	11	69*	900
4b/5b	150	12	84**	900
+MTX[e]	7			90
9a	43.3	8.5	30*	1300
9a	43.3	11	69**	1300
+MTX	7			90
Control (untreated)	–	6.5	–	–

* $P < 0.05$ vs. control; ** $P < 0.05$ vs. monotherapy.
[a] Dose administered the first, third and the fifth day after inoculation.
[b] MST.
[c] ILS.
[d] ALD.
[e] Methotrexate.

Table 3
Antitumor activity of derivatives **4b**/**5b**, **4c**/**5c** and **9a** in vivo against P388 mice leukemia

Compounds	Dose[a] (mg kg^{-1})	MST[b] (days)	ILS[c] (%)[d]	ALD_{50} (mg kg^{-1})
4b/**5b**	300	13	53*	900
	150	13	53*	
	50	9	6	
4c/**5c**	667	9	6	2000
	334	8.5	0	
	67	9	6	
9a	433	11	30*	1300
	217	11	30*	
	43.3	11	30*	
Control (untreated)	–	8.5	–	–

* $P < 0.05$.

4. Results and discussion

Methyl ester, chromone-3-carbonyl chloride and 3-formylchromone posses two electrophilic centers. As a consequence the diversity in nucleophilic reaction patterns was observed [38,44,45].

The chromone structure (4-oxo-4*H*-1-benzopyrane) ring is usually readily opened via nucleophilic attack at the two positions [45]. Such mechanism of reaction was observed in the case of reaction of methyl ester of chromone-3-carboxylic acid (**2**) with phosphorohydrazides (**1a**–**c**). After opening the benzopyrane structure of the compound **2** by phosphorohydrazides, a labile product undergoes intramolecular lactonization forming the 2,4-dione structure. Diones (**4a**–**c**) in the reaction environment (methanol or ethanol) undergo partial tautomerization to 4-hydroxycoumarin derivatives (**5a**–**c**). The ^{31}P NMR spectroscopic investigation in methanol solution of the product of reaction **2** with **1b** confirms the presence of ketoenamine form **4b** and iminoenol form **5b** in the ratio of 3.5:1. The compound **4b** with the structure of 2,4-dione crystallizes from the solution. The structure is stabilized by a strong intramolecular hydrogen bonds [18]. The case of the products of the reaction **2** and **1a**, **c**, the compounds with the structure of 4-hydroxycoumarin (**5a**, **c**) crystallized from the ethanol solution, what can be explained by the presence of the additional hydrogen bond (intramolecular) due to the presence hydrogen at N-2 of the hydrazone system [21,23].

The fact of obtaining identical final products from the reaction of 3-formyl-4-hydroxycoumarin signifies the occurrence of reversible tautomerism of **5a**–**c** ⇌ **4a**–**c** in the studied solutions.

According to the previous study [44,46] the aldehyde groups are not only targets for nucleophilic attack, but carbon atom C-2 as well. In the reaction examined in the present

study, no products of **1a–c** nucleophiles attack on chromone C-2 were formed. The reaction of 3-formylchromone and chromone-3-carbonyl chloride with **1a–b** gave only phosphorohydrazides **8b–c**, **9a–b**, respectively.

Former results could bear out hypothesis that coumarin and chromone derivatives posses antitumor activity, however the mechanism of the action is still unknown [5].

Our preliminary study demonstrates that in this class of compounds the derivatives of phosphorohydrazides constitute an interesting group of compounds with cytotoxic activity. The in vitro studies indicate that all the examined compounds **4a–c**/**5a–c**, **8b–c**, **9a–b** are strongly (++) alkylating agents. Compound **4b**/**5b** exhibits higher antitumor activity against leukemias L1210 and P388 than **9a**. Activity against leukemia L1210 increased considerably after therapy combined **4b**/**5b** and **9a** with the well known antitumor drug—methotrexate. On the basis of the presented spectroscopic analyses of **4a–c**/**5a–c** but 4-hydroxycoumarin system (**5b**) in the compound **4b**/**5b** is credited with being biologically active. Among chromone derivatives, cytotoxic activity against leukemia P388 has been recognized in phosphorohydrazinecarbonylic derivative (**9a**). Some of the investigated compounds (**4a**/**5a**, **4b**/**5b**, **8b** and **9a–b**) were evaluated for in vitro antibactierial activity using standard dilution method [43]. The MIC values are summarized in Table 4. The results indicated that the screened compounds showed similar activity against all the tested bacterial (MIC 150–300 μg ml^{-1}). Further biochemical and pharmacological investigations, due to the results of the present study, will focus on these groups of phosphorohydrazide derivatives since they exhibit interesting biological properties.

5. Experimental

5.1. General

The melting points were determined using a Bethius apparatus and they are uncorrected. The ^1H NMR and ^{13}C NMR spectra registered on a Varian Mercury spectrometer (^1H 300 MHz; ^{13}C 75.5 MHz). The ^{31}P NMR spectra were recorded on a Bruker HX360 and Varian 75 MHz spectrometry with H_3PO_4 as external standard. A Lambda 19 spectrophotometer (Perkin Elmer) was used for the colorimetric assay. Satisfactory elemental analyses (± 0.4% of the calculated values) were obtained for the new compounds using a Perkin–Elmer PE 2400 CHNS analyzer (Microanalytical Laboratory, Medical University of Lodz) and in Polish Academy of Science (Lodz).

Compounds **1a–c**, **2**, **3**, **6**, **7** used for the synthesis were obtained according to the described procedures. N^1-diethoxythiophosphoro- and N^1-dimethoxythiophosphoro-N^1-methylhydrazide (**1a**, **b**) according to Ref. [12], diethoxythiophosphorohydrazide (**1c**) according to Ref. [47], methyl 4-oxo-4*H*-1-benzopyran-3-carboxylate (**2**) according to Ref. [48], 4-hydroxy-2-oxo-2*H*-1-benzopyran-3-carboaldehyde (**3**) according to Ref. [32], 4-oxo-4*H*-1-benzopyran-3-carboaldehyde (**6**) according to Ref. [49], 4-oxo-4*H*-1-benzopyran-3-carbonyl chloride (**7**) according to Ref. [50].

5.2. Chemistry

5.2.1. Synthesis of compounds *4a–c*/*5a–c*

Path A.

Compounds **4a–c**/**5a–c** were synthesized according to the described general procedure [18,21].

Path B. General procedure for **4a–c**, **5a–c**.

Phosphorohydrazides (**1a–c**) (10 mmol) was added at room temperature to a solution of 4-hydroxy-2-oxo-2*H*-1-benzopyran-3-carboaldehyde (**3**) (10 mmol) in anhydrous methanol (15 ml). After 24 h the solid product was filtered off, dried, and crystallized from MeOH or EtOH.

3-{[(Diethoxythiophosphoryl)-metylhydrazino]-methylidene}-3,4-dihydro-2*H*-1-benzopyran-2,4-dione (**4a**); (E)-3-{(diethoxythiophosphoryl)-methylhydrazono]methylen}-4-hydroxy-2*H*-1-benzopyran-2-one (**5a**). Yield 2.78g (75%), m.p. 119–121 °C (ethanol).

5.2.1.1. ***5a*** *(major signals)*. ^1H NMR ($CDCl_3$): 1.35 (t, t, $J_{HH} = 7$ Hz, 6H, $2 \times CH_2$–CH_3), 3.21 (d, $^3J_{PH} = 9.1$ Hz, 3H, N–CH_3), 3.93–4.27 (m, $^3J_{PH} = 9.2$ Hz, $J_{HH} = 7$ Hz, 4H, $2 \times CH_2$), 7.23–7.33 (m, $J_{HH} = 1.6$and 8 Hz, 2H, C_6–H, C_8–H), 7.55–7.61 (m, $J_{HH} = 1.6$ and 8 Hz, 1H, C_7–H), 7.98–8.10 (dd, $J = 1.6$ Hz, 1H, C_5–H), 8.13 (s, 1H, CH=N), 13.60 (s, 1H, OH, disap. in D_2O).

(DMSO-d_6) δ: 1.28 (t, $J_{HH} = 7.1$ Hz, 3H, CH_3), 3.13 (d, $^3J_{PH} = 9.2$ Hz, 3H, N–CH_3), 3.96–4.27 (m, $^3J_{PH} = 9.4$ Hz,

Table 4
In vitro antibacterial activity of **4a**/**5a**, **4b**/**5a, 8b**, **9a–b**

Compounds	Concentration range (μg ml^{-1})	MIC (μg ml^{-1})						
		Gram-positive organisms				Gram-negative organisms		
		S. aureus ATCC 6538P	*S. aureus* ATCC 29213	*S. aureus* ATCC 25923	*E. faecalis* ATCC 29212	*E. coli* ATCC 35218	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
4a/5a	400–3.10	200	> 300	> 300	> 300	200	200	200
4b/5b	400–3.10	200	300	300	200	200	200	200
8b	400–3.10	200	200	200	200	200	200	200
9a	300–4.70	150	150	150	> 300	> 300	150	150
9b	400–4.70	200	> 300	> 300	200	200	200	200
Nalidixic acid	50–1.55	50	50	>50	> 50	6,2	3,1	> 50
Phosphomycin	100–3.10	6.25	12.5	25	50	50	50	6.25

J_{HH} = 7.1 Hz, 2 × CH_2), 7.33–7.73 (m, 3H, aromat), 7.92–7.99 (m, 1H, C_5–H), 8.17 (s, 1H, CH=N), 13.24 (s, 1H, OH, disap. in D_2O).

^{13}C NMR ($CDCl_3$): δ 15.93, 16.03 (CH_3–C), 33.82 (CH_3–N), 64.38 (CH_2O, $^2J_{P-C}$ = 5.1 Hz, 97.88, 116.82, 117.16, 124.15, 124.28, 124.56, 133.45, 134.69, 145.05, 145.20, 153.58, 162.18 (C–OH), 170.44 (2-CO).

^{31}P NMR ($CDCl_3$): δ 71.76.

(DMSO-d_6): δ 72.59.

*5.2.1.2. **4a** (minor signals).* ^1H NMR ($CDCl_3$): δ 3.15 (d, $^3J_{PH}$ = 10.9 Hz, 3H, N–CH_3), 8.59 (d, J_{HH} = 12.1 Hz, 1H, CH–NH), 10.75 (d, J_{HH} = 12.1 Hz, 1H, NH, disap. in D_2O).

(DMSO-d_6) δ: 1.06 (t, 3H, CH_2–CH_3), 8.40–8.64 (d, broad, 1H, CH–NH), 11.24–11.60 (d, broad, 1H, NH).

^{13}C NMR ($CDCl_3$): δ 39.50 (CH_3–N), 64.86 (CH_2–O, $^2J_{P-C}$ = 6 Hz), 97.11, 118.08, 120.37, 126.18, 126.71, 134.12, 154.51, 161.43, 164.42, 170.44 (2-CO), 178.08 (4-CO).

Ratio **5a**:**4a** ca. 8:1.

^{31}P NMR ($CDCl_3$): δ 73.10.

(DMSO-d_6): δ 73.31.

Solid state δ 69.31.

($CDCl_3$): ratio **5a**:**4a** ca. 8:1.

(DMSO-d_6): ratio **5a**:**4a** ca. 4:1.

(E)-3-{[dimethoxythiophosphoryl)-methylhydrazino]-methylidene}-3,4-dihydro-2*H*-1-benzopyran-2,4-dione (**4b**); 3-{[(dimethoxythiophosphoryl)-methylhydrazono]-methylen}-4-hydroxy-2*H*-1benzopyran-2-one (**5b**). Yield 2.94 g (86%), m.p. 123–124 °C (methanol).

*5.2.1.3. **5b** (major signals).* ^1H NMR ($CDCl_3$): δ 3.23 (d, $^3J_{PH}$ = 9.2 Hz, 3H, N–CH_3), 3.78 (d, $^3J_{PH}$ = 14 Hz, d, 6H, 2 × OCH_3), 7.24–7.35 (m, J_{HH} = 1.6 and 7.7 Hz, 2H, C_6–H, C_8–H), 7.56–7.86 (m, J_{HH} = 1.6 and 7.7 Hz, 1H, C_7–H), 8.00–8.11 (m, 1H, C_5–H), 8.14 (s, 1H, CH=N), 13.60 (s, 1H, OH, disap. in D_2O).

(DMSO-d_6): δ 3.15–3.30 (m, $^3J_{PH}$ = 8.7 Hz, 3H, N–CH_3), 3.73 (d, $^3J_{PH}$ = 14 Hz, 3H, CH_3), 7.32–7.40 (m, J_{HH} = 7.8 Hz, 2H, C_6–H, C_8–H 2H), 7.67–7.73 (m, J_{HH} = 1.7 and 7.3 Hz, 1H, C_7–H), 7.93–7.96 (m, J_{HH} = 1.7 Hz, 1H, C_5–H), 8.17 (s, 1H, CH=N), 13.18 (s, 1H, OH, disap. in D_2O).

^{13}C NMR ($CDCl_3$): δ 33.64 (CH_3–N), 54.47 (CH_3–O), 97.94, 116.83, 116.96, 124.20, 124.50, 133.48, 144.60 (d, CH=N, J = 11.7 Hz), 153.54, 162.12 (C–OH), 170.02 (2-CO).

^{31}P NMR ($CDCl_3$): δ 75.92.

(DMSO-d_6): δ 77.05.

(CD_3OD): δ 78.37.

*5.2.1.4. **4b** (minor signals).* ^1H NMR ($CDCl_3$): δ 3.17 (d, $^3J_{PH}$ = 10.7 Hz, 3H, N–CH_3), 8.58 (d, J_{HH} = 12.1 Hz, 1H, CH–NH), 10.71 (d, J_{HH} = 12.1 Hz, 1H, NH, disap. in D_2O).

(DMSO-d_6): δ 3.15–3.30 (m, $^3J_{PH}$ = 9.7 Hz, J_{HH} = 8.7 Hz, 3H, N–CH_3), 8.43 (d, J_{HH} = 12.1 Hz, 1H, CH–NH), 11.42 (d, J_{HH} = 12.1 Hz, 1H, NH, disap. in D_2O).

^{13}C NMR ($CDCl_3$): δ 39.65 (CH_3–N), 54.90 (CH_3–O, $^2J_{PC}$ = 6.0 Hz), 97.35, 117.28, 120.35, 124.33, 126.43, 134.77, 154.51, 170.02 (2-CO), 178.15 (4-CO).

Ratio **5b**:**4b** ca. 9:1.

^{31}P NMR ($CDCl_3$): δ 77.11.

(DMSO-d_6): δ 77.87.

(CD_3OD): δ 73.33.

($CDCl_3$): ratio **5b**:**4b** ca. 9:1.

(DMSO-d_6): ratio **5b**:**4b** ca. 3:1.

(CD_3OD): ratio **5b**:**4b** ca. 1:3.5.

Solid state δ 72.52.

3-{[(Diethoxythiophosphoryl)-hydrazino]-methylidene}-3,4-dihydro-2*H*-1-benzopyran-2,4-dione (**4c**); (E)-3-{[(diethoxythiophosphonyl)-hydrazono]-methylen}-4-hydroxy-2*H*-1-benzopyran-2-one (**5c**). Yield 2.35 g (66%) (ethanol).

*5.2.1.5. **5c** (major signals).* ^1H NMR ($CDCl_3$): δ 1.38 (t, J_{HH} = 6.9 Hz, t, 6H, 2 × CH_2–CH_3), 4.13–4.29 (m, $^3J_{PH}$ = 7.1 Hz, J_{HH} = 6.9 Hz, 4H, 2 × CH_2–CH_3), 7.10 (d, $^2J_{PH}$ = 25 Hz, 1H, NH–P), 7.24–7.32 (m, J_{HH} = 8.1 Hz, 2H, C_6–H, C_8–H), 7.55–7.63 (m, J_{HH} = 1.6 and 8.1 Hz, 1H, C_7–H), 8.01 (d, J_{HH} = 1.6Hz, 1H, C_5–H), 8.42 (s, 1H, CH=N), 13.00 (s, 1H, OH). Signals –OH and –NH disap. in D_2O.

(DMSO-d_6): δ 1.28 (t, J_{HH} = 7 Hz, 6H, 2 × CH_2–CH_3), 4.07–4.17 (m, J_{HH} = 7 Hz, $^3J_{PH}$ = 9.6 Hz, 4H, 2 × CH_2), 7.34–7.39 (m, J_{HH} = 7.9 Hz, 2H, C_6–H, C_8–H), 7.67–7.73 (m, J_{HH} = 1.8 and 7.9 Hz, 1H, C_7–H), 7.92–7.96 (m, J_{HH} = 1.8 Hz, 1H, C_5-H), 8.28 (s, 1H, CH=N), 9.79 (d, $^2J_{PH}$ = 25 Hz, 1H, NH–P), 13.07 (s, 1H, OH). Signals –OH and –NH disap. in D_2O.

^{13}C NMR ($CDCl_3$): δ 16.12, 16.22 (CH_3), 64.53 (CH_2), 97.51, 117.25, 124.41, 125.19, 134.28, 154.15, 154.82, 162.65 (C–OH), 174.95 (2-CO).

^{31}P NMR ($CDCl_3$): δ 65.88.

*5.2.1.6. **4c** (minor signals).* ^1H NMR ($CDCl_3$): δ 6.84 (d, $^2J_{PH}$ = 23 Hz, 1H, NH–P), 8.67 (d, J_{HH} = 12.1 Hz, 1H, CH–NH), 10.94 (d, J_{HH} = 12.1 Hz, 1H, CH–NH). Signals –NH disap. in D_2O.

(DMSO-d_6): δ 8.40 (d, J_{HH} = 12 Hz, 1H, CH–NH), 9.30 (d, $^2J_{PH}$ = 25 Hz, 1H, NH–P), 11.20 (d, J_{HH} = 12 Hz, 1H, NH). Signals -NH disap. in D_2O.

^{13}C NMR ($CDCl_3$): δ 64.59 (CH_2), 97.09, 118.19, 126.67, 154.93, 174.95 (2-CO), 178.10 (4-CO).

^{31}P NMR ($CDCl_3$): δ 67.11.

(DMSO-d_6): 68.92.

($CDCl_3$): ratio **5c**:**4c** ca. 5:1.

(DMSO-d_6): Ratio **5c**:**4c** ca. 3.5:1.

*5.2.2. Synthesis of compounds **8b**, **8c** and **9a**, **9b***

These compounds were synthesized according to the described general procedure [18,21,22].

(E)-3-{[(dimethoxythiophosphoryl)-methylhydrazono]-methylen}-4*H*-1-benzopyran-4-one (**8b**), m.p. 127–129 °C (methanol).

^{1}H NMR ($CDCl_3$): δ 3.30 (d, $^3J_{P-H}$ = 9.5 Hz, 3H, N–CH_3), 3.75 (d, $^3J_{PH}$ = 13.9 Hz, 6H, 2 × OCH_3), 7.42–7.51 (m, 2H, C_6–H, C_8–H), 7.67–7.73 (m, 1H, C_7–H), 7.84 (d, $^4J_{PH}$ = 1.4 Hz, 1H, CH=N), 8.26 (d, d, 1H, C_5-H), 8.52 (s, 1H, C_2–H).

^{13}C NMR ($CDCl_3$): δ 32.73 (d, $^2J_{PC}$ = 11.4 Hz, N–CH_3), 54.40 (d, $^2J_{PC}$ = 5.4 Hz, O–CH_3), 118.45 (C-8), 119.69 (C-3), 124.09 (C-4a), 125.66, 126.07 (C-5, C-6), 131.24 (d, ^3J = 14.3 Hz, CH), 133.98 (C-7), 153.01 (C-2), 156.28 (C-8a), 176.08 (C=O).

^{31}P NMR ($CDCl_3$): δ 74.91.

(E)-3-{[(diethoxythiophosphoryl)-hydrazono]-methylen}-4*H*-1-benzopyran-4-one (**8c**), m.p. 210–212 °C (ethanol).

^{1}H NMR ($CDCl_3$): δ 1.38 (t, t, 2 × CH_3, J = 7 Hz, 6H), 4.05–4.11 (m, J_{HH} = 7 Hz, $^3J_{PH}$ = 9.8 Hz, 4H, 2 × CH_2), 7.28–7.50 (m, 2H, C_6–H, C_8–H), 7.82 (m, 1H, C_7–H), 7.96 (d, $^2J_{PH}$ = 34 Hz, 1H, NH), 8.12 (m, 1H, C_5–H), 8.32 (s, 1H, CH=N), 8.56 (s, 1H, C_2–H). Signal –NH disapp. in D_2O.

(DMSO): δ 1.25 (t, t, J = 7.1 Hz, 6H, CH_3), 4.01-4.12 (m, J_{HH} = 7.1 Hz, $^3J_{PH}$ = 9.7 Hz, 4H, CH_2), 7.54 (m, 1H, C_6–H), 7.72 (d, 1H, C_8–H), 7.84 (m, 1H, C_7–H), 8.11(m, 1H, C_5–H), 8.12 (s, 1H, CH=N), 8.58 (s, 1H, C_2–H), 9.98 (d, $^2J_{PH}$ = 34.7 Hz, 1H, NH).

^{13}C NMR ($CDCl_3$) δ: 15.64 (CH_3–C), 64.52 (CH_2–OP, $^2J_{PC}$ = 5.2 Hz), 118.25 (C-8), 118.77 (C-3), 123.97 (C-4a), 125.27, 126.16 (C-5, C-6), 134.15 (CH=N), 138.11 (C-7), 150.50 (C-2), 156.25 (C-8a), 175.88 (4-CO).

^{31}P NMR ($CDCl_3$): δ 69.31; (DMSO): δ 64.68.

3-{[(Dietoxythiophosphoryl)-methylhydrazino]carbonyl}-4*H*-1-benzopyran-4-one (**9a**), m.p. 134–135 °C (ethanol).

^{1}H NMR ($CDCl_3$): δ 1.35 (t, t, J_{HH} = 7 Hz, 6H, 2 × CH_3), 3.16(d, $^3J_{PH}$ = 8.9 Hz, 3H, N–CH_3), 4.04–4.35 (m, J_{HH} = 7 Hz, $^3J_{PH}$ = 8.9 Hz, 4H, 2 × CH_2), 7.30–7.61 (m, 2H, C_6–H, C_8–H), 7.71–7.88 (m, 1H, C_7–H), 8.25–8.35 (m, 1H, C_5–H), 8.97 (s, 1H, C_2–H), 10.64 (s, 1H, NH disapp. in D_2O).

^{13}C NMR ($CDCl_3$): δ 15.84 (CH_3–C), 33.92 (d, $^2J_{PC}$ = 10.8 Hz, CH_3–N), 64.36 (d, $^2J_{PC}$ = 10.8 Hz, CH_2-OP), 64.36 (d, $^2J_{PC}$ = 5.2 Hz, CH_2–OP), 118.34 (C-8), 124.28 (C-4a), 126.15, 126.51 (C-6, C-5), 135.02 (C-7), 156.11 (C-8a), 162.73 (CONH), 176.55 (CO).

^{31}P NMR ($CDCl_3$): δ 73.51.

(E)-3-{[(dimethoxythiophosphoryl)-methylhydrazino]carbonyl}-4*H*-1-benzopyran-4-one (**9b**), m.p. 145–147 °C (methanol).

^{1}H NMR ($CDCl_3$): δ 3.15 (d, $^3J_{PH}$ = 8.5 Hz, 3H, N–CH_3), 3.78 (d, $^3J_{PH}$ = 13.9 Hz, 3H, O–CH_3), 7.50–7.59 (m, 2H, C_6–H, C_8–H), 7.76–7.82 (m, 1H, C_7–H), 8.29 (d, 1H, C_5–H), 8.98 (d, d, 1H, C_2–H), 10.65 (s, 1H, NH disapp. in D_2O).

^{13}C NMR ($CDCl_3$) δ: 38.21 (d, $^2J_{PC}$ = 10.6 Hz, CH_3–N), 54.29 (d, $^2J_{PC}$ = 4.9 Hz, CH_3–O), 115.62 (C-3), 118.52 (C-8), 124.15 (C-4a), 126.45, 126.56 (C-6, C-5), 135.01 (C-7), 156.03 (C-8a), 162.63 (C-2), 162.85 (CONH), 176.63 (4-C=O).

^{31}P NMR ($CDCl_3$): δ 77.75.

5.2.3. Alkylating properties (NBP test)

The tested compounds were dissolved in 2-methoxyethanol (1 ml, 0.005 mol) and NBP (1 ml, 5% 2-methoxyethanol solution) was added. The samples were heated at 100 ± 0.5 °C for 1 h and cooled quickly to temperature 20 °C. 2-Methoxyethanol (2.5 ml) and piperidine (0.5 ml) were added to the samples to a total volume of 5 ml. The final concentration was 2.5×10^{-5} mol l^{-1}. After 1.5 min, the absorbance was measured at λ = 560 nm in glass cells (1 ml) in the presence of 2-methoxyethanol (Table 1).

5.3. In vivo antitumor activity

The experiments were carried out in strict accordance to the Polish governmental regulations concerning experiment on animals (Dz. U. 97.111, 724) and rules followed by the Medical University of Lodz). Hybrid male CD2F1 (BALB)cx DBA(2) F1 mice weighting 22–30 g, 8–12 weeks old and leukemia cells (L1210, P388) were obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław). Leukemic cells from culture were resuspended in 0.9% NaCl so 3×10^5 L1210 or P388 cells were injected intraperitoneally (i.p.) into mice. The ALD_{50} of the tested compounds was determined by the method described by Deichmann and Le Blance [51]. Experiments were conducted using a group of five mice for each dose. The tested compounds were suspended in 1% aqueous solution of methylcellulose and were administered in a volume of 0.01 ml g^{-1} of mouse weight three times within the first, third and fifth day after inoculation. Control mice received equivalent volumes of 1% aqueous solution of methylcellulose. The mice were observed daily for survival. The median survival time (MST) according to Geran's method is: MST = (x = y)/2, where x denotes the earliest day when the number of dead animals is ≥ (N/2 + 1); N denotes the number of animals in the group [42]. The antileukemic effect of the tested compounds was assessed as the percentage ratio of MST of the treated group (MST_T) to that of the control group (MST_C): ILS = [(MST_T/MST_C) − 1] 100%. ILS of > 25% indicates activity. The comparison between antileukemic activity of a pure compound and combined with methotrexate was evaluated using Student's t-test for differences between means. The results of antitumor activity against P388 as average values ± S.D. (N = 5) were statistically analyzed by two-way analysis of variance (ANOVA) followed by the LSD Fisher part hoc test. Differences were considered signification when $P < 0.05$.

5.4. In vitro antibacterial activity

Tested compounds were examined towards in vitro antibacterial activity against Gram-positive (*S. aureus* ATCC 6538P, *S. aureus* ATCC 291213, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212) and Gram-negative (*E. coli* ATCC 35218, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) bacteria using standard agar dilution method. Agar dilution MIC testing was performed as described in the National Committee for Clinical Laboratory Standard (NCCLS) guidelines by using a steers replicator. The series of plates containing varying concentration (max. up to 400 μg ml^{-1} in DMSO) of each antimicrobial agent and growth controls plates without antimicrobial agent are prepared. Mueller–Hinton agar (Difco) was used for this test. Agar dilution plates were incubated at 35 °C in ambient air for 18 h. The MIC values of the tested compounds, nalidixic acid and phosphomycin were used as reference compounds as presented in Table 4.

6. Conclusions

The structures of several substituted chromone coumarin and chromon-2,4-dione derivatives (**4a**–**c**/**5a**–**c**, **8b**–**c**, **10a**–**b**) were confirmed by NMR spectroscopy. Three phosphorohydrazone derivatives **4a**–**c**/**5a**–**c** were obtained by the reaction of 3-formyl-4-hydroxycoumarin (new method) and chromone-3-carboxylic acid methyl ester with phosphorohydrazides (**1a**–**c**). The keto-enol tautomerism of 4-hydroxycoumarin (**5a**–**c**) and chromon-2,4-dione (**4a**–**c**) phosphorohydrazone derivatives was confirmed by spectral analyses. Compounds **4a**–**c**/**5a**–**c**, **8b**–**c** and **9a**–**b** were tested for their in vitro alkylating activity. Several selected compounds were tested for their in vivo antitumor activity. Compounds **4a**/**5a**, **4b**/**5b**, **8b** and **9a**–**b** showed moderate in vitro activity against Gram-positive and Gram-negative bacteria. Results of the present study showed that phosphorohydrazone derivatives exhibit significant and promising antineoplastic activity. Obviously further research is needed to evaluate their properties fully.

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