Structure/Activity and Molecular Modeling Studies of the Lophotoxin Family of Irreversible Nicotinic Receptor Antagonists

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Lophotoxin is a small cyclic diterpene that irreversibly inhibits agonist binding to nicotinic acetylcholine receptors by reacting covalently with Tyr^{190} in the α-subunits of the receptor. Structure/activity and molecular modeling studies were undertaken to investigate the structural and conformational features responsible for this unique biological activity. A total of 18 naturally occurring and 7 chemically modified analogues were evaluated for their ability to inhibit the binding of [^{125}I]-α-bungarotoxin to nicotinic acetylcholine receptors on membranes prepared from *Torpedo* electric organ. When the toxins were incubated with the receptor for short durations they did not slow the initial rate of binding of [^{125}I]-α-bungarotoxin, suggesting that they have relatively low reversible affinity. However, their ability to inhibit the equilibrium binding of [^{125}I]-α-bungarotoxin increased progressively with longer incubation times, consistent with an irreversible mechanism of action. Comparison of active and inactive analogues allowed identification of a conserved pharmacophore that appeared to be required for irreversible inhibition of the receptor. This pharmacophore contains lactone oxygens and an electron-deficient epoxide that may mimic the acetate oxygens and quaternary ammonium group of acetylcholine, respectively. Computer modeling of the toxins using molecular mechanics and dynamics revealed that the toxins have restricted conformational mobility, thus allowing identification of a minimum-energy conformation. The results allow speculation concerning the site of covalent reaction between Tyr^{190} and the toxins, the normal function of Tyr^{190} in binding acetylcholine, and the bound conformation of acetylcholine.

Introduction

Lophotoxin is one member of a family of neurotoxins with similar overall structure that can be isolated from gorgonian (soft) corals.[1-7] These toxins bind selectively and irreversibly within the acetylcholine-recognition site of nicotinic acetylcholine receptors, thereby preventing acetylcholine from activating the receptor.[1,2] The irreversible inhibition they produce results from a specific covalent reaction between the coral toxins and Tyr^{190} in the α-subunits of the receptor.[8,9] Thus these toxins have provided evidence that Tyr^{190} is located within the acetylcholine-recognition site. The fact that Tyr^{190} is uniquely conserved in all functional α-subunits whose sequence is known suggests that it plays an important role not only in reacting with lophotoxin but also in binding acetylcholine. More specifically, it has been suggested that Tyr^{190} forms part of the subsite involved in binding the quaternary ammonium group of acetylcholine.[9,10]

In addition to an unusual mechanism of action, the lophotoxin family of neurotoxins are unique nicotinic receptor antagonists in that they are devoid of nitrogen and thus do not contain a positive charge. Since they are relatively small and since their ring systems may provide conformational stability, a greater understanding of their site and mechanism of action may shed light on the three-dimensional topography of the amino acids comprising the acetylcholine-recognition site. Structure/activity studies were therefore undertaken to define further the structural features of the toxins that are involved in their chemical reactivity and biological activity. Structural analogues were obtained by isolation of naturally occurring congeners from different gorgonian species and by selective chemical modification of two of the naturally occurring toxins. In addition, the conformational mobility and the minimum-energy conformation of the toxins were investigated by molecular mechanics and molecular dynamics.

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Results

Irreversible Inhibition. Incubation of lophotoxin (1) or bipinnatin-B (2) with membranes prepared from *Torpedo* electric organ for short durations (2-5 min) did not appreciably slow the initial rate of binding of [^{125}I]-α-bungarotoxin. However, the apparent ability of either lophotoxin or bipinnatin-B to inhibit the equilibrium binding of [^{125}I]-α-bungarotoxin increased with increasing incubation time (Figure 1). The apparent affinity of lophotoxin increased from 1 through 16 h of incubation with the receptor, while the apparent affinity of bipinnatin-B did not continue to increase after ca. 4 h of incubation. Increases in apparent affinity with time of incubation were also observed for all of the other active lophotoxin analogues.

(1) Fenical, W.; Okuda, R. K.; Banurraga, M. M.; Culver, P.; Jacobs, R. S. *Science* **1981**, *26*, 1512.
(2) Culver, P.; Burch, M.; Potenza, C.; Wasserman, L.; Fenical, W.; Taylor, P. *Mol. Pharmacol.* **1985**, *28*, 436.
(3) Missakian, M. G.; Burreson, B. J.; Scheuer, P. J. *Tetrahedron* **1975**, *31*, 2513.
(4) Wright, A. E.; Burres, N. S.; Schulte, G. K. *Tetrahedron Lett.* **1989**, *30*, 3491.
(5) Look, S. A., Doctoral disseration under the direction of W. Fenical, 1983, University of California at San Diego. (X-ray crystallography was performed in collaboration with S. Rafii and J. Clardy at Cornell University.)
(6) Bandurraga, M. M., Doctoral dissertation under the direction of W. Fenical, 1982, University of California at San Diego.
(7) Burch, M., Masters thesis under the direction of W. Fenical, 1986, University of California at San Diego.
(8) Abramson, S. N.; Culver, P.; Kline, T.; Li, Y.; Guest, P.; Gutman, L.; Taylor, P. *J. Biol. Chem.* **1988**, *263*, 18568.
(9) Abramson, S. N.; Li, Y.; Culver, P.; Taylor, P. *J. Biol. Chem.* **1989**, *264*, 12666.
(10) Dennis, M.; Giraudat, J.; Kotzyba-Hibert, F.; Goeldner, M.; Hirth, C.; Chang, J.-Y.; Lazure, C.; Chretien, M.; Changeux, J.-P. *Biochemistry* **1988**, *27*, 2346.
(11) Beers, W. H.; Reich, E. *Nature* **1970**, *228*, 917.
(12) Sheridan, R. P.; Nilakantan, R.; Dixon, J. S.; Venkataraghavan, R. *J. Med. Chem.* **1986**, *29*, 899.
(13) Partington, J.; Feeney, J.; Burgen, A. S. V. *Mol. Pharmacol.* **1972**, *8*, 269.

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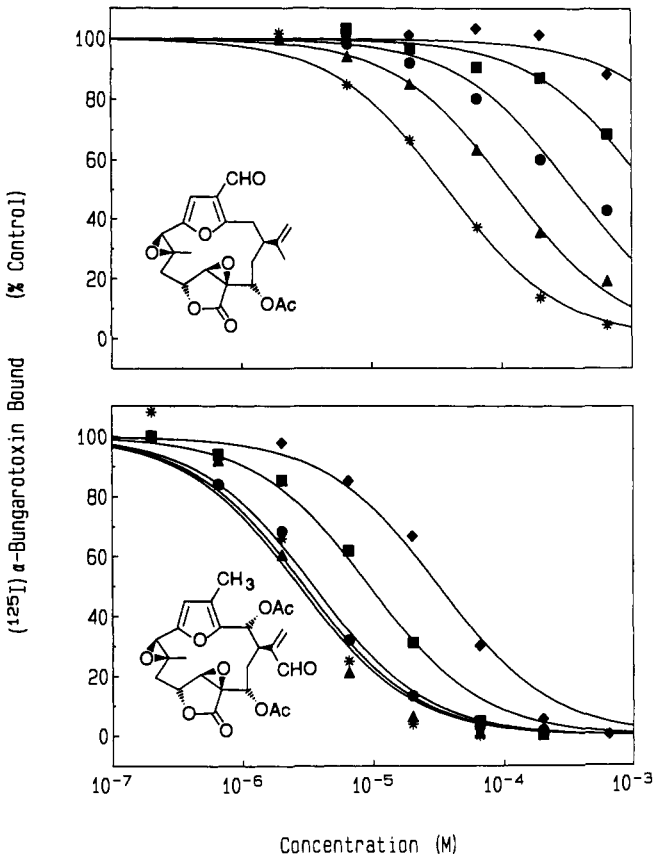


Figure 1. Effect of incubation time on the apparent affinities of lophotoxin and bipinnatin-B. Lophotoxin (upper panel) and bipinnatin-B (lower panel) were incubated with membranes containing nicotinic acetylcholine receptors for 1 (♦), 2 (■), 4 (●), 8 (▲), or 16 (*) h, and the presence of free receptors was then determined by equilibrium binding of [^{125}I]-α-bungarotoxin. The percent of specifically bound [^{125}I]-α-bungarotoxin is plotted versus the concentration of coral toxin prior to the addition of [^{125}I]-α-bungarotoxin. Each point represents the mean of three to seven separate experiments. The structures of lophotoxin (1) and bipinnatin-B (2) are shown as insets in the upper and lower panels, respectively.

Structure/Activity Relationships. Several naturally occurring analogues of lophotoxin, including bipinnatin-B (2), -A (3), -C (4), -E (5), and -F (6) (also known as lophotoxin analogue 1, 5, 4, 2, and 3, respectively[2,4]) have been evaluated previously for their ability to inhibit the binding of [^{125}I]-α-bungarotoxin.[2] However, to enhance the detection of weakly active analogues, these and other naturally occurring lophotoxin analogues were allowed to react with the receptor for 16 h prior to determination of the equilibrium binding of [^{125}I]-α-bungarotoxin. A total of 18 naturally occurring analogues isolated from different species of gorgonian corals were assayed (Table I). Active toxin analogues included lophotoxin (1), bipinnatin-B (2), bipinnatin-A (3), bipinnatin-C (4), bipinnatin-E (5), bipinnatin-F (6), lopholide (7), deoxylophotoxin (8), bipinnatin-G (9), bipinnatin-H (10), bipinnatin-I (11), and acetoxypukalide (12). These active analogues contained a variety of substituents at the C1, C2, and C4 positions and either an epoxide or a double bond between C11 and C12. Analogues which did not exhibit detectable activity included pukalide (13), pukalide aldehyde (14), hexahydropukalide (15), deoxypukalide (16), bipinnatolide-B (17), and bipinnatolide-E (18).

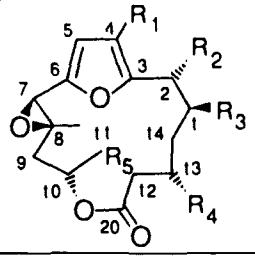
Since the congeneric series provided by the naturally occurring toxin analogues was necessarily limited, it was of interest to prepare additional analogues by chemical modification of the naturally occurring toxins. A total of six chemically modified toxin analogues were produced by reduction of lophotoxin and bipinnatin-B with $NaBH_4$ or $LiAlH_4$ (Table II). The modifications included reduction of the aldehydes (19–24), hydrolysis of the C2 (22) or C13 (21 and 24) acetate esters, and reduction of the lactone carbonyl (23 and 24). An ethanol adduct of lophotoxin (25), thought to be produced during extraction of *Lophogorgia* with ethanol,[2] was also tested. Each of the chemically modified analogues was allowed to react with the receptor for 2 h prior to determination of the equilibrium binding of [^{125}I]-α-bungarotoxin (Table II). The ethanol adduct of lophotoxin (25) did not exhibit detectable activity. However, the semisynthetic analogue produced by reduction of the aldehyde of bipinnatin-B (20) was the most potent of all of the lophotoxin analogues tested and was ca. 4–5 times more effective at irreversibly inhibiting nicotinic receptors than bipinnatin-B itself. Although lophotoxin showed only modest activity after a 2-h incubation with the receptor, reduction of the aldehyde of lophotoxin (19) also increased the apparent affinity, in this case ca. 31-fold. Hydrolysis of the C13 (21) or C2 (22) acetate esters decreased the apparent affinity ca. 12–39-fold, respectively, while reduction of the lactone carbonyl (23 and 24) essentially abolished the activity observed in a 2-h incubation. Reduction of the lactone carbonyl, however, did not completely impair the ability of toxin analogues to inhibit the receptor irreversibly, since a high concentration of 24 (660 μM) was able to reduce the number of [^{125}I]-α-bungarotoxin binding sites by ca. 24% after a 16-h incubation.

Molecular Modeling. Molecular mechanics and dynamics were utilized to investigate the conformational flexibility of the toxins and to determine their minimum-energy conformation. Molecular dynamics simulations were performed at 1000 K to maximize the number of different conformations visited during the dynamics simulation. Although this temperature was sufficient to allow complete 360° rotation of the aldehydes and acetate esters during the 30-ps simulation, the 14-carbon cembrane ring was only able to adopt a relatively limited range of conformations. Different random conformations generated during the dynamics simulation were subjected to molecular mechanics to obtain their minimum-energy conformation. All of them converged upon minimization to essentially similar conformations that differed by less than 1.1 kcal/mol (Figure 2). The most definitive feature of the minimum-energy conformation is that the furan and lactone rings are oriented at ca. 90° angles to one another, with the lactone carbonyl pointing in the opposite direction as the C7–C8 epoxide. The minimum-energy conformation of the cembrane ring was similar for lophotoxin, bipinnatin-B, and bipinnatin-C, and the results were independent of the initial conformation. The computer-generated minimum-energy conformations were also similar to the crystal conformations of bipinnatin-C and -E recently determined by X-ray crystallography.[4,5]

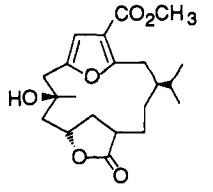
Discussion

In this study we investigated the ability of naturally occurring and chemically modified analogues of lophotoxin to inhibit the binding of [^{125}I]-α-bungarotoxin to nicotinic acetylcholine receptors on membranes prepared from the electric organs of *Torpedo californica*. This in vitro assay was chosen because it can be performed in relatively small volumes and it therefore requires very little of the toxin analogues, some of which cannot be isolated in large quantities. In addition, the [^{125}I]-α-bungarotoxin binding assay can be performed under "initial rate" conditions

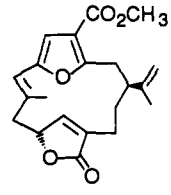
Table I. Structures and Activities of Naturally Occurring Lophotoxin Analogues[a]



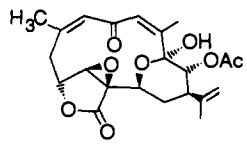
analogue	no.	R_1	R_2	R_3	R_4	R_5	log EC_{50} ± SEM	N
lophotoxin	1	-CHO	- H		-OCOCH$_3$		4.41 ± 0.02	7
bipinnatin-B	2	-CH$_3$	-OCOCH$_3$		-OCOCH$_3$		5.54 ± 0.04	7
bipinnatin-A	3	-CH$_3$	-OCOCH$_3$		-OCOCH$_3$		4.90 ± 0.03	7
bipinnatin-C	4	-CH$_3$	-OCOCH$_3$		-OCOCH$_3$		4.60 ± 0.07	12
bipinnatin-E	5	-CO_2CH_3	- H		-OCOCH$_3$		3.98 ± 0.04	7
bipinnatin-F	6	-CH$_3$	-OCOCH$_3$		-OCOCH$_3$		4.78 ± 0.04	6
lopholide	7	-CO_2CH_3	- H		-OCOCH$_3$		3.90 ± 0.05	7
deoxylophotoxin	8	-CHO	- H		-OCOCH$_3$		3.94 ± 0.04	5
bipinnatin-G	9	-CH$_3$	-OCOCH$_3$		-OCOCH$_3$		4.84 ± 0.03	7
bipinnatin-H	10	-CH$_3$	-OCOCH$_3$		-OCOCH$_3$		4.92 ± 0.02	7
bipinnatin-I	11	-CH$_3$	-OCOCH$_3$		-OCOCH$_3$		5.13 ± 0.04	7
acetoxypukalide	12	-CO_2CH_3	- H		-OCOCH$_3$		4.04 ± 0.04	10
pukalide	13	-CO_2CH_3	- H		- H		NA	9
pukalide aldehyde	14	-CHO	- H		-H		NA	8
hexahydropukalide	15						NA	6
deoxypukalide	16						NA	6
bipinnatolide-B	17						NA	6
bipinnatolide-E	18						NA	6



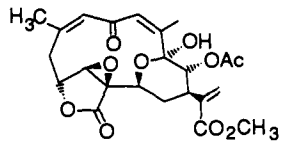
hexahydropukalide **15**



deoxypukalide **16**



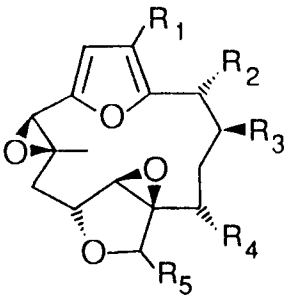
bipinnatolide-B **17**

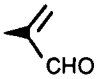
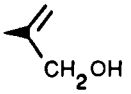
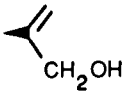
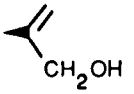
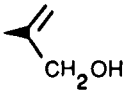
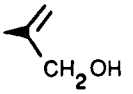


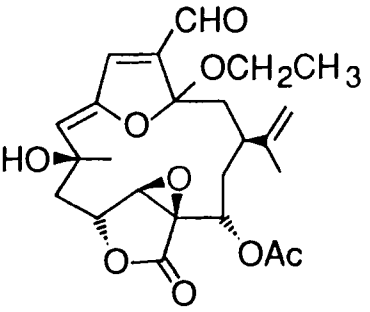
bipinnatolide-E **18**

[a] Toxin analogues were incubated with the receptor for 16 h prior to a 1-h incubation with a saturating concentration of [^{125}I]-α-bungarotoxin. The EC_{50} is the concentration of toxin (M) that inhibited 50% of the specifically bound [^{125}I]-α-bungarotoxin. Under similar assay conditions, the reversible antagonist *d*-tubocurarine exhibited a high apparent EC_{50} of ca. 3.2 mM. R5 represents either a C11–C12 unsaturated double bond or a C11–C12 epoxide.

Table II. Structures and Activities of Semisynthetic Analogues of Lophotoxin and Bipinnatin-B[a]



analogue	R_1	R_2	R_3	R_4	R_5	log EC_{50} ± SEM	*N*
lophotoxin	-CHO	- H		$-OCOCH_3$		2.86 ± 0.04	4
19	$-CH_2OH$	- H		$-OCOCH_3$		4.35 ± 0.13	4
bipinnatin-B	$-CH_3$	$-OCOCH_3$		$-OCOCH_3$		5.03 ± 0.03	6
20	$-CH_3$	$-OCOCH_3$		$-OCOCH_3$		5.68 ± 0.17	9
21	$-CH_3$	$-OCOCH_3$		-OH		4.59 ± 0.13	10
22	$-CH_3$	-OH		$-OCOCH_3$		4.09 ± 0.01	4
23	$-CH_3$	$-OCOCH_3$		$-OCOCH_3$		NA	2
24	$-CH_3$	$-OCOCH_3$		-OH		NA	6
25						NA	5



lophotoxin ethanol adduct **25**

[a] Toxin analogues were incubated with the receptor for 2 h prior to a 1-h incubation with a saturating concentration of [^{125}I]-α-bungarotoxin. The EC_{50} is the concentration of toxin (M) that inhibited 50% of the specifically bound [^{125}I]-α-bungarotoxin.

(where reversible drugs can be assayed for their ability to slow the initial rate of binding of [^{125}I]-α-bungarotoxin) or under "equilibrium" conditions (where irreversibly acting compounds can be assayed for their ability to inhibit the equilibrium binding of saturating concentrations of [^{125}I]-α-bungarotoxin).

When lophotoxin or bipinnatin-B were incubated with receptors for less than 5 min (in order to prevent significant accumulation of irreversibly inhibited receptor), they did not slow the initial rate of binding of [^{125}I]-α-bungarotoxin. Thus these toxins have relatively low reversible affinities for the receptor. This is perhaps somewhat surprising, since they exhibit a relatively high degree of specificity for the acetylcholine-recognition site of the nicotinic acetylcholine receptor.[1,2,8,9] Presumably their specificity arises in part from their specific covalent reaction with Tyr^{190}. In this sense, they may be similar to mechanism-based enzyme inhibitors that derive some of their specificity from a reversible affinity for a specific binding site and some from a specific catalytic event that transforms the inhibitor and irreversibly inhibits the enzyme.[14] This is in contrast

(14) Walsh, C. T. *Annu. Rev. Biochem.* 1984, *53*, 493.

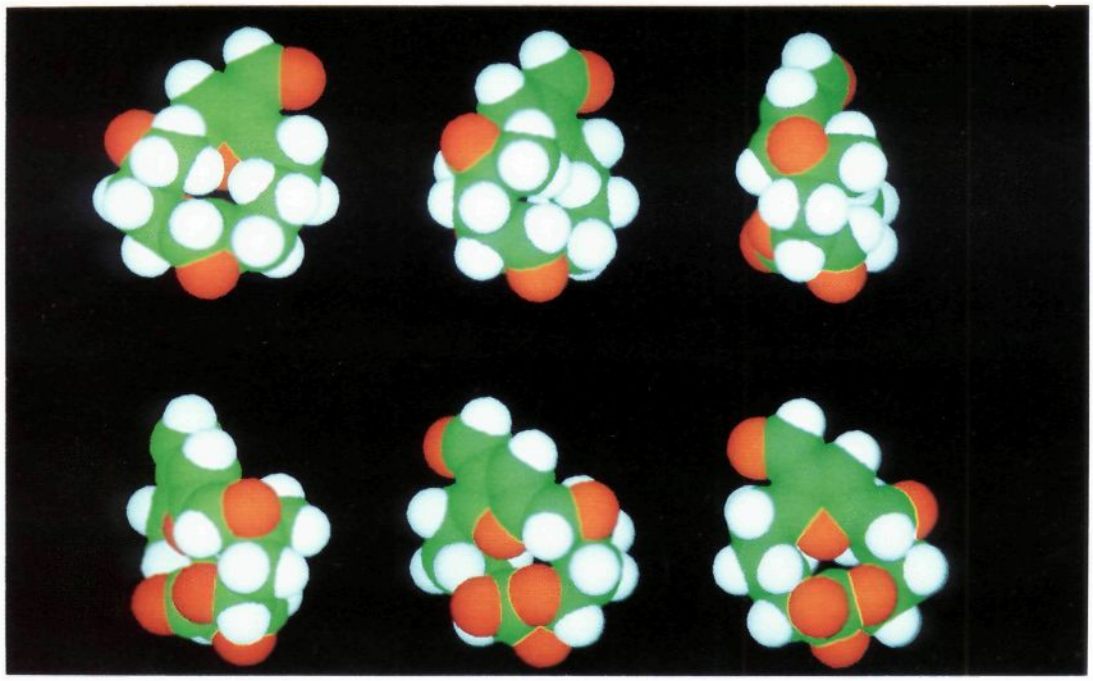


Figure 2. The minimum-energy conformation of lophotoxin. The minimum-energy conformation of lophotoxin was obtained by molecular mechanics and dynamics. Lophotoxin is shown being rotated at 36° intervals around the y axis to allow a 180° view of its three-dimensional conformation. The isopropenyl and acetoxy substituents at C1 and C13, respectively, were replaced with hydrogens to allow an unobstructed view of the 14-carbon cembrane ring. Note that the furan and lactone rings are oriented at ca. 90° angles to one another. Carbons are green, oxygens are red, and hydrogens are white.

to most active-site directed irreversible receptor antagonists, such as those containing acyl halides, isothiocyanates, or photoactive azides, that derive their specificity mainly from a high reversible affinity for the active site of the receptor.

Since the coral toxins do not have very high reversible affinity for the receptor, their ability to inhibit the equilibrium binding of [^{125}I]-α-bungarotoxin presumably reflects irreversible inhibition of the receptor. In support of this hypothesis, the apparent affinity of the active analogues increased with increasing incubation time. This observation is consistent with a progressive irreversible mechanism of action. The fact that the apparent affinity of lophotoxin increased from 1 to 16 h, while the apparent affinity of bipinnatin-B only increased from 1 to 4 h, suggests that lophotoxin is stable for at least 16 h and that bipinnatin-B is only stable for ca. 4 h.

All of the active toxins presumably inhibit the receptor by the same irreversible reaction mechanism involving covalent conjugation with Tyr^{190}. Thus the series of lophotoxin analogues tested in this study provided a means for evaluating the functional significance of different structural components of the toxins. Of the 25 analogues evaluated, 16 were found to inhibit the equilibrium binding of [^{125}I]-α-bungarotoxin while 9 were either completely inactive or exhibited only slight activity (less than 30% inhibition). Since the active analogues contained a variety of substituents at C1, C2, and C4, these regions are apparently not essential for either reversible association with the acetylcholine-recognition site or for covalent reaction with Tyr^{190}. In particular, this confirms earlier studies suggesting that the chemically reactive α,β-unsaturated aldehydes present in lophotoxin and bipinnatin-B are not required for the covalent reaction with Tyr^{190}.[9] In fact, reduction of these aldehydes to primary alcohols actually increased the apparent activity of both lophotoxin and bipinnatin-B. It is not known whether this increase in apparent activity resulted from an increase in reversible affinity, covalent reactivity, stability, or solubility.

Since the α,β-unsaturated aldehydes are clearly not required for covalent reaction with Tyr^{190}, attention was directed to the potentially reactive epoxides at C7–C8 and C11–C12. The role of the epoxide at C11–C12 was evaluated by comparison of lophotoxin, bipinnatin-A, bipinnatin-C, and bipinnatin-F, with deoxylophotoxin, bipinnatin-I, bipinnatin-H, and bipinnatin-G, respectively. The former four toxins have an epoxide at C11–C12 while the latter four toxins have a double bond at C11–C12. Since all eight analogues were active, the C11–C12 epoxide is presumably not required for binding to the acetylcholine-recognition site or for the covalent reaction with Tyr^{190}. However, since a double bond between C11 and C12 places the C11 carbon in conjugation with the lactone carbonyl, the C11 carbon cannot be completely ruled out as a potential site for nucleophilic attack of Tyr^{190}. In contrast to the C11–C12 epoxide, all of the active analogues contained an intact epoxide at C7–C8. The C7–C8 epoxide is therefore apparently essential for covalent reaction with the receptor, and it is therefore likely to be the site of nucleophilic attack by the hydroxyl group of Tyr^{190}.

Because the acetate ester at C13 is common to both lophotoxin and bipinnatin-B, and because it also shares structural similarity with the acetate ester of acetylcholine, it was hypothesized to be responsible for directing these toxins into the acetylcholine-recognition site on the nicotinic receptor.[2] This acetate ester, however, is apparently not absolutely required for activity, since hydrolysis of the ester only diminished the apparent activity about 12-fold (compare **20** and **21**). In contrast, reduction of the lactone carbonyl to a cyclic hemiacetal produced a much more dramatic decrease in activity (compare **20** and **23**), suggesting that the lactone carbonyl is more important for interaction with the acetylcholine-recognition site. The decrease in activity after reduction of the lactone carbonyl apparently resulted from a decrease in affinity rather than from a complete a loss of covalent reactivity, since **24**

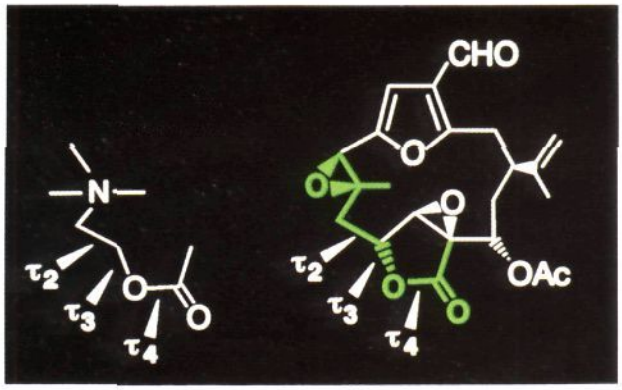


Figure 3. Comparison of the proposed lophotoxin pharmacophore with acetylcholine. The proposed pharmacophore region of lophotoxin is shown in green. It includes the C7–C8 epoxide and the lactone oxygens present in all of the active toxin analogues. The dihedral angles (τ_2–τ_4) that define the conformation of the toxin pharmacophore and the analogous dihedral angles that define the conformation of acetylcholine are identified.

displayed some modest activity after longer incubation times.

Comparison of active and inactive analogues allowed identification of a possible toxin pharmacophore extending from the epoxide at C7–C8 to the lactone carbonyl at C20 (Figure 3). This region is of interest because it is conserved in all of the active toxin analogues and because it reveals structural similarities to acetylcholine. For instance, the lactone oxygens of the toxins may mimic the acetate oxygens of acetylcholine, and the C7–C8 epoxide may have a steric volume, hydrophobicity, and charge distribution similar to the quaternary ammonium group of acetylcholine. The C7–C8 epoxide is also of interest because it provides a potential site for nucleophilic attack by Tyr^{190}.

Structural comparisons between the proposed toxin pharmacophore and acetylcholine suggest that the C8 carbon is analogous to the nitrogen of acetylcholine and that the C7 carbon is analogous to one of the three methyl groups on the nitrogen. The C7 carbon therefore represents the closest approach of Tyr^{190} to C8, and it is therefore the most likely site for nucleophilic attack by the hydroxyl group of Tyr^{190}. In support of this hypothesis, receptor modification and photoaffinity labeling studies have suggested that Tyr^{190} is located within the quaternary ammonium binding subsite of the acetylcholine-recognition site.[8,10] Since Cys^{192} and Cys^{193} are thought to be located in the vicinity of the acetate ester of acetylcholine,[15] these observations suggest that acetylcholine is oriented with its ester bond antiparallel to the amide bonds between Tyr^{190} and Cys^{192} (Figure 4).

Molecular modeling of the toxins provided a means for investigating their conformational mobility and for characterizing their minimum-energy conformation. The results suggest that the 14-carbon cembrane ring of the toxins is highly constrained, and that it can adopt only one well-defined minimum-energy conformation. Since the minimum-energy conformation of the toxins obtained by molecular mechanics and dynamics is similar to that found by X-ray crystallography,[4,5] this conformation is presumably the same as that adopted in solution. The dihedral angles and interatomic distances that define the proposed pharmacophore region are of particular interest. For instance, the carbonyl oxygen of the lactone is located ca. 4.8 Å away from the C8 carbon (Table III). This distance is similar to the proposed distance between the carbonyl oxygen and nitrogen in the active conformation of acetylcholine.[11,12] This observation supports the hypothesis that the proposed toxin pharmacophore mimics the conformation of acetylcholine as it is bound to the receptor.

Since the absolute conformation of the toxins is not yet known, the dihedral angles that define the toxin pharmacophore are only known relative to one another. The structural similarities between the toxin pharmacophore and acetylcholine, however, suggest that the dihedral angles of this region may provide important clues to the bound conformation of acetylcholine. For instance, τ_2 in the toxins is ca. 300° (Table III). This corresponds to one of the two (30° and 300°) symmetrical gauche conformations of acetylcholine. The gauche conformations have been shown to be the preferred low-energy conformations of acetylcholine in solution.[13] Although the low-energy

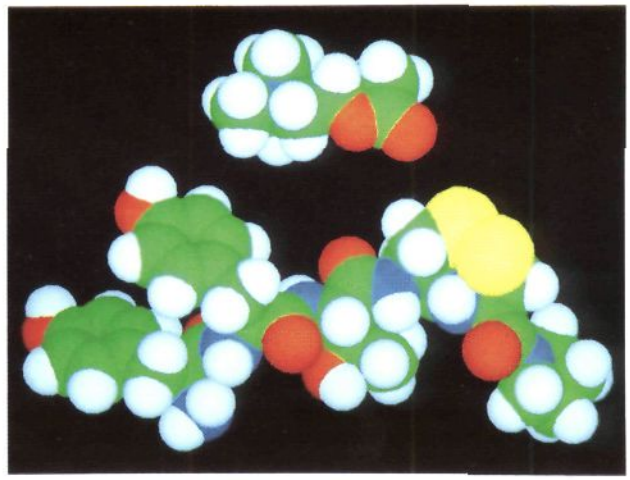


Figure 4. Orientation of acetylcholine and the α-subunit of the nicotinic acetylcholine receptor. Computer-generated images of acetylcholine (top) and amino acids Tyr^{189} through Pro^{194} from the acetylcholine-recognition site of the α-subunit of the *Torpedo* nicotinic acetylcholine receptor (bottom). Acetylcholine is shown in its presumed binding orientation, with the quaternary ammonium group near Tyr^{190} and the acetate ester group near Cys^{192}/Cys^{193}. This orientation dictates that the ester bond of acetylcholine is antiparallel to the amide bonds of the peptide. No specific conformation for acetylcholine or the α-subunit peptide is implied. Carbons are green, oxygens are red, nitrogens are blue, sulfurs are yellow, and hydrogens are white.

Table III. Dihedral Angles and Interatomic Distances That Define the Acetylcholine-like Pharmacophore of the Lophotoxin Family

structure	dihedral angles[a] τ_2	τ_3	τ_4	distance[b]
lophotoxin[c]	299	138	171	4.9
bipinnatin-B[c]	295	126	176	4.7
bipinnatin-C[c]	299	136	171	4.8
bipinnatin-C[d]	297	122	183	4.4

[a] Dihedral angles (in degrees) that define the conformation of the proposed toxin pharmacophore. The dihedral angles are identified in Figure 2 (τ_4 is directed toward the carbonyl oxygen). The dihedral angles are defined such that when looking down the bond between atoms 2 and 3 of the four atoms that compose a dihedral *angle, the magnitude of the angle represents the clockwise motion* of atom 1 relative to atom 4. [b] The distance (in angstroms) between the center of mass of the C8 carbon and the carbonyl oxygen of the lactone. [c] The minimum-energy conformations were obtained by molecular mechanics and dynamics as described in the Experimental Section. [d] The crystal structure of bipinnatin-C was determined by X-ray crystallography.[4]

(15) Kao, P. N.; Dwork, A. J.; Kaldany, R.-R. J.; Silver, M. L.; Wideman, J.; Stein, S.; Karlin, A. *J. Biol. Chem.* **1984**, *259*, 11662.

conformation for the acetate ester (τ_4) of acetylcholine is the extended conformation, the acetate ester of acetylcholine has been proposed to be bent in an almost lactone-like conformation when bound to the desensitized state of the receptor.[16] It is therefore of interest that the analogous oxygens of the toxin pharmacophore are bent in a lactone conformation.

In conclusion, structure/activity studies have helped to define structural regions of the lophotoxin family that are responsible for their unique biological activity. This has allowed identification of a potential toxin pharmacophore with structural similarity to acetylcholine. The toxin pharmacophore contains a potential hydrogen acceptor (the lactone carbonyl) and a potential site for reaction with Tyr^{190} (the C7 carbon). The structural similarities between the toxin pharmacophore and acetylcholine allow speculation concerning the bound conformation of acetylcholine. In addition, since it is possible to modify the toxins at positions remote from the proposed pharmacophore region (i.e. at C1, C2, C4, and C13) without resulting in a complete loss of covalent reactivity, it may be possible to prepare toxin analogues with improved solubility, affinity, or activity.

Experimental Section

Chemicals. [^{125}I]-α-Bungarotoxin (130 Ci/mmol) was obtained from New England Nuclear. Lophotoxin analogues were stored at −20 °C in either dimethyl sulfoxide or ethyl acetate.

Isolation of Naturally Occurring Lophotoxin Analogues. Corals were collected by hand using SCUBA. Lophotoxin and deoxylophotoxin were purified from *Lophogorgia chilensis* collected in Santa Barbara, CA. Lopholide was purified from *Lophogorgia rigida* collected in the Gulf of California. Pukalide, acetoxypukalide, and pukalide aldehyde were isolated from *Lophogorgia alba* collected in the Gulf of California. Bipinnatin-A, -B, -C, -E, and -F were isolated from *Pseudopterogorgia bipinnata* collected in the Bahamas. Bipinnatin-G, -H, and -I were isolated from an unidentified species of *Pseudopterogorgia* collected in the Bahamas.

Corals were dried and stored at −20 °C prior to being cut up into ca. 2-in. pieces and extracted twice with either 100% methylene chloride or 70:30 methylene chloride/methanol. The extracts were dried under vacuum and pure compounds were isolated by silica column chromatography under vacuum followed by normal-phase (silica) high-performance liquid chromatography (HPLC) or reverse-phase (C_{18}) HPLC.[1,3-7] During the isolation procedure the identity and the purity of the analogues were assessed by HPLC, thin-layer chromatography (TLC), ^{1}H NMR, and ^{13}C NMR.[1,3-8] However, because HPLC and TLC provided only limited information concerning the identity and purity of the analogues, the final determination of both structure and purity was made by inspection of the ^{1}H NMR and ^{13}C NMR spectra. Since the NMR spectra are unique, compounds with unassigned NMR peaks were subjected to further purification. The ^{1}H NMR and ^{13}C NMR results are summarized in the supplementary material. Most of the analogues were isolated on more than one occasion from separate extractions.

Preparation of Semisynthetic Lophotoxin Analogues. The semisynthetic analogue **19** was prepared by adding 4 mg of sodium borohydride to 10 mg of lophotoxin (**1**) dissolved in 5.0 mL of dry methanol. The reaction was stirred at 0 °C, and complete reduction was observed by thin-layer chromatography after 1 h. The methanol was evaporated and the mixture was partitioned between methylene chloride and water. Magnesium sulfate was added to the methylene chloride fraction, the solvent was removed under vacuum, and the reduction product (**19**) was isolated in 94% yield by normal-phase HPLC. The semisynthetic analogues **20–24** were prepared by adding a molar excess of lithium aluminum hydride to 160 mg of bipinnatin-B (**2**) dissolved in 30 mL of dry tetrahydrofuran. The reaction was stirred at −78 °C and the production of products was followed by thin-layer chromatography. After 15 min the reaction was quenched with water, acidified with 1 N HCl, and extracted twice with ethyl ether. The reaction products were isolated by silica-column chromatography under vacuum followed by normal-phase HPLC. Analogues **20–24** were isolated in 14%, 1.4%, 2.5%, 1.3%, and 0.6% yields, respectively. During the isolation procedure the identity and the purity of the analogues were assessed by HPLC, TLC, ^{1}H NMR, and ^{13}C NMR.[1,3-8] Final determination of both structure and purity was made by inspection of the ^{1}H NMR and ^{13}C NMR spectra. Compounds with unassigned ^{1}H NMR peaks were subjected to further purification. The ^{1}H NMR and ^{13}C NMR results are summarized in the supplementary material. The reductions were each performed more than once.

Radioligand Binding Assays. Membranes enriched in nicotinic acetylcholine receptors were purified from the electric organs of *T. californica* as described previously.[2,5,9] Membranes (ca. 1.0 pmol of α-toxin sites) were suspended in 100 μL of 10 mM sodium phosphate buffer (pH 7.4) containing 1.0 mM EDTA and 1.0 mM EGTA. Reaction with the coral toxin analogues was initiated by the addition of 1.0–2.5 μL of the analogue in 100% dimethyl sulfoxide. The coral toxin analogues were allowed to react with the receptor-containing membranes for various times at 23 °C. A 2–5-fold molar excess of [^{125}I]-α-bungarotoxin in 25 μL of buffer was then added, and the [^{125}I]-α-bungarotoxin was allowed to bind to the receptor for 15 s (initial rate assay) or for 60 min (equilibrium assay) at 23 °C. Duplicate 50-μL aliquots were removed and spotted onto 2.5 cm Whatman DE81 ion-exchange filters. The filters were washed twice for 10 min by immersion in 600 mL of 10 mM phosphate buffer (pH 7.4) containing 0.1% (w/w) Triton X-100. They were then blotted dry between paper towels and bound radioactivity was determined in a γ counter. The specifically bound [^{125}I]-α-bungarotoxin was usually 70–90% of the total [^{125}I]-α-bungarotoxin bound. Dose–response curves consisted of at least five different concentrations, and they were analyzed by least-squares nonlinear regression assuming a single noncooperative binding site. Specifically bound [^{125}I]-α-bungarotoxin was defined as the total amount bound minus the amount bound after incubation of the receptors with 200 μM bipinnatin-B for at least 2 h. The results are reported as the apparent EC_{50}, representing the concentration of toxin (M) that inhibited 50% of the specifically bound [^{125}I]-α-bungarotoxin.

Molecular Modeling. Lophotoxin, bipinnatin-B, and bipinnatin-C were constructed on a Silicon Graphics Iris Personal Workstation using the program insight (version 2.4) from Biosym (San Diego, CA). Although the relative stereochemistry of each of the asymmetric centers of the coral toxins is known, the absolute stereochemistry is not known.[1,3-7] Therefore, the structures constructed represented the commonly depicted isomer.[1] Minimum-energy conformations were obtained by molecular mechanics and molecular dynamics with the constant valence force field of the program Discover (version 2.4) from Biosym. The structures were initially minimized for 100 iterations with the steepest descents algorithm. This was followed by a 30-ps dynamics simulation using a step size of 1.0 fs at 1000 K. The structures obtained every picosecond along the dynamics trajectory were minimized with the quasi-Newton–Raphson (va09a) algorithm until they converged at less than 0.01 kcal/mol.

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Supplementary Material Available: ^{1}H NMR and ^{13}C NMR data for the naturally occurring and chemically modified lophotoxin analogues and Cartesian coordinates for the minimum-energy conformations of lophotoxin, bipinnatin-B, and bipinnatin-C (12 pages). Ordering information is given on any current masthead page.

(16) Behling, R. W.; Yamane, T.; Navon, G.; Jelinski, L. W. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6721.