TREATMENT OF T-CELL MEDIATED DISEASES

FIELD OF THE INVENTION

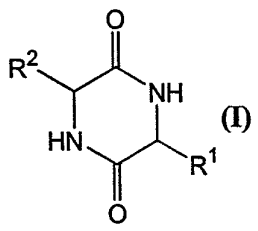
This invention relates to the treatment of T-cell mediated diseases and to the inhibition of the activation of T-cells using certain diketopiperazines. The invention also relates to pharmaceutical compositions comprising certain diketopiperazines and to methods of synthesizing diketopiperazines. The invention further relates to methods of making improved pharmaceutical compositions of proteins and peptides to either increase or decrease the content of diketopiperazines in the compositions and to the resultant improved pharmaceutical compositions.

BACKGROUND

T-cell mediated diseases represent a large number of immune system disorders. In particular, T-cells are thought to be the cells that start and perpetuate autoimmune diseases. Autoimmune diseases are a group of eighty serious, chronic illnesses that afflict millions of people in the United States alone. Autoimmune diseases are characterized by reactivity of the immune system to endogenous (self) antigens. These immune responses to self antigens are maintained by the persistent or recurrent activation of self-reactive T-cells and, directly or indirectly, the self-reactive T-cells are responsible for the characteristic tissue injury and destruction seen in autoimmune diseases. Although many treatments for autoimmune diseases and other T-cell mediated diseases have been proposed, there is still a need for additional treatments.

SUMMARY OF THE INVENTION

The present invention provides a method of treating T-cell mediated diseases. The method comprises administering to an animal in need thereof an effective amount of a diketopiperazine having the following formula:



(I)

wherein:

R^1 and R^2, which may be the same or different, each is:

(a) a side chain of an amino acid, wherein the amino acid is glycine, alanine, valine, norvaline, α-aminoisobutyric acid, 2,4-diaminobutyric acid, 2,3-diaminobutyric acid, leucine, isoleucine, norleucine, serine, homoserine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, homoarginine, citrulline, phenylalanine, *p*-aminophenylalanine, tyrosine, tryptophan, thyroxine, cysteine, homocysteine, methionine, penicillamine or ornithine; provided, however, that when R^1 is the side chain of asparagine or glutamine, then R^2 cannot be the side chain of lysine or ornithine, and when R^1 is the side chain of lysine or ornithine, then R^2 cannot be the side chain of asparagine or glutamine;

(b) R^1 is $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline and/or R^2 is $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline; or

(c) a derivative of a side chain of an amino acid, wherein the amino acid is one of those recited in (a), and the derivatized side chain has:

(i) an $-NH_2$ group replaced by an $-NHR^3$ or $-N(R^3)_2$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(ii) an -OH group replaced by an $-O-PO_3H_2$ or $-OR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iii) a -COOH group replaced by a $-COOR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iv) a -COOH group replaced by a $-CON(R^4)_2$ group, wherein each R^4 may independently be H or a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(v) an -SH group replaced by $-S-S-CH_2-CH(NH_2)-COOH$ or $-S-S-CH_2-CH_2-CH(NH_2)-COOH$;

(vi) a -CH_2- group replaced by a -$CH(NH_2)$- or a -CH(OH)- group;

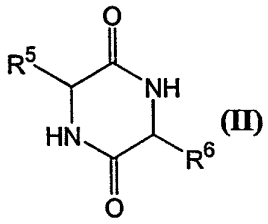
(vii) a -CH_3 group replaced by a -CH_2-NH_2 or a -CH_2-OH group; and/or

(viii) an H which is attached to a carbon atom replaced by a halogen; or

a physiologically-acceptable salt thereof.

The invention also provides a method of inhibiting the activation of T-cells. The method comprises administering to an animal in need thereof an effective amount of a diketopiperazine of formula I or a physiologically-acceptable salt thereof.

The invention further provides a pharmaceutical composition comprising a pharmaceutically-acceptable carrier and a diketopiperazine having the following formula:



(II)

wherein:

R^5 and R^6, which may be the same or different, each is:

(a) a side chain of an amino acid, wherein the amino acid is glycine, alanine, valine, norvaline, α-aminoisobutyric acid, 2,4-diaminobutyric acid, 2,3-diaminobutyric acid, leucine, isoleucine, norleucine, serine, homoserine, threonine, lysine, hydroxylysine, histidine, arginine, homoarginine, citrulline, phenylalanine, *p*-aminophenylalanine, tyrosine, tryptophan, thyroxine or ornithine; provided, however, that when R^5 is the side chain of asparagine or glutamine, then R^6 cannot be the side chain of lysine or ornithine, and when R^5 is the side chain of lysine or ornithine, then R^6 cannot be the side chain of asparagine or glutamine;

(b) R^5 is -CH_2-CH_2-CH_2- or -CH_2-CH(OH)-CH_2- and together with the adjacent ring nitrogen forms proline or hydroxyproline and/or R^6 is -CH_2-CH_2-CH_2- or -CH_2-CH(OH)-CH_2- and together with the adjacent ring nitrogen forms proline or hydroxyproline; or

(c) a derivative of a side chain of an amino acid, wherein the amino acid is one of those recited in (a), and the derivatized side chain has:

(i) an $-NH_2$ group replaced by an $-NHR^3$ or $-N(R^3)_2$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(ii) an -OH group replaced by an $-O-PO_3H_2$ or $-OR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iii) a $-CH_2-$ group replaced by a $-CH(NH_2)-$ or a -CH(OH)- group;

(iv) a $-CH_3$ group replaced by a $-CH_2-NH_2$ or a $-CH_2-OH$ group; and/or

(v) an H which is attached to a carbon atom replaced by a halogen; or

a physiologically-acceptable salt thereof.

The invention provides another method of treating a T-cell mediated disease. The method comprises administering to an animal in need thereof an effective amount of a pharmaceutical composition comprising a protein or peptide normally found in the animal, the protein or peptide having been treated so that the composition also comprises at least one diketopiperazine derived from the protein or peptide.

The invention further provides a method of inhibiting T-cell activation. The method comprises administering to an animal in need thereof an effective amount of a pharmaceutical composition comprising a protein or peptide normally found in the animal, the protein or peptide having been treated so that the composition also comprises at least one diketopiperazine derived from the protein or peptide.

In addition, the invention provides methods of synthesizing diketopiperazines. In one embodiment, the method comprises heating a solution of a protein or peptide under conditions effective to cause the formation of a diketopiperazine. In a second embodiment, the method comprises contacting a solution of a protein or peptide with an enzyme that cleaves the two N-terminal or the two C-terminal amino acids of the protein or peptide under conditions effective to produce a diketopiperazine.

The invention also provides an improved pharmaceutical composition of a protein or peptide. The improvement is that the composition comprises a decreased content of diketopiperazines.

In addition, the invention provides a method of making an improved pharmaceutical composition of a protein or peptide. The method comprises removing from the composition at least some of the diketopiperazines present in the composition.

The invention further provides a method of making an improved pharmaceutical composition of a protein or peptide. The method comprises treating a solution of the protein or peptide so as to increase the content of diketopiperazines in the composition.

The invention also provides an improved pharmaceutical composition of a protein or peptide. The improvement is that the composition comprises an increased content of diketopiperazines.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Tracings of counts versus concentration of ERK1/2 for TriPS cells (CD4+ T-cell line isolated from influenza-immunized donor which is specific for hemagglutinin) isolated on day 20 after stimulation with anti-CD3 OKT3 antibody and incubated with 25 ng phorbal myristic acid (PMA), HC-RBL (fraction of heated human colostrum of molecular weight less than 3 kD and containing MR-DKP) at a 1:10 dilution and 0.5 mM DA-DKP for 15 minutes at 37°C.

Figure 2. Bar graph showing inhibition of secretion of tumor necrosis factor α (TNFα) and IL-16 from TriPS cells 12 days after stimulation with anti-CD3 OKT3 antibody. Indicates the inhibition of both TNFα and IL-16 secretion by human colostrum (HC) 2626 (containing MR-DKP) band DA-DKP. The maximal release observed using HC 2626 at 1:100 and 1:1000 dilutions is due to the lytic effect of high concentrations of human colostrum. No lysis is observed using 0.5 mM DA-DKP, and TNFα and IL-16 secretion are decreased.

Figure 3. Bar graph showing inhibition of TNFα secretion from TriPS cells 10 days after stimulation with anti-CD3 OKT3 antibody. Indicates that HC RBL and DA-DKP need to be investigated further for titratable response as seen with HC 2626. May indicated a potent activity.

Figure 4. Bar graph showing inhibition of TNFα secretion from TriPS cells at varying times after stimulation with anti-CD3 OKT3 antibody. Indicates that early in the stimulation cycle, the effect of DA-DKP and HC RBL is inhibitory, while later in the cycle

(day 14) the effect is stimulatory. HC 2626 inhibits at all times, presumably due to other constituents.

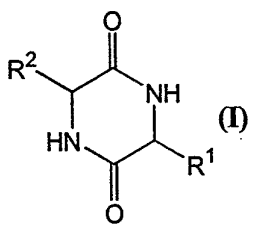
Figure 5. Bar graph showing inhibition of TNFα secretion from H4#9.25 cells (CD4+ T-cell line isolated from autopsy brain tissue of a multiple sclerosis patient which is specific for myelin basic protein) on day 7-10 after stimulation with anti-CD3 OKT3 antibody. Indicates that TNFα secretion from this T-cell line is also inhibited by HC 2626, HC RBL and DA-DKP.

DETAILED DESCRIPTION OF THE PRESENTLY-PREFERRED EMBODIMENTS

The present invention provides a method of treating T-cell mediated diseases. "Treat" is used herein to mean to reduce (wholly or partially) the symptoms, duration or severity of a disease, including curing the disease, or to prevent the disease.

T-cell mediated diseases include graft rejection, graft versus host disease, unwanted delayed-type hypersensitivity reactions (such as delayed-type allergic reactions), T-cell mediated pulmonary diseases, and autoimmune diseases. T-cell mediated pulmonary diseases include sarcoidosis, hypersensitivity pneumonitis, acute interstitial pneumonitis, alveolitis, pulmonary fibrosis, idiopathic pulmonary fibrosis and other diseases characterized by inflammatory lung damage. Autoimmune diseases include multiple sclerosis, neuritis, polymyositis, psoriasis, vitiligo, Sjogren's syndrome, rheumatoid arthritis, Type 1 diabetes, autoimmune pancreatitis, inflammatory bowel diseases (*e.g.*, Crohn's disease and ulcerative colitis), celiac disease, glomerulonephritis, scleroderma, sarcoidosis, autoimmune thyroid diseases (*e.g.*, Hashimoto's thyroiditis and Graves disease), myasthenia gravis, Addison's disease, autoimmune uveoretinitis, pemphigus vulgaris, primary biliary cirrhosis, pernicious anemia, and systemic lupus erythematosis.

The T-cell mediated disease are treated by administering to an animal in need thereof an effective amount of a diketopiperazine having the following formula:



(I)

wherein:

R^1 and R^2, which may be the same or different, each is:

(a) a side chain of an amino acid, wherein the amino acid is glycine, alanine, valine, norvaline, α-aminoisobutyric acid, 2,4-diaminobutyric acid, 2,3-diaminobutyric acid, leucine, isoleucine, norleucine, serine, homoserine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, homoarginine, citrulline, phenylalanine, *p*-aminophenylalanine, tyrosine, tryptophan, thyroxine, cysteine, homocysteine, methionine, penicillamine or ornithine; provided, however, that when R^1 is the side chain of asparagine or glutamine, then R^2 cannot be the side chain of lysine or ornithine, and when R^1 is the side chain of lysine or ornithine, then R^2 cannot be the side chain of asparagine or glutamine;

(b) R^1 is $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline and/or R^2 is $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline; or

(c) a derivative of a side chain of an amino acid, wherein the amino acid is one of those recited in (a), and the derivatized side chain has:

(i) an $-NH_2$ group replaced by an $-NHR^3$ or $-N(R^3)_2$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(ii) an -OH group replaced by an $-O-PO_3H_2$ or $-OR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iii) a -COOH group replaced by a $-COOR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iv) a -COOH group replaced by a $-CON(R^4)_2$ group, wherein each R^4 may independently be H or a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(v) an -SH group replaced by $-S-S-CH_2-CH(NH_2)-COOH$ or $-S-S-CH_2-CH_2-CH(NH_2)-COOH$;

(vi) a -CH_2- group replaced by a -$CH(NH_2)$- or a -CH(OH)- group;

(vii) a -CH_3 group replaced by a -CH_2-NH_2 or a -CH_2-OH group; and/or

(viii) an H which is attached to a carbon atom replaced by a halogen; or

a physiologically-acceptable salt thereof.

By "replaced" is meant that, with reference to the formula of an amino acid side chain, the specified group is replaced by the other specified group. For instance, the formula of the isoleucine side chain is -$CH(CH_3)$-CH_2-CH_3. If the terminal -CH_3 group is replaced with a

-CH_2-OH group, then the formula of the resulting derivatized isoleucine side chain would be

-$CH(CH_3)$-CH_2-CH_2-OH. As another example, the formula of the alanine side chain is -CH_3. If one of the hydrogen atoms is replaced by a chlorine atom, then the resulting derivatized alanine side chain would be -CH_2-Cl. Note that the side chain of glycine is -H and, if this H is replaced by a chlorine (or other halogen) atom, the resulting side chain will -Cl, with the chlorine atom attached to the ring carbon (*e.g.*, R^1 = -Cl)

Preferred are diketopiperazines wherein R^1, R^2 or both is the side chain of aspartic acid or glutamic acid or a derivative of such a side chain wherein the -COOH group is replaced by a

-$COOR^3$ group or a -$CON(R^4)_2$ group, wherein R^3 and R^4 are defined above. Of this group of compounds, most preferred are diketopiperazines comprising the side chains of aspartic acid and alanine (Asp-Ala DKP or DA-DKP), the side chains of glutamic acid and alanine (Glu-Ala DKP or EA-DKP), the side chains of tyrosine and aspartic acid (Tyr-Asp DKP or YD-DKP), the side chains of tyrosine and glutamic acid (Tyr-Glu DKP or YE-DKP) and derivatives of the aspartic acid or glutamic acid side chains of these four diketopiperazines wherein the -COOH group is replaced by a -$COOR^3$ group or a -$CON(R^4)_2$ group, wherein R^3 and R^4 are defined above.

Also, preferred are diketopiperazines wherein R^1 and R^2 are both hydrophobic side chains (*e.g.*, the side chain of phenylalanine) or hydrophobic side chain derivatives. By "hydrophobic side chain derivative" is meant that the derivatized side chain which is hydrophobic. In particular, preferred are diketopiperzines wherein R^1 and/or R^2, which may be the same or different, each is the side chain of glycine, alanine, valine, norvaline, α-aminobutyric acid, leucine, isoleucine, norleucine or phenylalanine, and/or R^1 and/or R^2 is

$-CH_2-CH_2-CH_2-$ and together with the adjacent nitrogen atom(s) form proline. Of this group of compounds, most preferred are the diketopiperazines comprising the side chains of glycine and leucine (Gly-Leu DKP or GL-DKP), proline and phenylalanine (Pro-Phe DKP or PF-DKP), and alanine and proline (Ala-Pro DKP or AP-DKP)

Additional preferred diketopiperazines are those wherein R^1, R^2 or both is the side chain of methionine, the side chain of arginine or a derivative of these side chains. Most preferred of this group is a diketopiperazine wherein R^1 is the side chain of methionine and R^2 is the side chain of arginine (Met-Arg DKP or MR-DKP).

By "side chain" of an amino acid is meant that portion of the amino acid attached to the common $NH_2-\underset{|}{C}H-COOH$ backbone of all of the amino acids listed above. For instance, the side chain of glycine is -H, the side chain of alanine is $-CH_3$, and the side chain of serine is

$-CH_2OH$.

By "hydrophobic" is meant a side chain or side chain derivative that is uncharged at physiological pH and is repelled by an aqueous solution.

By "alkyl" is meant a saturated straight-chain or branched hydrocarbon containing 1-10 carbon atoms, preferably 1-6, carbon atoms. "Lower alkyl" means a saturated straight-chain or branched hydrocarbon containing 1-6 carbon atoms.

By "cycloalkyl" is meant a saturated cyclic hydrocarbon containing at least one ring, each ring containing at least three carbon atoms. Preferably, the cycloalkyl contains one ring of 4-8 carbon atoms.

By "heterocycloalkyl" is meant a cycloalkyl having one or more of the ring carbon atoms of at least one of the rings replaced by an O, S or N.

By "aryl" is meant an aromatic group having at least one aromatic ring (*e.g.*, phenyl).

By "alkylaryl" is meant a lower alkyl having an H replaced by an aryl (*e.g.*, $-CH_2-C_6H_5$ or $-CH_3CH(C_6H_5)CH_3$).

By "arylalkyl" is meant an aryl having an H replaced by a lower alkyl (*e.g.*, $-C_6H_4-CH_3$).

By "heteroaryl" is meant an aryl having one or more of the ring carbon atoms of at least one of the rings replaced by an O, S or N.

By "substituted" is meant that the moiety is substituted with one or more substituents selected from the following group: -OH, NH_2, -SH, -COOH and/or a halogen atom.

By "halogen" is meant chlorine, fluorine, bromine or iodine. Preferred is chlorine or bromine.

The diketopiperazines of formula I are effective in treating T-cell mediated diseases because they inhibit the activation of T-cells. Accordingly, the diketopiperazines of formula I can also be used to treat inflammation and inflammatory diseases which are caused by, exacerbated by, or involve activated T-cells. "Inhibit" is used herein to mean to reduce (wholly or partially) or to prevent.

Methods of making diketopiperazines are well known in the art, and these methods may be employed to synthesize the diketopiperazines of the invention. See, *e.g.*, U.S. Patents Nos. 4,694,081, 5,817,751, 5,990,112, 5,932,579 and 6,555,543, US Patent Application Publication Number 2004/0024180, PCT applications WO 96/00391 and WO 97/48685, and Smith et al., *Bioorg. Med. Chem. Letters*, **8**, 2369-2374 (1998), the complete disclosures of which are incorporated herein by reference.

For instance, diketopiperazines can be prepared by first synthesizing dipeptides. The dipeptides can be synthesized by methods well known in the art using L-amino acids, D-amino acids or a combination of D- and L-amino acids. Preferred are solid-phase peptide synthetic methods. Of course, dipeptides are also available commercially from numerous sources, including DMI Synthesis Ltd., Cardiff, UK (custom synthesis), Sigma-Aldrich, St. Louis, MO (primarily custom synthesis), Phoenix Pharmaceuticals, Inc., Belmont, CA (custom synthesis), Fisher Scientific (custom synthesis) and Advanced ChemTech, Louisville, KY.

Once the dipeptide is synthesized or purchased, it is cyclized to form a diketopiperazine. This can be accomplished by a variety of techniques.

For example, U.S. Patent Application Publication Number 2004/0024180 describes a method of cyclizing dipeptides. Briefly, the dipeptide is heated in an organic solvent while removing water by distillation. Preferably, the organic solvent is a low-boiling azeotrope with water, such as acetonitrile, allyl alcohol, benzene, benzyl alcohol, n-butanol, 2-butanol, t-butanol, acetic acid butylester, carbon tetrachloride, chlorobenzene chloroform, cyclohexane, 1,2-dichlorethane, diethylacetal, dimethylacetal, acetic acid ethylester, heptane, methylisobutylketone, 3-pentanol, toluene and xylene. The temperature depends on the reaction speed at which the cyclization takes place and on the type of azeotroping agent used. The reaction is preferably carried out at 50-200°C, more preferably 80-150°C. The pH range

in which cyclization takes place can be easily determine by the person skilled in the art. It will advantageously be 2-9, preferably 3-7.

When one or both of the amino acids of the dipeptide has, or is derivatized to have, a carboxyl group on its side chain (*e.g.*, aspartic acid or glutamic acid), the dipeptide is preferably cyclized as described in U.S. Patent No. 6,555,543. Briefly, the dipeptide, with the side-chain carboxyl still protected, is heated under neutral conditions. Typically, the dipeptide will be heated at from about 80°C to about 180°C, preferably at about 120°C. The solvent will be a neutral solvent. For instance, the solvent may comprise an alcohol (such as butanol, methanol, ethanol, and higher alcohols, but not phenol) and an azeotropic co-solvent (such as toluene, benzene, or xylene). Preferably, the alcohol is butan-2-ol, and the azeotropic co-solvent is toluene. The heating is continued until the reaction is complete, and such times can be determined empirically. Typically, the dipeptide will be cyclized by refluxing it for about 8-24 hours, preferably about 18 hours. Finally, the protecting group is removed from the diketopiperazine. In doing so, the use of strong acids (mineral acids, such as sulfuric or hydrochloric acids), strong bases (alkaline bases, such as potassium hydroxide or sodium hydroxide), and strong reducing agents (*e.g.*, lithium aluminum hydride) should be avoided, in order to maintain the chirality of the final compound.

Dipeptides made on solid phase resins can be cyclized and released from the resin in one step. See, *e.g.*, U.S. Patent No. 5,817,751. For instance, the resin having an N-alkylated dipeptide attached is suspended in toluene or toluene/ethanol in the presence of acetic acid (*e.g.*, 1%) or triethylamine (*e.g.*, 4%). Typically, basic cyclization conditions are preferred for their faster cyclization times.

To prepare the diketopiperazine of formulas I and II wherein the amino acid side chains are derivatized, amino acid derivatives can be used in the synthesis of the dipeptides, the dipeptides can be derivatized and/or the diketopiperazines can be derivatized, as is known in the art. See, *e.g.*, those references cited above.

Other methods of cyclizing dipeptides and of making diketopiperazines are known in the art and can be used in the preparation of diketopiperazines useful in the practice of the invention. See, *e.g.*, those references listed above. In addition, many diketopiperazines suitable for use in the present invention can be made as described below from proteins and peptides. Further, diketopiperazines for use in the practice of the invention can be obtained commercially from, *e.g.*, DMI Synthesis Ltd., Cardiff, UK (custom synthesis).

The diketopiperazines of formulas I and II include all possible stereoisomers than can be obtained by varying the configuration of the individual chiral centers, axes or surfaces. In other words, the diketopierazines of formulas I and II include all possible diastereomers, as well as all optical isomers (enantiomers).

The physiologically-acceptable salts of the diketopiperazines of the invention may also be used in the practice of the invention. Physiologically-acceptable salts include conventional non-toxic salts, such as salts derived from inorganic acids (such as hydrochloric, hydrobromic, sulfuric, phosphoric, nitric, and the like), organic acids (such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, glutamic, aspartic, benzoic, salicylic, oxalic, ascorbic acid, and the like) or bases (such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation or organic cations derived from N,N-dibenzylethylenediamine, D-glucosamine, or ethylenediamine). The salts are prepared in a conventional manner, *e.g.,* by neutralizing the free base form of the compound with an acid.

As noted above, a diketopiperazine of the invention, or a physiologically-acceptable salt thereof, can be used to treat a T-cell mediated disease or to inhibit activation of T-cells. To do so, a diketopiperazine, or a physiologically-acceptable salt thereof, is administered to an animal in need of treatment. Preferably, the animal is a mammal, such as a rabbit, goat, dog, cat, horse or human. Effective dosage forms, modes of administration and dosage amounts for the compounds of the invention may be determined empirically, and making such determinations is within the skill of the art. It is understood by those skilled in the art that the dosage amount will vary with the particular compound employed, the disease or condition to be treated, the severity of the disease or condition, the route(s) of administration, the rate of excretion of the compound, the duration of the treatment, the identify of any other drugs being administered to the animal, the age, size and species of the animal, and like factors known in the medical and veterinary arts. In general, a suitable daily dose of a compound of the present invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. However, the daily dosage will be determined by an attending physician or veterinarian within the scope of sound medical judgment. If desired, the effective daily dose may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day.

Administration of the compound should be continued until an acceptable response is achieved.

The compounds of the present invention (*i.e.*, diketopiperazines and physiologically-acceptable salts thereof) may be administered to an animal patient for therapy by any suitable route of administration, including orally, nasally, rectally, vaginally, parenterally (*e.g.*, intravenously, intraspinally, intraperitoneally, subcutaneously, or intramuscularly), intracisternally, transdermally, intracranially, intracerebrally, and topically (including buccally and sublingually). The preferred routes of administration are orally and intravenously.

While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition). The pharmaceutical compositions of the invention comprise a compound or compounds of the invention as an active ingredient in admixture with one or more pharmaceutically-acceptable carriers and, optionally, with one or more other compounds, drugs or other materials. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the animal. Pharmaceutically-acceptable carriers are well known in the art. Regardless of the route of administration selected, the compounds of the present invention are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art. See, *e.g.*, *Remington's Pharmaceutical Sciences*.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, powders, granules or as a solution or a suspension in an aqueous or non-aqueous liquid, or an oil-in-water or water-in-oil liquid emulsions, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia), and the like, each containing a predetermined amount of a compound or compounds of the present invention as an active ingredient. A compound or compounds of the present invention may also be administered as bolus, electuary or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient (*i.e.*, one or more diketopiperazines of the invention and/or physiologically-acceptable salts thereof) is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose,

sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monosterate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in microencapsulated form.

Liquid dosage forms for oral administration of the compounds of the invention include pharmaceutically-acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active ingredient, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound. Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of compounds of the invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches, drops and inhalants. The active ingredient may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to the active ingredient, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch,

tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to the active ingredient, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder or mixtures of these substances. Sprays can additionally contain customary propellants such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of compounds of the invention to the body. Such dosage forms can be made by dissolving, dispersing or otherwise incorporating one or more compounds of the invention in a proper medium, such as an elastomeric matrix material. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate of such flux can be controlled by either providing a rate-controlling membrane or dispersing the compound in a polymer matrix or gel.

Pharmaceutical formulations include those suitable for administration by inhalation or insufflation or for nasal or intraocular administration. For administration to the upper (nasal) or lower respiratory tract by inhalation, the compounds of the invention are conveniently delivered from an insufflator, nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the composition may take the form of a dry powder, for example, a powder mix of one or more compounds of the invention and a suitable powder base, such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, *e.g.*, gelatin or blister packs from which the powder may be administered with the aid of an inhalator, insufflator or a metered-dose inhaler.

For intranasal administration, compounds of the invention may be administered by means of nose drops or a liquid spray, such as by means of a plastic bottle atomizer or

metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and Medihaler (Riker).

Drops, such as eye drops or nose drops, may be formulated with an aqueous or nonaqueous base also comprising one or more dispersing agents, solubilizing agents or suspending agents. Liquid sprays are conveniently delivered from pressurized packs. Drops can be delivered by means of a simple eye dropper-capped bottle or by means of a plastic bottle adapted to deliver liquid contents dropwise by means of a specially shaped closure.

Pharmaceutical compositions of this invention suitable for parenteral administrations comprise one or more compounds of the invention in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as wetting agents, emulsifying agents and dispersing agents. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like in the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monosterate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively,

delayed absorption of a parenterally-administered drug is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue. The injectable materials can be sterilized for example, by filtration through a bacterial-retaining filter.

The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampules and vials, and may be stored in a lyophilized condition requiring only the addition of the sterile liquid carrier, for example water for injection, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the type described above.

It has been found that diketopiperazines suitable for use in the present invention are present in some commercially-available intravenous pharmaceutical compositions containing albumin, immunoglobulin and erythropoietin. The diketopiperazines present in these pharmaceutical preparations are formed by the heating steps often used in the manufacture of these pharmaceutical compositions. The heating results in cleavage and cyclization of the two N-terminal and/or two C-terminal amino acids of the proteins to form diketopiperazines.

Accordingly, diketopiperazines for use in the present invention can be prepared by heating solutions of albumin, immunoglobulin, erythropoietin and other proteins and peptides. For example, a solution of albumin, immunoglobulin, erythropoietin or another protein or peptide in phosphate buffer at neutral pH is prepared. Preferably, the solution is a concentrated solution (*e.g.*, about 100-500 mM) to achieve protonation of the N-terminal and/or C-terminal amino acid. The solution is heated at 60°C for from about 2 hours to several days, preferably about 4 days, to cause formation of the diketopiperazines. Denaturation of the protein should, preferably, be avoided. This can be accomplished by using shorter times and/or by adding caprylic acid or N-acetyl tryptophan at about 0.02 M for each.

Diketopiperazines for use in the present invention can also be prepared by contacting a solution of albumin, immunoglobulin, erythropoietin or another protein or peptide with an enzyme that can cleave the two N-terminal amino acids from the protein or peptide (*e.g.*, dipeptidyl peptidases) or an enzyme that can cleave the two C-terminal amino acids from the protein or peptide (*e.g.*, carboxypeptidases). Suitable dipeptidyl peptidases and carboxypeptidases are available commercially from, *e.g.*, Sigma. The reaction should be conducted at pH 6-8, preferably in a buffer, such as phosphate buffer, at a temperature high enough to speed the reaction but not so high that the protein is denatured (*e.g.*, 37°C).

The amino acid sequences of numerous proteins and peptides are known, and a protein or peptide with the desired N-terminal and/or C-terminal sequence can be chosen to give the desired diketopiperazine(s) using either method. Also, peptides with a desired sequence can be synthesized by well known methods and used.

The diketopiperazines can be purified from solutions containing them, including from the commercially-available pharmaceutical compositions comprising albumin, immunoglobulin and erythropoietin, by well known methods, such as size-exclusion chromatography (*e.g.*, Centricon filtration), affinity chromatography (*e.g.*, using a column of beads having attached thereto an antibody or antibodies directed to the desired diketopiperazine(s) or an antibody or antibodies directed to the truncated protein or peptide), anion exchange or cation exchange. The purified diketopiperazines can be used and incorporated into pharmaceutical compositions as described above.

Instead of purifying the diketopiperazines, pharmaceutical compositions comprising albumin, immunoglobulin, erythropoietin and/or other proteins and/or peptides normally found in the animal recipient can be administered for treatment of a T-cell mediated disease and can be used to inhibit T-cell activation. Although compositions comprising these proteins and/or peptides which are currently available commercially can be used if they contain diketopiperazines, it is highly preferred to treat the albumin, immunoglobulin, erythropoietin and/or other proteins and/or peptides as described above to increase the content of the desired diketopiperzine(s) before administration of the thus improved compositions. The animal is preferably a human, and the proteins and/or peptides are preferably human proteins and/or peptides. Oral administration of the composition(s) is preferred.

Effective dosage amounts of the protein and/or peptide compositions can be determined empirically, and making such determinations is within the skill of the art. In particular, to determine an effective dosage amount of a protein and/or peptide composition, the quantity of one or more diketopiperazines present in the composition can be measured, and an amount of the composition sufficient to deliver an effective amount of the diketopiperazine(s) can be administered to the animal. It is understood by those skilled in the art that the dosage amount will vary with the particular composition employed, the disease or condition to be treated, the severity of the disease or condition, the route(s) of administration, the rate of excretion, the duration of the treatment, the identify of any other drugs being administered to the animal, the age, size and species of the animal, and like factors known in the medical and veterinary arts. In general, a suitable daily dose of a protein and/or peptide composition will be that amount which is the lowest dose effective to produce a therapeutic effect. However, the daily dosage will be determined by an attending physician or veterinarian within the scope of sound medical judgment. If desired, the effective daily dose may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day. Administration should be continued until an acceptable response is achieved.

As noted above, it has been found that diketopiperazines are found in commercially-available intravenous pharmaceutical compositions of albumin, immunoglobulin and erythropoietin where manufacture of these compositions involves one or more heating steps (*e.g.*, for sterilization). Diketopiperazines are also probably present in other pharmaceutical compositions of proteins and peptides where manufacture of the compositions involves heating steps. As described herein, many diketopiperazines have the ability to inhibit T-cell activation. Thus, it may not be desirable to administer compositions of albumin, immunoglobulin, erythropoietin or other proteins or peptides containing diketopiperazines to patients in many situations. For instance, albumin is often administered to patients suffering from trauma, immunoglobulin is often administered to patients suffering from infections or immune deficiencies, and erythropoietin is administered to anemic cancer or chronically ill patients whose immune systems are often compromised. Accordingly, the invention provides a method of removing at least some, preferably substantially all, of the diketopiperazines from such compositions. The diketopiperazines may be removed as described above (*e.g.*, by size-exclusion chromatography (*e.g.*, Centricon filtration), affinity

chromatography (*e.g.*, using a column of beads having attached thereto an antibody or antibodies directed to the desired diketopiperazine(s) or an antibody or antibodies directed to the albumin, immunoglobulin, erythropoietin or other protein or peptide), anion exchange or cation exchange) to produce improved compositions of albumin, immunoglobulin, erythropoietin and other proteins and peptides.

EXAMPLES

EXAMPLE 1: Absorption of Asp Ala DKP (DA-DKP) and Glu Ala DKP (EA-DKP) from rat intestine.

The rat intestine from the pyloric sphincter to the rectum was marginally isolated and perfused via the mesenteric artery with an erythrocyte based perfusate containing bovine serum albumin. The effluent perfusate from the gut was collected by cannulation of the portal vein and re-circulated (after re-oxygenation). After an equilibration period, a solution (approximately 1 ml) containing approximately 1 mg of Asp-Ala diketopiperazine (DA-DKP) or 1.4 mg of Glu-Ala diketopiperazine (EA-DKP) was administered by injection into the lumen of the duodenum.

After dosing, serial samples of the perfusate were collected at timed intervals up to 2 hours past dosing. Those samples were centrifuged and the plasmas assayed for both cyclic dipeptides by tandem liquid chromatography mass spectrometry (LC-MS).

The results showed that, after only 2 hours perfusion, the amounts of DA-DKP and EA-DKP which had been absorbed from the gut lumen into the circulation corresponded to 95% and 100% (actually 112%), respectively, of the dose administered.

Thus, both cyclic peptides are absorbed rapidly and efficiently from the gut lumen into blood, with no evidence of metabolism during transport across the gut wall. Hence these potential therapeutics may be given by mouth.

The rapid absorption of unchanged DA-DKP and EA-DKP from the gastrointestinal track into the blood combined with the lack of first pass hepatic clearance of both compounds in the isolated perfused rat liver (data not shown) shows that pre-systemic clearance is low. Consequently oral dosing will be an ideal route of administration.

Moreover, studies with isolated perfused rat kidney showed that, unlike many straight chain peptides, which are extensively metabolized by renal peptidases, the renal clearance of both cyclic dipeptides is relatively slow.

Collectively this data suggests that a dosing regimen of low daily doses of diketopiperazines is likely to be adequate for therapeutic purposes.

Preliminary pharmacokinetic data in rats after oral administration were consistent with the above for both cyclic dipeptides, with T_{max} values of 30-60 minutes and C_{max} values of 4-6 μg/ml (DA-DKP) and 0.6-1.1 μg/ml (EA-DKP) after oral dosing at 1.1-3.7 mg/kg body weight (DA-DKP) and 1.5 - 4.8 mg/kg body weight (EA-DKP) (T_{max} is the time when

the concentration reaches a maximum, and C_{max} is the maximum concentration reached; both were calculated from a curve fit equation for the data obtained).

Preliminary data suggest that DA-DKP and other diketopiperazines cross the blood-brain barrier. Thus, DA-DKP and other diketopiperazines of the invention should be useful for treating nervous system disorders, such as multiple sclerosis.

EXAMPLE 2: Inhibition of human T-lymphocyte cytokine production *in vitro* by fractions of human colostrum containing Met-Arg DKP (MR-DKP) and by Asp-Ala DKP (DA-DKP)

A. Materials

This example demonstrates that DA-DKP, human colostrum (HC 2626) containing MR-DKP, and a low-molecular weight fraction of human colostrum (HC RBL; a fraction of human colostrum containing components of molecular weights less than 3000 prepared by Centricon filtration of de-fatted colostrum) also containing MR-DKP, inhibited human T-lymphocyte cytokine production. DA-DKP and MR-DKP were obtained from DMI Synthesis, Ltd., Cardiff, UK. These two diketopiperazines are small naturally-occurring compounds generated during the physiological response to inflammation. They are also sometimes found in human intravenous immunoglobulin (IVIg), human albumin and other biological preparations.

B. Inhibition Of T-Cell Cytokine Production

Two different CD4-positive human T-lymphocyte clones were tested. One of the cell lines (TRiPS) was isolated from an influenza-immunized donor and is specific for hemagglutinin peptide 307-319. The other cell line (H4#9.25) was isolated from the autopsy brain tissue of a multiple sclerosis donor and is specific for myelin basic protein (amino acids 87-99). Both T-lymphocyte clones produce interleukin 8 (IL-8), IL-16, interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) after *in vitro* stimulation with either (1) specific antigen plus HLA-DR2-positive presenting cells or (2) anti-CD3 plus anti-CD28 antibodies.

The T-lymphocyte cell lines were stimulated for passage using approximately $4x10^5$ cells on day 18-20 after a previous stimulation. Cells were washed once in cold Iscove's Modified Dulbecco Minimal Essential Medium (IMDM, Sigma) plus 10% fetal bovine

serum (FBS; American Type Culture Collection (ATCC)) and resuspended in 1.0 ml cold IMDM medium containing a 1:500 dilution of anti-CD3 monoclonal antibody OKT3 (prepared from mouse ascites fluid). Cells were incubated with antibody for 30 minutes on ice, then washed with cold medium without FBS and combined with approximately $2x10^6$ 4000R-irradiated normal human donor peripheral blood leukocytes (PBL), as feeder cells, in medium plus 50 U/ml human IL-2 (Xenometrix). Cultures were expanded by the addtion of fresh IMDM medium with FBS plus IL-2 on day 3. Day of culture is measured from the day of stimulation with OKT3. Cells can be used for experiments starting on day 7 (at maximum proliferation), typically on day 14 (most sensitive to re-stimulation) and up until day 21 (resting cells approaching senescence).

Activation experiments were performed by withdrawing an aliquot of cells and washing twice with warmed (37°C) IMDM medium. For each specific assay, $2x10^5$ viable cells were pre-incubated in a total volume of 0.9 ml warmed IMDM medium containing the specified amount of treatment additive (*e.g.*, HC 2626, DA-DKP, PMA, etc.) for 15 minutes at 37°C. An aliquot of $2x10^5$ CD3/CD28 Dynabeads (Dynal), as activating stimulus, in 0.1 ml warmed IMDM was then added and the cultures incubated overnight (18 hours) at 37°C. Supernatants of the cell cultures were harvested after pelleting the cells by centrifugation. Cytokine content was assayed by specific ELISA (*e.g.*, TNFα, IFNγ, IL-8, IL-16; Endogen).

As shown in Figures 1-5, human colostrum (HC 2626) inhibited the *in vitro* cytokine production by both of the T-lymphocyte cell lines in a dose-dependent manner. As also shown in Figures 1-5, HC RBL and DA-DKP inhibited the *in vitro* cytokine production by both of the T-cell lines in a dose-dependent manner early in the stimulatory cycle. However, the effects of HC RBL and DA-DKP later in the cycle (day 14 or later) were stimulatory (see Figure 4). HC 2626 and HC RBL both contain MR-DKP (as determined by mass spectrometry), but HC 2626 contains other constituents (including caseins that are relatively dephosphorylated proteins which may, therefore, be anti-inflammatory, as described in co-pending application 10/723,247, filed November 25, 2003) besides MR-DKP which may be responsible for its inhibitory effects later in the cell cycle. Accordingly, HC RBL and HC 2626 (both containing MR-DKP), MR-DKP and DA-DKP should be useful in down-modulating the inflammatory cytokine response in T-cell-mediated and/or autoimmune diseases, such as multiple sclerosis, since they all inhibit cytokine production by T-cells early

in the stimulatory cycle. These results also suggest that HC RBL, HC 2626, MR-DKP and DA-DKP will selectively affect antigen-specific T-cells without affecting resting T-cells.

C. Mechanism Of Action

The mechanism of action of DA-DKP and HC 2626 (containing MR-DKP) was investigated. To do so, $1x10^6$ day 18 TRiPS cells were incubated for 30 minutes at 37°C, either with nothing added ("Nil"), with CD3/CD28 Dynabeads added (CD3/CD28 beads), with CD3/CD28 beads and 0.5 mM DA-DKP, or with CD3/CD28 beads and 1:500 dilution of HC 2626 added. After the incubation, the cells were lysed in Cell-Lytic Mammalian Cell Extraction Reagent (Sigma).

The cell extracts were then separately incubated with duplicate Hypromatrix Arrays for 2 hours at room temperature, followed by two washes following the manufacturer's (Hypromatrix) protocol. The Hypromatrix Array is a nylon membrane blotted with antibodies to the transcription factors listed in Table 1 (custom manufactured by Hypromatrix). An antibody cocktail specific for phosphorylated-tyrosine, phosphorylated-serine and phosphorylated-threonine (Zymed) was added, incubated for 1 hour. Then, an anti-immunoglobulin antibody labeled with biotin was added. After washing the anti-immunoglobulin-biotin away, streptavidin-peroxidase was added, and the arrays given a final wash before adding a peroxidase-reactive luminescent substrate.

The results were visualized by exposure to film and scored as 0 (negative) or + to ++++ (positive) as presented in Table 2. As shown in Table 2, some cytokine transcriptional factor activation (ERK1/2) and release of pre-formed cytokines were inhibited by HC 2626 (containing MR-DKP) and DA-DKP.

TABLE 1: HYPROMATRIX ARRAY (CUSTOM): PROTEINS FOR PHOSPHORYLATION

NUMBER	ACRONYM	COMPOUND
1	Akt 1/2	protein kinase B, anti-apoptotic kinase
2	c-Cbl	TcR inhibitory pathway; Tyr^{292} POation activates binding and inactivation of Syk and ZAP-70
3	CBP	csk-binding protein (PAG); *integral* membrane protein transiently (and at low level) Tyr-de-POated to release csk

NUMBER	ACRONYM	COMPOUND
4	CREB	cAMP response element binding protein; POated (unk) to activate/down-reg IL-2 promoter
5	csk	COOH-terminal src kinase; Ser^{364}-POated, also Tyr-POated (activity?) - POates and inactivates lck
6	ERK1	extracellular signal-related kinase
7	c-fos	AP-1 constituent activated by TcR stimulation; POated at both N- and C-unk residues
8	NFATC	nuclear factor of activated T-cells; intact in anergy
9	c-jun	AP-1 constituent activated by TcR activation; POated by JNK-MAPK at Ser^{63}
10	IκB-α	inhibitor of NFκB
11	pIκB-α	Ser-POated and inactivated NFκB inhibitor
12	p38 MAPK	mitogen-activated protein kinase
13	pI3 kinase/p85	activated by glucocorticoids and β2-adrenergic-R
14	pten	cytoplasmic 3'-inositol phosphatase; tumor suppressor gene antagonizes PI 3'kinase by converting PI-PO back to inactive forms
15	c-Raf-1	
16	Rap1	negative TcR regulatory GTPase
17	Ras	kinase; inactivated during anergy
18	fyn	cell membrane-bound immediate TcR signal kinase
19	lck	cell membrane-bound immediate TcR signal kinase, active form is Tyr^{395} POated; inactivated by csk POation at C-term Tyr
20	ZAP70kinase	signaller from CD3ζ; POated at ? by lck/fyn, ZAP70 POates LAT (linker for activation of T-cells) at Tyr's and Tyr's on SLP-76

TABLE 2: RESULTS

COMPOUND	NIL	CD3/CD28	DKP	HC2626
Akt 1/2	+	++	+++	++
c-Cbl	--	--	--	--
CBP	+	++	++	++
CREB	--	--	--	--
csk	+	++	+	+
ERK1	+	+	+	+
c-fos	--	--	--	--
NFATC	--	--	--	--
c-jun	++	+	+	+
IκB-α	++	++	+	+
pIκB-α	--	--	--	--
p38 MAPK	++	+++	+++	+++
pI3 kinase/p85	+	++	+	++
pten	--	--	--	--
c-Raf-1	--	--	--	--
Rap1	+	++	++	+
Ras	--	--	--	--
fyn	+	+	+	+
lck	--	--	--	--
ZAP70kinase	--	--	--	--

EXAMPLE 3: Inhibition of human T lymphocyte cytokine production *in vitro* by Gly-Leu DKP (GL-DKP) and Ala-Pro DKP (AP-DKP)

GL-DKP and AP-DKP (obtained from DMI Synthesis, Ltd., Cardiff, UK) were tested as described in Example 2 using TRiPS and H4#9.25 cell lines. GL-DKP and AP-DKP were found to inhibit the *in vitro* cytokine production by both of these T-lymphocyte cell lines in a dose-dependent manner. The mechanism of action is currently under investigation as described in Example 2, and both cytokine transcriptional factor activation and release of pre-formed cytokine appear to be affected.

EXAMPLE 4: Inhibition of human T lymphocyte cytokine production *in vitro* by Asp Ala DKP (DA-DKP) and Tyr Glu DKP (YE-DKP)

Normal human lymphocytes were isolated from the peripheral blood of a normal human donor with Histopaque (Sigma). Then, 3-4 x 10^5 of the lymphocytes were suspended in 1 ml of IMDM medium without serum. The cells were stimulated with by adding 25 µl of a 1:2000 dilution of anti-CD3 antibody (Pharmingen, San Diego, CA) and incubating for 18 hours at 37°C.

Then, one of three DKP preparations and dexamethasone (final concentration of 10^{-5} M) were added to triplicate cultures. The three DKP preparations were:

1. DA-DKP (obtained from DMI Synthesis, Ltd., Cardiff, UK; final concentration of 25 µg/ml in the cultures).

2. DKP-ZLB, a 25% albumin preparation (obtained from ZLB Bioplasma, AG 3000 Berne 22 Switzerland) heated for 4 days at 60°C, after which it was found to contain 0.5 mM DA-DKP, as determined by mass spectrometry (final concentration of 14 µg/ml DA-DKP in the cultures).

3. DKP-γ-glob --- a γ-globulin preparation (obtained from Sigma, number G-4386) containing 12 mg/ml γ-globulin in phosphate-buffered saline, pH 7.4, was filtered using a Centricon 3000 filter, and the filtrate (containing components having MW less than 3000) was used. The filtrate contained a mass of 292, which is the mass of Tyr-Glu DKP (YE-DKP), as determined by anion exchange HPLC coupled to negative electrospray mass spectrometry. The filtrate was used at a 1:4 final dilution in the cultures.

After addition of the DKP preparations or dexamethasone, the cultures were incubated for 18 hours at 37°C. Then, the amounts of IL-2, IFNγ and TNFα released into each culture were measured by ELISA (Pierce Biotechnology, Rockford, IL 61105).

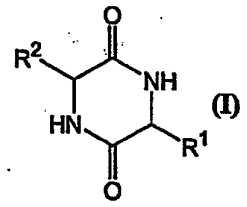
The results are presented in Table 3 below. As can be seen, the greatest reduction of release of all three cytokines was obtained with DKP-γ-glob. Flow cytometry looking at the number of CD69+ T-cells (CD69 is a marker found on activated T-cells) also showed that DKP-γ-glob reduced the number of CD69+ T-cells by about 90%, as compared to a reduction of about 50% by dexamethasone, despite the internalization of T-cell receptor complex.

TABLE 3

Stimulation	Treatment	U/ml IL-2	pg/ml IFNγ	pg/ml TNFα
Nil	---	0.24 ± 0.1	2.3 ± 0.9	2.8 ± 0.5
CD3	---	2.6 ± 0.5	289 ± 35	98 ± 3.2
CD3	DA-DKP	1.4 ± 0.3	306 ± 17	74 ± 4.7
CD3	DKP-ZLB	1.4 ± 0.4	311 ± 18	130 ± 2.9
CD3	DKP-γ-glob	0.24 ± 0.25 (91% reduction)	2.1 ± 0.1 (99% reduction)	1.6 ± 0.6 (98% reduction)
CD3	Dexamethasone	0.9 ± 0.1 (65% reduction)	76 ± 7.32 (74% reduction)	4.1 ± 0.3 (96% reduction)

WE CLAIM:

1. A compound for use in treating a T-cell mediated disease in an animal comprising a diketopiperazine having the following formula:



(I)

wherein:

R^1 and R^2, which may be the same or different, each is:

(a) a side chain of an amino acid, wherein the amino acid is glycine, alanine, valine, norvaline, α-aminoisobutyric acid, 2,4-diaminobutyric acid, 2,3-diaminobutyric acid, leucine, isoleucine, norleucine, serine, homoserine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, homoarginine, citrulline, phenylalanine, *p*-aminophenylalanine, tyrosine, tryptophan, thyroxine, cysteine, homocysteine, methionine, penicillamine or ornithine; provided, however, that when R^1 is the side chain of asparagine or glutamine, then R^2 cannot be the side chain of lysine or ornithine, and when R^1 is the side chain of lysine or ornithine, then R^2 cannot be the side chain of asparagine or glutamine;

(b) R^1 is $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline, R^2 is $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline, or both R^1 and R^2 are each independently $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogens form proline or hydroxyproline; or

(c) a derivative of a side chain of an amino acid, wherein the amino acid is one of those recited in (a), and the derivatized side chain has:

(i) an $-NH_2$ group replaced by an $-NHR^3$ or $-N(R^3)_2$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(ii) an -OH group replaced by an $-O-PO_3H_2$ or $-OR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iii) a -COOH group replaced by a $-COOR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iv) a -COOH group replaced by a $-CON(R^4)_2$ group, wherein each R^4 may independently be H or a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(v) an -SH group replaced by $-S-S-CH_2-CH(NH_2)-COOH$ or $-S-S-CH_2-CH_2-CH(NH_2)-COOH$;

(vi) a $-CH_2-$ group replaced by a $-CH(NH_2)-$ or a $-CH(OH)-$ group;

(vii) a $-CH_3$ group replaced by a $-CH_2-NH_2$ or a $-CH_2-OH$ group; and/or

(viii) an H which is attached to a carbon atom replaced by a halogen;

or a physiologically-acceptable salt thereof.

2. The compound of Claim 1 wherein R^1, R^2 or both is the side chain of aspartic acid, the side chain of glutamic acid, or a derivative of a side chain of aspartic acid or glutamic acid wherein the -COOH group is replaced by a $-COOR^3$ group or a $-CON(R^4)_2$ group.

3. The compound of Claim 2 wherein R^1 is the side chain of aspartic acid or a derivative of the side chain of aspartic acid wherein the -COOH group is replaced by a $-COOR^3$ group or a $-CON(R^4)_2$ group, and R^2 is the side chain of alanine.

4. The compound of Claim 2 wherein R^1 is the side chain of aspartic acid or a derivative of the side chain of aspartic acid wherein the -COOH group is replaced by a $-COOR^3$ group or a $-CON(R^4)_2$ group, and R^2 is the side chain of tyrosine.

5. The compound of Claim 2 wherein R^1 is the side chain of glutamic acid or a derivative of the side chain of glutamic acid wherein the -COOH group is replaced by a $-COOR^3$ group or a $-CON(R^4)_2$ group, and R^2 is the side chain of alanine.

6. The compound of Claim 2 wherein R^1 is the side chain of glutamic acid or a derivative of the side chain of glutamic acid wherein the -COOH group is replaced by a -$COOR^3$ group or a -$CON(R^4)_2$ group, and R^2 is the side chain of tyrosine.

7. The compound of Claim 2 wherein R^1 is the side chain of aspartic acid or glutamic acid and R^2 is the side chain of alanine.

8. The compound of Claim 2 wherein R^1 is the side chain of aspartic acid or glutamic acid and R^2 is the side chain of tyrosine.

9. The compound of Claim 1 wherein R^1 and R^2 are both a hydrophobic side chain or a hydrophobic side chain derivative.

10. The compound of Claim 9 wherein:

(a) R^1 and R^2, which may be the same or different, each is the side chain of glycine, alanine, valine, norvaline, α-aminobutyric acid, leucine, isoleucine, norleucine or phenylalanine;

(b) R^1 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline, and R^2 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline; or

(c) R^1 is the side chain of glycine, alanine, valine, norvaline, α-aminobutyric acid, leucine, isoleucine, norleucine or phenylalanine, and R^2 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline.

11. The compound of Claim 10 wherein R^1 is the side chain of glycine and R^2 is the side chain of leucine.

12. The compound of Claim 10 wherein R^1 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline and R^2 is the side chain of phenylalanine.

13. The compound of Claim 10 wherein R^1 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline and R^2 is the side chain of alanine.

14. The compound of Claim 1 wherein R^1, R^2 or both is the side chain of methionine, the side chain of arginine or a derivative of these side chains.

15. The compound of Claim 14 wherein R^1 is the side chain of methionine and R^2 is the side chain of arginine.

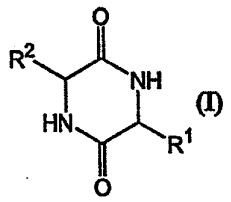
16. The compound of any one of Claims 1-15 wherein the animal is a human.

17. The compound of any one of Claims 1-15 wherein the T-cell mediated disease is graft rejection, graft versus host disease, an unwanted delayed-type hypersensitivity reaction, a T-cell mediated pulmonary disease or an autoimmune disease.

18. The compound of any one of Claims 1-15 wherein the T-cell mediated disease is multiple sclerosis, neuritis, polymyositis, psoriasis, vitiligo, Sjogren's syndrome, rheumatoid arthritis, Type 1 diabetes, autoimmune pancreatitis, inflammatory bowel diseases, Crohn's disease, ulcerative colitis, celiac disease, glomerulonephritis, scleroderma, sarcoidosis, autoimmune thyroid diseases, Hashimoto's thyroiditis, Graves disease, myasthenia gravis, Addison's disease, autoimmune uveoretinitis, pemphigus vulgaris, primary biliary cirrhosis, pernicious anemia, or systemic lupus erythematosis.

19. The compound of any one of Claims 1-15 wherein the T-cell mediated disease is pulmonary fibrosis or idiopathic pulmonary fibrosis.

20. A compound for use in inhibiting activation of T-cells in an animal comprising a diketopiperazine having the following formula:



(I)

wherein:

R^1 and R^2, which may be the same or different, each is:

(a) a side chain of an amino acid, wherein the amino acid is glycine, alanine, valine, norvaline, α-aminoisobutyric acid, 2,4-diaminobutyric acid, 2,3-diaminobutyric acid, leucine, isoleucine, norleucine, serine, homoserine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, homoarginine, citrulline, phenylalanine, *p*-aminophenylalanine, tyrosine, tryptophan, thyroxine, cysteine, homocysteine, methionine, penicillamine or ornithine; provided, however, that when R^1 is the side chain of asparagine or glutamine, then R^2 cannot be the side chain of lysine or ornithine, and when R^1 is the side chain of lysine or ornithine, then R^2 cannot be the side chain of asparagine or glutamine;

(b) R^1 is $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline, R^2 is $-CH_2-CH_2-CH_2-$ or $-CH_2-$

$CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline, or both R^1 and R^2 are each independently $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogens form proline or hydroxyproline; or

(c) a derivative of a side chain of an amino acid, wherein the amino acid is one of those recited in (a), and the derivatized side chain has:

(i) an $-NH_2$ group replaced by an $-NHR^3$ or $-N(R^3)_2$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(ii) an -OH group replaced by an $-O-PO_3H_2$ or $-OR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iii) a -COOH group replaced by a $-COOR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iv) a -COOH group replaced by a $-CON(R^4)_2$ group, wherein each R^4 may independently be H or a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(v) an -SH group replaced by $-S-S-CH_2-CH(NH_2)-COOH$ or $-S-S-CH_2-CH_2-CH(NH_2)-COOH$;

(vi) a $-CH_2-$ group replaced by a $-CH(NH_2)-$ or a -CH(OH)- group;

(vii) a $-CH_3$ group replaced by a $-CH_2-NH_2$ or a $-CH_2-OH$ group; and/or

(viii) an H which is attached to a carbon atom replaced by a halogen;

or a physiologically-acceptable salt thereof.

21. The compound of Claim 20 wherein R^1, R^2 or both is the side chain of aspartic acid, the side chain of glutamic acid, or a derivative of a side chain of aspartic acid or glutamic acid wherein the -COOH group is replaced by a $-COOR^3$ group or a $-CON(R^4)_2$ group.

22. The compound of Claim 21 wherein R^1 is the side chain of aspartic acid or a derivative of the side chain of aspartic acid wherein the -COOH group is replaced by a $-COOR^3$ group or a $-CON(R^4)_2$ group, and R^2 is the side chain of alanine.

23. The compound of Claim 21wherein R^1 is the side chain of aspartic acid or a derivative of the side chain of aspartic acid wherein the -COOH group is replaced by a -$COOR^3$ group or a -$CON(R^4)_2$ group, and R^2 is the side chain of tyrosine.

24. The compound of Claim 21 wherein R^1 is the side chain of glutamic acid or a derivative of the side chain of glutamic acid wherein the -COOH group is replaced by a -$COOR^3$ group or a -$CON(R^4)_2$ group, and R^2 is the side chain of alanine.

25. The compound of Claim 21wherein R^1 is the side chain of glutamic acid or a derivative of the side chain of glutamic acid wherein the -COOH group is replaced by a -$COOR^3$ group or a -$CON(R^4)_2$ group, and R^2 is the side chain of tyrosine.

26. The compound of Claim 21 wherein R^1 is the side chain of aspartic acid or glutamic acid and R^2 is the side chain of alanine.

27. The compound of Claim 21 wherein R^1 is the side chain of aspartic acid or glutamic acid and R^2 is the side chain of tyrosine.

28. The compound of Claim 20 wherein R^1 and R^2 are both a hydrophobic side chain or a hydrophobic side chain derivative.

29. The compound of Claim 28 wherein:

(a) R^1 and R^2, which may be the same or different, each is the side chain of glycine, alanine, valine, norvaline, α-aminobutyric acid, leucine, isoleucine, norleucine or phenylalanine;

(b) R^1 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline, and R^2 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline; or

(c) R^1 is the side chain of glycine, alanine, valine, norvaline, α-aminobutyric acid, leucine, isoleucine, norleucine or phenylalanine, and R^2 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline.

30. The compound of Claim 29 wherein R^1 is the side chain of glycine and R^2 is the side chain of leucine.

31. The compound of Claim 29 wherein R^1 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline and R^2 is the side chain of phenylalanine.

32. The compound of Claim 29 wherein R^1 is -CH_2-CH_2-CH_2- and together with the adjacent nitrogen atom forms proline and R^2 is the side chain of alanine.

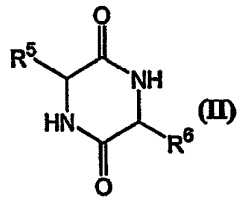
33. The compound of Claim 20 wherein R^1, R^2 or both is the side chain of methionine, the side chain of arginine or a derivative of these side chains.

34. The compound of Claim 33 wherein R^1 is the side chain of methionine and R^2 is the side chain of arginine.

35. The compound of any one of Claims 20-34 wherein the animal is a human.

36. The compound of any one of Claims 20-34 wherein the diketopiperazine is used to treat inflammation or an inflammatory disease which is caused or exacerbated at least in part by T-cell activation.

37. A pharmaceutical composition comprising a pharmaceutically-acceptable carrier and a diketopiperazine having the following formula:



(II)

wherein:

R^5 and R^6, which may be the same or different, each is:

(a) a side chain of an amino acid, wherein the amino acid is glycine, alanine, valine, norvaline, α-aminoisobutyric acid, 2,4-diaminobutyric acid, 2,3-diaminobutyric acid, leucine, isoleucine, norleucine, serine, homoserine, threonine, lysine, hydroxylysine, histidine, arginine, homoarginine, citrulline, phenylalanine, *p*-aminophenylalanine, tyrosine, tryptophan, thyroxine or ornithine; provided, however, that when R^5 is the side chain of asparagine or glutamine, then R^6 cannot be the side chain of lysine or ornithine, and when R^5 is the side chain of lysine or ornithine, then R^6 cannot be the side chain of asparagine or glutamine;

(b) R^5 is $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline, R^6 is $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogen forms proline or hydroxyproline, or both R^5 and R^6 are each independently $-CH_2-CH_2-CH_2-$ or $-CH_2-CH(OH)-CH_2-$ and together with the adjacent ring nitrogens form proline or hydroxyproline; or

(c) a derivative of a side chain of an amino acid, wherein the amino acid is one of those recited in (a), and the derivatized side chain has:

(i) an $-NH_2$ group replaced by an $-NHR^3$ or $-N(R^3)_2$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(ii) an -OH group replaced by an $-O-PO_3H_2$ or $-OR^3$ group, wherein each R^3 may independently be a substituted or unsubstituted alkyl, cycloalkyl, heterocycloalkyl, aryl, alkylaryl, arylalkyl or heteroaryl;

(iii) a $-CH_2-$ group replaced by a $-CH(NH_2)-$ or a -CH(OH)- group;

(iv) a $-CH_3$ group replaced by a $-CH_2-NH_2$ or a $-CH_2-OH$ group; and/or

(v) an H which is attached to a carbon atom replaced by a halogen;

or a physiologically-acceptable salt thereof.

38. The composition of Claim 37 wherein R^5 and R^6 are both a hydrophobic side chain or a hydrophobic side chain derivative.

39. The composition of Claim 38 wherein:

(a) R^5 and R^6, which may be the same or different, each is the side chain of glycine, alanine, valine, norvaline, α-aminobutyric acid, leucine, isoleucine, norleucine or phenylalanine;

(b) R^5 is $-CH_2-CH_2-CH_2-$ and together with the adjacent nitrogen atom forms proline, and R^6 is $-CH_2-CH_2-CH_2-$ and together with the adjacent nitrogen atom forms proline; or

(c) R^5 is the side chain of glycine, alanine, valine, norvaline, α-aminobutyric acid, leucine, isoleucine, norleucine or phenylalanine, and R^6 is $-CH_2-CH_2-CH_2-$ and together with the adjacent nitrogen atom forms proline.

40. The composition of Claim 39 wherein R^5 is the side chain of glycine and R^6 is the side chain of leucine.

41. The composition of Claim 39 wherein R^5 is $-CH_2-CH_2-CH_2-$ and together with the adjacent nitrogen atom forms proline and R^6 is the side chain of phenylalanine.

42. The composition of Claim 39 wherein R^5 is $-CH_2-CH_2-CH_2-$ and together with the adjacent nitrogen atom forms proline and R^6 is the side chain of alanine.

43. The composition of Claim 37 wherein R^5, R^6 or both is the side chain of methionine, the side chain of arginine or a derivative of these side chains.

44. The composition of Claim 43 wherein R^5 is the side chain of methionine and R^6 is the side chain of arginine.

45. A pharmaceutical composition for use in treating a T-cell mediated disease in an animal comprising a protein or peptide normally found in the animal, the protein or peptide having been treated so that the composition also comprises at least one diketopiperazine derived from the protein or peptide.

46. The composition of Claim 45 wherein the protein is albumin.

47. The composition of Claim 45 wherein the protein is immunoglobulin.

48. The composition of Claim 45 wherein the protein is erythropoietin.

49. The composition of any one of Claims 45-48 wherein the pharmaceutical composition is administered orally to the animal.

50. The composition of any one of Claims 45-48 wherein the animal is a human and the protein or peptide is a human protein or peptide.

51. A pharmaceutical composition for use in inhibiting T-cell activation in an animal comprising a protein or peptide normally found in the animal, the protein or peptide having been treated so that the composition also comprises at least one diketopiperazine derived from the protein or peptide.

52. The composition of Claim 51 where in the protein is albumin.

53. The composition of Claim 51 wherein the protein is immunoglobulin.

54. The composition of Claim 51 wherein the protein is erythropoietin.

55. The composition of any one of Claims 51-54 wherein the pharmaceutical composition is administered orally to the animal.

56. The composition of any one of Claims 51-54 wherein the animal is a human and the protein or peptide is a human protein or peptide.

57. A method of synthesizing a diketopiperazine comprising heating a solution of a protein or a peptide other than albumin under conditions effective to cause the formation of the diketopiperazine.

58. A method of synthesizing a diketopiperazine comprising heating a solution of a protein or peptide for four days at 60°C under conditions effective to cause the formation of the diketopiperazine.

59. The method of Claim 58 wherein the protein is albumin.

60. The method of Claim 57 or 58 wherein the protein is an immunoglobulin.

61. The method of Claim 57 or 58 wherein the protein is erythropoietin.

62. The method of Claim 57 or 58 wherein the diketopiperazine is purified from the solution.

63. A method of synthesizing a diketopiperazine comprising contacting a solution of a protein or peptide with an enzyme that cleaves the two N-terminal or the two C-terminal amino acids of the protein or peptide under conditions effective to produce the diketopiperazine.

64. The method of Claim 63 wherein the protein is albumin.

65. The method of Claim 63 wherein the protein is an immunoglobulin.

66. The method of Claim 63 wherein the protein is erythropoietin.

67. The method of Claim 63 wherein the enzyme is a dipeptidyl peptidase.

68. The method of Claim 63 wherein the enzyme is a carboxypeptidase.

69. The method of any one of Claims 63-68 wherein the diketopiperazine is purified from the solution.

70. An improved pharmaceutical composition of a protein or peptide, the improvement comprising a decreased content of diketopiperazines in the composition.

71. The composition of Claim 70 wherein the protein is albumin.

72. The composition of Claim 70 wherein the protein is an immunoglobulin.

73. The composition of Claim 70 wherein the protein is erythropoietin.

74. A method of making an improved pharmaceutical composition of a protein or peptide, the method comprising removing from the composition at least some of the diketopiperazines present in the composition.

75. The composition of Claim 74 wherein the protein is albumin.

76. The composition of Claim 74 wherein the protein is an immunoglobulin.

77. The composition of Claim 74 wherein the protein is erythropoietin.

78. A method of making an improved pharmaceutical composition of a protein or peptide, the method comprising treating a solution of the protein or peptide so as to increase the content of diketopiperazines.

79. The method of Claim 78 wherein the solution is heated under conditions effective to cause the formation of diketopiperazines.

80. The method of Claim 79 wherein the solution is heated for four days at 60°C.

81. The method of Claim 78 wherein the solution is contacted with an enzyme that cleaves the two N-terminal or the two C-terminal amino acids of the protein or peptide under conditions effective to produce the diketopiperazines.

82. The method of Claim 81 wherein the enzyme is a dipeptidyl peptidase.

83. The method of Claim 81 wherein the enzyme is a carboxypeptidase.

84. The method of Claim 78 wherein the protein is albumin.

85. The method of Claim 78 wherein the protein is an immunoglobulin.

86. The method of Claim 78 wherein the protein is erythropoietin.

87. An improved pharmaceutical composition of a protein or peptide, the improvement comprising an increased content of diketopiperazines in the composition.

88. The composition of Claim 87 wherein the protein is albumin.

89. The composition of Claim 87 wherein the protein is an immunoglobulin.

90. The composition of Claim 87 wherein the protein is erythropoietin.

91. The composition of any one of Claims 87-90 which is suitable for oral administration.