

# PATENT SPECIFICATION

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## (54) RADIOLABELED DERIVATIVES OF FOLIC ACID

(71) We, BECTON, DICKINSON AND COMPANY, a corporation organised and existing under the laws of the State of New Jersey, United States of America, of Rutherford, New Jersey, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to derivatives of folic acid (pteroylglutamic acid), and more particularly, to radiolabeled derivatives of folic acid, intermediates for the preparation of such radiolabeled derivatives; the assay of folates with such derivatives, and the method of preparing such folates.

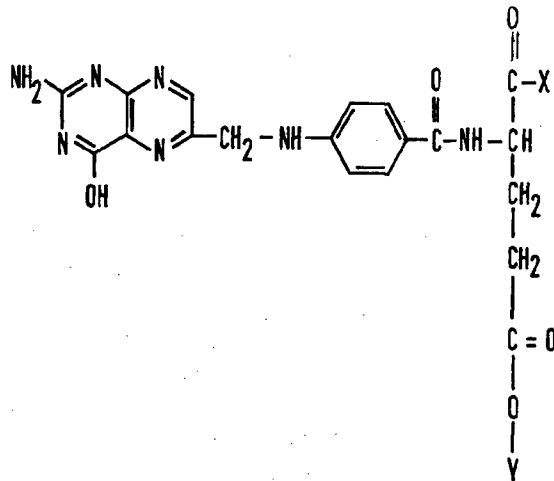
Folate (folic acid) deficiency is present in about one-third of all the pregnant women in the world, the vast majority of alcoholics, the majority of people who eat a diet of fresh uncooked fruits, or fresh uncooked vegetables or fresh fruit juices, many people with structural or functional damage in the upper third of the small bowel (including patients with tropical or nontropical sprue or a wide variety of malabsorption syndromes), and in many other people. Measurement of both serum and red cell folate levels constitutes the most direct and reliable means of determining the existence of folate deficiency, and these tests should be performed for every patient who has a megaloblastic anemia, as well as every patient who has anemia, hypersegmentation of granulocyte nuclei, and coincident evidence of iron deficiency.

The finding of low serum folate means that the patient's recent diet has been subnormal in folate content and/or that his recent absorption of folate has been subnormal, but does not prove the patient has or will develop tissue folate depletion requiring folate therapy. A low red cell folate can mean either that there is tissue folate depletion due to folate deficiency requiring folate therapy, or alternatively, that the patient has primary Vitamin B<sub>12</sub> deficiency blocking the availability of cells to take up folate, in which case the proper therapy would be with Vitamin B<sub>12</sub> rather than with folic acid. It is for these reasons that it is advisable to determine red cell folate in addition to serum folate, and thereby definitely determine that the diagnosis is folate deficiency for which the proper treatment is folic acid.

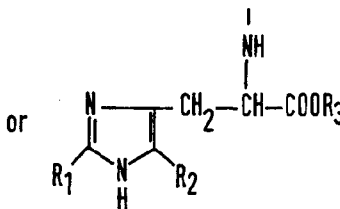
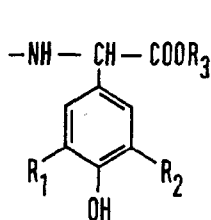
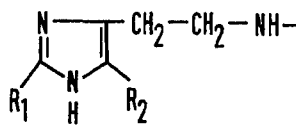
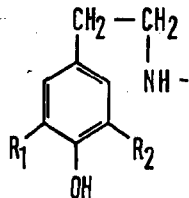
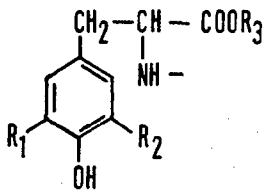
Endogenous folate is measured by a competitive binding technique which involves the ability of unlabeled folate in serum or other media to compete with labeled folic acid for a specific folate binder, present in usable concentrates, in such sources as cows milk, hog kidney, etc., and thereby inhibit the binding of labeled folic acid. As a result of the competitive inhibition, the ratio of bound labeled folic acid to free labeled folic acid diminishes as the concentration of unlabeled folate is increased. Accordingly, the concentration of folate in an unknown sample; e.g., patient's blood, is obtained by comparing the inhibition observed with that produced by known amounts of folate, as presented in a standard curve. The labeled folic acid generally employed in the assay is a radiolabeled folic acid, such as folic acid radiolabeled with tritium, and there is a need for improved radiolabeled compounds for the assay of folates by a radioassay technique.

In accordance with the present invention there is provided derivatives of folic acid wherein the  $\alpha$ -carboxyl group is substituted with an amino compound having an aromatic or heterocyclic ring substituent which is capable of being radiolabeled.

More particularly, in accordance with the present invention, there is provided novel derivatives of folic acid having the following structural formula:



wherein Y is —H, a C to C<sub>6</sub> alkyl (preferably C<sub>1</sub> or C<sub>2</sub>), an alkali metal or alkaline earth metal cation, a protonated amine or ammonium; X is one of the following:



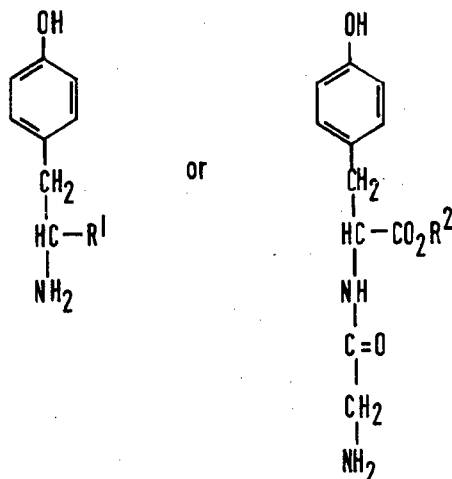
wherein R<sub>1</sub> and R<sub>2</sub> are each separately hydrogen, fluoro, chloro, bromo, nitro, C<sub>1</sub> to C<sub>6</sub> alkoxy, C<sub>1</sub> to C<sub>6</sub> alkyl, or a radioactive isotope of iodine, provided that at least one of R<sub>1</sub> and R<sub>2</sub> is hydrogen when the radical is unlabeled, and R<sub>3</sub> is hydrogen or C<sub>1</sub> to C<sub>6</sub> alkyl (preferably methyl or ethyl), an alkyl metal or alkaline earth metal cation, a protonated amine or ammonium

The radioactive isotope of iodine is preferably I<sup>125</sup>, I<sup>131</sup>, or I<sup>123</sup>.

The preferred radiolabeled compounds are the radioiodinated derivatives in which Y and R<sub>3</sub> are —H in that such derivatives are similar to folic acid with respect to lipophilicity, polarity, solubility and hydrophilicity, with the substituted and unsubstituted tyrosyl and histidyl derivatives being particularly preferred. Such preferred compounds are generally mono-radioiodinated.

In the above compounds, in the case where X has optically active isomers, X may be in the L-, D-, or DL-form, with the L-form being most preferred.

In Specification No. 1,504,263 there are described and claimed derivatives of folic acid comprising folic acid amide coupled through one or both of its carboxyl groups with the amino nitrogen of an amine compound having the formula:



(in which R<sup>1</sup> represents —H, —COOH or —COOR<sup>3</sup>, R<sup>3</sup> represents an alkyl group containing 1 to 5 carbon atoms and R<sup>2</sup> represents a hydrogen atom or an alkyl group containing 1 to 5 carbon atoms) and gamma emitting derivatives in which the benzene ring of the amine compound contains a gamma emitting isotope substituent, e.g. gamma emitting iodine.

It is explained in Specification No. 1,504,263, that the claimed derivatives can be prepared employing conventional techniques to couple folic acid to the amine compound and to introduce the isotopic iodine. In the Example I of the said specification, folic acid is coupled with tyramine hydrochloride to give a gel product which is ground after drying and used without further purification.

There is no disclosure or suggestion in the Specification No. 1,504,263 that the compounds of the present invention should be prepared and separated.

The compounds of the present invention are prepared by condensation of folic acid with the appropriate amine or amino acid derivative, preferably in the presence of peptide condensing agent, in a solvent system, followed by separation of the  $\alpha$ -substituted derivative. The radioiodinated compounds can be prepared by radioiodination of the  $\alpha$ -substituted derivative by one of the conventional procedures known in the art.

More particularly, folic acid is condensed with the appropriate amine or amino acid derivative; e.g. tyrosine or a substituted tyrosine in the presence of a condensing agent conventionally employed for the production of peptides. As representative examples of such agents, there may be mentioned: 1 - ethyl - 3 - (3 - dimethylaminopropyl)carbodiimide or its hydrochloride salt; dicyclohexylcarbodiimide; 1 - ethyl - 3 - (4 - morpholinyl)carbodiimide or its HCl salt; 1 - isopropyl - 3 - (3 - dimethylaminopropyl)carbodiimide or its HCl salt; 1 - cyclohexyl - 3 - (3 - dimethylaminopropyl)carbodiimide or its HCl salt. The process of the present invention is not limited to such condensing agents, and the selection of a suitable condensing agent is deemed to be within the scope of those skilled in the art from the teachings herein.

The condensation is effected in a suitable solvent for the folic acid and appropriate derivative. The preferred solvent system is a mixture of water and a water miscible organic solvent, such as pyridine, dioxane, tetrahydrofuran, dimethylformamide, dimethyl sulfoxide, or dimethylacetamide. The preferred system is pyridine and water. In some cases, it is possible to employ a mixture of water and a non-water miscible organic solvent, in combination with a suitable base, such as pyridine, triethylamine or N-methylmorpholine. The selection of a suitable solvent system is within the scope of those skilled in the art having regard to the teachings herein.

The condensation is generally effected at a temperature of from 0° to 65°C, preferably from 5° to 30°C. The pH of the reaction is generally from 5 to 10, preferably from 7—9.

The reaction mixture contains unreacted folic acid, disubstituted folic acid,  $\alpha$ -substituted folic acid and  $p$ -substituted folic acid. In accordance with the present invention, the disubstituted derivative is selectively removed from an aqueous solution of the mixture e.g. by appropriate adjustment of the pH, generally a pH of from 8—12, preferably 8.5 to 10.

After separating the disubstituted derivative, the desired  $\alpha$ -substituted product is selectively precipitated from the mixture e.g. by appropriate adjustment of the pH, generally a pH of from about 2—5, preferably 2.5—3.

The separated  $\alpha$ -substituted derivative can then be radioiodinated by conventional procedures to produce the radioiodinated derivatives of the present invention. Alternatively, the appropriate amine or amino acid derivative can be radioiodinated prior to the condensation with the folic acid, in which case, radioiodinated subsequent to the condensation is not necessary.

In accordance with a preferred aspect of the present invention, the preferred intermediates for preparing the radioiodinated derivatives are those in which one of  $R_1$  and  $R_2$  is hydrogen and one of  $R_1$  and  $R_2$  is fluoro, chloro, bromo, nitro,  $C_1$  to  $C_6$  alkyl or  $C_1$  to  $C_6$  alkoxy in that subsequent radioiodination produces a monoradioiodinated derivative. Alternatively, as a preferred procedure, the derivative to be condensed with folic acid is an amine or amino acid derivative in which one of  $R_1$  and  $R_2$  is a radioactive isotope of iodine and one of  $R_1$  and  $R_2$  is fluoro, bromo, chloro,  $C_1$  to  $C_6$  alkoxy,  $C_1$  to  $C_6$  alkyl or nitro.

As an alternative procedure for preparing the compounds of the present invention, folic acid is converted to the anhydride in the presence of a suitable condensing agent, such as dicyclohexylcarbodiimide, followed by condensation of the anhydride with the appropriate amine or amino acid derivative.

The radioiodinated derivatives of the present invention are particularly suitable to use as the tracer in a radioassay of folates in which a sample containing folate is incubated with a folate receptor and a folate tracer.

The invention will be further described with respect to the following Examples; however, the scope of the invention is not to be limited thereby.

#### Example I

A. A mixture of 230 mg of L-tyrosine methyl ester hydrochloride, 440 mg of pteroylglutamic acid and 250 mg of 1 - ethyl - 3 - (3 - dimethyl - aminopropyl)carbodiimide hydrochloride was dissolved in 10 ml of 1:1 pyridine water and stirred at room temperature for one hour and 4°C for 16 hours. The reaction mixture was diluted with 10 ml 0.5% sodium bicarbonate solution and filtered. The filtrate was acidified to pH 2.5 using 0.5N HCl solution. The solid  $\alpha$  - (pteroylglutamyl) - L - tyrosine methyl ester was filtered, washed with cold water and dried in vacuo. M.P. 255—56° (decomposition) Log  $\epsilon$  4.44 and 283 nm (0.1N NaOH)  $R_f$  0.46 (paper chromatography, 0.5%  $\text{NaHCO}_3$ ).

B. A mixture of 243 mg of DL-3-fluorotyrosine methyl ester hydrochloride, 443 mg of pteroylglutamic acid and 255 mg of 1 - ethyl - 3 - (3 - dimethylamino propyl)carbodiimide hydrochloride was stirred at 0°C for 2 hours and then 6 hours at room temperature. The reaction mixture was diluted with 15 ml of 0.4% sodium bicarbonate solution and was filtered. The solid was discarded. The filtrate was acidified and the separated solid  $\alpha$  - (pteroylglutamyl) - DL - 3 - fluorotyrosine methyl ester was filtered, washed with 50 ml cold water and dried in vacuo. M.P. >300°C. Log  $\epsilon$  4.44 at 282 nm (0.1N NaOH)  $R_f$  0.46 (paper chromatography, 0.5%  $\text{NaHCO}_3$ ).

The procedure of Example IB was also repeated with DL-3-fluorotyrosine ethyl ester, tyramine, histidine methyl ester and histamine to produce the corresponding derivatives of folic acid.

#### Example II

A. Nitrogen gas was bubbled through a mixture of 300 mg of  $\alpha$  - (pteroylglutamyl) - L - tyrosine methyl ester and 3 ml of 0.2N sodium hydroxide solution. After a few minutes the solution was filtered and acidified with 1N hydrochloric acid. The separated solid  $\alpha$  - (pteroylglutamyl) - L - tyrosine was filtered, washed with cold water and dried under vacuum. M.P. 275—285° (decomposition). U.V. Log  $\epsilon$  4.43 at 283 nm.  $R_f$  0.58 (paper chromatography, 0.5%  $\text{NaHCO}_3$ ).

B. Three milliliters of 0.2N sodium hydroxide was added to 312 mg of  $\alpha$  - (pteroylglutamyl) - DL - 3 - fluorotyrosine methyl ester) in the presence of

nitrogen gas. The mixture was quickly acidified and diluted with 10 ml cold water. The solid  $\alpha$  - (pteroylglutamyl) - DL - 3 - fluorotyrosine was filtered and dried. M.P. >300°C. U.V. Log  $\epsilon$  4.42 at 282 nm  $R_f$  0.59 (paper chromatography using 0.5% NaHCO<sub>3</sub>).

The procedure is repeated with  $\alpha$  - (pteroylglutamyl) - L - histidine methyl ester to produce the corresponding histidyl derivative of folic acid.

### Example III

A. Iodination of 60  $\mu$ g  $\alpha$  - (pteroylglutamyl) - L - tyrosine produced by the procedure of Example IIA with 10 mC<sup>125</sup>I is effected at pH 7.4 by the method of Hunger and Greenwood at a substrate to iodine ratio of 20 to 1. Unreacted iodine is removed by passage through a quaternary-amine anion exchange resin in the chloride form. The co-absorbed product is eluted and contains 8.5 mC in the two iodination products,  $\alpha$  - (pteroylglutamyl) - 3 - iodotyrosine and  $\alpha$  - (pteroylglutamyl) - 3,5 - diiodotyrosine which are formed in the ratio of 7 to 1. Also formed are some fragments of folic acid. The separation of the mono and diiodinated products was achieved by cellulose column chromatography.

This procedure is repeated with  $\alpha$  - (pteroylglutamyl) - L - tyrosine. The ratio of mono and diiodinated products obtained is 8 to 1.

B. Iodination of 21.5  $\mu$ g  $\alpha$  - (pteroylglutamyl) - DL - 3 - fluoro - tyrosine produced by the procedure of Example IIB with 5mC<sup>125</sup>I is effected at pH 7.4 by the method of Hunter and Greenwood at a substrate to iodine ratio of 20 to 1. Unreacted iodine is removed by passage through a quaternary amine exchange resin in the chloride form. The co-absorbed form is eluted with a mixture of tetrahydrofuran and hydrochloric acid and contains 4.3 mC in the monoiodination product  $\alpha$  - (pteroylglutamyl) - DL - 3 - fluoro - 5 - iodotyrosine. The pure product is separated from the fragments by passage through a cellulose column.

This procedure is repeated with  $\alpha$  - (pteroylglutamyl) - L - histamine and  $\alpha$  - (pteroylglutamyl) - L - histidine as produced by the procedure in Example IIB.

The radioiodinated derivatives of the present invention may be used as the labeled antigen in the radioassay of folic acid. A radioassay procedure which may be employed is one which is disclosed by Givas et al.; Clin. Chem., Vol. 21, pp 427-428 (March 1975) for tritium labeled folic acid as follows:

To 50 microliter of serum in disposable glass tube in 1.5 ml Lysine buffer (pH 9.2 $\pm$ .2) is added, with thorough mixing the calculated amount of radioiodinated derivative of the present invention. Folate binding protein is then added in sufficient amount to produce 50% $\pm$ 10% binding of the radioiodinated derivative in the absence of unlabeled drug, and the mixture is incubated at 25°C for 30 minutes. Competition between the radioiodinated compound and unlabeled 5-methyltetrahydrofolic acid for protein binding sites determines the amount of radioiodinated compound-antibody complex present at equilibrium. Separation of bound from free radioiodinated compound is achieved by the dextran coated charcoal technique, resulting in selective binding of the free labeled and unlabeled compound to the coated charcoal, which is then separated by centrifugation. The supernatant phase is decanted and counted in a gamma counter.

The radioiodinated folic acid derivatives of the present invention are an improvement over the tritiated folic acid presently employed in the art for the radioassay of folic acid for the following reasons:

1. An inexpensive well counter may be used as compared to the costly and complex liquid scintillation counter required for the tritiated compounds.

2. Liquid scintillation fluids and special vials are not needed.

3. No internal or external standardizations are needed as in the case of tritiated folic acid.

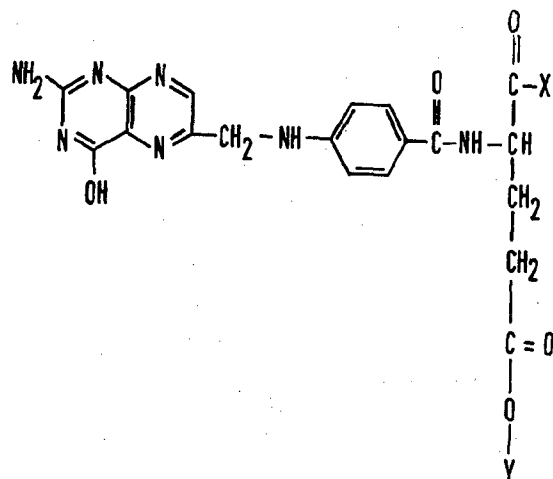
4. Counting efficiencies are higher, particularly in aqueous media.

More specifically, radioiodinated folic acid derivatives can be made at higher specific activity than tritiated folic acid. The relatively low specific activity and the lower counting efficiency of tritiated folic acid limit its commercial value.

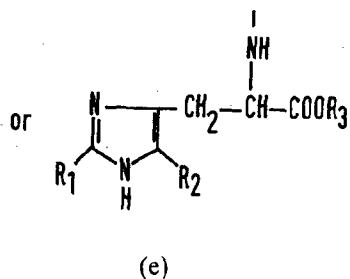
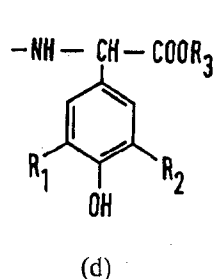
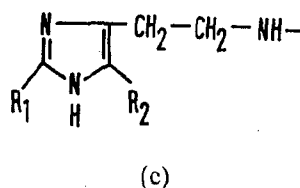
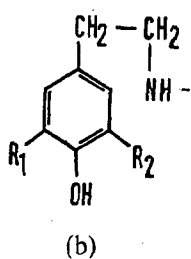
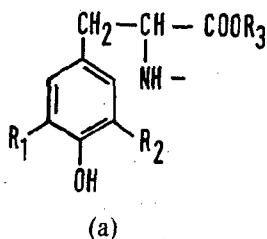
In addition, the radioiodinated derivatives, of the present invention in which the amino acid moiety is in the acid form, instead of the ester form, significantly increases the polarity, solubility and hydrophilicity of the compound whereby such derivatives are water soluble at physiological pH values; can be iodinated in aqueous media; possesses a side chain more nearly comparable in polarity to folic acid, exhibit superior binding to the binding protein, and shows lesser tendency toward absorption glass surface or lipophilic surfaces such as plastic test tubes.

WHAT WE CLAIM IS:—

1. A compound having the following structural formula:—



5 wherein Y is —H, a C<sub>1</sub> to C<sub>6</sub> alkyl, an alkali metal or alkaline earth metal cation, a protonated amine or ammonium; X is a radical having one of the following structural formulae: 5



10 wherein R<sub>1</sub> and R<sub>2</sub> are the same or different and each is hydrogen, fluoro, chloro, bromo-, nitro, C<sub>1</sub> to C<sub>6</sub> alkoxy, C<sub>1</sub> to C<sub>6</sub> alkyl, or a radioactive isotope of iodine, provided that at least one of R<sub>1</sub> and R<sub>2</sub> is hydrogen when the other of R<sub>1</sub> and R<sub>2</sub> is other than a radioactive isotope of iodine, and R<sub>3</sub> is hydrogen, C<sub>1</sub> to C<sub>6</sub> alkyl, an alkali metal or alkaline earth metal cation, a protonated amine, or ammonium. 10

15 2. A compound according to Claim 1 wherein X has structural formula (a). 15

3. A compound according to Claim 1 wherein X has structural formula (b).

4. A compound according to Claim 1 wherein X has structural formula (c).  
 5. A compound according to Claim 1 wherein X has structural formula (d).  
 6. A compound according to Claim 1 wherein X has structural formula (e).  
 7. A compound according to any one of the preceding claims wherein Y is  
 5 hydrogen.
8. A compound according to any one of the preceding claims wherein R<sub>3</sub> is  
 hydrogen.
9. A compound according to any one of the preceding claims wherein at least  
 one of R<sub>1</sub> and R<sub>2</sub> is a radioactive isotope of iodine.
10. A compound according to any one of the preceding claims wherein one of  
 10 R<sub>1</sub> and R<sub>2</sub> is a radioactive isotope of iodine and the other is other than a radioactive  
 isotope of iodine.
11. A compound according to Claim 1 wherein the compound is  
 radioiodinated  $\alpha$ -(pteroylglutamyl)-L-tyrosine.
12. A compound according to Claim 1 wherein the compound is  $\alpha$  -  
 15 (pteroylglutamyl) - DL - 3 - fluoro - 5 - <sup>125</sup>I - tyrosine.
13. A process for the radioassay of folates wherein a sample containing folate  
 is incubated with a folate receptor and a folate tracer, which comprises:  
 20 employing as the tracer a compound as defined in any one of the preceding  
 claims wherein at least one of R<sub>1</sub> and R<sub>2</sub> is a radioactive isotope of iodine.
14. A process for producing a compound as defined in Claim 1, comprising:  
 25 condensing in solution folic acid with an amine or amino acid derivative of  
 formula XH where X is as defined in Claim 1; adjusting the pH of the solution to  
 from 8—12 to precipitate and separate any di-substituted derivatives; and  
 thereafter adjusting the pH to from 2—5 to precipitate and separate an  $\alpha$ -mono-  
 substituted derivative of folic acid as defined in Claim 1.
15. The process of Claim 14 wherein the condensation is effected with a  
 peptide condensing agent at a pH of from 5—10.
16. The process of Claim 14 or 15 wherein X has structural formula (a), R<sub>1</sub> is  
 30 hydrogen R<sub>2</sub> is other than a radioactive isotope of iodine.
17. The process of Claim 14, 15 or 16 wherein R<sub>2</sub> is hydrogen.
18. A compound according to Claim 1 produced in accordance with the  
 Examples.
19. A process for producing a compound as defined in Claim 1 in accordance  
 35 with the Examples.
20. A process for the production of a compound as claimed in Claim 1 in which  
 R<sub>1</sub> and/or R<sub>2</sub> is a radioactive isotope of iodine which process comprises  
 radioiodinating a corresponding compound in which R<sub>1</sub> and/or R<sub>2</sub> is a hydrogen  
 atom.

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