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Synthesis and evaluation of novel phosphasugar anticancer agents*

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Abstract: Starting materials of phosphasugars, 1-phenyl-2-phospholene 1-oxides, were prepared from dienes and phenylphosphonous dichloride (dichlorophenylphosphine). Several substituted novel phosphasugars (3- or 4-halo-substituted)-1-phenyl-2-phospholene 1-oxides as well as 1-phenyl-2-phospholane 1-oxides were prepared from 2-phospholenes. The synthesized compounds were evaluated for their antitumor activities against the leukemia cell lines (U937 and K562) by in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 2,3,4-Tribromo-3-methyl-1-phenylphospholane 1-oxide showed superior antitumor activity against U937 and K562 cell lines in a comparative evaluation with Glivec. The analysis by flow cytometry implied that 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide induced apoptosis to leukemia cell lines.

Keywords: anticancer agents; leukemia cell lines; MTT in vitro evaluation; phosphasugar; phospholanes; tumors.

INTRODUCTION

Phosphorus compounds and sugar derivatives are deeply related to birth, growth, sustenance, reproduction, death, etc., of living things. The replacement of an oxygen–phosphorus bond of a phosphonate affords a carbon–phosphorus bond of a phosphonate, which bestows phosphonates on hydrolytically stable phosphorus derivatives to their phosphate analogues, and the mode of action of phosphonates is often brought by either competitive interaction with the substrate binding regions or as an analogue of the tetrahedral transition states. As a result, synthetic phosphonates were found to be applicable to medicines or agricultural medicines of antibiotics, antivirals, antiosteoclastics, and environmentally friendly herbicides [1]. The phosphonate moiety of the derivatives is also well represented in biologically active

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natural products and can be found in such diverse molecular entities as bialaphos (antifungal, antibiotic, herbicide) and fosfomycin (antibiotic) [2–4].

Sugar derivatives, whose oxygen atom in the hemiacetal ring is replaced by either carbon, nitrogen, or sulfur, are called carba, aza, or thia sugars, respectively, and are generally categorized as pseudo-sugars [5–9]. Pseudo-sugars of these classes are quite familiar as natural products, and many materials of naturally occurring materials as well as chemically synthesized derivatives are known to be biologically active compounds [1,10,11]. On the other hand, phosphasugars have not yet been discovered in nature, and have not received so much attention in the syntheses and the structure–activity studies, despite the reasonable expectations where biological activities might be manifested. Well-established general methodologies for pseudo-sugar synthesis are not necessarily applicable to phosphasugar targets, and we undertook to explore novel synthetic routes starting from phosphorus heterocyclic compounds, particularly 2- or 3-phospholene derivatives [12]. These characteristics of phosphorus compounds would introduce them into clinical use as new drugs. Given this potential for bioactivity of phosphorus compounds, phosphasugar chemistry is one of the interesting and rapidly developing areas of medicinal research [13–15].

We were challenging to develop the novel synthetic routes starting from phosphorus heterocyclic compounds, mainly 2- or 3-phospholene derivatives [16]. Deoxyphosphasugar analogues (2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide and 2,3,4-tribromo-3-methylphospholane 1-oxide), as well as some substituted phosphasugar analogues such as anhydrophosphasugar derivatives have been prepared and structurally characterized, and their bioactivity was investigated [2,11]. The potential of these phosphasugars as anticancer agents was evaluated with the aid of in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell cycle analysis, etc., and it has been demonstrated that they are potential anticancer agents [17], possessing (i) high levels of anticancer activity, (ii) wide spectrum of activity, and (iii) selectivity and specificity against leukemia cell lines, as well as solid tumor cells. Cell cycle analysis indicated that phosphasugars induce apoptosis similar to that observed with Glivec® (Imatinib mesylate), a well-known molecular targeting anticancer agent (Fig. 1) [18].

In this paper, we describe the synthesis and characterization of deoxyhalophosphasugar analogues as well as their properties as molecular targeted antitumor agents.

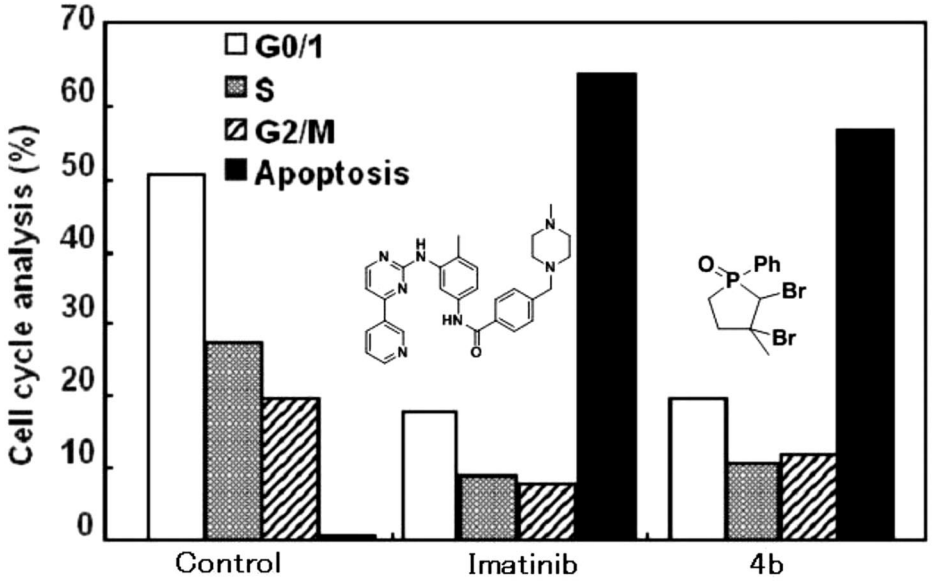
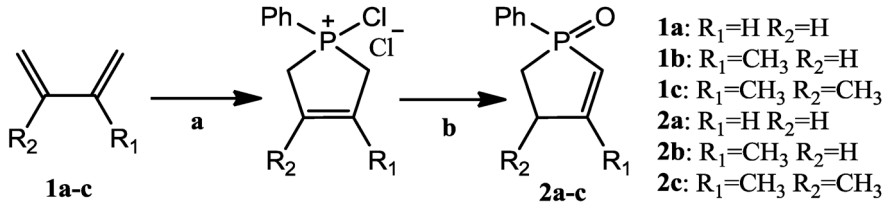


Fig. 1 Effect of compound **4b** and Glivec on cell cycles of DNA of K562 cells for 24 h. The cells were cultured without any compound (control: left), with Imatinib mesylate (Glivec) (center), and with phosphasugar **4b** (right).

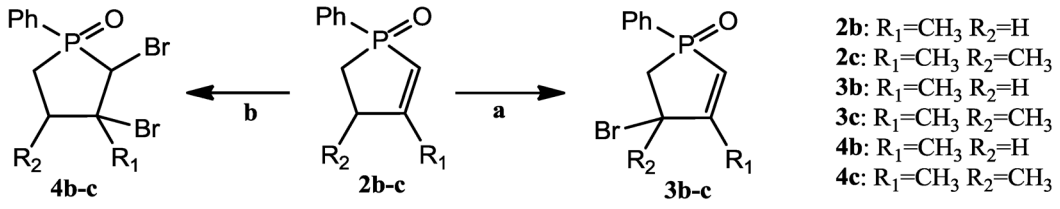
RESULTS AND DISCUSSION

Synthesis

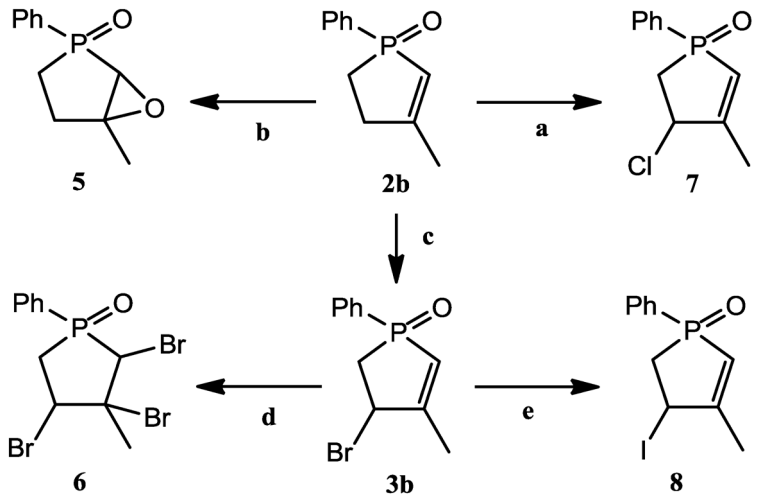
A series of branched phosphasugar analogues of 1-phenyl-2-phospholene 1-oxides **2a–c**; 4-halo-1-phenyl-2-phospholene 1-oxides **3b,c**, **7**, and **8**; 2,3-dibromo-1-phenyl-2-phospholane 1-oxides **4b,c**; 2,3-epoxy-3-methyl-1-phenylphospholane 1-oxide **5**; 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (**6**); and 1-(3-substituted phenyl)-2-phospholene 1-oxides **9–12** as described in Schemes 1–4 were prepared. While the synthesis of compounds **2a–c** was previously described [16], we report herein the more simplified and efficient synthetic methods to produce these compounds. Compounds **1a–c** were prepared from dienes and phenylphosphonous dichloride (phenyldichlorophosphine) by McComack reaction (Scheme 1). Compounds **3b,c** were prepared from compounds **2b,c** and *N*-bromosuccinimide (NBS) by substitution reaction of the hydrogen on the 4-position by a bromo radical in the presence of 2,2'-azobisisobutyronitrile (AIBN) catalyst (Scheme 2). Compounds **4b,c** and **6** were prepared from compounds **2b,c** and **3b**, respectively, by addition reaction of bromine in the presence of catalyst (Schemes 2 and 3). 2,3-Anhydrophosphasugar **5** was prepared by epoxidation of **2b** with *m*-chloroperbenzoic acid (*m*CPBA). 4-Deoxyhalophosphasugars **7** and **8** were prepared from **2b** and **3b**, respectively, by substitution reactions with chloro-radical and iodonium anion, respectively. 1-(3-Substituted phenyl)phosphasugars **9** and **10** were prepared from compound **2b** by nitration with HNO_3/H_2SO_4 and successive treatment with $SnCl_2$ and HCl. And then, compounds **11** and **12** were prepared by the same process as monobromides **3b,c** preparation. The structures of the phosphasugar derivatives were determined by analytical and spectroscopic measurements.



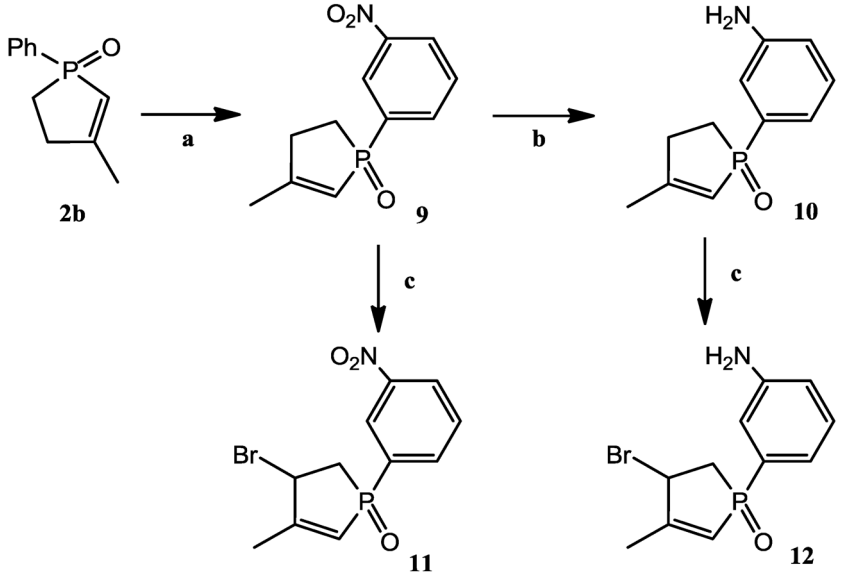
Scheme 1 Preparation of 1-phenyl-2-phospholene 1-oxides **2a–c** (reagents and conditions: (a) $PhPCl_2$; (b) H_2O).



Scheme 2 Preparation of 4-bromo-1-phenyl-2-phospholene 1-oxides **3b**,**c** and 2,3-dibromo-1-phenylphospholane 1-oxides **4b**,**c**. (Reagents and conditions: (a) NBS, AIBN, $CHCl_3$, reflux, 6 h; (b) Br_2, cat., $CHCl_3$, r.t., 12 h.)



Scheme 3 Preparation of 2,3-epoxy-3-methyl-1-phenylphospholane 1-oxide (**5**), 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (**6**), 4-chloro-3-methyl-1-phenyl-2-phospholene 1-oxide (**7**), and 4-iodo-3-methyl-1-phenyl-2-phospholene 1-oxide (**8**) (reagents and conditions: (a) NCS, AIBN, $CHCl_3$, reflux, 6 h; (b) mCPBA, $CHCl_3$, reflux, 24 h; (c) NBS, AIBN, $CHCl_3$, reflux, 6 h; (d) Br_2, CCl_4, reflux, 6 h; (e) NaI, acetone, reflux, 6 h).



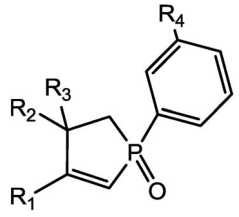
Scheme 4 Preparation of (3-substituted phenyl)phosphasugars **9–12** (reagents and conditions: (a) HNO_3, H_2SO_4, 0 °C -r.t., 24 h; (b) $SnCl_2$, HCl, $CHCl_3$, 0 °C -r.t., 24 h; (c) NBS, AIBN, $CHCl_3$, reflux, 6 h).

Biological activity

Synthesized compounds **2–12** were in vitro evaluated for their antileukemia activity against K562 (human chronic myelogenous leukemia) and U937 (human acute myelogenous leukemia) cell lines by the MTT method [17]. U937 and K562 cells of leukemia cell lines were incubated with the synthesized compounds at the indicated concentrations (0–1000 μM) at 37 °C for 48 h. Some of these phospha-

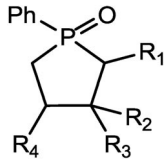
sugars have isomers, which were subjected to the isolation and the in vitro evaluation, otherwise, isomer separation is not mentioned. The results are summarized in Tables 1 and 2. As shown in Table 1, the proliferation of K562 and U937 cells were inhibited by compounds **3b,c** and **11**. Results showed that antileukemia activities for compounds **3b,c** were 87.3 and 18.5 μmol/l (μM) with regard to 50 % cell growth inhibition (IC_{50}) value against K562 cells. Besides, Tables 1 and 2 show that the effects of compounds **3b,c** and **11** on the inhibition of cell proliferation (IC_{50}) were 83.3, 5.9, and 19.8 μM, respectively, against U937 cells. On the other hand, compounds **2a–c**, **7–9**, **10**, and **12** did not inhibit the leukemia cells. The results suggested that dehydrophosphasugars **3b,c**, **11**, and **12** substituted by bromo group have higher activity against K562 and U937 cells than the other derivatives **2a–c** and **7–10**. Substituents of chloro and iodo groups have less antileukemia activity than the bromo group for the inhibition. Therefore, dibromides **4b,c** and tribromide **6** were synthesized and evaluated against the leukemia cells. Table 2 shows that the substituents of bromo or epoxy groups have high antitumor activity against K562 and U937. Interestingly, the antileukemia activities depended on the number of bromo substituents introduced into the phosphasugar molecules. Furthermore, the results showed that the deoxybromophosphasugars have remarkably high antileukemia activity against both K562 and U937 cells. On the contrary, Imatinib mesylate has no or less effect against U937 cells viability (IC_{50} > 500 μM). The methyl and/or bromo substituent effects of 2-phospholenes and phospholanes at C3 and C4 positions and/or C2, C3, and C4 positions, suggest that they are important substituents on the antiproliferative effects. The results imply that the phosphasugars with the larger number of those substituents should fulfill the excellent antileukemia activity.

Table 1 Inhibition of proliferation of K562 and U937 cells by phosphasugar derivatives, compounds **2a–c**, **3b,c**, and **7**.



Compound	Substituent				IC_{50} (μM)	
	R_1	R_2	R_3	R_4	K562	U937
2a	H	H	H	H	>200	>200
2b	CH_3	H	H	H	>200	>200
2c	CH_3	CH_3	H	H	>200	>200
3b	CH_3	H	Br	H	87.3	83.3
3c	CH_3	CH_3	Br	H	18.5	5.9
7	CH_3	H	Cl	H	N.D.	>200
8	CH_3	H	I	H	N.D.	160.0
9	CH_3	H	H	NO_2	N.D.	>200
10	CH_3	H	H	NH_2	N.D.	>200
11	CH_3	H	Br	NO_2	N.D.	19.8
12	CH_3	H	Br	NH_2	N.D.	>200

Table 2 Inhibition of proliferation of K562 and U937 cells by phosphasugar derivatives **4b,c**, **5**, and **6**.

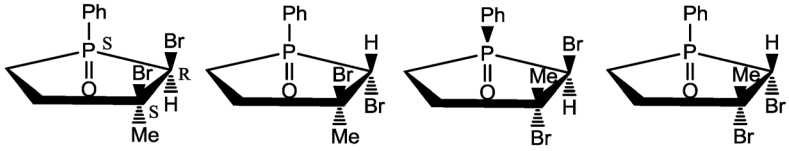


Compound	Substituent				IC_{50} (μM)	
	R_1	R_2	R_3	R_4	K562	U937
4b	Br	Br	CH_3	H	23.2	24.3
4c	Br	Br	CH_3	CH_3	16.4	5.4
5	-O-		CH_3	H	102.2	N.D.
6	Br	Br	CH_3	Br	3.2	2.3
Imatinib	–	–	–	–	0.48 [18]	500 [19]

Diastereoisomer

Some deoxybromophosphasugars such as dibromide **4** include diastereoisomers. Dibromides **4** have four diastereoisomers **4ba–bd** because the stereoselectivity of the addition reaction of bromine to the C=C double bond of the C2 and C3 positions of 2-phospholene derivatives was partially controlled by the substituents on the chiral center of the phenyl and phosphoryl groups. The structures of the diastereomers are shown in Table 3. In particular, ^{1}H NMR spectra of compounds **4b**, which have four isomers **4ba–bd**, showed different proton chemical shifts of H and Me groups on the C2 and C3 positions of the phosphorus heterocycles, and then each structure of the diastereomers is assigned to **4ba–bd** whose retention time for each isomer consists with the polarity and the structure (Table 3), and the ^{1}H NMR spectrum data also supports the structures of the diastereomers. The activities of these dibromo substituted phosphasugars **4ba–bd** were also evaluated by MTT in vitro method for antitumor agents against human leukemia K562 and U937 cell lines (Table 3).

Table 3 Diasteromeric structures and their properties of phosphasugars **4ba–4bd**.



4ba **4bb** **4bc** **4bd**

No.	HPLC[a] (min)	Dipole moment (Debye)	^{1}H NMR[b] δ (ppm)				Diastereomer yield (%)		IC_{50} (μM)	
			C(2)-H	C(3)-Me	C(4,5)-H	P(1)-Ph-H	MnO_2	$MnBr_2$	K562	U937
4ba	8.1	4.05	4.51	1.56	2.24–3.13	7.51–7.88	27	3	51	27
4bb	9.1	5.43	4.19	1.56	2.20–3.10	7.53–7.68	23	6	86	31
4bc	9.9	5.65	4.51	1.55	2.24–2.95	7.51–7.84	32	36	138	125
4bd	11.5	7.05	4.20	1.52	2.22–3.05	7.48–7.84	18	55	29	20

[a]The retention time was observed by HPLC analysis (column: Wakopak, Wakosil φ 4.6 × 250 mm; eluent: $CHCl_3$:MeOH = 30:1; flow rate: 0.5 ml/min).
[b]300 MHz, CD_3OD.

Among the four diastereomers **4ba–bd**, (2*S*,3*R*)-dibromo-(3*R*)-methyl-(1*S*)-phenylphospholane (1*S*)-oxide and the enantiomer, 1,2-*erythro*-2,3-*cis*-dibromo-3-methylphospholane 1-oxide and its enantiomer **4bd** had the largest retention time and the largest dipole moment, and its antileukemia activity against K562 and U937 cells was highest (the values of IC_{50} were 29 and 20 μM, respectively). The order of the antileukemia activities was 1,2-*erythro*-2,3-*cis* **4bd** > 1,2-*threo*-2,3-*cis* **4ba** > 1,2-*erythro*-2,3-*trans* **4bb** > 1,2-*threo*-2,3-*trans* **4bc**, depending on the structure of the diastereomers.

Flow cytometry

Cell cycle analysis for dibromide **4b** was carried out by flow cytometry, and the results are shown in Fig. 1. The distributions (%) of K562 cells obtained at each cell cycle stage are compared with that of Glivec. Treatment of K562 cells with **4b** for 24 h caused sub G1 (apoptosis) stage remarkably increased and G0/1, S, and G2 stages decreased. And the analyzed pattern of distribution of cells for Glivec and dibromide **4b** resembled each other. The cell cycle analysis indicates that phosphasugar **4b** induces apoptosis against K562 cells efficiently, and then kills leukemia cells selectively and specifically.

CONCLUSION

Phosphasugar analogues were synthesized and evaluated by in vitro MTT methods. Dehydrobromophosphasugars **3**, **4**, **6**, and **11** caused growth inhibition of cells against leukemia cell lines of K562 and U937. Among them, compound **6** may most plausibly be a novel antitumor agent or drug which acts on the gene at the new mitosis stage to induce apoptosis by controlling the mitosis at the lower concentration. By the further research on phosphasugars or phosphorus heterocycles, it may be more plausible that the present medicinal chemistry will lead to novel and wide spectral molecular targeted chemotherapeutic drugs for human cancers. Further research on these phosphasugars or phosphorus heterocycles and their bioactivities is now under development.

EXPERIMENTAL

Chemistry

General

TLC (silica gel: Wako Chromato Sheet and/or Merk Kieselgel 60; Eluent: $CHCl_3$:MeOH = 20:1, in R_f value); melting point appartus (Gallenkamp, in °C) and thermal analysis instrument (Shimazu: DTG-60A50AH, TGA and DSC, in °C); HPLC (GL Science: GL-7410 HPLC Pump and GL-7450 UV Detector); MS (MALDI-TOF-MS: GL Science, Voyager-DE Porimerix; Matrix: α-cyano-4-hydroxycinnamic acid, in *m/z*); and ^{1}H NMR (JEOL JNM-AL300 (300 MHz); Solvent: $CDCl_3$, in δ (ppm) from TMS) were used for analyzing the products.

*Preparation of 2-phospholenes **2***

Synthesis of 3,4-dimethyl-1-phenyl-2-phospholene 1-oxide (**2c**): 2,3-Dimethyl-1,3-butadiene 10 ml (90 mmol; 1.0 equiv) and phenylphosphonous dichloride 16 ml (120 mmol; 1.30 equiv) were mixed and reacted at room temperature for 2 weeks. The formed solid 3,4-dimethyl-1-phenyl-2-phospholenium dichloride was dissolved in chloroform (200 ml) and hydrolized at 0 °C by addition of ice. The reaction mixture was neutralized with sodium hydrogencarbonate and then filtered and extracted with chloroform (20 ml × 3). The chloroform extract was washed with water (50 ml), saturated sodium hydrogencarbonate solution (50 ml), and saturated sodium chloride solution (50 ml). Drying over the chloroform extract with anhydrous sodium sulfate followed by filtration, evaporation of the solvent in vacuo, and distillation under the reduced pressure afforded 3,4-dimethyl-1-phenyl-2-phospholene 1-oxide (**2c**; 5.6 g) in 30 % yield. B.p.: 130–132 °C /0.12 mmHg; HPLC (Wakosil 5SIL,

$CHCl_3$:MeOH = 20:1, flow rate 0.5 ml/min, λ = 254 nm); t_R = 11.04 min; R_f = 0.30 ($CHCl_3$:MeOH = 20:1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.30 (dd, J = 7.2 Hz, 3H, C4-CH_3), 1.80 (s, 3H, C3-CH_3), 2.07–2.17 (m, 1H, C4), 2.67–2.93 (m, 2H, C5), 5.93 (dd, J = 7.2 Hz, 1H, C2), 7.48–7.75 (m, 5H, Ph) MS (*m/z*): 205.7 (MH^+, 50), 207.7 (MH^+, 100).

Similarly, 1-phenyl-2-phospholene 1-oxide (**2a**) and 3-methyl-1-phenyl-2-phospholene 1-oxide (**2b**) were prepared.

1-Phenyl-2-phospholene 1-oxide (**2a**): Registry number: 703-03-7; R_f = 0.38 ($CHCl_3$:MeOH = 20:1); bp 145–152 °C (0.08 mmHg); HPLC (Wakosil 5SIL, $CHCl_3$:MeOH = 20:1, flow rate 0.5 ml/min, λ = 254 nm); t_R = 10.88 min; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 2.12–2.21 (m, 2H, C5), 2.78–2.94 (m, 2H, C4), 6.30 (d, J = 26.1, 1H, C3), 7.05–7.23 (m, 1H, C2), 7.47–7.71 (m, 5H, Ph); MS (*m/z*): 179.6 (MH^+, 100).

3-Methyl-1-phenyl-2-phospholene 1-oxide (**2b**): Registry number: 707-61-9; R_f = 0.32 ($CHCl_3$:MeOH = 20:1); bp 148–161 °C (0.10 mmHg); HPLC (Wakosil 5SIL, $CHCl_3$/MeOH = 20:1, flow rate 0.5 ml/min, λ = 254 nm); t_R = 10.06 min; ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 2.08 (s, 1H, C3-CH_3), 2.17–2.29 (m, 2H, C5), 2.59–2.83 (m, 2H, C4), 5.94 (d, J = 26.1 Hz, 1H, C2), 7.43–7.71 (m, 5H, Ph); MS (*m/z*): 193.7 (MH^+, 100).

*Preparation of 4-bromo-2-phospholenes **3***

Synthesis of 4-bromo-3,4-dimethyl-1-phenyl-2-phospholene 1-oxide (**3c**): To a chloroform (3 ml) solution of 3,4-dimethyl-1-phenyl-2-phospholene 1-oxide (**2c**; 206.1 mg, 1.00 mmol, 1.0 equiv) and NBS (213.6 mg, 1.20 mmol, 1.2 equiv) was added dropwise a chloroform (3 ml) solution of 2,2'-azobisisobutyronitrile (AIBN; 24.6 mg, 0.15 mmol, 0.15 equiv) at 60 °C and the reaction mixture was refluxed for 6 h under Ar atmosphere. The reaction mixture was neutralized with saturated $NaHCO_3$ aqueous solution (10 ml), washed with water (10 ml) and saturated NaCl solution (10 ml), and dried over anhydrous sodium sulfate. The solvent of the filtrate was evaporated under a reduced pressure to give an oily residual material. The residue was purified by column chromatography on silica gel by using chloroform and methanol (20:1) as the eluent to give 4-bromo-3,4-dimethyl-1-phenyl-2-phospholene 1-oxide (**3c**; 0.089 g, 0.314 mmol) in 31 % yield; R_f = 0.59 ($CHCl_3$:MeOH = 20:1); MS (*m/z*): 285.0 (MH^+, 100), 287.0 (MH^+, 90); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ (ppm) = 1.54 (s, 3H, C3-CH_3), 2.05 (d, J = 18.3 Hz, 3H, C4-CH_3), 2.12–2.27 (m, 2H, C5), 6.25 (d, J = 5.23 Hz, J = 17.4, 1H, C2), 7.48–7.79 (m, 5H, Ph).

Similarly, **3b** was prepared.

4-Bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (**3b**): Yield, 65 %; R_f = 0.42 ($CHCl_3$:MeOH = 20:1); MS (*m/z*), 271.5 (MH^+, 100), 273.5 (MH^+, 90); ^{1}H NMR ($CDCl_3$, 300 MHz), δ (ppm) = 2.22 (s, 3H, CH_3), 2.71–2.89 (m, 2H, C5), 2.96–3.15 (m, 1H, C4), 6.17 (d, J = 18.0 Hz, 1H, C2), 7.27–7.86 (m, 5H, Ph).

*Preparation of 2,3-dibromophospholanes **4***

Synthesis of 2,3-dibromo-3,4-dimethyl-1-phenylphospholane 1-oxide (**4c**): 2-Phospholene 1-oxide **2c** (0.206 g, 1.00 mmol) was dissolved in CH_2Cl_2 (5 ml). MnO_2 (0.24 g, 2.4 mmol) was added to the solution and then slow addition of CH_2Cl_2 solution of bromine (0.50 ml) was followed. The reaction mixture was stirred for 8 h at room temperature, and then the mixture was diluted with CH_2Cl_2 and vacuum filtered. The filtrate was collected, washed with 10 % Na_2SO_3 solution, water, and brine, and dried over anhydrous Na_2SO_4. Evaporation of the solvent followed by purification through silica gel column with chloroform and methanol (20:1) provided 2,3-dibromo-3,4-dimethyl-1-phenylphospholane 1-oxide (**4c**; 0.253 g, 0.691 mmol) in 69 % yield; R_f = 0.26 ($CHCl_3$:MeOH = 20:1); MS (*m/z*): 363.4 (MH^+, 30), 365.4 (MH^+, 65), 367.4 (MH^+, 100); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ (ppm) = 2.13 (s, 3H, C3-CH_3), 2.17–2.37 (m, 2H, C5), 2.42 (s, 3H, C4-CH_3), 2.71–2.79 (m, 1H, C4), 4.72 (d, J = 1.9 Hz, 1H, C2), 7.55–8.07 (m, 5H, Ph).

Similarly, **4b** was prepared.

2,3-Dibromo-3-methyl-1-phenylphospholane 1-oxide (**4b**): Yield, 56 %; R_f = 0.23 ($CHCl_3$:MeOH = 20:1); MS (*m/z*), 351.5 (MH^+, 60), 353.5 (MH^+, 100), 355.5 (MH^+, 40); ^{1}H NMR ($CDCl_3$, 300 MHz), δ = 2.17 (s, 3H, C3-CH_3), 2.44–2.58 (m, 2H, C5), 2.60–2.83 (m, 2H, C4), 4.67 (dd, J = 1.5 Hz, J = 7.2 Hz, 1H, C2), 7.32–7.56 (m, 5H, Ph).

*Preparation of 2,3-epoxyphospholane **5***

Synthesis of 2,3-epoxy-1-phenylphospholane 1-oxide (**5**): To a stirred mixture of 1-phenyl-2-phospholene 1-oxide (**2b**; 0.384 g, 2.00 mmol) in $CHCl_3$ (5 ml) was added *m*CPBA (0.587 g, 3.40 mmol), and then stirred for additional 24 h at 60 °C. The reaction mixture was neutralized with saturated $NaHCO_3$ aqueous solution (10 ml), washed with water (10 ml) and saturated NaCl solution (10 ml), and dried over anhydrous sodium sulfate. The solvent of the filtrate was evaporated under a reduced pressure to give an oily residual material. The residue was purified by column chromatography on silica gel by using chloroform and methanol (20:1) as the eluent to give product 2,3-epoxy-1-phenylphospholane 1-oxide (**5**; 0.168 g, 0.805 mmol) in 36 % yield; m.p.: 113–115 °C; R_f = 0.43 (AcOEt:MeOH = 20:1); MS (*m/z*): 209.54 (MH^+, 100); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ = 1.56 (s, 3H, C3-CH_3) 1.93–2.17 (m, 2H, C4), 2.34–2.50 (m, 2H, C5), 3.27 (d, J = 27.0 Hz, 1H, C1), 7.43–7.68 (m, 5H, Ph).

*Preparation of 2,3,4-tribromophospholane **6***

Synthesis of 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (**6**): To 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (**2b**; 0.751 g, 2.64 mmol) being dissolved in carbon tetrachloride (CCl_4, 10 ml) was heated to over 75 °C and bromine (1.03 ml, 20.0 mmol) in CCl_4 was added. After stirring the reaction mixture at 80 °C for 8 h, the reaction mixture was cooled, diluted with $CHCl_3$ (30 ml), washed with 10 % Na_2SO_3 solution, water, and brine, and then dried over anhydrous Na_2SO_4 to give 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (**6**; 0.250 g, 0.562 mmol) in 21 % yield; R_f = 0.62 ($CHCl_3$:MeOH = 20:1); MS (*m/z*): 429.0 (MH^+, 45), 431.1 (MH^+, 100), 433.1 (MH^+, 95), 435.1 (MH^+, 30); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ = 2.24 (s, 3H, C3-CH_3), 2.82–2.90 (m, 2H, C5), 3.06–3.15 (m, 1H, 4C), 5.20 (t, J = 9.1 Hz, 1H, C2), 7.56–7.88 (m, 5H, Ph).

*Preparation of 4-chloro-2-phospholene **7***

Synthesis of 4-chloro-3-methyl-1-phenyl-2-phospholene 1-oxide (**7**): Under Ar atmosphere, 3-methyl-1-phenyl-2-phospholene 1-oxide (**2b**; 0.178 g 1.0 mmol) was dissolved in chloroform ($CHCl_3$; 3.0 ml), *N*-chlorosuccinimide (NCS; 0.159 g 1.20 mmol) and AIBN (0.024 g, 0.15 mmol) were added to the reaction media. After stirring at 50 °C for 6 h, the reaction was cooled, diluted with $CHCl_3$, and vacuum filtered. The filterate was collected, washed by saturated sodium hydrogencarbonate solution, water, and brine, and dried over anhydrous Na_2SO_4. Evaporation of the solvent followed by purification through silica gel column with $CHCl_3$:MeOH (20:1) provided 4-chloro-3-methyl-1-phenyl-2-phospholene 1-oxide (**7**; 0.0546 g, 0.256 mmol) in 25 % yield; R_f = 0.55 ($CHCl_3$:MeOH = 20:1); MS (*m/z*): 227.3 (MH^+, 100), 229.3 (MH^+, 30); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ = 2.08 (s, 3H, C3-CH_3), 2.65–2.91 (m, 2H, C5), 4.67 (d, J = 9.0 Hz, 1H, C4), 5.73 (d, J = 9.86 Hz, 1H, C2), 7.49–7.82 (m, 5H, Ph).

*Preparation of 4-iodo-2-phospholene **8***

Synthesis of 4-iodo-3-methyl-1-phenyl-2-phospholene 1-oxide (**8**): To 4-bromo-3-methyl-1-phenyl-2-phospholane 1-oxide (**3b**; 1.05 g, 3.9 mmol) in acetone solution (10.0 ml) was added sodium iodide (NaI; 1.2 g, 7.8 mmol). After stirring the reaction mixture at 35 °C for 6 h to complete the reaction, the reaction mixture was cooled, added sodium hydrogensulfite solution, diluted with $CHCl_3$, and vacuum filtered. The filtrate was collected, washed with saturated sodium hydrogencarbonate solution, water, and brine, and dried over anhydrous Na_2SO_4. Evaporation of the solvent followed by purification through silica gel column with chloroform and methanol (20:1) provided 4-chloro-3-methyl-1-phenyl-2-phospholene 1-oxide (**8**; 0.107 g, 0.34 mmol) in 87 % yield; R_f = 0.36 ($CHCl_3$:MeOH = 20:1); MS (*m/z*): 317.7 (MH^+, 100); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ = 2.21 (s, 3H, C3-CH_3), 2.63–3.23 (m, 2H, C5), 4.35 (dd, J = 3.6 Hz, J = 9.0 Hz, 1H, C4), 5.25 (t, J = 9.3 Hz, 1H, C2), 7.55–7.86 (m, 5H, Ph).

*Preparation of 1-(3-nitrophenyl)-2-phospholene **9***

Synthesis of 3-methyl-1-(3-nitrophenyl)-2-phospholene 1-oxide (**9**): To a sulfuric acid solution (1 ml) of 2-phospholene **2b** (192 mg, 1.00 mmol) was added fuming nitric acid (0.089 ml), and then the mixture was kept at room temperature for 30 min with stirring. To the reaction miture was added ice/water and extracted with chloroform (10 ml × 3). The chloroform extract was washed with water (10 ml × 1) and saturated sodium chloride solution (10 ml × 1), and then dried over anhydrous sodium sulfate. Filtration of the chloroform extract and evaporation of the filtrate gave the residue, which was column chromatographed (silica gel; eluent: chloroform:methanol = 20:1) to give 3-methyl-1-(3-nitrophenyl)-2-phospholene 1-oxide (**9**; registry number 91207-34-0; 162 mg) in 68 % yield; R_f = 0.64 ($CHCl_3$:MeOH = 20:1); MS (*m/z*): 238.6 (MH^+); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ = 2.15 (s, 3H, C3-CH_3), 2.26–2.40 (m, 2H, C5), 2.76 (q, J = 5.0 Hz, 2H, C4), 5.98 (d, J = 25.3 Hz, 1H, C2), 7.68–8.42 (m, 4H, Ph).

*Preparation of 1-(3-aminophenyl)-2-phospholene **10***

Synthesis of 3-methyl-1-(3-aminophenyl)-2-phospholene 1-oxide (**10**): To chloroform solution (10 ml) of nitric acid (0.089 ml) of 2-phospholene (**2b**; 4.50 mg, 19.9 mmol) was added concentrated hydrochloric acid solution (5 ml) of Sn(II) chloride (4.5 g, 20 mmol) at 0 °C, and then the mixture was kept at 0 °C for 4 h and at room temperature for 4 days with stirring. The reaction miture was poured into ice/water and the diluted reaction mixture was neutralized with sodium hydrogencarbonate, and the mixture was filtrated and evaporated in vacuo. The residue was column chromatographed (alumina; eluent: chloroform:methanol = 10:1) to afford 3-methyl-1-(3-aminophenyl)-2-phospholene 1-oxide (**10**; 0.91 g) in 88 % yield; R_f = 0.41 ($CHCl_3$:MeOH = 20:1) ; MS (*m/z*): 208.5 (MH^+); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ = 1.73–1.81 (m, 2H, C5), 2.06 (s, 3H, C3-CH_3), 2.60–2.80 (m, 2H, C4), 5.88–5.96 (d, J = 22.5 Hz, 1H, C2), 6.78-6.80 (m, 2H, Ph-NH_2), and 6.83–7.27 (m, 4H, Ph).

*Preparation of 4-bromo-1-(3-nitrophenyl)-2-phospholene **11***

Synthesis of 4-bromo-3-methyl-1-(3-nitrophenyl)-2-phospholene 1-oxide (**11**): Under an Ar atmosphere, 3-methyl-1-(3-nitrophenyl)-2-phospholene 1-oxide (**9**; 0.237 g 1.0 mmol) was dissolved in chloroform ($CHCl_3$, 3.0 ml), and then NCS (0.236 g, 1.20 mmol) and AIBN (0.024 g, 0.2 mmol) were added to the reaction media. After stirring at 50 °C for 6 h, the reaction was cooled, diluted with $CHCl_3$, and vacuum filtered. The filtrate was collected, washed by saturated sodium hydrogencarbonate solution, water, and brine, and dried over anhydrous Na_2SO_4. Evaporation of the solvent followed by purification through silica gel column with $CHCl_3$:MeOH (20:1) provided 4-bromo-3-methyl-1-(3-nitrophenyl)-2-phospholene 1-oxide (**11**; 0.216 g, 0.684 mmol) in 68 % yield; R_f = 0.47 ($CHCl_3$:MeOH = 20:1); MS (*m/z*): 316.7 (MH^+, 100), 318.3 (MH^+, 90); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ = 2.22 (s, 3H, C3-CH_3), 2.87 (m, 1H, C4), 2.90–3.21 (m, 2H, C5), 5.90–6.05 (d, J = 26.4 Hz, 1H, C2), 7.49–7.87 (m, 4H, Ph).

*Preparation of 4-bromo-1-(3-aminophenyl)-2-phospholene **12***

Synthesis of 4-bromo-3-methyl-1-(3-aminophenyl)-2-phospholene 1-oxide (**12**): Under an Ar atmosphere, 3-methyl-1-(3-aminophenyl)-2-phospholene 1-oxide (**10**; 0.207 g, 1.0 mmol) was dissolved in chloroform ($CHCl_3$, 3.0 ml), and then *N*-chlorosuccinimide (NCS, 0.236 g, 1.20 mmol) and AIBN (0.025 g, 0.15 mmol) were added to the reaction media. After stirring at 50 °C for 6 h, the reaction mixture was cooled, diluted with $CHCl_3$, and vacuum filtered. The filtrate was collected, washed with saturated sodium hydrogencarbonate solution, water, and brine, and then dried over anhydrous Na_2SO_4. Evaporation of the solvent followed by purification through silica gel column with $CHCl_3$:MeOH (20:1) provided 4-bromo-3-methyl-1-(3-aminophenyl)-2-phospholene 1-oxide (**12**; 0.157 g, 0.548 mmol) in 55 % yield; R_f = 0.55 ($CHCl_3$:MeOH = 20:1); MS (*m/z*): 286.6 (MH^+, 100), 288.5 (MH^+, 90); ^{1}H NMR; ($CDCl_3$, 300 MHz), δ = 1.35–1.62 (m, 1H, C4), 1.90 (s, 1H, C3-CH_3), 2.37–2.68 (m, 2H, C5), 4.45–4.83 (m, 1H, C2), 5.74–5.99 (m, 2H, Ph-NH_2), 6.59–7.47 (m, 4H, Ph).

Biology

MTT method [17]

Compounds **2a–c**, **3a–c**, **4b,c**, and **5–12** were tested by the MTT method for antitumor activity against the K562 and U937 cell lines. Cells were seeded in 24-well flat-bottomed microplates at a density of 3×10^4 per well and incubated at various concentrations of phosphasugar analogues for 5 days. The cells were then washed with phosphate-buffered saline (PBS), harvested, and suspended in a 0.4 % trypan blue solution for the dye exclusion assay, in which viable cells were counted with a hemocytometer at the indicated incubation day. For the MTT assay, the cells were seeded in 96-well flat-bottomed microplates at a density of 5×10^5 per well. The cells were incubated at various concentration of phosphasugar analogues for 24 h. After incubation, 10 μL MTT solution (Sigma) was added to each well at a final concentration of 1 mg/ml. After the incubation at 37 °C for 4 h, absorbance was measured at a wavelength of 560 nm using a microplate reader.

Cell cycle analysis [17]

Propidium iodide (PI) (Sigma Chemical Company, St. Louis, MI) staining was used to analyze DNA content. Imatinib mesylate (Glivec) (1 μM) or phosphasugar **4b**-treated cells were cultured at 37 °C in 2 ml of complete medium containing 1×10^6 cells. After incubation for 48 h, the cells were washed twice with cold PBS, fixing with 70 % ethanol overnight, treated with 100 μg/ml RNase A, and then stained with 50 μg/ml PI. For apoptosis analysis, the relative DNA content per cell was measured by flow cytometry using an Epics Elite flow cytometer (Coulter Immunotech, Marseille, France). The percentage of cells in the apoptotic sub-G1 phase, as well as G1, S, and G2/M phases, was calculated using the Modfit program (Becton, Dickinson and Company, San Jose, CA).

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