Evidence for a Carbocation Intermediate during Conversion of Bipinnatin-A and -C into Irreversible Inhibitors of Nicotinic Acetylcholine Receptors

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The lophotoxins are naturally occurring antagonists of nicotinic acetylcholine receptors. These toxins are small diterpenes that irreversibly inhibit nicotinic receptors by specific covalent modification of Tyr^{190} in the α-subunits of the receptor. The naturally occurring lophotoxin analogs, bipinnatin-A and -C, are inactive protoxins. Activation of these toxins occurs spontaneously in buffer and involves replacement of the C2 acetate ester with a hydroxyl group. The mechanism involved in conversion of the inactive bipinnatins into their biologically active solvolysis products was investigated in this study. Solvolysis of bipinnatin-A in buffer containing [^{18}O]water demonstrated that the C2 hydroxyl of the biologically active solvolysis product originated from the solvent. The rates of solvolysis of bipinnatins-A and -C were not affected by sodium azide. However, in the presence of azide, solvent products decreased and new azide-containing products appeared. Thus azide acted as a nucleophile after a rate-limiting step, such as the formation of a carbocation intermediate. The k_{az}/k_s values for bipinnatin-A (2900 M^{-1}) and bipinnatin-C (1450 M^{-1}) suggest that the carbocation intermediates are relatively stable. Compounds capable of spontaneously generating carbocations may represent a novel new class of active-site-directed affinity reagents that can be applied to other receptors and enzymes.

Introduction

The lophotoxins (including bipinnatin-A and bipinnatin-C) are members of a family of nicotinic acetylcholine receptor antagonists isolated from marine soft coral.[1-4] These toxins are small diterpenes (less than 550 g/mol) with a notable absence of a positively charged center that might facilitate their interaction with the acetylcholine-binding site of nicotinic acetylcholine receptors. Nonetheless, the lophotoxins irreversibly inhibit nicotinic acetylcholine receptors by first interacting with the acetylcholine-binding sites and then forming a covalent bond with Tyr^{190} in the α-subunits (αTyr^{190}) of the receptor.[5,6] The lophotoxins are the only natural toxins known to irreversibly inhibit a neurotransmitter receptor by utilizing a covalent mechanism of action. A more complete understanding of their reaction mechanism may therefore provide new insights and direction in the development of novel irreversible receptor antagonists.

Irreversible inhibition of nicotinic acetylcholine receptors by bipinnatins-A and -C occurs after a delay of approximately 10–20 min, suggesting that these bipinnatins are inactive protoxins that are converted into active toxins during incubation with receptors.[6,7] In support of this hypothesis, preincubation of bipinnatin-A and -C in buffer alone results in an increase in their subsequent pseudo-first-order rate constants for irreversible inhibition of receptors. Solvolysis of these toxins in buffer alone follows a first-order exponential decay with a $t_{1/2}$ of approximately 1 h.[7] This initial solvolysis reaction appears to result from hydrolysis of a single specific acetate ester, since stoichiometric production of acetic acid during solvolysis also follows a first-order exponential function with a $t_{1/2}$ of about 1 h. Isolation and structural characterization of the biologically active solvolysis products of bipinnatin-A and -C revealed that hydrolysis of the C2 acetate ester is necessary and sufficient to convert the inactive protoxins into biologically active toxins.[7] The stereochemistry of the C2 carbon remained unchanged from inactive protoxin to active toxin, consistent with an S_N2 reaction involving nucleophilic attack of hydroxide at the carbonyl carbon of the C2 ester. However, the rate of solvolysis was relatively independent of pH, consistent with an S_N1 reaction mechanism.

Solvolysis of the C2 ester could occur by nucleophilic attack of hydroxide at the carbonyl carbon of the ester or at the C2 carbon (S_N2 mechanisms). Alternatively, an initial rate-limiting step involving elimination of the C2 ester and formation of a C2 carbocation could precede addition of hydroxide to the C2 carbon (S_N1 mechanism). The presence of a carbocation intermediate can be supported by several experimental criteria, including incorporation of ^{18}O from the solvent into the compound, a reaction rate that is relatively independent of pH, and racemization of an initially optically pure reactant. Reaction of a compound with an additional nucleophile under solvolysis conditions can often help to reveal the presence of a rate-limiting step involving a carbocation intermediate. For instance, sodium azide has been used as such a "trapping" agent to demonstrate the formation of diffusionally stable carbocations with mean lifetimes greater than 10^{-9} s.[8-10]

A variety of compounds exhibit biological activities associated with the formation of reactive carbocation intermediates. For instance, styrene oxide and the epoxy metabolites of polycyclic hydrocarbons undergo solvolysis reactions that involve rate-limiting carboca-

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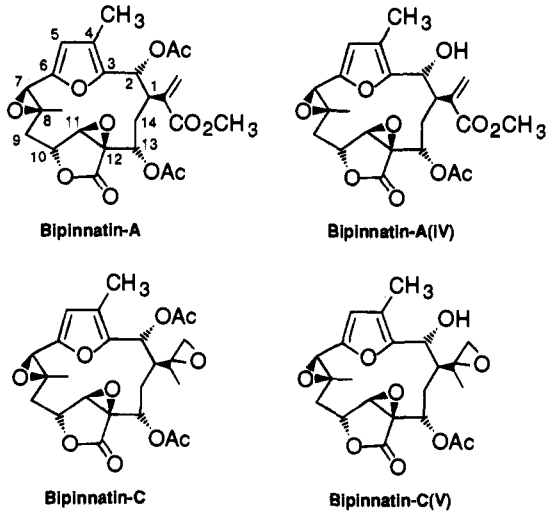

Figure 1. Structures of inactive protoxins bipinnatin-A and -C, and their active solvolysis products bipinnatin-A(IV) and -C(V).

tion intermediates that have been shown to react with nucleophiles on proteins and DNA.[9,11-13] In addition, a number of irreversible enzyme inhibitors have been designed to undergo mechanism-based deamination reactions resulting in carbocations that react covalently with amino acids in the active site of the enzyme.[14,15] Reactive carbocation intermediates may be involved in the biological activation of bipinnatin-A and -C, and in the irreversible inhibition of nicotinic acetylcholine receptors. This paper investigates the mechanistic steps involved in conversion of the inactive protoxins bipinnatin-A and -C into biologically active irreversible inhibitors.

Results

Bipinnatin-A and -C are inactive protoxins that require incubation in aqueous buffer to produce biologically active, irreversible inhibitors of nicotinic receptors (Figure 1).[6] Bipinnatin-A(IV) and -C(V) are the biologically active solvolysis products of bipinnatin-A and -C, respectively.[7] FAB-MS spectra of bipinnatin-A(IV) produced by solvolysis in unenriched and ^{18}O-enriched water were obtained in order to determine the origin of the C2 oxygen. FAB-MS spectra of bipinnatin-A in thioglycerol revealed strong signals from fragment ions consistent with the loss of both acetate esters from a compound with *m/z* of 504 (Table 1). The molecular ion for bipinnatin-A was not apparent when bipinnatin-A was dissolved in thioglycerol alone. However, addition of sodium or potassium to the thioglycerol resulted in ions consistent with the formation of sodium or potassium adducts with a compound of *m/z* of 504. FAB-MS of bipinnatin-A(IV) in thioglycerol revealed strong signals from fragment ions consistent with the loss of one acetate ester and one hydroxyl from a compound with *m/z* of 462. The molecular ion for bipinnatin-A(IV) was not apparent when bipinnatin-A(IV) was dissolved in thioglycerol alone. However, addition of sodium or potassium to the thioglycerol resulted in adduct ions consistent with a compound of *m/z* of 462.

Solvolysis of bipinnatin-A in buffer prepared with ^{18}O-enriched water did not alter the reverse-phase HPLC retention times for bipinnatin-A or bipinnatin-A(IV). In addition, the FAB-MS of bipinnatin-A isolated after incubation in ^{18}O-enriched water was similar to that of bipinnatin-A that had not been exposed to ^{18}O (Table 1). However, bipinnatin-A(IV) isolated from solvolysis of bipinnatin-A in ^{18}O-enriched water produced sodium and potassium adduct ions that were 2 mass units heavier then bipinnatin-A(IV) generated in unenriched buffer (464 versus 462, respectively). These results are consistent with an S_N1 reaction mechanism involving a carbocation intermediate, since the C2 oxygen of bipinnatin-A(IV) clearly originated from the solvent rather than from the acetate ester. However, it is possible that nucleophilic displacement of the C2 acetate ester takes place by an S_N2-mediated attack of hydroxide at the C2 carbon, resulting in incorporation of ^{18}O from solvent into the toxin at the C2 position.

In order to provide further evidence for a rate-limiting step in the solvolysis of bipinnatin-A and -C, the solvolysis reaction was carried out in the presence and absence of sodium azide. The amount of bipinnatin-A remaining after a 75 min incubation in buffer was not

Table 1. Ions Produced by FAB-MS of Bipinnatin-A and -A(IV) Isolated after Solvolysis of Bipinnatin-A in Phosphate Buffer Containing [^{16}O]Water, [^{18}O]Water, or Sodium Azide[a]

compound	condition[b]	*m/z*	assignment[c]
Solvolysis in Buffer Containing [^{16}O]Water			
bipinnatin-A	TG	445	504 − OAc
		385	504 − OAc − HOAc
	K + TG	543	504 + K
		483	504 − HOAc + Na
		445	504 − OAc
		385	504 − OAc − HOAc
	Na + TG	527	504 + Na
		467	504 − HOAc + Na
		445	504 − OAc
		385	504 − OAc − HOAc
bipinnatin-A(IV)	TG	445	462 − OH
		385	462 − OH − HOAc
	K + TG	501	462 + K
	Na + TG	485	462 + Na
		445	462−OH
		385	462−OH−HOAc
Solvolysis in Buffer Containing [^{18}O]Water			
bipinnatin-A	TG	445	504−OAc
		385	504−OAc−HOAc
	K + TG	543	504 + K
		527[d]	504 + Na
		445	504 − OAc
		385	504 − OAc − HOAc
	Na + TG	527	504 + Na
		467	504 − HOAc + Na
		445	504 − OAc
		385	504 − OAc − HOAc
bipinnatin-A(IV)	TG	487[d]	464 + Na
		445	464−^{18}OH
		385	464 − ^{18}OH − HOAc
	K + TG	503	464 + K
		445	464−^{18}OH
	Na + TG	487	464 + Na
Solvolysis in Buffer Containing 1.0 mM Sodium Azide			
bipinnatin-A(N_3)	TG	445	487 − N_3
		385	487 − N_3 − HOAc
	K + TG	526	487 + K
	Na + TG	510	487 + Na
		445	487 − N_3
		385	487 − N_3 − HOAc

[a] Summary of fast-atom bombardment mass spectroscopy (FAB-MS) for all mass fragments between *m/z* 360 and 650 that were at least 20% of the most abundant ion in that mass range. [b] Conditions include TG = thioglycerol alone; Na + TG = thioglycerol with sodium acetate, K + TG = thioglycerol with potassium acetate. [c] Calculated monoisotopic molecular weights for bipinnatin-A, bipinnatin-A(IV), and bipinnatin-A(N_3) are 504, 462, and 487, respectively. [d] Produced by contaminating amounts of sodium in the sample.

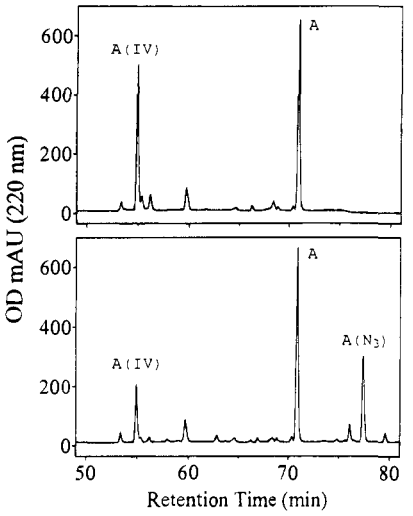

Figure 2. Separation of bipinnatin-A from solvolysis products by reverse-phase HPLC. Bipinnatin-A (20 mM in 100% DMSO) was diluted to 100 μM toxin and 1% DMSO in 0.5 mL of 25 mM phosphate buffer (pH 7.4) and incubated at 22–24 °C in the absence (upper panel) or presence (lower panel) of 1.0 mM sodium azide for 75 min. Solvolysis products were then separated by reverse-phase HPLC. Retention times for bipinnatin-A(IV), bipinnatin-A, and bipinnatin-A(N_3) (the azide adduct of bipinnatin-A) were 55, 71, and 77 min, respectively.

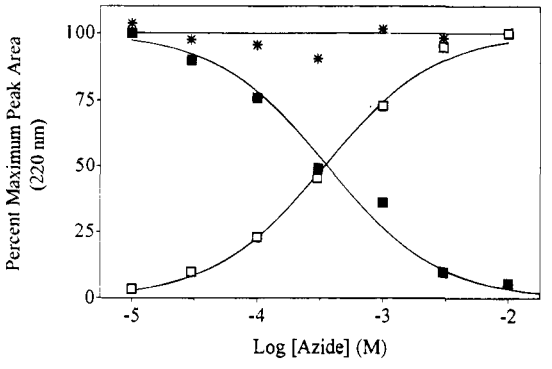

Figure 3. Effect of sodium azide on solvolysis of bipinnatin-A. Bipinnatin-A was incubated with the indicated concentrations of sodium azide as described for Figure 2. Bipinnatin-A (*) and bipinnatin-A(IV) (■) peak areas were normalized to bipinnatin-A and A(IV) peak areas in the absence of sodium azide. Peak area for the azide adduct (□) was normalized to the maximum peak area of the adduct produced with 10 mM sodium azide.

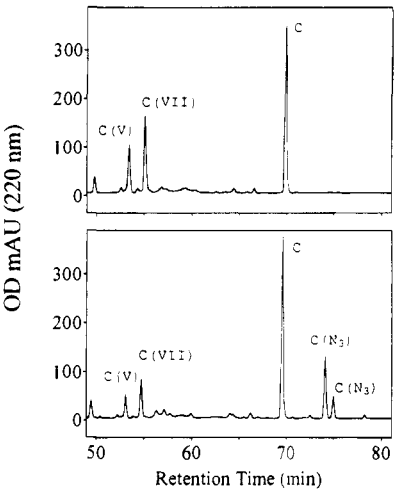

Figure 4. Separation of bipinnatin-C from solvolysis products by reverse-phase HPLC. Bipinnatin-C (20 mM in 100% DMSO) was diluted to 100 μM toxin and 1% DMSO in 0.5 mL of 25 mM phosphate buffer (pH 7.4) and incubated at 22–24 °C in the absence (upper panel) or presence (lower panel) of 1.0 mM sodium azide for 120 min. Solvolysis products were then separated by reverse-phase HPLC. Retention times for bipinnatin-C(V), bipinnatin-C(VII), bipinnatin-C, and bipinnatin-C(N_3) (the azide adducts of bipinnatin-C) were 53, 55, 70, 74, and 75 min, respectively.

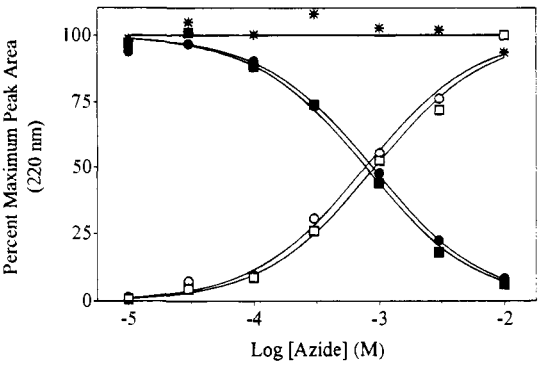

Figure 5. Effect of sodium azide on the solvolysis of bipinnatin-C. Bipinnatin-C was incubated with the indicated concentrations of sodium azide as described for Figure 4. Bipinnatin-C (*), bipinnatin-C(V) (■), and -C(VII) (●) peak areas were normalized to bipinnatin-C, -C(V), and -C(VII) peak areas in the absence of sodium azide. Peak areas for the azide adducts (□, ○) were normalized to the maximum peak areas of the adducts produced with 10 mM sodium azide.

affected by the presence of 1.0 mM azide (Figure 2). However, in the presence of azide, formation of the solvent product bipinnatin-A(IV) was suppressed and a new apparently more hydrophobic product was generated. Increasing concentrations of azide led to complete suppression of bipinnatin-A(IV) and a correlated increase in the more hydrophobic product (Figure 3). Although the product composition was dramatically altered by solvolysis of bipinnatin-A in the presence of 10 mM sodium azide, the overall rate of solvolysis of bipinnatin-A was unaffected by the presence of sodium azide. In contrast, sodium chloride (10 mM) had no affect on either the rate of solvolysis of bipinnatin-A or the amount of bipinnatin-A(IV) produced, indicating that the effects observed with sodium azide were due to the presence of azide and not sodium (data not shown). FAB-MS of the more hydrophobic solvolysis product produced in the presence of azide revealed sodium and potassium adduct ions of *m/z* 510 and 526, respectively, and fragment ions of 445 and 385 mass units, consistent with replacement of the C2 acetate with azide (Table 1).

Similar experiments were carried out with bipinnatin-C and sodium azide. Incubation of bipinnatin-C in buffer results in the formation of two major solvolysis products, bipinnatin-C(V) and -C(VII). However, only bipinnatin-C(V) appears to have biological activity.[7] The amount of bipinnatin-C remaining after a 120 min incubation in buffer was not affected by the presence of 1.0 mM azide (Figure 4). However, in the presence of azide, the formation of bipinnatin-C(V) and -C(VII) was suppressed and two new, apparently more hydrophobic, products were formed. Although the rate of solvolysis of bipinnatin-C was not affected by increasing concentrations of azide, the formation of bipinnatin-C(V) and -C(VII) decreased and that of the azide products increased (Figure 5).

Several other nucleophiles were investigated for their

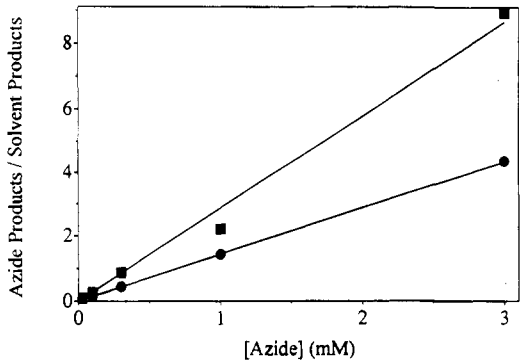

Figure 6. Ratio of solvolysis products versus azide concentration. The ratios of solvolysis products (azide adducts/solvent adducts) for bipinnatin-A (■) and bipinnatin-C (●) are plotted versus the concentration of azide. The linear relationship is consistent with pseudo-first-order conditions for the reaction of toxins with both solvent and azide. The slope of the lines (2900 and 1450 M^{-1} for bipinnatin-A and -C, respectively) gives k_{az}/k_s, where k_{az} is a second-order rate constant (in units of M^{-1} s^{-1}) and k_s is a pseudo-first-order rate constant (in units of s^{-1}).

ability to selectively scavenge a rate-limiting carbocation intermediate, but none were as selective as sodium azide. For instance, 100 mM pralidoxime decreased the apparent rate of formation of bipinnatin-A(IV) by 82%, but also increased the apparent rate of solvolysis of bipinnatin-A by 12%. *N*-Acetyl-L-cysteine was even less selective, affecting both solvolysis of bipinnatin-A and the production of bipinnatin-A(IV) to an equal extent (data not shown).

Reaction of solvent and nucleophiles with the toxins can be expected to follow pseudo-first-order reaction kinetics, provided that both solvent and nucleophiles are present at concentrations greater than the toxin (so that their concentrations remain essentially constant). Under these conditions, the ratio of the products formed by reaction of the toxin with azide and solvent at any given time should be equal to the ratio of the pseudo-first-order rate constants for reaction of the toxin with azide and solvent.[8] For example, this relationship for bipinnatin-A can be expressed as

$$[A(N_3)]/[A(IV)] = k_{az}[N_3^-]/k_s \qquad (1)$$

where $[A(N_3)]$ is the concentration of azide-containing product, [A(IV)] is the concentration of solvent product, k_{az} is a second-order bimolecular rate constant for the reaction of toxin with azide (in units of M^{-1} s^{-1}), $[N_3^-]$ is the concentration of azide, $k_{az}[N_3^-]$ is a pseudo-first-order rate constant for reaction of toxin with azide (in units of s^{-1}), and k_s is a pseudo-first-order rate constant for reaction of toxin with solvent (in units of s^{-1}). The ratio of the products formed under such pseudo-first-order reaction conditions should therefore be linear and directly proportional to the concentration of added nucleophile (azide), with a slope of k_{az}/k_s (in units of M^{-1}). As expected, the ratio of azide to solvent products was linear with increasing azide concentration for both bipinnatin-A and -C (Figure 6). The k_{az}/k_s ratios for bipinnatin-A and -C were 2900 and 1450 M^{-1}, respectively.

Discussion

Bipinnatin-A and -C are both inactive protoxins that contain an acetate ester at the C2 carbon.[3,4,6,7] Incubation of bipinnatin-A and -C in buffer results in an increase in biological activity which is attributed to the spontaneous production of the biologically active solvolysis products bipinnatin-A(IV) and -C(V). Bipinnatin-A(IV) and -C(V) are identical in structure and stereochemistry to bipinnatin-A and -C, respectively, except for the presence of a C2 alcohol rather then a C2 acetate ester.[7] The two mass unit increase observed for bipinnatin-A(IV) produced in [^{18}O]water compared to that produced in [^{16}O]water indicates that the C2 hydroxyl of bipinnatin-A(IV) originates from the solvent. This result is consistent with an S_N1 kinetic mechanism and a carbocation intermediate (Figure 7).

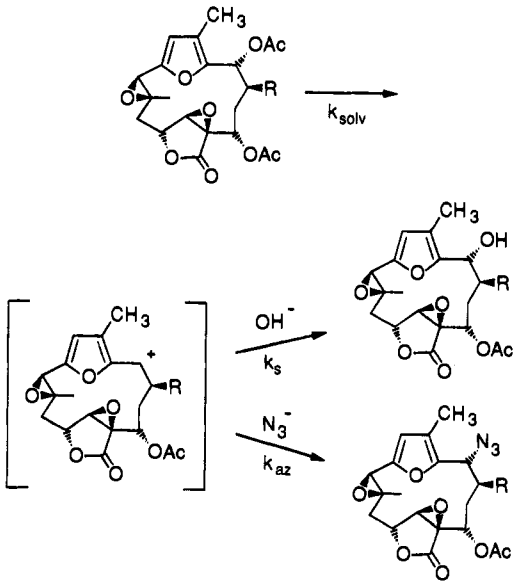

Figure 7. Proposed scheme for reaction of bipinnatin-A and -C with water or azide. The rate of solvolysis is represented by k_{solv}, while k_s and k_{az} represent the rates of reaction of the carbocation intermediate with solvent (OH^-) or azide (N_3^-), respectively. This S_N1 reaction mechanism predicts that k_{solv} will be similar for both bipinnatin-A and -C, that k_{solv} will be relatively independent of pH, that the presence of azide will affect the distribution of products without affecting k_{solv}, that k_{az}/k_s will be similar for both bipinnatin-A and -C, and that the C2 oxygen originates from the solvent. All of these predictions are consistent with the observed data. However, one or more of these predictions are not consistent with other possible reaction mechanisms (i.e. S_N2 or neighboring group mechanisms).

Nucleophilic reagents including sodium azide, N-acetyl-L-cysteine, aniline, and sodium thiocyanate have been used to "trap" proposed carbocations subsequent to their formation by a rate-limiting elimination reaction, thereby providing kinetic evidence for an S_N1-type reaction.[8,10,16-19] Carbocations that are relatively unstable in solution (those with large k_s values) will be expected to react rapidly with solvent, and higher concentrations of scavenging nucleophile will therefore be required to trap the unstable, highly reactive carbocation.[20,21] The concentrations of azide necessary to trap the carbocations generated by bipinnatin-A and -C were well below those needed to trap several other well characterized carbocations.[9,10,12,17,18] Therefore, the kinetic intermediate formed by elimination of the C2 acetate exists in solution as a diffusionally stable entity for a sufficiently long time, such that relatively low concentrations of "trapping" agent can react with it. The observation that a 10^6-fold change in pH produced less than a 2-fold change in the rate of solvolysis of bipin-

natin-A and -C is also consistent with an S_N1 and not an S_N2 reaction mechanism.[7]

The interaction of azide with carbocations can be assumed to be diffusion controlled with a bimolecular rate constant of $k_{az} = 5 \times 10^9$ M^{-1} s^{-1}, thus allowing the mean lifetime ($1/k_s$) of a carbocation to be estimated from the experimentally determined value of k_{az}/k_s.[12,22] The carbocation intermediates formed during solvolysis of bipinnatin-A and -C have estimated mean lifetimes of 0.58 and 0.29 ms, respectively. These carbocations are relatively long-lived when compared to aliphatic carbocations, presumably because they can be stabilized by resonance with the furan ring, an effect shown for other aryl carbocations.[16-23]

Bipinnatin-A and -C are clearly converted into bipinnatin-A(IV) and -C(V) by an S_N1 kinetic mechanism, and the rate-limiting reaction intermediate appears to be a relatively stable carbocation. However, it is possible that a neighboring group on the toxin acts as a nucleophile in a rate-limiting intramolecular reaction, resulting in displacement of the C2 ester. Although subsequent reaction of this intermediate with solvent or other nucleophiles could occur by an S_N2 reaction mechanism, the overall reaction would appear to be S_N1 with retention of stereochemistry. The only nucleophiles capable of such intramolecular reactions are the oxygens on the C1 substitutents (a methyl ester for bipinnatin-A and an epoxide for bipinnatin-C). However, the rate-limiting intermediates produced by solvolysis of bipinnatin-A and -C appear to form at similar rates and they appear to react with nucleophiles at similar rates, consistent with a common carbocation intermediate. These observations are inconsistent with the distinctly different intermediates that would be formed by a neighboring group mechanism. Alternatively, retention of stereochemistry at the C2 carbocation can be favored due to the relative inaccessibility of one side of the 14-carbon macrocyclic ring to solvent. While this is somewhat unusual for simple acyclic carbocations, it is not unexpected for macrocyclic compounds such as the lophotoxins, where the two sides of the toxin are not symmetrical.[4,23,24]

It is not clear why bipinnatin-A and -C are inactive while bipinnatin-A(IV) and -C(V) are active. It is possible that elimination of the bulky C2 acetate ester removes steric hindrance, thus allowing the toxins to fit into the acetylcholine-binding site of the receptor. Alternatively, it is possible that elimination of the C2 acetate allows for rotation of the furan ring such that the epoxide at C7–C8 is rendered more reactive. A C7 carbocation could then be stabilized by resonance with the furan ring and could subsequently undergo nucleophilic attack by the hydroxyl of αTyr^{190}. All of the known active lophotoxin analogs contain the C7-C8 epoxide, and based on structure–function studies, the C7 carbon was previously proposed as the site of reaction with αTyr^{190}.[4] Tyrosine residues in active sites of enzymes have been shown to be targets of irreversible inhibitors. For example, Tyr^6 and Tyr^{115} of glutathione transferase are thought to be involved in protonating epoxide-containing substrates and they can be alkylated by a variety of substrate analogs.[25] In addition, epoxide containing compounds have been shown to be specific irreversible inhibitors of several enzymes including phosphotyrosyl protein phosphatase, glucanohydrolase, cathepsin B, and HIV-1 protease.[26-30] In one case, the epoxide may react with a protein nucleophile via a carbocation intermediate.[30]

Most irreversible active-site directed inhibitors utilize one of two general mechanisms to form a covalent bond. For instance, some active-site directed affinity reagents contain relatively stable leaving groups (such as isothiocyanates or acyl halides) that are positioned near reasonably good nucleophiles in the active site of the receptor. Unfortunately, appropriate nucleophiles are not always available within the active site. Alternatively, many active-site-directed affinity reagents are relatively stable precursors (such as arylazides and aryldiazirines) that are photoactivated to produce highly reactive intermediates (such as nitrenes and carbenes). Unfortunately, not all preparations are amenable to photolysis. Spontaneously generated carbocations may provide an additional complementary route to the active-site-directed irreversible inhibition of receptors. It remains to be determined if a carbocation is actually involved in the covalent reaction between the lophotoxins and nicotinic acetylcholine receptors. If so, then this novel reaction mechanism could be exploited to design synthetic active-site-directed affinity reagents for other receptors and enzymes.

Experimental Section

Chemicals. All chemicals, buffers, and HPLC solvents were purchased from Sigma Chemical Co. or Fisher Scientific Co. Water enriched with ^{18}O was purchased from Cambridge Isotope Co.

Preparation of the Lophotoxins. Bipinnatin-A and -C were purified from extracts of *Pseudopterogorgia bipinnata* as described previously.[3,4,7] Purity was determined by TLC, normal- and reverse-phase HPLC, and proton NMR.[1,4] Biological activity was determined by inhibition of [^{125}I]-α-bungarotoxin binding to BC_3H-1 cells.[7] Unless otherwise indicated, toxins were dissolved in 100% DMSO (20 mM) and stored at −20 °C without loss of activity for up to 6 months.

Reverse-Phase HPLC. Separation of the native lophotoxins from the solvolysis products produced by incubation in buffer was performed by reverse-phase HPLC using a linear gradient of 100% water to 100% methanol in 100 min as described previously.[7] Peaks were detected at 220 or 235 nm and spectra from 200 to 600 nm were obtained for each peak. Peak areas were integrated using an algorithm supplied by Hewlett-Packard. When subsequent analysis was required, each peak was collected, immediately frozen in dry ice/methanol, and lyophilized before further analysis.

Purification of Bipinnatin-A Solvolysis Products. Bipinnatin-A (2 mg) was dissolved in 200 μL of DMSO, and then diluted to 5 mL with 25 mM phosphate buffer (pH 7.4), prepared with unenriched or ^{18}O-enriched water (96% ^{18}O). The solvolysis reaction was allowed to proceed for 2 h at 22–24 °C before separation of the solvolysis products by reverse-phase HPLC. Azide adducts of bipinnatin-A were prepared by allowing solvolysis to proceed for 1.25 h in the presence of 10 mM sodium azide. Bipinnatin-A, A(IV), and azide adducts of bipinnatin-A were collected, frozen in dry ice/methanol, and lyophilized before further analysis.

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). Approximately 100 μg of lyophilized sample dissolved in 20 μL of DMSO and a 2 μL aliquot was added to approximately 200 μL of thioglycerol or thioglycerol containing approximately 10 μg of potassium or sodium acetate, and the sample was then subjected to an ionized xenon gas plasma (3–6 keV).

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Supporting Information Available: Complete fast-atom bombardment mass spectra are provided for bipinnatin-A and bipinnatin-A(IV) produced in unenriched and ^{18}O-enriched water and for the product obtained by reaction of bipinnatin-A with 1.0 mM azide (16 pages). Ordering information is given on any current masthead page.

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