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Short Note

5-Methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ^5-phosphaspiro[4.4]nona-2,7-diene

Sławomir Kasperowicz [1,2], Jolanta Czerwińska [1], Bartosz Majchrzak [3], Barbara Tudek [1,3] and 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.); 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
* Correspondence: amiecz@ibb.waw.pl; Tel.: +48-22-592-35-06; Fax: +48-22-592-21-90

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Abstract: 5-Methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ^5-phosphaspiro [4.4]nona-2,7-diene was obtained by condensation of 2-amino-5-bromobenzohydrazide and methylphosphonyl dichloride in the presence of triethylamine. An initial biological screening was performed for the resulting product. The synthesized compound showed relatively strong cytotoxic activity, which was, however, similar for cancer and non-cancer cell lines.

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 effects. Bull reported [2] that some of the isoquino[2,1-*c*][1,3,2]benzodiazaphosphorine derivatives 2**a**–**c** exhibited promising anticancer effects 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 range against two leukemia cell lines (RPMI-8226 and SR), 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] 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, which are responsible for their antitumor activity [5].

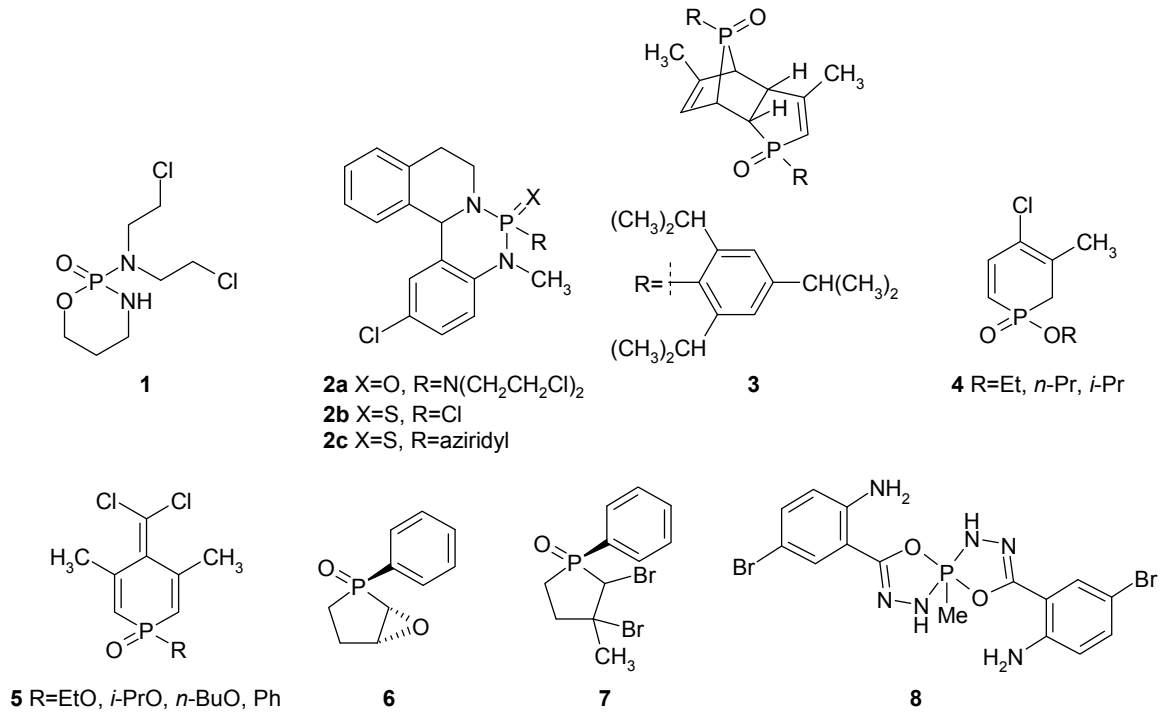
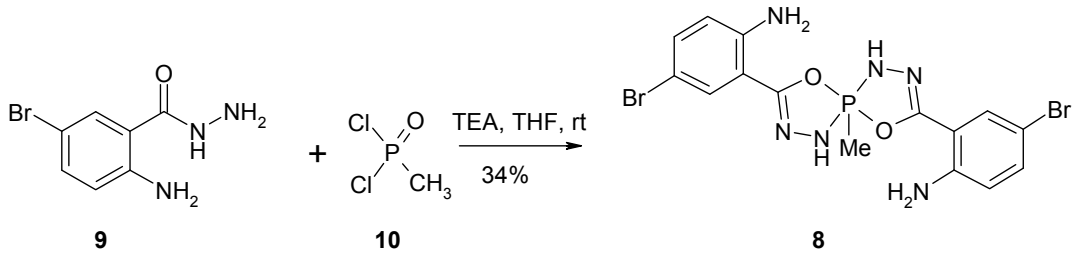


Figure 1. Examples of phosphoheterocycles exhibiting anticancer activity **1**–**7** and the newly synthesized compound **8**.

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, three equivalents of triethylamine were added dropwise in room temperature and the reaction mixture was stirred in at room temperature for 18 h. Low-resolution mass spectrometry showed the formation of a new product with molecular mass M = 502 g/mol, and the isotopic profile revealed a product with two bromine atoms. To complete the reaction, one more equivalent of methylphosphonyl dichloride **10** and three more equivalents of triethylamine were added, and the reaction mixture was stirred at room temperature for an additional 18 h. This led to complete consumption of **9**.



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

A literature search followed by the investigation of NMR spectra (see supplementary materials) led to a conclusion that product **8** possessed a 4,9-dioxa-1,2,6,7-tetraaza-5λ^5-phosphaspiro[4.4]nona-2,7-diene core resulting from the condensation of two molecules of

2-amino-5-bromobenzohydrazide **9** with 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 amine group of **9** remained intact. Compounds possessing 4,9-dioxa-1,2,6,7-tetraaza-5λ^5-phosphaspiro[4.4]nona-2,7-diene structure have been previously reported by Schmidpeter [10] and their synthesis has been 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 of the initially obtained products. The structure of **8** allows for further elaboration to more complex molecules.

The necessity of adding the 2 equivalents of phosphonyl dichloride **10** and the relatively low yield of the reaction could be explained by the formation of other by-products. The organic phase obtained after extraction with ethyl acetate, contained the main, relatively non-polar product **8**, and two minor, very polar and difficult to separate products, which have not been isolated and characterized thus far. Moreover, we cannot exclude a possibility that the water phase contained some additional polar products, insoluble in the organic phase. Our initial efforts to optimize the reaction conditions did not lead to a better yield or a more economic ratio of reagents. In addition, we recently found that although compound **8** is easily separated, purified, and characterized by physicochemical methods, its shelf-life (especially of the crude product before chromatographic purification), at room temperature, is rather limited. After the purification step, product **8** should be dried and stored at low temperature (−18 °C).

5-Methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ^5-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 (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, compound **8** seems not to be a candidate for selective anticancer treatment.

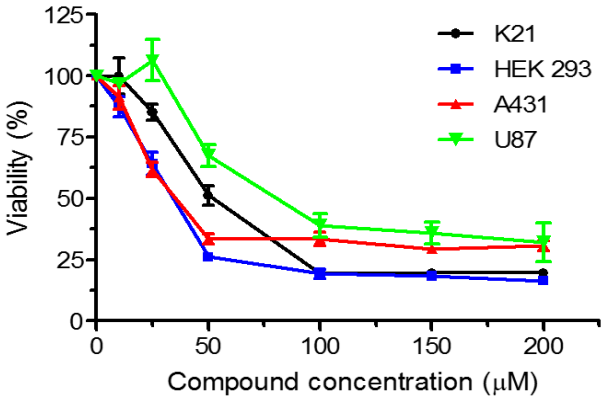


Figure 2. Viability plots of cell lines K21, HEK 293, A431 and U87 in response to 5-methyl-3,8-di-(2-amino-4-bromophenyl)-4,9-dioxa-1,2,6,7-tetraaza-5λ^5-phosphaspiro[4.4]nona-2,7-diene.

3. Materials and Methods

Commercially available chemicals were of reagent grade and used as received. The reactions were monitored by thin layer chromatography (TLC), using silica gel plates (Kieselgel 60F_{254}, E. Merck, Darmstadt, Germany). Column chromatography was performed on silica gel 60M (0.040–0.063 mm, E. Merck, Darmstadt, Germany). Melting points are uncorrected and were measured on a 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 a 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 (doublet), 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, on a LTQ Orbitrap Velos instrument, Thermo Scientific (Waltham, MA, USA). IR spectra were recorded with a Jasco 6200 (Easton, MD, USA) FT/IR spectrometer at the Laboratory of Optical Spectroscopy, Institute of Organic Chemistry PAS (Warsaw, Poland).

Cytotoxic activity of **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 3000 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, USA) 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-5λ^5-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, 3 equiv.) was added dropwise. The reaction mixture was stirred at room temperature for 18 h, then further portions of methylphosphonyl dichloride (**10**, 288 mg, 2.17 mmol, 1 equiv.) followed by 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 × 30 mL). The organic phase was dried over magnesium sulfate, filtered 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 yellow oil, which solidified during overnight storage. The resulting yellow crystals were washed with methanol to give a white powder. Yield: 184 mg (34%). M.p. 229.5–230.5 °C. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.66 (d, 2H, ^4J = 2.5 Hz, H_{Ar}), 7.22 (dd, 2H, ^4J = 2.5 Hz, ^3J = 8.5 Hz, H_{Ar}), 6.57 (d, 2H, ^3J = 8.5 Hz, H_{Ar}), 5.81 (d, 2H, $^3J_{(H\text{-}P)}$ = 31.0 Hz, 2 × NH); 5.43 (bs, 4H, 2 × NH_2), 2.08 (d, 3H, $^3J_{(H\text{-}P)}$ = 18.0 Hz, CH_3); ^{13}C-NMR (125 MHz, $CDCl_3$): 155.0 (d, $^2J_{C\text{-}P}$ = 10.2 Hz), 144.8, 133.1, 129.9, 117.4, 111.7 (d, $^3J_{C\text{-}P}$ = 0.8 Hz), 107.8, 22.9 (d, $^1J_{C\text{-}P}$ = 174.5 Hz); ^{31}P-NMR (202 MHz, $CDCl_3$): −34.45 (tq, $^3J_{(H\text{-}P)}$ = 31.0 Hz, $^3J_{(H\text{-}P)}$ = 18.0 Hz, coupled with 2 × NH and 1 × 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 http://www.mdpi.com/1422-8599/2018/1/M978/s1.

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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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