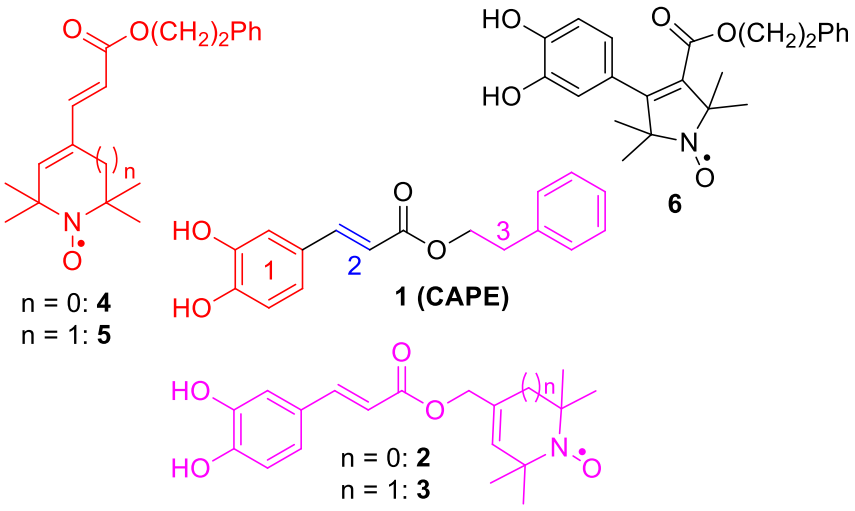
Detailed research report

We performed most of the studies planned. However, due to the COVID19 pandemic, certain experiments have not been finished yet, because the laboratories of our partners in the USA were closed for months and the acquisition of certain chemicals become very difficult during the last 1.5 year. Nevertheless, the ongoing studies will be finished during the next few months, then the results will be published (indicating the grant number in the funding/acknowledgements section).

During the 5 years of the grant, we published 15 research articles (ΣIF = 47.311; Q1: 6; Q2: 1; Q3: 5; Q4: 1) as a part of this research project. In addition, one more article is accepted by Arkivoc, two articles are under preparation and one further manuscript is in progress. We synthesized and characterized many new nitroxide containing biomolecules and paramagnetic building blocks. Our main results are summarized in the following 10 points.

1. Synthesis and study of paramagnetic alcohols and phenols.

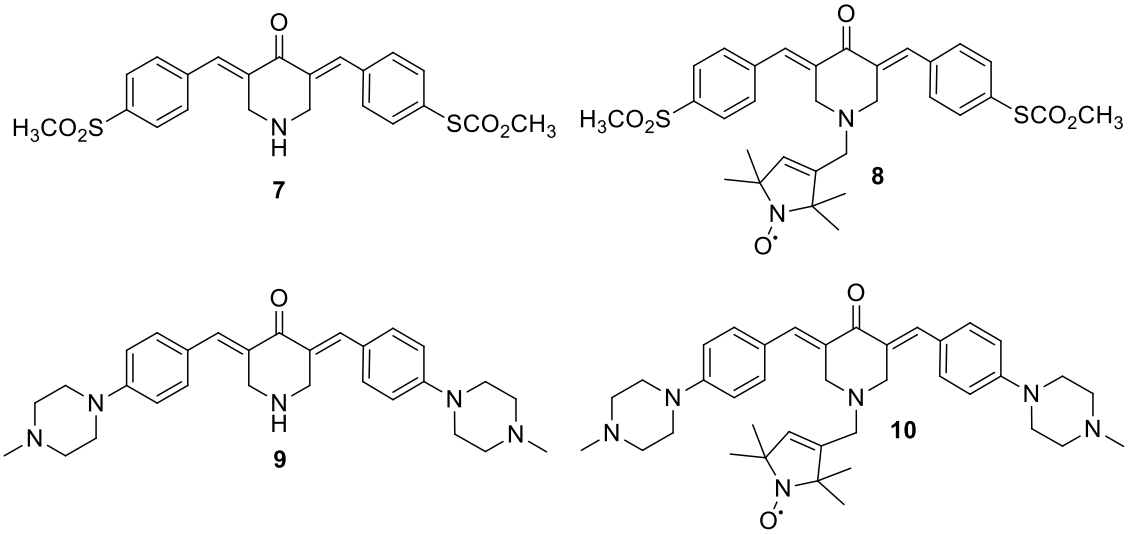
New paramagnetic caffeic acid phenethyl ester (**1**, CAPE) analogs have been synthesized. Three structural fragments (1, 2, 3) of the basic scaffold were modified with nitroxides (**2**-**6**). In collaboration with dr. Masaki Nagane (Laboratory of Biochemistry, School of Veterinary Medicine, Azabu University, Japan), the structure-activity relationships as potential antioxidants of these esters have been investigated in vitro and in vivo as well as their cytotoxicity and compared to CAPE and non-phenolic paramagnetic alcohols. The in vitro antioxidant activity was tested with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid radical ($ABTS^{\cdot+}$) scavenging, while the cell protection against reactive oxygen species (ROS) were carried out in the presence of 2′,7′-dichlorofluorescin diacetate and hydrogen-peroxide. Our results suggested that catechol ring is responsible for cytotoxicity, but it is necessary for great antioxidant property. Replacement of phenethyl group with nitroxide rings resulted compounds with lower cytotoxicity but similar antioxidant activity than CAPE. Further anti-cancer studies of compound **2** are on the way on rat experiments, but the data have not been published yet.[1]



2. Synthesis and study of dual-active curcuminoids.

In continuation of our previous research with diarylidienylpiperidones as potential antiproliferative compounds novel 4,4'-disulfonyldiarylidenyl piperidones have been synthesized and studied together with Prof. Kuppusamy's group (Department of Radiology and Medicine, Dartmouth College, USA). Cellular uptake and the metabolic conversion were followed by EPR spectroscopy, HCT-116 cells incubated with **8** (10 μM) at 37 °C exhibited a time-dependent EPR signal from the cell (pellet) as well as supernatant. Medium cytotoxicity was measured by MTT assay, the antiproliferative activity against HCT-116 colon cancer cells have been determined by bromodeoxyuridine assay. The western-blot analysis of some transcription factors in proliferative pathway has been performed as well. Compounds **7** and **8** showed great antiproliferative activity, however the nitroxide containing and non-containing piperidones have different mechanism. In addition, the nitroxide moiety decreased the cytotoxicity of the parent compound.[2]

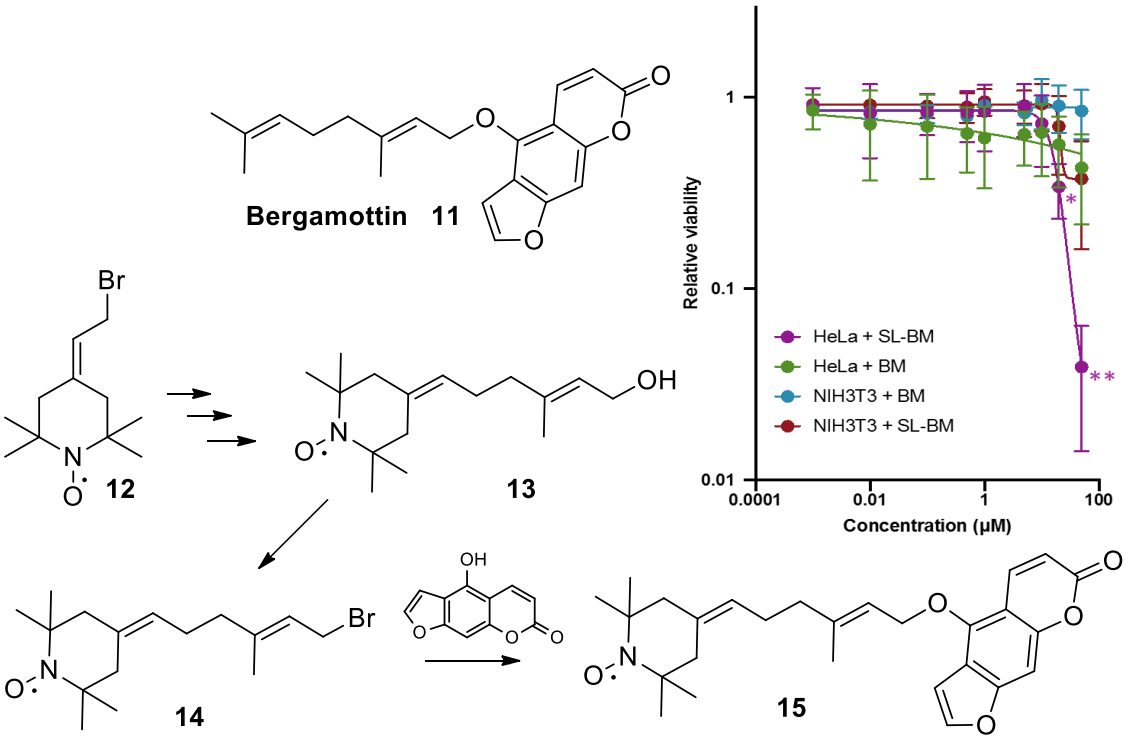
Pancreatic adenocarcinoma is an aggressive cancer with poor clinical prognosis and limited therapeutic options. The anticancer efficacy of diarylidenylpiperidones (**9**, **10**) in a human pancreatic cell line (AsPC-1) was studied in collaboration with Prof. Kuppusamy's group. We found that both the compounds exhibited potential cytotoxicity to AsPC-1 cells by inducing G2/M cell-cycle arrest, apoptosis, and cell death, by mitochondrial damage. Both compounds appear to act through p21 and the STAT3 pathway, by inhibiting the formation of p-STAT3 (signal transducer and activator of transcription 3), which protein is associated with the poor prognosis of this carcinoma. and inhibition of STAT3 phosphorylation.[3]



3. Synthesis and study of SL-bergamottin.

A new bergamottin (BM, **11**) containing a nitroxide moiety (spin labelled bergamottin, SL-BM, **15**) was synthesized in a multistep synthesis starting from allylic bromide (**12**), through SL-geraniol (**13**), and evaluated as a potential inhibitor of the CYP2C19, CYP3A4, and CYP2C9 enzymes and inhibitor of tumor cell proliferation in collaboration with dr. Masaki Nagane (Laboratory of Biochemistry, School of Veterinary Medicine, Azabu University, Japan), dr. Miklós Poór's group (Deparment of Pharmacology, University of Pécs, Hungary) and dr. Csaba Hetényi's group (Department of Pharmacology and Pharmacotherapy, University of Pécs). The CYP enzyme inhibitory activity of **15**

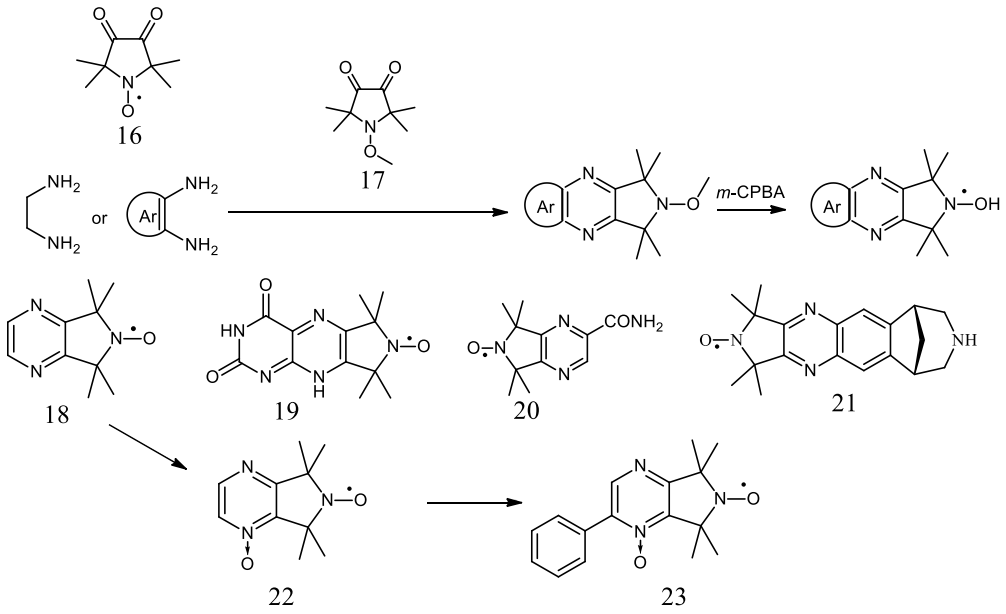
was compared to **11** and known inhibitors such as ketoconazole (3A4), warfarin (2C9), and ticlopidine (2C19). Among the compounds studied, BM showed the strongest inhibition of the CYP2C9 and 2C19 enzymes, nevertheless SL-BM is a more potent inhibitor of CYP3A4 than the parent compound. The enhanced inhibitory activity of SL-BM compared to that of BM was also supported by docking experiments, where the binding of SL-BM was more favourable than that of BM (ΔGbind(−10.4 vs. −9.2 kcal/mol)). The nitroxide moiety markedly increased the antitumor activity of BM toward HeLa cells and marginally increased its toxicity toward a normal cell line. [4]



4. Synthesis of 1,4-diazine-fused nitroxide free radicals

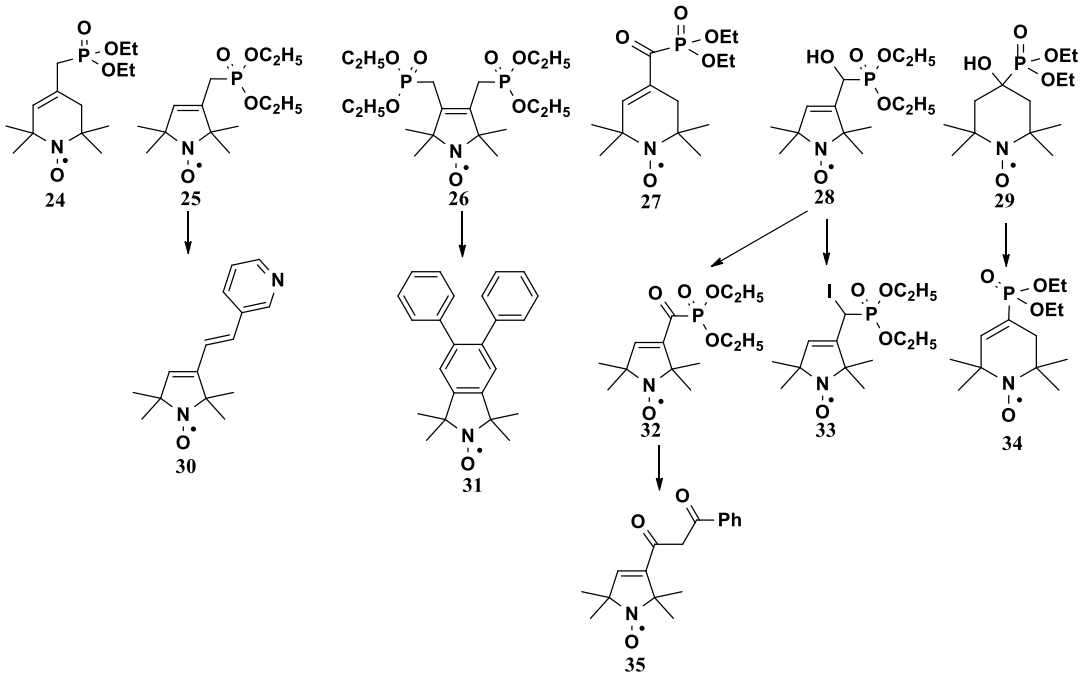
Diazines are important members of heterocycles with many biologically active compounds, nevertheless compound **16** (1-oxyl-2,2,5,5-tetramethylpyrrolidine-3,4-dione) proved to be unstable, so the direct reaction of diamines and the paramagnetic diketone wasn't an option in the synthesis of nitroxide fused diazines. We developed a new diamagnetic synthon, 1-methoxy-2,2,5,5-tetramethylpyrrolidine-3,4-dione (**17**) which was condensed with different aliphatic, aromatic or heteroaromatic 1,2-diamines followed by deprotection of the nitroxide function with m-CPBA, providing pyrroline nitroxide fused pyrazines (**18**, **20**), pteridine **19**, or quinoxalines. Overoxidation of **18** upon prolonged reaction time and excess m-CPBA we observed the formation of *N*-oxide **22**, which offered the possibility of C-H functionalization with benzene at C2 position by palladium catalysis and Ag_2CO_3 oxidation, yielding pyrazine **23**.[5]

In continuation of our previous research with spin-labeled drugs and biomolecules, we reported on a paramagnetic analogue of varenicline (Chantix, **21**), which, as a nicotinil receptor agonist, is used to treat nicotine addiction, by fusing the pyrazine ring of the original drug with a rigid 1-oxyl-2,2,5,5-tetramethylpyrrolidine nitroxide.[6]

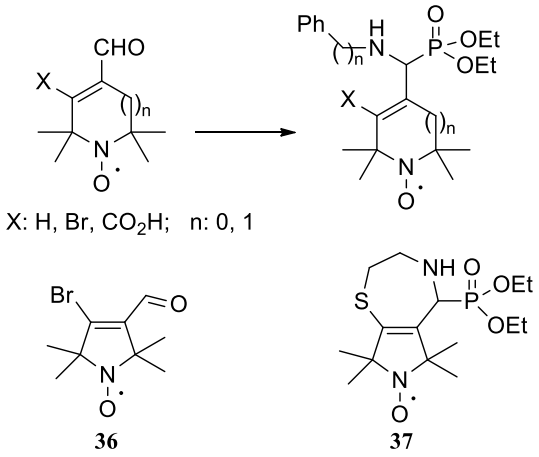


5. Synthesis and application of paramagnetic phosphorus compounds

Functionalized phosphonates are fascinating organophosphorus compounds used in biology, pharmacology, agriculture and organic chemistry, nevertheless a limited number of paramagnetic phosphorus compounds have been reported yet. Starting from paramagnetic aldehydes, ketones, acid chlorides and allylic bromides we reported the syntheses of pyrroline and piperidine nitroxide phosphonates (**24**-**29**) by known methods, such as the Pudovik, Arbuzov, Perkow reactions. Paramagnetic hydroxyphosphonates (**28**, **29**) proved to be useful starting compounds in the synthesis of **32** ketophosphonate, vinylphosphonate (**34**) and alfa-iodophosphonate (**33**). The synthesized paramagnetic phosphonates were useful synthetic building blocks for carbon-carbon bond-forming reactions such as Horner-Wadsworth-Emmons olefination (**30**, **31**). The Trolox equivalent antioxidant capacity (TEAC) of new phosphonates was also screened, among them **29** tertiary-hydroxyphosphonatate nitroxides exhibited remarkable antioxidant activity (Molecules 2020, 25, 2430). Compound **32** was successfully utilized as an acylation agent for the synthesis of paramagnetic 1,3-dicarbonyl compounds.[7,8]

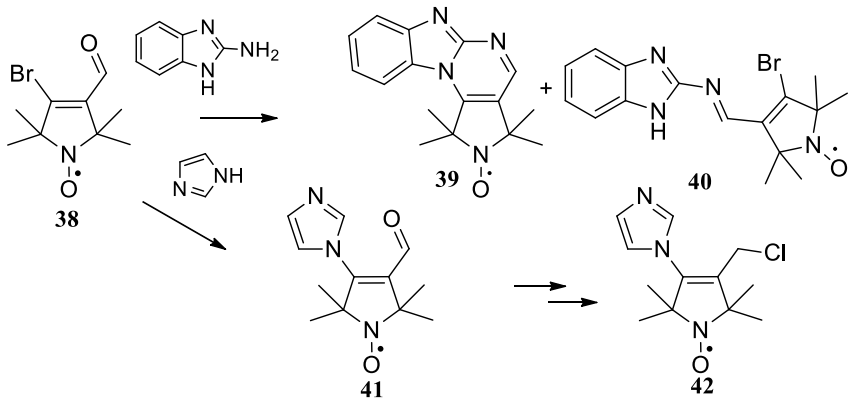


While paramagnetic α-amino acids are well known, as far as we know, this is the first report on paramagnetic α-aminophosphonate syntheses. In Kabachnik–Fields reaction we synthesized a variety of nitroxide containing α-aminophosphonates starting from five- and six-membered paramagnetic aldehydes, paramagnetic ketone and five- and six-membered paramagnetic amines as well. Starting from a 4-bromo-pyrroline nitroxide aldehyde, we constructed paramagnetic pyrrolo[3,4-f][1,4]thiazepine scaffold with a phosphonate ester substituent.[9]



6. Synthesis of *N*-heterocycles anellated with or connected to nitroxides

Nitroxides conjugated with imiadzoles or benzimidazoles were achieved by the Buchwald-Hartwig reaction of paramagnetic β-bromo-α,β-unsaturated aldehyde (**38**) with imidazole or benzimidazole. To explore the scope of this coupling reaction with the same conditions afforded polycondensed (1,1,3,3-Tetramethyl-1*H*-benzimidazo[1,2-a]pyrrolo[3,4-e]pyrimidin-2-yloxyl, **39**) derivative if 2-amino-benzimidazol was reacted with the aldehyde. Compound **41** in a two-step synthesis gave allylic chloride (**42**), which can be used in the synthesis of new spin-labelling methanethiosulfonate.[10]

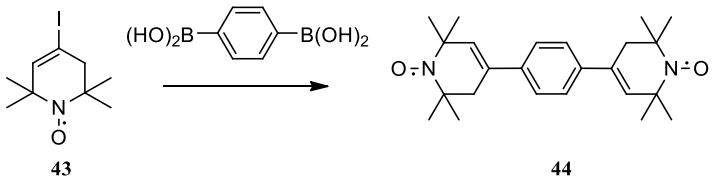


7. Interaction of Chrysin and Its Main Conjugated Metabolites Chrysin-7-Sulfate and Chrysin-7-Glucuronide with Serum Albumin

The complex formation of chrysin, which is found in nature and in several dietary supplements, chrysin-7-sulfate (C7S) and chrysin-7-glucuronide (C7G), which conjugates appear in the circulation at much higher concentrations than chrysin, with human (HSA) and bovine (BSA) serum albumins was investigated in a cooperation with dr. Poór Miklós's group (Deparment of Pharmacology, University of Pécs, Hungary), employing fluorescence spectroscopic, ultrafiltration, and modelling studies. Chrysin-7-sulfate was efficiently synthesized by the reaction of chlorsulfonic acid and chrysin in the presence of triethylamine. All tested flavonoids occupy Sudlow's Site I in HSA, compared to chrysin, C7S binds with a threefold higher affinity to HSA, while C7G binds with a threefold lower affinity. We concluded that the high intake of chrysin (e.g., through the consumption of dietary supplements with high chrysin contents) may interfere with the albumin-binding of several drugs, mainly due to the strong interaction of C7S with HSA. (International Journal of Molecular Sciences, 2018, 19, 4073-4087).[11]

8. The structure and internal dynamics of R6-p-C6H4-R6 biradical:

The biradical **44**, 4,4′-(1,4-phenylene)bis(2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-1-yloxyl), was prepared via the Suzuki coupling reaction of vinyl iodide (**43**) [and 1,4-phenylenebisboronic acid) and studied by X-band electron paramagnetic resonance (EPR) spectroscopy in the group of Prof. Kokorin (N. Semenov Institute of Chemical Physics, Russia). Hyperfine splitting (hfs) constants on the ^{14}N atoms, electron spin exchange integral |J|, and the distance between the two N–O fragments rNO–NO were experimentally measured. The optimized geometry was compared with X-ray crystallographic data and theoretical hfs constants were compared with the respective experimental EPR values. It is concluded that the current quantum chemical approaches provide good results in calculating hfs constants as well as some other EPR parameters. It is confirmed that the intramolecular electron spin exchange in biradicals is realized by the indirect mechanism rather than direct collision of the N–O·groups.[12]

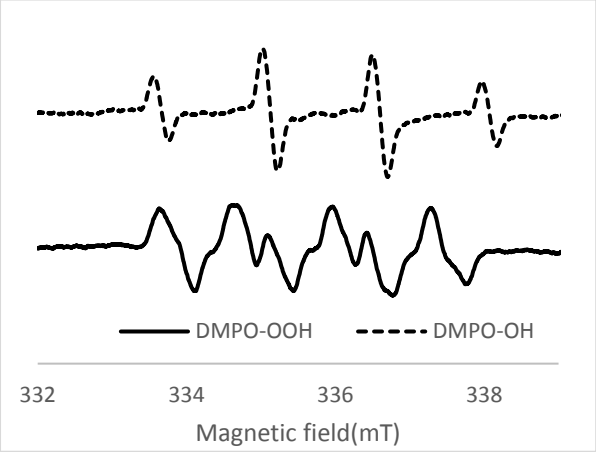


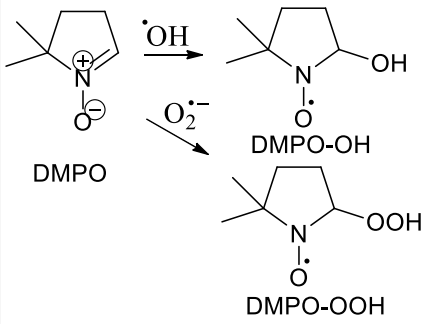
9. EPR spectroscopic detection of ROS formed on the surface of irradiated TiO_2

5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) forms a relatively stable paramagnetic adduct with both hydroxyl radical and superoxide radical anion, giving a characteristic coupling pattern in the EPR spectrum. After the TiO_2 samples were irradiated in an aqueous medium, we observed a quartet signal of DMPO-OH with an intensity ratio of 1:2:2:1, with hyperfine coupling constants aN = aH = 1.49 mT. When the solvent was changed to dimethyl sulfoxide, the EPR signal of DMPO-OOH was observed (aN = 1.37 mT, aH = 1.0 mT) (Figure 16). The kinetic studies of EPR-adducts showed good parallelism with the photodegradation of methyl orange.

The photocatalytic and antibacterial (Carbapenem-resistant Klebsiella pneumonia) activities of PF-co-doped anatase TiO_2 nanoparticles (NPs) were studied in collaboration with Dr. László Kőrösi (University of Pécs, Research Institute for Viticulture and Oenology) and his group. PF-co-doping increased the antibacterial activity, which result was also confirmed by quantitive EPR measurements of the forming hydroxyl radicals.[13]

In a collaboration with dr. László Kőrösi's group (Research Institute for Viticulture and Oenology, University of Pécs) we studied the photocatalytic stress responses of grapevine (Vitis vinifera L.) as model plant exposed to a well-known photocatalyst, Degussa P25 TiO2 NPs. Foliar exposure of five red cultivars (Cabernet sauvignon, Cabernet franc, Merlot, Kékfrankos and Kadarka) was carried out in blooming phenophase under field condition where plants are exposed to natural sunlight with relatively high UV dosage (in June). The presence of superoxide and hydroxyl radicals and singlet oxygen under UV illumination of Degussa P 25 were confirmed by EPR spectroscopy. The increasing level of polyphenols, K, Ca, Mg, Mn and B can also be related to the photocatalytically produced ROS.[14]





10. Reviews

a.: Synthesis and application of stable nitroxide free radicals fused with carbocycles and heterocycles

A review article has been published to present recent results with synthetic methodologies to achieve stable nitroxide free radicals fused with aromatic carbocycles. e.g., isoindoline- like nitroxides, phthalimides and heterocycles (furan, pyrrole, thiophene, 1,2-thiazole, selenophene, pyrazole, pyrimidine, pyridine, pyridazine, 1,5-benzothiazepine). The possible applications of these new stable nitroxide free radicals, such as covalent spin labels and noncovalent spin probes of proteins and nucleic acids, profluorescent probes, building blocks for construction of dual active drugs and electroactive materials, and substances for controlled free radical polymerization, are discussed as well.[15]

b.: Selections from the latest (2013-2021) research results of the Institute of Organic and Medicinal Chemistry of the PTE GyTK

The Institute of Organic and Medicinal Chemistry of PTE GYTK retained its main profile even after the death of its founders, Prof. Kálmán Hideg (1934-2018) and Dr. Olga H. Hankovszky (1934-2020), the synthesis, transformations and applications topic area. At the same time, his scope of research has also expanded with materials science research. In this Hungarian review we summarized our results from the last 8 years of publications, including the synthesis of new paramagnetic building blocks, heterocycles and carbocycles, synthesis and biological studies of new dual-drugs, EPR investigation of photoirradiated TiO_2 nanoparticles and the secondary chemical interactions of macromolecules/supramolecular systems and small molecules.[16]

Summary

The results shown above are based on synthetic organic chemistry research results, with diverse aims and in the course of OTKA 124331 we have published over 100 new compounds and intermediates. We wish to continue the research of stable nitroxide free radicals, on the field of dual-drugs, spin-labels (tetraethyl substituents) and paramagnetic building blocks. During this project we have published 16 research paper, (ΣIF: 47.311), we were participating 7 conferences presenting our results in 6 posters and 2 lectures. Based on these 2 BSc, 2 MSc and 2 PhD theses were manifested.

References:

1. Nagane, M.; Yamashita, T.; Vörös, P.; Kálai, T.; Hideg, K.; Bognár, B. Synthesis and evaluation of paramagnetic caffeic acid phenethyl ester (CAPE) analogs. *Monats. Chem*. **2019**, *150*, 1513–1522. **[IF: 1,501]** doi: 10.1007/s00706-019-02458-8
2. Prabhat, A.M.; Kuppusamy, M.L.; Bognár, B.; Kálai, T.; Hideg, K.; Kuppusamy, P. Antiproliferative Effect of a Novel 4,4'-Disulfonyldiarylidenyl Piperidone in Human Colon Cancer Cells. *Cell Biochem. Biophys.* **2019**, *77*, 61–67. **[IF: 2.073]** doi: 10.1007/s12013-018-0862-5
3. Mast, J.M.; Hinds, J.W.; Tse, D.; Axelrod, K.; Kuppusamy, M.L.; Kmiec, M.M.; Bognár, B.; Kálai, T.; Kuppusamy, P. Selective Induction of Cellular Toxicity and Anti-tumor Efficacy by N-Methylpiperazinyl Diarylidenylpiperidone and its Pro-nitroxide Conjugate through ROS-mediated Mitochondrial Dysfunction and G2/M Cell-cycle Arrest in Human Pancreatic Cancer. *Cell. Biochem. Biophys*. **2020**, *78*, 191-202. **[IF: 2.194]** doi: s12013-020-00919-0
4. Zsidó, B. Z.; Balog, M.; Erős, N.; Poór, M.; Mohos, V.; Fliszár-Nyúl, E.; Hetényi, Cs.; Masaki, N.; Hideg, K.; Kálai, T.; Bognár, B. Synthesis of Spin-Labelled Bergamottin: A Potent CYP3A4 Inhibitor with Antiproliferative Activity. *Int. J. Mol. Sci.* **2020**, *21*, 508. **[IF: 5.923]** doi: 10.3390/ijms21020508.
5. Isbera, M.; Bognár, B.; Gulyás-Fekete, G.; Kish, K.; Kálai, T. Syntheses of Pyrazine-, Quinoxaline-, and Imidazole-Fused Pyrroline Nitroxides. *Synthesis*, **2019**, *51*, 4463-4472. **[IF: 2.867]** doi: 10.1055/s-0039-1690678
6. Bognár, B.; Isbera, M.; Kálai, T.; Synthesis of a Nitroxide Spin-labeled Varenicline (Chantix) Derivative. *Org. Chem. Proced. Int.* **2021**, *53*, 311-315. **[IF: 1.628]** doi: 10.1080/00304948.2021.1877997
7. Isbera, M.; Bognár, B.; Jekő, J.; Sár, C.; Hideg, K.; Kálai, T. Syntheses and Reactions of Pyrroline, Piperidine Nitroxide Phosphonates. *Molecules* **2020**, *25*, 2430. **[IF: 3.06]** doi: 10.3390/molecules25102430
8. Isbera, M.; Bognár, B.; Sár, C.; Jekő, J.; Kálai, T. Syntheses and utilizations of pyrroline-nitroxide and tetrahydropyridine-nitroxide- based α- ketophosphonates, β-ketophosphonates, and a bisphosphonate. *Synth. Commun*. **2021**, *51*, 1353–1362. **[IF: 1.796**] doi: 10.1080/00397911.2021.1880595
9. Isbera, M.; Bognár, B.; Sár, C.; Jekö, J.; Kálai, T. Kabachnik–Fields reactions with stable nitroxide free radicals. Kabachnik–Fields reactions with stable nitroxide free radicals. Arkivoc, 2022 (In Press). **[IF: 0.57**] doi: 10.1007/s00723-018-1089-8doi: 10.24820/ark.5550190.p011.849
10. Úr, G.; Gulyás-Fekete, G.; Hideg, K.; Kálai, T. *N*-Vinylation of Imidazole and Benzimidazole with a Paramagnetic Vinyl Bromide. *Molbank* **2018**, M980; doi:10.3390/M980
11. Mohos, V.; Fliszár-Nyúl, E.; Schilli, G.; Hetényi, C.; Lemli, B.; Kunsági-Máté, S.; Bognár, B.; Poór, M. Interaction of Chrysin and Its Main Conjugated Metabolites Chrysin-7-Sulfate and Chrysin-7-Glucuronide with Serum Albumin. *Int. J. Mol. Sci*. **2018**, *19*, 4073. **[IF: 4.183]** doi: 10.3390/ijms19124073
12. Kokorin, A. I.; Gromov, O. I.; Dorovatovskii, P.V.; Lazarenko, V. A.; Khustalev, V. N.; Hideg, K.; Kálai, T. The Structure and Internal Dynamics of R6-p-C6H4-R6 Biradical: EPR, X-ray Crystallography and DFT Calculations. *Appl. Magn. Reason.* **2019**, *50*, 425-439. **[IF: 1.796**] doi: 10.1007/s00723-018-1089-8
13. Kőrösi, L.; Bognár, B.; Horváth, M.; Schneider, Gy.; Kovács, J.; Scarpellini, A.; Castelli, A.; Colombo, M.; Prato, M. Hydrothermal evolution of PF-co-doped TiO_2 nanoparticles and their

antibacterial activity against carbapenem-resistant Klebsiella pneumoniae. *Appl. Catal. B-Environ.* **2018**, *231*, 115-122. **[IF: 14.54]** doi: 10.1016/j.apcatb.2018.03.012

14. Kőrösi, L.; Bouderias, S.; Csepregi, K.; Bognár, B.; Teszlák, P.; Scarpellini, A.; Castelli, A.; Hideg, É.; Jakab, G. Nanostructured TiO_2-induced photocatalytic stress enhances the antioxidant capacity and phenolic content in the leaves of Vitis vinifera on a genotype-dependent manner. *J. Photochem. Photobiol.* **2019**, *190*, 137-145. **[IF: 2.073]** doi: 10.1016/j.jphotobiol.2018.11.010
15. Bognár, B.; Úr, G.; Sár, C.; Hankovszky, H. O.; Hideg, K.; Kálai, T. Synthesis and Application of Stable Nitroxide Free Radicals Fused with Carbocycles and Heterocycles. *Curr. Org. Chem.* **2019**, *23*, 480-501. **[IF: 2.029]** doi: 10.2174/1385272823666190318163321
16. Bognár, B.; Lemli, B.; Kőrösi, L.; Ameen, M.H.; Derdák, D.; Isbera, M.; Preisz, Zs.; Úr, Gy.; Sár, C.; Kunsági-Máté, S.; Kálai, T. Szemelvények a PTE GyTK Szerves és Gyógyszerkémiai Intézetének újabb (2013-2021) kutatási eredményeiből. Magyar kémiai folyóirat 2022, 128, 68-78. **[IF: 0.1**] doi: 10.24100/MKF.2022.02.68