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*Short note*

# 5-Methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ⁵-phosphaspiro[4.4]nona-2,7-diene – Synthesis and Preliminary Cytotoxic Screening

**Sławomir Kasperowicz,[1,2] Jolanta Czerwińska,[1] Bartosz Majchrzak,[3] Barbara Tudek,[1,3] Adam Mieczkowski[1*]**

[1] Institute of Biochemistry and Biophysics Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland; slawek.kasperowicz@gmail.com (S.K.); amiecz@ibb.waw.pl (A.M.); jczerwinska@ibb.waw.pl (J.C.); tudek@ibb.waw.pl (B.T.);

[2] Faculty of Chemistry, Warsaw University of Technology, Noakowskiego 3, 00-664 Warsaw, Poland

[3] Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Pawinskiego 5a, 02-106 Warsaw, Poland; bmajchrzak@student.uw.edu.pl (B.M.)

* Correspondence: amiecz@ibb.waw.pl; Tel.: +48-22-592-35-06; Fax: +48-22-592-21-90

**Abstract:** 5-Methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ⁵-phosphaspiro[4.4]nona-2,7-diene was obtained in a condensation reaction of 2-amino-5-bromobenzohydrazide and methylphosphonyl dichloride in the presence of triethylamine. An initial biological screening was performed for the obtained product. The synthesized compound possesses two aromatic primary amine groups and two bromine atoms within the structure, which are suitable for further structural modifications.

**Keywords:** aromatic hydrazide condensation; phosphorus heterocycles; cytotoxicity;

## 1. Introduction

After the development of cyclophosphamide **1** (Figure 1)—a potent antineoplastic agent [1]—medicinal chemists focused their attention on the application of phosphorus heterocycles as potential antiproliferative agents. Although no other phosphoheterocycle repeated the tremendous success of Cyclophosphamide **1**, some of them exhibited noticeable cytotoxic and antiproliferative effect. Bull reported [2] that some of the isoquino[2,1-*c*][1,3,2]benzodiazaphosphorine derivatives 2**a-c** exhibited promising anticancer effect against Ehrlich ascites carcinoma and P-388 lymphocytic leukemia cells. Hudson observed [3] that a dimer of 3-methyl-1(2,4,6-triisopropylphenyl)phosphole oxide **3** showed $GI_{50}$ values in the micromolar region against RPMI-8226 and SR leukemia cell lines, non-small cell lung cancer (NCI-H460), colon cancer (COLO 205), and melanoma (SK-Mel-5 and UACC-62). In the same article, he also observed a moderate antiproliferative effect of phosphinine 1-oxides **4** and **5** [3]. Ito reported [4,5] that phospholane derivatives **6** and **7** exhibited significant antitumor activities against leukemia cells such as the K562 and U937 cell lines, as well as solid cancer cells such as stomach cancer and lung cancer. Further mechanistic studies by Western blotting showed that such compounds could enhance the expression of IER5 and then suppressed the expression of Cdc25B, the effect responsible for their antitumor activity.

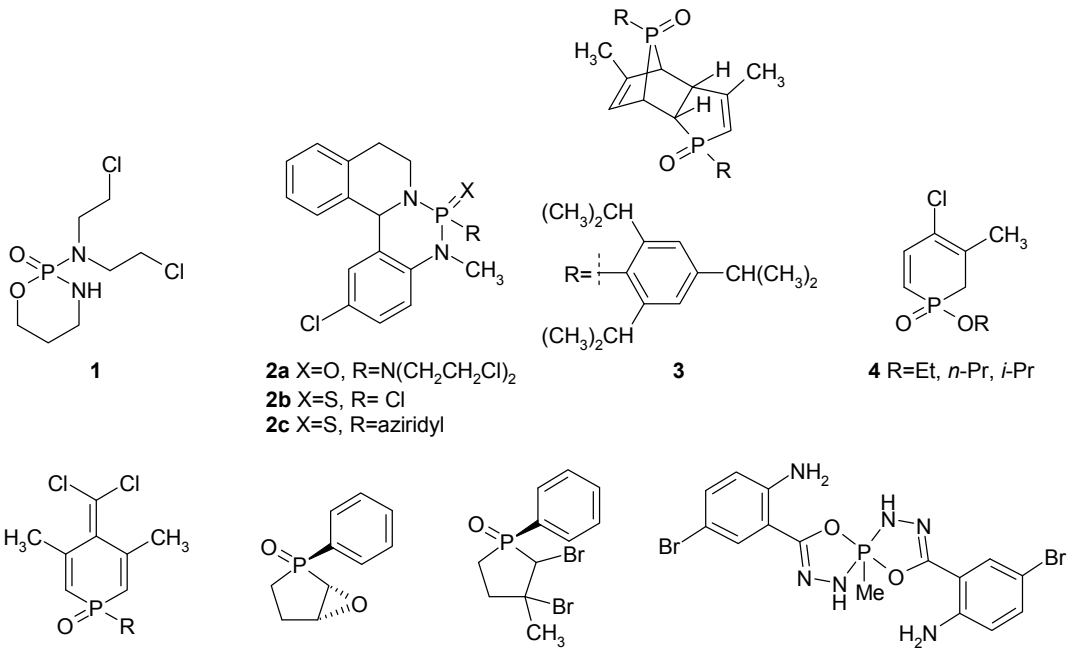
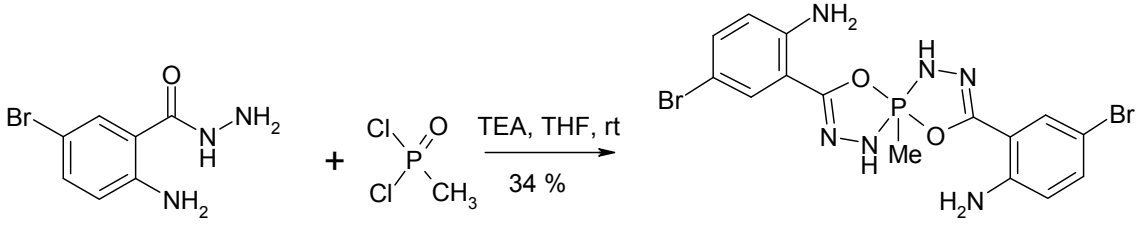



**Figure 1.** Examples of phosphoheterocycles exhibiting anticancer activity **1**-**7** and the newly synthesized compound **8**

## 2. Results and Discussion

During our continuous efforts toward development of anticancer-relevant, heterocyclic derivatives [6-8], we investigated a reaction between 2-amino-5-bromobenzohydrazide **9**, synthesized from 5-bromoisatoic anhydride and hydrazine hydrate according to the literature procedure [9], and methylphosphonyl dichloride **10** in the presence of triethylamine (Scheme 1). Equimolar amounts of **9** and **10** were dissolved in dry THF and three equivalents of triethylamine were added dropwise in room temperature. Low-resolution mass spectra showed the formation of new product with molecular mass M=502 g/mol, and the isotopic profile revealed the substitution of product with two bromine atoms. To complete the reaction, one more equivalent of methylphosphonyl dichloride **10** and three more equivalents of triethylamine were added, which led to complete exhaustion of 2-amino-5-bromobenzohydrazide **9**.




Scheme 1. Synthesis of 5-methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ⁵-phosphaspiro[4.4]nona-2,7-diene **8**

A literature search followed by the investigation of NMR spectra led to a conclusion that the obtained product **8** possesses 4,9-dioxa-1,2,6,7-tetraaza-5λ⁵-phosphaspiro[4.4]nona-2,7-diene core, resulted from the condensation of two molecules of 2-amino-5-bromobenzohydrazide **9** and one molecule of methylphosphonyl dichloride **10**. It was revealed that only the aromatic hydrazide group of **9** participated in the condensation with **10**, while the primary aromatic group of **9** remained intact. Compounds possessing 4,9-dioxa-1,2,6,7-tetraaza-5λ⁵-phosphaspiro[4.4]nona-2,7-diene structure were initially reported by Schmidpeter [10] and their syntheses were more recently investigated by Gholivand [11] and Hua [12]. Similar condensations between aromatic dihydrazides

and phosphonyl dichlorides were also published by Ali [13]. In previous reports, only simple aromatic hydrazides were condensed with phosphonyl dichlorides, which limited further modifications of obtained products. In contrast, the presence of two amine groups, as well as two bromide atoms within the structure of **8**, allows further modifications and development of more complex molecules.

5-Methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ5-phosphaspiro[4.4] nona-2,7-diene **8** was toxic to all studied cell lines: two cancer lines, epidermoid – A431, and glioblastoma – U87, as well as two non-cancer cell lines, embryonic kidney line HEK 293, and telomerase-immortalized fibroblasts, K21. The toxicity of the compound was similar for all cells and at 200 uM concentration survival dropped to about 25 % for non-cancer cell lines, and to about 30 % for cancer cell lines (Figure 2). Thus, 5-Methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5$\lambda^5$-phosphaspiro[4.4]nona-2,7-diene seems not to be a candidate for selective anticancer treatment.

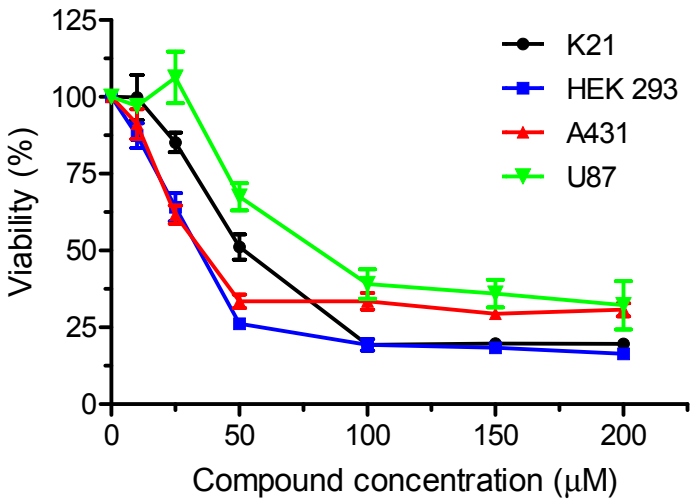



**Figure 2**. Viability plots of cell lines K21, HEK 293, A431 and U87 in response to 5-methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ5-phosphaspiro[4.4]nona-2,7-diene.

## 3. Materials and Methods

Commercially available chemicals were of reagent grade and used as received. The reactions were monitored by thin layer chromatography (TLC) analysis, using silica gel plates (Kieselgel 60$F_{254}$, E. Merck, Darmstadt, Germany). Column chromatography was performed on the Silica Gel 60M (0.040-0.063 mm, E. Merck, Darmstadt, Germany). Melting points are uncorrected and were measured on the Büchi (New Castle, DE, USA) Melting Point B-540 apparatus. The $^{1}H$, $^{13}C$ and $^{31}P$ NMR spectra in $CDCl_3$ were recorded at the Department of Chemistry, Warsaw University, using the Bruker AVANCE III HD (Billerica, MA, USA) 500 MHz spectrometer; shift values in parts per million are relative to the $SiMe_4$ internal reference. The resonance assignments are based on a peak integration, peak multiplicity, and 2D correlation experiments. Multiplets were assigned as bs (broad singlet), d (dublet), dd (doublet of doublet) and tq (triplet of quartet). High-resolution mass spectra were recorded by the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics PAS, with the LTQ Orbitrap Velos, Thermo Scientific (Waltham, MA, USA). IR spectra were recorded with the FT/IR 6200 Jasco (Easton, MD, USA) spectrometer at the Laboratory of Optical Spectroscopy, Institute of Organic Chemistry PAS (Warsaw, Poland).

Cytotoxic activity of 5-methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5$\lambda^5$-phosphaspiro[4.4]nona-2,7-diene **8** was verified against two cancer cell lines: A431 (human epidermoid carcinoma), U87 (human glioblastoma) and two non-cancer cell lines: K21 (human fibroblast) and HEK 293 (human embryonic kidney). Cells were seeded in 96-well plates at density

of 3,000 cells per well one day before treatment and then treated with increasing concentrations (10-200 μM) of tested compound in complete growth medium. After 48 h of incubation, cells were assayed to measure their viability using the alamarBlue assay (Invitrogen by Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Each experiment was repeated three times.

*Synthesis of 5-methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ⁵-phosphaspiro[4.4]nona-2,7-diene* **8**

2-Amino-5-bromobenzohydrazide (**9**, 500 mg, 2.17 mmol, 1 equiv.) and methylphosphonyl dichloride (**10**, 288 mg, 2.17 mmol, 1 equiv.) were dissolved in 15 ml of dry THF, then triethylamine (0.91 ml, 6.52 mmol, 1 equiv.) was added dropwise. The reaction mixture was stirred in rt for 18 h, then further portions of methylphosphonyl dichloride (**10**, 288 mg, 2.17 mmol, 1 equiv.) and then triethylamine (0.91 ml, 6.52 mmol, 1 equiv.) were added, and the reaction mixture was stirred for another 18 h. After addition of 30 ml of water, the solution was extracted with ethyl acetate (3 x 30 ml). The organic phase was dried over magnesium sulfate, filtrated, and evaporated with silica gel (2 g). The final product was purified by column chromatography, using hexane:ethyl acetate 8:2 v/v mixture. The fractions containing **8** were collected, the solvent was evaporated under the reduced pressure giving a yellowish oil, which solidified during overnight storage. The obtained yellow crystals were washed with methanol, which resulted in obtaining a white powder. Yield: 184 mg (34 %). M.p. 229.5-230.5°C. $^{1}$H NMR (500 MHz, $CDCl_3$): 7.66 (d, 1H, $^{4}J$=2.5 Hz, $H_{Ar}$), 7.22 (dd, 1H, $^{4}J$=2.5 Hz, $^{3}J$=8.5 Hz, $H_{Ar}$), 6.57 (d, 1H, $^{3}J$=8.5 Hz, $H_{Ar}$), 5.81 (d, 2H, $^{3}J_{(H-P)}$=31.0 Hz, 2 x NH); 5.43 (bs, 4H, 2 x $NH_2$), 2.08 (d, 2H, $^{3}J_{(H-P)}$=18.0 Hz, 2 x NH); $^{13}$C NMR (125 MHz, $CDCl_3$): 155.0 (d, $^{2}J_{C-P}$=10.2 Hz), 144.8, 133.1, 129.9, 117.4, 111.7 (d, $^{3}J_{C-P}$=0.8 Hz), 107.8, 22.9 (d, $^{1}J_{C-P}$=174.5 Hz); $^{31}$P NMR (202 MHz, $CDCl_3$): -34.45 (tq, $^{3}J_{(H-P)}$=31.0 Hz, $^{3}J_{(H-P)}$=18.0 Hz, coupled with 2 x NH and 1 x $CH_3$); HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{15}H_{15}Br_2N_6O_2P$: 500.94336, 502.94132, 504.93927, found: 500.94359, 502.94151, 504.93945; IR (KBr): $cm^{-1}$ 3473, 3446, 3415, 3346, 2923, 2852, 1882, 1737, 1611, 1586, 1554, 1489, 1423, 1339, 1314, 1300, 1250, 1163, 1130, 1114, 1059;

**Supplementary Materials:** Copies of the $^{1}$H-NMR, $^{13}$C-NMR, dept135, $^{31}$P-NMR, IR, HRMS-ESI mass spectra are available online at … .

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**Author Contributions:** A.M.: synthesis planning, writing of manuscript; S.K. experimental synthetic work; J.C, B.M.: screening of biological activity; B.T.: writing of manuscript;

**Conflicts of Interest:** The authors declare no conflict of interest.

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