Synthesis and structure optimization of double (fluorescent and spin) sensor molecules

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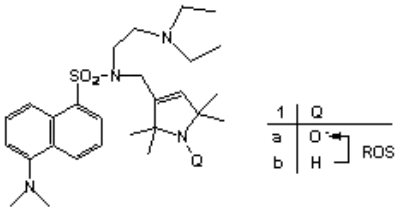
Dedicated to Professor Douglas Lloyd on the occasion of his 80th birthday

Abstract
Synthesis and fluorescence properties of stable nitroxide free radicals (**10a, 11a, 12a, 14a, 20a, 21a**) and their amine (**10b, 11b, 12b, 14b, 20b, 21b**) precursors covalently linked to dansyl or 3- and 4-aminophthalimide are reported. The best intramolecular quenching is achieved when the fluorophore and the nitroxide are in the closest possible position

Keywords: Fluorescence, sensor molecules, nitroxide, free radicals, aminophthalimide

Introduction

Fluorescence and spin double sensors are important probe molecules for detecting free radicals both in condensed-[1-4] and gas-phases[5]. Their sensing ability is based on the energy transfer from a donor moiety (fluorophore) to an acceptor moiety (nitroxide) which results in quenched fluorescence. The possible quenching mechanisms of these compounds are well discussed.[6-8] Absence of acceptor in the diamagnetic derivatives of these probe molecules, exhibit strong fluorescence. Utilizing these probes, Reactive Oxygen Species (ROS) production can be followed on the basis of either fluorescence quenching or the EPR detectable appearance of nitroxide. In case of biological application other requirements arise: to avoid the overlapping with background emission of intrinsic fluorophores, water solubility and permeability through membranes. Applications also require a large difference between the fluorescence emission of the amine and the corresponding nitroxide, in order to assure sensitive detection of ROS. Recently we have developed a sensor molecule for biological application, called DanePy[1] **1b**, which is readily oxidized by ROS to nitroxide **1a** (Scheme 1).

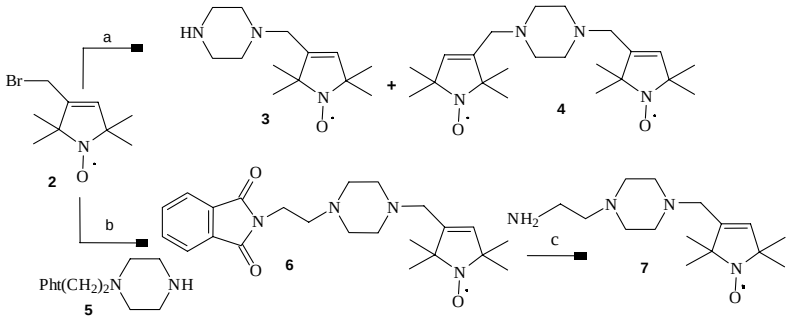


Scheme 1

Similar oxidation reaction was also observed earlier *in vivo*.[9] The diethylaminoethyl side-chain in DanePy **1b** ensures water solubility and penetration into chloroplasts.[10] In this paper we discuss the role of spacer group on quenching of fluorescence as well as extension of this idea to other aminophthalimide fluorophores to obtain more sensitive double sensors.

Results and Discussion

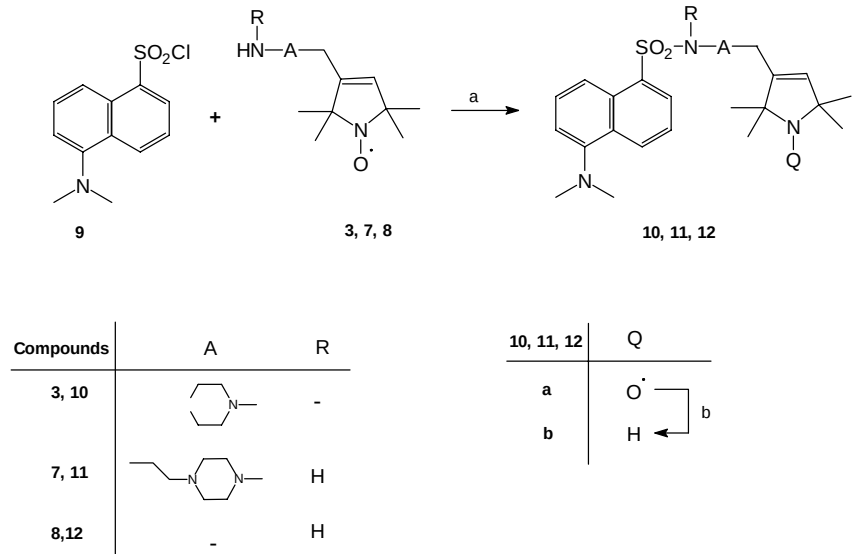
In our previous studies we experienced the advantage of a protonable amino group in spacer to increase of solubility in aqueous media. We achieved this by inserting of piperazine, or 1-(2-aminoethyl)piperazine as spacer group between nitroxide and fluorophore in double sensors. This could be accomplished by alkylating piperazine with allylic bromide[11] **2** in $CHCl_3$ in the presence of K_2CO_3 to give mixture of the monoalkylated compound **3** and the dialkylated compound **4**. The *N*-[2-(1-piperazidinyl)ethyl]phthalimide **5**[12] could be alkylated on the secondary nitrogen atom under the above conditions to yield compound **6**. Treatment of phthalimide derivative **6** with methylamine[13] in ethanol allowed the mild deprotection of the terminal amino group to give compound **7** (Scheme 2).



Scheme 2

Reagents and conditions: (a) piperazine (3 eq.), K_2CO_3 (1 eq.), $CHCl_3$, reflux, 1 h, **3** (40 %), **4** (37 %); (b) **5** (1 eq.), K_2CO_3 (1 eq.), $CHCl_3$, reflux, 2 h, (74 %); (c) CH_3NH_2, EtOH, r.t. 12 h, (33 %).

Amines **3** and **7** as well as allylic amine **8**[14] were treated with 5-dimethylamino-1-naphtalenesulfonyl chloride **9** in CH_2Cl_2 in the presence of triethylamine to get the paramagnetic dansyl derivatives **10a**, **11a** and **12a**. The paramagnetic compounds were reduced to sterically hindered amines **10b**, **11b** and **12b** by Fe powder in glacial acetic acid[15] (Scheme 3).



Scheme 3

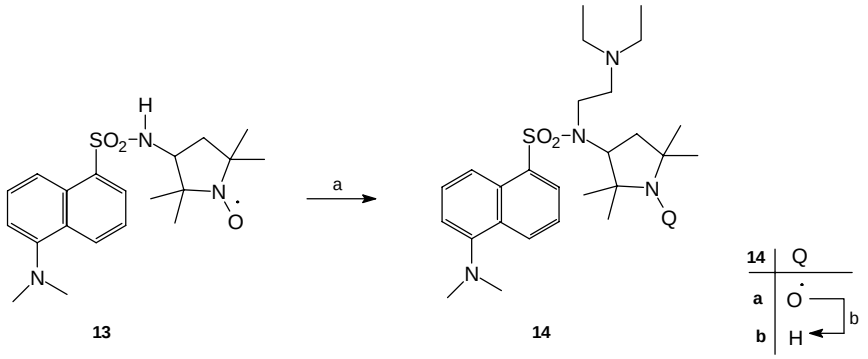
Reagents and conditions: (a) **8** (1.0 eq.) CH_2Cl_2, Et_3N (1.1 eq.), r.t., 4 h, (45-58 %); (b) Fe (5 eq.), AcOH, 80 °C, 30 min, then K_2CO_3, (32-48%).

However, comparing the ratios of fluorescence emission maxima of amines **10-12b** versus nitroxides **10-12a** in case of compound **10, 11** became less advantageous than **12b/12a** ratio (Table 1).

Table 1. Fluoresence emission data of compounds **1, 10, 11, 12, 14, 20, 21** in K-phosphate buffer (50mM, pH 7.2) containing 5% or 20 %* EtOH.

Compound	a (NO) emission max (nm)	b (NH) emission max (nm)	Peak intensity ratio (b/a)
1	530	551	5.56
10	560	556	1.57
11	545	550	1.10
12	540	550	6.39
14	519	523	28.13
20	546	503	25.00
21*	532	528	9.11

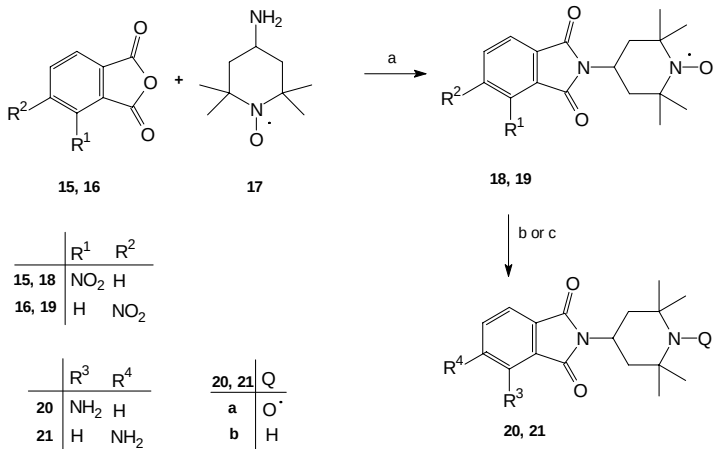
The introduction of a long spacer between the donor and the acceptor moieties decreased the rate of quenching of fluorescence, because the rate of Coulombic energy transfer inversely proportional to the sixth power of distance between donor and acceptor. While electron transfer or electron exchange rate decrease exponentially with increasing donor-acceptor distance.[16] To get better double sensor molecules, i. e. to achieve better ratio of emission maxima of amine versus nitroxide we investigated compound **13.**[17] The sodium salt of **13** was alkylated in dimethylformamide/THF mixture with freshly released 2-(diethylamino)ethyl chloride to give compound **14a,** which was then reduced to **14b** with Fe powder in AcOH (Scheme 4). Fluorescence emission maxima ratio of compounds **14b** and **14a** were the highest among the investigated dansyl derivatives (Table 1).



Scheme 4

Reagents and conditions: (a) NaH (2.0 eq.), dry THF, 0 °C, 15 min. then add benzene extract of aq. solution of 2-(diethylamino)ethyl chloride hydrochloride (5.0 eq.), K_2CO_3 (5.0 eq.), then add dry DMF, 0→65 °C, 3 h (34 %); (b) Fe (5 eq.), AcOH, 80 °C, 30 min, then K_2CO_3, (28 %).

This idea can be extended to other donor-acceptor pairs as we demonstrate in the case of 3- and 4-aminophthalimide as donor and 1-oxyl-2,2,6,6-tetramethyl piperidine as acceptor. We chose 3- and 4-aminophthalimides because beyond their fluorescence properties they exhibited the ability of recognizing CG Watson-Crick base pair, as reported very recently.[18] Reaction of 3-nitrophthalic anhydride **15** or 4-nitrophthalic anhydride **16** with 4-amino-1-oxyl-2,2,6,6-tetramethyl piperidine **17** in $CHCl_3$ followed by cyclocondensation of amides (not shown) in toluene in the presence of Et_3N gave paramagnetic 3-nitrophthalimide **18** and 4-nitrophthalimide **19** derivatives. Selective reduction of nitro compounds **18, 19** with ammonium formate in MeOH in the presence of Pd/C[19] yielded compounds **20a** and **21a**, respectively, although re-oxidation of hydroxylamines with PbO_2/O_2 was required. Treatment of paramagnetic 3- and 4-nitrophthalimide derivatives with Fe powder in AcOH resulted in simultaneous reduction of both nitro and nitroxide groups to the corresponding amines in order to give compounds **20b** and **21b**. In the case of compounds **20b** and **20a** we also got very good amine versus nitroxide fluorescence intensity ratio (Scheme 5).



Scheme 5

Reagents and conditions: (a) **15** or **16** (1 eq.) and **17** (1 eq.), $CHCl_3$, r.t., 1h, then evaporate, toluene, Et_3N (3 eq), 8 h, 110 °C (30-44 %); (b) HCO_2NH_4 (6 eq.), Pd/C (cat.), MeOH, 40 °C, 2 h, then PbO_2 (cat.)/O_2, 15 min., (28-36 %); (c) Fe (5 eq.), AcOH, 50 °C, 30 min, then K_2CO_3, (33-51 %).

In conclusion, the best double sensor reagents among the ones we tested were those with a minimal acceptor–donor distance. However, side-chains may prove to be advantageous in further biological applications.

Experimental Section

General Procedures. Melting points were determined with Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on Fisons EA 1110 CHNS elemental analyser. Mass spectra were recorded on a VG TRIO-2 instrument in EI mode (70 eV, direct inlet) or with thermospray technique (TSP). Samples were analyzed in bypass mode. 10 μL of the sample solution in MeOH was introduced via the thermospray interface. The mobile phase was MeOH/H_2O (1:1) containing 0.1 M NH_4OAc. The capillary tip temperature was 230 °C, the electrode voltage was 180 V and source temperature 210 °C. The ESR spectra were obtained from 10^{-5} molar solution ($CHCl_3$), using Bruker ECS-106 spectrometer. All monoradicals exhibit three equidistant lines with a_N = 14.3-14.8 G. Fluorescence emission spectra were recorded with Quanta Master QM-1 (Photon Technology International Inc.) using 345 nm excitation wavelength and 1 nm excitation and emission slits. The fluorescence emission peak intensities were normalised by concentration. Flash column chromatography was performed on Merck Kieselgel 60 (0.040-0.063 mm). TLC was carried out on commercially prepared plates (20x20x0.02 cm) coated with Merck Kieselgel GF_{254}. Compounds **9, 15, 16, 17** were purchased from Aldrich. Compounds **1**,[1] **5**,[12] **11**[17] were prepared according to published procedures.

Synthesis of 1-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)pipe-razine radical 3 and bis-[1,4-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)] piperazine biradical (4). A mixture of piperazine (2.58 g, 30.0 mmol), K_2CO_3 (1.38 g, 10.0 mmol) and allylic bromide **2** (2.33 g, 10.0 mmol) in $CHCl_3$ (30 mL) was stirred and refluxed for 1 h. After cooling the mixture was filtered, the organic phase was washed with brine (20 mL), dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash chromatography (hexane/EtOAc) to give compound **4** 720 mg (37%), mp 150-154 °C as a second band (the first band is compound **2**). Calc. for $C_{22}H_{38}N_4O_2$ C 67.66, H 9.81, N 14.34; found C 67.80, H 9.95, N 14.40; MS (EI) m/z: 390 (M^+, 23), 375 (10), 122 (94), 41 (100). Elution with $CHCl_3/Et_2O$ gave compound **3** 952mg (40%) as a third band, thick oil. Calc. for $C_{13}H_{24}N_3O$ C 65.51, H 10.15, N 17.63; found: C 65.60, H 10.20, N 17.50; MS (EI) m/z: 238 (M^+, 12), 224 (6), 138 (28), 99 (100).

Synthesis of *N*-{2-[4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl) piperazin-1-yl]ethyl}phthalimide (6). To a solution of compound **5** (2.59 g , 10.0 mmol) and K_2CO_3 (1.38 g, 10.0 mmol) in $CHCl_3$ (30 mL) allylic bromide **2** (2.33 g, 10.0 mmol) was added and the mixture was stirred and refluxed for 2h. After cooling the mixture was filtered, washed with brine (10 mL), dried ($MgSO_4$), evaporated. Flash column chromatography ($CHCl_3/Et_2O$) afforded compound **6** 3.04 g (74%), mp 127-132 °C. Calc. for $C_{23}H_{31}N_4O_3$ C 67.13, H 7.59, N 13.61; found: C 67.20, H 7.70, N 13.75 MS (TSP) m/z: 412 $(M+H)^+$.

Synthesis of 1-Oxyl-3-[4-(2-aminoethyl)piperazin-1-ylmethyl]-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole (7). To a solution of compound **6** (3.0 g, 7.30 mmol) in EtOH (10 mL) 40 % $MeNH_2$ solution in EtOH was (10 mL) added and the mixture was allowed to stay at rt for 12 h. The solvent was evaporated off, the residue was dissolved in $CHCl_3$ (25 mL), washed with brine, dried ($MgSO_4$), filtered, evaporated and the residue was purified by flash column chromatography ($CHCl_3$/MeOH) to give the title compound **7** as a thick yellow oil 676 mg (33%). Calc. for $C_{15}H_{29}N_4O$ C 64.02, H 10.39, N 19.91; found: C 64.15, H 10.35, N 20.05. MS (EI) m/z: 281 (M^+, 18), 251 (45), 221 (78), 99 (100).

Synthesis of paramagnetic dansyl derivatives 10a, 11a, 12a. General procedure

To a stirred solution of amines **3** or **7** or **8** (3.0 mmol) and triethylamine 333 mg (3.3 mmol) in CH_2Cl_2 (30 mL) dansyl chloride **9** (809 mg, 3.0 mmol) dissolved in dry CH_2Cl_2 (10 mL) was added. After stirring the reaction mixture for 4 h at r.t., the organic phase was washed with brine, separated, dried ($MgSO_4$), filtered, evaporated and the residue was purified by flash column chromatography (hexane/EtOAc, $Et_2O/CHCl_3$) to give compounds **10a** or **11a** or **12a** as yellow-green solids or oils.

***N*-[4-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)piperazin-1-yl]-(5-dimethylamino)-1-naphthalenesulfonamide radical (10a).** 734 mg (52 %), mp 143-145 °C, calc. for $C_{25}H_{35}N_4O_3S$: C 63.66, H 7.49, N 11.89, S 6.78; found: C 63.75, H 7.65, N 12.00, S 6.95; MS (EI) m/z: 471 (M^+, 3), 441 (4), 332 (10), 43 (100).

***N*-{2-[4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)piperazin-1-yl]ethyl}-(5-dimethylamino)-1-naphthalenesulfonamide radical (11a).** 894 mg (58 %), mp 62-65 °C, calc. for $C_{27}H_{40}N_5O_3S$: C 63.00, H 7.84, N 13.61, S 6.22; found: C 63.15, H 7.70, N 13.85, S 6.35; MS (EI) m/z: 514 (M^+, 14), 499 (16), 236 (100), 136 (86).

N-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)-5-dimethylamino-1-naphtalenesulfonamide radical (12a). 543 mg (45%), mp 128-129 °C, calc. for $C_{21}H_{28}O_3N_3S$: C 62.66, H 7.01, N 10.44, S 7.96; found: C 62.50, H 7.20, N 10.55, S 7.80 MS (EI) m/z: 402 (M^+, 16), 372 (10), 170 (42), 110 (100).

Synthesis of diamagnetic dansyl and phthalimidyl derivatives. General procedure

To a solution of nitroxide **10a** or **11a** or **12a** or **14a** or **18** or **19** (2.0 mmol) in glacial acetic acid (10 mL) Fe powder (560 mg, 10.0 mmol) or (1.12 g, 20.0 mmol in case of **18** or **19**) was added and the mixture was warmed up to 50 °C until the reaction started and the reaction was stirred for 30 min at r.t. After diluting water (30 mL), the solution was decanted from iron residue, and the solution was made alkaline (pH = 9) by adding solid K_2CO_3. The reaction mixture was filtered off, the filtrate was extracted with $CHCl_3$ (3 x 40 mL), and the separated organic phases were combined, dried ($MgSO_4$), filtered and evaporated in a vacuum. Flash column chromatography with $CHCl_3$/MeOH as eluent afforded the title amines **10b** or **11b** or **12b** or **14b** or **20b** or **21b** as yellow-green solids or oils.

***N*-[4-(2,2,5,5-Tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)piperazin-1-yl]-(5-dimethylamino)-1-naphthalenesulfonamide (10b).** 355 mg (39%), mp 67-72 °C, calc. for $C_{25}H_{36}N_4O_2S$: C 65.76, H 7.95, N 12.27, S 7.02; found: C 65.56, H 7.70, N 12.45, S 7.35, MS (EI) m/z: 456 (M^+, 9), 441 (35), 332 (25), 122 (100).

***N*-{2-[4-(2,2,5,5-Tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)piperazin-1-yl]ethyl}-(5-dimethylamino)-1-naphthalenesulfonamide (11b).** 320 mg (32%), mp 205-208, °C calc. for $C_{27}H_{41}N_5O_2S$: C 64.90, H 8.27, N 14.01, S 6.42; found: C 64.70, H 8.40, N 14.20, S 6.25, MS (EI) m/z: 499 (M^+, 12), 396 (10), 236 (76), 136 (100).

***N*-(2,2,5,5-Tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)-5-dimethylamino-1-naphtalenesulfonamide (12b).** 372 mg (48%), mp 125-126, °C, calc. for $C_{21}H_{29}O_2N_3S$: C 65.09, H 7.54, N 10.84, S 8.27; found: C 65.25, H 7.40, N 10.95, S 8.40. MS (EI) m/z: 387 (M^+, 3), 372 (19), 122 (58), 110 (100).

***N*-[(2-Diethylaminoethyl)-N-(2,2,5,5-tetramethylpyrrolidine-3-yl)]-5-dimethylamino-1-naphtalenesulfonamide (14b).** 266 mg (28%), yellow oil, calc. for $C_{26}H_{42}N_4O_2S$: C 65.79, H 8.92, N 11.80, S 6.75; found: C 65.65, H 9.05, N 11.70, S 6.90; MS (EI) m/z: 474 (M^+, 1), 170 (7), 124 (18), 86 (100).

3-Amino-*N*-(2,2,6,6-tetramethylpiperidine-4-yl)phthalimide (20b). 198 mg (33%), mp 169-171 °C, calc. for $C_{17}H_{23}N_3O_2$: C 67.75, H 7.69, N 13.94; found C 67.90, H 7.85, N 14.10; MS (TSP) m/z: 302 $(M+H)^+$.

4-Amino-*N*-(2,2,6,6-tetramethylpiperidine-4-yl)phthalimide (21b). 307 mg (51 %), mp 241-243 °C, calc. for $C_{17}H_{23}N_3O_2$: C 67.75, H 7.69, N 13.94; found C 67.70, H 7.60, N 13.90; MS (TSP) m/z: 302 $(M+H)^+$.

Synthesis of *N*-[(2-diethylaminoethyl)-*N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl)]-5-dimethylamino-1-naphthalenesulfonamide radical (14a). To a stirred solution of compound **13** (390 mg, 1.0 mmol) in dry THF (10 mL) NaH (48 mg, 2.0 mmol) was added in one portion and the suspension was further stirred for 15 min. at 0 °C under N_2. In an Erlenmeyer flask to a stirred solution of 2-(diethylamino)ethyl chloride hydrochloride (860 mg, 5.0 mmol) in water (15 mL) solid K_2CO_3 (690 mg, 5.0 mmol) was added at 0 °C and the mixture was stirred for 2 min., then extracted with benzene (2 x 10 ml). The organic phase was dried ($MgSO_4$), filtered, and the solution was added to mixture of the above sulfonamide – Na salt suspension at 0 °C. After adding dry dimethylformamide (20 mL), the mixture was allowed to warm to room temperature and then refluxed for 3h. After cooling, EtOH (1 mL) was added for destruction of the remaining NaH, then solvents were evaporated off, the residue was dissolved in $CHCl_3$ (30 mL), washed with brine (20 mL), dried ($MgSO_4$), filtered, evaporated. The residue was purified by flash column chromatography to give the title compound as a yellow oil 166 mg (34 %). Calc. for $C_{26}H_{41}N_4O_3S$: C 63.77, H 8.44, N 11.44, S 6.55; found: 63.80, H 8.55, N 11.60, S 6.45; MS (EI) m/z: 489 (M^+, 1), 170 (6), 124 (13), 86 (100).

Synthesis of 3-nitro-*N*-(1-oxyl-2,2,6,6-tetramethylpiperidine-4-yl)phthalimide radical (18) and 4-nitro-*N*-(1-oxyl-2,2,6,6-tetramethylpiperidine-4-yl)phthalimide radical (19). In a round-bottomed flask to a solution of amine **17** (855 mg, 5.0 mmol) in $CHCl_3$ (20 mL), 3-nitrophthalic anhydride **15** (965 mg, 5.0 mmol) or 4-nitrophthalic anhydride (**16**) (965 mg, 5.0 mmol) was added in one portion and after stirring at room temperature for 1 h the solvents were evaporated off. The gummy residue was suspended in toluene (50 mL), Et_3N (1.0 g, 10.0 mmol) was added and the mixture was heated at 110 °C under continuous removal of water with Dean-Stark apparatus. After 4 h further Et_3N (500 mg, 5.0 mmol) was added and the mixture was heated for further 4 h. After cooling, solvents were evaporated off and the residue was dissolved in $CHCl_3$ (30 mL), washed with brine (10 mL), dried ($MgSO_4$), filtered, evaporated. After purification with flash column chromatography (hexane/EtOAc) we got the title compounds, **18** 761 mg (44 %), mp 204-206 °C. Calc. for $C_{17}H_{20}N_3O_5$: C 58.95, H 5.82, N 12.13; found: C 58.80, H 6.00 N 12.15; MS (EI) m/z: 346 (M^+, 6), 332 (18), 316 (8), 41 (100), or **19** 519 mg (30 %), mp 242-244 °C. Calc. for $C_{17}H_{20}N_3O_5$: C 58.95, H 5.82, N 12.13; found: C 58.70, H 6.10 N 12.30; MS (EI) m/z: 346 (M^+, 7), 332 (22), 316 (12), 41 (100) as brown-red solids.

Synthesis of 3-amino-*N*-(1-oxyl-2,2,6,6-tetramethylpiperidine-4-yl)phthalimide radical (20a) and 4-amino-*N*-(1-oxyl-2,2,6,6-tetramethylpiperidine-4-yl) phthalimide radical (21a). To a stirred solution of compound 18 or 19 (692 mg, 2.0 mmol) and HCO_2NH_4 (756 mg, 12.0 mmol) in MeOH (30 mL) Pd/C (100 mg, 10%) was added in one portion at 40 °C and the mixture was further stirred for 2 h at this temperature under N_2. The mixture was filtered through Celite, the filter cake was washed with hot MeOH (2 x 10 mL) and the combined filtrates were evaporated to dryness. The residue was dissolved in $CHCl_3$ (40 mL), washed with brine, dried ($MgSO_4$), then PbO_2 (239 mg, 1.0 mmol) was added and O_2 was bubbled through mixture for 15 min. After filtration the mixture was evaporated and after flash column chromatography we got the title compounds as yellow solids 20a 176 mg (28%), mp 227-232 °C, calc. for $C_{17}H_{22}N_3O_3$: C 64.54, H 7.01, N 13.28; found C 64.70, H 7.00 N 13.10. MS (EI) m/z: 316 (M^+, 24), 302 (52), 175 (90),

124 (100) or compound 21a 227 mg (36%), mp 178-180 °C, calc. for $C_{17}H_{22}N_3O_3$: C 64.54, H 7.01, N 13.28; found C 64.40, H 7.10, N 13.15. MS (EI) m/z: 316 (M^+, 15), 302 (53), 175 (72), 124 (100).

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