Synthesis of New Double (Spin and Fluorescence) Sensor Reagents and Labels

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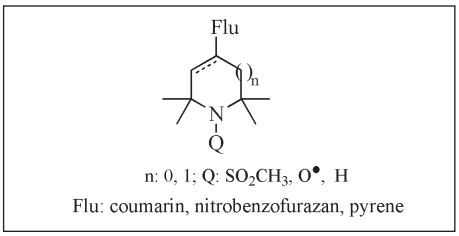
Dedicated to Prof. András Lipták on the occasion of his 70th birthday.



New double (spin and fluorescence) sensors were synthesized from aminocoumarin, pyrene and 4-nitrobenzofurazan dyes and from five- or six-membered nitroxides or their diamagnetic derivatives with aromatic nucleophylic substitution, Suzuki cross-coupling reaction and acylation reactions. The new compounds exhibit fluorescence emission between 382-529 nm affording various utilization possibilities.

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Introduction.

Fluorescent probes and sensors have attracted attention because of their high sensitivity and an exceptional ease of handling relative to their radioactive counterparts [1]. Connection of a fluorophore with a nitroxide free radical or its sterically hindered amine precursor results in a double (fluorescent and spin) sensor reagent, based on a nitroxide free radical (as an acceptor) fluorescence emission quenching of a fluorophore (as a donor) and the mechanism of the quenching has been well-documented in several cases [2,3]. The redox-status of the nitroxide or its amine precursor has a great influence on fluorescence emission intensity and this idea was utilized in design of new double sensor reagents. These reagents can be used in analytical chemistry to detect Reactive Oxygen Species (ROS) by electron spin resonance spectroscopy

(EPR) and by fluorescence spectroscopy [4,5] or fluorophore attached nitroxides can be utilized as redox-indicators [6]. In our laboratory several new type donor-acceptor pairs have been synthesized based on dansyl [7], aminophthalimide [8], benzazole [9] and 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) [10] type fluorophores. Till now, the most successful one has been the 3-[*N*-(β-diethylaminoethyl)-*N*-dansyl] aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole, so-called "DanePy", which is widely used in the singlet oxygen detection in plant physiology under oxidative stress conditions [11]. Another challenge in this area is the synthesis of double-label molecules combining advantages of the fluorescent labeling and the spin labeling. Because of the dynamic quenching in these donor-acceptor pairs, the lifetime of the fluorophore is drastically reduced and this is disadvantageous in the respect of fluorescence anisotropy measurements. Therefore we thought that supplying a spin label with a long-lifetime organic fluorophore can result in a double label, simultaneously applicable both in fluorescence and EPR spectroscopy. In this paper we report the synthesis of double sensor reagents based on easily available coumarin, 4-nitrobenzofurazan and pyrene fluorophores attached to nitroxides. The functionalized diamagnetic and paramagnetic forms of pyrene-nitroxide donor-acceptor pairs make possible the site-specific labeling of a protein with a reversible methanethiosulfonate (MTS) spin label and with a similar shape and polarity long-lifetime fluorophore MTS label offering biochemical and biophysical investigation of a protein with EPR and fluorescence.

Results and Discussions.

In the design of new double sensor reagents evident choice were coumarins, which generally show large Stokes shifts, high quantum yields and widely used in photoinduced electron transfer (PET) based chemosensors [12] and fluorescent derivatization reagents [13]. Treatment of 7-amino-4-methyl coumarin (**1**) with paramagnetic acid chloride (**2**) [14] gave paramagnetic coumarin derivative (**3a**) which exhibited a very weak fluorescence at short wavelength (382 nm). Paramagnetic compound **3a** was reduced to **3b** diamagnetic sterically hindered amine with Fe powder in acetic acid and could be basified with $NaHCO_3$ without opening the lactone ring. Interestingly, in the case of compound **3b** a similarly low quantum yield,

e.g. weak fluorescence was observed (Table). The quenching effect can be attributed to an intramolecular charge transfer as a band at 488 nm indicates in ultraviolet/visible (UV) spectrum of **3b** (and no such band in apolar dioxane). Because the advantageous effect of an amino or *N,N*-dialkylamino group in coumarin dyes regarding the fluorescence properties [15], paramagnetic amine (**5**) was treated with 3-carboxy-7-dimethylaminocoumarin imidazolide made *in situ* [16] from **4** [17] and 1,1'-carbonyldiimidazole (CDI) in CH_2Cl_2, affording compound **6a** which could also be converted to compound **6b** with Fe powder/glacial acetic acid. Compounds **6a** and **6b** exhibited longer (460 nm) wavelength fluorescence emission and there is a 25-fold difference in the fluorescence intensities of diamagnetic **6b** and paramagnetic **6a** forms. Reaction of 7-chloro-4-nitrobenzofurazan **7** with 4-amino-2,2,6,6-tetramethylpiperydin-1-oxyl (4-amino-TEMPO) (**8a**) in an aromatic nucleophilic substitution reaction [18] yielded compound **9a** with a weak yellow fluorescence. Because of the reduction of the aromatic nitro group to amine upon Fe/AcOH treatment, the diamagnetic pair **9b** was synthesized directly from 4-amino-2,2,6,6-tetramethylpiperidine (**8b**). The fluorescence intensity ratio of **9b** versus **9a** is the best (about 33-fold) among the studied examples, probably because of the vicinity of the donor and the acceptor (*e.g*., 4-amino group of nitroxide can be regarded as a part of the fluorophore) (Scheme 1).

Table

(Fluorescence and EPR data of compounds **3-17** prepared)

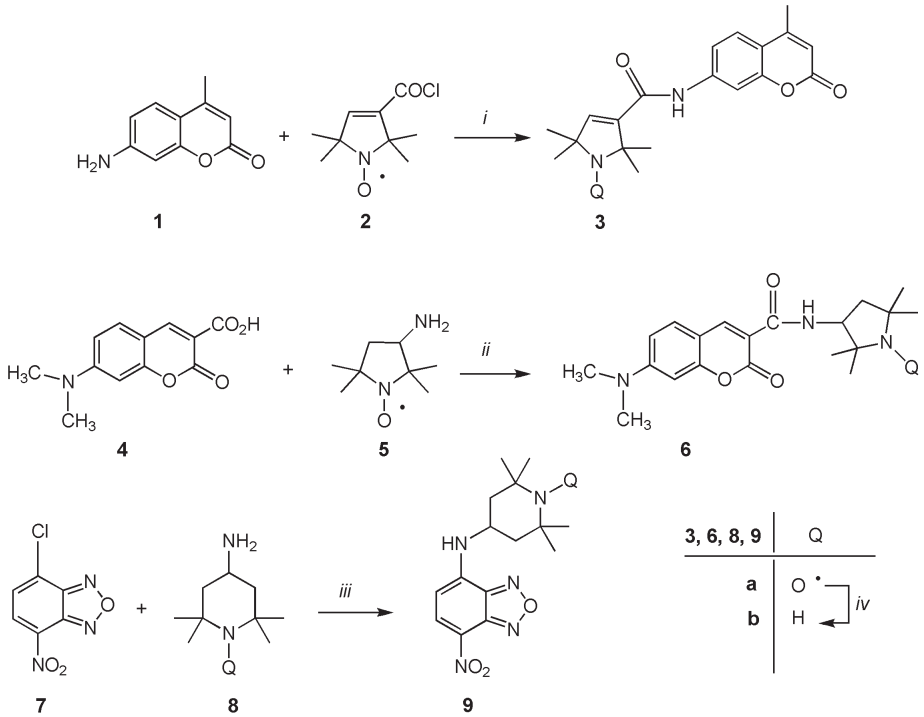
Compound	λ_{max} * [nm]	ε [L×mol^{-1}×cm^{-1}]	λ_{ex} [nm]	λ_{em} [nm]	QY**	a_N *** [G]
3a	329	2.10×10^4	322	382	0.008	14.4
3b	329	2.21×10^4	326	384	0.009	-
6a	412	4.49×10^4	413	462	0.015	14.4
6b	410	4.20×10^4	412	461	0.378	-
9a	460	2.21×10^4	470	526	0.024	15.5
9b	466	2.13×10^4	473	529	0.786	-
12	342	4.51×10^4	347	386	0.067	14.6
15	344	4.18×10^4	340	377	0.082	-
17	343	4.71×10^4	341	378	0.28	-

*Data were measured in acetonitrile, OD<0.05; ** ± 5 %, n= 3, referred to quinine, **9a, 9b** referred to fluorescein; *** Measured in $CHCl_3$.

Beyond the double sensor molecules mentioned above we were also interested in double labels synthesis, *e.g*., molecules supporting simultaneous EPR and fluorescence investigations.

The long-lifetime pyrene fluorophore was chosen and its 1-boronic acid derivative was reacted with β-bromo-α,β-unsaturated aldehyde (**10**) in a Suzuki-reaction [19] in dioxane and aq. Na_2CO_3 solution in the presence of $Pd(PPh_3)_4$ catalyst to give **11** aldehyde which was reduced to **12** alcohol

Scheme 1



Reagents and conditions: (*i*) THF/DMF, Et_3N (1 eq.), reflux, 2h, 25 °C, 8h, 45 %; (*ii)* CDI (1 eq.), CH_2Cl_2, 25 °C, 14h, then **5** (1 eq.), DBU (1 eq.) reflux 3h, 55 %; (*iii)* EtOAc, 25 °C, 2h, Et_3N (1 eq.) 78-83 %; (*iv)* Fe (10 eq.), AcOH, 70 °C, 1h, then $NaHCO_3$, 38-62 %.

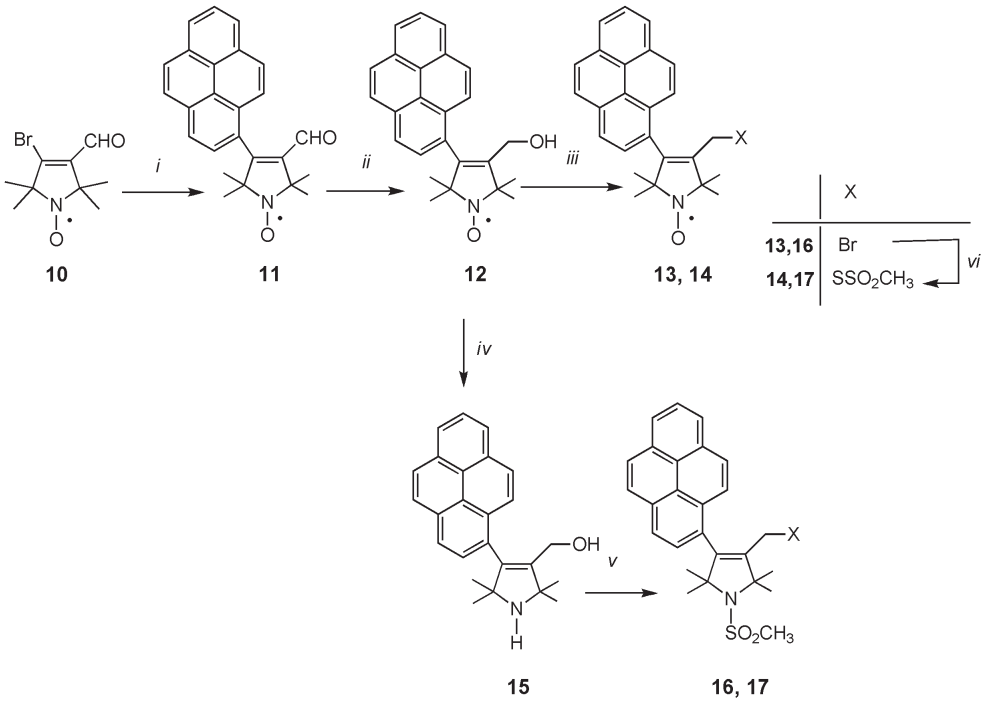
with $NaBH_4$ in ethanol. The paramagnetic bromide (**13**) was achieved *via* mesylate and nucleophilic substitution reaction with LiBr [20]. The treatment of compound **13** with $NaSSO_2CH_3$ in aq. acetone afforded compound **14** a reversible, thiolspecific methanethiosulfonate double (fluorescent and spin) label [21]. However, compound **14** exhibited low fluorescence quantum yield, and we checked its lifetime which was found to be very low (<1 ns) as well. Unfortunately, the reduction of compound **12** to diamagnetic amine (**15**) did not influence significantly either the fluorescence intensity or the lifetime (<1 ns) attributable to PET effect caused by the amino group in the pyrroline ring [22]. This meant that compound **14** could be applied only as an EPR active label therefore we decided to eliminate the PET effect by converting the amino group to an amide. In this case the nitrogen non-bonding electron pair is conjugated with a carbonyl or a sulfone group and cannot interact with the singlet state of the fluorophore. This was accomplished with an excess methanesulfonyl chloride treatment of **15** followed by a nucleophilic substitution with LiBr on the methylene group, affording compound **16** which could be converted to compound **17** with $NaSSO_2CH_3$. Compound **17** is a thiol-specific, reversible, pyrene-based fluorescent reagent with a 0.28 quantum yield and 2.9 ns lifetime, although this lifetime value may be decreased by excimer formation [1].

In conclusion, new coumarin, 4-nitrobenzofurazan based double sensor molecules were synthesized. Among them the 3-carboxamido-7-dimethylaminocoumarin derivative with blue fluorescence emission and 4-nitrobenzofurazan based sensors with yellow emission are the most promising ones. The recent results show that EPR and fluorescent labeling is hard to accomplish simultaneously with one label, seemingly a diamagnetic-paramagnetic pair must be used even in the case of application of a long-lifetime fluorophore such as pyrene. Further biological and photophysical studies of these compounds are in progress.

EXPERIMENTAL

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on Carlo Erba EA 1110 CHNS elemental analyzer. The IR (Specord 75) spectra were in each case consistent with the assigned structure. Mass spectra were recorded on an Automass Multi instrument in the EI mode (70 eV, direct inlet). EPR spectra were obtained from 10^{-5} molar solutions ($CHCl_3$), using a Magnettech MS200 spectrometer. Preparative flash column chromatography was performed on Merck Kieselgel 60 (0.040-0.063 mm). The UV spectra were taken with a Specord 40 (Jena Analytic) in acetonitrile using 1-cm quartz cells and solute concentrations (2-0.5) $\times 10^{-5}$ *M*. The molar

Scheme 2



Reagents and conditions: (*i*) 1-pyreneboronic acid (1 eq.), dioxane /aq. Na_2CO_3, $Pd(PPh_3)_4$ reflux, 2h, N_2, 77 %; (*ii)* $NaBH_4$ (3 eq.), EtOH, 30 min., 25 °C, 69 %; (*iii)* MsCl (1.1 eq.), Et_3N (1.1 eq), 0°C→25 °C, 1h, then LiBr (2 eq.), acetone, reflux, 1h, 45 %. (*iv)* Fe (10 eq.), AcOH, 70 °C, 1 h, then K_2CO_3, 35 %; *(v)* MsCl (2.2 eq.), Et_3N (2.2 eq), 0°C→25 °C, 1h, then LiBr (2 eq.), acetone, reflux, 36 %; (*vi*) $NaSSO_2CH_3$ (2 eq.), acetone/H_2O, reflux, 45 min., 39-49 %.

extinction coefficients (ε) at absorption maxima were obtained from slope of absorbance vs concentration using five solutions of different concentrations. Fluorescence spectra of compounds dissolved in acetonitrile was measured with Perkin Elmer LS50B spectrofluorimeter, with 5 nm slits, with correction of instrumental factors by means of a rhodamine B quantum counter and correction files supplied by the manufacturer. Quantum yields were referred to quinine dissolved in 0.1 *M* H_2SO_4 (Φ'= 0.53) or fluorescein dissolved in 0.1 *M* NaOH (Φ'= 0.95). The values were calculated on the equation Φ= (I/I')(A'/A)(n/n')Φ', where I', A', and Φ' are the integrated emission, absorbance (at the excitation wavelength), and quantum yield of the reference sample, respectively, n' is the refractive index of the solvent used for reference sample. I, A, n, Φ are related to sample with the same definitions applied to reference sample. Lifetimes were measured with ISS K2 multifrequency phase fluorimeter and referred to glycogen. Qualitative TLC was carried out on commercially prepared plates (20 x 20 x 0.02 cm) coated with Merck Kieselgel GF_{254}. ^1H NMR spectra of diamagnetic compounds were recorded with Varian Unity Inova 400 WB spectrometer; chemical shifts were referenced to TMS. Compounds **2** [14], **4** [17], **10** [23] were prepared according to published procedures and compounds **1**, **5**, **7**, **8a**, **8b** and 1-pyreneboronoc acid were purchased from Aldrich. The fluorescence data of all new compounds are listed in the Table.

1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-carboxylic Acid-(4-methyl-coumarin-7-yl)amide Radical (**3a**).

To a stirred solution of compound **1** (350 mg, 2.0 mmol) and Et_3N (202 mg, 2.0 mmol) in anhydrous 1:1 THF/DMF (10 mL) mixture acid chloride (**2**) (404 mg, 2.0 mmol) dissolved in dry THF (5 mL) was added dropwise and the mixture was stirred and refluxed for 2 h after which the mixture was allowed to stay overnight at room temperature. The solvents were evaporated off, the residue was dissolved in $CHCl_3$ (15 mL), washed with brine (10 mL) the organic phase was dried ($MgSO_4$), filtered and evaporated. After chromatographic purification ($CHCl_3$: Et_2O, 2:1) compound **3a** was obtained as a pale yellow solid (307 mg, 45 %), mp 259-260 °C, R_f : 0.3 ($CHCl_3$: Et_2O, 2:1). MS: *m/z* (%): 341 (M^+, 1), 311 (6), 205 (84), 147 (100). IR (nujol) ν = 3400, 1720, 1650, 1690, 1520 cm^{-1}.

Anal. Calcd. for $C_{19}H_{21}N_2O_4$: C 66.85, H 6.20, N 8.21. Found: C 66.80, H 6.07, N 8.03.

7-Dimethylaminocoumarin-3-carboxylic Acid-(1-oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl) amide Radical (**6a**).

A solution of **4** acid (932 mg, 4.0 mmol) and 1,1'-carbonyldiimidazole (648 mg, 4.0 mmol) in CH_2Cl_2 (10 mL) was stirred 14 h at room temperature, then amine **5** (628 mg, 4.0 mmol) dissolved in CH_2Cl_2 (5 mL) and DBU (608 mg, 4.0 mmol) was added and the mixture was refluxed for 3 h. After cooling the organic layer was washed with aq. $NaHCO_3$ solution (10 mL), brine (10 mL). The organic layer was separated, dried, ($MgSO_4$), filtered and evaporated. After flash column chromatography with (hexane/EtOAc and $CHCl_3$/Et_2O) compound **6a** was obtained as a yellow solid 818 mg (55 %), mp 215-217 °C, R_f : 0.32 ($CHCl_3$: Et_2O, 2:1). MS: *m/z* (%): 372 (M^+, 23), 299 (6), 216 (100). IR (nujol) ν = 3400, 1680, 1660, 1630, 1590, 1525 cm^{-1}.

Anal. Calcd. for $C_{20}H_{26}N_3O_4$: C 64.50, H 7.04, N 11.28. Found: C 64.50, H 7.00, N 11.20.

General Procedure for Reaction of 7-Chloro-4-nitrobenzofurazan (**7**) with Amines to give (**9a, 9b**).

To a stirred solution of compound **7** (997 mg, 5.0 mmol), Et_3N (505 mg, 5.0 mmol) in dry EtOAc (10 mL) amine **8a** or **8b** (5.0 mmol) was added and the mixture was stirred at room temperature for 2 h. The organic phase was washed with brine (10 ml) and the organic phase was separated, dried ($MgSO_4$), filtered and evaporated. Flash column chromatography purification ($CHCl_3$/Et_2O, $CHCl_3$/MeOH) afforded the orange-red solids **9a** or **9b**.

1-Oxyl-2,2,6,6-tetramethyl-4-(4-nitrobenzo[1,2,5]oxadiazol-7-ylamino)piperidine Radical **9a** was obtained in 83 % yield (1.38 g), mp 258-260 °C, R_f : 0.23 ($CHCl_3$: Et_2O, 2:1). MS: *m/z* (%): 334 (M^+, 14), 320 (16), 304 (7), 205 (100). IR (nujol) ν = 3420, 1610, 1580, 1545, 1510 cm^{-1}.

Anal. Calcd. for $C_{15}H_{20}N_5O_4$: C 53.87, H 6.03, N 20.95. Found: C 53.79, H 5.99, N 20.78.

2,2,6,6-Tetramethyl-4-(4-nitrobenzo[1,2,5]oxadiazol-7-ylamino)piperidine **9b** was obtained in 78 % yield (1.24 g), mp 193-195 °C, R_f : 0.12 ($CHCl_3$: MeOH, 9:1). MS: *m/z* (%): 319 (M^+, 32), 304 (100), 269 (3). IR (nujol) ν = 3400, 1600, 1580, 1550, 1510 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ: 8.33 (br d, 1H, arH), 6.37 (d, *J*= 8.2 Hz, 1H, ar H), 4.15 (br, 1H, N-CH), 1.86 (br, 2H, CH*H*), 1.42 (br, 2H, C*H*H), 1.30 (s, 6H, CH_3), 1.15 (s, 6H, CH_3).

Anal. Calcd. for $C_{15}H_{21}N_5O_3$: C 56.41, H 6.63, N 21.93. Found: C 56.28, H 6.62, N 21.82.

General Procedure for Reduction of Nitroxides to Amines [24] **3b, 6b, 15**.

To a solution of nitroxide **3a**, **6a**, **12** (2.0 mmol) in AcOH (10 mL) Fe powder (1.12 g, 20 mmol) was added and the mixture was warmed up to 70 °C until the reaction started. The mixture was stirred at this temperature for 1 h, after cooling diluted with water (15 mL), the decanted aq. solution was basified with solid $NaHCO_3$ or K_2CO_3. The mixture was extracted with $CHCl_3$ (3 x 15 mL), dried ($MgSO_4$), filtered, evaporated and after chromatographic purification ($CHCl_3$/MeOH) the title amines **3b, 6b** and **15** were obtained in 35-62 % yield.

2,2,5,5-Tetramethyl-2,5-dihydro-1*H*-pyrrole-3-carboxylic Acid-(4-methyl-coumarin-7-yl)amide (**3b**).

Compound **3b** was obtained as a white solid, 1.01 g (62 %), mp 281-283 °C, R_f : 0.23 ($CHCl_3$: MeOH, 9:1). MS: *m/z* (%): 326 (M^+, 1), 311 (11), 110(100). IR (nujol) = 3400, 1690, 1605, 1565, 1520 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ: 9.09 (bs, 1H, CONH), 7.67 (dd, $J_{5,6}$ = 8.7 Hz, $J_{6,8}$ = 2.0 Hz, 1H, C6-H), 7.56 (d, $J_{6,8}$ = 2.0 Hz, 1H, C8-H), 7.42 (d, $J_{5,6}$ = 8.7 Hz, 1H, C5-H), 6.42 (s, 1H, C3-H), 6.07 (s, 1H, C4'-H), 2.31 (s, 3H, C4-CH_3), 1.39 (s, 6H, CH_3), 1.24 (s, 6H, CH_3).

Anal. Calcd. for $C_{19}H_{22}N_2O_3$: C 69.92, H 6.79, N 8.58. Found: C 69.89, H 6.77, N 8.50.

7-Dimethylaminocoumarin-3-carboxylic Acid-(1-oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl) amide (**6b**).

Compound **6b** was obtained as a yellow solid, 271 mg (38 %), mp 210-212 °C, R_f : 0.58 ($CHCl_3$:MeOH, 9:1). MS: *m/z* (%): 357 (M^+, 24), 342 (6), 216 (64), 99 (100). IR (nujol) ν = 3450, 1690, 1620, 1520 cm^{-1}. ^1H NMR (DMSO-d_6): δ: 8.69 (d, *J* = 9.2 Hz, 1H, CONH), 8.66 (s, 1H, C4-H), 7.69 (d, $J_{5,6}$ = 8.2 Hz, 1H, C5-H), 6.81 (dd, $J_{5,6}$ = 8.2 Hz, $J_{6,8}$ = 2.4 Hz, 1H, C6-H), 6.62 (d, $J_{6,8}$

= 2.4 Hz, 1H, C8-H), 4.25 (dt, J_{NHCH} = 9.2 Hz, J_{CHCH} = 7.6 Hz, 1H,NCH), 3.06 (s, 6H, NCH_3), 2.00 (m, 1H, C*H*H), 1.65 (m, 1H, CH*H*), 1.14, 1.12, 1.09, 0.99 (4s, 4×3H, C-CH_3).

Anal. Calcd. for $C_{20}H_{27}N_3O_3$: C 67.20, H 7.61, N 11.76. Found: C 67.20, H 7.59, N 11.66.

3-Hydroxymethyl-4-(pyren-1-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole (**15**).

Compound **15** was obtained as a white solid, 249 mg (35 %), mp 185-187 °C, R_f: 0.2 ($CHCl_3$: MeOH, 9:1). MS: *m/z* (%): 355 (M^+, 11), 340 (100), 310 (46). IR (nujol) ν = 3500, 1600, 1555, 1540, 1510 cm^{-1}. ^1H NMR (DMSO-d_6): δ: 8.28-7.79 (m, 9H, pyrene CH's), 4.28 (bt, *J* = 5.0 Hz, 1H, OH), 3.74 and 3.65 (dAB, J_{CHOH} = 5.0 Hz, J_{AB} = 12.0 Hz, 2H CH_2OH), 1.50, 1.42, 1.33, 0.99 (4s, 4×3H, CH_3)

Anal. Calcd. for $C_{25}H_{25}NO$: C 84.47, H 7.09, N 3.94. Found: C 84.39, H 7.02, N 3.90.

3-Formyl-1-oxyl-4-(pyren-1-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole Radical (**11**).

A solution of compound **10** (988 mg, 4.0 mmol) and $Pd(PPh_3)_4$ (200 mg, 0.2 mmol) in dioxane (15 mL) was purged with N_2 stream for 5 min., then pyrene-1-boronic acid (984 mg, 4.0 mmol) and 10 % aq. Na_2CO_3 (10 mL) was added and the mixture was stirred and refluxed under N_2 for 2 h. After cooling the dioxane was evaporated off, water (10 mL) was added and the aqueous phase was extracted with EtOAc (2 x 20 mL). The organic phase was dried ($MgSO_4$), filtered, evaporated and after chromatographic purification (hexane/Et_2O, hexane/EtOAc) the title nitroxide **11** was obtained as a pale yellow solid 1.13 g (77%), mp 200-202 °C, R_f: 0.53 (hexane:EtOAc, 2:1). MS: *m/z* (%): 368 (M^+, 40), 338 (7), 323 (100). IR (nujol) ν = 1660, 1640, 1570, 1530 cm^{-1}.

Anal. Calcd. for $C_{25}H_{22}NO_2$: C 81.50, H 6.02, N 3.80. Found: C 81.40, H 5.97, N 3.72.

3-Hydroxymethyl-1-oxyl-4-(pyren-1-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole Radical (**12**).

To a stirred solution of compound **11** (736 mg, 2.0 mmol) in EtOH (10 mL) $NaBH_4$ (228 mg, 6.0 mmol) was added and the mixture was stirred at room temperature for 30 min. After evaporation of alcohol sat. aq. NH_4Cl solution (10 mL) was added to the residue and extracted with $CHCl_3$ (2 x 15 mL). The organic phase was dried ($MgSO_4$), filtered, evaporated and after chromatographic purification the title nitroxide **12** was obtained as a pale yellow solid 510 mg (69%), mp 183-185 °C, R_f : 0.16 (hexane:EtOAc, 2:1). MS: *m/z* (%): 370 (M^+, 31), 340 (12), 296 (46), 239 (100). IR (nujol) ν = 3500, 1600, 1550, 1540, 1510 cm^{-1}.

Anal. Calcd. for $C_{25}H_{24}NO_2$: C 81.05, H 6.53, N 3.78. Found: C 80.95, H 6.48, N 3.77.

General Procedure for Synthesis of Allylic Bromides (**13, 16**).

To a solution of alcohol **12** or **15** (2.0 mmol) in CH_2Cl_2 (15 mL) and Et_3N 222 mg (2.2 mmol in case of **12**) and 444 mg (4.4 mmol in case of **15**) methanesulfonyl chloride 252 mg (2.2 mmol in case of **12**) and 504 mg (4.4 mmol in case of **15**) dissolved in CH_2Cl_2 (3 mL) was added dropwise at 0 °C. After stirring the mixture at room temperature for 1 h, it was washed with water (15 mL), the organic phase was separated, dried ($MgSO_4$), filtered and evaporated, then the residue was dissolved in acetone (20 mL) and LiBr (348 mg, 4.0 mmol) was added and the reaction mixture stirred and refluxed for 1 h. After cooling the acetone was evaporated off under reduced pressure, the residue was partitioned between water (10 mL) and EtOAc (20 mL). The phases were then separated, the aqueous phase was washed again with EtOAc (10 mL). The combined organic phase was dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash column chromatography (hexane/EtOAc) to give compound **13** or **16**.

3-Bromomethyl-1-oxyl-4-(pyren-1-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole Radical (**13**).

This compound was obtained in 45 % yield (390 mg), mp 205-207 °C, R_f : 0.63 (hexane : EtOAc, 2:1). MS: *m/z* (%): 434/432 (M^+, 14/14), 402/404 (2/2), 338 (4), 297 (30), 239 (100). IR (nujol) ν =1600, 1550, 1520 cm^{-1}.

Anal. Calcd. for $C_{25}H_{23}BrNO$: C 69.29, H 5.35, N 3.23. Found: C 69.20, H 5.32, N 3.16.

3-Bromomethyl-1-methanesulfonyl-4-(pyren-1-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole (**16**).

Compound **16** 357 mg (36%) was obtained**,** mp 84-86 °C, R_f : 0.55 (hexane : EtOAc, 2:1). MS: *m/z* (%): 497/495 (M^+, 8/8), 482/480 (19/19), 436 (12), 281 (39), 79 (100). IR (nujol) ν =1600, 1560, 1520 cm^{-1}.

Anal. Calcd. for $C_{26}H_{26}BrNO_2S$: C 62.90, H 5.28, N 2.82. Found: C 62.84, H 5.22, N 2.78.

General Procedure for Synthesis of Methanethiosulfonates (**14, 17**).

A solution of allylic bromide **13** or **16** (1.0 mmol) and $NaSSO_2CH_3$ (268 mg, 2.0 mmol) in acetone (15 mL) and water (5 mL) was refluxed for 45 min. After cooling the acetone was evaporated off, water (5 mL) was added and the residue was partitioned between water (10 mL) and EtOAc (20 mL). The organic phase was separated, dried ($MgSO_4$), filtered and evaporated and flash column chromatography purification afforded methanethiosulfonates **14** or **17**.

3-Methanethiosulfonylmethyl-1-oxyl-4-(pyren-1-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole Radical (**14**).

This compound was obtained in 49 % yield (227 mg), mp 84-86 °C, R_f : 0.30 (hexane:EtOAc, 2:1). MS: *m/z* (%): 464 (M^+, 6), 434 (6), 239 (28), 43(100). IR (nujol) ν =1600, 1550, 1520 cm^{-1}.

Anal. Calcd. for $C_{26}H_{26}NO_3S_2$: C 67.21, H 5.64, N 3.01. Found: C 67.24, H 5.60, N 2.95.

3-Methanethiosulfonylmethyl-1-methanesulfonyl-4-(pyren-1-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole (**17**).

Compound **17** 205 mg (39%) was obtained, mp 245-248 °C, R_f : 0.22 (hexane : EtOAc, 2:1). MS: *m/z* (%): 527 (M^+, 26), 512 (43), 448 (17), 239(52), 79 (100). IR (nujol) ν =1600, 1560, 1510 cm^{-1}. ^1H NMR ($CDCl_3$): δ: 8.27-7.77 (m, 9H, pyrene CH's), 3.60 (s, 2H, CH_2), 3.17 (s, 3H, SSO_2CH_3), 2.43 (s, 3H, NSO_2CH_3), 1.94, 1.84, 1.78, 1.52 (4s, 4×3H, CCH_3).

Anal. Calcd. for $C_{27}H_{29}NO_4S_3$: C 61.45, H 5.54, N 2.65. Found: C 61.39, H 5.49, N 2.56.

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