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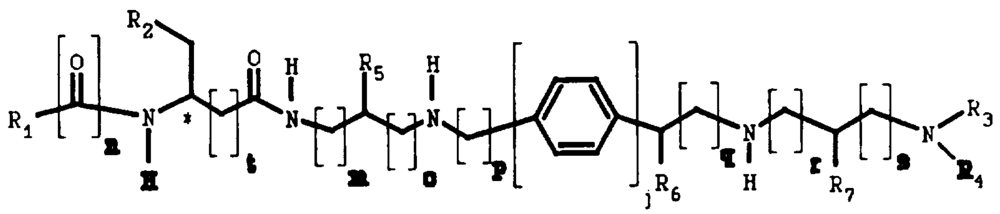

(I)

(57) Abstract

The present invention provides a compound having structure (I) wherein R_1 is a saturated or unsaturated linear or branched chain alkyl group, or a cholestanyl group; wherein R_2 ia s 2-indolyl, 3-indolyl, 4-indolyl, 5-indolyl, 4-hydroxyphenyl, 4-(arylalkyloxy)phenyl, 3,4-dihalophenyl, 4-hydroxy-3,5-dihalophenyl, 4-azidophenyl or 4-halophenyl group; wherein R_3 is H, a linear or branched chain alkyl or alkenyl group, or a phenyl, 2-azidophenyl, 3-azidophenyl, 4-azidophenyl group, or an alkenylacyl, 3-amino-3-butylpropyl, N-[N-(N-{4-azidobenzoyl}aminopropyl)aminopropyl], *cis*- or *trans*-cinnamyl, 2-amino-2-[(4'-azidophenyl)acetyl], (trifluoromethyl)-aminoacetyl or D- or L-arginyl group bonded through the α-carbonyl moiety thereof; R_4 is H, or a linear or branched chain alkyl group; wherein R_5, R_6 and R_7 are independently the same or different and are H, a linear or branched chain alkyl group, an aryl group or an arylalkyl group; wherein n, j and t are each 0 or 1; wherein m, o, p, q, r and s are independently the same or different and are 0, 1 or 2; wherein r+s and m+o are each equal to 2; wherein, j is 0, p+q is 2; wherein, if j is 1, then p is 1, q is 0 and R_6 is H; and wherein * denotes a D or L configuration. The invention also provides a method of synthesizing the compound. Another aspect of the invention concerns a method of treating a subject afflicted by a disorder associated with binding of an etiological agent to a glutamate receptor.

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BUTYRYL-TYROSINYL SPERMINE, ANALOGS THEREOF AND METHODS OF PREPARING AND USING SAME

The invention described herein was made in the course of work under Grant No. INT-8610138 from the National Science Foundation, and Grant Nos. AI 10187, ES 02594, and E504977 from the National Institute of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in the invention.

This application is a continuation-in-part of U.S. Serial No. 08/275,336, filed July 14, 1994, a continuation of U.S. Serial No. 07/701,223, filed May 16, 1991, now abandoned, a continuation-in-part of the U.S. Serial No. 07/153,151, filed February 8, 1988, now abandoned, the contents of which are hereby incorporated by reference into the present application.

Background of the Invention

Throughout this application various publications are referenced by arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed in this application.

Glutamate receptors are believed to be the principal excitatory neurotransmitter receptors in the central nervous system (CNS). Based on the chemicals that activate glutamate receptors, such receptors are generally divided into three major subtypes: quisqualate (QUIS-R), N-methyl-D-aspartate (NMDA-R), and kainate (KAIN-R). These receptors are involved in development, learning and neuropathology and likely mediate the neurodegenerative consequences of hypoxemia, epilepsy, Alzheimer's disease, and Huntington's disease (1-5). There is considerable interest in developing agents that block glutamate receptors, particularly antagonists of the NMDA type receptor because of their anticonvulsant action and possible protection from ischemic brain damage (7). NMDA receptors are involved in a variety of neurological and psychiatric disorders, and antagonists of this receptor may be therapeutically valuable in movement disorders, such as epilepsy, and in various acute and chronic neurodegenerative disorders.

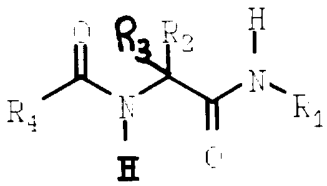
Studies of glutamate receptors, in particular studies employing biochemical techniques, have been made difficult by the relative paucity of potent antagonists for these receptor proteins. Selective, competitive and non-competitive antagonists of the NMDA receptor have become available during the past few years, but the search for antagonists of the L-quisqualate-sensitive receptor has only recently shown signs of success (8-10). Quisqualate-sensitive glutamate receptors are distributed widely in excitable tissues of multicellular animals (11) and studies of the effects of the venoms of certain wasps and spiders on vertebrate and invertebrate neurons and muscle fibers suggest that one source of antagonists for this class of receptor might be the venoms of some species of predaceous arthropods (12-17).

The solitary digger Philanthus triangulum F., which is a sphecid wasp that preys on honey bees, manufactures a venom which blocks glutamate receptors on locust skeletal muscle (16, 17). Piek and colleagues have shown that the venom of this wasp contains a component (termed δ-philanthotoxin) which exhibits a number of pharmacological properties including open-channel block of junctional glutamate receptors (18) and extrajunctional glutamate D-receptors (19) of locust leg muscle, most of which are quisqualate-sensitive (20). However, Piek and colleagues did not isolate or determine the active compound of the venom component.

In order to deduce the active ingredient contained in venom from the wasp Philanthus triangulum F., a series of extractions were performed to isolate an active fraction. Based on a structure deduced from chemical analysis of the fraction, a series of related compounds were synthesized and their activities and chemical properties compared to those of the venom extract fraction. This resulted in the unexpected discovery of the active compound of the venom. The present invention concerns the active ingredient contained in venom from the wasp Philanthus triangulum F., the chemical structure of this active ingredient, a method for synthesizing the active ingredient, designated philanthotoxin-433 (PhTX-433), and the use of PhTX-433 as a potent inhibitor of the glutamate receptors. In addition, the present invention involves the synthesis of pharmacologically active analogs of PhTX-433, e.g., PhTX-334, PhTX-343 and many others (wherein the numerals denote the number of methylenes between the amino groups of the spermine moiety).

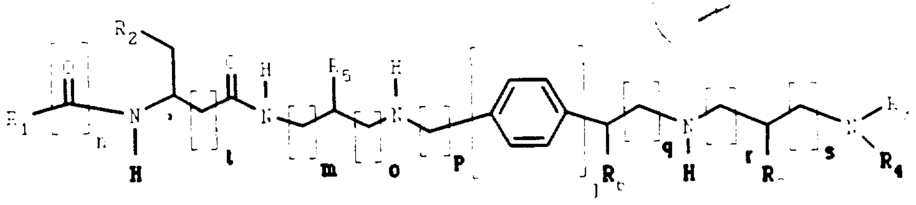
Summary of the Invention

The present invention provides a compound having the structure:



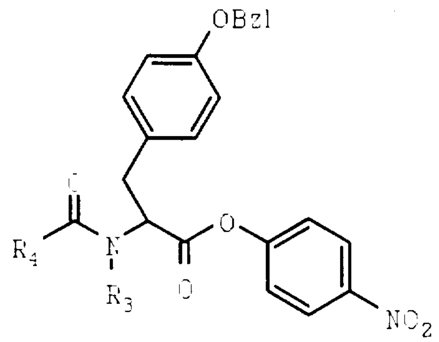
wherein R_1 is hydrogen or a branched or unbranched, substituted or unsubstituted aminoalkyl having from two to twenty atoms in the chain; R_2 is hydrogen, methyl, a branched or unbranched, substituted or unsubstituted alkyl having from two to twenty atoms in the chain or CH_2R_3; R_3 is hydrogen or a substituted or unsubstituted aryl; and R_4 is methyl, a branched or unbranched, substituted or unsubstituted alkyl, alkenyl, alkynyl, alkenynyl, or cycloalkyl having from two to twenty atoms in the chain, or a substituted or unsubstituted aryl group.

The subject invention further provides a compound having the structure:



wherein R_1 is a saturated or unsaturated linear or branched chain alkyl group, or a cholestanyl group; wherein R_2 is a 2-indolyl, 3-indolyl, 4-indolyl, 5-indolyl, 4-hydroxyphenyl, 4-(arylalkyloxy)phenyl, 3,4-dihalophenyl, 4-hydroxy-3,5-dihalophenyl, 4-azidophenyl or 4-halophenyl group; wherein R_3 is H, a linear or branched chain alkyl or alkenyl group, or a phenyl, 2-azidophenyl, 3-azidophenyl, 4-azidophenyl group, or an alkenylacyl, 3-amino-3-butylpropyl, N-[N-(N-{4-azidobenzoyl}aminopropyl)aminopropyl], *cis*- or *trans*-cinnamyl, 2-amino-2-[(4'-azidophenyl)acetyl, (trifluoromethyl)aminoacetyl or D- or L-arginyl group bonded through the α-carbonyl moiety thereof; R_4 is H, or a linear or branched chain alkyl group; wherein R_5, R_6 and R_7 are independently the same or different and are H, a linear or branched chain alkyl group, an aryl group or an arylalkyl group; wherein n, j and t are each 0 or 1; wherein m, o, p, q, r and s are independently the same or different and are 0, 1 or 2; wherein r+s and m+o are each equal to 2; wherein, if j is 0, p+q is 2; wherein, if j is 1, then q is 0 and R_6 is H; and wherein * denotes a D or L configuration.

The invention also concerns a method of preparing the compound which comprises treating venom, venom sacs or venom glands or the wasp <u>Philanthus</u> <u>triangulum F.</u> to produce an aqueous extract, and recovering the compound from the resulting aqueous extract. Additionally, the invention provides a method of preparing the compound which comprises contacting a branched- or unbranched-chain alkylamine having from two to twenty atoms in the chain and having hydrogen or a protection group attached to each nitrogen atom of the chain with a compound having the structure:



wherein R_3 and R_4 are the same or different and is hydrogen or a lower alkyl group so as to form a product, treating the product to produce the compound and recovering the compound.

Another aspect of the invention concerns a method of treating a subject afflicted by a disorder associated with binding of an etiological agent to a glutamate receptor which comprises administering to the subject an amount of the compound effective to inhibit binding of the etiological agent to the receptor. The invention also concerns a method of treating a subject afflicted by a stroke-related disorder associated with excessive binding of glutamate to glutamate receptors which comprises administering to the subject an amount of the compound effective to inhibit the excessive binding of the glutamate to the receptors. Lastly, the invention provides an insecticidal composition which comprises an effective amount of the compound and a suitable carrier and a method of combatting insects which comprises administering to the insects an amount of the insecticidal composition effective to produce paralysis in the insects.

Brief Description of Figures

Figure 1. Venom sac (VS), gland (VG) and the sting apparatus (Stg) dissected from Philanthus triangulum.

Figure 2. Fractionation of Philanthus venom by reverse phase high pressure liquid chromatography (HPLC). (A) Fractionation of lyophilized venom glands, extracted in 50% (vol/vol) acetonitrile/water. 450 μL (representing extracts of 225 wasps) were chromatographed on a YMC-ODS 20 x 280 mm column and developed by a linear gradient of 5% CH_3CN/0.1% TFA-95% CH_3CN/0.1% TFA for 30 minutes at a flow rate of 8 mL/min. UV absorption was monitored at 215 nm. (B) Fractionation of the main toxic fraction (hatched peak in Fig. 2A).

Figure 3. The chemical structures and synthesis of the natural philanthotoxin (PhTX-433) and two isomers PhTX-334 and PhTX-343. (A) The structures of the three toxins, compounds **1**, **2** and **3**. (B) Synthesis of intermediates of compounds **1** and **2**. (C) The final steps in the synthesis of the three toxins.

Figure 4. Effects of PhTX-433 (A) and PhTX-334 (B) on the neurally-evoked twitch contraction of locust metathoracic retractor unguis muscle. (A) and (B) are data from different nerve-muscle preparations dissected from the same adult, female locust (Schistocerca gregaria). The nerve-muscle preparations were superfused with standard locust saline for 30 minutes before the toxins were applied. The retractor unguis nerve was stimulated with single, brief (0.1 s), supramaximal stimuli applied at a constant, low frequency, before and after toxin application (in locust saline), but during the period

of toxin application, the stimulation frequency was sometimes increased temporarily to 0.6 Hz.

Figure 5. Scatchard analysis of glutamate-induced [^{3}H]MK-801 binding in absence (o), and presence of (●) 25μM PhTX.

Figure 6. Dose-dependent inhibition of the glutamate-induced [^{3}H]MK-801 binding to NMDA receptors of rat brain.

Figure 7. Method A and Method B for preparing analogs. a. *p*-nitrophenol, DCC, EtOAc; b. TFA, $CHCl_3$; c. Et_3N, butyryl chloride, $CHCl_3$; d. spermine, CH_3OH; e. H_2, 5% Pd/C, CH_3OH; f. N-α,ϵ-di-Cbz-L-lysine *p*-nitrophenol ester, DMF; g. NBS, KI, K_2HPO_4, CH_3OH/H_2O (5:1); h. $(Boc)_2O$, CH_3OH, cat. pyridine; i. cinnamoyl chloride, Et_3N, $CHCl_3$.

Figure 8. (a) halogens: I > Br > Cl > F; modifications to hydroxyl give variable activities; (b) *S* configuration is better than *R*; (c) polyamine chain essential; (d) longer chain with N^+ increases activity; (e) hydrophobicity and/or aromaticity increases activity but long aliphatic chains lead to insolubility; site for (photo)affinity labels; (f) *n*-butyl here increases activity. These structure-activity relationships reflect general trends. Simultaneous modifications in regions II and IV are multiplicative or better, while regions II and III are less than multiplicative. In addition, when III = *n*-$C_9H_{19}CONH$-, further change reduces activity.

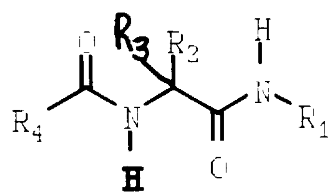
Figure 9. Synthesis of compound **7'**. a. $SOCl_2$, MeOH; b. butyl bromide, KF/Celite®, CH_3CN; c. $(Boc)_2O$,

CH_2Cl_2; d. DIBAL, CH_2Cl_2/hexane; e. $(CO)_2Cl_2$, DMSO, Et_3N, CH_2Cl_2; f. spermine, Na_2SO_4, $NaBH_4$; g. TFA, CH_2Cl_2. Compounds **8'**, **9'**, **10'** and **11'**.

Figure 10. Synthesis of compound **23'**. a. acrylonitrile, MeOH; b. $(Boc)_2O$, CH_2Cl_2; c. H_2 (50 psi), $Pd(OH)_2$, AcOH; d. acrylonitrile, MeOH; e. $(Boc)_2O$, CH_2Cl_2; f. H_2 (50 psi), $Ph(OH)_2$, AcOH; g. acrylonitrile, MeOH; h. $(Boc)_2O$, CH_2Cl_2; i. H_2 (50 psi), $Pd(OH)_2$, AcOH; j. O-benzyl-Boc-decanoyl amine-tyrosine aldehyde, Na_2SO_4, $NaBH_4$; k. TFA, CH_2Cl_2. Compounds **12'** and **13'**.

Detailed Description of the Invention

The present invention provides a compound having the structure:



wherein R_1 is hydrogen or a branched or unbranched, substituted or unsubstituted aminoalkyl having from two to twenty atoms in the chain; R_2 is hydrogen, methyl, a branched or unbranched, substituted or unsubstituted alkyl having from two to twenty atoms in the chain or CH_2R_3; R_3 is hydrogen or a substituted or unsubstituted aryl; and R_4 is methyl, a branched or unbranched, substituted or unsubstituted alkyl, alkenyl, alkynyl, alkenynyl, or cycloalkyl having from two to twenty atoms in the chain, or a substituted or unsubstituted aryl group.

Examples of R_1 include but are not intended to be limited to -H,

$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NH_2$,

$-CH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$,

$-CH_2(CH_2)_2NH(CH_2)_3NH(CH_2)_4NH_2$,

$-CH_2(CH_2)_3NH(CH_2)_3NH_2$,

$-CH_2(CH_2)_3NH_2$,

$-CH_2(CH_2)_3NHCH_2CH(CH_3)CH_2NH(CH_2)_3NH_2$,

$-CH_2(CH_2)_3NHCH_2CH(C_4H_9)CH_2NH(CH_2)_3NH_2$,

$-CH_2(CH_2)_3N^+(CH_3)_2(CH_2)_4N^+(CH_3)_2(CH_2)_3N^+(CH_3)_3$,

$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH_3$,

$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH_2NH_2$,

$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCO(CH_2)_3NH_2$,

$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH(NH_2)(CH_2)_4NH_2$,

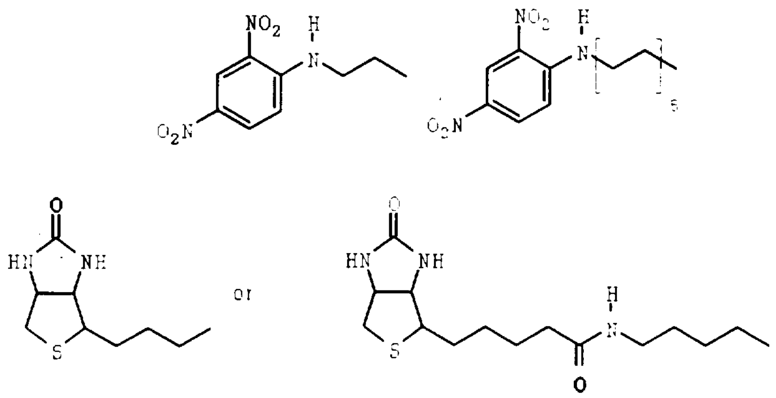
$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH(NH_2)(CH_2)_3NHCH(NH)NH_2$,

or

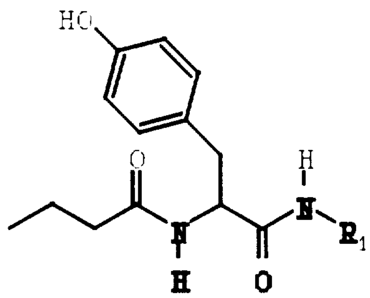
$-(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCOCH(NH_2)(CH_2)_4NHCOCH(NH_2)(CH_2)_4NH_2$

In addition, R_2 may be -H, $-CH_3$, $-CH_2CH(CH_3)_2$ or $-CH_2R_3$. When R_2 is $-CH_2R_3$, R_3 may be a hydroxyphenyl group, a phenyl group, an acetyloxyphenyl group, a benzyloxyphenyl group, 4-hydroxy-3,5-diiodophenyl, an indolyl moiety, 4-nitro-5-hydroxyphenyl, 4-fluoro-5-hydroxyphenyl, 4-hydroxy-3,5-dichlorophenyl, or 4-hydroxy-3,5-dibromophenyl.

R_4 may be $CH_3(CH_2)_2-$, CH_3-, $CH_3(CH_2)_5-$, $CH_3(CH_2)_8-$, $CH_3CH{=}CHCH{=}CH-$, a cyclohexyl group, a benzyl group, a benzylmethyl group, a benzylethenyl group, an N_3-benzyl group, $F_3CC(N_2)CONH(CH_2)_3-$,



In one embodiment of the invention, the compound above has the structure:



wherein R_1 is -H,

$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NH_2$,

$-CH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$,

$-CH_2(CH_2)_2NH(CH_2)_3NH(CH_2)_4NH_2$,

$-CH_2(CH_2)_3NH(CH_2)_3NH_2$,

$-CH_2(CH_2)_3NH_2$,

$-CH_2(CH_2)_3NHCH_2CH(CH_3)CH_2NH(CH_2)_3NH_2$,

$-CH_2(CH_2)_3NHCH_2CH(C_4H_9)CH_2NH(CH_2)_3NH_2$,

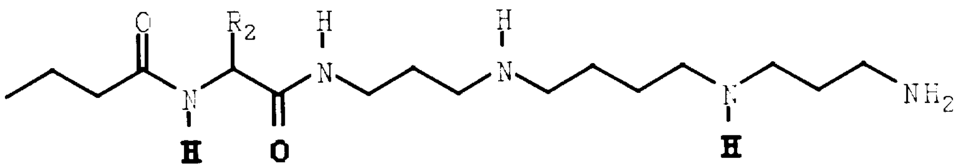
$-CH_2(CH_2)_3N^+(CH_3)_2(CH_2)_4N^+(CH_3)_2(CH_2)_3N^+(CH_3)_3$,

$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH_3$,

$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH_2NH_2$,
$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCO(CH_2)_3NH_2$,
$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH(NH_2)(CH_2)_4NH_2$,
$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH(NH_2)(CH_2)_3NHCH(NH)NH_2$, or
$-(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCOCH(NH_2)(CH_2)_4NHCOCH(NH_2)(CH_2)_4NH_2$

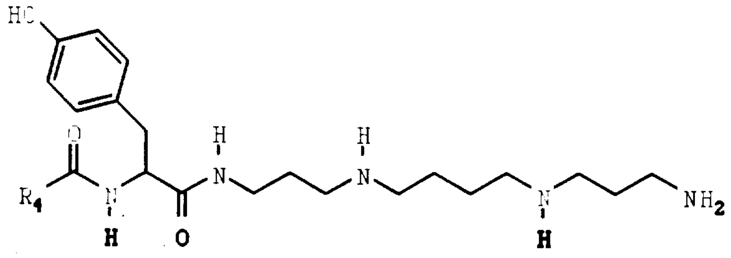
In the preferred embodiment of the above compound, R_1 is
$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NH_2$,
$-CH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$,
$-CH_2(CH_2)_2NH(CH_2)_3NH(CH_2)_4NH_2$, or
$-CH_2(CH_2)_3NHCH_2CH(C_4H_9)CH_2NH(CH_2)_3NH_2$.

In another embodiment of the invention, the compound above has the structure:

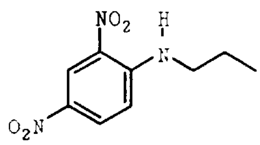


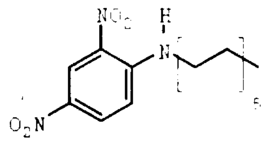
wherein R_2 is hydrogen, methyl, $-CH_2CH(CH_3)_2$ or $-CH_2R_3$; R_3 is hydrogen, a hydroxyphenyl group, a phenyl group, an acetyloxyphenyl group, a benzyloxyphenyl group, 4-hydroxy-3,5-diiodophenyl, an indolyl moiety, 4-nitro-5-hydroxyphenyl, 4-fluoro-5-hydroxyphenyl, 4-hydroxy-3,5-dichlorophenyl, or 4-hydroxy-3,5-dibromophenyl. In the preferred embodiment of the above compound, R_2 is CH_2R_3 and R_3 is a phenyl group, 4-hydroxy-3,5-diiodophenyl, or an indolyl moiety.

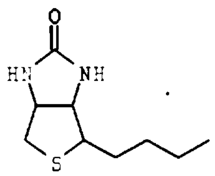
In yet another embodiment of the present invention, the compound above has the following structure:



wherein R_4 is $CH_3(CH_2)_2$-, CH_3-, $CH_3(CH_2)_5$-, $CH_3(CH_2)_8$-, $CH_3CH{=}CHCH{=}CH$-, a cyclohexyl group, a benzyl group, a benzylmethyl group,a benzylethenyl group, an N_3-benzyl group, $F_3CC(N_2)CONH(CH_2)_3$-,







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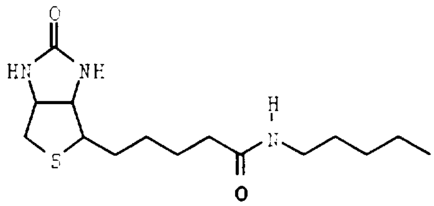
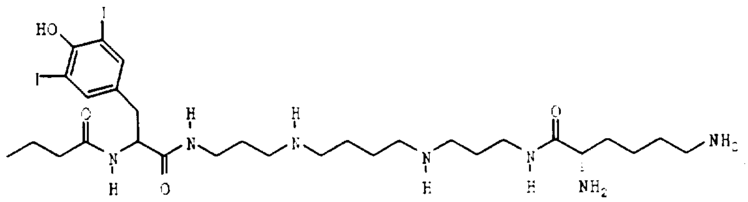


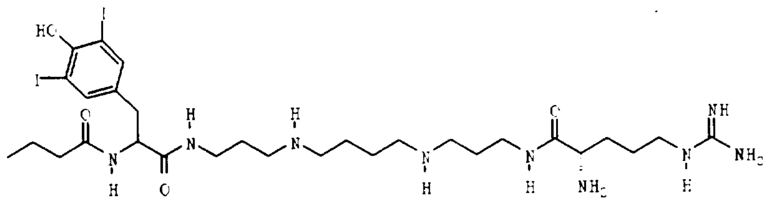
Preferably, R_4 is $CH_3(CH_2)_8$-, $CH_3CH{=}CHCH{=}CH$-, a benzyl group, a benzylethenyl group, or an N_3-benzyl group.

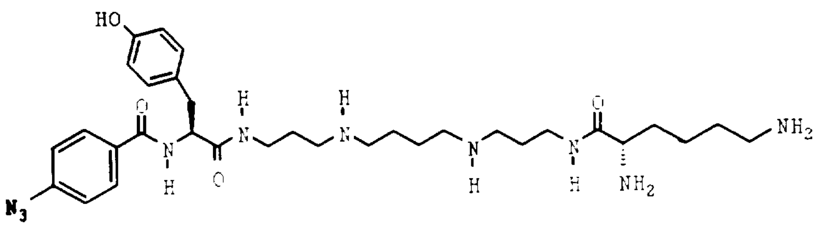
The present invention also provides a compound wherein R_1 is
$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH(NH_2)(CH_2)_4NH_2$ or
$-CH_2(CH_2)_2NH(CH_2)_4NH(CH_2)_3NHCOCH(NH_2)(CH_2)_3NHC(NH)NH_2$;

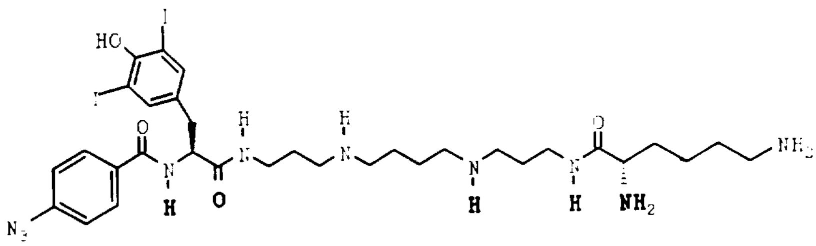
R_3 is 4-hydroxy-3,5-diiodophenyl, a hydroxyphenyl group, or an indolyl moiety; and R_4 is $CH_3(CH_2)_8$-, $CH_3(CH_2)$-, or an N_3-benzyl group.

Preferably, the compound above has one of the following structures:

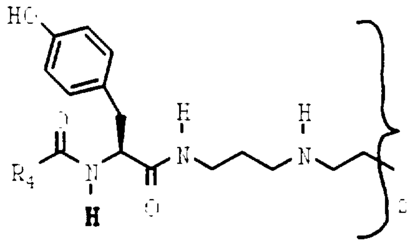






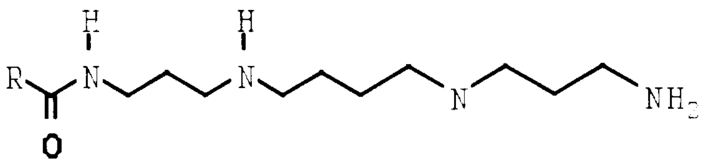


Additionally, the present invention provides for a compound having the structure:



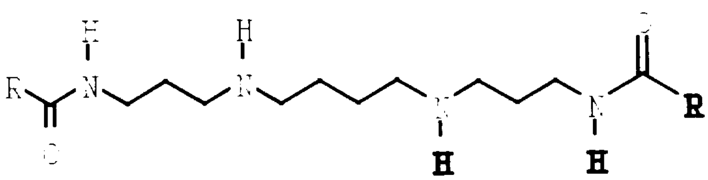
wherein R_4 is CH_3-, $CH_3(CH_2)_2$-, $CH_3(CH_2)_5$-, $CH_3(CH_2)_7CH_2$-, or a benzyl group.

The present invention also provides a compound having the structure:



wherein R is $CH_3(CH_2)_2$-, $CH_3(CH_2)_5$-, or $CH_3(CH_2)_8$-.

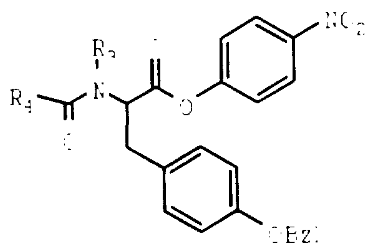
The present invention also provides a compound having the structure:



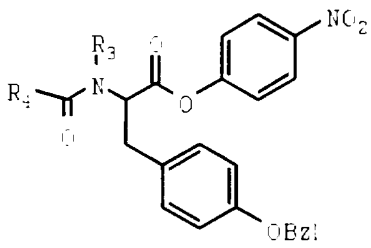
wherein R is $CH_3(CH_2)_2$-, $CH_3(CH_2)_5$-, or $CH_3(CH_2)_8$-.

The invention also provides a method of preparing or isolating the compound above. The method comprises treating venom, venom sacs or venom glands or the wasp Philanthus triangulum F. to produce an aqueous extract, and recovering the compound from the resulting aqueous extract. The recovery may be effected by a variety of separation techniques known to those skilled in the art to which the invention pertains, such as filtration, centrifugation, and chromatography. The treating of the venom, venom sacs, or venom glands may be effective by extraction with numerous organic solvents, such as 50% (vol/vol) CH_3CN/H_2O. Preferably, a series of extractions is performed wherein each subsequent extraction is performed on the fraction resulting from the previous extraction.

The invention also provides a method of synthesizing the compound described hereinabove which comprises contacting a branched- or unbranched-chain alkylamine, having from two to twenty atoms in the chain and having hydrogen or a protection group attached to each nitrogen atom of the chain, with a compound having the structure:



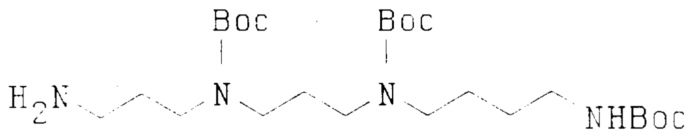
wherein R_3 and R_4 are the same or different and is hydrogen or a lower alkyl group, so as to form a product, treating the product to produce the compound and recovering the compound. The treating of the product may comprise deprotection with trifluoroacetic acid or hydrogen. Presently, the component having the structure:



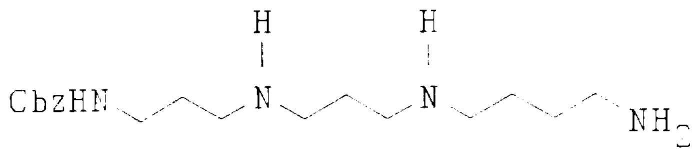
is obtained by the treatment of N-*tert*-butoxycarbonyl-O-benzyl-L-tyrosine *p*-nitrophenyl ester (preferably with trifluoroacetic acid) to remove the *tert*-butoxycarbonyl group followed by acylation (preferably with butyryl chloride). In certain embodiments, the alkylamine has the formula:

$$H_2N(CH_2)_xN(R_5)(CH_2)_yN(R_6)(CH_2)_zNH(R_7)$$

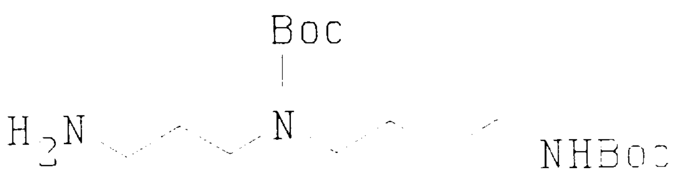
wherein each of *x*, *y*, *z* is the same or different and is an integer from 1 to 6 and each of R_5, R_6 and R_7 is the same or different and is hydrogen or a protection group. Several types of protection groups may be used in the practice of the present invention and these protection groups are well-known to those skilled in the art to which the invention pertains. Examples of useful protection groups include *tert*-butoxycarbonyl and carbobenzoxy groups and derivatives thereof. In the presently preferred embodiments, the alkylamine has the structure:



or



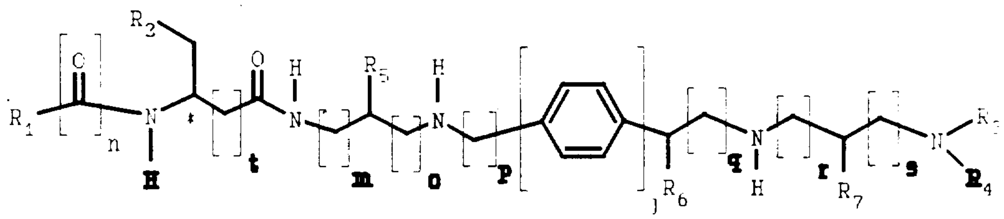
wherein Boc is a *tert*-butoxycarbonyl group and Cbz is a carbobenzoxy group. Such an alkylamine may be obtained by contacting acrylonitrile with a spermidine derivative having the structure:



so as to produce a nitrile, and reducing the nitrile and treating it with $(Boc)_2O$ or carbobenzoxy chloride. In another preferred embodiment, the alkylamine has the structure:

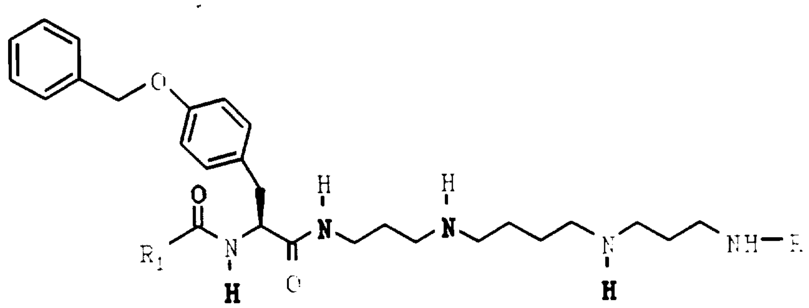
$$H_2N(-CH_2-)_3\overset{H}{\overset{|}{N}}(-CH_2-)_4\overset{H}{\overset{|}{N}}(-CH_2-)_3NH_2$$

The present invention provides a compound having the structure:

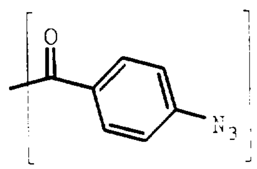


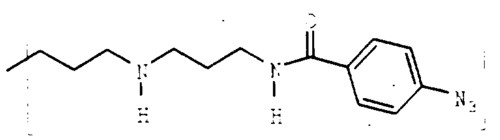
wherein R_1 is a saturated or unsaturated linear or branched chain alkyl group, or a cholestanyl group; wherein R_2 is a 2-indolyl, 3-indolyl, 4-indolyl, 5-indolyl, 4-hydroxyphenyl, 4-(arylalkyloxy)phenyl, 3,4-dihalophenyl, 4-hydroxy-3,5-dihalophenyl, 4-azidophenyl or 4-halophenyl group; wherein R_3 is H, a linear or branched chain alkyl or alkenyl group, or a phenyl, 2-azidophenyl, 3-azidophenyl, 4-azidophenyl group, or an alkenylacyl, 3-amino-3-butylpropyl, N-[N-(N-{4-azido-benzoyl}aminopropyl)aminopropyl], *cis*- or *trans*-cinnamyl, 2-amino-2-[(4'-azidophenyl)acetyl], (trifluoromethyl)-aminoacetyl or D- or L-arginyl group bonded through the α-carbonyl moiety thereof; R_4 is H, or a linear or branched chain alkyl group; wherein R_5, R_6 and R_7 are independently the same or different and are H, a linear or branched chain alkyl group, an aryl group or an arylalkyl group; wherein n, j and t are each 0 or 1; wherein m, o, p, q, r and s are independently the same or different and are 0, 1 or 2; wherein r+s and m+o are each equal to 2; wherein, if j is 0, p+q is 2; wherein, if j is 1, then q is 0 and R_6 is H; and wherein * denotes a D or L configuration. In a preferred embodiment, the present invention provides a compound having the above structure wherein j is 0 or 1. In another preferred embodiment, the present invention provides a compound having the above structure wherein k is 0 or 1.

The present invention also provides a compound having the structure:

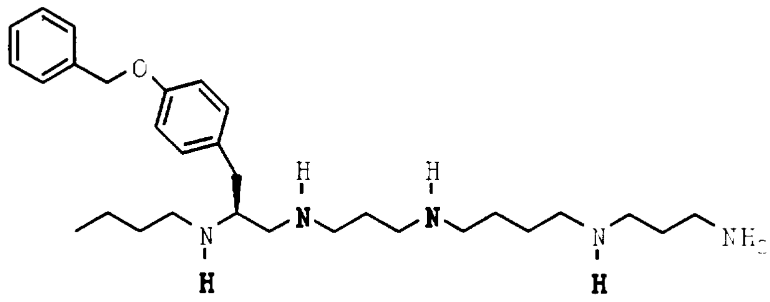


wherein R is selected from a group consisting of H, linear alkyl, linear acyl, arylalkyl, phenyl,

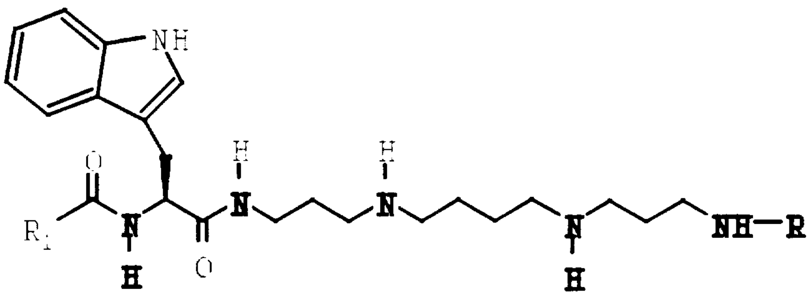
alkylacyl,  and

;

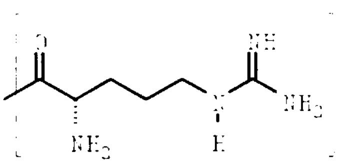
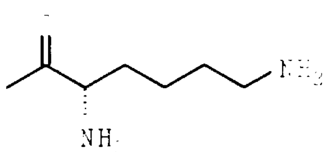
and wherein R_1 is a C_9 or C_{10} linear alkyl group. In a preferred embodiment, the present invention provides a compound having the above structure wherein R is H and R_1 is C_9H_{19}. In another embodiment, the present invention provides a compound having the structure:



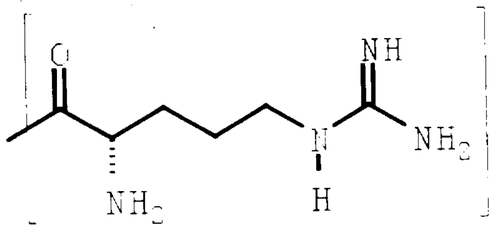
The present invention also provides a compound having the structure:



wherein R is selected from a group consisting of H,

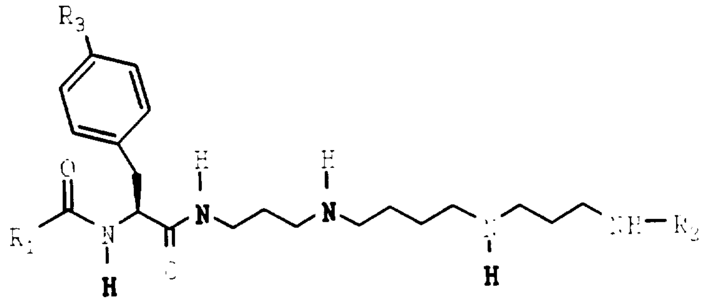
 and  ; and

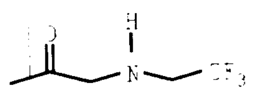
wherein R_1 is a C_9 or C_{10} linear alkyl group. In one embodiment, the invention provides a compound having the above structure wherein R is H and R_1 is C_9H_{19}. In another embodiment, the present invention provides a compound having the above structure wherein R is

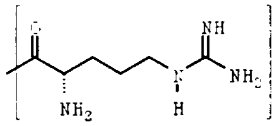


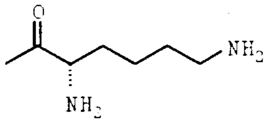
and R_1 is C_9H_{19}.

The present invention further provides a compound having the following structure:



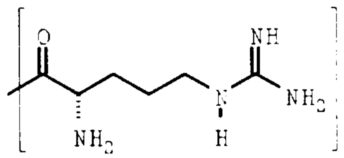
wherein R_1 is a C_9 or C_{10} linear saturated or unsaturated alkyl group; wherein R_2 is selected from a group consisting of H, H_2^+,  ,



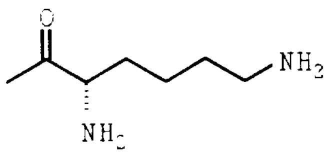
and


; and

wherein R_3 is selected from a group consisting of F, OH and N_3. In one embodiment, the present invention provides a compound having the above structure wherein R_1 is C_9H_{19};

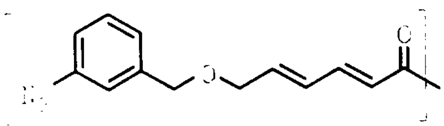
wherein R_2 is


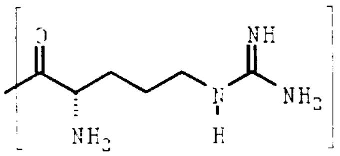
or



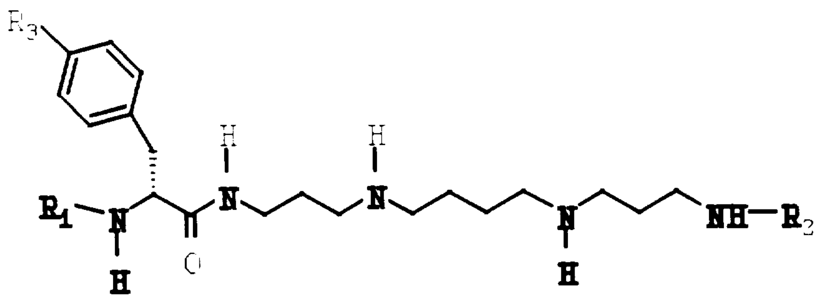
; and wherein R_3 is OH.

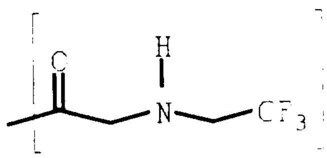
In a preferred embodiment, the present invention provides a compound having the above structure wherein R_1 is

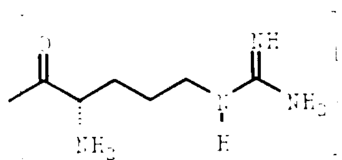
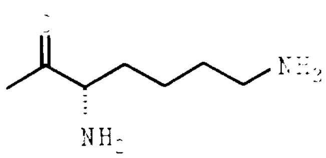

; wherein R_2 is

 ; and wherein R_3 is OH.

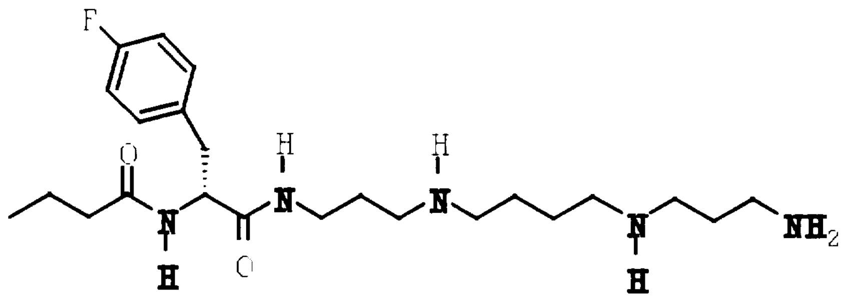
The present invention also provides a compound having the structure:



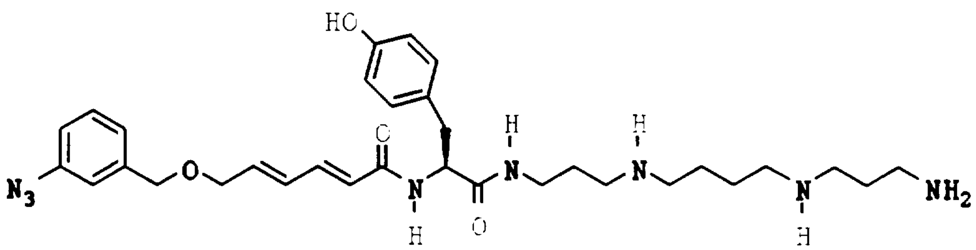
wherein R_1 is a C_9 or C_{10} linear saturated or unsaturated alkyl group; wherein R_2 is selected from a group consisting of H, H_2^+,  ,

 and  ;

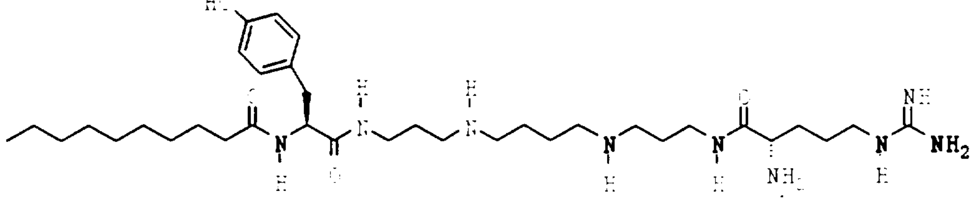
and wherein R_3 is selected from a group consisting of F, OH and N_3. In one embodiment, the invention provides a compound having the structure:



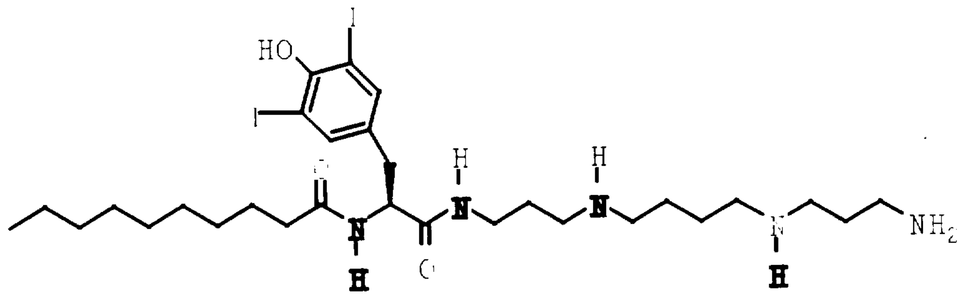
In another embodiment, the present invention provides a compound having the structure:



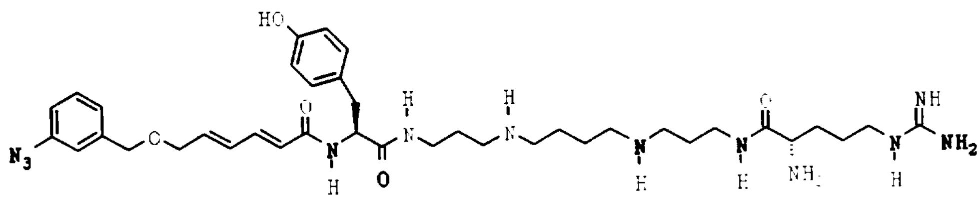
In another embodiment, the present invention provides a compound having the structure:



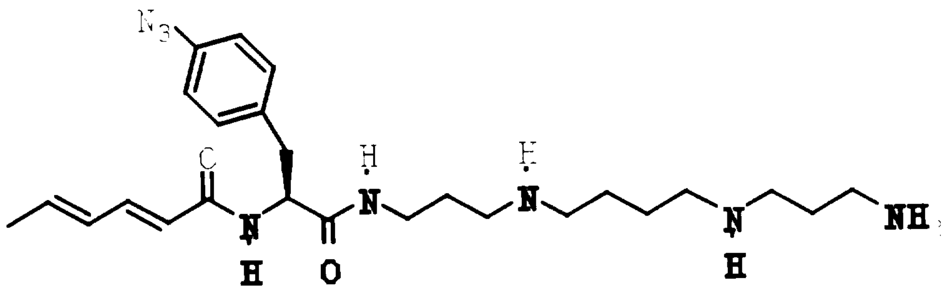
In another embodiment, the present invention also provides a compound having the structure:



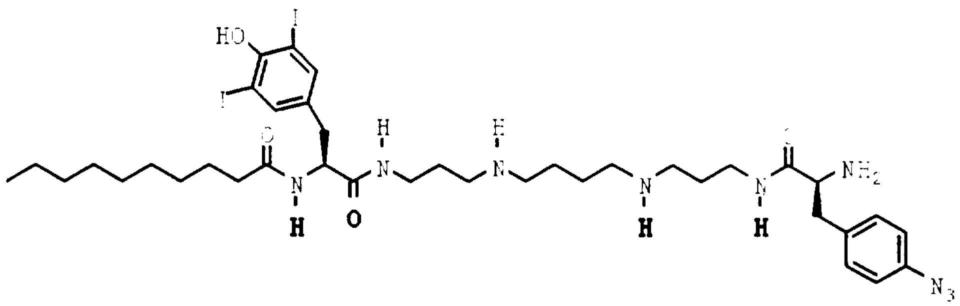
In an additional embodiment, the present invention provides a compound having the structure:



In another embodiment, the present invention provides a compound having the structure:



In another embodiment, the present invention also provides a compound having the structure:



It is also contemplated that any of the compounds of the present invention may be radioactively labelled or be formulated into a pharmaceutical composition or an insecticidal composition comprising an effective amount of any one of the compounds and a suitable carrier. The compounds may also be mixed with glutamate to form an admixture which in turn may be mixed with a carrier to provide a pharmaceutical composition. The compounds may also be useful as an anticonvulsant.

Another aspect of the invention concerns a method of inhibiting binding to a glutamate receptor which comprises contacting the receptor with a binding-inhibiting amount of any of the compounds described hereinabove or the admixture of the compounds with glutamate. Such methods of inhibiting binding to a glutamate receptor may prove useful in medical applications, agricultural applications or as research tools for the study of humans and animals. In one embodiment, the invention provides a method of treating a subject afflicted by a disorder associated with binding of an etiological agent to a glutamate receptor which comprises administering to the subject an amount of any one of the compounds or the admixture effective to inhibit binding of the etiological agent to the receptor.

The method is particularly useful where the receptor is quisqualate (QUIS-R) or NMDA receptor (NMDA-R). In medical applications, the present invention may have therapeutic value in epilepsy, in movement disorders, in protection from ischemic brain damage and in various other neurodegenerative disorders. The method may be useful where the neurodegenerative disorder is Huntington's disease, Parkinson's disease or Alzheimer's disease. Another embodiment provides a method of treating a subject afflicted by a stroke-related disorder associated with excessive binding of glutamate to glutamate receptors which comprises administering to the subject an amount of any one of the compounds or admixture effective to inhibit the excessive binding of the glutamate to the receptors.

As previously noted, the compounds may be mixed with a suitable carrier to form an insecticidal composition and the insecticidal composition may be used in a method of combatting insects which comprising administering to the insects an amount of the insecticidal composition effective to induce paralysis in the insects.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Experimental Details

A. Collection and Synthesis of PhTX-433

Collection of Wasp Venom and Bioassay. Female Philanthus triangulum F. were collected from the Dakhla oasis in the great Sahara desert in Egypt in the late summer when the population of this wasp is very high. The wasps were restrained by chilling at 4°C and their venom sacs and glands, with the sting apparati attached (Fig. 1), were removed and placed in liquid nitrogen before being lyophilized and stored at -20°C. To test the biological activity of the crude venom preparation (water extract of the lyophilized venom glands), it was injected into honey bees. Honey bee workers (1-3 weeks old) were restrained by chilling at 4°C then placed on their backs in a Lucite holder (16 bees to a holder) and injected in the ventral thorax behind the first pair of legs with 1 μL of water extract of the venom glands and immediately transferred to holding cages supplied with 40% sucrose solution. Controls received phosphate buffered Ringer's solution.

HPLC fractionation of venom extracts. Venom glands were extracted with 50% CH_3CN/H_2O and the extracts passed through a reverse-phase HPLC, YMC-ODS column 20 x 280 mm. A 5% to 95% linear gradient of CH_3CN/H_2O containing 0.1% TFA was used for 30 minutes at a flow rate of 8 mL/min. The fraction of highest pharmacological activity was further purified on a reverse-phase YMC-ODS column 4 x 280 mm, developed by 15% CH_3CN/H_2O containing 0.1% TFA for 15 minutes at a flow rate of 1 mL/min.

Electrophysiological studies. The metathoracic retractor unguis nerve-muscle preparation of the locust Schistocerca gregaria was dissected and mounted in a small Perspex® bath as described previously (21). The muscle apodeme was attached to a Grass FT 10-strain gauge with a short strand of terylene and the muscle stretched to maximal body length. The total volume of the bath, including inlet and outlet reservoirs, was about 2.2 mL and the contents could be replaced within 1 s. The dissection and setting up procedure were performed in continuously flowing saline. The muscles were stimulated indirectly through fine (40-80 μm) platinum wire electrodes, insulated to their tips and placed on the retractor unguis nerve. The venom fractions were dissolved in locust saline of the following composition: NaCl, 140 mM; KCl, 10 mM; $CaCl_2$, 2 mM; NaH_2PO_4, 4 mM; NaH_2PO_4, 6 mM, and buffered at pH 6.8. The nerve muscle preparation was perfused with this saline at a flow rate of 5-10 mL/min. at 19°C.

Experimental Results. Honey bees injected with water extracts of the venom glands were paralyzed in a dose-dependent manner. Time for recovery from paralysis was 15 ± 3 min. and 55 ± 8 min. for bees injected with 0.2 and 1.2 venom units (a unit is the extract from 1 wasp gland), respectively. Although all bees recovered within 1 hour, a dose-dependent mortality was evident after 24 hours (30, 80 and 100% mortality for bees injected with 0.4, 0.8 and 1.2 venom units, respectively). Polyacrylamide disc gel electrophoresis showed that the water extract of the venom glands contained a large number of proteins, all of which were precipitated by heating the extract 100°C for 10 minutes. The boiled extracts retained full activity when assayed on the locust nerve-muscle preparation or on honeybees.

The extract of each batch of 1000 venom glands was fractionated by reverse-phase HPLC and 30 fractions were collected. Each of the 30 fractions was tested for pharmacological activity on the locust nerve muscle preparation, using reduction in neurally evoked twitch amplitude as the measure of activity. Ten fractions were pharmacologically active. The most active fraction was the one collected at retention time 13 min. (hatched peak in Fig. 2A). Further purification of this fraction by reverse-phase HPLC gave four peaks (Fig. 2B), the most pharmacologically active was in the major peak (hatched Fig. 2B). This fraction gave 1.1 mg of toxin as amorphous powder.

The UV spectrum of **1** has a maximum of 274 nm, which shifts to 290 nm at pH 12, suggesting the presence of a tyrosine residue. This was supported by ^{1}H-NMR (250 MHz in D_2O): δ 3.00 (2H, d, J = 7.8 Hz), 4.43 (1H, t, J = 7.8 Hz), 6.88 (2H, d, J = 8.7 Hz). The presence of a butyryl group was also clear from ^{1}H-NMR, δ 0.83 (3H, t, J = 7.2 Hz), 1.57 (2H, quin. J = 7.2 Hz), 2.26 (2H, t, J = 7.2 Hz). The ^{1}H-NMR signal corresponding to six methylenes α to nitrogen at δ 3.0-3.3 (12H, m) and four methylenes β to nitrogen at δ 1.4-1.6 (4H, m) and 2.1-2.2 (4H, m) (22), together with the FAB-MS $(M+H)^+$ peak at m/z 436, showed the remainder of the molecule to be a polyamine of the spermine type. ^{1}H-NMR measured in DMSO-d_6 (500 MHz) clarified the connectivity of the butyryl, tyrosyl and polyamine moieties; namely, two amide protons were observed at δ 7.82 and 7.86 as a doublet and triplet, respectively, indicating that the former is due to tyrosine and the latter to polyamine. This leads to a butyryl/tyrosyl/polyamine sequence as shown as **1**, **2** and **3** of Figure 3A, but since spectroscopic evidence was ambiguous to differentiate the three, all isomers were synthesized. Chemical synthesis of the three isomers is illustrated in Figs. 3B and 3C. The protected polyamine **III** was obtained from spermidine derivative **I** in three steps: *(i)* by Michael addition to acrylonitrile (76%); *(ii)* Boc-protection (81%); and *(iii)* reduction of the nitrile (70%) as shown in Fig. 3B. Further Cbz-protection and Boc-deprotection of **III** yielded partially protected polyamine **IV** (Boc represents *tert*-butoxycarbonyl and Cbz represents carbobenzoxy). Deprotection of N-Boc-O-benzyl-L-tyrosine *p*-nitrophenyl ester **V** with trifluoracetic acid (TFA) followed by acylation with butyryl chloride gave key intermediate **VI** in 85% yield as shown in Fig. 3C. Coupling of **VI** protected polyamines **IV**, **III** and commercial spermine (1,12-diamino-4,9-diazadodecane) (24), ca. 65% yield, followed by deprotection gave PhTX **1** and analogs, **2** and **I** (ca. 80% yield). Synthetic material derived from **IV** was found to be identical with the natural product in all respects (^{1}H-NMR, MS, CD, HPLC and biological activities). Thus the chemical structure of the major naturally-occurring philanthotoxin is **1**, which is designated PhTX-433, the numerals denoting the number of methylene groups between the amino groups of the spermine moiety. All three of the synthetic end-products were biologically active, PhTX-334 having a higher potency than the natural PhTX-433 toxin, while PhTX-343 was somewhat less active.

Preliminary pharmacological studies with PhTX-433 suggested that its action on a locust nerve-muscle preparation was both time- and concentration-dependent. The effects of this toxin on the neurally-evoked twitch contraction of the locust retractor unguis muscle were investigated using toxin concentrations of 1-10 μM (21). It was clear from the data presented in Fig. 4A that PhTX-433 exerted a number of actions on the locust nerve-muscle system. There was an initial reduction in twitch amplitude, which was stimulus frequency independent. This was followed by a further reduction in the twitch height, the extent of this charge being directly proportional to the frequency at which the retractor unguis nerve was stimulated. Prolonged applications of PhTX-433 abolished the twitch. Immediately following removal of the toxin there was a brief period of repeated and prolonged contractions in response to a single stimulus applied to the retractor unguis nerve before the twitch slowly returned to normal. PhTX-433 at 10 μM concentration also reduced the response of the retractor unguis muscle to L-glutamate (0.1 mM; bath applied), which suggests that at least part of the reduction in twitch amplitude was due to the antagonism of postjunctional, quisqualate-sensitive glutamate receptors. PhTX-334 (Fig. 4B) and PhTX-343 influenced the twitch contraction in the same qualitative fashion as PhTX-433. The physiological activity of PhTX-343 and PhTX-334 were, respectively, 80% and 125% that of PhTX-433 as measured by the locust muscle twitch inhibition concentration.

The neutral philanthotoxin and its synthetic counterpart PhTX-433 and analogs PhTX-334 and PhTX-334 and PhTX-343 (Fig. 3A) represent a new class of chemicals that are active biologically and inhibit allosterically the quisqualate-sensitive glutamate receptor in insect skeletal muscle (Fig. 4). They are smaller in molecular weight (435 daltons) than the toxins isolated from orb web spider venoms, the argiotoxins (>600 daltons), and easier to synthesize. (14, 15, 17, 25).

<u>Binding PhTX-433 to NMDA Receptor</u>. The NMDA receptor is identified by its high affinity for the compound (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801). This compound is an anticonvulsant introduced by Merck Sharp & Dohme Co. and

is a potent non-competitive antagonist of the NMDA receptor. Binding of [^{3}H]MK-801 to synaptic membranes from rat brain after thorough washing is extremely poor. However, the binding (measured by a filtration assay) is potentiated by glutamate in a dose-dependent manner and reaches maximal potentiation at 10 μM glutamate (26). The increase in binding of [^{3}H]MK-801 resulting from addition of glutamate has been used as an index of NMDA receptor binding. Philanthotoxin (PhTX-433) inhibited the binding of [^{3}H]MK-801 to NMDA receptors (Fig. 6) with and EC_{50} (the concentration that inhibits 50% of binding) of 25 μM. Because of the difference in maximal glutamate-induced [^{3}H]MK-801 binding in the absence and presence of PhTX-433 (Fig. 5), it is suggested that MK-801 and PhTX-433 may affect the NMDA receptor by binding to distinct allosteric sites on the receptor protein.

B. <u>Synthesis of PhTX-343 Analogs</u>

A structure-activity study was undertaken in order to increase the inhibitory effect of an analog at a particular concentration (IC_{50}) in the locust muscle assay, operating on the assumption that an increase in activity would be observed as inhibition of muscle contraction at lower ligand concentrations. Spermine was used for the synthesis of most analogs because the biological activity of PhTX-343 was similar to that of natural PhTX-433 (80%) and because of its commercial availability. Furthermore, its symmetric structure makes it unnecessary to differentiate the two terminal amino groups when coupling to the *p*-nitrophenol activated esters; apparently only primary amines were reactive in this coupling since no product arising from the reaction of secondary amines could be detected.

The majority of analogs were synthesized according to

Method A shown in Fig. 7 with only slight modifications if necessary. Taking into account the structural similarity between PhTX-433 and other neurologically active spider toxins, the molecule was divided into four moieties, A, B, C and D in order to assess the structure-activity relation in a systematic manner. Moiety C, the butyryl moiety of the natural PhTX-433, suggests the possible necessity of a hydrophobic region in the molecule. Thus, analogs **18-24** were synthesized by exchanging butyryl chloride with the appropriate acyl chloride. In order to investigate the effect of the tyrosyl moiety in Moiety B, analogs **10-17** were prepared by the coupling of spermine and the *p*-nitrophenol esters of the corresponding N-butyryl amino acids. Analog **11** was obtained by treating N-Boc-tyrosine with acetic anhydride and triethylamine before proceeding with Method A. Analogs **4** and **5** of Moiety A were obtained through coupling of polyamine intermediates with the appropriate intermediate ester shown in Method A of Fig. 7 and then hydrogenolysis; mixing ammonium acetate and hydrogenolysis yielded **6**. Analogs **7** and **8** with branchings were prepared in order to examine the effects of alternating hydrophobic and hydrophilic regions and branching in the polyamine moiety; if active, a group suited for affinity binding to a solid support could be linked to the terminal of the branching. They were made by coupling of ester and an alkylated 433-type polyamine; such alkyl polyamines were synthesized in the same manner as thermospermine except that the an alkyl group was introduced by bromoalkyl quenching of the lithiated anion α to the nitrile of the Boc-protected Michael adduct of diaminobutane and acrylonitrile. These intermediates were then transformed into polyamines analogous to polyamine.

Arginine and other amino acids were linked to Moiety D, since in the spider toxins argiopine (28), NSTX-3 (29), argiotoxin 659 (25), and argiotoxin 673 (25), which show similar inhibition of locust muscle contraction (27), the polyamine moiety contains an additional arginine residue. Thus analogs **33-37** were synthesized by coupling O-benzyl-PhTX-343 with the *p*-nitrophenol esters of the corresponding amino acids, or in the case of **36**, with commercially available $(Cbz)_3$-arginine N-hydroxysuccinimide ester followed by deprotection under hydrogenolysis conditions.

Analogs **25**, **26**, **28**, **30**, **40**, and **41** were prepared to check the possibility of converting Moiety C into groups that could be used for photoaffinity labelling: **26**, **40**, **41**; or affinity labelling: **28**, **30**. As these functionalities are sensitive to the hydrogenolysis conditions employed for O-benzyl deprotection, they were synthesized according to Method B shown in Fig. 7. Thus N-carbonbenzyloxylation (Method B) instead of N-butoxycarbonylation (Method A) allowed hydrogenolysis to be performed prior to attachment of the functionality sensitive to reduction. N-tyrosyl acylation was achieved with either the free acid and diphenylphosphoryl azide or with the N-hydroxysuccinimide ester, depending on availability; in all cases the N-tyrosyl acylation proceeded the Boc deprotection step.

All of the compounds disclosed in Tables 1-9 were synthesized according to Method A or Method B shown in Fig. 7 with only slight modifications if necessary.

The coupling reaction of *p*-nitrophenol esters with spermine (Method A, Fig. 7) invariably led to some formation of bis adducts, i.e., spermines acylated on both amino terminals. These bis analogs were easily separated, and upon hydrogenolysis yielded a series of bis-type PhTX analogs **42-46**. These analogs were used in order to determine whether the effect of PhTX-type molecules on receptor/membrane complexes is a channel-blocking mechanism or a stabilization mechanism. It was also of interest to investigate the biological activities of simple mono- and bis-acylated spermine-343 molecules. Thus, three sets of mono- and bis-acyl spermine analogs **47-52** were similarly synthesized by reacting the appropriate *p*-nitrophenol esters with excess spermine.

Finally, radiolabelled analogs are necessary both for use in direct pharmacological characterization of receptors as well as for isolation of the glutamate receptor by photoaffinity labelling or affinity labelling. It was fortunate that introduction of iodine, which we had hoped to use for radiolabelling of the tryosyl moiety, also increased the biological activity approximately ten-fold. Cold iodinated analogs (**13**, **38**, **39**, **41**) were prepared by use of NBS and KI on a milligram scale, while radioactive ^{125}I analogs were prepared with $Na^{125}I$ and chloramine T in buffered solution on a micro-scale and purified by reverse phase HPLC.

Experimental

CI-MS (NH_3) spectra were obtained on a Nermag-10 while FAB-MS (3-nitrobenzyl alcohol matrix) spectra were obtained with a JOEL DX-303. Proton NMR spectra were recorded on a Brucker WM-250 instrument using residual proton solvent peaks of either $CDCl_3$ at 7.24 ppm or CD_3OD at 4.68 ppm as an internal standard. NMR spectra were measured in CD_3OD and as free bases unless otherwise specified. The solvents DMF and *i*-$PrNH_2$ and the reagents

Et_3N and pyridine were distilled neat at atmospheric pressure. HPLC was used to identify the correct isomer of the natural product with the following column and conditions. Column: YMC-ODS, 4.6 x 250 mm; solvent: (12.5% CH_3CN, 0.1% TFA)/H_2O; flow rate: 1 mL/min.; detection: 274 nm. A diatomaceous earth filtration aid sold under the trademark Celite® was used where indicated. All the solution ratios are (vol/vol) unless otherwise indicated.

2-(Diaminobutyl)ethylnitrile

Acrylonitrile (3.1 g, 58.4 mmol) in 1.5 mL CH_3OH solution was added to 1.5 mL CH_3OH solution of diaminobutane (4.3 g, 48.8 mmol) at 0°C and was stirred for 12 hours. The reaction was terminated by evaporation of the solvent and the oil was applied directly to a silica gel flash column, eluting with 3:1 $CHCl_3/CH_3OH$ and 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$. The product was obtained as a clear oil in 65% yield. CI-MS ($C_7H_{15}N_3$): m/z 142 $(M+1)^+$; NMR: δ 1.32 (4H, complex) 1.53 (2H, br s), 1.65 (1H, s), 1.82 (1H, s), 2.35 (2H, t, J = 6.6 Hz), 2.46 (3H, complex), 2.75 (2H, t, J = 6.6 Hz).

2-(N,N'-di-Boc-diaminobutyl)ethylnitrile

A solution of 2.82 g (20.0 mmol) of the above ethylnitrile and 4.8 g (22 mmol) of Boc anhydride in 70 mL of CH_2Cl_2 was stirred at room temperature for 12 hours. The reaction was worked up by pouring the mixture into water and extracting with EtOAc three times. The combined organic layers were washed with aqueous $NaHCO_3$ and saturated NaCl solutions. After drying the solution over $MgSO_4$ and evaporating the solvent, the crude oil was chromatographed on silica with $CHCl_3$ followed by 1%

$CH_3OH/CHCl_3$, yielding 4.2 g (75%) of the desired product. CI-MS ($C_{17}H_{31}N_3O_4$): m/z 342 $(M+1)^+$; NMR: δ 1.40 (9H, s) 2.57 (2H, br s), 3.08 (2H, q, J = 6.1 Hz), 3.24 (2H, t, J = 7.4 Hz), 3.42 (2H, t, J = 6.7 Hz), 4.58 (1H, br s).

N',N"-di-Boc-polyamine-34

To a suspension of 0.062 g (1.63 mmol) of lithium aluminum hydride (LAH) in 10 mL of Et_2O was added 0.158 g (0.463 mmol) of the above nitrile at 0°C, and the mixture was stirred at 0°C for 30 minutes. The excess LAH was quenched with 1 N NaOH at 0°C and the resulting white suspension was filtered through Celite® and washed with Et_2O. The filtrate was washed with water and the water layers were extracted with Et_2O. The combined Et_2O layers were washed with brine, dried over $MgSO_4$, and evaporated to yield the 0.108 g (68%) of the crude oil that was carried on to the next reaction without further purification. CI-MS ($C_{17}H_{35}N_3O_4$): m/z 346 $(M+1)^+$; NMR: δ 1.37 (9H, s), 1.38 (9H, s), 2.61 (2H, t, J = 6.7 Hz), 3.06 (4H, t, J = 6.7 Hz), 3.18 (2H, br, s), 4.65 (1H, br, s).

Di-Boc-polyamine-34-ethylnitrile

A mixture of 2.20 g (6.38 mmol) of the above N',N"-di-Boc-polyamine-34 and 0.63 mL (9.57 mmol) of acrylonitrile in 10 mL of CH_3OH was stirred at room temperature for 12 hours. The reaction was worked up by evaporation of solvent and chromatographed on silica gel with 1% up to 2% $CH_3OH/CHCl_3$ from which was obtained 2.47 g (97%) of the desired product. CI-MS ($C_{20}H_{38}N_4O_4$): m/399 $(M+1)^+$; NMR: δ 1.39 (9H, s), 1.40 (16H, s), 1.65 (2H, quintet, J = 6.9 Hz), 2.47 (2H, t, J = 6.8 Hz), 2.58 (2H, t, J = 6.8 Hz), 2.87 (2H, t, J = 6.8 Hz), 3.10 (4H, t, J = 6.3 Hz), 3.20

(1H, br s), 4.58 (1H, br s).

Tri-Boc-polyamine-34-ethylnitrile

To a 20 mL CH_3OH solution of the above di-Boc-polyamine-34-ethylnitrile 2.47 g (6.21 mmol) was added 1.62 g (7.45 mmol) of Boc anhydride. This mixture was stirred for 12 hours. The reaction was worked up on the same manner as for 3-(di-N,N'-Boc-diaminobutyl)ethylnitrile, yielding 3.06 g (98%). CI-MS ($C_{25}H_{46}N_4O_6$): m/z 499 $(M+1)^+$; NMR: δ 1.39 (9H, s), 1.40 (9H, s), 1.42 (9H, s), 1.71 (2H, quintet, J = 7.4 Hz), 2.57 (2H, br s), 3.12 (4H, complex), 3.22 (2H, t, J = 7.3 Hz), 3.43 (2H, t, J = 6.7 Hz), 4.61 (1H, br s).

2',3',4'-tri-N-Boc-thermospermine

A $CHCl_3$ solution of the above tri-Boc-nitrile, 5.2 g (10.1 mmol) was treated with 2.4 g of LAH as described in the procedures for synthesis of N',N"-di-Boc-polyamine-34. The polyamine was obtained after silica column chromatography using 1:10 $CH_3OH/CHCl_3$ in 91% yield (4.61 g).

4'-N-Cbz-1',2',3'-tri-N-Boc-thermospermine

To a 10 mL $CHCl_3$ of 1 g (2.0 mmol) of the above 2',3',4'-tri-N-Boc-thermospermine and Et_3N (0.33 mL, 2.4 mmol) was added 0.34 mL (2.4 mmol) of Cbz-Cl and this mixture was stirred for 30 minutes at room temperature. The mixture after evaporation of the solvent was directly chromatographed on silica with 1% $CH_3OH/CHCl_3$. The desired product was obtained in 94% (1.12 g) yield. CI-MS ($C_{33}H_{56}N_4O_8$): m/z 637 $(M+1)^+$; NMR: δ 0.79 (27H, s), 1.06 (4H, quintet, J = 6.8 Hz), 2.46 (12H, complex), 4.41 (2H, s), 6.68 (5H, m).

Method A

N-Boc-O-benzyl-L-tyrosine-*p*-nitrophenol ester

To a solution of 3.33 g (9.0 mmol) of N-Boc-O-benzyl-L-tyrosine (the starting compound in Fig. 7, Method A) in 35 mL of EtOAc was added 1.25 g (9.0 mmol) of *p*-nitrophenol and 1.95 g (9.45 mmol) dicyclodicarbodiimide. The solution was stirred at room temperature for 1.5 hours and then filtered through Celite®. The resulting filtrate was extracted with water and saturated $NaHCO_3$. The aqueous extracts were then extracted three times with EtOAc. The combined organic layers were shaken three times with saturated NaCl solution, dried over $MgSO_4$, filtered, and evaporated to a slightly yellow, white powder. The powder was recrystallized from EtOH to yield, after filtration and washing with cold EtOH, 3.44 g (78%) of a white powder. EI-MS ($C_{27}H_{28}N_2O_7$): m/z 492 (M^+); NMR: δ 1.44 (9H, s), 3.15 (2H, d, J = 5 Hz), 4.73 (3H, t, J = 5 Hz), 5.10 (2H, s), 6.94 (2H, d, J = 10.4 Hz), 7.12 (4H, d, J = 10.4 Hz), 7.38 (5H, m), 8.22 (2H, d, J = 10.4 Hz).

N-butyryl-O-benzyl-tyrosine-*p*-nitrophenol ester

To a solution of 2.95 g (6.0 mmol) of the previously made N-Boc-O-benzyl-L-tyrosine-*p*-nitrophenol ester in 30 mL of $CHCl_3$ was added 15 mL of trifluoroacetic acid (TFA) and this mixture was stirred at room temperature. After roughly 2 hours, when all of the starting material was consumed according to TLC (silica, 35% EtOAc/hexane), the solution was evaporated to dryness. The resulting solid was suspended in 10 mL of $CHCl_3$ with stirring and to this suspension was added simultaneously 0.75 mL (7.20 mmol) of butyryl chloride and 2.50 mL (18.0 mmol) of Et_3N. The slightly yellow solution was stirred at room temperature. After 90 minutes the solution was evaporated to a slightly yellow solid and recrystallized with EtOH or chromatographed on silica with $CHCl_3$ to yield 2.0 g (72%) of the desired product. CI-MS ($C_{26}H_{26}N_2O_6$): m/z 463 $(M+1)^+$; NMR: δ 1.05 (3H, t, J = 7.8 Hz), 1.80 (2H, m, J = 5.7 Hz), 2.58 (2H, t, J = 5.7 Hz), 3.3 (2H, m), 5.15 (1H, m), 5.18 (2H, s), 6.94 (2H, d, J = 10.4 Hz), 7.12 (4H, d, J = 10.4 Hz), 7.38 (5H, m), 8.25 (2H, d, J = 10.4 Hz).

<u>N-butyryl-O-benzyl-L-tyrosine-spermineamide and Bis[N-butyryl-O-benzyl-L-tyrosine]-spermineamide</u>

To a 10 mL CH_3OH solution of 0.36 g (0.78 mmol) of the previously made N-butyryl-O-benzyl-tyrosine-*p*-nitrophenol ester was added dropwise a 10 mL CH_3OH solution of 0.19 g (0.94 mmol) of spermine with stirring at room temperature. After 1 hour, the reaction mixture was evaporated to a yellow, semi-crystalline oil and 10 mL of $CHCl_3/CH_3OH$ (1:1) was added to enhance crystallization of the *p*-nitrophenol. This suspension was filtered through Celite® and washed with 10 mL of $CHCl_3/CH_3OH$ (1:1) solution. The filtrate was evaporated to a clear yellow oil and then chromatographed on 25 g of silica with a step gradient system of 9:1 $CHCl_3/CH_3OH$ and 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$, the elution yielding 0.003 g (20%) of the bis adduct. CI-MS ($C_{50}H_{68}N_6O_4$): m/z 849 $(M+1)^+$; NMR: δ 0.63 (6H, t, J = 5.2 Hz) 1.32 (4H, q, J = 6.8 Hz), 1.94 (4H, t, J = 7.8 Hz), 4.25 (2H, t, J = 7.8 Hz), 4.83 (4H, s), 6.70 (4H, d, J = 8.3 Hz), 6.93 (4H, d, J = 8.3 Hz), 7.19 (10H, m). The column elution with 4:4:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ yielded 0.127 g (38.4%) of a clear, light yellow oil. CI-MS ($C_{30}H_{47}N_5O_3$): m/z 526 $(M+1)^+$; NMR: δ 0.72 (3H, t, J= 5.2 Hz), 1.50 (10H, complex), 2.01 (2H, t, J = 5.2 Hz), 2.55 (12H, complex), 4.48 (1H, t, J = 7.8 Hz), 4.92 (2H, s), 6.88 (12H, complex), 4.48 (1H, t, J = 7.8 Hz), 4.92 (2H, s), 6.88 (2H, d, J = 8.3 Hz), 7.04 (2H, d, J = 8.3 Hz), 7.27 (5H, m).

PhTX-343 (compound **1**)

To a 15 mL CH_3OH solution containing 0.20 of the previously made N-butyryl-O-benzyl-L-tyrosine-spermineamide was added 0.2 g of 5% Pd/C. This solution was purged several times with hydrogen. The starting material was usually consumed after 2 to 3 hours. The reaction was terminated by filtration through Celite® and careful washing of the carbon with copious volumes of CH_3OH. After evaporation of the solvent, the clear oil was chromatographed on 10 g of silica with 10:1 $CHCl_3/CH_3OH$ and 4:4:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$. The desired product, 0.164 g (99%) was obtained as a clear oil. CI-MS ($C_{23}H_{41}N_5O_3$): m/z 436 $(M+1)^+$; NMR: δ 0.74 (3H, t, J = 5.2 Hz), 2.05 (2H, t, J = 5.2 Hz), 4.33 (1H, t, J = 5.2 Hz), 6.58 (2H, d, J = 7.8 Hz), 6.94 (2H, d, J = 7.8 Hz); HPLC retention time: 8.30 min., natural product 9.63 min.

Bis-PhTX-343 (compound **43**)

A 10 mL CH_3OH solution of 0.208 g (0.245 mmol) of the previously made Bis[N-butyryl-O-benzyl-L-tyrosine]-spermineamide was treated in the same manner as for the synthesis of **2** above with 0.05 g of 5% Pd/C. The reaction mixture was purified on a silica flash column with 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ to yield 0.124 g (76%) of the desired product. CI-MS ($C_{36}H_{56}N_6O_6$): m/z $(M+1)^+$; NMR: 0.68 (6H, t, J = 5.2 Hz), 1.98 (4H, t, J = 5.2 Hz), 4.52

(2H, t, J = 5.5 Hz), 6.77 (4H, d, J = 8.8 Hz), 7.12 (4H, d, J = 8.8 Hz).

O-benzyl-PhTX-2',3'-N,N-Boc-334

To a stirred solution of 0.022 g (0.44 mmol) of the previously made tri-Boc-polyamine-34-ethylnitrile in 0.4 mL of CH_3OH was added 0.018 g (0.04 mmol) of the previously made N-butyryl-O-benzyl-tyrosine-*p*-nitrophenol ester and the mixture was stirred for 15 minutes. After evaporation of the solvent, the mixture was loaded onto a silica flash column and eluted with 1% $CH_3OH/CHCl_3$ to yield 13 mg (39%) of the desired product.

PhTX-334 (compound **3**)

To a stirred solution of 0.195 g (0.25 mmol) of the previously made O-benzyl-PhTX-di-2',3'-N,N-Boc-334 in 3 mL of $CHCl_3$ was added 3 mL of TFA and this mixture was stirred at room temperature for 15 minutes. After evaporation of the solvent, the crude oil was loaded onto a silica flash column and eluted with a step gradient of 15:5:1 and 3:3:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ which yielded 0.072 g (67%) of the desired product. This pure free amine was dissolved in 3 mL of CH_3OH and this solution was stirred with 0.07 g of 5% Pd/C under hydrogen atmosphere at room temperature for 12 hours. The reaction was terminated by filtration and washing through Celite® with CH_3OH followed by removal of solvent *in vacuo* and then loading onto a silica flash column, eluting with a step gradient of 15:5:1 and 3:3:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ yielding 0.045 g (75%) of the desired product as a clear oil. CI-MS ($C_{23}H_{41}N_5O_3$): m/z 436 $(M+1)^+$; NMR: δ 0.67 (3H, t, J = 7.4 Hz), 1.35 (2H, sextet, J = 7.4 Hz), 1.99 (2H, t, J = 7.3 Hz), 4.20 (1H, t, J = 7.5 Hz), 6.52 (2H, d, J = 8.3 Hz), 6.86 (2H, d, J = 8.3 Hz); HPLC retention time: 8.43 min., natural product 9.63 min.

O-benzyl-PhTX-4'-N-Cbz-433

To a stirred solution of 244 mg (0.52 mmol) of N-butyryl-O-benzyl-tyrosine-*p*-nitrophenol ester in 1 mL of CH_3OH was added 200 mg (0.65 mmol) of 4'-N-Cbz-1',2',3'-tri-N-Boc-thermospermine in 1 mL of CH_3OH. This mixture was stirred for 15 minutes at room temperature. After evaporation of the solvent, the crude yellow oil was loaded onto a silica gel column and the desired product was eluted with a step gradient of 2% $CH_3OH/CHCl_3$ and 15:5:1 $CHCl_3/CH_3OH$/*i*-$PrNH_2$ which yielded 5 mg (23%).

PhTX-433 (compound **2**)

A mixture of 310 mg (0.47 mmol) of O-benzyl-PhTX-4'-N-Cbz-433 and 310 mg of 5% Pd/C in 1 mL of CH_3OH was stirred under hydrogen atmosphere for 12 hours. The mixture was then filtered and washed through Celite® with CH_3OH before loading onto a silica flash column and eluting with a step gradient of 15:5:1 and 3:3:1 $CHCl_3/CH_3OH$/*i*-$PrNH_2$. Thus 100 mg (49%) of the desired compound was obtained in the form of a clear oil. CI-MS ($C_{23}H_{41}N_5O_3$): m/z 436 $(M+1)^+$; NMR: δ 0.58 (3H, t, J = 7.4 Hz), 1.90 (2H, t, J = 7.2 Hz), 4.16 (1H, t, J = 8.4 Hz), 6.44 (2H, d, J = 4 Hz), 6.78 (2H, d, J = 8.4 Hz); HPLC retention time: 9.63 min., natural product 9.63 min., co-injection of synthetic and natural products eluted as one peak at 9.63 min.

Moiety D

O-benzyl-PhTX-343-N-α-N^G,$N^{G'}$-tri-Cbz-L-arginine-amide

To a 5 mL DMF solution of 0.452 g (0.816 mmol) of N-butyryl-O-benzyl-L-tyrosine-spermineamide was added 0.58 g (0.86 mmol) of N-α-N^G,$N^{G'}$-tri-Cbz-L-arginine-N-hydroxysuccinimide ester and the resulting solution was stirred overnight at room temperature. The reaction was worked up by evaporation of the solvent and extraction of the slightly yellow oil with $CHCl_3$ and washing the organic extracts with aqueous $NaHCO_3$, water, and brine. The crude product was chromatographed on silica with 9:1 $CHCl_3/CH_3OH/i$-$PrNH_2$ to yield 0.817 g (91%) of the desired product. NMR: δ 0.82 (3H, dt*, J = 1.5, 7.5 Hz), 2.11 (2H, complex*), 4.25 (1H, br s*), 4.52 (1H, br s*), 4.52 (1H, br s*). 5.0 -5.2 (8H, complex*), 6.85 (2H, d, J = 8.6 Hz), 7.10 (2H, d, J = 8.6 Hz), 7.30 (20H, complex*). *Complex couplings were due to a mixture of conformational isomers.

<u>PhTX-343-L-arginine-amide</u>

To a 10 mL CH_3OH solution of 0.81 g (0.74 mmol) of O-benzyl-PhTX-343-N-α-N^G,$N^{G'}$-tri-Cbz-L-arginine-amide was added 0.05 g of 5% Pd/C followed by hydrogenolysis at room temperature overnight. The reaction mixture was filtered and washed through a column of 12 g of silica, eluting with a step gradient of 1:1 $CH_3OH/CHCl_3$, 2:2:1 $CHCl_3/CH_3OH/i$-$PrNH_2$ and 2:2:1:1 $CHCl_3/CH_3OH/i$-$PrNH_2/H_2O$. After evaporation of the eluant solvent, the precipitated silica was filtered off and washed thoroughly with 1:1 $CH_3OH/CHCl_3$. The desired product was obtained as a clear foam, 0.25 g (56%). CI-MS ($C_{29}H_{58}N_9O_4$: m/z 592 $(M+1)^+$; NMR: δ 0.53 (3H, t, J = 7.4 Hz), 4.15 (1H, t, J = 7.5 Hz), 6.37 (2H, d, J = 8.2 Hz), 6.71 (2H, d, J = 8.2 Hz).

<u>Moiety C</u>

<u>N-decanoyl-O-benzyl-L-tyrosine-p-nitrophenol ester</u>

To a solution of 1.5 g (3.05 mmol) of N-Boc-O-benzyl-L-tyrosine-*p*-nitrophenol in 15 mL of $CHCl_3$ was added 8 mL of TFA and this mixture was stirred at room temperature. After roughly 2 hours when all of the starting material was consumed according to TLC (silica, 35% EtOAc/hexane), the solution was evaporated to dryness. The resulting solid was suspended in 15 mL of $CHCl_3$ with stirring and to this suspension was added simultaneously 0.697 g (3.66 mmol) of decanoyl chloride and 1.27 mL (9.15 mmol) of Et_3N. The slightly yellow solution was stirred at room temperature. After 90 minutes the solution was evaporated to a slightly yellow solid and recrystallized with EtOH or chromatographed on silica with $CHCl_3$ to yield 1.48 g (89%) of the desired product. NMR: δ 0.87 (3H, t, J = 7.7 Hz), 2.22 (2H, t, J = 7.8 Hz), 3.20 (2H, d, J = 6.8 Hz), 5.01 (1H, t, J = 6.8 Hz), 5.06 (2H, s), 6.96 (2H, d, J = 8.1 Hz), 7.12 (2H, d, J = 8.1 Hz), 7.17 (2H, d, J = 8.1 Hz), 7.41 (5H, complex), 8.24 (2H, d, J = 8.1 Hz).

<u>N-decanoyl-O-benzyl-L-tyrosine-spermine-amide and Bis[N-decanoyl-O-benzyl-L-tyrosine]-spermine-amide</u>

To a 10 mL CH_3OH solution of 1.45 g (2.38 mmol) of N-decanoyl-O-benzyl-L-tyrosine-*p*-nitrophenol ester was added dropwise a 10 mL CH3OH solution of 0.58 g (2.85 mmol) of spermine with stirring at room temperature. After 1 hour, the reaction mixture was concentrated to a yellow, semi-crystalline oil and 10 mL of $CHCl_3/CH_3OH$ (1:1) was added to enhance crystallization of the *p*-nitrophenol. This suspension was filtered through Celite® and washed with 10 mL of $CHCl_3/CH_3OH$ (1:1) solution. The filtrate was concentrated to a clear yellow oil and then chromatographed on 25 g of silica with a step gradient of 9:1 $CHCl_3/CH_3OH$ and 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$, the elution yielding 0.322 g (27%) of the bis adduct. NMR: δ 0.70 (6H, t, J = 6.5 Hz), 1.98

(4H, d, J = 8.7 Hz), 4.29 (2H, t, J = 8.7 Hz), 4.85 (4H, s), 6.73 (4H, d, J = 8.7 Hz), 6.95 (4H, d, J = 8.7 Hz), 7.16 (10H, complex). The column elution was continued with 4:4:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ which yielded 0.322 g (58%) of a clear, light yellow oil. NMR: δ 0.70 (3H, t, J = 5.2 Hz), 1.97 (2H, d, J = 7.3 Hz), 4.30 (1H, t, J = 7.2 Hz), 4.86 (2H, s), 6.72 (2H, d, J = 8.7 Hz), 6.97 (2H, d, J = 8.7 Hz), 7.18 (5H, complex).

C_{10}-PhTX-343 (compound **20**)

To a 2 mL CH_3OH solution containing 0.055 g (0.090 mmol) of N-decanoyl-O-benzyl-L-tyrosine-spermine-amide was added roughly 0.02 g of 5% Pd/C. This solution was purged several times with hydrogen and then stirred for 12 hours. The reaction was terminated by filtration through Celite® and careful washing of the carbon with copious volumes of CH_3OH. After evaporation of the solvent, the clear oil was chromatographed on 10 g of silica with 10:1 $CHCl_3/CH_3OH$ and 4:4:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$. The desired product was obtained as a clear oil in a yield of 0.26 g (55%). CI-MS ($C_{29}H_{53}N_5O_3$): m/z 520 $(M+1)^+$; NMR: δ 0.70 (3H, t, J = 6.6 Hz), 2.97 (2H, t, J = 6.3 Hz), 4.26 (1H, t, J = 7.6 Hz), 6.48 (2H, d, J = 7.9 Hz), 6.83 (2H, d, J = 7.9 Hz).

Bis-C_{10}-PhTX-343 (compound **45**)

A 3 mL solution of 0.110 g (0.108 mmol) Bis[N-butyryl-O-benzyl-L-tyrosine]-spermineamide was treated in the same manner as for the above synthesis of PhTX-343 above with roughly 0.05 g of 5% Pd/C. The product was purified on a silica flash column with 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ yielding 0.072 g (80%) of the desired product. CI-MS ($C_{48}H_{80}N_6O_6$): m/z 859 $(M+Na)^+$, 837 $(M+1)^+$; NMR: δ 0.67 (6H, t, J = 6.9 Hz), 2.97 (4H, br t, J = 6.3 Hz), 4.21 (2H, t, J = 7.7 Hz), 6.48 (4H, d, J = 8.4 Hz), 6.82 (4H, d, J = 8.4 Hz).

Moiety B

N-butyryl-L-glycine-*p*-nitrophenol

To a solution of N-Boc-L-glycine-*p*-nitrophenol ester (Sigma), 0.25 g (0.834 mmol) in 3 mL of $CHCl_3$ was added 2 mL of TFA room temperature with stirring. This solution was stirred for 30 minutes before the solvent was evaporated. The white powder was suspended in 3 mL of $CHCl_3$ and to this solution was added simultaneously 0.35 mL (2.5 mmol) of Et_3N and 0.10 mL (1.0 mmol) of butyryl chloride. This solution was stirred for 30 minutes before evaporation of the solvent and loading of the crude oil onto a silica flash column from which the pure product was eluted with $CHCl_3$ in 85% yield (0.188 g), NMR ($CDCl_3$): δ 1.54 (3H, t, J = 7.4 Hz), 2.25 (2H, m), 2.82 (2H, t, J = 7.5 Hz), 4.78 (2H, s) 7.95 (2H, d, J = 9.6 Hz), 8.84 (2H, d, J = 9.6 Hz).

N-butyryl-L-glycine-spermine-amide (compound **16**)

To a 7 mL CH_3OH solution of spermine 0.171 g (0.848 mmol) was added dropwise a 7 mL CH_3OH solution of N-butyryl-L-glycine-*p*-nitrophenol ester 0.188 g (0.71 mmol) with stirring at room temperature. This mixture was stirred for 30 minutes before evaporation of the solvent to a yellow, semi-crystalline oil. Roughly 10 mL of $CHCl_3/CH_3OH$ (1:1) was added to enhance crystallization of the *p*-nitrophenol. This suspension was filtered and washed through Celite® with 10 mL of $CHCl_3/CH_3OH$ (1:1) solution. The filtrate was evaporated to a clear yellow oil and then chromatographed on 6.8 g of silica with a step gradient system of 9:1 $CHCl_3/CH_3OH$ and 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ to yield 0.118 g (51%) of a clear, light yellow oil. FAB-MS ($C_{16}H_{35}N_5O_2$): m/z 352 $(M+Na)^+$, 330 $(M+1)^+$; NMR: δ 0.90 (3H, t, J = 7.4 Hz), 2.17 (2H, t, J = 7.5 Hz), 3.74 (2H, s).

Moiety A

PhTX-43 (compound **4**)

The corresponding O-benzyl-tyrosyl-amine was deprotected in the same manner as for PhTX-343 in 89% yield from 0.189 g of starting material. CI-MA ($C_{20}H_{34}N_4O_3$): m/z 379 $(M+1)^+$; NMR: δ 0.64 (3H, t, J = 7.4 Hz), 1.94 (2H, t, J = 7.6 Hz), 4.26 (1H, t, J = 7.6 Hz), 6.47 (2H, d, J = 8.4 Hz), 6.82 (2H, d, J = 8.4 Hz).

PhTX-4 (compound **5**)

The corresponding O-benzyl-tyrosyl-amine, 0.220 g (0.535 mmol) was deprotected in the same manner as above in 88% yield. CI-MS ($C_{17}H_{27}N_3O_3$): m/z 322 $(M+1)^+$; NMR: δ 0.66 (3H, t, J = 7.5 Hz), 1.96 (2H, t, J = 7.5 Hz), 4.28 (1H, t, J = 7.5 Hz), 6.49 (2H, d, J = 8.5 Hz), 6.84 (2H, d, J = 8.5 Hz).

PhTX-0 (compound **6**)

To a stirred solution of NH_4OAc 0.166 g (2.16 mmol) in 2 mL of DMF was added 0.200 g (0.433 mmol) of N-butyryl-O-benzyl-tyrosine-*p*-nitrophenol ester dissolved in 3 mL of DMF; this mixture was stirred for 5 minutes before terminating the reaction by pouring it into aqueous 0.1 N NaOH and extracting with EtOAC. The combined organic extracts were washed with brine and dried over $MgSO_4$ before evaporation of the solvent and elution from a silica flash column with 2% $CH_3OH/CHCl_3$ yielded 0.129 g (88%) of pure product. This clear oil was then dissolved in 15 mL of CH_3OH and treated with 0.130 g of 5% Pd/C and hydrogen for 1 hour. The reaction mixture was purified first by filtration and washing through Celite®, followed by evaporation of the solvent and recrystallization from 1:2 $CH_3OH/CHCl_3$ yielded 0.036 g (38%) of pure product. The mother liquid was re-evaporated and recrystallized from 1:1:2 $CH_3OH/Et_2O/CHCl_3$ to give another 0.02 g, a total yield of 50%. CI-MS ($C_{13}H_{18}N_2O_3$): m/z 251 $(M+1)^+$; NMR: δ 0.64 (3H, t, J = 7.4 Hz), 1.33 (2H, m), 1.94 (2H, t, J = 7.6 Hz), 2.58 (1H, dd, J = 13.9, 9.0 Hz), 2.86 (1H, dd, J = 13.9, 5.7 Hz), 4.37 (1H, dd, J = 9.0, 5.7 Hz), 6.50 (2H, d, J = 8.4 Hz), 6.87 (2H, d, J = 8.4 Hz).

N-[2-(1-(butyl)cyanoethyl)]-1,4-diamine

To a 10 mL dry THF solution at -78°C of 0.44 g (3.15 mmol) of 2-(diaminobutyl)ethylnitrile, was added 1.38 mL of 2.5 molar *n*-butyl lithium in hexane (Aldrich) (3.45 mmol) and this mixture was stirred for 5 minutes. To this suspension was added dropwise 0.33 mL of 1-bromobutane (3.1 mmol) and then the reaction temperature was raised to 0°C. After stirring for another 5 minutes, the reaction was allowed to rise to room temperature. After quenching by addition of H_2O, the solvent was evaporated and the residue suspended in water was extracted 3 times with $CHCl_3$. The combined organic layers were washed with brine, dried over $MgSO_4$, and then evaporated to yield a mixture of mono- and di-alkylation products (ca. 1:1) in 74% yield. This mixture was purified on silica gel with 10% $CH_3O/CHCl_3$. NMR ($CDCl_3$): δ 0.95 (3H, t, J = 6 Hz), 1.1-1.7 (11H, complex), 2.6-3.9 (5H, complex), 3.75 (1H, t, J = 6.3 Hz).

PhTX-(*n*-butyl)-433 (compound **8**)

N-butyryl-O-benzyl-L-tryrosyl-butyl-thermospermine (433) was deprotected in the same manner as for PhTX-343 yielding 0.081 g (28%) of pure product. FAB-MS ($C_{27}H_{49}N_5O_3$): m/z 492 $(M+1)^+$; NMR: δ 0.84 (3H, t, J = 7.5 Hz), 0.87 (3H, t, J = 5.9 Hz), 2.14 (4H, t, J = 7.7 Hz), 4.44 (1H, t, J = 7.7 Hz), 6.72 (2H d, J = 8.4 Hz), 7.00 (2H, m d, J = 8.4 Hz).

Method B

N-Cbz-L-tyrosyl-spermine-amide

To a 3 mL DMF solution of 0.56 g (2.75 mmol) of spermine was added dropwise a 3 mL DMF solution of 1.0 g (2.29 mmol) of N-Cbz-L-tyrosine *p*-nitrophenol ester, resulting in the instant formation of a bright yellow color. After completion of the ester addition, the solution was stirred for another 30 minutes. The desired product was obtained by evaporating the solvent, adding 20 mL of $CHCl_3$ and evaporating again. The bright yellow oily suspension was suspended in 10 mL of $CH_3OH/CHCl_3$ (1:1) and filtered through Celite® followed by rinsing with the same solution. Upon evaporation of the clear yellow solution, the yellowish crude oil was purified on 15 g of silica eluting the desired product with a gradient of 9:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$. This purification yielded 0.58 g (51%) of the desired product. FAB-MS ($C_{27}H_{41}N_5O_4$): m/z 500 $(M+1)^+$; NMR: δ 4.21 (H, br, s), 5.02 (2H, s), 6.67 (2H, d, J = 8.4 Hz), 6.96 (2H, d, J = 8.4 Hz), 7.25 (5H, s).

N-Cbz-L-tyrosyl-spermine-Na,Ne-di-Boc-L-lysine-diamide

To a 5 mL DMF solution of 0.62 g (1.2 mmol) of N-Cbz-L-tyrosyl-spermine amide was added dropwise a 4 mL DMF solution of 0.39 g (1.2 mmol) of Na,Ne-di-Boc-L-lysine *p*-

nitrophenol ester. This solution was stirred at room temperature for 30 minutes. The reaction was worked up by evaporation of DMF under high vacuum followed by addition of 6 mL of $CH_3OH/CHCl_3$ (1:1); this suspension was filtered and washed through Celite® with the same $CH_3OH/CHCl_3$ (1:1) solution. The clear yellow oil obtained after filtration and evaporation of the solvent was chromatographed on 32 g of silica and eluted with 9:1 $CHCl_3/CH_3OH$ and 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ to obtain 0.59 g (71%) of the desired product as a white foam. FAB-MS ($C_{43}H_{69}N_7O_9$): m/z 828 $(M+1)^+$; NMR: δ 1.33 (18H, s), 3.84 (1H, dd, J = 8.1, 4.7 Hz), 4.12 (1H, t, J = 7.5 Hz), 4.88 (1H, d, J = 13.0 Hz), 4.97 (1H, d, J = 13.0 Hz), 6.59 (2H, d, J = 8.2 Hz), 7.03 (2H, d, J = 8.2 Hz), 7.30 (5H, m).

N-Cbz-O-Boc-L-tyrosyl-di-Boc-spermine-Na,Ne-di-Boc-L-lysine-diamide

To a 10 mL CH_3OH solution containing 0.59 g (0.88 mmol) of the above diamide was added 0.81 mL (3.51 mmol) of Boc anhydride an 0.07 mL (0.88 mmol) of pyridine, and this mixture was stirred at room temperature for 12 hours. The clear oil was purified by evaporation of the solvent and by elution from 10.5 g of silica with 2% $CH_3OH/CHCl_3$. The resulting product was obtained as a clear oil in 82% yield (0.08 g). NMR: δ 1.38 and 1.45 (each s, total 45H), 3.87 (1H, br m), 4.24 (1H, br m), 4.92 (1H, d, J = 12 Hz), 5.00 (1H, d, J = 12 Hz), 6.95 (2H, d, J = 8.6 Hz), 7.18 (2H, d, J = 8.6 Hz), 7.20 (5H, m).

O-Boc-L-tyrosyl-di-Boc-spermine-Na,Ne-di-Boc-L-lysine-diamide

In a 10 mL of CH_3OH, 0.48 g (0.44 mmol) of the above N-

Cbz-O-Boc-diamide was dissolved and roughly 0.150 g of 5% Pd/C was added. This suspension was stirred under hydrogen atmosphere at room temperature for 12 hours. The reaction was worked up by filtration through Celite® and washing with copious volumes of CH_3OH. The filtrate was concentrated to leave a clear oil which was chromatographed on silica with a 1 to 5% $CH_3OH/CHCl_3$ step gradient. The desired product was obtained in pure form weighing 0.32 g (77%). NMR: δ 1.35 and 1.41 (each s, total 45H), 3.86 (2H, br m), 6.98 (2H, d, J = 8.5 Hz).

N-(*p*-azidobenzamide)-O-Boc-L-tyrosyl-di-2',3'-N,N-Boc-spermine-Na,Ne-di-Boc-L-lysine-triamide

In 6 mL of DMF containing 0.32 g (0.34 mmol) of the above per-Boc-diamide was added with stirring 0.06 g (0.37 mmol) of *p*-azidobenzoic azide. Finally, 0.08 mL (0.37 mmol) of Et_3N was added and this mixture was stirred overnight. The reaction was worked up by pouring the reaction mixture into 15 mL of water and extracting three times with EtOAc. The combined organic layers were washed twice with brine and dried over $MgSO_4$. After evaporation of the solvent, the crude oil was chromatographed on silica with $CHCl_3$ followed by 2.55 $CH_3OH/CHCl_3$. The product was obtained in 69% yield (0.25 g). NMR: δ 1.37 and 1.44 (each s, total 45H), 3.88 (1H, br s), 4.68 (1H, br s), 6.96 (2H, d, J = 8.6 Hz), 7.05 (2H, d, J = 8.6 Hz), 7.24 (2H, d, J = 8.6 Hz), 7.75 (2H, d, J = 8.6 Hz).

N-(*p*-azidobenzamide)-L-tyrosyl-spermine-L-lysine-triamide (compound **40**)

Boc deprotection of the above per-BOC-azido-triamide, 0.12 g (0.129 mmol) was effected in 4 mL $CHCl_3$ with 3 mL of repetitive evaporations of $CHCl_3$, the crude oil was chromatographed on silica with 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ and 5:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$. The desired product was obtained in 70% yield. FAB-MS ($C_{32}H_{50}N_{10}O_4$): m/z 661 $(M+Na)^+$, 639 $(M+1)^+$; NMR (TFA salt): δ 3.58 (1H, t, J = 7.6 Hz), 4.28 (1H, t, J = 7.6 Hz), 6.43 (2H, d, J = 8.6 Hz), 6.80 (2H, d, J = 8.6 Hz), 6.84 (2H, d, J = 8.6 Hz), 7.53 (2H, d, J = 8.6 Hz).

N-(*p*-azidobenzamide)-diiodo-L-tyrosyl-spermine-L-lysine triamide (compound **42**)

To a 1.3 mL solution H_2O/CH_3OH (5:1) containing 27.4 mg (0.025 mmol) of 40, 10.8 mg (0.065 mmol) of KI, and 17.4 mg (0.10 mmol) of K_2HPO_4 was added dropwise by pipette with rapid stirring 9.8 mg (0.065 mmol) of N-bromosuccinimide (NBS) dissolved in 1 mL of CH_3OH/H_2O (1:1). All solutions were degassed with argon. The reaction mixture was stirred for 30 minutes and then lyophilized to dryness. The brownish powder was loaded onto a silica pipette column and eluted with 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ and 4:4:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$. FAB-MS ($C_{32}H_{48}N_{10}O_4I_2$): m/z 891 $(M+1)^+$; NMR (D_2O, TFA salt): δ 3.72 (1H, br, s), 4.38 (1H, br s), 6.98 (2H, d, J = 7.5 Hz), 7.52 (2H, s), 7.70 (2H, d, J = 7.5 Hz).

Alternate Method

Heptanoylspermine-amide (compound **48**) and Bis-heptanoylspermine-amide (compound **51**)

To a solution of 0.126 g (0.50 mmol) of *p*-nitrophenol heptanoate in 3.0 mL of CH_3OH was added a 3 mL CH_3OH solution of 0.145 (0.717 mmol) of spermine. This solution was stirred at room temperature for 2 hours before evaporating the solvent, followed by filtration and washing of the cloudy, yellow suspension through Celite® with 1:1 $CH_3OH/CHCl_3$. The resulting filtrate was evaporated and the clear yellow oil loaded onto a silica flash column from which was eluted first a mixture of *p*-nitrophenol and the bis product **51** with 15:5:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$. The $CHCl_3$ solution of the impure bis product was washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$ before evaporation and re-elution of 20 mg (19%) of pure bis product **51** from a second silica flash column with 3:1:0 and 4:4:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$. CI-MS ($C_{24}H_{50}N_4O_2$): m/z 427 $(M+1)^+$; NMR: δ 0.72 (6H, t, J = 6.8 Hz), 1.112 (10H, br s), 1.43 (12H, complex), 1.98 (4H, t, J = 7.7 Hz), 2.40 (8H, m), 3.03 (4H, t, J = 6.8 Hz). The original flash column elution was continued with 4:4:1 and 1:1:1 $CHCl_3/CH_3OH/i\text{-}PrNH_2$ yielding pure mono-acylated product **48**, 0.099 g (63%). CI-MS ($C_{17}H_{38}N_4O$): m/z 315 $(M+1)^+$; NMR: δ 0.73 (3H, t, J = 6.8 Hz), 1.13 (8H, br s), 1.3-1.6 (10H, complex), 1.99 (2H, t, J = 7.6 Hz), 2.3 - 2.6 (4H, complex), 3.04 (2H, t, J = 6.8 Hz).

Synthesis of 7'

O-benzyl-L-tyrosine methyl ester (compound **1'**).

To 10 mL of cold methanol, 0.465 mL of thionyl chloride was added dropwise, followed by 1.0523 g of tyrosine. After stirring at 4°C for 2 hours, reaction was terminated by evaporating solvents. Water (20 mL) was added to dissolve the residue; *i*-$PrNH_2$ was added to neutralize the aqueous solution. After extracting with ether (3 x 20 mL), the organic layers were combined and dried over $MgSO_4$. Filtration and concentration *in vacuo* provided the expected product. NMR ($CDCl_3$): δ 7.3-7.45 (m, 5H), 6.85-7.15 (m, 4H), 5.3 (s, 1H), 5.0 (s, 2H), 3.7 (s, 3H), 2.8-3.1 (m, 2H), 1.65 (br s, 2H).

O-benzyl-butylamine-tyrosine methyl ester (compound **2'**).

To an acetonitrile solution of **1'** (0.7918 g) was added 0.54 g KF/Celite®. Butyl bromide (0.149 mL) was injected under an argon atmosphere. The mixture was refluxed for 4 hours, filtered through Celite®, concentrated, and then the residue was subjected to column chromatography to afford pure **2'**. NMR ($CDCl_3$): δ 7.3-7.5 (m, 5H), 6.9-7.1 (m, 4H), 5.05 (s, 2H), 3.6 (s, 3H), 3.5 (t, 1H), 2.9 (m, 2H), 2.4-3.6 (m, 2H), 1.4 (m, 3H), 1.3 (m, 2H), 0.9 (t, 3H).

<u>O-benzyl-Boc-butylamine-tyrosine methyl ester (compound **3'**)</u>.

To a dichloromethane solution of **2'** (98 mg) was added 150.1 mg $(Boc)_2O$. The mixture was stirred for 8 hours under argon and then concentrated. The residue was subjected to column chromatography to afford pure **3'**. NMR ($CDCl_3$): δ 7.3-7.5 (m, 5H), 6.9-7.1 (m, 4H), 5.05 (s, 2H), 3.7 (s, 3H), 3.0-3.3 (m, 3H), 2.5-2.7 (m, 1H), 1.45 (s, 9H), 1.3 (m, 2H), 1.2 (m, 2H), 0.85 (t, 3H).

<u>O-benzyl-Boc-butylamine-tyrosine alcohol (compound **4'**)</u>.

To a dichloromethane solution of **3'** (0.5749 g) at 0°C under argon was added excess DIBAL. After stirring for 1 hour, the reaction was quenched with 1N HCl, and then extracted with CH_2Cl_2 (3x). The organic layers were combined, washed with saturated NaCl and concentrated to provide a crude product suitable for use in the next step. NMR ($CDCl_3$): δ 7.3-7.5 (m, 5H), 6.9-7.1 (m, 4H), 5.05 (s, 2H), 3.5-3.8 (m, 4H), 2.7-3.1 (m, 4H), 1.45 (s, 9H), 1.2-1.4 (m, 4H), 0.9 (t, 3H).

<u>O-benzyl-Boc-butylamine-tyrosine aldehyde (compound **5'**)</u>.

To a solution of oxalyl chloride (9.8 mL) in 5 mL CH_2Cl_2 in a 25 mL round-bottom flask under argon was added DMSO (16 mL) at -50°C. The mixture was stirred for a few minutes, then the alcohol (50.8 mg) in CH_2Cl_2 was added dropwise in 5 minutes. The mixture was stirred for 15 minutes. Et_3N was then added, and the mixture was stirred for another 5 minutes. After warming to room temperature, water (10 mL) was added. The mixture was extracted with CH_2Cl_2, and the organic layer was concentrated to provide the expected product. NMR ($CDCl_3$): δ 9.6 (d, 1H), 7.3-7.5 (m, 5H), 6.9-7.1 (m, 4H), 5.05 (s, 2H), 2.9-3.6 (mm, 4H), 2.3-2.4 (m, 1H), 1.45 (ds, 9H), 1.2-1.4 (mm, 4H), 0.9 (t, 3H).

<u>O-benzyl-Boc-PhTX-C_4-amine-343 (compound **6'**)</u>.

To an ethanolic solution of **5'** (100 mg) and spermine (123 mg) was added 341 mg of Na_2SO_4. After stirring for 48 hours under argon, the mixture was filtered through glass wool. $NaBH_4$ (92 mg) was added to the filtrate. After stirring for 24 hours, 2 mL of H_2O was added quench the reaction. EtOH was removed by rotary evaporation. The residue was extracted by CH_2Cl_2 (3x). The organic layers were combined and dried over Mg_2SO_4. After concentrating, the residue was subjected to column chromatography to afford a pure product. NMR ($CDCl_3$): δ 7.3-7.4 (m, 5H), 6.8-7.1 (m, 4H), 5.0 (s, 2H), 2.5-3.0 (mm, 22H), 1.65 (m, 4H), 1.5 (m, 4H), 1.4 (s, 9H), 1.2 (m, 4H), 0.85 (m, 3H).

<u>O-Benzyl-PhTX-C_4-amine-343 (compound **7'**)</u>.

To a dichloromethane solution of **6'** was added TFA. After stirring for 30 minutes, the mixture was concentrated to provide a reaction product sufficiently pure for subsequent use. NMR ($CDCl_3$): δ 7.3-7.5 (m, 5H), 6.9-7.25

(m, 4H), 5.1 (s, 2H), 4.95 (s), 3.3 (s, 2H), 2.75-3.25 (mm, 12H), 2.0-2.2 (m, 2H), 1.8 (m, 3H), 1.65 (m, 4H), 1.45 (m, 10H), 1.3 (d, 2H), 0.95 (m, 3H). FAB-MS: $(M+H)^+$ 499

Synthesis of C_{10}-Trp-343 (compound 10').

N-Boc-L-tryptophan *p*-nitrophenyl ester (compound **8'**).

This compound was prepared as described above for N-Boc-L-tyrosine *p*-nitrophenyl ester.

N-decanoyl-L-tryptophan *p*-nitrophenyl ester (compound **9'**).

This compound as described for the corresponding tyrosine ester N-butyramide. Butyryl chloride and N-Boc-O-benzyl-L-tyrosine *p*-nitrophenyl ester were replaced by decanoyl chloride and N-Boc-L-tryptophan *p*-nitrophenyl ester, respectively. NMR ($CDCl_3$): δ 8.2 (d, 2H), 7.6 (d, 1H), 7.0-7.4 (m, 6H), 6.0 (d, 1H), 5.1 (q, 1H), 3.45 (t, 2H), 2.2 (t, 2H), 1.2 (s, 14H), 0.9 (t, 3H)

C_{10}-Trp-343 (compound **10'**).

To a 10 mL MeOH solution of 1.1 g (2.3 mmole) of N-decanoyl-L-tryptophan *p*-nitrophenyl ester **9'** was added dropwise a 10 mL MeOH solution of 0.65 g (3.2 mmole, 1.4 equivalents) of spermine with stirring at room temperature. After 3 hours, the reaction mixture was concentrated to a yellow oil and 10 mL of MeOH/CH_2Cl_2 (1:1) was added to enhance crystallization of *p*-nitrophenol. The resulting suspension was filtered and washed through Celite® with 10 mL of the same solution. The filtrate was concentrated to a clear yellow oil and then purified by silica gel flash column chromatography using a step gradient of CH_2Cl_2/MeOH (9:1) and

CH_2Cl_2/MeOH/*i*-$PrNH_2$ (15:5:1) eluting bis[N-decanoyl-L-tryptophan] spermine diamide. The column elution was continued with CH_2Cl_2/MeOH/*i*-$PrNH_2$ (4:4:1) which yielded 0.62 g (52%) of N-decanoyl-tryptophan spermine amide as a light yellow oil. DCI-MS (NH_3, $C_{31}H_{54}N_6O_2$): m/z 543 $(M+1)^+$; ^1H-NMR (250 MHz, CD_3OD): δ 7.45-7.40 (d, J = 7.5 Hz, 1H), 7.18-7.12 (d, J = 7.5 Hz, 1H), 6.95-6.80 (m, 3H), 4.46-4.42 (t, J = 7.2 Hz, 1H), 3.2-2.85 (m, 4H), 2.7-2.2 (m, 10H), 2.10-2.05 (t, J = 7.0 Hz, 2H), 1.6-0.9 (m, 22H), 0.80-0.75 (t, J = 7.0 Hz, 3H).

<u>Polyamine-3X3-$(Boc)_2$ (compound **11'**)</u>.

0.40 g (2.3 mmole) of mono N-Boc protected 1,3-diaminopropane in 7 mL EtOH was added to a suspension of 154 mg (1.15 mmole) *p*-terphthaldehyde and 0.3 mg of 3Å molecular sieves in 3 mL EtOH while stirring at room temperature. The reaction mixture immediately turned colorless. After stirring for 3 hours, a fresh $NaBH_4$ (350 mg) suspension in 10 mL EtOH was added to the previous reaction mixture, and the reduction allowed to proceed for 4 hours at room temperature. To quench the reaction, 5 mL of water was carefully added to the mixture. After stirring was continued for 1 hour, the precipitate was filtered through a pad of Celite®. The filter plug was washed with MeOH (5 mL); the filtrate was evaporated *in vacuo*, affording an opaque oily material. The crude product was pure enough by TLC (R_f = 0.65 in 8:4:1 CH_2Cl_2/MeOH/*i*-$PrNH_2$) and NMR analysis for the next step, and was used without further purification.

<u>Polyamine-3X3 (compound **12'**)</u>.

437 mg of polyamine-3X3-$(Boc)_2$ was dissolved in 9 mL of TFA/CH_2Cl_2 (1:2). The solution was stirred for 6 hours at

room temperature. The solvent was completely removed *in vacuo* to afford the expected product as an oily material in quantitative yield. After dissolving the residue in deionized water, and freezing, the frozen solid was lyophilized to provide a solid product. FAB-MS (3-nitrobenzyl alcohol matrix, $C_{14}H_{26}N_4$): m/z 251 $(M+1)^+$.

PhTX-3X3 (compound **13'**).

To a stirred suspension of 137 mg (0.37 mmole) N-butyryl-L-tyrosine *p*-nitrophenyl ester in 5 mL MeOH was added a 5 mL MeOH solution of 260 mg (0.37 mmole) of polyamine-3X3 TFA salt and 59 mg (1.5 mmole) of NaOH. The reaction mixture was stirred for 12 hours at room temperature. MeOH was evaporated to yield a yellow oily residue. The crude product was purified by silica gel flash chromatography using 16:4:1 CH_2Cl_2/MeOH/*i*-$PrNH_2$. The product was obtained as a pale yellow amorphous powder or oil in 45% yield (80 mg). FAB-MS (3-nitrobenzyl alcohol matrix, $C_{27}H_{41}N_5O_3$): m/z 484 $(M+1)^+$; ^1H-NMR (400 MHz, CD_3OD): δ 7.30 ($C_6\mathbf{H}_4$; s, 4H), 7.03-7.01 (d, J = 8.8 Hz, 2H), 6.70-6.67 (d, J = 8.8 Hz, 2H), 4.46-4.43 (HNC**H**CO; t, J = 8.4 Hz, 1H), 3.70-3.68 (HNC$\mathbf{H}_2$Ph; ds, 4H), 3.22-3.10 (CONHC$\mathbf{H}_2$; m, 2H), 2.93-2.88 (NHCHC$\mathbf{H}_2$Ar; dd, J = 7.2 Hz, 13.6 Hz, 1H), 2.76-2.71 (t, J = 7.2 Hz, 2H), 2.68-2.57 (t, J = 7.2 Hz, 2H), 2.48-2.45 (t, J = 7.2 Hz, 2H), 2.15-2.11 (COC$\mathbf{H}_2$$CH_2$; t, J = 7.6 Hz, 2H), 1.73-1.71 (quintet, J = 7.2 Hz, 2H), 1.70-1.57 (quintet, J = 7.2 Hz, 2H), 1.55-1.48 ($COCH_2$C$\mathbf{H}_2$; sextet, J = 7.6 Hz, 2H), 0.85-0.81 ($COCH_2CH_2$C$\mathbf{H}_3$; t, J = 7.6 Hz, 3H).

Synthesis of Compound 23'.

N-cyanoethyl-1,4-diaminobutane (compound **14'**).

To a methanol solution (containing 10 mL MeOH) of 1,4-

diaminobutane (3 mL) was added acrylonitrile by syringe pump over 5 hours. The mixture was stirred overnight, and then concentrated *in vacuo*. The residue was purified by column chromatography.

Di-Boc-N-cyanoethyl-1,4-diaminobutane (compound **15'**).

To a solution of 1.5983 g of **14'** in 10 mL CH_2Cl_2 was added excess $(Boc)_2O$. After stirring overnight, the reaction mixture was concentrated *in vacuo*. The residue was subjected to column chromatography to afford the desired product.

Di-Boc-4,3-amine (compound **16'**).

A solution of 0.6340 g of **15'** in 10 mL of glacial acetic acid was hydrogenated over $Pd(OH)_2$ at 50 psi hydrogen pressure for 2 hours. The catalyst was removed by filtration through Celite®. Evaporation of the solvent gave crude product which was dissolved in 50 mL of CH_2Cl_2, washed with 1 N NaOH (2x) and dried over Na_2SO_4. The solution was concentrated and subjected to column chromatography to afford the desired product.

cyanoethyl-di-Boc-4,3-amine (compound **17'**).

To a 10 mL MeOH solution of 0.5366 g of **16'** was added acrylonitrile by syringe under argon. After stirring for 8 hours, the reaction mixture was concentrated and subjected to column chromatography to afford the expected product. NMR ($CDCl_3$): δ 4.6 (br s, 1H), 3.5 (t, 2H), 3.25 (t, 2H), 3.15 (m, 8H), 2.6 (m, 2H), 1.75 (m, 2H), 1.45 (m, 29H).

cyanoethyl-tri-Boc-4,3-amine (compound **18'**).

The same general procedure followed to prepare compound **15'** was used to prepare compound **18'**. NMR ($CDCl_3$): δ 4.7 (br s, 1H), 3.0-3.3 (m, 12H), 2.7 (m, 2H), 2.2 (m, 2H), 1.7 (m, 4H), 1.4 (m, 32H).

tri-Boc-4,3,3-amine (compound **19'**).

The same general procedure followed to prepare compound **16'** was used to prepare compound **19'**.

cyanoethyl-tri-Boc-4,3,3-amine (compound **20'**).

The same general procedure followed to prepare compound **17'** was used to prepare compound **20'**. NMR ($CDCl_3$): δ 4.6 (br s, 1H), 3.1-3.3 (m, 12H), 2.9 (t, 2H), 2.6 (m, 2H), 2.5 (t, 2H), 1.7 (m, 4H), 1.4 (m, 29H).

tetra-Boc-4,3,3-amine (compound **21'**).

The same general procedures followed to prepare compounds **15'** and **16'** were used to prepare compound **21'**. NMR ($CDCl_3$): δ 4.7 (br s, 1H), 3.1-3.3 (m, 14H), 2.7 (t, 2H), 1.7 (m, 8H), 1.4 (m, 40H). MS: M^+ 659

O-benzyl-Boc-PhTX-C_{10}-amine-4333 (compound **22'**).

To an ethanolic solution containing equivalent amounts of O-benzyl-Boc-decanoyl amine-tyrosine aldehyde and compound **21'** was added excess solid anhydrous Na_2SO_4. After stirring for 36 hours, the reaction mixture was filtered through a glass wool plug. Solid $NaBH_4$ was added, and the resulting suspension was stirred overnight. Aqueous work-up gave crude product suitable for use in the next step. NMR ($CDCl_3$): δ 7.4 (m, 8H), 7.1 (m, 2H), 6.9 (d, 2H), 5.0 (s, 2H), 3.15 (m, 15H), ca. 2.7

(6H), 1.7 (m, 6H), 1.4 (s, 49H), 1.25 (s, 16H), 0.9 (t, 3H). FAB-MS: $(M+H)^+$ 1138

O-benzyl-PhTX-C_{10}-amine-4333 (compound **23'**).

To a dichloromethane solution of **22'** was added neat TFA by syringe. After stirring for 1 hour, the mixture was concentrated and purified by column chromatography. NMR ($CHCl_3$): δ 7.2-7.4 (m, 7H), 7.0 (d, 2H), 5.1 (s, 2H), 4.9 (s, 4H), 3.8 (m, 1H), 3.4 (dd, 1H), 3.3 (s, 7H) 2.9-3.2 (m, 10H), 2.1 (m, 6H), 1.6-1.6 (m, 6H), 1.3 (br s, 14H), 0.9 (t, 3H). FAB-MS: $(M+H)^+$ 640

Pharmacology

The retractor unguis muscle and its nerve were dissected from the metathoracic legs of adult, female locusts (Schistocerca gregaria) and mounted in a Perspex® perfusion chamber as described by Usherwood and Machili (21) and Bateman, et al. (14). The muscle was stretched to maximal body length and attached at its tendon or apodeme to a Grass FT 10-strain gauge by a short length of nylon thread. The volume of the muscle bath was about 0.5 mL and its contents could be exchanged within 1 s. The preparation was perfused continuously at the rate of 5-10 mL/min. (except during the application of toxin (see below) with standard locust saline of the following composition (mM) : NaCl, 180; KCl, 10; $CaCl_2$, 2; HEPES, 10; buffered to pH 6.8. The retractor unguis muscle is innervated by two excitatory motoneurons (30) and an inhibitory motoneuron (19,32), but the influence of the latter on the responsiveness of the muscle is slight. Maximal stimulation of the retractor unguis nerve at 0.2 Hz produced a series of twitch contractions of constant amplitude, which were maintained for many hours in good preparations. The test compounds were kept at

-20°C. They were dissolved in locust saline on the day of the assay and tested at room temperature. The compounds were applied by pipette to a nerve-muscle preparation such that the contents of the perfusion bath were completely replaced by the test solution. Exposure to the test compound lasted 20 minutes. Because of the limited availability of some of the compounds, the preparation was not perfused during this period. However, the amplitude of the retractor unguis muscle twitch rarely changed by more than 5-10% over a 20 minute period. During application of the test solutions the stimulation frequency was raised to 0.6 Hz for brief periods to test for stimulus frequency-dependent effects on twitch amplitude (31,37). Dose-inhibition data were obtained by testing the effects, on single retractor unguis muscle preparations, of a range of concentrations of the test compounds. Each concentration of the respective samples was tested at least three times, and each compound was assayed usually over a 100-fold concentration range (a total of 7-10 concentrations). The standard deviations for twitch inhibition for any given concentration of a toxin rarely exceed 10%. Those compounds for which the deviation was greater than this are identified in Tables 1-7. There was some variation in the potency of PhTX-343 and the test compounds between nerve-muscle preparations. In order to compensate for this, the following procedure was adopted: PhTX-343 was assayed at a variety of concentrations on eight retractor unguis nerve-muscle preparations to give a cumulative dose-inhibition relationship for this toxin. The concentration of PhTX-343 which reduced the twitch contraction by 50% (IC_{50}) was then determined by fitting a curve to the data using the following equation:

$$I = \frac{I_{max}}{1 + (IC_{50}/[T]^{n'})}$$

where: [T] = toxin concentration, I = % inhibition, I_{max} = maximum inhibition = 100% and n′ represents the Hill slope coefficient. PhTX-343, at its IC_{50} concentration (2.3×10^{-5} M) was then applied at an appropriate time during assay of each of the test compounds. The ratio of the actual reduction in twitch amplitude obtained with this single concentration of PhTX-343 and the 50% reduction that was anticipated from the cumulative dose-inhibition relationship for this toxin, was then used as a factor to normalize the data for the test compound. There were differences in the slopes of the dose-inhibition relationships for the different analogs. Also the different time-dependencies for inhibition exhibited by polyamine-containing toxins (33,37), further complicated attempts to accurately compare data for the different analogs. In some experiments the effects of the test compound on the response of the retractor unguis muscle to L-glutamic acid (100 μM) was investigated in an effort to more clearly identify postsynaptic action by the toxins. However, this approach was not used to quantitate the actions of the compounds because of considerable variations in responsiveness of individual nerve-muscle preparations to application of this amino acid alone. Despite these difficulties, the rank-order potencies described herein are reasonably accurate representations of the relative activities of the philanthotoxin analogs on the postsynaptic QUIS-R of locust leg muscle.

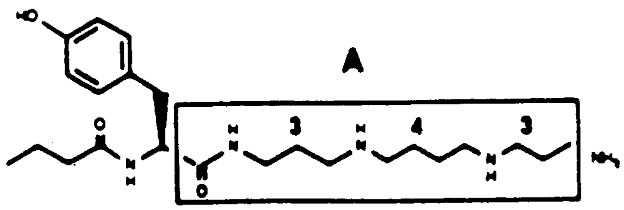
Results

Chemical modifications of PhTX-343 were performed at four regions: (A), spermine or polyamine moiety; (B), tyrosyl moiety; (C), butyryl moiety; and (D), spermine terminal amino group (Fig. 8). The structures of such compounds and their pharmacological activities are given in Tables 1-9.

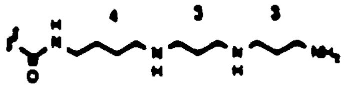
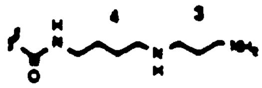
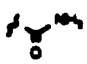
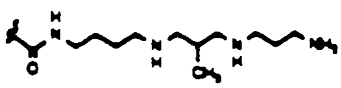
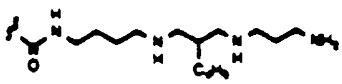
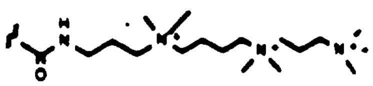
Modifications in Region A (Table 1)

The differences in the potencies of the PhTX-343 (compound **1**), PhTX-433 (natural, compound **2**) and PhTX-334 (compound **3**) analogs, although small, may reflect the distribution of negative charges on the receptor channel. Shortening of the polyamine chain from PhTX-343 (compound **1**) to PhTX-43 (compound **4**) and then to PhTX-4 (compound **5**), reduced potency, suggesting that within certain limits the number of protonated groups is important in determining the activity of these molecules. PhTX-0 (compound **6**) which is not protonated was slightly more active than PhTX-4 (compound **5**), which has a single protonated group. Addition of a methyl group to the middle carbon of the central C-3 moiety of spermine (compound **7**) did not greatly alter potency, whereas addition of a butyl group (compound **8**) increased potency by almost six-fold compared with PhTX-343. The synthesis of these two analogs was undertaken to test the feasibility of attaching various functionalities, such as affinity and photoaffinity labels, to the toxin at the end of a long alkyl chain. The permethyl analog which has three quaternary amines, exhibited a reduced potency compared with PhTX-343, suggesting possible steric hindrance of electrostatic interactions with anionic centers.

TABLE 1. MODIFICATIONS TO REGION A OF PhTX-343 AND THE EFFECTS OF ANTAGONISM OF THE NEURALLY-EVOKED TWITCH CONTRACTION OF THE LOCUST SCHISTOCERCA GREGARIA, METATHORACIC EXTENSOR TIBIAE MUSCLE



PhTX-343

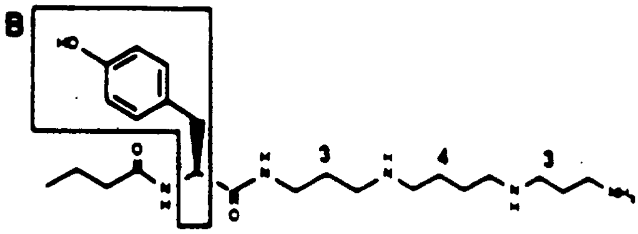
Cmpd. #		IC_{50} (M)	Relative Potency
1.		2.3×10^{-5}	1.0
2.		1.8×10^{-5}	1.3
3.		1.5×10^{-5}	1.5
4.		3.9×10^{-5}	0.6
5.		2.0×10^{-3}	0.01
6.		5.0×10^{-4}**	0.05
7.		4.0×10^{-5}	0.6
8.		4.0×10^{-6}	5.6
9.		1.3×10^{-4}	0.2

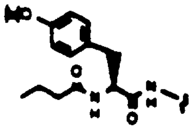
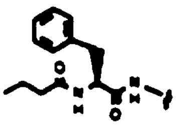
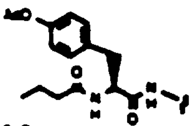
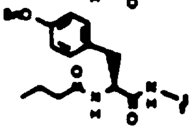
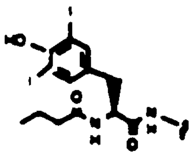
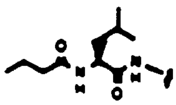
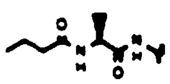
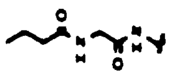
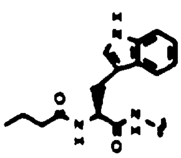
** SD $<$ 30 %.

Modifications in Region B (Table 2)

Removal of hydroxyl group in analog **10** increased potency about three-fold, whereas conversion of the tyrosyl analog to the 3,5-diiodo-tyrosyl analog **13** increased potency 4.5-fold (Table 2). The increased potency of analog **13** was especially useful since it allowed for radio-iodine labelling. It is conceivable that iodine atoms are assisting in hydrophobic binding. However, it is difficult then to account for the unchanged potencies of the O-acetyl analog **11** and the O-benzyl analog **12**. When the tyrosyl moiety was replaced by other amino acids, such as leucine analog **14** and alanine analog **15** the potency was either unchanged or slightly reduced, respectively. However, replacement by tryptophan as in analog **17** increased potency 10-fold: the tryptophan moiety may provide a subtle balance between hydrophilic (NH) and hydrophobic (aromatic) influences. The greatly reduced activity of the glycine analog **16** suggests the necessity of an anchoring functionality in this region.

TABLE 2. MODIFICATIONS TO REGION B OF PhTX-343 AND THE EFFECTS OF ANTAGONISM OF THE NEURALLY-EVOKED TWITCH CONTRACTION OF THE LOCUST <u>SCHISTOCERCA GREGARIA</u> METATHORACIC EXTENSOR TIBIAE MUSCLE



Cmpd. #		IC_{50} (M)	Relative potency
1.		2.3×10^{-5}	1
10.		6.7×10^{-6}	3.4
11.		3.3×10^{-5}	0.7
12.		2.4×10^{-5}	1.0
13.		5.3×10^{-6}	4.4
14.		2.3×10^{-5}	1.0
15.		4.5×10^{-5}	0.5
16.		1.3×10^{-4}*	0.2
17.		2.5×10^{-6}	9.2

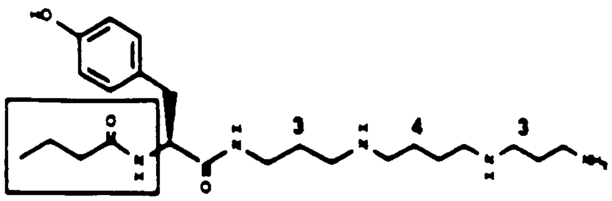
* SD < 20%

<u>Modifications in Region C (Table 3)</u>

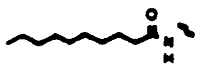
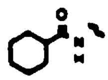
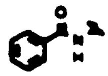
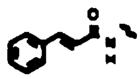
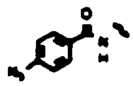
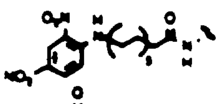
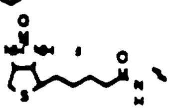
Shortening the butyryl group as in analog **18** resulted in slight but significant reduction in potency. However, increasing the length of the chain from 4 to 7 (compound **19**) and then 10 (compound **20**) produced successive increases in potency. Compound **21**, which contains two double bonds in six-carbon moiety was also very potent. The potency of the cyclohexane analog **22** was equal to that of compound **19**, whereas the phenyl analog **23** was equipotent with C_{10} analog **20**. These results indicate the importance of hydrophobicity in this region, the weak potency of compound **22** being attributable to the steric bulk of its cyclohexane moiety.

The phenyl acetyl derivative **24**, which differs from compound **23** by the presence of a methylene group located between the phenyl and carbonyl carbon, was less potent than PhTX-343. However, insertion of a double bond between the aromatic ring and the carbonyl moiety (compound **25**) led to a greatly increased potency. Substitution of an azido moiety on the aromatic ring (compound **26**), which nominally produces a photosensitive affinity label, produced reasonably active compounds compared with PhTX-343, whereas the diazo analog **27** was only weakly active. The potencies of the two dinitrophenyl analogs **28** and **29** were similar to PhTX-343. Introduction of hydrophilic groups in region C reduces activity. However, introduction of a hydrophobic spacer, as in compound **29**, compensates for this change. The 2,4-dinitrophenol moieties in compounds **28** and **29** were introduced in order to produce molecules for use in the preparation of IgG affinity columns. Compounds **30** and **31** containing biotin moieties were prepared as possible candidates for use in avidin affinity chromatography. However, the low solubility of these four analogs rendered them impracticable in this respect.

TABLE 3. MODIFICATIONS TO REGION C OF PhTX-343 AND THE EFFECTS OF ANTAGONISM OF THE NEURALLY-EVOKED TWITCH CONTRACTION OF THE LOCUST SCHISTOCERCA GREGARIA METATHORACIC EXTENSOR TIBIAE MUSCLE

C 

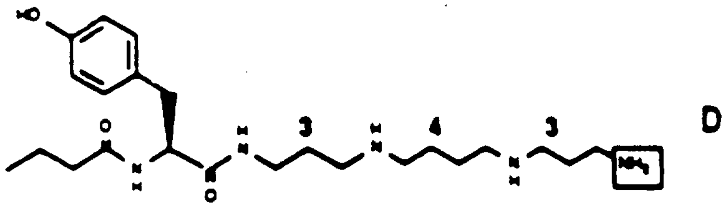
PhTX-343

Cmpd. #		IC$_{50}$ (M)	Relative Potency
1		2.3 x 10^{-5}	1.0
18.		5.0 x 10^{-5}*	0.7
19.		1.0 x 10^{-5}	2.7
20.		1.4 x 10^{-6}	16.0
21.		1.6 x 10-6	14.0
22.		9.3 x 10^{-6}	2.5
23.		2.83 x 10^{-6}	8.2
24		1.2 x 10^{-5}*	0.5
25.		2.0 x 10^{-6}	12.0
26.		4.2 x 10^{-6}	5.5
27.		2.9 x 10^{-4}	0.1
28.		5.3 x 10^{-5}**	0.4
29.		1.4 x 10^{-5}	1.6
30.		insol	
31		insol	

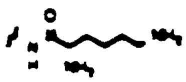
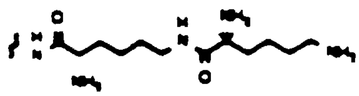
Modifications in Region D (Table 4)

The importance of a positive charge at the termination of the spermine moiety is emphasized by the low potency of compound **32**, the N-acetyl analog. Substitution in region D (Table 4) with either lysine (compound **35**) or arginine (compound **36**) increased potency, the latter being particularly efficacious. When glycine (compound **33**) or GABA (compound **34**) was substituted at this position, potency was reduced. Compound **37**, in which two lysine groups were substituted at the terminal amine, was more active than compound **35**, which contained only one lysine. This supports the contention that the number of protonated groups is an important determinant of potency. Compound **36** shares structural characteristics in common with certain polyamine-containing spider toxins. The enhanced potency of the arginine analog **36** could be accounted for if the guanidinium group were able to delocalize its positive charge over a wider area than a single point charge such as that associated with a primary amino group. In this way the guanidinium group may be better able to accommodate to the distribution of anionic centers on the wall of the receptor channel. The results with compound **36** indicate that a terminal guanidine is better than a terminal amine (compare **36** with **35**).

TABLE 4. MODIFICATIONS TO REGION D OF PhTX-343 AND THE EFFECTS OF ANTAGONISM OF THE NEURALLY-EVOKED TWITCH CONTRACTION OF THE LOCUST <u>SCHISTOCERCA GREGARIA</u> METATHORACIC EXTENSOR TIBIAE MUSCLE



PhTX-343

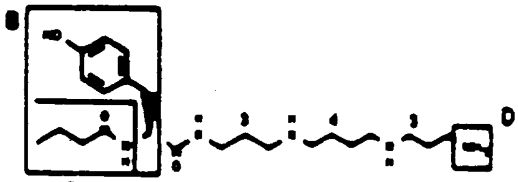
Cmpd. #		IC_{50} (M)	Relative Potency
1		2.3×10^{-5}	1.0
32.		3.0×10^{-4}	0.08
33.		1.0×10^{-4}	0.2
34.		6.8×10^{-5}	0.3
35.		1.3×10^{-5}	1.8
36.		6.3×10^{-6}	3.7
37		1.1×10^{-5} *	2.1

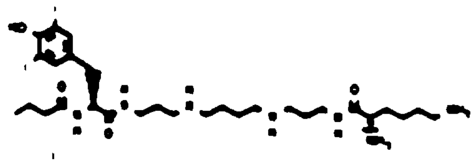
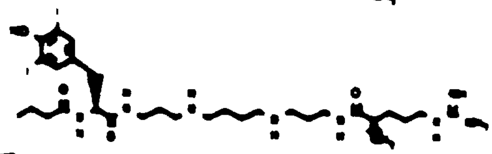
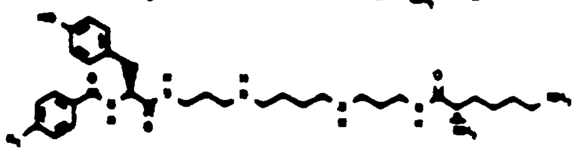
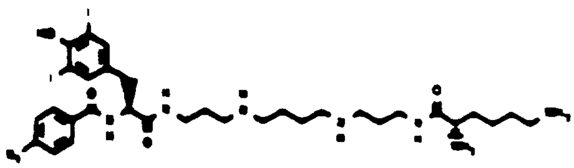
* SD < 20%

Modifications in more than one region (Table 5)

Interesting results were obtained when changes were made in more than one of the regions of PhTX-343. One such compound, diiodo-PhTX-lysine **38** is more potent than PhTX-343 as is diiodo-PhTX-arginine **39.** The triplet analog **41**, which contains changes in three regions of PhTX-343 was also more potent than the latter. These appears to be synergism between regional modifications, because the combination of diiodo substitution in region B and arginine substitution in region D raises potency much greater than was anticipated from the effects of single substitutions of this type.

TABLE 5. MODIFICATIONS TO MORE THAN ONE REGION OF PhTX-343 AND THE EFFECTS OF ANTAGONISM OF THE NEURALLY-EVOKED TWITCH CONTRACTION OF THE LOCUST SCHISTOCERCA GREGARIA METATHORACIC EXTENSOR TIBIAE MUSCLE



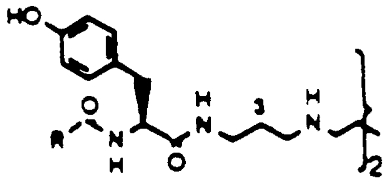
Cmpd #		IC_{50} (M)	Relative Potency
38		1.7 x 10^{-6}	14
39		0.7 x 10^{-6}	33
40		3.3 x 10^{-6}	7
41		1.8 x 10^{-6}	13

Other modifications (Tables 6 and 7)

The Bis-PhTX analogs surprisingly contained compound (Table 6) which excited rather than inhibited locust muscle. However, one must exercise some caution in interpreting data obtained from an assay which does not clearly differentiate between a variety of possible pre- and postsynaptic sites of action. Further studies on these molecules, using microelectrode recording techniques will, hopefully, shed further light on their excitatory or "agonist-like" properties.

The mono- and bis-spermine analogs **47-52** (Table 7) were prepared in order to check whether chain analogs with a hydrophobic end and a polyamine chain would have activity. The low potencies of the mono- and bis-spermine analogs (**48**, **49**, **50**) suggest that geometric constraints and the presence of both hydrophobic and hydrophilic moieties are essential for activity. However, compound **52**, bis-C_{10}-spermine is as active as PhTX-343. The potency results, ranging from agonistic to weak to moderate antagonism, as yet, cannot be of analogs **49** and **52** could result from the additional anchoring (either in a hydrophobic pocket in the QUIS-R or through association with the lipid bilayer) afforded to these molecules by their aliphatic chains, which compensates for the loss of the aromatic moiety.

TABLE 6. BIS ANALOGS OF PhTX-343: A SINGLE MOLECULE SYMMETRICALLY ACYLATED N-ALKYL-TYROSINE AND THE EFFECTS OF ANTAGONISM OF THE NEURALLY-EVOKED TWITCH CONTRACTION OF THE LOCUST *SCHISTOCERCA GREGARIA* METATHORACIC EXTENSOR TIBIAE MUSCLE



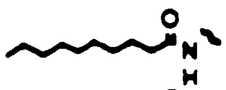
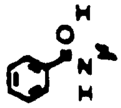
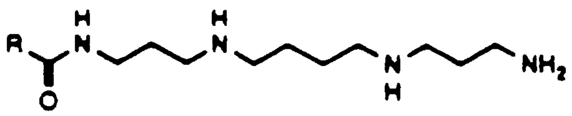
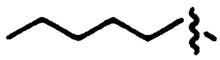
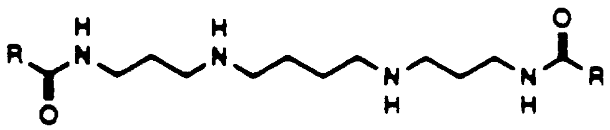
Cmpd. #	R	IC_{50} (M)	Relative Potency
42.		a-l	.
43.		a-l	.
44.		insol.	.
45.		insol.	.
46.		1.1×10^{-4}	0.2

TABLE 7. ANTAGONISTIC POTENCIES OF MONO- AND BIS-SPERMINE ANALOGS ON THE NEURALLY-EVOKED TWITCH CONTRACTION OF THE LOCUST SCHISTOCERCA GREGARIA METATHORACIC EXTENSOR TIBIAE MUSCLE



Cmpd. #	R	IC_{50} (M)	Relative Potency
47.		agonist	-
48.		1.0×10^{-4}	0.2
49.		1.0×10^{-5}	2.3



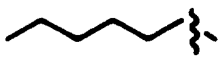
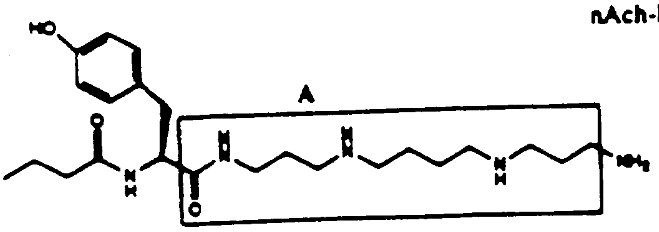
Cmpd. #	R	IC_{50} (M)	Relative Potency
50.		2.3×10^{-3}	0.1
51.		agonist	-
52.		2.3×10^{-5}	1.0

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

IC_{50} (rel. to PhTX343)
QUIS-R, muscle contraction: 1.0 [2.30 x 10^{-5}M]
nAch-R, [^{3}H]H_{12}-HTX binding: 1.0 [2.60 x 10^{-6}M]



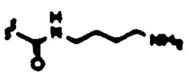
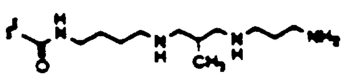
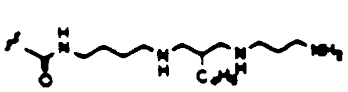
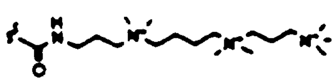
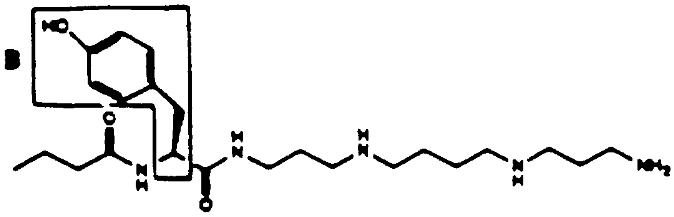
cmpd.	Name	Relative Potency Q	N	Reference
1. 	PhTX-343	1.0	1.0	1),3),5),7),8),9)
2. 	433	1.3	2.4	1),3),5),7),8),9)
3. 	334	1.5		1),3),5),7),8)
4. 	43	0.6	2.9	1),3),5),8),9)
5. 	4	0.01	0.05	1),3),5),8),9)
6. 		0.05	0.026	1),5),8),9)
7. 	Me-433	0.6	5.2	1),5),8),9)
8. 	Bu-433	5.6	18.6	1),3),5),8),9)
9. 	perMe	0.2		1),3),5),8)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS



IC_{50} (rel. to PhTX343)
QUIS-R, muscle contraction: 1.0 [2.30 x 10^{-5}M]
nAch-R, [^{3}H]H_{12}-HTX binding: 1.0 [2.60 x 10^{-6}M]

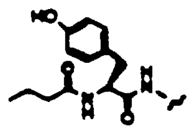
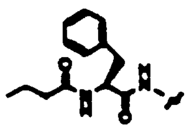
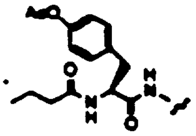
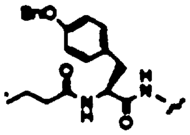
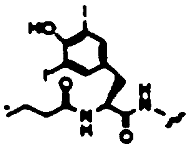
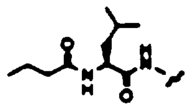
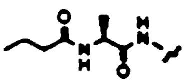
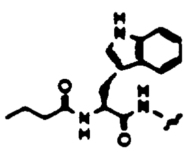
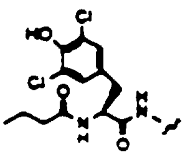
cmpd.#		Name	Relative Potency Q	N	Reference
1.		PhTX	1.0	1.0	1), 3),7),8),9)
10.		Phe	3.4	2.8	1), 3),4),7),8),9)
11.		OAc	0.7		1), 3),5),8)
12.		OBn	1.0		1), 3)
13.		I_2	4.4	8.7	1), 3),4),5),8),9)
14.		Leu	1.0	8.7	1), 3),4),5),8),9)
15.		Ala	0.5	4.6	1), 3),4),5),8),9)
16.		Gly	0.2	4.2	1), 3),4),5),8),9)
17.		Trp	9.2	9.0	1), 3),4),5),8),9)
1a.		Cl_2	1.3		3),4)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

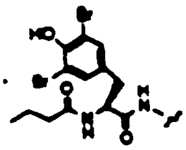
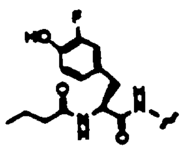
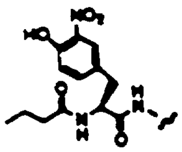
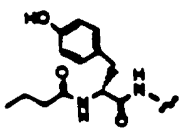
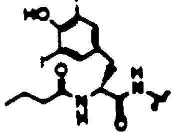
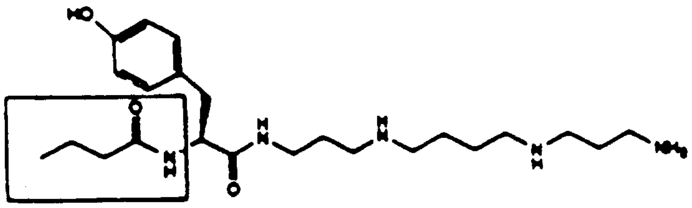
cmpd.		Name	Relative Potency Q	N	Reference
2a.		Br_2	2.0		3), 4)
3a.		F	0.6		3), 4)
4a.		NO_2	1.2		3), 4)
5a.		D	0.5		3), 4)
6a.		I_2-D	0.9		3), 4)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

C 

IC_{50} (rel. to PhTX343)
QUIS-R, muscle contraction: 1.0 [2.30 x 10^{-5}M]
nAch-R, [^{3}H]H_{12}-HTX binding: 1.0 [2.60 x 10^{-6}M]

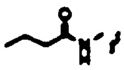
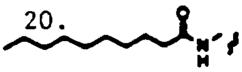
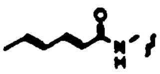
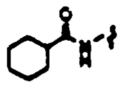
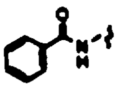
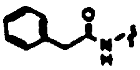
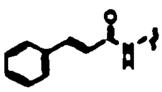
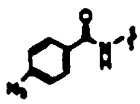
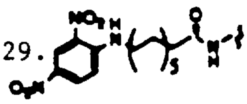
cmpd.	Name	Relative Potency Q	N	Reference
1. 	PhTX	1.0	1.0	1),7),8),9)
18. 	C_2	0.7		1),5),8),9)
19. 	C_7	2.7	6.5	1),3),4),5),8),9)
20. 	C_{10}	16.0	10.4	1),3),4),5),8),9)
21. 	Diene	14.0		1),3),4),8),9)
22. 	CH	2.5		1),3),4),5),8),9)
23. 	Ph	8.2		1),3),4),5),8),9)
24. 	Bn	0.5	18.4	1),3),4),5),8),9)
25. 	Cin	12.0	15.3	1),3),4),5),8),9)
26. 	N_3Ph	5.5	12.4	1),3),4),5),8),9)
27. 	Diazo	0.1		1),8)
28. 	DNP4	0.4	5.4	1),3),4),5),8),9)
29. 	DNP12	1.6	5.8	1), 3), 4),8),9)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

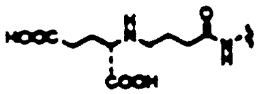
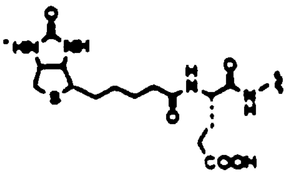
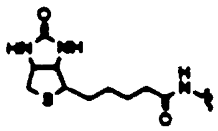
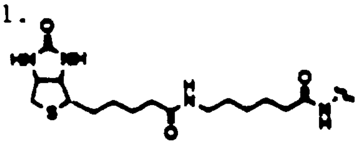
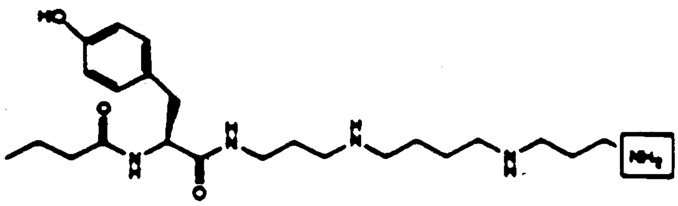
cmpd.	Name	Relative Potency Q	N	Reference
7a. 		0.15		3)
8a. 		<0.1		3)
9a. 		<0.05		3)
30. 	B-1	insol.		1),5),8)
31. 	B-2	insol.		1),8)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS



D

IC_{50} (rel. to PhTX343)
QUIS-R, muscle contraction: 1.0 [2.30 x 10^{-5}M]
nAch-R, [^{3}H]H_{12}-HTX binding: 1.0 [2.60 x 10^{-4}M]

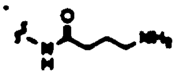
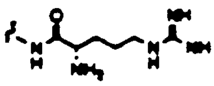
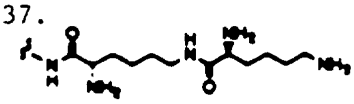
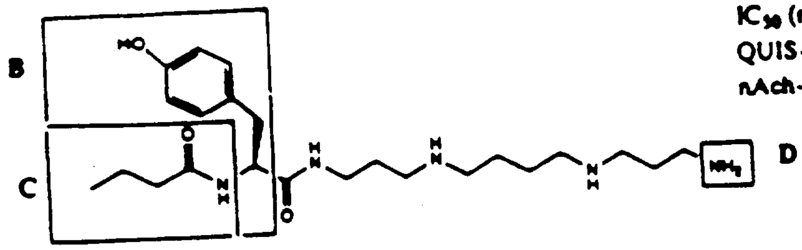
cmpd.		Name	Relative Potency Q	N	Reference
1.		PhTX-343	1.0	1.0	1),7),8),9)
32.		N-Ac	0.08		1),8),9)
33.		Gly	0.2	7.0	1),4),5),8),9)
34.		GABA	0.3		1),3),4),5),8),9)
35.		Lys	1.8	7.2	1),3),4),5),8),9)
36.		Arg	3.7	14.4	1),3),4),5),8),9)
37.		Lys_2	2.1	9.6	1),3),4),5),8),9)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS



IC_{50} (rel. to PhTX343)
QUIS-R, muscle contraction: 1.0 [2.30×10^{-5}M]
nAch-R, [^{3}H]H_{12}-HTX binding: 1.0 [2.60×10^{-6}M]

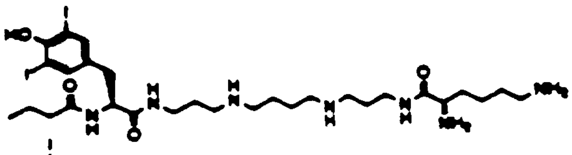
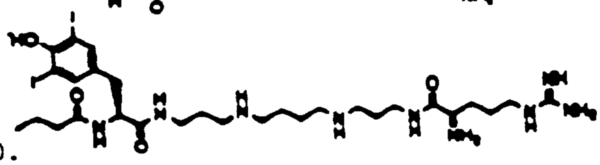
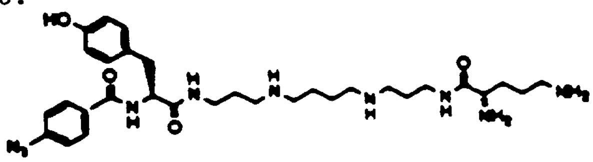
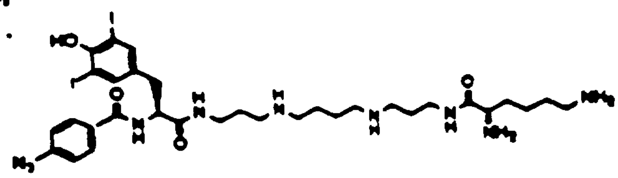
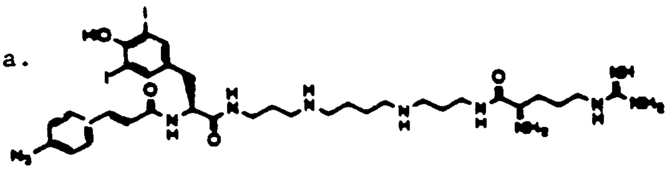
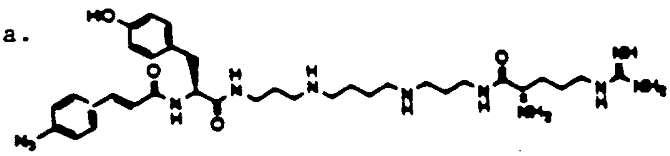
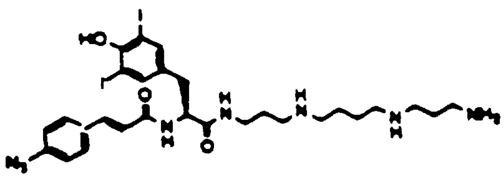
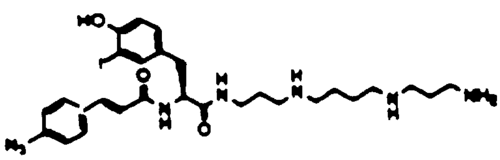
cmpd.		Name	Relative Potency Q	N	Reference
38.		I_2-343-Lys	14	8.1	1),5),8),9)
39		I_2-343-Arg	33	13.7	1),5),8),9)
40.		N_3Ph-343-Lys	7	6.5	1),5),8),9)
41.		N_3Ph-I_2-343-Lys	13	3.2	1),5),8),9)
10a.		N_3Cin-I_2-343-Arg	9.0		3)
11a.		N_3Cin-343-Arg	2.8		3)
12a.		N_3Cin-I_2-343	4		3)
13a.		N_3Cin-I-343	10		3)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

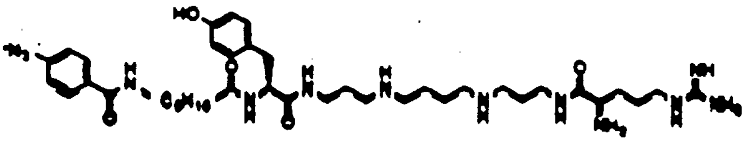
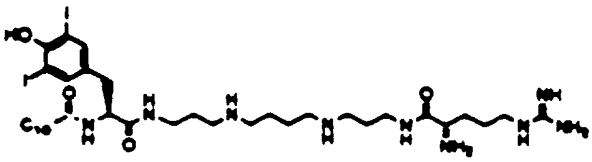
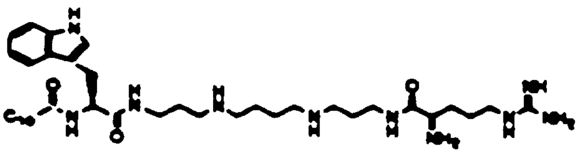
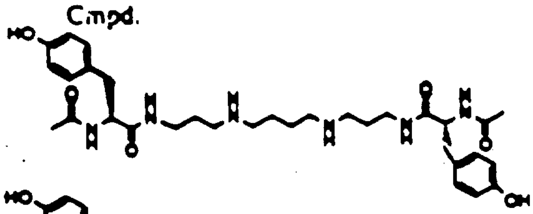
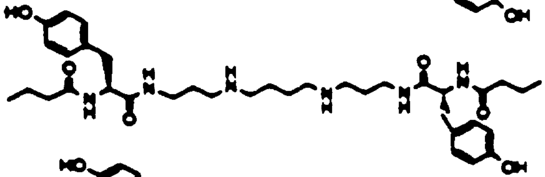
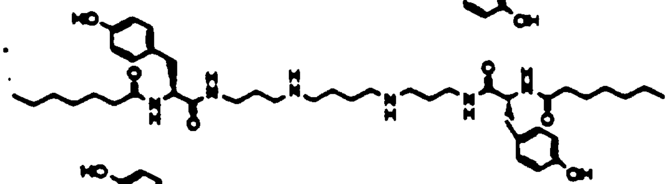
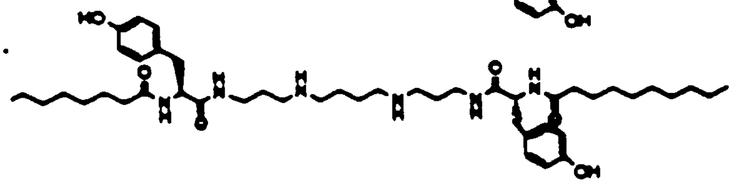
14a.		$N_3Ph-C_8-343-Arg$	2.8	3)
15a.		$C_{10}-I_2-343-Arg$	5.0	3)
16a.		C_{10}-Trp-343-Arg	10.0	3)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

Bisproduct

Cmpd.		Name	Relative Potency Q	Reference
42.		C_2-C_2		5),9)
43.		C_4-C_4	0.38	9)
44.		C_7-C_7	7.9	9)
45.		C_{10}-C_{10}	7.2	9)

Spermine Analogs

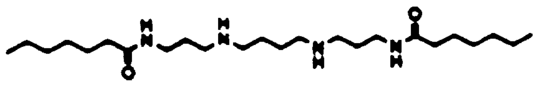
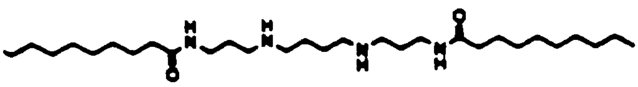
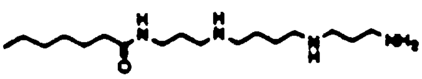
Cmpd.		Name	Relative Potency Q	Reference
50.		Bis-C_4	0.1	5),8)
51.		Bis-C_7	agonist	5),8)
52.		Bis-C_{10}	1.0	5),8)
47.		C_4	agonist	5),8)
48.		C_7	0.27	5),8)
49.		C_{10}	2.3	5),8)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

Hybrid analogs of argiotoxin-636

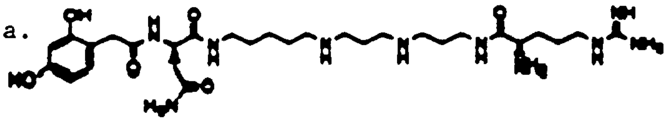
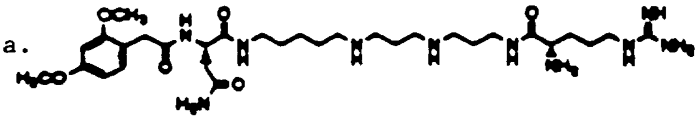
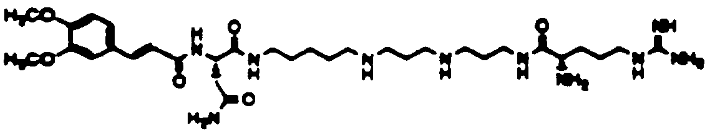
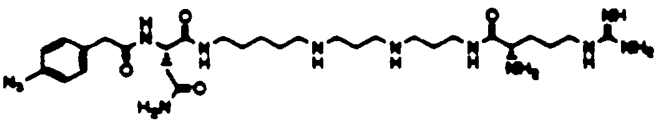
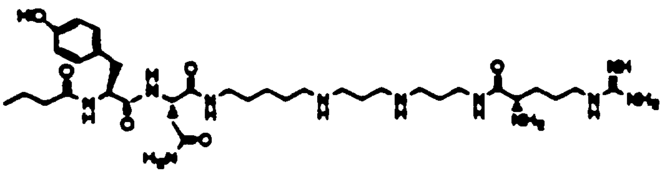
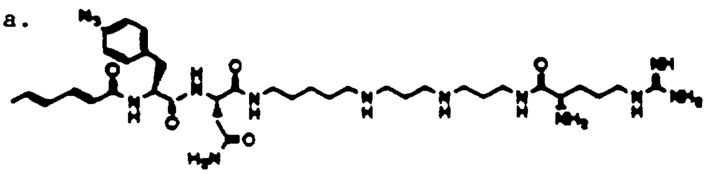
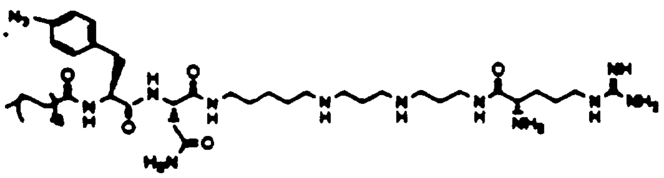
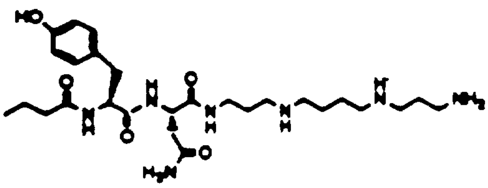
cmpd.		Name	Relative Potency Q	N	Reference
17a.		ArgTX-533	7		2)
18a.		OMe	8		2)
19a.			15		2)
20a.			6		2)
21a.			6		2)
22a.			8		2)
23a.			16		2)
24a.			0.2		2)

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

Unpublished data

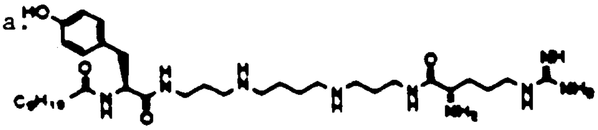
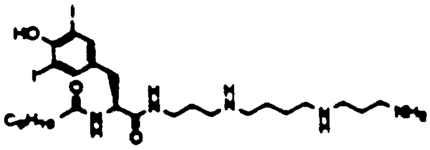
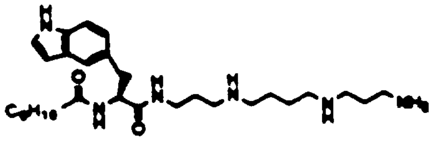
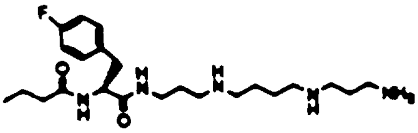
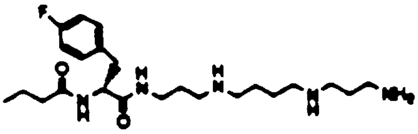
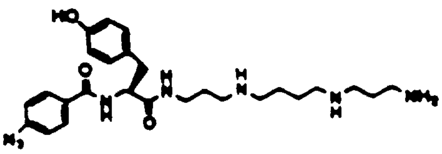
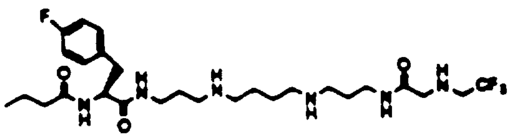
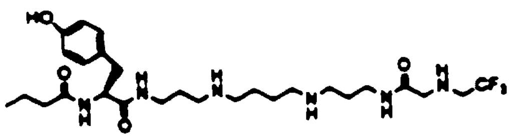
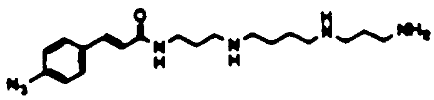
Cpmd.	Name	Relative Potency Q
25a. 	C_{10}-Arg	5.0
26a. 	C_{10}-I_2	7.0
27a. 	C_{10}-Trp	8.0
28a. 		1
29a. 		1
30a. 		
31a. 		
32a. 		
33a. 		0.6

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

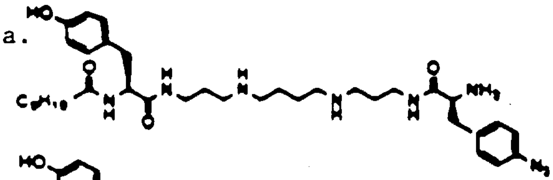
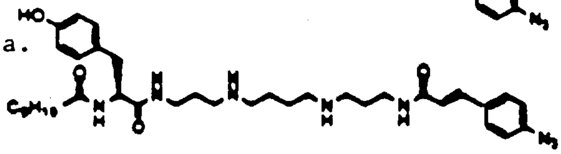
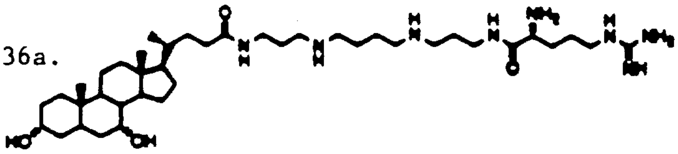
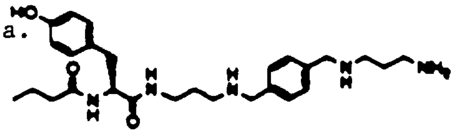
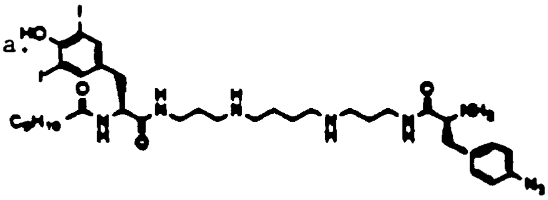
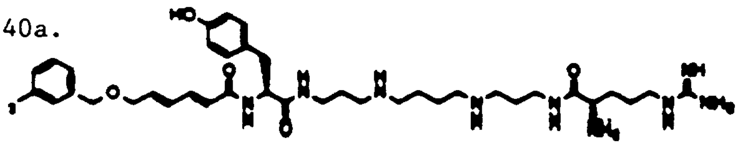
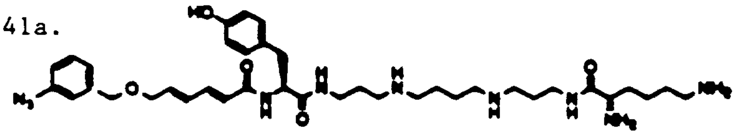
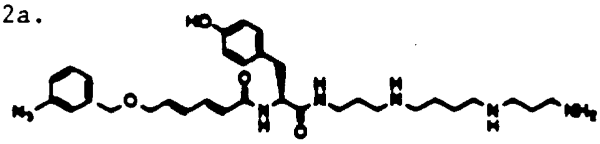
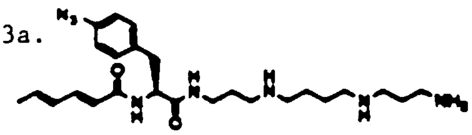
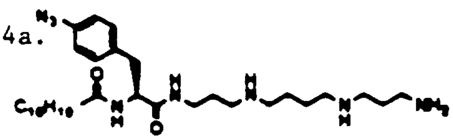
34a.			0.4
35a.		C_{10}-343-N_3Cin	0.55
36a.			1.5
37a.			
38a.			
39a.			>1-2
40a.			15
41a.			
42a.			1
43a.		Diene-N_3Ph-343	2
44a.		C_{10}-N_3Ph-343	

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

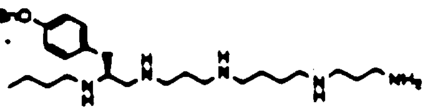
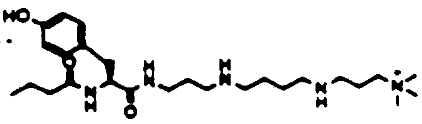
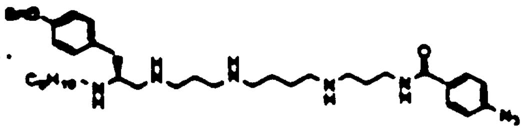
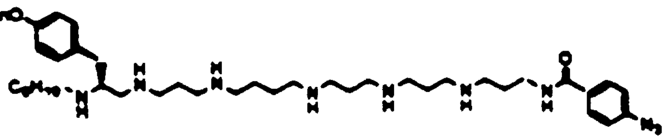
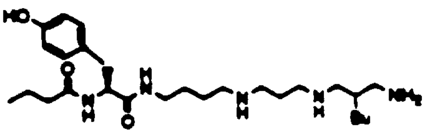
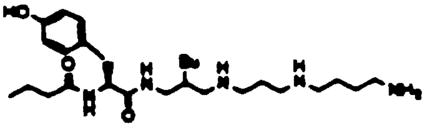
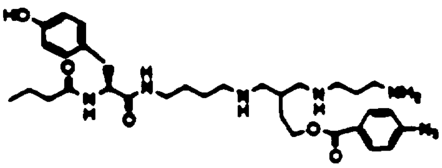
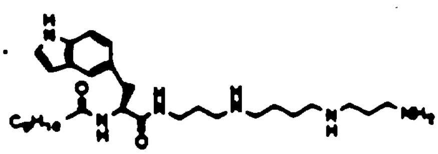
45a.			5.0
46a.			0.7
47a.			
48a.			
49a.			0.5
50a.			0.5
51a.			0.1
52a.		C10-Trp	8.0

TABLE 8. RELATIVE POTENCIES AT N AND Q RECEPTORS

REFERENCE:

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TABLE 9. ADDITIONALLY SYNTHESIZED PHILANTHOTOXIN ANALOGS AND THEIR RELATIVE ACTIVITIES

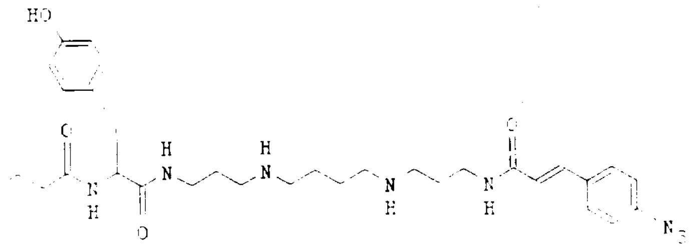
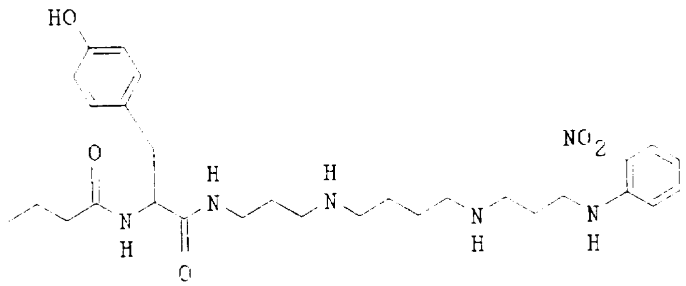
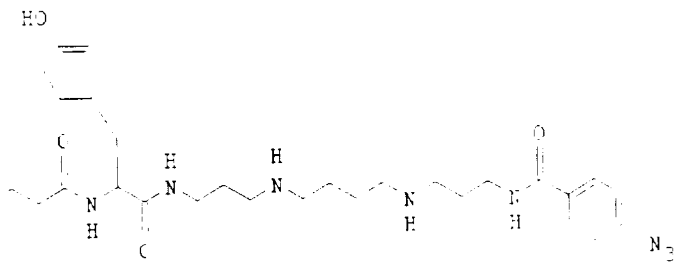
Compound and Name	Activity (PhTX-343 = 1.0)
1b. PhTX-N3-Cinn 	0.55
2b. Caged-PhTX 	4.3
3b. PhTX-343-benzoyl-N3 	0.77

TABLE 9. ADDITIONALLY SYNTHESIZED PHILANTHOTOXIN ANALOGS AND THEIR RELATIVE ACTIVITIES

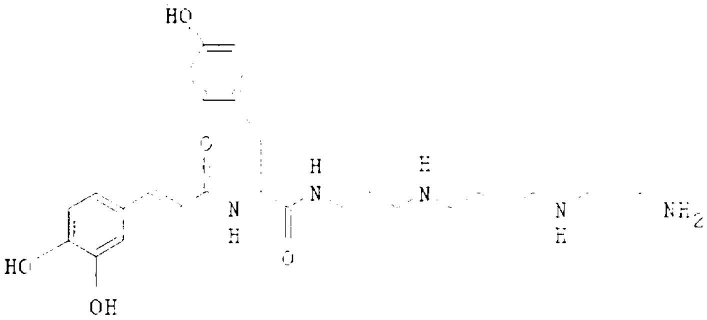
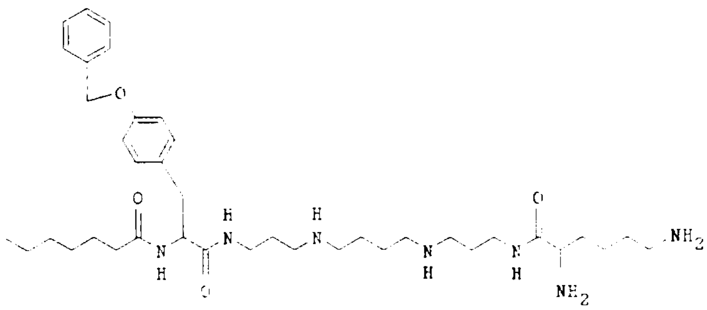
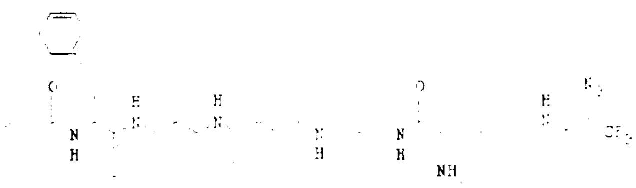
Compound and Name	Activity (PhTX-343 = 1.0)
4b. Cin-PhTX-343 	16.4
5b. C7-BnO-PhTX-343-Lys 	1.8
6b. C7-PhTX-Lys-N2-CF3-amide 	

TABLE 9. ADDITIONALLY SYNTHESIZED PHILANTHOTOXIN ANALOGS AND THEIR RELATIVE ACTIVITIES

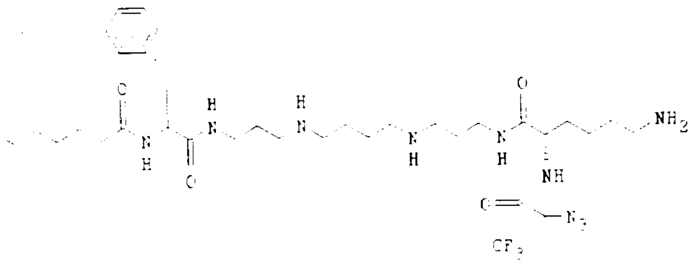
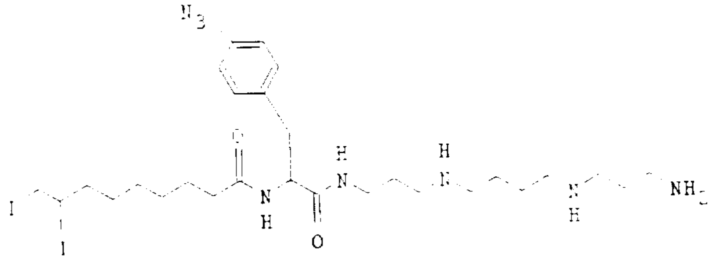
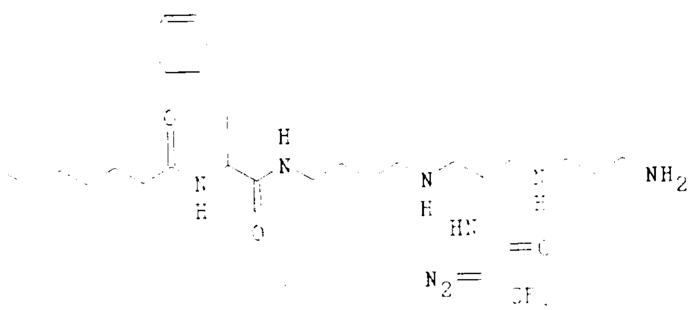
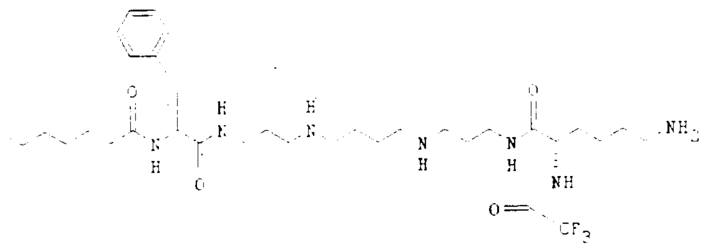
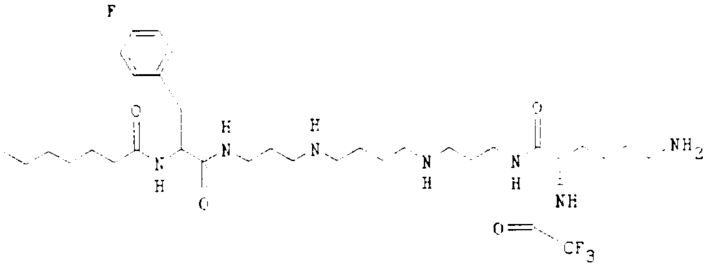
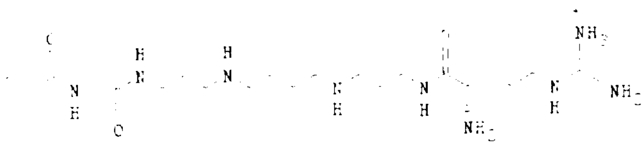
Compound and Name	Activity (PhTX-343 = 1.0)
7b. C7-Phe-343-Lys(a-CF3CN2CO) 	<< 0.33
8b. C7-N3-PhTX-343 	1.5
9b. C7-Phe-433-(N2-CF3)2 	

TABLE 9. ADDITIONALLY SYNTHESIZED PHILANTHOTOXIN ANALOGS AND THEIR RELATIVE ACTIVITIES

Compound and Name	Activity (PhTX-343 = 1.0)
10b. C7-Phe-343-Lys(a-CF3CO) 	< 0.5
11b. C7-Phe(p-F)-343-Lys(a-CF3CO) 	1.0
12b. Diene-N3-Phe-PhTX-343-Arg 	8.8

Discussion

In assessing the results of these studies it is important to bear in mind the limitations of the assay that was employed. Some of these problems have been alluded to under Materials and Methods. Although care was taken to show that all of the compounds identified as antagonists inhibited the muscle response to L-glutamate as well as that resulting from motor nerve stimulation, it would not be correct to assume that PhTX-343 and its analogs are simply non-competitive antagonists of the postsynaptic receptors unguis muscle. Reduction of twitch contraction amplitude by most of the analogs was enhanced when the nerve stimulation frequency was increased. This suggest that inhibition is use-dependent and lends support to the view that they are non-competitive antagonists of QUIS-R. However, the assay does not unequivocally differentiate between presynaptic and postsynaptic sites of action, both of which could, in principle, be influenced by stimulation frequency. Some of the compounds, particularly those in which the aromatic end of PhTX was made more hydrophobic, initially potentiated the neurally-evoked twitch contraction. This could have arisen from enhancement of transmitter uptake (34), but it is equally possible that the toxins interact with a site or sites distinct from those involved in their antagonism of QUIS-R (38).

This structure-activity study has produced several molecules which are most potent non-competitive antagonists of locust muscle QUIS-R than the natural philanthotoxin, PhTX-433. Hydrophobicity of the aromatic moiety is an important potency determinant and this is also true for the butyryl side chain, although in the latter case there is clear evidence that steric factors are also significant. Perhaps the role of these groups is to anchor the toxin in a hydrophobic pocket of the receptor channel to support the binding of the polyamine moiety to the channel wall (36). The increased potency seen in compound **8** is less easily reconciled with this model, although one might anticipate, perhaps, the presence of additional pockets of hydrophobicity in the region of the channel to which the polyamine moiety binds. If one were to seek a generalization from the results of these studies it would be the identification of a molecule which embraces the structures of the four moieties which produced the most potent ligands. This molecule, decanoyl-tryptophan-butylspermine-arginine is currently being synthesized.

PhTX-343 binds to the QUIS-R channel, possible at a site located within its selectively filter (35, 36). Other studies in our laboratories have shown that antagonism of locust muscle QUIS-R by PhTX-343 and compound **13**, the diiodo analog, is voltage-dependent, as one might expect for open channel blockers carrying a net positive charge. Interestingly, at high membrane potentials (above about -100 mV) the block is relieved, presumably as the toxin is dissociated from its binding site in the channel. Ashford, et al. (39) demonstrated a similar phenomenon when studying non-competitive antagonism of locust muscle QUIS-R by chlorisondamine, and Magazanik, et al. (40) have recently shown that high membrane potentials relieve channel block of insect muscle QUIS-R cause by the polyamine amide spider toxin, argiotoxin-636. Cation-selective membrane channels are generally envisaged as aqueous pores lined by fixed negative charges. In the case of the amphibian nicotinic acetylcholine receptor the latter are thought to be concentrated in three clusters in the vicinity of the selectively filter (41). Although we do not yet have equivalent information on the

QUIS-R channel, there is clear evidence from our studies that an increase in the number of protonated groups on the PhTX- blocks the QUIS-R channel by binding to the channel wall, then it seems likely that this results from interaction between the protonated groups in the toxin molecule and fixed negative charges on the wall of the channel. The relative disposition and number of protonated groups on the toxin seems to be important in determining potency, which appears to be maximized for a given number of protonated groups when these have a constant spacing of three methylenes on their equivalents.

It may be unwise to conclude from these and other data on the polyamine amide toxins that they interact exclusively with cation-selective channels of specific transmitter receptors (6, 8, 35). The polyamine, spermine, which is also a non-competitive antagonist of locust muscle QUIS-R (33), is known to stabilize membranes by cross-linking phospholipids (42). PhTX-343 and analogs might also bind thereby reducing membrane fluidity. The increased potency seen with increasing hydrophobicity of the PhTX-343 analogs could arise through the closer association of toxin molecule with the cell membrane lipid. The number and disposition of positive charges on the toxin relative to those on the membrane phospholipids would also play a role in determining the affinity of the toxin. In fact, the presence of proteins could, in principle, enable these compounds to bridge across the lipid-protein interface. If membrane stabilization reduces the capacity of receptor molecules to undergo conformational changed for required for channel gating, then one could envisage non-competitive antagonism of QUIS-R arising through relatively non-specific binding of PhTX-343 to membrane phospholipids, but it is difficult to understand how this model could account for the open channel block and the striking voltage dependencies associated with PhTX-343 antagonism.

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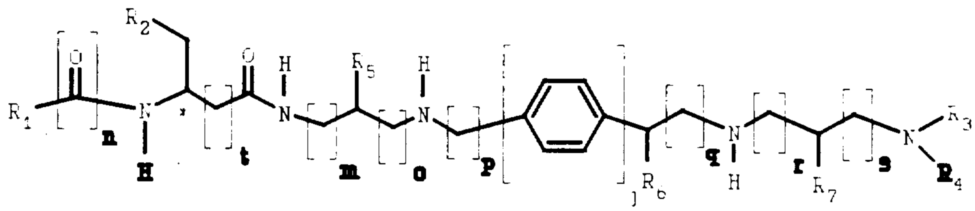
CLAIMS

1. A compound having the structure:

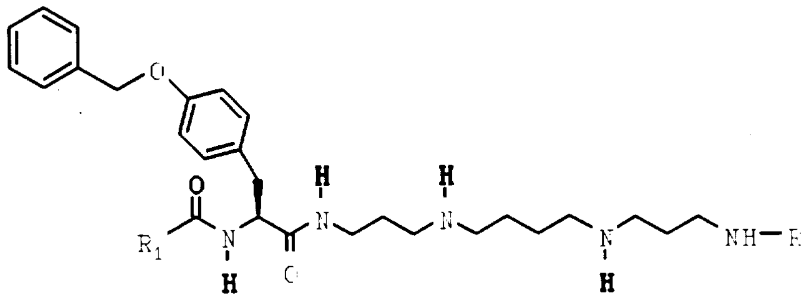


wherein R_1 is a saturated or unsaturated linear or branched chain alkyl group, or a cholestanyl group; wherein R_2 is a 2-indolyl, 3-indolyl, 4-indolyl, 5-indolyl, 4-hydroxyphenyl, 4-(arylalkyloxy)phenyl, 3,4-dihalophenyl, 4-hydroxy-3,5-dihalophenyl, 4-azidophenyl or 4-halophenyl group; wherein R_3 is H, a linear or branched chain alkyl or alkenyl group, or a phenyl, 2-azidophenyl, 3-azidophenyl, 4-azidophenyl group, or an a alkenylacyl, 3-amino-3-butylpropyl, N-[N-(N-{4-azidobenzoyl}aminopropyl)aminopropyl], cis- or trans-cinnamyl, 2-amino-2-[(4'-azidophenyl)acetyl], (trifluoromethyl)aminoacetyl or D- or L-arginyl group bonded through the α-carbonyl moiety thereof; R_4 is H, or a linear or branched chain alkyl group; wherein R_5, R_6 and R_7 are independently the same or different and are H, a linear or branched chain alkyl group, an aryl group or an arylalkyl group; wherein n, j and t are each 0 or 1; wherein m, o, p, q, r and s are independently the same or different and are 0, 1 or 2; wherein r+s and m+o are each equal to 2; wherein, if j is 0, p+q is 2; wherein, if j is 1, then p is 1, q is 0 and R_6 is H; and wherein * denotes a D or L configuration.

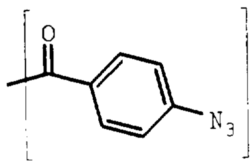
2. The compound of claim 1 wherein j is 0 or 1.

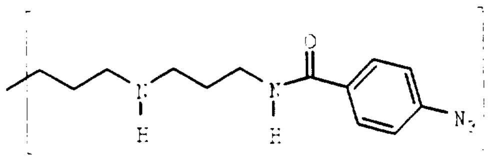
3. The compound of claim 2 wherein k is 0 or 1.

4. The compound of claim 1 having the structure:



wherein R is selected from a group consisting of H, linear alkyl, linear acyl, arylalkyl, phenyl,

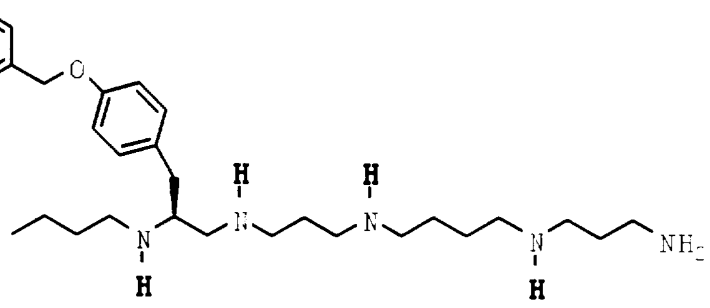
alkylacyl,  and

 ;

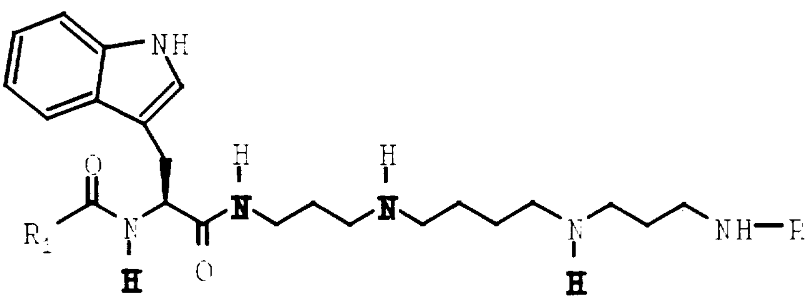
and wherein R_1 is a C_9 or C_{10} linear alkyl group.

5. The compound of claim 4 wherein R is H and R_1 is C_9H_{19}.

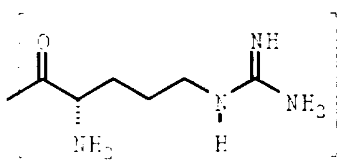
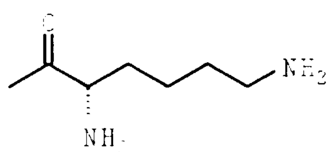
6. The compound of claim 4 having the structure:



7. The compound of claim 1 having the structure:

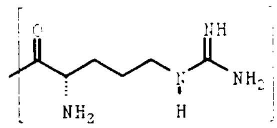


wherein R is selected from a group consisting of H,

 and  ;

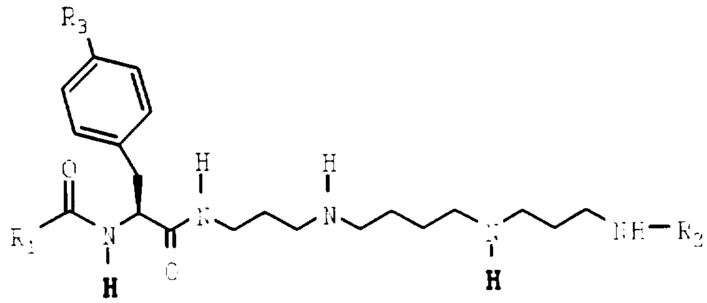
and wherein R_1 is a C_9 or C_{10} linear alkyl group.

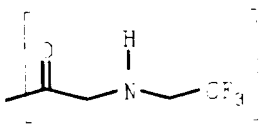
8. The compound of 7 wherein R is H and R_1 is C_9H_{19}.

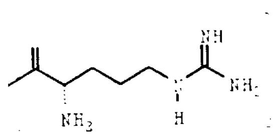
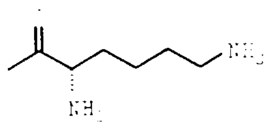
9. The compound of 7 wherein R is 

and R_1 is C_9H_{19}.

10. The compound of claim 1 having the structure:

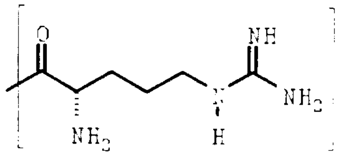


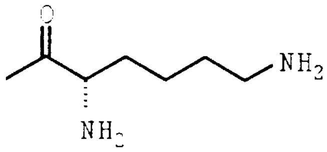
wherein R_1 is a C_9 or C_{10} linear saturated or unsaturated alkyl group; wherein R_2 is selected from a group consisting of H, H_2^+, ,

 and ; and

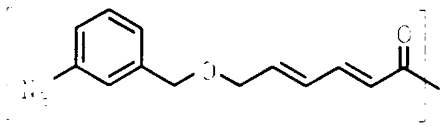
wherein R_3 is selected from a group consisting of F, OH and N_3.

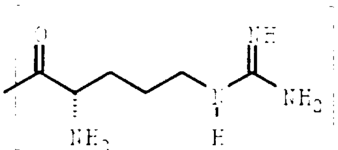
11. The compound of claim 10, wherein R_1 is a C_9H_{19};

wherein R_2 is  or

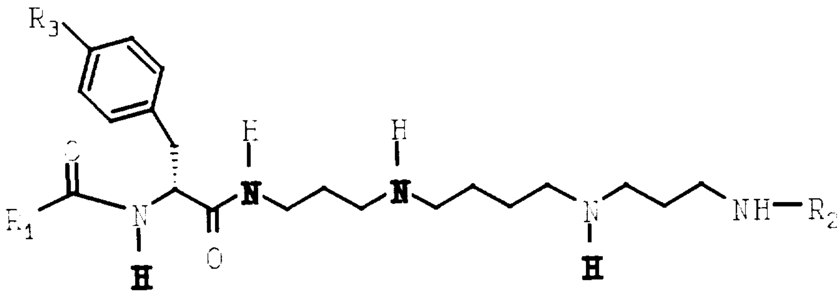
 ; and wherein R_3 is OH.

12. The compound of claim 10, wherein R_1 is

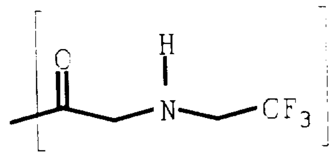
 ; wherein R_2 is

 ; and wherein R_3 is OH.

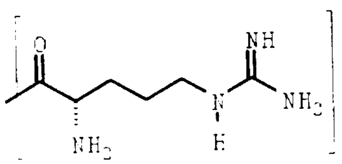
13. The compound of claim 1 having the structure:



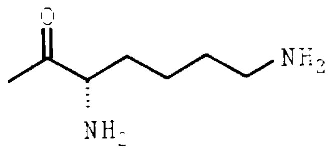
wherein R_1 is a C_9 or C_{10} linear saturated or unsaturated alkyl group; wherein R_2 is selected from a group consisting of H, H_2^+,



,



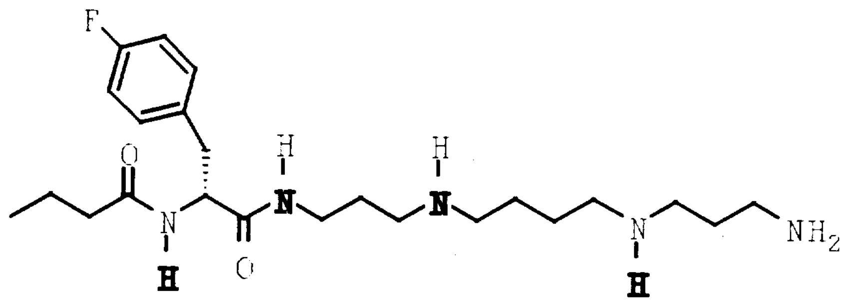
and



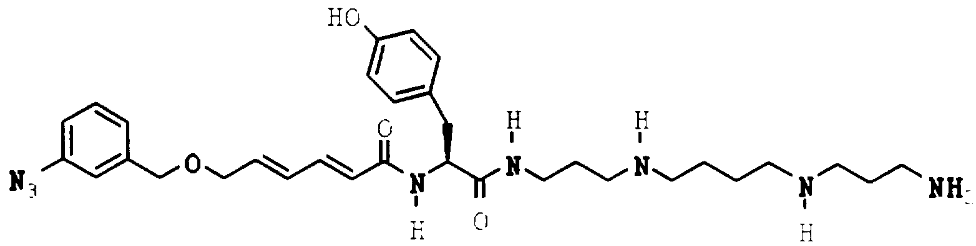
;

and wherein R_3 is selected from a group consisting of F, OH and N_3.

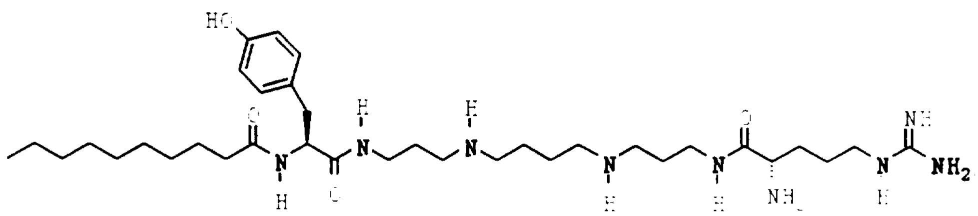
14. The compound of claim 1 having the structure:



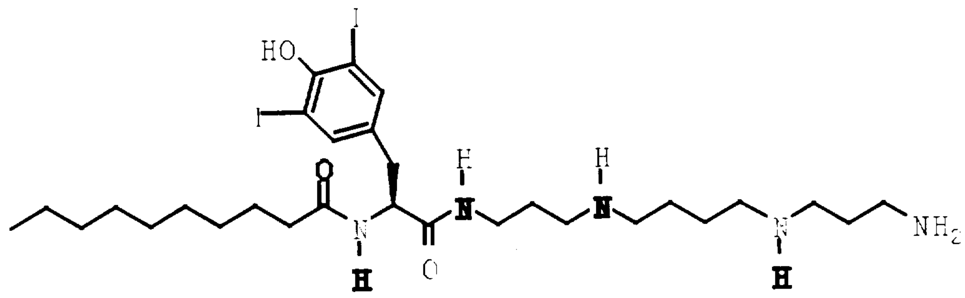
15. The compound of claim 1 having the structure:



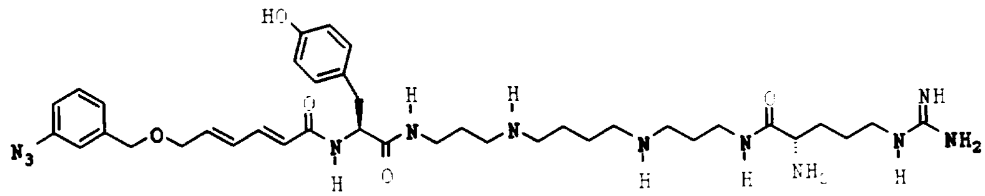
16. The compound of claim 1 having the structure:



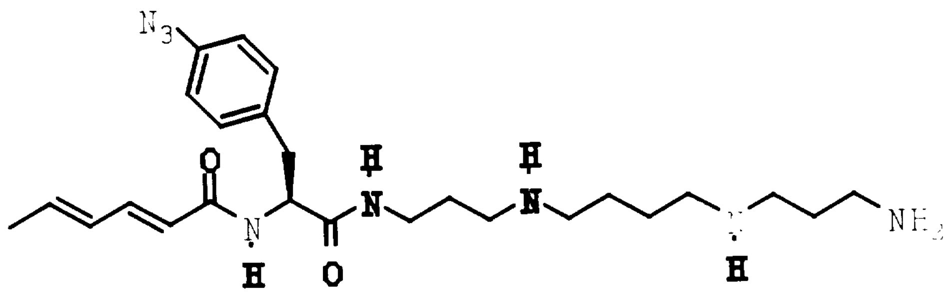
17. The compound of claim 1 having the structure:



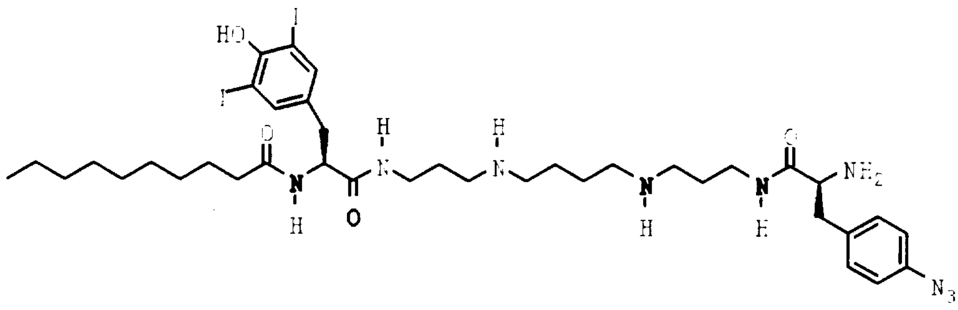
18. The compound of claim 1 having the structure:



19. The compound of claim 1 having the structure:



20. The compound of claim 1 having the structure:



21. A radioactively labeled compound of claim 1.

22. A pharmaceutical composition which comprises an effective amount of the compound of claim 1 and a pharmaceutically acceptable carrier.

23. A composition comprising the compound of claim 1 in admixture with glutamate.

24. A method of inhibiting binding to a glutamate receptor which comprises contacting the receptor with an effective binding-inhibiting amount of the compound of claim 1.

25. A method of claim 24, wherein the glutamate receptor is a quisqualate receptor.

26. A method of claim 24, wherein the glutamate receptor is a N-methyl-D-aspartate receptor.

27. A method of inhibiting binding to a glutamate receptor which comprises contacting the receptor with an effective binding-inhibiting amount of the composition of claim 22.

28. A method of claim 27, wherein the glutamate receptor is a quisqualate receptor.

29. A method of claim 27, wherein the glutamate receptor is a N-methyl-D-aspartate receptor.

30. A method of treating a subject afflicted by a disorder associated with binding of an etiological agent to a glutamate receptor which comprises administering to the subject an amount of the compound of claim 1 effective to inhibit binding of the etiological agent to the receptor.

31. A method of claim 30, wherein the disorder is a neurodegenerative disease or movement disorder.

32. A method of claim 30, wherein the neurodegenerative disease is Huntington's Disease, Parkinson's Disease, or Alzheimer's Disease.

33. A method of claim 30, wherein the movement disorder is epilepsy.

34. A method of treating a subject afflicted by a disorder associated with binding of an etiological agent to a glutamate receptor which comprises administering to the subject an amount of the composition of claim 116 effective to inhibit binding of the etiological agent to the receptor.

35. A method of claim 34, wherein the disorder is a neurodegenerative disease or movement disorder.

36. A method of claim 34, wherein the neurodegenerative disease is Huntington's Disease, Parkinson's

Disease, or Alzheimer's Disease.

37. A method of claim 34, wherein the movement disorder is epilepsy.

38. A method of treating a subject afflicted by a stroke-related disorder associated with excessive binding of glutamate receptors which comprises administering to the subject an amount of the compound of claim 1 effective to inhibit the excessive binding of the glutamate to the receptors.

39. An insecticidal composition which comprises an effective amount of the compound of claim 1 and a suitable carrier.

40. A method of combatting insects which comprises administering to the insects an amount of the composition of claim 39 effective to induce paralysis in the insects.

FIGURE 1

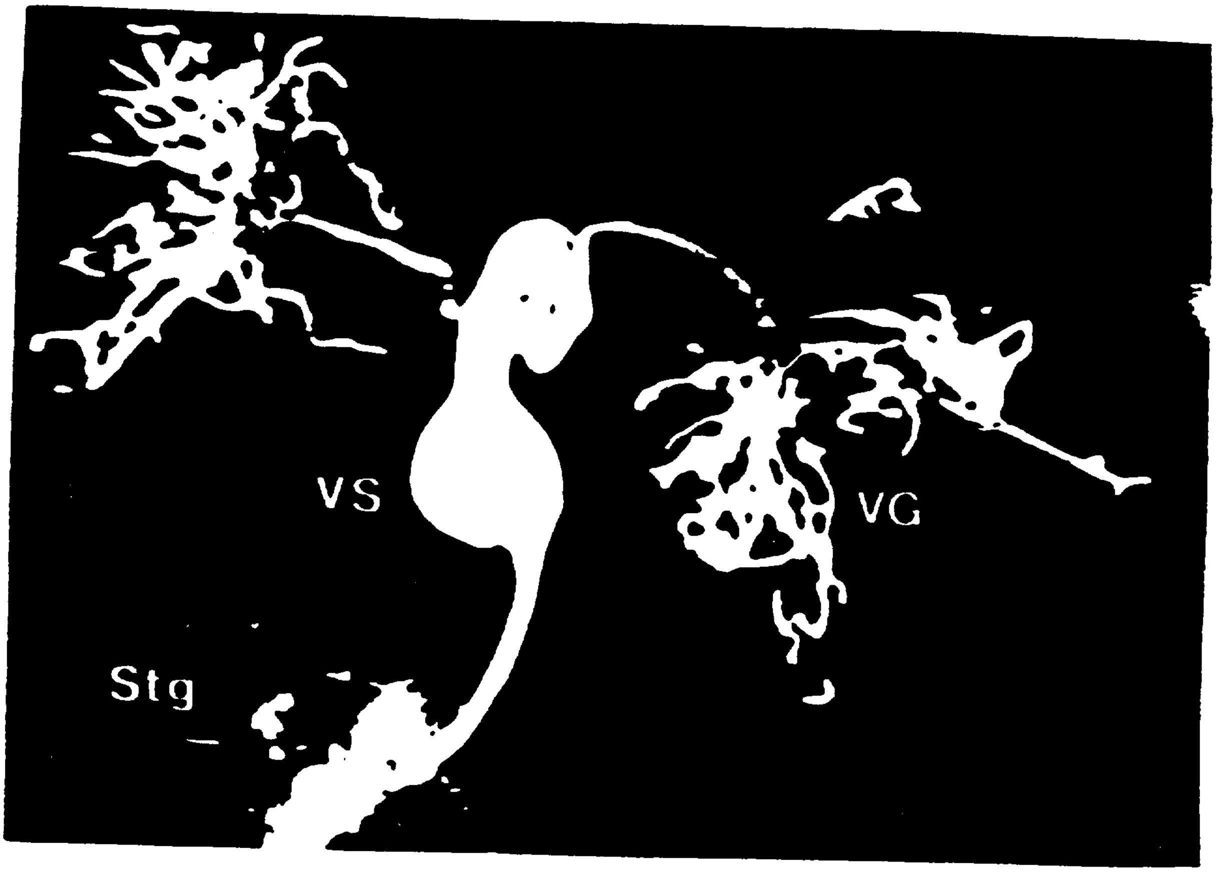


FIGURE 2A

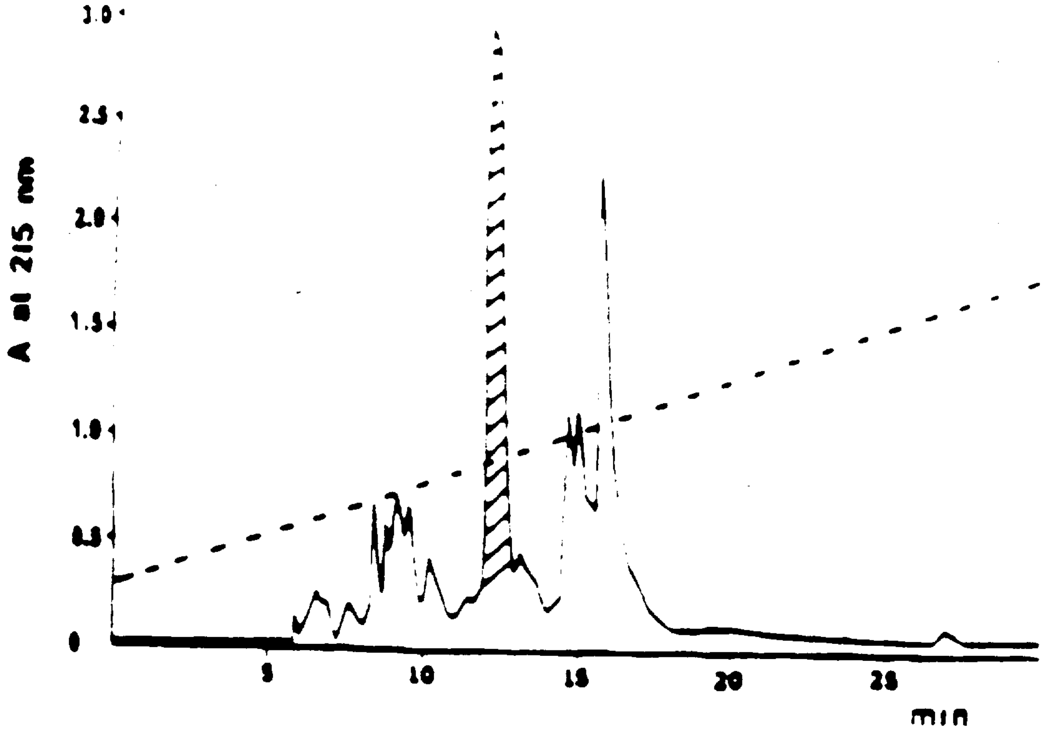


FIGURE 2B

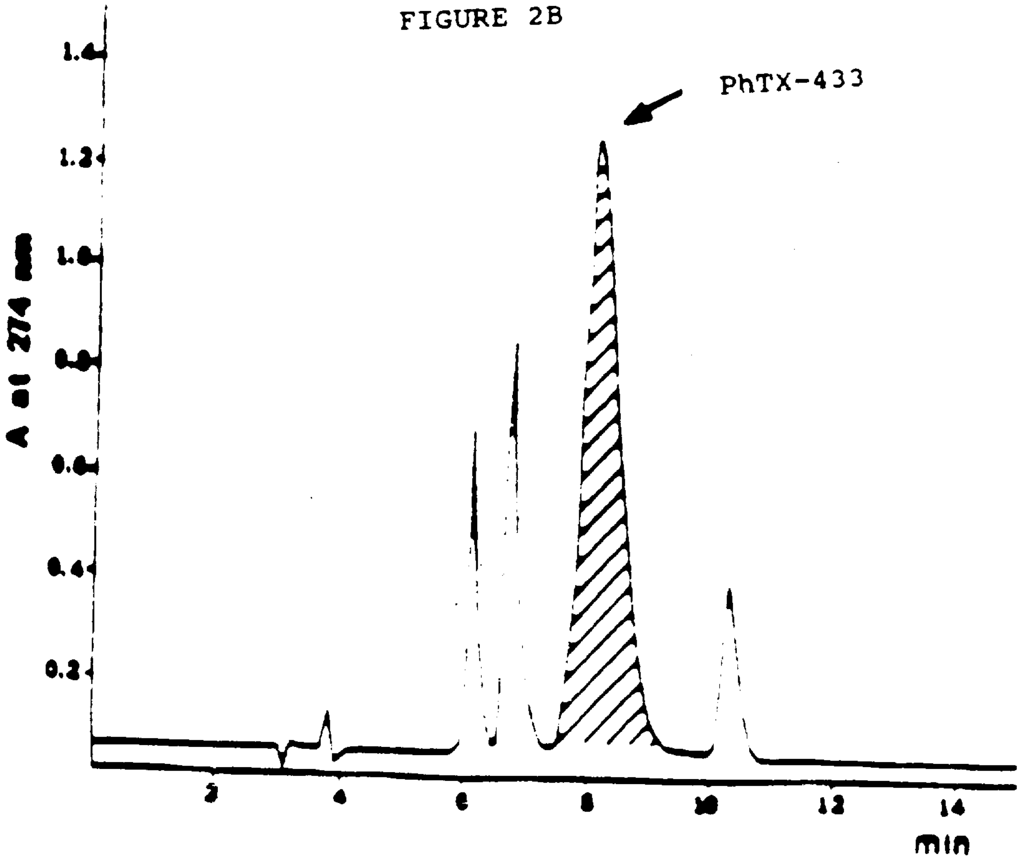
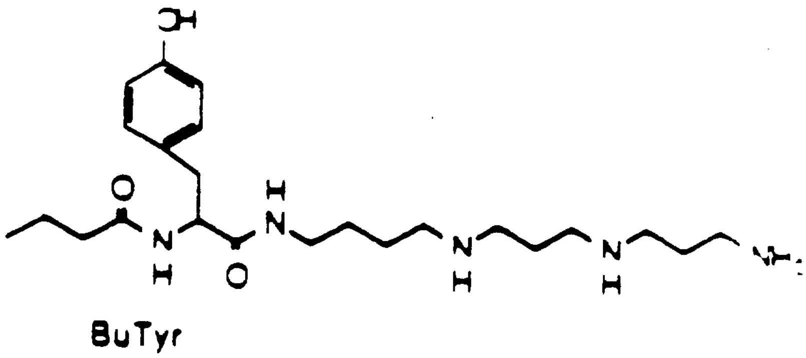
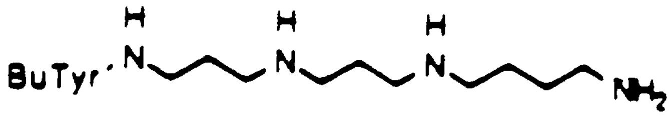


FIGURE 3A

Compound 1 PTX-433



Compound 2 PTX-334



Compound 3 PTX-343

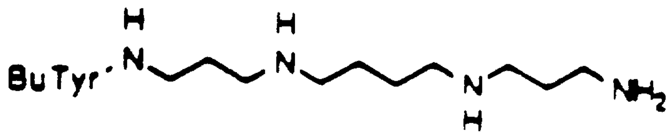
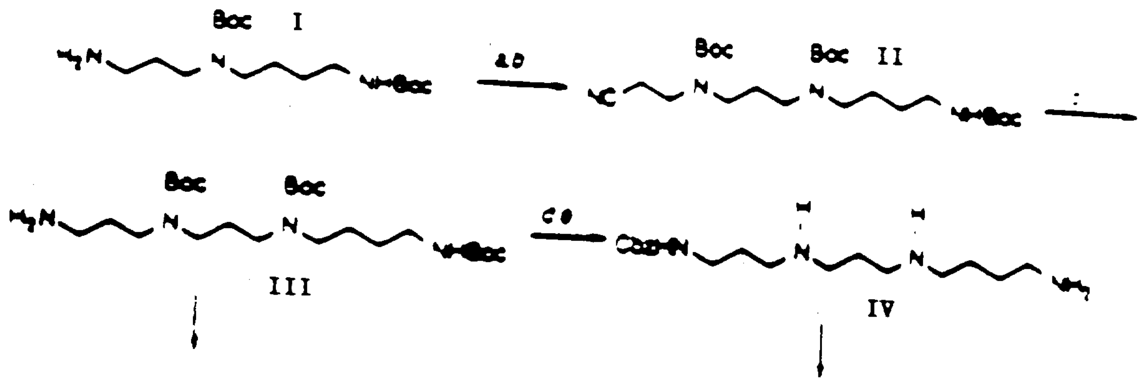


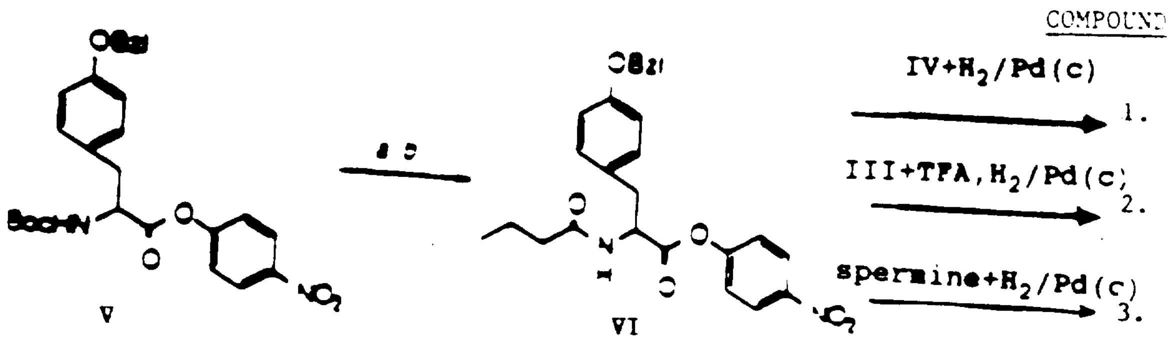
FIGURE 3B



reagents: a. CH_2=CHCN b $(Boc)_2O$ c. $LiAlH_4$.

d CbzCl/Et_3N e. TFA

FIGURE 3C



reagents a TFA b BuC.Et_3N

FIGURE 4A

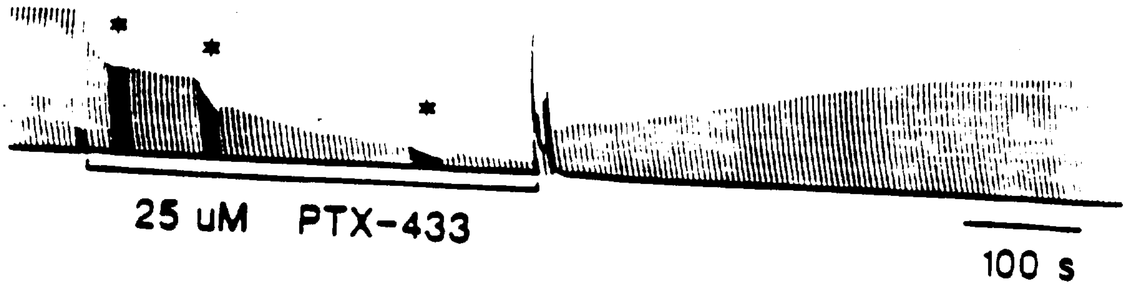


FIGURE 4B

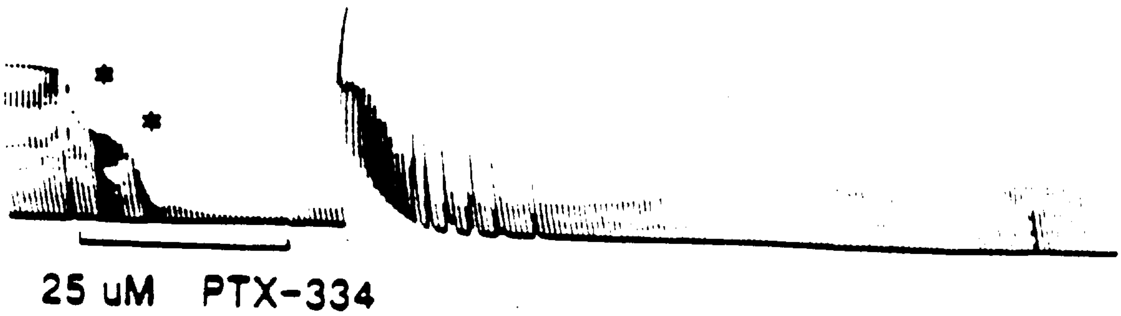


FIGURE 5

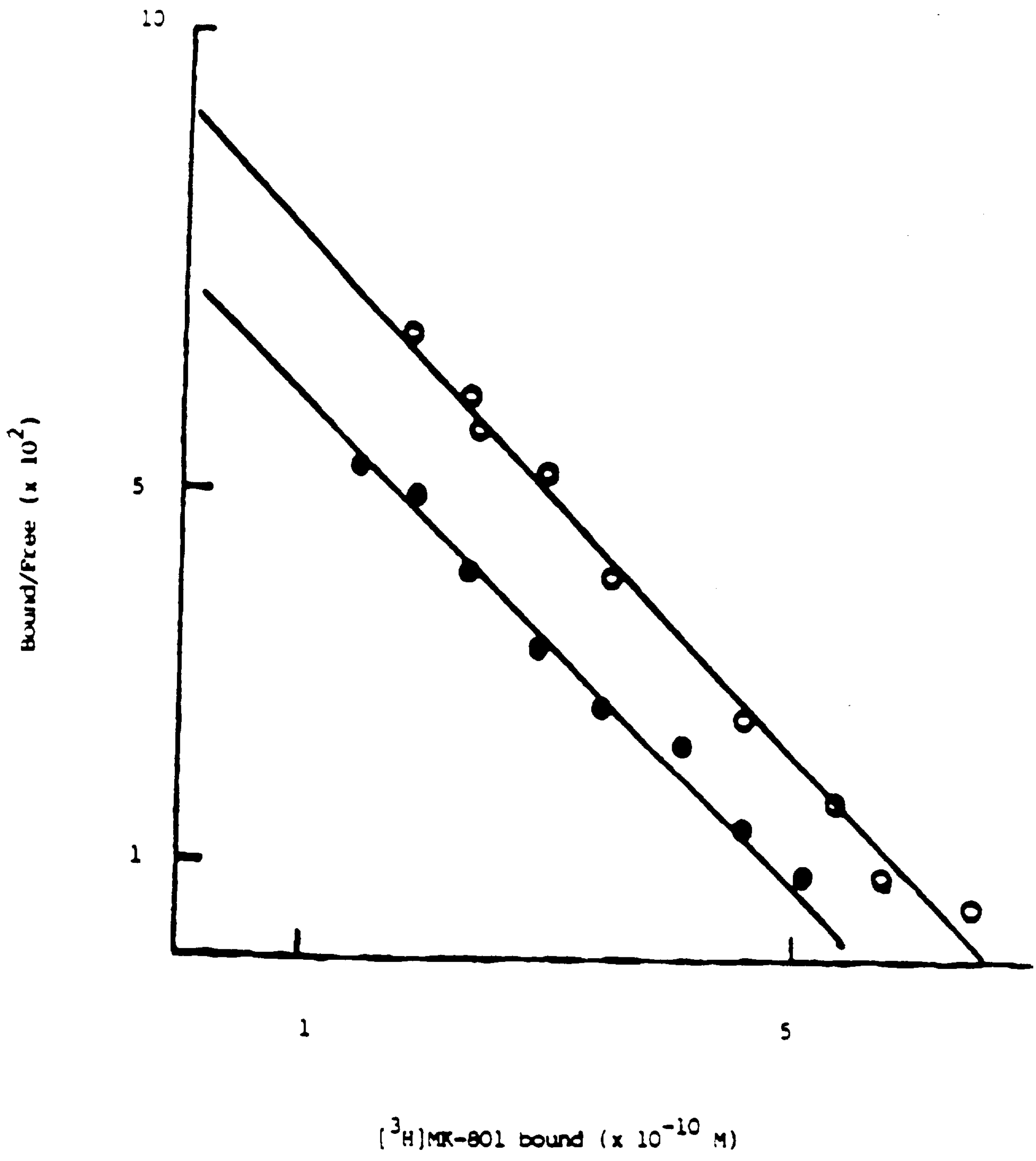


FIGURE 6

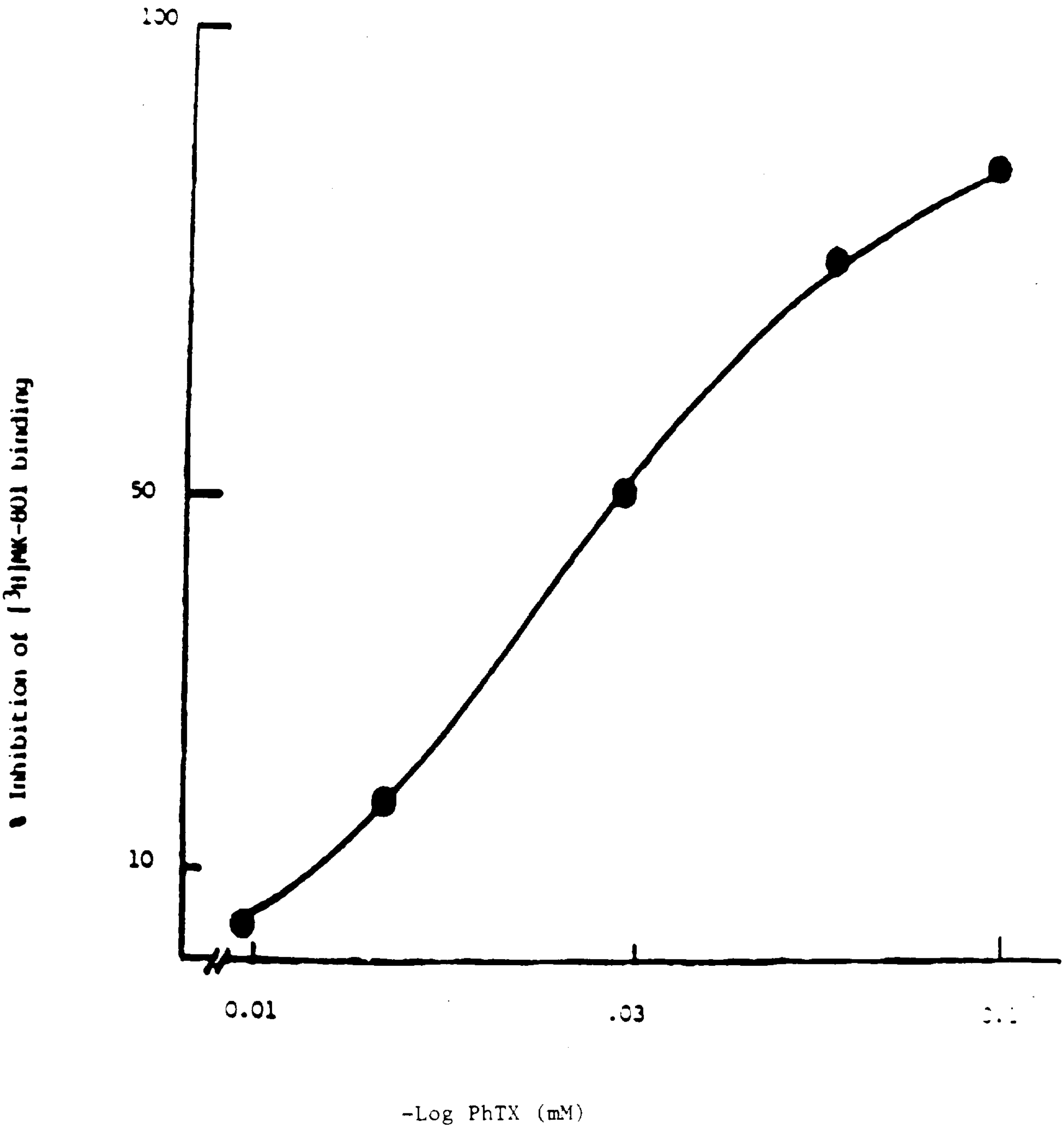
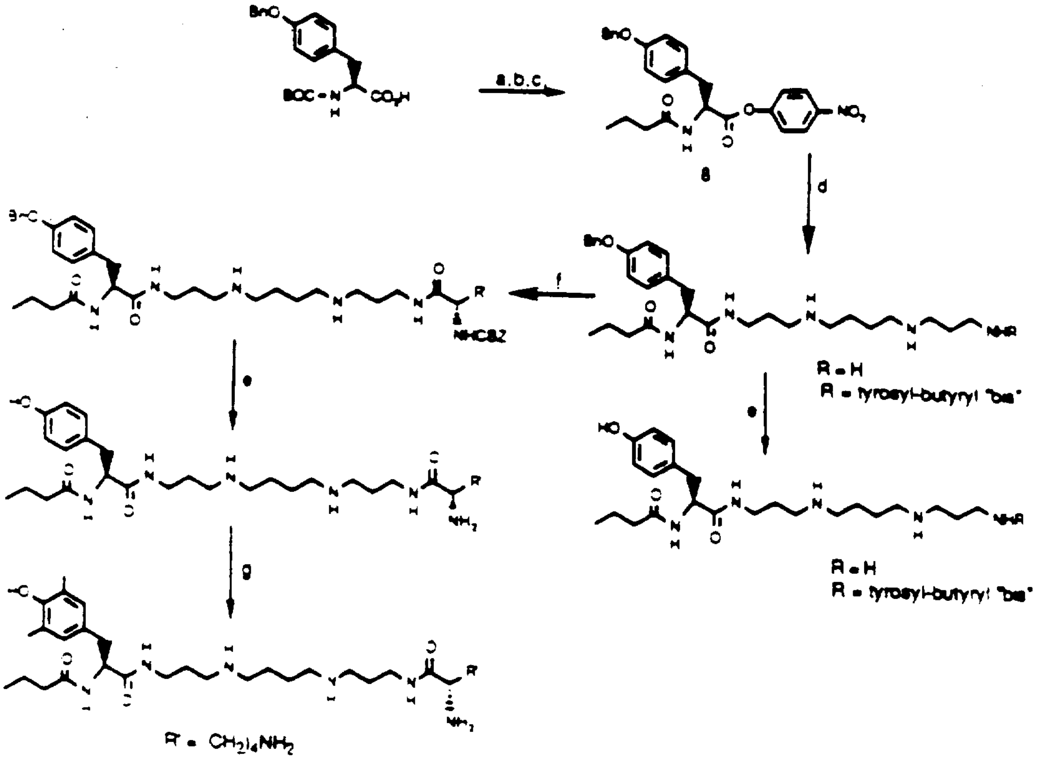


FIGURE 7

Method A



Method B

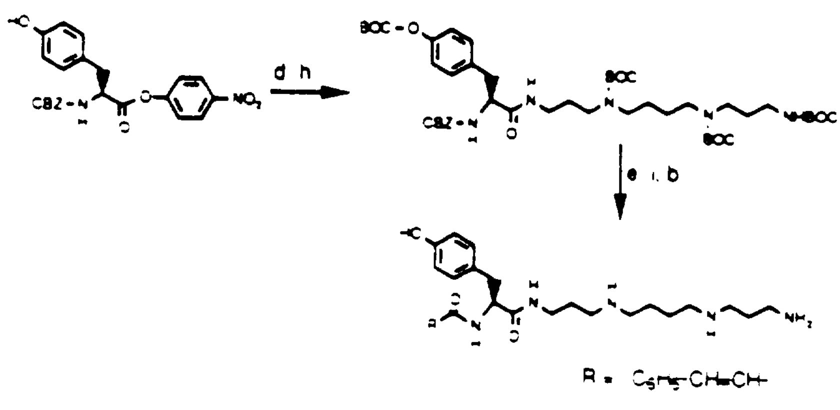


FIGURE 8

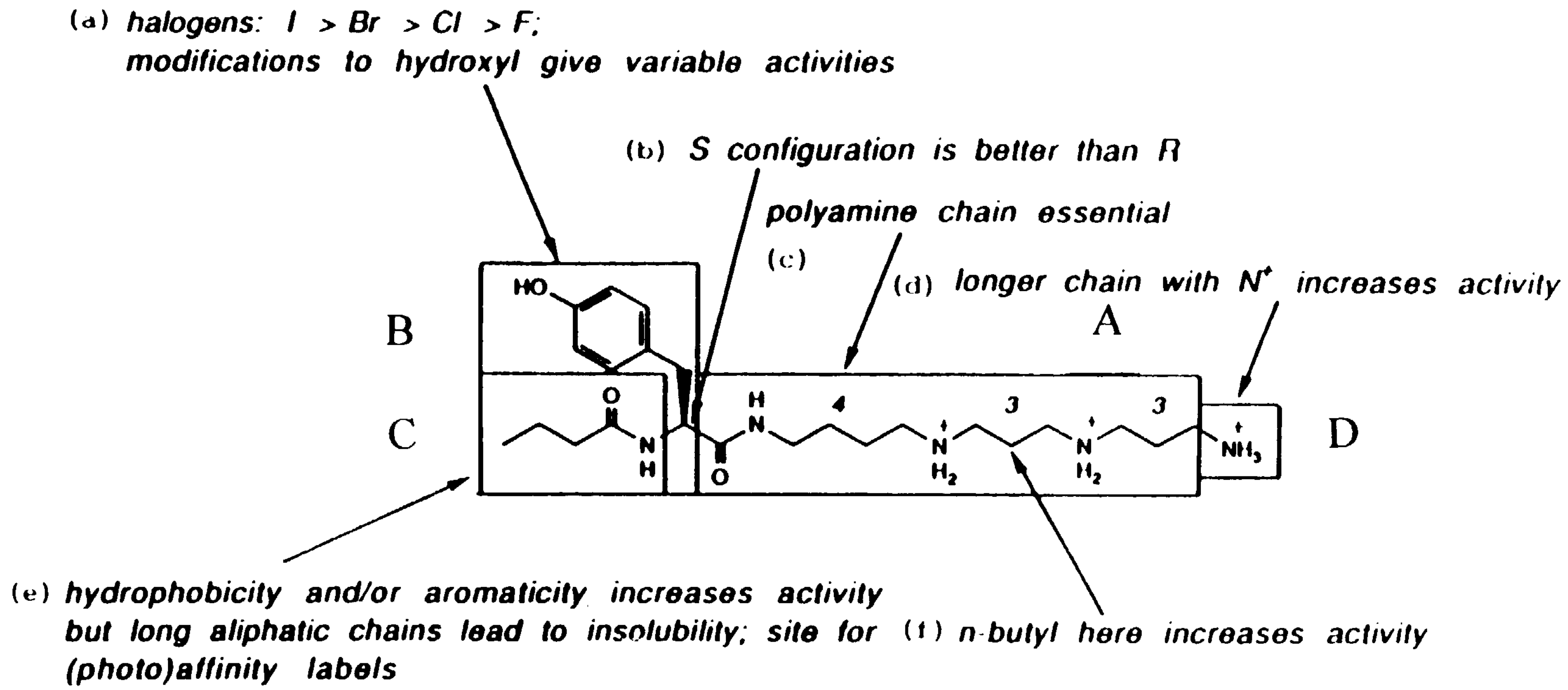
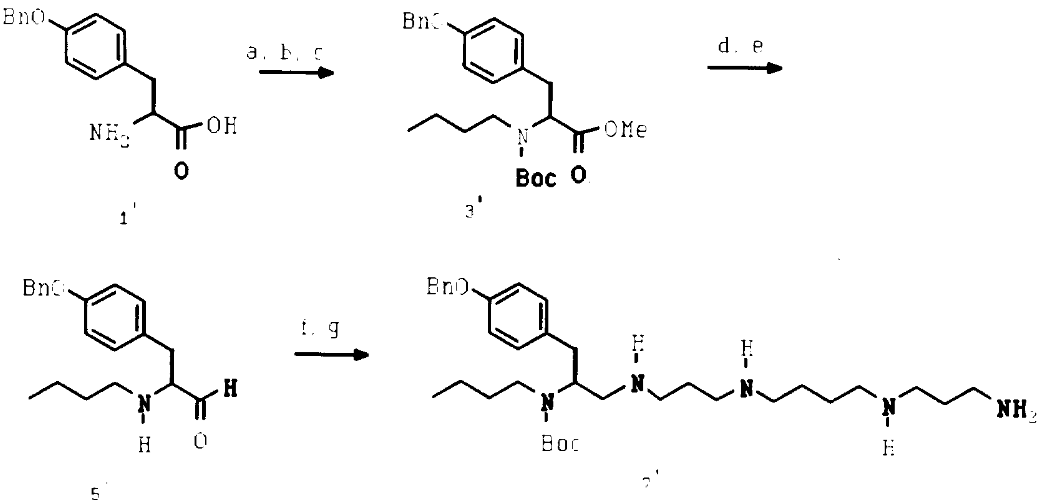
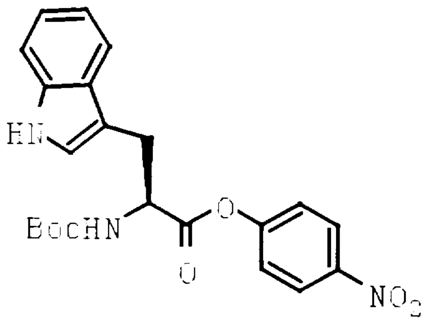
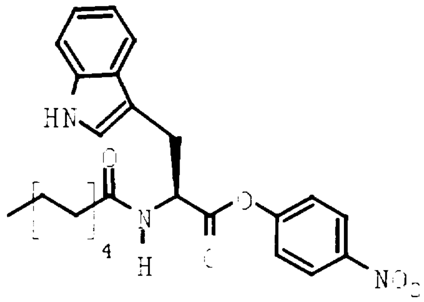

(a) halogens: I > Br > Cl > F;
modifications to hydroxyl give variable activities
(b) S configuration is better than R
polyamine chain essential
(c)
(d) longer chain with N+ increases activity
(e) hydrophobicity and/or aromaticity increases activity
but long aliphatic chains lead to insolubility; site for
(photo)affinity labels
(f) n-butyl here increases activity
A
B
C
D
HO
O
N
H
O
4
3
3
N
H2
NH3

FIGURE 9

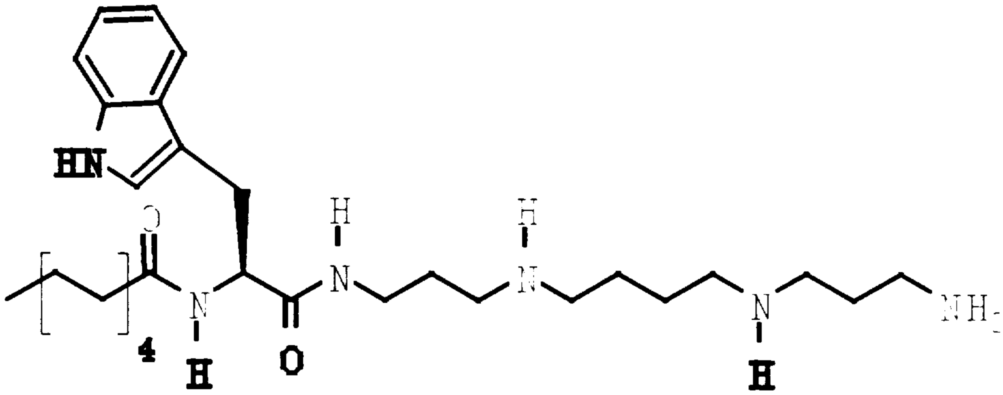




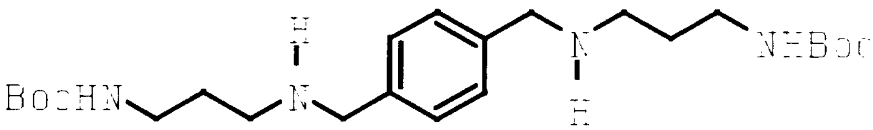
8′



9′

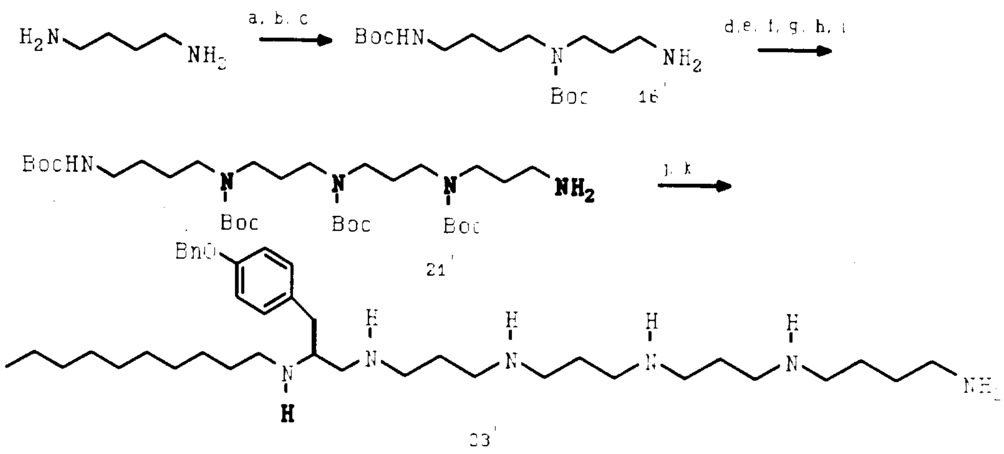


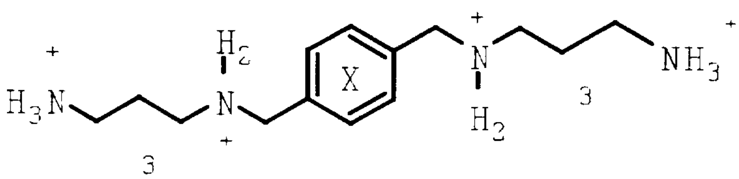
10′



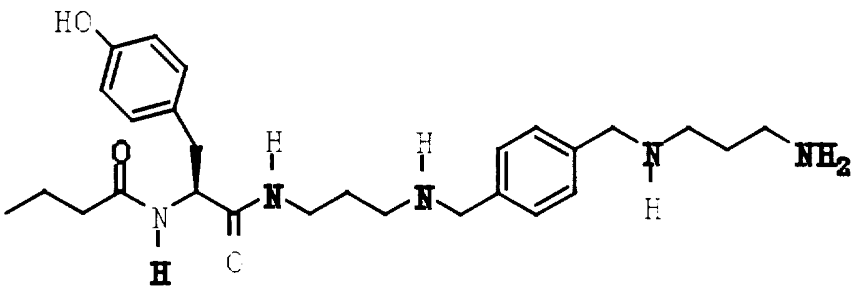
11′

FIGURE 10





12'



13'

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01128

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

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

NONE

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

CAS ONLINE SEARCH, STRUCTURE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Pure & Appl. Chem., Volume 62, No. 7, issued 1990, K. Nakanishi et.al., " Philanthotoxin-433(PhTX-433), a non-competitive glutamate receptor inhibitor, pages 1223-1230,especially pages 1226-1228.	1-3, 10-13, 22 and 39
X --- Y	The Journal of Pharmacology and Experimental Therapeutics, Volume 254, No. 3, issued September 1990, N. Anis et.al., "Structure-activity relationship of philanthotoxin analogs and polyamines on N-methyl-D-Aspartate and nicotinic acetylcholine receptors", pages, 764-773, especially pages 766-768.	1-3, 10-13, 22 and 39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 MAY 1996	13 JUN 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SHAILENDRA KUMAR
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1235

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01128

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Tetrahedron, Volume 46, No. 9, issued 1990, R.Goodnow et.al., "Synthesis of glutamate receptor antagonist philanthotoxin-433(PhTX-433) and its analogs." pages 3270-3286, especially pages 766-768.	1-3, 10-13, 22 and 39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01128

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-39

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01128

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07C 211/00, 235/00, 229/00; C07D 209/04; A61K 31/135, 31/16, 31/20, 31/405, 31/40

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/184; 260/404.5; 514/415, 419, 558, 559, 561, 617, 646, 616; 548/490, 491; 552/8;
564/153, 157

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/184; 260/404.5; 514/415, 419, 558, 559, 561, 617, 646, 616; 548/490, 491; 552/8;
564/153, 157

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-39, drawn to compounds, composition and method of inhibiting binding to the glutamine receptor.

Group II, claim(s) 40, drawn to a method of combatting insects.

The inventions listed as Groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under 37 CFR 1.475(d). Method of combatting insecticide is distinct than treating various diseases in human beings. Hence it can be seen that Groups I-II lack the same or special technical feature common to all the groups.