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Use of Yessotoxins in the Treatment of Allergic and Asthmatic Processes

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ABSTRACT

The present invention relates to the use of yessotoxins in the treatment of allergic and asthmatic processes. The mechanism of action of yessotoxin (YTX) and analogs thereof is related to cellular phosphodiesterase activation, and accordingly to the decrease of 5 cytosolic cAMP levels. The result of this activation after the administration of YTX is inhibition of the immunological activation of mast cells in rats and therefore of histamine release. This effect can be used as a strategy for the treatment of allergic and asthmatic processes.

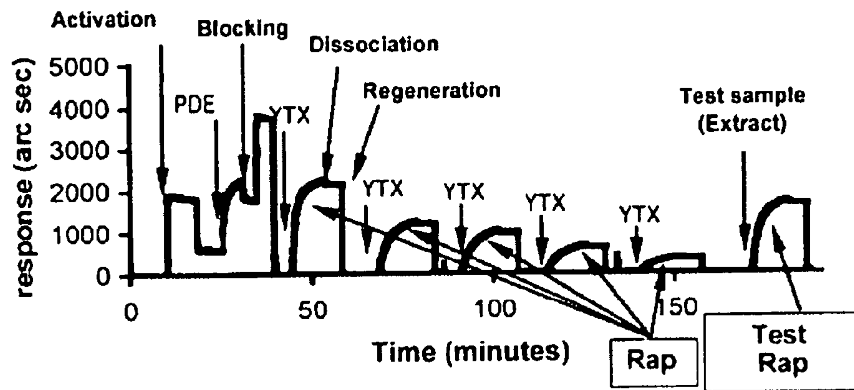

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Figure 2

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Australian Patents Act 1990 – Regulation 3.2A

ORIGINAL COMPLETE SPECIFICATION
STANDARD PATENT

Invention Title

"Use of Yessotoxins in the Treatment of Allergic and Asthmatic Processes"

The following statement is a full description of this invention, including the best method of performing it known to us:-

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USE OF YESSOTOXINS IN THE TREATMENT OF ALLERGIC AND ASTHMATIC PROCESSES

This is a divisional of Australian Patent Application No. 2004260895, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention describes the therapeutic use of yessotoxins in the treatment of allergy and asthma according to their ability to activate phosphodiesterase enzymes, one of the cellular targets of these toxins.

BACKGROUND OF THE INVENTION

Yessotoxin, hereinafter YTX, and its natural analogs are polycyclic ethers produced by the Protoceratium reticulatum and Lingolodinium polyedrum dinoflagellates, being originally isolated from the digestive apparatus of Patinopecten yessoensis (Murata, M. et al., *Tetrahedron Lett.*, 1987, 28, 5869-5872). As shown in Figure 1, it is a lipophilic molecule whose chemical structure consists of eleven rings with ether groups bound to an unsaturated side chain and two sulfonic esters. Figure 1 further shows some of the natural analogs of YTX which are differentiated in the side chain substituents, although recently more than 50 natural derivatives have been described, the structure of which has not yet been identified.

Some studies have been conducted in order to determine the toxicity of YTX. When it is orally administered to mice, no cases of acute toxicity have been observed (Aune, T. et al., *Toxicon*, 2002, 40, 77-82), however death of the rodents did occur after intraperitoneal injection (Tubaro, A. et al., *Toxicon*, 2003, 41, 783-792). Only some ultrastructural alterations in the myocardium have been observed after oral treatment and no accumulative toxin effects occurred after the repeated oral administration of this toxin (Tubaro, A. et al., *Toxicon*, 2004, 43, 439-445). The histological exam showed the presence of myocardial alterations which could be seen only with electron microscopy, with no damage to other organs. Therefore, based on these results, YTX and the derivatives thereof are considered non toxic, even at doses 100 times higher than those which a human being can consume daily.

YTXs were initially classified within the group of diarrhetic toxins since they are often detected together, however their mechanism of action is different from that of any other toxin. Further they do not produce diarrhea, nor do they affect cellular phophatases, the cellular target of diarrhetic toxins. Their mechanism of action is related to other enzymes.
5 In this sense, it has been described that YTX decreases cytosolic levels of cyclic adenosine monophosphate (cAMP), a second messenger, because it increases the activity of cellular phophodiesterases by a calcium-dependent mechanism (Alfonso, A. et al., *Biochem. Pharmacol.*, 2003, 65, 193-208). YTX further increases cytosolic calcium levels by stimulating its entry through a channel located in the cell membrane (De la Rosa, L.A. et
10 al., *Biochem. Pharmacol.*, 2001, 61, 827-833; De la Rosa, L.A., et al., *Cell Signal*, 2001, 13, 711-716). The effect on phosphodiesterases has been used to develop sensitive methods for detecting the presence of these toxins in contaminated mollusks, which have been recently been published (Alfonso, A. et al., *Analytical Biochem.*, 2004, 326, 93-99; Pazos, M.J. et al., *Analytical Biochem.*, 2004, 335, 112-118).

15 YTX has a singular structure and its mechanism of action does not seem to be very common, therefore it can be an interesting tool for biological and pharmacological studies. cAMP is a second messenger related to early pathways in the cell signal. Cells regulate cAMP levels by a balance between synthesis, through the activation of adenylate cyclases, and hydrolysis, by activation of phosphodiesterases. There are eleven families of
20 phosphodiesterases with different substrate specificity, sensitivity and location. Modulation of these enzymes is often used to regulate the activity of several types of inflammatory cells; in fact several treatments used in asthma interfere with this pathway. Therefore, it is very interesting to determine the effect that YTX has on inflammatory cells, since the toxin increases interleukin 2 levels in human lymphocytes (Alfonso, A. et
25 al., *Biochem. Pharmacol.*, 2003, 65, 193-208).

It has recently been observed that YTX is a histamine release inhibitor, which release is activated by immunological stimulus, in mast cells in rats. Therefore, it is a drug susceptible to being used as an antiallergic or antiasthmatic drug. The role of cAMP in histamine release is not very clear. Generally, an increase of cMAP levels inhibits
30 secretion, however contradictory effects have been observed in the presence of different stimuli (Alfonso, A. et al., *Cell Signal*, 2000, 12, 343-350; Alm, P.E., *Agents Actions*,

1983, 13, 132-137; Alm, P.E., *Int. Arch. Allergy Appl. Immunol.*, 1984, 75, 375-378; Teraoka, H. et al., *Gen Pharmacol.*, 1997, 28, 237-243). In this sense, it has been described that the immunological stimulation is accompanied by an initial increase of cAMP levels which disappears after ten minutes but which is required to activate events associated to
5 secretion since cAMP is part of these initial events (Alm, P.E., *Int. Arch. Allergy Appl. Immunol.*, 1984, 75, 375-378). This fact may explain the results obtained in the presence of YTX, since the toxin prevents the initial increase of cAMP, which in turns inhibits histamine release. Inhibition depends on toxin concentration, as does the decrease in cAMP levels (Alfonso, A. et al., *Biochem. Pharmacol.*, 2003, 65, 193-208). This fact is not
10 contradictory with the use of phosphodiesterases inhibition to suppress histamine release in the treatment of asthma since, in this case, cAMP levels remain high, which also prevents secretion. Therefore, the description of YTXs as histamine release modulators is an indication of the pharmacological importance of these molecules for their possible therapeutic application, in addition to being applied in HTS (high throughput screening)
15 protocols.

BRIEF DESCRIPTION OF THE INVENTION

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived
20 from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps
25 but not the exclusion of any other integer or step or group of integers or steps.

The authors of the present invention have found that YTXs can be used as inhibitors of the immunological activation of mastocytes and basophils due to their ability to activate cellular phophodiesterases.

Therefore, in a first aspect the present invention relates to a yessotoxin or an analog thereof
30 capable of activating cellular phosphodiesterases for its use in the treatment of of allergic or asthmatic processes.

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Preferably, analogs of yessotoxins are selected from the group consisting of hydroxyyessotoxin, noryessotoxin, homoyessotoxin, hydroxyl-homoyessotoxin, adriatoxin, carboxyyessotoxin and desulfoyessotoxin.

Particularly, the treatment of allergic or asthmatic processes involves promoting the
5 activation of cellular phosphodiesterases.

In another aspect, the present invention refers to the use of yessotoxin or an analog thereof capable of activating cellular phosphodiesterases in the manufacture of a medicament for the treatment of allergic or asthmatic processes.

Particularly, the treatment of allergic or asthmatic processes involves promoting the
10 activation of cellular phosphodiesterases.

Finally, another aspect of the present invention refers to the *in vitro* use of yessotoxin (YTX) or an analog thereof capable of activating cellular phosphodiesterases (PDEs) as mast cell modulators.

15 **DETAILED DESCRIPTION OF THE INVENTION**

The invention set forth describes three uses based on the discovery that YTX is a PDE activator and involves the conversion of this activation into a measurable signal.

Method for determining PDE-YTX biomolecular bonds using an affinity sensor.

Determining molecular interactions in real time using a biosensor is a new technique the
20 application of which is extending into different research fields (Hide, Tsutsui et al., *Anal. Biochem.*, 2002, 302(1), 28-37; Lee, Mozsolits et al., *J. Pept. Res.*, 2001, 370, 1-18; Mariotti, Minunni et al., *Anal. Chim. Acta*, 2002, 453(2), 165-172; Tsoi and Yang, *Biochem. J.*, 2002, 361(2), 317-325). A biosensor is used which is an equipment detecting molecular reactions between a biologically active molecule, called a ligand, and another
25 molecule it binds to, called a receptor. The ligand is bound to the support surface of the equipment, generally a cuvette or a plate. Created on the support surface where the bonds occur is an electromagnetic field, called an evanescent field, which is extremely sensitive to changes in mass. The biosensor transforms the changes in mass occurring on the support surface due to the ligand-receptor bond into an electrical signal. Commercial biosensor
30 models which can be used for this method are those marketed by Biacore and Thermo Labsystems companies. In the present invention, the PDEs function as the ligand and

samples with YTX, which acts as a receptor, are added thereto. The signal in the biosensor will be larger or smaller depending on the amount of toxin adhered to the PDEs and, therefore, depending on the YTX present in the sample.

Cellular PDEs which function as ligands bond to the support surface. Known
5 concentrations of YTX, which acts as a receptor, are subsequently added on this surface. The technique works well using planar surfaces or surfaces formed by a matrix. The PDE-YTX bond follows kinetics which adjust to an equation of pseudo-first order from which a constant is obtained which is called the apparent binding rate (Rap), and which is different for each concentration of toxin. A calibration line is drawn with the Rap and toxin
10 concentration data. A test sample (an extract of fishery products) is added on the surface, its Rap is calculated and the YTX concentration in the test sample can be obtained by placing this value on the calibration line.

Embodiment 1

The method is carried out at a temperature between 22 and 37°C.

15 a- A solution of PDEs at a concentration between 0.1-0.24 mg/mL at pH 7.7 is added onto an activated double compartment surface. These enzymes bind to the support surface by means of non-dissociable covalent bonds. The active groups the PDEs did not bind to were then blocked with different blockinfg solutions (BSA, ethanolamine, tris-HCl).

20 b- A solution with YTX at a known concentration is added to one compartment. The other compartment is used as a blank and the toxin solvent is added into it. The association kinetics between the PDEs and YTX are recorded for 15 minutes.

c- The ligand-receptor dissociation is then carried out by washing both compartments with buffer solution at pH 7.7, thus dissociating YTX from the PDEs.

25 d- The compartments are regenerated with an acid or base solution in order to completely remove YTX. Thus the PDEs will be accessible for a new addition of YTX.

e- Steps b. c and d are repeated with 5 different concentrations of YTX.

f- The apparent binding rates (Rap) are obtained from the association kinetics for
30 each concentration of toxin. The plotting of Rap values against YTX concentrations follows a linear fit with a regression coefficient greater than 0.9. A line is thus

obtained with which the concentration of YTX in a sample can be obtained if its Rap is known.

g- An extraction of the meat of the fishery product to be studied is performed.

This extraction is performed following Decision 2002/225/EEC of 15 March, 2002 or any other official method (D.O.G.A., 1986) for determining maximum levels and analysis methods for certain marine toxins present in different fishery products. An aliquot of the extract (test sample) is taken and is added in the PDE bound to the surface. Association kinetics are obtained from which its Rap is calculated. By placing this test Rap value on the regression line obtained, the YTX concentration present in the sample can be determined.

Figure 2 shows the graphic profile of the steps to be taken in this method, from surface activation to adding the test sample. The regression line is shown in Figure 3 with the Rap values against known concentrations of YTX.

Method for determining PDE activation by YTX using fluorescent molecule.

A usual way of detecting cellular PDE activity is to observe their ability to destroy cAMP. There is a fluorescent derivative of cAMP, anthraniloyl-cAMP (excitation wavelength: 350 nm, emission wavelength: 445 nm), the fluorescence of which decreases as it degrades. The decrease of fluorescence over time can be expressed as the destruction rate of cAMP. In the presence of PDEs, the destruction rate increases, and if these enzymes are activated, the degradation rate will be even greater. In the present invention, the degradation rate of the fluorescent indicator anthraniloyl-cAMP in the presence of PDEs is determined and its variation when samples with YTX are added is studied. Fluorescence is read with a fluorimeter that is prepared for reading microtritation plates. The destruction rate is determined in the presence of several known concentrations of YTX. The representation of the destruction rate against toxin concentration follows a linear fit with a regression coefficient greater than 0.9. A regression line is thus obtained in which the destruction rate value obtained with a sample from fishery products (test sample) can be transformed into YTX concentration.

Embodiment 2

The method is carried out in a microtirtation plate at a temperature range between 22 and 37°C and the fluorescence is measured at an excitation wavelength of 360 nm and am

emission wavelength of 460 nm.

There are four types of wells and each one of them is carried out in duplicate.

WELLS A: Wells for calculating the cAMP concentration. Anthraniloyl-cAMP (fluorescent indicator) is added thereto at 5 concentrations between 2 and 10 μM.

5 WELLS B: Control wells with 8 μM of fluorescent indicator and enzymes.

WELLS C: Calibration wells with 8 μM of fluorescent indicator, enzymes and known concentrations of YTX.

WELLS D: Test samples wells with 8 μM of fluorescent indicator, enzymes and samples from an extract of any fishery product.

10

a) Test buffer (10 mM Tris HCl + 1 mM $CaCl_2$ pH 7.4) is added in all the wells for a final incubation volume of 100 μL, and the corresponding amount, depending on the type of well, of anthraniloyl-cAMP. A first reading is performed for 2 minutes.

b) Between 2 and 5 μg of PDEs are added in wells B, C and D and a new reading is performed for 2 minutes.

15

c) YTX at a known concentration or a sample form a fishery product is added to wells C and D. YTX at concentrations between 0.1 and 10 μM is added. The samples from an extract are obtained following Decision 2002/225/EEC of 15 March, 2002 or any other official method (D.O.G.A., 1986) for determining maximum levels and analysis methods for certain marine toxins present in different fishery products.

20

d) After these additions, the plate is shaken and successive fluorescence measurements are performed for 15 minutes, acquiring data every minute.

e) A line is obtained with a regression coefficient greater than 0.999, by plotting the fluorescence data obtained with wells A against the concentration of indicator for each well.

25

f) The fluorescence data for the rest of the wells in an anthraniloyl-cAMP concentration is transformed using the previous line in an anthraniloyl-cAMP concentration. The amount of indicator destroyed per unit of time, i.e. the destruction rate of cAMP, is obtained from the cAMP concentration at toxin addition time zero and from the concentration after 10 minutes.

30

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g) The destruction rate data obtained with wells B is considered as a control destruction rate.

h) A YTX concentration standard line is obtained by plotting the destruction rate data of wells C against the YTX concentration. The YTX in that sample is
5 determined by substituting on this line the destruction rate obtained in wells D.

Figure 4 represents the fluorescence unit calibration line against a concentration of anthraniloyl-cAMP. Figure 5 represents the standard line for destruction rates of cAMP against YTX concentration for a standard assay.

Use of phosphodiesterases as a therapeutic target of YTX and compounds which
10 **induce the activation thereof**

Use of YTX as an inhibitor of the immunological activation of mastocytes and basophils

Immunological activation of mast cells and basophils requires a temporary increase of cAMP. This increase is indispensable for cell response activation (Botana, L.M. et al., *J. Leukocyte Biol.*, 1994, 55, 798-804). PDE activation cancels this initial cAMP peak, and
15 therefore prevents cell activation. In the presence of YTX, i.e. with activated PDEs, cell response will be inhibited. The inhibiting effect can be used in antiallergic or antiasthmatic therapeutic strategies, these being two pathologies in which mast cells play a predominant role (Metcalfe, D.D. et al., *Physiol. Rev.* 1997, 77, 1033-1079). The presence use describes the quantification of the inhibition that YTX produces on cell activation induced by
20 immunological stimulus in mast cells in rats. Cell response inhibition can be determined according to different protocols described in the literature. One in which the response is quantified according to the histamine released by mast cells in rats into the extracellular medium is set forth below (Alfonso, A., et al., *Br. J. Pharmacol.*, 2000, 130, 1809-1816; Estevez, M.D., et al., *Biochem. Pharmacol.*, 1994, 47, 591-593).

25 *Embodiment 3*

a. The rats are sensitized 15 days before conducting the experiment. Each rat is subcutaneously injected with 1 mL of physiological serum with 150 mg of ovalbumin and 10^9 *Bordetella pertussis* bacteria.

b. Mast cells are extracted from the chest and abdomen of a sensitized rat. The two
30 populations are mixed and a cell suspension is obtained.

c. The cell suspension is preincubated for 10 minutes with various concentrations of

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YTX and subsequently incubated 10 minutes in the presence of 5 mg/mL of ovalbumin.

d. The reaction is stopped in cold conditions and the released histamine is separated from the histamine remaining in the cells by means of centrifugation.

5 e. The supernatant is removed with the histamine released into the medium and the cells are cleaved with hydrochloric acid and ultrasound in order to release the histamine not sensitive to the action of the stimulus.

f. Both mediums are deproteinized with trichloroacetic acid.

g. The histamine is finally quantified, converting it into a fluorescent molecule by

10 reaction in a base medium with o-phthalic dialdehyde. The reaction is stopped with hydrochloric acid and the fluorescence is read at 360 nm excitation and 460 nm emission.

Figure 6 shows the percentage of inhibition of histamine release induced by ovalbumin in the presence of several concentrations of YTX.

15 Use of YTX as a neoplastic cell proliferation inhibitor

The proposed invention describes the use of YTXs as therapeutic agents according to their ability to activate cellular phosphodiesterases.

Neoplastic cell growth inhibition is an indicator of antitumor activity widely used to describe antineoplastic properties of new drugs. It has been found that YTX is cytotoxic

20 for human hepatocellular carcinoma cells, and it has further been described that this toxin induces apoptosis in neuroblastoma cells (Leira, F. et al., *Toxicology in vitro*, 2001, 15, 277-283), which all indicates that YTX is susceptible to being used as an antitumor drug. The ability of YTX as a cytotoxic drug for hepatic carcinoma tumor cells is quantified in the present invention. Cell growth inhibition can be determined according to different

25 protocols described in the literature. Once of these protocols is set forth below in which the response is quantified in the HEP-G2 cell line by means of staining with crystal violet and subsequent acetylation.

Embodiment 4

a. HEP-G2 cells are seeded on a microtritation plate with a density of 10000 cells per

30 well. They are incubated for 24 hours with growth medium at 37°C and 5% CO_2.

b. Different concentrations of YTX are added and it is incubated for 48 hours at 37°C

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and 5% CO_2.

c. 10 μL of 11% glutaraldheyde are added to fix the cells and it is incubated for 15 minutes. It is washed 3-4 times with distilled water.

d. A 0.1% solution of crystal violet is added and the plate is shaken for 15 minutes.

5 e. The dye is removed by washing with distilled water and it is subsequently dried.

f. 10% acetic acid is added and shaking is maintained for 15 minutes.

g. Absorbance is read in a spectrophotometer at 595 nanometers.

It was found with this protocol that 10 μM of YTX induce cell growth inhibition of about 82±1%.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Yessotoxin or an analog thereof capable of activating cellular phosphodiesterases (PDEs) for its use in the treatment of allergic or asthmatic processes.
2. Yessotoxin or an analog thereof as defined in claim 1, wherein the yessotoxin analog is selected from the group of hydroxyyessotoxin, noryessotoxin, homoyessotoxin, hydroxyl-homoyessotoxin, adriatoxin, carboxyyessotoxin and desulfoyessotoxin.
3. Yessotoxin or an analog thereof as defined in claim 1 or 2, wherein the treatment of allergic or asthmatic processes involves promoting the activation of cellular phosphodiesterases.
4. Use of yessotoxin or an analog thereof capable of activating cellular phosphodiesterases in the manufacture of a medicament for the treatment of allergic or asthmatic processes.
5. Use as defined in claim 4, wherein the yessotoxin analog is selected from the group of hydroxyyessotoxin, noryessotoxin, homoyessotoxin, hydroxyl-homoyessotoxin, adriatoxin, carboxyyessotoxin and desulfoyessotoxin.
6. Use as defined in claim 4 or 5, wherein the treatment of allergic or asthmatic processes involves promoting the activation of cellular phosphodiesterases.
7. The in vitro use of yessotoxin (YTX) or an analog thereof capable of activating cellular phosphodiesterases (PDEs) as mast cell modulators.
8. The use according to claim 7, wherein the yessotoxin analog is selected from the group of hydroxyyessotoxin, noryessotoxin, homoyessotoxin, hydroxyl-homoyessotoxin, adriatoxin, carboxyyessotoxin and desulfoyessotoxin.
9. A method of treating an allergic or asthmatic process including the step of administering a yessotoxin or an analog thereof capable of activating cellular phosphodiesterases to a patient in need thereof.
10. A method according to claim 9 wherein the yessotoxin analog is selected from the group of hydroxyyessotoxin, noryessotoxin, homoyessotoxin, hydroxyl-homoyessotoxin, adriatoxin, carboxyyessotoxin and desulfoyessotoxin.

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11. A method according to claim 9 or claim 10 wherein the treatment of allergic or asthmatic processes involves promoting the activation of cellular phosphodiesterases.

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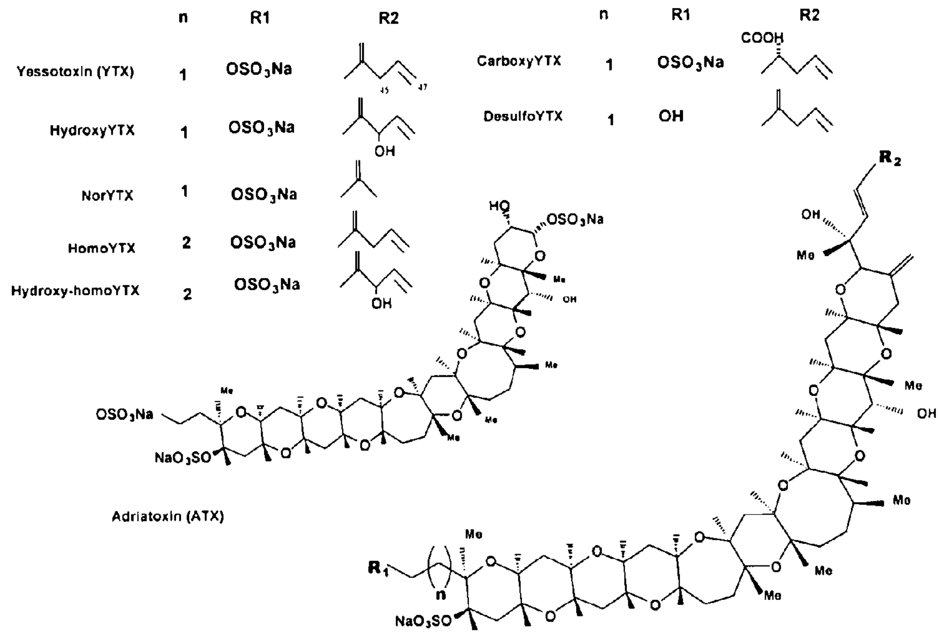

Figure 1

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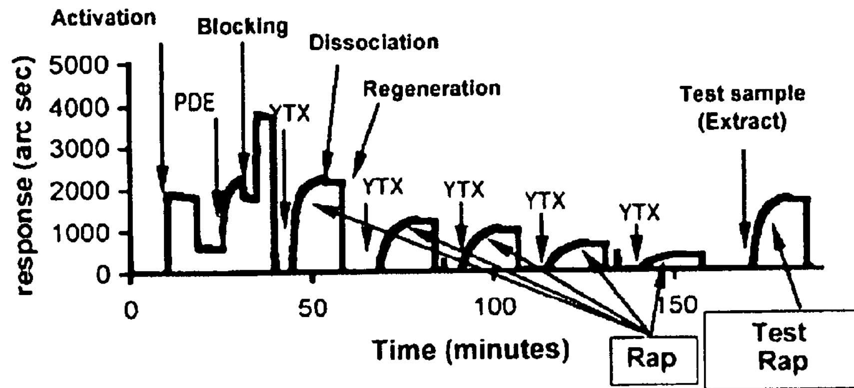

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Figure 2

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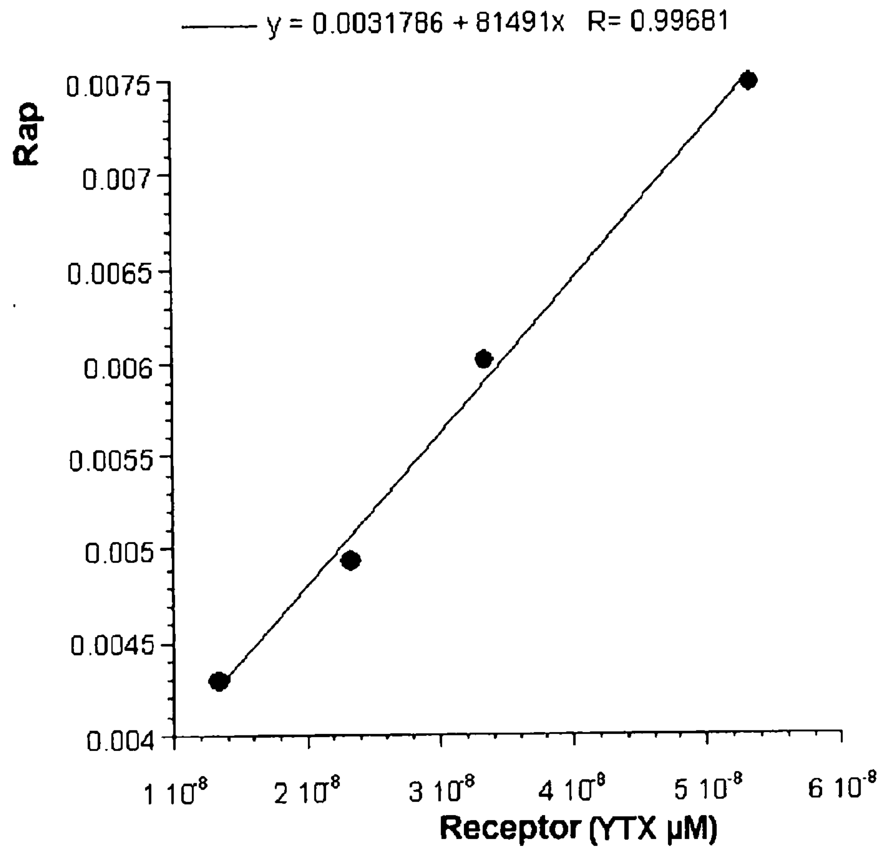

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Figure 3

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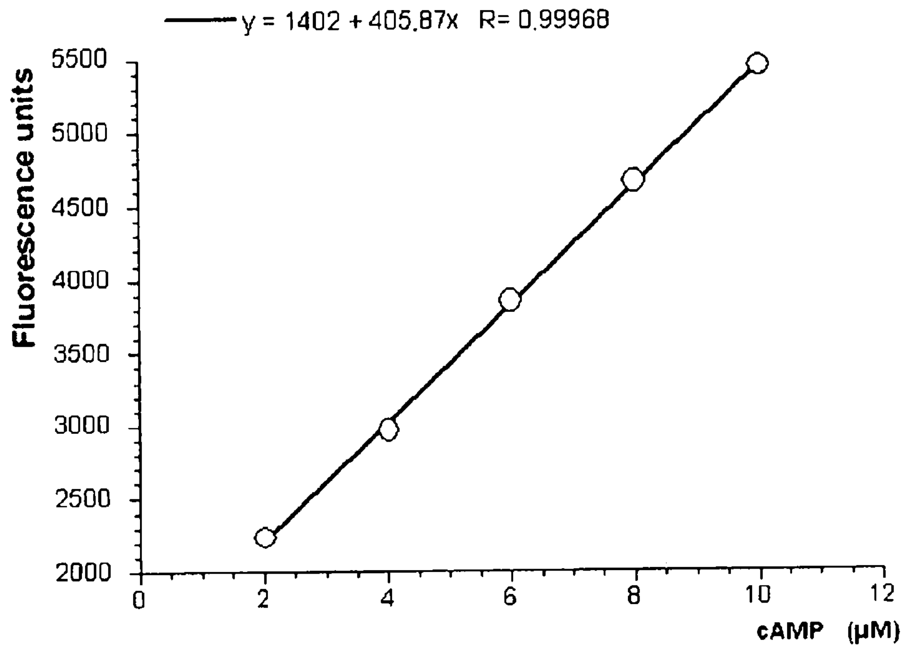

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Figure 4

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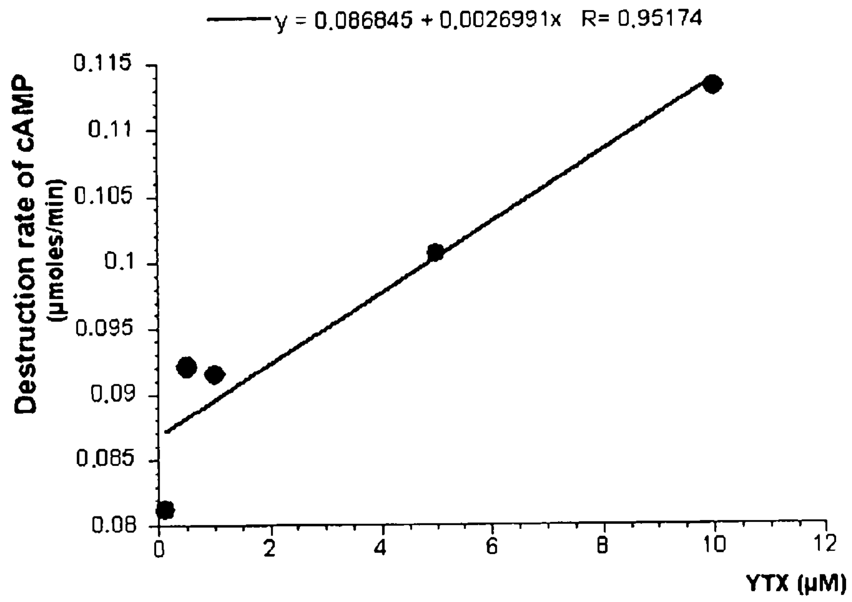

5 **Figure 5**

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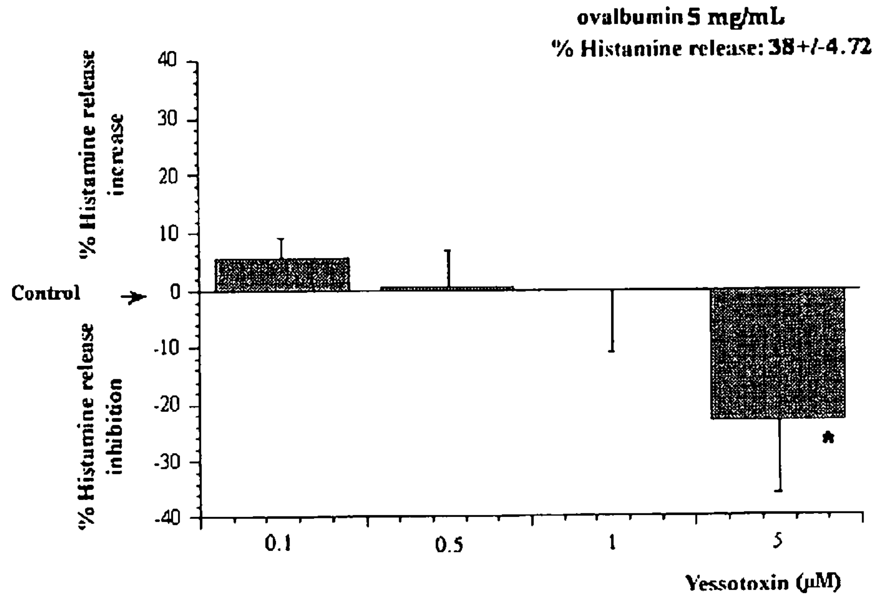

5 **Figure 6**