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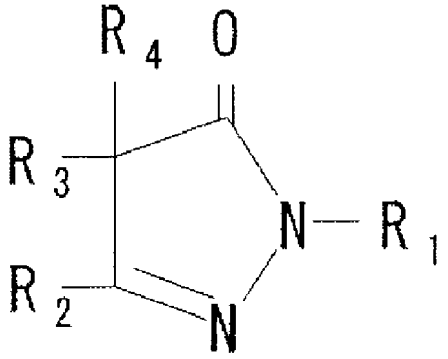

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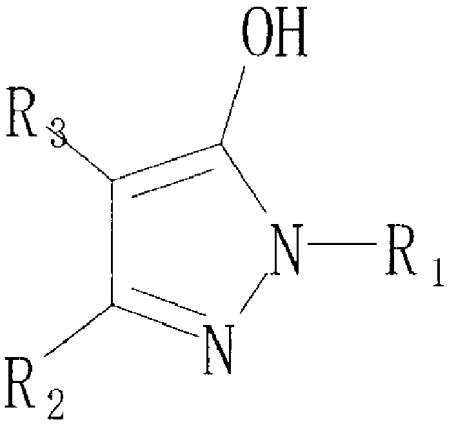

(II)

(57) Abrégé/Abstract:
[PLOBLEMS] To provide a inhibitor of protein modification products formation capable of inhibiting of vitamin B6 deficiency disease as a side effect, especially a renal protective agent. [MEANS FOR SOLVING PROBLEMS] There is provided a use, as an active

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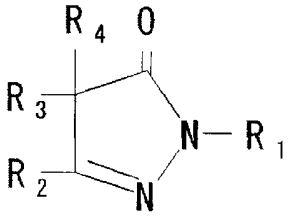
(57) Abrégé(suite)/Abstract(continued):
ingredient, of any of free or salt-form compounds of either of the formulae: (I) (II) [wherein R1 is substituted or unsubstituted aromatic ring; and each of R2, R3 and R4 is a hydrogen atom or monovalent organic group, or alternatively R2 and R3 cooperate to form a condensed ring or R3 and R4 cooperate to represent a divalent organic group, provided that R3 and R4 are not simulataneously hydrogen atoms]. (see formula I) (see formula II)

ABSTRACT OF THE DISCLOSURE

The compounds, at least in selected embodiments, are suitable as inhibitors of the formation of protein modification products capable of inhibiting vitamin B6 deficiency disease as a side effect, especially as a renal protective agent. There is provided a use, as an active ingredient, of any of free or salt-form compounds of either of the formulae: (I) or (II), wherein R1 is substituted or unsubstituted aromatic ring; and each of R2, R3 and R4 is a hydrogen atom or monovalent organic group, or alternatively R2 and R3 cooperate to form a condensed ring or R3 and R4 cooperate to represent a divalent organic group, provided that R3 and R4 are not simultaneously hydrogen atoms.



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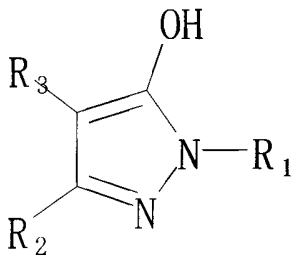

(II)

PROTEIN MODIFIER PRODUCTION INHIBITOR

TECHNICAL FIELD OF THE INVENTION

The present invention relates to an inhibitor of the formation of protein modification products, particularly to a medicament for inhibiting the formation of protein modification products such as advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs), which are formed by the reaction with carbonyl compounds under non-enzymatic conditions.

BACKGROUND OF THE INVENTION

Glycation encompasses the chain reactions starting from the non-enzymatic reaction between the amino moiety on peptides or proteins and the carbonyl moiety on reducing sugars (Maillard reaction; cf. Reference 1) and are divided roughly into the initial stage and the later stage. The initial stage comprises a reversible reaction, depending on the concentration of sugars and the reaction time, wherein the amino moiety and the carbonyl moiety are non-enzymatically reacted to form Schiff bases, followed by Amadori rearrangement to form Amadori compounds.

In the later stage, the Amadori compounds formed in the initial stage are irreversibly subjected to dehydration, condensation, cyclization, oxidation, fragmentation, polymerization, rearrangement, etc. to finally give protein modification products called "AGEs". By auto-oxidation of sugars and the like, highly reactive dicarbonyl compounds such as 3-deoxiglucosone (hereinafter referred to as "3-DG"), glyoxal (hereinafter referred to as "GO") and methylglyoxal (hereinafter referred to as "MGO") are produced, which may be further reacted with proteins to form AGEs modified at the lysine or arginine residues of the proteins in many cases.

Under the oxidation stress conditions, sugars, lipids, amino acids, etc., present abundantly in living bodies, are oxidized to highly reactive carbonyl compounds. The thus produced GO, MGO, arabinose, glycol aldehyde, etc. serve as precursors of AGEs. Dehydroascorbic acid, which is formed by oxidation of ascorbic acid, also serves as a precursor of AGEs. These precursors have a carbonyl group, which is non-enzymatically reacted with the amino moiety on proteins to give Schiff's bases and then form AGEs (cf. Reference 2).

Under the oxidation stress conditions, lipoperoxidation also proceeds to form various carbonyl compounds such as malondialdehyde, hydroxynonenal and acrolein (cf. Reference 3). These carbonyl compounds react with the amino moiety or the like on proteins to form protein modification products called ALEs such as malondialdehyde-modified lysine and hydroxynonenal modifier (cf. Reference 2).

In addition, amino acids such as serine and threonine are oxidized to form carbonyl compounds such as acrolein and GO, followed by conversion into protein modification products (cf. Reference 4). A large number of carbonyl compounds are formed by the oxidative pathway, but some carbonyl compounds, such as 3-DG, are formed through the non-oxidative pathway.

As examples of pathways for production of AGEs, there are (i) the pathway of conversion of Schiff's bases or Amadori compounds into AGEs through 3-DG, (ii) the pathway of oxidative conversion of Schiff's bases into glycolaldehyde alkylimines, followed by conversion of the latter into AGEs via aldoamines, (iii) the pathway conversion of aldoamines into AGEs via glyoxal monoalkylimines, (iv) the pathway of conversion of Amadori

compounds into MGO through 2,3-enediol, followed by conversion of said MGO into AGEs, and (v) others.

It has recently been revealed that carboxymethyl-lysine is one of the AGEs produced from GO, which is formed by lipoxidation of unsaturated fatty acids. It is thus considered that the glycation/oxidation and the lipoxidation reactions occur on a common basis.

As understood from the above, carbonyl compounds produced through the oxidative or non-oxidative pathway from sugars, lipids, amino acids and ascorbic acid, modify proteins non-enzymatically and finally give protein modification products such as AGEs and ALEs. In particular, increased protein modification reactions by carbonyl compounds formed via a plurality of reaction pathways is called protein modification due to excessive carbonyl, i.e. "carbonyl stress".

Known AGEs include pentosidine (cf. Reference 5), crossrine (cf. Reference 6), X1 (fluorolink), pyropyridine (cf. Reference 7), pyrarine (cf. Reference 8), carboxymethyl-lysine (cf. Reference 9), imidazolone compounds (cf. Reference 10), carboxyethyl-lysine (cf. Reference 11), MGO dimer (cf. Reference 12), GO dimer (cf. Reference 13),

imidazolysine (cf. Reference 14), argupyrimidine (cf. Reference 15), etc.

AGEs receptors as heretofore cloned include RAGE (cf. Reference 16), macrophage scavenger receptor class A (cf. Reference 17), galectin 3 (cf. Reference 18), OST-48 and 80K-H (cf. Reference 17), etc.

It is reported that in the blood vessel tissue, RAGE (a cellular membrane penetration type protein belonging to the immunoglobulin superfamily) is bonded to AGEs, thereby active oxygen is generated in the cell to activate the p21ras/MAPK pathway (cf. Reference 19), so that the activation of the transcription factor NF-κB is induced to lead the expression of angiopathy associated factors such as VCAM-1 (cf. Reference 20). It is also reported that AGEs control the proliferation of endothelial cells in finer vessels via RAGE, control the proliferation of pericytes, playing an important role in homeostasis, and produce a toxic effect (cf. Reference 21).

In addition, it is reported that AGEs act directly onto endothelial cells in finer vessels via RAGE to promote neoangiogenesis and inhibit the production of PGI2 causing thrombus tendency (cf. Reference 22). For further interests,

enhancement of the substrate production in mesanginal cells, enhancement of the monocyte migration ability, release of inflammatory cytokines from macrophages, acceleration of the collagenase production in synovial cells, activation of osteoclasts, proliferation of vascular smooth muscle cells, acceleration of platelet aggregation, NO activity and its suppression of the smooth muscle relaxation are reported as the physiological activities of AGEs and ALEs (cf. Reference 23).

Diseases associated with AGEs include (i) nephropathy as a complication of diabetes (cf. Reference 24), nervous disorder (cf. Reference 25), retinopathy (cf. Reference 21) and cataract, (ii) arteriosclerosis (cf. Reference 26), (iii) dialysis amyloidosis as a complication of dialysis (cf. Reference 27) and peritoneal sclerosis in peritodialysis patients, (iv) Alzheimer's disease as a central neurological disease (cf. Reference 28), Pick's disease and Parkinson disease, (v) rheumatoid arthritis (cf. Reference 29), (vi) sunlight elastic fibrosis, (vii) aging, (viii) renal failure (cf. Reference 30), etc. In addition, it is reported that in case of diabetes, AGEs prevent the vasodilation derived from blood vessel endothelial cells (cf. Reference 31), and promote renal sclerosis (cf. Reference 32).

From the above, it is understood that protein modification products such as AGEs provide an adverse effect on living bodies directly or via receptors.

On the other hand, it is known that the blood concentration of AGEs is increased with the reduction of the renal function. The reduction of the renal function results in the accumulation of carbonyl compounds, considered to have a molecular weight of no more than 5kDa. In case of pentosidine or pyrarine, those can be present in a free form, but a large portion of them are present in a binding form to serum albumin or the like (cf. Reference 33). In addition, it is reported that the blood level of pentosidine is strongly affected by the filtration function of glomeruli (cf. Reference 34).

Indeed, a large portion of AGEs is eliminated from kidney, and their blood concentration is kept lower while in good health. However, when the renal function is reduced, they act as uremic toxins to produce chronic bioactivities.

Dialysis therapy can remove AGEs in a free form, but hardly remove those in a binding form to proteins or in an intramolecular bridging form (cf. Reference 35). Therefore, the accumulation of modified forms in living bodies is

increased with the progression of renal failure. Further, in addition to the fundamental process where sugars are reacted in living bodies, AGEs in a free form, which are supplied by diets, as well as highly active intermediates such as 3-DG, GO and MGO formed from Amadori compounds and the like previously produced in living bodies, react with proteins in succession to enhance the production of AGEs. Furthermore, the contact of blood to a dialysis membrane results, for instance, in activation of complements and leucocytes to enhance the generation of free radicals. Thus, dialysis therapy itself enhances oxidation and represents one of the causes for production of AGEs.

Accordingly, it is important in dialysis therapy to remove free form substances at an early stage of dialysis and suppress the generation of AGEs in a binding form as much as possible. Since it is difficult to remove AGEs in a binding form by dialysis therapy as stated above, development of a medicament which suppresses formation of protein modification products is highly desired for dialysis therapy.

Further, it is believed that not only reduction of renal function but also reduction of anti-oxidation protective

mechanism associated with renal failure is concerned with accumulation of protein modification products. In patients with renal failure, unbalance of such anti-oxidation abilities is suggested (cf. Reference 40) as well as the increase of oxidized glutathione against reducing glutathione in blood (cf. Reference 36), the reduction of activity of glutathione dependent enzymes, the decrease of preservation term renal failure plasma glutathione peroxidase (cf. Reference 37), the decrease of blood glutathione (cf. Reference 38) and the increase of activity of plasma superoxide dismutase against the decrease of selenium concentration in plasma (cf. Reference 39).

Furthermore, it is reported that in patients with chronic renal failure, remarkable amounts of highly reactive carbonyl compounds and AGEs are generally accumulated in blood and tissues regardless of hyperglycemia (cf. Reference 41). In renal failure, carbonyl compounds are placed under a state of high load (carbonyl stress) by non-enzymatic chemical reaction so that protein modification products are increased. This is considered to have been caused by modification of proteins with carbonyl compounds produced from sugars and lipids (cf. Reference 42).

Accordingly, suppression of the production of protein modification products caused by various factors may provide alleviation of tissue injury, and prevent or treat the conditions associated with protein modification products such as AGEs.

Dialysis for patients with chronic renal failure includes hemodialysis and peritoneal dialysis. In case of peritoneal dialysis, debris in blood is excreted into peritoneal dialysate through a peritoneal membrane. Peritoneal dialysate of high osmotic pressure, which contains glucose, icodextrin, amino acid or the like, is effective in collecting highly reactive carbonyl compounds accumulated in blood of patients with renal failure such as carbonyl compounds derived from carbohydrates (e.g. arabinose, GO, MGO, 3-DG), carbonyl compounds derived from ascorbic acid (e.g. dehydroascorbic acid) and carbonyl compounds derived from lipids (e.g. hydroxynonenal, malondialdehyde, acrolein), through a peritoneal membrane therein.

Further, it is known that highly reactive carbonyl compounds (e.g. 3-DG, 5-hydroxymethylfurfural, formaldehyde, acetaldehyde, GO, MGO, levulinic acid, furfural, arabinose) are formed in a peritoneal dialysate

during the sterilization or storage of the peritoneal dialysate (cf. Reference 43).

Therefore, the concentration of carbonyl compounds in the peritoneal dialysate increases, and formation of protein modification enhances. As a result, the function of the peritoneal membrane is reduced, and thereby resulting in reduction of the water removing ability and progression to peritoneal sclerosis (cf. Reference 44).

In fact, it is demonstrated by the immunohistological study of endothelium and mesothelium that in patients with peritoneal dialysis, introducing glucose causes a carbonyl stress condition in the peritoneal cavity (cf. Reference 45).

In this way, it is presumed that in patients with dialysis, formation of protein modification products by carbonyl compounds causes the morphological alteration of peritoneum and the reduction of the function (i.e. water removal function) resulting therefrom.

Taking into consideration the above facts and various morbid conditions such as renal failure in combination, it is believed that the accumulation of carbonyl compounds is one of the causes for enhancement of the AGEs production

(cf. Reference 46). Thus, suppression of the AGEs production is considered as an effective measure for treatment of the conditions associated with AGEs.

A typical example of AGEs production inhibitors is aminoguanidine, which is considered to inhibit AGEs production by reaction with dicarbonyl compounds such as 3-DG generated from glucose, Schiff's bases or Amadori compounds to form thiazolines. Analysis using diabetes animal models confirmed that said compound is effective in delaying the progression of diabetic nephropathy (cf. Reference 47), retinopathy (cf. Reference 48) and cataract (cf. Reference 49).

Other examples are pyridoxamine derivatives (e.g. pyridorine). In case of OPB-9195 (i.e. (±)2-isopropylidene-hydrazon-4-oxo-thiazolydin-5-yl-acetanilide), the nitrogen atom in the hydrazine moiety is reacted with a carbonyl group to form a stable structure. Thus, it captures a reactive carbonyl group in a free form or a binding form to protein (cf. Reference 50) and therefore can prevent the production of not only AGEs but also ALEs in vitro. Since biguanide compounds such as metformin or buformin can also capture carbonyl compounds (cf. Reference 51), they may be used as AGEs forming inhibitors. Further, the use

of AGEs inhibitors capable of cleaving the bridge as a characteristic of AGEs and the enzymes capable of degrading Amadori compounds (i.e. amadoriase) are proposed.

Study is also made on the possibility of prevention of the AGEs and/or ALEs formation by removal of carbonyl compounds. For removal of carbonyl compounds, there are several enzymes and enzymatic pathways available, of which examples are aldol reducing enzymes, and aldehyde dehydrogenase and glyoxalase pathway (cf. Reference 52). Redox co-enzymes such as reducing glutathione (GSH) and NAD(P)H are important factors for activation of those pathways.

Lowering of these removing pathways simultaneously leads to increasing of numerous carbonyl compounds. Carbonyl compounds such as MGO and GO react with the thiol group of GSH and, as a result, are metabolized with an enzyme, i.e. glyoxalase. NAD(P)H activates the glutathione reducing enzyme and enhances the GSH level. Namely, it is believed that the removal system of carbonyl compounds is inhibited by lowering of GSH or NAD(P)H due to unbalance of the intracellular redox mechanism, which leads to accumulation of AGEs and ALEs. In case of

diabetes, it is suggested that the polyol pathway is activated by hyperglycemia, NAD(P)H and GSH are reduced and the removal system of carbonyl compounds is lowered.

If reduction in the concentration of thiols such as GSH and NAD(P)H lowers the removal of carbonyl compounds, and thereby causing the production of AGEs or ALEs as stated above, there is a possibility that carbonyl compounds would be decreased by increasing the thiol level. For this purpose, the supplementation of thiol groups with GSH, cysteine, acetylcysteine, etc., the lowering of the GSH demand with vitamin E, ubiquinol, etc. and the inhibition of the polyol system with aldose reducing enzyme inhibitors are proposed. Trapping of carbonyl compounds by using aminoguanidine, pyridoxamine, hydrazine, biguanide compounds or SH-containing compounds is also proposed (cf. Patent Reference 1).

As stated above in detail, the inhibition of the production of AGEs and ALEs is considered for prevention and treamtent of diseases associated with them.

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SUMMARY OF THE INVENTION

In one particular embodiment there is provided a compound selected from a group consisting of: 4-(3-hydroxy-5-hydroxymethyl-2-methylpyridin-4-yl-methylene)-1-phenyl-2-pyrazolin-5-one; and 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro[3,4-c]pyridin-7-ol, in a free or salt form thereof.

Problems

Based on the above findings, further studies were conducted to provide a medicament for preventing and treating a disease associated with a protein modification products(s) (i.e., AGEs and/or ALEs) produced by reacting with a carbonyl compound under non-enzymatic conditions. As a result, the present inventors found that 3-methyl-1-phenyl-2-pyrazolin-5-one and their pharmaceutically acceptable salts effectively inhibit the formation of protein modification products such as AGEs, ALEs, etc. On the basis of these findings, an inhibitor of the formation of protein modification products comprising said compounds as the active ingredient has been developed (Japanese Patent Application No. 2003-076955).

The present inventors conducted further studies and found that analogs which are converted phenyl moiety at 1-position and methyl moiety at 3-position of said 3-methyl-1-phenyl-2-pyrazolin-5-one to another substituent also represent similar activity and that said 3-methyl-1-phenyl-2-pyrazolin-5-one or analogs thereof cause vitamin B6 deficiency when they are administered in organisms. A further study was conducted to solve this problem. As a result, the present inventors found that such vitamin B6 deficiency is caused by the capture of vitamin B6 molecules in blood by 2-pyrazolin-5-one ring, which is the basic skeleton of 3-methyl-1-phenyl-2-pyrazolin-5-one or analogs thereof. Also it was found that such capturing is caused by the binding of methylene moiety at the 4-position of said 2-pyrazolin-5-one ring to vitamin B6 molecules.

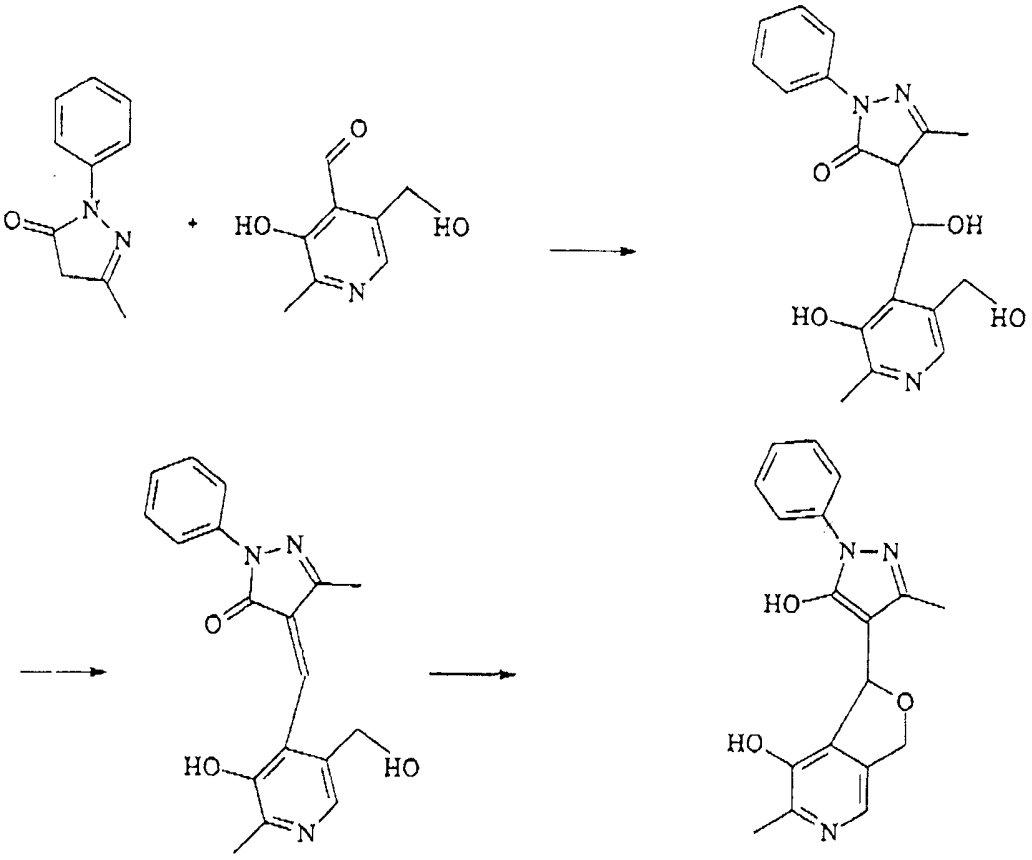
Means for solving problems

Based on these facts, the present invention was completed and its purpose is inhibiting the vitamin B6 deficiency, which is an unavoidable side effect relating to 3-methyl-1-phenyl-2-pyrazolin-5-one or analogs thereof, which are useful as inhibitor of the formation of protein modification products. This purpose was completed by the present invention, i.e. introducing a substituent which

inhibit the binding of vitamin B6 molecules to 3-methyl-1-phenyl-2-pyrazolin-5-one or analogs thereof on said methylene moiety at the 4-position.

Provided that, the substituents introduced to methylene at the 4-position are not always present, stably depending on the variation. For example, when to introduce a pyridoxal residue to methylene at the 4-position, 3-methyl-1-phenyl-2-pyrazolin-5-one is reacted with pyridoxal, 4-(3-hydroxy-5-hydroxymethyl-2-methylpyridin-4-yl-methylene)-1-phenyl-2-pyrazolin-5-one is not obtained, but 1-(5-hydroxy-3-methyl-1-phenyl-1H-4-yl)-6-methyl-1,3-dihydrofuro[3,4-c]pyridin-7-ol.

An explanation considered is that once the former is formed, it is followed by intramolecular rearrangement to form the latter, as depicted in the following scheme:



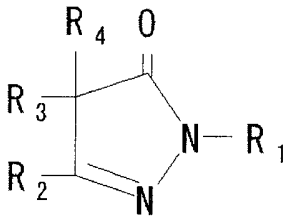
According to the previous study, the compounds introduced to the methylene moiety at the 4-position to prevent the binding of vitamin B6 molecules, generally represent the formation of protein modification products inhibiting effect, regardless of their intramolecular rearrangement as described above.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

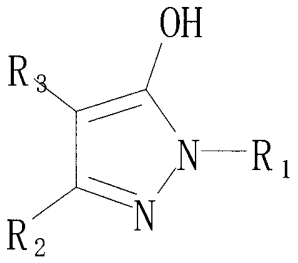
Namely, the present invention provides an inhibitor of the formation of protein modification products comprising as an active ingredient a compound having a formation of protein modification products inhibiting effect with suppression of the vitamin B6 deficiency adverse effect. The scope of this invention specifically covers the following technical embodiments:

(i) an inhibitor of the formation of protein modification products comprising as an active ingredient a compound to which is introduced a substituent that inhibits the binding of vitamin B6 molecules, comprising of that derived from vitamin B6 molecules itself, to 1-substituted-, unsubstituted-3-substituted- or unsubstituted-2-pyrazolin-5-one at the 4-position in free form, salt forms or intramolecular rearranged bodies thereof;

(ii) an inhibitor of the formation of protein modification products according to (i), wherein the compound as the active ingredient is selected from compounds of formula (I):



or formula (II)



wherein R1 is substituted or unsubstituted aromatic ring; and each of R2, R3 and R4 is a hydrogen atom or monovalent organic group, or alternatively R2 and R3 cooperate to form a condensed ring or R3 and R4 cooperate to represent a divalent organic group, provided that R3 and R4 are not simulataneously hydrogen atoms

in free or salt forms;

(iii) an inhibitor of the formation of protein modification products according to (ii), wherein R1 is an aromatic ring moiety of up to 20-membered carbocyclic or heterocyclic aromatic ring group optionally comprising up to 4 hetero atoms and optionally comprising up to 3 substituents;

(iv) an inhibitor of the formation of protein modification products according to (ii) or (iii), wherein each of R2, R3 or

R4 monovalent organic group is independently straight chain or cyclic aliphatic, alicyclic or aromatic hydrocarbon group having up to 30 carbon atoms optionally comprising up to 3 substituents, or halogen, nitro, amino, hydroxy, thiol, carboxy, carboxy (lower) alkyl, lower alkoxycarbonyl, formyl, lower alkanoyl, lower alkylamino, di(lower) alkylamino, lower alkanoylamino, aryl (lower) alkanoyl, aryloxy-amino, sulfonic acid or 3- to 7-membered heterocyclic group optionally comprising substituents;

(v) a protein modification products according to (ii) or (iii), wherein R2 and R3 cooperate to form a condensed ring which is 5- or 6-membered saturated carbocyclic ring optionally comprising substituents;

(vi) an inhibitor of the formation of protein modification products according to (ii) or (iii), wherein R3 and R4 cooperate to form bivalent organic group which is selected from phenylmethylene, phenyl-alkenylmethylene, quinolinyl-methylene, furanyl-methylene, diazolyl-methylene, aminomethylene, di (lower) alkylamino-methylene, pyridyl-methylene and thio-phenylmethylene, optionally comprising substituents;

(vii) an inhibitor of the formation of protein modification products according to any one of (iii) to (vii), wherein the substituents are selected from lower alkyl, lower alkenyl, lower alkoxy, lower alkenyloxy, lower alkanoyl, halo (lower) alkyl, carboxyl, lower alkoxycarbonyl, carboxy (lower) alkyl, halogen, nitro, amino, lower alkylamino, di (lower) alkylamino, lower alkanoylamino, hydroxy, thiol, hydroxysulfonyl, aminosulfonyl, aryl (lower) alkanoyl, aryloxyamino, aryl, aryl (lower)alkyl, cycro (lower) alkyl, cycro (lower) alkenyl, cycro (lower) alkyl (lower) alkyl and 3- to 7- membered heterocyclic group;

(viii) an inhibitor of the formation of protein modification products according to (ii), wherein R1 is phenyl group, R2 is methyl group, R3 and R4 cooperate to form 3-hydroxy-5-hydroxymethyl-2-methylpyridin-4-yl-methylene in formula (I);

(ix) an inhibitor of the formation of protein modification products according to (ii), wherein R1 is phenyl group, R2 is methyl group, and R3 is 6-methyl-1,3-dihydrofuro-[3,4-c]-pyridin-7-ol group in formula (II);

(x) an inhibitor of the formation of protein modification products according to any one of (i) to (ix), wherein the

protein modification products are selected from AGEs, ALEs and combinations thereof;

(xi) an inhibitor of the formation of protein modification products according to (x), wherein the protein modification products is AGEs;

(xii) an inhibitor of the formation of protein modification products according to (xi), wherein the AGEs is pentosidine;

(xiii) a renal tissue protecting agent comprising the inhibitor of the formation of protein modification products according to any one of (i) to (xii);

(xiv) a peritoneal dialysate comprising the inhibitor of the formation of protein modification products according to any one of (i) to (xii);

(xv) a hemodialysis fluid comprising the inhibitor of the formation of protein modification products according to any one of (i) to (xii);

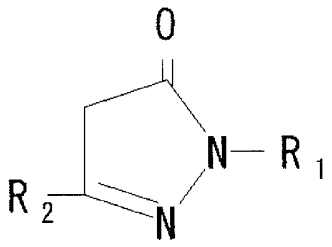
(xvi) a method for the reduction of the amount of a carbonyl compound(s) in a liquid sample, which comprises contacting

the inhibitor of the formation of protein modification products according to any one of (i) to (xii) with the liquid sample;

(xvii) a method for the suppression of the formation of protein modification products in the blood or peritoneal dialysate of a patient, which comprises contacting the inhibitor of the formation of protein modification products according to any one of (i) to (xii) with said blood or peritoneal dialysate;

(xviii) a method for the suppression of the vitamin B6 deficiency caused by said inhibitor of the formation of protein modification products, which comprises introducing a substituent that inhibits the binding of vitamin B6 molecules (comprising of that derived from vitamin B6 molecules itself) to 1-substituted-, unsubstituted-3-substituted- or unsubstituted-2-pyrazolin-5-one at the 4-position in free form or salt form to provide a compound that is useful as a protein modification products production inhibiting agent;

(xix) a method according to (xviii), wherein the 1-substituted-, unsubstituted-3-substituted- or unsubstituted-2-pyrazolin-5-one is of the formula:

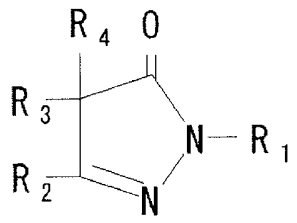


wherein R1 is hydrogen atom or substituted or unsubstituted aromatic ring; and R2 is hydrogen atom or monovalent organic group;

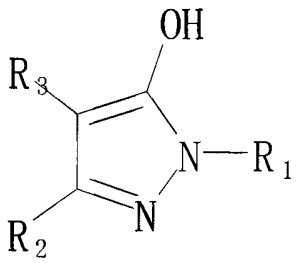
(xx) a method according to (xvi), wherein the substituent, which is introduced at the 4-position and inhibits binding of vitamin B6 molecules, is selected from an organic group;

(xxi) a compound on which is introduced a substituent that inhibits the binding of vitamin B6 molecules (comprising of that derived from vitamin B6 molecules itself) to 1-substituted-, unsubstituted-3-substituted- or unsubstituted-2-pyrazolin-5-one at the 4-position in free form or salt form, or intramolecular rearranged bodies thereof;

(xxii) a compound of formula (I)



or formula (II)



wherein R1 is substituted or unsubstituted aromatic ring; and each of R2, R3 and R4 is a hydrogen atom or monovalent organic group, or alternatively R2 and R3 cooperate to form a condensed ring or R3 and R4 cooperate to represent a divalent organic group, provided that R3 and R4 are not simulataneously hydrogen atoms

in free or salt forms;

(xxiii) a compound according to (xxii), wherein R1 is phenyl group, R2 is methyl, R3 and R4 cooperate to form 3-hydroxy-5-hydroxymethyl-2-methylpyridin-4-yl-methylene group;

(xxiv) a compound according to (xxii), wherein R1 is phenyl group, R2 is methyl group, and R3 is 6-methyl-1,3-dihydrofuro-[3,4-c]-pyridin-7-ol group;

(xxv) use of the compound according to any one of (xxi) to (xxiv) for the preparation of an inhibitor of the formation of protein modification products;

(xxvi) a method for treatment of a disease mediated by the formation of protein modification products, which comprises administering a therapeutically effective amount of the compound according to any one of (xxi) to (xxiv) to a patient in need of such treatment.

The term "protein modification products" herein used is intended to mean a protein modification products (e.g., AGEs, ALEs, etc.) produced by the reaction with a carbonyl compound under non-enzymatic conditions formed by the reaction with carbonyl compounds under non-enzymatic conditions and cover AGEs and ALEs, unless otherwise stated specifically. The protein modification products may be thus AGEs or ALEs, or their combination. Examples of AGEs are pentosidine, crossrine, X1 (fluorolink), pyropyridine, pyrarine, carboxymethyl-lysine, imidazolone compounds, carboxyethyl-lysine, MGO dimer, GO dimer, imidazolidine and argupyrimidine. Examples of ALEs are malondialdehydolysine, modified hydroxynonenal, etc.

The term "carbonyl compound" is intended to mean a compound having a carbonyl group causing protein modification regardless of being derived from organisms or non-organisms, and covers dicarbonyl compounds. Examples of the carbonyl compound include arabinose, GO,

MGO, 3-DG, glycolaldehyde, dehydroascorbic acid, hydroxynonenal, marondialdehyde, acrolein, 5-hydroxy-methylfurfural, formaldehyde, levulinic acid, furfural, etc.

The term "vitamin B6 deficiency" refers to several diseases caused by the deficiency of vitamin B6, and includes angular cheilitis, mouth inflammation, glossitis, chelitis, acute and chronic eczema, contact dermatitis, peripheral neuritis, anemia, hypolymphemia and nerve disorder.

An active ingredient of "inhibitor of the formation of protein modification products" is a compound on which is introduced a substituent that inhibits the binding of vitamin B6 molecules (comprising of that derived from vitamin B6 molecules itself) to 1-substituted-, unsubstituted-3-substituted- or unsubstituted-2-pyrazolin-5-one at the 4-position in free form or salt form, or rearranged bodies thereof, may finally suppress the production of protein modification products regardless of being in vivo, ex vivo and/or in vitro. By the term "finally suppress" it is meant that the compounds may, by their effect to trap the carbonyl compounds or by suppressing the reaction, cause the formation of protein modification products. It allows any mechanism to suppress finally the formation of protein

modification products, there is no limitation of their mechanism. In addition, the term "inhibitor" or "protectant" includes medicament for preventive and/or therapeutic use.

An active ingredient of inhibitor of the formation of protein modification products according to the present invention is a compound of formula (I) or (II).

In formula (I) and (II), R1 represents hydrogen atom or unsubstituted or substituted aromatic ring (includes heterocyclic ring) group. "Aromatic ring group" involves not more than 20 ring constituent atoms (hetero atoms such as oxygen, sulfate or nitrogen may be present therein and their number is not more than 4), in particular are preferred aryl comprising 6 to 10 ring constituent carbon atoms (for example phenyl or naphthyl).

The substituents may be selected from one or more of for example, lower alkyl, lower alkenyl, lower alkoxy, lower alkenyloxy, lower alkanoyl, halo (lower) alkyl, carboxyl, lower alkoxycarbonyl, carboxy (lower) alkyl, halo (such as chlorine, bromide, iodine, fluorine), nitro, amino, lower alkylamino, di (lower) alkylamino, lower alkanoylamino, hydroxy, thiol, hydroxysulfonyl, aminosulfonyl, aryl (lower) alkanoyl, aryloxyamino, aryl, aryl (lower) alkyl, cycro

(lower) alkyl, cycro (lower) alkenyl, cycro (lower) alkyl (lower) alkyl, 3- to 7-membered hetero cyclic (such as oxadiazolyl, thiadiazolyl). The number of substituents is not limited, but is usually not more than 3.

The substituted or unsubstituted aromatic ring group R1 covers the following examples: phenyl, naphthyl, o-, m- or p-lower alkylphenyl (such as o-methylphenyl, p-methylphenyl, p-ethylphenyl), o-, m- or p-lower alkoxyphenyl (such as o-, m- or p-methoxyphenyl, o-, m- or p-ethoxyphenyl), o-, m- or p-aminophenyl, o-, m- or p-nitrophenyl, o-, m- or p-halophenyl (such as o-, m- or p-chlorophenyl, o-, m- or p-fluorophenyl), o-, m- or p-halo-(lower)-alkylphenyl (such as o-, m- or p-trifluoromethyl-phenyl), o-, m- or p-sulfamoylphenyl, o-, m- or p-carboxyphenyl, o-, m- or p-lower alkoxycarbonyl-phenyl (such as o-, m- or p-methoxycarbonyl-phenyl, o-, m- or p-ethoxycarbonylphenyl, o-, m- or p-isopropoxy-carbonylphenyl), o-, m- or p-lower alkanoylphenyl (such as o-, m- or p-acetylphenyl), di (lower) alkylphenyl (such as 3,4-dimethylphenyl), dihydroxyphenyl (such as 2,4-dihydroxyphenyl), 2-amino-4-carboxyphenyl, 3-amino-5-carboxyphenyl, 3-lower alkoxy-4-hydroxyphenyl (such as 3-methoxy-4-hydroxyphenyl), 3-carboxy-4-halophenyl (such as 3-carboxy-4-chlorophenyl).

Each of R2, R3 and R4 represent independently hydrogen atom or monovalent organic group. The term "monovalent organic group" covers substituted or unsubstituted hydrocarbon, halo, nitro, amino, hydroxy, thiol, carboxy, carboxy (lower) alkyl, lower alkoxycarbonyl, formyl, lower alkanoyl, lower alkylamino, di (lower) alkylamino, lower alkanoylamino, aryl (lower) alkanoyl, aryloxyamino, sulfonic acid and 3- to 7-membered heterocyclic group. The term "hydrocarbon group" covers straight chain or cyclic aliphatic, alicyclic or aromatic hydrocarbon group comprising not more than 30, preferably not more than 8 carbon atoms. In particular, for example alkyl, alkenyl, alkynyl, cycroalkyl, cycroalkenyl and aryl groups are included. The term "3- to 7-membered heterocyclic group" comprises of not more than 3 hetero atoms as ring constituent atoms. For example, pyrrolidino, piperidino, morpholino and thiamorpholino are included. The variety and number of the substituent is as defined in R1, provided that R3 and R4 are not simultaneously hydrogen atoms.

Alternatively, R2 and R3 cooperate to form a condensed ring. In said condensed ring, 5- or 6-membered saturated carbon ring (i.e. R2+R3 = trimethylene or tetramethylene) are preferred, optionally comprising

substituents. Furthermore, R3 and R4 cooperate to form divalent organic group. In said divalent organic group, for example, methylene-type and spiro-type are included. In methylene-type, for example phenylmethylene, phenyl-alkenylmethylene, quinolinyl-methylene, furanyl-methylene, diazolyl-methylene, aminomethylene, di (lower) alkylamino-methylene, pyridyl-methylene and thiophenyl-methylene are included, optionally comprising substituents. The variety and number of substituent in such condensed ring or divalent organic group is as defined in R1.

The term "lower" in relation to alkyl, alkoxy, alkanoyl, etc. herein above means the group comprising up to 8 carbon atoms, preferably up to 5 carbon atoms.

The compounds (I) or (II) of this invention are exemplified as follows:

1. 2-(3-amino-5-oxo-1-phenyl-4,5-hydro-1H-pyrazol-4-yl)-2-oxo-N-phenyl-acetamide;
2. 2-(3-amino-5-oxo-1-phenyl-4,5-hydro-1H-pyrazol-4-yl)-2-oxo-N-thiazol-2-yl-acetamide;
3. 2-(3-amino-5-oxo-1-phenyl-4,5-hydro-1H-pyrazol-4-yl)-2-oxo-acetamide;
4. 2-(3-amino-5-oxo-1-phenyl-4,5-hydro-1H-pyrazol-4-yl)-N-(3,4-dimethyl-phenyl)-4-oxo-butylamide;

5. 2-(4-amino-phenyl)-4-(2-hydroxy-ethyl)-5-methyl-2,4-hydro-pyrazol-3-one;

6. 5-amino-2-phenyl-4-(1-phenyl-1H-tetrazol-5-yl-sulfanil)-2,4-dihydro-pyrazol-3-one;

7. 3-(3-methyl-5-oxo-1-penyl-4,5-dihydro-1H-pyrazol-4-yl)-propionic acid;

8. N-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-yl)-acetamide;

9. 4-[(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-phenyl-methyl]-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

10. 2-phenyl-3a,4,5,6-tetrahydro-2H-cycropentapyrazol-3-one;

11. 4-methyl-N-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-yl)-benzenesulfonamide;

12. N-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-yl)-acetamide;

13. 5-methyl-2-(3-nitro-phenyl)-4-(1-phenyl-1H-tetrazol-5-yl-sulfanil)-2,4-dihydro-pyrazol-3-one;

14. N-[5-oxo-1-phenyl-4-(1-phenyl-1H-tetrazol-5-yl-sulfanil)-4,5-dihydro-1H-pyrazol-3-yl]-benzamide;

15. 4-(hydroxy-phenyl-methyl)-2-phenyl-5-trifluoromethyl-2,4-dihydro-pyrazol-3-one;

16. 4-(1-hydroxyimino-ethyl)-2,5-diphenyl-2,4-dihydro-pyrazol-3-one;

17. 5,5'-dimethyl-2,2'-diphenyl-2,4,2',4'-tetrahydro-[4,4']-bipyrazol-3,3'-dione;

18. 2-(4-chloro-phenyl)-4-ethyl-5-methyl-2,4-dihydro-pyrazol-3-one;

19. 4-[4-(4-methoxy-phenyl)-thiazole-2-yl-sulfanil]-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

20. 4-(2-oxo-2-phenyl-ethyl)-2-phenyl-5-propyl-2,4-dihydro-pyrazol-3-one;

21. 5-methyl-2-phenyl-4-(4-p-toluyl-thiazole-2-yl-sulfanil)-2,4-dihydro-pyrazol-3-one;

22. 2-(4-fluoro-phenyl)-4-[[1-(4-fluoro-phenyl)-5-hydroxy-3-methyl-1H-pyrazol-4-yl]-(2-hydroxy-phenyl)-methyl]-5-methyl-2,4-dihydro-pyrazol-3-one;

23. N-(3,4-dimethyl-phenyl)-2-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-yl)-2-oxo-acetamide;

24. 5-(4-chloro-benzoyl)-4,4-dihydroxy-2-phenyl-2,4-dihydro-pyrazol-3-one;

25. sodium; 4-hydroxy-3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-sulfonate;

26. 5-methyl-4,4-di-morpholin-4-yl-2-phenyl-2,4-dihydro-pyrazol-3-one;

27. soduim 3-benzoylamino-4-hydroxy-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-sulfonate;

28. 3-methyl-1-phenyl-5-oxo-4-spiro-(3-oxo-2,3-dihydro-benzo[b]thiophen-2-yl)-4,5-dihydro-1H-pyrazol;

29. 4,4,5-trimethyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

30. 4,10-dimethyl-2,8,11-triphenyl-2,3,8,9-tetraza-dispiro [4.0.4.1]undeca-3,9-diene-1,7-dione;

31. 2-(2-chloro-phenyl)-4-(3-ethoxy-4-hydroxy-benzylidene)-5-methyl-2,4-dihydro-pyrazol-3-one;

32. 2-(2-chloro-phenyl)-4-(4-dimethylamino-benzylidene)-5-methyl-2,4-dihydro-pyrazol-3-one;

33. 5-methyl-4-(3-phenyl-allylidene)-2-(3-trifluoromethyl-phenyl)-2,4-dihydro-pyrazol-3-one;

34. 3-{5-[3-methyl-5-oxo-1-(4-sulfamoyl-phenyl)-1,5-dihydro-pyrazol-4-ylidene-methyl]-furan-2-yl}-benzoic acid;

35. 4-(4-dimethylamino-benzylidene)-2-(3-fluoro-phenyl)-5-methyl-2,4-dihydro-pyrazol-3-one;

36. 3-{4-[4-(3-chloro-4,5-dihydro-pyrazol-1-yl)-benzylidene]-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl}-benzoic acid;

37. 3-[4-(2-hydroxy-benzylidene)-5-oxo-3-phenyl-4,5-dihydro-pyrazol-1-yl]-benzoic acid;

38. 3-[1-(3-chloro-phenyl)-3-methyl-5-oxo-1,5-dihydro-pyrazol-4-ylidene-methyl]-1H-quinolin-2-one;

39. 3-{5-[3-methyl-5-oxo-1-(4-sulfamoyl-phenyl)-1,5-dihydro-pyrazol-4-ylidene-methyl]-furan-2-yl}-benzoic acid-methyl ester;

40. 4-(4-benzo[1,3]dioxol-5-ylmethylene-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl)-benzoic acid-methyl ester;

41. 4-{3-methyl-5-oxo-4-[5-(4-sulfamoyl-phenyl)-furan-2-yl-methylene]-4,5-dihydro-pyrazol-1-yl}-benzoic acid-methyl ester;

42. 2-(4-chloro-phenyl)-4-(2,4-dihydroxy-benzylidene)-5-methyl-2,4-dihydro-pyrazol-3-one;

43. 2-(4-chloro-phenyl)-4-(3-hydroxy-benzylidene)-5-methyl-2,4-dihydropyrazol-3-one;

44. 4-(3,4-dihydroxy-benzylidene)-5-methyl-2-p-toluyl-2,4-dihydro-pyrazol-3-one;

45. 3-[1-(4-acetyl-phenyl)-3-methyl-5-oxo-1,5-dihydro-pyrazol-4-ylidene]-1,3-dihydro-indol-2-one;

46. 2-(4-fluoro-phenyl)-4-(5-hydroxy-3-methyl-1-o-toluyl-1H-pyrazol-4-yl-methylene)-5-methyl-2,4-dihydro-pyrazol-3-one;

47. 2-(4-chloro-phenyl)-4-(4-hydroxy-3-methoxy-benzylidene)-5-trifluoromethyl-2,4-dihydro-pyrazol-3-one;

48. 2-(4-ethyl-phenyl)-4-(4-hydroxy-benzylidene)-5-methyl-2,4-dihydro-pyrazol-3-one;

49. 4-[4-(4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzenesulfonamide;

50. 4-(5-oxo-4-thiophene-2-yl-methylene-3-trifluoromethyl-4,5-dihydro-pyrazol-1-yl)-benzoic acid-ethyl ester;

51. 4-[4-(4-dimethylamino-benzylidene)-5-oxo-3-trifluoromethyl-4,5-dihydro-pyrazol-1-yl]-benzenesulfonamide;

52. 4-isopropylidene-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

53. 4-(4-hydroxy-benzylidene)-2-phenyl-5-trifluoromethyl-2,4-dihydro-pyrazol-3-one;

54. 4-(2,4-dihydroxy-benzylidene)-2-(3,4-dimethyl-phenyl)-5-methyl-2,4-dihydro-pyrazol-3-one;

55. 3-[4-(3-ethoxy-4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid;

56. 4-[4-(3,5-di-tert-butyl-4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid;

57. 3-[3-(4-hydroxy-3-methoxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid;

58. 3-[3-hydroxy-4-(4-hydroxy-3-methoxy-benzylidene)-5-oxo-pyrazolidin-1-yl]-benzoic acid;

59. 4-(3-hydroxy-2,4-dimethoxy-benzylidene)-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

60. 4-[4-(4-hydroxy-3-methoxy-benzylidene)-3-methyl-5-oxo-pyrazolidin-1-yl]-benzoic acid-isopropyl ester;

61. 2-chloro-5-[4-(2-chloro-4-hydroxy-5-methoxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid;

62. 4-[4-(4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid-ethyl ester;

63. 4-[4-(4-hydroxy-benzylidene)-5-oxo-3-trifluoromethyl-4,5-dihydro-pyrazol-1-yl]-benzoic acid-ethyl ester;

64. 4-[4-(4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid;

65. 4-dimethylaminomethylene-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

66. 4-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl-methylene)-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

67. 4-(4-chloro-benzylidene)-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

68. 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol;

69. 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol (hydrochloric acid salt);

70. 4-(4-hydroxy-benzylidene)-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

71. 2-(3-chloro-phenyl)-4-(4-hydroxy-benzylidene)-5-methyl-2,4-dihydropyrazol-3-one;

72. 4-(4-benzyloxy-benzylidene)-5-methyl-2-phenyl-2,4-dihydropyrazol-3-one;

73. 2-(3-chloro-phenyl)-5-methyl-2H-pyrazol-3,4-dione 4-oxym;

74. 5-(5-oxo-1,3-diphenyl-1,5-dihydro-pyrazol-4-ylidene)-4-phenyl-4,5-dihydro-[1,3,4]thiazole-2-carboxilic acid-ethyl ester;

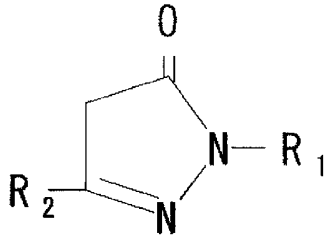
75. 4-[1,3]dithioran-2-ylidene-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one;

76. 5-(4-chloro-phenylsulfanilmethyl)-2-phenyl-4-[N'-(3-trifluoromethyl-phenyl)-hydrazino]-2,4-dihydro-pyrazol-3-one;

77. 4-(5-benzoyl-3-phenyl-3H-[1,3,4]thiadiazol-2-ylidene)-2,5-diphenyl-2,4-dihydro-pyrazol-3-one;

78. phosphoric acid mono-[5-hydroxy-6-methyl-4-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-pyrazol-4-ylidene-methyl)-pyridin-3-yl-methyl]ester.

To prepare the interest compound (I) of this invention, 1-substituted-, unsubstituted-3-substituted- or substituted-2-pyrazolin-5-one (III) as depicted in the following formula is generally subjected to appropriate chemical reactions known per se depending upon the variety of substituent which should be introduced on the 4-position:



[wherein R1 is hydrogen atom or substituted or unsubstituted aromatic ring; and R2 is hydrogen atom or monovalent organic group].

For example, the compound (III) is reacted with aldehyde of formula: R3-CHO (IV) to form the compound (I). The compound (I) may provide the compound (II) by intramolecular rearrangement. The reaction is usually carried out by treat the compound (III) and aldehyde (IV) in aqueous vehicle under alkali conditions, or in organic solvent (such as tetrahydrofuran, dioxan), in presence of organic or inorganic base at -120 to 100 °C.

The starting material compound (III) is known per se or may be prepared by any conventional chemical reactions. For example, 3-methyl-1-phenyl-2-pyrazolin-5-one (in the compound (III), R1 is phenyl and R2 is methyl) is referred to edaravone as its general name. The compound has free radical deleting activity, and is known as a medicament such as brain function normalizing agent (Japan patent publication No. 5-31523), peroxidized lipid production suppressing agent (Japan patent publication No. 5-35128), anti-ulcer agent (Japan patent No. 2906512), hyperglycemic suppressing agent (Japan patent No. 2906513). However, it has not been known before Japan patent application 2003-076955 that edaravone traps carbonyl compounds, improves carbonyl stress condition, therefore is effective to prevent or treat several diseases caused by carbonyl stress,

namely useful as inhibitor of the formation of protein modification products.

As described above, the compound (I) or (II) itself shows formation of protein modification products inhibiting effect without vitamin B6 deficiency as an adverse effect in organisms. This fact can be confirmed by the following test:

(A) Test to prove that the compound (I) itself exhibits a formation of protein modification products inhibiting effect:

(1) To a plasma sample taken from a dialysis patient without diabetes, the compound of this invention is added, and after a certain period of time, the amount of pentosidine formed is determined using pentosidine, a typical example of AGEs, as an index.

(2) Phenylalanine reacts with OH radical in presence of hydroxy radical to form o- or m-tyosine. Further, tyrosine reacts with NO radical in presence of peroxynitrite to form nitrotyrosine. A radical causes disorder of kidney in organisms. Accordingly, the radical capturing ability of the compound of this invention in phenylalanine-radical reacting system is determined.

(B) Test to prove that the compound (I) does not cause vitamin B6 deficiency:

(1) To a solution of vitamin B6, the compound of this invention is added, and after a certain period of time, the amount of vitamin B6 remaining is determined.

(2) To a normal rat, the compound of this invention is administered, and after a certain period of time, the presence or absence of vitamin B6 deficiency is determined.

The inhibitor of the formation of protein modification products of this invention comprising the compound (I) or (II) as an active ingredient is usable to prevent and/or treat the following conditions: renal failure, diabetic complications (nephropathy, nerve disorder, retinopathy, cataract, etc.), arteriosclerosis, dialysis amyloidosis which is a complication of dialysis, and peritoneal scrlosis in peritoneal dialysis patient, Alzheimer's disease which is a central neurological disease, Pick's disease and Parkinson disease, rheumatoid arthritis, sunlight erastic fibrosis, aging, etc. Said inhibitor is particularly useful to prevent and/or treat renal disorders.

The compound (I) of this invention, directly or with treatment such as dilution with water, may be used as

preventive agent or therapeutic agent, and may be used in combination with medicinal drugs or quasi drugs. The blending quantity in this case is selected depending on the condition or the product, and 0.001 to 50 % by weight, especially 0.01 to 10 % by weight of the compound is usually suitable when it is administered systemically. Since sufficient preventive or therapeutic effect may not be achieved when it comprises less than 0.001% by weight, or since property of the product such as stability or flavor may be impaired when it comprises more than 5% by weight, those quantities are not preferred.

The compound (I) of this invention may be present in free or salt form. The salt includes pharmaceutically acceptable salts, for example salt with inorganic or organic base, acid addition salt such as inorganic acid, organic acid, and basic or acidic aminoic acid addition salt. The salt with inorganic base includes for example alkali metal (such as sodium or potassium) salt, alkali earth metal (such as calcium, magnesium) salt, aluminum salt and ammonium salt. The salt with organic base includes for example salts with primary amine (such as ethanol amine), secondary amine (such as diethyl amine, diethanol amine, dicyclohexyl amine, N,N' -dibenzylethylen diamine), and tertiary amine

(such as trimethylamine, triethylamine, biridine, picoline, triethanol amine).

The salt with inorganic acid is exemplified salts with hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid and phosphoric acid, and the salt with organic acid is exemplified salts with formic acid, acetic acid, lactic acid, trifluoro acetic acid, fumaric acid, oxalic acid, tartaric acid, maleic acid, benzoic acid, citric acid, succinic acid, malic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid and p-toluenesulfonic acid. In addition, the salt with basic aminoic acid is exemplified salts with arginine, lysine and ornithine, and the acidic aminoic acid is exemplified salts with aspargine acid and glutamic acid.

The compound (I) or (II) of this invention is optionally used in combination with known agent such as amino guanidine, pyridoxamine derivative, OPB-9195, biguanide compound, bridge formation inhibitor, enzyme degrading Amadori compounds, GSH, cystein, acetyl cystein, vitamin E, ubiquinol, aldose reduction enzyme inhibitor, carbonyl compounds trapping agent to enhance sustentation of the formation of protein modification products inhibiting effect. In addition, identified material(s) which deactivate or

degrade the compound (I) or (II), selected materials which inhibit the identified materials and selected materials are used together to obtain stability of the active ingredient in the blended composition.

The administration route of the medicament of this invention may be selected from transmucosal, transdermal, intramuscular, subcutaneous or intrarectal administration other than oral or intravenous administration, and depending on the administration route, several preparations may be used. Each preparation is described herein after, but formulation used in this invention is not limited thereto, any kind of formulation used in pharmaceutical preparations may be used.

Used as preventive agent or therapeutical agent for condition associated with protein modification products, an oral dose of the compound (I) or (II) is generally in the range of preferably 0.3 mg/kg to 300 mg/kg, more preferably 1 mg/kg to 100 mg/kg. In systemic administration, especially in intravenous administration, dose will vary depending on the sex, age, body weight, etc. but usually administer to make available blood level in the range of 2 μg/mL to 200 μg/mL, more preferably 5 μg/mL to 100 μg/mL.

Dosage form of oral administration includes powder, granule, capsule, pill, tablet, elixir, suspension, emulsion and syrup. Further, dosage form of intraorally local administration includes masticatory, sublingual formulation, buccals, lozenge, ointment, adhesive preparation and liquid. Besides, some modifications such as sustained release, stable, ease disintegrate, hard disintegrate, enteric, ease absorption may be done.

Any known drug delivery system (DDS) may be adopted to each preparation listed above. DDS preparation herein means the most suitable preparation form such as sustained release preparation, topical applicable preparation (such as lozenge, buccals and sublingual formulation), controlled release preparation, enteric preparation and gasteric preparation based on administration route, bioavailability, adverse effect, etc.

The component of DDS includes essentially medicament, medicament release module, encapsulating body and therapeutic program. For each component, in particular, a medicament which blood level goes down quickly when the release is stopped and which has a short half life is preferred; the encapsulating body which does not react with living tissue of the administration site is

preferred; in addition, the therapeutic program which maintains the best drug level in a given period is preferred. The medicament release module comprise essentially medicament container, release controlling part, energy source and release hole or release surface. Such essential components are not all needed together, thus the best form can be selected by suitable addition or deletion.

Materials for DDS include high molecules, cyclodextrin derivative, and lecithin. The high molecules are selected from insoluble high molecule (such as silicon, ethylene-acetic vinyl copolymer, ethylene-vinylalcohol copolymer, ethylcellulose and cellulose acetate), soluble high molecule and hydroxyl gel forming high molecule (such as polyacrylamide, polyhydroxyethyl methacrylate cross-linked material, polyacryl cross-linked material, polyvinylalcohol, polyethylene oxide, water-soluble cellulose derivative, cross-linked poloxamer, chitin, chitosan), sustained soluble high molecule (such as ethylcellulose, partial ester of methylvinyl ether-maleic acid anhydride copolymer), gastric high molecule (such as hydroxypropylmethyl cellulose, hydroxy propylcellulose, carmellose sodium, macrogol, polyvinyl pyrolidone, methacrylic acid dimethylaminoethyl-methacrylic acid methyl copolymer), enteric high molecule (such as hydroxypropylmethyl cellulose phthalate, acetic

acid phthalcellulose, hydroxypropylmethyl cellulose acetate succinate, carboxymethylethyl cellulose, acrylic acid group polymer), biodegradable high molecule (such as thermocoagulation or cross-linked albumin, cross-linked gelatin, collagen, fibrin, polycyanoacrylate, polyglycolic acid, polylactic acid, poly β-hydroxy acetic acid, polycaprolactam).

Particularly, silicon, ethylene-acetic acid vinyl copolymer, ethylenevinylalcohol copolymer and partial ester of methylvinyl ether-maleic acid anhydride copolymer may be used for release control of drug, and cellulose acetate may be used as a material of osmotic pump, ethylcellulose, hydroxypropylmethyl cellulose, hydroxypropyl cellulose and methylcellulose may be used for membrane material of sustained release preparation, polyacryl cross-linked material may be used for adhesive preparation for oral or ocular mucosa.

In addition, depending on the formulation (such as formulation for oral administration, injection, suppository), suitable additives may be added, for example solvent, diluent, coating agent, base, binding agent, lubricant, disintegrant, solubilizing agent, suspending agent, thickener, emulsifier, stabilizer, buffer, tonicity agent,

soothing agent, preservative, flavoring agent, aromatic agent and/or colorant. For such additives, examples are listed herein after, but not limited to.

The solvent includes purified water, injection solvent, saline, arachis oil, ethanol and glycerin. The diluent includes starch, lactose, glucose, sucrose, crystalline cellulose, calcium sulfate, calcium carbonate, talc, titanic oxide, trehalose and xylitol. The coating agent includes sucrose, gelatin, cellulose acetate phthalate and high molecule as sited above. The base includes petrolatum, vegetable oil, macrogol, oil in water emulsifier base and water in oil emulsifier base.

The binding agent includes starch and derivatives thereof, cellulose and derivatives thereof, gelatin, alginate sodium, tragacanth, nature high molecules such as gum acacia, synthetic high molecules such as polyvinyl pyrrolidone, dextrin and hydroxypropyl starch. The lubricant includes stearic acid and salts thereof, talc, wax, wheat starch, macrogol, hydrogenated vegetable oil, sucrose fatty acid ester and polyethylene glycol. The disintegrant includes starch and derivatives thereof, agar, gelatin powder, sodium hydrogen carbonate, cellulose and derivatives thereof, carmellose calcium, hydroxypropyl

starch, carboxymethyl cellulose and salts thereof as well as cross-linked structures thereof and lower substituted hydroxypropyl cellulose.

The solubilizing agent includes cyclodextrin, ethanol, propylene glycol and polyethylene glycol. The suspending agent includes gum acacia, tragacanth, alginate sodium, aluminum monostearate, citric acid and several surfactants. The viscose includes carmellose sodium, polyvinyl pyrrolidone, methylcellulose, hydroxypropylmethyl cellulose, polyvinylalcohol, tragacanth, gum acacia and alginate sodium. The emulsifier includes gum acacia, cholesterol, tragacanth, methylcellulose, various surfactants and lecithin.

The stabilizer includes bisulfite sodium, ascorbic acid, tocopherol, chelate agent, innert gas and reducing material. The buffer includes hydrogen phosphate sodium, acetic acid sodium and boric acid. The tonicity agent includes sodium chloride and glucose. The soothing agent includes procaine hydrochloride, lidocaine and benzyl alcohol. The preservative includes benzoic acid and salts thereof, p-hydroxybenzoic esters, chloro butanol, cationic soap, benzyl alcohol, phenol and methyl salicylate. The flavoring agent includes sucrose, saccharine, licorice extract,

sorbitol, xylitol and glycerin. The aromatic agent includes spruce tinctura and rose oil. The colorant includes water solubility food colorant and lake dye.

As described above, continuance of available blood level, enhanced bioavailability, etc. may be predicted by preparing DDS preparations such as sustained release preparation, enteric preparation or controlled drug release preparation. However, the compound (I) or (II) is deactivated or degraded in an organism, and as a result, there is a possibility that the desired effect may be reduced or disappear. Accordingly, material(s) which inhibit the deactivator or degradator of the compound (I) or (II) are combined with the composition for prevention of treatment for the condition associated with protein modification products of this invention to continue the effect of the ingredient. Such material(s) may be mixed in the preparation, and may be administered separately. The material(s) which deactivate or degrade the compound (I) or (II) are identified, inhibitor of such material(s) may be selected, and mixed or used together by a person skilled in the art.

In the preparation, any ingredient used in normal composition as additives other than described above may

be used, and an amount of such ingredient is selected in a range such that the effect of this invention is not prevented.

The compound (I) or (II) of this invention may also be used to suppress the disorder from protein modification products in peritoneal dialysis and hemodialysis. Namely, the compound (I) or (II) as inhibitor of the formation of protein modification products is added to conventional peritoneal dialysate or hemodialysate.

The method to reduce the amount of carbonyl compounds in liquid sample according to this invention comprises the step that said liquid sample is contacted with the compound (I) or (II) as inhibitor of the formation of protein modification products.

In addition, the method to suppress the formation of protein modification products according to this invention comprises the step that blood from patient or peritoneal dialysate is contacted with the compound (I) or (II) as inhibitor of the formation of protein modification products. The protein modification products in dialysis include protein modification products that are formed by the carbonyl compounds derived from patient with peritoneal dialysis or hemodialysis and the protein modification products that are

formed by carbonyl compounds derived from peritoneal dialysate or hemodialysate themselves.

The composition of peritoneal dialysate or hemodialysate to which is added the compound (I) or (II) according to this invention is selected from known ones. Common peritoneal dialysate are composed of osmo-regulator (such as glucose), buffer (such as lactose, citric acid, malic acid, acetic acid, pyruvic acid, succinic acid and sodium hydrogencarbonate), and inorganic salts (such as sodium ion, potassium ion, magnesium ion, calcium ion and chloride ion). The peritoneal dialysate or hemodialysate to which was added the compound (I) or (II) may be sealed off directly to sterilize by heat. By doing so, the formation of protein modification products from main ingredients is accompanied with sterilization by heat or preservation.

In addition, liquid such as peritoneal dialysis is packed in separate containers that consist of first chamber and second chamber, reducing sugar is packed in the first chamber and the compound (I) or (II) is packed in the second chamber, and all of them may be mixed before use. When aminoic acid is comprised, a person skilled in the art may consider a better combination such as adding a third chamber.

Since the compound (I) or (II) suppresses the formation of protein modification products by intraperitoneal or intravascular administration, adverse effects such as peritoneal sclerosis may be alleviated. Furthermore, it can be expected to work as prevention and/or therapy for other conditions (such as diabetic complications). The dialysate may involve known agents such as amino guanidine other than the compound (I) or (II). Alternatively, it can be formulated in powdered dialysis agent.

The compound (I) or (II) may be injected to dialysis circuit that is equipped with suitable connector for coinjection. Alternatively, the compound (I) or (II) is directly injected into peritoneal cavity to mix with peritoneal dialysate in the peritoneal cavity. Alternatively, before peritoneal dialysate is injected to a patient or while it collects in a peritoneal cavity, the compound (I) or (II) may be injected intravenously to suppress the formation of protein modification products effectively.

The dialysate is filled in suitable sealing container and sterilized. The sterilization by high-pressure steam and by hot-water is effective. In this case, a container in which toxic substances are not eluted at high temperature and

that have enough hardness to endure carriage after sterilization is used. In particular, commutative plastic bag that is made from for example polyvinyl chloride, polypropylene, polyethylene, polyester, ethylene acetate vinyl copolymer are included. In addition, to avoid degradation of liquid due to the effect of ambient air, the container that is filled with dialysate is further packed by packing materials which has high gas barrier property.

When sterilization is carried out by heat including high-pressure heat, if the compound (I) or (II) used has enough stability against such treatment, the compound (I) or (II) is added previously to the dialysate and then the mixed dialysate is sterilized. If the compound (I) or (II) used does not have stability against sterilization by heat, sterilization without heat can be carried out. Such sterilization includes, for example sterilization by filtration.

For example, such sterilization can be carried out by filtration with fine filter that is equipped with membrane filter having pore diameter about 0.2 μm. The dialysate sterilized by filtration is filled in the container such as flexible plastic bag, and then it is sealed. In addition, to peritoneal dialysate that is previously sterilized by heat may be added the compound (I) or (II).

The timing of addition is not limited. Whether after or before sterilization, the compound (I) or (II) may be added, may be added just before or together with dialysis and may be injected into peritoneal cavity directly after the dialysate is injected.

The peritoneal dialysate of this invention is used for dialysis as current peritoneal dialysate or hemodialysate. Namely, for peritoneal dialysis, suitable amount of the peritoneal dialysate according to this invention is injected into peritoneal cavity of a patient with dialysis to transfer the low molecular weight ingredient in an organism into peritoneal dialysate through peritonea. The peritoneal dialysate is intermittently circulated and dialysis is continued depending on the condition of the patient. At this stage, the compound (I) or (II) suppresses the formation of protein modification products in the dialysate or the organism. The carbonyl compounds transfer from blood or intraperitonea to peritoneal dialysate together with dialysis ingredient such as creatinine, inorganic salts or chlorine ion. Accordingly, adverse effect on an organism by protein modification products is reduced.

The compound (I) or (II) is used for not only dialysate, but also any liquid medicament such as nutrient infusion, electrolyte infusion or enteral or tube feeding.

The compound of this invention is usable as therapy by injection, however, involves a compound which starts degrading immediately in solution and degrades about 40% after 12 hours (at 25 °C). The present inventors conducted a further study to provide injection which is administered in stable condition, comprisng the compound of this invention. Accordingly, the present invention also provides an injection which is administered in stable condition, comprisng the compound of this invention.

Effect of the present invention

The present invention provides an inhibitor of the formation of protein modification products, which effectively suppresses the formation of protein modification products, such as AGEs or ALEs. Particularly, the present invention provides a medicament for preventing and/or treating renal failure, diabetic complication such as nephropathy, nerve disorder, retinopathy, cataract, etc., arteriosclerosis, dialysis amyloidosis which is a complication of dialysis, and peritoneal screlosis in peritoneal dialysis patient,

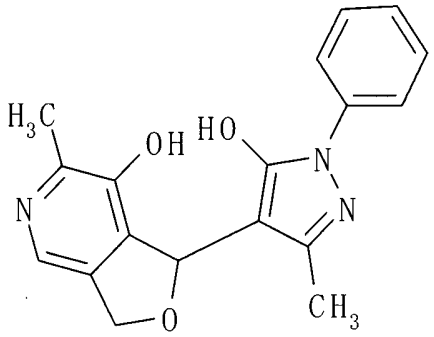
Alzheimer's disease which is central neurological disease, Pick's disease and Parkinson disease, rheumatoid arthritis, sunlight elastic fibrosis, aging, etc. In particular, a renal protective agent is provided which is applicable for renal failure and diabetic nephropathy, which is diabetic complication, or as a renal protective agent for depressor. The agent is useful to many patients in wide range, without blood pressure-lowering effect as its medicinal properties. In addition, from the point that the compound of this invention suppresses endoplasmic reticulum stress (ER stress), the compound of this invention may be used to treat diseases such as diabetes, Parkinson disease, or rheumatoid arthritis.

EXAMPLES

This invention will be hereinafter illustrated more in details by way of Examples, but the scope of this invention should not be understood to be limited to these Examples.

Preparation Example 1

Preparation of 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol



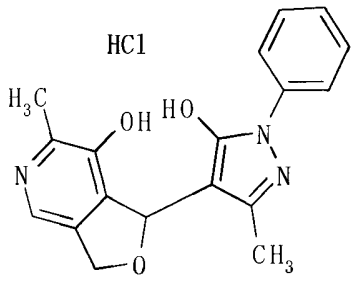
To a stirring solution of 3-methyl-1-phenyl-2-pyrazolin-5-one (1.74g) (hereinafter, referred to edaravone) in 0.1M of NaOH (100mL) at room temperature, were added dropwise a solution of pyridoxal hydrochloride (2.44 g) in water (100mL) with stirring. After the dropwise addition, the mixture was stirred for 30 minutes and the reaction was stopped, when it appeared that the deposition of a white precipitate was finished. The mixture was cooled to 4 °C in refrigerator to sufficiently deposit the precipitate. The reaction solution was allowed to cool overnight, the white precipitate was filtered off to give crude (wet) of 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol (23.7g).

Said crude was suspended in methanol (50mL) and stirred at 50 °C for 60 minutes in a supersonic stirrer, and the residual insoluble matter was filtered off and the filtrate was concentrated to 10 mL, followed by standing to cool at 4 °C in refrigerator overnight to precipitate crystals. Said

crystals were filtered off and dried in vacuum desiccator under light interception to give purified crystal of 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol (0.22g). Yield: 6.8%. Appearance: crystal powder, pale yellow white. Melt point: 207-209 °C (browning melting).

Preparation Example 2

Preparation of 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol hydrochloride



0.5006g of 1-(5-hydroxy-3-methyl-1-phenyl-1-H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol was dissolved in 150mL of methanol, and 2N of methanol hydrochloride (1.65mL) was added and stirred. The solution was concentrated to about 20mL, and when crystals were precipitate, 50 mL of ethanol was added and concentrated to substitute the crystal solvent, and repeated this operation twice and concentrated to about 5 mL. This concentrated solution was stood to cool in refrigerator

(4 °C) overnight, the precipitate was filtered off and dried in vacuum desiccator to give 1-(5-hydroxy-3-methyl-1-phenyl-1-H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol hydrochloride (0.5011g). Yield 90.0 %. Appearance: crystal powder, pale yellow white. Melting point: 247-249 °C (browning melt).

Test Example 1

Examination of the pentosidine formation inhibiting effect

For 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol (hereinafter referred to as "TM-2002"), the pentosidine, which is a typical AGEs, formation inhibiting effect was examined.

Fresh heparinized plasma samples were obtained after informed consent from hemodialysis patients prior to the dialysis session, and filtrated and sterilized. To the plasma (450μL), there was added the solution of TM-2002 in dimethylsurphoxide (50 μL) (final concentration: 0.8, 2.0, 5.0 mM) incubated at 37 °C for one week. Then, the amount of pentosidine was measured.

Measurement of the amount of pentosidine was made as follows: to each incubated sample (50μL) was added an

equal volume of 10% trichloroacetic acid, followed by centrifugation at 5000 g for 5 minutes; after removal of the supernatant, the pellet was washed by 5% trichloroacetic acid (300μL); the pellet was dried under reduced pressure and then subjected to hydrolysis in 6N HCl solution (100μL) at 110°C under nitrogen atmosphere for 16 hours; to the hydrolysate, 5N NaOH (100 μL) and 0.5 M phosphate buffer (pH 7.4) (200 μL) were added, followed by filtration through a porefilter with a pore size of 0.5 μm and dilution with PBS. The concentration of free pentosidine was determined by reversed-phase HPLC using a fluorescence detector (Miyata, T. et al.: Proc. Natl. Acad. Sci. USA, Vol. 93, p. 2353-2358, 1996). The effluent was monitored at 335/385 nm of excitation/emission wavelength. Synthetic pentosidine was used as the standard. The detection limit of pentosidine was 0.1 pmol/mg protein.

The inhibiting effect was estimated by comparing with positive control (pyridoxamine (Sigma)) reacted in the same manner as TM-2002. In addition, the inhibiting effects of aminoguanidine, olmesartan and edaravone were measured in the same manner. The result (the amount of pentosidine nmol/ml) was shown in Figure 1. (In the figure, the term "control" means negative control as only solvent. Hereinafter, as same.) It is understood from this result that

TM-2002 inhibits significantly the pentosidine formation compared to pyridoxamine as positive control.

Test Example 2

Phenylalanine hydroxylation inhibiting effect with hydroxy radical

Phenylalanine (final concentration: 1 mM), TM-2002 (final concentration: 0.1, 0.5, 2.5 mM), hydrogen peroxide (final concentration: 5mM) and cupric sulfate (final concentration: 0.1mM) were dissolved in 200 mM of phosphate buffer (pH7.4) (total volume 500 µL), followed by incubation at 37 °C for 4 hours. Then, DTPA (final concentration: 1mM) and 260 unit of catalase were added thereto to interrupt the reaction. The amounts of o-tyrosine and m-tyrosine formed were determined by HPLC in the following manner: after a predefined time, the reaction mixture was diluted to 100 folds; 20µL of the dilution was injected onto HPLC, separation was made with C18 column (4.6 x 250 mm, 5µm; Nomura Kagaku) and detection was effected using a fluorescence detector (RF-10A: Shimazu Seisakusho) under the condition of an excitation wavelength of 275nm and a fluorescence wavelength of 305nm. In the mobile phase, the flow rate was 0.6mL/min and the concentration of buffer B was varied from 6.5% to

10% in 25 minutes (buffer A: 0.10% trifluoroacetic acid; buffer B: 80% acetonitrile containing 0.08% trifluoroacetic acid). The results are shown in Figure 2 and 3 with the result of aminoguanidine, pyridoxamine and olmesartan.

Test Example 3

The suppressing effect on the nitration of tyrosine with peroxynitrite

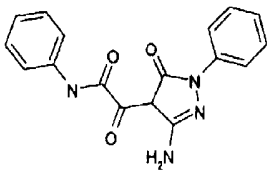
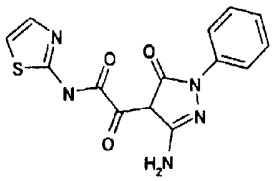
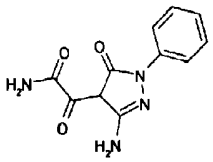
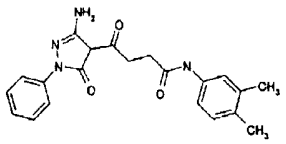
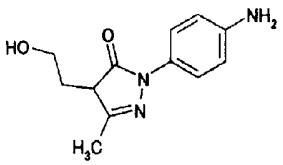
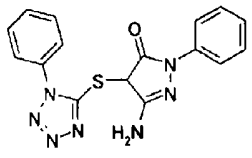
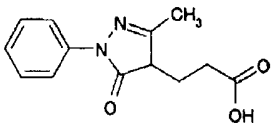
According to the method of Pannala, A.S. et al. (Free Radic. Biol. Med., 24:594-606, 1998), the examination was carried out. Namely, tyrosine (final concentration: 100 μM), TM-2002 (final concentration: 0.1, 0.5, 2.5 and 5mM) and peroxynitrite (Dojin Kagaku) (final concentration: 500μM) were dissolved in 200mM of phosphate buffer (pH7.4) (liquid volume 500μL) and incubated at 37 °C for 15 minutes. After incubation, the nitrotyrosine formation was determined with HPLC in the following manner: after a predefined time, the reaction mixture (20μL) was injected onto HPLC, separation was made with C18 column (4.6 x 250 mm, 5 μm: Waters) and detection was effected using a ultraviolet detector (RF-10A: Shimazu Seisakusho) at a wavelength of 280nm. In the mobile phase, the flow rate was 0.6 mL/min and the concentration of buffer B was varied from 5.0% to 30% in 30 minutes (buffer A: 0.10%

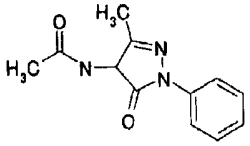
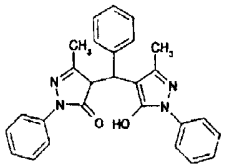
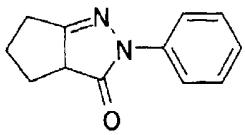
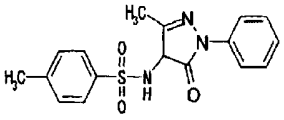
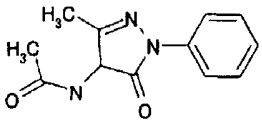
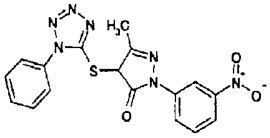
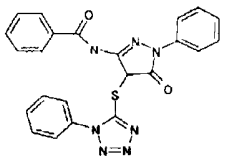
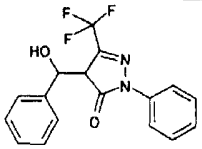
trifluoroacetic acid; buffer B: 80% acetonitrile containing 0.08% trifluoroacetic acid). 4-Hydroxy-3-nitrobenzoic acid (100μM) was used as the internal standard. The results are shown in Figure 4 with the result of aminoguanidine, pyridoxamine and olmesartan.

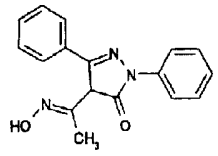
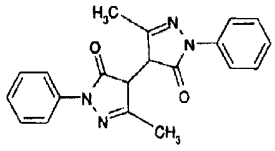
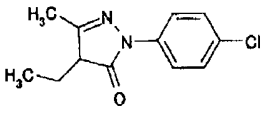
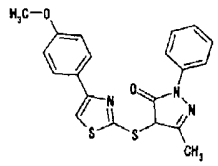
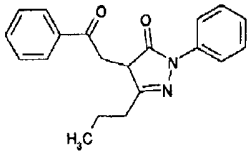
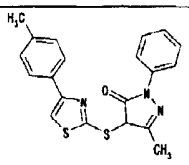
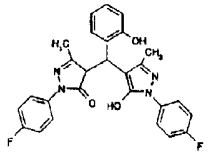
Test Example 4

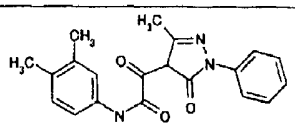
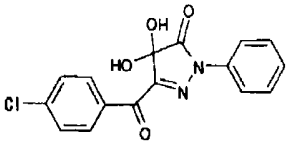
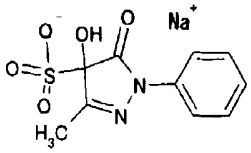
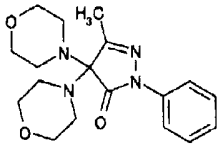
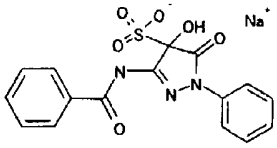
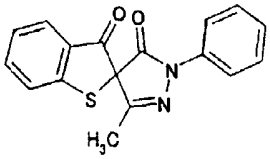
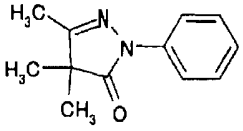
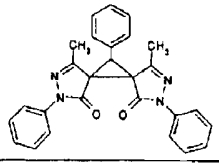
The plasma from patients was substituted to BSA and arabinose, and the pentosidine production inhibiting effect of another compounds (I) or (II) was measured in the same manner as [Test Examination 1]. The results are shown in Table 1. Therein, "-" means that the examination was not carried out.

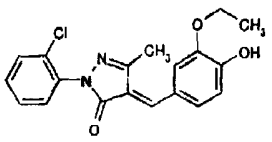
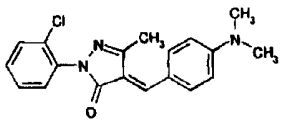
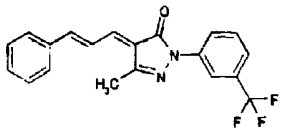
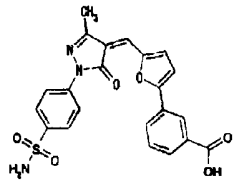
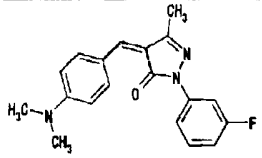
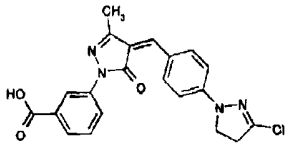
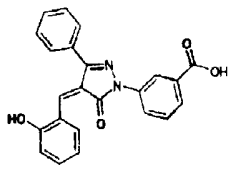
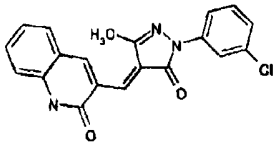
Table 1

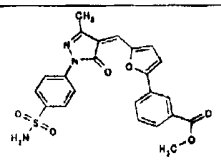
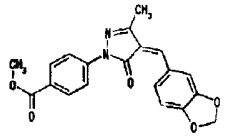
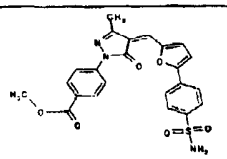
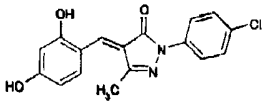
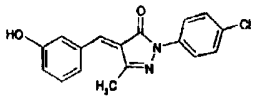
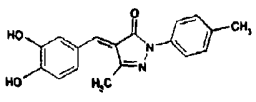
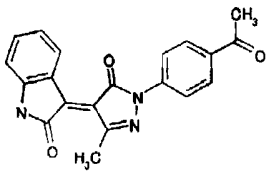
NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
1	2-(3-amino-5-oxo-1-phenyl-4,5-hydro-1H-pyrazol-4-yl)-2-oxo-N-phenyl-acetamide		-
2	2-(3-amino-5-oxo-1-phenyl-4,5-hydro-1H-pyrazol-4-yl)-2-oxo-N-thiazol-2-yl-acetamide		-
3	2-(3-amino-5-oxo-1-phenyl-4,5-hydro-1H-pyrazol-4-yl)-2-oxo-acetamide		-
4	2-(3-amino-5-oxo-1-phenyl-4,5-hydro-1H-pyrazol-4-yl)-N-(3,4-dimethyl-phenyl)-4-oxo-butylamide		-
5	2-(4-amino-phenyl)-4-(2-hydroxy-ethyl)-5-methyl-2,4-hydro-pyrazol-3-one		-
6	5-amino-2-phenyl-4-(1-phenyl-1H-tetrazol-5-yl-sulfanil)-2,4-dihydro-pyrazol-3-one		-
7	3-(3-methyl-5-oxo-1-penyl-4,5-dihydro-1H-pyrazol-4-yl)-propionic acid		64.64

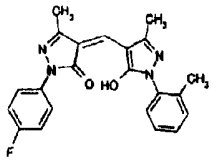
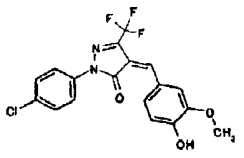
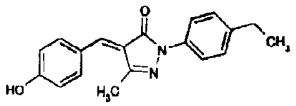
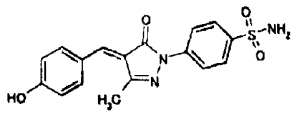
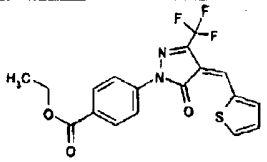
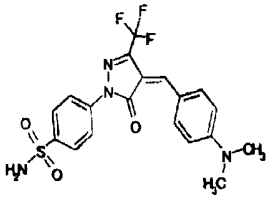
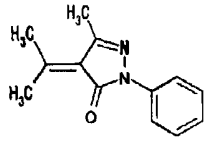
NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
8	N-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-yl)-acetamide		-
9	4-[(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-phenyl-methyl]-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one		7.52
10	2-phenyl-3a,4,5,6-tetrahydro-2H-cycropentapyrazol-3-one		-
11	4-methyl-N-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-yl)-benzenesulfonamide		-
12	N-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-yl)-acetamide		54.21
13	5-methyl-2-(3-nitro-phenyl)-4-(1-phenyl-1H-tetrazol-5-yl-sulfanil)-2,4-dihydro-pyrazol-3-one		74.66
14	N-[5-oxo-1-phenyl-4-(1-phenyl-1H-tetrazol-5-yl-sulfanil)-4,5-dihydro-1H-pyrazol-3-yl]-benzamide		61.27
15	4-(hydroxy-phenyl-methyl)-2-phenyl-5-trifluoromethyl-2,4-dihydro-pyrazol-3-one		-

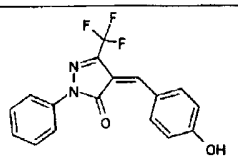
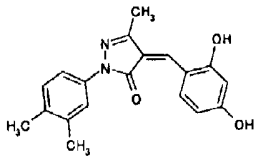
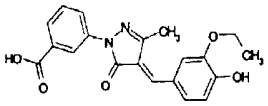
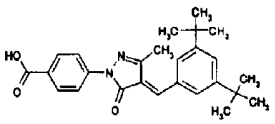
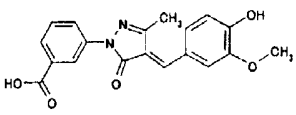
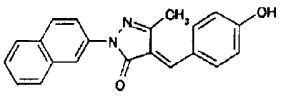
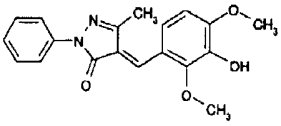
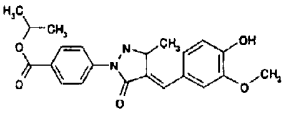
NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
16	4-(1-hydroxyimino-ethyl)-2,5-diphenyl-2,4-dihydro-pyrazol-3-one		-
17	5,5'-dimethyl-2,2'-diphenyl-2,4,2',4'-tetrahydro-[4,4']-bipyrazol-3,3'-dione		60.96
18	2-(4-chloro-phenyl)-4-ethyl-5-methyl-2,4-dihydro-pyrazol-3-one		-
19	4-[4-(4-methoxy-phenyl)-thiazole-2-yl-sulfanil]-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one		40.19
20	4-(2-oxo-2-phenyl-ethyl)-2-phenyl-5-propyl-2,4-dihydro-pyrazol-3-one		83.12
21	5-methyl-2-phenyl-4-(4-p-toluyl-thiazole-2-yl-sulfanil)-2,4-dihydro-pyrazol-3-one		42.07
22	2-(4-fluoro-phenyl)-4-[[1-(4-fluoro-phenyl)-5-hydroxy-3-methyl-1H-pyrazol-4-yl]-(2-hydroxy-phenyl)-methyl]-5-methyl-2,4-dihydro-pyrazol-3-one		2.9

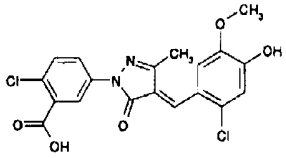
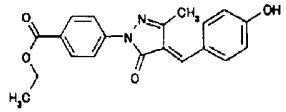
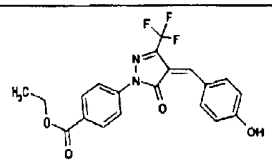
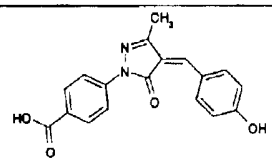
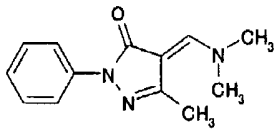
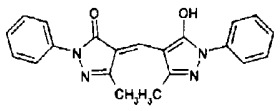
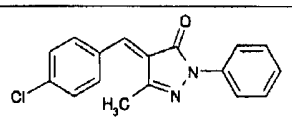
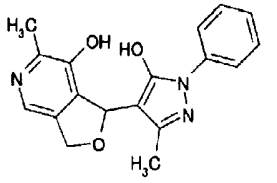
NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
23	N-(3,4-dimethyl-phenyl)-2-(3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-yl)-2-oxo-acetamide		-
24	5-(4-chloro-benzoyl)-4,4-dihydroxy-2-phenyl-2,4-dihydro-pyrazol-3-one		-
25	sodium; 4-hydroxy-3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-sulfonate		38.80
26	5-methyl-4,4-di-morpholin-4-yl-2-phenyl-2,4-dihydro-pyrazol-3-one		50.68
27	soduim 3-benzoylamino-4-hydroxy-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-sulfonate		2.83
28	3-methyl-1-phenyl-5-oxo-4-spiro-(3-oxo-2,3-dihydro-benzo[b]thiophen-2-yl)-4,5-dihydro-1H-pyrazol		22.83
29	4,4,5-trimethyl-2-phenyl-2,4-dihydro-pyrazol-3-one		-
30	4,10-dimethyl-2,8,11-triphenyl-2,3,8,9-tetraza-dispiro [4.0.4.1]undeca-3,9-diene-1,7-dione		62.43

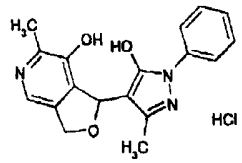
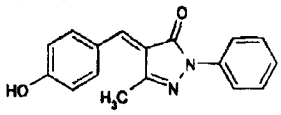
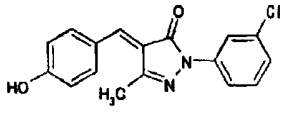
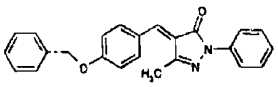
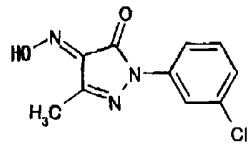
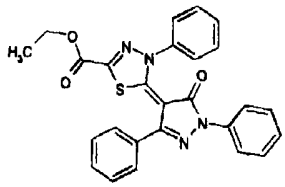
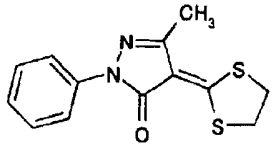
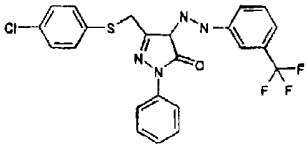
NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
31	2-(2-chloro-phenyl)-4-(3-ethoxy-4-hydroxy-benzylidene)-5-methyl-2,4-dihydro-pyrazol-3-one		15.57
32	2-(2-chloro-phenyl)-4-(4-dimethylamino-benzylidene)-5-methyl-2,4-dihydro-pyrazol-3-one		8.7
33	5-methyl-4-(3-phenyl-allylidene)-2-(3-trifluoromethyl-phenyl)-2,4-dihydro-pyrazol-3-one		80.08
34	3-{5-[3-methyl-5-oxo-1-(4-sulfamoyl-phenyl)-1,5-dihydro-pyrazol-4-ylidene-methyl]-furan-2-yl}-benzoic acid		-
35	4-(4-dimethylamino-benzylidene)-2-(3-fluoro-phenyl)-5-methyl-2,4-dihydro-pyrazol-3-one		76.31
36	3-{4-[4-(3-chloro-4,5-dihydro-pyrazol-1-yl)-benzylidene]-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl}-benzoic acid		26.37
37	3-[4-(2-hydroxy-benzylidene)-5-oxo-3-phenyl-4,5-dihydro-pyrazol-1-yl]-benzoic acid		0.02
38	3-[1-(3-chloro-phenyl)-3-methyl-5-oxo-1,5-dihydro-pyrazol-4-ylidene-methyl]-1H-quinolin-2-one		84.95

NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
39	3-{5-[3-methyl-5-oxo-1-(4-sulfamoyl-phenyl)-1,5-dihydro-pyrazol-4-ylidene-methyl]-furan-2-yl}-benzoic acid-methyl ester		16.87
40	4-(4-benzo[1,3]dioxol-5-ylmethylene-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl)-benzoic acid-methyl ester		32.37
41	4-[3-methyl-5-oxo-4-[5-(4-sulfamoyl-phenyl)-furan-2-yl-methylene]-4,5-dihydro-pyrazol-1-yl]-benzoic acid-methyl ester		37.81
42	2-(4-chloro-phenyl)-4-(2,4-dihydroxy-benzylidene)-5-methyl-2,4-dihydro-pyrazol-3-one		68.32
43	2-(4-chloro-phenyl)-4-(3-hydroxy-benzylidene)-5-methyl-2,4-dihydropyrazol-3-one		3.00
44	4-(3,4-dihydroxy-benzylidene)-5-methyl-2-p-toluyl-2,4-dihydro-pyrazol-3-one		66.19
45	3-[1-(4-acetyl-phenyl)-3-methyl-5-oxo-1,5-dihydro-pyrazol-4-ylidene]-1,3-dihydro-indol-2-one		19.22

NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
46	2-(4-fluoro-phenyl)-4-(5-hydroxy-3-methyl-1-o-toluyl-1H-pyrazol-4-yl-methylene)-5-methyl-2,4-dihydro-pyrazol-3-one		15.69
47	2-(4-chloro-phenyl)-4-(4-hydroxy-3-methoxy-benzylidene)-5-trifluoromethyl-2,4-dihydro-pyrazol-3-one		28.86
48	2-(4-ethyl-phenyl)-4-(4-hydroxy-benzylidene)-5-methyl-2,4-dihydro-pyrazol-3-one		0.02
49	4-[4-(4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzenesulfonamide		7.09
50	4-(5-oxo-4-thiophene-2-yl-methylene-3-trifluoromethyl-4,5-dihydro-pyrazol-1-yl)-benzoic acid-ethyl ester		63.17
51	4-[4-(4-dimethylamino-benzylidene)-5-oxo-3-trifluoromethyl-4,5-dihydro-pyrazol-1-yl]-benzenesulfonamide		41.68
52	4-isopropylidene-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one		-

NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
53	4-(4-hydroxy-benzylidene)-2-phenyl-5-trifluoromethyl-2,4-dihydro-pyrazol-3-one		66.76
54	4-(2,4-dihydroxy-benzylidene)-2-(3,4-dimethyl-phenyl)-5-methyl-2,4-dihydro-pyrazol-3-one		73.48
55	3-[4-(3-ethoxy-4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid		-
56	4-[4-(3,5-di-tert-butyl-4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid		-
57	3-[3-(4-hydroxy-3-methoxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid		-
58	3-[3-hydroxy-4-(4-hydroxy-3-methoxy-benzylidene)-5-oxo-pyrazolidin-1-yl]-benzoic acid		0.07
59	4-(3-hydroxy-2,4-dimethoxy-benzylidene)-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one		5.3
60	4-[4-(4-hydroxy-3-methoxy-benzylidene)-3-methyl-5-oxo-pyrazolidin-1-yl]-benzoic acid-isopropyl ester		9.02

NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
61	[2-chloro-5-[4-(2-chloro-4-hydroxy-5-methoxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid		46.25
62	4-[4-(4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid-ethyl ester		-
63	4-[4-(4-hydroxy-benzylidene)-5-oxo-3-trifluoromethyl-4,5-dihydro-pyrazol-1-yl]-benzoic acid-ethyl ester		-
64	4-[4-(4-hydroxy-benzylidene)-3-methyl-5-oxo-4,5-dihydro-pyrazol-1-yl]-benzoic acid		-
65	4-dimethylaminomethylene-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one		62.43
66	4-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl-methylene)-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one		15.17
67	4-(4-chloro-benzylidene)-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one		9.09
68	1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol		2.82

NO	Chemical name	Chemical structure	Pentosidine production rate in 5mM of medicament (%)
69	1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol (hydrochloric acid salt)		2.94
70	4-(4-hydroxy-benzylidene)-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one		0.04
71	2-(3-chloro-phenyl)-4-(4-hydroxy-benzylidene)-5-methyl-2,4-dihydropyrazol-3-one		4.63
72	4-(4-benzyloxy-benzylidene)-5-methyl-2-phenyl-2,4-dihydropyrazol-3-one		7.63
73	2-(3-chloro-phenyl)-5-methyl-2H-pyrazol-3,4-dione 4-oxym		66.42
74	5-(5-oxo-1,3-diphenyl-1,5-dihydro-pyrazol-4-ylidene)-4-phenyl-4,5-dihydro-[1,3,4]thiazole-2-carboxilic acid-ethyl este		87.24
75	4-[1,3]dithioran-2-ylidene-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one		-
76	5-(4-chloro-phenylsulfanilmethyl)-2-phenyl-4-[N'-(3-trifluoromethyl-phenyl)-hydrazino]-2,4-dihydro-pyrazol-3-on		-

Test Example 5

Examination with balloon injury model vascular endothelial thickening inhibiting effect in rat carotid balloon injury model, which is post-vasodilatory operation restenosis model.

TM-2002 was suspended in sodium carboxymethylcellulose aqueous solution (CMC) (0.5%) using mortar and prepared to the concentration of 12.5 mg/mL using measuring cylinder.

Aminoguanidine hydrochloride (Sigma) was used as positive control, suspended in CMC aqueous solution (0.5%) using mortar in the same manner, and prepare to the concentration of 11.25 mg/mL using measuring cylinder. Each preparation was formed to be administered orally coercively using disposable injection and oral sonde with suspending.

The vehicle group (n=10) was administered CMC aqueous solution (0.5%) of 4mL/kg/once, twice daily (8mL/kg/day). TM-2002 (test compound) group (n=10) was orally adiministered 50mg/kg/once of 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridin-7-ol hydrochloride, twice daily

(100mg/kg/day). The aminoguanidine group (n=10) was orally administered 45mg/kg/once of aminoguanidine hydrochloride, twice daily (90mg/kg/day).

The administration was started the day before balloon injury, and carried out 15 days (from injury day as 1 day to 14 day), morning and evening, twice daily, with more than 6 hour intervals (15 days after balloon injury, dissection was carried out; no administration on 15th day).

9 weeks aged (when balloon injury, 10 weeks aged) SD line male rats (Japan SLC) were used. When test animals were received, the health condition of each animal was checked by the naked eye and healthy animals were caged. After 6 days from coming as preparative breeding, good health individuals were subjected to the examination. 1 group involves 10 rats, and the rats were divided into 3 groups, i.e. vehicle, test compound, aminoguanidine group, depending on their body weight before administration.

The neck and femoral region of rats were incised under anesthesia by pentobarbital sodium (40mg/kg,i.p.), left carotid and arteria femoralis were exposed. The arteria femoralis was incised and inserted balloon catheter (2Fr, fogaty catheter; Baxter), and then the tip was leaded to

internal-external carotid branch of left carotid. The appearance of balloon catheter in the carotid was confirmed by the naked eye, then the balloon puffed by injection of air (0.3mL). While puffing the balloon, the balloon catheter was drawn forth to aortic arches. This operation was continued for a third and injured intima of the vessel. After drown of balloon catheter, arteria femoralis was tied up. The incision site was satured and the wound was mundified using isodine solution. The right carotid without injury was used as control of each individual.

While testing, the alive and the condition of wound were observed everyday. The body weight was measured once a day from the day before injury to 14 days after balloon injury. The dose of each individual was calculated by their body weight.

The blood samples were obtained from abdomen vena cava under anesthesia by ether on 15 days after balloon injury. After blood collection, left carotid was removed and divided into 3 sections. The 5mm slice from each section was obtained, right carotid was removed and picked out about 5mm slice from its center area, and each of them were fixed with 10% neutral buffered formalin. The fixed samples were prepared to paraffin block, followed by thin

slice and sustained with HE. The area of intravascular lumen, the area surrounded by internal elastic lamina and the area surrounded by external elastic lamina were calculated using image analyzer (VM-30, Olympus photology).

For each section (3 regions), the area of neointimal, media and neointimal/media rate of blood vessel were calculated from the measured area. The intimal thickening was estimated using the average of 3 regions from each individual. The results are shown in Figure 5 (A, B and C). It is understood from these results that TM-2002 group represents intimal thickening inhibiting effect equal to aminoguanidine group as positive control.

Test Example 6

Reactivity with vitamin B6

Vitamin B6 (pyridoxal-5'-phosphate) (50μM) and TM-2002 (0.5μM) were incubated in phosphate buffered saline (PBS), at 37°C. The concentration of residual pyridoxal-5'-phosphate was determined with HPLC to measure the kinetics between 0 to 20 hours in the following manner: after a designed period of time, the reaction mixture (10μL) was injected onto HPLC, separation was made with

Purecil™ C18 column (4.6 x 250 mm, 5 μm: Waters) and detection was effected using a fluorescence detector (RF-10A; Shimazu Seisakusho; excitation wavelength, 300 nm and fluorescence wavelength, 400nm). In the mobile phase, the flow rate was 0.6 ml/L and the concentration of buffer B was varied from 0% to 3% in 25 minutes (buffer A: 0.10% trifluoroacetic acid; buffer B: 80% acetonitrile containing 0.08% trifluoroacetic acid). Aminoguanidine, which is known for its vitamin B6 capturing effect was used as control.

After 20 hours, while pyridoxal-5'-phosphate residual rate of TM-2002 group was 97%, pyridoxal-5'-phosphate residual rate of aminoguanidine as control was only 0.2%. Accordingly, it is shown that TM-2002 does not react with vitamin B6.

In addition, other compounds (I) and (II) showed the same result as TM-2002.

Test Example 7

Vitamin B6 deficiency inhibiting effect

TM-2002 was administered to normal rat (WKY rat: Japan SLC) to examine the presence of vitamin B6

deficiency. Aminoguanidine, which is known for its vitamin B6 capturing effect, was used as control. Each group involves 10 rats. 13mg/kg/rat/once of TM-2002 or aminoguanidine suspended in carboxymethyl cellulose (0.5%) were respectively administered coercively using sonde twice daily. Administration term was 20 weeks. The conventional diet (CRF1: Oriental yeast) was used.

After 20 weeks, the appearance of WKY rat was observed. In TM-2002 group, condition caused by vitamin B6 deficiency such as angular cheilitis, mouth inflammation, glossitis, chelitis, acute and chronic eczema, contact dermatitis, peripheral neuritis, anemia, hypolymphemia and nerve disorder were not observed. On the other hand, in aminoguanidine group, skin inflammation, epilepsy and convulsion caused by cerebral disorder were observed.

In addition, other compounds (I) and (II) showed the same result as TM-2002.

Test Example 8

Renal protecting effect in SHR/NDmcr-cp rat and Wistar Kyoto rat

Renal protecting effect was observed using SHR/NDmcr-cp (Disease Model Cooperative Research Association) rat, which had been known for their high blood pressure, hyperglycemia, hyperlipemia, obesity, hyperinsulinemia and renal function disorder while aging.

SHR/NDmcr-cp rat and Wistar Kyoto rat (Disease Model Cooperative Research Association) of 9 weeks aged were conformed for 3 weeks and measured for their body weight and blood collection before administration. One group contains five rats (body weight 440g±32g). After division, excipient (negative control), positive control compound and test compound were administered for 20 weeks. Carboxymethylcellulose (carboxymethyl cellulose Na/ Wako) solution as excipient and olmesartane, which is one of the angiotensin II receptor antagonist having renal protective effect, as positive control compound were used to examine the effect of TM-2002 (test compound). The dosage of olmesartane was 3mg per 1kg of body weight and that of TM-2002 was 50mg per 1kg of body weight. Olmesartane was prepared by suspending or solving the designed amount in 1.0ml of 0.5% carboxymethylcellulose solution or purified water, and TM-2002 was prepared by mixing the designed amount in 30g of diet (for one day). Since the increase of body weight of test animals was

remarkable, the amount of positive control compound and test compound was modified depending on the result of body weight measures (every week). The dosage route was oral administration of 1 ml of 0.5% carboxymethylcellulose solution of excipients in case of negative control, and of designed amount of olmesartane in 0.5% carboxymethylcellulose solution in positive control respectively, using sonde. The dosage route of test compound group is diet with mixed designed amount of TM-2002. The amount of diet is 30 g daily for every group (excipient group as negative control, positive control compound group and test compound group). During the administration period, body weight was measured every week, and blood collection, urine collection and blood pressure measuring were done every 2 weeks before 4 weeks aged and every 4 weeks after 5 weeks aged. Blood collection was carried out after warming to 38°C by warming plate from caudal vein for 800μl (heparin treatment: the amount of heparin was determined by ratio 15μl to 1ml of blood). The urinary sample was collected using urine metabolizing cage (Japan Clea). The volume of daily urine was measured when urine was collected. The blood pressure was determined with the tail-cuff method blood pressure measure (Softron).

The blood samples were used to determine glucose level, the concentration of triglyceride, total cholesterol, hemoglobin A1c and insulin. The urine samples were used to determine the amount of urinary protein, creatinine and urinary nitrogen. Such examinations were carried out by Japan SRL.

The results of such examination for urine protective effect in 33 weeks aged provide that the negative control represents high blood pressure, high urinary protein and renal function disorder. In the positive control group, since olmesartane is a hypotensor, blood pressure lowering, urinary protein suppressing and renal function improving is shown. On the other hand, in the test compound group, urinary protein is suppressed remarkably without blood pressure lowering comparing to the negative control, and inhibiting effect is stronger than that of the positive control, thus, excellent renal protective effect is shown. The present invention is expected for the treatment of renal diseases without hyper tension, the treatment of renal disease combined with hypotensor without renal protective effect, and synergetic effect together with hypotensor with renal protective affect, thus is useful for medicament for renal diseases. The results are represented in Figure 7 and 8.

Test Example 9

Renal protective effect on Thy-1 nephritis model rat

1.2 mg/kg of OX-7, anti Thy-1 antibody, was administered to wister rat (male, body weight 150 g, 6 weeks aged) in caudal vein to prepare typical glomerulonephritis model with mesangial nephritis. After the administration of anti Thy-1 antibody, test compound (TM-2002, 50 mg/kg body weight, twice a day) was suspended in 0.5% carboxymethylcellulose, was coercively administered 5 days continuously using sonde, on the sixth day sampled kidney was obtained, and analysed pathologically (counting the number of glomerular cells). In particular, cells were stained by PAS according to conventional method, the stained image was captured by 3CCD camera (Olympus), and then analyzed using softwares, Image Graver PCI (FUJI shashin film) and Mac Aspect (Mitani kabushiki kaisha). Also, biochemical analysis of blood and urine were carried out (contract clinical analysis organization: SRL). As a result, urinary protein and BUN value were improved in TM-2002 group, and the number of glomerular cells, which increased along with disorder, was significantly suppressed ($p<0.0001$) and the renal protective effect was indicated. The results are indicated in Figure 8 to 10.

Test Example 10

Renal protective effect on ischemia-reperfusion renal failure model rat

This condition model is a typical acute renal failure model. To prepare the model, operated on wister rats (male, body weight 150 g, 6 weeks aged) to remove right kidney, and on the following day, the renal artery of the remaining left kidney was applied with a ligature with clip under general anesthesia. After the clipping, the rats were on warming plate not to reduce the body temperature and were observed for 45 minutes (ischemia), and then the clip was removed to allow re-perfusion. After preparing the ischemia-reperfusion model, test compound (TM-2002, 50 mg/kg body weight, twice daily) was suspended in 0.5% carboxymethylcellulose. The suspension was coercively administered 2 days continuously using sonde, on the third day kidney was obtained, and analysed pathologically (renal tubular stromal disorder score). In particular, the kidney was stained by PAS according to conventional method and the renal tubular stromal disorder was estimated for the presence or absence of renal tubular necrosis, renal tubular hypertrophy, renal tubular atrophia, renal tubular basal lamina thickening and cast in the sustained image of the kidney. As well as biochemical

analysis of blood was carried out (contract clinical analysis organization: SRL). As a result, urinary protein and BUN value were improved in TM-2002 group, and the number of glomerular cells, which increases along with disorder, was significantly suppressed ($p<0.0001$) and the renal protective effect was indicated. The results are indicated in Figures 11 to 13.

Test Example 11

Cerebroprotective action in middle cerebral artery ischemia-reperfusion model

CD(SD)IGS male rats (Japan Charles river Kabushiki kaisha, Hino farm, room No.22, specific product) (8 rats per one group) having body weight 270 to 350 g were anesthetized by 2% isoflurane (mixed bas comprising of 70% N_2O (laughter gas) and 30% O_2) to immobilize, and then the rats were put on warming plate to keep the rectal and brain temperature 37 to 38 °C. After that, to observe the stability in the examination, canula which was made by polyethylene (PE-50, Becton Dickinson) was inserted and left in caudal artery of said animals, and allowing blood drawing and blood pressure determining to monitor the biochemical parameters such as blood sugar level, hematocrit, CO_2 concentration, oxygen partial pressure, pH,

blood pressure, etc. In addition, cerebral blood flow in cortex was determined by laser doppler fluorometry (Neuroscience.inc: OMEGA FLOW (FLO-C1)), putting the detection site directly on cranium on the point left 4mm from bregma. Left neck of such animals was incised, and from internal and external carotid fork of common carotid artery to upstream of internal carotid, nylon surgical thread (length 16 mm, diameter 0.2 to 0.3 mm, with silicon coating on its tip 3 mm) was passed and left, middle cerebral artery was obstructed for 2 hours. After that, the thread was removed to release the middle cerebral artery, and blood was reperfused for 21 hours. To each animal, 3.0 mg/kg of edaravone (control) and 5.58 mg/kg of TM-2002 were respectively administered twice by cannula which is left in caudal artery 5 minutes and 5 hours after middle cerebral artery occlusion. After said operation, the brain was removed from said animals, and after preparing 7 brain slices with 2 mm thickness, to TTC stain (0.8 g of 2,3,5-triphenyltetrazolium chloride (Sigma) dissolved in 40 ml of saline) were soaked at 37°C for 15 minutes to stain the area of infarction, and fixed by 10% neutral formalin liquid to prepare specimens. Such specimens were created the image by CCD camera respectively, and analysed according to the method of Swanson et al. (J Cereb Blood Flow Metab 10:290-293; 1994). As a result, cerebral infarction nest

was significantly reduced by control agent edaravone and test agent TM-2002 comparing to excipient single administration. The results are indicated in Figure 13. In addition, nervous condition was estimated in the operated rat on horizontal table by grading system according to the method of Bederson et al. (Stroke 17:472-476, 1990) by 4 grades: Grade 0, when pushed from the side they walk normally without palsy; Grade 1, when pushed from the side they resist and walk straight to the front with forelimb flexion; Grade 2, when pushed from the side they do not resist and walk straight to the front; Grade 3, when pushed from the side they do not resist and cannot walk straight (spin or fall). Furthermore, function recovering was estimated before and after the operation by rotor rod test that estimates how much they can walk on rotating rotor. As a result, improvement of the nervous condition and recovering of the functions were remarkably shown in edaravone (control drug) and TM-2002 (test compound) comparing to excipient single administration. The results are indicated in Table 2.

Table 2

Test Name	Diluent single administration		Edaravone		TM-3001	
	Ave	SD	Ave	SD	Ave	SD
Nervous condition estimation test*						
after occlusion 10 min	2.9	0.3	2.8	0.4	2.8	0.7
after occlusion 2 hrs	2.9	0.3	2.7	0.5	2.6	1.1
after reperfusion 10 min	2.4	1.1	2.2	1.0	1.5	1.6
after reperfusion 3 hrs	2.3	1.1	2.1	1.0	1.3	1.5
after reperfusion 4 hrs	2.3	1.1	2.0	1.2	2.0	1.4
after reperfusion 22 hrs	1.6	1.4	1.5	1.2	2.0	1.4
rotor rod test**						
before occlusion	202.7	123.0	197.0	123.4	183.4	138.0
reperfusion	67.1	70.5	153.1	132.5	160.6	128.6
ratio to before occlusion (%)	81.4	131.8	160.4	190.0	78.1	30.6

*: Numerics in nervous condition estimation test is calculated by grading system of Bederson et al.
**: Numerics in rotor rod test is reading value of count from rotor rod machine.
Abbreviations: min: minute(s), hrs: hours, Ave: average, and SD: standard deviation

Test Example 12

Solubility test

TM-2002 was added in purified water to be 10mg per 1ml and added hydrochloride to adjust to pH2.0 and dissolved. Then, this solution was adjusted to ph 7.0, 8.0

and 2,0 (unadjust) by 1N sodium hydrate to prepare 3 solutions. These solutions were put on warming, dark cold place and after 24 hours, conditions of the solutions were observed. The pH2.0 solution kept clear solution; in the pH7.0 and pH8.0 solutions, however, precipitates were observed. In particular, the change of color was shown in pH7.0 and pH8.0 solutions. As a result, stable TM-2002 injectable formulation in its solubility may be prepared by keeping acidic conditions using inorganic acid such as hydrochloride, sulfuric acid, or various organic acids, or by using TM-2002 hydrochloride or sulfate as meterial which is made from said acids.

Test Example 13

Examination of stabilizing agent

Several kinds of stabilizing agent were usually used to stabilize the medicament and the effective manner is the addition of an antioxidant. Particularly, the material having lower oxidation-reduction potential is effective to stabilize highly oxidized compound. Accordingly, for sodium acid sulfite ($NaHSO_3$) and L-cystein ($C_3H_7O_2S \cdot HCl$), which are usually used in injection, the stability was examined by adding such materials to TM-2002 hydrochloride aqueous solution.

The solving state of TM-2002 hydrochloride solution added with sodium acid sulfite was examined in the following manner; 1ml of purified water, sodium acid sulfite (0.5mg) and sodium acid sulfite (1mg) solution was prepared respectively; to these solutions, there was added 63mg of TM-2002 hydrochloride, stirred and dissolved; the solutions were allowed to warm to room temperature to observe the solving state. As a result, crystal precipitates were deposited according to time in the solutions added with 0.5mg and 1mg of sodium acid sulfite and after 24 hours, remarkable precipitates were observed. On the contrary, pH was 2 to 2.5 in the solution solved in only purified water, after 24 hours, no change was observed in its solving state and significant suppression of TM-2002 degradation was observed. Accordingly, the stability of TM-2002 itself was increased by using hydrochloride.

Instead of sodium acid sulfite, sodium sulfite (Na_2SO_3), sodium pyrosulfite ($Na_2S_2O_5$) were used and carrying out the experiment in the same manner as above, crystal precipitate was deposited in all of the solutions added with these stabilizing agents.

The solving state of TM-2002 hydrochloride solution added L-cystein hydrochloride was examined in the

following manner; 1mg and 2mg of L-cystein hydrochloride were dissolved in 1ml of purified water respectively; pH was adjusted to 6.5; to these solutions, there was added 63mg of TM-2002 hydrochloride, stirred and dissolved; the solutions were allowed to warm to room temperature to observe the solving state. As a result, no precipitates and deposits was observed in each solution, and after 24 hours, no change was observed in solving state. In addition, the degradability was observed by thin layer chromatography (TLC/ Silica-gel 60F254 (Merck Japan) / developing solvent: chloroform:methanol=9:1/ detection:UV=254nm). The compound was degraded slowly in left over 24 hours at room temperature, however, it was significantly stabilized by addition of L-cystein. Under preservation at -20°C, this solution was stable after one week. Accordingly, the present invention provides lyophilized formulation comprising L-cystein by treating TM-2002 hydrochloride solution at low temperature and lyophilizing.

Preparation of TM-2002 injectable lyophilized formulation

L-cystein hydrochloride (160mg) was added to 80ml of purified water, stirred and dissolved. The solution was adjusted to pH6.7 with 1N NaOH aqueous solution. Then 1g of TM-2002 hydrochloride was added, stirred and dissolved, and the solution diluted with purified water to 100ml with

measuring flask. Such solution was divided into vial containers of 50ml by 6.3 ml each, freezed immediately with dry ice, and then stored at -80°C to freeze completely. This was subjected to lyophilized for 3 days using lyophilizer. After lyophilizing, covering by rubber cap and sealing by aluminum cap using fastener, TM-2002 injectable lyophilized formulation was prepared.

The solubility and stability of TM-2002 injectable lyophilized formulation

To TM-2002 injectable lyophilized formulation prepared above, there was added 20ml of purified water per 1 vial. The lyophilized material dissolved immediately and provided pale yellow, clear solution. 1.5ml of saline was added to this solution (1ml), and while keeping at room temperature, the solubility and stability was observed after 3 hours, 6 hours and 10 hours after solving. TLC (Kiezel gel 60F254/ developing solvent: $CHCl_3$:MeOH=9:1/ detection:UV=254nm) was carried out to examine the change of ingredient. As a result, there was no precipitate, crystals or insoluble materials after 10 hours from dissolution and noticeable change of color was not observed. For the stability of ingredients, while decomposition products were observed in the control solution of TM-2002 hydrochloride after 10 hours, no

decomposition product was observed in the present infectable formulation. The results of TLC are represented in Figure 16. When the solution was further diluted with saline, the stability was the same. In addition, for said injectable lyophilized formulation which was left at room temperature for 30 days, the solubility and stability were observed in the same manner and there was no difference from that soon after preparing. The formulation is suitable for injectable lyophilized formulation which is prepared before use, and stable and usable.

Test Example 14

Examination of in vivo endoplasmic reticula stress (ER stress) alleviation effect by TM-2002

Endoplasmic reticula stress alleviation effect by TM-2002 was examined using SHR/NDmcr-cp (Disease Model Cooperative Research Association) rats, which have been known for their high blood pressure, hyperglycemia, hyperlipemia, obesity and hyperinsulinemia and renal function disorder while aging, and immunostained utilizing anti-ORP150 antibody. The stained section samples which were used for staining were prepared in the following manner: SHR/NDmcr-cp rats which were administered with the desired amount of test compound TM-2002

(dose:100mg/1kg body weight) by diet (30g day), the rats which were administered no drug and SHR (hypertension) rats and Wistar Kyoto rats (control); kidney tissues of each rat were obtained after the end of drug administration period (33 weeks aged); all of tissues were fixed by Carnoy and embedded in paraffin. The staining of each sample was carried out by Catalyzed Sifnal Amplification (CSA) System (DAKO) according to their protocol. Briefly, deparaffinization was carried out by Histo-Clear™ (pational diagnotics) for 5 minutes 3 times, by 100% ethanol for 3 minutes 3 times and fitted in distilled water for 5 minutes; the samples were put into 10mM sodium citrate aqueous solution (pH. 6.0) and heated with microwave for 5 minutes with boiling to activate the antibody; the samples were cooled to room temperature, and washed by TBS-T (0.05M Tris-HCl pH7.6, 0.3M NaCl, 0.1% Tween™ 20) for 4 minutes 3 times; the samples were rinsed in 3% hydrogen peroxide solution (DACO) for 3 minutes to block endogenous peroxydase; the samples were treated with PROTEIN BLOCK (DACO) to inhibit non-specific reaction of the antibody, reacted for 15 minutes with anti-ORP150 antibody (primary antibody) which was diluted with 1.5% goat serum to 400 folds, washed by TBS-T for 4 minutes 3 times; reacted with biotin-labeled goat anti- leporine antibody (200 folds; secondary antibody) for 15 minutes and washed by

TBS-T for 4 minutes 3 times: reacted with Streptavidin-Biotin Complex (DACO) for 15 minutes and washed by TBS-T for 4 minutes 3 times; reacted with Amplification Reagent (DACO) for 15 minutes and washed with TBS-T for 4 minutes 3 times; reacted with Streptavidin-peroxidase (DACO) for 15 minutes and washed by TBS-T for 4 minutes 3 times; DAB coloring was carried out by Substrate-Chromogen Solution, washed by distilled water, anhydration was carried out by 100% ethanol for 3 minutes 3 times and by Histo-Clear™ (pational diagnotics) for 5 minutes 3 times, and embeded to complete the sample. The samples were observed by microscopic visualization with optical microscope (Olympus) and analyzed the result. As a result, there was no ORP150 positive staining site in Wistar Kyoto rats (control) and SHR rats (hypertension model) and there was positive staining site in SHR/NDmcr-cp rats (Type II diabetes hypertension model). The enhancing of ER stress was observed in SHR/NDmcr-cp rats. There was a decrease of positive staining site and suppressing of ER stress in SHR/NDmcr-cp rats administered TM-2002 (cf. Figure 17).

Test Example 15

Examination of drug efficiency on the variation in expression of ER stress inducing molecules of TM-2002

Rat pancreatic β cell line (RIN-5F) was seeded in 6 well culture plates to 1.0×10^5 cells/well, and after 24 hours, TM-2002 in DMSO (200mM, 50mM) were added to be final concentration 200μM, 50μM. After another 1 hour, 0.2μl of tunicamycin (Sigma) in methanol (1 mg/ml) was added. After culturing for 8 hours, the cells were washed with 2ml of PBS(+) twice, and the cells were dissolved in 100 μl of lysate (50mM Tris-HCl (pH7.5), 150mM NaCl, 100mM NaF, 100mM sodium phosphate (pH7.4), 2mM Na_3VO_4, 0.1% protease inhibitor cocktail (Sigma), 1% Triton™ X-100). To remove the insoluble fraction of this lysate, it was centrifuged at 12,000 x g for 10 minutes and obtained the supernatant to give cell extract. The extract was quantified for its protein concentration using DC protein assay kit (Bio Rad), and each sample (1μg) was electrophoresed by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% skimmed milk/0.1% Tween™20-TBS for 1 hour at room temperature, and reacted with anti-ORP150 antibody in 5% skimmed milk/0.1% Tween™20-TBS (2000 folds: specific antibody), anti-GRP78 antibody (Santa Cruz) in 5% skimmed milk/0.1% Tween™20-TBS (100 folds) and anti-actin antibody (Sigma) in 5% skimmed milk/0.1% Tween™20-TBS (200 folds) for 2 hours. Washed with 0.1% Tween™20-TBS for 10 minutes 3 times, and for the detection of ORP150 and actin, HRP-labeled anti-leporine

antibody (BIO-Rad; secondary antibody) in 5% skimmed milk/0.1% Tween™20-TBS (2000 folds), for the detection of GRP78 alkalisphosphatase-labeled anti-goat antibody in 5% skimmed milk/0.1% Tween™20-TBS (5000 folds) was used resectively, and reacted at room temperature for 1 hour. After washing with 0.1% Tween™20-TBS for 10 minutes 3 times, the detection was carried out using ECL western blotting detection reagent (Amersham Bioscience). The detected signal was analyzed by Lane analyzer (ATTO) and the signal strength was calculated. As a result, in tunicamycin added samples, ORP150 and GRP78 expression were enhanced respectively 1.88 folds and 1.46 folds comparing to that of the control sample, and this indicates that ER stress was caused. In the TM-2002 and tunicamycin co-added samples, hyperexpression of ORP150 and GRP78 were suppressed by tunicamycin, and it is indicated that ER stress caused by tunicamycin is reduced (cf. Figure 18 and 19).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a chart showing the inhibiting effect of TM-2002 on pentosidine production.

Figure 2 is a chart showing the inhibiting effect of TM-2002 on hydroxylation of phenylalanine by hydroxy radical (o-tyrosine production inhibiting is used as an index).

Figure 3 is a chart showing the inhibiting effect of TM-2002 on hydroxylation of phenylalanine by hydroxy radical (m-tyrosine production inhibiting is used as an index).

Figure 4 is a chart showing the inhibiting effect of TM-2002 on nitration of tyrosine by peroxynitrite.

Figure 5 is a photograph showing the vascular endothelial thickening inhibiting effect in rat carotid balloon injury model test (A is control; B is 50mg/kg of TM-2002 administration; C is 45mg/kg of aminoguanidine administration).

Figure 6 is a chart showing the absence of blood pressure-lowering effect in TM-2002.

Figure 7 is a chart showing the urinary protein inhibiting effect of TM-2002.

Figure 8 is a chart showing the BUN reducing effect of TM-2002 in Thy-1 nephritis model.

Figure 9 is a chart showing the urinary protein reducing effect of TM-2002 in Thy-1 nephritis model.

Figure 10 is a chart showing the number of glomerular cells reducing effect of TM-2002 in Thy-1 nephritis model.

Figure 11 is a chart showing the BUN reducing effect of TM-2002 in ischemia- reperfusion model.

Figure 12 is a chart showing the urinary protein reducing effect of TM-2002 in ischemia- reperfusion model.

Figure 13 is a chart showing the stromal disorder score of TM-2002 in ischemia-reperfusion model.

Figure 14 is a chart showing the reduction of the cerebral infarction nest with TM-2002.

Figure 15 is a chart showing the reduction of the cerebral infarction nest with TM-2002.

Figure 16 is a chart showing the stability of the solution of TM-2002 injectable lyophilized formulation.

Figure 17 is a chart showing the reduction of positive staining site of SHR/NDmcr-cp with TM-2002.

Figure 18 is a chart showing the OPR150 hyperexpression inhibiting effect of TM-2002 with tunicamycin.

Figure 19 is a chart showing the GRP78 hyperexpression inhibiting effect of TM-2002 with tunicamycin.

CLAIMS:

1. A compound selected from a group consisting of:
4-(3-hydroxy-5-hydroxymethyl-2-methylpyridin-4-yl-methylene)-1-phenyl-2-pyrazolin-5-one; and
1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro[3,4-c]pyridin-7-ol,
in a free or salt form thereof.

2. A composition for inhibition of the formation of protein modification products selected from the group consisting of advanced glycation end products (AGEs), advanced lipoxidation end products (ALEs) and combinations thereof, the composition comprising as the active ingredient the compound of claim 1 with a vitamin B6 molecule at the 4-position of the pyrazole ring, such that the compound is unable to bind vitamin B6 molecules, together with at least one excipient, carrier or diluent.

3. The composition of claim 2, wherein the active ingredient inhibits the formation of AGEs.

4. The composition of claim 3, wherein the active ingredient inhibits the formation of pentosidin.

5. Use of the composition of claim 2, 3 or 4, as a renal tissue protecting agent.

6. Use of the composition of claim 2, 3 or 4, as a peritoneal dialysate.

7. Use of the composition of claim 2, 3 or 4, as a hemodialysis fluid.

8. An *in vitro* method for reducing an amount of carbonyl compounds in a liquid sample, the method comprising a step of:

contacting *in vitro* the composition of claim 2, 3 or 4 with the liquid sample.

9. Use of a compound of claim 1 modified by the addition of a vitamin B6 molecule at the 4-position of the pyrazole ring, such that the compound is unable to bind vitamin B6 molecules, in the manufacture of a medicament for inhibition of the formation of protein modification products selected from the group consisting of advanced glycation end products (AGEs), advanced lipoxidation end products (ALEs) and combinations thereof.

Application number / numéro de demande: 2547887

Figures: 5, 15, 16, 17

Pages:

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au 10ème étage)

Figure 1

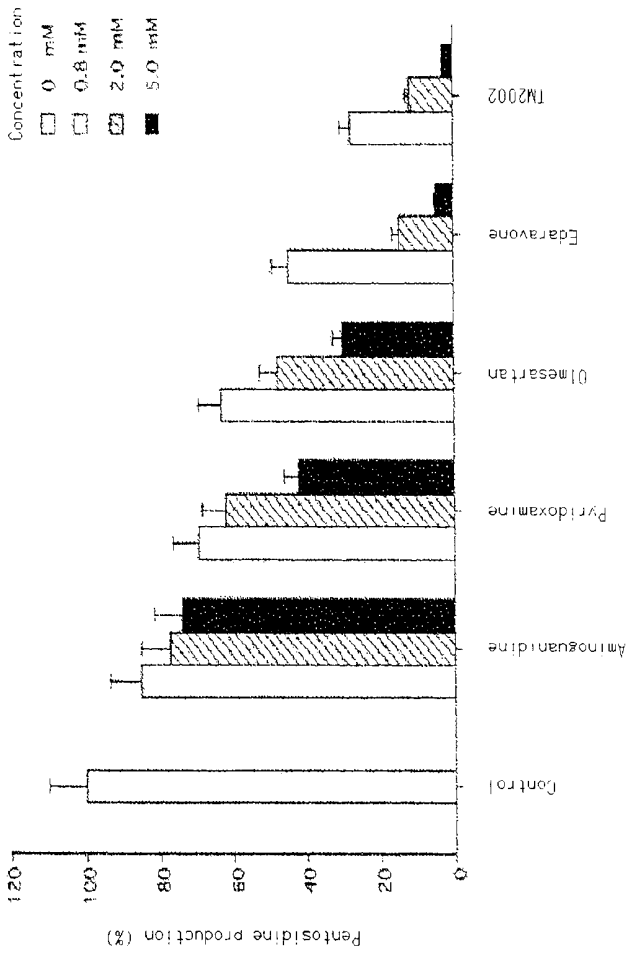
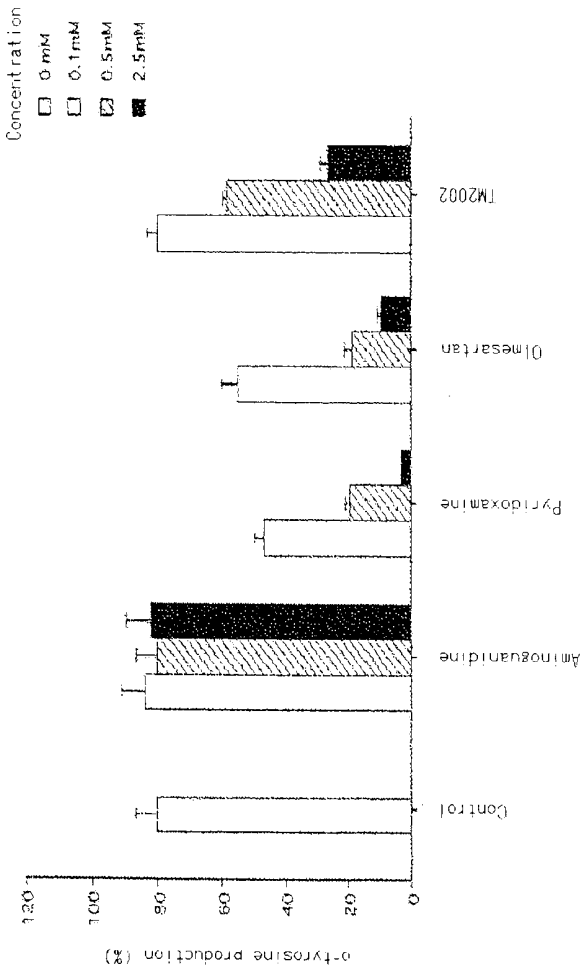

Concentration
0 mM
0.8 mM
2.0 mM
5.0 mM
Pentosidine production (%)
120
100
80
60
40
20
0
Control
Aminoguanidine
Pyridoxamine
Olmesartan
Edaravone
TM2002

Figure 2


Concentration
0 mM
0.1mM
0.5mM
2.5mM
α-tyrosine production (%)
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100
80
60
40
20
0
Control
Aminoguanidine
Pyridoxamine
Olmesartan
TM2002

Figure 3

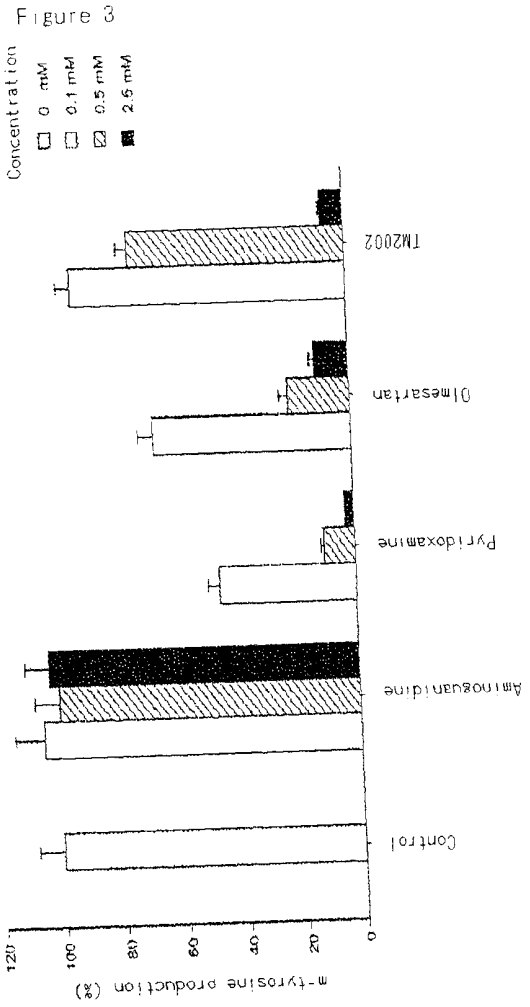

Concentration
0 mM
0.1 mM
0.5 mM
2.5 mM
m-tyrosine production (%)
120
100
80
60
40
20
0
Control
Aminoguanidine
Pyridoxamine
Olmesartan
TM2002

Figure 4

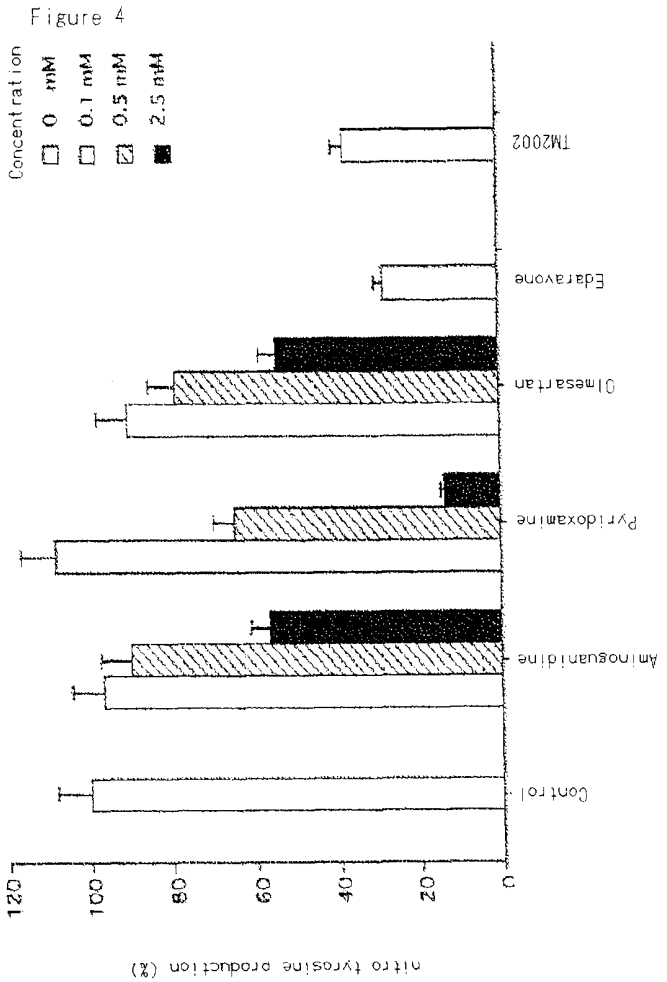

Concentration
0 mM
0.1 mM
0.5 mM
2.5 mM
TM2002
Edaravone
Olmesartan
Pyridoxamine
Aminoguanidine
Control
120
100
80
60
40
20
0
nitro tyrosine production (%)

Figure 6

Blood pressure-lowering effect by drug administration

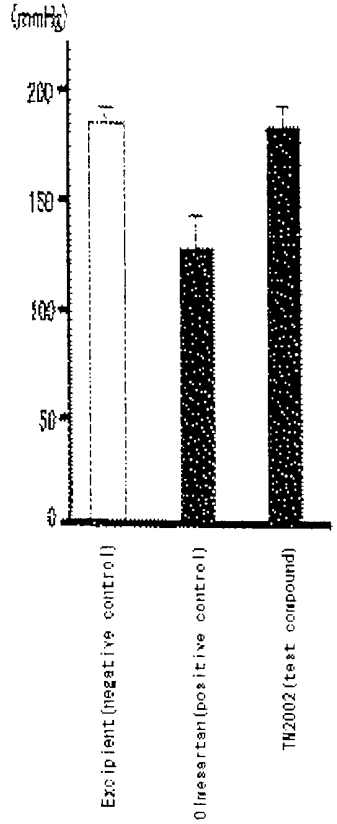


Figure 7

Urinary protein inhibiting effect by drug administration

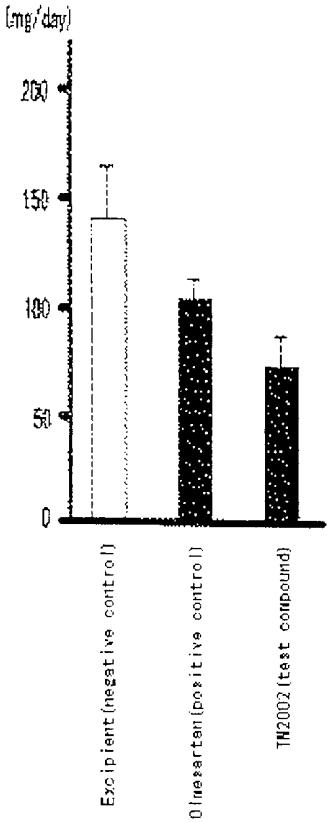


Figure 8

Thy-1 Nephritis model

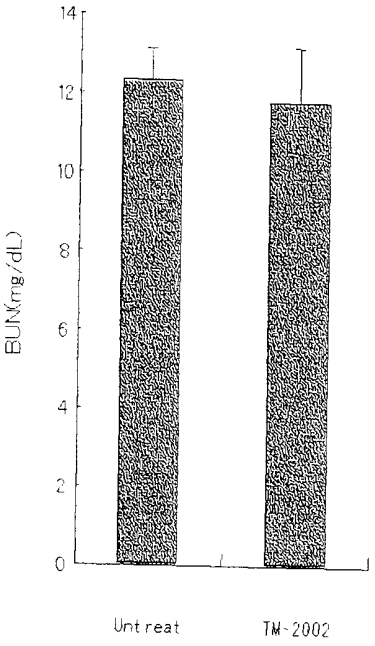


Figure 9

Thy-1 Nephritis model

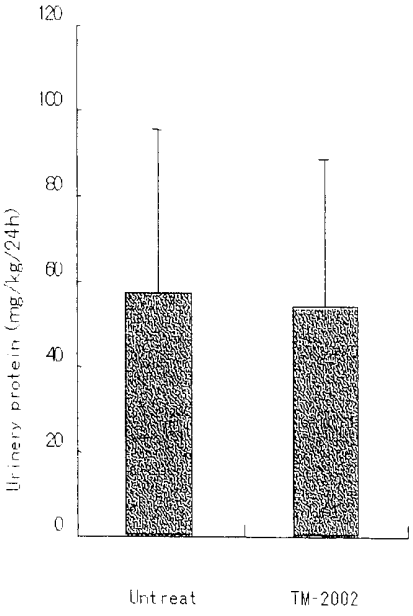


Figure 10

Thy-1 Nephritis model

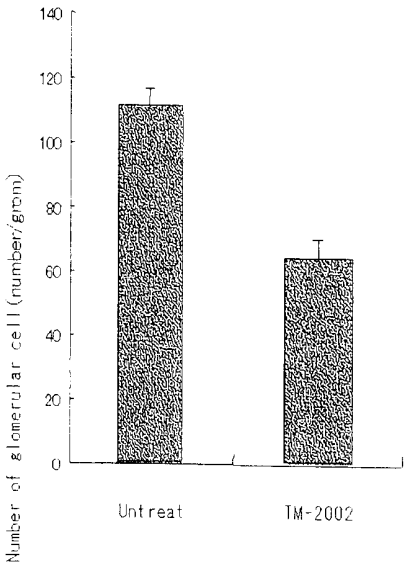


Figure 11

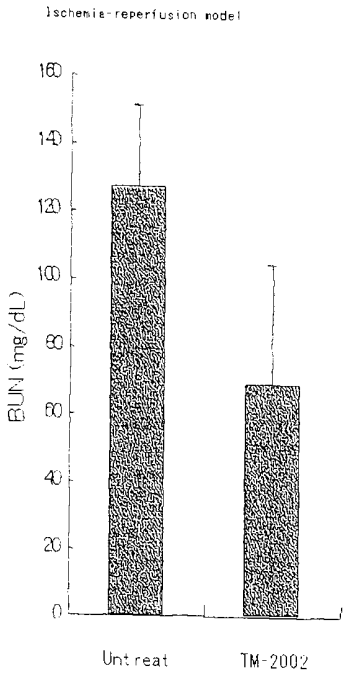

Ischemia-reperfusion model
BUN (mg/dL)
160
140
120
100
80
60
40
20
0
Untreat
TM-2002

Figure 12

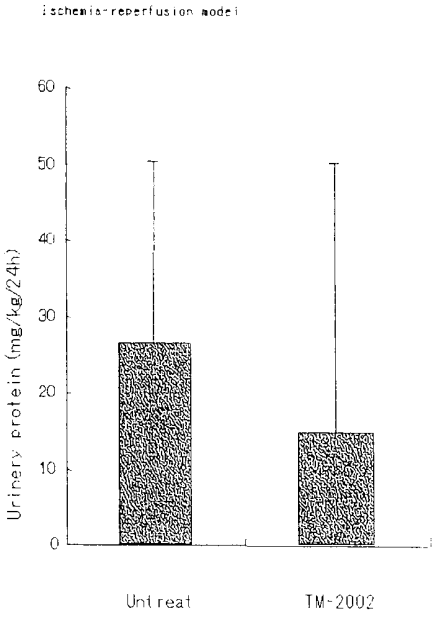

Ischemia-reperfusion model
Urinary protein (mg/kg/24h)
60
50
40
30
20
10
0
Untreat
TM-2002

Figure 13

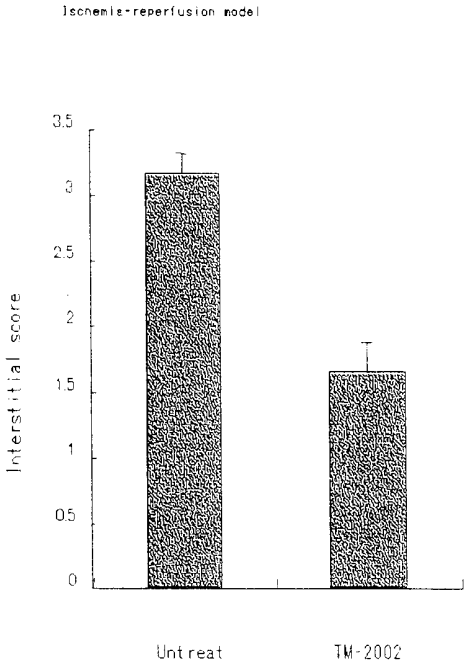

Ischemia-reperfusion model
Interstitial score
3.5
3
2.5
2
1.5
1
0.5
0
Untreat
TM-2002

Figure 14

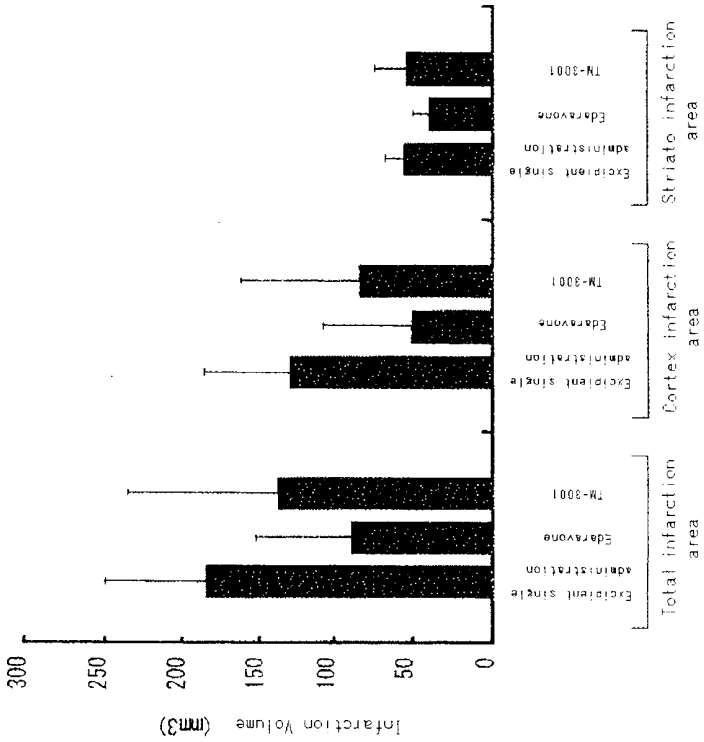

Infarction Volume (mm3)
300
250
200
150
100
50
0
Total infarction area
Cortex infarction area
Striato infarction area
Excipient single administration
Edaravone
TM-3001

Figure 18

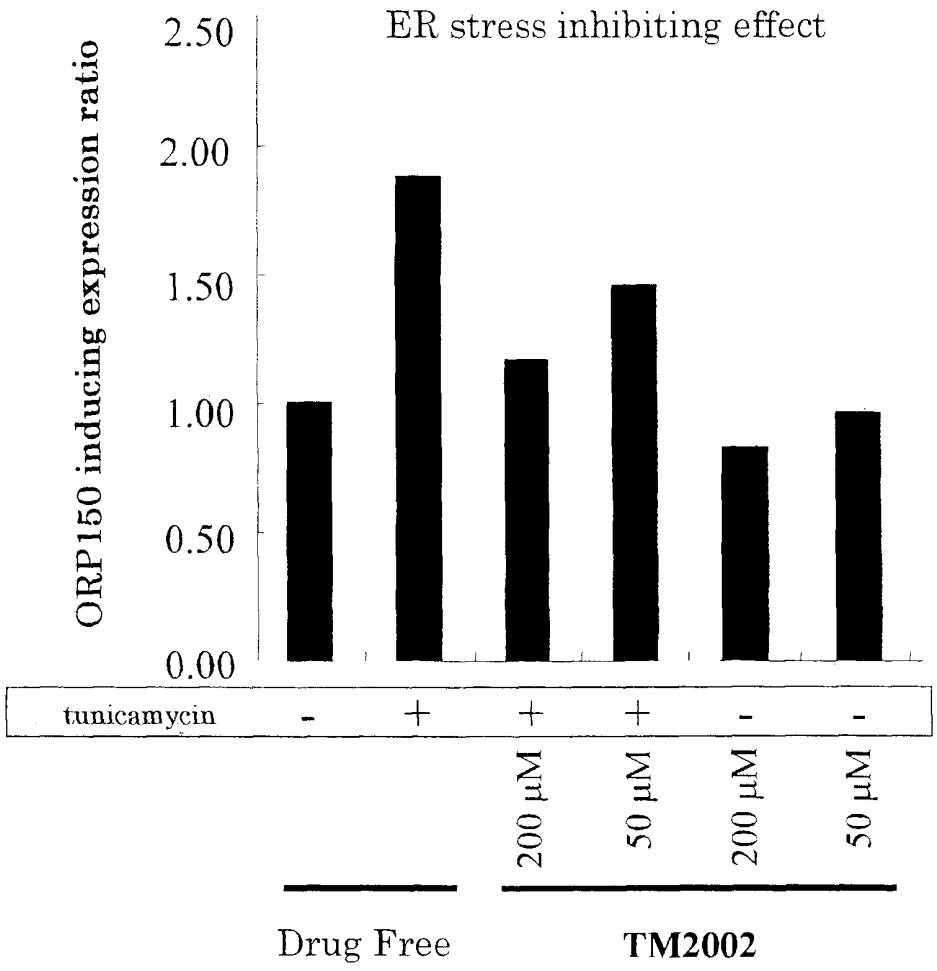
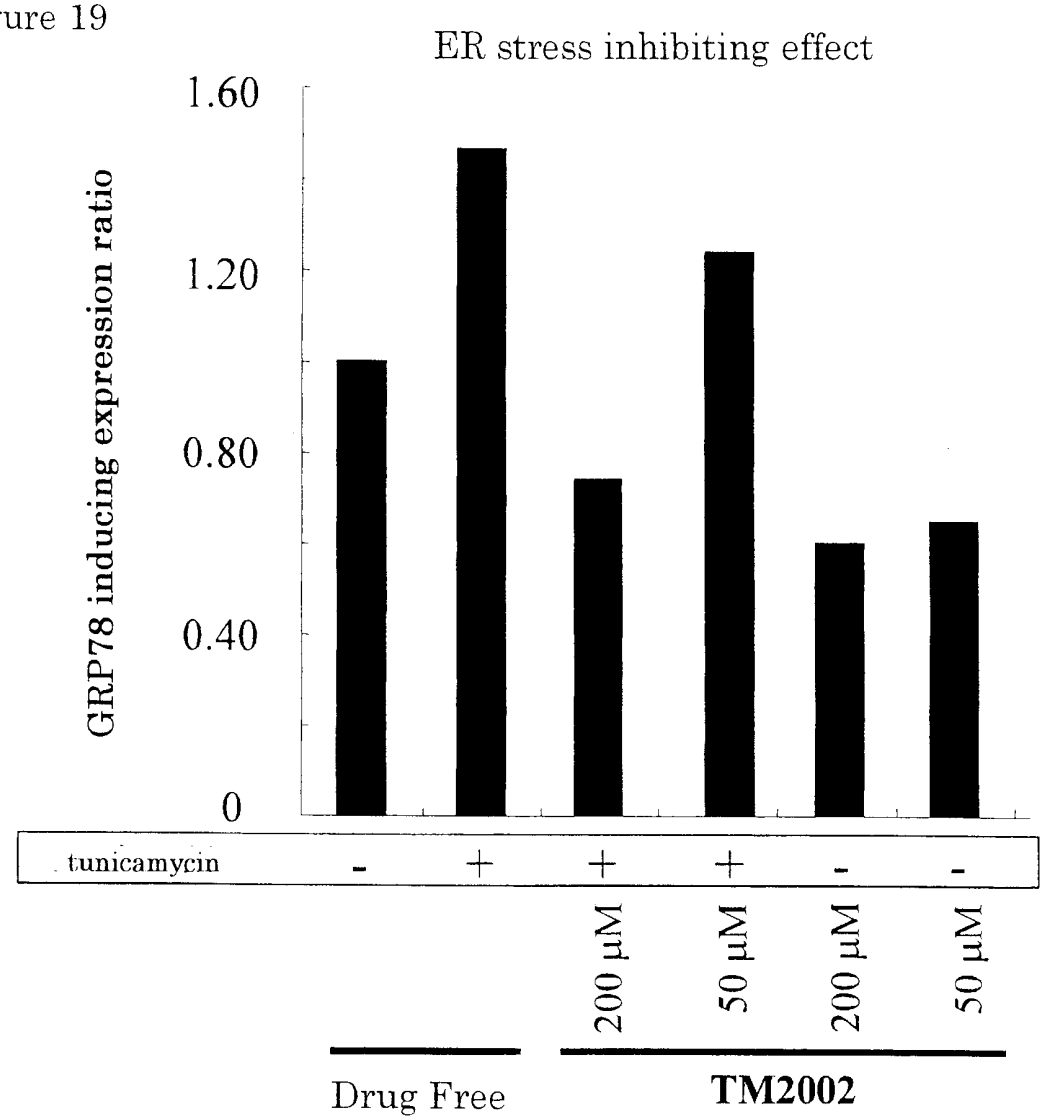
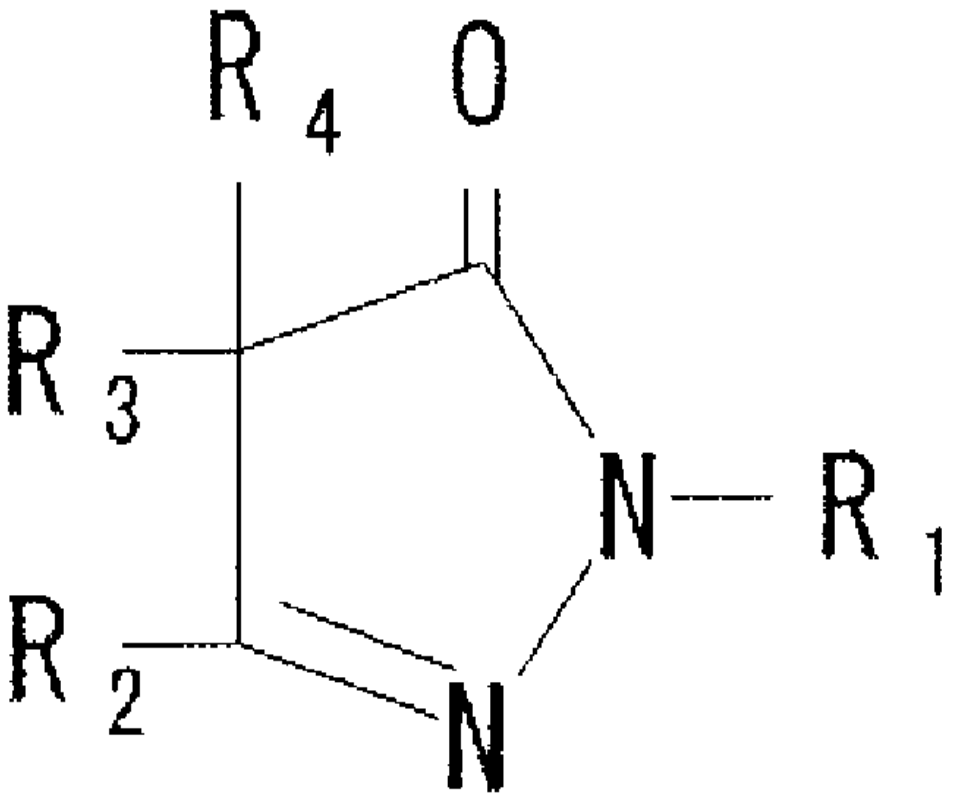
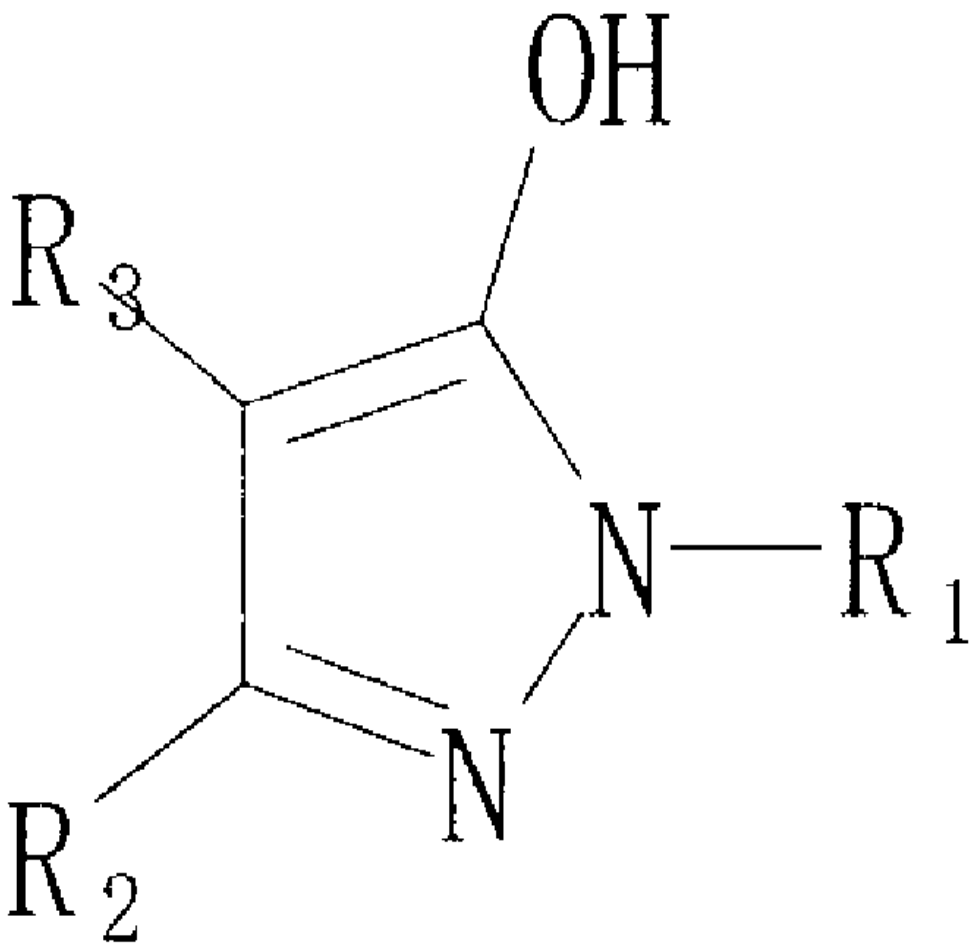

ER stress inhibiting effect
ORP150 inducing expression ratio
2.50
2.00
1.50
1.00
0.50
0.00
tunicamycin
-
+
+
+
-
-
200 μM
50 μM
200 μM
50 μM
Drug Free
TM2002

Figure 19


ER stress inhibiting effect
GRP78 inducing expression ratio
1.60
1.20
0.80
0.40
0
tunicamycin
-
+
+
+
-
-
200 μM
50 μM
200 μM
50 μM
Drug Free
TM2002



(I)



(II)