Recent results in chemistry and biology of nitroxides

Kálmán Hideg,* Tamás Kálai, Cecília P. Sár

Department of Organic and Medicinal Chemistry, University of Pécs,
H-7602 Pécs, P. O. Box 99, Hungary; E-mail: kalman.hideg@aok.pte.hu

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Introduction.

In the past two decades a main area of chemistry, biological and medical sciences has become the research of particles with unsaturated valence, the so-called free radicals. Today free radical chemistry and biology are considered as a developed discipline studying free radicals structure, physical and chemical properties including their useful or harmful role in biological systems [1-3]. One of the most interesting subjects of this field is the research of miscellaneous long-lived radicals, which can be isolated in a pure form. A main group of these radicals is nitroxide free radicals (nitroxides) - derivatives of nitrogen oxide with a disubstituted nitrogen atom containing one-valent oxygen as a third substituent with an unpaired electron. The presence of this unpaired electron provides paramagnetic properties to these nitrogen compounds, mainly nitrogen heterocycles, giving the possibility of their detection by electron paramagnetic/spin resonance spectroscopy (EPR/ESR) based on their ability of microwave energy absorption in external magnetic field [4, 5]. The first stable nitroxide free radicals were reported 45 years ago, almost independently by American, Russian and French scientists [6-8]. Several years after their discovery nitroxides were found to be useful as reporter molecules owing to their stability and detectability by EPR spectroscopy [9]. Using them as spin probes or spin labels is still a rapidly developing area [10,11] with the recent addition of EPR imaging [12].

The unpaired electron allows nitroxides to take part in one-electron transfer processes such as oxidation and reduction, therefore 1-oxyl-2,2,6,6-tetramethylpiperidine (TEMPO) is often used as a co-oxidant [13]. On the contrary nitroxides are also considered as potent antioxidants [14], SOD mimics [15] and are among the most effective non-thiol radioprotectants [16]. Nitroxide free radicals found a wide industrial application as a mediator of polymerization [17] and their study to construct new ferromagnetic organic materials is also a rapidly growing field [18]. Nitroxides became indispensable tools in analytical chemistry: nitronyl nitroxides are used to detect nitrogen monoxide [19] and isoindoline nitroxides proved to be useful in determining oxygen concentration in biological systems [20]. Fluorophore linked pyrrolidine (PROXYL) nitroxides were successfully used in determining alkyl and, indirectly, hydroxyl radicals in condensed phase [21], to mention a few examples.

The chemistry and utilization of nitroxides are regularly reviewed from the beginnings [22] by the scientists active in this field, so several excellent reviews [23-25] and monographs have been published in the past 15 years [26-28]. In this review we wish to summarize some of the main results of our work in our laboratory at the University of Pécs, emphasizing the synthetic chemistry aspects, since our latest summarizing works [29, 30].

Synthesis of piperidine, tetrahydropyridine, pyrroline and pyrrolidine nitroxides.

The key starting compound for the synthesis of piperidine, pyrroline and pyrrolidine nitroxides is triacetonamine [31]. The treatment of this ketone with bromine gave dibromoketone (**1**) as an important intermedier whose treatment with sodium methoxide or amines gave the pyrroline amines (**2a-c**) [22, 32] via Favorskii rearrangement while its oxidation with *m*-CPBA gave 3,5-dibromoketone stable free radical (**3)**. Oxidation of triacetonamine with hydrogen peroxide in the presence of Na_2WO_4 gives 4-oxo-TEMPO (**4**) the well-known nitroxide free radical, now available from almost all fine chemical companies [33]. This is a key intermediate in nitroxide chemistry, because this is the parent compound of all piperidine nitroxides via borohydride reduction [22] and all 2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridine nitroxides via cyanohydrine formation [34] followed by elimination to give compound (**5b).** This nitrile derivative can be converted further to aldehyde (**5c**), carboxylic acid (**5d**) and allylic bromide (**5e**) [35]. Although piperidine nitroxides are cheaper and they are more widely used in polymer chemistry and in organic chemistry as co-oxidants than pyrroline or pyrrolidine nitroxides, their stability is smaller towards putative reducing agents such as thiols and ascorbates [36]. Focusing on the synthesis of new spin label molecules we preferred working with the more stable pyrroline and pyrrolidine nitroxides. The paramagnetic starting material, ester (**6**) is available by metal catalyzed oxidation of sterically hindered amine **2a** with hydrogen peroxide. Hydrolysis of this ester to acid followed by reduction of its mixed anhydride gave allylic alcohol (**7a**) [37], which was converted to the corresponding allylic halogenides (**7b,c**) [38]. Compound **7c** as well as its mercury derivative (**7d**) [39] are thiolspecific non-cleavable spin label compounds while methanethiosulfonate (**7e**) available by nucleophilic substitution from **7b** is also a thiolspecific spin label, but reversible because this forms a

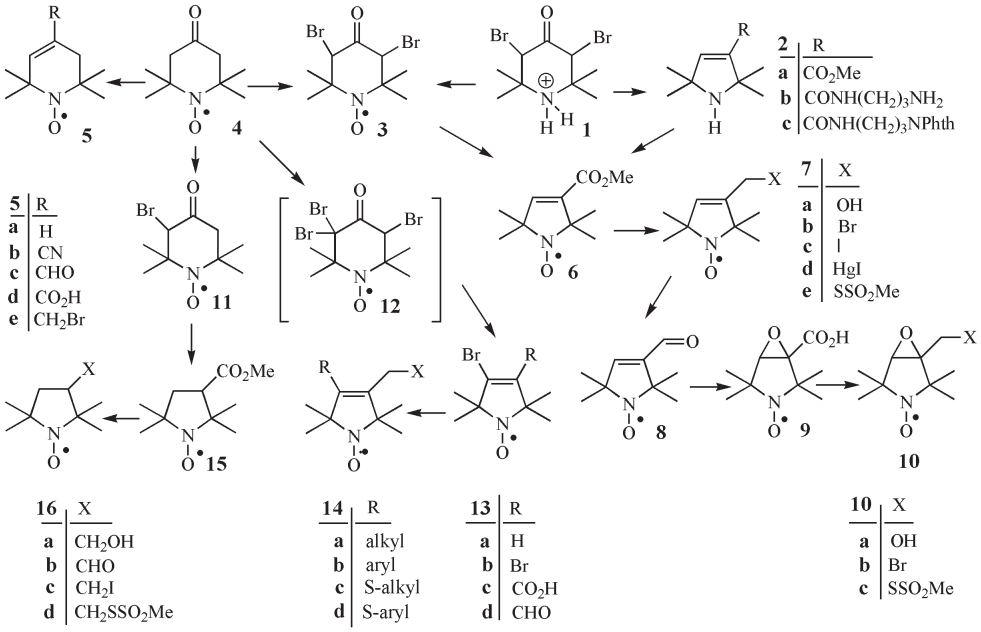
cleavable disulphide bond [40]. This compound abbreviated as MTSL (methanethiosulfonate spin label) is nowadays the most frequently used and commercialized spin label [41], due to its dominance in site directed spin labeling technique, which applies the incorporation of cysteine into any site of a peptide followed by labeling with MTSL, e. g. (**7e**). The oxidation of alcohol (**7a**) with activated MnO_2 gave aldehyde (**8**) of which double bond was oxidized with hydrogen peroxide in the presence of NaOH to epoxide (**9**). Fortunately, this epoxide was rather inert toward nucleophylic attack, therefore the carboxylic acid functional group could be converted further to **10a-c** derivatives [42].

An alternative route to pyrroline and pyrrolidine nitroxides is the bromination of **4** *N*-hydroxylamine salt with one equivalent or two equivalents of bromine [43, 44] to give compound **11** or **3,** respectively. The treatment of 4-oxo-TEMPO (**4**) with NaOBr solution gave compound **13c** via **12,** e. g. in a one-pot procedure bromination of **12** and then a Favorskii rearrangement takes place [27]. In this reaction, we observed the formation of decarboxylated products, such as **13a** and **13b**. Compound **13c** can be transformed to a valuable key intermediate **13d** aldehyde. This compound allowed us to introduce an arbitrarily chosen alkyl, aryl or hetaryl substituent by Pd-catalyzed Suzuki cross-coupling reaction **14a,b** with C-C bond formation and *S*-alkyl or *S*-aryl substituent in the presence of 1.1 equivalent DBU base to give **14c,d** with S-C bond formation or [45, 46]. The treatment of compound **11** with NaOMe yielded racemate ester (**15)** which was converted to the corresponding 3-substituted PROXYLs (**16a-c**) and thiolspecific spin label (**16d**) [39]. The resolution of compound **16a** has been published very recently allowing the synthesis of enantiomers of **16d** [47] (Scheme 1).

Synthesis and reactions of symmetric paramagnetic pyrrolidine diene and its 1,4-dibromo adduct.

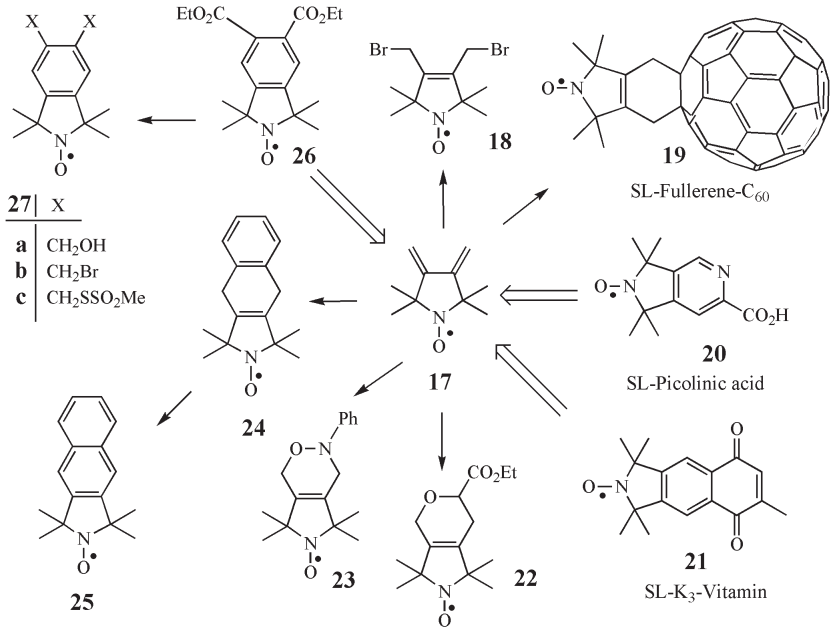
Michael addition reaction of **2a** with nitromethane [48] followed by Nef reaction and functional group transformation allowed the synthesis of paramagnetic **17** diene [49], which proved to be a key intermediate for further reactions. The 1,4-addition of bromine of **17** *N*-hydroxylamine HCl salt gave 3,4-bis(bromomethyl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole **18** another important key intermediate [49]. The Diels–Alder reaction is an important tool of organic chemistry to construct six-membered homo- and heteroaromatic rings [50, 51]. Utilization of asymmetrical paramagnetic dienes in Diels-Alder reactions is also well documented [52, 53], requiring long (several days) reaction time. However, compound **17** exhibited a very smooth and simple procedure to achieve the proper adducts. Reaction of **17** with fullerene gave compound **19** and hetero Diels-Alder with *N*-(butoxycarbonylmethylene)-p-toluenesulfonamide followed by hydrolysis leading to aromatization and esterification gave paramagnetic pyridine derivative (**20**) [54]. Another example of hetero Diels–Alder with ethyl glyoxalate in the presence of $LiClO_4$ resulted in formation of paramagnetic dihydropyrane derivative (**22**), and nitrosobenzene gave **23** adduct [55]. Reaction of **17** diene with 2-methylbenzoquinone, benzyne and diethyl acetylenedicarboxylate followed by an aromatization

Scheme 1



with DDQ yielded paramagnetic K_3-vitamin derivative (**21**) [54]. Reaction of **17** with benzyne followed by aromatization gave benzo[f]isoindoline nitroxide (**25**) and Diels-Alder reaction of diene (**17**) with diethyl acetylenedicarboxylate after DDQ oxidation yielded homobifunctional isoindoline nitroxide (**26**) which was converted further to **27a-c** derivatives as crosslinking reagents [49, 55]. This route is a new approach to isoindoline nitroxides and ensures greater variability than the synthesis from phthalimide published earlier [56] (Scheme 2).

Scheme 2

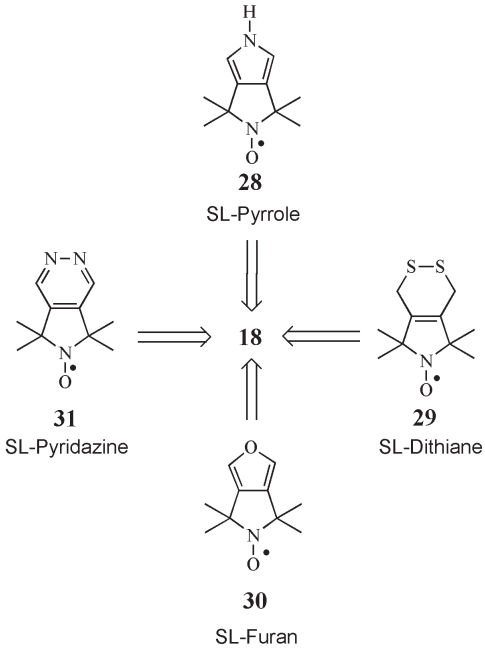


Alkylating *p*-toluenesulfonamide with compound **18** followed by aromatization with DDQ and basic hydrolysis gave **28** SL-pyrrole. The formation of bis(isothiouronium) salt from thiocarbamide and its hydrolysis afforded **29** SL-dithiane upon spontaneous oxidation during the work-up on air [49]. The significance of compound **29** is that it can be used as homobifunctional cross-linking spin label regent, capable of conjugation with cysteine residues. Conversion of compound **18** to bis(allylic alcohol) *via* 3,4-bisacetoxymethyl compound followed by partial oxidation gave 3-formyl-4-hydroxymethyl pyrroline and treatment of this latter compound with $BF_3{\cdot}Et_2O$ afforded **30** SL-furane [54]. The treatment of by-product dialdehyde with hydrazine hydrate offered **31** SL-pyridazine [49] (Scheme 3).

Reaction of paramagnetic β-bromo-α,β-unsaturated nitriles and aldehydes leading to pyrroline nitroxide fused heterocycles.

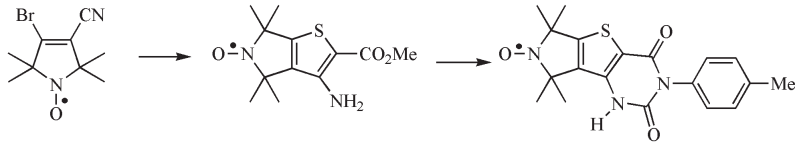
Reaction of **32** nitrile with methyl thioglycolate in acetonitrile in the presence of 1.1 eq. DBU gave paramagnetic 3-amino-2-methoxycarbonyl thiophene derivative (**33**), which, as a β-amino-ester, is a versatile synthetic building block [57]. The heating compound **33** with *p*-tolylisocyanate in toluene followed by the treatment with NaOMe

Scheme 3



in MeOH gave annulated pirimidine-2,4(1*H*,3*H*)-dione derivative (**34**) [55] (Scheme 4).

Scheme 4



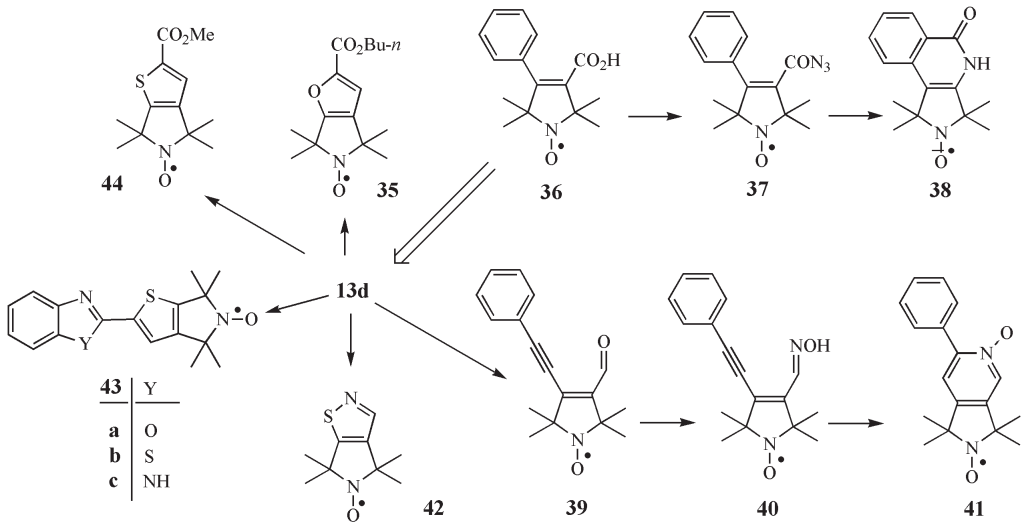
The β-bromo-α,β-unsaturated aldehyde **13d** was found a very valuable key compound: the treatment with sodium salt of *n*-butyl glycolate gave 4,4,6,6-tetramethyl-4,6-dihydro-5*H*-furo[2,3-c]pyrrole compound (**35**) [55]. The Suzuki cross-coupling of **13d** with phenylboronic acid followed by oxidation with Ag_2O to **36** carboxylic acid allowed us a more simple approach to β-substituted-α,β-unsaturated paramagnetic acids. This compound was converted to acid azide which was subjected to Curtius rearrangement and thermal Friedel-Crafts reaction to give 1,1,3,3-tetramethyl-1,3,4,5-tetrahydro-2,4*H*-pyrrolo[3,4-c]isoquinolin-5-one radical (**38**) [46]. Compound **13d** offered us another simple approach to pyrroline nitroxides annulated pyridine: Sonogashira reaction of **13d** with phenylacetylene gave aldehyde (**39**) of which oxime (**40**) spontaneously cyclized to pyridine *N*-oxide (**41**) with heating [46, 58]. The reaction of compound **13d** with 2 equivalent ammonium thiocyanate gave **42** isothiazole [55, 59], with 2-mercaptomethylbenzazoles (benzoxazole, benzothiazole, benzimidazole) in the presence of 1.1 eq. DBU gave the corresponding 2-thienylbenzazoles **43a-c** [55] and with thioglycolic acid methyl ester in the presence of 1.1 eq. DBU we got a thiophene annulated pyrroline nitroxide radical (**44**) [45]. (Scheme 5). These procedures were successfully applied for diamagnetic, thienyl ring containing polyheterocycles [60-62].

Synthesis of paramagnetic dihydropyridines.

Variations of the original Hantsch synthesis have been utilized to prepare a large number of the biologically active 1,4-dihydropyridine derivatives [63, 64] and numerous studies have been performed on their pharmacological activities [65, 66]. The most widespread Nifedipine is used therapeutically as calcium antagonist and vasodilator [67]. The 4-spin-labelled dihydropyridines (**46a-d**) were synthesized from aldehyde radical compounds (**8**, **16b**, **5c**) [68] and (**45**) [69] differing in the size and saturation of the hetero ring and alkyl aminocrotonate.

Dihydropyridines can be aromatized with several oxidizing reagents [70, 71] in one- or two-electron oxidation

Scheme 5

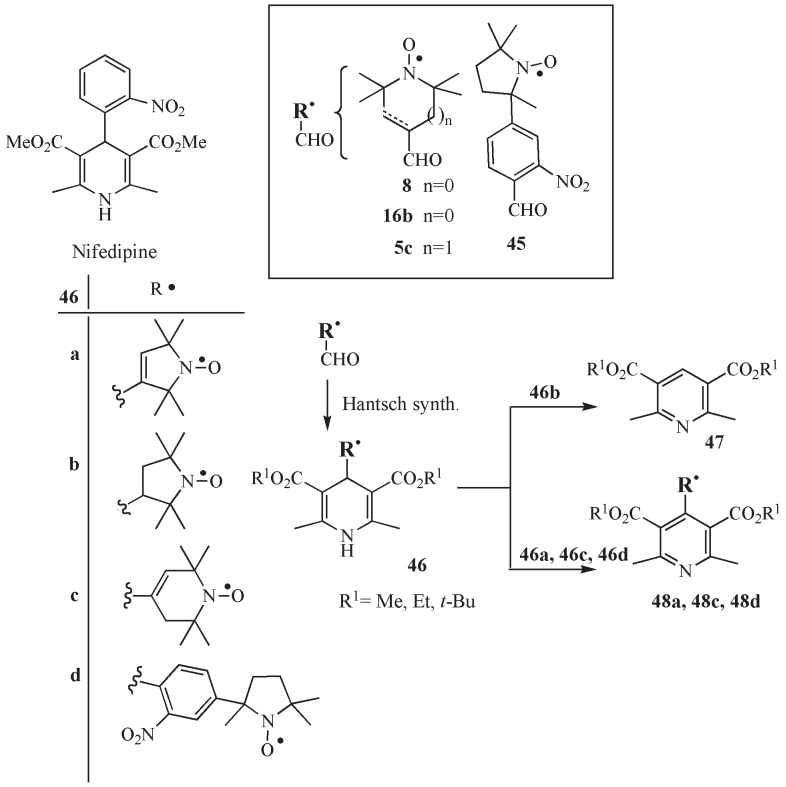


processes. The systematic investigations of the oxidation in cytochrome P-450 system showed that the reaction takes place *via* a radical mechanism, as shown by spin-trapping studies [72].

It was found that active MnO_2 is a convenient reagent for the aromatization of 1,4-dihydropyridines to pyridines. The oxidation takes place with the cleavage of 4-substituent when the connecting carbon has an sp^3 character (**46b**) and is part of the ring giving pyridine **47**. When the connecting carbon has an sp^2 character, R remains on the pyridine ring (**48a, 48c, 48d**) [68, 69] (Scheme 6).

Starting from acid chlorides (**49, 51**) [22] both by classic [73] and "one-pot" [74] Beaker-Venkataraman procedure, we got flavone (X=H) or 5-hydroxy flavone (X=OH) (**50**) derivatives with five- or six-membered nitroxides ring instead of aromatic "B" ring [75]. The reaction of 2-hydroxyacetophenone or 2,6-dihydroxy-acetophenone with paramagnetic benzoyl chloride (**51**) afforded compound **52** with a real flavone structure but with a pyrrolidine nitroxides on aromatic "B" ring [76]. We also considered introducing a paramagnetic label to "C" ring.

Scheme 6



Synthesis of paramagnetic flavones.

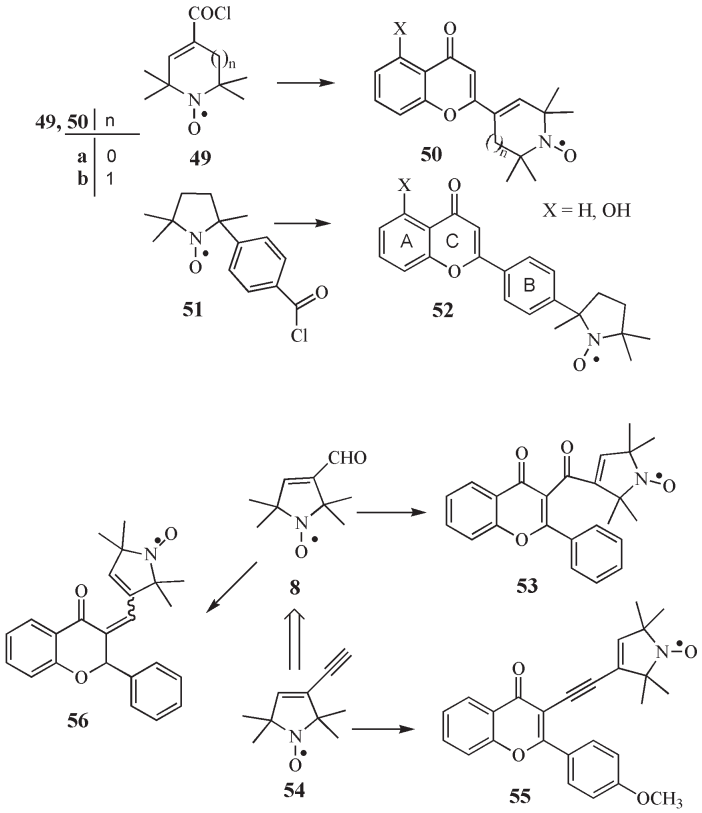
Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. Recently, much attention has been paid to different flavonoid derivatives as antioxidants, and dietary intake of these natural compounds has a significant effect on preventing a variety of diseases. Our idea was to improve the antioxidant properties of flavones and flavanones by combining them with nitroxides or nitroxide precursors capable of taking part in various redox cascades or eliminating Reactive Oxygen and Nitrogen Species (ROS, RNS).

The most evident procedure was an aldol condensation of paramagnetic aldehyde (**8**) with a chalcone derivative, which in low yields gave *Z/E* isomers of compound **56** [76]. The reaction of 3-Li salt of flavone with aldehyde (**8**) followed by oxidation resulted in formation of compound **53** and Sonogashira reaction of paramagnetic terminal acetylene **54** [49] with 3-iodo-flavone gave compound **55** (Scheme 7).

Drug modification with nitroxides or their precursors.

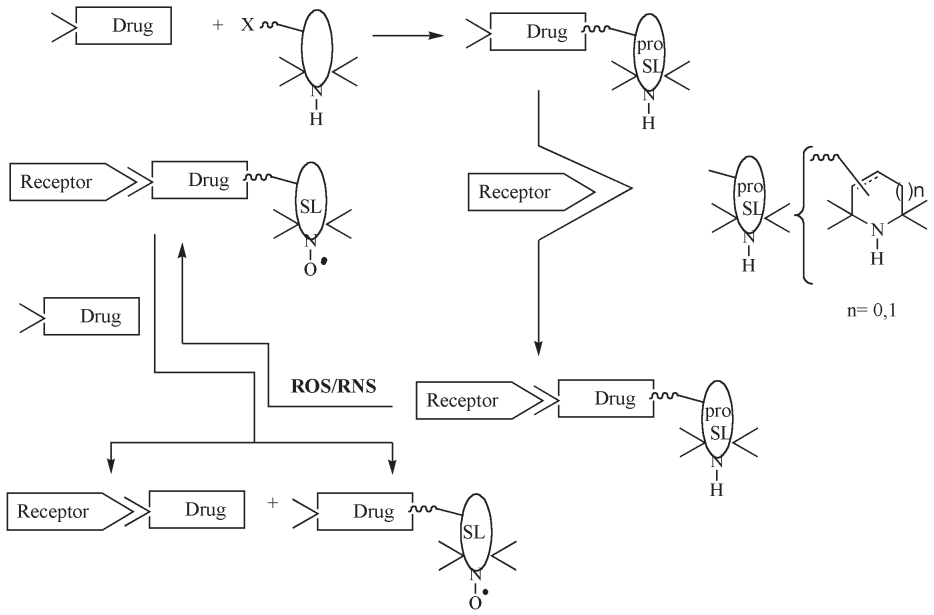
Spin labeled drugs were applied firstly in spin immunoassay in the early 70's, see references in [77]. Their utilization

Scheme 7



was based on the fact that the spin labeled drug in solution rotates freely and gives a typical three-line EPR spectrum. When a spin labeled drug is bound to an antibody or other proteins, it does not rotate freely anymore and the spectrum

Scheme 8



becomes perturbed. This idea can be extended to secondary amine nitroxide precursors also making the monitoring of oxidative stress possible *in situ* (Scheme 8).

In our laboratory, several spin-labeled drugs such as morphine [53], salicylic acid [69], dihydropiridines [68, 69], omeprazole-like compound [54], vitamin K_3 [54], warfarin [69] or local anesthetics [78] have been synthesized. During the last decade, it turned out that paramagnetically modified drugs have an additional more advantageous role beyond their original purpose. Namely, they have some antioxidant properties being capable of scavenging most of the toxic ROS and RNS highly damaging to biomolecules (e.g. proteins, lipids, and nucleic acids) [1].

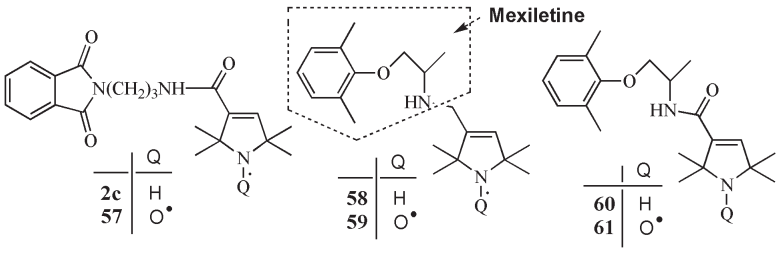
As several diseases are associated with elevated concentrations of these toxic species, ROS/RNS should be scavenged *in statu nascendi* to minimize the oxidative damage.

For this task, we have developed a group of new antiarrhythmic molecules which metabolize to non-toxic nitroxide. The lead molecule of this series is **2c** (H-2545) [32], metabolized to **57** (H-2954) (Scheme 9 and Scheme 10) [79].

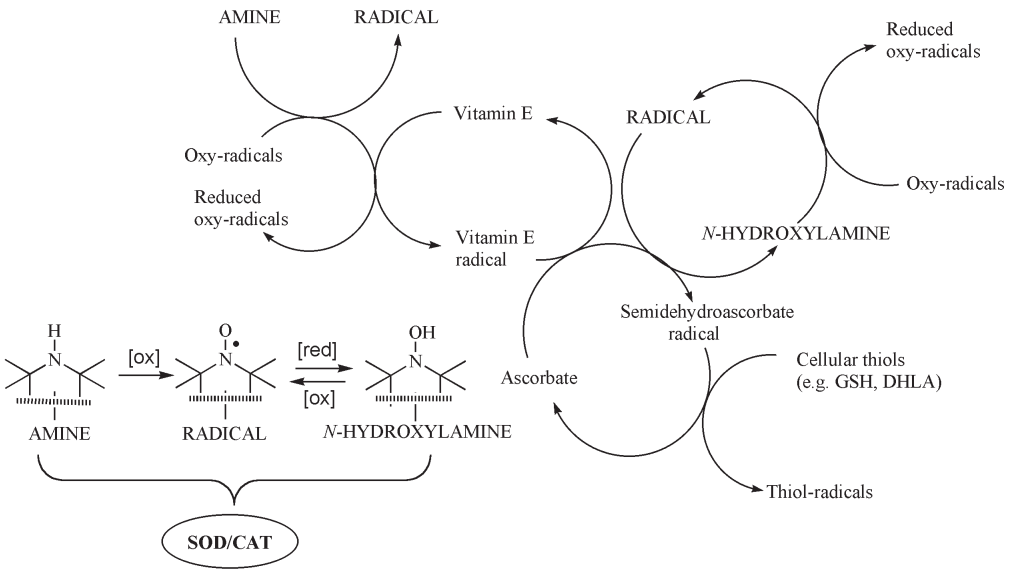
To minimize the damage by oxidative stress, the reactive non-toxic antioxidant has to be close to the site where these toxic species are formed. In order to confirm this concept, we have chosen the antiarrhythmic drug mexiletine accumulating in heart muscle membrane. After modifications with sterically hindered amine, i.e. the nitroxides precursor, **58** or **60** exhibited higher antiarrhythmic activity than the original drug molecule, so these modifications led to real cardioprotective molecules. Furthermore, the modified molecules featured higher inhibition of oxidative damage to proteins, lipids, and nucleic acids by oxidation to **59** and **61**, respectively [80-82].

Further applications of this principle may open new opportunities in designing new drugs for treatment of diseases associated with oxidative damage. The stable non-toxic nitroxide metabolite formed in the reaction between the drug and ROS/RNS is sensitive to reduction by ascorbate to diamagnetic hydroxylamine. This form can donate the proton of hydroxyl group to ROS/RNS molecules while being re-oxidized to the nitroxide form again (Scheme 10).

Scheme 9



Scheme 10



Synthesis of new pyrrolidine nitroxides modified at 2-position.

It was reported earlier that the C-alkylation of various 1-pyrroline *N*-oxides with alkyl, alkenyl and propargyl Grignard reagents led to higher substituted nitrones and ultimately to 1-oxyl-2,2,5,5-substituted pyrrolidine radicals **63**, **64** and **65** [83-85].

It is well known that 2,4,4-trimethylpyrrolin-1-oxide in a base catalyzed reaction with aromatic aldehydes (benzaldehyde and 4-nitrobenzaldehyde) gave 2-styryl nitrones [86].

However, we experienced that this aldol condensation takes place with a great variety of alkyl, alkenyl and aromatic aldehydes without any base catalyst. The reaction of nitrone (**62**) in boiling toluene with both aromatic and aliphatic aldehydes led to 2-*trans*-alkenylpyrrolin-1-oxides (**66**) in moderate to good yields [87]. In case of conjugated alkene aldehydes the nitrones (**66**) were the only products and we did not experience the formation of isoxazolidine ring. When nitrones **66** were reacted with MeMgI in Et_2O, only nitroxides **67** were formed in 1,2-addition reaction, without a conjugate addition.

The treatment of 2,5,5-trimethyl-2-pyrroline-1-oxide (**62**) with methyl acrylate in benzene afforded isoxazolidine (**68**) in high regioselectivity [88]. The isoxazolidine ring was opened to sterically hindered hydroxy amine and the oxidation of amine with H_2O_2/Na_2WO_4 resulted in the spin-labeled lactic acid, like α-hydroxy ester nitroxide (**69**) [87].

The 1,3-dipolar cycloaddition reaction of phenylacetylene with nitrone (**62**) in toluene afforded isoxazoline (**71**). Reductive cleavage of the N-O bond of isoxazoline (**71**) with Zn/AcOH afforded an amino alcohol which was then oxidized to paramagnetic benzyl alcohol (**72**)**,** followed by oxidation of alcohol to aryl ketone (**73**) with MnO_2 [89].

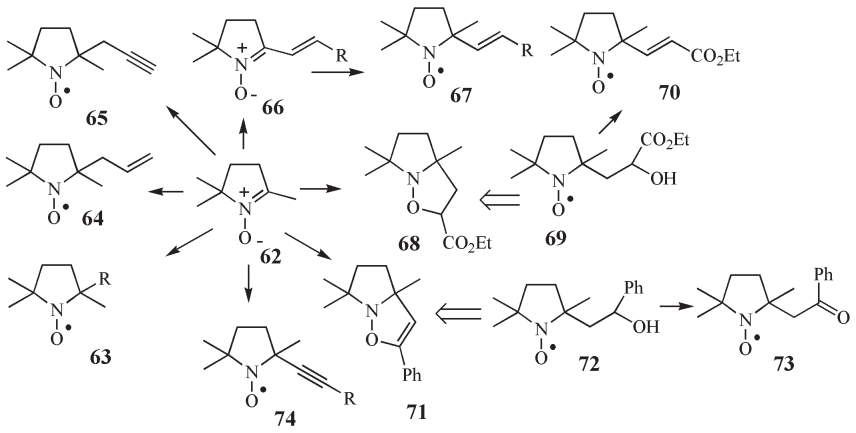
The synthesis of alkynyl substituted pyrrolidine nitroxide free radicals (**74**) demonstrated that the reaction of alkynylmagnesium bromides and pyrroline-1-oxide nitrone (**62**) could be carried out without dipolar cycloaddition [83, 89] (Scheme 11).

Synthesis of spin-labels.

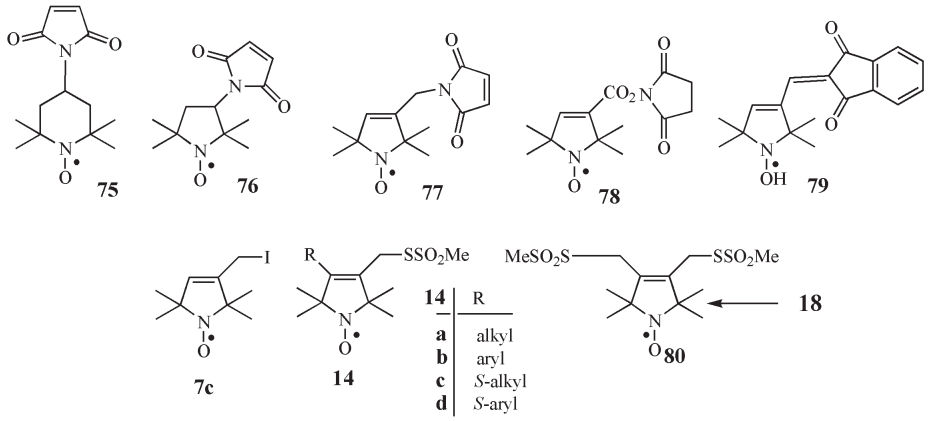
The first nitroxide spin labels were developed by combining the already well-known protein modification reagents with nitroxide heterocycles in order to target thiol and amino functions of proteins. Both five- and six-membered maleimide nitroxides (**75**) [90], (**76**) [91] and (**77**) [92] have been synthesized capable of making thioether bonds. The *N*-succinimidyl derivative (**78**) as an activated ester reacts with amines with amide bond formation [93]. At the end of the 80's, a new thiolspecific indanedione reagent (**79**) was developed starting from aldehyde (**8**) [94]. This indanedione reagent also makes thioether with cysteine side chain, similarly to the maleimides by Michael addition. The spin-label study of indanedione spin label on the myosin [95] and membrane proteins [96] were published in cooperation among American, Hungarian, German and Danish biophysicists. Although **75-79** reagents are still in use, some drawbacks of them should be considered. Namely, maleimides can react with amines at elevated pH, may hydrolyze to non-reactive maleamic acid form and thus undergo retro-Michael addition as well as the indanedione spin label. The *N*-succinimidyl group can also react with cysteine, tyrosine or histidine giving activated esters which are less stable than amides.

The above considerations urged us to develop novel thiolspecific spin labels, such as the aforementioned allylic iodide (**7c**)**,** making irreversible thioether bond without retro-Michael reaction possible and methanethiosulfonate (**7e)**, the classic MTS spin label which forms

Scheme 11



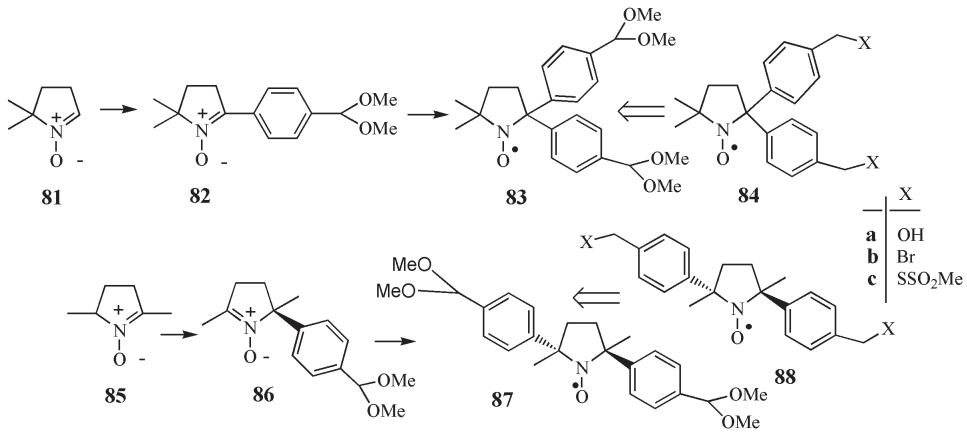
Scheme 12



cleavable disulphide bond with cysteine residues. The **14** MTS spin label was developed further by introducing a non-reactive substituent into the 4-position of the pyrroline ring [46]. These **14a-d** compounds were successfully applied in site-directed spin label studies at helix surface sites in T4 lysozyme [97].

Another challenge was to make homo-bifunctional crosslinking nitroxide reagents, particularly those reactive to sulfhydryl groups, promising to extend the capabilities of SDSL significantly. For example, bifunctional reagents that cross-link a pair of cysteine residues one or two turn apart in an α-helix are strongly immobilized with respect to the protein backbone, as expected. In this case, motion of the nitroxide group, reflected in the EPR spectrum, is dominated by fluctuations in the protein backbone. Thus, the EPR spectra of spin labels with dual attachment points may provide a direct and powerful measure of protein dynamics without the usual contributions from the internal motion of the side chain itself. This prompted us to synthesize compound **80** starting from bis(allylic bromide) (**18**) (Scheme 12) and **84c, 88c** bismethanethiosulfonates, the latter with a larger (10-15 Å) cross-bridge distances [98]. Numerous 2,5-diaryl pyrrolidine nitroxides have been published earlier [99, 100], however, synthesis of 2,2-diaryl pyrrolidines could also be executed by Grignard reaction of DMPO to give nitrone **82** followed by treatment with a more reactive aryllithium reagent (Scheme 13).

Scheme 13

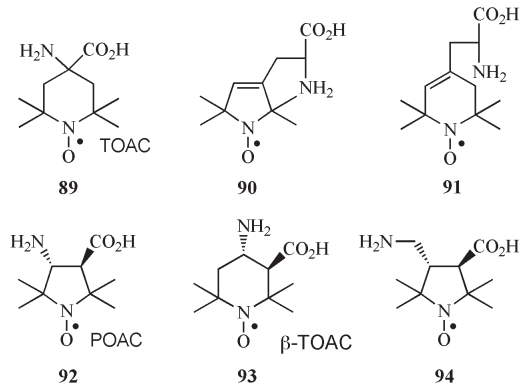


Synthesis of paramagnetic amino acids.

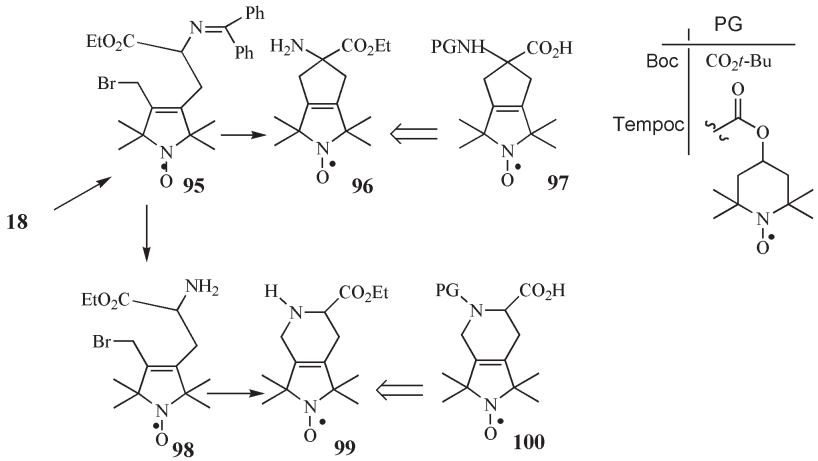
Besides the conventional spin labeling of protein side-chains with reactive nitroxides and SDSL technique, another approach to paramagnetically labeled proteins is solid phase protein synthesis. In this technique unnatural amino acids [101] with nitroxide moiety are incorporated in a step by step process [102]. A new trend with increasing significance is the nonsense suppression technology to build in unnatural amino acids [103, 104]. For ESR studies of proteins, a variety of paramagnetic α-amino acids (TOAC) (**89**) [105], (**90**) [106], (**91**) [53]; β-amino-acids (**92**) (POAC) [105], **93** (β-TOAC) [107] as well as γ-amino acid **94** [48] have been synthesized. In several cases, naturally occurring amino acids were modified by alkylation or acylation with functionalized nitroxides to obtain a paramagnetic protein building block [108]. TOAC, (4-amino-1-oxyl-2,2,6,6-tetramethyl-piperidine-4-carboxylic acid) by far the most popular among the above mentioned amino acids, is easily available from 1-oxyl-4-oxo-2,2,6,6-tetramethyl-piperidine (4-oxo-TEMPO) (**4**). This paramagnetic amino acid is considered to be a β-turn and 3_{10}-helix inducer and serves as a good reporter of backbone flexibility. TOAC was successfully incorporated into an α-melanocyte stimulating hormone without loss of biological activity [109, 110] (Scheme 14).

However, the six-membered ring of TOAC is sensitive to both reduction and oxidation as well as to acidic media, the latter being required for Merrifield solid phase synthesis. To eliminate this problem, we have very recently developed a new conformationally rigid paramagnetic α-amino acid synthesis by O'Donnell method [111] starting from dibromo compound (**18**). We observed that, depending on the reaction conditions different products were formed. The first alkylation step takes place at the α-carbon atom of ethyl *N*-diphenylmethylene glycine, giving compound **95**; while at low temperature and in the presence of NaHDMS, the second alkylation step occurs at the same carbon atom and after acidic hydrolysis we got **96** achiral α-amino acid with two fused five-membered rings. However, under conventional phase-transfer conditions, with dioxane, K_2CO_3, 18-crown-6 and heating, only one alkylation proceeds and during work-up (acidic and then base treatment) *N*-alkylation takes place, yielding a racemate of paramagnetic homoproline **99** amino acid [112]. Both amino acids can be further converted to *N*-protected derivatives, including a newly introduced paramagnetic protecting group (Tempoc), followed by ester hydrolysis as in compounds **97** and **100**, in order to fulfill the solid phase amino acid synthesis requirement (Scheme 15).

Scheme 14

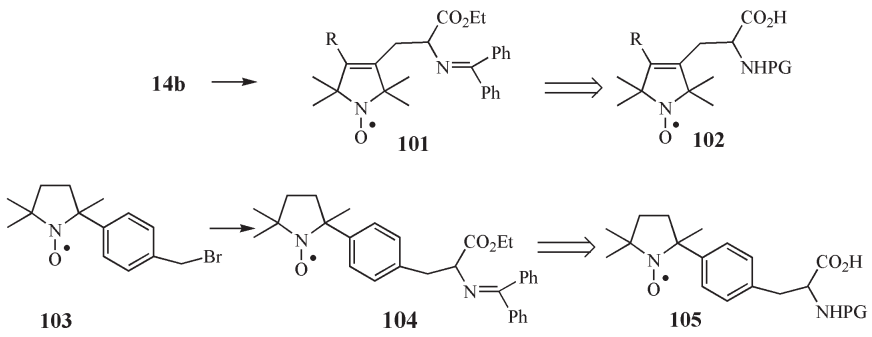


Scheme 15



The paramagnetic amino acids discussed above have the pyrroline or piperidine nitroxide moiety as the sole side chain. On the other hand, amino acids should mimic the shape and polarity of natural amino acids more precisely. Very recently, we have introduced new paramagnetic side chains taking advantage of 3,4-disubstituted pyrroline nitroxides, starting from **14** to get **102** amino acids with different alkyl and aromatic substituents following the above O'Donnell procedures. The amino acid synthesis worked well with 2-substituted pyrrolidine nitroxide **103** [113] affording **105,** which is not only a label with orientation different from pyrroline nitroxide, but it can also be considered as a proline-like side chain [114] (Scheme 16).

Scheme 16



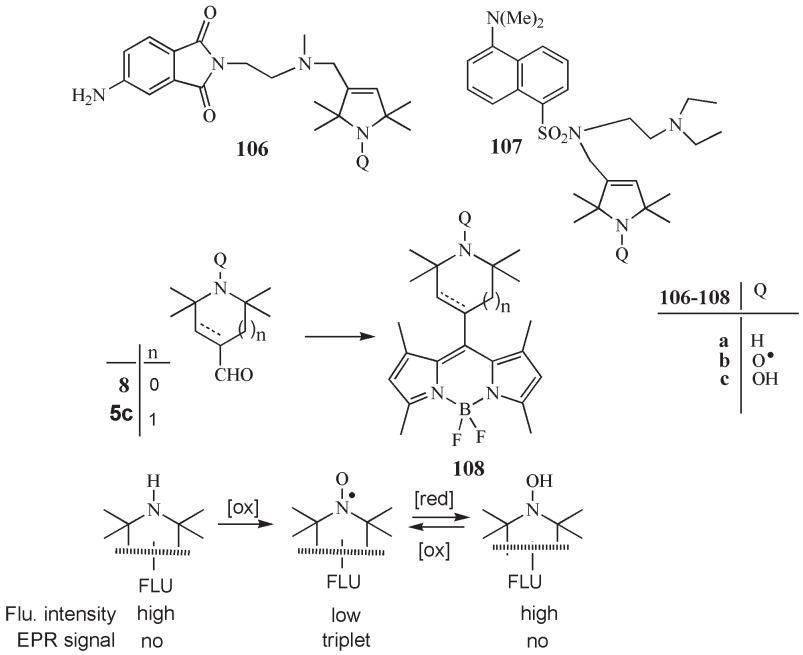
Synthesis of double (spin and fluorescence) sensor molecules.

The idea to connect a nitroxide to a fluorophore is quite an old one [115] and the fundamental recognition is that the fluorescence of the fluorophore as a donor is quenched by the nitroxide as an acceptor. The quenching rate is diffusion controlled, however the quenching mechanism is a question of debate, owing to the many conditions influencing it such as solvent polarity, spacer, nitroxides-ring size etc. [116]. The sensing of these donor-acceptor molecules is based on the reduction [117] or alkylation [21] of nitroxide moiety resulting in fluorescence enhancement or on fluorescence quenching when the sterically hindered secondary amine is oxidized to a nitroxide by ROS [118]. Several nitroxide-fluorophore donor acceptor pairs have been synthesized in our laboratory, mainly from the most stable pyrroline nitroxides [36]. Compound **106** is based on aminophthalimide fluorophore with a spacer containing an amino group. This and nitroxide moiety allows compound **106b** to behave as a logical "AND gate": the fluorescence is intense when nitroxide is reduced to *N*-hydroxylamine and the nitrogen in the spacer is protonated or complexed with a transition metal ion [119, 120]. Compound **107**, DanePy was used for *in vivo* detection of $^1O_2$ formation in broad bean leaves [121, 122]. The biological application of this molecule brings up further questions regarding the sensing mechanism as apparently not only the pyrroline ring participates in $^1O_2$ trapping [123]. Compound **108** was based on the successful incorporation of paramagnetic aldehydes **8**, **7c** into BODIPY [124]. The above molecules emit green/yellow fluorescence which does not overlap with the red chlorophyll fluorescence of plant leaves, making them ideal for such studies. With these compounds, ROS production and oxidation – reduction processes can be followed not only with fluorimetry but also with ESR, this is why these molecules are called double sensor molecules (Scheme 17).

Closing remarks.

In the present review, several aspects of the chemistry and biology of stable nitroxide free radicals have been complied mainly from our laboratory. During the chemical work, we often faced the problem: what kind of reactions can be done in the presence of a stable free radical? The problems can sometimes be solved easily thanks to organic chemists working out brilliant synthetic routes, but sometimes we need to go along roundabout ways to get the desired synthetic target. The real challenge in nitroxide chemistry is to create a “paramagnetic town” starting from triacetonamine or 4-oxo-TEMPO, as the only available building material to start with. The Russian and French nitroxide schools

Scheme 17



founded this town and we believe that chemists connected to this community all over the world improved the architecture further, but it will never be complete… Regarding the utilization of nitroxides: since the American scientists' pioneering work, spin labeling remained an important area in biophysical and biochemical research, providing means of time dependent analysis which cannot be substituted by NMR, MS or optical spectroscopy techniques. We believe that sooner or later nitroxide rings or their amine precursors will also appear as a new pharmacophore group in a drug available commercially.

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