Synthesis and utilization of nitroxide free radicals and their precursors as sensors and possibly biologically active molecules

Final report of OTKA 104956 (2012-2017)

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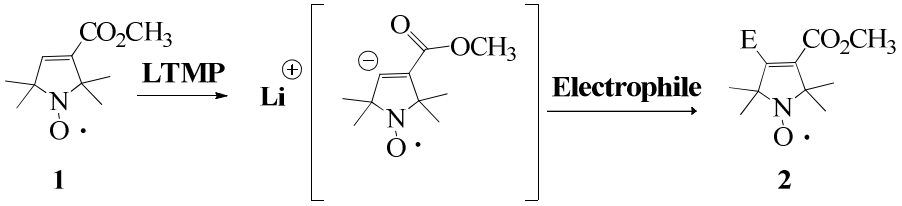
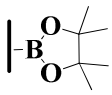
Introduction

Nitroxide stable free radicals have found broad application as spin labels, low molecular weight antioxidants, MRI contrast agents, co-oxidants, redox- and pH-sensitive probes and mediators of polymerization. The chemistry of nitroxides involves many challenging aspects, including reaction for carbon-carbon bond formation in the presence of amphiphilic nitroxide moieties or the synthesis of nitroxide containing hybrid molecules, for example, nitroxide incorporated biologically active molecules. In the frame of OTKA104956 project we aimed to achieve new double (spin and fluorescent) redox sensor molecules and accomplish the synthesis of new paramagnetically modified biomolecules, spin labels, complex forming agents. All these modifications had to be conducted with selective, but still more or less effective reactions.

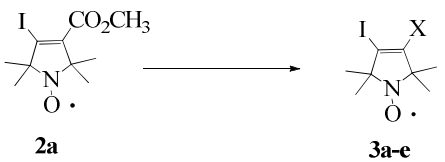
Results

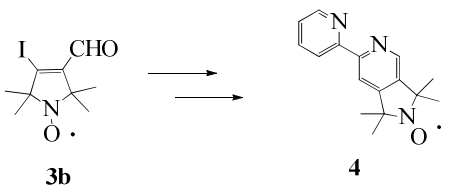
Novel approach for 3,4-disubstituted pyrroline nitroxides: Lithiation of an α,β-unsaturated pyrroline nitroxide ester **1** with LTMP at the β-carbon followed by treatment with electrophiles led to a new series of 3,4-disubstituted pyrroline nitroxides, which could be used as valuable paramagnetic building blocks.[1]

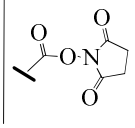


2	E
a	I
b	CO_2CH_3
c	$Sn(Bu)_3$
d	SPh
e	

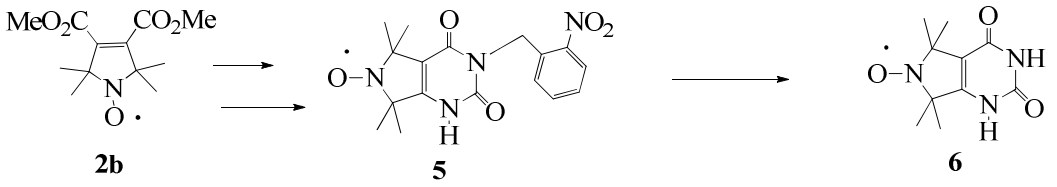
Among these compounds the most valuable were the **2a** and **2b** compounds. From **2a** compound we have worked out the selective synthesis of iodine derivatives (**3a-e**). Compounds **3d, 3e** are SH- and amine-specific spin labels, respectively, compound **3b** was used to synthesize the paramagnetic α,α'-dipyridyl **4**, probably capable for modifying transition metal centers of enzymes.[2]



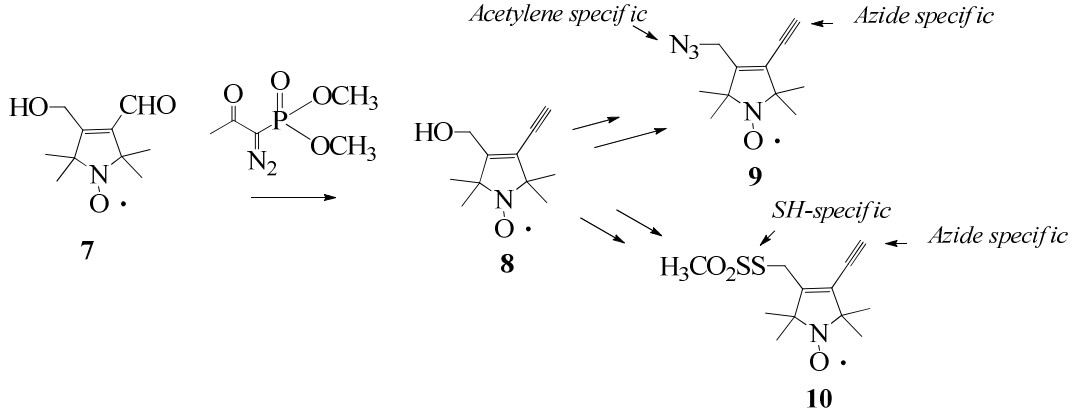


3	X
a	CH_2OH
b	CHO
c	CH_2Br
d	$CH_2SSO_2CH_3$
e	

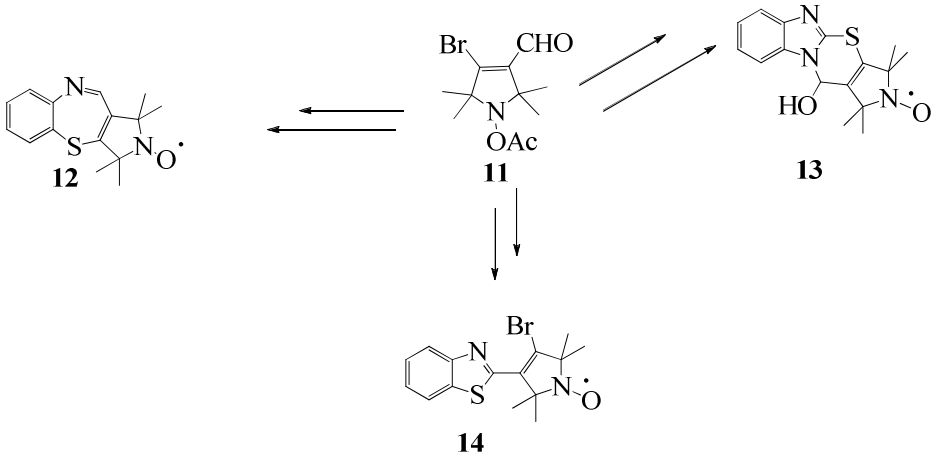
Compound **2b** gave an access to paramagnetic uracil **6**. During the synthesis we have found a new method to remove the 2-nitrobenzyl protecting group.[1]



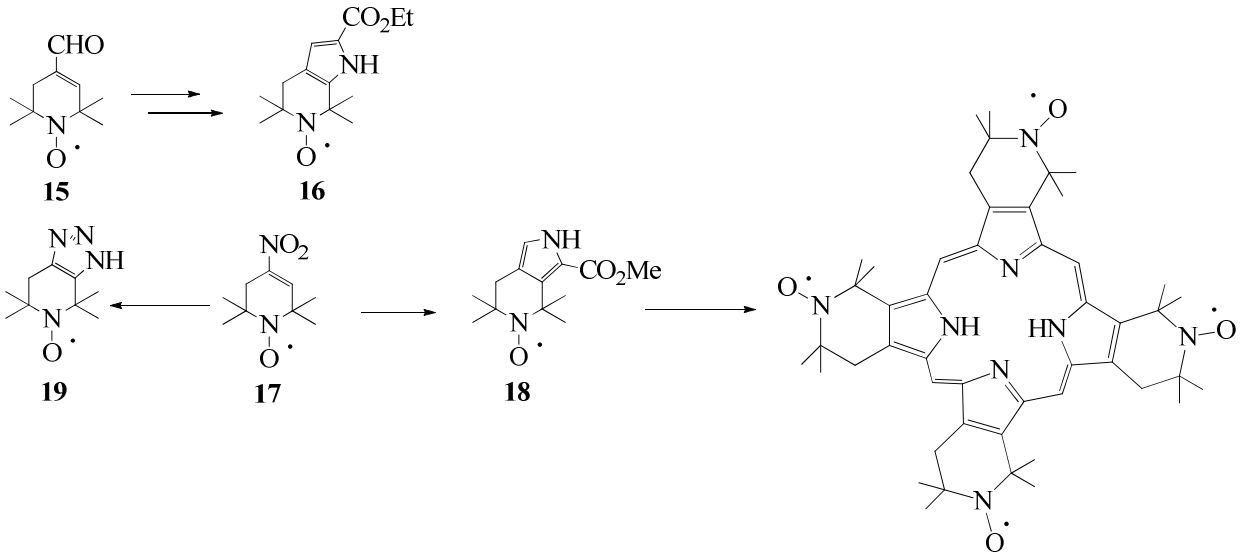
A new access for paramagnetic acetylenes were developed, Bestmann-Ohira reagent selectively transformed compound **7** to compound **8** which was used for synthesis of cross-linking spin labels such as **9** and **10**. The challenge in this subproject was to test, whether the generated carbene will react with the stable free radical itself or not.[3]



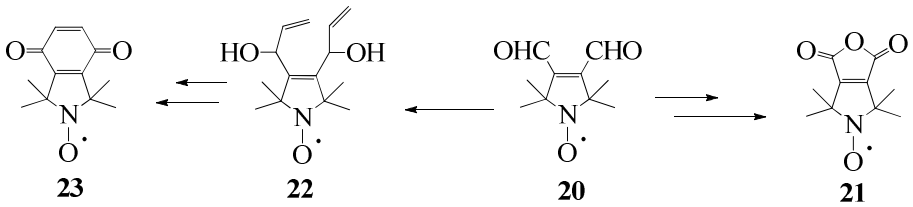
Synthesis of heterocycles, carbocycles condensed with pyrroline nitroxide radicals: The diamagnetic nitroxide derivative **11** in a ring-closing reactions with *S,N*-binucleophiles was evaluated, depending on reaction conditions and substrates 1,5-benzothiazepine **12** or 1,3-thiazine **13** or benzothiazole **14** were observed as products. The nitroxide protection is necessary to inhibit the oxidations of aromatic thiols to disulfide.[4]



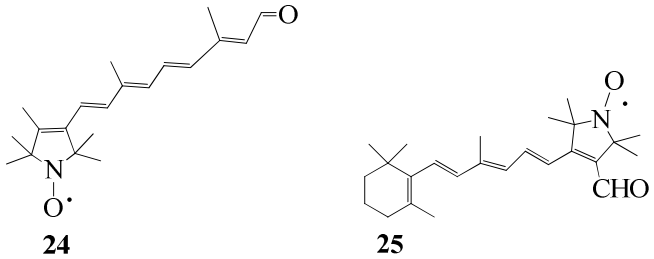
For pyrrole condensed nitroxides two approach was possible, from **15** aldehyde we achieved compound **16** with Hemetsberger-Knittel reactions and from unsaturated nitro compound **17** we got compound **18** with Barton-Zard pyrrole synthesis. Compound **17** also could be used for **19** triazol synthesis. Our aim to construct potential paramagnetic porphyrin building block was achieved, however these were not suitable for synthesis of porphyrin tetra radicals. We are working on other approaches.[5]



In the research of 3,4-disubstituted nitroxides the most important result was the synthesis of **21** anhydride from **20** dialdehyde with oxidation and dehydration. Reaction of **20** with vinyl magnesium bromide gave **22** bis-allylic alcohol of which metathesis reaction followed by oxidation furnished **23** paramagnetically modified 1,4-benzoquinone. As far as we know this was the first Grubbs II catalyst application in the presence of nitroxide free radicals.[6]

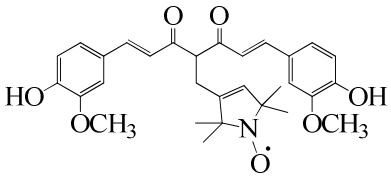


Synthesis of paramagnetic retinal and curcumin derivatives: The synthesis of a nitroxide-biomolecule-hybrid is a big challenge. We need to incorporate the pyrroline nitroxide ring into a biomolecule by leaving the essential functional groups intact.We accomplished this aim by means of Horner-Wadsworth-Emmons reactions and functional group interconversion. The nitroxide ring was incorporated into two ends of the molecule (see compounds **24, 25**).[7]



A series of paramagnetically modified curcumin analogues and 3,5-diarylidene-piperidones (DAP) have been designed, synthesized, and characterized on their anti-proliferative and antioxidant activity in cooperation with Prof. P. Kuppusamy group (Dartmouth College, USA). Biological characterization of the new compounds supported the earlier results that incorporation of a nitroxide moiety or its precursor into curcumin or diarylidenylpiperidone

(DAP) scaffolds resulted in anti-proliferative effect toward cancerous cell-lines in case of aryl hydroxyl and/or methoxy substituent containing derivatives suggesting their potential for targeted therapeutic applications. In case of basic side chain derivatives, nitroxide incorporation gave unambiguous results, however in tendency the more accessible DAP derivatives had stronger anti-proliferative effect. In most cases, the nitroxide incorporation increased the TEAC value (proton and electron donation capability) of DAP derivatives. Among the compounds synthesized and investigated the spin-labeled curcumin **26** and 3,5-bis(4-hydroxy-3-methoxybenzylidene)piperidin-4-one derivatives **27** were the most effective anti-proliferative and antioxidant analogs.[8]



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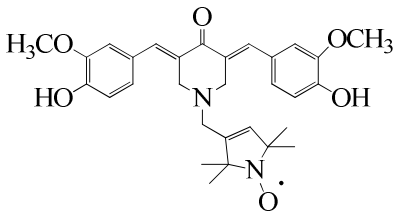
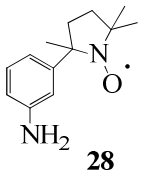


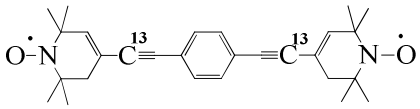
27

Utilization of spin labels in environmental research: In a cooperation with German researchers (Prof. H. J. Steinhoff, Prof. Matthies, M.) from University of Osnabrück (Germany) we carried out EPR experiments with labelled aniline **28** as a model compound in a proof-of-concept approach that the method can be used to identify strong binding of aromatic amines and determine their reaction kinetics to humic acid, an important soil constituent. Derivatives of nitroxide radicals are stable enough to study the interaction of specific functional groups to isolated humic substances as model compounds.[9]



28

Synthesis of biradicals: Synthesis of biradical compounds (**29-31**) were conducted for studying spin density distribution and spin exchange for EPR investigations and DFT calculations in cooperation with Russian researchers.[10,11] In case of compound **29** it was proven that intramolecular electron spin exchange is realized by indirect mechanism.



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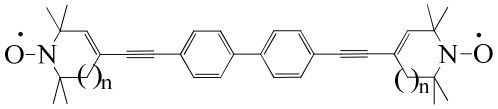
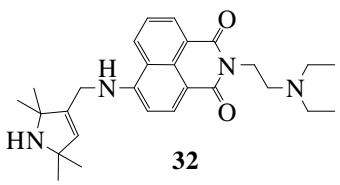


30 (n=0), 31 (n=1)

Synthesis of double (spin and EPR) sensor molecules: New double (fluorescent and spin) sensor molecules (**32, 33**) containing 4-amino substituted 1,8-naphthalimide as a fluorophore and a sterically hindered amine (pre-nitroxide) or pyrroline nitroxide as a quencher and radical capturing moiety were synthesized. All sensors were substituted with a diethylaminoethyl sidechain to increase the water solubility. Steady state fluorescence properties of these compounds and their responses to ROS in vitro are reported with perspectives of plant physiology use in vivo, in cooperation with researchers from University of Pécs, Natural Sciences Faculty (Prof. É. Hideg) and BRC HAS Szeged (Dr. F. Ayaydin).[12]



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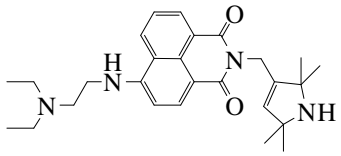


33

Among the synthesized compounds **33** was the best $^1O_2$ quencher with the diethylaminoethyl chain bound to the aromatic ring. Compound **32** has good penetrating properties, as illustrated in Fig.1 using tobacco leaves (popular models of plant stress physiology studies). Fluorescence from compound **32** (Fig.1A) is partly overlapping with native chlorophyll fluorescence (Fig.1B) indicating the presence of compound **32** in choloroplasts. The lack of complete special matching (Fig.1C) indicates that some of the compound remained outside the organelle, although it penetrated the cells. As probe delivery into plant cells and organelles is usually prone to more problems than delivery into animal tissues, these images suggest potential applicability. However the fluorescence change upon $^1O_2$ quenching was quite small even in response to relatively high ROS fluxes in vitro and is not expected to be responsive to small amounts in vivo. Compound **33**, on the other hand, would prove useful in experiments aimed at studying whether singlet oxygen can stimulate a response farther from its production site, as this compound was not co-localized with chlorophyll fluorescence but remained outside these organelles (Figs. 1D-F) which are the site of production in plants. This question is of special interest because in photosynthetic organisms the main source of $^1O_2$ is chlorophyll-sensitized photo-production in chloroplasts. Singlet oxygen is capable of activating nuclear genes but its role in this chloroplast to nucleus (a.k.a. retrograde) signaling has not been fully explored so far.

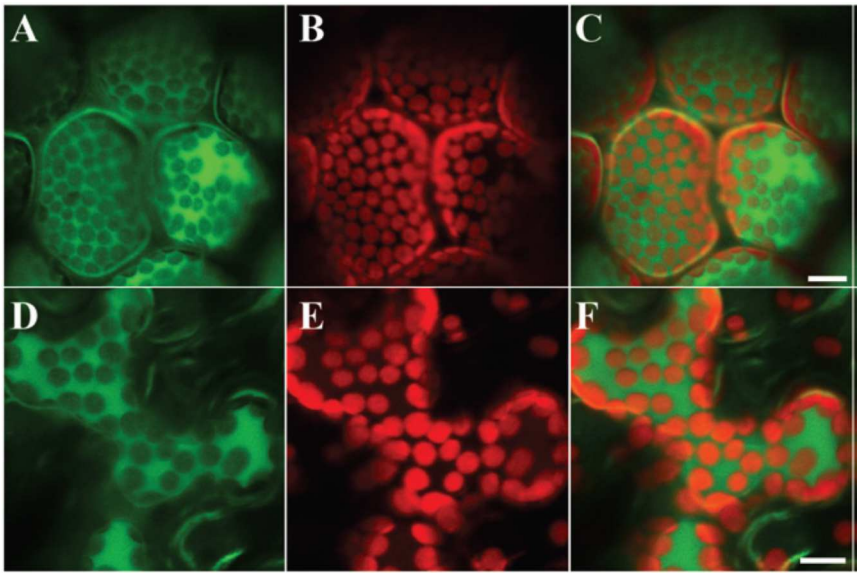
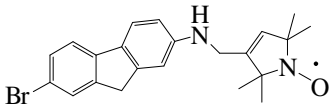


Figure 1. Subcellular localization of compounds **32** (upper panel) and **33** (lower panel). Merged images of compound (A, D) and chlorophyll fluorescence (B, E) are shown in panels C and F. Scale bars: 10 μm.

The utilization of similar sensors of $^1O_2$ and other EPR active methods were reviewed in a book chapter.[13]

Study of spin-labeled fluorene as inhibitors of Aβ oligomers growth: Spin-labeled fluorene **34** (SLF) was synthesized and in cooperation with Prof. J. Voss group (University of California,

Davis USA) it was investigated fluorescence correlation spectroscopy, EPR and CD spectroscopy that compound **34** can inhibit the growth of Aβ oligomers and disrupt existing oligomers, while retaining Aβ as a population of smaller, yet largely disordered oligomers (Figure 2).[14]



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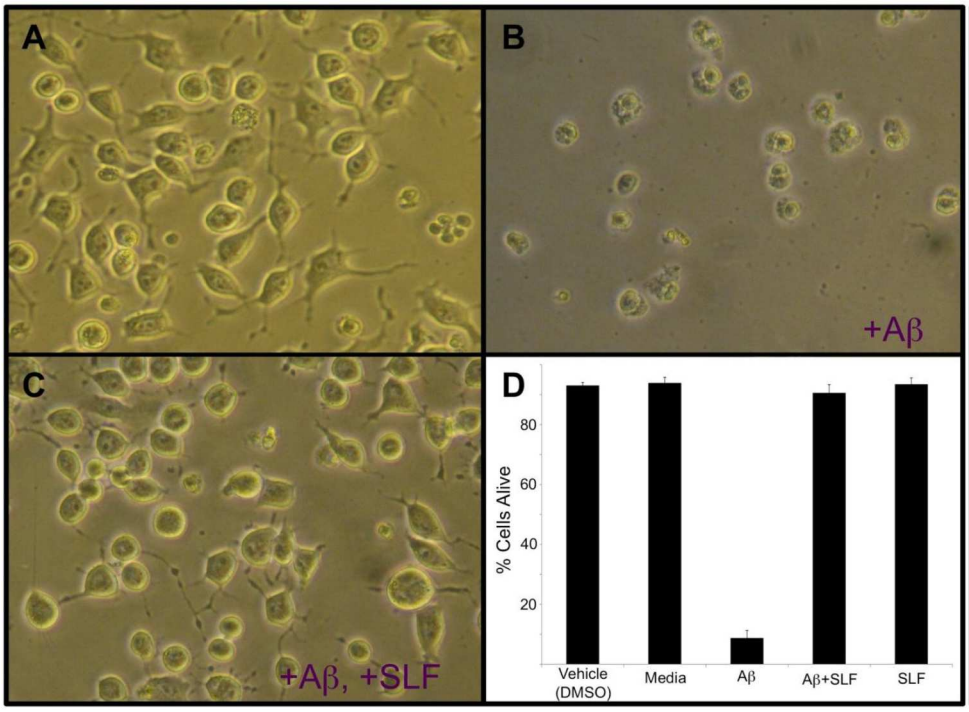

Figure 2: Shown are images of N2a cells after three days with treatment of either DMSO vehicle control (**A**) or addition of exogenous AβO (Aβ incubated for 24 hours in PBS to generate oligomers prior to addition to the culture media at a final concentration of 50 μM); **B**). Aβ toxicity is readily apparent from both the decreased cell count and the poor morphology of cells in panel **B**. When Aβ is combined with 4 μM compound **34** (SLF) prior to culture application (panel **C**), cell viability resembles the control in panel **A**. N2a cell viability was quantified using Trypan blue exclusion (panel **D**). Compound **34** alone was tested at 10 μM. Results are expressed as means ± SD of percent cells alive following treatment. * $p < 0.05$ compared to media treatment.

Summary

The results shown above are based on synthetic organic chemistry research results, with diverse aims and in the course of OTKA 104956 we have published ~ 120 new compounds and intermediates. Our publication activity slowed down in the recent years, because every journals require detailed NMR data, which is hard to access in case of paramagnetic compounds. Despite these difficulties, we wish to continue the research of stable nitroxide free radicals, however

many new structures have appeared in the recent years (for instance α,α'-cyclohexyl instead of tetramethyl substituents). In the future, we probably need to incorporate these new trends in our new molecules to be designed and synthesized.
During this project we have published 13 research paper, 1 book chapter (ΣIF:23.9), we were participating 10 conferences presenting our results in 11 posters and 7 lectures. Based on these 2 BSc and 1 MSc theses were accomplished and 1 pre-doctor will defend her PhD thesis, hopefully in 2018.

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11. Úr, Gy.; Kálai, T.; Balog, M.; Gulyás Fekete, G.; Hideg, K. Synthesis of 3,4-disubstituted pyrroline nitroxides with ethynyl functional group (p) *16th Blue Danube Symposium on Heterocyclic Chemistry, Balatonalmádi, Hungary, June 14-17, 2015.*
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