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Synthesis of 2,5-Bis(spirocyclohexane)-Substituted Nitroxides of Pyrroline and Pyrrolidine Series, Including Thiol-Specific Spin Label: An Analogue of MTSSL with Long Relaxation Time

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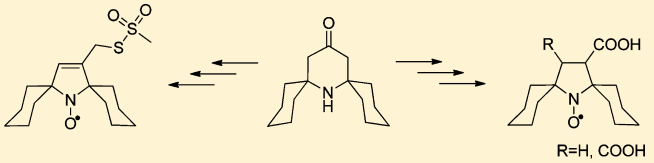
Supporting Information

ABSTRACT: The nitroxides of 7-azadispiro[5.1.5.2]-pentadecane and 7-azadispiro[5.1.5.2]pentadeca-14-ene series have been prepared, including thiol-specific methane thiosulfonate spin label for site-directed spin labeling. The effect of spirocyclohexane moieties on chemical and spectral properties has been studied. The obtained temperature dependencies of electron spin relaxation parameters demonstrate that new nitroxides may be suitable for PELDOR distance measurements at 80−120 K. Moreover, the new nitroxides demonstrated much higher stability toward reduction by ascorbate than spirocyclohexane-substituted nitroxides of piperidine series and showed 1.3−3.14 times lower reduction rates compared to corresponding 2,2,5,5-tetramethyl nitroxides.

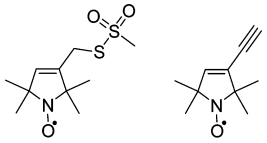


INTRODUCTION

Stable nitroxyl radicals (nitroxides) have found broad applications in various fields of science and technology. Numerous recent reviews demonstrate increasing importance of nitroxides in synthetic polymer chemistry,[1] magnetic materials,[2] reagents for selective oxidation,[1,3] biophysics,[4] structural and molecular dynamics studies,[5] therapeutic agents and biomedical research,[5,6] electrode active and charge storage materials in rechargeable batteries,[7] etc. Most of the widely used nitroxides have two pairs of methyl groups at α-carbon atoms of nitroxide moiety. However, it is known that introduction of spirocyclic moieties instead of the geminal methyl groups can improve properties of nitroxide with respect to nitroxide-mediated polymerization (NMP).[8] Moreover, an increase in steric requirements of substituents at α-carbon atoms produced by spirocylic moieties adjacent to nitroxide group may retard reduction of nitroxides with low-molecular biogenic reductants and enzymatic systems.[9−13] It has been found recently that piperidine nitroxides with spirocyclic moieties at α-carbons of nitroxide group may have serious advantages over 2,2,6,6-tetramethyl analogues in structural studies using pulse electron double resonance (PELDOR).[14,15] These studies imply application of site-directed spin labeling (SDSL) technique. Meanwhile, plenty of spin labels, e.g., MTSSL and TPA (Chart 1), which are often used in SDSL, belong to pyrroline nitroxides family.

It is also well-known that pyrrolidine nitroxides demonstrate much higher stability to reduction compared to piperidine derivatives.[16] Here we describe preparation of 7-azadispiro[5.1.5.2]pentadecane and 7-azadispiro[5.1.5.2]-pentadeca-14-ene nitroxides, including the spin label MTSSL

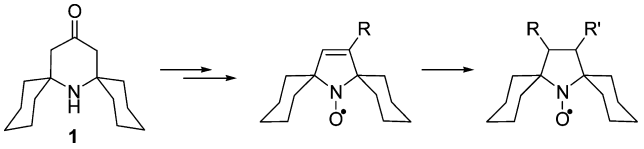
Chart 1



MTSSL TPA

analogue, from 7-azadispiro[5.1.5.3]hexadecan-15-one **1** (Scheme 1). The reduction rate constants of nitroxides with

Scheme 1



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ascorbate have been measured, and EPR parameters and temperature dependence of electron spin relaxation times of the new nitroxides are described.

RESULTS AND DISCUSSION

Synthesis. A classic pathway to 2,2,5,5-tetramethyl-3-pyrroline and 2,2,5,5-tetramethylpyrrolidine derivatives implies Favorskii rearrangement of brominated 2,2,6,6-tetramethylpi-

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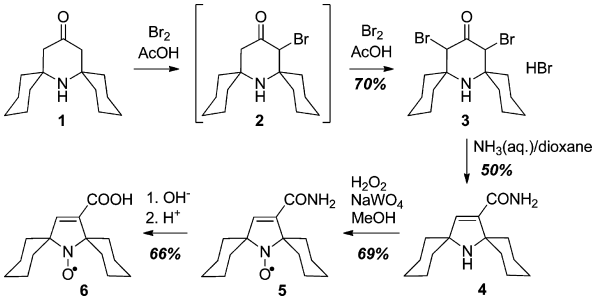
peridin-4-ones.[17,18] The bromination of **1** was performed in accordance with the procedure described for triacetonamine.[17] Addition of bromine to a solution of **1** in acetic acid rapidly leads to a bulky crystalline precipitate formation. The NMR ^{1}H spectrum of the crystalline precipitate showed that it contains no bromination products and consists of **1** hydrobromide. Stirring of **1** with 2 equiv of Br_2 after 24 h afforded a mixture of bromination products. Four equivalents of Br_2 were required for the reaction to be complete. The precipitate of **3** formed after 24 h of stirring was orange due to the presence of bromine. Recrystallization from $CHCl_3$ or from CCl_4 with a few drops of acetone gave colorless crystallosolvates of hydrobromide **3** with the chlorinated solvents.

Favorskii rearrangement was carried out in dioxane–aqueous ammonia mixture. The reaction afforded amide **4**, which was isolated with 50% yield (Scheme 2). IR spectrum of **4** showed

Scheme 2

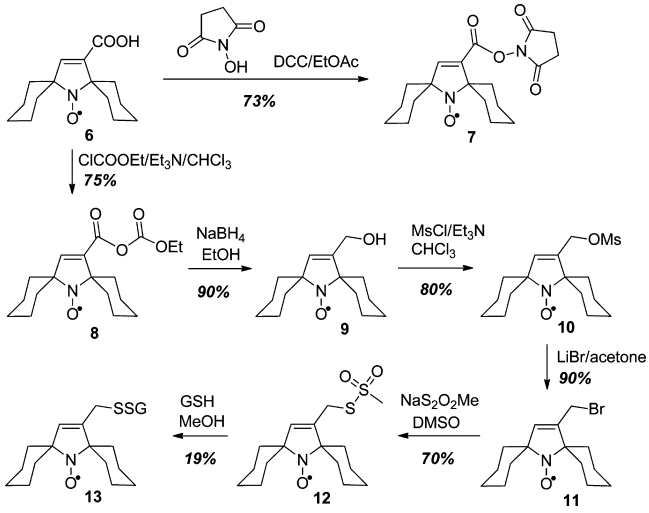


three strong absorption bands at 1652, 1624, and 1597 cm^{-1}, typical for vibrations of HC=C—CONH$_2$ moiety in 2,5-dihydropyrrol-3-carboxamides.[19] NMR spectra denote symmetrical structure of the molecule **4** with respect to plane of pyrroline ring. The two spirocyclohexane moieties are nonequivalent, showing the two sets of 3 different methylene group signals in the ^{13}C NMR spectra. The well-resolved multiplet (2H td) at 2.04 ppm in the ^{1}H NMR spectrum of **4** presumably belongs to axial protons at the positions 9 and 13 of 7-azadispiro[5.1.5.2]pentadeca-14-ene ring system with large triplet splitting (12.8 Hz) composed of equal couplings with geminal proton and with axial vicinal proton and small (4.8 Hz) doublet splitting due to interaction with equatorial vicinal proton. The signal downfield shift may result from unshielding effect of amide oxygen, denoting predominance of conformation with axial position of pyrroline nitrogen with respect to spirocyclohexane moiety at the position 8 of the tricyclic system. Spectral parameters for pyrroline ring atoms are close to those in the spectra of 2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-carboxamide.[20]

Oxidation of **4** with H_2O_2/Na_2WO_4 in water–methanol mixture gave corresponding nitroxide **5** with 69% yield. The C=C-conjugated amide bands in the IR spectrum of this nitroxide are slightly shifted into shorter wavelengths region, compared to those in the spectrum of **4** (1659, 1630, and 1601 cm^{-1}, cf.[19]). In this work we used ^{1}H NMR spectra recorded in the presence of thiophenol to prove the structure of nitroxides. Thiophenol is known to quench nitroxides efficiently.[21] Detailed study showed that the reaction gives a mixture of products, the main product being the corresponding amine.[22] In our experience, addition of PhSH to a solution of a nitroxide in $CDCl_3$ or in a mixture $CDCl_3$–CD_3OD leads to nearly quantitative reduction of nitroxide within a few minutes. The NMR spectra of the resulting mixtures are close to the spectra of corresponding amines in the presence of PhSH (see Figure S1, Supporting Information, for TEMPO as an example). Similarly, the ^{1}H NMR spectrum of **5**–PhSH mixture was similar to that of **4**.

Hydrolysis of the carboxamide group in the nitroxide **5** was complicated due to very low solubility of **5** in water (aqueous alkali). Almost no conversion was observed after heating of **5** under reflux in 10% aqueous NaOH for 24 h. Acceptable conversion (83%) was achieved upon heating of **5** in water–alcohol solution of NaOH in microwave oven (165 °C). The carboxylic acid **6** was then used in synthesis of spin labels in analogy with literature methods (Scheme 3).

Scheme 3



Treatment of **6** with *N*-hydroxysuccinimide in presence of DCC using the procedure described for 2,2,5,5-tetramethyl analogue[23] afforded the spin label **7**. Acylation of **6** with ethylchloroformate in presence of Et_3N afforded the anhydride **8**, which was then reduced with $NaBH_4$ to the 14-hydroxymethyl derivative **9**. The latter was converted into mesylate **10** and bromide **11**; remarkably, no modification of literature procedures was necessary.[24] Finally, treatment of **11** with sodium methanethiosulfonate in DMSO gave the spin label **12**. The nitroxide **12** is poorly soluble in water; nevertheless, it was found to be capable of reacting with glutathione in aqueous methanol. The nitroxide **13** is highly hydrophilic and attempts to extract it with EtOAc were not successful; nevertheless, it is poorly soluble in water and precipitates after the solution was concentrated in vacuum. This allowed us to isolate **13** as an individual compound.

It has been shown that pyrrolidine nitroxides demonstrate the highest stability to reduction.[25] Conversion of 3-pyrroline derivatives into pyrrolidines may be performed either by high-pressure catalytic hydrogenation[17] or using Michael nucleophilic addition to the double C=C bond.[26,27] The former reaction is normally applied to diamagnetic precursor before oxidation to nitroxide, because the nitroxide group could be reduced to amine under these conditions.

We have found that treatment of **5** with $LiBH_4$ leads to mild reduction of double C=C bond, the nitroxide and amide groups being not affected. Amide group vibrations are represented in the IR spectra of the nitroxide **14** with two strong bands at 1663 and 1633 cm^{-1} (amide I and amide II,

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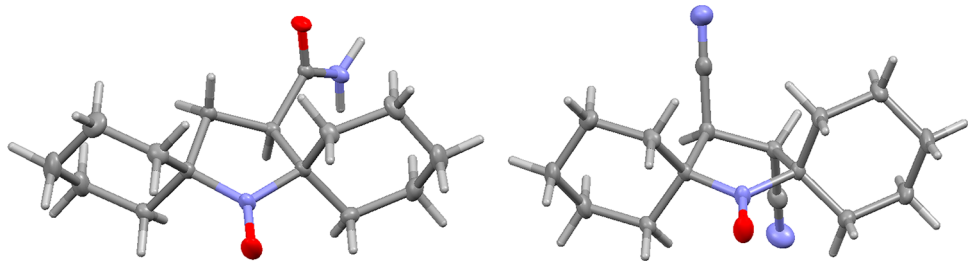
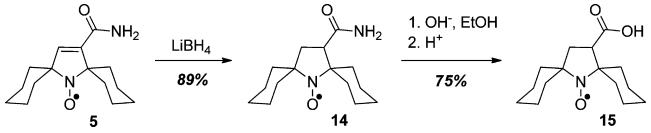


Figure 1. Structures of **14** (left) and **17** (right).

cf.[19]). NMR spectrum of the isolated nitroxide in presence of PhSH exhibited a methine proton multiplet at 2.66 ppm and superimposed multiplets of methylene protons of pyrrolidine ring at 2.01 and 2.04 ppm, similarly to that in the spectrum of 2,2,5,5-tetramethylpyrrolidine-3-carboxamide.[28] The structure of **14** was confirmed by X-ray analysis (Figure 1). Alkaline hydrolysis of **5** was performed in water−alcohol solution of KOH in a sealed ampule at 110−120 °C. The reaction afforded carboxylic acid **15** in 75% yield (Scheme 4).

Scheme 4



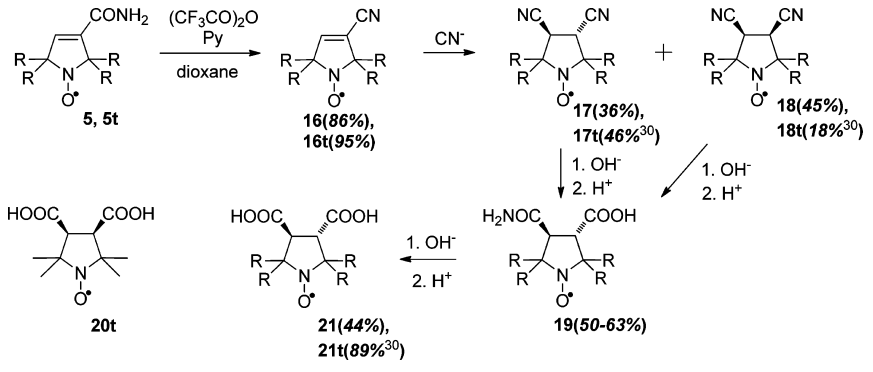
Nucleophilic addition to C═C bond of the pyrroline ring in pyrroline nitroxides usually requires conjugation with strong acceptor group, e.g., nitrile or ester. It has been shown that reaction of amides with trifluoroacetic anhydride in presence of base is a mild and efficient method for nitriles preparation.[29] Indeed, treatment of amides **5** and **5t** with this reagent afforded nitriles **16** and **16t** correspondingly with high yield (Scheme 5). Similarly to that described for 3-cyano-2,2,5,5-tetramethyl-2,5-dihydropyrrol-1-oxyl (**16t**),[30] addition of cyanide to **16** gives a mixture of isomeric dinitriles **17** trans (racemat) and **18** cis (meso-form), which were separated using column chromatography. The structure of trans-isomer **17** was confirmed by X-ray analysis (Figure 1). In contrast to that described for cyanide addition to **16t** where trans-isomer was predominant, the yield of **17** was a bit lower than that of **18**. Presumably this is a result of proton addition from the less hindered side of highly encumbered intermediate carbanion.

Alkaline hydrolysis of 2,2,5,5-tetramethyl-substituted dinitriles **17t** and **18t** was reported to give corresponding *trans*- and *cis*-dicarboxylic acids **20t** and **21t** (Scheme 4),[31] The structure of trans-isomer was later confirmed by X-ray analysis of corresponding diester.[32] It was also shown, that conversion of cis-dinitrile **18t** into trans-isomer **17t** occurs in the presence of *t*-BuONa.[32]

The dinitriles **17** and **18** were separately subjected to alkaline hydrolysis. Since these dinitriles were hydrophobic compounds, they were first heated in the sealed ampule to 100 °C with alcohol solution of NaOH (these conditions should cause both isomerization to more stable trans-isomer and conversion of nitrile groups to carboxamide) and then again heated under reflux in aqueous alkali for 48 h. The two samples obtained from *cis*- and *trans*-dinitriles showed identical IR spectra, and their identity and homogeneity were confirmed using HPLC analysis. The IR spectra of the samples showed strong absorption bands at 1715, 1670, and 1609 cm^{-1}. This pattern is more typical for monoamide of dicarboxylic acid. The data of element analysis were also closer to monoamide than to dicarboxylic acid. The ^1H NMR spectra of corresponding hydroxylamine prepared via hydrogenation in analogy to literature protocol[33] showed signals of AB system of non-equivalent methine protons of pyrrolidine ring at 3.45 and 3.50 ppm and a set of multiplets of cyclohexane ring protons, including low-field multiplet at 2.19−2.28 ppm (1H), denoting unsymmetrical structure. The structure of the compound prepared from *cis*-dinitrile **18** was determined on the basis of X-ray analysis data as crystallosolvate of **19** (Figure 2).

Remarkably, there are two pairs of **19** molecules with different conformation and ethyl acetate molecule in the independent lattice cell of the crystal. Several low-energy conformations of 7-azadispiro[5.1.5.2]pentadecane-7-oxyl ring system are possible, differing in inversion of cyclohexane ring

Scheme 5[a]



[a]R = Me (**t**), R + R = $(CH_2)_5$

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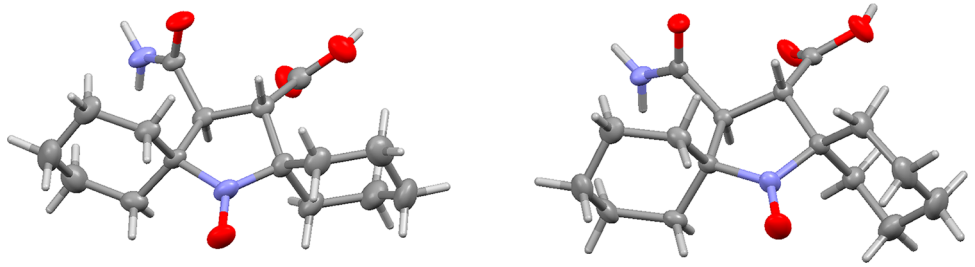

Figure 2. Structures of **19** *anti,anti* (left) and *anti,syn* (right) with respect to nitroxide N−O group.

"chair" conformations with respect to nitroxide group. According to simple DFT/PBE/3z calculations data (see the Supporting Information), the *anti,anti*-conformation is preferable for the structures without substituents at the positions 14 and 15 of the ring system. Introduction of carboxamide group into the position 14 of 7-azadispiro[5.1.5.2]pentadecane-7-oxyl makes the conformations *anti,anti* and *anti,syn* close in energy.

Hydrolysis of the remaining amide group requires much harder conditions; after 96 h of heating of **19** with 10% KOH in a sealed ampule at 150 °C, 60% conversion to **21** was achieved. The nitroxide **21** exhibited two strong carboxyl IR absorption bands at 1738 and 1703 cm^{-1} with no significant absorption between 1700 and 1500, denoting that hydrolysis is complete. A sharp singlet of methine protons of pyrrolidine ring at 3.46 ppm in the ^1H NMR of corresponding hydroxylamine prepared via hydrogenation supports symmetrical structure of the molecule.

EPR Studies. It has been shown that replacement of geminal methyl groups adjacent to nitroxide moiety with bulky substituents may result in significant line broadening in the EPR spectra.[11,34] In accordance to these observations, the linewidths in the spectra of nitroxides **5**, **6**, **9**, **14**, **15**, **17**−**19**, **21** were close to each other and equal to 0.45 mT. The parameters of the spectra obtained by simulations are listed in Table 1. A typical spectra of 7-azadispiro[5.1.5.2]pentadecane-7-oxyl and a proxyl nitroxide are shown in Figure 3.

Table 1. Values of g-Factors and Nitrogen *hfc* Constants a_N, mT[a]

nitroxide	g-factor (±0.00002)	a_N, mT (±0.3%)
5	2.00586	1.578
6	2.00581	1.596
9	2.00584	1.605
14	2.00586	1.598
15	2.00584	1.608
17	2.0059	1.483
18	2.00597	1.504
19	2.00598	1.580
21	2.00581	1.598
22	2.00594	1.663

[a]Spectra were recorded in 0.1 M phosphate buffer (pH 7.2) with 5% (v/v) of ethanol for better solubility.

Detailed study of line shapes in the spectra of imidazolidine nitroxides with geminal ethyl groups at the positions 2 and 5 of the heterocycle showed that introduction of each pair of geminal ethyl groups produced an additional splitting of about 0.2 mT in the EPR spectra, which arises from the hyperfine coupling (*hfc*) constant on only one of four methylene hydrogen atoms of two geminal ethyl substituents.[34] EPR

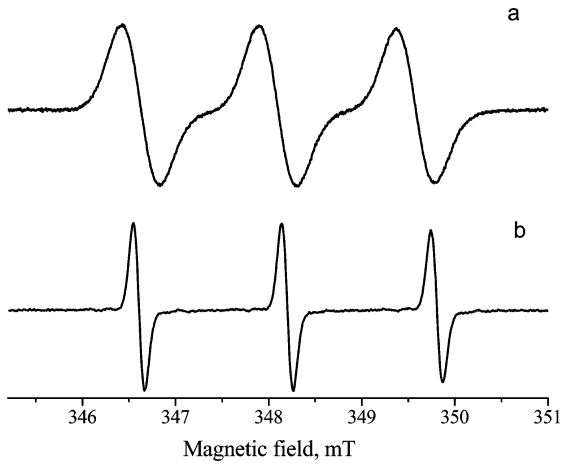

Figure 3. Experimental EPR spectra of 0.1 mM solutions of nitroxides **14** (a) and 3-carbamoyl proxyl (**14t**) (b) in 0.1 M phosphate buffer (pH 7.2) supplemented with ethanol 5% (v/v). Spectrometer settings were as follows: frequency, 9.87 GHz; mw power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.25 and 0.1 mT for **14** and **14t**, respectively; conversion time, 5.12 ms.

spectra of deoxygenated aqueous solution (5% of DMSO, pH 7.2) of 0.1 mM of nitroxide **6** also showed a partially resolved pattern, which was simulated using parameters of *hfc* on nitrogen, a_N = 1.6 mT, five a_H = 0.145 mT (5H), four a_H = 0.077 mT (4H); additional line width of 0.064 mT (Gaussian shape) and 0.074 mT (Lorentzian shape) (Figure 4c). The splitting may arise from *hfc* with four pairs of axial and equatorial hydrogen atoms at the positions 2 and 6 of the two cyclohexane rings; the latter may differ in spin density distribution due to steric and electronic effect of carbamoyl group. The last *hfc* a_H = 0.145 mT was attributed the H at position 3 in the ring simiar to the 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-l-oxyl, for which the H at position 3 in the ring has a coupling of about 0.05 mT.[35] Our simulation implies that the value of hfi with the hydrogen at the position 15 of 7-azadispiro[5.1.5.2] pentadeca-14-ene ring system is somewhat higher than expected. In our case, this value is equal to 0.145 mT instead of 0.05mT. This difference in *hfc* with hydrogens at the double bond may result from changes in pyrroline ring geometry, e.g., because of interaction of carboxamide group with cyclohexane ring. However, it is impossible to make absolutely definite assignments of the *hfc* constants on the basis of experimental data we have.

Note that EPR spectra of deoxygenated solution of 0.01 mM of nitroxide **15** and nitroxide **19** showed a much less resolved pattern and did not allow us to obtain reliable values of hydrogen atoms *hfc* (Figure 4a,b). This difference in spectral lines structure may result from higher conformational rigidity of

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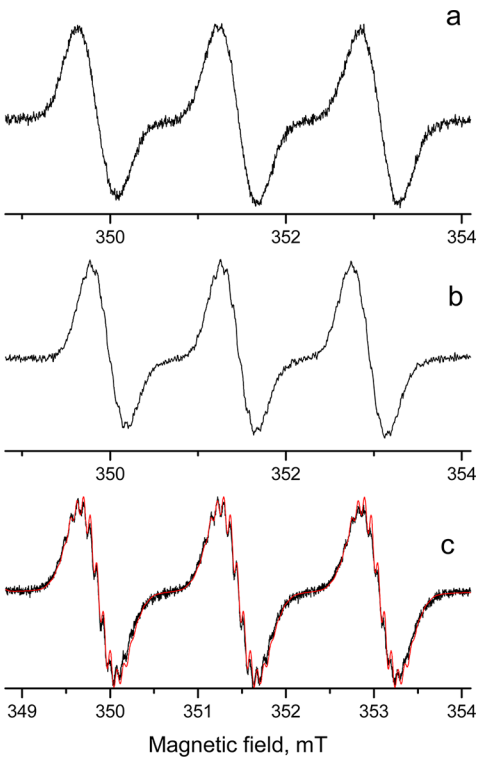

Figure 4. Experimental and simulated EPR spectra of 0.1 mM deoxygenated aqueous solution 5% DMSO of nitroxides **15** (a), **19** (b), and **6** (c) at pH 7.2 after three freezing–pumping–thawing cycles. Simulation parameters for radical **6**: a_N = 1.6 mT, five a_H = 0.145 mT (5H), four a_H = 0.077 mT (4H); additional line width of 0.064 mT (Gaussian shape) and 0.074 mT (Lorentzian shape). Spectrometer settings were as follows: frequency, 9.87 GHz; microwave power, 6.39 mW; modulation frequency, 100 kHz; modulation amplitude, 0.003 mT; conversion time, 655.36 ms.

7-azadispiro[5.1.5.2]pentadeca-14-ene ring system compared to 7-azadispiro[5.1.5.2]pentadecane one and/or from unsymmetrical structure of nitroxides **15** and **19** producing a set of small splittings with different *hfc*.

It was shown recently by Eaton and co-workers[15] that replacement of the geminal methyl groups at the carbons adjacent to the nitroxyl N–O group in conventional spin labels, such as TOAC, with spirocyclohexyl groups removes a major spin echo dephasing mechanism and makes electron spin echo dephasing time, T_m, the time constant for the echo decay as a function of the time delay between pulses, long enough to permit pulse electron double resonance (PELDOR)[36] measurements of interspin distance at the temperatures of liquid nitrogen range. This allows us to avoid using expensive liquid helium and makes applications of pulsed EPR spectroscopy in studying structure and conformation of peptides and proteins easier. The mechanism of spin echo dephasing of nitroxyl radicals with spirocylcohexyl groups at the positions 2 and 6 of piperidine ring was studied in details by Eaton coworkers.[14,15] It was shown that the observed spin echo decay curves in water–glycerol (1:1) solution at low temperature are described by $I = I_0 \exp(-(t/T_2))^n$, where $n > 1$, and are determined by nuclear spin diffusion of solvent protons.[37] The increase of different dynamic processes' contributions to spin–spin relaxation mechanism leads to a decrease of the value of n to 1. The accuracy of PELDOR measurements of the certain range of the distances depends on electron spin echo dephasing time, T_m.

In this work, temperature dependence of electron relaxation time T_1 and electron spin echo dephasing time T_m in water–glycerol (1:1) solutions was measured for nitroxides **5**, **6**, **9**, **12** and **13**. Typical spin echo decay curves observed for nitroxide **9** and MTSSL (for comparison) are shown on the inset of Figure 5a. On the contrary to MTSSL, spin echo decay curve for spiro-substituted nitroxides is not monoexponential. The same difference in the shape of spin echo decay was observed previously for piperidine nitroxides with spirocyclic moieties, and the origin of this difference was discussed in detail by Rajca et al.[14] Since in our experiments the spin echo decay curve is not monoexponential, the electron spin relaxation times were estimated as the time during which the spin echo signal decreases by 2.7. Temperature dependencies of T_1 and T_m for nitroxides **5**, **6**, **9**, **12** and **13** estimated using this procedure are shown in Figure 5. The estimated T_m for investigated nitroxides are practically independent of temperature between 50 and 120 K and are nearly the same for all nitroxides studied. The value

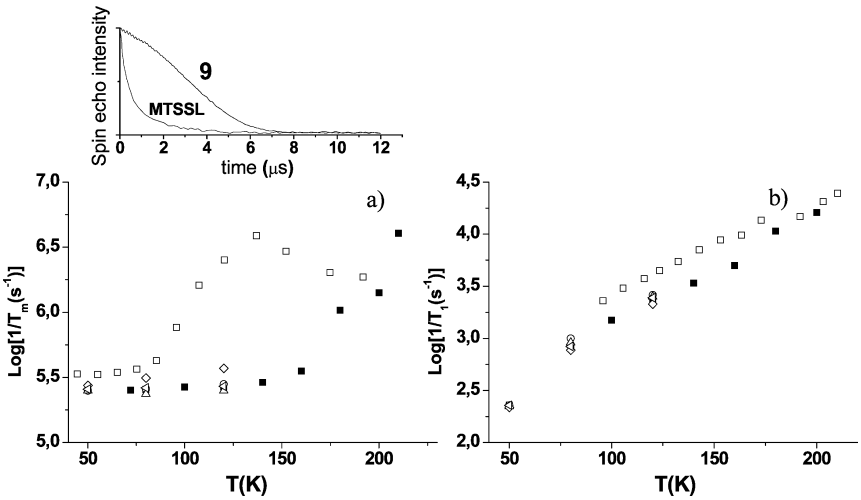

Figure 5. Temperature dependence of T_m (a) and T_1 (b) electron relaxation times in water–glycerol (1:1) solution for MTSSL (□),[14] **9** (■), **5** (○), **6** (Δ), **12** (◊), and **13** (▽). Accuracy of measurements is about 5%. The inset in (a) shows spin typical echo decay curves for MTSSL[14] and **9** in water–glycerol solution (1:1) at 80 K. Note that the obtained T_1 values could be effected by spectral diffusion.

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of T_m coincides (within measurements error) with those for nitroxides of 7-azadispiro[5.1.5.3]hexadecane series.[14,15]

Note that PELDOR allows the measurements of distances between paramagnetic particles in chaotically oriented systems—pairs and supramolecular ensembles—in the range of 1.5−8.0 nm. At 80 Å one needs to measure a dipolar oscillation at up to 10 μs in order to accumulate data describing a full oscillation, allowing accurate conversion to a distance distribution.[38−45] Because of high T_m value at 80−120 K, spin echo intensity for spirocyclohexane-substituted nitroxides is significantly larger at the same time delay (5−10 μs) in comparison to conventional spin labels such as MTSSL. This makes these nitroxides suitable for the PELDOR measurements of interspin distance in liquid nitrogen temperature range.[46]

The obtained relaxation rates T_1 (Figure 5b) for spirocyclohexane-substituted nitroxides in the temperature range 50−120 K (Figure 5b) are determined by Raman process and are proportional to T^2 at high temperature limit as for most of nitroxides.[14,47] At temperature range 120−200 K additional process (a local mode) contributes to relaxation rates. This allows one to increase the repetition rate with increase of the temperature and, as a result, to compensate for the reduction of spin echo intensity, which is proportional to the Boltzmann factor ~1/T.

Attachment of the spin label **12** to glutathione did not lead to significant changes in the values of T_1 and T_m for **13** (Figure 5, Table 2).

Table 2. Electron Spin Echo Dephasing Time, T_m, and Electron Relaxation Time, T_1, in Water−Glycerol (1:1) Solution for Investigated Radicals at 80 and 120 K[a]

radical	$\log[1/T_m]$ (s^{-1}) at 80 K	$\log[1/T_1]$ (s^{-1}) at 80 K	$\log[1/T_m]$ (s^{-1}) at 120 K	$\log[1/T_1]$ (s^{-1}) at 120 K
5	5.37	2.96	5.44	3.42
6	5.41	2.96	5.40	3.40
9	5.41	3.00	5.42	3.38
12	5.49	2.87	5.57	3.33
13	5.42	2.92	5.43	3.39
MTSSL	5.57 [ref 14]	3.2	6.4 [ref 14]	3.58 [ref 14]

[a]Experimental error was about 5%. Note that the obtained T_1 values could be effected by spectral diffusion.

Figure 6 shows EPR spectra of **12** and **13** in the DMSO/water mixture. The tumbling correlation times τ_{corr} = 0.125 ns for **12** and τ_{corr} = 0.316 ns for **13** were obtained by simulation of experimental spectra (isotropic and fast motion model was used in EasySpin program; for more details concerning simulation parameters see caption to Figure 6). The changes of the line shape in EPR spectra correspond to the two times increase of rotational correlation time of **13** in comparison with **12**. This is in a good agreement with estimation ($\tau_{corr} \propto R^3$) of molecular radius of both molecules: R (**12**) ~ 0.75 R (**13**). Rotational correlation time can be estimated using formula $\tau_{corr} = (4\pi\eta R^3)/(3k_BT)$, where η = 3.5 cP,[48] viscosity of water−DMSO mixture (1:1) at T = 298 K; k_B= 1.38 × 10^{23} JK^{-1}, Boltzmann constant and hydrodynamic radius R of nitroxides.

Taking into account the values of R = 4.5 Å for **12** estimated by ChemBio3D program, we obtained τ_{corr} = 0.32 ns. Note that evaluation rotational correlation time for **12** from hydrodynamic radius gives higher value than that obtained from simulations. The reason for that could be the not spherical shape of molecules **12**, which can lead to inaccuracy of evaluated hydrodynamic radius. Another possible reason is that *hfc* and g-factor anisotropy is averaged by motion of nitroxide fragment of molecule with shorter correlation time than rotational correlation time of whole molecule (especially for **13**).

Reduction of Nitroxides with Ascorbate and Lipophilicities. Solubility in water/partition coefficients and stability are important parameters for various biological applications of the nitroxides, such as MRI and EPR studies (redox status and distribution) and possible pharmacological applications (SOD-mimetics, antioxidants, etc). Bulky nonpolar cyclohexane rings apparently make the new set of nitroxides more hydrophobic compared to their tetramethyl analogues. In particular, the nitroxides **16**−**18** are practically insoluble in water, while **5**−**10** showed low solubility. The partition coefficients of water-soluble nitroxides in *n*-octanol −0.1 M phosphate buffer mixture (pH 7.2) were measured for three nitroxides **14**, **15**, **21**. Among these nitroxides the amide **14** showed predictably the highest lipophilicity ($P_{o/w}$= 99.6). The nitroxides **15** and **21** are expected to exist in the anionic carboxylate form at pH 7.2. Nevertheless **15** was located mainly in organic phase ($P_{o/w}$= 3.2), which makes the nitroxide similar

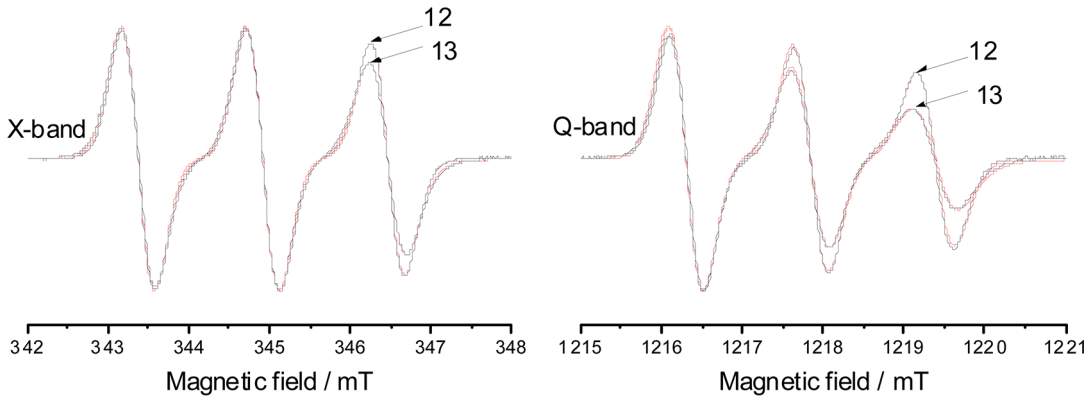

Figure 6. EPR spectra of 0.5 mM of **12** and **13** in the DMSO/water mixture (1:1). Black line, experimental spectra; red line, simulated spectra. The tumbling correlation times τ_{corr} = 0.125 ns for **12** and τ_{corr} = 0.316 ns for **13** were obtained by EasySpin spectra simulation. Simulations were performed with taking into account modulation of *hfc* anisotropy and g-factor anisotropy by fast motion with correlation time τ_{corr} for both spectra in Q- and X-band. Simulation parameters are as follows: HFC tensor a_N = [0.5 0.5 3.9] mT and g-tensor = [2.0091 2.0061 2.0022]; additional line width was 0.07 mT in X-band and 0.13 mT in Q-band (Lorentzian line shape).

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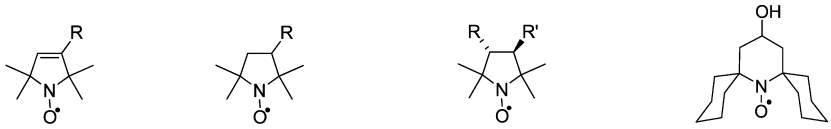
Chart 2



R=$CONH_2$ (**5t**); R=$CONH_2$ (**14t**); R,R'= COOH (**21t**); **22**

COOH (**6t**); COOH (**15t**);

CH_2OH (**9t**);

to 4-hydroxy-TEMPO ($P_{o/w}$= 3.14), and oxo-TEMPO ($P_{o/w}$= 1.8).[11] Dianionic **21** is hydrophilic ($P_{o/w}$= 0.004).

Bulky substituents are known to retard nitroxides reduction in biological systems, increasing the lifetimes of the spin probes. Here we studied the reaction of water-soluble nitroxides **5**, **6**, **9**, **14**, **15**, **19**, **21** and their 2,2,5,5-tetramethyl analogues (Chart 2) with ascorbic acid.

It is known that the ascorbic acid plays a significant role in reduction of nitroxides in biological samples.[49] Reaction of nitroxides with ascorbate has a complex mechanism with several reversible steps, and observed kinetics of nitroxides reduction may differ from exponential decay.[50] These deviations were shown to be significant for nitroxides with very low reduction rate constant and/or at high degree of conversion. It has been shown, however, that glutathione can scavenge ascorbate radical suppressing the reverse steps and leading the kinetics to monoexponent pattern.[40] This method was used to improve the accuracy of the rate constant measurements for some of the new nitroxides. Note that the second order decay rate constant is very sensitive to multiple factors: temperature, pH, metal impurities in buffers or in nitroxides, which catalyze the ascorbate kinetic, etc., on the reduction rate constants of nitroxides by ascorbic acid.[51] In our experiments we used double deinonized water, and we checked experimentally that addition of DTPA did not affect the kinetics. Temperature is the main factor that leads to changes in kinetics. We did not perform precise control of the temperatures (experiments were performed at room temperature and temperature changes could be 4−5 degrees); thus, the error for rate constant measurements may reach 15%. The examples of nitroxide decay kinetics in presence of ascorbate are shown in Figure 7, and the rate constants are listed in Table 3.

Redox properties of nitroxides are obviously influenced by both steric and electronic effects of the substituents. Replacement of methyl groups in 4-oxo-TEMPO with spirocyclohexane moieties was shown to decrease the nitroxide reduction rate; however, effect of spirocyclohexane moieties was smaller than that of ethyl groups.[13] Similarly, replacement of geminal methyl groups in the positions 2 and 5 of pyrrolidine or pyrroline ring with spirocyclohexane moieties leads to decrease of the nitroxide reduction rate (Table 3), although the effect is much smaller than those observed upon replacement the four methyl groups to ethyl in nitroxides of isoindoline, imidazoline, imidazolidine and piperidine series.[9,10,12] The value of this effect depends on nitroxide structure. The largest effect (from 2.6 to 3.14 fold) was observed for pyrroline nitroxides, whereas oxidant activity of 3-monosubstituted pyrrolidine nitroxides showed much weaker dependence on the substituents (from 1.3 to 1.7 fold). This difference in the substituents effect may result from peculiarities of conformational population of 7-azadispiro[5.1.5.2]pentadecane and 7-azadispiro[5.1.5.2]pentadeca-14-ene ring systems (see the quantum chemical calculations data in the Supporting Information). Apparently, the *anti,anti* conformation of the spirocyclohexane rings with respect to nitroxide group (which is predominant in 14-substituted 7-azadispiro[5.1.5.2]-pentadecane-7-oxyls) favors the reaction of nitroxide with ascorbate anion. In 14-substituted 7-azadispiro[5.1.5.2]-pentadeca-14-ene-7-oxyls the *anti,syn*-conformation is predominant, and in nitroxide group is partly protected with cyclohexane moiety. As a result, 2,5-bis-spirocyclohexane derivatives demonstrate almost no difference between oxidant activities of nitroxides of pyrroline and pyrrolidine series, while 2,2,5,5-tetramethyl substituted nitroxides of pyrrolidine series are reduced ca. twice slower than corresponding pyrroline nitroxides.

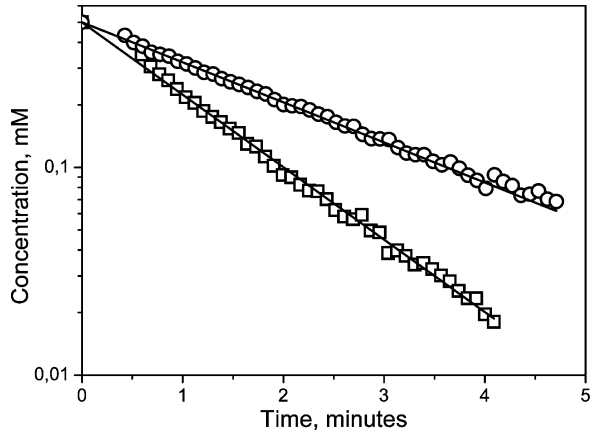


Figure 7. The decay of nitroxides **15** (○) and **19** (□) in carbonate-buffered solutions of ascorbic acid (0.1 M) and glutathione (50 mM) at pH 7.2 in logarithmic scale (see the Experimental Section). The corresponding rate constants are listed in Table 3.

Table 3. Values of the Reduction Rate Constants of Nitroxides with Ascorbic Acid, $k_{asc} \times 10^{-2}$, M^{-1} s^{-1} at pH 7.2[a]

nitroxide	k_{II}, 10^{-2} M^{-1} s^{-1}	nitroxide	k_{II}, 10^{-2} M^{-1} s^{-1}	k(Nt)/k(N)
5	22	**5t**	58	2.63
6	7	**6t**	22	3.14
9	9	**9t**	25	2.78
14	18	**14t**	30	1.67
15	7.4	**15t**	9.7	1.3
19	13			
21	7.7	**21t**	20	2.6
22	125			

[a]Experimental error was 15%.

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The oxidant activities of the nitroxides listed in Table 3 within 2,2,5,5-tetramethyl and 2,5-bis-spirocyclohexane series are clearly dependent on electronic effects of the substituents. Electron-rich anionic carboxylate groups decrease the reduction rates, whereas electron-acceptor carboxamide groups make the nitroxides stronger oxidants. It should also be noted that both 2,2,5,5-tetramethyl- and 2,5-bis-spirocyclohexane substituted 5-membered ring nitroxides demonstrate much higher stability to reduction than sterically hindered nitroxide of piperidine series **22**.

We also would like to note the potential suitability of these labels to in-cell PELDOR measurements, which have been of great interest during the past years.[52−54]

CONCLUSIONS

In this work, we have described convenient methods for synthesis of spin labels and spin probes of 7-azadispiro[5.1.5.2]-pentadecane and 7-azadispiro[5.1.5.2]pentadeca-14-ene series. The new spin labels demonstrated clear advantages over 2,2,5,5-tetramethylpyrroline nitroxides with respect to electron spin relaxation rates, which should favor PELDOR distance measurements at liquid nitrogen temperature range. The new nitroxides also demonstrated higher stability to reduction compared to their 2,2,5,5-tetramethyl analogues, which makes them promising spin probes for EPR and MRI redox status measurements in biomedical research.

EXPERIMENTAL SECTION

7-Azadispiro[5.1.5.3]hexadecan-15-one **1**[12] and the nitroxides **5t**,[17] **6t**,[17] **9t**,[24] **14t**,[18] **15t**,[18] **21t**[26,30] and **22**[55] were prepared according to the literature protocols. ^{1}H NMR spectra were recorded at 300 or 400 MHz, and ^{13}C NMR spectra were recorded at 75 or 100 MHz, as indicated next to each NMR analysis. To confirm structure of nitroxides, PhSH (10−20 mg) was added into NMR tube with nitroxide solution, and the sample was heated to 40−50 °C for ca. 30 s prior to recording the spectra. Alternatively, the nitroxide (10−30 mg) was dissolved in methanol (2−3 mL) and subjected to hydrogenation on 5% Pd/C in accordance to previously published protocol.[33] After the reaction was complete, the solution was acidified to pH 1 with concentrated HCl, filtered, and evaporated to dryness under reduced pressure. The residue (the hydroxylamine chlorohydrate) was used for spectra recording. ^{1}H and ^{13}C chemical shifts (δ) were internally referenced to the residual solvent peak. IR spectra were acquired on FT-IR spectrometer in KBr and are reported in wave numbers (cm^{-1}). The HPLC analyses were performed on a C8 column (150 × 4.6 mm 5 μm) with a gradient from 30% H_2O (0.1% TFA)/70% MeOH (0.1% TFA) to 10% H_2O (0.1% TFA)/90% MeOH (0.1% TFA) over 15 min with a flow of 0.8 mL/min. Reactions were monitored by TLC carried out using UV light as visualizing agent and/or aqueous permanganate. Column chromatography was performed on silica gel 60 (70−230 mesh). HRMS were recorded on double-focusing, high resolution mass spectrometer equipped with high performance toroidal ESA.

7-Azadispiro[5.1.5.3]hexadecan-15-one hydrobromide (1). A solution of Br_2 (0.554 g, 3.46 mmol) in acetic acid (2 mL) was added dropwise to a stirred solution of **1** (0.8 g, 3.4 mmol) in acetic acid (2 mL). The crystalline precipitate started to form within few minutes. The precipitate formed was immediately filtered off, washed with diethyl ether, then with a Et_2O−acetone−EtOH mixture and dried at room temperature. **1 × HBr** yield 0.6 g, 56%, colorless crystals: ^{1}H NMR (400 MHz, $CDCl_3$−CD_3OD 4:1) 1.04−1.20 (m, 2H), 1.20−1.32 (m, 4H), 1.50−1.58 (m, 2H), 1.58−1.66 (m, 4H), 1.84 (td, J = 12.5, 3.5, 4H), 2.16 (d, J = 12.5, 4H); 2.83 (s, 4H). Basification with aqueous $NaHCO_3$ recovers the starting material.

14,16-Dibromo-7-azadispiro[5.1.5.3]hexadecan-15-one hydrobromide (3). A solution of Br_2 (21 g, 136 mmol) in acetic acid (20 mL) was added dropwise to a stirred solution of **1** (8 g, 34 mmol) in acetic acid (20 mL). The mixture was left overnight, the precipitate was filtered off, washed with acetic acid, then with diethyl ether and dried at room temperature to yield **3** (11 g, 70%) as pale orange crystalline powder. The resulting crude **3** was used without further purification: mp 157−159 °C ($CHCl_3$) colorless crystals; IR ν_{max} 2935, 2866, 2784, 2723, 2665, 1743, 1686, 1633, 1558, 1456, 1365, 1265, 1178, 1151, 1139, 1119, 606, 543; ^{1}H NMR (400 MHz; $CDCl_3$−CD_3OD 4:1) 1.33−1.55 (m, 8H), 1.76- 1.87 (m, 2H) 1.88−2.04 (m, 4H), 2.16−2.25 (m, 4H), 2.29−2.40 (m, 2H), 5.22 (s, 2H); ^{13}C NMR (75 MHz; $CDCl_3$−CD_3OD 10:1) 20.0 (CH_2), 20.4 (CH_2), 22.9 (CH_2), 31.6 (CH_2), 33.6 (CH_2), 53.6 (CH), 67.6 (C), 189.3 (C). Anal. Calcd for $C_{15}H_{24}Br_3NO \times CHCl_3$: C, 32.38; H, 4.25; N, 2.36. Found: C, 32.57; H, 4.33; N, 2.60. In a separate experiment, bromination with 2 equiv of Br_2 for 24 h afforded the mixture, presumably containing **3** and **2** in a molar ratio ca. 2:5; **2**: ^{1}H NMR (400 MHz; $CDCl_3$−CD_3OD 4:1) 4.62 (s, 1H), 3.45 and 2.90 (AB, J = 14, each 1H).

14-Carbamoyl-7-azadispiro[5.1.5.2]pentadeca-14-ene (4). The crude hydrobromide **3** (3.5 g, 7.4 mmol) was added portionwise to a mixture of 25% aqueous ammonia (33 mL) and 1,4-dioxane (30 mL) upon vigorous stirring. After the precipitate was dissolved completely the mixture was heated to 40 °C and stirred for 0.5 h. Then solid NaOH (1 g, 25 mmol) was added, and the mixture was evaporated under reduced pressure. The residue was triturated with chloroform, inorganic precipitate was filtered off and washed with chloroform. The chloroform solutions were evaporated under reduced pressure, the residue was triturated with cold benzene, and crystalline precipitate was filtered off to give **4**: yield 0.9 g (50%), colorless crystals, mp 145−147 °C (ethyl acetate−hexane 4:1); IR ν_{max} 3439, 3337, 3265, 3227, 3204, 3184, 2936, 2856, 1652, 1624, 1597, 1450, 1387, 1313, 1277, 1163, 1144, 1099, 932, 912, 887, 820, 773, 709, 665, 636; ^{1}H NMR (400 MHz; $CDCl_3$) 1.17 (dtt J_d = 11.6, 11.9, 4.8, 1H), 1.33−1.62 (m, 17H), 2.04 (td, J_t = 12.6, 4.8, 2H), 5.81 (br. s, 2H), 6.30 (s, 1H); ^{13}C NMR (100 MHz; $CDCl_3$) 22.3 (CH_2), 23.2 (CH_2), 25.0 (CH_2), 25.3 (CH_2), 37.6 (CH_2), 39.8 (CH_2), 66.2 (C), 68.5 (C), 140.2 (CH), 142.6 (C), 167.9 (C). Anal. Calcd for $C_{15}H_{24}N_2O$: C, 72.54; H, 9.74; N, 11.28. Found: C, 72.58; H, 10.05; N, 11.53. HRMS EI (m/z) calcd for $C_{15}H_{24}N_2O$ 248.1883, found 248.1880.

14-Carbamoyl-7-azadispiro[5.1.5.2]pentadeca-14-ene-7-oxyl (5). A solution of $Na_2WO_4 \times 2\ H_2O$ (0.3 g, 0.9 mmol) and EDTA disodium salt (0.3 g, 0.89 mmol) in water (5 mL) was poured into a solution of amine **4** (3.5 g, 14 mmol) in methanol (25 mL). Then 30% hydrogen peroxide (5 mL) was added, and the solution was stored in a dark place at room temperature (20 °C) for 30 days. The solution was cooled to 0 °C, and crystalline precipitate of nitroxide **5** (1.8 g, 49%) was filtered off. A new portion of 30% hydrogen peroxide (5 mL) was added to the filtrate, and the solution was again allowed to stand for 30 days. The methanol was distilled off under reduced pressure, the residue was extracted with chloroform, and the chloroform solution was washed with 5% solution of $NaHSO_4$ and dried with Na_2CO_3. The solvent was distilled off under reduced pressure to give **5** (0.75 g, 20%). Total yield of **5**: 69%, yellow crystals, mp 197−199 °C (hexane); IR ν_{max} 3418, 3341, 3312, 3271, 3233, 3213, 2932, 2858, 1659, 1630, 1601, 1449, 1400, 1331, 1283, 1256, 1175, 1126, 1094, 910, 773, 706, 633; ^{1}H NMR (400 MHz; $CDCl_3$−CD_3OD 10:1 + PhSH) 0.95−1.09 (m, 2H), 1.17−1.59 (m, 12H), 1.60−1.73 (m, 4H), 2.08 (dt, J_d = 4, 14, 2H), 6.53 (s, 1H). Anal. Calcd for $C_{15}H_{23}N_2O_2$: C, 68.41; H, 8.80; N, 10.64. Found: C, 68.54; H, 9.09; N, 10.82. HRMS EI (m/z) calcd for $C_{15}H_{23}N_2O_2$ 263.1754, found 263.1758.

14-Carboxy-7-azadispiro[5.1.5.2]pentadeca-14-ene-7-oxyl (6). A mixture of amide **5** (1.2 g, 4.6 mmol), NaOH (1.5 g, 37.5 mmol), ethanol (15 mL) and water (3 mL) was placed into single-mode microwave oven CEM Discover S-class vessel and heated to 165 °C using microwave power 260 W for 25 min. After cooling of the reaction mixture to room temperature, ethanol was distilled off under reduced pressure, and the residue was triturated with water (50 mL). The precipitate of unreacted amide **5** (0,2 g) was filtered off, the solution was extracted with diethyl ether to remove remaining trace of **5**, and then acidified to pH 2−3 using $NaHSO_4$ and again extracted

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with Et_2O. The extract was dried with $MgSO_4$, and the solvent was distilled off under reduced pressure to give **6** (0.8 g, 80%, conversion 83%): light-yellow crystals, mp 196−198 °C (ethyl acetate); IR ν_{max} 2955, 2932, 2862, 1722, 1626, 1449, 1421, 1294, 1231, 1171, 1117, 995, 933, 908, 860, 845, 779, 760, 685; ^{1}H NMR (400 MHz; $CDCl_3$−CD_3OD 4:1 + PhSH) 1.21 (m, 2H), 1.42 (m, 2H), 1.50−1.90 (m, 12H), 1.99 (m, 2H), 2.36 (m, 2H), 6.83 (s, 1H). Anal. Calcd for $C_{15}H_{22}NO_3$: C, 68.15; H, 8.39; N, 5.30. Found: C, 68.27; H, 8.47; N, 5.11. HRMS EI (*m*/*z*) calcd for $C_{15}H_{22}NO_3$ 264.1594, found 264.1596.

14-((2,5-Dioxopyrrolidin-1-yloxy)carbonyl)-7-azadispiro-[5.1.5.2]pentadeca-14-ene-7-oxyl (7). A mixture of the carboxylic acid (100 mg, 0.38 mmol), *N*-hydroxysuccinimide (45 mg, 0.39 mmol) and ethyl acetate (2 mL) was placed into ice bath, and a solution of DCC (80 mg, 0.39 mmol) in ethyl acetate (1 mL) was added upon stirring. The mixture was stirred for 5 h, the precipitate was filtered off and washed with ethyl acetate, the solution was concentrated under reduced pressure, and the residue was separated using column chromatography on silica gel, eluent $CHCl_3$−CCl_4 1:1, to a give **7**: yield 100 mg (73%), yellow crystals, mp 203−204 °C (methanol); IR ν_{max} 2934, 2856, 1767, 1738, 1620, 1450, 1427, 1371, 1306, 1225, 1203, 1084, 1069, 993, 964, 895, 766, 743, 648, 594. Anal. Calcd for $C_{19}H_{25}N_2O_5$: C, 63.14; H, 6.97; N, 7.75. Found: C, 62.88; H, 7.06; N, 7.74. HRMS EI (*m*/*z*) calcd for $C_{19}H_{25}N_2O_5$ 361.1763, found 361.1762.

14-((Ethoxycarbonyloxy)carbonyl)-7-azadispiro[5.1.5.2]-pentadeca-14-ene-7-oxyl (8). Ethyl chloroformate (0.25 mL, 2.6 mmol) was added dropwise to a stirred cold (−10 °C) solution of **6** (0.5 g, 1.9 mmol) in a mixture of dry chloroform (15 mL) and triethylamine (0.35 mL, 2,4 mmol). After stirring for 0.5 h the solvent was distilled off under reduced pressure and the residue was triturated with dry diethyl ether. The precipitate was filtered off, washed with dry diethyl ether, the combined ether solutions were evaporated under reduced pressure and the residue was recrystallized from hexane to give **8** (0.48 g, 75%): light-yellow crystals, mp 105−107 °C; IR ν_{max} 2926, 2855, 1794, 1744, 1624, 1449, 1367, 1302, 1265, 1246, 1200, 1150, 1084, 1036, 984, 943, 908, 866, 744. Anal. Calcd for: C, 64,27; H, 7.79; N, 4.16. Found: C, 64.45; H, 7.71; N, 4.30. HRMS EI (*m*/*z*) calcd for $C_{18}H_{26}NO_5$ 336.1806, found 336.1803.

14-Hydroxymethyl-7-azadispiro[5.1.5.2]pentadeca-14-ene-7-oxyl (9). A solution of $NaBH_4$ (70 mg, 1.85 mmol) in ethanol (3 mL) was placed in an ice bath, and nitroxide **8** (0.3 g, 0,89 mmol) was added portionwise upon stirring. After stirring for 2 h, the solvent was distilled off under reduced pressure, and the residue was diluted with water (3 mL) and extracted with diethyl ether. The extract was dried with Na_2CO_3, the solvent was distilled off under reduced pressure and the residue was separated using column chromatography on silicagel, eluent chloroform to give **9** (200 mg, 90%): yellow crystals, mp 106−108 °C; IR ν_{max} 3362, 3059, 2930, 2858, 1454, 1443, 1408, 1356, 1207, 1194, 1173, 1130, 1047, 1018, 1007, 905, 841, 673, 638, 608, 554; ^{1}H NMR (400 MHz; $CDCl_3$ + PhSH) 1.16 (m, 1H), 1.31 (m, 1H), 1.49 (m, 4H), 1.65 (m, 14H), 4.27 (s, 2H), 5.90 (s, 1H). Anal. Calcd for $C_{15}H_{24}NO_2$: C, 71,96; H, 9.66; N, 5.59. Found: C, 72.01; H, 9.76; N, 5.63. HRMS EI (*m*/*z*) calcd for $C_{15}H_{24}NO_2$ 250.1802, found 250.1799.

14-((Methylsulfonyloxy)methyl)-7-azadispiro[5.1.5.2]-pentadeca-14-ene-7-oxyl (10). A solution of **9** (250 mg, 1.02 mmol) and triethylamine (140 μL, 1 mmol) in dry chloroform (3 mL) was cooled to −10 °C, and then methanesulfonyl chloride (100 μL, 1.3 mmol) was added dropwise upon vigorous stirring. The solution was stirred for 3 h at room temperature, and then washed with water and with 5% $NaHCO_3$ solution and dried with $MgSO_4$. The solvent was evaporated under reduced pressure, and the residue was crystallized from hexane to give **10** (260 mg, 80%): yellow crystals, mp 125−127 °C; IR ν_{max} 3024, 3003, 2945, 2926, 2856, 1445, 1414, 13556, 1173, 972, 961, 908, 841, 808, 737, 673, 528, 492. Anal. Calcd for $C_{16}H_{26}NO_4S$: C, 58.51; H, 7.98; N, 4.26; S, 9.76. Found: C, 58.75; H, 7.95; N, 4.29; S, 9.86. HRMS EI (*m*/*z*) calcd for $C_{16}H_{26}NO_4S$ 328.1577, found 328.1578.

14-Bromomethyl-7-azadispiro[5.1.5.2]pentadeca-14-ene-7-oxyl (11). The mixture of **10** (250 mg, 0.76 mmol), LiBr (0,5 g, 5.76 mmol) and acetone (5 mL) was stirred under reflux for 0.5 h. The solvent was evaporated under reduced pressure, and the residue was diluted with water (10 mL) and extracted with diethyl ether. The extract was dried with $MgSO_4$, and the solvent was evaporated under reduced pressure. The residue was crystallized from hexane to give **11** (216 mg, 90%): yellow crystals, mp 122−124 °C; IR ν_{max} 2982, 2926, 2856, 1449, 1356, 1414, 1292, 1236, 1169, 1126, 1059, 1001, 939, 904, 842, 818, 781, 708, 615, 563. Anal. Calcd for $C_{15}H_{23}BrNO$: C, 57.51; H, 7.40; N, 4.47; Br, 25.51. Found: C, 57.67; H, 7.30; N, 4.38; Br, 25.28. HRMS EI (*m*/*z*) calcd for $C_{15}H_{23}BrNO$ 312.0958, found 312.0957.

14-(((Methansulfonyl)thio)methyl)-7-azadispiro[5.1.5.2]-pentadeca-14-ene-7-oxyl (12). The mixture of **11** (250 mg, 0.8 mmol), sodium methanetiosulfonate (0.2 g, 1,5 mmol) and DMSO (3 mL) was stirred at room temperature for 3 h, diluted with saturated NaCl solution (20 mL) and extracted with ethyl acetate. The extract was washed with saturated NaCl solution and dried with $MgSO_4$. The solvent was evaporated under reduced pressure, and the residue was crystallized from mixture hexane−ethyl acetate 4:1 to give **12** (195 mg, 70%): yellow crystals, mp 131−133 °C; IR ν_{max} 3022, 2997, 2936, 2862, 1449, 1406, 1315, 1128, 960, 908, 743, 544, 482. Anal. Calcd for $C_{16}H_{26}NO_3S_2$: C, 55.78; H, 7.61; N, 4.07; S, 18.61. Found: C, 56.02; H, 7.66; N, 4.07; S, 18.67. HRMS EI (*m*/*z*) calcd for $C_{16}H_{26}NO_3S_2$ 344.1349, found 344.1352.

14-(((2-(4-amino-4-carboxybutanamido)-3-((carboxymethyl)amino)-3-oxopropyl)disulfanyl)methyl)-7-azadispiro[5.1.5.2]pentadeca-14-ene-7-oxyl (13). A solution of glutathione (12 mg, 39 μM) in 50% aqueous methanol (2 mL) was added dropwise to a solution of **12** (14 mg, 40.6 μM) in methanol (2 mL). The solution was allowed to stand at ambient temperature for 15 min, and then methanol was distilled off in vacuum, and the yellowish resin precipitated was separated, washed with water, and dried in vacuum. The resin was dissolved in a hot mixture methanol−isopropanol (1:4) (ca. 1.5 mL) and filtered. The solvents were removed in the stream of air, and the semicrystalline residue was triturated with dry diethyl ether. The crystalline precipitate of **13** was filtered off and washed with ether. Yield: 11 mg (19%), yellowish powder, mp 171−173 °C; IR ν_{max} 3062, 2930, 2856, 1730, 1652, 1533, 1452, 1409, 1225, 907, 545. Anal. Calcd for $C_{25}H_{39}N_4O_7S_2$: C, 52.52; H, 6.88; N, 9.80; S, 11.22. Found: C, 52.29; H, 6.84; N, 9.57; S, 10.93.

14-Carbamoyl-7-azadispiro[5.1.5.2]pentadecane-7-oxyl (14). A mixture of suspended $NaBH_4$ (182 mg, 4.8 mmol) and LiCl (202 mg, 4.8 mmol) in dry THF (15 mL) was stirred and heated under reflux for 0.5 h. Then amide **5** (0.5 g, 1.9 mmol) was added, and the reaction mixture was stirred and heated under reflux for 7 days. The end of reaction was controlled with TLC (Silicagel, ether). The THF was distilled off under reduced pressure, and then water (30 mL) was added to the residue, and the mixture was extracted with chloroform. The chloroform solution was dried with Na_2SO_4, the solvent was distilled off under reduced pressure, and the residue was crystallized from hexane−ethyl acetate 1:1 to give **14** (445 mg, 89%): yellow crystals mp 133−135 °C; IR ν_{max} 3481, 3423, 3309, 3182, 2937, 2858, 1662, 1633, 1433, 1404, 1328, 1292, 1259, 1172, 1145, 906, 729, 653, 580, 501. ^{1}H NMR (300 MHz; $CDCl_3$ + PhSH) 1.13−1.76 (br m, 20H), 2.01 (dd, J_{vic} = 8.0, J_{gem} = 13.2, 1H), 2.04 (dd, J_{vic} = 9.5, J_{gem} = 13.2, 1H), 2.66 (dd *J* = 8.0, 9.5, 1H), 5.94 and 6.55 (both br s). Anal. Calcd for $C_{15}H_{25}N_2O_2 \times H_2O$ (the composition is in agreement with X-ray analysis data): C, 63.67; H, 8.48; N, 9.71. Found: C, 63.57; H, 9.60; N, 9.89. HRMS EI (*m*/*z*) calcd for $C_{15}H_{25}N_2O_2$ 265.1911, found 265.1912. The structure was confirmed by X-ray analysis data.

14-Carboxy-7-azadispiro[5.1.5.2]pentadecane-7-oxyl (15). A solution of amide **14** (150 mg, 0.57 mmol) in ethanol (8 mL) was added to 10% KOH water solution (2 mL) and placed into thick-walled glass ampule, sealed and placed into oil bath heated to 110−120 °C for 48 h. After cooling of the reaction mixture to room temperature, the ampole was carefully opened, ethanol was distilled off under reduced pressure, and the residue was diluted with water (50 mL). The solution was acidified to pH 2−3 using 3% HCl and

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extracted with $CHCl_3$. The extract was dried with Na_2SO_4, the solvent was distilled off under reduced pressure, and the residue was crystallized from hexane−ethyl acetate 1:3 to give **14** (113 mg, 75%): yellow powder, mp 173−175 °C; IR ν_{max} 3427, 2937, 2856, 1724, 1450, 1411, 1313, 1269, 1207, 1178, 1159, 1136, 908, 688, 671, 576, 507. Anal. Calcd for $C_{15}H_{24}NO_3$: C, 67.64; H, 9.08; N, 5.26. Found: C, 67.58; H, 9.00; N, 5.24. HRMS EI (*m*/*z*) calcd for $C_{15}H_{24}NO_3$ 266.1751, found 266.1749.

14-Cyano-7-azadispiro[5.1.5.2]pentadeca-14-ene-7-oxyl (16). A solution of **5** (500 mg, 1.9 mmol) in a mixture of 1,4-dioxane (5 mL) and pyridine (0,6 mL, 7.5 mmol) was cooled to 5 °C, and then TFAA (1 mL, 7.2 mmol) was added, and the reaction mixture was allowed to stand overnight at room temperature. The solution was diluted with chloroform (15 mL), carefully washed with water and dried with $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was crystallized from hexane to give **16** (0.4 g, 86%): yellow crystals, mp 141−143 °C; IR ν_{max} 3073, 2938, 2862, 2220, 1607, 1456, 1425, 1315, 1259, 1200, 1175, 1136, 1063, 997, 943, 910, 860, 847, 775, 671, 632, 596; ^{1}H NMR (400 MHz; $CDCl_3$ + PhSH) 1.20−1.57 (m, 5H), 1.57−1.90 (m, 15H), 6.72 (s, 1H). Anal. Calcd for $C_{15}H_{21}N_2O$: C, 73.43; H, 8.63; N, 11.42. Found: C, 73.49; H, 8.66; N, 11.43. HRMS EI (*m*/*z*) calcd for $C_{15}H_{21}N_2O$ 245.1648, found 245.1647. Similarly, 3-cyano-2,2,5,5-tetramethyl-2,5-dihydropyrrol-1-oxyl (**16t**) was prepared from 3-carbamoyl-2,2,5,5-tetramethyl-2,5-dihydropyrrol-1-oxyl (**5t**): yield 95%, light orange crystals, mp 60−62 °C (hexane); IR ν_{max} 3071, 2980, 2934, 2870, 2228, 1801, 1622, 1462, 1439, 1375, 1364, 1325, 1281, 1223, 1161, 1088, 952, 928, 903, 862, 758, 681, 635, 615, 571, 530; ^{1}H NMR (300 MHz; $CDCl_3$ + PhSH) 1.35 (s, 6H), 1.44 (s, 6H), 6.42 (s, 1H).

14,15-Dicyano-7-azadispiro[5.1.5.2]pentadecane-7-oxyls (17 and 18). A mixture of nitrile **16** (350 mg, 1.43 mmol), NaCN (400 mg, 8.1 mmol), ammonium chloride (400 mg, 7.5 mmol), ethanol (25 mL) and water (5 mL) was stirred and heated under reflux for 48 h. Ethanol was evaporated under reduced pressure, and the residue was separated using column chromatography on neutral Al_2O_3. Elution with $CHCl_3$−hexane 1:1 afforded consequently **16**, 50 mg (conversion 86%); (14*R*(*S*),15*R*(*S*))-14,15-dicyano-7-azadispiro-[5.1.5.2]pentadecane-7-oxyl (**17**), 140 mg (42%); and (14*R*,15*S*)-14,15-dicyano-7-azadispiro[5.1.5.2]pentadecane-7-oxyl (**18**), 176 mg (53%). **17**: yellow crystals, mp 175−177 °C (hexane); IR ν_{max} 2943, 2864, 2245, 1454, 1415, 1342, 1309, 1263, 1215, 1196, 1161, 1136, 993, 931, 837, 727, 644, 490, 455. ^{1}H NMR (400 MHz; $CDCl_3$ + PhSH) 1.18−1.33 (m, 2H), 1.48−1.78 (m, 18H), 3.03 (s, 2H). Anal. Calcd for $C_{16}H_{22}N_3O$: C, 70.56; H, 8.14; N, 15.43. Found: C, 70.78; H, 7.94; N, 15.45. HRMS EI (*m*/*z*) calcd for $C_{16}H_{22}N_3O$ 272.1757, found 272.1755. The structure was confirmed by X-ray analysis data. **18**: yellow crystals, mp 138−140 °C (hexane); IR ν_{max} 2945, 2916, 2864, 2247, 1456, 1412, 1333, 1298, 1263, 1227, 1165, 1136, 1063, 995, 907, 866, 789, 733, 677, 501. ^{1}H NMR (400 MHz; $CDCl_3$ + PhSH) 1.26−1.37 (m, 2H), 1.43−1.69 (m, 14H), 1,77−1.92 (m, 4H), 3.17 (s, 2H). Anal. Calcd for $C_{16}H_{22}N_3O$: C, 70.56; H, 8.14; N, 15.43. Found: C, 70.68; H, 7.95; N, 15.29. HRMS EI (*m*/*z*) calcd for $C_{16}H_{22}N_3O$ 272.1757, found 272.1755.

(14*R*(*S*),15*R*(*S*))-14-Carbamoyl-15-carboxy-7-azadispiro-[5.1.5.2]pentadecane-7-oxyl (19). A solution of dinitrile **17** (100 mg, 0.37 mmol) and KOH (1 g, 17 mmol) in 80% ethanol (6 mL) was placed into thick-walled glass tube, sealed and heated to 100 °C for 7 h as described for hydrolysis of **14**. Then the tube was open, and the reaction mixture was analyzed using TLC (silica gel, eluent $CHCl_3$−EtOH 25:2). Comparison of alkaline and acidified samples of the reaction mixture showed no significant difference, indicating that hydrolysis did not proceed. Therefore ethanol was evaporated, and the residue was diluted with water (5 mL) and heated under reflux. After heating for 48 h the TLC analysis showed nearly complete formation of acidic (carboxylate-containing) products. Then the solution was acidified to pH 2 with $NaHSO_4$ and extracted with diethyl ether−isopropanol mixture 4:1. The extract was dried with $MgSO_4$ and evaporated under reduced pressure. The residue was crystallized from ethyl acetate to give **19**: 60 mg (50%) light-yellow powder, mp 166−168 °C (ethyl acetate); IR ν_{max} 3391, 3200, 2934, 2870, 1715, 1668, 1605, 1443, 1356, 1296, 1261, 1215,1202, 1171, 1140, 907, 874, 690, 640, 607, 455, 436. ^{1}H NMR (after hydrogenation on Pd/C) (400 MHz; CD_3OD) 1.37−1.55 (m, 2H), 1.56−1.87 (m, 11H), 1.88−2.02 (m, 3H), 2.03−2.17 (m, 3H), 2.18−2.28 (m, 1H), 3.46 and 3.50 (AB, *J* = 10.7, each 1H). Anal. Calcd for $C_{16}H_{25}N_2O_4 \times 1/4$ EtOAc (the composition is in agreement with X-ray analysis data): C, 61.61; H, 8.21; N, 8.45. Found: C, 61.57; H, 7.99; N, 8.54. The described above procedures with **18** afforded the compound identical to **19**, with no difference in IR spectra: R_f (silica gel, eluent $CHCl_3$−AcOH−EtOH 25:1:1), HPLC t_R = 3.235 min, and mp 168−170 °C, yield 63%; the structure was confirmed by X-ray analysis data.

(14*R*(*S*),15*R*(*S*))-Dicarboxy-7-azadispiro[5.1.5.2]-pentadecane-7-oxyl (21). A solution of **19** (80 mg, 0.57 mmol) in 10% aqueous KOH solution ethanol (8 mL) was placed into round-bottom thick-walled glass tube and sealed. The tube with reaction mixture was loaded into oil bath and heated to 150 °C for 96 h. After cooling of the reaction mixture to room temperature, the tube was opened, and the solution was acidified to pH 2 with $NaHSO_4$ and extracted with diethyl ether−isopropanol mixture 4:1. The extract was dried with $MgSO_4$ and evaporated under reduced pressure. The residue was separated using column chromatography on silica gel, eluent $CHCl_3$−AcOH−EtOH 25:1:1 to give consequently dicarboxylic acid **19** (36 mg) and unreacted **19** (30 mg, conversion 63%). **21**: yellow crystalline powder, yield 71%, mp 197−199 °C (hexane−ethyl acetate 1:3); IR ν_{max} 2938, 2864, 2677, 1738, 1703, 1442, 1412, 1296, 1265, 1217, 1191, 1165, 1138, 933, 908, 700, 673, 461, 438. ^{1}H NMR (after hydrogenation on Pd/C) (400 MHz; CD_3OD) 1.40−1.51 (m, 2H), 1.61−1.87 (m, 12H), 1.90−2.03 (m, 2H), 2.04−2.14 (m, 2H), 2.17−2.27 (m, 2H), 3.46 (s, 2H). Anal. Calcd for $C_{16}H_{24}NO_5$: C, 61.92; H, 7.79; N, 4.51. Found: C, 61.86; H, 7.74; N, 4.41. HPLC t_R = 4.519 min

Determination of the Partition Coefficients. A solution of a nitroxide (1 mM) in 0.1 M phosphate buffer solution pH 7.2 was mixed with 0.2 mL of *n*-octanol for 10 s with a vortex mixer, and then the mixture was centrifuged at 12000*g* for 2 min. Both the *n*-octanol and PBS layers were separately transferred to 50-μL disposable capillaries. The capillaries were set at a constant position in the EPR cavity, and the EPR spectrum was recorded. Double integration of EPR spectrum of studied nitroxide was corrected with double integration of EPR spectrum of 1 mM TEMPO solution in the same capillaries. The partition coefficient was expressed as the ratio of the double integration of EPR spectrum of the *n*-octanol layer to that of the PBS layer: $P_{o/w} = [A_{oct}]/[A_{PBS}]$.

Reduction of Nitroxides with Ascorbic Acid (AsA). A solution of a nitroxide (0.2−0.5 mM) in ethanol (10 μL) was mixed with 0.1 M carbonate buffer solution containing ascorbic acid (100 mM) and glutathione (50 mM); the resulting pH was 7.2. The mixture immediately was transferred to 50-mL disposable capillary for EPR measurements, and EPR spectra were recorded at 25 °C at intervals of 5 s. Decay of the double integral over the EPR spectrum was monitored as a function of time. The nitroxide concentrations were calculated from double integration of EPR spectrum using 1 mM TEMPO solution for calibration. The second order rate constant between nitroxyl radicals and AsA was calculated from the decay of EPR spectra under conditions of pseudofirst order reactions.

EPR Measurement. EPR spectra were recorded on X-band spectrometer using the following settings: frequency, 9.87 GHz; power, 6.38 mW; modulation frequency, 100 kHz; modulation amplitude, 0.05−0.01 mT; conversion time, 5.12 ms.

Electron spin relaxation time, T_1, and electron spin echo dephasing time, T_m, were measured using the X/Q-band EPR spectrometer equipped with the cryogenic system. For measurement of the longitudinal electron spin relaxation times, T_1, we used the inversion recovery pulse sequence $\pi-\tau_{var}-\pi/2-\tau-\pi-\tau-echo$. For the measurement of electron spin echo dephasing time, T_m, we used the two-pulse echo sequence $\pi/2-\tau_{var}-\pi-\tau_{var}-echo$, where the interpulse delay τ_{var} was varied. The pulse lengths were 10 and 20 ns (for $\pi/2$ and π pulses, respectively). The pulse power was adjusted to maximize the two-pulse echo intensity. The both T_1 and T_m values were measured in magnetic field correspondent to the center of the EPR spectrum of nitroxide. All

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samples were prepared in water−glycerol (1:1) solution, and to avoid the effect of exchange interaction on the relaxation times, the concentration of nitroxides radicals was less than 0.5 mM.

■ ASSOCIATED CONTENT

Supporting Information

Copies of the ^{1}H and ^{13}C NMR spectra of products, Figure S1, the ^{1}H NMR of the reaction mixture of TEMPO and PhSH, DFT/BPE/3z calculations data, and CIF file with X-ray data for **14**, **17**, and **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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