(12) UK Patent Application (19) GB (11) 2 282 815 (13) A

(43) Date of A Publication 19.04.1995

(21) Application No 9420293.4

(22) Date of Filing 07.10.1994

(30) Priority Data
(31) 138522 (32) 15.10.1993 (33) US

(71) Applicant(s)
Merck & Co Inc

(Incorporated in USA - New Jersey)

P O Box 2000, 126 East Lincoln Avenue, Rahway, New Jersey 07065-0900, United States of America

(72) Inventor(s)
Barry Cunningham
John Hannah
Stephen Marburg
Richard L Tolman

(51) INT CL[6]
C07K 14/16 , A61K 39/21

(52) UK CL (Edition N)
C3H HA3 HA4 H309 H310 H314 H365
U1S S1524 S2410 S2419

(56) Documents Cited
EP 0469701 A2 EP 0467699 A2

(58) Field of Search
UK CL (Edition M) C3H HA3 HA4
INT CL[5] C07K 7/04 7/06 7/08 15/04 15/12
ONLINE DATABASES: WPI,CLAIMS, DIALOG/BIOTECH,CAS ONLINE,CHABS

(74) Agent and/or Address for Service
J Thompson
Merck & Co Inc, European Patent Department, Terlings Park, Eastwick Road, HARLOW, Essex, CM20 2QR, United Kingdom

(54) **HIV peptide sulfides cyclised via a thioether linkage**

(57) Cyclic human immunodeficiency (HIV) peptides of the V3 region of the HIV envelope are produced by ring closure through a thioether linkage, which include the conserved sequence Gly Pro Gly Arg of the V3 loop and may include flanking aminoacids. Effective vaccine constructs include the outer membrane protein complex (OMPC) of Neisseria meningitidis, lipopeptides, multiple antigen peptides, and annular antigen scaffolds. Once incoporated into a suitable vaccine construct, these epitopes raise high titers of antibodies which are capable of neutralizing the HIV virus.

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TITLE OF THE INVENTION

HIV CYCLIC PEPTIDE SULFIDES

BACKGROUND OF THE INVENTION

This patent disclosure relates to novel cyclic peptides which provide useful epitopes to raise immune responses against human immunodeficiency virus (HIV). Antibodies raised against this new class of stably cyclic HIV PND peptides recognize the principal neutralizing determinant (PND) on the HIV virus and are useful in vitro in enzyme linked immunosobent assays (ELISA). In addition, upon conjugation of these peptides to appropriate immune enhancing carriers, anti-HIV immune responses, including neutralizing immune responses, are generated.

It has now become standard practice in the art of vaccine production to prepare conjugates of poorly immunogenic epitopes and strongly immunogenic carriers for the purpose of enhancing the immunogenicity of the poorly immunogenic epitopes. For a recent review of conjugate vaccines, see Dintzis, R. Z., Pediatric Res., 32:376-356 (1992). Thus, capsular bacterial polysaccharides are conjugated to proteins, see for example US Patent 4,695,624, and peptidyl epitopes have likewise been conjugated, see for example, EP 0 467 700. A new annular antigen scaffold core is described in patent application USSN , (Attorney Docket number 19008, cofiled with the instant application), in which peptides of the instant invention are conjugated to form an annular antigen scaffold which is effective at inducing anti-peptide, anti-HIV, and HIV-neutralizing immune responses.

There are many reports in the literature describing the third variable region, V3, of the HIV envelope protein (env). For example, LaRosa et al., described the sequence of this region in 245 isolates of HIV [Science 249:932-935 (1990)].The gp120 V3 domain of the HIV-1 envelope protein has been shown to be a disulfide-linked closed loop of approximately 30 amino acid residues [Leonard et al., (1990), J. Biol. Chem., 265, pp.10373-82]. The loop, either in the context of intact gp120 or as a synthetic peptide fragment, binds and elicits anti-HIV 1 type-specific virus-neutralizing antibodies [Goudsmit et

al., (1988), AIDS, 2, pp.157-164; Goudsmit et al., (1988), Proc. Natl. Acad. Sci. USA, 85, pp.4478-4482; Ho et al., (1987), J. Virol., 61, pp.2024-2028; Javaherian et al., (1989), Proc. Natl. Acad. Sci. USA, 86, pp.6768-6772; Kenealy et al., (1989), AIDS Res., 5, pp.173-182; Rusche et al., (1988), Proc. Natl. Acad. Sci. USA, 85, pp.3198-3202]. Accordingly, the V3 domain has been termed the principal neutralization determinant.

The in vitro characteristics of anti-V3 loop antibody include relatively potent virus-neutralizing activity, ability to neutralize following binding of the virus to the host cell CD4 receptor and ability to prevent fusion of virus-infected and uninfected cells [Linsley et al., (1988), J. Virol., 62, pp.3695-3702; Skinner et al., (1988), J. Virol., 62, pp.4195-4200]. Berman et al. immunized chimpanzees with recombinantly expressed mammalian cell-derived gp120 or its gp160 precursor [Berman et al., (1990), Nature, 345, pp.622-625]. Upon virus challenge of the animals, protection from infection was noted solely in those chimpanzees that had been inoculated with gp120. The only measured immune response that correlated with the protection was anti-V3 loop antibody. Girard et al. inoculated several chimpanzees with a series of immunogens, the last of which were V3 loop-specific synthetic immunogens [Girard et al., (1991), Proc. Natl. Acad. Sci. USA, 88, pp.542-546]. Significant virus-neutralizing activity was elicited only after this final inoculation. Upon challenge, the chimpanzees were either completely protected or exhibited delayed infection. Finally, Emini et al. reported an in vitro neutralization of chimpanzee infectivity study in which protection from or delay of infection also correlated with the presence of anti-V3 loop virus-neutralizing antibody [Emini et al., (1990), J. Virol., 64, pp.3647-3678]. Emini et al. have recently shown that a mouse/human chimeric monoclonal antibody specific for a single HIV-1 strain (IIIb) could prevent HIV-1 IIIb infection in chimpanzees both pre- and post-exposure to the virus [Emini et al., (1992), Nature, 355, pp.728-730]. Thus, the utility of V3-loop specific antibodies has been demonstrated.

While most HIV V3 epitopes disclosed in the prior art are linear, a few publications exist where cyclic peptides are disclosed. In EP 0471453 (2/19/1992, Merck Docket number 18149), HIV peptides cyclized through amide bonds were disclosed. In EP 0467699 (1/22/92, Merck Docket number 18150), HIV peptides cyclized through

dithioether containing linkers were described. In EP 0467701 (1/22/91, Merck Docket number 18068IB), HIV peptides cyclized through disulfide bonds were disclosed. In USSN 7283849, Robey et al., (NIH) disclose a method to incorporate bromoacetyl and chloroacetyl moieties on amino groups of synthetic peptides using an automated peptide synthesizer. In WO 91/01331, cyclic peptide sulfides useful as platelet aggregation inhibitors were disclosed.

SUMMARY OF THE INVENTION

Stably cyclic human immunodeficiency (HIV) principal neutralizing determinant (PND) peptides are produced by ring closure through a thioether linkage. The conserved sequence Gly Pro Gly Arg (SEQ. ID:1:) of the V3 loop of HIV is included in the sequence of these peptides, which may optionally include flanking aminoacids. Ring closure is accomplished by bromoacetylation of the amino-terminus of synthetic peptides and allowing capture of a cysteine or homocysteine thiol residue incorporated near the carboxy-terminus of the peptide. This method of ring closure is superior to other methods used to create cyclic HIV PND peptides because the process is simpler, the products are stably cyclic, and are constrained to remain in much fewer molecular conformations, including neutralizing conformations, than if the peptides are linear.

The carboxy-terminus of the cyclic HIV PND sulfide may also incorporate a residue which has a pendant amino group, such as lysine or ornithine, which can function to facilitate attachment to a vaccine carrier or carrier surrogate. Analysis and characterization of the vaccine is aided by the presence of "marker residues" which are easily recognized in an amino acid analysis. Effective vaccine constructs include, but are not limited to, the outer membrane protein complex (OMPC) of Neisseria meningitidis, lipopeptides, multiple antigen peptides, and annular antigen scaffolds. Once incoporated into a suitable vaccine contruct, these epitopes are shown to raise high titers of antibodies which are capable of neutralizing the HIV virus.

DETAILED DESCRIPTION OF THE INVENTION

Stable cyclic human immunodeficiency (HIV) peptide sulfides containing the Gly Pro Gly Arg [SEQ. ID:1:] conserved residues of the the V3 loop of HIV are prepared by standard solid phase peptide chemistry. Once a linear peptide has been produced incorporating the conserved V3 loop sequence and a cysteine or other sulfhydryl contributing group at the carboxy terminal side of the Gly-Pro-Gly-Arg sequence, sulfide formation is accomplished by bromoacetylation of the amino terminus which then captures the thiol presented at the carboxy terminal end of the peptide.

Accordingly, this invention provides a method for making a cyclic HIV peptide, cyclized through a sulfide, comprising the conserved HIV V3 sequence Gly Pro Gly Arg [SEQ. ID:1:], which comprises:

(a) Synthesizing a linear peptide comprising the sequence Gly Pro Gly Arg [SEQ.ID:1:] and a protected thiol at the carboxy terminal end of the Gly Pro Gly Arg sequence;

(b) Bromoacylating or chloroacylating a free amine at the amino-terminal end of the Gly Pro Gly Arg sequence;

(c) Cleaving the peptide from the resin and deprotecting the protected side chains;

(d) Cyclizing the peptide by basifying a dilute solution of the peptide.

Primary sequences for many isolates of the HIV V3 region are known in the art. Peptides containing the conserved Gly Pro Gly Arg [SEQ. ID:1:] sequence have heretofore been referred to as principal neutralizing determinants (PND) and these terms are used interchangeably herein to define peptides comprising this conserved sequence.

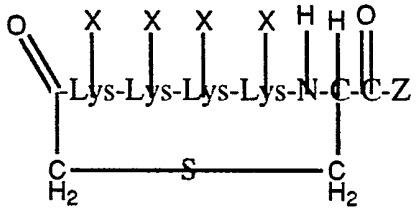
Peptides of this invention have been shown to bind with high affinity to a monoclonal antibody, r447, which is effective at neutralizing HIV. In a preferred method, the peptide is immobilized and antibody is allowed to flow over the immobilized peptide. In this manner, the on and off rates of various peptides may be accurately

measured. Use of the BIAcore™ machine for this purpose has been reported [see Pharmacia Biosensor AB, Application Notes 101 and 102]. In the present invention, this technique was used to confirm the binding affinity of cyclic HIV V3 loop sulfides. This is accomplished by binding cyclic HIV PND peptide sulfides to the gold CM5 chip provided by the manufacturer and binding a recombinant monoclonal anti-HIV PND (V3 loop specific) antibody to the immobilized peptide. Any of a number of known HIV-V3 loop specific monoclonal antibodies may be utilized for this purpose. Preferably, a broadly reactive antibody having a range of neutralizing activity against heterologous HIV strains is used. Thus, a preferred antibody for this purpose is human mAb 447-52D (ATCC#HB 10725) and 447-52DIV (ATCC #HB 10891) disclosed in WO93/08216, (see page 19, lines 1-10) herein incorporated by reference for the purpose of teaching monoclonal antibodies useful for showing the binding affinitiy of the HIV-V3 loop compounds). Alternatively, a recombinantly produced monoclonal antibody, having the attributes described hereinabove, is used. Such an antibody was used to generate the following binding data:

Peptide Sequence	logKa	logkd	logKaff
[SEQ.ID:2:] s(CH_2CO)Arg Ile His Ile Gly-Pro Gly Arg Ala Phe Tyr Thr-Cys Gly	5.492	-3.495	8.987
[SEQ.ID:3:] s(CH_2CO)Ile His Ile Gly-Pro Gly Arg Cys Lys	5.62	-4.17	9.79

To produce immunogenic antigens, the cylic HIV PND sulfides are conjugated to the outer membrane protein complex (OMPC) of Neisseria meningitidis b or to an annular antigen scaffold core (AASC, disclosed and separately claimed in USSN , Attorney docket

number 19008, filed concurrently herewith) having the general formula:



wherein:

S is a sulfur atom, contributed by the cysteine shown, forming a thioether bond;

X is an antigen or receptor ligand with each X being the same or different;

Z is a moiety linked to the ligand scaffold, such as ß-alanine, a lipopeptide adjuvant, or another T-cell epitope.

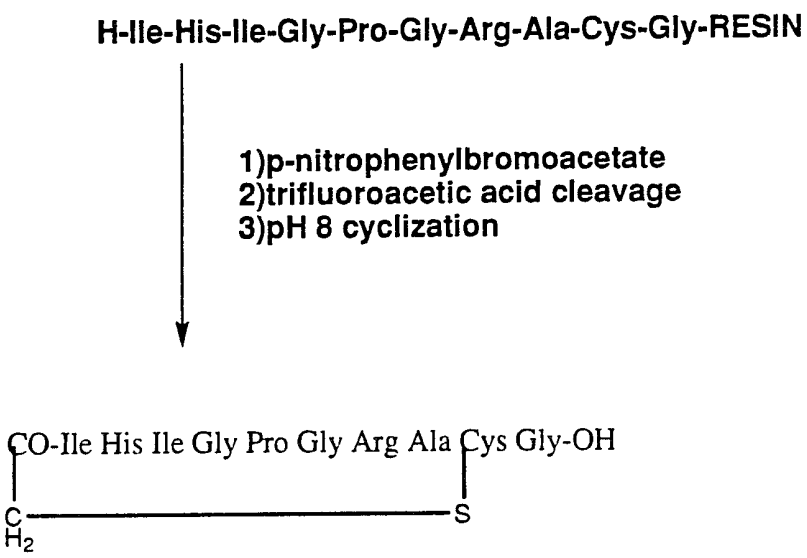
The AASC provides a compact and easily synthesized structure which, once the antigens have been linked, can display the antigens surrounding the annulus, thus essentially obscuring the presence of the core from the immune system as completely as possible. In this fashion, minimal immune responses against the core structure are elicited.

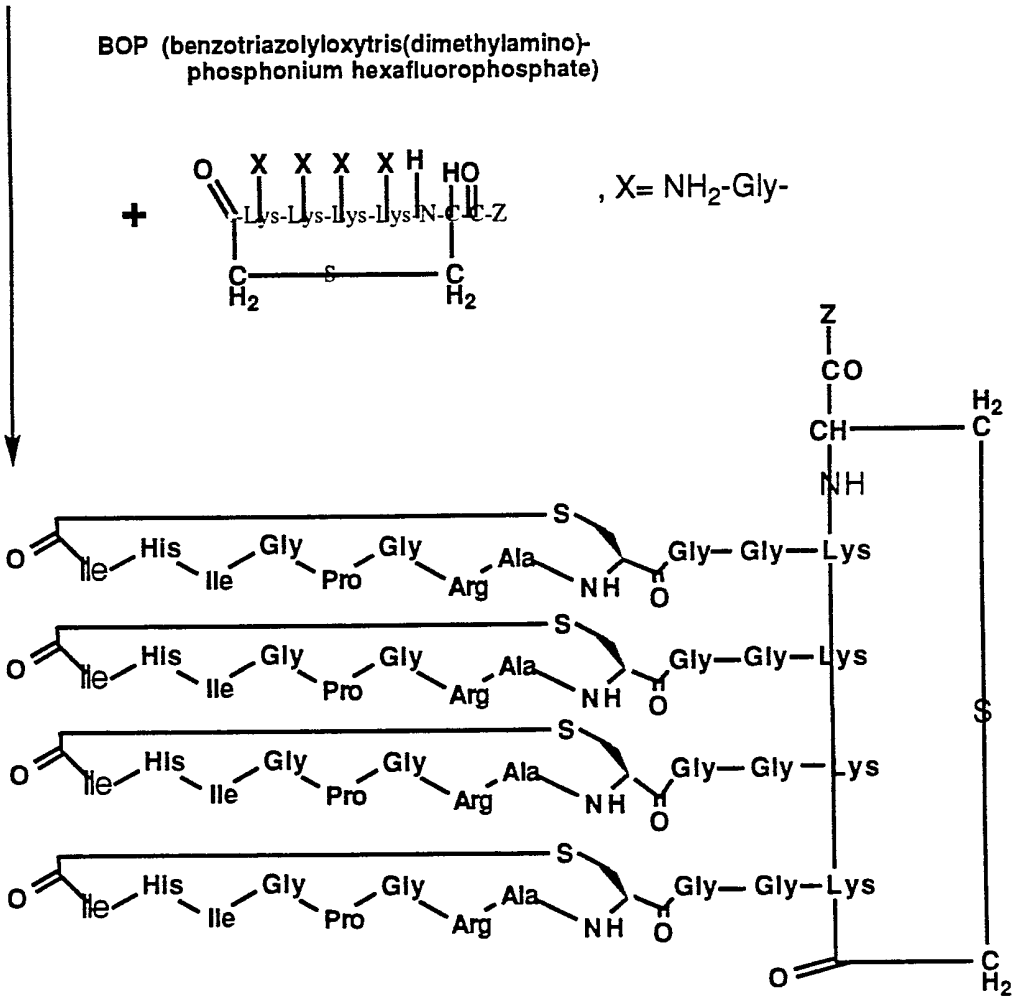
Once the core has been synthesized as described above, it is purified according to standard peptide purification procedures known in the art. A particularly preferred method is to purify the core by reverse-phase high performance liquid chromatography (RPHPLC) as shown in the examples below.

According to this embodiment of the invention, antigenic scaffolds are prepared by coupling purified cyclic HIV PND sulfide epitopes to a purified AASC. Thus, a tetraepitope scaffold which could be purified by HPLC and verified by electrospray mass spectrometry is provided as an example. The process involves synthesizing a linear HIV PND peptide on a solid resin support, bromoacetylating the N-terminal amine, deprotecting and cleaving peptide from the resin, cyclizing the peptide at about pH 8 between the bromoacetyl and the sulfur on a

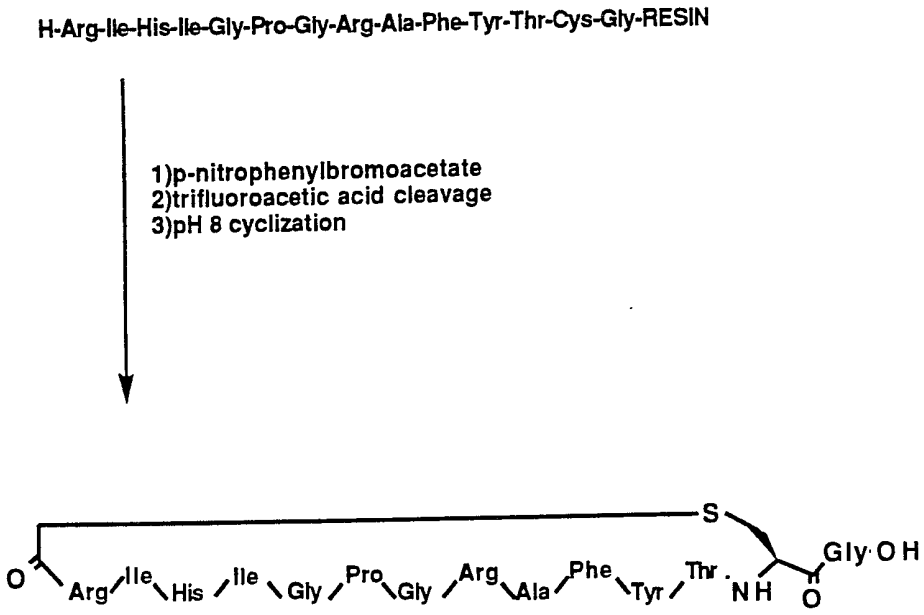
cysteine to form a cyclic thioether, and coupling the cyclic thioether to the AASC directly, via a Gly-Gly peptide bond for the purpose of eliminating steric hindrance and racemization problems. In another example of this embodiment, the solubility of the scaffold is enhanced by introducing a hydrophilic linker of either glycine or serine between the scaffold and the peptide epitope. These syntheses are shown in Schemes I-III respectively:

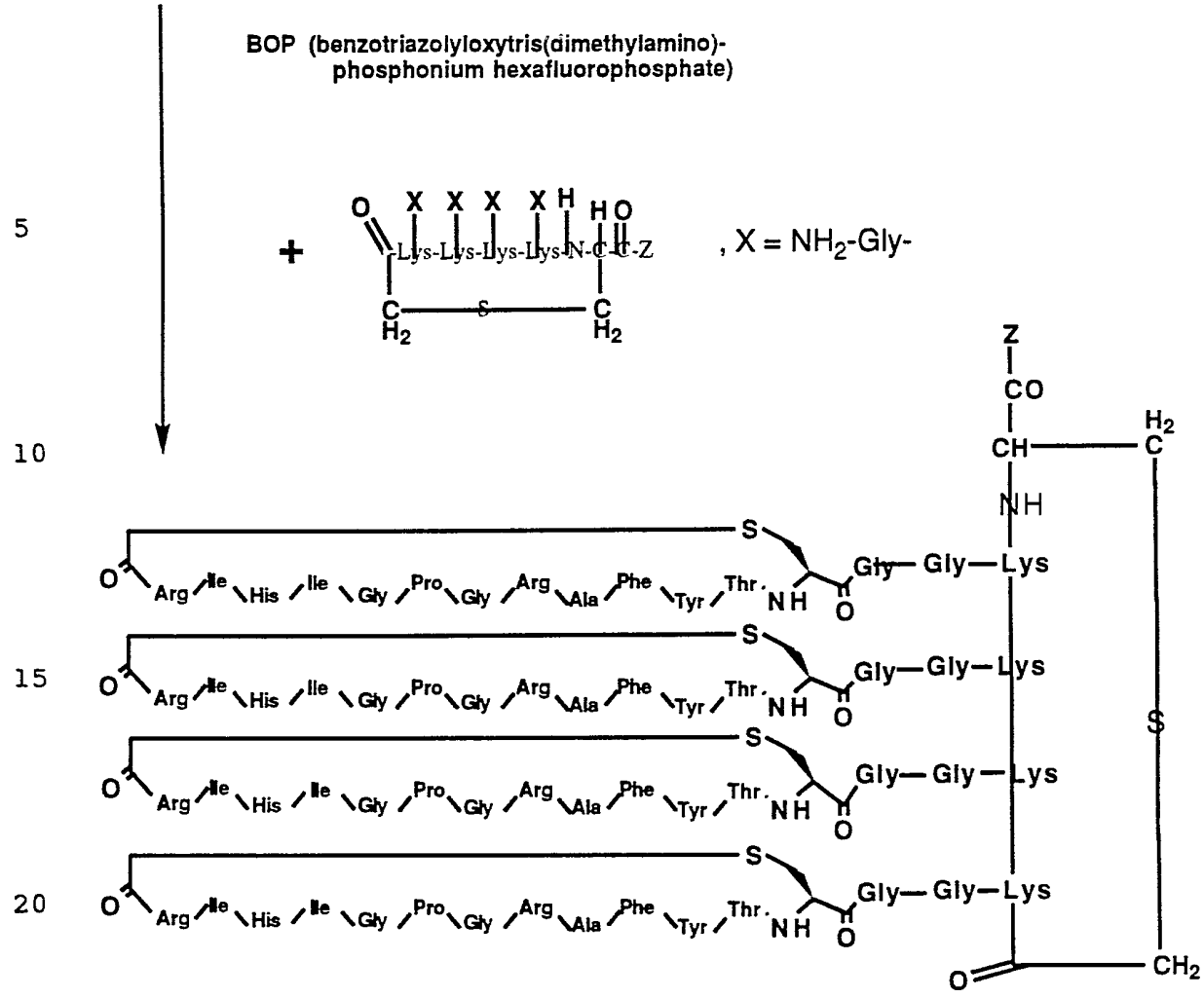
Scheme I, [SEQ.ID:4:1]:



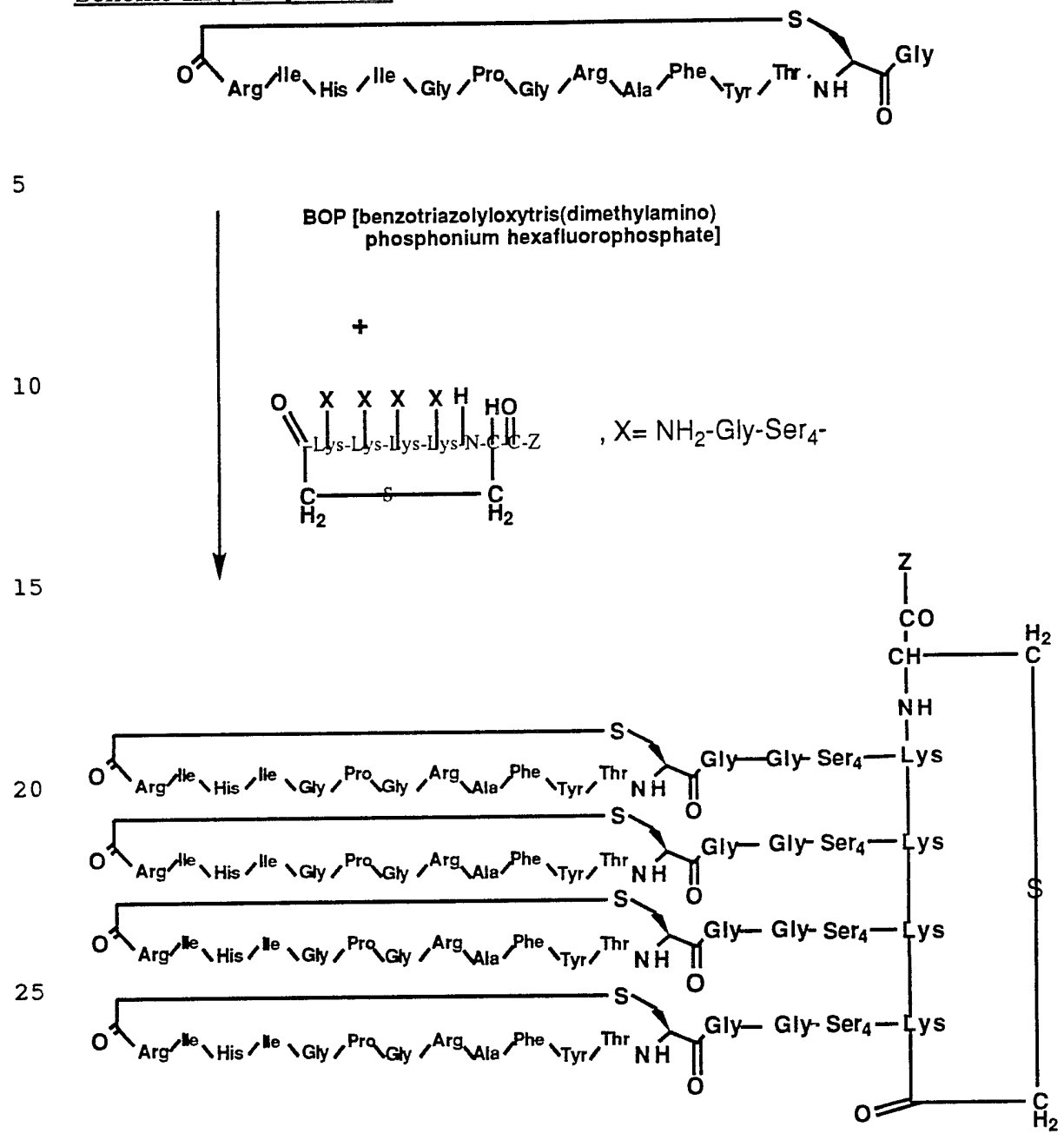

BOP (benzotriazolyloxytris(dimethylamino)-
phosphonium hexafluorophosphate)
, X= NH2-Gly-
Lys-Lys-Lys-Lys-N-C-C-Z
Ile His Ile Gly Pro Gly Arg Ala NH
Gly— Gly— Lys
CO
CH
NH
H2
C
S
O
H2

Scheme II, [SEQ.ID:2:]:




BOP (benzotriazolyloxytris(dimethylamino)-
phosphonium hexafluorophosphate)
, X = NH2-Gly-
Lys-Lys-Lys-Lys-N-C-C-Z
Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr
Gly—Gly—Lys
CO
CH
NH
CH2
S
O

Scheme III, [SEQ.ID:2:]:



In a preferred embodiment of this invention, in addition to the unhindered groups and solubilizing groups, the group Z is appended which has adjuvant activity. To this end, lipopeptide moieties may be

attached at the position shown for Z in the structure for AASC. Lipopeptides useful in this invention include synthetic lipopeptide analogs of the E. coli or S. willmorei lipopeptide. In general, these synthetic lipopeptide analogs are triacyl-glyceryl-cysteinyl-seryl-serine [SEQ.ID:5:], and are preferably $(Palmitoyl)_3$glycerylCys Ser Ser.

The cyclic HIV V3 sulfides of this invention are administered as vaccines by incorporating the peptide-carrier conjugate, such as the peptide-OMPC or the peptide-annular antigen scaffold conjugate with a liposome, by adsorbing it onto aluminum hydroxide, or by first incorporating it with a liposome and then adsorbing the AAS/liposome to aluminum hydroxide. Alternatively, the scaffold is linked to a lipopeptide so as to yield a self-adjuvanting complex.

The peptide-conjugate is admininstered at a dose of between about 1μg to 1mg per kilogram. It may be administered subcutaneously, intravenously, or intramuscularly. The scaffold may be administered to a vertebrate to generate immune responses against the conjugated epitopes. Preferably, the vertebrate is a mammal, and most preferably, the mammal is a human.

The following examples are provided to further define but not to limit this invention.

EXAMPLE 1

PREPARATION OF CYCLIC HIV PND PEPTIDE EPITOPES:

General: All reagents were used as received by the supplier. In the case of solvents, HPLC-grade are used where available. HPLC (binary gradient) is performed on a Waters 600E system with Waters 484 tunable U.V. detector (Aufs=0.1 analytical or 2.0 preparative scale) and recorded on a Waters 746 Data Module. A Waters WISP™712 autosampler (2000 mL sample loop) is used for analytical samples. A Rheodyne 7125 manual injection port (5000 mL sample loop) is used for preparative samples. A = H_2O, 0.1% TFA; B=CH_3CN, 0.1% TFA. Mass spectra are taken on a Finnegan MAT 90, spectrophotometer (positive ion, NBA matrix).

Abbreviations: Standard amino acid abbreviations are used. RT, room temperature; DCC, 1,3-dicyclo-hexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; DIEA, N,N-diisopropylethyl-amine; DTT, dithiothreitol; EDTA-2Na, ethylenediaminetetraacetic acid disodium salt; NBA, 3-nitro-benzyl alcohol; DMAP, 4-dimethylaminopyridine; NMP, 1-methyl-2-pyrrolidinone; DCM, dichloromethane; NMM, N-methylmorpholine; BOP, benzotriazol-1-yloxy-tris(dimethyl-amino) phosphonium hexafluorophosphate.

Stably cyclic human immunodeficiency (HIV) principal neutralizing determinant (PND) peptides are produced by ring closure through a thioether linkage. The conserved sequence Gly Pro Gly Arg (SEQ. ID:1:) of the V3 loop of HIV is included in the sequence of these peptides, which may optionally include flanking aminoacides. Ring closure is accomplished by bromoacetylation of the amino-terminus of synthetic peptides and allowing capture of a cysteine or homocysteine thiol residue incorporated near the carboxy-terminus of the peptide.

The linear peptide Ile-His-Ile-Gly-Pro-Gly-Arg-Ala-Cys-Gly [SEQ.ID:4:] was prepared by standard methods of solid phase synthesis on a Milligen 9050 synthesizer, using 0.41 g of Fmoc-Gly-Wang Resin (Penninsula Labs Lot # 018347, 0.5 meq/g). The resin was mixed with three volumes of glass beads. Double couplings were conducted at Ile^3, Gly^4, Pro^5, Gly^6, and Arg^7. Piperidine (20%) was used to remove Fmoc at each cycle. Coupling was with the pentafluorophenyl amino acids.

At the end of synthesis, a ninhydrin reaction on the resin was dark blue. The peptide was bromoacetylated on the resin using para nitrophenyl bromoacetate (0.25 g), DMAP (0.028 g) in NMP. The sample was washed with NMP and then dried. The peptide was cleaved/deprotected by addition of 90% TFA, 5 % thioanisole, 5 % thiocresol at room temperature for three hours.

The resin was filtered off and washed with 100% TFA. The combined filtrates were evaporated in vacuo to yield the crude peptide,

which was washed with diethylether and dichloromethane to remove scavangers. The peptide was recovered by scraping and filtering (0.446 g).

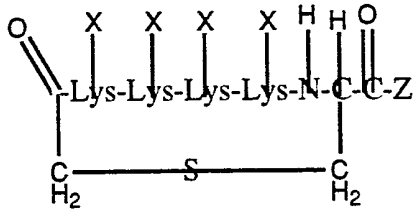
Peptide (144.2 mg) was dissolved in water (600 mL) and the pH was raised to 8 by addition of sodium bicarbonate (1.45 g). The sample was stirred for twelve hours to allow cyclization to occur, and then dried by lyophilization.

The cyclized peptide was dissolved in 20 % acetonitrile (12 mL). A yellow precipitate was removed by centrifugation prior to preparative HPLC (0-25% acetonitrile over 70 minutes, followed by isocratic elution at 25 % acetonitrile, in 2xC18 preparative columns in tandem, monitored at 215 nm, with 2 absorbance units full scale). The cyclized peptide eluted at 74 minutes. The sample was lyophilized (19.7 mg). An aliquot was measured by FAB-MS and gave the predicted mass of 1020.

Other cyclic HIV PND sulfides are prepared in essentially identical fashion to the method used above. Thus, the linear peptide Arg-Ile-His-Ile-Gly-Pro-Gly-Arg-Ala-Phe-Tyr-Thr-Cys-Gly [SEQ.ID:2:] was prepared by standard solid phase synthesis using two couplings at Ile^2, Ile^4, Gly^5, Pro^6, Arg^8, Phe^{10}, Tyr^{11}. Sixty minutes was provided per cycle (90 minutes for the final arginine). Piperidine deprotection was allowed to run for ten minutes. FAB-MS gave the predicted 1588 for the cyclic sulfide.

EXAMPLE 2

PREPARATION OF AN ANNULAR ANTIGEN SCAFFOLD CORE:



wherein:

S is a sulfur atom, contributed by the cysteine shown, forming a thioether bond;

X is -NH_2;
Z is ß-alanine-OH.

1. Synthesis of the linear peptide: Lys-Lys-Lys-Lys-Cys, [SEQ.ID:6:]:

Boc-ßAla-PAM Resin (Peninsula Labs, Lot 027925, 0.15 meq/g, 1 g) was washed three times with DCM, deprotected with 50% TFA/DCM for five minutes, and again for twenty minutes. The resin was washed again with DCM twice, five times with 5%DIEA, and then five more times with DCM.

Onto this resin, the active ester of N-Fmoc-S-Trityl-L-Cys [Bachem lot 2J541, 0.26g, prepared in NMP with BOP (0.19g) and NMM (0.45g, 0.49 mL)] was coupled to a negative ninhydrin. The amino terminus was then deprotected with 20% Piperidine in NMP followed by five NMP washes. Next, N-ε-Boc-N-α-Fmoc-L-Lys (Bachem lot 2J724, 0.211g) was prepared in NMP with BOP (0.19g) and NMM (0.45g, 0.49 mL) and was acylated onto the peptide to a negative ninhydrin. An additional three cycles of lysine addition, following almost exactly the above procedure for addition of the first lysine, was then conducted, and the amino terminus deprotected by treatment with 20% piperidine in NMP for twenty minutes, followed by five NMP washes.

2. Cyclization of the linear peptide to form a thioether linked annular antigen scaffold core:

The deprotected amino terminus was bromoacetylated by addition of bromoacetic acid (Aldrich, AW03119ET, 0.45mmoles, 63 mg) plus dicyclohexylcarbodiimide (DCC, 0.225 mmoles, 46 mg) in DCM. This bromoacetylation was allowed to run for 1.5 hours and then repeated, by addition of the same reagents, to ensure complete bromoacetylation, for another 20 minutes.

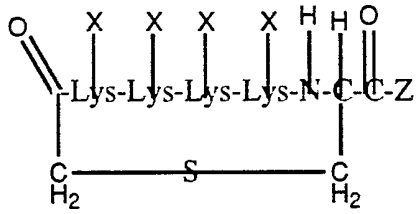
The resin was washed five times with DCM. The Boc protection was removed using 50% TFA for 20 minutes, in the presence of 0.5 mL triethyl silane as a scavanger. Four DCM washes and one diethyl ether wash followed.

The peptide was cleaved from the resin in dry hydrofluoric acid (HF), without any scavenger, for one hour at 0°C. The HF was then evaporated. The peptide was separated from the resin by dissolution in TFA, filtering off the resin, and removing the TFA in a rotary evaporator. Cyclization of the peptide was allowed to occur by dissolving the peptide in 800 mL of double deionized water, and adding solid $NaHCO_3$ until the pH reached 8. The solution was stirred, and cyclization was allowed to proceed over approximately 48 hours. The liquid was then removed by rotary evaporation.

The peptide was dissolved in 8 mL of double deionized water and the cyclized AASC was recovered by loading onto a preparative Vydac C18 HPLC column at 3 mL/minute, and then eluting at 10 mL/minute using a gradient from 0-25% CH_3CN over ten minutes. Sample elution was monitored at 215 nm, with full scale deflection set at 2. Two peaks were collected and rechromatographed (2.5% CH_3CN-12.5% CH_3CN over 60 minutes) after drying in a rotary evaporator. A peak eluting at 23.4 minutes was collected and lyophilized, to yield 17.6 mg of peptide. The mass was analyzed by FAB-MS, and the predicted mass of 746 for the AASC was confirmed.

EXAMPLE 3

PREPARATION OF BROMOACETYLATED CORE:



wherein:

S is a sulfur atom, contributed by the cysteine shown, forming a thioether bond;

Z is ß-alanine-OH; and

X= bromoacetyl.

The HIV PND cyclic sulfides of this invention may be conjugated to an AASC by bromoacetylating the free amino groups on the AASC and reacting the bromoacetylated groups with thiolated HIV PND sulfides. For this purpose, the AASC is bromoacetylated as follows:

Bromoacetic anhydride, $(BrAc)_2O$ was prepared by dissolving 15.2 mg of bromoacetic acid (BrAcOH) in 2 mL of dry methylene chloride. Dicyclohexyl carbodiimide (DCC, 8 equivalents, 11.3 mg) was added and the mixture stirred at room temperature for one hour in the dark. At the same time, tetralysine cyclic core, TLCC (5.1 mg, 6.8 μmol) was dissolved in dry, degassed DMF. The $(BrAc)_2O$ was filtered through glass wool to remove dicyclohexylurea, DCU, and added to the solution of TLCC. After one hour, a 2μL aliquot was removed for Kaiser analysis which was negative. A second 2μL aliquot was removed, diluted with 100μL water and 10μL was injected into an analytical, reverse phase HPLC (RP HPLC, 1.5 mL/min, Vydac C18, 10-60%CH_2CN over 20 minutes, λ=215nm, 0.1 abs. units full scale). Four peaks were observed.

An additional equivalent of $(BrAc)_2O$ (7.6 mg BrAcOH, 5.6 mg DCC) was prepared in 0.5 mL methylene chloride. This was filtered, added to the reaction mix, and the bromoacetylation was allowed to proceed overnight. When RP HPLC analysis showed no change after overnight reaction, the reaction was concentrated in vacuo and an additional two equivalents of $(BrAc)_2O$ in 500μL methylene chloride was prepared. The concentrated reaction was taken up in 1 mL of 1:1 DMF/methylene chloride, and the filtered $(BrAc)_2O$ was added. The reaction was stirred at room temperature for 5 hours. RPHPLC analysis revealed 3 major product peaks.

The reaction was concentrated in vacuo and products were isolated in a single RP HPLC run on a RCM25X10 C18 delta pak column with a gradient from 20-26% over 30 minutes at 10 mL/min. Four pooled fractions were collected, peaking at the indicated times: A-11 minutes; B-15 minutes; C-18 minutes; D-21 minutes. The C fraction

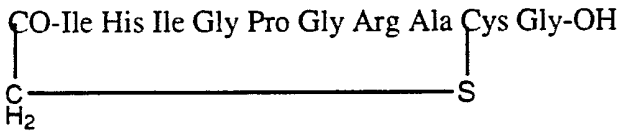
was identified as having the correct mass of 5818 for tetrabromoacetylated product by electrospray mass spectrometry.

For conjugation of HIV PND cyclic sulfides with the bromoacetylated core, the HIV PND sulfide is thiolated by addition of a cysteine at the carboxy terminus. This is accomplished by coupling a cysteine protected at the SH by Trt and a tBu protection of the carboxyl in the presence of DCC and DCU. The protecting groups are then removed with 50% TFA and the free SH reacted with the bromoacetylated core.

EXAMPLE 4

PREPARATION OF AAS WITH CYCLIC HIV PND PEPTIDE ANTIGENS:

The cyclic HIV PND peptide, (SEQ. ID:4:):



(24.2 mg, 23.7 μmoles) was added to a solution containing BOP (10.5 mg), NMM (3.5 mg, 4 μL), DMF (4 drops, dry). After 15 minutes, HOBt (3.2 mg), and AASC (wherein X=NH_2-Gly- ; 2.2 mg) were added. DMF (9 drops, dry) was added and the mixture was sonicated for 30 minutes to a final bath temperature of 58°C. The mixture was then stirred for 15 minutes, sonicated for an additional 10 minutes, and then NMM (3 μL) was added and the mixture was stirred an additional 45 minutes. The DMF was removed by drying in a rotary evaporator. The conjugate was then solubilized in water (2 mL) and frozen.

The conjugate was thawed and purified by preparative HPLC using a gradient of 12.5% to 27.5% CH_3CN over 70 minutes. A peak eluting at about 25% CH_3CN was collected, and dried to yield 6.9 mg.

This material, by electrospray mass spectrometry, gave the predicted mass of 4753.7 for AAS-1.

EXAMPLE 5

BIAcore™ BINDING OF HIV PND SULFIDES

The Pharmacia BIAcore™ was loaded with a CM5 Sensor Chip coated with HIV PND cyclic sulfides, followed by binding with recombinant monoclonal antibody R447, according to the manufacturer's instructions. Real time binding data was obtained according to the manufacturer's instructions and the following data was generated:

Peptide Sequence	logKa	logkd	logKaff
[SEQ.ID:2:] s(CH_2CO)Arg Ile His Ile Gly-Pro Gly Arg Ala Phe Tyr Thr-Cys Gly	5.492	-3.495	8.987
[SEQ.ID:3:] s(CH_2CO)Ile His Ile Gly-Pro Gly Arg Cys Lys	5.62	-4.17	9.79

From this data, peptides of this invention clearly display high affinity binding to HIV PND specific monoclonal antibody R447.

EXAMPLE 6

Preparation of a Recombinant 447-52D Antibody

A recombinant antibody, 447-52D was produced and used to generate data in the BIAcore™ system. This antibody was produced as follows:

An antibody was produced in which the variable domain of the light chain contains a signal sequence and light chain intronic sequence-appended version of the heterohybridoma 447-52D light chain variable region fused to a DNA fragment containing a short intronic segment of the human lambda 2

constant region, and the lambda 2 constant encoding domain. The variable domain of the heavy chain is similarly derived from the heterohybridoma 447-52D heavy chain V-region to which the same signal sequence and a heavy chain intronic sequence are appended, fused to a fragment containing a short intronic segment of the human gamma 1 constant region, and the human gamma 1 encoding domain in its genomic form.

Plasmids encoding the antibody portions were made and designated pHIV447VH for the heavy chain-containing plasmid, and pHIV447Vl for the light chain-containing plasmid. Both plasmids in their respective E. coli host have been deposited before the filing date of this application, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville MD, without restriction as to availability and in accordance with the terms and requirements of the Budapest Treaty. The E. coli hosts containing the plasmids have been assigned the ATCC accession numbers 68945 for pHIV447VHCg1 and 68943 for pHIV447Vl/Cl.

Equal amounts (10mg) of the plasmids encoding the heavy chain and the light chains were transfected by standard calcium phosphate precipitation procedures into human 293 cells (DeMartino, J.A. et al., supra.), except co-transfection with an HIV-1 TAT encoding plasmid was found not to be required for transactivation and resultant high level transcription of the HIV-1 LTR expression vector described herein. The culture supernatant fluids were assayed by trapping or solid-phase ELISAs (described below) for the secretion of a human lambda light chain containing IgG1 immunoglobulin.

ELISAs were developed for the quantitation of the amounts of 447-52D recombinant antibody expressed in conditioned mammalian cell growth medium. Immulon-2 (Dynatech Labs) 96-well plates are coated overnight with a 10mg/ml solution of mouse anti-human lambda chain constant domain monoclonal antibody (cat. #05-4101, Zymed Laboratories, Inc.) in phosphate-buffered saline (PBS) at 4°C, and blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C. After repeated washing with PBS, samples (conditioned medium containing recombinant 447-52D antibody or a human lambda/IgG1 standard antibody obtained from Sigma Chemical) diluted in PBS containing 1% BSA were added in duplicates and

incubated for 1 hour at 37°C. Standard calibration curves were constructed using IgG1 concentrations ranging from 7.8 ng/ml to 500 ng/ml. Bound and fully assembled human IgG1 were detected with 50ml aliquots of a 1:400 dilution of mouse anti-human IgG1 Fc monoclonal antibody conjugated to horseradish peroxidase (cat #05-3320, Zymed Laboratories, Inc.) in (PBS) containing about 1% BSA. After incubation for 1 hour at 37°C and subsequent washing, the quantities of bound conjugate were detected by addition of 1mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 0.1M sodium citrate, pH4.2, containing 0.03% hydrogen peroxide and incubation at room temperature for 20 minutes. The adsorbance of the wells was determined with a ELISA plate reader (Bio-Rad, Inc.) set at 415 nm. Alternatively, solid-phase ELISAs were carried out on plates coated with a 26-residue peptide based on the sequence of the MN isolate. The peptide (NleCSYNKRKRIHIGPGRAFYTTKNIIGCS [SEQ.ID: 7:] is synthesized by solid-phase Fmoc chemistry using preactivated pentafluorophenyl esters and hydroxybenzyltriazine activation. Immulon-2 plates were coated with 1 mg/ml peptide overnight, and blocked with 1% fetal bovine serum in PBS. Detection of bound 447-52D antibody is carried out as described above. The antibody secreted by the transfected human 293 cells following transient expression was subsequently purified by standard protein A chromatography (DeMartino, J.A. et al., supra.).

The concentration of recombinant 447-52D antibody was determined by the ELISAs described above, and tested for efficacy by demonstrating its capacity to neutralize the infectivity of unique serotypes of HIV-1.

The following assay was performed to quantitate the neutralization of cell-free HIV-1 virus infection as well as the inhibition of cell-to-cell spread by measuring cell survival after exposure of cultures to antibody and virus for 7-8 days. Two-fold serial dilutions of the antibody under test were made in cell growth medium (RPMI1640 + 10% fetal calf serum), and 100 ml volumes were placed in the wells of a 96-well dish (Costar, Corp.). 100 ml of virus stock (prepared from chronically infected H9 cells or from newly established chronically infected FDA/H9 cells; 3 day conditioned medium from the chronically infected cell population plated at a cell density of 2 x 10^5 cells/mL is clarified and 10 fold more than the last dilution of virus

stock which kills all MT-4 cells in a 7 day assay is chosen as the challenge dose) was added to each well and the virus-antibody mixtures were incubated at 37°C for 1 hour. MT-4 cells were added to each well (1 x 10^4 cells/well) in 50 ml of culture medium and the dish was incubated for 7 days at 37°C at which time the endpoint was determined. The concentration of the last antibody dilution which prevented MT-4 cell killing is reported as the neutralization endpoint.

The results of the neutralization assays are shown below and indicate that the potency of the recombinant human 447-52D antibody was equal to that of the human heterohybridoma-derived 447-52D antibody for each HIV-1 serotype investigated. This result shows that the recombinantly constructed antibody, expessed with human lambda and gamma 1 constant domains, has not been modified in such a way as to alter its interactions with the V3 PND loop and the recombinant antibody retains the biological activities of the native antibody.

NEUTRALIZATION ENDPOINT (µg/mL) IN in vitro
MT-4 CELL KILLING

	RECOMBINANT	HETEROHYBRIDOMA
HIV-1 ISOLATE	447	447
III_B	0.78	1.29*
MN	0.19	0.37
AL-1	0.09	0.15
SF-2	0.04	0.04
DU 6587-5	0.09	0.62
DU 7887-7	0.37	0.78
WMJ-2	0.78	1.35
RF	nd	0.62
SF-162	nd	1.98+

* = Geometric mean titer

+ = Macrophage/monocyte primary cultures (2 x 10^5 cells/microtiter well) were fed at days 5, 8, 12, 14, 21 with fresh medium. Conditioned medium was assayed for the presence of p24 virus core antigen by ELISA (Coulter Immunology, Hialiah, FL) and at day 24 the cells of each well were lysed and subjected to the same assay. The endpoint was determined to be the last dilution of antibody that prevented the appearance of p24 within the cell culture.
nd = not done.

EXAMPLE 7

An expression system was constructed and employed to produce large quantities of the recombinant 447 antibody. The expression system utilized the Cytomegalovirus immediate early (CMVIE) transcriptional promoter and the glutamine synthetase (GS) selection and amplification cassette, and is applicable to various different mammalian cell lines (Bebington et al., Biotechnology 10: 169-175, 1992). The basic vectors, pEE12 and pEE6, were obtained from Celltech, Ltd. and used to create a single plasmid, designated p63.79r447, which transcribed both the heavy and light chain immunoglobulin peptides, in addition to the GS gene product. The manner in which this r447 expression vector was constructed utilized existing basic vectors which contained other immunoglobulin sequences.

The final plasmid p63.79r447 in its E. coli host, containing both the heavy chain and light chain coding regions of human monoclonal antibody 447, has been deposited before the filing date of this application, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, without restriction as to availability and in accordance with the terms and requirements of the Budapest Treaty. The E. coli host containing plasmid p.63.79r447 has been assigned the ATCC accession number ATCC 68944.

EXAMPLE 8

Analysis of Sera for Anti-HIV cPND IgG Antibodies:

Each serum sample is analyzed by enzyme-linked immunoadsorbent assay (ELISA). Polystyrene microtiter plates are

coated with 0.5 mg per well of the synthetic peptide in phosphate-buffered physiological saline (PBS) at 4°C. Each well is then washed with PBS containing 0.05% TWEEN-20 (PBS-T). Test serum, diluted serially in PBS-T, is added to the peptide-containing wells and allowed to react with the adsorbed peptide for one hour at 36°C. After washing with PBS-T, alkaline phosphatase-conjugated goat anti-human IgG is added to the test wells and allowed to react for one hour at 36°C. The wells are then washed extensively in PBS-T. Each well receives 0.1% p-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, containing 0.5 mM $MgCl_2 \cdot 6H_2O$. The ensuing reaction is allowed to proceed at room temperature for 30 minutes, at which time it is terminated by the addition of 3.0 N NaOH.

The greater the interaction of antibodies in the test serum with the peptide substrate, the greater is the amount of alkaline phosphatase bound onto the well. The phosphatase enzyme mediates the breakdown of p-nitrophenyl phosphate into a molecular substance which absorbs light at a wavelength of 405 nm. Hence, there exists a direct relationship between the absorbance at 405 nm of light at the end of the ELISA reaction and the amount of peptide-bound antibody.

EXAMPLE 9

Analysis of Sera for Activity which Specifically Neutralizes HIV Infectivity:

Virus-neutralizing activity is determined with an assay described by Robertson et al., J. Virol. Methods 20: 195-202 (1988). The assay measures specific HIV-neutralizing activity in test serum. The assay is based on the observation that MT-4 cells, a human T-lymphoid cell line, are readily susceptible to infection with HIV and, after a period of virus replication, are killed as a result of the infection.

The test serum is treated at 56°C for 60 minutes prior to the assay. This treatment is required to eliminate non-specific inhibitors of HIV replication. Heat treated serum, serially diluted in RPMI-1640 cell culture medium, is mixed with a standard infection dose of HIV. The dose is determined prior to the assay as containing the smallest

quantity of virus required to kill all the MT-4 cells in the assay culture after a period of 7-8 days. The serum-virus mixture is allowed to interact for one hour at 37°C. It then is added to 1.0×10^5 MT-4 cells suspended in RPMI-1640 growth medium supplemented with 10% fetal bovine serum. The cultures are incubated at 37°C in a 5% CO_2 atmosphere for 7 days.

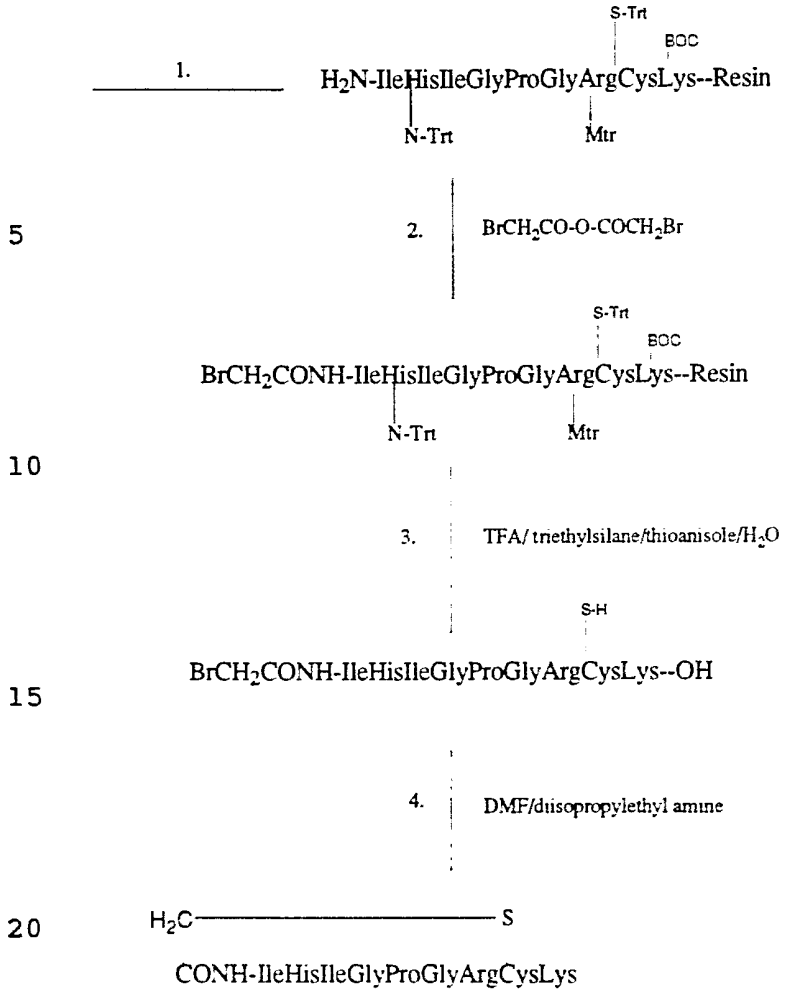
At the end of the incubation period, a metabolic dye, DTT, is added to each culture. This dye is yellow in color upon visual inspection. In the presence of live cells, the dye is metabolically processed to a molecular species which yields a blue visual color. Neutralized HIV cannot replicate in the target MT-4 cells and therefore does not kill the cells. Hence, positive neutralization is assessed by the development of blue color following addition of the metabolic dye.

The cyclic HIV PND peptide sulfides of this invention are used to induce HIV neutralizing antibodies which are tested according to this method and are useful in identifying HIV PND peptides which bind with high affinity.

EXAMPLE 10

PREPARATION OF ADDITIONAL CYCLIC HIV PND SULFIDES

The synthesis of a cyclic peptide sulfide is further exemplified below [SEQ.ID:3:]:



In general, peptides of this invention may be prepared as follows: 1. The linear peptide is synthesized on an ABI instrument using Wang resin and FMOC chemistry. 2. Bromoacetylation is effected on the resin using bromoacetic anhydride generated from bromoacetic acid using DCC. 3. The peptide is removed from the resin by TFA using triethylsilane and thioanisole as scavengers followed by water to scavenage MTR and hydrolyze silyl groups. 4. Cyclization is effected in DMF by basifying with diisopropylethyl amine (DIEA).The final product is isolated by reverse phase HPLC.

These procedures were carried out for the specific compound shown above as follows:

1. After synthesis of the linear peptide was complete the resin was washed, in the ABI, twice with methanol and once with methylene chloride. The resin was then vacuum dried in a dessicator and a weight gain of 392 mg was noted (the starting weight of resin was 625 mg). This is 96% of the theoretical weight gain. A workup of an aliquot of the resin afforded a 42% peptide recovery assuming a 70% peptide content in the isolated material.

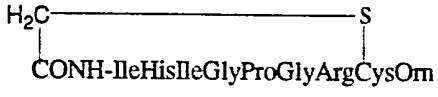
2. Bromoacetylation: A bromoacetic anhydride solution was prepared by dissolving 139 mg of bromoacetic acid (1 mmol) in 5 mL of methylene chloride and adding 106 mg of dicyclohexylcarbodiimide (DCC). The mixture was stirred overnight and then the dicyclohexyl urea was filtered and the resulting filtrate diluted to 5 mL and used as such (estimated as a 0.1M solution).

The peptidyl resin (203.5 mg est 47 μmol) was covered with 2 mL of N-methylpyrrolidone (NMP) and stirred for 1 h to effect swelling. To this was added 8μL of DIEA (46 μmol) and 0.5 mL of the above bromoacetic anhydride solution. After stirring 2.3 h the resin still afforded a positive Kaiser test. An additional 0.3 mL of the bromoacetic anhydride solution was added and after stirring an additional 1.5 h the Kaiser test was negative. After filtration , washing with methylene chloride and drying in vacuo a weight gain of 21 mg was observed.

3. Cleavage of peptidyl resin: The above resin was covered with 9 mL of trifluoroacetic acid (TFA) and to it added 0.4 mL of thioanisole and 0.4 mL of triethylsilane. The mixture was stirred for 16 h and then 0.2 mL of H_2O added and stirring continued for an additional 2 h. The mixture was filtered and the TFA solution concentrated to dryness. The residue was stirred for 2 h with 30 mL of ether. The ether decanted affording a residue of 59 mg (theory : 51 mg bromoacetylated peptide).

4. The bromoacetylated peptide is cyclized according to the method disclosed in example 1.

In similar fashion to the above, the peptide [SEQ.ID:8:]



, wherein Orn is ornithine, was prepared, (M_r =1006) was prepared. Thus, essentially any peptide having a Gly-Pro-Gly-Arg [SEQ.ID:1:] core and a ring size of between about 5 and 15 amino acids may be prepared conveniently according to the method disclosed above, and will predictably bind to HIV V3 specific HIV neutralizing monoclonal antibodies, as described above (BIAcore™). In addition, these compounds, when presented in the correct immunizing contexts, as in a conjugated state, predictably raise antibodies which are HIV neutralizing. These peptides may therefore be used in analyzing useful eptiopes for production of anti-HIV immune responses in vitro and for producing such responses in vivo.

EXAMPLE 11

BIAcore™ ANALYSIS OF CYCLIC HIV PEPTIDE BINDING AFFINITY

Determination of the kinetic rate and affinity constants for the interaction of monoclonal antibody with peptide antigen by Surface Plasmon Resonance (SPR) analysis on BIAcore system (Pharmacia Biosensor): The objective of the assay is to identify peptides as HIV vaccine candidates. The BIAcore system (Pharmacia Biosensor) allows a quantitative analysis of molecular interaction in real time. Therefore, association and dissociation rate constants can be readily calculated. The system uses surface plasmon resonance (SPR)(Kretschmann and Raether, 1968), a quantum mechanical phenomenon, which detects changes in optical properties at the surface of a thin gold film on a glass support (sensor surface (Lofas and Johnson, 1990). The sensor surface carries a carboxylated dextran matrix on which one of the two molecules (a peptide in this assay) is covalently attached. The other (an antibody in this assay) is introduced in a flow passing over the surface. Resonance occurs at specific angle of incident light. The resonance

angle depends on the refractive index in the vicinity of the sensor surface and is monitored continuously, thus allowing the association or dissociation of molecules from the sensor surface to be followed in real time. No labeling of the ligands is required.

Theory of measuring the kinetic rate constants (Karlson et al., 1991):

During a kinetic run, the antibody solution flowing over the sensor surface is constantly replenished. The reaction between matrix-immobilized peptide and antibody in solution therefore can be assumed to follow pseudo-first-order kinetics. Thus the rate of antibody binding is

$$dR_A/dt=\text{constant} - (k_aC+k_d)R_A$$

where R_A is the resonance response, representing the antibody bound; C is the concentration of injection antibody; k_a is the association rate constant and k_d is the dissociation rate constant. Both dR_A/dt and R_A values are collected by the instrument. When several concentrations of antibody are injected, the slope value, k_S, obtained from each dR_A/dt vs R_A plot can be used in a plot vs antibody concentration to obtain the association rate constant, k_a:

$$K_S=k_aC+k_d$$

Theoretically the dissociation rate constant also is obtained as the intercept at the y-axis. However, this intercept cannot be determined accurately when k_d is low. A more favorable experimental situation is obtained by measuring the dissociation of bound antibody separately in continuous buffer flow. The rate equation is

$$dR_A/dt=-k_dR_A$$

If dissociation starts at time t_1, from a high response level R_{A1}, the dissociation rate constant is obtained from the solution of the rate equation

$$\ln(R_{A1}/R_{An})=k_d(t_n-t_1)$$

and the slope value, obtained from $\ln(R_{A1}/R_{An})$ vs(t_n-5_1) plot, is the k_d. The affinity of the interaction between antibody and peptide antigen is given by the equilibrium constant;

$$K_{eq}=k_a/k_d$$

Assay Procedure:

(a) Immobilization of peptides on the sensor surface: The immobilization is performed at a flow rate of 5 uL/min in HBS, pH 7.4[10 mM Hepes, 0.15 M NaCl, 3.4 mM ethylenediaminetetracetic acid disodium salt, 0.05% surfactant 20]. The peptide is concentrated on the carboxylated dextran matrix by an ion exchange effect at pH below the isoelectric point of the peptide and covalently coupled via either primary amino or thiol groups. Primary amine coupling: The carboxylated dextran matrix is first activated with 35 uL of an N-ethyl-N'-(3'dimethylaminopropyl) carbondimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) mixture. Twenty five-250 mcg/Ml of peptide (20-40 uL) is injected in a buffer with pH lower than that of the peptide, eg. 0.01 M acetic acid, pH 4. Unreacted groups on the sensor surface are blocked by 30 uL of ethanolamine-HCl, pH 8.5. Thiol coupling: The carboxylated matrix is similarly activated with EDC/NHS mixture. A reactive disulfide, 2-(2-pyridinyldithio) ethaneamine (PDEA), 80 mM, pH 8.5, is introduced. Twenty five-250 mcg/mL of a thiol containing peptide is coupled onto the surface via the exchange of the disulfide bonds. The excess disulfides are deactivated with 50 mM cystein in 1 M NaCl, pH 4.3.

(b) Measurement of kinetic rate constants: Recombinant 447 monoclonal HIV antibody at concentrations ranging from 5 nM to 1 uM is HBS, is allowed to interact with sensor surfaces on which the different HIV gp120 V3 loop-tip peptides have been immobilized. The runs are performed at 25°C, at a flow rate of 5 uL/min for 7 min. (35-uL injection), taking report points (for dR_A/dt and R_A values) every 5 s. After the run, the surface is regenerated by injection 5 uL of a 10-20 mM HCl solution. The instrument software produces a table of dR_A/dt and R_A values that can be directly used in a plotting program (Microsoft Excel).

Indication of result(s) type (%INH.IC50, Zone size, etc.)

K_a: association rate constant (M^{-1} s^{-1})
k_d: dissociation rate constant (s^{-1})
k_{eq}; equilibrium constant (M^{-1})

Interpretation of the results of the basis of activity

Peptides which show high affinity ($K_{eq}>10^{10}M^{-1}$) for the 447 antibody, a strong HIV neutralizing antibody, may induce the production of a 447 like antibody when used as a vaccine antigen.

References:

Karlsson, R., Michaelsson, A. and Mattsson, L. Kinetic analysis of monoclonal antibody-antibody interactions with a new biosensor based analytical system. J. Immunol. Methods 145: 229-240, 1991.

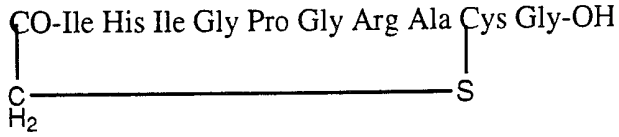
Kretschmann, E. and Raether, H. Radiative decay on non radiative surface plasmons excited by light. Z. Naturforsch., Teil A23, 2135-2136, 1968.

Lofas, S. and Johnsson, B. A novel hydorgel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobiliation of ligands. J. Chem. Soc., Chem. Commun. 21, 1526-1528, 1990.

WHAT IS CLAIMED IS:

1. A cyclic HIV peptide, cyclized through a sulfide linkage, comprising the conserved HIV V3 sequence Gly Pro Gly Arg [SEQ. ID:1:] .

2. The cyclic HIV V3 sulfide of Claim 1 having the formula:



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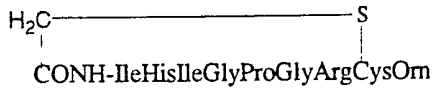
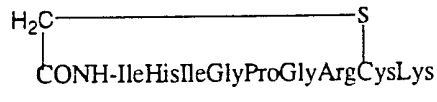
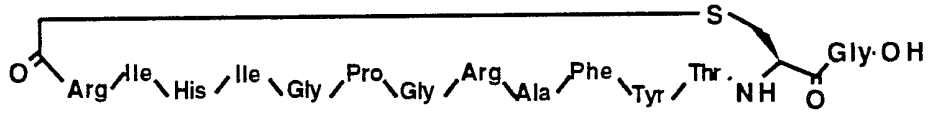


, wherein Orn is ornithine,



,

or



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3. A method for making a cyclic HIV peptide, cyclized through a sulfide, comprising the conserved HIV V3 sequence Gly Pro Gly Arg [SEQ. ID:1:], which comprises:

(a) Synthesizing a linear peptide comprising the sequence Gly Pro Gly Arg [SEQ.ID:1:] and a protected thiol at the carboxy terminal end of the Gly Pro Gly Arg sequence;

(b) Bromoacylating a free amine at the amino-terminal end of the Gly Pro Gly Arg sequence;

(c) Cleaving the peptide from the resin and deprotecting the protected side chains;
(d) Cyclizing the peptide by basifying a dilute solution of the peptide.

Examiner's report to the Comptroller under Section 17 (The Search report)

35

GB 9420293.4

Relevant Technical Fields

(i) UK Cl (Ed.M) C3H (HA3, HA4)

(ii) Int Cl (Ed.5) C07K 7/04, 7/06, 7/08, 15/04, 15/12

Search Examiner
C SHERRINGTON

Date of completion of Search
3 JANUARY 1995

Databases (see below)
(i) UK Patent Office collections of GB, EP, WO and US patent specifications.

(ii) ONLINE DATABASES: WPI, CLAIMS, DIALOG/BIOTECH, CAS ONLINE, CHABS

Documents considered relevant following a search in respect of Claims :-
1 TO 3

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family; corresponding document.

Category	Identity of document and relevant passages	Relevant to claim(s)
X	EP 0467699 A2 (MERCK & CO INC) whole document	1
X	EP 0467701 A2 (MERCK & CO INC) whole document	1

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