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Increasing the Persistency of Stable Free-Radicals: Synthesis and Characterization of a Nitroxide Based [1]Rotaxane

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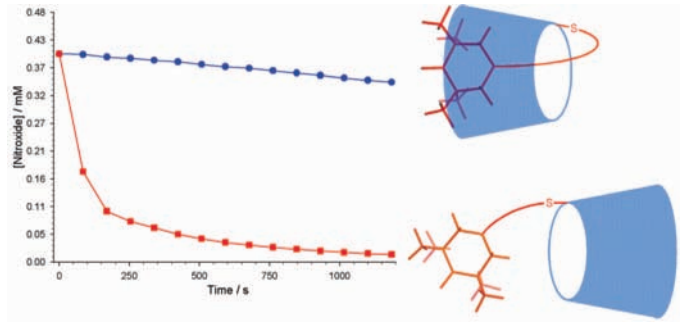
ABSTRACT



A persistent paramagnetic [1]rotaxane based on β-cyclodextrin showing an increased persistency under reductive conditions has been prepared and characterized for the first time.

EPR is the most useful and popular method for the detection of free radicals both *in vitro* and *in vivo* conditions. Free radicals can be naturally present (endogenous radicals) or intentionally added (exogenous radicals) to the system under investigation. As exogenous free radicals, nitroxides are certainly the most commonly employed in biology and medicine where they are used as spin labels,[1] contrast agents for magnetic resonance imaging,[2] superoxide dismutase mimics,[3] oximetry,[4] and also to measure the spectra of a living body.[5] The recent development of substituted triarylmethyl radicals has significantly expanded the potential for using EPR and related techniques *in vivo* conditions.[6] Nevertheless, nitroxide radicals still maintain several advantages because of variability of structure, solubility, and ability to be targeted.

Nitroxides can be converted in biological systems to EPR silent compounds by reductants or oxidants. In general, nitroxides are reduced by enzyme mediated pathways to the corresponding hydroxylamines,[7] while their reaction with oxygen centered radical (•OH, •OOH) afford an oxoammonium cat-

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(4) Krishna, M. C.; English, S.; Yamada, K.; Yoo, J.; Murugesan, R.; Devasahayam, N.; Cook, J. A.; Golman, K.; Ardenkjaer-Larsen, J. H.; Subramanian, S.; Mitchell, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2216–2221.

(5) As an example see: (a) Berliner, L. J.; Fujii, H. *Science* **1985**, *227*, 517–519. (b) He, G.; Shankar, R. A.; Chzhan, M.; Samouilov, A.; Kuppusamy, P.; Zweier, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4586–4591. (c) Eaton, G. R.; Eaton, S. S. In *Handbook of Electron Spin Resonance*; Poole, C. P., Jr., Farach, H. A., Eds.; AIP Press: Secaucus, 1999; pp 327−343.

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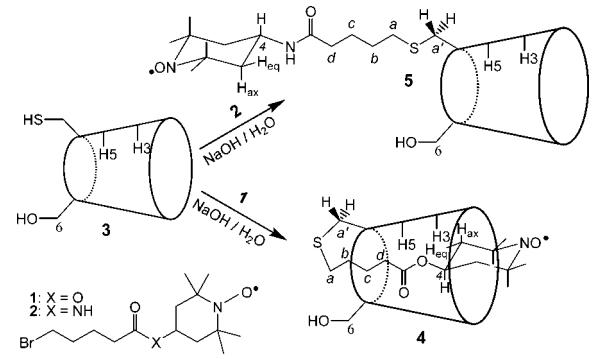
ion.[3,8] This unfavorable nitroxides feature has established the need for development of new structures with enhanced persistency to obtain strongly and clearly resolved EPR signals. It has been reported that cyclodextrins (CDs) play an important role in stabilizing nitroxides, leading to EPR signal enhancement and in increasing the partial resistance of the radical against biomimetic reductive conditions.[9] However, the need for high CD concentrations can constitute an important disadvantage for *in vivo* EPR experiments. Moreover, once the complex is dissolved in a biological environment, the higher affinity of biomolecules toward the hydrophobic compartment of CD, can result in separation of the radical guest from the host. These limitations can be overcome if the nitroxide functionality is mechanically trapped inside the cavity of CD by a covalent link with one of the CD rims, that is, by formation of [1]rotaxane.[10] CD-labeled nitroxides have already been described in the literature.[11] In all cases, however, the paramagnetic label was found to be located outside the ring cavity. Presumably, this behavior is due to the fact that in all cases the reaction between the CD and the nitroxide moiety were carried out in organic solvents which are known to strongly reduce the affinity of an organic guest for the CD cavity, if compared to water. Actually, [1]rotaxanation reaction with CD has been recently shown to be very effective when performed in water respect to DMSO.[12]

Based on this, we decided to prepare a nitroxide-based [1]rotaxane by reacting nitroxide **1** or **2** with 6-mercapto-β-cyclodextrin (**3**, β-CD-SH) using alkaline water as reaction medium. After the reaction, the target molecules **4** and **5** were purified by using reverse phase and exclusion chromatographies (see Supporting Information).

As indicated in Scheme 1, different results were obtained depending on the nature of paramagnetic unit that has been reacted with β-CD-SH. While with **2** only the product with the paramagnetic arm located outside the cavity was obtained (**5**), with nitroxide **1** the reaction afforded [1]rotaxane **4** in 7.6% yield.

The fortuitous formation of **5** and the comparison of its NMR and EPR spectra with those of **4** notably helped us to the structurally assignment of the latter one as [1]rotaxane. To render the paramagnetic nitroxides suitable to NMR

Scheme 1



analysis they were quantitatively converted into the analogous *N*-hydroxy amines (**4-OH**, **5-OH**) by adding directly inside the NMR tube a stoichiometric amount of phenylhydrazine.[13]

The presence of the nitroxide moiety inside the β-CD cavity in **4** has been confirmed using 2D ROESY ^{1}H NMR both in DMSO (Figure 1) and water (see Supporting Information). The

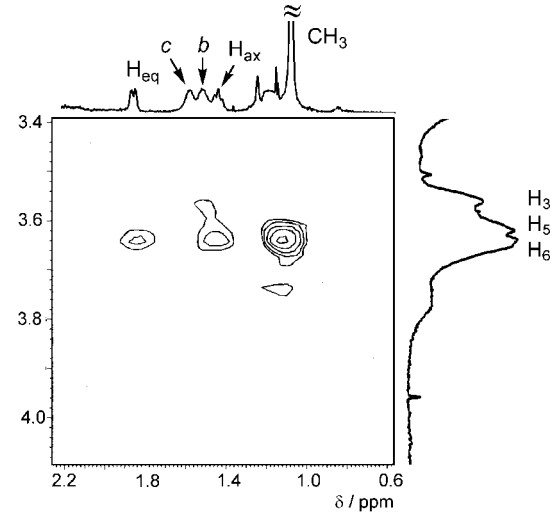


Figure 1. Partial 2D ROESY ^{1}H NMR spectrum of **4-OH** (1 mM) in DMSO-d_6. The sample recorded in D_2O shows similar interactions (see Supporting Information).

NOEs between the H_{ax}, H_{eq}, and CH_3 protons and the H3 and H5 protons on the interior of β-CD showed that the heterocycle is embedded in the β-CD cavity to form [1]rotaxane **4-OH**. On the basis of the results mentioned above, it is concluded that the nitroxide moiety should be included with its symmetry axis nearly parallel to that of the CD and with the geminal methyl groups pointing toward the larger rim of the CD (see Scheme 2).

The 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) fragment has an approximately cylindrical shape whose diameter is about 8.5 Å,[1] well above the internal diameter of the β-CD

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(10) Very few example of 1[rotaxane] has been reported in the literature: Hiratani, K.; Kaneyama, M.; Nagawa, Y.; Koyama, E.; Kanesato, M. *J. Am. Chem. Soc.* **2004**, *126*, 13568–13569.
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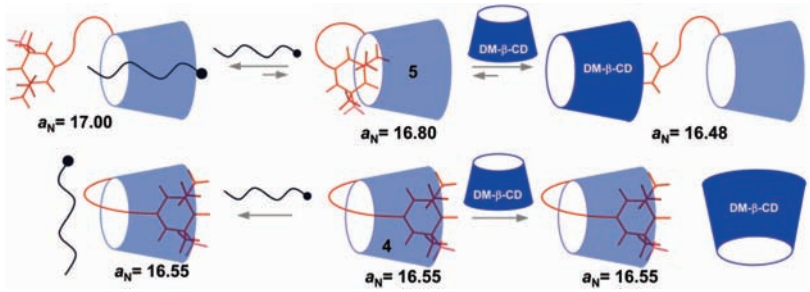
Scheme 2



smaller rim (~6.2 Å).[14] Based on this, it seems reasonable to conclude that the barrier for the excape of the nitroxide moiety from the CD cavity must be significantly high and, thus, to exclude the presence of a reversible equilibrium between the open and threaded forms of self-complex **4**. Support for this conclusion comes from the observation of strong NOE interactions even in the presence of an organic solvent like DMSO. Because organic solvents are known to strongly reduce the affinity of an organic guest for the CD cavity,[12,14] the presence of a reversible equilibrium between the open and threaded forms, should lead under this condition to a dramatic increase in the amount of the former one and, thus, to a diseapearance of NOE interactions. This is actually the case of **5** for which 2D ROESY ^{1}H NMR spectrum do not show obvious NOEs between the CD internal H3 and H5 and the piperidine ring protons both in water and DMSO (see Supporting Information), this being a clear indication of the exclusion of the thread from the CD cavity.

The water solutions of **4**−**5** shows typical nitroxide EPR spectra with the high field line broadened due to restricted tumbling.

Inspection of the ^{14}N hyperfine splittings (a_N) values reported in Table 1 confirms the proposed geometry for **4** and **5**. The 0.45 G Δa_N decrease between **4** and the free thread **1** is compatible with a geometry in which the piperidine ring is strongly self-included within the hydrophobic cavity of the β-CD with the nitroxyl pointing toward the bulk water. On the other hand, the smaller Δa_N (0.20 G) between **2** and **5** does not agree with an interlocked radical species but, presumably, with a weak self-complexing nitroxide. According to recent results reported by Bardelang et al.,[11d] this loose complex **5** may be likely represented by a structure in which the nitroxide moiety is located among the hydroxy crown of the narrow rim of the CD cavity, (see Scheme 2). This hypothesis is supported by the analysis of the EPR spectra variation observed after the addition of 2,6-di-*O*-Me-β-cyclodextrin (DM-β-CD) or sodium dodecyl sulfate (SDS) as external competing host or guest, respectively. Whereas the EPR spectrum of **5** recorded in the presence of DM-β-CD 0.1 M showed a significant decrease in the a_N value, that recorded in the presence of SDS is characterized by a_N value comparable to that of the free thread. This indicates that in **5** the nitroxide fragment can be reversibly trapped by another host or displaced by guest competing for the CD cavity. Differently, the spectrum of **4** did not show any variation in the presence of the competing species, thus confirming the irreversible nitroxide trapping inside the cavity of CD.[15]

Table 1. Nitrogen hfsc of Nitroxides in 0.1 mM Water Solution at Room Temperature

nitroxide	cosolute	a_N/G
1	–	17.00
2	–	17.02
4	–	16.55
4	DM-β-CD 0.1 M	16.55
4	SDS 5 mM	16.60
5	–	16.80
5	DM-β-CD 0.1 M	16.48
5	SDS 5 mM	17.00

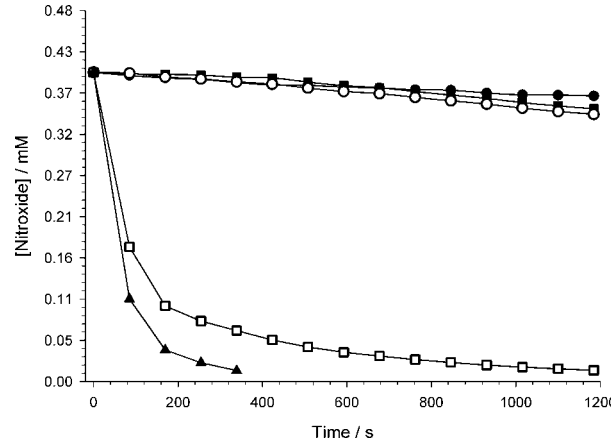


Figure 2. The kinetics of the reduction of 0.4 mM nitroxides **4** (●), **5** (■), and 4-hydroxy TEMPO (▲) by GSH 2.1 mM in the absence (filled symbols) and in the presence (empty symbols) of SDS 5 mM at 298 K in water.

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The persistency of nitroxides under reductive condition was tested in the presence of glutathione[16] (GSH) by EPR. The signal of the reference nitroxide 4-hydroxy TEMPO vanishes rather rapidly in the presence of GSH 2.1 mM (see Figure 2). On the contrary, the EPR signals of **4** or **5** could be recorded up to several hours after the addition of 2.1 mM GSH. Addition of SDS 5 mM to the above solution, however, resulted in the rapid decrease of EPR signal of **5** whereas that of **4** remains practically constant. This result can be justified only by admitting that GSH is able to react with the nitroxide fragment only when it has been displaced from the CD cavity by SDS, this being possible in the weakly CD/nitroxide complex **5** and not in the mechanically interlocked [1]rotaxane **4**. It must be mentioned, however, that no protection of paramagnetic unit toward ascorbate monoanion reduction was observed with **4**. This observation indicates that the nitroxyl group in **4** is still accessible to small-sized reducing agents like the ascorbate. Nevertheless, *in vivo* where nitroxide reduction is generally carried out by enzyme mediated pathways the proposed [1]rotaxane is expected to show a significant increased persistency with respect to *unprotected* radicals. Experiments to test the stability in biological tissues are under progress in our laboratory.

Acknowledgment. Financial support from MIUR (Rome; research project "Free Radical Processes in Chemistry and Biology: Fundamental Aspects and Applications in Environment and Material Sciences") are gratefully acknowledged.

Supporting Information Available: Synthesis, spectral characterization of **1**−**5** and kinetics of reduction. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Although the EPR spectrum of **4** did not show any dependence of the spectroscopic parameters on the radical concentration, the value of the nitrogen splitting in **5** decreases by increasing the radical amount in solution. This last observation can be related to the formation of intermolecular inclusion complex involving the non-trapped thread of **5** and the CD cavity.

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