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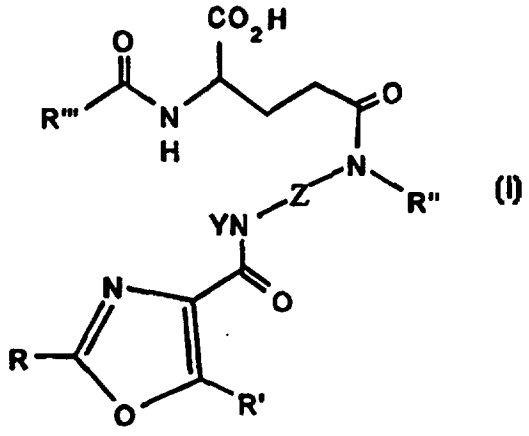
(54) Title: PHOSPHATASE INHIBITORS AND METHODS OF USE THEREOF

(57) Abstract

The present invention is directed to compounds having formula (I), wherein R, R' and R''' are the same or different and are preferably hydrophobic groups, Z represents one or two bivalent segments and Y is H or is absent when Z represents two bivalent segments. R'' may be the same or different as R, R' and R''', and is absent when Z represents two bivalent segments. The invention further provides a method of making compounds according to formula (I). The compounds are useful as inhibitors of protein phosphatases, for example PP1, PP2A, PP3, CDC25A, CDC25B and CDC25C. The invention is further directed to a method of inhibiting a protein phosphatase, a method of inhibiting cell proliferation, and pharmaceutical compositions comprising the subject compounds.



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Description

Phosphatase Inhibitors And Methods Of Use Thereof

The present application is a continuation-in-part of and claims priority rights based on United States Patent Application Serial No. 08/688,530, filed on July 30, 1996.

The regulation of protein phosphorylation by kinases and phosphatases controls many eukaryotic cell functions, including signal transduction, cell adhesion, gene transcription and cell proliferation. The identification and characterization of kinases, phosphatases, and inhibitors thereof thus allows pharmaceutical regulation of a variety of cellular functions. The present invention provides inhibitors of protein phosphatases, and methods of making and using the inhibitors. The compounds of the present invention are useful, for example, as inhibitors of cell proliferation.

Background of the Invention

Many eukaryotic cell functions, including signal transduction, cell adhesion, gene transcription, RNA splicing, apoptosis and cell proliferation, are controlled by protein phosphorylation. Protein phosphorylation is in turn regulated by the dynamic relationship between kinases and phosphatases. Considerable research in synthetic chemistry has focused on protein kinases. However, recent biological evidence for multiple regulatory functions of protein phosphatases has triggered further investigation of phosphatases. The protein phosphatases represent unique and attractive targets for small-molecule inhibition and pharmacological intervention.

Most eukaryotic amino acid phosphate derivatives in polypeptides and proteins are found on serine, threonine and tyrosine residues. Three basic types of eukaryotic protein phosphatases have been defined: serine/threonine protein

phosphatases (PSTPases), tyrosine protein phosphatases (PTPases), and dual-specificity phosphatases (DSPases). The DSPases dephosphorylate tyrosine and threonine residues on the same polypeptide substrate.

The serine/threonine protein phosphatases (PSTPases) are further classified into subfamilies (PP1, PP2A, PP2B, PP2C and PP3) by substrate specificity, metal ion dependence and sensitivity to inhibition. At least forty different enzymes of this type have been identified through DNA cloning. Potent inhibitors of the serine/threonine phosphatases have been identified, including proteins designated Inhibitor-1, Inhibitor-2, DARPP-32, and NIPP-1, which are reviewed for example by Honkanen et al. in Protein Kinase C, Kuo, ed., Oxford Univ. Press, Oxford, 1994, p.305. In addition, several toxins, mostly from marine organisms, have been identified as potent inhibitors of the serine/threonine phosphatases. The natural product inhibitors are depicted in Figs. 1(a) and 1(b), and discussed for example by Fujiki et al. (1993) Gazz. Chim. Ital. 123: 309.

Okadaic acid, a polyether fatty acid produced by several species of marine dinoflagellates, reversibly inhibits the catalytic subunits of serine/threonine phosphatase subtypes PP1, PP2A and PP3. However, okadaic acid does not rapidly penetrate cell membranes and accumulates within cells slowly, making it difficult to control the intracellular concentration of the compound. Further, okadaic acid is not very chemically stable.

Other natural product inhibitors have been identified that are more stable, may penetrate some cell types better, are more potent, and exhibit selectivity toward different PSTPase isotypes. Calyculin A is a cytotoxic component of the marine sponge Discodermia calyx. It has an extremely high affinity to PP1, PP2A and PP3, with an inhibitory concentration$_{50}$ (hereinafter "IC_{50}") of about 0.3 nM (IC_{50} represents the concentration that causes 50% inhibition compared to untreated control preparation) . Microcystins are potent cyclic hepta- and pentapeptide toxins of the general structure cyclo[D-Ala-X-D-*erythro*-b-methyl-*iso*-Asp-Y-Adda-D-*iso*-Glu-N-methyldehydro-Ala] wherein X and Y are variable L-amino

acids. Microcystins are known to promote tumors *in vivo*, but, with the exception of hepatocytes, are impermeable to most cells *in vitro*. Compounds of the nodularin series exhibit IC_{50}'s for PP1 and PP3 of about 2 and 1 nM, respectively. Motuporin, which has been recently isolated from a New Guinea sponge, is even more potent, with an IC_{50} of less than 1 nM for PP1. Tautomycin is produced by a terrestrial *Streptomyces* strain, and inhibits PP1, PP2A and PP3 indiscriminately with an IC_{50} in the 15 nM range. The remaining natural product inhibitors, thyrsiferal-23-acetate and cantharidine, are somewhat selective, but weak (IC_{50} of 0.16-10 μM), inhibitors of PP2A.

High toxicity, especially hepatotoxicity, is commonly found with the naturally occurring serine/threonine phosphatase inhibitors. The high toxicity appears to be intrinsically associated with non-specific phosphatase activity, and often limits the range of feasible pharmacological studies. Honkanen (1994) Toxicon 32: 339. Further, the chemical diversity of compounds obtained from natural sources is limited. Accordingly, there is a need in the art to diversify the chemical complexity of the natural products and to optimize biochemical and pharmacological effects.

However, only limited structure-activity relationship (hereinafter "SAR") studies have been reported on naturally occurring serine/threonine phosphatase inhibitors. For example, SAR studies of okadaic acid indicate that the carboxyl group as well as the four hydroxyl groups are important for activity. Nishiwki et al. (1990) Carcinogenesis 11:1837; Takai et al. (1992) Biochem J. 284:539; Sasaki et al. (1994) Biochem J. 288:259.

A limited SAR study of naturally occurring microcystins was performed by Rinehart et al. (1994) J. Appl. Phycol. 6: 159. It was found that the substitution of alanine for arginine has little effect on phosphatase inhibitory potency, but does result in a difference in relative cytotoxicity. The dehydroamino acid residue and the N-methyl substituents were also found to be noncritical. Esterification of the glutamic acid residue led to inactive compounds, and the (6Z) Adda isomer was

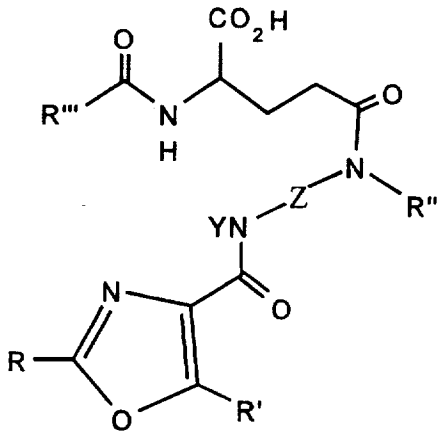
inactive, suggesting the criticality of the glutamic acid unit and the overall shape of the Adda residue. However, some variations in the Adda unit, for example the O-dimethyl and the O-dimethyl-O-acetyl analogs, exerted little effect on bioactivity. The general SAR of the nodularin series appears similar to the microcystins, although fewer compounds are available for testing. SAR studies have not been reported to date for calyculin A, tautomycin or thyrsiferyl acetate.

The DSPase class of phosphatases has recently been defined, and its members are emerging as important regulators of cell cycle control and signal transduction. The first documented DSPase, VH1, as described by Guan et al. (1992) Proc. Natl. Acad. Sci. USA 89: 12175, corresponds to the H1 open reading frame of Vaccinia virus. Other members of the DSPase class have been identified and generally fall into two substrate motifs, the VH1 type and the CDC25 type. Mammalian cells contain at least three CDC25 homologues (CDC25A, CDC25B and CDC25C). The CDC25 phosphatases are positive regulators of cell cycle progression, and are reviewed by Hunter et al. (1994) Cell:573. Further, there is a strong link between overexpression of the CDC25 phosphatases and oncogenic transformations, particularly in human breast cancer. Galaktinov et al. (1995) Science 269: 1575. Four compounds with anti-DSPase activity have been isolated from natural sources. Two benzoquinoid antitumor antibiotics, dnacin A1 and dnacin B1 (See Fig. 1(a)), have been reported to inhibit CDC25B DSPase activity non-competitively with IC_{50} of 141 and 64 μM, respectively. (See Horiguchi et al. (1994) Biochem. Pharmacol. 48: 2139. Also, dysidolide has been reported to inhibit the dephosphorylation of p-nitrophenol phosphate by CDC25A with an IC_{50} of 9.4 μM. (See Fig. 1(a)). Recently, the Streptomyces isolate, RK-682, was shown to be an inhibitor of the dual specificity phosphatase VHR. The IC_{50} was 1μM. However, no potent inhibitors of the DSPases which are easily synthesized and have relatively low molecular weights are known. Moreover, none are known to selectively inhibit DSPases.

Since nearly all forms of human neoplasias have altered cell cycle control, the role of phosphatases in cell cycle control makes these molecules attractive targets for pharmaceutical intervention. The ability of phosphatase inhibitors to interfere with aberrant cell activity has been demonstrated. For example, the naturally occurring PSTPase inhibitor okadaic acid has been shown to induce apoptosis in myeloid leukemia cells (Ishida et al. (1992) J. Cell. Physiol. 150: 484) and in rat hepatocytes, rat pituitary adenoma cell, human mammary carcinoma cells and human neuroblastoma cells (Boe et al. (1991) Exp. Cell Res. 195: 237). Thus there is a significant need to design and synthesize selective modulators of this family of enzymes in order to identify useful therapeutic agents.

Summary of the Invention

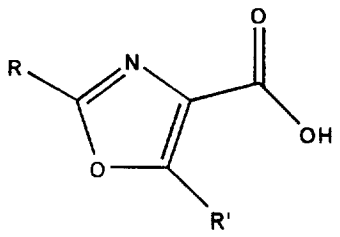
The present invention provides a compound having the formula I:



I

wherein R, R′ and R‴ are the same or different and are preferably hydrophobic groups. Z represents one or two bivalent segment(s) which are bound to the two central nitrogen atoms as shown in formula I. If Z represents a single bivalent segment Y is H. If Z represents two bivalent segments Y is absent. R″ is absent if Z represents two bivalent segments. If Z represents a single bivalent segment, R″ is the same as or different from R, R′ and R‴ and is preferably a hydrophobic group.

In another embodiment, the present invention provides a method of synthesizing a compound having formula I. The method of synthesis comprises coupling glutamate to a solid support, adding the hydrophobic residue R''' COX, wherein X is a leaving group, adding diamine having the formula -NYZNH(R''), adding oxazole carboxylic acid having the formula II



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wherein R and R' are the same or different and are preferably hydrophobic groups, and cleaving the resulting compound from the solid support.

The present invention further provides a method of inhibiting a protein phosphatase comprising contacting a phosphatase-inhibiting effective amount of a compound of formula I with a protein phosphatase under conditions whereby the activity of the protein phosphatase is inhibited.

The present invention also provides a method of inhibiting cell proliferation comprising introducing into cells a proliferation-inhibiting amount of a compound of formula I. In a preferred embodiment the cells are human breast cancer cells or murine SCCVII/SF head and neck cancer cells.

Pharmaceutical compositions comprising a compound of formula I and a pharmaceutically acceptable carrier are also provided by the present invention.

Brief Description of the Drawings

Fig. 1(a) depicts the formulae of naturally occurring inhibitors of the serine/threonine phosphatases.

Fig. 1(b) compares the structures of compounds according to formula I, wherein Y is H and Z is a bivalent ethylene segment, with the structures of the natural products Microcystin-LR and calyculin A.

Fig. 2 presents a representative scheme for the synthesis of compounds having formula I.

Fig. 3 presents a representative scheme for a synthetic route to monoprotected diamines.

Fig. 4(a) shows a scheme for the synthesis of a heterocyclic moiety from N-benzoyl threonine.

Fig. 4(b) shows an alternate scheme for the synthesis of a heterocyclic moiety.

Fig. 5 shows a scheme for the solution phase synthesis of model compound II.

Fig. 6 is a graph of inhibition of PP2A activity by compound 1d. The catalytic subunit of PP2A was incubated with vehicle alone (control), calyculin A (10 nM) or compound 1d (100 uM), and the dephosphorylation of the substrate fluorescein diphosphate determined spectrophotometrically. Mean results of two independent experiments are shown.

Fig. 7 presents a graph depicting the ability of compounds 1(a)-(r) to inhibit CDC25A and CDC25B activity. Results are presented as percentage of control (100%).

Fig. 8 presents a dose-response curve of the ability of compound 1f to inhibit CDC25A.

Fig. 9 is a graph showing the anti-proliferative effect of compound 1f against human MDA-MB-231 breast cancer cells.

Figs. 10A-D depict cell cycle distribution of human breast cancer cells after treatment with compound 1f determined by flow cytometry. Fig. 10A shows flow cytometry analysis of MDA-MB-231 cells treated with vehicle alone. Fig. 10B shows flow cytometry analysis 48 hours after treatment with 88 μM compound 1f.

Fluorescence channel measures intracellular propidium iodide concentration, an index of DNA content. Horizontal bars are the gating positions that allow for cell cycle analysis. Fig. 10C shows MDA-MB-231 cell cycle distribution 48 hours after continuous treatment with 88 μM compound 1f, and is the result of one experiment. Open bars are control cells and black bars are cells treated with compound 1f. Fig. 10D shows cell cycle distribution 72 hours after continuous treatment with 88 μM compound 1f. The mean values were obtained from three independent determinations. Open bars are control cells and black bars are cells treated with 88 μM compound 1f. The standard errors of the means are displayed.

Fig. 11 depicts a general formula of compounds according to formula I synthesized via the combinatorial methods described herein wherein Z represents various bivalent segments.

Figs. 12(a)-12(n) depict the specific structural formulae of fourteen compounds according to formula I having various Z substitutions. These compounds were synthesized via the combinatorial methods as described herein.

Fig. 13 presents a graph depicting the ability of compounds 1(a)-1(r) from Table I, compounds SC-ααδ9 and SC-αα69, and the compounds depicted in Figs. 12(a)-12(n) to inhibit PTP1B. Results are presented as percentage of inhibition.

Fig. 14 presents a graph depicting the ability of the compounds depicted in Figs. 12(a)-12(n) and the compounds SC-ααδ9 and SC-α1δ9 (i.e., compound 1f and 1c, respectively, of Table I resynthesized by solution phase methods) to inhibit the activities of CDC25C, PP-1, PP-2A and PTP1B. The potential inhibitors were tested at 100 μM.

Figs. 15(a)-15(d) present graphs depicting the concentration dependance of the ability of compounds 1e and 1f and their solution chemistry analogues, which are SC-αα69 and SC-ααδ9, respectively, to inhibit CDC25A and CDC25B.

Fig. 16 presents a graph depicting the cytotoxicity of compounds 1a-1r to murine SCCVII/SF cells. Exponentially growing SCCVII cells were exposed to two ten-fold different concentrations of each drug continuously for 48 hours. The

average drug concentrations were 80 μM (black bars) and 800 μM (open bars). The total cell number was determined by the assay described in Example III. Each value is the mean of three determinations performed in quadruplicates.

Fig. 17 presents a graph depicting the concentration-dependant cytotoxicity response of murine SCCVII/SF cells in culture to SC-$\alpha\alpha$69 (i.e., compound 1e of Table I prepared by solution chemistry techniques) and SC-$\alpha\alpha\delta$9 (i.e., compound 1f of Table I prepared by solution chemistry techniques).

Fig. 18 presents a graph depicting the results of an SCCVII/SF tumor excision clonogenic assay for compounds SC-$\alpha\alpha$69 and SC-$\alpha\alpha\delta$9.

Fig. 19 presents a Western blot of SV-40 large T-antigen (Panel A), CDC25B (Panel B) and CDC25A (Panel C) expression in mouse embryonic fibroblasts (hereinafter "MEF") and MEF/SV40 cells. Cell lysates were isolated from MEF and SV-40/MEF cells as described in Example XII, separated by and immunoblotted with antibodies against SV40 large T antigen (Ab-2, Oncogene) or CDC25B (C-20, Santa Cruz). The data shown are representative of three independent experiments.

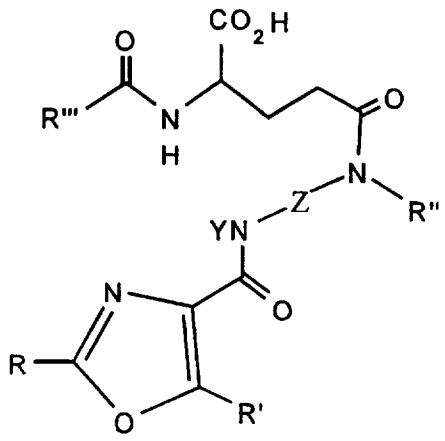
Figs 20(a)-20(c) present a concentration-dependent cytotoxicity response of MEF and MEF/SV40 cells to okadaic acid, vanadate or compound 1(f). MEF and SV40/MEF were plated in 96 well plates. After 24 hours, various concentrations of okadaic acid (Panel A), vanadate (Panel B) or SC-$\alpha\alpha\delta$9 were added. Cell viability was determined 48 hours later using the microtitration assay as described in Example XII. Each value is the mean of 3-4 independent experiments performed in quadruplicate. The bars stand for SEM.

Fig. 21 depicts a graph of plating efficiency versus concentration of compound SC-$\alpha\alpha\delta$9. MEF and SV40/MEF were plated in triplicate in 6 well plates and treated with vehicle or inhibitor as described in Example XII. After 10-12 days of continuous exposure to the drug, colonies were stained with crystal violet and were counted. Data are representative of five independent experiments.

This data shows that compound SC-ααδ9 selectively inhibited transformed cell clonogenic growth

Detailed Description of the Invention

The present invention provides compounds having the formula I as follows:



I

wherein R, R′ and R‴ are the same or different and are preferably hydrophobic groups. Z represents one or two bivalent segment(s) which are bound to the two central nitrogen atoms as shown in formula I. The shortest path through Z between the two central nitrogen atoms is preferably less than ten atoms. If Z represents a single bivalent segment, Y is H. If Z represents two bivalent segments, Y is absent. R″ is absent if Z represents two bivalent segments. If Z represents a single bivalent segment R″ is the same as or different from R, R′ and R‴ and is preferably a hydrophobic group.

In preferred compounds according to formula I, Y is H; Z is a bivalent segment selected from the group consisting of an alkylene segment, an alkenylene segment, an alkynylene segment, a cycloalkylene segment, an aromatic hydrocarbon segment, and heteroatom-substituted variants of these same bivalent segments; and R, R′, R″ and R‴ are independently H, alkyl, alkenyl, alkynyl, cycloalkyl, phenyl (Ph), oxetanyl, azetidinyl, furanyl, pyrrole, indolyl, oxazolyl,

isoxazolyl, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, pyranyl, pyridyl, pyridonyl, piperidyl, piperazinyl, quinolyl, azepinyl, and diazepinyl.

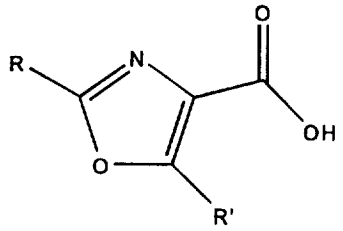
More preferably, in the compounds according to formula 1, Y is H; Z is a bivalent segment selected from the group consisting of an alkylene segment, an alkenylene segment, an alkylidenylene segment, and alkynylene, a cycloalkylene segment, an aromatic hydrocarbon segment, and heteroatom-substituted variants of these same bivalent segments; R and R‴ are independently Ph, CH_3, n-C_5H_{11}, n-C_7H_{15}, n-C_9H_{19}, PhCHCH, $PhCH_2CH_2$, $Ph(CH_2)_2CC(CH_3)$, (p-MeO)Ph, (p-MeNHCO)Ph, $PhCHC(CH_3)CH_2CH_2$, $Ph(CH_2)_2CHCHCHC(CH_3)$, $Ph(CH_2)_2CHCHCHCH$, $Ph(CH_2)_3CHC(CH_3)CHCH$, $C_6H_{13}CH(CH_3)CHC(CH_3)CHCH$, or $C_4H_9CH(CH_3)CHC(CH_3)CHC(CH_3)$; and R′ is H, CH_3 or Ph, and R″ is H, CH_3, benzyl (Bn), $CH_2CH(CH_3)$, n-C_6H_{13}, CH_2CH_2NHBn, CH_2CH_2Ph, or $(CH_2)_3Ph$.

Most preferably, Y is H; Z is a bivalent ethylene segment (i.e., -CH_2CH_2-); and R is Ph, R′ is Ph, R″ is Bn or CH_3 and R‴ is n-C_9H_{19}.

In an alternative embodiment, Y is absent; R″ is absent; Z is two bivalent segments which together form the atoms necessary to form a fused piperidine ring which incorporates the two central nitrogen atoms shown in formula I; and R, R′, and R‴ are independently H, alkyl, alkenyl, alkynyl, cycloalkyl, phenyl (Ph), oxetanyl, azetidinyl, furanyl, pyrrole, indolyl, oxazolyl, isoxazolyl, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, pyranyl, pyridyl, pyridonyl, piperidyl, piperazinyl, quinolyl, azepinyl, and diazepinyl.

The compounds of the present invention may be synthesized by solid-phase combinatorial chemistry techniques. The present invention provides a method of synthesis of the compounds of the invention by a single-bead combinatorial strategy whereby the structure of each compound is known. The present method thus avoids the difficulties inherent in other prior art combinatorial syntheses, for example the need for sophisticated tagging schemes or extensive analytical techniques to identify the synthesized compounds.

The method of synthesizing the compounds of the present invention comprises coupling a diprotected glutamate moiety to a solid support, deprotecting the glutamate amino-terminus, adding the hydrophobic residue R‴ COX, wherein X is a leaving group such as chloride, anhydride, active ester, pentafluorophenyl, phosphate derivative or phosphonate derivative, deprotecting the glutamate carboxy-terminus, adding protected diamine having the formula A-NYZNH(R″) wherein A is a protecting group, deprotecting the amino-terminus of the diamine, adding an oxazole carboxylic acid having the formula II:



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and cleaving the resulting compound from the solid support. By coupling protected glutamate to the solid support on a large scale, and distributing batches of the growing compound to different vessels at each step, for example after the removal of each protecting group, a large number of compounds can be synthesized during each reaction run by varying the R, R′, R″ and R‴ groups. A representative synthetic scheme is set forth in Fig. 2.

Solid supports for combinatorial synthesis are known to those of ordinary skill in the art. In a preferred embodiment of the present invention, the solid support is a polystyrene resin. In a more preferred embodiment, the solid support is the polystyrene resin described by Wang (1973) J. Amer. Chem. Soc. 95: 1328, and also known as the Wang resin. The Wang resin is commercially available, for example from Advanced Chemtech. Other suitable solid supports include polyethylene glycol-polystyrene graft or Rink resins.

Diprotected glutamate may be prepared by methods known in the art. The skilled artisan is aware of suitable protecting groups for the amino- and carboxy-termini of glutamate, which are reviewed for example by Greene et al. Protective

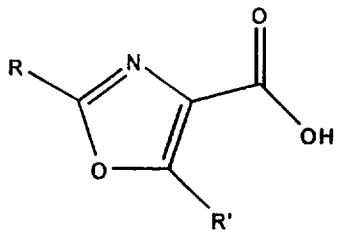
groups in organic synthesis, 2nd edition, Wiley, New York, 1991. In a preferred embodiment the amino-terminal protecting group is Fmoc and the carboxyl-terminal protecting group is a γ-allyl ester group. This preferred diprotected glutamate may be synthesized by protecting the carboxylic function with the allyl ester as described by Belshaw et al. (1990) Syn Comm. 20: 3157, followed by treatment with Fmoc-Cl. According to the method of Belshaw et al., chlorotrimethylsilane is added to a suspension of L-glutamic acid in dry allyl alcohol under N_2 and stirred for 18 hours, followed by the addition of ethyl ether.

Diprotected glutamate is coupled to a solid support by methods known to those of ordinary skill in the art and appropriate for the desired support. In a preferred embodiment, diprotected glutamate is coupled to the Wang resin with 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDCl) on a large scale to provide a supply of solid phase beads coupled to diprotected glutamate. Other suitable coupling methods are reviewed for example by Bodanszky, Principles of peptide synthesis, 2nd edition, Springer, Berlin, 1993, and include DCC, HOBt, BOC reagents, and others.

The glutamate amino-terminus is deprotected by a method appropriate to the protecting group. For example, a base labile group such as Fmoc may be removed by treatment with piperidine and tetrahydrofuran (THF). At this point the resin may be distributed to a number of separate vessels in order that compounds having different R‴ substituents may be prepared. For example, the resin may be distributed to multiple filters equipped with inert gas inlets for maintaining steady bubbling and suction adapters. After the addition of solvent, hydrophobic residues R‴ COX are added to each flask. Thus different amide derivatives are provided, the number of which is determined by the number of vessels to which the resin has been distributed and the number of different R‴ substituents. By adding R‴ COX having differing R‴ groups to each vessel, the final compounds can be conveniently identified. R‴ CO_2H or R‴ COX may be prepared by standard solution synthesis or obtained commercially.

After filtration and rinsing of the solid support, the carboxy-terminal protecting group is removed by a method suitable for the protecting group. For example, allyl esters may be removed by Pd(0) chemistry as described by Dangles et al. (1987) J. Org. Chem. 52: 4984. The resin may again be divided into batches at this point and distributed into separate vessels such as the filters described above so that compounds containing different R″ groups may be prepared. The protected diamine is then added with a suitable coupling agent so that the side chain carboxyl terminus of glutamate is extended. In a preferred embodiment the diamine is ethylene diamine, and the protecting group of the diamine is an N-allyloxycarbonyl group such that the diamine has the formula Alloc-$NHCH_2CH_2NH(R'')$. Suitable coupling agents include for example PyBroP[60] and CloP[61]. Alloc-$NHCH_2CH_2NH(R'')$ may be conveniently prepared by carbamoylation of chloro-ethylamine followed by treatment with sodium iodide and commercially available amine $R''NH_2$, as depicted in Fig. 3. Protected diamines other than ethylene diamine may be prepared similarly.

The resulting compounds are deprotected, at which point the compounds may be again distributed to different vessels for the addition of different R and R′ substituents. Coupling with oxazole carboxylic acids having the formula II



II

wherein R and R′ are as defined above for formula I, in the presence of CloP, followed by rinsing with solvent provides the compounds of the invention attached to the solid support. The oxazole carboxylic acids may be prepared separately in solution phase from carboxylic acids having the structure R-CO_2H wherein R is as defined above for formula I and serine methyl ester, threonine methyl ester and phenyl serine methyl ester. An oxidation-cyclodehydration protocol depicted in

Fig. 4(a) and described by Wipf et al. (1993) J. Org. Chem. 58: 3604, followed by saponification yields the desired carboxylic acid segments. The intermediate oxazole esters may be purified by column chromatography on SiO_2.

In an alternative synthesis, which is a modification of protocols by Vorbrüggen et al. (1993) Tetrahedron 49: 9353 and Williams et al. (1997) Tetrahedron 38: 331, a one-pot condensation/dehydration of carboxylic acids and serine methyl esters provides oxazoles in >50% yield. Bromination at the C(5) position and Palladium-catalyzed coupling can provide a large variety of substitution patterns for these moieties. This synthesis method is depicted in Fig. 4(b).

The carboxylate may be released from the support by complete or partial cleavage with 50% trifluoroacetic acid to provide the compounds of the invention. After filtration of the solid support and evaporation of the resulting mother liquor, the compounds of Formula I are chemically pure and structurally well-defined.

It has been found in accordance with the present invention that compounds of formula I are capable of inhibiting serine/threonine protein phosphatases. Inhibition of serine/threonine protein phosphatases is defined herein as inhibition of the activity of one or more of PP1, PP2A or PP3 at a concentration of 100 μM or less of the inhibitor compound. In a preferred embodiment, the activity of the phosphatase is inhibited by at least 10%. In more preferred embodiments, the activity of the phophatase is inhibited by at least 25%, or even more preferably, by at least 50%. Inhibition of PP1, PP2A or PP3 can be assessed by methods known to one of ordinary skill in the art. Suitable assays are described, for example by Honkanen et al. (1994) Toxicon 32:339 and Honkanen et al. (1990) J. Biol. Chem. 265: 19401, the disclosures of which are incorporated herein by reference. Briefly, phosphatase activity is determined by quantifying the [^{32}P] released from a ^{32}P-labeled substrate such as phosphohistone or phosphorylase-a. Decreased [^{32}P] release in the presence of the compounds of the present invention relative to

control samples provides a measure of the ability of the compounds of the invention to inhibit PP1, PP2A or PP3.

In another suitable assay, the ability of the compounds of the present invention to inhibit the activity of protein phosphatase PP2A is assessed. The activity of the catalytic subunit of bovine cardiac muscle PP2A (Gibco-BRL, Gaithersburg, MD) is measured in 96-well microtiter plates using the substrate fluorescein diphosphate as follows. Inhibitors are resuspended in DMSO, which is also used as the vehicle control. An incubation mixture of 150 μL is prepared containing 25 mM Tris, pH 8.0, 5 mM EDTA, 33 μg/mL BSA, 20 μM fluorescein diphosphate, and 100 μM inhibitor or DMSO control. Reactions are initiated by adding 0.2 units of PP2A, and incubated at room temperature overnight. Fluorescence emission from the product is measured spectrofluorometrically, for example with Perseptive Biosystems Cytofluor II (Framingham, MA) (excitation filter, 485 nm; emission filter, 530 nm). The rate of increase in absorbance due to formation of dephosphorylated substrate is proportional to phosphatase activity. Thus, decreased absorbance relative to control samples provides a measure of the ability of the present compounds to inhibit the phosphatase.

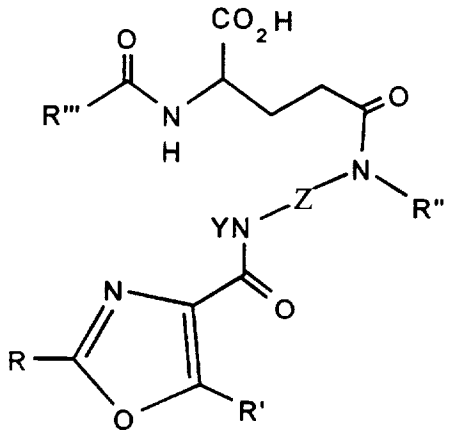
The compounds of the present invention are also capable of inhibiting dual specificity phosphatases, for example CDC25A, CDC25B and CDC25C. Phosphatases CDC25A and CDC25B are disclosed in U.S. Patent 5,441,880. Methods of isolating recombinant GST-CDC25A, GST-CDC25B, GST-CDC25C vaccinia human related phosphatase (hereinafter "VHR") and mitogen activated protein kinase phosphatases (hereinafter "MAPKP") are described by Baratte et al. (1992) Anticancer Res. 12: 873, the disclosure of which is hereby incorporated by reference herein. Inhibition of dual specificity phosphatases is defined herein as inhibition of the activity of CDC25A, CDC25B and CDC25C at a concentration of 100 μM of the inhibitor compound. In a preferred embodiment, the activity of the phosphatase is inhibited by at least 10%. In more preferred embodiments, the activity of the phophatase is inhibited by at least 25%, or even more preferably, by

at least 50%. The ability of the compounds of the present invention to inhibit the phosphatases can be determined by measuring the effect of the compounds on the ability of CDC25A, CDC25B, CDC25C, VHR on MAPKP to dephosphorylate a substrate. Appropriate methods are known to those of ordinary skill in the art, and include, for example, colorimetric assays. A suitable assay is described in U.S. Patent 5,441,880, the disclosure of which is incorporated herein by reference. As disclosed therein, the compound to be tested is combined with CDC25A, CDC25B or CDC25C and an appropriate CDC25 substrate, such as p-nitrophenyl phosphate or inactive cyclin/cdc2, and the ability of the compound to inhibit the phosphatase activity of CDC25 is assessed. Phosphatase activity may be assessed by known techniques, such as measuring optical density and comparing it to the optical density of a control sample that does not contain the inhibitor. The assay may be performed as a rapid colorimetric microtitration plate assay. It was found that a suitable assay employed a 20μM solution of 3,6 fluorescein diphosphate as a substrate incubated with ~250ng of recombinant protein and 1mM DTT at room temperature for 60 minutes. The fluorescence emission was monitored as described above.

The compounds of the present invention are useful as inhibitors of protein phosphatases. It is known that inhibition of protein phosphatases results in increased protein phosphorylation in vitro and in cells. Sassa et al. (1989) Biochem. Biophys. Res. Comm. 159: 939; Yatsunami et al. (1991) Biochem. Biophys. Res. Commun. 177: 1165. Thus the compounds are useful to inhibit protein dephosphorylation, for example in in vitro assays in which phosphorylated proteins are measured or detected, or in methods in which proteins are labeled by phosphorylation. For example, proteins are commonly labeled with ^{32}P to facilitate detection. Inclusion of a compound of the present invention is useful to prevent dephosphorylation by endogenous phosphatases. Further, phosphatases are known to have multiple functions, including but not limited to regulation of signal transduction, cell adhesion, gene transcription, RNA splicing, apoptosis,

mitosis and cell proliferation. Thus inhibitors of protein phosphatases are useful in the alteration of various cellular functions. In particular, the present compounds are useful as inducers of apoptosis and inhibitors of cell proliferation.

Accordingly, the present invention provides a method of inhibiting a protein phosphatase comprising contacting a phosphatase-inhibiting effective amount of a compound having the formula:



I

with a protein phosphatase under conditions whereby the activity of the protein phosphatase is inhibited. The substituents Z, Y, R, R′, R″ and R‴ are as previously described.

Compounds having a bulky moiety in the R″ position are believed to be superior inhibitors. Examples of bulky moieties include substituents such as isobutyl, isopropyl, benzyl and neopentyl.

In a preferred embodiment the protein phosphatase is a serine/threonine phosphatase. In a more preferred embodiment the protein phosphatase is PP1, PP2A or PP3. In another preferred embodiment the phosphatase is a dual specificity phosphatase, including, for example, CDC25A, CDC25B, CDC25C, VHR and MAPKP. The skilled artisan can readily determine the amount of the phosphatase inhibitor that is required to inhibit protein phosphatase by measuring

phosphatase activity in the presence and absence of the inhibitor. Phosphatase activity can be determined by assessing the dephosphorylation of a substrate as described hereinabove, or by measuring parameters that are known to result from phosphatase activity. For example, phosphatase inhibition by the compounds of the present invention can be assessed by measuring inhibition of cell proliferation in response to treatment of cells with the present compounds.

The compounds of the present invention are also useful as antiproliferative agents. The CDC25 enzymes are positive regulators of cell cycle progression. For example, CDC25C drives entry into mitosis by dephosphorylating and thereby activating the mitotic inducer cdc2. U.S. Patent No. 5,294,538 discloses that dephosphorylation of cdc2 by the CDC25 phosphatase activates the M phase-promoting factor (MPF) that triggers the G2/M transition of the cell cycle, and that inhibition of the CDC25 phosphatase activity inhibits entry of cells into mitosis. Further, CDC25A and CDC25B act as oncogenes, and CDC25B is over-expressed in one third of primary human breast cancers (Galaktionov et al., (1995) Science 269: 1575) and is elevated in other human tumor cell types and in virally transformed cells (Nagata et al. (1991) New Biologist 3:959). It is also known that cell proliferation is coordinated by cyclin-dependent kinases, and tightly controlled by both kinases and phosphatases. The cell cycle regulating activity of CDC25 is controlled by PP2A. Hunter et al. (1994) Cell 79: 573. Thus, inhibition of PP1 or PP2A may also result in disrupted cell cycle transition.

It has been demonstrated in accordance with the present invention that compounds of the invention inhibit proliferation of cells by reducing the number of cells in inhibitor-treated versus untreated cell cultures. Accordingly, the present invention further provides a method of inhibiting cell proliferation comprising introducing into cells a proliferation-inhibiting amount of a compound of formula I. In a preferred embodiment the compound has formula I wherein Z is an ethylene segment, Y is H, R is Ph, R′ is Ph, R″ is Bn and R‴ is n-C_9H_{19}. In another preferred embodiment the cells are tumor cells. In still another preferred

embodiment the cells are human breast cancer cells. In yet another preferred embodiment, the cells are murine SCCVII/SF head and neck cancer cells which grow readily as tumors in mice and have been shown by Western blotting to be CDC25A positive.

The ability of the compounds of the present invention to inhibit proliferation can be assessed by methods known to those of ordinary skill in the art. For example, proliferating cells can be contacted with a compound of the invention, and cell numbers determined. A reduction in cell number in treated versus untreated cells provides a measure of the ability of the present compounds to inhibit proliferation. In a preferred embodiment the cells to be treated are cancer cells, for example breast cancer cells such as the CDC25B positive breast cancer cells MDA-MB-231, which are available from the American Type Culture Collection (Accession No. HTB-26). The antiproliferative activity of the present compounds can also be measured by the assay described by Lazo et al. (1995) J. Biol. Chem. 270: 5506, the disclosure of which is incorporated herein by reference. The microtiter-based colorimetric assay is based upon the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide by living cells, and permits evaluation of the cytostatic or cytotoxic actions of large numbers of compounds quickly in mouse embryonic fibroblasts and human CDC25B positive breast cancer cells. Briefly, exponentially growing CDC25B positive MDA-MB-231 cells are treated continuously with from 0 to 100 μM of a compound of the invention, and cell proliferation is determined colorimetrically after 72 hours by assessing the ability of the cells to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide.

The antiproliferative ability of the compounds of the present invention can also be measured in an in vivo tumor reduction assay. Athymic (nu/nu) and severe combined immunodeficiency (SCID) mice provide well-recognized models for antitumor activity. Mice are injected subcutaneously (s.c.) with 10^6 MDA-MB-231 cells in 100 μl phosphate buffered saline (PBS). Once tumors reach a palpable

size (100 mm^2), mice are treated once daily orally (p.o.), intraperitoneally (i.p.) or subcutaneously (s.c.) with 0, 0.1, 1, 10 or 30 mg/kg of a compound of the present invention for five days. Tumor mass is measured with a vernier caliper and calculated as described by Jani et al. (1992) Cancer Res. 52: 2931. Reduction in tumor mass in treated versus untreated mice is indicative of antiproliferative activity of the compounds of the invention. In another in vivo tumor assay, C3/HeJ mice with established subcutaneous tumors (14 days post implant) were treated with compounds according to the present invention in accordance with a clonogenic survival assay described by Fu et al. (1984) Int. Radical Oncol. Biol. Phys. 10: 1473. Pharmacological principles may be used to optimize the dose and schedule of administration and are readily within the skill of those in the art.

The present invention also provides pharmaceutical compositions comprising a compound of the present invention and a pharmaceutically acceptable carrier or diluent. As used herein, the term "pharmaceutically acceptable carrier or diluent" means any and all solvents, dispersion media, antibacterial and antifungal agents, microcapsules, liposomes, cationic lipid carriers, isotonic and absorption delaying agents and the like which are not incompatible with the active ingredients. The use of such media and agents for pharmaceutically active substances is well known in the art. Supplementary active ingredients may also be incorporated into the compositions and used in the methods of the invention.

The formulation of pharmaceutical compositions is generally known in the art and reference can conveniently be made to Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa. Formulation of the compounds of the present invention must be stable under the conditions of manufacture and storage and also must be preserved against the contaminating action of microorganisms such as bacteria and fungi. Prevention against microorganism contamination can be achieved through the addition of various anti-bacterial and antifungal agents.

The pharmaceutical forms of the compounds of the invention suitable for administration include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Typical carriers include a solvent or dispersion medium containing, for example, water buffered aqueous solutions (i.e., biocompatible buffers), ethanol, polyols such as glycerol, propylene glycol, polyethylene glycol, suitable mixtures thereof, surfactants, and vegetable oils. Isotonic agents such as sugars or sodium chloride may be incorporated into the subject compositions.

The compounds of the invention are compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier, preferably in dosage unit form. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for a subject to be treated, each unit containing a predetermined quantity of a compound of the invention calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The compositions may be administered in a manner compatible with the dosage formulation, in such amount as will be therapeutically effective, and in any way which is medically acceptable. Possible administration routes include oral route and parenteral administration such as intravascular, intravenous, intraarterial, subcutaneous, intramuscular, intratumor, intraperitoneal, intraventricular and intraepidural. The compositions may also be directly applied to tissue surfaces. Sustained release administration, for example by depot injections or erodible implants, is also specifically included.

The invention is further illustrated by the following specific examples which are not intended in any way to limit the scope of the invention.

EXAMPLE I

A method for the combinatorial synthesis of compounds of formula I (Fig. 2) was developed by optimizing a solution phase synthesis of model compound II

(i.e., compound 1(a) of table I). The solution phase synthesis of model compound II is depicted in Fig. 5 and proceeded as follows:

All glassware was dried in an oven at 150 °C prior to use. THF and dioxane were dried by distillation over Na/benzophenone under a nitrogen atmosphere. Dry CH_2Cl_2, DMF and CH_3CN were obtained by distillation from CaH_2.

L-Glutamic acid (**3**) was protected in 62% yield as the γ-allyl ester using allyl alcohol and chlorotrimethylsilane to provide 2-amino-pentanedioic acid 5-allyl ester **4** as follows:

To a stirred suspension of 2.5 g (16.9 mmol) of L-glutamic acid (**3**) in 40 mL of dry allyl alcohol was added dropwise 5.4 mL (42.3 mmol) of chlorotrimethylsilane. The suspension was stirred at 22 °C for 18 h and poured into 300 mL of Et_2O. The resulting white solid was filtered off, washed with Et_2O, and dried in vacuo to provide 3.80 g (62%) of ester **4**: Mp 133-134.5 °C (Et_2O); IR (KBr) 3152, 2972, 2557, 1738, 1607, 1489, 1450, 1289, 1366, 1264, 1223, 1177, 1146, 121, 1084 cm^{-1}; ^1H NMR (D_2O) δ 5.8-5.7 (m, 1 H), 5.14 (dd, 1 H, J = 1.4, 17.3 Hz), 5.09 (dd, 1 H, J = 1.0, 10.4 Hz), 4.44 (d, 2 H, J = 5.6 Hz), 3.92 (t, 1 H, J = 6.8 Hz), 2.48 (t, 2 H, J = 7.0 Hz), 2.1-2.0 (m, 2 H); ^{13}C NMR (DMSO-d_6) δ 171.5, 170.6, 132.7, 117.9, 64.7, 51.2, 29.3, 25.2; MS (EI) m/z (relative intensity) 188 (63), 142 (72), 128 (27), 100 (21), 85 (100), 74 (32), 56 (73).

Treatment with Fmoc as follows provided 2-(9-H-Fluoren-9-ylmethoxycarbonylamino)-pentanedioic acid 5-allyl ester **5**. To 20 mL of dioxane was added 1.5 g (6.7 mmol) of ester **4**. The resulting suspension was treated with 16.8 mmol (17.7 mL of a 10% solution) of sodium carbonate at 0 °C, stirred for 5 min and treated with 1.74 g (6.7 mmol) of Fmoc-Cl dissolved in 10 mL of dioxane. The reaction mixture was warmed to 22 °C, stirred for 3 h, poured into 50 mL of H_20 and extracted with Et_2O (2X 25 mL). The aqueous layer was cooled to 0 °C, acidified to pH 1 with concentrated HCl, and extracted with EtOAc (3X25 mL). The resulting organic layer was dried (Na_2SO_4) and concentrated in vacuo to give

2.72 g (99%) of 5 as a viscous oil: $[\alpha]_D$+8.5° © 2.8, $CHCl_3$, 21 °C); IR (neat) 3312, 3061, 2951, 2361, 2349, 2332, 1725, 1528, 1447, 1414, 1325, 1254, 1117, 1078, 1049 cm^{-1}; ^1H NMR δ 11.09 (bs, 1 H), 7.73 (d, 2 H, J = 7.5 Hz), 7.57 (d, 2 H, J = 5.1 Hz), 7.4-7.25 (m, 4 H), 6.0-5.85 (m, 1 H), 5.76 (d, 1 H, J = 8.1 Hz), 5.30 (d, 1 H, J = 19.5 Hz), 5.21 (d, 1 H, J = 10.5 Hz), 4.6-4.35 (m, 5 H), 4.19 (t, 1 H, J = 6.6 Hz), 2.5-2.2 (m, 4 H); ^{13}C NMR δ 175.6, 172.6, 156.2, 143.7, 143.5, 141.2, 131.7, 127.6, 127.0, 125.0, 119.9, 118.4, 67.1, 65.4, 53.1, 46.9, 30.2, 27.1; MS (El) m/e (relative intensity) 409 (7), 351 (19), 338 (12), 280 (11), 239 (11), 196 (12), 178 (100), 165 (40); HRMS (El) calculated for $C_{23}H_{23}NO_6$: 409.1525, found: 409.1501.

Treatment with Fmoc-Cl followed by coupling to benzyl alcohol using 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide [by] hydrochloride (EDCl) provided 2-(9H-Fluoren-9-ylmethoxycarbonylamino)-pentanedioic acid 5-allyl ester 1-benzyl ester **6** in 82% yield. To a solution of 1.5 g (36.6 mmol) of **5** in 5 mL of CH_2Cl_2 was added 0.42 mL (40.3 mmol) of benzyl alcohol, 0.912 g (47.6 mmol) of EDCI, and 45 mg (3.66 mmol) of dimethylaminopyridine (DMAP). The reaction mixture was stirred at 22 °C for 6 h, diluted with 20 mL of CH_2Cl_2, and extracted with H_20 (1x15 mL), 0.1 M HCl (2x15 mL), and brine (2x10 mL). The organic layer was dried (Na_2SO_4), concentrated in vacuo, and chromatographed on SiO_2 (Hexanes/EtOAc, 5:1) to give 1.83 g (82%) of **6** as a white solid: Mp 66.2-67.1 °C (EtOAc/Hexanes); $[\alpha]_D$ +1.4° © 1.64, $CHCl_3$, 21 °C); IR (neat) 3314, 1726, 1682, 1527, 1443, 1414, 1383, 1254, 1173, 1099, 1082, 980, 754, 735 cm^{-1}; ^1H NMR δ 7.75 (d, 2 H, J = 7.4 Hz), 7.59 (d, 2 H, J = 7.1 Hz), 7.41-7.27 (m, 9 H), 5.95-5.85 (m, 1 H), 5.44 (d, 1 H, J = 8.2 Hz), 5.34-5.19 (m, 4 H), 4.56 (d, 2 H, J = 5.6 Hz), 4.5-4.4 (m, 3 H), 4.21 (t, 1 H, J = 7.0 Hz), 2.5-2.0 (m, 4 H); ^{13}C NMR δ 172.2, 171.6, 155.8, 143.7, 143.5, 141.1, 135.0, 131.8, 128.5, 128.3, 128.1, 127.6, 126.9, 124.9, 119.8, 118.3, 67.2, 66.9, 66.2, 53.3, 47.0, 28.0, 27.3; MS (FAB, MNBA/MeOH) m/z (relative intensity) 500 ($[M+H]^+$, 40), 465 (8), 448 (14), 433 (12), 413 (8), 386 (38), 371 (24), 349 (9), 324 (16), 309 (26), 293 (11), 265 (10),

247 (24), 231 (56), 215 (39), 202 (26), 191 (24), 179 (67), 165 (48), 154 (67), 143 (31), 133 (71), 117 (100).

The Fmoc protective group was subsequently removed by exposure to DMAP and the free amine was acylated *in situ* with decanoyl chloride to give 2-Decanoylamino-pentanedioic acid 5-allyl ester 1-benzyl ester (7) in 63% yield as follows. To a suspension of 1 g (2.0 mmol) of **6** in 10 mL of CH_2Cl_2 was added 1 g (8.2 mmol) of DMAP. The reaction mixture was stirred at 22 °C for 24 h, treated with 0.62 mL (3.0 mmol) of decanoyl chloride, stirred for 2 h at 22 °C, and extracted with saturated sodium bicarbonate solution (2x10 mL). The organic layer was dried (Na_2SO_4), evaporated to dryness, and the residue was chromatographed on SiO_2 (Hexanes/EtOAc, 5:1) to give 548 mg (63%) of 7 as a viscous oil: IR (neat) 3293, 3063, 2924, 2855, 1740, 1649, 1534, 1453, 1379, 1175, 986, 930 cm^{-1}; ^1H NMR δ 7.26 (s, 5 H), 6.68 (d, I H, J = 7.8 Hz), 5.85-5.75 (m, 1 H), 5.22 (d, 1 H, J = 17.3 Hz), 5.14 (d, 1 H, J = 10.4 Hz), 5.08 (s, 2 H), 4.63-4.57 (m, 1 H), 4.48 (d, 2 H, J = 5.6 Hz), 2.38-2.28 (m, 2 H), 2.2-2.1 (m, 3 H), 2.0-1.9 (m, 1 H), 1.55 (t, 2 H, J = 6.9 Hz), 1.20 (bs, 12 H), 0.82 (t, 3 H, J = 5.9 Hz); ^{13}C NMR δ 173.0, 172.1, 171.6, 135.0, 131.7, 128.2, 128.1, 127.8, 117.9, 66.8, 64.9, 51.3, 36.0, 31.6, 29.9, 29.1, 29.0, 26.8, 25.3, 22.3, 13.8; MS (El) m/z (relative intensity) 431 (12), 319 (21), 296 (51), 142 (100), 124 (31), 91 (91); HRMS (El) m/z calculated for $C_{25}H_{37}NO_5$: 431.2672, found: 431.2673.

Pd(O)-catalyzed deprotection of the allyl ester proceeded as follows to yield 2-Decanoylamino-pentanedioic acid 1-benzyl ester (8). To a solution of 752 mg (1.74 mmol) of 2-decanoylamino-pentanedioic acid 7 in 10 mL of CH_2Cl_2 was added 100 mg (0.087 mmol) of tetrakistriphenylphosphine Pd(O) followed by 0.52 mL (1.9 mmol) of tributyltin hydride. After 15 min, the reaction mixture was quenched with 10 mL of a 10% HCl solution. The aqueous layer was reextracted with 15 mL of CH_2Cl_2 and the organic layer dried (Na_2SO_4), concentrated in vacuo, and chromatographed on SiO_2 (Hexanes/EtOAc, 9:1) to provide 545 mg (79.9%) of 8 as a thick oil: $[\alpha]_D$ +2.8° © 1.2, $CHCl_3$, 21 °C); IR (neat) 3351, 3064, 2995,

2852, 1738, 1712, 1657, 1536, 1454, 1380, 1364, 1265, 1209, 1183, 1121, 739 cm^{-1}; ^1H NMR δ 10.9-10.7 (bs, 1 H), 7.22 (s, 5 H), 6.58 (d, 1 H, J = 7.8 Hz), 5.09 (s, 2 H), 4.63 (dd, 1 H, J = 8.1, 12.9 Hz), 2.4-2.25 (m, 2 H), 2.2-2.1 (m, 3 H), 2.0-1.9 (m, 1 H), (m, 6 H), 1.53 (t, 2 H, J = 6.6 Hz), 1.19 (bs, 12 H), 0.81 (t. 3 H, J = 6.0 Hz); ^{13}C NMR δ 176.9, 174.0, 171.8, 134.9, 128.5, 128.4, 128.1, 67.3, 51.4, 36.2, 31.7, 29.9, 29.3, 29.2, 29.1, 27.0, 25.5, 22.5, 14.0; MS (El) m/z (relative intensity) 391 (54), 373 (62), 279 (13), 256 (19), 178 (27), 178 (23), 155 (13), 146 (6), 130 (7), 102 (100); HRMS (El) m/z calculated for $C_{22}H_{33}NO_5$:391.2358, found: 391.2350.

Coupling to ethylene diamine (9) yielded 4-[(2-Allyloxycarbonylamino-ethyl)-methyl-carbamoyl]-2-decanoylamino-butyric acid benzyl ester (10). To a solution of 526 mg (1.3 mmol) of **8** in 10 mL of CH_2Cl_2 was added 225 μL (1.61 mmol) of triethylamine and 320 mg (2.0 mmol) of secondary amine **9**. The solution was stirred at 22 °C for 5 min, treated with 710 mg (1.61 mmol) of benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP reagent), stirred at 22 °C for 10 min, concentrated in vacuo, dissolved in 15 mL of EtOAc, and extracted with 2 M HCl solution. The organic layer was chromatographed on SiO_2 (Hexanes/EtOAC, 1:3) to give 715 mg (94%) of **10** as a clear oil: $[\alpha]_D$ + 5.3 © 0.58, $CHCl_3$, 21 °C); IR (neat) 3420, 3250, 2924, 1713, 1680, 1657, 1642, 1632, 1537, 1495, 1470, 1455, 1252, 845 cm^{-1}; ^1H NMR δ 7.35-7.2 (bs, 5 H), 6.97 (d, 0.3 H, J = 7.5 Hz), 6.82 (d, 0.7 H, J = 7.3 Hz), 5.9-5.6 (m, 2 H), 5.3-5.1 (m, 4 H), 4.65-4.5 (m, 1 H), 4.50 (d, 2 H, J = 4.9 Hz); 3.55 (t, 1 H, J = 7.0 Hz), 3.35-3.1 (m, 3 H), 2.85 (s, 3 H), 2.4-1.8 (m, 6 H), 1.65-1.5 (m, 2 H), 1.22 (bs, 12 H), 0.84 (t, 3 H, J = 6.1 Hz); ^{13}C NMR (MEOD) δ 176.4, 176.3, 174.4, 174.2, 173.2, 158.6, 137.1, 134.3, 134.2, 132.9, 129.5, 129.2, 129.1, 117.6, 117.4, 67.8, 66.3, 66.2, 53.5, 53.3, 39.6, 39.3, 36.7, 36.6, 34.2, 32.9, 30.5, 30.4, 30.3, 30.2, 29.7, 27.6, 26.8, 23.6, 14.5; MS (El) m/z (relative intensity) 531 (16), 473 (37), 418 (16), 396 (26), 374 (38), 361 (17), 338 (87), 220 (54), 184 (52), 155

(36), 130 (29), 101 (37), 91 (100); HRMS (EI) m/z calculated for $C_{29}H_{45}N_3O_6$:531.3308, found: 531.3316.

Monoprotected ethylene diamines **9** were easily achieved by carbamoylation of 2-chloroethylamine monohydrochloride (**12**), Finkelstein reaction, and aminolysis (Fig. 3).

(2-Chloro-ethyl)-carbamic acid allyl ester (13) was synthesized as follows. A solution of 2.5 g (22 mmol) of chloroethylamine hydrochloride in 10 mL of 6 M NaOH was cooled to 0 °C and treated dropwise with 2.7 mL (25.9 mmol) of allyl chloroformate while keeping the pH at 9 by addition of 6 M NaOH solution. The reaction was then warmed to 22 °C, stirred for 2 h, and extracted with THF. The organic layer was dried (Na_2SO_4, concentrated in vacuo, and chromatographed on SiO_2 (Hexanes/EtOAc,9:1) to give 3.1 g (88%) of **13** as a yellow oil: IR (neat) 3333, 2949, 2348, 1705, 1647, 1529, 1433, 1368, 1248, 1190, 1144, 1061, 991, 929, 776 cm^{-1}; ^1H NMR δ 6.05-5.85 (m, 1 H), 5.55-5.35 (bs, 1 H), 5.26 (dd, 1 H, J = 1.5, 17.1 Hz), 5.18 (dd, 1 H, J = 1.0, 10.4), 4.54 (d, 2 H, J = 5.5 Hz), 3.57 (t, 2 H, J = 5.5 Hz), 3.5-3.35 (m, 2 H); ^{13}C NMR δ 156.0, 132.5, 117.7, 65.6, 43.8, 42.7.

To produce (2-Methylamino-ethyl)-carbamic acid allyl ester (9), a solution of 14 g (86 mmol) of **13** and 25 g (172 mmol) of sodium iodide in 40 mL of acetone was refluxed for 18 h, concentrated in vacuo, dissolved in H_2O, and extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4) and cooled to 0 °C. Methyl amine was bubbled through the reaction mixture until the solution was saturated. The reaction mixture was warmed to 22 °C, stirred for 36 h, concentrated in vacuo and chromatographed on SiO_2 (EtOAc) to produce 6.14 g (45%) of **9** as a yellow oil: IR (neat) 3306, 2938, 2313, 1844, 1703, 1651, 1525, 1460, 1383, 1256, 1144, 995, 927, 775 cm^{-1}; ^1H NMR δ 5.95-5.8 (m, 1 H), 5.28 (dd, 1 H, J = 1.4, 17.3 Hz), 5.18 (d, 1 H, J = 10.4 Hz), 4.54 (d, 2 H, J = 5.3 Hz), 4.9-4.6 (bs, 1 H), 3.34 (q, 2 H, J = 5.6 Hz), 2.79 (t, 2 H, J = 5.6 Hz), 2.47 (s, 3 H); ^{13}C NMR δ 157.22, 132.8, 117.6, 65.5, 50.7, 39.7, 35.4; MS (EI) m/e (relative intensity) 158 (32), 138 (17), 129 (25), 101 (13), 84 (12), 73 (13), 57 (100).

The heterocyclic moiety **11** was prepared from N-benzoyl threonine (14) as follows and as depicted in Fig. 4.

5-Methyl-2-phenyl-oxazole-4-carboxylic acid methyl ester (15) was synthesized by treating a solution of 750 mg (3.2 mmol) of **14** in 10 mL of CH_2Cl_2 with 1.61 g (3.8 mmol) of Dess-Martin reagent. The reaction was stirred at 22 °C for 10 min, concentrated in vacuo, and chromatographed on SiO_2 (Hexanes/EtOAc, 3:2) to give 658 mg (89%) of 2-benzoylamino-3-oxo-butyric acid methyl ester. Alternatively, a solution of 9.12 g (38 mmol) of **14** in 80 mL of CH_2Cl_2 was cooled to -23 °C and treated with 16.1 mL (1 15-mmol) of triethylamine and a solution of 18.3 g (115 mmol) of SO_3-pyridine complex in 60 mL of dry DMSO. The reaction mixture was warmed to 22 °C, stirred for 30 min, then cooled to -48 °C and quenched with 20 mL of saturated $NaHCO_3$. The solution was extracted with 50 mL of Hexanes/EtOAc, 2:1. The aqueous layer was reextracted with Hexanes/Et_2O, 2:1, and the combined organic layers were washed with brine, dried (Na_2SO_4), and chromatographed (Hexanes/EtOAc, 3:2) to give 7.1 g (79%) of 2-benzoylamino-3-oxo-butyric acid methyl ester as a white solid: Mp 112.7-113.3 °C (Hexanes/EtOAc); IR (neat) 3402, 1734, 1662, 1599, 1578, 1510, 1478, 1435, 1354, 1269, 1156, 1121, 912, 804, 714 cm^{-1}; ^1H NMR 8.2-8.1 (bs, 1 H), 8.0-7.4 (m, 5 H), 5.49 (s, 1 H), 3.86 (s, 3 H), 2.33 (s, 3 H); ^{13}C NMR δ 168.2, 167.2, 132.6, 132.5, 132.1, 128.7, 127.3, 83.9, 54.2, 23.2; MS (El) m/e (relative intensity) 235 (13), 208 (18), 192 (8), 121 (7), 105 (100), 77 (58).

A solution of 277 mg (1.06 mmol) of triphenylphosphine, 268 mg (1.06 mmol) of iodine, and 0.29 mL (2.11 mmol) of triethylamine in 5 mL of CH_2Cl_2 was cooled to -48 °C and treated with a solution of 124 mg (0.528 mmol) of 2-benzoylamino-3-oxo-butyric acid methyl ester in 5 mL of CH_2Cl_2. The reaction mixture was warmed to 22 °C, stirred for 20 min, transferred to a separatory funnel and extracted with aqueous sodium thiosulfate followed by saturated sodium bicarbonate. The organic layer was concentrated in vacuo and chromatographed on SiO_2 (Hexanes/EtOAc, 9:1) to give 84.4 mg (74%) of **15** as a white solid: Mp

89.3-89.9 °C (Hexanes/EtOAc); IR (neat) 3025, 1717, 1610, 1561, 1485, 1436, 1348, 1323, 1302, 1285, 1235, 1188, 1103, 1072, 1057, 1022 cm^{-1}; ^1H NMR 8.1-7.95 (m, 2 H), 7.5-7.3 (m, 3 H), 3.92 (s, 3 H), 2.68 (s, 3 H); ^{13}C NMR δ 162.7, 159.5, 156.3, 130.8, 128.8, 128.6, 128.3, 126.4, 51.9, 11.98; MS (El) m/z (relative intensity) 231 (6), 217 (51), 185 (55), 105 (100), 77 (4 1), 44 (64); HRMS (El) m/z calculated for $C_{12}H_{11}NO_3$:217.0739, found: 217.0729.

5-Methyl-2-phenyl-oxazole-4-carboxylic acid **11** we produced by stirring a solution of 2.07 g (9.5 mmol) of 15 in 20 mL of 3 M NaOH and 12 mL of MeOH at 22 °C for 2 h and extracting with Et_2O. The aqueous layer was acidified to pH 1 with concentrated HCl and extracted with EtOAc. The organic layer was dried (Na_2SO_4), and concentrated in vacuo to give 1.84 g (95%) of 11 as an off-white solid: Mp 182.3-182.6 °C (EtOAc/Hexanes); IR (neat) 3200, 2950, 2932, 2890, 2363, 2336, 1694, 1682, 1611, 1563, 1483, 1450, 1337, 1255, 1192, 1117, 1053, 1020 cm^{-1} ; ^1H NMR δ 10.2-9.9 (bs, 1 H), 8.2-7.9 (m, 2 H), 7.6-7.4 (m, 3 H), 2.75 (s, 3 H); ^{13}C NMR (CD_3OD) δ 164.6, 160.7, 157.4, 131.9, 129.8, 129.6, 127.3, 127.2, 12.1; MS (El) *m/z* (relative intensity) 203 (53), 185 (24), 157 (13), 116 (17), 105 (100), 89 (21), 77 (33), 63(16); HRMS calculated for $C_{11}H_9NO_3$: 203.0582, found: 203.0583.

2-Decanoylamino-4-(methyl-{3-[5-methyl-2-phenyl-oxazole-4-carbonyl]-ethyl}-carbamoyl)-butyric acid benzyl ester (formula II) was then provided as follows: To a solution of 193 mg (0.363 mmol) of 10 in 15 mL of CH_2Cl_2 was added 20 mg (0.018 mmol) of tetrakistriphenylphosphine Pd(0), 127 μL (0.472 mmol) of tributyltin hydride, and 20 μL of H_20. The reaction mixture was stirred at 22 °C for 5 min, filtered through a plug of basic Al_2O_3 and treated with 150 mg (0.726 mmol) of oxazole 11, 60 μL (0.436 mmol) of triethylamine, and 192 mg (0.436 mmol) of BOP reagent. The reaction mixture was stirred for 30 min at 22 °C, diluted with 10 mL of CH_2Cl_2, and extracted with saturated $NaHCO_3$ solution, 1M HCl, and brine. The organic layer was concentrated in vacuo and chromatographed on SiO_2 (Hexanes/EtOAc, 1:1) to give 131 mg (57%) of 2 as a

viscous oil: $[\alpha]_D$ -0.8° © 1.32, $CHCl_3$, 21 °C); IR (neat) 3476, 3415, 3311, 3065, 2925, 2854, 1741, 1649, 1526, 1491, 1379, 1338, 1264, 1240, 1200, 1174, 1070, 711 cm^{-1}; ^1H NMR 8.0-7.95 (m, 2 H), 7.5-7.4 (m, 2 H), 7.33 (bs, 6 H), 6.93 (d, 0.3 H, J = 7.0 Hz), 6.85 (d, 0.7 H, J = 7.2 Hz), 5.18-5.07 (m, 2 H), 4.65-4.55 (m, 1 H), 3.7-3.3 (m, 4 H), 2.98 (s, 1 H), 2.96 (s, 2 H), 2.71 (d, 3 H, J = 2.6 Hz), 2.6-2.0 (m, 6 H), 1.58 (t, 2 H, J = 6.8 Hz), 1.3-1.1 (bs, 12 H), 0.86 (t, 3 H, J = 6.9 Hz); ^{13}C NMR δ 173.3, 172.8, 172.0, 171.9, 182.5, 158.6, 153.2, 152.8, 135.9, 130.7, 130.6, 129.7, 128.8, 128.5, 128.3, 128.2, 126.7, 126.5, 126.2, 66.9, 52.2, 52.1, 48.9, 47.6, 37.2, 37.1, 36.4, 36.3, 36.2, 34.1, 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 28.9, 26.8, 26.6, 25.5, 22.8, 14.1, 11.8; MS (El) *m/z* (relative intensity) 632 (38), 497 (9), 405 (18), 374 (22), 260 (21), 220 (42), 186 (56), 105 (18), 91 (100); HRMS calculated for $C_{36}H_{48}N_4O_6$: 632.3574, found: 632.3572.

EXAMPLE II

The solution phase synthesis of compound 1a (formula II) in Example I established the necessary general protocols for the preparation of a library of structural variants of the compounds of formula I on a solid support. A representative solid phase synthesis of compounds of formula I wherein Y is H and Z is a bivalent segment corresponding to ethylene diamine is depicted in Fig. 2 and proceeded as follows.

Coupling of diprotected glutamate **5** to the polystyrene-based Wang resin described by Wang (1973), J. Am. Chem. Soc. 95:1328, with EDCI was performed on large scale and provided a supply of solid phase beads. The base-labile Fmoc protective group was removed by treatment with piperidine and THF, and the resin was distributed to three specially designed Schlenk filters equipped with suction adapters and inert gas inlets for maintaining steady bubbling. After the addition of solvent, hydrophobic residues R'''COCl were added to each flask, which provided three different amide derivatives **17**. After filtration and rinsing of the resin, allyl esters **17** were deprotected via Pd(0) chemistry and each batch was distributed over

three modified Schlenk filters, providing nine different reaction sites for acylation. Addition of three different *N*-allyloxycarbonyl protected diamines in the presence of PyBroP[39] or CloP[40] as coupling agents extended the side chain carboxyl terminus of glutamic acid toward the desired heterocyclic moiety in **1**. The resulting nine compounds **18** were each deprotected at the *N*-terminus and distributed over two additional Schlenk filters for the final segment condensation. Coupling with two different oxazole carboxylic acids in the presence of CloP and final purification by rinsing with solvent provided the phosphatase library (formula I) still attached to the solid support. Complete or partial cleavage with 50% trifluoroacidic acid was necessary to release the carboxylate which is required for biological activity. After filtration of the solid support and evaporation of the resulting mother liquor, the desired compounds of formula I were obtained in a chemically pure and structurally well defined fashion ready for rapid throughput biological screening. In each case, the purity of the final compound was >60% according to spectroscopic analysis (^{1}H NMR, MS). The contamination was derived from incomplete couplings to the sterically hindered secondary amine moiety of Alloc-NHCH$_2$CH$_2$NH(R").

The foregoing synthesis is provided in more detail as follows:

Step 1, 5 → 16. In a medium porosity Schlenk filter apparatus was placed 750 mg of Wang resin (0.96 mmol/g, 0.72 mmol of active sites). The resin was suspended in 12 mL of dry DMF and a stream of nitrogen was forced up through the filter at a rate which allowed the solvent to gently bubble. To this reaction mixture was added 1.47 g (3.6 mmol) of **5**. The suspension was agitated for 5 min and treated with 26 mg (0.216 mmol) of DMAP and 550 mg (2.88 mmol) of EDCI, agitated at 22 °C for 18 h and filtered, and the resin was washed with DMF (2x10 mL), H_20 (3x10 mL), THF (3x10 mL), and CH_2Cl_2 (3x10 mL). The resin was dried under vacuum and the remaining active sites were capped by addition of 10 mL of CH_2Cl_2 and 10 mL of acetic anhydride along with 26 mg (2.88 mmol) of DMAP to the resin. Bubbling was continued at 22 °C for 3 h and the resin was

then washed with CH_2Cl_2 (6x15 mL) and dried in vacuo. To test the loading on the resin, 30 mg of resin was removed and suspended in 2 mL of trifluoroacetic acid for 5 min at 22 °C, filtered and washed (3x3 mL) with CH_2Cl_2. The filtrate was concentrated in vacuo to give 7.3 mg (85%) of **5**.

Step 2, 16 → 17. A suspension of 690 mg (0.576 mmol) of 2-(9H-fluoren-9-ylmethoxycarbonylamino)-pentanedioic acid 5-allyl ester linked to Wang resin **16** in 15 mL of THF was treated with 6 mL (57.6 mmol) of piperidine, agitated by bubbling for 30 min, filtered and washed with CH_2Cl_2 (6X 10 mL). The resin was dried in vacuo. A suspension of this resin in 10 mL of CH_2Cl_2 was treated with 0.48 mL (2.31 mmol) of decanoyl chloride and 14 mg (0.115 mmol) of DMAP. The reaction mixture was agitated at 22 °C for 6 h, filtered and the resin was washed with CH_2Cl_2 (6x10 mL) and dried in vacuo.

Step 3, 17 → 18. A suspension of 690 mg (0.576 mmol) of 2-decanoylamino-pentanedioic acid 5-allyl ester linked to Wang resin **17** in 10 mL of THF was treated with 67 mg (0.0576 mmol) of tetrakis(triphenylphosphine) palladium (0) and 806 mg (5.75 mmol) of dimedone, and agitated by bubbling at 22 °C for 18 h. The resin was then filtered, washed with THF (2x10 mL), CH_2Cl_2 (2x10 mL), MeOH (2x10 mL), H_2O (2x10 mL), 1% acetic acid solution (2x10 mL), H_2O (2x10 mL), MeOH (2x10 mL), CH_2Cl_2 (2x10 mL), and dried in vacuo. Cleavage and examination of 40 mg of resin by ^1H NMR showed full deprotection of the allyl ester.

A suspension of this resin in 12 mL of DMF was treated with 0.22 mL (1.572 mmol) of triethylamine and 414.1 mg (2.62 mmol) of Alloc-$NHCH_2CH_2NHMe$. After agitating the reaction mixture for 5 min to ensure proper mixing, 540 mg (1.572 mmol) of CloP was added. The reaction mixture was agitated with bubbling for 18 h at 30 °C, cooled to 22 °C, and the resin was filtered and washed with DMF (2x10 mL), CH_2Cl_2 (2x10 mL), MeOH (2x10 mL), H_2O (2x10 mL), THF (2x10 mL), and CH_2Cl_2 (2x10 mL). The resin was dried

in vacuo and 40 mg of resin was cleaved with CF_3CO_2H. The ^1H NMR of the residue showed that coupling had occurred to nearly 100%.

Step 5, 18 → 19. A suspension of 200 mg (0.192 mmol) of 4-[(2-allyloxycarbonylaminoethyl)-methyl-carbamoyl]-2-decanoylamino-butyric acid linked to Wang resin **18** in 6 mL of CH_2Cl_2 was treated with 12 mg (0.0096 mmol) of tetrakistriphenylphosphine Pd(0), 62 µl (0.230 mmol) of tributyltin hydride, and 10 µl of H_2O. The reaction mixture was agitated with bubbling N_2 for 15 min, filtered, and the resin was washed with 10 mL portions of CH_2Cl_2, THF, acetone, MeOH, H_2O, acetone, EtOAc, hexanes, THF, and CH_2Cl_2. The resin was then dried in vacuo and 15 mg was removed for testing. The ^1H NMR of the TFA-cleaved residue showed full deprotection as well as full removal of all tin side products.

A suspension of 185 mg (0.190 mmol) of this resin in 8 mL of CH_2Cl_2 was treated with 117 mg (0.576 mmol) of oxazole carboxylic acid, 198 mg (0.576 mg) of CloP, and 80 µl (0.576 mmol) of triethylamine. The reaction mixture was agitated by bubbling with N_2 for 3 h, filtered, and washed with 20 mL of CH_2Cl_2, acetone, water, acetone, and CH_2Cl_2. The resin was dried in vacuo and 15 mg was removed for testing. The ^1H NMR of the residue showed that the reaction had gone to 60% completion. The resin was subsequently submitted to a second coupling cycle.

Step 6, 19 → 1. A suspension of 115 mg (0.12 mmol) of 2-decanoylamino-4-(methyl-{3-[5-methyl-2-phenyl-oxazole-4-carbonyl]-ethyl}-carbamoyi)-butyric acid linked to Wang resin **19** in 3 mL of TFA was stirred for 5 min, filtered, and washed with 5 mL of CH_2Cl_2. The extract was concentrated in vacuo to provide 33.1 mg (100% for step 2 to step 6) of 1. A ^1H NMR showed the product to be 66% pure with 2-acylamino-pentanedioic acid as the major impurity. Acid 1a was dissolved in 3 mL of CH_2Cl_2 and treated with 0.016 mL (0.138 mmol) of benzyl bromide and 0.02 mL (0.138 mmol) of DBU to provide material identical with the benzyl ester **2** prepared by solution phase chemistry.

Compounds 1b-1r have also been prepared via the same general method. The most important difference in the solution phase synthesis from the solid bead synthesis is in the use of 2,2,2-trichlorethoxycarbonyl and allyloxycarbonyl protective groups and amide coupling with BopCl(_) and DPPA(_) coupling agents. All combinatorial compounds were a minimum of 60% pure based on ^{1}H NMR and mass spectral analysis, while the corresponding compounds prepared by solution chemistry were > 90% pure based on ^{1}H NMR mass spectral and ^{13}C NMR analyses. The compounds prepared by solution chemistry were racemic, while the combinatorial compounds were enriched in the L-stereoisomer.

Compounds wherein Z is not ethylene may be prepared in a similar manner to that described above by simply substituting a different protected amine with the same protective groups. Compounds wherein Y is absent are also prepared similarly by substituting different protected amines.

EXAMPLE III

The following compounds 1a - r (Table 1) corresponding to formula I wherein Y = H and Z is a bivalent ethylene segment were tested for their ability to inhibit PP1, PP2A and PP3.

TABLE 1

Compound	R	R′	R″	R‴
1a	Ph	CH_3	CH_3	n-C_9H_{19}
1b	Ph	CH_3	n-C_6H_{13}	n-C_9H_{19}
1c	Ph	CH_3	Bn	n-C_9H_{19}
1d	Ph	Ph	CH_3	n-C_9H_{19}
1e	Ph	Ph	n-C_6H_{13}	n-C_9H_{19}
1f	Ph	Ph	Bn	n-C_9H_{19}
1g	Ph	CH_3	CH_3	$PhCH_2CH_2$
1h	Ph	CH_3	n-C_6H_{13}	$PhCH_2CH_2$
1i	Ph	CH_3	Bn	$PhCH_2CH_2$
1j	Ph	Ph	CH_3	$PhCH_2CH_2$
1k	Ph	Ph	n-C_6H_{13}	$PhCH_2CH_2$
1l	Ph	Ph	Bn	$PhCH_2CH_2$
1m	Ph	CH_3	CH_3	PhCH=CH
1n	Ph	CH_3	n-C_6H_{13}	PhCH=CH
1o	Ph	CH_3	Bn	PhCH=CH
1p	Ph	Ph	CH_3	PhCH=CH
1q	Ph	Ph	n-C_6H_{13}	PhCH=CH
1r	Ph	Ph	Bn	PhCH=CH

Phosphatase activity and the inhibitory activity of the compounds of Table 1 were determined by the method of Honkanen et al. (1994) Toxicon 32:339. Briefly, phosphatase activity against phosphorylase-a or phosphohistone was determined by the quantification of liberated [^{32}P]. Assays, 80 µl total volume, containing 50 mM Tris-HCl, pH 7.4, 0.5 mM DTT, 1 mM EDTA (assay buffer) and [^{32}P]phosphoprotein (1-2 µM PO_4), were conducted as described previously (Honkanen et al., 1991 Mol. Pharmac. 40:577). Dephosphorylation reactions were

routinely conducted for 5-10 min. using phosphorylase-a as a substrate and for 10-20 min. using phosphohistone. In all assays, the dephosphorylation of substrate was kept to less than 10% of the total phosphorylated substrate available, and the reactions were adjusted to ensure that enzyme activity was linear with respect to enzyme concentration and time. [^{32}P]Phosphate liberated by the enzymes was extracted as a phosphomolybdate complex and measured according to the methods of Killilea et al. (1978) Arch. Biochem. Biophys. 191:638. Inhibition of protein phosphatase activity by inhibitors was determined by adding the 100 μM of inhibitors to the enzyme mixture 5-10 min. prior to initiating the reaction with the addition of substrate.

Results are presented in Table 2 as the percent inhibition relative to control (100%).

TABLE 2			
Compound	**PP1**	**PP2A**	**PP3**
none	100	100	100
1a	121	100	76
1b	59	135	32
1c	ND	129	28
1d	53	69	33
1e	153	152	71
1f	117	156	85
1g	53	109	21
1h	63	88	17
1i	ND	80	14
1j	80	72	38
1k	48	67	24
1l	59	69	22
1m	111	108	60
1n	40	87	26
1o	64	88	13
1p	65	99	68
1q	85	60	28
1r	53	68	15

Compound 1d (R=Ph, R′=Ph, R″=CH_3, R‴=n-C_9H_{19}) was further assessed for its ability to inhibit PP2A, and compared to calyculin A, a known inhibitor of PP2A.

The activity of the catalytic subunit of bovine cardiac muscle PP2A (Gibco-BRL, Gaithersburg, MD) was measured with fluorescein diphosphate (Molecular Probes, Inc., Eugene, OR) as a substrate in 96-well microtiter plates. The final incubation mixture (150 μL) composed 25 mM Tris (pH = 7.5), 5 mM EDTA, 33 μg/mL BSA, and 20 μM fluorescein diphosphate. Inhibitors were resuspended in DMSO, which was also used as the vehicle control. Reactions

were initiated by adding 0.2 units of PP2A and incubated at room temperature overnight. Fluorescence emission from the product was measured with Perseptive Biosystems Cytoflour II (exciton filter, 485 nm; emission filter, 530 nm) (Framingham, MA).

As demonstrated in Fig. 6, calyculin A inhibited PP2A activity at 10 nM, and compound 1d caused 50% inhibition at 100 μM.

The above-described assays were repeated in a second test series using catalytic subunits of PP1 and PP2A which were purified from a different source. Specifically, the catalytic subunits of PP1 and PP2A were purified from oysters and phosphatase activity was determined with a radiolabeled phosphohistone substrate (histone HI), by the liberation of [^{32}P] as described above. Okadaic acid (1 nM) was included in some PP1 preparations to suppress endogenous PP2A activity and had no apparent affect on the inhibitory activity of the compounds tested. Dephosphorylation of [^{32}P]-labeled histone was determined after a 10-20 min incubation with or without 100 μM combinatorial compounds by extraction as a phosphomolybdate complex. The reaction was directly dependent on enzyme concentration and time under these conditions. The inhibitory activity of SC-$\alpha\alpha\delta$9 and SC-$\alpha\alpha$69 (i.e., the solution chemistry analogs of compounds 1e and 1f of Table I, respectively) against PP1 and PP2A was also measured with rabbit skeletal muscle PP1 and PP2A and [^{32}P] labeled phosphorylase A as a substrate by the commercially available method of (GIBCO, BRL) (Grand Island, N.Y.).

The results of this second test series are presented in table 3, below.

TABLE 3. Inhibition of PSTPase activity with 100μM combinatorial compound.

Compound	PP1		PP2A	
	% Inhibition			
	Mean	SEM	Mean	SEM
1a	0	0	0	0
1b	4	10	14	15
1c	11	3	33	13
1d	0	0	0	0
1e	0	0	0	0
1f	9	7	5	11
1g	12	6	15	11
1h	11	5	31	10
1i	13	1	48	8
1j	22	2	20	4
1k	1	19	32	21
1l	32	5	47	6
1m	11	4	13	4
1n	27	9	23	2
1o	25	3	35	4
1p	27	5	8	11
1q	17	9	40	9
1r	24	9	46	8

Each value is the % inhibition from untreated control and the mean from three independent determinations.

Although Table 2 shows that compound 1d caused an approximate 50% inhibition of enzyme activity with the PP2A catalytic subunit from bovine cardiac muscle, no significant inhibition of oyster PP1 and PP2A was observed. (Table 3). Okadaic acid (100 μM) and calyculin A (10 nM) were highly effective in this assay and caused > 99% inhibition of PP1 or PP2A activity. Although no compound at 100 μM produced > 60% inhibition of PP1 or PP2A (Table 3), a preference for compounds with aromatic substituents on R″ and R‴ with this pharmacophore emerged for these PSTPases.

EXAMPLE IV

Compounds 1a-1r were assessed for ability to inhibit CDC25A and CDC25B activity. Recombinant human CDC25A, CDC25B and CDC25C were obtained as a glutathione-S-transferase (GST) fusion protein using human cDNA and standard molecular biological methods. The cDNA constructs are in a plasmid that is expressed in E. coli under the control of isopropyl-beta-D-thiogalactosidase (IPTG). The bacterial pellet was disrupted by sonication, and centrifuged at 10,000 x g. Using glutathione-agarose beads, the fusion protein was purified from postmicrosomal supernatant fraction as described by Baratte et al. (1992) Anticancer Res. 12: 873-880. Phosphatase activity was assayed with a spectrofluorimeter under the following conditions: 1 unit (1 U=amount of protein that induces 33 fluorescence units/minute of product) of fusion protein in a final incubation mixture (150 μL) comprised of 25mM Tris (pH=8.0), 5 mM EDTA, 33 μg/ml BSA, and 20 μM fluorescein diphosphate in 96-well microtiter plates. Plates were preincubated for 1 hour with 0 (control), 0.3, 1, 3, 10, 30, 100 μM compounds at room temperature. After the 1 hour incubation at room temperature, fluorescence of the fluorescein product (Ex. 485 nm; Em. 530 nm) were measured with a Biosystems Cytofluor II (Framingham, MA).

Results are presented in Fig. 7 and Table 4 as percent inhibition relative to control. A dose-response curve for compound 1f is presented in Fig. 8.

TABLE 4		
Compound	**CDC25A**	**CDC25B**
none	100	100
1a	67	106
1b	14	44
1c	11	42
1d	99	91
1e	7	17
1f	3	15
1g	50	81
1h	36	69
1i	31	63
1j	107	90
1k	83	76
1l	62	69
1m	19	45
1n	29	66
1o	35	66
1p	71	87
1q	55	62
1r	42	56

The foregoing results demonstrate that compounds 1a-1c and 1e-1r are capable of inhibiting the activity of CDC25A and/or CDC25B.

A second series of assays was performed for compounds 1a-1r to repeat these experiments and to assess their ability to inhibit CDC25A, CDC25B, CDC25C and MAPKP. In particular, the compounds' ability to inhibit CL100, which is a type of MAPKP, was tested.

CL100, CDC25A, CDC25B and CDC25C were obtained by a procedure similar to that described above. Specifically, E. *coli* strain BL21 (DE3) was used for transfection with plasmids containing the fusion constructs encoding GST and

CDC25A, B or C under the transcriptional control of isopropyl β-D-thiogalactopyranoside (IPTG). E. *coli* were first grown overnight at 37°C for 405 h. IPTG (1 mM final concentration) was then added and the cultures incubated at 37°C for an additional 3 h. Cells were harvested by centrifugation at 3,500 *g* for 10 min at 4°C. The resultant bacterial pellets were kept frozen at -80°C until extraction. His_6 tagged CL100 was produced similarly except the E. *coli* strain DH5α was used in place of GL21 (DE3).

The proteins were then purified as follows. The bacterial pellets were disrupted by sonication at 4°C in lysis buffer containing 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 100 μg/ml AEBSF and 10 mM DTT. The homogenate was then centrifuged for 10 min at 4°C at 100,000 *g*. The resulting supernatant fraction was immediately mixed and rotated with glutathione beads (equilibrated with lysis buffer) for 1 h at 4°C (5 vol of supernatant/1 vol of 50% bead slurry). The glutathione beads were washed two times with 10 vol of lysis buffer and then twice with 10 vol of 2x reaction buffer (60 mM Tris, pH 8.5, 145 mM NaCl, 1.34 mM EDTA, 0.066% BSA) containing 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 100 μg/ml AEBSF and 10 mM DTT. The fusion protein was eluted with 3 successive washes using 10 mM glutathione in 2x reaction buffer. The efficiency of the elution was monitored by the phosphatase assay described below. Active fractions were pooled and supplemented with 20% glycerol prior to storage at -80°C. His_6 tagged CL100 was purified using the same procedure except 20 mM β-mercaptoethanol was used in place of DTT for all steps of the purification and 100 mM imidazole was used instead of 10 mM glutathione for the elution.

The activity of the above DSPases was measured with FDP (Molecular Probes, Inc., Eugene, OR), which is readily metabolized to the fluorescent fluorescein monophosphate, as a substrate in a 96-well microtiter plates. The final incubation mixture (150 ml) comprised 30 mM Tris (ph 8.5), 75 mM NaCl, 0.67 mM EDTA, 0.033% bovine serum albumin, 1mM DTT and 20 μM FDP. Inhibitors were resuspended in DMSO and all reactions including controls were

performed at a final concentration of 7% DMSO. Reactions were initiated by adding ~ 0.25 μg of fusion protein and incubated at ambient temperature for 1 h. Fluorescence emission from the product was measured with a multiwell plate reader (Perseptive Biosystems Cytofluor II; Framingham, MA; excitation filter, 485/20; emission filter, 530/30). For all enzymes the reaction was linear over 2 h of incubation, well within the 1 h used in the experiments, and was directly proportional to both the enzyme and substrate concentration. The experimental results are presented in Table 5 below:

Table 5. Inhibition of DSPase activity with 100 μM combinatorial compound.

	Cdc25												MAPKP			
	Cdc25A		Cdc25A		Cdc25B		Cdc25B		Cdc25C		Cdc25C		CL100		CL100	
	% Inhibition															
Compound	Exp. 1		Exp. 2		Exp. 1		Exp. 2		Exp. 1		Exp. 2		Exp. 1		Exp. 2	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1a	35	3	30	1	0	0	0	0	6	3	0	0	10	3	5	6
1b	86	2	86	2	53	1	60	1	11	1	28	1	16	4	21	5
1c	90	1	88	3	55	1	60	2	44	2	35	5	19	3	0	0
1d	0	0	3	2	9	2	8	1	33	2	21	2	0	0	18	6
1e	95	4	92	3	82	1	84	2	64	3	56	1	26	4	27	6
1f	97	2	97	1	84	1	87	5	56	3	60	2	0	0	12	10
1g	48	3	52	5	42	10	0	0	18	1	2	1	0	0	0	0
1h	70	2	58	4	44	4	18	3	17	2	14	2	0	0	0	0
1i	77	2	62	2	35	1	38	1	21	8	28	2	0	0	0	0
1j	0	0	0	0	9	1	11	6	46	2	31	1	0	0	0	0
1k	20	4	15	2	24	1	24	2	27	4	40	4	0	0	4	6
1l	48	1	29	1	30	2	32	3	57	5	51	2	0	0	0	0
1m	85	4	77	1	53	1	57	1	54	1	52	2	0	0	0	0
1n	78	5	64	1	34	1	34	3	30	2	33	1	0	0	0	0
1o	76	3	55	1	34	1	34	3	37	4	36	1	0	0	0	0
1p	31	3	27	4	7	5	20	3	46	2	51	2	0	0	4	6
1q	48	2	43	2	46	3	29	2	52	6	51	1	25	5	21	1
1r	73	2	43	3	57	3	31	6	66	3	56	3	0	0	0	0

Each value is the % inhibition from untreated control and the mean from an experiment done in triplicate.

Table 4 confirms that the compounds significantly inhibited CDC25A, CDC25B and CDC25C phosphatase activity at 100 μM. Specifically, compounds 1e and 1f caused > 90% inhibition of CDC25A at 100 μM and compounds 1b, 1c and 1m

caused > 80% inhibition of CDC25A. These compounds also produced > 50% inhibition of CDC25B and CDC25C. However, inhibition of MAPKP CL100 was limited.

A comparison of the data in Tables 3 and 4 shows that there are structural characteristics which may be associated with successful inhibitors of PP2A and CDC25. Specifically, modification of the R′ position produced minor changes with no obvious overall preference for phenyl versus methyl among the congeners. The best inhibitors of this series were found when both R″ and R‴ contained hydrophobic moieties, such as aromatic or extended alkyl species. Compounds 1e and 1g produced approximately 30% inhibition of CL100 activity at 100 μM. In contrast, the other members of the combinatorial library consisting of compounds 1a-1r had little or no effect on this MAPKP DSPase. Thus, several compounds, such as 1f, appeared to have some selectivity for CDC25 DSPases compared to either the MAPKP DSPase CL100 or PSTPases. Moreover, distinct specificities emerged between PP2A and CDC25. The nonyl moiety has greater steric bulk and is more hydrophobic (logP > 4) compared to either the phenethyl (logP=3.15) or styryl (logP=2.95) moieties. Substitution of the nonyl moiety, which has a greater steric bulk than the phenethyl or styryl moieties, at the R‴ site of a compound containing a phenyl at R′ and a benzyl at R‴ caused a significant increase in CDC25 phosphatase inhibition and a complete loss of PSTPase inhibition as compared to the substitution of the phenethyl or styryl moiety at R‴. This may reflect a hydrophobic region on CDC25 near the active site. In contrast to CDC25 phosphatases, the PSTPases were much less tolerant of bulky, hydrophobic substitutions at R‴ and none of the R‴ nonyl compounds were effective inhibitors of PP1 or PP2A while compounds 1b and 1c were respectable inhibitors of CDC25A and B.

EXAMPLE V

The inhibition constants for SC-ααδ9 and SC-αα69 were determined via steady-state kinetics as follows. Reactions with GST-CDC25A, B, and C were conducted in 30 mM Tris (pH 8.5), 75 mM NaCl, 0.67 mM EDTA, 0.033% bovine serum albumin, and 1 mM DTT. Reactions with CL100 were conducted in 30 mM Tris (pH 7.0), 75 mM NaCl, 0.67 mM EDTA, 0.033% BSA, 1 mM DTT, and 20 mM imidazole. DMSO was kept at 7% in reaction mixtures to ensure compound solubility. All were carried out at room temperature and product formation determined in a multiwell plate reader (Perseptive Biosystems Cytofluor II; Framingham, MA; excitation filter, 485/20; emission filter, 630/30). Data were collected at 10 min intervals for 1 h. The V_o was determined for each substrate concentration and then fit to the Michaelis-Menten equation (Equation 1):

$$V_o=V_{max}[S]/K_m+[S]) \qquad \text{(Eq. 1)}$$

using Prism 2.0 (GraphPad Software Inc.). The correlation coefficient for each experiment and substrate was always >0.9. The substrate concentrations used to determine the steady-state kinetics for CDC25A, B and C, respectively, were 10, 20, 30, 40, 50, 75, 100 and 200 μM FDP while for CL100 the concentrations were 75, 100, 200, 300, 400, 500, and 750 μM FDP.

At least four concentrations of SC-ααδ9 and SC-αα69, ranging from 0 to 30 μM, were used with CDC25A or B. The K_i of CDC25C was calculated using at least 4 concentrations of drugs that ranged from 0 to 100 μM of SC-ααδ9 and SC-αα69. The K_i of CL100 was determined using 3 different concentrations of SC-ααδ9: 30, 100 and 300 μM. The results of these experiments are depicted in Table 6 below.

Table 6. K_m and inhibition constant of SC-SC-ααδ9 and SC-αα69 for DSPases

Compound	K_m (μM)		K_i for SC-ααδ9 (μM)		K_i for SC-αα69 (μM)	
	Average	SEM	Average	SEM	Average	SEM
Cdc25A	45	3	9	2	8	3
Cdc25B	12	3	6	2	7	3
Cdc25C	22	1	11	3	11	2
CL100	192	72	229	15	ND	ND

As shown in Table 6, the K_m determined with FDP for CDC25A, CDC25B, CDC25C and CL100 were 45 ± 3 (SEM, n=10), 12 ± 3, 22 ± 1, and 192 ± 72 μM, respectively. Therefore, FDP was a much better substrate for CDC25 phosphatases than for the MAPKP CL100. Kinetic studies using SC-ααδ9 and SC-αα69 with CDC25B were most consistent with a competitive inhibition model. It was also found that SC-ααδ9 competitively inhibits CDC25A, CDC25C and CL100 and SC-αα69 competitively inhibits CDC25A and CDC25C. SC-αα69 had a K_i of 7 ± 3 μM for CDC25B phosphatase. The K_i for CDC25A and CDC25C were 8 ± 3 μM and 11 ± 2 μM, respectively. The K_i for SC-ααδ9 for the MAPKP phosphatase CL100 was 229 ± 115.

EXAMPLE VI

The concentration dependence of the inhibitory effect of compounds 1e and 1f, and their analogues which have been prepared by solution chemistry: namely, SC-αα69 and SC-ααδ9, were examined. As illustrated in Figs. 15A-D, both solid-phase derived 1f and solution phase derived SC-ααδ9 demonstrated concentration-dependent inhibition of recombinant human CDC25A and B activity. The half-maximal inhibitory concentration (IC_{50}) for CDC25A and B was 75 μM when treated with compound 1f, while SC-ααδ9 showed an IC_{50} of approximately 15 μM for CDC25A and B. Thus, the compounds prepared by solution chemistry displayed a 5-fold greater inhibitory activity compared to the compounds

synthesized by solid-phase methods, which could reflect the increased purity, the inclusion of R-stereoisomers in the racemic solution chemistry compounds, or both. Similarly, both compound 1e and SC-αα69 samples resulted in concentration-dependent inhibition of CDC25A and B, with the racemic solution chemistry compound being approximately 5-6 fold more potent (Figs. 15(b) and (d)). The widely used PTPase inhibitor vanadate had an IC_{50} of 1 μM for CDC25A and B in this assay.

EXAMPLE VII

Several of the compounds listed in Figs. 12a-12n were also tested by the methods described in Examples III and IV to determine whether they were effective inhibitors of PP1, PP2A, CDC25C and PTP1B. These compounds, wherein Z is not a bivalent ethylene segment, showed significant differences in inhibitory activity at 100 μM with FY-αα69, FY-α109, FY6-α109, FY9-α109 and FY10-α109 lacking any anti-CDC25C activity. (See Fig. 14). These compounds also showed significant differences in anti-PTP1B activity at 3 μM. Constrained structures, such as FY2-αα69, FY2-α109, FY3-αα69, FY-αα69, FY7-αα69, FY7-α109 and FY8-αα69, retained CDC25C inhibitory activity at this lower concentration. (See Fig. 14.) The two cis-metaphenyl compounds, FY7-αα69 and FY7-α109, were the only compounds that at 100 μM caused a >50% inhibition of PP1 and/or PP2A.

EXAMPLE VIII

Compounds 1a-1r were tested for antiproliferative activity against human MDA-MB-231 breast cancer cells.

Human MDA-MB-231 breast carcinoma cells were obtained from the American Type Culture Collection at passage 28 and were maintained for no longer than 20 passages. The cells were grown in RPMI-1640 supplemented with 1% penicillin (100 μg/mL) and streptomycin (100 μg/mL), 1% L-glutamate, and

10% fetal bovine serum in a humidified incubator at 37 °C under 5% CO_2 in air. Cells were routinely found free of mycoplasma. To remove cells from the monolayer for passage or flow cytometry, cells were washed two times with phosphate buffer and briefly (< 3 min) treated with 0.05% trypsin/2 mM EDTA at room temperature. After the addition of at least two volumes of growth medium containing 10% fetal bovine serum, the cells were centrifuged at 1,000 x g for 5 min. Compounds were made into stock solutions using DMSO, and stored at -20 °C. All compounds and controls were added to obtain a final concentration of 0.1-0.2% (v/v) of the final solution for experiments.

The antiproliferative activity of the compounds was determined by the method of Lazo et al. (1995) J. Biol. Chem. 270:5506. Briefly, cells (6.5 x 10^3 cells/cm^2) were plated in 96 well flat bottom plates for the cytotoxicity studies and incubated at 37 °C for 48 h. The plating medium was aspirated off 96 well plates and 200 μL of growth medium containing the compound was added per well. Compounds were used at from O to the highest available concentration which ranged from 30 to 100 μM. Plates were incubated for 72 h, and then washed 4x with serum free medium. After washing, 50 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide solution (2 mg/mL) was added to each well, followed by 150 μL of complete growth medium. Plates were then incubated an additional 4 h at 37 °C. The solution was aspirated off, 200 μL of DMSO added, and the plates were shaken for 30 min at room temperature. Absorbance at 540 nm was determined with a Titertek Multiskan Plus plate reader. Biologically active compounds were tested at least 3 independent times.

Administration of compound 1h caused 50% growth inhibition at 20 μM but had no further cytotoxicity at higher drug concentrations. Compound 1f caused 50% growth inhibition at 100 μM and had a clear concentration-dependency (Figure 9).

EXAMPLE IX

The cell cycle distribution of human breast cancer cells after treatment with compound 1f was determined by flow cytometry.

MDA-MB-231 cells (6.5 x $10^5/cm^2$) were plated and incubated at 37 °C for 48 h. The plating medium was then aspirated off, and medium containing a concentration of compound 1f that caused approximately 50% growth inhibition (88-100 μM) was added for 48 to 72 h. Untreated cells at a similar cell density were used as control populations. Single cell preparations were fixed in ice-cold 1% paraformaldehyde, centrifuged at 1,000 x g for 5 min, resuspended in Puck's saline, centrifuged, and resuspended in ice-cold 70% ethanol overnight. The cells were removed from fixatives by centrifugation (1,000 x g for 5 min) and stained with a 5 μg/mL propidium iodide and 50 μg/mL RNase A solution. Flow cytometry analyses were conducted with a Becton Dickinson FACS Star. Single parameter DNA histograms were collected for 10,000 cells, and cell cycle kinetic parameters calculated using DNA cell cycle analysis software version C (Becton Dickinson). Experiments at 72 h were performed at least 3 independent times.

Results are presented in Figs. 10A-D.

Exponentially growing human MDA-MB-231 breast cancer cell populations (population doubling time of approximately 30-35 h) typically have approximately 30% of all cells in the S or DNA synthetic phase of the cell cycle (Figs. 10A and C). In contrast, when MDA-MB-231 cells were incubated for 48 h with 88 μM compound 1f, there was prominent accumulation in the G1 phase with a concomitant decrease in both S and G2/M phases (Figs. 10B and C). Incubation of MDA-MB-231 cells for 72 h with 88 μM 1f also caused a prominent accumulation in the G1 phase (Figure 10D).

These results demonstrate that compound 1f exhibits a concentration-dependent inhibition in proliferation of MDA-MB-231 cells, and that blockage in cell cycle progression is at the G1 checkpoint.

EXAMPLE X

The compounds listed in Figs. 12(a)-12(n), together with SC-ααδ9 and SC-α1δ9 were tested for antiproliferative activity against SCCVII/SF cells.

Murine SCCVII/SF squamous cell carcinoma tumors were produced by subcutaneous inoculation of 5 x 10^5 exponential growth-phase cells from culture in the right flank of 6- to 10- week-old female C3/HeJ mice as described by Fu et al. (1984) Int. Radiat. Oncol. Biol. Phys. 10: 1473, the disclosure of which is hereby incorporated by reference. SCCVII/SF cells were generated from tumors by routine dissection and resuspension in Dulbecco's Minimum Essential Medium containing 20% fetal bovine serum, Penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO at 37°C.

Exponentially growing murine SCCVII/SF head and neck cancer cells, which grow readily as tumors in mice, and are CDC25A positive based on Western blotting, were exposed to library compounds for 48 hours and the cell number was determined by the titration procedure described in Example VIII. (See Fig. 16) At 80 μM, 6 of the 12 compounds produced >50% growth inhibition (see, e.g., compounds 1e and 1f). Thus, the SCCVII/SF tumor cells were more sensitive to the growth inhibition by the library compounds than MDA-MB-231 cells as described in Example VIII. The IC_{50} for growth inhibition of the compounds 1e and 1f were 20 and 25 μM, respectively (Fig. 16). After 24 hours of exposure of the SCCVII/SF cells to 30 μM SC-ααδ9, they were found to be arrested in the G_1 phase. In studies with MDA-MB-231 cells, the IC_{50} for FY8-α109 and FY8-αα69 was approximately 100 μM, and the IC_{50} for FY7-αα69 was 50 μM. The other compounds shown in Figures 12(a) - 12(n) displayed little or no growth inhibition in this initial study, which suggests some selectivity in cytotoxicity and confirms entry into the cell for the compounds shown in Figures 12(a) - 12(n).

EXAMPLE XI

In vivo antitumor studies were performed by treating mice having tumors with compounds according to the invention.

To facilitate these studies, the toxicity of compound SC-ααδ9, which corresponds to combinatorial compound 1f, was determined. In a preliminary study, three age-matched female C57B1/6 mice (average weight = 24g) were injected with 0 or 30 mg/kg of this drug i.p. once a day for 5 days. No significant weight loss was observed and no mortality was seen almost two months after the injection.

Mice having tumors were treated with SC-ααδ9 to determine if the compound would inhibit tumors. The particular clonogenic survival assay employed has been described by Fu et al., (1984) Int. Radiat. Oncol. Biol. Phys. 10:1473, which is incorporated by reference herein.

Female mice (3/4 treatment group) with established subcutaneous (s.c.) tumors (14 days postimplant) were treated with a single intraperitoneal dose of either SC-ααδ9, SC-αα69 or vehicle (Cremophore and ethanol). Tumors were then harvested after 24 h and the numbers of clonogenic tumor cells determined. Fig. 18 shows the dose-dependent reduction in the clonogenicity of SCCVII/SF tumor cells after a single dose of either agent. The maximum effect seen with both SC-ααδ9 and SC-αα69 was a 50% decrease at 45 mg/kg. This decrease is comparable to the effects seen with a single dose of 30 mg/kg cyclophosphamide, 30 mg/kg doxorubicin, 75 mg/kg carboplatin or 4 mg/kg cisdiaminedichloroplatinum in this tumor model.

EXAMPLE XII

SC-ααδ9 was tested to determine if it would show preferential cytotoxicity for transformed cells.

First, MEF cells were isolated from fetuses of 14.5-day pregnant mice (129 Ola x C57Bl/6) using previously described methods. Cells were maintained in

Dulbecco's Minimum Essential Medium (GIBCO, BRL) containing 20% fetal bovine serum (HyClone, Logan, UT), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO at 37°C. Primary cultures of MEF cells were never extended beyond passage 15 to avoid entering of crisis. MEF cells were transformed using plasmid containing SV-40 large T antigen using lipfectamine (GIBCO, BRL) according to the manufacturer's instructions.

Western blotting was performed as follows. Cells were grown to subconfluency in 100 mm dishes, harvested, and lysed in HEPES lysis buffer as described by Sun et al. (1995) Cancer Res. 55:4243, which is hereby incorporated by reference. Lysates were electrophoresed on 4-20% SDS-PAGE, transferred to nitrocellulose and immunoblotted with antibodies against SV40 large T Antigen (Ab-2 Oncogene Science, Manhasset, NY), CDC25B (C-20), or anti-CDC25A (144) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Positive antibody reactions were visualized using peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection system as described by Vogt et al. (1995) J. Biol. Chem. 270:660, which is hereby incorporated by reference.

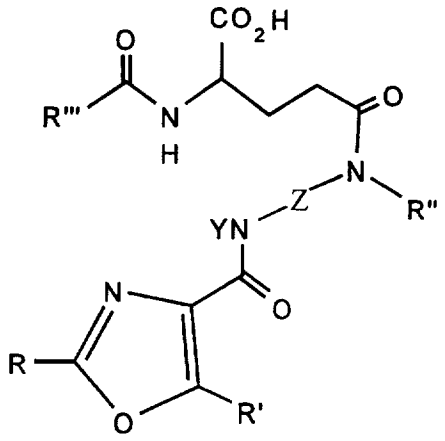
The colony formation assay was performed as follows. MEF and SV40/MEF cells were plated (300 and 200 cells/well, respectively) in triplicate in 6 well plates and treated the next day with vehicle (DMSO) or various concentrations of SC-ααδ9 in DMSO without change of medium in order to not disturb the cell attachment process. After 10 to 12 days, when visible colonies had formed, colonies were fixed and stained with crystal violet/formaldehyde solution. Plating efficiency was determined as the percentage of cells that attached to the support and grew into colonies larger than 1 mm in diameter.

The results of Western blotting showed that the transformed cells produced increased levels of CDC25B (Figs. 19(a)-(c)). After treatment with SC-ααδ9, normal and transformed MEF were found to exhibit preferential cytotoxicity (IC_{50} in SV40 MEF: 180 μM; Figure 20C). In contrast, neither okadaic acid nor vanadate preferentially affected cell proliferation. These results indicate that

SC-ααδ9 has antiproliferative activities distinct from the PSTP inhibitor and the broad PTP inhibitor vanadate. It was also found that continuous exposure to SC-ααδ9 more greatly affects the colony formation of SV40 transformed MEF compared to nontransformed MEF cells (Fig. 21).

Claims

1. A compound having the formula I:



I

wherein,

Z represents one or two bivalent segments which are bound to two central nitrogen atoms as shown wherein a shortest path between the two central nitrogen atoms has less than ten atoms;

R, R′ and R‴ are each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, phenyl, oxetanyl, azetidinyl, furanyl, pyrrole, indolyl, oxazolyl, isoxazolyl, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, pyranyl, pyridyl, pyridonyl, piperidyl, piperazinyl, quinolyl, azepinyl, and diazepinyl;

R″ is absent when Z represents two bivalent segments, and if Z represents a single bivalent segment R″ is selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, phenyl, oxetanyl, azetidinyl, furanyl, pyrrole, indolyl, oxazolyl, isoxazolyl, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, pyranyl, pyridyl, pyridonyl, piperidyl, piperazinyl, quinolyl, azepinyl, and diazepinyl;

Y is absent when Z represents two bivalent segments and if Z represents a single bivalent segment Y is H.

2. The compound of Claim 1 wherein Z represents a single bivalent segment, R and R‴ are independently phenyl, CH_3, n-C_5H_{11}, n-C_7H_{15}, n-C_9H_{19}, PhCHCH, $PhCH_2CH_2$, $Ph(CH_2)_2CC(CH_3)$, (p-MeO)Ph, (p-MeNHCO)Ph, $PhCHC(CH_3)CH_2CH_2$, $Ph(CH_2)_2CHCHCHC(CH_3)$, $Ph(CH_2)_2CHCHCHCH$, $Ph(CH_2)_3CHC(CH_3)CHCH$, $C_6H_{13}CH(CH_3)CHC(CH_3)CHCH$, or $C_4H_9CH(CH_3)CHC(CH_3)CHC(CH_3)$, R′ is H, CH_3 or Ph, and R″ is H, CH_3, benzyl, $CH_2CH(CH_3)$, n-C_6H_{13}, CH_2CH_2NHBn, CH_2CH_2Ph, or $(CH_2)_3Ph$.

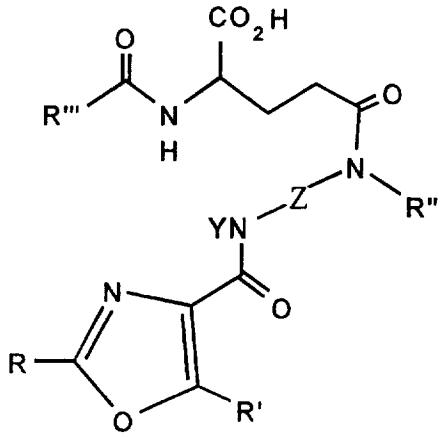
3. The compound of Claim 1 wherein Z is a bivalent ethylene segment, Y is H, R is phenyl, R′ is phenyl, R″ is benzyl or CH_3 and R‴ is n-C_9H_{19}.

4. The compound of Claim 1 wherein Y is H and Z is a single bivalent segment selected from the group consisting of an alkylene segment, an alkenylene segment a alkynylene segment, a cycloalkylene segment, an aromatic hydrocarbon segment, and heteroatom-substituted variants of these same segments.

5. The compound of Claim 4 wherein Z is selected from the group consisting of an alkylene segment, a cyclohexylene segment and a phenylene segment.

6. The compound according to Claim 2 wherein Y is H and Z is a single bivalent segment selected from the group consisting of an alkylene segment, an alkenylene segment a alkynylene segment, a cycloalkylene segment, an aromatic hydrocarbon segment, and heteroatom-substituted variants of these same segments.

7. A method of making a compound having the formula I



I

comprising in order the steps of:

(a) coupling a diprotected glutamate to a solid support;

(b) deprotecting the glutamate amino-terminus;

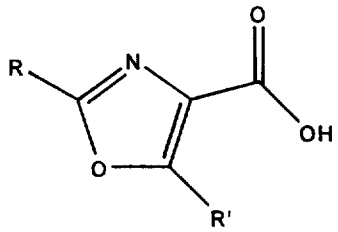
(c) adding R‴ COX, wherein X is a leaving group;

(d) deprotecting the glutamate carboxy terminus;

(e) adding diamine having the formula A-NYZNH(R″), wherein A is a protecting group, and a coupling agent;

(f) deprotecting the amino-terminus of said diamine;

(g) adding



and

(h) cleaving the resulting compound from the solid support.

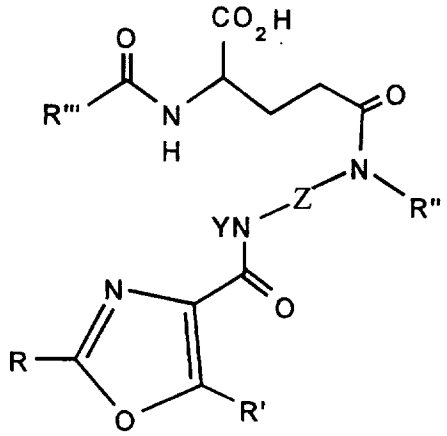
8. The method of Claim 7 wherein said solid support is a polystyrene resin.

9. The method of Claim 7 wherein said diprotected glutamate is 2-(9-H-fluoren-9-ylmethoxycarbonylamino)-pentanedioic acid 5-allyl ester.

10. The method of Claim 7 wherein A is an N-allyloxycarbonyl group.

11. The method of Claim 7 wherein said compound is cleaved from said solid support with trifluoroacetic acid.

12. A method of making a compound having the formula I



I

comprising in order the steps of:

(a) coupling diprotected glutamate to a solid support;

(b) deprotecting the glutamate amino-terminus;

(c) distributing the solid support to multiple vessels;

(d) adding R‴ COX, wherein X is a leaving group, to each vessel;

(e) deprotecting the glutamate carboxyl terminus;

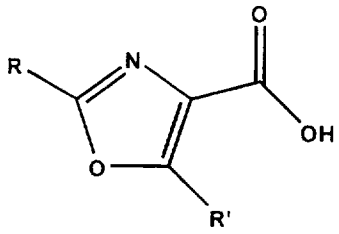
(f) distributing the solid support to additional multiple vessels;

(g) adding diamine having the formula A-NYZNH(R″), wherein A is a protecting group, and a coupling agent, to each vessel;

(h) deprotecting the amino terminus of said diamine;

(i) distributing the solid support to additional multiple vessels;

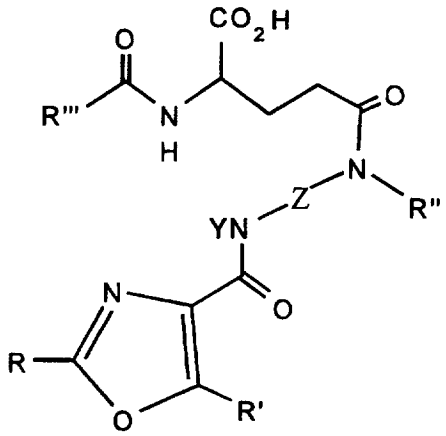
(j) adding



and

(k) cleaving the resulting compound from the solid support.

13. A method of inhibiting a protein phosphatase comprising contacting a protein phosphatase with a protein phosphatase-inhibiting effective amount of a compound having the formula



I

wherein,

Z represents one or two bivalent segments which are bound to two central nitrogen atoms as shown wherein a shortest path between the two central nitrogen atoms has less than ten atoms;

R, R′ and R‴ are each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, phenyl, oxetanyl, azetidinyl, furanyl, pyrrole, indolyl, oxazolyl, isoxazolyl, imidazolyl,

pyrazolyl, triazolyl, tetrazolyl, pyranyl, pyridyl, pyridonyl, piperidyl, piperazinyl, quinolyl, azepinyl, and diazepinyl;

R″ is absent when Z represents two bivalent segments, and if Z represents a single bivalent segment R″ is selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, phenyl, oxetanyl, azetidinyl, furanyl, pyrrole, indolyl, oxazolyl, isoxazolyl, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, pyranyl, pyridyl, pyridonyl, piperidyl, piperazinyl, quinolyl, azepinyl, and diazepinyl;

Y is absent when Z represents two bivalent segments and if Z represents a single bivalent segment Y is H.

14. The method of Claim 13 wherein said protein phosphatase is a serine/threonine protein phosphatase.

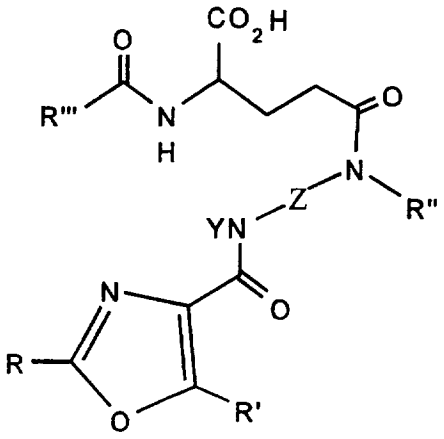
15. The method of Claim 14 wherein said protein phosphatase is PP1, PP2A or PP3.

16. The method of Claim 13 wherein said protein phosphatase is a dual specificity phosphatase.

17. The method of Claim 16 wherein said protein phosphatase is CDC25A or CDC25B.

18. The method of Claim 13 wherein said protein phosphatase is VHR or MAPKP.

19. A method of inhibiting cell proliferation comprising introducing into cells a proliferation-inhibiting amount of a compound having the formula:



I

wherein Y is H, Z is an ethylene segment, R is phenyl, R′ is phenyl, R″ is benzyl, and R‴ is n-C_9H_{19}.

20. The method of Claim 19 wherein said cells are tumor cells.

21. The method of Claim 19 wherein said cells are breast cancer cells.

22. A pharmaceutical composition comprising the compound of Claim 1 and a pharmaceutically acceptable carrier.

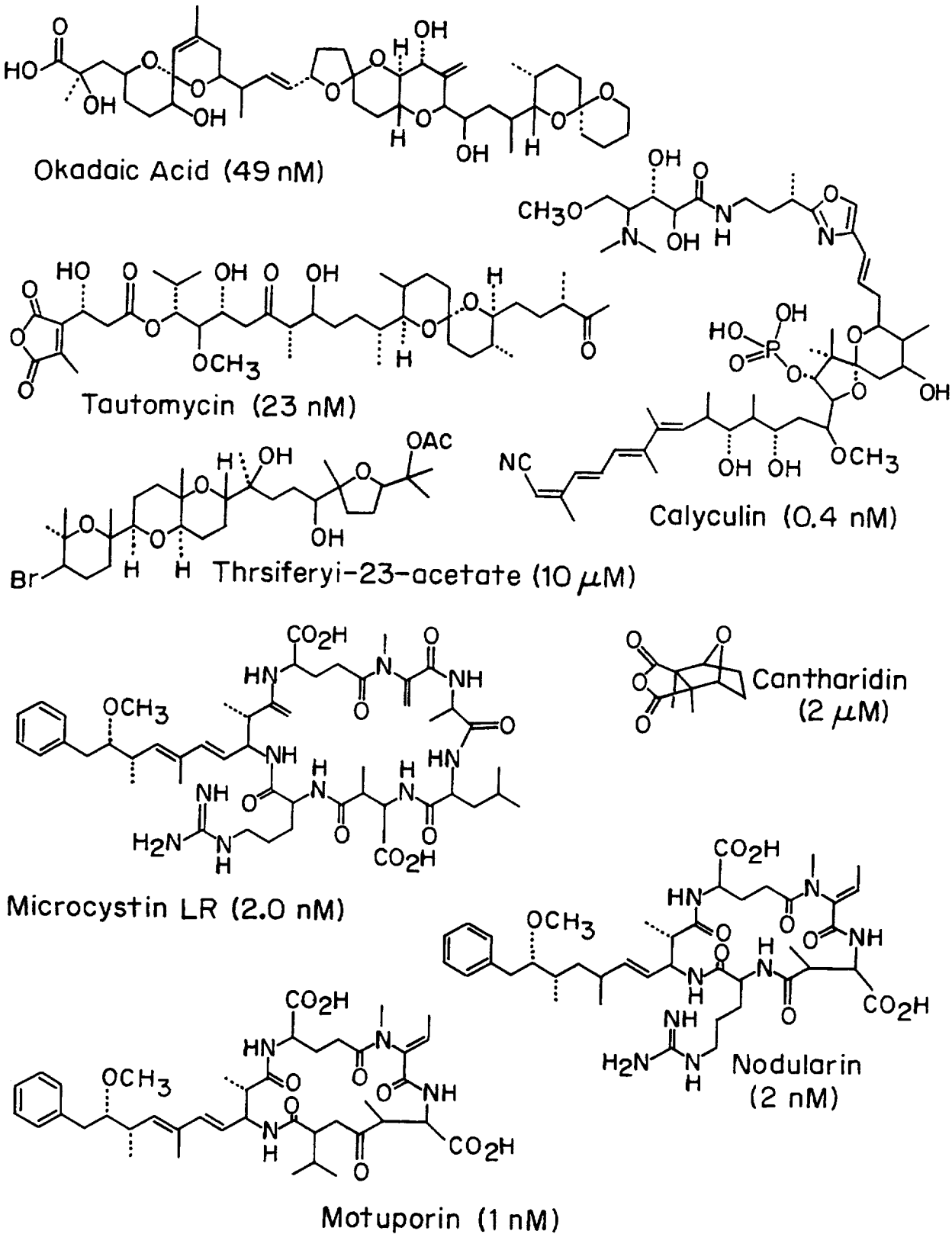

Okadaic Acid (49 nM)
Tautomycin (23 nM)
Calyculin (0.4 nM)
Thrsiferyi-23-acetate (10 μM)
Cantharidin (2 μM)
Microcystin LR (2.0 nM)
Nodularin (2 nM)
Motuporin (1 nM)

FIG. 1(a)

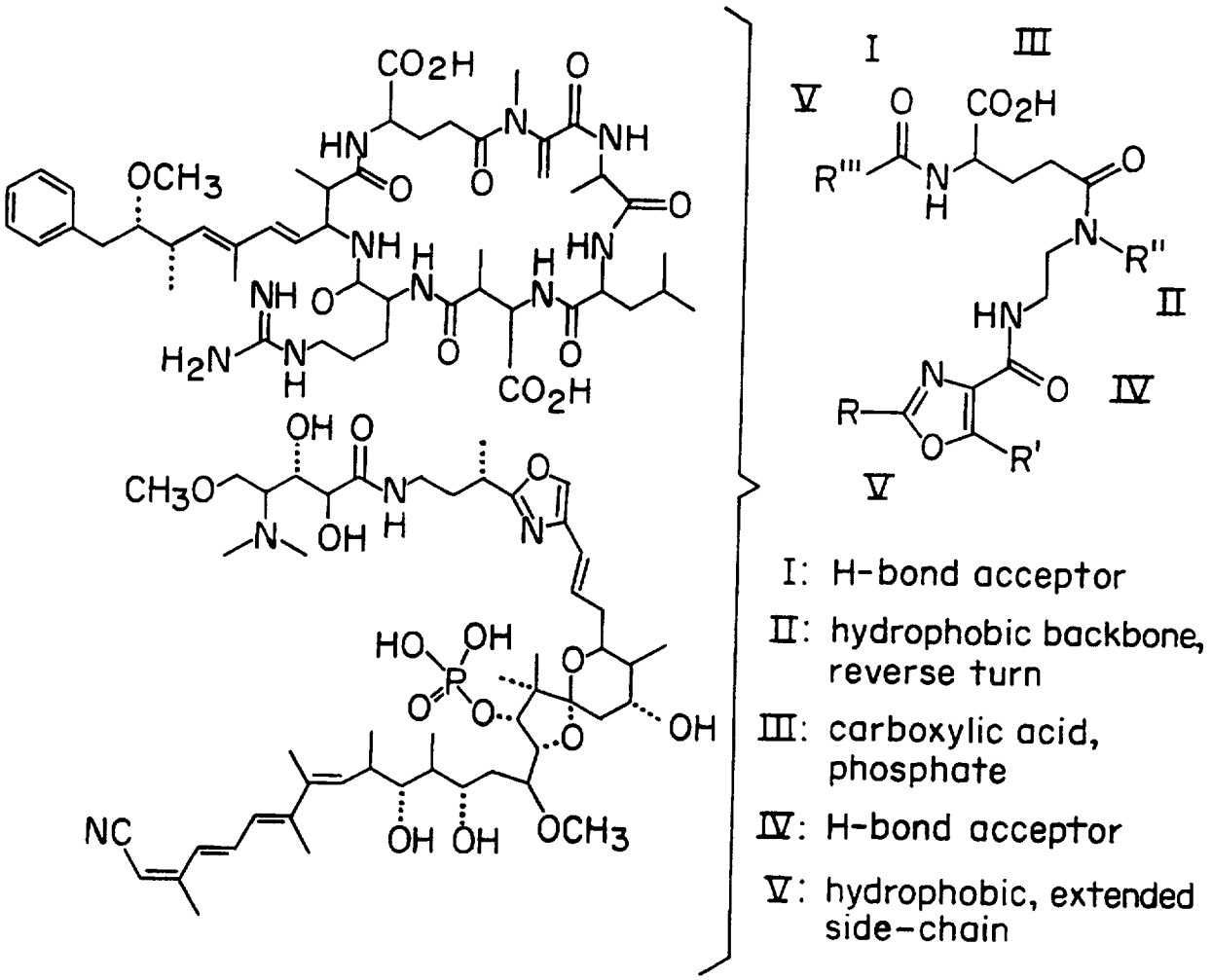

I
III
V
II
IV
V
I: H-bond acceptor
II: hydrophobic backbone, reverse turn
III: carboxylic acid, phosphate
IV: H-bond acceptor
V: hydrophobic, extended side-chain

FIG. 1(b)

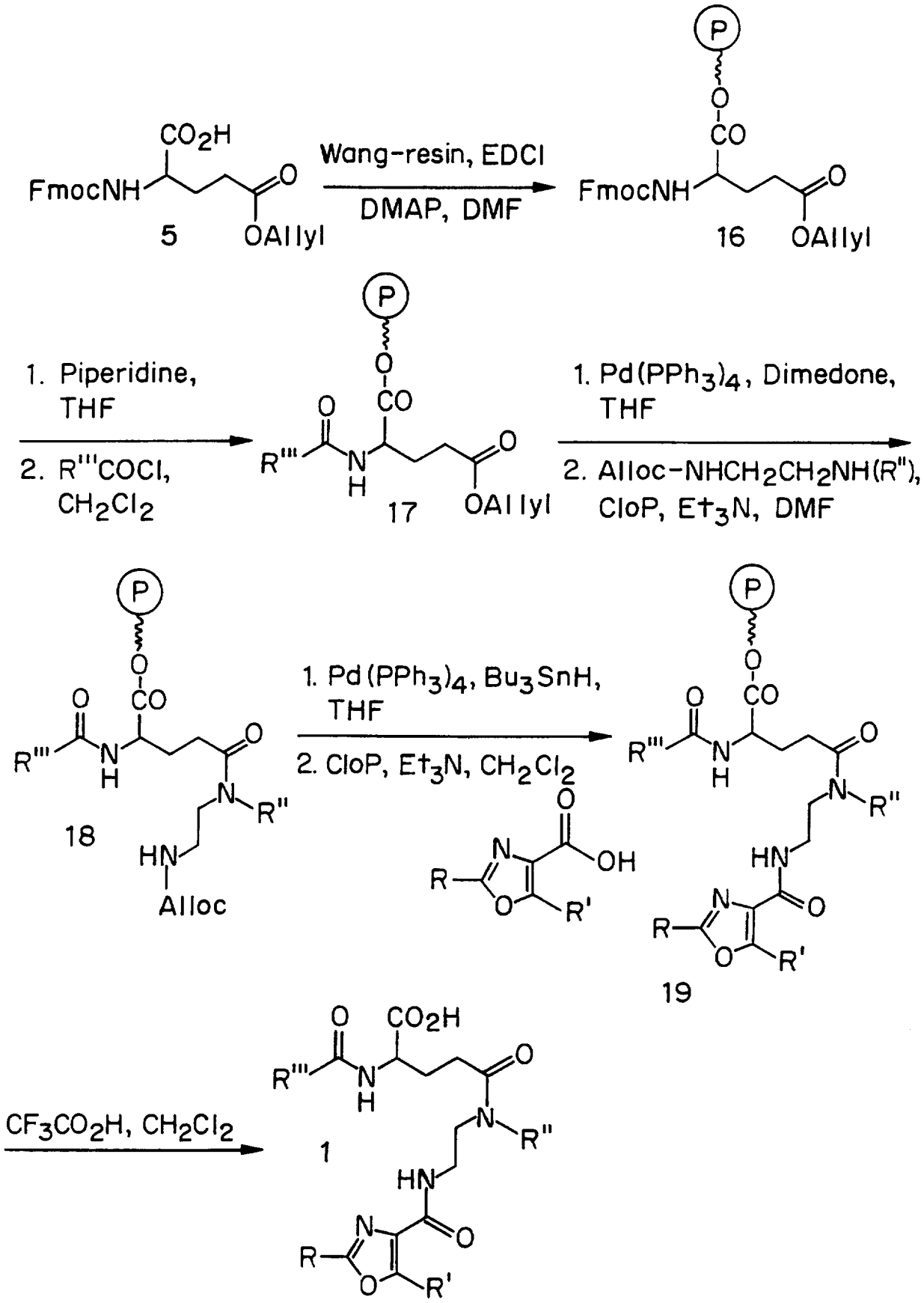

FmocNH
CO2H
OAllyl
5
Wang-resin, EDCI
DMAP, DMF
16
1. Piperidine, THF
2. R'''COCl, CH2Cl2
17
1. Pd(PPh3)4, Dimedone, THF
2. Alloc-NHCH2CH2NH(R''), ClOP, Et3N, DMF
18
Alloc
1. Pd(PPh3)4, Bu3SnH, THF
2. ClOP, Et3N, CH2Cl2
19
CF3CO2H, CH2Cl2
1

FIG. 2

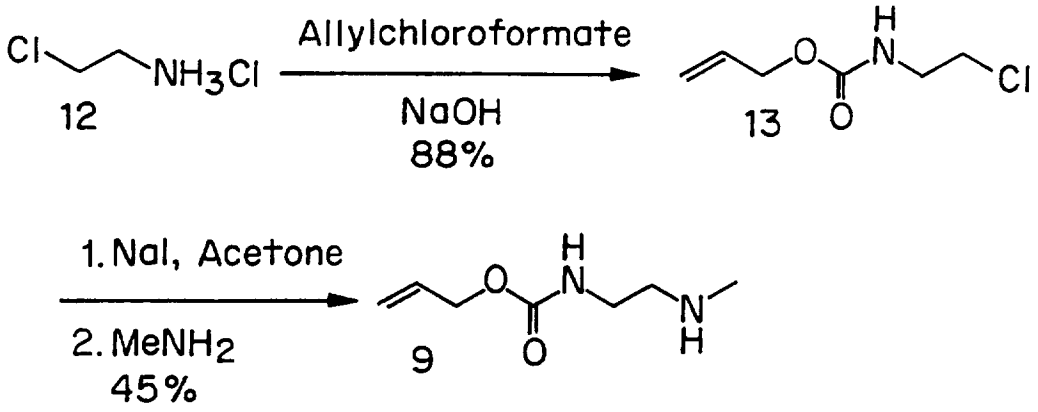


FIG. 3

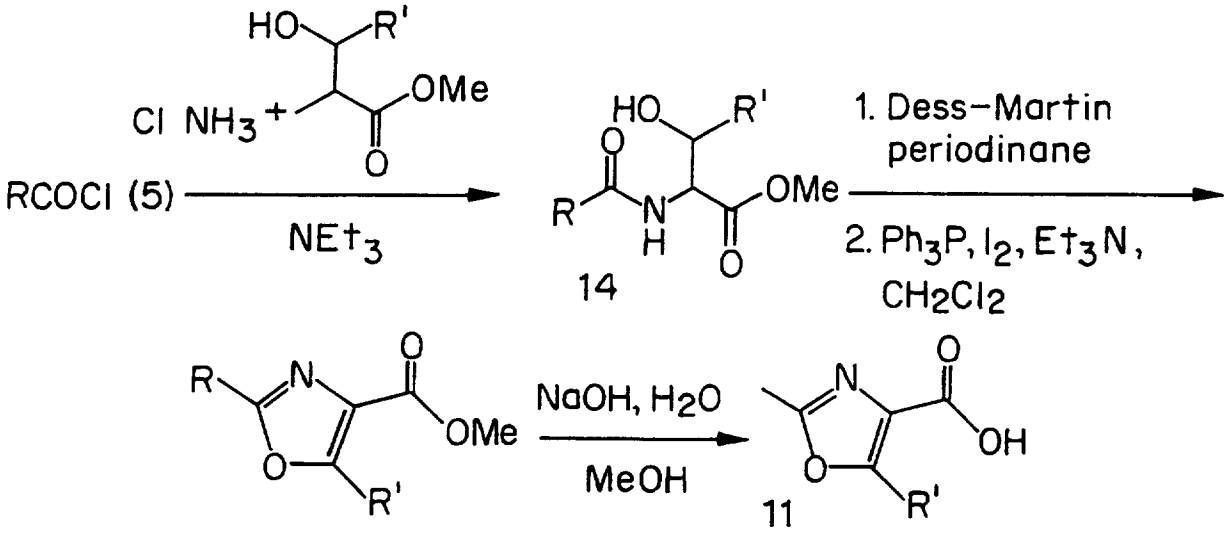


FIG. 4(a)

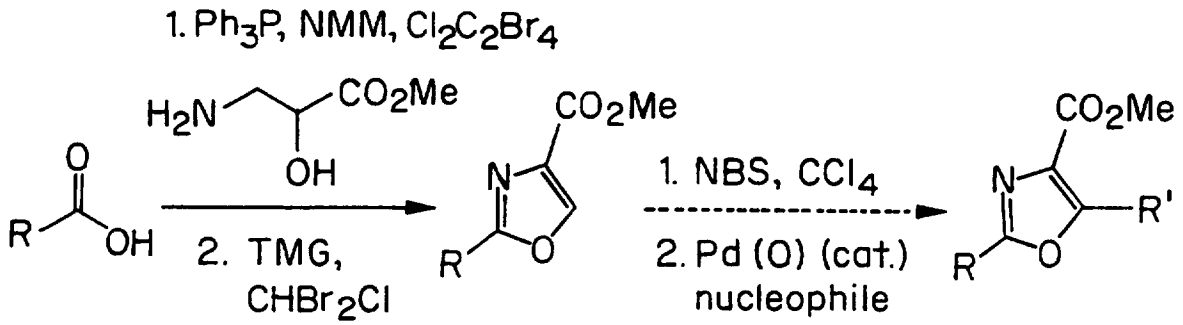


FIG. 4(b)

SCHEME 1

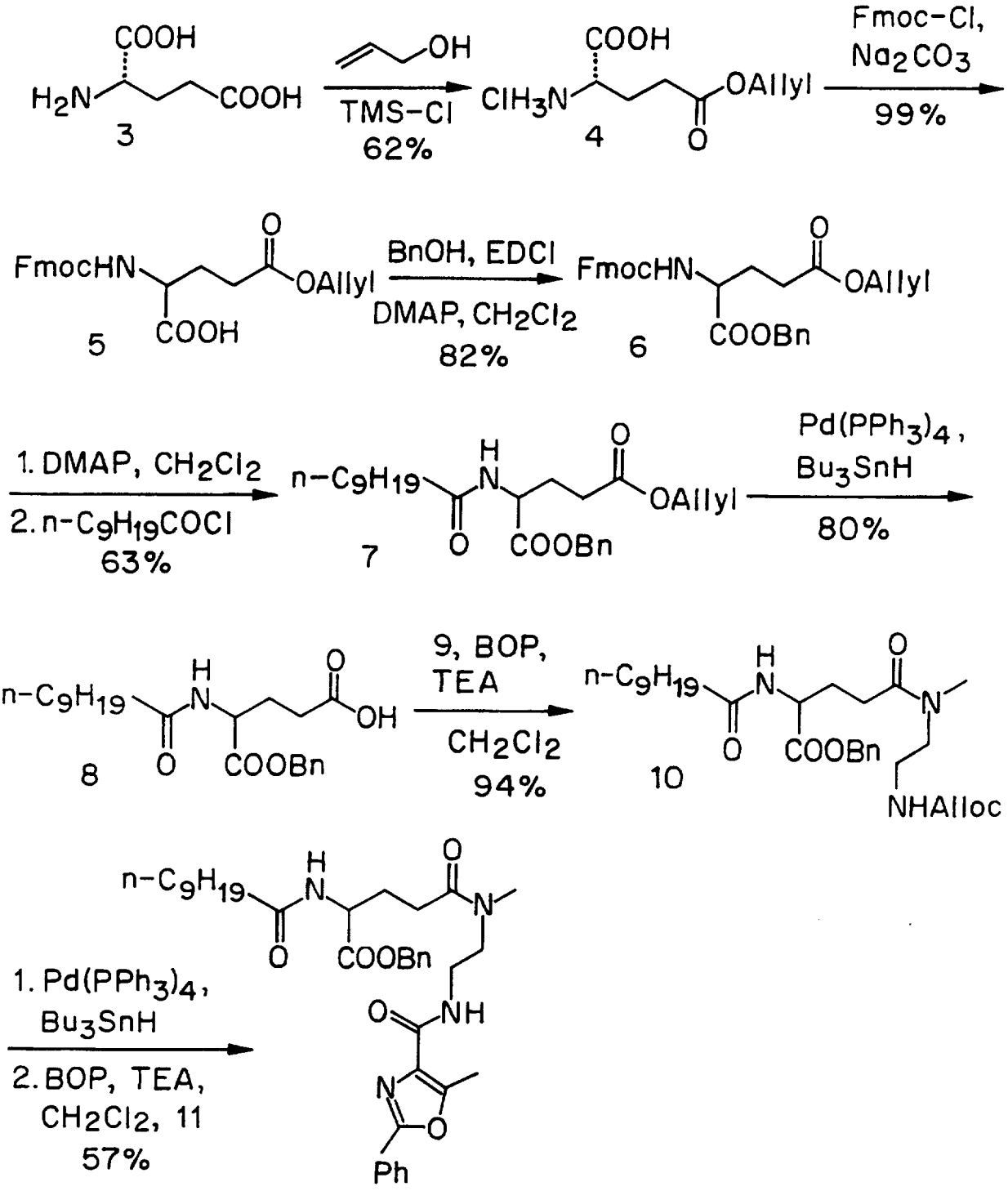


FIG. 5

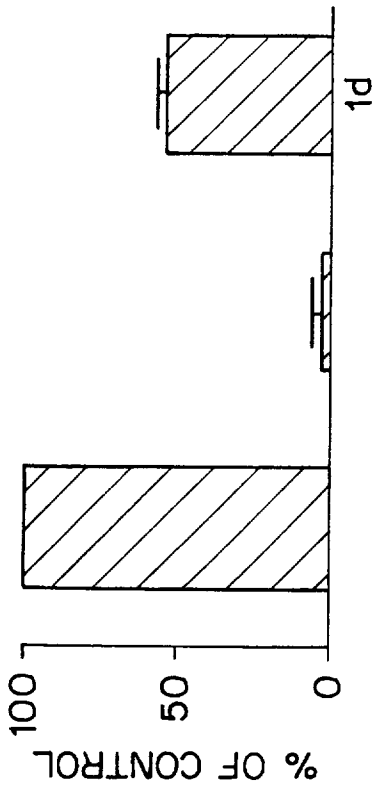

% OF CONTROL
100
50
0
1d

FIG. 6

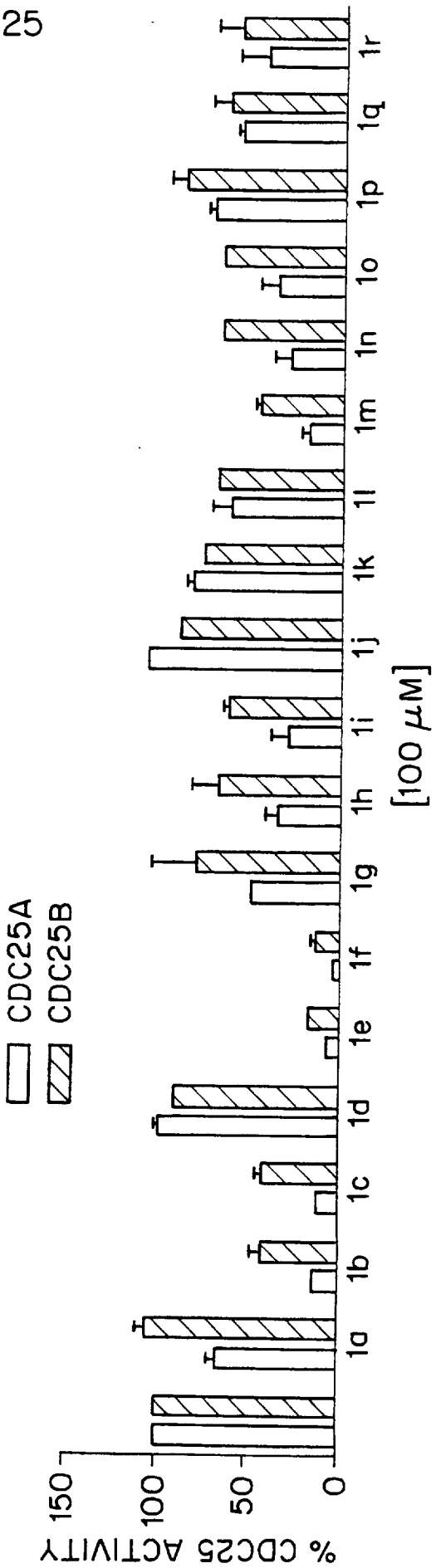

% CDC25 ACTIVITY
150
100
50
0
CDC25A
CDC25B
1a
1b
1c
1d
1e
1f
1g
1h
1i
1j
1k
1l
1m
1n
1o
1p
1q
1r
[100 μM]

FIG. 7

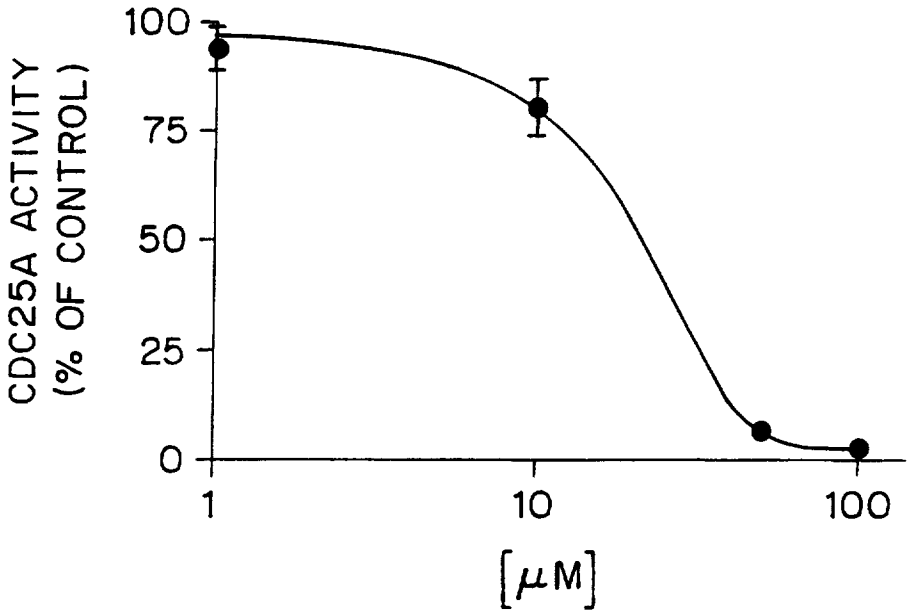

CDC25A ACTIVITY
(% OF CONTROL)
100
75
50
25
0
1
10
100
[μM]

FIG. 8

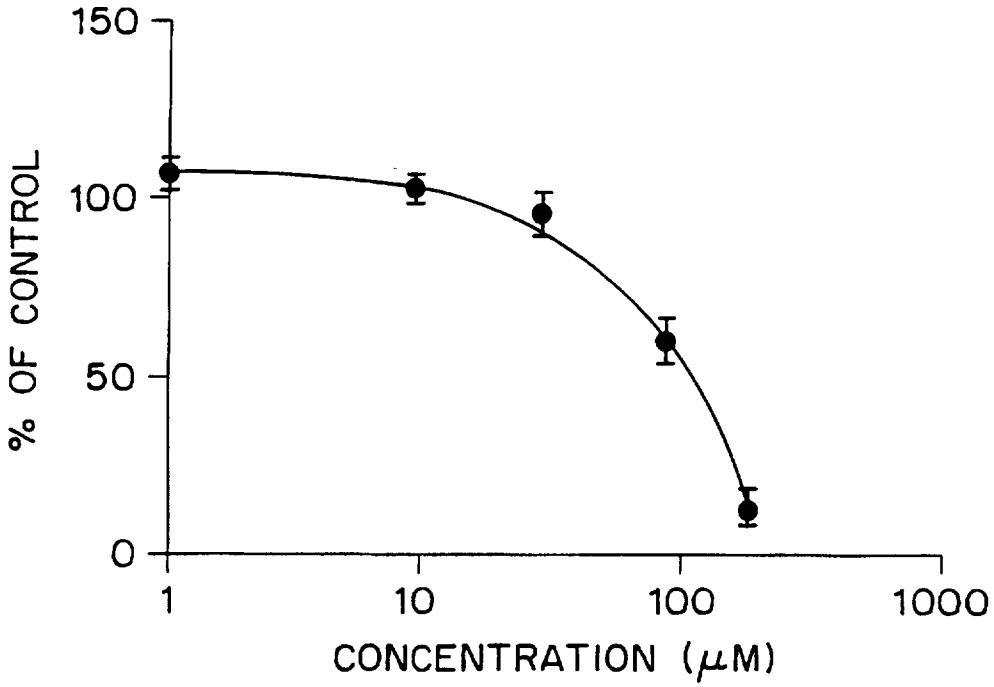

% OF CONTROL
150
100
50
0
1
10
100
1000
CONCENTRATION (μM)

FIG. 9

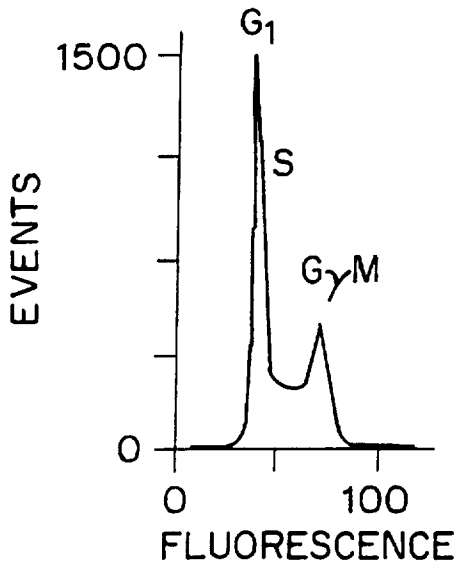


FIG. 10A

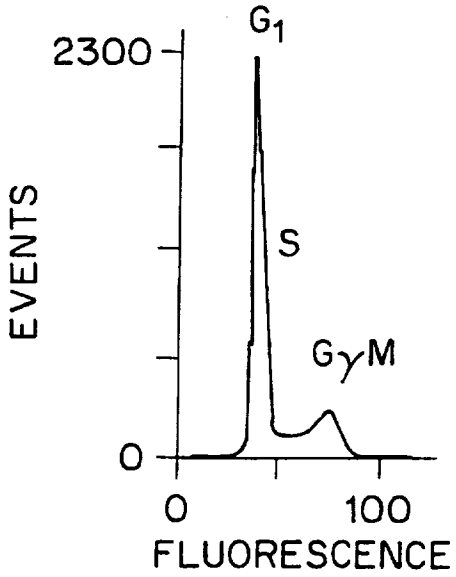


FIG. 10B

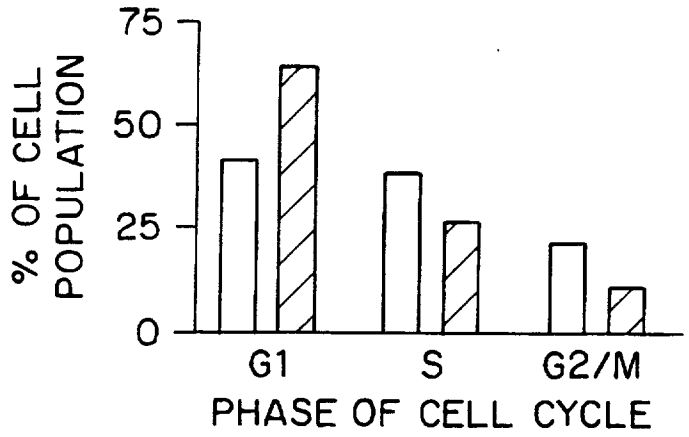


FIG. 10C

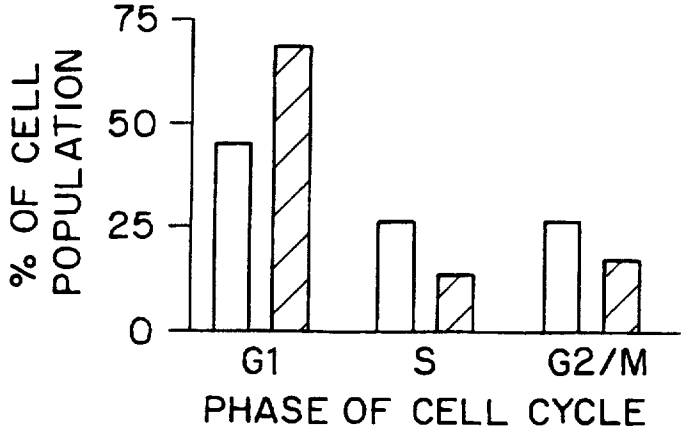


FIG. 10D

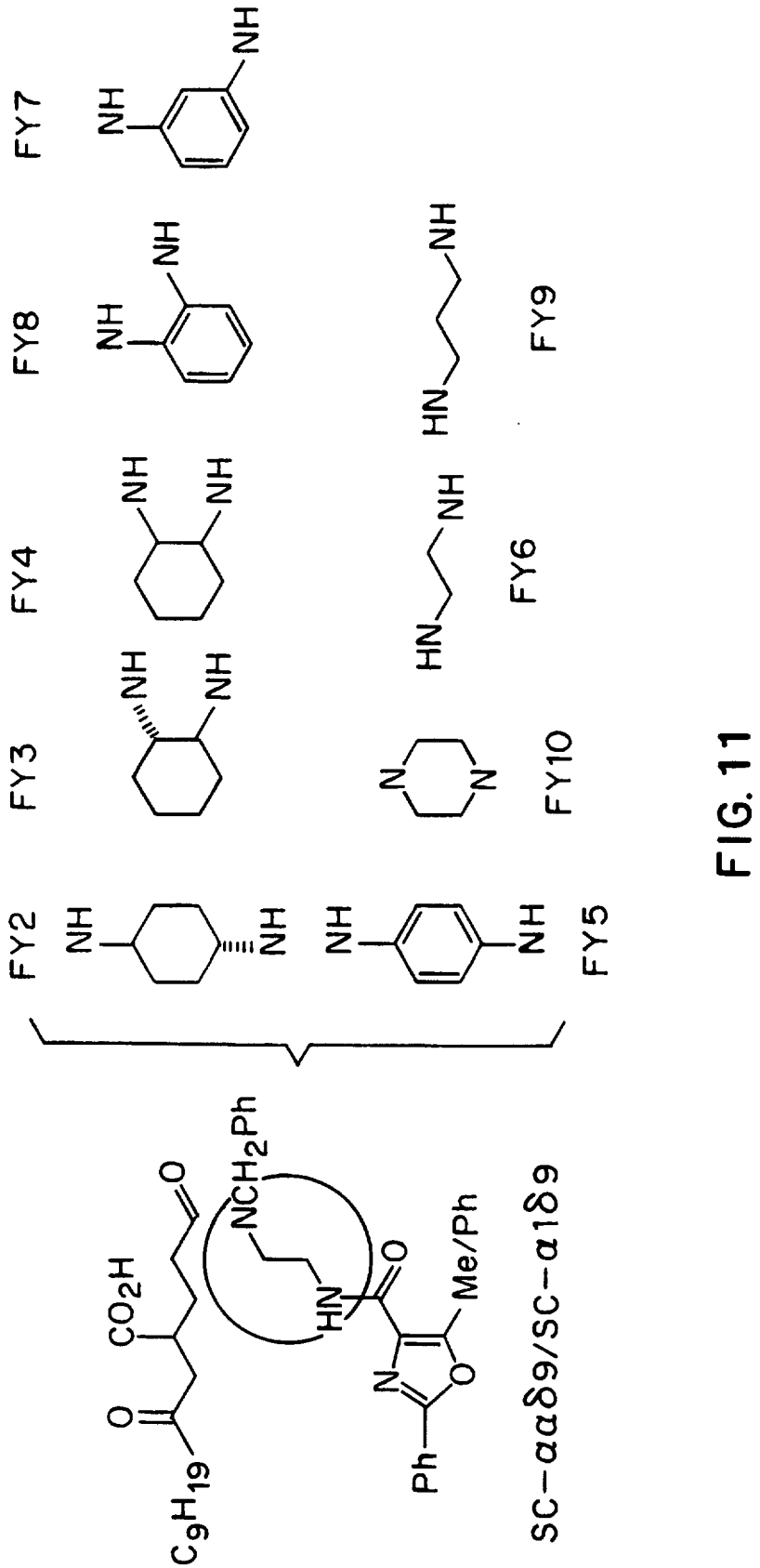
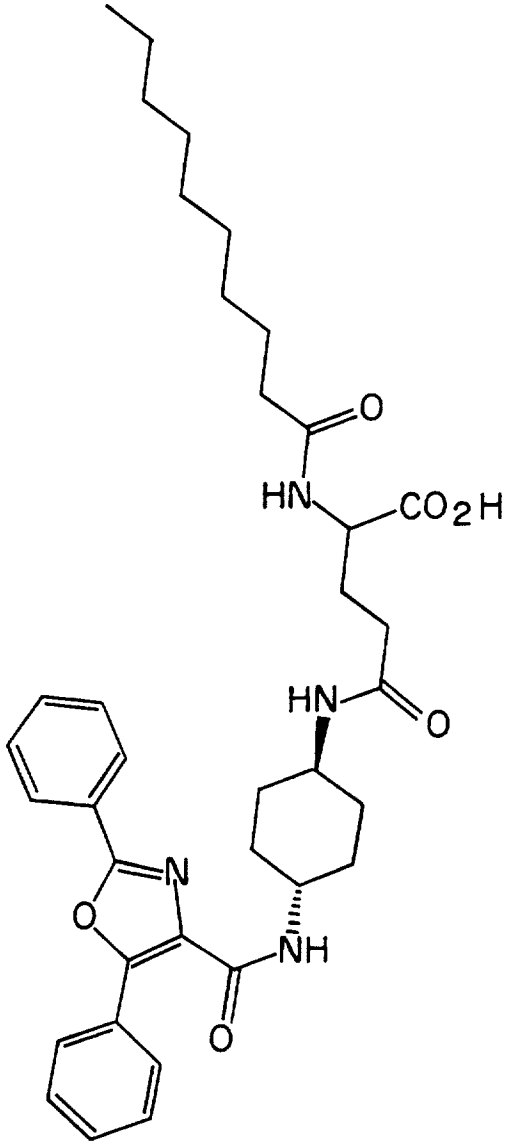


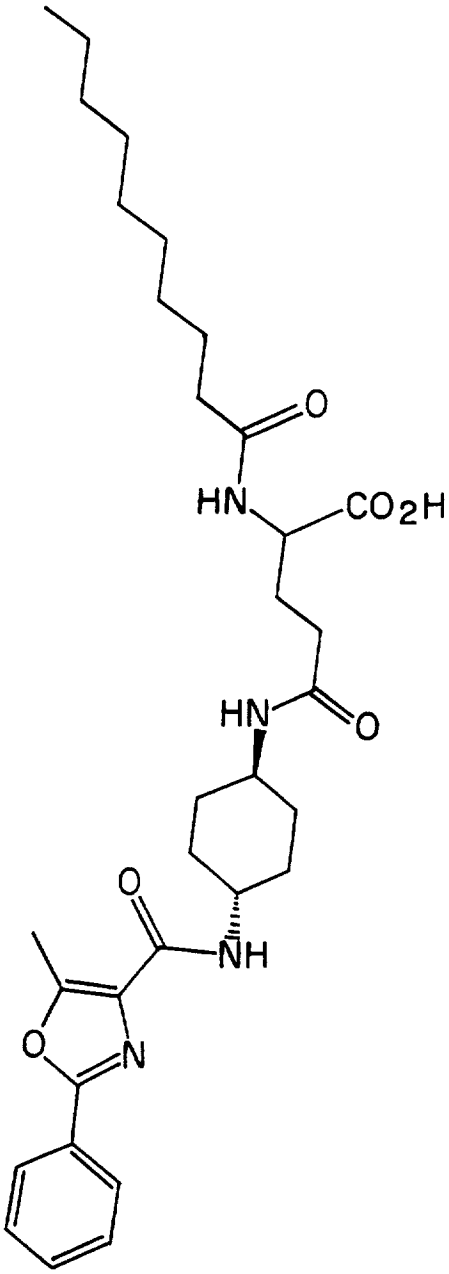
FIG. 11



$C_{37}H_{48}N_4O_6$
Exact Mass: 644.36
Mol. Wt.: 644.81
C, 68.92; H, 7.50; N, 8.69;
O, 14.89

FY2-αα09

FIG. 12(a)



$C_{32}H_{46}N_4O_6$
582.34
Mol. Wt.: 582.74
C, 65.96; H, 7.96; N, 9.61;
O, 16.47

FY2-α109

FIG. 12(b)

$C_{37}H_{48}N_4O_6$
Exact Mass: 644.36
Mol. Wt.: 644.81
C, 68.92; H, 7.50; N, 8.69;
O, 14.89
FY3-αα09

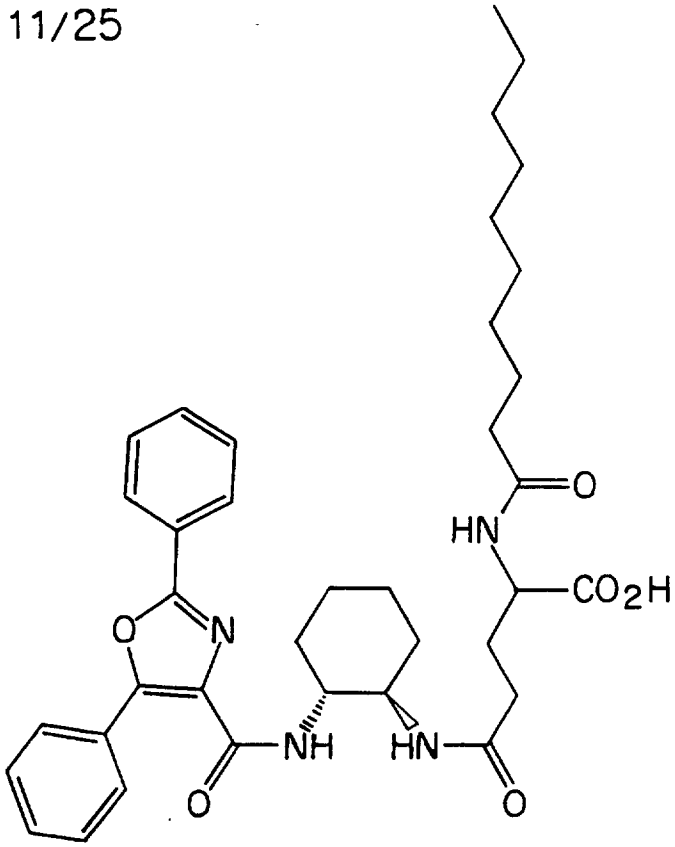


FIG. 12 (c)

$C_{32}H_{46}N_4O_6$
Exact Mass: 582.34
Mol. Wt.: 582.74
C, 65.96; H, 7.96; N, 9.61;
O, 16.47
FY3-α109

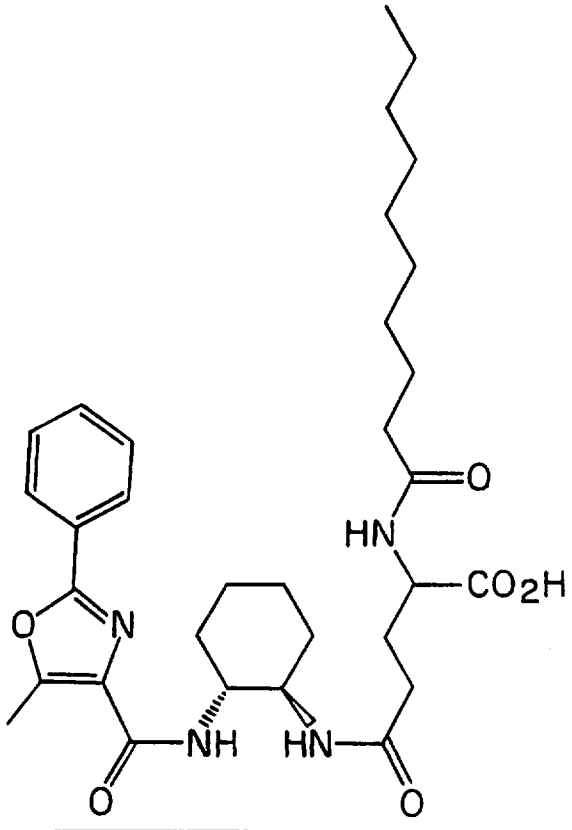


FIG. 12 (d)

$C_{32}H_{46}N_4O_6$
Exact Mass: 582.34
Mol. Wt.: 582.74
C, 65.96; H, 7.96; N, 9.61;
O, 16.47
FY4-α109

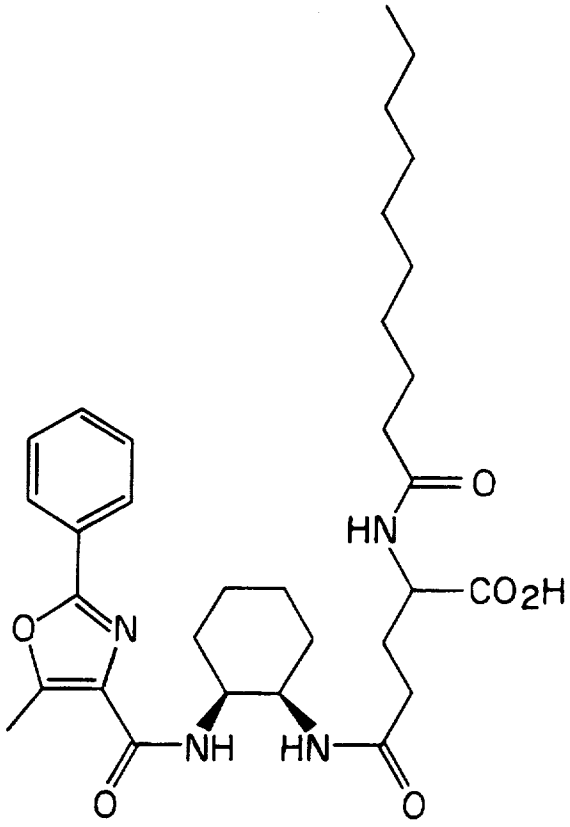


FIG. 12(e)

$C_{37}H_{48}N_4O_6$
Exact Mass: 644.36
Mol. Wt.: 644.81
C, 68.92; H, 7.50; N, 8.69;
O, 14.89
FY4-αα09

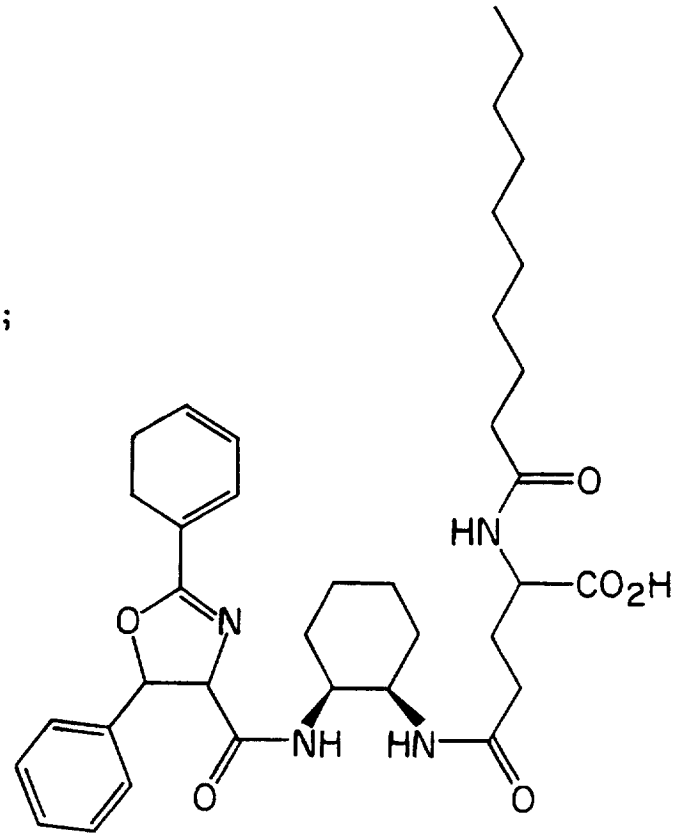
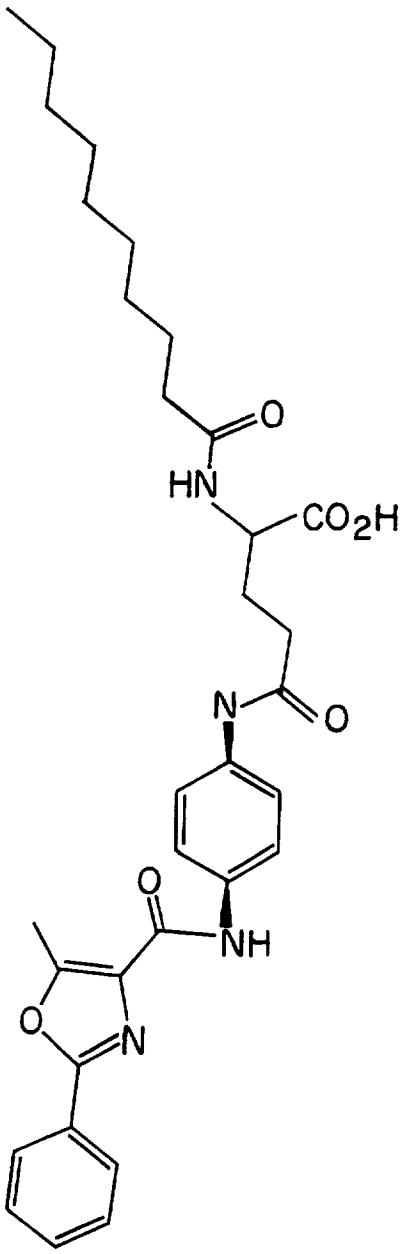


FIG. 12(f)



$C_{32}H_{40}N_4O_6$

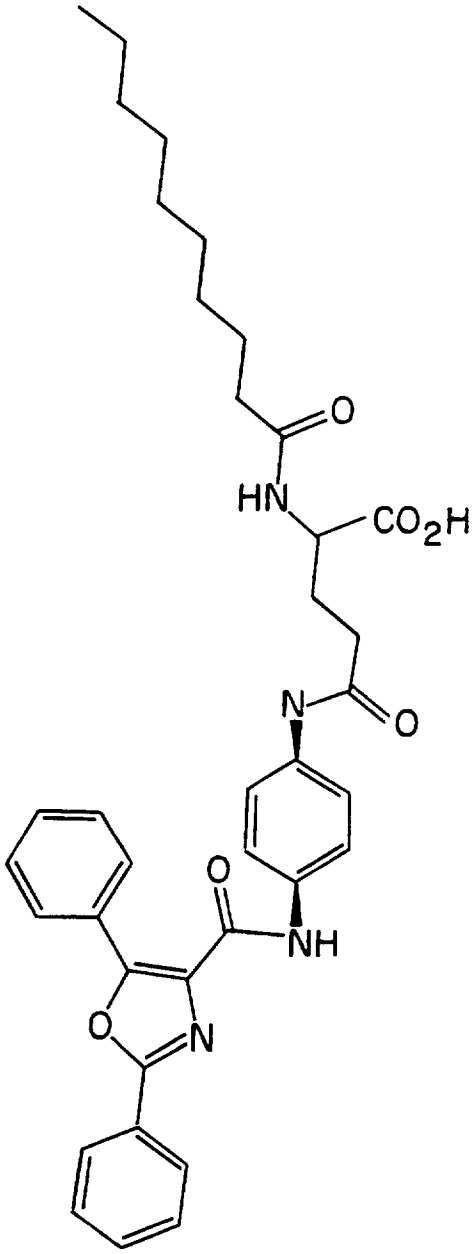
Exact Mass: 576.29

Mol. Wt.: 576.69

C, 66.65: H, 6.99; N, 9.72; O, 16.65

FY5-α109

FIG. 12(g)



$C_{37}H_{42}N_4O_6$

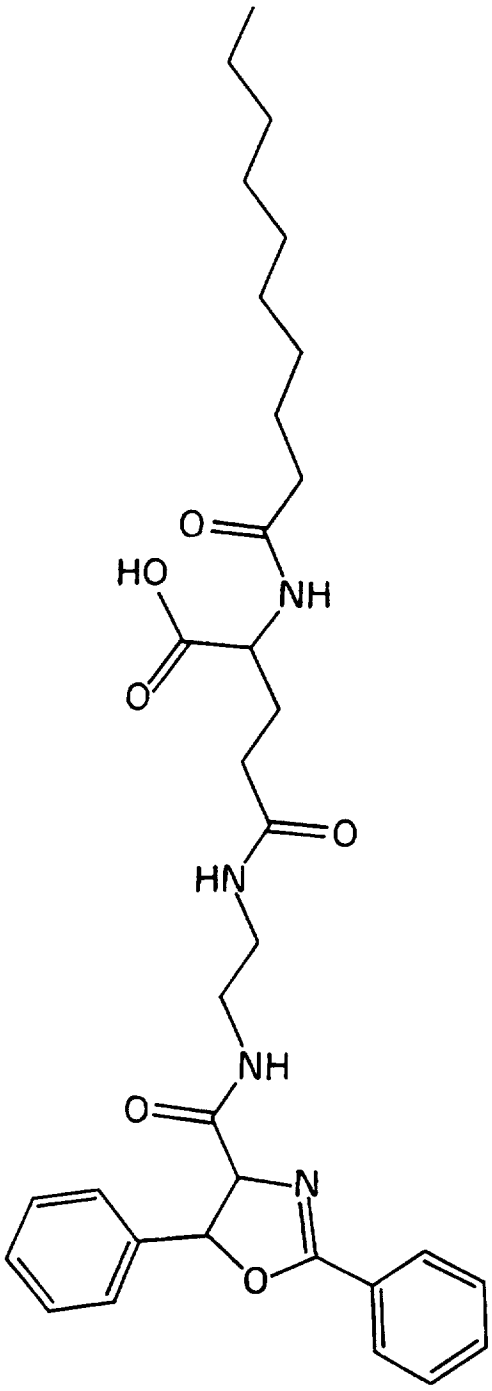
Exact Mass: 638.31

Mol. Wt.: 638.76

C, 69.57; H, 6.63; N, 8.77; O, 15.03

FY5-αα09

FIG. 12(h)



$C_{33}H_{42}N_4O_6$

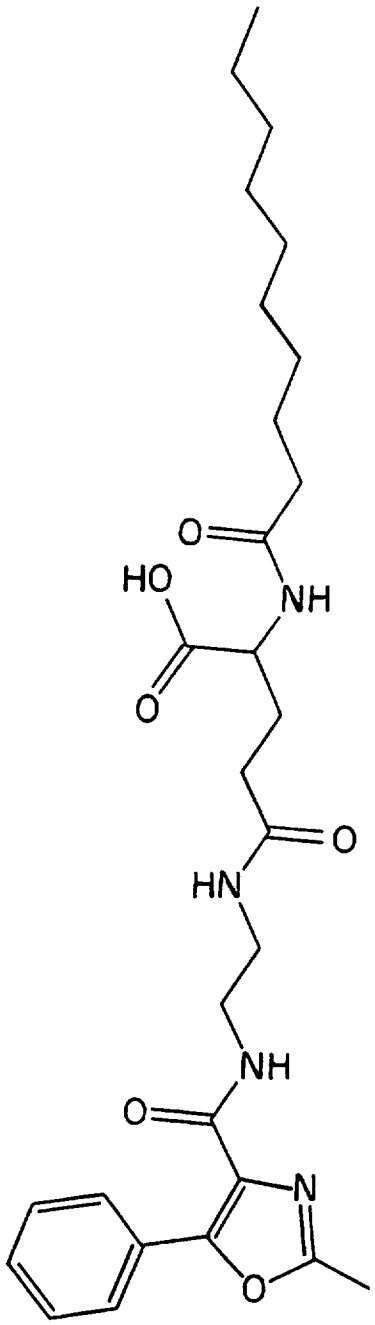
Exact Mass: 590.31

Mol. Wt.: 590.72

C, 67.10; H, 7.17; N, 9.48;
O, 16.25

FY6-αα09

FIG. 12(i)



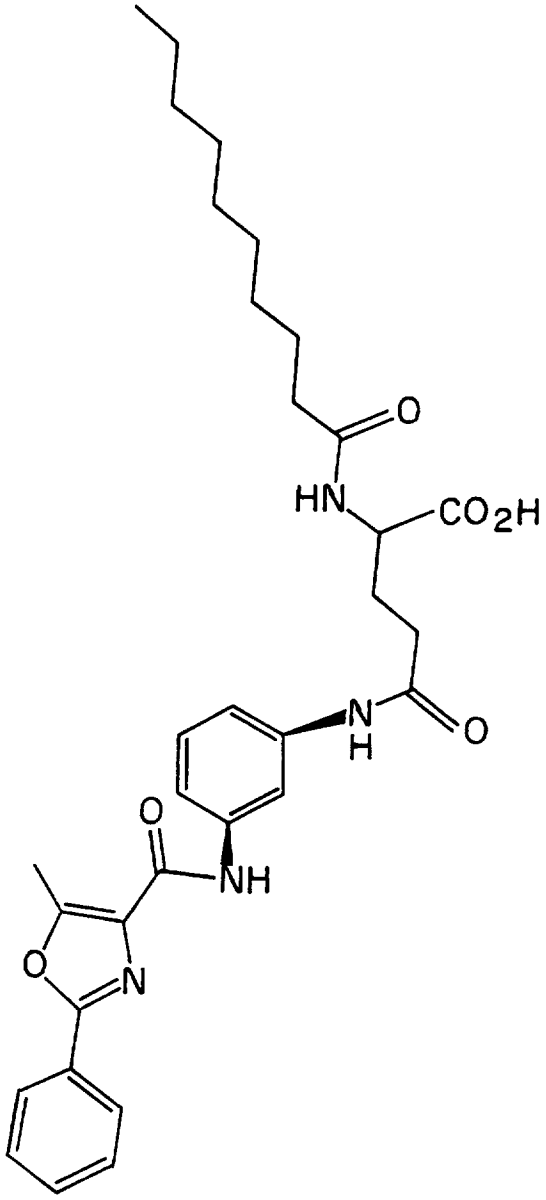
Exact Mass: 528.29

Mol. Wt.: 528.65

C, 63.62; H, 7.63; N, 10.60
O, 18.16

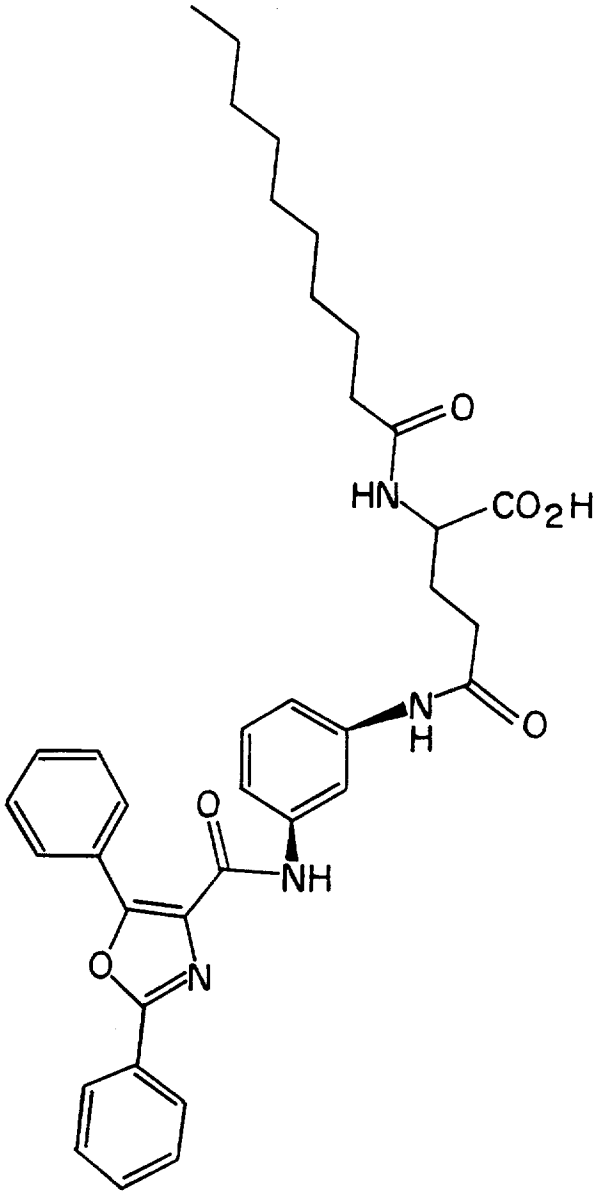
FY6-α109

FIG. 12(j)



$C_{32}H_{40}N_4O_6$
Exact Mass: 576.29
Mol. Wt.: 576.69
C, 66.65; H, 6.99; N, 9.72;
O, 16.65
FY7-α109

FIG. 12(k)



$C_{37}H_{40}N_4O_6$
Exact Mass: 638.81
Mol. Wt.: 638.76
C, 69.57; H, 6.63; N, 8.77;
O, 15.03
FY7-αα09

FIG. 12(l)

$C_{37}H_{42}N_4O_6$
Exact Mass: 638.31
Mol. Wt.: 638.76
C, 69.57; H, 6.63; N, 8.77;
O, 15.03
FY8-αα09

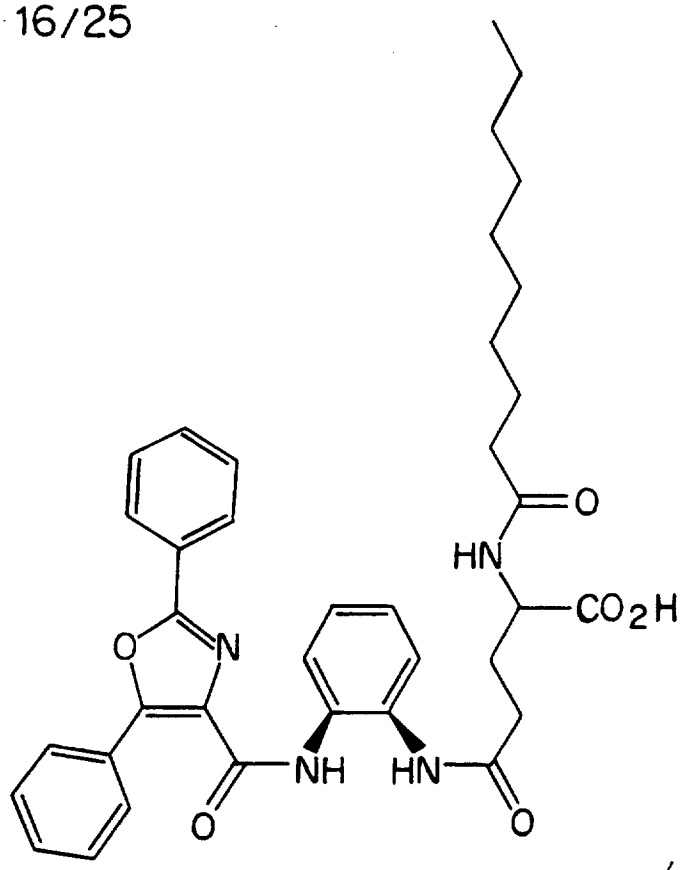


FIG. 12(m)

$C_{32}H_{40}N_4O_6$
Exact Mass: 576.29
Mol. Wt.: 576.69
C, 66.65; H, 6.99; N, 9.72;
O, 16.65
FY8-α109

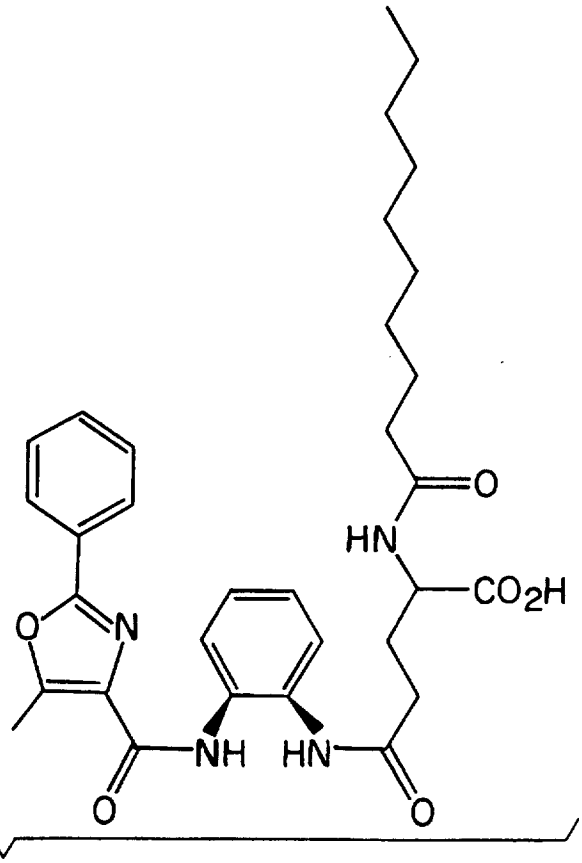


FIG. 12(n)

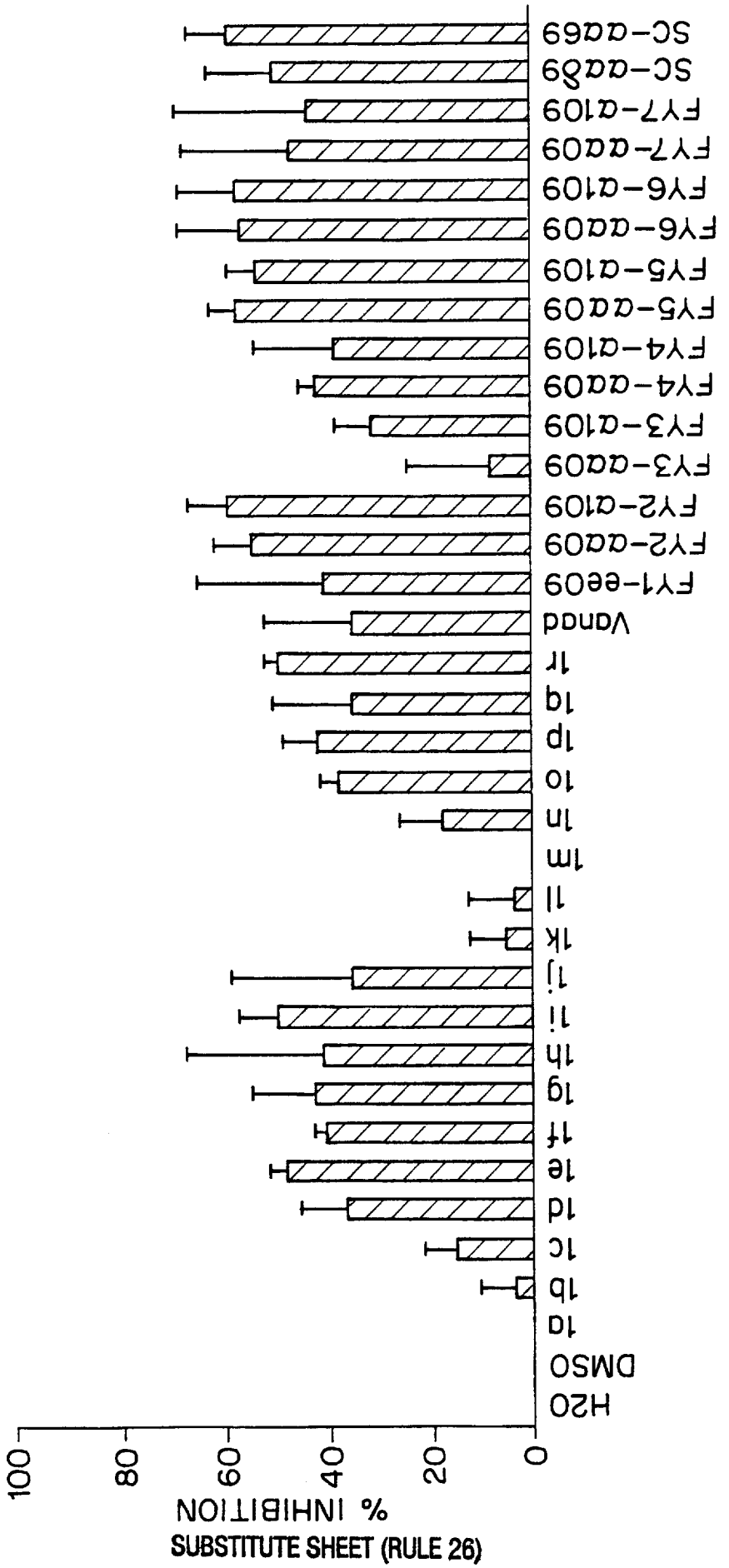

H2O
DMSO
1a
1b
1c
1d
1e
1f
1g
1h
1i
1j
1k
1l
1m
1n
1o
1p
1q
1r
Vanad
FY1-ee09
FY2-αα09
FY2-α109
FY3-αα09
FY3-α109
FY4-αα09
FY4-α109
FY5-αα09
FY5-α109
FY6-αα09
FY6-α109
FY7-αα09
FY7-α109
SC-αβ69
SC-αα69
0
20
40
60
80
100
% INHIBITION

FIG. 13

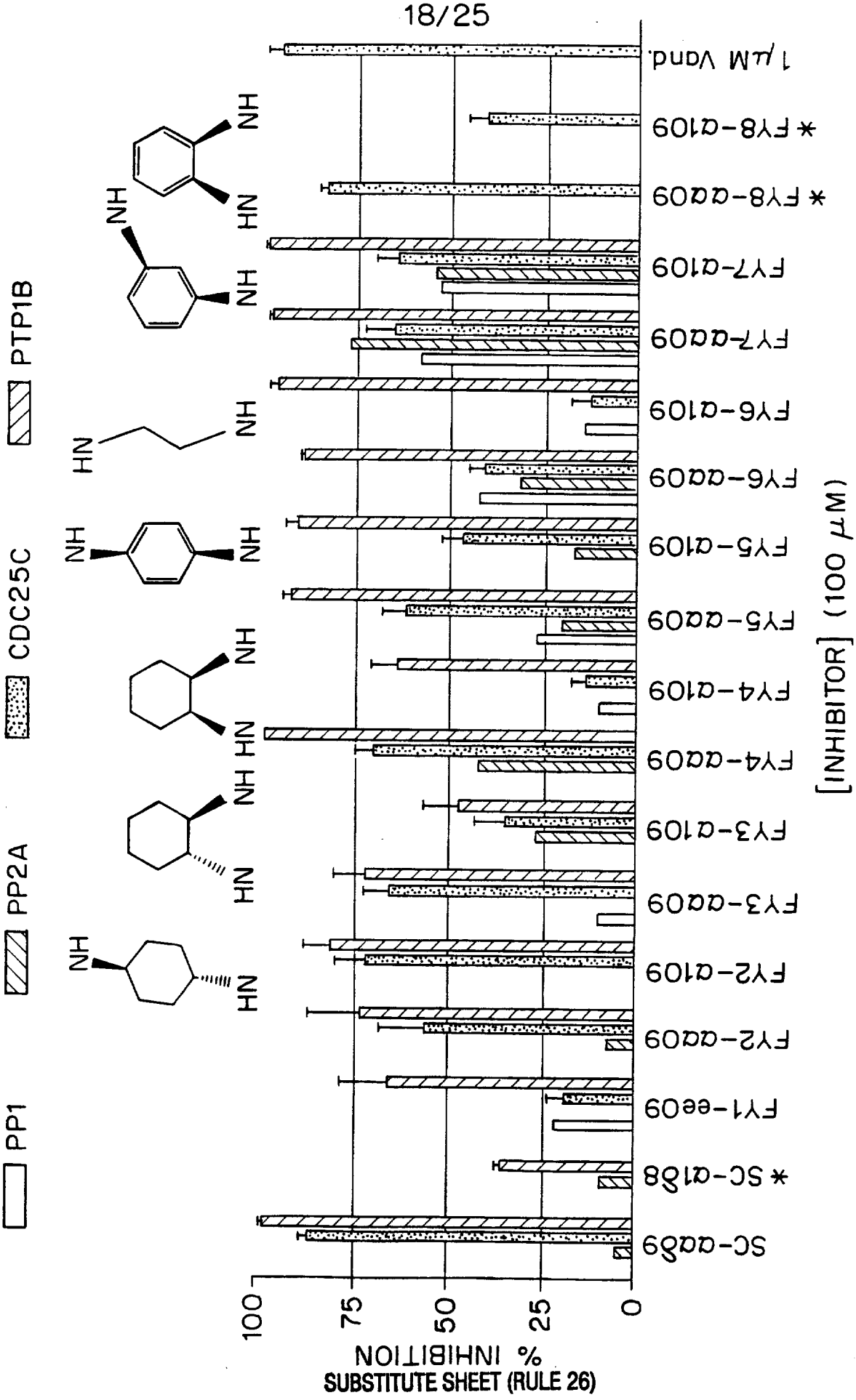

PP1
PP2A
CDC25C
PTP1B
% INHIBITION
100
75
50
25
0
SC-aaδ9
*SC-α1δ8
FY1-ee09
FY2-aa09
FY2-α109
FY3-aa09
FY3-α109
FY4-aa09
FY4-α109
FY5-aa09
FY5-α109
FY6-aa09
FY6-α109
FY7-aa09
FY7-α109
*FY8-aa09
*FY8-α109
1 μM Vand.
[INHIBITOR] (100 μM)

FIG. 14

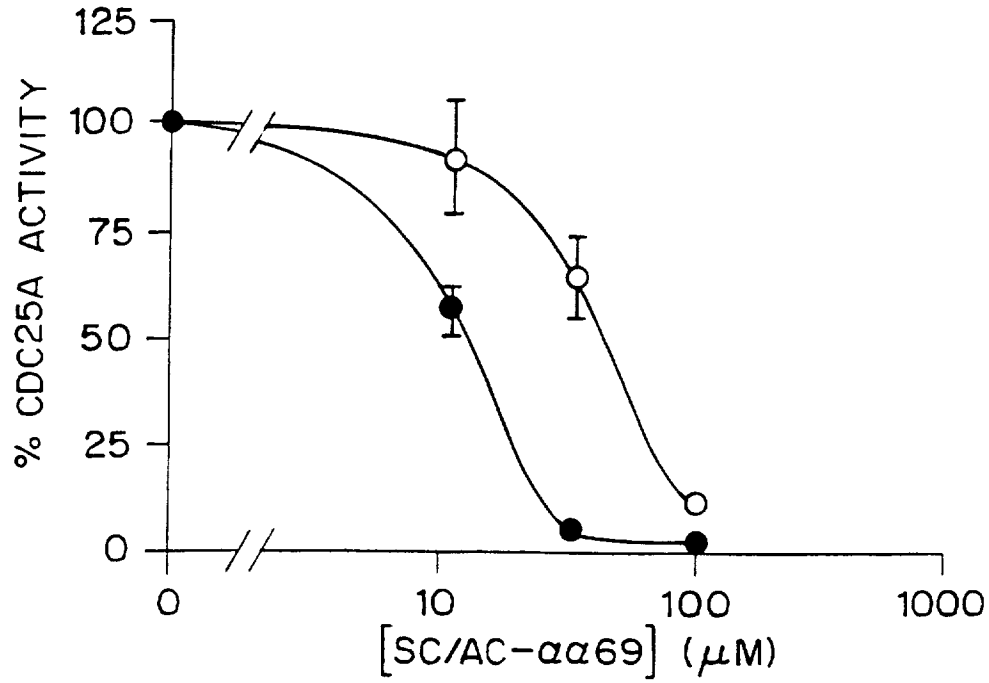


FIG. 15A

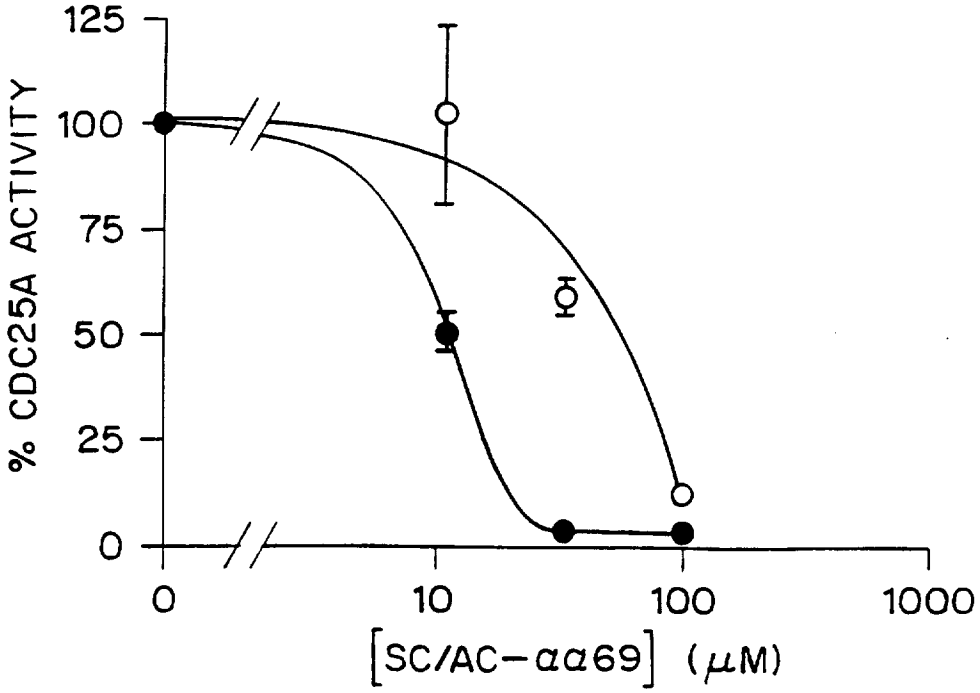


FIG. 15B

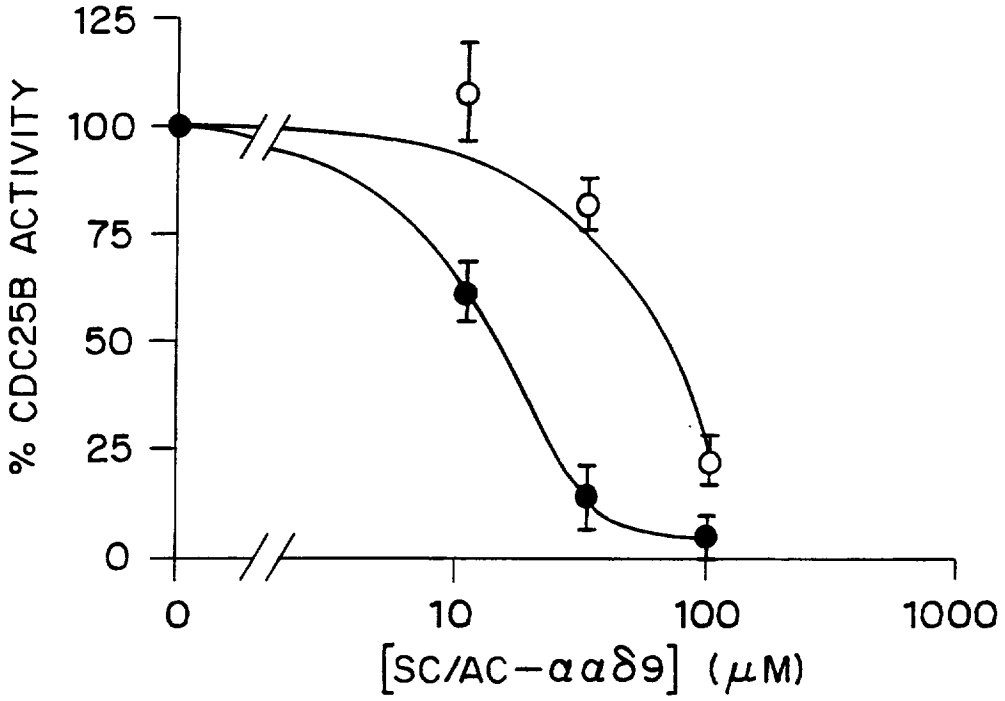


FIG. 15C

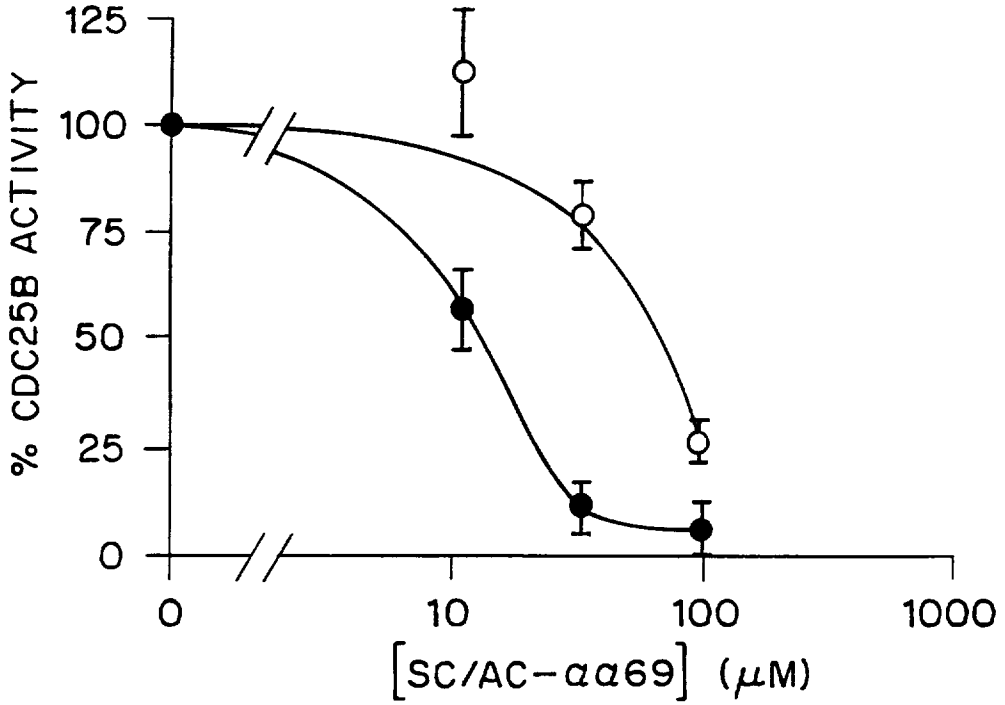


FIG. 15D

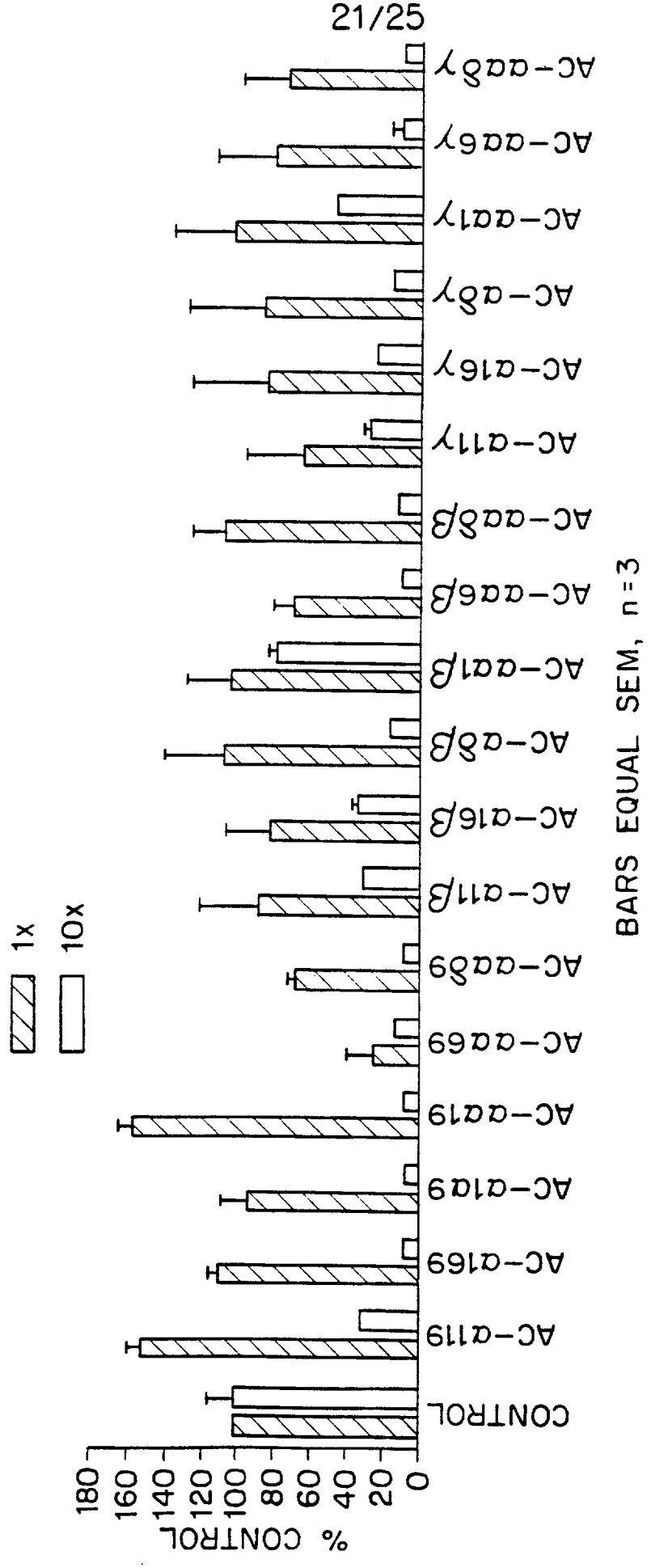

% CONTROL
180
160
140
120
100
80
60
40
20
0
CONTROL
AC-α11γ
AC-α16γ
AC-αδγ
AC-α1γ
AC-α6γ
AC-ααγ
AC-αδβ
AC-α11β
AC-α16β
AC-αδβ
AC-α1β
AC-α6β
AC-ααβ
AC-α11γ
AC-α16γ
AC-αδγ
AC-α1γ
AC-α6γ
AC-ααγ
1x
10x
BARS EQUAL SEM, n=3

FIG. 16

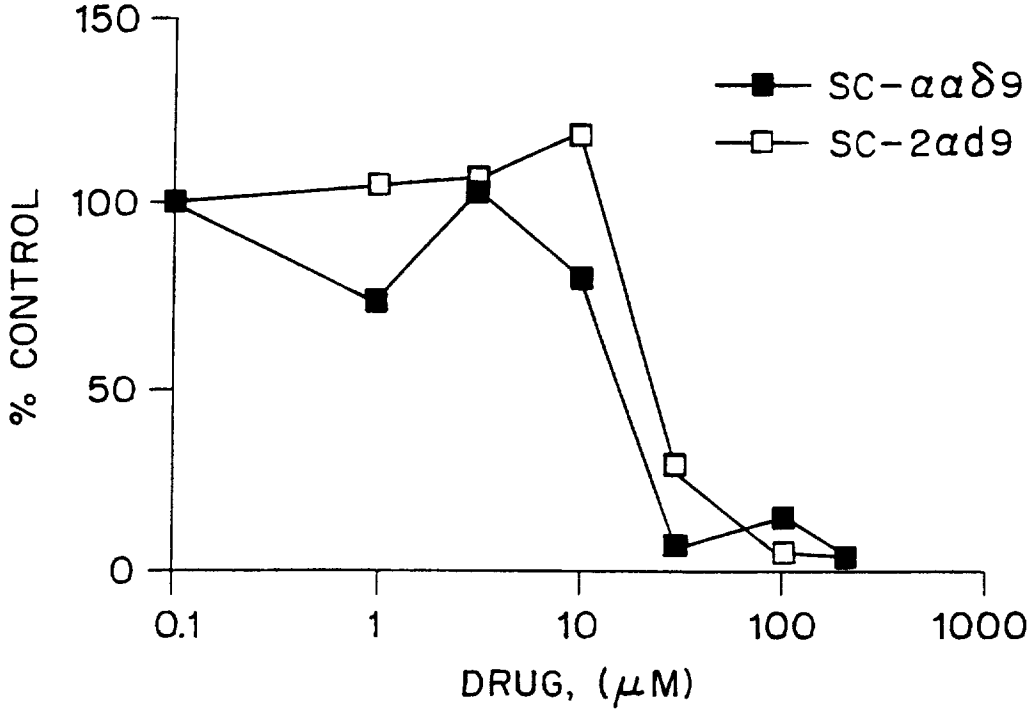

150
100
50
0
% CONTROL
SC-ααδ9
SC-2αd9
0.1
1
10
100
1000
DRUG, (μM)

FIG. 17

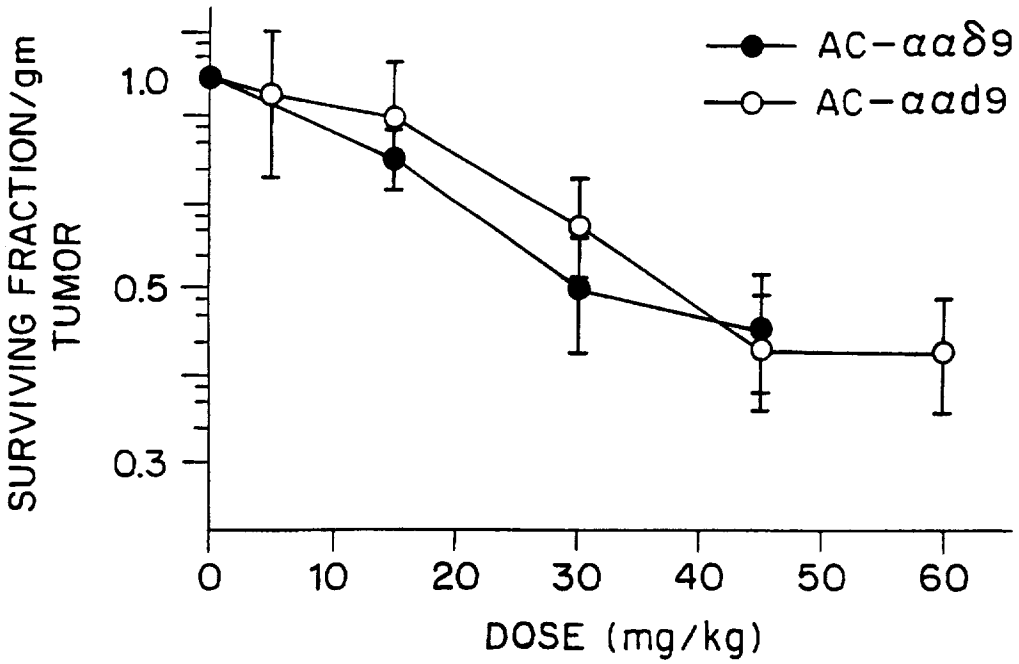

SURVIVING FRACTION/gm TUMOR
1.0
0.5
0.3
AC-ααδ9
AC-ααd9
0
10
20
30
40
50
60
DOSE (mg/kg)

FIG. 18

FIG. 19A

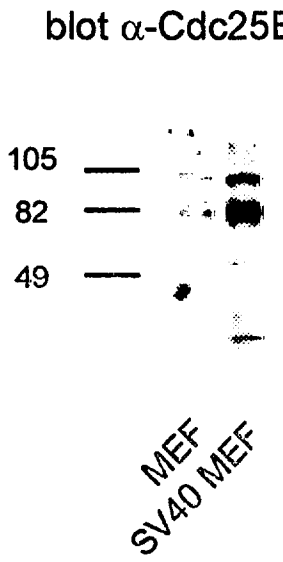


FIG. 19B

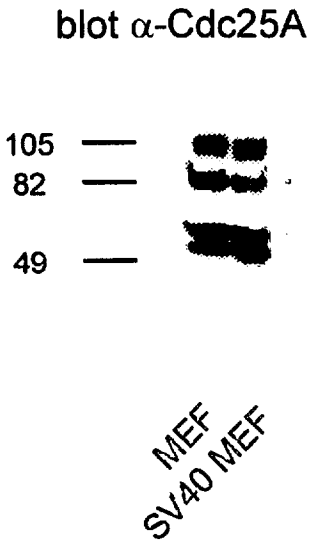


FIG. 19C

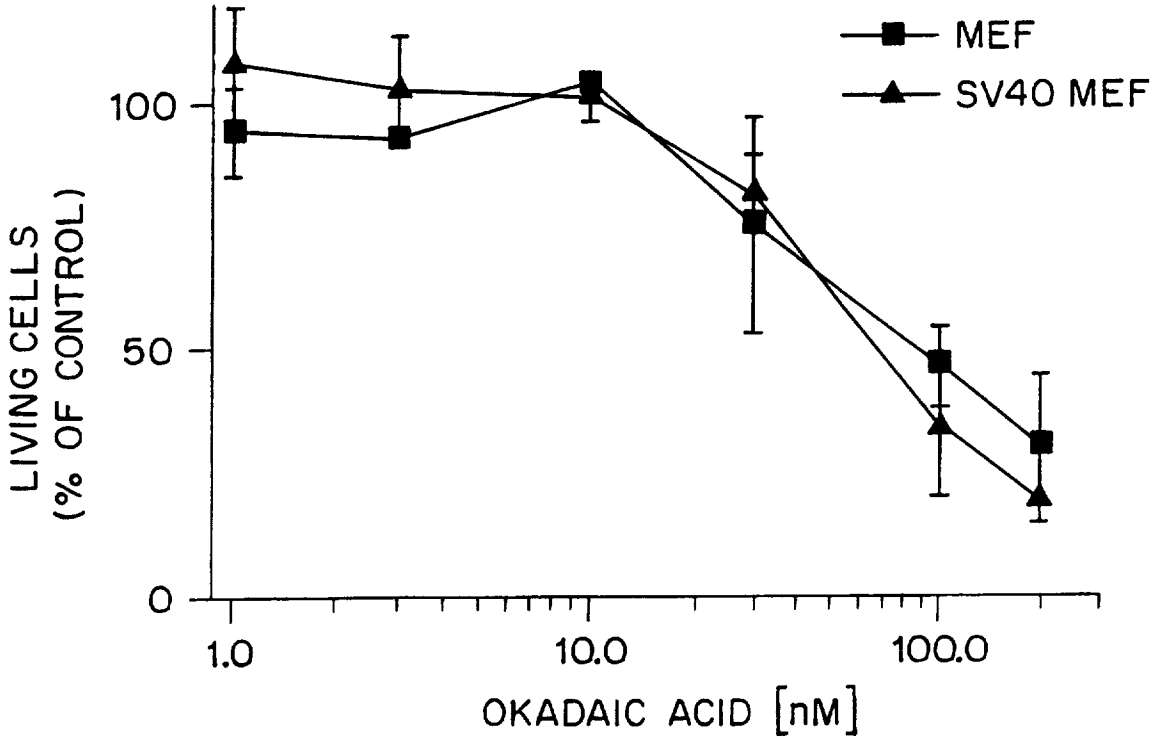


FIG. 20A

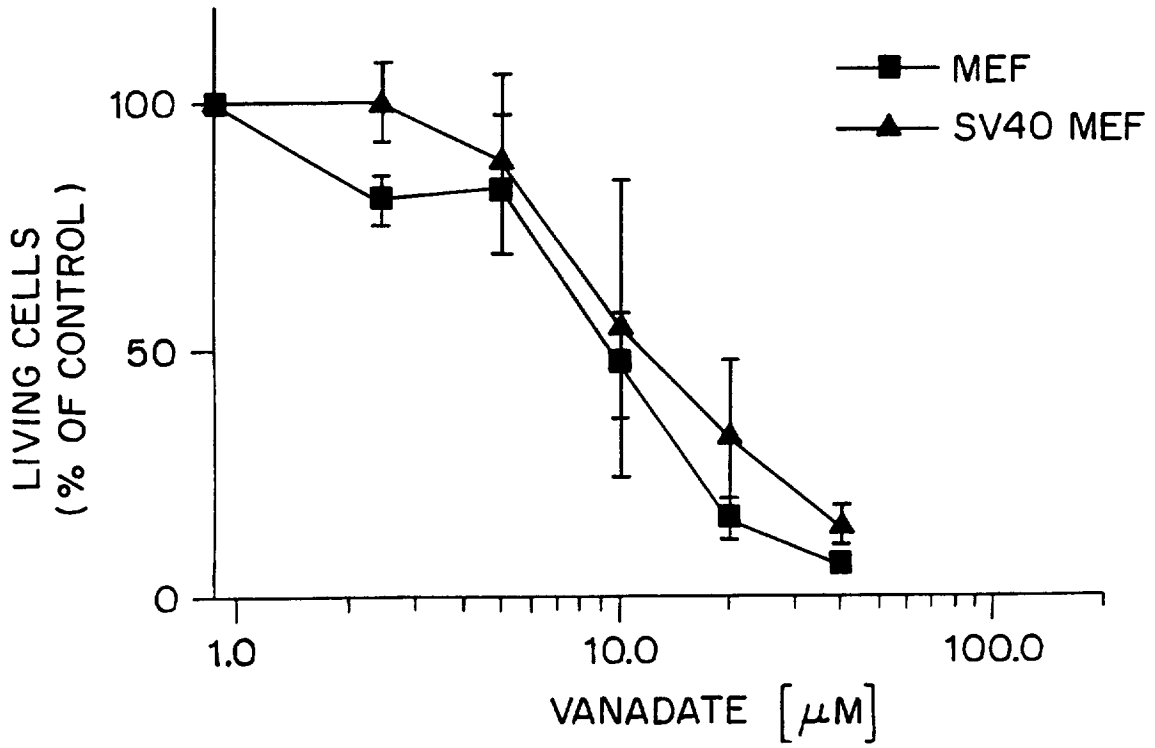


FIG. 20B

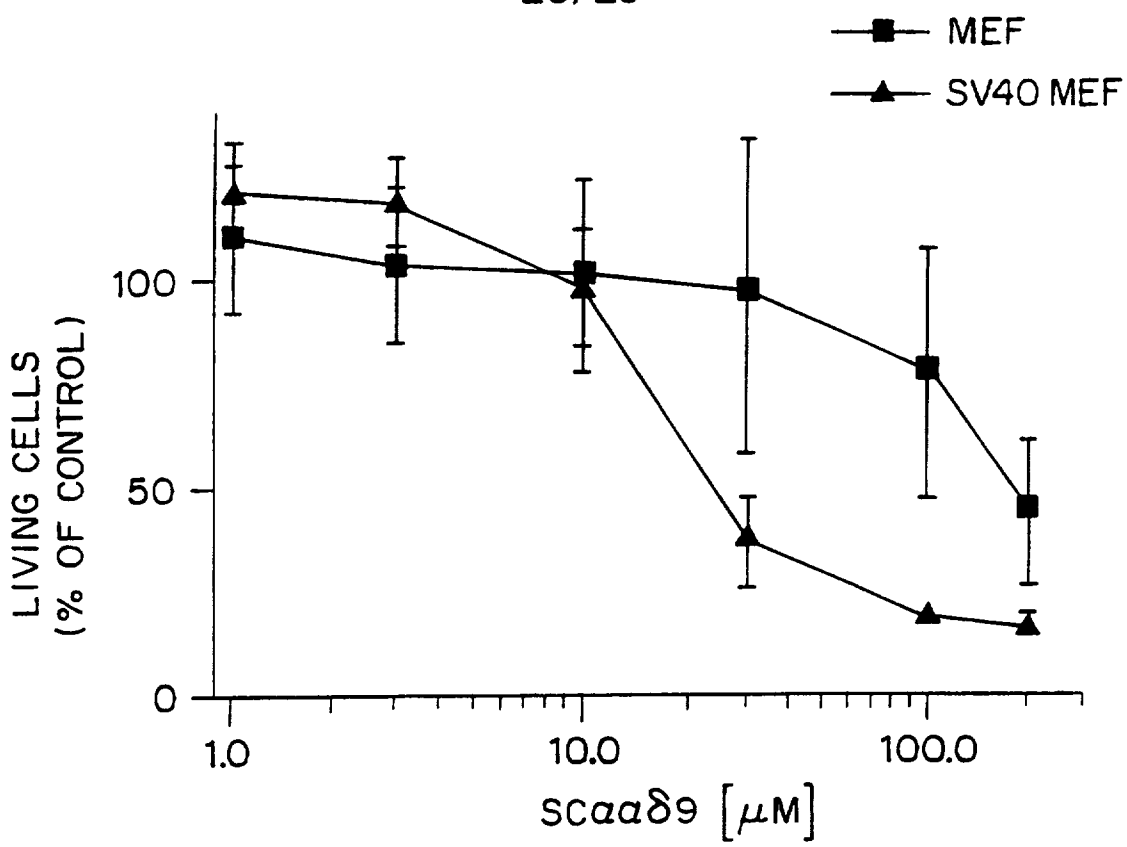


FIG. 20C

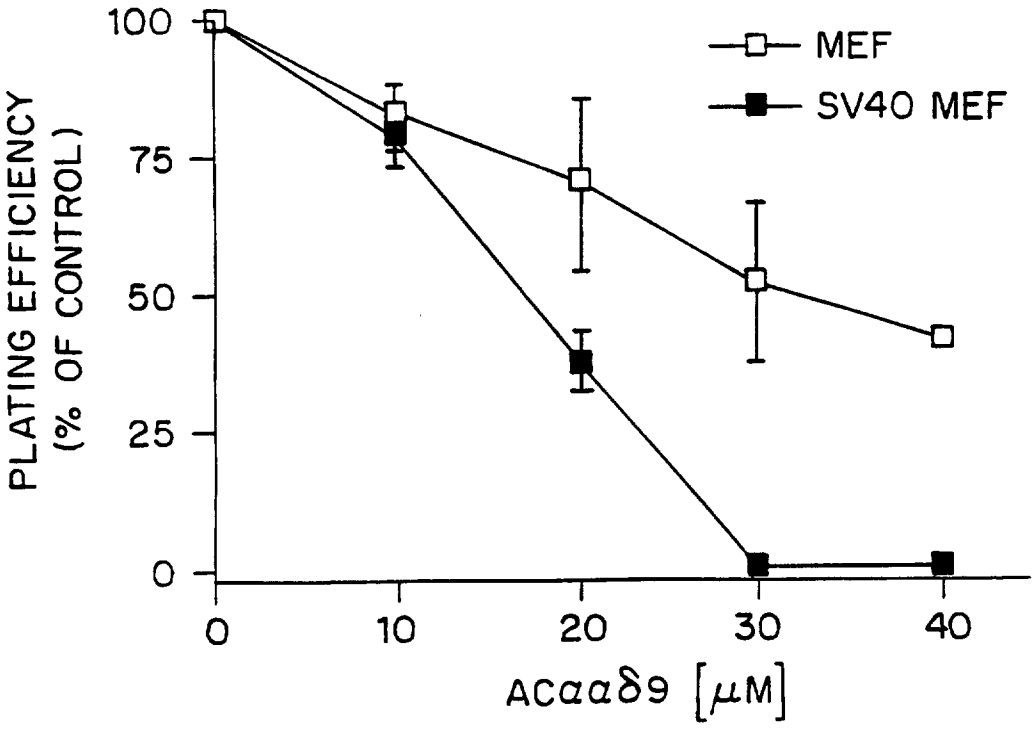


FIG. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/13408

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/42; C07D 263/32
US CL :514/374; 548/236

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/374; 548/236

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	US 5,583,232 A (KEMPF et al) 10 December 1996, see entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search 10 SEPTEMBER 1997	Date of mailing of the international search report 26 SEP 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOSEPH K. MCKANE Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/13408

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/13408

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-11 and 13-22, drawn to compounds, methods of use, pharmaceutical compositions and a first method for preparing the compounds.

Group II, claim 12, drawn to a second method for preparing instant compounds.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The method for preparing instant compounds of instant formula I is drawn to different reactive steps and conditions. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.