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# SPIN LABELING IN PHARMACOLOGY

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EDITED BY

JORDAN L. HOLTZMAN

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Jordan L. Holtzman

*Medical Service*

*Veterans Administration Medical Center*

*Minneapolis, Minnesota*



1984



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## Preface

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Electron spin resonance spectrometry (ESR) is a technique for the detection and quantitation of free radicals. Like nuclear magnetic resonance spectrometry (NMR), the development of this form of spectrometry is in part an outgrowth of the massive advances that have occurred in electronics since the outbreak of World War II. Even though the two techniques have many features in common and are based on the same physical principles, ESR has not achieved nearly as widespread usage as NMR. Much of this relates to the fact that whereas NMR is useful for studying a wide range of stable chemical compounds, ESR can only detect free radicals, that is, chemical species containing one or more free electrons. But by their very nature, free radicals represent a much narrower group of chemicals, many of which are highly reactive species. In spite of the limited number of chemical structures that can be detected by ESR, the method still has major advantages over NMR in the study of certain problems. Among these advantages are that the ESR has significantly higher sensitivity for a comparably priced instrument. Further, the shorter lifetime of the excited state of the electron when compared to that of the proton makes it possible to study better many problems in molecular motion. Finally, that organic free radicals are generally unstable and most transition metal free radicals cannot be detected at room temperature mean that in many biological systems there is essentially no background. This can simplify many of the studies to be discussed. In an attempt to utilize many of the inherent benefits of ESR spectrometry, in the 1960s a number of workers synthesized a family of stable organic free radicals, the spin labels that can serve as probe molecules in a wide range of studies. Although the application of these probes is potentially a powerful and useful technique for the study of biological problems, it has yet to be utilized to its full potential in biological studies.

One major reason that spin labeling and ESR have not achieved their full potential is that many workers, in areas such as pharmacology, are not familiar with the methodology involved and therefore are hesitant to embark on expensive and complex studies utilizing a relatively unknown methodology. It is the objective of this book to bring together reviews by a number of workers who have investigated the application of spin labels to a variety of problems. From these summaries the reader may gain insights into the application of spin labels to pharmacology and related fields. I have intentionally excluded a detailed discus-

sion of the basic physics of ESR spectrometry but have rather tried to emphasize the chemistry and potentially important biological problems that may be of interest to the biochemical pharmacologist or those workers in related fields.

# **Spin Labeling in Pharmacology**

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# Synthesis and Chemistry of Nitroxide Spin Labels

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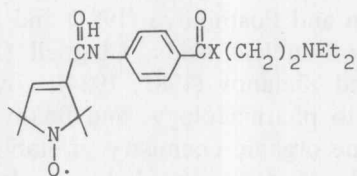
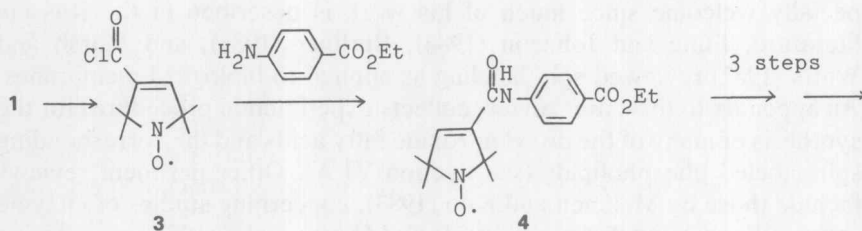
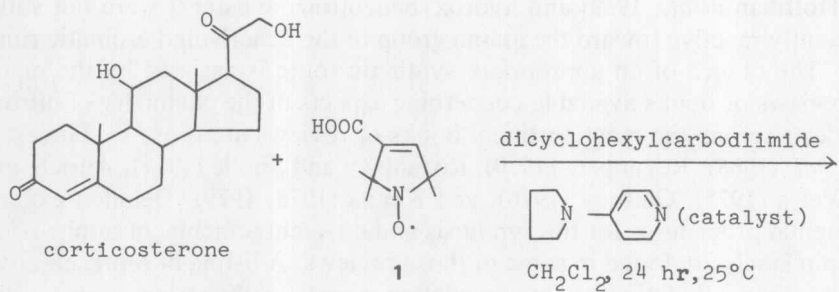
## I. Introduction

The nitroxide spin-labeling approach has much to offer the field of pharmacology, particularly within the broad area of the nature of drug-receptor interactions. Central to nitroxide spin-labeling methodology is ready access to the desired nitroxide spin-labeled analog in chemically pure form through laboratory synthesis. One purpose of this chapter is to apprise potential users of the spin-labeling method of the array of available stable nitroxide free radicals. Another is to provide examples and guidance for the synthesis of new nitroxide spin labels and for their attachment to, or incorporation into, molecules of interest.

This chapter is also relevant to the design and synthesis of new nitroxides that may serve as contrast enhancers in NMR whole-body medical imaging applications (Brasch *et al.*, 1983a,b). Pharmacology will play an important role in the development of clinically safe and effective nitroxide-based imaging agents. It is quite possible that a nitroxide spin-labeled drug prepared with other objectives in mind could well become an effective NMR contrast-enhancing agent.

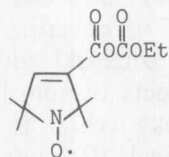
The synthesis problem can be approached in two ways. The simplest approach, and therefore the most popular, involves a single chemical coupling reaction between an appropriately functionalized and activated nitroxide, often commercially available (see Appendix) and the substrate of interest. Conventional reactions such as acylation or alkylation are used. Mixtures will, of course, be produced in this approach if specificity for a particular site on the target molecule is not high. This approach is illustrated by a synthesis of spin-labeled derivatives of the hydroxy steroids testosterone, corticosterone, deoxycorticosterone, and estrone using nitroxide carboxylic acid **1** as the acylating agent (Kirley and Halsall, 1982). Note that had the saturated nitroxide acid **2** been used instead of **1**, a difficultly separable mixture of diastereomers would have likely been produced owing to the racemic nature of **2** (contains a chiral atom).

The second general approach to a desired spin-labeled molecule involves a multistage chemical synthesis in which either the nitroxide portion, or the substrate, or both, must be first synthesized or modified chemically before the two pieces are assembled into the target. These operations can be a real chore in a laboratory not accustomed to doing moderately involved organic synthesis. A synthesis of spin-labeled "active" procaine and some derivatives is illustrative (Hideg *et al.*, 1979). It was noted that freshly prepared acid chloride **3** (details of the preparation from **1** are given by Rosantsev, 1970) gave the spin-labeled benzocaine **4** in good yield, as did the mixed anhydride **5** (Griffith *et al.*, 1967). However, other active esters of **1** such as *N*-hydroxysuccinimide ester **6**

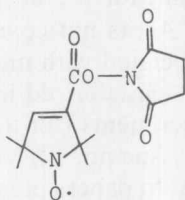


X = O (spin-labeled procaine)

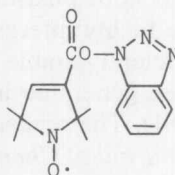
X = S and NH also prepared



**5**



**6**



**7**

(Hoffman *et al.*, 1969) and hydroxybenzotriazole ester **7** were not sufficiently reactive toward the amino group of the deactivated aromatic ring.

The choice of an appropriate synthetic route is assisted by the many reviews or books available concerning aspects of the chemistry of nitroxides. Among the most pertinent books or reviews are those by Forrester *et al.* (1968), Rozantsev (1970), Rozantsev and Sholle (1971), Aurich and Weiss (1975), Gaffney (1976), and Keana (1978, 1979). Detailed experimental procedures for the synthesis and covalent attachment of nitroxide spin labels are found in some of those reviews. A listing of representative nitroxide alkylating agents, acylating agents, sulfonating agents, and phosphor(n)ylating agents is included within the comprehensive review by this author (Keana, 1978). Konieczny and Sosnovsky (1981) provided a highly useful review of methods for the preparation of spin-labeled phosphorus compounds and their application as phosphorylative spin labels. Volodarskii *et al.* (1980) summarized his outstanding research in the synthesis and chemistry of stable imidazoline nitroxides (see Section VI,E) and included experimental procedures. Volodarskii's review is especially welcome since much of his work is described in the Russian literature. Fung and Johnson (1984), Berliner (1983), and Marsh and Watts (1982) reviewed spin labeling as applied to biological membranes. An appendix to this latter review collects experimental procedures for the synthesis of many of the doxyl nitroxide fatty acids and the corresponding spin-labeled phospholipids (see Section VI,A). Other pertinent reviews include those by Makinen and Kuo (1983), concerning studies of enzyme action, Wenzel and Tschesch (1983) and Hankovszky (1984), concerning spin-labeled amino acids, Kamzolova and Postnikova (1981) and Bobst (1979), covering the spin labeling of nucleic acids, Chignell (1979), Gwozdinski and Bartosz (1980), and Zhdanov (1981, 1984), covering aspects of spin labeling as applied to pharmacology, and finally, two books written in Russian covering the organic chemistry of stable free radicals (Rozantsev and Sholle, 1979) and stable organic biradicals (Parron *et al.*, 1980).

The scope of this chapter will be confined to that segment of the chemistry of stable nitroxide free radicals important for the development of new spin labels of pharmacological interest. Areas not covered include much of the highly interesting chemistry associated with nitroxides that are not sufficiently stable to be isolated and characterized in pure form, such as those generated in spin trapping experiments (for a review, see Perkins, 1981). The reader is also referred to issue no. 12, Vol. 60 of the *Canadian Journal of Chemistry* (1982) devoted to papers presented at the International Conference on Spin Trapping and Nitroxyl Chemistry held in Guelph, Ontario, Canada, in July 1981. Literature coverage for the

present chapter extends through about May 1984 and is representative rather than exhaustive.

## II. Nitroxide Nomenclature

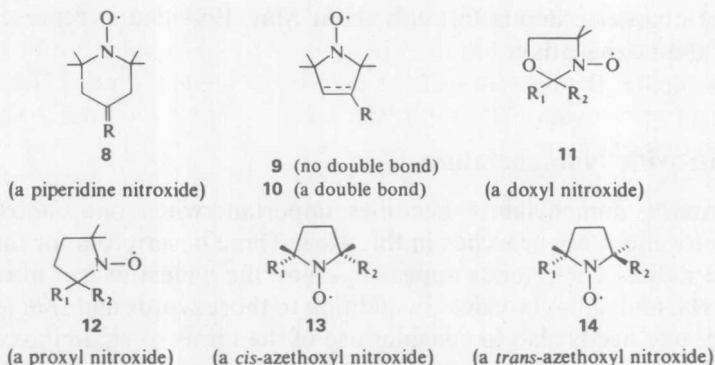
Nitroxide nomenclature becomes important when one chooses key words for literature searches in this area. Three descriptors for this class of free radical compounds appear to enjoy the widest usage: nitroxides, nitroxyls, and aminyl oxides. In addition to those words and *spin label*, of course, one needs also to consider use of the terms oxyl, aminoxyl, aminooxyl, and monodehydroaminoxid (a term preferred by Beilstein) in keyword literature searches. P. A. S. Smith (personal communication) summarized the nomenclature scene as follows: The August 1981 meeting of the IUPAC Commission of Organic Nomenclature produced a recommendation, yet to be approved by the IUPAC, that the terms aminooxyl or aminoxyl be included as the parent name for naming nitroxides. By way of illustration, nitroxide carboxylic acid **2** would be named 3-carboxy-2,2,5,5-tetramethylpyrrolidinoxyl. It is seen that the *amin* portion of *aminoxyl* is replaced by whatever the parent amine turns out to be, in this case, a substituted pyrrolidine. Another recommendation of the Commission was that the term nitroxide be retained as a class name.

## III. Structural Features Characteristic of a Stable Nitroxide

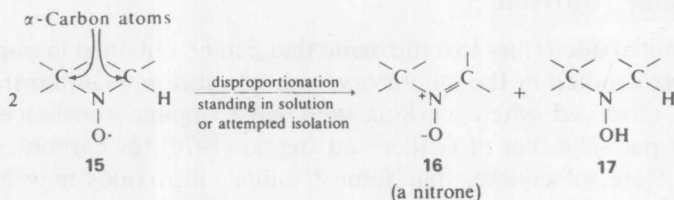
*Stable* nitroxide refers to a nitroxide that can be obtained in pure form, stored and handled in the laboratory with no more precaution than that normally observed when working with most organic substances. This definition parallels that of Griller and Ingold (1976) for carbon-centered radicals. Note, of course, that some "stable" nitroxides may be more stable than others.

Most of the stable nitroxide free radicals in use today as spin labels are members of one of the following seven classes of nitroxides: the 2,2,6,6-tetramethylpiperidinoxyl nitroxides **8**, in which the substituents are typically at the 4 position; the 2,2,5,5-tetramethylpyrrolidinoxyl nitroxides **9**; the 2,2,5,5-tetramethylpyrrolinoxyl nitroxides **10**; the 4,4-dimethyl-2,2-disubstituted-oxazolidinoxyl (doxyl) nitroxides **11**; the 5,5-dimethyl-2,2-disubstituted-pyrrolidinoxyl (proxyl) nitroxides **12**; and the *cis*- and *trans*-2,5-dimethyl-2,5-disubstituted-pyrrolidinoxyl (azethoxyl) nitroxides **13** and **14**, respectively.

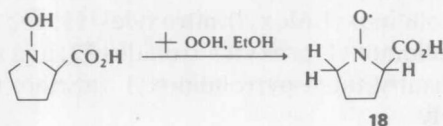




It is immediately apparent that all these stable nitroxides are secondary amine *N*-oxides that bear no hydrogen atoms on the carbons attached to the nitrogen atom (i.e., the  $\alpha$ -carbon atoms). When one or more hydrogen atoms are present on an  $\alpha$ -carbon atom, the nitroxide is normally not stable, although it may "persist" (Griller and Ingold, 1976) for hours, days, or longer in dilute solution. Such nitroxides typically undergo a disproportionation reaction (Bowman *et al.*, 1971; Briere and Rassat, 1976), producing the corresponding nitron and *N*-hydroxyamine (**15**  $\rightarrow$  **16** + **17**), either or both of which may undergo further chemical reactions. The kinetics and product distribution of the self-reactions of several dialkyl nitroxides have been examined (Briere and Rassat, 1976; Bowman *et al.*, 1971). The rate of the disproportionation reaction strongly depends on the degree of substitution of the  $\alpha$ -carbon atoms and on the solvent.



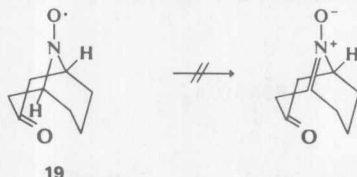
A structural feature that impedes the disproportionation reaction may give rise to a stable nitroxide. For example, Lin *et al.* (1974) synthesized proline nitroxide **18** by oxidation of *N*-hydroxyproline with *tert*-butyl hydroperoxide in ethanol. Despite the fact that **18** has three  $\alpha$  hydrogens, it





is a light yellow, crystalline solid that is stable indefinitely in the solid state and shows a half-life of  $\sim 16$  hr in pH 7 phosphate buffer at  $24^\circ\text{C}$ . Undoubtedly, the presence of the carboxylate anion at pH 7 slows down the disproportionation reaction, since two anions would not want to approach one another.

The bicyclic nitroxides, the first example (19) of which was synthesized by Dupeyre and Rassat (1966), constitute an interesting class of stable



nitroxides that also bear  $\alpha$ -hydrogen atoms. Disproportionation is impeded in these nitroxides by the strain inherent with a (nitron) double bond involving a bridgehead carbon atom (Bredt's rule). The reader is referred to earlier reviews (Keana, 1978, 1979) for additional examples of stable bicyclic nitroxides. As a class, these nitroxides have not been exploited as spin labels for several reasons: they are not commercially available, relatively few have been prepared with functional groups appropriate for attachment to another molecule, the ESR spectra are complicated by additional splitting by the  $\alpha$  hydrogens, and finally, many bicyclic nitroxides are not as "rock stable" as those of general structures 8–14. Nevertheless, this class does have potential as spin labels, particularly in studies where spin labels of novel molecular shape are desired.

All the nitroxides discussed to this point have had saturated ( $sp^3$ -hybridized) carbon atoms flanking the nitroxide group. Another group of stable (and unstable) nitroxides are those in which the nitroxide group is attached to an  $sp^2$ -hybridized carbon atom, such as that of an aromatic ring, a carbonyl group or some other carbon atom involved in a double bond. These nitroxides owe their stability to a combination of two factors, delocalization of the unpaired electron through conjugation and a degree of steric hindrance toward dimerization, disproportionation, etc. inherent in a given structure. The ESR spectra of many nitroxides in this group can be quite complex, however, owing to easily resolved splittings by nuclei involved in the conjugated system.

Interest to date in conjugated nitroxides has been more from a generation-reactions perspective rather than directed toward their potential as spin labels. The exquisitely wide variety of structure variation among this group of nitroxides would suggest a careful look by those interested in spin labels with structures similar to known DNA intercalators, enzyme