Synthesis of 3-Substituted 2,5-Dihydro-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radicals, Useful for Spin-Labelling of Biomolecules

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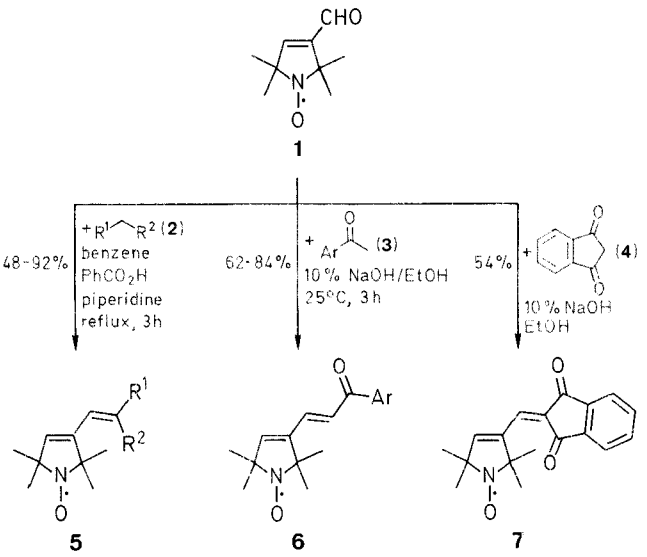
Synthesis and some reactions of 3-substituted 2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl radicals (nitroxyl radicals, nitroxides) activated with carbonyl, cyano, or ester electron-withdrawing groups have been described. These compounds are useful new SH-reagents for spin-labelling of biomolecules.

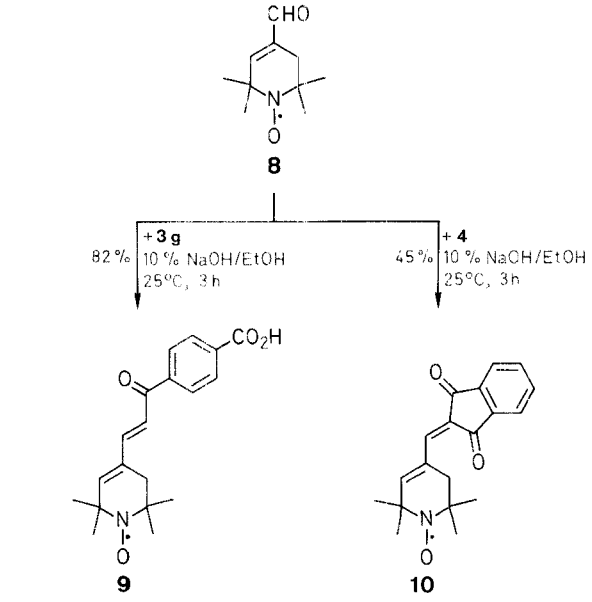
Although the α,β-unsaturated aldehydes **1**[1-5] and **8**[5,6] are very useful synthons for preparing various nitroxide spin-label reagents, they are not reactive enough themselves to undergo conjugate addition with nucleophilic groups of biomolecules such as thiol or amino groups. We have now condensed the nitroxide aldehydes **1** and **8** with compounds containing active methylene group such as **2**, **3** or **4** to give α,β,γ,δ-dienes **5**–**7**, **9** or **10** conjugated with carbonyl, nitrile, or ester groups, which are electrophilically activated, and therefore excepted to accept nucleophiles in a Michael type addition. (Schemes **A** and **B**).



2, 5	R^1	R^2	3, 6	Ar
a	CN	CN	**a**	Ph
b	CO_2Me	CN	**b**	4-FC_6H_4
c	CO_2Me	$COCH_3$	**c**	2-HOC_6H_4
d	CO_2Bu-*t*	$COCH_3$	**d**	4-HOC_6H_4
e	COPh	COPh	**e**	2,4-$(HO)_2C_6H_3$
			f	2,6-$(HO)_2C_6H_3$
			g	4-$HO_2CC_6H_4$
			h	2-indolyl
			i	2-benzimidazolyl

Scheme **A**

Compound **6a** described earlier[2] has a considerably reduced segmental mobility relative to the protein than the normally used sulfhydryl spin-labels,[7,8] e.g. the 1-oxyl-2,2,6,6-tetramethyl-4-(*N*-maleimidyl)piperidine (SL-NEM) and therefore could better be used to study the overall rotational motion of the Na^+–K^+)-ATPase in the membrane.[9] It seems likely that the activated dienes will offer considerable advantages in the study of other proteins using saturation transfer electron spin resonance (STESR) spectroscopy.[10-12]



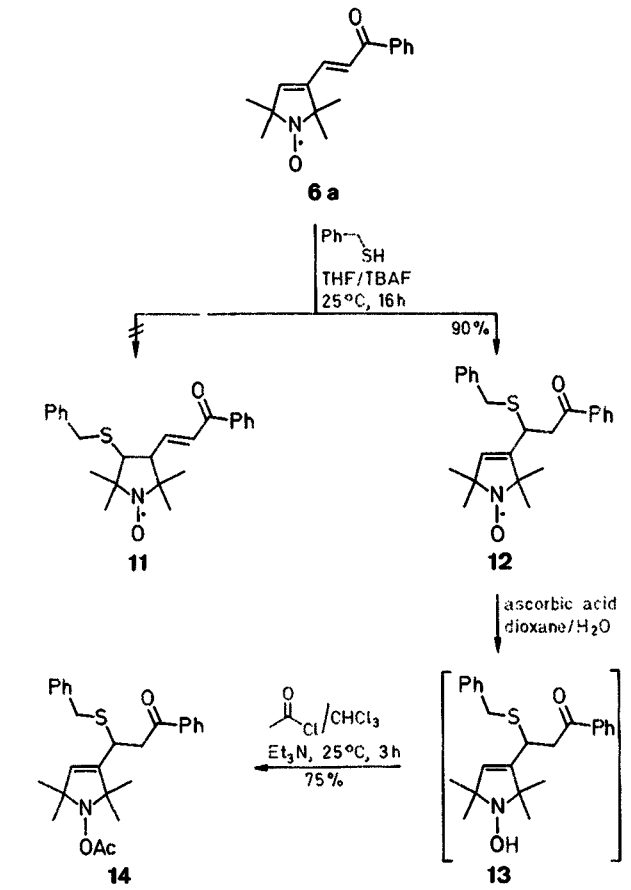
Scheme **B**

The indane-1,3-dione derivative **7** has the highest reactivity amongst the ketones with less segmental mobility than SL-NEM when covalently attached to the Ca^{2+}-ATPase.[13] An additional advantage of compounds **5**–**7** is that five-membered nitroxide free radicals are more resistant toward reduction than six-membered ones.[14-16]

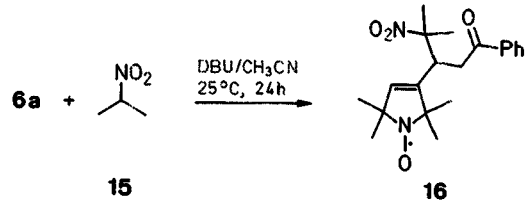
The conjugate addition of **6a** with phenylmethanethiol gave only one product **12**. This was confirmed by its reduction to the *N*-hydroxy compound **13**, which was found to be unstable and characterized as the *N*-acetyl derivative **14** (Scheme **C**). This indicates that a 1,4-addition with retention of the γ,δ-double bond in the heteroring has taken place, and not a 1,6-addition to give **11**.

A 1,4-addition of 2-nitropropane **(15)** to **6a** could be carried out by generating the anion of **15** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to form the adduct **16** (Scheme **D**).

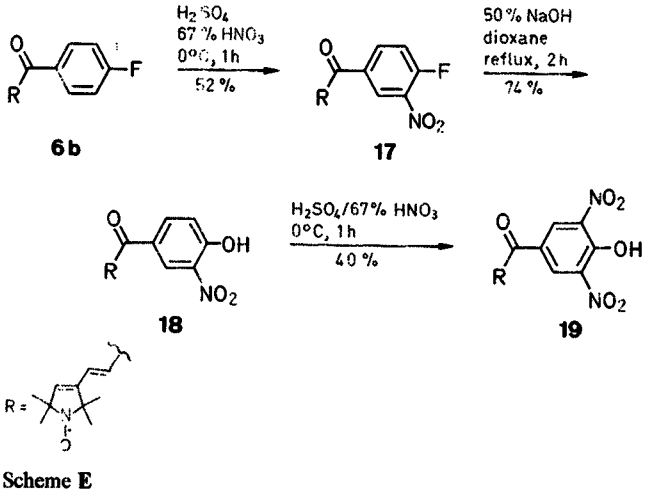
In a recent work from our laboratory the nitration of 3-benzoyl-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl radical to form the corresponding mononitrated 3-(3-nitrobenzoyl) derivatives was reported.[5] The dienone analogue **17** was obtained now by nitration of **6b**. Hydrolysis of **17** to **18** followed by a second nitration afforded the highly acidic and water-soluble 3-(3,5-dinitro-4-hydroxybenzoyl) derivative **19** (Scheme **E**). The spin-label reagent **19** could also be prepared by nitration of **6d** in one step (Scheme **F**).



Scheme C

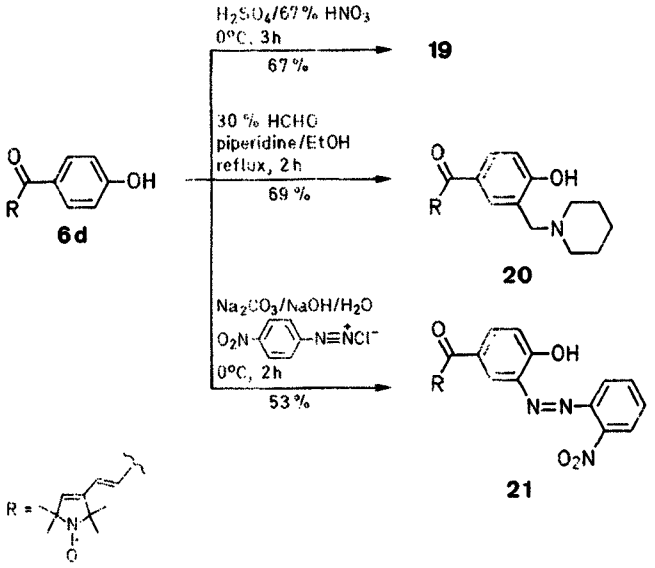


Scheme D



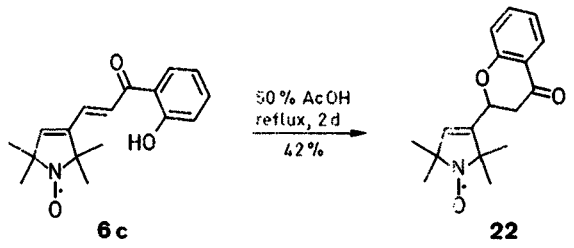
Scheme E

The Mannich reaction of **6d**, even with an excess of formaldehyde and piperidine, gave only mono-aminomethylated product **20**. The spin-labelled azo compound **21** could be prepared by coupling of 2-nitrobenzenediazonium ion with **6d** (Scheme F).



Scheme F

The ring closure reaction of a 3-(2-hydroxybenzoyl) ketone **6c** in acidic media was selected to illustrate the feasibility of preparing a spin-labelled chromanone **22** (Scheme G).



Scheme G

Melting points were determined on a Boetius micro-mp apparatus and are not corrected. IR spectra were measured in Nujol suspensions or neat with a Zeiss Specord 75 type instrument. ^{1}H-NMR spectrum of **14** was recorded in CCl_4 (internal standard: TMS) with a Perkin-Elmer R-12 spectrometer. The mass spectra were taken on a Finnigan MAT 8430 mass spectrometer applying the direct insertion technique. Operation conditions: R = 1250, $T_{ion\ source}$ = 250 C, U_{acc} = 3 kV, E_{el} = 70 eV, I_{el} = 500 µA. Evaporation temperatures of the samples varied between 150 and 350°C, and were controlled in each case within ±1°C accuracy. Flash column chromatography on silica gel was performed using Merck Kieselgel 60 (0.040–0.063 mm). Qualitative TLC was carried out on commercially prepared plates coated with Merck Kieselgel GF_{254}. Preparative TLC was performed on plates (20 × 20 × 0.2 cm) coated with the same material.

The aldehydes **1**[1] and **8**[2] were prepared as described earlier in the literature. Tetrabutylammonium fluoride (TBAF) catalyst was prepared as follows. A THF solution of commercially available TBAF trihydrate was adsorbed on silica gel (10 mol%), the solvent evaporated and the silica gel was dried at 80°C.

3-Substituted 2,5-Dihydro-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radicals 5–7; General Procedures:

For Dienonitriles and Esters **5a–e**: A solution of (1.68 g, 10 mmol) and the appropriate active methylene compound **2a–e** (10 mmol) in benzene (80 mL) are heated to reflux with benzoic acid (40 mg) and piperidine (35 mg) in a Dean–Stark apparatus for 3 h, during which time

water (0.2 mL) is collected (calc. 0.18 mL). The mixture is allowed to cool to room temperature and washed with 5% aq. $NaHCO_3$ solution and brine. The benzene layer is dried ($MgSO_4$), filtered, and evaporated to dryness to yield a deep orange solid. Recrystallization from $CHCl_3$/*n*-hexane gives **5** as red **(5a)** or orange yellow **(5b–e)** crystals.

For Dieno Ketones **6a–i, 7, 9,** *and* **10**: To a solution of the aldehyde **1** or **8** (5 mmol) and ketone **3** (5.0 mmol) in EtOH (20 mL), 10% aq. NaOH (10 mL) is added at room temperature in 3 h. The mixture is acidified with 5% H_2SO_4 to pH 2, washed with brine, dried ($MgSO_4$), and evaporated to dryness. The yellow solid is purified by flash column chromatography

Table. Compounds **5**, **6**, **7**, **9–12**, **14**, **16–22** Prepared

Product	Yield (%)	mp (°C)[a]	Molecular Formula[b] or Lit. mp (°C)	IR (neat or nujol) ν (cm^{-1})	MS (70 eV)[c] *m/z* (%, rel. int.)
5a	92	108–110	$C_{12}H_{14}N_3O$ (216.3)	2215 (CN)	216 (M^+, 65.5); 201 (*a*, 22.5); 186 (*b*, 42); 171 (*c*, 100); 144 (*c*-HCM, 24.5)
5b	67	98–99	$C_{14}H_{19}N_2O_3$ (263.3)	2220 (CN), 1730 (CO)	263 (M^+, 100); 248 (*a*, 76); 233 (*b*, 80); 218 (*c*, 100); 190 (*c*-C_2H_4, 40)
5c	80	98–100	$C_{14}H_{20}NO_4$ (266.3)	1735 (CO), 1670 (CO)	266 (M^+, 11); 236 (*b*, 40); 221 (*c*, 100); 193 (*b*-$COCH_3$, 37); 189 (*c*-CH_3OH, 62); 43 (91)
5d	82	124–126	$C_{17}H_{26}NO_4$ (308.4)	1710 (CO), 1675 (CO)	308 (M^+, 15); 222 (*b*-C_4H_8, 85); 207 (*c*-C_4H_8, 100); 189 (207-H_2O, 53); 43 (CH_3CO^+, 73)
5e	48	94–95	$C_{24}H_{24}NO_3$ (374.5)	1670, 1640 (CO)	374 (M^+, 6); 344 (*b*, 15); 329 (*c*, 50); 105 ($PhCO^+$, 100)
6a	68	126–128	106–108[2]	1660 (CO)	270 (M^+, 90); 255 (*a*, 23); 240 (*b*, 31); 225 (*c*, 100); 199 (*d*, 41)
6b	77	112–113	$C_{17}H_{24}FNO_2$ (288.4)	1665 (CO)	288 (M^+, 85); 273 (*a*, 18); 258 (*b*, 25); 243 (*c*, 100); 217 (*d*, 38); 123 ($ArCO^+$, 50)
6c	73	90–92	$C_{17}H_{20}NO_3$ (286.4)	3600–3200 (OH), 1638 (CO)	286 (M^+, 64); 241 (*c*, 39); 215 (*d*, 10); 147 ($ArCOCH{=}CH^+$, 100); 121 ($ArCO^+$, 45)
6d	84	201–102	$C_{17}H_{20}NO_3$ (286.4)	3200–2900 (OH), 1645 (CO)	286 (M^+, 68); 256 (*b*, 42); 241 (*c*, 73); 215 (*d*, 36); 121 ($ArCO^+$, 100)
6e	68	203–205	$C_{17}H_{20}NO_4$ (302.4)	3500–2900 (OH), 1635 (CO)	302 (M^+, 31); 257 (*c*, 23); 231 (*d*, 8); 163 ($ArCOCH{=}CH^+$, 100); 137 ($ArCO^+$, 57)
6f	72	174–175	$C_{17}H_{20}NO_4$ (302.4)	3600–3100 (OH), 1620 (CO)	302 (M^+, 13); 257 (*c*, 100); 231 (*d*, 20.4)
6g	79	198–199	$C_{18}H_{20}NO_4$ (314.4)	3600–3200 (OH), 1690, 1660 (CO)	314 (M^+, 72); 284 (*b*, 39); 269 (*c*, 100); 243 (*d*, 67); 149 ($ArCO^+$, 98); 135 (*b*-ArCO, 79)
6h	72	206–207	$C_{19}H_{21}N_2O_2$ (309.4)	3400–3100 (NH), 1645 (CO)	309 (M^+, 100); 279 (*b*, 28); 264 (*c*, 68); 236 (*d*, 15)
6i	62	196–198	$C_{18}H_{20}N_3O_2$ (310.4)	3400–3000 (NH), 1670 (CO)	310 (M^+, 100); 280 (*b*, 35); 265 (*c*, 30); 237 (*d*, 88); 119 ($ArCO^+$, 60)
7	54	157–158	$C_{18}H_{18}NO_3$ (296.4)	1720, 1685 (CO)	296 (M^+, 16); 266 (*b*, 100); 251 (*c*, 65); 223 (*c*-CO, 20)
9	82	> 240	$C_{19}H_{22}NO_4$ (328.4)	3600–3200 (OH), 1720, 1690 (CO)	328 (M^+, 12); 314 ($[M+H]^+$-CH_3, 19)[d]; 298 (*b*, 31); 149 ($ArCO^+$, 100); 121 (Ar^+, 34)
10	45	175–176	$C_{19}H_{20}NO_3$ (310.4)	1720, 1680 (CO)	310 (M^+, 27); 280 (*b*, 97); 265 (*c*, 60); 237 (*c*-CO, 100); 98 ($C_5H_8NO^+$, 51)
12	90	91–92	$C_{24}H_{28}NO_2S$ (394.5)	1680 (CO)	394 (M^+, 25); 241 (*b*-SCH_2Ph, 25); 105 ($PhCO^+$, 100)
14	75	oil	$C_{26}H_{31}NO_3S$ (437.6)	1740, 1680 (CO)	437 (M^+, 0.4); 422 (*a*, 29); 380 (*a*-CH_2O, 34); 105 ($PhCO^+$, 100)
16	68	128–129	$C_{20}H_{27}N_2O_4$ (359.5)	1695 (CO), 1525, 1370 (NO_2)	359 (M^+, 16); 298 (*b*-NO_2, 12); 105 ($PhCO^+$, 100)
17	52	122–123	$C_{17}H_{18}FN_2O_4$ (333.3)	1665 (CO), 1535, 1340 (NO_2)	333 (M^+, 63); 318 (*a*, 32); 303 (*b*, 31); 288 (*c*, 100); 262 (*d*, 32); 242 (*c*-NO_2, 30)
18	74	142–144	$C_{17}H_{19}N_2O_5$ (331.4)	3600–3200 (OH), 1660 (CO), 1540, 1320 (NO_2)	331 (M^+, 82); 316 (*a*, 27); 301 (*b*, 31); 286 (*c*, 100); 260 (*d*, 40); 166 ($ArCO^+$, 34)
19	67	155–156	$C_{17}H_{18}N_3O_7$ (376.4)	3600–3250 (OH), 1660 (CO), 1540, 1320 (NO_2)	376 (M^+, 56); 361 (*a*, 27); 346 (*b*, 32); 331 (*c*, 100); 305 (*d*, 34); 135 (*b*-ArCO, 54)
20	69	142–143	$C_{23}H_{31}N_2O_3$ (383.5)	3600–3200 (OH), 1650 (CO)	383 (M^+, 73); 353 (*b*, 50); 268 (*b*-$C_5H_{11}N$, 30); 84 ($C_5H_{10}N^+$, 100)
21	53	202–203	$C_{23}H_{23}N_4O_5$ (435.5)	3600–3200 (OH), 1655 (CO), 1520, 1320 (NO_2), 1380 (N=N)	435 (M^+, 84); 405 (*b*, 100); 390 (*c*, 67); 270 (*c*-Ar, 27); 120 (Ar^+, 54)
22	42	79–80	$C_{17}H_{20}NO_3$ (286.4)	1680 (CO)	286 (M^+, 70); 241 (*c*, 82); 147 (M^+-pyrrolinyl, 100); 121 ($ArCO^+$, 88)

[a] Solvents for recrystallization: **5**, **6**, **7**, **9**, **10**, **18** ($CHCl_3$/*n*-hexane); **12**, **14**, **16**, **17**, **20–22** (ether/*n*-hexane); **19** ($CHCl_3$/ether).

[b] Satisfactory microanalyses obtained: C ±0.21, H ±0.25, N ±0.19, S ±0.12 (Exceptions: **6h**, H +0.54; **6i**, H −0.46).

[c] Most frequently occuring types of ions are denoted as; *a* = $(M-CH_3)^+$; *b* = $(M-NO)^+$; *c* = $(M-NO-CH_3)^+$; *d* = $(M-C_3H_5NO)^+$.

[d] Mass spectra of nitroxides almost invariably contain this type of ion,[17] though usually less significant. As past experiences indicate, the origin of the $[M+H]^+$ species must at least partly be a slow reaction of the nitroxides with moisture present in the air.

with *n*-hexane/ether (6:1) as eluent: If necessary, further purification is effected by crystallization from $CHCl_3$/*n*-hexane.

3-(1-Benzylthio-3-oxo-3-phenylpropyl)-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radical (12):

To a solution of **6a** (1.35 g, 5 mmol) and phenylmethanethiol (621.5 mg, 5.0 mmol) in dry THF (10 mL), TBAF (1.0 g, 10 mol% on silica gel) is added and stirred at room temperature for 16 h and filtered. The filtrate is evaporated and chromatographed on silica gel column, using *n*-hexane/EtOAc (10:1) as eluent to give **12** as a yellow crystalline product.

1-Acetyl-3-(1-benzylthio-3-oxo-3-phenylpropyl)-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrole (14):

To a solution of the paramagnetic adduct **12** (394.6 mg, 1 mmol) in dioxane (5 mL), an aqueous solution (ca. 5 mL) of ascorbic acid (880.0 mg, 5.0 mmol) is added. The originally yellow solution decolorizes soon (ca. 30 min), and is extracted after 1 h with $CHCl_3$ (3 × 10 mL). The organic phase is collected and dried [K_2CO_3 (3 g) + $MgSO_4$ (3 g)]. To this stirred mixture under argon atmosphere, a solution of Et_3N (TEA) (202.4 mg, 2 mmol) in dry $CHCl_3$ (20 mL) followed by acetyl chloride (157.0 mg, 2 mmol) in dry $CHCl_3$ (20 mL) are added, the stirring is maintained for 3 h, and then the mixture is filtered. The filtrate is washed subsequently with aq. 5% H_2SO_4, 5% $NaHCO_3$, brine, and dried ($MgSO_4$). The solvent is evaporated, and the crude product is chromatographed on silica gel using *n*-hexane/EtOAc (10:2) as eluent to give **14** as an oil.

^{1}H-NMR (CCl_4/TMS): δ = 1.02 (s, 6H, $2CH_3$); 1.12 (s, 6H, $2CH_3$); 1.94 (s, 3H, $COCH_3$); 3.21 (d, 2H, J = 7.2 Hz, CH_2); 3.5–3.8 + 3.6 (m + s, 3H, CH + SCH_2); 5.34 (s, 1H, H_{pyrr}); 7.0–7.9 (m, 10H, H_{arom}).

2,5-Dihydro-3-[1-(1-nitro-1-methylethyl)-3-oxo-3-phenylpropyl]-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radical (16):

To a solution of **5a** (540.8 mg, 2 mmol) and 2-nitropropane (270.0 mg, 3 mmol) in dry CH_3CN (5 mL), DBU (30.5 mg, 0.2 mmol) is added at room temperature. After 1 d the cherry pink solution is diluted with EtOAc (20 mL) and washed with 5% H_2SO_4, brine, dried ($MgSO_4$), and evaporated to dryness. The yellow solid residue is purified by flash chromatography on silica gel using *n*-hexane/EtOAc (4:1) as eluent to give the pure yellow adduct **16**.

2,5-Dihydro-3-[3-(4-fluoro-3-nitrophenyl)-3-oxo-1-propenyl]-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radical (17); Typical Procedure for Nitration:

To a stirred solution of **6b** (1.44 g, 5 mmol) in conc. H_2SO_4 (10 mL) at −5 °C is added dropwise a mixture of conc. H_2SO_4 (2 mL) and 67% HNO_3 (1 mL). The deep red mixture becomes colorless, then turns pink. It is stirred at 0 °C for 1 h, and then poured onto stirred crushed ice (ca. 100 g) and extracted with EtOAc (3 × 10 mL). The organic phase is washed with 50% aq. $NaHCO_3$, brine, dried ($MgSO_4$), and evaporated to dryness. The solid residue is purified on silica gel column with *n*-hexane/EtOAc (8:2) as eluent. The strong yellow band of product is isolated and proved to be the mononitrated compound **17**.

2,5-Dihydro-3-[3-(4-hydroxy-3-nitrophenyl)-3-oxo-1-propenyl]-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radical (18):

A solution of **17** (333.3 mg, 1.0 mmol) in dioxane (5 mL) is refluxed with 50% aqueous NaOH (1 mL) for 2 h, cooled, acidified with 5% H_2SO_4 (10 mL) and extracted with EtOAc (3 × 10 mL). The organic phase is dried ($MgSO_4$) and evaporated to dryness. The residue yellow solid is purified by preparative TLC ($CHCl_3$/ether, 1:1).

2,5-Dihydro-3-[3-(4-hydroxy-3,5-dinitrophenyl)-3-oxo-1-propenyl]-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radical (19):

By Nitration of **18**: A solution of the hydroxyketone **18** (1.65 g, 5 mmol) in conc. H_2SO_4 (10 mL) is stirred with a mixture of conc. H_2SO_4 (2 mL) and 67% HNO_3 (1 mL) below 0 °C for 1 h, then worked up as above but without washing the organic phase with aq. $NaHCO_3$.

Column chromatography on silica gel using $CHCl_3$/acetone/MeOH (1:1:0.1) as eluent affords pure **19**.

By Nitration of **6d**: A solution of **6d** (1.43 g, 5.0 mmol) in concentrated H_2SO_4 (10 mL) is stirred at 0 °C with a larger amount of the nitrating mixture [concentrated H_2SO_4 (8 mL) and 67% HNO_3 (4 mL)] for 3 h, then worked up as above. Chromatography with $CHCl_3$/acetone/MeOH (1:1:0.1) as eluent yields a minimal amount of **18** (less then 10%) and as a major product the dinitrophenol **19**. The slowly moving band is isolated to give 4-hydroxy-3,5-dinitrobenzoic acid, which is identical with an authentic sample; yield: 240 mg (21%); mp 240–243 °C (Lit.[18] mp 243 °C).

2,5-Dihydro-3-[3-(4-hydroxy-3-piperidinomethylphenyl)-3-oxo-1-propenyl]-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radical (20):

A solution of **6d** (592.8 mg, 2 mmol), 30% formaldehyde (1 mL, 5 mmol), and piperidine (425 mg, 5 mmol) in EtOH (10 mL) is refluxed for 2 h, and then evaporated to dryness. The residue is dissolved in 5% H_2SO_4 (10 mL) and extracted with EtOAc (20 mL) to remove any unreacted **6d**. The aqueous phase is basified with 25% aq. ammonia, extracted with EtOAc (3 × 20 mL), the organic phase is washed with water, and dried ($MgSO_4$). The solvent is evaporated in vacuum, and the residue is purified by preparative TLC (*n*-hexane/ether, 2:1).

2,5-Dihydro-3-[4-hydroxy-3-(2-nitrophenylazo)-3-oxo-1-propenyl]-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radical (21):

A solution of 2-nitroaniline (138.1 mg, 1.0 mmol) in 4% HCl (10 mL) is diazotized at 0 °C with a solution of $NaNO_2$ (69 mg, 1.0 mmol). The cold solution of 2-nitrobenzenediazonium chloride is added dropwise with stirring to a solution of **6d** (286.3 mg, 1.0 mmol), Na_2CO_3 (318 mg, 3.0 mmol), and NaOH (80 mg, 2.0 mmol) in water (10 mL) cooled to 0 °C. The mixture is stirred for 2 h, then neutralized with 5% $NaHCO_3$, and extracted with $CHCl_3$ (3 × 10 mL). The dried ($MgSO_4$) $CHCl_3$ phase is evaporated, and the dark semisolid residue is purified by preparative TLC *n*-hexane/EtOAc, 2:1) to yield the azocompound **21**.

3-(4-chromanon-2-yl)-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl Radical (22):

A solution of **6c** (286.3 mg, 1.0 mmol) in 50% AcOH (10 mL) is refluxed for 2 d, then cooled and extracted with EtOAc (3 × 5 mL). The organic phase is washed with 5% aq. $NaHCO_3$, brine, dried ($MgSO_4$), and evaporated to dryness. The residual solid consists of unreacted **6c** and product **22**, which is separated by preparative TLC on silica gel *n*-hexane/EtOAc, 2:1).

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