Irreversible Inhibitors of Nicotinic Acetylcholine Receptors: Isolation and Structural Characterization of the Biologically Active Solvolysis Products of Bipinnatin-A and Bipinnatin-C

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The lophotoxins irreversibly inhibit nicotinic acetylcholine receptors by covalent modification of Tyr^{190} in the α-subunits of the receptor. Previous studies have shown that the naturally occurring lophotoxin analogs bipinnatin-A, -B, and -C are actually inactive protoxins and that their ability to irreversibly inhibit nicotinic receptors is enhanced by preincubation in buffer. However, the ability of lophotoxin to irreversibly inhibit nicotinic receptors does not appear to be enhanced by preincubation in buffer. These observations led to the current effort to isolate and determine the structures of biologically active bipinnatins. Disappearance of the lophotoxins from solution followed a simple first-order exponential decay function. Lophotoxin, however, was approximately 40-fold more stable then bipinnatin-A, -B, or -C. Solvolysis of the bipinnatins, but not of lophotoxin, resulted in production of an equimolar amount of acetic acid at a rate similar to the rate of solvolysis, suggesting that the initial event in solvolysis of these toxins involves hydrolysis of an acetate ester. Proton NMR and fast-atom bombardment mass spectroscopy were used to confirm the structures of the active solvolysis products of bipinnatin-A and -C. Their structures and the relative pH insensitivity of the solvolysis reaction suggest that biological activation of the bipinnatins may proceed through an S_N1 type of substitution reaction involving elimination of acetate followed by reaction of a carbocation intermediate with solvent.

Introduction

The lophotoxins are a family of naturally occurring nicotinic acetylcholine receptor antagonists that irreversibly inhibit both neuronal and muscle subtypes of the receptor.[1–9] Lophotoxin and the bipinnatins (including bipinnatin-A, -B, -C, -D, -E, -F, -G, -H, and -I) are all members of this family (Figure 1). All of the active lophotoxins inhibit nicotinic acetylcholine receptors irreversibly by covalent modification of Tyr^{190} in the α-subunits (αTyr^{190}) of the receptor.[10] These are the only known naturally occurring toxins that utilize a covalent mechanism of action to irreversibly inhibit a neurotransmitter receptor. Structure–activity and molecular modeling studies have lead to the hypothesis that the conserved lactone oxygens and the C7–C8 epoxide are important for the covalent reaction between αTyr^{190} and these toxins.[7] The structural motif defined by the lactone oxygens and the C7–C8 epoxide was proposed to allow for correct orientation of the lophotoxins in the acetylcholine-binding pocket and for the subsequent nucleophilic attack of the αTyr^{190} hydroxyl toward the C7 carbon of the epoxide. However, the actual mechanism involved in covalent modification of αTyr^{190} by the lophotoxins remains unknown.

The apparent affinity (IC_{50}) of lophotoxin for *Torpedo* nicotinic acetylcholine receptors increases as the duration of incubation of lophotoxin with the receptors increases from 1 to 16 h.[7] This observation is consistent with a relatively stable irreversible inhibitor. In contrast, the apparent affinity (IC_{50}) of bipinnatin-B for *Torpedo* nicotinic receptors does not continue to increase after approximately 4 h of exposure to receptors, suggesting that the bipinnatins are unstable in buffer. Additional experiments investigated and defined the kinetics of irreversible inhibition of nicotinic acetylcholine receptors on intact BC_3H-1 cells.[11,12] Preincubation of bipinnatin-A, -B, and -C in buffer results in an increase in the subsequent rate of irreversible inhibition of nicotinic receptors, demonstrating that these toxins are actually inactive protoxins that are converted into biologically active toxins by incubation in buffer.[11] Analysis of the pseudo-first-order rate constants for irreversible inhibition reaches a maximum within approxi-

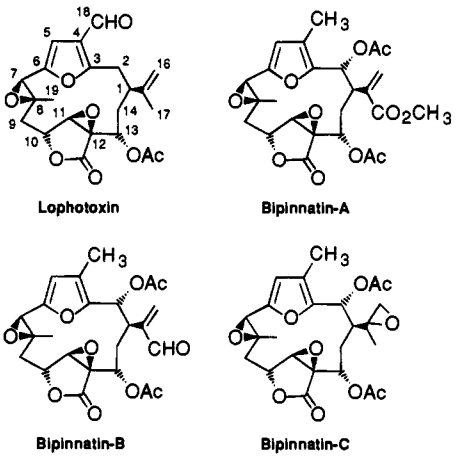

Figure 1. Structures of lophotoxin and bipinnatin-A, -B, and -C.

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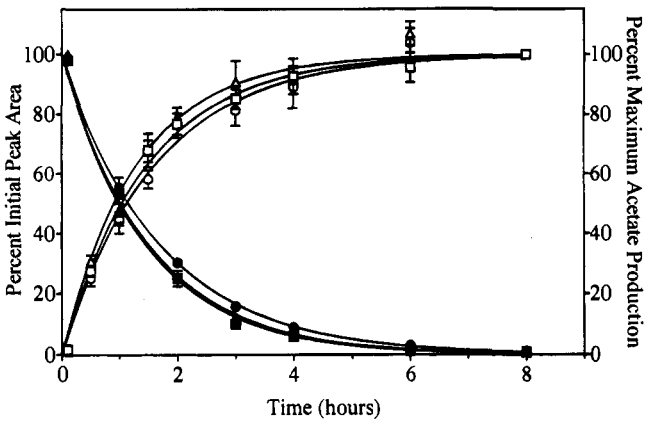

Figure 2. Solvolysis and acetic acid production during incubation of the bipinnatins in aqueous buffer. Bipinnatin-A (●), -B (■), and -C (▲) were incubated for the indicated times in phosphate buffer (pH 7.4) prior to separation by reverse-phase HPLC. Peak areas associated with the toxins were integrated and are presented as a percent of the initial peak area. The data were fit by nonlinear regression to a single-exponential decay function. Each data point represents the mean ± SEM of three to nine independent experiments. Bipinnatin-A (○), -B (□), and -C (△) were incubated in phosphate buffer (pH 7.4) for the indicated times, and the amount of acetic acid produced was determined over the next 5 min. The data are presented as a percent of the maximum amount of acetic acid produced and were fit by nonlinear regression to a simple exponential growth function. Each data point represents the mean ± SEM of three to five independent experiments.

mately 2 h of preincubation in buffer. In contrast to the bipinnatins, lophotoxin appears to be relatively stable since there is no change in its activity after 8 h of preincubation in buffer.[12]

Previous structure–activity studies of the lophotoxins were performed before it was determined that some of the lophotoxins are actually inactive protoxins.[5,7,11] The proposed mechanism for covalent modification of αTyr^{190} involving the toxin C7–C8 epoxide was therefore based on structures of inactive protoxins.[7] If the C7–C8 epoxide is modified in the biologically active toxins, then this mechanism may not be valid. A better understanding of the structures of the biologically active toxins and the reaction mechanism involved in activation of the bipinnatins is a prerequisite for understanding the novel mechanism by which these toxins irreversibly inhibit nicotinic acetylcholine receptors. The present study was therefore undertaken to isolate and characterize the structures of biologically active toxins produced by incubation of the bipinnatins in buffer.

Results

Earlier work has shown that bipinnatin-A, -B, and -C are inactive protoxins that are converted into active irreversible inhibitors of nicotinic acetylcholine receptors by preincubation in aqueous buffer.[11] In contrast to the bipinnatins, the biological activity of lophotoxin is relatively unaffected by preincubation in buffer.[12] The chemical stability of lophotoxin and bipinnatin-A, -B, and -C in aqueous buffer was therefore investigated directly. The toxins were incubated in 25 mM phosphate buffer (pH 7.4) containing 1% DMSO and then analyzed by reverse-phase HPLC. Solvolysis of the toxins followed a simple first-order exponential decay function (Figure 2). Each of the three bipinnatins

Table 1. Rate Constants of Solvolysis and Acetate Production for the Lophotoxins

toxin	k_{solv} (h^{-1})[a]	half-life (h)	acetate production (h^{-1})	half-life (h)
bipinnatin-A	0.638 ± 0.013	1.08 ± 0.02	0.670 ± 0.030	1.04 ± 0.05
bipinnatin-B	0.719 ± 0.030	0.97 ± 0.04	0.735 ± 0.076	0.97 ± 0.11
bipinnatin-C	0.596 ± 0.025	1.17 ± 0.05	0.589 ± 0.062	1.24 ± 0.16
lophotoxin	0.017 ± 0.001	40.0 ± 0.83	ND[b]	ND

[a] Rates were determined by nonlinear regression of the data to a single-exponential decay function for solvolysis of the lophotoxins (k_{solv}) or to a single-exponential growth function for the production of acetic acid. All reported values are the mean ± SEM of three to nine separate and independent experiments. [b] ND = not determined.

disappeared from solution at approximately the same rate with $t_{1/2}$'s of approximately 1 h (Table 1). Similar results were obtained in 2.5 and 0.25 mM phosphate buffer and 25 mM Hepes (data not shown). In contrast, lophotoxin was approximately 40 times more stable with a $t_{1/2}$ of approximately 40 h (Table 1). These results are consistent with earlier observations demonstrating that the biological activity of the bipinnatins is enhanced by preincubation in buffer, whereas the activity of lophotoxin is relatively unaffected by preincubation in buffer.[7,11,12]

Solvolysis of the lophotoxins could conceivably result from several different reactions, including reactions that involve the epoxides, the acetate esters, or the lactone. However, incubation of bipinnatin-A, -B, or -C in buffer resulted in the generation of stoichiometric amounts of acetic acid at rates similar to their rates of solvolysis (Figure 2, Table 1). These results suggest that the initial event in the transformation of the bipinnatins into biologically active toxins involves hydrolysis of either the C2 or C13 acetate ester. Hydrolysis of either acetate ester could occur by S_N2-mediated attack of hydroxide at the carbonyl carbon of the ester or by S_N1-mediated cission of the C–O bond between the ester oxygen and the toxin. An S_N2 mechanism predicts that the rate of solvolysis should be directly proportional to the concentration of nucleophile. However, a 10^6-fold change in the concentration of hydroxide (from pH 4 to pH 10) resulted in less than a 2-fold change in the rate of solvolysis of bipinnatin-C (Figure 3). Similar results were obtained with bipinnatin-A (data not shown). These results suggest that solvolysis of the bipinnatins may not be mediated by S_N2 attack of base at the carbonyl carbon of one of the esters.

Lophotoxin and bipinnatin-A, -B, and -C each contain an acetate ester adjacent to the lactone ring at the C13 carbon (Figure 1). Unlike lophotoxin, however, the bipinnatins also contain a second acetate ester adjacent to the furan ring at the C2 carbon. Therefore, comparison of acetic acid generated by solvolysis of lophotoxin versus the bipinnatins can be used to determine which of the two bipinnatin acetate esters is more labile. After 8 h of incubation at pH 7.4, each of the bipinnatins produced approximately 1 mol of acetic acid/mol of toxin (Figure 4). In contrast, lophotoxin and acetylcholine produced only 0.17 and 0.22 mol of acetic acid/mol, respectively. Incubation in buffer for 8 h at pH 10 resulted in only modest increases in the amount of acetic acid generated from either the bipinnatins or lophotoxin but caused the complete hydrolysis of acetylcholine. However, incubation for 8 h in 5 mM NaOH resulted in

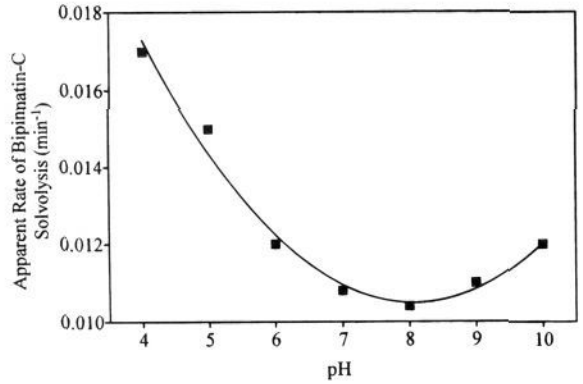

Figure 3. Effect of pH on the solvolysis of bipinnatin-C. Bipinnatin-C (100 μM) was incubated for 2 h in 25 mM phosphate buffer (pH 4–8) or 25 mM borate buffer (pH 9 and 10) prior to separation by reverse-phase HPLC. First-order rates of solvolysis were determined by quantitating peak areas corresponding to bipinnatin-C assuming a simple exponential decay.

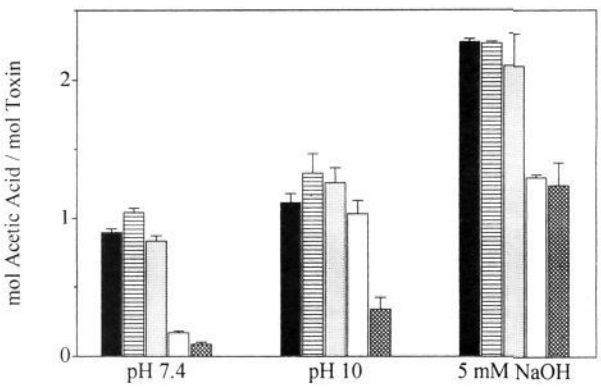

Figure 4. Effect of pH on the generation of acetic acid by the bipinnatins, lophotoxin, and acetylcholine. Bipinnatin-A, -B, and -C, acetylcholine, and lophotoxin (represented from left to right in the figure) were each incubated at 100 μM in phosphate buffer (pH 7.4 or 10) or 5 mM NaOH for 8 h before determination of acetic acid over the next 2 h. Each bar represents the mean ± SEM of three independent experiments.

approximately 2 mol of acetic acid/mol of bipinnatin and approximately 1 mol of acetic acid/mol of lophotoxin. Thus all four toxins contain one acetate ester (at the C13 carbon) that is more stable to base hydrolysis then the acetate ester of acetylcholine. In addition, the three bipinnatins also contain a second acetate ester (at the C2 carbon) that is more labile then the acetate ester of acetylcholine.

Although hydrolysis of the C2 acetate ester appears to be the initial solvolysis reaction of the bipinnatins, incubation of each of the bipinnatins in aqueous buffer resulted in a complex mixture of several unique solvolysis products that could be separated by reverse-phase HPLC (Figure 5). Except for bipinnatin-B(X), all of the solvolysis products appeared to be more hydrophilic than the native bipinnatins since they were retarded to a lesser extent on the reverse-phase HPLC column. Examination of the chromatograms for each of the bipinnatins after 0.5–8 h of preincubation in buffer revealed the appearance and disappearance of each product over time (data not shown). Some products, such as bipinnatin-A(IV), were more abundant earlier in the solvolysis, while other products, such as bipinnatin-A(VIII), were more abundant at later times.

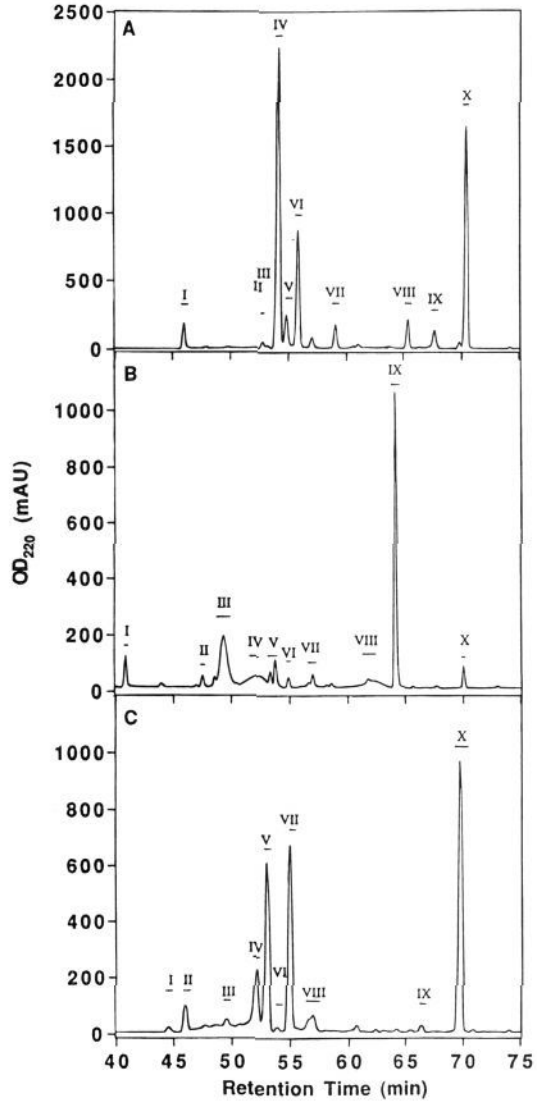

Figure 5. Representative reverse-phase HPLC chromatograms of the bipinnatins after 2 h of incubation in buffer. Bipinnatin-A (panel A), -B (panel B), and -C (panel C) were incubated for 2 h in phosphate buffer (pH 7.4), and the solvolysis products were separated by reverse-phase HPLC. The HPLC chromatograms were monitored at 220 nm from 0 to 120 min, but peaks were only detected between 40 and 75 min. Each of the roman numerals and bars denotes the peaks that were collected, immediately frozen in dry ice–methanol, and lyophilized for subsequent analysis. Peaks A(X), B(IX), and C(X) were identified as bipinnatin-A, -B, and -C, respectively, on the basis of retention time and UV–vis spectra.

These results demonstrate that the bipinnatins contain more than one labile bond and that several different solvolysis reactions occur over a similar time frame (1–8 h).

Although, the initial event in solvolysis of the bipinnatins appears to involve the C2 acetate ester, it is possible that biologically active toxins are the result of more than one solvolysis reaction. To determine which solvolysis products were responsible for the biological activity associated with the bipinnatins, each product was isolated by reverse-phase HPLC and assayed for its ability to irreversibly inhibit the binding of [^{125}I]-α-bungarotoxin to nicotinic receptors on BC_3H-1 cells. The bipinnatins were each preincubated in buffer for 0, 1, 2, 4, or 8 h to ensure that the maximum possible amount

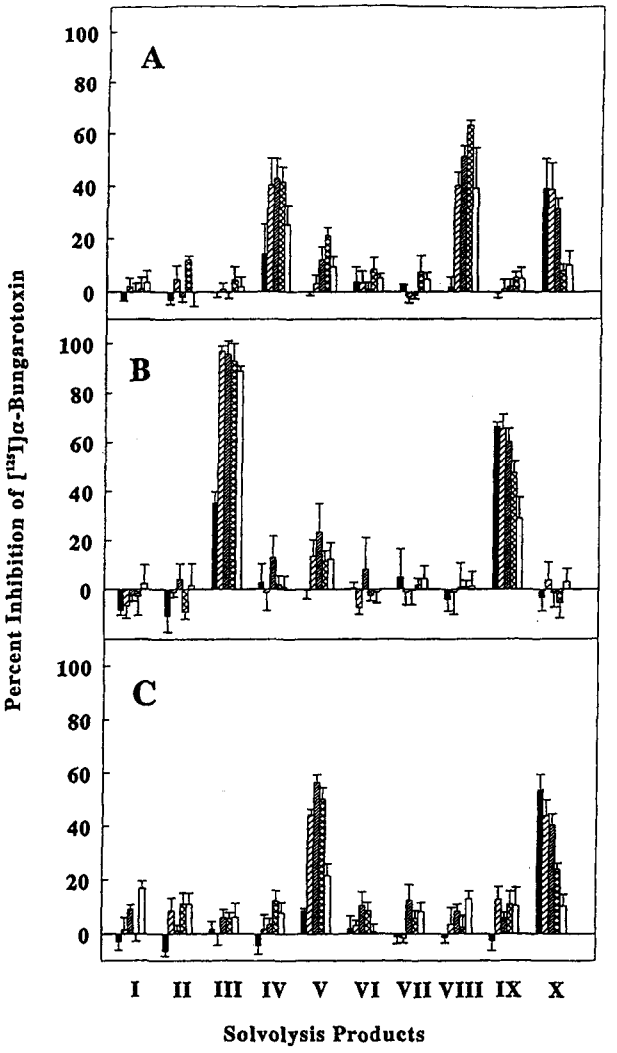

Figure 6. Identification of biologically active solvolysis products. Bipinnatin-A (panel A), -B (panel B), and -C (panel C) were preincubated in buffer for 0 (solid bar), 1 (hatched bar), 2 (double-hatched bar), 4 (crossed bar), and 8 (open bar) h prior to separation of solvolysis products by reverse-phase HPLC. Each of the solvolysis products (I–X, as indicated in Figure 5) was assayed for its ability to irreversibly inhibit the binding of [^{125}I]-α-bungarotoxin to nicotinic acetylcholine receptors on BC_3H-1. Each bar represents the mean ± SEM of three to five independent experiments.

of each product was collected and assayed for biological activity (Figure 6). Bipinnatin-B and -C each appeared to produce only one active product, labeled B(III) and C(V), respectively, while bipinnatin-A apparently produced two active products, labeled A(IV) and A(VIII). The active solvolysis products eluted at distinct retention times indicating that they were all structurally unique compounds. As expected, the apparent activity associated with bipinnatin-A, -B, and -C decreased as the preincubation time increased, whereas the apparent activity associated with the active solvolysis products A(IV), A(VIII), B(III), and C(V) increased with an increase in the preincubation time.

Incubation of bipinnatin-A in buffer resulted in two biologically active solvolysis products. Bipinnatin-A(IV) predominated and reached a maximum concentration during the initial phase of solvolysis (within approximately 2 h), while bipinnatin-A(VIII) accumulated later. The stability and activity of bipinnatin-A(IV) was therefore investigated in greater detail to ensure that

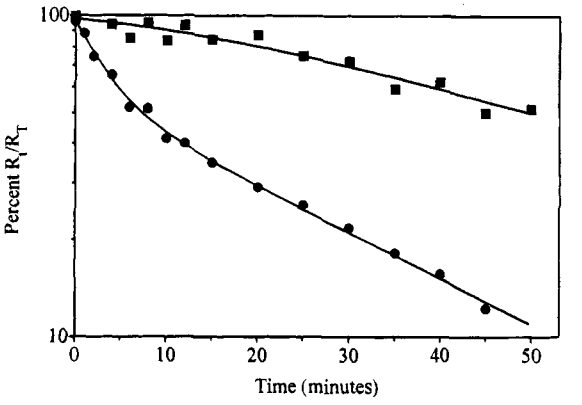

Figure 7. Irreversible inhibition of [^{125}I]-α-bungarotoxin binding to nicotinic acetylcholine receptors on intact BC_3H-1 by bipinnatin-A and -A(IV). Bipinnatin-A (■) and -A(IV) (●) were diluted to 100 μM toxin (1% DMSO) with assay buffer and added immediately to cells. At the indicated times, the toxin solution was removed and the number of remaining receptors determined with a saturating concentration of [^{125}I]-α-bungarotoxin. The *y*-axis represents the log of the percentage of specifically bound [^{125}I]-α-bungarotoxin at each time point (R_t) to the amount of [^{125}I]-α-bungarotoxin specifically bound at zero time (R_T). Data points shown are the means of at least two independent experiments. The data for bipinnatin-A(IV) were fit by nonlinear regression to a double-exponential decay function, where 41.5 ± 0.7% of the receptors were inhibited at a rate of 0.29 ± 0.14 min^{-1} and 57.5 ± 2.1% were inhibited at a rate of 0.033 ± 0.007 min^{-1} (mean ± SD).

it was in fact a biologically active compound and that it did not require further solvolysis to bipinnatin-A(VIII). Bipinnatin-A(IV) was more stable than bipinnatin-A with an apparent first-order rate of solvolysis of 0.399 ± 0.081 h^{-1} ($t_{1/2}$= 1.74 ± 0.36 h). As expected for an inactive protoxin, bipinnatin-A was initially inactive, and the rate of irreversible inhibition of nicotinic receptors increased with increasing duration in buffer (Figure 7). In contrast, bipinnatin-A(IV) was immediately active as an irreversible inhibitor without any evidence for a delay in activity. Thus further solvolysis of bipinnatin-A(IV) was not required for this compound to irreversibly inhibit nicotinic receptors. Lophotoxin and bipinnatin-A, -B, and -C have been shown previously to react preferentially with the acetylcholine-binding site located at the α/δ subunit interface.[11–14] This selective interaction resulted in two distinct apparent rates of irreversible inhibition that differed by almost 10-fold (Figure 7). The results obtained with purified bipinnatin-A(IV) were similar to the rates of irreversible inhibition reported for the unfractionated solvolysis mixture of bipinnatin-A.[11]

The two most abundant biologically active solvolysis products, bipinnatin-A(IV) and -C(V), were isolated in sufficient quantities for structural characterization by FAB-MS and proton NMR. Although the molecular ions were not detected, FAB-MS of bipinnatin-A(IV) and -C(V) in thioglycerol resulted in strong signals from fragment ions consistent with the loss of a hydroxyl and an acetate ester from compounds of mass 462 and 434, respectively. Addition of either sodium or potassium acetate to the thioglycerol resulted in strong signals from adducts of sodium or potassium with compounds of mass 462 and 434. These results are consistent with the hypothesis that bipinnatin-A(IV) and -C(V) differ

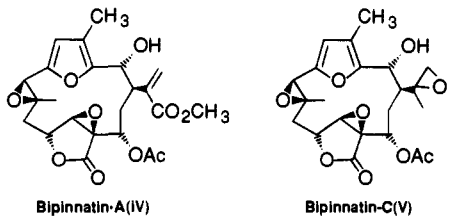

Figure 8. Structures of the active solvolysis products bipinnatin-A(IV) and -C(V).

Table 2. Proton NMR Chemical Shifts and Coupling Constants for Bipinnatin-A, -A(IV), -C, and -C(V)[a]

	bipinnatins			
proton	A	A(IV)	C	C(V)
1	4.53 dd (10, 10)	4.42 dd (10, 10)	3.99 dd (11, 11)	3.37 dd (11,11)
2	6.12 d (10)	4.74 m	5.75 d (11)	4.65 dd (11, 4)[b]
5	5.93 s	5.94 s	5.99 s	5.99 s
7	4.11 s	4.10 s	4.11 s	4.07 s
9(α)	2.05 dd (15,4)	2.05 dd (15,4)	2.05 dd (15, 4)	2.04 dd (15, 3)
9(β)	2.51 dd (15,3)	2.49 dd (15, 4)	2.50 dd (15, 3)	2.49 dd (15, 3)
10	4.75 dd (4, 3)	4.76 dd (4, 4)	4.76 dd (4, 3)	4.94 dd (3, 3)
11	4.13 s	4.12 s	4.15 s	4.14 s
13	4.85 d (7)	4.86 d (7)	5.08 d (7)	5.04 d (7)
14(α)	2.79 ddd (16, 10, 7)	2.68 ddd (14, 10, 7)	1.90 ddd (15, 11, 7)	2.04 ddd (15, 11, 7)
14(β)	1.35 d (16)	1.48 d (14)	1.00 d (15)	1.23 d (15)
16(α)	6.32 s	6.39 s	2.76 d (4)	3.22 d (5)
16(β)	6.00 s	6.04 s	2.53 d (4)	2.70 d (5)
17			1.60 s	1.46 s
18	1.96 s	2.01 s	1.99 s	2.05 s
19	1.11 s	1.09 s	1.06 s	1.07 s
CH_3COO-	2.03 s	2.04 s	2.19 s	2.17 s
CH_3COO-	1.99 s		2.09 s	
-CO_2CH_3	3.79 s	3.82 s		

[a] Protons are numbered as in Figure 1. Chemical shifts (δ) are given in ppm relative to tetramethylsilane. Coupling constants (J) are given in parentheses in hertz (Hz), where s = singlet, d = doublet, and m = unresolved multiplet. The NMR spectra of bipinnatin-A and -C were similar to previously published results.[6,7] [b] Addition of D_2O converts this signal to a doublet with J = 11 Hz.

from bipinnatin-A and -C, respectively, by substitution of a hydroxyl for one of the two acetate esters (Figure 8).

The active solvolysis products, bipinnatin-A(IV) and -C(V), were further analyzed by proton NMR and their spectra compared to those of bipinnatin-A and -C (Table 2). The most prominent difference in the NMR spectra of the products was the absence of a methyl resonance associated with one of the two acetate esters. A second difference in the spectra of the products was an upfield shift of approximately 1.3 ppm for the resonance of the C2 proton. In bipinnatin-A and -C, the acetate ester at the C2 carbon causes the C2 proton to resonate at approximately 6 ppm. However, in the solvolysis products bipinnatin-A(IV) and -C(V), the C2 proton signal was observed at approximately 4.7 ppm. In addition, the C2 proton of bipinnatin-C is a doublet (J = 11 Hz), since it is coupled to the C1 proton.[7,10] However, in bipinnatin-C(V), the C2 proton was a double doublet (J = 11, 4 Hz). NMR spectra obtained in the presence of deuterated water (to eliminate coupling with solvent exchangeable protons) removed the additional 4 Hz coupling of the C2 proton. These results indicate that solvolysis of the bipinnatins results in replacement of the C2 acetate ester with a hydroxyl group. All of the other proton resonances and coupling constants were similar, indicating that there were no additional structural alterations. In particular, it is notable that the coupling constant between the C1 and C2 protons (J = 11 Hz) did not change, indicating that the stereochemistry at the C2 carbon is the same in both the parent toxins and the active solvolysis products (Figure 8).

Discussion

Lophotoxin and bipinnatin-A, -B, and -C undergo solvolysis in aqueous solution by what appear to be first-order processes. However, the rates of solvolysis of the bipinnatins were similar (approximately 0.65 h^{-1}), whereas lophotoxin was approximately 40-fold more stable with a solvolysis rate constant of 0.017 h^{-1}. The lophotoxins are highly constrained, and they contain several potentially reactive bonds, including the C2 and C13 acetate esters, the C7–C8 and C11–C12 epoxides, and the lactone ring. It is impossible to identify *a priori* the most labile bonds. However, the C2 ester and the C7–C8 epoxide might be expected to be particularly reactive due to their proximity to the furan ring.[15,16] In fact, since solvolysis of simple phenyl and furyl epoxides at neutral pH is faster than that of the corresponding phenyl and furyl acetates, solvolysis of the C7–C8 epoxide might be expected to occur prior to solvolysis of the C2 acetate ester.[15–17] However, the rates of solvolysis and production of acetic acid were nearly identical, suggesting that the initial event in solvolysis of the bipinnatins involves hydrolysis of either the C2 or C13 acetate ester. Comparison of acetic acid production by lophotoxin and the bipinnatins suggested that the C2 acetate ester was the more labile of the two acetate esters. In fact, the C13 ester actually appeared to be more resistant to hydrolysis than the ester of acetylcholine, suggesting reduced accessibility of the C13 ester to solvent.

Earlier work on the kinetics of irreversible inhibition of nicotinic acetylcholine receptors by the bipinnatins used an unfractionated solvolysis mixture to demonstrate that the rate of irreversible inhibition of nicotinic receptors increases after preincubation of the toxins in buffer.[11] However, only isolation and structural characterization of biologically active solvolysis products can define the chemical transformation(s) responsible for this phenomenon. For instance, although hydrolysis of the C2 acetate ester appears to be the initial solvolytic event, these toxins clearly undergo several different solvolysis reactions, and additional chemical transformations could be required to produce biologically active toxins. Isolation of each of the products produced by solvolysis of the bipinnatins allowed identification of the active solvolysis products responsible for irreversible inhibition of nicotinic receptors. Bipinnatin-B and -C each produced only one active solvolysis product (bipinnatin-B(III) and -C(V), respectively), whereas bipinnatin-A produced two active products (bipinnatin-A(IV) and -A(VIII)). Since bipinnatin-A(IV) appeared prior to bipinnatin-A(VIII), and since bipinnatin-A(IV) was immediately active as an irreversible inhibitor, the transformation that produced bipinnatin-A(IV) was clearly *necessary and sufficient* to produce an active irreversible inhibitor. Bipinnatin-A(VIII) presumably results from an additional solvolysis reaction that does not result in diminished biological activity.

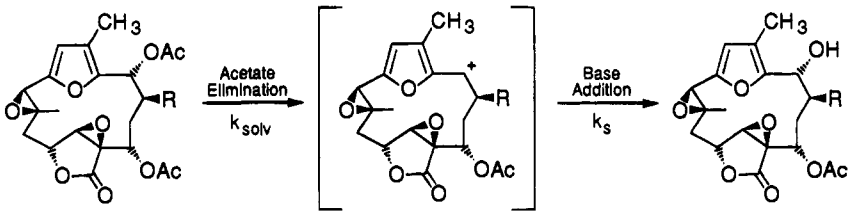

Figure 9. Proposed S_N1 substitution reaction mechanism for the generation of active bipinnatins from inactive protoxins. The rate of solvolysis is represented by k_{solv}, while k_s represents the rate of reaction of a carbocation intermediate with hydroxide. The reactive carbocation intermediate could be stabilized by resonance with the adjacent furan ring.

The structures of the most abundant active solvolysis products, bipinnatin-A(IV) and -C(V), were deduced from FAB-MS and proton NMR spectroscopy. Bipinnatin-A(IV) and -C(V) contain only the more stable acetate ester at the C13 carbon and not the more labile acetate ester at the C2 position. Because the C2 acetate is not conserved in all active lophotoxin analogs, previous structure–activity studies concluded that it is not required for biological activity.[7] In fact, the current results demonstrate that the presence of this ester actually inhibits the activity of these toxins. Release of this acetate ester may facilitate association of the bipinnatins with the receptor by removing steric hindrance. Alternatively, removal of the bulky ester may allow for an orientation of the furan ring that makes the C7–C8 epoxide more reactive. This is an attractive hypothesis, since a covalent reaction between αTyr[190] and the C7–C8 epoxide was proposed previously.[7] In addition, replacement of an acetate ester with a hydroxyl may convert a relatively hydrophobic protoxin into a more hydrophilic toxin, thereby increasing the effective concentration of the soluble toxin. An increase in hydrophilicity may also facilitate release and solubilization of these toxins from their presumably hydrophobic storage environment in the coral after ingestion by predators.

Solvolysis of the bipinnatins was relatively independent of pH, suggesting that the reaction mechanism is not a simple base-mediated S_N2 reaction. Several possible reaction mechanisms are consistent with the relative lack of dependence on pH, including an S_N1 mechanism involving a rate-limiting elimination reaction and a carbocation intermediate followed by reaction of the carbocation with hydroxide (Figure 9). The carbocation intermediate of an S_N1 type of substitution reaction could presumably be stabilized by resonance between the C2 carbon and the adjacent electron-rich furan ring.[15] This phenomenon is well documented for a variety of aromatic compounds, including epoxy benzo[α]pyrenes, styrene oxides, 1-arylethyl acetates, substituted 1-phenylethyl esters, and furfuryl acetates.[15–19] Such a mechanism would also be consistent with the increased reactivity of the C2 ester compared with the C13 ester or the acetate ester of acetylcholine. The rates of solvolysis of bipinnatin-A, -B, and -C did not differ significantly, suggesting a common mechanism of solvolysis where the rate-limiting step is not affected by different substituents on the C1 carbon. The similar structures of the two active solvolysis products, bipinnatin-A(IV) and -C(V), further supports the hypothesis of a common solvolysis reaction mechanism. Therefore, isolation and structural characterization of two representative active solvolysis products yield information pertinent to all of the lophotoxins that contain a C2 ester.

S_N1 type substitution reactions involving carbocation intermediates often result in significant loss of stereochemistry (i.e., racemization), since solvent and other nucleophiles can usually react at similar rates with either face of the presumably planar carbocation intermediate. However, proton NMR spectroscopy revealed that the stereochemistry of the C2 carbon is the same in bipinnatin-C and -C(V). Retention of stereochemistry during an S_N1 reaction is unusual for simple acyclic compounds. However, steric hindrance to nucleophilic attack at one face of a carbocation intermediate in the case of the macrocyclic bipinnatins may not be unexpected and may be due to orientation of the furan and lactone rings or to the presence of a bulky substituent at C1 that shields one side of the carbocation intermediate. In addition, it is possible that the oxygens of the C1 substituents (the carbonyl oxygen of bipinnatin-A, the aldehyde oxygen of bipinnatin-B, and the epoxide oxygen of bipinnatin-C) actually participate in stabilizing a carbocation intermediate by neighboring group effects.[20] In either case, nucleophilic attack by hydroxide would be expected to occur only on the side of the toxin that previously contained the acetate ester, resulting in retention of stereochemistry at the C2 carbon.

The lophotoxins are very specific, selective, irreversible inhibitors of nicotinic acetylcholine receptors. Concentrations that irreversibly inhibit nicotinic receptors do not inhibit nerve conduction, release of neurotransmitter, or related enzymes and receptors, such as acetylcholinesterase, muscarinic acetylcholine receptors, histamine receptors, or γ-aminobutyric acid receptors.[2–4,9] This specificity is presumably due to a productive combination of reversible affinity coupled with a unique irreversible inhibitory mechanism. Previous structure–activity and molecular modeling studies led to the suggestion that αTyr[190] reacts covalently with the lophotoxins by nucleophilic attack of the αTyr[190] hydroxyl at the C7 carbon of the C7–C8 epoxide.[7] It is important to note that the biologically active bipinnatins retain an intact C7–C8 epoxide, and thus covalent reaction between αTyr[190] and the C7 carbon remains a possible mechanism by which these toxins could irreversibly inhibit nicotinic receptors. Additional direct evidence in support of the hypothesized carbocation intermediate at the C2 carbon may lead to a greater understanding of the mechanism involved in covalent modification of αTyr[190]. This information could potentially be applied to the design and synthesis of irreversible inhibitors of other neurotransmitter receptors.

Experimental Section

Preparation of the Lophotoxins. The lophotoxins were extracted from the soft coral *Lophogorgia chilensis* and *Pseudopterogorgia bipinnata* with methylene chloride and purified by flash chromatography and normal-phase HPLC.[7]

Identity and purity of the lophotoxins were determined using thin-layer chromatography, reverse-phase HPLC, normal-phase HPLC, and proton NMR. The lophotoxins (solid powder) were dissolved in 100% dimethyl sulfoxide (DMSO) to 10 mM and stored at −20 °C for at least 6 months without significant loss of material (as monitored by reverse-phase HPLC) or biological activity (as monitored by irreversible inhibition of [^{125}I]-α-bungarotoxin binding to BC_3H-1 cells). The lophotoxins were diluted from 10 mM stocks in 100% DMSO to 100 μM toxin and 1% DMSO in 25 mM sodium phosphate buffer (pH 7.4) and allowed to incubate at 22−24 °C unless otherwise indicated. After incubation in buffer, the toxin solutions were either separated by reverse-phase HPLC or assayed directly for acetic acid.

Reverse-Phase HPLC. Separation of the native lophotoxins from the solvolysis products produced by incubation in buffer was performed using reverse-phase HPLC (0.46 × 25 cm Vydac C_{18} column, Hewlett-Packard Model 1050 quaternary pump, and Hewlett-Packard Model 1040 diode array detector). The lophotoxins (0.25−5 mL of 100 μM toxin) were separated from their solvolysis products using a linear gradient of 100% water to 100% methanol in 100 min. A 10 min water wash preceded the methanol gradient, and a 10 min methanol wash followed completion of the gradient. Peaks were detected at 220 or 235 nm, and spectra from 200 to 600 nm were obtained for each peak. Peaks were integrated using an algorithm supplied by Hewlett-Packard. Each peak was collected, immediately frozen in dry ice−methanol, and lyophilized before further analysis.

Determination of Acetic Acid. Acetic acid generated by the lophotoxins was measured at 22−24 °C using a sensitive coupled enzyme assay.[21,22] The lophotoxins (100 μM) were incubated in 0.5 mL of 25 mM sodium phosphate buffer (pH 7.4) and 1% DMSO for up to 8 h at 22−24 °C before addition of 0.05 mL of an 11-fold concentrated solution of the coupled enzyme assay mix. The coupled enzyme assay contained (final concentration in 0.55 mL): 150 mM triethanolamine buffer (pH 8.0), 10 mM malate, 3 mM $MgCl_2$, 170 μM coenzyme A, 1 mM NAD, 2.7 mM ATP, 5 units of acetate kinase, 7 units of phosphotransacetylase, 8 units of malate dehydrogenase, and 4 units of citrate synthase. In this coupled enzyme assay system, any acetic acid liberated from the toxins was converted into citrate with the concomitant reduction of NAD to NADH. The reduction of NAD to NADH was monitored as an increase in absorbance at 340 nm. The amount of NADH formed was monitored immediately after addition of the coupled enzyme assay mix to the toxin solution. For equilibrium experiments, the coupled enzyme assay was monitored every 60 s for 2 h. Conversion of NAD to NADH usually reached a maximum within 90 min and was stable for 2 h. The final equilibrium concentration of acetic acid was determined from the absorbance at 340 nm after 2 h. For more rapid determination of acetic acid, NADH production was monitored every 5 s for 5 min, and the initial rate of NADH formation was determined by linear regression. Correlation coefficients for the linear regression were always greater than 0.8. All absorbance readings were obtained using a Hewlett-Packard Model HP5492A UV−vis diode array spectrophotometer. Experimental absorbance values were compared with standard curves obtained using known concentrations of acetic acid between 25 and 200 μM constructed under identical experimental conditions.

Irreversible Inhibition of [^{125}I]-α-Bungarotoxin Binding to BC_3H-1 Cells. Maintenance of BC_3H-1 cells was as described previously.[11] For experiments, BC_3H-1 cells were plated into 24-well multiwell plates that were pretreated with gelatin and the cells were grown as described previously.[11,12] Each isolated, lyophilized HPLC peak was dissolved in 1.0 mL of assay buffer (140 mM KCl, 25 mM Hepes, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgSO_4$, and 0.06 mg/mL BSA, pH 7.4) containing 1% DMSO. Cells were allowed to equilibrate in assay buffer for 20 min at 22−24 °C before removal of the assay buffer and addition of 250 mL of the dissolved toxin solution. Toxins were incubated with the cells for 1 h (bipinnatin-A and -B) or 4 h (bipinnatin-C), and the cells were then washed twice with 2.0 mL of assay buffer to remove free unbound toxin. A saturating concentration of [^{125}I]-α-bungarotoxin (250 μL of 15−20 nM) in assay buffer was then added to each well and the mixture allowed to incubate for 1 h. Free, unbound [^{125}I]-α-bungarotoxin was removed with two washes of 2.0 mL of assay buffer. Bound [^{125}I]-α-bungarotoxin was removed from the assay wells with two 0.5 mL washes of 1% Triton X-100. The amount of bound [^{125}I]-α-bungarotoxin was determined using an LKB CliniGamma Automatic γ-Counter (Model 1272). Total binding of [^{125}I]-α-bungarotoxin was determined by incubating cells with assay buffer alone for 30 min before replacing the solution with [^{125}I]-α-bungarotoxin. Nonspecific binding of [^{125}I]-α-bungarotoxin was determined by incubating cells with 100 nM α-bungarotoxin in assay buffer for 30 min before replacing the solution with [^{125}I]-α-bungarotoxin. Specific binding of [^{125}I]-α-bungarotoxin was calculated by subtracting the nonspecific binding of [^{125}I]-α-bungarotoxin from the total binding of [^{125}I]-α-bungarotoxin. All assays were performed in triplicate.

Purification of Active Solvolysis Products from Bipinnatin-A and -C. Bipinnatin-A (30 mg) was dissolved in 10 mL of DMSO, diluted to 200 mL with 25 mM sodium phosphate buffer (pH 7.4), incubated at 22−24 °C for 3 h, and then frozen in dry ice−methanol and lyophilized. The lyophilized material was resuspended in 5.0 mL of DMSO. Aliquots (0.5 mL) were diluted to 5.0 mL with buffer, and the active solvolysis product of bipinnatin-A was isolated by reverse-phase HPLC using a Vydac C_8 column. Bipinnatin-C (40 mg) was dissolved in 4.0 mL of DMSO. Aliquots (100 μL) were diluted to 5.0 mL with 25 mM sodium phosphate buffer (pH 7.4) and allowed to incubate at 22−24 °C for 90 min before isolation of the active solvolysis product of bipinnatin-C by reverse-phase HPLC using a Vydac C_{18} column.

Fast-Atom Bombardment Mass Spectroscopy. Fast-atom bombardment mass spectroscopy (FAB-MS) was performed on an Extrel ELQ-400 single-quadrapole, turbo-pumped mass spectrometer (Extrel, Pittsburgh, PA). Mass spectra were collected in positive ion mode over a mass range of 250−650 amu. Approximately 10 μg of bipinnatin-A, -A(IV), -C, or -C(V) in 2 μL of DMSO was added to approximately 200 μL of thioglycerol or thioglycerol containing approximately 10 μg of potassium or sodium acetate.[23] The solution was subjected to an ionized xenon gas plasma (3−6 keV).

Proton NMR Spectroscopy. Proton NMR spectra were obtained using an Osuka spectrometer equipped with a 9.4 T magnet (401.102 MHz for proton resonances). A single pulse sequence was used with a 90° pulse duration of 26 μs and a repetition delay of 20 s. All samples (2−5 mg of toxins) were dissolved in 0.75 mL of deuterated chloroform (99.96%). All spectra were obtained at 24 °C, and data were collected into 4096 complex points and zero-filled once before Fourier transformation. Chemical shifts were referenced to that of tetramethylsilane.

Materials. All chemicals, buffers, and HPLC solvents were purchased from Sigma Chemical Co. or Fisher Scientific Co. [^{125}I]-α-Bungarotoxin (12−15 Ci/mmol) was purchased from DuPont/NEN. BC_3H-1 cells were a gift from Dr. P. Taylor, University of California, San Diego, CA, and also obtained from the American Type Culture Collection.

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