Bulgarian Chemical Communications, Volume 55, Issue 3 (pp. 273-277) 2023 DOI: 10.34049/bcc.55.3.NCNTC07

Study of the toxicity of benzanthrone luminescent dyes

A. Kırılova, M. Savıcka, T. Grıgorjeva, E. Kırılova*

Institute of Life Sciences and Technology, Daugavpils University, Daugavpils, Latvia

Accepted: August 07, 2023

Many dyes, including luminescent ones, are widely used in our everyday consumption, therefore they are potentially dangerous for the environment, as they can get into sewage and groundwater. In this regard, a sensitive, fast, reliable and inexpensive toxicity assay is needed to assess toxicity to eukaryotic cells. To identify the toxicity of compounds, it is necessary to use various bioassay methods that complement each other in the spectrum of analytes. For this purpose, the toxicological effect of new benzanthrone aminophosphonates was previously evaluated using wheat germ (*Triticum aestivum*) as a test organism and it was found that the studied compounds exhibit a toxic effect on the growth of wheat seedlings to varying degrees, depending on the concentration and substituent on α-carbon atom. The study of the biotoxicity of these substances was then extended to a new test object. As the test organism, yeast *Saccharomyces cerevisiae* was used. The biological toxicity test used the *S. cerevisiae* lethality test based on the detection of changes in yeast viability. The studied dyes in various concentrations were exposed to yeast cells for various durations. After the selected exposure time, the number of live and dead cells was counted and analyzed to assess the toxicity of the studied luminescent dyes.

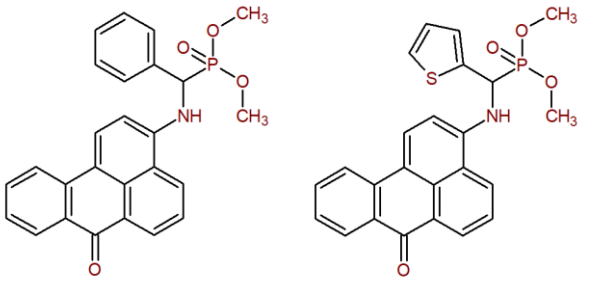
Keywords: Luminescent dyes; Benzanthrone derivatives; Toxicity; Yeast *Saccharomyces cerevisiae*; Viability

INTRODUCTION

Today, luminescent dyes are an important research tool in many fields of science. Every year they are increasingly used in natural science and biotechnology: to study biological objects, visualize specific organs and tissues of the body, identify biomolecules - proteins, peptides, nucleic acids and other biologically important compounds [1]. Therefore, scientists are developing new luminescent dyes for various purposes, which makes it necessary to study the properties of newly synthesized substances. Some luminescent dyes are widely used in our daily consumption, therefore they are potentially dangerous for the environment, as they can get into sewage and ground water. Therefore, in order to assess toxicity to eukaryotic cells, it is necessary to carry out toxicity analysis for the substances used. Current microbial and biochemical assays to assess the chemical toxicity of various substances include insects [2], fish [3], freshwater microalgae [4], aquatic crustaceans [5, 6], and tumor cell lines [7] to assess molecular toxicity to eukaryotic cells and tissues. To identify the toxicity of compounds, it is necessary to use various bioassay methods that complement each other in the spectrum of analytes.

EXPERIMENTAL

The studied dyes were synthesized according to Kabachnik-Fields reaction from 3-amino-benzanthrone and appropriate aldehydes and dimethylphosphite, The structures of these compounds are presented in Scheme 1. Synthesis details, as well as spectral data were given earlier [8].



Scheme 1. Chemical structure of the studied compounds.

Instant dehydrated baker's yeast, Saf-Instant, produced in Poland (*S. cerevisiae*) was used.

The scanning of visual field by vision camera and the use of image analysis to evaluate results enabled a significant speed-up and simplification of the work. Axiolab 5 microscope (× 1000) with Axiocam

* To whom all correspondence should be sent:
E-mail: jelena.kirilova@du.lv

© 2023 Bulgarian Academy of Sciences, Union of Chemists in Bulgaria

208 color camera (Zeiss) was used in these experiments (programme Zeiss Labscope).

The microscopic images of colored yeast cells were obtained using a buffered methylene blue solution [9]. For microscopic studies approximately 0.1 g of yeast was suspended in 10 ml of studied substance solution. A water-yeast suspension without any other substance was prepared as a control sample. The quantity of yeast used corresponds to the requirement of 50–150 cells in the visual field of the microscope. A suspension with a larger number of cells in the visual field is difficult to count, a smaller number of cells leads to inaccurate results. After the selected exposure time, one drop of the suspension and one drop of methylene blue were mixed on a slide and examined microscopically. The number of live (colourless) and dead (coloured) cells was immediately calculated in at least 5 visual fields. The same procedure was performed with the control suspension.

The conductivity was determined using a conductivity meter with conductivity cell 980-K19/120 (Mettler Toledo). 20 ml of the examined water were pipetted in five test tubes and 2.0 g of sugar were added in each tube. Other five test tubes with sugar solution without any toxic substances were prepared as control samples. After dissolution, the specific conductivity of the sugar solution was measured in all test tubes.

1.0 g of yeast was added into each of the test tubes and stirred well. After 30 and 60 min of exposure the specific conductivity was measured again in each test tube. It was indispensably necessary to keep the same temperature in all test tubes during the test.

RESULTS AND DISCUSSION

α-Aminophosphonates have received considerable attention in recent years, mainly due to their interesting biological properties, some of which have already been commercialized [10, 11]. Aryl aminophosphonates are used as starting materials for the synthesis of biologically active compounds [12-14]. Some of them have pronounced fluorescent properties, in connection with which it is proposed to use them as luminescent dyes [15-18].

Since these compounds have been found to be effective protease and phosphatase inhibitors, genotoxicity and cytotoxicity studies have been conducted for some of them. Anthracene-derived aminophosphonates show moderate genotoxic and antiproliferative activity *in vivo* and low toxicity to mouse embryo cells, some of derivatives were potent cytotoxic agents towards colon carcinoma cell line [19]. Pyrene-derived aminophosphonates were found to be cytotoxic for colon cancer cell lines, while exhibiting virtually no toxicity to lymphocytes [20].

Previously, the effect of some alkyl aminophosphonates on the membrane potential and electrical conductivity of the internodal cells of *Nitellopsis obtusa* cells and hemolysis of erythrocytes was studied [21]. It has been established that these organophosphorus compounds at concentrations of 10^{-4} - 10^{-5} M cause depolarization and increase in the electrical conductivity of algae membranes. When used at higher concentrations, aminophosphonates caused hemolysis of erythrocytes. The observed changes are the result of direct interaction of aminophosphonates with the lipids of the plasma membrane. Thus, as was established earlier [22], the biological activity of organophosphorus compounds is associated with their interaction with the lipid phase of biological membranes.

It was of interest to evaluate the toxicity of newly synthesized benzanthrone aminophosphonates [8], which showed pronounced luminescent properties, like many other N-containing benzanthrone derivatives [23-25]. Convenient objects of toxicity research are various plants, especially their sprouts growing in seedlings. For plants under any stressful conditions, the dominant goal is to reduce the effects of stressors in order to survive and continue to grow. The literature describes studies of the stress effect of various chemical substances on plant development, using different concentrations of substances and exposure times. Athinarayanan *et al*. studied the effect of natural dyes on the germination of *Phaseolus aureus* seeds, showing as a result that these natural dyes do not have a toxic effect on the growth of aquatic plants within the investigated concentrations 50-100 ppm [26]. Studies of the physiological effect of dimethyl sulfoxide (DMSO) on rice seeds (*Oryza sativa L.*) have been carried out [27]. As a result, it was found that a linear decrease in the relative growth rate of rice seedlings was observed as the concentration of DMSO increased from 0.56 to 13.54 mM.

The evaluation of toxicity of compounds **1** and **2** on the wheat seedlings was performed using dye concentrations from 10^{-7} M to 6×10^{-5} M [8]. Both dyes were found to cause significant oxidative damage to wheat seedlings, as evidenced by elevated levels of malondialdehyde (as the indicator of oxidative stress [28]) content and electrolyte leakage compared to control wheat sprouts. Compound **2** showed a very pronounced toxic effect on wheat growth (see Figure 1). As a result of the study, it was concluded that dye **1** and **2** solutions cause

significant oxidative damage to wheat seedlings. It was also established that the toxic properties of the studied substances differed: a substance with a thiophene group is more toxic to plants than a substance containing a phenyl group. It can be concluded that wheat germ is a good object for assessing the toxicity of the studied substances. However, this method requires 6-7 days for one experiment and three weeks for three independent experiments.

Fig. 1. Wheat sprouts cultivated in the presence of dye **2** with concentrations: a (0 M), b (6×10^{-5} M), c (3×10^{-6} M).

The search for other faster methods has led to yeast. Previously, several bioassays using yeast have been proposed [29-31]. The yeast *S. cerevisiae* is a simple eukaryotic model organism for evaluating toxic effects, since its cellular structure and functional organization share many similarities with animal and plant cells [29, 32]. Thus, it is a convenient model organism for assessing toxic effects in human cells and tissues. The study of cell death in *S. cerevisiae* can potentially be productive for understanding cell death. Therefore, we continued our study of toxicity using a new test object - yeast *Saccharomyces cerevisiae*.

The used biological toxicity test – *S. cerevisiae* lethal test is based on detection of yeast viability changes. Viability is a term used to describe whether a cell is alive or dead and refers to the percentage of living cells.

The most common approaches to detect yeast cell viability and concentration is to identify and manually count live yeast cells in a sample stained with methylene blue. To distinguish living cells from dead cells, they were examined under a microscope using methylene blue: dead cells stained blue, while living cells did not stain. Cells constantly die during normal physiological processes such as development and differentiation-based cell turnover. Cells can also die in response to various external (pathogens, toxins, environmental changes) or internal (mutations or epigenetic malfunctions) factors.

The studied dyes at various concentrations were exposed to yeast cells for various durations. After the selected exposure time the number of live (colorless) and dead (colored) cells were calculated and analyzed for evaluation of toxicity of the investigated luminescent dyes.

For the yeast viability test, dye concentrations of 6×10^{-5} M and 10^{-4} M were used. The results are presented in Table 1. No statistically significant increase in the yeast cell mortality after 15 min treatment with different studied dyes in a concentration of 6×10^{-5} M could be detected. The number of living cells of *S. cerevisiae* decreased by about 3% in the presence of dye **1** and **2** and it reached 6% at the end of the investigated period compared with the control. An increase in the concentration of the studied dyes (10^{-4} M) slightly reduced the number of living yeast cells: by about 7% compared with a lower concentration. However, there were no changes in the number of living cells of yeast after 24-h exposure compared to control. Recent studies showed that dead yeast cells can provide surviving cells with nutrients or induce their stress response by transmitting an alarm signal [33, 34]. Dead yeast cells absorbed more chemicals than control cells to protect living cells from stressors. Perhaps our results are another proof of the "altruistic" death hypothesis [35], and the death of a part of yeast cells can contribute to the survival of other cells. Similar results were obtained on other microorganisms, e.g., *E. coli* [36], algal cells [37], etc.

Table 1. Viability of yeast in the presence of dyes **1** and **2**.

	Concentration 6×10^{-5} M			Concentration 10^{-4} M		
	After 15 min	After 30 min	After 60 min	After 15 min	After 30 min	After 60 min
Standard	100	100	100	100	100	100
Dye **1**	96.5	94.4	94.4	94.7	92.7	93.3
Dye **2**	97.2	94.5	94.4	93.7	94.9	92.6

Further in the work, a conductometric test for water toxicity was carried out, based on the observation of a change in the specific electrical conductivity of a suspension of the yeast *S. cerevisiae* as a result of inhibition of the yeast fermentation activity under toxic conditions [30]. As can be seen from the results (see Table 2), there is no significant change in the electrical conductivity of the yeast suspension, which would indicate cell destruction and electrolyte leakage as a result of this. These data also support the "altruistic" death hypothesis, according to which the death of some yeast cells may contribute to the survival of other cells.

Table 2. Viability of yeast in the presence of dyes **1** and **2 (**concentration 10^{-4} M).

	Conductivity, μS/cm	
	After 30 min	After 60 min
Standard	155.7	182.8
Dye **1**	153.8	184.1
Dye **2**	155.8	189.7

CONCLUSION

It was shown that yeast cells, unlike wheat germs, are weakly sensitive to the action of new benzanthrone aminophosphonates, the number of dead cells slightly increases with increasing dose and duration of exposure. Thus, despite the simplicity and speed of obtaining results, yeast is poorly suited for assessing the toxicity of the studied compounds.

Acknowledgement: *The current study was supported by Daugavpils University Students Research Project (Project No: 14-89/2023/2).*

REFERENCES

1. X. Fei, Y. Gu, *Prog. Nat. Sci.: Mater.*, **19**, 1 (2009).
2. M. A. Nascarella, J. G. Stoffolano Jr., E. J. Stanek, P. T. Kostecki, E. J., Calabrese, *Environ. Pollut.*, **124,** 257 (2003).
3. S. Pichardo, A. Jos, J. L. Zurita, M. Salguero, A. M. Camean, G. Repetto, *Toxicol. in Vitro*, **21,** 1460 (2007).
4. A. Krishna Moorthy, B. Govindarajan Rathi, S. P. Shukla, K. Kumar, V. Shree Bharti, *Environ. Toxicol. Pharmacol.* **82**, 103552 (2021)
5. J. L. Zurita, A. Jos, A. del Peso, M. Salguero, A. M. Camean, Lopez-Artiguez, G. Repetto, *Sci. Total Environ.*, **387,** 155 (2007).
6. S. W. Reddy, J. W. Osborne, *Biocatal. Agric. Biotechnol.*, **25**, 101574 (2020).
7. N. Yang, J. Pang, Z. Huang, Q. Zhang, Z. Wang, D. Sun, *Food Chem. Toxicol.*, **173**, 113612 (2023).
8. A. Maleckis, E. Griskjans, M. Cvetinska, M. Savicka, S. Belyakov, E. Kirilova, *J. Molec. Struct.*, **1277**, 134838 (2023).
9. K. Painting, B. Kirsop, *World J. Microbiol. Biotechnol.*, **6**, 346 (1990).
10. A. Mucha, P. Kafarski, Ł. Berlicki, *J. Med. Chem.*, **54**, 5955 (2011).
11. A. Amira, Z. Aouf, H. K'tir, Y. Chemam, R. Ghodbane, R. Zerrouki, N.-E. Aouf, *ChemistrySelect*, **6**, 6137 (2021).
12. R. Karpowicz, J. Lewkowski, M. Stasiak, A. Czopor, P. Tokarz, A. Król, D. Rogacz, P. Rychter, *Phosphorus Sulfur Silicon Relat. Elem.*, **193**, 423 (2018).
13. B. Zhang, X. T. Hu, K. M. Zhou, Y. S. Yang, H. L. Zhu, *Bioorg. Chem.*, **102**, 104096 (2020).
14. X. C. Yang, C. M. Zeng, S. R. Avula, X. M. Peng, R. X. Geng, C. H. Zhou, *Eur. J. Med. Chem*., **245**, 114891 (2023).
15. A. Maleckis, M. Cvetinska, E. Griskjans, L. Mezaraupe, M. Kirjusina, V. Pavlova, E. Kirilova, *J. Photochem. Photobiol*. A., **444**, 114918 (2023).
16. A. Kusnierz, E. Chmielewska, *Phosphorus Sulfur Silicon Relat. Elem.*, **192**, 700 (2017).
17. M. G. Gorniak, P. Kafarski, *Phosphorus Sulfur Silicon Relat. Elem.*, **191**, 511 (2016).
18. M. G. Gorniak, A. Czernicka, P. Młynarz, W. Balcerzak, P. Kafarski, *Beilstein J. Org. Chem.*, 10, 741 (2014).
19. I. Kraicheva, I. Tsacheva, E. Vodenicharova, E. Tashev, T. Tosheva, A. Kril, M. Topashka-Ancheva, I. Iliev, T. Gerasimova, K. Troev, *Bioorg. Med. Chem.*, **20**, 117 (2012).
20. J. Lewkowski, M. Rodriguez Moya, A. Wrona-Piotrowicz, J. Zakrzewski, R. Kontek, G. Gajek, *Beilstein J. Org. Chem*., **12**, 1229, (2016).
21. Z. Trela, H. Kleszczynska, J. Sarapuk, *Z. Naturforsch. C J. Biosci*., **56**, 838 (2001).
22. R. H. Shimabukuro, *Pestic. Biochem. Physiol*., **48,** 85 (1994).
23. M. Olipova, A. Maleckis, A. Puckins, A. Kirilova, E. Romanovska, E. Kirilova, *Bulg. Chem. Commun.*, **54**, 253 (2022).
24. U. Tarabara, E. Kirilova, G. Kirilov, K. Vus, O. Zhytniakivska, V. Trusova, G. Gorbenko, *J. Mol. Liq.*, **324**, 115102 (2021).
25. N. Orlova, I. Nikolajeva, A. Puckins, S. Belyakov, E. Kirilova, *Molecules*, **26**, 2570 (2021).
26. G. Athinarayanan, R. Mariselvam, A. J. A. Ranjitsingh, A. U. R. Nanthini, *Int. J. Sci. Res.*, **4**, 2061 (2015).
27. X.-H. Zhang, X.-Z. Yu, D-M. Yue, *Int. J. Environ. Sci. Technol.*, **13**, 607 (2016).
28. M. Khoubnasabjafari, K. Ansarin, A. Jouyban, *Curr. Pharm. Anal.*, **12**, 4 (2016)
29. L. Rumlova, J. Dolezalova, *Environ. Toxicol. Pharmacol.*, **33**, 459 (2012).
30. J. Dolezalova, L. Rumlova. *Environ. Toxicol. Pharmacol.*, **38**, 977 (2014).
31. A. L. Valimaa, A. Kivisto, M. Virta, M. Karp, *Sensors (Basel)*, **8**, 6433 (2008).
32. E. V. Grosfeld, V. A. Bidiuk, O. V. Mitkevich, E. S. M. O. Ghazy, V. V. Kushnirov, A. I. Alexandrov, *J. Fungi*, **7**, 886, (2021).

33. J. M. Hardwick, *mBio*, **9**, 4 (2018).
34. D. Carmona-Gutierrez, T. Eisenberg, S. Buttner, C. Meisinger, G. Kroemer, F. Madeo. *Cell Death Differ*., **17**, 763 (2010).
35. N. A. Kireeva, S. S. Sokolov, E. A. Smirnova, K. V. Galkina, F. F. Severin, D. A. Knorre, *ASM Journals mSphere*, **6**, 6 (2021).
36. M. Snoussi, J. P. Talledo, N-A. Del Rosario, S. Mohammadi, B-Y. Ha, A. Kosmrlj, S. Taheri-Araghi, *Elife*, **7** (2018).
37. N. F. Y. Tam, A. M. Y. Chong, Y. S. Wong, *Mar. Pollut. Bull.*, **45**, 362 (2002).