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-phases5. 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 DanePy1 1b, which is readily oxidized by ROS to nitroxide 1a (Scheme 1).
Similar oxidation reaction was also observed earlier in vivo. The diethylaminoethyl side-chain in DanePy 1b ensures water solubility and penetration into chloroplasts. 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 \( 2 \) in CHCl\(_3\) in the presence of K\(_2\)CO\(_3\) to give mixture of the monoalkylated compound \( 3 \) and the dialkylated compound \( 4 \). The \( N-[2-(1-piperazinyl)ethyl]phthalimide \( 5 \) 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\(_2\)CO\(_3\) (1 eq.), CHCl\(_3\), reflux, 1 h, \( 3 \) (40 %), \( 4 \) (37 %); (b) \( 5 \) (1 eq.), K\(_2\)CO\(_3\) (1 eq.), CHCl\(_3\), reflux, 2 h, (74 %); (c) CH\(_3\)NH\(_2\), EtOH, r.t. 12 h, (33 %).
Amines 3 and 7 as well as allylic amine 8\textsuperscript{14} were treated with 5-dimethylamino-1-naphtalesulfonyl chloride 9 in CH\textsubscript{2}Cl\textsubscript{2} in the presence of triethylamine to get the paramagnetic dansyl derivatives 10\textsubscript{a}, 11\textsubscript{a} and 12\textsubscript{a}. The paramagnetic compounds were reduced to sterically hindered amines 10\textsubscript{b}, 11\textsubscript{b} and 12\textsubscript{b} by Fe powder in glacial acetic acid\textsuperscript{15} (Scheme 3).

Scheme 3

**Reagents and conditions:** (a) 8 (1.0 eq.) CH\textsubscript{2}Cl\textsubscript{2}, Et\textsubscript{3}N (1.1 eq.), r.t., 4 h, (45-58 %); (b) Fe (5 eq.), AcOH, 80 °C, 30 min, then K\textsubscript{2}CO\textsubscript{3}, (32-48%).

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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>a (NO) emission max (nm)</th>
<th>b (NH) emission max (nm)</th>
<th>Peak intensity ratio (b/a)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>530</td>
<td>551</td>
<td>5.56</td>
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<tr>
<td>10</td>
<td>560</td>
<td>556</td>
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<td>11</td>
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<tr>
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<td>546</td>
<td>503</td>
<td>25.00</td>
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<tr>
<td>21\textsuperscript{*}</td>
<td>532</td>
<td>528</td>
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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.\textsuperscript{16} To get better double sensor molecules, i.e. to achieve better ratio of emission maxima of amine versus nitroxide we investigated compound 13.\textsuperscript{17} The sodium salt of 13 was alkylationed in dimethylformamide/THF mixture with freshly released 2-(diethylamino)ethyl chloride to give compound 14\textsubscript{a}, which was then reduced to 14\textsubscript{b} with Fe powder in AcOH (Scheme 4). Fluorescence emission maxima ratio of compounds 14\textsubscript{b} and 14\textsubscript{a} were the highest among the investigated dansyl derivatives (Table 1).

\textbf{Scheme 4}

\textbf{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\textsubscript{2}CO\textsubscript{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\textsubscript{2}CO\textsubscript{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.\textsuperscript{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\textsubscript{3} followed by cyclocondensation of amides (not shown) in toluene in the presence of Et\textsubscript{3}N 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\textsuperscript{19} yielded compounds 20\textsubscript{a} and 21\textsubscript{a}, respectively, although re-oxidation of hydroxylamines with PbO\textsubscript{2}/O\textsubscript{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 20\textsubscript{b} and 21\textsubscript{b}. In the case of compounds 20\textsubscript{b} and 20\textsubscript{a} we also got very good amine versus nitroxide fluorescence intensity ratio (Scheme 5).
Reagents and conditions: (a) 15 or 16 (1 eq.) and 17 (1 eq.), CHCl₃, r.t., 1h, then evaporate, toluene, Et₃N (3 eq), 8 h, 110 °C (30-44 %); (b) HCO₂NH₄ (6 eq.), Pd/C (cat.), MeOH, 40 °C, 2 h, then PbO₂ (cat.)/O₂, 15 min., (28-36 %); (c) Fe (5 eq.), AcOH, 50 °C, 30 min, then K₂CO₃, (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/H₂O (1:1) containing 0.1 M NH₄OAc. 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 (CHCl₃), 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 GF₂₅₄. Compounds 9, 15, 16, 17 were purchased from Aldrich. Compounds 1₁, 5₁₂ 11₁⁷ were prepared according to published procedures.
Synthesis of 1-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethyl)piperazine radical 3 and bis-[1,4-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethyl)] piperazine biradical (4). A mixture of piperazine (2.58 g, 30.0 mmol), K$_2$CO$_3$ (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$_4$O$_2$ 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$_2$O gave compound 3 952mg (40%) as a third band, thick oil. Calc. for C$_{13}$H$_{24}$N$_3$O 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-1H-pyrrol-3-ylmethyl)piperazin-1-yl]ethyl}phthalimide (6). To a solution of compound 5 (2.59 g, 10.0 mmol) and K$_2$CO$_3$ (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$_2$O) afforded compound 6 3.04 g (74%), mp 127-132 °C. Calc. for C$_{23}$H$_{31}$N$_4$O$_3$ 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-1H-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$_4$O 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$_2$Cl$_2$ (30 mL) dansyl chloride 9 (809 mg, 3.0 mmol) dissolved in dry CH$_2$Cl$_2$ (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$_2$O/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-1H-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$_4$O$_3$S: 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-(1-oxy-2,2,5,5-tetramethyl-2,5-dihydro-1H-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_{5}O_{3}S: 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-oxy-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethyl)-5-dimethylamino-1-naphtalenesulfonamide radical (12a). 543 mg (45%), mp 128-129 °C, calc. for C_{21}H_{28}O_{3}N_{3}S: 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_{2}CO_{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-1H-pyrrol-3-ylmethyl)piperazin-1-yl][ethyl]}-(5-dimethylamino)-1-naphthalenesulfonamide (10b). 355 mg (39%), mp 67-72 °C, calc. for C_{25}H_{36}N_{4}O_{2}S: 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-tetramethylpyrrolidine-3-yl)]ethyl}-(5-dimethylamino)-1-naphthalenesulfonamide (11b). 320 mg (32%), mp 205-208 °C, calc. for C_{27}H_{41}N_{5}O_{2}S: 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-1H-pyrrol-3-ylmethyl)-5-dimethylamino-1-naphtalenesulfonamide (12b). 372 mg (48%), mp 125-126 °C, calc. for C_{21}H_{29}O_{2}N_{3}S: 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-(2,2,6,6-tetramethylpiperidine-4-yl)]-5-dimethylamino-1-naphthalenesulfonamide (14b). 266 mg (28%), yellow oil, calc. for C_{35}H_{44}N_{4}O_{2}S: 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_{3}O_{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_{3}O_{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)^{+}. 

ISSN 1424-6376 Page 118 ©ARKAT USA, Inc
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 N₂. In an Erlenmeyer flask to a stirred solution of 2-(diethylamino)ethyl chloride hydrochloride (860 mg, 5.0 mmol) in water (15 mL) solid K₂CO₃ (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 (MgSO₄), 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 CHCl₃ (30 mL), washed with brine (20 mL), dried (MgSO₄), filtered, evaporated. The residue was purified by flash column chromatography to give the title compound as a yellow oil 166 mg (34 %). Calc. for C₂₆H₄₁N₄O₃S: C 63.77, H 8.44, N 11.44, S 6.55; found: C 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 CHCl₃ (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), Et₃N (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 Et₃N (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 CHCl₃ (30 mL), washed with brine (10 mL), dried (MgSO₄), 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 C₁₇H₂₀N₃O₅: 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 C₁₇H₂₀N₃O₅: 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 HCO₂NH₄ (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 N₂. 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 CHCl₃ (40 mL), washed with brine, dried (MgSO₄), then PbO₂ (239 mg, 1.0 mmol) was added and O₂ 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 C₁₇H₂₂N₃O₃: 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_{3}O_{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)

Acknowledgements

This work was supported by grants from the Hungarian National Research Foundation (OTKA T 034307). The authors wish to express thanks to M. Balog for technical assistance and M. Szabó (ICN Hungary Ltd.) for mass spectral measurements.

References and Notes