A NOVEL PHOSPHA SUGAR ANALOGUE:SYNTHESIS AND EVALUATION OF 2,3-DIBROMO-3- METHYL- 1-PHENYLPHOSPHOLANE 1-OXIDE AS A NEW CLASS OF POTENTIAL ANTI-PROLIFERATIVE MATERIALS FOR LEUKEMIA CELLS

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Abstract: A novel dideoxydibromophospha sugar analogue, 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (**1**), was prepared from 1-phenyl-3-methyl-2-phospholene 1-oxide (**2**) and evaluated by MTT *in vitro* methods for leukemia cells. The evaluation of dibromide **1** (mixture of diastereomers) as well as the separated diastereomer of dibromides **1a** to **1d** from the diastereomer mixtures revealed clearly that the synthesized phospha sugar analogues have competent potentials and excellent anti-tumor activities for the human leukemia cells of K562 and U937 in selective and specific manner. The cell cycle analyses by flow cytometry for K562 and U937 cells clearly demonstrated that the mechanism of the anti-proliferative effect on the human tumor cells is apoptosis induced by the phospha sugar.

Introduction

Well known typical pseudo sugars are *carba*-, *aza*-, and *thia*-sugars,[1-3] with a carbon, nitrogen, and sulphur atom, respectively, instead of the oxygen atom in the hemiacetal ring of the normal sugars. These pseudo sugars are known to exist in nature and are also prepared by synthetic sugar chemistry. Pseudo sugars exert important biological activities, therefore, many studies on the isolation, synthesis, characterization, etc., are actively performed. On the other hand, phospha sugars, one new category of the pseudo sugars which have a phosphorus atom in the hemiacetal ring of sugars, are not yet found in nature and the synthesis of them are rather difficult compared with the typical pseudo sugars.[4-7]

We have been searching biologically active phospha sugars and/or phosphorus heterocycles, and we have first found new anti-tumor phospha sugar derivatives by using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) *in vitro* evaluation methods for some of these phospha sugars or phosphorus heterocycles against leukemia cells.[8] In this paper we will deal with the research on the synthesis, separation, and *in vitro* evaluation for 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (**1**), one of dibromodideoxyphospha sugars, against human

leukemia cell lines of K562 and U937. From the results of the research we may say that dibromide **1** may lead to develop a new type of carcinostatic drugs being useful in oncostasis.

Results and Discussion

2,3-Dibromo-3-methyl-1-phenylphospholane 1-oxide (**1**) was prepared by an addition reaction of bromine with 3-methyl- 1-phenyl-2-phospholene 1-oxide (**2**). The addition reaction of bromine to the electron deficient C=C double bond of phospholene **2** was accelerated by a manganese catalyst and the results are summarized in Table 1.

Table 1. Preparation of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (**1**) by addition reaction of **2** with bromine.

Run	Catalyst		Reaction Condition			Yield of Dibromide **1**/%
	Compound	Equivalent	Temperature[a]	Time/h	Solvent	
1	None	0	R.t.	48	CH_2Cl_2	33
2	MnO_2	2.0	R.t.	8	CH_2Cl_2	75
3	$MnBr_2$	0.50	R.t.	4	CH_2Cl_2	94

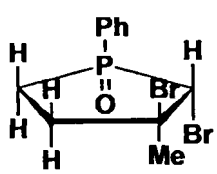
a) R.t. means reaction temperature at room temperature.

The diasteromers of **1** in the product (Run 2 of Table 1) were separated by HPLC (column: Wakopak Wakosil Φ 20.0 mm × 250 mm; eluent: $CHCl_3$: MeOH = 40 : 1; flow rate: 5.0 ml/min) into the four diasteremers **1a** to **1d** (Table 2). The structure of the four diastereomers **1a** to **1d** are rationalized by the chemical shift value of C(2)-H and C(3)-Me on the 2- and 3-positions, respectively, of the phospholane ring whose protons are suffured from the magnetic anisotropy effect caused by the phenyl group on the phosphorus heterocycle, P(1)-Ph. By the effect, the same side of hydrogen on the numbering two, C(2)-H, with the phenyl group was shifted to the relatively higher maginetic field, on the other hand, the hydrogen on the opposite side with the phenyl group shifted to the relatively lower field. The methyl group on the numbering three, C(3)-Me, was also shifted by the anisotropy effect to the higher or lower magnetic field depending on the same or opposite side of the phenyl group, respectively.[9] Therefore, the structures of the diastereomers **1a** to **1d** of the fractions with the retention times of 8.1, 9.1, 9.9, and 11.5 min should be assigned to the following structures (Figure 1). Addition of bromine to a C=C double bond is known to proceed via a nucleophilic attack of bromo cation to the bromonium intermediate, then the two vicinal bromo substituents should add *trans*-fasshion. The *cis* dibromide must be formed by the isomerization of the *cis*-fused dibromide via a stable tertially carbonium intermediate by S_N1 mechanism, which was observed for 3-methylphospholane 1-oxides.[10]

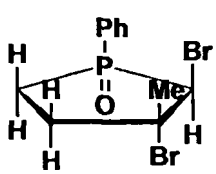
Table 2. Diastereomers **1a** to **1d** of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (**1**).

Diastereomer of Dibromide **1** [b]/%	Retention Time (RT)[a] of HPLC/min	^{1}H-NMR Chemical Shift δ/ppm (Coupling Constant J_{HP}/Hz) C(2)-H	C(3)-Me	C(4,5)-H	P(1)-Ph-H	Ratio of diastereomer
1a	8.1	4.19 (7.8)	1.56	2.20-3.10	7.53-7.86	27
1b	9.1	4.51 (7.3)	1.55	2.24-2.95	7.51-7.84	32
1c	9.9	4.51 (5.1)	1.56	2.24-3.13	7.51-7.88	23
1d	11.5	4.20 (7.2)	1.52	2.22-3.05	7.48-7.84	18

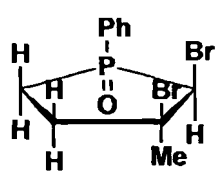
a) RT means the retention time observed by HPLC analysis (colum: Wakopak, Wakosil Φ 4.6 mm × 250 mm; eluent: $CHCl_3$: MeOH = 30 : 1; flow rate: 0.5 ml/min).b) Diastereomer ratio of HPLC peak area.



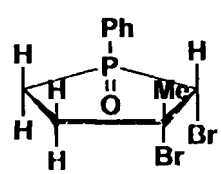
1a



1b



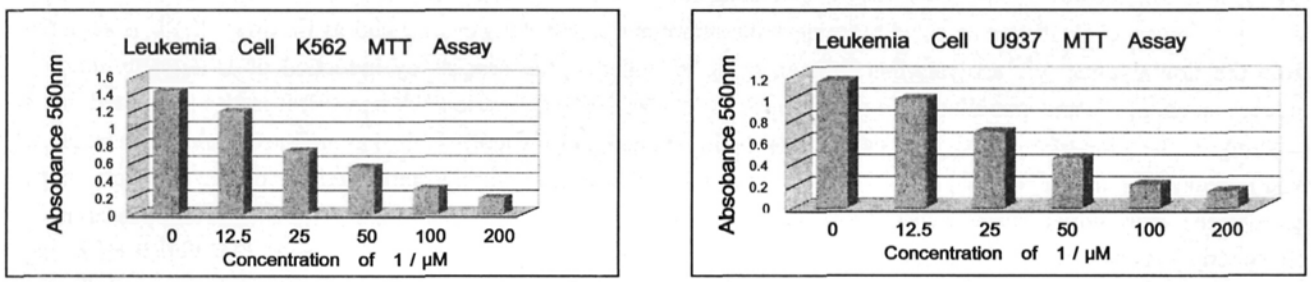
1c



1d

Figure 1. Structures of diastereomers **1a** to **1d**.

Evaluation of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (**1**; mixture of diastereomers) as inhibitors on proliferation of leukemia cells was carried out by *in vitro* MTT assay method. The results of K562 and U937 cells are shown in Figure 2. K562 and U937 cells of leukemia cell lines were incubated with the mixture of dibromide **1** at the indicated concentrations (0-1000 μM) at 37 °C for 24 h and 48 h. The cell proliferation or inhibition was measured as the function of the absorbance at 560 nm visible light and evaluated by MTT *in vitro* assay. Dibromide **1** strongly suppressed the cell proliferation of K562 cells in a dose-dependent manner and the intensity of absorbance at 560 nm decreased. The decrease of the absorbance means the death of the cell and clearly indicates that dibromide **1** possesses the growth inhibitory effect on K562 cells and that half of the absorbance intensity was achieved by 25 μM of **1** (Figure 2 (Left)). Similar growth inhibition by **1** was also shown for U937 cells (Figure 2 (Right)). The observed anti-proliferative effect of dibromide **1** on U937 cells observed was much more efficient than that of Gleevec.[11]



2,3-dibromo-3-methyl-1- phenylphospholane 1-oxide (**1**) at 37 °C for 48 h.

The separated four diastereomers **1a** to **1d** were also evaluated by MTT *in vitro* method against K562 cell lines. The results are shown in Figure 3. From the figure the most active component **1d** (LD_{50} = 31 μM) has higher anti-proliferative effect than component **1b** (LD_{50} = 125 μM) by four times in the activity.

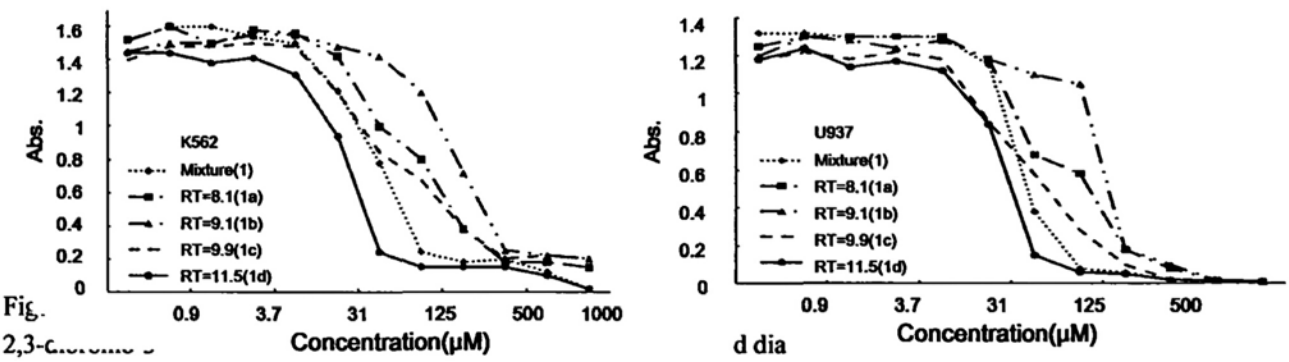


Fig. 2,3-c... d dia
(**1a** to **1d**), for K562 cells (Left) and U937 cells (Right) at 37 °C for 48 h.

Flow cytometric analysis for the action of **1** against leukemia cells was performed to elucidate the mechanism of **1** as the new anti-cancer agent. Leukemia cells (K562 and U937) were treated at 37 °C for 24 h and/or 48 h, and then the treated cells were subsequently stained with propidium iodide (PI) and analyzed by flow cytometry.[12,13] After treatment of leukemia cells with diastereomer mixture of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (**1**), induction of apoptosis[12] was certainly observed and the observations were reproduced. The results of the cell cycle analyses for K562 and U937 leukemia cells are summarized in Table 3 and the count data and the analyzed results for K562 cells are shown in Figures 4 and 5.

Table 3. Cell cycle analysis for K562 and U937 cells treated with 50 and 100 μM of **1** at 37 °C for 24 and 48 h.

Human Tumor Cell Lines	Flow Cytometry					
	% Apoptotic Cells at 24 h[a)]			% Apoptotic Cells at 48 h[a)]		
Concentration of **1**/μM						
	Concentration of **1**/μM					
0 (Control)	50	100	0 (Control)	50	100	
K562	2.3±0.4	64.8±1.5	80.4±3.2	2.5±0.4	84.3±4.1	92.4±4.1
U937	2.5±0.4	65.6±2.3	79.2±3.9	2.2±0.4	80.2±3.5	89.6±3.6

a) Data represent the mean value with standard deviation (± SD) of three independent experiments.

The cell cycle data of Table 3 and Figures 4 and 5 show that the concentration of dibromide **1** of 50 and 100 μM effectively caused the leukemia cells' death by apoptosis induction. Furthermore, these data show that the action mechanism on the K562 leukimia cells by dibromide **1** is quite similar to that of Gleevec ("Imatinib Mesylate"), which is known as a molecule targeting chemotherapeutical agent.[11]

By these *in vitro* evaluation data for dibromide **1** the important conclusions can be said as follows: (i) It is acquired from the flow cytometryic analysis that the cell cycle is stoped at the process by induction of **1**; (ii) Dibromide **1** induces an apoptosis and annihilates the canner cells at the Gap 2/Mitosis period;[13,14] and (iii) It is also suggested that **1** is acting on the gene of a mitosis (the detailed data will be shown elsewhere).[13] It may be mostly plausible from the result obtained by the *in vitro* evaluation that dibromide **1** can be a novel carcinostatic agent or drug which acts on the gene at the new mitosis stage by controlling the mitosis at the lower concentration. By the further research on phosphorus heterocycles' or phospha sugars' anti-tumor agents, it may be more possible that the medicinal chemistry will lead to find novel phospha sugars or phosphorus heterocycles as molecular targeting therapeutic drugs for the human cancers. Researches on these phospha sugars or phosphorus heterocycles chemistry and the evaluation of new molecular targeting chemotherapeutic anti-tumor agents are now under developing.

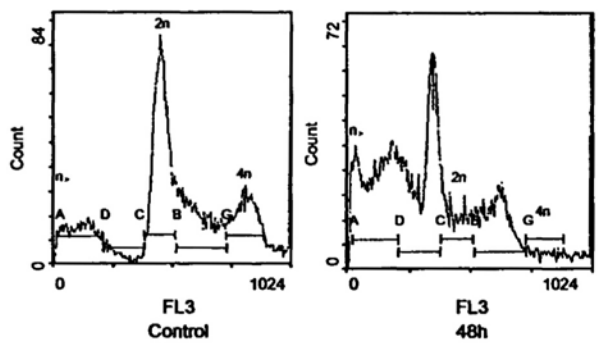


Figure 4. Cell cycle analysis of K562 cells with dibromide **1** (100 μM) for 48 h.

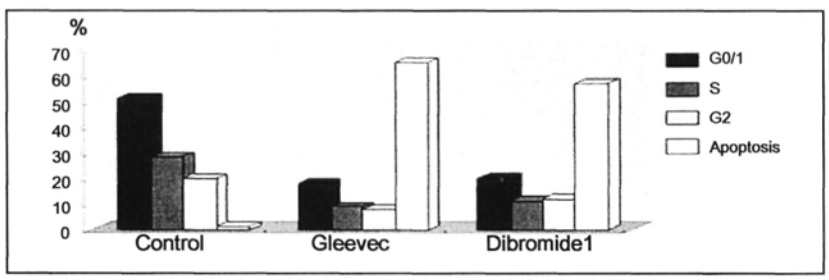


Figure 5. Comparison of results of cell cycle analysis of K562 with dibromide **1** and Gleevec for 48 h at the

concentration of 100 μM.

Experimental Section

Synthesis of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (1)

To CH_2Cl_2 (10ml) solution of 3-methyl-1-phenyl-2-phosholene 1-oxide (**2**; 0.27 g, 1.4 mmol) and Mn(IV) dioxide (0.24 g, 2.8 mmol; 2.0 eq.) was added drop wise CH_2Cl_2 (10 ml) solution of bromine (0.40 ml, 7.8 mmol; 5.6 eq.) and the reaction mixture was stirred for 8 h at room temperature. The reaction was quenched by addition of saturated sodium sulfite aqueous solution. The aqueous mixture was extracted with chloroform (10 ml x 3). The organic layer was neutralized with saturated $NaHCO_3$ aqueous solution, washed with saturated NaCl solution and dried over with anhydrous sodium sulfate. The solvent of the filtrate was evaporated under a reduced pressure to give an oily mixture of product **1**. The mixture was purified by column chromatography on silica gel by using chloroform and methanol (30 : 1) as the eluent to give 2,3-dibromo-3-methyl-1-phenylphosholane 1-oxide (**1**, 0.37 g) in 75% yield; m.p. (Shimadu Simultaneous DTA-TG Apparatus (DTG-60A50AH)) 189.20 °C; b.p. 280.24 °C; TLC (Silica gel: Wako Chromato Sheet and/or Merk Kieselgel 60; Eluent: $CHCl_3$: MeOH = 20 : 1), Rf = 0.42; MS (MALDI-TOF-MS: GL Science (Voyager-DE Porimerix); Matrix: α-Cyano-4-hydroxycinnamic acid (m/z)), 349.29 (M - H^+ (Molecular peak - 1); isotope peaks: 349.29, 351.29, and 353.29) and 351.29 (M + H^+ (Molecular peak + 1); isotope peaks: 351.29, 353.29, and 355.29); IR (JASCO FT/IR 410 (KBr)): 1126 cm^{-1} (P=O), 748 cm^{-1}, 1396 cm^{-1} (C-Br); ^{1}H-NMR (JEOL JNM-AL300 (300 MHz) and Hitach R90H (90 MHz); Solvent: $CDCl_3$, δ (ppm)); 1.67 (s, 3H, CH_3), 2.36-2.46 (m, 2H, H-4), 2.97-3.02 (m, 2H, H-5) 4.28-4.31 (m, 1H, C-2), 7.51-7.70 (m, 5H, Ph-H). HPLC (Apparatus: JASCO HPLC Set (JASCO 860-CO, 880-PU, 875-UV, RI-930, and 807-IT; Column: Silica gel (Analysis: Wakopac, Wakosil Φ 4.6 mm × 250 mm, Eluent: $CHCl_3$: MeOH = 30 : 1, Flow rate: 0.5 ml/min), RT (retention time: min) values of diastereo isomers were 8.1, 9.1, 9.9, and 11.5.

The diastereoisomers of product **1** were separated by preparative HPLC (column: Wakopac, Wakosil Φ 20.0 mm × 250 mm (Wako Gel); eluent: chloroform : methanol = 40 : 1; Flow rate: 5.0 ml/min) to give the four components **1a** to **1d** (Figure 1).

Reagent and solvent for the *in vitro* MTT evaluation

2,3-Dibromo-3-methyl-1-phenylphospholane 1-oxide (**1**), the reagent being evaluated by the *in vitro* MTT method, was dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical Company, St. Louis, MO, USA) as the solvent, and was diluted into appropriate concentration with DMSO in culture medium immediately before use. The final concentrations of **1** in DMSO in all experiments were less than 0.010%, and all the treatment conditions were compared with vehicle controls. The control experiments for the evaluation were carried out by using DMSO, and the absorption change in the MTT method was not observed for K562 and U937 cells at 37 °C for 48 h.

Human tumor cell lines and culture

Chronic myeloid leukemia (K562) and promyeloid leukemia (U937) cells were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) 292 mg/l (or 2.0 mM) L-glutamine, 100 μg/ml streptomycin, and 200 U/ml penicillin (GIBCO-BRL, Gaithersburg, MD, USA). All cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C.

MTT cell proliferation assay [12,14]

For the MTT cell proliferation assay, the cells were seeded in 96-well flat bottomed microplates at a density of 5×10^4 per well. Cells were incubated with or without 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (**1**), at 37 °C for 72 h, and then 10 μl 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) solution (Sigma Chemical Co., St. Louis, Mo, USA) was added to each well at a final concentration of 1.0 μg/ml/well. Cells grown in the presence of the medium alone were used as the controls. After incubation at 37 °C for 4 h, absorbance was measured at a wavelength of 560 nm by using a microplate reader for *in vitro* evaluation.

Apoptosis analysis [13-15]

DNA content analysis at the each stage of the cell cycles was performed by using propidium iodide (PI) staining. Cells (K562) were cultured in 2 ml complete medium containing 1 x 10^6 cells in the presence of 50, 100, and 200 μM of 2,3-dibromo-3- methyl-1-phenylphospholane 1-oxide (**1**), at the indicated concentrations in the Table 3 and incubated at 37 °C. After 24 h and 48 h of incubation, the cells were washed twice with cold PBS, fixed with 70% ethanol for overnight before treatment with 100 μg/ml RNase A, and then stained with 50 μg/ml of PI. The relative DNA content per cell was analysed by flow cytometry by using an Epics Elite flow cytometer (Coulter Immunotech, Marseille, France).

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