(54) Title: REACTIVE LIGANDS AND COVALENT LIGAND-PROTEIN COMPLEXES

(57) Abstract

Ligands bind to proteins according to particular kinetic profiles. The reversible kinetics of binding lead to release of the ligand from the binding site, thereby affecting the recognition of the ligand–protein complex. By changing the kinetics of the reversible binding, it is possible to alter the response to the cellular immune system. Reactive ligands can be used to change the kinetics of binding by reacting with the protein when bound.

REACTIVE LIGANDS AND COVALENT LIGAND-PROTEIN COMPLEXES

BACKGROUND OF THE INVENTION

Ligand protein interactions influence many factors
5 of biological systems. Ligands bind to proteins
according to particular kinetic profiles. For example,
antigenic peptides of T cells first bind to class I or
class II major histocompatibility complex (MHC) molecules
before interacting with T cell receptors on CD^{8+} T cells
10 or CD^{4+} T cells to elicit immune responses. Stern, L.J. &
Wiley, D.C. (1994), Structure 2, 245-251. In general,
conservative residue substitutions in the middle portion
of antigenic peptides of T cells are introduced to
selectively inhibit activation of T cells without
15 significantly altering binding affinity to the relevant
MHC molecules. See, Sloan-Lancaster, J., Evavold, B.D. &
Allen, P.M. (1993) Nature 363, 156-159; De Magistris,
M.T., Alexander, J., Coggeshall, M., Altman, A., Gaeta,
F.C.A., Grey, M.H. & Sette, A. (1992) Cell 68, 625-634;
20 and Alexander, J., Snoke, K., Ruppert, J., Sidney, J.,
Wall, M., Southwood, S., Oseroff, C., Arrhenius, T.,
Gaeta, F.C.A., Colon, S.M., Grey, H.M. & Sette, A. (1993)
J. Immunol. 150, 1-7. Other designs of MHC-blocking
peptides have included the replacement of several
25 residues from the middle portion of antigenic peptides
with moieties such as 4-aminobutyric acid,
6-aminohexanoic acid, and phenanthridine to act as
spacers. E.g., see Rognan, D., Scapozza, L., Folkers, G.
& Daser, A. (1995) Proc. Natl. Acad. Sci. USA 92,
30 753-757.

The reversible kinetics of binding lead to the
release of the ligand from the binding site, thereby
influencing recognition of the ligand-protein complex.
By changing the kinetics of binding it is possible to
35 alter the response to the cellular immune system. For
example, covalent complexes of peptides with MHC

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molecules have been constructed previously by generating
recombinant proteins with elongated N-termini that
extended into the binding grooves of MHC class I (see,
e.g., Mottez, E., Langlade-Demoyen, P., Gournier, H.,
5 Martinon, F., Maryanski, J., Kourilsky, P. & Abastado,
J.-P. (1995) Journal of Experimental Medicine **181**, 493-
502) and MHC class II (see, e.g., Kozono, H., White, J.,
Clements, J., Marrack, P. & Kappler, J. (1994) Nature
369, 151-154) molecules. These molecules are active and
10 have been suggested as possible therapeutics to induce T-
cell tolerance in cases where the peptide autoantigens
can be identified. See, for example, Kozono, H., White,
J., Clements, J., Marrack, P. & Kappler, J. (1994) Nature
369, 151-154 and Sharma, S. D., Nag, B., Su, X.-M.,
15 Green, D., Spack, E., Clark, B.R. & Sriram, S. (1991)
Proc. Natl. Acad. Sci. USA **88**, 11465-11469.

SUMMARY OF THE INVENTION

The present invention features compounds that bind
as ligands to a binding region of a protein. The
20 compounds react with residues in the binding region,
selectively forming a covalent bond between the protein
and the ligand. The formation of the covalent bond
changes the reversibility of the binding kinetics between
the ligand and the protein.
25   In one aspect, the invention relates to a compound
of the formula (I)

$$R_2$$

$$R_1$$ (I)

$$X$$ $$R_3$$

$$R_4$$ $$R_5$$
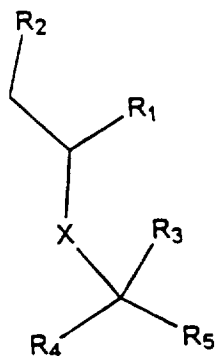
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where each of R_1 and R_2, independently, is hydroxy,
halogen, haloalkyl, haloalkyl carbonyl, alkyl sulfonate,
aryl sulfonate, alkyl carboxylate, a Michael acceptor,
amino, alkylamino, ammonium, or alkyl ammonium, or
5 together R_1 and R_2 are O, NH, $NH_2 \cdot$, or NZ, where Z is alkyl
or aryl; X is C_1 to C_6 alkyl, substituted aryl, or
deleted; R_3 is H, alkyl, or aryl; R_4 is alkyl, aryl, -NH-
W, or -C(=O)-Y, where W is H, alkyl, aryl, an amino acid
residue bonded at its carbonyl group, or a peptide moiety
10 bonded at its terminal carbonyl group, and Y is alkyl,
alkoxy, amino, substituted amino, hydroxyl, a peptide
moiety bonded at its terminal amino group or an amino
acid residue bonded at its amino group; and R_5 is H,
alkyl, aryl, -NH-W', or -C(=O)-Y', where W' is H, alkyl,
15 aryl, an amino acid residue bonded at its carbonyl group,
or a peptide moiety bonded at its terminal carbonyl
group, and Y' is alkyl, alkoxy, amino, substituted amino,
hydroxyl, a peptide moiety bonded at its terminal amino
group, or an amino acid residue bonded at its amino
20 group.

An alkyl group is a branched or unbranched C_1 to
C_6 alkyl or benzyl, and aryl is a substituted or
unsubstituted C_6 to C_{14} carbocyclic or heterocyclic
aromatic having one or two rings. A substituted aryl is
25 an aryl bearing one or more C_1 to C_6 substituents
including alkyl, alkoxy, or acyl groups, or halogen,
hydroxy, amino, or amido groups. A substituted amino is
an amino group having one or two branched or unbranched C_1
to C_6 alkyl or benzyl groups. A Michael acceptor (e.g.,
30 $H_2C=CH-C(=O)-$, and related unsaturated groups) is a group
that undergoes conjugate addition with a nucleophile.

Preferably, R_1 is halogen, alkyl sulfonate,
carboxylate, or aryl sulfonate and R_2 is hydroxy, amino,
alkylamino, ammonium, or alkyl ammonium, or together R_1
35 and R_2 are O, $NH_2 \cdot$, or NH; X is a C_2 to C_4 alkyl; R_3 is H;

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R_4 is -NH-W, where W is an amino acid residue bonded at its carbonyl group or a peptide moiety bonded at its terminal carbonyl group; and R_5 is a -C(=O)-Y', where Y' is a peptide moiety bonded at its terminal amino group or

5   an amino acid residue bonded at its amino group.

In preferred embodiments, W is an amino acid residue bonded at its carbonyl group and Y' is a peptide moiety bonded at its terminal amino group. In other preferred embodiments, R_1 is alkyl sulfonate and R_2 is an

10 ammonium, or together R_1 and R_2 are O, or NH; X is C_4 alkyl; and the amino acid residue is a glycine residue.

In preferred embodiments, a portion of the peptide moiety is identical to a portion of a selected peptide or an antigenic peptide. The peptide moiety can preferably

15 contain between 2 and 10 amino acid residues. Most preferably, the compound is G(az)IDKPILK or G(az)AFVTIGK, where (az) is an unnatural amino acid residue where the side chain is a C_6 alkyl having a terminal aziridine group.

20   A further aspect of this invention relates to a method of forming a ligand-protein complex. The method includes the steps of providing a protein having an arginine-specific binding region, the binding region including a nucleophilic residue, and adding a ligand to

25 bind with the protein at the arginine-specific binding region, the ligand having a moiety which reacts with the nucleophilic residue. The moiety of the ligand and the nucleophilic residue of the protein form a covalent bond between the ligand and the protein.

30   In preferred embodiments, the arginine-specific binding region includes a thiol, a thiol and a carboxyl, or two carboxyl groups, and the moiety is halogen, haloalkyl, haloalkyl carbonyl, alkyl sulfonate, aryl sulfonate, carboxylate, a Michael acceptor, aziridine, or

35 epoxide. Most preferably, the arginine-specific binding

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region includes a thiol and a carboxyl and the moiety is aziridine, or epoxide.

In other preferred embodiments, the step of providing the protein includes the step of altering a binding region of the protein to introduce the arginine-specific binding region.

Another aspect of this invention relates to a covalently linked ligand-protein complex including a protein having an arginine-specific binding region and a ligand covalently bonded to the binding region of the protein, said ligand having the formula

$$\begin{array}{c} R_2 \\ \diagdown \\ \diagup \\ \diagdown R_1 \\ \bigg| \\ X \diagdown \diagup R_3 \\ R_4 \diagup \diagdown R_5 \end{array}$$
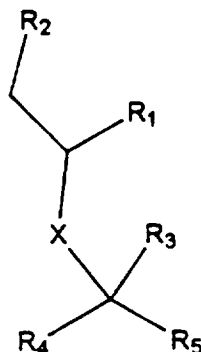
where one of R_1 and R_2 is hydroxy, alkyl, alkyl carbonyl, amino, alkyl amino, ammonium, or alkyl ammonium, and the other of R_1 and R_2 is the covalent bond to the protein; X is C_1 to C_6 alkyl, substituted aryl, or deleted; R_3 is H, alkyl, or aryl; R_4 is alkyl, aryl, -NH-W, or -C(=O)-Y, where W is H, alkyl, aryl, an amino acid residue bonded at its carbonyl group, or a peptide moiety bonded at its terminal carbonyl group, and Y is alkyl, alkoxy, amino, substituted amino, hydroxyl, a peptide moiety bonded at its terminal amino group or an amino acid residue bonded at its amino group; and R_5 is H, alkyl, aryl, -NH-W', or -C(=O)-Y', where W' is H, alkyl, aryl, an amino acid residue bonded at its carbonyl group, or a peptide moiety bonded at its terminal carbonyl group, and Y' is alkyl, alkoxy, amino, substituted amino, hydroxyl, a peptide

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moiety bonded at its terminal amino group, or an amino
acid residue bonded at its amino group.

　　　In preferred embodiments, the arginine-specific
binding region includes a thiol, a thiol and carboxyl, or
5　two carboxyl groups; R_1 is the covalent bond between the
ligand and the protein; R_2 is hydroxy, alkyl, alkyl
carbonyl, amino, alkyl amino, ammonium, or alkyl
ammonium; R_3 is H; and X is a C_2 to C_4 alkyl. Most
preferably, R_2 is the covalent bond between the ligand and
10　the protein (i.e., a thioether or ester linkange).

　　　The invention includes one or more of the
following advantages. The ligand selectively reacts with
groups in the binding region of the protein. Thus,
undesired reactions outside of the binding region are
15　unlikely to occur. In addition, the covalent nature of
the linkage between the ligand and the protein changes
the kinetics of ligand binding dramatically by making the
ligand binding essentially irreversible.

　　　A "selected peptide" is any peptide or variant
20　thereof that is associated with an undesired physical
condition, such as, but not limited to, an immune system-
selective peptide (i.e., a peptide that induces an immune
response). Note that "an antigenic peptide" mentioned
herein can be either a naturally occurring peptide or a
25　variant thereof (e.g., containing an acetylated alpha-
amino group, an amidated alpha-carboxyl group, a D-
and/or beta- or gamma-amino acid residue, or any suitable
organic moiety); indeed, there are only two criteria,
i.e., it is a peptide and it binds to a class I or class
30　II MHC molecule in the manner described in Stern, L.J. &
Don C. Wiley, Structure (1994) 2, 245-251.

　　　Other features or advantages of the present
invention will be apparent from the following drawings
and detailed description of representative examples.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic diagram depicting the synthesis and structure of a compound according to the invention.

Fig. 2 is a drawing of a model of the P2 pocket of HLA-B27 binding arginine (Fig. 2A), a model of an aziridine-containing ligand (Fig. 2B) and a covalently bonded residue (Fig. 2C).

Fig. 3 is a graph of three HPLC runs depicting the stability of the aziridine ligand to free thiols in solution.

Fig. 4 is an image of an SDS-PAGE (15%) of HLA-B27 complexed with the aziridine-containing peptide (G(az)IDKPILK) (lane 1) and the control peptide (GRIDKPILK) (lane 2).

DETAILED DESCRIPTION OF THE INVENTION

Ligands bind to proteins according to particular kinetic profiles. Short peptides containing arginine at peptide position 2 (P2) bind to arginine-specific binding regions of class I major histocompatibility complex (MHC) glycoproteins, such as HLA-B27. The HLA-B27/peptide complex is recognized by particular T cells. This recognition leads to both the development of the repertoire of T cells in the cellular immune system and the activation of cytotoxic T cells. The reversible kinetics of binding lead to release of the ligand from the binding site, thereby affecting the recognition of the ligand-protein complex. By changing the kinetics of the reversible binding, it is possible to alter the response to the cellular immune system. Reactive ligands can be used to change the kinetics of binding by reacting with the protein when bound.

Suitable reactive ligands for changing the kinetics of binding with protein substrates are compounds

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of the formula I. Each of R_1 and R_2, independently, is
hydroxy, halogen, haloalkyl, haloalkyl carbonyl, alkyl
sulfonate, aryl sulfonate, carboxylate, a Michael
acceptor, amino, alkylamino, ammonium, or alkyl ammonium.

5 Alternatively, together R_1 and R_2 are O, NH, NH_2^+, or NZ,
where Z is alkyl, or aryl, thus forming an epoxide,
aziridine or substituted aziridine ring. X, a spacer
group, is C_1 to C_6 alkyl, substituted aryl, or deleted.
R_3 is H, alkyl, or aryl. R_4 is alkyl, aryl, -NH-W, or

10 -C(=O)-Y, where W is H, alkyl, aryl, an amino acid
residue bonded at its carbonyl group, or a peptide moiety
bonded at its terminal carbonyl group, and Y is alkyl,
alkoxy, amino, substituted amino, hydroxyl, a peptide
moiety bonded at its terminal amino group or an amino

15 acid residue bonded at its amino group. R_5 is H, alkyl,
aryl, -NH-W', or -C(=O)-Y', where W' is H, alkyl, aryl,
an amino acid residue bonded at its carbonyl group, or a
peptide moiety bonded at its terminal carbonyl group, and
Y' is alkyl, alkoxy, amino, substituted amino, hydroxyl,

20 a peptide moiety bonded at its terminal amino group, or
an amino acid residue bonded at its amino group.

In particular, one of R_1 and R_2 is a reactive
group that will react with a nucleophile (e.g., -SH, $-NH_2$,
-OH, or $-COO^-$). Thus, one of the groups is a leaving

25 group or contains a leaving group. The leaving group can
be a halogen, haloalkyl, haloalkyl carbonyl, alkyl
sulfonate, aryl sulfonate, alkyl carboxylate, ammonium,
alkyl ammonium, epoxide, aziridine, or substituted
aziridine, where the alkyl group is a branched or

30 unbranched C_1 to C_6 alkyl or benzyl and aryl is a
substituted or unsubstituted C_6 to C_{14} carbocyclic or
heterocyclic aromatic having one or two rings.
Alternatively, R_1 and R_2 is a Michael acceptor (e.g.,
$H_2C=CH-C(=O)-$, and related unsaturated groups) which

35 undergoes conjugate addition with a nucleophile. The

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reactions between the group at R_1 or R_2 and the
nucleophile take place at a binding region of a protein.
        The compounds of the invention can be used to
prepare reactive ligands, or are themselves reactive
5   ligands that can be used to make covalent ligand-protein
complexes *in vitro* or *in vivo*. At R_4 and R_5, is a
reactive group or the peptide moiety or amino acid
residue is ultimately a group that binds selectively to a
protein. In particular, a portion of the peptide moiety,
10 the amino acid residue, or a combination thereof is a
selected peptide or an antigenic peptide, which can be
either a naturally occurring peptide or a variant thereof
(e.g., containing an acetylated alpha-amino group, an
amidated a-carboxyl group, a D- and/or beta- or gamma-
15 amino acid residue, or any suitable organic moiety). In
particular, a portion of the peptide moiety, the amino
acid residue, or their combination, is a peptide and it
binds to a class I or class II MHC molecule in the manner
described in Stern, L.J. & Wiley, D.C., Structure (1994)
20 **2**, 245-251. For example, short peptides containing
arginine at peptide position 2 (P2) bind to arginine-
specific binding regions of class I major
histocompatibility complex (MHC) glycoproteins, such as
HLA-B27. The HLA-B27/peptide complex is recognized by
25 particular T cells.
        Fig. 1 is a schematic diagram depicting the
synthesis and structure of a compound according to the
invention. Abbreviations are Boc, butoxycarbonyl; Ph,
phenyl; DEAD, diethyl azodicarboxylate; THF,
30 tetrahydrofuran; DMS, dimethyl sulfide; TFA,
trifluoroacetic acid; Gly, glycine; BOP, benzotriazol-1-
yloxy-tris(dimethylamino)phosphonium hexafluorophosphate;
HOBT, 1-hydroxybenzotriazole; DIEA, diisopropyl
ethylamine; MCPBA, *m*-chloroperoxybenzoic acid; EtOAc,
35 ethyl acetate; Ms, mesyl; Me, methyl; HBTU 2-(1H-

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benzotriazol-1-yl)-1,1,3,3-tetramethyluronium
hexafluorophosphate; and DMF, dimethylformamide.

The first two steps shown in Fig. 1 can be
modified to prepare variants of Compound 1, where the
5   length of the alkenyl group can be varied, or other
functionalities can be added using ordinary synthetic
methods. Boc-amine (Compound 1) was prepared according
to the methods described in Arnold, L.D., Kalantar, T.H.
& Vederas, J.C. (1985) J. Am. Chem. Soc. 107, 7105-7109,
10   and Arnold, L.D., Drover, J.C. G. & Vederas, J.C. (1987)
J. Am. Chem. Soc. 109, 4649-4659 and the dipeptides were
prepared according to the methods described in Arnold,
L.D., Kalantar, T.H. & Vederas, J.C. (1985) J. Am. Chem.
Soc. 107, 7105-7109, and Arnold, L.D., Drover, J.C.G. &
15   Vederas, J.C. (1987) J. Am. Chem. Soc. 109, 4649-4659.
The olefinic group of Compound 1 can be transformed by
ordinary methods, e.g., addition reactions. The
subsequent reactions of Compound 1 and its analogues can
be carried out to link other natural or unnatural amino
20   acid residues or peptide moieties.

A ligand-protein complex is formed by exposing a
protein having an arginine-specific binding region which
includes a nucleophilic residue to a ligand which binds
with the protein at the arginine-specific binding region.
25   The ligand is a compound of the invention, thus having a
moiety which reacts with the nucleophilic residue. Upon
binding, the reactive moiety of the ligand and the
nucleophilic residue of the protein form a covalent bond
between the ligand and the protein, resulting in a
30   covalently linked ligand-protein complex where the
protein and ligand become linked at R_1 or R_2, changing the
kinetics of release of the ligand from the binding site.
As an example, this influences the recognition of the
ligand-protein complex by the cellular immune system,
35   making it possible to alter immune responses.

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Cytotoxic T-lymphocytes (CTLs) recognize antigens
as short peptides bound to MHC class I molecules that
consist of a heavy chain (H) and β_2-microglobulin (β_2-m).
For a review of CTLs, see Germain, R.N. & Margulies, D.H.
5 (1993) A. Rev. Immun. **11**, 403-450. The structure of HLA-
B27 is known. See, Madden, D.R., Gorga, J.C.,
Strominger, J.L. & Wiley, D.C. (1991) Nature **353**, 321-
325, Gorga, J.C., Madden, D.R., Prendergast, J.K., Wiley,
D.C. & Strominger, J.L. (1992) Proteins **12**, 87-90, and
10 Madden, D.R., Gorga, J.C., Strominger, J.L. & Wiley, D.C.
(1992) Cell **70**, 1035-1048.

The involvement of HLA-B27 has been implicated in,
for example, the autoimmune disorder ankylosing
spondylitis. See, Brewerton, D.A., Caffrey, M., Hart,
15 F.D., James, D.C.O., Nicholls, A. & Sturrock, R.D. (1973)
Lancet **1**, 904-907, Schlosstein, L., Terasaki, P.I.,
Bluestone, R. & Pearson, C.M. (1973) N. Engl. J. Med.
288, 704-706, and Hammer, R.E., Maika, S.D., Richardson,
J.A., Tang, J.-P. & Taurog, J.D. (1990) Cell **63**, 1099-
20 1112. The autoimmune response involves binding of an
autoantigen peptide by HLA-B27. Endogenous and viral
peptides that bind to HLA-B27 have an arginine in their
P2 position, as described by Jardetzky, T.S., Lane, W.S.,
Robinson, R.A., Madden, D.R. & Wiley, D.C. (1991) Nature
25 **353**, 326-329. The d-guanidinium group of the arginine is
bound within a deep pocket where four polymorphic
residues project toward it. See, Madden, D.R., Gorga,
J.C., Strominger, J.L. & Wiley, D.C. (1991) Nature **353**,
321-325, and Madden, D.R., Gorga, J.C., Strominger, J.L.
30 & Wiley, D.C. (1992) Cell **70**, 1035-1048.

Activation of T cell receptors on CD8+ and CD4+ T
cells requires the recognition of antigenic peptides
bound in the groove of MHC molecules on the surface of
antigen-presenting cells. Antigenic peptides bind in an
35 extended conformation to class I and class II MHC

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molecules. X-ray crystallography studies have revealed
that for all class I MHC complexes, the peptide N- and C-
termini form hydrogen bonds with conserved MHC amino acid
residues located at both ends of the binding site, some
5 peptide side chains are buried in deep pockets along the
binding site, and the central region of the peptide arch
away from the floor of the binding site with side chains
generally pointing toward T cell receptors. Antigenic
peptides of 8- to 10-amino acids long have been shown to
10 adopt this mode of binding. A decamer has been shown to
extend out of the binding groove at the C-terminus and an
octamer has been shown to preferentially leave the N-
terminal end of the binding site unoccupied. On the
basis of these observations, other modes of binding can
15 be described: extension of a decamer out of the N-
terminal end of the binding site and binding of an
octamer preferentially at the N-terminal end of the
groove leaving the C-terminal end of the groove
unoccupied.

20   In contrast to class I MHC molecules, class II MHC
molecules bind to longer antigenic peptides. This
difference in length can be ascribed to structural
differences between the two binding sites and the
position of key MHC amino acid residues that interact
25 with the peptide backbone. The few X-ray structures of
class II MHC complexes that have been determined so far
indicate that the antigenic peptide is stretched out
along most of its length and adopts a bound conformation
characteristic of a type II polyproline helix. The
30 peptide's N- and C-termini extend out of the binding
groove. Side chains of some amino acid residues bind
specifically in pockets made of polymorphic MHC amino
acid residues while other side chains, spaced
approximately three amino acid residues apart, point away
35 from the binding site.

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Interaction of T cell receptors with both types of MHC complexes involves amino acid residues on both the peptide and MHC molecules. For class I MHC complexes, amino acid residues from the central portion of the

5 peptide, where side chains are most exposed, play a more crucial role in this recognition event, whereas for class II MHC complexes, amino acid residues having side chains pointing up are found along the entire sequence. In designing peptides of this invention, it is preferable to

10 select as anchor amino acid residues those residues with side chains pointing toward T cell receptors.

References for the structures of class I MHC molecules include: Madden, D.R., Garboczi, D.N. & Wiley, D.C. (1993) Cell **75**, 693-708; Bjorkman, P.J., Saper,

15 M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. & Wiley, D.C. (1987) Nature **329**, 506-512; Madden, D.R., Gorga, J.C., Strominger, J.L. & Wiley, D.C. (1991) Nature **353**, 321-325; Silver, M.J., Guo, H.-C., Strominger, J.L. & Wiley, D.C. (1992) Nature **360**, 367-369; Guo, H.-C.,

20 Jadertzky, T.S., Garrett, T.P.J., Lane, W.S., Strominger, J.L. & Wiley, D.C. (1992) Nature **360**, 364-366; and Madden, D.R., Gorga, J.C., Strominger, J.L. & Wiley, D.C. (1992) Cell **70**, 1035-1048. References for the structures of class II MHC molecules include Brown, J.H., Jardetzky,

25 T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1993) Nature **364**, 33-39; Stern L.J. and Wiley, D.C. (1994) Structure 2, 245-251; and Stern, J.L., Brown, J.H., Jardertzky, T.S., Gorga, J.C., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1994)

30 Nature **368**, 215-221.

According to the structural model of the binding site shown in Fig. 2A, His-9, Thr-24, Glu-45, and a bound water form a hydrogen bond network with the guanidinium group of the ligand. The P2 pocket of HLA-B27 forms a

35 planar network of hydrogen bonds to the guanidinium group

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of arginine at P2 of a bound peptide. Perpendicular to
the planar network of hydrogen bonds, the free thiol of
Cys-67 is separated from the guanidinium group by about
3.7 Å. The sulfhydryl group of Cys-67 is located
5 directly above the plane of the guanidinium group of the
ligand.

       The arrangement of protein side chains (amino acid
residues) suggested that it might be possible to induce
the pocket to act as an enzyme active site, by designing
10 a suitable substrate. A ligand was synthesized with a
reactive side chain designed to mimic arginine. Although
other reactive groups at this position can be used, an
aziridine is most preferred. The reactive ligand
contains an aziridine and binds covalently by reaction of
15 the aziridine upon binding in the arginine-specific P2
pocket of HLA-B27. A peptide was designed such that a
covalent bond with HLA-B27 will form between the ligand
and the protein spontaneously and selectively upon
binding. An aziridine-containing amino acid mimic of
20 arginine was selected (Fig. 2B). According to Fig. 2B,
the aziridine-containing ligand can bind in place of the
guanidinium group with the aziridine amine, forming a
hydrogen bonded salt bridge to Glu-45. In this model
with one diastereomer of the aziridine depicted, the free
25 thiol of Cys-67 is poised for in-line nucleophilic attack
of the aziridine group.

       Fig. 2C shows the expected geometry of the
covalent complex between Cys-45 and the aziridine-
containing peptide that results from the nucleophilic
30 attack of the thiol which opens the aziridine group. The
positively charged secondary amine of the aziridine ring
is expected to form a hydrogen-bonded charged-pair with
Glu-45 in the arginine-specific pocket, positioning the
eta-carbon of the aziridine ring for an in-line, ring-
35 opening attack by the thiolate of Cys-67 (Fig. 2C).

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Selectivity is expected from the polarizability of the aziridine by Glu-45 and the propinquity of the Cys-67 thiol to the bound aziridine ring.

Using tryptic digestion followed by mass
5   spectrometry and amino acid sequencing, the aziridine-containing ligand is shown to alkylate specifically cysteine 67 of HLA-B27. Neither free cysteine in solution nor an exposed cysteine on a class II MHC molecule can be alkylated, showing that specific
10 recognition between the anchor side-chain pocket of an MHC class I protein and the designed ligand (propinquity) is necessary to induce the selective covalent reaction with the MHC class I molecule.

The aziridine within the ligand was cyclized from
15 the mesylate in solution, before addition to the folding buffer containing recombinantly expressed, purified HLA-B27 heavy chain and β_2-m.

In general, conservative residue substitutions in the middle portion of antigenic peptides of T cells are
20 introduced to selectively inhibit activation of T cells without significantly altering binding affinity to the relevant MHC molecules. See, Sloan-Lancaster, J., Evavold, B.D. & Allen, P.M. (1993) Nature 363, 156-159; De Magistris, M.T., Alexander, J., Coggeshall, M.,
25 Altman, A., Gaeta, F.C.A., Grey, M.H. & Sette, A. (1992) Cell 68, 625-634; and Alexander, J., Snoke, K., Ruppert, J., Sidney, J., Wall, M., Southwood, S., Oseroff, C., Arrhenius, T., Gaeta, F.C.A., Colon, S.M., Grey, H.M. & Sette, A. (1993) J. Immunol. 150, 1-7. Activation and
30 regulation of immune responses (e.g., autoimmune responses, responses to viral infection, and responses to cancerous cells) can be influenced by the compounds of the invention, since the compounds bind to specific protein binding pockets irreversibly. It is possible to
35 activate T cell responses by treatment with a preformed

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ligand-protein complex or by treatment with the reactive
ligand or a precursor thereof.

Without further elaboration, it is believed that
one skilled in the art can, based on the description
5   herein, utilize the present invention to its fullest
extent. All publications disclosed herein are
incorporated by reference. The specific examples set
forth below are, therefore, to be construed as merely
illustrative, and not limitative of the remainder of the
10   disclosure in any way whatsoever.

Ligand Synthesis

Nonamer peptides containing the aziridine were
synthesized by coupling the dipeptide to synthetic
heptamers corresponding to the seven C-terminal amino
15   acids of two peptides known to bind to HLA-B27, GRIDKPILK
and GRAFVTIGK. See, Jardetzky, T.S., Lane, W.S.,
Robinson, R.A., Madden, D.R. & Wiley, D.C. (1991) Nature
353, 326-329. The peptide sequence of the ligand shown
in Fig. 1 and described here is based upon a ribosomal
20   peptide GRIDKPILK. Other peptide sequences and analogues
can be substituted in the scheme shown in Fig. 1 to
afford other reactive ligands. Diastereomers of the
aziridine were unresolved. All reactions followed
standard laboratory practices. All intermediates and
25   products were characterized by ^{1}H NMR, ^{13}C NMR, IR and
fast atom bombardment mass spectrometry (FAB MS).

Boc-amine (Compound 1). Boc-amine Compound 1 was
prepared according to the methods described in Arnold,
L.D., Kalantar, T.H. & Vederas, J.C. (1985) J. Am. Chem.
30   Soc. **107**, 7105-7109, and Arnold, L.D., Drover, J.C.G. &
Vederas, J.C. (1987) J. Am. Chem. Soc. **109**, 4649-4659.
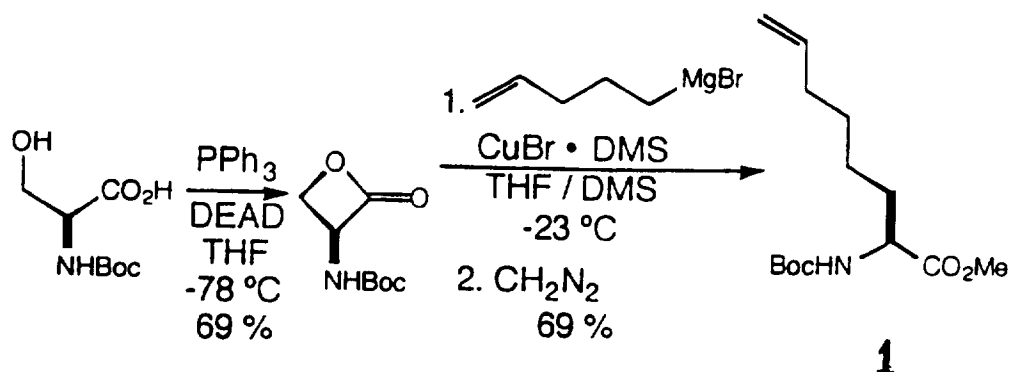
**Boc-Glycine-(2-amino-hept-6-enoic acid) dipeptide
methyl ester (Compound 2).** To a solution of Compound 1
(211 mg, 0.911 mmol) in 5.0 mL of CH₂Cl₂ was added 5.0 mL
of trifluoroacetic acid. The mixture was swirled,

5   allowed to stand for 20 minutes, diluted with 4 mL of
toluene, and concentrated *in vacuo* to afford the crude
deprotected amine. The deprotected amine was dissolved
in 5.0 mL of CH₂Cl₂. Diisopropyl ethylamine (0.476 mL,
0.273 mmol) was added to the solution containing the

10 deprotected amine, followed by Boc-glycine (175 mg, 1.00
mmol) and benzotriazol-1-yloxy-
tris(dimethylamino)phosphonium hexafluorophosphate (442
mg, 1.00 mmol). The mixture was stirred for 6 hours and
then quenched by adding 5 drops isopropyl amine. The

15 mixture was concentrated *in vacuo* and chromatographed
(40% ethyl acetate:hexanes) to give 178 mg (0.542 mmol,
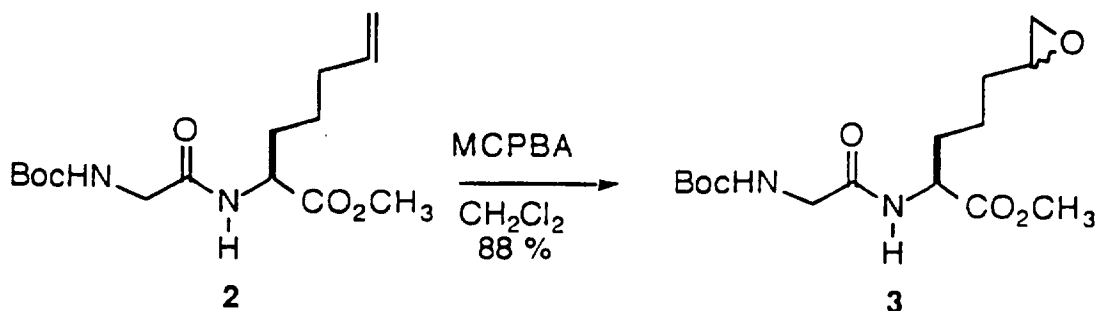59%) of Compound 2.
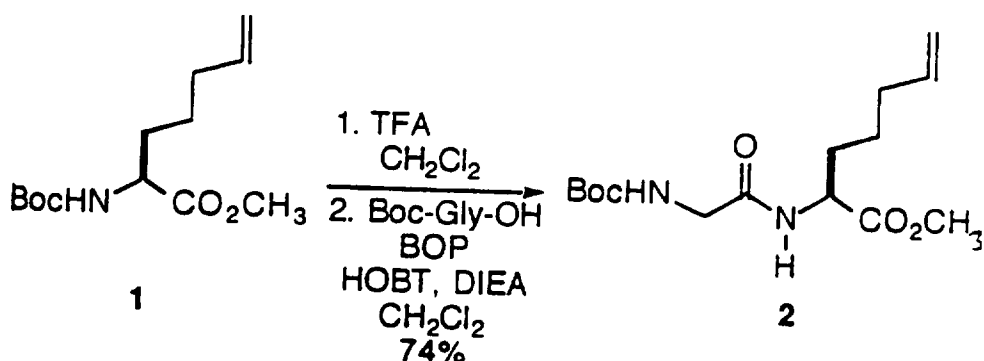
Epoxy Dipeptide (Compound 3). To a solution of
Compound 2 (145 mg, 0.441 mmol) in 7.0 mL of CH₂Cl₂ was

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added *m*-chloroperoxybenzoic acid (91.4 mg, 0.529 mmol).
The mixture was stirred for 9 hours and then diluted with
40 mL of ethyl acetate, washed with 10% aqueous NaS_2O_5 (20
mL), washed with saturated $NaHCO_3$ solution (2 x 20 mL),
5 washed with brine (10 mL), dried over $MgSO_4$, concentrated
in vacuo, and chromatographed (60% ethyl acetate:hexanes)
to give 91.0 mg (0.264 mmol, 50%) of epoxy dipeptide
Compound 3.

       Compound 3 was characterized by: IR (Film): n
10 2934, 1744, 1719, 1678, 1524, 1456, 1437, 1412, 1391,
1368, 1250, 1211, 1171, 1051, 1030, 939, 864, 835, 781,
615 cm^{-1}; ^{1}H NMR ($CDCl_3$): d 1.41 (s), 1.48 (m), 1.52 (m),
1.62 (m), 1.82 (m), 2.40 (q), 2.69 (t), 2.84 (t), 3.69
(s), 3.78 (m), 4.57 (q), 5.26 (d), 6.71; and ^{13}C NMR
15 ($CDCl_3$): d 24.82, 25.32, 25.38, 28.16, 31.94, 44.10,
46.77, 51.87, 51.96, 52.20, 79.92, 156.02, 169.41,
172.59.

Azido Dipeptide (Compound 4). To a solution of
Compound 3 (66.0 mg, 0.191 mmol) in 5.0 mL of a 9:1
20 methanol:water mixture was added NH_4Cl (80 mg, 1.49 mmol)
and NaN_3 (300 mg, 4.61 mmol). The mixture was heated to
reflux and stirred for 2 hours. The mixture was then
cooled to 25°C, diluted with 40 mL of ethyl acetate,
washed with water (2 x 20 mL), washed with brine (10 mL),
25 dried over $MgSO_4$, and concentrated *in vacuo* to give 71.0
mg (0.191 mmol, 100%) of azido dipeptide Compound 4.

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3 → **4**
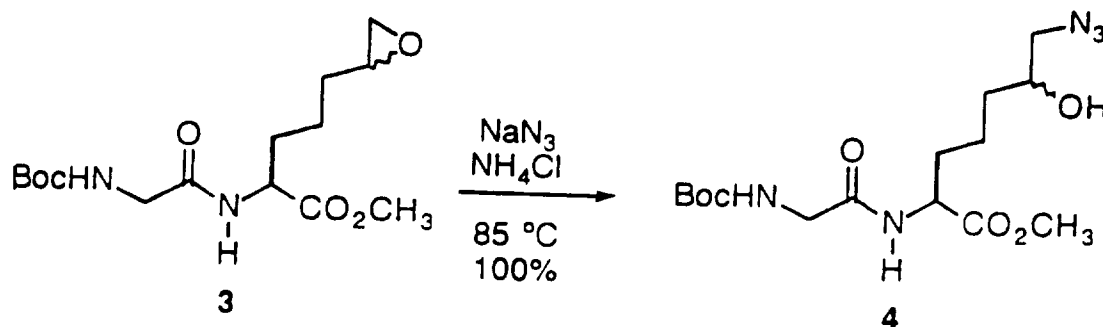
Compound 4 was characterized by: IR (Film): n 3328, 2976, 2936, 2865, 2101, 1742, 1671, 1524, 1439, 1393, 1368, 1279, 1252, 1217, 1167, 1051, 1030, 941, 864, 632 cm^{-1}; ^{1}H NMR (CDCl$_3$): d 1.28 (m), 1.38 (s), 1.61 (m), 1.79 (m), 1.82 (d), 3.17 (m), 3.24 (m), 3.66 (s), 3.74 (m), 4.52 (m), 5.49 (t), 6.90 (d); and ^{13}C NMR (CDCl$_3$): d 24.60, 24.72, 24.82, 28.19, 32.01, 33.80, 44.09, 51.75, 51.89, 52.34, 56.86, 64.84, 70.12, 70.38, 80.18, 156.16, 169.59, 172.72.

Boc-amine Dipeptide (Compound 5). A 5% solution of Pd on carbon in ethyl acetate (6.0 mL) was stirred at room temperature, under atmospheric pressure of hydrogen gas, for one hour. Addition of Compound 4 (82.3 mg, 0.24 mmol) and Boc-anhydride (104.3 mg, 0.48 mmol) required 1.0 mL ethyl acetate, followed by two ethyl acetate washes of 1.0 mL each. After stirring 12 hours, and Celite was added. The solution was filtered and concentrated *in vacuo*. The residue was purified by flash chromatography, running neat ethyl acetate, to yield 86.6 mg (0.188 mmol, 78%) of Boc-amine dipeptide Compound 5.


4 → **5**
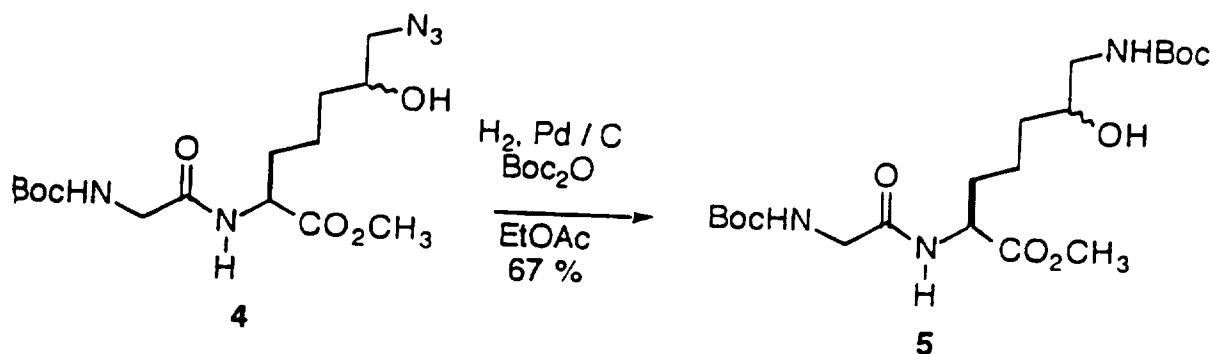
- 20 -

Compound 5 was characterized by: IR (Film): n
3328, 2978, 2934, 2865, 1744, 1686, 1524, 1456, 1439,
1412, 1393, 1368, 1275, 1250, 1169, 1051, 1030, 864, 781,
735 cm⁻¹; ¹H NMR (CDCl₃): d 1.39 (s), 1.41 (s), 1.48 (t),
5   1.64 (m), 1.81 (m), 2.92 (m), 3.22 (br s), 3.60 (d), 3.69
(s), 3.77 (d), 4.55 (m), 5.13 (br s), 5.43 (d), 6.86 (br
s); and ¹³C NMR (CDCl₃): d 24.56, 24.73, 24.95, 28.08,
31.71, 33.90, 43.82, 46.35, 51.75, 52.15, 70.38, 70.63,
79.06, 79.84, 156.11, 156.56, 169.76.

10   **Mesylate Dipeptide Methyl Ester (Compound 6).**
Compound 5 (96.0 mg, 0.208 mmol) was weighed into a pear
shaped flask and azeotroped with xylenes, before being
dissolved in dry methylene chloride (3.5 mL, 0.06 M).
After cooling to 0°C, mesyl anhydride (174.2 mg, 0.312
15   mmol) was added. Approximately 20 minutes later, the
reaction was complete and was quenched by the addition of
water. Excess ethyl acetate was added. The organic
phase was washed with 5% aqueous HCl, saturated aqueous
sodium bicarbonate, and brine. Following drying over
20   MgSO₄, the mesylate dipeptide methyl ester Compound 6 was
isolated *in vacuo*.

5

6
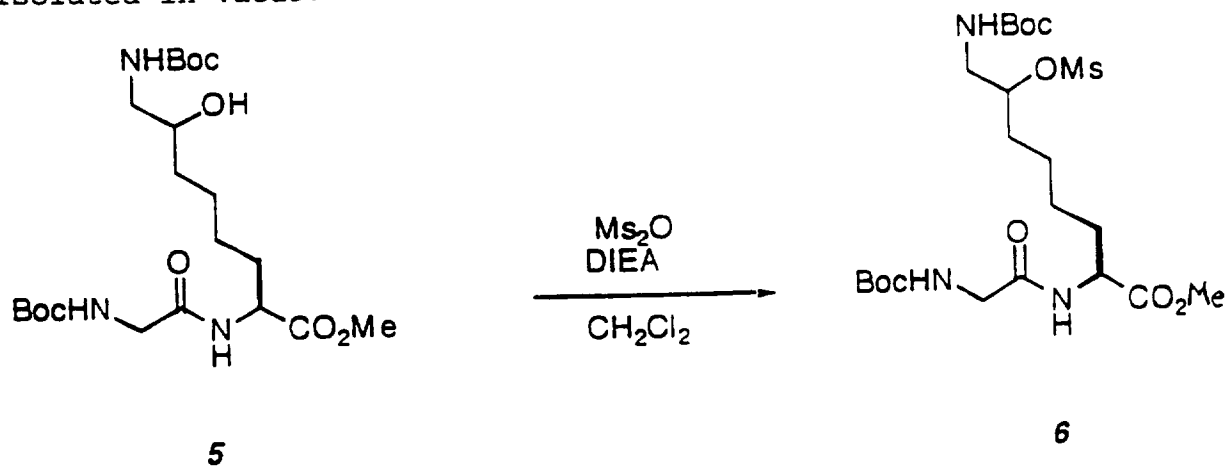
Mesylate Dipeptide Carboxylic Acid (Compound 7).
Compound 6 was dissolved in methanol (0.56 mL) and
tetrahydrofuran (2.25 mL), and cooled to 0°C. An aqueous

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solution of 1N LiOH (0.56 mL) was added, and the reaction was stirred for half an hour. The reaction was quenched by the addition of excess ethyl acetate and 1N aqueous HCl (0.59 mL). The organic phase was isolated *in vacuo*
5 and azeotroped with xylenes to afford the mesylate dipeptide carboxylic acid Compound 7.

LiOH
H_2O / MeOH / THF
───────────►
0 °C
65 %

6

7
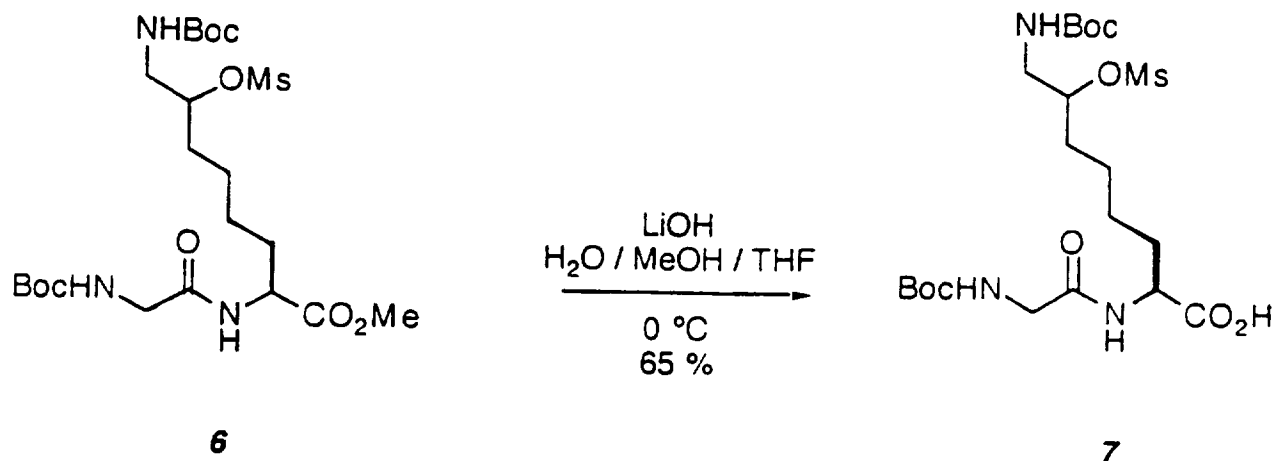
Mesylate Polypeptide (Compound 8). Compound 7, prepared in the step above, was added in N-methyl pyrrolidone to Wang resin coupled to the sequence
10 H_2N-IDKPILK. 1-Hydroxybenzotriazole (61.2 mg, 0.4 mmol), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (83.4 mg, 0.22 mmol), and Hunig's base (0.061 mL, 0.47 mmol) were added, and the resin was shaken for one hour at room temperature to give the
15 resin-bound mesylate polypeptide. The polypeptide was cleaved from the resin using 95% trifluoroacetic acid in water to afford the mesylate polypeptide Compound 8. Compound 8 was purified by reverse phase HPLC, running a gradient from 85:15 to 50:50 (water with 0.1%
20 trifluoroacetic acid:methanol). Mass spectrometry confirmed the identity of Compound 8 (MH⁺ 1133).

7
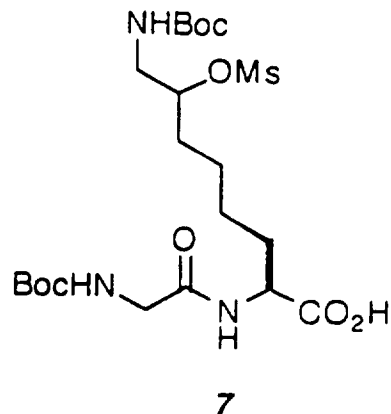
1. H₂N-IDKPILK-Resin
   HBTU / HOBT
   DIEA
   DMF

2. 2. 95 % TFA

8
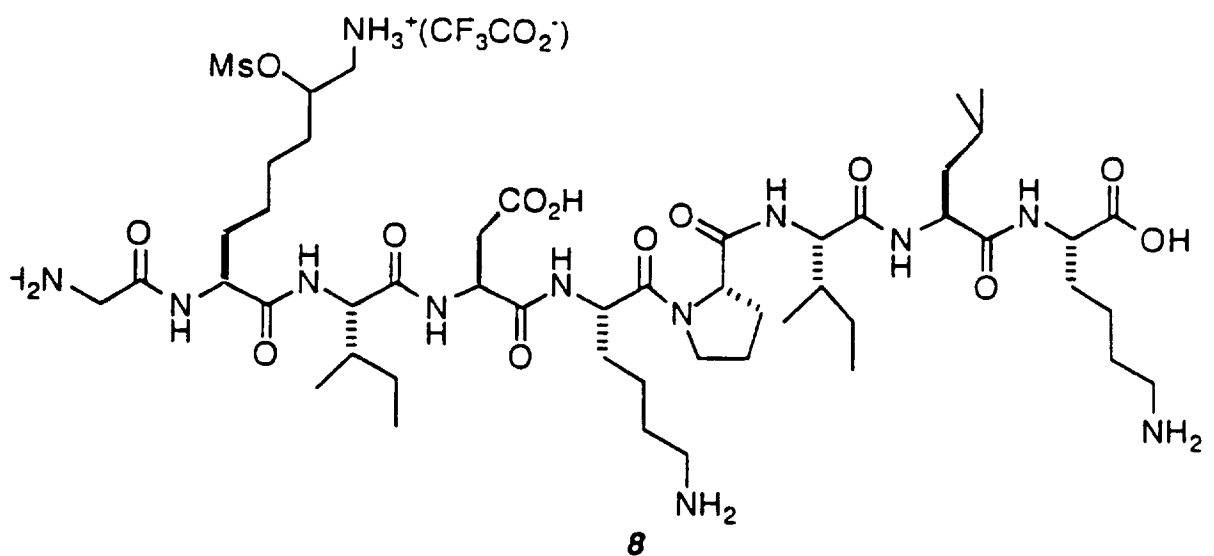
Aziridine Polypeptide (Compound 9). Compound 8
was treated with sodium hydroxide (10 equivalents at room
temperature for half an hour), or lithium hydroxide in
methanol, to afford aziridine polypeptide Compound 9.
5 Mass spectrometry confirmed the identity of Compound 9
(MH⁺ 1055). Additionally, Compound 9 was subjected to

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Edman degradation and microsequencing, which confirmed
its composition to be G(az)IDKPILK, where (az) is the
unnatural aziridine amino acid residue.

     The polypeptide G(az)AFVTIGK was prepared using a
5 different resin-bound peptide. The aziridine within a
peptide was cyclized from the mesylate in solution before
addition to the folding buffer containing recombinantly
expressed, purified HLA-B27 heavy chain and β_2-m.

8

LiOH
MeOH

9
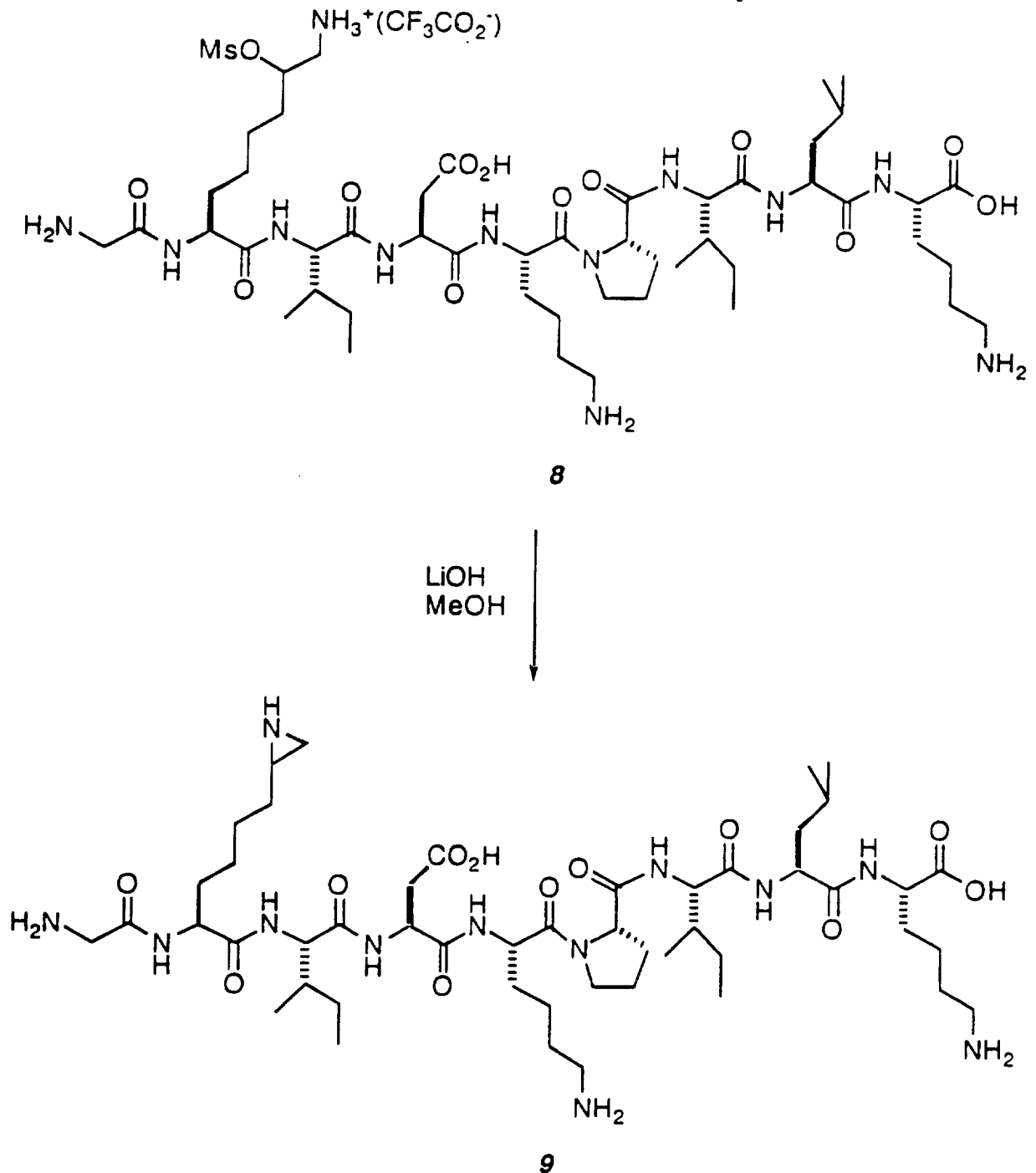
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<u>Ligand-Protein Complexation and Complexes</u>

Specificity of the Aziridine-containing Ligand for HLA-B27. The selectivity of the aziridine-containing peptide for the free thiol on HLA-B27 was demonstrated in
5   three ways.

First, neither the aziridine-peptide nor its mesylate precursor reacted with a 200 molar equivalent excess of the amino acid components of the P2 binding pocket: the free amino acids cysteine, threonine, or
10   glutamic acid. To 950 mL of HLA-B27 folding buffer, the unprotected amino acids cysteine, threonine and glutamic acid were added each to a final concentration of 20 mM. 220 mg of mesylate Compound 8 was made basic in 50 mL methanol by the addition of 2 mL 1N NaOH. After 15
15   minutes, the solution contained approximately equimolar distributions of mesylate precursor Compound 8 and aziridine-containing ligand Compound 9 (verified by reverse phase HPLC) and the solution was added to the folding buffer (both Compound 8 and Compound 9 have a
20   final concentration of 0.1 mM in the folding buffer), which was incubated at 10°C. After half an hour and approximately every 12 hours later, 100 mL of the folding buffer was removed and subjected to reverse phase HPLC, running a gradient of 100:0 to 40:60 (0.1%
25   trifluoroacetic acid in water:acetonitrile) over 30 minutes and monitoring at 214 nm.

Elution profiles of reverse phase HPLC after designated incubation times show constant levels of the aziridine and mesylate containing peptides, as shown in
30   Fig. 3. The reactivity was accessed in a time course experiment over three days. The stabilities of the ligands (Compound 8 and Compound 9) were established in aqueous solution over that time period, although a small fraction of the mesylate (Compound 8) gradually converted
35   to the aziridine (Compound 9). The aziridine-nonameric-

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peptide (G(az)IDKPILK) and its mesylate precursor are
stable and do not react with free cysteine, threonine, or
glutamic acid. The unprotected amino acids cysteine,
threonine, and glutamic acid (each present in 200-fold

5 molar excess) were incubated with equal molar amounts of
the aziridine-containing ligand and its mesylate
precursor in the HLA-B27 folding buffer containing
reduced glutathione (50-fold molar excess of free thiol).
Over three days, the sum of the mesylate precursor and

10 aziridine-containing ligand remained approximately the
same. No degradation or reaction products of the
aziridine or mesylate were observed. Peaks at 15.63 and
16.05 minutes correspond to the aziridine-containing
ligand and its mesylate precursor, respectively, other

15 peaks belong to the folding buffer. Peaks were assigned
by injection of standards or FAB-MS. The ordinate is
proportional to absorbance with arbitrary units.

        Second, the aziridine-containing-peptide failed to
alkylate the free cysteine residue in the peptide-binding

20 groove of the class II MHC molecule HLA-DR1. See, Stern,
L.J. & Wiley, D.C. (1992) Cell 68, 465-477. Following
standard peptide loading procedures for class II MHC
molecules described in Stern, L.J. & Wiley, D.C. (1992)
Cell 68, 465-477, empty, folded HLA-DR1 was incubated at

25 37°C with an eighteen fold molar excess of the aziridine-
containing-peptide, G(az)IDKPILK, Compound 9. Aliquots
of the mixture were removed daily for 3 days, denatured
in 6M guanidine hydrochloride, and tested for the
presence of free thiol using Ellman's reagent.

30   The portion of HLA-DR1 containing the only free
cysteine, Cys-30, does not bind arginine and lacks the
glutamate and threonine of the arginine binding pocket in
HLA-B27 (see, Stern, L.J., Brown, J.H., Jardetzky, T.S.,
Gorga, J.C., Urban, R.G., Strominger, J.L. & Wiley, D.C.

35 (1994) Nature 368, 215-221), nevertheless it represents a

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free thiol accessible on the surface of a protein. The
free thiol of Cys-30 remained unalkylated and its
concentration was constant, throughout the course of the
experiment. As a control the addition of one molar
5   equivalent of iodoacetic acid to the denatured HLA-DR1,
after 2 hours, was shown to decrease measured amounts of
free thiol by greater than 50%, demonstrating that the
response to Ellman's reagent was due to the presence of a
free thiol.

10   Third, the aziridine ligand alkylates Cys-67
during the folding of recombinant HLA-B27 despite a 5000
molar equivalent excess of cysteine in the glutathione of
the refolding buffer. Each complex was folded by
incubation at 10°C of HLA-B27 heavy chain (1 mM) and β_2-m
15   (2 mM) in a N_2(g) sparged solution of 20 mM TES (pH =
8.0), 0.2 M L-arginine (pH = 8.0), 2 mM Na_2EDTA, 5 mM
reduced glutathione, 0.5 mM oxidized glutathione, and 20
mM ligand. The ligand was described in Garboczi, D. N.,
Hung, D. T. & Wiley, D. C. (1992) Proc. Natl. Acad. Sci.
20   USA **89**, 3429-3433. Yields of the complex were sometimes
enhanced with rigorously anaerobic folding conditions.

Gel filtration HPLC with a running buffer of 150
mM NaCl, 50 mM Tris (pH = 8.0) was used to purify folded
HLA-B27 complexes from aggregates and excess β_2-m. Folded
25   complexes had a retention time consistent with their
molecular weight of 44 kD. Lower yields were
consistently obtained with the aziridine ligand Compound
9; typical yields, confirmed by amino acid analysis, were
approximately 60% or less than yields obtained with the
30   control peptide (GRIDKPILK). The lower yields might
result from less efficient hydrogen bonding to the
aziridine moiety compared to the planar guanidinium group
of the control peptide, or decreased reactivity of one
diastereomer of the aziridine ligand Compound 9 (kinetic
35   resolution).

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As in previous folding studies of recombinant
class I MHC molecules (Garboczi, D.N., Hung, D.T. &
Wiley, D.C. (1992) Proc. Natl. Acad. Sci. USA 89, 3429-
3433), the folding of HLA-B27 was dependent upon a
5 peptide ligand, in this case containing aziridine
(Compound 9). HLA-A2, an MHC molecule lacking
specificity for arginine at P2, failed to fold in the
presence of the aziridine-containing peptide, as
expected.

10 **Characterization of Covalent Ligand-Protein Bond.**
The aziridine-containing ligands were shown to form
covalent bonds with HLA-B27 by a shift in mobility of the
heavy chain on SDS-PAGE; reaction with Cys-67 was shown
by tryptic digestion, mass spectrometry (MS), and Edman
15 microsequencing.

In all preparations of the MHC class I complex
based on the aziridine-containing ligand Compound 9,
there was an additional SDS-PAGE band corresponding to
unmodified heavy chain. This band became more prominent
20 with decreasing concentrations of the aziridine-
containing ligand Compound 9 in the folding buffer and
low yields of MHC class I molecules, suggesting that the
band is due to empty MHC class I molecules.

Fig. 4. depicts the results of an SDS-PAGE (15 %)
25 of HLA-B27 complexed with the aziridine-containing
peptide (G(az)IDKPILK (lane 1) and the control peptide
(GRIDKPILK) (lane 2). The SDS-PAGE of HLA-B27 complexed
with aziridine-peptides revealed a mobility shift of the
heavy chains of the complex due to addition of the
30 aziridine-containing ligand (Fig.4, lane 1), when
compared to heavy chain from complexes with the same
peptides with arginine in the P2 position (Fig. 4, lane
2). The mobility of β_2-m remained unchanged.

In order to identify the alkylated amino acid, the
35 aziridine-modified complex was digested with trypsin and

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the fragments separated by reverse phase HPLC. The carboxyamidomethylated tryptic peptides were separated by narrow-bore HPLC using a Vydac C18 2.1 x 150 mm reverse phase column on a Hewlett-Packard 1090 HPLC/1040 diode

5 array detector. Putative difference peaks from the chromatogram were chosen based on differential UV absorbance at 210, 277, and 292 nm. These were further screened by matrix-assisted laser desorption time-of-flight spectrometry (MALDI-MS) on a Finnigan Lasermat

10 2000 (Hemel, England), and submitted to Edman microsequencing on an Applied Biosystems 477A (Foster City, CA). Details of strategies for the selection of peptide fractions and their microsequencing have previously been described by Lane, W.S., Galat, A.,

15 Harding, M.W. & Schreiber, S.L. (1991) J. Prot. Chem. 10, 151-160. Microcapillary HPLC/electrospray ionization/tandem mass spectrometry on a Finnigan TSQ7000 triple quadrupole mass spectrometer (San Jose, CA) as described in Hunt, D. F., Henderson, R.A., Shabanowitz,

20 J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A.L., Appella, E. & Engelhard, V.H. (1992) Science 255, 1261-1263.

The chromatogram of the proteolyzed fragments was compared with a tryptic digest of the MHC class I protein

25 formed with the arginine-containing parent-peptide (GRIDKPILK). Matrix-assisted laser desorption time-of-flight (MALDI) MS was used to screen rapidly putative difference peaks, which were deficient in UV absorbance at 277 and 292 nm (lacking tyrosine and tryptophan). A

30 fraction was found with a singly charged ion (M^{H+}) of the expected mass (m/z = 1759) of the tryptic fragment resulting from alkylation of Cys-67 by the aziridine-containing ligand. Edman microsequencing revealed this fraction to contain the peptide ligand in equimolar

35 amounts with the HLA-B27 heavy chain fragment containing

- 29 -

Cys-67. Consistent with the specific alkylation of Cys-67 by the aziridine, no PTH derivatives were observed at either the sequence position of the aziridine-amino acid in the peptide or Cys-67 in the heavy chain.

5   Microcapillary LC electrospray mass spectrometry (LC-ESIMS) (11, 12) further confirmed the expected mass of the aziridine-modified fragment (m/z=879.8 M^{2H++}).

    Stability of Covalent MHC-peptide Complexes. The thermodynamic stability of the class I MHC molecules from
10 mouse and human have been shown to depend on the sequence of the bound peptide. See, Bouvier, M. & Wiley, D.C. (1994) Science **265**, 398-402, Fahnestock, M.L., Tamir, I., Narhi, L.O. & Bjorkman, P.J. (1992) Science **258**, 1658-1662, and Fahnestock, M.L., Johnson, J.L., Renny Feldman,
15 R.M., Tsomides, T.J., Mayer, J., Narhi, L.O. & Bjorkman, P.J. (1994) Biochemistry **33**, 8149-8158. The thermal transition of the HLA-B27 complexes was measured using circular dichroism (CD).

    An Aviv 62DS (Lakewood, NJ) equipped with
20 thermoelectric temperature controller was used to obtain thermal denaturation curves in triplicate, from 25-95°C, as described in Bouvier, M. & Wiley, D.C. (1994) Science **265**, 398-402. The addition of 50 mM NaCl to the typical 10 mM MOPS (pH = 8.0) buffer was found to prevent
25 aggregation of HLA-B27. The additional salt had no effect on the measured melting temperature (T_m) of the HLA-A2 complex formed with a hepatitis B-derived peptide.

    The covalent complexes of HLA-B27 with two aziridine-containing-peptides, G(az)IDKPILK (Compound 9)
30 and G(az)AFVTIGK, both denature at approximately the same temperature, 62°C. G(az)AFVTIGK was prepared by coupling a different peptide to Compound 8 The non-covalent complexes of HLA-B27 with the cognate peptides (GRIDKPILK and GRAFVTIGK) melted at 72°C and 60°C, respectively.

- 30 -

The data indicate that covalently bound peptides
with an aziridine replacing arginine stabilize HLA
molecules approximately like non-covalently bound
peptides, in one case stabilizing slightly more and in
5 one less. Although the stability to thermal denaturation
is comparable for non-covalent and covalent complexes,
the covalently bound peptides should resist dissociating
at 37°C for much longer than the non-covalently bound
peptides.

10   Covalent MHC-peptide complexes like those reported
here may find use generating anergy if administered alone
(see, Kozono, H., White, J., Clements, J., Marrack, P. &
Kappler, J. (1994) Nature 369, 151-154) or activation if
administered with co-ligands for inducing costimulatory
15 signals as suggested by Schwartz, R.H. (1992) Cell 71,
1065-1068. A non-peptide ligand capable of crossing
cellular membranes to block selectively HLA-B27, based on
the same aziridine specificity can also be prepared.

20   OTHER EMBODIMENTS
From the above description, one skilled in the art
can easily ascertain the essential characteristics of the
present invention, and without departing from the spirit
and scope thereof, can make various changes and
25 modifications of the invention to adapt it to various
usages and conditions.
For example, the strategy can be used to form
covalent complexes of peptides or other ligands with HLA-
B27 does not add any new amino acids to the surface of
30 the MHC molecule (that might be immunogenic), but instead
forms the covalent attachment deep in a pocket buried by
the bound peptide. In addition, the aziridine strategy
can be generalized to other proteins (i.e., recombinant
class I MHC molecules) by mutating residues in the
35 binding region (i.e., the P2 pocket residues) to those in

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HLA-B27. The reverse experiment, engineering an HLA-A2 P2 specificity pocket into HLA-B27 by mutating residues 9, 24, 45, 66, 67, and 70 has been reported by Colbert, R. A., Rowland-Jones, S. L., McMichael, A. J. &
5 Frelinger, J. A. (1993) Proc. Natl. Acad. Sci. USA 90, 6879-6883, resulting in the desired effect of altering the specificity for the P2 position of bound peptide but retaining the same ability to interact with T cells as the unmodified protein. The reverse can be similarly
10 accomplished, HLA-B27 P2 pocket residues can be incorporated into HLA-A2. The success of the Colbert et al. experiment further suggests that any small adjustments in the local shape of the MHC molecule that might accompany covalent bond formation will not affect
15 T-cell recognition, since the covalent bond formation (Figure 1) is a much smaller modification than six amino acid substitutions.

        Also contemplated within the scope of this invention are peptide analogs obtained by back
20 modification of the above-described MHC-blocking peptides, e.g., replacement of at least one of the peptide bonds with $-CH_2-NH-$, CH_2-S-, or the like. Similarly, alkyl groups can be readily substituted by alkenyl, alkynyl or alkyl ether groups. Thus, other
25 embodiments are also within the claims.

        What is claimed is:

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CLAIMS

1.   A compound of the formula
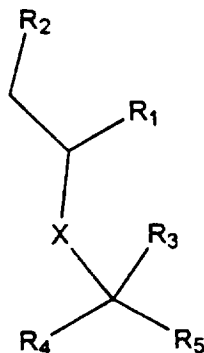
wherein

       each of R_1 and R_2, independently, is hydroxy,
5   halogen, haloalkyl, haloalkyl carbonyl, alkyl sulfonate,
aryl sulfonate, alkyl carboxylate, a Michael acceptor,
amino, alkylamino, ammonium, or alkyl ammonium, or
together R_1 and R_2 are O, NH, NH_2^+, or NZ, where Z is alkyl
or aryl;
10   X is C_1 to C_6 alkyl, substituted aryl, or deleted;
       R_3 is H, alkyl, or aryl;
       R_4 is alkyl, aryl, -NH-W, or -C(=O)-Y, where W is
H, alkyl, aryl, an amino acid residue bonded at its
carbonyl group, or a peptide moiety bonded at its
15   terminal carbonyl group, and Y is alkyl, alkoxy, amino,
substituted amino, hydroxyl, a peptide moiety bonded at
its terminal amino group or an amino acid residue bonded
at its amino group; and
       R_5 is H, alkyl, aryl, -NH-W', or -C(=O)-Y', where
20   W' is H, alkyl, aryl, an amino acid residue bonded at its
carbonyl group, or a peptide moiety bonded at its
terminal carbonyl group, and Y' is alkyl, alkoxy, amino,
substituted amino, hydroxyl, a peptide moiety bonded at
its terminal amino group, or an amino acid residue bonded
25   at its amino group.

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2.   The compound of claim 1, wherein:

R₁ is halogen, alkyl sulfonate, carboxylate, or aryl sulfonate and R₂ is hydroxy, amino, alkylamino, ammonium, or alkyl ammonium, or together R₁ and R₂ are O,
5 NH₂⁺, or NH; and

X is a C₂ to C₄ alkyl.

3.   The compound of claim 2, wherein:

R₃ is H;

R₄ is -NH-W, where W is an amino acid residue
10 bonded at its carbonyl group or a peptide moiety bonded at its terminal carbonyl group; and

R₅ is a -C(=O)-Y', where Y' is a peptide moiety bonded at its terminal amino group or an amino acid residue bonded at its amino group.

15   4.   The compound of claim 3, wherein:

W is an amino acid residue bonded at its carbonyl group; and

Y' is a peptide moiety bonded at its terminal amino group.

20   5.   The compound of claim 4, wherein:

R₁ is alkyl sulfonate and R₂ is an ammonium, or together R₁ and R₂ are O, or NH;

X is C₄ alkyl; and

the amino acid residue is a glycine residue.

25   6.   The compound of claim 4, wherein a portion of the peptide moiety is identical to a portion of a selected peptide.

7.   The compound of claim 4, wherein a portion of the peptide moiety is identical to a portion of an
30 antigenic peptide.

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8. The compound of claim 7, wherein the peptide moiety contains between 2 and 10 amino acid residues.

9. A method of forming a ligand-protein complex comprising the steps of:

    providing a protein having an arginine-specific binding region, the binding region including a nucleophilic residue; and

    adding a ligand to bind with the protein at the arginine-specific binding region, the ligand having a moiety which reacts with the nucleophilic residue,

    whereby the moiety of the ligand and the nucleophilic residue of the protein form a covalent bond between the ligand and the protein.

10. The method of claim 9, wherein:

    the arginine-specific binding region includes a thiol, a thiol and a carboxyl, or two carboxyl groups; and

    the moiety is halogen, haloalkyl, haloalkyl carbonyl, alkyl sulfonate, aryl sulfonate, carboxylate, a Michael acceptor, aziridine, or epoxide.

11. The method of claim 10, wherein the arginine-specific binding region includes a thiol and a carboxyl.

12. The method of claim 11, wherein the moiety is aziridine, or epoxide.

13. The method of claim 9, wherein the step of providing the protein includes the step of altering a binding region of the protein to introduce the arginine-specific binding region.

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14. The method of claim 13, wherein:
the arginine-specific binding region includes a thiol, a thiol and a carboxyl, or two carboxyl groups; and

5   the moiety is halogen, haloalkyl, haloalkyl carbonyl, alkyl sulfonate, aryl sulfonate, alkyl carboxylate, a Michael acceptor, aziridine, or epoxide.

15. The method of claim 14, wherein the arginine-specific binding region includes a thiol and a carboxyl.

10   16. The method of claim 15, wherein the moiety is aziridine, or epoxide.

17. A covalently linked ligand-protein complex comprising a protein having an arginine-specific binding region and a ligand covalently bonded to the binding
15 region of the protein, said ligand having the formula
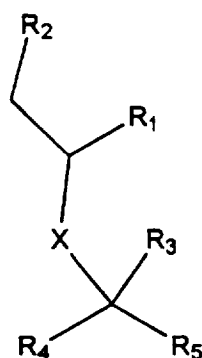
wherein
one of R_1 and R_2 is hydroxy, alkyl, alkyl carbonyl, amino, alkyl amino, ammonium, or alkyl ammonium, and the other of R_1 and R_2 is the covalent bond
20 to the protein;
X is C_1 to C_6 alkyl, substituted aryl, or deleted;
R_3 is H, alkyl, or aryl;

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R4 is alkyl, aryl, -NH-W, or -C(=O)-Y, where W is
H, alkyl, aryl, an amino acid residue bonded at its
carbonyl group, or a peptide moiety bonded at its
terminal carbonyl group, and Y is alkyl, alkoxy, amino,
5   substituted amino, hydroxyl, a peptide moiety bonded at
its terminal amino group or an amino acid residue bonded
at its amino group; and

R5 is H, alkyl, aryl, -NH-W', or -C(=O)-Y', where
W' is H, alkyl, aryl, an amino acid residue bonded at its
10 carbonyl group, or a peptide moiety bonded at its
terminal carbonyl group, and Y' is alkyl, alkoxy, amino,
substituted amino, hydroxyl, a peptide moiety bonded at
its terminal amino group, or an amino acid residue bonded
at its amino group.

15   18.   The complex of claim 17, wherein:
the arginine-specific binding region includes a
thiol, a thiol and carboxyl, or two carboxyl groups;
R1 is the covalent bond between the ligand and the
protein;
20   R2 is hydroxy, alkyl, alkyl carbonyl, amino, alkyl
amino, ammonium, or alkyl ammonium;
R3 is H; and
X is a C2 to C4 alkyl.

19.   The complex of claim 17, wherein:
25   the arginine-specific binding region includes a
thiol, a thiol and carboxyl, or two carboxyl groups;
R1 is hydroxy, alkyl, alkyl carbonyl, amino, alkyl
amino, ammonium, or alkyl ammonium;
R2 is the covalent bond between the ligand and the
30 protein;
R3 is H; and
X is a C2 to C4 alkyl.

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20.   The complex of claim 19, wherein:

R$_4$ is -NH-W, where W is an amino acid residue
bonded at its carbonyl group or a peptide moiety bonded
at its terminal carbonyl group; and

R$_5$ is a -C(=O)-Y', where Y' is a peptide moiety
bonded at its terminal amino group or an amino acid
residue bonded at its amino group.

21.   The complex of claim 20, wherein:

W is an amino acid residue bonded at its carbonyl
group; and

Y' is a peptide moiety bonded at its terminal
amino group.

22.   The complex of claim 21, wherein:

R$_1$ is hydroxy, amino, or ammonium;

X is C$_4$ alkyl; and

the amino acid residue is a glycine residue.

23.   The complex of claim 22, wherein the
arginine-specific binding region includes a thiol and
carboxyl.

24.   The complex of claim 21, wherein a portion of
the peptide moiety is identical to a portion of a
selected peptide.

25.   The complex of claim 21, wherein a portion of
the peptide moiety is identical to a portion of an
antigenic peptide.

26.   The complex of claim 25, wherein the peptide
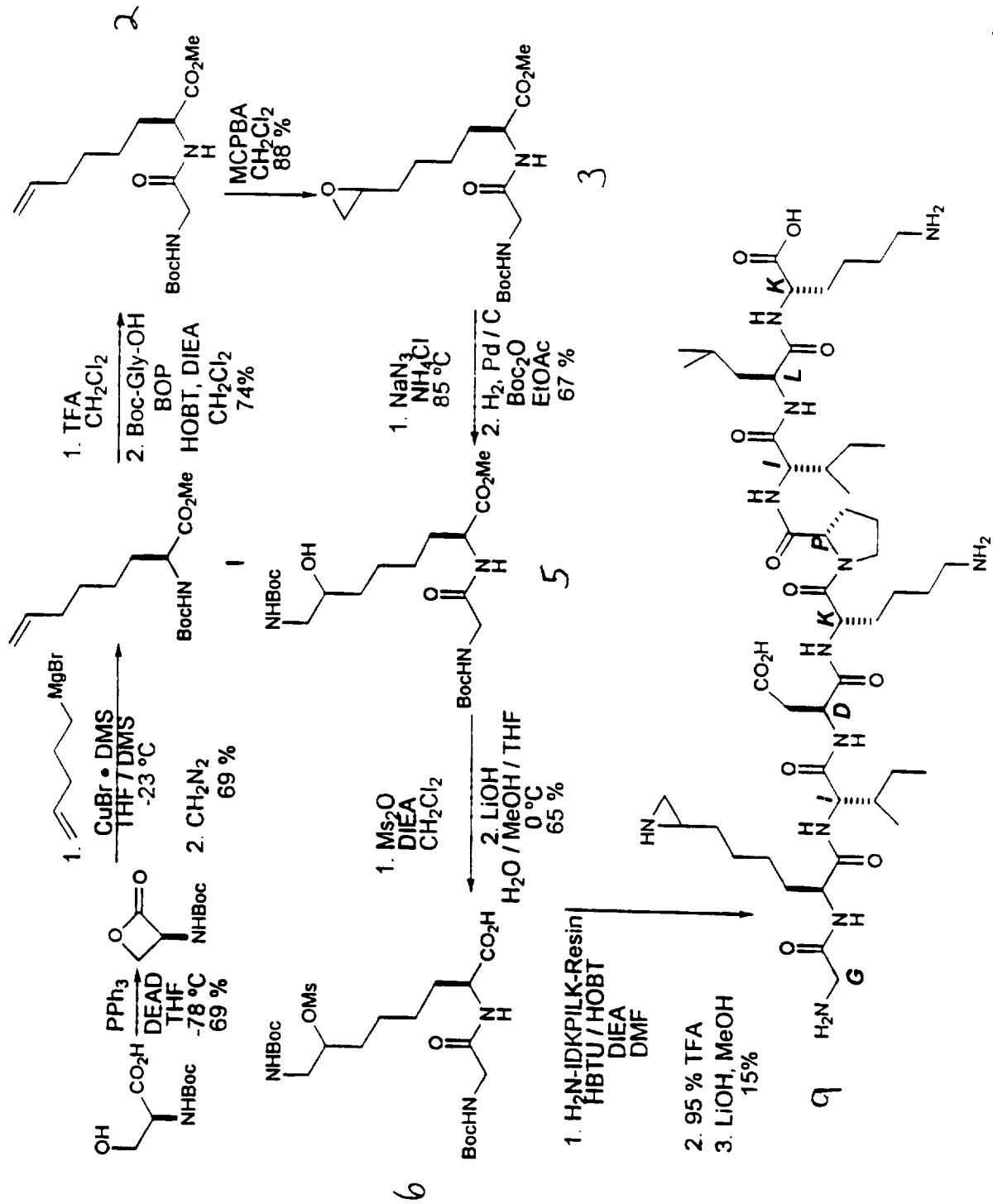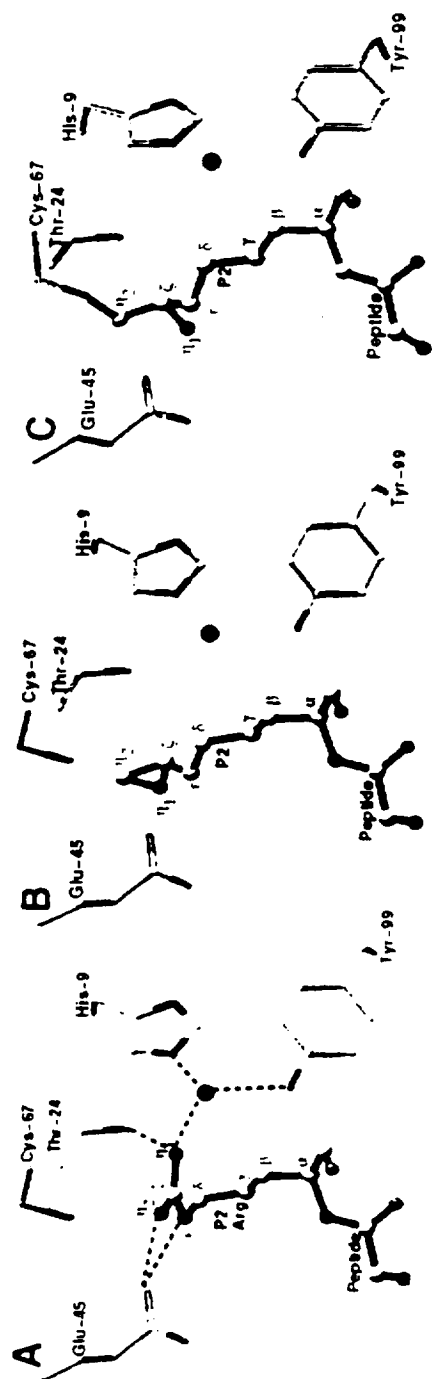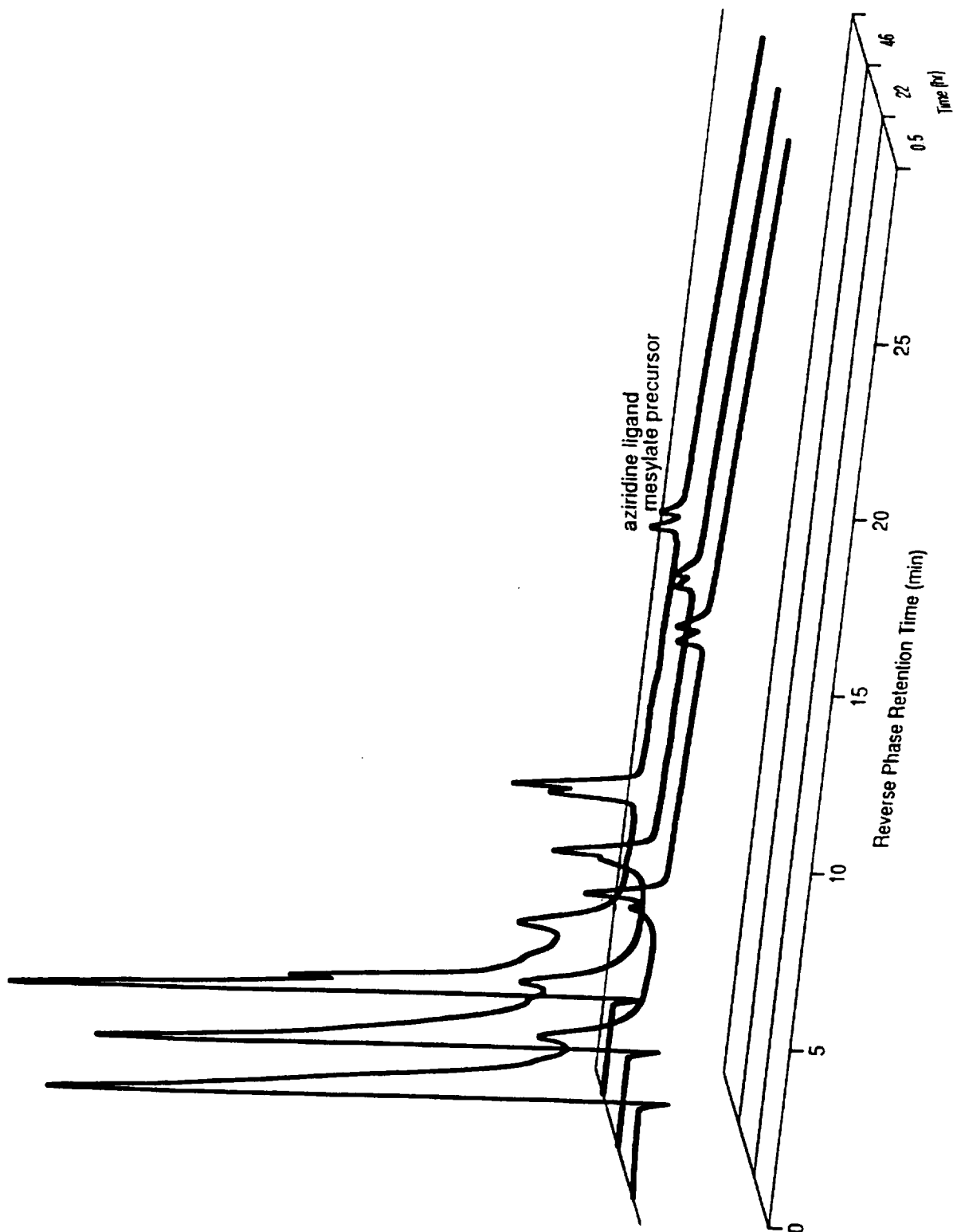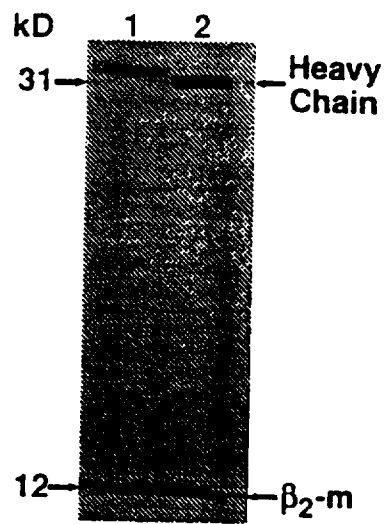moiety contains between 2 and 10 amino acid residues.

Fig. 1

Fig. 2

FIG. 3

Fig. 4

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/00; C07K 7/06
US CL :424/184.1; 530/328, 402, 405

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1; 530/328, 402, 405

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

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

CAS, MEDLINE, USPAT
search terms: aziridin?, arginin?, cysteine?, pentanediol, 2 chloro 1 pentanol

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	WEISS et al. Covalent HLA-B27/peptide Complex Induced by Specific Recognition of an Aziridine Mimic of Arginine. Proc. Natl. Acad. Sci. (USA). October 1996, Vol. 93, pages 10945-10948, see entire document.	1-26
X	US 5,137,929 A (DEMMIN et al.) 11 August 1992, column 6, lines 35-44.	1, 2
X	US 3,625,986 A (FELDMAN et al.) 07 December 1971, column 8, lines 63-68.	1
A	ROGNAN et al. Rational Design of Nonnatural Peptides as High-affinity Ligands for the HLA-B*2705 Human Leukocyte Antigen. Proc. Natl. Acad. Sci. (USA). January 1995, Vol. 92, pages 753-757, see entire document.	1-26

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

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"B"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

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