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New phthalimide-methionine dyad-based fluorescence probes for reactive oxygen species: Singlet oxygen, hydrogen peroxide, and hypochlorite

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Abstract

Different reactive oxygen species were detected by the molecular probes **1-3** that were composed of the phthalimide fluorophore as reporter and a methionine-derived thioether side-chain as receptor part. The sulfoxides that were formed as the primary oxidation products show strong fluorescence in the blue-green (430-540 nm) spectral region. Self-sensitized oxidation by singlet oxygen is in general inefficient indicating rapid electron-transfer quenching of the excited probe molecules. With hydrogen peroxide as thermal oxidant conversion to the sulfoxides is slow but can be accelerated by addition of titanium(IV) catalysts, whereas hypochlorite as oxidant behaves much more reactive even under uncatalyzed conditions. Singlet oxygen that is generated by energy transfer from the photosensitizer Rose Bengal was detected by sensor **1a** with rate constants of $>10^7 M^{-1}\ s^{-1}$, a typical rate constant for the oxidation of thioethers to sulfoxides.

1 | INTRODUCTION

Reactive oxygen species (ROS) constitute a class of biologically highly relevant molecules that exhibit diverse cellular effects and are formed in substantial amounts under the conditions of oxidative stress.[1] The 4 most important ROS are hydroxyl and the superoxide radicals, hydrogen peroxide, and singlet molecular oxygen ($^1\Delta_g$ $^1O_2$), species with distinct different intrinsic reactivities. Singlet oxygen is formed by several thermal peroxide decay processes or by photosensitization.[2] The later route is the basis for photodynamic therapy, one of the direct tumor therapeutic tools with high future potential.[3] The detection of ROS in low concentration with high spatial resolution is a challenging task not only because of the low concentrations of ROS in cellular media but also because of similar reaction profile of the species involved.[4] For singlet oxygen, the direct detection by the weak 1270 nm phosphorescence has been intensively studied and used for intracellular detection and lifetime determination.[5] This photophysical tool is highly specific for the electronically excited state of molecular oxygen but technically demanding due to the short lifetimes of singlet oxygen in aqueous media (roughly 3 μs) and the low phosphorescence quantum yields.

Several chemical probes were developed that are based on the high cycloaddition reactivity of $^1O_2$ with anthracenes (Figure 1). The commercial available singlet oxygen sensor green (**A**, SOSG, λ_{em}^{ox} = 530 nm)[7] and the recently developed Aarhus sensor green (**B**, ASG, λ_{em}^{ox} = 537 nm)[8] probes are based on modified fluoresceine dyes coupled to fluorescence-quenching anthracene group. These sensors are highly sensitive for $^1O_2$ because no other oxidant is known to convert arenes to endoperoxides. A related silicon-containing rhodamine dye was recently described for far-red detection of $^1O_2$ in cells (λ_{em}^{ox} = 680 nm).[9] Prefluorescent dyes that are sensitive towards cycloadditions or oxidative double bond cleavage are compounds **C** (λ_{em}^{ox} = 400 nm)[10] and **D** (λ_{em} = 420 nm).[11] These fluorescence-switching principles were also used in tetracene-containing polymers that respond to $^1O_2$.[12] All

Dedicated to Professor Waldemar Adam on occasion of his 80th birthday.

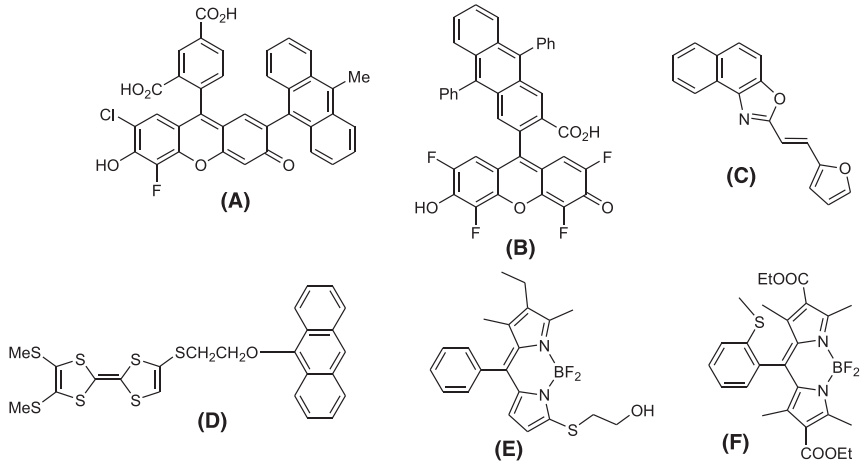


FIGURE 1 Molecular probes for $^1O_2$ **A-D**, and hypochlorite **E,F** based on the oxidation of anthracenes, furans, thiofulvenes, and thioethers

probes having these reporting properties are, however, structurally unrelated to biologically relevant structures and the singlet oxygen reactions that serve as sensoring processes are also not occurring under physiological conditions. Furthermore, the singlet oxygen detection is irreversible under the reaction conditions. In several cases, the product from probe oxygenation itself is a singlet oxygen sensitizer, clearly a severe disadvantage.[13] Another target structure that can be addressed by reactive oxygen species are thioethers that are known to be good donors for excited state electron transfer and thus efficient fluorescence quenchers. Probes (**E**,**F**) for hypochlorite were published that use the BODIPY chromophore and thioether-containing side-chains.[14,15] In this paper, we describe our probe design using a combination of the highly fluorescent 4,5-dimethoxy- and 3-amidophthalimides, respectively, and thioether-containing amino acids and dipeptides (Figure 2).

2 | EXPERIMENTAL SECTION

2.1 | *meta*-Meconin (5)

Ten grams (54.89 mmol, 1 eq.) veratric acid (**4**) and 15.33 g (0.51 mol, 9.3 eq.) of paraformaldehyde were suspended in 330 ml of hydrochloric acid solution (37%) and the mixture was heated at 85°C until the suspension became a clear brown solution.[16] The reaction mixture was cooled with an ice bath and neutralized with aqueous ammonia solution

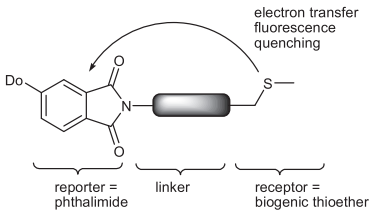


FIGURE 2 Our molecular reporter-spacer-receptor concept for reactive oxygen species detection

(25%). The beige precipitate was collected by suction filtration, washed with water several times, and then recrystallized from ethanol to yield (**2**) as fine, colorless crystals (7.87 g, 43.2 mmol, 74%). m.p. 155°C. R_f (cyclohexane/EtOAc, 1:1) = 0.32. ^{1}H-NMR (300 MHz, DMSO): δ [ppm] = 7.25 (s, 1H), 7.21 (s, 1H), 5.26 (s, 2H), 3.87 (s, 3H), 3.83 (s, 3H). ^{13}C-NMR (75 MHz, DMSO): δ [ppm] = 171.3, 155.0, 150.4, 142.20, 116.8, 106.1, 105.2, 69.6, 56.30. GC-MS: τ_R: 13.32 min; *m/z*: [%] 194 [M] (35%), 166 (11%), 165 (100%), 137 (8%), 122 (8%), 95 (18%), 92 (8%), 77 (23%), 74 (7%), 51 (17%). FT-IR: [cm^{-1}] = 3863 (m), 3833 (m), 3778 (w), 3703 (w), 3686 (w), 2934 (w), 2373 (w), 2337 (w), 1768 (s), 1748 (s), 1714 (w), 1603 (w), 1503 (m), 1471 (m), 1451 (w), 1345 (s), 1256 (m), 1225 (m), 1193 (w), 1124 (m), 995 (m), 975 (w), 861 (m).

2.2 | 4,5-Dimethoxyphthalic acid (6)

Thirty grams (154.5 mmol, 1 eq.) of *m*-Meconin (**5**) was suspended in 750 ml water.[14] Then 7% aqueous NaOH (350 ml) and 26.87 g (170 mmol, 1.1 eq.) $KMnO_4$ were added and stirred 4 days at room temperature. The reaction mixture was filtered through celite and the filtrate was acidified to pH 1 with concentrated hydrochloric acid solution (37%) and extracted with EtOAc (3 × 400 ml). The combined organic phases were extracted with brine, dried over $MgSO_4$, and the solvent was removed under reduce pressure. A total of 33 g (145.9 mmol, 94%) of 4,5-dimethoxyphthalic acid (**3**) optained as a colorless solid. m.p: 176°C. ^{1}H-NMR (300 MHz, DMSO): δ [ppm] = 7.18 (s, 2H), 3.83 (s, 6H). ^{13}C-NMR (75 MHz, DMSO): δ [ppm] = 168.8, 150.3, 126.2, 111.70, 56.3.

2.3 | 4,5-Dimethoxyphtalic acid anhydride (7)

Five grams (22.1 mmol) of the acid **6** were suspended in 15 ml acetic anhydride and stirred for 2 hours under reflux.[14] After cooling, the solvent was removed under

reduced pressure and 4.6 g (22.1 mmol, 99%) of the anhydride **7** were obtained as a beige solid. m.p: 164°C. ^{1}H-NMR (300 MHz, DMSO): δ [ppm] = 7.18 (s, 2H), 3.82 (s, 6H). ^{13}C-NMR (75 MHz, DMSO): δ [ppm] = 165.0, 157.4, 126.5, 111.7, 56.2. GC-MS (EtOAc, EI): τ_R: 13.21 min; *m/z*: [%] = 208 [M] (17%), 165 (4%), 164 (66%), 137 (7%), 136 (100%), 121 (12%), 93 (39%), 62 (9%), 50 (27%). FT-IR: [cm^{-1}] = 3900 (w), 3851(w), 3749 (w), 3734 (w), 3689 (w), 3648 (w), 3586 (w), 3566 (w), 2925 (w), 2853 (w), 2368 (w), 2337 (w), 1868 (w), 1843 (m), 1772 (s), 1732 (w), 1716 (w), 1589 (m), 1506 (m), 1456 (w), 1423 (w), 1321 (s), 1231 (m), 1134 (w), 1090 (m), 983 (m), 888 (s), 817 (m), 817 (m), 700 (w).

2.4 | Synthesis of the dimethoxyphthalimides 1a, 1b, 8

A mixture of 1 eq. of the phthalic anhydride (**7**) and 1.05 eq. of the amine was dissolved in acetic acid (0.05 mM) and stirred for 4 hours under reflux. After cooling, the reaction mixture was diluted with water and a precipitate was formed, which was collected by suction filtration.

2.5 | 5,6-Dimethoxy-2-(3-(methylthio)propyl) isoindoline-1,3-dione (1a)

Yield 1.2 g (4.1 mmol, 82%). Colorless solid. m.p. 179°C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.30 (s, 2H), 4.00 (s, 6H), 3.75 (t, *J* = 7.0 Hz, 2H), 2.53 (t, *J* = 7.4 Hz, 2H), 2.10 (s, 3H), 2.03-1.90 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ [ppm] = 168.5, 153.8, 125.5, 105.3, 56.6, 37.1, 31.4, 28.1, 15.4. GC-MS (EtOAc, EI): τ_R: 13.79 min; *m/z*: [%] = 295.26 [M] (22%), 248.19 (100%), 220.24 (70%), 207.17 (20%), 190.09 (24%), 177.17 (22%), 164.16 (32%), 136.19 (29%), 121.12 (34%), 93.13 (31%), 75.13 (38%), 61.05 (36%). FT-IR: [cm^{-1}] = 3081 (w), 1763 (w), 1733 (m), 1697 (m), 1598 (w), 1504 (m), 1475 (w), 1460 (w), 1422 (w), 1390 (s), 1339 (s), 1311 (s), 1278 (w), 1225 (m), 1210 (m), 1190 (m), 1163 (m), 1137 (m), 1131 (m), 1115 (m), 1104 (m), 1088 (m), 1027 (m), 1015 (m), 994 (m), 947 (w), 910 (w), 891 (w), 861 (w), 842 (m), 835 (m), 797 (s), 778 (w), 751 (s), 714 (w), 702 (w), 662 (w), 652 (w). UV (CH_3CN, 10^{-5} M): λ_{max} = 347 nm, ε (347 nm) = 1700 L mol^{-1} cm^{-1}.

2.6 | 2-(5,6-Dimethoxy-1,3-dioxoisoindolin-2-yl)-4-(methylthio)butanoic acid (1b)

Yield 7.25 g (21.4 mmol, 89%). Colorless solid. m.p. 236°C. ^{1}H-NMR (300 MHz, DMSO): δ [ppm] = 7.41 (s, 2H), 4.86 (t, *J* = 7.3 Hz, 1H), 3.93 (s, 6H), 2.39-2.30 (m, 4H), 2.01 (s, 3H). ^{13}C-NMR (300 MHz, DMSO): δ [ppm] = 171.0, 168.1, 154.1, 124.8, 106.2, 56.7, 50.8, 30.42, 28.0, 14.8. ESI-MS: 340 [M + H^+]. FT-IR: [cm^{-1}] = 3931 (w), 3902 (w), 3778(m), 3648 (m), 3566 (m), 3081 (w), 2923 (w), 2387 (w), 2337 (w), 1733 (m), 1697 (s), 1616 (m), 1506 (m), 1473 (m), 1419 (w), 1370 (s), 1311 (s), 1225 (m), 1163 (m), 1087 (m), 998 (m), 891 (w), 861 (w), 751 (s), 667 (w). UV (CH_3CN, 10^{-5} M): λ_{max} = 347 nm, ε (347 nm) = 2440 L mol^{-1} cm^{-1}.

2.7 | 2-(5,6-Dimethoxy-1,3-dioxoisoindolin-2-yl)acetic acid (8)

Yield 0.84 g (3.16 mmol, 44%). Colorless solid. m.p. 289.5°C. ^{1}H-NMR (300 MHz, DMSO) δ [ppm] = 7.43 (s, 2H), 4.26 (s, 2H), 3.93 (s, 6H). ^{13}C-NMR (75 MHz, DMSO) δ [ppm] = 169.6, 167.8, 154.3, 125.1, 106.3, 56.9. FT-IR: [cm^{-1}] = 3293 (w), 2330 (w), 1749 (m), 1710.0 (s), 1599 (m), 1506 (m), 1458 (w), 1423 (m), 1393 (m), 1306 (m), 1306 (m), 1289 (w), 1221 (m), 1190 (w), 1161 (m), 1120 (w), 1088 (m), 995 (s), 930 (w), 903 (m).

2.8 | Methyl 2-(2-(5,6-dimethoxy-1,3-dioxoisoindolin-2-yl)acetamido)-4-(methylthio) butanoate (3)

A solution of 8 (0.53 g, 2 mmol) in dry THF (4 ml) was cooled to –25°C. A total of 0.28 ml (2 mmol, 1 eq.) of NEt_3 and 0.28 ml (2.2 mmol, 1.1 eq.) of isobutylchloroformate were added and stirred 30 minutes at –25°C. A total of 0.40 g (2 mmol, 1 eq.) *L*-methionine methyl ester hydrochloride were suspended in 4 ml chloroform and added to reaction and stirred 90 minutes at –25°C. The reaction was diluted with 5% $NaHCO_3$-solution and extracted. The organic phase was washed with 10% HCl-solution, brine and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and 0.82 g (1.99 mmol, quant.) of **3** were observed as colorless solid. m.p. 227°C. ^{1}H-NMR (300 MHz, DMSO) δ [ppm] = 8.67 (d, *J* = 7.7 Hz, 1H; NH), 7.41 (s, 2H), 4.39 (m, 1H), 4.21 (d, *J* = 1.7 Hz, 2H), 3.93 (s, 6H), 3.64 (s, 3H), 2.50 (s, 2H), 2.04 (s, 3H), 0.91 (d, *J* = 6.7 Hz, 2H), 0.83 (dd, *J* = 9.3, 6.7 Hz, 2H). ^{13}C-NMR (75 MHz, DMSO) δ [ppm] = 172.4, 167.9, 167.1, 154.1, 125.3, 106.2, 75.9, 56.9, 52.5, 51.4, 31.1, 29.8, 15.0. FT-IR: [cm^{-1}] = 3294 (w), 2947 (w), 2358 (w), 1747 (m), 1716 (s), 1705 (s), 1662 (w), 1597 (w), 1558 (w), 1506 (w), 1474 (w), 1463 (w), 1417 (s), 1394 (m), 1381 (w), 1310 (s), 1224 (m), 1211 (m), 1116 (w), 1090 (m), 1000 (s), 926 (w), 891 (w), 870 (w). HR-MS: [M + H]$^+$: 411.12205 (calc.), 411.12191 (measured). Elemental analysis: calc.: 52.67% C, 5.40% H, 6.83 % N, measured: 52.66% C, 5.86% H, 5.77% N. UV (CH_3CN, 10^{-5} M): λ_{max} = 345 nm, ε (345 nm) = 1940 L mol^{-1} cm^{-1}.

2.9 | 4-Amino-2-butylisoindoline-1,3-dione (10)

According to the procedure described above for the synthesis of phthalimides, 7.2 g (29 mmol, 94%) of 2-butyl-4-nitroisoindoline-1,3-dione was obtained as colorless solid. m.p. 72°C. ^{1}H-NMR (300 MHz, DMSO) δ = 8.26 (d, *J* = 8.0 Hz, 1H), 8.15 (d, *J* = 7.3 Hz, 1H), 8.04 (t, *J* = 7.8 Hz, 1H), 3.57 (t, *J* = 7.1 Hz, 2H), 1.63-1.50 (m, 2H), 1.36-1.23 (m, 2H), 0.89 (t, *J* = 7.3 Hz, 3H). ^{13}C-NMR (75 MHz, DMSO) δ = 166.5, 163.8, 144.7, 136.5, 134.0, 128.6, 127.2, 123.5, 38.1, 30.2, 19.9, 13.9. To a solution of 5 g (20.1 mmol) of the nitro compound in 300 ml ethanol was added Pd/C 10% and saturated with hydrogen gas. This mixture was stirred under hydrogen atmosphere at room temperature overnight. Then this reaction mixture was filtered through celite to remove the catalyst. Next, the filtrate was concentrated in vacuum to get 4.37 g (20 mmol, 99%) of **10** as a green solid. m.p. 72°C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.40 (t, *J* = 7.8 Hz, 1H), 7.13 (d, *J* = 7.1 Hz, 1H), 6.85 (d, *J* = 8.3 Hz, 1H), 3.63 (t, *J* = 7.2 Hz, 2H), 1.69-1.57 (m, 2H), 1.43-1.32 (m, 2H), 0.94 (t, *J* = 7.3 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 170.4, 168.8, 145.1, 135.0, 132.9, 120.9, 112.6, 111.4, 37.4, 30.7, 20.1, 13.7.

2.10 | *N*-(2-Butyl-1,3-dioxoisoindolin-4-yl)-3-(methylthio)propanamide (2)

A total of 1.34 g (10 mmol) of methyl 3-(methylthio) propanoate was dissolved in 25 ml methanol. About 0.8 g (20 mmol) of NaOH dissolved in 10 ml water was added to the alcoholic solution and stirred for 1 hour at 65°C. Next, the solvent was removed under reduced pressure and the residue was acidified with hydrochloric acid to pH 1. This mixture was extracted 3 times with EtOAc, the organic phases were washed with brine and dried over $MgSO_4$. The solvent was removed under reduced pressure to obtain 1.19 g (9.9 mmol, 99%) of the acid as a colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$) δ [ppm] = 2.77-2.70 (m, 2H), 2.68-2.61 (m, 2H), 2.10 (s, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ [ppm] = 178.2, 34.2, 28.6, 15.4. FT-IR: [cm^{-1}] = 1711 (w), 906 (m), 730 (s). The acid (0.24 g, 2 mmol) was dissolved in 10 ml dry DCM under argon atmosphere. This solution was cooled to 0°C and 0.86 ml (10 mmol, 5 eq.) oxalyl chloride and one drop of DMF were added and stirred 2 hours at room temperature. The solvent was evaporated under reduced pressure and the chloride was used without further purification. Next 0.22 g (1 mmol) of **10** were dissolved in 10 ml of dry THF and to this solution 2 mmol of the chloride were added. This reaction mixture was stirred at 80°C for 16 hours. After this solvent was removed under reduced pressure and the solid was purified by silica gel flash chromatography using cyclohexane and ethyl acetate (4:1) as eluent to afford 0.27 g (0.85 mmol, 85%) of **2** as colorless solid. m.p 71.5°C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ [ppm] = 9.57 (s, 1H; NH), 8.71 (d, *J* = 8.4 Hz, 1H), 7.66-7.58 (m, 1H), 7.45 (d, *J* = 6.9 Hz, 1H), 3.61 (t, *J* = 7.2 Hz, 2H), 2.93-2.82 (m, 2H), 2.80-2.70 (m, 2H), 2.15 (s, 3H), 1.62 (q, *J* = 7.5 Hz, 2H), 1.33 (dq, *J* = 14.5, 7.3 Hz, 2H), 0.92 (t, *J* = 7.3 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ [ppm] = 170.3, 170.3, 167.7, 136.9, 135.7, 131.5, 124.6, 117.9, 115.8, 37.7, 30.6 (, 29.3, 20.0, 15.7, 13.6. FT-IR: [cm^{-1}] = 3352 (w), 2960 (w), 2357 (w), 1763 (w), 1700 (s), 1616 (m), 1531 (m), 1476 (w), 1439 (w), 1397 (m), 1345 (m), 1286 (w), 1239 (w), 1177 (m), 1155 (m), 1053 (m), 943 (m), 824 (w). HR-MS: $[M + H]^+$: 321.12674 (calc.), 321.12660 (measured). Elemental analysis: calc.: 59.98% C, 6.29% H, 8.74% N, measured: 59.91% C, 6.53% H, 8.68% N. UV (CH_3CN, 10^{-5} M): λ_{max} = 342 nm, ε (342 nm) = 4460 L mol^{-1} cm^{-1}.

2.11 | Photooxidation of the probes

1a^{ox}: 30 mg (0.1 mmol) of **1b** were dissolved in 0.6 ml $CDCl_3$ and filled into a NMR tube. A catalytic amount of the photosensitizer meso-tetraphenylporphyrine (1 mg of TPP, leading to a 2×10^{-3} M solution) was added. During the irradiation with a 50 W LED lamp at room temperature, air was bubbled through the solution. After 5 minutes of irradiation, the reaction was controlled by NMR spectroscopy. The solvent was removed under reduced pressure and the product was purified by silica gel chromatography (*c*-hex: EtOAc/4:1). Yield 26 mg (0.08 mmol, 85%). Colorless solid. m.p. 189°C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.29 (s, 2H), 4.00 (s, 6H), 3.81 (t, *J* = 6.4 Hz, 2H), 2.86 - 2.66 (m, 2H), 2.58 (s, 3H), 2.26-2.08 (m, 2H).^{13}C-NMR (75 MHz, $CDCl_3$): δ [ppm] = 168.4, 154.0, 125.3, 105.4, 56.5, 52.0, 38.7, 36.8, 22.4. FT-IR: [cm^{-1}] = 2959 (w), 2927 (w), 2873 (w), 2858 (w), 1761 (s), 1728 (m), 1699 (s), 1647 (w), 1653 (w), 1600 (w), 1541 (w), 1505 (m), 1489 (m), 1475 (m), 1458 (m), 1401 (s), 1375 (m), 1364 (w), 1339 (m), 1310 (m), 1283 (s), 1223 (m), 1200 (w), 1192 (w), 1123 (s), 1087 (s), 1072 (m), 1039 (m), 1017 (m), 992 (m), 969 (w), 920 (w), 876 (m), 859 (w), 829 (w), 799 (w), 784 (s), 776 (s), 766 (s), 743 (s), 706 (w), 694 (m), 673 (w), 661 (w).

1b^{ox}: 40 mg (0.1 mmol) of **1b** were dissolved in 0.6 ml DMSO-d6 and filled into a NMR tube. A catalytic amount of the photosensitizer Rose Bengal (1 mg of RB, leading to a 1.6×10^{-3} M solution) was added. During the irradiation with a 50 W LED lamp at room temperature, air was bubbled through the solution. After 60 minutes of irradiation, the reaction was controlled by NMR spectroscopy. A full conversion was obtained and further purification of the **1b^{ox}** was not performed. ^{1}H-NMR (300 MHz, DMSO): δ [ppm] = 7.40 (s, 2H), 4.84 (dd, *J* = 9.6, 5.4 Hz, 1H), 3.92 (s, 6H), 2.89-2.62 (m, 2H), 2.55-2.28 (m, 5H). ^{13}C-NMR (75 MHz, DMSO):

δ [ppm] = 170.6, 167.9, 154.3, 124.9, 106.3, 56.9, 51.2, 50.3, 38.4, 22.5.

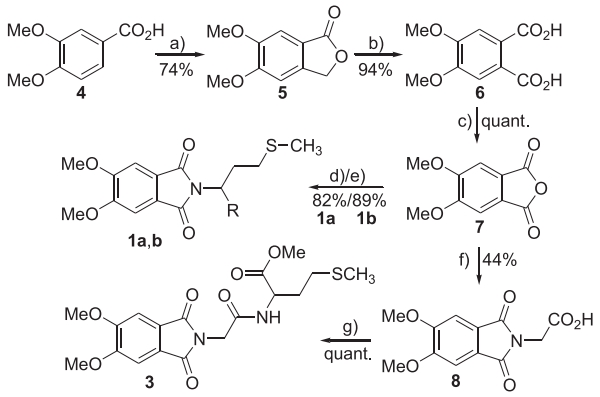
3 | RESULTS AND DISCUSSION

We have initially designed a new family of selective $^1O_2$ probes **1**-**3** (Figure 3) based on the well-known ability of $^1O_2$ to oxidize thioethers to the corresponding sulfoxides.[17] These probes take advantage of the highly reactive natural amino acid methionine that is converted to the sulfoxide, a process that can also be reversed by methionine sulfoxide reductase. In intermolecular quenching studies, we have already identified the strong intermolecular fluorescence quenching of fluorescent phthalimides by thioethers.[18] In the new probes, donor-substituted phthalimides are used as fluorophores and methionine derivatives as the $^1O_2$-sensitive parts.[19] The synthetic routes to the three model compounds **1a**,**b** and **3** use well-known aromatic substitution and coupling steps (Scheme 1) starting from veratric acid (**4**).[6]

3.1 | Singlet oxygen

Probe molecule **1a** is a molecular combination of the strongly fluorescent 4,5-dimethoxyphthalimide[14] with the biogenic amine of methionine and shows a weak fluorescence signal in aqueous media centered at 450 nm. The addition of 0.1 eq. of the dye Rose Bengal (resulting in a 10^{-6}M sensitizer solution in acetone/H_2O) as an external singlet oxygen energy-transfer sensitizer causes an additional weak emission at 570 nm that originates from the weak fluorescence of the singlet oxygen sensitizer (Figure 4). Irradiation with white light under an air atmosphere rapidly led to an increase of a new red-shifted emission band at 502 nm with an isoemissive point at 445 nm indicating the formation of the oxidation product **1a**^{ox}.

After 60 minutes, **1a** was completely (NMR detection) converted to the sulfoxide. No further oxidation was detected, ie, no sulfone was formed after complete conversion of **1a** (and also the other probes **2** and **3**). Identical results were observed for the methionine derivative **1b** (free acid). From the chiral probe **1b**, 2 diastereoisomeric sulfoxides were formed in a 1:1 ratio. Irradiation of probe **1a** in the absence of the singlet oxygen sensitizer Rose Bengal resulted in a very slow buildup of the sulfoxide **1a**^{ox}. From comparison



SCHEME 1 Synthesis of the dimethoxyphthalimides **1** and **3**[6]

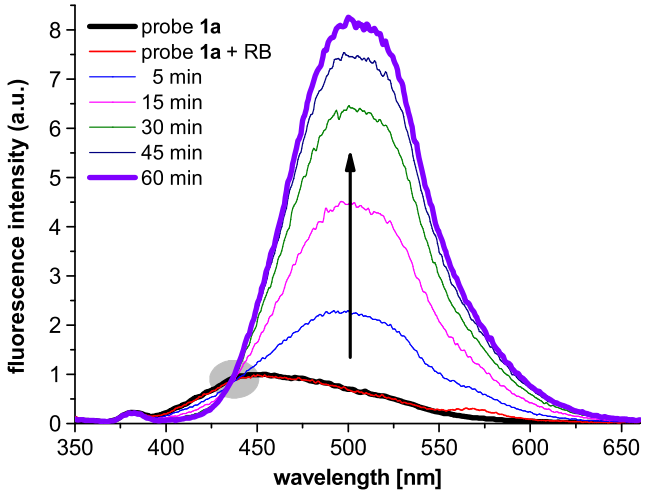


FIGURE 4 Fluorescence spectra of probe **1a** (10^{-5}M in acetone/H_2O 1:1, λ_{ex} = 340 nm) during irradiation with RB/air

of the singlet oxygen quantum yield of Rose Bengal (Φ_Δ = 0.75)[20] and the initial **1a**^{ox}-formation kinetics, Φ_Δ for **1a** of 0.02 was estimated.[21]

The rate constant of the singlet oxygen reaction with **1a** was estimated by comparison with the well-known standard substrate, 2,3-dimethyl-2-butene, with a bimolecular rate constant of 5.4 × 10^7 L/mol/s in chloroform.[22] From the pseudo first-order kinetics of product formation (Figure 5). the rate constant for the reaction with **1a** was determined as 2.4 × 10^7 L/mol/s under the same solvent conditions as used in the fluorescence studies. The literature value for the singlet

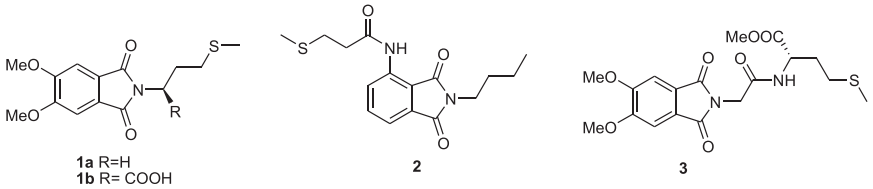


FIGURE 3 New singlet oxygen probe molecules **1**-**3**

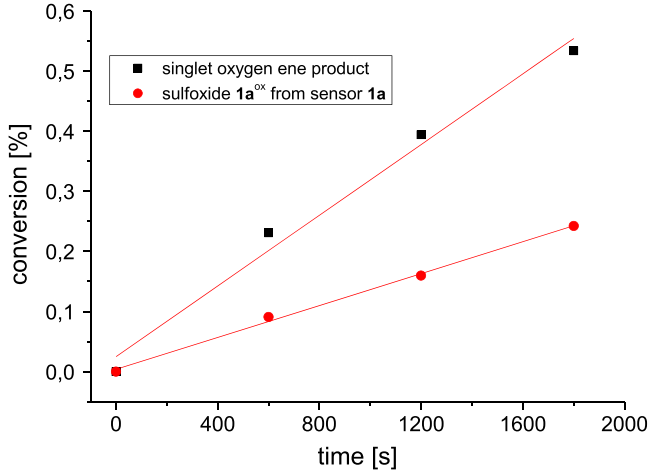
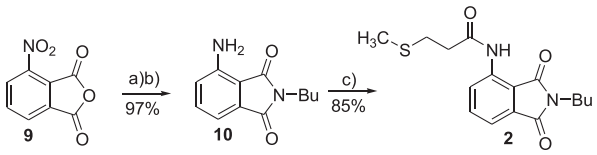


FIGURE 5 Oxygen uptake from the photooxygenation of probe **1a** versus the standard substrate 2,3-dimethyl-2-butene under identical conditions (10 mM Rose Bengal in acetone/H_2O 1:1, oxygen)

oxygen reaction with the free amino acid methionine in water/methanol is 3×10^7 L/mol/s.[21]

As an alternative connection between fluorophore and thioether group, probe molecule **2** was investigated. This compound is a 3-thioethylamido phthalimide with a weak fluorescence band at 425 nm available by a 3-step synthesis from 3-nitrophthalic anhydride (Scheme 2).[23] During $^1O_2$ generation, the low initial probe fluorescence increases in intensity by a factor of 15, however without notable shift



SCHEME 2 Synthesis of the 3-amidophthalimide **2**[23]

in emission wavelength (Figure 6). Both absorption and emission bands of **2** and the sulfoxide $\mathbf{2}^{ox}$ are blue-shifted by 80 nm in comparison with the dimethoxy probes **1**. Because of the conformationally unrestricted and close contacts between the phthalimide chromophores and the fluorescence quenching group in probes **1** and **2**, similar effects were obtained with respect to quenching magnitudes. The observed red-shift in emission for **1a**,**b**, however, makes these probes more powerful because the amplification is much higher in the wavelength region between 550 and 580 nm.

To investigate the quenching ability of the thioether group as a function of distance between quencher and fluorophore, the glycine-containing dipeptide Gly-Met was connected to the 4,5-dimethoxyphthalimide (**3**). In this case, the original probe fluorescence itself is already strong and centered at 480 nm (Figure 7). When irradiated in the presence of Rose Bengal under air, a red-shift and increase in fluorescence intensity is observed similar to **1**. Methionine can therefore only be applied as fluorescence quenching and singlet oxygen reporting unit when directly linked to the chromophore either by the imide nitrogen (in **1a**,**b**) or at the C3- or C4-positions of the aromatic core (in **2**).

3.2 | Hydrogen peroxide

Another crucial aspect beside sensitivity of a $^1O_2$ probe is the ROS selectivity. We therefore investigated the oxidation of all probes with hydrogen peroxide. As shown for **1a** (Figure 8). H_2O_2 also leads to the formation of the emissive $\mathbf{1a}^{ox}$, albeit very inefficient: a 10^4 molar excess of H_2O_2 was used and complete oxidation took more than 3 hours. As product, again solely the sulfoxide was formed without over-oxidation products. Equimolar amounts of H_2O_2 or 10- to 20-fold

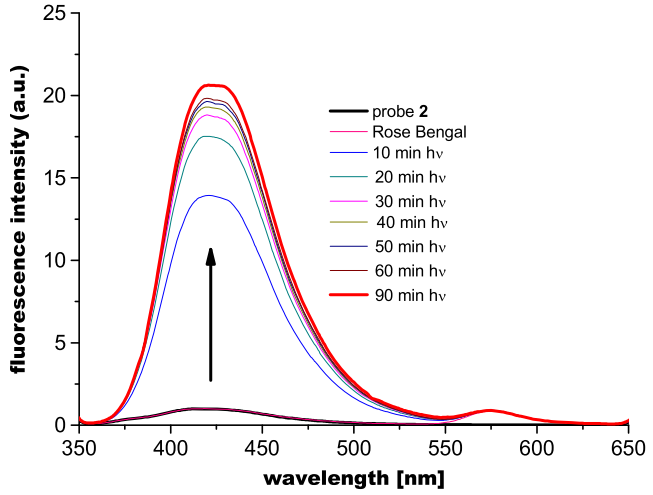


FIGURE 6 Fluorescence spectra of probe **2** (10^{-5}M in acetone/H_2O 1:1, λ_{ex} = 338 nm) during irradiation with RB/air

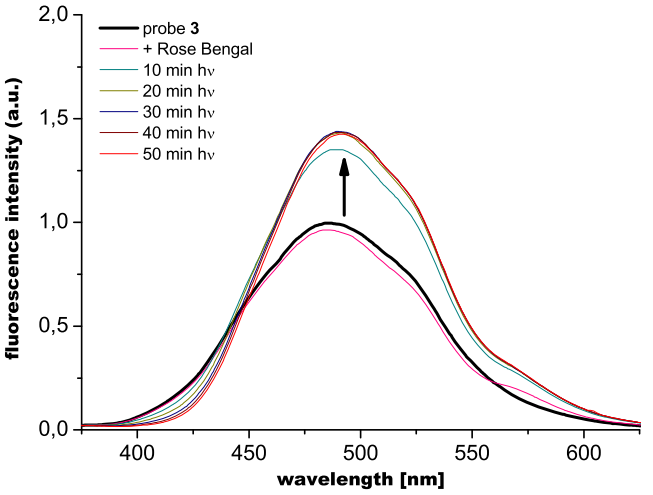


FIGURE 7 Fluorescence spectra of probe **3** (10^{-5}M in acetone/H_2O 1:1, λ_{ex} = 338 nm) during irradiation with RB/air

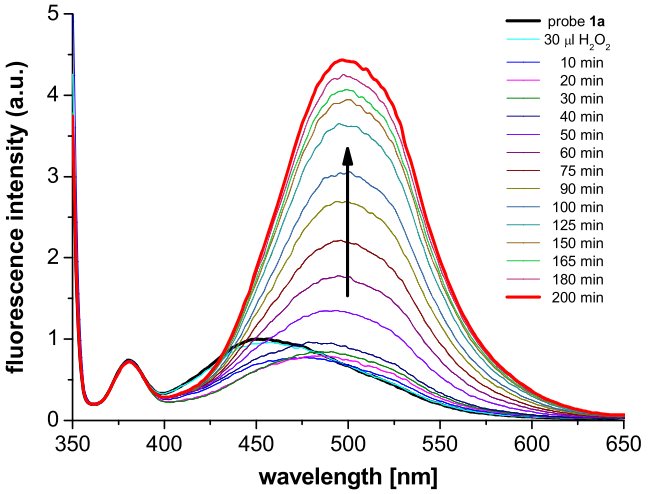


FIGURE 8 Fluorescence spectra of probe **1a** (10^{-5}M in acetone/H_2O, λ_{ex} = 340 nm) after treatment with 10^4 eq. H_2O_2

excess of H_2O_2 did not result in any conversion indicated by the unchanged probe fluorescence.

This reaction can however be accelerated by the addition of catalytic amounts of titanium(IV)alkoxides as known from the Kagan enantioselective thioether oxidation (Figure 9).[24] In this process, a titanium-peroxide complex is formed that can efficiently transfer oxygen atoms to thioethers. This process is known to proceed with high enantioselectivity in the presence of chiral ligands. All probe molecules did show rapidly increased fluorescence in the presence of H_2O_2 and catalytic amounts of $Ti(OiPr)_4$. Only one diastereosisomeric sulfoxide was formed from the chiral methionine-derived probe molecules **1b**.

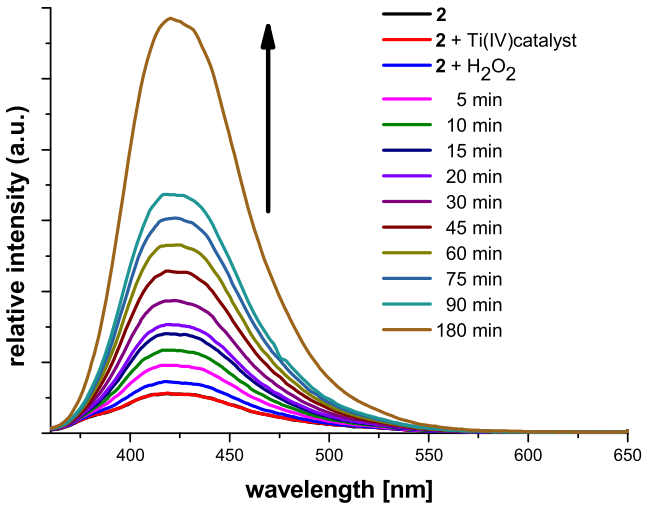


FIGURE 9 Fluorescence spectra of probe **2** (10^{-5}M in acetone/H_2O, λ_{ex} = 340 nm) after treatment with 10^3 eq. H_2O_2 after treatment with catalytic amounts of $Ti(OiPr)_4$

3.3 | Hypochlorite

Another important oxygen transfer species and ROS that is efficient in thioether oxidation is the hypochlorite anion. In contrast to the results with hydrogen peroxide where a large excess of the oxidant has to be used, hypochlorite has to applied only in a 20-fold excess for complete oxidation of the probe as shown for the 3-amido phthalimide **2** in Figure 10. This oxidation needs only short reaction times is immediately finished after addition of the oxidant (no change in fluorescence intensity over time) and stops at the sulfoxide oxidation stage.

4 | CONCLUSION

In summary, we have shown that $^1O_2$ can be detected in water by the molecular probes **1**-**3** that consist of a phthalimide fluorophore and a methionine-derived thioether side-chain by bluegreen emission of the sulfoxide products. Self-sensitized oxidation of the probes is inefficient for **1** and **2** indicating rapid electron-transfer quenching of the excited probe molecules (Scheme 3). Probe **3** is inefficient because already strong initial fluorescence indicates slower electron-transfer

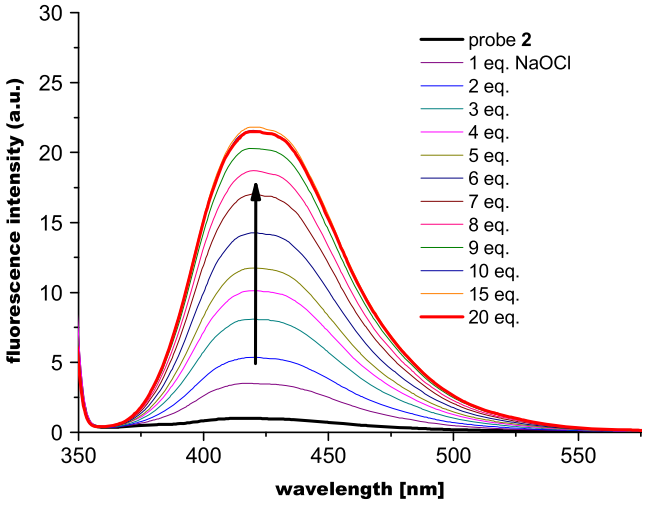
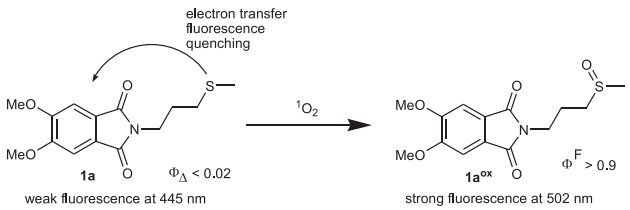


FIGURE 10 Fluorescence spectra of probe **2** (10^{-5}M in acetone/H_2O 1:1, λ_{ex} = 338 nm) directly after treatment with NaOCl



SCHEME 3 Fluorescence quenching and photooxygenation principle of probe **1a**

quenching. Oxidation with H_2O_2 is much less effective and can be accelerated by titanium(IV) catalysts. The probe molecules **1a**,**b**, **2** and **3** are also rapidly oxidized with hypochlorite in aqueous solution.

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