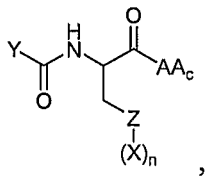
Tripeptide and tetraeptide sulfones

Technical Field

This invention relates to tripeptide and tetrapeptide sulfones, pharmaceutical compositions containing them, their pharmaceutical use, and their preparation.

Background Art

US Patents Nos. 5599903; 5763570; 5767086; 5786336; and 5955432; European Patent Publication No. 0 645 397; and PCT International Publications Nos. WO 95/08563 and WO 96/40205 disclose various tripeptide and tetrapeptide compounds that are analogs of reduced glutathione (L-γ-glutamyl-L-cysteinylglycine), including compounds of the formula [WO 95/08563]:



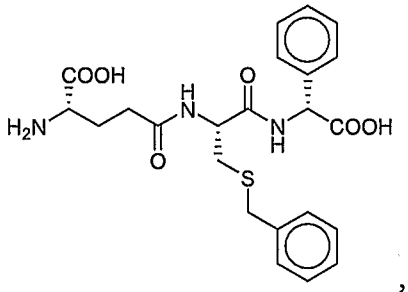
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and their C_{1-10} alkyl or alkenyl or C_{7-12} aralkyl esters, amides, and mixed ester/amides, where:
Z is S, O, or C;
n is 1 to 3;
when Z is S or O and n is 1, X is a C_{1-20} hydrocarbyl optionally containing 1 or 2 non-adjacent O, S, or N heteroatoms, unsubstituted or mono- or disubstituted with halo, –NO, $-NO_2$, $–NR_2$, –OR, or –SR, where R is H or C_{1-4} alkyl ;
when Z is S and n is 2, one X is as above defined and the other X is C_{1-4} alkyl; and
when Z is C and n is 3, one X is as above defined and the other two X are independently H or C_{1-4} alkyl;
YCO is γ-glu, β-asp, glu, asp, γ-glu-gly, β-asp-gly, glu-gly, asp-gly ; and
AA_c is an amino acid coupled through a peptide bond to the remainder of the compound.

The compounds are described as having various uses, including as reagents useful in characterizing glutathione *S*-transferase (GST) isoenzymes, in determining the GST complements of cells and tissues, as chromatographic affinity ligands, binding agents, and enzyme inhibitors; and therapeutically to: potentiate the cytotoxic effects of chemotherapeutic agents in tumor cells, selectively exert cytotoxicity in tumor cells, elevate

the production of granulocyte-macrophage (GM) progenitors in bone marrow, stimulate the differentiation of bone marrow, mitigate the bone marrow-destructive effects of chemotherapeutic agents, and modulate hematopoiesis in bone marrow.

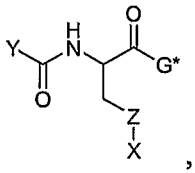
TLK117, identified in those patents and publications as TER 117 and named variously as γ-Glu-Cys(Bz)-phenylGly, γE-C(Bz)-φG, γE-C(Bz)-PG, γE-C(benzyl)-φG, and benzyl PG, is one of these compounds. TLK117 is the compound of the formula



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and may be named L-γ-glutamyl-S-(phenylmethyl)-L-cysteinyl-D-phenylglycine. TLK117 inhibits GST P1-1 with an IC_{50} of approximately 400 nM. TLK199, identified in those patents and publications as TER 199, is the diethyl ester of TLK117; while TLK261 and TLK262 are the dimethyl and diisopropyl esters, respectively.

US Published Application No. 2003/0100511 and PCT International Publication No. WO 00/44366 disclose lipid formulations, including liposomal formulations, of diesters of compounds of the formula:



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where:

each ester is 1-25C;

YCO is γ-glu or β-asp;

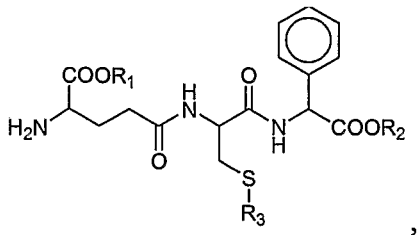
G* is phenylglycine;

Z is CH_2, O, or S; and

X is 6-8C alkyl, benzyl, or naphthyl,

or a pharmaceutically acceptable salt thereof; or

a compound of the formula:



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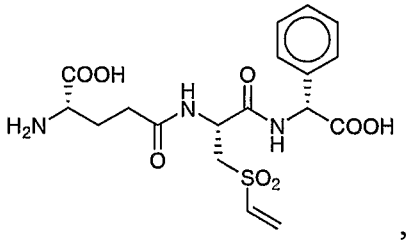
where:

R_1 and R_2 are independently chosen from linear or branched alkyl groups (1-25C), cycloalkyl groups (6-25C), substituted alkyl groups (2-25C), heterocycles (6-20C), ethers or polyethers (3-25C), or where R_1-R_2 (2-20C) together form a macrocycle with the formula; and

R_3 is 6-8C alkyl, benzyl, or naphthyl,

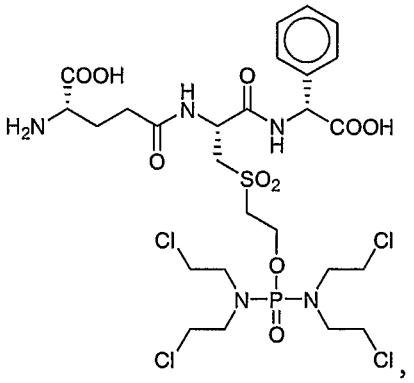
or a pharmaceutically acceptable salt thereof.

Townsend et al., *Mol. Cancer Ther.*, **1**(12), 1089-1095 (2002) disclose the compound of the formula



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the "vinyl sulfone", as one of the two products of the GST-mediated cleavage of canfosfamide, a GST-activated anticancer agent of the formula



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which is disclosed in US Patent No. 5556942 and PCT International Publication No. WO 95/09866, where it is referred to as TER 286.

Many conditions are characterized by depleted bone marrow, including myelodysplastic syndrome (MDS), a form of pre-leukemia in which the bone marrow

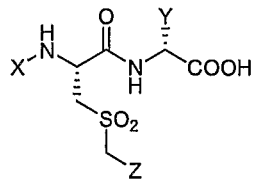
produces insufficient levels of one or more of the three major blood elements (white blood cells, red blood cells and platelets). Myelosuppression, a reduction in blood cell levels and in the generation of new blood cells in the bone marrow, is also a common, toxic effect of many standard chemotherapeutic drugs.

TLK199 has been shown to induce the differentiation of HL-60 promyelocytic leukemia cells *in vitro*, to potentiate the activity of cytotoxic agents both *in vitro* and *in vivo*, and to stimulate colony formation of all three lineages of hematopoietic progenitor cells in normal human peripheral blood. In preclinical testing, TLK199 has been shown to increase white blood cell production in normal animals as well as in animals in which white blood cells were depleted by treatment with cisplatin or fluorouracil. Similar effects may provide a new approach to treating MDS. TLK199 is currently being evaluated in a Phase II clinical trial for the treatment of MDS. Interim results from this trial, reported at the 2004 and 2005 American Society of Hematology meetings, demonstrated that TLK199 was well tolerated and resulted in multilineage hematologic improvement. These results also suggest a potential role for TLK199 in treating chemotherapy-induced cytopenias.

It would be desirable to develop potent inhibitors of GST P1-1 for use in humans to: potentiate the cytotoxic effects of chemotherapeutic agents in tumor cells, selectively exert cytotoxicity in tumor cells, elevate the production of GM progenitors in bone marrow, stimulate the differentiation of bone marrow, mitigate the myelosuppressive effects of chemotherapeutic agents, and modulate hematopoiesis in bone marrow.

Disclosure of the invention

In a first aspect, this invention is compounds of the formula



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where:

X is L-γ-glutamyl or L-γ-glutamylglycyl;

Y is H, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, or optionally substituted

heteroaralkyl; and

Z is C_{5-9} alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, or optionally substituted heteroaralkyl;

and their C_{1-10} alkyl, phenyl-C_{1-3} alkyl, or (C_{5-6} heteroaryl)-C_{1-3} alkyl mono- and di-esters;

and salts of the compounds and their mono- and di-esters.

The compounds (especially in the acid form) are inhibitors, typically selective inhibitors, of GST P1-1 *in vitro*. In the diester form, they have also been shown to be effective in the immunoprecipitation of GSTπ from HL-60 cells *in vitro*, and to be cytotoxic to HL-60 cells *in vitro*. They are also active in enhancing the differentiation of HL-60 cells *in vitro*, and one has been shown to enhance granulocyte/monocyte colony formation from murine bone marrow cells *ex vivo*. From this and from the structural similarity to TLK117, TLK199, and related compounds, the compounds of this invention are therefore expected to act therapeutically in humans to: potentiate the cytotoxic effects of chemotherapeutic agents in tumor cells, selectively exert cytotoxicity in tumor cells, elevate the production of GM progenitors in bone marrow, stimulate the differentiation of bone marrow cells, mitigate the myelosuppressive effects of chemotherapeutic agents, and modulate hematopoiesis in bone marrow.

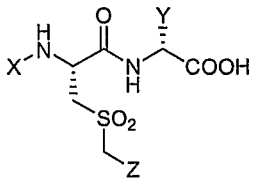
In a second aspect, this invention is pharmaceutical compositions comprising compounds of the first aspect of this invention, and optionally one or more excipients.

In a third aspect, this invention is therapeutic methods, particularly in a human, of one or more of: potentiating the cytotoxic effects of chemotherapeutic agents in tumor cells, selectively exerting toxicity in tumor cells, elevating the production of GM progenitors in bone marrow, stimulating the differentiation of bone marrow cells, mitigating the myelosuppressive effects of chemotherapeutic agents, and modulating hematopoiesis in bone marrow, by the administration of compounds of the first aspect of this invention or a pharmaceutical composition of the second aspect of this invention, and the use of the compounds of the first aspects of this invention in the manufacture of a medicament for one or more of: potentiating the cytotoxic effects of chemotherapeutic agents in tumor cells, selectively exerting toxicity in tumor cells, elevating the production of GM progenitors in bone marrow, stimulating the differentiation of bone marrow cells, mitigating the

myelosuppressive effects of chemotherapeutic agents, and modulating hematopoiesis in bone marrow.

In a fourth aspect, this invention is methods of preparing compounds of the first aspect of this invention.

In a fifth aspect, this invention is compounds of the formula



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where:

X is *N*-α-R^1-L-γ-glutamyl or *N*-α-R^1-L-γ-glutamylglycyl, where R^1 is an amine-protecting group; and

Y and Z are as defined for the compounds of the first aspect of this invention;

and their C_{1-10} alkyl, phenyl-C_{1-3} alkyl, or (C_{5-6} heteroaryl)-C_{1-3} alkyl mono- and di-esters;

and salts of the compounds and their mono- and di-esters.

These compounds are useful as intermediates in the preparation of the compounds of the first aspect of this invention.

Preferred embodiments of this invention are characterized by the specification and by the features of claims 2-20 of this application as filed, and of pharmaceutical compositions, methods, and uses of these compounds.

Modes for Carrying Out the Invention

Definitions

"Alkyl" means a monovalent group derived from a saturated or unsaturated (but not aromatically unsaturated) C_1-C_{10}, preferably C_{1-6}, hydrocarbon that may be linear, branched, or cyclic by removal of one hydrogen atom from a carbon atom, such as methyl, ethyl, propyl, 1-propenyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, hexyl, cyclopentyl, cyclopenten-1-yl, cyclopropylmethyl, cyclohexyl, and cyclohexylmethyl. Saturated alkyls and C_1-C_6 alkyls are exemplary. Note that the definition of "alkyl" in this application is broader than the conventional definition and includes groups more commonly referred to as "cycloalkyl", "cycloalkylalkyl", "alkenyl", and "alkynyl".

A "substituted alkyl" is an alkyl substituted with up to three halogen atoms and/or a substituent selected from -CN, -NO_2, -OR, and -NR_2 (where each R is independently H or C_{1-3} alkyl). Thus, for example, substituted alkyl groups include such groups as trifluoromethyl and 3-chloropropyl. Preferred substituted C_{5-6} cycloalkyl groups are cyclopentyl and cyclohexyl, substituted with 1 or 2, especially 1, groups selected from halo, hydroxy, methyl, trifluoromethyl, and methoxy, especially fluoro, chloro, or methyl.

"Heteroalkyl" means "alkyl" (as defined above) in which 1 or 2 of the methylene groups are replaced by O, S, SO_2, or NR (where R is H or C_{1-3} alkyl), including linear groups such as 3-oxapentyl; monocyclic rings containing 5 or 6 ring atoms such as 2-tetrahydrofuranyl, 2-pyrrolidinyl, 3-piperidinyl, 2-piperazinyl, 4-dihydropyranyl, and 3-morpholinyl; and groups such as tetrahydrofuran-2-ylmethyl and piperidin-3-ylethyl. Preferred C_{5-6} cycloheteroalkyl groups are cyclopentyl or cyclohexyl in which 1 of the ring methylene groups is replaced by O, S, SO_2, NH, or N-methyl; and include 2- and 3-tetrahydrofuranyl, 3- and 4-tetrahydropyranyl, 4-tetrahydrothiopyranyl and its *S,S*-dioxide, and 2-, 3-, and 4-piperidinyl. A "substituted heteroalkyl" is a heteroalkyl substituted in the manner described above for substituted alkyl.

"Aryl" means a monovalent group derived from an aromatic hydrocarbon containing 6-14 ring carbon atoms by removal of one hydrogen atom from a carbon atom, and may be monocyclic (such as phenyl), condensed polycyclic (such as naphthyl), or linked polycyclic (such as biphenylyl).

"Substituted aryl" means aryl substituted with substituted with up to 3 substituents selected from halo, -CN, -NO_2, -OH, optionally halo-substituted C_{1-3} alkyl (e.g. ethyl, trifluoromethyl), optionally halo-substituted C_{1-3} alkyloxy, phenoxy, (C_{5-6} heteroaryl)oxy, formyl, carboxy, C_{1-3} alkoxycarbonyl, and -NR_2 (where each R is independently H or C_{1-3} alkyl).

"Aralkyl" means alkyl substituted with aryl, such as benzyl and phenethyl. "Substituted aralkyl" means aralkyl in which one or both of the aryl and the alkyl are substituted in the manner described above for substituted aryl and substituted alkyl.

"Halogen" or "halo" means F, Cl, or Br.

"Heteroaryl" means a monovalent group derived from an aromatic hydrocarbon containing 5-14 ring carbon atoms by removal of one hydrogen atom from a carbon atom, in which 1 to 4 of the ring carbon atoms are replaced by O, S, N, or NR (where R is H or C_{1-3} alkyl), and may be monocyclic containing 5 or 6 ring atoms (such as furyl, thienyl, pyrrolyl, oxazolyl, imidazolyl, pyridinyl, pyrazinyl, pyridazinyl, and pyrimidinyl); condensed bicyclic (such as benzimidazolyl, benzotriazolyl, and quinolyl); or linked bicyclic, especially (C_{5-6} heteroaryl)-phenyl such as 4-(2-pyridyl)phenyl. Preferred C_{5-6} heteroaryl groups are 2– and 3-furyl, 2– and 3-thienyl, and 2–, 3–, and 4-pyridinyl. "Substituted heteroaryl" means heteroaryl substituted in the manner described above for substituted aryl.

"Heteroaralkyl" means alkyl substituted with heteroaryl, such as 2-pyrrolylmethyl. "Substituted heteroaralkyl" means heteroaralkyl substituted in the manner described above for substituted alkyl.

"Salts" are described in the section entitled "Compounds of this invention".

An "amine-protecting group" is a group capable of protecting the glutamyl α-amine group of a compound of the first aspect of this invention or an intermediate thereto during the synthesis of that compound, and subsequently removable without affecting the remainder of the compound of the first aspect of this invention. Common such groups include *tert*-butoxycarbonyl and benzyloxycarbonyl, conveniently removable by acidolysis; with benzyloxycarbonyl also being conveniently removable by catalytic hydrogenation. Amine-protecting groups are well known in the field of organic synthesis and particularly peptide synthesis. Suitable such groups, and the conditions for their removal, are described in books such as *Synthesis of Peptides and Peptidomimetics, Workbench edition*, M. Goodman, ed., Georg Thieme Verlag, Stuttgart, Germany, 2004, and *Protective groups in organic synthesis*, 3 ed., T.W. Greene and P.G.M. Wuts, eds., John Wiley & Sons, Inc., New York, New York, U.S.A., 1999, and will be well known to a person of ordinary skill in the art.

A "therapeutically effective amount" means the amount that, when administered to a mammal, especially a human, for effecting treatment by one of the therapeutic methods, is sufficient to effect treatment for the condition that is the object of that therapeutic treatment. "Treating" or "treatment" of a condition in a mammal includes one or more of:

(1) inhibiting development of the condition, e.g., arresting its development,

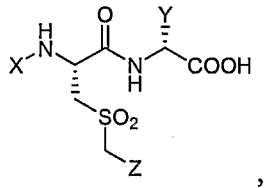
(2) relieving the condition, e.g., causing regression of or curing the condition,

(3) preventing recurrence of the condition, and

(4) palliating symptoms of the condition.

The compounds of this invention and their preparation

The compounds of the first aspect of this invention are compounds of the formula



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where:

X is L-γ-glutamyl or L-γ-glutamylglycyl; and

Y is H, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, or optionally substituted heteroaralkyl;

Z is optionally substituted C_{5-9} alkyl, optionally substituted C_{5-9} heteroalkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, or optionally substituted heteroaralkyl;

and their C_{1-10} alkyl, phenyl-C_{1-3} alkyl, or (C_{5-6} heteroaryl)-C_{1-3} alkyl mono- and di-esters;

and salts of the compounds and their mono- and di-esters.

Preferred compounds of the first aspect of this invention include those compounds having one or more of the following features:

1. X is L-γ-glutamyl;

2. Y is optionally substituted C_{5-6} cycloalkyl, optionally substituted C_{5-6} cycloheteroalkyl, optionally substituted phenyl, or optionally substituted C_{5-6} heteroaryl; especially Y is cyclopentyl, cyclohexyl, thienyl, furyl, pyridinyl, or optionally substituted phenyl; more especially cyclohexyl or more especially phenyl, optionally substituted with 1 or 2 fluoro, chloro, methyl, hydroxy, methoxy, or trifluoromethyl groups; such as where the phenyl is unsubstituted, or is substituted and one of the substituents is in the 4-position, particularly where there is only one substituent;

3. Z is C_{5-9} alkyl, or is phenyl, 1- or 2-naphthyl, 2-, 3-, or 4-biphenylyl, or 2-, 3-, or 4-(C_{5-6} heteroaryl)phenyl, each optionally substituted with 1 to 3 halo, cyano, hydroxy, carboxy, trifluoromethyl, or -R, -OR, -COOR, or -NR_2 groups (where R is C_{1-3} alkyl);

especially phenyl or 2-, 3-, or 4-biphenylyl, or 2-, 3-, or 4-(C_{5-6} heteroaryl)phenyl, each optionally substituted with fluoro, chloro, cyano, hydroxy, methoxy, methyl, or trifluoromethyl;

4A. the compound is a diacid; or

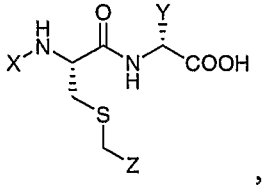
4B. the compound is a diester, especially a C_{1-6} alkyl diester, more especially a C_{1-3} alkyl diester.

Generally, a compound having a greater number of these features is preferred over a compound having a lesser number of these feature; in particular, addition of one of these features to a compound having less than all the features will generally result in a compound that is preferred over the compound without that feature.

Suitable salts (*see* Berge et al., *J. Pharm. Sci.*, **66**:1 (1971) for a nonexclusive list) of the compounds of this invention are those formed when inorganic bases (e.g. sodium, potassium, and calcium hydroxide) or organic bases (e.g. ethanolamine, diethanolamine, triethanolamine, ethylenediamine, tromethamine, N-methylglucamine) react with the carboxyl groups, and those formed when inorganic acids (e. g hydrochloric, hydrobromic, sulfuric, nitric, and chlorosulfonic acids) or organic acids (e.g. acetic, propionic, oxalic, malic, maleic, malonic, fumaric, or tartaric acids, and alkane- or arenesulfonic acids such as methanesulfonic, ethanesulfonic, benzenesulfonic, substituted benzenesulfonic such as chlorobenzenesulfonic and toluenesulfonic, naphthalenesulfonic and substituted naphthalenesulfonic, naphthalenedisulfonic and substituted naphthalenedisulfonic, and camphorsulfonic acids) react to form acid addition salts of the amine groups, of the compounds. Such salts are preferably formed with pharmaceutically acceptable acids and bases. A suitable salt for the compounds is an acid addition salt, especially with an inorganic acid, such as the hydrochloride salt.

The preparation of the compounds of the first aspect of this invention by the process of this invention involves:

(a) oxidizing a compound (a "sulfide compound") of the formula



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where:

X is L-γ-glutamyl or L-γ-glutamylglycyl, or is *N*-α-R^1-L-γ-glutamyl or *N*-α-R^1-L-γ-glutamylglycyl, where R^1 is an amine-protecting group and

Y and Z are as defined for the compounds of the first aspect of this invention;

or its C_{1-10} alkyl, phenyl-C_{1-3} alkyl, or (C_{5-6} heteroaryl)-C_{1-3} alkyl mono- or di-ester;

to give a compound of the first or fifth aspect of this invention;

(b) if Y is *N*-α-R^1-L-γ-glutamyl or *N*-α-R^1-L-γ-glutamylglycyl, deprotecting the resulting sulfone that is a compound of the fifth aspect of this invention to give a compound of the first aspect of this invention; and optionally

(c) if the compound of the first aspect of this invention prepared in step (a) or step (b) is a diacid, forming a mono- or diester of the compound; or

(d) if the compound prepared in step (a) or (b) is a mono- or diester, de-esterifying the compound to prepare the diacid of the compound; and

(e) forming a salt of the compound prepared in any of steps (a) to (d).

In step (a), the sulfur atom is oxidized to prepare a compound of the first aspect of this invention (if the glutamyl amine group is unprotected) or a compound of the fifth aspect of this invention (if the glutamyl amine group is R^1-protected).

Typically, the sulfide compound is dissolved in a suitable solvent, an oxidizing agent is added, and the reaction mixture is held at a sufficient time and sufficient temperature to complete the oxidation. Suitable solvent/oxidant combinations include C_{2-3} alkanoic acid/hydrogen peroxide/peroxyalkanoic acid combinations such as acetic acid/peroxyacetic acid and acetic acid/hydrogen peroxide/peroxyacetic acid, and the use of C_{2-3} peroxyalkanoic acids or peroxytrifluoroacetic acid as both solvent and oxidant. After quenching excess oxidant with a reagent such as dimethyl sulfide if desired, the compound of the first (or fifth) aspect of this invention is isolated simply by concentration. Particularly suitable solvent/oxidant combinations include combinations where the oxidation takes place in a biphasic system, with the sulfide compound dissolved in a non-polar aprotic solvent such as an ester (e.g. isopropyl acetate) and the oxidant being in aqueous solution. Suitable oxidants for these biphasic oxidations include borates and peroxy compounds such as perborates and persulfates, e.g. ammonium, sodium, or potassium persulfate, and a particularly suitable oxidant is potassium monopersulfate, such as OXONE™ ($2KHSO_5.KHSO_4.K_2SO_4$). After

separation of the aqueous and non-aqueous phases, and optional washing of the non-aqueous phase to ensure complete removal of any oxidant, the compound the first (or fifth) aspect of this invention is isolated from the non-aqueous phase by concentration.

In optional step (b), the R^1-protected amine group of a compound of the fifth aspect of this invention is deprotected to prepare a compound of the first aspect of this invention.

The compound of the fifth aspect of this invention may be deprotected by any method suitable for removal of the amine-protecting group that does not also affect the remainder of the molecule. When the amine-protecting group is an acidolytically removable group, it is conveniently deprotected by the use of a strong acid, such as trifluoroacetic acid optionally in the presence of dichloromethane. When the amine-protecting group is a catalytically removable amine-protecting group, it is conveniently deprotected by catalytic reduction or isomerization, though even such groups as benzyloxycarbonyl, generally considered catalytically removable, may also be removed acidolytically.

For catalytic reduction, typically, the compound is dissolved in a suitable solvent such as a C_{1-4} alkanol, or a polar protic solvent, or in a non-polar aprotic solvent, especially in the presence of water, e.g. isopropyl acetate in the presence of water, and contacted with hydrogen or a hydrogen donor such as cyclohexene or 1,4-cyclohexadiene in the presence of a reduction catalyst, typically a palladium catalyst such as palladium black, palladium on barium sulfate, and palladium on carbon. For catalytic isomerization, a zerovalent palladium complex such as tetrakis(triphenylphosphine)palladium(0) is used, typically in the presence of a nucleophilic allyl group scavenger such as a secondary amine. After removal of the catalyst, the compound of the first aspect of this invention is isolated, conveniently as an acid addition salt (in optional step (e)).

In optional steps (c) and (d), the compound of the first aspect of this invention is esterified (if a diacid and an ester is desired), or de-esterified (if an ester and the diacid is desired). These esterifications or de-esterifications are conventional and may be carried out by any of the methods well known to persons of ordinary skill in the art; in particular, a convenient esterification technique involves the reaction of the diacid compound of the first aspect of this invention with the alcohol that produces the desired ester, using that alcohol as solvent, in the presence of a halosilane such as chlorotriethylsilane at temperatures between room temperature and the boiling temperature of the alcohol.

In optional step (e), a salt of the compound of the first aspect of this invention is formed. This will typically be an acid addition salt (see the definition of "salts" above). Conveniently, this is done immediately after the compound of the first aspect of this invention is formed; and may be accomplished by addition of a solvent that will become an anti-solvent for the salt, such as an aprotic solvent, e.g. a hydrocarbon or halogenated hydrocarbon such as dichloromethane, followed by addition of the acid chosen to form the salt, especially in the form of the anhydrous acid alone or in an aprotic solvent, e.g. hydrogen chloride gas. Further anti-solvent for the salt, e.g. ethers such as diethyl ether, methyl *tert*-butyl ether, and tetrahydrofuran, especially methyl *tert*-butyl ether, may be added if necessary or desired.

The starting materials for the process (the "sulfide compounds") are tripeptides and tetrapeptides, optionally mono- or di-esterified, optionally protected at the glutamyl amine, and with the cysteine sulfur alkylated with a Z-CH_2– group.

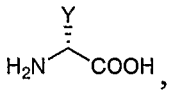
These S-alkylated glutathione analogs are conveniently prepared by conventional methods of peptide synthesis well-known to persons of ordinary skill in the art.

Where an appropriately S-alkylated cysteine is not readily available for the peptide synthesis, the peptide synthesis may be performed using one of the many standard S-protecting groups, such as triphenylmethyl, and the resulting S-protected peptide then deprotected, for example by acidolysis. Typically, the S-protected peptide is dissolved in an acid that is strong enough to remove the acidolytically removable sulfur-protecting group but not strong enough to remove any amine-protecting group, optionally in the presence of a scavenger. Suitable such acids include trifluoroacetic acid and other strong acids such as trifluoromethanesulfonic acid, optionally in the presence of a cosolvent such as dichloromethane; suitable scavengers include aromatic ethers and sulfides such as anisole and thioanisole, phenols such as cresol, and, most efficiently, silanes including trialkylsilanes such as triethylsilane and triisopropylsilane and silane polymers such as poly(methylhydrosiloxane); and a particularly suitable deprotection reagent is trifluoroacetic acid in the presence of poly(methylhydrosiloxane). The S-deprotected peptide can be isolated from the reaction mixture by addition of an anti-solvent, for example an aprotic non-polar solvent such as a hydrocarbon or an ether.

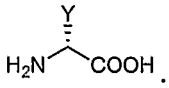
Then, the thiolate anion of the S-deprotected peptide is alkylated with a compound of the formula Z-CH_2-L, where L is a leaving group such as a halogen (chlorine or bromine) or an optionally substituted alkane- or arenesulfonate to prepare the appropriately S-alkylated peptide. This is conveniently done when the glutamyl amine is protected, to minimize side reactions, but can be done even when the amine is unprotected.

Typically, the S-deprotected peptide is dissolved in a solution of a strong base [e.g. an alkali metal or alkaline earth metal hydroxide, carbonate, bicarbonate, phosphate, or alkoxide, or ammonium hydroxide; or an organic amine base such as tetramethylguanidine, DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), and the like] in a suitable solvent (e.g. a C_{1-6} alkanol, a diol such as 1,2-ethanediol or 1,3-propanediol, an ether such as 2-methoxyethanol, 1,2-dimethoxyethane, or tetrahydrofuran, and the like) to form the thiolate anion, the compound of the formula Z-CH_2-L is added (typically in excess), and the reaction mixture is held at an appropriate temperature and time until completion. Convenient reaction conditions are the use of 20% tetrahydrofuran in water and an approximately ten-fold excess of sodium bicarbonate.

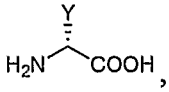
The sulfide compounds may also be prepared as follows:
an (optionally protected) cysteine is alkylated with an appropriate Z-CH_2- group, and the tripeptide or tetrapeptide then formed by standard methods of peptide synthesis, coupling first with an amino acid of the formula



and then with an (optionally protected) L-γ-glutamine or L-γ-glutamylglycine; or coupling first with the (optionally protected) L-γ-glutamine or L-γ-glutamylglycine and then with the amino acid of the formula



Many of the amino acids of the formula



such as D-phenylglycine, D-(4-chlorophenyl)glycine, D-(4-hydroxypheny)glycine, D-(3-thienyl)glycine, and D-cyclohexylglycine, are commercially available in resolved form,

and in some cases as the esters. Others are available in racemic form, but may be resolved by any of the methods known for the resolution of amino acids. Yet others may be prepared by methods known for the synthesis of amino acids.

The sulfide compounds may be prepared as diacids (though this may involve intermediate protection of the glutamyl α-carboxyl as well as the amine) or as mono- or diesters, depending on the state of the starting amino acids. If the sulfide compound is prepared as a diacid, it may be mono- or di-esterified (typically while amine-protected) before oxidation of the sulfide to the sulfone, and the esterification conditions will typically be similar to those that may be used for esterification of a compound of the first aspect of this invention.

A person of ordinary skill in the art will have no difficulty, considering that skill and this disclosure (including the Examples), in preparing compounds of the first and fifth aspects of this invention.

Uses of the compounds and process

The compounds of the first aspect of this invention (especially in the acid form) are inhibitors, typically selective inhibitors, of GST P1-1. They are also active in enhancing the differentiation of HL-60 cells *in vitro*, and one has been shown to enhance granulocyte/monocyte colony formation from murine bone marrow cells *ex vivo*. The compounds are therefore expected to act therapeutically in humans to: potentiate the cytotoxic effects of chemotherapeutic agents in tumor cells (because GST, especially GST P1-1, which is elevated in many tumor tissues, is implicated in the resistance of tumor cells to chemotherapeutic agents; so that a GST inhibitor will reduce the ability of the tumor cells to clear the chemotherapeutic agent), selectively exert cytotoxicity in tumor cells (again because of GST isoenzyme inhibition), elevate the production of GM progenitors in bone marrow, stimulate the differentiation of bone marrow, mitigate the myelosuppressive effects of chemotherapeutic agents, and modulate hematopoiesis in bone marrow.

The compounds of the fifth aspect of this invention and the process of the fourth aspect of this invention are useful in the preparation of compounds of the first aspect of this invention.

The second aspect of this invention is pharmaceutical compositions comprising a compound of the first aspect of this invention, optionally also including an excipient, such as a pharmaceutically acceptable excipient, therapeutic methods involving administering these compounds or a pharmaceutical composition containing them, typically in a therapeutically effective amount, and the use of these compounds for the treatment of cancer and for the manufacture of medicaments for the treatment of cancer.

The compounds may be administered by any route suitable to the subject being treated and the nature of the subject's condition. Routes of administration include, but are not limited to, administration by injection, including intravenous, intraperitoneal, intramuscular, and subcutaneous injection, by transmucosal or transdermal delivery, through topical applications, nasal spray, suppository and the like or may be administered orally. Pharmaceutical compositions containing these compounds may optionally be liposomal formulations, emulsions, formulations designed to administer the drug across mucosal membranes or transdermal formulations. Suitable formulations for each of these methods of administration may be found, for example, in *Remington: The Science and Practice of Pharmacy*, 20 ed., A. Gennaro, ed., Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, U.S.A., 2003. Typical compositions will be either oral or solutions for intravenous infusion and will contain the compound and typically will also contain one or more pharmaceutically acceptable excipients. Typical dosage forms will be tablets, solutions for intravenous infusion, lyophilized powders for reconstitution as solutions for intravenous infusion, and liposomal formulations and lyophilized liposomal formulations also for intravenous administration.

A therapeutically effective amount of a compound of the first aspect of this invention is about 10 - 2000 mg/m^2 body surface area, especially 50 - 1000 mg/m^2. Administration may be at 1 - 35 day intervals; for example, about 250 - 1000 mg/m^2 at 1 - 5 week intervals, especially at 1, 2, 3, or 4 week intervals, or at higher frequencies including as frequently as once/day for several (e.g. 5 or 7) days, with the administration repeated every 2, 3, or 4 weeks, or constant infusion for a period of 6 – 72 hours, also with the administration repeated every 2, 3, or 4 weeks. A typical regimen may involve administration once/day for five days every three weeks.

Preparations and Examples

The following examples show the preparations of useful intermediates and the syntheses of the compounds of this invention by the process of this invention, and the utility of the compounds of the first aspect of this invention as therapeutic agents.

Preparations 1 and 2 describe preparations of reagents for S-alkylation of cysteine or the cysteine of the tripeptides and tetrapeptides (the "sulfide compounds"). For convenience, the reagents are named as derivatives of benzyl bromide (α-bromotoluene).

Preparation 1: Preparation of 4-(2-pyridyl)benzyl bromide.

4-(2-Pyridyl)toluene, 2 mL (1.98 g, 11.7 mmol), *N*-bromosuccinimide (NBS), 2.5 g (14.0 mmol), and α,α'-azobis(isobutyronitrile) (AIBN), 23 mg (0.14 mmol) were suspended in 35 mL tetrachloromethane, and refluxed for 24 hours. The product was filtered, and the solvent removed from the filtrate by rotary evaporation. Equal amounts of chloroform and water were added and the product extracted. The chloroform layer was separated and dried, and the solvent removed. 4-(2-Pyridyl)benzyl bromide, 2 g, was obtained as a sticky yellow solid.

Preparation 2: Preparation of 4-(2-methoxyphenyl)benzyl bromide.

4-bromotoluene, 171 mg (1 mmol), 2-methoxyphenylboronic acid, 152 mg (1 mmol), palladium diacetate, 1 mg (4 μmol), *N*-butylammonium bromide, 322 mg (1 mmol), and 2 M aqueous Na_2CO_3, 1.9 mL (3.8 mmol), were placed in a microwaveable pressure vial and heated to 150 C for 5 minutes. After cooling to room temperature, the product was poured into a separatory funnel and 30 mL each of water and diethyl ether were added. After extraction, the layers were separated and the aqueous layer washed with another 30 mL diethyl ether. The ether extracts were combined, dried over $MgSO_4$, and the solvent removed by rotary evaporation under vacuum. 2-methoxy-4'-methylbiphenyl, 160 mg (81% yield) was obtained as a solid product, 95% purity by NMR.

2-methoxy-4'-methylbiphenyl, 160 mg (0.81 mmol), NBS, 158 mg (0.91 mmol), and AIBN, 1 mg (0.006 mmol) were suspended in 7 mL tetrachloromethane, and refluxed for 24 hours. The product was filtered, and the solvent removed from the filtrate by rotary evaporation. Equal amounts of diethyl ether and water were added and the product

extracted. The ether layer was separated and dried over $MgSO_4$, filtered, and the solvent removed. 4-(2-Methoxyphenyl)benzyl bromide, 128 mg, was obtained as an oil.

4'-(2-cyanophenyl)benzyl bromide may be prepared from 2-cyanophenylboronic acid by this method.

Other benzyl bromides substituted with optionally substituted phenyl or optionally substituted C_{5-6} heteroaryl may readily be prepared by the methods of these preparations.

Preparation 3 describes the preparation of an S-alkylated cysteine.

Preparation 3: Preparation of *N*-α-(*tert*-butoxycarbonyl)-*S*-(4-biphenylylmethyl)-L-cysteine.

N-α-(*tert*-Butoxycarbonyl)-L-cysteine, 1.0 g (4.52 mmol), is dissolved in a mixture of 9 mL tetrahydrofuran and 9 mL 1 M aqueous NaOH which had been degassed by bubbling argon through it. A solution of 4-(bromomethyl)biphenyl, 1,12 g (4.53 mmol), in 5 mL tetrahydrofuran (THF) was added and the solution stirred under argon bubbling for 8 hours at room temperature. The THF was removed by rotary evaporation under vacuum and the residue diluted with water and washed with diethyl ether. The aqueous phase was acidified to pH 3 with 6 M aqueous HCl, and extracted three times with ethyl acetate. The combined organic extracts were washed with water, saturated aqueous $NaHCO_3$, water, and brine, and then dried and concentrated to give 1.44 g *N*-α-(*tert*-butoxycarbonyl)-*S*-(4-biphenylylmethyl)-L-cysteine (82% yield) as a white solid.

Other (optionally protected) S-alkylated cysteines may be readily prepared by the method of this preparation.

Preparations 4 to 6 describe the preparation of some "sulfide compounds".

Preparation 4: Preparation of L-γ-glutamyl-*S*-[(4-biphenylyl)methyl]-L-cysteinyl-D-phenylglycine.

L-γ-glutamyl-*S*-[(4-biphenylyl)methyl]-L-cysteinyl-D-phenylglycine, as the hydrochloride salt, was prepared from 330 mg *N*-α-(benzyloxycarbonyl)-L-γ-glutamyl-L-cysteinyl-D-phenylglycine by dissolving the amine-protected peptide in acetonitrile/water, adding an approximate 10-fold excess of sodium bicarbonate, and adding a slight excess of 4-(bromomethyl)biphenyl, then maintaining the reaction under nitrogen until completion. The reaction mixture was diluted with water, washed with diethyl ether, and the aqueous phase

separated and acidified to pH 2-3 with hydrochloric acid, then extracted with ethyl acetate. The organic phase was dried over anhydrous $MgSO_4$, filtered, and the volume reduced to give *N*-α-(benzyloxycarbonyl)-L-γ-glutamyl-*S*-[(4-biphenylyl)methyl)-L-cysteinyl-D-phenylglycine. The amine-protected sulfide compound, approx. 300 mg, was dissolved in 2 mL 95:5 trifluoroacetic acid(TFA):dichloromethane with 1 drop water, and stirred at 45°C overnight, and purified by preparative HPLC to give L-γ-glutamyl-*S*-[(4-biphenylyl)methyl)-L-cysteinyl-D-phenylglycine. The L-γ-glutamyl-*S*-[(4-biphenylyl)methyl)-L-cysteinyl-D-phenylglycine was isolated as the hydrochloride salt. If desired, the amine-protected sulfide compound could have been taken directly into oxidation step (a), to prepare a compound of the fifth aspect of this invention.

Compounds such as L-γ-glutamyl-*S*-{[4-(2-pyridyl)phenyl]methyl}-L-cysteinyl-D-phenylglycine, and their amine-protected analogs, were prepared by similar methods.

Other sulfide compounds, including sulfide compounds that are mono- and diesters, may readily be prepared by similar methods.

Preparation 5: Preparation of L-γ-glutamyl-*S*-[(4-biphenylyl)methyl]-L-cysteinyl-D-phenylglycine diethyl ester.

L-γ-glutamyl-*S*-[(4-biphenylyl)methyl]-L-cysteinyl-D-phenylglycine diethyl ester, as the hydrochloride salt, was prepared from L-γ-glutamyl-*S*-[(4-biphenylyl)methyl]-L-cysteinyl-D-phenylglycine, 30 mg, by esterification with ethanol and chlorotrimethylsilane, giving L-γ-glutamyl-*S*-[(4-biphenylyl)methyl]-L-cysteinyl-D-phenylglycine diethyl ester. The L-γ-glutamyl-*S*-[(4-biphenylyl)methyl]-L-cysteinyl-D-phenylglycine diethyl ester was isolated as the hydrochloride salt.

Compounds such as L-γ-glutamyl-*S*-{[4-(2-pyridyl)phenyl]methyl}-L-cysteinyl-D-phenylglycine diethyl ester were prepared by similar methods. Other esters, particularly diesters, of the sulfide compounds may readily be prepared by similar methods.

Preparation 6: Preparation of L-γ-glutamyl-*S*-[(4-biphenylyl)methyl]-L-cysteinyl-D-cyclohexylglycine.

D-Cyclohexylglycine ethyl ester was prepared from D-cyclohexylglycine, 500 mg (3.1 mmol), by suspending the acid in 15 mL ethanol, adding chlorotrimethylsilane, 2.02 mL (5 equivalents), and heating the mixture at 50°C for 18 hours. The volatile components were

removed under vacuum and the residue dissolved in ethyl acetate, washed with aqueous $NaHCO_3$ and twice with brine, dried over $MgSO_4$, filtered, and evaporated to give 401 mg D-Cyclohexylglycine ethyl ester as an oily solid.

N-α-(*tert*-butoxycarbonyl)-*S*-(4-biphenylylmethyl)-L-cysteine, 588 mg (1.9 mmol), was dissolved in 5 mL dimethylformamide (DMF), and *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium hexafluorophosphate (HBTU), 860 mg (1.2 equiv.), and di(isopropyl)ethylamine, 990 μL (3 equiv.), were added. After 3 minutes, D-Cyclohexylglycine ethyl ester, 360 mg (1 equiv.), dissolved in 4 mL DMF, was added, and the mixture stirred at room temperature for 2 hours, then the crude *N*-α-(*tert*-butoxycarbonyl)-*S*-(4-biphenylylmethyl)-L-cysteinyl-D-cyclohexylglycine ethyl ester isolated. The dipeptide was dissolved in 10 mL 20% TFA/dichloromethane, and stirred at room temperature for 2 hours, then the crude product isolated by removal of volatiles under vacuum. The crude *S*-(4-biphenylylmethyl)-L-cysteinyl-D-cyclohexylglycine trifluoroacetate may be used directly in the next step, or may be further processed.

N-α-(*tert*-butoxycarbonyl)-*O*-α-*tert*-butyl-L-γ-glutamine was dissolved in 2 mL DMF, and HBTU, 174 mg (1.2 equiv.), and di(isopropyl)ethylamine, 330 μL (5 equiv.), were added. After 5 minutes, crude *S*-(4-biphenylylmethyl)-L-cysteinyl-D-cyclohexylglycine trifluoroacetate, 200 mg (0.38 mmol), dissolved in 2 mL DMF, was added, and the mixture stirred at room temperature for 2 hours. The mixture was poured into 0.1 N aqueous citric acid, and extracted with ethyl acetate. The organic layer was washed four times with brine, dried over $MgSO_4$, and the volatiles evaporated to give *N*-α-(*tert*-butoxycarbonyl)-*O*-α-*tert*-butyl-L-γ-glutamyl-*S*-(4-biphenylylmethyl)-L-cysteinyl-D-cyclohexylglycine as a brown oil. This was dissolved in 10 mL 20% TFA/dichloromethane and stirred at room temperature for 24 hours. The volatiles were removed under vacuum and the product purified by reverse phase HPLC, then the L-γ-glutamyl-*S*-(4-biphenylylmethyl)-L-cysteinyl-D-cyclohexylglycine isolated as the hydrochloride salt, 47 mg. If desired, the protected sulfide compound could also have been taken directly into oxidation step (a).

L-γ-glutamyl-*S*-(4-biphenylylmethyl)-L-cysteinyl-D-(4-chlorophenyl)glycine was prepared as the hydrochloride salt by a similar method.

Synthetic Example 1. Synthesis of L-γ-glutamyl-3-[(phenylmethyl)sulfonyl]-L-alanyl-D-phenylglycine, **1A**.

To 100 mg (0.2 mmol) L-γ-glutamyl-*S*-(phenylmethyl)-L-cysteinyl-D-phenylglycine hydrochloride (TLK117 hydrochloride) dissolved in acetic acid was added 200 μL of a 32% solution of peracetic acid in acetic acid (0.95 mmol). The reaction mixture was maintained at room temperature, following the reaction by LCMS. On completion of the reaction, the reaction mixture was concentrated under vacuum to a solid, then taken up in 50% acetonitrile/water, filtered, and the residue washed with more acetonitrile/water. The filtrate was lyophilized, then the residue and filtrate purified by preparative HPLC, and the product-containing fractions again lyophilized. After dissolution in acetonitrile/water and lyophilization, then trituration with methanol/ether and drying under vacuum, L-γ-glutamyl-3-[(phenylmethyl)sulfonyl]-L-alanyl-D-phenylglycine, **1A**, was obtained as a solid product, with 96% purity by LC (ELSD detection). The proton NMR spectrum (DMSO-d_6) was consistent with the proposed structure. Two further lots were similarly prepared on a larger scale (0.4 mmol and 11 mmol, respectively), using a 2.3-2.5-fold excess of peracetic acid, and altering solvent ratios and quantities slightly. The same product was obtained in each case.

Synthetic Example 2. Synthesis of L-γ-glutamyl-3-[(phenylmethyl)sulfonyl]-L-alanyl-D-phenylglycine diethyl ester, **1B**.

To 123 mg (0.22 mmol) L-γ-glutamyl-*S*-(phenylmethyl)-L-cysteinyl-D-phenylglycine diethyl ester hydrochloride dissolved in 3 mL acetic acid was added 225 μL of a 32% solution of peracetic acid in acetic acid (1.06 mmol). The reaction mixture was maintained at room temperature, following the reaction by LCMS. On completion of the reaction, the reaction mixture was concentrated under vacuum to a solid, then taken up in methanol/dimethyl sulfoxide/water and purified by preparative HPLC, and the product-containing fractions again lyophilized. L-γ-glutamyl-3-[(phenylmethyl)sulfonyl]-L-alanyl-D-phenylglycine diethyl ester, **1B**, was obtained as a solid product. The proton NMR spectrum (DMSO-d_6) was consistent with the proposed structure.

Synthetic Example 3. Synthesis of L-γ-glutamyl-3-[(phenylmethyl)sulfonyl]-L-alanyl-D-phenylglycine diisopropyl ester, **1D**.

To 309 mg (0.61 mmol) L-γ-glutamyl-*S*-(phenylmethyl)-L-cysteinyl-D-phenylglycine hydrochloride suspended in 6 mL isopropyl alcohol was added 767 μL chlorotrimethylsilane

(6.05 mmol). The reaction mixture was stirred under nitrogen at room temperature, when a white precipitate gradually formed. The mixture was heated to 50°C, an additional 300 μL chlorotrimethylsilane was added, and the reaction mixture maintained at elevated temperature. After several hours, the reaction was found to be 85% complete (with the remainder being the monoester); and the precipitate that had formed was filtered, washed with isopropanol, dried (240 mg yield), dissolved in methanol, and purified by preparative HPLC using 95:5 water/acetonitrile with 0.01% hydrochloric acid as the solvent. The diisopropyl ester, 87 mg (0.16 mmol), was dissolved in 2 mL acetic acid, and oxidized with the use of 82 μL peracetic acid solution (2.5 equivalents. On completion of the reaction, 2 mL methanol was added, the product purified by preparative HPLC using 95:5 water/acetonitrile with 0.01% trifluoroacetic acid as the solvent, and the product-containing fractions lyophilized. L-γ-glutamyl-3-[(phenylmethyl)sulfonyl]-L-alanyl-D-phenylglycine diisopropyl ester hydrochloride was obtained as a solid product, 98% purity by HPLC. The proton NMR spectrum (DMSO-d_6) was consistent with the proposed structure.

Synthetic Example 4. Synthesis of L-γ-glutamyl-3-[(phenylmethyl)sulfonyl]-L-alanyl-D-phenylglycine dimethyl ester, **1C**.

L-γ-glutamyl-3-[(phenylmethyl)sulfonyl]-L-alanyl-D-phenylglycine dimethyl ester, **1C**, as the hydrochloride salt, was prepared from 500 mg (0.98 mmol) L-γ-glutamyl-*S*-(phenylmethyl)-L-cysteinyl-D-phenylglycine hydrochloride using the method of Synthetic Example 3, using methanol instead of isopropanol in the esterification, and oxidizing the dimethyl ester in the same way as for the diisopropyl ester, with three dissolution/lyophilization cycles used for purification. Purity 98% by HPLC.

Synthetic Example 5. Synthesis of L-γ-glutamyl-3-[(4-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine, **11A**.

L-γ-glutamyl-3-[(4-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine, **11A**, as the hydrochloride salt, was prepared from in three steps from 330 mg *N*-α-(benzyloxycarbonyl)-L-γ-glutamyl-L-cysteinyl-D-phenylglycine by dissolving the amine-protected peptide in acetonitrile/water, adding an approximate 10-fold excess of sodium bicarbonate, and adding a slight excess of 4-(bromomethyl)biphenyl, then maintaining the reaction under nitrogen until completion. The reaction mixture was diluted with water, washed with diethyl ether, and the aqueous phase separated and acidified to pH 2-3 with hydrochloric acid, then extracted

with ethyl acetate. The organic phase was dried over anhydrous magnesium sulfate, filtered, and the volume reduced to give *N*-α-(benzyloxycarbonyl)-L-γ-glutamyl-3-[(4-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine. The amine-protected sulfide, approx. 300 mg, was dissolved in 2 mL 95:5 trifluoroacetic acid:dichloromethane with 1 drop water, and stirred at 45°C overnight, and purified by preparative HPLC to give L-γ-glutamyl-3-[(4-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine. The deprotected sulfide was oxidized in the same manner as in the previous Synthetic Examples to give L-γ-glutamyl-3-[(4-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine ester, **11A**, as the hydrochloride salt, purity 92% by HPLC.

Synthetic Example 6. Synthesis of L-γ-glutamyl-3-[(2-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine, **4A**.

L-γ-glutamyl-3-[(2-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine, **4A**, as the hydrochloride salt, was prepared in three steps from 500 mg *N*-α-(benzyloxycarbonyl)-L-γ-glutamyl-L-cysteinyl-D-phenylglycine using the method of Synthetic Example 5, with 2-(bromomethyl)-biphenyl as the alkylating agent for the protected peptide. Purity 99% by HPLC.

Synthetic Example 7. Synthesis of L-γ-glutamyl-3-[(2-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine diethyl ester, **4B**.

L-γ-glutamyl-3-[(2-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine dimethyl ester, **4B**, as the hydrochloride salt, was prepared from 143 mg L-γ-glutamyl-*S*-(2-biphenylylmethyl)-L-cysteinyl-D-phenylglycine hydrochloride (an intermediate in Example 6) by esterification with ethanol and chlorotrimethylsilane, then oxidizing the diethyl ester, as in Synthetic Example 3. Purity 94% by HPLC.

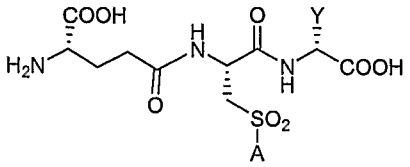
Synthetic Example 8. Synthesis of L-γ-glutamyl-3-[(2-naphthylmethyl)sulfonyl]-L-alanyl-D-phenylglycine, **3A**.

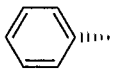
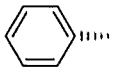
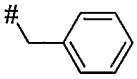
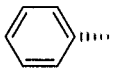
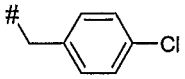
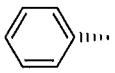
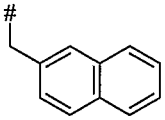
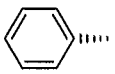
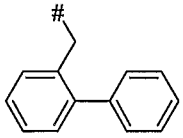
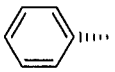
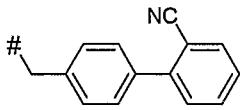
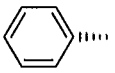
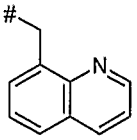
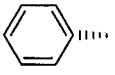
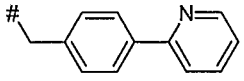
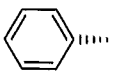
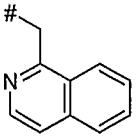
L-γ-glutamyl-3-[(2-naphthylmethyl)sulfonyl]-L-alanyl-D-phenylglycine, **3A**, as the hydrochloride salt, was prepared in three steps from *N*-α-(benzyloxycarbonyl)-L-γ-glutamyl-L-cysteinyl-D-phenylglycine using the method of Synthetic Example 5, with 2-(bromomethyl)naphthalene as the alkylating agent for the protected peptide. Purity 92% by HPLC.

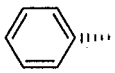
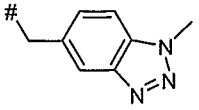
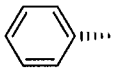
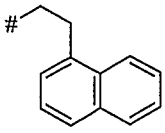
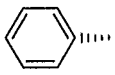
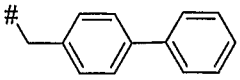
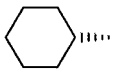
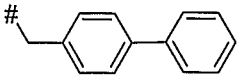
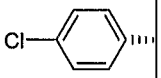
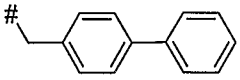
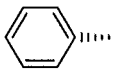
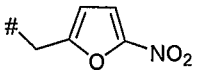
L-γ-glutamyl-3-[(5-nitrofuran-2-ylmethyl)sulfonyl]-L-alanyl-D-phenylglycine, **14A**, as the hydrochloride salt, was prepared by a similar method. Purity 99% by HPLC.

Representative compounds of the first aspect of this invention are given in Table 1 below:

Table 1. Diacid compounds of the first aspect of this invention



Compound No.	Y	A	Exact Mass (M)	MS (m/z)
"Vinyl sulfone"				
1A			505	506 (M+H)
2A			539	540 (M+H)
3A			555	556 (M+H)
4A			581	580 (M–H)
5A			606	607 (M+H)
6A			556	557 (M+H)
7A			582	583 (M+H)
8A			556	557 (M+H)

Compound No.	Y	A	Exact Mass (M)	MS (m/z)
9A			560	561 (M+H)
10A			569	570 (M+H)
11A			581	582 (M+H)
12A			587	586 (M–H)
13A			615	616 (M+H)
14A			540	541 (M+H)

# denotes the point of attachment of A to the sulfonyl sulfur atom of the compound

The diethyl esters **1B** to **12B** of compounds **1A** to **12A** were prepared by one of the methods discussed above; and analyzed by mass spectrometry, with each compound giving a mass peak (M/z) corresponding to its mass, typically M+H with positive ionization, or M-H with negative ionization. The dimethyl and diisopropyl esters **1C** and **1D** of diacid **1A** were also prepared and analyzed by mass spectrometry, with each compound giving a mass peak of M+H with positive ionization.

Other compounds of this invention may be similarly prepared, using methods well known to a person of ordinary skill in the art having regard to that skill and this disclosure.

In vitro assay examples.

These examples illustrate the effects of compounds of this invention (and one or other of the comparator compounds TLK117 and TLK199, and the "vinyl sulfone" and its diethyl ester) in predictive *in vitro* assays.

Recombinant human GST P1-1, A1-1, and M1-1 isoenzymes, and rabbit polyclonal anti-human GSTπ antibody were obtained from EMD Biosciences, Inc., San Diego, California, U.S.A. Mouse monoclonal anti-human GSTπ antibody was obtained from was

obtained from Dakoctyomation, Inc., Carpinteria, California, U.S.A. IRDye800 conjugated, affinity purified goat anti-rabbit IgG antibody was obtained from Rockland Immunochemicals, Inc., Gilbertsville, Pennsylvania, U.S.A. The human cancer cell line HL-60 (promyeloid myelocytic leukemia) was obtained from the National Cancer Institute, Bethesda, Maryland, U.S.A. RPMI 1640 medium and Iscove's Modified Dulbecco's Medium (IMDM) were obtained from Invitrogen, Inc., Carlsbad, California, U.S.A. The CellTiter-Glo™ assay kit was obtained from Promega Corporation, Madison, Wisconsin, U.S.A., and was used in accordance with manufacturer's directions. Phycoerythrin-conjugated anti-CD11b antibody and phycoerythrin-conjugated isotype control antibody were obtained from BD Biosciences, Inc., San Jose, California, U.S.A. MethoCult™ M3234 methylcellulose medium (2.5% methylcellulose in IMDM, supplemented with fetal bovine serum, bovine serum albumin, rh insulin, h transferrin, 2-mercaptoethanol, and L-glutamine) was obtained from StemCell Technologies, Inc., Vancouver, Canada, and was diluted (to 1% methylcellulose concentration) in accordance with the manufacturer's directions.

In vitro Example 1. Inhibition of glutathione *S*-transferase isoenzymes. This example illustrates the effect of compounds of this as inhibitors of the glutathione *S*-transferase isoenzymes GST P1-1, A1-1, and M1-1, *in vitro*. Inhibition of GST P1-1 is considered predictive of efficacy in the therapeutic methods of the third aspect of this invention, because TLK199 (the diethyl ester of TLK117 tested in these assays) has shown myelostimulant activity in humans and chemopotentiation activity in an animal model.

Recombinant human GST P1-1, A1-1, and M1-1 isoenzymes were used at final concentrations of 0.02, 0.011, and 0.0083 units/mL, respectively [1 unit is the amount of enzyme that will conjugate 1 μmol of 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione (GSH) per minute at 20°C and pH 6.5], in assay buffer (100 mM KH_2PO_4/K_2HPO_4, 0.1 mM EDTA, pH 6.5 in water). GSH was prepared as a 100 mM stock solution in water, and CDNB as a 100 mM stock solution in ethanol, and both were diluted with assay buffer to the appropriate concentration (to achieve a final concentration of $2\times K_m$ for the isoenzyme being evaluated). The test compounds (diacids, compounds **1A** to **12A** and TLK117) were dissolved in dimethyl sulfoxide (DMSO) and diluted with DMSO to the appropriate concentration for the assay (8 concentrations over range with midpoint near expected IC_{50} – the concentration that causes 50% inhibition of enzyme activity). For each

assay, the test compound and GSH were added to the isoenzyme. Immediately after the addition of CDNB solution to the isoenzyme/compound/GSH solution, the absorbance at 340 nm was recorded continuously for 5 min on a SpectraMax plate reader (Molecular Devices, Inc., Sunnyvale, California, U.S.A.). The GST inhibitory activity of each compound was calculated from the slopes of the absorbance/time curves at the various concentrations of test compound. All assays were conducted in duplicate wells, with DMSO control, with the final DMSO concentration maintained at 2%. Results of these assays are given in Table 2 below.

In vitro Example 2. Cytotoxicity in HL-60 cells. This example illustrates the cytotoxicity of compounds of this invention against the HL-60 human leukemia cell line *in vitro*.

Log-phase cells were seeded in 96-well plates at 1500 cells/well in 150 μL RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine, and incubated at 37°C under air/5% CO_2 for 4-5 hours. The test compounds (diethyl esters, compounds **1B** to **12B** and TLK199) were dissolved and serially diluted in DMSO (8 concentrations over range with midpoint near expected CC_{50}). After a further 1:50 dilution in the supplemented RPMI 1640 medium, 50 μL of the diluted compounds were added to achieve a final DMSO concentration of 0.5%. The cells were then incubated for 72 hours (approximately three doubling times). The cells were then harvested by centrifugation (1200 rpm for 5 min at 20°C), and 100 μL of the culture supernatant was removed and replaced by the same volume of the CellTiter-Glo reagent. After incubation for 10 minutes at room temperature with constant mixing, the plate was read with a luminometer, with the number of live cells being proportional to the observed luminescence. All assays were conducted in triplicate wells, with DMSO solvent control. The CC_{50} (concentration that causes 50% growth inhibition) was calculated from the luminescence/concentration curve. Results of this assays are given in Table 2 below.

In vitro example 3. GSTπ immunoprecipitation assay. This example illustrates the beneficial effects of compounds of this invention in causing GSTπ immunoprecipitation. These results are considered predictive of efficacy in the therapeutic methods of the third aspect of this invention, because TLK199 (the diethyl ester of TLK117 tested in these assays) has shown myelostimulant activity in humans and chemopotentiation activity in an animal model.

HL-60 cells were seeded at 6×10^5 cells/mL in 20 mL RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mM glutamine and 20 μM gentamycin in a T75 flask and cultured overnight in 5% CO_2 atmosphere at 37°C. The cells were treated with 0.1% DMSO or 20 μM of the test compounds (diethyl esters, compounds **1B** to **12B** and TLK199) in culture medium containing 0.1% DMSO for 2 hours. Following the treatment, the cells were collected by centrifugation and washed twice with phosphate-buffered saline. The cell pellets were resuspended in 1 mL lysis buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% NP-40 surfactant, 100 mM NaF; to which protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, Indiana, U.S.A.) had been added before use) and incubated with gentle agitation for 30 minutes at 4°C. The cell lysates were cleared by centrifugation at 13,000 rpm for 10 minutes to remove cell debris. For immunoprecipitation, 2.1 μg of mouse monoclonal anti-human GSTπ antibody was added to 500 μL cell lysate of 1 mg/mL total protein. Following overnight incubation at 4°C, 30 μL of Protein A/G agarose beads (Pierce, Rockford, IL) were added to the mixture and incubated for 1 hour at 4°C. The beads were then collected by centrifugation, washed three times in lysis buffer, and resuspended in 10 μL non-reducing lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, California, U.S.A.). The suspended samples were heated at 95°C for 5 minutes and subjected to SDS-PAGE on a 4 - 12% gel, followed by Western blot analysis using rabbit polyclonal anti-human GSTπ antibody followed by IRDye 800 conjugated, affinity purified goat anti-rabbit IgG antibody. GSTπ protein bands were visualized and band intensity quantified on Odyssey infrared scanner (Li-Cor, Lincoln, Nebraska, U.S.A.). The band intensity from a compound treatment was compared to its corresponding DMSO control and expressed as percent inhibition. The results are shown in Table 2 below.

Table 2. GST isoenzyme inhibition by diacid compounds of this invention, and HL-60 cytotoxicity and GSTπ immunoprecipitation of diethyl ester compounds of this invention.

Compound	GST P1-1 IC_{50}, nM	GST A1-1 IC_{50}, μM	GST M1-1 IC_{50}, μM	HL-60 CC_{50}, μM	GSTπ inhibition, %
TLK117	409	17	70	16	90
"vinyl sulfone"	22700	69	66	83	NM
1	690	5.8	76	25	88
1C	NM	NM	NM	72	NM
1D	NM	NM	NM	14	NM
2	600	NM	NM	11	84
3	310	0.12	5.5	4.7	92
4	98	0.13	19	16	99
5	78	1.9	23	6.8	95
6	280	0.46	79	25	95
7	430	NM	NM	13	87
8	240	0.27	110	16	90
9	400	19	155	42	38
10	120	NM	NM	8	77
11	43	2.2	6.3	5.2	50
12	12	NM	NM	3.7	31
13	88	NM	NM	5.5	6.1
14	3200	52	100	*	*

NM – value not measured; * – diethyl ester not made

The diacid compounds of this invention are potent inhibitors of GST P1-1, and are also generally selective for GST P1-1 over GST A1-1 and GST M1-1, the two other human GST isoenzymes. The diethyl esters all show good cytotoxicity in HL-60 cells, and many show significant inhibition of GSTπ as measured in the immunoprecipitation assay.

Some of the diethyl ester compounds of this invention were also tested for their ability to enhance the differentiation of HL-60 cells. This illustrates the beneficial effect of

the compounds of this invention in stimulating differentiation of the HL-60 human leukemia cell line *in vitro*. These results are considered predictive of efficacy in human myelostimulation, because TLK199, which enhances differentiation in this assay, has shown myelostimulant activity in humans. Log-phase cells (1×10^5 cells/mL) were incubated at 37°C under air/5% CO_2 for 24 hours in 2 mL of the supplemented RPMI 1640 medium used in *In vitro* Example 2 and the test compounds dissolved in DMSO, with a final DMSO concentration of 0.1%. The cells were harvested by centrifugation (1250 rpm for 5 min at 20°C) and washed with 2 mL ice-cold phosphate-buffered saline (PBS) containing 2% FBS, then stained with 10 μL phycoerythrin-conjugated anti-CD11b antibody or 10 μL phycoerythrin-conjugated isotype control antibody. After standing for 30 min on ice, the cells were re-suspended in 2 mL ice-cold PBS containing 2% FBS. The cells were harvested by centrifugation (1250 rpm for 5 min at 20°C) and resuspended in 0.4 mL PBS containing 2% FBS. The specific cell surface expression of CD11b, a marker for granulocytes and monocytes, was detected by flow cytometry using a FACSCalibur analyzer (BD Biosciences). All assays were conducted in triplicate, with DMSO control. The extent of differentiation was expressed as a multiple of the signal from the DMSO control. Several of the tested compounds enhanced the differentiation of HL-60 cells.

From these results and the structural similarity of these compounds to TLK199, the compounds, especially as the diesters, are expected to act therapeutically to: potentiate the cytotoxic effects of chemotherapeutic agents in tumor cells, selectively exert cytotoxicity in tumor cells, elevate the production of GM progenitors in bone marrow, stimulate the differentiation of bone marrow, mitigate the myelosuppressive effects of chemotherapeutic agents, and modulate hematopoiesis in bone marrow.

Formulation Example. This example illustrates suitable formulations for the compounds of this invention, especially the diesters.

A solid formulation for oral administration is prepared by combining the following:

Compound	25.0% w/w
Magnesium stearate	0.5% w/w
Starch	2.0% w/w
Hydroxypropylmethylcellulose	1.0% w/w
Microcrystalline cellulose	71.5% w/w

and the mixture is compressed to form tablets or filled into hard gelatin capsules containing, for example, 250 mg of the compound. Tablets may be coated, if desired, by applying a suspension of a film-forming agent (for example, hydroxypropylmethylcellulose), pigment (for example, titanium dioxide), and plasticizer (for example, diethyl phthalate), and drying the film by evaporation of the solvent.

A formulation for IV administration is prepared by dissolving the compound, for example as a pharmaceutically acceptable salt, to a concentration of 1% w/v in phosphate-buffered saline; and the solution is sterilized, for example by sterile filtration, and sealed in sterile containers containing, for example, 250 mg of a compound of this invention.

Alternatively, a lyophilized formulation is prepared by dissolving the compound, again for example as a pharmaceutically acceptable salt, in a suitable buffer, for example the phosphate buffer of the phosphate-buffered saline mentioned above, sterilizing the solution and dispensing it into suitable sterile vials, lyophilizing the solution to remove the water, and sealing the vials. The lyophilized formulation is reconstituted by the addition of sterile water, and the reconstituted solution may be further diluted for administration with a solution such as 0.9% sodium chloride intravenous infusion or 5% dextrose intravenous infusion.

Alternatively, a liposomal formulation is prepared by the method of US Published Application No. 2003/0100511 (e.g. Examples 4 and 5) using a compound of this invention in place of compound 9 of that publication, and may be lyophilized as described in that publication.

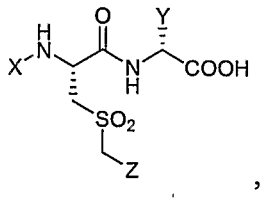
Therapeutic Example. This example illustrates a suitable therapeutic method for the compounds of this invention.

A compound of this invention, in an intravenous formulation as described in the Formulation Example above, is administered intravenously over 30 minutes to a patient suffering from myelodysplastic syndrome at an initial dose of 50 mg/m^2; and this dose is increased to 100 mg/m^2, 200 mg/m^2, 400 mg/m^2, and 600 mg/m^2. The compound is administered once per day for five days every three weeks.

While this invention has been described in conjunction with specific embodiments and examples, it will be apparent to a person of ordinary skill in the art, having regard to that skill and this disclosure, that equivalents of the specifically disclosed materials and methods will also be applicable to this invention; and such equivalents are intended to be included within the following claims.

Claims:

1. A compound of the formula



where:

X is L-γ-glutamyl or L-γ-glutamylglycyl;

Y is H, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, or optionally substituted heteroaralkyl; and

Z is C_{5-9} alkyl, optionally substituted aryl, optionally substituted aralkyl, optionally substituted heteroaryl, or optionally substituted heteroaralkyl;

or its C_{1-10} alkyl, phenyl-C_{1-3} alkyl, or (C_{5-6} heteroaryl)-C_{1-3} alkyl mono- or di-ester;

or a salt of the compound or its mono- or di-ester.

2. The compound of claim 1 where X is L-γ-glutamyl.

3. The compound of claim 1 or 2 where Y is optionally substituted C_{5-6} cycloalkyl, optionally substituted C_{5-6} cycloheteroalkyl, optionally substituted phenyl, or optionally substituted C_{5-6} heteroaryl.

4. The compound of claim 3 where Y is cyclopentyl, cyclohexyl, thienyl, furyl, pyridinyl, or optionally substituted phenyl.

5. The compound of claim 4 where Y is cyclohexyl.

6. The compound of claim 4 where Y is phenyl, optionally substituted with 1 or 2 fluoro, chloro, methyl, hydroxy, methoxy, or trifluoromethyl groups.

7. The compound of claim 6 where the phenyl is unsubstituted.

8. The compound of claim 6 where the phenyl is substituted and one of the substituents is in the 4-position.

9. The compound of claim 8 where there is only one substituent.

10. The compound of any one of claims 1 to 9 where Z is C_{5-9} alkyl, or is phenyl, 1- or 2-naphthyl, 2-, 3-, or 4-biphenylyl, or 2-, 3-, or 4-(C_{5-6} heteroaryl)phenyl, each optionally substituted with 1 to 3 halo, cyano, hydroxy, carboxy, trifluoromethyl, or -R, -OR, -COOR, or $-NR_2$ groups (where R is C_{1-3} alkyl).

11. The compound of claim 10 where Z is phenyl, optionally substituted with fluoro, chloro, cyano, hydroxy, methoxy, methyl, or trifluoromethyl.

12. The compound of claim 11 where Z is phenyl.

13. The compound of claim 10 where Z is 2-, 3-, or 4-biphenylyl, or 2-, 3-, or 4-(C_{5-6} heteroaryl)phenyl, each optionally substituted with fluoro, chloro, cyano, hydroxy, methoxy, methyl, or trifluoromethyl.

14. The compound of claim 13 where Z is 2-, 3-, or 4-biphenylyl or 2-, 3-, or 4-(C_{5-6} heteroaryl)phenyl.

15. The compound of any one of claims 1 to 14 that is a diacid.

16. The compound of any one of claims 1 to 14 that is a diester.

17. The compound of claim 16 that is a C_{1-6} alkyl diester.

18. The compound of claim 17 that is a C_{1-3} alkyl diester.

19. The compound of claim 18 that is a diethyl ester.

20. The compound of claim 1 that is selected from:
L-γ-glutamyl-3-[(phenylmethyl)sulfonyl]-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-{[(4-chlorophenyl)methyl]sulfonyl}-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-[(2-naphthylmethyl)sulfonyl]-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-[(2-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-{[(2'-cyano-4-biphenylyl)methyl]sulfonyl}-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-{[(8-quinolyl)methyl]sulfonyl}-L-alanyl-D-phenylglycine,

L-γ-glutamyl-3-{[4-(2-pyridyl)phenylmethyl]sulfonyl}-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-{[(1-isoquinolyl)methyl]sulfonyl}-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-{[(1-methylbenzotriazol-5-yl)methyl]sulfonyl}-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-{[2-(1-naphthyl)ethyl]sulfonyl}-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-[(4-biphenylylmethyl)sulfonyl]-L-alanyl-D-phenylglycine,
L-γ-glutamyl-3-[(4-biphenylylmethyl)sulfonyl]-L-alanyl-D-cyclohexylglycine, and
L-γ-glutamyl-3-[(4-biphenylylmethyl)sulfonyl]-L-alanyl-D-(4-chlorophenyl)glycine,
and their diethyl esters,
and the salts of the compounds and their diethyl esters.

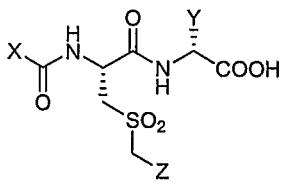
21. A pharmaceutical composition comprising a compound of any one of claims 1 to 20.

22. The pharmaceutical composition of claim 21 further comprising an excipient.

23. The use of a compound of any one of claims 1 to 20 for the manufacture of a medicament for one or more of: potentiating the cytotoxic effects of chemotherapeutic agents in tumor cells, selectively exerting toxicity in tumor cells, elevating the production of GM progenitors in bone marrow, stimulating the differentiation of bone marrow cells, mitigating the myelosuppressive effects of chemotherapeutic agents, and modulating hematopoiesis in bone marrow.

24. A therapeutic method of one or more of: potentiating the cytotoxic effects of chemotherapeutic agents in tumor cells, selectively exerting toxicity in tumor cells, elevating the production of GM progenitors in bone marrow, stimulating the differentiation of bone marrow cells, mitigating the myelosuppressive effects of chemotherapeutic agents, and modulating hematopoiesis in bone marrow, comprising administering a compound of any one of claims 1 to 20 or a composition of any one of claims 21 to 22.

25. A compound of the formula



,

where:

X is *N*-α-R^1-L-γ-glutamyl or *N*-α-R^1-L-γ-glutamylglycyl, where R^1 is an amine-protecting group; and

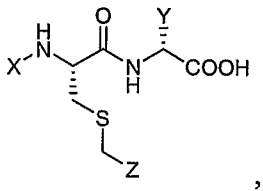
Y and Z are as in claim 1;

or its C_{1-10} alkyl, phenyl-C_{1-3} alkyl, or (C_{5-6} heteroaryl)-C_{1-3} alkyl mono- or di-ester;

or a salt of the compound or its mono- or di-ester.

26. A method of preparing a compound of claim 1, comprising:

(a) oxidizing a compound of the formula



,

where:

X is L-γ-glutamyl or L-γ-glutamylglycyl, or is *N*-α-R^1-L-γ-glutamyl or *N*-α-R^1-L-γ-glutamylglycyl, where R^1 is an amine-protecting group; and

Y and Z are as in claim 1;

or its C_{1-10} alkyl, phenyl-C_{1-3} alkyl, or (C_{5-6} heteroaryl)-C_{1-3} alkyl mono- or di-ester;

(b) if Y is *N*-α-R^1-L-γ-glutamyl or *N*-α-R^1-L-γ-glutamylglycyl, deprotecting the resulting sulfone to give a compound of claim 1; and optionally

(c) if the compound of claim 1 prepared in step (a) or step (b) is a diacid, forming a mono- or diester of the compound; or

(d) if the compound prepared in step (a) or (b) is a mono- or diester, de-esterifying the compound to prepare the diacid of the compound; and

(e) forming a salt of the compound prepared in any of steps (a) to (d).

27. The method of claim 26 where R^1 is a catalytically removable amine-protecting group.