Pure & Appl. Chem., Vol. 62, No. 7, pp. 1223–1230, 1990.
Printed in Great Britain.

Philanthotoxin-433 (PhTX-433), a non-competitive glutamate receptor inhibitor

Koji Nakanishi,[a] R. Goodnow,[a] K. Konno,[a] M. Niwa,[a] Rudolph Bukownik,[a] Thomas A. Kallimopoulos,[a] Peter Usherwood,[b] Amira T. Eldefrawi[c] and Mohyee E. Eldefrawi.[c]

[a]Department of Chemistry, Columbia University, New York, NY 10027, USA
[b]Department of Zoology, University of Nottingham, Nottingham NG7 2RD, UK
[c]School of Medicine, University of Maryland, Baltimore, MD 21201, USA

Abstract - A polyamine toxin present in the venom sac of the solitary digger wasp *Philanthus triangulum*, first noted by T. Piek, has been isolated, characterized, and synthesized. This toxin, philanthotoxin-433 (PhTX-433), having a butyryl/tyrosyl/polyamine structure, is a potent noncompetitive inhibitor of the quisqualate sub-type glutamate receptor as assayed by the twitch contraction of locust leg muscles. It is also an allosteric inhibitor of acetylcholine receptors of vertebrates. About sixty analogs of PhTX have been synthesized in order to clarify structure/activity relationships and to select candidates suitable for isolating the glutamate receptor by photoaffinity label and/or affinity label.

INTRODUCTION

L-Glutamate is a major chemical mediator in the central nervous system of man and other animals and also serves this function in the peripheral nervous system of insects. Glutamate receptors are believed to be involved in higher neural functions such as memory and learning, and in neurological disorders, e.g., epilepsy, Huntington's disease, Alzheimer's disease, Parkinson's disease (ref.1,2,3). Glutamate receptors are divided into three major subtypes: N-methyl-D-aspartate (NMDA), quisqualate, and kainate, based on their response to agonists (ref.4). Although other neuronal receptors such as the acetylcholine receptor (ref.5) and γ-amino-butyric acid (GABA) receptor (ref.6) have been isolated and partially characterized, studies on glutamate receptors have been limited because of the relative paucity of potent and selective antagonists. Selective competitive and noncompetitive antagonists for the NMDA receptor have become available during the past few years (ref.7), but those for the quisqualate and kainate receptors are still insufficient.

Piek had shown (ref.8) that the solitary digger wasp *Philanthus triangulum* F., a wasp found in the Sahara Desert that preys on honeybees, makes a venom that blocks glutamate receptors on locust muscles. It had also been shown by Piek and other workers that the toxin exhibits a number of pharmacological properties, and that it is an antagonist against the quisqualate-sensitive glutamate receptor (ref.9). This toxin, philanthotoxin 433 (PhTX 433; numerals denote the number of methylenes in the polyamine moiety), has been isolated from the venom sacs of the female wasp (Fig.1) and assigned the structure shown from spectroscopic data and by synthesis; two other analogs, PhTX 343 and 433 were also synthesized (ref.10). PhTX-433 was found to be an efficient noncompetitive antagonist of vertebrate central quisqualate receptors and in insect muscles. However, it has been found that it is also an allosteric inhibitor on acetylcholine receptors of vertebrates (ref.11). Recently argiotoxins, also quisqualate receptor antagonists, have been isolated from orb web spider venoms (refs.12-14); the structures, although similar to PhTX, are somewhat more complex (Fig.5). The purpose of the following structure/activity study is to find synthetic analogs with higher binding affinity than natural

ISOLATION AND STRUCTURE

Isolation (Fig. 1):

venom glands (1,000 female *Philanthus triangulum* F.)

↓ *50% MeCN/H_2O*

extracts

↓ *HPLC, YMC ODS*
5-95% MeCN/H_2O (0.1% TFA)

peak with ret. time 13 min

↓ *HPLC, YMC ODS*
15% MeCN/H_2O (0.1% TFA)

PhTX-433, 1.1 mg

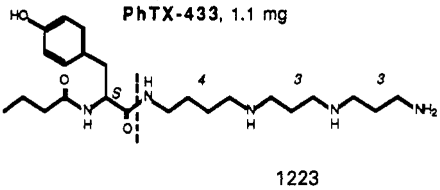


Fig. 1

PhTX, and eventually to isolate the glutamate receptor. Two routes are conceivable for isolation of the quisqualate subtype glutamate receptor. (a) *Photoaffinity isolation*: the radioactive toxin is covalently bound to the protein by a photoaffinity label. (b) *Affinity chromatography*: the toxin carrying a 2,4-dinitrophenyl group is passed through a Sepharose-IgG column that has strong specific affinity for the group; alternatively, the combination of a biotin group and a Sepharose-avidin column could also be used (see ref.15).

One thousand female *P. triangulum* were collected from the Dakhla oasis in the great Sahara Desert in the late summer when the population of this wasp is high, their venom sacs and glands with the sting apparati attached were removed, placed in liquid nitrogen, lyophilized, and sent to New York for isolation and characterization. At the early stage, the isolation was monitored by injection of honeybees with aqueous extracts of the glands which led to dose-dependent reversible paralysis. Recovery time from paralysis was 15 and 55 min for bees injected with 0.2 and 1.2 venom units (a unit is the extract from one wasp gland), respectively. Polyacrylamide gel electrophoresis shoed the presence of a large number of proteins, all of which were precipitated by boiling 10 min at 100° C; boiled extracts retained full activity. The extraction was later monitored by a more reliable pharmacological test on the locust retractor unguis nerve-muscle preparation, using reduction in neurally evoked twitch amplitude as the measure of activity.

The lyophilized venom glands were extracted with 50% CH_3CN/H_2O and the extracts were passed through a reverse-phase HPLC, YMC-ODS, 5 → 95% linear gradient, CH_3CN/H_2O containing 0.1% TFA. The most active fraction was further purified by reverse-phase HPLC, YMC-ODS, 4 x 280 mm. 15% CH_3CN/H_2O containing 0.1% TFA, to yield four peaks. The pharmacological activity resided in the major peak which gave 1.1 mg of the toxin, amorphous powder. Two other peaks are active but the structures still remain to be investigated.

Structure

Its UV spectrum has a maximum at 274 nm, which shifts to 290 nm at pH 12, suggesting the presence of a tyrosine residue. The ^1H-NMR signals of PhTX trifluoroacetate showed the presence of a butyryl group, six methylenes α to N^+, and four methylenes β to N^+; this 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. ^1H-NMR measured in DMSO-d_6 (500 MHz) clarified the connectivity of the butyryl, tyrosyl and polyamine moieties. However, it was not possible to differentiate the connectivity of methylene groups within the polyamine moiety, i.e., 433, 343 or 334. Syntheses of all three possibilities showed the 433 sequence to be correct (ref. 10)(all three are active, Fig. 6).

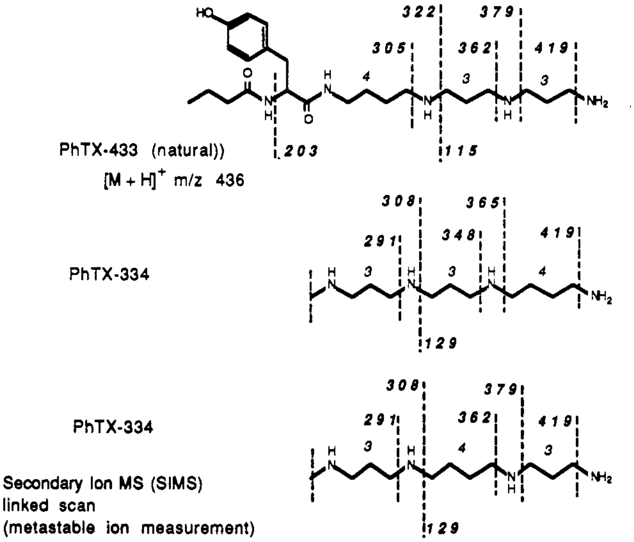


Fig. (2)

It has subsequently been found that a MS method employing liquid secondary-ion MS (SIMS) in conjunction with metastable-ion measurements (linked scanning at constant B/E) leads to sequence-specific differentiation among the three isomers (ref. 16) (Fig. 2). This should be a powerful method for the structural characterization of other polyamines which are frequently found in nature.

SYNTHESIS

Chemical synthesis of the three isomers is illustrated in Fig. 3. The protected polyamine **6** was obtained from spermidine derivative **4** (ref. 17) through: (1) Michael addition to acrylonitrile (76%); (2) Boc-protection (81%); and (3) reduction of the nitrile (70%). Further Cbz-protection and Boc-deprotection of **6** yielded partially protected polyamine **7**. Deprotection of N-Boc-O-benzyl-*L*-tyrosine p-nitrophenylester **8** (Fig. 3B) with TFA followed by acylation with butyryl chloride gave key intermediate **9** in 85% yield (Fig. 3C). Coupling of **9** with protected polyamines **7**, **6** and commercial spermine at ca. 65% yield, followed by deprotection, gave PTX and analogs 334 and 343 (ca. 80% yield)(ref. 10). Spermine (343) was used for the syntheses of most analogs (ref. 18) because the biological activity of PhTX-343 was similar to that of natural PhTX-433 (80%)

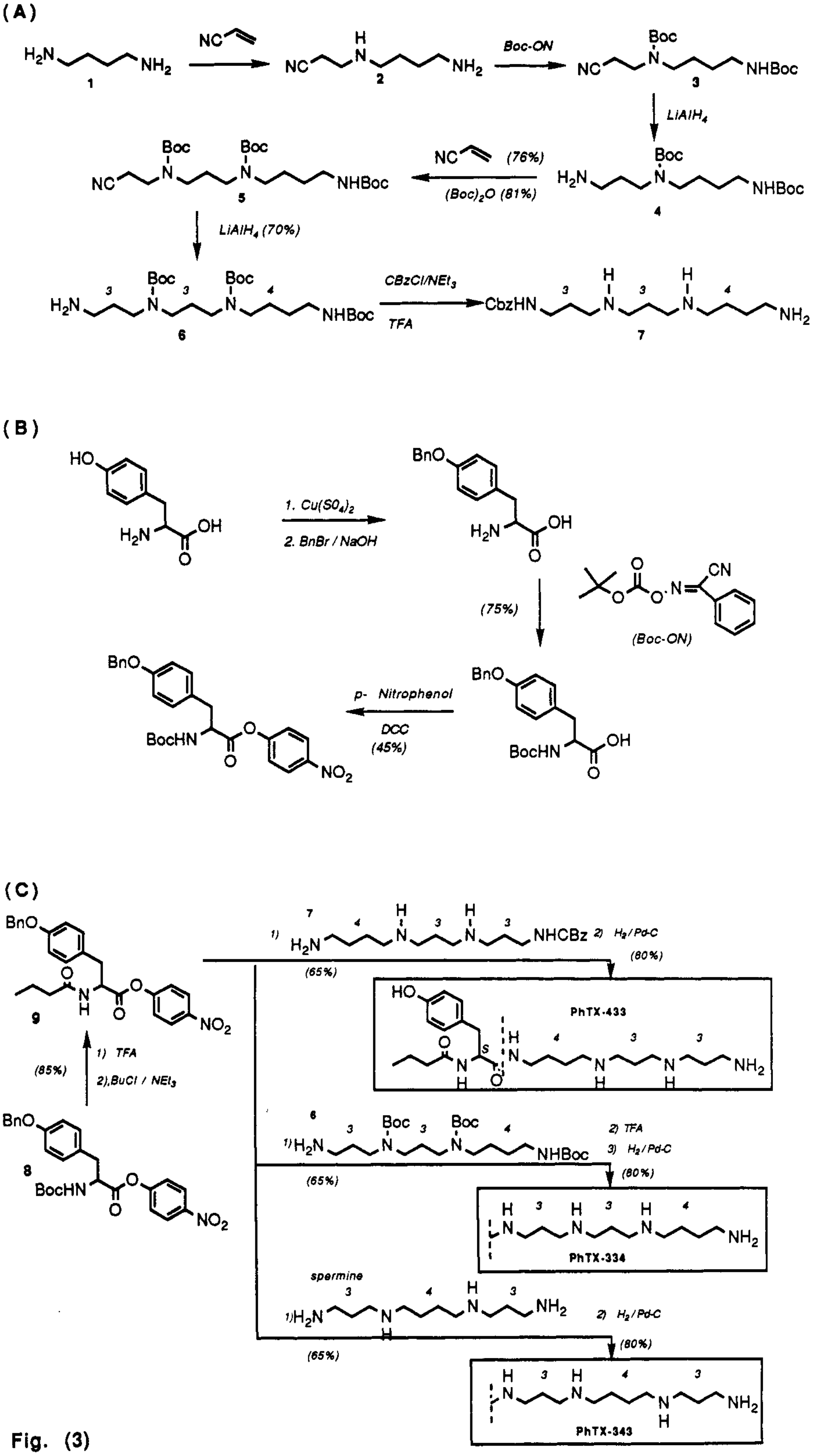
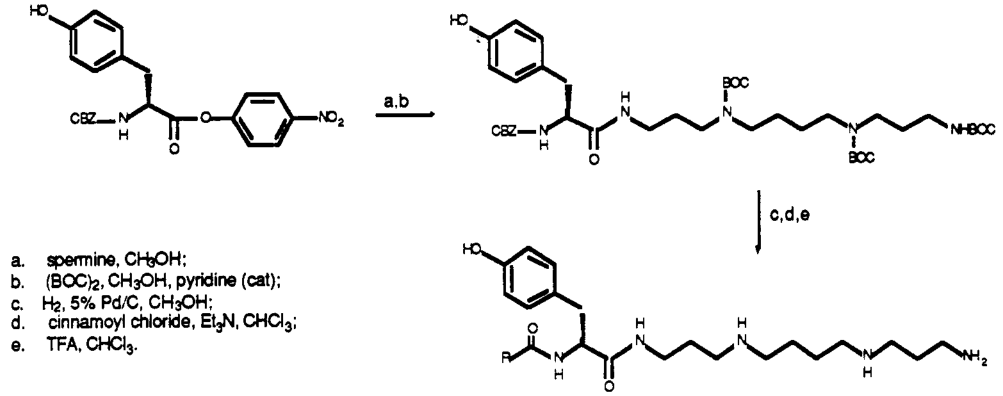

(A)
1
NC
2
Boc-ON
3
LiAlH4
4
(76%)
(Boc)2O (81%)
5
LiAlH4 (70%)
6
CBzCl/NEt3
TFA
7
(B)
1. Cu(SO4)2
2. BnBr / NaOH
(75%)
(Boc-ON)
p- Nitrophenol
DCC
(45%)
(C)
9
8
1) TFA
2),BuCl / NEt3
(85%)
1)
2) H2/Pd-C
(80%)
(65%)
PhTX-433
2) TFA
3) H2/Pd-C
PhTX-334
spermine
PhTX-343

Fig. (3)

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 amimes 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 the method shown in Fig. 3 with only slight modifications if necessary. In the few cases where functionalities are sensitive to the hydrogenolysis conditions employed for O-benzyl deprotection, they were synthesized according to scheme shown in Fig. 4. Thus for the preparation of compounds such as the cinnamyl or azidophenyl analogs (Fig. 8), N-carbobenzyloxylation instead of N-butoxycarbonylation 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-hydoxysuccinimide ester, depending on availability.



a. spermine, CH_3OH;
b. $(BOC)_2$, CH_3OH, pyridine (cat);
c. H_2, 5% Pd/C, CH_3OH;
d. cinnamoyl chloride, Et_3N, $CHCl_3$;
e. TFA, $CHCl_3$.

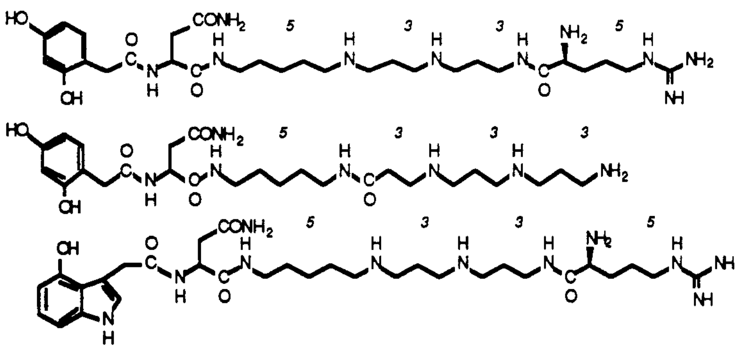
Fig. 4

12 R: C_6H_5-CH=CH-

STRUCTURE ACTIVITY RELATIONSHIP

As depicted in Fig. 5, the structures of typical spider toxins and PhTX-433 (δ-PTX, ref. 19) possess similar structures. In order to simplify the systematic study of structure/activity relationships, the PhTX structure was divided into four regions, I-IV, and each region was modified separately. Based on the results of single modification, analogs with multiple modifications were then prepared for the purpose of selecting potent analogs suited for receptor isolation studies, by chemical and/or genomic approaches. Reduction in amplitude of the neurally evoked twitch contraction of locust meththoracic retractor unguis muscle was used to construct dose-inhibition relationships from which IC_{50}'s were estimated. In the following figures the activities on the postsynaptic quisqualate receptor of locust leg muscles are listed relative to the *unnatural* PhTX-343 with the spermine-derived polyamine moiety; higher the numerals, higher the activity ref.20).

<u>*Spider Toxins*</u>

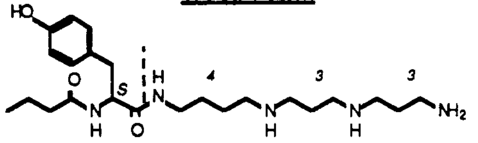


argiopine
Grishin et al. , 1986
argiotoxin 636
Adams et al., 1987

JSTX-3
Aramaki et al., 1986

argiotoxin 659
Adams et al., 1987

<u>*Wasp Toxin*</u>



PhTX-433 (δ-PTX)

Fig. (5)

Region I (Fig. 6)

Structure Activity Relationship
activity relative to PhTX-343 = 1

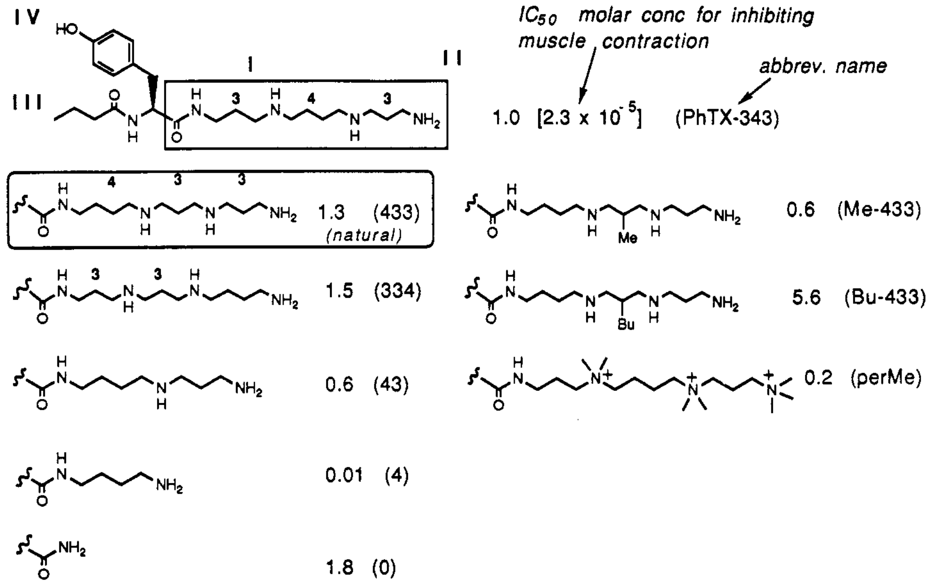


Fig. (6)

The differences seen in the potencies of the 433 (natural), 343 and 334 analogues, although small, may reflect the subtle difference in the distances between the distribution of positive charges of the three analogs and the distribution of negative charges on the receptor channel. A model analog to check this possibility is being synthesized. Shortening of the polyamine chain from PhTX-334 → 43 → 4 reduced potency, suggesting that the number of protonated groups is important. For unknown reasons, PhTX-0 with no positive charge is slightly more active than singly protonated PhTX-4. Addition of a methyl group to the middle carbon of the central C-3 moiety of spermine as in Me-433 did not greatly alter potency, whereas addition of a butyl group (Bu-433) increased potency by almost 6-fold compared. The synthesis of these alkylated analogs was undertaken to test the feasibility of attaching to the polyamine moiety, long alkyl chains that carry at their terminus functionalities suited for preparing affinity columns. The reduced activity of perMe with three quaternary amines may be the result of steric hindrance of electrostatic interactions with anionic centers.

Region II (Fig. 7)

activity relative to PhTX-343 = 1

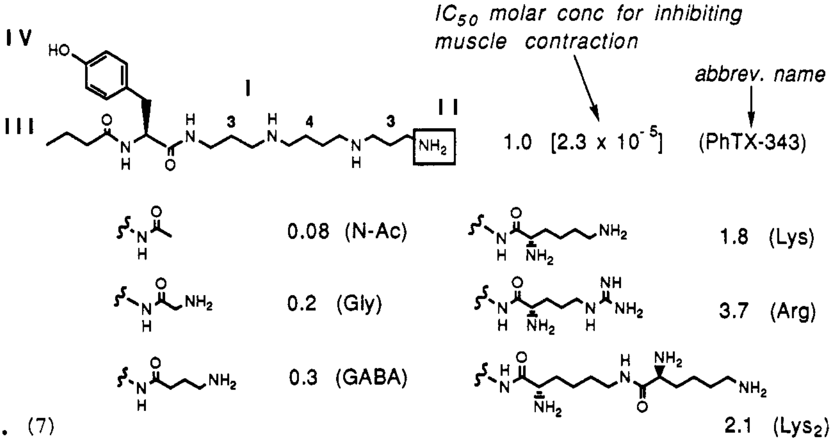


Fig. (7)

The low activities of N-Ac, Gly and GABA analogs suggests that the presence of a positive charge about ten (3+4+3) atoms removed from the tyrosyl amide is necessary. The Lys, Arg and Lys_2 analogs do carry such positive charges; The increased activity of Lys and Arg implies that longer polyamine chains interact more efficiently; however, similar activity of Lys and Lys_2 supports the contention that longer protonated are not necessary. The Arg analog shares structural characteristics in common with spider toxins. The enhanced potency of the arginine analogue could be accounted for by the fact that the guanidinium group can delocalize its positive charge over a wider area than a primary amino group, thus being able to better accommodate to the distribution of anionic centers on the wall of the receptor channel.

Region III (Fig. 8)

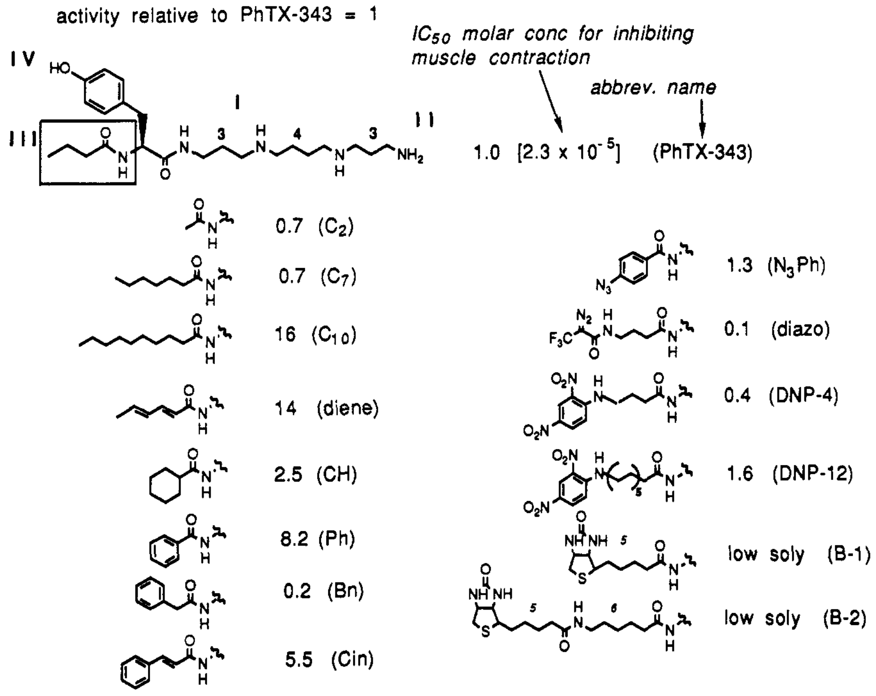


Fig. (8)

Shortening the butyryl group to an acetyl group reduces the potency. However, increasing the length of the chain from 4 → 7 → 10 produced successive increases in potency. The diene is also very potent. The increased potency of the phenyl analog over the cyclohexyl analogue may be attributable to the steric bulk of its cyclohexane moiety. Modification results in Region III indicate the importance of hydrophobicity in this region. It should be noted that insertion of a double bond between the aromatic ring and the carbonyl moiety (Cin) leads to a greatly increased potency. It is important that the azidophenyl group with a photosensitive affinity label produced reasonably active compound; a p-azidocinnamoyl analog is currently being tested. The potency of the dinitrophenyl analogue DNP-12 its usage in IgG linked affinity columns. The analogs containing biotin moieties were prepared as possible candidates for use in avidine affinity chromatography, but their low solubility rendered them impractical in this respect; if the biotin moiety were to be used, it should be attached to oxygen or sulfur containing spacers.

Region IV (Fig. 9)

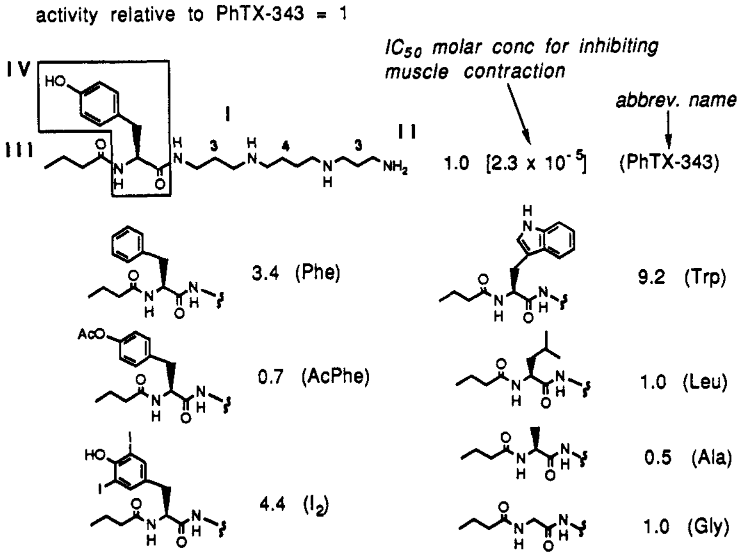


Fig. (9)

Removal of hydroxyl group (Phe) increased potency about 3-fold, whereas conversion of the tyrosyl analogue to the 3,5-diiodo-tyrosyl analogue increased potency 4.5-fold. The increased potency is especially useful since it allows for radio-iodine labelling. It is concievable that the iodine atoms are assisting in hydrophobic binding. However, it is difficult to rationalize the unchanged potencies of the O-acetyl analogue. Replacement of Tyr by other amino acids, e.g., Leu and Ala, did not affect the potency. However, replacement by tryptophan 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 alanine and glycine analogues suggests the necessity of an anchoring group in this region.

Doublets/triplets and summary (Figs 10, 11)

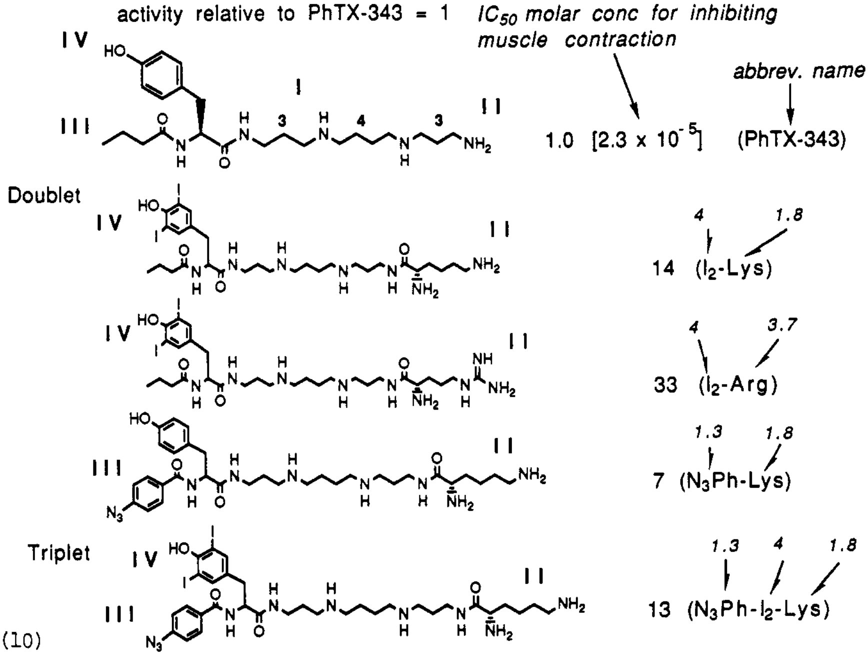


Fig. (10)

The italicized numerals denote the potency relative to PhTX-343 accompanying the specified modifications. The diiodo analog has a factor of 4 (Fig. 9) and the Lys analog a factor of 1.8 (Fig. 7); however, the diiodo-lys analog has an increased potency of 14. The other cases also show that there is synergism between regional modifications, an important result for making active analogs.

The results of above-mentioned structure/activity relationship studies are summarized in Fig.11.

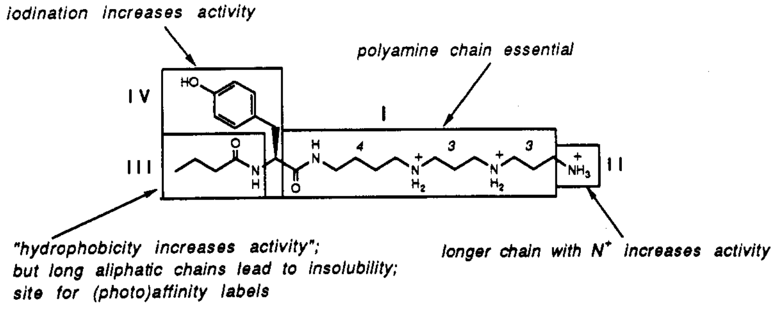
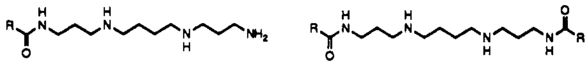


Fig. (11)

Mono- and bis-spermine analogs (Fig. 12)



R	IC₅₀ relative to *PhTX-343*	IC₅₀ relative to *PhTX-343*
	agonist (C_4)	0.1 (bis-C_4)
	0.2 (C_7)	agonist (bis-C_7)
	2.3 (C_{10})	1.0 (bis C_{10})

Fig. (12)

The mono- and bis-spermine analogs were prepared to check whether straight chain analogs with a hydrophobic end and a polyamine chain would suffice for exertion of activity. Of the numerous possibilities for the structure of the receptor, Fig. 13 depicts two conceptually conceivable models. In *A* the polyamine chain inserts itself into the channel with negative groups, whereas in *B* the polyamine chain rests on the membrane surface. The low potencies of C$_7$ and bis-C$_{10}$ suggest that geometric constraints and the presence of both hydrophobic and hydrophilic moieties are essential for activity. However, bis-C$_{10}$-spermine is as active as PhTX-343. The activities, ranging from agonistic to moderate antagonism, as yet, cannot be accounted for, but the relatively high potencies of analogues C$_{10}$ and bis-C$_{10}$ could result from the additional anchoring capabilities (either in a hydrophobic pocket or through association with the lipid bilayer) of these molecules by their aliphatic chains.

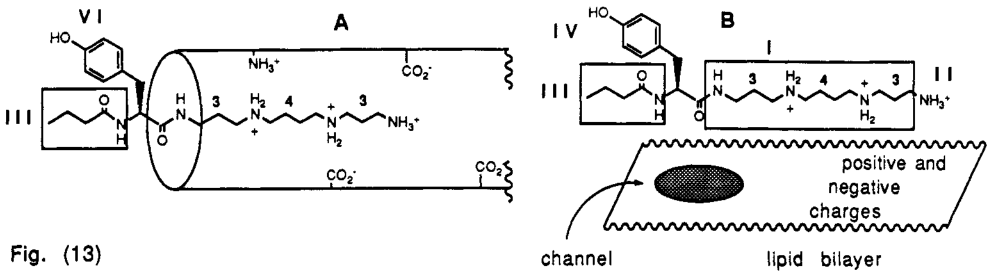


Fig. (13)

CONCLUSION

Several molecules which are more potent non-competitive antagonists of the locust quisqualate receptor than the natural PhTX-433 have been made. Hydrophobicity of regions III and IV is an important potency determinant, although for region III 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 (ref. 21). The increased potency seen in Bu-433 (Fig. 6) is less easily reconciled, although one might anticipate, perhaps, the presence of additional pockets of hydrophobicity in the region of the channel to which the polyamine moiety binds. Several molecules embracing the structures of the four moieties which produced the most potent ligands are currently being synthesized. However, in view of the ubiquitous presence of polyamines it is still possible that further structural modifications will be required to further increase the specificity of the PhTX analog for the glutamate receptor.

Acknowledgements
The studies were supported by NIH grant AI 10187 (to KN), British Agriculture and Food Research Council (to PNRU) and NSF INT-8610138 (to ME).

REFERENCES

1. C.W. Cotman, L.L. Iversen, *Trends Neurosci.*, **10**, 263 (1987).
2. M.B. Robinson, J.T. Coyle, *FASEB J.*, **1**, 446 (1987).
3. H. Jackson, P.N.R. Usherwood, *Trends Neurosci.*, **11**, 278-283 (1988).
4. A.C. Foster, G.E. Fagg, *Brain Res. Rev.*, **7**, 103 (1984).
5. J.P. Changeux, F. Revah, *Trends Neurosci.*, **10**, 245 (1987).
6. E.A. Barnard, M.GF. Darlson, P. Seeburg, *Trends Neurosci.*, **10**, 502 (1987).
7. J.C. Watkins, H. Olverman, *Trends Neurosci.*, **10**, 265 (1987).
8. T. Piek, P. Mantel, E. Engels, *Comp. Gen. Pharmacol.* **2**, 317 (1971).
9. T. Pie k, R.H. Fokkens, H. Karst, C. Kruk, A. Lind, J. van Marle, T. Nakajima, N.M.M. Nibering, H. Shinozaki, W. Spanner, T.C. Tong, *Neurotox '88: Molecular Basis of Drug & Pesticide Action*; G.G. Lunt ed., pp 61-76, Elsevier, Amsterdam (1988).
10. A.T. Eldefrawi, M.E. Eldefrawi, K. Konno, N.A. Mansour, K. Nakanishi, E. Oltz, P.N.R. Usherwood, *Proc. Natl. Acad. Sci. USA*, **85**, 4910 (1988).
11. R. Rozenthal, G.T. Scoble, E.X. Albuquerque, M. Idriss, S. Sherby, D.B. Sattelle, K. Nakanishi, K. Konno, A.T. Eldefrawi, M.E. Eldefrawi, *J. Pharm. Experimental Therapeutics*, 249, 123-130 (1989).
12. L.G. Grishin, T.M. Voldova, A. Arsoniev, A.S. Reshetova, V.V. Onoprienko, L.G. Magazanic, S.M. Antonov, I.M. Fedorova, *Bioorg. Khim.* **12**, 1121 (1986).
13. Y. Aramaki, T. Yasuhara, T. Higashjima, M. Yoshioka, M. Miwa, N. Kawai, T. Nakajima, *Proc. Jpn. Acad.* **Ser. B 62**, 359 (1986).
14. M.E. Adams, R.L. Candy, F.E. Enderlin, T.E. Fu, M.A. Jarema, J.P. Li, C.A. Miller, D.A. Schooley, M.J. Shapiro, V.J. Venema, *Biochem. Biophys. Res. Commun.* **348**, 678 (1987).
15. P.R. Schofield, M.G. Darlison, N. Fujita, D.R. Burt, A.A. Stephenson, H. Rodriguez, L.M. Rhee, J. Ramachandran, V. Reale, T.A. Glencorse, P.H. Seeburg, E.A. Barnard, *Nature*, **328**, 221 (1987).
16. P.T.M. Kenny, R.A. Goodnow, K. Konno, K. Nakanishi, *Rapid Comm. in MS*, **3**, 295-297 (1989).
17. M. Humora, J. Quick, *J. Org. Chem.*, **44**, 1166-1168 (1970).
18. R.Goodnow,K.Konno, M.Niwa, T.Kallimopoulos, R.Bukownik, D.Lenares, K.Nakanishi,*Tetrahedron*,in press.
19. W. Spanjer, T.E. May, T. Piek, H. de Hann, Comp. Biochem. Physiol., 71C, 149-157 (1982).
20. M. Bruce, A.T. Eldefrawi, M.E. Eldefrawi, R. Bukownik, R. Goodnow, T. Kallimopoulos, K. Konno, M. Niwa, K. Nakanishi, P.N.R. Usherwood, *Toxicon*, in press.
21. P.N.R. Usherwood, I.S. Blagborough, *Insecticide Action: From Molecule to Organism*, T. Narahashi, ed., Plenum Press, in press.