A new series of 2-substituted 3-phosphonic derivatives of chromone. Part II. Synthesis, *in vitro* alkylating and *in vivo* antitumour activity

Elzbieta Budzisz,*[a] Julita Graczyk-Wojciechowska,[b] Remigiusz Zieba[b] and Barbara Nawrot[c]

[a] *Chair of Medical Chemistry, Faculty of Pharmacy, Medical University of Lodz, Muszynskiego 1, 90-151, Lodz, Poland. E-mail: elora@ich.pharm.am.lodz.pl; Fax: +48 42 678 93 83; Tel: +48 42 677 92 17*

[b] *Institute of Pharmacology, Faculty of Pharmacy, Medical University of Lodz, Muszynskiego 1, 90-151, Lodz, Poland. E-mail: julita@mail.bp.am.lodz.pl, remusz@wp.pl; Fax: +48 42 678 93 83; Tel: +48 42 677 91 79, 677 92 98*

[c] *Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Department of Bioorganic Chemistry, Sienkiewicza 112, 90-363, Lodz, Poland. E-mail: bnawrot@bio.cbmm.lodz.pl; Fax: +48 42 681 54 83; Tel: +48 42 681 69 70*

Received (in Montpellier, France) 14th June 2002, Accepted 5th September 2002
First published as an Advance Article on the web 31st October 2002

Products of the reaction of (±)-*O*-acetylmandeloyl chloride with, respectively, sodium 2-hydroxy- or 2-hydroxy-5-methyl-acetophenone were brominated and coupled with trimethyl phosphite to give the Perkov products **4a** and **4b**, the Wittig-type products **6a** and **6b** and the title 3-phosphonic derivatives of chromone, **7a** {2-[1-(±)-acetoxybenzyl]-3-(dimethoxyphosphoryl)-4-oxo-4*H*-chromene} and **7b** {2-[1-(±)-acetoxybenzyl]-3-(dimethoxyphosphoryl)-4-oxo-6-methyl-4*H*-chromene}. Esters **7a** and **7b** were subjected to acidic hydrolysis to give the corresponding phosphonic acids **8a** and **8b**, and the unexpected phosphonolactones **9a** and **9b**. They were also treated with benzylamine forming the corresponding salts of the cyclic phosphonolactones (**10a** and **10b**). Derivatives **4a**,**b**, **6a**,**b**–**10a**,**b** were tested for *in vitro* alkylating activity while compounds **7a**, **7b** and **9a** were tested for *in vivo* antitumor activity. As determined by *in vitro* Preussmann tests, compounds **4**, **6** and **7** possess strong alkylating activity. Compounds **10** have moderate potential for alkylation, whereas the remaining compounds **8** and **9** are only weakly active. The derivatives **7a**, **7b** and **9a** demonstrated low *in vivo* antitumour activity against lymphocytic leukaemia L1210, whereas compound **7b** exhibited significant antitumour activity against leukaemia P388 in mice.

Chromones and their structural analogues are of great interest on account of their anticonvulsant,[1] antimicrobial,[2,3] and antitumour activities.[4] On the other hand phosphonic acids, and their salts and esters, attract significant attention because of their role as both agrochemical (herbicides, pesticides, growth regulators in plants) and medical (antibiotics, antivirals, enzyme inhibitors) products with a broad spectrum of applications.[5–9] The alkylating properties of phosphonic esters have been exhaustively described in many studies[10,11] and have been a subject of many patents.[12–14] Incorporation of a C–P bond into the chromone system offers a new class of compounds of potential importance. Since alkylating agents have a long history in the treatment of cancer, recent interest has focused on these aspects of their activity.

This work is a continuation of the earlier studies of our groups focused on the synthesis and biological properties of 3-phosphonic derivatives of chromone.[15–17] Here we present the synthesis, isolation and characterisation of a new series of 2-substituted 3-phosphonic derivatives of chromone, **7a**,**b**–**9a**,**b**, and studies on their *in vitro* alkylating and *in vivo* antitumour activity.

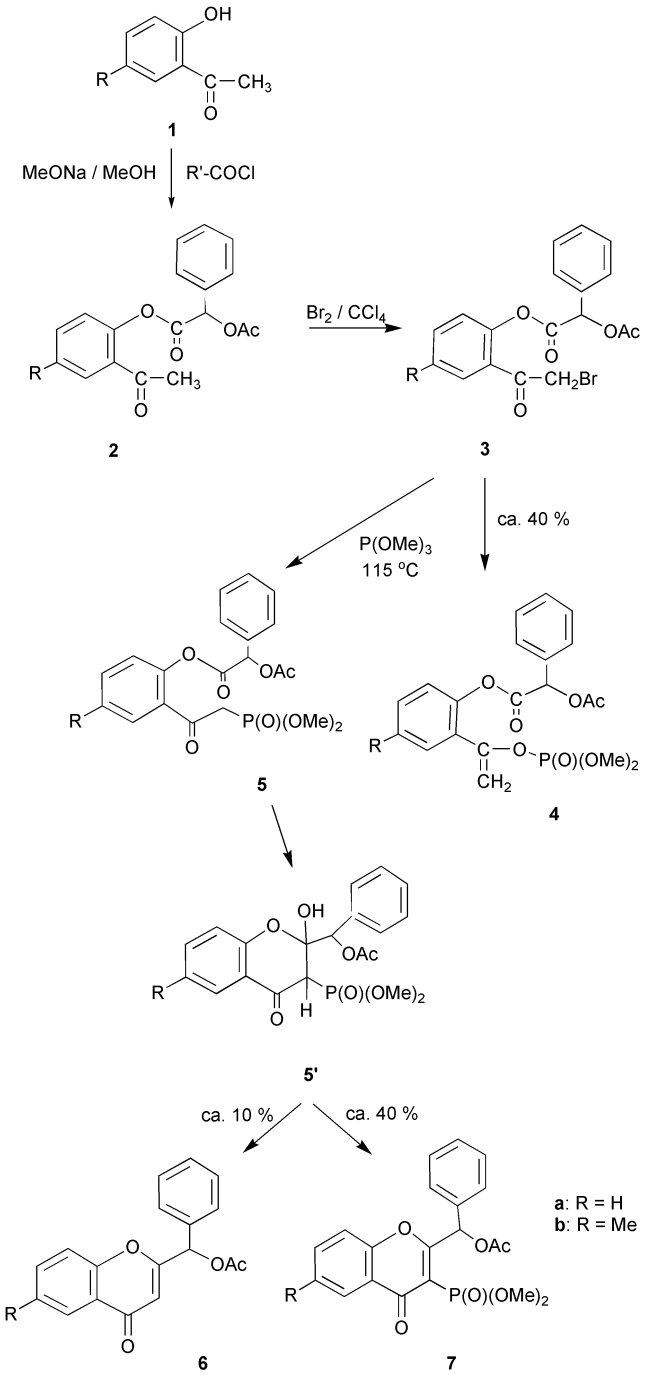
Results

Chemistry

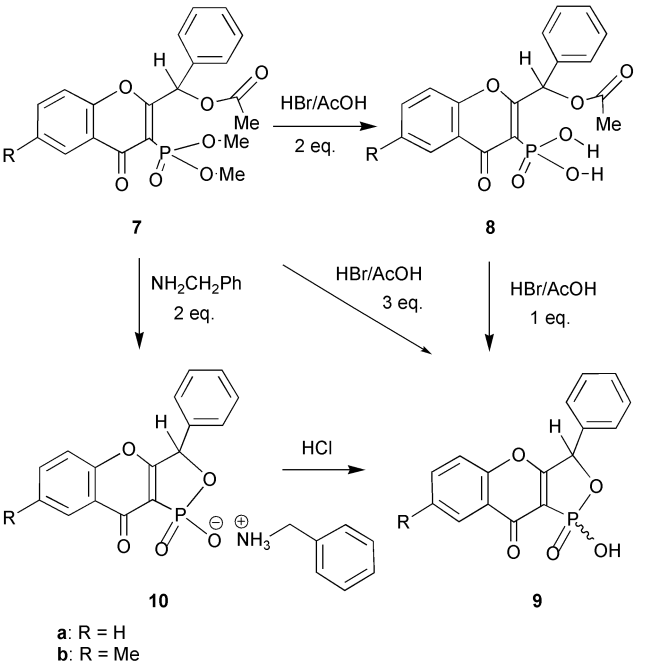
Reaction of the sodium salt of 2-hydroxyacetophenone (**1a**) or 2-hydroxy-5-methyl-acetophenone (**1b**) with racemic (±)-*O*-acetylmandeloyl chloride, carried out according to the method previously reported by us,[18] gave excellent yields of the esters **2a** and **2b**, respectively (Series a: R = H; series b: R = Me, Scheme 1). Selective bromination of the methyl group of the acetophenone moiety of **2**, performed in carbon tetrachloride according to Hercouet *et al.*,[19] led to the bromo derivatives **3**. On treatment with trimethyl phosphite at 110–115 °C for 30 min these compounds were transformed into a mixture of at least three major products (**a**: R_f 0.86, 0.63, 0.39 and **b**: R_f 0.84, 0.62, 0.33), as detected by TLC analysis (chloroform–acetone, 9 : 1 v/v mixture). Traces of products of R_f 0.55 (**a**) and 0.57 (**b**) were also visible. After removal of excess trimethyl phosphite, the reaction mixture was chromatographed over silica gel eluting with a chloroform–acetone, 5 : 1 v/v mixture. The two fast-eluting components of the mixture were isolated in *ca.* 10 and 40% yield. Their structures were assigned as **6a/b** (R_f 0.86 and 0.84) and **4a/b** (R_f 0.63 and 0.62) (Scheme 1) on the basis of their spectroscopic properties (see Experimental). However, the products of $R_f = 0.39$ (**a**) and 0.33 (**b**) were not eluted from the column. Instead, we isolated derivatives of much higher mobility [R_f 0.55 (**a**) and 0.57 (**b**)] in *ca.* 40% yield. The ^{1}H, ^{13}C and ^{31}P NMR analysis, as well as IR spectra and MS data, indicate that these compounds are depicted by structures **7a** and **7b**, respectively (Scheme 1). It seems probable that compounds **7a/b** are formed by transformation of the polar components of the reaction mixtures on the column (see Discussion).

The racemic mixtures **7a** (R = H) and **7b** (R = Me) were subjected to acidic hydrolysis with 30% HBr in acetic acid

This journal is © The Royal Society of Chemistry and the Centre National de la Recherche Scientifique 2002



Scheme 1



Scheme 2

(Scheme 2). When the molar ratio of hydrobromic acid to the substrate **7a** or **7b** was 2 : 1 we obtained the corresponding acids **8a** and **8b**. When the molar ratio was 3 : 1 we obtained a cyclic derivative **9a** or its methyl analogue **9b**. Treatment of compounds **7** with 2 equiv. of benzylamine gave the benzylammonium salts **10**, which, when treated with concentrated (*ca.* 20%) aqueous hydrochloric acid produced the acid derivatives **9**.

Pharmacology

Alkylating activity. For determination of the alkylating properties of the new compounds **4**, **6**–**10** we chose an *in vitro* Preussmann[20] test employing 4-(4-nitrobenzyl)pyridine (NBP) as the target molecule. Alkylation of the nitrogen atom of the pyridine ring of NBP results in formation of a quaternary salt, which under the alkaline conditions is transformed into a neutral coloured compound. The extent of alkylation can be quantified spectrophotometrically at 560 nm. Thus, the compounds **4a**,**b**, **6a**,**b**–**10a**,**b** were screened for their alkylating activity toward NBP at a concentration of 1.67×10^{-4}–1×10^{-3} M in 2-methoxyethanol. For comparison we quote the data of alkylating activity of trimethyl phosphite, trimethyl phosphate, and the widely accepted alkylating drug cyclophosphamide.[20,21] The results obtained are presented in Table 1. Compounds **4**, **6** and **7** possess strong alkylating properties (+++) while compounds **10** are comparable to cyclophosphamide. The remaining derivatives **8** and **9** are very poor alkylating agents.

Antitumour activity. The two phosphonic derivatives of chromone, **7a** and **7b**, demonstrating the strongest alkylating activity *in vitro*, and the derivative **9a**, representing a new class of phosphonolactone derivatives of chromone, were selected for biological studies in an animal model (hybrid male CD2F1 mice, weighing 22–30 g, 8–12 weeks old). The antineoplastic activity of the test compounds was examined against two murine leukaemias: L1210 and P388. L1210 or P388

Table 1 NBP test results

Compound	Conc/10^{-3} M	Absorbance A^a	Alkylation activity[b]
4a	0.5	0.7243	+++
4b	0.5	0.7633	+++
6a	0.167	0.5438	+++
6b	0.167	0.5693	+++
7a	1	0.9840	+++
7b	1	1.1268	+++
8a	1	0.0627	+
8b	1	0.0626	+
9a	1	0.0680	+
9b	1	0.0745	+
10a	1	0.1025	++
10b	1	0.1037	++
Cyclophosphamide	1	0.1200	++
Trimethyl phosphite	1	0.5300	+++
Trimethyl phosphate	1	0.9000	+++

[a] Means from 3 determinations. [b] According to Preussmann[20]: (–) $A < 0.05$, (+) $A = 0.05$–0.10, (++) $A = 0.10$–0.50, (+++) $A > 0.50$.

Table 2 Antitumour activity of derivatives **7a**, **7b** and **9a** in *in vivo* tests against L1210 and P388 leukaemias in mice

	L1210				P388		
Compd.	Dose[a] /mg kg^{-1}	MST[b] /days	ILS[c] (%)	ALD_{50}[d] /mg kg^{-1}	MST/days	ILS (%)	ALD_{50}/mg kg^{-1}
7a	750	14	0		10	−9	
	500	14	0	1000	10	−9	1000
	250	14	0		11	0	
7b	1500	11	−21		10	−9	
	1000	14	0	2000	10	−9	2000
	500	14	0		14	+27	
9a	800*	2	Toxic		2	Toxic	
	400*	4	Toxic	1200	2	Toxic	1200
	200	14	0		11	0	
Control (untreated)	–	14	–		11	–	

[a] Dose administered the 1st, 3rd and the 5th day after inoculation, except for *, where the drug was administered once on the first day after inoculation. [b] Median survival time. [c] Increase of lifespan. [d] Approximate lethal dose.

leukaemia cells (3×10^5) were intraperitoneally implanted. The test compounds were administered three times: on the first, third and fifth day after inoculation of the leukaemia cells. The results are collected in Table 2. Compounds **7a** and **9a** are not effective in the treatment of the L1210 and P388 leukaemias in mice. Those mice treated with various doses of **7a** and **9a** lived as long as or shorter than the mice of the control group. In contrast, compound **7b** exhibits significant antitumour activity against P388 leukaemia after administration of 500 mg kg^{-1} three times (the 1st, 3rd and 5th day after inoculation). Increase of the lifespan (ILP) of the P388 infected mice is estimated at around 27%. Compound **7b** demonstrates antitumour activity only in the case of the cell line P388 at a dose of 500 mg kg^{-1}. The results of treating infected mice with **9a** indicate that it is significantly more toxic than **7a** and **7b**. These experiments also demonstrate that the approximate lethal dose (ALD_{50}) of derivative **7b** is 2000 mg kg^{-1}.

Discussion

Alkyl phosphites react with bromomethylketones by two possible mechanisms.[22,23] One mechanism involves nucleophilic attack either at halogen, to give an intermediate salt that rearranges, or at the methylene carbon (the Arbusov reaction[24]). Both routes lead to the same product, a phosphonate. Alkyl phosphites can also react with the carbon of the carbonyl group of bromomethylketones according to the Perkov mechanism, resulting in formation of enol-phosphate derivatives.[25] In our experiments the reaction of trimethyl phosphite with bromoderivatives **3** (**a**: R = H, **b**: R = Me, Scheme 1) led to products **4**, **6** and **7**. Product **4**, obtained in *ca.* 40% yield, is a typical Perkov product while **6** and **7** (*ca.* 10% and 40% yield, respectively), are the products of intramolecular cyclisation and elimination. The structures of **6** and **7** suggest that the first step of the reaction involves nucleophilic attack of phosphite at either halogen or at the methylene carbon of **3**, in a typical Arbusov process, leading to the unstable phosphonate **5**. This compound cyclises to a mixture of diastereoisomers **5′** that subsequently either lose dimethyl phosphate to give the Wittig-type[26] product **6** or undergo 1,2-*trans*-elimination of water on the column to give the 3-phosphonic chromone derivative **7**. A stable Arbusov product of type **5** was isolated when derivative **3** had a benzoyl instead of an *O*-acetylmandeloyl moiety.[15,16] However, the presence of the *O*-acetyl substituent in **5** significantly increases the partial positive charge on the carbonyl carbon atom of the mandelic residue, facilitating intramolecular cyclisation of this derivative. Cyclisation of **5** to **5′** is additionally facilitated by the presence of a carbonyl group at the beta position to the phosphonic residue, making the alpha protons more acidic. The presence of intermediate **5′** was observed by ^{31}P magnetic resonance spectroscopy of a crude reaction mixture in series **b**. A resonance signal at 23.72 ppm (integration of 46%) does not appear in any of the isolated products. Instead, a new signal at 15.94 ppm appears in the spectrum of product **7**, isolated in 35.5% yield after column chromatography.

In general alkylating agents are the first class of cytostatics used for therapy.[27] Under *in vivo* conditions these agents alkylate nucleophilic centres of nucleobases and amino acids, resulting either in cleavage or cross-linking of double-stranded DNA molecules or proteins. Such cleavage causes damage of DNA while covalent cross-links prevent unwinding of nucleic acids, functionally important in replication and transcription processes.[28] The alkylating properties of the test derivatives can be determined by an *in vitro* Preussmann test.[20] This test permits the estimation of the direct ability of the compound to alkylate the model target molecule 4-(4-nitrobenzyl)pyridine and it provides a useful indication of alkylation potential for nucleophilic centres of aminoacids and nucleobases. However, due to its simplicity, the Preussmann test does not allow the determination of the mode of DNA alkylation. Despite this drawback, this test has been used to determine the alkylating potential of several trialkyl phosphate, phosphite and phosphonate derivatives as well as cyclophosphamide toward NBP.[20] We used this test to determine the alkylating properties of the derivatives **4**, **6**–**10** (Table 1). Compounds **4**, **6**, and **7** possess strong alkylating activity. Compounds **10** have moderate potential for alkylation, whereas the remaining compounds **8** and **9** are only weakly active. The test compounds **4, 6** and **7** exhibit higher alkylating activity *in vitro* than trimethyl phosphite and cyclophosphamide, and comparable alkylating properties to trimethyl phosphate.[20] It should be noted that cyclophosphamide, which is a widely known alkylating drug, has significantly stronger alkylating activity *in vivo* due to its metabolic transformation into an active alkylating species.[21]

It is interesting to correlate the alkylating properties of the test compounds with their *in vivo* antitumour activity. The phosphonic derivatives of chromone, **7a** and **7b**, demonstrating the strongest alkylating activity *in vitro*, and compound **9a**, representing a new class of phosphonolactone derivatives of chromone, were selected for antitumour studies in leukaemia L1210 and P388 infected mice. Only compound **7b** at a dose of 500 mg kg^{-1} demonstrates antitumour activity in the P388 cell line (Table 2). The results of treating infected mice with **9a** indicate that it is significantly more toxic than **7a** and **7b**.

The interesting biological activity of **7b** encouraged us to investigate further this class of compounds. Thus, we asked the question whether the pure enantiomers of compound **7b** would differ in their biological properties. Starting from

enantiomerically pure mandelic acid precursors, we obtained both enantiomers of **7a** and both enantiomers of **7b**. X-Ray analysis showed that the levorotatory product has the *R* configuration of the chiral centre. The results of preliminary evaluation of biological activity indicate significant differences between the enantiomers of both derivatives **7a** and **7b**. These results will be published elsewhere.

Conclusions

The 3-dimethoxyphosphonyl derivatives **7a** and **7b** have been prepared. Hydrolysis afforded the corresponding acetates **8a** and **8b** or phosphonolactones **9a** and **9b**. These compounds were tested for their *in vitro* alkylating and selected ones for their *in vivo* antitumour activity. Most of the tested compounds possess strong or moderate alkylating activity, while compound **7b** demonstrates significant *in vivo* antitumour activity against leukaemia P388 cells in an animal model.

Experimental

Chemistry: materials and methods

The (±)-*O*-acetylmandelic acid used in this study was purchased from Aldrich. Purity of all compounds was analysed on silica gel PF_{254} plates (Merck). Column chromatography was carried out using silica gel 30–60 μm (Baker), and a mixture of chloroform–acetone (5 : 1, v/v) as eluent. 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 solid KBr. NMR spectra were registered on a Varian Mercury spectrometer (^{1}H 300 MHz; ^{13}C 75.5 MHz; ^{31}P 121.5 MHz). In the ^{31}P spectra positive chemical shifts are assigned to resonances downfield of phosphoric acid. The MS data were obtained on a Finnigan Matt mass spectrometer (100 eV ionisation energy) with isobutane as reagent. UV/VIS spectra were obtained at 800–400 nm on a Lambda 19 Perkin Elmer instrument. Satisfactory elemental analyses (±0.4% of the calculated values) were obtained for the new compounds. Elemental analyses were performed by the Microanalytical Laboratory of the Institute of Chemistry using a Perkin Elmer PE 2400 CHNS analyser.

Syntheses

Synthesis of compounds 2a and 2b. Racemic *O*-acetylmandeloyl chloride (22.6 mmol) was added dropwise with intensive stirring into a cooled (ice-bath) solution of sodium 2-hydroxy- or 2-hydroxy-5-methyl-acetophenone **1a** or **1b** (22.6 mmol) dissolved in 150 ml of diethyl ether. The stirring was continued for an additional hour. Then water (20 ml) was added to the reaction mixture and it was extracted with chloroform (4 × 50 ml) and with aqueous sodium bicarbonate. The collected organic layers were dried with $MgSO_4$ and concentrated under reduced pressure. Products **2a** and **2b** were obtained as pale yellow oils.

*(±)-Acetoxyphenylacetic acid 2-acetylphenyl ester (***2a***).* Yield 84.2%. Oil. IR (KBr): ν = 1781.7, 1741.1, 1709.1 (C=O) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.17 (s, 3H, CH_3); 2.35 (s, 3H, CH_3); 6.71 (s, 1H, CH); 7.21–8.21 (m, 9H, aromat.). EI MS *m*/*z* (%): 312 (13, M^+), 253 (20), 225 (52), 177 (43), 135 (45), 121 (53), 107 (61). Anal. found: C, 69.34; H, 5.30. Calcd. for $C_{18}H_{16}O_5$ (312.31): C, 69.22; H, 5.16%.

*(±)-Acetoxyphenylacetic acid 2-acetyl-4-methyl-phenyl ester (***2b***).* Yield 92.6%. Oil. IR (KBr): ν = 1746.1, 1687.7 (C=O), 1643.0 (C=O), 1050 (C–O–C) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.24 (s, 3H, CH_3), 2.25 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 6.18 (s, 1H, CH), 7.02–7.81 (m, 8H, aromat.). EI MS *m*/*z* (%): 327 (3, M^+), 239 (17), 225 (10), 177 (78), 151 (29), 149 (100), 135 (43). Anal. found: C, 69.56; H 5.53. Calcd. for $C_{19}H_{18}O_5$ (326.33): C, 69.92; H 5.56%.

Synthesis of compounds 3a and 3b. Bromine (0.01 mol) was added dropwise to a carbon tetrachloride solution (50 ml) of compound **2a** or **2b** (0.01 mol). The mixture was refluxed for 15 min. The solvent was evaporated under reduced pressure and the semisolid residue was suspended in 50 ml of diethyl ether. The resultant colourless crystalline product **3a** or **3b** was filtered off and washed with diethyl ether.

*(±)-Acetoxyphenylacetic acid 2-(2-bromoacetyl)phenyl ester (***3a***).* Yield 79%. M.p. = 90.1–93.1 °C. IR (KBr): ν = 1773.4 (C=O); 1741.2 (C=O); 1702.3 (C=O) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.23 (s, 3H, CH_3); 4.09 (s, 2H, CH_2); 6.17 (s, 1H, CH); 7.08–7.82 (m, 6H, arom.). EI MS *m*/*z* (%): 391 (9, M^+), 333 (6), 304 (9), 211 (22), 177 (96), 135 (100), 107 (13). Anal. found: C, 55.46; H, 3.91. Calcd. for $C_{18}H_{15}O_5Br$ (391.20): C, 55.26; H, 3.86%.

*(±)-Acetoxyphenylacetic acid 2-(2-bromoacetyl)-4-methylphenyl ester (***3b***).* Yield 73.6%. M.p. = 89.1–91.0 °C. IR (KBr): ν 1779.9 (C=O); 1737.4 (C=O); 1700.0 (C=O); 1040.0 (C–O–C) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.24 (s, 3H, CH_3); 2.36 (s, 3H, CH_3); 4.05 (s, 2H, CH_2); 6.14 (s, 1H, CH); 6.96–8.01 (m, 8H, aromat.). EI MS *m*/*z* (%): 406 (58, M^+), 335 (100), 228 (35), 177 (63), 149 (13), 107 (78), 77 (16). Anal. found: C, 56.04; H, 3.99. Calcd. for $C_{19}H_{17}O_5Br$ (405.23): C, 56.31; H, 4.23%.

Synthesis of compounds 4, 6 and 7. To 10 mmol of the corresponding acetate **3a** or **3b** melted in a flask, 12 mmol of trimethyl phosphite were added dropwise at 110–115 °C. After 30 min of 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 chloroform–acetone (5 : 1, v/v). Compounds **4a,b** were obtained as oils while the crude products **6** and **7** were purified by crystallisation.

*(±)-Acetoxyphenylacetic acid 2-[1-(dimethoxyphosphoryloxy)-vinyl]phenyl ester (***4a***).* Yield 38%; R_f = 0.63, oil. IR (KBr): ν = 1744 (C=O), 1258 (P=O), 1104 (P–O–C) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.20 (s, 3H, CH_3); 3.71 (d, 3H, O–CH_3, $^3J_{PH}$ = 11.31 11.31 Hz); 3.74 (s, 3H, O–CH_3, $^3J_{PH}$ = 11.31 Hz); 4.77 (t, 1H, CH_2, $^4J_{PH}$ = 2.4 Hz); 5.02 (t, 1H, CH_2, $^4J_{PH}$ = 2.4 Hz); 6.17 (s, 1H, CH); 7.02–7.58 (m, 9H, aromat.). ^{13}C NMR ($CDCl_3$): δ 20.65, 54.74 (d, $^2J_{PC}$ = 6.01 Hz); 103.0; 102.94; 122.36, 127.41; 167.1 (C=O); 170.3 (C=O). ^{31}P NMR ($CDCl_3$): δ −3.9. EI MS (70 eV) *m*/*z* (%): 244 (100) 245 (8), 135 (19), 127 (96), 118 (35), 109 (21), 107 (37), 105 (8), 90 (17); Anal. found: C, 56.89; H, 5.03, P, 7.70. Calcd. for $C_{20}H_{21}O_8P$ (420.34): C, 57.14; H, 5.04, P, 7.37%.

*(±)-Acetoxyphenylacetic acid 2-[1-(dimethoxyphosphoryloxy)-vinyl]-4-methylphenyl ester (***4b***).* Yield 36%, R_f = 0.62; oil. IR (KBr): ν = 1774.2, 1746.9 (C=O), 1232.9 (P=O), 1048.9 (C–O–C), 1006 (P–O–C) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.20 (s, 3H, CH_3); 2.32 (s, 3H, CH_3); 3.72 (d, 3H, O–CH_3, $^3J_{PH}$ = 11.31 Hz); 3.74 (d, 3H, O–CH_3, $^3J_{PH}$ = 11.31 Hz); 4.72 (t, 1H, CH_2, $^4J_{PH}$ = 2.4 Hz); 4.99 (t, 1H, CH_2, $^4J_{PH}$ = 2.4 Hz); 6.15 (s, 1H, CH); 6.89–7.58 (m, 8H, aromat.); ^{13}C NMR ($CDCl_3$): δ 20.67, 20.88; 54.74 (d, $^2J_{PC}$ = 6.01 Hz); 103.0; 102.94; 122.36, 127.41; 167.1 (C=O); 170.3 (C=O). ^{31}P NMR ($CDCl_3$): δ −3.93. EI MS *m*/*z* (%): 258 (100), 243 (3), 149 (12), 132 (62), 127 (22), 107 (16); Anal. found: C, 57.75; H, 5.33, P, 7.33. Calcd. for $C_{21}H_{23}O_8P$ (434.36): C, 58.06; H, 5.34, P, 7.13%.

*(±)-(4-Oxo-4H-chromen-2-yl)phenylmethyl acetate (***6a***).* Yield 9%, M.p. = 131.4–132.0 °C (diethyl ether); R_f = 0.86. IR (KBr): ν = 1764.1, 1658.8 (C=O), 1620 (C=C), 1034 (C–O–C) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.22 (s, 3H, CH_3); 6.48 (s, 1H, CH); 6.62 (s, 1H, CH); 7.42–7.58 (m, 9H, aromat.).

^{13}C NMR ($CDCl_3$): δ 20.54 (CH_3); 74.43 (CH); 76.59 (CH); 123.98; 128.08; 131.16; 146.22; 170.39 (C=O); 189.82 (C=O). EI MS m/z (%): 295 (100, $M^+ + 1$); 245 (16). Anal. found: C, 73.57; H, 4.89. Calcd. for $C_{18}H_{14}O_4$ (294.29): C, 73.46; H, 4.79%.

*(±)-(6-Methyl-4-oxo-4H-chromen-2-yl)phenylmethyl acetate (**6b**).* Yield 10.5%, M.p. = 73.5–75.0 °C (diethyl ether); $R_f = 0.84$. IR (KBr): ν = 1751.2, 1652.3 (C=O), 1616 (C=C), 1045 (C–O–C) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H, CH_3); 2.23 (s, 3H, CH_3); 6.45 (s, 1H, CH); 6.62 (s, 1H, CH); 7.27–7.95 (m, 8H, aromat.). ^{13}C NMR ($CDCl_3$): δ 20.98 (CH_3); 21.1 (CH_3); 74.89 (CH); 76.80 (CH); 122.95; 128.08; 130.16; 167.22; 170.60 (C=O); 191.02 (C=O). CI MS m/z (%): 309 (100, $M^+ + 1$); 251 (27). Anal. found: C, 73.88; H, 5.40. Calcd. for $C_{19}H_{16}O_4$ (308.32): C 74.01, H, 5.23%.

*(±)-2-(1-Acetoxybenzyl)-3-(dimethoxyphosphoryl)-4-oxo-4H-chromene (**7a**).* Yield 40.5%, M.p. 128.6–129.1 °C (ethyl ether–methanol, 25 : 2), $R_f = 0.56$. IR (KBr): ν = 1746.1 (C=O); 1648.2 (C=O); 1238.6 (P=O); 1024 (P–O–C); 1045 (C–O–C) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.20 (s, 3H, CH_3); 3.85 (d, 3H, –OCH_3, $^3J_{PH}$ = 11.7 Hz); 3.92 (d, 3H, –OCH_3, $^3J_{PH}$ = 11.7 Hz), 6.75 (s, 1H, CH), 7.26–7.78 (m, 9H, aromat.). ^{31}P NMR ($CDCl_3$): δ 16.78. ^{13}C NMR ($CDCl_3$): δ 20.69 (CH_3); 53.34 (d, O–CH_3, $^2J_{PC}$ = 5.70 Hz); 72.28 (CH); 117.49; 125.42; 127.70; 170.10 (C=O); 172.42 (d, C=O, $^2J_{PC}$ = 25.35 Hz). EI MS (70 eV) m/z (%): 402 (0.3, M^+); 359 (9); 342 (27); 329 (25); 328 (100); 313; 295; 105. Anal. Found: C, 59.88; H, 4.70; P, 7.81. Calcd. for $C_{20}H_{19}O_7P$ (402.32): 59.70; H, 4.76; P, 7.70%.

*(±)-2-(1-Acetoxybenzyl)-3-(dimethoxyphosphoryl)-6-methyl-4-oxo-4H-chromene (**7b**).* Yield 35.5%, M.p. 137.0–137.9 °C (acetone), $R_f = 0.57$. IR (KBr): ν = 1743 (C=O); 1649 (C=O); 1236 (P=O); 1024 (P–O–C); 1045 (C–O–C) cm^{-1}. ^{1}H NMR: ($CDCl_3$) δ 2.19 (s, 3H, CH_3); 2.44 (s, 3H, CH_3); 3.74 (d, 3H, –OCH_3, $^3J_{PH}$ = 11.7 Hz); 3.92 (d, 3H, –OCH_3, $^3J_{PH}$ = 11.9 Hz). ^{31}P NMR ($CDCl_3$): δ 16.78. ^{13}C NMR ($CDCl_3$): δ 20.69 (CH_3); 21.00 (CH_3); 53.34 (d, O–CH_3, $^2J_{PC}$ = 5.70 Hz); 72.28 (CH); 117.49; 125.42; 127.70; 170.10 (C=O); 172.42 (d, C=O, $^2J_{PC}$ = 25.35 Hz). EI MS (70 eV) m/z (%): 416 (100, M^+), 384 (40), 342 (70), 105 (3). Anal. found: C, 60.77; H, 5.28; P, 7.54. Calcd. for $C_{21}H_{21}O_7P$ (416.35): C, 60.58; H, 5.08; P, 7.44%.

Synthesis of the racemic acids 8a and 8b. A 30% solution of hydrobromic acid in acetic acid was added at room temperature to 0.7 mmol of the corresponding ester **7a** or **7b**. The reaction mixture was stirred at room temperature for 24 h. The precipitated solid was filtered and washed with water and acetone.

*(±)-[4-Oxo-3-phosphono-4H-chromen-2-yl]phenylmethyl acetate (**8a**).* Yield 68%, M.p. 208.5–209 °C (acetone). IR (KBr): ν 3100–2560 (OH), 1752.8, 1620 (C=O); 1244 (P=O) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.22 (s, 3H, CH_3); 6.22 (s_{broad}, 2H, OH); 7.42–7.58 (m, 9H, aromat.); 8.39 (s, 1H, CH). ^{13}C NMR (DMSO-d_6): δ 20.51 (CH_3); 71.06 (CH); 114.07 (d, C–P, $^1J_{PC}$ = 177.71 Hz); 121.45; 122.01; 128.37; 152.78; 167.54 (d, C=O, $^2J_{PC}$ = 20.94 Hz); 169.24; 176.09. ^{31}P NMR (DMSO-d_6): δ 7.9. FAB-MS: 375 $[M]^+$. Anal. found: C, 58.10; H, 3.83; P, 8.15. Calcd. for $C_{18}H_{15}O_4P$ (374.27): C, 57.76; H, 4.04; P, 8.27%.

*(±)-[6-Methyl-4-oxo-3-phosphono-4H-chromen-2-yl]phenylmethyl acetate (**8b**).* Yield 62.96%, M.p. 201.5–202.5 °C (acetone). IR (KBr): ν 1761.2, 1630.3 (C=O); 1225.4 (P=O) 1003 (P–O); 3070.1–2813.0 (OH) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.18 (s, 3H, CH_3); 2.50 (s, 3H, CH_3); 7.33–7.78 (m, 8H, aromat.); 8.32 (s, 1H, CH). ^{13}C NMR (DMSO-d_6): δ 20.37 (CH_3), 20.68 (CH_3), 71.12 (CH), 112.03 (d, C–P, $^1J_{PC}$ = 177.71 Hz), 117.82, 124.11, 126.76, 128.43, 135.82, 135.91, 136.47, 152.73, 169.14 (d, C=O, $^2J_{PC}$ = 20.86 Hz). ^{31}P NMR (DMSO-d_6): δ 6.49. FAB-MS: 388 $[M]^+$. Anal. found: C, 59.02; H, 4.32; P, 7.93. Calcd. for $C_{19}H_{17}O_7P$ (388.30): C, 58.77; H, 4.41; P, 7.98%.

Synthesis of compounds 9a and 9b. Method A. A 30% hydrobromic acid solution in glacial acetic (2.1 mmol) was added to 0.7 mmol of the corresponding acetate **7a** or **7b**. The reaction mixture was kept at room temperature for 24 h. The precipitated product was collected and washed with acetone.

*(±)-1-Hydroxy-1-oxo-3-phenyl-1,3-dihydro-1λ^5-2,1-oxaphospholo[4,5-b]-4**H**-1-benzopyran-4-one (**9a**).* Yield 67.5%, M.p. 261.5–262 °C (acetone). IR (KBr): ν = 3349 (P–OH); 1759.4 (C=O); 1249.2 (P=O) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 6.49 (d, 1H, CH, $^3J_{P-H}$ = 8.3 Hz); 7.28–8.13 (m, 8H, aromat.); 12.01 (s_{broad}, 1H, OH). ^{13}C NMR (DMSO-d_6): δ 76.78 (CH–Ph); 107.72 (d, C–P, $^1J_{PC}$ = 173.49 Hz), 118.55; 128.89; 173.12 (d, C=O, $^2J_{PC}$ = 12.31 Hz). ^{31}P NMR (DMSO-d_6): δ 25.1. FAB-MS: 315 $[M+H]^+$. Anal. found: C, 60.87; H, 3.19; P, 9.71. Calcd. for $C_{16}H_{11}O_5P$ (314.14): C, 61.18; H, 3.50; P, 9.86%.

*(±)-1-Hydroxy-7-methyl-1-oxo-3-phenyl-1,3-dihydro-1λ^5-2,1-oxaphospholo-[4,5-b]-4**H**-1-benzopyran-4-one (**9b**).* Yield 80.5%, M.p. 207.8–209.5 °C (acetone). IR (KBr): ν = 3419.8 (P–OH); 1762.2, 1645 (C=O); 1226.1 (P=O) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.21 (s, 3H, CH_3); 6.41 (d, 1H, CH, $^3J_{P-H}$ = 8.3 Hz); 6.98–7.91 (m, 8H, aromat.); 12.01 (s_{broad}, 1H, OH). ^{13}C NMR (DMSO-d_6): δ 20.19 (CH_3), 77.18 (CH–Ph); 107.52 (d, C–P, $^1J_{PC}$ = 173.50 Hz); 118.35; 128.29; 172.12 (d, C=O, $^2J_{PC}$ = 12.31 Hz). ^{31}P NMR (DMSO-d_6): δ 24.31. FAB-MS: 388 $[M]^+$. Anal. C, 62.38; H, 4.08; P, 9.48. Calcd. for $C_{19}H_{17}O_7P$ (388.30): C, 62.20; H, 3.99; P, 9.44%.

Synthesis of compound 9a. Method B. To a 50% aqueous methanolic solution of benzylammonium salt **10a** (0.1 mmol), a solution of hydrochloric acid was added to pH 6. The precipitated solid was filtered off, dried and crystallised from acetone. Compound **9a** was obtained in 56% yield.

Synthesis of compounds 10a and 10b. To a solution of **7a** or **7b** (1.00 mmol) in methanol (2 ml), 2.0 mmoles of benzylamine were added. The mixture (after standing overnight at room temperature in the case of **7b**) was then cooled to −10 °C to initiate crystallisation. The colourless product was obtained in 75–78% yield. The crude product was purified by further crystallisation from acetone.

*1-Hydroxy-1-oxo-3-(±)-3-phenyl-1,3-dihydro-1λ^5-2,1-oxaphospholo-[4,5-b]-4**H**-1-benzopyran-4-on benzylammonium salt (**10a**).* Yield 67%, M.p. 214–216 °C (methanol). IR (KBr): ν = 3442.03–3035.67 (–NH_3^+–CH_2Ph); 1648.74 (C=O), 1204.2 (P=O) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 4.04 (s, 2H, NH–CH_2), 5.96 (d, 1H, $^3J_{HP}$ = 8.22 Hz), 7.28–7.67 (m, 9H, arom.), 8.71 (s, 3H, –NH_3^+). ^{31}P NMR (DMSO-d_6): δ 20.23. MS (70 eV): m/z (%) 421 (5, M^+), 258 (100), 149 (19), 132 (62), 127 (21), 43 (15). Anal. found: C, 65.78; H, 4.98; N, 3.43; P, 7.56. Calcd. for $C_{23}H_{20}NO_5P$ (421.37): C, 65.56; H, 4.78; N, 3.32; P, 7.35%.

*1-Hydroxy-7-methyl-1-oxo-3-(±)-3-phenyl-1,3-dihydro-1λ^5-2,1-oxaphospholo-[4,5-b]-4**H**-1-benzopyran-4-on benzylammonium salt (**10b**).* Yield 75%, M.p. 176–177 °C (acetone). IR (KBr): ν = 3393.3–3035.67 (–NH_3^+–CH_2Ph); 1597.2 (C=O), 1578 (C=C) 1204.8 (P=O), 1047.0 (P–O–C) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.20 (s, 3H, CH_3), 3.80 (s, 2H, NH–CH_2), 5.91 (d, 1H, $^3J_{HP}$ = 11.12 Hz), 7.03–7.79 (m, 13H, aromat.), 10.05 (s, 3H, –NH_3^+). ^{31}P NMR (DMSO-d_6): δ 29.88. ^{13}C NMR (DMSO-d_6): δ 20.19 (CH_3), 40.07 (CH_2–N), 74.77 (CH), 119.5, 127.15, 128.32, 130.14, 134.93, 154.13, 179.1 (C=O). MS (70 eV): m/z (%) 435 (3, M^+), 328 (9), 310 (20), 248 (15), 107 (51), 106 (88), 91 (100), 77 (40). Anal. found: C, 66.48; H, 4.98; N, 3.43; P, 7.36. Calcd. for $C_{24}H_{22}NO_5P$ (435.40): C, 66.20; H, 5.09; N, 3.22; P, 7.13%.

Pharmacology

Alkylating properties (NBP test). The test compounds (0.835–5.00 μmol) were dissolved in 2-methoxyethanol (1 ml) and a solution of NBP in 2-methoxyethanol (5% solution, 1 ml) was added. The samples were heated at $100 \pm 0.5\,^{\circ}C$ for 1 h and then quickly cooled to 20 °C. 2-Methoxyethanol (2.5 ml) and piperidine (0.5 ml) were added to the samples to give a total volume of 5 ml. The final concentration of the test compound was 1.67×10^{-4}–1×10^{-3} M. After 90 s the absorbance was measured at $\lambda = 560$ nm in a glass cell (1 cm), in the presence of 2-methoxyethanol (Table 1).

Animals. Hybrid male CD2F1(BALB/c × DBA/2)F1 mice weighing 22–30 g, 8–12 weeks old, purchased form the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw), were used in experiments allowing the determination of the antitumour activity of the test compounds against selected leukaemia. Mice were fed with standard laboratory food and water *ad libitum*.

Leukaemia cells. Leukaemia cells L1210 and P388 were purchased from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw) and were maintained by serial passages *in vivo*. Leukemic cells from the fluid were resuspended in 0.9% NaCl so that 3×10^5 L1210 or P388 cells were injected intraperitoneally (i.p.) into mice.

Therapeutics. The compounds were administered in a volume of 0.1 ml g^{-1} of mouse weight (1% aqueous solution of methylcellulose). Control mice received equivalent volumes of a 0.9% NaCl solution.

Toxicity determination. The approximate lethal dose (ALD_{50}) was determined by the method described by Deichmann and Le Blanck[29] using male CD2F1 mice.

Antileukaemia assay. L1210 or P388 leukaemia cells (3×10^5) were implanted i.p. into CD2F1 mice. Five mice were used for group. The tested compounds were administered three times within the first, third and fifth day after inoculation. The mice of the control group received saline the same day of treatment. The mice were observed daily for survival. The median survival time (MST) according to the method of Geran *et al.*[30] is: $MST = (x+y)/2$, where x denotes the earliest day when the number of dead animals is $> N/2$, y denotes the earliest day when the number of dead animals is $> (N/2+1)$ and N denotes the number of animals in the group. The antileukaemic effect of the drug was assessed as percentage ratio of MST of the treated group (T) to that of the control group (C): ILS (increase of the lifespan) $= [(MST_T/MST_C)-1] \times 100\%$).

Statistical analysis. The results were evaluated using the Student's test for differences between means. Differences were considered significant when $p < 0.05$.

Acknowledgements

Financial support from the Medical University of Lodz (grant No 502-13-755 to E.B. and 502-13-613 to J. G.-W.) is gratefully acknowledged. We thank Mrs. Agnieszka Zdolska for skillful experimental assistance.

References

1 A. M. El-Nagger, A. M. Abdel-El-Salam, F. S. M. Latif and M. S. A. Ahmed, *Pol. J. Chem.*, 1981, **55**, 793.
2 K. A. Ohemeng, C. F. Schwender, K. P. Fu and J. F. Barrett, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 225.
3 M. Okuhara and T. Goto, *Drugs Exp. Clin. Res.*, 1981, **7**, 559.
4 E. Middleton and C. Kandaswami, in The Impact of Plant Flavonoids on Mammalian Biology. Implication for Immunity, Inflammation and Cancer. *The Flavonoids Advances in Research Since 1986*, ed. J. B. Harborne, Chapman Hall, London, 1994, pp. 619–652.
5 P. Kafarski and B. Lejczak, *Phosphorus, Sulfur Silicon Relat. Elem.*, 1991, **63**, 193.
6 E. Bayer, K. H. Gugel, K. Heagele, H. Hagenmeier, S. Jessipow, W. A. Koening and R. M. Zahmer, *Helv. Chim. Acta*, 1972, **55**, 224.
7 Y. Ogawa, T. Tsuruaka, S. Inouye and T. Niida, *Sci. Rep. Meiji Seika Kaisha*, 1973, **13**, 42.
8 V. P. Kukhar and H. R. Hudson, in *Aminophosphonic and Aminophosphinic Acids. Chemistry and Biological Activity*, ed. V. P. Kukhar and H. R. Hudson, John Wiley & Sons Ltd, London, 2000, ch. 1.
9 H. P. Kuemmerle, T. Murakawa, H. Sakamoto, N. Sato, T. Konishi and F. De Santis, *Int. J. Clin. Pharmacol. Therapy Toxicol.*, 1985, **23**, 515.
10 M. Gray and D. Smith, *Tetrahedron Lett.*, 1980, **21**, 859.
11 K. Troev and G. Botisov, *Phosphorus, Sulfur Relat. Elem.*, 1987, **29**, 129.
12 J. D. Zech, *US Pat.* 2815345, 1957 (*Chem. Abs.*, 1958, **52**, 5485).
13 J. D. Zech, *US Pat.* 2824113, 1958 (*Chem. Abs.*, 1958, **52**, 11 110).
14 R. B. Crawford, *US Pat.* 3253036, 1966 (*Chem. Abs.*, 1966, **65**, 5397).
15 E. Budzisz, E. Nawrot and M. Malecka, *Arch. Pharm.*, 2001, **334**, 381.
16 K. Kostka, S. Pastuszko and M. Porada, *Phosphorus, Sulfur Silicon Relat. Elem.*, 1992, **71**, 67.
17 E. Budzisz and S. Pastuszko, *Tetrahedron*, 1999, **55**, 4815.
18 K. Kostka, S. Pastuszko, A. Kotyński and E. Budzisz, *Phosphorus Sulfur Silicon Relat. Elem.*, 1998, **134/135**, 199.
19 A. Hercouet, M. Le Corre and Y. Le Floc'h, *Synthesis*, 1982, **7**, 597.
20 R. Preussmann, H. Schneider and F. Epple, *Arzneim.-Forsch.*, 1969, **19**, 1059.
21 R. F. Borch and G. W. Canute, *J. Med. Chem.*, 1991, **34**, 3044.
22 L. M. Peuzner, V. M. Ignat'ev and B. I. Ionin, *Russ. J. Gen. Chem.*, 1998, **68**, 542.
23 V. F. Mironov, L. M. Burnaeva, D. K. Khusnutdinova and I. V. Konovalova, *Russ. J. Gen. Chem.*, 1993, **63**, 1551.
24 B. J. Walker, *Organophosphorus Chemistry*, Penguin Books Ltd., Harmondsworth, Middlesex, England, 1972, pp. 67–68.
25 D. C. E. Corbridge, *Inorganic Chemistry 10. Phosphorus. An Outline of its Chemistry, Biochemistry and Technology*, Elsevier, Amsterdam, 1990, p. 352.
26 J. March, *Advanced Organic Chemistry: Reaction, Mechanisms and Structure*, John Wiley and Sons, New York, USA, 4th edn., 1992, pp. 956–963.
27 G. Zon, *Prog. Med. Chem.*, 1982, **19**, 205.
28 H. Lindermann and E. Harbers, *Arzneim.-Forsch.*, 1980, **30**, 2075.
29 W. B. Deichmann and T. Le Blanck, *J. Ind. Hyg. Toxicol.*, 1945, **25**, 415.
30 R. J. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher and B. J. Abbot, *Cancer Chemother.*, 1992, **3**, 1.