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(54) Title: DEOXYHYPUSYL HYDROXYLASE INHIBITORS

(57) Abstract

This invention relates to competitive and syncatalytic inhibitors of deoxyhypusyl hydroxylase. Such inhibitors prevent cell proliferation and permit cell differentiation, and thus are useful for synchronizing cell populations and for treating diseases caused by altered cell proliferation.

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DEOXYHYPUSYL HYDROXYLASE INHIBITORS

Background of the Invention

This invention relates to inhibitors of the
5 enzymatic activity of deoxyhypusyl hydroxylase.

One residue of the amino acid hypusine [N^{ϵ} -(4-amino-2R-hydroxybutyl)-lysine] forms a part of each molecule of the eukaryotic translation initiation factor, eIF-4D. Hypusine is the product of two consecutive
10 modifications of a lysine side chain in the peptide sequence -Gly-Lys-His-Gly-. A butylamine moiety from spermidine is transferred to the lysine residue to produce a deoxyhypusine moiety [N^{ϵ}- (4-aminobutyl)lysine]. The deoxyhypusine moiety is then
15 hydroxylated to give the hypusine residue. This hydroxylation is catalyzed by the enzyme deoxyhypusyl hydroxylase (DH).

Inhibitors of DH include ferrous ions, which are potent inhibitors of DH *in vitro*; pyridine 2,4-
20 dicarboxylate, which causes inhibition only in millimolar quantities; and some metal chelating agents, e.g., α,α-dipyridyl, picolinic acid, and desferal (Abbruzzese et al., 261 J. Biol. Chem. 3085, 1986).

Paz et al., 33 Bioc. Pharm. 779, 1984, describe
25 hydralazine as an inhibitor of hydroxylation of hypusine. They state that hydralazine has an inhibitory effect on DNA synthesis and cell growth.

Park et al., 257 J. Biol. Chem. 7217, 1982, describe the biosynthesis of hypusine, and inhibition
30 of its synthesis by metal chelators.

Cooper et al., 80 Proc. Natl. Acad. Sci. USA 1854, 1983 state that:

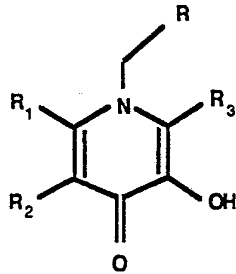
> Since increased protein synthesis is a major component of activation of quiescent
> 35 lymphocytes or of growth-arrested fibroblasts,

it follows that eIF-4D, and the possible modulation of its activity by post-translational formation of hypusine, may play an essential role in the regulation of cell
5 growth.

Summary of the Invention

In certain aspects, the invention features competitive and syncatalytically inactivating inhibitors of deoxyhypusyl hydroxylase.

10 The agents of the competitive class of compounds physically attach to the active site metal ion to form a complex. These agents have the following formulae:



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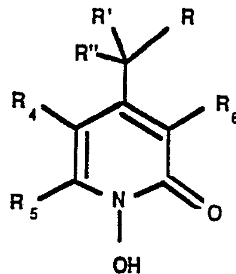

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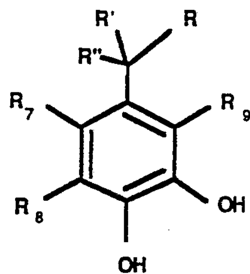

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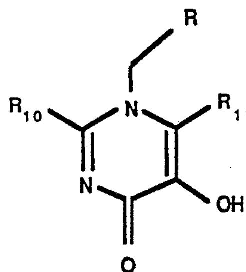

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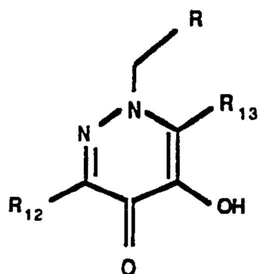

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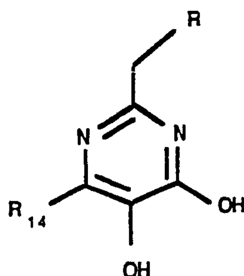

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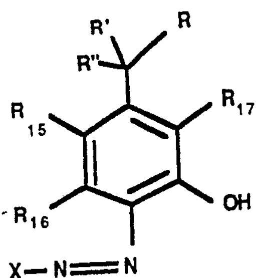

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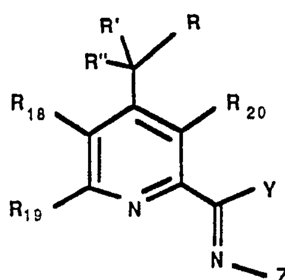

where R includes a group chosen from aryl or alkyl moieties, in particular

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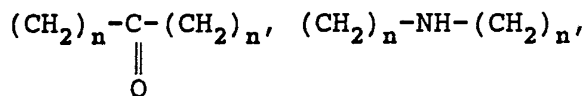

$(CH_2)_n$-C(=O)-$(CH_2)_n$, $(CH_2)_n$-NH-$(CH_2)_n$,

or [$(CH_2)_n$-branched or -unbranched peptide of amino acids]

10 where each n independently is between 1 and 10; each R_1 to R_{20} is chosen independently from H, CH_3, NH_3, $(CH_2)_nNH_3$, $(CH_2)_nCH_3$, or a halogen; R' and R'' are independently H, or CH_3, or together form a double bond

to an oxygen; R_{21}, R_{22}, and R_{23} independently represent aryl or alkyl moieties.

The agents in the syncatalytically inactivating class of compounds do not physically attach to the metal ion at the active site of the enzyme, but mimic the deoxyhypusyl substrate, only to disintegrate into labile fragments upon hydroxylation. These fragments, in turn, irreversibly inactivate the enzyme. These agents have the following general structure:

where X is CH_2, CF_2, or Si; Y is O, S or NH; and Z is NH, CH_2, or Si; or X is O, S, or NH; Y is CH_2, CF_2, or Si; and Z is NH or CH_2; and W is an alkyl or aryl residue or represents the α carbon atom within the backbone structure of a peptide, i.e., a branched or unbranched chain of amino acids.

The inhibitors of this invention reversibly inhibit the cell-growth cycle between G1- and S-phase, so that they inhibit proliferation but allow cellular differentiation. Thus, they are useful for synchronizing cell populations and for treating various diseases caused by altered cellular proliferation. For example, they are useful for treatment of cancer, cosmetic hirsutism, and non-physiological formation of blood vessels. In addition, they are useful for preventing transplant or graft rejection.

In a related aspect, the invention features a method for allowing cell differentiation and preventing cellular proliferation in a population of cells. The method includes contacting the population of cells with

an inhibitor as described above; and culturing the cell population with the inhibitor.

Preferably, the subpopulation is present in a living organism, and the contacting step involves administering the inhibitor to the organism. The method is also suitable for treating a disease caused by altered cell proliferation in a patient (and includes the step of identifying a patient having such a disease prior to contacting the population with the inhibitor), for preventing rejection of a graft or a transplant in a patient (and includes performing the transplant or graft after contacting the population with the inhibitor), and for treatment of cancer (and includes, after the contacting step, killing growing cells and then removing the inhibitor from contact with the cell population).

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Fig. 1 is a diagrammatic representation of the interaction of DH (shown as a shaded structure) with its substrate;

Fig. 2 is the structural chemical formula of mimosine;

Fig. 3 is the structural chemical formula of ciclopirox olamine;

Figs. 4, 5, 6, and 7 are diagrammatic representations of the interaction of various inhibitors with DH;

Fig. 8 is a diagrammatic representation of the interaction of Nα-acetyl-NΔ- (3,4,dihydroxy-benzoyl-L-Orn-L-Pro-Gly with DH; and

Figs. 9, 10, and 11 are graphical representations of the inhibitory action of Nα-acetyl-NΔ-(3,4,dihydroxy-benzoyl)-L-Orn-L-Pro-Gly and Nα-acetyl-NΔ-(2,3,dihydroxy-benzoyl)-L-Orn-L-Pro-Gly on DH activity; and

5 Fig. 12 is a time course of release from mimosine and aphidicolin, the % cells in S phase for each release time point was calculated from the number of cells with G1 DNA content having positive BrdU fluorescence by standard procedure.

10 Inhibitors

Inhibitors useful in this invention have the general formulae described above in the Summary of the Invention. Suitable inhibitors are designed by analysis of the structure of DH and its interaction with its
15 substrate.

The inhibitors are also designed to mimic the action of DH-inhibitory chemicals such as mimosine (see Fig. 2) and ciclopirox olamine (see Fig. 3). As will be described below, these two compounds inhibit DH activity
20 and inhibitors based upon the active parts of those molecules which interact with the active site of DH are useful in this invention.

Those substances which are useful in this invention have sufficiently high DH inhibitory activity
25 *in vivo* to cause a majority of cells in a population to stop proliferating (i.e., at least 50% of cells in a population) while allowing cellular differentiation. The extent to which any particular inhibitor is able to inhibit cell proliferation and allow cell differentiation
30 can be assayed by methods well known to those of ordinary skill in the art, examples of which are provided below. These examples are not limiting to this invention and those of ordinary skill in the art will readily understand that equivalent assays may be used to
35 determine useful inhibitors of this invention.

The inhibitors can also be tested to ensure that they can be administered to an animal, e.g., a human, without significant side effects, by procedures well known to those of ordinary skill in this art. The amount of inhibitor which must be administered to treat any particular animal or disease can be determined by standard protocols and will depend upon the efficacy of inhibition of DH activity, and upon the ability of that inhibitor to contact and inhibit DH within the animal or within a cell. It is desirable that the inhibitors be designed to have little, if any, antigenic activity within the animal being treated.

Referring to Fig. 1, the structure of DH and its substrate is shown diagrammatically. The enzyme is shown generally by shaded portion 10 having an active site in region 12, shown as a space within shaded portion 10. Substrate 14 lies within this site. The enzyme contains a metal ion 13 which is arbitrarily displayed in the figure in octahedral symmetry with ligands L1, L2, L3, L4, and L5 representing apo-enzyme side chains that hold the metal in a discrete orientation relative to the substrate binding site. Also present in enzyme 10 is a negatively charged site 16 which interacts with a primary amine (NCH_3+) at the end of the substrate.

Although the substrate for DH is generally a peptide, inhibitors of this invention need not be peptides. The inhibitors need only mimic the deoxyhypusine side chain shown in Fig. 1. For example, referring to Fig. 4, inhibitors may simply have a primary amine group, adapted to react with negatively charged site 16, attached to a side chain similar in structure to deoxyhypusine. Such inhibitors are competitive reversible inhibitors of DH, and may coordinate the active site metal ion of DH. These inhibitors are stable under biological conditions.

Alternatively, referring to Fig. 5, the inhibitor may be based upon the structure of mimosine, being designed to interact with metal site 13.

Referring to Fig. 6, the inhibitor may have a
5 modified mimosine-structure having a primary amine group designed to interact with negatively-charge site 16 to form a salt bridge. Such an inhibitor will be a competitive reversible inhibitor which acts by chelation of the active site metal ion 13, and by salt bridge
10 formation to site 16. Referring to Fig. 7, in yet another example, the inhibitor may be formed as an irreversible suicide inhibitor. Suicide inhibitors undergo proton abstraction and hydroxylation to disintegrate into reactive fragments within the enzyme
15 catalytic pocket center. In this inhibitor, the bond between a carbon and oxygen is at the center of hydroxylation which upon oxidation causes the bond to break. These inhibitors are particularly useful in this invention since only one molecule of inhibitor is
20 necessary to completely inhibit a DH molecule. The other inhibitors have lower efficiency since they act in a competitive manner.

In all of the above examples, the critical part of the inhibitory molecule is that part which interacts with
25 the anion moiety 16 or with the active site metal 13. The side chain attached to that reactive moiety is less relevant so long as it allows the inhibitor to enter the active site of DH. The greater the similarity of this side chain to deoxyhypusine the better the inhibitory
30 effect and greater the specificity of that inhibitor.

There follow several examples of inhibitors of this invention, along with methods for assay of the inhibitory activity. In some of these examples, the inhibitory effect of mimosine or ciclopirox olamine is
35 tested on DH activity. These examples are used only by

way of illustration, and the substitution of inhibitory compounds of the invention in these methods or tests is a suitable way to measure inhibitory activity of an inhibitor.

5 The inhibitory compounds described above may be synthesized by standard procedures, examples of which are provided below. Again, these examples are not limiting in this invention since those of ordinary skill in the art can readily determine equivalent or alternate methods
10 by which inhibitors can be synthesized.

Example 1: Inhibitor Compounds I and II

Short peptides identical in sequence to the physiological substrate of DH, e.g., Lys-Thr-Gly-X-His-Gly-His-Ala-Lys, where X is a lysine or deoxyhypusine
15 residue, fail to provide significant inhibition of DH. The lysine-containing peptide gives essentially no inhibition (even at 2 mM) while the deoxyhypusine-containing peptide showed a K_i of only 0.33 mM. In view of this, bifunctional molecules were designed that
20 combined the substrate-like peptide sequence with a metal binding domain. This dual structural feature allows alignment of the inhibitor in the peptide site of DH, and coordination to metal ion 13 of the catalytic center. In this way the enzyme bound metal ion acts to stabilize an
25 inhibitor-enzyme complex. A catechol nucleus was chosen as a metal binding moiety since it has a high formation constant and acts as an obligatory bidentate ligand. Further, the active site metal ion of DH appears to be uncharged and acts as an obligatory bidentate ligand.
30 The two chelating tripeptides studied were termed compound I [Nα-acetyl-NΔ-(3,4-dihydroxy-benzoyl)-L-Orn-L-Pro-Gly] and compound II [Nα-acetyl-NΔ-(2,3-dihydroxy-benzoyl)-L-Orn-L-Pro-Gly].

The compounds were synthesized using conventional
35 methods (see U.S. Patent No. 4,797,471). After initial

synthesis of the C-terminally protected component Nα-acetyl-tripeptide, the appropriate, derivatized dihydroxybenzoate isomer was attached to the ornithine residue. All protecting groups were then removed in a single step. Briefly, Nα-acetyl-L-Orn-L-Pro-Gly-benzylester and the 2,3- or the 3,4-dibenzyloxybenzoic acid were dissolved in dimethylformamide in the presence of 1-hydroxybenzotriazole and N-ethylmorpholine. After addition of dicyclohexylcarbodiimide, the reaction was allowed to proceed for 20 hours. The dicyclohexylurea was removed by filtration, the solvent evaporated in vacuo, and the residue, dissolved in ethyl acetate, chromatographed on silica gel, with chloroform:methanol as mobile phase. The purified derivative was hydrogenated using paladium on activated charcoal as a catalyst. This catalyst was removed by filtration and the solvent evaporated in vacuo. The products, compound I or compound II, respectively, were homogeneous on thin layer chromatography.

The DH used in the tests described below was a partially purified preparation obtained from rat testis homogenate by ammonium sulfate fractionation, ion exchange chromatography, and exclusion chromatography. Biosynthetically radiolabeled unhydroxylated protein substrate ([^{3}H]-deoxyhypusine protein) was prepared by culturing Chinese hamster ovary cells in the presence of [2,3-^{3}H]-putrescine (4 μCi/ml) and the metal chelating agent 2,2'-dipyridyl (0.1 mM).

DH enzyme activity was measured by an ion exchange chromatographic procedure. Acid hydrolysates of aliquots of the protein fraction of assay mixtures were chromatographed to separate out hypusine and deoxyhypusine. The amounts of hypusine and deoxyhypusine could then be separately measured for radioactivity. Compounds were tested as enzyme inhibitors using assay

mixtures containing about 40 nM [^{3}H]-deoxyhypusine protein substrate, 6 mM dithiothreitol, appropriate amounts of test compound and about 2 units of enzyme in a total volume of 0.2 ml of 0.02 M Tris-HCl buffer, pH 7.5. Reactions were conducted at 37°C for 60 min.

In kinetic inhibition studies, assays were carried out under the same conditions, except that the concentrations of both protein substrate and inhibitor were varied. The data obtained were fitted to the equation for competitive inhibition.

$$V = \frac{V_{max} S}{K_m\left(1 + \frac{I}{K_i}\right) + S}$$

Referring to Figs. 9, 10, and 11, inhibition of DH by compound I and compound II, and the effects of their individual building blocks, the carrier peptide and the chelating molecules 2,3-dihydroxybenzoic acid (2,3-DHBA), 3,4-dihydroxybenzoic acid (3,4-DHBA), and their ester derivatives are shown.

All of the compounds tested showed dose-dependent suppression of DH activity. The inhibitory concentrations extended from the micromolar to the millimolar range, and was closely correlated with structural modifications of the molecules tested. Whether studied as the free acid, the ester, or the catecholpeptide, the 3,4-isomer gave a greater degree of inhibition then the 2,3-isomer (compare Figs. 9 and 10), despite the first formation constant for metal-ligand complexes in solution being higher for 2,3-DHBA than for

3,4-DHBA, ($10^{20.5}$ L mol^{-1} versus $10^{18.9}$ L mol^{-1} with Fe(III) as metal ion). The superior inhibitory effectiveness of the less efficient metal chelator makes it unlikely that these agents exert their inhibitory
5 action via complex formation in solution. Rather, the data suggest a direct interaction of the catechol moiety in both isomers with the tightly-bound metal ion at the enzyme active site. The preference for the 3,4-isomer, whether in the form of the free acid, the ester
10 derivative, or the catecholpeptide, may reflect restricted steric accessibility imposed by the protein.

The Lineweaver-Burk plot for the inhibition of the deoxyhypusyl hydroxylase by compound I is shown in Fig. 11. The mode of inhibition is competitive with
15 respect to unhydroxylated precursor of eIF-4D. The K_i was 0.032 ± 0.0034 mM.

Referring to Fig. 8, the interaction of compound I with DH is shown. Distance D is that distance between the C2 carbon atom and a catechol chelated metal center.
20 This distance is about 12 angstroms for compound I, and 10 angstroms for compound II. The metal ion-containing locus of the enzyme is situated inside the apo-protein. This hydrophobic environment allows access to uncharged molecules such as the ester-derivatives of the
25 dihydroxybenzoate class of agents.

Both compounds I and II inhibit DH activity. Although the peptide backbone of compounds I and II differs from elF-4D precursor sequence (having a proline instead of a histidine), inhibitory potency of these
30 tripeptides is superior to Lys-Gly-deoxyhypusine-His-Gly-His-Ala-Lys. This demonstrates that the catechol moiety imparts inhibitory character to the two compounds.

The peptide moiety of the inhibitors appears to
35 serve as a directing force for the catechol moiety, and

the catechol moiety by chelation to the metal ion at the catalytic center facilitates alignment of the peptide in the peptide substrate site.

Inhibitor assays

5 The following are examples of assays for inhibitory compounds of this invention. Other equivalent assays are also suitable. In general, these assays determine the time of blockage of a cell cycle by an inhibitor. This time is between G1- and S-phase (i.e., 10 late G1-phase) for inhibitors of this invention. Inhibitors may also be assayed by determining inhibition of DH activity, as discussed above.

Example 2: Primary mouse lymphocyte assay

In this assay, inhibitors of this invention allow 15 activated T lymphocyte precursors to differentiate to cytotoxic T effector cells but do not allow them to proliferate.

Mouse spleens were excised and a unicellular suspension prepared. The cells were stimulated with the 20 mitogen Concanavalin A in the presence of increasing concentrations of mimosine (MIMO, 25 to 400 μM). After 42 hours, the cells were harvested and their ability to lyse target cells in a standard chromium (Cr) release assay was measured. This assay evaluates the acquisition 25 of differentiated effector function by precursor cytotoxic T cells. The stimulated lymphocytes were co-cultured with 10^3 ^{51}Cr-labelled target cells and PHA to agglutinate effector and target cells without regard to specificity. The effector to target (E/T) ratio was 100, 30 30 and 10. The assay was carried out for 4 hours. Effector function results in target cell lysis and ^{51}Cr release. The lysis of control mitogen-stimulated spleenocytes which are not drug-treated varies from 7.6 to 3.8%, which is comparable to unstimulated (UNST) 35 spleenocytes cultured without mitogen. The addition of

mimosine at 75 and 100 µM concentration results in a significant increase in lytic activity (ranging from 20% to 7%) despite the block in proliferation which is induced by mimosine which is measured by flow cytometric
5 analysis of DNA content as described below.

Example 3: Leukemia cell line

The inhibitory activity of mimosine and ciclopirox olamine is demonstrated by their reversible blocking of the cell cycle of HL-60 promyeloid leukemia cells. Such
10 blocking causes arrest of cell proliferation in the late G1-phase.

In these experiments cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 20% fetal calf serum (FCS) and
15 penicillin/streptomycin. Cells were seeded at approximately 10^6 cells/ml. Two hours later, mimosine at 300 µM or ciclopirox olamine at 10 µM was added. After a 16 hour incubation period in the compounds, the cells were collected by centrifugation and either prepared for
20 analysis with the flow cytometer or resuspended in fresh medium without inhibitor compound for release and re-addition experiments.

Flow cytometric analysis of DNA content was performed five hours after the start of release. In this
25 analysis, cells were harvested by centrifugation and resuspended in phosphate buffered saline (PBS) at 1 to 2 x 10^6 cells/ml. Triton X-100 detergent was added to a final concentration of 0.1% in order to permeabilize the cell membrane. Hoechst 33342 dye, which binds
30 stoichiometrically to DNA, was added to a final concentration of 20 µg/ml. After 15 to 60 minutes on ice, the samples were subjected to flow cytometric analysis using a FACS IV instrument equipped with a 6 W argon-ion laser (Innova 90-6) adjusted to emit 200 mW at
35 the combined 351 and 364 nm lines. The fluorescence of

the Hoechst 33342 was collected using a 460 nm long pass and a 580 short pass filter combination. The DNA histogram parameters such as the cell cycle phase fractions and the peak means are calculated by the
5 computer model of Dean (13 Cell Tissue Kinetics 299, 1980).

The two compounds are effective in arresting cell proliferation and act as synchronization agents. The compounds arrest cell proliferation in the late G1 phase
10 of the cell cycle, as compared to the exponentially growing control. After a 5 hour release, a cohort of synchronously growing cells progress approximately midway through S-phase. Both compounds appear to act at the same point in the late G1-phase, prior to beginning of S-
15 phase.

Example 4: EBV-Transformed cell line

In this example, blockage of cell proliferation by mimosine in late G1-phase is demonstrated by flow cytometry and Northern analysis. A human Epstein Barr
20 virus-transformed cell line, LAZ463 (Lalande et al., 5 Cytometry 101, 1984) was cultured in RPMI 1640 medium supplemented with 10% FBS. Cells were seeded at roughly 10^5/ml. Eight hours later, mimosine at 200 μM or aphidicolin at 5 μg/ml was added. After a 16 hour
25 incubation in the cell cycle inhibitor, cells were collected by centrifugation and the lymphocytes prepared for flow cytometry or RNA extraction, or resuspended in fresh medium without inhibitor compound for release and re-addition experiments.

30 Flow cytometric analysis of DNA was performed as described above. Northern blots were performed on total cellular RNA prepared by the guanidium method of Chirgwin et al. (18 Biochemistry 5294, 1979). Cell pellets were resuspended in 3.5 ml of guanidium solution (4 M
35 guanidium isothiocyanate, 20 mM sodium acetate (pH 5.2),

0.1 mM dithriothreitol and 0.5% N-lauryl sarcosine). The lysate was then layered over 1.5 ml of 5.7 M cesium chloride and pelleted by centrifugation at 35,000 rpm for 20 hours at 18°C. The RNA was resuspended in diethylpyrocarbonate-treated water and quantitated by reading the relative absorbance at 260 nm. Ten μg of RNA were then electrophoresed in a 1% (w/v) agarose-6.6% (v/v) formaldehyde gel. The RNA was then transferred by standard procedure to a nylon filter membrane (GeneScreen Plus, New England Nuclear) in 20X SSC (3 M NaCl, 0.3 M sodium citrate). Prehybridization, hybridization, and washing were performed by standard procedures.

Mimosine arrested cell proliferation in the G1 phase of the cell cycle. The mimosine-induced cell cycle arrest point occurs before the block affected by aphidicolin (APH) which was measured in a manner similar to that described above. This result was obtained by initially blocking cell cycle with mimosine or with APH and then subsequently blocking with the alternative reagent. The levels of mRNAs for histone 4, thymidine kinase, p53 and esterase D genes were determined by Northern blot as described above. Both H4 and TK mRNA levels are increased in cells blocked with APH relative to those arrested with mimosine while p53 mRNA levels are increased with mimosine relative to that with APH. These data show that the mimosine arrest point occurs at most 2 hours prior to the G1/S boundary defined by APH.

The effects of different cell cycle inhibitors on active DNA replication was also determined using an antibody to bromodeoxyuridine (BrdU) and a flow sorter. Cells were seeded at roughly 10^5/ml, and, after four hours, either aphidicolin at 10 μg/ml or mimosine at 400 μM were added. Following a one hour incubation in the cell cycle inhibitors, BrdU at 20 μM was added, cells were incubated for an additional 16 hours and collected

by centrifugation. For release experiments, cells were washed once in PBS and suspended either in 1 ml PBS containing either 10 μg/ml aphidicolin, 400 μM mimosine or no inhibitor. Each sample was put on ice until all
5 released samples were collected. The cells were then prepared for flow cytometric analysis as follows. Cells were centrifuged for 5 minutes at 500g, resuspended in 20μl cold PBS and fixed in 70% ethanol. After a 10 minute centrifugation (500g), cells were resuspended in 1
10 ml of 5% Triton X-100 in 5M HCl and incubated for 30 minutes at 20°. The cells were then again centrifuged (500g) for 10 minutes at 20° and were resuspended in 1 ml of 0.1 M $Na_2B_4O_7$, pH 8.5. Cells were collected by centrifugation and resuspended in 50 μl of 0.5% Tween 20
15 (v/v) and 1% BSA (w/v) in PBS. Next 20 μl of fluorescein isothiocyanate (FITC)-conjugated murine monoclonal anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA) at a concentration of approximately 7 μg/ml was added, followed by vortexing. After a 30
20 minute incubation at 20°, cells were centrifuged (500g, 10 min), resuspended in PBS, and propidium iodide was added to a final concentration of 5 μg/ml. Samples were subjected to flow cytometric analysis using a FACS IV instrument equipped with a 5 Watt Argon ion laser
25 adjusted to emit at 488nm. The FITC fluorescence emission was collected from 515 to 540nm while that of the PI was collected in a second photomultiplier tube at wavelengths abve 580nm. The results of this experiment are shown in Fig. 12. The results show that aphidicolin
30 blocks cell cycle progression in early S phase whereas mimosine appears to act at or near G1/S interface, since a larger proportion of aphidicolin-treated cells have undergone active DNA replication during the drug incubation period than in the case with mimosine. The
35 data indicate that the inhibitors of the present

invention blocks cell synthesis before the cells enter active DNA synthesis.
This method is useful for assay of compounds of the present invention to determine whether they cause
5 inhibition of the cell cycle prior to onset of active DNA replication.

Example 5: Normal human T lymphocytes

The inhibitory activity of mimosine and ciclopirox olamine on mitogen-stimulated human T lymphocytes can be
10 shown in vitro. As with the above examples, this assay facilitates identification of inhibitors useful in this invention. The assay demonstrates blockage of the proliferative response and of DNA synthesis indicative of blockage at the G1/S-phase of the cell cycle.
15 T lymphocytes are stimulated and DNA content assayed as described above. Northern blots were performed as described above.

The addition of inhibitor, e.g., mimosine (250 to 400 μM) or CPX (7.5 μM), can be delayed up to 24 hours
20 after initiation of in vitro stimulation. Addition of mimosine at this time blocks the proliferative response. The expression of thymidine kinase and DNA polymerase alpha are not affected by addition of the drug. Blockage of DNA synthesis is shown by reduction of histone 4 (H4)
25 mRNA levels. The effects of the drug are reversible in primary T lymphocytes, and MIMO-treated T cells express normal levels of both the cell surface interleukin 2 and the transferrin receptors.

Example 6: Primary human tumors

30 A further assay suitable for determination of useful inhibitors of the present invention involves measuring the effect of DH inhibitors on clonogenic growth of human solid tumors. As described by Jahner et al. (31 Proc. Am. Ass. Cancer. Res. Preclinical
35 Pharmacology/Experimental Therapeutics, Abstract No.

2473, 1990), the effects of the inhibitors can be studied on *in vitro* colony formation of primary human solid tumors. In this assay system, DH inhibitors give a concentration-dependent carcinostatic effect.

5 Use

Inhibitors of this invention can be used to suppress DH activity *in vitro* and *in vivo*, as measured by standard enzyme assay procedures (Abbruzzese et al. 261 J. Biol. Chem 3085, 1986).

10 Inhibitors of this invention reversibly or irreversibly inhibit cell cycle progression in late G1 phase and thus are important in both fundamental studies of cell growth and the control of clinical disorders of cell proliferation. These inhibitors may be used in vivo

15 or in vitro to study and indentify key transcriptional and post transcriptional processes which regulate the initiation of DNA synthesis in mammalian cells.

The inhibitors are also useful in clinical chemotherapy to block tumor cell growth, or to induce

20 differentiation, or as immunosuppressive compounds.

Because these inhibitors act before the onset of DNA synthesis is induced but nonetheless allow cell differentiation to occur, they are useful agents to use in conjunction with transplants or grafting techniques.

25 In such uses, they will allow lymphocytes to differentiate but not to grow. These cytotoxic T lymphocytes are responsible in large part for rejection of grafts and transplants. These graft-selective lymphocytes can be specifcally killed prior to or at the

30 onset of synchronized clonal expansion and their loss may irreversibly incapacitate the individual's rejection of the transplanted tissue, thus resulting in transplant tolerance without generalized immunoparalysis. Thus the inhibitors allow specific killing of any activated

35 lymphocyte. In addition, the inhibitors allow treatment

of diseases which are caused by altered cell proliferation and for immunosuppression.

When used in conjunction with other killing or inhibitory agents, inhibitors of this invention are extremely useful. They can aid targeting of compounds to a specific cell population and thus enhance the potency of other inhibitors. For example, in the treatment of cancers an inhibitory agent can be administered to a patient to cause proliferating cells to arrest at the end of G1 phase. Since tumor cells are actively growing, many of the tumor cells will arrest at G1 phase. Removal of the agent will allow synchronous release of the tumor cells into S phase, and agents which are active in S phase (e.g., Cytosine Arabinoside, Adriamycin, Vinblastine, Vincristine and Methotrexate) can therefore be added to specifically kill those tumor cells. A lower amount of such inhibitors of tumor cells can be used since the efficacy of that inhibitor will be increased by the synchronization of the tumor cells.

Inhibitors of this invention can also be administered locally to protect specific cells from destruction. For example, the inhibitor may be added systemically to cause cell growth to stop at late G1 phase and a second agent (e.g., Cytosine Arabinoside, Adriamycin, Vinblastine, Vincristine and Methotrexate) added which will kill cells after they are released from the blockage in G1. Cells may then be protected by local administration of an inhibitory agent of this invention to maintain those cells in G1 phase while allowing the killing agent to kill those cells not protected by this inhibitor. One example of such use is in the protection of the mucus membranes of the gastrointestinal tract using an exclusively locally active, orally administered form of the inhibitors of this invention.

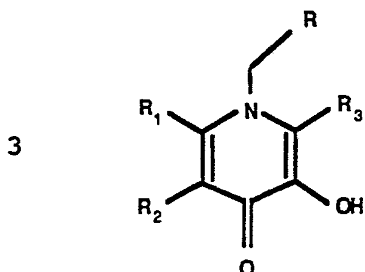
Other examples of diseases which can be treated by this inhibitory agent include those in which the immune system regulation is disrupted or the nonphysiological formation of blood vessels and for cosmetic hirsutism.

5 Other embodiments are within the following claims.

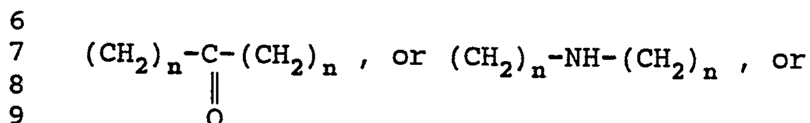
Claims

1 1. An inhibitor of deoxyhypusyl hydroxylase,
2 having the formula:

3



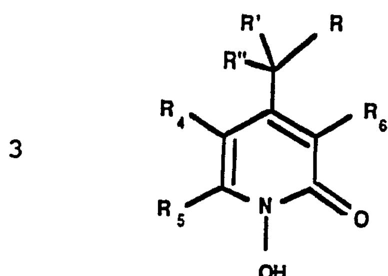
4 where R includes a group chosen from aryl or alkyl
5 moieties, or



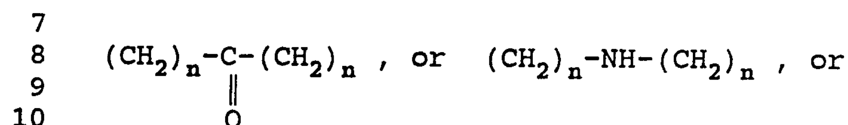
6–9 $(CH_2)_n\text{-}C(=O)\text{-}(CH_2)_n$, or $(CH_2)_n\text{-}NH\text{-}(CH_2)_n$, or

10 [$(CH_2)_n$-branched or -unbranched peptide of amino acids]
11 where each n independently is between 1 and 10; R_1, R_2,
12 and R_3 are each chosen independently from H, CH_3, NH_3,
13 $(CH_2)_nNH_3$, $(CH_2)_nCH_3$, or a halogen.

1 2. An inhibitor of deoxyhypusyl hydroxylase,
2 having the formula:

3 

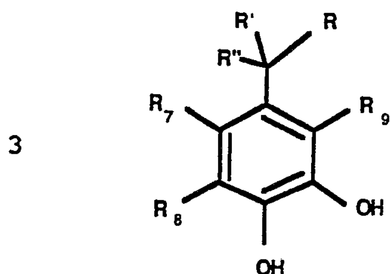
4 where R includes a group chosen from aryl or alkyl
5 moieties,
6 or
7
8
9
10



11 [$(CH_2)_n$-branched or -unbranched peptide of amino acids]
12 where each n independently is between 1 and 10; R_4, R_5,
13 and R_6 are each chosen independently from H, CH_3, NH_3,
14 $(CH_2)_nNH_3$, $(CH_2)_nCH_3$, or a halogen; R' and R'' are
15 independently H or CH_3, or together form a double bond to
16 an oxygen.

1 3. An inhibitor of deoxyhypusyl hydroxylase,
2 having the formula:

3



4 where R includes a group chosen from aryl or alkyl
5 moieties, or

6
7
8
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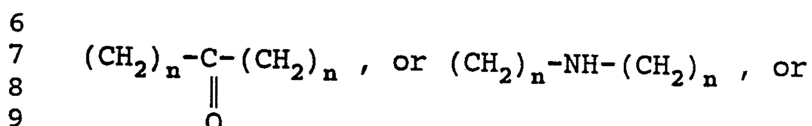
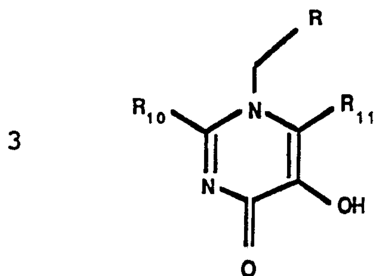


10 [$(CH_2)_n$-branched or -unbranched peptide of amino acids]
11 where each n independently is between 1 and 10; R_7 R_8, and
12 R_9 are each chosen independently from H, CH_3, NH_3,
13 $(CH_2)_nNH_3$, $(CH_2)_nCH_3$, or a halogen; R' and R'' are
14 independently H or CH_3, or together form a double bond to
15 an oxygen.

1 4. An inhibitor of deoxyhypusyl hydroxylase,
2 having the formula:

3



4 where R includes a group chosen from aryl or alkyl
5 moieties, or
6
7
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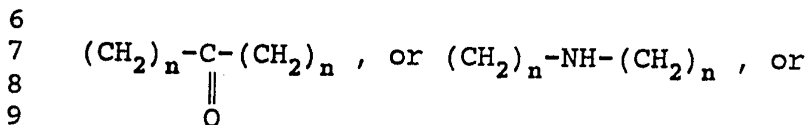
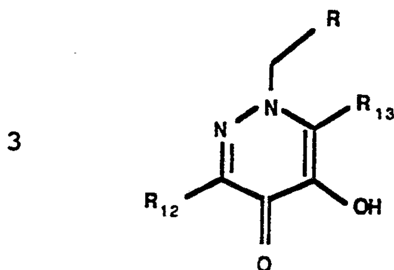
9



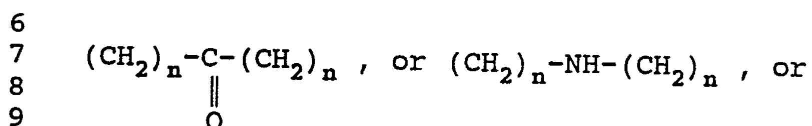
10 [$(CH_2)_n$-branched or -unbranched peptide of amino acids]
11 where each n independently is between 1 and 10; R_{10} and
12 R_{11} are each chosen independently from H, CH_3, NH_3,
13 $(CH_2)_nNH_3$, $(CH_2)_nCH_3$, or a halogen.

1 5. An inhibitor of deoxyhypusyl hydroxylase,
2 having the formula:

3



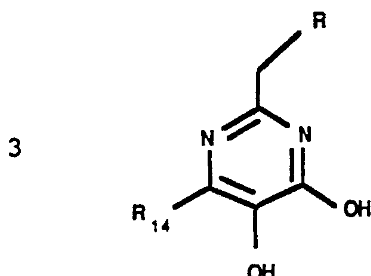
4 where R includes a group chosen from aryl or alkyl
5 moieties, or
6
7
8
9



10 [$(CH_2)_n$-branched or -unbranched peptide of amino acids]
11 where each n independently is between 1 and 10; R_{12} and
12 R_{13} are each chosen independently from H, CH_3, NH_3,
13 $(CH_2)_nNH_3$, $(CH_2)_nCH_3$, or a halogen.

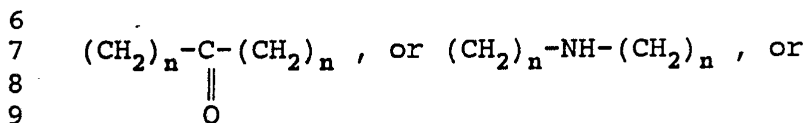
1 6. An inhibitor of deoxyhypusyl hydroxylase,
2 having the formula:

3



4 where R includes a group chosen from aryl or alkyl
5 moieties, or

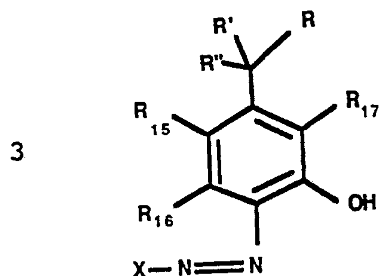
6
7
8
9


$(CH_2)_n-\underset{\underset{O}{\|}}{C}-(CH_2)_n$, or $(CH_2)_n-NH-(CH_2)_n$, or

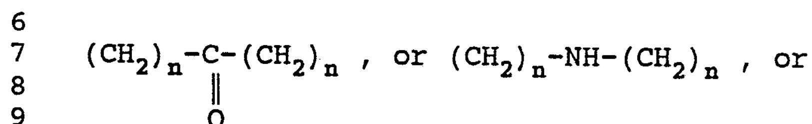
10 [$(CH_2)_n$-branched or -unbranched peptide of amino acids]
11 where each n independently is between 1 and 10; R_{14} is
12 chosen from H, CH_3, NH_3, $(CH_2)_nNH_3$, $(CH_2)_nCH_3$, or a
13 halogen.

1 7. An inhibitor of deoxyhypusyl hydroxylase,
2 having the formula:

3



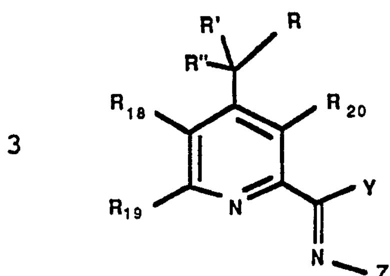
4 where R includes a group chosen from aryl or alkyl
5 moieties, or
6
7
8
9



10 [$(CH_2)_n$-branched or -unbranched peptide of amino acids]
11 where each n independently is between 1 and 10; each R_{15},
12 R_{16}, and R_{17} are each chosen independently from H, CH_3,
13 NH_3, $(CH_2)_nNH_3$, $(CH_2)_nCH_3$, or a halogen; R_{21} is an aryl or
14 alkyl moieties; R' and R'' are independently H or CH_3, or
15 together form a double bond to an oxygen.

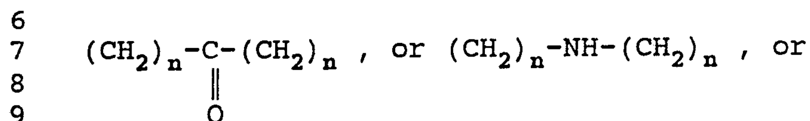
1 8. An inhibitor of deoxyhypusyl hydroxylase,
2 having the formula:

3



4 where R includes a group chosen from aryl or alkyl
5 moieties, or

6
7
8
9



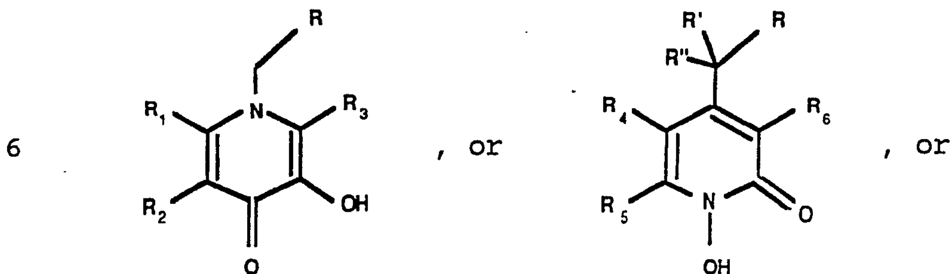
10 [$(CH_2)_n$-branched or -unbranched peptide of amino acids]
11 where each n independently is between 1 and 10; R_{18}, R_{19},
12 and R_{20} are each chosen independently from H, CH_3, NH_3,
13 $(CH_2)_nNH_3$, $(CH_2)_nCH_3$, or a halogen; R_{22} and R_{23} are aryl or
14 alkyl moieties; R' and R'' are independently H or CH_3, or
15 together form a double bond to an oxygen.

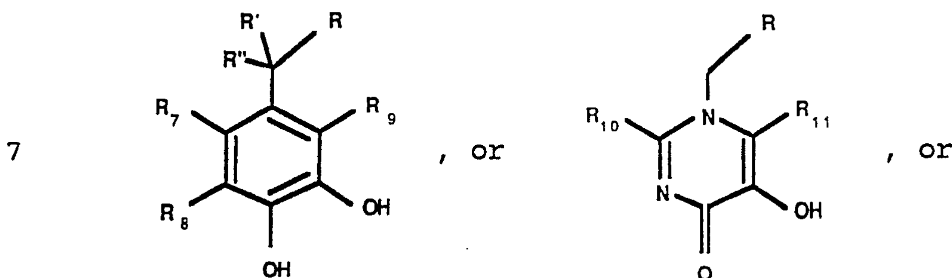
1 9. An inhibitor of deoxyhypusyl hydroxylase,
2 having the formula:

3

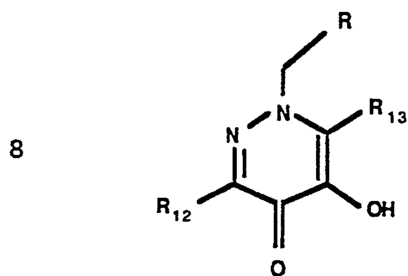
4 where X is CH_2, CF_2, or Si; Y is O, S or NH; and Z is
5 NH, CH_2, or Si; or X is O, S, or NH; Y is CH_2, CF_2, or
6 Si; and Z is NH or CH_2; and W is an alkyl or aryl
7 moiety or represents the α carbon atom within the
8 backbone structure of a peptide, i.e., a branched or
9 unbranched chain of amino acids.

1 10. A method for allowing cell
2 differentiation and preventing cellular proliferation
3 in a population of cells, comprising the steps of:
4 contacting the population of cells with an
5 inhibitor having the formula:

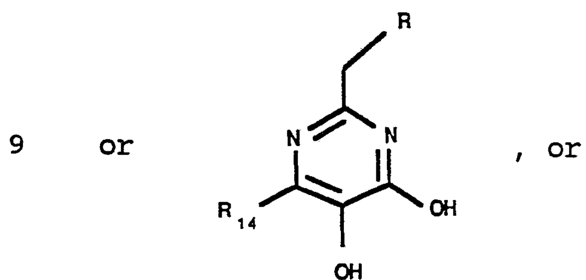
6 

7 

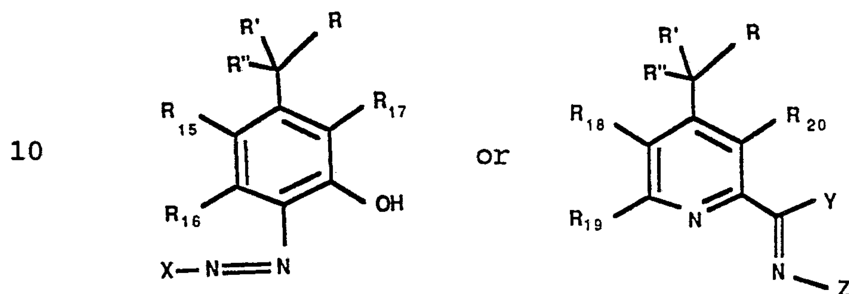
SUBSTITUTE SHEET

8 

,

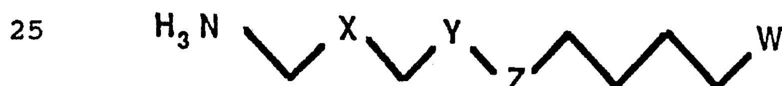
9 or 

, or

10 

11 where R
12 includes a group chosen from aryl or alkyl moieties,
13 or
14
15 $(CH_2)_n-\underset{\underset{O}{\|}}{C}-(CH_2)_n$, or $(CH_2)_n-NH-(CH_2)_n$, or
16
17

18 [$(CH_2)_n$-branched or -unbranched peptide of amino acids]
19 where each n independently is between 1 and 10; R_1 to R_{20}
20 are each chosen independently from H, CH_3, NH_3, $(CH_2)_nNH_3$,
21 $(CH_2)_nCH_3$, or a halogen; R_{21}, R_{22}, and R_{23} are aryl or
22 alkyl moieties; R' and R'' are independently H or CH_3, or
23 together form a double bond to an oxygen;
24 or

25 

26 where X is CH_2, CF_2, or Si; Y is O, S or NH; and Z is
27 NH, CH_2, or Si; or X is O, S, or NH; Y is CH_2, CF_2, or
28 Si; and Z is NH or CH_2; and W is an alkyl or aryl
29 moiety or represents the α carbon atom within the
30 backbone structure of a peptide, i.e., a branched or
31 unbranched chain of amino acids; and
32 culturing the population with said inhibitor.

1 11. The method of claim 10, wherein said
2 subpopulation is present in a living organism and said
3 contacting step comprises administering the inhibitor
4 to said organism.

1 12. The method of claim 11, wherein said
2 method is suitable for treating a disease caused by

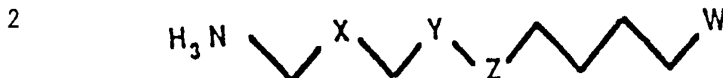
3 altered cell proliferation in a patient, and said
4 method comprises the step of identifying a said
5 patient prior to contacting said population with said
6 inhibitor.

1 13. The method of claim 12, wherein said
2 method is suitable for preventing rejection of a graft
3 or a transplant in the patient, and said method
4 includes the step of performing said transplant or
5 said graft after contacting said population with said
6 inhibitor.

1 14. The method of claim 12, wherein said
2 method is suitable for treatment of cancer, and said
3 method further comprises, after said contacting step,
4 killing growing cells and then removing said inhibitor
5 from contact with said population.

1 --15. The inhibitor of claim 9 having formula:

2



3 where X is O, S, or NH; Y is CH_2, CF_2 or Si; Z is NH or CH_2; and
4 W is an alkyl or aryl moiety or represents the α carbon atom
5 within the backbone of a branched or unbranched peptide, i.e., a
6 branched or unbranched chain of amino acids.--

1 --16. A method for allowing cell differentiation and
2 preventing cellular proliferation in a population of cells,
3 comprising the step of
4 contacting the population of cells with an inhibitor
5 having the formula:

6

7 , or salts thereof.

1 --17. A method for allowing cell differentiation and
2 preventing cellular proliferation in a population of cells,
3 comprising the step of:
4 contacting the population of cells with an inhibitor
5 having the formula:

6

7 , or salts thereof.

1 --18. The method of claim 16 wherein said population
2 is present in a living organism and said contacting step
3 comprises administering said inhibitor to said living organism.

1 --19. The method of claim 18, wherein said method is
2 suitable for treating a disease caused by altered cell
3 proliferation in a patient, said method further comprising the
4 step of identifying said patient prior to administering said
5 inhibitor to said patient.

1 --20. The method of claim 19, wherein said method is
2 suitable for preventing rejection of a graft or a transplant in
3 said patient, said method further comprising the step of
4 performing said transplant or said graft after contacting said
5 population with said inhibitor.

1 --21. The method of claim 19, wherein said method is
2 suitable for treatment of cancer, said method further comprising,
3 after said contacting step, killing growing cells and then
4 removing said inhibitor from contact with said population.--

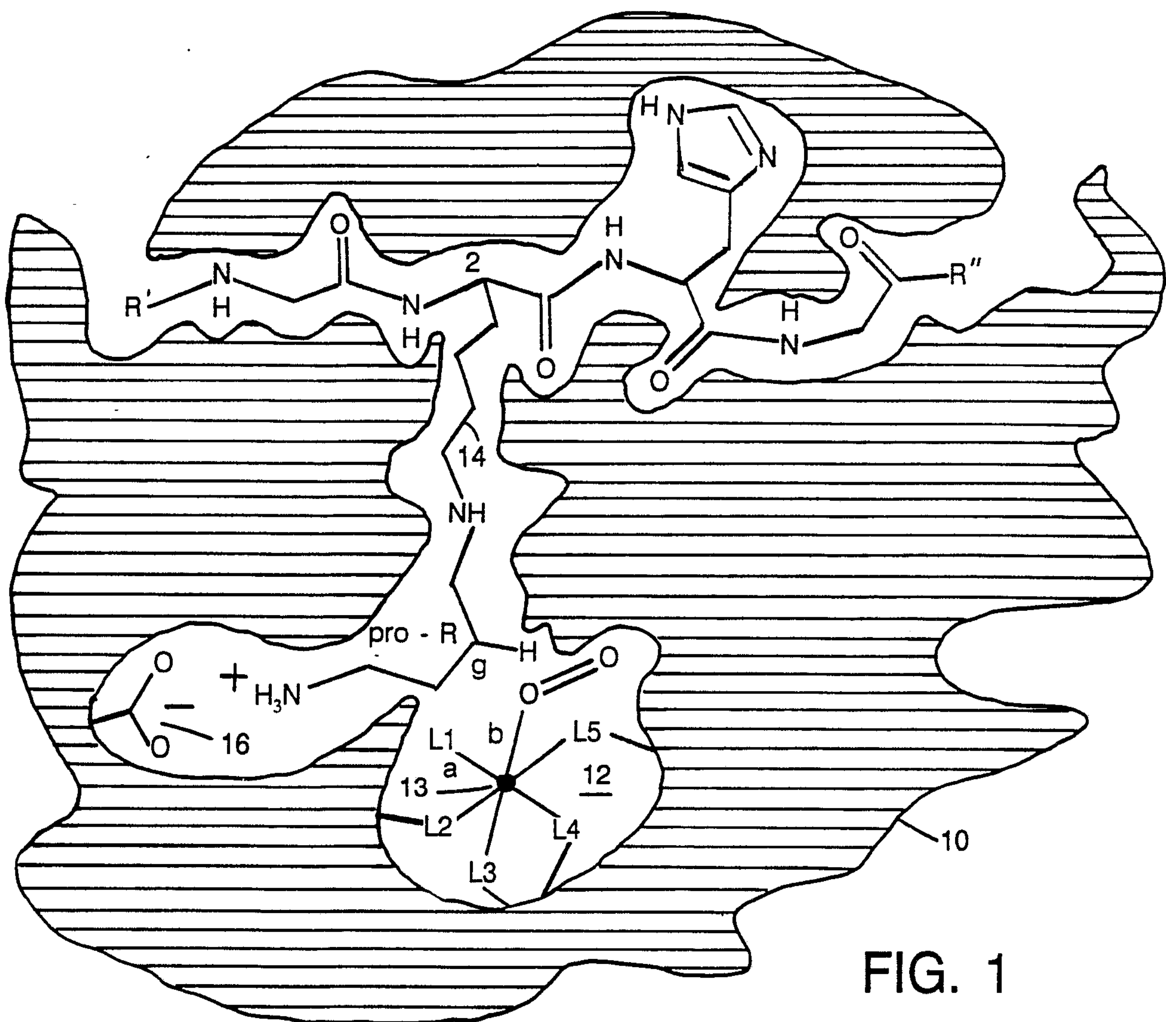


FIG. 1

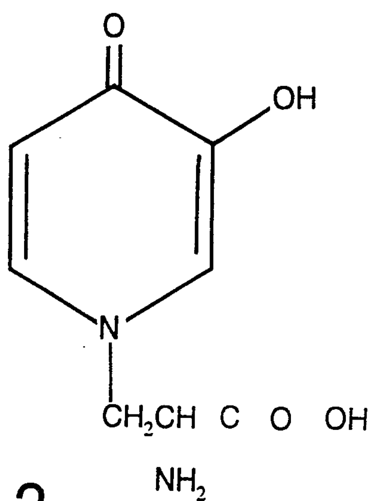


FIG. 2

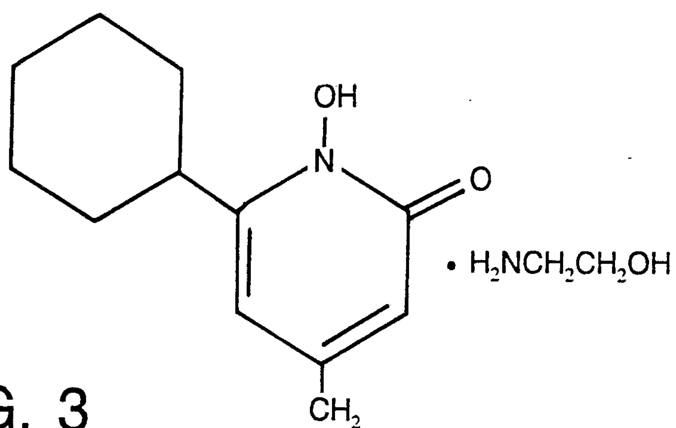


FIG. 3

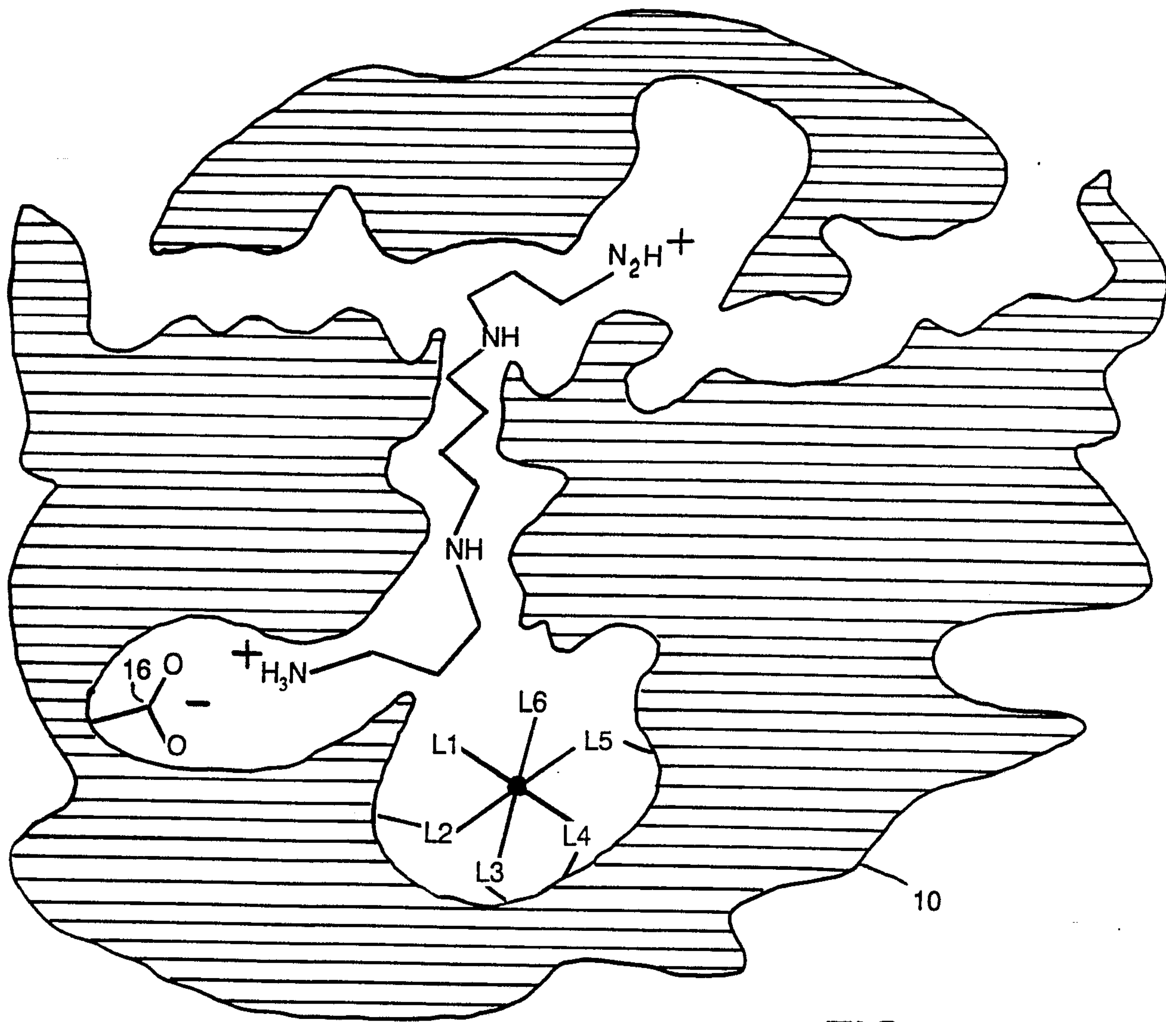


FIG. 4

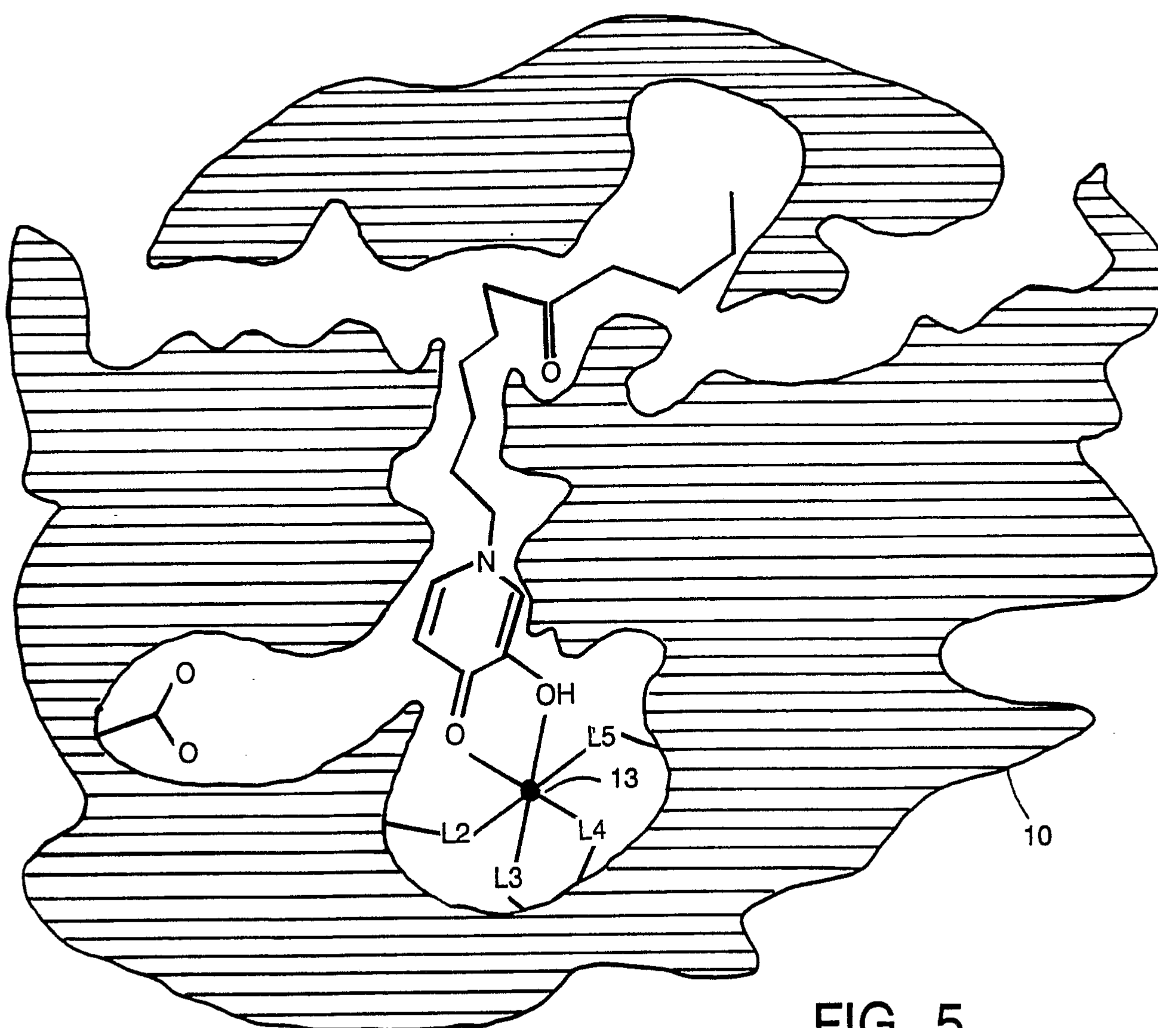


FIG. 5

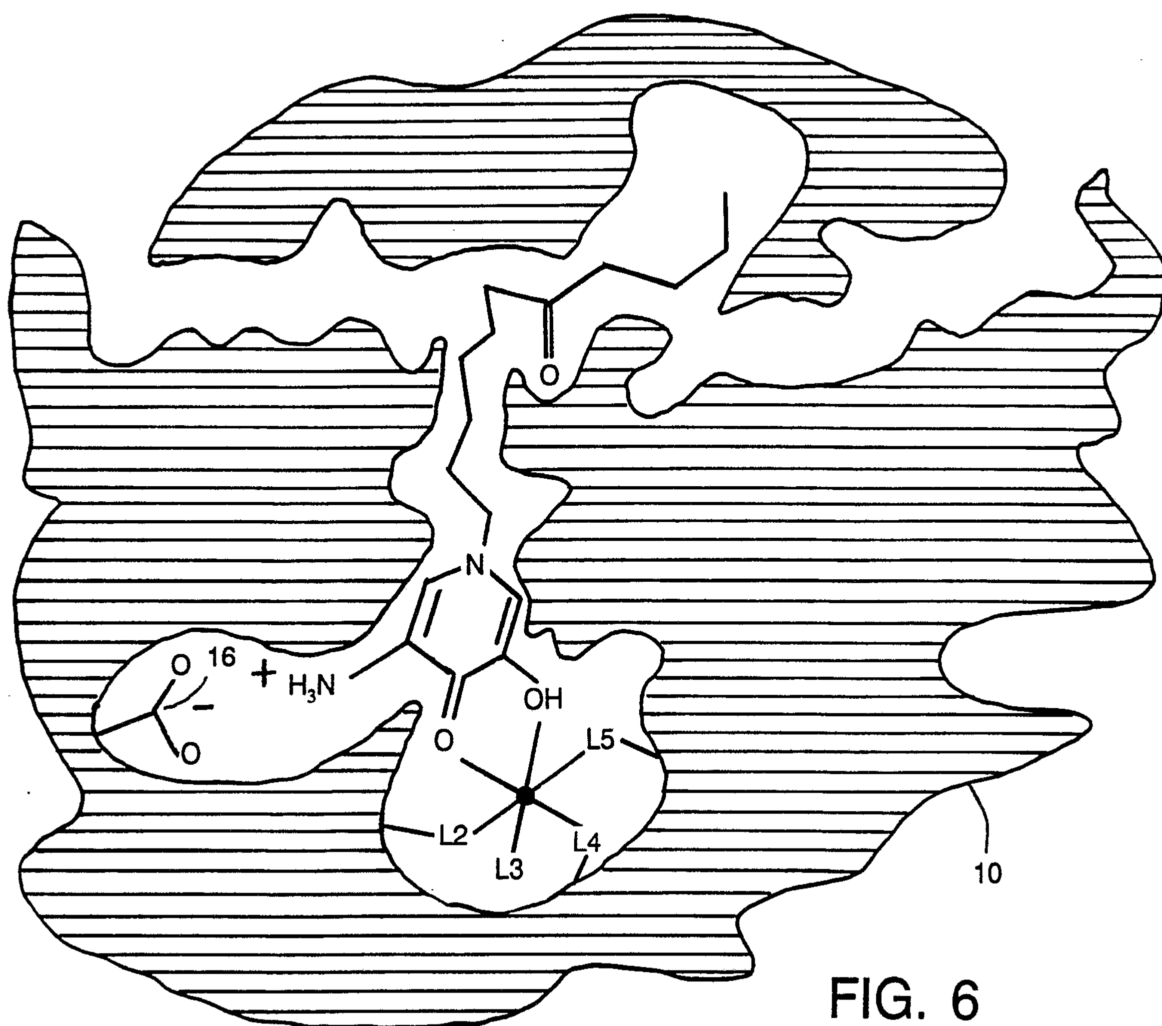


FIG. 6

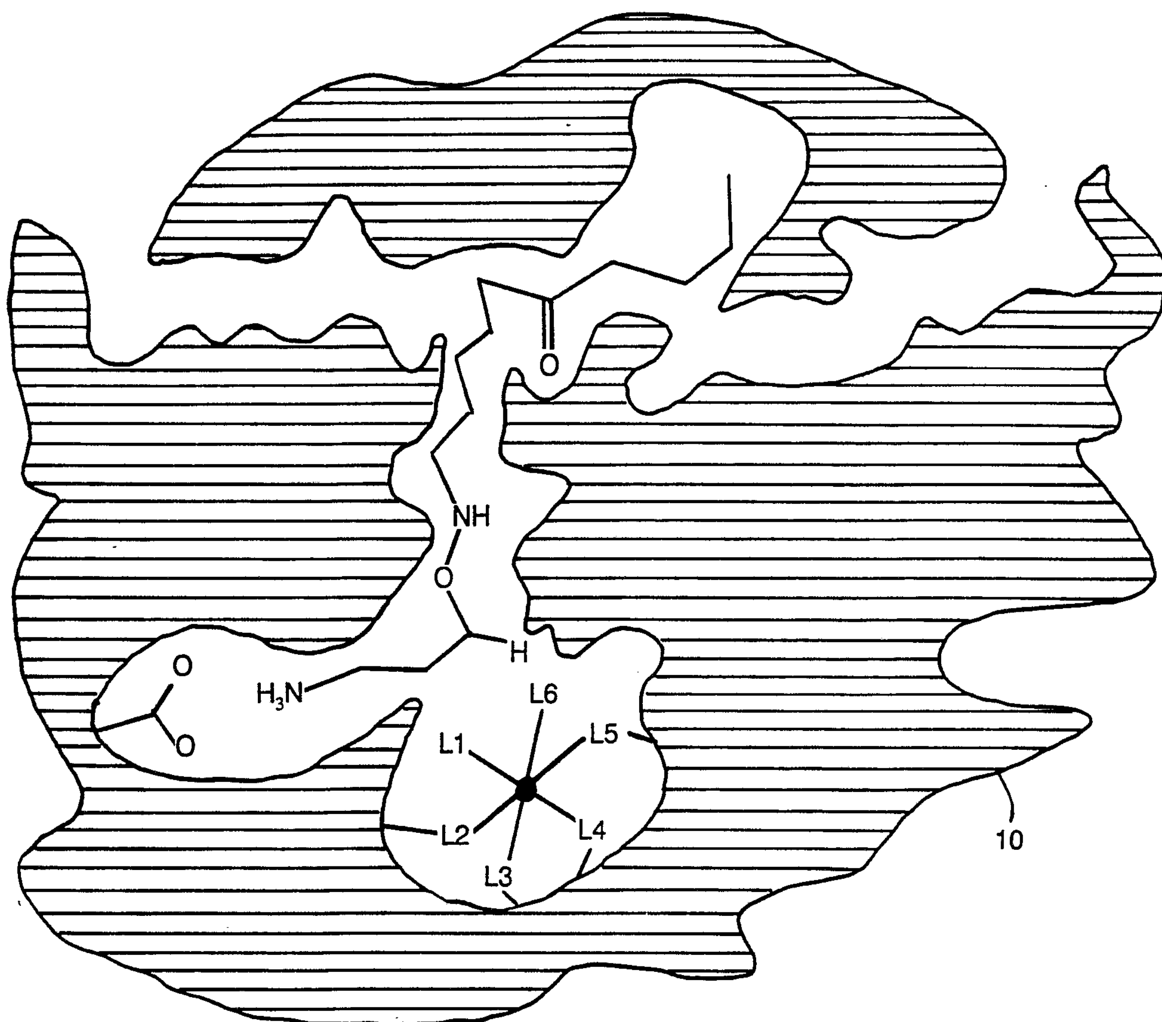


FIG. 7

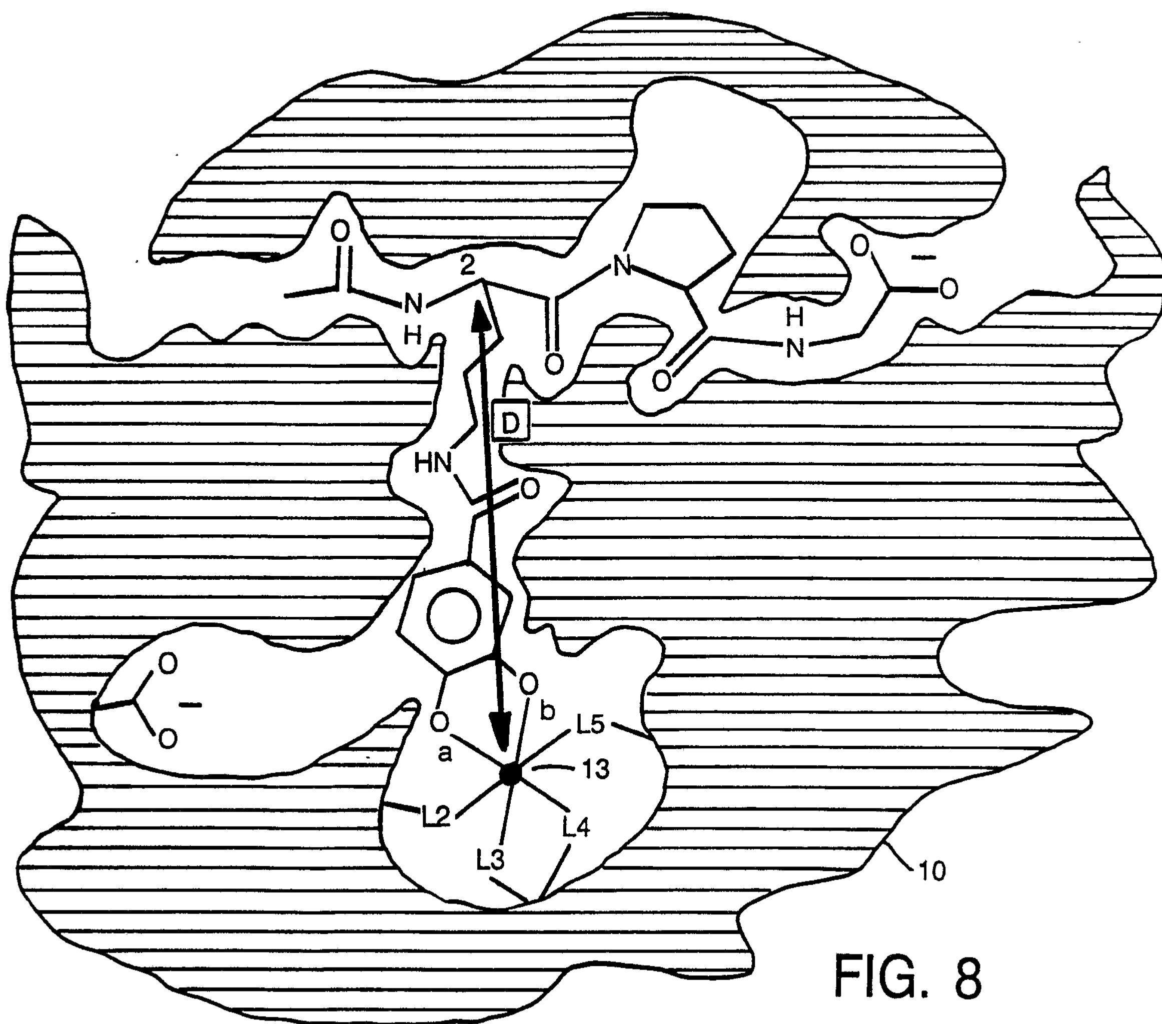


FIG. 8

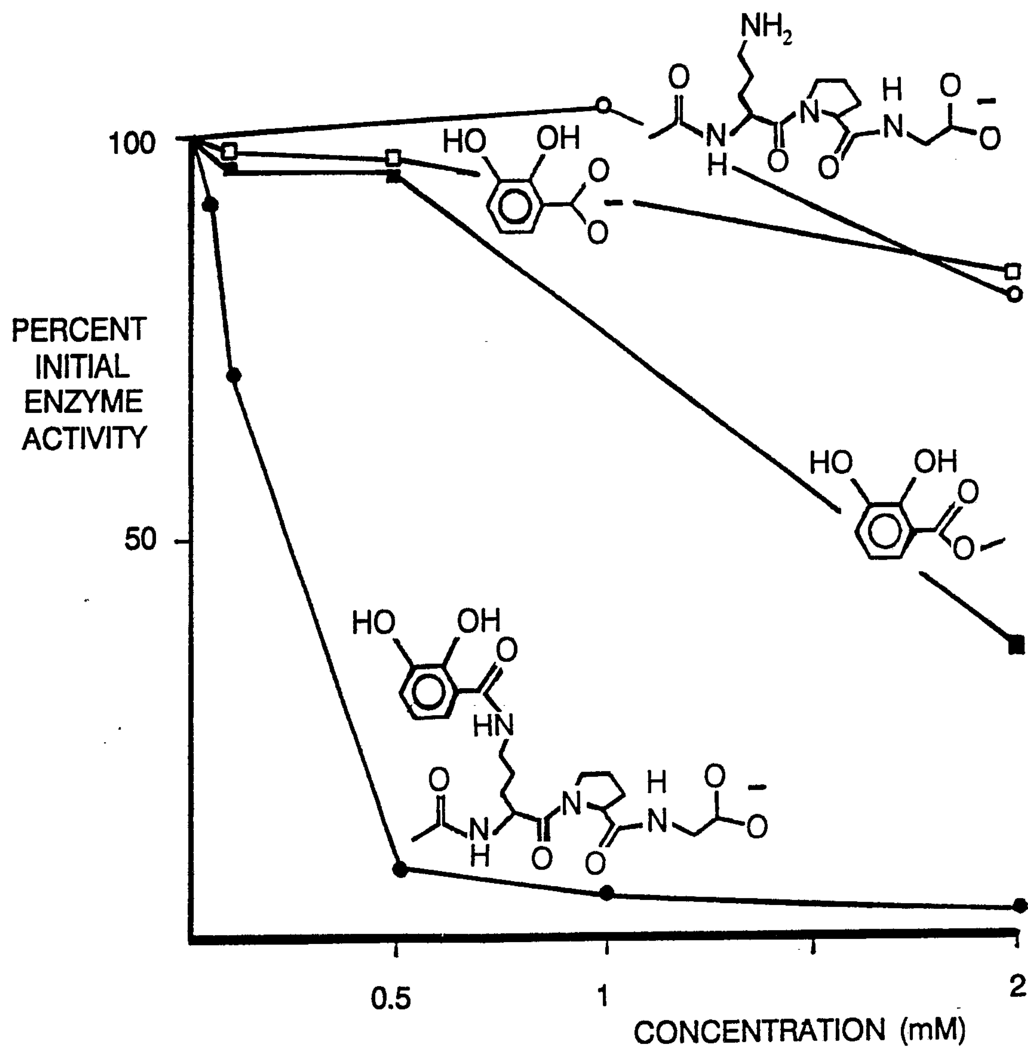


FIG. 9

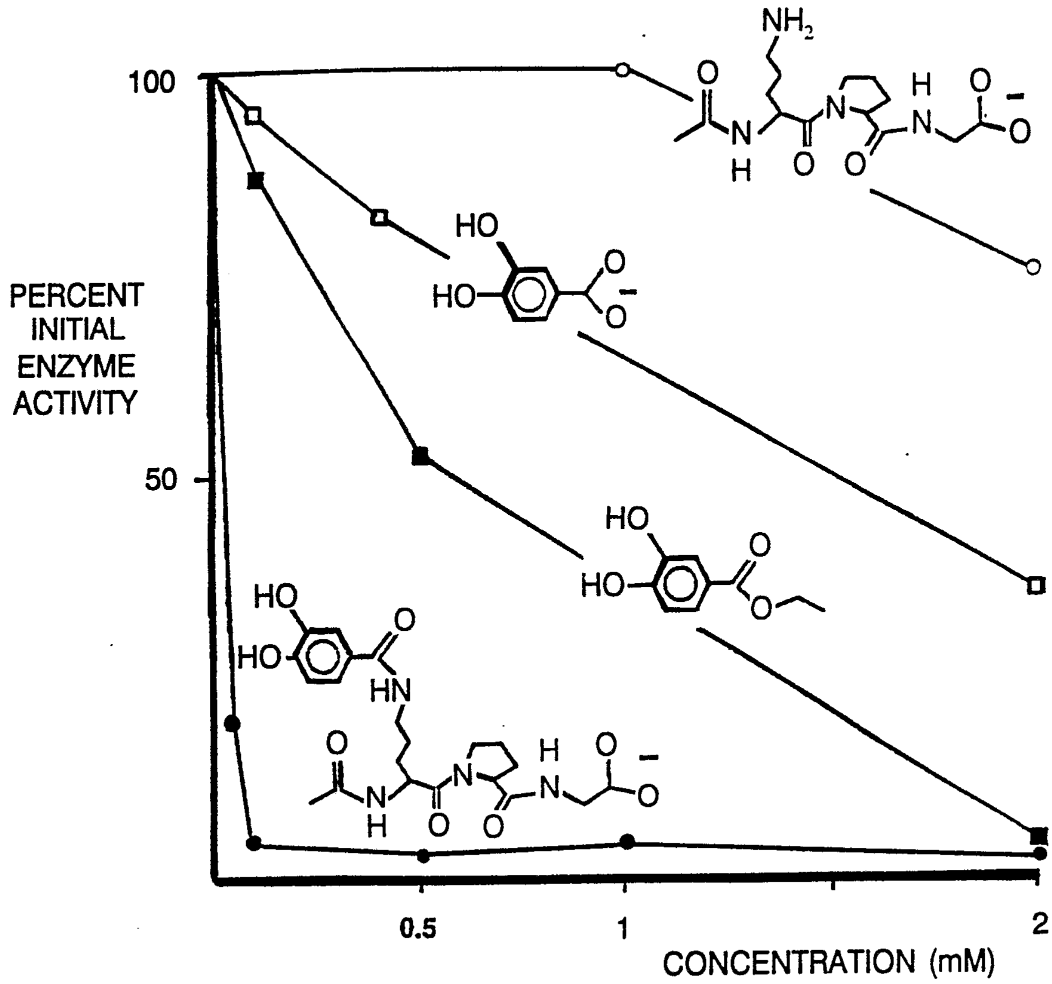


FIG. 10

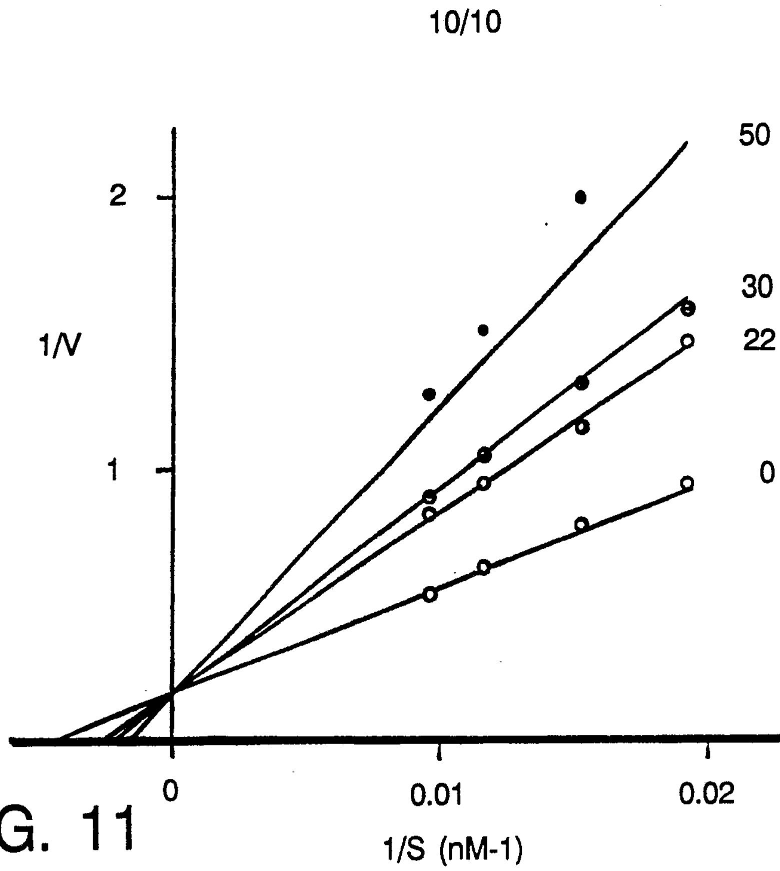


FIG. 11

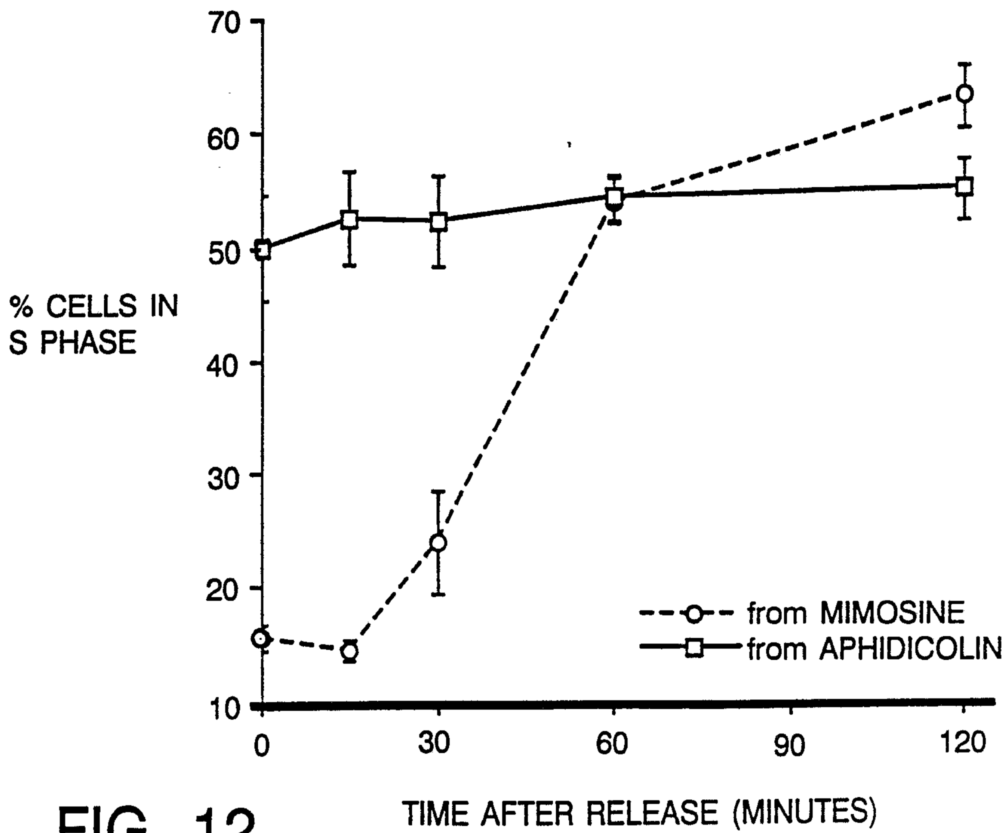
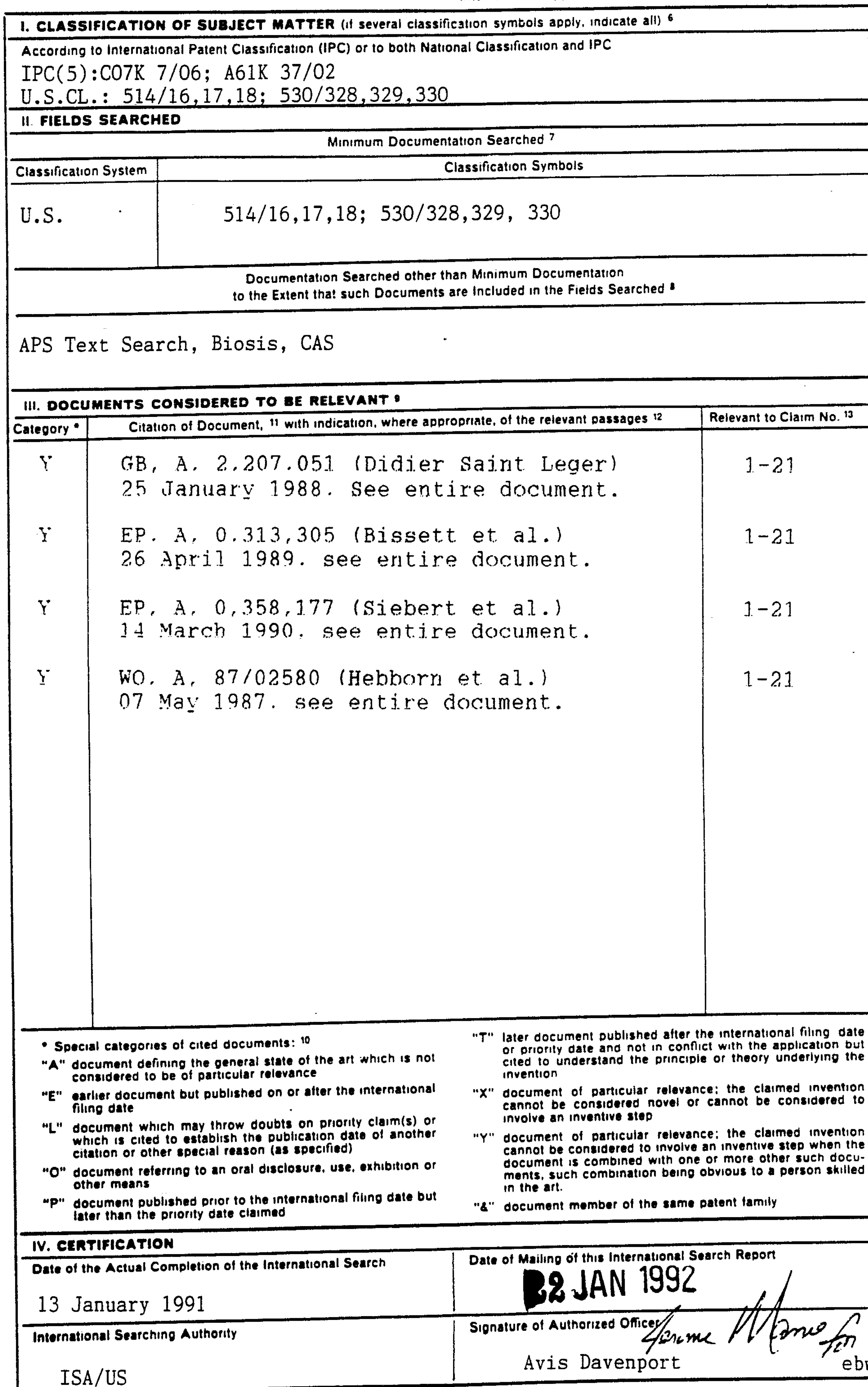


FIG. 12



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06721

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5):C07K 7/06; A61K 37/02
U.S.CL.: 514/16,17,18; 530/328,329,330

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/16,17,18; 530/328,329, 330

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS Text Search, Biosis, CAS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	GB, A, 2,207,051 (Didier Saint Leger) 25 January 1988. See entire document.	1-21
Y	EP, A, 0,313,305 (Bissett et al.) 26 April 1989. see entire document.	1-21
Y	EP, A, 0,358,177 (Siebert et al.) 14 March 1990. see entire document.	1-21
Y	WO, A, 87/02580 (Hebborn et al.) 07 May 1987. see entire document.	1-21

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 January 1991	22 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Avis Davenport ebw