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N-(1-OXIDE-2,2,5,5-TETRA LOWER ALKYL PYRROLIDINYL-3)-MALEIMIDES

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Int. Cl. C07d 27/18, 27/06
U.S. Cl. 260—326.5 2 Claims

ABSTRACT OF THE DISCLOSURE

Free radical organic nitroxides in which the nitroxide nitrogen atom forms part of a heterocyclic ring. A maleimide ring is attached to the heterocyclic ring and is useful for forming a bond with a biologically active molecule to "spin label" the biologically active molecule. Nitroxides containing a maleimide ring are particularly good spin labels in that they are not rapidly hydrolyzed by water in competition with protein and synthetic polypeptides.

The present invention relates in general to electron spin resonance (ESR) labeling of biologically active molecules and more particularly to an improved organic spin labeling compound containing the paramagnetic nitroxide group, and containing the maleimide ring for attachment to the bimolecule.

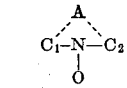
Heretofore, a limited number of synthetic organic ESR labels have been used for labeling biomolecules. One example of such a prior label is the positive ion radical of the tranquilizer drug chlorpromazine (CPZ^+) which forms the subject matter of and is claimed in the pending application Ser. No. 496,682 filed Oct. 15, 1965. CPZ^+ attaches predominantly only to DNA and RNA type biomolecules for labeling same. A more general type of spin label for biomolecules employs the nitroxide radical group, which provides a strong electron resonance line spectrum having a simple triplet hyperfine structure. Preferred molecular structures involving this radical group are remarkably stable and inert, and show ESR spectral features that are sensitive to molecular motion, and to a lesser extent, sensitive to the polarity of the molecular environment. This sensitivity in turn permits certain chemical, structural and kinetic information concerning biomolecules to be obtained from the paramagnetic resonance of the attached spin labels. The use of nitroxide groups for spin labeling is described in the pending application, Ser. No. 496,682 filed Oct. 15, 1965, and Ser. No. 496,683 filed Oct. 15, 1965. In one preferred embodiment of the above invention, the nitroxide radical spin label is attached to the biomolecule via the intermediary of an isocyanate group.

The principal object of the present invention is to provide still another improved organic spin label for biologically active molecules, especially a label that is not rapidly hydrolyzed by water in competition with the labeling of proteins and synthetic polypeptides.

One feature of the present invention is the provision of a maleimide ring system as the intermediary for attaching the spin label to proteins and synthetic polypeptides, and the methods for synthesizing such spin labels.

In the preferred embodiment improved nitroxide compounds are synthesized to contain at least one maleimide ring which serves to form a covalent bond with atoms of the biologically active molecule to be labeled. Maleimide ring-containing nitroxide compounds are especially useful for labeling most proteins through reaction between the moleimide ring and sulfhydryl (—SH) and ϵ=amino (ϵ=NH_2) groups of the protein molecules.

Thus, in accordance with this invention, a class of improved nitroxide compounds exhibiting ESR and useful for spin labeling biologically active molecules are those organic free radicals of the general formula,

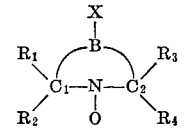


I

where C_1 and C_2 are tertiary carbon atoms; C_1 and C_2 are bonded directly to carbon or fluorine atoms; A represents at least one independent organic group and has a total valency of 6 for bonding to said C_1 and C_2 tertiary carbon atoms. (The broken lines between A and C_1 and C_2 representing 6 saturated bonds); and where A containing at least one maleimide ring, which is operative to form a bond with a biologically active molecule.

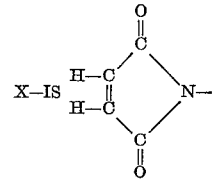
As will appear more fully hereinafter, A may represent one or more independent organic groups up to a total of 6 and the maleimide functional group serving to form the bond with the biologically active molecule may be present on any one or more of these groups.

Present work has shown that much useful information can be gained where A in the above formula includes a plurality of carbon atoms arranged to form a closed ring with C_1 and C_2 and where C_1 and C_2 are further substituted with lower alkyl groups so as to provide the requisite tertiary character for C_1 and C_2. These materials may be defined as having the general formula,



II

wherein R_1, R_2, R_3 and R_4 are lower alklyl groups i.e., each having 1–5 carbon atoms; B represents a plurality of carbon atoms in a partial cycloalkyl chain, and X is the maleimide ring:



III

In this latter situation it will be appreciated that A in the general formula previously discussed comprises four independent organic groups, namely R_1, R_2, R_3 and R_4, and B, the five groups having a total valency of 6 since B is divalent, whereas the R's are monovalent.

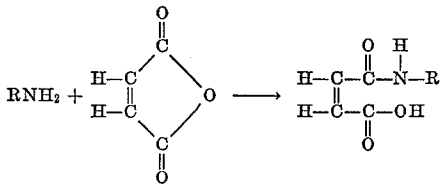
Within the group of materials covered by Formula II, an especially useful material is that obtained where R_1, R_2, R_3 and R_4 are each a methyl group, B represents an ethylene or propylene group so as to form a five or six membered saturated heterocyclic ring, respectively, with the nitrogen atom, and X is the maleimide ring attached to one of the carbon atoms in the ethylene or propylene group.

It is to be noted that whereas the preferred materials include a heterocyclic ring as provided by Formula II the ring structure is not essential so long as the tertiary character of C_1 and C_2 is retained. Further, and as already mentioned, C_1 and C_2 in addition to being tertiary must have all of their valencies satisfied by saturated bonds to either carbon atoms or fluorine atoms. For example, in Formula IV given above, the replacement of the methyl groups by fluorine atoms would provide typical compounds contemplated within the scope of this invention.

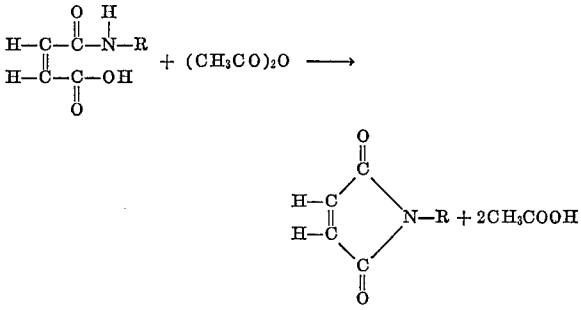
The maleimide group is especially useful for labeling proteins and some synthetic polypeptides, since the male-

imide group forms covalent bonds by reaction with both sulfhydryl groups and ε-amino groups of proteins. The maleimide ring also is not rapidly hydrolyzed by water in competition with the labeling reaction with a great variety of proteins.

Preparation of labels having the maleimide ring follows conventional organic synthesis procedures. In general, there are a number of compounds of various structures known and available containing the requisite nitroxide group with adjacent tertiary carbon atoms. The present materials may be conveniently prepared from corresponding compounds having the structure of the end product sought with the difference being the presence of an amino group at the site where the maleimide group is desired. With such a corresponding amino compound the maleimide is obtained by conventional condensation with maleic anhydride to form the maleamic acid



and then water is eliminated by heating with a dehydrating agent such as acetic anhydride.

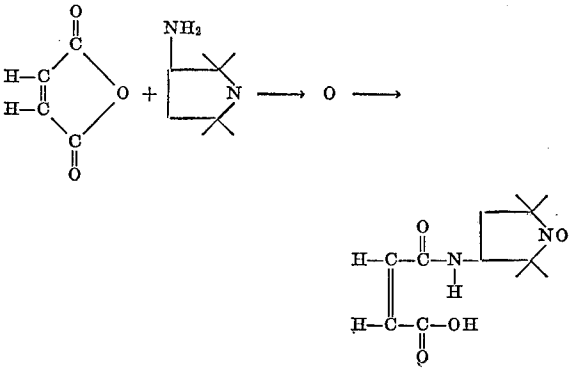


EXAMPLE

2,2,5,5 - tetramethyl - 3 - amino - pyrrolidine - 1 - oxyl is used as a starting material and may be synthesized from triacetoneamine by the method of Rozantzev and Krivitzkaya, Tetrahedron 21, 491 (1965). An equimolar amount (0.40 gm.) of the starting material in one ml. anhydrous ether was slowly added with stirring to a room-temperature solution of 0.25 gm. of maleic anhydride in 5 ml. anhydrous diethyl ether. The N-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-maleamic acid (V) immediately precipitated and after three hours of stirring at room temperature the precipitate was filtered, washed ten times with 0.4 ml. of anhydrous diethyl ether, and dried; yield 97%.

Analysis.—Calc. for $C_{12}H_{19}N_2O_4$: C, 56.5; H, 7.5; N, 11.0%. Found: C, 56.0; H, 7.5; N, 11.3%.

The reaction is

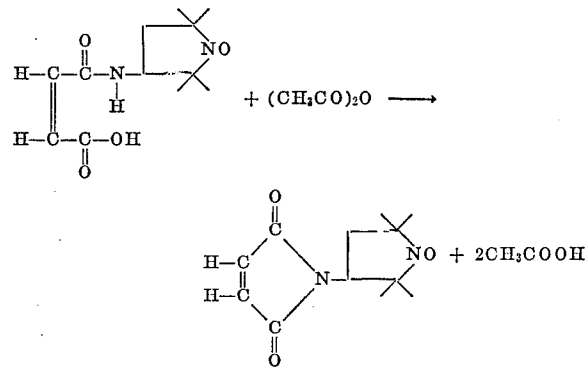


V

The desired improved spin-label, N-(1 - oxyl - 2,2,5,5-tetramethylpyrrolidinyl)-maleimide was obtained from the above acid, as follows. A mixture of 0.63 gm. V, 5 ml. acetic anhydride, and 0.2 gm. sodium acetate were stirred in a tightly closed container for twenty-four hours at 25–35° C. The acetic anhydride was removed in vacuo at room temperature and the crude product was obtained as a viscous oil which solidified on standing. N-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-maleimide VI was purified by sublimation or by recrystallization from benzene (compound V is insoluble in benzene).

Analysis.—Calc. for $C_{12}H_{17}N_2O_3$: C, 60.7; H, 7.2; N, 11.8%. Found: C, 60.6; H, 7.3; N, 11.8%.

The reaction is



VI

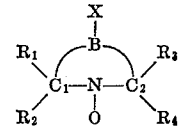
In typical labeling experiments involving proteins, the solid labeling compound VI is added to a stirred solution of the protein, and non-attached paramagnetic radicals removed from the solution by dialysis, or by use of a sephadex column. In this way the following proteins and synthetic polypeptides have been spin labeled using VI:

alcohol dehydrogenase
aldolase
catalase
carboxypeptidase
ceruloplasmin
α-chymotrypsin
creatin kinase
cytochrome C
haemoglobin
lactate dehydrogenase
lipase
lysozyme
papain
poly-L-lysine
trypsin
soluble rat brain proteins
tobacco mosaic virus.

Attachment of the spin label VI to poly nucleic acids is also possible, as, for example, by first reacting VI with an aromatic dye (having amino groups) or antibiotics that in turn bind to nucleic acids.

What is claimed is:

1. A nitroxide of the formula



wherein R_1, R_2, R_3 and R_4 are lower alkyl groups, B is an alkylene group having 2–3 carbon atoms forming a heterocyclic ring with C_1 and C_2; and X is a maleimide ring.

2. A nitroxide in accordance with claim 1 wherein said molecule is N-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl-3)-maleimide.

References Cited

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U.C. Cl. X.R.

23—230; 195—103.5; 260—294.7, 112, 112.5; 424—2

PO-1050
(5/69)

UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 3,481,952 Dated December 2, 1969

Inventor(s) Harden H. McConnell et al. Assignee: Synvar Associa

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 1, line 6, the word "Synbar" should be -- Synvar --

SIGNED AND
SEALED
MAY 5 1970

(SEAL)
Attest:

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