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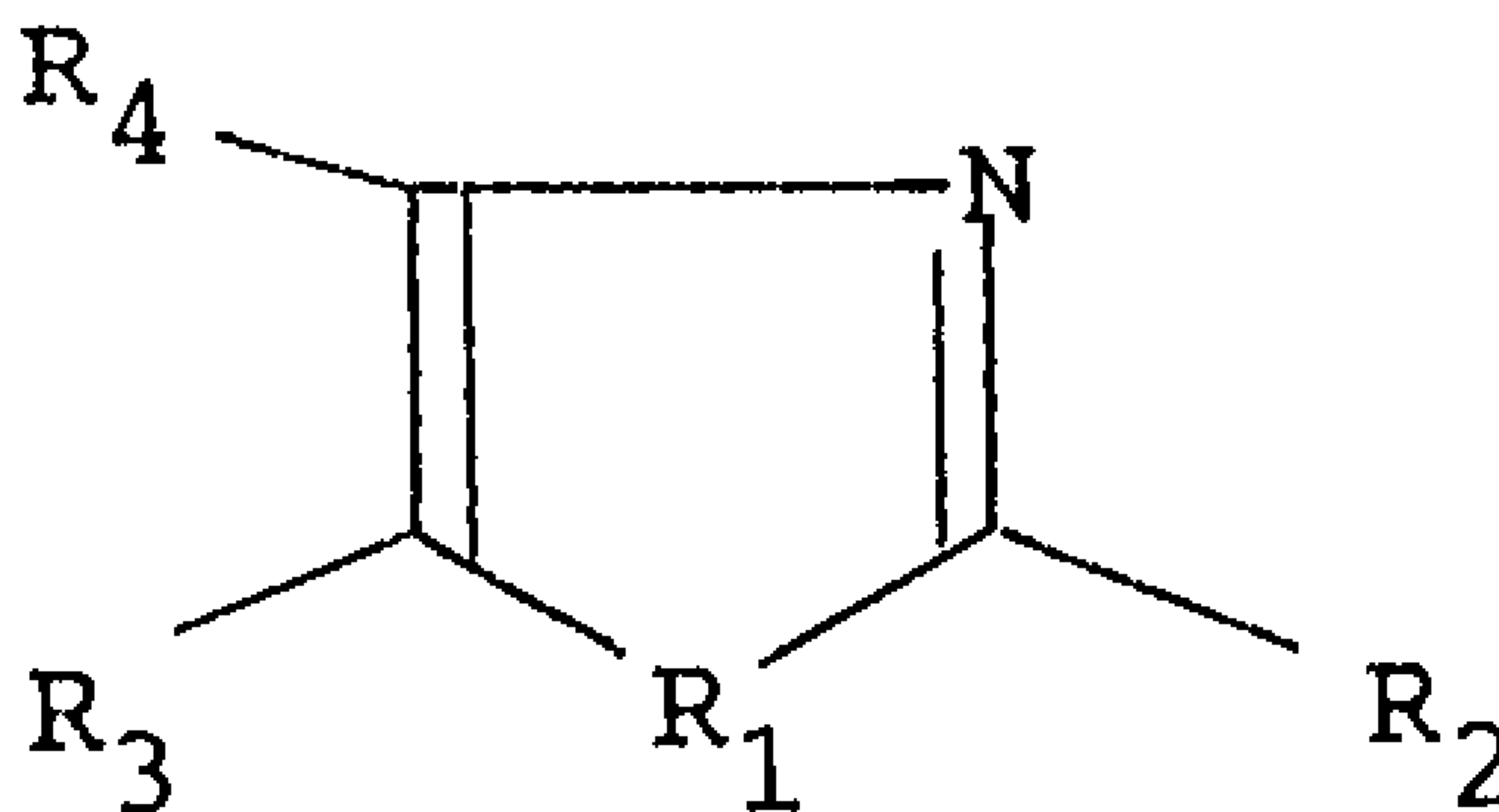
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(54) Titre : METHODE D'AUGMENTATION DE LA CHIMIOLUMINESCENCE

(54) Title: METHOD FOR ENHANCEMENT OF CHEMILUMINESCENCE



(57) Abrégé/Abstract:

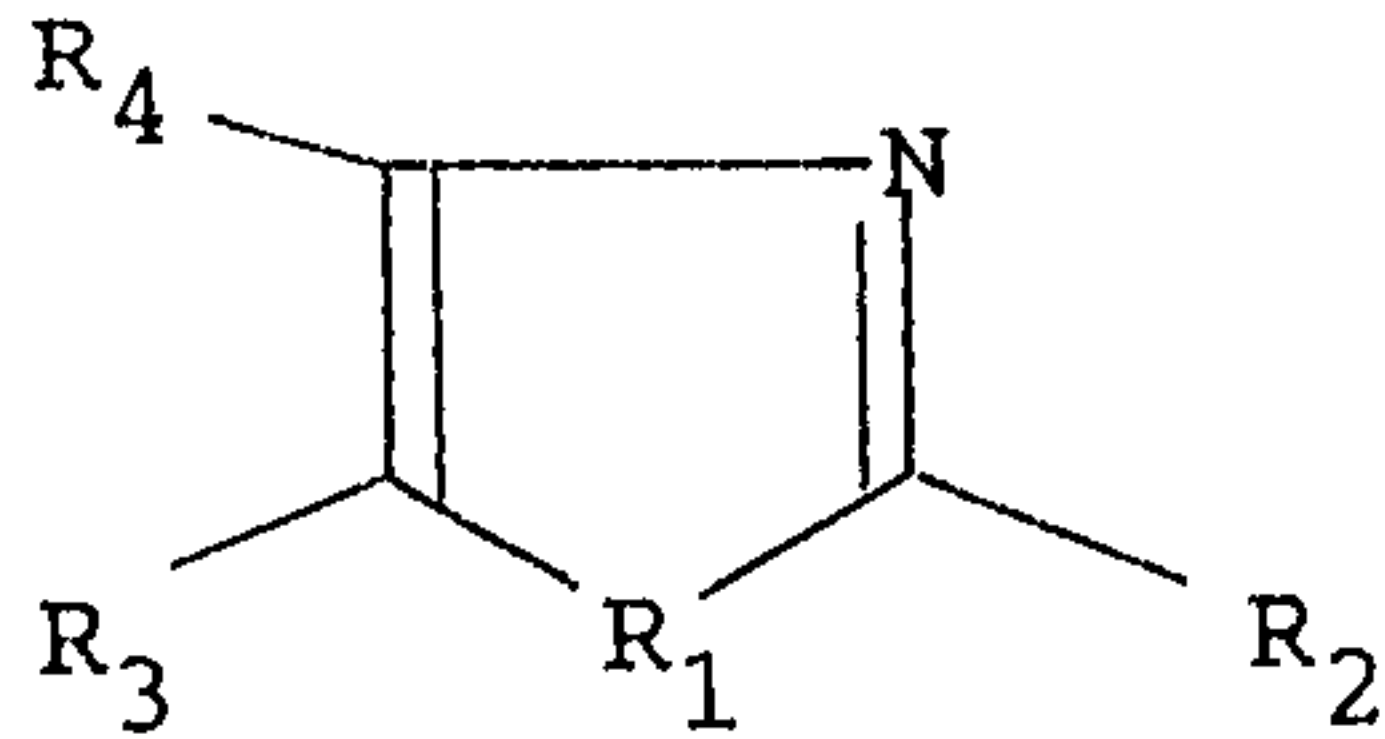
A method for enhancing chemiluminescence which uses a heterocyclic compound of the formula: (see above formula) wherein R₁ is an oxygen or sulfur atom or an imino group optionally substituted by 4-hydroxyphenyl, and R₂, R₃ and R₄ are a hydrogen or halogen atom, an optionally substituted hydrocarbon residue, a heterocyclic group or the like in a luminescence system.



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ABSTRACT OF THE DISCLOSURE

A method for enhancing chemiluminescence which uses a heterocyclic compound of the formula:



wherein R₁ is an oxygen or sulfur atom or an imino group optionally substituted by 4-hydroxyphenyl, and R₂, R₃ and R₄ are a hydrogen or halogen atom, an optionally substituted hydrocarbon residue, a heterocyclic group or the like in a luminescence system.

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SPECIFICATION

TITLE OF THE INVENTION

Method for enhancement of chemiluminescence

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a method for enhancing chemiluminescence.

Particularly, this invention relates to a method for detecting a chemically luminous substance, especially to a method for enhancing chemiluminescence which can achieve an improvement of sensitivity of immunological quantitative analysis, enzymatic quantitative analysis or the like based on the quantitative analysis of chemically luminous substance and measuring of chemiluminescence.

2. Description of the Prior Arts

Analytical method based on chemical luminous reaction has a possibility to become an extremely high-sensitive method for detecting a chemical substance and has been intensively studied. An attention has been focussed to this analytical method especially in the field of immunological analysis, to which various kind of techniques have been tried.

In the immunological analysis, a method using radioactive isotope (RIA) as a label has been developed at first, which however shows the disadvantages due to the use of radioactive isotope, namely, short half-valued period, bio-hazard problem and occasionally insufficient sensitivity. And then, a detecting method by colorimetric analysis or fluorescence analysis such as enzyme-immunological analysis using enzyme as a label has been developed for overcoming the disadvantages accompanied by RIA. However, development of new techniques was sought to achieve improvement on sensitivity. Among the new techniques, a method utilizing chemiluminescence as detecting means is expected to be the most sensitive method and accordingly has been intensively studied.

Immunochemical analysis method utilizing chemical luminous reaction are generously classified into the following 4 methods [Akio Tsuji et al., Peptide, Nucleic Acid and Enzyme, Extra Issue, Vol. 31, pages 255 - 263 (1988)].

(1) A method labeling a chemically luminous substance such as luminol, isoluminol, acridinium derivative, etc., as a label compound on antibody or antigen.

(2) A method utilizing chemical luminous reaction for measuring enzyme activity in the enzyme immunological analysis (EIA) labeling enzyme on antibody or antigen.

(3) A homogeneous immunological analysis utilizing phenomenon of decrease of coenzyme activity by antigen-antibody reaction in which coenzyme, namely, NAD or ATP is labeled.

(4) Enzyme immunological analysis utilizing biological luminous reaction.

Among these methods, the method (1) involves some problems. That is, the sensitivity can not be improved as expected due to decline of luminous quantum yield caused by chemical combination of luminous substance, and measuring in a very short time of chemiluminescence is extremely difficult.

Improvement of sensitivity by the above method (3) can not be expected, and the method (4) can not be generalized due to use of specific enzyme in many cases.

On the other hand, improvement of sensitivity by the above method (2) can be expected, because the chemically luminous substance exists in a free form in the solution and the luminous reaction is catalyzed by labeled enzyme. In this case, however, the luminous reaction continued generally for a very short time and

could not result in substantial improvement of the sensitivity.

Recently, it was found that total luminous quantum by chemical luminous reaction can be extremely improved by coexistence of a third compound in a chemical luminous reaction system. That is, G. H. G. Thorpe and L. J. Kricka reported a group of compound improving a luminous reaction of luminol using peroxidase as a catalyst in their general remarks in "Methods in Enzymology", Vol. 133, pages 331 - 353 (1986, Academic Press, New York). As described on page 335, strong activity as an enhancer was recognized for 6-hydroxybenzothiazole and phenol derivatives. Chemical luminous reaction can be delayed and chemical luminous quantum can be increased by using those enhancers, and accordingly difficulty to check the reaction in a few seconds was dissolved and the measurement can be conducted under easy conditions.

Following enhancers have been found.

- (1) 6-Hydroxybenzothiazole
- (2) Phenol derivatives such as p-iodophenol or naphthol derivatives (Japanese Patent Kokai No. 171,839/1984)
- (3) Ammonia and water-soluble organic amines (Japanese Patent Kokai No. 124,446/1987)

(4) Aromatic amine compounds (Japanese Patent

Kokai No. 54,453/1986)

(5) Amino acids having cyclic group

(6) Fluorescence substances

Among these enhancers, the above (1) and p-iodophenol in the above (2) were selected as the superior ones.

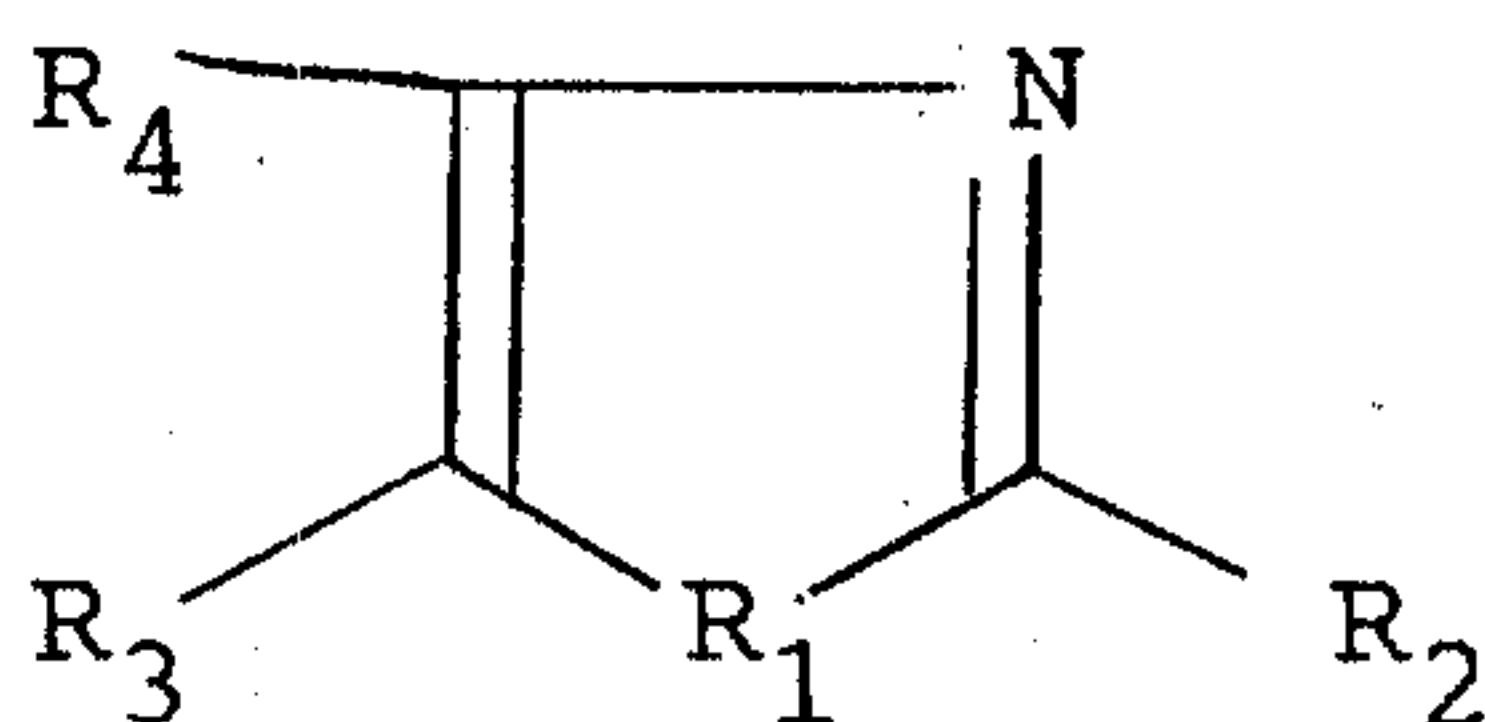
However, it was necessary to find out more excellent enhancer for improving sensitivity of the analysis. That is, if sensitivity could be successfully improved, very small amount of a biologically active substance in a living body can be easily detected, and accordingly any substance which has not been able to be measured due to its too small amount will be able to be detected. Then, it will be possible to know a normal value of a very small amount of a substance, and also to detect an increase of concentration of the substance at an early stage of a disease, and accordingly to make a diagnosis of the disease at an early stage. And further, it will be possible to detect a substance which further decreases in accordance with a disease, and a new method to make a diagnosis of a disease will be developed. Very useful means for managing recuperation of a disease will also be developed. These methods can be utilized not only

in diagnosis of a disease, but also in a field where microanalysis is essential such as environmental analysis, and further in detection of a bioactive substance and elucidation of movement of a substance in a living body.

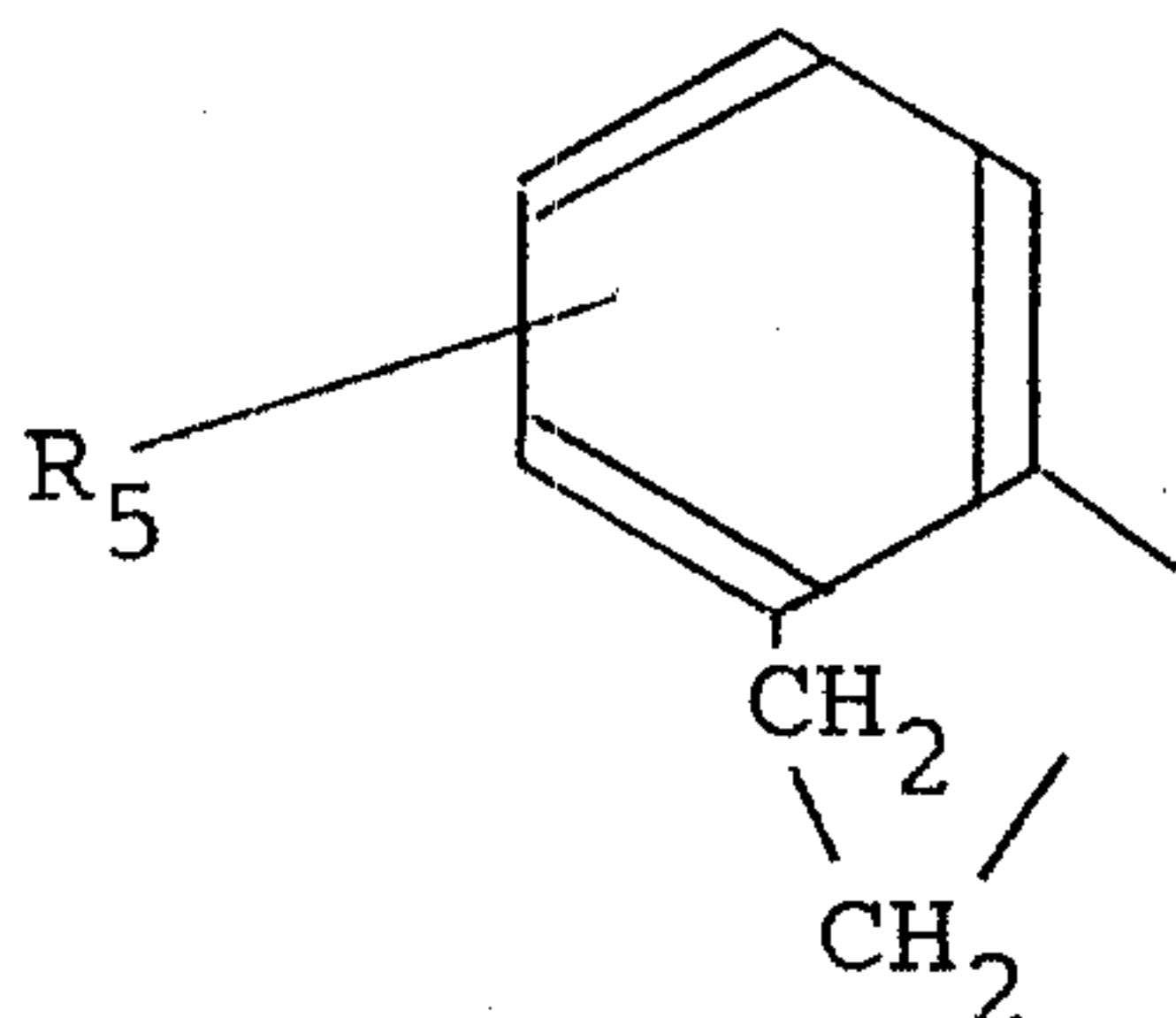
SUMMARY OF THE INVENTION

The inventors of this invention studied intensively for the purpose as mentioned above and found that oxazole derivatives, thiazole derivatives and imidazole derivatives possess strong activity to enhance chemiluminescence.

This invention relates to a method for enhancing chemiluminescence, which comprises using a compound of the general formula (I):



wherein R_1 is an oxygen or sulfur atom or an imino group which may be substituted by 4-hydroxyphenyl, R_2 , R_3 and R_4 are each a hydrogen atom, a halogen atom, a hydrocarbon residue which may be substituted or a heterocyclic group which may be substituted, or R_3 and R_4 may combine to form a di-valent group of the formula:



(wherein, R_5 is a hydrogen atom or a hydroxy group, provided that when R_1 is an oxygen or sulfur atom or an imino group, R_4 is a 4-hydroxyphenyl group and when R_1 is 4-hydroxyphenylimino group, R_4 is a hydrogen atom) in a luminescence system consisting of a chemically luminous substance, an oxidizing agent and a peroxidase.

That is, one characteristic feature of the present invention resides in letting the compound of the general formula (I) co-exist in the known luminescence system.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows a relation between the chemiluminescent signal and HRP concentration.

DESCRIPTION OF PREFERRED EMBODIMENTS

In the above general formula (I), the halogen atom may be fluorine, chlorine, bromine or iodine. The hydrocarbon moiety in the "hydrocarbon residue which

may be substituted" may be alkyl group (preferably C_{1-10} alkyl group), alkenyl group (preferably C_{2-10} alkenyl group), aryl group (preferably C_{6-14} aryl group), or aralkyl group (preferably C_{7-19} aralkyl group). The suitable heterocyclic group may be 5- to 7-membered heterocyclic group, for example, 5- to 7-membered heterocyclic group containing a sulfur atom, nitrogen atom or oxygen atom, 5- to 6-membered heterocyclic group containing 2 to 4 nitrogen atoms, 5- to 6-membered heterocyclic group containing 1 to 2 nitrogen atom(s) and a sulfur atom, and these heterocyclic groups may be condensed with a 6-membered ring containing 1 or 2 nitrogen atom(s), a benzene ring or a 5-membered ring containing a sulfur atom.

The C_{1-10} alkyl group may be methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, isopentyl, neopentyl, hexyl, heptyl, octyl, nonyl, decyl or the like. The C_{2-10} alkenyl group may be, for example, allyl, 2-butenyl, 2-pentenyl, 3-hexenyl, 2-cyclopentenyl, 2-cyclohexenyl, or the like. The suitable aryl group may be the one of 6 to 14 carbon atoms, for example, phenyl, anthryl, biphenyl or the like. The suitable C_{7-19} aralkyl group may be a combination of a C_{6-14} aryl group and a

C₁₋₄ alkyl group, for example, benzyl, phenethyl or the like.

Suitable examples of the above 5- to 7-membered heterocyclic groups may be 2-pyridyl, pyrimidyl, pyrazinyl, pyridazinyl, pyrazolyl, imidazolyl, thiazolyl, oxazolyl, pyrido[2,3-b]pyrimidyl, benzo-pyranyl, 1,8-naphthyridinyl, quinolyl, thieno[2,3-b]pyridyl, tetrazolyl, thiadiazolyl, oxadiazolyl, triazinyl, triazolyl, thienyl, pyrrolyl, pyrrolinyl, furyl, pyrrolidinyl, benzothienyl, indolyl, imidazolidinyl, piperidyl, piperidino, piperazinyl, morpholinyl, morpholino, or the like.

The hydrocarbon residue represented by R₂, R₃ and R₄ may have 1 to 3 optional substituent(s) such as a halogen (e.g., fluorine, chlorine, bromine or iodine), hydroxy group, a C₁₋₆ alkoxy group, an amino group which may be substituted with a C₁₋₆ alkyl (e.g., dimethylamino, diethylamino, dipropylamino, etc.), an amino group substituted with an acyl group derived from an organic carboxylic acid (e.g., C₁₋₁₀ alkanoyl, etc.) (e.g., acetylamino, propionylamino, benzoylamino, etc.), a carbamoyl group which may be substituted with a C₁₋₆ alkyl group (e.g., dimethylcarbamoyl, ethylcarbamoyl, dipropylcarbamoyl, etc.), a mono- or dialkoxyphosphoryl group, an esterified carboxy group,

for instance, an alkoxycarbonyl group, preferably a C_{1-6} alkoxycarbonyl group (e.g., methoxycarbonyl, ethoxycarbonyl, propoxycarbonyl, butoxycarbonyl, etc.), an aryloxy carbonyl group, preferably a C_{6-14} aryloxy carbonyl group (e.g., phenoxycarbonyl, etc.), an aralkyloxy carbonyl group, preferably a C_{7-19} aralkyloxy carbonyl group (e.g., benzyloxy carbonyl, etc.), and the like.

Examples of substituents on the heterocyclic group which may be substituted of R^2 , R^3 and R^4 are a halogen atom (e.g., fluorine, chlorine, bromine or iodine), a C_{1-6} alkyl group, a halogenated C_{1-6} alkyl group substituted by the above mentioned halogen, hydroxy group, a C_{1-6} alkoxy group, a C_{1-10} acyl group, an amino group which may be substituted by a C_{1-6} alkyl or a C_{1-10} acyl (e.g., dimethylamino, diethylamino, dipropylamino, acetylamino, propionylamino or benzoylamino), a carbamoyl group which may be substituted by a C_{1-6} alkyl (e.g., dimethylcarbamoyl, diethylcarbamoyl or dipropylcarbamoyl), a C_{1-6} alkoxy carbonyl group (e.g., methoxycarbonyl, ethoxycarbonyl or propoxycarbonyl) or the above mentioned heterocyclic group.

The compounds represented by the formula (I) may be oxazole derivatives where R_1 is an oxygen atom, thiazole derivatives where R_1 is a sulfur atom or imidazole derivatives where R_1 is an imino group.

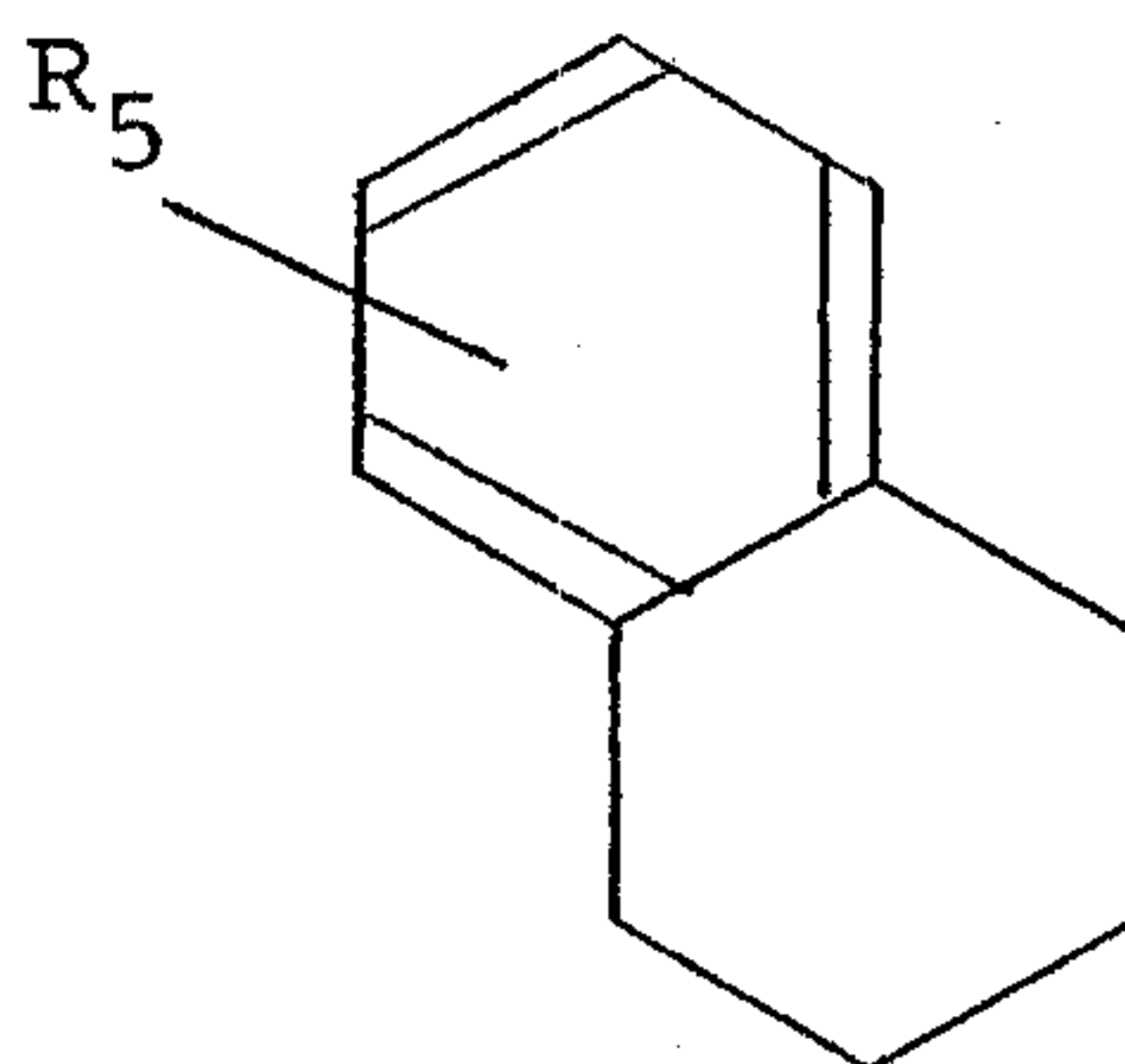
Among these derivatives, preferable compounds are the ones having 4-hydroxyphenyl group for R_4 and hydrogen atom, a halogen atom, a hydrocarbon residue which may be substituted, preferably an alkyl, alkenyl or aromatic group, or a heterocyclic group for R_2 and R_3 . Especially, suitable derivatives are compounds where R_2 and R_3 are hydrogen atoms, compounds where R_3 is a hydrogen atom and R_2 is a lower alkyl group such as methyl group or ethyl group, compounds where R_3 is a hydrogen atom and R_2 is a halogen atom, compounds where R_3 is a lower alkyl group such as methyl group or ethyl group and R_2 is a halogen atom, compounds where R_3 is a halogen atom and R_2 is a hydrogen atom, compounds where R_3 is a halogen atom and R_2 is a lower alkyl group, compounds where R_3 is a pyridyl group and R_2 is a hydrogen atom, compounds where R_3 is a pyridyl group and R_2 is a lower alkyl group such as methyl group or ethyl group, compounds where R_3 is a hydrogen atom and R_2 is a pyridyl group, compounds where R_3 is a lower alkyl group (C_{1-4} alkyl group) such as methyl group or ethyl group and R_2 is a pyridyl group, compounds where

R_3 is a hydrogen atom and R_2 is an alkoxycarbonylalkyl group such as ethoxycarbonylethyl group, and the like.

And further, suitable derivatives are the compounds where R_1 is an imino group substituted by a 4-hydroxyphenyl group, R_3 is a hydrogen atom, R_2 and R_4 are each hydrogen atom, a halogen atom, an alkyl group, alkenyl group or aromatic group which may be substituted or a heterocyclic group, especially the compounds where R_2 and R_4 are hydrogen atoms, compounds where R_2 is a hydrogen atom and R_4 is a lower alkyl group such as methyl group or ethyl group, compounds where R_2 is a hydrogen atom and R_4 is a halogen atom, compounds where R_2 is a lower alkyl group such as methyl group or ethyl group and R_4 is a halogen atom, compounds where R_2 is a halogen atom and R_4 is a hydrogen atom, compounds where R_2 is a halogen atom and R_4 is a lower alkyl group such as methyl group or ethyl group, compounds where R_2 is a pyridyl group and R_4 is a hydrogen atom, compound where R_2 is a pyridyl group and R_4 is a lower alkyl group such as methyl group or ethyl group, compounds where R_2 is a hydrogen atom and R_4 is a pyridyl group, compounds where R_2 is a lower alkyl group such as methyl group or ethyl group and R_4 is a pyridyl group, compounds where R_2 is a hydrogen

atom and R_4 is an alkoxycarbonylalkyl group such as ethoxycarbonylethyl group, and the like.

And further, the compounds where R_3 and R_4 are combined to form a di-valent group of the formula:

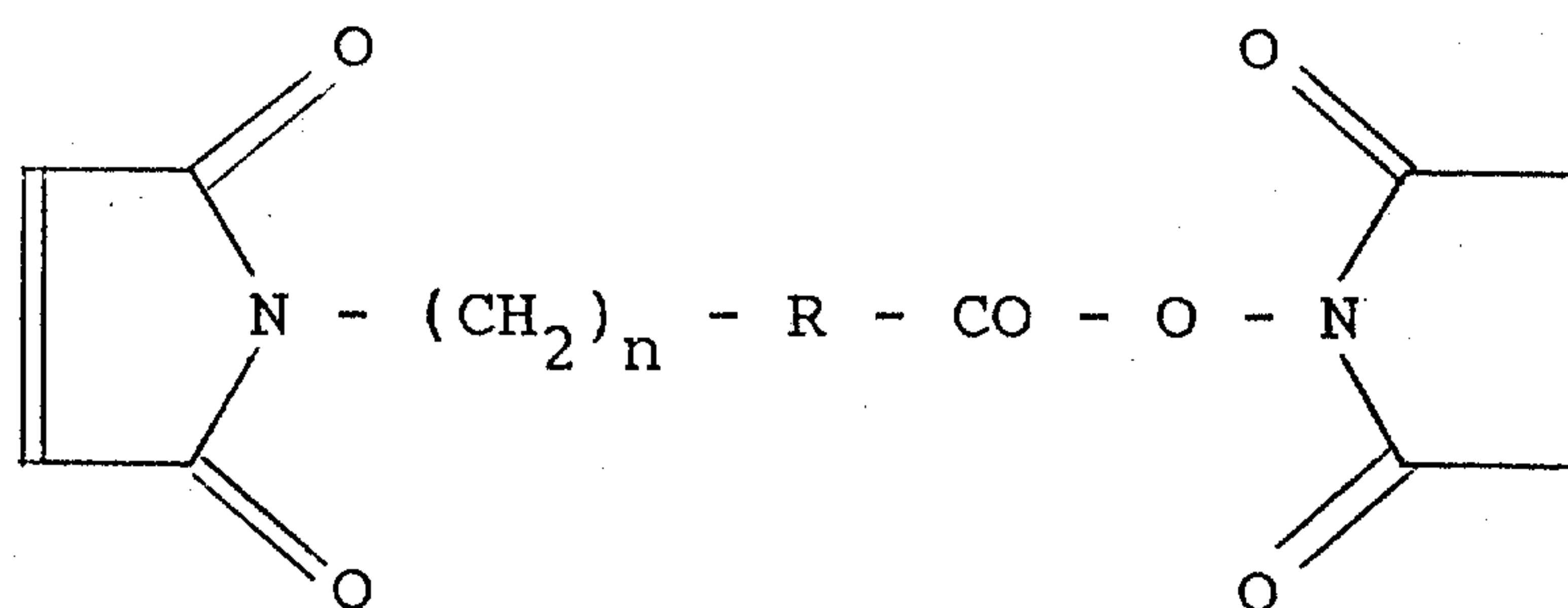


, and R_2 is a hydrogen atom, a halogen atom, an alkyl, alkenyl or aromatic group which may be substituted or a heterocyclic group, especially a hydrogen atom, a halogen atom, lower alkyl group such as methyl group or ethyl group, pyridyl group or an alkoxycarbonylalkyl group such as ethoxycarbonylethyl group are also suitable derivatives.

The peroxidase used also in this invention according to the known technique may be the ones derived from various kinds of origins such as horseradish, pineapple, fig, sweat potato, broad bean, corn and the like, and especially horseradish peroxidase extracted from horseradish is suitable.

Peroxidase is used in a free form (non-labeled form) according to analysis system, but it is necessary to be bonded with an immunochemically active substance such as antigen, hapten, antibody or the like in case of immunochemical analysis using a method of the present invention.

The combination of peroxidase with an immunochemically active substance can be achieved by using the known methods. For instance, glutaraldehyde cross linking method [Immunochemistry, Vol. 6, page 43 (1969); bid. Vol. 8, page 1175 (1971)], periodic acid cross linking method [J. Histochem. Cytochem., Vol. 22, page 1084 (1974)], and especially the method using a bonding agent of the formula:



wherein, n is an integer of 0 to 5, and R is a chemical bond or 6-membered, cyclic hydrocarbon residue [Japanese Patent Kokai 149,700/ 1983] can be preferably applied.

Luminol and its derivative, lophin, lucigenin and the like have been known as chemically luminous substances.

Suitable substance may be luminol and its derivatives as described in "Methods in Encymology", vol. 57, pages 409 - 423 (1987), for example, luminol, isoluminol, N-(4-aminobutyl)-N-ethylisoluminol hemi-succinimide, N-(6-aminohexyl)-N-ethylisoluminol, N-ethylisoluminol or the like. Among these substances, luminol and isoluminol are preferably used.

10 The oxidizing agent to be used also in this invention according to the known technique may be the one which can yield luminous energy by chemical luminous reaction. For example, oxidizing agents such as hydrogen peroxide, perboric acid salt, etc., can be preferably used. And further, a system in which an oxidizing agent is indirectly produced, for example, a system in which hydrogen peroxidase is produced from glucose by using an enzyme such as glucose oxidase can
20 be applied.

A suitable measuring system to be employed in this invention comprises

1. peroxidase or an immunochemically active substance labeled by peroxidase,
2. oxidizing agent (hydrogen peroxide or its

analogue),

3. luminol or its derivative and

4. compound (I) (enhancer)

In general, peroxidase is used in chemical combination with hapten, antigen, antibody or the like. The above reagents 2, 3 and 4 are used in a fixed amount, and a condition where the chemical luminous quantum changes in accordance with a change of amount of peroxidase in very small amount domain thereof is selected. Chemical luminous reaction is delayed and luminous quantum is extremely enhanced by using the enhancer. Through these phenomena, it becomes possible to detect a very small amount of substance in comparison with the prior arts.

The method of this invention using the compound (I) as an enhancer in the chemical luminous reaction is preferably conducted under the following conditions.

The reaction temperature may be 0 to 60°C, preferably 5 to 30°C. The pH value of a buffer to be used may be within the range from around neutral to alkaline, for instance, pH 7 to 10, preferably pH 8 to 9. Various kinds of buffers can be used as a buffer, and borate buffer, phosphate buffer, carbonate buffer, Tris buffer, etc. can be preferably used.

The concentrations of the reagents can vary in accordance with the enhancer to be used, but following concentrations are usually preferable.

- | | |
|---|--------------------|
| (1) peroxidase | 0 - 1000 μ g/l |
| (2) oxidizing agent (hydrogen peroxide) | 1 μ M - 30mM |
| (3) enhancer | 0.1 μ M - 10mM |
| (4) luminous substance (luminol) | 1 μ M - 10mM |

Especially, the concentration of 0 to 1 μ g/liter for the above (1), 20 μ M to 2mM for the above (2), 4 μ M to 1mM for the above (3) and 10 μ M to 2mM for the above (4) are respectively preferable.

The chemical luminous reaction is carried out under the co-existence of the substances in the above (1) - (4), and usually the chemical luminous reaction is conducted by adding a solution containing any one among the above (1) - (4) into a system which contains the remaining three of the above (1) - (4). The luminous quantum produced in the reaction solution can be measured by a measuring instrument which is marketed or one's own made (e.g., a photometer equipped with a high sensitive photoelectron multiplier). That is, a quantitative analysis can be conducted by measuring luminous quantum in term of few seconds to few minutes

at the stage of few seconds to several tens minutes after the addition of the last solution.

Namely, a reliable relationship between the luminous quantum actually measured and the amount of the peroxidase can be recognized, based on which the analysis is possible. Usually, the peroxidase makes co-valent bond with an immunochemically active substance.

Test sample to be analyzed may be bioliquids such as blood serum, blood plasma, urine, medullary liquid, etc., a liquid extracted from organ, or the like.

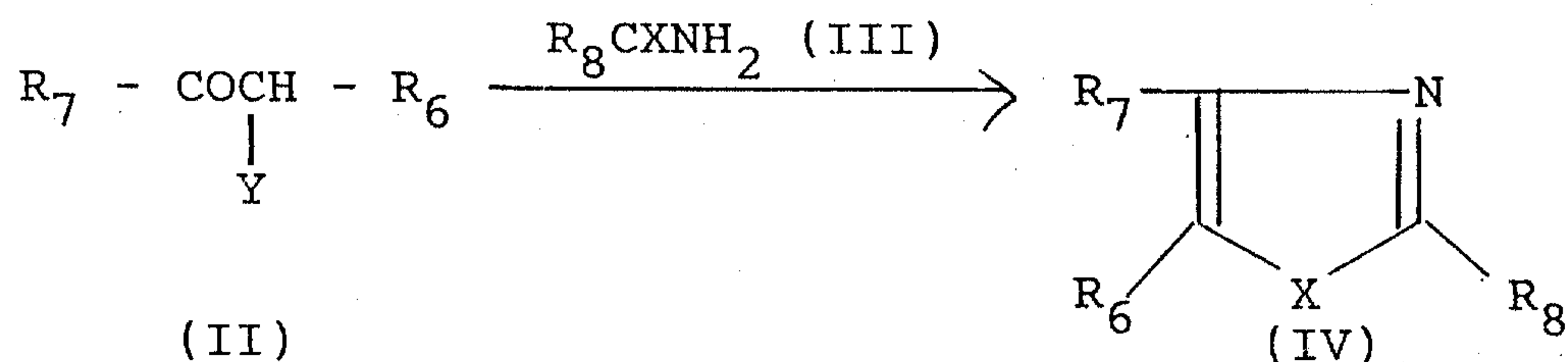
The chemical luminous quantum can be measured by a known method, for example, by a marketed or one's own made luminometer equipped with a photoelectron multiplier. In case such an instrument is used, the luminous quantum produced in a few seconds to several tens seconds after a few seconds to several hundreds seconds from mixing of the above reagents is measured.

The compounds represented by the formula (I) can be prepared by an appropriate known method. The methods for preparing thiazole, oxazole or imidazole derivatives are well known in this field, and can be referred to the standard methods described in, for example, "Shin-Jikken-Kagaku-Koza", Vol. 14, Synthesis and Reaction of Organic Compounds [IV] (1978) published

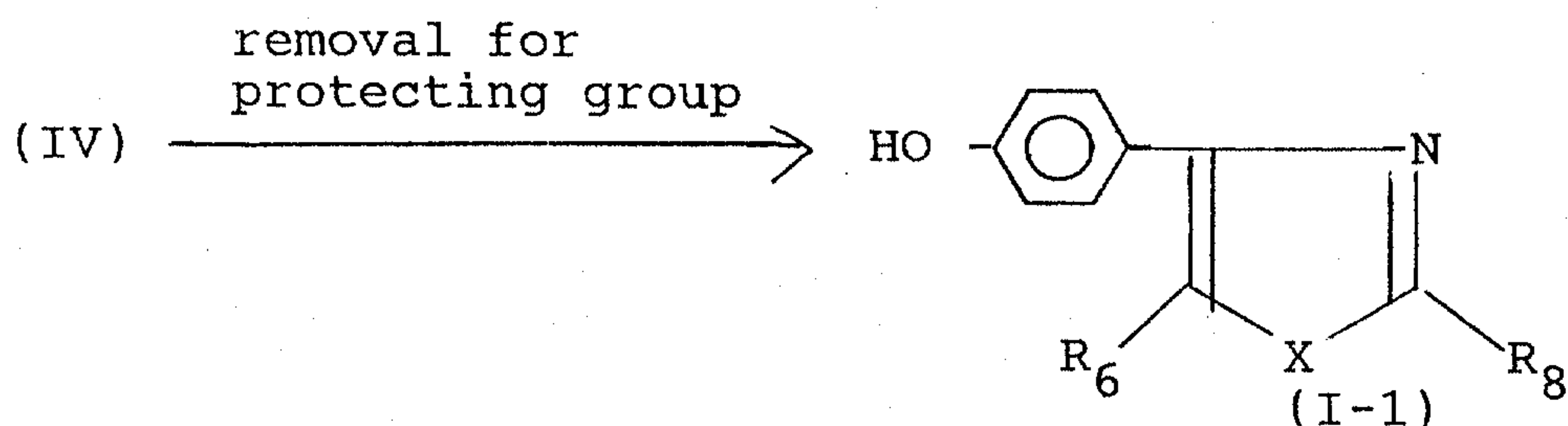
by Maruzen Co., Ltd of Japan. The methods for preparing the compounds of the formula (I) are explained in detail in the following.

Method A

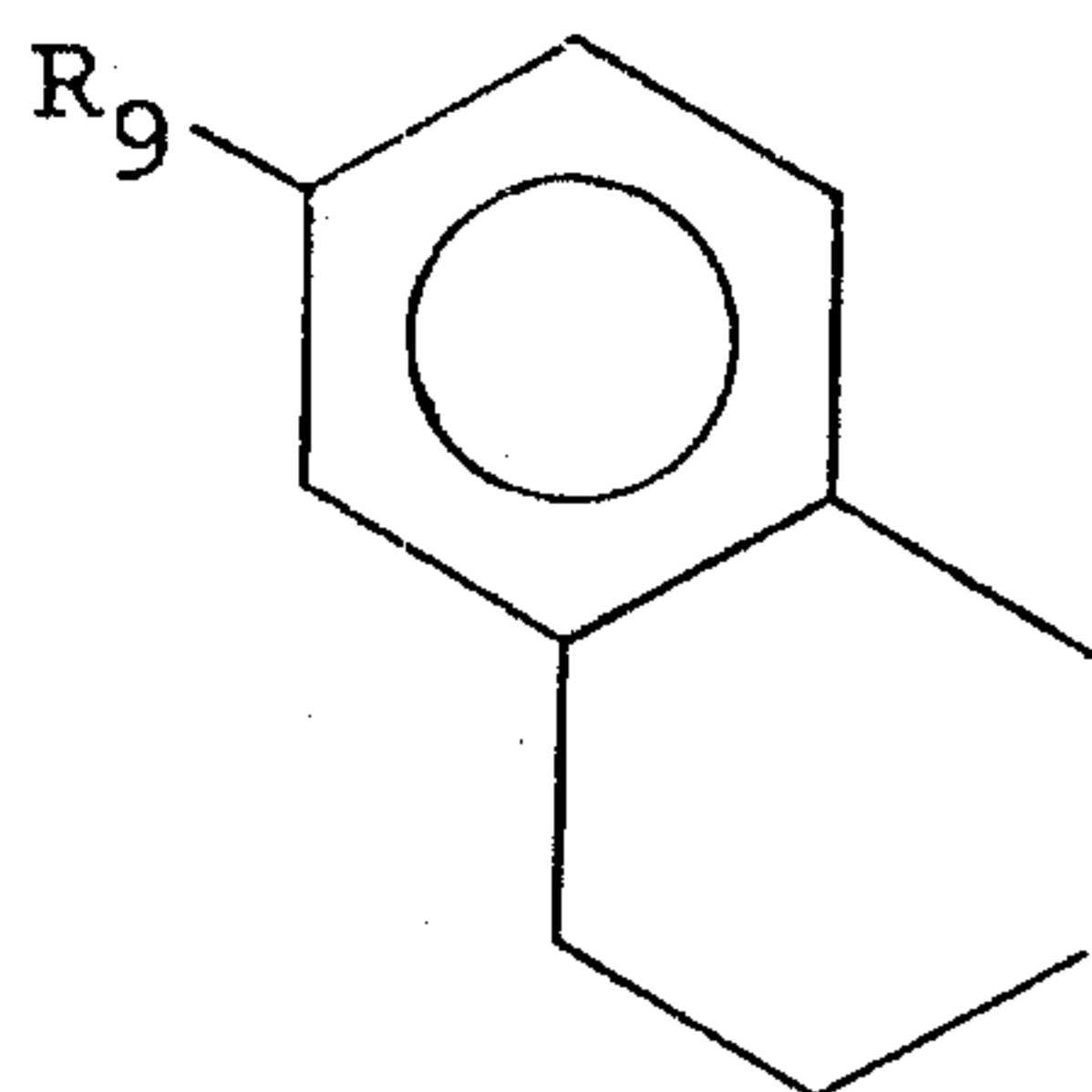
Step 1



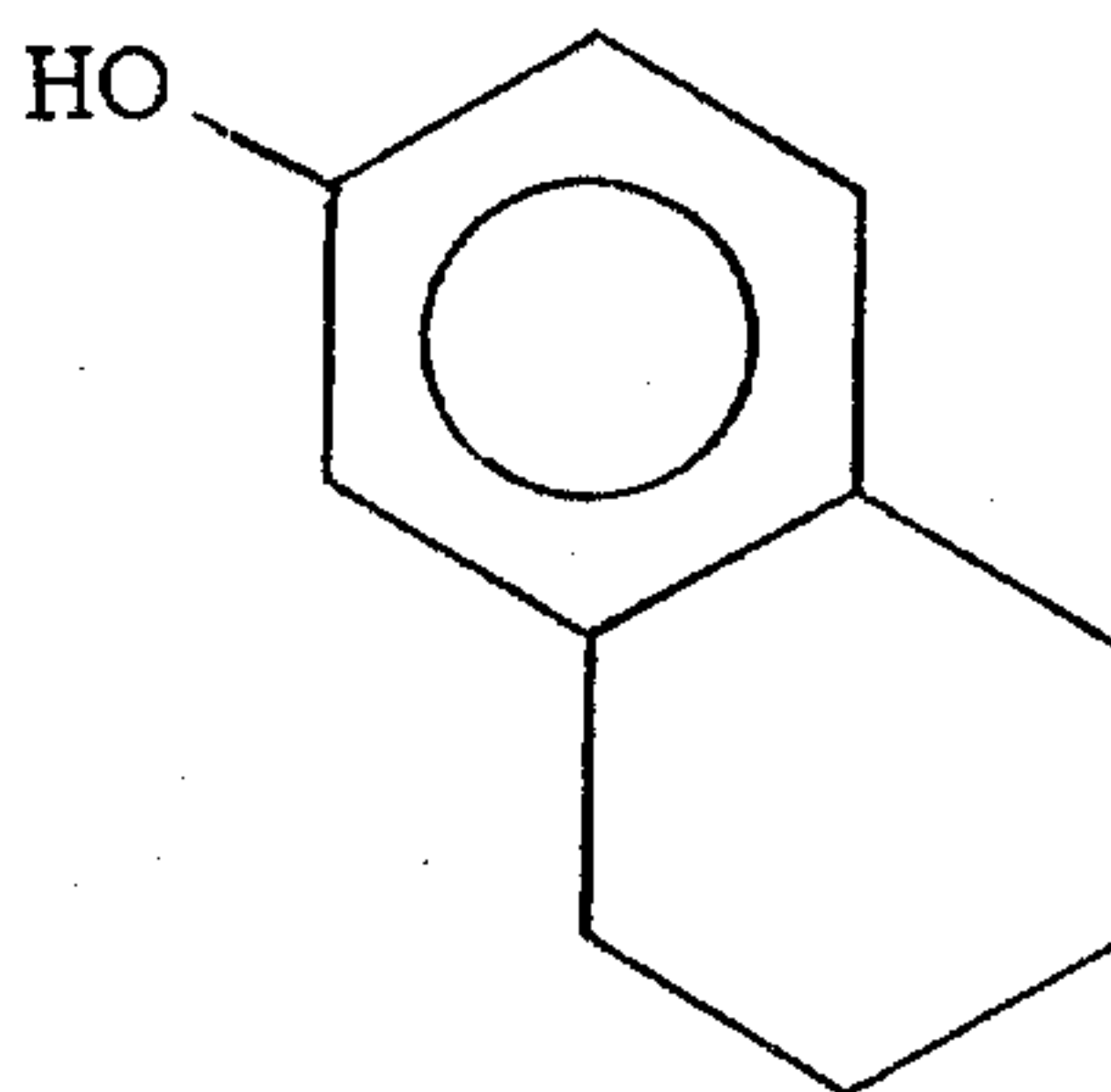
Step 2



In the formula, R_6 and R_8 are each hydrogen atom, a hydrocarbon residue which may be substituted or a heterocyclic group, R_7 is a phenyl group having a hydroxy group at the 4th position, or R_7 and R_6 are combined to form a group of the formula:



(wherein R_9 is a lower alkoxy or aralkyloxy), or R_6 and the 4-hydroxyphenyl group are combined to form a group of the formula:



, X is an oxygen atom or a sulfur atom, and Y is a removable group.

The hydrocarbon residue which may be substituted and the heterocyclic group represented by R_6 and R_8 are the same as exemplified for R_2 and R_3 . The phenyl group having a protected hydroxy group at the 4th position represented by R_7 may be 4-lower alkoxyphenyl such as 4-methoxyphenyl, 4-ethoxyphenyl, 4-isopropoxyphenyl, etc., or 4-aralkyloxyphenyl such as 4-benzyloxyphenyl, 4-(4-chlorobenzyloxy)phenyl, etc. The lower alkoxy for R_9 may be methoxy, ethoxy, propoxy, isopropoxy, butoxy, etc., and the aralkyloxy for R_9 may be benzyloxy, phenethyloxy, 4-chlorobenzyloxy, etc. The removable group for Y may be halogen (e.g., chlorine, bromine, iodine, etc.), methanesulfonyloxy, benzenesulfonyloxy, p-toluenesulfonyloxy, or the like.

(Step 1)

In this method, a compound (II) and a thioamide or amide derivative of the general formula (III) are reacted at first to give a thiazole or oxazole derivative (IV). This reaction (Step 1) is carried out in an appropriate solvent (e.g., toluene, xylene, pyridine, 1,2-dichloroethane, 1,1,2,2-tetrachloroethane, N,N-dimethylformamide, dimethyl sulfoxide, ethanol, etc.) or without solvent, by heating at 50 to 200°C. The amount of the compound (III) to be used is 1 to 10 moles, preferably 1.5 to 4 moles, to one mole of the compound (II). The reaction time is 0.5 to 30 hours, preferably 1 to 5 hours.

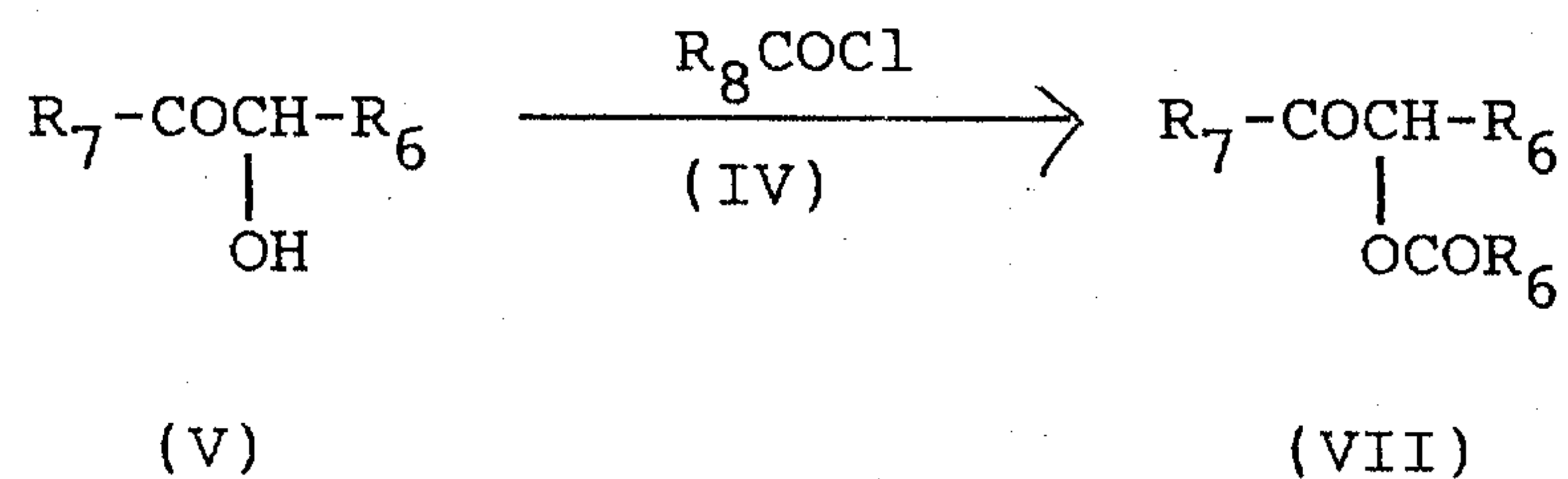
(Step 2)

The compound (IV) obtained in the above Step 1 is then subjected to removal reaction of the protective group to give a compound (I-1). This reaction for removing the protecting group is conducted by using an inorganic acid such as hydrobromic acid, hydrochloric acid or sulfuric acid, Lewis acid such as aluminum chloride, etc., pyridine hydrochloride, or the like. This reaction is preferably carried out by catalytic hydrogenation when R₇ is a benzyloxy derivative. In case the inorganic acid is used, alcohol (e.g.,

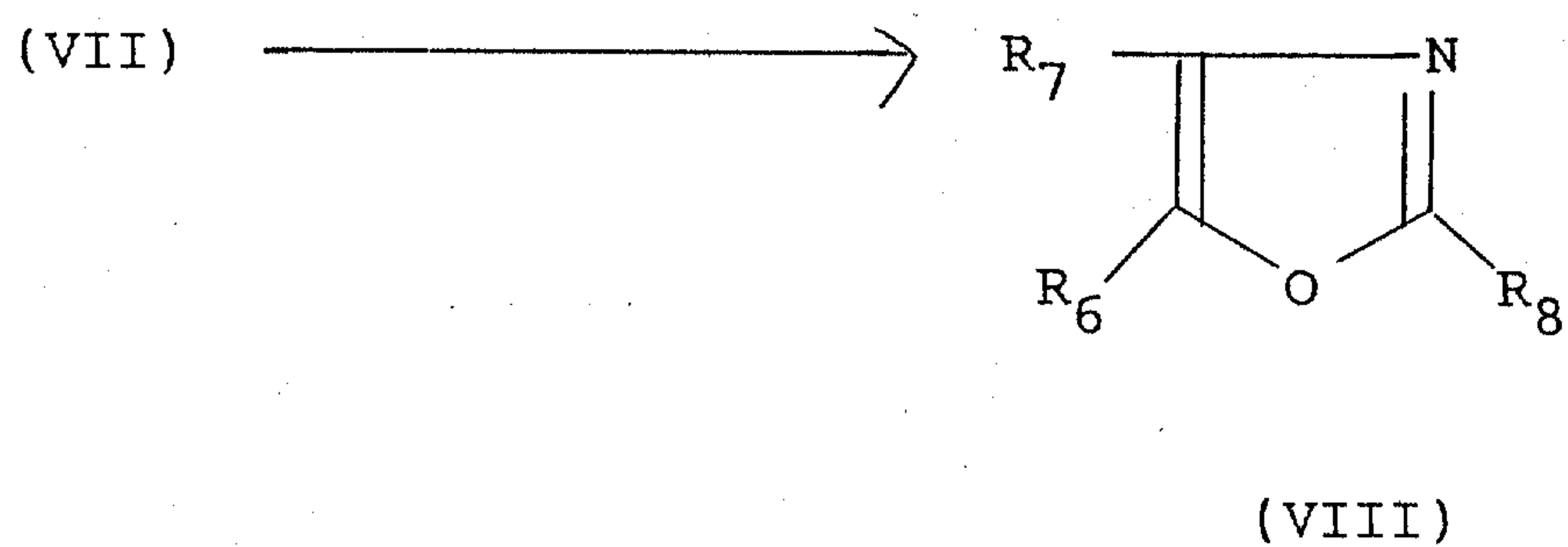
ethanol, propanol, ethyleneglycol, 2-methoxyethanol, etc.), water or a mixture thereof is used as a solvent. The inorganic acid is usually used in large excess (5 to 100 equivalents) for the compound (IV), preferably 10 to 50 equivalents. The reaction is carried out at 50 to 150°C for 0.5 to 20 hours. The reaction for removing the protecting group by using Lewis acid is conducted in an appropriate inert solvent against Lewis acid (e.g., chloroform, dichloromethane, benzene, carbon disulfide, etc.) at -10 to 100°C. The amount of the Lewis acid to be used is 1 to 5 moles, preferably 1 to 3 moles to one mole of the compound (IV). The reaction time is 0.5 to 10 hours. The reaction using pyridine hydrochloride is carried out by heating a mixture of the compound (IV) and pyridine hydrochloride at 50 to 200°C. The amount of the pyridine hydrochloride to be used is 1 to 5 moles, preferably 1 to 3 moles, to one mole of the compound (IV). The reaction time is 0.5 to 10 hours.

Method B

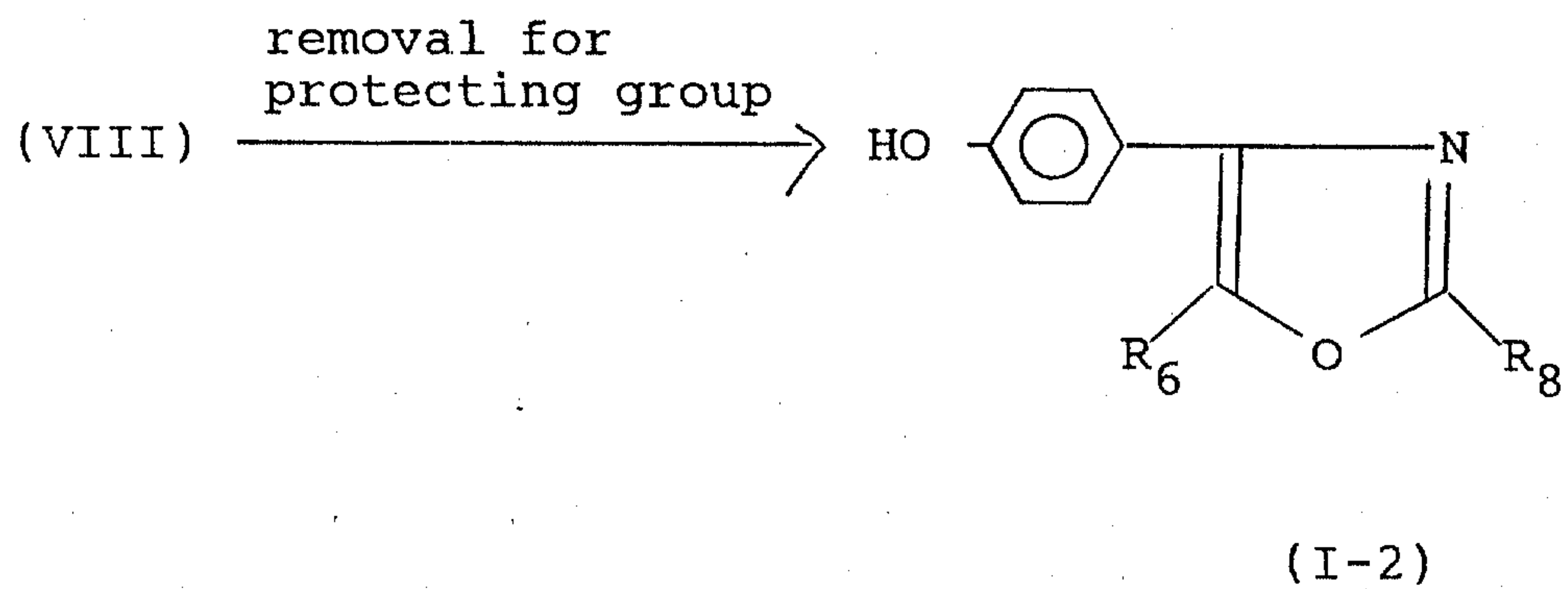
Step 1



Step 2



Step 3



[wherein, R₆, R₇ and R₈ are each as defined in the above]

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(Step 1)

In this method, a compound (VI) is at first acylated to give a compound (VII). The acylation reaction can be carried out by a known method, for example, in a proper solvent (e.g., chloroform, dichloromethane, ethyl acetate, tetrahydrofuran, water or a mixture thereof), in the presence of a base (e.g., triethylamine, N-methylmorpholine, sodium bicarbonate, potassium bicarbonate, sodium carbonate, potassium carbonate, etc.), at - 10 to +50°C for 0.1 to 5 hours. The amount of the compound (VI) to be used is 1 to 1.2 moles to one mole of the compound (V).

(Step 2)

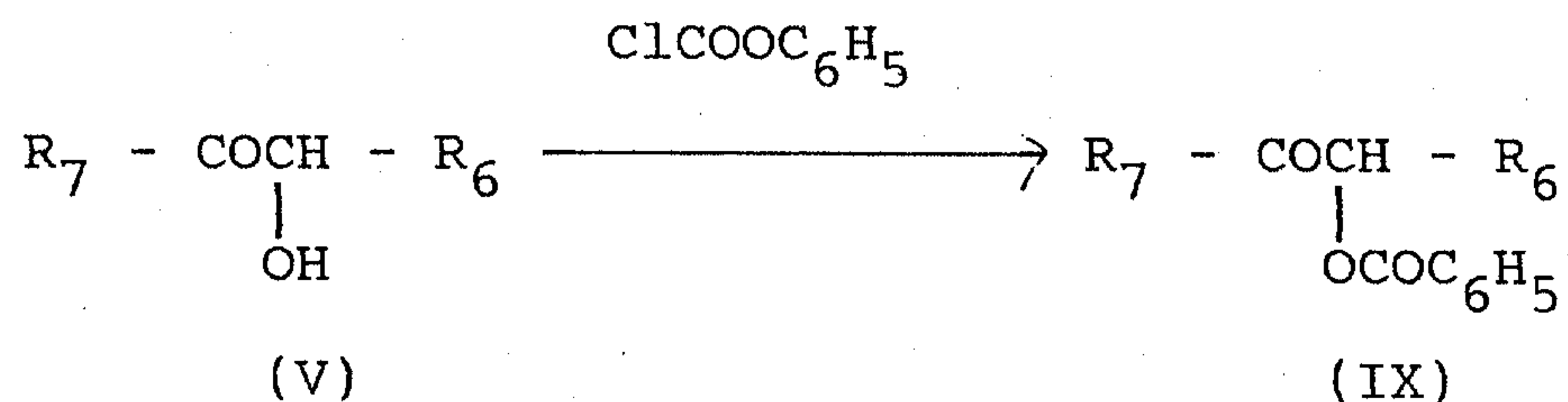
Thus prepared compound (VII) is then reacted with a cyclizing agent containing nitrogen atom(s) such as urea or ammonia to give an oxazole derivative (VIII). In case ammonia is used as a reagent, the reagent is preferably in a form of an ammonium salt, for example, in a form of acetate in acetic acid. This reaction is carried out, for example, by heating a compound (VII) with 1 to 5 equivalents of ammonium acetate in acetic acid at 50 to 110°C for 0.5 to 10 hours.

(Step 3)

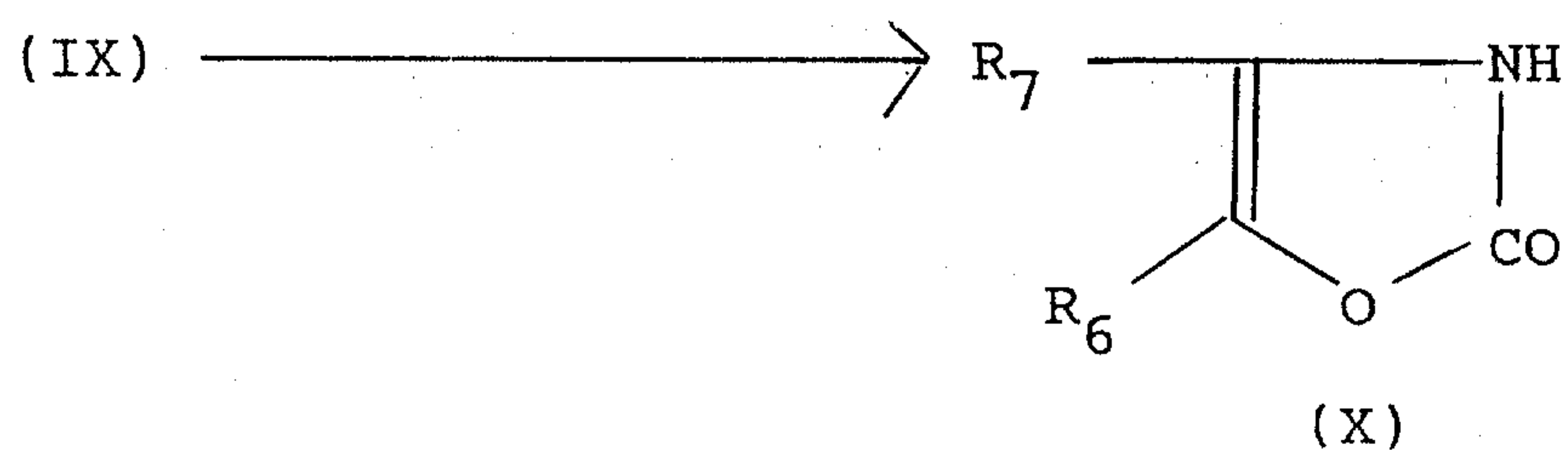
Thus prepared compound (VIII) is then subjected to removing reaction of the protective group to give a compound (I-2). The removing reaction of the protective group can be carried out in the same manner as the Step 2 in the Method A.

Method C

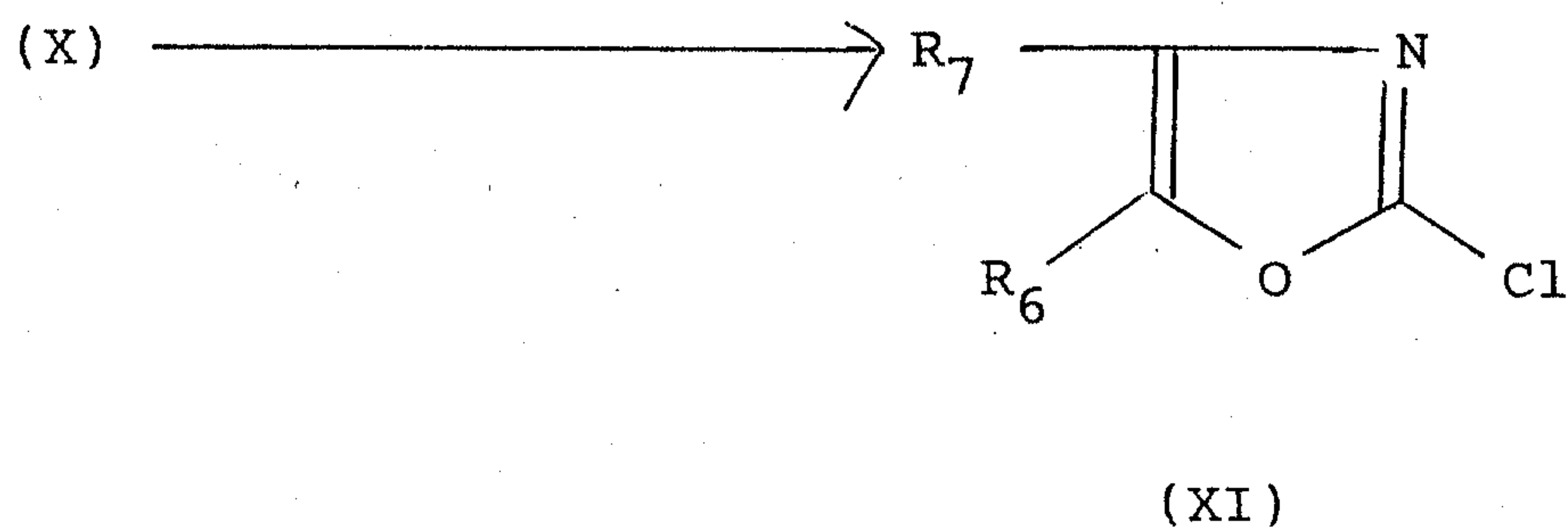
Step 1



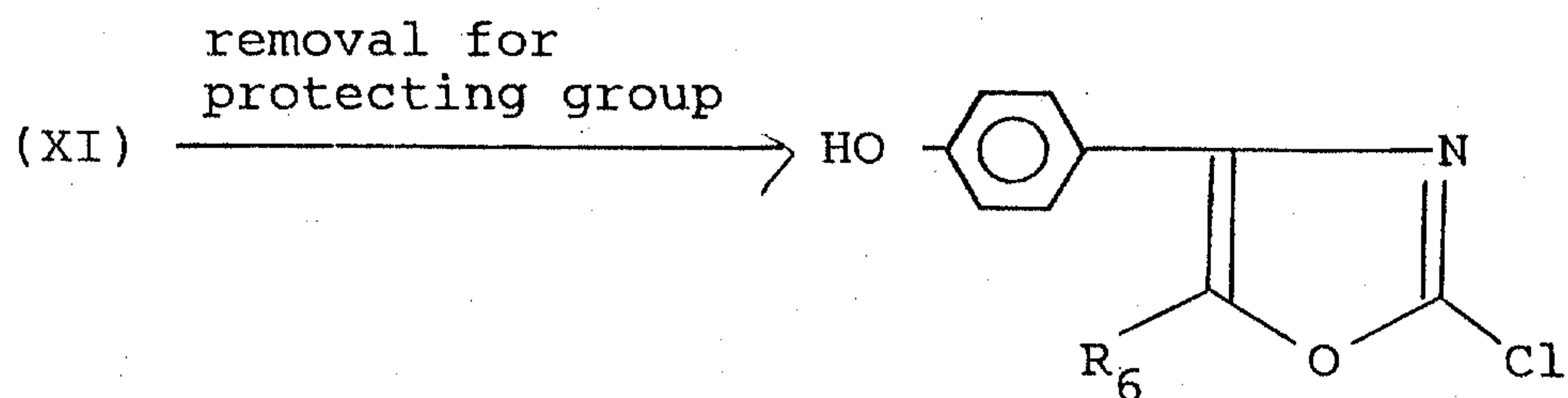
Step 2



Step 3



Step 4



(I-3)

[wherein, R_6 and R_7 have the same meanings as defined above]

(Step 1)

In this process, a compound (V) is acylated by phenyl chlorocarbonate to give a compound (IX) at first. The acylation reaction can be conducted by a know method, for example, in a proper solvent (e.g., chloroform, dichloromethane, ethyl acetate, tetrahydrofuran, water or a mixture thereof), in the presence of a base (e.g., triethylamine, N-methylmorpholine, sodium bicarbonate, potassium bicarbonate, sodium carbonate, potassium carbonate, etc.), at -10 to $+50^\circ\text{C}$, for 0.1 to 5 hours. The amount of phenyl chlorocarbonate to be used is 1 to 1.2 moles to one mole of the compound (V).

(Step 2)

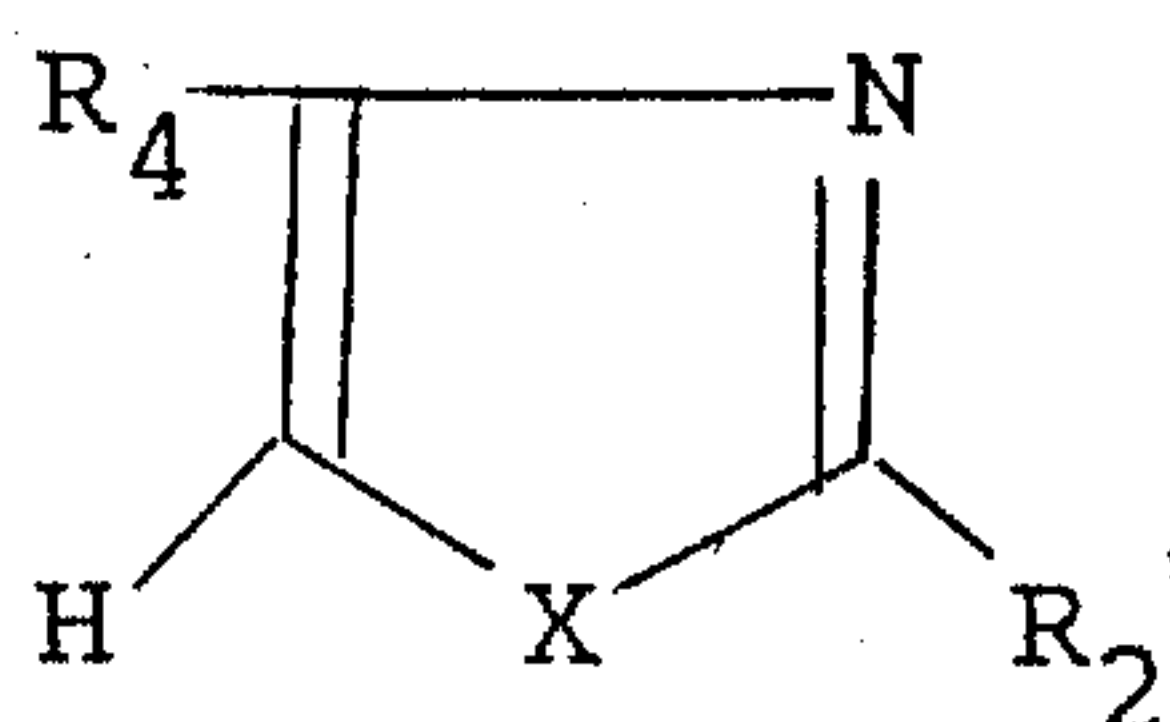
The compound (IX) is then reacted with a cyclizing agent containing nitrogen atom(s) such as urea or ammonia to give an oxazolone derivative (X). In case ammonia is used, the reagent is preferably in a form of ammonium, for example, in a form of acetate in acetic acid. This reaction is carried out, for example, by heating a compound (IX) with 1 to 5 equivalents of ammonium acetate in acetic acid at 50 to 110°C for 0.5 to 10 hours.

(Step 3)

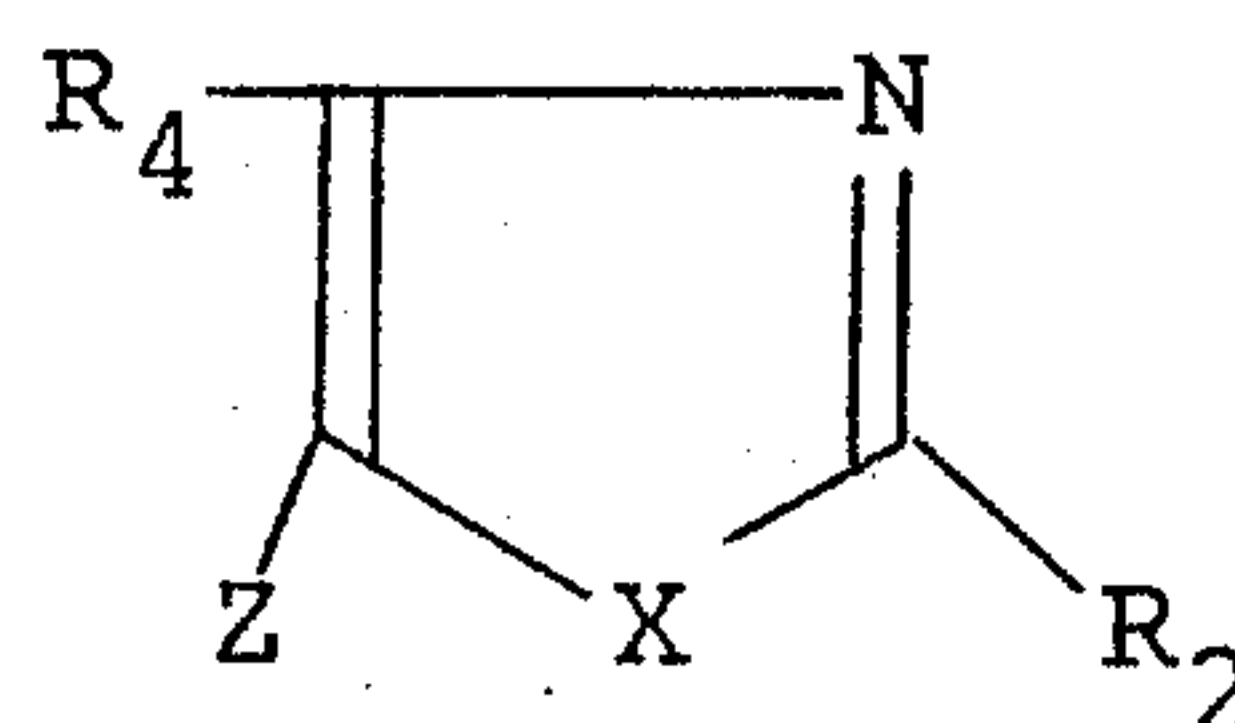
The compound (X) is then derived to a 2-chlorooxazole derivative (XI) by a known reaction using phosphorus oxychloride-N,N-dimethyl formamide (Vilsmeier reagent), phosphorus pentoxide, or the like.

(Step 4)

The compound (XI) is then subjected to a reaction for removing the protective group to give a compound (I-3). The reaction for removing the protective group can be conducted in the same manner as the Step 2 in the Method A.

Method D

(I-4)



(I-5)

[wherein, R_2 , R_4 and X have the same meanings as above, and Z is a halogen atom]

In this method, a compound (I-4) having a hydrogen atom for R_3 in the general formula (I) is halogenated to give a compound (I-5) having a halogen atom (e.g., chlorine, bromine, iodine) for R_3 . The halogenating reaction can be carried out by a known method, for example, in an inert solvent under the halogenating reaction conditions (e.g., chloroform, dichloromethane, carbon tetrachloride, tetrahydrofuran or a mixture thereof), in the presence of chlorine, bromine or iodine, at 0 to 100°C for 0.1 to 5 hours. The amount of the chlorine, bromine or iodine to be used is 1 to 5 moles, preferably 1.2 to 3 moles for 1 mole of the compound (I-4).

Among the compounds represented by the general formula (I), the compounds having an imino group substituted by 4-hydroxyphenyl for R_1 can be

synthesized by a method described in Journal of American Chemical Society Vol. 71, page 383 (1949).

In a luminescence system consisting of chemically luminous substance, oxidizing agent and peroxidase, the luminescence reaction can be delayed and the luminescence reaction can be delayed and the luminescence can be extremely enhanced by using a kind of the compounds (I) or a mixture thereof.

Following Preparations and Examples are given for illustrating the present invention in more detail, but it should be noted that the scope of this invention is not restricted to these Preparations and Examples.

Reference Example 1

Synthesis of 4-(4-hydroxyphenyl)thiazole

(1) 4'-Benzyloxyacetophenone

4'-Hydroxyacetophenone (40g) and potassium carbonate (60.9g) were mixed in dimethyl formamide (250ml). Benzyl bromide (36.7ml) was added dropwise to the mixture, and the mixture was stirred at room temperature for 1 hour and further at 50 to 60°C for 1 hour. The reaction mixture was poured into ice-water, and the precipitating crystals were collected by filtration and recrystallized from methanol to give

4'-benzyloxyacetophenone (55.4g, 83.3%). m.p. 93 - 94°C.

NMR(CDCl₃)δ: 2.5(3H,s), 5.1(2H,s),
6.97(2H,d,J=9Hz), 7.36(5H,m),
7.17(2H,d,J=9Hz)

(2) 4'-Benzyloxy-2-bromoacetophenone

4'-Benzyloxyacetophenone (55.0g) was stirred in chloroform (500ml), and bromine (13.0ml) was added dropwise thereto at room temperature in 30 minutes. The mixture was stirred for 10 minutes, and the reaction mixture was washed with aqueous solution of sodium sulfate, water and saturated saline in turn, dried with anhydrous sodium sulfate and then concentrated. The precipitating crystals were recrystallized from acetone-diisopropyl ether to give 4'-benzyloxy-2-bromoacetophenone (25.7g, 34.6%). m.p. 81 - 82°C

NMR(CDCl₃)δ: 4.4(2H,s), 5.1(2H,s),
7.0(2H,d,J=9Hz), 7.4(5H,m),
7.95(2H,d,J=9Hz)

(3) Thioformamide

Phosphorus pentasulfide (5g) was added slowly to formamide (10g) in 20 minutes under stirring and ice-

cooling. After stirring at room temperature for 1 hour, diethyl ether (75ml) was added thereto, and the mixture was stirred for 20 hours. The ether layer was concentrated to give oily product (5.2g, 38.2%). The product was purified by column chromatography on silica gel [eluting solvent: ethyl acetate-hexane (1:1)] to give thioformamide (1.65g).

(4) 4-(4-Benzyloxyphenyl)thiazole

A mixture of 4'-benzyloxy-2-bromoacetophenone (3.0g) and thioformamide (0.63g) in ethanol (50ml) was refluxed under heating for 30 minutes. The reaction mixture was poured into ice-water, and the precipitating crystals were collected by filtration, extracted with diethyl ether and purified by column chromatography on silica gel [eluting solvent: methylene chloride-hexane (1:9)] to give 4-(4-benzyloxyphenyl)thiazole (720mg, 27.7%), which was further purified by recrystallization from diethyl ether-hexane. m.p. 109 - 110°C.

NMR(CDCl₃) δ : 5.1(2H,s), 7.0(2H,d,J=9Hz),
7.4(1H,d,J=2Hz), 7.4(5H,m),
7.85(2H,d,J=9Hz), 8.8(1H,d,J=2Hz)

IR(KBr) : 1610cm⁻¹

Elementary analysis: C₁₆H₁₃NOS

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Calculated: C, 71.88; H, 4.90; N, 5.24

Found : C, 71.94; H, 4.85; N, 5.07

(5) 4-(4-Hydroxyphenyl)thiazole

To 4-(4-benzyloxyphenyl)thiazole (1.1g) was added 47% aqueous hydrogen bromide (10ml), and the mixture was stirred at 100°C for 30 minutes. Ice-water was added to the reaction mixture and extracted with ethyl acetate. The ethyl acetate layer was washed with saturated saline, dried by anhydrous sodium sulfate and then concentrated. Crystals were collected by filtration by using hexane to give 4-(4-hydroxyphenyl)thiazole (466mg, 63.8%). The crystals were purified by recrystallization from methylene chloride-hexane. m.p. 161.5 - 162°C

NMR(CDCl₃) δ : 5.4(1H,s), 6.85(2H,d,J=9Hz),
7.35(1H,d,J=2Hz), 7.8(2H,d,J=9Hz),
8.8(1H,d,J=2Hz)

IR(KBr) : 1610, 3100cm⁻¹

Elementary analysis: C₉H₇NOS

Calculated: C, 60.99; H, 3.98; N, 7.90

Found : C, 61.00; H, 3.85; N, 7.78

Reference Example 2

Synthesis of 4-(4-hydroxyphenyl)oxazole

(1) 4-(4-Benzyloxyphenyl)oxazole

Formamide (1.0ml) was added to 4'-benzyloxy-2-bromoacetophenone (3.0g) and heated at 130 to 135°C for 1 hour. Water was added to the reaction product, and the mixture was alkalified with aqueous solution of sodium bicarbonate and then extracted with ethyl acetate. The ethyl acetate layer was washed with saturated saline, treated with activated charcoal, dried with anhydrous sodium sulfate and then concentrated. The residue was purified by column chromatography on silica gel [eluting solvent: ethyl acetate-hexane (1:9)] to give 4-(4-benzyloxyphenyl)oxazole (410mg, 16.6%). The product was further purified by recrystallization from diethyl ether-hexane. m.p. 101 - 102°C

NMR(CDCl₃)δ: 5.1(2H,s), 7.0(2H,d,J=9Hz),
7.4(5H,m), 7.7(2H,d,J=9Hz),
7.8(1H,s), 7.9(1H,s)

(2) 4-(4-Hydroxyphenyl)oxazole

4-(4-Benzyloxyphenyl)oxazole (360mg) was dissolved in methanol (10ml). 5% Palladium on carbon (containing 50% water) (300mg) was added to the solution, and the mixture was subjected to catalytic reduction for 80 minutes. The catalyst was filtered off from the

reaction mixture, and the filtrate was concentrated. The residue was purified by column chromatography on silica gel [eluting solvent: ethyl acetate-hexane (1:2)] to give 4-(4-hydroxyphenyl)oxazole (105mg, 45.5%). The product was further purified by recrystallization from diethyl ether-hexane. m.p. 147.5 - 148°C

NMR(CDCl₃) δ : 5.7(1H,s), 6.9(2H,d,J=9Hz),
7.7(2H,d,J=9Hz), 7.9(1H,s), 8.0(1H,s)

IR(KBr) : 3350, 3130⁻¹

Elementary analysis: C₉H₇NO₂

Calculated: C, 67.08; H, 4.38; N, 8.69

Found : C, 67.06; H, 4.42; N, 8.57

Example 1

Reagent

Sodium luminol was used after recrystallization from aqueous sodium hydroxide. Horseradish peroxidase (HRP) Type VI manufactured by Sigma Chemical was used.

Analysis equipment

Chemical luminescence reaction was conducted in a disposable glass culture tube (12mm x 75mm, manufactured by Corning). The luminescence was measured by

using a luminometer (BLR-201, manufactured by Aloka).

1. Screening of enhancer

Luminol solution was prepared by adding sodium luminol (12.5mg) and aqueous hydrogen peroxide (15 μ l, 31%) to borate buffer (0.2 molarity, pH 8.5, 50ml). HRP solution was prepared by diluting HRP with borate buffer (0.2 molarity, pH 8.5). Both solution were prepared about 2 hours before the use and cooled with ice.

A solution (10 μ l) of a sample as an enhancer in dimethylsulfoxide was added to a luminol solution (990 μ l) just before the use and mixed. The mixture (100 μ l) of luminol/hydrogen peroxide/enhancer and HRP solution (1ng/ml, 100 μ l) were mixed in a culture tube, and improvement of ratio of signal/background at 5 minutes later was measured. The results are shown in the following Table 1.

Table 1

Enhancer sample	Additive concentration (mM)	Improvement of ratio of signal/background
P-iodophenol * (manufactured by Wako Pure Chemical Industries, Ltd.)	40 4 0.4	72.0 5.3 1.0
4-(4-hydroxyphenyl)oxazole (Reference Example 2)	40 4	12.8 49.7
4-(4-hydroxyphenyl)thiazole (Reference Example 1)	40 4	163.3 403.1
2-ethyl-4-(4-hydroxyphenyl)-5-(3-pyridyl)thiazole	40 4	381.3 46.1
4-(imidazol-1-yl)phenol	40 4 0.4	110.3 6.0 1.3
4-(4-hydroxyphenyl)-2-methylthiazol (Reference Example 2)	40 4	10.7 119.2
4-(4-hydroxyphenyl)-5-(3-pyridyl)thiazole	40 4	146.4 21.4

* control enhancer

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Table 1 (continued)

Enhancer sample	Additive concentration (mM)	Improvement of ratio of signal/background
4-(4-hydroxyphenyl)- 2-(3-pyridyl) thiazole	40 4	33.5 87.5
4-(4-hydroxyphenyl)- 2-(4-pyridyl) thiazole	40 4	10.8 44.8
2-amino-4,5-dihydro- naphto[1,2-d]thiazole	40 4 0.4	0.3 5.0 8.0

2. Measurement for curve relating to amount and activity

Luminol solution was prepared by adding sodium luminol (5mg) and aqueous hydrogen peroxide (12 μ l, 31%) to borate buffer (0.2 molarity, pH 8.5, 50ml). HRP solution was prepared by diluting HRP with borate buffer (0.2 molarity, pH 8.5). These solutions were prepared about 2 hours before the use and cooled with ice.

A solution (10 μ l) of a sample as an enhancer in dimethyl sulfoxide was added to a luminol solution (990 μ l) just before the use and mixed. [p-Indophenol 40mM, 4-(4-hydroxyphenyl)oxazole 4mM, 4-(4-hydroxyphenyl)thiazole 4mM, 2-ethyl-4-(4-hydroxyphenyl)-5-(3-pyridyl)thiazole 40mM were used, respectively.] The mixture (100 μ l) of luminol/hydrogen peroxide/enhancer and HRP solution diluted in 10 stages (0 - 10ng/ml) (100 μ l) were mixed in a culture tube, and the signal at 5 minutes later was measured. The results are shown in the following Table 2.

Table 2

HRP (pg/ml)	Sample of Enhancer			
	(1)	(2)	(3)	(4)
0	63	28	99	99
0.01	73	25	90	38
0.1	63	28	97	38
1	74	30	122	37
10	80	85	241	49
10 ²	323	192	1,044	506
10 ³	8,738	3,427	19,832	14,641
10 ⁴	108,089	51,896	120,041	139,346

Sample of enhancer

(1): p-iodophenol

(2): 4-(4-hydroxyphenyl)oxazole

(3): 4-(4-hydroxyphenyl)thiazole

(4): 2-ethyl-4-(4-hydroxyphenyl)-5-(3-pyridyl)
thiazole

The relation between the HRP concentration and the signal are shown in Figure 1.

The limits for detecting HRP by using each of these compounds were judged from Figure 1, and the results are shown in the following.

(1) p-iodophenol	50 pg/ml
(2) 4-(4-hydroxyphenyl)oxazole	10 pg/ml
(3) 4-(4-hydroxyphenyl)thiazole	5 pg/ml
(4) 2-ethyl-4-(4-hydroxyphenyl)- 5-(3-pyridyl)thiazole	10 pg/ml

Example 2

Measurement for hCG

1. Purification of anti-hCG- β -CTP

- (1) Saturated aqueous solution of ammonium sulfate (3.3ml) was added to rabbit antiserum (5ml) which was rendered immune by P1 peptide [hCG- β C end peptide (123-145)], and the mixture was stirred at room temperature. After centrifugalizing with 10,000 rpm for 10 minutes, precipitate was dissolved in saline (5ml).
- (2) Saturated aqueous solution of ammonium sulfate (2.7ml) was added to the solution, and the mixture was stirred at room temperature for 30 minutes and

centrifugalized with 10,000rpm for 10 minutes.

Precipitate was dissolved in saline (5ml).

- (3) The solution was dialyzed with 0.02M borate buffer (pH 8.0) overnight and cnetrifugalized with 10,000 rpm for 10 minutes to remove the precipitate.
- (4) The solution was subjected to an affinity column on P1 peptide solidified on sepharose 4B. The dialyzed substance was put on the column, washed with 0.02M borate buffer (pH 8.0) and then with 0.17M glycine buffer (pH 3.0), and eluted with 3M sodium thiocyanate buffer (pH 7.4).
- (5) Eluate fractions were collected, and dialyzed with saline (containing 0.01% sodium azide) overnight to give an anti-hCG- β -CTP solution. Absorption at 280nm was measured and protein concentration was determined to 219.2 μ g/ml and the total amount to 1.64mg.

2. Preparation of anti-hCG- β -CTP solidified on polystyrene beads

- (1) Polystyrene beads (4.4mm diameter) were stirred in 2N aqueous sodium hydroxide for 2 hours, washed with distilled water and then dried. The beads were stirred in 2N hydrochloric acid for 2 hours, washed with distilled water and then dried.

- (2) Anti-hCG- β -CTP was dissolved in 0.1M carbonate buffer (pH 9.5). Polystyrene beads were added to the solution and allowed to stand at 4°C for 3 days (1 μ g of anti-hCG- β -CTP 1 μ g/per one piece of polystyrene bead).
- (3) The beads were washed with saline. 1% BSA solution was added to the beads and the mixture was allowed to stand at 4°C for 1 day.
- (4) The beads were washed with saline and stored at 4°C in 0.1% BSA solution.

3. Measurement

- (1) Each sample (100 μ l) containing various amount of hCG and 0.02M phosphate buffer (containing 10% calf serum) (100 μ l) were mixed. Anti-hCG- β -CTP solidified on polystyrene beads were added to the mixture and allowed to stand at room temperature overnight.
- (2) The beads were washed with distilled water. Diluted solution of anti-hCG/HRP compound (200 μ l) was added to the beads, and the mixture was allowed to stand at room temperature for 2 hours.
- (3) The beads were washed with distilled water and put in a test tube for measuring the luminescence. A solution containing luminol (100 μ M), hydrogen

peroxide (300 μ M) and enhancer, 4-(4-hydroxyphenyl) thiazole (40 μ M) was added to the beads, and the luminous reaction was started. Luminous quantum at 1 minute later was measured. Limits for detecting hCG was 0.005 mIU/ml.

Example 3

Measurement for Endothelin

Procedure

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Determined wells of Microtiter plate with 96 wells (DYNATECH micro FLUOR[®], Dynatech Lab. Ltd., Chantilly, Va. USA) were coated with monoclonal anti-endothelin antibody as described in Suzuki et al., J. Immun. Methods 118, 245 - 250 (1989).

20

One hundred μ l of a solution of endothelin (1-21, Peptide Institute Inc., Osaka, Japan) (0, 0.2, 0.5 and 1 pg/me) in 0.02M phosphate buffer (pH 7.0) containing 10% Block Ace[®] (Snow Brand Milk Products Co., Ltd., Japan), 0.4M NaCl and 1mM EDTA were added to the wells of the plate and incubated for 16 hours at 6°C. The wells were washed with 0.05M phosphate buffer containing 0.8% NaCl. Then, 100 μ l of a solution of polyclonal anti- endothelin antibody-HRP complex (see the above Suzuki et al.) were added to each well of the plate. The plate was incubated for 24 hours at 6°C and washed

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with a phosphate buffer. Then, HRP activity present in the plate was measured by a chemiluminescence method using an enhancer of the present invention. That is, 100 μ l of 0.1M Tris buffer (pH 8.7) containing 2mM luminol, 500 μ M H_2O_2 and 20 μ M 4-(4-hydroxyphenyl)thiazole, 0.25% ethanol and 0.02mM EDTA were added to each well of the plate, and the light was measured on an automated microtiter plate chemiluminescence machine (Model ML 1000, Dynatech Lab. Inc.) adjusted for high sensitivity and 0.25 second measurements.

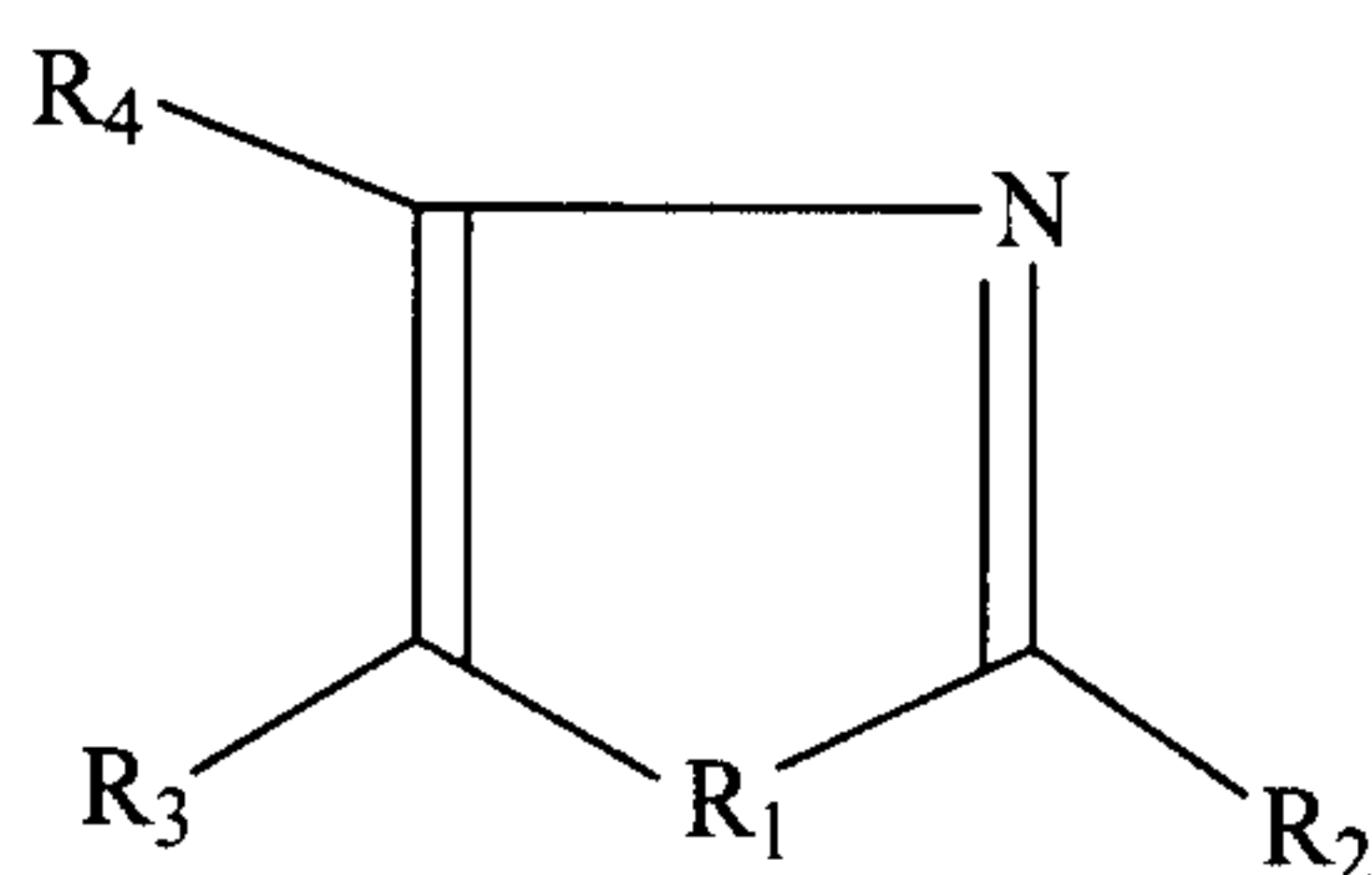
The detection limit was 0.03pg of endothelin, and thus the method of the present invention can be said to show every high sensitivity.

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CLAIMS:

1. . A method for enhancing chemiluminescence, which comprises in a luminescence system containing a chemically luminous substance, an oxidizing agent and a peroxidase, using
5 a compound of the formula:

10



(I)

15

wherein:

R₁ is an oxygen or sulfur atom,

R₂ and R₃ are each independently

a hydrogen atom,

20

a halogen atom,

a hydrocarbon group which is selected from the group consisting of (a) a C₁₋₁₀ alkyl group, (b) a C₂₋₁₀ alkenyl group, (c) a C₆₋₁₄ aryl group and (d) a C₇₋₁₉ aralkyl group and

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which may be substituted with 1 to 3 substituents selected from the group consisting of halogen, hydroxyl, C₁₋₆alkoxy, amino, C₁₋₆alkylamino, C₁₋₁₀alkanoylamino, carbamoyl, C₁₋₆alkylcarbamoyl, mono- or di-alkoxyphosphoryl, 5 C₁₋₆alkoxycarbonyl, C₆₋₁₄aryloxycarbonyl and C₇₋₁₉aralkyloxycarbonyl or

a pyridyl group which may be substituted with a substituent selected from the group consisting of halogen, C₁₋₆alkyl, halogenated C₁₋₆alkyl, hydroxyl, C₁₋₆alkoxy, 10 C₁₋₁₀acyl, amino, C₁₋₆alkylamino, C₁₋₁₀acylamino, carbamoyl, C₁₋₆alkylcarbamoyl, and C₁₋₆alkoxycarbonyl, and

R₄ is a 4-hydroxyphenyl group.

2. The method of claim 1, wherein R₁ is a sulfur atom.
3. The method of claim 2, wherein R₃ is a hydrogen atom.
- 15 4. The method of claim 2 or 3, wherein R₂ is a C₁₋₁₀ alkyl group or a pyridyl group.
5. The method of any one of claims 1 to 4, in which the luminescence system contains luminol as the chemically luminous substance.
- 20 6. The method of any one of claims 1 to 4, in which the luminescence system contains the peroxidase that is in the form of a chemical combination with an imuno-active substance.
7. The method of claim 1 in which the compound (I) is 4-(4-hydroxyphenyl)thiazole.
- 25 8. The method of claim 1 in which the compound (I) is

4-(4-hydroxyphenyl)-2-methylthiazole.

9. The method of claim 1 in which the compound (I) is
4-(4-hydroxyphenyl)-2-(3-pyridyl)thiazole.

10. A method of any one of claims 1 to 9, wherein:
the use of the compound of the formula (I) in a luminescence system comprises adding a solution of one of (1) the peroxidase, (2) the oxidizing agent, (3) the compound of the formula (I) as an enhancer and (4) the chemically luminous substance, into a system which contains the remaining three of the above members (1) through (4) and a sample to be analyzed, at a temperature of 0 to 60°C at a pH of 7 to 10, in a buffer solution so as to cause a chemical luminous reaction; the members (1) through (4) are employed in the following concentrations:

- | | |
|----------------------------|-----------------------|
| (1) the peroxidase | up to 1000 μ g/l, |
| (2) the oxidizing agent | 1 μ M-30mM, |
| (3) the enhancer | 0.1 μ M-10mM, and |
| (4) the luminous substance | 1 μ M-10mM. |

11. A method of claim 10, wherein the sample to be analyzed is a bioliquid selected from the group consisting of blood serum, blood plasma, urine medullary liquid and a liquid extracted from an organ.

12. A method of claim 1, 2, 3, 4, 5, 7, 8 or 9 wherein the peroxidase is bonded to an immunochemically active substance which is selected from the group consisting of an antigen, a hapten and an antibody and which reacts with an ingredient to be detected in a bioliquid sample.

13. A method according to claim 12, wherein:
the use of the compound of the formula (I) in a

luminescence system comprises adding a solution of one of (1) the peroxidase, (2) the oxidizing agent, (3) the compound of the formula (I) as an enhancer and (4) the chemically luminous substance, into a buffer solution system which contains the remaining three of the above members (1) through (4) and the bioliquid sample, so as to cause a chemical luminous reaction.

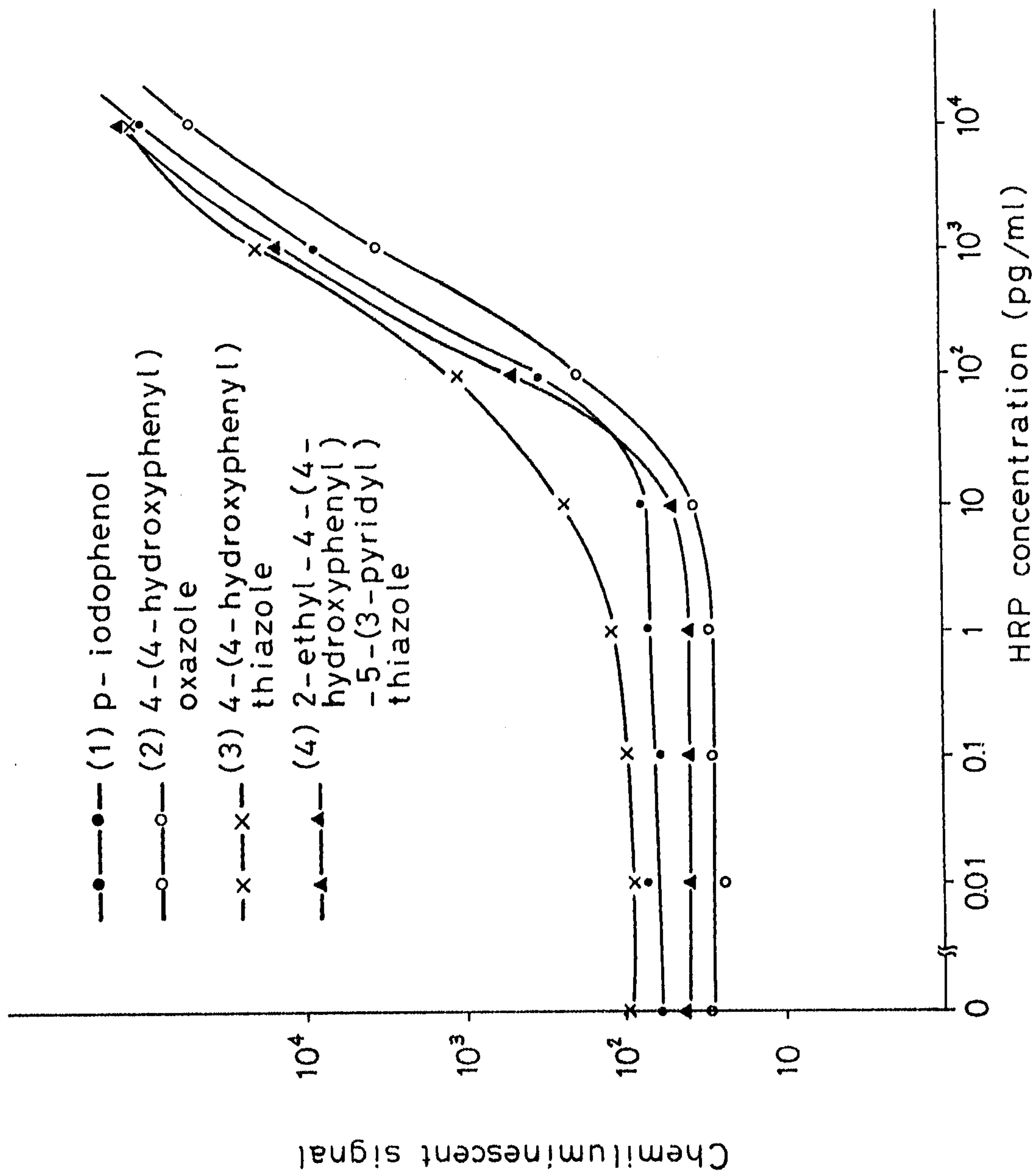
14. A method according to claim 13, wherein the chemical luminous reaction is conducted at a temperature of 0 to 60° C at a pH of 7 to 10 using the members (1) - (4) in the following concentrations:

- | | |
|----------------------------|------------------|
| (1) the peroxidase | up to 1000µg/l, |
| (2) the oxidizing agent | 1µM-30mM, |
| (3) the enhancer | 0.1 µM-10mM, and |
| (4) the luminous substance | 1µM-10mM. |

FETHERSTONHAUGH & CO.
OTTAWA, CANADA

PATENT AGENTS

FIG. 1



Patent Agents
Fetherstonhaugh & Co.

