(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 782 820 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
30.07.2014 Bulletin 2014/31

(21) Application number: **05755811.6**

(22) Date of filing: **28.06.2005**

(51) Int Cl.:
A61K 38/00 *(2006.01)* ***A61P 1/00*** *(2006.01)*
A61P 9/00 *(2006.01)* ***A61P 31/04*** *(2006.01)*
A61P 43/00 *(2006.01)* ***C07K 7/02*** *(2006.01)*

(86) International application number:
PCT/JP2005/012286

(87) International publication number:
WO 2006/001542 (05.01.2006 Gazette 2006/01)

(54) **METHOD OF SCREENING TOXIN-NEUTRALIZING PEPTIDE, STX2 INHIBITORY PEPTIDE AND VERO TOXIN-NEUTRALIZING AGENT**

SCREENING-VERFAHREN FÜR TOXIN-NEUTRALISIERERNDES PEPTID, STX2-HEMMENDES PEPTID UND VERO TOXIN-NEUTRALISIERENDES MITTEL

PROCÉDÉ DE RECHERCHE PAR CRIBLAGE D'UN PEPTIDE NEUTRALISANT UNE TOXINE, PEPTIDE INHIBITEUR DE STX2 ET AGENT NEUTRALISANT UNE VERO-TOXINE

(84) Designated Contracting States:
CH DE FR GB IT LI

(30) Priority: **28.06.2004 JP 2004189801**
07.10.2004 JP 2004295405

(43) Date of publication of application:
09.05.2007 Bulletin 2007/19

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Printed by Jouve, 75001 PARIS (FR)

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Description

TECHNICAL FIELD

[0001] This invention relates to a method for screening for a toxin neutralizing peptide which can inhibit verotoxin.

[0002] This invention further relates to an STX2 inhibiting peptide and a verotoxin neutralizing agent. More specifically, it relates to an STX2 inhibiting peptide which can competitively inhibit adhesion of verotoxin to cells to effectively inhibit verotoxin, and a verotoxin neutralizing agent capable of oral administration.

BACKGROUND ART

[0003] Verotoxins that enterohemorrhagic Escherichia coli 0157:H7 produces are proteins belonging to the AB_5 family of bacterial toxins analogous to Shiga toxin derived from dysentery bacillus, and it has been known that these toxins are incorporated into cells by recognizing and bonding a globo3 sugar moiety of globotriaosylceramide (Gb_3, Galα1-4Galβ1-4G1cβ1-Cer) in vascular endothelial cells of various target organs to show a toxicity.

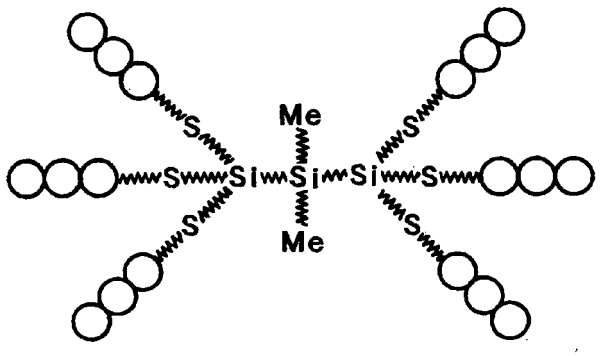
[0004] Such Shiga-like toxins include two types, and these toxins induce hemorrhagic colitis, severe complications as a series of consequential microvascular disorders (for example, hemolytic uremic syndrome (HUS)) and the like. A toxin expressed as STX1 has the same amino acid sequence as Shiga toxin produced by Shigella dysenteriae (Shigella dysenteriae Type I). Meanwhile, a toxin expressed as STX2 has an amino acid sequence which is identified to be homologous to that of STX1 by 50 to 60%. Although there is a slight difference in amino acid sequence, toxicities thereof show activities such as a cytotoxicity and an intestinal toxicity by inhibition of protein synthesis. STX is an AB_5-type toxin comprising two types of subunits (A and B) in which one molecule of A-subunit is surrounded by five molecules of B-subunit via hydrophobic binding. It is A-subunit that plays a role in toxicity, and B-subunit plays a role in binding to a sugar chain receptor present on the surface of the cell. Through detailed examination by analysis of X-ray crystal structure of the toxin, it has been clarified that three binding sites of a sugar chain are present in one molecule of B-subunit. That is, since five molecules of B-subunit are present in one molecule of STX2, it is presumed that 15 binding sites in total are presented.

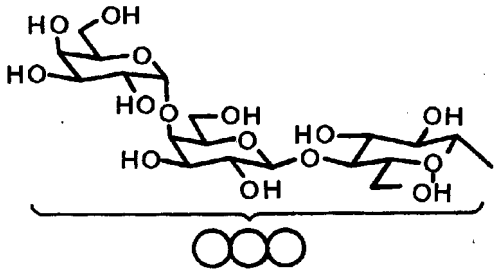
[0005] STX is classified in two families, STX1 and STX2. It is mainly STX2 producing bacteria that induce severe complications, and STX2 is more significant clinically. For this reason, the development of an inhibitor of STX2 is urgently required. These STXs are A-B_5-type toxins, and incorporated into cells such that B-subunit is bound to Gb_3 (globotriaosylceramide: Ga1α(1-4)-Ga1β(1-4)-Ga1cβ1-Ceramide), a receptor on the cell membrane. A B-subunit pentamer specifically recognizes a Gb3 sugar chain moiety (globo3 sugar: Ga1α(1-4)-Ga1β(1-4)-G1cβ1-). Accordingly, a compound in which globo3 sugar is accumulated at high density is bound to STX with high affinity, and becomes an STX inhibitor of STX function.

[0006] Since the subunit structure of the toxin and its function have been clarified, a method for selectively inhibiting the binding of B-subunit having a function of binding to the sugar chain receptor on the cell surface has attracted much interest, and studies thereof have been made from various aspects.

[0007] The inventors of this application have also conducted construction of an artificial sugar chain cluster which effectively binds to the sugar chain binding site of the toxin to inhibit the adhesion of the toxin to the host cell. They have so far proposed a dendrimer compound group having carbosilane as a sugar chain-supporting structure or water-soluble polymer compounds (Patent Documents 1 and 2, and non-Patent Document 1).

[0008] Examples thereof include SUPER TWIG (1)6 represented by the following formula, and the like. This is the first compound whose effectiveness has been verified in an O157:H7 infection experiment.





[0009] A similar compound is the oligovalent, water soluble carbohydrate ligand (named STARFISH) with subnanomolar inhibitory activity against STX-1 and -2 disclosed in Kitov, P. I. et al., 1999, Nature, Vol. 403, 669-672.

[0010] The past development of STX inhibitors including SUPER TWIG has been conducted on the basis of the concept of how the globo3 sugar as an STX-binding unit is accumulated for exhibiting an inhibitory activity in vivo. However, an affinity (Kd) for STX with the globo3 sugar alone is 10^{-3} M which is not necessarily high, and its chemical synthesis is quite difficult. This is a great barrier to clinical application. Accordingly, for the development of clinically applicable therapeutic agents, the development of a new STX-binding unit which is easier to synthesize than the globo3 sugar and excellent in bindability to STX is required.

[0011] Further, it is presumed that the foregoing problem is not only peculiar to the verotoxin but also common to, other than the verotoxin STX, cholera toxin (A-B5 type) receptor: GM1, enterotoxigenic Escherichia coli heat-labile diarrhea causal toxin LT (A-B5 type) receptor: GM1, pertussis toxin (A-B5 type) receptor, Bacillus anthracis toxin (heptamer type) receptor: protein having VWA domain (anthrax toxin receptor) in which a receptor binding portion is considered to have a subunit structure, and the like.

[0012] Under these circumstances, the inventors of this application have conducted investigations to construct a substance formulated as a receptor of STX2.

Patent Document 1: WO 02/02588
Patent Document 2: Japanese Patent Application No. 2004-108483
Non-Patent Document 1: Proc. Natl. Acad. Sci. USA 2002; 99; 7669-74

[0013] Also disclosed is Songyang, Z. et al., 1994, Current Biology, Vol. 4(11), 973-982, which discloses the use of an orientated peptide library to determine the optimal substrates of protein kinases.

DISCLOSURE OF THE INVENTION

[0014] Under these circumstances, on the basis of the studies which have been so far conducted by the inventors, this invention aims to provide a new screening method for realizing a verotoxin neutralizing agent which is easy to synthesize and can effectively inhibit verotoxin.

[0015] Further, on the basis of the studies which have been so far conducted by the inventors, this invention aims to provide a new STX2 inhibitor which is easy to synthesize and can effectively inhibit verotoxin, namely a verotoxin neutralizing agent.

[0016] For solving the foregoing problems, the inventors of this application have conducted studies on a possibility of realizing verotoxin neutralizing peptides. Attention has been drawn to peptides mainly because synthesis thereof is relatively easy and a safety is generally high in application to drugs.

[0017] As a result of the assiduous investigations by the inventors, this application is to provide first, for solving the foregoing problems, the following method for screening a polyvalent STX2 toxin neutralizing peptide, and more specifically to provide an STX2 inhibiting peptide and a verotoxin neutralizing agent.

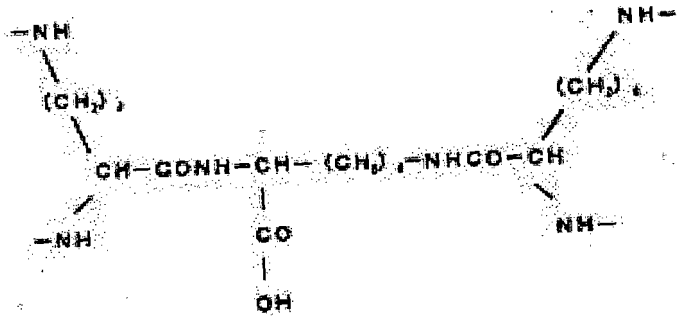
[0018] The present invention therefore provides:

1st: A method for screening for a polyvalent STX2 toxin-neutralizing peptide which can neutralize STX2, the method comprising the following steps:

(a) providing STX2,
(b) providing a mutant of STX2 which is functionally deficient in the site by which it binds to its receptor due to the presence of a mutation in the receptor-binding site of STX2,
(c) providing a first polyvalent peptide library, wherein each entity in the first polyvalent peptide library comprises a core structure comprising a plurality of lysines and wherein degenerate peptides are bound to the terminal amino groups of the lysines,

(d) contacting said first polyvalent peptide library with STX2 and the mutant,
(e) contrasting a peptide motif bound to STX2 with a peptide motif bound to the mutant to obtain an amino acid selection ratio where the amino acid selection ratio can be used to specify a receptor-binding site-specific binding peptide motif, and

wherein the STX2-neutralizing peptide comprises the receptor-binding site-specific binding peptide motif.
2nd: The method for screening for a polyvalent STX2 toxin neutralizing peptide as described above, wherein the step (e) is conducted plural times to specify the binding site-specific peptide motif having the higher amino acid selection ratio in order.
3rd: The method for screening for a polyvalent STX2 toxin neutralizing peptide as described above, wherein in the 1st polyvalent peptide library, the peptide library is bound to the terminal amino group via a spacer molecule.
4th: An STX2 inhibiting peptide comprising a molecular core portion which has three molecules of lysine (Lys) having the structure:



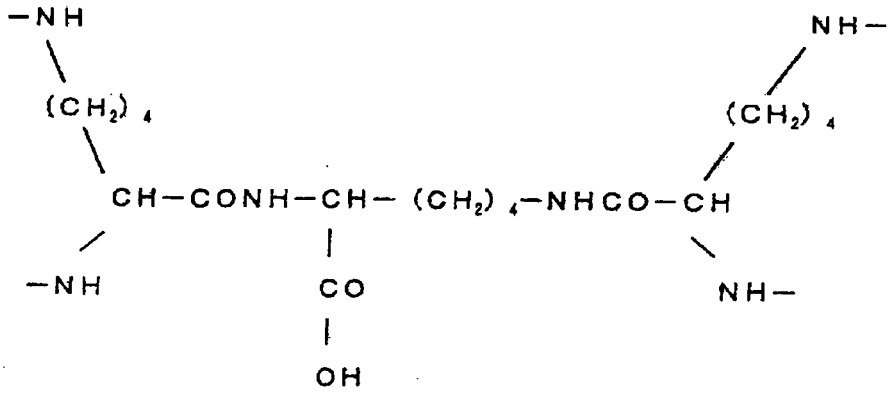
wherein each terminal amino group in the Lys core structure is bound to a peptide motif, wherein each peptide motif is identical and is selected from the group consisting of:

(1) FRRNRRN (SEQ ID NO: 1)
(2) PPPRRRR (SEQ ID NO: 2)
(3) PPRRNRR (SEQ ID NO: 3)
(4) KRRNPRR (SEQ ID NO: 4).
5th: The STX2 inhibiting peptide as described above, wherein the peptide motif is incorporated via a spacer molecule.
6th: The STX2 inhibiting peptide as described in the 5th embodiment, wherein the spacer molecule is a molecule having a peptide or an amino group and a carboxyl group and having a hydrocarbon chain structure with from 4 to 10 carbon atoms.
7th: The STX2 inhibiting peptide as described above, wherein the peptide motif has a terminal modification molecule.
8th: The STX2 inhibiting peptide as described in the 7th embodiment, wherein the terminal modification molecule is an uncharged molecule.
9th: The STX2 inhibiting peptide as described above, wherein the peptide motif may have a molecule for amino acid sequencing.
10th: The STX2 inhibiting peptide as described above represented by the following formula (a) or (b),

$$(\text{Met-Ala-Xo-Ala-AHA-})_4\text{-3Lys} \quad \text{(a)}$$

$$(\text{acetyl-Xo-AHA-})_4\text{-3Lys} \quad \text{(b)}$$

[wherein AHA represents an aminohexanecarboxylic acid group, Xo represents any of the foregoing peptide motifs (1), (2), (3) and (4), and 3Lys represents a structure of the following formula (1)



16th: A verotoxin neutralizing agent comprising the foregoing peptide as an active ingredient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

Fig. 1 is a mass spectrum of a compound of peptide motif (1) FRRNRRN (SEQ ID NO: 1).
Fig. 2 is a mass spectrum of a compound of peptide motif (2) PPPRRRR (SEQ ID NO: 2).
Fig. 3 is a mass spectrum of a compound of peptide motif (3) PPRRNRR (SEQ ID NO: 3).
Fig. 4 is a mass spectrum of a compound of peptide motif (4) KRRNPRR (SEQ ID NO: 4).
Fig. 5 is a mass spectrum of a peptide motif-free Met-Ala compound.
Fig. 6 is a graph showing an affinity for STX2B-subunit.
Fig. 7 is a graph showing an effect of inhibiting a cytotoxicity of STX2 of a vero cell.

<Screening of a toxin neutralizing peptide>

[0020] The following section is provided merely as useful background to the invention. Viewed generally, the screening method of the invention comprises two steps:

(1) specifying a receptor binding site by introduction of mutation, and
(2) specifying a binding site-specific peptide motif on the basis of an amino acid selection ratio by contrast between a peptide motif bound to a wild-type subunit and a peptide motif bound to a mutant functionally deficient in the binding site according to a peptide library method.

[0021] It is preferable that step (2) is conducted a plurality of times in order to specify the binding site-specific peptide motif having the higher amino acid selection ratio.

[0022] Since it is known that a cluster effect exists in the binding between the subunit and the receptor, it is advisable that an initial (1st) peptide library for the step (2) has a core structure formed by binding to a plurality of amino acids to comply with the cluster effect.

[0023] With respect to such a core structure, in view of, for example, its design, ease of synthesis and, among others, a molecular size for an actual cluster effect, a core structure having bound thereto a plurality of lysines (Lys), for example, preferably from 2 to 5 lysines may be used to prepare a peptide library which is itself polyvalent.

[0024] That is, in the step (2), a 1st polyvalent peptide library may be used in which a core structure having a plurality of lysines (Lys) bound thereto is formed and a plurality of peptide motifs are bound to the terminal amino groups thereof. In the 1st polyvalent peptide library, the peptide motifs may be bound to the terminal amino groups via a spacer molecule. In this case, it may be preferable that, for example, the spacer molecule is a molecule having a peptide or an amino group and a carboxyl group and having a hydrocarbon chain structure with from 4 to 10 carbon atoms.

[0025] The receptor binding site in step (1) may be a known site or may be specified as a site in which mutation is actually introduced to have a great influence on the binding to the receptor. According to the inventors of this application, in the case of verotoxin STX, the binding site of the receptor has been already found to be specified in the binding of globo3 sugar.

BEST MODE FOR CARRYING OUT THE INVENTION

<Screening of a verotoxin neutralizing peptide and an STX2 inhibiting peptide>

[0026] The invention is described in more detail below by referring to an example of screening a verotoxin inhibiting peptide.

[0027] Infection of enterohemorrhagic Escherichia coli such as 0157:H7 not only induces hemorrhagic colitis but also sometimes accompanies hemolytic uremic syndrome (HUS) and encephalopathy, and these complications can lead to death. Verotoxin (Shiga toxin: STX) is a main pathogenic factor produced by enterohemorrhagic Escherichia coli, and a microvascular endothelium disorder of kidneys or brain due to verotoxin invasion into the blood is considered to cause the above described complications. Accordingly, an STX absorbing agent which strongly absorbs STX produced in the intestinal tract to inhibit the invasion of STX into the blood and an STX neutralizing agent which is bound to a trace amount of STX already invaded into the blood to inhibit its function have been expected as effective therapeutic agents against infection by enterohemorrhagic Escherichia coli.

[0028] The screening of the Escherichia coli verotoxin neutralizing peptide can be based on the inventors' detailed investigations on the relation between the molecular structure of SUPER TWIG (1)6 and the functional mechanism thereof.

[0029] The detailed description is as follows.

1) Identification of binding sites of STX1B- and 2B-subunits of SUPER TWIG (1)6

[0030] Three types of globo3 sugar binding sites called sites 1, 2 and 3 exist in one B-subunit monomer. Accordingly, it is known that fifteen globo3 sugars in total can be bound to a B-subunit pentamer. For developing a new STX binding unit, what site is targeted is first a serious problem. Therefore, single, double and triple mutations were introduced in the respective sites to prepare various mutant B-subunits, and binding affinities between these mutants and SUPER TWIG (1)6 were comparatively examined. Consequently, it was found that "sites 1 and 2" or "site 3 alone" are/is used in the binding between SUPER TWIG (1)6 and STX 1B-subunit and that "site 3 alone" is used in the binding between SUPER TWIG (1)6 and STX2B-subunit. That is, for developing a new binding unit to STX2 which is clinically more significant, it was found that site 3 may be targeted.

2) Development of a new STX binding unit

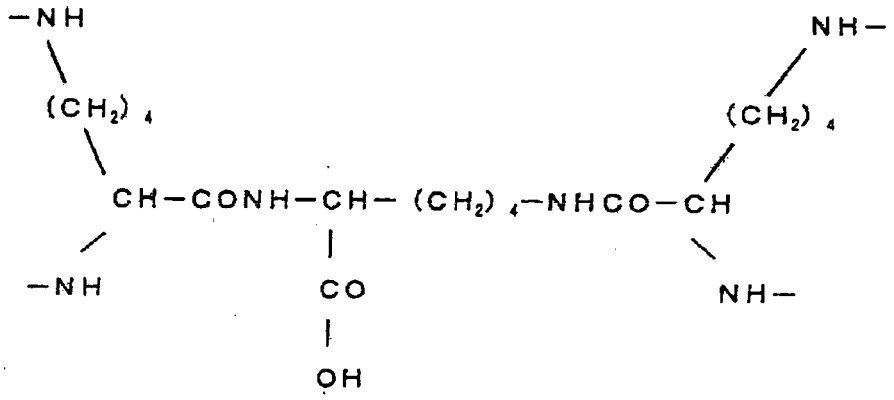
[0031] In consideration of the clinical significance and the specificity of the binding site clarified in (1), a new substance which can be bound to STX2B-subunit to inhibit the toxicity of STX2 has been decided to be investigated as a substance having a peptide structure. Attention has been drawn to peptides mainly because their synthesis is relatively easy, they have been generally applied as a drug without a great barrier and there is a strong possibility of the development of variants and derivatives.

[0032] In search of an active peptide from these aspects, peptide libraries have been newly developed.

[0033] That is, on the basis of the findings obtained in 1), a binding motif specific to site 3 of STX2B-subunit has been determined by subtracting a peptide motif bound to mutant STX2B-subunit functionally deficient in site 3 from a peptide motif bound to wild-type STX2B-subunit.

[0034] That is, first, the inventors have already developed a peptide library method which is a method for determining a motif directly bound to a functional domain such as a catalytic site of a protein kinase (K. Nishikawa et al., Mol. Cell, 6, 969-2000). Thus, on the basis of the knowledge of this peptide library method, a peptide library based on a new concept that a peptide library per se is polyvalent has been developed using the fact that a cluster effect exists in the binding of the B-subunit pentamer and the globo3 sugars in the verotoxin neutralizing agent as described above.

[0035] First, in consideration of the size and the like of the core molecule structure in the foregoing SUPER TWIG (1)6, a compound in which four peptide motifs are bound via spacers has been synthesized using a structure having three lysines (Lys) bound thereto as represented by the following formula



as a core structure used for making the peptide library polyvalent. This compound is one in which four peptide motifs (Xaa-Xaa-Xaa-Xaa) represented by the following formula
MAXXXXA-AHA- (SEQ ID NO:5)
are bound to the terminal amino groups in the Lys core molecule structure in the foregoing formula, namely (MAXXXXA-AHA)$_4$-3Lys. In this compound, AHA in the formula indicates aminohexanoic acid constituting the spacer molecule.

[0036] AHA (aminohexanoic acid) has a length of 6 carbon atoms, and it is used to comply with the optimum conditions of SUPER TWIG. Terminal MA (Met-Ala) is introduced at the time of screening for checking whether the amino acid sequencing is surely conducted in the sequencing. A (Ala) before AHA- is also introduced for the same reason. The design complies with the optimum structure of SUPER TWIG in all of its shape, the distance between branched chains present in the core structure, the valence of the peptide motifs and a distance between the peptide motifs. When the resulting compound was subjected to amino acid sequencing, it was confirmed that the 19 types of amino acids (except Cys) used were efficiently randomized in positions where the amino acids were degenerate.

[0037] Upon using this 1st peptide library, a peptide motif bound to wild-type STX2B-subunit was first determined, and a peptide motif bound to site 3 mutant STX2B-subunit was then determined. Specifically, each of 2B and 2B-mutant (W32A) put on beads is prepared in an amount of from 3 to 5 mg, and filled in a column to prepare an affinity column.

[0038] Approximately 50 mg of a multiple peptide library for 1st screening: (MAXXXXA-AHA-)$_4$-3Lys is added and fully bound thereto. Thereafter, washing was conducted well with PBS or the like to wash out an unbound library.

[0039] Finally, the library put on the column is eluted with 30% acetic acid. The recovered fraction is dried up, and subjected to analysis of amino acid sequence. Consequently, numerical results of what amino acid is selected with what intensity are obtained. Normalization is conducted such that all the values of the 19 amino acids add up to 1.

[0040] The value of each amino acid in each degenerate position as obtained above when using 2B is divided by the value of each corresponding amino acid in each corresponding degenerate position as obtained above when using the mutant: W32A. Consequently, how many times the amino acid in 2B is selected in comparison to the amino acid in W32A is a numerical value. That is, the binding motif specific to site 3 of STX2B-subunit can be determined by dividing the selection ratio of each amino acid present in the former motif by the selection ratio of each corresponding amino acid present in the latter motif.

[0041] The other 18 amino acids are examined in the same manner, and normalization is finally conducted such that all the values of the 19 amino acids add up to 19. At this time, when there is no difference in selectivity between the amino acids, the value becomes 1. Generally, when this value exceeds 1.5, it is judged that a strong selectivity is observed. The results are shown in Table 1. Incidentally, in the following Tables 1 to 4 and Figs. 6 and 7, amino acids are expressed by single character code.

Table 1
1 st screening of the binding motif for 2 B-subunit

Degenerate position			
1	2	3	4
R (2.1)		N (2.0)	
K (1.7)	R (1.8)	R (1.7)	R (1.8)
F (1.6)	K (1.7)	K (1.7)	K (1.8)
	N (1.7)		N (1.6)

[0042] On the basis of the resulting motif, the 2nd library shown in Table 2 was then prepared. In practice, amino acids such as Arg, Asn and Phe were selected in the 1st screening. Accordingly, these amino acids are introduced in the 2nd screening in fixed positions. Then, since the bindability to 2B-subunit as the overall library is increased, a more specific motif is easily obtained.

Table 2

Peptide libraries for the 2nd screening

Degenerate position	1	2	3	4	5	6	7
(MA-X X X X X X X-A-AHA)$_4$-3 L y s							
	X	X	R	X	N	X	X
	X	X	X	R	X	X	X
	X	X	X	N	X	X	X

[0043] Upon using the 2nd peptide libraries, a peptide motif bound to wild-type STX2B-subunit and a peptide motif bound to site 3 mutant STX2B-subunit were determined in the foregoing manner. The binding motif specific to site 3 of STX2B-subunit could be determined with a higher selectivity than the motif obtained using the 1st peptide library by dividing the selection ratio of each amino acid present in the former motif by the selection ratio of the corresponding amino acid present in the latter motif. The results are shown in Table 3.

Table 3

2nd screening of the binding motif for 2B-subunit

	position						
Libraries	1	2	3	4	5	6	7
R X N	P (1.5)	P (1.3) R (1.2)	R	R (1.4) I (1.3)	N	R (2.1) W (1.4)	R (3.0) W (1.4)
X R X	P (1.4) I (1.3) W (1.2) V (1.2)	P (1.4) R (1. 2) W (1. 2) V/I (1.2)	P (1.3) R (1.2) W (1.2) V/I (1.2)	R	R (1.5)	R (1.9)	R (2.3)
XNX	K (1.7) R (1.6) P (1.5) F (1.4)	R (1.8) P (1.4) N (1.4)	R (1.8) N (1.5) P (1.4)	N	P (1.4) R (1.3)	R (1.8) N (1.4) D (1. 3)	R/N (1.8) D (1.3)

[0044] The amino acid sequences of the resulting peptide motifs are as follows.

(1) FRRNRRN (SEQ ID NO: 1)
(2) PPPRRRR (SEQ ID NO: 2)
(3) PPRRNRR (SEQ ID NO: 3)
(4) KRRNPRR (SEQ ID NO: 4)

[0045] Incidentally, with respect to motif (1) FRRNRRN (SEQ ID NO: 1), a basic amino acid and Pro, a hydrophobic amino acid are selected in the 2nd screening also. However, when an amino acid with the largest value in each position is withdrawn in the best motif, an information that a hydrophobic amino acid is preferable is not reflected. For this reason, Phe is introduced in the consensus sequence obtained in each library.

[0046] Thus, a compound was synthesized in which the resulting motif was incorporated into the molecular core structure of the foregoing formula having three lysines (Lys) bound thereto as

Met-Ala-Xo-Ala-AHA-

(Xo represents any of the motifs, and AHA is as defined above).

In this instance, since the core structure of Lys3 is commercially available in a state bound to beads, synthesis is conducted in sequence from the C terminal with a usual amino acid synthesizer, that is, four chains are extended at once in the formula of 3Lys. Since AHA has also an amino group and a carboxyl group, it is also possible to use the

amino acid synthesizer.

[0047] These compounds were identified by mass spectrometry. Figs. 1, 2, 3 and 4 show mass spectrums of the foregoing peptide motifs (1), (2), (3) and (4).

[0048] Fig. 5 shows a mass spectrum of a compound free of any of the peptide motifs, namely (Met-Ala):Met-Ala-Ala-AHA as a control.

<STX2 inhibiting peptide and verotoxin neutralizing agent>

[0049] With respect to each of the foregoing compounds, the affinity for STX2B-subunit was examined, and the compound has been found to be bound thereto with a high affinity. It has been further found that the cytotoxicity of STX2 of vero cells is efficiently inhibited.

[0050] Table 4 and Fig. 6 show the affinity for STX2B-subunit. The measuring method is as follows.

[0051] That is, first, the synthetic peptide in an amount shown in Fig. 6 is coated on a plastic plate for ELISA. After blocking with 1% BSA, 0.1 microgram/ml of wild-type 2B-His or site3 mutant W32A-His is added and bound thereto at room temperature for 1 hour. After washing, each 2B-subunit bound is detected by ELISA using anti-STX2 polyclonal antibody.

[0052] A small view in Fig. 6 shows that an anti-STX2 polyclonal antibody is likewise reacted with a known amount of wild-type 2B-His or site 3 mutant W32A-His.

[0053] Fig. 7A shows an effect of inhibiting an STX2 cytotoxicity of vero cells. In the measurement of the results in Fig. 7, 1 pg/ml of STX2 and each synthetic peptide at each concentration are caused to exist in cultured vero cells, and cultured for 3 days. Viable cells after the culturing are quantitatively determined by WST-assay (cell viability assay kit).

[0054] Values are shown on condition that a value in STX2(-) is defined as 100% and a value in 1 pg/ml of STX2 alone (namely, inhibitor-free) as 0%.

[0055] Fig. 7B shows the results of examination using a mouse infected with E. coli O157:H7. The procedure is as follows. That is, Day 0: A mouse deficient in protein calory was gastrically infected with a lethal dose of E. coli O157:H7 N-9 strain. Day 2 to Day 4: A sample peptide, a trisaccharide analog (75 μg/g of body weight) or saline alone was gastrically administered twice a day. Day 2 to Day 5: PPR-tet or PPP-tet (225 μg/g of body weight) was gastrically administered twice a day.

[0056] The excellent functional effect of the sample peptide of this invention is confirmed.

[0057] MA indicated in Figs. 6 and 7 refers to a control of reference compound (Met-Ala):Met-Ala-Ala-AHA which does not contain any of the foregoing peptide motifs (1), (2), (3) and (4) and whose mass spectrum is shown in Fig. 5. PPR-tet and the like indicated in Fig. 7 correspond to those shown in Table 4.

Table 4

Kinetic analysis of the binding of synthetic Peptides to His-tagged Stx 28-subunit

	K_b (μM of unit)	RUmax (AU)
P P R R N R R	2.7	1,350
P P P R R R R	3.2	1,250
K R R N P R R	2.1	1,290
F R R N R R N	1.7	1,490
M A	ND	ND
SUPER TWIG $(1)_4$6	1.1	640

[0058] In the above-described compounds, AHA (aminohexanoic acid) is used as the spacer molecule, and Ala present between AHA and peptide motif Xo and Met-Ala as a terminal modification molecule of peptide motif Xo are introduced for confirming the amino acid sequencing. These may be various types. AHA as the spacer molecule is one selected from the comparative examination with SUPER TWIG as a substance containing an amino group and a carboxyl group and having a chain length of 6 carbon atoms. Although the carbon number is preferably 6, it may be from 4 to 10.

[0059] As the spacer molecule, other types of molecules are also available unless impairing the activity of inhibiting STX2 with peptide motif Xo and molecular core structure portion 3Lys.

[0060] Terminal Met-Ala and Ala bound to AHA introduced for amino acid sequencing at the time of screening may be other appropriate amino acids. After the screening, these are unnecessary because of lack of the activity of inhibiting STX2. However, when NH_2 is exposed at the terminal of the motif, a plus charge is provided. From the standpoint of controlling the charge, it is preferable that terminal MA or other types are present. These are generally uncharged amino

acids, and preferably amino acids which have no great influence on hydrophobicity.

[0061] The NH_2 of Met at the N-terminus may be protected with an acetyl group to suppress gastrointestinal decomposition with a protease following oral administration.

[0062] According to the inventors' confirmation, the acetylation increases the function (activity) of inhibiting the in-vivo cytotoxicity of STX2 by approximately five times. In the infection experiment as well, the increase in effect is confirmed.

[0063] From the foregoing as well, in the case of, for example, (acetyl-Xo-AHA)$_4$3Lys, replacement of acetyl with another protecting group, replacement of AHA with another spacer or the absence of the spacer is advantageously considered.

[0064] According to the inventors' investigations, with respect to the method for screening for a polyvalent STX2 inhibiting peptide it has been found more commonly that the STX2 inhibiting peptides may be peptides in which a peptide motif, which is formed by peptide linkage of at least seven amino acids, whose sequence has two cluster portions each having bound thereto at least two basic amino acids, for example, asparagine (Arg), lysine (Lys) and histidine (His) and whose C-terminal side is a basic amino acid, is incorporated in a molecular core structure portion having three molecules of lysine (Lys) peptide-linked thereto.

[0065] It is considered preferable that arginine (Arg) is contained as the basic amino acid constituting the cluster portion. Accordingly, a preferable cluster portion is, for example, -Arg-Arg- or -Arg-Arg-Asn-.

[0066] The C-terminal side is preferably a basic amino acid, for example, arginine (Arg) and the N-terminal side is a hydrophobic amino acid, for example, proline (Pro).

[0067] The reason for the C-terminal side preferably being a basic amino acid is considered to be that an acidic amino acid cluster is present near the globo3 sugar binding site 3 of the STX2B-subunit to be bound and both would electrostatically interact to increase the affinity. The reason for the N-terminal side preferably being a hydrophobic amino acid is considered to be that it hydrophobically interacts with tryptophan (Trp) which plays a main role in the globo3 sugar binding site 3 of the STX2B-subunit.

[0068] In view of the foregoing, motifs (1), (2), (3) and (4) are provided in this invention as a preferable example.

[0069] The peptide motif of the aspect of this invention relating to the screening method may comprise at least seven amino acids as described above in consideration of the molecular size effect on the STX2 inhibiting function. However, the number of amino acids may be more than 7 unless the STX2 inhibitory activity is greatly impaired, and the larger number of amino acids is available for application to drugs or the like. Further, needless to say, various spacers or terminal modification groups may be provided as stated above.

[0070] In this invention, as the verotoxin neutralizing agent containing the peptide having the STX2 inhibitory activity as an active ingredient, various dosage forms may be employed. In the oral administration, the agent may be formulated with a vehicle and the like to provide tablets or a powder, or a liquid preparation as a composition with purified water and the like. In the composition and the dosage forms, various ingredients including known ones may be used. Various methods may be employed for this purpose.

[0071] Regarding the dose as the verotoxin neutralizing agent, it may be considered to be used generally at a dose of from 5 to 500 mg/kg-body weight since the discovery of the infection with E. coli O157:H7. Of course, it may properly be determined according to the symptom.

[0072] In recent years, a large number of peptides having a high physiological activity in trace amounts have been discovered, and supply of peptides in large quantities has been enabled by the rapid progress of biotechnologies such as gene recombination technology and cell fusion. Attempts have been made to apply these physiologically active peptides to therapy of diseases as pharmaceutical preparations. It has been however known that even though such peptide pharmaceutical preparations are orally administered, no sufficient absorption ratio is obtained. This is presumably because these peptide pharmaceutical preparations undergo rapid decomposition with a digestive enzyme or a protease in the digestive tract or poorly permeate the mucous membrane of the digestive tract owing to water solubility and high molecular weight. For this reason, the administration of these pharmaceutical preparations is mostly limited clinically to the administration by injection such as muscular administration, subcutaneous administration or intravenous administration. However, these administrations by injection have defects that they give pains to patients and severe side effects such as allergic reaction and anaphylactic shock are developed. Accordingly, permucosal administration including oral administration has lately attracted much interest as an administration route to replace the injection. However, no sufficient absorption ratio is obtained in comparison to the injection. Therefore, at present, for improving the absorption ratio of physiologically active peptides after oral administration and permucosal administration, various methods have been attempted. These can be classified in (1) use of pharmaceutical additives such as an absorption enhancer and a protease inhibitor, (2) development of a new administration route of drugs, (3) modification of a molecular structure of drugs and (4) dosage form modification of drugs.

[0073] For oral administration it is possible to adopt appropriate approaches, for example, use of typical surfactants, bile acid, chelating agents and hydrocarbons such as fatty acids as an absorption enhancer, addition of sodium glycocholate, bacitracin, soybean trypsin inhibitor, camostat, aprotinin and the like as a protease inhibitor, inclusion in liposome or emulsion and use by encapsulation.

INDUSTRIAL APPLICABILITY

[0074] According to the method of this invention, it is possible, as stated above, to screen for polyvalent STX2 toxin neutralizing peptides, which have the property of inhibiting verotoxin whose synthesis is easy as peptide synthesis and which are effective as a therapeutic agent, and to provide these peptides.

[0075] The foregoing peptides of this invention have the STX2 inhibiting property, are easy to synthesize as peptide synthesis, and can provide the verotoxin neutralizing agent which is effective as a therapeutic agent of enterohemorrhagic Escherichia coli infectious diseases.

SEQUENCE LISTING

[0076]

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<120> A method for screening a toxin-neutralizing peptide, STX2-inhibiting peptide and vorptoxin-neutralizing agent

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Claims

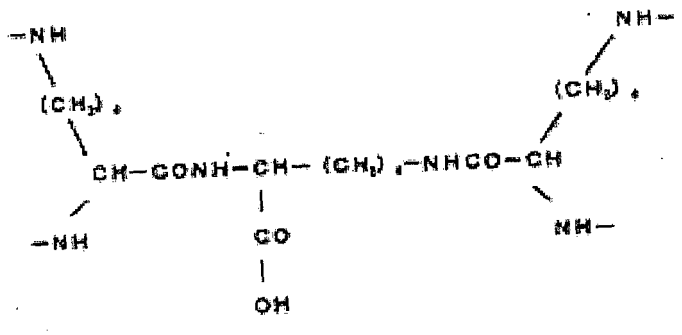
1. A method for screening for a polyvalent STX2 toxin-neutralizing peptide which can neutralize STX2, the method comprising the following steps:

(a) providing STX2,
(b) providing a mutant of STX2 which is functionally deficient in the site by which it binds to its receptor due to the presence of a mutation in the receptor-binding site of STX2,
(c) providing a first polyvalent peptide library, wherein each entity in the first polyvalent peptide library comprises a core structure comprising a plurality of lysines and wherein degenerate peptides are bound to the terminal amino groups of the lysines,

(d) contacting said first polyvalent peptide library with STX2 and the mutant,
(e) contrasting a peptide motif bound to STX2 with a peptide motif bound to the mutant to obtain an amino acid selection ratio where the amino acid selection ratio is used to specify a receptor-binding site-specific binding peptide motif, and

wherein the STX2-neutralizing peptide comprises the receptor-binding site-specific binding peptide motif.

2. The method of claim 1, wherein said core structure has three molecules of lysine (Lys) having the structure:



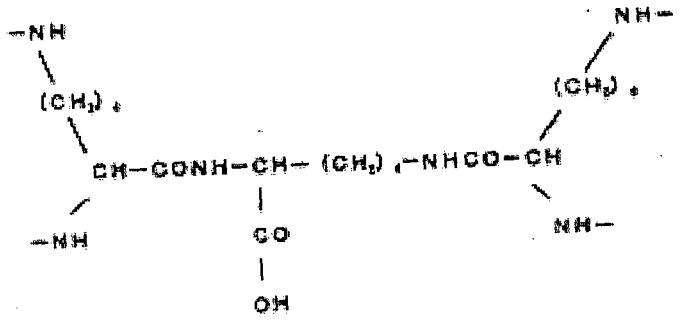
and said peptides are represented by MAXXXXA-AHA (SEQ ID NO: 5), to give a molecule of formula $(MAXXXXA\text{-}AHA)_4$-3Lys,
wherein X is an amino acid which is not cysteine and AHA is aminohexanoic acid, and
wherein said STX2 mutant comprises a tryptophan to alanine mutation at position 32 of the STX2B subunit and said contrasting step comprises:

(a) determining a peptide motif within the peptides of the library that binds to STX2,
(b) determining a peptide motif within the peptides of the library that binds to the mutant,
(c) specifying a receptor-binding site-specific peptide motif by determining a first normalised value for each amino acid in the STX2 toxin-bound peptides based on the number of times each amino acid is present at each position in the STX2 toxin-bound peptides, and a second normalised value for each amino acid in the mutant-bound peptides based on the number of times each amino acid is present at each position in the mutant-bound peptides, and
(d) dividing the first normalised value by the corresponding second normalised value, in order to obtain a selection ratio for each amino acid position.

3. A method as claimed in claim 2, wherein a second polyvalent peptide library is screened wherein the sequences of the peptides in the second peptide library are based on the sequences of the previously-obtained receptor-binding site-specific peptide motifs, and wherein the amino acids in the receptor-binding site-specific peptide motifs with high selection ratios are fixed in the peptides of the second library, in order to obtain a receptor-binding site-specific peptide motif with a higher selectivity for the receptor.

4. A method for screening for a toxin-neutralizing peptide according to claim 1, wherein in the first polyvalent peptide library, the peptides are bound to the terminal lysine amino groups via a spacer molecule.

5. An STX2 inhibiting peptide comprising a molecular core portion which has three molecules of lysine (Lys) having the structure:



wherein each terminal amino group in the Lys core structure is bound to a peptide motif, wherein each peptide motif is identical and is selected from the group consisting of:

(1) FRRNRRN (SEQ ID NO: 1)
(2) PPPRRRR (SEQ ID NO: 2)
(3) PPRRNRR (SEQ ID NO: 3)
(4) KRRNPRR (SEQ ID NO: 4).

6. An STX2 inhibiting peptide according to claim 5, wherein the peptide motif is incorporated via a spacer molecule.

7. An STX2 inhibiting peptide according to claim 6, wherein the spacer molecule is a molecule having a peptide or an amino group and a carboxyl group and having a hydrocarbon chain structure with from 4 to 10 carbon atoms.

8. An STX2 inhibiting peptide according to any of claims 5 to 7, wherein the peptide motif has a terminal modification molecule.

9. A peptide according to claim 8, wherein the terminal modification molecule is an uncharged molecule.

10. A peptide according to claim 9, wherein the terminal modification molecule has Met-Ala in the N-terminal.

11. An STX2 inhibiting peptide according to claim 5, wherein the molecular core structure has one of the following formulae attached to each terminal amino group:

Met-Ala-Xo-Ala-AHA- (a)

Acetyl-Xo-AHA- (b)

wherein AHA is an aminohexanecarboxylic acid group,
and Xo represents any one of the peptides (1), (2), (3) or (4) as defined in claim 5.

12. A verotoxin neutralising agent comprising the peptide according to any one of claims 5 to 11 as an active ingredient.

Patentansprüche

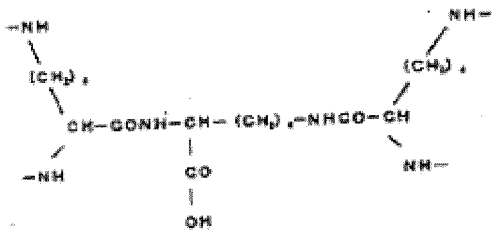
1. Verfahren zum Screenen nach einem polyvalenten STX2 Toxin-neutralisierenden Peptid, das STX2 neutralisieren kann, wobei das Verfahren die folgenden Schritte umfasst:

(a) Bereitstellen von STX2,
(b) Bereitstellen einer Mutante von STX2, die an der Stelle, mit der sie an den Rezeptor bindet, aufgrund des Vorliegens einer Mutation in der Rezeptor-bindenden Stelle von STX2 funktionell fehlerhaft ist,
(c) Bereitstellen einer ersten polyvalenten Peptidbibliothek, wobei jede Einheit in der ersten polyvalenten Peptidbibliothek eine Kernstruktur umfasst, die eine Vielzahl von Lysinen umfasst und wobei degenerierte Peptide an die endständigen Aminogruppen der Lysine gebunden sind,
(d) in Kontakt bringen der ersten polyvalenten Peptidbibliothek mit STX2 und der Mutante,
(e) Gegenüberstellen eines an STX2 gebundenen Peptidmotivs mit einem Peptidmotiv, das an die Mutante

gebunden ist, um ein Aminosäureselektionsverhältnis zu erhalten, wobei das Aminosäureselektionsverhältnis verwendet wird, um ein bindendes Peptidmotiv festzulegen, das für eine Rezeptorbindungsstelle spezifisch ist, und

wobei das STX2-neutralisierende Peptid das bindende Peptidmotiv umfasst, das für eine Rezeptorbindungsstelle spezifisch ist.

2. Verfahren nach Anspruch 1, wobei die Kernstruktur drei Moleküle Lysin (Lys) aufweist, welche die folgende Struktur aufweisen:



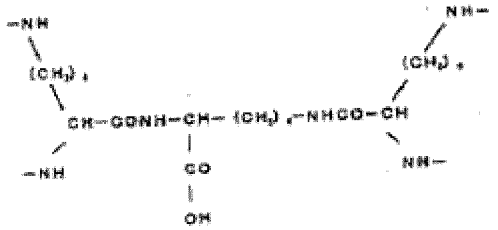
und die Peptide durch MAXXXXA-AHA (SEQ ID NO:5) dargestellt werden, um ein Molekül der Formel $(MAXXXXA\text{-}AHA)_4$-3Lys zu erhalten,
wobei X eine Aminosäure ist, die nicht Cystein ist und AHA Aminohexansäure ist,
und
wobei die STX2 Mutante eine Tryptophan zu Alanin Mutation an Position 32 der STX2B Untereinheit umfasst und der Schritt des Gegenüberstellens Folgendes umfasst:

(a) Bestimmen eines Peptidmotivs unter den Peptiden der Bibliothek, das an STX2 bindet,
(b) Bestimmen eines Peptidmotivs unter den Peptiden der Bibliothek, das an die Mutante bindet,
(c) Festlegen eines Peptidmotivs, das für eine Rezeptorbindungsstelle spezifisch ist durch Bestimmen eines ersten normalisierten Werts für jede Aminosäure in den STX2 Toxin-gebundenen Peptiden auf Basis der Häufigkeit, mit der jede Aminosäure an jeder Position in den STX2 Toxin-gebundenen Peptiden vorkommt sowie eines zweiten normalisierten Werts für jede Aminosäure in den Mutanten-gebundenen Peptiden auf Basis der Häufigkeit, mit der jede Aminosäure an jeder Position in den Mutanten-gebundenen Peptiden vorkommt
und
(d) Teilen des ersten normalisierten Werts durch den entsprechenden zweiten normalisierten Wert, um ein Selektionsverhältnis für jede Aminosäureposition zu erhalten.

3. Verfahren wie in Anspruch 2 beansprucht, wobei eine zweite polyvalente Peptidbibliothek gescreent wird, wobei die Sequenzen der Peptide in der zweiten Peptidbibliothek auf den Sequenzen der zuvor erhaltenen Peptidmotive beruhen, die für eine Rezeptorbindungsstelle spezifisch sind, und wobei die Aminosäuren mit hohen Selektionsverhältnissen in den Peptidmotiven, die für eine Rezeptorbindungsstelle spezifisch sind, in den Peptiden der zweiten Bibliothek fixiert werden, um ein Peptidmotiv, das für eine Rezeptorbindungsstelle spezifisch ist, mit einer höheren Selektivität für den Rezeptor zu erhalten.

4. Verfahren zum Screenen nach einem Toxin-neutralisierenden Peptid nach Anspruch 1, wobei die Peptide in der ersten polyvalenten Peptidbibliothek an die endständigen Lysin-Aminogruppen über ein Abstandhaltermolekül gebunden sind.

5. STX2 hemmendes Peptid, das einen molekularen Kernanteil umfasst, der drei Moleküle Lysin (Lys) aufweist, welche die folgende Struktur aufweisen:



wobei jede endständige Aminogruppe in der LysKernstruktur an ein Peptidmotiv gebunden ist, wobei jedes Peptidmotiv identisch ist und ausgewählt ist aus der Gruppe bestehend aus:

(1) FRRNRRN (SEQ ID NO:1)
(2) PPPRRRR (SEQ ID NO:2)
(3) PPRRNRR (SEQ ID NO:3)
(4) KRRNPRR (SEQ ID NO:4)

6. STX2 hemmendes Peptid nach Anspruch 5, wobei das Peptidmotiv über ein Abstandhaltermolekül eingebaut ist.

7. STX2 hemmendes Peptid nach Anspruch 6, wobei das Abstandhaltermolekül ein Molekül ist, das ein Peptid oder eine Aminogruppe und eine Carboxylgruppe aufweist und eine Kohlenwasserstoffkettenstruktur mit 4 bis 10 Kohlenstoffatomen aufweist.

8. STX2 hemmendes Peptid nach einem der Ansprüche 5 bis 7, wobei das Peptidmotiv ein endständiges Modifizierungsmolekül aufweist.

9. Peptid nach Anspruch 8, wobei das endständige Modifizierungsmolekül ein ungeladenes Molekül ist.

10. Peptid nach Anspruch 9, wobei das endständige Modifizierungsmolekül ein Met-Ala am N-Ende aufweist.

11. STX2 hemmendes Peptid nach Anspruch 5, wobei die molekulare Kernstruktur eine der folgenden Formeln, gebunden an jede endständige Aminogruppe, aufweist:

Met-Ala-Xo-Ala-AHA- (a)

Acetyl-Xo-AHA- (b)

wobei AHA eine Aminohexancarbonsäuregruppe ist,
und Xo eines der Peptide (1), (2), (3) oder (4) wie in Anspruch 5 definiert darstellt.

12. Verotoxin-neutralisierendes Mittel, welches das Peptid nach einem der Ansprüche 5 bis 11 als aktiven Bestandteil umfasst.

Revendications

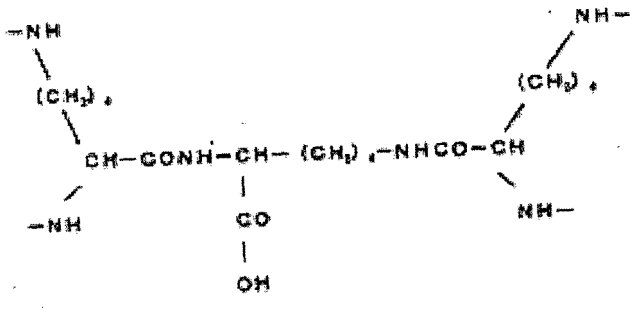
1. Procédé de criblage d'un peptide neutralisant la toxine STX2 polyvalent qui peut neutraliser la STX2, le procédé comprenant les étapes suivantes :

(a) fournir la STX2,
(b) fournir un mutant de la STX2 qui est fonctionnellement déficient dans le site par lequel il se lie à son récepteur en raison de la présence d'une mutation dans le site de liaison au récepteur de la STX2,
(c) fournir une première banque de peptides polyvalents, chaque entité dans la première banque de peptides polyvalents comprenant une structure centrale comprenant une pluralité de lysines et des peptides dégradés étant liés aux groupes amino terminaux des lysines,
(d) mettre en contact ladite première banque de peptides polyvalents avec la STX2 et le mutant,
(e) mettre en contraste un motif peptidique lié à la STX2 avec un motif peptidique lié au mutant pour obtenir un rapport de sélection d'acides aminés où le rapport de sélection d'acides aminé est utilisé pour préciser un motif

peptidique de liaison spécifique au site de liaison au récepteur, et

dans lequel le peptide neutralisant la STX2 comprend le motif peptidique de liaison spécifique au site de liaison au récepteur.

2. Procédé selon la revendication 1, dans lequel ladite structure centrale a trois molécules de lysine (Lys) ayant la structure :



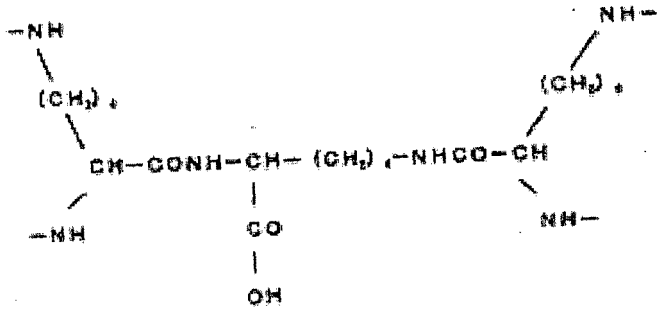
et lesdits peptides sont représentés par MAXXXXA-AHA (SEQ ID N° : 5), pour donner une molécule de formule $(MAXXXXA\text{-}AHA)_4$-3Lys,
dans laquelle X est un acide aminé qui n'est pas la cystéine et AHA est l'acide aminohexanoïque, et
dans laquelle ledit mutant de la STX2 comprend une mutation tryptophane en alanine en position 32 de la sous-unité STX2B et ladite étape de mise en contraste comprend :

(a) la détermination d'un motif peptidique dans les peptides de la banque qui se lie à la STX2,
(b) la détermination d'un motif peptidique dans les peptides de la banque qui se lie au mutant,
(c) la spécification d'un motif peptidique spécifique au site de liaison au récepteur en déterminant une première valeur normalisée pour chaque acide aminé dans les peptides liés à la toxine STX2 sur la base du nombre de fois où chaque acide aminé est présent à chaque position dans les peptides liés à la toxine STX2, et une deuxième valeur normalisée pour chaque acide aminé dans les peptides liés au mutant sur la base du nombre de fois où chaque acide aminé est présent à chaque position dans les peptides liés au mutant, et
(d) la division de la première valeur normalisée par la deuxième valeur normalisée correspondante, afin d'obtenir un rapport de sélection pour chaque position d'acide aminé.

3. Procédé selon la revendication 2, dans lequel une deuxième banque de peptides polyvalents est criblée, dans lequel les séquences des peptides dans la deuxième banque de peptides sont basées sur les séquences des motifs peptidiques spécifiques au site de liaison au récepteur obtenus précédemment, et dans lequel les acides aminés dans les motifs peptidiques spécifiques au site de liaison au récepteur ayant des rapports de sélection élevés sont fixés dans les peptides de la deuxième banque, afin d'obtenir un motif peptidique spécifique au site de liaison au récepteur ayant une plus grande sélectivité pour le récepteur.

4. Procédé de criblage d'un peptide neutralisant une toxine selon la revendication 1, dans lequel dans la première banque de peptides polyvalents, les peptides sont liés aux groupes amino lysine terminaux via une molécule d'espacement.

5. Peptide inhibant la STX2 comprenant une partie centrale moléculaire qui a trois molécules de lysine (Lys) ayant la structure :



dans laquelle chaque groupe amino terminal dans la structure centrale Lys est lié à un motif peptidique, chaque motif peptidique étant identique et étant sélectionné dans le groupe constitué par:

(1) FRRNRRN (SEQ ID N° : 1)
(2) PPPRRRR (SEQ ID N° : 2)
(3) PPRRNRR (SEQ ID N° : 3)
(4) KRRNPRR (SEQ ID N° : 4).

6. Peptide inhibant la STX2 selon la revendication 5, dans lequel le motif peptidique est incorporé via une molécule d'espacement.

7. Peptide inhibant la STX2 selon la revendication 6, dans lequel la molécule d'espacement est une molécule ayant un peptide ou un groupe amino et un groupe carboxyle et ayant une structure de chaîne hydrocarbonée ayant de 4 à 10 atomes de carbone.

8. Peptide inhibant la STX2 selon l'une quelconque des revendications 5 à 7, dans lequel le motif peptidique a une molécule à modification terminale.

9. Peptide selon la revendication 8, dans lequel la molécule à modification terminale est une molécule non chargée.

10. Peptide selon la revendication 9, dans lequel la molécule à modification terminale a Met-Ala dans la terminaison N.

11. Peptide inhibant la STX2 selon la revendication 5, dans lequel la structure centrale moléculaire a l'une des formules suivantes rattachée à chaque groupe amino terminal :

Met-Ala-Xo-Ala-AHA- (a)

Acétyl-Xo-AHA- (b)

dans lesquelles AHA est un groupe acide aminohexanecarboxylique,
et Xo représente l'un quelconque des peptides (1), (2), (3) ou (4) tels qu'ils sont définis dans la revendication 5.

12. Agent neutralisant la vérotoxine comprenant le peptide selon l'une quelconque des revendications 5 à 11 en tant que principe actif.

Fig. 1

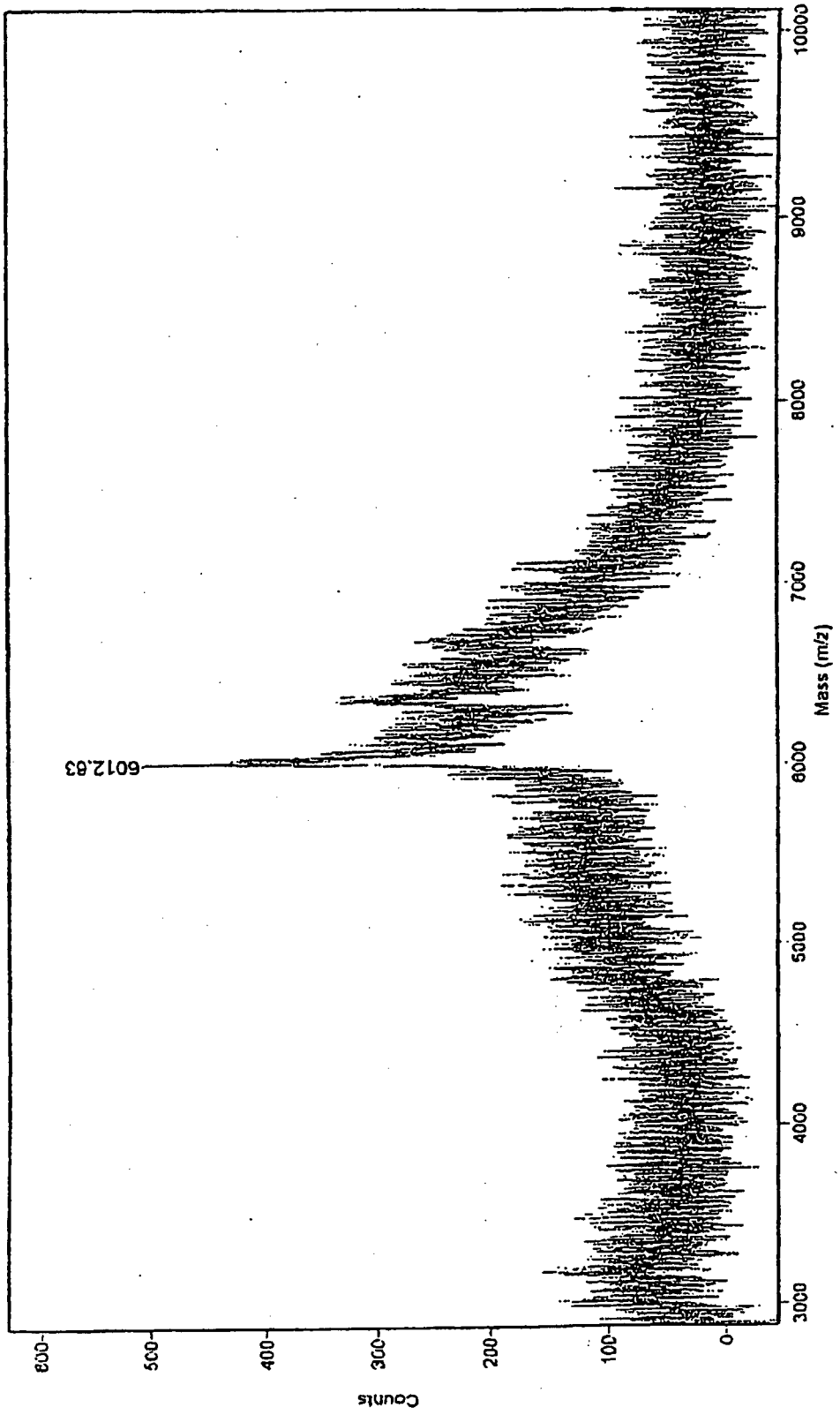

6012.63
Counts
0
100
200
300
400
500
600
3000
4000
5000
6000
7000
8000
9000
10000
Mass (m/z)

Fig. 2

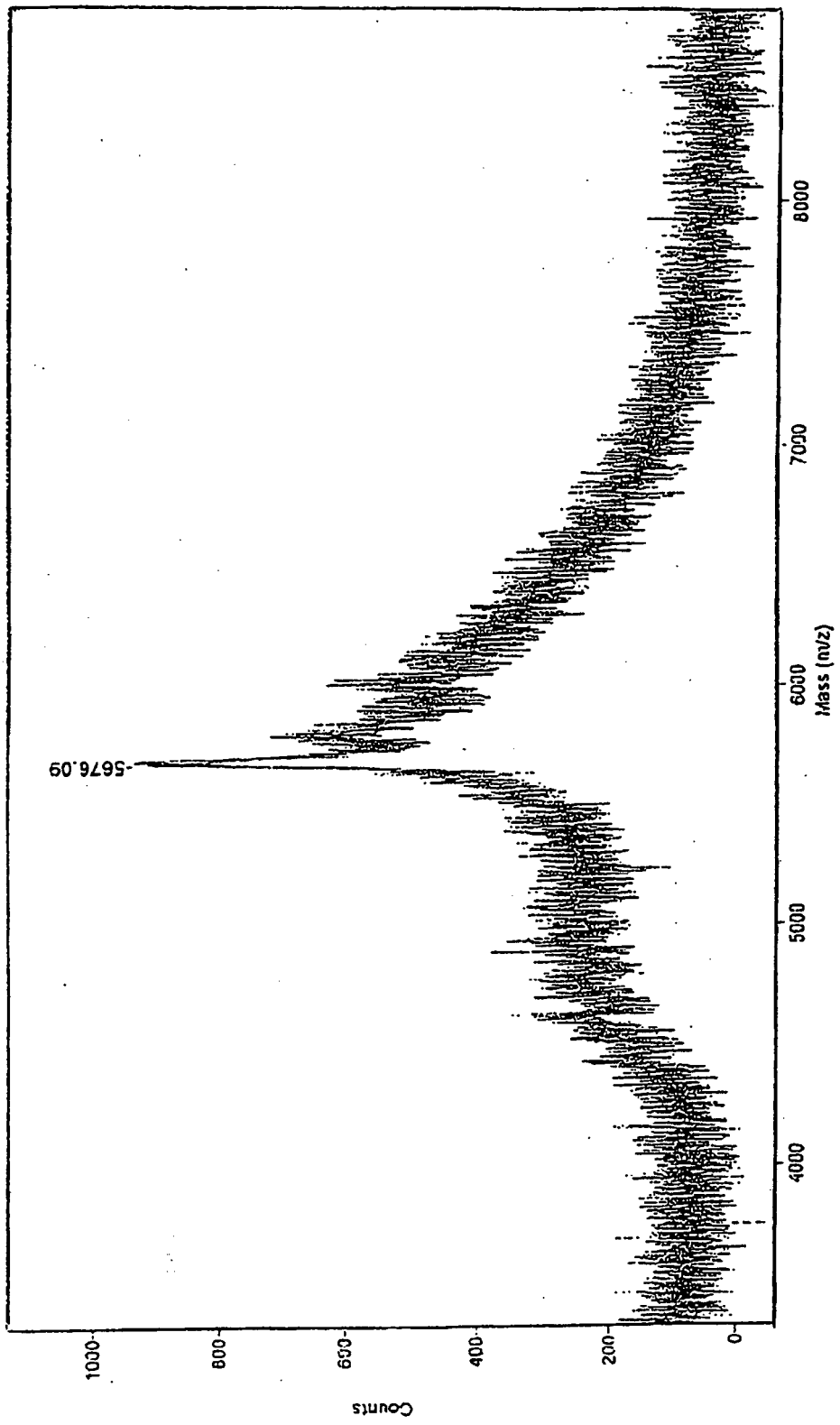

5676.09
Counts
0
200
400
600
800
1000
4000
5000
6000
7000
8000
Mass (m/z)

Fig. 3

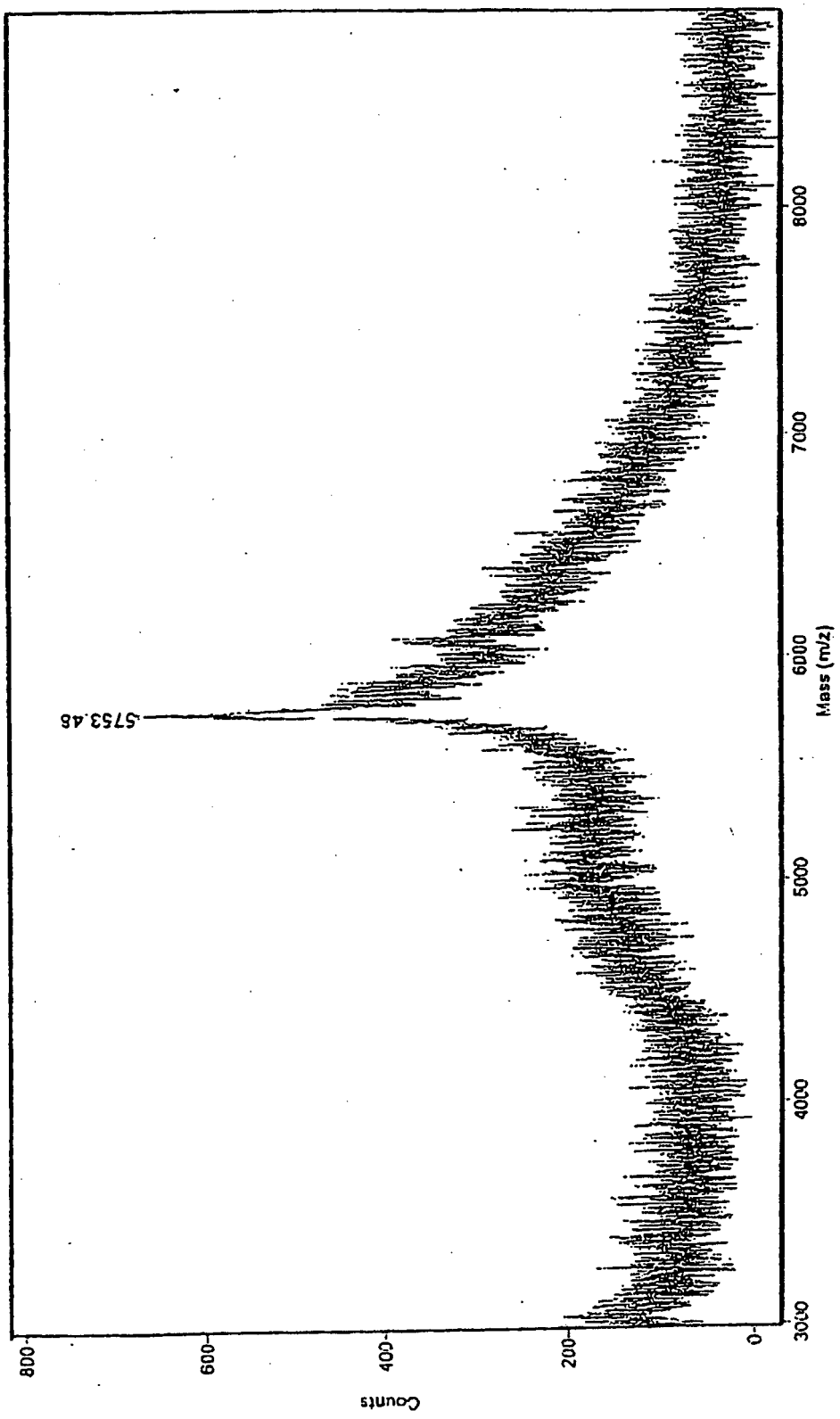

5753.45
Counts
800
600
400
200
0
3000
4000
5000
6000
7000
8000
Mass (m/z)

Fig. 4

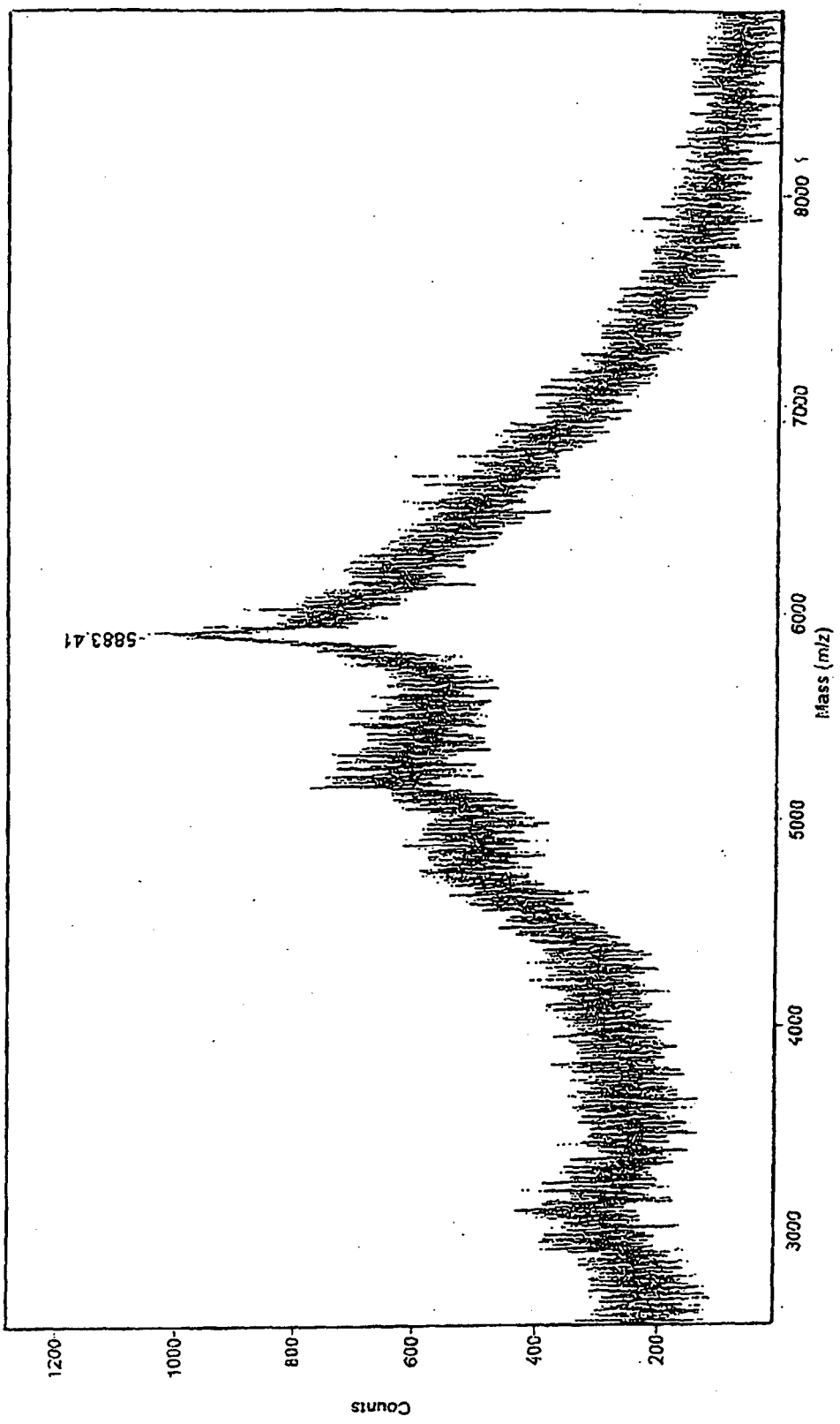

5883.41
Counts
1200
1000
800
600
400
200
3000
4000
5000
6000
7000
8000
Mass (m/z)

Fig. 5

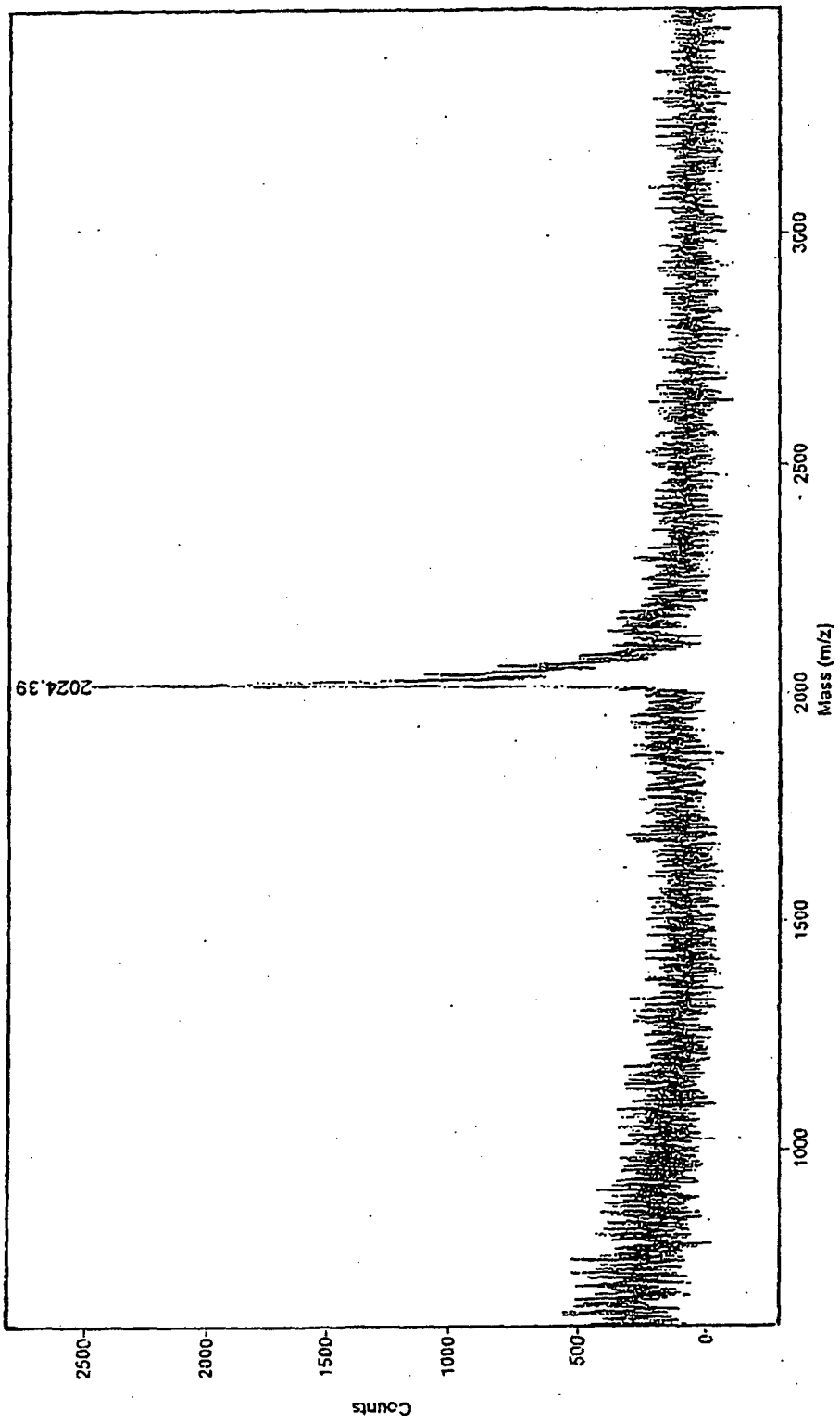

2024.39
Counts
2500
2000
1500
1000
500
0
1000
1500
2000
2500
3000
Mass (m/z)

Fig. 6

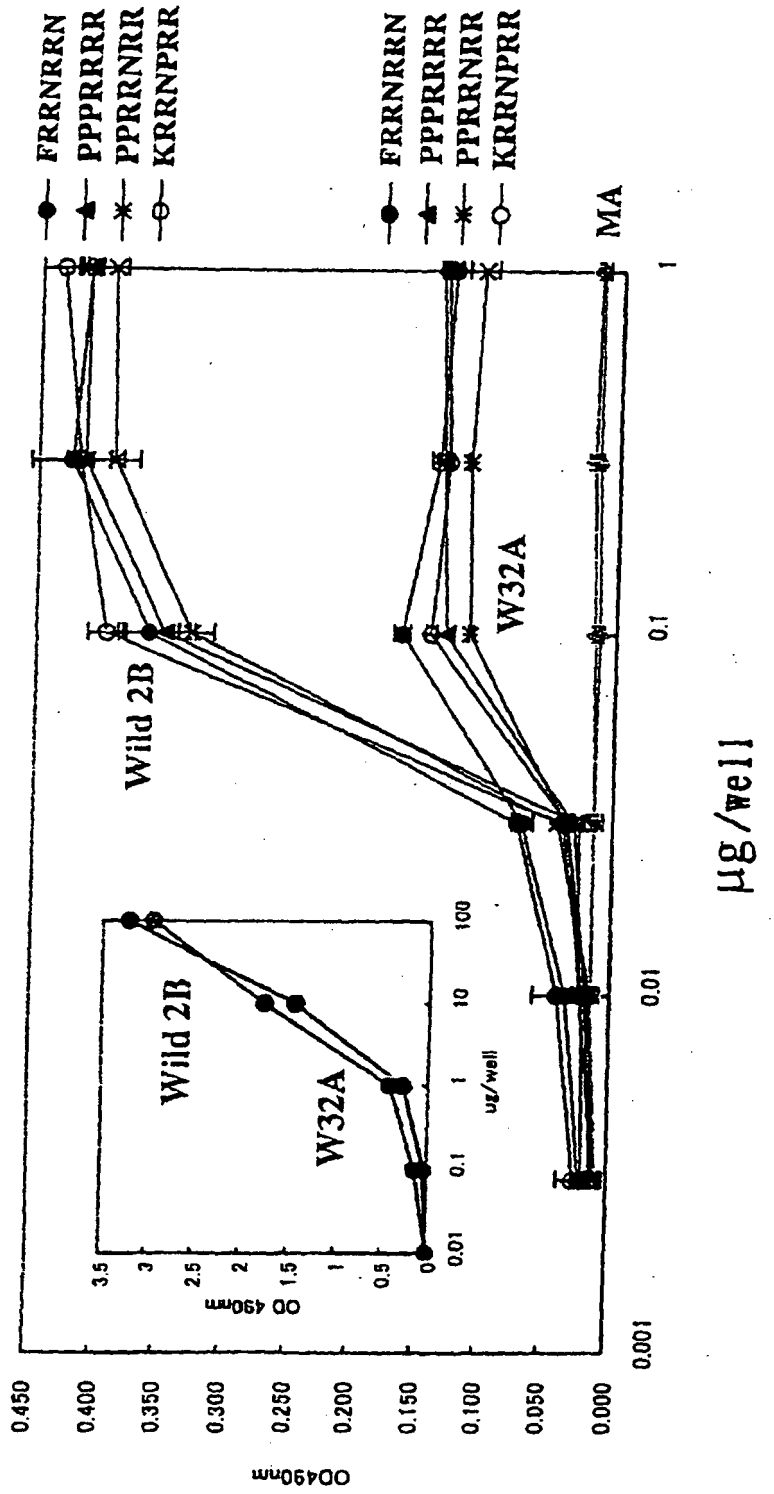
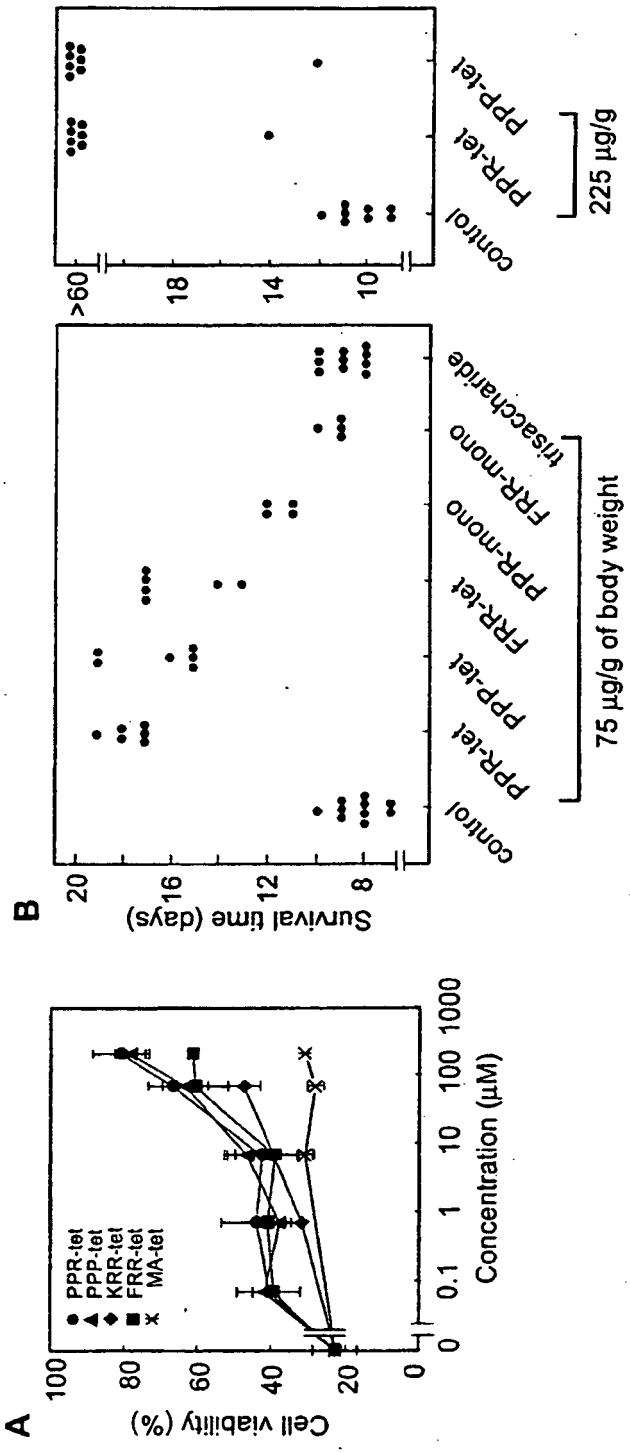

FRRNRRN
PPPRRRR
PPRRNRR
KRRNPRR
MA
W32A
Wild 2B
μg/well
OD490nm
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0.001
0.01
0.1
1
3.5
3
2.5
2
1.5
1
0.5
0
0.01
0.1
1
10
100
ug/well

Fig. 7


A
Cell viability (%)
100
80
60
40
20
0
PPR-tet
PPP-tet
KRR-tet
FRR-tet
MA-tet
0
0.1
1
10
100
1000
Concentration (μM)
B
Survival time (days)
20
16
12
8
control
PPR-tet
PPP-tet
FRR-tet
PPR-mono
FRR-mono
trisaccharide
75 μg/g of body weight
>60
18
14
10
control
PPR-tet
PPP-tet
225 μg/g

REFERENCES CITED IN THE DESCRIPTION

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