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(54) **Amyloid Beta oligomers for use in treatment, alleviation or prevention of Alzheimer's Disease**

Amyloid Beta Oligomere zur Behandlung, Linderung oder Vorbeugung der Alzheimers Krankheit

Amyloid Beta Oligomères pour le traitement, le souagement ou la prévention de la maladie d'Alzheimer

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Description

FIELD OF THE INVENTION

[0001] This invention relates to methods and compositions for the treatment or alleviation of Alzheimer's disease and of other conditions related to abnormal protein aggregation. In particular, the invention relates to : amyloid β peptides with cross-linked tyrosine, for use in treating, alleviating or preventing Alzheimer's disease.

BACKGROUND OF THE INVENTION

[0002] The characteristic amyloid lesions of Alzheimer's disease (AD) are primarily composed of Amyloid β (Aβ) (Glenner & Wong, 1984), a 39-43 amino acid protein which is a normally soluble protein found in biological fluids. Amyloid formation is linked to the pathogenesis of the disease, so identifying the neurochemical changes which lead to the inhibition of Aβ catabolism and its accumulation in the neocortex would be an important clue to the pathogenesis of AD.

[0003] Although the fundamental pathology, genetic susceptibility and biology associated with AD are becoming clearer, a rational chemical and structural basis for developing effective drugs to prevent or cure the disease remains elusive. While the genetics of AD indicate that the metabolism of Aβ is intimately associated with the pathogenesis of the disease as indicated above, drugs for the treatment of AD have so far focused on "cognition enhancers", which do not address the underlying disease processes. These drugs have met with only limited success.

[0004] The nature of the deranged neurochemical environment in AD can be partly deduced from the post-translational modifications of amyloid Aβ. Aβ extracted from biological systems normally migrates as an apparent ~4 kD monomer on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; (Shoji et al., 1992)); however, Aβ extracted from specimens of AD-affected post-mortem brain migrates on SDS-PAGE as SDS-, urea- and formic acid-resistant oligomers (Masters et al., 1985; Roher et al., 1996; Cherny et al., 1999).

[0005] Matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) of these SDS-resistant oligomers extracted from neuritic plaque and vascular amyloid indicates the presence of covalently cross-linked dimeric and trimeric Av species (Roher et al., 1996).

[0006] Synthetic $A\beta_{1-40}$ and $A\beta_{1-42}$ normally migrate as apparent monomers on SDS-PAGE, but form apparent higher molecular weight species upon incubation (Burdick et al., 1992). This process is accelerated by exposure to oxidative systems (Dyrks et al., 1992; Atwood et al., 1997).

[0007] Tyrosine cross-linking has been proposed as a mechanism of Aβ oligomerization *in vivo*, since tyrosine residues in synthetic human Aβ can be cross-linked by peroxidase-catalyzed oxidation systems (Galeazzi et al., 1999). As Rat Aβ, unlike human Aβ, lacks a tyrosine residue (Atwood et al.,1997), it is therefore resistant to metal-catalyzed oxidative oligomerization, and this perhaps explains the rarity of amyloid deposits in these animals (Vaughan and Peters, 1981).

[0008] Tyrosine cross-linking in proteins is a sensitive marker of oxidative stress. Covalent carbon-carbon bridges or carbon-oxygen bridges are formed between single tyrosyl residues and/or dityrosyl residues, resulting in a number of stable, fluorescent reaction products (Gross and Sizer, 1959; Amado et al., 1984, Jacob et al., 1996). The major reaction products of the free tyrosyl radical are the intensely fluorescent amino acids 3,3'-dityrosine (DT), 3,3',3'-trityrosine (TT) and pulcherosine (P), and the non-fluorescent isodityrosine (iso-DT) (Gross and Sizer, 1959; Amado et al., 1984, Jacob et al., 1996; Heinecke et al., 1993). DT and 3-nitrotyrosine levels are elevated in the hippocampus and neocortical regions of brains of patients with AD compared to the same regions of normal brain, and are also elevated in ventricular cerebrospinal fluid in AD patients (Hensley et al., 1998).

[0009] Tyrosine cross-linking may also be important in other neurodegenerative diseases such as Parkinson's disease, and other conditions in which α-synuclein fibrils are deposited. These include Parkinson's disease itself, dementia with Lewy body formation, multiple system atrophy, Hallerboden-Spatz disease, and diffuse Lewy body disease. Exposure of recombinant α-synuclein to nitrating agents results in nitration of tyrosine residues as well as oxidation of tyrosine to form DT; this results in cross-linking of α-synuclein to form stable aggregates (Souza et al, 2000). The same authors also found that monoclonal antibodies raised against nitrated synuclein bound specifically to Lewy bodies and to glial cell inclusions in a variety of synucleinopathies (Duda et al., in preparation referred to in Souza et al., 2000).

[0010] We have now found that human amyloid-derived Aβ contains tyrosine cross-links, and includes both dityrosine and trityrosine cross-linked species. These cross-links can be replicated *in vitro,* for example by incubating synthetic human Aβ with peroxidase and H_2O_2, or with H_2O_2 in the presence of copper ions. These modifications are protease-resistant, and therefore we propose that tyrosine cross-linkage in AD caused by abnormal interaction of Aβ with H_2O_2 and peroxidases or copper ions contributes to the formation of neurotoxic Aβ oligomers, and to the deposition of Aβ. Immunization against low molecular weight tyrosine cross-linked compounds rather than with whole Aβ can therefore be used for treatment or prevention of AD, without the risk of provoking autoimmune complications which could otherwise be induced by immunization with intact Aβ or large fragments thereof. By restricting the target for immunotherapy to an abnormal fragment or portion of the molecule, it may be possible to minimise undesirable interference with the normal

function of the molecule, while providing an active therapy against the abnormal molecule. It will be appreciated that either active or passive immunization may be used.

[0011] The oxidative processes which give rise to covalent cross-linking of proteins via tyrosine are also associated with other disorders which are characterised by pathological aggregation and accumulation of specific proteins. It is therefore considered that these conditions also will be amenable to prevention or treatment by the method of the invention.

SUMMARY OF THE INVENTION

[0012] The invention provides an Aβ peptide comprising a cross-linked tyrosine moiety for use in treating, preventing or alleviating Alzheimer's disease. The peptide may be an immunogenic portion of the pathologically aggregated form of Aβ, the peptide comprising a cross-linked tyrosine moiety linked to residues upstream and downstream of the cross-linked tyrosine, a dimer, trimer or tetramer of the Aβ peptide monomer.

[0013] Also disclosed herein is a method of prophylaxis, treatment or alleviation of a condition in which the condition is characterised by pathological aggregation and accumulation of a specific protein associated with oxidative damage and formation of tyrosine cross-links, the method comprising the step of immunizing a subject in need thereof with an immunizing-effective dose of one or more compounds selected from the group consisting of dityrosine, trityrosine, tetratyrosine (also known as pulcherosine), oxidised tyrosine orthologues such as o-tyrosine and m-tyrosine, nitrotyrosine, and peptides comprising tyrosine cross-links, and optionally also comprising copper ions complexed to the compound. These compounds are collectively referred to herein as "tyrosine cross-linked compounds".

[0014] A person of ordinary skill in the art will recognise that an immunizing-effective dose of the compound is one which will elicit antibody which is able to bind to a tyrosine cross-linked compound. Such a person will also be able to determine whether a particular tyrosine cross-linked compound elicits an antibody.

[0015] The pathologically aggregated form of the specific protein may comprise a tyrosine cross-linked moiety. The tyrosine cross-linked compound may be a peptide which is an immunogenic portion of the pathologically aggregated form of the specific protein, the peptide comprising a cross-linked tyrosine moiety linked to residues upstream and downstream of the cross-linked tyrosine.

[0016] The tyrosine cross-linked compound may be a dityrosine cross-linked compound.

[0017] Up to 3 equivalents of copper per equivalent of dityrosine may be used, provided that each dose administered contains no more than 1 μM copper.

[0018] Optionally the compound used for immunisation is coupled to a carrier protein which is itself immunogenic, such as tetanus toxoid, keyhole limpet haemocyanin, or albumin. Also optionally the compound may be administered together with an adjuvant such as alum, monophosphoryl lipid, a muramyl peptide, an iscom such as QS21 and the like. Persons skilled in the art will be well aware of suitable carriers and adjuvants.

[0019] The peptide comprising tyrosine cross-links is preferably a minimal and immunogenic portion of the particular protein associated with the condition, which is constituted by the dityrosine moiety linked to residues upstream and downstream and downstream of the cross-linked tyrosine. Preferably the peptide comprising tyrosine cross-links is derived from the sequence surrounding tyrosine 10 in the amino acid sequence of human $A\beta_{1-40}$ or $A\beta_{1-42}$.

[0020] It is preferred that the tyrosine cross-links are obtainable by oxidation in the presence of copper ions.

[0021] More preferably the peptide also comprises copper ions complexed to dityrosine.

[0022] Immunization according to the method disclosed above, or immunication of the Aβ peptide of the invention, may be by any convenient route, including subcutaneous, intramuscular or intravenous injection, application to mucosal surfaces, or topical administration, for example in an ointment.

[0023] The dose of the compound to be administered will vary, depending on the nature of the individual compound, the weight, age and general state of health of the patient, and whether an adjuvant is used. It is contemplated that the dose will be in the region of 0.1 μg to 200 mg of DT, more preferably 1 to 50 mg, most preferably 10 to 20 mg.

[0024] Although a single immunization may be given, preferably multiple immunizations are administered, for example once a week for one to twelve months, more preferably for four months. A booster series may be given after six to twelve months. The immune response is monitored by measuring DT antibodies; any convenient assay system may be used, such as ELISA.

[0025] In an optional embodiment of the method disclosed herein said method also comprises the additional steps of identifying the predominant forms of the tyrosine cross-links in the pathologically aggregated specific protein; and synthesising one or more tyrosine cross-linked compounds comprising one or more of the predominant forms of tyrosine cross-links. According to the invention, the Aβ peptide may preferably comprise one or more of the predominant forms of tyrosine cross-links in the pathologically aggregated form of Aβ.

[0026] Immunization may be passive in methods disclosed herein. Thus prophylaxis, treatment or alleviation of a condition, in which the condition is characterised by pathological aggregation and accumulation of a specific protein associated with oxidative damage and where the pathologically aggregated form of the specific protein comprises a tyrosine cross-link, the method comprising the step of administering an effective amount of an antibody or an antibody

fragment,
said antibody or antibody fragment is raised against a tyrosine cross-linked compound,
said compound being an immunogenic portion of the pathologically aggregated form of the specific protein and comprising a tyrosine cross-link ,
and which antibody or antibody fragment is capable of specifically binding the pathologically aggregated form of the specific protein,
to a subject in need of such treatment is disclosed.

[0027] The present invention provides the antibody or antibody fragment of claim 10 for use in preventing, treating or alleviating Alzheimer's disease wherein said antibody or antibody fragment is capable of specifically binding the pathologically aggregated form of Aβ.

[0028] The antibody may be polyclonal or monoclonal. Where the antibody is polyclonal, it is preferably of human origin, and may for example be derived from pooled human serum from normal healthy individuals. Alternatively serum from individuals who have been hyperimmunized against a tyrosine cross-linked compound may be used. Protocols for hyperimmunization are known in the art. The antibody may be isolated from serum by any convenient method; a variety of suitable methods is known in the art. Where the antibody is monoclonal, it is preferably humanized. It will be clearly understood that the use of antigen-binding fragments of antibodies, such as F(ab'), $F(ab')_2$ Fv or monoclonal scFv, are within the scope of the invention. Methods for production and purification of polyclonal and monoclonal antibodies and for recombinant production of humanized monoclonal antibodies or of scFv fragments are well known in the art. See for example Harlow and Lane (1988); WO90/07861; and WO92/01047. Humanized monoclonal antibodies may also be produced in transgenic mammals; see for example WO91/10741 and WO93/12227.

[0029] It is preferred that the antibody reacts specifically with the pathologically aggregated form of the specific protein, and does not react significantly with the unaggregated form of the protein.

[0030] Following either active or passive immunization, the patient is monitored for clinical improvement, which may commence within as little as one week, but more probably may be observed at six weeks, and may take as long as 12 months. The normal clinical indices which are used in the monitoring of patients with the relevant condition are used. The attending clinician will be aware of the most suitable tests to use.

[0031] Where the treatment is prophylactic, the patient is monitored for signs of development of the condition. The patient may be at risk as a result of genetic linkage, e.g. in familial Alzheimer's disease or Huntington's disease.

[0032] In a second aspect, therefore, the invention provides a prophylactic or therapeutic composition for use as a medicament, comprising an Aβ peptide comprising a cross-linked tyrosine moiety, and optionally further comprising an adjuvant, and/or copper ions complexed to the peptide, or an antibody which is raised against said Aβ peptide or a fragment thereof together with a pharmaceutically acceptable carrier. Also disclosed is a prophylactic or therapeutic composition for use in the method disclosed herein or as a medicament, comprising a tyrosine cross-linked compound, together with a pharmaceutically acceptable carrier and optionally further comprising an adjuvant, and/or copper ions complexed to the compound.

[0033] Further, a prophylactic or therapeutic composition for use in the passive immunization method disclosed herein or as a medicament, comprising an antibody directed against a tyrosine cross-linked compound as defined above, or a fragment thereof which is capable of binding to the tyrosine cross-linked compound, together with a pharmaceutically acceptable carrier is disclosed.

[0034] Also disclosed is a method of diagnosis of a condition, in which the condition is characterised by pathological aggregation and accumulation of a specific protein associated with oxidative damage and formation of tyrosine cross-links, the method comprising the step of assaying a sample of a biological fluid from a subject suspected of suffering from the condition for the presence of a compound selected from the group consisting of dityrosine, trityrosine, tetratyrosine, oxidised tyrosine orthologues such as o-tyrosine and m-tyrosine, nitrotyrosine, and peptides comprising tyrosine cross-links.

[0035] The method alternatively comprises the step of assaying a biological fluid from a subject suspected of suffering from the condition for the presence of antibody directed against a tyrosine cross-linked compound.

[0036] The invention provides a method of diagnosis of Alzheimer's disease the method comprising the step of assaying in vitro a sample of a biological fluid from a subject suspected of suffering from the condition for the presence of a molecule comprising tyrosine cross-links or an antibody directed against a molecule comprising tyrosine cross-links.

[0037] Preferably the biological fluid is selected from the group consisting of blood, plasma, serum, cerebrospinal fluid, urine, and saliva. Preferably the compound is di tyrosine.

[0038] The assay may be performed by any suitable means, but is most conveniently performed by an ELISA assay using antibody directed against tyrosine cross-linked compounds. Such an assay may conversely be used to detect antibody directed against a tyrosine cross-linked compound. Preferably the antibody is a monoclonal antibody, or a mixture of monoclonal antibodies. Alternatively the assay may be performed by measuring fluorescence at an excitation wavelength of 325 nm and an emission wavelength of 350-500 nm.

[0039] In the methods disclosed herein, preferably the condition is selected from the group consisting of Alzheimer's

disease, amyotrophic lateral sclerosis, motoneuron disease, cataract, Parkinson's disease, Creutzfeldt-Jacob disease, Huntington's disease, dementia with Lewy body formation, multiple system atrophy, Hallerboden-Spatz disease, and diffuse Lewy body disease, or cataract.

[0040] More preferably the condition is Alzheimer's disease or Parkinson's disease. The methods of the invention relate to Alzheimer's disease.

[0041] For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

BRIEF DESCRIPTION OF THE FIGURES

[0042]

Figure 1 shows that human Aβ, but not rat Aβ, develops fluorescence and SDS-resistance following peroxidase-catalyzed oxidation. Human $A\beta_{1-40}$. human $A\beta_{1-42}$, or rat $A\beta_{1-40}$ (50 μM) was incubated in 50 mM borate, pH 9.5 ± H_2O_2 (1 mM) and peroxidase (7.5 μg/ml), for 1 day at 37°C.

(A) fluorescent spectra (λ_{ex}325, λ_{exm}350-500);
(B) migration on SDS-PAGE (by Western blot using 4G8) ;
(C) $A\beta_{1-42}$ (10 nM) was incubated with H_2O_2 (1 μM)and peroxidase (7.5 μg/ml) for 5 days at 37°C in phosphate buffered saline, pH 7.4. The product (lane 2) was compared to peptide incubated under the same conditions in the absence of H_2O_2 /peroxidase (lane 1) by SDS PAGE and Western blot (4G8).

Figure 2 shows that human amyloid-derived Aβ contains tyrosine cross-linked oligomers. Human amyloid-derived Aβ (20 μM)(Roher et al., 1996) was analysed by fluorescence spectroscopy compared to a pure DT standard (λ_{ex} 325, λ_{ero} 350-500) (A), and Western blot (4G8) (B).

Figure 3 shows that dityrosine and trityrosine cross-links are present in human amyloid-derived Aβ, and that they bind copper.

(A) Human amyloid was purified, hydrolyzed and the mass spectrum determined after chromatographic separation. Two individual scans reflecting analyses of the same sample eluting at different chromatographic retention times (RT) are shown.
(B) Absorbances at 280 nm and 315 nm of purified DT in the presence of increasing concentrations of $CuSO_4$ or NaCl.

Figure 4 shows that soluble human Aβ binds copper with high affinity.

(A) Silver stain of crude soluble extract (1) and pH 1 eluate from the copper-chelating Sepharose column (2).
(B) Western blot of pH 1 eluate probed with WO2, G211 and G210.

Figure 5 shows the results of LC-MS analysis, confirming that human Aβ binds copper.

(A) LC-MS analysis of crude and IMAC purified soluble extracts.
(B) Mass spectra of $A\beta_{1\,41}$ and $A\beta_{1-40}$ with two bound copper atoms.
The IMAC and LC-MS data demonstrate that brain- derived Aβ can bind copper.

Figure 6 shows the detection of dityrosine in cross-linked $A\beta_{1-40}$ and $A\beta_{1-42}$ in Western blots.
Two techniques to create the dityrosine linkages are also compared.'
The top Western blot (A) demonstrates the presence of Aβ using the W02 antibody. The bottom blot (B) demonstrates the presence of dityrosine linkages recognised by the monoclonal antibody IC3. This antibody was raised against a form of dityrosine prepared using borate/H_2O_2 horseradish peroxidase.

Lane	1	$A\beta_{1-40}$	- borate cross linking
Lane	2	$A\beta_{1-42}$	- borate cross linking
Lane	3	$A\beta_{1-40}$	- copper cross linking
Lane	4	$A\beta_{1-42}$	- copper cross linking

(continued)

Lane	5	$A\beta_{1-40}$	- untreated
Lane	6	$A\beta_{1-42}$	- untreated
Lane	7	Dityrosine conjugated to KLH	

Figure 7 shows examples of the forms of tyrosine cross-links produced as potential immunogens. These structures contain tyrosine cross-links and have the carboxy- and amino-termini acetylated to mimic the presence of additional amino acid residues that would normally be present on either side of a tyrosine cross-linked moiety in a tyrosine cross-linked peptide. The presentation of multiple copies of the dityrosine antigen is designed to improve the strength of the immune response generated.

7A Tyrosine, Dityrosine and Atee
7B DiAtee, IsoDiAtee and TriAtee
7C TetraAtee and Alternate form of TriAtee with one iso bond.

Figure 8 shows the detection of dityrosine bonds in a variety of tyrosine cross-linked species in Western Blots. The DT-containing species include dityrosine cross-linked $A\beta_{9-16}$ is dimer or trimer linked to BSA, and various poly-DT species linked to either BSA or KLH carrier proteins. The top Western blot (A) demonstrates the ability of the sample to bind to a polyclonal rabbit anti serum raised against DT which was prepared using the borate/H_2O_2/peroxidase technique and linked to KLH using glutaraldehyde (discussed in Example 7). The bottom Western blot (B) demonstrates the presence of dityrosine linkages recognised by the monoclonal antibody IC3. This antibody was raised against a form of dityrosine also prepared using the borate/H_2O_2/peroxidase technique.

Lane 1 Aβ 9-16 DT dimer - BSA
Lane 2 Abeta 9-16 DT trimer - BSA
Lane 3 Crude ATEE - BSA
Lane 4 PolyTyr - BSA
Lane 5 BSA
Lane 6 Abeta trimer - KLH
Lane 7 Crude ATEE - KLH
Lane 8 PolyTyr - KLH
Lane 9 KLH

DETAILED DESCRIPTION OF THE INVENTION

[0043] The invention will now be described in detail by way of reference only to the following non-limiting examples and drawings.

[0044] Abbreviations used herein are as follows:

AD	Alzheimer's disease
DT	3,3'-dityrosine
TT	3,3'3'-trityrosine
P	pulcherosine
iso-DT	isodityrosine

EXPERIMENTAL PROCEDURES

Reagents and Aβ Peptide Preparation

[0045] Oligomeric Aβ was extracted from amyloid plaques of human AD-affected brains as previously described (Roher et al., 1996). The purified amyloid Aβ was solubilized in formic acid, and then immediately dialyzed with 5 changes of 100 mM ammonium bicarbonate, pH 7.5 before use.

[0046] Human $A\beta_{1-40}$, $A\beta_{1-42}$ and rat $A\beta_{1-40}$ were synthesized, purified and characterized by HPLC analysis, amino acid analysis and mass spectroscopy by W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT), and corroborative studies were performed using peptide synthesized by Quality Control Biochemicals,

Inc. (Hopkinton, MA).

[0047] Each peptide was identified as a single peak by HPLC. Synthetic Aβ peptides were dissolved in doubly deionized water at a concentration of 0.5-1.0 mg/ml, sonicated for 3 min. and then centrifuged for 20 min. at 10 000g and the supernatant (stock Aβ) used on the day of the experiment. The concentrations of stock Aβ peptides were determined by spectrophotometric absorbance at 214 nm or by Micro BCA protein assay (Pierce, Rockford, IL) as previously described (Atwood et al., 1998).

[0048] Prior to use, all buffers and stock solutions of metal ions were filtered though a 0.22 μm filter (Gelman Sciences, Ann Arbor, MI) to remove particulate matter. All other reagents were analytical grade or purer. Horseradish peroxidase was obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation and Fluorescence Analysis of Dityrosine and Tyrosine Cross-linked Aβ

[0049] DT standards were generated by incubating L-tyrosine (1 mg/ml) solubilized in borate buffer (50 mM, pH 9.5) with H_2O_2 (5 mM) and horseradish peroxidase (7.5 μg/ml) for 1 day at 37°C (Amado et al., 1984).

[0050] Cross-linked Aβ was generated by incubating Aβ (50 μM) in borate buffer (50 mM, pH 9.5) and with H_2O_2 (1 mM) and peroxidase (7.5 μg/ml) for 5 days at 37°C. In a separate experiment to study this reaction under conditions which approached physiological, $Aβ_{1-42}$ was diluted to 10 nM in phosphate-buffered saline (PBS, pH 7.4), and incubated with 1 μM H_2O_2 and peroxidase (7.5 μg/ml) for 5 days at 37 °C. Following the incubation, the samples were lyophilized to bring the peptide into a concentration range which could be detected by Western blot (see below).

[0051] Reaction products were separated by fast phase liquid chromatography (FPLC). Excess borate was first precipitated from samples prior to chromatography by centrifugation at 0°C. Samples were then acidified by addition of 0.25% TFA and remaining insoluble material removed by filtration (0.22 μm pore size). Samples were loaded on to a 3 ml Resource RPC column (Pharmacia, Uppsala, Sweden) and the column washed with water containing 0.1% TFA. Bound species were eluted with a 0-100% linear gradient of acetonitrile containing 0.1% TFA at 1 ml/min over 45 min and collected in 0.5 ml fractions. Fractions were dried, reconstituted in water and assayed for dityrosine by fluorescence (excitation 330 nm; emission 400 nm) and UV absorbance (284 nm). Peak fractions were further characterized by mass spectrometry, and dityrosine quantitated using the extinction coefficient (E_{315} nm = 8380 M^{-1} cm^{-1}; Malencik et al., 1996).

[0052] Solutions were analyzed for the presence of fluorescent compounds using a Hitachi F-4500 spectrofluorometer. DT, TT and P have characteristic emission spectra (λ_{ex} 325 nm, λ_{em} 350-500 nm), which are quite distinct from those of tyrosine and tryptophan, which do not fluoresce at these wavelengths. There was a linear increase in fluorescence at this emission range with increasing dityrosine concentration between 0-50 μM.

MALDI-TOF Mass Spectrometry

[0053] Samples of SDS-resistant, oligomeric, human amyloid-derived Aβ were hydrolyzed *in vacuo* with 6N HCl for 48 h at 105 °C. Following this, samples were analyzed by liquid chromatography MALDI-TOF mass spectrometry (LC-MS) at the Harvard University Mass Spectrometry Facility.

[0054] Mass spectra were obtained using a LCT mass spectrometer (Micromass Inc, Beverly MA) interfaced with a HP 1100 liquid chromatograph, attached to a C18 reversed-phase column (2.1mm x 250 mm). LC-MS was performed using a gradient of buffer A (water-0.1% formic acid (FA)), and buffer B (acetonitrile-0.1% FA). The gradient was from 2 % B (0- 2 min), to 100 % B (20 - 23 min).

Western Blot Analysis

[0055] Aliquots of each reaction (2 ng peptide) were collected into 15 μl sample buffer (containing 4% SDS, 5 % β-mercaptoethanol) and heated to 95°C (5 min). Samples were run on PAGE (Tricine gels, 10-20%; Novex, San Diego, CA), transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA), fixed with glutaraldehdye (1%, v/v), blocked with milk (10 %, w/v) and then probed with the anti-Aβ monoclonal antibody 4G8 (Senetek, Maryland Heights, MI) overnight at 4°C. In one experiment the monoclonal antibodies WO2 (epitope:residues 5-8), G211 (epitope:residues 35-42) or G210 (epitope:residues 33-40) were used. The blot was then incubated with anti-mouse horseradish peroxidase (HRP) conjugate (Pierce, Rockford, IL) for 2 h at room temperature, and developed with ECL reagent (Amersham, Little Chalfont, UK) or Supersignal Ultra (Pierce, Rockford, IL). The chemiluminescent signal was captured using the Fluoro-S Image Analysis System (Bio-Rad, Hercules, CA) and electronic images analyzed using Multi-Analyst Software (Bio-Rad, Hercules, CA). Molecular size markers were from Amersham (Arlington Heights, IL).

Example 1: Peroxidase-catalyzed Aβ polymerization is accompanied by formation of tyrosine cross-links

[0056] We initially tested whether peroxidase-catalyzed oxidative conditions could promote Aβ polymerization by

measuring the fluorescence of human $A\beta_{1-40}$, human $A\beta_{1-42}$, and rat $A\beta_{1-40}$ (50 μM) incubated with or without H_2O_2 and peroxidase for 1 day. Fluorometric analysis of these samples indicated a marked increase in fluorescence in samples containing $A\beta_{1-40}$ and $A\beta_{1-42}$, as illustrated in Figure 1A. These results are similar to those previously reported for synthetic human Aβ, achieved at a much higher peptide concentration, 1.25 mM (Galeazzi et al., 1999). In contrast to the behaviour of the human-sequence Aβ peptide, no increase in the fluorescence signal of rat $A\beta_{1-40}$ was observed after incubation with H_2O_2 and peroxidase, as also shown in Figure 1A. This suggested that the fluorescent signal was specific for tyrosine.oxidation products of Aβ, since rat Aβ lacks tyrosine (Shivers et al., 1988).

[0057] To confirm that these reactions resulted in Aβ polymerization, $A\beta_{1-40}$ and $A\beta_{1-42}$ treated as described above were run on SDS-PAGE and analyzed by Western blot. Both human synthetic $A\beta_{1-40}$ and $A\beta_{1-42}$ incubated with H_2O_2 and peroxidase displayed marked increases in apparent SDS-resistant polymers compared to untreated Aβ, as shown in Figure 1B. Neither polymerization nor increased fluorescence was observed when Aβ was incubated with either H_2O_2 or peroxidase alone.

Example 2: polymerization occurs under physiological condi tions

[0058] To determine whether H_2O_2 /peroxidase- induced polymerization of synthetic Aβ occurs under conditions which approached physiological, we also incubated $A\beta_{1-42}$ at 10 nM with H_2O_2 at 1μM and peroxidase (7.5 μg/ml) in PBS at pH 7.4. We observed that SDS-resistance of the peptide was again induced, as shown in Figure 1C; however, oligomers of lower apparent molecular weight than those generated by using higher concentrations of substrates were generated, as illustrated in Figure 1B. The migration on SDS-PAGE of the apparent Aβ polymers under these conditions suggested the formation of dimers (8 Kd), trimers (13 kD,) and tetramers (17 kD).

[0059] As shown in Figure 2A and Figure 2B respectively, fluorescent analysis of Aβ purified from AD-affected post-mortem brain tissue revealed the characteristic spectrofluorometric pattern of tyrosine cross-linked species; this purified protein migrated as apparent oligomers on SDS-PAGE, as previously described (Roher et al., 1996).

Example 3: Tyrosine cross-linking of oligomers

[0060] To confirm that the apparently oligomeric human amyloid-derived Aβ was tyrosine cross-linked, a sample was hydrolyzed and then analyzed by MALDITOF-MS. This analysis, illustrated in Figure 3A, indicated a peak corresponding to 361 Da (m/z 361, representative of M + H), thereby confirming the existence of DT or iso-DT in the sample. A smaller peak corresponding to 540 Da was also detected, consistent with the presence of TT or P. Other prominent peaks were detected at 247, 263, 307, 309 and 538 Da; these may represent other modifications to Aβ amino acids, such as carbonylation (Atwood, 1999) and other amino acid cross-links.

[0061] More abundant fragments from the hydrolysis of human Aβ were also detected at 423 and 425 Da (ratio 3:2), suggestive of Cu binding to DT or iso-DT (Cu mass = 63 & 65 Da, =2:1 natural isotope abundance).

Example 4: Binding of copper by dityrosine

[0062] In order to test whether the peaks at 423 and 425 could be due to DT binding to Cu, we examined the interaction of Cu^{2+} with DT by spectroscopic analysis. Dityrosine (50 μM) was solubilized in phosphate buffer (50 mM, pH 7.4) and the absorbance spectra (200 - 1000 nm) measured on a SPECTRAmax Plus (Molecular Devices). A trough (280 nm) and peak (315 nm) were apparent. Dityrosine was then incubated with increasing concentrations of $CuNO_3$ (0-200 μM) or NaCl (0-200 μM), and changes in absorbance at both 280 nm and 315 nm were monitored.

[0063] We found that as DT was incubated with increasing concentrations of Cu^{2+} its characteristic absorbance peak at 315 nm diminished, whereas a new absorbance peak developed at 280 nm. The spectroscopic changes reached a plateau at a stoichiometric ratios between 1:1 - 2:1 (Cu:DT), and then saturated at 3:1, suggesting that DT can bind up to 3 equivalents of Cu. Dichloride binding would also produce a similar p + 2 mass unit increment (Cl mass = 35 and 37 Da, ≈3:1 natural isotope abundance), but coincubating DT with NaCl induced no spectroscopic absorbance changes. These results are shown in Figure 3C.

Example 5: Dityrosination of Aβ increases its copper-binding capacity

[0064] We predicted that a proportion of the Aβ found in the soluble fraction of human brain would display enhanced copper binding properties due to dityrosination. To test whether this was in fact the case, we passed a portion of soluble extract of AD-affected brain over a chelating Sepharose column charged with copper. 0.5 g of cerebral cortex grey matter from frozen AD and control brains (AC) was homogenised in 3 ml of .ice cold phosphate buffered saline (PBS). Samples were centrifuged at 175 000 g for 1 hour and the supernatant retained for analysis of Aβ content. 10ml of supernatant was loaded onto a chelating Sepharose column charged with 1mg/ml copper sulphate. Unbound proteins were washed

through using a 0.05M Na acetate buffer with 0.5M NaCl at pH 8. The bound material eluted in a stepwise gradient of increasing acidity, using successive steps of pH 5.5, 3 and 1, followed by a wash with 50mM EDTA to strip the column. Eluates were subjected to exhaustive dialysis to remove free copper and salts using a size cutoff of 2kDa, freeze-dried and subjected to SDS-PAGE, Western blot and LC-MS analyses. ESI mass spectra (+ ve ion) were acquired on a Quatro II triple quadrupole (Micromass). Mass spectra were collected in continuum mode every 8 seconds from 650 to 1650 m/z. Samples were introduced to the ion source in 5mM ammonium acetate buffer. Slot blot analysis showed no WO2 immunoreactivity in the pH3 eluate, and a further elution was performed at pH 1. Strong immunoreactivity was detected at this pH, and the dialysed sample was blue in colour.

[0065] Western blot analysis revealed the presence of Aβ in the pH 1 and EDTA fractions; this suggested very high-affinity binding to copper, since pH 3 is usually sufficient to elute most copper-binding protein from such a column. Material in these fractions was shown to be highly enriched in oligomeric Aβ. These results are illustrated in Figure 4.

[0066] Silver staining (Figure 4A) demonstrated substantial metal affinity-based purification (lane 1 vs. 2), and Western blot analysis displayed immunoreactive bands which appear to correspond to multiples of monomeric Aβ (Figure 4B). Figure 5 shows LC (top) and MS (bottom) traces from crude and IMAC-purified supernatant extracts from AD brain tissue. It is noticeable that the LC and MS spectra are substantially cleaner for the IMAC purified sample. LC-MS analysis of the IMAC purified sample produced signals corresponding to Aβ species, including $A\beta_{1-40}$ bearing 2 copper atoms, as confirmed by LC-MS analysis of synthetic peptide in the presence or absence of copper. Highlighted peak clusters on representative mass spectra indicate mass/charge ratios consistent with parent ions of masses 4515.1 ($A\beta_{1-42}$) and 4457.9 ($A\beta_{1-40}$ +2 Cu).

[0067] In order to confirm whether this strongly copper-binding Aβ fraction contained DT, we employed the monoclonal antibody IC3 raised against DT generated by a process using H_2O_2 and horseradish peroxidase (Kato et al. (1998); this was the gift of Dr. Yoji Kato of the Himeji Institute of Technology, Himeji, Japan.). We found that the higher molecular weight oligomers of Aβ observed on Western blot co-localised with positive staining for DT.

[0068] The Aβ containing fractions also exhibited fluorescence emission spectra characteristic of the presence of the dityrosine moiety. This emission was quenched by the addition of copper in a fashion predicted for the enhanced copper binding due to this modification.

*Example 6: Further characterisation of dityrosinated A*β

[0069] DT-enriched Aβ is isolated from the soluble fraction of human brain in sufficient quantity to carry out further characterisation. These studies include toxicity studies in tissue culture, amino acid sequencing, metal binding studies, and experiments to determine whether DT-enriched Aβ has enhanced electrochemical activity, for example induction of hydrogen peroxide formation and copper reduction.

Example 7: Effect of immunization against dityrosine

[0070] We attempted to raise an immune response to DT in wild-type mice. In this experiment the DT was prepared by mixing tyrosine in borate buffer with H_2O_2, and incubating this mixture with horseradish peroxidase, as described in the Experimental Procedures.

[0071] DT was conjugated to the carrier protein Keyhole Limpet Haemocyanin (KLH) using glutaraldehyde and according to standard protocols. An emulsion of each of DT-KLH, KLH alone or untreated tyrosine was prepared in Freund's complete adjuvant, and two animals each were inoculated intraperitoneally with an inoculum containing 100mg of either DT-KLH, or unreacted tyrosine or KLH alone. Pre-immune serum was taken at this time. The first immune sera were collected 10 days after immunization. Two booster immunizations were given at fortnightly intervals thereafter. Blood samples were taken at each inoculation and at one week following the final boost.

[0072] An ELISA was adapted to assay the immune response to DT. We found that the immune responses to DT of the mice which were immunized with either DT-KLH or unreacted tyrosine were never greater than the responses of mice immunized with KLH alone. The DT monoclonal antibody IC3 obtained from Dr. Kato was used as a positive control, and produced a modest positive reaction against DT in this assay.

[0073] In a second experiment, two rabbits were immunized with DT-KLH in the manner described above. The ELISA results for sera produced by these animals demonstrated a moderate immune response against DT.

[0074] We also attempted to demonstrate the presence of endogenous antibodies to DT in individual sera from four human patients who were diagnosed with Alzheimer's disease by post mortem histopathology. No immunoreactivity against DT was observed in these sera by ELISA or by Western blot.

[0075] In a further experimental iteration, we examined whether the mouse or rabbit antisera raised against the DT-KLH described above, recognised DT moieties in the dimeric and higher order oligomers of Aβ extracted from human brain. Surprisingly, none of the sera demonstrated activity against DT moieties in human brain Aβ. The positive control antibody IC3 was also negative in this assay.

Example 8: Effect of the method of producing dityrosine moieties on immunogenicity and antibody reactivity.

[0076] We suspected that the unexpected lack of an immune response might be due to poor antigenicity of the dityrosine moieties.

[0077] To investigate this hypothesis, we prepared tyrosine cross-linked synthetic $A\beta_{1-40}$ and $A\beta_{1-42}$ by two different methods. The first method involved incubation of the Aβ peptides in borate buffer with horseradish peroxidase and H_2O_2, as described in the Experimental Procedures above.

[0078] In the second method, a 2.5μM solution of Aβ was prepared in double deionised water containing 30μM $CuCl_2$ and 200μM H_2O_2, and incubated for one to five days at room temperature.

[0079] Samples of each variety of cross-linked Aβ were subjected to PAGE, and Western blotting was performed using the Aβ-specific antibody W02 or the positive control anti-DT antibody IC3. The results of these blots are presented in Figure 6.

[0080] The IC3 antibody detected DT in the cross-linked Aβ in both ELISA and Western blot assays. In addition, in Western blots the antibody recognised the presence of dityrosine in the DT-KLH produced in Example 7. From these results it appears that $A\beta_{1-42}$ is more efficiently cross-linked by either the borate or copper methods than is $A\beta_{1-40}$. In addition, $A\beta_{1-40}$ loses immunoreactivity to WO2 when cross-linked with the method involving copper. This may be due to greater susceptibility of the peptide to free radical damage or the modification, masking or hindering of the antibody binding site after crosslinking.

[0081] Surprisingly, it is also evident from the differential staining with IC3 that the pattern of Aβ cross-linking through dityrosine depends on the different reactions used to produce the crosslinking. The IC3 monoclonal antibody did not detect DT produced by the boric acid method, but did detect DT produced by the copper method.

[0082] Also surprisingly, the IC3 antibody detected DT cross-linking in $A\beta_{1-40}$ in preference to $A\beta_{1-42}$. This pattern is the inverse of that observed with the anti Aβ antibody W02.

[0083] These results demonstrate that the method of inducing DT cross-linking and the structure of the polypeptide being cross-linked are crucial variables in recognition of DT by an antibody. In this case, the addition of two amino acid residues to dityrosine-linked $A\beta_{1-40}$ resulted in a dramatic decrease in the ability of an anti-dityrosine antibody to bind. This result may be extrapolated to the *in vivo* situation, suggesting that the selection of antigen is critical to eliciting a physiologically-relevant immune response.

Example 9: Effect of *the form of tyrosine cross-link on antibody recognition*

[0084] It was anticipated that a DT inoculum must be conjugated to a large carrier protein to provoke an immune response. Furthermore, the quality of the immune response generated would also be in part dependent upon the selection of an appropriate carrier. To examine this we selected two alternative carriers for various DT species, Bovine Serum Albumin (BSA) and Keyhole Limpet Haemocyanin (KLH).

[0085] In addition, to investigate the role of different forms of dityrosine in immuno-recognition, we prepared a crude mixture which contained variety of forms of DT, including numerous oligomers and branched forms of DT. The tyrosine cross-links in this crude mixture were created using the borate/H_2O_2/peroxidase method described above. The resulting DT mixture contained molecules with linkages at a variety of positions on the ring and backbone of the tyrosine molecule. Examples of the structures produced are illustrated in Figure 7.

[0086] The crude mixture was then separated by reverse phase HPLC into fractions which contained predominantly mono-dityrosine, dityrosine, trityrosine and polytyrosine.

[0087] Two important characteristics of the oligomeric structures are that they can present multiple copies of desired antigen to improve immunogenicity and enhance the immune response, and that they can allow the presentation of alternative forms of chemical bonds between the tyrosine residues.

[0088] To investigate the nature of the tyrosine cross-links which comprise the oxidative modifications to Aβ in vivo in AD, we also prepared tyrosine cross-linked Aβ fragments. Using the same technique, we prepared molecules consisting of two or more $A\beta_{9-16}$ peptide chains cross-linked by dityrosine (structures not shown). The resultant cross-links most probably represent a racemic mixture of a variety of forms of tyrosine cross-links.

[0089] A number of the novel structures described above were characterised in Western blots using the anti-DT monoclonal IC3 or the immune serum from a rabbit which was immunized with DT-KLH (described in Example 7). These results are presented in Figure 8.

[0090] The results demonstrated that the dimer but not the trimer of $A\beta_{9-16}$ linked to BSA was immunoreactive to both the rabbit immune serum and the monoclonal antibody IC3.

[0091] The presence of KLH was recognised by the rabbit immune serum in the blots irrespective of whether it was conjugated to an additional tyrosine cross-link antigen. Polytyrosine-BSA and polytyrosine-KLH were recognised by IC3, but the rabbit immune serum could not distinguish between KLH alone and polytyrosine-KLH.

[0092] It is clear from these results that the rabbit immunization elicited an antibody which was reactive with some

forms of dityrosine but not others, as predicted from the data presented in Figure 6.

Example 10: Effect of immunization with dityrosine on Aβ deposits in transgenic animals.

[0093] Transgenic mouse models are available for a number of neurological disorders, including Alzheimer·s disease (Games et al., 1995; Hsiao et al., 1996); Parkinson's disease (Masliah et al., 2000); familial amyotrophic lateral sclerosis (ALS) (Gurney et al., 1994); Huntington's disease (Reddy et al., 1998); and Creutzfeld-Jakob disease (CJD) (Telling et al., 1994).

[0094] We have found that one of the transgenic models for Alzheimer's disease, the APP2576 tg mouse (Hsiao et al., 1996) also has a high incidence of cataract. These animal models are suitable for testing the methods of the invention.

[0095] Transgenic mice of the Strain APP2576 (Hsiao et al 1996) are used. Eight to nine month old female mice are selected and divided into groups for treatment.

[0096] Tyrosine cross-linked antigens are prepared using a variety of techniques to generate different forms of tyrosine cross-links. Antigens used include:

Antigen	Carrier protein
$A\beta_{9-16}$ dimer	BSA
$A\beta_{9-16}$ trimer	BSA
(crude) ATEE	BSA
poly-tyrosine	BSA
Aβ trimer	KLH
(crude) ATEE	KLH
poly-tyrosine	KLH

[0097] Each immunisation comprises 25μg of antigen in Freund's complete adjuvant, in a total volume of 0.5ml, given subcutaneously.

[0098] Control animals received carrier protein without the tyrosine cross-linked antigen.

[0099] Samples of serum are taken at 14 day intervals, with booster immunizations given at 28 days. Serum samples are assayed for the presence of anti-DT antibody, using the ELISA method of Kato et al for example. It is expected that high antibody titres are obtained by about five weeks following the final booster injection. The levels of Aβ in the blood are also determined.

[0100] Once high titre antibody is present, mice are sacrificed at intervals, and their brains examined to determine whether the immunization decreases brain amyloid formation, and to identify the most effective immunization protocol. The levels of soluble and insoluble Aβ in the brain and serum is determined using calibrated Western blots. The Aβ plaque burden in the brain is examined immunohistochemically.

[0101] Other mice in each group are tested over a period of up to eight months for cognitive performance using a Morris water maze according to standard methods. The general health and well being of the animals is also measured every day by a blinded operator using a five point integer scale that subjectively rates a combination of features including motor activity, alertness and general health signs.

Example 11 : Effect of treatment with antibodies against dityrosine

[0102] Normal mice are hyperimmunized by standard procedures well known in the art with one or more of the immunogens described in Example 7. The mice are bled at intervals and their sera assayed for anti-DT as described above. Upon detection of high titre antibody, sera are harvested and the antibody component isolated and/or enriched using methods commonly available in the art.

[0103] These antibodies are injected intravenously or directly into the CSF of APP2576 transgenic mice, either in a single dose or repeated dosages over a course of days or weeks.

[0104] The transgenic mice are sacrificed at intervals following treatment with anti-dityrosine antibodies, and their brains examined to determine whether antibody treatment decreases brain amyloid formation.

Example 12: Diagnosis of conditions associated with tyrosine cross-linking

[0105] Samples of sera and cerebrospinal fluid (CSF) from patients confirmed to be suffering from AD and from age-

matched controls are assayed for the presence of tyrosine cross-linked compounds using fluorescence analysis as described above. In one set of samples, tyrosine cross-linked compounds in the sample are first enriched by passing the sample over a solid support coupled to nitrilotriacetic acid, as described in U.S. Patent No. 5972674.

[0106] Similar assays are performed using samples from patents suffering from ALS, Parkinson's disease, and CJD.

[0107] It is possible that patients may also have circulating antibodies directed against tyrosine cross-linked compounds, and so in an alternative assay such antibodies are directed in either sera or CSF using an ELISA assay, employing monoclonal antibodies directed against DT (Kato et al., 1998).

*Example 13: Identification of the forms of dityrosine present in oxidatively-modified A*β

[0108] In order to identify the predominant form or forms of DT present in oxidatively modified Aβ, enzymatic digestion fragments of copper-catalysed Aβ oligomers are generated, and the fragments analysed by mass spectrometry. This technique has recently been applied to the analysis of copper-catalysed oxidative modifications to the prion protein (Requena, J.R., et al. 2001 PNAS 98: 7170-7175)

[0109] This enables the identification of the antigen most likely to be effective in eliciting monoclonal antibodies suitable for use in passive immunization, as described in Example 11. Methods for generating highly specific monoclonal antibodies against any specific antigen are well known in the art. Once the antigen has been selected, a systematic analysis of the most effective means of antigen presentation is carried out using known methods.

DISCUSSION

[0110] The neuronal damage in AD is associated with soluble Aβ rather than insoluble Aβ which is immobilized in neuritic plaques (McLean et al., 1999). We have now shown for the first time that the neurotoxic Aβ oligomers extracted from AD-affected brains contain tyrosine cross-links), which may be DT, iso-DT, TT and/or P. These modifications were emulated *in vitro* by incubating Aβ with peroxidase and H_2O_2, or by oxidation of Aβ in the presence of copper ions. These modifications could interfere with the metabolism of Aβ, may contribute to the neurotoxicity seen in AD, and is indicative of the neurochemical derangement in the disease.

[0111] The formation of the carbon-carbon bridge between DT, T and P is thought to be irreversible; DT cross-links are very resistant to hydrolytic cleavage by 6N HCl at 110°C for 24h, and to protease digestion (Smail et al., 1995). Pathologically, the catabolic resistance of DT modifications of proteins could explain the contribution of tyrosine polymers to lipofuscin formation (Kato et al., 1998), and to the cross-linking of α-crystallin in fluorescent cataract formation (Kikugawa et al., 1991). Clearly, tyrosine cross-linkage of Aβ would be expected to inhibit its catabolism, and so may be an important step in the evolution of amyloid plaque deposits in AD.

[0112] The formation of tyrosine cross-links necessitates that molecules containing tyrosyl radicals come into contact. Our results suggest that the tyrosine residue of Aβ must be accessible to peroxidase(s), and that tyrosyl residues between Aβ subunits of amyloid must, at some stage, be in apposition.

[0113] Since H_2O_2 is required for DT formation, the detection of DT modifications in AD-derived brain Aβ implies that H_2O_2 is elevated in the brain in AD. Without wishing to be bound by any proposed mechanism, we believe that phagocytic activation of the microglial cells in the brain parenchyma, which is closely associated with amyloid formation in AD (Sheng et al., 1997), could contribute peroxidase activity and H_2O_2 to cause tyrosine cross-linkage of Aβ. Activated rat microglia have been observed to have increased peroxidase levels (Lindenau et al., 1998), and in vitro experiments have demonstrated the capacity of Aβ to prime and/or trigger the respiratory burst of cultured rat microglia and human phagocytes (Van Muiswinkel et al., 1996). Activated phagocytes release myeloperoxidase (Pember et al., 1983), and generate reactive oxygen species during the respiratory burst. This response is designed to kill invading pathogens or tumor cells; however, this environment has also been shown to promote the oxidation of surrounding proteins and lipids (Byun et al., 1999). A similar microenvironment may be generated in the vicinity of activated microglia. In vitro, myeloperoxidase-H_2O_2 systems promote the synthesis of tyrosine cross-linked species such as DT, TT, P and isoDT (Jacob et al., 1996).

[0114] Thus the activation of microglia in response to Aβ accumulation may promote tyrosine cross-linkage of the Aβ, inhibiting its clearance and leading to a vicious cycle. Contributing to this possible vicious cycle, a proximate source of H_2O_2 for DT formation may be generated by Aβ itself, since Aβ forms H_2O_2 by reacting with O_2 through the reduction of substoichiometric amounts of Cu^{2+} or Fe^{2+} (Huang, Atwood, et al., 1999; Huang, Cuajungco, et al., 1999). Therefore, it is highly significant that Aβ was purified intact, together with bound copper, from human amyloid (Fig. 3A). Synthetic $A\beta_{1-42}$ binds Cu^{2+} with attomolar affinity, and since copper is enriched in AD amyloid (Lovell et al., 1998), we had suspected that Aβ might bind copper in vivo. The finding that amyloid-derived Aβ contains copper is also relevant to AD pathophysiology, because Cu^{2+} precipitates Aβ (Atwood et al., 1998), and the toxicity of the peptide is potentiated by Cu^{2+} (Huang, et al., 1999).

[0115] Intriguingly, Cu^{2+} remained bound to DT after acid hydrolysis of the human amyloid-derived Aβ, as well as under the acidic conditions of the mass spectrometry (Fig. 3A). This unusual affinity for Cu^{2+} could be the result of an

adventitious high-affinity Cu^{2+} binding site on Aβ being formed by the DT modification. As a consequence of this exaggerated affinity for cu^{2+}, the neurotoxicity of DT-modified Aβ or its electrochemical activity may be increased compared to non-modified Aβ. Adventitious Cu^{2+} binding caused by the DT modification could also exaggerate the precipitation of Aβ into amyloid, which would explain why treatment with chelators at pH 7.4 promoted the release of dimeric Aβ to a greater extent than that of monomeric Aβ (assayed by Western blot) from post-mortem AD brain tissue (Cherny et al., 1999). The combination of increased proteolytic resistance and adventitious metal binding may be particularly pernicious consequences of the tyrosine cross-linking of Aβ which contribute to the pathology of AD.

[0116] PDAPP transgenic mice overproduce the human form of $Aβ_{1-42}$ and show extensive cerebral amyloid plaque deposition with aging, as well as behavioural and cognitive deficits (Games et al., 1995; WO96/40896). Immunisation of mature PDAPP mice with synthetic $Aβ_{1-42}$ results in a striking diminution in the number and intensity of amyloid plaques, while PDAPP mice immunised with this antigen fail to develop amyloid plaques (Schenk et al., 1999 and WO99/27944). It appeared that a successful immune response to $Aβ_{1-42}$ had been induced, with evidence of scavenging microglial cells in the immediate vicinity of the remnant amyloid plaques, and the presence in blood of antibodies directed against $Aβ_{1-42}$ The authors suggested that immunization with Aβ could be used for prevention or treatment of AD. However, it is widely thought that it is unlikely that an immunotherapy for AD is feasible, because a human recipient would be unable to mount a significant immune response to a self protein because of immunological tolerance. The results obtained by Schenk et al. suggest that the brain may have the capacity to resorb and clear otherwise intractable amyloid deposits, given the appropriate stimulus. However, it is undesirable to use immunisation with Aβ itself, because of the potential for induction of harmful autoimmune responses, and/or the induction of an inadequate, non plaque-clearing response. By immunising with non-native dityrosine or dityrosine-containing compounds according to the present invention, this problem can be avoided.

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Claims

1. An Aβ peptide oligomer comprising a cross-linked tyrosine moiety for use in treating, preventing or alleviating Alzheimer's disease.

2. The Aβ peptide oligomer for use according to claim 1, which is an immunogenic portion of the pathologically aggregated form of Abeta, the peptide comprising a cross-linked tyrosine moiety linked to residues upstream and downstream of the cross-linked tyrosine.

3. The Aβ peptide for use according to claim 1 or 2, which is a dimer, trimer or tetramer of an Abeta peptide monomer.

4. The Aβ peptide for use according to any one of claims 1 to 3, which is a dimer of a peptide monomer selected from the group consisting of human Abeta $_{1-40}$, Abeta $_{1-42}$, and Abeta $_{9-16}$.

5. The Aβ peptide oligomer for use according to any one of claims 1 to 4, in which the tyrosine cross-links in the peptide are obtainable by oxidation in the presence of copper ions.

6. The Aβ peptide oligomer for use according to any one of claims 1 to 5 which is coupled to a carrier protein which is itself immunogenic.

7. The Aβ peptide oligomer for use according to claim 6, in which the carrier protein is selected from the group consisting of tetanus toxoid, keyhole limpet haemocyanin, and albumin.

8. The Aβ peptide oligomer for use according to any one of claims 1 to 7, in which the peptide is formulated for administration together with an adjuvant.

9. The Aβ peptide oligomer for use according to any one of claims 1 to 8, wherein said peptide comprises one or more of the predominant forms of tyrosine cross-links in the pathologically aggregated form of Abeta.

10. An antibody which is raised against an Aβ peptide as defined in any one of claims 1 to 7 or 9, or an antibody fragment thereof for use in preventing, treating or alleviating Alzheimer's disease, wherein said antibody or antibody fragment is capable of specifically binding the pathologically aggregated form of Aβ.

11. The antibody or antibody fragment for use according to claim 10, wherein said antibody is polyclonal.

12. The antibody or antibody fragment for use according to claim 10 or 11, wherein said antibody is of human origin.

13. The antibody or antibody fragment for use according to claim 10, in which the antibody is monoclonal.

14. The antibody or antibody fragment for use according to claim 16, in which the antibody is humanized.

15. A prophylactic or therapeutic composition for use as a medicament comprising an peptide was defined in any one of claims 1 to 9, or an antibody or antibody fragment of any one of claims 10 to 14, together with a pharmaceutically acceptable carrier.

16. A prophylactic or therapeutic composition for use according to claim 15, further comprising an adjuvant.

17. The use of an Aβ peptide as defined in any one of claims 1 to 9 , or an antibody or antibody fragment of any one of claims 10 to 14 in the manufacture of a medicament for treating, preventing or alleviating Alzheimer's disease.

18. A method of diagnosis of Alzheimer's disease, comprising the step of assaying in vitro a sample of a biological fluid from a subject suspected of suffering from the condition for the presence of a molecule comprising tyrosine crosslinks or an antibody directed against a molecule comprising tyrosine crosslinks.

19. The method of claim 18 wherein said molecule comprising tyrosine crosslinks is an Aβ peptide as defined in any one of claims 1 to 6 or 9.

20. The method according to claim 19, wherein the Aβ peptide comprises dityrosine.

21. The method according to any one of claims 18 to 20, in which the biological fluid is selected from the group consisting of blood, plasma, serum, cerebrospinal fluid, urine and saliva.

Patentansprüche

1. Aß-Peptid-Oligomer, umfassend einen vernetzten Tyrosinrest für die Verwendung bei der Behandlung, Vorbeugung oder Linderung von Morbus Alzheimer.

2. Aß-Peptid-Oligomer für die Verwendung nach Anspruch 1, wobei es sich um einen immunogenen Abschnitt der pathologisch aggregierten Form von Abeta handelt, wobei das Peptid einen vernetzten Tyrosinrest umfasst, der an Reste stromaufwärts und stromabwärts des vernetzten Tyrosins gebunden ist.

3. Aß-Peptid für die Verwendung nach Anspruch 1 oder 2, wobei es sich um ein Dimer, Trimer oder Tetramer eines Abeta-Peptid-Monomers handelt.

4. Aß-Peptid für die Verwendung nach einem der Ansprüche 1 bis 3, wobei es sich um ein Dimer eines Peptid-Monomers, ausgewählt aus der Gruppe bestehend aus human-$Abeta_{1-40}$, $Abeta_{1-42}$ und $Abeta_{9-16}$ handelt.

5. Aß-Peptid-Oligomer für die Verwendung nach einem der Ansprüche 1 bis 4, bei dem die Tyrosin-Vernetzungsstellen in dem Peptid durch Oxidation in Gegenwart von Kupferionen erhältlich sind.

6. Aß-Peptid-Oligomer für die Verwendung nach einem der Ansprüche 1 bis 5, das mit einem Trägerprotein, das selbst immunogen ist, gekoppelt ist.

7. Aß-Peptid-Oligomer für die Verwendung nach Anspruch 6, wobei das Trägerprotein aus der Gruppe bestehend aus Tetanus-Toxoid, Keyhole Limpet Haemocyanin und Albumin ausgewählt ist.

8. Aß-Peptid-Oligomer für die Verwendung nach einem der Ansprüche 1 bis 7, wobei das Peptid für die Verabreichung gemeinsam mit einem Hilfsstoff formuliert ist.

9. Aß-Peptid-Oligomer für die Verwendung nach einem der Ansprüche 1 bis 8, wobei das Peptid eine oder mehr der vorherrschenden Formen von Tyrosinvernetzungen in der pathologisch aggregierten Form von Abeta umfasst.

10. Antikörper gegen ein Aß-Peptid wie in einem der Ansprüche 1 bis 7 oder 9 definiert oder Antikörperfragment davon, für die Behandlung, Vorbeugung oder Linderung von Morbus Alzheimer, wobei der Antikörper oder das Antikörperfragment spezifisch an die pathologisch aggregierte Form von Aβ zu binden vermag.

11. Antikörper oder Antikörperfragment für die Verwendung nach Anspruch 10, wobei der Antikörper polyklonal ist.

12. Antikörper oder Antikörperfragment für die Verwendung nach Anspruch 10 oder 11, wobei der Antikörper menschlichen Ursprungs ist.

13. Antikörper oder Antikörperfragment für die Verwendung nach Anspruch 10, wobei der Antikörper monoklonal ist.

14. Antikörper oder Antikörperfragment für die Verwendung nach Anspruch 16, wobei der Antikörper humanisiert ist.

15. Prophylaktische oder therapeutische Zusammensetzung für die Verwendung als Arzneimittel, umfassend ein Aß-Peptid wie in einem der Ansprüche 1 bis 9 definiert oder einen Antikörper oder ein Antikörperfragment nach einem der Ansprüche 10 bis 14 zusammen mit einem pharmazeutisch unbedenklichen Träger.

16. Prophylaktische oder therapeutische Zusammensetzung für die Verwendung nach Anspruch 15, die weiterhin einen Hilfsstoff umfasst.

17. Verwendung eines Aß-Peptids wie in einem der Ansprüche 1 bis 9 definiert oder eines Antikörpers oder Antikörperfragments nach einem der Ansprüche 10 bis 14 in der Herstellung eines Arzneimittels für die Behandlung, Vorbeugung oder Linderung von Morbus Alzheimer.

18. Verfahren zum Diagnostizieren von Morbus Alzheimer, umfassend den Schritt, dass man eine Probe einer biologischen Flüssigkeit von einem Probanden, von dem vermutet wird, dass er an der Krankheit leidet, auf das Vorhandensein eines Moleküls mit Tyrosinvernetzungen oder eines Antikörpers, der gegen ein Molekül mit Tyrosinvernetzungen gerichtet ist, in einem invitro-Assay prüft.

19. Verfahren nach Anspruch 18, wobei es sich bei dem Molekül mit Tyrosinvernetzungen um ein Aß-Peptid wie in einem der Ansprüche 1 bis 6 oder 9 definiert handelt.

20. Verfahren nach Anspruch 19, wobei das Aß-Peptid Dityrosin umfasst.

21. Verfahren nach einem Ansprüche 18 bis 20, wobei die biologische Flüssigkeit aus der Gruppe bestehend aus Blut, Plasma, Serum, Zerebrospinalflüssigkeit, Harn und Speichel stammt.

Revendications

1. Oligomère de peptide Aβ comprenant un fragment tyrosine réticulé pour utilisation dans le traitement, la prévention ou l'atténuation de la maladie d'Alzheimer.

2. Oligomère de peptide Aβ pour utilisation selon la revendication 1, qui est une partie immunogène de la forme pathologiquement agrégée d'Abeta, le peptide comprenant un fragment tyrosine réticulé lié à des résidus en amont et en aval de la tyrosine réticulée.

3. Peptide Aβ pour utilisation selon la revendication 1 ou 2, qui est un dimère, trimère ou tétramère d'un monomère de peptide Abeta.

4. Peptide Aβ pour utilisation selon l'une quelconque des revendications 1 à 3, qui est un dimère d'un monomère peptidique choisi dans le groupe constitué de $Abeta_{1-40}$, $Abeta_{1-42}$, $Abeta_{9-16}$ humain.

5. Oligomère de peptide Aβ pour utilisation selon l'une quelconque des revendications 1 à 4, dans lequel les réticulations de tyrosine dans le peptide peuvent être obtenues par oxydation en présence d'ions de cuivre.

6. Oligomère de peptide Aβ pour utilisation selon l'une quelconque des revendications 1 à 5 qui est couplé à une protéine porteuse qui est elle-même immunogène.

7. Oligomère de peptide Aβ pour utilisation selon la revendication 6, dans lequel la protéine porteuse est choisie dans le groupe constitué de l'endotoxine tétanique, l'hémocyanine de patelle, et l'albumine.

8. Oligomère de peptide Aβ pour utilisation selon l'une quelconque des revendications 1 à 7, dans lequel le peptide est formulé pour administration conjointement avec un adjuvant.

9. Oligomère de peptide Aβ pour utilisation selon l'une quelconque des revendications 1 à 8, dans lequel ledit peptide comprend une ou plusieurs des formes prédominantes de réticulations de tyrosine dans la forme pathologiquement agrégée d'Abeta.

10. Anticorps dirigé contre un peptide Aβ tel que défini dans l'une quelconque des revendications 1 à 7 ou 9, ou un fragment d'anticorps de celui-ci pour utilisation dans la prévention, le traitement ou l'atténuation de la maladie d'Alzheimer, ledit anticorps ou fragment d'anticorps étant capable de se lier spécifique à la forme pathologiquement agrégée de Aβ.

11. Anticorps ou fragment d'anticorps pour utilisation selon la revendication 10, ledit anticorps étant polyclonal.

12. Anticorps ou fragment d'anticorps pour utilisation selon la revendication 10 ou 11, **caractérisé en ce que** ledit anticorps est d'origine humaine.

13. Anticorps ou fragment d'anticorps pour utilisation selon la revendication 10, **caractérisé en ce que** l'anticorps est monoclonal.

14. Anticorps ou fragment d'anticorps pour utilisation selon la revendication 16, **caractérisé en ce que** l'anticorps est humanisé.

15. Composition prophylactique ou thérapeutique pour utilisation en tant que médicament comprenant un peptide Aβ tel que défini dans l'une quelconque des revendications 1 à 9, ou un anticorps ou fragment d'anticorps de l'une quelconque des revendications 10 à 14, conjointement avec un véhicule pharmaceutiquement acceptable.

16. Composition prophylactique ou thérapeutique pour utilisation selon la revendication 15, comprenant en outre un adjuvant.

17. Utilisation d'un peptide Aβ tel que défini dans l'une quelconque des revendications 1 à 9, ou d'un anticorps ou fragment d'anticorps de l'une quelconque des revendications 10 à 14 dans la fabrication d'un médicament pour traiter, prévenir ou atténuer la maladie d'Alzheimer.

18. Procédé de diagnostic de la maladie d'Alzheimer, comprenant l'étape d'analyse *in vitro* d'un fluide biologique d'un sujet suspecté de souffrir de la maladie pour déterminer la présence d'une molécule comprenant des réticulations de tyrosine ou un anticorps dirigé contre une molécule comprenant des réticulations de tyrosine.

19. Procédé de la revendication 18 dans lequel ladite molécule comprenant des réticulations de tyrosine est un peptide Aβ tel que défini dans l'une quelconque des revendications 1 à 6 ou 9.

20. Procédé selon la revendication 19, dans lequel le peptide Aβ comprend la dityrosine.

21. Procédé selon l'une quelconque des revendications 18 à 20, dans lequel le fluide biologique est choisi dans le groupe constitué du sang, du plasma, du liquide céphalorachidien, de l'urine et de la salive.

Figure 1

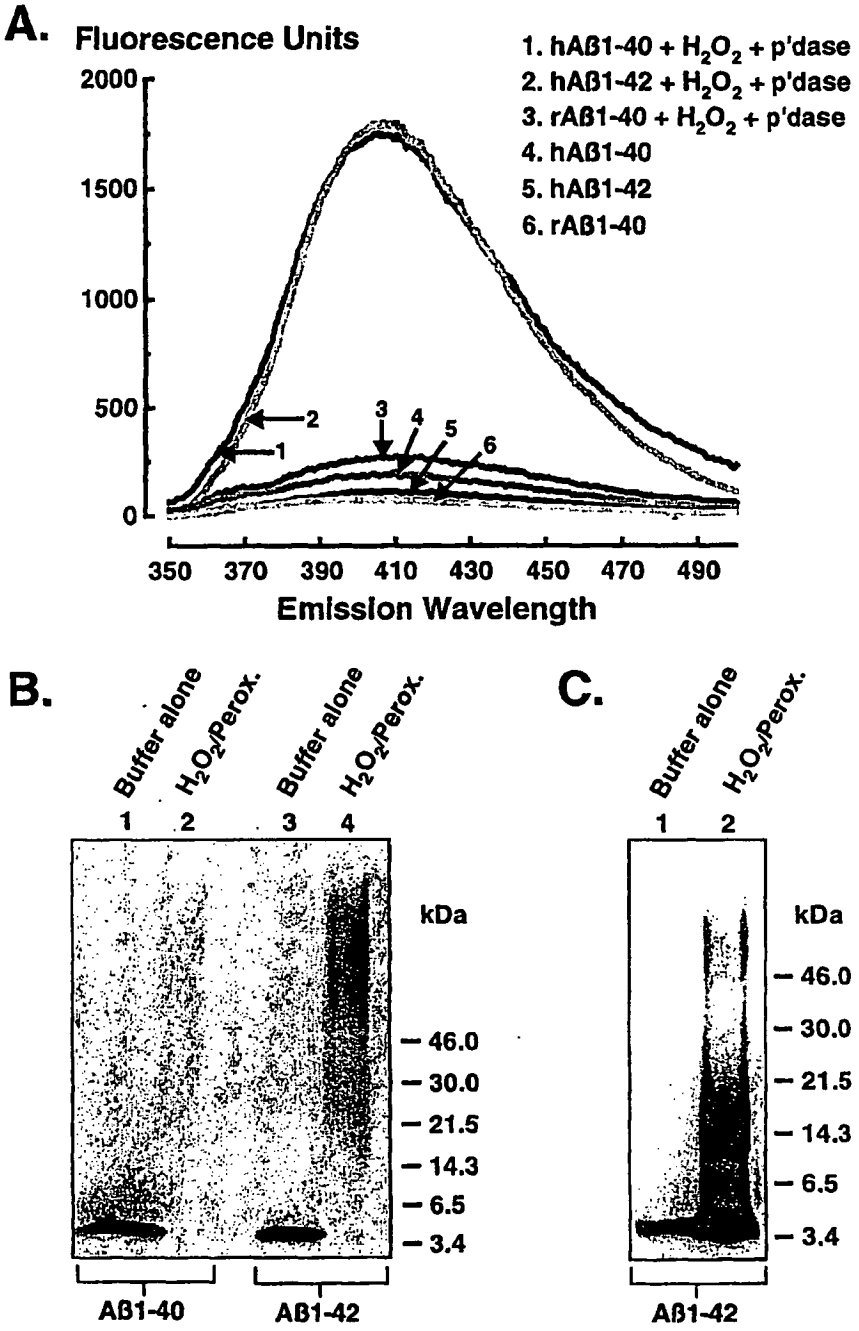

A.
Fluorescence Units
2000
1500
1000
500
0
1. hAß1-40 + H2O2 + p'dase
2. hAß1-42 + H2O2 + p'dase
3. rAß1-40 + H2O2 + p'dase
4. hAß1-40
5. hAß1-42
6. rAß1-40
1
2
3
4
5
6
350 370 390 410 430 450 470 490
Emission Wavelength
B.
Buffer alone
H2O2/Perox.
Buffer alone
H2O2/Perox.
1 2 3 4
kDa
46.0
30.0
21.5
14.3
6.5
3.4
Aß1-40
Aß1-42
C.
Buffer alone
H2O2/Perox.
1 2
kDa
46.0
30.0
21.5
14.3
6.5
3.4
Aß1-42

Figure 2

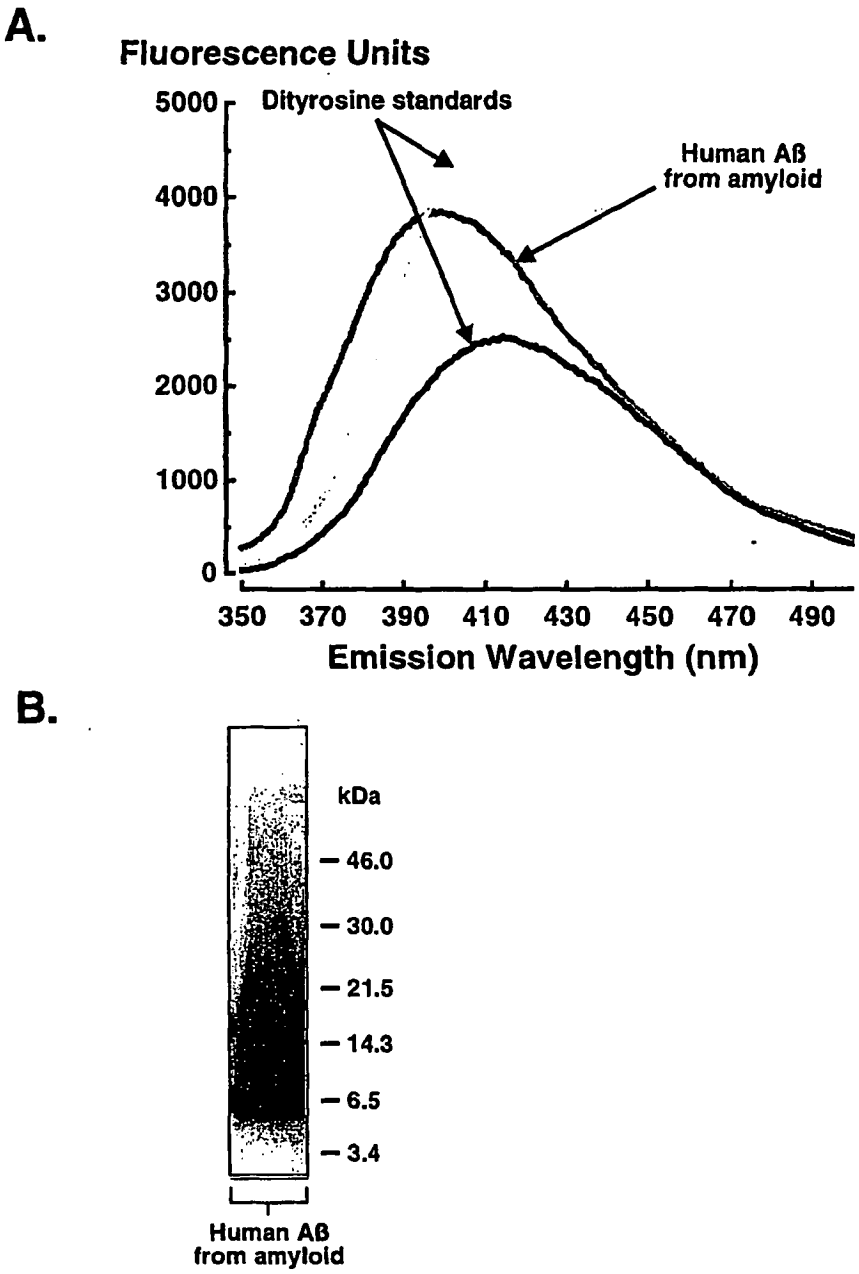

A.
Fluorescence Units
5000
4000
3000
2000
1000
0
Dityrosine standards
Human Aß
from amyloid
350
370
390
410
430
450
470
490
Emission Wavelength (nm)
B.
kDa
46.0
30.0
21.5
14.3
6.5
3.4
Human Aß
from amyloid

Figure 3A

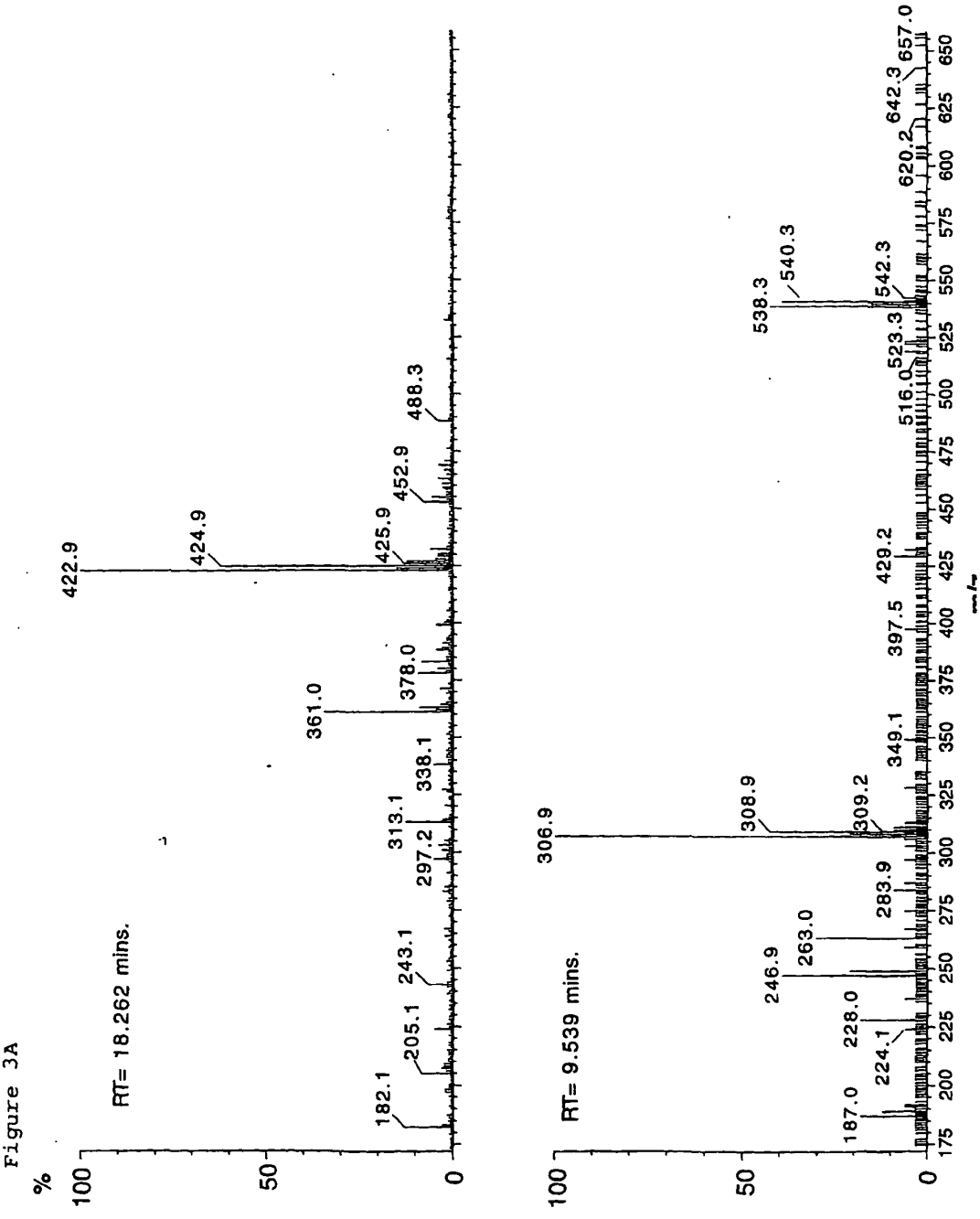
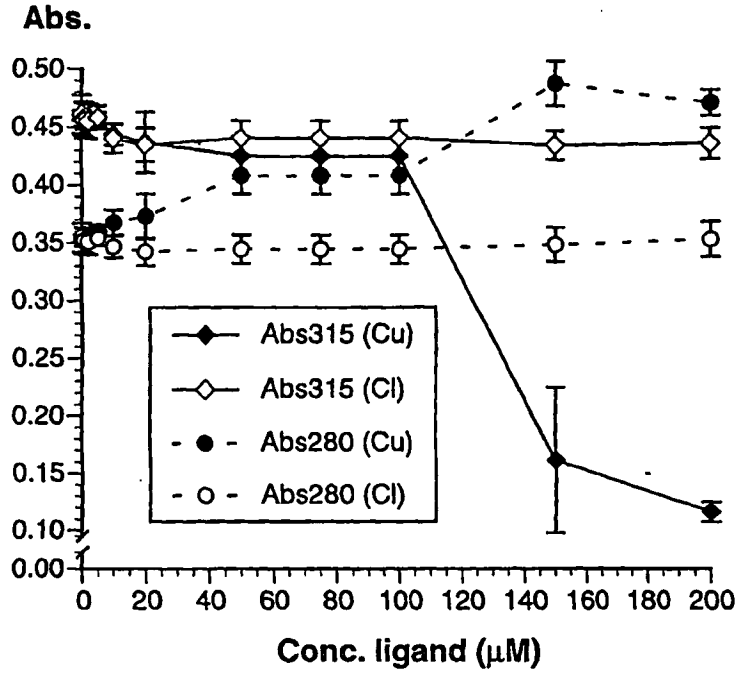

%
100
50
0
RT= 18.262 mins.
422.9
424.9
425.9
452.9
488.3
361.0
378.0
338.1
313.1
297.2
243.1
205.1
182.1
RT= 9.539 mins.
306.9
308.9
309.2
349.1
397.5
429.2
246.9
263.0
283.9
228.0
224.1
187.0
538.3
540.3
542.3
523.3
516.0
620.2
642.3
657.0
175
200
225
250
275
300
325
350
375
400
425
450
475
500
525
550
575
600
625
650

Figure 3B


Abs.
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.00
0
20
40
60
80
100
120
140
160
180
200
Abs315 (Cu)
Abs315 (Cl)
Abs280 (Cu)
Abs280 (Cl)
Conc. ligand (µM)

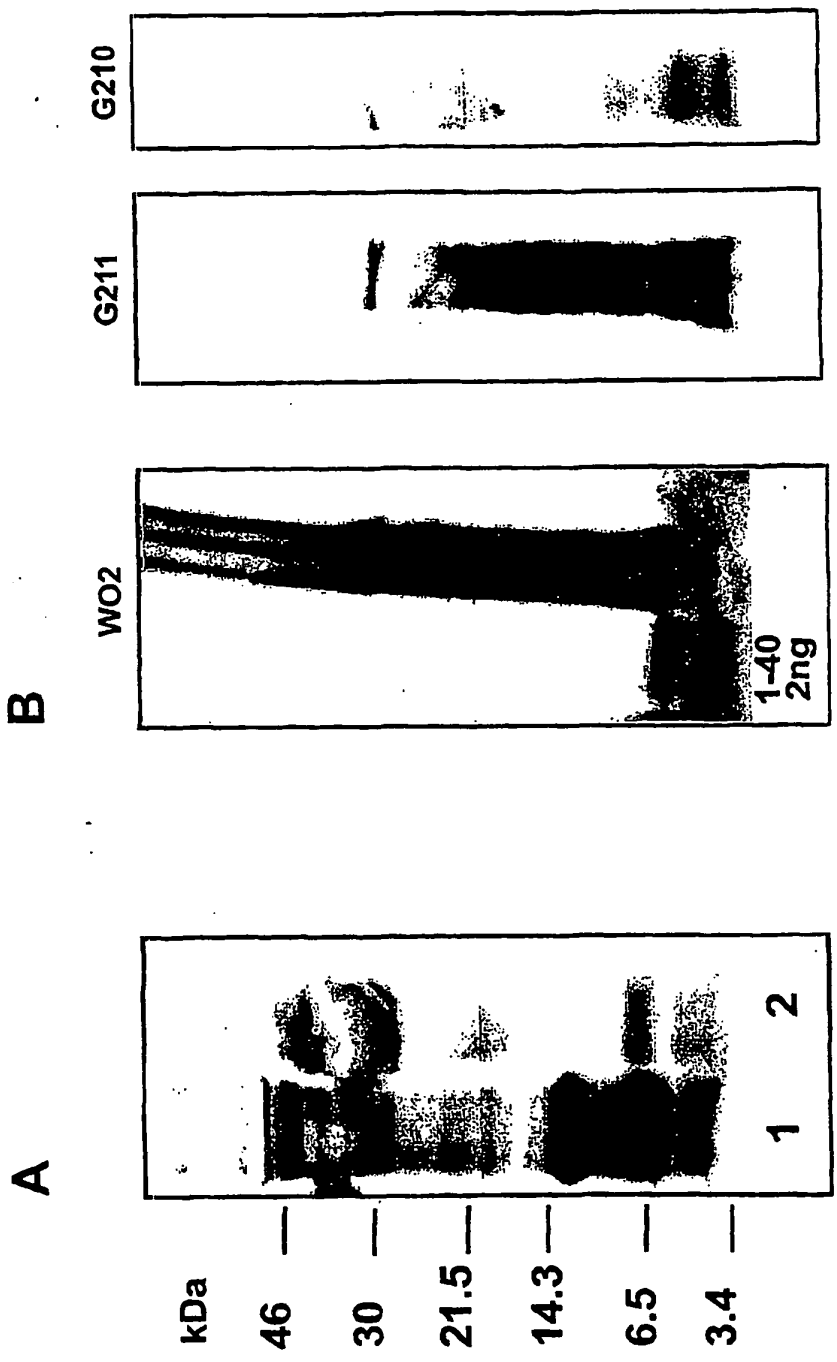
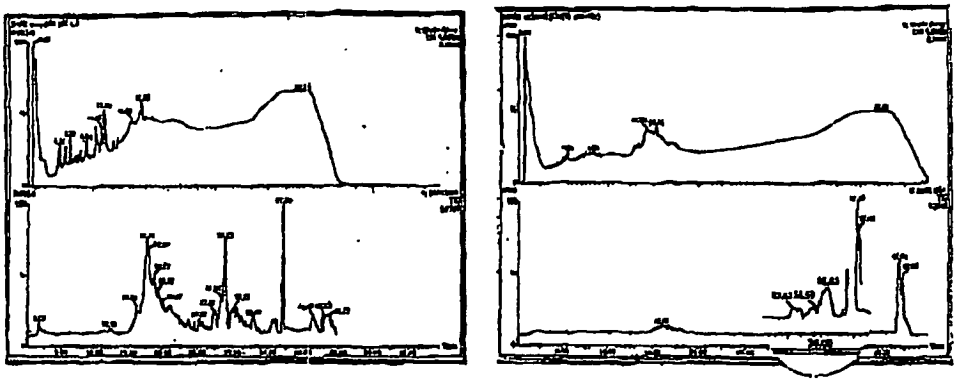


Figure 4

Figure 5


Unpurified
IMAC - purified

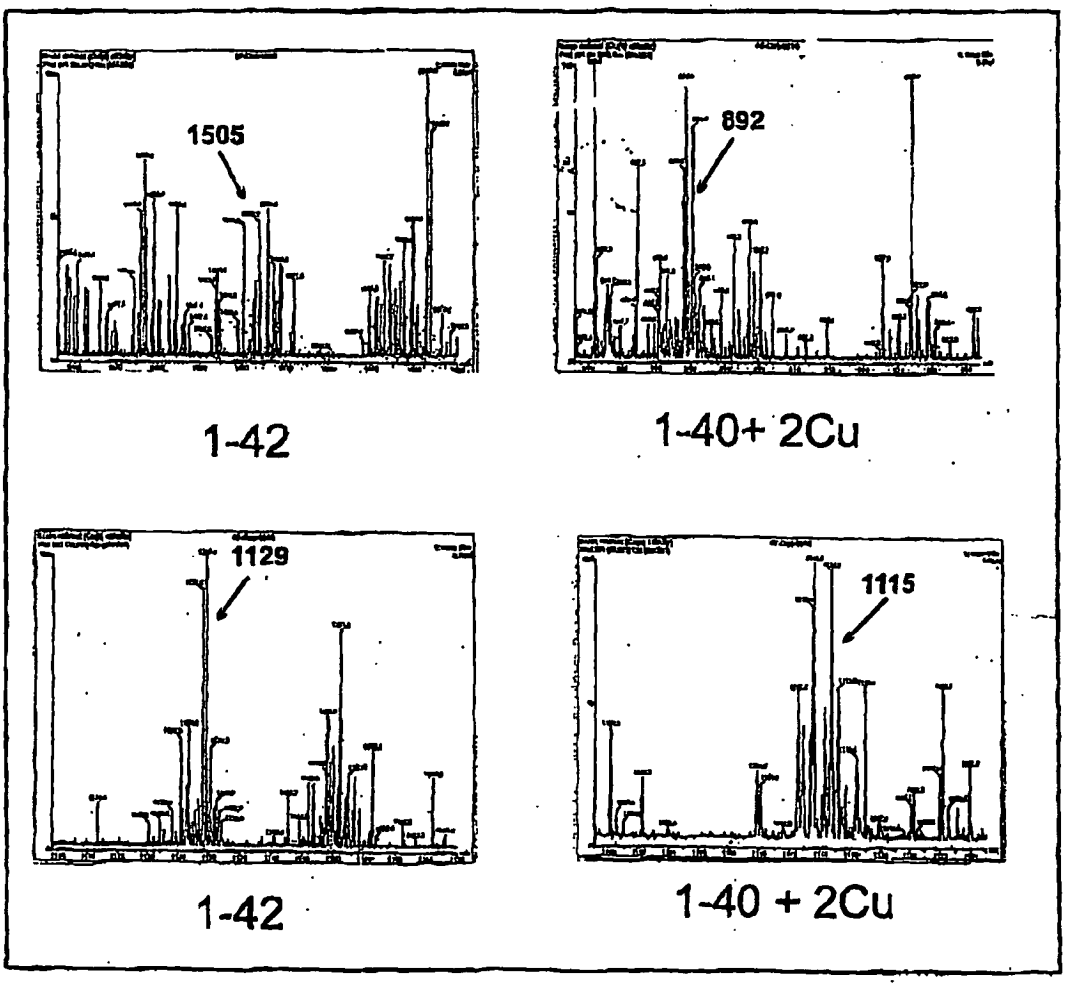
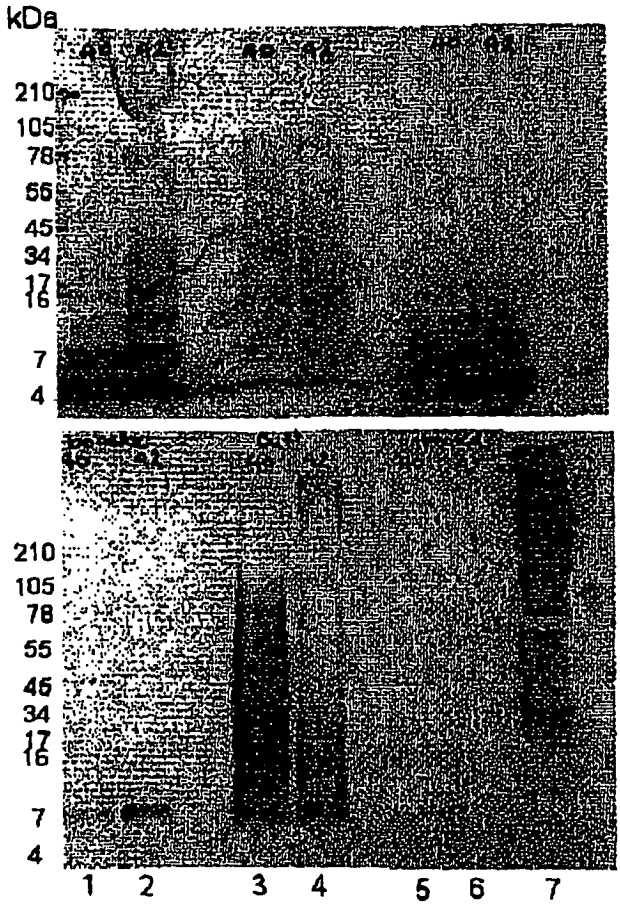
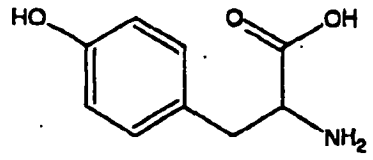

1505
1-42
892
1-40+ 2Cu
1129
1-42
1115
1-40 + 2Cu

Figure 6

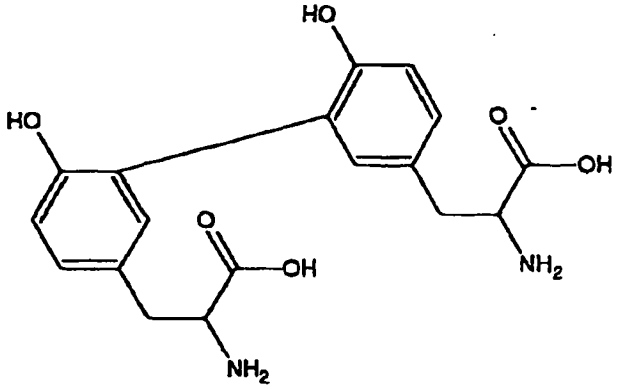


1. Aβ 1-40 - borate crosslinking
2. Aβ 1-42 - borate crosslinking
3. Aβ 1-40 - copper crosslinking
4. Aβ 1-42 - copper crosslinking
5. Aβ 1-40 - untreated
6. Aβ 1-42 - untreated
7. Dityrosine conjugated to KLH

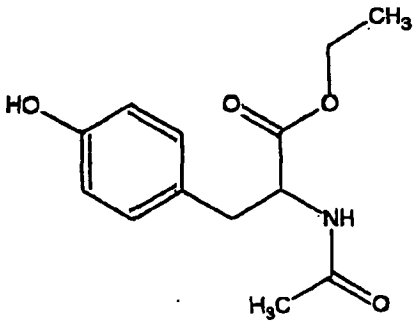
Figure 7A



Tyrosine

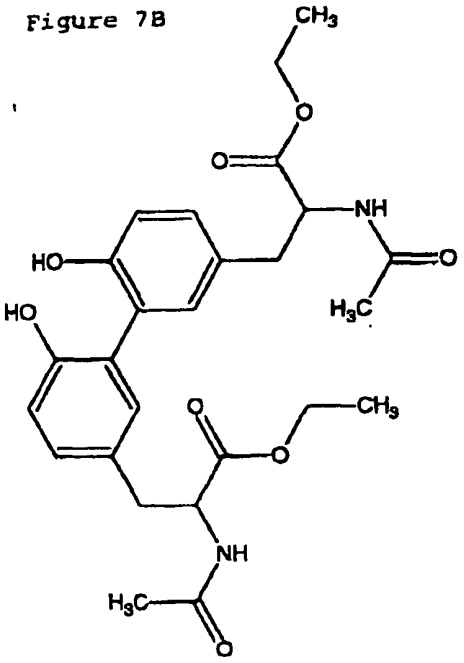


Dityrosine

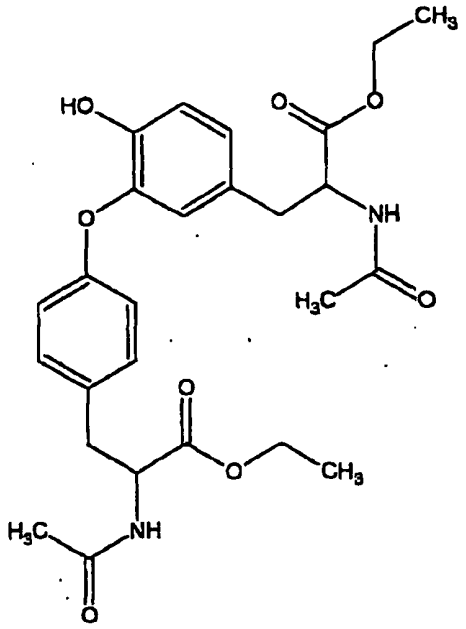


Atee

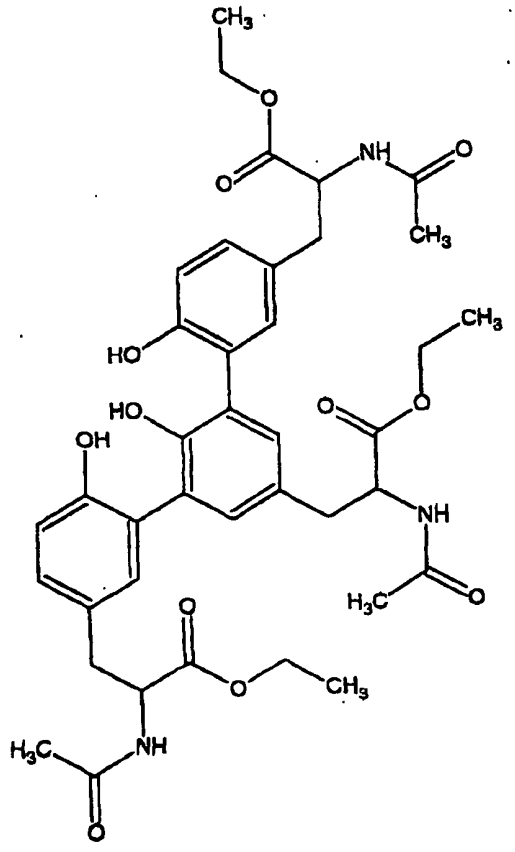
Figure 7B



DiAtee

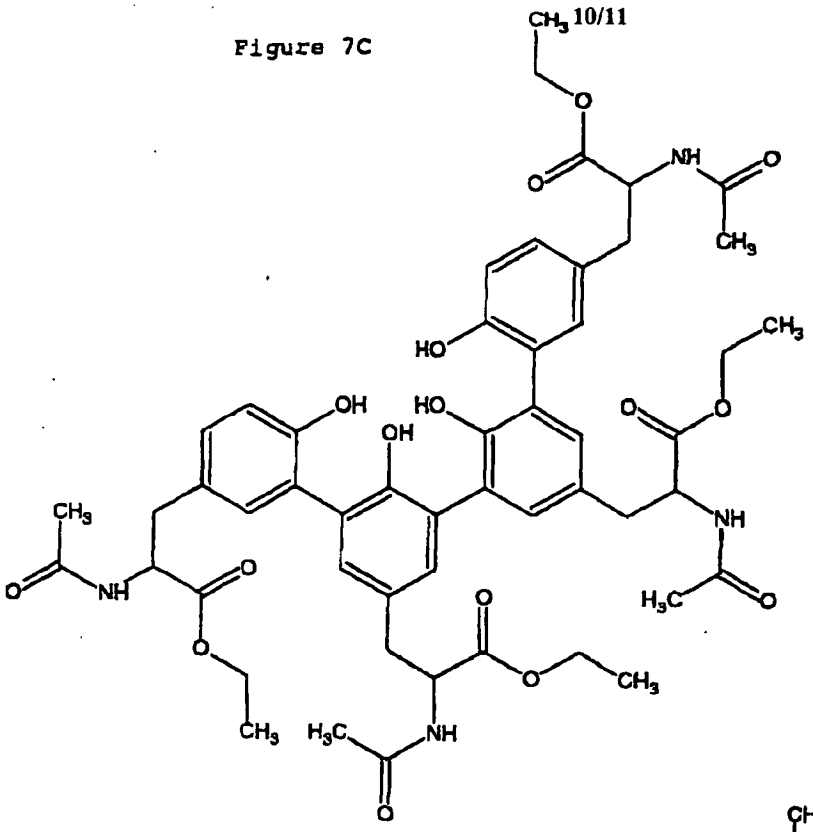


IsoDiatee

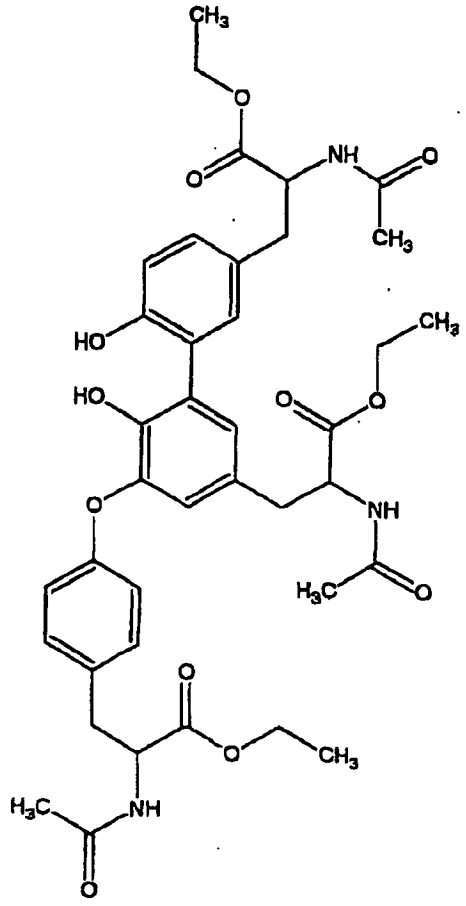


TriAtee

Figure 7C



TetraAtee



Alternative form of TriAtee with one iso bond

Figure 8

Rabbit serum innoculated with DT-KI

A

IC3 +ve control Ab

B

1. Abeta 9-16 DT dimer -BSA
2. Abeta 9-16 DT trimer -BSA
3. crude ATEE -BSA
4. polyTyr - BSA
5. BSA
6. Abeta trimer - KLH
7. crude ATEE - KLH
8. polyTyr - KLH
9. KLH

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专利名称(译)	淀粉样蛋白β寡聚体用于治疗，缓解或预防阿尔茨海默病		
公开(公告)号	EP1296705B1	公开(公告)日	2012-06-13
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申请(专利权)人(译)	PRANA生物技术有限公司 总医院CORPORATION		
当前申请(专利权)人(译)	PRANA生物科技有限公司 总医院CORPORATION		
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摘要(译)

本发明涉及用于治疗或减轻阿尔茨海默氏病和与异常蛋白质聚集有关的其他病症的方法和组合物。 特别地，本发明涉及用于阿尔茨海默氏病，帕金森氏病和白内障的免疫疗法的方法和组合物。 在一个方面，本发明提供了一种预防，治疗或减轻以免疫有效剂量的一种或多种酪氨酸交联的化合物有关的特定蛋白质的病理性聚集和蓄积为特征的疾病的方法，并且任选地还包含铜离子 与化合物复合。 或者，可以使用针对酪氨酸交联化合物的被动免疫。 预防或治疗组合物和诊断方法也被公开和要求保护。