Structural Studies of Sodium Channel Neurotoxins. 3. Crystal Structures and Absolute Configurations of Grayanotoxin III and α-Dihydrograyanotoxin II[1]

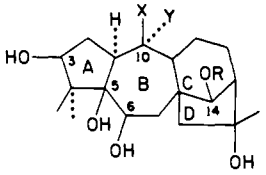
Penelope W. Codding

Contribution from the Departments of Chemistry and Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta, Canada T2N 1N4. *Received May 3, 1984*

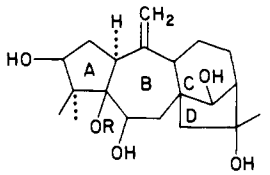
Abstract: Crystal structure analyses have been carried out on two neurotoxic grayanotoxins: grayanotoxin III and α-dihydrograyanotoxin II. The space group is monoclinic in each case: grayanotoxin III is $C2$, a = 21.941 (10) Å, b = 8.762 (2) Å, c = 18.504 (8) Å, β = 143.50 (3)°; and α-dihydrograyanotoxin II is $P2_1$, a = 9.7493 (2) Å, b = 6.8596 (4) Å, c = 14.670 (3) Å, β = 107.635 (9)°. The absolute configuration was determined by anomalous dispersion and is consistent with that predicted by chemical correlations. Comparison of the two molecular structures indicates a common conformation for these active compounds. Structural features match those found in the sodium channel neurotoxins: batrachotoxin, veratridine, and aconitine. Two potential binding domains are found in grayanotoxins, a triangle of three oxygen atoms (cationic group binding) and a hydroxyl group (a hydrogen bond donor). The present model for receptor binding is examined in terms of the available structure–activity data for grayanotoxins.

Grayanotoxins are one of four structural classes of lipid-soluble polycyclic compounds that bind to a single site on sodium ion channels.[2] These neurotoxins do not bind to the ion pore itself; yet, they shift the activation of channels to more negative membrane potentials and block inactivation. This produces a lasting depolarization of the excitable membrane. The binding of the four types of neurotoxins—batrachotoxin, aconitine, veratridine, and grayanotoxin—is competitive; thus, these diverse chemical structures all probably bind to a single site.

The structural requirements for the toxicity of grayanotoxins are complex, involving stereochemistry, position, and type of functional groups.[3] An active grayanotoxin must have the 10-methyl substituent on the same side of the molecule as the hydroxyl groups that form a triangle of oxygen atoms. Thus, α-dihydrograyanotoxin II (**1**) and grayanotoxins I (**2**) and III (**3**) are active, while β-dihydrogranotoxin II (**4**), grayanotoxin II (**5**), and the 6-acetyl and 2,3 epoxide derivatives are not. From these data,



1, X = CH_3; Y = H; R = H
2, X = CH_3; Y = OH; R = Ac
3, X = CH_3; Y = OH; R = H
4, X = H; Y = CH_3; R = H



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Matsutani et al.[4] identified a triangle of oxygen atoms for the neurotoxins and a second attachment point that is a specific binding site for the methyl group on carbon atom 10 and for similar methyl groups on the alkaloid neurotoxins. Recent work by Brown and Daly[5] has shown that batrachotoxin does not need the methyl group for activity, so this binding site needs re-examination. We have developed a model for the binding of the alkaloid neurotoxins to the same site through the analysis of three-dimensional structural information.[1,3] This model has two binding sites: a triangle of oxygen atoms on one side of the molecule and, about 6 Å away, a nitrogen atom that participates in a hydrogen bond. Our model did not explain the stereospecificity of grayanotoxin binding, nor had we correlated our proposed second binding site with a specific part of the non-nitrogenous grayanotoxins. For the differences between these two models to be resolved, the crystal and molecular structures of two of the more *active* derivatives—grayanotoxin III (GTXIII) and α-dihydrograyanotoxin II (α-H_2GTXII)—have been determined and compared to the closely related structure of *inactive* grayanotoxin II (GTXII).[6]

Table I. Crystal Data

	GTXIII	α-H_2GTXII
mol form	$C_{20}H_{34}O_6 \cdot 2.5H_2O$	$C_{20}H_{34}O_5$
mol wt	370.49	354.49
cell dimensions		
a, Å	21.941 (10)	9.7493 (2)
b, Å	8.762 (2)	6.8596 (4)
c, Å	18.504 (8)	14.670 (3)
β, deg	143.50 (3)	107.635 (9)
V, Å	2116 (1)	934.9 (3)
space group	$C2$, Z = 4	$P2_1$, Z = 2
density		
obsd, g cm^{-3}	1.23	
calcd, g cm^{-3}	1.30	1.259
cryst size, mm^{-1}	0.2 × 0.2 × 0.3	0.25 × 0.20 × 0.25
linear abs coeff, cm^{-1}	1.20	7.25
wavelength	0.71069, Mo Kα, graphite monochromator	1.5418, Cu Kα, Ni filter
max θ, deg	27	70
unique reflns measured	2587	1932
% > 2.5σ	72.7	60
temp, °C	−100 (5)	22
wtng scheme w	$(1.0/\sigma^2(F_o) + 0.001F_o^2)^{1/2}$	$(1.0/\sigma^2(F_o) + 0.0015F_o^2)^{1/2}$
max shift/error	0.068	0.008
std dev of obs unit wt	1.4	1.0

Experimental Section

Grayanotoxin III and α-dihydrograyanotoxin II were generous gifts of Dr. T. Narahashi, Northwestern University Medical School. Crystals were grown for both samples by slow evaporation of a water/methanol mixture. Crystal data are given in Table I. Each data set was collected on an Enraf-Nonius CAD4F diffractometer with use of the $\omega/2\theta$ technique. The data collection procedure was the same as that described in ref 1. Two octants of data were collected and averaged for GTXIII. Lorentz and polarization corrections were applied. No absorption corrections were made.

The correct space group for the GTXIII data set was not identified by the automatic indexing routine of the CAD4F software nor by the cell

(1) Part 2 of this series: Codding, P. W. *J. Am. Chem. Soc.* **1983**, *105*, 3172–3176.
(2) Three recent interviews of the pharmacology of sodium channels have appeared: (a) Catterall, W. A.; Hartshorne, R. P.; Beneski, D. A. *Toxicon* **1982**, *20*, 27–40. (b) Catterall, W. A. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 15–43. (c) Narahashi, T. *Adv. Cytopharm.* **1979**, *3*, 293–303.
(3) Codding, P. W. *Acta Crystallogr., Sect. B* **1982**, *B38*, 2519–2522.
(4) Matsutani, T.; Seyama, I.; Narahashi, T.; Swasa, J. *J. Pharmacol. Exp. Ther.* **1981**, *217*, 812–819.
(5) Brown, G. B.; Daly, J. W. *Cell. Mol. Neurobiol.* **1981**, *1*, 361–371.

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Table III. Atomic Coordinates (×10⁴) and $B_{equivalent}$ (×10) for the Carbon and Oxygen Atoms of Grayanotoxin III[a]

atom	x/a	y/b	z/c	B_{ew} or B_{iso}[b]
C(1)	138 (4)	4436 (0)	7679 (4)	7
C(2)	67 (4)	6133 (8)	7365 (4)	9
C(3)	-934 (4)	6256 (8)	5986 (4)	11
C(4)	-1084 (4)	4648 (8)	5519 (4)	12
C(5)	-805 (4)	3617 (8)	6447 (4)	10
C(6)	-494 (4)	1991 (8)	6540 (4)	9
C(7)	-380 (4)	926 (8)	7288 (4)	8
C(8)	478 (4)	1169 (8)	8678 (4)	9
C(9)	332 (3)	2640 (8)	9014 (4)	9
C(10)	357 (4)	4262 (7)	8709 (4)	8
C(11)	1080 (4)	2532 (7)	10380 (4)	11
C(12)	2157 (4)	1991 (8)	11211 (4)	11
C(13)	2156 (4)	602 (7)	10691 (4)	9
C(14)	1581 (4)	1098 (8)	9448 (4)	10
C(15)	399 (4)	-244 (8)	9116 (4)	10
C(16)	1518 (4)	-747 (8)	10354 (4)	10
C(41)	-325 (4)	4459 (8)	5612 (5)	19
C(42)	-2180 (4)	4421 (8)	4176 (5)	15
C(101)	-363 (4)	5243 (7)	8508 (4)	11
C(161)	1699 (4)	-1225 (8)	11300 (5)	16
O(3)	-1770 (3)	6704 (6)	5652 (3)	13
O(5)	-1621 (3)	3630 (6)	6182 (3)	9
O(6)	-1311 (3)	1357 (6)	5314 (3)	15
O(10)	1380 (2)	4889 (6)	9778 (3)	7
O(14)	1657 (3)	-46 (6)	8968 (3)	11
O(16)	1771 (3)	-2077 (6)	10165 (3)	12
O(1W)	1572 (5)	416 (9)	-3103 (6)	21
O(2W)	-263 (0)	-396 (0)	-4750 (0)	36*
O(3W)	85 (0)	-809 (0)	-4188 (0)	39*
O(4W)	644 (0)	-352 (0)	-4127 (0)	41*
O(5W)	766 (0)	-890 (0)	-3629 (0)	39*

[a] The estimated standard deviations are in parentheses. The positions (and isotropic thermal parameters) of the disordered solvent atoms (W) were not refined. B_{eq} is calculated from $^1/_3$ the trace of the B_{ij} matrix. [b] An asterisk indicates B_{iso}.

reduction program TRACER. The space group and initial model were determined by Dr. Charles F. Campana of the Nicolet Corp. using the SHELXTL programs. After initial refinement, difference electron density calculations indicated the positions of the hydrogen atoms; they were included in the model at calculated positions with isotropic thermal parameters but were not refined. Five disordered solvent molecules were found clustered around the twofold axis. Four of these were modeled as oxygen atoms with fixed positional and isotropic thermal parameters; only their occupancy parameters were refined. The other atom O(1W) was more static and was refined with an anisotropic thermal parameter. Weighted full-matrix least-squares refinement with anisotropic thermal parameters for the carbon and oxygen atoms gave final residuals of $R = 0.068$ and $Rw = 0.085$.

α-H_2GTXII was solved with use of MULTAN78.[7] All carbon and oxygen atoms were identified in the first Fourier synthesis, and all hydrogen atoms were identified in subsequent difference electron density syntheses. The hydrogen atoms were included in the model at calculated positions with isotropic thermal parameters and were not refined. Weighted full-matrix least-squares refinement with anisotropic thermal parameters for the carbon and oxygen atoms and isotropic extinction gave final residuals of $R = 0.039$ and $Rw = 0.049$. The extinction parameter,[8] g, refined to 2.1 (1) × 10^{-3}.

Absolute Configuration Determination. The absolute configuration of α-H_2GTXII was determined from the anomalous scattering due to the 5 oxygen atoms with use of Cu radiation. The structural parameters, including positions, anisotropic thermal parameters, and an isotropic extinction correction, were refined to convergence for each enantiomer. The R value for enantiomer one was 0.03936 and for enantiomer two was 0.03955. These values give an R value ratio of 1.0048; according to the Hamilton R-factor test,[9] this ratio indicates a significant difference at the 99% confidence level for 226 parameters and 1572 reflections. Thus, the correct enantiomer (one) is uniquely determined. As a check on this determination, the $|F_o - F_c|$ values for the 180 reflections that had a significant contribution from the oxygen atoms (F_c' calculated only from the oxygen atoms was $\geq 0.5F_o$) were tabulated (see Table II[10]) and used to calculate R values for these most sensitive reflections. The R values

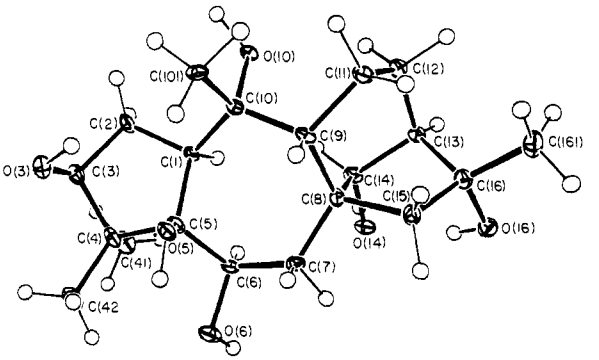

Figure 1. The absolute configuration and atomic labeling for grayanotoxin III. The thermal ellipsoids are drawn at the 50% probability level by plotting program ORTEP.[15]

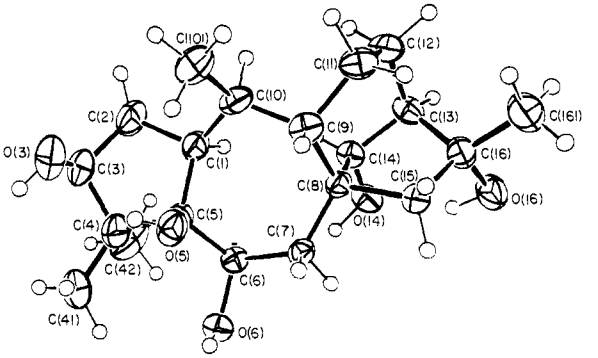

Figure 2. The absolute configuration and atomic labeling for α-dihydroganotoxin II. The thermal ellipsoids are drawn at the 50% probability level.[15] The difference in ellipsoid size between Figures 1 and 2 reflects the reduced degree of atomic vibration at -100 °C.

Table IV. Atomic Coordinates (×10⁴) and $B_{equivalent}$ (×10) for the Carbon and Oxygen Atoms of α-Dihydrograyanotoxin II[a]

atom	x/a	y/b	z/c	B_{eq}
C(1)	3852 (4)	-5786 (0)	2449 (3)	34
C(2)	2286 (5)	-5938 (10)	2514 (4)	50
C(3)	1660 (5)	-7828 (11)	2020 (4)	50
C(4)	2406 (5)	-8105 (10)	1243 (3)	42
C(5)	4006 (4)	-7550 (8)	1811 (3)	29
C(6)	4926 (4)	-6964 (8)	1187 (2)	25
C(7)	6501 (4)	-6514 (8)	1704 (3)	26
C(8)	6995 (4)	-4988 (8)	2510 (3)	24
C(9)	6604 (4)	-5514 (9)	3423 (3)	29
C(10)	5003 (4)	-5500 (9)	3410 (3)	35
C(11)	7465 (5)	-4204 (10)	4261 (3)	34
C(12)	7399 (5)	-2060 (9)	3986 (3)	35
C(13)	7700 (4)	-1756 (8)	3031 (3)	30
C(14)	6564 (4)	-2832 (9)	2264 (3)	26
C(15)	8676 (4)	-4863 (9)	2760 (3)	29
C(16)	9122 (4)	-2722 (9)	2997 (3)	33
C(41)	2235 (6)	-10196 (12)	860 (5)	67
C(42)	1740 (5)	-6677 (14)	432 (4)	65
C(101)	4869 (5)	-6879 (11)	4210 (3)	54
C(161)	10400 (5)	-2395 (10)	3874 (3)	46
O(3)	2011 (4)	-9366 (8)	2720 (3)	60
O(5)	4710 (3)	-9166 (7)	2379 (2)	35
O(6)	4924 (3)	-8472 (7)	498 (2)	32
O(14)	6647 (3)	-2226 (7)	1340 (2)	33
O(16)	9565 (3)	-1860 (8)	2229 (2)	45

[a] The estimated standard deviations are in parentheses. The y coordinate of C(1) was fixed to define the origin. B_{eq} is calculated from $^1/_3$ the trace of the B_{ij} matrix.

(6) Furusaki, A.; Hamanaka, N.; Matsumoto, T. *Bull. Chem. Soc. Jpn.* **1980**, *53*, 1956.
(7) Germain, G.; Main, P.; Woolfson, M. M. *Acta Crystallogr., Sect. A* **1971**, *A27*, 368-376.
(8) Larson, A. C. In "Crystallographic Computing"; Ahmed, F. R., Ed.; Munksgaard: Copenhagen, 1969; pp 291-294.
(9) Hamilton, W. C. *Acta Crystallogr.* **1965**, *18*, 502-510.
(10) See paragraph at the end of the paper regarding supplementary material.

Table IX. Hydrogen Bond Parameters for Both Structures[a]

atoms	distance, Å H···O	distance, Å O···O	angle, deg O–H···O	equiv. position
		Grayanotoxin III		
		Intramolecular Bonds		
O(3)–H(03)···O(5)	2.40	2.794 (8)	100	
O(16)–H(016)···O(14)	1.92	2.691 (9)	143	
O(5)–H(05)···O(6)	2.48	2.960 (10)	113	
		Intermolecular Bonds		
O(10)–H(010)···O(16)	1.68	2.707 (7)	165	$(x, 1 + y, z)$
O(14)–H(014)···O(10)	1.98	2.805 (8)	150	$(^1/_2 - x, y - ^1/_2, 2 - z)$
O(5)–H(05)···O(3)	1.91	2.749 (6)	152	$(-x - ^1/_2, y - ^1/_2, 1 - z)$
O(6)–H(06)···O2W	1.87	2.848	172	$(x, y, 1 + z)$
O(6)–H(06)···O3W	1.91	2.749	152	$(x, y, 1 + z)$
		α-Dihydrograyanotoxin II		
		Intramolecular Bonds		
O(5)–H(05)···O(3)	2.20	2.827 (5)	143	
O(16)–H(016)···O(14)	1.92	2.758 (4)	149	
		Intermolecular Bonds		
O(14)–H(014)···O(6)	1.80	2.800 (4)	160	$(1 - x, ^1/_2 + y, -z)$
O(3)–H(03)···O(16)	2.21	2.845 (6)	131	$(x - 1, y - 1, z)$
O(6)–H(06)···O(14)	2.37	3.118 (6)	164	$(x, y - 1, z)$

[a] Values without an estimated standard deviation were calculated from atomic coordinates that were not refined.

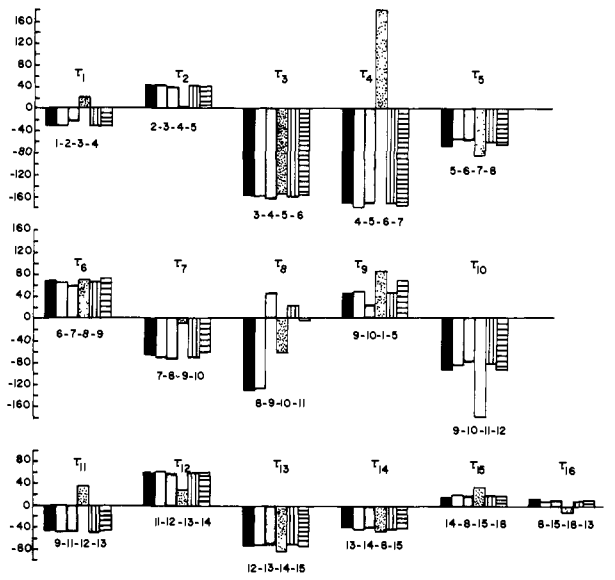

Figure 3. Torsion angle histogram for the skeletal atoms of five grayanotoxins. The compounds are grayanotoxin III (solid bar), α-dihydrograyanotoxin II (open bar), grayanotoxin II[6] (shaded bar), 6,14-di-*O*-acetyl-3-*O*-(2-chlorobenzyl)grayanotoxin II[16] (dotted bar), and two examples of grayanotoxin XVI[17] (lined bar).

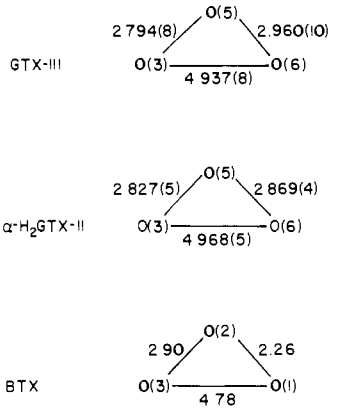

Figure 4. Distances (Å) between the important oxygen atoms in grayanotoxin III, α-dihydrograyanotoxin II, and batrachotoxin.[18]

were 0.0272 for enantiomer one and 0.0283 for enantiomer two. These results confirm that the correct enantiomer is one; this configuration is the same as that predicted from chemical studies. The absolute configuration of GTXIII was determined by correlation with α-H_2GTXII.

Results and Discussion

Figures 1 and 2 show the conformation, absolute configuration, and atomic labeling scheme for GTXIII and α-H_2GTXII, respectively. The fractional atomic coordinates for the carbon and oxygen atoms in the two structures are given in Tables III and IV. The anisotropic thermal parameters (Table V), hydrogen atom parameters (Table VI), and bond distances and angles (Tables VII and VIII) are available[10]. Hydrogen bond dimensions are given in Table IX.

These two neurotoxic grayanotoxins have almost identical shapes as is evident in the molecular diagrams given in Figures 1 and 2 and in the similarity of the skeletal torsion angles shown in the first two bars for each torsion angle in Figure 3. A least-squares fit[11] of the positions of the 16 skeletal carbon atoms in the two structures gives an average deviation of only 0.098 Å.

All of the lipid soluble neutrotoxins must have similar three-dimensional distributions of groups that bind to the receptor. A common structural feature, a triangle of oxygen atoms, has been found in the structures of aconitine, batrachotoxin, and veratridine.[1] As previously suggested by Matsutani et al.[4] from model building studies, a triangle of oxygen atoms is also found in the two toxic grayanotoxins examined in our work. The three atoms are the hydroxyl oxygen atoms bonded to C(3), C(5), and C(6). The dimensions of the triangle for each compound are shown in Figure 4 as are the dimensions found in batrachotoxin. It has recently been observed by Kosower[12] that this group of oxygen atoms could be a binding site for a cationic group on the receptor, possibly the ε-ammonium group of lysine. The separation between O(3) and O(6) in the grayanotoxins, ~4.9 Å, is similar to the oxygen–oxygen separation found in crown ether complexes of ammonium cations.[13]

In the analysis[1] of the similarities of the alkaloid neurotoxins, aconitine, batrachotoxin, and veratridine, an additional binding group, the nitrogen atom about 6 Å from the triangle, was identified. Although it was originally thought that the unprotonated form of batrachotoxin was the active species, Brown and Daly[5] have shown that batrachotoxin has a $pK_a \geq 8.2$ and

(11) The fit was done by using the program PROFIT written by Dr. G. D. Smith of the Medical Foundation of Buffalo, Buffalo, NY.

(12) Kosower, E. M. *FEBS Lett.* **1983**, *163*, 161–164.

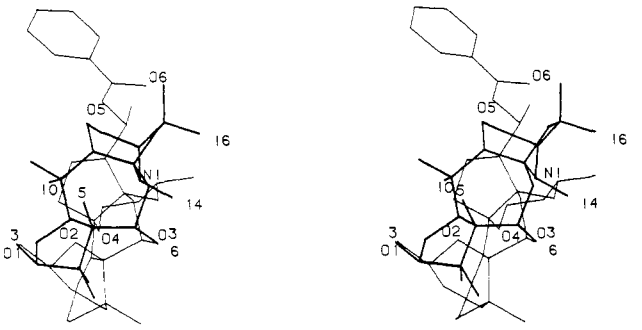

Figure 5. Stereodrawing of the superposition of grayanotoxin III and batrachotoxin[18] drawn with the computer program PLUTO.[19] The GTXIII molecule is drawn with heavy lines. The oxygen atoms of this molecule are labeled only with numbers.

is protonated at physiological pH. The pK_a values for aconitine and veratridine allow for a significant concentration of the protonated form in physiological conditions. These data would lead to the hypothesis that the nitrogen atom in each molecule is a hydrogen bond donor. The grayanotoxins do not have a nitrogen atom and are not protonated. However, the oxygen atom bonded to C(14) is approximately the same distance from the triangle of oxygen atoms as is the nitrogen atom in each of the other toxins. The average distance is 5.83 Å for α-H_2GTXII and 5.72 Å for GTXIII. Although the contribution of this oxygen atom to the overall toxicity of grayanotoxins has been discounted,[4] any modification of this group that diminishes its ability to *donate* a hydrogen atom also diminishes its toxicity. Thus, it is possible that O(14) is one of the pharmacophoric groups for the grayanotoxins along with the triangle of oxygen atoms and is the correlate of the nitrogen atom in the alkaloid neurotoxins.

When the two groups (triangle and O(14)) are superimposed[11] on the groups of similar function in BTX, a good fit can be obtained for the long edge of the triangle, O(3)–O(6), and the hydrogen bond donor atom. Figure 5 is a stereodiagram of this superposition. The nitrogen atom of BTX and O(14) of GTXIII are within 1.5 Å of each other and could donate to the same acceptor group on the sodium channel receptor with slight adjustments from the positions shown. These adjustments could certainly be accommodated in small receptor movements.

It is notable that some *inactive* grayanotoxins contain the minimal binding features of neurotoxins that have just been described. One example is grayanotoxin II[6] (GTXII) (**5**) which differs from the active compounds only by the hybridization at C(10). The dimensions of the triangle of oxygen atoms in GTXII are within the range of the values in Figure 4, and the through-space separation of the triangle from O(14) is similar to the distances in GTXII and α-H_2GTXII; yet, GTXII is inactive. There are two possible explanations for the lack of toxicity in GTXII: either the change in geometry at C(10), from tetrahedral to triangular planar, distorts the relative orientations of the reactive groups or the receptor is in intimate contact with the C(10) area of the molecule and is sensitive to the geometry in this region.

For the first possibility, changes in relative orientation, to be examined, the positions of the triangle of oxygen atoms in GTXII and α-H_2GTXII were fit by least-squares[11] and the separations between like atoms in the two structures were calculated. The largest separations were for the atoms that were the most distant from the triangle, i.e., for O(16), 1.30 Å, and for C(16), 1.15 Å; the proposed binding site, O(14), was 0.88 Å in GTXII from the atomic position found in the active molecule. Although it is not certain that this change in position is sufficient to account for the inactivity of GTXII, it is certain that hydrogen bond strength is affected by the distance between donor and acceptor atoms. Thus the strength of an interaction could be diminished. This distortion in the framework of grayanotoxin molecules is evident in the last four bars of the torsion angle histogram in Figure 3. Each of the last four compounds has an exocyclic double bond at position C(10); torsion angles τ_8(C(8)–C(9)–C(10)–C(11)) and τ_9(C(9)–C(10)–C(1)–C(5)) show large deviations from the values observed in the active compounds, GTXIII and α-H_2GTXII.

Distortion of the molecular framework caused by an accumulation of effects from several substitutions of functional groups explains some of the other structure–activity data on grayanotoxins. Multiple acetyl substitutions for the hydroxyl groups in these compounds reduce activity[4] even when the corresponding single substitutions have no effect. The torsion angle histogram shows the effect of acetyl substitution. The change from GTXII (shaded bar) to the two observations of a 6-acetyl derivative, grayanotoxin XVI (striped bars), produces only minor changes in torsion angles. Yet, the addition of a second acetyl group on C(14) (dotted bar) produces significant distortions in the six- and seven-membered rings as is shown in Figure 3. These torsion angle comparisons suggest that several small perturbations produce a large cumulative change in the relative positions of important groups.

The second explanation for the inactivity of GTXII, the recognition of the C(10) region of the molecule, has been used by Matsutani et al.,[4] who postulate a binding site for the β-methyl group on C(10) as discussed earlier. It is more probable that a bulky group in the α position at C(10) prevents close approach of the toxin to the binding site. GTXIII has an α-OH group on C(10) and is only one-quarter as active as α-H_2GTXII which has a hydrogen atom in the α position. This decrease in activity with bulk in the α position is consistent with steric restriction in the region of the binding site that binds this part of the toxin.

Hydrogen bonding is an important component of the molecular interactions in these crystals. Since all of the hydrogen atoms were identified in these determinations (except those attached to the disordered solvent molecules), the hydrogen bonds can be described in detail. Table IX contains the parameters for the hydrogen bonds found in both crystal structures. In each structure, O(14) forms two of the strongest hydrogen bonds. Apparently the conformations of rings C and D are ideal for the formation of the intramolecular hydrogen bond between O(16)H and O(14) that is observed in both structures. Newton[14] has shown with ab initio calculations that when an oxygen atom has a proton-acceptor function it forms a *stronger* proton-donor bond; this is demonstrated by a calculated 0.07 Å shortening of the OH···O distance. Such an enhancement of hydrogen bonding is evident in both of the structures; in each case O(14) forms a strong intermolecular donor bond to either O(10) in GTX3 or O(6) in α-H_2GTXII. The enhancement of the proton donor function of O(14) arising from the molecular proximity of O(16) is important to its potential as a receptor-binding site, particularly since comparison with BTX strongly suggests that this site acts as a proton donor. This intramolecular hydrogen bond is present in two inactive grayanotoxins as well, GTX II and GTX-XVI. The O···H distances are longer in the inactive compounds, 2.07 and 2.04 Å, respectively, indicating slightly weaker bonding. The O···O distances are approximately the same as those found in the active compounds. In the inactive compounds, the weaker intramolecular hydrogen bond will diminish the donor function of O(14), thus amplifying the poor interaction caused by the distorted molecular framework found in these compounds.

In summary, these structural analyses confirm the presence of a triangle of oxygen atoms in the grayanotoxins that parallels a similar grouping in other sodium channel neurotoxins. In addition, a second binding site, a hydrogen-bonding donor atom (O(14)), is found to be similar in position and possible function to the nitrogen atom in alkaloid neurotoxins. Structural comparisons

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predict that grayanotoxin II derivatives are inactive (nontoxic) because of a distortion of the relative positions of the two sites mentioned above or because there is a sterically restricted site near the C(10) position. Structural evidence does not as yet discriminate between these possibilities. These findings yield a model for the minimal features of a binding site for the lipid-soluble neutrotoxins. Further investigations must examine the differences between the fully active batrachotoxin compounds and the other partially active toxins. Attempts to model the cationic group interaction with the triangle of oxygen atoms are in progress.

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Supplementary Material Available: Fractional atomic coordinates for the hydrogen atoms, all temperature factors, bond distances and bond angles, observed and calculated structure factors for both structures, and calculated structure factors for the two enantiomers of α-H_2GTXII (40 pages). Ordering information is given on any current masthead page.

Synthesis of PHI (Peptide Histidine Isoleucine) and Related Peptides and Immunochemical Confirmation of Amino Acid Residue in Position 24 of PHI with use of the Synthetic Peptides†

K. Nokihara,‡ C. Yanaihara,‡ K. Iguchi,‡ S. Fukata,‡ M. Tanaka,‡ T. Mochizuki,‡ K. Tatemoto,⊥ J. M. Lundberg,⊥ V. Mutt,⊥ and N. Yanaihara*‡§

Contribution from the Laboratory of Bioorganic Chemistry, Shizuoka College of Pharmacy, Shizuoka 422, Japan, Department of Biochemistry II, Karolinska Institute, Stockholm, Sweden, and Laboratory of Cellular Metabolism, National Institute for Physiological Sciences, Okazaki, Aichi 444, Japan. *Received March 8, 1984*

Abstract: An immunochemical approach, using synthetic peptides, was employed to establish the nature of residue 24 in the amino acid sequence of PHI (peptide histidine isoleucine). PHI(20–27) and [Gln^{24}]-PHI(20–27) were synthesized by conventional solution methods and were used as haptenic immunogens for production of antisera. An antiserum raised against [Gln^{24}]-PHI(20–27), R8304, was shown to recognize specifically the glutaminyl residue in position 24. With use of this antiserum together with an anti-PHI(20–27) serum, R8201, the amino acid residue in position 24 of natural PHI preparation was radioimmunologically demonstrated to be a glutamyl and not a glutaminyl residue. Crossreactivity of a crude extract of porcine duodenum in radioimmunoassays with the two PHI antisera, R8201 and R8304, again confirmed the presence of a glutamyl residue in position 24 of the intact PHI molecule in the tissue. On the basis of these results, a heptacosapeptide amide corresponding to the proposed [Glu^{24}] sequence of PHI was synthesized by the azide fragment condensation method in solution. The synthetic preparation was identical with the natural preparation of PHI in high-performance liquid chromatography, radioimmunoassay, and bioassay. The present study not only provided an immunochemical method for confirmation of a single amino acid residue among the 27 constituent amino acids of the PHI molecule but also led to a synthetic PHI preparation identical with that of the natural peptide. This synthetic peptide provides a tool for the investigation of the physiological significance of PHI.

Porcine PHI (peptide histidine isoleucine) is a peptide of 27 amino acid residues having amino terminal histidine and carboxyl terminal isoleucine. The peptide was isolated by Tatemoto and Mutt[1] from porcine upper intestinal tissue. The peptide shows considerable sequence homology with porcine secretin and VIP (Figure 1). Tatemoto and Mutt directed their attention to the amino acid residues in position 24 in these three peptides. In secretin position 24 is occupied by a glutaminyl residue and in VIP by asparagine. These findings suggested the possibility that glutaminyl residue originally present in position 24 had been deamidated during the purification of the PHI.[1] In this study, we used a novel immonochemical approach to confirm the presence of a glutamyl residue in position 24 in the intact molecule of the PHI.

The first objective of this study was to develop a radioimmunoassay using specific antisera which could unequivocally identify the amino acid residue in position 24 of PHI. For the purpose of producing such antisera, PHI(20–27) and [Gln^{24}]-PHI(20–27) were chosen as haptenic immunogens and these two peptides were prepared by conventional solution methods (Figure 2). We employ the azide fragment condensation procedure with minimum side-chain protection for the synthesis of peptides. This strategy was used extensively in our previous studies on the syntheses of various peptides.[2a–e] The present synthesis of the two octapeptide amides proceeds along the same line and afforded homogeneous materials without resorting to complicated purification procedures.

† The amino acids except glycine are of the L configuration. The conventions and nomenclature used are those recommended by IUPAC: *Pure Appl. Chem.* **1974**, *40* 317–331.

‡ Shizuoka College of Pharmacy.

⊥ Karolinska Institute.

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