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Synthesis and Cytostatic Activity of *4-bromo-5-ethyl-2-(ethylamino)-5-methyl-5H-1,2-oxaphosphole 2-oxide*

Dobromir D. Enchev[1], Vanya P. Koleva[1*], Asya P. Dragoeva[1], Leman B. Behchet-Ibryam[1], Mihail N. Mihaylov[1], Nikolay M. Uzunov[1,2], Laura Melendez-Alafort[3], Antonio Rosato[3,4], Valery Brel[5]

[1]Faculty of Natural Sciences, Konstantin Preslavski University of Shumen, 115, Universitetska Str., 9712 Shumen, Bulgaria.
[2]INFN, Laboratori Nazionali di Legnaro, Legnaro (Padova), Italy.
[3]Oncological Institute of Veneto, Via Gattamelata 64, 35128 Padua, Italy.
[4]Department of Surgery, Gastoenerological and Oncological Sciences, University of Padua, Via Giustiniani 2, 35128 Padova, Italy.
[5]Laboratory of Organophosphorus Compounds, A. N. Nesmeyanov Institute of Organoelement Compounds of Russian Academy of Sciences, Moscow, Vavilova St. 28, 119991, Russian Federation.

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ABSTRACT

Synthesis of phosphorus heterocycles has gained increasing interest in recent years. Here we wish to report our results on synthesis and on studying of biological activity of *4-bromo-5-ethyl-2-(ethylamino)-5-methyl-5H-1,2-oxaphosphole 2-oxide* (Br-oxph-1) using *in vivo* and *in vitro* approaches. Br-oxph-1 represents a structural analogue of *4-bromo-2-(diethylamino)-5-ethyl-5-methyl-5H-1,2-oxaphosphole 2-oxide* (Br-oxph). Root growth inhibition test using *Triticum aestivum* L. seeds was provided in order to determine general toxicity of Br-oxph-1. The effective concentration that cause 50% of root length as compared to control was determined – 8.23×10^3 µM. Cytotoxic potential of Br-oxph-1 (8.23×10^3 µM) was evaluated using *Allium cepa* L.-test. Significant decline in mitotic activity and disturbances of cell cycle kinetics were observed. Further, effects of Br-oxph-1 on human hepatoma cell line SK-HEP-1 *in vitro* were evaluated by means of cell proliferation assay. The results revealed concentration-dependent and statistically significant inhibitory effect. Concentration of Br-oxph-1 showing 50% reduction in cell proliferation/viability was 0.642×10^3 µM. In conclusion, plant test-systems used in this study revealed growth inhibitory and cytostatic effects of Br-oxph-1. Data *in vitro* was in accordance with these results. Results of present study revealed stronger cytostatic effect of Br-oxph-1 in comparison with Br-oxph. Obviously, modifications in chemical structure of Br-oxph influence its biological effects.

INTRODUCTION

The importance of organophosphorus compounds as intermediates in organic synthesis is well documented. Phosphorus heterocycles have received considerable interest because of their possible therapeutic application, including uses as genotoxic and anticancer agents (Hudson and Keglevich, 2008). Numerous of reports deals with the scope and limitation of synthesis of phosphorus heterocycles (Mcreinolds *et al*., 2004; Christau *et al*., 2005; Hah *et al*., 2003; Peng and Ding, 2005; Ma *et al*., 2007; Yu *et al*., 2007) and especially to those concerning the synthesis of oxaphosphole derivatives. Among other promising synthetic protocols, the heterocyclization of phosphorylated allenes has been established as one of the most fruitful ones. The combination of double bonds and a phosphoryl group in the molecules of those compounds, each with different reactivity, makes them useful substrates for the synthesis of different organophosphorus compounds. The organic synthesis with participations of phosphorylated allenes lead to the formation of five- or six-membered phosphorus heterocycles with certain biological activity (Enchev, 2010).

Nevertheless, the large number of investigations devotet to the chemistry of oxaphosphole derivatives, data of their biological activity is scarce (Berger and Montchamp, 2014; Virieux *et al*., 2012). Compounds with structural similarity to oxaphospholes

*Corresponding Author
Vanya P. Koleva, Faculty of Natural Sciences Shumen University, 115 Universitetska Str., Shumen, Bulgaria.
E-mail: vanyakolleva @ gmail.com

are used as antiviral drugs (Tam *et al*., 2000; Kamiya, 2003; Pescovitz, 2008; Okano, 2009) and anticancer agents (Kanno *et al*., 2009; Sharabi and Haran-Ghera, 2011). Our studies in this field demonstrate mitodepressive, clastogenic and turbagenic effects on plant and animal test–systems of this class of phosphorus heterocycles as well as inhibition of growth of human lung carcinoma cells *in vitro* (Kalcheva *et al*., 2009a; Koleva *et al*., 2014).

Here we wish to report our results on synthesis and on studying of biological activity of certain oxaphosphole derivative using *in vivo* and *in vitro* approaches. *In vivo* tests were carried out on plant test-systems. Plant bioassays are widely used for toxicity evaluation of different compounds nowadays (Lerda *et al*., 2010; Andrioli and Mudry, 2011; Herrero *et al*., 2012; Nefic *et al*., 2013). Among them, *Allium cepa*-test is a sensitive tool for toxicity evaluation and shows good correlation with the results of other test systems (Leme and Marin-Morales, 2009). *In vitro* tests are used in order to estimate the influence of chemical compounds on proliferation/viability of human cancer cells.

MATERIALS AND METHODS

Chemistry

The titled compound was synthesized according procedures described earlier (Enchev, 2015), namely from the corresponding 1,2-alkadienephosphonates and bromine in nonpolar media (Fig. 1).

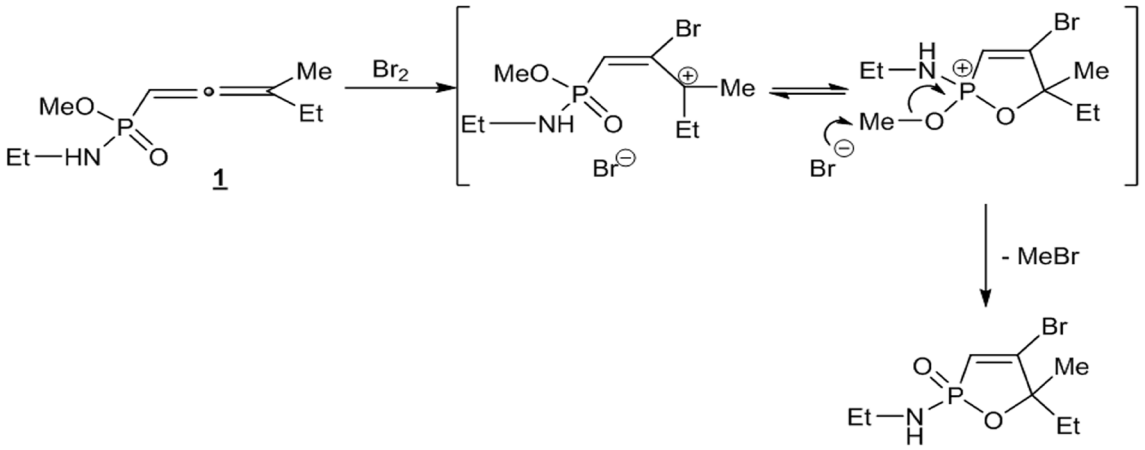

Fig. 1: Synthesis of ***4-bromo-5-ethyl-2-(ethylamino)-5-methyl-5H-1,2-oxaphosphole 2-oxide (2)*** (Br-oxph-1). *Pail-red cryst., m.p. (°C) uncorrected 120-122; Anal. Calcd. for $C_{10}H_{19}BrNO_2P$; (Mr = 268.07): P 11.55, N 5.22, Hal. 29.80 Found: P 11.38, N 5.12, Hal. 29.43%; 1.21 g (82%), IR(KBr): ν_{max}/cm^{-1} 1590, 1260, 1000; ^1H NMR($CDCl_3$): δ/ppm 5.35 (dd, $^2J_{HP}$ 27.7Hz, $^3J_{HH}$ 3.75Hz, ^1H), 3.79 (m, ^1H); 2.93 (m, $^3J_{HP}$ 13.6Hz, 2H), 1.00 (tr, $^3J_{HH}$ 7.0Hz, 3H); 1.70 (m, 2H), 0.69 (tr, $^3J_{HH}$ 6,5Hz, 3H); 1.23 (s, 3H); ^{31}P NMR($CDCl_3$) δ/ppm: ^{31}P 30.09.*

Root growth inhibition test

Root growth inhibition test was conducted as described in (Kalcheva *et al*., 2009b). Br-oxph-1 was tested at concentrations 1 × 10^3 μM; 5 × 10^3 μM; 1 × 10^4 μM; 5 × 10^4 μM; 1 × 10^5 μM. The solutions were prepared just before each treatment.

For root growth inhibition test, seeds of *Triticum aestivum* L. cv. GTW were used. Wheat seeds were thoroughly rinsed with tap water and sterile distilled water. After that seeds were placed between two sheets of filter paper and dried at 25˚C. Twenty seeds were placed on filter paper in each of ten Petri dishes (10 cm in diameter). Five mL of each solution or distilled water, as a control, were applied to the seeds. The dishes were sealed and incubated at 25 ± 1˚C for 96 h. The length of the roots of germinated seeds was measured. The percentage of root growth inhibition caused by each extract in relation to the control was determined. Seeds failed to germinate were excluded from the root elongation test. A growth curve was drawn: root length as percent of control (ordinate) against test concentrations (abscissa). From the growth curve the effective concentration that decreased root growth about 50% (EC50) when compared to the negative control group (distilled water, 100%) were obtained. Three replications of each treatment were done.

Allium cepa-test

Allium cepa-test was conducted as described in (Kalcheva *et al*., 2009a). To assess the possible cytotoxic and genotoxic effects of Br-oph-1 EC50 concentration was tested. *Allium cepa* L. cv. Shtuttgarter Riesen seeds (2*n* = 16) were used. Thirty seeds were placed on filter paper in each of three Petri dishes (10 cm in diameter), containing 5 mL of distilled water. The Petri dishes were sealed and incubated at 25 ± 1˚C for 72 h. Twenty germinated seeds with equal length of roots (~1 cm) were removed and placed on filter paper in each of another three Petri dishes. Five mL of solution tested (at concentration corresponding to EC50 value) were added, and incubated at 25 ± 1˚C for 24 h. Distilled water was used as a negative control. After treatment, the roots were fixed in Clarke's fixative (95% ethanol: acetic acid glacial, 3:1) for 90 min, hydrolysed in 2N HCl for 8 min and in 45% acetic acid (CH_3COOH) for 30 min at room temperature and stained for 40 min in 2% aceto-orcein. After staining, the terminal root tips (1-2 mm) were cut off and squashed in 45% CH_3COOH. Each sample consisted of six root meristems.

At least 600 cells of each root meristem were analyzed. The microscopic analysis included estimation of the mitotic indices and aberrant cells. The mitotic index was determined as a ratio between the number of cells in mitosis and the total number of

analyzed cells. The index of each phase of mitotic division was calculated as a ratio between the cell number in the respective period and the number of dividing cells.

In vitro cell proliferation/viability assay

Cell lines and culture conditions

The SK-HEP-1 was obtained from National Bank for industrial Microorganisms and Cell Cultures (Bulgaria). The cells were maintained as adherent in controlled environment: MEM medium (Sigma-Aldrich), supplemented by 10% heat-inactivated fetal calf serum (Sigma-Aldrich), in incubator at 37°C, 5% CO_2 and humidified atmosphere. In order to keep cells in log phase, the cultures were refed with fresh medium two or three times/week.

In vitro cytotoxicity assay (dose-response relationship)

In vitro cytotoxicity assay was conducted as described in (Koleva *et al*., 2014). Cell proliferation/viability was assessed using water-soluble tetrazolium salt (WST-1) (Roche Applied Science). Exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 µL/well) at a density of 1×10^5 cells per mL. Time of treatment was 24 hours. Four hours before the end of incubation time, cell proliferating reagent WST-1 (10 µL/well) was added to the culture media. Microplates were further incubated for 4 hours at 37°C. The absorbance of formazan product was quantitated at 450 nm using an microplate reader. The cell survival fractions were calculated as a percentage of the untreated control (untreated control = 100%). Dose response curves were created by plotting the percent of proliferating/viable cells versus the test concentrations. Concentration of Br-oxph-1 showing 50% reduction in cell proliferation/viability (half maximal inhibitory concentration value, IC50) was then calculated.

Statistics

The calculations were carried out as described in (Kalcheva *et al*., 2009a, Kalcheva *et al*., 2009b) and (Koleva *et al*., 2014). Experimental data were processed by Student's t-test with $P \leq 0.05$ taken as significance level. In root inhibition test we chose as an experimental unit the root. The calculations were carried out on the assumption that roots used in each treatment made one sample, and each sample was tested against the control sample. In *Allium cepa* test we chose as an experimental unit the cell, instead of the root. The calculations were carried out on the assumption that all the cells of the six root meristems made one sample, and each sample was tested against the negative control. The cytotoxicity assays were carried out in 9 replicate wells. The values for each concentration tested represent the average (mean ± standard deviation).

RESULTS AND DISCUSSION

Root growth inhibition test

In present study root growth inhibition has been used as an index of general toxicity (Çelik and Aslantürk, 2010). The results revealed statistically significant ($P \leq 0.05$) inhibitory effect of compound tested relative to the untreated control (Fig. 2). Treatment with Br-oxph-1 at concentration of 1×10^3 µM inhibited wheat root elongation by 13% as compared with the control. This effect increased with concentrations – growth inhibition by 35.3% (5×10^3 µM), 57.91% (1×10^4 µM) and 77.5% (5×10^4 µM). Almost complete inhibition of root growth was observed at the maximal concentration tested (1×10^5 µM). The effective concentration of Br-oxph-1 that cause 50% of root length as compared to control (EC50) was determined – 8.23×10^3 µM.

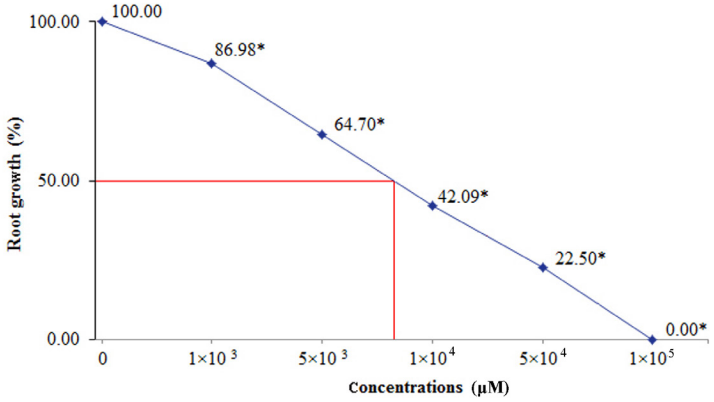

Fig. 2: Inhibition of root growth of *Triticum aestivum* induced by treatment with different concentrations of Br-oxph-1 for 96 h; *$P \leq 0.05$.

Allium cepa–test

The macroscopic effect (inhibition of root growth) appears to be a sensitive parameter for general toxicity, while microscopic studies could provide definitive information regarding the extent of cytotoxic action of compound tested (Bhattacharya and Haldar, 2010). We evaluated microscopic effects of Br-oph-1 at concentration 8.23×10^3 µM (EC50 value) using *Allium cepa*–test. Time of exposure was 24 hours, corresponding to one cell cycle duration time (Trivedi and Ahmad, 2013).

Table 1 presents the effect of Br-oxph-1 on the rate of cell divisionin root meristematic cells of *A. cepa*. Upon the treatment the mitotic index was decreased about 2.4 fold ($P \leq 0.05$) in comparison with the negative control. These resuts revealed stronger cytostatic effect of Br-oxph-1 in

comparison with Br-oxph (Kalcheva *et al*., 2009a). The treatment also changed the mitotic phase distribution relative to untreated cells (Table 2). The notable effect of Br-oph-1 was an increased number of cells in metaphase (by 11.22 percent units) and simultaneous decrease of cells in telophase (by 14.68 percent units).

Table 1: Effect of treatment with Br-oxph-1 (8.23×10^3 µM, for 24 h) on mitotic index in root tip meristematic cells of *Allium cepa* L.

Sample	Number of cells analysed	Number of dividing cells	MI % (mean ± SD)
NC	4176	137	3.30 ± 0.56
EC50	5193	68	1.39 ± 0.47*

NC: negative control (distilled water); EC50: Br-oxph-1 at concentration, corresponding to EC50 value; MI: Mitotic Index; Data are expressed as means ± SD (standard deviation); *P ≤ 0.05.

Table 2: Effects of treatment with Br-oxph-1 (8.23×10^3 µM, for 24 h) on phase indices in root tip meristematic cells of *Allium cepa* L.

Sample	*n*	Prophase Ph	Metaphase Ph	Anaphase Ph	Telophase Ph
		I% ± SD	I% ± SD	I% ± SD	I% ± SD
NES	137	36.00 ± 10.02	25.72 ± 6.27	11.12 ± 5.66	27.17 ± 6.92
EC50	68	28.46 ± 21.42	36.94 ± 31.95	14.15 ± 13.07	12.49 ± 17.13

NC: negative control (distilled water); EC50: Br-oxph-1 at concentration, corresponding to EC50 value; *n* – number of dividing cells; PhI: Phase Index; Data are expressed as means ± SD (standard deviation); *P ≤ 0.05.

Inhibition of mitotic division is considered as an index for cytotoxicity (Konuk *et al*., 2007; Sousa *et al*., 2010; Leme and Marin-Morales, 2009). So, the decline of the mitotic index corroborates a cytotoxic effect of Br-oxph-1. Plant growth maybe affected in different ways: one of the principal mechanisms is the alteration of the mitotic division (Mohamed and El-Ashry, 2012). General toxicity test revealed dose-dependent inhibition of root growth of wheat seeds treated with Br-oxph-1. Root growth is due to cell division of meristematic cells, followed by cell elongation which involves independent events (Shishkova *et al*., 2008; Obroucheva, 2008). In our study root growth inhibition of wheat seeds was accomplished by decreased mitotic division in *Allium cepa* root tips, confirming strong cytostatic effect of Br-oxph-1.

Disturbance of cell cycle kinetics also indicates that compound tested has a cytotoxic potential (Liman *et al*., 2012; Amin, 2002). Br-oxph-1 represents a structural analogue of a clastogenic oxaphosphole derivative (Kalcheva *et al*., 2009a). As described by (Yoshiyama *et al*., 2013) "In response to DNA damage, cell-cycle progression is delayed or arrested at critical stages before or during DNA replication and before cell division". This activation of cell–cycle checkpoints in interphase allows DNA repair system to correct disturbances in DNA or activates apoptosis (Harrison and Haber, 2006). Blocking of cells in G2 phase, which prevents entry into mitosis, could be a reason for the observed decrease in prophase index in treated with Br-oxph-1 cells. Another important checkpoint that ensures proper segregation of chromosomes is blocking spindle assembly checkpoint (Espeut *et al*., 2012). This mechanism prevents anaphase onset until all chromosomes are properly attached to the spindle (Musacchio and Salmon, 2007). Moreover, there is evidence that other phases of mitosis are also monitored since a checkpoint at anaphase–telophase transition has been described (Scolnick and Halazonetis, 2000; Afonso *et al*., 2014; Hopkins *et al*., 2017). In addition of clastogenic properties, a structural analogue of Br-oxph-1 (titled Br-oxph) was found to induce spindle disturbances (Kalcheva *et al*., 2009a). The possible turbagenic action of Br-oxph-1 could explain the increase in the rate of metaphase and anaphase (Andrioli and Mudry, 2011).

A marked individual variation in the responses to Br-oxph-1 was observed. Because of it, the changes in phase indices values were not significant. The same regularity has been observed previously in studies about biological activity of Br-oxph (Kalcheva *et al*., 2009a). A great varibility in the specific response to different chemicals has been reported also in other studies (Tate *et al*., 2005; Hoskins *et al*., 2008; Allen and Stewart, 2009; Marsh and Van Booven, 2009). According to (Lovell, 2013), statistical significance is not the only obligatory factor to be declared a positive result.

In present study the significant decrease in root length of *T. aestivum* seeds was combined with strong inhibition of mitotic division of *Allium cepa* root meristematic cells. The confirmation of macroscopic effects by microscopic events is in accordance with statement of other authors (Aybeke *et al*., 2008; Kaymak and Muranli, 2006) that *T. aestivum* is a suitable test plant for toxicity testing.

In *Allium cepa*-test the evaluation of different kinds of aberrations allows assessing the action mechanisms of the tested compounds. In cytogenetic studies analysis of chromosome aberrations is possible only if there are enough dividing cells (Herrero *et al*., 2012). As can be seen in Table 1, mitotic index value of treated cells was significantly lowered in comparison with untreated control. For this reason, chromosome aberrations were not scored in our study.

In vitro cell proliferation/viability assay

Data obtained using plant test-systems could serve as a basis for further investigations. Taking into account the significant cytotoxic potential of Br-oxph-1, as a next step in this study we evaluated effects on human hepatoma cell line SK-HEP-1 *in vitro*.

Experiments aimed to determine the dose response effect of Br-oxph-1 on SK-HEP-1 cells proliferation/viability were carried out by means of WST-1 cell proliferation assay. The results revealed concentration-dependent and statistically

significant $P \leq 0.05$ inhibition effect of Br-oxph-1 (concentration range 0.338×10^3 µM – 1.013×10^3 µM, for 24 h) (Fig. 3). As can be seen, treatment with 0.338×10^3 µM Br-oxph-1 showed reduction in cell viability. After addition of 0.507×10^3 µM Br-oxph-1 viability was reduced by 36.79 percent units in comparison to untreated control. The inhibitory effect of the compound tested was notable at dose range 0.675×10^3 µM – 0.844×10^3 µM. After treatment with 1.013×10^3 µM Br-oxph-1 no viable cells were detected. From the curve based on the obtained values plotted as percentage of viable cells against Br-oxph-1 test concentrations the IC50 was obtained – 0.642×10^3 µM.

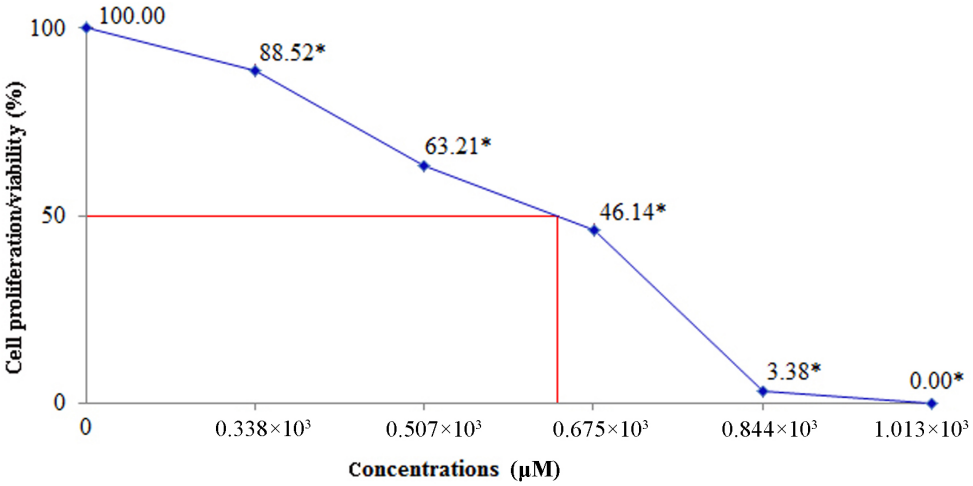

Fig. 3: Effect of Br-oxph-1 on proliferation/viability of human hepatoma cell line SK-HEP-1; *P ≤ 0.05.

Selectivity of action of new antitumor drugs on different type cancer cells is of importance (Burger and Fiebig, 2004; Abu-Surrah and Kettunen, 2006). Our previous studies revealed different cytotoxic influence of analogue of Br-oxph-1 (Br-oxph) on different type cancer cells (Koleva *et al*., 2014; Dragoeva *et al*., 2014). The preliminary results revealed strong inhibitory effect of Br-oxph on SK-HEP-1 cells: after treatment with 10.6×10^3 µM survived cells were 2.27% in comparison with control (data is not presented). Stronger effect of the modified compound (Br-oxph-1) was observed at 10-time lower concentration (1×10^3 µM) - survived SK-HEP-1 cells were 0% in comparison with control. It can be concluded that modifications in chemical structure of Br-oxph lead to significant increasement of antiproliferative activity against tested cell line. Further modifications could increase this activity.

CONCLUSION

Br-oxph-1 represents a newly synthesized by us structural analogue of a cytotoxic and genotoxic Br-oxph. Plant test-systems used in this study revealed growth inhibitory and cytotoxic effects of Br-oxph-1. Data *in vitro* was in accordance with the results from plant tests – Br-oxph-1 exerted strong cytotoxic effects on human hepatoma cell line SK-HEP-1. Cytotoxic effects *in vitro* of Br-oxph-1 were stronger in comparison with Br-oxph. Thus, additional investigations on biological activity of Br-oxph-1 are a matter of interest.

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