Synthesis of 2,2- and 2,5-Disubstituted 1-Oxyl Pyrrolidine Radicals as New Homobifunctional Cross-Linking Spin Labels

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Abstract: The synthesis of 2,2- and 2,5-disubstituted pyrrolidine nitroxide radicals starting from readily available nitrones **1**, **11** is described. The stable radicals are acylating (**10**, **20**), alkylating (**7**, **17**) and thiol-specific (**8**, **18**) reagents capable of producing cross-links in proteins over distances in the range of 10–15 Å.

Key words: cross linking reagents, proteins, free radicals, Grignard reactions, nitrones, lithium

Nitroxides have received considerable attention as co-oxidants,[1] spin labels,[2] MRI agents,[3] antioxidants[4] and double sensor molecules.[5] Among these uses, spin labeling is the most important and wide-spread application. A major advance in the field of protein spin labeling was the introduction of site directed spin labeling (SDSL). SDSL is proving to be a general strategy for determining the structure of both soluble and membrane proteins of any molecular weight.[6–8] Moreover, recent work indicates that protein dynamics, an important determinant of protein function, can be extracted from the motion of nitroxide side chains.[9,10] Homo-bifunctional nitroxide reagents, particularly those with reactive functionality toward sulfhydryl groups, promise to significantly extend the capabilities of SDSL. For example, bifunctional reagents that cross-link a pair of cysteine residues spaced by one or two turns along an α-helix are strongly immobilized with respect to the protein backbone, as expected. In this case, motion of the nitroxide group, reflected in the EPR spectrum, is dominated by rigid body motions of the helix. Thus, the EPR spectra of spin labels with dual attachment points may provide a direct and powerful measure of protein dynamics without the usual contributions from the internal motion of the side chain itself.[10] Because the orientation of the nitroxide ring is necessarily fixed by the cross-link, the angular dependence of the EPR spectrum in an oriented system (i.e., membranes) can be used to deduce the orientation of the helical axis with respect to the director of the system. Finally, the rate of cross-link formation, easily monitored by EPR in real time, will provide direct measures of both cysteine proximity and protein flexibility. In our laboratory we have synthesized several heterobifunctional (thiol-specific and photoactivatable)[11,12] and thiol-specific homobifunctional cross-linking reagents[13,14] containing reactive substituents only in the 3,4-positions of the pyrroline nitroxide ring. In this paper, we present an extension of the above idea with the synthesis of novel 2,2- and 2,5-disubstituted pyrrolidine nitroxide homobifunctional reagents that are designed to serve the purposes mentioned above.

Treatment of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (**1**) or 2,5-dimethyl-1-pyrroline *N*-oxide[15] (**11**) with 4-dimethoxymethylphenylmagnesium bromide yielded trisubstituted nitrones[16] **2** and **12**, respectively, after oxidation of the hydroxylamines to the nitrones by activated MnO_2. Further treatment of nitrone **12** with 4-dimethoxymethylphenylmagnesium bromide gave *trans* diphenyl nitroxide **13** analogously to earlier cases.[17] On the other hand, similar treatment of nitrone **2** yielded only the unsubstituted hydroxylamine. Apparently, the second phenyl substituent cannot be easily introduced into this sterically hindered position, as was observed earlier by Iwahashi et al.[18] Treatment of compound **2** with the more reactive 4-dimethoxymethylphenyllithium obtained by translithiation[19] of 4-bromobenzaldehyde dimethyl acetal gave the desired nitroxide **3** after oxidation with activated MnO_2.

Dimethylacetals **3** and **13** could be deprotected by treatment with aqueous H_2SO_4 in THF to give aldehydes **4** and **14**,[20] which were in turn reduced with $NaBH_4$ in ethanol to give alcohols **5** and **15**. The alcohols were converted to the corresponding mesylates, which yielded the benzylic bromides **6** and **16** upon treatment with LiBr in acetone.[21,22] To obtain the more reactive benzylic iodides for use as alkylating agents,[23] compounds **6** and **16** were treated with NaI to give compounds **7** and **17**. The thiol-specific bismethanethiosulfonate reagents **8** and **18** were obtained by treatment of bromides **6** and **16** with $NaSSO_2CH_3$ in aqueous acetone.[24] Reagents **8** and **18** are capable of making cross-links between SH of cysteine residues. Dialdehydes **4** and **14** were oxidized to dicarboxylic acids **9** and **19** by Ag_2O in an aq NaOH (10%)–THF mixture.[25] The dicarboxylic acids were converted to disuccinimidyl esters[26] **10** and **20** by treatment with *N*-hydroxysuccinimide and *N*,*N*′-dicyclohexylcarbodiimide (Schemes 1 and 2). The resulting disuccinimides are acylating reagents for amino groups and capable of making cross-link between lysine side chains.

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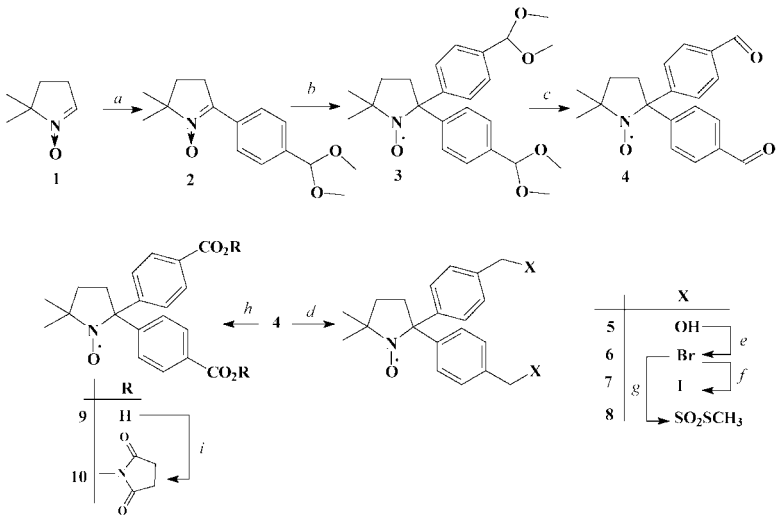
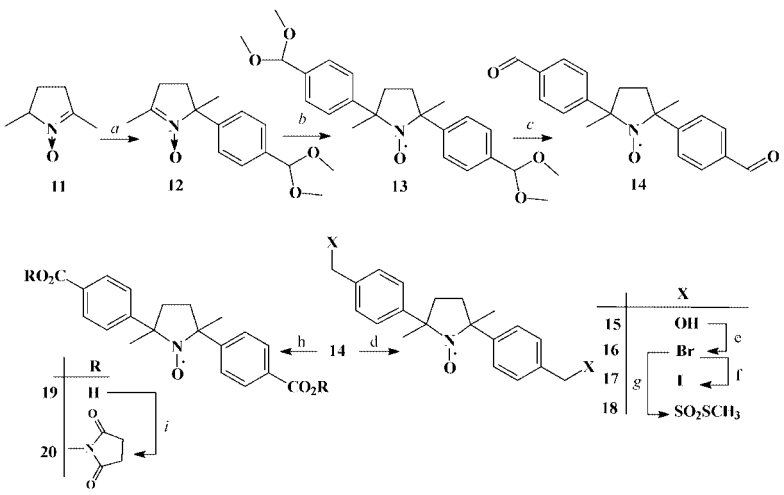


Scheme 1 *Reagents and conditions*: (a) 4-dimethoxymethylphenylmagnesium bromide (1.2 equiv) THF, 0 °C→reflux, 3 h, under N_2, then activated MnO_2 (1.0 equiv), O_2, 10 min, $CHCl_3$, reflux, 1 h, 70%; (b) 4-dimethoxymethylphenyllithium (1.2 equiv), THF, –78 °C, 20 min, then **2,** reflux 5 h, then activated MnO_2, $CHCl_3$, O_2, 30 min, 65%; (c) THF, aq H_2SO_4 (5%), r.t., 8 h, 92%; (d) $NaBH_4$ (2.5 equiv) EtOH, 40 °C, 30 min, 78%; (e) MsCl (2.2 equiv), Et_3N (2.2 equiv), CH_2Cl_2, 0 °C →r.t., 1 h, then LiBr (4.0 equiv), acetone, reflux, 1 h, 55%; (f) NaI, 4.0 equiv, reflux, 30 min, 68%; (g) $NaSSO_2CH_3$ (4.0 equiv), acetone–H_2O, 30 min, reflux, 43%; (h) Ag_2O (4.0 equiv), aq NaOH (10%)–THF, 60 °C, 40 min, then H^+, 45%; (i) *N*-hydroxysuccinimide (2.0 equiv), DCC (2.0 equiv), EtOAc, 0 °C ∅ r.t., 12 h, 61%.

In summary, new 2,2- and 2,5-disubstituted stable pyrrolidine radicals were obtained with simple procedures starting from readily available nitrones. These homobifunctional amino-specific and thiol-specific reagents serve as cross-linkers between lysine and cysteine residues, respectively. The general synthetic procedure is readily extendable to include spacer groups other than benzyl to vary the cross-linking distance. Applications of the reagents will be reported separately.

Mps 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. IR (Specord 75) spectra were in each case consistent with the assigned structure. Mass spectra were recorded on a VG TRIO-2 instrument in the EI mode (70 eV, direct inlet). ESR spectra were obtained from 10^{-5} molar solutions ($CHCl_3$), using a Bruker ECS-106 spectrometer. All monoradicals exhibited three equally spaced lines with a_N = 15.1–15.5 G. Preparative flash column chromatography was performed on Merck Kieselgel 60 (0.040–0.063 mm). Qualitative TLC was carried out on commercially prepared plates



Scheme 2 *Reagents and conditions*: (a) 4-dimethoxymethylphenylmagnesium bromide (1.2 equiv) THF, 0 °C →reflux, 3 h, under N_2, then activated MnO_2 (1.0 equiv), O_2, 10 min, $CHCl_3$, reflux, 1 h, 55%; (b) 4-dimethoxymethylphenylmagnesium bromide (1.2 equiv), THF, 0 °C, then **12**, reflux, 3 h, under N_2, then activated MnO_2, $CHCl_3$, O_2, 30 min, 60%; (c) THF, aq H_2SO_4 (5%), r.t., 8 h, 85%; (d) $NaBH_4$ (2.5 equiv) EtOH, 40 ºC, 30 min, 70%; (e) MsCl (2.2 equiv), Et_3N (2.2 equiv), CH_2Cl_2, 0 °C →r.t., 1 h, then LiBr (4.0 equiv), acetone, reflux, 1 h, 53%; (f) NaI (4.0 equiv), acetone, reflux, 30 min, 77%; (g) $NaSSO_2CH_3$ (4.0 equiv), acetone–H_2O, 30 min, reflux, 28%; (h) Ag_2O (4.0 equiv), aq NaOH (10%)–THF, 60 °C, 40 min, then H^+, 40%; (i) *N*-hydroxysuccinimide (2.0 equiv), DCC (2.0 equiv), EtOAc, 0 ºC →r.t., 12 h, 55%.

Table 1 New Compounds (**2**–**10**, **12**–**20**) Prepared[a]

Compound	Yield (%)	Mp (ºC)	IR (cm^{-1}) Neat or Nujol	MS (*m*/*z*)
2	70	60–62	1600, 1500 (C=C)	263 (M^+, 27), 248 (5), 232 (100), 216 (20)
3	60	oil	1600, 1500 (C=C)	414 (M^+, 2), 297 (22), 246 (83), 75 (100)
4	92	133–135	1680 (C=O), 1590 (C=C)	322 (M^+, 8), 308 (35), 292 (23), 236 (100)
5	78	107–109	3300 (OH), 1500 (C=C)	326 (M^+, 10), 312 (23), 296 (15), 240 (100)
6	55	174–176	1500 (C=C)	450/452/454 (M^+, 3/6/3), 436/438/440 (8/16/8), 364/366/368 (6/12/6), 285/287 (100/100)
7	68	183–185	1520 (C=C)	546 (M^+, 16), 473 (6), 420 (6), 333 (100)
8	43	115–117	1510 (C=C)	514 (M^+, <1), 484 (2), 404 (2), 65 (100)
9	45	143–145	1680 (C=O), 1600, 1560 (C=C)	354 (M^+, 9), 340 (43), 324 (55), 268 (100)
10	61	103–105	1760, 1720 (C=O), 1550 (C=C)	548 (M^+, <1), 435 (1), 115 (35), 55 (100)
12	55	oil	1600, 1510 (C=C)	263 (M^+, 38), 232 (76), 161 (77), 75 (100)
13	65	55–57	1600, 1500 (C=C)	414 (M^+, 3), 384 (18), 192 (13), 161 (100)
14	85	181–183	1680 (C=O), 1590, 1560 (C=C)	322 (M^+, 5), 308 (16), 292 (23), 146 (100)
15	70	163–165	3300 (OH), 1500 (C=C)	326 (M^+, 4), 312 (9), 296 (14), 148 (100)
16	53	161–162	1500 (C=C)	450/452/454 (M^+, 1/2/1), 420/422/424 (2/4/2), 210/212 (14/14), 131 (100)
17	77	155–157	1510 (C=C)	546 (M^+, 2), 389 (4), 258 (8), 131 (100)
18	28	138–139	1500 (C=C)	514 (M^+, <1), 484 (1), 80 (55), 65 (100)
19	40	242–244	1680 (C=O), 1590 (C=C)	354 (M^+, 2), 340 (6), 310 (20), 148 (100)
20	55	222–224	1760, 1720 (C=O), 1550 (C=C)	548 (M^+, <1), 435 (1), 115 (35), 55 (100)

[a] All compounds gave satisfactory microanalyses: C, H, N, S (where appropriate) ± 0.3.

($20 \times 20 \times 0.02$ cm) coated with Merck Kieselgel GF_{254}. All reagents and **1** were purchased from Sigma-Aldrich, Hungary. Compound **11** was prepared according to published procedures.[15] The physical and spectral data of all new compounds are listed in Table 1.

5-(4-Dimethoxymethylphenyl)-2,2-dimethyl-1-pyrroline *N*-Oxide (2) and 2-(4-Dimethoxymethylphenyl)-2,5-dimethyl-1-pyrroline *N*-Oxide (12)

To a stirred solution of 4-dimethoxymethylphenylmagnesium bromide [made from Mg turnings (1.44 g, 60.0 mmol) and 4-bromobenzaldehyde dimethyl acetal (13.86 g, 60.0 mmol)] in THF (40 mL), nitrone **1** or **11** (5.65 g, 50.0 mmol) was added dropwise at 0 °C and the mixture was stirred and refluxed for 3 h under N_2. After cooling, sat. aq NH_4Cl solution (20 mL) was added, the organic phase was separated, and the aq phase was extracted with $CHCl_3$ (2×20 mL). The combined organic phases were dried ($MgSO_4$), filtered and evaporated. The residue was dissolved in $CHCl_3$ (40 mL), activated MnO_2 (4.35 g, 50.0 mmol) was added in one portion, O_2 was bubbled through for 10 min, and the solution was stirred and refluxed for 1 h. The MnO_2 was filtered off, the solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography ($CHCl_3$–Et_2O) to give nitrone **2** or **12**.

Nitrone 2

Yield: 9.20 g (70%); off-white solid; mp 60–62 ºC; R_f 0.28 ($CHCl_3$–Et_2O, 2:1).

Nitrone 12

Yield: 7.23 g (55%); oil; R_f 0.28 ($CHCl_3$–MeOH, 9:1).

2,2-Bis-(4-dimethoxymethylphenyl)-5,5-dimethylpyrrolidin-1-yloxyl Radical (3)

To a stirred solution of 4-dimethoxymethylphenyl bromide (6.93 g, 30.0 mmol) in THF (30 mL), BuLi (2.5 M in hexane; 14 mL, 35 mmol) was added dropwise at –78 °C and the mixture was stirred for 20 min at this temperature. Nitrone **2** (6.57 g, 25.0 mmol) dissolved in THF (20 mL) was added dropwise and the mixture was stirred and refluxed for 5 h under N_2. After cooling, the reaction mixture was quenched with sat. aq NH_4Cl solution (20 mL), the organic phase was separated and the aq phase was extracted with $CHCl_3$ (2×20 mL). The combined organic phases were dried ($MgSO_4$), activated MnO_2 (870 mg, 10.0 mmol) was added, O_2 was bubbled through for 30 min, and the mixture was filtered and evaporated. The residue was purified by flash column chromatography (hexane–EtOAc) to give compound **3**.

Yield: 6.72 g (65%); red oil; R_f 0.28 (hexane–EtOAc, 2:1).

2,5-*trans*-Bis-(4-dimethoxymethylphenyl)-2,5-dimethylpyrrolidin-1-yloxyl Radical (13)

To a stirred solution of 4-dimethoxymethylphenylmagnesium bromide [made from Mg turnings (720 mg, 30.0 mmol) and 4-bromobenzaldehyde dimethyl acetal (6.93 g, 30.0 mmol)] in THF (25 mL), nitrone **12** (6.57 g, 25.0 mmol) dissolved in THF (20 mL) was added dropwise at 0 °C and the mixture was stirred and refluxed for 3 h under N_2. After cooling, sat. aq NH_4Cl solution (20 mL) was added, the organic phase was separated, and the aq phase was extracted with $CHCl_3$ (2 × 20 mL). The combined organic phases were dried ($MgSO_4$), activated MnO_2 (870 mg, 10.0 mmol) was added, O_2 was bubbled through for 30 min, and the mixture was filtered and evaporated. The residue was purified by flash column chromatography (hexane–EtOAc) to give compound **13**.

Yield: 6.21 g (60%); orange solid; mp 55–57 ºC; R_f 0.35 (hexane–EtOAc, 2:1).

2,2-Bis-(4-formylphenyl)-5,5-dimethylpyrrolidin-1-yloxyl Radical (4) and 2,5-*trans*-Bis-(4-formylphenyl)-2,5-dimethylpyrrolidin-1-yloxyl Radical (14)

To a stirred solution of bis(dimethyl acetal) **3** or **13** (8.28 g; 20.0 mmol) in THF (40 mL),
aq H_2SO_4 (5%; 10 mL) was added and the mixture was stirred for 8 h at r.t. Brine (30 mL) and Et_2O (30 mL) were then added, the organic phase was separated, dried ($MgSO_4$), filtered, evaporated and the residue was purified by flash column chromatography (hexane–EtOAc) to give the aldehyde **4** or **14**.

Aldehyde 4

Yield: 5.92 g (92%); yellow solid; mp 133–135 ºC; R_f 0.16 (hexane–EtOAc, 2:1).

Aldehyde **14**

Yield: 5.47 g (85%); yellow solid; mp 181–183 ºC; R_f 0.19 (hexane–EtOAc, 2:1).

2,2-Bis-(4-hydroxymethylphenyl)-5,5-dimethylpyrrolidin-1-yloxyl Radical (5) and 2,5-*trans*-Bis-(4-hydroxymethylphenyl)-2,5-dimethylpyrrolidin-1-yloxyl Radical (15)

To a stirred solution of aldehyde **4** or **14** (6.44 g, 20.0 mmol) in EtOH (50 mL), $NaBH_4$ (1.89 g, 50.0 mmol) was added and the mixture was stirred for 30 min at 40 ºC. The solvents were evaporated, the residue was dissolved in brine (30 mL), the aq phase was extracted with $CHCl_3$ (2 × 20 mL), dried ($MgSO_4$), filtered and the solvent evaporated. The residue was purified by flash column chromatography ($CHCl_3$–MeOH) to give compound **5** or **15**.

Compound 5

Yield: 5.08 g (78%); yellow solid; mp 107–109 ºC; R_f 0.26 ($CHCl_3$–MeOH, 9:1).

Compound 15

Yield: 4.56 g (70%); yellow solid; mp 163–165 ºC; R_f 0.33 ($CHCl_3$–MeOH, 9:1).

2,2-Bis-(4-bromomethylphenyl)-5,5-dimethylpyrrolidin-1-yloxyl Radical (6) and 2,5-*trans*-Bis-(4-bromomethylphenyl)-2,5-dimethylpyrrolidin-1-yloxyl Radical (16)

To a solution of bis(benzylic alcohol) **5** or **15** (3.26 g, 10.0 mmol) and Et_3N (2.22 g, 22 mmol) in CH_2Cl_2 (40 mL), methanesulfonyl chloride (2.52 g, 22.0 mmol) was added at 0 °C and the mixture was stirred at r.t. for 1 h. The reaction mixture was then washed with H_2O (30 mL), dried ($MgSO_4$), filtered, and the solvent evaporated. The residue was dissolved in anhyd acetone (50 mL), LiBr (3.48 g, 40.0 mmol) was added, and the mixture was heated at reflux and stirred for 1 h. After cooling, the acetone was evaporated, the residue was dissolved in Et_2O (40 mL), washed with H_2O (20 mL), the organic phase was separated, dried ($MgSO_4$), filtered, and the solvent evaporated. The residue was purified by flash column chromatography (hexane–EtOAc) to give compound **6** or **16**.

Compound 6

Yield: 2.48 g (55%); mp 174–176 ºC; R_f 0.56 (hexane–EtOAc, 2:1).

Compound 16

Yield: 2.39 g (53%); mp 161–162 ºC; R_f 0.60 (hexane–EtOAc, 2:1).

2,2-Bis-(4-iodomethylphenyl)-5,5-dimethylpyrrolidin-1-yloxyl Radical (7) and 2,5-*trans*-Bis-(4-iodomethylphenyl)-2,5-dimethylpyrrolidin-1-yloxyl Radical (17)

A solution of bis(benzylic bromide) **6** or **16** (904 mg, 2.0 mmol) and NaI (1.20 g, 8.0 mmol) in acetone was stirred and refluxed for 30 min. The NaBr was filtered off, and the acetone was evaporated under reduced pressure. The residue was dissolved in EtOAc (20 mL) and washed with aq $Na_2S_2O_3$ solution (5%; 10 mL). The organic phase was separated, dried ($MgSO_4$), filtered, evaporated under reduced pressure and the residue was purified by flash column chromatography (hexane–Et_2O) to give compound **7** or **17**.

Compound 7

Yield: 742 mg (68%); yellow solid; mp 183–185 ºC; R_f 0.58 (hexane–EtOAc, 2:1).

Compound 17

Yield: 841 mg (77%); yellow solid; mp 155–157 ºC; R_f 0.65 (hexane–EtOAc, 2:1).

2,2-Bis-(4-methanethiosulfonylmethylphenyl)-5,5-dimethylpyrrolidin-1-yloxyl Radical (8) and 2,5-*trans*-Bis-(4-methanethiosulfonylmethylphenyl)-2,5-dimethylpyrrolidin-1-yloxyl Radical (18)

To a solution of dibromo compound **6** or **16** (452 mg, 1.0 mmol) in acetone (10 mL) and H_2O (5 mL), $NaSSO_2CH_3$ (536 mg, 4.0 mmol) was added and the mixture heated under reflux for 30 min. After cooling, the acetone was evaporated, and the aq phase was extracted with $CHCl_3$ (2 × 10 mL). The organic phase was separated, dried ($MgSO_4$), filtered, evaporated and the residue was purified by flash column chromatography ($CHCl_3$–Et_2O) to give compound **8** or **18**.

Compound 8

Yield: 221 mg (43%); mp 115–117 ºC; R_f 0.46 ($CHCl_3$–Et_2O, 2:1).

Compound 18

Yield: 144 mg (28%); mp 138–139 ºC; R_f 0.47 ($CHCl_3$–Et_2O, 2:1).

2,2-Bis-(4-carboxyphenyl)-5,5-dimethylpyrrolidin-1-yloxyl Radical (9) and 2,5-*trans*-Bis-(4-carboxyphenyl)-2,5-dimethylpyrrolidin-1-yloxyl Radical (19)

To a vigorously stirred suspension of freshly precipitated Ag_2O (9.26 g, 40.0 mmol) in aq NaOH solution (10%; 40 mL), aldehyde **9** or **19** (3.22 g, 10.0 mmol) in THF (15 mL) was added at 60 ºC and the mixture was stirred and refluxed for 40 min. After cooling, the solution was filtered through a Celite pad and washed with MeOH (20 mL). The organic solvents were evaporated off, the aq phase was acidified with aq H_2SO_4 (5%) to pH 2, and extracted with $CHCl_3$ (3 × 20 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash column chromatography ($CHCl_3$–MeOH) to give dicarboxylic acid **9** or **19**.

Dicarboxylic Acid 9

Yield: 1.59 g (45%); mp 143–145 ºC; R_f 0.13 ($CHCl_3$–MeOH, 9:1).

Dicarboxylic Acid 19

Yield: 1.41 g (40%); mp 242–244 ºC; R_f 0.16 ($CHCl_3$–MeOH, 9:1).

2,2-Bis-(4-carboxyphenyl)-5,5-dimethylpyrrolidin-1-yloxyl Bis(succinimide Ester) Radical (10) and 2,5-*trans*-Bis-(4-carboxyphenyl)-2,5-dimethylpyrrolidin-1-yloxyl Bis(succinimide Ester) Radical (20)

To a stirred solution of carboxylic acid **9** and **19** (354 mg, 1.0 mmol) and *N*-hydroxysuccinimide (230 mg, 2.0 mmol) in EtOAc (10 mL), DCC (412 mg, 2.0 mmol) dissolved in EtOAc (5 mL) was added dropwise at 0 ºC. The mixture was stirred at r.t. overnight, the precipitated dicyclohexyl urea was filtered off, the filtrate was evaporated and purified by flash column chromatography ($CHCl_3$–Et_2O) to give active ester **10** or **20**.

Ester 10

Yield: 335 mg (61%); mp 103–105 ºC; R_f 0.39 ($CHCl_3$–MeOH, 9:1).

Ester 20

Yield: 301 mg (55%); mp 222–224 ºC; R_f 0.35 ($CHCl_3$–MeOH, 9:1).

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